DNA ISOLATION AND SEQUENCING

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ABBREVIATIONS

Amp	ampicillin
APS	ammonium persulfate
BSA	bovine serum albumin
c^7dGTP	7-deaza-dGTP
CIAP	calf intestinal alkaline phosphatase
CPG	controlled-pore glass
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DMT	dimethyloxytrityl
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
EtBr	ethidium bromide
FSB	frozen storage buffer
IPTG	isopropyl β-D thiogalactopyranoside
KDB	Klenow dilution buffer
MTBE	modified Tris-borate EDTA
PCR	polymerase chain reaction
PEG	polyethylene glycol
RF	replicative form
SDS	sodium dodecylsulfate
SSC	standard saline-citrate
STS	sequence-tagged sites
TB	Terrific Broth
TEMED	*N,N,N',N'*-tetramethylenediamine
Tet	tetracycline
Tris	Tris(hydroxymethyl)aminomethane
UV	ultraviolet
X-gal	5-bromo-4-chloro β-D-galactopyranoside

PREFACE

This manual is a compilation of many of the everyday methods used in the average molecular biology laboratory, with emphasis on the techniques for large-scale DNA sequencing. Various forms of the manual have been in use in our laboratory for several years, and this latest version has been updated to include more detailed DNA sequencing protocols and DNA sequencing automation techniques.

This manual has been written in a protocol format, with little theoretical discussion. For theory and additional information, users of this manual are referred back to the original literature, or to other textual manuals such as that published by Sambrook, Fritsch and Maniatis (1989) see reference 1, Chapter 1.

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Bruce A. Roe

SAFETY

Attention to safety aspects is an integral part of all laboratory procedures and national legislations impose legal requirements on those persons planning or carrying out such procedures. While the authors, editor and publisher believe that the recipes and practical procedures, as set forth in this book, are in accord with current recommendations and practice at the time of publication, they accept no legal responsibility for any errors or omissions, and make no warranty, expressed or implied, with respect to material contained herein. It remains the responsibility of the reader to ensure that the procedures which are followed are carried out in a safe manner and that all necessary safety instructions and national regulations are implemented.

In view of ongoing research, equipment modifications and changes in governmental regulations, the reader is urged to review and evaluate the information provided by the manufacturer, for each reagent, piece of equipment or device, for any changes in the instructions or usage and for added warnings and precautions.

These are freely accessible using:
http://joule.pcl.ox.ac.uk/MSDS/.

Other safety infomation on the Internet can be accessed on:
gopher://atlas.chem.utah.edu/11/MSDS
gopher://ginfo.cs.fit.edu:70/lm/safety/msds
http://physchem.ox.ac.uk/MSDS
http://www.fisher1.com/Fischer/Alphabetical Index.html
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Radiation. The use of radioisotopes is subject to legislation and requires permission in most countries. Furthermore, national guidelines for their use and disposal must be rigorously adhered to. The procedures in protocols that use radioisotopes must only be carried out by individuals

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All procedures mentioned within this book must be carried out under conditions of good laboratory practice in accordance with local and national guidelines. Some procedures involve specific hazards, including but not limited to hazards in the following categories:

Chemical. A number of the reagents are known to be carcinogenic, mutagenic, toxic, inflammable, highly reactive or otherwise hazardous. Substances known to be hazardous have been marked with the symbol ⚠ in the list of reagents (but not subsequently) for each protocol, or if they appear as alternatives to the main protocol, the *first time* they appear in the notes. The reader should consult the safety notes on these pages before embarking on any of the procedures covered. This is in no way meant to imply that undesignated chemicals are nonhazardous, and all laboratory chemicals should be handled with extreme caution. Information is not available on the possible hazards of many compounds. The criteria we have generally used for denoting a substance with ⚠ is based upon a hazard level of 2 or more (on a scale 0–4) in any of the categories in the Baker Saf-T-Data™ system used in the material safety data sheets (MSDS) held at the University of Oxford, UK.

Biological. Antibodies, sera and cells (particularly, but not exclusively, those of human and nonhuman primate origin) pose a significant biological hazard. All such materials, whatever their origin, may harbor human pathogens and should be handled as potentially infectious material in accordance with local guidelines. Any recombinant DNA work associated with protocols is likely to require permission from the relevant regulatory body and you must consult your local safety officer before embarking upon this work.

Electrical. Many of the procedures in this book use electrical equipment. Electrophoresis techniques may present particular hazards of this nature.

Lasers. Flow cytometers and certain other types of laboratory equipment contain lasers. Users should ensure they are fully aware of the potential hazards of using such equipment.

CONTENTS

I GENERAL METHODS

Methods available

Phenol extraction of DNA samples (see *Protocol 1*)
Phenol extraction is a common procedure used to purify a DNA sample [1]. Typically, an equal volume of TE-saturated phenol is added to an aqueous DNA sample in a microcentrifuge tube. The mixture is vortexed vigorously, and then centrifuged to achieve phase separation. The upper, aqueous layer is removed carefully to a new tube, avoiding the phenol interface, and is then subjected to two ether extractions to remove residual phenol. An equal volume of water-saturated ether is added to the tube, the mixture is vortexed and the tube is centrifuged to allow phase separation. The upper, ether layer is removed and discarded, including phenol droplets at the interface. After this extraction is repeated, the DNA is concentrated by ethanol precipitation.

Concentration of DNA by ethanol precipitation (see *Protocol 2*)
Typically, 2.5–3 volumes of an ethanol/acetate solution is added to the DNA sample in a microcentrifuge tube, which is placed in an ice-water bath for at least 10 min. Frequently, this precipitation is performed by incubation at –20°C overnight [1]. To recover the precipitated DNA, the tube is centrifuged, the supernatant discarded, and the DNA pellet is rinsed with a more dilute ethanol

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solution. After a second centrifugation, the supernatant again is discarded, and the DNA pellet is dried in a Savant Speed-Vac.

Restriction digestion (see *Protocol 3*)
Restriction enzyme digestions are performed by incubating double-stranded DNA molecules with an appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier, and at the optimal temperature for the specific enzyme. The optimal sodium chloride concentration in the reaction varies for different enzymes, and a set of three standard buffers containing three concentrations of NaCl are prepared and used when necessary. Typical digestions include a unit of enzyme per microgram of starting DNA, and one enzyme unit usually (depending on the supplier) is defined as the amount of enzyme needed to digest one microgram of double-stranded DNA completely in 1 h at the appropriate temperature. These reactions usually are incubated for 1–3 h, to ensure complete digestion, at the optimal temperature for enzyme activity, typically 37°C.

Agarose gel electrophoresis (see *Protocol 4*)
Agarose gel electrophoresis [2] is employed to check the progression of a restriction enzyme digestion, to determine quickly the yield and purity of a DNA isolation or polymerase chain reaction (PCR) and to size-fractionate DNA molecules, which then could be eluted from

Protocols provided

1. *Phenol extraction of DNA samples*
2. *Concentration of DNA by ethanol precipitation*
3. *Restriction digestion*
4. *Agarose gel electrophoresis*
5. *Elution of DNA fragments from agarose*
6. *Kinase end labeling of DNA*
7. *Bacterial cell maintenance and storage*
8. *Fragment purification on Sephacryl S-500 spin columns*

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the gel. Prior to gel casting, dried agarose is dissolved in buffer by heating, and the warm gel solution then is poured into a mold (made by wrapping clear tape around and extending above the edges of an 18 cm × 18 cm glass plate), which is fitted with a well-forming comb. The percentage of agarose in the gel varies. Although 0.7% agarose gels typically are used, in cases where the accurate size-fractionation of DNA molecules smaller than 1 kb is required, a 1%, 1.5% or 2% agarose gel is prepared, depending on the expected size(s) of the fragment(s). Ethidium bromide is included in the gel matrix to enable fluorescent visualization of the DNA fragments under ultraviolet (UV) light. Agarose gels are submerged in electrophoresis buffer in a horizontal electrophoresis apparatus. The DNA samples are mixed with gel tracking dye and loaded into the sample wells. Electrophoresis is usually at 150–200 mA for 0.5–1 h at room temperature, depending on the desired separation. When low-melting agarose is used for preparative agarose gels, electrophoresis is at 100–120 mA for 0.5–1 h, again depending on the desired separation, and a fan is positioned such that the heat generated is dissipated rapidly. Size markers are co-electrophoresed with DNA samples, when appropriate for fragment size determination. Two size markers are used, πX174 cleaved with restriction endonuclease *Hae*III to identify fragments between 0.3 and 2 kb and λ phage cleaved with restriction endonuclease *Hin*dIII to identify fragments between 2 and 23 kb. After electrophoresis, the gel is placed on a UV light box

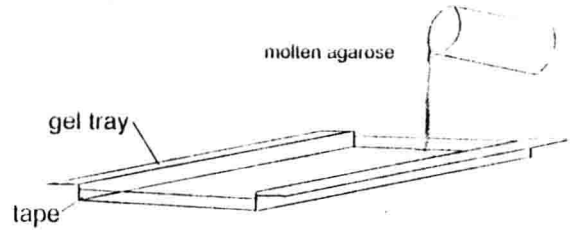

Figure 1. Preparation of a mold tray for agarose gels using plastic tape. Figure reproduced from Jones, P., Qui, J. and Rickwood, D. (1994) *RNA Isolation and Analysis* published by BIOS Scientific Publishers, Oxford.

and a picture of the fluorescent ethidium bromide stained DNA separation pattern is taken with a Polaroid camera.

Elution of DNA fragments from agarose (see *Protocol 5*)
DNA fragments are eluted from low-melting temperature agarose gels using an unpublished procedure first developed in our laboratory. Here, the band of interest is excised with a sterile razor blade, placed in a microcentrifuge tube, frozen at –70°C and then melted. Then, TE-saturated phenol is added to the melted gel slice, and the mixture is again frozen and then thawed. After this second thawing, the tube is centrifuged and the aqueous layer removed to a new tube. Residual phenol is removed with two ether extractions, and the DNA is concentrated by ethanol precipitation.

Kinase end-labeling of DNA (see *Protocol 6*)
Typical 5′-kinase labeling reactions include the DNA to be labeled, [γ-^{32}P]dATP, T4 polynucleotide kinase and buffer [3]. After incubation at 37°C, reactions are heat inactivated by incubation at 80°C. Portions of the reactions are mixed with gel loading dye and loaded into a well of a polyacrylamide gel and electrophoresed. The gel percentage and electrophoresis conditions vary depending on the sizes of the DNA molecules of interest. After electrophoresis, the gel is dried and exposed to X-ray film for radiolabeled DNA sequencing.

Bacterial cell maintenance and storage (see *Protocol 7*)

Three strains of *Escherichia coli* are used in these studies: JM101 for M13 infection and isolation [4], XL1BMRF′ (Stratagene) for M13 or pUC-based DNA transformation [5] and ED8767 for cosmid DNA transformation [6, 7 and H. Revel, personal communication]. To maintain their respective F′ episomes necessary for M13 viral infection [8], JM101 is streaked on to a M9 minimal media (modified from that given in ref. 1) plate and XL1BMRF′ is streaked on to an LB plate [1] containing tetracycline. ED8767 is streaked on to an LB plate containing ampicillin. These plates are incubated at 37°C overnight. For each strain, 3 ml of appropriate liquid media are inoculated with a smear of several colonies and incubated at 37°C with shaking at 250 r.p.m. for 8 h, and those cultures are then transferred into 50 ml of respective liquid media and incubated for an additional 12–16 h. Glycerol is added to a final concentration of 20%, and the glycerol stock cultures are distributed in 1.3 ml aliquots and frozen at −70 °C until use [1].

Fragment purification on Sephacryl S-500 spin columns
(see *Protocol 8*)

DNA fragments larger than a few hundred base pairs can be separated from smaller fragments by chromatography on a size exclusion column such as Sephacryl S-500. To simplify this procedure, a mini-spin column method has been developed.

Notes on precipitation of nucleic acids

General rules

Most nucleic acids may be precipitated by addition of monovalent cations and 2–3 vol. of cold 95% ethanol, followed by incubation at 0 to –70°C. The DNA or RNA may then be pelleted by centrifugation at 10000–13000 r.p.m. for 15 min at 4°C. A subsequent wash with 70% ethanol, followed by brief centrifugation, removes residual salt.

The general procedure for precipitating DNA and RNA is:

1 Add 0.1 vol. of 3 M sodium acetate, pH 4.5 to the nucleic acid solution to be precipitated. 5 M ammonium acetate, pH 7.4, NaCl and LiCl may be used as alternatives to sodium acetate. DNA also may be precipitated by addition of 0.6 vol. of isopropanol.
2 Add 2 vol. of cold 95% ethanol⚠.
3 Place at –70°C for at least 30 min, or at –20°C overnight.

or alternatively:

1 Combine 95 ml of 100% ethanol⚠ with 4 ml of 3 M sodium acetate, pH 4.5 and 1 ml of sterile water. Mix by inversion and store at –20°C.
2 Add 2.5 vol. of cold ethanol/acetate solution⚠ to the nucleic acid solution to be precipitated.
3 Place at –70°C for at least 30 min or –20°C for 2 h to overnight.

Oligonucleotides

Add 0.1 vol. of 3 M sodium acetate, pH 4.5, and 3 vol. of cold 95% ethanol⚠. Place at –70°C for at least 1 h.

RNA

Add 0.1 vol. of 1 M sodium acetate, pH 4.5, and 2.5 vol. of cold 95% ethanol. Precipitate large volumes at –20°C overnight. Small volume samples may be precipitated by placing in powdered dry ice or a dry ice–ethanol bath for 5–10 min.

Isobutanol concentration of DNA

DNA samples may be concentrated by extraction with isobutanol. Add slightly more than one volume of isobutanol, vortex vigorously and centrifuge to separate the phases. Discard the isobutanol (upper) phase, and extract once with water-saturated diethyl ether to remove residual isobutanol. The nucleic acid then may be ethanol precipitated as described previously.

Notes on phenol extraction of nucleic acids

The standard and preferred way to remove proteins from nucleic acid solutions is by extraction with neutralized phenol or phenol:chloroform. Generally, samples are extracted by addition of 1.0 vol. of neutralized (with TE buffer, pH 7.5) phenol to the sample, followed by vigorous mixing for a few seconds to form an emulsion. Following centrifugation for a few minutes, the aqueous (top) phase containing the nucleic acid is recovered and transferred to a clean tube. Residual phenol is then removed by extraction with an equal volume of water-saturated diethyl ether. Following centrifugation to separate the phases, the ether (upper) phase is discarded and the nucleic acid is ethanol precipitated as described previously.

A 1:1 mixture of phenol and chloroform is also useful for the removal of protein from nucleic acid samples. Following extraction with phenol:chloroform, the sample should be extracted once with an equal volume of chloroform, and ethanol precipitated as described above.

Protocol 1. Phenol extraction of DNA samples

Reagents

95% Ethanol/0.12 M sodium acetate (ethanol/acetate⚠)
TE-saturated phenol⚠: add an equal amount of 10 mM Tris–HCl, pH 7.5–8.0, 1 mM Na_2EDTA, pH 8.0 to ultrapure phenol(i), mix well, allow phases to separate, remove and discard upper (aqueous) phase. Repeat until the pH of the aqueous phase is between 7.5 and 8.0 (store at 4°C)
1.0 M Tris–HCl, pH 7.6
1.0 M Tris–HCl, pH 8.0
Water-saturated diethyl ether

Equipment

Microcentrifuge
1.5 ml Microcentrifuge tubes
Vortex mixer

Procedure

1 Add an equal volume of TE-saturated phenol to the DNA sample contained in a 1.5 ml microcentrifuge tube and vortex for 15–30 sec.
2 Centrifuge the emulsion for 5 min at 12 000 r.p.m. at room temperature to separate the phases.
3 Remove about 90% of the upper, aqueous layer to a clean tube, carefully avoiding proteins. Add an equal volume of 1:1 TE-saturated phenol:chloroform, centrifuge and remove to a clean tube as above. This additional extraction is not usually necessary if care is taken during the first phenol extraction.
4 Add an equal volume of water-saturated ether to the phenol extracted

Notes

This procedure will take about 15 min.

(i) Pure phenol is colorless, a pinky color indicates oxidation and in this case the phenol needs to be distilled before use.

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