PCR突变检测手册

PCR Mutation Detection Protocols

Bimal D.M.Theophilus
Ralph Rapley

Humana Press
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METHODS IN MOLECULAR BIOLOGY

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Preface

As we enter the new millennium, it is tempting to speculate what may lie ahead in future years, decades, and even centuries. In the area of the medical and life sciences at least, we can speculate with perhaps more certainty than may be possible in other areas. The exciting stage at which we find ourselves in the field of molecular genetics means that we can be in no doubt that the application of DNA technology will underlie many major advances in medicine in the coming decades. While international research efforts seek to demonstrate the viability of gene therapy, a major present application of human molecular genetics is the identification of disease-causing mutations. This information may be used for prenatal and carrier diagnoses, or to aid early detection and determine appropriate treatment of various disease states. While, traditionally, progress has been in diseases caused by mutations in single genes, present research is unraveling the underlying molecular basis of multigene disorders such as cancers, as well as identifying increasing numbers of disease-associated single nucleotide polymorphisms (SNPs). In addition, the completion of the human genome project will no doubt advance the pace of discovery even further, and also provide new possibilities for diagnosis and treatment.

The rapidly increasing applications of DNA technology to disease diagnosis has spawned numerous molecular diagnostic laboratories with an interest in mutation detection methodology. Such laboratories would like the availability of a single mutation method that is cheap, fast, with 100% detection in kilobase lengths of DNA, and does not require specialized equipment or harmful reagents. However, because no such universally applicable method exists, the present state of play is a plethora of methodology, from which the user makes a choice based on facilities, expertise, frequency of use, detection rate demanded, and whether the application purpose is diagnostic (detection of the presence or absence of a known mutation) or involves screening a candidate gene for a new unidentified mutation.

PCR Mutation Detection Protocols comprises a comprehensive step-by-step guide that brings together the large number of PCR-based mutation detection methods described to date. Many of the earlier chapters describe the basic technology and techniques, e.g., the principles and methodology of PCR, labeling DNA probes, restriction fragment length polymorphism analysis, and Southern blotting. Further techniques are then presented covering both categories of

mutation detection: detection of the presence of a known mutation and screening for new mutations. The techniques presented in each involve different approaches appropriate to different mutation types: point mutations (e.g., ASO-PCR, SSCP, DGGE, chemical cleavage), deletions (multiplex PCR, FISH, blotting), nonsense mutations (PTT), etc. The new and exciting techniques of DNA array analysis are also presented. The final chapters deal with different approaches to DNA sequencing as a detection method in its own right, or for characterizing mutations previously located by one of the other screening techniques. Recently developed and experimental methods, such as conformation sensitive gel electrophoresis, are presented in addition to the more established methods.

Each chapter includes the underlying basis of the techniques, and enables the reader to select the optimum method to use in relation to the above criteria. Particularly useful are the Notes sections containing the small details necessary for the successful execution of the technique. *PCR Mutation Detection Protocols* is aimed at postgraduate scientists and researchers in diagnostic and research laboratories. In addition, the basic techniques covered in the introductory chapters will ensure the book constitutes a fitting initiation to molecular techniques for individuals in related medical and scientific fields.

Bimal D. M. Theophilus
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Contents

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Agarose and Polyacrylamide Gel Electrophoresis

Andrea M. Guilliatt

1. Introduction

Electrophoresis through agarose or polyacrylamide gels is a standard method used to separate, identify, and purify nucleic acids. The technique is simple, rapid to perform and capable of resolving fragments that differ by as little as 0.2% in size. Electrophoresis occurs under the influence of an electric field: Charged molecules such as nucleic acids migrate in the direction of the electrode having the opposite charge (anode). The electrophoretic mobility of nucleic acids is determined by a number of parameters, but molecules of linear double-stranded DNA migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs *(1)* and therefore larger molecules migrate more slowly because of the greater frictional drag (*see* **Note 1**). Other factors affecting electrophoretic mobility include the p*K* value, base composition, concentration of gel matrix, composition and ionic strength of the electrophoresis buffer, temperature and the use of intercalating dyes such as ethidium bromide.

The matrix used for electrophoresis should have adjustable but regular pore sizes and be chemically inert, and the choice of which gel matrix to use depends primarily on the sizes of fragments being separated. Agarose gels are the most popular medium for the separation of moderate and large-sized nucleic acids and have a wide range of separation but a relatively low resolving power. Polyacrylamide gels are most effective for separating smaller fragments, and although the gels are generally more difficult to prepare and handle, they have three major advantages over agarose gels. They have a greater resolving power, can accommodate larger quantities of DNA without significant loss in resolution, and the DNA recovered from polyacrylamide gels is extremely pure.

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Two electrophoresis buffers are commonly used and contain EDTA and Tris-acetate (TAE) or Tris-borate (TBE) at a concentration of approx 50 m*M*. For historical reasons, TAE is the most commonly used buffer for agarose gel electrophoresis, but its buffering capacity is low and may become exhausted during extended electrophoresis. TBE is slightly more expensive, but it offers significantly higher buffering capacity. Although the resolving power of the buffers is almost identical, double-stranded linear DNA migrates approx 10% faster in TAE than in TBE. Electrophoresis buffers are routinely prepared as concentrated solutions and stored at room temperature (*see* **Note 2**).

The most convenient method for visualizing DNA in agarose and polyacrylamide gels is by staining with the fluorescent dye ethidium bromide (3,8-diamino-6-ethyl-5-phenyl-phenanthridium bromide), which contains a fixed planar group that intercalates between the stacked bases of the DNA *(2)*. The fixed position and the close proximity to the bases causes the bound dye to display an increased fluorescent yield compared to that of the free dye in solution. Ultraviolet (UV) radiation at a range of 260–360 nm is absorbed by the DNA and transmitted to the dye, and the energy is re-emitted at 590 nm in the red–orange region of the visible spectrum. Because the fluorescent yield of ethidium bromide: DNA complexes is greater than that of unbound dye, small amounts of DNA can be detected in the presence of free ethidium bromide in the gel. Ethidium bromide promotes damage of the nucleic acids when viewed under UV light (photonicking); therefore, if the nucleic acid is to be used in reactions following visualization, the gel should be viewed using long-wavelength UV light (300 nm).

1.1. Agarose Gel Electrophoresis

Agarose is a linear polymer extracted from seaweed that forms a gel matrix by hydrogen-bonding when heated in a buffer and allowed to cool. Many chemically modified forms of agarose are available commercially that gel or melt at different temperatures without any significant loss of mechanical strength. Although these different forms of agarose can be useful in both the qualitative and preparative electrophoresis of DNA, the resolving power is still not comparable to that of polyacrylamide gels.

The density and porosity of the gel matrix is determined by the concentration of agarose used, referred to as the percentage of agarose (w/v) in buffer (*see* **Note 3**). Typical agarose gel concentrations fall within the range of 0.3 to 2.5% (w/v), depending on the size of DNA fragments to be separated (**Table 1**). For most applications, only a single-component agarose is needed and no polymerization catalysts are required and they are, therefore, quick and easy to prepare. This coupled, with the lack of toxicity (unless in the buffers), is largely responsible for the popularity of agarose gel electrophoresis.

Table 1
Range of Separation of Linear DNA Molecules in Different Agarose Gel Concentrations

Concentration of agarose (% [w/v])	Efficient range of separation of linear DNA molecules (kb)
0.3	5–60
0.6	1–20
0.7	0.8–10
0.9	0.5–7
1.2	0.4–6
1.5	0.2–3
2.0	0.1–2

Many configurations and sizes of agarose gel electrophoresis tanks are available, of which the most common is the horizontal slab gel. Because of their relatively poor mechanical strength, agarose gels are cast in clear plastic UV-transparent trays allowing handling and transfer of the gel once set (*see* **Note 4**). Electrophoresis is carried out with the gel submerged just beneath the surface of the buffer, and as the resistance of the gel is similar to that of the buffer, a current passes through the gel. The principle advantage of submarine gel electrophoresis is that the thin layer of buffer prevents the gel from drying out and provides some degree of cooling.

The electrophoretic behavior of DNA in agarose gels is not significantly affected by temperature or the base composition of the DNA *(3)*; therefore, agarose gels are generally run at room temperature unless low-melting-temperature agarose is used or the agarose concentration is less than 0.5% (w/v), when the mechanical strength can be improved by running at 4°C.

1.2. Polyacrylamide Gel Electrophoresis

Polyacrylamide gels are formed by the vinyl polymerization of acrylamide monomers, (CH_2=CH-CO-NH_2) crosslinked by the bifunctional co-monomer *N,N'*-methylene-bis-acrylamide (CH_2=CH-CO-NH-CH_2-NH-CO-CH=CH_2). The resulting crosslinked chains form a gel structure whose pore size is determined by the initial concentrations of both acrylamide and the crosslinker. The nomenclature introduced by Hjertén et al. *(4)* is now widely used to describe gel composition, the term *T* being the total monomer concentration (acrylamide and Bis) in grams/100 mL and *C* being the percentage (by weight) of total monomer *T* that is contributed by the crosslinker (Bis). The pore size of the gel can be altered in an easy and controllable fashion by changing the concentrations of the two monomers. The polymerization proceeds by a free-radi-

Table 2
Range of Separation of Linear DNA Molecules in Agarose and Polyacrylamide Gels and the Position of Migration of Bromophenol Blue and Xylene Cyanol

Agarose gel concentration (%[w/v])	Effective range of resolution (bp)	Xylene cyanol migration (bp)	Bromophenol blue migration (bp)
0.5–1.5	1000–3000	4000–5000	400–500
Acrylamide gel concentration (%[w/v])			
3.5	1000–2000	460	100
5.0	80–500	260	65
8.0	60–400	160	45
12.0	40–200	70	20
15.0	25–150	60	15
20.0	6–100	45	12

cal mechanism and the most common method of initiation is with ammonium persulfate, which produces oxygen free radicals by a base-catalyzed mechanism, typically tertiary aliphatic amines such as *N,N,N'N'*-tetramethylethylenediamine (TEMED) (*see* **Note 5**). The length of the chains is determined by the concentration of the acrylamide in the polymerization reaction (between 3.5% and 20%). One molecule of crosslinker is included for every 29 monomers of acrylamide. The effective range of separation in nondenaturing gels containing different concentrations of acrylamide is shown in **Table 2**.

Polyacrylamide gels are usually run between two glass plates, ensuring uniform electrical conditions across the slab so that comparison between different sample zones is far more accurate and a large number of samples may be run on the gel.

Polyacrylamide gels are poured and run in 1X TBE at low voltages to prevent denaturation of small fragments of DNA by heat generated by passage of the electric current. Most species of double-stranded DNA migrate through the gel at a rate approximately inversely proportion to the $\log_{10}$ of their size, however, their electrophoretic mobility is affected by their base composition and sequence, so that two DNAs of exactly the same size can differ in mobility by up to 10%, as a result of secondary structures that may form at specific sequences in the double-stranded DNA *(5)*.

Denaturing polyacrylamide gels are used for the separation and purification of single-stranded fragments of DNA and are polymerized in the presence of

an agent that suppresses base-pairing in nucleic acids, usually urea. Denatured DNA migrates though these gels at a rate that is almost completely dependent on its base composition and sequence and is discussed elsewhere in this volume (*see* Chapters 14–16).

2. Materials

2.1. Agarose Gel Electrophoresis

All of the chemicals used are of molecular biology grade, and solutions are prepared with double-distilled water unless otherwise stated.

1. Agarose gel apparatus, comprising:
   a. Gel tank and safety lid
   b. Gel tray
   c. Comb
   d. Gel caster (optional)
2. Power supply capable of at least 100 V, 100 mA.
3. Powdered agarose.
4. Electrophoresis buffer (*see* **Note 2** for formulations).
5. 10X Gel loading buffer: The loading buffer for sample application should contain 0.25% bromophenol blue (BPB) and 0.25% xylene cyanol as tracking dyes and 30% sucrose, glycerol, or Ficoll to increase the sample solution density (*see* **Note 6**).
6. Ethidium bromide solution is generally prepared as a stock solution at a concentration of 10 mg/mL in water and stored at room temperature protected from light. Ethidium bromide is toxic and a powerful mutagen; therefore, gloves should always be worn. Solutions containing ethidium bromide should be disposed of appropriately as discussed in the Material Safety Data Sheets.
7. Microwave oven or hot plate.
8. UV transilluminator and gel documentation system.

2.2. Polyacrylamide Gel Electrophoresis

All of the chemicals used are of molecular biology grade and solutions are prepared with double-distilled water unless otherwise stated.

1. Polyacrylamide gel apparatus, comprising:
   a. Gel tank and safety lid
   b. Glass plates
   c. Spacers and combs of the same thickness
   d. Clamps or gel caster assembly (optional)
2. 30% Acrylamide stock, prepared by the addition of 29 g of acrylamide and 1 g *N,N'*-methylene-bis-acrylamide to 100 mL water (*see* **Note 7**).
3. 10X TBE (*see* **Note 2** for formulation).
4. 10% Ammonium persulfate, prepared by adding 1 g ammonium persulfate to 10 mL water. This solution may be kept at 4°C for several weeks.
5. TEMED.

6. Power supply.
7. Siliconizing solution (dimethyl dichlorosilane [e.g., Sigmacote®]).

3. Methods

3.1. Agarose Gel Electrophoresis

3.1.1. Assembly and Pouring of the Gel

1. Seal the edges of the UV-transparent plastic casting tray with strong masking tape or use a commercial gel casting system (*see* **Note 8**).
2. Place the tray/gel caster onto a horizontal section of bench, using a glass leveling plate if necessary, and place the comb(s) in the appropriate position(s) so that wells are formed at the cathode end of the gel.
3. Add the desired amount of powdered agarose to a measured quantity of 1X electrophoresis buffer in an Erlenmeyer flask or beaker and cover with Saran-Wrap. Heat the mixture in a microwave oven swirling every 30 s until the agarose is visibly seen to have dissolved. Alternatively, the agarose can be heated using a hot plate. Any undissolved agarose appears as small translucent particles (*see* **Note 9**).
4. Allow the solution to cool to 50°C, unless a high concentration of agarose or high-gelling-temperature agarose is used where gelation will occur more rapidly. A low level (0.5 µg/mL) of ethidium bromide can be added at this stage, allowing the progression of the electrophoresis to be analyzed during electrophoresis by illuminating the gel with UV light (*see* **Note 10**).
5. Pour the agarose into the gel mold, ensuring that no air bubbles form between the teeth of the comb, and allow the gel to set at room temperature for 30–40 min.

3.1.2. Running the Gel

1. Carefully remove the comb and place the gel and tray into the gel tank oriented with the wells at the cathode end, and add sufficient 1X electrophoresis buffer to cover the gel to a depth of approx 1 mm (*see* **Note 11**).
2. Mix the DNA samples with gel loading buffer to produce a 1X concentration of buffer and load into the wells through the thin layer of running buffer. Placing a black piece of paper behind the wells may facilitate in the loading process by making the wells more visible (*see* **Note 12**).
3. Load a DNA size standard to allow the determination of the sizes of the DNA fragments, because although the tracking dyes in the loading buffer give a rough estimate of the migration of the DNA, they do not give the exact size. Size standards can be purchased commercially or prepared by restriction enzyme digestion of plasmid DNA, producing DNA fragments of known sizes.
4. Place the lid onto the gel tank, being careful not to disturb the samples, and begin electrophoresis (*see* **Note 13**).
5. When the dyes have migrated the appropriate distance on the gel as shown in **Table 2**, turn off the power supply and proceed with visualization of the DNA.