Basic Cloning Techniques

A MANUAL OF EXPERIMENTAL PROCEDURES

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Preface

This manual was written for a practical course at the University of Leicester designed to teach cloning techniques to those who had no first hand experience of them. The popularity of the course, and demand for the course manual, prompted us to edit it for publication to a wider audience.

The techniques set out in protocol form in the Manual have in common that they are all regularly used in the laboratories of the School of Biological Sciences. First hand experience of them rather than an attempt to be comprehensive was an important criterion for inclusion. Consequently the Manual does not lay claim to offer a complete coverage of the subject. A knowledge of microbiological technique and basic microbial genetic skills is assumed and a popular guide is available—*Experiments in Molecular Genetics*, by J.H. Miller, published by Cold Spring Harbor Laboratory, N.Y., 1972. Other important techniques, such as DNA sequencing were included in the course at Leicester as demonstrations backed up by lectures and seminars. No practical work was performed however and none have been added to the published manual. Recent practical reviews can be consulted by those who need these methods (Maxam & Gilbert 1980; Bankier & Borrell, 1983).

The different sections were designed and written by different groups. Consequently there were inevitably differences in style and layout, and some repetition of descriptions of commonly used techniques. In preparing the manual for publication we have endeavoured to bring a measure of uniformity to the style and layout. But we have tried to do this with a light touch so that the spontaneity of the original versions has not been edited out. Procedures do not always succeed when they are translocated to different laboratories. The introductory section which precedes each of the experiments contains references which, wherever possible, provide background information that should help to identify any problems. The authors of the various sections may also be able to help and would welcome being told of difficulties that have been encountered.

The experimental procedures are set out in protocol form with an indication of the time required to complete them. They can all be fitted into an intensive 2-week course by running

different sections of a complete procedure in parallel. This was the format of the course at Leicester. Under a more leisurely time-table the longer procedures can be carried through from start to finish in sequence.

There are numerous references to specific manufacturers in the text. In many cases there will be acceptable alternative manufacturers who can supply similar products and the one cited here is simply a matter of local preference at the time of writing, or habit.

The number of strains of bacteria, and their viruses and plasmids, employed in the course is not large. Many of them are widely used and will be found in laboratories all over the world. The authors of the different sections of the course have also agreed to supply those used in their Sections to readers of this manual who cannot obtain them locally.

The list of people who have helped to teach this course on two occasions inevitably omits to name many people at Leicester who have contributed to its success. We would like to record our gratitude and thanks to them.

We are most grateful to the Biotechnology Directorate of the Science and Engineering Research Council for providing a substantial grant towards the cost of this course since 1981, and to many industrial companies who have also generously supported us. We are also greatly indebted to the many people who have helped with the development, over several years, of many difficult techniques which we are now able to present as relatively routine.

I.B. Holland
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Maxam, A.M. & Gilbert, W. (1980) Sequencing end-labelled DNA with base specific chemical cleavages. *Meth. Enzym.*, **65**, 499.

Bankier, A.T. & Borrell, B.G. (1983) Shotgun DNA sequencing. *Techniques in the Life Sciences*, B5: Ch. B508; Nucleic Acid Biochem. pp. 1–34. Elsevier, Amsterdam.

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Section E Electronmicroscopy of nucleic acids

Section A Mammalian cDNA cloning

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Introduction

Section A is devoted to the synthesis and cloning of DNA copies of eukaryotic messenger RNAs (mRNAs). For an explanation of why cloning copy DNA (cDNA) is distinct from cloning genomic DNA and why it is often advantageous, see the excellent review by Williams (1981). The essential points are that mRNAs represent the contiguous protein-coding functional domain of a gene and that their relative abundance within a given cell is determined by the state of differentiation of that cell. Most eukaryotic mRNAs have a 3′ polyA tract, which can be used to advantage in their purification and the subsequent synthesis of cDNA.

This section of the course attempts to cover all the essential stages in cloning cDNAs of eukaryotic mRNAs in a plasmid vector. The experiments to be carried out would normally form a continuum, and realistically could be expected to take 2–4 weeks. In order to compress them into a 4-day timetable this section has been broken into three subsections which can run concurrently. They cover the following topics:

1 Isolation, purification and characterization of polyA-containing messenger RNA from animal tissue.

Table A.1.

	Subsection A.1	*Subsection A.2*	*Subsection A.3*
Day 1			
a.m.	—	First-strand cDNA synthesis	Competent cell preparation
p.m.	RNA extraction	G50 column	Transformation
Day 2			
a.m.	(i) RNA extraction (ii) Measure absorbance	Second-strand cDNA synthesis	Colony picking
p.m.	Oligo dT-cellulose chromatography	G50 column	Colony picking
Day 3			
a.m.	mRNA precipitation	S1 nuclease treatment	Prepare filters—cell lysis
p.m.	*In vitro* translation of mRNA	(i) Homopolymer tailing—pilot (ii) cDNA gel	(i) Hybridization of filters (ii) Inoculate minicultures
Day 4			
a.m.	—	(i) Homopolymer tailing (ii) Develop cDNA autoradiograph	(i) Plasmid minipreps (ii) Wash and expose filters
p.m.	—	Annealing	(i) Digestion and gel analysis of plasmid DNAs (ii) Develop filter autoradiograph

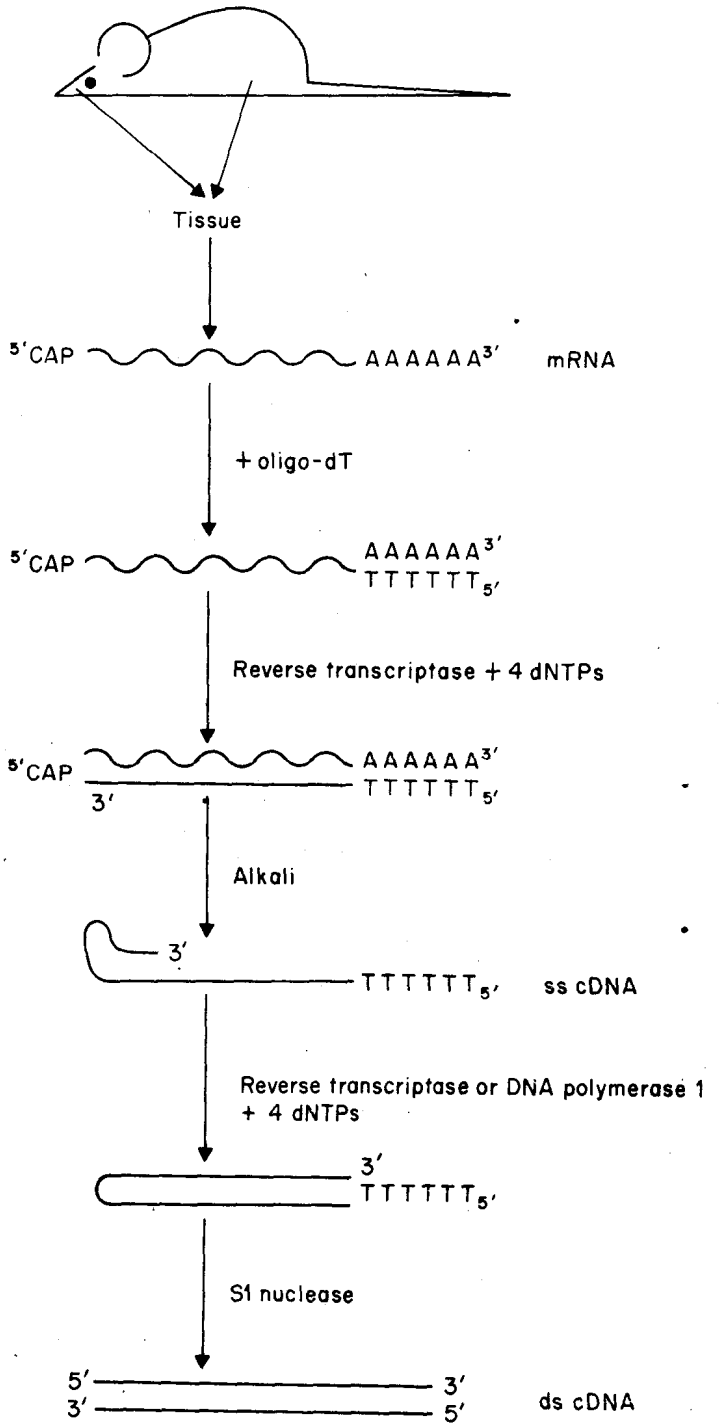

Fig. A.1 Preparation of cDNA

2 Synthesis of homopolymer-tailed double-stranded cDNA and construction of hybrid plasmids by annealing to complementary-tailed vector DNA.

3 Cloning of hybrid plasmids and analysis of clones by insertional inactivation of antibiotic-resistance genes, colony hybridization and restriction endonuclease digestion.

These stages are outlined in Figs A.1 and A.6 and a sample timetable for a 4-day programme is given in Table A.1.

Reference

Williams, J.G. (1981). The preparation and screening of a cDNA clone bank. In: *Genetic Engineering. Vol. 1.* (Ed. Williamson, R.), pp. 1–61. Academic Press, London.

Section A.1 Isolation of mRNA and translation *in vitro*

Background

Many methods are currently in use for isolation of ribonucleic acids from mammalian cells, tissues and organs. It is particularly important to obtain a good quality, undergraded mRNA preparation, since this ultimately determines the quality of cDNA synthesized and the size and usefulness of clones.

The method described here (Noyes *et al.* 1979) is particularly appropriate for isolation of mRNA from animal tissues.

To prevent degradation of the RNA by cellular ribonucleases, it is important to use either extremely fresh tissue or tissue placed immediately into liquid nitrogen at the time of collection and stored at −70°C until required.

It is equally imperative to introduce the tissue to the phenol/chloroform/isoamyl alcohol (PCI) mixture as soon as possible, since this should protect the RNA from further degradation by RNases. Several PCI extractions and back extractions are required in order to achieve thorough deproteinization of the total cellular RNA. The RNA is then separated from DNA by passage through a caesium chloride 'pad' gradient. We find this method excellent for extracting RNA from soft animal tissues. However, alternative methods may be better for RNA extraction from plant cells, solid tissue or tissue culture cells. A general review of extraction methods for RNA is given by Taylor (1979).

References

Noyes, B.E., Mevarech, M., Stein, R. & Agarwal, K.L. (1979) Detection and partial sequence analysis of gastrin mRNA by using an oligodeoxynucleotide probe. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 1770.

Taylor, J.M. (1979) The isolation of eukaryotic messenger RNA. *Ann. Rev. Biochem.*, **48**, 681.

Important note

RNA is very susceptible to degradation by nucleases which can easily contaminate apparatus and solutions. Virtually all biological cells and tissues, especially human skin, are rich in these nucleases. When working with RNA the user should

take every precaution to avoid the presence of nucleases. Gloves should be worn at all times and all glass apparatus used should be autoclaved sterile. It is also advisable that all water to be used for solutions containing RNA should be treated with diethyl pyrocarbonate (DEPC) before use (see p. 8).

Experiment A.1 Isolation of RNA

Materials needed (all sterile)

TLE buffer (0·2 M Tris-HCl, pH 7·5; 0·1 M LiCl; 25 mM EDTA; 0·1% SDS)
PCI mixture (50: 48: 2, phenol: chloroform: isoamyl alcohol)
CE solution (5·7 M $CsCl_2$, 0·1 M EDTA, pH 7·5)

All solutions are prepared with DEPC-treated H_2O which is produced as follows:
To 1 litre distilled water add 1 ml diethyl pyrocarbonate (DEPC). Shake well and allow to stand for 10 min.

Autoclave at 15 psi for 15–20 min to remove unreacted DEPC. This step is particularly important as any remaining DEPC will poison subsequent enzyme reactions.

Procedure

1 Mix together equal volumes of TLE buffer and PCI mixture in the homogenizer flask. (We suggest 30 ml of each for a maximum of 10 g of tissue.)
2 Weigh tissue provided as quickly as possible and add to the flask.
3 Homogenize tissue thoroughly in a Dounce (or equivalent) homogeniser.
4 Divide homogenate equally between glass universal centrifuge bottles. Shake vigorously for a few minutes. Spin at 5000 rpm for 15 min in a bench centrifuge.
5 Remove aqueous supernatant to conical flask containing 20 ml of PCI mixture and shake vigorously for 5 min.
6 Re-extract the lower phenol phase twice with 15 ml TLE buffer, each time spinning as in step 4 and pool the supernatant with that of step 5.
7 Shake the flask with the pooled supernatants vigorously for 5 min. Spin at 5000 rpm for 15 min.
8 Remove aqueous phase to sterile measuring cylinder. N.B. If the aqueous phase is not translucent at this stage carry out a further PCI extraction.
9 Carefully layer the aqueous phase over 0.2 vol CE solution. Top up the tubes with liquid paraffin if necessary, and balance the tubes. Load into a swing out rotor and centrifuge for 18 h at 25000 rpm (83 000 *g*) at 25°C.

10 Next day, remove paraffin and supernatant with Pasteur pipette. Identify the DNA band above the CsCl pad and remove this carefully.

11 Invert the tube, allow any remaining CsCl solution to drain away.

12 Take up the translucent RNA pellet in 1–2 ml of DEPC-treated water and allow to redissolve over about an hour. Transfer to Corex tube.

13 Add 0·1 vol 2 M sodium acetate and 2 vol absolute ethanol. Precipitate in a dry-ice bath for 30 min.

14 Centrifuge at 10000 rpm in Sorval SS34 rotor (or equivalent) for 30 min at 0°C and carefully decant the ethanol supernatant.

15 Cover tube with Parafilm. Prick several holes and dry under vacuum for 5 min (do not over-dry). This procedure prevents loss of dry pellet through air turbulence.

16 Resuspend pellet in 0·4 ml DEPC water.

17 Remove a small aliquot (say 2·5 μl) and dilute to 500 μl. Estimate the RNA concentration by reading the absorbance at A_{260} (1 mg ml^{-1} RNA = 20 A_{260} units).