EDVOTEK®

The Biotechnology Education Company ®

EDVO-Kit #

207

Southern Blot Analysis

See Page 3 for storage instructions

EXPERIMENT OBJECTIVE:

The objective of this experiment is to introduce the use of a Southern Blot as a tool for "DNA Fingerprinting" in a hypothetical paternity determination.

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207.140702

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All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

THIS EXPERIMENT DOES NOT CONTAIN HUMAN DNA.
None of the experiment components are derived from human sources.

Experiment Components

This experiment module contains reagents for 5 laboratory groups to perform agarose gel electrophoresis, Southern blot transfer and detection of the transferred DNA.

Store DNAs (A - E) in the freezer. Store all other components at room temperature.

DNA samples do not require heating prior to gel loading.

Store DNAs (A - E) in the freezer. Store all other components at room temperature.

- DNA Samples for Electrophoresis
  - A Standard DNA fragments
  - B Mother DNA cut with Enzyme
  - C Child DNA cut with Enzyme
  - D Father 1 DNA cut with Enzyme
  - E Father 2 DNA cut with Enzyme
- Practice Gel Loading Solution
- UltraSpec-Agarose™
- Electrophoresis Buffer (50x)
- 1 ml Pipet
- 100 ml Graduated Cylinder
- Transfer pipets
- Pre-cut Nylon Membrane (7 x 7 cm)
- Pre-cut Blotting Filter Paper (7 x 7 cm)
- Bottle Blue-Blot DNA Stain™ Solution (10x)

Requirements

- Horizontal gel electrophoresis apparatus
- DC power supply
- Water bath (65°C)
- DNA visualization system
- Staining net and tray
- Automatic micropipets
- 80°C oven (optional)
- 250 ml beakers or flasks
- 10 ml graduated cylinder, or 5 or 10 ml pipets/pumps
- Hot plate, Bunsen burner or microwave oven
- Hot gloves
- Small plastic tray to soak gel
- Distilled or deionized water
- NaCl
- NaOH
- Concentrated HCl
- Plastic wrap
- Paper towels
- Forceps

Background Information

DNA fingerprinting (also called DNA typing) is a recently developed method that allows for the identification of the source of unknown DNA samples. The method has become very important in forensic laboratories where it has been used to provide evidence in paternity and criminal cases. In contrast to the more conventional methodologies, such as blood typing, which can only exclude a suspect, DNA fingerprinting can provide positive identification with great accuracy.

Restriction Enzyme	Organism
Bgl I	*Bacillus globigii*
Bam HI	*Bacillus amyloliquefaciens* H
Eco RI	*Escherichia coli* RY13
Eco RII	*Escherichia coli* R 245
Hae III	*Haemophilus aegyptius*
Hind III	*Haemophilus influenzae* R4

Figure 1

DNA fingerprinting involves the electrophoretic analysis of DNA fragment sizes generated by restriction enzymes. Restriction enzymes are endonucleases which catalyze the cleavage of the phosphate bonds within both strands of DNA. They require Mg^{+2} for activity and generate a 5 prime (5') phosphate and a 3 prime (3') hydroxyl group at the point of cleavage. The distinguishing feature of restriction enzymes is that they only cut at very specific sequences of bases called recognition sites. Restriction enzymes are produced by many different species of bacteria (including blue-green algae). Over 1500 restriction enzymes have been discovered and catalogued.

Restriction enzymes are named according to the organism from which they are isolated. This is done by using the first letter of the genus followed by the first two letters of the species. Only certain strains or sub-strains of a particular species may be a producer of restriction enzymes. The type of strain or substrain sometimes follows the species designation in the name. Finally, a Roman numeral is always used to designate one out of possibly several different restriction enzymes produced by the same organism or by different substrains of the same strain.

Restriction enzymes recognize specific double stranded sequences in DNA. Most recognition sites are 4 to 8 base pairs in length. Cleavage occurs within or near the site. The cleavage positions are indicated by arrows. Recognition sites are frequently symmetrical, i.e., both DNA strands in the site have the same base sequence when read 5' to 3'. Such sequences are called palindromes. It is at such sites that restriction enzymes cut DNA (i.e. G'AATTC is the site for *Eco* RI).

The size of the DNA fragments generated by restriction enzyme cleavage depends on the distance between the recognition sites. In general, the longer the DNA molecule, the greater the probability that a given recognition site will occur. The DNA of an average human chromosome is very large, containing over 100 million base pairs. A restriction enzyme having a 6 base pair recognition site, such as *Eco* RI, would be expected to cut human DNA into approximately 750,000 different fragments. To determine the number of times a restriction enzyme cleaves double stranded DNA we use this equation.

4^N= Expected occurrence of a restriction site

N= Number of bases in restriction enzyme recognition site.

For *Eco* RI, N=6 bases, therefore 4^6=4096. *Eco* RI will cut DNA once every 4096 bases.

Background Information

No two individuals have exactly the same pattern of restriction enzyme recognition sites. There are several reasons for this fact. A large number of alleles exist in the population. Alleles are alternate forms of a gene. Alleles result in alternative expressions of genetic traits which can be dominant or recessive. Chromosomes occur in matching pairs, one of maternal and the other of paternal origin. The two copies of a gene (which can be alleles) at a given chromosomal locus, and which represent a composite of the parental genes, constitute an individual's unique genotype. It follows that alleles have differences in their base sequences which consequently creates differences in the distribution and frequencies of restriction enzyme recognition sites. Other differences in base sequences between individuals can occur because of mutations and deletions. Such changes can also create or eliminate a recognition site.

The example in Figure 2 shows how a silent mutation can eliminate a recognition site but leave a protein product unchanged.

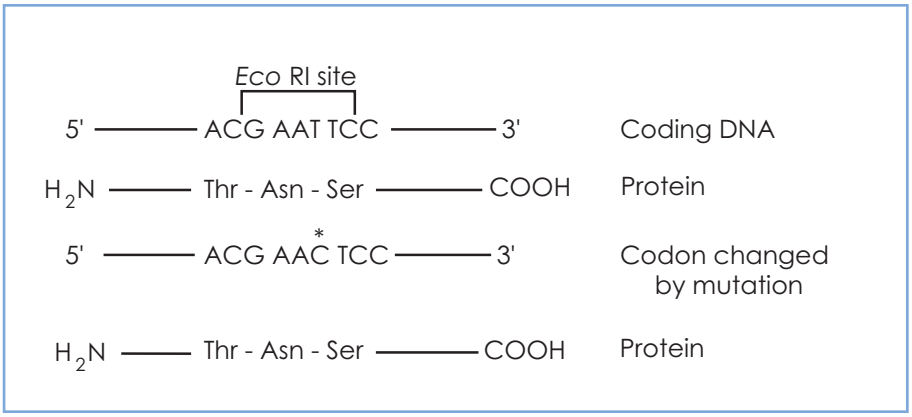


Figure 2:
Silent mutation (T → C) changes the *Eco R*I site.

Individual variations in the distances between recognition sites in chromosomal DNA are often caused by intervening repetitive base sequences. Repetitious sequences constitute a large fraction of the mammalian genome and have no known genetic function. These sequences can occur between genes or are adjacent to them. They are also found within introns. Ten to fifteen percent of mammalian DNA consists of sets of repeated, short sequences of bases that are tandemly arranged in arrays. The length of these arrays (the amount of repeated sets) varies between individuals at different chromosomal loci.

TGTTTA|TGTTTA|TGTTTA|.....variable number

When these arrays are flanked by recognition sites, the length of the repeat will determine the size of the restriction enzyme fragment generated. Variations in the length of these fragments between different individuals, in a population, are known as restriction fragment length polymorphisms (RLFP). RFLPs are a manifestation of the unique molecular genetic profile, or "fingerprint", of an individual's DNA. As shown in Figure 3, there are several types of these short, repetitive sequences that have been cloned and purified.

There are two types of probes commonly used for genetic identification. The single-locus probes (SLPs) which detect a single segment of the repetitive DNA located at a specific site on a single chromosome. This will result in one or two DNA bands corresponding to one or both chromosomes. If the segments recognized on the chromosome pairs are the same, then there will be one band. On the other hand, if they are different, it will appear as two bands. Several (SLPs) are available. Since more than one person can exhibit the same exact pattern for a specific SLP, several SLPs are used for a single test. Multiple-

Background Information

locus probes (MLPs) detect multiple repetitive DNA segments located on many chromosomes yielding 20 to 30 bands. Because of the multiband patterns, the chances of two people chosen at random is enormously remote. For example, it is calculated that two unrelated individuals having the identical DNA pattern detected by MLPs as an average is 1 in 30 billion. It should be kept in mind that the total human population on earth is between 5-6 billion.

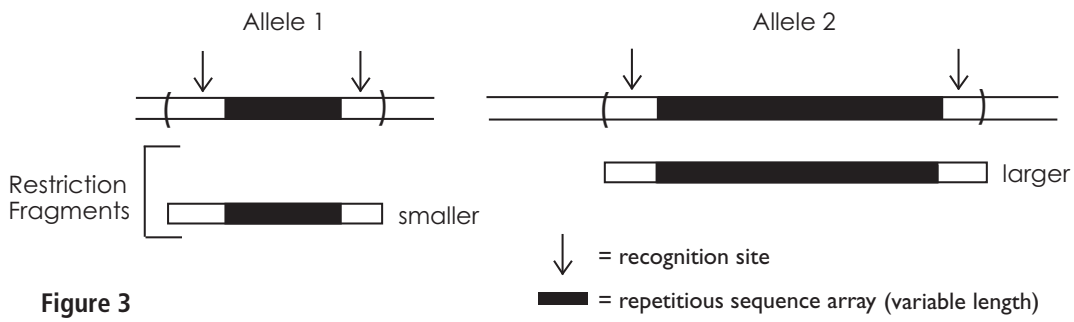


Figure 3

RFLP analysis of complex DNA, is facilitated by Southern blot analysis. After electrophoresis, the gel is sequentially treated in HCl and NaOH. The HCl treatment introduces apurinic sites in DNA which makes phophodiester bonds at these sites labile and therefore introduces nicks in double-stranded DNA. Apurinic sites result when the purine base is removed, such as an adenine residue from the A=T base pair. The NaOH treatment disrupts the the interstrand hydrogen bonds between the base pairs. The sequential acid and base treatments therefore result in the formation of small fragments from large DNA fragments. This facilitates the transfer of DNA fragments onto the nylon membrane. This procedure causes the double stranded DNA restriction fragments to be converted into single stranded form.

A replica of the electrophoretic pattern of DNA fragments in the gel is made by transferring (blotting) to a membrane of treated nylon. This is done by placing the nylon membrane on the gel after electrophoresis and transferring the fragments to the membrane by capillary action or suction by vacuum. The DNA becomes permanently adsorbed to the membrane, which can be manipulated much more easily than the gel. At this point the DNA is not visible on the nylon membrane.

Analysis of the transferred DNA is often done by hybridization with a radioactive DNA probe. Alternatively, a non-isotopic detection system is employed to detect DNA bound to the sheet. In RFLP analysis, the probe is a DNA fragment that contains base sequences which are complementary to the variable arrays of tandemly repeated sequences found in the human chromosomes. Because these probes are chemically synthesized or cloned and purified, it is relatively easy to label them with radioactive isotopes. A solution containing the single-stranded form of the probe is incubated with the membrane containing the transferred, single-stranded DNA fragments. Under the proper conditions, the probe will only base pair (hybridize) to those fragments containing the complementary repeated sequences. The membrane is then washed to remove excess probe and is exposed to a sheet of x-ray film. Only those DNA fragments that have hybridized to the probe will reveal their positions on the film because the localized areas of radioactivity cause exposure of the x-ray film. This process is known as autoradiography. The hybridized fragments appear as discrete bands (fingerprint) on the film and are in the same relative positions as they were in the agarose gel after electrophoresis. The reason that well-defined bands can now be visualized is because only a small fraction of the hundreds of thousands of fragments present contain sequences complementary to the probe. Since autoradiography is an extremely sensitive technique, only small amounts of DNA samples are required.

This experiment demonstrates the use of Southern Blot Analysis to determine paternity identification. In such a determination, DNA fingerprinting samples obtained from the mother, the child and possible fathers are fingerprinted. A child's DNA is a composite of its parent DNAs. Therefore, comparison of DNA fragmentation patterns obtained from the mother and child will give a partial match. Bands in the child's fingerprint that are not present in the mother's must have been contributed by the father. Because of allelic differences, not all of the bands present in the parents' fingerprint will appear in the child's fingerprint. However, as shown in Figure 4, the bands that do appear in the child's fingerprint must be found in either the father's or mother's fingerprint.

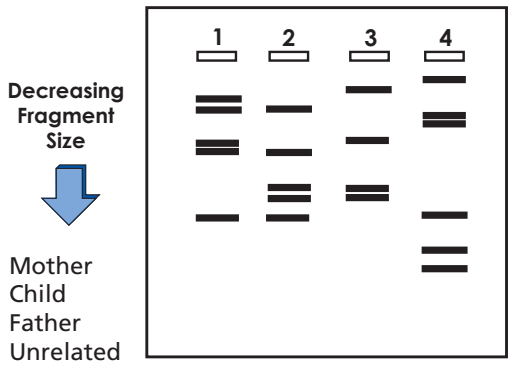


Figure 4: The child's (lane 2) DNA pattern contains DNA from the mother (lane 1) and the biological father (lane 3).

In DNA fingerprinting laboratories, the two most commonly used restriction enzymes are *Hae* III (GGCC) and *Hin*f I (G'ANTC), which are 4-base and 5-base cutting enzymes respectively. In recent years, the presentation of DNA analysis as evidence has become increasingly significant in court cases involving murder, rape, and other types of crimes as well as in paternity determinations. To ensure greater accuracy, scientists incorporate standardization procedures in DNA analysis, such as the use of Standard DNA Fragments. Such standards are used to determine the exact size of individual DNA fragments in a DNA fingerprint.

In this hypothetical case, DNA was extracted from samples obtained from two possible fathers, which were cleaved with the same restriction enzymes in separate reactions. The objective is to analyze and match the DNA fragmentation patterns after agarose gel electrophoresis and determine if Father 1 or Father 2 is the biological parent of the child.

To avoid the hazards and special techniques associated with radioactive analysis, this experiment employs a non-isotopic DNA detection system (EDVOTEK Blue Blot™) specifically designed for classroom use. Non-isotopic labeling in a research or diagnostic laboratory is also much more complex. In a conventional DNA fingerprint analysis, the DNA samples are transferred to the nylon membrane or sheet after electrophoresis. An isotopic or non-isotopic (i.e. biotinylated) probe would be used in the Southern Blot Analysis to detect complimentary sequences transferred onto the membrane.

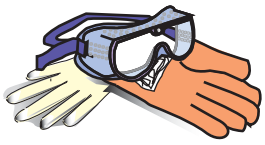
THIS KIT DOES NOT CONTAIN HUMAN DNA.

Experiment Overview and General Instructions

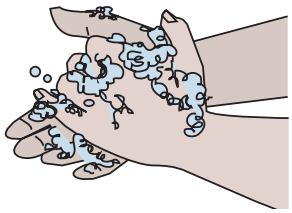
EXPERIMENT OBJECTIVE:

The objective of this experiment is to introduce the use of a Southern Blot as a tool for "DNA Fingerprinting" in a hypothetical paternity determination.

LABORATORY SAFETY



1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.



LABORATORY NOTEBOOK RECORDINGS:

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

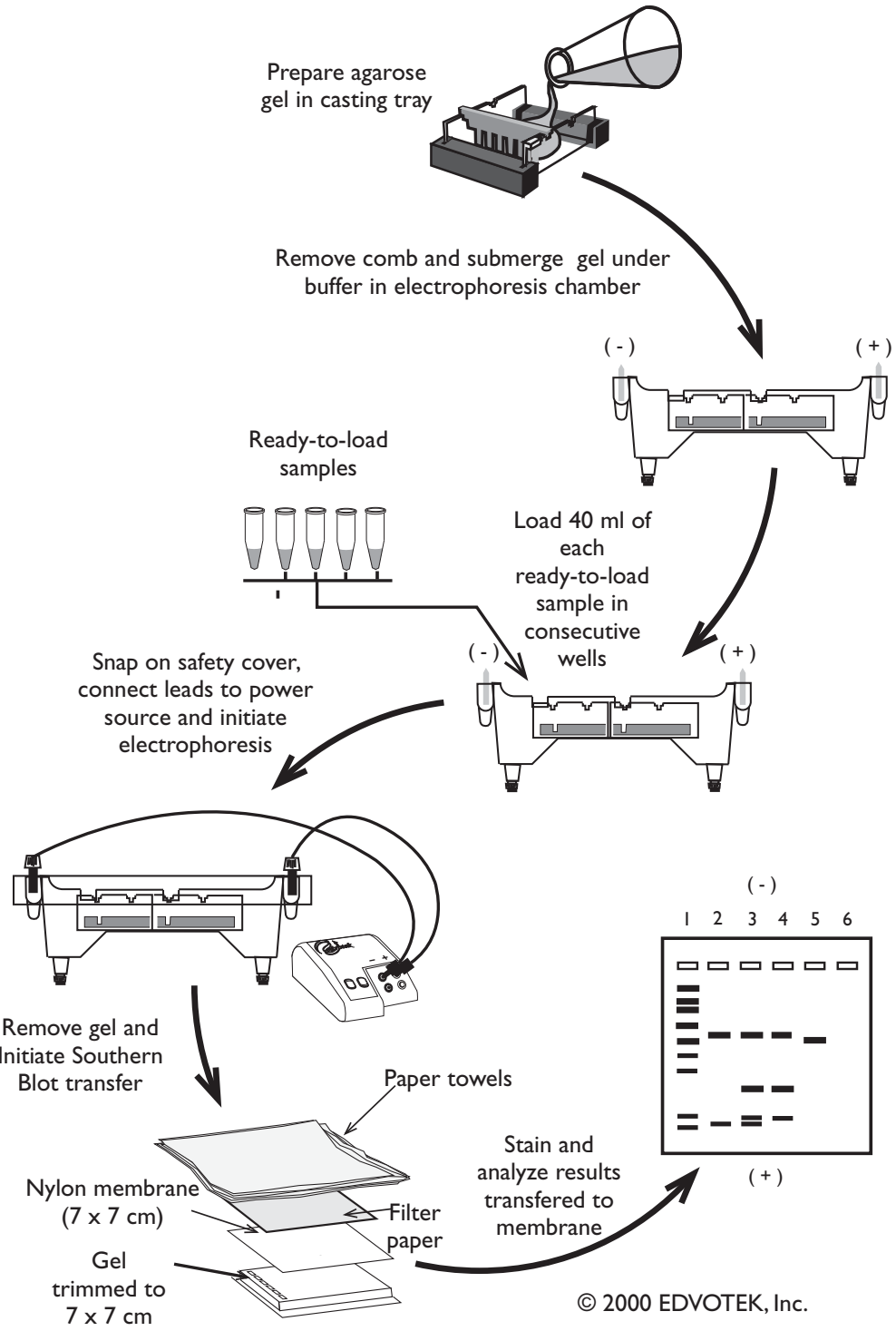
During the Experiment:

- Record (draw) your observations, or photograph the results.

Following the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

Experiment Overview: Flow Chart



Agarose Gel Electrophoresis

Prepare the Gel

1. Prepare a 0.8% agarose gel for electrophoresis of Ready-to-load DNA samples. Refer to Appendix A.

   - Recommended gel size: 7 x 7 cm or 7 x 14 cm
   - Number of sample wells required: 6
   - Placement of well-former template: first set of notches
   - Agarose gel concentration required: 0.8%

Wear gloves and safety goggles

Before Loading DNA Samples

2. Have a waterbath or beaker of water warmed to 65°C for heating the tubes containing DNA fragments before gel loading. At 65°C, non-specific aggregation due to sticky ends generated by restriction enzyme digestions will melt. This will result in sharp individual DNA bands upon separation by agarose gel electrophoresis.

3. Heat the DNA samples A - E for two minutes at 65°C. Allow the samples to cool for a few minutes.

Load the Samples

4. Load each sample in tubes A - E into the wells in consecutive order. The amount of sample that should be loaded is 35-38 µl.

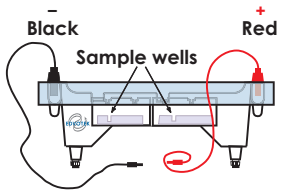
Run the Gel

5. After the DNA samples are loaded, set the power source at the required voltage and conduct electrophoresis for the length of time specified by your instructor.

6. After electrophoresis is completed, proceed to the Southern Blot Analysis.

Reminders:

During electrophoresis, the DNA samples migrate through the agarose gel towards the positive electrode. Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.



After connecting the apparatus to the D.C. power source, check that current is flowing properly - you should see bubbles forming on the two electrodes.

Experiment Procedure

Unit II: Southern Blot Analysis

Quick Reference:

The depurination soak must be brief (no more than 8 min). Prolonged exposure to HCl would completely depurinate DNA strands. Subsequent treatment with a denaturation solution would fragment the completely depurinated DNA molecules into very short oligonucleotides, which are poor targets for probes.

Wear gloves and safety goggles

DO NOT TOUCH THE NYLON MEMBRANE WITH BARE HANDS.

During this procedure you will transfer DNA fragments from agarose gel to a nylon membrane. After the transfer, the membrane will be baked for a short time to fix the DNA to the membrane. To avoid the hazards and special techniques associated with radioactive analysis, this experiment employs a non-isotopic DNA detection system (EDVOTEK Blue Blot™) specifically designed for classroom use. Non-isotopic labeling in a research or diagnostic laboratory is also much more complex. In a conventional DNA fingerprint analysis, the DNA samples are transferred to the nylon membrane or sheet after electrophoresis. An isotopic or non-isotopic (i.e. biotinylated) probe would be used in the Southern Blot Analysis to detect complimentary sequences transferred onto the membrane.

Depurination/Denaturation

After electrophoresis, the gel is sequentially treated in HCl and NaOH. The HCl treatment introduces apurinic sites in DNA which makes phophodiester bonds at these sites labile and therefore introduces nicks in double-stranded DNA. Apurinic sites result when the purine base is removed, such as an adenine residue from the A=T base pair. The NaOH treatment disrupts the the interstrand hydrogen bonds between the base pairs. The sequential acid and base treatments therefore result in the formation of small fragments from large DNA fragments. This facilitates the transfer of DNA fragments onto the nylon membrane. This procedure causes the double stranded DNA restriction fragments to be converted into single stranded form.

1. After electrophoresis, depurinate the agarose gel by placing it in a small tray containing 100 ml of 0.25 N HCl.
   - Incubate at room temperature for 8 minutes. Make sure the gel is immersed in the liquid and agitate periodically.
   - Carefully discard the HCl solution; do not reuse.
   - Rinse the gel with several changes of 100 ml distilled water.
2. Soak agarose gel 15 min. in 100 ml of DNA Denaturation Solution (0.5 M NaOH/0.6 M NaCl). Make sure gel is immersed in liquid.
   - Periodically shake the tray to immerse the gel, which will float because of the density of the solution.
   - Discard the solution.
3. In a second 100 ml of DNA Denaturation Solution, continue soaking the gel for 15 minutes.

Unit II: Southern Blot Analysis

Setting up the Southern Blot Transfer

When the second soaking of gel in DNA Solution is complete:

4. Place a sheet of plastic wrap (such as Saran Wrap or Reynolds Film) on a flat level lab bench.
    - Remove the gel from the tray and place it (well side down) directly onto the plastic wrap. Inverting the gel places the smooth surface on top for contact with the membrane.
    - Do not discard the DNA Denaturation Solution. Save the solution to wet the nylon membrane in Step 6.
5. Wearing gloves and using forceps and scissors, trim the nylon membrane to the size of the gel.
6. Carefully pick up membrane at edges with two clean forceps. Slightly bend membrane in middle and slowly wet (from the middle out) in DNA Denaturation Solution contained in tray from step 4.
7. Release the membrane and gently submerge it for 5 minutes until it is thoroughly saturated with DNA Denaturation Solution.
    - Use forceps to remove saturated membrane from DNA Denaturation Solution and place it on top of inverted agarose gel.
8. Trim the blotting filter paper to the same size as the gel and the membrane. Place the filter paper on top of the membrane.
    - Roll a 5 or 10 ml pipet across filter paper to remove air bubbles.
    - Carefully place a stack of paper towels approximately 4 - 5 cm thick on top of the filter paper.
    - Place an empty tray on top of the paper towels. Put an empty 400 ml beaker inside the tray for weight.
9. Allow the blot transfer to progress 3-4 hours or overnight.

Areas of the membrane touched by ungloved hands will leave oil residues and will not bind DNA during transfer. Many gloves contain powder, which will increase the background on the membrane. Put on gloves and wash them under tap water to remove any residual powder.

After incubating for 3-4 hours (or overnight):

10. Remove the tray, beaker, and all the paper towels.
11. Wearing rinsed gloves and using forceps, flip the stack (gel - nylon membrane - filter paper) over to lie on the filter paper.

Unit II: Southern Blot Analysis

12. Using a blue ink pen, draw through the six sample wells and trace their positions on the nylon membrane.

13. Using forceps, remove the gel from the membrane.

    Note thickness and consistency of the now dehydrated gel. The gel can be discarded since all further processing takes place with the nylon membrane.

14. Lay the membrane on a dry paper towel with the DNA side up (the side which was in direct contact with the gel).

15. Using a blue ink pen, label the DNA side of the membrane with your lab group number or initials.

16. For optimal results, completely dry and fix DNA to the membrane:
    - Place the membrane between two small sheets of filter paper.
    - Place into an 80°C oven for 30 minutes.

OPTIONAL STOPPING POINT:

The baked membrane can be stored in a dry area between two sheets of filter paper and stained at a later time.

Unit III: Non-Isotopic Detection of DNA

During this procedure you will process and visualize the DNA on the membrane. Blue Blot DNA Stain™ is a non-isotopic reagent, developed by EDVOTEK for classroom use, that eliminates all the associated hazards of working with radioactive isotopes or chemicals used in non-isotopic labeling.

1. Place the membrane with the DNA side up in 60 ml of dilute BlueBlot™ solution. If necessary, use a ziploc bag to completely submerge the membrane.
2. Soak the membrane at room temperature for 10 to 15 minutes.
3. Remove the membrane with forceps and rinse in 200 ml of distilled water.
4. Replenish the distilled water 3 to 4 times, or until the membrane is destained and DNA bands are clearly visible.

The dried membrane can be stored at room temperature, away from moisture, and between two sheets of Whatman filter paper until you are ready to continue with the non-isotopic detection procedure.

Study Questions

Observe and record the results of your experiment in your laboratory notebook or on a separate worksheet. Continue with answering the following study questions:

1. Why do different individuals such as siblings have different restriction enzyme recognition sites?
2. What is the function of probes in DNA paternity analysis?
3. Why is there more than one single locus probe used in an actual paternity DNA test?
4. Why is a Southern Blot Analysis required for forensic and paternity DNA fingerprinting testing?
5. Why do we not use probes in this DNA paternity simulation and still obtain results?

Instructor's Guide

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in planning and implementing this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances. If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK web site. Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

EDUCATIONAL RESOURCES, NATIONAL CONTENT AND SKILL STANDARDS

By performing this experiment, students will learn to load samples and run agarose gel electrophoresis. Experiment analysis will provide students the means to transform an abstract concept into a concrete explanation.

EDVOTEK Ready-to-Load Electrophoresis Experiments are easy to perform and are designed for maximum success in the classroom setting. However, even the most experienced students and teachers occasionally encounter experimental problems or difficulties. EDVOTEK web site resources provide suggestions and valuable hints for conducting electrophoresis, as well as answers to frequently asked electrophoresis questions.

Laboratory Extensions and Supplemental Activities

Laboratory extensions are easy to perform using EDVOTEK experiment kits. For example, a DNA sizing determination activity can be performed on any electrophoresis gel result containing DNA markers run in parallel with other DNA samples. For DNA Sizing instructions, and other laboratory extension suggestions, please refer to the EDVOTEK website.

Notes to the Instructor & Pre-Lab Preparations

Store DNAs (A - E) in the freezer. Store all other components at room temperature.

Each standard Experiment # 207 contains biologicals and reagents for five groups. The experimental procedures consist of three major parts:

1. Agarose gel electrophoresis
2. Southern Blot Transfer
3. Non-isotopic detection of DNA.

Because the electrophoresis time for this experiment is relatively short, Gemini split trays (Cat. # 535), or trays approximately 7 cm in length are recommended for this experiment.

The Southern Blot Transfer procedure should be set up immediately following electrophoresis. Reagents for five blots are included in this experiment. Prepare only the amounts required for the number of blots to be performed.

APPROXIMATE TIME REQUIREMENTS

- For this experiment, the approximate time for electrophoresis is approximately 60 minutes at 75 volts or 2 hours at 50 volts. Electrophoresis should be stopped when the orange tracking dye has migrated approximately 4.5 cm from the sample wells.
- Prelab preparation of biologicals and reagents for Southern Blot Transfer can be done either the day before, or on the day of the lab. Reagent preparations take approximately one-half hour.
- Approximately 90 minutes is required for students to prepare the Southern Blot Transfer procedure, which can progress overnight. The following day the blot is disassembled and baked for 30 minutes in an oven.
- The non-isotopic detection of DNA will require approximately 20 to 25 minutes for staining and destaining.
- The approximate time for electrophoresis will vary from 30 minutes to 2 hours.

Quick Reference:

DNA Samples for Southern Blot

A Standard DNA fragments
B Mother DNA cut with Enzyme
C Child DNA cut with Enzyme
D Father 1 DNA cut with Enzyme
E Father 2 DNA cut with Enzyme

Pre-Lab Preparations

GENERAL PREPARATIONS

1. For each gel to be processed for Southern blotting, gather the following items:

   1 piece of pre-cut nylon membrane (7 x 7 cm)
   1 piece of pre-cut blotting filter paper (7 x 7 cm)
   20 paper towels (should be large enough to cover the gel)

2. On day two of the Southern Blot transfer, set up an 80°C oven.

PREPARATION OF SOLUTIONS FOR SOUTHERN BLOT TRANSFER (FOR 5 BLOTS)

3. Prepare 1.0 liter of approximately 0.25 N HCl. Mix together:

21	ml	Concentrated HCl (12 N)
979	ml	Distilled/deionized water

4. Prepare 2.0 liters of the alkaline/salt DNA denaturation solution, 0.5 M NaOH/0.6 M NaCl.

   Add NaOH and NaCl to the water. Use a magnetic stir plate to dissolve. Add distilled water to a final volume of 2.0 liters.

1.8	L	Distilled or deionized water
40.0	g	NaOH pellets
70.0	g	NaCl

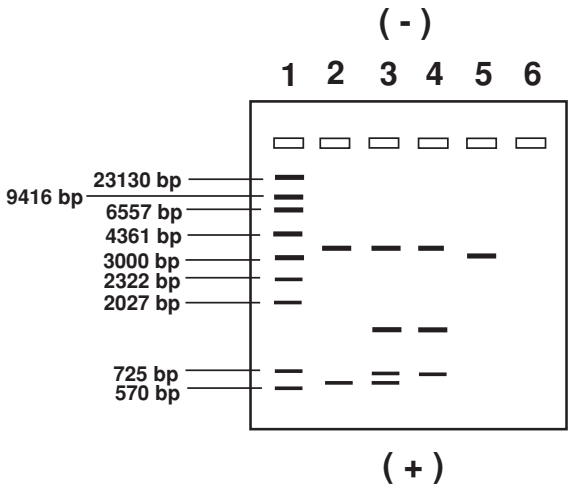
PREPARATION OF BLUE-BLOT™ DNA STAINING SOLUTION

5. On the day the blots are to be stained for visualization, prepare Blue-Blot™ DNA stain by mixing the following:

30 ml	Blue-Blot™ Stain (10x concentrate)
270 ml	Distilled or deionized water

Expected Results

Actual results will yield broader bands of varying intensities. The idealized schematic at left shows the approximate relative positions of the bands, but the results are not depicted to scale. Some of the smaller bands may not be visible.



Lane 1	A	Standard DNA fragments
Lane 2	B	Mother DNA cut with Enzyme
Lane 3	C	Child DNA cut with Enzyme
Lane 4	D	Father 1 DNA cut with Enzyme
Lane 5	E	Father 2 DNA cut with Enzyme

The sizes of the Standard DNA fragments in base pairs, from largest to smallest are:

23130 9416 6557 4361 3000
2322 2027 725* 570*

The largest fragments migrate the slowest, the smallest migrate the fastest.

*These fragments are not always visible due to their small size.

Please refer to the kit insert for the Answers to Study Questions

Appendix **A**

0.8% Agarose Gel Electrophoresis Reference Tables for Southern Blot Analysis

If preparing a 0.8% gel with concentrated (50x) buffer, use Table A.1

If preparing a 0.8% gel with diluted (1x) buffer, use Table A.2

Table A.1 Individual 0.8%* UltraSpec-Agarose™ Gel

Size of Gel (cm)	Amt of Agarose (g)	+ Concentrated Buffer (50x) (ml)	+ Distilled Water (ml)	= Total Volume (ml)
7 x 7	0.23	0.6	29.4	30
7 x 14	0.46	1.2	58.8	60

Table A.2 Individual 0.8%* UltraSpec-Agarose™ Gel

Size of Gel (cm)	Amt of Agarose (g)	+ Diluted Buffer (1x) (ml)
7 x 7	0.23	30
7 x 14	0.46	60

* 0.77 UltraSpec-Agarose™ gel percentage rounded up to 0.8%

Table B Electrophoresis (Chamber) Buffer

EDVOTEK Model #	Total Volume Required (ml)	Dilution: 50x Conc. Buffer (ml)	+ Distilled Water (ml)
M6+	300	6	294
M12	400	8	392
M36	1000	20	980

For DNA analysis, the recommended electrophoresis buffer is Tris-acetate-EDTA, pH 7.8. The formula for diluting EDVOTEK (50x) concentrated buffer is one volume of buffer concentrate to every 49 volumes of distilled or deionized water. Prepare buffer as required for your electrophoresis unit.

Time and Voltage recommendations for EDVOTEK equipment are outlined in Table C.1 for 0.8% agarose gels. The time for electrophoresis will vary from approximately 15 minutes to 2 hours depending upon various factors. Conduct the electrophoresis for the length of time determined by your instructor.

Table C.1 Time and Voltage Guidelines (0.8% Gel)

	EDVOTEK Electrophoresis Model M6+	EDVOTEK Electrophoresis Model M12 & M36
Volts	Minimum / Maximum	Minimum / Maximum
150	15 / 20 min	25 / 35 min
125	20 / 30 min	35 / 45 min
70	35 / 45 min	60 / 90 min
50	50 / 80 min	95 / 130 min

Quantity Preparations for Agarose Gel Electrophoresis

Appendix B

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities for sharing by the class. Unused diluted buffer can be used at a later time and solidified agarose gel solution can be remelted.

Bulk Electrophoresis Buffer

Quantity (bulk) preparation for 3 liters of 1x electrophoresis buffer is outlined in Table D.

Table D

Bulk Preparation of Electrophoresis Buffer

Concentrated Buffer (50x) (ml)	+	Distilled Water (ml)	=	Total Volume (ml)
60		2,940		3000 (3 L)

Batch Agarose Gels (0.8%)

For quantity (batch) preparation of 0.8% agarose gels, see Table E.1.

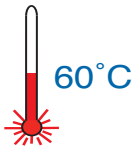
Table E.1

Batch Preparation of 0.8% UltraSpec-Agarose™

Amt of Agarose (g)	+	Concentrated Buffer (50X) (ml)	+	Distilled Water (ml)	=	Total Volume (ml)
3.0		7.5		382.5		390

Note: The UltraSpec-Agarose™ kit component is often labeled with the amount it contains. In many cases, the entire contents of the bottle is 3.0 grams. Please read the label carefully. If the amount of agarose is not specified or if the bottle's plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.

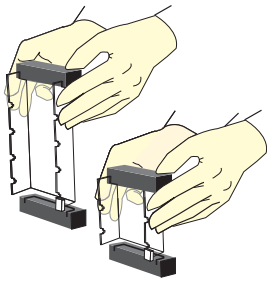
1. Use a 500 ml flask to prepare the diluted gel buffer
2. Pour 3.0 grams of UltraSpec-Agarose™ into the prepared buffer. Swirl to disperse clumps.
3. With a marking pen, indicate the level of solution volume on the outside of the flask.
4. Heat the agarose solution as outlined previously for individual gel preparation. The heating time will require adjustment due to the larger total volume of gel buffer solution.
5. Cool the agarose solution to 60°C with swirling to promote even dissipation of heat. If evaporation has occurred, add distilled water to bring the solution up to the original volume as marked on the flask in step 3.



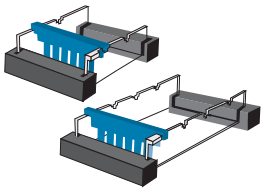
6. Dispense the required volume of cooled agarose solution for casting each gel. The volume required is dependent upon the size of the gel bed and DNA staining method which will be used. Refer to Appendix A or B for guidelines.
7. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Then proceed with preparing the gel for electrophoresis.

Appendix C

Agarose Gel Preparation Step by Step Guidelines



EDVOTEK agarose gel electrophoresis units include 7 x 7 cm or 7 x 14 cm casting trays.



Preparing the Gel bed

1. Close off the open ends of a clean and dry gel bed (casting tray) by using rubber dams or tape.

   A. Using Rubber dams:

   - Place a rubber dam on each end of the bed. Make sure the rubber dam fits firmly in contact with the sides and bottom of the bed.

   B. Taping with labeling or masking tape:

   - Extend 3/4 inch wide tape over the sides and bottom edge of the bed.
   - Fold the extended tape edges back onto the sides and bottom. Press contact points firmly to form a good seal.

2. Place a well-former template (comb) in the first set of notches at the end of the bed. Make sure the comb sits firmly and evenly across the bed.

If gel trays and rubber end caps are new, they may be somewhat difficult to assemble. Here is a helpful hint:

Place one of the black end caps with the wide "u" shaped slot facing up on the lab bench.

Push one of the corners of the gel tray into one of the ends of the black cap. Press down on the tray at an angle, working from one end to the other until the end of the tray completely fits into the black cap. Repeat the process with the other end of the gel tray and the other black end cap.

Casting Agarose Gels

3. Use a 250 ml flask or beaker to prepare the gel solution.

4. Refer to the appropriate Reference Table (i.e. 0.8%, 1.0% or 2.0%) for agarose gel preparation. Add the specified amount of agarose powder and buffer. Swirl the mixture to disperse clumps of agarose powder.

5. With a lab marking pen, indicate the level of the solution volume on the outside of the flask.

6. Heat the mixture to dissolve the agarose powder.

   A. Microwave method:

   - Cover the flask with plastic wrap to minimize evaporation.
   - Heat the mixture on High for 1 minute.
   - Swirl the mixture and heat on High in bursts of 25 seconds until all the agarose is completely dissolved.

   At high altitudes, use a microwave oven to reach boiling temperatures.

   B. Hot plate method:

   - Cover the flask with aluminum foil to minimize evaporation.
   - Heat the mixture to boiling over a burner with occasional swirling. Boil until all the agarose is completely dissolved.

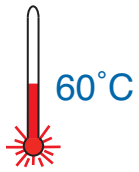
   Continue heating until the final solution appears clear (like water) without any undissolved particles. Check the solution carefully. If you see "crystal" particles, the agarose is not completely dissolved.

Appendix C

Agarose Gel Preparation
Step by Step Guidelines, continued

7. Cool the agarose solution to 60°C with careful swirling to promote even dissipation of heat. If detectable evaporation has occurred, add distilled water to bring the solution up to the original volume marked in step 5.

DO NOT POUR BOILING HOT AGAROSE INTO THE GEL BED.

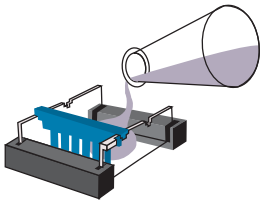


Hot agarose solution may irreversibly warp the bed.

After the gel is cooled to 60°C:

- **If you are using rubber dams, go to step 9.**
- **If you are using tape, continue with step 8.**

8. Seal the interface of the gel bed and tape to prevent agarose solution from leaking.
   - Use a transfer pipet to deposit a small amount of the cooled agarose to both inside ends of the bed.
   - Wait approximately 1 minute for the agarose to solidify.



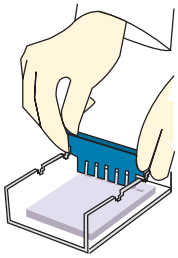
9. Place the bed on a level surface and pour the cooled agarose solution into the bed.

10. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes.

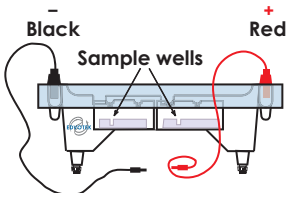
Preparing the gel for electrophoresis

11. After the gel is completely solidified, carefully and slowly remove the rubber dams or tape from the gel bed. Be especially careful not to damage or tear the gel wells when removing the rubber dams. A thin plastic knife, spatula or pipet tip can be inserted between the gel and the dams to break possible surface tension.

12. Remove the comb by slowly pulling straight up. Do this carefully and evenly to prevent tearing the sample wells.



13. Place the gel (on its bed) into the electrophoresis chamber, properly oriented, centered and level on the platform.



During electrophoresis, the DNA samples migrate through the agarose gel towards the positive electrode.

14. Fill the electrophoresis apparatus chamber with the appropriate amount of diluted (1x) electrophoresis buffer (refer to Table B on the Appendix page provided by your instructor).

15. Make sure that the gel is completely submerged under buffer before proceeding to loading the samples and conducting electrophoresis.