PCR Product Analysis

a guide to using semidry flatbed gel electrophoresis

Amersham
Biosciences

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PCR Product Analysis: Using Semidry Flatbed Gel Electrophoresis

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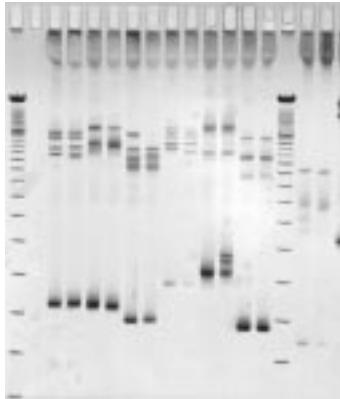
Figure 1 BRCA1 mutants unanalyzed by SSCP using GeneGel Excel 12.5/24 with the GenePhor electrophoresis unit.

Introduction

Polymerase chain reaction (PCR) has revolutionized the field of molecular biology due to its exceptional speed, specificity, and sensitivity as a tool for genetic analysis. Of note also is the exceptional versatility of PCR and the readiness with which users of the technique have learned to adapt and modify its fundamental feature of targeted deoxyribonucleic acid (DNA) amplification to achieve diverse experimental goals.

Typical DNA fragment sizes that can be reliably generated after PCR amplification tend to be less than 500 to 600 base pairs in length, although these limitations are rapidly disappearing. There are certainly commercially available polymerases and specialized reaction conditions that allow for much greater processivity, but these are typically reserved for highly specific applications. Thermostable polymerases with fairly moderate processivity are all that is required to perform the majority of widely used PCR applications today.

Convenient upstream and downstream analysis of relatively small DNA fragments is important to take full advantage of the convenience, sensitivity, and efficiency of most PCR strategies. This guidebook aims to bring together in one place fundamental and applied concepts of how to approach some of these widely used PCR-based assays. As you may note, making the separation and detection of small DNA fragments as rapid and convenient as possible is vital to the ease and efficiency of most of the assays described. In that regard, Chapter 3 describes the benefits of using semidry flatbed polyacrylamide gel electrophoresis over standard vertical PAGE systems for these types of experiments. These systems are unique in that they require no liquid acrylamide or running buffer handling and no concern about gel distortion, because of the exclusive use of plastic-backed precast gels. We begin with an overview of the fundamental concepts of PCR and gel electrophoresis and then move into essential descriptions of the applications themselves.

Chapter 1: PCR

PCR is a method for in vitro amplification of DNA. It has substantially accelerated the pace of research in many fields of biology, both by reducing the time required to perform routine manipulations of DNA and by making new manipulations possible. In essence, PCR is multiple rounds of primer extension reactions in which complementary strands of a defined region of a DNA molecule are simultaneously synthesized by a thermostable DNA polymerase. During repeated rounds of these reactions, the number of newly synthesized DNA strands increases exponentially so that after 20 to 30 reaction cycles, the initial template DNA will have been replicated several million–fold. This power to faithfully amplify, along with the low cost and simplicity of the method, have made PCR an indispensable tool.

A number of excellent practical handbooks are available that provide detailed guidelines for specific applications of PCR. Among the variables that must be considered for a particular application of PCR are the quality and quantity of the DNA template, the polymerase used, primer sequence and concentration, nucleoside triphosphate and magnesium concentration, and the cycle parameters. Although PCR is a robust and reliable procedure, all of these factors can affect its efficiency and fidelity, and reaction conditions must be adjusted according to the application. (See the list of references at the end of this chapter.)

The remarkable ability of PCR to amplify specific DNA sequences has, along with its obvious benefits, some practical pitfalls that require careful attention. First among these is the ability of PCR to amplify DNA inadvertently introduced into the reaction. Precautions against contamination are especially important in forensic and clinical applications, but must be considered in every laboratory using the technique. Some principles used in sterile culture of microorganisms are applicable, but additional precautions— such as strict segregation of sample preparation, reaction assembly, thermocycler, and analysis work areas, and the use of positive displacement pipettes or aerosol preventive tips—may also be necessary. The use of ultraviolet light and chemical decontamination procedures as well as of enzymatic methods to prevent the amplification of "carryover" templates should be employed in some situations.

For many purposes, the PCR-induced mutation rate is not of serious consequence. But where the sequence of the reaction product itself, or the activity of the message or protein it encodes, is important, a number of factors must be considered. These include the inherent fidelity and proofreading activity of the polymerase, as well as a number of the reaction parameters discussed below.

Parameters Influencing the Yield and Specificity of PCR

DNA Polymerase

Many thermostable DNA polymerases are now commercially available, each with different characteristics of thermal stability, exonuclease activity, processivity, fidelity, and reverse transcriptase activity. *Taq* DNA polymerase, with its heat stability, high optimal temperature, and broad pH tolerance, is the most widely used thermostable DNA polymerase. The recommended concentration range for *Taq* DNA polymerase is between 1 and 2.5 units per 100 l reaction. Enzyme requirements may vary with respect to individual target templates, primers, and suppliers. *Taq* DNA polymerase from different suppliers may behave differently because of different formulations, assay conditions, and unit definitions (especially for non-licensed products). If the enzyme concentration is too high (>4 units/ml), nonspecific background products may accumulate; if it is too low, an insufficient amount of desired product is made.

PCR Buffer

Each thermostable polymerase has unique buffer requirements for optimal activity, most requiring Tris-Cl between 10 mM and 50 mM at pH 8.3–9.0. A stabilizing protein such as BSA or gelatin, and a non-ionic detergent such as Tween™ 20, NP-40, or Triton™ X-100, is also usually required. Concentrated reaction buffers are normally provided by the supplier of the enzyme, but for certain applications other additives such as formamide or glycerol may be essential for an efficient reaction.

Magnesium Concentration

Magnesium concentration has a significant effect on the efficiency and specificity of the PCR reaction. In addition to its direct influence on enzyme activity and fidelity, magnesium also affects the T_m of the various hybrids that form during cycling, including primer-template, template-template, and primer-primer. It is also important to remember that free nucleotides and any EDTA present in the reaction influence the effective magnesium concentration. If the magnesium concentration is too low, little or no product may be produced. If it is too high, artifactual products may result from non-specific priming of template DNA or from primer-dimers. For each new primer pair, it is generally worthwhile to titrate the magnesium concentration in 0.5-mM steps over the range of 0.5 mM to 5 mM.

dNTPs

A total dNTP concentration between 80 mM and 800 mM (20 mM to 200 mM each dNTP) is recommended for most PCR reactions. It is tempting to believe that more is better, but it should be recognized that 5μg of PCR product can be synthesized from only 15 nmoles of dNTP. Concentrations higher than 800 mM increase the rate at which *Taq* DNA polymerase misincorporates nucleotides. This effect can be exploited to generate mutants, but should usually be avoided.

Template

In some respects the quality of template DNA is not as critical for PCR as it is for certain other enzymatic manipulations. Evidence for this can be found in the wide variety of sources of DNA that can be successfully amplified, including "fossil DNA," entire bacterial colonies, paraffin-embedded tissue, and single human hairs. For some applications, however, the quality of the DNA template is a very important factor. For example, amplification of long pieces of DNA requires that the template be prepared in such a way as to minimize single-strand nicks; and a GC-rich template may require higher or prolonged denaturation steps and the use of a more thermostable polymerase.

A 10^7-fold excess of primer to template is a good rule of thumb for a standard PCR reaction. Because primer concentrations above about 0.5 mM can result in a detrimental amount of primer-dimer formation, the concentration of template DNA is limited to about 50 fM. For a 100 μl reaction, this concentration represents pg amounts of plasmid DNA, ng amounts of bacterial DNA, and μg amounts of human genomic DNA. Under specific reaction conditions, however, genomic DNA from a single cell can be amplified.

Cycle Parameters

It is critical that complete strand separation occurs during the denaturing step. A temperature of 94 to 96 °C for 1 minute is usually sufficient, but GC-rich templates may require a higher temperature. Due to the high molar excess of primer to template, the optimal annealing temperature is dependent on the rate of cooling from the denaturing temperature. It is thus usually several degrees higher than the calculated T_m for the primer-template. The extension reaction is fairly temperature-tolerant, and 72 °C is optimal for many thermostable polymerases. The duration of the extension step should be adjusted for the length of the amplified region, with 1 minute recommended for targets less than 500 bp in length, and 2 to 3 minutes for targets greater than 500 bp. To reduce the chance of mispriming that may result from non-specific hybridization occurring during the addition of reaction components, so-called hot-start PCR, in which *Taq* DNA polymerase is added only after the other reactants are raised to a temperature of 70 to 80 °C, may be recommended.

Primer Design and Concentration

Primers from 10 to 30 nucleotides in length with a GC composition of 40 to 60% are used in most PCR reactions. Obviously, self-complementarity and complementarity between primers should be avoided, as should continuous runs of the same nucleotide. Primers corresponding to regions of the template containing a high degree of potential secondary structure should also be avoided. Specificity and efficiency of the reaction is in many cases improved if the 3'-end of each primer terminates in two or more G or C residues. Due to the increased likelihood of mispriming, however, it is best to avoid an unusually high GC content at the 3'-end of the primer. Many applications depend on the use of primers incorporating special features in their design, either to control specificity or to facilitate subsequent manipulations of the amplified product. These features may include modified nucleotides or non-complementary sequence.

Primer concentrations between 0.1 M and 0.5 mM are generally recommended. Higher primer concentrations may promote mispriming and accumulation of non-specific product as well as increase the probability of generating a template-independent artifact *(primer-dimer)*. These side products are themselves substrates for PCR and compete with the desired product for enzyme, dNTPs, and primers, resulting in lower yield of the desired fragment.

Ready-To-Go™ Reaction with Components

Amersham Biosciences offers premixed PCR reaction components in the form of room-temperature stable beads, a formulation that minimizes pipetting steps and reduces potential for pipetting errors and contamination. Ready-To-Go PCR Beads and Ready-To-Go RAPD Analysis Beads contain all of the reaction components except water, template, and primer, predispensed in 0.5-ml or 0.2-ml tubes. PCR results are comparable or superior to those obtained with traditional "wet reagents" with regard to reproducibility, yield, specificity, and performance of the amplification product. Amersham Biosciences is licensed to sell this product for use in PCR for research and development. Its shelf life, if it is stored properly, is 2 years.

Ready-To-Go PCR Beads are optimized for standard PCR conditions and can be used with a variety of templates, including genomic DNA, viral DNA, plasmid DNA, and cDNA. Ready-To-Go RAPD Analysis Beads are optimized for the low-stringency PCR conditions utilized in the RAPD reaction. Six different RAPD Analysis Primers designed to produce useful numbers of PCR products with a variety of template sources are available. Ready-To-Go RAPD Analysis Beads were developed to address the problem of irreproducibility sometimes associated with RAPD analysis, much of which stems in part from difficulty in standardizing PCR reaction mixtures.

Because RNA cannot be amplified directly, it has to be converted to cDNA using reverse transcriptase. Ready-To-Go RT-PCR Beads are premixed, predispensed individual reactions for performing RT-PCR. Each bead contains all the necessary components, except primers and template, for performing reverse transcriptase and PCR sequentially as a one-tube, one-step 50-l reaction. The kit also provides control beads as well as pd(N)6 and pd(T)12-18 for priming reverse transcriptase. This product is useful for applications such as detection of RNA viruses, RACE, cDNA sequence analysis, comparative RNA analysis, and high-throughput screening.

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Chapter 2
Electrophoresis of Nucleic Acids

Introduction

Simply put, *electrophoresis* is the movement of charged molecules in an aqueous environment due to the effect of an applied electric field. Electrophoresis of macromolecules is normally carried out by applying a sample in a narrow zone to a solution stabilized by a porous matrix. Under the influence of an applied voltage, different species of molecules in the sample move through the matrix at different velocities. At the end of the separation, the different species are detected as bands at different positions in the matrix. The separated molecules can be detected in position in the gel by staining or by autoradiography, or they may be transferred to a membrane surface for detection by various methods.

A matrix is required because the electric current passing through the electrophoresis solution generates heat, which increases the rate of diffusion and causes convective mixing of the bands in the absence of a stabilizing medium. Historically, media such as paper, starch, and cellulose acetate were widely used, but today polyacrylamide and agarose gels are the most common stabilizing media used in electrophoresis. The widespread use of agarose and acrylamide gels stems from the fact that these matrices also act as "molecular sieves" during electrophoresis, restricting the movement of biomolecules according to their size and structure. Agarose and polyacrylamide gels are cross-linked, spongelike structures. Although they are up to 99.5% water, the size of the pores of these gels is similar to that of many proteins and nucleic acids. As molecules are forced through the gel by the applied voltage, larger molecules are retarded by the gel to a greater extent than are smaller molecules. For any particular gel, molecules smaller than a matrix-determined size are not retarded at all; they move almost as if in free solution. At the other extreme, molecules larger than a matrix-determined size cannot enter the gel at all. Gels can be tailored to sieve molecules of a wide range of sizes by appropriate choice of matrix concentration. The average pore size of a gel is determined by the percentage of solids in the gel and, for polyacrylamide, by the amount of cross-linker as well.

Although there are practical limits to the range of gel densities possible with agarose and polyacrylamide, these two matrices allow the electrophoretic separation of DNA strands anywhere from oligonucleotides only a few base pairs in length to chromosomes or chromosomal fragments as large as several million base pairs long. Polyacrylamide, which makes a small-pore gel, is used to separate polynucleotides from fewer than 5 bases up to approximately 2,000 base pairs in size. Agarose gels, with their large pore size, can be used to separate nucleic acids from 50 to 30,000 base pairs, and, with pulsed-field techniques, up to chromosome- and similar-sized pieces greater than 5×10^6 base pairs long.

Agarose Gels

Agarose is a highly purified polysaccharide derived from agar. It is available in a number of grades with various levels of electroendosmosis, melting temperature, gel strength, and clarity. *Electroendosmosis (EEO)* is the bulk flow of water toward the cathode that results from the presence of fixed charges in the agarose matrix. Agarose sold as "molecular biology grade" is generally of low enough EEO for routine purposes. A variety of agaroses are available for particular applications, such as for separation of very large DNAs by pulsed-field techniques, "in-gel" enzymatic digestions, or extraction of separated material from the gel.

Agarose gels are prepared by boiling a suspension of agarose powder in buffer and pouring the dissolved agarose into a mold. Within the mold it remains liquid until it gels, or "sets," as the temperature drops to about 40 °C. Once set, the gel is stable at temperatures below approximately 100 °C. There are types of agarose with melting and gelling temperatures considerably lower than those of standard agarose. These properties allow recovery of material from a gel after separation and permit subsequent enzymatic treatments of the separated material if desired. The pore size and sieving characteristics of a gel are determined by adjusting the concentration of agarose in the gel: The higher the concentration, the smaller the pore size. Working concentrations are normally in the range of 0.4 to 4% (w/v). Concentrations outside this range result in gels with mechanical properties that make their use impractical.

Electrophoresis in agarose gels is usually performed in a flatbed format, submerged in buffer. Continuous buffer systems based on Tris and acetate or borate are suitable for most purposes. The principal advantages of using agarose gel electrophoresis are its simplicity and versatility, and the wide separation ranges that are possible. Applications for which agarose is most suitable include subcloning, where it is necessary to purify fragments and check their orientation within a vector; restriction mapping and restriction fragment length polymorphism (RFLP) analysis; northern and southern mapping; and pulsed-field gel electrophoresis (PFGE).

Polyacrylamide Gels

In applications where high resolution of DNA strands below about 1-kilobase length is required, the more restrictive polyacrylamide gel is necessary to retard the migration of the molecules. A polyacrylamide gel forms when a dissolved mixture of acrylamide and cross-linker monomers polymerize into long chains that are covalently linked. The gel structure is held together by the cross-linker. The most common cross-linker is N, N'-methylenebisacrylamide (*"bis"* for short). Because polymerization of acrylamide is a free-radical catalyzed reaction, preparation of polyacrylamide gels is somewhat more complex than that of agarose gels. Atmospheric oxygen is a free-radical scavenger that can inhibit polymerization. For this reason polyacrylamide gels are nearly always cast between glass plates.

The size of the pores in a polyacrylamide gel is determined by two parameters: total solids content (%T) and the ratio of cross-linker to acrylamide monomer (%C). The %T is the ratio of the sum of the weights of the acrylamide monomer and the cross-linker to the volume of solution, expressed as % w/v. For example, a 20%T gel would contain 20% w/v of acrylamide plus bis. As the %T increases, the pore size decreases. The second way to adjust pore size is to vary the amount of cross-linker. The %C is the weight/weight percentage of total cross-linker weight in the sum of monomer and cross-linker weights. Thus, a 20%T 5%C bis gel would have 20% w/v of acrylamide plus bis, and the bis would account for 5% of the total solids weight (acrylamide plus bis). Occasionally, gel compositions are given as ratios of acrylamide to cross-linker (such as 19:1 for the 20%T 5%C mixture). It has been found that for any single %T, 5% cross-linking creates the smallest pores in a gel. Above and below 5%, the pore size increases.

Determination of %T and %C for acrylamide gels:

> **%T = {g(acrylamide + bisacrylamide) / 100ml} 100**
> **%C = {g(bisacrylamide) / g(acrylamide + bisacrylamide)} 100**

If the sample material is a mixture with species having a wide range of molecular weights, you may want to use a pore-gradient gel. In these gels the pore size is larger at the top of the gel than at the bottom; the gel becomes more restrictive as the run progresses. The presence of the gradient yields a gel with a wider range of size resolution and also keeps bands tighter than in uniform-concentration gels.

The polymerization of acrylamide gel can be initiated either by a chemical peroxide or by a photochemical method. The most common method uses ammonium persulfate as the initiator peroxide and the quaternary amine, N,N,N',N'-tetramethylethylenediamine (TEMED) as the catalyst. For photochemical polymerization, riboflavin and long-wave ultraviolet (UV) light are the initiator and TEMED is the catalyst. The photochemical reaction is started by shining long-wavelength ultraviolet light on the gel mixture, usually from a fluorescent light. Polymerization of acrylamide generates heat. Rapid polymerization can generate too much heat, causing convection inconsistencies in the gel structure and occasionally even breaking glass plates. This is a problem particularly for high-concentration gels (>20%T). To prevent excessive heating, the concentration of initiator-catalyst reagents should be adjusted so that complete polymerization requires 20 to 60 minutes.

Electrical Parameters

The velocity (v) in cm/sec of a charged species in an electric field is given by:

$$v = \mu E$$

where μ is electrophoretic mobility in cm^2/(vsec) and E is field strength in v/cm. *Electrophoretic mobility* is a term accounting for a number of variables associated with the ion (net charge, size, and shape) and the medium in which the electrophoresis takes place (viscosity, temperature, and pH). Although not a generally useful equation, this relationship shows that the mobility of the biomolecule is directly proportional to the voltage applied to the electrophoresis system.

Two equations—Ohm's Law and the power equation—relate the electrical parameters involved in electrophoresis. *Ohm's Law,*

$$V = IR$$

relates volts (V), current in amperes (I), and resistance in ohms (Ω). A power supply provides the electrical potential that moves the molecules through the gel, while the leads, electrodes, buffer, and gel all act as simple resistors.

The second fundamental equation in electrophoresis is the *power equation*, which describes the amount of heat produced in a circuit. This heat is also referred to as *Joule heat*. In its simplest form,

$$W = VI$$

the power equation relates power in watts (W) to voltage (V) and current (I).

During the course of a separation, however, the resistance of the electrophoresis circuit does not remain constant. Resistance depends on such factors as ionic strength and capacity of the buffer, gel concentration, salts present in the sample, and temperature. Buffer resistance declines with increasing temperature caused by Joule heating, and may also change as discontinuous buffer ion fronts move through a gel, generally increasing as the run progresses.

Power supplies used for electrophoresis can hold constant at least one electrical parameter (current, voltage, or power). If, for example, the power supply is set for constant voltage, Ohm's Law and the power equation tell us that as the resistance of the circuit declines, the current and power will increase. In some circumstances the current may increase until the current or power limit of the power supply is met. With a high-capacity power supply, this could lead to a level of Joule heating that could ruin an experiment or, worse, represent a severe safety hazard. High-performance power supplies, including those sold by Amersham Biosciences, offer an automatic crossover function in which limits can be set for two or all three electrical parameters, preventing any of them from exceeding safe limits.

Electrophoresis and the Structure of PCR Products

Unlike proteins, which can be either positively or negatively charged because they contain both acidic and basic residues, nucleic acids remain negatively charged at the pH used for most electrophoresis buffers because of the strong acidic nature of the phosphate groups in the backbone. Furthermore, because the charge of a nucleic acid resides in the phosphate backbone, the charge-density of nucleic acids is constant. Practically, this means that the relative mobilities of nucleic acids within agarose and polyacrylamide gels are strictly determined by their size and structure. Which of these features most influences the rate of migration within the gel depends on conditions that can be easily manipulated by the researcher.

Under "native" conditions of pH, ionic strength, and buffer composition, double-stranded DNAs adopt essentially the same simple structure, and their rate of migration through a gel matrix is based primarily on size. Sample preparation and electrophoresis under such conditions are straightforward, and, for many purposes, such simple size-based separations are adequate. In DNA sequencing, however, single-base resolution is achieved by entirely eliminating the effects of structure through complete denaturation of the DNA with such agents as formamide and urea at elevated temperatures. On the other hand, experimental conditions can be manipulated in ways that favor the formation of structure to the extent that DNA strands of identical length can be resolved exclusively on the basis of structure. Single-stranded conformational polymorphism (SSCP) and heteroduplex analysis are methods by which differences in DNA structure resulting from single-base mutations provide the basis for electrophoretic separation. Two other important methods of mutation detection are based on structural transitions that occur during electrophoresis. In temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE), separation of the DNA strands initially occurs according to size; but as electrophoresis proceeds, melting of the DNA strands occurs and separation begins to depend more on structure. The point in the gradient at which the melting occurs is sequence-dependent, and a particular mutation can often be identified by its affect on the mobility of the DNA in which it occurs.

Temperature Regulation

For most electrophoresis applications, it is desirable to remove heat from the system. Excessive heat can cause agarose gels to melt and glass plates to break; it can also damage the electrophoresis unit and cause nucleic acids to denature. As discussed in other sections of this guidebook, however, certain applications require that the temperature of the gel be raised above ambient temperature. Non-uniform heat distribution distorts the gel pattern, because electrophoretic mobility is proportional to temperature. Slab gels are described as "smiling" when the samples in the centre lanes move faster than those in the outside lanes. This effect is due to more-rapid loss of heat from the edges of the gel than from the centre. Bands may appear broader than expected, or even as doublets, when the front and rear vertical glass plates or the top and bottom of a horizontal slab are at different temperatures.

To maintain acceptable temperature control and uniformity throughout the gel and for the duration of the separation, the electrophoresis equipment must allow for efficient distribution of heat within the gel. The buffer covering the gel in a horizontal submarine unit for agarose gels may be an adequate heat sink for many purposes, but some applications require that the buffer be circulated and that the entire system be cooled with an external thermostatic circulator such as the MultiTemp™ III. A proven design for vertical slab gel units also uses the buffer as a heat sink, and high-performance units include a heat exchanger for connection to an external thermostatic circulator. Many DNA sequencing units use an aluminum plate in contact with one side of the slab assembly to distribute heat evenly across the gel and reduce smiling. The most precise method for controlling temperature in an electrophoresis system is through the use of solid-state devices—Peltier elements. The use of Peltier elements allows for direct feedback through which the temperature of the gel bed determines the output of the Peltier elements. Peltier elements also provide both heating and cooling capabilities in a compact unit.

High-Resolution Polyacrylamide Gel Electrophoresis

Resolution is a measure of the ability of a method to separate molecules from one another by some specified criterion. In the analysis of PCR products, resolution must often be measured in base pairs. One of the factors that influences resolution in electrophoresis is the initial width of the applied sample. Gels and buffers supplied by Amersham Biosciences constitute what are called *discontinuous buffer systems*. The electrophoretic mobilities of ions present in the gel and the cathodal buffer are formulated so that as electrophoresis begins, the sample zone, which is initially several millimeters deep, is progressively compressed between two moving fronts of ions. By the time the sample zone reaches the region of the gel in which the mobility of the molecules is retarded by the gel matrix, the sample zone is only a fraction of a millimeter wide.

Another factor limiting electrophoretic resolution is the thickness of the gel: the thicker the gel, the greater the potential for a temperature differential to exist across that thickness. Because there is an inverse relationship between the resistance and the temperature, by Ohm's Law a temperature differential results in a non-uniform electrical potential across the gel thickness. Molecules in the warmer region of the gel experience a greater field strength and migrate faster that identical molecules in the cooler region of the gel. The result is that molecules move in a front that is tilted with respect to the plane of the gel, so when viewed from above, the band appears broader than it actually is. This has been termed the "venetian blind effect." Gels supplied by Amersham Biosciences are thinner than conventional slab gels (0.4–0.5 mm). This makes it easier to maintain a uniform temperature across the gel, and so minimizes the problem.

The method of detection can also limit the resolution of which an electrophoresis system is capable. This is particularly true of fluorescent detection of DNA in agarose gels. Unlike polyacrylamide, which is transparent, agarose gels are semiopaque. Thus the light emitted from a fluorescently stained sample is scattered by the surrounding agarose medium, and the light appears to originate from an area that is larger than that actually occupied by the sample. Light is scattered to a much lesser extent in polyacrylamide gels, so the detected sample area more closely matches the true sample area. Silver staining of DNA in polyacrylamide gels does not suffer from this light-scattering effect on resolution, because what is actually visualized is atoms of silver bound directly to the DNA sample within the gel. Another advantage of silver staining over fluorescent detection methods is increased sensitivity. This allows the application of smaller amounts of a sample which may contain contaminants that could adversely effect resolution. Amersham Biosciences supplies convenient-to-use silver staining kits and an automated silver staining device.

Finally, *diffusion* is an effect that influences the resolution of which an electrophoresis system is capable. Although it may seem counterintuitive, molecules undergo diffusion even when they are moving through a gel in the presence of an electric field. A significant factor affecting the rate of diffusion is temperature, and electrophoresis systems supplied by Amersham Biosciences allow for efficient temperature control. In the case of PhastSystem™ and GenePhor System, very precise temperature control is provided by solid-state Peltier elements in the gel platform. Another way to limit the loss of resolution due to diffusion is to reduce the amount of time during which it can occur, by running the separation quickly. The provision of efficient temperature control in Amersham Biosciences electrophoresis systems through thin gels and cooling of the gel platform makes it possible to perform the electrophoretic separation at very high field strengths. This in turn reduces the time required to perform a separation.

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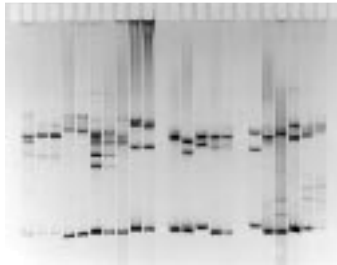
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Chapter 3
Flatbed PAGE

Figure 3.1 Identification of animal species by SSCP analysis on CleanGel™ 36S with subsequent silver staining.

Introduction

The GenePhor, Multiphor,™ and PhastSystem are highly versatile instruments for rapid, convenient, high-resolution electrophoresis of both proteins and nucleic acids. Each system is based on a horizontal acrylamide gel format that features semidry running buffer designs for fast setup and cleanup. Each system is based on a select group of precast gel products for a wide range of key applications. Sensitive sample detection, easy handling, and convenient gel archiving features also set these systems apart from standard vertical gel electrophoresis. Each precast gel is manufactured as a thin slab that has been immobilized onto a flexible, transparent plastic film backing. This simple feature makes the gels extremely easy to handle and prevents the common problems of tearing or distortion during the staining and drying processes. The plastic backing combined with the automated silver staining feature of the complete systems allows for easy data archiving, because silver-stained bands can be directly identified and recovered from the gels at any time.

Figure 3.2

Figure 3.3

Figure 3.4

GenePhor

Comparable to PhastSystem in terms of speed and temperature control, the GenePhor System features twice the separation distance and resolving power and three times the sample-number capacity (24 samples per gel). Fine temperature control, doubled separation length, increased sample-number capacity, and uniform precast gels make the GenePhor System perfect for highly reproducible single-strand conformational polymorphism (SSCP) characterization and screening. The capacity of the GenePhor gel for large sample numbers and high resolving power also make it a complete system for screening small double-stranded DNA fragments generated by a broad number of PCR-based analyses, including many DNA typing methods.

Multiphor II

The largest format of the three flatbed systems, the complete Multiphor II System features temperature control and the versatility of large-sample-number capacity (up to 48 samples per gel) where throughput and resolution are critical.

PhastSystem

The smallest format of the three flatbed systems, PhastSystem features speed, automated sample application, and temperature control for oligonucleotide (15–150 mer) and analyses of small double-stranded DNA fragments (20–1,300 base pairs). This makes PhastSystem extremely effective where short run times or temperature control are required. Ideal for checking the quality of oligonucleotides and performing simple SSCP analysis, PhastSystem offers a convenient range of horizontal precast acrylamide gels for DNA sizing.

Table 3.1 System and Components

Instrument	Temperature control unit	Gels self/precast	Detection	Power supply
GenePhor	Built-in Peltier	Precast	Automated Gel Stainer*	External
Multiphor	External water circulation*	Precast	Automated Gel Stainer*	External
PhastSystem	Built-in Peltier	Precast	Development Unit	Built-in and programmable

*Not built in (manual silver staining is optional).

Table 3.2 Systems and Features

Instrument	Temperature control	Gel size (millimeters)	Typical run times*	Sample numbers	DNA gels	Special kits
GenePhor	Built-in Peltier	$122 \times 110 \times 0.5$	1.5–3 hrs.	24/run	GeneGel Excel, GeneGel Clean	—
Multiphor	External water circulation	$90 \times 80 \times 0.5$ and $260 \times 200 \times 0.5$	3–6 hrs.	25–48/run	ExcelGel,*** CleanGel	—
PhastSystem	Built-in Peltier	$50 \times 43 \times 0.45$	45 min.–1 hr.	6–12/gel**		BandShift

*Includes silver staining with the Automated Gel Stainer.
**Capacity to run two gels at the same time.
***Format options: no wells, gradient (8–18%) and homogeneous with wells (7.5%, 12.5%, and 15%).

Gel and Buffer Systems

A wide variety of gels are available for PhastSystem, including three acrylamide gradient gels and four homogeneous acrylamide concentration gels. Gradient gels may give better results when the sample contains DNA strands in a broad range of sizes. Use the chart on page 16 to select the appropriate gel according to the size range of DNA strands in the particular sample. Two different ready-made buffer systems are available for use with PhastGel,™ DNA Buffer Strips are recommended for most applications, as they provide the sharpest bands and the shortest run times. Native Buffer Strips may be useful in detecting differences among particular SSCP conformers that are not stable in the buffer supplied by DNA Buffer Strips. For analysis of PCR products shorter than approximately 600 bp when using Native Buffer Strips, the gel must be pre-run for 100 Vh before the sample is applied, because DNA strands below this size will run with the ion front in this buffer system. This pre-run is unnecessary with DNA Buffer Strips. If resolving very small PCR products or oligonucleotides is necessary, it is recommended that a MOPS buffer system be used for best results. Details on how to prepare these, along with other specific information on analysis of PCR products, is provided in the PhastSystem Application Note "DNA Analysis Using PhastGel Media" (18-1112-39).

For GenePhor and Multiphor Systems, two types of gel are available. GeneGel Excel for GenePhor and Excel-Gel DNA Analysis Kit for Multiphor contain ready-to-use gels and Buffer Strips that constitute a discontinuous buffer system suitable for most applications. Both are 12.5% acrylamide gels; GeneGel Excel contains 24 sample wells, and ExcelGel DNA contains 48 sample wells. GeneGel Clean and the gels supplied in the Clean-Gel™ DNA Analysis Kit are dried acrylamide gels that may be rehydrated with the buffers included, or they may be rehydrated with any buffer the researcher chooses. GeneGel Clean are 15% acrylamide gels with 24 sample wells, and the gels in the CleanGel DNA Analysis Kit are 10% acrylamide gels with 48 sample wells.

For most applications, add at least 2 µl 10 mM Tris, 1 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol, pH 7.5 to the DNA sample, for a total volume of 6 µl. Other buffers of low ionic strength (<0.1 M) can also be used. The amount of DNA to load on the gel depends on the detection method, but for silver staining a good rule of thumb is 6 ng per band. Whether using PhastSystem, GenePhor, or Multiphor II, it is important to apply kerosene or silicone oil between the gel and the separation bed of the electrophoresis unit to ensure good thermal contact, and to precool (or preheat) the gel before applying the sample. See the User Manual for the specific gel kit and electrophoresis unit for details on how to accomplish this, as well as for specific run parameters.

Literature

Please refer to pages 00–00 for complete list of relevant product literature.

GenePhor References

AN #1 SSCP analysis in exons 7-9 and 9-10 of the tumor suppressor gene p53 (18-1118-15)

AN #8 Detection of ApoB mutations by SSCP (18-1118-10)

SSCP screening for breast cancer BRCA1 (breast cancer) point mutations (18-1118-11)

Multiphor II References

Identification of animal species by SSCP analysis of amplified cytochrome b gene fragments (18-1108-90)

SSCP analysis on CleanGel: A screening method for mitochondrial DNA (mtDNA) point mutations (18-1108-89)

Ubiquitin SSCP: Analysis as a differentiation tool for ground beetles (*Carabus* species) (18-1108-88)

PhastSystem References

Detection of SSCP as applied to exon 11 of the CFTR gene (18-1108-51)

Detection of SSCP in exons 5 and 7 of the PAH gene (18-1108-50)

Detection of SSCP: A non-radioisotopic technique as applied to exon 8 of the F9 gene (18-1108-52)

DNA fragment analysis using PhastGel media (18-1112-39)

Non-radioactive SSCP applied to exon 1 of the *Ki-ras* gene (18-1108-86)

PCR-SSCP analysis (18-1035-40)

SSCP analysis of PCR products to identify clones in lymphoid leukaemia and lymphoma (18-1108-54)

SSCP analysis of PCR products to type human papillomavirus (18-1108-53)

Table 3.3 Multiphor II Gels and Features

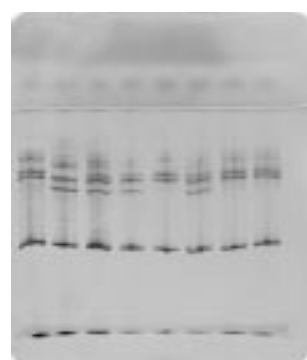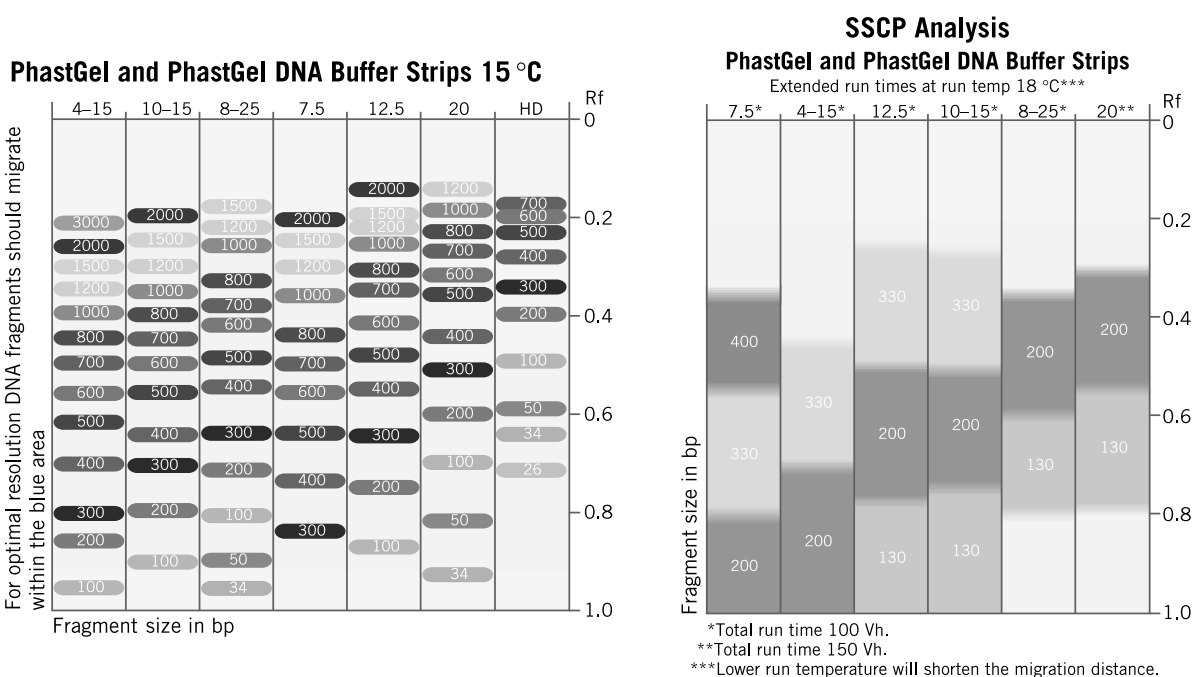|	ExcelGel DNA analysis	CleanGel DNA analysis	ExcelGel SDS homogeneous	CleanGel SDS, Native
% matrix	12.5% (T = 12.5%; C = 2%)	10% (T = 10%; C = 2%)	7.5% (T = 7.5%; C = 3%) 12.5% (T = 12.5%; C = 2%) 15% (T = 12.5%; C = 3%)	10%
Key features	Ready-to-load samples (48 samples)	Rehydrate with any buffer (48 samples)	Homogenous (25 samples)	25 samples 36 samples 48 samples
Applications	All general	All general	All general	All general
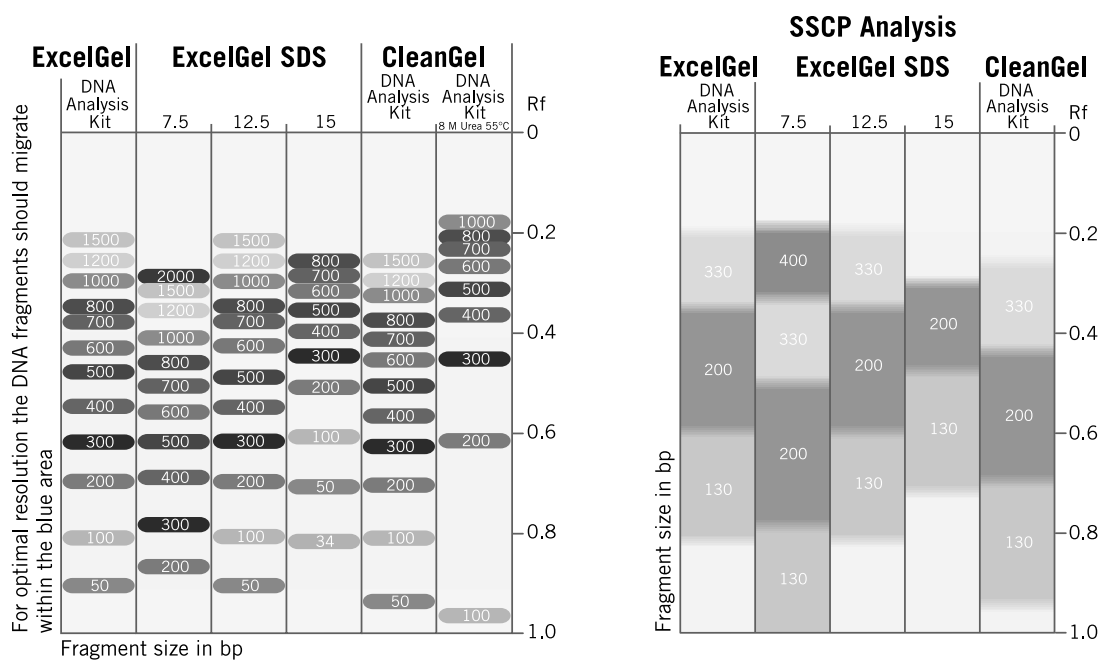
Table 3.4 PhastSystem Gels/Kits and Features*

	PhastGel homogeneous	PhastGel gradient	PhastGel Buffer Strips	Bandshift Kit
% matrix	7.5% (T = 7.5%; C = 3%) 12.5% (T = 12.5%; C = 2%) 20% (T = 20%; C = 3%) High density	4–15% 10–15% 8–25%	DNA SDS Native	N/A
Features	Ready to use	Ready to use	Buffer system options	Positive controls: EBNA-1 Oct-1
Applications analysis	All general	All general	All general	protein-DNA binding

*Sample combs (6, 8, or 12 samples).

PhastGel and PhastGel DNA Buffer Strips 15 °C

SSCP Analysis
PhastGel and PhastGel DNA Buffer Strips
Extended run times at run temp 18 °C***

*Total run time 100 Vh.
**Total run time 150 Vh.
***Lower run temperature will shorten the migration distance.

SSCP analysis of normal factor V and mutated factor V (heterozygous, homozygous), on PhastGel homogeneous 12.5% with PhastGel DNA Buffer Strip.
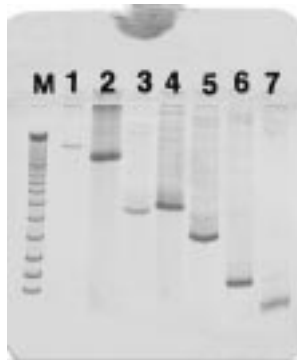

Analysis of crude PCR reaction products on PhastGel homogeneous 12.5% silver stained.

Table 3.5 GenePhor Gel Kits and Features

	% matrix	Wells/ sample volume	Key feature	Resolution	Key applications
GeneGel Excel 12.5/24	12.5% (T = 12.5%; C = 2%)	24/6 l	Ready-to-load samples (no rehydration)	6–8 bp up to 150 bp	SSCP RAPD STR (5–10 bp)
GeneGel Clean 15/24	15% (T = 15%; C = 2%)	24/6 l	Rehydrate with any buffer	6–8 bp up to 200 bp	SSCP (stock buffer or your own buffer) RAPD STR (5–10 bp)

GeneGel
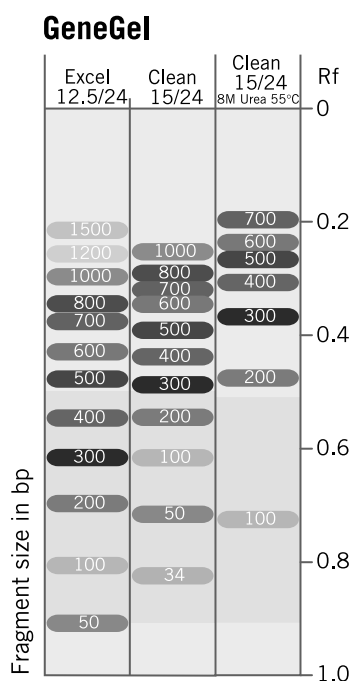
For optimal resolution the DNA fragments should migrate within the blue area

SSCP Analysis GeneGel
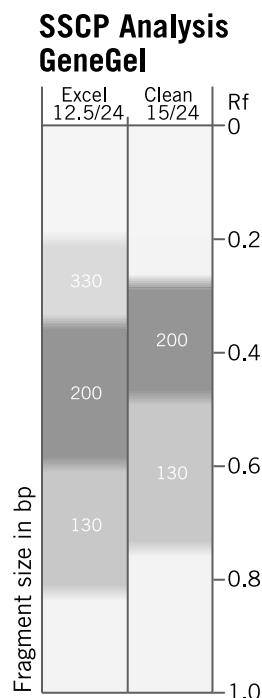
Chapter 4
Detection and Imaging

Nucleic acids do not absorb light in the range of wavelengths to which the human eye is sensitive and so cannot be visualized directly. A number of indirect methods have been employed to detect nucleic acids following electrophoresis, both within gels and on the surface of membranes after their transfer from gels. The three most common ways of detecting nucleic acids in gels are autoradiography, fluorescent dyes, and silver staining.

Autoradiography

Autoradiography is presently the most sensitive method for detecting nucleic acids following electrophoresis. For many situations the need for sensitivity outweighs the obvious disadvantages associated with handling radioactive materials. The fact that the useful life of the labeled DNA is governed by the half-life of the incorporated isotope is another disadvantage that must be considered. The most common isotopes for labeling nucleic acids are ^{32}P, ^{35}S, and ^{33}P.

Traditionally, autoradiography is performed by placing photographic film in contact with the gel surface. Radioactive emission of ß-particles forms a latent image of the bands in the gel by activating silver-halide crystals suspended in the film's emulsion layer. Chemical reduction of the silver ions within the activated crystals to metallic silver produces a visible image of the bands in the gel. Phosphorescent intensifying screens placed behind the film can be used to amplify the signal several-fold by converting into light energy those particles passing through the film. Disadvantages of film-based autoradiography are the non-linearity and narrow dynamic range of the film's response and the requirement for a darkroom and film-processing facility.

In the past decade, highly sensitive instruments based on phosphor-storage technology have become widely available. Rather than form a latent image of the gel within silver salts in a film emulsion, energy from radioactive decay is stored within phosphorescent minerals embedded in a reusable plate. Within the instrument this stored energy is released as light energy, which is then measured and converted to digital information. The broad linear dynamic range of phosphor-based imaging allows quantitative analysis in a single exposure of both very weak and very strong signals. These advantages, along with the immediate generation of a digital image format, are seen by many to outweigh the disadvantage of the high initial cost of a phosphor-storage instrument.

Fluorescent Dyes

Where sensitivity is not of utmost importance, detection of nucleic acids using *fluorescent dyes* has traditionally been the method of choice, its chief virtues being its simplicity and speed. The dye is in many cases simply added to the gel and electrophoresis buffer, so staining takes place during electrophoresis. After the separation is complete, the gel is exposed to light of a suitable wavelength; the light emitted by the dye molecules is either viewed directly or captured by some sort of image-recording device. As in all fluorescent events, the light emitted by the dye is of a longer wavelength than that used to excite the dye. Optical filters can be used to prevent light of the exciting wavelength from entering the imaging system, thereby improving sensitivity. The dye/DNA complex often exhibits enhanced fluorescence relative to the unbound dye, a property of the dye that also leads to greater sensitivity.

In recent years the development of more-sensitive fluorescent dyes has been spurred by the rising costs associated with handling radiolabeled nucleic acids. There have also been improvements in imaging devices. Whereas using photographic film and an ultraviolet lightbox was previously the only way to record the images of fluorescently stained gels, charge-coupled device (CCD) cameras now offer greater sensitivity than film, and a number of very sensitive dedicated fluorescent imagers are also available.

A serious objection to the use of fluorescent dyes for nucleic acid detection is that they represent a safety hazard and must be handled and disposed of accordingly. It should come as no surprise that molecules exhibiting a high affinity for DNA should have the potential to interfere with DNA function: Most dyes used for detecting nucleic acids are mutagens and are either suspected or confirmed carcinogens.

Because it is simple to use and is sufficiently sensitive for most routine procedures, ethidium bromide has long been the standard fluorescent dye for staining nucleic acids in gels. Agarose gels are most conveniently cast and run with buffers already containing the dye at a concentration of 0.5 µg/ml. Ethidium bromide is an intercalating dye: The planar molecule fits with high affinity between adjacent base pairs in the double helix of dsDNA, but it also binds ssDNA and RNA. If dye is included in the gel and running buffer, the progress of the separation can be monitored during electrophoresis by using a handheld UV lamp, but ethidium bromide–stained gels are most commonly viewed by placing them on an ultraviolet lightbox. The image can be recorded using either a conventional or a CCD camera.
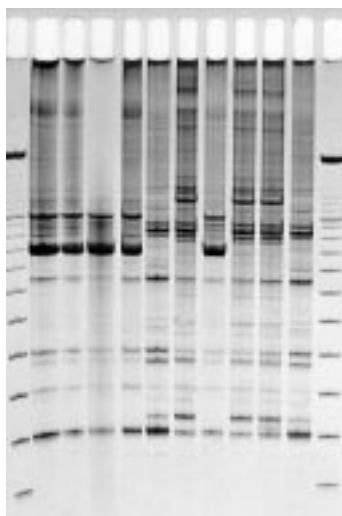
Figure 4.1 Different isolates of *E. coli* on ExcelGel 48S DNA (12.5%T 2%C) using RAPD analysis.

Silver Staining

Silver staining is the most sensitive non-radioactive method for permanent staining of nucleic acids in polyacrylamide gels. It is permanent, and DNA can be excised for reamplification years after electrophoresis. It creates a record of the electrophoresis result that can be viewed without any special equipment. It is, however, a complex, multistep process, and many variables can influence the result. High-purity reagents and precise timing are necessary for reproducible, high-quality results.

In silver staining, polyacrylamide gels are impregnated with soluble silver ion (Ag^+) and developed by treatment with a reductant. Macromolecules such as DNA fragments in the gel promote the reduction of silver ion to metallic silver (Ag^0), which is insoluble and visible, allowing nucleic acid–containing bands to be seen. The initial deposition of metallic silver promotes further deposition in an autocatalytic process, resulting in exceptionally high sensitivity.

Amersham Biosciences produces kits for silver staining of nucleic acids in polyacrylamide gels. PlusOne™ DNA Silver Staining Kit will allow visualization of as little as 20 to 50 pg of DNA/band with an essentially colorless background.

The inherent high sensitivity of silver staining may also lead to interference from a variety of sources. These interferences can result in over- and understaining, high background, smearing, and uneven staining. Exceptional cleanliness must be practiced in preparing the electrophoresis apparatus, in subsequent handling of the gel, and in preparation of the staining tray. All equipment used for running and staining the gel must be cleaned with detergent and thoroughly rinsed, as detergents can interfere with silver staining. Gloves should be worn when handling the electrophoresis apparatus, the gel, and the staining tray. High-quality water must be used to prepare the reagents, as impurities have a strong effect on silver staining. Ideally, the water used should be either glass-distilled or deionized. For best results, use water with a resistivity of 35 mega ohms. Temperature also affects silver staining—higher temperatures promote faster development and darker background. This effect is more pronounced when staining DNA than when staining protein. Staining with the PlusOne DNA Silver Staining Kit is best performed on gels between 0.75 and 1.5 mm thick if unbacked, or between 0.5 and 1.0 mm if gels are on plastic backing. Thicker gels stain less efficiently due to incomplete penetration of staining reagents. In addition, the background can be higher due to incomplete washing out of reagents between steps.

Amersham Biosciences Literature

ImageMaster AN #1 Imaging DNA agarose gels stained with ethidium bromide (80-6323-77)

ImageMaster AN #2 Imaging DNA agarose gels stained with SYBR Green 1 (80-6338-78)

ImageMaster AN #3 Quantitation of PCR products in ethidium bromide–stained agarose gel (80-6333-65)

ImageMaster AN #4 Image analysis of silver stained polyacrylamide gels/species identification by RAPD analysis (80-6334-41)

ImageMaster Automated Gel Stainer (80-6343-34)

ImageMaster High-resolution DNA analysis: Application Note file (18-1108-06)

Molecular Dynamics Literature

AN #50: Quantitative double-label autoradiography using storage phosphor imaging

AN #53: Quantitative PCR using storage phosphor technology 1: mRNA quantitation

AN #55: Genetic typing using fluorescently labeled polymorphic STR markers

AN #56: Oncogene mRNA profiling using fluorescent quantitative PCR

Fluorescence Imaging Applications Guide

TN #52: Improving quantitation in autoradiography

TN #57: Understanding fluorescence

References

Bonner, W. M. "Autoradiograms: ^{35}S and ^{32}P" in Berger, Shelby L., and Kimmel, Alan R., eds., *Methods in Enzymology,* vol. 152, Academic Press, San Diego, 1987.

Figure 5.1 DyNA Quant™ 200 fluorometer

Chapter 5
Quantification of Nucleic Acids

Molecular biologists can use several methods to quantify nucleic acids in solution. One approach to determining DNA concentrations is to separate an aliquot of the DNA solution on an agarose gel, stain the bands with ethidium bromide, and compare the intensity of the stained bands with that of marker bands of known mass. While only semi-quantitative, this method is adequate for some purposes. For many applications involving PCR, however, greater accuracy is required, and spectrophotometric or fluorometric methods of DNA quantification should be used.

Spectrophotometric Quantification of Nucleic Acids

Due to their molecular structures, nucleic acids absorb light of certain wavelengths. For both DNA and RNA, maximal absorption occurs at 260 nm. Over a wide range of concentrations, a linear relationship exists between the absorption of light and the nucleic acid concentration. Inside a spectrophotometer, the sample is exposed to ultraviolet light at 260 nm, and a photodetector measures the light that passes through the sample. The more light absorbed by the sample—that is, the less light sensed by the photodetector—the higher the nucleic acid concentration in the sample.

The amount of light absorbed by a nucleic acid is a function of the molar extinction coefficients of the substituent nucleotide bases. The Beer-Lambert Law relates the amount of light absorbed at a given wavelength to the molar extinction coefficient and concentration of an absorbing molecule:

A = εcl

where A is absorbance, ε is the molar extinction coefficient, c is the concentration of the solute, and l is the length of the light path in solution measured in centimeters (usually 1 cm). By entering the molar extinction coefficient (in $M^{-1}cm^{-1}$), concentration is obtained in mol / l. Alternatively, by entering the specific absorption coefficient (in $(\mu g/ml)^{-1}cm^{-1}$), concentration is expressed in µg/ml. Assuming the cuvette has a path length of 1 cm, different nucleic acids are characterized by the following specific absorption coefficients:

ssDNA: 0.027 $(\mu g/ml)^{-1}cm^{-1}$

dsDNA: 0.020 $(\mu g/ml)^{-1}cm^{-1}$

ssRNA: 0.025 $(\mu g/ml)^{-1}cm^{-1}$

By entering these values into the Beer-Lambert equation, an absorbance at 260 nm of 1.0 corresponds to:

ssDNA: 37 µg/ml

dsDNA: 50 µg/ml

ssRNA: 40 µg/ml

For ssDNA this conversion factor applies only to longer nucleic acid molecules. For oligonucleotides, an absorbance of 1.0 corresponds to a concentration of 20 to 33 µg/ml, depending on the oligonucleotide length and base composition. The following formula provides an estimate of the concentration, where N equals length in bases:

c (pmol/µl) = A_{260} / (10 × N)

A more accurate formula accounts for base composition as well as length:

c (pmol/µl) = A_{260} / (100 / [1.5 NA + 0.71 NC + 1.2 NG + 0.84 NT])

where N equals number of each of the four bases: A, C, G, and T.

A number of methods for estimating the purity of nucleic acid samples based on spectrophotometric measurement have been devised over the years. The simplest uses the ratio of absorbance at 260 nm to absorbance at 280 nm to indicate contamination by protein or phenol. An A_{260}/A_{280} ratio greater than 1.9 for DNA, or greater than 1.8 for RNA is indicative of samples that are free of these contaminants. Other methods for detecting the presence of impurities involve measurements of absorbance at 230 nm and 320 nm.

The GeneQuant™ II is an instrument that uses the light absorption of a nucleic acid solution at four wavelengths (230 nm, 260 nm, 280 nm, and 320 nm) to calculate the amount and purity of the nucleic acid. It offers direct readings of absorbance and concentration (in µg/ml, µg/µl, pmol/µl, or pmol phosphate). In addition, after entering the sequence of an oligonucleotide, the GeneQuant II calculates its molecular weight, melting temperature, and theoretical absorption. These calculations are based on the results of theoretical and experimental studies that have analyzed thermodynamic parameters for each base, taking into consideration adjacent bases within the oligonucleotide. Using the ultramicrovolume cuvette, as little as 35 ng of DNA can be detected in a 7-µl volume.

Fluorometric Quantification of DNA

Another approach—quantification using a fluorometer, such as the DyNA Quant 200—is recommended for applications in which only a small amount of material is available, or when the DNA is mixed with other absorbing substances, such as proteins, RNA, primers, and nucleotides. During fluorometric measurement, light of a particular wavelength excites dye molecules bound to the DNA. A photodetector then measures the increased emitted fluorescent light, which has a different wavelength than the exciting light. The stronger the fluorescence—that is, the more light that is sensed by the detector—the higher the DNA concentration in the solution. The fluorescent dye Hoechst 33258 binds very specifically to double-stranded DNA and can be used with the DyNA Quant 200 to measure DNA even in cellular extracts containing large amounts of protein and RNA. As little as 10 ng of DNA in a 2-ml volume can be detected using the Hoechst 33258 assay in the DyNA Quant 200. This corresponds to less than 1% of the DNA synthesized in a typical 100-µl PCR reaction.

In addition to double-stranded DNA product, a completed PCR reaction contains unincorporated nucleotides and primers. Because these also absorb ultraviolet light, spectrophotometric measurement cannot be used to determine the yield of a PCR reaction. On the other hand, because Hoechst 33258 binds dsDNA but not small oligonucleotide primers or nucleotides, fluorometry can be used to accurately measure the amount of product synthesized in PCR. The ability to directly measure the yield of a PCR reaction makes it much easier to optimize PCR conditions. This is especially important if large amounts of

product are required for subsequent direct sequencing or subcloning, or for the amplification of DNA greater than about 2,000 base pairs in length. It should be remembered, however, that the values provided by fluorometry will include any non-specific dsDNA produced by the reaction. The absence of any non-specific reaction products should be confirmed by electrophoresis of an aliquot of the reaction on an agarose gel.

To both determine the exact amount of DNA and identify potential contaminants in a sample, the combination of photometric and fluorometric analyses offers the ideal solution. If the photometric measurement indicates a higher nucleic acid concentration than the fluorometric measurement, the DNA solution contains RNA or other substances absorbing light of the same wavelengths as DNA. This dual approach is more reliable and accurate than the more time-consuming quantification using agarose gels and ethidium bromide staining.

Literature

DyNA Quant 200 AN #6 Fluorescence assay for DNA quantitation (35092 80-6240-74)

DyNA Quant 200 AN #7 Fluorescence quantitation of PCR products before and after EasyPrep purification (80-6329-09)

DyNA Quant 200 AN #8 Fluorescence quantitation of plasmid DNAs/calf thymus as calibration standards (80-6323-58)

DyNA Quant 200 AN #9 Fluorescence quantitation of double-stranded DNA after cDNA synthesis (80-6333-46)

DyNA Quant 200 AN #10 Fluorescence quantitation of PCR products prior to re-amplification and direct sequencing (80-6338-59)

DyNA Quant 200 AN #11 Fluorescence quantitation of single-stranded M13 DNA (80-6370-89)

Nucleic Acid Quantification Guide (18-1125-25)

Chapter 6
Applications: Mutation Detection and Genetic Typing

Introduction

Mutation detection typically refers to the identification of allelic variants of single or low-copy-number gene sequences. It is of tremendous value in the diagnosis and understanding of heritable diseases as well as the understanding of complex biochemical processes having no easily measurable phenotype. Direct sequencing of specific sequence regions is still the most reliable and accurate method of mutation detection, but it is exceptionally expensive and labor intensive as a method of analysis. As indicated in this chapter, PCR has greatly facilitated the development of alternative methods that provide standards of accuracy and convenience that complement direct sequencing methods.

Although there are few current methods, short of direct sequencing and specialized techniques of hybridization, that can directly pinpoint the specific identity of all single nucleotide variations in a limited segment of DNA, some of the recently developed PCR-based mutation detection assays offer highly reliable methods of identifying a single nucleotide variation in a given fragment. As a diagnostic tool, this offers the powerful advantage of allowing one to conveniently prescreen large numbers of unknown samples of which only implicated variants would need to be directly sequenced.

Individual identification through genetic typing represents a broader scope of the concept of mutation detection as a method of differentiating individuals in a closely related population of organisms. Based on the ability to easily identify repeated polymorphisms of high copy number, DNA typing methods allow questions of simple genetic linkage, paternity, evolutionary taxonomy, and population genetics to be conveniently addressed through a simple assay.

Forensic applications of genetic typing have caused a small revolution in the field of genome characterization. The need for accurate, reliable, and efficient methods of individual identification has spurred rapid growth in the identification of polymorphic sequences that are now used in numerous DNA typing methods. The rapid diversification of these techniques also reflects the growing value of being able to type genomic DNA in a wide range of organisms and their subspecies. The discovery of small, abundant, highly polymorphic repetitive sequences is the critical basis of powerful genetic typing methods, and PCR has led to dramatic innovations in this field.

The use of ever smaller polymorphic sequences combined with sensitive PCR-based sequence amplification techniques has significantly reduced the restrictions of sample quality, quantity, and sensitivity that were early obstacles to the rapid development and application of these methods. Collectively, the development of advanced mutation detection and genetic typing methods has dramatically expanded the ease and accuracy of genetic analysis without the need for direct or complete sequence knowledge of the organism being studied.
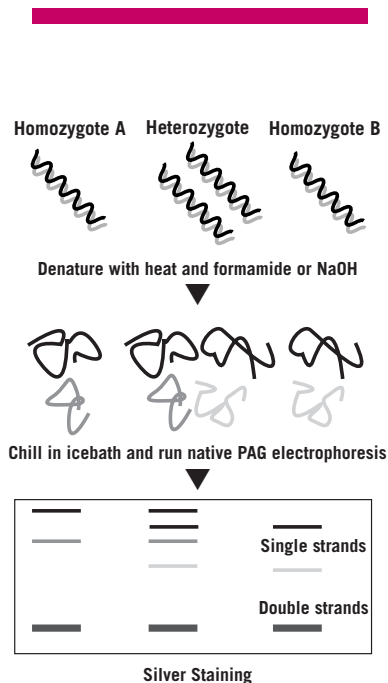
Homozygote A   Heterozygote   Homozygote B

Denature with heat and formamide or NaOH

Chill in icebath and run native PAG electrophoresis

Single strands

Double strands

Silver Staining

**Figure 6.1** Mutation detection with SSCP

SSCP

Single-strand conformational polymorphism (SSCP) is one of the most widely used mutation scanning techniques because of its simplicity. The principle of this method is based on the fact that the electrophoretic mobility of nucleic acids in a non-denaturing gel is sensitive to both size and shape. Unlike double-stranded DNA, single-stranded DNA is flexible and will adopt a conformation determined by intramolecular interactions and base stacking that is uniquely dependent on sequence composition (Orita et al., 1989). This conformation can be affected when even a single base is changed. Conformational changes can be detected as alterations in the electrophoretic mobility of the single-stranded DNA in non-denaturing polyacrylamide gels.

SSCP is still largely empirical because of the absence of a robust theory for predicting the dependence of conformation on sequence, and of mobility on conformation. SSCP is, nonetheless, a convenient mutation screening method. Detection levels of 70% to greater than 95% in certain model systems have been achieved. Achieving such high detection rates by SSCP may require running gels under several conditions, because the conformation of single-stranded DNA is sensitive to a number of parameters, including ionic strength, the type of gel matrix, fragment length, and temperature.

Originally, SSCP was performed on large sequencing gels in a cold room to control temperature and using radioactive nucleotides. The procedure was cumbersome and not always reproducible. The development of non-radioactive techniques and temperature-controlled electrophoretic units, and the modification of the technique for standard and mini-gels, have increased the simplicity and reproducibility of this method.

Basic Protocol

The target sequence from genomic DNA or cDNA is amplified by PCR. The amplicon is denatured to single-stranded DNA and electrophoresed on a non-denaturing gel. Bands of the single-stranded DNA at positions in the gel different from the wild-type indicate the presence of a mutation. In general, a wild-type sample will generate two SSCP bands. Similarly, a homozygous mutant sample will also generate two SSCP bands, but these will migrate differently from the wild-type. If it is a heterozygous mutant, four bands will be generated: two with the same mobility as the wild-type and two with different mobility. There may be only three bands if the mutation changes the conformation of one strand but not the other.
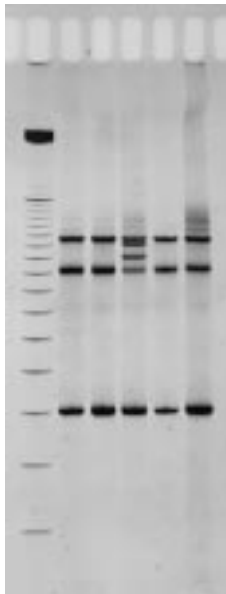
Figure 6.2 Detection of BRCA1 point mutations by SSCP analysis.
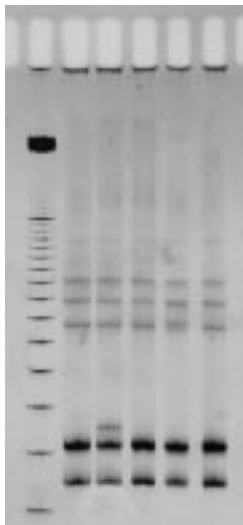
Figure 6.3 SSCP analysis of ApoB point mutations.

Generating the Product

DNA products used for SSCP are generally amplified by PCR. The sensitivity of PCR-SSCP tends to decrease as fragment length increases. For fragments of about 200 bp, greater than 90% of single-base changes can be detected; whereas for fragments of 400 bp, the detection rate is lowered to 80%. Although mutations have been detected in fragments as large as 800 bp, the sensitivity of the assay will probably not be as high. Sequences longer than 400 bp should be divided into shorter segments before analysis by SSCP. This can be done by generating overlapping subfragments separately or by amplifying the intact fragment and then digesting it with restriction enzymes. It is important that PCR conditions be optimized for stringency, as artifacts will interfere with the assay. The PCR product should be checked prior to SSCP analysis.

Electrophoresis

The choice of running conditions is rather arbitrary. The advantage of a flatbed system is that temperature can be easily controlled with Peltier cooling, which makes optimizing conditions easier.

References

Hayashi, K., and Yandell, D. W. How sensitive is PCR-SSCP? *Human Mutation* 2: 338–346, 1993.

Hennessy, L., Teare, J., and Ko, C. PCR conditions and DNA denaturants affect reproducibility of single-strand conformation polymorphism patterns for BRCA1 mutations. *Clin Chem* 44: 879–882, 1998.

Markoff, A., Savov, A., Vladimirov, V., Bogdanova, N., Kremensky, I., and Ganev, V. Optimization of single-stranded conformation polymorphism analysis in the presence of polyethylene glycol. *Clin Chem* 43: 30–3, 1997.

Orita, M., Suzuki, Y., Sekiya, T., and Hayashi, K. Rapid and sensitive detection of point mutations and SNA polymorphisms using the polymerase chain reaction. *Genomics* 5: 874–9, 1989.

PCR-SSCP (18-1112-40)

Heteroduplex Analysis

Mutations are detected by *heteroduplex analysis* based on the retardation of the heteroduplex compared with the corresponding homoduplex on a non-denaturing polyacrylamide gel. Heteroduplexes migrate more slowly than their corresponding homoduplexes due to a more open double-stranded configuration surrounding the mismatched bases.

Basic Protocol

Heteroduplexes are formed by mixing wild-type and mutant DNA amplified by PCR. The samples are denatured and reannealed (usually by heating and cooling). Four distinct species are generated by this reassortment: wild-type homoduplex, mutant homoduplex, and two heteroduplexes. Heteroduplexes can also

be formed during standard PCR if the DNA has two different alleles. The formation of the heteroduplexes and their stability depend primarily on the type of mutation in the fragment. Large deletions and insertions create relatively stable heteroplexes. Single-base changes are more sensitive to temperature, solvents, and ionic strength of the buffer. There is no way to predict the influence of these parameters on the stability of the heteroduplex, and thus electrophoretic conditions must be optimized.

References

PhastSystem AN #1: Heteroduplex mobility assay for subtyping HIV-1 (80-6375-26)

DGGE

The separation principle of *denaturing gradient gel electrophoresis (DGGE)* is based on the melting (denaturation) properties of DNA in solution. DNA molecules melt in discrete segments called *melting domains,* when the temperature or denaturant concentration is raised. The melting temperature (T_m) of a melting domain depends on its nucleotide sequence. As DNA fragments are electrophoresed through a linear gradient of increasing denaturing concentration, the separation of double-stranded DNA into single-stranded segments increases. This causes the DNA segment to form a less uniform three-dimensional structure that moves through a polyacrylamide matrix at a reduced rate. The gradient itself can be arranged in either a perpendicular or a parallel orientation relative to the electric field, depending on whether a broad or a narrow range of denaturing conditions is required.

As a matter of practice, a perpendicular gradient gel, where the gradient is perpendicular to the electric field, is typically composed of a broad denaturing gradient range, often spanning anywhere from 0 to 100%. In parallel DGGE the denaturing gradient is parallel to the electric field, and the range of denaturant is somewhat narrower, so fragments can separate from one another more readily in a given distance.

As a DNA fragment enters the concentration of denaturant where its lowest temperature domain melts, the molecule begins branching and, hence, slowing down at a unique position in the gel. This results in separation of different fragments at the end of the run. The attachment of a GC-rich segment, called a *GC clamp,* which never denatures at the conditions chosen for the experiment, allows for a branched-shaped molecule whose shape is anchored as a double-stranded molecule by the GC clamp. Using this strategy, detection of almost all single-base changes in the fragment can be analyzed. To determine the theoretical melting domains of the DNA fragment of interest, a computer program developed by Lerman is typically used.

Although DGGE can require significant preparation and analysis time compared with similar techniques such as SSCP, it offers the unique benefit of not having to actually sequence, prepare, or test primers for the DNA segment to be studied. Still, the rate of polymorphism detection is somewhat lower than with SSCP (Sheffield et al., 1993). Similar techniques related to DGGE include genomic denaturing gradient gel electrophoresis (gDGGE) (Burmeister et al., 1991). In this method restricted genomic DNA is run in denaturing gradient gels and then transferred to a nylon membrane; sequences of interest are detected by standard hybridization methods much like RFLP.

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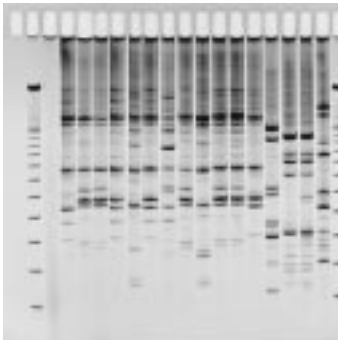
RAPD

Figure 6.4 RAPD analysis of bacterial strains, using GeneGel Excel 12.5/24 kit.

Random amplification of polymorphic DNA (RAPD) is a very general method for obtaining a molecular fingerprint of a strain or species. It is a convenient and sensitive method that is finding increasing application in such fields as epidemeology, molecular genetics, microbial ecology, molecular evolution, and taxonomy. Low-stringency PCR amplification of genomic DNA using a single short primer (10–22 bases) of arbitrary sequence is used to generate a set of fragments that is characteristic of the species or strain from which the DNA was prepared. Each fragment in the set results from the fortuitous hybridization of a pair of primers on opposite DNA strands in the appropriate orientation, and separated by such a distance allowing efficient PCR (<1,500 bp). Following electrophoresis and staining, the number of bands in common between a known and an unknown DNA sample can be used for visual estimation of the molecular relatedness of two individuals.

By performing similar experiments with different primers and many strains, quantitative data can be derived which can then be used to prepare dendrograms for taxonomic studies. A particular band can also be considered to be a Mendelian "trait" of an organism and can be used as a molecular marker to study the segregation of other traits of economic or clinical significance. In food production and clinical settings, RAPD has been found to be a particularly quick and sensitive alternative to standard bacteriological methods for strain identification, which may take days to perform.

The principal advantage of RAPD is that, unlike most other methods of strain identification, it requires no prior knowledge of the organism under investigation. A primer of arbitrary sequence and low-stringency PCR conditions is all that is required to generate information. Of course, some effort is necessary to optimize the amount of information obtained in an experiment, since a particular primer and set of PCR conditions may result in too few or too many bands to be particularly useful. The method is sufficiently simple and rapid, however, that such optimization can be performed in a short time.

Because RAPD depends on the identification of common bands between two samples based on their size, the spatial resolution of the electrophoresis method employed contributes significantly to the method's accuracy. RAPD has traditionally been performed using ethidium bromide–stained agarose gels. The thin polyacrylamide gels and discontinuous buffer systems of ExcelGel,™ GeneGel Excel, CleanGel,™ and GeneGel Clean, however, offer significantly improved resolution over agarose gels for the size range of fragments generated in RAPD. Furthermore, unlike ethidium bromide staining, in which optical effects can lead to an apparent loss of resolution, silver staining better reflects the

actual physical resolution of which polyacrylamide gels are capable. Silver staining is generally recognized to offer greater sensitivity than ethidium bromide. The temperature control available in the GenePhor™ and Multiphor™ II electrophoresis units, together with quality-controlled precast gels and Buffer Strips, ensures a high level of reproducible electrophoresis results.

A more serious problem, related to the sensitivity of the method, is reproducibility. Amplification under low-stringency conditions is very sensitive to such factors as quality and quantity of the DNA, primer and magnesium concentration, quality of the polymerase, and even the brand of thermal cycler. Amersham Biosciences offers a number of products that reduce the potential variability in the RAPD technique. In addition to genomic DNA preparation kits for isolation of genomic DNA from cells and tissue, and the DyNA Quant 200 minifluorometer for accurate quantification of small amounts of DNA, Ready-To-Go™ RAPD Beads represent a convenient route to minimizing variability in the procedure. Ready-To-Go RAPD Beads are quality-controlled, room temperature–stable, premixed reaction components designed to minimize the number of manual steps involved in setting up a RAPD reaction, each of which can lead to loss of reproducibility.

Literature

GenePhor AN #5: Characterization of bacterial strains by RAPD Analysis (18-1118-12)

Multiphor II AN: Identification of different isolates of *E. coli* by RAPD analysis (18-1115-56)

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Berg, D. E., Akopyants, N. S., and Kersulyte, D. Fingerprinting microbial genomes using the RAPD or AP-PCR method. *Methods in Molecular and Cellular Biology* 5: 13–24, 1994.

AFLP

Amplified fragment length polymorphism (AFLP) is a fingerprinting technique that allows one to distinguish closely related species or subspecies that may be difficult or impossible to differentiate on the basis of morphological or biochemical characteristics. Like RAPD it requires no sequence information from the organism to be investigated, but obtaining the most useful data from AFLP analysis does involve some amount of optimization. Because stringent conditions for PCR are employed in AFLP, it is inherently more reproducible than the RAPD assay.

In essence, an AFLP fingerprint represents a subset of the population of restriction fragments resulting from a complete digest of the target genome. Characteristic differences in the fingerprints of two genomes result from polymorphisms that occur within or immediately adjacent to restriction sites, as well as from insertions and deletions occurring between restriction sites. The amount of information that would result if the entire population of restriction fragments were displayed would be overwhelming, and the AFLP procedure provides several means for adjusting the complexity of the information that is ultimately displayed on a gel.

The first step in AFLP is complete digestion of genomic DNA with restriction enzymes. In the analysis of plant genomes, where AFLP is currently finding wide use, the genomic DNA is digested with the "six-cutter" *Eco*RI and the "four-cutter" *Mse*I. In a genome of 2×10^9 bp, such a digestion would produce approximately 8×10^6 fragments. *Mse*I and *Eco*RI linkers are then ligated to the ends of the resulting fragments. These linkers provide the sequences for hybridization of primers in subsequent "pre-amplification" and AFLP-PCR steps. The "pre-amplification" step, in which PCR is performed using only a primer complementary to the *Eco*RI end, has been found useful for improving specificity. In the final AFLP-PCR step, primers complementary to *Eco*RI ends and *Mse*I ends are used. Only the *Eco*RI primer is labeled, however, so only *Eco*RI–*Eco*RI and *Eco*RI–*Mse*I fragments are represented in the final AFLP pattern. The complexity of the pattern is reduced further through the use of primers containing one or more additional bases at the 3'-end. A single 3'-extension on both primers will reduce the number of fragments amplified by a factor of 16, a two-base extension will reduce the number by 256, and a three-base extension will reduce it by 4,096.

The challenge in optimizing AFLP is in obtaining an amount of information on the gel that is sufficient for the purpose, but not so great as to overcomplicate the analysis. This challenge is obviously related to the complexity of the target genome and the amount of genetic variability that exists within the group of organisms being examined. There are a number of steps at which the number of amplified fragments appearing on the gel can be limited:

- In the choice of restriction enzymes
- By including 3'-extensions on one or both linkers
- By including 3'-extensions on the "pre-amplification" primer
- In the labeling of the AFLP-PCR primers
- By including 3'-extensions on the AFLP-PCR primers

Unless one can apply an established procedure to the particular genome being investigated, a certain degree of "tuning" of the AFLP procedure must be expected.

AFLP analysis is usually performed on denaturing gels, and the fragments are visualized by either autoradiography or fluorescent imaging. For highly complex patterns, the length of a standard sequencing gel is required, but the resolution obtained with CleanGel is adequate for many purposes. Using silver staining for detection is in many cases advantageous over the use of radiolabeled primers.

Ribotyping

Traditional *ribotyping* is modeled after RFLP analysis and typically used for analyzing bacteria and other microorganisms. It uses so-called universal probes targeted at specific conserved domains of ribosomal RNA coding sequences, allowing characterization with only limited sequence information. Depending on the protocols used, the resultant band patterns can be compared with known species and strains of organisms to determine genetic and evolutionary relationships.

Basis of Methodology

Strategies can vary widely depending on the sequences selected, the level of information required, and the target organism. Here are some of the key advantages that differentiate this method of DNA typing:

- The genes for rRNA appear in several different copies at different loci within the genome having different flanking restriction site locations.

- There is variability among the rRNA genes (16S and 23S).

- There is variability in the spacer region between 16S and 23S rRNA genes.

Related Strategies

Standard ribotyping protocols based on restriction digests of genomic DNA samples are now commonly modified to use PCR primers to amplify conserved rRNA coding regions. The amplified fragments are subsequently cut with one or more restriction enzymes and separated by native polyacrylamide gel electrophoresis. The band patterns are compared by either radioactive or simple non-radioactive detection. This method is also called *amplified ribosomal DNA restriction analysis (ARDRA)*.

Direct sequencing of 16S RNA is also commonly employed for similar species and subspecies identification purposes, because it is fairly direct and uncomplicated. Direct sequencing of 16S ribosomal RNA, however, has the disadvantage of the highly conserved nature of the 16S sequence. Thus useful polymorphism in the 16S region is frequently absent at the subspecies level. Sequencing is also relatively expensive and labor intensive compared with newer PCR-based strategies (see ARDRA and RAPD analysis).

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Multiphor AN: Analysis of 16 rDNA digested with different enzymes (18-1115-55)

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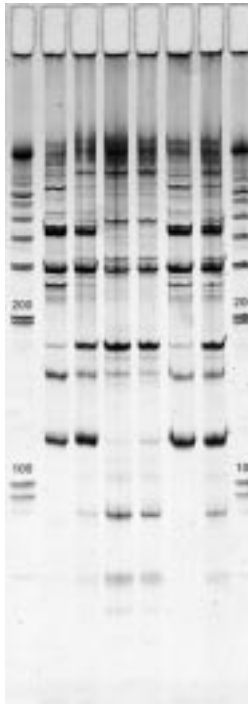
DDRT

Introduction

Differential display reverse transcription (DDRT) PCR is among the most effective methods of identifying and isolating genes that are uniquely or differentially expressed based on cell type or cell condition. For example, it is a powerful method for identifying genes that are expressed in specific tissues and tumors in response to drug treatment and virus infections, as well as identifying transcripts that are differentially expressed due to environmental stress or shock. The method was first reported in 1992 by Liang and Pardee; it has since flourished as a tool that has directly lead to the characterization and cloning of hundreds of unique gene sequences. Well over 500 articles have been published since 1992, describing the successful identification of differentially expressed genetic sequences and their contexts. Many of these articles include improvements upon the principal method itself.

Advantages of DDRT

Only a fraction (perhaps 8,000 to 15,000) of all human genes (100,000–150,000) are expressed in any given cell type (Ito and Sakaki, 1996). Prior to the invention of differential display PCR, changes in an organism's gene expression patterns were examined by differential hybridization at the mRNA/cDNA level and by 2-D electrophoresis on the protein level. Although differential hybridization and 2-D protein electrophoresis have evolved into powerful technologies, differential display PCR continues to offer the following critical advantages:

- Small tissue sample requirement

- Fast analysis

- Simultaneous display of all differences in gene expression patterns between different cell fractions, which include mostly novel, unknown sequences

- Simultaneous detection of both up-regulated and down-regulated genes

- Low to modest cost requirement

Fundamentals of the Protocol

The basic objective of differential display is to analyze the total pool of cDNA of a given mRNA pool by DNA fragment size and intensity analysis on a sequencing gel. The fundamental strategy of DDRT can be described as a method of reducing the number of bands that would appear on such a gel by using selective PCR amplification. This is achieved through reverse transcription with a specific subset of oligo-dT primers ($T_{12}NN$), where *NN* represents any given two-nucleotide combination.

Figure 6.5 Differential display analysis of PCR products derived from two species of hydra, and from interspecies grafts (in duplicate), performed on GeneGel Clean 15/24.

The dinucleotide combination serves two critical functions: (1) It anchors the oligo-dT portion of the primer directly at the junction between the poly-A tail and the end of the transcript and (2) it anchors the oligo-dT primer only to the subset of transcripts that are complementary at the dinucleotide position. Because the 5'-end of the tail is already composed of poly-dT, primer sets containing dinucleotide anchors beginning with dT nearest to the poly-dT tail are omitted, as they would more typically behave as single nucleotide anchors, which bind transcripts at much lower specificity than dinucleotide-anchored primers.

Dinucleotide-anchored oligo-dT primers are still considered the standard for differential display for two basic reasons: (1) The dinucleotide combination reduces the number of reactions that must be generated per given mRNA pool being analyzed, and (2) each anchored-primer subset identifies a low enough complexity of transcripts that can be easily interpreted after electrophoresis on standard manual sequencing gels. What's most important, however, is that the anchoring effect of the dinucleotide anchor supports size uniformity of DNA fragments that are generated through the next step—PCR amplification.

PCR amplification of the first strand cDNA is achieved using random primers (typically 10 mers) as the amplification primer at the 5'-end. Therefore, a total of 12 reactions per mRNA pool are generated. Matching (by anchored-primer sequence) amplification reactions from each mRNA pool are then separated in adjacent lanes using denaturing gel electrophoresis. Differential expression is determined by identifying differential intensities of parallel bands in neighboring lanes. Typically, fragments are detected by autoradiography by incorporating radiolabeled nucleotides into each PCR reaction mix or by using end-labeled primers.

Bands of differential intensity can then be cut directly from the acrylamide gel and eluted from the matrix for amplification and subsequent confirmation and analysis. Because there are a number of factors that can create a bias in each step of the process, every prospective fragment that is isolated should first be confirmed through standard northern blot hybridization or by RNase protection analysis. Northern blotting offers the benefit of using unamplified pools of mRNA and secondary internal control standards for confirming that apparent differential expression is not due to artifacts in the DDRT process. RNase protection assays offer greater sensitivity and specificity.

Common Issues

The most common issues with the DDRT method are the frequency of false positives and the sensitivity for transcripts that are expressed at very low copy numbers. False positives first begin to manifest themselves by the inability of selected fragments to reamplify once they have been isolated from the gel. Others fail at the point of northern hybridization, and so on.

Additionally, DDRT offers only partial sequence information that resides primarily in the 3'-non-coding regions of the gene. Although this can be a great benefit in some respects, because these sequences represent regions of greater variance which can help to distinguish closely related genes or members of small gene families, the downstream analysis of putative positive fragments is relatively time-consuming and laborious. In that respect there have been many successful efforts to reduce the frequency of false positives and the number of steps during and after the display process.

Last, there is no certainty of minimizing sequence-dependent bias at each step in the process. DDRT makes many assumptions that the relative abundance of certain sequences for priming sites is fairly equal (factoring primer degeneracy) and that there is no preferential bias for amplification of certain primer pairs during the first and second PCR steps, real or artifactual.

Variations

In the six years since its invention, more than 70 articles have been published that describe improvements and powerful derivatives of the differential display method as well as standard protocols. Improvements and derivative methods include the following:

- **Reduction of false positives:** numerous strategies primarily based on primer selection, amplification protocols, different reverse transcriptase combinations, and novel methods of isolating differentially expressed signals from unrelated contaminating sequences

- **Primer design optimization:** numerous methods that describe strategies involving the use of gene-specific primers, deoxyinsoine, and primer compatibility software

- **Purification and direct sequencing:** numerous protocols based on optimized purification, nested primer selection, and PCR-based sequencing methods

- **Reamplification improvements:** protocols that describe optimized fragment purification procedures as well as optimized PCR reamplification recipes

- **Fluorescence detection methods:** fluorescent end-labeling of primers and detection, using automated sequencers and scanning software which increases the throughput and eliminates the need to handle radioisotopes

- **Silver staining detection:** protocol based on an optimized staining procedure and upstream and downstream primer concentration that eliminates the need to handle radioisotopes

- **Low-abundance transcript identification:** rapid, efficient, and non-radioactive (REN) method and variations

- **Multiplexing and high throughput:** Sophisticated methods for high-throughput strategies that are generally target-specific

Literature

GenePhor AN #2: REN (rapid, efficient, and non-radioactive) display of DDRT-PCR products (18-1118-14)

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rRNA and tRNA Spacer Analysis

16S/23S rRNA Spacer Analysis

Species-specific polymorphism of the intergenic region between the 16S and 23S rRNA coding regions offers another valuable target for DNA typing and identification. Using ARDRA strategies targeted to this intergenic sequence domain, band patterns can be analyzed after polyacrylamide electrophoresis of the resultant fragments. Higher organisms tend to show significant interspecies differentiation within this intergenic region, but it is not uncommon for bacterial templates to show only limited variations in band patterns. Although this complexity can vary anywhere from one to two bands to greater than seven size differentials of the individual fragments can be very small and most often require fragment separation using polyacrylamide gel electrophoresis.

tRNA Spacer Analysis

Like rRNA spacer analysis between the 16S and 23S rRNA coding sequences, tRNA spacer analysis is based on amplification of the intergenic regions between the different tRNA genes using the T3 and T5 primers. This region of DNA is fairly rich in polymorphism while providing the benefit of conserved flanking sequences that can be used as priming sites, thus eliminating the need for extensive sequence information of the target organism.

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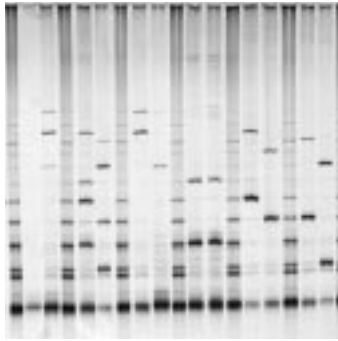
Figure 6.6 VNTR analysis of human blood specimens for YNZ 22 alleles performed on GeneGel Excel 12.5/24.
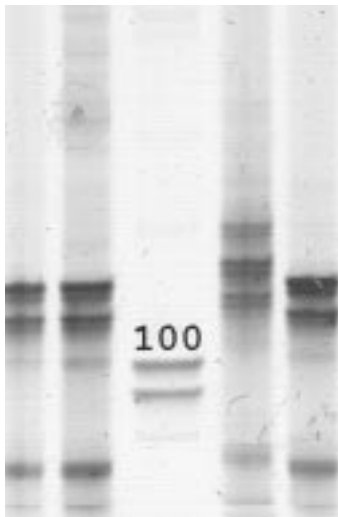
Figure 6.7 Separation of amplified microsatellite sequences from different cancer tissues performed on GeneGel Clean 15/24.

VNTR/STR

Tandemly repeated sequences are powerful tools for genotyping and linkage analysis. The sequences that are the core of the repeated units represent a relatively wide diversity of size (2–80 bp), sequence, and genome distribution. They are extremely valuable as genetic markers because of their highly polymorphic and abundant presence in the genomes of higher organisms. Tandemly repeated sequences can be broadly categorized into two groups— VNTRs and STRs—based primarily on the size range of the core sequence that is repeated.

Variable-number tandem repeats (VNTRs) describe allelic variants of tandemly repeated core sequences. VNTRs represent simple head-to-tail repeated sequences that can typically range in length from 10 to 80 bps and are highly polymorphic as to the number of repeats at a given locus. They occur fairly frequently in the genome, but there are relatively few different types. Not surprisingly, longer VNTRs tend to show greater polymorphism and thus greater utility for genotyping. Occurring every few kilobases on average, they are not evenly dispersed throughout the genome, tending to cluster toward the telomeric ends of chromosomes.

A working VNTR fragment size range is 50 to 1,500 base pairs. These sequences are typically identified using a strategy similar to that of RFLP analysis, where genomic DNA is digested with an endonuclease and separated by electrophoresis. The gels are then analyzed by southern blotting, using sequence-specific probes to the repeated core sequence.

Short tandem repeat (STR) sequences, or microsatellites, consist of much shorter (2–10 bp) core sequences whose allelic variants are tandemly repeated as many as hundreds of times at different genetic loci. They are typically more evenly dispersed throughout the genome as compared with the larger VNTRs, and they represent ideal tools as genetic markers because of their rich diversity, wide distribution, and polymorphism. Common repeats used for typing and linkage analysis are "CA" or "ACTT" sequences.

Specifically designed as amplification-based detection methods, STR and microsatellite-based DNA typing offer some practical advantages over typing methods based on larger repeat sequences. For example, PCR amplification using primers targeted to a specific STR sequence typically generates 50-to-500-bp-sized fragments without compromising allelic diversity. This allows for easier sizing of a wider range of alleles on a single electrophoretic separation, as compared with larger tandem repeat sequences that typically produce an order-of-magnitude greater range in fragment size diversity. In addition, the smaller average size of STR and microsatellite alleles allows the technique to exhibit a greater tolerance for crude or partially degraded genomic DNA samples, because the need for longer intact sequence domains is reduced. As a corollary, these techniques also show a greater tolerance for rapid and simplified sample preparation methods that make analysis faster and easier.

Literature

GenePhor AN #7: SSCP analysis on GenePhor: A screening method for BRCA1 point mutations (18-1118-11)

GenePhor AN #8: Detection of ApoB mutations by SSCP (18-1118-10)

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Appendix

PCR Data: Standard Calculations and Genome Information

Molecular weights of nucleic acids

Average molecular weight of a deoxynucleotide base	= 324.5 daltons
Average molecular weight of a deoxynucleotide base pair	= 649 daltons
Average molecular weight of a ribonucleotide base	= 340.5 daltons
1 kb of dsDNA (sodium salt)	= 6.5×10^5 daltons
1 kb of ssDNA (sodium salt)	= 3.3×10^5 daltons
1 kb of ssRNA (sodium salt)	= 3.4×10^5 daltons
1 megadalton of ds DNA (sodium salt)	= 1.54 kb
λ DNA	= 3.1×10^7 daltons[1]
pBR322 DNA	= 2.8×10^6 daltons[1]
φX-174 DNA	= 3.6×10^6 daltons[1]
E. coli DNA	= 3.1×10^9 daltons[1]

[1] Ausubel, F. M., et al., eds. *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 1994.

Sizes of representative genomes (megabases)

SV40	0.005243
Bacteriophage lambda	0.048502
Escherichia coli	4.7
Saccharomyces cerevisiae	16.5
Caenorhabditis elegans	80
Drosophila melanogaster	170
Xenopus laevis	2,900
Homo sapiens	3,300
Zea mays	15,000

Spectrophotometric quantification of DNA or RNA

Spectrophotometric measurements of nucleic acid solutions are typically taken at wavelengths of 260 nm and 280 nm. The A_{260} reading is used to determine the concentration of nucleic acid in solution. For a solution with an A_{260} = 1.0, the following approximations hold:

1 A_{260} unit of double-stranded DNA = 50 µg/ml

1 A_{260} unit of single-stranded DNA = 37 µg/ml*

1 A_{260} unit of single-stranded RNA = 40 µg/ml

*For oligonucleotides an A_{260} of 1.0 represents anywhere from 20 to 33 µg/ml with the actual conversion factor dependent on the length and base sequence of the oligonucleotide.

The ratio between measurements at 260 nm and 280 nm provides an indication of the purity of a nucleic acid. In solution, pure DNA and RNA typically have A_{260}/A_{280} ratios of 1.8 and 2.0, respectively. If the absorbance ratio is significantly less than the values above, the nucleic acid is probably contaminated with protein or phenol. Accurate quantitation of a contaminated nucleic acid is not feasible without prior purification, and the efficacy of this can be established by the A_{260}/A_{280} ratio.

Most of the lyophilized polynucleotides are sold as A_{260} units.[2] For an approximation of quantity, use the conversion factors provided above to convert the A_{260} units into micrograms, which must be known if a certain concentration is desired.

[2] Unit definition: One unit is that quantity of oligonucleotide or polynucleotide that has an absorbance of 1.0 at a given wavelength when dissolved in 1 ml of buffer and measured in a 1-cm cuvette at 20 °C. The wavelength at which the absorbance is measured is printed on the Certificate of Analysis that accompanies the product. For nucleic acids typically an absorbance is taken at 260 nm in 20 mM sodium phosphate (pH 7.0), 0.1 M NaCl.

Mass-to-moles conversions

1 µg/ml of DNA	= 3.08 µM phosphate
1 µg/ml of a 1-kb DNA fragment	= 3.08 nM 5'-ends
1 µg of a 1-kb DNA fragment	= 1.5 pmol = 9.1×10^{11} molecules
1 µg of a 1-kb DNA fragment	= 3.0 pmol 5'-ends
1 pmol of a 1-kb DNA fragment	= 0.65 µg
1 µg of pUC18/19 DNA (2,686 bp)	= 0.57 pmol = 3.4×10^{11} molecules
1 pmol of pUC 18/19 DNA	= 1.77 µg
1 µg of pBR322 DNA (4,361 bp)	= 0.35 pmol = 2.1×10^{11} molecules
1 µg of linear pBR322 DNA	= 0.70 pmol of 5'-ends
1 pmol of pBR322 DNA	= 2.83 µg
1 pmol of 5'-ends of linear pBR322	= 1.4 µg
1 µg of M13mp18/19 DNA (7,249 bp)	= 0.21 pmol = 1.3×10^{11} molecules
1 pmol of M13mp18/19 DNA	= 4.78 µg
1 µg of λ DNA (48,502 bp)	= 0.033 pmol = 1.8×10^{10} molecules
1 pmol of λ DNA	= 32.01 µg

Determining the molecular weight and moles of ends of a double-stranded DNA

Molecular weight (MW) of a dsDNA fragment	= (# of bp) × (649 daltons/bp)
Moles of ends of linear DNA	= 2 x (g of DNA)/(# of bp) × (649 daltons/bp)
Moles of ends generated by a restriction digest:	
for linear DNA	= (# of cuts) × (moles of DNA) × 2 (ends per cut) + 2 (ends of linear DNA) × (moles of DNA)
for circular DNA	= (# of cuts) × (moles of DNA) × 2 (ends per cut)

Storage of oligonucleotides

Oligonucleotides are stable for years when stored as lyophilized powders at –20 °C. They are less stable in solution but can be kept for several months if stored at –20 °C. Oligonucleotides can be stored in sterile water or dilute buffers at or near pH 7.0.

Preparation of oligonucleotides

Oligonucleotides may be dissolved in water or dilute buffers at or near pH 7.0. Because laboratory distilled water is often slightly acidic, some addition of dilute sodium hydroxide or ammonia solution may be necessary to raise the pH to 7.0.

Determining the molecular weight of an oligonucleotide

The molecular weight (MW) of a DNA oligonucleotide can be calculated with the following formula*:

MW(g/mole) = [(dA × 312.2) + (dC × 288.2) + (dG × 328.2) + (dT × 303.2)]
+ [(MW counter-ion) × (length of oligo in bases)]

The molecular weight calculated using this equation must be adjusted for the contribution of the atoms at the 5'- and 3'-ends of the oligonucleotide. This adjustment is necessary because the weight of the repeat units is calculated for nucleotides located in internal positions within the oligonucleotide.

Phosphorylated oligos, add: 17 + (2 × MW counter-ion)
Non-phosphorylated oligos, subtract: 61 + (MW counter-ion)

The molecular (atomic) weights of the most common oligonucleotide counter-ions are:

Counter-ion	MW (g/mole)
Sodium	23.0
Potassium	39.1
Triethylammonium	102.2

*The molecular weight listed for each nucleotide is the molecular weight of that nucleotide incorporated in the oligo.

Determining the number of micromoles of an oligonucleotide

1. Determine the number of μg of the oligonucleotide: 1 A_{260} unit of single-stranded DNA = 20–33 μg.
2. Determine the molecular weight of the oligonucleotide: see previous page.
3. μmol = μg/MW

Estimating the concentration of an oligonucleotide

N = number of bases in the oligonucleotide

$\varepsilon_{260} \approx 10^4 \times N$ ($M^{-1}cm^{-1}$)

MW $\approx$ 324.5 daltons $\times$ N

$\dfrac{A_{260}}{\varepsilon_{260}} \times 10^6$ = concentration (μM)

concentration (μM) $\times$ MW = concentration (ng/ml)

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General References

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Amersham Biosciences Literature

Use our convenient literature list here to identify the document that best answers your instrument and application questions. For your greater benefit, the documents below are inclusive of all featured applications for each instrument and are not limited to only DNA analysis applications.

GenePhor

Characterization of bacterial strains by RAPD analysis (18-1118-12)

Identification of different isolates of *E. coli* by RAPD analysis (8-1115-56)

User Manual (18-1116-08 Edition AA)

Technical Manuals (gels)

GeneGel Clean 15/24 (71-5000-9 Edition AA)
GeneGel Excel 12.5/24 (71-5000-90 Edition AA)

Application Notes

#1 SSCP analysis in exons 7-9 and 9-10 of the tumor suppressor gene p53 (18-1118-15)

#2 REN (rapid, efficient, and non-radioactive) display of DDRT-PCR products (18-1118-14)

#3 Microsatellite analysis of cancer tissue (18-1118-16)

#4 VNTR analysis of alleles in the AMPFLP system YNZ and pMCT (D1S80) system (18-1118-17)

#5 Characterization of bacterial strains by RAPD analysis (18-1118-12)

#6 Analysis of plasmid insert size by PCR amplification: Screening deletion plasmids for sequencing

#7 SSCP analysis on GenePhor: A screening method for BRCA1 point mutations (18-1118-11)

#8 Detection of ApoB mutations by SSCP (18-1118-10)

SSCP screening for breast cancer BRCA1 (breast cancer) point mutations (18-1118-11)

Multiphor II

DNA Fragment Analysis Kit (18-1108-05)

User Manual (18-1103-43 Edition AF)

Technical Manual (gels): ExcelGel SDS, Homogeneous 7.5, 12.5, and 15 (71-7126-00 Edition AC)

Brochures

Behind Every Great… (18-1101-95)

Precast Gel Media Guide (18-1108-77)

You Don't Need Magic to Get Good Results (18-1115-58)

Application Notes

#1 Two-dimensional phosphopeptide mapping (80-6375-45)

#2 Phosphoamino acid analysis by thin layer electrophoresis (80-6413-07)

Analysis of 16 rDNA digested with different enzymes (18-1115-55)

Analysis of PCR fingerprint products (18-1108-44)

Identification of animal species by SSCP analysis of amplified cytochrome b gene fragments (18-1108-90)

Identification of different isolates of *E. coli* by RAPD analysis (18-1115-56)

SSCP analysis in exon 6 of the tumor suppressor gene p53 (18-1115-54)

SSCP analysis of PCR products to type the coagulation factor V gene (18-1115-53)

SSCP analysis on CleanGel: A screening method for mitochondrial DNA (mtDNA) point mutations (18-1108-89)

Ubiquitin SSCP: Analysis as a differentiation tool for ground beetles (*Carabus* species) (18-1108-88)

PhastSystem

Analysis of labelled and unlabelled oligonucleotides (18-1108-47)

Analysis of *lysU* gene from *E. coli* (18-1108-49)

Analysis of restriction fragments and PCR products on PhastSystem (18-1108-46)

Analysis of synthetic oligonucleotides and short PCR products (18-1108-45)

Analysis of various restriction enzyme digests of cloned miniprep plasmid DNA (18-1108-48)

Detection of SSCP as applied to exon 11 of the CFTR gene (18-1108-51)

Detection of SSCP in exons 5 and 7 of the PAH gene (18-1108-50)

Detection of SSCP: A non-radioisotopic technique as applied to exon 8 of the F9 gene (18-1108-52)

DNA fragment analysis using PhastGel media (18-1112-39)

Heteroduplex mobility assay for subtyping HIV-1 (80-6375-26)

Non-radioactive SSCP applied to exon 1 of the *Ki-ras* gene (18-1108-86)

PCR-SSCP analysis (18-1035-40)

Recovery and PCR amplification of DNA fragments separated on PhastSystem (18-1108-43)

SSCP analysis of PCR products to identify clones in lymphoid leukaemia and lymphoma (18-1108-54)

SSCP analysis of PCR products to type human papillomavirus (18-1108-53)

Data Files

PhastGel high density (18-1013-41)

PhastGel separation media (18-1010-58)

PhastSystem instrumentation (18-1022-23)

PhastTransfer (18-1010-08)

Separation Technique Files

#100 IEF and electrophoretic titration curve analysis (80-1311-94)

#101 IEF with PhastGel dry IEF (18-1018-20)

#110 SDS PAGE (80-1311-95)

#111 SDS PAGE in homogeneous media (18-1010-63)

#120 Native PAGE (80-1311-96)

#121 Native PAGE in homogeneous media (80-1312-26)

#130 Native SDS of HMW proteins using PhastGel gradient 4-15 (18-1011-67)

#131 PCR-SSCP analysis (18-1035-40)

#132 DNA analysis using PhastGel homogeneous media (18-1100-58)

Development Technique Files

#200 Fast Coomassie staining (80-1311-97)

#210 Sensitive silver staining (80-1311-98)

#211 Silver staining of peptides using PhastGel high density (80-1312-31)

#222 Blotting of peptides (80-1312-32)

Technical Notes

2-D electrophoresis with PhastGel separation media (80-1312-28)

Choosing the optimal gel for a separation with the help of selectivity curves (18-1016-66)

Crossed immunoelectrophoresis (CIE) and rocket immunoelectrophoresis (RIE) (18-1010-60)

Diamine silver staining for PhastGel gradient media (80-1313-81)

Gel casting guide for isoelectric focusing, SDS-PAGE, PAGE, and agarose (18-1011-54)

Brochures

Let PhastSystem Do the Work (18-1105-43)

PCR-SSCP (18-1112-40)

Precast Gel Media Guide (18-1108-76)

Application Notes

#1 Heteroduplex mobility assay for subtyping HIV-1 (80-6375-26)

#300 PAGE of basic proteins (18-1010-39)

#301 Analysis of glycoproteins (18-1313-73)

#371 Protein mapping of mycoplasmas by 2-D electrophoresis and immunoblotting (18-1016-05)

#372 Protein sequencing of cyanogen bromide digested myoglobin... (10-1016-62)

#373 Typing of haptoglobin using PhastSystem (18-1019-69)

#374 Use of PhastGel dry IEF for rapid detection of bovine milk... (18-1022-50)

#375 Analysis of isozyme variability in cucumber… (18-1022-52)

#376 Phenotyping of a1-antitrypsin using PhastGel dry IEF (18-1022-73)

#377 Detection of abnormal hemoglobins using PhastGel dry IEF (18-1022-74)

#378 Detection of oligoclonal IgG in cerebral spinal fluid (CSF) (18-1034-01)

#379 Identifying fish species by IEF with PhastSystem (18-1034-08)

#380 Identifying meat species by IEF with PhastSystem (18-1034-09)

#381 Diagnostic detection of SSCP: …exons 5 and 7 of the PAH gene (18-1034-04)

#383 Diagnostic detection of SSCP: …exon 8 of the F9 gene (18-1034-06)

#384 SSCP analysis of PCR products to type human papillomavirus (18-1039-81)

#385 Single-strand conformation polymorphism…lymphoid leukemia and lymphoma (18-1039-85)

DNA analysis using PhastGel media (18-1112-39)

Automated Gel Stainer

Application Note: #1 Automated colorimetric immunodetection on western blots (80-6371-65)

Brochure: Stains with the Push of a Button (18-1118-87)

Protocol Guide: Automated Gel Stainer (80-6343-34)

DyNA Quant 200

BioMedical Reprint: Low-cost Quantitation A (80-6321-87)

Fluorometer Product Sheet: DQ 200 DyNA Quant (35078 80-6230-86)

Flyer: Gus Assay (35086 80-6231-05)

Nucleic Acid Quantification Guide (18-1125-25)

Application Notes

#1 Protease assay A 0.30 (35080 80-6236-37)

#2 B-Galactosidase assay (35081 80-6236-56)

#3 B-Glucuronidase assay (35082 80-6236-75)

#4 D-B-Hydroxybutyrate (BHB)/NADH-coupled assay (35083 80-6236-94)

#5 Fluorescent probe studies of proteins (35084 80-6237-13)

#6 Fluorescence assay for DNA quantitation (35092 80-6240-74)

#7 Fluorescence quantitation of PCR products before and after EasyPrep purification (80-6329-09)

#8 Fluorescence quantitation of plasmid DNAs/calf thymus as calibration standards (80-6323-58)

#9 Fluorescence quantitation of double-stranded DNA after cDNA synthesis (80-6333-46)

#10 Fluorescence quantitation of PCR products prior to re-amplification and direct sequencing (80-6338-59)

#11 Fluorescence quantitation of single-stranded M13 DNA (80-6370-89)

General

Acrylamide Gel Casting Handbook (18-1102-95)

Data File: PlusOne high quality (18-1103-56)

Electrophoresis Buyer's Guide Brochure: Everything for Electrophoresis (18-1116-82)

High-resolution DNA analysis: Application Note file (18-1108-06)

ImageMaster

Data Files

VDS Software (80-6254-61)

Sharp JX-330 Desktop Scanner (80-6361-01)

Flyer: 1D & 2D Gel Evaluation Software (Elite, Prime, Database) (80-6361-20)

Brochures

ImageMaster (18-1108-08)

VDS Software (80-6334-2)

Application Notes

#1 Imaging DNA agarose gels stained with ethidium bromide (80-6323-77)

#2 Imaging DNA agarose gels stained with SYBR Green 1 (80-6338-78)

#3 Quantitation of PCR products in ethidium bromide–stained agarose gel (80-6333-65)

#4 Image analysis of silver stained polyacrylamide gels/species identification by RAPD analysis (80-6334-41)

Power Supplies

Data Files

PS 3000 Power Supply (35022 80-6004-38)

PS 500XT Power Supply (35021 80-6004-19)

Brochure: EPS Power Supply Line (18-1060-89)

Molecular Dynamics Literature

AN #50: Quantitative double-label autoradiography using storage phosphor imaging

AN #53: Quantitative PCR using storage phosphor technology 1: mRNA quantitation

AN #55: Genetic typing using fluorescently labeled polymorphic STR markers

AN #56: Oncogene mRNA profiling using fluorescent quantitative PCR

Fluorescence Imaging Applications Guide

TN #52: Improving quantitation in autoradiography

TN #57: Understanding fluorescence

Ordering Information

product name	product code
GenePhor System	
GenePhor Electrophoresis Unit	18-1115-82
GeneGel Excel 12.5/24 Kit	
Includes: 5 gels and 6 pairs Buffer Strips	17-6000-14
GeneGel Clean 15/24 Kit	17-6000-13
Includes: 5 gels, paper Buffer Strips, and gel rehydration and electrode buffers	
GeneGel cover sheets, 25/pack	17-6000-49
Rehydration tray for GeneGel Clean	18-1117-60
EPS 1000 Power Supply	18-1123-96
Multiphor II System	
Multiphor II Electrophoresis Unit	18-1018-06
MultiTemp III Thermostatic Circulator	
115 VAC	18-1102-77
230 VAC	18-1102-78
ExcelGel DNA Analysis Kit	
Includes: 5 gels and 6 pairs Buffer Strips	17-1198-07
CleanGel DNA Analysis Kit	17-1198-06
Includes: 5 gels, paper Buffer Strips, and gel rehydration and electrode buffers	
ExcelGel SDS homogenous	
7.5%	80-1260-01
12.5%	80-1261-01
15%	80-1262-01
ExcelGel SDS gradient	
8–18%	80-1255-53
XL 12–14%	17-1236-01
ExcelGel SDS Buffer Strips	17-1342-01
CleanGel, 10%	
25S	18-1031-54
36S	18-1031-55
48S	18-1031-56
Buffer Kits	
SDS, pH 8.0	18-1031-60
Native, pH 5.5	18-1031-61
Native, pH 8.9	18-1031-62
Includes: gel buffer, electrode buffers, and electrode strips for 5 gels	
GelPool	18-1031-58
CleanGel electrode strips	8-1035-33
PaperPool	18-1031-59
EPS 1000 Power Supply	18-1123-96

product name	product code
Automated Gel Staining and Silver Staining Kit	
Hoefer Automated Gel Stainer	
with 19 × 29 cm tray	80-6395-02
with 29 × 35 cm tray	80-6396-16
PlusOne DNA Silver Staining Kit	17-6000-30
PhastSystem	
PhastSystem Separation-Control and Development Units	
120 VAC	18-1018-23
220 VAC	18-1018-24
PhastGel Sample Applicators	
12/0.3 µl	18-1614-01
8/0.5 µl	18-1617-01
8/1 µl	18-1618-01
6/4 µl	18-0012-29
Sample well sample	18-0097-01
PhastGel gradient	
4–15	17-0678-01
10–15	17-0540-01
8–25	17-0542-01
PhastGel homogeneous	
7.5	17-0622-01
12.5	17-0623-01
20	17-0624-01
PhastGel Buffer Strips, DNA	17-1599-01

product name	quantity	product code
Ready-To-Go PCR Beads (0.5-ml tubes)	100 reactions	27-9555-01
Ready-To-Go PCR Beads (0.2-ml tubes/plate)	96 reactions 5 × 96 reactions	27-9553-01 27-9553-02
Ready-To-Go You-Prime First-Strand Beads	50 reactions	27-9264-01
Ready-To-Go RAPD Analysis Beads (100 reactions)	1 kit	27-9500-01
RAPD Analysis Primer Set (6 primers)	1 set	27-9501-01
Ready-To-Go RAPD Analysis Kit (100 reactions and 6 primers)	1 kit	27-9502-01
DyNA Quant 200 Fluorometer	1	80-6406-80
GeneQuant II RNA/DNA Calculator	1	80-2105-98