

Edvo-Kit #

S-55

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Decoding Fragile X Syndrome

Experiment Objective:

In this multi-part experiment, students will gain an understanding of the screening process for Fragile X syndrome. First, students will analyze simulated patient PCR samples to determine CGG repeat expansions in the FMR1 gene. Next, simulated Southern blot analysis will be used to confirm repeat size and methylation status.

See page 3 for storage instructions.

Version S-55.260421

EDVOTEK®

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Experiment Components

READY-TO-LOAD™ SAMPLES FOR ELECTROPHORESIS

Components (in QuickStrip™ format)

Store QuickStrip™ samples in the refrigerator upon receipt.

- A FMR1 CGG Repeat Ladder
- B Patient 1 PCR Sample
- C Patient 2 PCR Sample
- D Patient 3 PCR Sample
- E Patient 4 PCR Sample
- F Patient 5 PCR Sample
- G Patient 6 PCR Sample
- H Patient 7 PCR Sample

Check (✓)

- ☐
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- ☐

Experiment #S-55 is designed for 10 groups.

STORAGE:

Store Ready-to-Load™ QuickStrip™ samples in the refrigerator immediately upon receipt. All other components can be stored at room temperature.

REAGENTS & SUPPLIES

Store the following at room temperature.

- UltraSpec-Agarose™
- Electrophoresis Buffer (50x)
- Practice Gel Loading Solution

- ☐
- ☐
- ☐

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

- Horizontal gel electrophoresis apparatus ([Cat. 500](#) recommended)
- D.C. power supply
- Automatic micropipettes with tips
- Balance
- Microwave, hot plate or burner
- Blue light transilluminator (Built-in with [Cat. 500](#) or [Cat. 555](#) or [557](#) recommended)
- Pipet pump
- 250 mL flasks or beakers
- Hot gloves
- Safety goggles and disposable laboratory gloves
- Distilled or deionized water

Background Information

WHAT IS FRAGILE X SYNDROME?

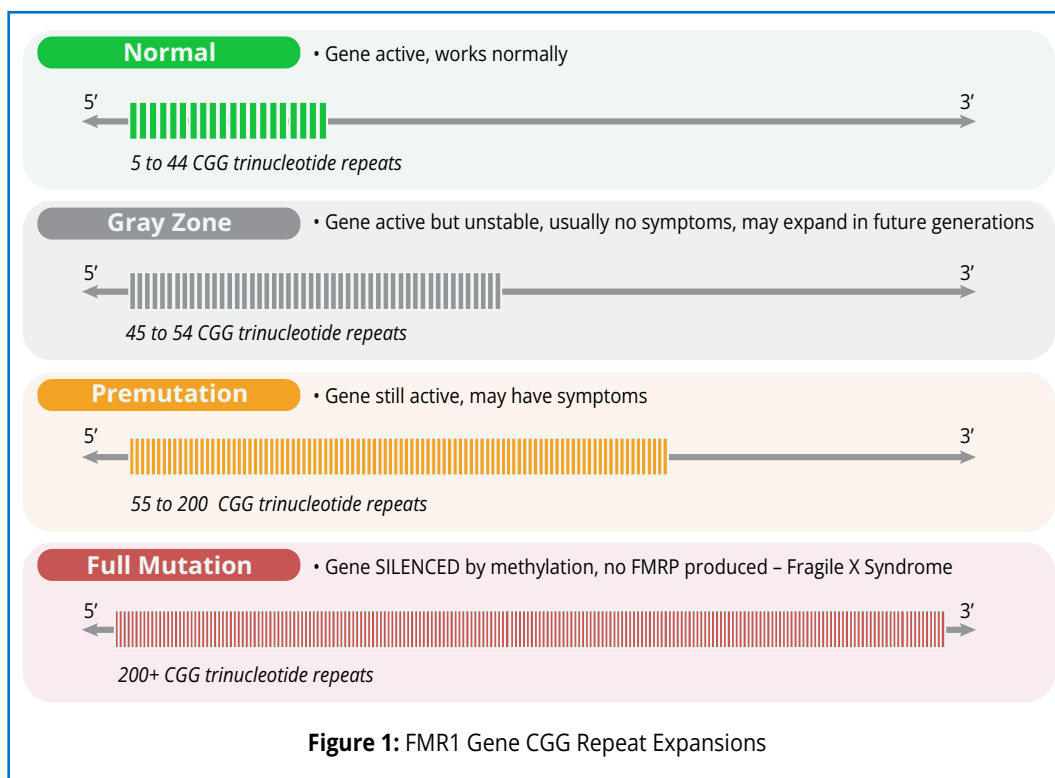
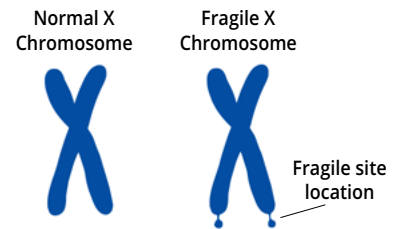
Imagine your DNA as a very long instruction manual for building and running your body. Tucked inside that manual is a gene called **FMR1**, sitting on the **X chromosome**. This gene's job is to produce a protein — called **FMRP** — that is essential for normal brain development and learning. In most people, a small section of the FMR1 gene contains a short repeating sequence: **CGG CGG CGG...** Usually the sequence repeats **fewer than 45 times**, and everything works just fine (Figure 1). But in some people this sequence grows, sometimes to hundreds of repeats. When that happens, the gene can stop working, and the result is **Fragile X Syndrome (FXS)**: the most common inherited cause of intellectual disability and a leading genetic cause of autism spectrum disorder.

Think of the CGG repeat like a stuck record player. Normally the record plays cleanly (5–44 repeats). But sometimes the needle gets stuck and the same note repeats over and over. The more it repeats, the bigger the problem. When a person has 200 or more CGG repeats (full mutation), something extra happens: the gene gets "silenced" through a process called methylation. This silencing of the FMR1 gene prevents the body from making FMRP, and that's what causes Fragile X syndrome.

People with 55–200 repeats (premutation) don't have classic Fragile X syndrome, but they aren't completely in the clear. The extra CGG repeats cause the cell to produce too much FMR1 "message" (**mRNA**). This overload can, over time, become toxic to certain cells.

Quick Fact: Little Feet

FXS is called "Fragile X" because under a microscope, the X chromosome of affected individuals looks like it has a fragile, pinched spot near the tip where the FMR1 gene lives.



Why Does It Affect Males and Females Differently?

- Males (XY)** have only **one copy** of the FMR1 gene because they have just one X chromosome. If that single copy contains a full mutation (more than 200 CGG repeats), the gene becomes silenced and the cell cannot produce the FMRP protein. Because males do not have a second copy of the gene to compensate, the **effects are usually more severe**.

Males with a full mutation commonly experience:

- Intellectual disability and learning difficulties
- Anxiety and hyperactivity
- Features of autism spectrum disorder
- Physical traits like an elongated face or prominent ears

FXTAS (Fragile X-associated Tremor/Ataxia Syndrome): Some older adult males with a **premutation** develop FXTAS — a neurological condition with hand tremors, balance problems, and memory difficulties.

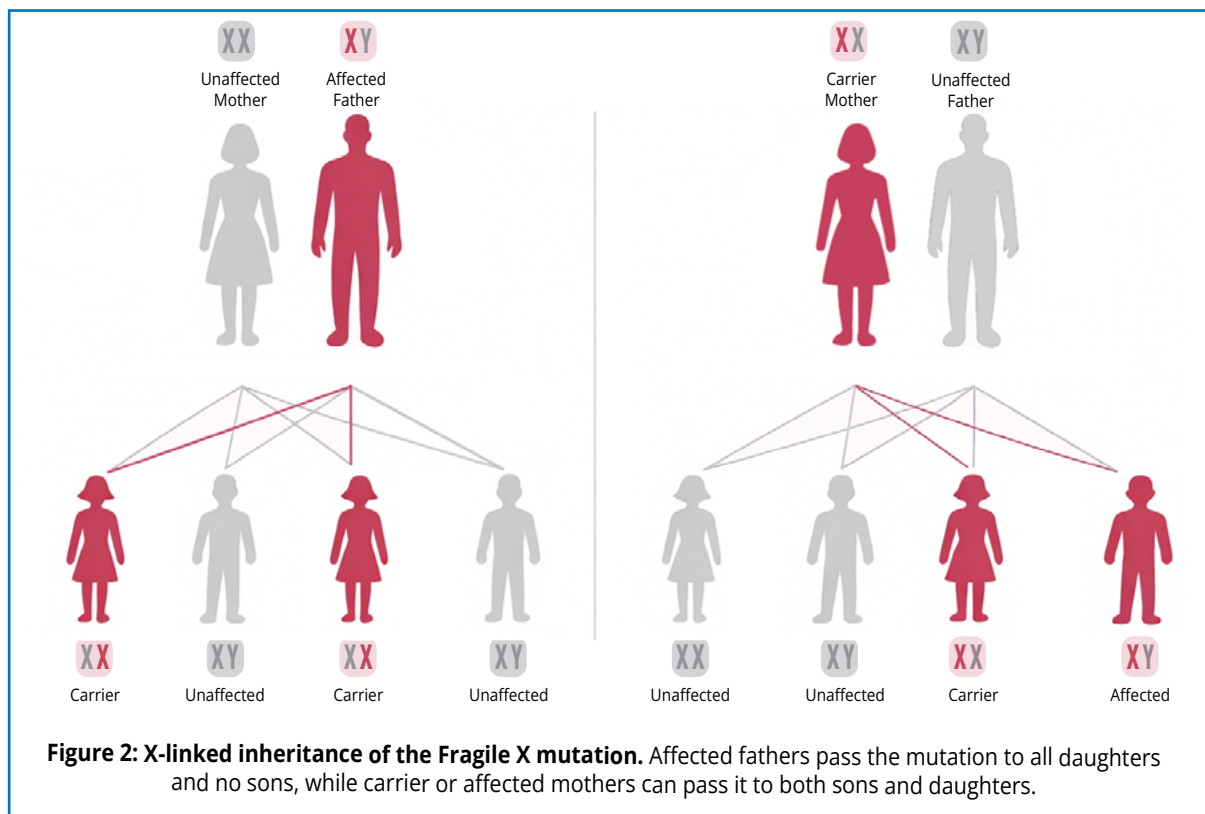
- Females (XX)** have **two copies** of the FMR1 gene — one on each X chromosome. Even if one copy has the mutation, the other copy can often still make some FMRP. This partial "backup" is why females with Fragile X usually have **milder or even no symptoms**.

Early in development, every cell in a female's body randomly turns off one of its two X chromosomes, a process called **X-inactivation**. Some cells will silence the normal copy, while others silence the mutated copy. This random patchwork means each female carrier can experience a unique range of effects, from no symptoms at all to mild learning differences or anxiety.

FXPOI (Fragile X-associated Primary Ovarian Insufficiency): Female **premutation** carriers can develop FXPOI, which can cause irregular menstrual cycles, difficulty becoming pregnant, or early menopause. Some female carriers may also experience anxiety or subtle differences in memory and focus.

Quick Fact: A Common Condition

Fragile X syndrome affects approximately 1 in 4,000 males and 1 in 8,000 females worldwide. The premutation is even more common — roughly 1 in 150 females and 1 in 450 males carry it. This means that in a city of 1 million people, thousands of individuals may carry the Fragile X premutation without even knowing it.



HOW IS FRAGILE X PASSED DOWN THROUGH FAMILIES?

One of the most unique — and sometimes surprising — things about Fragile X is the way it travels through families. Being that FXS is an X-linked genetic disease (Figure 2), it doesn't always show up the same way in every generation. In fact, a family might carry the mutation for years without anyone knowing, until one day a child is born with symptoms that finally prompt testing. When doctors look back at the family history, they often find the mutation has been quietly growing for generations. To understand how this happens, we need to revisit the idea of the CGG repeat — and a key rule: the more repeats a person has, the more unstable the sequence becomes when it is passed to the next generation.

Meet the Rivera Family: A Story of Three Generations

Let's follow one fictional family to see how this works in real life!

Generation 1 — Grandma Rosa
Rosa is 68 years old. She has 62 CGG repeats — a premutation. She has lived her whole life without ever being diagnosed with Fragile X. She had some anxiety and went through early menopause in her 40s, but no one connected those things to a genetic condition. Rosa has no idea she is a carrier.
Generation 2 — Rosa's Daughter, Carmen
Carmen inherited her mother's premutation X chromosome — but when it was passed from Rosa to Carmen, the repeat count grew. Carmen now has 110 CGG repeats. She still doesn't have Fragile X syndrome, but her premutation is larger than her mother's. She also doesn't know she is a carrier.
Generation 3 — Carmen's Son, Marco
Carmen passed her premutation X chromosome to her son Marco. But this time, when the repeat was passed on, it expanded dramatically — from 110 repeats all the way past 200. Marco was born with a full mutation. His FMR1 gene is silenced, he produces no FMRP, and he is diagnosed with Fragile X syndrome at age 3.

In this scenario, Rosa and Carmen both had the mutation their whole lives but showed little to no symptoms. It wasn't until Marco was born that the family learned about Fragile X at all, and genetic testing later revealed the full picture going back to Grandma Rosa. This is called anticipation: when a genetic mutation tends to become more severe (or more noticeable) with each passing generation.

The Rules of X-Linked Inheritance

To understand who can pass Fragile X to whom, it helps to remember how X chromosomes are inherited. Every person gets one X chromosome from their mother. Males (XY) also get a Y chromosome from their father, while females (XX) get a second X chromosome from their father.

Parent with mutation	Can they pass it on?	What happens to the mutation?
Mother (carrier)	Yes — to sons AND daughters	Repeat count often expands when passed to children. Sons who inherit the mutation X are at high risk for full mutation.
Father (premutation only)	Yes — to daughters only (never to sons)	Fathers pass their X chromosome only to daughters. Sons get the father's Y, so they cannot inherit FXS from dad.
Father (full mutation)	Yes — to daughters only	Interestingly, the repeat does NOT expand when passed by a father. Daughters receive the premutation range, not the full mutation.

Table 1: X-Linked Inheritance of Fragile X Syndrome.

Why Can't Sons Inherit FXS From Their Father?

When a father has a premutation or full mutation in his FMR1 gene, it is located on his X chromosome. Fathers pass their X chromosome exclusively to their daughters, and their Y chromosome to their sons. Since the FMR1 gene is on the X chromosome, sons can only inherit it from their mother. So, a son with Fragile X syndrome always gets the mutation from his mother. His father may be completely unaffected and carry no mutation at all, or his father may carry a premutation himself (in which case he got it from his own mother). Because fathers can only pass FXS to daughters, and because mothers can pass it to both sons and daughters, the pattern of who is affected in a family can sometimes seem confusing. A grandfather with a premutation might have a daughter who is a carrier, and that daughter's sons might have Fragile X syndrome, while the grandfather himself never showed any major symptoms. Genetic counselors use family history and testing to trace these patterns!

Expansion Risk by Premutation Size:

- **55-59 repeats:** Low risk of expanding to full mutation in one generation
- **60-89 repeats:** Moderate risk — expansion becomes more likely
- **90-199 repeats:** High risk — very likely to expand to full mutation in the next generation

The Expansion Problem: Why Do Repeats Grow?

Scientists are still working to fully understand why the CGG repeat region tends to grow when passed from parent to child, but we know a few things. The repeat region forms unusual shapes in the DNA that can cause copying errors during cell division. The larger the premutation already is, the more likely it is to expand further in the next generation. This is why a grandmother with 60 repeats might have a daughter with 120 repeats, and that daughter might have a son with 350 repeats. The instability of the gene builds over time.

HOW DO SCIENTISTS TEST FOR FRAGILE X?

Because we can't see CGG repeats with the naked eye, scientists use two powerful molecular tools: PCR and Southern blot analysis. Together, they help us figure out exactly how many repeats a person has and whether the FMR1 gene has been silenced.

Polymerase Chain Reaction

PCR is a highly utilized molecular biology technique that amplifies DNA with the use of designed primers that target a specific sequence, such as the FMR1 gene, allowing for the detection of genetic mutations. In fragile X carrier testing, PCR is used to measure the number of CGG repeats found in the FMR1 gene, allowing us to determine if an individual has a normal allele, premutation, or full mutation. Individuals with normal alleles typically have less than 45 repeats, whereas premutation have between 55 and 200 repeats, and individuals with full repeats have over 200 repeats. Additionally, there are individuals that contain between 45 and 54 repeats, which is classified as a "gray zone". These individuals can be thought of as pre-premutation carriers, as the number of repeats will increase with subsequent generations.

The PCR process involves three key steps: denaturation, annealing, and extension (Figure 3). During the denaturation step, double stranded DNA is separated by heating the sample, typically at 94°C. DNA primers then bind to the target sequence on these separated strands in the annealing step, at temperatures ranging from 40-65°C. Lastly, in the extension step, the thermostable DNA polymerase synthesizes new DNA strands at 72°C. This process is typically repeated 35 to 40 times, allowing for millions of copies of a targeted DNA sequence to be amplified. After amplification, the resulting fragments are analyzed through gel electrophoresis, which separates the DNA fragments by size. The size of the PCR product corresponds directly to the number of CGG repeats found in the FMR1 gene.

One of the primary advantages of PCR in fragile X carrier testing is its sensitivity and speed, requiring minimal amounts of DNA to provide results within hours. PCR is highly effective in detecting normal and premutation alleles (especially in males, where there is only one X chromosome), however is not the most reliable when detecting full mutation. This is due to the highly repetitive, high GC-rich nature of the CGG expansion region that can cause structures that hinder amplification of full mutation alleles. As a result, PCR alone can yield ambiguous results, especially in women. In these instances, a woman with one normal allele and one full mutation allele may be mis-classified as a non-carrier by PCR alone due to the expanded allele going undetected. To combat this, additional testing using southern blot analysis is used for result confirmation.

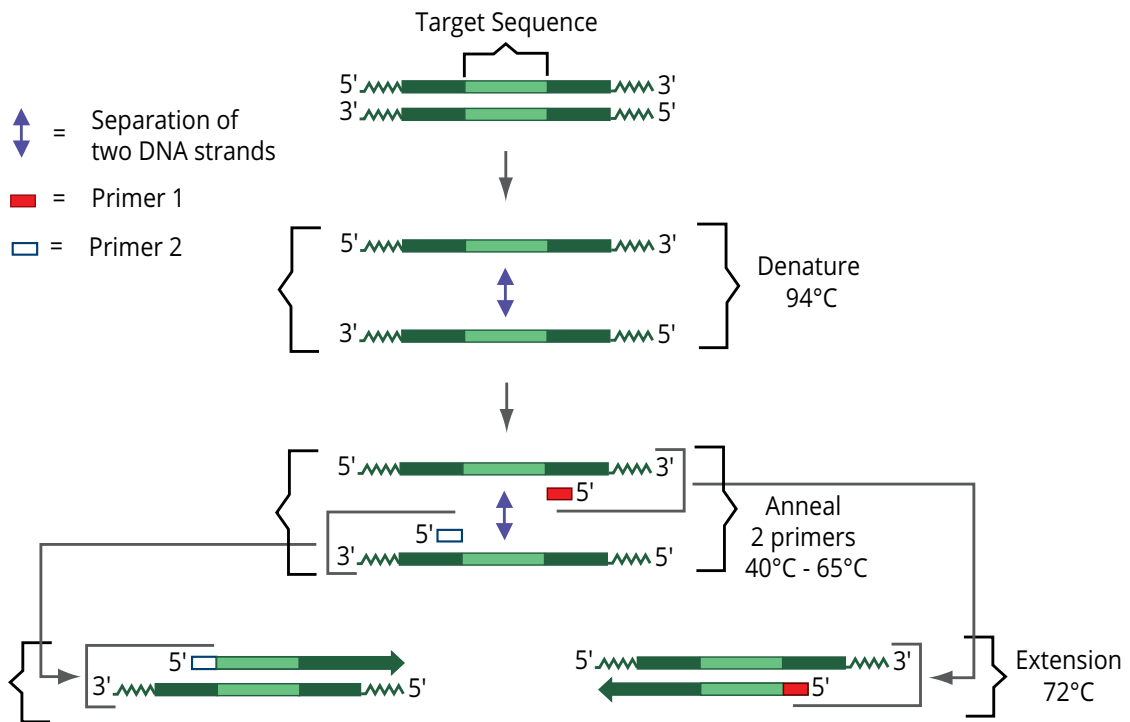


Figure 3: The Polymerase Chain Reaction

In your experiment, you'll look for colored bands on the gel to determine each patient's repeat count. The color of the band tells you what category the allele falls into: normal (green), gray zone (yellow), or premutation (red). You'll also look at how many bands appear for each patient:

- One band = likely male (one X chromosome, one FMR1 allele)
- Two bands = likely female (two X chromosomes, two FMR1 alleles — possibly different sizes)

For example, a female carrier might show one green band (normal allele) and one red band (premutation allele) — two different-sized alleles, one on each X chromosome. A male with a premutation would show just one red band, because he only has one X.

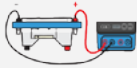
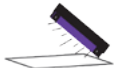
Quick Fact: When PCR Can't Get the Job Done

PCR has an important limitation for Fragile X testing: it struggles to copy very long, repetitive sequences. The full mutation (200+ CGG repeats) creates a region that is so long and GC-rich that the DNA copying machinery tends to stall or skip it entirely. This creates a tricky situation. A female with one normal allele and one full mutation allele may show only ONE band on the gel — because PCR amplified the normal allele but completely missed the full mutation. Without knowing this, you might mistakenly classify her as a non-carrier. This is exactly why Southern blot analysis is needed as a follow-up test.

Southern Blot Analysis

Southern blot analysis is a molecular biology technique invented in 1975 by a British scientist named Edwin Southern, that provides crucial information when PCR alone is insufficient – particularly in the context of fragile X carrier testing. Recall that when someone has 200+ CGG repeats, the cell responds by methylating the FMR1 promotor. Once this happens, the gene is silenced and no FMRP protein is produced, leading to Fragile X Syndrome.

Southern blot can detect this methylation directly by using a special restriction enzyme that will only cut DNA if it is unmethylated. If the enzyme cuts the DNA at the FMR1 region, that tells us the gene is active (unmethylated). If the enzyme does NOT cut there, that tells us the gene has been silenced (methylated). The southern blot allows for the manipulation of DNA in several **key steps**, done across roughly 18 hours:

Step		What Happens	Why It Matters for FXS
1. Digest		Two restriction enzymes act as molecular scissors and cut the DNA at known locations around the FMR1 gene. One enzyme only cuts unmethylated DNA.	The methylation-sensitive enzyme is the key to detecting gene silencing. If it can't cut, the FMR1 gene is methylated and likely silenced.
2. Separate		The cut DNA fragments are loaded into a gel and separated by size using electrophoresis. Smaller fragments travel farther.	A full mutation allele produces a much larger DNA fragment than a normal allele, too large for PCR to copy, but visible here as a band high up on the gel.
3. Denature		The gel is treated with chemicals that "unzip" the double-stranded DNA into single strands.	Single-stranded DNA is needed so the probe can attach to it in the next steps.
4. Transfer		The single-stranded DNA is transferred from the gel onto a sturdy nylon membrane — like pressing a stamp onto paper.	The membrane is easier to work with than a fragile gel, and the DNA keeps its size-separated pattern.
5. Fix		The DNA is permanently locked onto the membrane using UV light or heat.	This ensures the DNA doesn't wash away during the next steps.
6. Hybridize		A DNA probe (a short, labeled piece of DNA designed to match the FMR1 region) is washed over the membrane.	The probe will only bind to the FMR1 fragments on the membrane, ignoring anything else.
7. Visualize		The membrane is treated to make the probe-labeled bands glow or appear as dark marks.	The position of each band reveals the size of the FMR1 fragment. Large bands = large expansions. The presence or absence of bands from the methylation-sensitive enzyme tells us if the gene is silenced.

This approach allows for the accurate sizing of large CGG expansions that may not be visible with PCR alone, and it helps to assess the methylation status of the FMR1 gene. For carrier testing, southern blot analysis is extremely important when a woman's PCR shows a single allele, as it can confirm if both alleles are normal, or if the second X chromosome carries an expanded and methylated allele. This is crucial for accurately identifying the carrier status of a woman that may be at risk of passing a full mutation on to her children. Additionally, southern blot can help resolve complex cases that involve mosaicism, where individuals may have cells with varying sizes of methylation patterns of the CGG repeat region. While southern blot analysis is more time consuming and requires more DNA than PCR, its ability to provide information on band size and methylation makes it an important step in fragile X carrier testing.

Reading the Results: What Do the Bands Mean?

When you look at a Southern blot result for Fragile X testing, you're essentially reading a map of each patient's FMR1 alleles. Here's how to decode what you see:

- **Band position (high vs. low):** Higher on the blot = larger DNA fragment = more CGG repeats. A band sitting at 2.8 kb indicates a normal-sized allele. A band around 6 kb suggests a premutation. A band around 8 kb or higher points to a full mutation.
- **Number of bands:** A unaffected female will have two bands, one smaller (2.8 kb) and one larger (5.2kb) methylated band that directly correlates to her inactivated X chromosome. The presence of a third band on the membrane will indicate whether she has a partial (6 kb) or full mutation (8 kb+). Males will have one band regardless of carrier status, and status will be determined directly based on size.

In this experiment you will act as a Medical Laboratory Scientist (MLS) investigating Fragile X carrier status by analyzing simulated patient DNA samples. You will begin by interpreting PCR results labeled with fluorescent markers that represent different FMR1 allele sizes, allowing you to estimate CGG repeat number, infer genetic sex based on allele patterns, and identify samples that require additional testing. For selected cases, you will then analyze a simulated Southern blot to detect large repeat expansions and determine methylation status, a key factor in FMR1 gene silencing. Together, these analyses will guide you in making evidence-based conclusions about carrier status and illustrate how PCR and Southern blotting are used together in real-world Fragile X testing.

Experiment Overview

EXPERIMENT OBJECTIVE

In this multi-part experiment, students will gain an understanding of the screening process for Fragile X syndrome. First, students will analyze simulated patient PCR samples to determine CGG repeat expansions in the FMR1 gene. Next, simulated Southern blot analysis will be used to confirm repeat size and methylation status.

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 NOTEBOOKS

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Module I Overview

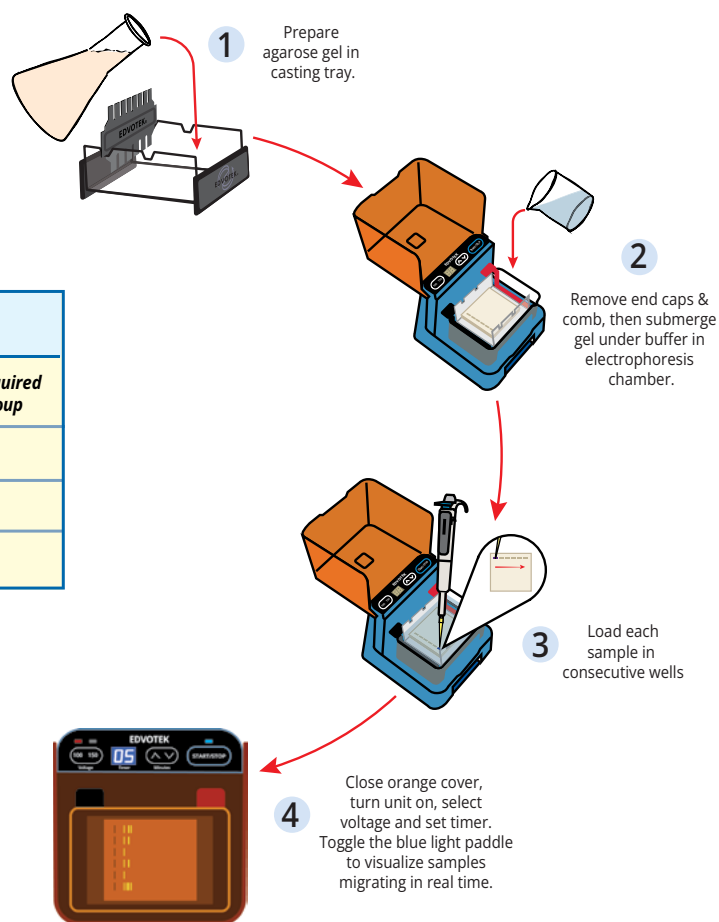
In this module, you will analyze PCR samples from seven patient DNA samples to assess FMR1 CGG repeat size and identify potential Fragile X carriers. The FMR1 gene contains a region of repeating CGG DNA sequences, and the number of these repeats determines whether an individual has a normal allele, an intermediate (gray zone) allele, or a premutation associated with Fragile X-related conditions.

As you examine the gel, you will interpret fluorescent band positions to determine each patient's FMR1 status. You will also use the number of bands present to infer whether a patient is genetically male or female. Because males have one X chromosome and females have two, the number of bands observed can be used to infer genetic sex. Some samples may produce ambiguous or incomplete results due to large repeat expansions that do not amplify efficiently by PCR. Identifying these cases is an important part of the analysis and will guide decisions about whether follow-up testing is needed. By the end of this module, you will have screened all seven patients and determined which samples can be confidently interpreted and which require confirmatory testing using Southern blot analysis in Module II.

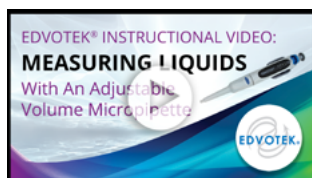
Agarose Gel Electrophoresis

Time required: See Table C

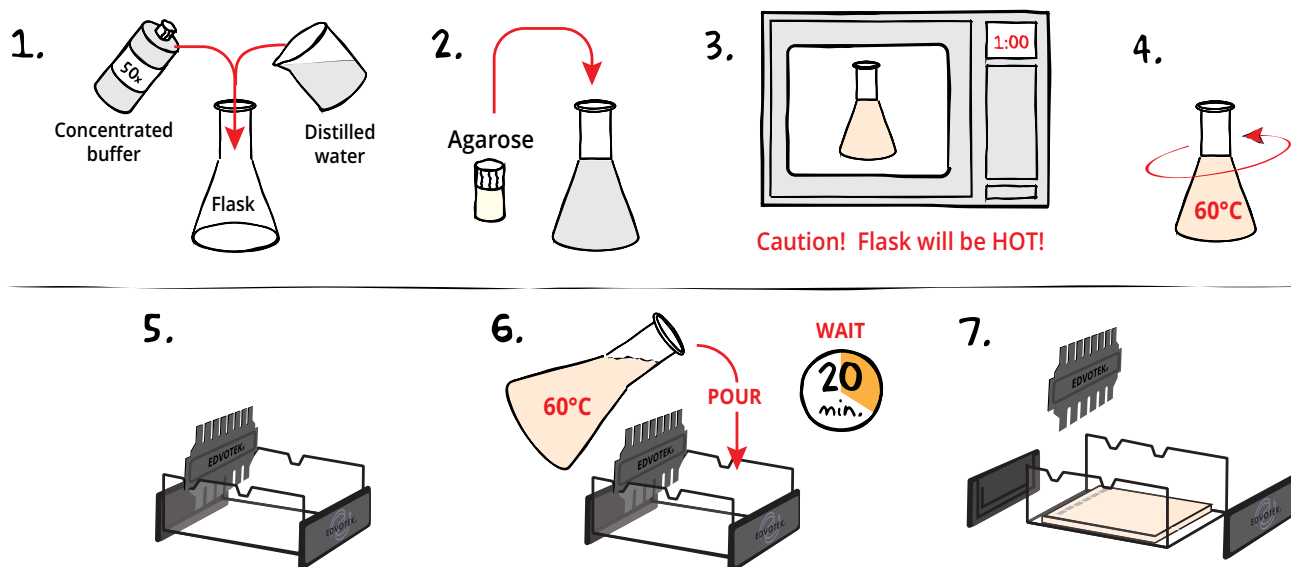
Quick Reference for EDVO-Kit #S-55			
Size of gel casting tray	Groups per gel	Placement of comb	Wells required per group
7 x 7 cm	1 group	1st set of notches	8
10 x 7 cm	1 group	1st set of notches	8
14 x 7 cm	2 groups	1st and 3rd sets of notches	8



Related EDVOTEK® Instructional Videos:



Module I-A: Agarose Gel Electrophoresis



CASTING THE AGAROSE GEL

- DILUTE** concentrated 50X Electrophoresis buffer with distilled water (refer to Table A for correct volumes depending on the size of your gel casting tray).
- MIX** agarose powder with buffer solution in a 250 mL flask (refer to Table A).
- 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).
- COOL** agarose to 60 °C with careful swirling to promote even dissipation of heat.
- 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.
- 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.
- REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.



Wear gloves
and safety goggles

REMINDER:

This experiment
requires 2.0%
agarose gels cast
with 8 wells.

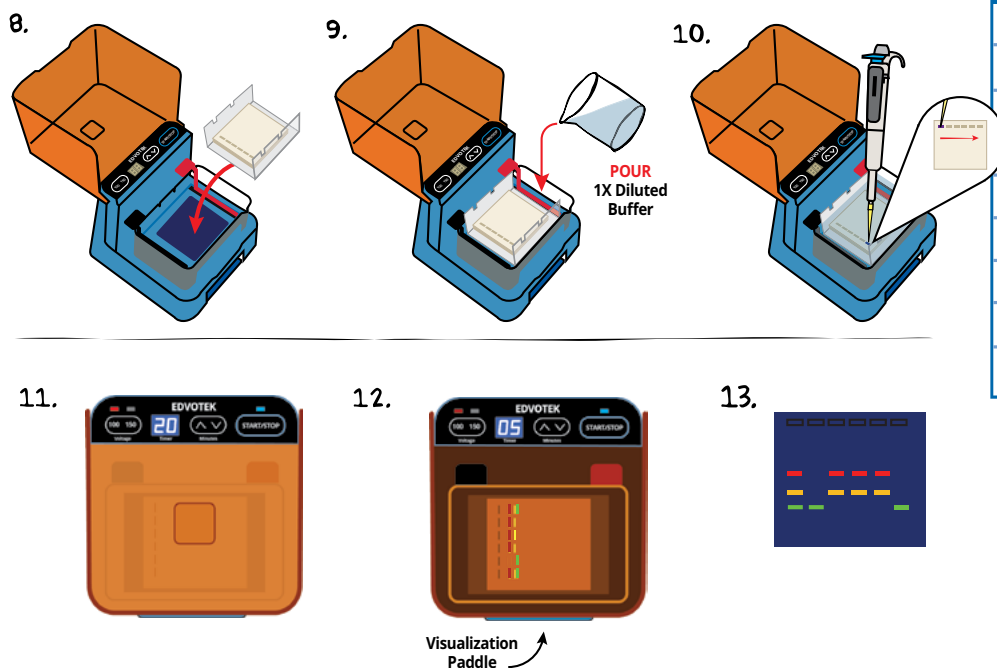
Table
A

Individual 2.0% UltraSpec-Agarose™ Gels

Size of Gel Casting tray	Concentrated Buffer (50x)	+ Distilled Water	+ Amt of Agarose	= TOTAL Volume
7 x 7 cm	0.6 mL	29.4 mL	0.6 g	30 mL
10 x 7 cm*	0.9 mL	44.1 mL	0.9 g	45 mL
14 x 7 cm	1.2 mL	58.8 mL	1.2 g	60 mL

*Recommended gel volume for the EDGE™ Integrated Electrophoresis System. (Cat. #500).

Module I-A: Agarose Gel Electrophoresis



Gel Loading Table for #S-55		
Lane	Tube	Sample
1	A	FMR1 CGG Repeat Ladder
2	B	Patient 1
3	C	Patient
4	D	Patient 3
5	E	Patient 4
6	F	Patient 5
7	G	Patient 6
8	H	Patient 7

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

LOADING AND RUNNING THE GEL

8. **PLACE** the gel (still on the tray*) into the electrophoresis chamber.
9. **COVER** the gel with 1X Electrophoresis Buffer (See Table B for recommended volumes). The gel should be completely submerged.
10. **PUNCTURE** the foil overlay of the QuickStrip™ with a pipet tip. **LOAD** the entire sample (35 µL) into the well in the order indicated by the Gel Loading Table.
11. **CLOSE** orange safety cover. (*NOTE: The EDGE™ will not operate while cover is open.*) **ENSURE** the unit is plugged in and switch is turned ON. **SELECT** desired voltage (100 V or 150 V) and **SET** timer. *NOTE: Reference your experiment protocol for recommended Voltage Guidelines.* **PRESS** the START/STOP button to start the current.
12. As you are performing electrophoresis, **TOGGLE** the visualization paddle **ON** to illuminate the blue LED light. **VISUALIZE** the samples migrating in real time. Remember, the samples will migrate toward the positive (red) electrode.
13. After electrophoresis is complete, **PRESS** the START/STOP button to stop the current, **DOCUMENT** the results of the experiment by using a camera or smartphone to take an image through the orange safety lid. Dimming the lights in the room can increase the visibility if needed.

Table
B

1x Electrophoresis Buffer (Chamber Buffer)

EDVOTEK Model #	Total Volume Required	50x Conc. Buffer	Dilution + Distilled Water
EDGE™	150 mL	3 mL	147 mL
M12	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

Table
C

Time and Voltage Guidelines (2.0% Agarose Gel)

Electrophoresis Model		
EDGE™		M12 & M36
Volts	Min/Max (minutes)	Min/Max (minutes)
150	10/15	10/15
125	N/A	10/15
100	15/20	15/20

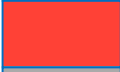
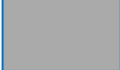
*Gels that have previously been removed from their trays should be "anchored" back to the tray with a few drops of molten agarose before placing into the electrophoresis chamber. This will prevent the gels from sliding around in the trays and the chambers.

Module I-B: PCR Results Analysis

Now that your gel has run, it's time to read the results! Each lane contains a patient's simulated PCR sample. Your job is to figure out what each patient's band pattern is telling you. Work through Steps 1–4 for every lane before filling in your results table.

BAND COLOR GUIDE

The bands on your gel are color-coded to show the category of each allele. Use this guide every time you look at a lane:

Color	Category (# Repeats)	What It Means
	Green: Normal (5–44)	The FMR1 gene has a typical number of CGG repeats. This person is not a carrier.
	Yellow: Gray Zone (45–54)	Slightly elevated repeat count. Not a carrier now, but the repeat may grow in future generations.
	Red: Premutation (55–200)	This person is a premutation carrier. They don't have FXS but could pass a larger mutation to their children.
	No Band: Possible Full Mutation (200+)	PCR may have failed to amplify the allele because the repeat is too large. This patient needs Southern blot follow-up.

STEP-BY-STEP: HOW TO ANALYZE EACH LANE

For every patient lane (Lanes 2–8), work through each step in order. Use your answers to fill in the Results Table on the next page.

Step 1: Find the Ladder (Lane 1)

Before analyzing any patient, look at the FMR1 CGG Repeat Ladder in Lane 1. This lane contains known reference bands at each category level (normal, gray zone, and premutation). Use the ladder to calibrate your eye: take note of where each color sits on the gel so you can compare patient bands to the same positions.

Step 2: Count the Bands in the Patient Lane

Look carefully at your patient's lane and count the number of distinct bands you see.

- **One band:** The patient is likely male (one X chromosome, one FMR1 allele). It could also be a female whose two alleles happen to be the same size — but treat one band as male unless told otherwise.
- **Two bands:** The patient is likely female (two X chromosomes, two FMR1 alleles of different sizes).
- **No band:** PCR may have failed to copy a very large allele (full mutation). Flag this patient for Southern blot follow-up.

Step 3: Identify the Color(s) of Each Band

For each band you found in Step 2, match its color to the Band Color Guide above. Record the category for each band. If a lane has two bands, record the color of each one separately as they may be different categories (e.g., one green and one red means a normal allele + a premutation allele).

Module I-B: PCR Results Analysis

Step 4: Determine Carrier Status and Flag for Follow-Up

Use your band count (Step 2) and band colors (Step 3) to determine each patient's carrier status. Follow this logic:

What you see on the gel:	Likely sex:	Carrier status:	Southern blot needed?
One green band	Male OR Female	Not a carrier OR Possible full mutation on second allele, inconclusive	YES
One yellow band	Male	Gray zone	No
One red band	Male	Premutation carrier	No
No band	Male	Suspected full mutation	YES
One green + one yellow band	Female	Gray zone carrier	No
One green + one red band	Female	Premutation carrier	No

Key Tip: Always combine band number + band color to make your conclusion — neither alone is sufficient.

PCR RESULTS TABLE: RECORD YOUR FINDINGS

Complete the table below using your analysis from Steps 1–4. Write the band color(s) you observed, your determination of sex, carrier status, and whether this patient needs Southern blot follow-up.

PCR Results Analysis				
Lane	Sample	Sex (M/F)	Carrier Status	Notes / Southern Blot Needed?
1	FMR1 CGG Repeat Ladder	—	Reference only	—
2	Patient 1			
3	Patient 2			
4	Patient 3			
5	Patient 4			
6	Patient 5			
7	Patient 6			
8	Patient 7			

Module II: Determining Methylation Status via Southern Blot Analysis

In this module, you will analyze Southern Blot results to further investigate the FMR1 alleles from Module 1. While PCR is an effective screening tool for estimating CGG repeat size, very large repeat expansions may not amplify efficiently. Southern blot analysis is used in these cases to detect large DNA fragments and to determine whether the FMR1 gene is methylated, a key factor in gene silencing and disease expression. Larger band sizes correspond to larger, methylated CGG repeat expansions, while smaller bands correspond to normal, unmethylated repeat ranges.

Using the provided blot, you will interpret band position, number, and intensity to resolve ambiguous PCR results from Module I. You will determine whether large FMR1 expansions are present, assess methylation status, and confirm whether each patient carries a full Fragile X mutation.

STEP-BY-STEP: HOW TO ANALYZE THE SOUTHERN BLOT

Use the simulated Southern blot membrane provided and the band sizes in the table below. Work through Steps 1–4 for each patient lane.

Step 1: Draw Your Bands on the Membrane

Using the band sizes provided in Table 3, draw a horizontal line in the correct position in each patient's lane on the simulated Southern blot membrane. Using the ladder in Lane 1 as your ruler, find the matching kb marker on the left side of the membrane and draw the band at that height. *Tip: A band at 2.8 kb sits near the bottom of the blot (small fragment, traveled far). A band at 10 kb sits near the top (large fragment, didn't travel far).*

Step 2: Identify What Each Band Size Means

For each patient, look at the size of the band(s) you drew and use this key to determine the allele type:

- **2.8 kb band:** Normal FMR1 allele — gene is unmethylated and working.
- **5.2 kb band:** FMR1 allele on second inactivated X chromosome in females.
- **6 kb band:** Premutation allele — elevated CGG repeats, but gene is not fully silenced.
- **10 kb band:** Full mutation allele — very large CGG expansion. This band only appears because the methylation-sensitive enzyme COULD NOT cut the DNA, which means this region is methylated and the FMR1 gene is silenced.

Step 3: Determine Methylation Status

For each patient, decide whether their FMR1 allele is methylated or unmethylated, based on the band size:

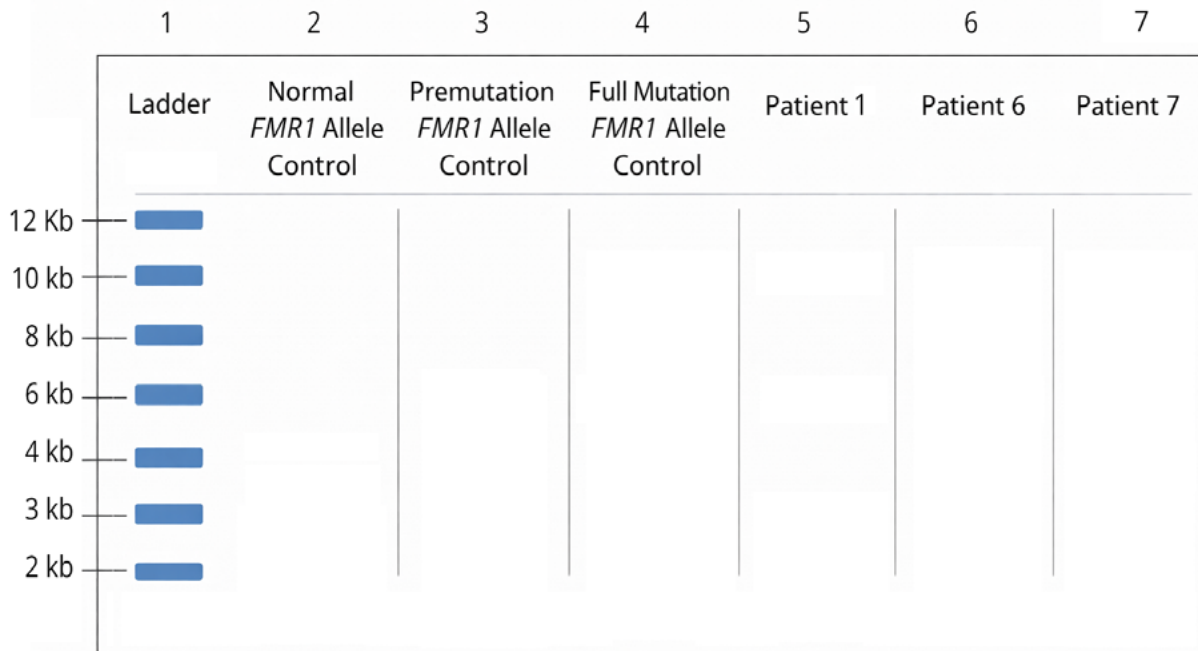
- **2.8 kb or 6 kb band:** The methylation-sensitive enzyme successfully cut the DNA here, meaning this allele is UN-METHYLATED. The FMR1 gene is active and producing FMRP.
- **10 kb band:** The enzyme could NOT cut this region — meaning it is METHYLATED. The FMR1 gene has been chemically silenced. This is the defining feature of a full mutation.

Step 4: Draw Conclusions About Each Patient

Combine your band size (Step 2) and methylation status (Step 3) to make a final determination for each patient.

Table 3: Band sizes for each sample

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
12kb Ladder	Normal FMR1 Allele Control	Premutation FMR1 Allele Control	Full Mutation FMR1 Allele Control	Patient 1	Patient 6	Patient 7
Band sizes provided on membrane	2.8kb	6kb	10kb	2.8kb	10kb	10kb, 5.2kb, 2.8kb



Southern Blot Membrane: Fragile X Testing Results

SOUTHERN BLOT RESULTS TABLE: RECORD YOUR FINDINGS

Based on the results of the southern blot analysis, what conclusions can be made about the sex, methylation status (methylated or unmethylated), and carrier status of the three patients?

Southern Blot Analysis Results			
Patient	Sex (M/F):	FMR1 allele methylation status:	Fragile X carrier status:
Patient 1			
Patient 6			
Patient 7			

Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. What is the normal CGG repeat range in unaffected individuals, and why is it considered stable?
2. Define premutation and full mutation alleles in Fragile X syndrome.
3. Why are symptoms of Fragile X syndrome generally more severe in males than in females?
4. What Fragile X-associated conditions are linked to premutation carriers, and what is their proposed molecular cause?
5. What role does PCR play in Fragile X carrier testing?
6. What is the “gray zone” in Fragile X testing, and why is it clinically relevant?
7. Why is Southern blot analysis necessary in Fragile X carrier testing?
8. Why is Southern blot particularly important when a woman’s PCR result shows only one allele?

Instructor's Guide

ADVANCE PREPARATION:

PREPARATION FOR:	WHAT TO DO:	WHEN?	TIME REQUIRED:
Module I: Agarose Gel Electrophoresis	Prepare QuickStrips™.	Up to one day before performing the experiment.	45 min.
	Prepare diluted electrophoresis buffer.		
	Prepare molten agarose and pour gels.		

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- Online catalog
- Order products
- Experiment protocols
- Tech support
- Additional resources



Pre-Lab Preparations: Module I

AGAROSE GEL ELECTROPHORESIS

This experiment requires 2.0% agarose gels. Enough reagents are provided to cast either eight 7 x 7 cm gels, eight 10 x 7 cm gels, or four 14 x 7 cm gels. You can choose whether to prepare the gels in advance or have students prepare their own. Allow approximately 30 minutes for this procedure.

Quick Reference for EDVO-Kit #S-55			
Size of gel casting tray	Groups per gel	Placement of comb	Wells required per group
7 x 7 cm	1 group	1st set of notches	8
10 x 7 cm	1 group	1st set of notches	8
14 x 7 cm	2 groups	1st and 3rd sets of notches	8

FOR MODULE I Each group will need:

- 50x concentrated buffer
- Distilled Water
- UltraSpec-Agarose™
- QuickStrip™ Samples

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.

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 B.

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.

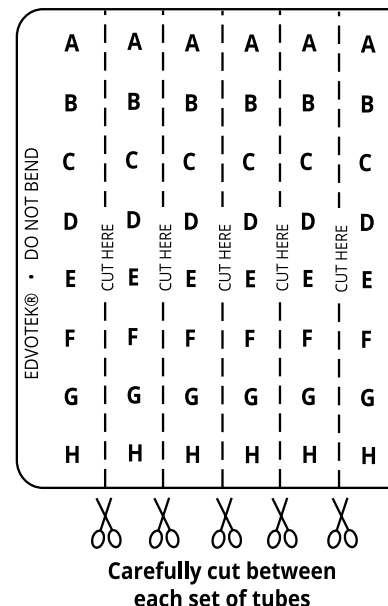
Gels that have been removed from their trays for storage should be "anchored" back to the tray with a few drops of molten agarose before being placed into the tray. This will prevent the gels from sliding around in the trays and the chambers.

SAMPLES FORMAT: PREPARING THE QUICKSTRIPS™

In this experiment, the QuickStrip™ samples contain the simulated PCR samples. (PCR amplification is not performed in this experiment.) Students will analyze these PCR products using gel electrophoresis. QuickStrip™ tubes consist of a microtiter block covered with a protective foil overlay. Each well contains pre-aliquoted sample.

Using sharp scissors, carefully divide the block of tubes into individual strips by cutting between the rows (see diagram at right). Take care not to damage the foil overlay while separating the samples.

Each student group will receive one set of QuickStrip tubes containing eight samples (one ladder and seven patient PCR samples). Before loading the gel, remind students to tap the tubes to collect the sample at the bottom of the tube. Puncture the foil overlay of the QuickStrip™ with a pipet tip to aspirate the sample. **DO NOT REMOVE THE FOIL AS SAMPLES CAN SPILL.**



Experiment Results and Analysis

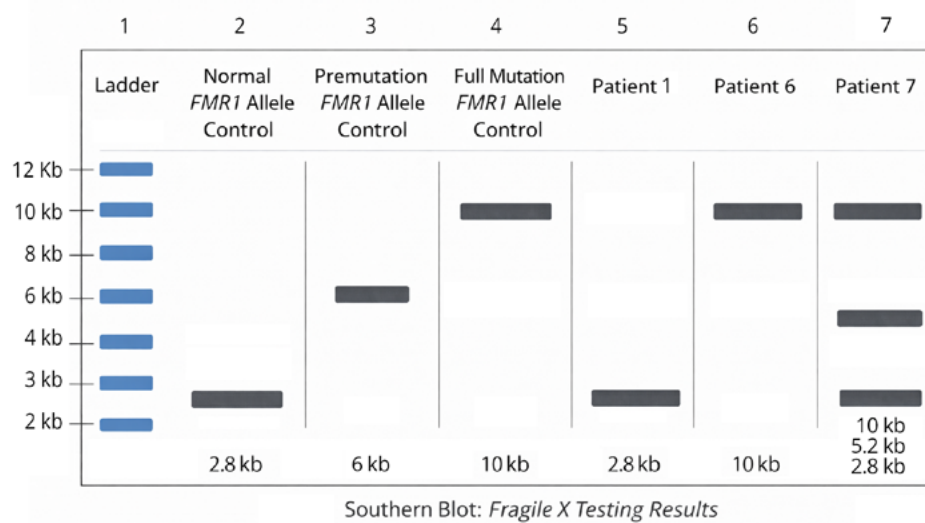
MODULE I



PCR Results Analysis				
Lane	Sample	Sex (M/F)	Carrier Status	Notes / Southern Blot Needed?
1	<i>FMR1</i> CGG Repeat Ladder	—	Reference only	—
2	Patient 1	Male OR Female	Inconclusive	YES, Southern Blot needed
3	Patient 2	Female	Gray zone carrier	No, Southern Blot not required
4	Patient 3	Female	Premutation carrier	No, Southern Blot not required
5	Patient 4	Male	Gray zone carrier	No, Southern Blot not required
6	Patient 5	Male	Premutation carrier	No, Southern Blot not required
7	Patient 6	Male	Suspected full mutation	YES, Southern Blot needed
8	Patient 7	Male OR Female	Inconclusive	YES, Southern Blot needed

Experiment Results and Analysis, continued

MODULE II



Based on the results of the southern blot analysis, what conclusions can be made about the sex, methylation, and carrier status of the three patients?

Southern Blot Analysis Results			
Patient	Sex (M/F):	<i>FMR1</i> allele methylation status:	<i>Fragile X</i> carrier status:
Patient 1	Male	1 normal <i>FMR1</i> allele	Non carrier
Patient 6	Male	Methylated	Full mutation carrier
Patient 7	Female	1 normal <i>FMR1</i> allele, 1 methylated <i>FMR1</i> allele	Full mutation carrier

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

Appendices

- A EDVOTEK® Troubleshooting Guide
- B Bulk Preparation of Electrophoresis Buffer and Agarose Gels

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

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- Online catalog
- Order products
- Experiment protocols
- Tech support
- Additional resources



Appendix A

EDVOTEK® Troubleshooting Guides

PROBLEM:	CAUSE:	ANSWER:
Bands are not visible on the gel.	The electrophoresis buffer was not prepared properly.	Ensure that the electrophoresis buffer was correctly diluted.
	The dyes ran off the gel because the polarity of the leads was reversed.	Ensure that leads are attached in the correct orientation.
	Malfunctioning electrophoresis unit or power source.	Contact the manufacturer of the electrophoresis unit or power source.
Very light colored bands seen after electrophoresis.	Pipetting error.	Make sure students pipet 35 µL of dye sample per well.
Poor separation of bands.	Gel was not prepared properly.	Make sure to prepare a 2.0% gel.
Dye bands disappear when gels are kept at 4 °C.	The dye molecules are small and will diffuse out of the gel.	The results must be analyzed upon the completion of electrophoresis.

Visit www.edvotek.com for additional troubleshooting suggestions.

Appendix B

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.

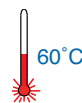
Table D Bulk Preparation of Electrophoresis Buffer			
50x Conc. Buffer	+	Distilled Water	Total Volume Required
60 mL		2,940 mL	3000 mL (3 L)

Batch Agarose Gels (2.0%)

For quantity (batch) preparation of 2.0% agarose gels, see Table E.

Table E Batch Prep of 2.0% UltraSpec-Agarose™					
Amt of Agarose	+	Concentrated Buffer (50X)	+	Distilled Water	Total Volume
7.5 g		7.5 mL		367.5 mL	375 mL

1. Use a 500 mL flask to prepare the diluted gel buffer.
2. Pour 7.5 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 30 mL for a 7 x 7 cm tray, 45 mL for a 10 x 7 cm tray, and 60 mL for a 14 x 7 cm tray. **For this experiment, 10 x 7 cm gels are recommended.**
7. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Solidified gels can be stored under buffer in the refrigerator for up to 2 weeks. Do not freeze gels.



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.

PROCEED to Loading and Running the Gel (page 12).