EDVOTEK®

THE **BIOTECHNOLOGY**
EDUCATION COMPANY®

Edvo-Kit #
311

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DNA Fingerprinting with Southern Blot Analysis

Experiment Objective:

In this advanced experiment, students will perform a Southern blot for a contested paternity case. Southern blotting is an important technique that is useful to both clinicians and researchers. Students will understand DNA fingerprinting, restriction enzymes, and Southern blots.

See page 3 for storage instructions.

Table of Contents

Safety Data Sheets can be found on our website:
https://www.edvotek.com/safety-data-sheets

Experiment Components

Component		Storage	Check ✓
Biotin Labeled DNA Samples for Electrophoresis			
A	Standard DNA Fragments	-20 °C Freezer	❑
B	Mother's DNA cut with Enzyme 1	-20 °C Freezer	❑
C	Mother's DNA cut with Enzyme 2	-20 °C Freezer	❑
D	Child's DNA cut with Enzyme 1	-20 °C Freezer	❑
E	Child's DNA cut with Enzyme 2	-20 °C Freezer	❑
F	Father 1's DNA cut with Enzyme 1	-20 °C Freezer	❑
G	Father 1's DNA cut with Enzyme 2	-20 °C Freezer	❑
H	Father 2's DNA cut with Enzyme 1	-20 °C Freezer	❑
I	Father 2's DNA cut with Enzyme 2	-20 °C Freezer	❑
Components for Membrane Transfer			
•	Pre-cut Nylon Membranes (7 x 14 cm)	Room Temp.	❑
•	Pre-cut Filter Papers (7 x 14 cm)	Room Temp.	❑
Components for Detection			
J	Detection Buffer, 20x concentrated	Refrigerator	❑
K	Shielding Buffer	Refrigerator	❑
L	Termination Buffer, 20x concentrated	Refrigerator	❑
M	NBT/BCIP Tablets	-20 °C Freezer	❑
N	Streptavidin-Alkaline Phosphatase, SAAP	-20 °C Freezer	❑
Other Reagents & Supplies			
•	UltraSpec-Agarose™ Powder	Room Temp.	❑
•	Concentrated Electrophoresis Buffer	Room Temp.	❑
•	Practice Gel Loading Solution	Room Temp.	❑

This experiment contains enough reagents for 5 lab groups.

All experiment 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.

Experiment Requirements

- Concentrated HCl (12 N)
- NaCl
- NaOH
- Distilled Water
- Horizontal Gel Electrophoresis Apparatus (Cat. #502 recommended)
- D.C. Power Supply
- Microwave or Hot Plates
- Balance
- Incubator Oven(s)
- Automatic Pipettes with Tips
- 10 mL and 100 mL Graduated Cylinders
- 50 mL, 250 mL, and 500 mL Flasks or Beakers
- Small Trays with Lids (non-acrylic and 7x14 cm or larger)
- Forceps
- Scissors
- Plastic Wrap
- Foil
- Tape
- Several Packs of Paper Towels
- Filter Paper (Optional)
- Latex or Vinyl Lab Gloves
- Safety Goggles

Background Information

The human genome is 99.9% identical between individuals. However, there are regions of variation within our genome called polymorphisms. Each of us inherits a unique combination of polymorphisms from our parents. By focusing on several polymorphic regions, scientist can uncover a person's "DNA fingerprint". Like a regular fingerprint, a DNA fingerprint can be used to identify and distinguish between individuals. Because polymorphisms are inherited, DNA fingerprints can also be used to determine family relationships.

DNA fingerprinting was first used as a forensic tool in the United Kingdom, following the pioneering work of Dr. Alex Jeffreys. Fingerprint analysis by Jeffreys led to the apprehension of a murderer in the first forensic DNA case in 1987. Since then, fingerprinting has been used in thousands of convictions. Additionally, hundreds of convicted prison inmates have been exonerated from their crimes. DNA fingerprinting is also used to determine parentage, identify missing people, identify human remains, and diagnose certain inherited disorders.

Today there are many ways to observe similarities and differences in people's DNA. DNA fingerprinting is a general term that applies to them all. Three of the most popular technologies are sequencing, polymorphic chain reactions (PCR), and Southern blots. In this experiment, you will perform the key steps of a Southern blot in order to visualize DNA collect from a mother, child, and two possible fathers. By creating and comparing the DNA fingerprints of these four individuals you will help resolve this simulated paternity case.

SOUTHERN BLOTS

Southern blots are a popular way to search an individual's genome for specific DNA sequences of interest and then determine the size of these sequences and part of their nucleotide makeup. The method's name comes from its inventor (Dr. Edward Southern) and from one of the procedure's key steps (transferring or "blotting" DNA from an agarose gel to a nylon membrane). This procedure is advanced and demanding - it uses several different biotechnologies, involves multiple steps, and can take up to 18 hours to complete! However, its accuracy, specificity, and accessibility make it a valuable genetics tool.

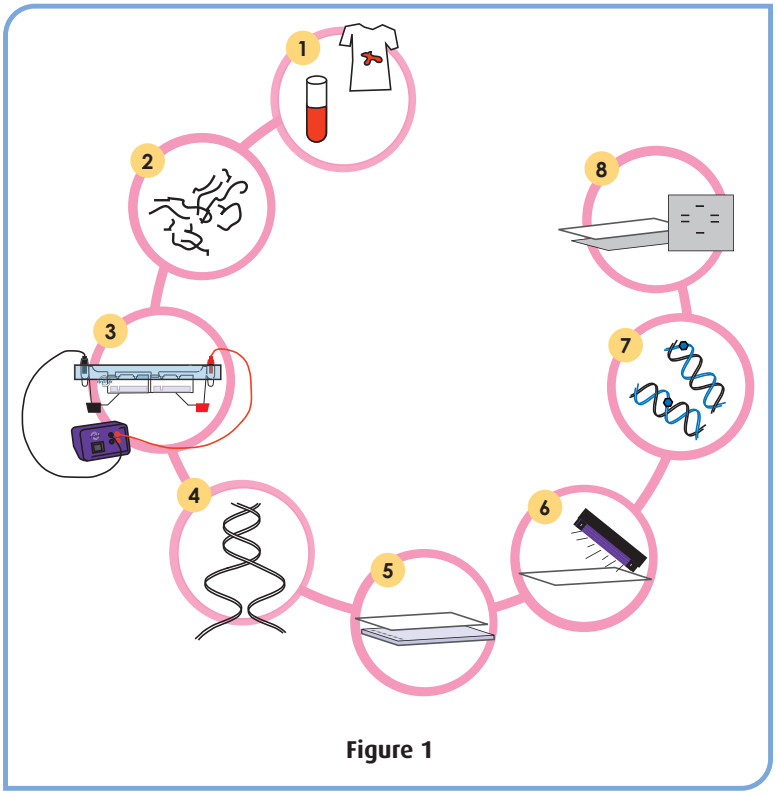


Figure 1

During a Southern blot, an individual's DNA is manipulated and modified in several ways so that differences in DNA can be observed by the human eye (Figure 1). These manipulations and modifications are described below:

1. **Collect and Extract:** In a forensic setting, blood or other tissue samples are collected from a crime scene or victim

and cell samples are collected from potential suspects. For paternity determinations, cell samples are obtained from the mother, child, and possible fathers. These samples are then treated with a reagent mixture that contains a detergent to rupture the cell membranes and obtain the DNA for further analysis.

2. **Digest:** Long strands of DNA are broken into smaller fragments using proteins called restriction enzymes that act like molecular scissors. These enzymes - and the essential role that they plan in fingerprinting - are described in detail in the next section.

3. **Separate:** The mixture of digested DNA is separated by size using agarose gel electrophoresis. The DNA is added into depressions within a gel and an electrical current is then passed through the gel. Because DNA has a strong negative charge, the current drives the restriction fragments through the gel towards the positive electrode. Small DNA fragments move through the gel easily, but large DNA fragments have a more difficult time. This causes differently sized fragments to separate and form discrete "bands" within the gel.

4. **Denature:** The gel is first submerged in an acid solution (often HCl). This acid treatment "nicks" the DNA molecules that are in the gel by removing certain nucleotide bases called purines. Next, the gel is submerged in a base solution (often NaOH). This base treatment disrupts the bonds between all the base pairs causing the double-stranded DNA to divide into single-stranded halves. Timing is key during this step. Overexposure – especially to the acid – can cause the DNA molecules to break down into very short pieces that are much more difficult to visualize.

5. **Transfer:** DNA is transferred from the gel to a nylon membrane using a small electric current or using capillary action. This experiment will use capillary action by creating a stack comprised of the gel, a nylon membrane, dry filter paper, and paper towels which causes the buffer to flow from the gel - a region of high water potential - to the paper towels - a region of low water potential. The DNA follows the current but then gets stuck on the membrane whose minor positive charge serves to both attract and capture negatively charged molecules like DNA.

6. **Fix:** The DNA is securely attached to the membrane in the same pattern that it was on the gel. This is done by exposing the membrane to UV rays, incubating it in a vacuum, or baking it for 2 hours at 80°C.

7. **Hybridize:** The membrane is incubated in a solution containing small fragments of single-stranded DNA called probes. Probes have a very specific sequence of nucleotides, which is chosen by the experimenter, as well as an attached color changing, fluorescent or radioactive molecule that serves as a label. During incubation probes bind to any DNA molecules on the membrane that have complementary - or close to complementary - sequences. This "DNA-to-DNA" binding, called hybridization, is strong. It's also very selective. Only a small fraction of the hundreds of thousands of differently sized DNA fragments fixed on the membrane will contain a complementary sequence. Consequently, when the membrane is then washed only a few bands of hybridized probes stay attached to the membrane.

8. **Visualize:** Finally, the membrane is treated to highlight the probe labeled DNA bands. As the majority of Southern blot probes are radioactively labeled this is usually done using x-ray film. However, it can also be accomplished by exposing the membrane to UV light (in cases where fluorescently labeled probes are used) or by soaking the membrane in color-changing solutions (in cases where chromogenically labeled probes are used). Scientists can then determine key parts of an individual's genotype based on which bands appear and their size.

RESTRICTION ENZYMES

One of the most significant discoveries of molecular biology is a class of enzymes known as restriction endonucleases. These endonucleases (also known as restriction enzymes) are produced by many species of bacteria to protect themselves from invading viral DNA. Restriction enzymes act like molecular scissors, cutting double-stranded

DNA at specific sequences. The utility of restriction enzymes has made molecular cloning, DNA mapping, sequencing and various genome-wide studies possible, launching the era of biotechnology.

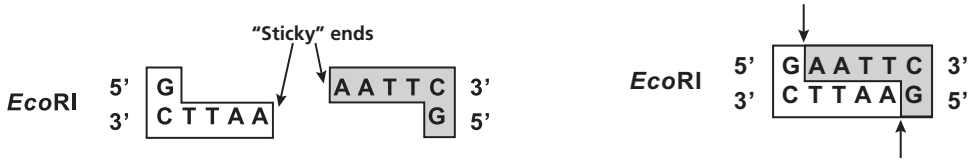
Since they were first discovered in the 1970s, over 3,000 restriction enzymes have been identified, each one given a unique acronym describing the organism from which it was first isolated. The first letter of the acronym is the first letter of the genus, the next two letters are the first two letters of the species name of the organism, and additionally, letters and numerals indicate specific strains and order of discovery. For example, *Eco*RI was the first restriction enzyme isolated from the RY13 strain of the bacterium *Escherchia coli*. (More examples are shown in Table 1.)

Table 1: Common Restriction Enzymes

Restriction Enzyme	Organism	Species	Strain	Recognition Site
*Ava*I	*Anabaena*	*variabilis*	N/A	C^YCGUG
*Bgl*I	*Bactillus*	*globigii*	N/A	GCCNNNN^NGGC
*Eco*RI	*Escherichia*	*coli*	RY13	G^AATTC
*Hae*III	*Haemophilus*	*aegyptius*	N/A	GG^CC
*Hin*dIII	*Haemophilus*	*influenzae*	R_4	A^AGCTT
*Sac*I	*Streptomyces*	*achromogenes*	N/A	GAGCT^C

Many restriction enzymes require Mg2+ for activity and recognize palindromic stretches of DNA, generally 4-8 base pairs in length. The probability that a given enzyme will cut, or "digest", a piece of DNA is directly proportional to the length of its recognition site. Statistically, an enzyme will average one cut for every 4^n base pairs, where n is the length of the recognition site. For instance, an enzyme that recognizes a four base pairs long sequence (e.g., *Hae*III) will cut DNA once every 256 (or 4^4) base pairs, while an enzyme that recognizes a six base pairs long site (e.g., *Eco*RI) will cut once every 4096 (or 4^6) base pairs. Therefore, the longer a DNA molecule is, the greater the probability is that it contains one or more restriction sites. For example, if *Eco*RI is used to digest human chromosomal DNA containing 3 billion base pairs and a plasmid containing 5,000 base pairs, it will cut the chromosomal DNA over 700,000 times (3 billion base pairs, cut every 4096 base pairs), but may only cut the plasmid once (5,000 base pairs, cut every 4096 base pairs).

Digestion by a restriction enzyme generates DNA fragments with one of two types of DNA ends - "sticky" or "blunt". To illustrate this, first consider the recognition site and cleavage pattern of *Eco*RI.



*Eco*RI cleaves between the G and neighboring A, as indicated by the arrows in the left side of the figure. It is important to note that the positions of the cleavage are staggered, so the resulting fragments project short overhangs of single-stranded DNA with complementary sequences. Such overhangs are referred to as "sticky" ends because the single-strands can interact with—or stick to—other overhangs with a complementary sequence (Figure 2). Digestion of the same piece of DNA using different enzymes can produce sticky ends of different lengths and strand orientation (5′ vs. 3′).

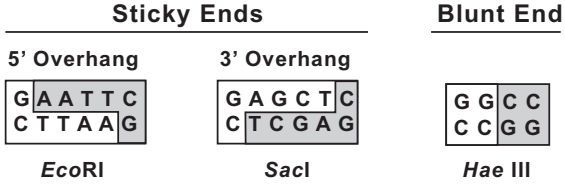
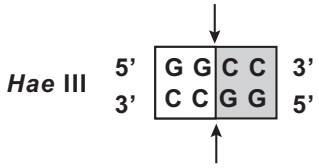
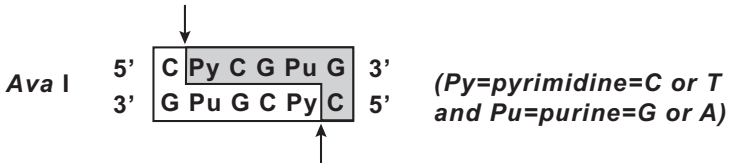


Figure 2: Different types of DNA ends produced by Restriction Enzymes.

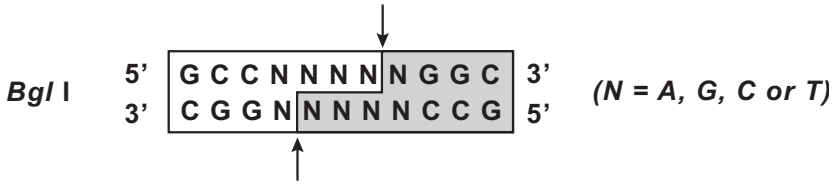
In contrast to *Eco*RI, *Hae*III cuts both DNA strands at the same position, which generates fragments without an overhang. These so-called "blunt" ends can be joined with any other blunt end without regard for complementarity.



Some restriction enzymes, such as *Ava*I, recognize "degenerate" sites, which contain one or more variable positions.



Consequently, there are four possible sites that *Ava*I will recognize and cut: CCCGGG, CCCGAG, CTCGGG and CTCGAG.



There are even enzymes like *Bgl*I that recognize "hyphenated" sites, which are palindromic sequences separated by a number of completely variable bases.

The six G-C base pairs that *Bgl*I specifically recognizes must be separated by five base pairs of DNA; otherwise the enzyme cannot properly interact with the DNA to cleave its backbone. Because these five base pairs are not required to make up a specific sequence, *Bgl*I can recognize and cleave up to 625 possible sequences!

Depending on the distances between recognition sites, digestion of DNA by a restriction enzyme will produce DNA fragments of varying length. These fragments are called RFLPs (Restriction Fragment Length Polymorphisms). To separate these fragments, scientists use agarose gel electrophoresis.

In some cases – such as when a person's entire DNA sample (their genome) is being tested – the restriction enzyme digestion generates so many differently sized fragments that they exceed the resolving capacity of agarose gels. When this happens, the digests appear as a smear after staining and the banding pattern in unreadable. Southern blots overcome this challenge by pairing enzyme digestion with a probe hybridization step that highlights a much smaller subset of RFLPs.

DNA FINGERPRINTING IN PATERNITY CASES

Humans are diploid organisms with 23 pairs of chromosomes. This means that every DNA region comes in two copies - one that was inherited from the individual's mother and one that was inherited from their father. These two copies can be identical (in which case the individual is said to be "homozygous" at that location) or different (in which case the individual is said to be "heterozygous" at that location).

Some polymorphic DNA region have multiple potential copies leading to many possible combinations. Alleles are alternate forms of a gene that are found at the same place on a chromosome. At the molecular level alleles are

created by mutations – base pair deletions, insertions, replacements, or repeats – that modify the nucleotide sequence. Figure 3 is an example of a mutation caused by a single nucleotide substitution of thymine to cytosine. In this case, the mutation is "silent" because the resulting amino acid code does not change the final protein. However, this difference would be identified by digesting the two DNA samples with the enzyme *Eco*RI.

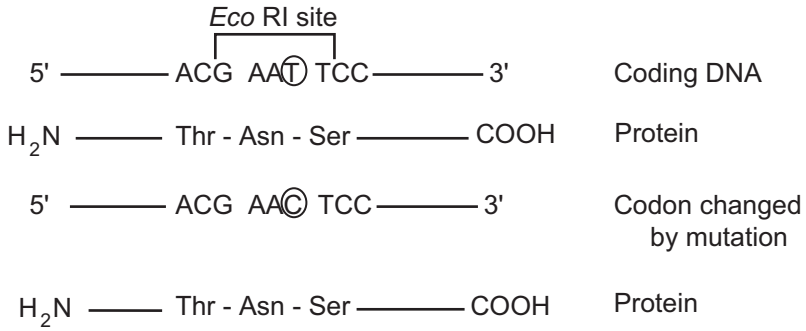


Figure 3: Effect of a point mutation results in an altered amino acid code.

Mutations also occur in noncoding DNA regions. Some of the most variable regions of human DNA are noncoding regions of DNA that contain repeating units of nucleotides. These are called VNTRs (Variable Number of Tandem Repeats) when the nucleotide repeats are 15 to 70 bp long and STR (Short Tandem Repeats) when the nucleotide repeats are 2 to 6 bp long. These polymorphisms can also be detected by restriction enzymes provided that an enzyme recognition site is on either end of the variable region (Figure 4). Although the term allele was originally used to describe genes, it now also refers to this type of non-coding DNA sequence variation.

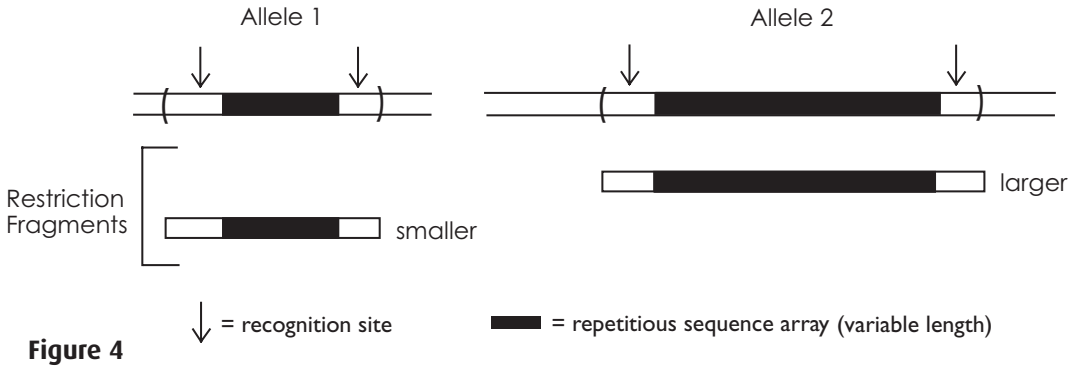


Figure 4

For paternity determinations, DNA is obtained from the mother, child, and possible fathers. A child's DNA is a composite of both parent's DNA. Not all of the bands present in the parent's fingerprint will appear in the child's fingerprint. However, as shown in Figure 5, DNA bands that appear in the child's fingerprint must be found in the fingerprint from either the father or mother. Therefore, a comparison of DNA fragmentation patterns obtained from the mother and child will give a partial match. DNA bands in the child's fingerprint that are not present in the mother's profile must match with those from the biological father.

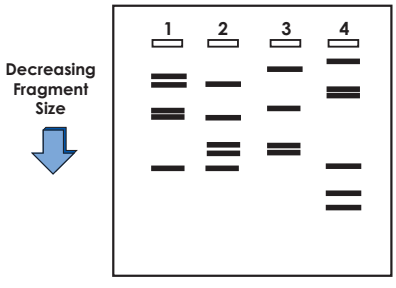


Figure 5

Lane 1 Mother
Lane 2 Child
Lane 3 Father
Lane 4 Unrelated

Experiment Overview

EXPERIMENT OBJECTIVE

In this advanced experiment, students will perform a Southern blot for a contested paternity case. Southern blotting is an important technique that is useful to both clinicians and researchers. Students will understand DNA fingerprinting, restriction enzymes, and Southern blots.

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. Exercise caution when using any electrical equipment in the laboratory.
4. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.


Wear gloves and safety goggles

LABORATORY NOTEBOOKS

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

Before starting the Experiment:

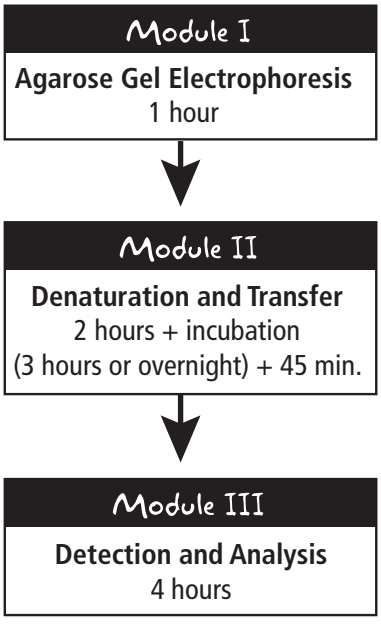
- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

During the Experiment:

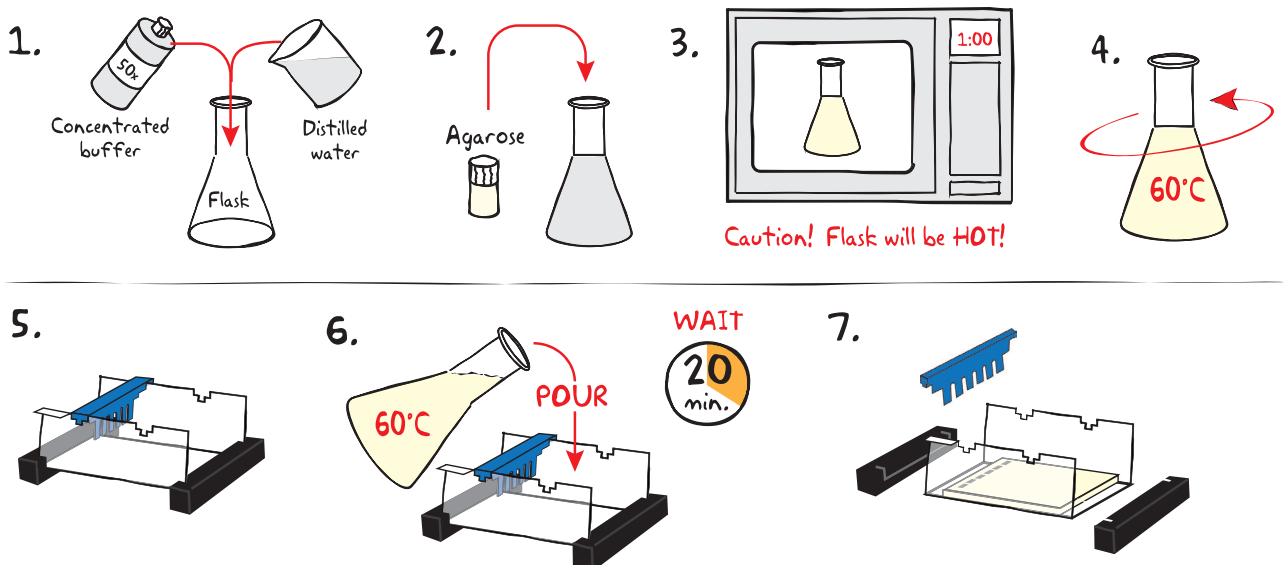
- Record (draw) your observations, or photograph the results.

After 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.



Module I: Agarose Gel Electrophoresis



CASTING THE AGAROSE GEL


Wear gloves and safety goggles

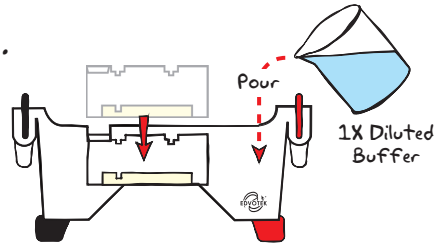
1. **DILUTE** concentrated 50X Electrophoresis buffer with distilled water (Table A).
2. **MIX** agarose powder with buffer solution in a 250 mL flask (Table A).
3. **DISSOLVE** agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
4. **COOL** agarose to 60 °C with careful swirling to promote even dissipation of heat.
5. While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the well template (comb) in the appropriate notch.
6. **POUR** the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
7. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

Table A — Individual 0.8% UltraSpec-Agarose™ Gel

Size of Gel Casting tray	Concentrated Buffer (50x)	+ Distilled Water	+ Amt of Agarose	= TOTAL Volume
7 x 14 cm	1.2 mL	58.8 mL	0.46 g	60 mL

Module I: Agarose Gel Electrophoresis, continued

8.



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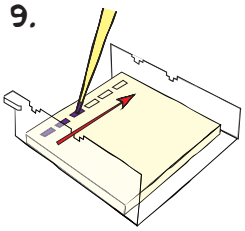
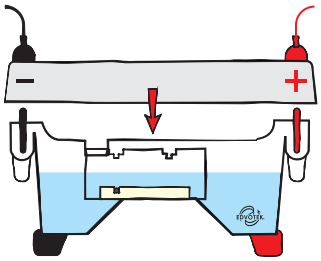
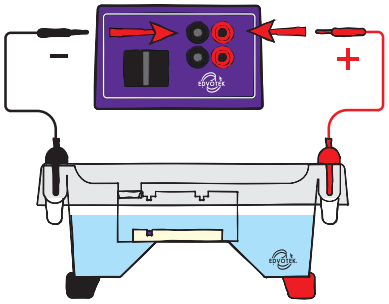


REMINDER:
Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.

10.



11.



In this experiment the DNA samples have been predigested with two different restriction enzymes as well as biotinylated for later detection.

RUNNING THE GEL

8. **PLACE** the gel (still on the tray) into the electrophoresis chamber. **COVER** the gel with 1X Electrophoresis Buffer (See Table B for recommended volumes). The gel should be completely submerged.
9. **LOAD** 35-38 µL of each DNA sample according to Table 1, at right.
10. **PLACE** safety cover on the unit. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
11. **CONNECT** leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines). Allow the tracking dye to migrate at least 3.5 cm from the wells.
12. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber and *proceed immediately* to Module II instructions for **DENATURATION AND TRANSFER**.

Table 1: Gel Loading

Row	Lane	Tube	Sample
1	1	A	Standard DNA fragments
1	2	B	Mother's DNA cut with Enzyme 1
1	3	C	Mother's DNA cut with Enzyme 2
1	4	D	Child's DNA cut with Enzyme 1
1	5	E	Child's DNA cut with Enzyme 2
1	6	-	---
2	1	A	Standard DNA fragments
2	2	F	Father 1's DNA cut with Enzyme 1
2	3	G	Father 1's DNA cut with Enzyme 2
2	4	H	Father 2's DNA cut with Enzyme 1
2	5	I	Father 2's DNA cut with Enzyme 2
2	6	-	---

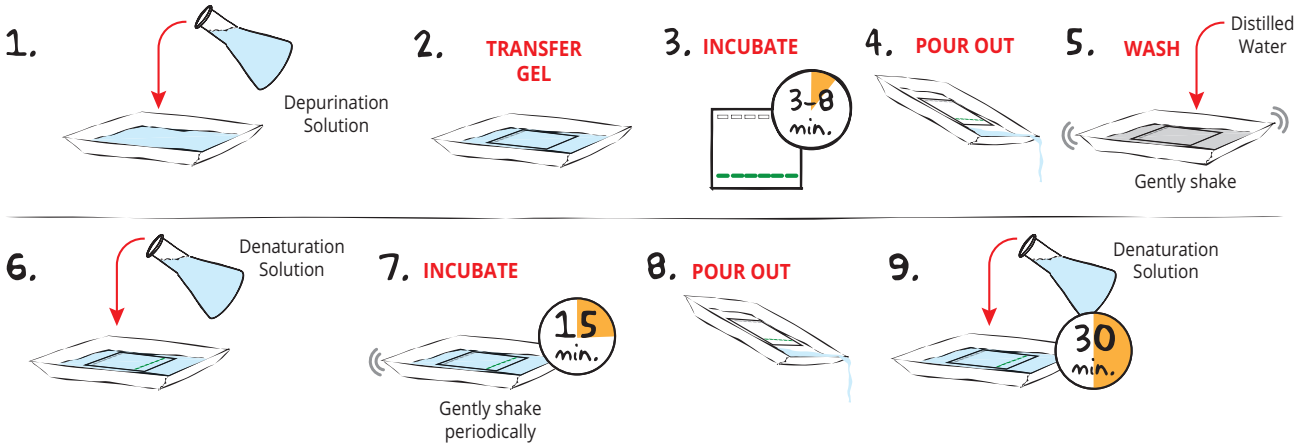
Table B: 1x Electrophoresis Buffer (Chamber Buffer)

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

Table C: Time & Voltage Guidelines (0.8% Agarose Gel)

	Electrophoresis Model		
Volts	M6+ Min. / Max.	M12 (new) Min. / Max.	M12 (classic) & M36 Min. / Max.
150	15/20 min.	20/30 min.	25 / 35 min.
125	20/30 min.	30/35 min.	35 / 45 min.
75	35 / 45 min.	55/70 min.	60 / 90 min.

Module II: Denaturation and Transfer



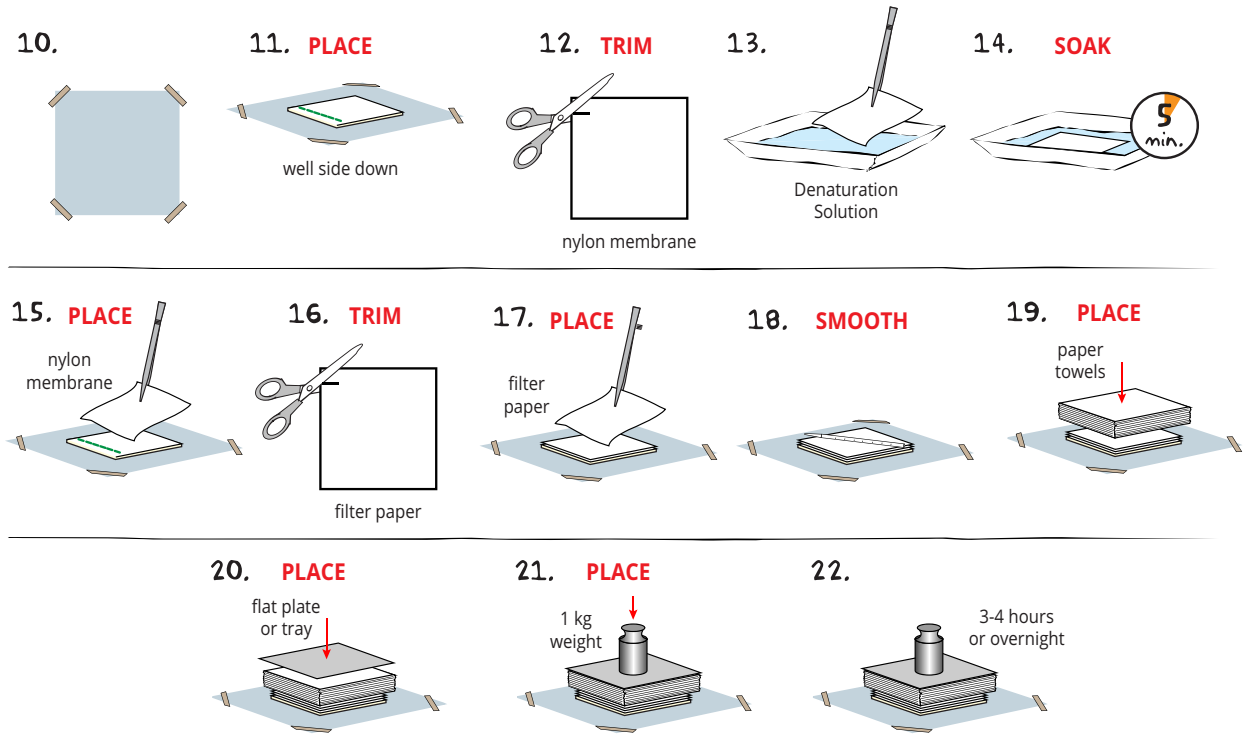
DEPURINATION

1. **ADD** 200 mL of depurination solution (0.25 N HCl) to a plastic tray.
2. **TRANSFER** the gel from Module I to this tray. Make sure the gel is immersed in the solution.
3. **INCUBATE** at room temperature for 3-8 minutes. **STOP** incubation and continue to step 3 *as soon as* the blue tracking dye in the gel changes to a greenish yellow. **WATCH** the time and this color change carefully as over-incubation will fragment the DNA and make later detection difficult.
4. Immediately, **POUR OUT** the depurination solution.
5. **WASH** the gel by adding 200 mL of distilled water to the tray, gently shaking the tray, and then pouring the water out. **REPEAT** this wash 2 times.

DENATURATION

6. **ADD** 200 mL of denaturation solution (0.5 M NaOH/0.6 M NaCl) to the tray.
7. **INCUBATE** at room temperature for 15 minutes. The density of the solution will cause the gel to float, periodically **SHAKE** the tray to keep it immersed.
8. **POUR OUT** the denaturation solution.
9. **ADD** 200 mL of new denaturation solution to the tray and **INCUBATE** at room temperature for 30 minutes. **DO NOT POUR OUT** this second volume of denaturation solution and keep the tray—you will need both for steps 13 and 14.

Module II: Denaturation and Transfer, continued



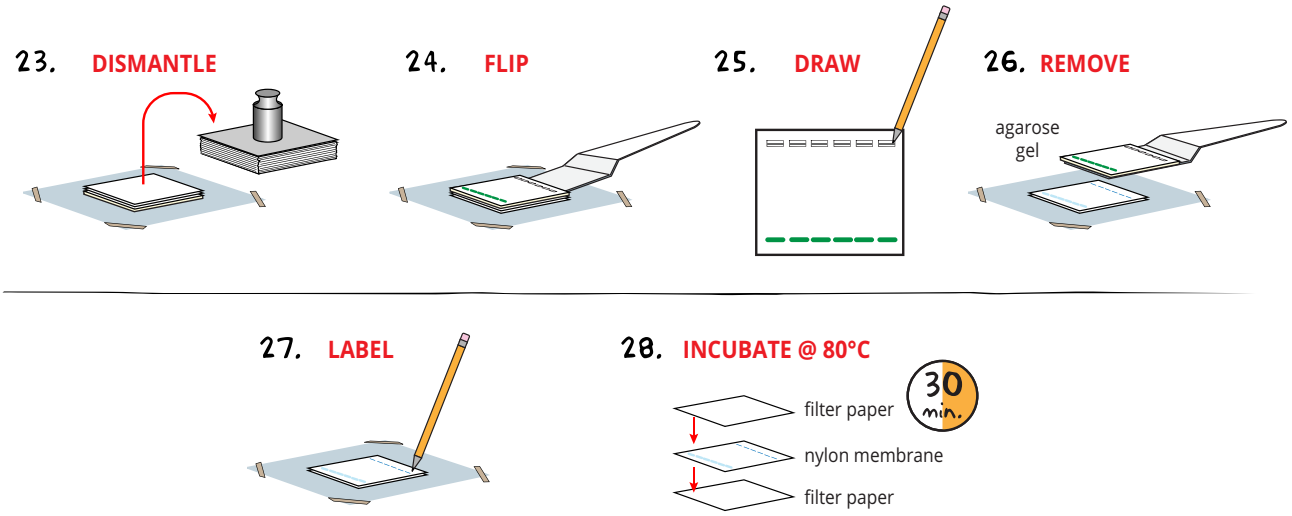
SOUTHERN BLOT TRANSFER

10. **PREPARE** your lab bench by spreading out a small sheet of plastic wrap. To keep the wrap tight and stable, **TAPE** the edges to the bench.
11. **PLACE** your gel well side down onto the plastic wrap so that the exposed (top side) of the gel is smooth.
12. Using forceps and scissors, carefully **TRIM** the nylon membrane to the size of the gel. A membrane that is larger than the gel will compromise the transfer process.
13. Carefully **SLIDE** the membrane out of the blue covers and **TRANSFER** to the denaturation solution from step 9. For best results, **ADD** the membrane to the solution in a U shape so that the middle comes in contact with the solution first. The solution can then slowly spread out to both edges.
14. **SOAK** the membrane for 5 minutes.
15. Using forceps, **REMOVE** the membrane from the tray and **PLACE** it on top of the inverted agarose gel.
16. **TRIM** the filter paper to the same size as the gel and membrane.
17. **PLACE** the filter paper on top of the nylon membrane.
18. **REMOVE** any air bubbles from the top of the gel, membrane, and filter paper using a large pipette tip (5 mL–10 mL) or similar cylindrical object.
19. **PLACE** a 5-6 cm stack of paper towels on top of the filter paper.
20. **PLACE** an empty tray or flat plate on top of the paper towels.
21. **PLACE** a 1 kg weight on top—an empty 500 mL beaker works well.
22. **ALLOW** the transfer to progress for 3-4 hours or overnight.

NOTE:
When handling the nylon membrane, be gentle and try to touch only the outer edges.

Oils from your hands and powders from gloves can interfere with the transfer. For steps 10-22 wear rinsed and dried lab gloves and for steps 12-15 use clean forceps.

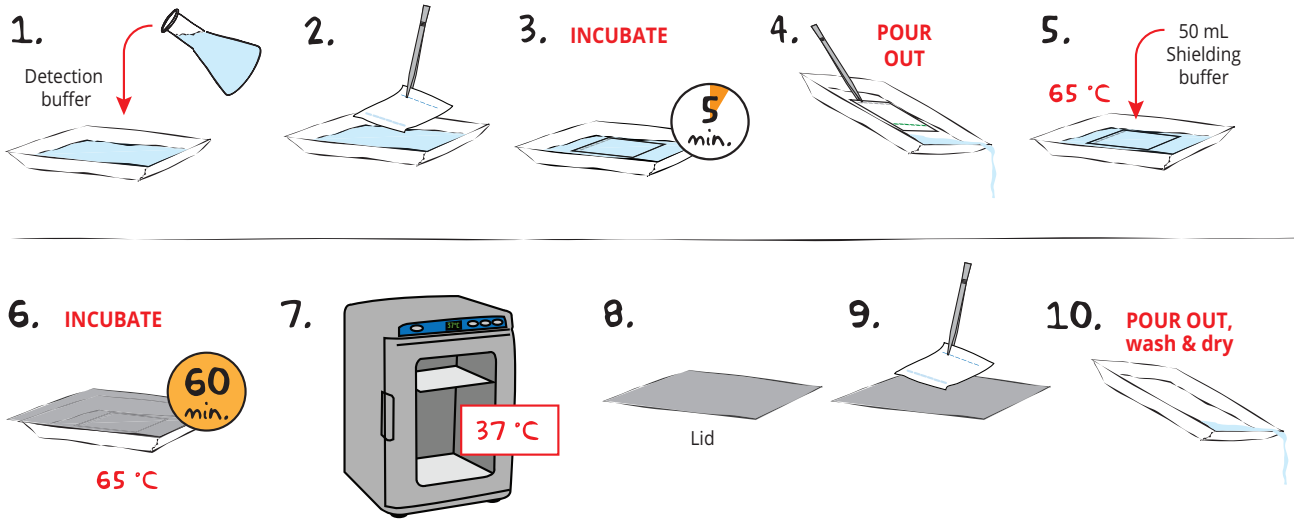
Module II: Denaturation and Transfer, continued



23. **DISMANTLE** the stack above the filter paper by removing the weight, tray, and paper towels.
24. **FLIP** the remaining stack (filter paper-membrane-gel) so that the gel is now facing up. This can be done with a spatula or with forceps and rinsed gloves.
25. Use a pencil to **DRAW** through the six sample wells on the gel and mark their position on the nylon membrane.
26. Carefully **REMOVE** and **DISCARD** the gel with forceps or a spatula.
27. **VERIFY** that the transfer has occurred by the presence of the blue tracking dye on the membrane. Using a pencil, gently **LABEL** this side of the membrane with your group's ID. This is the side that the DNA is on so keep the label small.
28. For best results, **PLACE** the membrane between two sheets of filter paper or paper towels and **INCUBATE** at 80 °C for 30 minutes.

OPTIONAL STOPPING POINT:
Once you have performed the fixation step (Step 28), then the membrane can be stored until you are ready to continue to Module III. Keep it in between the two sheets of filter paper, at room temperature, and away from moisture.

Module III: Detection and Analysis

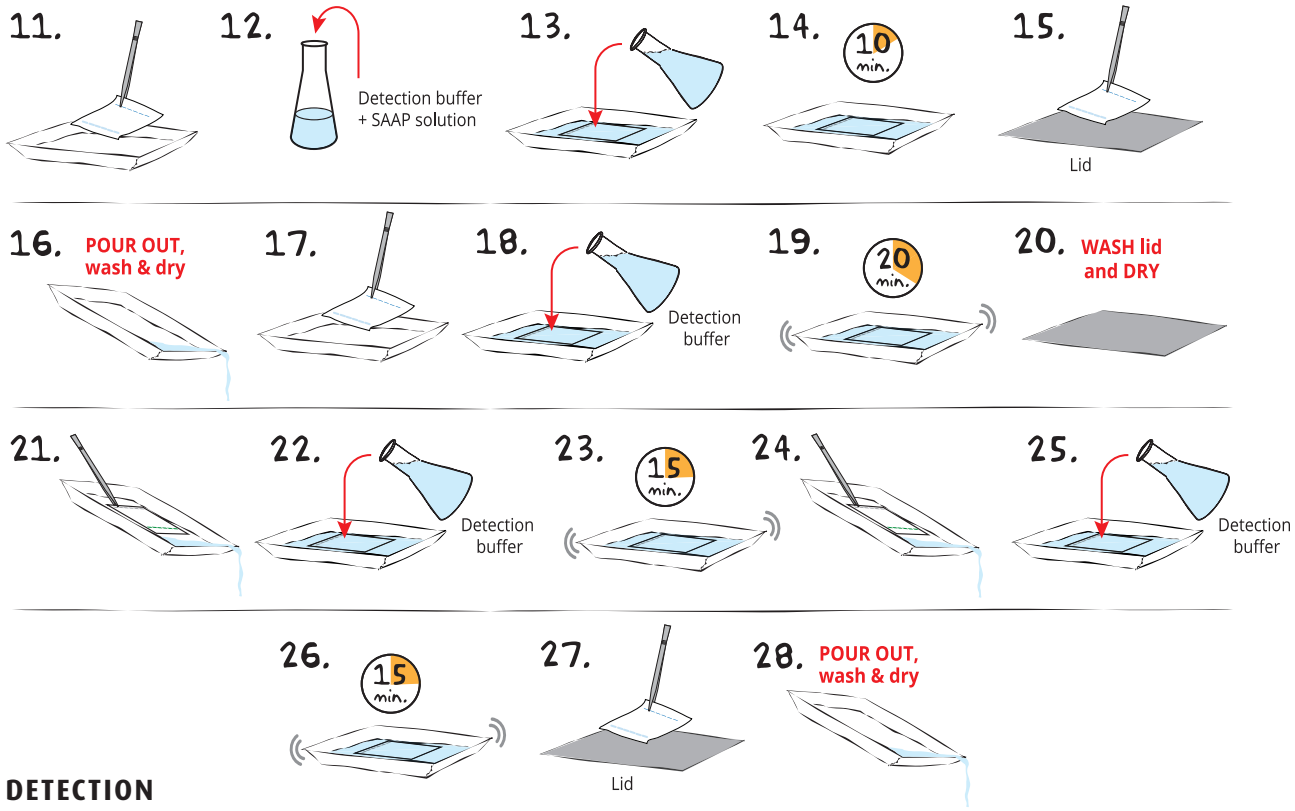


REHYDRATION AND SHIELDING

1. **ADD** 100 mL of detection buffer to a tray.
2. **PLACE** the membrane in the detection buffer (DNA side UP).
3. **INCUBATE** for 5 minutes at room temperature.
4. Use forceps to **HOLD** the membrane and then POUR OUT the detection buffer.
5. Slowly **ADD** 50 mL of warm (65 °C) shielding buffer to the tray so that it gently coats the DNA side of the membrane.
6. **COVER** the tray with a lid and **INCUBATE** for 1 hour at 60-65 °C. Make sure that the temperature ***DOES NOT EXCEED*** 65 °C.
7. **REMOVE** the tray from incubator. **RESET** the incubator's temperature to 37 °C.
8. **REMOVE** the tray's lid and place the lid upside down on your lab bench.
9. Use forceps to **MOVE** the membrane from the tray to the lid.
10. **POUR OUT** the shielding buffer, **WASH** the tray several times with distilled water, and **DRY** the tray by shaking. Do not use paper towels to dry the tray as lint may interfere with later detection.

NOTE:
While resilient, nylon membranes can get scratched. To avoid this: (1) handle the membranes gently, (2) touch only their edges when possible, (3) use only one membrane per tray, and (4) ***keep the DNA side of the membrane facing up*** unless instructed otherwise.

continued



DETECTION

11. Use forceps to **MOVE** the membrane back to the tray.
12. In a 50 mL flask, **COMBINE** 10 mL of detection buffer and 8 µL of concentrated SAAP solution. **MIX**.
13. Slowly **ADD** 10 mL of the diluted SAAP solution to the tray so that it gently coats the DNA side of the membrane.
14. **INCUBATE** for 10 minutes at room temperature. Periodically **ROCK** the tray from side to side to keep the membrane covered in solution.
15. Use forceps to **MOVE** the membrane from the tray to the lid.
16. **POUR OUT** the diluted SAAP solution, **WASH** the tray several with distilled water, and **DRY** the tray by shaking.
17. Use forceps to **MOVE** the membrane back to the tray.

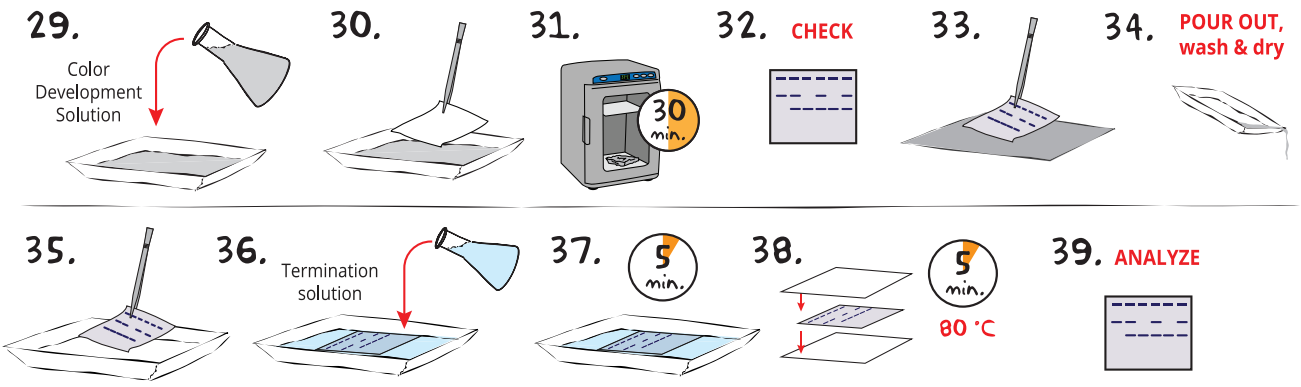
SAAP stands for streptavidin-alkaline phosphatase complex. The streptavidin part of this complex binds to biotinylated DNA during step 14. While the alkaline phosphatase part of this complex reacts with BCIP (5-Bromo-4-chloro-3-indolyl phosphate) in the color development solution to form an insoluble blue precipitate during step 31. This two-step detection system is used in Southern blots that use biotinylated probes to detect specific DNA sequences.

Washing the Membrane

18. **ADD** 400 mL of detection buffer to tray.
19. **INCUBATE** for 20 minutes with occasional rocking. If its available, use a shaking platform.
20. During the incubation (step 19), **WASH** the lid with distilled water and **DRY** by shaking.
21. Use forceps to **HOLD** the membrane and **POUR OUT** the detection buffer.
22. **ADD** 200 mL of new detection buffer to tray.
23. **INCUBATE** for 15 minutes with occasional rocking. If it's available, use a shaking platform.
24. Use forceps to **HOLD** the membrane and **POUR OUT** the detection buffer.
25. **ADD** 200 mL of new detection buffer to tray.
26. **INCUBATE** for 15 minutes with occasional rocking. If it's available, use a shaking platform.
27. Use forceps to **MOVE** the membrane from the tray to the lid.
28. **POUR OUT** the detection buffer, **WASH** the tray several with distilled water, and **DRY** the tray by shaking.

continued

Module III: Detection and Analysis, continued



COLOR DEVELOPMENT

29. **ADD** 8 mL of color development solution to the tray or to a sealable, leak-proof plastic bag.
30. Use forceps to **MOVE** the membrane back to the tray *DNA side down* and cover with the lid. (Alternatively, **PLACE** the membrane into the bag, remove all air pockets, and seal.) Make sure the membrane is in contact with the solution.
31. **INCUBATE** for 30 minutes at 37 °C and *in the dark*. To protect the membrane from light, cover the incubator door with foil or wrap the tray/plastic bag with foil.
32. **CHECK** the membrane. If the DNA bands are visible, continue to step 23. If the DNA bands are not visible, continue incubation for another 30 minutes. ***NOTE: Depending on the transfer an extended incubation time of up to 4 hours may be needed. After 4 hours it is recommended that you proceed to termination to avoid having too much background color.***
33. Use forceps to **MOVE** the membrane from the tray/plastic bag to the lid.
34. **POUR OUT** the color development solution, **WASH** the tray several times with distilled water, and **DRY** the tray by shaking.

TERMINATION

35. Use forceps to **PLACE** the membrane back into the tray DNA side up.
36. **ADD** 100 mL of termination solution.
37. **INCUBATE** for 5 minutes at room temperate and away from strong direct light.
38. To best preserve the membrane, **REMOVE** the membrane from the tray and place between two sheets of filter paper or between two paper towels. **DRY** for 5 minutes at 80 °C or for 30-60 minutes at room temperature. Dried membranes can be stored in the dark for several months.
39. **ANALYZE** the banding pattern. Based on the RFLP fingerprints of the Mother, Child, Father 1, and Father 2 who is the father of the child?

Study Questions

1. What is polymorphic DNA? How is it used for identification purposes?

2. What are the key steps of a typical Southern blot?

3. What is the most likely cause of Restriction Fragment Length Polymorphisms?

4. What is the advantage of using a Southern blot when analyzing an individual's full genome over simply using restriction enzyme digestion followed by gel electrophoresis and DNA gel staining?

Instructor's Guide

NOTES TO THE INSTRUCTOR

In this experiment Module I must be immediately followed by Module II. Module III can be completed any time as the DNA is highly stable once it is fixed on the nylon membrane. Module III is a 4 hour lab with no stopping points. Plan accordingly. If you have any questions, please call or email us!

Preparation for:	What to do:	When?	Time Required:
Module I: Agarose Gel Electrophoresis	(Optional) Prepare diluted electrophoresis buffer	One day before Module I (or up to two weeks with proper storage)	40 min.
	(Optional) Prepare molten agarose and pour gels		
	Hand out supplies	Day of Module I	10 min.
Module II: Denaturation and Transfer	Prepare solutions	Up to 24 hours before Module II	15 min.
	Hand out supplies	Day of Module II	10 min.
	Set incubator to 80 °C	Anytime before performing Module II	5 min.
Module III: Detection and Analysis	Prepare detection buffer and termination solution	Up to 24 hours before Module III	15 min.
	Hand out supplies	Day of Module III	10 min.
	Set incubator(s) to 65°C, 37°C, and 80°C	Before/during Module III	5 min.
	Heat and aliquot shielding buffer	During Module III	10 min.
	Prepare color development solution	During Module III	5 min.

Red = Prepare immediately before or during module.

Yellow = Prepare shortly before module.

Green = Flexible / prepare up to a week before the module.

Pre-Lab Preparations

MODULE I: AGAROSE GEL ELECTROPHORESIS

This experiment requires a 0.8% agarose gel per student group. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow approximately 30-40 minutes for this procedure.

This experiment also requires a 7 x 14 cm agarose gel or a gel that can accommodate 10 samples. Before beginning, make sure that both your electrophoresis equipment (Module I) and the container trays with lids (Modules II and III) accommodate this sized gel. Both the nylon membrane and filter papers included in this experiment have been precut to fit 7x14 cm gels. To ensure a good transfer your class's gels must be 7x14 cm or must be easily cut to meet this size requirement before Module II.

NOTE:
Accurate pipetting is critical for maximizing successful experiment results. If students are unfamiliar with using micropipettes, we recommended performing Cat. #S-44, Micropipetting Basics or Cat. #S-43, DNA DuraGel™ prior to conducting this advanced level experiment.

Individual Gel Preparation:
Each student group can be responsible for casting their own individual gel prior to conducting the experiment. See Module I in the Student's Experimental Procedure. Students will need 50x concentrated buffer, distilled water and agarose powder.

FOR MODULE I
Each Group should receive:
- 50x concentrated buffer
- Distilled Water
- UltraSpec-Agarose™
- DNA Samples

Batch Gel Preparation:
To save time, a larger quantity of agarose solution can be prepared for sharing by the class. Electrophoresis buffer can also be prepared in bulk. See Appendix A.

Preparing Gels in Advance:
Gels may be prepared ahead and stored for later use. Solidified gels can be stored under buffer in the refrigerator for up to 2 weeks. Do not freeze gels at -20° C as freezing will destroy the gels. Gels that have been removed from their trays for storage should be "anchored" back to the tray with a few drops of molten agarose before being placed into the tray. This will prevent the gels from sliding around in the trays and the chambers.

DNA Samples:
Components A through I are predigested DNA in a gel loading solution that are ready to be loaded directly into each group's gel. Student groups can share these tubes or you may aliquot the solutions to additional tubes. In this case each group should receive ~40 µL of each sample.

OPTIONAL GEL STAINING AFTER ELECTROPHORESIS

Visualizing the DNA samples after electrophoresis is not required to complete a Southern blot but can illustrate why this procedure is essential for complex DNA samples. The materials provided in this lab allow students to first stain their gels with ethidium bromide and then continue to the denaturation, transfer, and detection without compromising the results. In addition, the provided samples have been intentionally spiked with chromosomal DNA to show a smear pattern following electrophoresis which will contrast with the specific "probe" bands visible at the end of the experiment. Running this added step will require additional lab time on day 1 of the experiment, additional reagents (InstaStain® Ethidium Bromide Cat #2001), and access to a UV transilluminator (Cat. #558 recommended). See Appendix B for instructions on gel staining with InstaStain® Ethidium Bromide.

Pre-Lab Preparations

MODULE II: DENATURATION AND TRANSFER

1. Prepare 1 L of depurination solution (0.25 N HCl) by mixing 21 mL of concentrated HCl (12 N) and 979 mL distilled water. Provide each group with 200 mL of this mixture. Label each beaker either as "Depurination Solution" or as "0.25 N HCl".

2. Prepare 2 L of denaturation solution (0.5 M NaOH/0.6 M NaCl) by adding 40 g of NaOH and 70 g of NaCl to 1.8 L of distilled water, mixing, and then brining to a final volume of 2 L. Provide each group with 400 mL of this mixture. Label each beaker either as "Denaturation Solution" or as "0.5 M NaOH/0.6 M NaCl".

3. Distribute the remaining Module II items. For the nylon membranes, keep both the upper and lower protective cover sheets around the membranes. Use rinsed and dried lab gloves for this step as powdered gloves my interfere with the transfer.

4. Set an incubator to 80°C. (If this will be after an overnight transfer, wait until the second day of Module II.)

FOR MODULE II
Each Group should receive:
- Depurination Solution
- Denaturation Solution
- Distilled Water
- Tray large enough to hold a 7x14 cm gel
- Plastic wrap and tape
- 1 pre-cut nylon membrane (7x14 cm), protected by two blue sheets
- 1 pre-cut filter paper (7x14 cm)
- Stack of paper towels (around 20)
- 2 forceps, 1 spatula (optional), and lab gloves
- Pencil
- Filter Paper*

** Filter paper provides additional protection during the final fixation step. If you do not have filter paper students can carefully sandwich the membrane between two paper towels.*

Pre-Lab Preparations

MODULE III: DNA DETECTION AND ANALYSIS

The SAAP solution and the color change solution must be prepared immediately before use. All other buffers can be prepared and aliquoted before the experiment.

1. Prepare 5 L detection buffer by mixing 250 mL concentrated detection buffer (J) and 4.75 L distilled water. Provide each group with 1 L of this mixture.

2. Prepare 500 mL termination solution by mixing 25 mL concentrated termination solution (L) and 475 mL distilled water. Provide each group with 50 mL of this mixture.

FOR MODULE III
Each Group should receive:
- Detection buffer
- Shielding buffer (warmed to 65°C)
- Color development solution (prepared during Module III)
- Termination solution
- Access to SAAP tube
- Distilled water
- Their nylon membrane from Module II
- Tray and Lid
- Forceps
- Graduated cylinder
- Empty 50 mL flask
- Access to incubator at 65°C, 37°C, and 80°C
- Access to shaking platform (optional)
- Foil
- Filter paper or paper towels

Day of Module III

3. Set the incubator oven(s). If you have one incubator, set it to 65°C, following step 6 of the student experiment change to 37°C, and following step 21 of the student experiment change to 80°C. If you have more than one incubator, set one to each temperature (65°C, 37°C, and 80°C).

4. For each group, aliquot 50 mL of shielding buffer (K) and warm to 65°C. ***Do not allow the temperature of the solution to exceed*** 65°C as this may cause the solution to thicken and/or solidify. (Alternatively, each student group can be responsible for carefully heating their shielding buffer at the start of Module III.)

5. For step 12, students will need access to the tube of concentrated SAAP solution (N). Because of the small volumes involved we suggest having the five student groups pipette this solution directly from this original tube.

6. During step 26 of the student protocol, prepare the color development solution. ***This solution must be used within 30 minutes of preparation.*** Dissolve the two BCIP/NBT tablets in 40 mL of distilled water and then vortexing until the tablets are completely dissolved. Provide each group with 8 mL of solution.

Expected Results and Analysis

EXPECTED AGAROSE GEL RESULTS

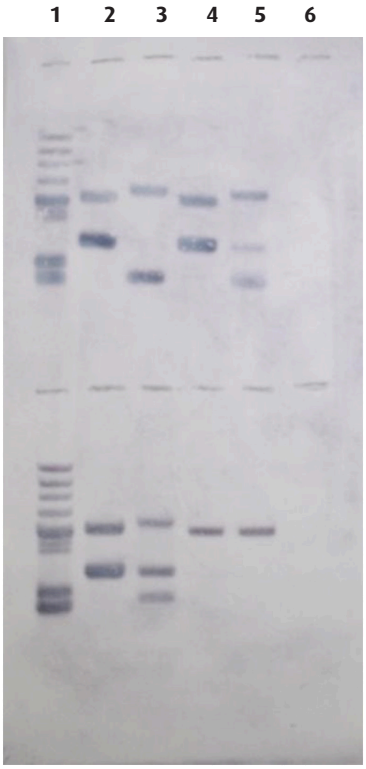
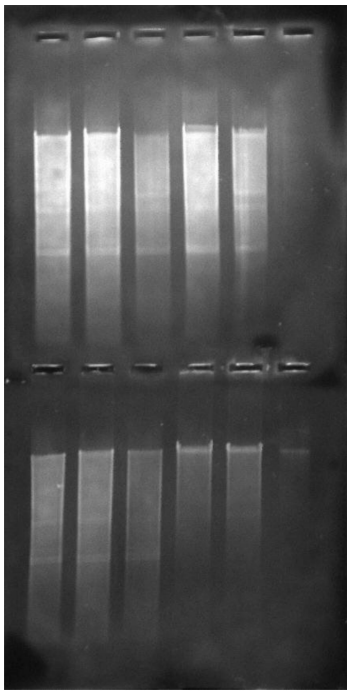


Table 1: Gel Loading

Row	Lane	Tube	Sample
1	1	A	Standard DNA fragments
1	2	B	Mother's DNA cut with Enzyme 1
1	3	C	Mother's DNA cut with Enzyme 2
1	4	D	Child's DNA cut with Enzyme 1
1	5	E	Child's DNA cut with Enzyme 2
1	6	-	---
2	1	A	Standard DNA fragments
2	2	F	Father 1's DNA cut with Enzyme 1
2	3	G	Father 1's DNA cut with Enzyme 2
2	4	H	Father 2's DNA cut with Enzyme 1
2	5	I	Father 2's DNA cut with Enzyme 2
2	6	-	---

Standard DNA Fragment Sizes: 23130 bp, 9416 bp, 6557 bp, 4361 bp, 3000 bp, 2322 bp, 2027 bp, 725 bp*, 570 bp*.
*These fragments are not always visible due to their small size.

While the results from the digestion with enzyme 1 are inconclusive the results from the digestion with enzyme 2 indicate that the child's father in Father 1. This is because the middle sized bands visible in row 1 lane 5 (Child's DNA cut with Enzyme 2) is not present in the mother's DNA and could only be inherited from Father 1.



Optional gel staining after electrophoresis produces a smear. Such smears occur when there are too many restriction fragment length polymorphisms in a sample to be resolved on an agarose gel.

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

Appendix A
Bulk Preparation of Electrophoresis Buffer and Agarose Gels

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.

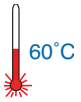
Table D — Bulk Preparation of Electrophoresis Buffer

50x Conc. Buffer	+	Distilled Water	Total Volume Required
60 mL		2,940 mL	3000 mL (3 L)

BATCH AGAROSE GELS (0.8%)

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

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. Measure 60 mL for a 7 x 14 cm tray. ***For this experiment, 7 x 14 cm gels are recommended.***
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.

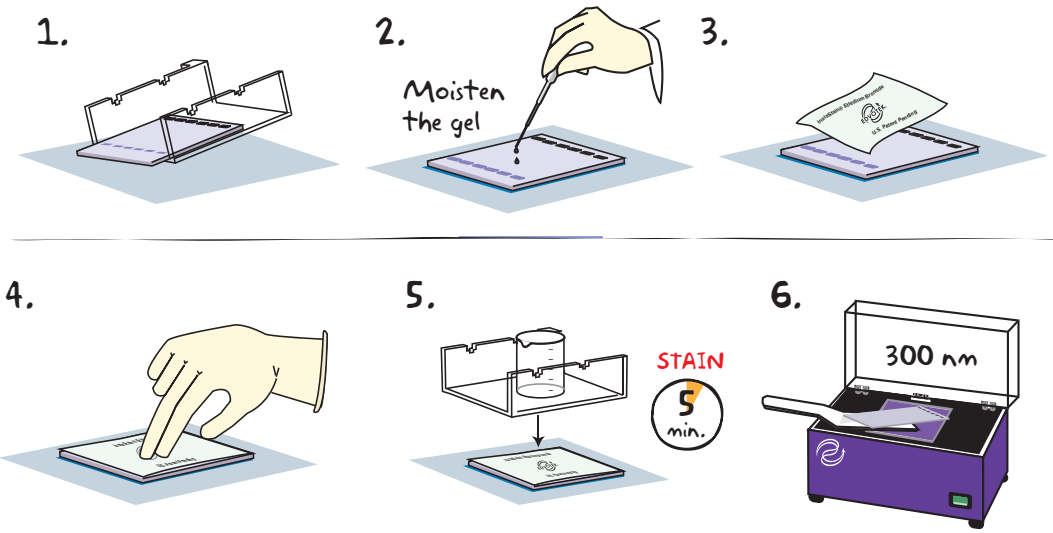
NOTE:
The UltraSpec-Agarose™ kit component is usually labeled with the amount it contains. Please read the label carefully. If the amount of agarose is not specified or if the bottle's plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.

Table E — Batch Prep of 0.8% UltraSpec-Agarose™

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

Appendix B

Staining Agarose Gels with InstaStain® Ethidium Bromide



Wear gloves and safety goggles

1. Carefully **REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off of the casting tray on to a piece of plastic wrap on a flat surface.

   DO NOT STAIN GELS IN THE ELECTROPHORESIS APPARATUS.

2. **MOISTEN** the gel with a few drops of electrophoresis buffer.
3. Wearing gloves, **REMOVE** and **DISCARD** the clear plastic protective sheet from the unprinted side of the InstaStain® card(s). **PLACE** the unprinted side of the InstaStain® Ethidium Bromide card(s) on the gel. You will need 2 cards to stain a 7 x 14 cm gel.
4. With a gloved hand, **REMOVE** air bubbles between the card and the gel by firmly running your fingers over the entire surface. Otherwise, those regions will not stain.
5. **PLACE** the casting tray on top of the gel/card stack. **PLACE** a small weight (i.e. an empty glass beaker) on top of the casting tray. This ensures that the InstaStain® Ethidium Bromide card is in direct contact with the gel surface. **STAIN** the gel for 3-5 minutes.
6. **REMOVE** the InstaStain® Ethidium Bromide card(s). **VISUALIZE** the gel using a long wavelength ultra-violet transilluminator (300 nm). DNA should appear as bright orange bands on a dark background.

   BE SURE TO WEAR UV-PROTECTIVE EYEWEAR!