Gene Cloning and Analysis

A LABORATORY GUIDE

Compiled by the staff of
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Gene Cloning Course
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Preface

This manual is based on the experimental protocols for an intensive, practical course on gene cloning and analysis held in Leicester during September 1985 and July 1986. As such it is a much updated version of Leicester's previous manual entitled *Basic Cloning Techniques: A manual of experimental procedures* (Eds. R.H. Pritchard and I.B. Holland, 1985, Blackwell Scientific Publications).

As for the previous manual, we do not claim to have provided a comprehensive manual but rather hope to have focussed on the key methodologies. All the methods described are used on a day-to-day basis in the School of Biological Sciences at Leicester. Where reference is made to a particular manufacturer or supplier this should not be taken as the only source of a given item.

A number of different groups at Leicester have been involved in the production of this manual. Consequently, there are inevitable differences in style and layout, and some duplication was unavoidable. Where possible an attempt has been made to highlight particular difficulties and possible ways to solve problems. Also, alternative methods have been indicated where appropriate. Again, however, we have not attempted to be comprehensive in listing all possible alternative methods and protocols.

The practical courses held at Leicester, and hence this manual, would not have been possible without the hard work, dedication and enthusiasm of many research workers. Our thanks must go to the numerous post doctoral research associates, graduate students and technicians who, over the years, have contributed to the establishment of these methods.

We are also grateful to the Biotechnology Directorate of the Science and Engineering Research Council of the United Kingdom for continued support and the many companies who have generously supported the practical course.

Graham Boulnois

Abbreviations

Amp (Ap)	ampicillin
BCIG (Xgal)	5-bromo-4-chloro-3-indolyl-β-D-galactoside
BSA	bovine serum albumen
BPB	Bromophenol Blue
DEPC	diethyl pyrocarbonate
DTT	*dithiothreitol*
ETOH	ethanol
HAc	acetic acid
IMS	industrial methylated spirit
IPTG	isopropylthio-β-galactoside
KAC	potassium acetate
L-agar/broth	Luria agar/broth
PAGE	polyacrylamide gel electrophoresis
RF	replicative form
SDS	sodium dodecyl sulphate
Tc/Tet	tetracycline
TCA	trichloro acetic acid
XC	xylene cyanol

Contents

1 Translation of mRNA in a rabbit reticulocyte lysate cell-free protein synthesizing system

1.1 Introduction

In vitro translation provides a powerful method for assaying specific mRNAs prior to cDNA cloning, for monitoring their purity, for studying their properties and for identifying sequences complementary to mRNA in cDNA plasmid clones. The above techniques are essentially variations on a standard theme which is illustrated below. There are two types of *in vitro* translation systems in common use; those derived from rabbit reticulocytes (Bonner and Laskey, 1974) and those from wheatgerm extracts (Hames, 1981). Messages which can be translated well in one system may be poorly translated in the other for no readily apparent reason. The reticulocyte system may be better for the synthesis of larger proteins (up to 200 000 daltons) than wheatgerm. Other factors may enter into the choice of cell free system. Both extracts can be 'home-made' or are available commercially. In this chapter the use of a commercial lysate prepared from rabbit reticulocytes will be described. For most mRNAs this lysate requires no adjustment of potassium or magnesium concentration. The optimum poly(A)$^+$ mRNA concentration and incubation time must first be determined (see section 1.2 and 1.3).

An experiment is described to compare the translation products of poly(A)$^+$ mRNA extracted from light grown *Petunia* seedlings and light grown albino suspension cultured cells (section 1.4). These are examples only of two types of eukaryotic mRNA used to demonstrate the basic principles of the *in vitro* translation technique. Identical procedures may be used for the analysis of most eukaryotic mRNA samples. A commercially available mRNA (Tobacco Mosaic Virus) is convenient to use as a simple control to check the fidelity of the translation system. The analysis of translation products in this experiment is performed using gel electrophoresis and autoradiography. For analysis of specific mRNAs, especially those present at a low abundance, more sensitive techniques are required. For instance an antibody raised against the protein under study can be used in an immunoprecipitation assay of translation products (Marcu and Dudock, 1974). Hybrid arrested *in vitro* translation techniques are commonly used to analyse specific cDNA clones (Parnes, *et al.*, 1981), whilst hybrid release (hybridselect) *in vitro* translation procedures are used both to screen cDNAs, and to select out specific transcripts from complex mRNA populations to synthesize fairly large amounts of a specific protein, coded for by a cDNA of interest, for further analysis (Pelham and Jackson, 1976).

1.2 Determine the time course of ^{35}S incorporation into protein

1.2.1 Materials required

Rabbit Reticulocyte Lysate (Amersham N.90).
^{35}S methionine (>800 Ci/mM, Amersham S.J.204).

Poly(A)$^+$ mRNA from seedling leaves and albino *Petunia* cell suspension culture (0.5 μg/ml) in sterile distilled H_2O.
Sterile H_2O.
100% ethanol.
5% trichloroacetic acid (TCA).
10% TCA containing casein hydrolysate (0.5%, BDH, Biochemical grade).
Filter paper squares (Whatman^{TM} No.1).
Cracking buffer — 2% SDS (specially purified for electrophoresis, BDH), 5% 2-mercaptoethanol (Sigma), 10% sucrose, 0.002% Bromophenol Blue (Biorad) in distilled H_2O.
Scintillation fluid 4.0 g PPO, 0.8 g POPOP made up to 1 litre with toluene.
Radioactive protein mol.wt markers (Amersham CFA 626).
TMV RNA (Amersham N–149).

1 All reactants must be kept on ice except the cracking buffer.
2 The lysate must be kept in a dry-ice/ethanol bath until immediately before use. The lysate must be thawed and gently mixed before use and must not be refrozen and reused.
3 A general procedure for the preparation of poly(A)$^+$ mRNA is outlined elsewhere (sections 2.9 and 2.10).

1.2.2 Procedure

1 Prepare a reaction mixture as follows:
14 μl rabbit reticulocyte lysate.
1 μl poly(A)$^+$ mRNA (0.5 μg/ml).
2 μl ^{35}S methionine.
3 μl sterile H_2O.
Mix gently and place in a water bath at 28°C.
Note: if required a 2-s spin in microfuge will be sufficient to collect mixture into 1 volume.
2 At time intervals (from 0–75 min) remove 2 μl aliquots and spot onto a filter. Place filter in 10% TCA at 0°C containing casein hydrolysate. After adding the final filter allow to stand for 10 min.
3 Transfer filters to boiling 5% TCA for 10 min and then to cold 5% TCA (0°C) for 10 min. Wash twice in 100% ethanol and dry.
4 Place filters into scintillation vials with 6 ml of scintillation fluid and count them in a scintillation counter.
5 Plot a graph of counts incorporated *vs* time.

1.3 Optimization of poly(A)$^+$ mRNA concentration

1.3.1 Procedure

Materials required are outlined in section 1.2.1.

1 Prepare five translation mixtures with a range (0.1, 0.2, 0.5, and 1.0 μg) of poly(A)$^+$ mRNA concentrations as follows:

7 μl RRL
1 μl ^{35}S Methionine.
Poly(A)$^+$ mRNA.
Sterile H_2O (to give a final reaction of volume of 10 μl).
Mix gently and incubate at 28°C for 40 min.

2 Add 10 μl cracking buffer to stop reaction.

3 Spot 4 μl from each reaction onto a filter and add the filters to 10% TCA containing casein hydrolysate cooled to 0°C. Leave for 10 min.

4 Transfer filters to boiling 5% TCA for 10 min and then to cold (0°C) 5% TCA for a further 10 min. Wash the filters twice in 100% ethanol and dry, either under an infra-red lamp or allow to air dry.

5 Place filters in scintillation vials together with 6 ml of scintillation fluid and count in a scintillation counter.

6 Plot a graph of counts incorporated *vs* mRNA concentration.

1.4 Comparison of the translation products of poly(A)$^+$ mRNA extracted from seedling leaves and albino suspension cultured cells of *Petunia*

Using the procedures described above (sections 1.2 and 1.3) the optimum conditions for translation with respect to poly(A)$^+$ mRNA concentration, and incubation time will have been determined. In general an optimum translation efficiency is achieved with an incubation time of around 45 min and a poly(A)$^+$ mRNA concentration of between 0.1–0.5 μg/reaction. Thus, translate samples of poly(A)$^+$ mRNA extracted from light grown seedling leaves, light grown albino *Petunia* suspension cultured cells and a marker mRNA (TMV) as outlined above (sections 1.2 and 1.3). Use water as a control and remember that the reaction mixture should have a total volume of 10 μl of which 7 μl should be lysate. After 45 min stop the reaction with 10 μl of cracking buffer and mix. For each of the mRNA translations, remove a 4 μl sample and determine the number of TCA precipitatable counts incorporated as outlined above (section 1.3). Remove samples that contain equal amounts of radioactivity (at least 50 000 cpm) for analysis by SDS polyacrylamide gel electrophoresis as outlined below (sections 1.5–1.7). An optimal autoradiographic exposure will be given by 20 000 cpm, if a complex population of mRNA has been translated. If only a few protein bands are expected on the gel then a fewer number of counts need to be loaded on the gel. Include also radioactively labelled proteins on gels as mol.wt markers.

1.5 Analysis of translation products by SDS polyacrylamide gel electrophoresis (PAGE)

1.5.1 Background

This technique provides a relatively rapid and simple method for protein analysis. Proteins are dissociated under reducing conditions to constituent polypeptide chains by the strongly anionic detergent SDS which binds to proteins. Dissociated polypeptides are separated more or less on the basis of their mol.wt. This system uses a discontinuous buffer which employs different buffer ions in the gel compared to the one in the electrode reservoirs.

The samples for electrophoresis are mixed with an equal volume of cracking buffer and boiled for 2 min before loading onto the gel. The samples are loaded onto a large-pore 'stacking' gel polymerized into a small-pore resolving gel. The proteins are concentrated into very narrow zones during migration through the stacking gel prior to separation during electrophoresis in the small-pore resolving gel. This allows large volumes of dilute protein samples to be applied to the gel without loss of resolution.

1.5.2 Materials required

Slab gel apparatus (Studier type) and power pack.
Stock of acrylamide/bisacrylamide 30:0.8 solution (BDH specially purified electrophoresis grade). Deionize before use by stirring for 1 h with 5 g of mixed bead resin per 100 ml of solution (Amberlite monobed resin MG-1, BDH). Filter (Whatman™ No. 1) into a dark glass bottle and store at 4°C.
10% SDS solution (BDH specially purified for biochemical work).
1 M Tris-HCl, pH 8.8.
1 M Tris-HCl, pH 6.8.
1.5% ammonium persulphate, freshly prepared.
TEMED (Sigma).
Distilled water.
Cracking buffer (see 1.2.1).
x 5 Reservoir buffer — Tris glycine pH 8.3 For 1.0 litre of x 5; 139.6 g glycine, 5 g SDS, 15.15 g Tris-HCl. Dilute to x 1 with distilled water.
1:1 propan-1-ol and water.
Fixing solution — 7% acetic acid in distilled water.
Plastic gel boxes (Steward Plastics™).

1.5.3 Procedure

Wear disposable rubber gloves throughout.

1 Clean glass plates firstly with detergent then with ethanol/acetone mix (1:1). Allow to air dry.

2 Assemble apparatus using 1.5 mm gel spacers held in place by clamping the glass plates together with fold-back clips. Seal the edges and bottom of the gel with a couple of millilitres of molten 1% agarose. Allow the agarose to set.

3 Mix the following in order adding the SDS and TEMED after degassing and persulphate last. This mixture will be used to pour a 12% resolving gel:

16.0 ml 30:0.8% acrylamide/bisacrylamide.
14.8 ml 1 M Tris-HCl pH 8.8.

6.8 ml distilled H_2O.
Degas for 1 min.
0.4 ml 10% SDS.
20 μl TEMED.
2.0 ml 1.5% ammonium persulphate.

4 Pour gently into glass plates, trying to avoid bubbles.

5 Gently overlay with 1–2 ml of propanol and water (1:1) using a Pasteur pipette. Try not to break the smooth surface of the acrylamide.

6 Make sure the plates are upright and level and then allow the resolving gel to polymerize for 30–60 min. Polymerization is usually indicated by a sharp line between the gel and overlay. It is usually a good idea to insert the well former (comb) to stop the plates from pulling too close together as the gel polymerizes.

7 Remove propanol and dry the surface.

8 Mix the following to make a stacking gel:
2.5 ml 30:0.8% acrylamide/bisacrylamide.
2.5 ml 1 M Tris-HCl pH 6.8.
13.8 ml distilled H_2O.
Degas for 1 min.
0.2 ml 10% SDS.
15 μl TEMED.
1.0 ml 1.5% ammonium persulphate.

9 Rinse top of resolving gel with a small amount of stacking gel solution. Load the stacking gel solution into the glass plates.

10 Insert well former and leave for around 15 min to allow polymerization, again as evidenced by a distinct interface on the surface of the gel.

11 Remove comb immediately after polymerization taking care to minimize sideways movement. Rinse wells with reservoir buffer and straighten out with a syringe needle if required. Carefully remove the bottom spacer and mount in the electrophoresis tank with the slotted plate against the apparatus. Hold in position with fold-back clips. Fill top reservoir tank and make sure the wells are flooded. Lower the top chamber until the plates rest on the supports in the bottom chamber. Fill bottom chamber with reservoir buffer and remove any air bubbles between the two plates using a syringe with a bent needle.

12 Heat the samples in a boiling water bath for 2 min.

13 Carefully load samples into wells with capillary tubing or automatic pipette. Unused wells may be filled with an equal volume of cracking buffer.

14 Electrophorese at 50 V overnight (18 h).

Note: the negative and positive terminals should be connected to the top and bottom reservoir respectively.

15 When the Bromophenol Blue marker dye reaches the bottom of the gel, turn off the power, turn the gel out into fixing solution and leave for 15–20 min.

1.6 Visualization of radioactive proteins in PAGE gels

The protein bands in a gel may be visualized directly by staining with Coomassie Brilliant Blue or, for radioactive translation products, indirectly by autoradiography and/or fluorography of gels.

1.6.1 Fluorography of PAGE gels

The impregnation of gels with scintillant before drying and exposure to X-ray film results in an enhancement of soft emission by a factor of around 15-fold, thus reducing autoradiography time.

1 Remove gels into a fixative containing 7% acetic acid in distilled water for approximately 5 h with two changes of solution.
2 Pour off fixing solution into a radioactive waste container. Soak the gel in a volume of 'Amplify' (Amersham) sufficient for the gel to be free floating. Agitate gently for 20 min.
3 Remove gel from solution and dry under vacuum at 80°C on a gel drier (Biorad) for 2–3 h.
4 Expose the gel to X-ray film (Fuji™) at −80°C in a light proof container (e.g. X-ray film cassette). Autoradiography time will in general depend upon the radioactivity of the gel and may vary from overnight for samples with $>10^6$ cpm to 2 weeks for samples with 10^4 cpm.
5 Develop film according to manufacturers' instructions.

1.6.2 Coomassie Brilliant Blue staining

1 Remove gels to a staining solution containing 0.1% Coomassie Brilliant Blue in distilled water, methanol, glacial acetic acid (5:5:2). Before use the stain is filtered through Whatman™ No. 1 paper.
2 Gently agitate gel at 40–50°C for 1 h.
3 Remove excess dye from gel by destaining under the same conditions in a solution containing 30% methanol and 10% acetic acid in water for at least 1 h.

1.7 References

Bonner W.M. and Laskey R.A. (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83–88.

Hames, B.D. (1981) In: *Gel Electrophoresis of Protein. A Practical Approach.* Ed. B.D. Hames and D. Rickwood. IRL Press: Oxford.

Marcu K. and Dudock B. (1974) Characterization of a highly efficient protein synthesising system derived from commercial wheat germ. *Nucleic Acids Res.* **1**, 1385–1397.

Parnes J.R., Velan B., Felsenfeld A., Ramanathan L., Ferrini U., Appella, E. and Seidman, J.G. (1981) Mouse β_2 microglobulin cDNA clones: a screening procedure for cDNA clones corresponding to rare mRNAs. *Proc. Natl. Acad. Sci. USA.* **78**, 2253–2257.

Pelham H.R.B. and Jackson R.J. (1976) An efficient mRNA-dependent translation system for reticulocyte lysates. *Eur. J. Biochem.* **67**, 247–256.

2 Synthesis and cloning of cDNAs from eukaryotic mRNAs

2.1 Introduction

The availability of complementary DNA (cDNA) to mRNAs has provided a means of analysing the structure, organization and expression of eukaryotic genes in both plant (e.g. Broglie *et al.*, 1983; Greenland, Thomas and Walden, 1987; Hershey *et al.*, 1984; Nelson *et al.*, 1984) and animal systems (e.g. Jeffreys and Flavell, 1979; March *et al.*, 1985; Sood *et al.*, 1981). Consequently, the first step in many projects in molecular biology is to produce a library of cDNA sequences which faithfully represents the mRNA populations within a particular tissue and from which cDNAs of interest can be selected.

The technique used to produce cDNA libraries should generate large numbers of clones from small amounts of template mRNA so that sequences representing mRNAs of low abundance are present in the library. It is also desirable that such techniques yield full-length cDNA transcripts of the mRNAs. The method used in this laboratory to produce cDNA libraries has been modified from existing techniques (Gubler and Hoffman, 1983; Okayama and Berg, 1982) and relies on classic first-strand synthesis with reverse transcriptase primed with oligo (dT) annealed to the poly(A)$^+$ tail of the mRNA followed by RNAase H — DNA polymerase I mediated second-strand synthesis. The double-stranded cDNAs so produced are then cloned into plasmid vectors by homopolymer tailing. This method avoids the hairpin-loop cleavage by S1 nuclease, common to many earlier techniques and which was responsible for low cloning efficiencies and truncated cDNAs.

Table 2.1. Size of some cDNA libraries made at the Leicester Biocentre, 1985–1986

Organism	Tissue	Library size no. of recombinants × 10^5 per μg cDNA	per μg mRNA
Cucumber	Cotyledons	0.55	0.14
Pea	Apices	1.40	0.34
Wheat	Immature embryos	0.72	0.18
Yeast	—	2.20	0.25

Table 2.1 gives details of the size of a number cDNA libraries synthesized at the Leicester Biocentre from plant and fungal mRNAs using the above method. The efficiencies given in Table 2.1 would potentially allow isolation of cDNAs to comparatively rare mRNAs, for example those occurring at a frequency of >0.1% of a poly(A)$^+$ enriched RNA fraction. By optimizing conditions during preparation of competent bacterial cells and transformation (the use of commercially available competent cells, which claim very high transformation efficiencies, would be an alternative), or by cloning into a suitable lambda (λ) vector and taking advantage of *in vitro* packaging, a 10-fold increase in library size might reasonably be expected.