Protein Expression

A PRACTICAL APPROACH

Edited by
S. J. HIGGINS and B. D. HAMES

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Protein Expression

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Preface

Some years ago we edited a book for The Practical Approach series entitled *Transcription and translation: a practical approach*. When the time came to consider organizing a second edition, it rapidly became clear that no one book of the desired size could include in sufficient detail the myriad of important new techniques for investigating gene expression. As a result, a decision was taken to produce a collection of books to cover this important area. *Gene transcription: a practical approach* and two volumes of *RNA processing: a practical approach* have since been published. Now, this book, *Protein expression: a practical approach*, and its companion volume, *Post-translational processing: a practical approach*, complete the 'mini-series' by providing a comprehensive and up-to-date coverage of the synthesis and subsequent processing of proteins.

Protein expression: a practical approach describes in detail the expression of cloned DNA or RNA templates in all the major *in vitro* and *in vivo* systems, both prokaryotic and eukaryotic, as well as methods for monitoring expression. The *in vivo* systems include cultured mammalian cells (described comprehensively by Marlies Otter-Nilsson and Tommy Nilsson), yeast (by Mick Tuite and his colleagues), baculovirus (Bob Possee *et al.*), and *Xenopus* (Glenn Matthews). Expression *in vivo* in prokaryotes is covered by Ed Appelbaum and Allan Shatzman. On the *in vitro* side, the chapter by Mike Clemens and Ger Pruijn focuses on the purification of eukaryotic mRNA and its translation in cell-free extracts. The prokaryotic *in vitro* systems of note are those that offer coupled transcription–translation and hence these are the subject of the chapter by Boyd Hardesty's group. Finally, John Colyer's chapter provides essential techniques for monitoring protein expression.

Those researchers who wish to fully characterize the expressed protein product, and to follow its post-translational fate, are advised to also consult the companion volume, *Post-translational processing: a practical approach*, which covers protein sequence analysis, protein folding and import into organelles, protein modification (phosphorylation, glycosylation, lipid modification), and proteolytic processing.

The overriding goals of *Protein expression: a practical approach* are to describe, in precise detail, tried and tested versions of key protocols for the active researcher, and to provide all the support required to make the techniques work optimally, including hints and tips for success, advice on potential pitfalls, and guidance on data interpretation. We thank the authors for their diligence in writing such strong chapters and for accepting the editorial changes we suggested. The end-result is a comprehensive compendium of the best of current methodology in this subject area. It is a book designed both to be used at the laboratory bench and to be read at leisure to gain insight into future experimental approaches.

Leeds S.J.H.
August 1998 B.D.H.

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Abbreviations

5-FOA	5-fluoro-orotic acid
A_{260}	absorbance at 260 nm
αAA	α-aminoadipate
AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
ATP	adenosine 5′-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BFP	blue fluorescent protein
BSA	bovine serum albumin
cAMP	adenosine 3′,5′-cyclic monophosphate
CAT	chloramphenicol acetyltransferase
CIP	calf intestinal alkaline phosphatase
DAB	diaminobenzidine
DDAB	dimethyldioctadecyl ammonium bromide
DEAE	diethylaminoethyl
DEPC	diethyl pyrocarbonate
DHF	dihydrofolate
DHFR	dihydrofolate reductase
dia.	diameter
DMEM	Dulbecco's modified Eagle's medium
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DOC	deoxycholate
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium chloride
dsRNA	double-stranded RNA
DTE	dithioerythritol
DTT	dithiothreitol
ε	molar extinction coefficient
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-*O*,*O*′*bis*(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid
eIF	eukaryotic initiation factor
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ER	endoplasmic reticulum
FA	folinic acid
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate

G-6-P	glucose 6-phosphate
GFP	green fluorescent protein
GST	glutathione *S*-transferase
GTP	guanosine 5′-triphosphate
GUS	β-glucuronidase
HBS	Hepes-buffered saline
h.p.i.	hours post-infection
hyg	hygromycin
HzSNVP	*Heliothis zea* single nucleopolyhedrovirus
IgG	immunoglobulin class G
IPTG	isopropyl-β-D-thiogalactoside
KLH	keyhole limpet haemocyanin
LB	Luria broth
LM	low molecular weight mixture
MBS	modified Barth's saline
MCS	multiple cloning site
MFα1	mating factor α1
MNPV	multiple nucleopolyhedrovirus
m.o.i.	multiplicity of infection
MOPS	3-(*N*-morpholino)propane sulfonic acid
M_r	relative molecular mass
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NBT	nitroblue tetrazolium
NCYC	National Collection of Yeast Cultures
nd	not determined
neo	neomycin
Ni-NTA	nitrilo-triacetic acid chelated with Ni^{2+} ions
NMR	nuclear magnetic resonance
NPV	nucleopolyhedrovirus
OD_{650}	optical density (at 650 nm)
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
p.f.u.	plaque-forming units
pI	isoelectric point
P_i	inorganic phosphate
PMSF	phenylmethylsulfonyl fluoride
P-ser	phosphoserine
p.s.i.	pounds per square inch
P-thr	phosphothreonine

PTT	protein truncation test
P-tyr	phosphotyrosine
PVDF	polyvinylidene difluoride
RBS	ribosome binding site
rDNA	ribosomal DNA
RNase	ribonuclease
S30	supernatant from 30 000 *g* centrifugation
SDS	sodium dodecyl sulfate
Sf	*Spodoptera frugiperda*
SFV	Semliki forest virus
SNPV	single nucleopolyhedrovirus
ssRNA	single-stranded RNA
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing Tween
TCA	trichloroacetic acid
TE	Tris-EDTA
TLC	thin-layer chromatography
tRNA	transfer RNA
UTP	uridine 5′-triphosphate
UTR	untranslated region
V	volts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YGSC	Yeast Genetics Stock Center

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Protein expression in mammalian cells

MARLIES OTTER-NILSSON and TOMMY NILSSON

1. Introduction

Protein expression has become a major tool to analyse intracellular processes, both *in vitro* and *in vivo*. The choice of expression system depends entirely on the purpose of the study. For some cases, transient transfection is the most obvious choice because of its relatively short time investment. In other cases, homogeneous populations and large quantities of cells may be required, which involves making cell lines stably expressing the desired protein. It may also be advantageous to express proteins under inducible promotors; this is particularly true if the protein exerts pathological effects on the cell. There are several inducible systems available but none, so far, is easy and straightforward. Expression through virus infection is also possible. Here, large quantities of cells can be infected at the same time and the protein assayed for shortly after infection. The drawback, however, with this method is the need for special precautions when making and handling virus stocks and likely side-effects exerted by the virus on the cellular machinery upon infection. Protein expression can also be achieved directly via microinjection of plasmid DNA directly into the nucleus of the host cell. This allows for protein expression in single cells which may be desirable when performing video microscopy. Thus the choices of expression systems are multiple and one should carefully consider the range of these available before investing time and other resources in the experiments themselves. It is the goal of this chapter to describe the various expression systems so as to make this choice easier.

2. Viral and plasmid vectors

2.1 Semliki forest virus

A vector based on Semliki forest virus (SFV) has been developed by Liljeström and co-workers (1) and has turned out to be a very efficient expression system in mammalian cells. The virus has a genome of ssRNA which is

capped and polyadenylated and has a positive polarity, acting as a direct mRNA upon infection. It encodes its own RNA polymerase producing viral RNA transcripts. Vectors pSFV1, 2, and 3, which lack the structural protein genes of the virus, and helper vectors 1 and 2, encoding the structural viral proteins have all been described (1, 2). The pSFV1, 2, and 3 all have a polylinker with unique restriction sites, followed by stop codons in all three reading frames. The three vectors have minor differences with respect to their cloning sites. The pSFV3 vector has an additional ribosome binding site and initiation codon within the vector and is therefore the most convenient vector to use. The DNA encoding the protein of interest is cloned into one of the pSFV vectors under control of the viral promotor. The recombinant pSFV viral DNA and the helper vector are then linearized by *Spe*I (note: the insert should not contain a *Spe*I site!) and then used for *in vitro* transcription to obtain RNAs. Co-transfection of the helper RNA and the pSFV RNA into cells yields both protein and virions containing the recombinant RNA. Production of these virions is described in *Protocol 1*.

The recombinant pSFV virions can now be used to infect an appropriate mammalian cell line such as BHK, Vero, HeLa, or MDCK II (3), during which the gene of interest cloned into the recombinant virus is expressed (*Protocol 2*).

Protocol 1. Transfection of recombinant viral RNA into mammalian cells by electroporation

Equipment and reagents

- Electroporation equipment, e.g. Gene-pulser (Bio-Rad)
- Electroporation cuvettes
- Cell scraper (rubber policeman)
- Mammalian cells in tissue culture dishes in the logarithmic phase of growth
- Growth medium supplemented with 10% FCS
- PBS: 1.37 mM NaCl, 2.7 mM KCl, 8.2 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4
- Recombinant RNA and helper RNA encoding the structural viral proteins (1–5 μg)
- 0.1% crystal violet in 20% ethanol
- Trypsin/EDTA: 0.5 mg/ml trypsin, 0.2 mg/ml EDTA

A. *Infection of the cells*

1. Grow the cells to 80% confluency, then pour off the growth medium, and wash the cells with PBS.
2. Scrape the cells off the tissue culture dish using a rubber policeman, or detach the cells using trypsin/EDTA.
3. Centrifuge the cells at 400 *g* for 5 min.
4. Wash the cells with PBS by centrifugation (400 *g* for 5 min).
5. Resuspend the cells at 10^7 cells/ml in PBS, add 1–5 μg recombinant pSFV RNA, and 1 μg helper RNA in a total volume not exceeding 800 μl.

6. Add the mixture to the electroporation cuvette and pulse twice at 850 V/25 μF (the time constant should be 0.4).
7. Dilute the cell suspension 20-fold with growth medium containing FCS, and plate the cells.
8. Incubate the cells for 36 h. During this time, virions are produced and released from the cells and the protein of interest is expressed.
9. To collect the virions, centrifuge at 400 *g*, 4°C for 10 min to remove cell debris. Recover the supernatant.
10. Snap-freeze aliquots of the supernatant (virus stock) and store at −70°C or in liquid N_2.

B. *Titration of the virus stock*

1. Grow the cells to 80% confluency on coverslips.
2. Make virus dilutions in serum-free medium. Spot small droplets of the diluted virus onto Parafilm and place the coverslips over the virus droplets. Incubate for 1 h at 37°C.
3. Transfer the coverslips to growth medium containing 10% FCS.
4. After 4–6 h, clear plaques of virus-infected cells in the monolayer of cells can be detected.
5. Stain the cells with 0.1% crystal violet in 20% ethanol.

Protocol 2. Infection of mammalian cells with recombinant SFV virions

Reagents

- Mammalian cells in logarithmic phase of growth (e.g. BHK, Vero, HeLa, or MDCKII)
- Growth medium without FCS
- Growth medium supplemented with 10% FCS
- PBS (see *Protocol 1*)
- *Recombinant SFV virus stock* (10^6 to 10^{10} infectious units/ml)

Method

1. Aspirate the growth medium from the cells and add 10 ml PBS to the cells in a 100 mm tissue culture dish. Swirl the PBS gently in the dish and aspirate the PBS.
2. Make dilutions of the virus stock in serum-free medium. Usually this means dilutions of 1:100 to 1:1000 of the stock to give a final 10^5 infectious units/ml.
3. Incubate the cells for 1 h with the virus stock in serum-free medium.
4. Remove the virus suspension. Do not wash the cells.

Protocol 2. *Continued*

5. Add new fresh medium with 10% FCS to the cells and incubate for at least 3–4 h.
6. Monitor the morphology of the cells. Usually it will not change until 6–8 h after infection. Then cells might round up or even detach.
7. The protein of interest can be detected after 2–3 h[a] (see also Section 4).

[a] Depending on the DNA insert and the cell type, some cells might require a higher concentration of virus and longer incubation times for adequate expression levels.

2.2 Vaccinia virus

Vaccinia virus has been widely used as a means of expressing proteins in eukaryotic cells. The virus grows rapidly, is easy to handle, and is relatively safe to work with when safety guide-lines are followed. However safety precautions **must** be strictly observed:

(a) When handling virus stocks, use protective clothing, gloves, and eye glasses (*contamination of your eyes with virus may lead to blindness*).

(b) Most laminar flow-hoods blow air into the hood to create a positive pressure but also to create a barrier to prevent contamination with virus outside of the hood. However items such as pipette canisters placed onto the vents upset and breach this barrier and can easily expose the user to aerosol containing virus. Therefore, it is essential to use the laminar flow-hood correctly.

(c) Most laboratories require that their personnel obtain vaccination against small pox before starting work with the virus. If sharing the work space with other colleagues, they also require vaccination.

(d) Before starting work with the virus, consult with the local safety representative to ensure that all appropriate safety procedures have been implemented.

The introduction of a non-replicating vaccinia vector based on a modified vaccinia strain, the Ankara strain, reduces some of the risks involved. This highly attenuated virus cannot replicate in human cells but nevertheless leads to a high expression level of recombinant protein when introduced into human cell lines (4). The recombinant vaccinia protein expression has been developed and described in detail by Moss and co-workers (5, 6). The production, purification, and titration of vaccinia virus stocks are described in *Protocol 3*.

There are two systems for protein expression available with vaccinia virus:

(a) The first (direct) method requires the generation of a recombinant vaccinia virus. The gene of interest is inserted into a transfer vector, such as pSC11ss or pSC65, carrying a non-essential gene of the virus (i.e. thymi-

dine kinase, *tk*) under an early/late vaccinia promotor ($P_{7.5}$). Eukaryotic cells, such as BHK, HeLa, or CV-1 cells, are infected with vaccinia virus and then, after 30 minutes of incubation, the plasmids containing the DNA of interest are also transfected into these cells using a suitable transfection protocol. Recombinant vaccinia virus is formed in the host cells by plasmid and the vaccinia genome, and this can then be isolated from the cell lysate. To do this, the lysate is used to infect another cell line to screen for recombinants of interest. For example, when using thymidine kinase as the marker, a *tk*$^-$ cell line such as the osteosarcoma 143B is infected with the lysate in the presence of bromodeoxyuridine.

(b) The second method (*Protocol 4*) is an indirect one but is less laborious than the above procedure. The gene of interest is cloned into a plasmid such as pBluescript or pAR2529 under the control of the T7 phage promoter. The vaccinia virus used in this case is a recombinant virus (vTF7-3) containing the bacteriophage T7 RNA polymerase under a vaccinia virus promoter (7). Both the plasmid DNA and the vaccinia virus are introduced into the host cells. This procedure and the subsequent screening of the cells for the expressed protein are easy and rapid to perform, usually taking only a day or two.

Protocol 3. Production, purification, and titration of vaccinia virus stocks

Equipment and reagents

- Sonication water-bath
- Cell scraper (rubber policeman)
- Dounce homogenizer fitted with tight pestle
- Host cells in tissue culture dishes (100 mm diameter)
- DMEM medium, serum-free
- DMEM medium supplemented with 10% FCS
- PBS (see *Protocol 1*)
- 10 mM Tris–HCl pH 9
- 36% sucrose (w/v) in 10 mM Tris–HCl pH 9
- 0.1% crystal violet in 20% ethanol
- 0.25 mg/ml trypsin

A. *Infection of cells with vaccinia virus*

1. Grow the cells to 80% confluency.
2. Infect the cells for 30 min at 37 °C with recombinant vaccinia virus at 0.05–0.1 plaque-forming units/cell (p.f.u.). For a 100 mm dish containing 10^7 cells, 10^5 to 10^6 p.f.u. are needed. Occasional rocking of the plates during incubation is recommended.
3. Add 10 ml DMEM containing 10% FCS to the cells and incubate for two to three days.

B. *Production of cell lysates containing vaccinia virus*

1. Recover the cells from part A, step 3 by scraping with a rubber policeman, and then centrifuge at 400 *g* for 5 min at 4 °C.

Protocol 3. *Continued*

2. Resuspend the cell pellet in DMEM medium containing 10% FCS and lyse the cells by freeze–thawing (five cycles). The lysate can be stored at –70°C.
3. Prior to the use of cell lysates for infection of the cells, add an equal amount of 0.25 mg/ml trypsin and incubate for 30 min at 37°C with occasional vortexing.

C. *Production of purified virus stocks*

1. Harvest the cells from part A, step 3 by scraping using a rubber policeman, and then centrifuge at 400 *g* for 5 min at 4°C.
2. Add 5–10 ml of 10 mM Tris–HCl pH 9 to the cell pellet and homogenize in a Dounce homogenizer with a tight pestle using at least 50 strokes.
3. Centrifuge the homogenate for 5 min at 400 *g* at 4°C. Keep the supernatant.
4. Wash the pellet once with 10 mM Tris–HCl pH 9 by centrifugation. Keep the supernatant.
5. Pool the two supernatants and centrifuge at 800 *g* for 10 min at 4°C.
6. Recover the supernatant and sonicate it in a sonication water-bath for 2 min.
7. Add an equal volume of 36% (w/v) sucrose in 10 mM Tris buffer pH 9 (equal to the volume of the supernatant) to an ultracentrifuge tube. Layer the supernatant carefully onto the sucrose layer. Centrifuge for 90 min at 4°C at 100 000 *g*.
8. Resuspend the pellet in an appropriate volume (1–2 ml) of 10 mM Tris–HCl pH 9. Make aliquots and freeze them at –70°C. This will usually yield 10^9 to 10^{10} p.f.u.
9. Freeze aliquots and store at –70°C.

D. *Titration of vaccinia virus stocks*

1. Grow the cells to 80–90% confluency.
2. Dilute the virus stock in serum-free medium to a virus concentration in the range 10^{-5} to 10^{-9} p.f.u./ml.
3. Wash the cells once with PBS and then pour this off.
4. Add the diluted virus to the cells and incubate for 30 min at 37°C.[a] Also set up a negative control (cells with no virus) and a positive control (known number of p.f.u.).
5. Aspirate the supernatant. Replace it with DMEM medium containing 10% FCS. Incubate for two to three days.

6. Remove the medium.
7. Stain the cells with 0.1% crystal violet in 20% ethanol.

[a] Incubate for 2 h at 37 °C if a cell lysate is used instead of purified virus.

Protocol 4. Indirect method of vaccinia virus-mediated protein expression

Equipment and reagents

- Host cells in tissue culture dishes
- Vaccinia recombinant virus vFT7-3
- Plasmid DNA containing the gene of interest under control of the T7 phage promoter
- Cationic lipid solution (see *Protocol 7*)
- DMEM medium, serum-free
- Reagents to monitor expression of the protein of interest (see Section 4)

Method

1. Grow the cells to 80–90% confluency.
2. Wash the cells once with serum-free medium.
3. Add the vFT7-3 recombinant virus to the cells (use 10^6 p.f.u. for 10^7 cells).
4. Incubate for 30–45 min (or for 2 h when a cell lysate is used) at 37 °C. Occasional rocking is recommended.
5. Prepare a plasmid DNA/lipid mixture according to *Protocol 7*, step 2.
6. Aspirate the virus and add the plasmid DNA/lipid mixture to the cells. Incubate the cells with the DNA/lipid mixture at 37 °C according to *Protocol 7*, step 3.
7. Remove the DNA/lipid mixture and add fresh growth medium to the cells. (Over)expression of the protein is detectable after 2–2.5 h.

2.3 Retroviral vectors

The use of retroviral transduction systems has proliferated rapidly over the past five years. These have now become major tools in gene therapy, where long-term stable expression of (exogenous) proteins is required. The procedures for using retroviral vectors can be summarized as follows:

(a) A retroviral packaging cell line is required that is capable of producing the proteins necessary for viral assembly.

(b) The retroviral vector is then introduced into this cell line.

(c) Cell clones are then screened to find those that produce sufficiently high virus titres.

Several variants of this procedure have been published:

(a) In 1993, Pear and co-workers (8) described a protocol for transient transfection of retroviral vectors into a producing cell line BOSC 23 (which is an adenovirus-transformed embryonic human kidney cell line). Using the calcium phosphate method, they obtained 10^7 infectious particles/ml 48–72 hours post-transfection. Their virus stocks were essentially helper-free and could even be prepared using genes that appeared toxic to stable cell lines.

(b) Almost simultaneously, another highly efficient transduction system for retroviral vectors was described by Finer *et al.* (9). High levels of retroviral transcripts were harvested 48 hours post-transfection of specially designed *kat* expression vectors into NIH 3T3 cells using calcium phosphate. This protocol avoids the lengthy procedure of making stable clones.

(c) A third innovative method, called virofection, allows the production of stable mammalian cell lines expressing proteins as a one-step method (10). It relies on the co-transfection of two vectors, one of which is essentially replication defective and carries the gene of interest, and the other carrying the *gag/pol* and *env* genes to ensure proper assembly of virus particles. Virus particles are assembled a few days after co-transfection of these two vectors into the host cells and will then infect neighbouring cells. The viral genome is reverse transcribed and stably integrated into the host DNA by a (retroviral) integrase.

(d) Most recently a method has been reported by Bilbao *et al.* (11). This uses an adenovirus/retroviral chimera that incorporates the better characteristics of both viral systems to achieve stable transduction in mammalian cells. In several cases, adenoviruses or adeno-associated viruses have proved to give the highest levels of direct gene transfer (12–15). However, adeno-associated viruses are not able to achieve long-term (stable) high titres upon transfection *in vivo*, since they have a limited integrating capacity as compared to a retroviral system. The chimera allows use of the integrative functions of the retrovirus combined with the high titre characteristic of the adenovirus. Adenovirus replication deficient (E1A–B deleted) vectors were constructed that contained either the retroviral packaging functions (*gag/pol* and *env* sequences) or retroviral vector functions in combination with the gene of interest and/or a reporter gene such as green fluorescent protein (GFP). Upon co-transfection of these vectors into the producer cell line NIH 3T3, retroviral particles were produced and stably transducted neighbouring cells *in situ*.

2.4 Plasmid pCMUIV

For optimal and consistent protein expression, it is important to consider the best expression vector to use, the choice of which is mainly dependent on

which cell type is used. For example, a human promotor such as the α-globin promotor used in pCMUIV (16) gives rise to very high expression in human cells upon transient transfection. This vector (see *Figure 1*) has been optimized for expression by incorporating strong SV40 enhancer elements, an α-globin promotor, and an intron/exon splicing cassette terminating with a poly(A) addition sequence. A leader sequence from *Xenopus laevis* has been inserted between the promotor and the *Bam*HI site which maximizes expression (17). In this way, only the coding region of the cDNA is needed plus three extra bases (GCC) prior to the ATG. In fact, it is recommended to remove all 5′ and 3′ untranslated regions from the cDNA before inserting it into the expression vector since these are likely to contain regulatory regions affecting expression. This vector is a low copy one and is best propagated in

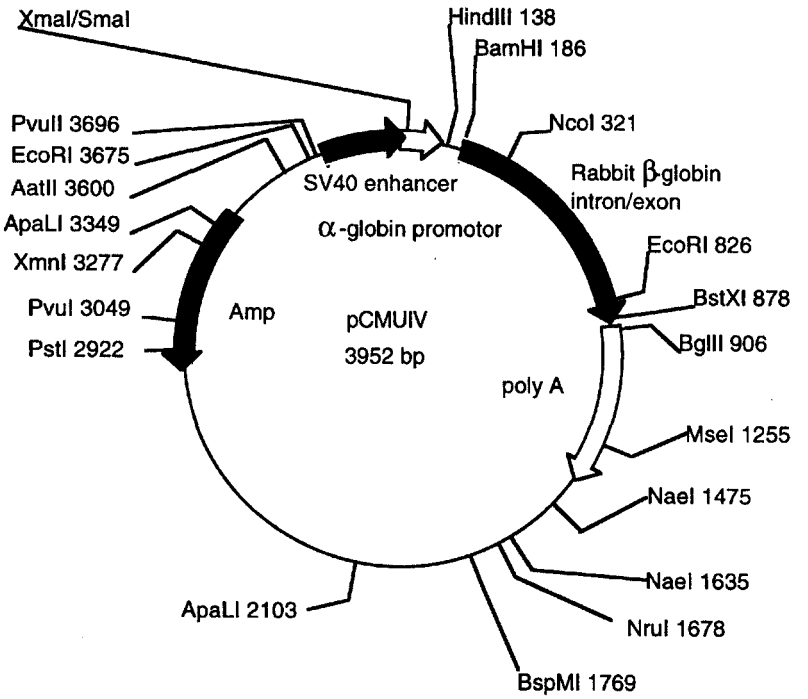

Figure 1. pCMUIV has been designed for high levels of expression of cDNAs in mammalian cells (17). cDNAs are inserted into the *Bam*HI site. Only the coding region plus three additional bases (to form a Kozak consensus sequence (G/ACCATG..) should be used to ensure optimal expression. The inserted cDNA is under the control of the 72 bp enhancer element of SV40 which has been fused to the human α-globin promotor. The sequence between the *Hin*dIII (nt 138) and the *Bam*HI (nt 186) site encodes the 5′ untranslated region of *Xenopus laevis* β-globin. Immediately after the *Bam*HI site, a rabbit β-globin intron/exon splicing cassette ensures proper processing of the transcribed RNA and a poly(A) addition site ensures its stability as a mRNA.

rich media in the presence of ampicillin. It is non-replicating in mammalian cells. It is not commercially available but can be optained free of charge from Tommy Nilsson, Cell Biology Programme, EMBL, Heidelberg.

2.5 Plasmid pSRα

Plasmid pSRα (see *Figure 2*) has been designed for high expression levels in mammalian cells and was constructed by Yutaka Takebe. The vector is a high copy one and is grown in the presence of ampicillin. It is non-replicating in mammalian cells and is commercially available from DNAX.

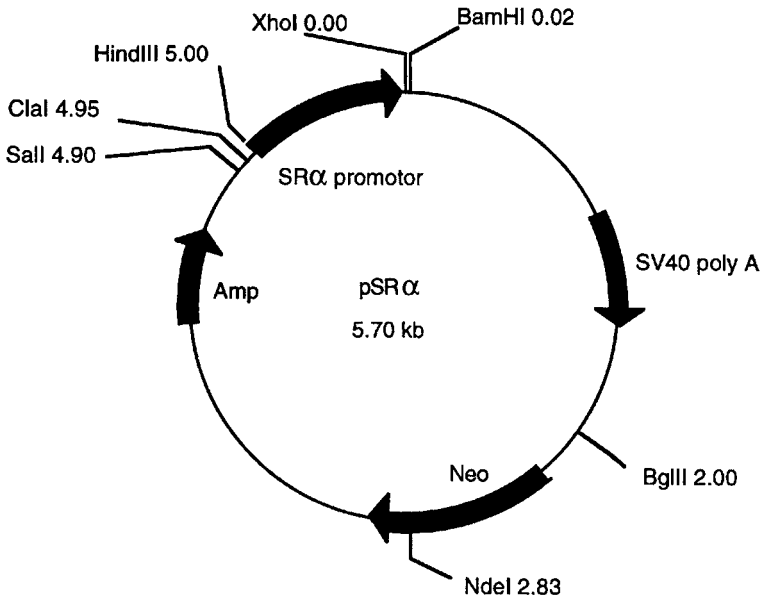

Figure 2. cDNAs are inserted into the *Xho*I or the *Bam*HI site of pSRα. Only the coding region plus three additional bases (to form a Kozak consensus sequence (G/ACCATG..) should be used to ensure optimal expression. The inserted cDNA is transcribed from the SRα promotor which is composed of an early SV40 promotor fused with an R segment and part of the U5 sequence of the long terminal repeat of HTLV-1. Downstream of the *Bam*HI site, an SV40 poly(A) addition site is present to stabilize the mRNA produced. A *neo* selection marker is present to allow for selection in the presence of G418.

3. Transient and stable transfection methods

3.1 Calcium phosphate

The most common transfection technique is the one based on the formation of calcium phosphate–DNA precipitates. This technique, ideally suited for adherent cells, was originally described by Graham and Van der Eb (18) and

traps DNA inside a calcium phosphate precipitate where it is protected from degradation. This precipitate is actively taken up by cells and transported to the nucleus where the DNA is released and transcribed. In its original form, the protocol mixed DNA and phosphate prior to addition of calcium. In later protocols (2) this is reversed; calcium is added to the DNA and is allowed to bind in excess. Limited amounts of phosphate are then added and a fine precipitate is formed.

There are several parameters to consider in order to optimize the system for high and reproducible transfection:

(a) Cells should be in the log phase of growth and at a density not exceeding 40% of the stationary phase density.

(b) It is possible to trigger cells to enter mitosis by trypsinization and it is likely that this enhances transfection efficiency. This conclusion is based on the observation that synchronized cells show enhanced transfection efficiency 6–10 h after mitosis (Nilsson, unpublished results).

(c) It is imperative that the plasmid DNA is supercoiled and of highest purity, that is, it must be devoid of any contaminating bacterial DNA. The latter has an adverse effect on protein expression in mammalian cells, whereas the former is a prerequisite for the formation of a uniform precipitate. The best way to ensure high purity and supercoiled plasmid DNA is purification on a CsCl gradient. Purification of plasmid DNA with commercially available pre-made columns is very rapid though expensive. Furthermore, these columns do not yet yield the same high purity of DNA as CsCl gradients and DNA isolated using CsCl is generally more stable at 4 °C. This is an important factor since repeated freezing and thawing nicks and linearizes plasmid DNA resulting in a dramatic decrease in transformation efficiency.

(d) Some protocols suggest the use of carrier DNA to enhance transfection. However this is not recommended unless the plasmid DNA must be diluted for experiments when lower expression levels are required. The carrier DNA should be plasmid DNA of the same quality as the expression plasmid.

(e) The precipitate should be formed under precise conditions to ensure that it is of a reproducible size. The pH should be pH 6.7 in both the calcium and phosphate mix. The concentration of the DNA and the temperature at which the precipitate is formed are also very important factors to obtain reproducible results.

Protocol 5 describes a procedure for transient transfection of mammalian cells using the calcium phosphate method. In this procedure, 20 μg plasmid DNA is used in a final volume of 640 μl. This is sufficient to transfect about 10^7 cells at an efficiency exceeding 70%.

Protocol 5. Transient transfection of mammalian cells by the calcium phosphate method

Equipment and reagents

The pH of the following solutions must be adjusted and solutions must be sterilized by passage through a 0.22 μm filter.

- Tissue culture dishes (100 mm diameter)
- Water-bath at 37 °C
- Mammalian cells of choice in logarithmic phase of growth[a]
- Plasmid DNA (20 μg)
- Calcium mixture: 0.5 M $CaCl_2$, 280 mM NaCl, 100 mM Hepes pH 6.74
- 2 × HBS: 50 mM Hepes pH 6.74, 0.75 mM Na_2HPO_4, 0.75 mM NaH_2PO_4
- 10 × TBS: 250 mM Tris pH 7.4, 1.37 M NaCl, 50 mM KCl, 7 mM $CaCl_2$, 5 mM $MgCl_2$
- 15 mM Na_2HPO_4
- Dilute the 10 × TBS tenfold, adding 15 mM Na_2HPO_4 to a final concentration 0.6 mM (note: these concentrated solutions are made for practical reasons, since it is difficult to make the required dilute solutions from dry reagents, and because the solutions have to be sterilized separately)
- Growth medium: DMEM containing 10% FCS
- TE buffer: 10 mM Tris–HCl pH 7, 1 mM EDTA

Method

1. Day 0. Split the cells into tissue culture dishes (100 mm diameter), aiming for 30–40% confluency. Add 10 ml growth medium per plate.
2. Day 1. Pre-warm all solutions to 37 °C.
   (a) Prior to use, add 20 μg DNA to a final volume of 160 μl of 0.1 TE buffer.
   (b) Incubate in water-bath for 5 min at 37 °C.
   (c) Add 160 μl calcium mixture. Mix carefully and leave in the 37 °C water-bath for 10 min.
   (d) Add 320 μl of 2 × HBS and leave for 15 min in the 37 °C water-bath. When adding the 2 × HBS, put the tube on the vortex at full speed, add the 2 × HBS slowly, and then vortex four or five times. During the 15 min incubation do not rock or move the tubes. A calcium phosphate–DNA precipitate now forms.
   (e) Add the calcium phosphate–DNA precipitate to the cells and mix carefully. Incubate at 37 °C and 5% CO_2.
3. Day 2.
   (a) Look at the cells after 16–20 h. Note the fine precipitate which should be moving by Brownian motion. Pour off the medium and wash the cells two or three times with 1 × TBS or until all of the precipitate has been removed.
   (b) Add fresh medium to the cells. Incubate at 37 °C.
4. Day 3–4. The cells are now ready for assaying, as described in Section 4.

[a] Not all cell lines are transfectable by calcium phosphate; this is particularly true for lymphoid cell lines.

Variations of *Protocol 5* can be found throughout the literature. These include passing CO_2 through the culture medium following the addition of the calcium phosphate–DNA precipitate. Presumably, this serves to lower the pH but it is a rather crude and inconsistent alternative to having the right pH to start with. Cells in suspension can also be transfected sometimes with higher efficiencies than those obtained using adherent cells. Simply resuspend 10^6 to 10^7 cells in the transfection mixture containing the preformed precipitate and incubate for 10–15 min at room temperature. Then add growth medium and plate out the cells. Incubate for 16–20 h at 37 °C, and then wash as in *Protocol 5*, step 3.

3.2 DEAE–dextran

Another transfection procedure is to bind the DNA to DEAE–dextran and then for cells to internalize this via endocytosis. This method (*Protocol 6*) is a common alternative to the calcium phosphate technique and is easy, inexpensive, and quick. Good results are achieved when performing transient transfections but reports from several research groups show that stable transfection is less successful when using this method. Transfection with DEAE–dextran was first described by McCutchan and Pagano (19) and later modified and improved by different groups (20–23). These modifications often include a shock treatment of the cells following the transfection, using 10% DMSO, 15% glycerol, or 10% polyethylene glycol (PEG). The period for the shock treatment is usually very short, 1–2 min. Lengthening this period will increase the transfection efficiency but will also decrease cell viability (22). Many protocols include chloroquine in the transfection mix to prevent lysosomal degradation of the DNA/DEAE–dextran complex.

Protocol 6. Transient transfection of mammalian cells using DEAE–dextran

Equipment and reagents

- Mammalian cells in logarithmic phase of growth
- Pure plasmid DNA (1–5 μg per 100 mm dish of cells)
- PBS (see *Protocol 1*)
- 10 mg/ml DEAE–dextran in PBS
- 10 mM chloroquine in PBS
- 1% DMSO in PBS
- Growth medium containing 10% FCS

Method

1. Add the desired amount of DNA (1–10 μg/ml) to one volume of the growth medium supplemented with 10% FCS.
2. Dilute the DEAE–dextran in growth medium with 10% FCS to a final concentration of 100 μg/ml.
3. Pre-warm equal volumes of the DNA solution and the DEAE–dextran solution to 37 °C, then mix these together, and vortex to mix.

Protocol 6. *Continued*

4. Add 10 mM chloroquine in PBS to the mixture to a final concentration of 100 μM.
5. Pour the culture medium off the cells and wash them with PBS.
6. Pour off the PBS and add the DNA/DEAE–dextran mixture containing the chloroquine. Incubate at 37 °C for 4 h.
7. Aspirate the medium and add 1% DMSO in PBS for 1 min maximum. Wash away the DMSO with PBS (twice).
8. Add fresh growth medium containing 10% FCS to the cells and incubate them at 37 °C for two to three days before assaying.

3.3 Lipid-mediated transfection

Since Felgner and co-workers (24) developed a highly reproducible and quick liposome method, based upon a synthetic cationic lipid called DOTMA (*N*-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethyl ammonium chloride), many laboratories and (bio)chemical companies have synthesized similar structures to transfect DNA into a variety of cell lines. The method is not based on encapsulation of DNA as might be assumed, but rather on positive/negative charge interactions as in the DEAE technique. Positively charged lipids such as DOTMA or DDAB (dimethyldioctadecyl ammonium bromide) bind to the negatively charged DNA. In general, several liposomes bind to one DNA molecule. The entire DNA/liposome complex then binds to the cell and is endocytosed. A suitable procedure is described in *Protocol 7*. As with the DEAE–dextran method, chloroquine may be included in the transfection mixture to prevent degradation of the DNA. Although cationic lipid is an efficient transfection medium, it is also toxic to cells but this can be overcome by incorporating other positively charged molecules such as the cholesterol analogue (3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol) into liposomes. Note that since most lipids have overlapping fluorescence spectra with GFP, it is not recommended to use this transfection procedure when GFP is the reporter gene for expression.

Protocol 7. Transient transfection of mammalian cells using liposomes

Equipment and reagents

- Mammalian cells in logarithmic phase of growth
- Plasmid DNA (1–10 μg)
- Hepes buffer: 150 mM NaCl, 20 mM Hepes pH 7.4
- Lipid solutions (Lipofectin or Transfectase from Gibco BRL, DOTAP from Avanti Polar Lipids or Boehringer Mannheim, or DDAB from Sigma)
- Growth medium containing 10% FCS

Method

1. Grow the cells to 80% confluency. Remove the medium and then wash the cells with Hepes buffer.
2. Add 1–10 μg DNA to 1.5–2 ml Hepes buffer. Next add the cationic lipid (about 5 μg lipid solution per μg DNA) and mix. Incubate for 5 min at 37 °C to allow the binding of DNA and lipid.
3. Add the lipid/DNA mixture to the cells and incubate for 3–5 h at 37 °C.
4. Add another 10 ml growth medium containing 10% FCS to the cells and incubate overnight (16–20 h).
5. Replace the medium with fresh growth medium containing 10% FCS and leave for two to three days before assaying.

3.4 Electroporation

Biomembranes are transiently permeable when submitted to short electrical pulses and so take up extracellular molecules. This method (electroporation) was first reported by Neumann and collaborators (25) and has since been optimized and fine-tuned for many different cell lines (1, 2, 26–28). Some authors claim high transfection efficiency up to 100% whereas others state that 50% mortality of the cells is quite common (1, 26, 28). Generally, DNA or RNA (10–50 μg) is transferred to a calcium- and magnesium-free buffer (PBS) and mixed with cells (10^6 to 10^7 cells) in a cuvette with two small metal plates on the inside of either side of the cuvette. The strength of the electrical field and duration of the pulse(s) largely determine the efficiency of the electroporation. The voltage applied can vary between 100 V to 2000 V. To prevent any unnecessary damage to the cells, high voltage is used only in combination with short pulses and low voltage with longer pulses. The pulse length used varies from a few μsec to 10–20 msec. The duration of the pulse is controlled with the electroporation device and should be around 25–30 μF. It is important to minimize salt concentrations in the sample as much as possible to avoid exploding the sample during electroporation. Some investigators have argued that several short pulses improve the efficiency of transfection (29). *Protocol 8* describes the electroporation of DNA into mammalian cells but the voltage used and/or duration of the pulse should be optimized for the particular cell line under study.

Protocol 8. Transient transfection of mammalian cells by electroporation

Equipment and reagents

- Rubber policeman
- Electroporation cuvettes (0.4 cm)
- Electroporation equipment, e.g. Gene-pulser (Bio-Rad)

Protocol 8. *Continued*

- Mammalian cells in logarithmic phase of growth
- Growth medium supplemented with 10% FCS
- DNA (1–5 μg)
- PBS (see *Protocol 1*)
- Trypsin/EDTA: 0.5 mg/ml trypsin, 0.2 mg/ml EDTA

Method

1. Grow the cells to 80% confluency. Then pour off the growth medium and wash the cells with PBS.
2. Scrape the cells off the tissue culture dish using a rubber policeman or detach the cells with trypsin/EDTA.
3. Centrifuge the cells at 400 *g* for 5 min.
4. Wash the cells with PBS by centrifugation (400 *g* for 5 min).
5. Resuspend the cells in 750 μl PBS at a density of 10^7 cells/ml and add 1–5 μg DNA. The final volume should not exceed 800 μl.
6. Add the mixture to the electroporation cuvette and mix well by pipetting up and down.
7. Pulse twice at 850 V/25 μF (the time constant should be 0.4).
8. Dilute the cells 20–25 times with growth medium containing 10% FCS.
9. Incubate the cells at 37 °C for two to three days before assaying.

3.5 Microinjection

Microinjection, first developed by Graessman (30), is one of the most elegant, efficient, and reliable methods available for transfection of DNA or RNA. Perhaps most importantly, it is also possible to transfect large DNA molecules efficiently (up to 500 kb) (31). However, microinjection requires expensive equipment and some skill. Whereas DNA is best injected directly into the nucleus, *in vitro* transcribed mRNA is injected directly into the cytoplasm where it is translated with an efficiency exceeding 50%. The DNA integrates into the genomic DNA of the host cell in a 'head-to-tail' concatamer and remains stable even in the absence of selection. Furthermore, the frequency of stable integration upon microinjection well exceeds those of conventional transfection methods. Usually, 0.1–20% of microinjected cells stably integrate the linearized plasmid as compared to 1 in 10^6 cells for other methods. The microinjection procedure is described in *Protocol 9*. The DNA or RNA to be injected is usually dissolved in a buffer lacking calcium and magnesium and then mixed with a fluorescent marker such as FITC–dextran or Cascade Blue albumin to visualize and monitor the injected cells. Microinjection into the nucleus or cytoplasm is performed with glass capillary needles containing very small volumes (1–2 μl) of the DNA or RNA solution. These microglass needles are easily pulled starting with glass capillaries and using a more or less auto-

mated pulling device. Within seconds two identical glass needles can be made with a tip diameter ranging from 0.2–1 μm. The glass needle is inserted into a microinjector which is available from different companies (e.g. Eppendorf) and easily mounted onto a light microscope. Pressure controlled microinjectors have shown highly reproducible results with respect to the injected volumes, 0.01 picolitres for the nucleus and 0.1 picolitres for the cytoplasm (32). The needles are manoeuvred using a micromanipulator (Eppendorf). All devices (manipulator, injector, inverted fluorescence microscope, and video camera) can be computer controlled. Such an automated (micro)injection system, AIS, was developed at EMBL by the group of Ansorge and can be obtained commercially from Zeiss. With training, more than 1000 cells an hour can be microinjected using AIS. A second manipulator may be desirable if injecting oocytes and embryos as well as non-adherent cells (33, 34).

Protocol 9. Microinjection of DNA or RNA into cells

Equipment and reagents

- Inverted fluorescence microscope: Axiovert 100 TV (Zeiss) optimally fitted with a video camera (Hamamatsu)
- Microinjector (Eppendorf)
- Micromanipulator (Zeiss or Eppendorf)
- Glass capillary puller (Sutter Instruments)
- Glass capillaries (Clark Electromedical Instruments)
- Mammalian cells in tissue culture plates
- 25–100 μg/ml DNA or 1 mg/ml RNA solution in PBS (lacking Ca^{2+} or Mg^{2+})
- 5% FITC–dextran (≥ 10000 Da, Sigma) in PBS, or 5% Cascade Blue albumin (Molecular Probes) in PBS
- Growth medium supplemented with 10% FCS

Method

1. Grow cells to 40–50% confluency, and then replace the growth medium with fresh medium before microinjection.
2. Mix the DNA or RNA solution 1:1 with 5% FITC–dextran in PBS.
3. Mount the culture dish firmly in place on the microscope.
4. Take up a small volume (max. 1 μl) into the micropipette and transfer this to the rear open end of a glass capillary needle.
5. Insert the capillary needle into the holder and (most importantly) quickly lower the capillary to the growth medium in the culture dish so that the small volume does not dry out. Make sure that back pressure does not allow medium to be sucked up into the capillary thereby mixing the DNA with the medium. If this happens, it will increase the volume to be injected and decrease the concentration of the DNA solution.
6. Using a low magnification lens (× 10 or × 20), focus above the cells. Bring the place where the capillary enters the medium into focus. A ring will appear in the field. Bring the tip into focus. Follow the tip downwards until the cell is in focus.

Protocol 9. *Continued*

7. Use a higher magnification and repeat step 6.
8. Repeat this a few times in order to reach the maximal magnification, usually × 300–500.
9. Record the Z-limits as specified by the microinjection system. These are the injection level and the panning level.
10. Select the cells and proceed with the injection. A positive pressure is best maintained by allowing the sample to slowly leave the capillary during the process. A successful injection leaves a small field where the capillary deposited the sample.

3.6 Stable transfection and selection

In order to establish a stable cell line expressing the protein of interest, it is convenient to co-transfect using a plasmid carrying a selection marker together with the plasmid containing the relevant cDNA (*Protocol 10*). This usually results in a high frequency of positive clones and negates the need for additional subcloning. Several different selection markers exist but the most widely used ones are neo, hyg, and gpt. These are dominant selection markers encoded by *E. coli* genes (*hyg* and *gpt*) or by a bacterial transposon (*neo*) which upon transfection render the expressing cells resistant to specific antibiotics or mycophenolic acid. Both *neo* and *hyg* can be used simultaneously as selection markers allowing for co-transfection studies. Clearly, when selecting for stable clones, it is important not to start the selection until the respective selection marker is expressed phenotypically.

Protocol 10. Stable transfection and selection of mammalian cells

Equipment and reagents

- 24-well and 6-well microtitre plates plus coverslips
- Cloning rings (optional) made by cutting off small slices from plastic tubing
- Mammalian cells in logarithmic phase of growth
- Plasmid containing a cDNA insert encoding the protein of interest
- Plasmid containing *neo* gene
- Growth medium: DMEM containing 10% FCS
- Geneticin or G418: 0.4 mg/ml final concentration (Gibco BRL)
- Trypsin/EDTA: 0.5 mg/ml trypsin, 0.2 mg/ml EDTA

Method

1. Co-transfect the cells with the plasmids containing the selection marker and the relevant cDNA according to one of the previous protocols (*Protocols 5–9*).

2. Following transfection, incubate the cells for at least 24 h at 37°C in growth medium. If needed, split the cells to a final confluency of 30%.
3. Add growth medium containing the appropriate selection agent (e.g. 0.4 mg/ml G418 for selection of *neo* expressing cells) and incubate for three days. Replace the medium every three days in the first week and every five days for another two weeks with fresh medium containing the selection marker. Take care not to dislodge cells during the medium changes since this results in secondary colonies.
4. After two to three weeks, the cells will either have formed a monolayer or colonies which should be clearly visible. In the case of the former, a gentle tap releases dead cells and these are then washed off during the medium change (resistant cells will need additional time to grow until they are ready for isolation). Usually, colonies or foci or colony should be 1–2 mm in diameter when they are picked. If the colony has a coloured (e.g. red) centre, the cells have been incubated too long and must be picked immediately or else discarded.
5. Picking the colonies or foci is the most laborious part of the procedure and is also the point where the risk of cross-contamination is highest. Two alternative procedures are available.
   (a) Stick a cloning ring onto the dish using grease so that the colony is at its centre. Then fill the cloning ring with trypsin/EDTA and, when the cells have detached, transfer them to the well of a microtitre dish for expansion.
   (b) An alternative and quicker method is to pick each colony directly off the dish using a glass capillary. Use a 50 µl disposable capillary to scrape the colony off the dish and allow it to float. Then suck up the colony into the capillary and transfer it to a well of a 24-well plate containing 300 µl trypsin/EDTA per well. After 10 min, add 2 ml culture medium and resuspend the colony using a Pasteur pipette. This procedure allows for the isolation of 48 foci per hour.
6. At some point, it may be necessary to subclone the stable cell line. This is done by diluting the cells to 10 cells/ml and adding 100 µl per well into a 96-well plate. Those wells in which growth occurs will contain clones that arose from a single cell and hence represent subclones of the original stable cell line.

Established cell lines are best maintained in the presence of the selection agent. It is not advisable to culture cell lines longer than 20–30 passages. Therefore, freezing cells at an early stage is recommended, to act as stocks that can be thawed and cultured in future as required.

3.7 Inducible protein expression in stable cell lines

3.7.1 Tetracycline-induced system

For some purposes, it may be required to express proteins under controlled (inducible) conditions. This may be to obtain a phenotypic dose-dependent response or to avoid negative effects on the host cell by the expressed protein. Recently, a tetracycline-inducible system has been developed which offers advantages over other systems, particularly the very low basal level of expression and non-toxicity for the host cells. The tetracycline-inducible system was originally developed by Bujard and co-workers (35), has been modified (36) so that the key regulatory molecule, the transactivator (tTA), is inducible as well as the expressed protein of interest. This is important since the tTA itself is toxic for cells at certain levels. Briefly, the tTA is a fusion protein consisting of a bacterial (*E. coli*) tetracycline repressor fused to the C-terminal part of herpes simplex virus VP16. The tTA is expressed on a separate plasmid (pTet-tTAk) and several cell lines have now been established (e.g. HeLa) which can be used for transfection of the second plasmid (pTet-Splice) expressing the gene of interest. When tetracycline is present in the culture medium, it binds to tTA and prevents binding to the *tet* resistance promotor (a hybrid between the tetracycline resistance operator and a CMV promotor). Since both the tTA and the gene of interest are under the regulation of the *tet* resistance promotor, not only is expression of the gene of interest blocked but also very little tTA is produced, circumventing any toxic effects which may otherwise be exerted by this protein on the cell. When tetracycline is removed, the small basal level of tTA ensures a rapid and massive production of tTA in an autoregulatory manner as well as rapid expression of the gene of interest in the second plasmid. The key characteristics of low basal expression and high inducibility make this system by far the best currently available. *Protocol 11* describes the use of the tetracycline system. Expression of the gene of interest upon removal of tetracycline is very rapid but the optimal time should be determined for each protein of interest.

Protocol 11. Protein expression in stably transfected cells using a tetracycline-inducible expression system

Equipment and reagents

- Equipment and reagents for transfection (see *Protocols 5–9* as appropriate)
- Equipment and reagents for picking colonies/foci (see *Protocol 10*)
- Cell line: HeLa, Vero, CHO
- pTet-Splice plasmid (Life Technologies) containing the gene to be expressed
- pTet-tTAk plasmid (Life Technologies) containing the tTA gene
- Plasmid containing first selection marker (e.g. *neo*)
- Plasmid containing second selection marker (e.g. *hyg*)
- 1 mg/ml tetracycline (stock solution)

Method

1. Establish the cell line stably expressing the tTA. To do this, co-transfect the cells (using one of the procedures described in *Protocols 5–9*) with a ratio 1:5–1:10 of pTet-tTAk to the plasmid containing the first selection marker (e.g. *neo*).
2. Select for co-transfectants by incubating the cells in growth medium containing 0.5–1 μg/ml tetracycline plus the appropriate selection agent (e.g. 0.4 mg/ml G418 for *neo*-expressing cells).
3. Grow the cells for two to three weeks under this selection pressure and then pick colonies/foci as described in *Protocol 10.*
4. Screen the clones for their ability to both activate and express tTA. This is important since some clones may express tTA in a non-regulated manner and must be discarded. Two approaches can be used to screen the cells: either PCR for the tTA mRNA or Western blotting for tTA protein.
5. Expand and plate the selected clones.
6. Co-transfect the recombinant pTet-Splice plasmid, containing the gene of interest, and a second plasmid bearing a selection marker different from that used initially (e.g. *hyg*).[a]
7. Grow the cells for two to three weeks under the appropriate selection pressure (e.g. with 0.4 mg/ml hygromycin) and then pick colonies/foci as described in *Protocol 10.*
8. Screen the selected clones both for their ability to activate and express tTA as well as the expression and inducibility of the gene of interest.

[a] *Immediately following transfection (48–72 h) a portion of the cells can be tested for expression and inducibility.*

Expression of the gene of interest upon removal of the tetracycline is very rapid but the optimal time (e.g. 12–20 h) should be determined for each protein of interest.

3.7.2 *Lac* promotor

In the *E. coli lac* operon, the lac repressor binds to the *lac* operator switching off transcription of the *lacZ* gene. The action of the repressor is impaired by the addition of IPTG so that the addition of IPTG results in transcriptional activation of any downstream cDNA. This feature can be utilized for expression in mammalian cells. First, an expression vector encoding the *lac* repressor is introduced into the cells so that it is stably expressed. A 'mother' cell line is then selected for and this is transfected with the plasmid encoding the cDNA of interest under the control of the *lac* operator. A stable cell line expressing the desired cDNA is now selected for. However, expression will be obtained

only upon the addition of IPTG to the culture medium. The induction is quick (around 4 h). Commercial systems (e.g. LacSwitch™ II from Stratagene) have improved expression by adapting the prokaryotic components of the system for use in mammalian cells.

3.7.3 Use of sodium butyrate

The addition of sodium butyrate to the culture medium immediately following transfection (37), for example upon removal of the calcium phosphate–DNA precipitate, allows for stable integration of transfected DNA into regions of the genome which are relatively inactive. Removal of the sodium butyrate 24 hours post-transfection will in some cases silence the introduced gene which is then activated by the re-addition of the sodium butyrate. This is a straightforward way to establish stable cell lines although the inducibility is normally only two- to fivefold. One should also be aware that the addition of sodium butyrate to cells in general will switch on multiple genes, thus possibly causing phenotypical changes.

4. Detection of expressed protein

4.1 GFP as a tool in protein expression

The green fluorescent protein (GFP) can be excited with wavelengths ranging between 395 nm and 475 nm and the molecule then emits light at a wavelength around 508 nm. By fusing GFP to molecules of interest, hybrid molecules can be examined directly in live cells. The original GFP from Chalfie *et al.* (38) has been altered extensively so that expression and visualization of GFP hybrid proteins has become routine. Initial reports suggested that GFP could not be expressed in the lumenal space of many organelles (e.g. Golgi) and that GFP itself was toxic for cells in general. However several groups have now successfully expressed GFP both as cytosolic or lumenal fusion proteins in stable cell lines without noticeable effects on both the GFP or the viability of the cell. For most purposes, GFP is fused to the molecule of interest so that it either precedes or terminates the fusion construct. GFP variants have been produced by point mutation and have excitation/emission spectra that have shifted to the blue, yellow, or the red region of the spectrum to produce blue fluorescent protein (BFP), yellow fluorescent protein, and red fluorescent protein. Whereas the former is truly blue, the latter is only shifted towards the red. Nevertheless, these variants can, with appropriate filters, be used simultaneously to reveal different hybrid molecules (39). For practical purposes, though, in terms of stability and brightness, GFP is by far better and easier to use (BFP bleaches about 100 times faster than GFP). Although GFP is easily revealed in fixed cells, its expression and detection in living cells allow for a rapid and convenient way to select for stable cell lines. Two weeks following transfection and selection, the cell population can be subjected to

fluorescence activated cell sorting (FACS) (40) to enrich the GFP expressing population. This not only permits their isolation but also the selection of populations expressing GFP hybrid molecules at different levels. Isolated cells can then be expanded and examined. If required, the cell population can be resorted until it is sufficiently homogeneous.

4.2 Epitope tags

The insertion of epitope tags such as VSV-G, myc, or HA into the coding region of the gene to be expressed allows for rapid and convenient detection of the protein. These tags are inserted in the gene so that they are expressed either at the N-terminus, internally, or at the extreme C-terminus of the protein. If placed at one of the termini of the protein, the tag should be separated from the protein by a spacer of one or two glycine or proline residues, to provide a flexible separation of the tag from the rest of the protein. The expressed protein can then be detected using an antibody directed against the tag; both monoclonal (anti-myc, anti-HA, anti-VSV-G) and polyclonal antibodies exist for these tags. In all cases, these antibodies work well by immunofluorescence, electron microscopy, precipitation, and Western blotting, and are available commercially. The most convenient way to detect the expressed proteins is by immunofluorescence (*Protocol 12*) or, if the protein is secreted, by Western blotting (see Chapter 8). It is important to follow the time course of expression to determine when protein expression is optimal and this depends much on the choice of promotor. Usually, optimal expression is achieved 24–48 hours following transient transfection or 6–24 hours following induction. *Protocol 12* is based on refs 41–43 and describes two alternative fixation protocols. Antibodies, both primary and secondary, can have different responses depending on the fixation procedure used and hence each antibody must be tested for optimal use.

Protocol 12. Indirect immunofluorescence

Equipment and reagents

- Glass slides, coverslips, 6-well microtitre plates
- Mammalian cells expressing the protein of interest
- Primary antibody to the protein of interest or to the relevant epitope tag. Prepare the antibody as follows: centrifuge the antibody for 10 min at full speed in a microcentrifuge (4°C). Dilute the antibody in PBS/0.2% gelatin and store on ice.
- Secondary antibody conjugated to FITC (TAGO Inc.) or Texas Red (Vector Lab): prepare the secondary antibody for use as described for the primary antibody
- PBS (see *Protocol 1*)
- 3% (w/v) formaldehyde solution: add 15 g *p*-formaldehyde to 500 ml PBS. Heat the solution to 92°C. (Do this in the fume-hood and wear protective clothing and gloves. Pay careful attention to the temperature.) Once the formaldehyde has dissolved, allow the solution to cool to room temperature, then add 0.5 ml of 0.5 M $CaCl_2$ and 0.5 ml of 0.5 M $MgCl_2$. Store in small aliquots at −20°C. When thawed, filter the solution through a 0.22 μm filter to remove any aggregates.
- 50 mM NH_4Cl in PBS: dissolve 0.156 g NH_4Cl in 50 ml PBS
- 0.1% Triton X-100 in PBS: make up fresh each time

Protocol 12. *Continued*

- Moviol mounting medium: place 6 g analytical grade glycerol in a 50 ml Falcon tube with 2.4 g Moviol 4-88 (Hoechst). Stir well until mixed (but not dissolved). Add 6 ml water and leave the suspension for 2 h at room temperature. Add 12 ml of 0.2 M Tris–HCl pH 8.5 and incubate at 50°C for 10 min with occasional stirring to dissolve the Moviol. Remove insoluble material by centrifugation at 5000 *g* for 15 min.
- PBS/0.2% gelatin: dissolve 5 g fish skin gelatin (Sigma) in 100 ml PBS. Dilute to 1 litre in PBS. Filter the final solution through a 0.22 μm filter. Store at 4°C.

A. *Formaldehyde/Triton X-100 fixation*

1. Seed the cells onto glass coverslips in a 6-well microtitre plate and allow them to grow for two days to about 75% confluency.
2. Remove the growth medium. Add 2 ml of 3% formaldehyde solution and incubate for 20 min at room temperature to fix the cells. After the cells have been treated with formaldehyde they should not be allowed to dry out.
3. Wash the fixed cells three times with 2 ml PBS. For each wash, aspirate the previous solution and rapidly (but carefully) add 2 ml PBS using a repeater pipette.
4. Quench aldehyde groups by washing the cells with 2 ml of 50 mM NH_4Cl in PBS for 10 min.
5. Wash the cells three times with 2 ml PBS each time.
6. Place the coverslips in 2 ml of 0.1% Triton X-100 in PBS for 4 min.
7. Wash the cells three times with 2 ml PBS each time.
8. Wash the cells three times with PBS/0.2% gelatin over 5 min.
9. Wash the cells three times with 2 ml PBS over 5 min.
10. Incubate the cells with the specific (primary) antibody as follows:
    (a) Place a few drops of water on the bench and place Parafilm on top. Press away excess water with a tissue. This creates a flat level hydrophobic surface.
    (b) Place 100 μl antibody solution on the Parafilm.
    (c) Remove excess liquid from the coverslip by touching the edge of the coverslip onto tissue paper.
    (d) Carefully place the coverslip, cell side down, onto the drop of antibody solution.
    (e) Place a cover (e.g. the cover of a 6-well microtitre plate) on top to prevent drying. Incubate for 20 min at room temperature.
11. Return the coverslips to the wells of the microtitre plate containing 2 ml fresh PBS and wash three times with PBS/0.2% gelatin over 5 min.
12. Wash the cells twice with 2 ml PBS over 5 min.

13. Incubate the cells with the second antibody, following the same procedure as described in step 10.
14. Wash the cells twice with 2 ml PBS/0.2% gelatin over 5 min.
15. Wash the cells twice with 2 ml PBS over 5 min.
16. Mount the coverslips onto slides as follows. Wash the coverslip in water then dry off by touching the edges onto tissue paper. Mount each coverslip onto a slide using a 25 μl drop of Moviol mounting medium. Then place a double layer of tissue paper on top of the coverslip together with a small light weight (e.g. the cover of the 6-well plate) to blot excess Moviol from the slide. Leave for 2–3 h to allow the Moviol to set. Then store the slide at 4°C.

B. *Methanol/acetone fixation*

1. Place two glass Petri dishes, one containing methanol and the other acetone into a –20°C freezer or onto an aluminium sheet on dry ice.
2. Transfer the coverslips bearing the cells directly from the growth medium to the methanol (cell side up) for 4 min.
3. Transfer the coverslips to acetone for a further 4 min.
4. Air dry.
5. Continue as from part A, step 7.

The precise intracellular location of the expressed protein can be determined by electron microscopy (EM) on thin frozen sections. A detailed procedure for this has been described recently by Rabouille (43). When using this method, it is important that homogeneous protein expression is occurring throughout the cell population since, at this level of resolution, the overall picture of protein localization in the population is not determined. A combination of light microscopy, immuno-EM and, perhaps, subcellular fractionation will localize the expressed protein accurately within the cell, at steady state. Still more information can be gained through metabolic labelling of the expressed protein followed by immunoprecipitation and SDS–PAGE. This will allow quantification of the expression and monitoring of the time course of expression. It may also allow determination of the kinetics of secretion of the protein and whether it is modified post-translationally (e.g. glycosylation).

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2

Expression in *Xenopus* oocytes and cell-free extracts

GLENN M. MATTHEWS

1. Introduction

Adult female *Xenopus laevis* carry approximately 40 000 oocytes at various stages of development. During growth, oocytes accumulate cellular components which carry the developing embryo through many cell cycles. Although much of the content of the fully grown oocyte consists of yolk proteins absorbed from the maternal circulation, there is also a large reserve of translational components required for the high level of protein synthesis which occurs during the period immediately after fertilization. The oocyte also contains a store of each of the three RNA polymerases sufficient to support zygotic transcription up to the 30 000 cell stage (1).

To accommodate these stores, the oocyte grows to a diameter of > 1 mm. This combination of size and protein synthetic capacity has allowed its use as an expression system to translate proteins encoded on microinjected nucleic acids (2–4). The same properties of the *Xenopus* egg have also been exploited to prepare a cell-free translation extract which efficiently translocates secretory and membrane proteins into membrane compartments derived from the endoplasmic reticulum (5).

1.1 Translation in oocytes

Many *in vivo* expression experiments are now conducted using cultured eukaryotic cells. Although this approach has many benefits, microinjection of mRNA into *Xenopus* oocytes provides a simple system which enables many sequences to be analysed in a single experiment. Oocytes are less prone than cultured cells to microbial contamination in culture and relatively few cells need to be analysed per experimental procedure. Since oocytes are in meiotic arrest, it is possible to express proteins which interfere with cell growth or division. Oocytes have been used extensively for electrophysiological studies, exploiting the ease of manipulation, and the endogenous electrophysiological responses have, therefore, been characterized extensively (6, 7).

Oocytes synthesize about 20 ng of protein per hour (8) and microinjected mRNAs compete with the endogenous pool (9), directing up to 10% of translation. Since most mRNAs are stable after microinjection and will continue to translate for a number of days in culture, this enables sufficient recombinant protein to be produced from a single cell for functional analysis.

1.2 *Xenopus* egg extracts

Translation *in vitro*, using rabbit reticulocyte lysate (10) or wheat germ extract (11), has proven a useful technique not only for the analysis of translation products but also for the study of the process of translation. However, both of these translation systems lack membrane components, and so do not post-translationally modify secretory and membrane proteins. Addition of canine pancreatic membranes (12) to these systems can reconstitute primary events such as signal sequence cleavage and *N*-glycosylation, but the quality and fidelity of these events can be highly variable unless conditions are optimized carefully for each experiment. In practice, this precludes the use of these systems for the early analysis of newly isolated sequences.

Centrifugal lysates of *Xenopus* eggs have been used for a variety of purposes including studies of the cell cycle (13) and DNA replication (14, 15). A key feature of these preparations is the high concentration of cytoplasmic components, including the translational apparatus and the membrane compartments of the secretory pathway. Consequently, these extracts show both high translational activity (200 μg protein/ml/h from endogenous mRNA) and efficient translocation of secretory proteins into membrane vesicles derived from the endoplasmic reticulum where they undergo a range of primary post-translational modifications. Extracts can be rendered mRNA-dependent by treatment with RNase A and frozen for storage (5).

In addition to quantitative signal sequence cleavage, *N*-linked glycosylation, accurate folding, and the assembly of multimers, appropriate secretory proteins also undergo some *O*-linked glycosylation and mannose-6-phosphorylation. Since these activities are associated with the *cis*-Golgi, it is possible that some transport can occur, although endoglycosidase H resistance has not been observed, indicating that proteins do not experience a medial Golgi environment.

The methionine pool of the *Xenopus* egg extract is approximately 35 μM which enables most individual translation products to be detected by overnight autoradiography after translation in the presence of 1 mCi/ml [^{35}S]methionine. Products are, however, labelled to a lower specific activity than in reticulocyte lysate or wheat germ extract, which have negligible methionine pools.

1.3 Maintaining *Xenopus laevis* stocks

Note that the use of animals in the laboratory is regulated by law and it is necessary to obtain the appropriate permits before animals are obtained.

Xenopus laevis can be purchased from laboratory animal suppliers as wild, laboratory-conditioned, or laboratory-bred animals. Laboratory-conditioned individuals tend to be larger and adapt to a new environment more quickly. Mature (over two years old) females should be specified when ordering.

Xenopus may be maintained under a variety of regimes. The simplest arrangement is to use plastic tanks approximately 60 cm (l) × 40 cm (w) × 25 cm (d) containing water to a depth of around 10 cm and covered with soft plastic mesh. These can be stocked with 10–15 frogs each and, ideally, should be fitted with a drain tap and water supply to facilitate water changes, which should be performed twice weekly after feeding. *Xenopus* do not require live food so can be maintained on a variety of diets including chopped beef or liver and maggots, but specifically formulated pellets are also available and these are easy to store and use. The quality of the water is important. Chlorinated tapwater should not be used directly but should be dechlorinated by standing in a holding tank for 24 hours. The plumbing between this and the frog tanks should be plastic since soft water will dissolve sufficient metal from copper pipes to be toxic in the long-term. This is particularly important for the production of good quality eggs.

Maintaining a 12 hourly light/dark cycle improves consistency throughout the year but even with this and good temperature control (ideally 20°C), some seasonal variations in oocyte and egg quality are inevitable.

The maintenance of *Xenopus* is covered in greater detail in ref. 16.

2. *Xenopus* oocyte microinjection

2.1 Equipment

(a) *Stereomicroscope.* A good quality stereomicroscope with a continuously variable magnification range of × 5 to × 40, a working distance of at least 10 cm, and a good depth and width of field is required. Since an experiment may require a number of hours of work at the microscope, a comfortable location is also important.

(b) *Light source.* A fibre-optic light source gives even illumination of the working area without heating the oocytes.

(c) *Microinjector.* Although manual microinjection systems have been used widely in the past, automated injectors simplify the task greatly, improving productivity and consistency of delivery. A popular model is the Drummond 'Nanoject variable' which delivers volumes in multiples of 4.6 nl. This is equipped with a manual control box but adding a foot-operated switch leaves both hands free for manipulating the needle and oocytes during injection. The injector should be mounted at 45° on a three plane micromanipulator, such as the Brinkmann MM33, which allows 30–50 mm of movement in each plane. A typical arrangement of microscope and injector is shown in *Figure 1.*

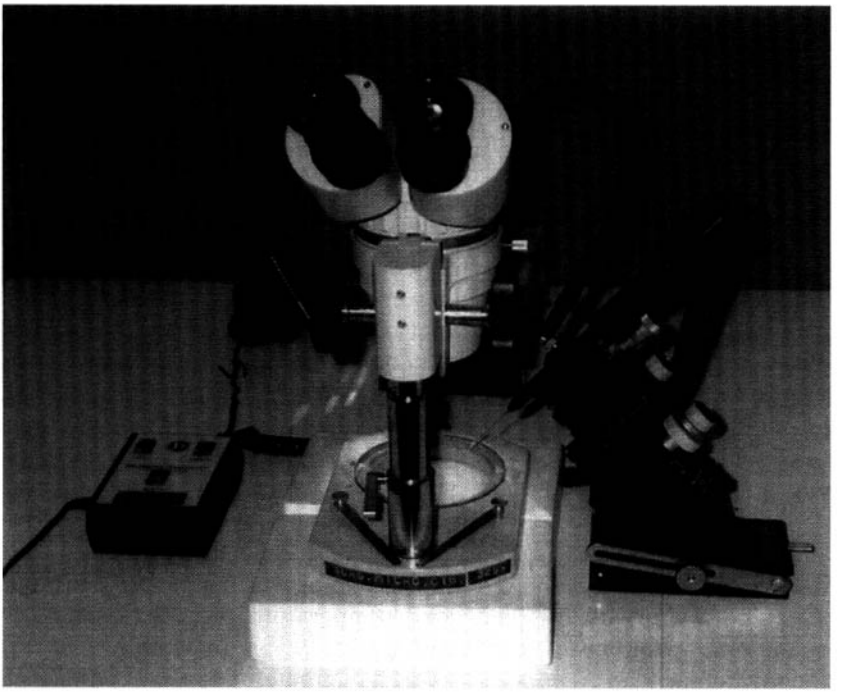

Figure 1. Microinjection equipment. The microscope and injector are shown in their normal positions. Placing the control box to the left of the microscope enables the position of the needle to be adjusted whilst filling.

(d) *Needle puller.* Many commercial micropipette pullers, such as the Narashige PN3, can be used to generate needles for automatic injectors. Since sufficient needles for many injection sessions can be produced quite rapidly, it is not strictly necessary to have constant access to a puller.

(e) *Incubator. Xenopus* oocytes should be maintained at 20°C. A small refrigerated incubator, or a normal incubator sited in a cold room, is adequate for most experiments.

2.2 Obtaining and culturing oocytes

A mature frog contains many tens of thousands of oocytes and only a few hundred would normally be required for most experiments. In the past, it was possible to operate on individual frogs many times, removing a small lobe of ovary on each occasion. This is no longer possible under UK legislation and an animal must be sacrificed for each batch of oocytes. The procedure for obtaining oocytes is described in *Protocol 1*, and *Figure 2* shows ovary dissection.

A normal ovary contains oocytes at all stages of development. Dumont (17) defined six stages from the small pre-vitellogenic (transparent) precursors to

Table 1. Preparation of modified Barth's saline (MBS)

MBS contains 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM Hepes–NaOH pH 7.6, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 U/ml penicillin, 10 µg/ml streptomycin, 50 µg/ml gentamycin, 10 U/ml nystatin. In view of the range of components, it is convenient to prepare this from concentrated stock solutions as indicated below.

High salt stock

Dissolve 128 g NaCl, 2 g KCl, 5 g $NaHCO_3$, and 89 g Hepes in 800 ml water and adjust to pH 7.6 using 1 M NaOH. Make up to 1 litre and store as 40 ml aliquots at –20 °C.

Divalent cation stock

Dissolve 1.9 g $Ca(NO_3)_2.4H_2O$, 2.25 g $CaCl_2.6H_2O$, and 5 g $MgSO_4.7H_2O$ in 1 litre of water. Store as 40 ml aliquots at –20 °C.

Antibiotics

Purchase the necessary antibiotics as solutions at 1000-fold the final working concentration (10 000 U/ml penicillin, 10 mg/ml streptomycin, 50 mg/ml gentamycin, 10 000 U/ml nystatin).

To prepare MBS

Mix, in the following order: 900 ml water, 40 ml high salt stock, 40 ml divalent cation stock, 1 ml each antibiotic stock solution. Make the volume up to 1 litre and use within two days. Store at 4 °C.

Sterile MBS

Filter MBS through a pre-rinsed 0.22 µm filter and add fresh antibiotics.

the fully-grown cell. Although all stages have sufficient translational capacity to be used for expression experiments, only oocytes at stages 5 (> 1 mm diameter) and 6 (> 1.2 mm) should be selected as these are more robust to injection and culture.

The composition of modified Barths' saline (MBS), the standard medium for isolated oocytes, is given in *Table 1*. At 20 °C in MBS, oocytes are viable for at least a week providing they do not become contaminated by micro-organisms. Oocytes can be exposed to temperatures as high as 25 °C for short periods but temperatures beyond this cause heat shock.

Protocol 1. Obtaining *Xenopus* oocytes

Equipment and reagents

- Small surgical scissors, blunt forceps, and two pairs of watchmakers' forceps
- 20 °C incubator
- Petri dishes (9 cm diameter)
- Glass Pasteur pipettes, shortened by scribing with a diamond pencil, snapping, and polishing the tip briefly in a flame, to produce an orifice of approx. 2 mm diameter

Protocol 1. *Continued*

- 2% MS-222 (anaesthetic solution): dissolve 20 g of 3-aminobenzoic acid ethyl ester (MS-222) (Sigma) in 1 litre water and use immediately
- Stereomicroscope (Section 2.1)
- Modified Barths' saline (MBS) (see *Table 1*)
- *Xenopus laevis* frog (sexually mature, female)

Method

1. Anaesthetize a frog by immersion in 2% MS-222 for 30–60 min.
2. Place the frog on its back on a bed of ice and cut open the body wall at the side of the abdomen. Avoid the mid-line, where there is a major blood vessel.
3. Remove the ovary through the incision using blunt forceps, snip off connective tissue using scissors, and transfer the ovary immediately to a Petri dish containing MBS.
4. Pith the frog to ensure that it cannot recover. Then dispose of the carcass.
5. Wash the ovary in MBS to remove blood and break it into small (pea-sized) clumps by tearing it apart with two pairs of watchmakers' forceps. A fragment of dissected ovary is shown in *Figure 2A*.
6. Working under the stereomicroscope, hold a small clump of ovary with one pair of watchmakers' forceps and gently pull off individual oocytes using a second pair. This is shown in *Figure 2B*.
7. Transfer isolated large (= 1 mm diameter) oocytes to a Petri dish containing fresh MBS using a Pasteur pipette and store them at 20°C.

2.2.1 Oocyte quality

Although oocytes can be injected immediately after isolation, mechanical damage does not become evident for the first few hours. After dissection, therefore, oocytes should be stored in MBS for at least two hours or, preferably, overnight before use. When oocytes die in culture, the uniform pigmentation of the animal hemisphere breaks up into a mottled pattern and may even disappear. This process occurs quite rapidly relative to the time scale of most experiments, allowing healthy cells to be selected for analysis. Some oocytes display small areas of pigment loss as concentric rings centred on the injection site but these are normally quite healthy.

Oocytes can be damaged mechanically during the isolation or injection processes, or can be poisoned by toxic contaminants of RNA preparations or defective media. The mRNA contaminants most likely to cause problems are residual phenol and ethanol and these can be removed easily by reprecipitating the RNA. Not all toxic RNA preparations can be rescued, however, and often the simplest solution is to prepare a fresh batch.

Some ovaries appear more fragile than others during oocyte isolation and

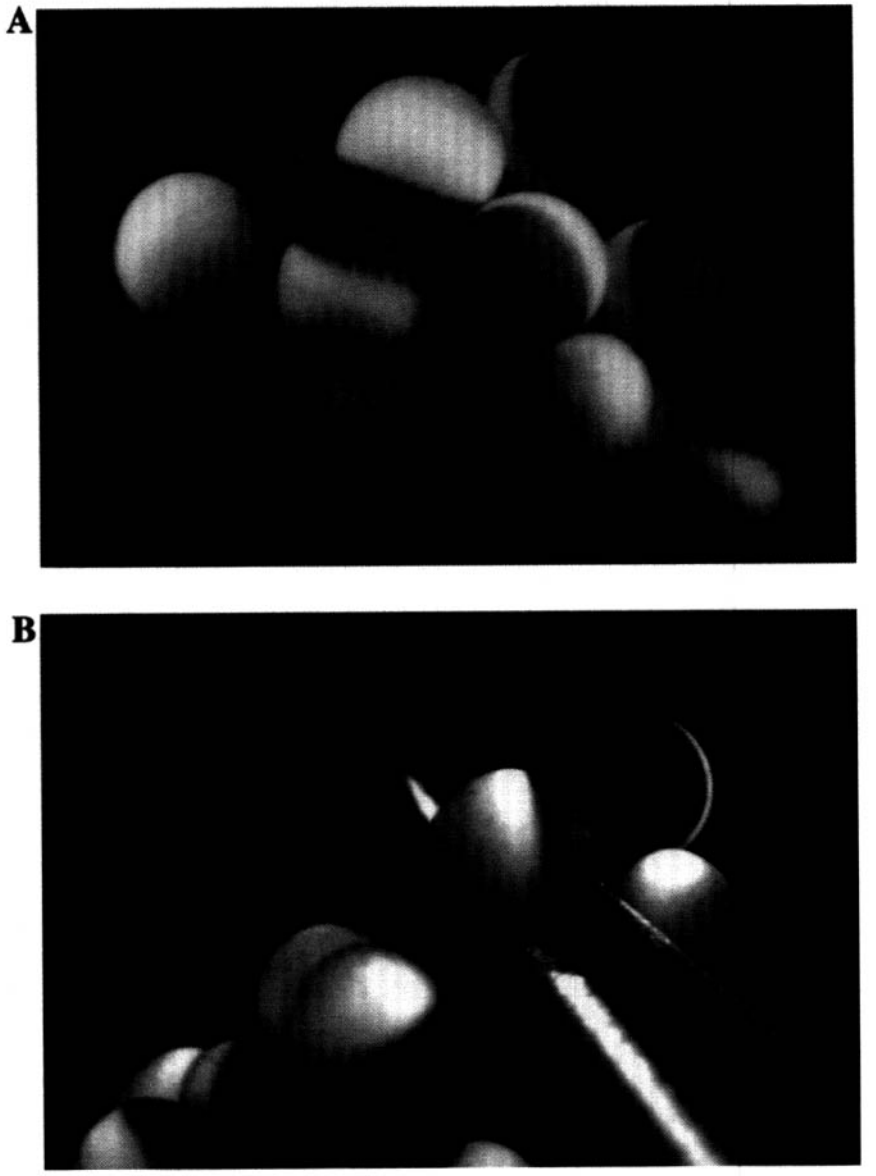

Figure 2. Ovary dissection. (A) A fragment of ovary, comprising oocytes at various stages of development. (B) The fragment is held with one pair of fine forceps (out of view) whilst individual oocytes are separated using a second pair.

these are more often prone to injection damage. This is often because the connective tissue is tougher and frequently the force required to detach oocytes causes many to burst during isolation, whilst all but the finest needles will cause injection damage. It may be productive to treat such ovaries with 200 μg/ml collagenase in MBS for 30 min at 20°C. Under these conditions, connective tissue will be softened but the ovary will not be disaggregated. Alternatively, such ovaries often improve upon storage overnight, presumably as the result of the action of endogenous matrix-degrading proteases.

When first attempting oocyte microinjection, it is advisable to inject some oocytes with a known solution, such as sterile water, to distinguish between injection damage and toxicity. A high death rate after injection with water probably indicates that the needles are too coarse. Similarly, it is always advisable to retain some uninjected oocytes. A high death rate amongst these may indicate a problem with the MBS stocks.

2.3 mRNA

2.3.1 Isolation of mRNA

The RNA to be translated may be purified from a natural source or transcribed *in vitro* from a cloned cDNA. Although total RNA can be used, most messenger RNAs are around 50 times more concentrated in the poly(A)$^+$ fraction. Isolation of poly(A)$^+$ RNA may be precluded, however, if very little RNA is available. It is also possible that the mRNA of interest may lack a poly(A) tail. Many protocols and commercial kits are available for RNA preparation and fractionation and the choice between these will be guided by the nature of the source tissue. The purified RNA should be dissolved at a minimum concentration of 1 mg/ml in sterile water.

2.3.2 *In vitro* transcription

Transcribing mRNA *in vitro* gives greater control over the activity of the final product. One strategy for obtaining a reliably high translational activity is to subclone the coding sequence of the cDNA into the plasmid pSP64T (18). This contains sequences derived from the *Xenopus* β-globin 5′ and 3′ UTRs, followed by an oligo(A) and an oligo(G) stretch, flanking the cloning site. These provide both mRNA stability and a strong translational context. When subcloning into pSP64T, it is often beneficial to remove as much of the natural untranslated regions as possible, particularly if strong start codons are present upstream of the coding sequence. If it is necessary to transcribe from other recombinant constructs, multiple restriction sites between the promotor and the translational start should be avoided, as should the use of T3 polymerase, which may not produce stable capped transcripts.

The activities of mRNAs synthesized *in vitro* vary widely, according to the sequence and the protocol used for transcription. *Protocol 2* is a simple procedure which has been proven to be effective over many experiments involv-

ing expression in oocytes, embryos, and the cell-free extract. Successful transcription depends largely upon the quality and concentration of the DNA template and can be predicted from the percentage incorporation of the radioactive tracer. The RNA from transcription reactions that incorporate significantly less than 20% of the tracer is generally inactive whilst that from very successful reactions (60–80% incorporation) has the highest specific activity.

Protocol 2. *In vitro* transcription

Reagents[a]

- Linearized DNA template:[b] 1 mg/ml in sterile water
- 10 × transcription buffer: 400 mM Tris, 60 mM $MgCl_2$, 20 mM spermidine pH 7.5
- 1 mg/ml acetylated BSA (BRL)
- 5 mM rNTPs: 5 mM each of ATP, CTP, GTP, UTP (Pharmacia Biotech)
- 5 mM cap dinucleotide: $m^7G(5')ppp(5')G$ (Pharmacia Biotech)
- 1 mCi/ml [α-^{32}P]CTP (diluted from stock in water)
- 1 M dithiothreitol (DTT)
- 50 000 U/ml RNase inhibitor (Boehringer Mannheim)
- 60 000 U/ml SP6 or T7 RNA polymerase (Pharmacia Biotech)
- 5 M ammonium acetate
- 1:1 (v/v) phenol:chloroform
- Ethanol (absolute)
- DE81 paper (Whatman)
- DE81 wash buffer: 150 mM NaH_2PO_4
- Sterile water

Method

1. At room temperature, mix in the following order:
   - Water 30 µl
   - 1 mg/ml linear DNA template 10 µl
   - 1 mg/ml BSA 10 µl
   - 5 mM rNTPs 10 µl
   - 5 mM cap dinucleotide 10 µl
   - 1 mCi/ml [α-^{32}P]CTP 5 µl
   - 10 × transcription buffer 10 µl
   - 1 M DTT 5 µl
   - 50 000 U/ml RNase inhibitor 5 µl
   - 60 000 U/ml SP6 or T7 RNA polymerase 5 µl

   Incubate at 37 °C for 2 h.
2. Remove 0.5 µl of the reaction mixture and add 9.5 µl water. Store on ice or at –20 °C for later determination of the percentage incorporation.
3. Add 100 µl sterile water and 28 µl of 5 M ammonium acetate to the remaining reaction mixture, and extract twice with an equal volume of phenol:chloroform (1:1).
4. Add 2 vol. ethanol and store at –20 °C for 1 h.
5. Using a pencil, mark out 4 × 5 mm squares on a piece of DE81 paper.
6. Spot 2 µl of the diluted product from step 2 onto the centre of each square.

Protocol 2. *Continued*

7. Wash two of the DE81 squares for 5 min in 10 ml DE81 wash buffer. Repeat this four times. Rinse briefly in sterile water, then ethanol, and then acetone, and air dry.
8. Determine the level of radioactivity on each filter by scintillation counting. The counts from a pair of filters should agree within 5%.
9. Calculate the percentage of total counts (determined from the unwashed filters) remaining on the washed filters.
10. Calculate the yield of RNA on the basis that 100% incorporation would equate to 72 μg RNA.
11. Recover the RNA transcripts from step 4 by centrifugation for 10 min in a microcentrifuge. Wash the pellet twice with 70% ethanol. Remove the ethanol completely, allow the pellet to air dry briefly, and redissolve it at 1 mg/ml in sterile water. Store the RNA solution at −20°C in 5 μl aliquots.

[a] Although RNase inhibitor is used in this procedure, it is important to ensure that solutions are free of contamination. The previous history of each component must be known and the reagents reserved solely for use in transcription reactions. Use autoclaved 18 MΩ water throughout.

[b] DNA templates may be prepared by any standard method but must be free of residual RNase. Quantification of DNA must be reasonably accurate since template concentration is a key factor in the outcome of the transcription reaction.

2.4 Use of the injector

Before attempting a microinjection experiment, it is advisable to become familiar with the operation of the injection apparatus. The injector and manipulator are not complex and it should not take long to become sufficiently familiar with the controls to be able to perform all necessary operations whilst looking through the microscope. The key variable is the injection needle. If this is too coarse a high proportion of the oocytes will be killed by the injection, whilst a fine needle will become blocked frequently and may not deliver the set volume consistently due to the pressure bleeding through the seal at the seat of the needle. The preparation of microinjection needles is described in *Protocol 3*.

In principle, the apparatus should not require calibration since the plunger displaces a set volume on each stroke. The volume expelled can be measured, however, by delivering water or dye into a 3–4 μl droplet of mineral oil and measuring the diameter of the bubbles formed using a calibrated eyepiece graticule. A simpler check on the system is to set the injector to deliver the minimum volume (4.6 nl) and to verify by eye that similar-sized bubbles are formed consistently in the mineral oil.

Needles can be loaded with relatively large volumes but, since it is difficult

to recover the contents of a blocked or broken needle, it is safer to load and inject 1–2 μl before refilling.

Protocol 3. Preparation of microinjection needles

Equipment and reagents

- Glass capillaries (Drummond 3-00-203-G/X, Laser Laboratory Systems)
- 1 ml syringe with 26 gauge spinal needle
- Needle puller (Narashige PN 3) (see Section 2.1)
- Microinjector (see Section 2.1)
- Stereomicroscope with fibre-optic light source (see Section 2.1)
- Watchmakers' forceps
- Mineral oil (Sigma)
- *Storage tray: strip of modelling clay in a* 100 mm square Petri dish

Method

1. Sterilize the capillaries by baking them at 160°C for at least 2 h in a glass test-tube, or a small measuring cylinder, capped with aluminium foil.
2. Set up the needle puller according to the manufacturer's instructions. On the Narashige PN 3, with all controls set to maximum values, the resulting needles have the profile shown in *Figure 3A*. Although needles can be produced very rapidly with this apparatus, it is advisable to make a number (10–20) at a session, so they are available immediately during an injection session. Needles can be stored by pressing the shaft into a strip of modelling clay in a 100 mm square Petri dish.
3. Working under the stereomicroscope, trim a needle back to the point where the diameter is 20–30 μm by breaking the tip with the watchmakers' forceps.
4. Fill the needle from the back with mineral oil, using a 26 gauge spinal needle, and mount it onto the microinjector according to the manufacturer's instructions. Avoid introducing air bubbles during these operations as these will compress and damp the action of the injector. Take care to ensure that all seals are in the correct location and orientation.
5. Mount the injector to one side of the microscope stage, in a position which allows the full range of movement of the manipulator to be used (see *Figure 1*). The tip of the needle is very fragile, so care should be taken not to bring it into contact with the microscope, etc.
6. To fill the needle, pipette 1–2 μl of the injection solution onto a piece of sealing film ('Parafilm' or 'Nescofilm'), lower the tip of the needle into this, and draw the solution up. In order to avoid taking any air into the system, it is normally necessary to leave some liquid on the film.

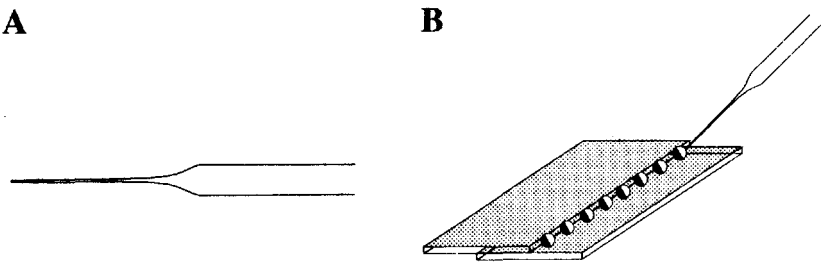

Figure 3. Microinjection needles. (A) The profile of a needle narrows sharply before tapering off to a fine point. (B) Aligning oocytes along the step made by a pair of overlapping microscope slides keeps them in position during the injection procedure.

2.5 Microinjection

There are many different ways of positioning oocytes on the microscope stage for injection. The approach described here (see *Protocol 4* and *Figure 3B*) has the advantages that they are firmly supported against the injector needle and that 10–20 oocytes can be mounted in the field of the microscope within the travel of the manipulator.

mRNAs should be injected into the vegetal (unpigmented) pole of the oocyte. Not only do injected macromolecules diffuse more readily from this position but this also reduces the chance of damaging the nucleus and killing the cell. Even after vegetal injection, membrane proteins are inserted initially into the plasma membrane close to the site of injection and it may take several days for expression products to become distributed evenly around the oocyte surface. If the experiment involves the interaction of two or more proteins, it is important, therefore, to either co-inject the mRNAs or culture the oocytes for a number of days after the last injection.

The total volume of the injection should be 20–30 nl. Some descriptions of the procedure using a manual system indicate a volume around 50 nl but this damages the oocyte. Once the injector is set for the required volume, using the dip switches on the control box, it should not require further adjustment.

Protocol 4. Microinjection

Equipment and reagents

- Stereomicroscope with fibre-optic light source and microinjector mounted in position (*Figure 1*)
- mRNA to be injected (see Section 2.3)
- *Xenopus* oocytes (see *Protocol 1*)
- Microscope slides
- Watchmakers' forceps
- Shortened Pasteur pipettes (see *Protocol 1*)
- MBS (see *Table 1*)

Method

1. Fill the needle with the mRNA to be injected (*Protocol 3*, step 6).

2. Transfer five to ten oocytes onto the step between two offset microscope slides (see *Figure 3B*) using a shortened Pasteur pipette.
3. Remove excess medium by blotting using a tissue.
4. Under the microscope, using forceps, manoeuvre the oocytes so that the vegetal (unpigmented) poles face the needle.
5. Gently drive the needle into an oocyte, until the tip is approximately in the middle of the vegetal half, and inject the mRNA.
6. Withdraw the needle and move to the next oocyte.
7. When all oocytes on a slide have been injected, wash them into a Petri dish containing MBS.

2.6 Expression from microinjected DNA

Although it is more common to express proteins from microinjected mRNA, an alternative approach is to inject DNA constructs into the nucleus for transcription and translation. The DNA should be circular and should contain the natural transcriptional start site and a strong promotor but, ideally, no introns, since the degree of accurate splicing appears to be variable in *Xenopus* oocytes.

The oocyte nucleus is quite large, occupying 5–10% of the cell volume and is located in the middle of the animal (pigmented) pole. Many workers inject 'blind' into this region but it is also possible to bring the nucleus to the surface by centrifuging oocytes in a swing-out rotor at around 500 *g* for 10 min after orienting them animal pole uppermost on a 0.5 mm square plastic mesh in a Petri dish.

Needles for nuclear injection should be finer than for RNA injection, ideally about 10 μm diameter at the tip. Although this risks more frequent blockage, it reduces damage to the nucleus. About 10 nl of a 1 mg/ml solution of DNA should be injected. Injecting a 0.2% solution of Trypan Blue in a trial experiment provides a simple means of checking nuclear injection technique. After injection, a small slit can be made in the surface of the animal pole and the nucleus extruded by gently squeezing the oocyte with a pair of forceps. Injected nuclei have a definite colour, whilst uninjected nuclei are transparent. DNA microinjection is discussed in greater detail in ref. 19.

2.7 Radioactive labelling

Most routine experiments are followed by labelling newly synthesized proteins with [^{35}S]methionine or [^{35}S]cysteine, since the internal pool sizes of these amino acids are small (around 30 pmol/oocyte for methionine, 35 pmol/oocyte for cysteine) and ^{35}S is a moderately energetic β emitter. Alternatively, a ^{35}S-labelled amino acid mixture (which contains [^{35}S]cysteine and [^{35}S]methionine) can be used, but this is less versatile since it cannot be used for *in vitro*

translation. Oocytes are freely permeable to amino acids in the incubation medium and so labelling is carried out by adding the radioactive amino acid(s) to the medium (*Protocol 5*). A concentration of 1 mCi/ml is sufficient for most routine experiments but this can be increased to 5 mCi/ml without toxicity to increase the specific activity of the labelled product.

The simplest format for labelling is to incubate five oocytes in 30 μl of medium in a 1.5 ml microcentrifuge tube (*Protocol 5*) or a well of a microtitre plate. Where significant oocyte mortality is a problem, particularly if the medium is to be analysed for secreted proteins, oocytes can be incubated individually in 6 μl of medium in a 0.5 ml tube to avoid cross-contamination.

When the protein to be labelled contains no methionine, or for analyses such as protease mapping or N-terminal sequencing, it may be necessary to use alternative amino acids labelled with ^{3}H. ^{14}C-labelled amino acids are rarely used since they are expensive and available only at relatively low specific radioactivities. ^{3}H-labelled amino acids are frequently supplied at low concentrations and often need to be concentrated by freeze-drying before use. When using tritiated amino acids, the pool size of the particular amino acid in the oocyte will determine the specific radioactivity of the protein product. Arginine, histidine, isoleucine, leucine, phenylalanine, proline, tyrosine, and valine all have pool sizes of less than 100 pmol/oocyte, so can be used without difficulty, whereas oocytes contain between 200–800 pmol of alanine, aspartate, glutamate, glycine, lysine, serine, and threonine, which will reduce labelling significantly (20).

It is difficult to perform accurate pulse-chase experiments with oocytes, since radioactive precursors take about an hour to equilibrate with the internal pool when added to the medium, although this can be reduced by microinjecting the labelled precursor. The duration of labelling then depends upon the specific radioactivity of the label. High specific radioactivity [^{35}S]methionine at 1 mCi/ml is effectively depleted after 6 hours, whilst the addition of non-radioactive methionine to 60 μM will allow linear incorporation over 24 hours. Labelling can be chased using 1 mM methionine in MBS but, again, this takes up to an hour to equilibrate within the oocyte.

The labelling strategy used must take account of the stability of both the mRNA and the protein product. Poly(A)$^{+}$ mRNAs are very stable (21, 22) and persist in the oocyte for many days whilst some poly(A)$^{-}$ species can be degraded very rapidly (23, 24). Translation products of unstable RNAs may be detected more easily if the radioactive amino acid is co-injected with the RNA but this will not give maximal labelling of products from stable RNAs since there is normally a lag of several hours before mRNAs are translated fully (25). Similarly, some proteins are very stable and can be detected after many days of chase (26) whilst unstable species are best analysed by harvesting within the effective labelling period.

For most routine experiments, the simplest scheme is to perform the isolation, microinjection, labelling, and harvesting on separate days.

Protocol 5. Metabolic labelling of oocytes

Equipment and reagents

- Stereomicroscope with fibre-optic light source (see Section 2.1)
- 1.5 ml homogenizer tubes (Anachem)
- Microinjected oocytes (see *Protocol 4*)
- Sterile MBS (*Table 1*)
- 1 mg/ml BSA
- Labelling medium: add [^{35}S]methionine to sterile MBS to give a final concentration of 1 mCi/ml—prepare 6 μl per oocyte to be labelled

Method

1. Prepare 1.5 ml microcentrifuge tubes by filling with 1 mg/ml BSA, standing at room temperature for 1 h, then washing repeatedly with sterile water.
2. Wash the microinjected oocytes in two to three changes of sterile MBS.
3. Transfer five oocytes to each microcentrifuge tube[a] and remove the medium using a 200 μl pipette.
4. Add 30 μl labelling medium. Ensure the oocytes are completely submerged.
5. Incubate at 21°C overnight.
6. Collect the medium using a micropipette and store this if it is to be analysed (see Section 2.8, and step 7).
7. Immediately add fresh unlabelled MBS to the oocytes and then transfer the oocytes to a Petri dish containing MBS. Examine the oocytes under the stereomicroscope to check that they are healthy. Good oocytes from batches containing dead ones can normally be analysed for intracellular products, but the media from these batches should be discarded.
8. If the oocytes are to be analysed immediately (*Protocol 6*), transfer them to 1.5 ml homogenizer tubes. Alternatively, completely drain the oocytes of medium, stand them on dry ice to freeze, and store at −70°C.

[a] If it is necessary to label individual oocytes, 0.5 ml microcentrifuge tubes can be used without increasing the amount of medium per oocyte (see Section 2.7).

2.8 Analysis of radioactive translation products

At the end of the labelling period, the radioactive medium is removed and the oocytes washed into fresh MBS (see *Protocol 5*). If the medium is to be analysed, the condition of the oocytes should be checked before pooling media from different tubes, since protein can be released from dead oocytes.

Suspended matter in the medium can be pelleted in a microcentrifuge for 5 min. This will reduce the background resulting from very light microbial contamination but a clearly visible pellet indicates severe contamination and such samples may be compromised either by having high backgrounds or by bacterial degradation of secreted proteins.

Uninjected oocytes secrete very little protein (3). Consequently, some proteins which are efficiently translated and secreted can be analysed directly by SDS–PAGE (Chapter 6, *Protocol 7*) and fluorography (27) of unfractionated medium. The fluorogram of a gel lane loaded with 5 μl of medium should be virtually blank, whilst a strong 'ladder' of labelled protein indicates bacterial or fungal contamination. Unlike secreted proteins, the majority of labelled proteins that remain within the oocyte require purification and/or specific detection, normally by immunoprecipitation with a specific antibody.

Many protocols have been used for immunoprecipitation and the choice is dictated by the properties of the antibody to be used. Oocytes do not seem to contain significant amounts of 'sticky' proteins, but some antisera can cross-react to an extent with endogenous proteins. The method given in *Protocol 6* is simple and sensitive but may give high backgrounds with such antibodies. In general, buffers which are designed to reduce background problems reduce sensitivity. Experiments should include controls such as replacing the antibody with appropriate pre-immune serum or a non-specific antibody and immunoprecipitating from uninjected labelled oocytes to distinguish between specific signals, insoluble proteins, and background. Some examples of proteins immunoprecipitated from radiolabelled oocytes are shown in *Figure 4*.

Protocol 6. Immunoprecipitation of translation products

Equipment and reagents

- 1.5 ml homogenizer tubes (Anachem)
- Rotating wheel mixer
- 100 mM PMSF (phenylmethylsulfonyl fluoride) in isopropanol
- Homogenization buffer: 20 mM Tris–HCl pH 7.6, 0.1 M NaCl, 1% Triton X-100
- NET buffer: 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM Tris pH 7.5—add 100 mM PMSF to 1 mM final concentration immediately before use
- Antibody or antiserum (see text)
- NET-gel buffer: 0.25% gelatin in NET buffer; heat to 37°C for 15 min to dissolve the gelatin, add 100 mM PMSF to 1 mM final concentration immediately before use
- Protein A–Sepharose (Pharmacia): 10% suspension in NET buffer
- Radiolabelled oocytes (from *Protocol 5*)
- SDS sample buffer, polyacrylamide gel, and other reagents for SDS–PAGE (see Chapter 6, *Protocol 7*)

Method

1. Place drained fresh or frozen oocytes in a 1.5 ml homogenization tube on ice.
2. Add homogenization buffer at 40 μl per oocyte and homogenize immediately. If a large number of oocytes is to be processed in one microcentrifuge tube, it may be easier to add only a fraction of the buffer, break the oocytes, then add the remainder.

3. Pellet the yolk[a] in a microcentrifuge at 13000 r.p.m. at 4°C for 10 min, and transfer the supernatant to a fresh tube. The homogenate (supernatant) can be stored frozen at −20°C at this stage, if required.
4. Add 200 μl oocyte homogenate (five oocyte equivalents) to 800 μl NET-gel buffer containing the appropriate antiserum (usually 1–5 μl of whole serum, depending on the titre). Incubate at 4°C for 3 h to overnight.
5. Pellet any aggregates which have formed by centrifuging in a microcentrifuge at 13000 *g* at 4°C for 10 min. Transfer the supernatant to a fresh tube.
6. Add 100 μl of a 10% Protein A–Sepharose suspension and incubate at 4°C in a capped microcentrifuge tube on a slowly rotating wheel for 1–3 h.
7. Pellet the Protein A–Sepharose beads in a microcentrifuge for 30 sec at 4°C and remove the supernatant. The supernatant may now be reanalysed using a second antibody.
8. Wash the Protein A–Sepharose beads at least three times with 1 ml NET buffer. Drain as much buffer as possible after the last wash and add 90 μl SDS sample buffer. Heat to 95°C for 2 min and analyse 20 μl (one oocyte equivalent) by SDS–PAGE (see Chapter 6, *Protocol 7*).

[a]The yolk fraction of the oocyte is Triton X-100 insoluble and is removed from the initial homogenate by centrifugation, reducing the protein content significantly.

2.9 Fractionation of oocytes

Quantitative recovery of membrane-bound and membrane-associated proteins can be achieved simply by homogenizing oocytes in detergent-free buffer and centrifugation through neutral sucrose (28), as described in *Protocol 7*. Integral membrane proteins can be identified by treatment of this pellet with alkali, as described, but high resolution density-based subfractionation of membrane compartments has not yet proved possible.

Protocol 7. Membrane fractionation

Equipment and reagents

- Appropriate centrifuge tubes (1–5 ml), swing-out rotor, and preparative ultracentrifuge
- Sterile 0.22 μm membrane filters (Millipore)
- 10% Triton X-100
- Concentrated HCl
- T + 10 buffer: 10% sucrose in 50 mM KCl, 10 mM magnesium acetate, 100 mM NaCl, 20 mM Tris pH 7.6—filter sterilize and store at −20°C
- 200 mM sodium carbonate
- T + 20 buffer: 20% sucrose in 50 mM KCl, 10 mM magnesium acetate, 100 mM NaCl, 20 mM Tris pH 7.6—filter sterilize and store at −20°C
- Carbonate sucrose: 20% sucrose, 100 mM sodium carbonate pH > 11—prepare fresh for each use
- Reagents for SDS–PAGE (see Chapter 6, *Protocol 7*)

Protocol 7. *Continued*

Method

1. Homogenize ten oocytes in 0.5 ml T + 10 buffer on ice.
2. Layer the homogenate onto a 1 ml cushion of T + 20 buffer in a small (1–5 ml) centrifuge tube and centrifuge at 20 000 *g* for 30 min at 4°C in a swing-out rotor. After centrifugation, cytosolic proteins will be in the T + 10 layer, mitochondria at the T + 10/T + 20 interface, and membrane components of the secretory pathway will be in the pellet, together with yolk platelets and other insoluble matter. The pellet can now be analysed as a total membrane fraction or further fractionated to separate integral membrane proteins from lumenal secretory proteins, by the steps described below.
3. On ice, suspend the membrane pellet in 0.5 ml T + 10 buffer then add 0.5 ml of 200 mM sodium carbonate.
4. Split the sample into two halves and add 50 μl of 10% Triton X-100 to one.[a] Incubate both on ice for 30 min.
5. Layer each sample onto a separate 0.5 ml cushion of carbonate sucrose and centrifuge at 100 000 *g* for 1 h at 4°C.
6. In each case, recover 250 μl from the top layer and neutralize the pH by adding 6 μl concentrated HCl. Discard the carbonate sucrose layer and dissolve the pellet in oocyte homogenization buffer.
7. Analyse the fractions directly by SDS–PAGE (Chapter 6, *Protocol 7*) or after immunoprecipitation (see *Protocol 6*).

[a] The sample treated with Triton X-100 provides a control to distinguish between membrane-bound and insoluble proteins.

3. Preparation and use of *Xenopus* egg cell-free extracts

This approach (5) exploits the large size and fragility of *Xenopus* eggs to generate a lysate which is, effectively, highly concentrated cytoplasm. The method is relatively rapid and simple. The two key elements of a successful preparation are a good yield of healthy eggs and minimal dilution of the extract with buffer.

3.1 Equipment

(a) *Centrifuge.* The centrifuge must be refrigerated and able to achieve an RCF of 15 000 *g* within 90 sec of starting. The procedure for preparing

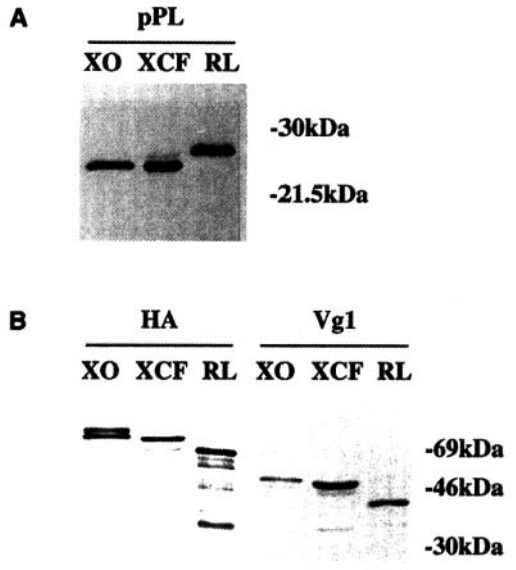

Figure 4. Comparison of secretory translation products. Pre-prolactin (pPL), influenza haemagglutinin (HA), and *Xenopus* Vg1 (Vg1) were translated by microinjection into *Xenopus* oocytes (XO) (*Protocols 4* and *5*), in the *Xenopus* cell-free extract (XCF) (*Protocol 13*), and in rabbit reticulocyte lysate, and analysed by SDS–PAGE and fluorography. The microinjected samples were recovered by immunoprecipitation (*Protocol 6*) from the medium (A) or whole oocyte homogenate (B), whilst the *in vitro* translation products were analysed directly. (A) Prolactin signal sequence cleavage leads to a distinct increase in mobility after translation in the *Xenopus* systems relative to reticulocyte lysate whilst (B) *N*-linked glycosylation decreases the mobilities of HA and Vg1. The least mobile form of HA after translation in the oocyte, resulting from carbohydrate modifications in the Golgi apparatus, is not seen in the product from the *Xenopus* cell-free extract.

extracts described in this chapter (see *Protocol 10*) was optimized using a Beckman TL100 bench-top ultracentrifuge but this is not absolutely necessary. Active extracts have been produced using a standard microcentrifuge in a cold room, but the fixed angle rotor significantly reduces the amount of material which can be recovered. Although a number of swing-out microcentrifuges are available, most of these are inappropriate as they have reduced top speeds. One microcentrifuge which meets these specifications, however, is the Hettich Mikro 24-48R (Thistle Scientific).

(b) *Cold room.* It is advised that the procedure is performed in a 4°C cold room since this will ensure not only that the product is kept cold at all times but also that items such as pipette tips and tubes will be cold and so will not warm the extract during manipulations.

3.2 *Xenopus* eggs

Collect *Xenopus* eggs as described in *Protocol 8* and remove their jelly coats (*Protocol 9*). Although egg quality could be assessed by *in vitro* fertilization, this is time-consuming and does not appear to predict the outcome of the procedure since batches which are refractory to fertilization will often yield good extracts. The simplest way of assessing egg quality is by appearance. To the eye, eggs appear essentially identical to oocytes, except that they are covered by a thick, transparent jelly coat. Unhealthy oocytes give rise to unhealthy eggs with similarly disrupted pigmentation; batches containing significant numbers of these should be discarded. Eggs can be damaged during shedding, particularly if the frog is squeezed, and many of these lyse within the jelly coat, liberating yolk proteins to give the jelly coat a distinct opaque/white appearance. Most damaged and lysed eggs are lost during the cysteine treatment to remove jelly coats (*Protocol 9*) as their contents are washed away once the jelly coat is dissolved but if they are present at high proportions (> 10%) the batch should be discarded. Sometimes fragile eggs lose pigmentation and lyse after the cysteine is removed. Batches containing significant numbers of these should be discarded also.

Protocol 8. Collecting *Xenopus* eggs

Equipment and reagents

- Plastic boxes (20 × 30 cm)
- 25 ml disposable plastic pipette with the tip cut back to give an orifice of > 3 mm
- Salt water: 110 mM NaCl in dechlorinated water
- Large adult female *Xenopus laevis*
- Folligon and chorionic gonadotropin (Intervet), reconstituted according to the supplier's instructions

Method

1. Prime large adult female *Xenopus laevis* three to five days before eggs are required by injection of 50–100 IU of Folligon into the dorsal lymph sac.
2. 12–16 h before preparing an extract, induce ovulation by injecting 750 IU of chorionic gonadotropin into the dorsal lymph sac.
3. Keep the frogs individually overnight in the plastic boxes, in 1.5 litres of salt water.
4. Remove the frog from the plastic box which should now contain eggs. Discard any obvious contaminants (e.g. shed skin, faeces), together with any eggs which are obviously necrotic.
5. Collect the good eggs into a 250 ml glass beaker using the 25 ml shortened plastic pipette.

Protocol 9. Removal of jelly coats

Reagents

- *Xenopus* eggs (from *Protocol 8*)
- 2% cysteine, 110 mM NaCl: adjust to pH 7.7 with NaOH and use within 1 h of preparation
- Extract buffer: 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM sucrose, 10 mM Hepes pH 7.7. Prepare this from stock solutions as follows. Prepare a 20 × salt stock by dissolving 149 g KCl, 0.29 g $CaCl_2.2H_2O$, and 4.1 g $MgCl_2.6H_2O$ in 1 litre water, dispense into 50 ml portions, and store at –20°C. 1.5 M sucrose by dissolving 513.5 g in 1 litre water and freezing 40 ml portions at –20°C. 1 M Hepes by dissolving 47.7 g in 200 ml water and freezing 10 ml portions at –20°C. To prepare extract buffer, mix 50 ml of 20 × salts, 33 ml of 1.5 M sucrose, and 10 ml of 1 M Hepes in 800 ml water, adjust to pH 7.7 with 10 M KOH, and make up to 1 litre. Chill on ice before use. If the water used to make this has been pre-cooled by storing at 4°C overnight, less time on ice is required to cool the solution to working temperature.

Method

1. Transfer the eggs to approx. 4 vol. 2% cysteine, 110 mM NaCl by decanting the medium, and adding 200 ml of cysteine/NaCl. Allow the eggs to settle and repeat this. Swirl gently every 30 sec or so.
2. After a few minutes, removal of the jelly coats is indicated by the fact that the eggs pack closely together when allowed to settle.[a]
3. Terminate the process by washing the eggs four or five times with ice-cold extract buffer. Immediately prepare the extract (*Protocol 10*).

[a] The loss of the jelly coats reduces the volume of 50 ml of eggs to 8–10 ml.

3.3 Preparation of extract

3.3.1 Basic method

The method for preparation of cell-free extract from *Xenopus* eggs is described in *Protocol 10*. Excess buffer is removed by the first, low speed, centrifugation step (*Figure 5*). This should compress the eggs to give a regular, snakeskin-like pattern and cause little if any lysis. It is important to remove as much buffer as possible since any that remains will end up in the extract. Any lysed or badly damaged eggs will often rise to the surface and can be aspirated with the buffer.

After the first high speed spin, the lysate separates into three major fractions: a dark pellet, a pale yellow cytoplasm layer, and a supernatant lipid pellicle (see *Figure 5*). Sometimes there is a minor layer of greyish granular material on the surface of the pellet. This appears to contain yolk and must be avoided as it will lead to a substantial expansion of the amino acid pool and seriously reduce the specific activity of translation products. The cytoplasm is most easily recovered using a Pasteur pipette inserted through the lipid. This fraction is relatively viscous and should be aspirated gently to avoid both

introducing air bubbles and contaminating it with material from the pellet interface. The second high speed centrifugation essentially clarifies the product, pelleting any particulate matter carried over and separating the small amount of lipid remaining (*Figure 5*).

Time is the most important factor in preparing a successful extract. Once jelly coats have been removed (*Protocol 9*), it is important to perform the subsequent steps quickly and to keep the extract cold at all times. In addition to performing all manipulations on ice, ideally in a cold room, all reagents and items which will come into contact with the extract (tips, tubes, Pasteur pipettes, etc.) must be cooled. The one exception to this is the cytochalasin B in DMSO (used in *Protocol 10*) which is solid at 4°C so must be warmed to room temperature.

Protocol 10. Preparation of the *Xenopus* extract

Equipment and reagents

- Cold room
- Refrigerated centrifuge fitted with a swing-out rotor and 2 ml centrifuge tubes (see Section 3.1)
- Dejellied *Xenopus* eggs (from *Protocol 9*)
- 10 mg/ml cytochalasin B in DMSO
- 10 mg/ml aprotinin in water: store as 50 µl aliquots at –20°C

Method

This procedure should ideally be performed on ice, in a cold room. If this is not possible, the extract should at least be kept on ice at all stages.

1. Pipette the dejellied eggs into 2 ml centrifuge tubes. Allow them to settle for a minute or so, remove excess buffer, and top the tubes up with any remaining eggs.[a]
2. Centrifuge at 500 *g* for 1 min at 4°C to pack the eggs.
3. Remove the supernatant buffer using a micropipette.
4. Centrifuge the eggs at 15 000 *g* for 15 min at 4°C.
5. Recover the 'cytoplasm' fraction (*Figure 5*), by inserting a Pasteur pipette through the lipid pellicle. Pool the cytoplasm into fresh chilled 2 ml tubes.
6. Estimate the volume recovered, add 5 µl/ml of 10 mg/ml cytochalasin B, and mix gently with a Pasteur pipette.
7. Recentrifuge at 15 000 *g* for 15 min at 4°C.
8. Recover the cytoplasm, which now occupies most of the tube (*Figure 5*), avoiding the viscous pellet.
9. Add 1 µl/ml of 10 mg/ml aprotinin.
10. Use the extract immediately for translation (*Protocol 13*), or first treat it with RNase A (see Section 3.3.2), or freeze the extract as described below.

11. To freeze *Xenopus* extracts, add 0.15 vol. of 1.5 M sucrose,[b] mix thoroughly, and dispense aliquots of 50–100 µl into 0.5 ml microcentrifuge tubes, on ice. Freeze in liquid nitrogen, then store at –70°C or under liquid nitrogen.

[a] If the number of eggs available is limited, it is better to reduce the number of tubes used as it is easier to collect fractions from full tubes.
[b] Addition of sucrose before freezing reduces the activity of most extracts slightly but appears to rescue some which would otherwise be much less active when subsequently thawed.

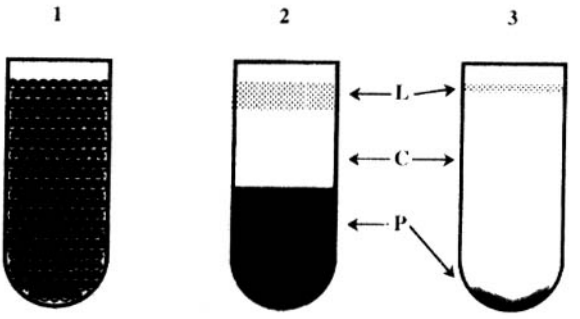

Figure 5. Appearance of the extract after each of the centrifugation steps. The first, low speed, spin (1) should compact the eggs and produce a small amount of supernatant buffer. Lysis at 15000 *g* (2) generates three layers: a solid pigmented pellet (P), a lipid pellicle (L), and the cytoplasm (C). When the cytoplasm is recovered and recentrifuged (3) only a relatively small amount of lipid and pellet should remain.

3.3.2 Ribonuclease treatment

Xenopus cell-free extracts may be used untreated for *in vitro* translation experiments, but prior treatment with RNase A is used to degrade endogenous mRNAs and hence minimize background translation. Experience has shown that different batches of RNase A differ in activity to a great extent. This probably reflects differences in purity, formulation, and storage. On the other hand, the concentration of RNase required to treat different extracts appears to be similar. Once a stock of RNase has been titrated, therefore, it can be used for all subsequent extracts, providing it is stored at –20°C. Titration of RNase is described in *Protocol 11*. RNase-treated extracts should show no translation at the highest level of RNase used and significant translation at the lowest level, irrespective of added mRNA. At least two samples of extract treated with intermediate RNase concentrations should show minimal background activity but a strong signal from the mRNA added. Use these for subsequent *in vitro* translation experiments. When this treated extract has been exhausted, treat new batches of *Xenopus* extract with the same final concentration of RNase A as described in *Protocol 12*, using the titred RNase stock solution.

Protocol 11. Titration of ribonuclease stocks

Equipment and reagents

- Water-bath at 10°C
- *Xenopus* extract (from *Protocol 10*)
- 1 mg/ml RNase A[a]
- 5 mg/ml calf liver tRNA
- Purified mRNA to act as a control
- Ribonuclease inhibitor (Boehringer Mannheim): 50 U/μl
- 200 mM DTT
- Materials for SDS–PAGE (*see Protocol 6*)

Method

1. Using the stock 1 mg/ml RNase solution, set up five working RNase solutions to give final RNase concentrations in step 2 in the range 2–0.12 μg/ml.
2. For each RNase concentration to be tested, prepare a 200 μl aliquot of *Xenopus* extract in a 0.5 ml microcentrifuge tube, on ice. To each, add 1 μl of the appropriate RNase solution from step 1. Mix gently but thoroughly by pipetting. Incubate at 10°C for 15 min. Set up a control extract sample with water in place of RNase.
3. Place the tubes on ice and add 1 μl of 200 mM DTT and 2 μl (100 U) ribonuclease inhibitor to each tube. Mix by pipetting gently and incubate at 10°C for a further 10 min.
4. Add 2 μl of 5 mg/ml tRNA to each tube and mix gently.
5. For each RNase concentration being tested, assemble two translation reactions (see *Protocol 13*); to one add purified control mRNA as template but add no RNA to the duplicate (control reaction). Freeze the remainder of the RNase treated extract (see *Protocol 10*, step 11) for future use.
6. Analyse the translation products by SDS–PAGE (see *Protocol 6*) to determine the concentration of RNase which leads to the best signal (protein products from the exogenous mRNA) over background (endogenous protein synthesis).

[a]To reduce the frequency of titrating RNase stocks, it is advisable to prepare a reasonable quantity (> 1 ml) of 1 mg/ml RNase A stock specifically for this application and store it at –20°C in small aliquots.

Protocol 12. Treatment of extracts using titred RNase A

Equipment and reagents

- Water-bath at 10°C
- *Xenopus* extract (from *Protocol 10*)
- Titrated RNase A stock (from *Protocol 11*)
- 200 mM DTT
- Ribonuclease inhibitor: 50 U/μl (Boehringer Mannheim)
- 5 mg/ml calf liver tRNA

Method

1. On ice, dispense 1 μl of the titred stock of RNase A, diluted so as to be at 200-fold the final concentration of RNase desired, into 0.5 ml microcentrifuge tubes. Then add 200 μl of extract to each tube, mixing gently but thoroughly by pipetting. Incubate at 10°C for 15 min.
2. Return the tubes to ice and add 1 μl of 200 mM DTT then 100 U (2 μl) RNase inhibitor to each. Mix by pipetting and incubate at 10°C for a further 10 min.
3. Add 2 μl of 5 mg/ml tRNA to each tube and mix gently. The extract should now be used directly or frozen for storage as described in *Protocol 10*, step 11.

3.4 *In vitro* translation using *Xenopus* cell-free extracts

3.4.1 Translational activity

In vitro translation is carried out as described in *Protocol 13*. *Xenopus* extracts are most active when fresh; about half of the original activity is recovered after storage at –70°C. When using extracts without RNase treatment, or when total poly(A)$^+$ mRNA is supplied, the translation proceeds rapidly and should be complete in less than an hour. When programmed by a single synthetic mRNA, however, limiting factors are depleted more slowly and translation products can continue to accumulate over many hours. Hence the extract may be incubated overnight for maximum signal (see *Figure 6A*). Overnight reactions should be performed in an incubator and contain a volume of at least 20 μl to prevent them drying out by evaporation. Some reactions have shown high backgrounds upon extended incubation but this appears to be incorporation into bacterial proteins as a result of reagent contamination. Reactions incubated for more than 4 h may form a gel to some extent. This does not appear to affect translation, translocation, or the integrity of the membranes, and the gel can be dispersed easily in either SDS–PAGE sample buffer (see *Protocol 6*) or T + 10 buffer (see *Protocol 7*).

Similar considerations with respect to mRNA preparation and radioactive labelling as those described in Sections 2.3 and 2.7, respectively, for oocytes also apply to cell-free extracts.

Protocol 13. Translation reactions

Equipment and reagents

- Water-bath at 20°C
- mRNAs to be translated
- Rabbit reticulocyte lysate (BRL)
- 500 mM creatine phosphate
- 100 mM spermidine
- [^{35}S]methionine > 1000 Ci/mmol (Amersham)
- 1 mg/ml RNase A (optional)

Protocol 13. *Continued*

Method

1. Use fresh *Xenopus* extract or thaw a frozen extract at room temperature, then place on ice.
2. To 50 μl extract, add 5 μl reticulocyte lysate, 1 μl of 500 mM creatine phosphate, 0.5 μl of 100 mM spermidine, and 50 μCi [^{35}S]methionine. Mix gently.
3. Divide this mixture between a series of microcentrifuge tubes containing the mRNAs to be translated. The volume of each reaction should be in the range 5–50 μl, containing a final concentration of 10–100 μg/ml mRNA, depending on the nature of the experiment and the translational activity of the mRNA being used. Include a control without mRNA to verify the absence of background translation.
4. Incubate for 1–2 h at 20°C.
5. If highly radioactive mRNA has been used (from *in vitro* translation), add RNase A to a final concentration of 10 μg/ml and incubate for 15 min at 20°C.
6. Analyse the translation products immediately (see Section 3.5) or freeze the samples and store them at –70°C.

3.4.2 Added factors

Fresh (not frozen) extracts are active without supplementation; added mRNAs compete with endogenous transcripts for the translational machinery. When a single mRNA is translated for one hour in a RNA-dependent extract, spermidine doubles the translational activity in some extracts but has no effect upon others, whilst creatine phosphate and reticulocyte lysate increase activity by factors of about six and three respectively. In extracts that have been frozen and then thawed, the relative effects of each of these additions are similar but little, if any, translation can be detected in the absence of creatine phosphate.

It is not clear which factors are contributed by the reticulocyte lysate but it is likely to be soluble initiation factors since an S-100 fraction, which is depleted of ribosomes, has an identical effect to whole lysate. Whole lysate, however, has a similar effect and is simpler to prepare. Reticulocyte lysate has always been obtained by the author from BRL/Life Technologies, and it is possible that the preparations from other sources may not stimulate the extract to the same extent.

3.4.3 Quality control

Preparation of an extract must be performed 'blind', since it is not possible to check the quality during the procedure. It is still informative, however, to perform some control reactions during the process in order to identify retro-

spectively any faulty reagents. Translation reactions should be performed using whole and RNase-treated extract before and after freezing, using mRNAs known to be active. Fresh (not frozen) untreated extract should incorporate about 30% of added [^{35}S]methionine into TCA precipitable counts in one hour in the absence of other additions. The addition of creatine phosphate should increase this to 50–60%. After freezing and thawing, the unsupplemented extract should be inactive but creatine phosphate should restore around 30% incorporation. Reticulocyte lysate appears to affect the rate of incorporation rather than the maximum yield so its effects are more easily observed upon the translation products of a single mRNA in RNase treated extracts.

3.5 Analysis of translation products

3.5.1 Analysis of total translation products

Ideally, the only radioactive proteins present after translation should be those programmed by the added mRNA. Standard SDS–PAGE followed by fluorography is therefore the most common means of analysis. Since *Xenopus* extracts contain about 50 mg/ml of protein, the amount to be loaded onto a gel must be controlled to prevent overloading. The choice of gel system is dictated by the experiment. Although denaturing SDS–PAGE is often used, the *Xenopus* extract can support legitimate assembly of oligomeric proteins which must be followed by non-reducing or native gel electrophoresis.

For SDS–PAGE, prepare extract samples by adding 4 vol. 1% Triton X-100, 1 mM PMSF, on ice. Then add an equal volume of the appropriate 2 × SDS–PAGE sample buffer. Either store the samples at –20°C for subsequent analysis or denature and run SDS–PAGE immediately (Chapter 6, *Protocol 7*). No more than 1 μl equivalent of *Xenopus* extract should be loaded per 5 mm × 0.75 mm lane of a mini gel.

3.5.2 Analysis of translated secretory and membrane proteins

Where secretory and membrane proteins are being analysed, these can be analysed as described in *Protocol 7* (see *Figure 6B*) or, more simply, recovered in the membrane fraction as described in *Protocol 14*. This procedure can also be applied to translation reactions which have been stored at –70°C after freezing in liquid nitrogen. The membrane fraction contains about 20% of the protein content of the complete extract, enabling proportionately more product to be analysed than is the case for cytosolic translation products. In common with their natural counterparts, membrane proteins generated by translation in the *Xenopus* extract are often rendered insoluble by heating in SDS–PAGE sample buffer and the best results are often obtained by simply incubating the mixture at room temperature for 10 min before loading the gel.

Several post-translational modifications of secretory and membrane proteins may be inferred simply from the migration of a protein translated in the

Xenopus extract relative to its behaviour after translation in non-translocating systems such as reticulocyte lysate. In most cases, signal sequence cleavage causes a measurable increase in mobility (*Figure 4A*), whilst glycosylation invariably causes a decrease (*Figure 4B*). *N*-linked glycosylation occurs rapidly upon translocation and is therefore apparent in samples from short translation periods, whilst *O*-glycosylation requires extended incubation in the extract and normally results in a more diffuse band on the gel. *N*-linked glycosylation can also be inferred by differences in gel mobility between translations performed in the presence or absence of 2 mM (acetyl)-Asn-Tyr-Thr-(amide), which crosses membranes and competitively inhibits *N*-linked glycosylation (29). Careful titration of this peptide in the range 0.2–1 mM can be used to generate partial inhibition, allowing the number of *N*-linked carbohydrate groups normally bound to the protein to be estimated from the number of partial products obtained.

Protocol 14. Recovery of the membrane fraction

Reagents

- T + 10 buffer (see *Protocol 7*)
- 1% Triton X-100, 1 mM PMSF
- Reagents for SDS–PAGE (Chapter 6, *Protocol 7*)

Method

1. After translation, add 10 vol. T + 10 buffer to each reaction mixture and mix well.
2. Centrifuge in a microcentrifuge at 6500 r.p.m. for 5 min then 13000 r.p.m. for 10 min, at 4°C.
3. Remove the supernatant.
4. Resuspend the membrane pellet in 1% Triton X-100, 1 mM PMSF, then add an equal volume of 2 × SDS–PAGE sample buffer before electrophoresis (see Chapter 6, *Protocol 7*).

3.5.3 Protease protection analysis

Whilst recovery in the pellet after centrifugal fractionation (*Protocol 14*) implies that a protein is associated with membranes, protease protection analysis (*Protocol 15*) provides a useful method for determining whether this is due to translocation into the lumen of the membrane vesicle or binding to the cytosolic face. This approach can also be used to explore the membrane topology of a transmembrane protein as shown in *Figure 6C*. The stability of the membrane fraction in *Xenopus* extracts enables quantitative recovery of protected fragments from this procedure.

Ideally, translation reactions should be programmed with mRNAs encoding cytosolic and lumenal proteins in addition to the sequence being tested, to

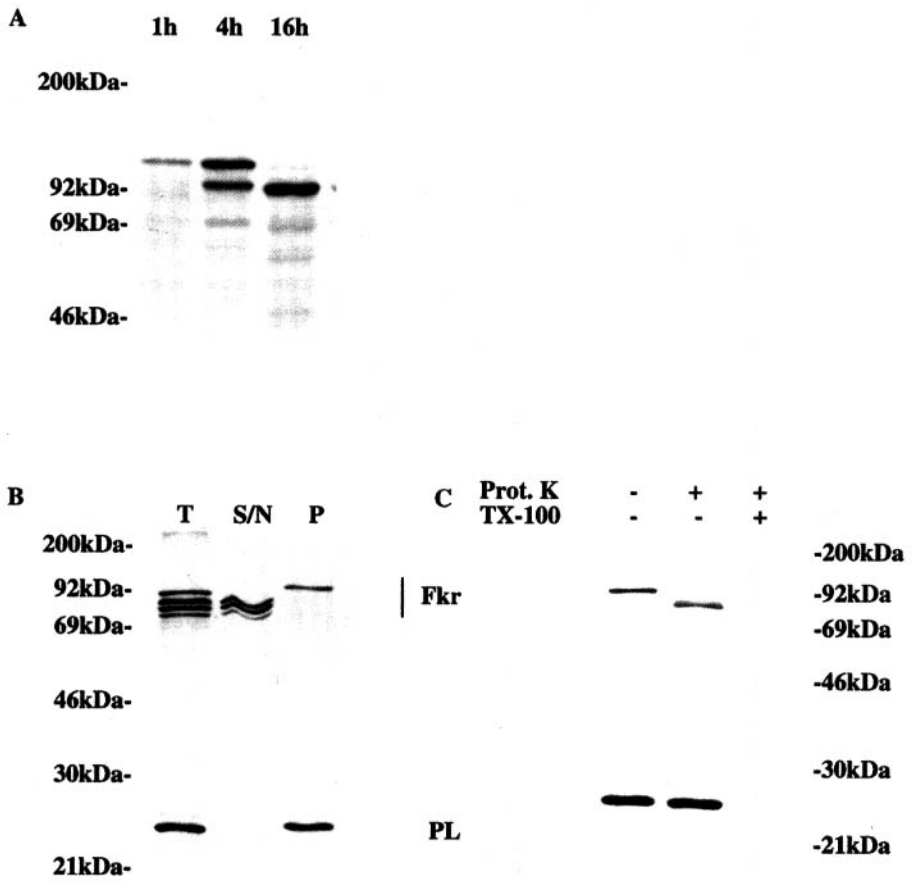

Figure 6. Analysis of proteins expressed in the *Xenopus* cell-free extract. (A) *Xenopus* furin was translated in the cell-free extract for 1, 4, and 16 h and equal portions of each reaction analysed by SDS–PAGE and fluorography. The stepwise increase in mobility of the translation product over time was confirmed by radio-sequencing to be the result of autocatalytic cleavage of the propeptide, demonstrating accurate folding of the enzyme to its active conformation. (B) The *Xenopus* furin-related protein Fkr was co-translated with prolactin in the *Xenopus* cell-free extract before membrane fractionation as described in *Protocol 7*. Equivalent amounts of the total translation mixture (T), the supernatant (S/N), and the membrane pellet (P) were analysed by SDS–PAGE and fluorography. Three of the four Fkr translation products (which originate from alternative translational starts) are not translocated into the endoplasmic reticulum and remain in the sucrose supernatant, whilst the fourth co-purifies with prolactin in the membrane fraction. (C) Protease protection analysis was performed on the sucrose pellet from (B) as described in *Protocol 15*. Whilst prolactin (which is a soluble protein), is completely protected from digestion by its inclusion within membrane vesicles, Fkr (which is a type I membrane protein), undergoes an increase in mobility after Proteinase K (Prot. K) treatment, due to the removal of its cytosolic 'tail'. Disruption of the membranes by addition of Triton X-100 (TX-100) leads to complete digestion of both translation products.

provide internal positive and negative controls. Cytosolic species should be completely degraded whilst lumenal proteins remain unaffected.

Protocol 15. Protease protection analysis

Reagents

- 30 μl translation mixture (prepared as described in *Protocol 13*)
- 20 mg/ml Proteinase K in water: incubate at 37°C for 15 min to digest any contaminating lipases—store at −20°C in 5 μl aliquots
- 15 mM PMSF in 10% sucrose: immediately before use, mix 15 μl of 100 mM PMSF with 85 μl of 10% sucrose; discard any unused material
- 2 × SDS sample buffer and other reagents for SDS–PAGE (see Chapter 6, *Protocol 7*)
- Two Proteinase K solutions: 200 μg/ml Proteinase K in 10% sucrose, and 200 μg/ml Proteinase K, 2% Triton X-100, 10% sucrose. Prepare these solutions immediately before use as follows. Add 0.4 ml of 10% sucrose to a 5 μl aliquot of Proteinase K and mix well. Transfer half of this to a tube containing 50 μl of 10% Triton X-100 in 10% sucrose, and add a further 50 μl of 10% sucrose to the remainder.

Method

1. On ice, divide the 30 μl translation mixture into three 10 μl portions in microcentrifuge tubes.
2. Add 10 μl of 10% sucrose to the first portion, 10 μl of 200 μg/ml Proteinase K in 10% sucrose to the second portion, and 10 μl of 200 μg/ml Proteinase K, 2% Triton X-100 in 10% sucrose to the third.
3. Leave on ice for 1 h.
4. Add 5 μl of 15 mM PMSF, 10% sucrose to each tube and leave on ice for a further 15 min.
5. Add 25 μl of 2 × SDS sample buffer to each tube and analyse by SDS–PAGE (Chapter 6, *Protocol 7*).

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3

Expressing cloned genes in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*

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1. Introduction

When considering whether or not to express a heterologous gene in yeast, one has not only to decide which strategy should be used—vector, strain, promoter, etc.—but also which yeast. The term 'yeast', although widely regarded to be synonymous with the yeast *Saccharomyces cerevisiae*, is in fact an umbrella term covering approximately 500 different yeast species. The reason for this laxity in terminology is perhaps simple laziness, but there are ever increasing numbers of researchers turning to *S. cerevisiae* as a model organism for their studies on gene structure, function, and regulation. The completion and release of the entire genome sequence of *S. cerevisiae* will ensure that such interest is maintained.

There is a well-documented battery of vector–transformation systems that can be exploited in engineering the *S. cerevisiae* genome (1). These can be used to remove genes altogether from the genome or simply to replace endogenous genes either with modified forms of the same gene or with heterologous genes. This impressive genetic technology is supported by a wealth of knowledge of the physiology, metabolism, genetics, and cytology of *S. cerevisiae*. Yeasts are also attractive species to choose for the controlled production of recombinant proteins because, by-and-large, they are non-pathogenic, rapidly dividing, unicellular eukaryotes that can be grown easily by researchers with only a modicum of training in microbiology. *S. cerevisiae* has become a workhorse for both fundamental studies and for biotechnological applications. It will be the major focus of this chapter. Nevertheless, there has been an increasing tendency towards using other yeast species—particularly the methylotrophic yeast species, *Pichia pastoris*—for expressing heterologous genes at high levels. This change has been facilitated by the 'off-the-shelf' availability of an expression system for *P. pastoris*. In this chapter we will, therefore, review the basic strategies and techniques for high level expression of cloned genes in both *S. cerevisiae* and *P. pastoris*. However, these are not the only

two yeast species that have been successfully exploited in this context. For example, there has been some success with a variety of other yeast species including *Hansenula polymorpha*, *Kluyveromyces lactis*, and *Yarrowia lipolytica* (2). Very recently, the food yeast *Candida utilis* has also been successfully engineered to express heterologous proteins (3).

It is beyond the scope of this chapter to review all of the vector systems and strategies that have been used to express heterologous genes in *S. cerevisiae* and *P. pastoris* and readers are referred to several comprehensive reviews should they wish to explore the sometimes daunting array of possibilities (4, 5). Rather, we will orientate this chapter towards the researchers who may, for the first time, wish to work with yeasts and to harness their capacity to express genes from other species. The protocols we provide are those that the non-yeast researcher may be coming across for the first time when embarking on such a project. The chapter does not include protocols for the generic molecular biological procedures associated with gene cloning, amplification, mutagenesis, etc. since these can be found in any number of volumes including several in this series. This is not an exhaustive review, however, and the reader will find many variations in the published literature in terms of host strains, promoters, signal sequences, etc.

The primary objectives in expressing a heterologous gene in yeast, as with any other expression host, are usually twofold: to achieve as high a level of expression of the recombinant protein as is possible, and to ensure that it is authentic in terms of both its primary amino acid sequence and, where it is appropriate, its post-translational modifications. Secondary objectives will include the generation of an expression system that shows the necessary genetic stability for scaling up protein production whilst ensuring cost-effectiveness with regard to the use of media and inducers. These objectives are not always achievable and we recognize that there are increasing numbers of researchers who may want to express heterologous genes in yeast simply to see whether they can functionally complement a defect in an homologous yeast gene and thus high level expression may not be a requirement.

Before starting to build a suitable expression system (i.e. an expression vector plus a yeast host strain) the following have to be taken into account:

(a) While approximately 5% of genes in the *S. cerevisiae* genome have one intron (very occasionally more), this species, like other yeast species, is unable to remove introns from the primary transcripts of heterologous genes. Therefore, it is imperative that a cDNA copy of the heterologous coding sequence is used as the starting point for any expression project.

(b) If the target protein requires post-translational modification (e.g. disulfide bond formation, glycosylation), then the protein needs to be targeted to the secretory pathway. However, many yeast species, in particular *S. cerevisiae*, have a very low secretory capacity and thus the chances of obtaining a high yielding secretion system are limited (but not impossible).

(c) One must always consider the possibility that the target heterologous protein may be toxic to the host yeast cell, even though the encoded protein may not have any associated toxicity in its normal cellular environment. If there are such concerns, then it is essential to use an expression system that can be tightly regulated. Particularly problematical in this context are membrane proteins and membrane-associated proteins.

(d) There is no guarantee that there will be a successful outcome to a yeast expression project. There are numerous examples of failure (generally unpublished) where, in spite of optimizing all of the necessary and obvious parameters, only low levels of expression can ever be achieved for a given heterologous gene or cDNA. Yeasts are not alone in this unpredictability and that is why researchers interested in generating high yielding systems will often carry out expression in yeast in parallel with equivalent studies in other expression hosts such as the bacterium, *Escherichia coli* (Chapter 6), mammalian cells (Chapter 1), or baculovirus (Chapter 4).

2. *Saccharomyces cerevisiae* expression systems

The potential of *S. cerevisiae* as an expression host was first realized in 1981 when it was engineered to express human interferon-α1 using the efficient promoter from the alcohol dehydrogenase (*ADH1*) gene (6). This development came only three years after the first description of effective plasmid-based transformation systems (7, 8). Subsequently an impressive array of vector systems has been developed that allows the researcher significant scope when considering how to introduce a heterologous gene into *S. cerevisiae* and then to get it expressed.

2.1 Plasmid-based vectors

There is a bewildering array of vector systems compatible with *S. cerevisiae* to choose from (1), but those which are used to establish heterologous expression systems essentially fall into three major classes:

- autonomous, multicopy plasmids (e.g. YEp or YRp)
- autonomous, single copy plasmids (e.g. YCp)
- integrative (usually single copy) plasmids (e.g. YIp)

Each class of plasmid is circular and contains sequences for replication and selection in both *S. cerevisiae* and *E. coli*. The *E. coli* sequences simply serve to facilitate the engineering and amplification of the plasmid in *E. coli* and are usually derived from one or other of the standard *E. coli* vectors.

For selection of plasmids after transformation in *S. cerevisiae*, it is usual to employ a host strain that has a mutation in a biosynthetic gene, such that the strain depends on one or more nutritional supplements to grow on a fully

defined medium. The plasmid should carry a normal (wild-type) copy of the gene which is defective in the host strain and therefore, when transformed into that strain, will complement the growth deficiency of the host. Examples of genes widely used in this context are the *URA3* gene (required for uracil biosynthesis), *LEU2* (required for leucine biosynthesis), and *HIS3* (required for histidine biosynthesis). Selection of a plasmid carrying, for example, the *URA3* gene can be thus achieved by growing the transformed strain in a defined medium lacking uracil.

To ensure that a vector is able to replicate in *S. cerevisiae*, it must carry either the origin of DNA replication from the endogenous 2 μm plasmid (to generate a YEp vector) or from the chromosomes themselves, the so-called ARS (autonomously replicating sequence) elements (to generate a YRp vector). Both YEp and YRp plasmids are multicopy (between 5–100 copies per haploid cell depending on the vector configuration), but in the absence of selective growth of a strain carrying them, the plasmids are readily lost from the cell. This is particularly problematical when using YRp plasmids. Hence most multicopy expression plasmids are based on YEp vectors because between 80–95% of certain YEp plasmid-containing cells will still contain the plasmid (9) when grown over 15–20 generations in non-selective media.

Inclusion of a centromere from one of the 16 *S. cerevisiae* chromosomes can lead to a further increase in the mitotic stability of a plasmid when cells are grown under non-selective conditions. YCp plasmids have the disadvantage of having very low copy numbers (one or two copies per cell) irrespective of the origin of replication present on the plasmid. Therefore, they are rarely used where high levels of expression are sought. However, because of their mitotic stability, they are useful when expressing heterologous genes to check for functional complementation.

A particularly versatile set of basic autonomously replicating vectors has recently been described for *S. cerevisiae*, the pRS series of plasmids (10). These allow for variation in replication mode, copy number, genetic stability, and selectable marker.

If a high degree of mitotic stability of an expression system is required in cells grown in non-selective media, then an alternative approach is to integrate the heterologous gene and its associated expression signals into the yeast genome. Integration requires that the plasmid vector carries sequences that are homologous to a region of the *S. cerevisiae* chromosome, since integration occurs almost exclusively via homologous recombination. Relatively efficient targeted integration can be achieved by using either a linearized plasmid or a linear DNA fragment excised from a plasmid prior to transformation. Using the uncleaved circular form of a plasmid directly for integration is not to be recommended because it:

(a) Results in the entire plasmid (including the bacterial sequences) being integrated into the genome.

(b) Creates a duplication of the target sequence, which in turn may result in 'looping out' of the plasmid from the genome.

(c) Often gives rise to more than one copy integrated into the genome.

The preferred route for integration is to create an integration cassette (*Figure 1*) in which the heterologous gene and its associated expression sequences are in turn flanked by sequences from the 5′ and 3′ ends of a target endogenous gene. Transformation of such a fragment into *S. cerevisiae* will result in its efficient integration into the genome at the target locus providing the flanking sequences on the integration cassette are large enough to promote efficient and specific recombination. The minimal length of such flanking sequences is about 45–50 bp.

How does one select for integration of an expression cassette when the heterologous gene may not confer a new selectable phenotype on the transformed cell? The solution is to target the cassette to one of two loci which are dispensable for normal growth but which, when rendered non-functional by the integration event, give rise to a selectable phenotype. Two such endogenous genes have proven useful in this respect: *URA3*, integration into which results in resistance to 5-fluoro-orotic acid (5-FOA) (11), and *LYS2*, integration into which results in resistance to α-aminoadipate (αAA) (12).

Should one wish to integrate multiple copies of an expression cassette,

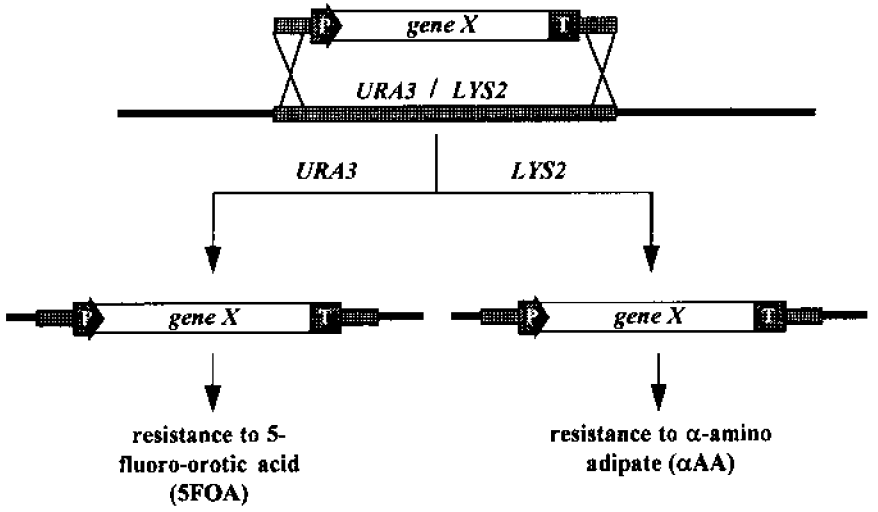

Figure 1. Targeted integration of a gene or gene expression cassette into the genome of *Saccharomyces cerevisiae*. The sequence to be integrated must be flanked by 45–50 bp of DNA (cross-hatched) which is homologous to sequences located 5′ and 3′ respectively of the site at which integration is desired. To allow for positive selection of integration, the target site is *URA3* or *LYS2* since disruption of either gene gives rise to a readily identifiable resistance phenotype as indicated in the figure.

there are several strategies available which exploit reiterated sequences naturally present in the genome. These include strategies for integration into the dispersed retrotransposon Ty sequence (13) or the ribosomal DNA (rDNA) cluster on chromosome XII (14).

2.2 Transformation of *S. cerevisiae*

There are three basic methods for introducing plasmid DNA into *S. cerevisiae*:

(a) Removing the cell wall enzymatically in osmotically-buffered medium to generate sphaeroplasts which can then take up DNA in the presence of polyethylene glycol and Ca^{2+} (7, 8).

(b) Electroporation of intact cells with plasmid DNA (15).

(c) Transformation of whole cells that have been incubated with a lithium salt (16).

While the sphaeroplast-based method is labour-intensive, it is regarded by most researchers as the most efficient method. Nevertheless, the two whole cell-based methods are much simpler, if less efficient, and suitable for a researcher with limited experience of working with *S. cerevisiae*. The lithium method is described in *Protocol 1*. All three methods can be used for transformation with either circular or linearized DNA templates, although in the case of a linearized DNA template, the frequency of transformation will be approximately one order of magnitude lower than for a circular template. Prepare the plasmid DNA for transformation by any standard, rapid, small scale method (17). The choice of *S. cerevisiae* strain is discussed in Section 2.3.

Protocol 1. Transformation of *S. cerevisiae* using the lithium method

Equipment and reagents

- *S. cerevisiae* strain to be used (see Section 2.3)
- Sterile double distilled or deionized water[a]
- Filters for filter sterilizing solutions (Whatman, 0.2 μm, cellulose nitrate filters)
- 20% (w/v) D-glucose[a]
- YPD medium: dissolve 10 g yeast extract (Difco, 0127-01-7) and 20 g Bactopeptone (Difco, 0118-01-8) in 1 litre of distilled water and autoclave. Cool the medium to room temperature and aseptically add 0.1 vol. 20% autoclaved or filter sterilized glucose.
- Shaker incubator at 30°C
- Sterile 250 ml conical flask (Corning, 25600-250)
- TE buffer: 10 mM Tris–HCl pH 7.4, 1 mM EDTA[a]
- Sterile 50 ml centrifuge tubes (Falcon, 2098)
- LA buffer: 0.1 ml lithium acetate in TE buffer[a]
- Sterile 15 ml polypropylene snap-top tubes (Falcon, 2059)
- Plasmid DNA: $\geq$ 0.5 μg/μl in sterile water or TE buffer
- 50% (w/v) PEG (polyethylene glycol 4000, BDH, 44273 4X): dissolve the PEG in TE buffer, then filter sterilize. Store the solution for up to one week only.
- Sterile plastic Petri dishes (Sterilin, Triple Vent, 109132P)
- 10 × YNB medium: dissolve 6.7 g yeast nitrogen base without amino acids (Difco, 0919-15-3) in 100 ml distilled water[a]

- 100 × stock solutions of each of the following medium supplements: adenine sulfate, uracil, L-tryptophan, L-histidine–HCl, L-arginine–HCl, L-threonine–HCl (each at 2 mg/ml); L-tyrosine, L-leucine, L-isoleucine, L-lysine–HCl (each at 3 mg/ml); L-phenylalanine (5 mg/ml); L-glutamic acid and L-aspartic acid (each at 10 mg/ml); L-valine (15 mg/ml); L-threonine (20 mg/ml); and L-serine (37.5 mg/ml). Filter sterilize each of these stock solutions.
- YNBD plates: dissolve 0.67 g agar in 100 ml 10 × YNB medium and 984 ml sterile water by autoclaving. Cool the molten agar to about 50°C and aseptically add 1 ml of each of the 100 × stock solutions of the medium supplements listed above. Mix, pour the agar into Petri dishes, and allow them to set.

Method

1. Prepare a 50 ml overnight starter culture in YPD medium of the *S. cerevisiae* strain to be transformed. Grow the cells at 30°C with shaking. Measure the A_{600}.
2. Dilute the overnight starter culture into 50 ml YPD medium in a 250 ml conical flask to an A_{600} = 0.25 (typically a 20-fold dilution). Incubate the culture at 30°C with shaking until the A_{600} = 1. This usually takes 4–5 h.
3. Harvest the cells in a sterile 50 ml centrifuge tube by centrifugation at room temperature (2000 *g*, for 5 min). Resuspend the cell pellet in 50 ml TE buffer and centrifuge again.
4. Resuspend the cells in 2.5 ml LA buffer and incubate them at 30°C with shaking for 1 h. This volume of competent cells is sufficient for eight transformations.
5. For each transformation, dispense 0.3 ml competent cells into a 15 ml polypropylene snap-top tube. Add up to 10 μg vector DNA (in a maximum of 20 μl) and 0.7 ml of 50% PEG.[b] Mix gently and incubate at 30°C for 1 h, without shaking.
6. Heat shock the cells by heating them for 5 min at 42°C.
7. Collect the cells by centrifugation as in step 3 and remove the excess PEG from the pellet.
8. Resuspend the cells in 200 μl TE buffer and plate them out on YNBD plates.[c] Incubate the plates at 30°C. Transformants should appear after three to seven days.

[a] These solutions should be sterilized by either autoclaving them (120°C, 20 min) or passing them through a sterile filter.

[b] Transformation frequencies can be increased by adding 200 μg single-stranded carrier DNA, but this is not usually necessary. If this is required, dissolve salmon sperm DNA to 10 mg/ml in sterile water. Shear the DNA by passing the solution several times through a 25 gauge needle using a disposable sterile syringe. Boil the sheared DNA for 10 min and store the solution at –20°C.

[c] If selecting for the *LEU2*-d marker (see Section 2.3) it is necessary to incubate transformants overnight at 30°C with shaking in YPD medium before selection on the YNBD plates.

2.3 Choice of strain of *S. cerevisiae*

There is a bewildering choice of strains of *S. cerevisiae* that are suitable for transformation by plasmid DNA. However, if one is considering expression of a plasmid-borne gene following transformation then the number of strains one can reliably use is somewhat reduced. The properties one would need for such a strain are as follows:

(a) It should carry at least two different non-revertible mutations that can be complemented by an appropriate plasmid-borne gene. The most commonly used in this context are the *ura3-52* mutation (complemented by the *URA3* gene) and the *leu2-3,112* mutation (complemented by the *LEU2* gene).

(b) If the vector to be used carries a significant part of the endogenous 2 μm plasmid, then a strain lacking the 2 μm plasmid (2 μm^0) should be used to prevent plasmid rearrangement by intermolecular recombination between the incoming plasmid and the endogenous 2 μm plasmid. However, if the plasmid only carries the *ORI-STB* sequence of the 2 μm plasmid, then the host strain must contain the endogenous 2 μm plasmid in order to supply the necessary *trans*-acting replication and maintenance factors.

(c) The inclusion of a mutation in one or more of the genes known to encode a protease may increase the final yield of some proteins. For example, *pep4* mutants, which are defective in a variety of vacuolar proteases, and *pra1 prb1* double mutants, which are deficient in specific vacuolar endopeptidases, can be used.

It is beyond the scope of this article to review all the strains that have been used for expressing heterologous genes in *S. cerevisiae*. Based on our personal experiences, the following have proved useful:

- S150-2B (*a leu2-3,112 ura3-52 trp1-289 his3Δ1*)
- MD40/4c (*a leu2-3,112 his3-11,15 ura2 trp1*)
- JRY188 (α *leu2-3,112 ura3-52 his3 trp1 sir3*)
- BJ5457 (*a leu2Δ1 ura3-52 trp1 lys2-801 his3Δ200 pep4::HIS3 prb1Δ1 can1 GAL*)

These and other suitable strains can be obtained from either the National Collection of Yeast Cultures (NCYC), or the Yeast Genetics Stock Center (YGSC) whose addresses are in the Appendix.

While the described and/or published genotype of a strain can be used as a guide to the choice of the strain, the extensive genealogy of laboratory strains unfortunately means that there are often other undefined mutations present which may influence achievable expression levels for a heterologous protein. For example, several standard strains carry the *gal2* mutation which results in

a defect in galactose permease and hence cannot be used for expression from the galactose-regulated *GAL* promoter (Section 2.4.1).

2.4 Transcription and translation of heterologous genes and cDNAs

Any gene or cDNA that one wishes to express in *S. cerevisiae* must be efficiently transcribed and translated. While a great deal is known about the processes of transcription and translation in *S. cerevisiae*, it is clear that in many aspects both processes show subtle differences to those of higher eukaryotes. This necessitates careful consideration of how one optimizes both processes with a heterologous sequence, since either transcription or translation can be a rate limiting step in the expression pathway. The rest of this section discusses how this may be done.

2.4.1 Transcription of heterologous sequences

To get a heterologous gene/cDNA transcribed in *S. cerevisiae,* it must be cloned downstream of an *S. cerevisiae* promoter, because almost invariably heterologous promoters are not recognized by the *S. cerevisiae* RNA polymerase II and associated transcription factors. As with strain choice, many options are available that exploit either natural promoters or promoters that have been artificially created by genetic engineering.

The two most desirable properties of a promoter are that it is tightly regulated, such that its induction can be separated from the growth phase of the culture, and that when induced it shows a high level of transcriptional activity. Regulation is particularly desirable where the heterologous protein is known, or suspected, to be toxic to yeast.

Table 1 lists the most commonly utilized promoters for expressing heterologous genes in *S. cerevisiae.* Of these, the galactose-regulated promoters (from either the *GAL1*, *GAL7*, or *GAL10* genes) are the most popular choice: they are strongly repressed by glucose and, in the absence of glucose, can be induced up to 1000-fold by the addition of galactose. Furthermore, the upstream activation sequence (UAS_{GAL}) from the *GAL* gene promoters, through which the glucose/galactose regulation is mediated, can be used to transfer suitable regulatory properties to a high efficiency promoter that is itself poorly regulated. An example of one such fusion promoter is the *PGK-GAL* promoter (18).

The galactose-regulated promoters can only be effectively induced following depletion of glucose from an initial glucose-based growth medium, or by initially growing the cells on a non-repressing carbon source such as glycerol, raffinose, or lactate. While such non-repressing carbon sources lead to poor growth relative to that achievable with glucose as a carbon source, the addition of galactose to such cells results in rapid induction of transcription. This induction strategy, described in *Protocol 2*, is ideal if one is expressing an

Table 1. Promoters commonly used for the expression of heterologous proteins in *Saccharomyces cerevisiae*

Promoter	Properties
Natural promoters	
PGK	High efficiency, moderately inducible by glucose.
GAP	High efficiency, moderately inducible by glucose.
TEF1	High efficiency, constitutive.
GAL1	Efficient, 1000-fold induction by galactose.
ADH2	Efficient, repressed by glucose.
PHO5	Moderately efficient, repressed by inorganic phosphate.
CUP1	Low-to-moderate efficiency, induced by Cu^{2+}.
MFα1	Low–to-moderate efficiency, constitutive.
TRP1	Very low efficiency, constitutive.
Engineered promoters	
PGK/GAL (PAL)	High efficiency, induced by galactose, consisting of the *PGK* promoter with the UAS sequence from the GAL1 promoter inserted.
GAP/GAL	As for the *PGK/GAL* promoter, but based on the *GAP* promoter.
GAP/ADH2	High efficiency, repressed by glucose, consisting of the *GAP* promoter with the UAS sequence from the *ADH2* promoter inserted.
CYC1/GRE	Moderate efficiency, induced by deoxycorticosterone, consisting of the *CYC1* promoter with a glucocorticoid response element (GRE) inserted. NB: a host strain must express the glucocorticoid receptor.
PGK/ARE	High efficiency, induced by dihydrotestosterone, consisting of the *PGK* promoter with the androgen response element (ARE) inserted. NB: the host strain must express the androgen receptor.

unstable protein. There are alternative strategies which can be used for induction of *GAL* promoters; for example, cells can be grown on a mixture of glucose and galactose with induction occurring once the glucose (which is metabolized in preference to galactose) has been utilized. Such alternative strategies are less controllable than the one described in *Protocol 2*, but are simple and usually adequate for pilot studies. In addition, this alternative strategy may be preferable to the use of raffinose grown cells for large scale cultures.

Protocol 2. Induction of *GAL* promoters

Equipment and reagents

- Appropriate strain of *S. cerevisiae*
- YPD medium and sterile water (see *Protocol 1*)
- 20% (w/v) raffinose: autoclave or filter sterilize (see *Protocol 1*)
- YPD medium plus 2% raffinose
- Sterile plastic 25 ml Universal containers (Nunc, 3-64211)
- 20% (w/v) galactose (Sigma, G0750, less than 0.01% glucose): autoclave or filter sterilize (see *Protocol 1*)

Method[a]

1. Prepare an overnight starter culture of the transformant in YPD medium as described in *Protocol 1*, step 1.
2. Dilute the starter culture into 10 ml YPD medium plus 2% raffinose in a 25 ml Universal container to an A_{600} = 0.25 (typically a 20-fold dilution). Incubate the culture at 30°C with shaking until A_{600} = 1. This usually takes 4–5 h.
3. Add 1 ml 20% galactose and incubate with shaking at 30°C for 8–24 h (typically overnight). Measure the A_{600} and harvest the cells by centrifugation (see *Protocol 1*, step 3) when the A_{600} = 10–15.

[a] If one is dealing with a strain carrying a plasmid which requires selection, then a synthetic YNB-based medium can be used. For step 1, use a synthetic complete medium. Dissolve 6.7 g yeast nitrogen base without amino acids (Difco, 0919-15-3), 10 g succinic acid, 6 g NaOH, 5 g low salt Casamino acids (Difco, 0230-01-1), and 20 g glucose in 1 litre of distilled water, and then autoclave. For step 2, use a non-inducing synthetic medium (synthetic complete medium with 0.5 g glucose, 20 g sodium lactate, and 30 g glycerol as the carbon source).

In addition to considering the initiation of transcription, one must also ensure that transcription of the heterologous sequence is correctly terminated and that formation of the 3′ end of the mRNA is able to take place. As with promoter sequences, the termination/polyadenylation sequences of *S. cerevisiae* genes are different to those of both higher eukaryotes and prokaryotes, even though the basic process of 3′ end formation and polyadenylation has been conserved. It is therefore recommended to use an expression vector that contains a yeast terminator sequence downstream of the cloning site (see *Figure 1*). Because the exact identity and/or location of the necessary sequences for termination of transcription are poorly defined in *S. cerevisiae* genes, this means that expression vectors usually include the 200–300 bp of DNA located downstream of the coding sequence of a yeast gene. The most commonly used gene sequences in this context are from the *CYC1*, *PGK1*, or *URA3* genes, or from one or other of the genes of the 2 μm plasmid.

2.4.2 Translation of heterologous mRNAs

In engineering a heterologous gene or cDNA for expression in *S. cerevisiae*, not only must consideration be given to the optimization of transcription, but also to ensuring that the encoded mRNA is efficiently translated. Critically, attention must be paid to the 5′ untranslated region (5′ UTR), i.e. the mRNA sequence from the 5′ capped end of the mRNA up to the initiation codon. This is particularly so if the target gene has been subcloned out of a polylinker cloning site since this will often involve the inclusion of vector sequences in the 5′ UTR.

For the 5′ UTR of an mRNA to present no translational barriers, it should *not* have any of the following features:

(a) An AUG codon 5′ to the required initiation codon. As with mammalian mRNAs, yeast ribosomes initiate translation usually (but not invariably) from the first AUG encountered by the scanning ribosomal initiation complex.

(b) Runs of Gs since their presence in yeast 5′ UTRs is rare, and, when introduced, lead to a significant reduction in the efficiency of translation. It is preferable to have an AU-rich 5′ UTR since this is a common feature of yeast 5′ UTRs (19).

(c) Sequences that can potentially form a stable stem structure by intramolecular base pairing either with other sequences in the 5′ UTR of the mRNA or with sequences elsewhere in the mRNA. Stems with thermodynamic stabilities of > –28 kcal/mol can lead to 98% inhibition of translation of a heterologous mRNA in *S. cerevisiae* (20).

Unlike mammalian mRNAs there is little evidence for an optimal nucleotide context for the AUG codon to be efficiently recognized by yeast ribosomes although analysis of yeast mRNA sequences suggest that five A nucleotides usually precede the initiation codon (19). In addition, yeast 5′ UTRs are generally 25–50 nucleotides long although some longer unstructured 5′ UTRs are naturally found in efficiently translated mRNAs (19).

There is limited evidence that the use of non-preferred codons reduces the overall efficiency of translation of an mRNA in *S. cerevisiae* (2). The inclusion of runs of non-preferred codons may result in elevated levels of mistranslation at those codons with either the incorrect amino acids being introduced, or the ribosomes shifting to another reading frame (21). Similarly, there is no clear consensus sequence environment for the termination codon (UAA, UAG, or UGA) for optimal efficiency of termination. However, recent studies have demonstrated that base changes in the codon preceding the termination codon can have dramatic effects on the efficiency of termination (22). Strains of yeast carrying the yeast prion [*PSI*$^+$] have a reduced efficiency of termination *in vivo* and, therefore, non-prion [*psi*$^-$] strains should be used. If the [*PSI*] status of a cell is unknown, then simply growing a strain in YPD medium containing 2% glucose (see *Protocol 1*) and 5 mM guanidine hydrochloride at 30°C for approximately eight to ten generations will result in a [*PSI*$^+$] cell becoming [*psi*$^-$] (23)

2.5 Directing the extracellular synthesis of heterologous proteins

Many of the heterologous proteins whose synthesis is sought in *S. cerevisiae* are secretory or membrane-associated proteins. Yet *S. cerevisiae*, unlike many fungi, naturally secretes very few proteins and even those that are secreted

are present at only very low levels in the culture medium. Nevertheless, there has been considerable success in engineering *S. cerevisiae* to secrete heterologous proteins, particularly small proteins such as epidermal growth factor or unglycosylated proteins such as human serum albumin where levels of up to 2 g/litre of cells have been reported (24).

The following parts of this section discuss some of the factors involved in secretion of heterologous proteins from *S. cerevisiae.*

2.5.1 Signal sequences

An absolute requirement for a protein to enter the *S. cerevisiae* secretory pathway is the presence of an N-terminal hydrophobic signal peptide. Although the majority of heterologous signal peptides will not direct efficient secretion of a protein from *S. cerevisiae*, there are some that do. For example, the signal peptide of human serum albumin is very effective in this context and has been used to direct the secretion of a number of heterologous proteins (25). However, the majority of secretion vectors for use with *S. cerevisiae* contain an homologous signal peptide from the yeast prepro-α-factor (MFα1). The *MFα1* gene encodes a 165 amino acid protein, prepro-α-factor, which carries a 22 residue signal (pre)peptide and a 61 residue pro-region linked to four tandem copies of the 13 residue mating factor and pheromone. The pre-peptide is cleaved by the ER membrane-associated signal peptidase, while the pro-region, which contains three *N*-linked glycosylation sites, is subsequently cleaved within the Golgi complex by the Kex2p enzyme. This cleaves on the C-terminal side of the Lys–Arg pair that separates the pro-region from the four tandem repeats of the α-factor (*Figure 2A*).

Many vectors have been built that facilitate the in-frame attachment of the *MFα1* prepro region to the target heterologous protein. In such vectors, it is important that careful attention is paid to the junction between the prepro region and the heterologous protein sequence. Cleavage by Kex2p at the Lys–Arg sequence must generate an authentic N-terminal sequence on the heterologous proteins. One vector we have found useful for this is pGS4 (26) which contains the *GAL1* promoter fused to the *MFα1* prepro sequence, followed by a *Stu*I site (*Figure 2B*). The coding sequence of the mature heterologous protein will abut the Lys–Arg junction of the signal sequence, and so cleavage by Kex2p will generate an authentic N-terminus on the secreted protein.

In some instances, there is an additional requirement for inclusion of a pair of Glu–Ala 'spacers' C-terminal to the Lys–Arg residues. These 'spacers' are present in the *MFα1*-encoded prepro-α-factor (*Figure 2A*) and, in some cases, are necessary (but not essential) for efficient Kex2p-mediated cleavage after the Lys–Arg residues. If the need for the Glu–Ala spacers is unclear for a specific chimeric protein, a plasmid such as pWYG82 (*Figure 2C*) may be used. In this, the heterologous gene sequence can be inserted between the *Xho*I site and the other unique restriction sites in the polylinker using

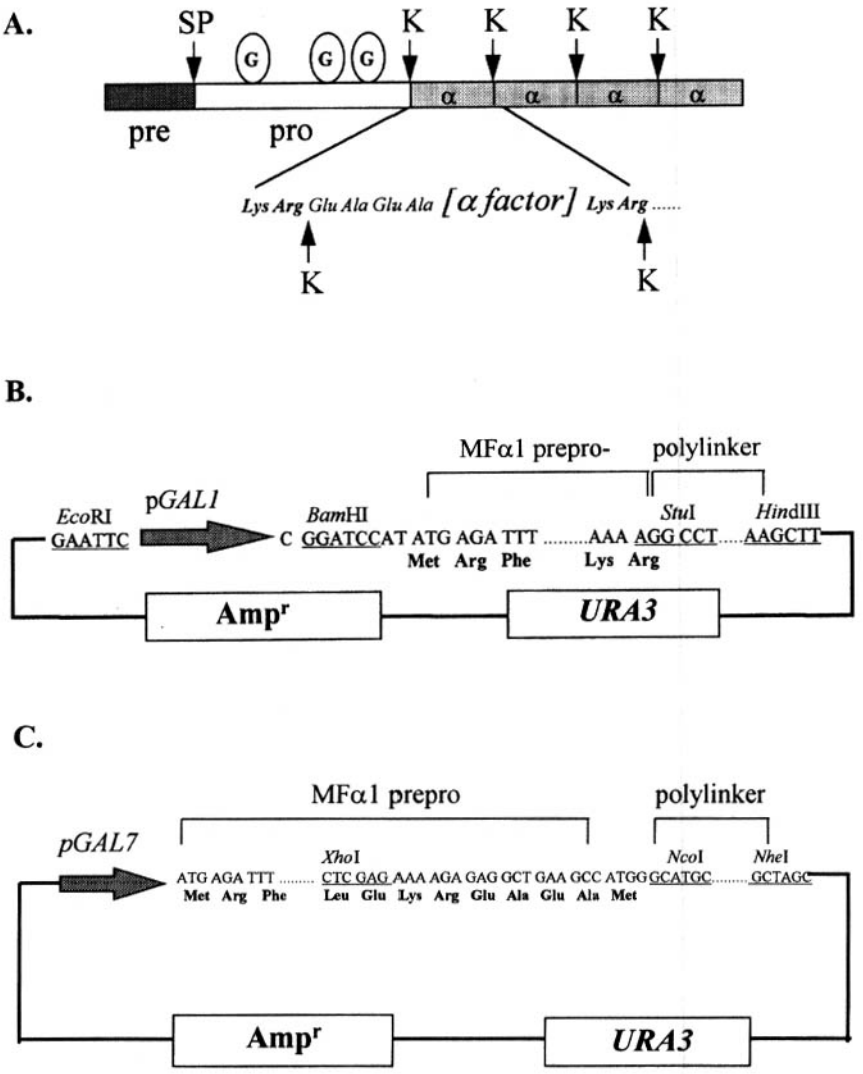

Figure 2. The prepro-α-factor encoding gene (MFα1) of *Saccharomyces cerevisiae* and its exploitation in the generation of expression vectors for the secretion of heterologous proteins. (A) Organization of prepro-α-factor of *S. cerevisiae* indicating the location of the 22 amino acid pre (or signal) sequence, the 61 amino acid pro-region (including three *N*-linked glycosylation sites, G), and the four copies of the α-factor sequence (α) each of which is contained within a larger 19 amino acid sequence. The cleavage sites for signal peptidase (SP) and Kex2p (K) are indicated. (B) Organization of plasmid pGS4 showing the location of the *GAL1* promoter (*pGAL1*), the MFα1 prepro region, and the polylinker. Full details of the plasmid and its use are given in the text. (C) Organization of plasmid pWYG82 showing the location of the *GAL7* promoter (*pGAL7*), the MFα1 prepro region, and the polylinker. Full details of the plasmid and its use are in the text.

synthetic oligonucleotides to reconstruct the required junction, i.e. Lys–Arg or Lys–Arg $(Glu\text{-}Ala)_2$.

Ideally, the secretion vectors based on the *MFα1* prepro region should utilize a promoter other than the *MFα1* promoter, since this promoter is only active in cells of α mating type. For example, both pGS4 (*Figure 2B*) and pWYG82 (*Figure 2C*) utilize *GAL* promoters.

Other than the *MFα1* prepro sequence, another homologous signal sequence used successfully for secreting heterologous proteins is that of invertase, encoded by the *SUC2* gene (27).

2.5.2 Post-translational modification

After a secretory protein has been translocated into the lumen of the ER, it immediately becomes available to a number of lumenal proteins that facilitate the correct folding of the protein prior to its continuation through the secretory pathway. The lumenal ER proteins include Kar2p/BiP, the product of the *KAR2* gene (a highly conserved molecular chaperone), and protein disulfide isomerase (PDI) which mediates the efficient and accurate formation of disulfide bonds (28). With one exception, the lumenal components of the yeast ER are similar to those of the mammalian ER. The one exception is endoplasmin (also called Grp94), a molecular chaperone with homology to the Hsp90 family of stress proteins, which is missing from yeast's protein folding repertoire.

Correct folding of a heterologous secretory protein in the ER is essential if one is to achieve efficient secretion of the heterologous protein. Failure of a protein to fold correctly will result in its accumulation in the ER and the subsequent activation of the 'unfolded protein response' in which genes encoding the major soluble components of the ER (e.g. *KAR2*, *PDI1*) are transcriptionally activated, leading to higher levels of the corresponding folding factors (29). While this response may ensure the correct folding of homologous secretory proteins, it remains to be determined whether heterologous proteins need their homologous protein folding factors to facilitate their efficient folding. Engineered overexpression of the PDI-encoding gene *PDI1* does lead to enhanced secretion from yeast of secretory proteins with multiple disulfide bonds (30).

In addition to the folding activities within the lumen of the ER, a number of additional post-translational modifications may be required to a heterologous protein before it can be transported via the complex network of membrane structures to the outer surface. Particularly important is *N*- and *O*-linked glycosylation in which sugars are covalently attached to the protein. In *N*-linked glycosylation, a core sugar structure is transferred to the *N*-amide group of Asn if it lies within the amino acid sequence Asn–X–Ser/Thr where X is any amino acid apart from proline. In this sense, *N*-linked glycosylation in *S. cerevisiae* follows the same rules as in higher eukaryotic cells. Following the addition of the core sugars to the secretory protein in the ER, this structure undergoes additional modification in both the ER and the Golgi complex.

However, in *S. cerevisiae* these additional modifications are simply an expansion of the mannose scaffold rather than the formation of shortened side branches ending with complex sugars such as sialic acid and fucose. Consequently, there may be significant differences in the nature of the glycosylation between the yeast-derived and native glycoprotein—differences that will be immediately apparent by comparison of their M_r by SDS–PAGE.

The authenticity of glycosylation of heterologous proteins is an important issue, and one which can limit the use of *S. cerevisiae* as an expression host. The addition of large numbers of mannose residues to heterologous proteins is clearly undesirable since these non-native sugars may represent immunogenic determinants.

In addition to *N*-linked glycosylation, *S. cerevisiae* is also able to carry out *O*-linked glycosylation although again there may be differences in the mechanism and/or outcome of their modification between yeast and mammalian cells. *S. cerevisiae* is also able to carry out a variety of other post-translational modifications including N-terminal acetylation, phosphorylation, myristylation, and isoprenylation (2).

Ultimately, it must be recognized that *S. cerevisiae* does not efficiently secrete its own proteins and therefore, because of this low natural capacity, it is very easy to saturate the system by overexpressing and targeting a heterologous protein to the secretory pathway. It is, therefore, advisable to utilize an expression system that does not employ a high efficiency promoter; for example the *MFα1* promoter. As indicated above, the engineered overexpression of specific components of the secretory machinery may alleviate one or two specific bottlenecks, but it is unlikely that all rate limiting steps can be overcome by such a rational approach. An alternative, more empirical, approach can be employed, namely using standard mutagenesis protocols to produce mutant strains that show enhanced levels of secretion of the target protein. For example, this has been successfully exploited to produce strains that efficiently secrete human serum albumin (24).

Once a secretion system has been set up for a target protein, one must also pay due attention to optimization of the physiological conditions employed. For example, the use of buffered medium may be beneficial when growing cells at temperatures below 28 °C, while slowing down the growth rate of the yeast often results in increased efficiency of secretion.

2.6 Analysis of heterologous gene expression

Following transformation of an expression system into *S. cerevisiae*, successful expression of the target gene or cDNA should be checked at one or all of three different levels:

- mRNA synthesis
- protein synthesis
- biological activity

2.6.1 mRNA synthesis

The most straightforward check for expression is to use Northern blot analysis to confirm that the heterologous sequence has been transcribed. There are several suitable protocols for preparing intact mRNA from *S. cerevisiae* that is suitable for blotting. The protocol we describe here (*Protocol 3*) generally gives good yields and intact mRNA. Ideally, the RNA should be prepared from exponentially growing cells since in non-growing cells transcription will be repressed and consequently the steady state level of mRNA will be significantly reduced via RNA turnover in the absence of ongoing transcription. With the average half-life of yeast mRNA being of the order of 20–30 min (31), it would only require 1–2 h of transcriptional inactivity to lead to a significant reduction in the steady state levels of mRNA. There is no evidence that heterologous mRNAs are turned over any more rapidly in *S. cerevisiae* than are homologous mRNAs.

Protocol 3. RNA preparation

Equipment and reagents

- 50 ml culture *S. cerevisiae*, exponentially growing (A_{600} = 1) (see *Protocol 1*, steps 1–2)
- Guanidinium isothiocyanate solution: dissolve 50 g guanidinium isothiocyanate and 0.5 g sodium *N*-laurylsarcosinate in 80 ml water. Add 2.5 ml of 1 M sodium citrate pH 7 and 0.7 ml of 2-mercaptoethanol. Make up the solution to 100 ml with water and filter. Store the solution at 4°C.
- 15 ml polypropylene tubes with caps (Sarstedt, 55.510): soak these overnight in 0.1% diethyl pyrocarbonate (DEPC), and then autoclave them at 120°C for 20 min
- Acid washed glass beads (0.45 mm dia., Braun): soak them for 16 h in concentrated HCl, rinse thoroughly in distilled water, and bake them for 16 h at above 150°C
- Phenol, equilibrated with 1 M Tris–HCl pH 8
- Platform vortex mixer (IKA-Vibrax-VXR) with test-tube rack (Merck BDH, 330/0360/00)
- Phenol:chloroform:isoamyl alcohol (25:24:1, by vol.)
- Chloroform:isoamyl alcohol (24:1, v/v)
- DEPC treated water: add DEPC to distilled water (final concentration 0.1%, v/v) in a screw-capped bottle. Mix by shaking. Loosen the lid, leave the solution overnight, then autoclave it at 120°C for 20 min.
- 1 M acetic acid (in DEPC treated water)
- Ethanol: absolute and 70% (v/v) solution (in DEPC treated water)
- 1.5 ml microcentrifuge tubes treated with DEPC as for the polypropylene tubes
- 3 M sodium acetate (pH 4.5 with acetic acid)

Method

1. Harvest the yeast cells by centrifugation at 4°C (2000 *g*, 10 min). Resuspend the pellet in 10 ml ice-cold water and recentrifuge.
2. Resuspend the pellet in 3 ml guanidinium isothiocyanate solution and transfer the suspension to a DEPC treated 15 ml polypropylene tube.
3. Add acid washed glass beads to two-thirds the height of the meniscus, place the tubes in a platform vortex mixer in a cold room, and vortex at full speed for 15 min.
4. Add 1 vol. phenol:chloroform:isoamyl alcohol to the lysate, vortex

Protocol 3. *Continued*

again (see step 3), and centrifuge at 4°C (10000 *g*, 15 min). Transfer the upper (aqueous) phase to a new polypropylene tube.

5. Re-extract the aqueous phase repeatedly with phenol:chloroform: isoamyl alcohol, as in step 4, until there is little or no material left at the interface between the two phases.
6. Extract the aqueous phase once with 1 vol. chloroform:isoamyl alcohol, as in step 5.
7. Transfer the aqueous phase to a clean DEPC treated polypropylene tube. Precipitate RNA by adding 0.025 vol. 1 M acetic acid and 0.75 vol. absolute ethanol. Leave the mixture at –20°C for at least 30 min.
8. Collect the RNA precipitate by centrifugation at 4°C (10000 *g*, 15 min) and drain off the supernatant.
9. Vortex the pellet in 70% ethanol and centrifuge, as in step 8.
10. Dissolve the RNA precipitate in 0.4 ml DEPC treated water at 4°C. Transfer the solution to a DEPC treated 1.5 ml microcentrifuge tube. Vortex it with 0.4 ml phenol:chloroform:isoamyl alcohol, as in step 4. Re-extract the aqueous phase once with an equal volume of chloroform:isoamyl alcohol.
11. Reprecipitate the RNA by adding 0.1 vol. 3 M sodium acetate and 2.5 vol. absolute ethanol at –20°C (see step 7).
12. Collect the precipitate by centrifugation in a microcentrifuge (12000 *g*) at 4°C for 15 min.
13. Remove the supernatant and resuspend the RNA pellet in 100 μl DEPC treated water. Store the RNA solution at –70°C.[a]

[a]The original 50 ml culture grown to A_{600} = 1 should yield 150–300 μg RNA. For gel electrophoresis and Northern blotting, use 5–20 μg per gel track.

2.6.2 Protein synthesis

The presence of an abundant heterologous transcript does not guarantee that the translation product will be equally abundant or even synthesized. To check whether a non-secreted heterologous protein is synthesized, cell-free extracts must be prepared from the appropriate transformed strain and analysed by a combination of SDS–PAGE and Western blot analysis using a monoclonal or polyclonal antibody raised against the heterologous protein.

Several mechanical methods have been developed for disrupting yeast cells to obtain extracts suitable for analysis. Although a French Press or Eaton Press can be used, the most rapid and efficient method for preparations on an analytical or preparative scale is disruption with glass beads (*Protocol 4*). In preparing lysates, steps must be taken to minimize the likelihood of proteolysis.

The two most important are to carry out the procedure at 2–4 °C and in the presence of a cocktail of protease inhibitors (see *Protocol 4*).

An alternative approach to disruption with glass beads is to prepare sphaeroplasts from the cells and then lyse these at a high concentration in a hypotonic buffer. This approach, while perhaps giving higher yields of protein, has a number of drawbacks: these include the need to remove all traces of the lytic enzymes used to prepare the sphaeroplasts (since they are usually rich in proteases), the need to have a high yield of sphaeroplasts (which can be negatively influenced by a variety of factors, including growth phase, genotype, and quality of the lytic enzyme preparation), and the need to employ mechanical means to ensure effective disruption of the sphaeroplasts in the hypotonic buffer.

Once a concentrated protein extract has been prepared, the most straightforward method of analysis is one-dimensional SDS–PAGE followed by visualization of the proteins with either Coomassie Blue or silver staining. To detect and identify the target protein, expression levels must be relatively high (> 1% total cell protein) and the protein pattern must be compared with that of an extract prepared from the same strain transformed with the non-recombinant expression vector. An approximate quantification of the level of expression can be obtained by densitometric analysis of the stained gel. A further problem in detection may arise if the heterologous protein co-migrates with an abundant endogenous protein, although this can usually be overcome by resorting to two-dimensional gel electrophoresis (32).

However, the most direct and effective means of detecting the target heterologous protein is by Western blotting. This will of course require an appropriate antibody, although in the absence of a suitable antibody one can 'tag' the target heterologous protein with a short protein sequence against which an antibody is available, e.g. the FLAG epitope tag or the HA epitope tag (33, 34). However, if the objective is to express an authentic heterologous protein, the latter is not an option. Low level synthesis (i.e. less than 1% total cell protein) of a heterologous protein can usually be detected by Western blot analysis, and sensitivity can be increased by radiolabelling proteins *in vivo* with [^{35}S]methionine, immunoprecipitating the heterologous protein with a suitable antibody, and detecting its presence by autoradiography.

Protocol 4. Preparation of protein extracts

Equipment and reagents

- 10 ml culture *S. cerevisiae* grown to A_{600} = 10 (see *Protocol 1*, step 1)
- Distilled or deionized water (autoclaved)
- Sterile 15 ml polypropylene snap-top tubes (Falcon, 2059)
- Extraction buffer: 50 mM Tris–HCl pH 7.4, 100 mM NaCl, 2 mM EDTA—cool this on ice
- Platform vortex mixer and acid washed glass beads (*Protocol 3*)
- 5 × protease inhibitor cocktail: 20 mM EDTA, 20 mM EGTA, 20 mM PMSF, 10 mg/ml pepstatin, 10 mg/ml leupeptin, 10 mg/ml chymostatin, 10 mg/ml antipain—store this at –20 °C
- Phase-contrast microscope
- Pasteur pipettes (fine tipped, e.g. Volac)
- Materials for assaying total protein (Bio-Rad protein assay kit, or similar)

Protocol 4. *Continued*

Method

1. Harvest the cells from a 10 ml culture by centrifugation (4°C, 2000 *g*, 5 min).
2. Resuspend the cell pellet in 10 ml water, transfer the cell suspension to a 15 ml polypropylene snap-top tube, and centrifuge (as in step 1). Remove excess supernatant from the pellet.[a]
3. Resuspend the cell pellet in 0.5 ml ice-cold extraction buffer containing 125 μl 5 × protease inhibitor cocktail.
4. Add glass beads to two-thirds the height of the meniscus, place the tubes on a platform vortex mixer in a cold room, and vortex at full speed for 10 min.[b,c]
5. Check the cells for breakage by phase-contrast light microscopy. Intact cells appear bright, while broken cells are phase-dark 'ghosts'.
6. Remove the cell extract from the glass beads and store it in a clean tube.
7. Use a fine-tipped plastic Pasteur pipette to recover as much of the extract as possible. Re-extract the beads with 0.5 ml ice-cold extraction buffer.[d]
8. Pool the extracts (steps 6 and 7) and determine the total protein concentration using the assay kit. The yield of total protein from a 10 ml culture grown to A_{600} = 10 is approx. 10 mg.
9. If necessary, remove any insoluble protein by centrifugation for 15 min at 4°C in a microcentrifuge (12000 *g*).[e] Transfer the supernatant (soluble protein) to a new tube and determine the protein concentration (as in step 8). Keep the supernatant and the pellet (insoluble proteins) for subsequent analysis.[f]
10. Store the protein extract (and the soluble and insoluble fractions, if prepared in step 9) frozen at –20°C until analysed by SDS–PAGE or Western blotting.[g]

[a] At this point, the washed cell pellet may be stored at –70°C, if desired.
[b] Alternatively the samples can be vortexed by hand using a standard laboratory vortex mixer in six 30 sec bursts interspersed by cooling for 30 sec on ice.
[c] Breakage is most efficient in wide round-bottom tubes such as those recommended here. For larger volumes use 30 ml centrifuge tubes or a 'Bead Beater' (Biospec Products).
[d] An alternative method for recovering the extract (steps 6 and 7) is to centrifuge the suspension for 2 min at 1000 *g* through a plastic disposable filter (Poly-prep chromatography column; Bio-Rad, 731-1550). It will be necessary to check in a pilot run that the protein of interest is not retained by the filter.
[e] For large scale purification of proteins, the preparation may be centrifuged at 100000 *g* for 1 h to precipitate the cellular membranes.
[f] To determine the proportion of a protein that is soluble, resuspend the insoluble protein pellet in the same volume of buffer as the soluble protein preparation, and analyse equal volumes of 'total', 'soluble', and 'insoluble' protein by SDS–PAGE.
[g] Upon thawing, a precipitate, largely consisting of cell membranes, may be seen. This should be dispersed prior to SDS–PAGE by placing the tube in a boiling water-bath for 1–2 min.

Protocol 4 describes the preparation of yeast cell extracts suitable for analysis by SDS–PAGE or Western blotting. These techniques are described in Chapter 6 (*Protocols 7* and *8*) and Chapter 8 (*Protocols 2–5*).

2.6.3 Secretory proteins and their analysis

Heterologous proteins targeted to the secretory pathway are usually subjected to one or more post-translational modifications (Section 2.5) and end up largely in the culture medium. Fortunately, because of the relatively low efficiency of secretion of homologous proteins from the yeast cell, the secreted heterologous protein will usually account for most of the proteins present in the culture medium. Nevertheless, the heterologous protein will be present at a relatively low concentration (usually 5–50 μg/ml) which precludes one-dimensional SDS–PAGE analysis of the culture medium. Therefore, it is usually necessary to concentrate the culture medium either by using ultrafiltration membranes or by TCA precipitation. *Protocol 5* describes both of these methods.

As with heterologous proteins retained inside the cell, it is important to compare the protein profile with that of culture medium from the same strain carrying the expression vector without the heterologous gene. It is very rare that all of the heterologous secretory protein is secreted into the culture medium. Usually some is retained within the secretory pathway or by the cell wall. Therefore, it is important also to analyse the total cell-associated protein sample for the heterologous protein (*Protocol 4*, excluding step 9).

The three principal post-translational modifications to which heterologous proteins may be subjected in *S. cerevisiae* are *N*-linked glycosylations, *O*-linked glycosylation, and disulfide bond formation (22). Heavily glycosylated proteins usually migrate as diffuse bands on SDS–PAGE with apparent molecular masses significantly higher than those predicted from their protein sequences. Glycosylated proteins will often react poorly with antibodies and therefore it is important that a deglycosylated sample of the secreted glycoprotein is prepared for comparison. Deglycosylation can be achieved either *in vitro* by use of an enzyme such as endoglycosidase H (which removes *N*-linked sugars from proteins), or *in vivo* by growing the cells in the presence of tunicamycin (an antibiotic which blocks the addition of sugars to asparagine residues) (34).

There are direct and relatively simple physical methods that can be used to assess whether or not a recombinant protein contains inter- or intrachain disulfide bonds (35). The simplest is to compare the electrophoretic mobility of the recombinant protein on SDS–PAGE under reducing and non-reducing conditions. Proteins with intramolecular disulfide bonds unfold to a smaller hydrodynamic volume in SDS proteins without disulfide bonds (36). Thus, by carrying out SDS–PAGE under non-reducing conditions (but where formation of artefactual disulfide bonds are blocked) (35), a disulfide bonded protein will have a greater electrophoretic mobility than under reducing conditions.

Similarly, under non-reducing conditions a disulfide bonded protein will show a greater electrophoretic mobility than a protein of the same molecular mass but without disulfide bonds. SDS–PAGE under non-reducing conditions can therefore be used to resolve and distinguish reduced and oxidized forms of a protein and hence to assay formation of intramolecular disulfide bonds.

Protocol 5. Preparation of secreted proteins

Equipment and reagents

- *S. cerevisiae* strain expressing the heterologous protein of interest
- YNB selective medium or YEP medium (see *Protocol 1*)[a]
- Centricon concentrators (Amicon): 3, 10, 30, or 100, depending on the molecular mass of the foreign protein under investigation
- TCA/deoxycholate: 50% (w/v) TCA in 0.2% (w/v) sodium deoxycholate
- 10% (w/v) TCA
- Acetone (at –20°C)
- SDS sample buffer: 50 mM Tris–HCl pH 6.8, 100 mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol

A. *Growth of cells*

1. Grow a 50 ml culture of *S. cerevisiae* in either a YNB-selective medium or YEP medium as described in *Protocol 1.*[a]
2. Pellet the cells by centrifugation (4°C, 2000 *g*, 5 min). Remove the supernatant (culture medium). Retain the pellet.[b]
3. Concentrate the proteins in the culture medium by either of the following methods.

B. *Concentration of secreted proteins by ultrafiltration*

1. Concentrate the culture medium using the appropriate Centricon concentrator, according to the manufacturer's instructions. Use an angle rotor to prevent complete dehydration of the sample. Since the length of centrifugation required may vary depending on the molecular mass of the heterologous protein, centrifuge for a shorter period than recommended by the manufacturer at first, and then repeat if necessary. The following are guide-lines for a Centricon 30 concentrator: using cells grown in YEP medium, 2.5 ml of culture medium can be concentrated to 250 μl by centrifuging for 40 min at 5000 *g*. With YNB medium, 2.5 ml of culture medium can be concentrated to 50 μl in 25 min.
2. Store the concentrated culture medium at –20°C until required.

C. *Concentration of secreted proteins by TCA precipitation*

1. Add 0.25 vol. TCA/deoxycholate to the culture medium (part A, step 2), and leave the mixture on ice for 30 min.
2. Collect the TCA precipitate by centrifugation (4°C, 20000 *g*, 20 min).

3. Resuspend the precipitate in 10% TCA then centrifuge as in part C, step 2.
4. Resuspend the pellet in acetone (at –20°C) to remove the TCA. Centrifuge as in part C, step 2. Drain the pellet and allow the acetone to evaporate.
5. Redissolve the washed protein pellet in SDS sample buffer for SDS–PAGE.[c]

[a] Several secreted proteins accumulate to lower levels in minimal YNB medium than in rich YEP medium. Yields in YNB medium may be improved by either adding casamino acids to 5 g/litre or buffering the medium to pH 6 using potassium phosphate (unbuffered media may be pH ~ 4).
[b] The cell pellet should be stored at –70°C for analysis of protein that has accumulated inside the cell.
[c] Protein from up to 10 ml culture medium may be applied to one sample well of a typical SDS–PAGE gel.

3. *Pichia pastoris* expression systems

3.1 Introduction

Pichia pastoris is an industrial methylotrophic yeast initially chosen for production of single cell protein because of its ability to grow to very high cell density in simple defined media. This fermentation technology formed the basis of a highly efficient expression system using the methanol-inducible *AOX1* promoter and vectors that integrate into the *P. pastoris* genome (37, 38). *P. pastoris* can routinely achieve percentage yields (5–40%) of cell protein much higher than *S. cerevisiae*, and often equivalent to *E. coli* or baculovirus systems. Additionally, the growth of *P. pastoris* to high cell density is simple and has resulted in enormous yields, e.g. > 12 g/litre for tetanus toxin fragment C (39) and > 3 g/litre for secreted human serum albumin (40). As a result of this, and its availability in kit form from Invitrogen, *P. pastoris* has now become a very widely-used expression system both in the biotechnology industry and in the laboratory.

The main advantages of *P. pastoris* are:

(a) Extremely high yields of intracellular proteins.
(b) Very high levels of secretion into the medium.
(c) Ease of fermentation to high cell density.
(d) Genetic stability and scale up without loss of yield.

P. pastoris is also proving valuable for producing large amounts of protein for analytical studies; one interesting recent application is in the efficient *in vivo* isotopic labelling of proteins for NMR (41).

However, as with any expression system, examples of low yields or failure

of expression are also accumulating, though many remain unpublished. Probably the commonest problem encountered is proteolysis of secreted polypeptides (38), though ways of overcoming this have become available. Another common problem is inefficient secretion of complex foreign proteins, e.g. HIV-1 gp120 (42). Finally, some heterologous genes do not give any detectable protein expression, and this is often due to fortuitous yeast transcriptional terminators resulting in truncated mRNA. This problem was first described for highly AT-rich genes in *S. cerevisiae*, where it can be solved by gene synthesis using alternative (not AT-rich) codons to increase the GC content, but there are suggestions that it may be more frequent in *P. pastoris* (38).

3.2 Expression strategies

3.2.1 Effect of gene dosage

Since no stable episomal vectors have been developed for *P. pastoris*, integrating vectors are generally used. The first strategy adopted for generating recombinant strains was single copy transplacement of the foreign gene at the *AOX1* site, since this type of transformant is the most stable and in some early studies yielded reasonable levels of product. However, numerous examples have now accumulated (*Table 2*) where multicopy transformants have been used to increase yields dramatically. For intracellular expression, vector copy number is usually the most important factor affecting product yield (38).

With secreted proteins, the effects of gene dosage are not as simple. There are many examples where product yield has been improved using multiple copies of the vector, and indeed in several cases the maximum copy number

Table 2. Some examples of high level expression resulting from multicopy integration in *Pichia pastoris*[a]

Protein expressed[b]	Intracellular or secreted?	Copy number	Yield[c]	Increase in yield[d]	Ref.
TNF	Intracellular	> 20	25%	200 ×	45
Tetanus toxin fragment C	Intracellular	14	27%	6 ×	39
Pertactin	Intracellular	21	10%	n/a	47
Aprotinin	Secreted	5	0.9 g/litre	7 ×	48
Murine EGF	Secreted	19	0.45 g/litre	13 ×	43
IGF-1	Secreted	6	0.5 g/litre	5 ×	49

[a] Data are for *P. pastoris* grown and induced in fermenter cultures.
[b] Abbreviations: TNF, tumour necrosis factor; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1.
[c] For intracellular proteins the yield is expressed as per cent of total protein; for secreted proteins it is expressed as g/litre of culture medium.
[d] Expressed as the increase in yield over single copy clones in comparable fermenter inductions. These increases over single copy clones are usually more pronounced when inductions are carried out in shaking flask cultures.

tested was optimal, e.g. with murine EGF (39). However, too high a copy number usually reduces yield, i.e. an *optimal* rather than maximum copy number is required (38).

3.2.2 Types of transformant

Although most expression vectors for *P. pastoris* are very similar to each other, there is a choice of host phenotypes and chromosomal sites for vector integration. Vectors can be integrated into the genome in one of two general ways, depending on where the DNA is cut prior to transformation.

(a) Integration at *AOX1* or *HIS4*. Linearization of the vector, by cutting either 5′ to the *AOX1* promoter (e.g. at the *Sac*I site) or within the *HIS4* marker, directs integration of the plasmid to the homologous sites in the genome. In addition to single copy transformants, rarer multicopy transformants (up to ten copies/transformant) can arise from repeated recombination events. Integration at *AOX1*, using *Sac*I digestion, is an efficient, straightforward way to generate recombinant clones for expression. We would *not* recommend using *HIS4* integrants since they can eliminate the foreign gene by recombination while still retaining the His^+ phenotype.

(b) Transplacement at *AOX1*. Digestion of the vector to give a fragment with both ends homologous to *AOX1*, e.g. using *Bgl*II, leads to replacement of the genomic *AOX1* by the foreign gene (transplacement), generating an *aox1* strain with the Mut^s phenotype (methanol utilization slow) instead of the Mut^+ phenotype of the wild-type strain. This should yield only single copy Mut^s transformants. However, a detailed analysis (43) showed that extreme clonal variation in expression levels occurs, resulting from a diverse set of transformants which differed from each other in the site of vector integration, vector copy number, and Mut phenotype. This diversity arises from a complex set of events including *in vivo* ligation and repeated recombination of the transplacing fragment. Because of this complexity and the lower frequency of transformation, we would not recommend this strategy for routine use. However, the highly divergent population of transformants it yields can be useful for detailed optimization studies. Note also that strains with disrupted *AOX1* (i.e. the Mut^s phenotype), such as KM71, can also be used for integration. KM71 and other Mut^s strains are physiologically different in that they grow very slowly on methanol as a carbon source, e.g. during induction. The different types of transformants and their uses are summarized in *Table 3*.

3.2.3 Recommended strategies

The range of recombinant strains that can be generated can make the *P. pastoris* expression system appear complex. However, in the majority of cases it is not necessary to consider all the options. For routine intracellular expression, we recommend generating *AOX1* integrants (e.g. using a *Sac*I digested vector),

Table 3. Types of *Pichia pastoris* transformant

Host strain and Mut phenotype	Restriction enzyme(s) used for vector digestion[a]	Phenotype of resulting His$^+$ transformants	Comments
GS115 (Mut$^+$)	*Sac*I *AOX1* integration	Mut$^+$ Vector integrated 5′ to genomic *AOX1* gene.	High frequency transformation, using either sphaeroplasts or electroporation. Ideal for routine use. Multicopy integrants (up to ten copies) arise at low frequency.
	*Sal*I, *Stu*I *HIS4* integration	Mut$^+$ Vector integrated within genomic *his 4* locus.	High frequency transformation, using either sphaeroplasting or electroporation. Potential to generate His$^+$ 'pop-outs' lacking foreign gene.[b] Multicopy integrants (up to ten copies) arise at low frequency.
	*Bgl*II *AOX1* transplacement	Mut^s *Bgl*II fragment replaces genomic *AOX1* gene.	Low frequency transformation, sphaeroplast method preferable. Best method for multicopy clones (1–10% frequency and up to 30 copies). Generates a heterogeneous pool of Mut^s and Mut$^+$ transformants, including some non-expressers.
KM71 (Mut^s)	*Sac*I or *Sal*I+/*Stu*I	All transformants Mut^s *AOX1* already disrupted.	Higher transformation frequency than with GS115, especially using electroporation.

[a] Since their sites are common to all vectors, the restriction enzymes indicated can generally be used unless the sites are also present in the foreign gene.
[b] This problem does not occur often in small scale cultures.

and isolating transformants with high vector copy number (> five). This can be achieved simply by drug selection using vectors, such as pPIC3K and pPIC9K (*Figure 3*), that contain the *Tn903 kan*^{r} gene which confers dose-dependent resistance to the antibiotic G418 (44).

In some cases one may consider that there is further scope for improvement or that a more detailed optimization is required, for example, prior to scale up for commercial production of a protein. For intracellular proteins, we suggest using transplacement to isolate clones with very high copy numbers (10–30). In cases where the Mut phenotype may also have an effect on yield (e.g. in secretion), transplacement can be used to generate a population of transformants varying both in copy number and Mut phenotype for subsequent

detailed comparisons. The best method for screening the resulting transformants is a rapid semi-quantitative DNA dot blot of whole cell lysates (45) since this can identify the very high copy number 'jackpot' clones, unlike selection using G418.

For secreted proteins, initial studies can be carried out with single copy transformants in order to assess the efficiency of secretion and authenticity of the product. However, for optimization of the yield, it is preferable to test transformants with a range of copy numbers. These can then be tested empirically for expression, or the precise vector copy numbers can first be determined so as to identify a series with progressively increasing copy number for a rigorous optimization. An alternative to isolating multicopy transformants is to utilize a plasmid that can be used to generate tandem copies (up to eight) of the expression cassette *in vitro* (37). This method has the potential advantage of predetermining the foreign gene copy number prior to transformation although recombination can occur to alter the number of copies. The disadvantage is that several sequential DNA cloning steps are required and these can become increasingly difficult to achieve with high efficiency.

In cases where a good assay is available for the secreted protein, optimization can be carried out empirically by initial high throughput expression screening of transformants, without any prior knowledge of copy number (41).

3.3 Host–vector systems and transformation methods for *P. pastoris*

3.3.1 Host strains

The host strains of *P. pastoris* that are most commonly used are GS115 (*his4*) and KM71 (*his4 aox1::ARG4*). The more recently available protease-deficient strains (e.g. SMD1168; *his4 pep4*) are finding increasing use in reducing proteolytic cleavage of secreted proteins. Strain KM71 gives transformation frequencies that are two- to fourfold higher than for GS115.

3.3.2 Vectors

There are now many variations of the original *P. pastoris AOX1* expression vectors listed in ref. 38, some of which are available through Invitrogen Corp. *Figure 3* illustrates the structures of the typical intracellular expression vector pPIC3 and the α-factor secretion vector pPIC9, together with their kanamycin resistance counterparts pPIC3K and pPIC9K for G418 selection (44). The multicloning site polylinkers in pPIC3K and pPIC9K are designed for ligation of the 5′ end of the foreign gene to the *Bam*HI or the *Nco*I site (which contains the ATG initiation codon), and the 3′ end to one of the remaining restriction sites. The secretion vectors pPIC9 and pPIC9K comprise an *S. cerevisiae* α-factor leader sequence with an engineered *Xho*I site just 5′ to the DNA encoding the Kex2 cleavage site. Foreign genes are ligated to this site but the Kex2 cleavage site must be reconstructed using a synthetic oligo-

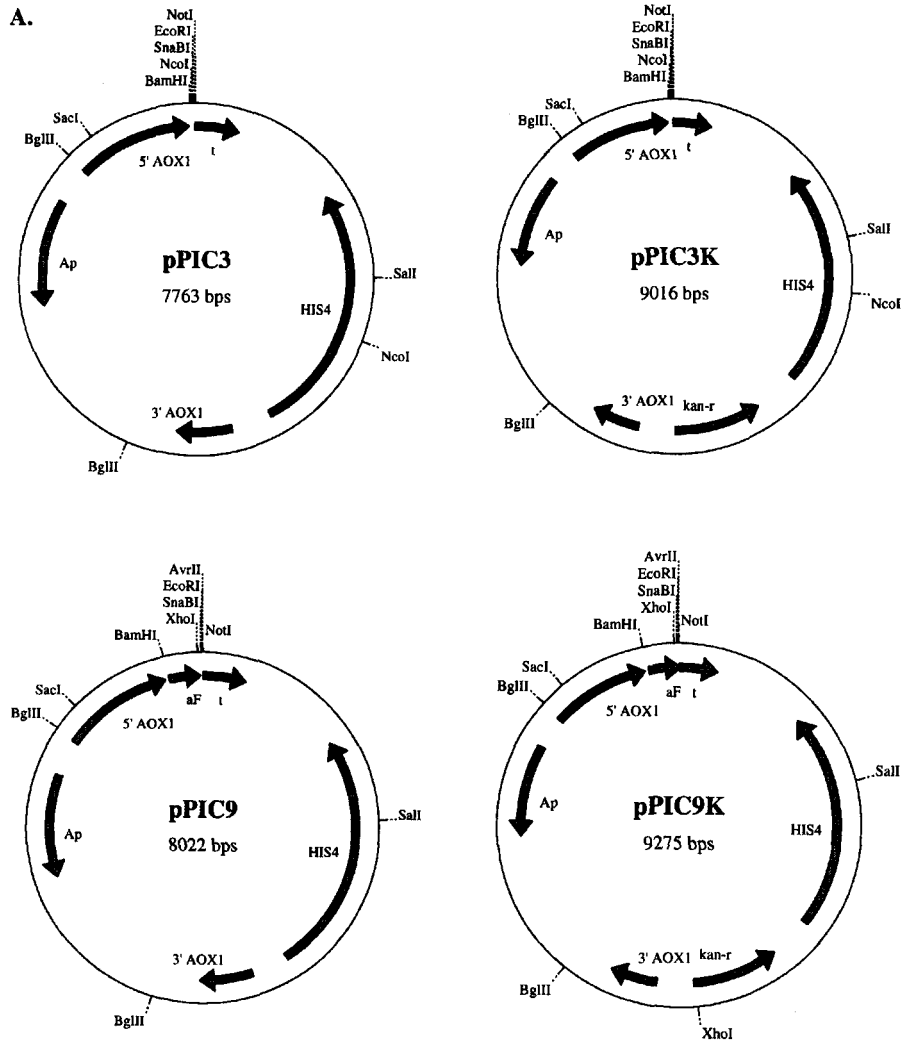

Figure 3. Maps of intracellular expression and secretion vectors for *Pichia pastoris*. (A) The figure shows maps of the intracellular expression vector pPIC3 and the secretion vector pPIC9, together with their counterparts pPIC3K and pPIC9K containing the kanamycin-resistance gene (kan^r) for selection of transformants based on resistance to the antibiotic G418. The maps also show the locations of the ampicillin-resistance (Ap) gene, the 5′ *AOX1* region that includes the promoter, the *AOX1* transcription terminator (t), the 3′ region of *AOX1*, the *HIS4* gene, and the α-factor leader (αF) of *S. cervisiae*. (B) Sequences of the multicloning sites (MCS) in pPIC3, pPIC3K, pPIC9, and pPIC9K, showing the available restriction sites. The initiation codon (ATG) is in bold for pPIC3 and pPIC3K. The α-factor leader peptide sequence is shown pPIC9 and pPIC9K.

B. pPIC3/pPIC3K MCS

*Bam*HI *Nco*I *Sna*BI *Eco*RI *Avr*II *Not*I

GGATCCAAACC**ATG**GAAAATACGTAGAATTCCCTAGGGCGGCCGC

pPIC9/pPIC9K MCS

*Xho*I *Hin*dIII *Sna*BI *Eco*RI AvrII *Not*I

CTCGAGAAAAGAGAGGCTGAAGCTTACGTAGAATTCCCTAGGGCGGCCGC

nucleotide. The *Xho*I site in pPIC9 is unique, but in pPIC9K there is an additional site located in the *kan*^{r} gene. Versions of pPIC9K are now available where the *Xho*I cloning site is unique. By cloning at the *Hin*dIII site (not unique) of the polylinker, the Glu–Ala spacers of the native prepro-α-factor peptide may also be included.

Recently Invitrogen have introduced some useful additional vectors for *P. pastoris*. The pPICZ series utilizes selection with Zeocin in both *E. coli* and *P. pastoris*. They are compact in size and offer the possibility of selection for multicopy clones. The pPICZ series contains the same α-factor leader sequence as pPIC9. All these vectors encode C-terminal *myc* epitopes and His_6 tags. Plasmids pGAPZ and pGAPZα are the analogous plasmids with the glyceraldehyde 3-phosphate dehydrogenase promoter for constitutive expression.

3.3.3 Transformation by electroporation

Electroporation is a simple and fast method for transforming *P. pastoris*. Although it gives a lower frequency of multicopy transformants than the sphaeroplast technique, electroporation combined with G418 selection is ideal for the rapid isolation of multicopy transformants for routine laboratory use (44). However, G418 cannot readily be used for *direct* selection because the level of resistance shows a dependence on plating density above 10^5 cells/plate. Thus, cells must be selected at $< 10^5$ cells/plate. Therefore, His^+ colonies are first selected on minimal medium after electroporation of approximately 10^8 cells and these are then pooled for a secondary selection on G418.

In practice, the electroporation/G418 selection method works best using *AOX1* integration in the strain KM71, because two- to fourfold higher transformation frequencies (e.g. 1000–2000 colonies/μg DNA) can be achieved with this strain. For G418 selection, use pPIC3K or pPIC9K (or similar vectors)

and digest the plasmid with *Sac*I except where a *Sac*I site is present in the foreign gene (in which case use another unique site in the 5′ *AOX1* fragment). *Protocol 6* describes a procedure for electroporation of *P. pastoris*.

Protocol 6. Electroporation of *P. pastoris*

Equipment and reagents

- Plasmid DNA (e.g. pPIC3 or PIC9)
- *Sac*I and the appropriate digestion buffer as supplied by the enzyme manufacturer
- Phenol:chloroform:isoamyl alcohol, 3 M sodium acetate, and absolute ethanol (see *Protocol 3*)
- Low speed centrifuge and sterile 50 ml tubes (see *Protocol 1*)
- $TE^{0.1}$ buffer: 10 mM Tris–HCl pH 7.4, 0.1 mM EDTA (see *Protocol 1*)
- Suitable strain of *P. pastoris*, e.g. KM71 or GS115 (Invitrogen)
- Sterile 25 ml Universal tubes (see *Protocol 2*)
- YPD medium (see *Protocol 1*)
- Sterile disposable 50 ml and 500 ml conical flasks (Corning)
- Ice-cold sterile double distilled water (see *Protocol 1*)
- 1 M sorbitol: filter sterilize (see *Protocol 1*), store at room temperature, but cool on ice before use
- Electroporation apparatus (Gene-Pulser with Pulse Controller from Bio-Rad)
- Sterile electroporation cuvettes (0.2 cm, Bio-Rad): pre-cool at 4°C
- Sterile Pasteur pipettes (Volac)
- 2 × YNBD agar plates:[a] 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4 × 10^{-5}% (w/v) biotin, 1% (w/v) glucose, 1.5% (w/v) agar. Prepare the YNBD agar from stock filter sterilized 20 × YNB (13.4% yeast nitrogen base without amino acids), and 500 × biotin (0.02%).

Method

1. Digest the plasmid DNA with *Sac*I according to the enzyme supplier's instructions.
2. Add an equal volume of phenol:chloroform:isoamyl alcohol. Vortex and centrifuge at 4°C (10 000 *g*, 15 min) to separate the phases.
3. Transfer the upper (aqueous) phase to a clean microcentrifuge tube, add 0.1 vol. 3 M sodium acetate, 2.5 vol. absolute ethanol, and mix. Leave at –70°C for 15 min to precipitate the DNA.
4. Collect the DNA by centrifugation (10 000 *g*, 15 min, 4°C). Redissolve the DNA in $TE^{0.1}$ buffer (at least 1–3 μg/5 μl) and store at –20°C or on ice until required (step 13).[b]
5. Prepare an overnight starter culture of *P. pastoris* KM71 or GS115. Inoculate 5 ml YPD medium in a 25 ml Universal tube with *P. pastoris* and incubate the tube overnight at 30°C with shaking (300 r.p.m.).
6. Use 20–100 μl of this starter culture to inoculate 100 ml YPD medium in a sterile 500 ml flask. Grow the culture overnight at 30°C to A_{600} = 1.3–1.5.[c]
7. Harvest the cells by low speed centrifugation (4°C, 1500 *g*, 5 min) in sterile 50 ml centrifuge tubes.
8. Resuspend the cell pellet in 100 ml ice-cold sterile double distilled water and harvest by centrifugation (4°C, 1500 *g*, 5 min).

9. Repeat step 8 with 50 ml ice-cold sterile double distilled water.
10. Resuspend the washed pellet in 5 ml ice-cold sterile 1 M sorbitol and harvest by centrifugation (4°C, 1500 *g*, 5 min).
11. Resuspend these electrocompetent cells in 0.2 ml ice-cold sterile 1 M sorbitol.
12. Aliquot 40 μl electrocompetent cells per transformation into a pre-cooled sterile 0.2 cm electroporation cuvette, avoiding the creation of air bubbles.
13. Add 1–3 μg of the *Sac*I digested plasmid DNA (from step 4) in a maximum volume of 5 μl. Mix gently and place the cuvette on ice for 5 min.
14. Pulse at 1.5 kV, 25 μF, 400 Ω in the electroporation apparatus. The expected time constant is in the range 7–9.[d]
15. Immediately add 1 ml ice-cold 1 M sorbitol to the cuvette using a sterile Pasteur pipette. Mix by pipetting up and down then transfer to a culture tube.
16. Spread 200 μl aliquots of the transformed cells on to 2 × YNBD agar plates and incubate the plates at 30°C for two to four days until the colonies appear.

[a] YNB plates for *P. pastoris* have twice as much YNB as for *S. cerevisiae*.
[b] An alternative to phenol purification of the DNA is to use one of the many proprietary resin-based purification kits.
[c] If difficulty is experienced in obtaining overnight cultures of the correct density, then inoculate 100 ml YPD medium to A_{600} = 0.3, using the overnight starter culture (step 5), and incubate for 4–5 h until A_{600} = 1.3–1.5. There is little or no loss in electroporation efficiency in the later steps.
[d] If the time constant is outside this range, discard the electroporation mixture and repeat steps 11–14. Problems may arise if the DNA is too concentrated or has too high a salt content, so the DNA may need to be diluted or reprecipitated (steps 3–4).

Although we have found the frequency of His^+ colonies lacking the expression vector to be negligible, others have reported a high rate of non-expressing transformants following electroporation. The reason for this discrepancy is unclear but it could be due to differences in the electroporation methods or the amounts of DNA used. However, subsequent G418 selection eliminates this problem by selecting for true transformants. *Protocol 7* describes selection with G418.

The frequencies achieved for transplacement are typically 20-fold lower when compared to those obtained using integration. Therefore, we recommend the sphaeroplast transformation method for transplacement, especially where very high copy number transformants are required. A sphaeroplast kit is now available from Invitrogen.

Protocol 7. Selection for multicopy transformants using the antibiotic G418

Equipment and reagents

- *P. pastoris* His^+ transformants on 2 × YNBD plates (see *Protocol 6*)
- YPD medium (see *Protocol 1*)
- Sterile cell spreaders (Cherwell Labs)
- Haemocytometer
- Sterile Petri dishes (9 cm dia., Falcon)
- G418 sulfate (Geneticin, Gibco): prepare stock solution (50 mg/ml in water), filter sterilize (see *Protocol 1*), and store at –20°C
- Glycerol (Sigma): make a 75% (v/v) stock, then autoclave at 120°C for 20 min
- Sterile Pasteur pipettes (Volec)
- 1–2 ml sterile screw-cap vials and 25 ml Universal containers (Nunc)
- YPD agar plates containing different concentrations of G418. Prepare YPD agar by autoclaving YPD medium plus 1.5% agar at 120°C for 20 min. Cool the molten agar to 50°C and aliquot it into quantities sufficient for each Petri dish. Add the appropriate volume of stock G418 solution to give the final G418 concentrations of 0.25, 0.5, 0.75, 1, 1.5, 2 mg/ml. Pour each plate, allow the agar to cool, and store the plates at 4°C for up to two months.

Method

1. Pool His^+ transformants selected on 2 × YNBD plates. Do this by resuspending them in 2–3 ml sterile YPD medium using a cell spreader.
2. Determine the cell density of the resuspended transformants using a haemocytometer (typically 5–50 × 10^8 cells/ml).[a] Dilute the cell suspensions in YPD medium to a final density of approx. 10^6 cells/ml (typically a 1000-fold dilution).
3. Spread 100 μl of the cell suspension (~ 10^5 cells) on each 9 cm YPD agar plate containing the G418.
4. Incubate the plates at 30°C. Resistant colonies should take two to five days to appear. The numbers of resistant colonies declines steeply above 0.5–1 mg G418/ml.
5. Pick several resistant colonies for further analysis using sterile Pasteur pipettes. For example, a total of six, including colonies selected on the highest G418 concentration and a range of colonies from plates with lower G418 concentrations.
6. Store as glycerol stocks at –70°C. To make these, inoculate 10 ml YPD in a sterile 25 ml Universal container using a single isolated colony. Grow for one to two days at 30°C, shaking at 300 r.p.m. Place 0.8 ml of this culture into a 1–2 ml sterile screw-cap vial and add 0.2 ml 75% (v/v) glycerol. Cool as slowly as possible to –70°C (e.g. by placing the tubes in an expanded polystyrene box in a –70°C freezer). Frozen cultures remain viable for several years.
7. Analyse the production of the heterologous protein by these representative transformants as described in *Protocol 4*.

8. If desired, determine the vector copy number in each transformant using Southern blot analysis and/or quantitative DNA dot blot hybridization (see Section 3.4).

[a] Alternatively, measure the A_{600}. One A_{600} unit is equivalent to about 5×10^7 cells/ml.

Although G418 selection is effective in isolating multicopy transformants, experience has shown that it is not possible to make comparisons of copy number for transformants selected at the same G418 concentration in different experiments. The reason for this is not clear. In addition, we find that transformants with five to seven copies per cell can be selected at the highest concentrations of G418 that can be made (2–4 mg/ml), so it is not possible to distinguish transformants with higher copy numbers. However, DNA dot blotting can be used to screen cells cultured in microtitre plates (39).

3.4 Analysis of DNA from *P. pastoris* transformants

Although it is possible to use the *P. pastoris* system purely empirically by simply screening transformants for sufficient levels of heterologous protein expression, it is valuable to determine the number of copies of the integrated plasmid. For intracellular expression this will give an indication of how much potential there is for further increasing the expression level, while for secreted proteins optimizing the copy number can dramatically affect the yield.

To determine the absolute copy number of the vector it is necessary to isolate total DNA from transformed strains as described in *Protocol 8*. Southern blot analysis method is then used to determine the chromosomal structure of integrated vector DNA (i.e. the site of integration, *AOX1* or *HIS4* genes vector copy number, and whether *AOX1* gene replacement has occurred).

Protocol 8. Preparation of total DNA from *P. pastoris*

Equipment and reagents

- G418-resistant transformants of *P. pastoris* (see *Protocol 7*)
- Untransformed *P. pastoris* strain (GS115 or KM71, as appropriate)
- YPD medium, TE buffer, sterile 250 ml conical flasks, sterile 50 ml centrifuge tubes, sterile water, absolute ethanol (see *Protocol 1*)
- SCE buffer: 1 M sorbitol, 10 mM trisodium citrate, 10 mM EDTA—autoclave at 120°C for 20 min
- 1 M dithiothreitol stock (store at –20°C)
- SCED buffer: SCE containing 10 mM DTT
- 3 mg/ml zymolyase (Zymolyase-100T) (Sigma, available as Lyticase, Cat. No. L5263): make this up just before use
- 1% (w/v) SDS
- 5 M potassium acetate (pH 8 with acetic acid)
- 10 mg/ml ribonuclease A (DNase-free): store at –20°C
- Propan-2-ol
- Fine glass rod (2 mm dia.)
- Rocking platform shaker (Luckham)

Method

1. Prepare an overnight culture of each transformant and of the untransformed GS115 or KM71 parent strain as a control. Do this by

Protocol 8. *Continued*

inoculating each into 50 ml YPD medium in sterile 250 ml conical flasks and incubating at 30°C.

2. Harvest the cells by low speed centrifugation (1500 *g* for 10 min) in 50 ml sterile centrifuge tubes.
3. Wash the cells by resuspending cell pellet in 10 ml sterile water and centrifuging again.
4. Resuspend the cells in 5 ml SCED buffer. Add 100 μl of 3 mg/ml zymolyase and incubate the cells for 1 h at 37°C to prepare sphaeroplasts.
5. Add 5 ml of 1% SDS. Mix gently by inversion and place the sphaeroplasts on ice for 5 min.
6. Add 3.75 ml of 5 M potassium acetate pH 8.9 and mix gently.
7. Remove the precipitate by centrifugation (10000 *g* for 20 min).
8. Add 2 vol. absolute ethanol to the supernatant and mix. Leave the mixture at room temperature for 15 min, and then centrifuge at 10000 *g* for 20 min.
9. Drain the pellet well then redissolve it in 3 ml TE buffer. This may require gentle agitation on a rocking platform shaker for several hours.
10. Remove undissolved material by centrifugation (10000 *g* for 10 min).
11. Add 15 μl of 10 mg/ml RNase to the supernatant and incubate the mixture at 37°C for 30 min to digest the RNA.
12. Add 1 vol. propan-2-ol to form a separate layer. Mix gently so that the DNA forms a fibrous precipitate at the interface.
13. Remove the DNA 'spooling' it around a glass rod, then place it in 250 μl TE.[a]
14. Allow the DNA to dissolve and store the DNA solution at 4°C.

[a] This spooling technique, which yields very pure DNA, may not work if the DNA is too dilute after step 11. If this is the case, the alternative is to extract the DNA with phenol: chloroform:isoamyl alcohol precipitation as described in *Protocol 3*, steps 10–12.

The simplest method for determining the copy number is by Southern blot analysis using any of the protocols described in standard laboratory manuals including those of this series (46). As a guide for the type of transformant generated in *Protocol 7*, with standard *P. pastoris* vectors, it is always informative to digest the chromosomal DNA with *Bgl*II and hybridize with a *HIS4*-specific probe. Digest 20 μl DNA from each transformant with *Bgl*II in a 50 μl reaction. Include a control digest of GS115 or KM71 DNA. Probe the blot using a random primed ^{32}P-labelled *HIS4* probe (e.g. the 0.6 kb *Kpn*I fragment of

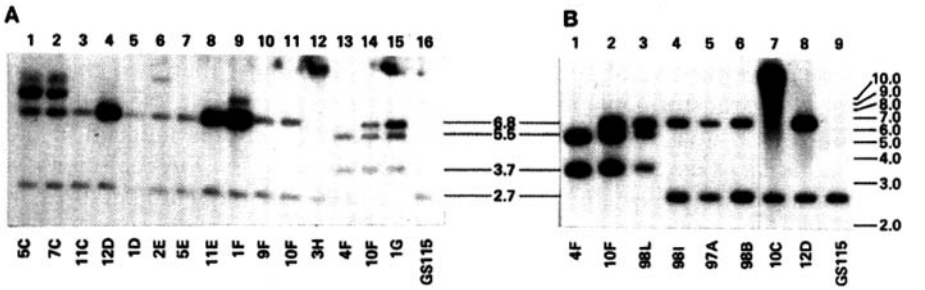

Figure 4. Southern blot analysis of chromosomal DNA from different integrant clones expressing tetanus toxin fragment C from a transplacement transformation. (A) Mut^s and (B) Mut$^-$ transformants from transplacement experiments were analysed. The designations of the clones are shown along the bottom of the autoradiograph. Band sizes (kb) are indicated in the centre and molecular weight markers (kb) at the right. The DNA was restriction digested with *Bgl*II and the blot hybridized with an *HIS4* probe. (A) For the Mut^s transformants this gave the expected two bands: a 2.7 kb band containing the chromosomal *his4* gene and a 6.8 kb band containing both the fragment C gene and the wild-type *HIS4* gene. In single copy transformants (A; lanes 3, 5, 7, 10, 11) these two bands have similar intensity, whereas in multicopy transformants the intensity of the 6.8 kb band is greater (A; lanes 1, 2, 4, 8, 9). (B) Similar patterns are seen for Mut$^-$ transformants from 'transplacements'. In both Mut^s and Mut$^+$ transformants some bands larger than 6.8 kb can be seen (A; lanes 1, 2, 6, 9, 12) (B; lane 7), which are consistent in size with the loss of one or more *Bgl*II sites, possibly due to exonucleolytic trimming prior to integration of the fragment C gene. For comparison, some clones that have vector fragments integrated at *his4* are shown, in which the 2.7 kb fragment is disrupted to give bands of 3.7 kb or 5.5 kb (A; lanes 13–15) (B; lanes 1–3). Control digestions of the vector GS115 are shown in (A; lane 16) and (B; lane 9).

HIS4). Hybridize using a standard protocol and visualize the blot by autoradiography. The resulting Southern blots usually show two bands, a 2.7 kb band corresponding to the single copy chromosomal *HIS4* gene and a larger band equivalent to the *HIS4* containing *Bgl*II fragment of the expression vector (*Figure 4*). A direct comparison of the intensities of these two bands (e.g. using a PhosphorImager) gives the copy number of the foreign gene. The majority of transformants are expected to be single copy and should give two bands of similar intensity. It should be pointed out that with transplacements the *Bgl*II sites can be lost so that the larger fragment has a much larger size than expected. In some cases, therefore, quantitative dot blot analysis is the preferred analytical method (43).

3.5 Induction of foreign protein expression in *P. pastoris*

One of the major advantages of *P. pastoris* is the very high volumetric yield that can be obtained in fermenters due to the very high cell densities that are possible with this organism. This advantage is not obtained in shake-flask inductions. Moreover, shake-flask inductions can be suboptimal due to lack of

aeration and inability to control the levels of the methanol inducer. Therefore, it is advisable, where possible, to carry out preliminary optimization experiments with several (perhaps six) transplacements, then test the best two or three transformants in a fermenter as soon as possible. Nevertheless, *P. pastoris* can give very good yields in small scale cultures induced in the shaking flasks as described in *Protocol 9*. Aeration of *P. pastoris* cultures is a key consideration since the efficiency of growth and induction of protein expression are reduced when oxygen is limited. To avoid this in small scale inductions, incubate the culture with vigorous shaking, keep the culture volume to a minimum (e.g. 5–10 ml), and use large flasks (e.g. at least 100 ml, and preferably fitted with baffles). The optimal induction period may vary for different proteins and it is recommended that a time course be carried out in a pilot experiment to maximize yields.

Protocol 9. Induction of small scale *P. pastoris* cultures

Equipment and reagents

- *P. pastoris* transformants (see *Protocol 7*)
- YNB medium
- YNBG medium: 2 × YNB medium containing 4 ng/ml biotin and 2% (w/v) glycerol
- Sterile 25 ml Universal containers (see *Protocol 2*)
- Shaking incubator set at 30°C (New Brunswick)
- Sterile 100 ml conical flasks (Corning)
- Methanol
- YNBM medium: 2 × YNB medium containing 4 mg/ml biotin and 1% (v/v) methanol

Method

1. Prepare a starter culture of each transformant by inoculating 10 ml 2 × YNBG medium in a 25 ml Universal container with a single colony of the transformant and shaking the culture overnight at 30°C.
2. Read the A_{600} which should be about 5.
3. Dilute the starter culture into 10 ml of 2 × YNBG medium in a 100 ml conical flask (A_{600} = 0.25). Incubate the culture at 30°C with vigorous shaking for 6–8 h to obtain an exponentially growing culture.
4. Harvest the cells by centrifugation (4°C, 2000 *g*, 5 min) and resuspend the cell pellet in 10 ml sterile water.
5. Harvest the cells by centrifugation (see step 4). Resuspend the cells in 10 ml of 2 × YNBM medium in a 100 ml conical flask.
6. Incubate the culture at 30°C with vigorous shaking for 24 h.
7. After 24 h, add methanol to 0.5% and continue the induction for up to four days more. The A_{600} should be 5–10.
8. Harvest the cells by centrifugation (4°C, 2000 *g*, 5 min).

When using *Protocol 9* for inducing secreted proteins, the following points should be noted:

(a) It is advisable to concentrate the cells 20-fold before induction (e.g. by growing a 100 ml culture and inducing the cells in 5 ml) since this gives an almost proportionate increase in protein concentration in the medium. If necessary, the protein can be further concentrated by ultrafiltration (see *Protocol 5*).

(b) Retain both the culture medium and the cell pellet (see step 8) for analysis to determine the efficiency of secretion.

(c) Proteolytic degradation is a serious problem for some proteins. Buffering the induction medium with 0.1 M sodium phosphate pH 6 and adding 1% casamino acids has been found to reduce this problem in some cases, and we would recommend that this is done routinely. The use of the protease-deficient strain SMD1168 and buffering the induction medium to another pH (determined in a pilot experiment) have reduced proteolysis in some cases (37, 42).

Although shaking flask cultures can be used successfully (*Protocol 9*), optimal expression in *P. pastoris* really requires the use of fermenters since this increases both the efficiency of induction and the culture density. Extremely high culture densities (e.g. 130 g dry weight per litre) can be achieved very readily in only a basic fermenter with monitors and controls for pH, dissolved O_2, stirring speed, temperature, and air flow. Cultures are grown in a defined medium consisting of basal salts, trace elements, and glycerol. The pH is maintained by the addition of ammonium hydroxide which also provides the source of nitrogen. For optimal growth and induction, it is important to maintain dissolved O_2 above 20%. Fermentations are carried out in three phases:

(a) An initial batch growth phase.

(b) A limited glycerol feed during which the culture reaches high cell density.

(c) A methanol feed of several days during which product accumulates.

The procedure given in *Protocol 10* is for a one litre 'bench-top' fermenter but very similar conditions have been used up to a 240 litre scale.

To achieve optimal yield, the duration of the glycerol-limited phase (*Protocol 10*, step 4), and thus the cell density at induction, can be varied depending on the type of protein produced and the Mut phenotype of the host. Mut^+ strains can be induced at low cell density since they continue to grow during the induction phase. Mut^s strains should be allowed to achieve high density before induction. The yield of secreted proteins may be improved by early induction, allowing secretion to occur during a period of active growth.

Protocol 10. Large scale fermentation of *P. pastoris*

Equipment and reagents

- *P. pastoris* transformant to be tested (see *Protocol 7*)
- 2 × YNBG medium and shaking incubator (see *Protocol 9*)
- 2 × YNB broth medium, sterile 250 ml conical flask, membrane filters, sterile distilled water (see *Protocol 1*)
- Fermenter equipped with monitors and controls for pH, temperature, dissolved O_2, airflow, and stirring speed (Braun)
- 5 × basal salts solution: 42 ml phosphoric acid, 1.8 g $CaSO_4.2H_2O$, 28.6 g K_2SO_4, 23.4 g $MgSO_4.7H_2O$, 6.5 g KOH per litre[a]
- 500 × biotin solution: 0.2 mg biotin dissolved in 100 ml distilled water. Filter sterilize this through a 0.2 μm membrane filter. Store the stock solution at 4°C (stable up to one year).
- PTM1 salts: 6 g $CuSO_4.5H_2O$, 80 mg KI, 3 g $MnSO_4.H_2O$, 0.2 g sodium molybdate, 0.02 g boric acid, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 65 g $FeSO_4.7H_2O$, 0.2 g biotin, 5 ml H_2SO_4 per litre[a]
- 75% (v/v) glycerol[a]
- 50% (v/v) ammonium hydroxide in sterile distilled water
- Glycerol/PTM1: 50% (v/v) glycerol containing 12 ml/litre PTM1
- Methanol/PTM1: 100% methanol with 12 ml/litre PTM1—do not autoclave or filter sterilize this
- Bead mill and glass beads (Dyno Mill) for preparation of intracellular protein only

Method

1. Prepare a starter culture by inoculating 10–50 ml of 2 × YNBG in a 250 ml flask with the transformant. Incubate overnight at 30°C with shaking.
2. Add this culture to the fermenter containing one litre 5 × basal salts solution plus 4 ml PTM1 salts and 5% (v/v) glycerol.
3. Allow the culture to grow at 30°C until the glycerol is exhausted (24–30 h). Maintain the dissolved O_2 above 20% by adjusting the aeration and agitation, and maintain the pH at 5 by the addition of 50% (v/v) ammonium hydroxide. The dissolved O_2 level will begin to rise sharply when the glycerol becomes exhausted.
4. Start adding the glycerol/PTM1 solution at 12 ml/h and continue this limited glycerol feed for 4–24 h while maintaining the pH, temperature, and dissolved O_2 as before. Monitor cell growth at A_{600} and continue the glycerol-limited growth until the cell density reaches 30–100 g dry weight per litre. One A_{600} unit is approx. equivalent to 0.33 g/litre.
5. Induce the culture by replacing the glycerol PTM1 solution with methanol/PTM1 solution feed at 1 ml/h. Gradually increase this feed rate over a period of 6 h to 6–10 ml/h and continue the fermentation for a further 46–92 h. Continue monitoring the dissolved oxygen. For the efficient induction of Mut^+ strains, the aim is to achieve maximal methanol feed rates (after the initial 6 h period to adapt the culture to methanol utilization) while maintaining sufficient aeration. If dissolved oxygen levels cannot be maintained above 20%, the methanol feed rate should be reduced. This will also avoid the accumulation of toxic

levels of methanol. To avoid this problem with Mut^s strains, the methanol feed rate should be reduced after 48 h to 1 ml/h.

6. For secreted proteins harvest the medium and analyse it as described. For intracellular proteins, break open the cells using glass beads in a bead mill with efficient cooling as described by the manufacturer.

[a] Autoclave solutions at 120°C for 20 min.

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4

Baculovirus expression systems

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1. Introduction

The baculovirus expression system has provided an efficient and effective way to synthesize foreign proteins in eukaryotic cells. Analysis of the baculovirus genome has identified genes which are dispensable for the propagation of the virus in cell culture. Additionally, the promoters associated with these genes are exceptionally active, leading to the production of high levels of protein in virus-infected cells. As a result, baculoviruses have been successfully employed as vehicles for the production of large amounts of recombinant proteins which are structurally, functionally, and antigenically authentic. The material produced using the baculovirus expression system is therefore suitable for a number of applications, such as structure–function studies, therapeutics, and vaccines.

Baculoviruses have been isolated only from invertebrates, primarily insect species. They are not infectious for vertebrates and plants. Therefore, baculoviruses present few safety problems when used as expression vectors. Baculoviruses are classified according to their structure and are included in two subgroups: nucleopolyhedroviruses (NPVs) and granuloviruses (1). Each virus is named after the species from which it was first isolated. Viruses of the NPV subgroup are mainly used as expression vectors; in particular, the *Autographa californica* NPV (AcMNPV), which was isolated from the larva of the alfalfa looper. This is regarded as the prototype baculovirus and has been the focus for molecular studies and for expression work.

The baculovirus genome is a circular, covalently closed, double-stranded DNA molecule which, in the case of AcMNPV, has a size of approximately 134 kbp (2). It is complexed with a small, basic DNA binding protein inside the rod-shaped nucleocapsid. The nucleocapsid is surrounded by a lipoprotein envelope which delineates the virus particle. The number of nucleocapsids enclosed within the envelope subdivides the NPVs into MNPVs (multiple nucleopolyhedroviruses) with many nucleocapsids per envelope (e.g. AcMNPV), and SNPVs (single nucleopolyhedroviruses), with only one nucleocapsid per envelope, e.g. *Heliothis zea* (HzSNPV). The virus particles are further

embedded within a proteinaceous matrix, comprising the polyhedrin protein, to form occlusion bodies or polyhedra.

2. Baculovirus life cycle

The occlusion body serves to protect the baculovirus from degradation, and so the virus can persist for long periods in the natural environment, such as on leaf surfaces and in the soil. The polyhedra are ingested by susceptible larvae as they feed on contaminated foliage. They dissolve in the alkaline contents of the insect midgut, releasing the virus particles which traverse the peritrophic membrane and initiate a primary round of replication in the columnar epithelial cells. Studies of viral gene expression *in vitro* have revealed two temporally distinct phases: early and late. Early gene transcription is carried out by host RNA polymerase II, whereas late and very late transcription is undertaken by a modified RNA polymerase which is thought to be encoded by the virus. Late gene expression is further subdivided into the expression of late and, subsequently, very late genes. Late gene expression occurs concomitant with, or subsequent to, DNA replication and requires the presence of viral-encoded gene products. These products, known as late expression factors (3, 4), probably direct DNA replication and may constitute the modified RNA polymerase. Late gene products include structural proteins which constitute the virus particle. During the very late phase, two virus-encoded proteins are produced in copious amounts, eventually accounting for some 50% of total cell protein. These are polyhedrin, described above, which is the major component of the occlusion body, and P10, which is thought to be involved with polyhedron formation and cell lysis (5). Both polyhedrin and P10 proteins are not essential for propagation of AcMNPV in cell culture (5–7), as they are not involved with the formation of infectious virus particles. These genes, therefore, have been targeted as sites for the insertion of foreign genes into the baculovirus genome (Section 4).

Baculoviruses produce two structurally distinct, but genetically identical, forms during the replication cycle. From about 12 hours post-infection (h.p.i.), nucleocapsids bud through the plasma membrane, acquiring the viral envelope and the virus-encoded glycoprotein, GP64. This form of virus is known as budded virus and serves to establish a systemic infection throughout the insect via the respiratory system. Later in infection (24 h.p.i.), the nucleocapsids are enveloped *de novo* in the nucleus of the infected cell and become embedded within polyhedrin protein to form occlusion-derived virus. These remain within the nuclei of infected cells until the larva liquifies, releasing the polyhedra which spread the infection to other insects. In cell culture, polyhedra are non-infectious unless the virus particles are released by alkaline treatment prior to inoculation; the acidity of the cell culture medium (pH 6.2) prevents spontaneous dissolution of the polyhedra. Upon infection of insect cells in culture, budded viruses are released into the medium and may be stored as a

stock of infectious virus. Polyhedra are clearly visible by light microscopy as highly refractile bodies in the nuclei of infected cells.

3. Insect cell culture

The baculovirus expression system is based upon the ability to propagate AcMNPV in cell culture. Cell lines derived from the pupal ovaries of the fall army worm, *Spodoptera frugiperda* (Sf), are commonly used and include IPLB-Sf-21 (8) and ATCC-Sf-9 (9). Sf-9 cells have been adapted to grow in serum-free medium—Sf900II medium which is available from Gibco. We generally use Sf-21 cells between passages 150 and 180 to ensure reproducibility. However, higher passages are not detrimental to the cell line and, with care, cells may be maintained indefinitely. *Trichoplusia ni* TN-368 and BTI-TN-5B1-4 (otherwise known as High 5) also support the replication of AcMNPV and produce recombinant proteins to higher levels and with more authentic post-translational modification than Sf-9 cells (10, 11). However, these cell lines are not easily adaptable to growth in suspension culture and tend to form large aggregates (10). These cells must, therefore, be grown in monolayer culture to achieve the optimum level of protein production.

One of the advantages of using insect cells is that very simple facilities are required. These comprise a hood for the handling of cells in a sterile atmosphere and an incubator which can be set at 28 °C. The incubator does not need to be charged with carbon dioxide to maintain the pH of the insect cell culture medium. A warm room set at the same temperature is also an advantage when scaling up cell culture volumes in suspension. If this is not available, insect cells (particularly Sf-9) can be propagated as cultures shaken in heated orbital incubators such as those normally used for bacterial or yeast cells. However, it is advisable not to mix eukaryotic and prokaryotic cells in the same incubator for obvious reasons.

Cell lines are routinely maintained in TC100 medium which is supplemented before use with 5% or 10% fetal calf serum (FCS). Antibiotics (500 U/ml penicillin, and 500 μg/ml streptomycin) may be added; we prefer not to use them in routine cell culture. Stocks of cells are grown either in monolayer or suspension culture at room temperature or 28 °C in atmospheric air. Monolayer cultures may be maintained in glass or plastic flasks and are harvested by gentle scraping into the medium with a rubber or plastic scraper. Trypsin should not be used for *S. frugiperda* cells as it drastically reduces their viability. For a monolayer culture, it is advisable to seed cells at a density of 2×10^4 cells/cm^2 and allow them to reach confluence before subculturing. Suspension cultures are particularly useful for producing large stocks of virus or recombinant protein. Cells are seeded at 1×10^5/ml into sterilized, flat-bottomed flasks containing a magnetic stir bar and stirred at 100 r.p.m. It is vital to maintain the oxygen supply to the cells, so the flasks must not be filled to more than 50% of their total volume to maximize the surface area exposed

to the air. Cultures of up to 2 litres may be grown in this way, although cell numbers do tend to decrease due to oxygen limitation, unless oxygen is bubbled through the medium.

For long-term storage, *S. frugiperda* cells may be maintained in liquid nitrogen (*Protocol 1*). This permits maintenance of a defined passage range of cells and, therefore, reproducibility of results.

Protocol 1. Storage of *Spodoptera frugiperda* cells in liquid nitrogen

Equipment and reagents

- Liquid nitrogen storage facilities, protective gloves, and face mask
- Tissue culture facilities (see text)
- Tissue culture flasks (25 cm^2; Gibco)
- *S. frugiperda* cells: monolayer culture in TC100 growth medium in the logarithmic phase of growth (5×10^6 cells/25 cm^2 flask)
- TC100 growth medium (Gibco) supplemented with 10% (v/v) FCS
- Rubber or plastic cell scrapers (Gibco)
- Haemocytometer
- Trypan Blue solution (Gibco)
- Light microscope
- Cryotubes (2 ml; Gibco)
- 2 × freezing medium: TC100 medium containing 20% (v/v) FCS and 20% (v/v) dimethylsulfoxide (DMSO)
- Water-bath (at 28°C)

Method

NB: ensure that protective gloves and face mask are worn when using liquid nitrogen.

1. Decant the growth medium from the flask. Add 10 ml TC100 growth medium and harvest the cells using a cell scraper.
2. Determine the cell density using a haemocytometer.
3. Determine the cell viability by diluting a small sample of the cell suspension with an equal volume of Trypan Blue solution. Examine the cells by light microscopy and determine the proportion of clear (viable) and blue (non-viable) cells. The cells should be at least 90–95% viable.
4. Adjust the cell concentration to 2×10^6 cells/ml with TC100 growth medium.
5. Label 2 ml cryotubes with the cell type, passage number, and date.
6. Add an equal volume of 2 × freezing medium to the cell suspension and mix. Dispense 1 ml aliquots into the prepared vials. Immediately chill the cells on ice, as DMSO is cytotoxic.
7. Freeze the cells overnight in the vapour phase of liquid nitrogen, then transfer the vials to the liquid phase.
8. To revive the cells,[a] thaw the frozen suspension rapidly in a water-bath at 28°C, and seed the cells into a 25 cm^2 tissue culture flask containing 5 ml TC100 growth medium. Incubate the flasks at 28°C for 5 h.

9. Decant the medium and replace it with 5 ml fresh TC100 growth medium. Incubate the flasks at 28°C until the cells reach confluence, then subculture for use in experimental protocols.

[a] It is advisable to thaw one vial one week after freezing to determine the viability of the cells.

Virus propagation may be carried out in *S. frugiperda* cells on either a small or large scale. Small scale production (e.g. the amplification of a plaque isolate of virus) may be performed conveniently in 35 mm diameter Petri dishes (tissue culture grade); this procedure is described in *Protocol 2*. Titration of the virus produced is performed by plaque assay (*Protocol 3*). For the production of larger amounts of virus, the cells may be grown in 60 mm diameter dishes or 25 cm^2 or 75 cm^2 flasks. Seeding densities for these vessels are given in *Table 1*. The production of virus on a large scale is described in *Protocol 4*.

Table 1. Seeding densities for *S. frugiperda* cells

Dish/flask	Seeding density[a] (cell number)	Volume of medium (ml)
35 mm diameter Petri dish	1×10^6	1.5–2
60 mm diameter Petri dish	2×10^6	3–4
25 cm^2 flask	1×10^6	3–4
75 cm^2 flask	$0.5–1 \times 10^7$	10
Spinner cultures	$1–2 \times 10^5$/ml[b]	40–500

[a] Incubate the cells at 28°C prior to seeding.
[b] Grow the cells to a density of 5×10^5/ml before inoculating with virus for the production of high titre stocks; $1–2 \times 10^6$/ml for the production of recombinant protein.

Protocol 2. Preparation of virus stocks in *S. frugiperda* cells

Equipment and reagents

- 35 mm diameter Petri dishes (tissue culture grade; Gibco)
- *S. frugiperda* cells (logarithmic phase) (see *Protocol 1*)
- TC100 growth medium and tissue culture facilities (see *Protocol 1*)
- Incubator at 28°C
- Virus inoculum (preferably > 1×10^7 p.f.u./ml)[a]
- Rocking platform shaker
- Sterile Pasteur pipettes
- Sandwich box lined with moist tissue paper and a tight-fitting lid

Method

1. Seed 35 mm diameter Petri dishes, one or more for each virus stock as required, each with 1×10^6 cells in 1.5–2 ml TC100 growth medium. Incubate the dishes overnight at 28°C.

Protocol 2. *Continued*

2. Remove the medium from the cells and add the virus inoculum (100 μl) at a multiplicity of infection (m.o.i.) of 0.1 p.f.u./cell.
3. Incubate the cells for 1 h at room temperature (21°C) on a level surface. It is important to ensure the surface is level so that the virus suspension evenly covers the cell monolayer. Gently rock the dishes every 15–20 min to bathe the cell monolayer.
4. Remove the inoculum with a Pasteur pipette and discard safely, according to local rules. Replace the inoculum with 1.5–2 ml TC100 growth medium.
5. Incubate the cells in the enclosed humidified sandwich box at 28°C and examine the cells daily, using a light microscope, for signs of infection. In general, virus stocks will need to be harvested four to five days after infection.
6. Harvest the cells and medium together using a Pasteur pipette to dislodge the cells from the dish. Virus-infected cells tend to detach easily from the substratum and float in the medium.
7. Remove the cells by low speed centrifugation (2500 *g* for 10 min) and store the clarified medium at 4°C (for viability from days to weeks) or −70°C (for viability from months to years). Titrate the virus as described in *Protocol 3*.
8. To amplify the virus further, repeat the procedure with larger flasks (75 cm^2 or greater) or use the method described in *Protocol 4* for suspension cultures.

[a] If using virus isolated from a single plaque (see *Protocol 9*) inoculate the cells with 100 μl of the 500 μl plaque stock.

Protocol 3. Titration of virus by plaque assay

Equipment and reagents

- *S. frugiperda* cells (logarithmic phase) and tissue culture facilities (see *Protocol 1*)
- TC100 growth medium (with 10% FCS but no antibiotics) (see *Protocol 1*)
- 35 mm diameter Petri dishes (see *Protocol 2*)
- Virus stock to be titred (see *Protocol 2*)
- TC100 growth medium with antibiotics: TC100 medium (Gibco) supplemented with 5% (v/v) FCS, 500 U/ml penicillin, and 500 μg/ml streptomycin
- Overlay medium: 2% (w/v) low gelling temperature agarose (FMC Sea-plaque) dissolved and sterilized by autoclaving (120°C, 20 min) in distilled water, cooled to 37°C, and mixed 1:1 with TC100 growth medium with 10% FCS, pre-warmed to 37°C
- 0.5% (w/v) neutral red stain in water: filter sterilized and stored in the dark
- Phosphate-buffered saline (PBS): 140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4 pH 7.2

Method

1. Seed the *S. frugiperda* cells (1.5×10^6 cells in 2 ml TC100 growth medium without antibiotics) into a series of 35 mm diameter Petri dishes.[a] Incubate the dishes at 28°C for 2–4 h.
2. Prepare tenfold dilutions of the virus in TC100 growth medium with antibiotics. The dilutions used are determined by the source of the virus. For example, to titrate virus from an individual plaque isolate (*Protocol 9*), use the undiluted stock plus 10^{-1}, 10^{-2}, and 10^{-3} dilutions. To titrate other virus stocks, assay 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions.
3. Remove the medium from the *S. frugiperda* cells (step 1) and inoculate them with 100 μl of each virus dilution (two or three replicate plates for each dilution). Set up a similar number of dishes plus 100 μl TC100 growth medium with antibiotics as controls. Incubate the dishes at room temperature for 1 h to allow the virus to adsorb to the cells.
4. Remove the virus inoculum carefully from each dish and cover the cells with 2 ml overlay medium.
5. When the overlay medium has set, add 1 ml TC100 growth medium with antibiotics. Incubate the dishes at 28°C for three to four days.
6. Stain the cells with neutral red. To do this dilute the stock neutral red solution 1:20 with PBS and add 1 ml to each dish. Incubate at 28°C for 2–4 h, then drain off the stain, and leave the dishes in the dark overnight to allow the plaques to clear.[b]
7. Examine the dishes and count the plaques. Use the values (*c.* 10–30 plaques per dish) from the most appropriate dilution to calculate the p.f.u./ml of the virus stock suspension.

[a] The number of dishes required will be determined by the number of virus samples to be assayed (see steps 2 and 3).
[b] The virus plaques may be stained with X-gal to permit detection of β-galactosidase (see Section 5.3.1).

Protocol 4. Purification of virus particles and preparation of viral DNA

Equipment and reagents

- Spinner culture vessels: round, flat-bottomed glass 1 litre flasks
- TC100 growth medium (see *Protocol 1*)
- *S. frugiperda* cells (see *Protocol 1*)
- Titrated virus stock (see *Protocol 3*)
- Low speed centrifuge (e.g. Beckman J2-HS) and bottles
- Centrifuge, swing-out rotors for 15 ml and 38 ml tubes (e.g. Beckman SW41, SW28, and Ultraclear tubes)
- TE buffer: 10 mM Tris–HCl pH 8, 1 mM EDTA
- 10% (w/v) and 50% (w/v) sucrose in TE buffer: sterilize by autoclaving (120°C, 20 min)
- Microcentrifuge tubes (1.5 ml; autoclaved at 120°C, 20 min)
- Sarkosyl solution: 20% (w/v) *N*-lauryl sarcosine in 10 mM Tris–HCl pH 8, 10 mM EDTA
- 50% (w/v) CsCl solution in TE buffer containing 25 μg/ml ethidium bromide
- Liquid paraffin
- Butan-1-ol

Protocol 4. *Continued*

Method

1. Aseptically transfer 400–500 ml TC100 growth medium into the spinner culture vessels and seed each with 1–2 × 10^5 *S. frugiperda* cells in TC100 growth medium. Incubate the cells with stirring at 28°C for two to three days until the cell density has reached 5 × 10^5 cells/ml.
2. Inoculate the spinner vessels with virus (m.o.i. = 0.1 p.f.u./cell).
3. When all the cells are infected (four or five days post-infection), decant the cultures into centrifuge bottles and remove cell debris by centrifugation at low speed (2500–3000 *g*) for 10 min at 4°C.
4. Transfer the clarified supernatant to 38 ml ultracentrifuge tubes and pellet the virus (100 000 *g*, 1 h, 4°C).
5. Decant the supernatant and soak the virus pellet overnight in 1–2 ml TE buffer at 4°C.
6. Prepare discontinuous sucrose gradients by layering 5 ml 10% (w/v) sucrose in TE buffer on top of 5 ml 50% (w/v) sucrose in TE buffer in 15 ml swing-out centrifuge tubes. Cool the tubes in ice.
7. Gently resuspend the pellet (step 5). If necessary, remove any remaining cell debris as in step 3.
8. Using a Pasteur pipette layer 0.5–1 ml virus suspension onto the discontinuous sucrose gradients. Centrifuge at 100 000 *g* for 1 h at 4°C.
9. The virus will band at the interface between the two sucrose layers. Harvest the virus from above using a Pasteur pipette.
10. Dilute the virus 1:5 with TE buffer and centrifuge the suspension at 100 000 *g*, 30 min, 4°C.
11. Soak the virus pellet overnight at 4°C in 1 ml TE buffer. Gently resuspend the virus and store the suspension at 4°C. Do not freeze the virus as this causes lysis of the virus particles and subsequent difficulty in preparing the DNA.
12. Transfer 400 μl of the purified virus suspension into a sterile 1.5 ml microcentrifuge tube and add 100 μl Sarkosyl solution. Incubate at 60°C for 30 min to lyse the virus particles.
13. Layer the lysate onto 5 ml of 50% (w/w) CsCl in TE buffer containing 25 μg/ml ethidium bromide in a 15 ml swing-out centrifuge tube. Top up the tube with liquid paraffin and centrifuge at 250 000 *g* for 18 h at 20°C.
14. The DNA will be visible as two orange bands, corresponding to supercoiled and nicked–circular species. Harvest the DNA by puncturing the bottom of the tube and collecting both of the bands. Mix the two bands and extract the ethidium bromide by gently shaking the

solution with an equal volume of butan-1-ol.[a] Allow the layers to separate. Remove and discard the upper butanol phase. Repeat the extraction two or three times.

15. Dialyse the DNA overnight against 1 litre TE buffer at 4°C, changing the buffer once. Transfer the DNA into a sterile container and store it at 4°C.[b] Determine the concentration of DNA by measuring its A_{260}. The viral DNA may be used for transfection and should be kept sterile. Do not freeze the DNA as this reduces infectivity.

[a] During the butanol extraction, do not shake the DNA vigorously. From this stage onwards care must be taken to avoid shearing the DNA when using pipettes, etc.
[b] The DNA must not be precipitated as this may cause shearing.

4. Baculovirus expression vectors

4.1 Manipulating the baculovirus genome

The large size of the AcMNPV baculovirus genome (134 kbp) (2) limits its direct manipulation, although it is possible to insert foreign genes directly into the viral DNA (12, 13). However, for most workers, it is easier to employ a plasmid vector for manipulation of a portion of the baculovirus genome, designated the transfer vector. The transfer vector serves as a vehicle for insertion of the foreign gene into the virus genome by means of cell-mediated homologous recombination. This is achieved by co-transfection of the transfer vector and viral DNA into insect cells, and selection of recombinant virus from the background of parental virus. The site of insertion for the foreign gene is determined by the sequence of the regions flanking the gene in the transfer vector. For the majority of baculovirus transfer vectors, this site is based on the polyhedrin gene region. Replacement of the polyhedrin coding sequences in the virus results in the production of a recombinant virus which does not form polyhedra. Additional improvements to both the transfer vectors and the viral DNA itself greatly facilitate the selection of viruses containing the foreign gene (Section 5.1).

4.2 Baculovirus transfer vectors

The majority of baculovirus transfer vectors are based on the polyhedrin gene region and utilize the polyhedrin promoter. Other loci in the viral genome have also been employed as sites for foreign gene insertion. A summary of some of the transfer vectors currently available is shown in *Table 2*.

4.2.1 Polyhedrin gene promoter-based transfer vectors

There are several transfer vectors available which permit the insertion of a foreign gene at the polyhedrin locus under the control of the polyhedrin

Table 2. Examples of baculovirus transfer vectors[a]

Vector	Size (kbp)	Features	Reference or source of information
Polyhedrin gene locus			
Single expression vectors			
pAcYM1 (polyhedrin)	9.2	Retains complete 5′ leader and first nucleotide of polyhedrin ATG codon.	14
pAcCL29 (polyhedrin)	7.8	As pAcYM1, less sequence flanking polyhedrin. Has single-stranded DNA capability.	15
pBacPAK8/9 (polyhedrin)	5.5	MCS[b] in place of polyhedrin coding sequences. Identical except for MCS order.	Clontech
pAcMP1 (basic)	10.1	Basic protein promoter in place of polyhedrin gene and promoter. Expression in late phase.	21
pAcSurf2 (polyhedrin)	8.5	Fusion of foreign gene with duplicate copy of *gp64*. Permits display of ligands on virion surface.	24
BaculoGEX vectors (polyhedrin)	8.5	Fusion of foreign gene with *sj26*. GST–fusion protein[c] produced.	22
pBacPAKHis (polyhedrin)	4.9	Fusion of foreign gene to His_6 sequence. Histidine-tagged fusion protein produced.	Clontech
Multiple expression vectors			
pAcUW2B (dual promoter)	10.7	*p10* promoter upstream of normal polyhedrin gene and promoter. Polyhedrin positive virus produced.	16
pAcUW31 (dual promoter)	8.5	Polyhedrin and *p10* promoters at polyhedrin locus. Has single-stranded DNA capability.	Pharmingen
pAcAB3 (triple promoter)	10.1	Two *p10* promoters, one polyhedrin promoter at polyhedrin locus.	19
pAcAB4 (quadruple promoter)	10.1	Two copies each of polyhedrin and *p10* promoters at polyhedrin locus.	19
p10 gene locus			
pAcUW1	4.5	*p10* gene deleted. Normal polyhedrin gene and promoter. Polyhedrin positive virus produced.	16

[a] All these vectors may be used in conjunction with linearized BacPAK6, apart from pAcUW1 which is used with linearized AcUW1.*lacZ* (*lacZ* at *p10* locus; normal polyhedrin locus) (16). Various baculovirus laboratory manuals are available to supplement this list (25, 55).
[b] MCS: *Bam*HI, *Pst*I, *Xho*I, *Xba*I, *Bgl*II, *Kpn*I, *Sac*I, *Eco*RI, *Sma*I, *Not*I, *Pac*I.
[c] GST, glutathione *S*-transferase.

promoter. Vectors which retain the complete 5′ mRNA leader sequence of the promoter express proteins most efficiently, such as pAcYM1 (14) (*Table 2*). The vector pAcCL29 (15) (*Table 2*) is also useful as it contains the bacteriophage M13 intergenic region, which permits the formation of single-stranded DNA in *Escherichia coli* after superinfection with the helper phage, M13 KO7. This is particularly useful for procedures such as site-directed mutagenesis. The vectors pBacPAK8 and pBacPAK9 (commercially available from Clontech) are used in conjunction with linearized viral DNA (Section 5.1).

4.2.2 *p10* promoter-based transfer vectors

The role of the P10 protein in virus replication is poorly understood, but it may be involved with polyhedron formation (5, 7). Transfer vectors which utilize the *p10* promoter include pAcUW1 (16) (*Table 2*). This plasmid is based on the *p10* locus, where foreign genes are inserted into the virus genome in place of native *p10*. Consequently, recombinant viruses constructed using this transfer vector produce polyhedra, since the polyhedrin gene region remains unmodified. This is a useful vector to use with improved selection methods (Section 5.1).

4.2.3 Multiple promoter vectors

These vectors permit the simultaneous expression of up to five foreign proteins by a single recombinant virus (17–20) (*Table 2*). They contain multiple copies of one or more very late promoters and are useful for the study of protein complex assembly by permitting the co-expression of more than one protein in a single cell.

4.2.4 Transfer vectors employing late promoters

It may sometimes be advantageous to express recombinant proteins at earlier stages in virus infection if extensive post-translational modification is required. At earlier times post-infection, the cellular modification systems are less compromised by the virus infection and, therefore, more authentic post-translational modification may be attained. Use of late, rather than very late, promoters may assist this end. However, the level of expression from these promoters may not be as high as with the very late promoters. Late gene promoters which have been employed in transfer vectors include the basic protein and *gp64* promoters, pAcMP1 (21) and pAcATM3, respectively (*Table 2*). Since these genes are required for the production of infectious virus in cell culture, the promoters must be duplicated at alternate loci in the baculovirus genome, namely the polyhedrin locus.

4.2.5 Vectors facilitating the purification of recombinant proteins

Vectors have been designed that yield recombinant viruses expressing foreign proteins fused with glutathione *S*-transferase (22, 23) (*Table 2*). This facilitates purification of the protein fusion using glutathione–Sepharose. Similarly,

vectors are available which permit fusion of the expressed protein with poly-histidine tags. In addition, a system has been developed for AcMNPV which permits the display of foreign proteins on the surface of the virion (24) (*Table 2*). This is achieved by fusion of the foreign gene with a duplicated copy of *gp64*, which leads to the recombinant fusion protein oligomerizing with wild-type GP64 protein and subsequent display of the fusion protein on the virion envelope. This system is analogous to phage display systems.

4.3 Preparation of recombinant transfer vectors

A method suitable for the preparation of transfer vectors is described in *Protocol 5*. This protocol assumes that pure preparations of vector and foreign DNA are available. Methods for preparing DNA are described in several laboratory manuals (e.g. ref. 26). Optimum results are obtained using DNA which has been prepared using CsCl gradients (25) or commercially available DNA preparation kits (e.g. from Qiagen). Competent *E. coli* cells for transformation may be prepared by the calcium chloride method (25) or obtained commercially. Recombinant clones isolated at the end of *Protocol 5* should be analysed by restriction mapping to verify the presence of the DNA insert and to determine its orientation (see *Protocol 11* and ref. 26). It is advisable to sequence across the junction between the virus gene promoter and the foreign DNA insert to confirm the predicted nucleotide sequence. The recombinant vector prepared as in *Protocol 5* may be used subsequently in co-transfections with viral DNA.

Protocol 5. Preparation of recombinant transfer vector

Equipment and reagents

- Transfer vector (e.g. pBacPAK8 or 9) (Clontech)
- Restriction endonuclease and restriction enzyme buffer appropriate for the cloning site being used in the vector
- Calf intestinal phosphatase (CIP): 1 U/μl (Boehringer)
- 10% (w/v) SDS
- 0.5 M EDTA
- Phenol:chloroform (50:50, v/v) saturated with 50 mM Tris–HCl pH 7.5
- 3 M sodium acetate pH 5.2
- Ethanol: absolute and 75% (v/v)
- TE buffer (see *Protocol 4*)
- 10 × ligation buffer: 660 mM Tris–HCl pH 7.5, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP
- T4 DNA ligase: 1 U/μl (Gibco)
- Foreign DNA with ends compatible with the cloning site in the vector
- Sterile deionized water
- Competent *E. coli* cells for transformation (see text)
- Selective Luria broth (LB) agar plates containing ampicillin (25)

Method

1. Digest 5 μg of the transfer vector DNA with the restriction endonuclease as recommended by the enzyme supplier.
2. Add 5 U CIP and incubate at 37 °C for 15 min.
3. Add 10% SDS to 1% and 0.5 M EDTA to 25 mM (final concentrations). Extract the sample twice with an equal volume of phenol:chloroform.

4. Recover the upper phase and add 3 M sodium acetate to 0.3 M final concentration and 2.5 vol. absolute ethanol to precipitate the DNA.
5. Centrifuge at 10000 *g* for 10 min, wash the DNA pellet with 75% ethanol, and then resuspend it in 50 μl TE buffer.
6. Ligate the foreign DNA insert to the digested transfer vector in a total volume of 10–20 μl. For example, mix 10 ng transfer vector, 50 ng foreign DNA, 1 μl of 10 × ligation buffer, 1 μl T4 DNA ligase, and sterile H_2O to a total volume of 10 μl. Incubate the mix overnight at 16°C.
7. Transform competent *E. coli* cells with the recombinant transfer vector (see text). Spread the transformed cells onto selective LB agar plates, and then incubate them at 37°C for 16–18 h.
8. Isolate plasmid DNA sample from selected colonies and characterize them by restriction enzyme analysis as described in *Protocol 11* and ref. 26.

5. Preparation of recombinant virus

5.1 Optimizing the selection of recombinant virus

When the baculovirus expression system was first developed, selection of recombinant virus was undertaken by visual inspection of progeny virus plaques for the absence of polyhedra. For the inexperienced user of this system, this technique was time-consuming and laborious, as the frequency of recombination was low, 1% (27). In addition, polyhedra negative plaques could frequently be obscured by wild-type virus plaques. One solution to the problem was the development of transfer vectors which contained the *E. coli lacZ* gene in addition to the foreign DNA insert. Insertion of this gene into the baculovirus genome results in the recombinant virus plaques staining blue when X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) is added to the plaque assay (see *Protocol 8*) against a background of colourless parental plaques. However, this method did not address the problem of the low recombination frequency and also resulted in the contamination of the recombinant protein with β-galactosidase.

A method effective in improving the recombination frequency was introduced with the use of linearized viral DNA. A unique restriction enzyme site (*Bsu*36I) was introduced into the AcMNPV genome at the polyhedrin locus to generate AcRP6-SC (28). Linearization of the viral genome using *Bsu*36I reduces the infectivity of the DNA, but increases the proportion of recombinant virus produced to 30% (29). On co-transfection of the linearized viral DNA and the transfer vector, a double recombination event repairs the break in the viral DNA, resulting in the restoration of infectivity. Thus, the proportion of recombinant virus is enhanced. This technique was modified by

the use of virus DNA containing *lacZ* at either the polyhedrin locus, as in AcRP23.*lacZ* (28), or the *p10* locus, as in AcUW1.*lacZ* (16). *LacZ* contains a *Bsu*36I site which permits linearization of the genome within *lacZ*. Co-transfection with the transfer vector (polyhedrin-based for AcRP23.*lacZ*, e.g. pAcYM1, or *p10*-based for AcUW1.*lacZ*, e.g. pAcUW1) results in progeny virus which remain colourless against a background of blue parental plaques in the presence of X-gal.

A further modification of this system resulted in the ability to produce almost 100% recombination frequencies (27). Two extra *Bsu*36I sites were introduced into AcRP23.*lacZ* in the regions flanking the polyhedrin locus to generate BacPAK6. Digestion of BacPAK6 with *Bsu*36I results not only in linearization of the viral genome, but also disrupts ORF 1629, a gene essential for replication of AcMNPV in cell culture (28). Thus, infectious virus can only be produced by recombination with a transfer vector containing an intact ORF 1629 gene, along with the foreign gene under the control of the polyhedrin promoter. Colourless recombinant virus plaques are then selected on X-gal medium against a very low background of blue parental virus plaques. The background is usually due to incomplete *Bsu*36I digestion of BacPAK6. The transfer vectors pBacPAK8 and pBacPAK9 permit insertion of foreign genes at a multiple cloning site downstream from the polyhedrin promoter and are then used in co-transfection with the linearized BacPAK6. These vectors and viral DNA are commercially available from Clontech. However, most transfer vectors based on the polyhedrin locus contain ORF 1629 and can, therefore, be used with linearized BacPAK6 (*Table 2*). This system drastically reduces the time taken to prepare recombinant virus, since only a few plaques need to be selected and characterized.

We recommend the use of the BacPAK system when preparing recombinant virus. A method for the linearization of viral DNA is given in *Protocol 6*.

Protocol 6. Preparation of linearized viral DNA

Equipment and reagents

- Infectious viral DNA from BacPAK6 (for polyhedrin-based vectors) or AcUW1.*lacZ* (for *p10*-based vectors) (see *Protocol 4*)
- *Bsu*36I and 10 × enzyme buffer (New England Biolabs)
- Sterile deionized water
- Water-baths at 37 °C and 60 °C
- 0.6% agarose gel for DNA analysis, appropriate electrophoresis equipment (see *Protocol 11*)

Method

1. Mix 2 μg viral DNA with 10 μl of 10 × enzyme buffer and 10 U *Bsu*36I in a total volume of 100 μl (made up with sterile water).[a] Also set up a control incubation where the enzyme is replaced by H_2O.
2. Incubate the mixtures at 37 °C for from 6 h to overnight and then store them at 4 °C.

3. Remove a 100 ng sample from each and analyse the DNA on a 0.6% agarose gel (for details, see *Protocol 11*). Check that all the DNA has been linearized by comparing the enzyme-digested and control samples. If linearization is not complete, add more enzyme to the main sample (step 2) and continue the incubation for a few more hours.
4. Terminate the reaction by heating at 60°C for 15 min, then store the linearized DNA at 4°C.

[a] Sterility of the DNA is essential, since it will be used to transfect insect cells in culture.

5.2 Co-transfection of insect cells with linearized viral DNA and recombinant transfer vectors

Co-transfection may be carried out using either the calcium phosphate precipitation method or lipofectin. Lipofectin is more efficient in promoting the transfection of insect cells than calcium phosphate. It has the disadvantage that the lipofectin reagent is expensive and has a limited shelf-life. If few transfections are to be performed in a six month period most of the lipofection may be wasted. For those users concerned about costs, the calcium phosphate method offers considerable savings over the lipofectin-based method since all of the reagents required are usually available in most laboratories. The major disadvantage of using calcium phosphate-based methods is that more DNA is required to perform the transfection. If virus DNA is to be purchased, rather than prepared from purified virus particles (*Protocols 5* and *6*), the savings may be outweighed by this additional expense. Cells are also more prone to be damaged by the heavy precipitate formed during calcium phosphate-mediated transfection. This reduces virus yields. Both methods are described in *Protocol 7* but we recommend the use of lipofectin.

Protocol 7. Co-transfection of insect cells

Equipment and reagents

- *S. frugiperda* cells grown to logarithmic phase in 35 mm Petri dishes (see *Protocol 2*)
- TC100 growth medium and TC100 medium lacking FCS or antibiotics (see *Protocol 1*)
- Linearized viral DNA (see *Protocol 6*)
- Recombinant transfer vector (prepared as in *Protocol 4*)
- Filters (0.2 μm) for sterilizing solutions (Millipore)
- 2 × Hepes-buffered saline (HBS): 40 mM Hepes–NaOH pH 7.05, 2 mM Na_2PO_4, 10 mM KCl, 280 mM NaCl (filter sterilized)
- 100 mM glucose (filter sterilized)
- Sterile deionized water
- 2 M $CaCl_2$ (filter sterilized)
- Sterile 6 ml polystyrene containers (Gibco)
- Lipofectin (Gibco)

A. *Calcium phosphate precipitation*

1. Seed 35 mm diameter Petri dishes with 1×10^6 *S. frugiperda* cells/dish in 2 ml TC100 growth medium. Incubate at 28°C for 2 h to allow the cells to attach to the plastic surface.

Protocol 7. *Continued*

2. Mix 1 μg linearized viral DNA, 5 μg transfer vector, 0.5 ml of 2 × HBS, 100 μl of 100 mM glucose, and make up to 935 μl with sterile water.
3. While vortexing the mixture, add 65 μl 2 M $CaCl_2$ dropwise over a period of 10 sec or less.[a] Incubate the mixture at room temperature for 30 min to allow a fine precipitate to form. Gently resuspend the precipitate to generate the transfection mix.
4. Remove the medium from the cells and add the transfection mix dropwise onto the surface of the cells. Incubate the cells at room temperature for 30 min.
5. Add 2 ml TC100 growth medium to each dish and incubate them at 28°C for 24 h.
6. Remove the medium from the dishes, wash the cells with 2 ml TC100 growth medium, then add a further 2 ml of this medium to each dish. Incubate the dishes at 28°C for a further 48 h.
7. Harvest the cell culture medium and titrate the virus using the plaque assay described in *Protocol 3.*

B. *Lipofectin-mediated transfection*

1. Carry out part A, step 1.
2. Mix 100 ng linearized viral DNA with 500 ng recombinant transfer vector in a sterile polystyrene container. Add 500 μl TC100 medium lacking FCS and antibiotics.[b]
3. Mix 5 μl lipofectin with 500 μl TC100 lacking FCS in a separate polystyrene container. Add the mixture to the DNA solution (part B, step 2). Incubate at room temperature for 15 min.
4. Remove the medium from the cells and wash the cells twice with 2 ml TC100 lacking FCS and antibiotics. Add 1 ml TC100 lacking FCS and antibiotics to the cells.
5. Add the DNA/lipofectin mixture (volume 1 ml) from part B, step 3 to the cells and swirl gently. Incubate the dishes at 28°C for at least 5 h or overnight.
6. Remove the medium from the cells and add 2 ml TC100 growth medium (containing FCS and antibiotics). Incubate at 28°C for 48 h.
7. Carry out part A, step 7.

[a] Vortexing must not be carried out for longer than 10 sec to avoid shearing the viral DNA.

[b] The presence of antibiotics can reduce the efficiency of the transfection.

5.3 Identification and purification of recombinant viruses

5.3.1 Plaque titration and staining with neutral red or X-gal

To distinguish recombinant from parental virus, the co-transfection medium from *Protocol 7* (part A, step 7 or part B, step 7) is used in a plaque assay in the same way as for the propagation of virus stocks (*Protocol 3*). When linearized DNA has been used, the undiluted medium and dilutions of 10^{-1}, 10^{-2}, and 10^{-3} should be assayed. A method for the selection of *lacZ* positive or *lacZ* negative viruses is given in *Protocol 8*. If *lacZ* positive viruses are required, use X-gal alone; if *lacZ* negative viruses are required, use both X-gal and neutral red for dual staining.

Protocol 8. Distinguishing between *lacZ* positive and *lacZ* negative viruses

Equipment and reagents

- Equipment and reagents for the standard plaque assay (see *Protocol 3*)
- Neutral red stain solution (prepared in *Protocol 3*, step 6)
- X-gal solution: 2% (w/v) 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside in dimethyl formamide (DMF)—store the X-gal at –20°C in the dark

Method

1. Carry out the plaque assay as described in *Protocol 3*, steps 1–5.
2. Replace the 1 ml liquid overlay with 1 ml TC100 growth medium containing antibiotics and 15 μl X-gal solution per ml medium. Incubate the dishes at 28°C for 5 h or overnight.
3. To stain the plaques with both X-gal and neutral red, add 1 ml neutral red stain 3 h after the start of the incubation with X-gal (step 2). Continue the incubation for a further 2 h at 28°C. If neutral red stain is not required, omit this step.
4. Drain the stain solution from the dishes and invert them in the dark to permit the plaques to clear.
5. Examine the plaques and pick the desired plaques as described in *Protocol 9*.

5.3.2 Picking and purifying plaques

Once plaques have been identified as containing recombinant virus (*Protocol 8*), they must be isolated and independently plaque purified. It is essential that several plaques are obtained to avoid selection of false positive viruses (although this is less of a problem when employing BacPAK6 as the parental virus). It is best to select well-isolated plaques and it may be helpful to ring them with a marker pen on the underside of the dish before picking. Plaques

picked in this way may be used directly in the next round of amplification. The procedures for picking, and amplifying plaques and purification are described in *Protocol 9.*

Protocol 9. Plaque purification and amplification

Equipment and reagents

- Plates containing recombinant virus plaques (see *Protocol 8*)
- Sterile Pasteur pipettes and 6 ml polystyrene containers (Gibco)
- TC100 growth medium (see *Protocol 1*)
- *S. frugiperda* cells in the logarithmic growth phase (see *Protocol 1*)
- Equipment and reagents for the plaque assay (see *Protocol 3*) and large scale preparation of virus (see *Protocol 4*)

Method

1. Take a plug of agarose from the plate into a Pasteur pipette so it contains the plaque you have identified. Wash the plug into 500 μl TC100 growth medium in a sterile polystyrene container.
2. Vortex the container to release the virus particles from the plug. Store the virus suspension at 4°C until required for the next round of plaque purification or amplification.
3. To purify the virus by replating, dilute 100 μl of the virus suspension to create 10^{-1}, 10^{-2}, and 10^{-3} dilutions in TC100 growth medium. Carry out several rounds of the plaque assay as described in *Protocol 3.*[a]
4. To amplify the purified virus, first propagate a small virus stock using the method described in *Protocol 2.* Titre this stock (*Protocol 3*) and then prepare virus on a large scale in suspension culture, as described in *Protocol 4*, steps 1–3.

[a]For the isolation of genetically homogeneous recombinant virus, at least three rounds of plaque purification are normally employed to generate a pure stock of virus. If linearized DNA has been used for co-transfection, the number of rounds of may be reduced.

6. Characterization of recombinant virus DNA

The presence of the foreign gene in the purified recombinant virus may be determined by restriction enzyme analysis of the genome followed by Southern blot hybridization and sequencing, if necessary. A method for the extraction of DNA from virus which is suitable for these analyses is given in *Protocol 10.* Restriction enzyme analysis of this DNA and separation of the fragments by agarose gel electrophoresis, described in *Protocol 11*, will identify changes in pattern due to the insertion of the foreign sequence. Subsequent blotting of the fragments onto a nitrocellulose membrane and probing with an appropriate radiolabelled probe will confirm the presence and correct orientation of the foreign gene in the recombinant viral genome.

Protocol 10. Extraction of DNA from virus-infected cells

Equipment and reagents

- *S. frugiperda* cells grown to 1 × 10^6 cells/35 mm Petri dish (see *Protocol 2*)
- 35 mm diameter Petri dishes (see *Protocol 2*)
- Virus stocks prepared and titred as described in *Protocols 2* and *3*, respectively
- TC100 growth medium (see *Protocol 1*)
- Sandwich box lined with moist tissue paper and a tight-fitting lid
- Cell scrapers (see *Protocol 1*)
- PBS (see *Protocol 3*)
- TE buffer (see *Protocol 4*)
- Lysis buffer: 50 mM Tris–HCl pH 8, 5% (v/v) 2-mercaptoethanol, 0.4% (w/v) SDS, 10 mM EDTA
- 10 mg/ml Proteinase K in TE buffer (freshly made and incubated at 37°C for 30 min prior to use)
- 10 mg/ml RNase A in TE buffer (boiled for 10 min prior to initial use and subsequently stored at –20°C)
- Phenol:chloroform (see *Protocol 5*)
- Rocking platform shaker
- 3 M sodium acetate pH 5.2
- Ethanol: absolute and 75% (v/v)

Method

1. Remove the medium from the cells and inoculate them with virus using a high m.o.i. (10 p.f.u./cell). Other dishes of cells should also be infected with unmodified AcMNPV to serve as a wild-type virus control. Also set up mock-infected controls inoculated with the same volume of TC100 growth medium without virus. Incubate all the dishes for 1 h at room temperature to allow the virus to adsorb.
2. Remove the inoculum and the mock inoculum from the dishes and overlay the cells with 2 ml TC100 growth medium. Incubate the cells in the closed sandwich box for 18 h at 28°C.
3. Scrape the cells off the surface of the dish into the medium. Transfer the cell suspension to a microcentrifuge tube and pellet the cells (2500–3000 *g*, 1 min). Wash the pellet with 500 μl PBS, then centrifuge again. The washed pellets may be stored at –20°C or used immediately.
4. Resuspend the pellet in 250 μl TE buffer and add 250 μl lysis buffer. Mix gently.
5. Add 12.5 μl of 10 mg/ml Proteinase K and 2.5 μl of 10 mg/ml RNase A. Incubate the mixture at 37°C for 30 min.
6. Extract the lysate with an equal volume of phenol:chloroform by rocking gently for 2–3 min. Separate the phases by microcentrifugation. Re-extract the aqueous phase twice more.
7. Add 50 μl of 3 M sodium acetate and 1 ml absolute ethanol. Pellet the DNA by microcentrifugation for 5 min. Recover the DNA precipitate (see *Protocol 5*, step 5).

Protocol 10. *Continued*

8. Wash the DNA pellet twice with 75% (v/v) ethanol, then air dry it, and add 100 μl TE buffer. Store the DNA overnight at 4°C to allow the DNA to rehydrate.
9. Incubate the DNA at 37°C for 10 min, then gently resuspend using a micropipette tip. Store the DNA solution at 4°C.

Protocol 11. Analysis of DNA from virus-infected cells

Equipment and reagents

- DNA extracted from virus-infected cells (see *Protocol 10*)
- Restriction endonuclease and restriction enzyme buffer as appropriate for the DNA to be analysed
- 10 × TBE buffer: 108 g/litre Tris base, 55 g/litre boric acid, 9.4 g/litre EDTA
- 5 × DNA loading dye: 40% (w/v) sucrose, 2.5% (w/v) SDS, 0.25% (w/v) bromophenol blue in 5 × TBE
- Electrophoresis size markers (e.g. phage λ DNA digested with *Hin*dIII and *Eco*RI)
- 0.7% (w/v) agarose gel in 1 × TBE containing 0.5 μg/ml ethidium bromide
- ^{32}P-labelled nucleic acid probe (for methods of preparation see ref. 26)
- Electrophoresis equipment for PAGE
- UV transilluminator
- Photographic facilities
- Equipment and solutions for Southern blotting and nucleic acid hybridization
- Nitrocellulose or nylon membranes (Amersham-Pharmacia)

Method

1. Digest 5–10 μl DNA solution with an appropriate restriction enzyme in a volume of 20–40 μl at 37°C for 2 h (see the instructions provided by the enzyme supplier).
2. Add 5–10 μl of 5 × DNA loading dye to the restricted DNA. Prepare a sample of the DNA size markers in the same way.
3. Load the samples on the agarose gel and start the electrophoresis using 1 × TBE buffer.
4. When the dye front is at the bottom of the gel, stop the electrophoresis and then photograph the gel using a UV transilluminator.
5. Transfer the DNA by blotting the gel onto a nitrocellulose or nylon membrane.
6. Identify recombinant DNA sequences using the radiolabelled probe.

7. Analysis of protein synthesis in virus-infected cells

Polyacrylamide gel electrophoresis (PAGE) is the method most commonly used to detect the synthesis of foreign proteins in recombinant virus-infected cells. When the foreign gene is under the control of the very strong polyhedrin

or *p10* promoters, it may be possible to visualize the protein in the PAGE gel using Coomassie Blue staining as described in *Protocol 12*. Alternatively, the cells may be pulse labelled during infection with a radiolabelled amino acid such as [^{35}S]methionine (the most common choice), [^{35}S]cysteine, or [^{3}H]leucine (less widely used) followed by autoradiography of the gel as described in *Protocol 13*.

To achieve a synchronous infection of the cells and, therefore, an accurate profile of the temporal synthesis of the recombinant protein, a high m.o.i. must be used. Additionally, control samples from wild-type infected cells and uninfected cells should always be included in the analysis to facilitate comparison of the protein synthesis profiles. Initially, samples produced at 24 and 48 h.p.i. should be analysed, then a more extensive time course employed to examine the optimum time of foreign protein synthesis. Procedures for running polyacrylamide gels are given in Chapter 6 (*Protocol 7*) or ref. 30.

Protocol 12. Analysis of protein synthesis in virus-infected cells

Equipment and reagents

- Virus stocks prepared and titred as described in *Protocols 2* and *3*, respectively
- Reagents and cells for virus propagation in 35 mm diameter dishes (see *Protocol 10*)
- PBS (see *Protocol 3*)
- TE buffer (see *Protocol 4*)
- 5 × dissociation mix: 10% (w/v) SDS, 25% (v/v) 2-mercaptoethanol, 50 mM Tris–HCl pH 6.8, 25% (w/v) glycerol, 0.25% (w/v) bromophenol blue
- Polyacrylamide gel: 12% single concentration or 10–30% gradient (30)
- Electrophoresis equipment for PAGE
- Coomassie Blue stain mix: 0.2% (w/v) Coomassie Brilliant Blue in destain mix
- Destain mix: 5% (v/v) glacial acetic acid, 50% (v/v) methanol, 45% (v/v) water

Method

1. For each time point desired set up one Petri dish (1×10^6 *S. frugiperda* cells/dish) for AcMNPV infection and one for mock infection. Allow the cells to attach. Inoculate the experimental cells with virus (10 p.f.u./cell) as described in *Protocol 10*, step 1. Incubate at 28°C for the predetermined times post-infection.
2. Scrape the cells into the medium[a] and transfer the suspension to a microcentrifuge tube. Pellet the cells (2500–3000 *g*, 2 min). Wash the pellet with 500 μl PBS.
3. Centrifuge the cells again and resuspend them in 100 μl TE buffer. Add 25 μl of 5 × dissociation mix and heat them at 100°C for 5 min. Cool the samples to room temperature.
4. Load the samples onto the polyacrylamide gel and electrophorese at 50 V overnight or until the bromophenol blue has reached the bottom of the gel.

Protocol 12. *Continued*

5. Stain the gel for 1 h with Coomassie Blue stain mix. Destain it in destain mix to visualize the separated proteins.

[a] After 18 h.p.i., the infected cells will begin to detach from the substratum and will be easy to dislodge from the plate.

Protocol 13. Radiolabelling the proteins of virus-infected cells

Equipment and reagents

- Equipment and reagents for analysis of protein synthesis in infected cells (see *Protocol 12*)
- [^{35}S]methionine: 1000 Ci/mmol (Dupont)[a]
- Starvation medium: TC100 medium lacking both the amino acid used for labelling and bactotryptose broth

Method

1. Infect the cells as described in *Protocol 12*, step 1. Incubate the cells at 28 °C for the required period, usually 12–72 h.p.i.
2. Remove the medium from the cells. Add 0.5 ml starvation medium to each dish and continue the incubation at 28 °C for 30 min.
3. Add a further 0.5 ml starvation medium containing 15–30 μCi [^{35}S]methionine and incubate at 28 °C for 1 h.[b]
4. Harvest the cells and analyse by SDS–PAGE as described in *Protocol 12*, steps 2–4.
5. Fix the proteins in the gel using destain mix (see *Protocol 12*, step 5), then dry the gel, and expose it to X-ray film.

[a] [^{35}S]cysteine (600 Ci/mmol), [^{14}C]arginine (300 mCi/mmol), or [^{3}H]leucine (140 Ci/mmol), supplied by Dupont, may be substituted depending on the protein to be labelled and the type of labelling desired.
[b] Use 1–5 μCi [^{14}C]arginine or 30–50 μCi [^{3}H]leucine if these precursors are being used.

8. Post-translational modification of proteins synthesized using the baculovirus expression system

An advantage of using the baculovirus expression system, instead of prokaryotic expression systems, is that insect cells have the capacity to perform post-translational modifications to proteins such as glycosylation, phosphorylation, intracellular targeting, and signal peptide cleavage, and the formation of

tertiary and quartenary complexes (14, 30–33). A summary of selected post-translational modification characteristics found with proteins expressed using this system is given below:

(a) Glycosylation. Proteins expressed in insect cells tend to possess different oligosaccharide side chains compared to their mammalian counterparts (34, 35). Insect cells, unlike mammalian cells, use high mannose oligosaccharides for glycosylation (36). These differences in glycosylation sometimes cause glycoproteins expressed in insect cells to appear smaller than those produced in mammalian cells.

(b) Phosphorylation. This appears to be carried out by an endogenous insect protein kinase, with phosphoserine acting as the major phosphoamino acid (37, 38). Fidelity of recombinant protein phosphorylation in insect cells has been demonstrated (39–42).

(c) Intracellular targeting and signal peptide cleavage. Most proteins synthesized by baculovirus vectors appear to be targeted within the insect cell in a manner analogous to the native host cell. Signal peptide cleavage has been shown to be performed correctly for a number of proteins including human interleukin-2 (43) and influenza virus haemagglutinin (34).

(d) Formation of complexes. Recombinant proteins expressed using baculovirus vectors have the ability to form tertiary and quaternary structures. The multiple expression vectors described in Section 4.2.3 permit the simultaneous expression of several proteins in a single cell (19, 20), allowing the investigation of complex formation.

It is evident from these examples that the baculovirus expression system can be used to produce foreign proteins which are correctly modified and exhibit biological activity. In addition, the proteins are antigenically similar to their native counterparts (44–46).

9. Scaling up recombinant protein production

Once a foreign protein is being successfully expressed by a recombinant baculovirus, in most cases it will be necessary to scale up protein synthesis. There is no standard procedure for this since several factors affect how much infected cell culture is required. These factors include how well the protein is expressed by the baculovirus, the ease of recombinant protein purification, the purity of protein required and, ultimately, how the protein is to be used. For procedures which require only small amounts of protein, the use of monolayer cultures in flasks may be sufficient. To produce larger amounts of protein, suspension cultures must be employed, as they have the capacity to generate higher cell densities. Volumes of up to 500 ml may be grown in this way, with cell densities typically reaching 2–3 $\times$ 10^6 cells/ml. Simple suspension cultures may be set up in round flat-bottomed flasks containing a

magnetic stir bar. Establishment of a synchronous infection in suspension culture is more difficult to achieve than in monolayer culture. There are two ways in which a suspension culture may be inoculated with virus:

(a) Add a virus inoculum directly to the culture. This means that only one manipulation is required, but not all the cells may be infected synchronously.

(b) Harvest the cells, resuspend them in a small volume, and then infect them. After the virus adsorption period, dilute the cells into a large volume to prevent any further virus adsorption. This method efficiently establishes a synchronous infection, but may be impractical for very large volumes of culture.

For culture volumes above 500 ml, the ratio of surface area to culture volume decreases, leading to a restriction in the supply of oxygen to the cells. If air is passed through an insect cell culture (known as gas sparging), many of the cells are lysed when they come into contact with the small air bubbles. Gas sparging also leads to foaming of the culture. However, success has been achieved by growing insect cells in large scale culture in bioreactors and airlift fermenters (47, 48). However, the practical details of these techniques are beyond the scope of this chapter.

10. Alternative methods for producing recombinant baculoviruses

The BacPAK system is one of the most powerful tools available to simplify selection of recombinant viruses. However, other methods are available which also aim to simplify selection. These are directed towards reducing the number of plaque purifications required, a technique which appears troublesome to workers inexperienced in the field.

10.1 Baculovirus–yeast system

This system involves using baculovirus–yeast shuttle vectors (49). Yeast-specific sequences are incorporated into the baculovirus genome to enable it to replicate to a low copy number extrachromosomally. Selectable markers are also included in the viral genome to facilitate the detection of yeast clones containing the baculovirus replicon. Replacement of one selectable marker by homologous recombination with a transfer vector carrying the foreign gene permits the identification of yeast cells carrying the recombinant viral genome. Subsequently, viral DNA is extracted from the yeast cells and used to transfect insect cells. This facilitates the clonal selection of virus and obviates the need for repeated rounds of plaque purification. For methods pertaining to the baculovirus–yeast system, see ref. 50.

10.2 Bacmid system

In a similar way to the baculovirus–yeast system, the baculovirus genome has also been engineered so that it may be harboured as a replicon in *E. coli* (51). Incorporation of antibiotic resistance genes into the baculovirus genome permits the identification of bacterial cells containing the replicon. In addition, a bacterial transposon site is inserted into the viral genome. This allows transposon-mediated recombination to take place with a transfer vector in which the foreign gene is flanked by transposon sequences. Clones are selected by antibiotic resistance, the viral DNA is extracted from these clones, and used to transfect insect cells. Again, this method means that no rounds of plaque purification are required to generate a pure recombinant virus. The system is available as a kit from Gibco Life Technologies.

11. Future developments of the baculovirus expression system

The baculovirus expression system is based upon the fact that expression from the polyhedrin and *p10* promoters is very high. How this expression is regulated is still an ongoing area of research. Elucidation of how polyhedrin and P10 are hyperexpressed would permit the development of vectors which express to this high level, but at an earlier stage in the virus replication cycle, thus providing a longer period for foreign protein production. The nature of the virus-induced RNA polymerase and viral genes controlling late and very late gene expression are central to this question.

Of the 154 open reading frames identified by analysis of the DNA sequence of the AcMNPV genome, only a few have been fully characterized. From this, it has been found that a significant proportion of the genes is not essential for virus replication in cell culture. It is possible that deletion of these non-essential genes could lead to the production of an improved baculovirus expression vector. This is because the transcriptional load on the virus would be reduced, leading to an enhancement of foreign gene expression. In addition, the deleted genes may render the virus non-viable *in vivo*, creating an even safer vector in terms of risk assessment.

Baculoviruses regulate their host range by controlling programmed cell death or apoptosis (52). In AcMNPV, various genes have been implicated to have a role in this control (53, 54). As these genes inhibit apoptosis, it follows that they may also affect cell viability. Manipulation of these genes could lead to the extension of the productive life of a baculovirus-infected cell.

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Protein synthesis in eukaryotic cell-free systems

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1. Introduction

During the last two decades cell-free protein synthesizing systems have been used extensively for the translation of eukaryotic mRNAs, for studies on the mechanism and regulation of the translation process, and the factors involved. The primary advantages of production of a protein in an *in vitro* translation system versus *in vivo* production are:

(a) The flexibility of *in vitro* systems.

(b) The possibility of using a defined template to direct protein synthesis.

In combination with the development of *in vitro* transcription systems, cell-free translation systems have allowed us to demonstrate a direct correlation between a DNA sequence and the encoded polypeptide. For translation of eukaryotic mRNA the rabbit reticulocyte lysate and the wheat germ extract have received the most attention as highly efficient *in vitro* translation systems. This chapter will focus on the preparation and applications of these two systems, although other eukaryotic cell-free translation systems will also be discussed briefly.

The choice of the system to use for any particular application will depend on a variety of factors. In general, eukaryotic systems should be used to translate eukaryotic sequences (whereas prokaryotic systems must be chosen for prokaryotic sequences—see Chapter 7). If the protein to be synthesized may already be present as an endogenous component of the translation system, this may complicate immunological or functional applications. Thus, in these cases it may be wise to choose a system evolutionarily most distant from the biological source of the mRNA to be translated. When post-translational modifications of the protein product are desired, this may be achieved by using microsomal membranes; these work most efficiently in rabbit reticulocyte lysates. Of additional importance in choosing a particular system are the possibilities of using DNA rather than RNA as template (coupled transcription–translation systems; see Section 7.2), and preference for certain detection

methods for the products (e.g. radioactivity versus chemiluminescence; a non-radioactive detection procedure has been developed only for the rabbit reticulocyte lysate system so far).

2. Preparation of messenger RNAs

The utility of cell-free translation systems for the analysis of gene expression has been made possible by the development of procedures for obtaining undegraded, biologically active mRNAs from a variety of eukaryotic sources. It should be noted, however, that for many purposes the need to prepare such mRNA has been eliminated by the development of coupled transcription–translation systems (see Section 7.2).

Because of the great diversity of cells and tissues from which eukaryotic mRNAs need to be extracted, there are many different preparative procedures. The principal methods have been described in detail in an earlier volume of the Practical Approach series (1), and elsewhere (2), and will not be reiterated here. A few general points need to be made, however, the most important of which concern the need to avoid contamination with ribonucleases at all stages in order to obtain good results.

2.1 Precautions against RNase-mediated degradation

Ribonucleases are very robust enzymes and only minute amounts are sufficient to destroy mRNA activity. Both the problem of endogenous ribonuclease activity in the biological material itself and the introduction of ribonuclease contamination from other sources must be avoided. Disposable gloves should be worn at all times when dealing with RNA in order to protect the samples from ribonucleases on the skin, all glass and plasticware should be clean and sterile, and solutions should be prepared from the purest possible reagents, using autoclaved, double glass-distilled water. Solutions should not be stored for long periods of time at room temperature. Many specialist suppliers sell ultra pure, ribonuclease-free reagents. There are also specific reagents which can be used to inhibit ribonuclease activity during handling of mRNA. Heparin is effective in many applications since it adsorbs nucleases and competitively inhibits them. Addition of this compound to buffers at a concentration of approximately 1 mg/ml during subcellular fractionation and polysome preparation (see below) can substantially improve the yield and translational activity of purified mRNA. However, the heparin must be removed from the RNA (with which it co-purifies) before translation assays are carried out since it is an inhibitor of polypeptide chain initiation. This can be achieved by washing ethanol precipitated RNA with 3 M sodium acetate or 2 M LiCl. A ribonuclease inhibitor protein which binds ribonuclease and inhibits it has been purified from human placenta—this is available commercially as RNase inhibitor, RNasin, RNase Block, and ribonuclease inhibitor (from companies such as

Boehringer Mannheim, Promega, Stratagene, Life Technologies, or Clontech). It is useful not only for preventing polysome degradation during subcellular fractionation, and RNA degradation during extraction, but also for improving the yield of translation products when added with exogenous mRNA to the wheat germ cell-free system (Section 4). Proteinase K is also useful as an anti-ribonuclease reagent since it is active in the presence of SDS during RNA extractions and rapidly inactivates nucleases from many sources. It can readily be removed during the subsequent phenol extraction stage.

2.2 Preparation of intact RNA from ribosomal and polysomal fractions

2.2.1 High speed centrifugation of polysomes

If it is necessary to examine the nature and composition of the mRNA population being translated in cells (as opposed to the total cellular mRNA) then isolation of polysomes must precede the RNA extraction itself (3). There are numerous descriptions in the literature of methods of polysome preparation by differential centrifugation. A post-mitochondrial supernatant of a cell lysate or homogenate is layered on a 1 M sucrose cushion in an alkaline buffer (pH 8.5) and centrifuged for 300 000–400 000 *g*.hours. The polysome pellets are then resuspended in buffer for further fractionation or analysis. *Protocol 1* describes a general procedure for polysome preparation. Cells must be lysed under conditions that inhibit ribonuclease activity, do not shear the mRNA population, and do not allow 'run-off' of ribosomes from the mRNA due to translational termination. Cell lysis should be rapid, and all manipulations must be carried out at 0–4 °C. Nuclei, mitochondria, and other cellular organelles are removed by low speed centrifugation, and the polysomes are then obtained from the cytoplasmic fraction by high speed centrifugation. The presence of a sucrose cushion minimizes the contamination of the ribosomal pellet with soluble cytoplasmic proteins.

Protocol 1. General procedure for preparation of polysomes

Equipment and reagents

All reagents must be free of nuclease contamination (see Section 2.1).

- Ice-cold 0.15 M NaCl
- Lysis buffer: 200 mM Tris–HCl pH 8.5,[a] 100 mM KCl, 40 mM NaCl, 5 mM magnesium acetate
- 10% (v/v) Triton X-100
- 10% (w/v) sodium deoxycholate
- 1 M sucrose in lysis buffer
- Small sterile glass homogenizer

Method

1. Wash the cells or tissue in ice-cold 0.15 M NaCl, centrifuging the cells at 150 *g* for 10 min to pellet them.
2. Resuspend the cells in lysis buffer (200 μl/10^7 cells) and lyse the cells

Protocol 1. *Continued*

by adding Triton X-100 to 0.1% and sodium deoxycholate to 0.5%.[b] Vortex the suspension and leave on ice for 5 min.

3. Centrifuge at top speed in a microcentrifuge at 4°C for 5 min.
4. Remove the supernatant and layer it over an equal volume of 1 M sucrose in lysis buffer. Centrifuge at 260 000 *g* for 2 h at 4°C.
5. Remove and discard the supernatant and gently rinse the pellet with lysis buffer (without sucrose).
6. Drain the pellet for a few minutes and then resuspend it in an appropriate buffer (the composition of which will depend on whether RNA is to be extracted or the polysomes are to be analysed on sucrose gradients), using a small sterilized glass homogenizer.

[a] An alkaline pH helps to inhibit endogenous ribonucleases present in cell extracts.
[b] Triton X-100 effectively disrupts cell membranes, and the additional presence of sodium deoxycholate disrupts the association between polysomes and the cytoskeleton.

Although this method of preparing polysomes often works well, there can be problems in some cases. The relatively long time involved in polysome preparation may allow some degradation of the mRNAs by endogenous ribonucleases. There may be some shearing, particularly of long mRNAs, by mechanical damage during pelleting and resuspension of the polysomes. Some non-polysomal RNP particles may also contaminate the pellet. The latter two problems can be overcome by preparing the polysomes by sucrose density gradient centrifugation (see Section 2.2.2) rather than by differential centrifugation. Finally, the recovery of polysomes from some tissues is often less than quantitative, with considerable (perhaps differential) losses during the initial low speed centrifugations of the homogenate.

2.2.2 Fractionation of polysomes

It is often desirable or essential to separate different classes of polysomes before extraction of the mRNA species they contain. This may be necessary in order to determine whether particular proteins are synthesized by specific populations of polysomes (e.g. membrane-bound versus free). One commonly used procedure is the fractionation of polysomes by size on sucrose gradients. This is most useful for analysing mRNA species which are abundant in the total population. Alternatively, if a specific antibody is available against the protein encoded by a particular mRNA, an extremely powerful technique is to utilize the antigenic specificity of the nascent polypeptide chains in the polysomes containing that mRNA.

A number of good procedures have been published for the near quantitative fractionation of polysomes into membrane-bound and free populations and for the extraction of intact mRNAs from these fractions (4–6). The most

thoroughly studied systems have been tissues and cells which are active in protein secretion. One problem is the potential contamination of the bound polysomes with lysosomes, which are rich in ribonuclease activity. Methods have been developed to separate rough microsomal membranes from lysosomes without disrupting the latter (7, 8).

Provided that suitable precautions are taken to prevent the breakdown of polysomes, which can result from either ribonuclease action or ribosome 'run-off' during cell lysis, different size classes of mRNA can be fractionated by centrifugation of the corresponding polysomes through continuous sucrose density gradients. In general, the largest mRNAs occur in the largest polysomes and vice versa, although exceptions to this rule may occur if a particular mRNA species can initiate protein synthesis more or less efficiently than average, resulting in more or less dense packing of ribosomes, respectively. The usual conditions for size fractionation of polysomes are centrifugation through linear gradients of 10–50% sucrose in a buffer such as 0.2 M Tris–HCl pH 8.5, 0.1 M KCl, 40 mM NaCl, 5 mM magnesium acetate. In some cases, for example muscle polysomes, an even higher salt concentration is necessary to prevent protein aggregation and precipitation. The time period and speed of centrifugation and the type of sucrose gradient to be used will depend on the size of the polysomes of interest and must be determined empirically. A good starting point for a 10–50% gradient is to run it at 165000 *g* in a Beckman SW50.1 rotor for 90 min at 4°C. The gradient is then analysed by passing it through the flow cell of a recording spectrophotometer (e.g. ISCO) set to monitor optical density at 254 nm.

RNA can be extracted from preparations of total polysomes, or from polysomal fractions separated on the basis of their association with membranes, their size, or their ability to be immunoprecipitated with specific antibodies.

2.3 Oligo(dT) affinity chromatography for isolation of poly(A)$^+$ mRNA

This is the most widely used method for the purification of mRNA molecules which have a 3′ poly(A) tract and again has been described previously in the Practical Approach series (1). Polysomes can be dissolved in buffer containing SDS and the RNA successfully fractionated on oligo(dT)–cellulose directly, without prior deproteinization. Using this method, mRNA from up to 10 mg of total RNA can be isolated at one time, and then recovered by precipitation with ethanol in the presence of 0.3 M sodium acetate pH 5.2.

2.4 Isolation of individual mRNA species

The isolation of particular mRNA species from total RNA preparations is possible if an antisense sequence complementary to the required mRNA is available for hybridization selection. The antisense DNA or RNA is immobilized on a solid support and then used to bind individual mRNAs under

conditions of high ionic strength (e.g. 1 M NaCl, 50 mM Pipes pH 7, 2 mM EDTA). The mRNA can subsequently be eluted under low salt conditions. A convenient means of probe immobilization is to synthesize the sequence in a biotinylated form and then bind it to streptavidin–agarose (available from Sigma and other suppliers) or streptavidin coated magnetic beads (Dynabeads, Dynal). For further details see ref. 2.

2.5 Transcription of mRNA *in vitro*

Recombinant DNA technology in combination with the characterization of highly efficient and structurally simple prokaryotic promoters, as well as the bacteriophage RNA polymerases that transcribe genes under the control of such promoters, has allowed relatively straightforward preparation of templates for *in vitro* transcription and translation of almost any cloned gene. During the generation of such templates one should take care that the appropriate signals for translation are present in the transcribed RNA. Obviously introns should be absent from the gene that is being transcribed and therefore cDNAs are the most appropriate starting points for the preparation of plasmids for *in vitro* transcription. In the case of eukaryotic translation systems, the presence of optimal sequences around the start codon ('Kozak sequences') (9, 10) and the presence of an m^7GpppG cap at the 5′ end or of an internal ribosome entry site in an mRNA will have a significant impact on the translatability of the RNA (see Section 7.3). The length of the 5′ untranslated region (UTR) and the relative absence of secondary structure within this sequence can affect translational efficiency (11, 12), and the structure of the 3′ UTR may also be important (13).

The enzymes that are most frequently used for *in vitro* mRNA transcription are T7, T3, and SP6 RNA polymerases. Many general cloning vectors, such as pGEM and pBluescript vectors, contain promoters for these polymerases flanking the multiple cloning sites. In general, cloning of a eukaryotic cDNA including 5′ and 3′ UTR sequences into such a vector will allow the synthesis of a suitable RNA template by one of the RNA polymerases mentioned above. *Protocol 2* describes the procedure to be followed.

Protocol 2. mRNA preparation by *in vitro* transcription

Equipment and reagents

It is important that all reagents are RNase-free (see Section 2.1).

- Template DNA: this will be the cDNA of interest cloned into a vector containing T3, T7, and/or SP6 promoters flanking the cloning site. Prior to the transcription reaction, linearize the recombinant DNA by digesting it with a restriction enzyme[a] that cuts at a position downstream of the cDNA coding sequence or (if only the N-terminal part of the encoded polypeptide is to be made) at an appropriate site within the coding sequence. Purify the linearized DNA by phenol:chloroform extraction and concentrate it by ethanol precipitation. Dissolve the DNA template in 10 mM Tris–HCl pH 8, 1 mM EDTA at approx. 1 μg/μl. An alternative to preparing a linearized DNA template is to use a PCR product that contains the required cDNA sequence and the T3, T7, or SP6 promoter.

- 5 × transcription buffer: 200 mM Tris–HCl pH 7.9, 50 mM NaCl, 30 mM $MgCl_2$, 10 mM spermidine–HCl
- Ribonucleotide mixture: 5 mM each of ATP, CTP, and UTP, 1 mM GTP
- Phenol:chloroform:isoamyl alcohol (25:24:1, by vol.)
- Sephadex G50 spin column, equilibrated with 10 mM Tris–HCl pH 7.9, 1 mM EDTA
- 100 mM DTT
- 5 mg/ml BSA
- 5 mM $m^7G(5')ppp(5')$[b]
- 40 U/μl RNasin (Promega)
- 15 U/μl T3, T7, or SP6 RNA polymerase (Boehringer Mannheim)
- 3 M sodium acetate (pH 5.2 with acetic acid)
- Ethanol (absolute)

Method

1. Mix the following components at room temperature:
   - Sterile water 14 μl
   - 5 × transcription buffer 10 μl
   - 100 mM DTT 5 μl
   - 5 mg/ml BSA 1 μl
   - Ribonucleotide mixture 10 μl
   - 5 mM $m^7G(5')ppp(5')G$[b] 5 μl
   - Template DNA (1–2 μg) 2 μl
   - 40 U/μl RNase inhibitor (RNasin) 1.5 μl
   - 15 U/μl T7, T3, or SP6 RNA polymerase 1.5 μl
2. Incubate for 60 min at 37°C.
3. Extract with 50 μl phenol:chloroform:isoamyl alcohol and recover the aqueous phase.
4. Centrifuge the aqueous sample through a Sephadex G50 spin column at 4°C to remove unincorporated nucleotides.
5. Precipitate the nucleic acids by adding 0.1 vol. 3 M sodium acetate pH 5.2 and 2.5 vol. ethanol. Leave at –70°C for 20 min or at –20°C overnight. Centrifuge at 10000 *g* for 15 min and discard the supernatant.
6. Dissolve the nucleic acid pellet in 50 μl (or other appropriate volume) of sterile water and store at –70°C.
7. If necessary,[c] template DNA can be removed by digestion with RNase-free DNase, followed by an acid phenol extraction step, and ethanol precipitation. To do this, add 125 U DNase I and incubate at 37°C for 30 min.

[a] Avoid the use of restriction enzymes producing 3′ protruding ends, because these may inhibit termination at the correct site and result in the transcription of sequences on the complementary strand of the template. If no alternative is available, treatment of the template DNA with Klenow DNA polymerase in the absence of deoxynucleotides prior to transcription will lead to removal of the protruding ends.

[b] In view of the importance of a methylated cap structure for the binding of translation initiation factors and mRNA stability the use of m^7GpppG is highly recommended. Uncapped mRNAs or mRNAs carrying a non-methylated cap (GpppG) will also be translated in *in vitro* translation systems, although the efficiency may be lower, and optimal conditions may be different.

[c] Removal of the DNA template is usually desirable to prevent possible interference with subsequent translation. However it is possible to carry out protein synthesis without this step, as in coupled transcription–translation systems.

3. The reticulocyte lysate cell-free translation system

Although reticulocyte lysates may conveniently be obtained from a number of commercial sources (see *Table 1*), and these provide preparations already optimized for translation of mRNA, this is an extremely expensive way of obtaining such lysates. Provided the appropriate facilities and expertise are available, it makes considerable economic sense to prepare lysates in-house.

3.1 Preparation and storage of reticulocyte lysate

Reticulocyte lysates are obtained by lysing red blood cells from rabbits that are recovering from experimentally-induced anaemia (14). Different regimes have been used to induce anaemia. The recommended procedure is described in *Protocol 3* and consists of four daily injections of fresh acetylphenylhydrazine subcutaneously into 2–2.5 kg male rabbits. This is then followed by five days of recovery before the blood is taken. This schedule allows the blood

Table 1. Commercial sources for cell-free translation systems

Translation system	Supplier
Reticulocyte lysate (untreated)	
Untreated reticulocyte lysate kit	Ambion
Rabbit reticulocyte lysate, untreated	Promega
Reticulocyte lysate (micrococcal nuclease-treated)	
Retic lysate IVT kit	Ambion
Rabbit reticulocyte lysate system	Amersham
Translation kit, reticulocyte	Boehringer Mannheim
Red nova lysate	Novagen
Rabbit reticulocyte lysate, nuclease-treated	Promega
Flexi rabbit reticulocyte lysate system	Promega
In vitro translation system	Sigma
In vitro express translation kit	Stratagene
Wheat germ extract (micrococcal nuclease-treated)	
Wheat germ IVT kit	Ambion
Translation kit, wheat germ	Boehringer Mannheim
Wheat germ extract	Promega
Non-radioactive translation systems (rabbit reticulocyte lysate)	
ECL *in vitro* translation system	Amersham
Biotin *in vitro* translation kit	Boehringer Mannheim
tRNA^{nscend} non-radioactive translation setection system	Promega
Single tube coupled transcription–translation (rabbit reticulocyte lysate)	
PROTEINscript	Ambion
Linked SP6/T7 transcription–translation system	Amersham
Single tube protein system 2	Novagen
TNT coupled reticulocyte lysate system	Promega
TNT T7 quick coupled transcription/translation system	Promega
Single tube coupled transcription–translation (wheat germ extract)	
TNT coupled wheat germ extract system	Promega

haematocrit to rise, thus giving more red blood cells and hence more lysate per rabbit, and it also gives time for the acetylphenylhydrazine and its breakdown products to disappear from the circulation. It is convenient to treat several rabbits at once to save time. The animals should remain healthy (although anaemic) throughout.

Protocol 3. Preparation of reticulocyte lysates

Equipment and reagents

- Rabbits (male, 2–2.5 kg)
- 5 mg/ml acetylphenylhydrazine solution in water (prepared fresh)
- 1% (w/v) heparin in 0.9% (w/v) NaCl
- Washing buffer: 0.14 M NaCl, 5 mM KCl, 5 mM magnesium acetate, 5 mM glucose, 5 mM Hepes–KOH pH 7.2
- Double distilled water
- Anaesthetic
- Large needle attached to flexible plastic tubing
- Corex tubes (Sorvall)
- Liquid nitrogen storage facilities

Method

NB: ensure that personnel and procedures are fully licensed for work with experimental animals by the relevant regulatory authorities, and that all necessary animal welfare procedures are scrupulously followed.

1. Inject each rabbit subcutaneously with 5 ml acetylphenylhydrazine solution daily for four days.
2. Allow the rabbits to recover from the induced anaemia for five days.
3. On the ninth day, completely anaesthetize each animal with an appropriate dose of a proprietary anaesthetic by injection into an ear vein together with 1 ml of the heparin solution. The vein is readily visible at the ear margin when a little fur is shaved off. The injection requires some experience and is easier if an assistant holds the rabbit. Anaesthesia should occur rapidly but the heart should still beat firmly.
4. With the animal on its back, collect the blood by cardiac puncture using a large needle attached to flexible tubing leading to a flask on ice.[a]
5. Centrifuge the blood in a swing-out rotor at 1200 *g* for 10 min at 4°C.
6. Aspirate and discard the plasma, wash the cells by resuspension in 200 ml ice-cold washing buffer, followed by centrifugation as in step 5.
7. Repeat step 6 twice more, finally centrifuging the cell suspension at 1600 *g* for 15 min at 4°C.
8. Aspirate the supernatant and measure approximately the volume of the packed cells.
9. Lyse the red cells by adding an equal volume of cold double distilled water and mixing thoroughly.

Protocol 3. *Continued*

10. Centrifuge the lysed cells at 21 000 *g* for 20 min at 4°C in glass Corex tubes. This will produce a very large pellet of cell debris.
11. Carefully remove the dark red supernatant (the lysate), avoiding the material in the pellet, and immediately dispense the lysate into 1 ml aliquots in pre-cooled microcentrifuge tubes.
12. Freeze the aliquots rapidly and store them in liquid nitrogen.

[a] The heart should pump the blood out quite rapidly. If this does not work, or the heart stops, the blood can still be collected by opening the thorax, cutting the aorta, and then removing the blood with a syringe. It will not clot because of the heparin present in the injection, and should be collected and kept on ice at this stage.

There is often considerable variation in the relative activities of reticulocyte lysates prepared from different rabbits, and so it is advisable to keep the blood from each rabbit separate in case the least active preparations contain inhibitors which would impair the activity of the better lysates. The yield of blood should be up to 100 ml per rabbit. The reticulocyte count can be determined by mixing a few drops of blood with 1% Brilliant Cresyl Blue in saline and examining a smear under a microscope using an oil immersion lens. It is a common experience, however, that the reticulocyte count does not always correlate with the translational activity of the lysate obtained.

3.1.1 Preparation of lysates containing endogenous mRNA

The blood cells are harvested by centrifugation, washed, and then lysed with cold water (*Protocol 3*). After centrifugation to remove the stroma, the lysate can either be frozen immediately in aliquots and stored in liquid nitrogen (*Protocol 3*, steps 11–12) or first treated with micrococcal nuelease (Section 3.1.2) before storage. Reticulocyte lysates retain their activity during storage in liquid nitrogen for very long periods (at least two years). At –70°C, activity is retained for several months, but storage at –20°C is not recommended. Lysates should be sealed in plastic bags before transportation in dry ice, to prevent loss of activity due to exposure to high concentrations of carbon dioxide. Once thawed for use, it is best not to refreeze lysates, although this may be done once without too much loss of activity.

Each lysate that is prepared should be characterized by monitoring its endogenous protein synthetic activity in the presence of different concentrations of haemin. The latter prevents activation of a translational inhibitor, the protein kinase HCR (HRI) (15). Such an experiment will indicate not only how much amino acid incorporation occurs during translation of the lysate's own mRNA under standard conditions, but also the extent to which the lysate is stimulated by haemin, and the optimal haemin concentration which produces this effect. In general, the most active lysates also show the greatest response to haemin. Haemin stock solutions are difficult to make and store

unless the correct procedure is followed. The solutions are stable only if prepared in 90% ethylene glycol buffered with Tris–HCl pH 7.8. *Protocol 4* describes the correct procedure for preparation of a 1 mM haemin stock solution that is stable for prolonged times on storage at –20°C. In order to determine the haemin optimum, a lysate is incubated with various amounts of this stock solution to give haemin concentrations up to 40 μM, under conditions suitable for protein synthesis to take place (Section 3.2.1). After 60 min at 30°C, the amount of radioactive amino acid incorporated into protein is measured. In most cases, good lysates show an approximately tenfold stimulation of translation by haemin, with an optimum of 10–20 μM.

Protocol 4. Preparation of haemin stock solution

Reagents

- Haemin (solid)
- 1 M KOH
- 0.2 M Tris–HCl pH 7.8
- Ethylene glycol
- 1 M HCl

Method

1. Dissolve 6.5 mg haemin by adding it slowly to 0.25 ml of 1 M KOH solution.
2. Slowly add 0.5 ml of 0.2 M Tris–HCl pH 7.8 to the haemin solution, followed by 8.9 ml ethylene glycol, and 0.25 ml of 1 M HCl.
3. Store the haemin stock solution (1 mM) at –20°C; it does not freeze at this temperature.

3.1.2 Preparation of micrococcal nuclease-treated lysates

It is possible to eliminate endogenous mRNA by treatment of reticulocyte lysates with micrococcal nuclease (14). The procedure is elegantly simple since the enzyme (from *Staphylococcus aureus*) is entirely calcium-dependent for its activity and may therefore be subsequently inactivated by addition of the Ca^{2+} chelating agent, EGTA. Conditions are established under which the nuclease destroys mRNAs without significantly damaging the biological activity of the tRNA or ribosomes in the lysate. Thus a cell-free system can be obtained in which the background level of amino acid incorporation is virtually eliminated but which will translate added mRNAs with high activity.

Protocol 5 summarizes the procedure for obtaining nuclease-treated, mRNA-dependent reticulocyte lysate. In essence, the method involves just two steps. In the first, the lysate is incubated with micrococcal nuclease in the presence of 1 mM $CaCl_2$ for 15–20 min at 20°C. Then EGTA is added to a concentration of 2 mM and the mixture is placed on ice. The lysate can either be used immediately for a translation assay or stored in aliquots in liquid

nitrogen. Haemin (40 μM) should be included in the incubation with nuclease to prevent activation of HCR. This gives a final haemin concentration of 20 μM when the nuclease-treated reticulocyte lysate is used for *in vitro* translation since the lysate is diluted so as to comprise 50% of the final volume of the incubation mixture. If the haemin optimum of the untreated lysate is less than 20 μM, a correspondingly lower haemin concentration should be used during micrococcal nuclease treatment. Creatine phosphokinase can also be added during nuclease treatment although it is not essential at this stage.

Protocol 5. Preparation of micrococcal nuclease-treated (mRNA-dependent) reticulocyte lysates

Reagents

- 0.2 M $CaCl_2$
- 1 mM haemin stock solution (from *Protocol 4*)
- Micrococcal nuclease (5000 U/ml)
- 0.1 M EGTA pH 7
- Reticulocyte lysate (from *Protocol 3*)

Method

1. Thaw an aliquot of reticulocyte lysate in the presence of sufficient haemin solution to give a final concentration of 40 μM.[a]
2. Add stock 0.2 M $CaCl_2$ to 1 mM final concentration and micrococcal nuclease to 25–100 U/ml.[b]
3. Incubate for 15–20 min at 20°C.
4. Add 0.1 M EGTA pH 7 to 2 mM final concentration and return the lysate to ice.
5. Use the mRNA-dependent lysate immediately or store as aliquots in liquid nitrogen.

[a] Twice the final concentration of haemin that is usually optimal for protein synthesis.
[b] The optimal concentration of nuclease may vary between lysates and should be determined experimentally (see text).

The optimal concentration of nuclease to be used may vary between lysates and should be determined for each new preparation. Too low a concentration will not destroy all endogenous mRNA and will leave a high background of protein synthesis. Too much enzyme may damage ribosomes and tRNAs and will result in less stimulation of amino acid incorporation by added mRNAs.

3.2 Assays of protein synthesis in reticulocyte lysates

3.2.1 Amino acid incorporation using endogenous mRNAs

Reticulocyte lysate contains substantial amounts of endogenous mRNAs (mainly those for α- and β-globins) which can be translated under suitable

conditions. In this mode, the reticulocyte lysate is now mainly used to study the mechanism and regulation of protein synthesis. For the translation of exogenous mRNAs the lysate is usually first treated with micrococcal nuclease (Section 3.1.2) to reduce the translation due to the endogenous globin mRNAs. The incubation conditions described below are optimal both for endogenous mRNA translation and, with minor modifications (Section 3.2.2), for the translation of exogenous mRNAs by nuclease-treated lysates.

When the reticulocyte lysate is used in a cell-free protein synthesis assay, it is important that the lysate itself should remain as concentrated as possible to ensure maximum translational activity. Once the optimal haemin concentration is known, the frozen lysate is best thawed in the presence of twice this haemin concentration and the lysate should comprise 50% of the final volume of the assay mixture. A convenient way of setting up the incubations is described in *Protocol 6*. The final concentration of each component added to the lysate is given in *Table 2* but, in addition, the lysate itself contributes K^+ ions (up to 20 mM) and Mg^{2+} ions (1 mM). Some investigators omit ATP and GTP from the salts–amino acid–energy mixture since these are already present in the lysate. If this is done, the amount of magnesium acetate added is reduced so that its final concentration is 0.5 mM.

Table 2. Concentrations of components in the reticulocyte lysate translation assay

10 mM Tris–HCl pH 7.6
75 mM KCl[a]
2 mM magnesium acetate
3 mM glucose
50–200 μM amino acids
4 μCi/ml [^{14}C]leucine or up to 500 μCi/ml [^{35}S]methionine
1 mM ATP
0.2 mM GTP
7 mM creatine phosphate
1 mg/ml creatine phosphokinase
20 μM haemin[b]
Up to 100 μg/ml exogenous mRNA (optional)

[a] Higher concentrations of K^+ ions can be tolerated if potassium acetate is used instead of KCl.
[b] The haemin optima of some lysates may be lower than 20 μM and should be determined experimentally.

It is important to ensure thorough mixing in the tubes (without frothing) since the lysate is very dense. All components should be kept on ice before commencing the incubations, which are best performed at 30°C for prolonged protein synthesis. Under optimal conditions, amino acid incorporation should be linear for at least 60 minutes. If it ceases before this, it is worth checking the fraction of the labelled amino acid which has been incorporated because very active lysates translating their own mRNAs can rapidly exhaust the

amino acid pool unless sufficient amino acids are added. Thus the use of high specific radioactivity precursors at low concentrations is not always an advantage in this system, although this is more of a problem with radiolabelled leucine than with [^{35}S]methionine.

Protocol 6. Setting up a reticulocyte lysate protein synthesis assay

Reagents

All reagents must be free of nuclease contamination (see Section 2.1).

- Reticulocyte lysate (see *Protocols 3* and *5*)
- 10 × creatine phosphate–creatine phosphokinase mixture:[a] 70 mM creatine phosphate, 10 mg/ml creatine phosphokinase
- Pure nuclease-free water
- mRNA preparation
- 1 mM haemin stock solution (see *Protocol 4*)
- 5 × salts–amino acids–energy mixture:[a] 50 mM Tris–HCl pH 7.6, 0.375 M KCl,[b] 10 mM magnesium acetate, 15 mM glucose, 0.25–1 mM of each of the 20 common amino acids,[c] 20 μCi/ml [^{14}C]leucine (20–30 mCi/mmol) or up to 2.5 mCi/ml [^{35}S]methionine (*c.* 1200 Ci/mmol), 5 mM ATP, 1 mM GTP

Method

1. Thaw an aliquot of reticulocyte lysate after adding sufficient 1 mM haemin stock solution to give twice the final optimal concentration (usually 40 μM). Replace on ice.
2. Set up the assays in microcentrifuge tubes. To each tube at 4°C, add 50 μl reticulocyte lysate with 20 μl of 5 × salts–amino acids–energy mixture, 10 μl creatine phosphate–creatine phosphokinase mixture, and 20 μl water or mRNA solution (depending on whether the translation of endogenous or exogenous mRNA is to be measured). Exogenous mRNAs should be added to give final concentrations of 5–100 μg/ml.
3. Mix the contents of each tube thoroughly but without frothing.
4. Incubate the mixtures at 30°C for various times up to 2 h.

[a] The 5 × salts–amino acid–energy mix can be stored at –20°C in aliquots. Radioactive amino acids should be added just prior to use.

[b] Potassium acetate can be substituted for KCl, allowing a higher final K^+ ion concentration to be used if desirable (as in the case of certain mRNAs with a high K^+ ion optimum), since high concentrations of Cl^- ions inhibit the initiation of protein synthesis.

[c] The unlabelled amino acid corresponding to the radioactive one to be used can be omitted from the amino acid mixture, but take care that amino acid concentrations do not become rate limiting for protein synthesis when very active reticulocyte lysates are used.

3.2.2 Amino acid incorporation using exogenous mRNAs

When exogenous mRNAs are to be translated, these are added to the reticulocyte lysate (usually nuclease-treated; see Section 3.1.2) in place of an equivalent volume of water to give a final concentration usually in the range of 5–100 μg/ml (*Protocol 6*). Nuclease-treated reticulocyte lysates are used with

exogenous mRNAs under exactly the same conditions as for untreated lysate (Section 3.2.1). Sometimes it may be advantageous to add extra tRNA (calf liver) to a concentration of 50 μg/ml, not because of destruction of endogenous tRNA, but rather because the composition of the reticulocyte tRNA population is related to the amino acid content of rabbit globins and may not be optimal for translation of other mRNAs. The optimal salt conditions for other mRNAs may also vary from those given in *Table 2* (16) and therefore should be determined in preliminary experiments. It may also be necessary to reoptimize the Mg^{2+} ion requirement of the system, to compensate for the weak chelating ability of excess EGTA towards this ion.

3.2.3 Efficiency of the translation assay

The extent of amino acid incorporation at the end of an incubation is easily assessed by pipetting small aliquots (2–10 μl) on to numbered Whatman No. 1 filter paper discs followed by precipitation with TCA (*Protocol 7*). Charged tRNA is hydrolysed by heating the discs in hot TCA and the acid soluble radioactivity is then removed by washing the filters in 5% TCA. The extent of amino acid incorporation is determined by measuring the radioactivity of the precipitated polypeptide products using a liquid scintillation counter. As an alternative to this disc method, some investigators transfer a sample of each incubation mixture into 0.5 ml of water to which is added NaOH and hydrogen peroxide (*Protocol 7*). In this case, the NaOH hydrolyses the charged tRNA and bleaching with hydrogen peroxide occurs simultaneously. The sample proteins are then recovered by precipitation with TCA followed by filtration on to glass fibre filters, with determination of radioactivity as before.

Protocol 7. Measurment of amino acid incorporation into proteins in the reticulocyte lysate protein synthesis assay[a]

Equipment and reagents

- Reticulocyte lysate reactions (from *Protocol 6*)
- Whatman No. 1 filter paper discs (25 mm diameter)
- Whatman GF/C glass fibre filter discs (25 mm diameter)
- 50% (w/v) and 5% (w/v) TCA
- Acetone
- Absolute ethanol or industrial methylated spirit (IMS)
- Non-aqueous scintillation fluid
- 1 M NaOH
- 30% (w/v) hydrogen peroxide

A. *Whatman filter method*

1. Pipette 2–10 μl aliquots of the reticulocyte lysate incubation mix (*Protocol 6*) onto separate Whatman No. 1 filter discs, numbered in pencil and laid out on a sheet of aluminium foil.

Protocol 7. *Continued*

2. Place the discs in a beaker containing a large volume of 5% TCA and leave for 15 min, with occasional swirling of the beaker.
3. Replace the TCA with a fresh solution and incubate the discs at 90°C for 15 min to hydrolyse aminoacyl-tRNAs.
4. Wash the discs successively with similar volumes of fresh cold 5% TCA, alcohol, and acetone, with much swirling to ensure good mixing.
5. Allow the discs to dry at room temperature (or in an oven at 50°C) on a fresh sheet of foil and then place them individually in non-aqueous scintillation fluid.
6. Measure the acid insoluble radioactivity in a liquid scintillation counter.

B. *NaOH–hydrogen peroxide method*

1. Pipette 2–10 μl aliquots of the reticulocyte lysate incubation mix (*Protocol 6*) into 0.5 ml water and add 0.5 ml of 1 M NaOH (to hydrolyse the aminoacyl-tRNA) and 50 μl hydrogen peroxide solution (to bleach the samples).
2. When bleaching is complete, add 0.25 ml of 50% TCA and allow the protein to precipitate on ice for 30 min.
3. Filter each precipitate onto a Whatman GF/C glass fibre filter using a filtration manifold attached to a vacuum line.
4. Wash each filter with three 10 ml washes of 5% TCA and one 10 ml wash of alcohol.
5. Proceed as in part A, steps 5 and 6.

[a] Two alternative procedures are given. Both are equally effective.

As a guide to the endogenous protein synthetic activity of a typical lysate, TCA precipitation of 2 μl from an assay containing 100 μCi/ml of [^{35}S]methionine should give about 150 000 c.p.m. after a 60 minute incubation. When calculating the amount of protein synthesized, remember the contribution of the endogenous amino acids (about 5 μM in the case of methionine) to the final specific radioactivity of the labelled precursor. Nuclease-treated reticulocyte lysate is exquisitely sensitive to low concentrations of mRNAs and will translate as little as 0.3 μg/ml to give a detectable product using the assay described here. At the other extreme, up to 200 μg/ml of some mRNAs can be translated well.

A plot of amino acid incorporation against mRNA concentration should be constructed with each new preparation of RNA, including a negative control with no added mRNA and a positive control with a known amount of a stan-

dard mRNA. Incubations should be allowed to continue for up to 2 h at 30°C, to ensure complete synthesis of large products. Different RNA preparations vary considerably in their ability to stimulate amino acid incorporation. Several viral RNAs and globin mRNA are good templates for the reticulocyte lysate system, giving several hundred-fold stimulations over background when added at 5–20 μg/ml. Many cellular mRNAs are less efficient. Both total cytoplasmic RNA and selected poly(A)$^+$ RNA preparations stimulate translation. However, much higher amounts of total cytoplasmic RNA (100–200 μg/ml) are required since most of this is rRNA. Provided the RNA solutions do not contain too high a concentration of salts (especially Mg^{2+} ions), quite large volumes can be added to assays. Alternatively, very dilute solutions (in water) can be lyophilized in the incubation tubes and the RNA dissolved directly in the assay mixture. Some RNA preparations may contain inhibitors of translation such as sulfated polysaccharides or double-stranded RNA (dsRNA). The presence of inhibitors may be checked by adding the preparations to assays containing a known amount of a standard translatable mRNA. Double-stranded RNA can be a particular problem when RNAs transcribed *in vitro* by bacteriophage RNA polymerases are used as templates for translation (17, 18). This is probably because of low level non-specific transcription from both strands of the DNA, producing complementary sequences that can hybridize with each other. The reticulocyte lysate is exquisitely sensitive to inhibition by concentrations of dsRNA in the ng/ml range due to activation of the inhibitory protein kinase PKR (19). This problem can be at least partially overcome by including the PKR inhibitor 2-aminopurine at 10–20 mM in the protein synthesis assay (20, 21). Alternatively, addition of high concentrations of a synthetic dsRNA such as poly(I):poly(C) paradoxically can be used to block the activation of PKR by low concentrations of other dsRNA species (21).

3.3 Advantages and disadvantages of the reticulocyte lysate system

The reticulocyte lysate system, either in its native form or after micrococcal nuclease treatment, is still the most widely used eukaryotic cell-free protein synthesizing system. There are many reasons for this, notably the high translational activity with endogenous or exogenous mRNAs, the relative ease of preparation, and the stability of the system on storage. Multiple rounds of protein synthesis occur on mRNAs, each active mRNA being translated 40–70 times in a 90 minute incubation, with a polypeptide chain elongation rate of around one amino acid per second per ribosome.

To be set against these valuable characteristics, it should be noted that reticulocyte lysates are initially somewhat unpredictable in their activity and the success rate in preparing good ones is variable. In addition, they are uniquely sensitive to certain inhibitors, notably low concentrations of dsRNA

and oxidized thiol compounds which may sometimes be present in added components. Thus, for example, some RNA preparations which are translated well in the wheat germ system (Section 4) are poorly utilized, and even inhibitory, in the reticulocyte lysate system (21). The reticulocyte lysate also exhibits unique regulatory features, such as the stringent requirement for haemin, which make it atypical in studies of translational control mechanisms. Another drawback is that initiation can occur at spurious sites on some mRNA species, producing a complex pattern of translation products (16). In some cases this may be overcome by adding high concentrations of RNA binding proteins which presumably block access to internal regions of the RNA, making initiation more strictly dependent on cap-mediated ribosomal binding and scanning from the 5′ end (22).

Micrococcal nuclease-treated reticulocyte lysate is the most common cell-free system for the translation of exogenous eukaryotic cellular and viral mRNAs. Not surprisingly, therefore, many such preparations are now commercially available (see *Table 1*). Not only can these be used to examine specific translation products, but they are also valuable in studies on mRNA competition and structure–function relationships, as well as in analysis of regulatory aspects of the protein synthetic process itself. Messenger-dependent reticulocyte lysates can also be used in coupled transcription–translation systems (see Section 7.2). These systems also allow the requirements for post-transcriptional modifications to produce active RNA templates to be determined.

With all these advantages it is important to bear in mind one possible problem associated with nuclease-treated lysates. It is likely that significant amounts of mRNA fragments remain after enzymatic digestion, including sequences which contain initiation sites. Thus the formation of ribosomal initiation complexes may not be entirely dependent on exogenous mRNA, even though little translation of the fragments occurs. Added mRNAs have to compete with these fragments for ribosome binding, and weakly initiating species may not do so efficiently. It should also be stressed that the activity of the mRNA-dependent system will only be as good as the original lysate allows and a poorly initiating extract will remain poor even after nuclease digestion and supplementation with exogenous mRNAs.

4. The wheat germ cell-free translation system

Several factors may make the wheat germ cell-free translation system preferable to the reticulocyte lysate system for translation of a particular protein. For example, the presence of functional or antigenic cross-reacting components in the reticulocyte lysate or the presence of low concentrations of compounds (e.g. dsRNA, oxidized thiols) that are inhibitory to the reticulocyte lysate in the mRNA preparation can be problems.

4.1 Sources of wheat germ

Wheat germ extracts are commercially available (see *Table 1*). However, such extracts are extremely easy to prepare (23) and wheat germ is readily available from a number of sources, e.g. 'health food' shops (note that the wheat germ should be untoasted and with no preservatives added) or from companies such as Sigma or General Mills. It may be necessary to prepare extracts from several different sources and to compare their activities, since both the level of endogenous amino acid incorporation and the extent of stimulation by added mRNAs vary considerably between batches (24).

4.2 Preparation of wheat germ extracts

Wheat germ can be stored for long periods of time under vacuum at 4°C. The detailed preparation procedure for the extract is described in *Protocol 8*. The extracts can be stored in aliquots at –70°C or under liquid nitrogen for several years.

Protocol 8. Preparation of wheat germ extract

The complete procedure should be carried out at 4°C (using nuclease-free sterile materials).

Equipment and reagents

- Pestle and mortar (cold)
- Ground glass (e.g. a glass Pasteur pipette, crushed into small pieces)
- Extraction buffer: 20 mM Hepes–KOH pH 7.6, 100 mM potassium acetate, 1 mM magnesium acetate, 2 mM $CaCl_2$, 0.1 mM EDTA, 1 mM DTT (prepare this buffer without DTT, autoclave it, and then add DTT after cooling)
- Column buffer: 20 mM Hepes–KOH pH 7.6, 120 mM potassium acetate, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT (prepare this buffer without DTT, autoclave it, and then add DTT after cooling)
- A column (2 cm × 30 cm) packed with Sephadex G25 coarse and equilibrated with column buffer
- Centrifuge tubes

Method

1. Grind 5 g wheat germ for 1–2 min in a mortar in the presence of 1 g ground glass.
2. Add 7.5 ml extraction buffer and grind for an additional 30 sec.
3. Transfer the resulting paste into centrifuge tubes, rinse the mortar with 5 ml extraction buffer, and add this to the paste.
4. Centrifuge at 30 000 *g* for 10 min.
5. Transfer the central portion of the supernatant (avoiding the floating layer of lipid) to a new centrifuge tube and centrifuge again at 30 000 *g* for 10 min.
6. Load the supernatant (approx. 5 ml) on the Sephadex G25 column equilibrated with column buffer, and elute with column buffer.

Protocol 8. *Continued*

7. Collect fractions. The wheat germ extract will separate into a fawn-coloured band which elutes first from the column, and a yellow band of pigment which elutes much later. Measure the absorbance at 260 nm of the fractions containing the first band and pool those containing at least 100 absorbance units/ml.
8. Store the extract in aliquots at –70°C or under liquid nitrogen.

4.2.1 Micrococcal nuclease treatment

Although background incorporation in wheat germ extracts in general is low it can be reduced even further by micrococcal nuclease treatment. The nuclease treatment protocol described earlier for the reticulocyte lysate (*Protocol 5*) can be used without modification (except for the omission of haemin) to prepare a nuclease-treated wheat germ extract.

4.3 Assays of protein synthesis in wheat germ extracts

A procedure for use of wheat germ extracts for protein synthesis assays is described in *Protocol 9*. The optimal conditions are broadly similar to those for the reticulocyte lysate (Section 3.2). However, unlike the reticulocyte lysate, the endogenous translational activity of the wheat germ system is low. Therefore, translation in this system is largely dependent on added mRNAs and the precise requirements may vary with different mRNAs. The final concentration of added mRNA is usually in the range of 20–80 μg/ml. A concentration curve should be run for each mRNA preparation at an incubation temperature of 25°C. Amino acid incorporation should be linear for at least one hour. Addition of the human placental ribonuclease inhibitor (RNasin, Promega) may allow translation to continue up to 3 h. The incubation conditions for use of nuclease-treated wheat germ extracts are as described for untreated extracts.

Protocol 9. Protein synthesis in wheat germ extract

Reagents

- Wheat germ extract (from *Protocol 8*; see also Section 4.2.1)
- mRNA
- 10 × salts–energy mixture:[a] 200 mM Hepes–KOH pH 7.6, 0.5 M potassium acetate, 10 mM magnesium acetate, 2 mM spermidine, 10 mM ATP, 4 mM GTP, 25 mM DTT
- L-[^{35}S]methionine (15 mCi/ml, > 1000 Ci/mmol) (or another radiolabelled amino acid)
- 300 mM potassium acetate pH 7.6
- 15 mM magnesium acetate pH 7.6
- Amino acid mixture minus methionine (or minus another amino acid depending on which radiolabelled amino acid is being used): concentration of each amino acid (except methionine) 1.5 mM
- 10 × creatine phosphate–creatine kinase: 160 mM creatine phosphate, 7 mg/ml (or 240 U/ml) creatine phosphokinase

Method

1. Thaw an aliquot of wheat germ lysate sufficient for the number of assays to be carried out.
2. Set up the assays in microcentrifuge tubes. To each tube on ice, add:
   - 10 × salts–energy mixture 1.5 μl
   - 300 mM potassium acetate (final concentration 50–170 mM)[a] 0–6 μl
   - 15 mM magnesium acetate (final concentration 1–3.5 mM)[a] 0–2.5 μl
   - Amino acid mixture minus methionine 1 μl
   - L-[^{35}S]methionine (15 μCi/μl) 1 μl
   - 10 × creatine phosphate–creatine kinase 1.5 μl
   - mRNA[b] 1 μl
   - Wheat germ extract 6 μl
   - Sterile water to a final volume of 15 μl
3. Mix well and incubate at 25°C for 60 min.

[a] The exact requirements for K^+ and Mg^{2+} ions will depend on the mRNA being translated and must be determined experimentally.
[b] The optimal mRNA concentration may vary with different mRNAs and is usually in the range of 20–80 μg/ml. This optimal concentration should be determined for each mRNA preparation.

4.3.1 Amino acid incorporation

The extent of amino acid incorporation at the end of an incubation can be determined as described in Section 3.2 for translation in reticulocyte lysates (see *Protocol 7*).

4.4 Advantages and disadvantages of the wheat germ system

The most obvious benefits of the wheat germ system are its ease of preparation and low cost. Unlike the reticulocyte lysate system, no animal handling facilities are required and little time need be invested in preparing many different extracts for comparison of their activities. Furthermore, wheat germ extracts are sensitive to stimulation of overall protein synthesis by exogenous mRNAs without prior micrococcal nuclease treatment, unlike the untreated reticulocyte lysate. Under optimal conditions, an efficiently utilized mRNA will stimulate amino acid incorporation as much as 400-fold.

Potential disadvantages of this system are that the ionic optima for translation are quite sensitive to the nature and concentration of the mRNA, and should be determined for each template. There is also some evidence that, at low levels of stimulation, added RNA can enhance endogenous protein synthesis so that the products observed may not be entirely coded by the mRNA which is being characterized (25). However, perhaps the biggest criticism of the wheat germ system is its tendency to produce incomplete products, especially in the case of relatively long mRNAs. The incomplete products are either due to premature termination and the release of peptidyl tRNA or to

internal initiation of translation. This can be a problem with large mRNAs, coding for polypeptides in excess of 60 000 Da. Premature termination may be overcome by addition of the polyamines spermidine and spermine, which stimulate the rate of chain elongation (26) and lower the Mg^{2+} ion optimum. Aberrant internal initiation may (at least in part) be due to leaky ribosomal scanning, especially at relatively high mRNA concentrations, and might be relieved by optimization of the mRNA concentration.

5. Cell-free translation systems from other eukaryotic cell types

In addition to the widely used reticulocyte lysate and wheat germ systems, extracts from a variety of eukaryotic cell types can be prepared for the translation of exogenous mRNAs or to study aspects of the regulation of protein synthesis. However such extracts are generally far less active in translation than the reticulocyte or wheat germ systems. The extracts are prepared as 10 000–30 000 *g* supernatants. They may be pre-incubated or nuclease-treated to eliminate endogenous mRNA, and/or dialysed or treated with Sephadex G25 to standardize ionic conditions. A procedure appropriate for preparing extracts from tissue culture cells is given in *Protocol 10.* This method is based on that described by Carroll and Lucas-Lenard (27). It uses lysolecithin as a means of lysing cells and this has been reported to give much more active extracts than homogenization of cells. Alternative detergents for cell lysis are Triton X-100 or NP-40 (0.2–1%, v/v) and the best reagent for a given cell type will need to be determined experimentally. All manipulations must be carried out at 4°C.

The cells from which the supernatants are made are usually grown as large scale suspension cultures. Extracts with the ability to translate added cellular and viral mRNAs can be prepared from mouse L cells, Ehrlich ascites tumour cells, HeLa cells, and Chinese hamster ovary (CHO) cells, all of which can be grown in multilitre quantities. Procedures for translation assays using extracts from such cell types are very simlilar to those for the reticulocyte lysate and wheat germ systems (*Protocols 6* and *9*), but conditions may need to be optimized to obtain the best results.

Protocol 10. Preparation of translationally active extracts from cultured cells

Equipment and reagents

- Tissue culture cells in the exponential phase of growth
- Microcentrifuge and tubes
- 26 gauge syringe needle
- 0.15 M NaCl, 20 mM Hepes–KOH pH 7.2
- Lysis buffer: 20 mM Hepes–KOH pH 7.4, 100 mM potassium acetate, 2.2 mM magnesium acetate, 2 mM DTT, 0.1 mg/ml lysolecithin

- Incubation buffer: 25 mM Hepes–KOH pH 7.4, 125 mM potassium acetate, 2.8 mM magnesium acetate, 2.5 mM DTT, 1.25 mM ATP, 0.25 mM GTP, 6.25 U/ml creatine phosphokinase, 37.5 mM creatine phosphate, and 0.125 mM amino acids minus methionine

Method

Carry out all manipulations at 4°C.

1. Harvest cells that are in exponential growth by centrifugation at 1000 *g* for 5 min at 4°C.
2. Decant the supernatant and wash the cells by resuspension in 0.15 M NaCl, 20 mM Hepes–KOH pH 7.2 followed by centrifugation as in step 1.
3. Repeat step 2, using a much smaller volume of washing buffer, and pellet the cells by brief centrifugation in a microcentrifuge. Do not allow the cells to remain in the cold buffered saline solution for more than 10 min in total.
4. Suspend the cells in lysis buffer at 8×10^7 cells/ml. Leave on ice for a maximum of 1 min.
5. Immediately centrifuge the permeabilized cells in a microcentrifuge.
6. Resuspend the pellet in incubation buffer at 4×10^8 cells/ml.[a]
7. Lyse the cells by multiple passages through a 26 gauge needle or by repeated micropipetting. Leave on ice for 5 min.
8. Centrifuge for 20 sec in a microcentrifuge and collect the supernatant.

[a] Some procedures recommend the addition of 20% glycerol and/or 1 mM spermidine to cell extracts to stabilize proteins and improve the translational activity of the extracts.

6. Methods for analysis of translation products

Translation products can be analysed by a variety of methods, including gel electrophoresis followed by autoradiography or chemiluminescence, immunological methods, and ligand binding assays.

6.1 Radioisotopic methods

The most common way to characterize *in vitro* translation products is based upon the incorporation of at least one radioactive amino acid into the translated polypeptide(s). This allows the product(s) to be visualized by autoradiography or PhosphorImaging following SDS–PAGE. In principal, any radiolabelled amino acid can be added to the lysate/extract to produce radiolabelled polypeptides, but [^{35}S]methionine, [^{35}S]cysteine, [^{14}C]leucine, and [^{3}H]leucine are most frequently used. If the translated protein is relatively small or contains a relatively low number of one of these amino acids, one

should choose a radioactive amino acid that occurs more frequently in the protein's sequence to guarantee efficient labelling of the protein. Alternatively a sequence encoding, for example, a methionine tag consisting of a stretch of four or five methionines may be cloned in-frame with the open reading frame of the template, to facilitate efficient labelling. If, on the other hand, the translated protein contains a large number of residues of the amino acid used for labelling and the amount of label is used up too quickly, premature termination may result. The addition of small amounts of the corresponding unlabelled amino acid may solve this problem. A disadvantage of using [^{35}S]methionine in the reticulocyte system is that it may bind to a 42 kDa reticulocyte protein which then appears as an apparent translation product on SDS–PAGE analysis (28). The use of 'translation grade' [^{35}S]methionine may reduce this phenomenon, but it can be controlled for by conducting an incubation containing all the components of the assay mixture except the exogenous mRNA.

^{35}S-labelled amino acids are easily oxidized to translation inhibiting sulfoxides and should usually be stored in aliquots with buffer containing DTT or 2-mercaptoethanol at –70°C. A convenient alternative form of [^{35}S]methionine that does not require freezing is Redivue L-[^{35}S]methionine (Amersham). The use of radioactive amino acids requires that radiological health precautions be taken. Especially with ^{35}S-labelled amino acids, which are known spontaneously to produce volatile decomposition products, measures must be implemented to minimize contamination of facilities, equipment, and personnel (29).

6.2 Chemiluminescence

The use of lysine-tRNA^{Lys}, modified at the ε-NH_2 group of lysine with biotin, for the non-radioactive detection of *in vitro* translation products was described as early as 1988 (30), but has received more attention during the last few years. This is due to the development of sensitive chemiluminescence detection methods and the availability of commercial kits based upon these technologies, such as the 'Biotin *in vitro* Translation Kit' of Boehringer Mannheim and the 'tRNA^{nscend} Non-Radioactive Translation Detection System' of Promega. In these systems, biotinylated lysine residues are incorporated into nascent proteins during translation using an amino acid mixture containing all amino acids except lysine. Lysine is added to the translation reaction in the form of a pre-charged biotinylated lysine-tRNA complex. This complex can be prepared by coupling of lysine to tRNA^{Lys} (e.g. from brewer's yeast) using aminoacyl-tRNA synthetases (e.g. from rat liver) and subsequent modification of the lysine by biotinylating reagents such as D-biotinoyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester. After the *in vitro* translation reaction the products can be analysed by SDS–PAGE, Western blotting, and incubation with streptavidin conjugated to alkaline phosphatase followed by

chemiluminescent (or colorimetric) detection. The electrophoretic mobility of biotinylated *in vitro* translated proteins does not significantly differ from that of the same proteins translated in the presence of a radioactive amino acid, suggesting that the number of biotin labelled lysines incorporated per molecule is relatively low. This is most likely due to the presence of some endogenous lysine as well as $tRNA^{Lys}$ in cell extracts. In spite of this, the sensitivity of detection is comparable to that obtained by incorporation of [^{35}S]methionine. Approximately 1 ng of protein can be detected by these methods.

The major advantages of biotin *in vitro* translation over radioactive *in vitro* translation are:

(a) The detection method is much faster.

(b) It avoids the inconvenience, hazards, and stability problems associated with radioactivity.

(c) It allows immobilization of the translation products by binding to solid supports containing (strept)avidin (30).

6.3 Immunoprecipitation of translation products

If specific antibodies are available against *in vitro* translated proteins, they provide a powerful means of identifying and quantifying these products, especially when mixtures of many mRNA species are translated and yield a large number of different polypeptides. Although the most suitable method for immunoprecipitation of cell-free translation products will depend on the characteristics of the particular antibody and antigen in question, a generally applicable immunoprecipitation procedure is described in *Protocol 11*.

Protocol 11. Immunoprecipitation of translation products

Equipment and reagents

- Protein A beads or Protein G beads: Protein A or Protein G immobilized on agarose, Sepharose, or Trisacryl (Sigma)
- IPP500 buffer: 10 mM Tris–HCl pH 8, 500 mM NaCl, 0.1% Nonidet P-40, 0.1% Tween 20
- IPP150 buffer: 10 mM Tris–HCl pH 8, 150 mM NaCl, 0.1% Nonidet P-40, 0.1% Tween 20
- Antibody or antiserum
- End-over-end rotation apparatus (Stuart Scientific)
- SDS sample buffer: 62.5 mM Tris–HCl pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.002% bromophenol blue
- *In vitro* translation reaction mixture (see *Protocol 6* or *9*)

Method

1. Centrifuge the *in vitro* translation reaction mixture for 15 min at 12 000 *g*.
2. Wash the immobilized Protein A beads or Protein G beads twice with IPP500 buffer. To do this, resuspend the beads in a volume of buffer at least tenfold the volume of beads. Centrifuge briefly to collect the

Protocol 11. *Continued*

beads, then remove the supernatant by gentle aspiration. In general 5 μl beads (10 μl of a 50% suspension) is sufficient per immunoprecipitation.

3. Resuspend the beads in 0.5 ml IPP500 and add an appropriate volume of antiserum (or culture supernatant/ascites fluid in the case of a monoclonal antibody). Incubate for 1 h by end-over-end rotation at 4°C.
4. Wash the beads three times with IPP500 and once with IPP150 buffer.
5. Resuspend the beads in 0.5 ml IPP150 and add 1–10 μl of centrifuged translation reaction mixture (step 1). Incubate for 1 h by end-over-end rotation.
6. Wash the beads four times with IPP150. To prevent contamination by protein adsorbed to the walls of the tube during immunoprecipitation, the beads should be transferred to a fresh tube before the final wash step.
7. Remove the final supernatant as fully as possible (e.g. by using a drawn-out Pasteur pipette).
8. Resuspend the final pellet in 20 μl SDS sample buffer and heat at 90°C for 3 min.
9. Remove the beads by centrifugation and analyse the solubilized immunoprecipitate by SDS–PAGE (Chapter 6, *Protocol 7* or ref. 31).

The inclusion of at least one non-ionic detergent in the immunoprecipitation buffers (IPP500 and IPP150) used in *Protocol 11* helps to reduce non-specific contamination of the immunoprecipitated complexes but it should be noted that some antigen–antibody combinations may dissociate in the presence of detergents. If the antigen–antibody complex is sufficiently stable, raising the salt concentration in the immunoprecipitation buffer may also lead to a reduction of non-specific contamination. If a murine monoclonal antibody of the IgG1 isotype is used for immunoprecipitation it is advisable to use a 'bridge', e.g. a polyclonal rabbit antibody against murine immunoglobulins, for coupling the antibody to immobilized Protein A to stabilize the binding to the beads. When culture supernatants are used as a source of monoclonal antibodies, the use of such a 'bridge' may also be helpful, especially when the titre of monoclonal antibody is relatively low and the culture medium contains calf serum. Alternatively, a serum-free culture medium can be used to produce the monoclonal antibody.

If the translated proteins are being used for immunoprecipitation, ligand binding and/or biological activity assays one should realize that the translation system contains many proteins from the source of the lysate or extract. Such proteins might include the endogenous homologues of the proteins that are being translated and/or proteins that interact with the translated proteins. In

case these endogenous proteins interfere with the translated protein in the assays mentioned, an alternative procedure must be chosen to obtain a preparation that is applicable in the assay of interest. If a mammalian protein is to be translated the choice of wheat germ extract rather than reticulocyte lysate to express the protein may solve the problem. Alternatively, it may be possible to fuse a sequence encoding a tag to that of the protein of interest, to allow specific isolation of the expressed protein from the translation reaction mixture.

Immunoprecipitation of *in vitro* translated proteins has been used extensively to perform epitope mapping studies (32, 33). The major advantages of this epitope mapping method are the speed of obtaining (mutant) protein from (mutant) cDNA and the fact that the protein is expressed in a properly folded, soluble form.

6.4 Ligand binding assays

Proteins produced in cell-free translation systems have been used in a variety of ligand binding assays, such as metal ion binding assays, nucleic acid binding assays, and protein–protein interaction analyses. These analyses can be broadly divided into two types:

(a) Solution assays in which one of the interacting molecules can be precipitated specifically using either specific antibodies or tags introduced in one of the interacting components.

(b) Assays in which the protein component is immobilized, typically on a nitrocellulose membrane.

In view of the limited amount of protein that is produced in cell-free systems, these assays strongly rely on the specificity and affinity of the interaction to be studied. Frequently it is difficult to distinguish specific bands from non-specific or background reactivities. If a protein is immobilized on a nitrocellulose membrane after electroblotting from SDS gels one should realize that a method will only be successful when at least the domain of interest is renaturable on the membrane. Note that for these types of applications, PVDF (polyvinylidene difluoride) membranes might be superior to nitrocellulose due to the hydrophobic nature of the interaction between the protein and the membrane. The most frequently used ligand binding methods that are based upon the use of *in vitro* translated proteins are listed in *Table 3*.

6.5 *In vitro* synthesis of membrane and secretory proteins

Most eukaryotic membrane and secretory proteins are synthesized as precursors with a cleavable amino-terminal signal peptide responsible for targeting the protein to the endoplasmic reticulum. After translocation across the ER membrane, most signal peptides are cleaved by a signal peptidase on the lumenal side of the ER membrane. This implies that, for the proper production of membrane and secretory proteins by *in vitro* translation, the *in vitro*

Table 3. Ligand binding assays

Interacting molecules	Method	Reference
Protein–DNA	Immunoprecipitation	Section 6.3, ref. 64
	Epitope tagging; immunoprecipitation	65, 66
	Mobility shift assay	67
	South-Western blotting	68
Protein–RNA	Immunoprecipitation	Section 6.3, ref. 64
	Biotinylated RNA; streptavidin agarose	69
	Epitope tagging; immunoprecipitation	65, 66
	North-Western blotting	70
Protein–protein	Immunoprecipitation	Section 6.3, ref. 63
	Epitope tagging; immunoprecipitation	65, 66
	Far-Western blotting	71

translation system has to be modified in such a way that membrane translocation/insertion and processing can occur. This can be achieved by the addition of microsomal vesicles to the translation reaction (see Section 7.4.1). In the wheat germ cell-free system, the recognition of signal peptides of nascent proteins by signal recognition particles may cause translational arrest (34), which can be released by the addition of ER membranes (microsomal vesicles), i.e. under conditions where translocation can occur (35–37). The synthesis of membrane and secretory proteins in the presence of microsomal membranes can be monitored by SDS–PAGE and the association of (nascent) polypeptides with microsomal membranes can be determined by centrifugation through sucrose step gradients (35–37).

7. Specialized procedures

7.1 Synthesis of biotinylated proteins

As described in Section 6.2, the availability of pre-charged biotinylated lysine-tRNA complexes allows the *in vitro* synthesis of biotinylated proteins. Presently, the only commercially available systems are based upon rabbit reticulocyte lysates, but in principle the wheat germ system can also be modified in such a way that synthesis of biotinylated proteins in this system is possible. Although in this procedure the reticulocyte lysate system has been set up using an amino acid mixture without lysine, not all lysines in a translated protein will be biotinylated due to the presence of endogenous lysine as well as $tRNA^{Lys}$ in the lysate. The total number of biotinylated residues in a polypeptide will be determined by the length of the polypeptide and the number of lysines it contains, but it is likely that only every third or fourth lysine will be labelled with biotin (38). An important advantage of the incorporation of biotinylated lysines in a polypeptide, in addition to the possibility of employing

non-radioactive detection methods, is the ability to immobilize the translated protein by binding to a solid support, such as streptavidin–agarose.

In spite of the fact that not all lysines in the translated protein will be biotinylated, the biotin labelled products are chemically different from the native proteins. Especially when the translation products are intended to be used for biological activity studies, this might be an important problem. Although it has been demonstrated in some cases that the biotinylation does not interfere with post-translational processing of the translation product (such as proteolytic removal of a signal peptide or glycosylation) or with functional activities of the translation product (such as DNA binding or enzymatic activity) (38, 39), one should always bear in mind that in other cases such activities may be abolished by biotinylation. Lysines are frequently involved in proper folding of proteins and are often located at positions on the surfaces of folded proteins that are directly involved in biological activities. If such problems arise, the addition of some non-labelled lysine to the translation reaction may increase the percentage of molecules which are properly folded and/or biologically active.

7.2 Coupled *in vitro* transcription–translation systems

The increased need for the expression of cloned cDNAs and PCR products by *in vitro* translation has encouraged researchers to develop coupled *in vitro* transcription–translation systems (40). These are faster, less laborious, and influenced to a lesser extent by RNA degradation than procedures in which the RNA has first to be extracted and purified before being translated. Originally, due to differences in optimal conditions for *in vitro* transcription and *in vitro* translation, these systems were based upon two sequential reactions: after the transcription reaction was terminated an aliquot was transferred directly to the translation reaction. Recently however, coupled systems have been developed in which all of the components are mixed in a single tube and in which transcription and translation proceed in a single reaction. Coupled *in vitro* transcription–translation systems have been described for all of the three most commonly used RNA polymerases (T7, T3, and SP6), and the majority of these systems are based on the rabbit reticulocyte lysate. In view of the fact that lysates/extracts from different sources may have different properties, it is difficult to provide a general protocol for coupled *in vitro* transcription–translation. Therefore, we recommend either following the guide-lines for optimization of the system described by Craig *et al.* (40) or using a coupled system from a commercial source (see *Table 1*).

7.3 Cap-dependent versus internal initiation of translation

Investigation of mRNA structure and the mechanism by which a given mRNA is translated (41) may require that assays be established for the 5′ cap-dependence of the initiation of protein synthesis. There is growing evidence

that some cellular mRNAs, like those of several picornaviruses and other classes of virus, can be translated by a process of internal ribosome binding rather than scanning from the 5′ end of the RNA (42). Moreover, the high efficiency with which some picornavirus internal ribosome entry sites are used for translation has allowed the development of vectors that allow high level expression of proteins whose open reading frames are cloned just downstream of these sites. One such class of vectors are the pCITE-4 plasmids (Novagen), incorporating part of the encephalomyocarditis virus 5′ untranslated region that directs efficient ribosome binding. Such vectors also possess other useful features such as poly(A) sequences, that confer mRNA stability, and fusion sequences for the easy purification of translation products.

7.3.1 Use of picornavirus-encoded proteases

Some picornaviruses produce specific proteases (43) that cleave the eukaryotic protein synthesis initiation factor eIF4G, rendering it unable to interact with the mRNA cap binding protein eIF4E. Because of an absolute requirement for eIF4E, the initiation of protein synthesis on capped mRNAs is therefore strongly inhibited in cells infected by these viruses. In contrast, initiation on mRNAs that possess internal ribosome entry sites is not inhibited (and may even be enhanced) by cleavage of eIF4G, since internal initiation does not require cap recognition. The discrimination between cap-dependent and internal initiation that occurs *in vivo* can be mimicked in cell-free translation systems if picornavirus enzymes such as the L protease of foot-and-mouth disease virus are added. Thus the L protease (which is available in recombinant form) is a useful reagent to distinguish between the different possible pathways of initiation on specific mRNAs. It should be noted however that the addition of L protease *in vitro* may also stimulate the translation of uncapped mRNAs which do not depend on internal initiation (44, 45) so it is important that the effect of *in vitro* capping on the translatability of transcripts in the presence and absence of L protease should be tested where possible.

7.4 Assays for post-translational processing

Although most eukaryotic proteins are synthesized on cytoplasmic polysomes, many function in specialized compartments and must be specifically modified and translocated co- or post-translationally. These processes are guided by a variety of signals specified by the amino acid sequence. The most common processing events include signal peptide cleavage, membrane insertion, translocation, glycosylation, and phosphorylation. Accordingly, the use of cell-free protein synthesizing systems is not confined to the characterization of the initial products of translation. Under appropriate conditions, a number of post-translational modifications of primary products can occur, including proteolytic cleavages (46) and both *N*- and *O*-linked glycosylation (46–48). Since in an intact cell many of these events occur at or inside the endoplasmic

reticulum, supplementation of a translationally active lysate or extract with microsomal membranes allows the *in vitro* production of processed polypeptides (49, 50). A potential disadvantage of processing of an *in vitro* translated polypeptide by the addition of dog pancreas microsomes (the most frequently used type of microsomes) to a rabbit reticulocyte lysate or wheat germ extract can be the divergent sources of assay components, which may influence the validity of the processing event(s) observed for a particular polypeptide. As an alternative an intact cell translation/processing system from a single source can be used, for example unfertilized eggs of *Xenopus laevis* (51). The latter system (see Chapter 2) is capable of the translation and segregation into membranes of microgram per millilitre levels of proteins from added mRNAs.

7.4.1 Processing by microsomal membranes

Cell-free translation studies have provided important evidence for the existence of N-terminal signal sequences for the insertion of proteins into or through membranes of the endoplasmic reticulum (52, 53). Such a process is characteristic of the biosynthesis of many secreted and membrane proteins and occurs as the nascent polypeptide chain emerges from the ribosome in the early stages of its synthesis. Transmembrane insertion of nascent polypeptides is an important factor in the binding of polysomes to the membranes of the rough endoplasmic reticulum. Signal sequences are commonly 20–30 amino acids long, usually have highly hydrophobic regions within them and, in most cases, are cleaved off by a membrane located enzyme before the polypeptide chain is completed.

In order to study these processes *in vitro* it is necessary to prepare not only the mRNAs for the appropriate proteins and the systems in which to translate them, but also the membranes which recognize the signal sequences. Choice of suitable incubation conditions, and the development of assays for the signal sequences and for the passage of nascent chains into membrane bounded vesicles, must also be considered (54).

A method for preparing microsomal membranes from dog pancreas is described in *Protocol 12*. Similar microsomal membrane preparations are also commercially available (from Boehringer Mannheim or Promega) for studies on the processing of nascent secretory proteins *in vitro*. The preparations are nuclease-free and have high processing activity. It is possible to make similar preparations of microsomal membranes, with signal sequence recognition and signal peptidase activities, from rat liver or other mammalian sources. Since there is no specificity in the recognition of signal sequences by microsomal membranes, these may be equally satisfactory. However, the relative activities of such preparations and especially the extent of their contamination with ribonucleases may vary and should be thoroughly checked before adopting these membranes for routine use.

Protocol 12. Preparation of microsomal membranes

Equipment and reagents

- Buffer A: 50 mM triethanolamine–HCl pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 250 mM sucrose
- Buffer B: 50 mM triethanolamine–HCl pH 7.5, 50 mM KCl, 1 mM DTT, 250 mM sucrose
- Buffer C: 20 mM Hepes–KOH pH 7.3, 100 mM KCl, 3 mM $MgCl_2$, 2 mM DTT, 250 mM sucrose
- 0.2 M EDTA pH 7
- Dog pancreas (surgically removed from the animal and placed in cold buffer A)
- Potter–Elvehjem homogenizer (motor-driven)
- Dounce homogenizer (A pestle)
- Solutions of 1.3 M, 1.5 M, 1.75 M, and 2.2 M sucrose in buffer A
- 10–55% sucrose gradients in buffer B

Method

1. Remove connective tissue, fat, and large blood vessels from the pancreas and rinse it repetitively with cold buffer A.
2. Chop the pancreas and mince the pieces extensively with a razor blade or pass the pieces through a stainless steel tissue press (1 mm diameter mesh).
3. Add 3 vol. buffer A and homogenize the tissue in a motor-driven Potter–Elvehjem homogenizer (five to ten strokes at 400 r.p.m.).
4. Centrifuge at 13 000 *g* for 10 min at 4°C.
5. Remove floating fatty material by aspiration and layer the supernatant onto a discontinuous gradient of 1.5 M, 1.75 M, and 2.2 M sucrose (5 ml each) in buffer A.
6. Centrifuge at 140 000 *g* overnight at 4°C.
7. Recover the 1.75 M sucrose layer ('rough' microsomes). Dilute this with an equal volume of buffer A and layer over 2 ml of 1.3 M sucrose in buffer A.
8. Centrifuge at 100 000 *g* for 30 min at 4°C.
9. Decant the supernatant. The pellet (rough microsomes) may be stored at −70°C or may be stripped of endogenous polysomes by treatment with EDTA as in steps 10–14.
10. Resuspend the rough microsome pellet by manual homogenization in a Dounce homogenizer (two or three strokes) in buffer B to a concentration of 100 A_{260} U/ml. Add 0.2 M EDTA pH 7 to give 3 μmol of EDTA per 10 A_{260} units of rough microsomes.
11. Layer 0.5 ml aliquots onto 12.5 ml gradients of 10–55% sucrose in buffer B and centrifuge at 190 000 *g* for 2 h at 4°C.
12. Collect the turbid band (stripped microsomes) located at ~ 40–45% sucrose and dilute this with 2 vol. buffer B.

13. Centrifuge at 100 000 *g* for 2 h at 4°C.
14. Decant the supernatant and store the pellet at –70°C.
15. Before using stored rough microsomes (step 9) or stripped microsomes (step 14), resuspend the pellet by brief sonication in buffer C.

The conditions under which mRNAs are translated, and their products processed by added preparations of microsomal membranes, are essentially the same as those described earlier (Sections 3 and 4). The membranes themselves are added at concentrations of up to 5 A_{260} U/ml. A typical method is described in *Protocol 13*. It has frequently been noted that high concentrations of membranes (necessary to drive processing reactions to completion) can inhibit overall protein synthesis, although this is more of a problem in the wheat germ system than in the micrococcal nuclease-treated reticulocyte lysate. Choice of incubation times will depend on the kinetics of protein synthesis and the post-translational processing events. The latter can be synchronized, and their temporal relationship to initiation of polypeptide synthesis determined, by deliberately inhibiting further initiation shortly after the beginning of the incubation. This may be achieved with specific agents such as the cap analogue m^7GDP. Similarly, the fate of pre-existing nascent chains on polysomes bound to rough endoplasmic reticulum membranes (not stripped by EDTA treatment) can be studied *in vitro* under conditions where initiation is completely blocked and only elongation of polypeptides occurs.

Protocol 13. Use of microsomal membranes in translation systems

Reagents

- *In vitro* translation system: either rabbit reticulocyte lysate or wheat germ extract (see *Protocols 6* and *9*, respectively)
- Rough or stripped microsomal membranes (see *Protocol 12*)[a]
- 40 U/μl RNase inhibitor (RNasin) (Promega)
- 100 μg/ml cycloheximide
- 30 mM tetracaine in 20 mM Hepes–KOH pH 7.6, 70 mM KCl, 1 mM $MgCl_2$
- Trypsin/chymotrypsin solution: 500 μg/ml each in 20 mM Hepes–KOH pH 7.6, 70 mM KCl, 1 mM $MgCl_2$
- 10 mM PMSF in DMSO
- 20 TIU/ml aprotinin

Method

1. Mix all components of the translation system together (see *Protocols 6* and *9*) except the microsomal membranes and the mRNA.
2. Add RNase inhibitor (3 μl for reticulocyte lysate or 0.5 μl for wheat germ extract).
3. Add microsomal membranes at concentrations of up to 5 A_{260} U/ml and mix.

Protocol 13. *Continued*

4. Add the mRNA and incubate under standard conditions (see *Protocols 6* and *9*).
5. Either analyse the translation products directly by SDS–PAGE (Chapter 6, *Protocol 7*) or, if translocation events are to be assayed, proceed as follows.
6. Add cycloheximide to a concentration of 10 μg/ml and tetracaine to a concentration of 3 mM.[b]
7. Incubate for 5 min at 22°C and cool to 0°C by placing the tube in an ice water-bath.
8. Add 0.1 vol. trypsin/chymotrypsin solution (i.e. to a concentration of 50 μg/ml each) and incubate for 3 h at 0–2°C.
9. Terminate proteolysis by the addition of PMSF to 0.5 mM and aprotinin to 2 TIU/ml.
10. Analyse the reaction products by SDS–PAGE (Chapter 6, *Protocol 7*).

[a] While both rough and stripped microsomal membranes can be used with the wheat germ system, it is recommended to use only stripped microsomal membranes with reticulocyte lysate in view of the polypeptide elongation activity of the endogenous membrane-bound polysomes on rough microsomes in the latter translation system.
[b] Cycloheximide is added to prevent further protein synthesis and tetracaine is added to stabilize the microsomal membranes during the subsequent incubation.

The existence of additional N-terminal signal sequences was first revealed by the observation that primary translation products of mRNAs coding for secretory proteins, synthesized in the absence of microsomal membranes, are slightly larger than the authentic proteins. The presence of an extra 20–30 amino acids on a protein is sufficient for it to be resolved from the normal size product by SDS–PAGE, particularly in the case of small proteins. Electrophoretic mobility can therefore be used as a simple assay for the presence or absence of a signal sequence. However, it should be noted that additional modifications to a primary translation product, especially extensive glycosylations, can also change its behaviour on gels (sometimes in an unpredictable way). Cleavage of signal sequences is normally a co-translational process which occurs concomitantly with the translocation of the nascent polypeptides across the microsomal membranes. Since the proteins become sequestered within the lumen of the microsomal vesicles they are protected from attack by exogenous proteases. This has provided a method for assaying such translocation events. Addition of proteases at the end of a cell-free translation reaction can be used to destroy newly synthesized products that are not protected within membrane vesicles. Precipitation with TCA, combined with SDS–PAGE and autoradiography or fluorography, will then indicate which proteins are resistant to proteolysis under these conditions (see *Protocol 13*).

Both negative controls (no protease treatment) and positive controls (protease treatment of a detergent solubilized incubation) should be included in such an analysis.

The signal recognition particle that becomes associated with microsomal membranes and mediates the selective binding of nascent secretory proteins to the membranes during translation *in vitro* has the ability to arrest the chain elongation of polypeptides with signal sequences, particularly in the wheat germ system (55). This effect is alleviated in the presence of the membranes themselves. Thus the use of cell-free translation systems can play important roles in the characterization of the mechanism of protein translocation across membranes.

7.4.2 Proteolysis of primary products of translation

In addition to the removal of N-terminal signal sequences in the presence of microsomal membranes, primary translation products can undergo other proteolytic processing events in cell-free systems. This is particularly common in the case of the polyproteins of several animal and plant viruses, which are synthesized by complete translation of large mRNAs. Polypeptide chain cleavages can occur at various specific sites along the primary translation products. The proteases responsible are soluble enzymes, distinct from the membrane-bound signal peptidase activity, and can be present as endogenous components of the translation system used. Alternatively, they may themselves be products of viral mRNA translation which are formed during the course of the *in vitro* incubations.

Co-translational and post-translational processing of newly synthesized products in cell-free systems are best studied by kinetic analysis of the appearance and disappearance of specific polypeptides, using SDS–PAGE and autoradiography or fluorography of [^{35}S]methionine labelled material. Many experimental approaches are possible for pulse-labelling and pulse-chasing (using excess unlabelled methionine in the chase), employing various incubation times. Initiation of protein synthesis may be synchronized by the addition of initiation inhibitors such as cap analogues shortly after the mRNA is added.

It is also possible to induce specific proteolysis of translation products if elevated levels of particular proteases are present in translation systems. This is the case, for example, when extracts from virus-infected cells or clone-derived viral proteases are used to carry out processing of homologous virus proteins synthesized *in vitro* (56). Active recombinant versions of some viral proteases can be expressed in bacteria and purified (57), or synthesized *in vitro* (44).

A cautionary note is necessary concerning the interpretation of labelled polypeptide patterns after SDS–PAGE. Because of the tendency of some cell-free systems to terminate polypeptide synthesis prematurely (see Section 4.4), a complex set of products often arises from the translation of a single large

mRNA. The appearance of these incomplete proteins may obscure, or complicate the analysis of, the proteolytic processing of large polyproteins to give smaller species. This problem is least acute in the reticulocyte lysate system and this is therefore the system of choice for most studies of this type. On the other hand, the reticulocyte system can in some cases carry out aberrant initiation at non-physiological sites on long viral RNAs (16) and this too can complicate the interpretation of gel patterns.

7.4.3 Folding of newly-synthesized proteins and formation of disulfide bonds

Although the amino acid sequence of a polypeptide chain contains the information that determines the three-dimensional structure of the functional protein, the folding of many proteins *in vivo* requires the assistance of a pre-existing machinery of molecular chaperone proteins. However, proteins expressed in *in vitro* translation systems in general are soluble and functionally active, suggesting that they are properly folded during their synthesis. This might be related (at least in part) to the presence of molecular chaperones in the cell-free translation systems and indeed evidence has been obtained that such factors display biological activity in these translation systems (58).

In contrast to the incorporation of a radiolabelled amino acid in the nascent protein, the substitution of an amino acid with a biotinylated amino acid as an alternative method of labelling (see Section 6.2) does chemically change the polypeptide and thus might interfere with protein folding and transport into microsomes. Nevertheless, several examples have been reported in which the incorporation of biotinylated lysine residues did not negatively affect these processes (39). Apparently, the molecular chaperones present in the lysate/extract or microsomes are able to interact faithfully with biotinylated substrate proteins. However, one should realize that this might not be true for all proteins and that even when the biotinylated protein is properly folded this does not exclude the possibility that the biotin moieties might interfere with the biological activity of the translated protein.

Extracellular proteins often contain several disulfide bonds, in contrast to intracellular proteins which usually lack these. Protein disulfide isomerase is an abundant resident endoplasmic reticulum protein that is the major catalyst of native disulfide formation in nascent proteins. Microsomes have been demonstrated to support post-translational oxidative folding in the presence of oxidized glutathione (glutathione is the major redox buffer in the endoplasmic reticulum), leading to a relatively efficient formation of native oxidized product (59).

7.4.4 Oligomerization of newly synthesized proteins

Proteins that are able to form dimers, trimers, or other oligomers might also associate into oligomeric complexes when expressed in *in vitro* translation systems (60). Gel filtration chromatography of the translation products can

be performed to investigate the formation of oligomers. The production of functionally active proteins in *in vitro* translation systems can also be employed to study protein–protein interactions other than the formation of homo-oligomers. This can be achieved by the simultaneous expression of two (or more) genes in a single (coupled transcription–)translation reaction, which allows the complexes to be formed within the lysate or extract (61). Formation of such complexes can be easily studied by co-immunoprecipitation when specific antibodies against at least one of the interacting components are available. An alternative to this procedure that might be successful is the expression of the proteins to be studied in separate translation reactions followed by mixing the translation products to allow the complexes to be formed and analysed.

7.5 The protein truncation test

An interesting and popular application of cell-free translation is the protein truncation test (PTT) (62, 63). In this test *in vitro* transcription–translation is performed to identify translation terminating mutations in the gene of interest. In some cancer genes many of the inherited or acquired mutations are either nonsense or frameshift mutations, resulting in the expression of a truncated protein, and the PTT therefore provides a rapid and reliable test for routine screening of such genetic diseases. Their ease of use makes coupled transcription–translation systems (Section 7.2) ideal for performing PTTs.

A gene (segment) of interest is first amplified from either genomic DNA by PCR or from mRNA by RT-PCR. The sense PCR primer should contain signals for transcription (T7, T3, or SP6 RNA polymerase promoter) and translation (Kozak consensus sequence and start codon). The PCR product is used directly as template in the transcription–translation system and the resulting protein(s) are analysed by SDS–PAGE.

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6

Prokaryotic *in vivo* expression systems

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1. Introduction

Bacterial expression systems are commonly used for production of heterologous gene products of both eukaryotic and prokaryotic origin. This chapter will discuss strategies and provide protocols for the expression of heterologous proteins in *Escherichia coli*, which is the bacterial system that is most widely and routinely used for this purpose. While many specialized vectors are available for gene expression in other Gram-negative and Gram-positive bacteria, such systems are usually employed for the genetic analysis and manipulation of specific organisms, rather than for high level expression of proteins to be purified and used for research. The emphasis of this chapter is on protocols that are suitable for producing proteins in the laboratory for research purposes. There are additional considerations in the selection of systems for large scale production (i.e. hundreds of litres or more of culture) and for production of proteins for clinical investigation or commercial use.

In this chapter we briefly review some of the major factors to consider in selecting an *E. coli* expression strategy and provide protocols that have been successful in our laboratories in a variety of expression applications. Additional details and extensive literature citations are available in several recent reviews (1–6). Descriptions of commercially available systems are readily accessible over the Internet and in protocols supplied by appropriate vendors (see Section 7).

2. General considerations in selecting an *E. coli* expression system

2.1 Choosing between *E. coli* and other expression systems

The advantages of using *E. coli* for heterologous gene expression include the ease of growth and manipulation of this organism using simple laboratory equipment, the availability of dozens of vectors and host strains that have

been developed for maximizing expression, a wealth of knowledge about the genetics and physiology of *E. coli*, and the influence of specific genetic and environmental factors on expression of heterologous proteins. Moreover, expression can often be achieved quite rapidly: beginning with a eukaryotic cDNA clone, it is possible to express a protein in *E. coli* and purify it in milligram quantities in less than two weeks. Shaken-flask cultures can produce many tens of milligrams of a heterologous protein per litre of culture. In fermenters, where much higher cell densities can be achieved, it is possible to produce more than a gram per litre of the heterologous protein. Recombinant proteins can be accumulated at levels up to 50% of total cell protein. Nevertheless, there are situations where other expression systems, such as those based on yeast, insect, or mammalian cells, are more likely to be successful. The most important limitation is the inability of *E. coli* to carry out complex post-translational modifications typical of eukaryotes, such as glycosylation, myristylation, phosphorylation, specfic protoeolytic processing, etc., and its limited ability to carry out extensive disulfide bond formation and assembly of heterologous proteins into multisubunit assemblies. Another limitation of *E. coli*, which can require a considerable effort to overcome, is that some proteins are made in insoluble form, a consequence of protein misfolding, aggregation, and sequestration into 'inclusion bodies'. It is sometimes difficult to produce a protein with an authentic N-terminus, that is, one that has the appropriate presence or absence of an N-terminal methionine as in the eukaryotic cell of origin. Finally, it is sometimes difficult to achieve a sufficiently high level of expression because of problems such as protein degradation, or inefficient translation due to mRNA structural features or eukaryotic codon usage that are not optimal for *E. coli*.

In deciding when to use *E. coli*, a critical consideration is the intended use of the protein. *E. coli* expression is generally a first choice for expression of a protein whose use does not require a native conformation, such as an immunogen for raising an antiserum that will be used for Western blotting. *E. coli* is also often useful for production of a protein with a native conformation and activity, with the likelihood of success being greatest when the protein is small (preferably less than about 70 kDa), does not have unpaired cysteines or more than three or four intramolecular disulfide bonds, and does not require post-translational modification for activity or solubility.

The protocols described in the rest of this chapter have been successful in expressing many proteins. The two most common problem areas are inadequate expression levels and poor product solubility. Several simple manipulations, discussed in Sections 2.2 and 2.3, are frequently successful in overcoming these problems. Fusion proteins and tags (Section 2.4) are a generally useful approach to improve both the level of expression and the solubility, as well as aiding in purification. Approaches to engineering an authentic N-terminus are discussed in Section 2.5.

2.2 Improving the level of expression

The levels of expression are sometimes inadequate to meet all the needs of an investigator, even when expression systems are used that employ strong transcriptional and translational signals (discussed in Section 3). Simple approaches to this problem, discussed in this section, are: changing the induction conditions, changing the coding sequence of the heterologous gene, and changing the host strain to overcome proteolysis of the product. Use of gene fusions to improve expression is discussed in Section 2.4.

2.2.1 Induction conditions

Varying the time period and/or temperature of induction of expression of the heterologous protein is always worthwhile to find the optimal conditions for product accumulation. Some expression systems allow induction under alternative physiological conditions. For example, the P_L system allows induction by temperature shift, by addition of nalidixic acid, or by addition of tryptophan (Section 3.1). The levels of specific cellular proteases may vary in these different conditions. Changing the composition of the growth medium has also been reported to improve expression in some cases (6, 7).

2.2.2 Coding sequence of the heterologous gene

Expression is often improved by making changes to the nucleotide sequence of the coding region that do not change the amino acid sequence of the expressed product. Improvements have been reported by changing G and C residues in the first few codons to A or T, by replacing codons rarely found in highly-expressed *E. coli* genes with more favourable codons throughout the heterologous gene, and by making changes that reduce mRNA secondary structure in the translation intiation region. Plasmids that overexpress tRNA molecules that recognize rare codons in the heterologous gene have also been described, and can be introduced into the strain being used for expression as an alternative to changing rare codons in the coding sequence. Codons that have been associated with translation problems in *E. coli* are AGG, AGA, CGA, and CGG (arginine), AUA (isoleucine), CUA (leucine), GGG and GGA (glycine), and CCC (proline) (1, 3, 6).

2.2.3 Use of protease-deficient host strains

The use of host strains carrying mutations which eliminate production of cellular proteases can sometimes enhance product accumulation by reducing degradation (reviewed in refs. 1 and 5). For recombinant proteins expressed in the cytoplasm, mutations in the *lon*, *clp*, and *rpo*H (*htp*R) genes have reportedly been helpful. The *lon* and *clp* genes encode cytoplasmic proteases. The *rpo*H (*htp*R) gene encodes a sigma factor which stimulates production of heat shock proteins, some of which are proteases. For recombinant proteins

secreted to the periplasm, mutations which eliminate the HtrA (DegP), OmpT, protease III, and/or Prc (Tsp) proteases can be helpful.

2.3 Improving the solubility of a protein expressed in *E. coli*

For many proteins, the most significant obstacle to the use of *E. coli* is protein insolubility under conditions of high level expression. Several techniques, discussed below, are available for improving the solubility of non-fusion proteins (the use of gene fusions is discussed in Section 2.4).

2.3.1 Secretion of the heterologous protein

In Gram-negative bacteria such as *E. coli*, the cytoplasm is surrounded by two membranes. The compartment bounded by these inner and outer cell membranes is referred to as the periplasm. The periplasmic space comprises about 10–40% of the total cell volume under normal growth conditions (according to various estimates), and contains 4% of the total cell protein or about 100 proteins (1, 5). Fusion of a heterologous protein to an *E. coli* secretion signal sequence directs the protein across the cellular inner membrane into the periplasm. The signal sequence (usually 18–25 amino acids in length) is removed during secretion, and refolding of the secretory protein into a native conformation occurs resulting in accumulation of soluble protein in the periplasm. In the case of proteins that have intramolecular disulfide bonds in the native conformation, the oxidizing environment of the periplasm allows disulfide bond formation, which does not occur in the reducing environment of the cytoplasm. This partitioning of the protein away from cytoplasmic proteins can be helpful for purification, may reduce proteolysis, and may allow the accumulation of proteins that are toxic in the cytoplasm (discussed in refs. 1, 3, and 5). Secretion can also be useful in engineering an authentic N-terminus (see Section 2.4).

In some cases, release of a protein from the periplasm into the extracellular growth medium can also be achieved. This can occur spontaneously with some proteins, or as a result of genetic or physiological manipulations which increase the permeability of the outer membrane. Release of the expressed periplasmic protein into the extracellular growth medium may confer further advantages in purification. However, the process by which some products cross the outer membrane is not well understood and success is difficult to predict.

Several bacterial signal sequences have been used to drive secretion of heterologous proteins in *E. coli*, including sequences from the *E. coli ompA*, *lamB*, *phoA*, β-lactamase, and *STII* genes, from *Erwinia carotovara pelB*, and from *Staphylococcus aureus* Protein A (1, 5) (*Table 1*). Mammalian signal sequences may function in *E. coli* (5), but bacterial sequences are generally used to achieve the highest possible efficiency. For some proteins, there are significant differences in the efficiency of different bacterial signal sequences.

The efficiency of signal sequence removal by the *E. coli* secretion machinery is also influenced by the amino acid sequence of the heterologous protein, especially the first few amino acids following the cleavage site, so efficient secretion and processing may not be possible with some proteins. The chance of success in achieving *E. coli* secretion is highest when the protein is one that is naturally secreted by its eukaryotic cell of origin.

2.3.2 Growth temperature

One of the simplest approaches to improving the solubility of proteins expressed in the cytoplasm is to reduce the growth temperature of the culture (to 30°C or lower) during induction (1, 3). Reducing the rate of protein synthesis by use of weaker promoters or partial induction conditions can also disfavour protein misfolding and aggregation, allowing the accumulation of larger amounts of soluble protein.

2.3.3 Co-expression of chaperones and enzymes influencing folding of the heterologous protein *in vivo*

Post-translational folding of proteins, assembly into oligomers, and transport to the periplasm are facilitated by molecular chaperones. Co-expression of a protein with *E. coli* chaperones GroES-GroEL or DnaJ and DnaK, or with eukaryotic protein disulfide isomerase, has sometimes proven useful (1, 3, 8). Overexpression of thioredoxin, and null mutations in *trxB* encoding thioredoxin reductase, have also been found to improve the solubility of some proteins (1).

2.3.4 Refolding of the heterologous protein *in vitro*

Proteins that are made in insoluble form in inclusion bodies can often be solubilized and refolded. This approach to obtaining soluble protein offers some advantages. Inclusion bodies can be easily separated by centrifugation from soluble proteins and other cellular components as a first purification step, and proteins sequestered into inclusion bodies may be less susceptible to degradation. The major disadvantage is that conditions for refolding into a fully active form may be difficult to find.

Solubilization is usually accomplished with denaturants such as urea or guanidine hydrochloride, and reducing agents to break disulfide bonds. Removal of the denaturant and refolding may then be accomplished by methods such as dilution or dialysis. The conditions which allow refolding and reformation of disulfide bonds vary with different proteins and must be determined empirically in each case. Some of the factors which may be varied to improve the yield of active protein are the protein concentration and purity, the pH and ionic strength of the buffer, and the disulfide oxidizing conditions which may be modulated by addition of agents such as cysteine, arginine, glutathione, or dithiothreitol (9).

2.4 Expression of heterologous proteins as fusion proteins or with protein tags

Many vectors are available which allow expression of heterologous proteins which are fused at their N- or C-terminus to polypeptides or short peptide sequences (*Table 1*). Such fusion partners offer several potential advantages:

(a) Improved expression. Fusion of the N-terminus of a heterologous protein to the C-terminus of a highly-expressed fusion partner often allows high level expression of the fusion protein (see Section 3.1).

(b) Improved solubility. Fusion of the N-terminus of a heterologous protein to the C-terminus of a soluble fusion partner often improves solubilty of a protein (see Section 3.1).

(c) Improved detection. Fusion of a protein at either terminus to a short peptide (epitope tag) or a polypeptide which is recognized by an antibody or binding protein allows Western blot analysis of a protein during expression and purification.

(d) Improved purification. Simple purification schemes have been described for proteins fused at either end to tags which bind affinity resins. Available tags include His_6 (six tandem histidine residues, which bind to Ni-NTA; nitrilo-triacetic acid chelated with Ni^{2+} ions), GST (glutathione *S*-transferase, which binds to glutathione–Sepharose), thioredoxin (binds to ThioBond resin), maltose binding protein (binds to amylose resin), Protein A (binds to immunoglobulin G–Sepharose), polyarginine (binds to thiopropyl–Sepharose), biotinylation domain (binds to avidin resin), chitin binding domain (binds to chitin resin), or any defined epitope (such as the FLAG peptide) for which a corresponding monoclonal antibody is available. Further information about these tags and their use for protein purification may be found in ref. 1 and at the World Wide Web sites listed in *Table 1*.

Specific excision sites are usually added to the junction of a protein with the fusion partner to allow eventual enzymatic or chemical removal (1, 4, 10) (also see information sources listed in *Table 1*). Two commonly used proteolytic sites are those recognized by bovine enterokinase and Factor Xa. These enzymes are commercially available and are fairly specific for their recognition sequences, often allowing specific cleavage without protein degradation under gentle conditions where protein solubility and activity are preserved. Enterokinase and Factor Xa cleave on the C-terminal sides of their recognition sequences (DDDDK and IEGR, respectively), and have a fairly wide tolerance for different amino acids in the position following the cleavage site. Thus, fusion of a protein's N-terminus to the C-terminus of one of these protease recognition sequences may allow generation of a cleaved protein with an authentic N-terminus.

2.5 Nature of the N-terminus of the heterologous protein

Translation in *E. coli* is initiated with *N*-formylmethionine which is usually deformylated during protein synthesis. The N-terminal methionine is sometimes removed from the translated protein by an endogenous methionine aminopeptidase. In other cases it remains on some or all of the expressed protein. This extraneous amino acid may be undesirable if it changes the biological activity or immunogenicity of the protein.

The efficiency of removal of the N-terminal residue is greatly influenced by the side chain length of the next amino acid after methionine (11). Met is usually removed from most or all of the expressed protein when followed by a residue with a short side chain, such as Gly, Ala, Pro, Ser, Thr, Val, or Cys. In cases where the methionine is retained and is deleterious, several strategies are available to generate a protein with the desired N-terminus. Expression in a secretion system allows endogenous removal of the signal sequence, leaving a protein with the desired N-terminus. Expression as a fusion protein, in which the fusion partner is followed by a cleavage site and then the desired N-terminus of the protein, may allow specific enzymatic or chemical cleavage of the purified protein to remove the fusion partner and generate the desired product.

3. Features of *E. coli* expression systems

All high-level expression vectors for *E. coli* have the following features:

(a) Regulatory elements which control transcription and translation.

(b) Restriction endonuclease cleavage sites for convenient insertion of coding sequences.

(c) A region encoding vector replication functions.

(d) A selectable marker (usually an antibiotic resistance gene) for maintaining selection pressure for cells carrying the vector.

Many vectors encode additional optional components such as signal sequences to direct secretion, or peptide tags that are added to the N- or C-terminus of the protein. Some expression systems require the use of specialized host strains which provide regulatory elements. Several widely utilized systems are listed in *Table 1*.

3.1 Promoters and other transcription regulatory elements

A promoter is ideally:

(a) Strong enough to allow product accumulation up to half of total cellular protein.

Table 1. Examples of *E. coli* expression systems and Internet addresses for further information

Vector system	Promoter/induction method	Special host strains required?[a]	Protein tag[c]	Secretion signal sequences	Source, Web site, or reference
Pinpoint	*tac*/IPTG *or* T7/IPTG	Yes	Biotin binding domain		Promega Corp. `http://www.promega.com`
pET	T7/IPTG	Yes	His_6, T7 gene 10	*ompT*, *pelB*	Novagen Inc. `http://www.novagen.com`
pGEX	*tac*/IPTG	No	GST		Pharmacia Biotech `http://www.biotech.pharmacia.se`
pTRX	P_L	Yes	Thioredoxin		Invitrogen `http:/www.invitrogen.com`
pKK	*trc*/IPTG	Yes			Clontech `http://www.clontech.com`
IMPACT I	*tac*/IPTG	No	Chitin binding domain		New England Biolabs `http://www.neb.com`
IBI FLAG	*tac*/IPTG	No	FLAG peptide	*ompA*	Kodak Scientific Imaging `http://www.kodak.com`
pPROEX-1	*trc*/IPTG	No	His_6		Life Technologies `http://www.lifetech.com`
pQE	T5/IPTG	Yes	His_6		Qiagen Inc. `http://www.qiagen.com`
		TOPP host strains[b]			Stratagene `http://www.stratagene.com`
pAS	P_L	Yes	NS1-81		refs 10, 12, 13

[a] Specialized host strains are needed for some systems to supply transcription elements such as *lacI*^{q}, T7 polymerase under *lac* operator control, or a repressor of the P_L promoter. In other systems, a *lacI* gene is carried by the vector itself.
[b] The TOPP strains are non-K12 *E. coli* strains carrying *lacI*^{q} that improve expression of some proteins. Vector systems carrying *tac* or *trc* promoters may be used in these strains.
[c] Tag-specific affinity resins and endoproteinases for tag removal are generally available for use with these vectors.
Abbreviations: IPTG (isoproplyl-β-D-thiogalactopyranoside), GST (glutathione *S*-transferase).

(b) Tightly-regulated to prevent product toxicity from basal level expression during routine propagation.

(c) Easy to induce in a variety of physiological conditions and host genetic backgrounds.

Many natural and engineered promoters have been described that satisfy all or most of these criteria, including *lac*UV5, *trp*, *tac*, *trc*, phage lambda P_L and P_R, and phage T7 promoters (1) (*Table 1*). The *lac*UV5, *tac*, and *trc* promoters are repressed by the *lac* repressor (usually overexpressed from a *lacI* or *lacI*^{q} gene on the vector or in the host strain), and chemically induced with isopropyl-β-D-thiogalactopyranoside (IPTG). The *trp* promoter is repressed by the *trp* repressor, and induced by removal of tryptophan from the growth medium or addition of the chemical inducer, indole-3-acetic acrylic acid. Induction of the phage T7 promoter requires expression of a phage RNA polymerase, which is usually accomplished using a host strain in which the polymerase is expressed from a *lac*UV5 promoter induced by addition of IPTG.

The P_L promoter, which is used in the protocols in Sections 5.1, 5.2, and 6, exibits a level of high maximal expression when induced, and extremely low basal expression in host strains carrying a chromosomal copy of the phage cI repressor gene. Induction conditions depend on the host strain used; temperature shift-up in strains carrying the temperature-sensitive cI857 repressor, or addition of nalidixic acid in strains carrying the wild-type cI repressor. P_L can also be induced by addition of tryptophan in strains carrying cI under the control of the *trp* operon (available from Invitrogen Corp.) (*Table 1*), which allows induction at lower temperatures than with heat shift or nalidixic acid. However, significant leakiness (basal expression under non-inducing conditions) is observed under these conditions. Disadvantages of the P_L system include a requirement for specific host strains, and use of a relatively low copy number vector, which is necessary to maintain tight regulation but reduces the yield of plasmid DNA.

In addition to the promoter, many vectors have transcriptional terminators or anti-terminators. For example, in the P_L system used in the protocols described in later sections, appropriately positioned anti-termination regulatory elements ensure that transcription completely traverses the gene to be expressed, while a transcriptional terminator reduces unwanted transcription through the replication region and may help to stabilize the mRNA, thus increasing mRNA half-life (10, 12, 13).

3.2 Translation initiation and termination signals

The efficiency of translation initiation at the AUG initiator methionine codon is influenced by the mRNA nucleotide sequence, especially in the region preceding the AUG that is generally referred to as the Shine–Dalgarno sequence or ribosome binding site (discussed in refs. 1 and 3). *E. coli* expression vectors

typically contain an efficient translation initiation sequence at an optimal distance upstream of the AUG. Most expression vectors contain a restriction site, such as *Nde*I (CAT*ATG*) or *Nco*I (CC*ATG*G), that overlaps the ATG initiation site which is positioned at an optimal distance from the Shine–Dalgarno sequence. Genes to be expressed in *E. coli* usually need to be engineered at the 5′ end for insertion into these sites.

Any of the three possible termination codons will suffice, but TAA is preferred because it is less prone to read-through than TAG or in TGA in commonly used host strains (1). Tandem termination codons may be engineered at the 3′ end of a gene to improve termination efficiency.

3.3 Host strain

As mentioned above, some vectors require specialized host strains carrying regulatory elements. In addition, expression may be enhanced through use of host strains carrying mutations in proteases, or which have been engineered to overexpress or underexpress gene products that affect the solubility of the recombinant protein.

In all expression systems, the host strain supplies many essential functions for protein production, including the basic machinery for transcription, translation, and vector replication, and the energy and building blocks needed for synthesizing and folding a foreign protein. Additional host functions are involved in transport of proteins to the periplasm in secretion systems. Accordingly, the genotype of a strain (including known loci and any unknown mutations acquired through many generations of laboratory propagation) potentially can influence both the growth characteristics of the organism and the ability to accumulate high levels of a heterologous protein.

High level expression systems usually use descendants of the K12 strain of *E. coli*. K12 strains have been used more extensively than other strains for genetic investigation of this organism, and are favoured by governmental guide-lines and regulations for recombinant DNA work. *E. coli* B derviatives and other *E. coli* strains are also used on occasion, and reportedly offer advantages in expression of some genes (1) (also note TOPP strains listed in *Table 1*). The simple expedient of switching strains can occasionally result in improved expression of a particular protein.

4. Protocols for expression: general comments

The protocols described in later sections are examples of how to do the following:

(a) Add a small protein tag to a heterologous protein using a generic PCR cloning procedure.

(b) Construct an expression vector.

(c) Induce expression in the cytoplasm or periplasm.

(d) Use the tag for both detection and purification.

The same protocols can be used to express an untagged protein. Similar procedures are applicable to a variety of other expression systems and tags (for which sources of specific protocols are shown in *Table 1*).

The vector employed in these protocols is illustrated in *Figure 1*. It is a derivative of the pAS and pSKF vectors that have been discussed in detail elsewhere (10, 12, 13). Transcription is controlled by elements of bacteriophage lambda, including the strong and tightly-regulated P_L promoter, and

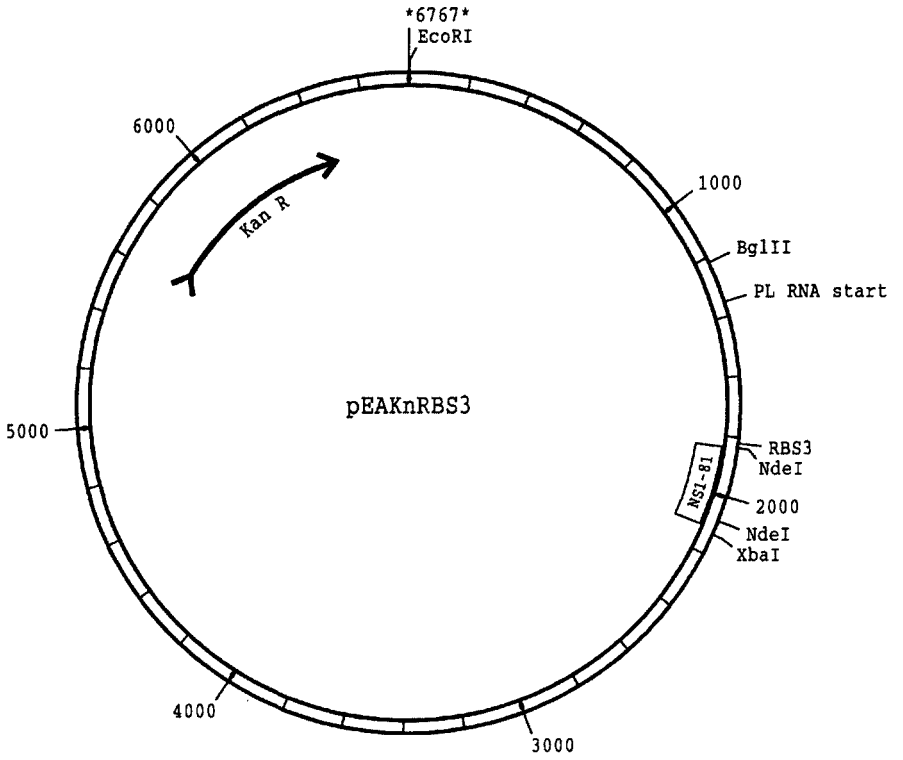

Figure 1. Vector pEA181KnRBS3. Vector pEA181KnRBS3 contains a phage lambda P_L promoter (PL RNA start), a strong ribosome binding site (RBS3), and a kanamycin resistance (Kan R) gene for selection. Other transcriptional regulatory elements and plasmid pBR322 replication functions are not shown here for simplicity but are unchanged from the parental vectors pAS1 and the pSKF series (10, 12, 13). The NS1-81 segment is an 81 amino acid portion of influenza virus non-structural gene NS1. This segment (from the *Nde*I site at position 1835 to the *Xba*I site at 2121) is removed and replaced by heterologous genes in the protocols in this chapter. As indicated in Section 5.2, expression of the NS1 polypeptide serves as a useful control when attempting expression of foreign genes cloned into this site.

a repressor of P_L transcription carried by specially constructed host strains (Section 3.1). Expression is induced by temperature shift in a strain carrying a repressor with a temperature-sensitive mutation.

In these protocols, a gene is inserted into the *Nde*I and *Xba*I restriction sites of the vectors at positions 1835 and 2131. The *Nde*I restriction site (CATATG) overlaps an initiator methionine AUG codon that is located at an appropriate distance from a ribosome binding site (RBS3). Genes may be modified by a variety of cloning strategies to allow insertion of tagged and untagged genes into these sites, with or without signal sequences. Addition of a His_6 tag is employed in the protocols, which allows detection by Western blot using an anti-His_6 monoclonal antibody (*Protocol 8*), and purification by metal chelate affinity chromatography (*Protocols 10* and *11*). A *pelB* signal sequence to drive secretion is used in *Protocols 12* and *13*.

5. Expression, detection, and purification of a His_6-tagged protein

In the sequence of protocols described in this section, the vector pEA181KnRBS3 is used, but any other vector in the pAS or pSKF series containing *Nde*I and *Xba*I sites may be substituted (10, 13).

5.1 Construction of the recombinant vector and transformation of host cells

In *Protocol 1* the vector DNA is digested with restriction enzymes *Nde*I and *Xba*I to generate ends compatible for ligation with the coding sequence to be cloned. It includes an electrophoretic purification step which is advisable in order to separate the 6491 and 296 bp *Nde*I/*Xba*I fragments of the vector. The former contains regulatory elements essential for expression; the latter is not needed for expression. *Protocol 1* starts with purified vector. The vector DNA may be purified from host strain MM294cI+ containing this plasmid (10, 13) using a Qiagen plasmid purification kit (No. 12143) as recommended by the kit manufacturer for low copy number vectors.

Protocol 1. Restriction endonuclease digestion and electrophoretic purification of vector

Equipment and reagents

- Vector pEA181KnRS3 DNA (at least 50 μg/ml)
- 10 × TA buffer: 0.3 M Tris–acetate pH 7.8, 0.65 M potassium acetate, 0.1 M magnesium acetate, 40 mM spermidine trihydrochloride, 5 mM DTT
- Restriction enzymes: *Nde*I (Promega, R680A) and *Xba*I (Promega, R618G)
- Sterile deionized or distilled water (autoclaved at 121°C for 20 min)
- 10 × DNA loading buffer: 50% (v/v) glycerol, 1% (w/v) SDS, 10 mM Tris–HCl pH 7, 0.25% (w/v) bromophenol blue
- 1 mg/ml DNA size markers (Life Technologies, 15615–016)

- Agarose (Mallinckrodt GeneAR, 77–36)
- *Agarose gel electrophoresis apparatus (e.g.* a Life Technologies Horizon 58 apparatus) and power supply
- 50 × TAE buffer: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8, water to one litre
- 0.5 μg/ml ethidium bromide[a] in 1 × TAE buffer
- Long wavelength hand-held UV light (Fisher, FB-UVLS-80) and UV safety visor or goggles[b]
- Scalpel or razor blade
- Qiexll kit (Qiagen)
- *DNA Dipstick kit (Invitrogen)*

Method

1. To a 1.5 ml microcentrifuge tube, add 0.8 μg vector DNA, 2 μl of 10 × TA buffer, 5 U each of *Nde*I and *Xba*I, and water to a final volume of 20 μl.
2. Incubate the mixture at 37 °C for 2 h.
3. Add 2 μl of 10 × DNA loading buffer.
4. Make up the DNA size markers by diluting to 100 μg/ml in 1 × TAE buffer and then adding 0.1 vol. 10 × DNA loading buffer. This solution may be stored for six months at 4 °C.
5. Make a 0.8% agarose gel in 1 × TAE buffer in the agarose gel electrophoresis apparatus in accordance with instructions of the apparatus manufacturer. Use a well-forming comb sufficiently large to allow 20 μl to be loaded per lane.
6. Load the gel, with the reaction mixture in one lane and the DNA size markers in another. Run at 60 V toward the positive electrode until the dye is near the bottom (~ 1–2 h).
7. Stain the gel by incubating for 15 min in 0.5 μg/ml ethidium bromide.[a] Rinse the gel briefly in water.
8. Locate the DNA bands using a long wavelength UV light held above the gel.[b]
9. Use a scalpel or razor blade to excise the 6491 bp vector band.[b]
10. Extract the DNA from the agarose fragment using the Qiexll kit in accordance with the manufacturer's instructions. This will result in a DNA sample in 20 μl TE buffer.
11. Quantify the amount of DNA extracted with the DNA Dipstick kit, following the supplier's instructions.

[a] Caution: ethidium bromide is mutagenic, so wear disposable gloves when working with it, and dispose of all ethidium bromide solutions and contaminated materials as specified by local safety rules.

[b] Protect your eyes when using UV light (steps 8 and 9) by wearing appropriate goggles or a visor.

Protocol 2 uses the polymerase chain reaction (PCR) to generate a DNA sequence for cloning that consists of six tandem histidine codons fused in-frame to the C-terminus of the coding sequence of the protein to be

expressed, with dual stop codons following the histidine tag, and with *Nde*I and *Xba*I restriction sites flanking the tagged protein coding region to allow cloning into the vector. Normally this engineered sequence would be added to the PCR reaction in the form of supercoiled plasmid DNA or a DNA fragment derived from a plasmid by restriction enzyme digestion and electrophoretic purification (see *Protocol 1*). The PCR reaction requires forward and reverse oligonucleotide primers, normally synthesized using a commercial DNA synthesizer. In the procedures described in this section the 24 nt forward oligonucleotide primer contains the sequence 5′-TTC-CAT-ATG-XXX-XXX-XXX-XXX-XXX, where the sequences XXX refer to nucleotides at the 5′ end of the gene to be expressed, and the *Nde*I site is underlined. The 50 nt reverse oligonucleotide primer has the sequence 5′-CG-TCT-AGA-ATC-ATT-GTG-GTG-GTG-GTG-GTG-GTG-YYY-YYY-YYY-YYY-YYY-YYY, where the sequences YYY correspond to the non-coding strand at the C-terminus of the protein to be expressed, and the *Xba*I site is underlined. The series of six GTG sequences on the non-coding strand will result in the addition of the His_6 tag, and the ATC-ATT sequence will add two stop codons UAA-UAG immediately following the His_6 tag. The number of X and Y nucleotides and the annealing conditions below can be adjusted to optimize the amplification reaction using computer software such as *Oligo*™ (version 4.0 for Macintosh available from W. Rychlik, National Biosciences, Inc., Plymouth, MN) that is designed for selecting and evaluating PCR primers. However, this is usually unnecessary in the amplification and subcloning situation described in *Protocol 2* as long as there are at least 18 nt of homology (X's and Y's) included at each end of the gene to be amplified. Additional modifications in conditions may be needed to amplify fragments longer than 1 kb. A typical amplification protocol would consist of 25 cycles each involving a denaturing step at 94 °C for 30 sec, an annealing step at 52 °C for 30 sec, and an extension step at 72 °C for 1 min, followed by a final 5 min extension cycle at 72 °C. These reaction conditions can be adjusted if necessary to maximize the yield of reaction products. Electrophoretic purification of the PCR amplified fragment is not usually necessary, but may be helpful if the PCR reaction produces other sequences besides the desired amplified coding sequence fragment.

Protocol 2. Amplification of gene coding sequence and modification of the 3′ end to attach a His_6 tag

Equipment and reagents

- TE buffer, *Nde*I and *Xba*I restriction endonucleases, sterile water, and DNA Dipstick kit (see *Protocol 1*)
- Coding sequence DNA to be amplified: 10–20 ng/μl TE buffer, carried in a purifed DNA fragment, or in a vector in circular or linear form
- A pair of forward and reverse oligonucleotide primers suitable for the coding sequence to be amplified (1 μg/μl H_2O)
- 10 × PCR reaction buffer containing Mg^{2+} ions (Boehringer Mannheim, 1271318)

- Deoxyribonucleotide mixture: 10 mM each of dATP, dCTP, dGTP, and dTTP
- 5 U/μl *Taq* DNA polymerase (Boehringer Mannheim, 1435094)
- Mineral oil (Perkin-Elmer, 186–2302)
- Perkin-Elmer Thermocycler 480
- 0.5 M EDTA pH 8
- 3 M sodium acetate (pH 5.2 with acetic acid)
- Equipment and reagents for agarose gel electrophoresis of DNA (see *Protocol 1*)
- Phenol:chloroform:isoamyl alcohol (25:24:1, by vol.) (Boehringer Mannheim, 101001)
- Ethanol: absolute and 70% (v/v) solution at −20°C
- Freeze-drier or vacuum desiccator

Method

1. To a 0.4 ml microcentrifuge tube on ice, add 84.5 μl sterile water, 1 μl DNA (10–20 ng), 10 μl of 10 × PCR reaction buffer, 2 μl deoxyribonucleotide mixture, 1 μl of each of the forward and reverse oligonucleotides primers (1 μg each), and 0.5 μl *Taq* DNA polymerase. Also set up negative control reactions in which either the forward or the reverse primer, or the DNA substrate is omitted from the reaction and is replaced by the equivalent volume of water.
2. Add one drop of mineral oil to the tube, close the top, and label the top of the tube (advisable because ink labels on the sides of the tubes may be lost when exposed to mineral oil).
3. Add one drop of mineral oil to each hole in the thermocycler block, and then place the tubes in the holes.
4. Amplify the DNA for 25 cycles consisting of:
   - 94°C for 30 sec (denaturation)
   - 52°C for 30 sec (annealing)
   - 72°C for 1 min (extension)

   Finish the amplification stage at 72°C for 5 min.
5. Place the tubes on ice and add 2 μl of 0.5 M EDTA to each.
6. Remove 10 μl from each tube and analyse the DNA by agarose gel electrophoresis (as in *Protocol 1*) to verify the size of the amplified fragment and estimate its yield.
7. Transfer the remainder of each reaction mixture to a 1.5 ml microcentrifuge tube and add an equal volume of phenol:chloroform: isoamyl alcohol. Vortex for 10 sec. Centrifuge the tubes at 10000 *g* for 5 min to separate the phases. Remove the aqueous (upper) phase to a fresh tube.
8. Precipitate the DNA by adding 10 μl of 3 M sodium acetate pH 5.2, followed by 200 μl absolute ethanol at −20°C. Centrifuge at 16000 *g* for 15 min in a microcentrifuge at 4°C.
9. Wash the DNA pellet gently with 500 μl of 70% ethanol, decanting the ethanol carefully without disturbing the pellet. Dry the pellet under vacuum for 2–3 min.

Protocol 2. *Continued*

10. Resuspend the pellet in 20 μl TE buffer. The yield is usually 0.5–1 μg of amplified DNA fragment.
11. Determine the concentration of the amplified DNA using the DNA Dipstick kit in accordance with the manufacturer's instructions.
12. To a 1.5 ml microcentrifuge tube, add enough water to give a final reaction volume of 20 μl, and then add the following restriction enzyme reaction components: 0.2 μg of the PCR product (from step 10), 2 μl of 10 × TA buffer, and 2 U each of *Nde*I and *Xba*I. Incubate at 37 °C for 2 h.
13. Add 2 μl of 0.5 M EDTA pH 8 followed by 80 μl TE buffer, and then repeat steps 7–11.
14. Proceed to the ligation step (*Protocol 3*).

Ligation of the restriction vector DNA and the PCR amplified coding sequence is described in *Protocol 3*.

Protocol 3. Ligation of vector and gene fragments

Equipment and reagents

- *Nde*I/*Xba*I digested vector DNA, and sterile deionized water (see *Protocol 1*)
- PCR amplified DNA sequence to be cloned (see *Protocol 2*)
- Water-baths at 65 °C and 16 °C
- 5 × ligation buffer: 250 mM Tris–HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol 8000
- 3 U/μl T4 DNA ligase (Promega, M180B)

Method

1. To a 0.4 ml microcentrifuge tube, add 50–100 ng *Nde*I/*Xba*I digested vector DNA and PCR amplified DNA fragment (sufficient to give a five-fold molar ratio of fragment DNA to vector DNA), and sterile deionized water to a final volume of 10 μl.
2. Heat the mixture to 65 °C for 10 min, then place on ice.
3. Add 4 μl of 5 × ligation buffer, and 1 μl T4 DNA ligase.
4. Incubate at 16 °C for 4–16 h.
5. Store the ligation mixture at 4 °C until used for transformation of the host (*Protocol 4*).

5.1.2 Transformation of *E. coli* and selection of recombinants

The recombinant vector ligated DNA (from *Protocol 3*) is introduced into *E. coli* host strain AR58 (10) as described in *Protocol 4*. This host carries a defective phage lambda lysogen that expresses a temperature-sensitive repressor (cI857) of the vector P_L promoter. During growth at 32 °C, basal

expression from the P_L promoter is very low, which is important in preventing expression of toxic gene products and in preventing destabilization of the vector that may occur during prolonged growth under inducing conditions. *It is essential to maintain this strain at a temperature no higher than 32 °C* at all times during propagation on plates or in liquid culture, except when inducing expression by temperature shift as in *Protocol 6*.

The host is made competent for transformation by a simple treatment with $CaCl_2$. Include two controls to monitor transformation, one with undigested (circular) vector DNA (50 ng) as a positive control, and the other with TE buffer containing no DNA as a negative control. The positive control should produce thousands of colonies per transformation.

Protocol 4. Transformation of *E. coli* host

Equipment and reagents

- *E. coli* strain AR58 grown overnight in LB
- LB: 1% tryptone, 0.5% yeast extract, 0.5% NaCl
- LB containing 50 μg/ml kanamycin
- Shaking water-bath at 32 °C
- Beckman table-top centrifuge (or equivalent) and microcentrifuge
- 100 mM $CaCl_2$ (filter sterilized through a 0.45 μm or 0.2 μm filter), ice-cold
- 50% (v/v) glycerol (sterilized by autoclaving at 120 °C for 15 min)
- Storage vials for cells (1.8 ml cryotube; Fisher, 12–565–171N)
- Recombinant vector DNA (ligation mixture from *Protocol 3*)
- Native vector
- TE buffer (see *Protocol 2*)
- Water-bath at 37 °C
- LB agar plates: 1% agar in LB containing 50 μg/ml kanamycin
- Incubator for plates set at 32 °C

Method

1. Prepare competent *E. coli* AR58 cells by diluting 2 ml of an overnight culture of AR58 into 200 ml LB (no antibiotic). Grow the cells with shaking at 32 °C.
2. When the OD_{650} reaches 0.5–0.6, harvest the cells by centrifugation at 3000 *g* for 10 min at 4 °C.
3. Resuspend the cell pellet by vortexing vigorously in 30 ml ice-cold 100 mM $CaCl_2$. After 10 min at 0 °C, centrifuge the cells again at 3000 *g* for 10 min at 4 °C, and resuspend them as gently as possible in 5 ml ice-cold 100 mM $CaCl_2$.
4. After 2–4 h on ice, add 2.5 ml sterile 50% glycerol and gently mix. Aliquot the cells into separate storage vials, and either use them immediately or store them at –70 °C for future use.[a]
5. Add 7.5 μl recombinant vector DNA (ligation mixture from *Protocol 3*) to 100 μl competent cells. Set up positive and negative control samples in which the recombinant vector is replaced by intact native vector or by TE buffer, respectively (see text).
6. Incubate the samples on ice for 20 min, place them in a 37 °C water-bath for 5 min, and then return them to room temperature.

Protocol 4. *Continued*

7. Add 1 ml LB (containing no antibiotic) to each tube, invert the tubes to mix their contents, and then place the tube in a 37 °C water-bath for 60 min.
8. Vortex each sample for 1 sec, and then spread 50–100 μl from each sample on an LB agar plate containing 50 μg/ml kanamycin.
9. Microcentrifuge the remaining cells for 10 sec at 16000 *g* and pour off all but 50–100 μl liquid. Resuspend the cell pellets by vortexing. Separately spread the entire cell suspension from each tube on another LB agar plate containing kanamycin.
10. Incubate the plates overnight at 32 °C. Analyse colonies as in *Protocol 5*.

[a] Cells prepared in this way are competent for transformation for many months.

Colonies containing vector with an inserted gene (recombinants) must be distinguished from those containing vector with no inserted gene, and vector with multiple inserts of the gene, or other undesirable cloning products. This is most conveniently done by analysis of colonies by PCR (*Protocol 5*). It is preferable to use a pair of PCR primers that includes one vector-specific primer and one gene-specific primer. For example, the gene-specific forward primer used in *Protocol 2* can be used in combination with a vector-specific reverse primer that is located downstream of the vector *Xba*I site (sequence 5′-GAA-GGA-GCT-GAC-TGG-GTT-G, for example). For colonies with the correctly inserted fragment, this PCR reaction will produce a PCR product that is slightly larger (46 bp longer in the case of the oligonucleotide described above) than the *Nde*I-*Xba*I gene fragment used for cloning. However, if a vector-specific primer is not available, the analysis can be carried out with the same pair of primers as was used for fragment amplification in *Protocol 2*. This will distinguish clones with inserts from those without, but will not distinguish clones with single inserts from those with multiple inserts. Screening of 20 independent colonies is usually more than sufficient to identify several candidates with the correctly inserted fragment.

Protocol 5. Identification of colonies by PCR

Equipment and reagents

- LB (containing 50 μg/ml kanamycin), LB agar plates (containing kanamycin), and plates containing transformants for analysis (see *Protocol 4*)
- Toothpicks, sterilized by autoclaving (120 °C, 15 min)
- Incubator at 32 °C
- Reagents for PCR (see *Protocol 2*)
- Agarose gel electrophoresis apparatus, Qiagen plasmid purification kit, 10 × DNA loading buffer, sterile H_2O, UV safety goggles or visor, 50 × TAE buffer, ethidium bromide (see *Protocol 1*)
- Short wavelength UV transilluminator (IBI, 46400) and photographic unit (IBI, 46524)
- DNA sequencing system (Applied Biosystems Automated DNA Sequencer, model 377)

Method

1. Pick medium-to-large sized colonies with sterile toothpicks. In each case touch the toothpick onto an LB agar plate (with kanamycin) to form a master plate. Then inoculate 14 μl sterile water in a 0.4 ml microcentrifuge tube. Incubate the master plate overnight at 32°C.
2. Make up sufficient colony screen reaction mixture (for 20 colonies) by mixing 40 μl of 10 × PCR reaction buffer, 8 μl deoxyribonucleotide mixture, 4 μg each of the forward and reverse primers, 2 μl *Taq* polymerase with sterile water (final volume of 120 μl).
3. Add 6 μl of the colony screen reaction mixture to each inoculation tube. PCR amplify the DNA in each sample as in *Protocol 2,* steps 2–5. Remember to set up positive and negative PCR controls.
4. Add 2 μl of 10 × DNA loading buffer to each tube.
5. Analyse 10 μl of each PCR reaction by agarose gel electrophoresis and ethidium bromide staining as in *Protocol 1,* steps 4–7. Expose the gel to short wavelength UV light using the transilluminator and photograph the DNA bands. **Caution**: protect your eyes with UV goggles or visor.
6. Restreak colonies that yield a fragment of the expected size onto LB agar plates to obtain single colonies.
7. Pick the colonies using toothpicks and inoculate them into LB with kanamycin. Grow them overnight at 32°C.
8. Prepare plasmid DNA using a Qiagen plasmid purification kit as in *Protocol 1.*
9. Sequence the inserted gene in the plasmid DNA using a commercial DNA sequencing system.

DNA sequencing is essential to identify mutations that occasionally are generated during the PCR reaction. However, DNA yields are low with plasmids of this low copy number; 100 ml of culture typically produces sufficient DNA for two to four sequencing runs.

5.2 Expression of the heterologous sequence

Expression from the vector P_L promoter in host strain AR58 is induced by raising the temperature. The higher temperature inactivates the temperature-sensitive cI857 phage lambda repressor carried in the host genome. Induction temperatures in the range 37°C to 42°C allow expression, with maximum product accumulation occurring over a period of 45–180 min. The optimal temperature and time for maximum product level and solubility varies with different gene products.

Protocol 6 describes a general procedure for the growth of recombinant

cells and the induction of expression of the heterologous protein. It can be run on an analytical scale to check the efficiency and authenticity of expression. Alternatively, it can be run on a preparative scale as described in *Protocol 6*; samples are taken for analysis while the bulk of the cells are stored at –70°C to await purification of the heterologous protein once the results of the analysis are known. In this procedure it is desirable to induce a control culture in parallel to check the induction conditions. A useful control strain is *E. coli* transformed with the parent vector, pEA181KnRBS3, containing no foreign gene insert. Such cells will express an 81 amino acid (9 kDa) polypeptide derived from the influenza virus NS1 gene.

Protocol 6. Growth of cells and induction of expression

Equipment and reagents

- Two gyrorotary shaking water-baths (or warm air shakers) set to 32°C and 40°C
- *E. coli* transformed with a recombinant vector consisting of pEA181KnRBS3 containing the desired heterologous gene (see *Protocol 5*)
- *E. coli* control strain transformed with parent vector, pEA181KnRBS3
- LB containing 50 μg/ml kanamycin (see *Protocol 4*)
- Dry ice

Method

1. Grow the recombinant and control *E. coli* strains overnight at 32°C in LB containing kanamycin.
2. Dilute the overnight culture 1:20 into fresh LB containing kanamycin. Grow the cultures at 32°C in a gyrorotary shaker at 250 r.p.m. until the OD_{650} reaches 0.6–0.8.
3. Remove a 1 ml sample from each culture for analysis. Spin these samples for 30 sec at 16000 *g* in a microcentrifuge, decant the medium, then place the microcentrifuge tubes containing the pellets on dry ice.
4. Move the cultures to the 40°C water-bath.[a]
5. Continue growing the cultures at 40°C for 2 h.
6. Remove another 1 ml aliquot from each and process them as in step 3.[b] Record the OD_{650}; typically it will be 1.3 or higher if the gene product is not toxic to the cell.
7. Harvest the remaining cells by centrifugation and freeze them at –70°C until ready for purification of the heterologous protein (Section 5.4).
8. Analyse the retained samples (steps 3 and 6) as described in Section 5.3.

[a] For cultures larger than 100 ml, ensure a rapid rise in temperature to 40°C by adding 1/3 vol. LB with kanamycin which has been pre-warmed to 65°C.
[b] Save a duplicate sample if information on product solubility (see *Protocol 9*) is needed.

5.3 Analysis of expression of the heterologous protein

Expression is coveniently monitored by lysing the cells, solubilizing the proteins with detergent, fractionating the proteins by PAGE, and visualizing the proteins with a protein stain such as Coomassie Brilliant Blue. A product comprising more than 10% of the total cellular protein can usually be visualized in this way. *Protocol 7* describes SDS–PAGE of cellular proteins, and *Figure 2* shows an example.

Protocol 7. Analysis of cellular proteins by SDS–PAGE

Equipment and reagents

- Cell (or protein) samples (frozen) for analysis (e.g. from *Protocol 6*, steps 3 and 6)
- Protein sample buffer: 50 mM Tris–HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 0.1% (w/v) bromophenol blue
- Boiling water-bath
- Rocking platform shaker
- PAGE apparatus that uses pre-cast gels, and a power supply (e.g. the XCell II from Novex)[a]
- Stain solution: 125 ml propan-2-ol, 50 ml acetic acid, 1.25 g Coomassie Brilliant Blue R-250, and water to 325 ml
- Destain solution: 1 litre methanol, 1.4 litres acetic acid, made up to 20 litres with water

Method

1. Add protein sample buffer to the frozen cell pellets. Calculate the volume of protein loading buffer to be added according to the OD_{650} of the culture from which the samples were taken; 100 μl of protein loading buffer is added per OD unit of cells.[b]
2. Vortex the pellets for 5 sec, then immediately place the tubes in a boiling water-bath for 5 min. Vortex again for 10 sec, and centrifuge the tubes in a microcentrifuge for 1 min to pellet any insoluble material. Carefully remove the supernatant (lysate) for analysis.
3. Load 10 μl of the lysates onto the pre-cast gel in accordance with the manufacturer's instructions.
4. Electrophorese the samples towards the positive electrode at 150 V for 1–2 h, until the bromophenol blue dye front approaches the bottom of the gel.
5. Separate the gel plates, remove the gel using gloved hands, immerse it in stain solution, and rock it slowly on a platform shaker for 1 h.
6. Discard the stain solution and replace it with destain solution. Rock the gel on a platform shaker for several hours with two or three changes of destain solution until the areas of the gel lacking protein are free of stain and the stained protein bands are clearly visible.

[a] Pre-cast gels of varying compositions and buffers are commercially available. The manufacturer will recommend different gels and buffers for the different sizes of the proteins to be analysed.
[b] For example, 130 μl is added to a pellet from 1 ml of cells harvested at OD_{650} = 1.3.

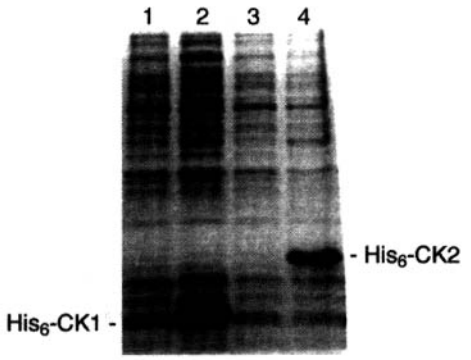

Figure 2. Analysis of heterologous protein expression by SDS–PAGE. *E. coli* cells carrying pEA181KnRBS3-derived vectors encoding His_6-tagged human proteins CK1 (lanes 1, 2) or CK2 (lanes 3, 4) were grown and induced for expression at 40°C as described in Section 5.2. Cell lysates were then analysed on a Coomassie Blue stained gel as described in Section 5.3. Aliquots were taken for analysis at the start of induction (lanes 1, 3) and after 2 h (lanes 2, 4). The induced proteins are visible as major bands in lanes 2 and 4 (labelled His_6-CK1 and His_6-CK2).

Lysates can be also run on a polyacrylamide gel as in *Protocol 7*, but then examined by immunoblotting (Western blot) rather than by staining. Much smaller amounts of the lysates can be run on a gel intended for Western blotting than on a gel intended for staining; one-fourth the amount as is usually sufficient. The protein can be detected with a protein-specific antiserum or monoclonal antibodies, or with an antibody specific for the His_6 tag. *Protocol 8* describes immuno (Western) blotting.

Protocol 8. Detection of expressed protein by immuno (Western) blotting

Equipment and reagents

- Nitrocellulose filter (e.g. Schleicher & Schuell, Protean BA85, 0.45 μm pore size, No. 00860),[a] cut to the same size as the gel (NB: wear plastic or latex disposable gloves to prevent contamination of the nitrocellulose)
- Six pieces of Whatman 3MM paper, cut to the same size as the gel
- Container for incubating and washing the nitrocellulose filter; the plastic lid from a box of micropipetter tips is suitable and will require 20 ml volumes of solutions
- SDS–PAGE gel for analysis (see *Protocol 7*)
- 4 × Tris–glycine buffer: 60 g Tris base, 288 g glycine, water to 5 litres
- Transfer buffer: 250 ml of 4 × Tris–glycine buffer, 200 ml methanol, water to 1 litre
- Western blotting apparatus (Bio-Rad Transblot, or equivalent) and power supply
- PBS: 120 mM sodium chloride, 6 mM potassium phosphate pH 6.8
- Blocking buffer: 0.5% (w/v) gelatin in PBS, autoclaved (120°C, 15 min)
- Wash buffer: 1 g non-fat dried milk, 2.5 g Triton X-100, in 1 litre PBS

- Plastic wrap (e.g. Saran Wrap)
- ECL detection kit (Amersham)
- Primary antibody against the protein to be detected. Dilute this in wash buffer just before use.[b] An antiserum or monoclonal antibody specific for the expressed protein may be used. The concentration of antiserum or antibody required must be determined empirically. Alternatively, use a primary antibody that recognizes the His_6 tag (e.g. 6xHis Monoclonal Antibody; Clontech, 8904–1; dilute this 1:5000 in wash buffer just before use).
- Secondary antibody: a horseradish peroxidase-conjugated antibody that recognizes the primary antibody. Dilute this in wash buffer just before use.[b] If the primary antibody is the 6xHis Monoclonal Antibody, a suitable secondary antibody is the sheep anti-mouse IgG-HRP (Amersham, NA 9XA931) diluted 1:2500 in wash buffer just before use.
- X-ray film, cassette for holding the film during exposure, and an X-ray processor machine (or facilities for developing X-ray film)

Method

NB: wear disposable gloves when handling the gel and nitrocellulose filters.

1. Soak the 3MM paper and nitrocellulose filter in transfer buffer. The nitrocellulose should wet evenly; if it does not, cut a new piece.
2. Remove the SDS–PAGE gel from the glass plates. The stacking gel can be removed or left in place. Rinse the gel briefly in transfer buffer.
3. Place three sheets of the wetted 3MM paper on one side of the blot holder of the Transblot apparatus (the side that will face the negative electrode). Place the gel on the 3 MM paper (either side up). Press a gloved finger wetted in transfer buffer across the top of the gel to remove any bubbles trapped under it. Then place the nitrocellulose filter on the gel. Remove any bubbles again. Place the three remaining 3MM sheets on the nitrocellulose, and again remove any bubbles.
4. Close the blot holder and place it in the apparatus, orientated so transfer will be from the gel through the nicrocellulose towards the positive electrode. Attach the power supply and run the transfer for 2 h at 300 mA.
5. Open the blot holder, remove the nitrocellulose filter, and rinse it twice for 3 min each time in water.[c]
6. Place the nitrocellulose filter (protein side up) in 20 ml blocking buffer for 1 h or overnight.
7. Wash the nitrocellulose filter in 20 ml wash buffer, once for 15 min and then twice for 5 min, changing the buffer between washes.
8. Add the diluted primary antibody to the nitrocellulose filter. Incubate with slow rotation for 60 min.
9. Repeat step 7.
10. Add the diluted secondary antibody. Incubate for 60 min with slow rotation.

Protocol 8. *Continued*

11. Repeat step 7, but add two extra 5 min washes.
12. Place the nitrocellulose filter (protein side up) on plastic wrap. Mix equal volumes of the two ECL detection reagents and immediately pour the mixture onto the nitrocellulose filter (2.5 ml of each is enough for a 7.5 × 7.5 cm filter). Incubate for exactly 1 min. Lift the filter out with tweezers and allow excess fluid to run off.
13. Place the nitrocellulose filter on a fresh piece of plastic wrap and cover the filter with plastic wrap. Immediately expose the filter to X-ray film in a film casette holder, and then develop it in an X-ray processor. Exposure time may vary from 1 sec to 5 min, depending on the amount of protein blotted and the antibody titre.

[a] Other forms of protein binding paper may be used.
[b] The dilutions required for the primary and secondary antibodies should be determined in pilot experiments. For primary antisera, optimal dilutions are typically in the range 1:1000 to 1:50 000; 1:5000 is a good dilution to try first. Secondary antisera usually give good results at a 1:2500–1:5000 dilution.
[c] At this point, the filter can be hung up to dry and stored indefinitely at room temperature. If dried, the filter must be rehydrated in water before restarting the protocol at step 6.

Should it be necessary to determine the solubility of the expressed heterologous protein, follow the procedure described in *Protocol 9*. The presence of the protein in the supernatant or pellet from step 3 will indicate whether it is soluble or insoluble, respectively.

Protocol 9. Determination of product solubility

Equipment and reagents

- Frozen cell pellets from 1 ml of culture (see *Protocol 6*)
- Buffer A: 50 mM Tris–HCl pH 8, 2 mM EDTA, 0.1 mM DTT, 5% (v/v) glycerol
- Lysozyme (Sigma, L-6876)
- Buffer A containing lysozyme: add lysozyme to 0.2 mg/ml shortly before use and mix gently to dissolve it
- Sonicator (Branson Cell Disruptor-350 with cup horn)
- 2 × protein sample buffer: 100 mM Tris–HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 0.2% (w/v) bromophenol blue

Method

1. Place the microcentrifuge tubes containing the cell pellets on ice. Add buffer A containing lysozyme to each cell pellet (0.25 ml per OD_{650} unit of cells at the time of harvest).[a] Vortex the cell suspensions and then incubate them for 20 min on ice.
2. Sonicate the samples twice, 20 sec each time (in pulse mode, 50% duty cycle, at the maximum output setting permitted for the cup horn). Cool the tubes in ice during sonication.

3. Centrifuge the tubes at 16000 *g* in a microcentrifuge for 30 min at 4°C.
4. Remove each supernatant and mix it with an equal volume of 2 × protein sample buffer.
5. Resuspend each pellet in the original volume of buffer A (step 1), and then add an equal volume of 2 × protein sample buffer.
6. Analyse the solubilized samples by SDS–PAGE and Western blotting as in *Protocols 7* and *8*.

[a] For example, add 0.2 ml buffer A if the OD_{650} = 0.8, or 0.3 ml if the OD_{650} = 1.2, and so on.

5.4 Purification of His_6-tagged proteins

Ni-NTA agarose is able to bind a His_6 tag fused to the N- or C-terminus of recombinant proteins. The binding is stable in the presence of denaturants, such as urea or guanidine hydrochloride, which are used to solubilize the protein. The protein can be eluted from the matrix by either lowering the pH (to pH 5.9 or lower) or adding imidazole (which binds to the Ni-NTA and displaces the tagged protein). This procedure (described in *Protocol 10*) typically yields at least 1 mg of protein, most of which consists of the tagged protein, even when the expression level is quite modest, i.e. when only a faint induced band is visible on a Coomassie Blue stained gel of a total cell lysate (*Protocol 7*). In *Protocol 10* the addition of protease inhibitors may be helpful in reducing protein degradation, but their effect on the biological activity of the recombinant protein should be considered and explored in pilot experiments, if necessary.

Protocol 10. Purification of a *soluble* His_6-tagged protein using Ni-NTA agarose[a]

Equipment and reagents

- Cells harvested from a 1 litre culture of *E. coli* cells induced as in *Protocol 6*
- Sonication buffer: 50 mM sodium phosphate, 300 mM NaCl pH 8[b,c]
- Sonicator and lysozyme (see *Protocol 9*)
- Disposable plastic syringe and needle (20 gauge or narrower)
- Ni-NTA agarose (Qiagen, R9605) equilibrated in sonication buffer
- Chromatography column: polypropylene, 20 ml bed volume (Bio-Rad, 732–1010)
- Wash buffer: 50 mM sodium phosphate, 300 mM NaCl, 30 mM imidazole pH 8[b,c]
- Elution buffer: 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole pH 8[b]
- Protein sample buffer and materials for SDS–PAGE (see *Protocol 7*)

Method

1. If the cells have been stored at –70°C, thaw them for 15 min in ice.
2. Resuspend the cells in 10 ml sonication buffer.

Protocol 10. *Continued*

3. Add lysozyme to 0.2 mg/ml (final concentration) and incubate the cells in ice for 30 min.
4. Sonicate the cells in ice (as in *Protocol 9*, step 2).
5. If the lysate is very viscous, draw the lysate through a 20 gauge syringe needle several times to shear the DNA.
6. Centrifuge the lysate at 10 000 *g* (or higher) for 20 min at 4 °C, and collect the supernatant.
7. Add 8 ml of a 50% (v/v) slurry of Ni-NTA agarose (equilibrated in sonication buffer) to the supernatant. Stir the mixture on ice for 60 min.
8. Load the agarose into the chromatography column. Run the column at room temperature. Wash the column with 20 ml wash buffer and collect 5 ml fractions. Read the A_{280} of the wash fractions. If necessary, wash the column with more wash buffer until the A_{280} of the flow-through is 0.01 or less.
9. Elute the protein from the agarose with 20 ml elution buffer. Collect 2 ml fractions.
10. Analyse 5 μl aliquots of the fractions by SDS–PAGE. To do this incubate the aliquots for 10 min in protein sample buffer at 37 °C instead of boiling them to avoid imidazole-mediated cleavage of labile peptide bonds.
11. Load the samples on the gel and proceed as in *Protocol 7*.

[a] This protocol is adapted from protocols supplied with the Qiagen, Inc. QIAexpress™ system.
[b] Sonication buffer, wash buffer, and elution buffer can be supplemented with non-ionic detergents such as NP-40, with protease inhibitors, with low concentrations of reducing agents (0.1 mM DTT or up to 20 mM of 2-mercaptoethanol), or with glycerol (up to 50%, v/v) if necessary to improve purification. Addition of protease inhibitors (such as Protease Inhibitor Cocktail, Boehringer Mannheim, 1697 498) often reduces proteolysis but may affect subsequent biological assays of the purified product.
[c] The sonication and wash buffers can be made with a range of imidazole concentrations (5–10 mM for the sonication buffer, 5–40 mM for the wash buffer) to improve removal of co-purifying contaminant proteins. The optimal imidazole concentration varies with different expressed proteins and must be determined empirically.

If the heterologous protein is expressed in an insoluble form, it must be solubilized before purification. This is described in *Protocol 11*. Most insoluble proteins can be solubilized in 6 M guanidine hydrochloride, but 8 M urea, a milder denaturant, may be substituted in many cases. Fractions containing urea can be analysed directly by SDS–PAGE, whereas samples containing guanidine hydrochloride must be diluted with water at least sixfold to prevent the formation of precipitates in the presence of SDS. In *Protocol 11* guanidine hydrochloride is used for the initial solubilization, and urea is then substituted in subsequent purification steps. All steps of the protocol should be performed at room temperature unless otherwise indicated. As discussed in Section 2.3.4,

conditions for removal of the denaturant and refolding of the protein must be determined empirically for each protein, and are not readily amenable to a generalized protocol. *Figure 3* shows an example of the purification of an insoluble His_6-tagged protein using Ni-NTA agarose.

Protocol 11. Purification of *insoluble* His_6-tagged protein using Ni-NTA agarose[a]

Equipment and reagents

- Cells and chromatography column (see *Protocol 10*)
- Buffer AQ: 6 M guanidine–HCl, 0.1 M sodium phosphate, 0.01 M Tris–HCl pH 8[b]
- Ni-NTA agarose resin (Qiagen, R9605) equilibrated in buffer AQ
- Buffer B: 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris–HCl pH 8[b,c]
- Buffer C: 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris–HCl pH 6.3[b,c]
- Buffer C containing 250 mM imidazole
- Materials for SDS–PAGE (see *Protocol 7*)

Method

1. If the cells have been stored at –70°C, thaw them for 15 min in ice.
2. Resuspend the cells in 10 ml buffer AQ. Stir the cell suspension for 1 h at room temperature.
3. Centrifuge the lysate at 10000 *g* for 15 min at 4°C. Collect the supernatant.
4. Add 8 ml of a 50% (v/v) slurry of Ni-NTA agarose (equilibrated in buffer AQ) to the supernatant. Stir at room temperature for 45 min, then load the agarose carefully into the chromatography column.
5. Wash the column with 40 ml buffer AQ, and then 20 ml buffer B. Read the A_{280} of the final washings. If necessary, wash the column with more buffer B until the A_{280} of the flow-through is 0.01 or less.
6. Wash the column with buffer C until the A_{280} is < 0.01.
7. Elute the protein with 50 ml buffer C containing 250 mM imidazole. Collect 5 ml fractions, and analyse 5 μl aliquots by SDS–PAGE (see *Protocol 7*). Incubate aliquots for the gel for 10 min at 37°C instead of boiling to avoid imidazole-mediated cleavage of labile peptide bonds.

[a] This protocol is adapted from protocols supplied with the Qiagen, Inc. QIAexpress™ system.
[b] Buffers AQ, B, and C may be supplemented with 5–40 mM imidazole to improve removal of co-purifying contaminant proteins. The optimal imidazole concentration varies with different expressed proteins and must be determined empirically.
[c] 8 M urea is used instead of 6 M guanidine–HCl in buffers B and C because 6 M guanidine–HCl precipitates in the presence of SDS, which makes the subsequent SDS–PAGE analysis difficult. Fractions which contain guanidine–HCl must be either diluted 1:6 with water before analysis or separated from the guanidine–HCl by dialysis or TCA precipitation.

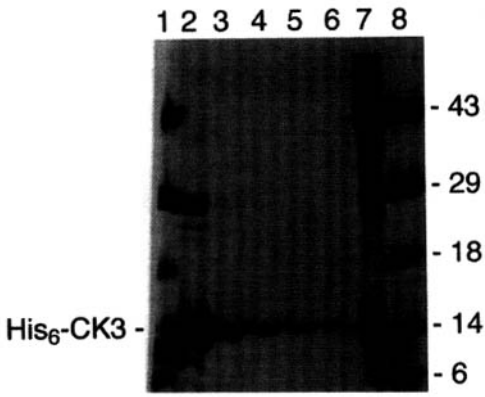

Figure 3. Purification of insoluble His_6-tagged protein using Ni-NTA agarose. *E. coli* cells expressing His_6-tagged human protein CK3 were induced and lysed with guanidine hydrochloride. The lysate was mixed with Ni-NTA agarose, and the bound proteins were then eluted with 250 mM imidazole as described in Section 5.4. Aliquots from the first five column fractions are shown in a Coomassie Blue stained SDS–PAGE gel (lanes 2–6). Unpurified total cell lysate is shown for comparison in lane 7. Molecular weight markers (indicated by mass in kDa) were run in lanes 1 and 8. Most of the His_6-CK3 protein eluted in fraction 1 (lane 2). Both a monomeric (14 kDa) CK3 major band (labelled His_6-CK3) and a dimeric (28 kDa) minor band (unlabelled) are visible in this fraction. The identities of the monomeric and dimeric forms were confirmed by Western blot analysis which showed immunoreactivity of both forms with anti-His_6 monoclonal antibody (data not shown). Comparison of lane 2 with lane 7 indicates that the purification procedure provided a great enrichment for His_6-CK3, but that relatively low levels of some co-purifying contaminant proteins (non-reactive with anti-His_6 antibody; data not shown) were detectable in this fraction.

6. Expression of heterologous proteins in a secretion system

Expression of a secreted protein employs the same basic vector system as the cytoplasmic expression protocols in Section 5. However the coding sequence is modified to fuse a secretion signal sequence to the N-terminus of the expressed protein. For example, the signal sequence shown below is the 22 amino acid *pelB* signal sequence, which has been successfully used to drive the secretion of Fab antibody fragments and other gene products by *E. coli* (14). A DNA fragment encoding an *Nde*I cloning site (underlined in the sequence below) and the signal sequence in-frame with the coding region of the protein of interest may be constructed by conventional cloning strategies, and this fragment may then be cloned as an *Nde*I-*Xba*I fragment into a pAS or pSKF vector such as pEA181KnRBS3 as in *Protocol 2*.

The *pelB* signal sequence coding region and the first codon (designated XXX) of the gene encoding the secreted product will therefore be:

```
      Met  Lys  Tyr  Leu  Leu  Pro  Thr  Ala  Ala  Ala  Gly  Leu  Leu
CAT   ATG  AAA  TAC  CTA  TTG  CCT  ACG  GCA  GCC  GCT  GGA  TTG  TTA

Leu   Leu  Ala  Ala  Gln  Pro  Ala  Met  Ala  Xxx
TTA   CTC  GCT  GCC  CAA  CCA  GCC  ATG  GCC  XXX
```

The *E. coli* secretory machinery can accommodate most amino acid residues at the Xxx position shown above (15), and will lead to a correctly processed secreted product with an Xxx N-terminus. Gene products secreted by *E. coli* generally are found in the periplasm, but in some cases may accumulate in the extracellular growth medium (14), especially after long induction periods. *Protocol 12* describes procedures for inducing expression, for determining whether the product is in the cells or in the medium, and for determining the solubility of any product found in the cell. *Protocol 13* describes a small scale osmotic shock procedure for determining whether the intracellular protein is in the cytoplasm or in the periplasm and is in a soluble form. The periplasmic fraction is a potentially enriched source of a secreted protein. The small scale osmotic shock may be scaled up to provide material for purification (*Protocols 10* and *11*). Purification of soluble proteins can be carried out as described in *Protocol 10*, starting with cell pellets, periplasmic material generated by osmotic shock, or extracellular medium.

Protocol 12. Expression from a vector carrying a signal sequence for directing secretion

Equipment and reagents

- AR58 host strain carrying a pAS or pSKF vector with the *pelB* gene fusion (see text)
- Dry ice
- Materials for SDS–PAGE (see *Protocol 7*) and Western blotting (see *Protocol 8*)

Method

1. Induce the cells by temperature shift to 40°C as in *Protocol 6*, steps 1–4.
2. Continue growing the culture at 40°C for 5 h, removing triplicate 1 ml aliquots at 1, 2, and 5 h after induction.
3. Use one aliquot to determine whether the protein is in the cells (cytoplasm plus periplasm) or secreted into the medium. Spin the 1 ml aliquot for 30 sec in a microcentrifuge at 16000 *g*. Save 500 μl of the supernatant in a separate tube, and freeze it on dry ice. Remove and discard the rest of the supernatant from the pellet, and freeze the cell pellet on dry ice.
4. Analyse the samples of supernatant and cell pellet (step 3) by SDS–PAGE (see *Protocol 7*) followed by Western blotting (*Protocol 8*). The appearance in the cell pellet sample of two immunopositive bands

Protocol 12. *Continued*

differing in size by about 2 kDa most likely indicates that the signal sequence was not removed from all of the expressed protein. The unprocessed protein, if detectable at all, is usually insoluble and will be membrane associated or in the cytoplasm rather than in the periplasmic compartment.

5. Examine the solubility of the cell-associated material in the second aliquot of cells (step 2). Process the sample to obtain the cell pellet (step 3) and analyse it as in *Protocol 9*.
6. Use the third aliquot (step 2) to determine if the cell-associated product is in the periplasm or the cytoplasm/membrane fraction. This procedure should be carried out with freshly harvested cells, not frozen cell pellets, so proceed as in step 3 but do not freeze the cell pellet. Subject the cells to osmotic shock (*Protocol 13*).

Protocol 13. Osmotic shock procedure for the localization of the expressed protein in the periplasm or the cytoplasm/membrane fraction[a]

Equipment and reagents

- Freshly harvested (not frozen) cell pellets from 1 ml culture (*Protocol 12*, step 3)
- Sucrose/Tris: 10 mM Tris–HCl pH 7.6, 20% (w/v) sucrose (ice-cold)
- 0.5 M EDTA pH 8
- 1 M Tris–HCl pH 7.6 (ice-cold)
- Buffer A (see *Protocol 9*)
- 2 × protein sample buffer (see *Protocol 9*)
- Materials for SDS–PAGE (see *Protocol 7*) and Western blotting (see *Protocol 8*)

Method

1. Add 0.15 ml ice-cold sucrose/Tris to the cell pellet. Vortex well. Incubate on ice.
2. After 5 min, add 5 μl of 0.5 M EDTA pH 8. Continue incubation for 10 min on ice.
3. Centrifuge at 16000 *g* for 5 min at 4°C. Collect the supernatant ('sucrose fraction'). Mix a 15 μl aliquot with 85 μl buffer A and 100 μl of 2 × protein sample buffer.
4. Resuspend the cell pellet in 0.1 ml ice-cold water and incubate on ice for 10 min.[b]
5. Centrifuge at 16000 *g* for 10 min. Collect this supernatant ('water fraction'). Mix a 10 μl aliquot with 90 μl buffer A and 100 μl of 2 × protein sample buffer.
6. Resuspend the cell pellet in 0.5 ml ice-cold 1 M Tris–HCl pH 7.6 and incubate in ice for 30 min.[c]

7. Centrifuge at 16000 *g* for 10 min. Collect this supernatant ('1 M Tris–HCl fraction'). Mix a 50 μl aliquot with 50 μl of 2 × protein sample buffer.
8. Resuspend the final pellet by vortexing vigorously in 0.5 ml buffer A, and mix a 50 μl aliquot with 50 μl of 2 × protein sample buffer for analysis.
9. Analyse 20 μl each of the sucrose fraction (step 3), water fraction (step 5), 1 M Tris–HCl fraction (step 7), and the final pellet (step 8) by SDS–PAGE and Western blotting (*Protocols 7* and *8*). The sucrose, water, and 1 M Tris–HCl fractions are all operationally defined as periplasmic preparations.

[a] Based on ref. 16.
[b] This step shocks the cells osmotically, disrupting the outer membrane and forcing the release of periplasmic proteins.
[c] This high ionic strength treatment sometimes releases proteins that are 'insoluble' in earlier steps due to aggregation or weak adherence to cellular components.

7. Sources of information on expression systems

Detailed protocols for the use of the commercially available expression systems listed in *Table 1* are available from the manufacturers. World Wide Web sites (see *Table 1*) are particularly convenient sources of information such as vector maps and sequences, and specific suggestions and sources of reagents for their use. These Web sites are regularly updated as new features are added to these systems. Some manufacturers also publish protocols in hard copy, or supply diskettes with information such as vector DNA sequences.

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7

Cell-free coupled transcription–translation systems from *Escherichia coli*

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1. Background

1.1 Bacterial cell-free expression systems

Development of a bacterial cell-free coupled transcription–translation system in which a specific polypeptide was synthesized and could be identified was pioneered by Zubay (1). *E. coli* genomic DNA or bacteriophage DNA was used as the template for protein expression. Zubay (1) also pointed out that *E. coli* extracts are unsuitable for translation of isolated mRNA because of their rapid degradation. This statement still holds true despite the development of RNase-deficient *E. coli* strains.

The development of plasmid expression vectors provided the basis for a large improvement over the system described by Zubay. High yield expression of only one specific protein is possible by the use of such engineered plasmids. It should be emphasized, however, that the synthesis of a given protein depends to a large extent on the specific plasmid and construct that are used. Identical coding sequences may give very different amounts of protein in different plasmids. Secondly, the expression of each coding sequence from a given plasmid, as well as the expression of different coding sequences from the same plasmid, may require slightly different conditions for optimal expression in the coupled transcription–translation system.

The preparation of the *E. coli* extract deficient in DNA and mRNA is carried out essentially as described by Zubay (1). The *E. coli* extract prepared for coupled transcription–translation (designated S30) can be further fractionated by ultracentrifugation to yield crude ribosomes. The resuspended ribosome fraction contains all components necessary for efficient translation (2). This fraction has the advantage of having lower levels of many *E. coli* soluble degradative enzymes. Further extraction of the crude ribosomes by salt washing results in salt washed ribosomes and a fraction rich in translational activity.

This latter fraction can be subjected to size exclusion chromatography to make it deficient in molecular chaperones (3).

In this chapter we present detailed protocols for the preparation of the *E. coli* S30 and fractions derived from it, as well as descriptions of the other components necessary for efficient protein synthesis by coupled transcription–translation.

1.2 Usefulness of cell-free coupled transcription–translation systems

In vitro expression of coding sequences engineered into plasmids has a variety of applications:

(a) They are especially useful in those cases where *in vivo* expression is impractical or not possible. Examples include toxicity to the host cell (4), formation of inclusion bodies (5, 6), or rapid proteolytic degradation of the product formed (7).

(b) Since the cell-free system has the advantage of allowing a labelled amino acid to be incorporated into a single protein, the amount of the protein synthesized and its specific activity can be easily determined. This may greatly facilitate the determination of specific enzymatic activities in a large number of samples, such as encountered in screening the effects of amino acid changes on the specific activity of an enzyme.

(c) Coupled transcription–translation can be used for rapid screening of mutagenized sequences where *in vivo* screening would have required much more material and would have been time-consuming. '*In vitro* scanning saturation mutagenesis' has been coined as the descriptive term for this method (8). In these situations, PCR products can be used directly in the *in vitro* coupled transcription–translation assay. Examples of applications of this technology include the investigations of mutagenizing coding sequences, such as single chain antibody coding sequences (8), and the effects of mutagenizing sequences in the 5′ and 3′ untranslated regions (UTRs) of mRNAs on the translatability of the mRNA. In an extension of this type of analysis, we have constructed synthetic tRNAs and tested their effects as suppressor tRNAs (9).

(d) The folding of nascent polypeptides has also been studied and the effects that molecular chaperones may have on this process were analysed (10–12).

(e) Another important application of the *E. coli* cell-free system is the co-translational incorporation of a fluorophore at the N-terminus of the synthesized protein. This is done by using methionine, derivatized at its amino group with coumarin, in initiator Met-tRNA, i.e. N-(coumarin)-Met-$tRNA_f$ rather than fMet-$tRNA_f$, to start protein synthesis (12, 13). This method can only be applied in the bacterial cell-free system, but both

prokaryotic and eukaryotic derivatized proteins can be synthesized in high yield and used subsequently in fluorescence experiments (11–14; and unpublished results).

It is important to have an assay specific for the protein synthesized that will not be affected by other components in the cell extract used in the coupled transcription–translation. In ref. 8 this was done by ELISA but has been achieved in other situations by enzyme assays (9–11) (Section 3.2.5). The details of the assay system to be used and analysis of the product will depend on the particular research questions asked. Methods of analysing the product formed as well as representative results will be given in subsequent sections.

2. Preparation of extracts and components for coupled transcription–translation systems

All components needed for coupled transcription–translation can be prepared in large batches and stored (in most cases frozen) in small aliquots until they are used. The quality of the results obtained depends to a large degree on the proper preparation of these components. The protocols described for these preparations (Sections 2.2–2.6) include cautionary notes to avoid pitfalls.

Protein synthesis is carried out on ribosomes aided by associated translation factors. For coupled transcription–translation, a plasmid carrying the appropriate coding sequence under the control of a bacteriophage promoter and the necessary bacteriophage RNA polymerase are added exogenously.

2.1 Preparation of the bacterial cell-free extract (S30)

The most commonly used cell-free system and easiest to prepare is the S30 fraction, an *E. coli* extract depleted in DNA and mRNA. It is important that the cells used to prepare the S30 are grown in rich medium and are harvested in mid-log phase. *Protocol 1* describes how this is done. The cells can be stored frozen until the S30 is prepared as described in *Protocol 2*.

Protocol 1. Growth of cells for S30 extract

Equipment and reagents

- Luria broth (LB; Sigma), sterilized by autoclaving
- 37°C shaking incubator
- Centrifuge (e.g. Beckman J2-21 with a JA-14 rotor) and corresponding centrifuge tubes
- 4 litre Erlenmeyer flasks each containing 2 litres autoclaved LB
- 20% (w/v) glucose (sterile)
- 2 litre glass Erlenmeyer flask containing 500 ml autoclaved LB
- Bacteriological culture tubes with 5 ml autoclaved LB
- LB agar plate with *E. coli* K12 strain A19[a]
- Solution 1: 10 mM Tris–acetate pH 8.2, 14 mM magnesium acetate, 60 mM KCl, 6 mM 2-mercaptoethanol
- Saran Wrap and aluminium foil

Protocol 1. *Continued*

Method

1. Inoculate 5 ml LB in a bateriological tube with a single colony from the LB agar plate and incubate with vigorous shaking at 37°C for about 8 h.
2. Transfer the culture to 500 ml LB containing 0.4% glucose in a 2 litre flask. Incubate overnight with shaking at 37°C.
3. Next morning, determine the A_{600} of a 1:10 dilution of the overnight culture.
4. Inoculate four 4 litre flasks containing 2 litres LB with 0.4% glucose from the overnight culture to give a calculated A_{600} of about 0.05.
5. Incubate at 37°C with vigorous shaking.
6. Monitor the growth by measuring the A_{600} of 1 ml samples taken hourly for 3 h and then at 30 min intervals.
7. Quickly stop growth in mid-log phase (A_{600} = 0.75) by placing the culture flasks in iced water.
8. Centrifuge for 15 min at 6000 r.p.m. at 4°C.
9. Resuspend cell pellets in cold solution 1 (pool all cells from the 8 litres of culture in about 500 ml solution 1).
10. Centrifuge as in step 8.
11. Scrape cell pellets as a wet paste onto Saran Wrap on aluminium foil. Determine the weight and freeze the cells at –70°C.[b]

[a] Preferably freshly prepared, not older than one month.
[b] The yield of cells should be about 50 g.

Protocol 2. Preparation of S30

Equipment and reagents

- 50 g frozen cells (prepared as in *Protocol 1*)
- Centrifuge (e.g. Beckman J2-21, with JA-14 and JA-20 rotors or equivalent) and corresponding centrifuge tubes
- French press and cell (that holds about 40 ml)
- 1 M dithiothreitol (DTT)[a]
- 75 mM phenylmethylsulfonyl fluoride (PMSF) in propan-2-ol[a]
- Solution 1 (see *Protocol 1*)
- Solution 2: solution 1, containing 1 mM DTT in place of the 2-mercaptoethanol
- Solution 3: 75 mM Tris–acetate pH 8.2, 7.5 mM DTT, 21 mM magnesium acetate, 500 μM of each of all 20 L-amino acids, 6 mM ATP, 67 mM phosphoenolpyruvate (PEP), 20 μg/ml pyruvate kinase—make this fresh just before use from stock solutions (at least 5 × concentrated) stored individually at –20°C

Method

NB: keep the cells and lysate on ice as much as possible, and use cold solutions.

1. Partially thaw the frozen cells for 30 min at 4°C and resuspend them in 500 ml solution 1.

2. Centrifuge the cell suspension for 15 min at 8000 r.p.m. in the JA-14 rotor.
3. Resuspend the cell pellet in 200 ml solution 1 and recentrifuge.
4. Resuspend cell pellet in 65 ml solution 2 and add 75 mM PMSF to a final concentration of 0.5 mM.[b]
5. Break the suspended cells in the French press (maximum pressure 800 p.s.i.).
6. Immediately add DTT to lysate (1 μl of 1 M DTT/ml lysate).
7. Pass the lysate through the French press again as in step 5.
8. Centrifuge lysate for 30 min at 16000 r.p.m. in the JA-20 rotor.
9. Recover the supernatant and recentrifuge it as in step 8.
10. Mix the supernatant with 8 ml fresh solution 3.
11. Incubate the mixture in a light-protected vessel at 37°C for 80 min.
12. Dialyse the mixture against solution 2 (first against 500 ml for about 2 h, then against a fresh 500 ml for about 3 h, then against 1 litre overnight).
13. Centrifuge the dialysed lysate as in step 8.
14. Freeze the supernatant rapidly in liquid nitrogen in small aliquots (0.1–1 ml).

[a] Store these stock solutions at –20°C.
[b] PMSF is unstable in aqueous solution ($t_{1/2}$ ~ 30 min or less above pH 8), so break the cells as soon as possible after the addition of PMSF.

2.2 Construction and preparation of plasmids

2.2.1 Construction of plasmids

For coupled transcription–translation, we have used expression vectors into which the coding sequence is inserted under the T7 promoter or the SP6 promoter. For efficient translation, a strong Shine–Dalgarno (ribosome binding) sequence is required about five to ten base pairs upstream from the ATG codon where translation of the transcribed mRNA will begin. This sequence can either be part of the natural 5′ UTR of the gene to be transcribed and translated, or, in a commercially available vector, upstream of an *Nco*I or *Nde*I restriction site into which the coding sequence with its translation start codon is ligated.

Since we do not linearize the plasmid used in the coupled transcription–translation assay (2), we have obtained the best results when the ampicillin resistance gene (the coding sequence for β-lactamase) is localized in the opposite direction on the strand from which the coding sequence of interest is transcribed, for example pGEM-3Z (Promega) with the coding sequence

under the control of the T7 promoter or in SP65 (Promega) under the control of the SP6 promoter.

2.2.2 Preparation of plasmid DNA

There are many procedures available to purify plasmids from *E. coli* cells. It is essential that RNases are not used in the procedure. The method we use for preparing a typical plasmid is described in *Protocol 3*. We obtain the best results with *E. coli* strain XLIB as the host.

Protocol 3. Large scale plasmid preparation

Equipment and reagents

- LB in bacteriological culture tubes and 4 litre flasks (see *Protocol 1*)
- Autoclaved distilled H_2O
- Ampicillin (Sigma): 100 mg/ml in autoclaved H_2O
- Sterile disposable syringes (10 ml)
- 0.25 μm filters (Schleicher & Schuell) for use with disposable syringes
- *E. coli* strain XLIB
- Beckman J2-21 centrifuge (or equivalent), with JA-14 and JA-20 rotors, and plastic centrifuge tubes
- Corex glass bottles (150 ml) and glass centrifuge tubes (30 ml) with adapters for J2-21 centrifuge
- Lysozyme (Sigma)
- Tris/EDTA stock solution: 0.25 M Tris–HCl pH 7.8, 0.1 M EDTA
- Tris/EDTA/sucrose: 25 mM Tris–HCl pH 7.8, 10 mM EDTA, 15% (w/v) sucrose—make this fresh using the Tris/EDTA stock solution
- NaOH/SDS: 0.2 M NaOH containing 1% (w/v) SDS
- 3 M sodium acetate pH 4.8
- Tris/EDTA buffer: 10 mM Tris–HCl pH 7.6, 1 mM EDTA
- 96% (v/v) ethanol at –20°C
- 8.4 M ammonium acetate
- 0.3 M NaCl/MOPS: 50 mM MOPS–NaOH pH 6.8, 0.3 M NaCl, 15% (v/v) ethanol
- 1 M NaCl/MOPS: 50 mM MOPS–NaOH pH 6.8, 1 M NaCl, 15% (v/v) ethanol
- EconoPac Q cartridge (Bio-Rad): before use equilibrate it in 0.3 M NaCl/MOPS applied by using a syringe and 0.25 μm filter
- 1 M NaOH

Method

1. Inoculate 5 ml LB containing 50 μg/ml ampicillin (2.5 μl of the 100 mg/ml ampicillin stock solution) with the *E. coli* cells containing the plasmid of interest. Grow this culture at 37°C with shaking for 6–8 h.
2. Add 1 ml ampicillin (100 mg/ml) to 2 litres LB in a 4 litre flask. Inoculate the flask with the 5 ml culture. Let the cells grow overnight at 37°C with shaking.
3. Harvest the cells in plastic centrifuge tubes at 5000 r.p.m. in the JA-14 centrifuge for 15 min.
4. Resuspend and combine all the cell pellets in one tube in 50 ml Tris/EDTA/sucrose solution using a glass Pasteur pipette.
5. Add 0.1 g lysozyme. Mix slowly and incubate for 20 min on ice.
6. Add 50 ml NaOH/SDS and incubate for 10 min on ice.
7. Add 25 ml of 3 M sodium acetate pH 4.8. Gently mix by inversion and incubate for 20 min on ice.

8. Carefully transfer the highly viscous solution into plastic centrifuge tubes for the JA-20 rotor. Centrifuge for 20 min at 17 000 r.p.m.
9. Decant and save the supernatant, carefully avoiding the viscous DNA and white denatured protein.
10. Divide the supernatant between two 150 ml Corex glass bottles, then add 2.5 vol. ethanol. Mix, and incubate at −20°C for at least 1 h.
11. Centrifuge for 30 min at 5000 r.p.m. in the JA-14 rotor.[a]
12. Resuspend the pellets in Tris/EDTA buffer to give a final volume of 15 ml. Transfer the suspension to a 30 ml glass Corex tube.
13. Add 6 ml 8.4 M ammonium acetate, mix, and incubate for 10 min on ice.
14. Centrifuge (with adapter) in the JA-20 rotor for 20 min at 10 000 r.p.m.[a]
15. Divide the supernatant between two 30 ml Corex glass tubes and add 2 vol. ethanol. Mix, and keep it at −20°C for at least 1 h.
16. Centrifuge the mixture in the JA-20 rotor for 30 min at 10 000 r.p.m.
17. Resuspend the pellet in 3 ml 0.3 M NaCl/MOPS.
18. Slowly load the plasmid preparation onto the EconoPac Q cartridge using a 10 ml syringe without filter.
19. Collect the flow-through at about 2 ml/min in 1 ml aliquots. Wash the cartridge with 0.3 M NaCl/MOPS until about ten fractions are obtained.
20. Determine the A_{260} and A_{280} of each fraction. Combine the three or four fractions with the highest A_{260} values (usually between 1–2 A_{260} units/ml, starting with fractions three or four).
21. Combine the fractions and add 2.5 vol. 96% ethanol. Mix, and keep at −20°C for at least 4 h to precipitate the DNA.
22. Centrifuge the precipitated DNA as in step 16. Resuspend it in 300–500 μl autoclaved H_2O.
23. Dilute 5 μl of the DNA into 0.795 ml H_2O. Measure the A_{260}, and calculate the DNA concentration assuming that 1 A_{260} U/ml is equivalent to 50 μg DNA/ml.
24. Regenerate the EconoPac Q cartridge immediately after use according to the manufacturer's instructions, by washing successively with 1 M NaOH, distilled H_2O, 1 M NaCl/MOPS, and 0.3 M NaCl/MOPS.[b]

[a] Tubes break at higher speeds.
[b] If treated in this way, the cartridge can be reused at least ten times.

The yield of plasmid DNA when using *Protocol 3* is usually about 2–3 mg, but can be as high as 5–6 mg. It is recommended that the purity of the plasmid obtained is analysed by agarose gel electrophoresis in the presence of ethidium bromide. Good quality preparations consist predominantly of super-

coiled DNA with no RNA contamination. It is critically important not to include RNase treatment in the preparation procedure.

2.3 Preparation of RNA polymerases

In the coupled transcription–translation assay, endogenous *E. coli* RNA polymerase is suppressed by the addition of rifampicin (15) (Section 3.1). The coding sequence of interest is transcribed from the plasmid by either T7 or SP6 RNA polymerase (depending on which promoter is in the correct orientation for the cloned sequence). Both of these RNA polymerases are available commercially. However, the SP6 RNA polymerase is approximately five times more expensive than T7 RNA polymerase. Therefore, if we wish to use the SP6 RNA polymerase, we prepare it according to the procedure described in *Protocol 4*, which is a modification of a procedure described by Davanloo *et al.* (16) for the isolation of T7 RNA polymerase.

Protocol 4. Preparation of SP6 RNA polymerase

Equipment and reagents

- *E. coli* strain DH5α with the coding sequence for the SP6 RNA polymerase inserted into the genome (this may be obtained from Dr A. Spirin, Russian Academy of Sciences, Pushchino, Moscow Region, Russia)
- LB, bacteriological culture tubes, and three 2 litre flasks containing LB (see *Protocol 1*)
- Ampicillin stock solution (see *Protocol 3*)
- Beckman J2-21 centrifuge with JA-14 and JA-20 rotors (or equivalent), plus centrifuge tubes
- Solution A: 50 mM Tris–HCl pH 7.8, 100 mM NH_4Cl, 1 mM EDTA
- Solution B: 50 mM Tris–HCl pH 8.1, 20 mM NaCl, 2 mM EDTA, 1 mM DTT
- Solution C: 20 mM phosphate pH 7.7, 50 mM NaCl, 1 mM EDTA, 1 mM DTT
- Solution D: as solution C, but containing 250 mM NaCl
- Solution E: 20 mM phosphate pH 7.7, 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% (v/v) glycerol
- Solution F: solution E with 0.4 M NaCl
- Solution G: 20 mM Tris–HCl pH 7.7, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol
- 75 mM PMSF (see *Protocol 2*)
- Lysozyme (15 mg/ml in H_2O)
- 4% (w/v) sodium deoxycholate (DOC)
- Solid ammonium sulfate: grind it to a powder in a mortar
- 2 M ammonium sulfate
- Polymin P (polyethyleneimine, Sigma): this is sold as a 50% aqueous solution—adjust the pH to ~ 8 with concentrated HCl, and then dilute it with distilled H_2O to 10% final concentration
- S-Sepharose (Sigma): make a 30 ml bed volume column and equilibrate it in solution C; check the pH is 7.7 just before use
- Q-Sepharose (Sigma): make a 10 ml bed volume column and equilibrate it in solution E; check the pH is 7.7 just before use
- Fraction collector, chromatography columns, gradient former
- Autoclaved tubes for storing the polymerase at –20°C

Method

NB: the volumes of solutions used in the procedure are for 20 g (wet weight) of *E. coli* cells, the usual yield in steps 1–2.

1. Grow and harvest the *E. coli* cells as described in *Protocol 3*, steps 1–3, but start with three 5 ml cultures (step 1) and then inoculate three 2 litre cultures (step 2).

2. Wash the cells with 200 ml solution A. Centrifuge again as in *Protocol 3*, step 3.
3. Resuspend the cell pellets in 48 ml solution B, add 0.7 ml PMSF, and 1.1 ml lysozyme. Incubate for 30 min at 37°C.
4. Add DOC to 0.04% final concentration. Incubate for 45 min on ice.
5. Add 10 ml of 2 M ammonium sulfate, then add 12 ml of 10% Polymin P slowly with stirring. Stir on ice for 20 min.
6. Centrifuge in the JA-20 rotor for 15 min at 16000 r.p.m.
7. Decant the supernatant and measure its volume.
8. Stir the supernatant on ice while slowly adding solid ammonium sulfate to give 45% saturation.[a] Stir on ice for another 10 min.
9. Centrifuge as in step 6. Discard the supernatant and save the pellet.
10. Resuspend the pellet in a minimum volume of solution C and dialyse it twice against two 2 litre lots of solution C for at least 4 h each.
11. If a precipitate has formed, centrifuge as in step 6. Decant and save the supernatant.
12. Load the supernatant onto the S-Sepharose column.
13. Wash the column with solution C until all non-adsorbed material has been removed ($A_{280} < 0.05$).
14. Elute adsorbed protein with solution D. Collect fractions of about 2.5 ml.
15. Determine the A_{280} of the fractions and combine those with $A_{280} > 0.3$.
16. Dialyse the eluate (combined fractions from step 15) twice against 1 litre each of solution C.[b]
17. Load the dialysed sample onto the Q-Sepharose column.
18. Wash column with about four column volumes of solution E.
19. Apply a linear gradient (about 80 ml total volume) of solution E to solution F.
20. Combine the earliest fractions with high A_{280} (which will elute at around 100 mM NaCl) and dialyse this against three 1 litre lots of solution G (each for at least 5 h).
21. Determine the protein concentration of the RNA polymerase preparation from the A_{280}/A_{260}. Store the preparation in aliquots in autoclaved tubes at –20°C.[c]

[a] Remember that the suspension already contains ammonium sulfate from step 5.
[b] The enzyme may precipitate if the NaCl concentration is less than 25 mM.
[c] The glycerol prevents the solution from freezing, which is detrimental to the enzyme.

2.4 Preparation of the low molecular weight mix (LM) for coupled transcription–translation

The low molecular weight components required for the coupled assay are prepared as indicated in *Protocol 5*. We usually make a 10 ml batch and store it frozen at –70°C in 100 µl aliquots. The composition of LM is summarized in *Table 1*. Since the coupled assay itself (Section 3.1) uses radioactive leucine to label the expressed protein, LM contains all the normal amino acids except leucine.

Table 1. Composition of the low molecular weight mixture (LM) for coupled transcription–translation

Component	Concentration (mM, except where indicated)
Tris–acetate	165
Potassium acetate	216
Ammonium acetate	108
Amino acids (except leucine)	1.5 (each amino acid)
PEG	6%
DTT	6
ATP	3.6
GTP	2.4
CTP	2.4
UTP	2.4
PEP	91
G-6-P	1
cAMP	1.2
EDTA	1.5
FA	0.1 mg/ml

Protocol 5. Preparation of the low molecular weight mix (LM) for coupled transcription–translation

Reagents

- Leucine-minus amino acid mixture: the 19 natural amino acids except leucine (each at 5 mM); add 1 M DTT to 0.5 mM final concentration, and adjust to pH 7 with KOH
- Tris base
- 2 M Tris–acetate pH 7.8
- 2 M ammonium acetate
- 2 M potassium acetate
- 100 mM cAMP (pH 7 with KOH)
- 0.5 M EDTA
- Stock solutions of ATP, GTP, CTP, and UTP: each at 100 mM (pH 6.5 with 2 M Tris base)
- 0.1 M glucose 6-phosphate (G-6-P)
- 1 M DTT
- 0.5 M phosphoenolpyruvate (trisodium salt) (PEP)
- 40% polyethylene glycol (PEG), M_r 6000
- 2.7 mg/ml folinic acid (FA)

Method

1. Mix together, in the order listed, the following volumes of the prepared stock solutions:

• Tris–acetate	0.825 ml
• Potassium acetate	1.08 ml
• Ammonium acetate	0.54 ml
• Leucine-minus amino acid mixture	3 ml[a]
• PEG	1.5 ml
• DTT	0.06 ml
• ATP	0.36 ml
• GTP	0.24 ml
• CTP	0.24 ml
• UTP	0.24 ml
• PEP	0.82 ml
• G-6-P	0.1 ml
• cAMP	0.12 ml
• EDTA	0.03 ml
• FA	0.39 ml
• H_2O	0.4 ml

2. Check the pH and adjust it if necessary to 7.8 at room temperature using Tris base.
3. Adjust the final volume to 10 ml with H_2O.
4. Store the LM at –70°C in 100 µl aliquots.

[a] Vortex the leucine-minus amino acid mixture just before use as Asn, Gln, Trp, and Tyr may precipitate in the stock solution.

3. The coupled transcription–translation assay

3.1 The basic assay

The general assay is described in *Protocol 6*. We usually carry out the assay in a total volume of 30 µl. It is necessary to optimize the concentration of plasmid in the assay. This is described in *Protocol 6* and typical results are shown in *Figure 1*, where the amount of protein (in this case GroES) synthesized was quantified from the incorporation of [^{14}C]leucine as described in Section 3.2. The magnesium ion concentration used in *Protocol 6* is satisfactory for most batches of S30. If necessary, it may be optimized using an adaptation of *Protocol 6*, in which smaller volumes of master solution are set up, each with a different concentration of magnesium acetate. Assay these with a standard amount of plasmid and calculate the radioactivity incorporated as in Section 3.2.

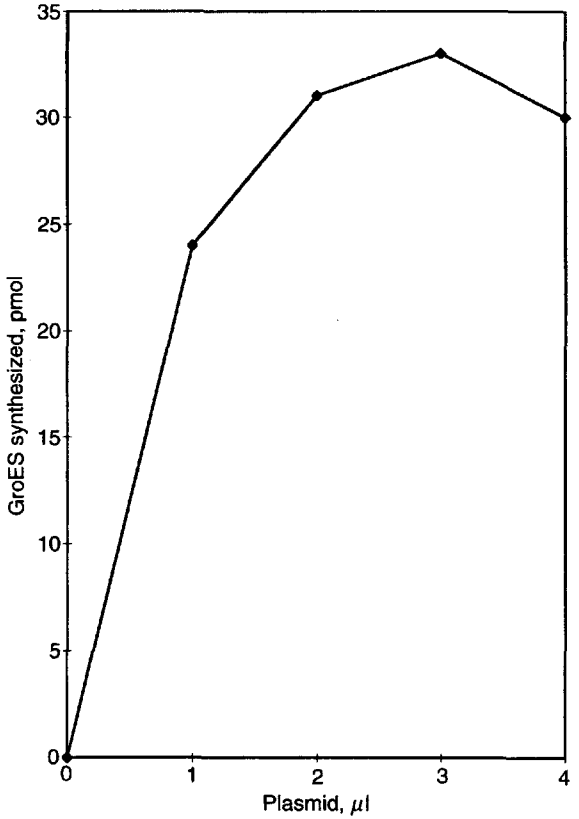

Figure 1. Optimizing the plasmid concentration for coupled transcription–translation. An assay was set up as in *Protocol 6*, and the amount of protein synthesized was determined according to *Protocol 7* and Section 3.2.4.

Protocol 6. Coupled transcription–translation assay

Equipment and reagents

- Shaking water-bath at 37°C
- Glass test-tubes (1 cm × 7 cm)
- LM (see *Protocol 5*)
- tRNA from *E. coli* MRE 600 (Boehringer): 10 mg/ml in H_2O
- Rifampicin (Sigma): 5 mg/ml neutralized with 1 M KOH
- [^{14}C]leucine: 40 Ci/mol, 0.5 mM
- 120 mM magnesium acetate
- Pyruvate kinase (Sigma): dissolve this at 1 mg/ml in 10 mM Tris–HCl pH 7.5, 50% (v/v) glycerol, 1 mM DTT, and store it at –20°C
- SP6 RNA polymerase (see *Protocol 4*)[a]
- S30 (see *Protocol 2*)
- Plasmid to be tested (0.3 mg/ml)[b]

Method

NB: work at 4°C except where stated otherwise.

1. Prepare a master solution[c] by mixing together, in the order listed, the following volumes of the prepared stock solutions:
   - LM 60 μl
   - [^{14}C]leucine 30 μl
   - tRNA 12 μl
   - Magnesium acetate 15 μl
   - Rifampicin 6 μl
   - SP6 RNA polymerase 1 μl[d]
   - Pyruvate kinase 2.1 μl
2. Set up five test-tubes to enable the amount of the plasmid to be optimized. Add H_2O and plasmid to each tube as follows:

Tube	H_2O	Plasmid
1	4 μl	0 μl
2	3 μl	1 μl
3	2 μl	2 μl
4	1 μl	3 μl
5	0 μl	4 μl

3. Thaw the S30 and add 30 μl of it to the master solution (step 1). Mix by gentle pipetting. Immediately add 26 μl aliquots of this master solution plus S30 to the five test-tubes (step 2). Mix by gentle pipetting.
4. Incubate the tubes immediately in a shaking water-bath at 37°C for 30 min.
5. Place the tubes on ice and assay the amount of protein synthesized (*Protocol 7*) to determine the optimal amount of plasmid required in the assay.

[a] Use the T7 RNA polymerase (available commercially) if the sequence inserted in the plasmid is under the control of the T7 promoter. SP6 RNA polymerase is also available commercially.
[b] A plasmid concentration between 0.25–0.35 mg/ml is normally used provided the plasmid preparation is pure and does not contain contaminating RNA (see Section 2.2.2).
[c] This master solution is calculated for six tubes; it is necessary to prepare a slight excess.
[d] A volume of SP6 RNA polymerase between 0.3–1 μl/per tube is usually optimal.

Once the assay has been optimized for the magnesium ion concentration and the plasmid DNA concentration, it may be used for further studies on the expression of the sequence of interest.

3.2 Analysis of the product formed in the coupled transcription–translation assay

3.2.1 The amount of protein synthesized

The reaction mixture after coupled transcription–translation is routinely analysed for the amount of protein synthesized. This is accomplished by determining the amount of radioactive amino acid that is incorporated into

protein (*Protocol 7*). Subsequently, additional analyses are performed (Sections 3.2.2–3.2.4) depending on the objectives of the experiment.

Protocol 7. Quantification of polypeptide synthesized

Equipment and reagents

- Coupled transcription–translation assay samples (see *Protocol 6*) for analysis
- Glass tubes (1 cm × 7 cm)
- 1 M NaOH
- Shaking water-bath set at 37 °C
- Cold 5% (w/v) TCA, keep on ice
- Glass fibre filters (Schleicher & Schuell, No. 34)
- Millipore manifold vacuum filtration apparatus fitted with a water pump or other vacuum pump
- *Drying oven at ~ 160 °C*
- Scintillation counting fluid (5 g of 2,5-diphenyl oxazole per litre of toluene or equivalent), vials, and scintillation counter

Method

1. Set up sufficient tubes for the numbers of samples to be assayed. Add 20 μl H_2O to each tube.
2. Pipette 10 μl aliquots of the coupled assay reaction samples after incubation (*Protocol 6*, step 5) into the glass tubes.
3. Add about 100 μl of 1 M NaOH to each tube. Mix.
4. Incubate for 5 min at 37 °C with shaking.
5. Add about 2 ml of 5% TCA to each tube. Mix, and leave on ice for 5 min to precipitate the proteins.
6. Prepare the manifold filtration apparatus by putting the glass fibre filters over as many holes as needed. Block the other holes with rubber stoppers. Rinse the filters with cold 5% TCA.
7. Filter the precipitated protein through the glass fibre filters by applying a vacuum. Rinse the tubes twice with about 2.5 ml of 5% TCA each time, and pass the washings through the filters. Then wash the filters once with 5% TCA.
8. Dry the filters in the oven; 160 °C for about 10 min.[a]
9. Place each filter into a counting vial containing scintillation fluid, and determine its radioactivity.
10. Calculate the amount of protein synthesized as described in Section 3.2.4.

[a] It is important that the filters are completely dry before being submerged in the counting fluid. Wet filters will remain white in the counting fluid while dry filters become translucent.

3.2.2 Analysis of proteins by SDS–PAGE

Analysis of the synthesized protein by SDS–PAGE followed by autoradiography (*Protocol 8*) is important for two reasons:

(a) To ensure that a polypeptide of the right size has been formed. Occasionally, proteins run more slowly in SDS–PAGE than expected from their

known molecular mass. However, if a polypeptide runs faster than expected, it is likely that the polypeptide product is smaller than anticipated. This may be due to part of the coding sequence being missing in the plasmid, to the premature termination or proteolytic degradation of the protein, or even to the synthesis of a different protein.

(b) SDS–PAGE may reveal that more than one polypeptide has been formed. This may occur with non-linearized plasmids, when large primary transcripts are synthesized, and the coding sequence of a plasmid gene, e.g. β-lactamase, is located on the same DNA strand downstream from the coding sequence for the protein of interest.

Protocol 8. Analysis of the product formed by SDS–PAGE and autoradiography

Equipment and reagents

- Coupled transcription–translation assay samples (*Protocol 6*, step 5) for analysis
- Loading buffer: 125 mM Tris–HCl pH 6.8, 4% (w/v) SDS, 200 mM DTT, 0.01% (w/v) bromophenol blue, 20% (v/v) glycerol
- 10% polyacrylamide gel (10 cm × 10 cm) (either home-made or purchased, e.g. from Novex)[a]
- PAGE apparatus and power supply
- Molecular weight markers: 2 μg each of lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin; 3 μg each of BSA and phosphorylase *b*
- Tris–glycine buffer: 25 mM Tris, 192 mM glycine pH 8.3, 0.1% (w/v) SDS
- Destaining solution: 45% methanol, 10% glacial acetic acid, 45% H_2O (by vol.)
- 0.25% (w/v) Coomassie Brilliant Blue R in destaining solution
- Gel dryer (Bio-Rad model 224)
- X-ray film (Hyperfilm from Amersham) and X-ray cassette
- Kodak developer and fixer (diluted according to manufacturer's specification)
- Diluted acetic acid: 2 ml acetic acid, 500 ml H_2O

Method

1. Remove 15 μl of each coupled transcription–translation assay sample and add 5 μl loading buffer. Heat for 5 min at 90°C.
2. Load the samples onto the 10% polyacrylamide gel. Separately load an appropriate amount of molecular weight markers in another lane. Run the electrophoresis with Tris–glycine buffer at no more than 30 mA or 200 V until the tracking dye has migrated to the bottom of the gel.
3. Fix the gel for 10 min in destaining solution.
4. Stain the gel for 20 min in Coomassie Brilliant Blue R in destaining solution.
5. Destain the gel until the background is clear.
6. Dry the gel using the gel drier.
7. Place the dried gel under X-ray film and tape them together. Punch holes at the corners through both the X-ray film and the gel so that the autoradiogram can later be oriented properly over the gel.

Protocol 8. *Continued*

8. Expose the gel for 24–48 h at –70°C in the X-ray cassette.
9. Develop the X-ray film in Kodak developer for 1–5 min. Rinse briefly in diluted 500 ml diluted acetic acid. Fix the film in Kodak fixer. Rinse it in distilled H_2O and dry.

[a] The buffer system of Laemmli (17) is used in this protocol.

Results of a typical SDS–PAGE analysis are shown in *Figure 2*.

3.2.3 Release of proteins from ribosomes for analysis

Protocol 9 describes a method by which radioactively labelled protein released from the ribosomes can be separated from those polypeptides that

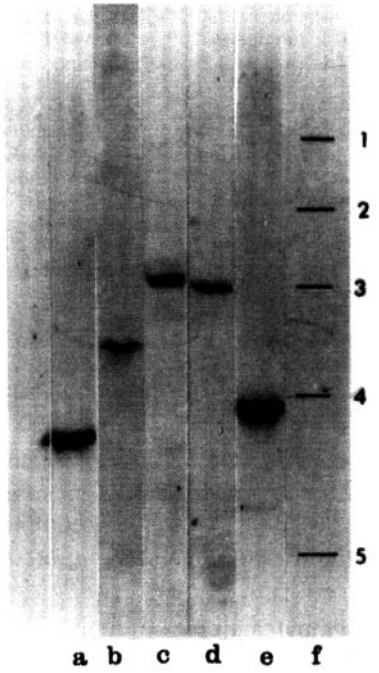

Figure 2. A single polypeptide of the expected size is released from the ribosomes. Coupled transcription–translation was carried out according to *Protocol 6*, then the reaction mixture was centrifuged as described in *Protocol 9*. An aliquot of the resulting soluble fraction was analysed by SDS–PAGE and autoradiography (*Protocol 8*). The figure shows the autoradiogram. Lane a, *E. coli* DHFR. Lane b, chloramphenicol acetyltransferase (CAT). Lane c, bovine rhodanese. Lane d, ricin. Lane e, GroES. Lane f, molecular weight markers: BSA (1; 67 kDa), ovalbumin (2; 43 kDa), carbonic anhydrase (3; 30 kDa), soybean trypsin inhibitor (4; 22 kDa), and lysozyme (5; 14 kDa).

remain bound to the ribosomes after protein synthesis was stopped. In our experience, if an enzyme is being synthesized, full-length enzymatically active protein will be found only in released form. The exception to this is artificially C-terminally extended proteins (18).

Protocol 9. Separation of ribosomes from the soluble fraction after coupled transcription–translation

Equipment and reagents

- Coupled transcription–translation assay carried out as in *Protocol 6* but using an increased volume (90 μl)
- Airfuge (Beckman) and corresponding centrifuge tubes
- Solution 1: 20 mM Tris–HCl pH 7.5, 100 mM NH_4Cl, 10 mM magnesium acetate, 1 mM dithioerythritol (DTE), 50% (w/v) sucrose
- Solution 2: solution 1 without sucrose

Method

1. Remove 50–60 μl from the coupled transcription–translation reaction mixture and layer this over a cushion of 50 μl solution 1 in a centrifuge tube.
2. Centrifuge for 40 min at room temperature at 140000 *g*.
3. Carefully remove and retain the supernatant (including the sucrose solution).
4. Rinse the ribosome pellet with about 50 μl solution 2, then resuspend the pellet in 50 μl of fresh solution 2.
5. Analyse 20 μl of the supernatant fraction and 10 μl of the resuspended ribosomes for TCA insoluble radioactivity (*Protocol 7*).
6. Retain the remaining supernatant and ribosomes for further analysis.

3.2.4 Quantification of full-length product formed

The radioactivity incorporated into the protein product synthesized in the coupled assay may be used to calculate the amount of protein formed. The incorporation is measured by TCA precipitation as described in *Protocol 7*. Assuming that [^{14}C]leucine has a specific radioactivity of 40 Ci/mol and the counting efficiency is 90%, the calculation is performed as follows:

(a) Subtract the radioactivity of the assay blank (the reaction mix in *Protocol 6* from which the plasmid was omitted, i.e. tube 1 in step 2) from the radioactivity determined for the full assay.

(b) Divide the net radioactivity by 80 to obtain the pmol leucine incorporated.

(c) Divide the pmol leucine incorporated by the number of leucine residues in the protein (obtained from the amino acid or nucleotide sequences) to calculate the pmol protein formed.

(d) Multiply pmol protein by ($M_r \times 10^{-3}$) to obtain ng protein. This step is only valid if analysis by SDS–PAGE and autoradiography (*Protocol 8*) revealed one radioactive band corresponding to the M_r of the desired protein.

3.2.5 Enzyme assays to determine specific enzymatic activity

As mentioned in Section 3.2.3, enzymatic activity is usually associated only with polypeptides released from the ribosomes. *Protocols 10* and *11* give two examples of how we determine the enzymatic activity of *in vitro* synthesized proteins—a eukaryotic enzyme (rhodanese), and the bacterial enzyme dihydrofolate reductase (DHFR). In the case of rhodanese, the enzyme assay should contain 20–30 ng rhodanese. Calculate this as described in Section 3.2.4. The rate of reaction should be linear over the incubation time used in *Protocol 10* for valid calculations of enzyme activity.

Protocol 10. Rhodanese assay[a]

Equipment and reagents

- Shaking water-bath at 30°C
- Glass test-tubes (1 cm × 10 cm)
- Coupled transcription–translation reaction mixture (see *Protocol 6*) or ribosomal supernatant (see *Protocol 9*)
- 0.15 M $Na_2S_2O_3$: this is degraded by bacteria; prepare it using autoclaved H_2O, and store at –20°C
- 0.12 M KH_2PO_4
- 18% (v/v) formaldehyde
- 0.15 M KCN (NB: highly toxic especially at low pH such as at the end of the procedure; handle all KCN solutions with the utmost care)
- Ferric nitrate solution: add 404 g $FeNO_3.9H_2O$ to 800 ml 65% (v/v) HNO_3. Dilute to 3 litres and filter if turbid. Dilute small amounts of this stock solution 1:1 with H_2O for use.

Method

1. Combine equal volumes of $Na_2S_2O_3$, KCN, and KH_2PO_4 solutions. Make this solution within 30 min of use.
2. Add 15 μl of the coupled transcription–translation reaction mixture or of the ribosomal supernatant to 0.5 ml of the mixture prepared in step 1.
3. Prepare a reagent blank with 15 μl H_2O in place of the assay samples in step 2.
4. Incubate the tubes for 10 min at 30°C.
5. Add 0.25 ml formaldehyde, then 0.75 ml ferric nitrate to each tube. Mix carefully by vortexing.
6. Read the A_{460}.
7. Convert A_{460} to μmol product formed/min using the molar extinction coefficient (4.2×10^3) for the complex formed between thiocyanate and ferric nitrate.

[a] Based on ref. 19.

Protocol 11. Dihydrofolate reductase assay[a]

Equipment and reagents

- Shaking water-bath at 30°C
- Glass test-tubes (1 cm × 10 cm)
- Coupled transcription–translation reaction mixture (see *Protocol 6*) or ribosomal supernatant (see *Protocol 9*)
- 1 M imidazole–HCl pH 7
- 1 M 2-mercaptoethanol
- 9 mM dihydrofolate (DHF)
- 1 mM NADPH

Method

1. On ice prepare an enzyme reaction mixture containing the following in a total volume of 1 ml: 100 μl 1 M imidazole–HCl, 10 μl of 1 M 2-mercaptoethanol, 5 μl of 9 mM DHF, 60 μl of 1 mM NADPH, and 5–15 μl of the coupled transcription–translation reaction mixture or the ribosomal supernatant.
2. Determine the A_{340} of the enzyme reaction mixture.
3. Incubate the enzyme reaction mixture for 10 min at 30°C.
4. Briefly place it on ice and determine the A_{340} again.
5. Determine the DHFR enzyme activity. One unit of DHFR activity is defined as the amount of DHFR required to reduce 1 μmol of DHF/min based on a molecular extinction coefficient of 12.3×10^3 (ref. 21).

[a] Based on ref. 20.

Table 2 summarizes the analysis of several proteins synthesized in the coupled assay and analysed as described in this section.

4. Modified coupled transcription–translation assays

Crude ribosomes prepared as described in *Protocol 12* can be substituted for the S30 extract in coupled transcription–translation (2). Using only the ribosome fraction may be especially useful when degradation of plasmid DNA (2) or of the protein product is encountered in the standard assay (*Protocol 6*) using the S30 fraction. This may become a necessity for instance when β-lactamase is transcribed from the DNA strand as the coding sequence.

Salt washed ribosomes can be derived from crude ribosomes as described in *Protocol 13*. These salt washed ribosomes cannot support protein synthesis by themselves; they must be supplemented by either the ribosomal wash fraction (*Protocol 13*) or by a translation factor-rich fraction prepared as in *Protocol 12*. A fractionated system such as this is useful in answering questions concerning the dependency of the protein synthesized on specific components present in the reaction mixture. In one application, the translation factor-rich

Table 2. Analyses of products formed by *in vitro* coupled transcription–translation[a]

Protein	Plasmid used	Amount of product[b] pmol	Released protein in supernatant[c] pmol	Enzyme activity ($U \times 10^{-3}$)
Bacterial				
DHFR	pSP65	3.8	3	5.74
GroES	pET14b	58	43	n/a[d]
RF-1	pGEM	24	14.6	n/a
CAT	pSP65	6	4.7	9.3
Eukaryotic				
Rhodanese	pSP65	26.1	12.3	70.1
Rhodanese	pGEM	15.2	6.1	46.2
Ricin	pGEM	15	5.1	n/a

[a] Calculated for a 30 μl reaction mixture as in *Protocol 6*.
[b] Determined for the total reaction mixture (*Protocol 7*).
[c] After centrifugation of the reaction mixture (*Protocol 9*).
[d] Not applicable.

fraction was further purified by size exclusion chromatography to eliminate the molecular chaperone GroEL (3).

Protocol 12. Preparation of ribosomes, post-ribosomal supernatant, and translational factor-rich fraction

Equipment and reagents

- Beckman L5–65 ultracentrifuge and Ti-50 rotor (or equivalent)
- Centrifuge tubes
- Teflon–glass homogenizer
- Ribosome buffer A: 20 mM Tris–HCl pH 7.6, 10 mM magnesium acetate, 30 mM NH_4Cl, 1 mM DTT
- S30 (see *Protocol 2*)

Method

1. Centrifuge 3 ml of the S30 in the fixed-angle Ti-50 rotor at 100 000 *g* for 3 h.
2. There will be three distinct fractions present in the centrifuge tube after this step: the upper post-ribosomal supernatant fraction (containing most of the soluble components), the lower relatively viscous, slightly amber-coloured fluid phase (the translational factor-rich fraction), and the gelatinous ribosomal pellet. Pour off and retain the supernatant fraction from the upper part of the tube.
3. Carefully remove the viscous, coloured, factor-rich fraction from above the ribosomal pellet.

4. Resuspend the pellet of ribosomes in 1–1.5 ml ribosome buffer A using a Teflon–glass homogenizer. Adjust the resulting suspension to about 1600 A_{260} U/ml.
5. Store all three fractions in small aliquots at –70°C.

Protocol 13. Preparation of salt washed ribosomes and ribosomal wash fraction

Equipment and reagents

- Crude ribosomal pellet (*Protocol 12*, step 4)
- Other equipment and reagents as described in *Protocol 12*
- Ribosome buffer B: 20 mM Tris–HCl pH 7.6, 10 mM magnesium acetate, 500 mM NH_4Cl, 1 mM DTT

Method

1. Resuspend the crude ribosomal pellet in ribosome buffer B to half the original volume of the S30 used in *Protocol 12*, step 1.
2. Pellet the ribosomes at 100 000 *g* for 1.5 h.
3. Separate the supernatant (ribosomal salt wash fraction) from the ribosomal pellet.
4. Resuspend the salt washed ribosomes in ribosome buffer A (to give 1500–1600 A_{260} U/ml).
5. Clarify the ribosome suspension by low speed centrifugation (10 000 *g*, 10 min).
6. Store the ribosomes (step 5) and the ribosomal salt wash fraction (step 3) in small aliquots at –70°C.

5. Further developments

In an *in vitro* transcription–translation system using the *E. coli* S30 extract, expression of coding sequences under the control of a promoter for a bacteriophage RNA polymerase can be partially controlled by adjusting the amount of the RNA polymerase added. As a convenient alternative, S30 fractions may be prepared from *E. coli* cells that contain a phage RNA polymerase regulated by an inducible promoter/operator in the genome, such as Novagen's *E. coli* strain BL21 (DE3) or on a plasmid (15). Further improvements of the S30 itself have been suggested recently in ref. 22. It was demonstrated by the authors that concentrating the S30 fraction will increase the yield of the expressed protein.

For studies involving mechanisms or regulation of protein synthesis, mutant strains of bacteria may be used for the preparation of the S30 fraction that

lack a specific protein assumed to be involved in optimal protein expression. One such example is the *E. coli* strain PK101 developed in E. Craig's laboratory (23). In these *E. coli* cells, the *dnak* operon has been deleted that contains the genes coding for the molecular chaperones DnaK and DnaJ. Thus, by using an S30 derived from this mutant, the involvement of DnaJ and DnaK in folding and expression of active protein can be tested by measuring specific enzymatic activity of an enzyme synthesized *in vitro*.

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8

Monitoring protein expression

JOHN COLYER

1. Introduction

1.1 General considerations

The methods available to monitor protein expression are numerous and can be employed in combination to explore a particular aspect of protein expression. A cursory examination of the issue of protein expression might leave one with the impression that there are only a few, somewhat straightforward objectives. However, upon more detailed consideration, it becomes apparent that monitoring protein expression involves a multitude of interrelated objectives, including:

(a) Identification of cells or tissues expressing the protein of interest.
(b) Quantification of the steady state level of expression.
(c) Description of the kinetics of synthesis and degradation.
(d) Definition of the post-translational modifications *en route* to the mature protein.
(e) Identification of the location of the protein within the cell.
(f) Exploration of the targeting of the protein to its cellular destination.
(g) Description of the modulation of any of the above by stress, hormones, stage of development, etc.
(h) Identification of signals within the primary sequence which control each of the above.
(i) Optimization of the expression of the protein in an heterologous system.

Achieving each of these objectives is possible through the application of the techniques described in this chapter. Well-tried and successful protocols for the following techniques are described:

- immunoblotting (dot blots, Western blots)
- pulse-chase labelling
- immunoprecipitation
- epitope tagging

- immunohistochemistry
- surrogate reporter protein detection

In some instances, comparable techniques are available for studying the expression of mRNA transcripts rather than the protein itself. The sensitivities of the techniques used to detect the RNA transcript on the one hand, and the protein on the other, are not always equivalent. Thus, investigators must decide, on a case-by-case basis, which strategy best serves the goals of the experiment. Protocols for the analysis of mRNA transcript expression have been published elsewhere (1).

1.2 Basic strategies for monitoring protein expression

Three basic strategies for the monitoring of protein expression have been defined. These may be distinguished from each other in terms of the techniques employed:

(a) Immunological techniques using antibodies to the native protein.

(b) Techniques using a foreign reporter sequence inserted into the protein of interest for immunological or other detection methods.

(c) Deployment of a surrogate reporter protein, either in addition to the protein of interest or as a fusion partner with that protein.

The development of these three strategies has been largely sequential in time, driven by the limitations of each preceding technology. While there is an element of duplication between these strategies, the methodologies of strategy (b) and strategy (c) extend our experimental capability over that achieved by the methods of strategy (a) alone.

The immunological techniques useful for the detection of protein expression comprise immunoblotting (dot blotting and Western blotting), immunoprecipitation, and immunohistochemistry. The type of issue which can be addressed by each of these varies. Immunoblotting of cell or tissue extracts will establish whether the expression of a protein occurs in a particular cell or tissue, and can be used to quantify the steady state level of that expression. It can also be used to assess the rate of protein degradation by analysing extracts from cells exposed to inhibitors of protein synthesis for different periods of time. However, it cannot define the cellular origin of the protein detected in a tissue extract, nor will it allow recovery of the native or enzymatically active protein from the sample. These latter objectives are met by the techniques of immunohistochemistry, immunolocalization (or microscopy), and immunoprecipitation, respectively.

It is implicit in strategy (a) that an antibody with the necessary properties has to be available for each protein of interest. The antibody must be wholly specific for the protein of interest and suitable for use ideally in immunoblotting, immunoprecipitation, and immunomicroscopy. This is not always the case and

the raising and characterization of such an antibody may impose an unacceptable delay or may simply be unachievable. Strategy (b) provides an alternative route in which the epitope for a pre-existing antibody is inserted into the primary sequence of the protein of interest, thus circumventing the need for a new antibody specific to the whole protein. Once 'tagged' in this manner, the protein of interest can be monitored through the detection of this additional epitope. Epitope tagging lends itself to the same techniques as are applicable in strategy (a), but it also offers the ability to discriminate between endogenous (wild-type) and mutant forms of the protein, since experiments can be designed where only the mutant protein contains the epitope tag. In this way the contribution of individual amino acids or segments of primary sequence to the expression of a particular protein (e.g. synthesis, stability, or cellular location) can be investigated.

The third strategy abandons completely the immunorecognition of the protein of interest and utilizes a reporter protein instead. Reporter proteins either possess an enzyme activity, which is simple to measure (e.g. chloramphenicol acetyltransferase, CAT; β-glucuronidase, GUS; or luciferase), or possess a distinctive intrinsic fluorescence (e.g. green fluorescent protein, GFP, from *Aequoria victoria*) which again is easy to measure. These reporters may be used in two ways:

(a) The expression of the reporter protein is controlled by the promotor from the protein of interest (protein X). Thus, the reporter protein acts as a surrogate for protein X. This allows one to explore the synthesis of protein X (e.g. using CAT or luciferase) and the histological pattern of expression (e.g. employing GUS).

(b) A protein X–reporter fusion protein is used to investigate the subcellular location and dynamics of protein X movement throughout the cell (e.g. exploiting a protein X–GFP fusion protein).

The remainder of this chapter describes the background to the methods listed earlier in this section, and provides detailed protocols for their use.

2. Immunodetection of protein expression

2.1 Considerations affecting the choice of antibody

2.1.1 Preparation of the antibody

The production of an antibody to a native protein, or to a portion thereof, can facilitate the detailed study of the expression of that protein within a particular cell, throughout the tissues of an organism, or between organisms. The production of such an antibody can be achieved in three ways:

(a) By immunization of animals (usually rabbit, goat, sheep, or chicken) to produce a polyclonal immune serum.

(b) By immunization of animals (mouse or rat) to produce a monoclonal antibody.

(c) By screening a recombinant antibody library to isolate immunoglobulin fragments able to interact with the antigen under study (2).

In each case, it is necessary to choose the antigen to illicit or identify the immunoglobulin of interest. This can be:

(a) The entire sequence of a protein.

(b) A domain from the protein (either alone or as a fusion partner with an irrelevant protein).

(c) A peptide derived from the sequence of the protein of interest.

The final option has become increasingly popular for reasons of speed, ease, and economy. In essence, a peptide of eight or more residues (typically 10–20 residues), conjugated to a carrier protein (e.g. BSA, KLH, or thyroglobulin), is able to illicit a humoral immune response, producing antibodies which are frequently able to recognize the protein from which the peptide sequence was derived. The carrier protein chosen should be the one least likely to be encountered in the experiments planned, since antibodies to this protein will also be abundant in the polyclonal serum obtained. The choice of peptide sequence from protein X that is selected for immunization is influenced by the application for which the anti-peptide antibody is intended, and by the likelihood that this segment of the native parent protein will be accessible to an immunoglobulin. Segments rich in polar and charged amino acids are more likely to be exposed on the surface of a protein than areas lacking these types of residue. Several algorithms are available for predicting the antigenicity and surface accessibility of protein segments, e.g. *PREDICT STRUCTURE* (3).

2.1.2 Intended use of the antibody

The intended use of the anti-peptide antibody is an entirely separate matter. Is it required to distinguish between isoforms of a protein? If so, a polyclonal serum to the entire native protein is unlikely to suffice, since this immunogen will stimulate antibody production to determinants shared by different isoforms as well as to determinants exclusive to the isoform of interest. Peptide sequences, however, can be selected which exhibit significant sequence divergence between the isoforms of a protein. Polyclonal antibodies raised to such material have an improved likelihood of discriminating between the isoforms of a protein. Conversely, is the antibody required to identify all isoforms? If so, the regions of minimum sequence divergence between isoforms should be selected.

Where a number of segments within the protein of interest satisfy the above criteria, the occurrence of each intended peptide sequence within other proteins must be considered. Interrogation of protein sequence databases can

provide warning of potential cross-reactivity of the planned antibodies (i.e. immunorecognition of proteins other than the one intended). Then one must assess the likely consequence of this. For example, cross-reactivity with proteins not encountered in the planned experimental situation presents less concern than cross-reactivity with a second protein expressed in the tissue under study. An account of the methods for antibody production is not presented in this chapter, but instead the reader is directed to another source which deals with this (4).

2.1.3 Choice between an affinity purified antibody and a crude polyclonal serum

A second consideration when working with polyclonal antibodies is the merit of affinity purification of the immunoglobulin of interest. A polyclonal immune serum will contain a large number of immunoglobulins, only about 10% of which will react with the antigen of interest. The others will react with proteins other than the one under study, and might, thereby, complicate interpretation of the data obtained. Two strategies are available to manage this situation:

(a) Affinity purification of the immunoglobulin of interest.
(b) Use of competitive inhibition techniques as a means of distinguishing specific interactions from non-specific.

In the first of these strategies, the immunoglobulin of interest is purified from the polyclonal serum by virtue of its ability to interact with the antigen (i.e. protein X or the peptide from protein X) immobilized on a solid support. Typically this is a straightforward and successful procedure. However, the recovery of the affinity purified antibody and the avidity of its interaction with the antigen are low compared to the original polyclonal serum. Strategy (b) can be adopted in many instances, particularly when it is intended to use Western blots in the planned experimentation. In Western blotting, both the apparent molecular weight of the protein and its recognition by the antibody are used as independent measures to interpret the data. Thus, immunoreactivity with proteins of unexpected molecular weights can be treated with suspicion. Furthermore, specific anti-peptide recognition can be confirmed by competitive inhibition of the immunosignal upon inclusion of excess synthetic peptide antigen in the antibody:antigen reaction. In this situation, the specific interaction of the immunoglobulin with protein X will be inhibited by the excess peptide present, but the unrelated interactions of other immunoglobulins with irrelevant antigens will be unaffected. My own practice is to evaluate each polyclonal serum for unwanted immunoreactivity. In cases where this is high, I would affinity purify the immunoglobulin of interest but, in cases where unwanted reactivity is low (5), affinity purification is unnecessary and peptide competition strategies would be employed to verify the specificity of any antibody:antigen interactions.

2.2 Immunodot blots

In many studies of protein expression, the initial stages involve screening a large number of samples to identify those containing the protein of interest. These might be homogenates from tissues of an organism, extracts from a variety of cells in culture, or various culture media from experiments where optimization of the heterologous expression of the protein is the objective. In each case, the technique chosen to screen for samples of interest must be able to accommodate large numbers of independent samples, must be rapid, and must involve simple technology. The dot blot technique is ideal for this purpose.

Protocol 1 describes the basic procedure for preparing a dot blot. It uses a commercial blotting apparatus that allows one to apply large numbers of samples simultaneously to the membrane in the form of circles ('dots') or rectangles ('slots') depending on the design of the apparatus. This enables one to screen many samples at a time. In addition, the uniform size and shape of the dots (or slots) will aid the quantification of the immunological signals by densitometry when the blot is subsequently subjected to immunological detection methods (described in Section 2.4). In this respect, 'slot' blots provide a more suitable signal geometry, less subject to orientation artefacts, than the traditional 'dot' blot.

Dot blots can also be set up without a commercial apparatus. Samples (2–10 μl) may be spotted onto the membrane with a micropipette in a regular grid pattern and the membrane allowed to air dry. However, it is difficult to control the size of the dots and ensure comparable geometry for subsequent quantitative analysis, particularly if different volumes of each sample are involved.

The technique described in *Protocol 1* uses a nitrocellulose membrane for the blot, but dot blots can be performed with a range of membranes to suit the individual application, such as nylon, PVDF, ion exchange, and covalently activated membranes.

Protocol 1. Preparation of a protein dot blot

Equipment and reagents

- Nitrocellulose membrane (0.2 μm pore size; Schleicher & Schuell)[a]
- TBS: 50 mM Tris–HCl pH 7.4, 150 mM NaCl
- Plastic storage container (domestic sandwich box; 22 × 16 cm)
- Dot blot apparatus (Bio-Rad or Schleicher & Schuell)
- Vacuum source (water aspirator or vacuum pump)
- Protein samples for analysis[b]

Method

NB: wear latex (or similar) disposable gloves when handling nitrocellulose for your own protection and to prevent contamination by proteins from the surface of your hands. Carry out all steps at room temperature.

1. Cut the nitrocellulose membrane to the dimensions of the dot blot

apparatus, cutting off one corner of the membrane to aid orientation of the blot at the end of the experiment.

2. Soak the nitrocellulose membrane in 100 ml TBS in a clean plastic storage container for 5 min.
3. Assemble the dot blot apparatus with the nitrocellulose membrane beneath the sample slots to form a seal between the two halves of the apparatus, as described in the manufacturer's instructions.
4. Without connecting the vacuum, pipette 50 μl TBS into each sample well.
5. Add the protein samples (2–50 μl)[c] to the TBS in separate wells. Mix the samples with the TBS by gently tapping the side of the apparatus.
6. Connect the vacuum, and draw the samples through the nitrocellulose membrane. Maintain the vacuum until the membrane appears to be dry.
7. Dismantle the dot blot apparatus and recover the nitrocellulose membrane. Process it as described in *Protocol 4* or *5* to detect the protein of interest by probing with a specific antibody.[d]

[a] Alternative membranes include nylon, PVDF, ion exchange, and covalent activated membranes.
[b] Samples can be tissue homogenates, cell lysates, chromatography column fractions, etc. Most of the commonly used buffers are compatible with binding of the proteins to the standard membranes used in dot blots.
[c] The volume of sample used will depend on the concentration of the protein under investigation and the titre of the specific antibody to be used in the subsequent detection (*Protocols 4* or *5*); pilot studies may be required to establish the quantities to use.
[d] If the blot is not to be analysed immediately, store it dry and flat in a plastic bag.

Methods are available for immunodetection and quantification of protein X on dot blots (see Section 2.4). Quantification of the signal is important, as a comparison of the relative amounts of protein X in two samples is frequently of interest. However, although quantification of immunoblots is possible, it should be recognized that the range of signal intensities that can be distinguished is somewhat limited, particularly when using colorimetric substrates (discussed fully in Section 2.4). When quantification is the objective, it is preferable to apply spots of uniform size using a dot blot microfiltration apparatus onto a membrane with a high protein binding capability (e.g. PVDF membranes are better than nitrocellulose) and small pore size (0.2 μm). In addition, each blot should be set up to contain a full calibration curve of purified protein X to ensure that the signals from the experimental samples all fall within the range of detection and quantification.

A derivative of the dot blot technique is utilized in the screening strategy for expression cloning of proteins. In this case, cDNA fragments from a DNA library are inserted into expression cloning vectors which are then introduced

into bacteria. The expression of a protein of interest in a bacterial colony can be identified by taking a replica print of the bacterial colonies growing on an agar plate onto a sheet of sterile nitrocellulose. The nitrocellulose is then placed, colony side up, on a fresh agar plate and the expression of the cDNA insert within each bacterium is induced. Protein X is detected immunologically following lysis of the cells (6).

While dot blots are very convenient for the high throughput screening of samples in the initial stages of a study, the amount of detailed information obtained concerning protein X is rather limited. More detailed information may be obtained using Western blots which are described in the following section.

2.3 Western blotting

Once the samples of greatest interest have been identified in an initial screen based on dot blots, more advanced aspects of the process of protein expression can be investigated. The first of these is typically confirmation that the identification of protein X in the dot blots is justified. Once this has been confirmed, it is possible to determine the steady state level of expression of the protein, the kinetics of synthesis and destruction, and the post-translational events *en route* to the mature functional enzyme.

The first step is to evaluate the accuracy of evidence which suggests protein X is present in the sample under study. As will be explained in Section 2.4, false positive responses can be a problem with dot blots, and thus several independent assessments of the immunoreactivity should be sought to ensure that it is generated by the presence of protein X. The molecular weight or isoelectric point of protein X is frequently known and either of these can be used as a second independent characteristic to confirm the specific recognition of the protein of interest in immunological assays. Alternatively, there may be other features of the protein which can be exploited to confirm its identity if the former parameters are not available, for example, covalent attachment of a specific (labelled) ligand or use of multiple antibodies to different regions of the protein. However, to exploit any of these properties the protein components of a sample must be separated, typically by electrophoresis. The specific immunorecognition of a protein of the appropriate molecular weight or isoelectric point, or bearing a radiolabelled ligand, can then be examined by immunoblotting or Western blotting, which are alternative names for the same technique (7). In Western blotting, the electrophoretic step of protein separation is coupled to the transfer of proteins to an inert membrane (such as nitrocellulose or PVDF) to allow for the subsequent immunodetection of a protein of interest.

The electrophoretic separation can be isoelectric focusing, non-denaturing (native) PAGE, or (denaturing) SDS–PAGE. Respectively, these separate proteins according to their isoelectric points (pI), as a function of charge/size/shape of the native protein, and the apparent molecular weight of its individual polypeptides. The transfer process produces an exact copy of the electrophoretic gel onto the inert membrane which can then be probed with antibodies.

The transfer of proteins from the electrophoretic gel to the inert membrane is performed either by electrotransfer in a tank or by semi-dry electrotransfer. In conventional tank electrotransfer techniques, the original electrophoretic gel (usually SDS–PAGE) and the inert membrane (PVDF, nitrocellulose) are sandwiched between filter paper supports, immersed in transfer buffer (as depicted in *Figure 1*). The transfer buffer provides the conducting medium and the means of heat dissipation during the electrophoretic transfer. In this technique, the field strength (V/cm), which is the driving force for the transfer process, can be varied usually by adjusting the voltage, but also by altering the distance between electrodes. Tank electrotransfers are efficient but slow, and require relatively large volumes of buffer reagents. Semi-dry electrotransfer is an alternative, horizontal format where the buffer component is supplied via filter papers impregnated with the transfer buffer. In this format, the small distances between the electrodes translates into a reduced field strength at any operating current. Efficient transfer of proteins at a current of 0.8 mA/cm^2 of gel over 60 minutes is usual, which is advantageous in laboratories with a high throughput of Western blots. However, it is reported that poor transfer of high molecular weight proteins and loss of small proteins (driven through the membrane) can

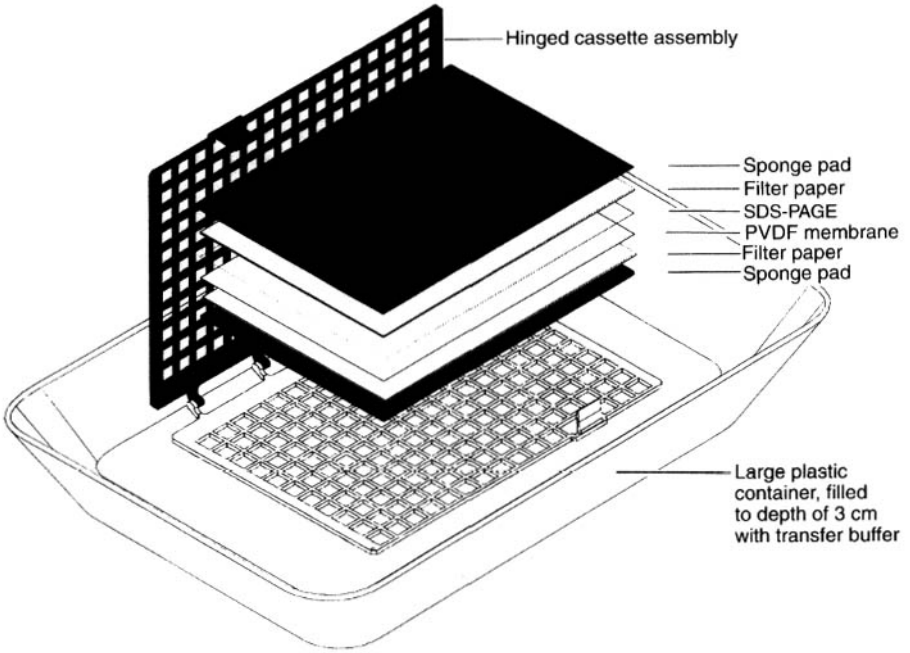


Figure 1. Assembly of the tank electrotransfer apparatus. A sandwich of the SDS–PAGE gel and the PVDF membrane is made between filter paper and sponge pad supports beneath the surface of a reservoir of transfer buffer. Reproduced with permission from Pharmacia Biotech (8).

be associated with this version of the technique. This has not been my own experience: use of PVDF membranes (0.2 μm) with a small pore size prevents loss of small proteins (down to 6 kDa) through the membrane, and results in the quantitative transfer of components up to 110 kDa within 60 minutes (unpublished data). PVDF membranes also capture the proteins on one face of the membrane, with no apparent penetration of protein through the membrane to the other face. This is ideal for subsequent immunodetection and quantification of the signal.

Tank electrotransfer is described in *Protocol 2* and the semi-dry electrotransfer is in *Protocol 3*. In each case, it is assumed that the proteins in the samples to be screened will have been separated by SDS–PAGE. Methods for this will be found elsewhere (Chapter 6, *Protocol 7*) (9, 10). Immunoscreening of the blots generated will be described in Section 2.4. To provide a means of estimating the M_r of proteins detected by antibody screening of the blots, molecular weight markers must be run on the gel, and these will be transferred to the blot. Pre-stained markers are ideal for this purpose since they also enable one to confirm that the electrotransfer from the gel to the blot has been achieved. Where more than one gel is being Western blotted, run pre-stained markers in different lanes so that the blots may be easily distinguished from each other.

In both techniques, several gels may be transferred simultaneously depending on the design of the electrotransfer apparatus. In the case of semi-dry electrotransfer apparatus, up to six gels may be accommodated at a time. However, my laboratory imposes a limit of two gels to ensure efficient and even transfer from the gels (S. Calaghan, G. A. Drago, and J. Colyer, unpublished observations).

Protocol 2. Tank method for the electrotransfer of proteins from an SDS–PAGE gel to a PVDF membrane

Equipment and reagents

- SDS–PAGE gel containing the protein samples for analysis with pre-stained molecular weight size markers (Bio-Rad, BDH, Sigma)
- Transfer buffer A: 25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol[a]
- Two plastic storage containers (16 × 20 cm) (see *Protocol 1*)[b]
- Filter paper (Whatman No. 1)
- PVDF membrane (0.2 μm pore size; Amersham, Bio-Rad, Pall, or Schleicher & Schuell)
- Methanol
- Plastic tray (30 × 40 cm and about 5 cm deep)
- Electrotransfer tank (Bio-Rad or Pharmacia Biotech) complete with plastic assembly cassette, two sponge pads, and a cooling block or coil[c]
- Glass rod or test-tube for use as a roller
- Magnetic stirrer

Method

NB: wear latex (or similar) disposable gloves during all stages of this procedure for your own protection and to prevent contamination of the blot by proteins from your hands.

1. Remove the SDS–PAGE gel from the glass electrophoresis plates upon completion of the electrophoresis.[d]
2. Soak the gel in 200 ml transfer buffer A in a clean plastic container for 15 min at room temperature.
3. Measure the dimensions of the gel accurately; the gel will shrink in size due to the methanol in transfer buffer A.
4. Cut four pieces of filter paper to dimensions larger than the gel (by about 1 cm all round), and one piece of PVDF membrane to the exact dimensions of the gel.
5. Wet the PVDF membrane with methanol. The PVDF membrane will change in appearance from opaque/white to translucent/grey as it becomes impregnated with the methanol.[e] Now immerse it in 200 ml transfer buffer A in a clean plastic container for 10 min at room temperature.
6. Fill the large plastic tray with transfer buffer A to a depth of 3 cm. Open the plastic assembly cassette and submerge one-half of the cassette in the transfer buffer (see *Figure 1*).
7. Build up the transfer stack in the cassette one layer at a time (see *Figure 1*) keeping each layer fully submerged below the transfer buffer. In order, the layers consist of:
   - one sponge pad
   - two sheets of filter paper
   - the PVDF membrane
   - the SDS–PAGE gel
   - two sheets of filter paper
   - the second sponge pad

   Exclude air bubbles from between the layers by using a glass rod or test-tube as a roller to smooth each layer and expel visible air bubbles.
8. Close the plastic assembly cassette below the level of the transfer buffer, and secure it with the locking clips.
9. Pour cold (4°C) transfer buffer A into the transfer tank until it is three-quarters full. Insert the loaded cassette (from step 8), orientated with the PVDF membrane on the anodal side (towards the positive terminal) of the SDS–PAGE gel.
10. Add more transfer buffer A to the tank to cover the electrode panels by 2 cm.
11. Insert a magnetic stirrer bar and place the tank on the magnetic stirrer.
12. Insert the cooling block or cooling coil and set it to 15°C. [f]
13. Connect the tank to the power pack and transfer the proteins onto the PVDF membrane at 100 V for 1–3 h at 15°C, or at 10 V for 16 h (4°C).

Protocol 2. *Continued*

14. At the end of the transfer, switch off the power and disconnect the electrodes. Check the efficiency of transfer by visualization of the pre-stained molecular weight markers on the PVDF but not in the gel.
15. Proceed to the visualization stage (*Protocol 4* or *5*) or store the membrane (*Protocol 1*, footnote *d*) for later analysis.

[a] Transfer buffer A can be reused three to five times. Store it at 4°C for reuse.
[b] If more than one gel is to be run, a separate container is required for each gel.
[c] Most commercial apparatus can run several gels at once. If more than one gel is to be processed, a complete assembly cassette is required for each gel.
[d] Where more than one gel is involved, process them in parallel through steps 1–8.
[e] The translucent appearance should remain throughout the procedure.
[f] Alternatively, place the tank in a container of crushed ice.

Protocol 3. Semi-dry electrotransfer of proteins from an SDS–PAGE gel to a PVDF membrane

Equipment and reagents

- Two plastic storage containers, filter paper, PVDF membrane, methanol, and SDS–PAGE gel (see *Protocol 2*)
- Transfer buffer B: 39 mM Tris base, 48 mM glycine, 0.0375% (w/v) SDS, 20% (v/v) methanol[a]
- Semi-dry electrotransfer apparatus (Pharmacia or Bio-Rad)[b]
- Cellophane membrane (Pharmacia): this is required only if more than one gel is being processed

Method

NB: wear latex (or similar) disposable gloves during all stages of this procedure for your own protection to prevent contamination of the blot by proteins from your hands.

1. Remove the SDS–PAGE gel from the glass electrophoresis plates upon completion of the electrophoresis. Soak the gel in 200 ml transfer buffer B in a clean plastic container for 10 min at room temperature.[c]
2. Measure the dimensions of the gel accurately; the gel will shrink in size due to the methanol in transfer buffer B.
3. Cut 18 pieces of filter paper and one piece of PVDF membrane to the exact dimensions of the gel.
4. Wet the PVDF membrane with methanol. The PVDF membrane will change in appearance from opaque/white to translucent/grey as it becomes impregnated with the methanol.[d] Now immerse it in 200 ml transfer buffer B in a clean plastic container for 10 min at room temperature.
5. Soak the anode and cathode electrodes in distilled water for 10 min at room temperature.

6. Remove the anode plate from the water, and wipe off excess water with a soft paper towel.
7. Impregnate the first filter paper sheet (from step 3) with transfer buffer B. To do this, lay the paper on the surface of some transfer buffer B in a clean plastic storage container. Within seconds the paper will darken as it becomes impregnated with buffer. No white spots of dry filter paper should be evident.
8. Drain off the excess buffer and place the filter paper on the anode plate. Remove air bubbles from between the anode and the filter paper using a glass rod or test-tube as a roller to expel visible air bubbles.
9. Repeat steps 7 and 8 eight times ensuring exact superimposition of each sheet on the previous one.
10. Drain excess transfer buffer from the PVDF membrane (from step 4) and place it on top of the filter paper stack. Likewise, drain excess liquid from the SDS–PAGE gel (from step 1) and place it on top of the PVDF membrane. Again, ensure that no air bubbles are trapped between the layers.
11. Complete the stack with a further nine pieces of impregnated filter paper, laid on top of the SDS–PAGE gel, one sheet at a time as in steps 7 and 8.
12. Remove the cathode plate from the distilled water and wipe off excess water with a soft paper towel. Place the plate on top of the transfer stack.
13. Connect the stack to the power pack and transfer the proteins for 60 min at room temperature at 0.8 mA/cm^2 (calculated from the dimensions of gel, step 2).
14. After transfer, turn off the power and disconnect the electrodes. Recover the PVDF membrane and check the efficiency of transfer (*Protocol 2*, step 14).
15. Visualize the protein of interest (*Protocol 4* or *5*) or store the blot (*Protocol 1*, footnote *d*).

[a] Transfer buffer B can be reused up to about ten times. Store it at 4°C for a maximum of one month.
[b] As in *Protocol 2* more than one gel may be processed at the same time (see text).
[c] If processing more than one gel, use a separate plastic container for each gel.
[d] The translucent appearance should remain throughout the procedure.
[e] Orientate the gel so that lane 1 transfers to the left-hand side of the PVDF membrane.
[f] If more than one gel is to be transferred in the same stack, the order of the layers should be: (i) anode; (ii) three layers of impregnated filter paper; (iii) six layers of impregnated filter paper; (iv) PVDF membrane; (v) first SDS–PAGE gel; (vi) six layers of impregnated filter paper; (vii) cellophane membrane soaked in transfer buffer B; (viii) layers (iii)–(vii) repeated for the second gel; (ix) three layers of impregnated filter paper; (x) cathode.

2.4 Immunodetection of proteins on dot blots and Western blots

2.4.1 General strategy

The basis of identification of a protein of interest on a dot blot is immunological. Similarly, once the proteins in a sample have been separated by electrophoresis and an exact print of the gel has been captured on a Western blot membrane, it is also possible to investigate the properties of individual proteins using an immunological strategy. In each case, the presence of protein X in any sample is identified by exposure of the dot blot or Western blot membrane to an antibody specific for that protein (the primary antibody). The interaction of the primary antibody with protein X is identified by the sub-

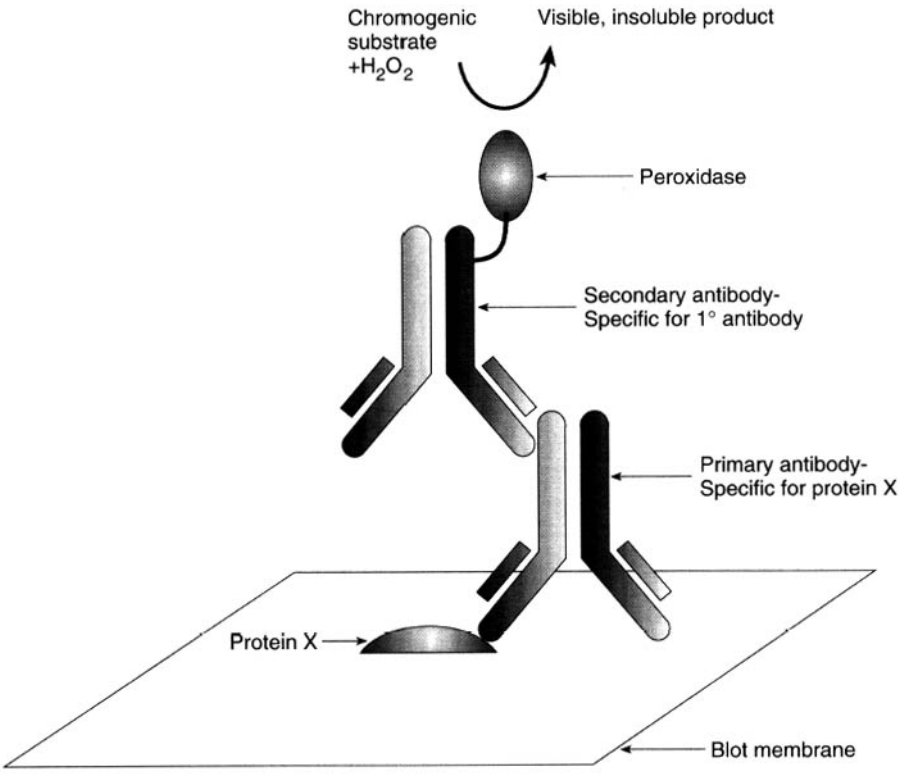


Figure 2. Schematic representation of immunoblot detection. The presence of an antigen on a membrane is identified using a small cascade of protein–protein interactions which results in the deposition of a coloured insoluble product (or other signal) at that location on the membrane. The cascade of interactions comprises a primary antibody binding to the antigen and a subsequent secondary antibody (with covalently attached marker enzyme) binding to the primary antibody.

sequent interaction of an additional detecting protein with the primary antibody. The detecting protein can be Protein A or Protein G, both of which are IgG binding proteins from bacteria (11) or a secondary antibody which binds to the primary immunoglobulin. In each case, the detecting protein is conjugated to a marker such as ^{125}I, alkaline phosphatase, or peroxidase. The complex is then visualized by exposure to an X-ray film (in the case of ^{125}I) or by the use of a suitable chromogenic substrate in the case of the two enzyme tags to generate an insoluble product, which precipitates at the site of production, thereby marking the location of the immune complex with a visible stain. This is displayed in cartoon form in *Figure 2*.

Protein A and Protein G interact non-covalently with a subset of immunoglobulin isoforms from a variety of species, as summarized in *Table 1*. ^{125}I-labelled forms of Proteins A and G are commercially available from ICN.

A band on a blot is typically the first piece of evidence for the expression of the protein of interest in the system under study. The classification of a sample as one which contains the protein of interest is tentative. False positive immunoreactivity is the most significant problem, particularly when using crude polyclonal serum as the primary antibody source. False positive reactions can arise as a consequence of the immunorecognition by the primary (or secondary) antibody of components other than the one intended.

Where the cross-reacting component is unrelated in structure to protein X, it should not be identified when using an affinity purified polyclonal antibody, or if monoclonal antibodies are employed. However, where the cross-reacting components exhibit primary sequence similarities with protein X, they may continue to cross-react even with an affinity purified antibody or a monoclonal antibody.

Table 1. Immunoglobulins recognized by Protein A and Protein G[a]

Immunoglobulin	Protein A	Protein G
Human IgG1, IgG2, IgG4	++	++
Human IgG3	–	++
Mouse IgG1	+	+
Mouse IgG2a, IgG2b, IgG3	++	++
Rat IgG1	+	+
Rat IgG2a	–	++
Rat IgG2b	++	+
Rat IgG2c	++	++
Rabbit IgG	++	++
Sheep IgG1	–	++
Sheep IgG2	+	++
Goat IgG1	+	++
Goat IgG2	++	++

[a] Adapted from ref. 12. No interaction (–); weak interaction (+); strong interaction (++).

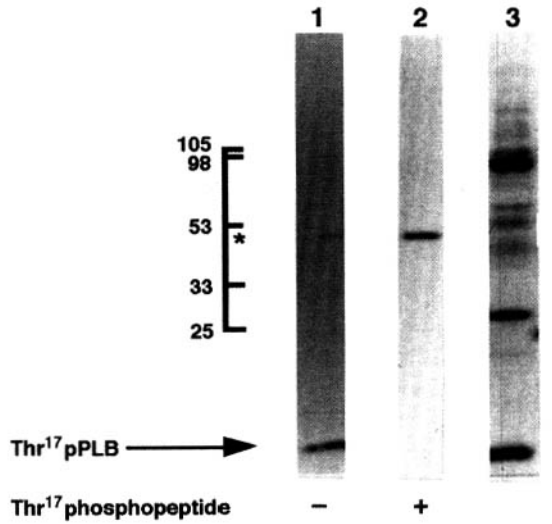


Figure 3. Immunological identification of a protein on a Western blot: differentiation between specific and 'unrelated' immunoreactivity. Cardiac muscle homogenates were phosphorylated *in vitro* by calmodulin-dependent kinase II using [γ-^{32}P]ATP, the proteins (100 μg) separated by SDS–PAGE, and transferred to a PVDF membrane. The phosphorylation of the protein, phospholamban, on Thr-17 (Thr^{17}pPLB) was detected with a polyclonal antibody PT-17 (lane 1) using the chemiluminescent detection protocol (*Protocol 5*). Two bands are apparent in lane 1 at M_r 48 kDa (*) and ~ 6 kDa (arrow). The inclusion of 1 μM phospholamban peptide phosphorylated at Thr-17 with the PT-17 antiserum (lane 2) prevents recognition of Thr^{17}pPLB (6 kDa) but not the 48 kDa protein. This confirms the specific nature of the recognition of Thr^{17}pPLB, and identifies the interaction of antiserum with the 48 kDa protein as unrelated. The total repertoire of ^{32}P-labelled phosphoproteins is displayed in lane 3 by exposure of a segment of the blot to X-ray film (ten days at –70°C). Adapted from data in ref. 5.

The use of two identical (replica) copies of the blot, one developed with the primary antibody alone and the other with the primary antibody plus excess (1–10 μM) antigen (pure protein X) is worthwhile as a means of investigating the nature of the immunorecognition by a polyclonal serum. A signal on the first blot, which is absent from the second, provides some confidence that the immunoreactivity is truly related to the presence of the antigen of interest (protein X). This is illustrated in Section 2.4.3 and *Figure 3*.

2.4.2 Immunodetection of antigens on blots using a peroxidase-linked antibody

Protocols 4 and *5* describe methods for detecting a protein of interest on a dot blot or Western blot using a secondary antibody linked to the enzyme peroxidase as the detection system. *Protocol 4* uses a colorimetric end-point, whereas *Protocol 5* employs enhanced chemiluminescence, which can be used

for quantitative estimations (Section 2.4.3). Both protocols use the same basic detection system. The primary antibody is often 'home-made', i.e. an antibody is raised in a laboratory animal (usually a rabbit or mouse) by injecting the purified antigen (protein X) in one of a number of well-established protocols (4). Alternatively, if one is investigating the expression of a previously characterized protein, antibodies may be available commercially (e.g. from UBI, Sigma, or Transduction Labs). In each case, the optimal dilution of the primary antibody required for detection of the antigen on the blot must be established empirically in pilot experiments. If the antibody is obtained commercially, the supplier may be able to provide guidance. Generally, one should start by trying dilutions of 1:100, 1:1000, and 1:5000.

High quality peroxidase-conjugated secondary antibodies for the detection of the primary antibody are available commercially (Jackson Inc. or Sigma). These need to be specific for the host species and immunoglobulin class of the primary antibody. Thus, if a rabbit IgG is the primary antibody, one would use an anti-rabbit IgG antibody as the secondary antibody. As with the primary antibody, the optimal dilution of the secondary antibody has to be determined empirically in a pilot experiment; the supplier of the antibody can usually provide guidance as to the dilution range to try.

Protocol 4. Immunodetection of proteins on a blot using a peroxidase-linked antibody: colorimetric end-point

Equipment and reagents

- Nitrocellulose or PVDF membrane (the blot) for analysis (see *Protocols 1–3*)[a]
- TBS/milk solution: TBS (see *Protocol 1*) containing 2% (w/v) non-fat dried milk powder
- Plastic storage containers (two per blot), filter paper, TBS (see *Protocol 1*)
- Orbital shaker (Stuart)
- Primary antibody (see text)
- Polythene sheeting (25 cm wide)
- Electric heat sealer (BDH)
- Peroxidase-conjugated secondary antibody (Jackson Inc. or Sigma)
- Peroxidase substrate: 50 mg 4-methoxy-1-naphthol (Aldrich)[b]
- Methanol (ice-cold)
- Hydrogen peroxide: 30% as supplied; store at 4°C

Method

NB: wear latex (or similar) disposable gloves when handling the blot for your own protection and to prevent contamination by proteins from your hands.

1. Incubate the blot in 200 ml TBS/milk solution in a clean plastic container for 30 min at room temperature with constant shaking at 30 r.p.m.[a]
2. Dilute the primary antibody to the required working concentration in 10 ml of TBS/milk solution in a test-tube.[c]
3. Remove the blot from the TBS/milk solution and lay it on a piece of

Protocol 4. *Continued*

polythene sheeting (~ 4 cm larger than the blot in both dimensions). Lay a second sheet of polythene (same size) on top and heat seal three edges within 3 mm of the blot to form a bag.

4. Pour the diluted primary antibody solution (from step 2) into the polythene bag. Expel air bubbles from the solution through the unsealed edge of the bag. Heat seal the fourth edge to close the bag.
5. Incubate the sealed bag on a flat surface overnight at 4°C, or for 3 h at room temperature.
6. Cut off three sides of the bag and open it out. Remove the blot from the bag and discard the antibody solution.
7. Wash the blot in 100 ml TBS/milk solution in a clean plastic container for 5 min at room temperature with constant shaking at 30 r.p.m. Discard the wash solution and repeat the wash twice more.
8. Dilute the peroxidase-conjugated second antibody to the appropriate working concentration in 100 ml TBS/milk solution. Pour this onto the blot and incubate it at room temperature for 90 min with constant shaking at 30 r.p.m.
9. Wash the blot as described in step 7.
10. As soon as the washing has started, make up the peroxidase substrate. Dissolve 50 mg 4-methoxy-1-naphthol in 10 ml ice-cold methanol. Store it on ice for at least 10 min.[d]
11. When the blot has been washed, add the peroxidase substrate solution to 100 ml TBS containing 0.027% hydrogen peroxide.
12. Pour the substrate solution onto the blot and leave it for 5 min at room temperature with constant shaking at 30 r.p.m.
13. Discard the substrate solution and wash the blot with 100 ml deionized water as described in step 7.
14. Dry the blot between two layers of filter paper laid flat with a suitable weight on top to keep the blot flat. For a permanent record, photograph the blot.[b]
15. Quantify the immunosignals if desired (see Section 2.4.4).

[a] If several blots are being analysed, process each separately and in parallel.
[b] 4-methoxy-1-naphthol is superior to 4-chloronaphthol. It generates a more intense colour which does not fade as badly with time.
[c] This quantity of antibody solution is sufficient for 200 cm^2 PVDF membrane. Reduce the volume for smaller blots to conserve antibody.
[d] A flocculant precipitate will form as the methanolic solution of 4-methoxy-1-naphthol is mixed with the TBS in the next step if the 10 min incubation on ice is omitted.

Protocol 5. Immunodetection of proteins on a blot using a peroxidase-linked antibody: enhanced chemiluminescence

Equipment and reagents

- Nitrocellulose or PVDF membrane (the blot) for analysis (from *Protocols 1–3*)[a]
- TBST (Tris-buffered saline/Tween™): 50 mM Tris, 150 mM NaCl, 0.1% Tween 20™, pH 7.4
- TBST/milk solution: TBST containing 5% (w/v) non-fat dried milk
- Plastic storage containers, electric heat sealer, polythene sheeting, orbital shaker, primary and secondary antibodies (see *Protocol 4*)
- Chemiluminescence substrate (Amersham, Boehringer Mannheim, or Pierce)
- Saran Wrap (Dow Chemical Co.)
- X-ray film (Kodak; X-Ograph)
- Developer and fixer (Kodak; X-Ograph) diluted according to the manufacturer's instructions
- Photographic dark-room and associated facilities

Method

NB: wear latex (or similar) disposable gloves when handling the blot for your own protection and to prevent contamination by proteins from your hands.

1. Incubate the blot in 200 ml TBST/milk solution in a clean plastic container for 30 min at room temperature with constant shaking at 30 r.p.m.[a]
2. Dilute the primary antibody to the required working concentration in 10 ml TBST/milk in a test-tube.[b]
3. Follow *Protocol 4*, steps 3–6 but use TBST/milk solution instead of TBS/milk solution.
4. Rinse the blot twice with 100 ml TBST.
5. Wash the blot with 100 ml TBST in a clean plastic container for 5 min at room temperature with shaking at 30 r.p.m. Repeat the wash.
6. Dilute the peroxidase-conjugated secondary antibody to the appropriate working concentration in 100 ml TBST. Pour it onto the blot and incubate the blot for 90 min at room temperature with shaking at 30 r.p.m.
7. Rinse the blot twice with 100 ml TBST.
8. Wash the blot at room temperature with shaking at 30 r.p.m. once for 15 min and four times for 5 min, each time using 100 ml TBST.
9. Mix the chemiluminescence substrate solutions as directed by the manufacturer.[c]
10. Remove the blot from the TBST and drain off the excess liquid. Place the blot on a sheet of Saran Wrap on a level, flat surface. Layer 6 ml substrate solution (from step 9) evenly across the blot and cover it with a second piece of Saran Wrap to ensure that a film of substrate covers the entire surface of the blot. Incubate for 60 sec at room temperature.

Protocol 5. *Continued*

11. Remove the blot and drain off the excess substrate solution. Place the blot on a new piece of Saran Wrap and cover it with a second layer of clean Saran Wrap, taking care to avoid trapping air between the blot and the Saran Wrap. Immediately take the blot to the dark-room.
12. Working with only a dark-room safe light for illumination, place a sheet of X-ray film[d] on top of the blot. Leave it for 5 sec to 10 min, depending on the intensity of the chemiluminescence.
13. Develop the X-ray film for 4 min in the developer, rinse it for 30 sec in water, and fix it for 2 min in fixer. After this, the film is light-safe and can be washed extensively in water under normal illumination.
14. Hang the X-ray film from a clothes line (or similar) to dry for approx. 1 h.
15. If desired, quantify the immunosignal(s) as described in Section 2.4.4.

[a] If several blots are being analysed, process each separately and in parallel.
[b] This volume of solution is sufficient for 200 cm^2 PVDF membrane.
[c] For example, with Amersham reagents, mix 3 ml solution A with 3 ml solution B in a clean disposable vial for a blot of 12 cm × 16 cm.
[d] The dynamic range of the X-ray film can be extended by brief exposure to light from a photographic flash gun placed at a distance of 50–150 cm (to be optimized by the investigator). This pre-flashing will discolour the film slightly upon subsequent development (in step 13) but extends its sensitivity at the low end of the signal range.

2.4.3 Interpretation of Western blot data

An example of the type of data generated by Western blotting is given in *Figure 3*. In this example (5), total cardiac muscle ^{32}P-labelled phosphoproteins generated by activation of calmodulin-dependent protein kinase II have been separated by SDS–PAGE and transferred to a PVDF membrane. The separation of proteins as a function of their apparent molecular weight (M_r) has been achieved and a multitude of phosphoproteins is visualized in lane 3 by means of an autoradiograph. The phosphorylated form of phospholamban (Thr^{17} pPLB; $M_r \sim 6$ kDa) is identified by a crude polyclonal antiserum in lane 1, along with a second protein ($M_r \sim 48$ kDa). It was concluded that this second immunoreactive protein has no relation to the protein under study (phospholamban) by developing a second identical blot in the presence of excess soluble antigen (in this case, 1 μM of the Thr-17 phosphorylated phospholamban peptide). This is displayed in lane 2, in which the PLB-directed immunoreactivity ($M_r \sim 6$ kDa) is lost, but the unrelated component ($M_r \sim 48$ kDa) is still identified by the crude polyclonal antiserum.

2.4.4 Quantification of the immunosignals in Western and dot blots

In most applications, the ability to relate immunosignal intensity to the amount of protein X present is an advantage. Calibration of immunoblot data is feasible using simple calibration graphs which require standard amounts of purified antigen (protein X) to be included in each blot.

Table 2. Sensitivity ranges for different detection methods for the primary antibody[a]

	Visualization system				
Method of detection of primary antibody	**Radiolabel ^{125}I or ^{35}S**	**Peroxidase: colorimetric[b]**	**Peroxidase: chemiluminescence**	**Alkaline phosphatase: colorimetric[c]**	**Alkaline phosphatase: chemiluminescence**
Protein A or Protein G	10–100 ng	nd[d]	5 ng	nd[d]	nd[d]
α-IgG	1–10 ng	500 pg	< 1 pg	100 pg	~ 10 pg

[a] Data taken from refs. 12 and 13.
[b] Using 4-chloronaphthol and DAB.
[c] Using BCIP and NBT.
[d] nd; not determined.

The sensitivity of immunodetection in Western blot (or dot blot) format is affected by the method chosen for the detection of the primary antibody. *Table 2* shows the sensitivity ranges for a variety of detection routes in common use. Detection routes using radiolabelled components (e.g. ^{125}I- or ^{35}S-labelled secondary antibodies or Proteins A or G) offer the simplest means of quantification as the bands of interest can be excised from the blot and the associated radioactivity measured by gamma or scintillation counting as appropriate. More conveniently, densitometric analysis of autoradiographs can be used. Densitometry of colorimetric and chemiluminescent immunosignals can be employed. Whole band densitometry, where both the optical density and area of the immunostain are computed, is the preferred method, and several commercial densitometry packages are available for this purpose (e.g. from Phoretix International Ltd.). Colorimetric end-points (*Protocol 4*) have a limited dynamic range—up to about a ~ 60-fold range in optical density but closer to a tenfold range in terms of antigen quantity (14). Chemiluminescence output (*Protocol 5*) displays a greater dynamic range, but in our experience the signal does not show a linear relationship to antigen concentration. *Figure 4* shows a calibration curve for the Thr-17 phosphorylated forms of phospholamban (see Section 2.4.3) probed with a polyclonal antibody specific for this antigen. The dynamic range in terms of antigen concentration approaches three orders of magnitude (0.2–63 pmol), although the immunosignal intensity to antigen relationship is sigmoid in nature over this range (15).

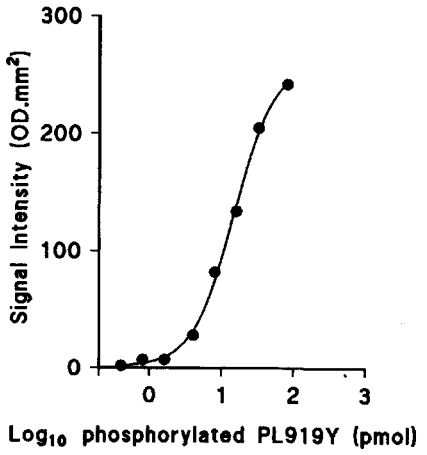


Figure 4. Relating immunosignal intensity to antigen quantity. Phosphorylated peptide antigen (Thr^{17}pPLB peptide, 0.2–63 pmol) was detected using polyclonal antibody PT-17 (5) following SDS–PAGE and electrotransfer to a PVDF membrane. Immunorecognition was performed using chemiluminescent substrates (*Protocol 5*) and the signal intensity analysed by density and area, displayed as the product of these values. A logistic sigmoidal relationship over two to three log units of antigen concentration is observed.

2.4.5 Use of Western blotting to follow the post-translational chemical modification of a protein

Monitoring protein expression involves the description of the stages through which a protein must pass *en route* to the mature form, and the definition of any chemical modification of the protein which might control its function. Specific examples of the chemical modification of proteins are discussed in a companion volume (16, 17). An initial indication of the presence of chemical modification of the protein (e.g. phosphorylation, ADP ribosylation, nitrosylation) can sometimes be made by Western blotting since the M_r of the protein under study will be influenced by its state of modification (18, 19). Post-translational events such as proteolysis, glycosylation, are more difficult to investigate by Western blotting, since only the steady state level of any intermediates can be measured, rather than the chronological sequence of events. The chronology of post-translational modifications is best described using pulse-chase labelling techniques in combination with immunoprecipitation of the protein of interest. These techniques are described in detail in Section 2.5. However, samples from such experiments typically are also analysed by SDS–PAGE and Western blotting to provide additional data on post-translational mechanisms.

2.5 Pulse-chase labelling and immunoprecipitation of proteins

2.5.1 Strategy

Pulse-chase labelling is a technique in which a radiolabelled precursor (normally an amino acid, but it could be a specific monosaccharide or fatty acid if glycosylation or lipid modification of the protein is being studied) is made available to a cell for a short period of time. Proteins synthesized during this period will incorporate the radiolabelled precursor and will thereby be detectable (e.g. by sequential immunoprecipitation, electrophoresis, and autoradiography). This 'pulse' is followed by a chase period in which the radiolabelled precursor is removed and the cells are exposed to an excess of unlabelled precursor (a simple method for doing this is to add a large excess of unlabelled precursor to 'dilute out' the labelled precursor). Proteins synthesized during the chase will be unlabelled and, therefore, remain undetected by autoradiographic techniques. Thus, the fate of labelled proteins synthesized during the brief period of radioactive precursor administration can be followed as a function of time.

Since large numbers of radioactive proteins are synthesized simultaneously by the cell, the individual protein of interest needs to be separated from the others using immunoprecipitation. In this technique antibodies specific to the protein of interest (protein X) are immobilized on an insoluble particulate support (e.g. agarose, Sepharose) to form an affinity matrix for the recovery of protein X. After interaction with protein X, the antibody affinity particles

are harvested by centrifugation and washed free of unrelated cellular components prior to analysis by SDS–PAGE and autoradiography.

2.5.2 Basic methods

Protocol 6 describes pulse-chase labelling of proteins. It can also be used simply to label proteins with a radioactive precursor, in which case the chase is omitted.

Protocol 7 describes the immunoprecipitation of specific antigens using a Protein A or Protein G matrix—the choice of Protein A or Protein G depends on the nature of the primary antibody against protein X (see *Table 1*). As in the case of the other techniques utilizing specific antigen–antibody recognition, the quantity of immune serum required for the preparation of the immune beads must be optimized in pilot experiments (compare Section 2.4.2). Similarly, the amount of pre-immune or non-immune serum used in the preparation of the pre-clearing beads must also be optimized. If non-immune serum is used instead of pre-immune serum, it must be from the same animal species as the immune serum and of the same immunoglobulin isoform.

Protocol 7 uses autoradiography and densitometry to quantify the immunoprecipitated antigen. PhosphorImage technology represents an alternative to autoradiography. To use this, follow the PhosphorImager manufacturer's instructions. Expose the PhosphorImage plate to bright light, wrap the SDS–PAGE gel in a single layer of Mylar film, and lay the gel face down on the PhosphorImage plate positioned within a plate cassette. Close the cassette and store it flat at room temperature for one to seven days. Develop the plate using the PhosphorImage reader. The sensitivity of PhosphorImaging is about ten times that of autoradiography.

Protocol 6. The pulse-chase method for labelling cellular proteins

Equipment and reagents

- Pulse medium lacking the specific amino acid being used for labelling (e.g. media for metabolic labelling from Life Technologies)
- Radiolabelled amino acid for labelling cellular proteins: [^{35}S]cysteine or [^{35}S]methionine (ICN Flow)
- Membrane filters (0.22 μm pore size, sterile) (Life Technologies)
- Appropriate tissue culture cells growing in 6-well tissue culture dishes[a] (Gibco, Nunc)
- PBS: 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl pH 7.2
- Tissue culture incubator with humidified 95% air:5% CO_2 atmosphere
- Chase medium: pulse medium containing 1 mM of the non-radioactive amino acid used for labelling
- Lysis buffer: 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM sodium vanadate, 0.1 mM PMSF, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40
- Rubber policeman
- Reagents for protein assay[b]

Method

1. Supplement the pulse medium with ~ 1 MBq/ml ^{35}S-labelled amino acid. Pass the medium through a sterile 0.22 μm filter membrane into a sterile receptacle and warm this radioactive pulse medium to 37 °C.

2. Aspirate the culture medium from the cells in the culture dishes. Dispense 5 ml per well of PBS (pre-warmed to 37°C) onto the cells. After 30 sec remove the PBS by aspiration.
3. Dispense ~ 5 ml of warm radioactive pulse medium onto the cells in each well. Incubate the dishes at 37°C in an atmosphere of 95% air:5% CO_2 in a humidified incubator for 0.5–2 h.[c]
4. Remove the radioactive medium by aspiration.[d]
5. If the proteins are simply being labelled, and no chase is involved, proceed directly to step 9.
6. If a chase is involved, wash the cells with the warm (37°C) chase medium (5 ml/well).
7. After 30 sec, remove the chase medium and replace it with fresh, warm chase medium (5 ml/well). Place the dishes in the incubator at 37°C.
8. At predetermined intervals after starting the chase, take a dish of cells from the incubator. Remove the medium.
9. Wash the cells with ice-cold PBS (5 ml/well).
10. Remove the PBS and add 300 µl lysis buffer per well to lyse the cells. Scrape the cells from the plastic culture dish using a rubber policeman. Transfer the suspensions to microcentrifuge tubes and vortex for 15 sec. Incubate the tubes at 4°C for 30 min to ensure the solubilization of the cells.
11. Centrifuge the lysates at 13000 *g* for 5 min at 4°C. Transfer each supernatant to a new microcentrifuge tube.
12. Determine the protein concentration of each lysate.[b]
13. Proceed to the analysis of the radiolabelled proteins by immunoprecipitation (*Protocol 7*).

[a] Cells can also be grown in plastic Petri dishes; organ culture can also be used.
[b] Any standard method may be used (20).
[c] The exact labelling period must be optimized for each application; shorter or longer times than indicated here may be appropriate.
[d] Disposal methods for this and other radioactive materials must comply with local radiochemical regulations.

Protocol 7. Immunoprecipitation of proteins[a]

Equipment and reagents

- Protein G– or Protein A–Sepharose or agarose (Pharmacia Biotech or Sigma)
- PBS and lysis buffer (see *Protocol 6*)
- Microcentrifuge, with refrigeration or positioned in a cold room (Eppendorf, Heraeus, or Sorvall)

Protocol 7. *Continued*

- Pre-immune or non-immune serum (see text)
- Primary antibody raised against the protein of interest
- Rotary wheel mixer (Stuart)
- Cell lysates for analysis from radioactive pulse-chase experiments (see *Protocol 6*)
- Wash buffer: lysis buffer (see *Protocol 6*) without Triton X-100 or Nonidet P-40
- Materials and equipment for SDS–PAGE
- 3MM filter paper (Whatman)
- 2 × SDS sample buffer: 125 mM Tris–HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 0.05% (w/v) bromophenol blue
- Mylar wrap (Raytech)
- X-ray cassette fitted with intensifying screens (Kodak; X-Ograph)
- Materials for autoradiography (see *Protocol 5*)

Method

1. Resuspend Protein G– or Protein A–Sepharose in PBS to form a 50% (v/v) slurry. Dispense 200 μl into a microcentrifuge tube. Centrifuge at 13000 *g* for 60 sec at room temperature to sediment the Sepharose beads.
2. Remove the supernatant and add 1 ml lysis buffer to the pellet of beads. Resuspend the beads using gentle manual agitation. Centrifuge as in step 1, and repeat the wash procedure twice more.
3. Resuspend the washed beads as a 50% (v/v) slurry in lysis buffer.
4. Dispense 100 μl aliquots of the slurry into microcentrifuge tubes.
5. To one aliquot of the beads add 11 μl pre-immune serum. These will be the pre-clearing beads. To another aliquot of beads, add 20 μg primary antibody.[b] These will be the immune beads.
6. Incubate both samples for 90 min at 4°C on a rotary wheel mixer.
7. Wash the pre-clearing beads and the immune beads separately as described in step 2. Resuspend the washed beads in lysis buffer as 50% (v/v) slurries.[c]
8. For each radioactive cell lysate, mix an aliquot equivalent to 100 μg protein with 20 μl of the pre-clearing bead slurry, in a final volume of 100 μl made up with lysis buffer. Incubate the samples for 90 min at 4°C on a rotary wheel mixer.
9. Centrifuge the samples at 13000 *g* for 1 min at 4°C. Transfer the supernatants to fresh microcentrifuge tubes containing 20 μl of the immune bead slurry. Incubate the samples for at least 90 min at 4°C on a rotary wheel mixer.[d]
10. Centrifuge the sample at 13000 *g* for 1 min at 4°C and remove the supernatants.[e] Wash the beads twice in 1 ml lysis buffer and twice in 1 ml wash buffer using gentle manual agitation to resuspend the pellet of beads, and centrifugation at 13000 *g* for 1 min at 4°C to recover the beads.
11. Remove the final wash buffer. Add 40 μl of 2 × SDS sample buffer.

Vortex for 10 sec and then boil for 2 min. Centrifuge at 13000 *g* for 1 min at room temperature.

12. Remove the supernatants and load an aliquot of each (the same volume for all the samples)[f] in the wells of an SDS–PAGE gel.
13. Run the electrophoresis as advised by the electrophoresis apparatus manufacturer.
14. Stain the gel with Coomassie Blue to reveal the protein bands (9).
15. Dry the stained gel onto a sheet of Whatman 3MM paper.
16. Working in a dark-room under safe light illumination, place a sheet of X-ray film inside an X-ray cassette fitted with intensifying screens.
17. Place the dry SDS–PAGE gel, in a single layer of Mylar wrap, face down on the X-ray film. Secure the film to the gel with sticky tape. Draw several lines from the 3MM paper backing of the SDS–PAGE gel onto the X-ray film with a permanent marker pen (for future alignment of the gel and its autoradiogram).
18. Close the light-tight X-ray cassette. Store it at –70°C for 7–14 days.
19. Remove the cassette from the freezer and leave it at room temperature for 30 min. Working in a dark-room under safe light illumination, open the cassette and take out the SDS–PAGE gel. Remove the sticky tape from the X-ray film and develop it as described in *Protocol 5*, steps 13 and 14. Autoradiographic signals may be quantified by densitometry (see Section 2.4.4).

[a] Method adapted from ref. 21.
[b] Optimization of the quantity of primary antibody required for the immunoprecipitation needs to be evaluated empirically in pilot experiments.
[c] The beads may be stored at this point for 24–48 h at 4°C without loss of activity.
[d] An increase in incubation time (up to 20 h) may increase the efficiency of the immunoprecipitation. However, non-specific interactions of cell lysate proteins with the beads will also increase.
[e] Retain the supernatant for further analysis. Proteins may be recovered by TCA precipitation and the precipitates subjected to SDS–PAGE if the recovery of precipitable material is of interest.
[f] The sample volume for SDS–PAGE analysis must be determined in pilot experiments, and is usually based on the amount of radioactive protein present. This can be determined by scintillation counting of an aliquot.

2.5.3 Use of pulse-chase and immunoprecipitation to study post-translational processing of the insulin receptor

Post-translational modification of the insulin receptor (summarized in *Figure 5A*) is extensive and will be used as a specific example to describe how post-translational events may be studied in general. The insulin receptor is a plasma membrane protein containing two types of subunit, the α-subunit of 135 kDa which possesses the ligand (insulin) binding site and a β-subunit of

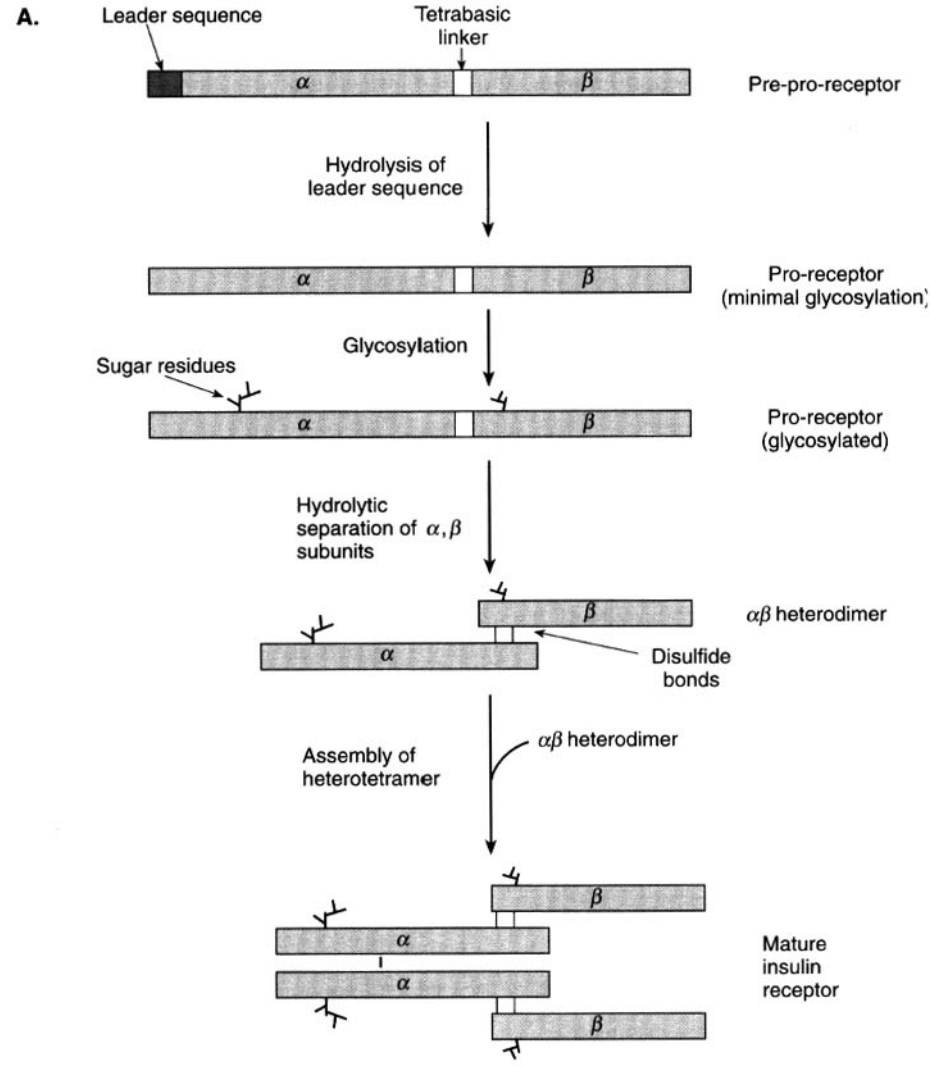
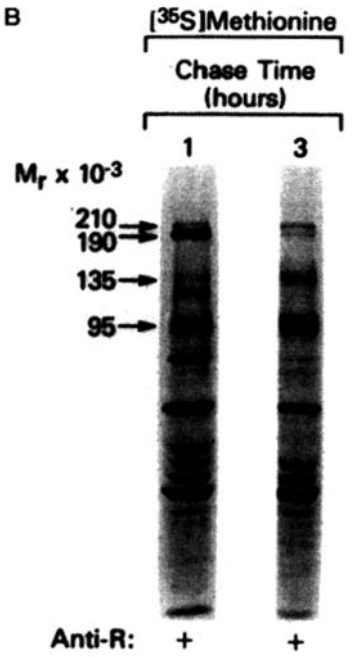


Figure 5. Post-translational processing of the insulin receptor. (A) Schematic representation of the pathway of synthesis of the insulin receptor. (B) Immunoprecipitates of the insulin receptor after 1 h and 3 h of chase. Proteins of 210, 190, 135, and 95 kDa (arrows) were evident in immune serum precipitates only. All other bands were present in both immune and non-immune precipitates (and are thus non-specific). Reproduced with permission from ref. 22.



90 kDa, which transduces the signal of insulin binding into the cell. Two copies of each subunit assemble to form a heterotetramer linked by disulfide bonds. The synthesis of the insulin receptor initially involves a single polypeptide encompassing both subunits arranged in tandem. The processing of this polypeptide precursor involves the removal of a pre-sequence (or leader sequence), glycosylation, proteolytic separation of the α- and β-subunits, and targeting of the receptor to the plasma membrane.

The temporal relationship between these molecular species was explored using a pulse-chase and immunoprecipitation strategy (22). An example of the data generated by this approach is shown in *Figure 5B*. This reveals the intermediates involved throughout the processing of the insulin receptor. Protein species of 95 kDa, 135 kDa, 190 kDa, and 210 kDa were specifically immunoprecipitated with an antibody to the insulin receptor; the other bands detected in this experiment were also seen following precipitation with non-immune serum and, therefore, can be disregarded. Shortly after synthesis of the insulin receptor the concentration of the 190 kDa species is at its highest. This represents the minimally glycosylated pro-receptor which is generated following removal of the pre-sequence in the lumen of the endoplasmic reticulum. This decays in abundance with time and this decrease is accompanied by a relative increase in abundance of the 210 kDa species (the fully glycosylated pro-receptor) and substantial increases in the concentrations of the 135 kDa (α-subunit) and 95 kDa (usually denoted 90 kDa, β-subunit) species.

In this way, the sequence of intermediates in the maturation of the insulin receptor was described. The processing of any other protein of interest can be analysed in a manner similar to this example. Furthermore, the state of glycosylation of the intermediates may be examined in a Western blot probed with an appropriate lectin and a labelled monosaccharide ligand.

2.6 Examination of protein expression by immunomicroscopy

The final stage in the investigation of protein expression using antibodies to the protein of interest is immunomicroscopy. This lends itself to two major types of investigation, one of which aims to establish the exact cellular source of protein X in an isolated tissue, and the second seeks to establish the location of protein X within individual cells. These can both be addressed by light microscopy, although higher resolution definition can be obtained using immunoelectron microscopy. Individuals interested in this latter technique are directed to ref. 23 for detailed descriptions and methods.

The identification of protein X expression in a tissue extract (by dot blot or Western blot) does not provide information concerning the cell types expressing the protein within that tissue. Histological examination of sections of the tissue probed with the specific protein X antibody and counterstained with independent markers of tissue structure (e.g. haematoxylin and eosin or cell type-specific monoclonal antibodies) is required to identify the cellular origin of protein X expression. This can be accomplished using techniques such as immunohistochemistry or immunocytochemistry. A version of it, immunoperoxidase microscopy, is described in *Protocol 8*. It uses a similar immunoperoxidase staining technique, as outlined in *Figure 2*, but on thin sections of a tissue rather than on a blot. *Protocol 8* also utilizes an antibody–biotin–streptavidin detection system that provides amplification of the peroxidase signal due to the multivalent interactions of biotin and streptavidin. As in the case of immunoprecipitation (Section 2.5.2), care has to be exercised in choosing the primary and secondary antibodies; the latter must react with the Ig isoform and host species of the primary antibody. Moreover, it should not react with Ig endogenous to the tissue specimen.

Protocol 8. Immunoperoxidase microscopy

Equipment and reagents

- Tissue sample for investigation
- Mould for cryopreservation of tissue
- O.C.T. embedding compound (BDH)
- Isopentane
- Liquid nitrogen
- Cryostat
- Coverslips and slides (Phillip Harris)
- Coverslip carrier (Phillip Harris)
- Paraformaldehyde: 4% solution in water
- 50 ml plastic container (e.g. multichannel pipette reservoir)
- TBS (see *Protocol 1*)
- TBS/gelatin: TBS plus 0.2% (w/v) gelatin
- Phenylhydrazinium chloride: 0.01% (w/v) solution in TBS

- Primary antibody
- Unconjugated serum from the host species of origin of the secondary antibody (Sigma or Jackson Immunochemicals)
- Parafilm (BDH)
- 10 cm × 10 cm square plastic Petri dish: line the base of the dish with damp tissue paper to form a humidified chamber when closed
- Biotinylated secondary antibody (Sigma or Jackson Immunochemicals)
- Streptavidin biotinylated peroxidase (Vector, Sigma, or Jackson Immunochemicals)
- 3-amino-3-carbazole
- *N,N*-dimethylformamide
- 50 mM sodium acetate pH 5
- Hydrogen peroxide (30% as supplied)
- Substrate solutions: 0.02% (w/v) 3-amino-3-carbazole in 5% (v/v) *N,N*-dimethylformamide in 50 mM sodium acetate buffer pH 5 plus 0.01% H_2O_2—make this up just before use
- Nail varnish
- Light microscope fitted with photography attachments

Method

1. Remove the tissue of interest from the organism under study and place it in a mould containing a drop of O.C.T. embedding compound. Immerse the mould in isopentane cooled in liquid N_2.[a]
2. Cut sections (8–15 μm) of the frozen tissue on a cryostat. Mount the sections on coverslips by thawing them on the glass and allowing them to dry in the air. Place the coverslips in a coverslip carrier for ease of handling multiple samples. Incubate the sections with 50 ml of 4% paraformaldehyde in a plastic container at room temperature for 20 min to fix the specimens.
3. Discard the paraformaldehyde and wash the specimens with 50 ml TBS for 5 min at room temperature with occasional gentle agitation (e.g. raise and lower the coverslip carrier from time to time). Discard the TBS and repeat the wash step twice more.
4. Incubate the specimens with 50 ml TBS/gelatin for 30 min at room temperature to block non-specific protein binding sites.
5. Discard the TBS/gelatin solution, and add 50 ml of 0.01% phenylhydrazinium chloride in TBS. Incubate at room temperature for 30 min to inactivate endogenous peroxidase activity.
6. Discard the phenylhydrazinium chloride solution, add 50 ml TBS/gelatin, and leave the sections for 1 min at room temperature.
7. Dilute the primary antibody[b] in TBS/gelatin containing 1% (v/v) unconjugated serum. Lay the coverslips, specimen uppermost, on a layer of Parafilm on top of the damp tissue paper in the square Petri dish. Pipette sufficient of diluted primary antibody onto the coverslips to cover the specimens (500 μl is generally sufficient, less if the specimen is small). Replace the Petri dish lid and incubate at room temperature for 2 h.
8. Remove the coverslips from the Petri dish and place them in the coverslip carrier. Rinse them twice in 50 ml TBS. Wash the coverslips three times by immersing them in 50 ml TBS at room temperature for 5 min on each occasion. Agitate as in step 3.

Protocol 8. *Continued*

9. Dilute the biotinylated secondary antibody 1:50 in TBS/gelatin. Place the coverslips in the Petri dish chamber as in step 7 ensuring the tissue paper is still damp. Cover the specimens with 500 μl diluted secondary antibody. Replace the lid and incubate at room temperature for 1 h.
10. Wash the coverslips as described in step 8.
11. Dilute the streptavidin biotinylated peroxidase 1:400 in TBS/gelatin. Place 500 μl of this on each specimen as described in step 7. Incubate as before at room temperature for 1 h.
12. Wash the specimens as in step 8.
13. Immerse the coverslips in 50 ml substrate solution at room temperature for 5–20 min. Stop the enzyme reaction by immersing the coverslips in deionized water.
14. Mount the coverslips on slides with the specimen sandwiched between the two glass surfaces. Dry the glass around the edge of the coverslip and seal it with nail varnish. View the specimens using a light microscope. Photograph appropriate areas to record the results.

[a] *The moulds can be stored at –70°C for several months, if required.*
[b] The dilution required must be predetermined in pilot experiments; a 1:50–1:500 dilution is usually required.

One problem which can arise with immunomicroscopy is a failure of the primary antibody to interact with protein X. The fixation of tissue in paraformaldehyde (*Protocol 8*) can alter the chemical structure of protein X (e.g. by extensive covalent cross-linkage) and thus mask antigenic determinants, thereby preventing recognition by the antibody. A variety of other fixation protocols is available (24) and alternative procedures should be investigated if the standard method (*Protocol 8*) proves unsuccessful.

The localization of protein X within a cell is the second worthwhile objective which can be satisfied by immunomicroscopic localization. Although the immunoperoxidase technique can be applied to single cells, it is not the preferred detection technique as the diffusion of the peroxidase products prevents precise immunolocalization. Instead, detection using fluorescently labelled secondary antibodies is preferred (immunofluorescence microscopy). In this technique, cells in suspension, or those growing as a monolayer on a coverslip, are fixed using ethanol or glutaraldehyde, permeabilized and exposed to the primary antibody. The interaction of this antibody with protein X is then visualized by the subsequent binding of a secondary antibody specific for the primary antibody, to which a fluorescent tag (such as fluorescein or rhodamine) has been attached. These fluorescent secondary antibodies are available commercially (Sigma or Jackson). The location of protein X within the cell

can be determined by viewing the sections in a fluorescence microscope, and the resolution of this technique can be improved still further by application of confocal microscopy in which thin (~ 1 μm) planes of focus of the specimen can be examined by precision focusing of the laser light illumination.

3. Monitoring of protein expression by epitope tagging

In some instances, the need to develop a specific, versatile, and well-characterized antibody to the protein of interest presents a limit to experimental progress. In this situation, one solution is the insertion of a new epitope, for which a monoclonal antibody of the desired quality is already available, into the primary sequence of the protein of interest. The epitope for an antibody (i.e. the amino acids which form the binding site for the antibody) usually comprises relatively few amino acids from the target protein, and in some cases these can be contiguous in the primary sequence. Thus, the insertion of an extra 4–12 amino acids (depending on the example) is often sufficient to create an epitope for a pre-existing monoclonal antibody (an epitope tag). Several of these epitopes are described in *Table 3*.

Once the epitope tag has been inserted in the protein of interest then the full range of immunological techniques for studying protein expression (Section 2) become available for this protein.

Insertion of an epitope tag is performed by addition of an oligonucleotide encoding the epitope tag to the cDNA for the protein of interest. This extended form of the protein of interest can then be expressed in appropriate cell lines using a suitable plasmid vector. Most frequently, epitopes are inserted at the ends of the coding region of the protein of interest to generate either an N-terminal or a C-terminal epitope tag. Several companies offer vectors containing the tag sequence juxtaposed with a multiple cloning site to facilitate insertion in tandem of the cDNA of interest (Novagen and Invitrogen). Alternative sites of insertion have also been described, for instance, at a unique restriction site within the cDNA sequence of the protein of interest (31).

The insertion of the additional primary sequence (the epitope tag) is assumed to have no bearing on the enzymatic or structural properties of the protein under study. This has been confirmed in many examples, but needs to be considered with each application. The BTAG insert (*Table 3*) is noteworthy in that it introduces an uncharged segment of amino acid sequence as the epitope tag, and may, therefore, produce the least perturbation to the structure of protein X (26).

In addition to the practical advantage of obviating the need for a good specific antibody to protein X, epitope tagging facilitates the examination of the behaviour of mutant forms of a protein in cells which also express the endogenous wild-type protein. The epitope tag can be inserted exclusively in the mutant forms of the protein, thereby allowing a comparison in the

Table 3. Epitope tag sequences

Tag and source	Epitope sequence[a]	Specific antibody (commercial source)	Reference
Bovine papillomarvirus L1 protein	DTYRYI	AU1 (BabCo)	25
BTAG	QYPALT	D11, F10	26
FLAG	DYKD	α-FLAG M1 (International Biotechnology Inc.)	27
His tag	HH_nH[b]	α-His tag (Invitrogen)	
Influenza virus haemagglutinin	YPYDVPDYA	12CA5 (BabCo)	28
Myc	QQKLISEEDLN	9E10 (Invitrogen)	29
SV5	GKPIPNPLLGLD	α-SV5 (Invitrogen)	
T7 gene 10	MASMTGGQQMG	α-T7 gene 10 (Novagen)	30
Xpress	DLYDDDDK	α-Xpress (Invitrogen)	
Heart muscle kinase phosphorylation site (cAMP-dependent protein kinase)	RRAS	HMK phosphorylation site	

[a] Amino acid sequence (single letter designations).
[b] The His tag antibody recognizes oligohistidine, where the number of histidine residues within the peptide has not been established.

behaviour of mutant and wild-type proteins in the same cell. This provides the ability to examine the role of individual segments of protein sequence in a number of aspects of protein expression, for instance, rates of synthesis and destruction, post-translational processing, and location in the cell.

4. Surrogate reporter systems for monitoring protein expression

4.1 General principles

The preceding sections (Sections 2 and 3) have defined two strategies capable of addressing all the issues surrounding protein expression. There is, however, a third strategy which is gaining in popularity. This abandons the immunological monitoring of the expression of protein X and employs a surrogate protein marker instead. The surrogate protein marker possesses either an enzyme activity which is simple to measure (e.g. CAT, GUS, luciferase), or intrinsic optical properties, which can be detected *in vivo* (e.g. GFP).

The surrogate reporter protein technology can be used in three ways:

(a) In a quantitative approach, to determine the steady state level of expression of protein X in a cell. This is achieved by controlling the expression of the reporter protein via the promotor sequence from the gene for protein X. The reporter protein is expressed in addition to protein X in the cell and it is assumed that the concentration of surrogate reporter protein (which is measured using a simple assay) reflects the steady state concentration of protein X. For this relationship to hold, the surrogate protein must display rapid synthesis (and post-translational processing steps to a mature active protein) and degradation, in order to respond quickly to changes instructed by the prevailing experimental conditions (e.g. exogenous stimulus, stage of cell cycle). GUS and GFP fail to satisfy these conditions; in the case of GUS due to slow degradation (32) and in the case of GFP because of both slow processing to its active form, and slow degradation (33). Consequently, they are not used for this quantitative application.

(b) In a qualitative assessment of the histological pattern of expression. The GUS reporter activity lends itself to this application, which has been particularly useful in plant biology via the generation of transgenic plants containing a protein X promotor–GUS fusion constructs (34).

(c) In the non-destructive monitoring of protein expression and subcellular distribution of the protein of interest. GFP possesses a natural chromophore which absorbs light at a particular wavelength and emits light at a longer wavelength (fluorescence). This represents a considerable advance over other methods; it provides a non-destructive output for the presence and location of GFP in a live cell or transparent organism. This has found

particular favour when GFP expression is in the form of a fusion protein between protein X and GFP (in which both elements appear to retain their independent function) and so the location of GFP in the cell (and hence the location of protein X) can be monitored in real time.

4.2 Quantification of protein X expression using the CAT reporter assay

CAT is a highly stable prokaryotic enzyme absent from eukaryotes. This makes it an ideal reporter molecule, which has been used for the quantitative assessment of the level of expression of eukaryotic genes since the early 1980s (35). Expression of CAT in eukaryotic cells of choice, under the control of the promotor from gene X, provides an assessment of the level of protein X expression. Cell extracts are prepared and endogenous eukaryotic deacylation enzymes are inactivated by brief exposure to heat (65 °C for 10 min). The acetylation of [^{14}C]chloramphenicol, catalysed by CAT, yields acetylated products which can be resolved from the substrate by ascending thin-layer chromatography. They can then be quantified by scintillation counting or by densitometric analysis of an autoradiograph of the TLC plate.

Protocol 9 describes the CAT assay. To facilitate comparison of CAT activities in different cell extracts, one must have an independent measure of the transfection efficiencies. Therefore, it is usual to co-transfect the cells with a second reporter gene (e.g. luciferase, from Promega) to determine the efficiency of transfection. Normalization of the CAT activities of the samples for differences in transfection efficiency is carried out using the activities of the second reporter rather than relying on the protein concentrations of the extracts for the comparison.

Protocol 9. CAT reporter gene assay

Equipment and reagents

- Promoter sequence from the gene of protein X (e.g. a restriction fragment from a genomic clone or a synthetic oligonucleotide spanning the protein X promoter)
- CAT vector (e.g. pCAT-basic from Promega, or pBLCAT3) (36)
- T4 DNA ligase
- Dulbecco's modified Eagle's medium (DMEM)
- Eukaryotic cells for transfection with promotor–CAT construct growing in DMEM in tissue culture dishes
- Tissue culture incubator and dishes, PBS, rubber policeman, and materials for protein assay (see *Protocol 6*)
- Fetal calf serum (FCS)
- Plastic culture tubes (15 ml, Falcon)
- Microcentrifuge tubes (1.5 ml)
- Microcentrifuge, Mylar film, X-ray film, X-ray cassette, photographic materials (see *Protocol 7*)
- 0.25 M Tris–HCl pH 7.8
- Reporter lysis buffer (Promega)
- Heater blocks for 1.5 ml microcentrifuge tubes (Sigma Techware) set at 37 °C and 65 °C
- Acetyl CoA (Sigma): 13 mM solution stored on ice until used
- [^{14}C]chloramphenicol: 0.1 μCi/μl (ICN Flow)
- Ethyl acetate (at 4 °C)
- Speed vacuum concentrator (SpeedVac from Jouan)
- Silica gel TLC plates (Sigma or Merck)
- Hair drier
- Glass chromatography tank for TLC plates
- Chloroform:methanol (95%:5%, v/v)

Method

1. Following the instructions provided with the CAT vector and the DNA ligase, ligate the DNA fragment carrying the promotor region from the gene of interest to the CAT gene in the CAT vector, so that the expression of CAT can be controlled by the promotor of interest.
2. Grow the experimental cells in culture in DMEM to 50–90% confluence.
3. Transfect the cells with the recombinant CAT vector from step 1 as described in ref. 37. Replace the culture dishes in the incubator.
4. 6 h after transfection, remove the medium and replace it with fresh DMEM containing 10% FCS. Incubate the cultures for an additional 42 h.
5. Aspirate the medium from the cells and replace it with 5–10 ml warm (37 °C) PBS. Scrape the cells from the plastic surface of the dish using a rubber policeman. Transfer the cells to a sterile 15 ml plastic culture tube.
6. Centrifuge the tubes at 100 *g* for 10 min at room temperature.
7. Discard the supernatant, resuspend the cells in 1 ml PBS, and transfer them to a microcentrifuge tube. Centrifuge the tubes at 13000 *g* for 3 min at room temperature.
8. Discard the supernatant. Add 80 μl of 0.25 M Tris–HCl pH 7.8 to each cell pellet, followed by 20 μl reporter lysis buffer. Vortex the cells for 20 sec. Centrifuge the lysates at 13000 *g* for 3 min.
9. Transfer the supernatants to fresh microcentrifuge tubes. Measure the protein concentration of each lysate by a standard method.
10. Dilute samples of each lysate (equivalent to 100–150 μg protein) in 0.25 M Tris–HCl pH 7.8, to a final volume of 100 μl. Incubate for 10 min in a heater block at 65 °C to inactivate endogenous deacetylation enzymes.
11. Add 20 μl of 13 mM acetyl CoA and 1 μl [^{14}C]chloramphenicol to each sample. Incubate the samples at 37 °C in the heater block for 2–16 h.[a]
12. Remove the samples from the heating block, and stop the CAT reaction by the addition of 500 μl cold ethyl acetate. Vortex the tubes for 20 sec and then centrifuge them at 13000 *g* for 3 min at room temperature.
13. Remove the ethyl acetate (upper layer) from each sample into a fresh microcentrifuge tube. Dry down the samples in a speed vacuum concentrator for about 45 min.
14. Redissolve the residue in 20 μl ethyl acetate. Spot this onto a TLC plate by making four applications each of 5 μl to the same place on the plate, and dry each application with a hair dryer.

Protocol 9. *Continued*

15. Place the plate in a glass chromatography tank containing 95% chloroform:5% methanol and close the lid. Stand the tank in a fume-cupboard and allow the solvent to ascend the plate for 45–60 min at room temperature.
16. Remove the plate and leave it in the fume-cupboard to evaporate the solvent.
17. Wrap the plate in Mylar film and identify spots of radioactivity by autoradiography. Expose the TLC plate to X-ray film in the dark for 24–48 h. Develop and fix the film following the manufacturer's instructions.[b]

[a] The time of incubation should be selected to combine assay sensitivity with linearity of product formation with time. This should be determined in pilot experiments.
[b] PhosphorImaging may also be used (Section 2.5.2).

4.3 Histological examination of protein expression using GUS reporter activity

GUS from the *E. coli gusA* gene is a homotetrameric enzyme of subunit M_r about 68 kDa (32) which serves as a useful reporter protein, particularly in plant biology. With a few exceptions, plants lack GUS activity, while the different pH optima of eukaryotic and prokaryotic GUS enzymes provide the basis for the unambiguous assay of prokaryotic GUS as a surrogate marker for protein X expression (38). Various vectors containing GUS are available commercially (Clontech) or can be obtained from academic groups (see the appendix to ref. 32). Both fluorogenic (4-methylumbelliferyl-β-D-glucuronide) and non-fluorescent (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) substrates are available to monitor GUS activity. The latter generates an insoluble blue product which is suited to histological staining of tissue sections. Methods detailing the use of GUS in plant and animal studies can be found in ref. 32.

4.4 Monitoring expression and cellular location using GFP

Fluorescent proteins from a number of marine organisms have been identified. These exhibit considerable potential as research probes. GFP from *Aequorea victoria* is the best characterized example of this class of photoprotein; it is a protein of 238 residues which contains a natural chromophore comprising a cyclic (and oxidized) configuration of residues Ser65–Tyr66–Gly67 (ref. 33). This chromophore is part of an extended α-helix, protected from the aqueous phase by virtue of a surrounding β-barrel (39). The excitation spectrum for GFP shows a major peak at 395 nm and a minor peak at 475 nm, and the emission maximum at 507 nm. Repeated excitation of GFP at 395 nm causes

photoisomerization (with a shift in the absorption maximum from 395 nm to 475 nm) and photobleaching (i.e. a decrease in absorption at all wavelengths) both of which considerably limit the versatility of GFP. However, site-directed mutagenesis of the chromophore residues has provided several GFP mutants with altered spectral properties (33). For instance GFP-S65T exhibits a number of improvements over wild-type GFP. It possesses single absorption (488 nm) and emission (511 nm) maxima, does not undergo photoisomerization upon repeated excitation, and is less prone to photobleaching. This variant of GFP is used by most workers as the GFP reporter protein of choice.

In principle, GFP can be used in the same way as CAT for the measurement of steady state protein X expression. However, while qualitative confirmation of the expression of protein X in a particular cell line may be an acceptable use of GFP, quantitative analysis of expression is not recommended for the following reasons:

(a) Although the synthesis of GFP (the wild-type or the S65T mutant) is fast, the production of an active chromophore, and hence the means of detecting its expression, is slow (33).

(b) GFP protein is extremely stable, and thus the protein cannot serve as a sensitive dynamic reporter of steady state levels of expression.

GFP is of most value in the study of protein expression as a fusion partner with protein X to monitor the location of X in a cell at any given time. N-terminal and C-terminal fusions of GFP with proteins of interest have been performed with numerous individual proteins (33). It appears that both protein components of this fusion arrangement fold normally and act independently. Vectors containing GFP or spectral mutants with adjacent multiple cloning sites into which a cDNA of interest can be inserted are available commercially (Clontech's Living Colors vectors). The fluorescence output from GFP can then be used to define the position of protein X in a living cell, and monitor changes in this distribution as a function of a particular stimulus. However, when studying the expression of GFP fusion proteins with TGN38 (a Golgi complex-specific protein) Girotti and Banting (40) have identified one shortcoming of this approach which may be of general concern: that high levels of expression of a GFP fusion partner protein can alter the subcellular distribution of the new protein by exceeding the capacity of normal protein targeting mechanisms in the cell. Thus the expression of GFP–TGN38 fusion proteins during transient expression experiments generated high levels of fusion protein which appeared to exceed the sorting mechanisms for protein targeting in the cell. As a consequence, GFP–TGN38 was observed at inappropriate subcellular locations. More modest levels of expression using the same constructs were generated in stable transformed cell lines, and here the distribution of GFP–TGN38 was consistent with that of TGN38 alone (40).

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