Edvo-Kit # 330

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PCR Amplification of DNA

Experiment Objective:

The objective of this experiment is for students to gain hands-on experience of the principles and practice of Polymerase Chain Reaction (PCR). The PCR products are then analyzed by agarose gel electrophoresis.

See page 3 for storage instructions.

Version #330.240214

PROTOCOL HAS BEEN UPDATED!
Please review before beginning experiment!

EDVOTEK®

Table of Contents

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

Experiment Components

Component		Storage	Check (✓)
•	PCR EdvoBeads™ PLUS*	-20°C Freezer	❑
A	TE buffer	-20°C Freezer	❑
B	Ultra-pure Water	-20°C Freezer	❑
C	LyphoPrimer™ Mix	-20°C Freezer	❑
D	LyphoTemplate™	-20°C Freezer	❑
E	DNA Standard Marker	-20°C Freezer	❑
•	SYBR® Safe DNA Stain	-20°C Freezer	❑

NOTE: Components C and D are supplied in our LyphoPrimer™ and LyphoTemplate™ format. They will require reconstitution before use. Be sure to review Page 21 in the Instructor's Guide for more details.

**Each PCR EdvoBead™ PLUS contains: dNTP Mixture, Taq DNA Polymerase Buffer, Taq DNA Polymerase, $MgCl_2$, and Reaction Buffer).*

This experiment is designed for 10 lab groups of 2-4 students each.

NEW Update:
PCR EdvoBeads™ are now conveniently stored at -20°C with the other perishable components.

Reagents & Supplies

Store all components below at room temperature.

- UltraSpec-Agarose™ ❑
- 50X Electrophoresis Buffer ❑
- FlashBlue™ Stain ❑
- Microcentrifuge Tubes ❑
- PCR Tubes ❑

NOTE: Check that your PCR program matches the protocol found in the student and instructor's guides before starting the experiment!

Requirements

- Thermal cycler (EDVOTEK® Cat. #540 or #541-542 highly recommended) *
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Balance
- Microcentrifuge
- UV Transilluminator or Blue light visualization (EDVOTEK® Cat #557 recommended)
- UV safety goggles
- White light visualization system (use if staining with FlashBlue™) (EDVOTEK® Cat. #552 recommended)
- Automatic micropipettes (5-50 µL) with tips
- Microwave
- Pipet pump
- 250 mL flasks or beakers
- Hot gloves
- Disposable vinyl or latex laboratory gloves
- Ice buckets and ice
- Distilled or deionized water

**If you do not have a thermal cycler, this experiment can be conducted using three water baths with proper care. However, a thermal cycler assures a significantly higher rate of success. See Appendix B for detailed instructions.*

Background Information

THEORY OF PCR

The polymerase chain reaction (PCR) is a DNA amplification technique that has revolutionized almost all aspects of biological research. PCR was invented in 1984 by Dr. Kary Mullis at the Cetus Corporation in California. The enormous utility of the PCR method is based on its ease of use and its ability to allow the amplification of small DNA fragments. For this ground breaking technology, Mullis was awarded the Nobel Prize in Chemistry in 1993.

Before performing PCR, template DNA is extracted from various biological sources. Because PCR is very sensitive, only a few copies of the gene are required. Nevertheless, freshly isolated DNA will provide better amplification results than older DNA specimens that may have become degraded. In order to amplify the specific DNA or target sequence, two primers (short, synthetic DNA molecules) are designed to correspond to the ends of the target sequence. The primers hybridize to the DNA template, which marks this sequence to be copied by DNA Polymerase. Starting from the primer, DNA Polymerase builds a new strand of DNA in the 5' –-> 3' direction, using the DNA template as a guide.

To perform PCR, the template DNA and a molar excess of primers are mixed with the four "free" deoxynucleotides (dATP, dCTP, dGTP, and dTTP), and a thermostable DNA polymerase. The most commonly used DNA polymerase is *Taq* DNA polymerase. This enzyme, originally purified from a bacterium that inhabits hot springs, is stable at very high temperatures. These components (template DNA, primers, the four deoxynucleotides, and *Taq* DNA polymerase) are mixed with a buffer that contains Mg^{+2}, an essential cofactor for *Taq* polymerase. The PCR reaction mixture is subjected to sequential heating/cooling cycles at three different temperatures in a thermal cycler.

- In the first step, known as "denaturation", the mixture is heated to near boiling (94°C - 96°C) to "un-zip" (or melt) the target DNA. The high temperature disrupts the hydrogen bonds between the two complementary DNA strands and causes their separation.

- In the second step, known as "annealing", the reaction mixture is cooled to 45°C - 65°C, which allows the primers to base pair with the target DNA sequence.

- In the third step, known as "extension", the temperature is raised to 72°C. This is the optimal temperature at which *Taq* polymerase can add nucleotides to the hybridized primers to synthesize the new complementary strands.

These three steps - denaturation, annealing, and extension - constitute one PCR "cycle" (Figure 1). Each PCR cycle doubles the amount of the target DNA in less than five minutes. In order to produce enough DNA for analysis, twenty to forty cycles may be required. To simplify this process, a specialized machine, called a "thermal cycler" or a "PCR machine", was created to rapidly heat and cool the samples.

PRACTICE OF PCR

Mathematically, PCR can be expressed as an exponential relationship – if we begin with a starting copy number of m, then after n cycles, we will have $m \times 2^n$ copies of our DNA target. For example, in Figure 1 we start with one copy of our template DNA. After three rounds of PCR, we end up with eight copies of the DNA template. If the amplification continued for a total of 20 cycles, the polymerase chain reaction would have produced over one million copies of the original DNA template. In theory, this process could continue indefinitely. In practice, after many cycles (regardless of the amount of DNA present in the starting material) the amount of DNA produced reaches a maximum where a product curve flattens out, known as the plateau (Figure 2). This leveling off of the curve is due to the depletion of reaction components like primers and nucleotides and the loss of *Taq* polymerase activity.

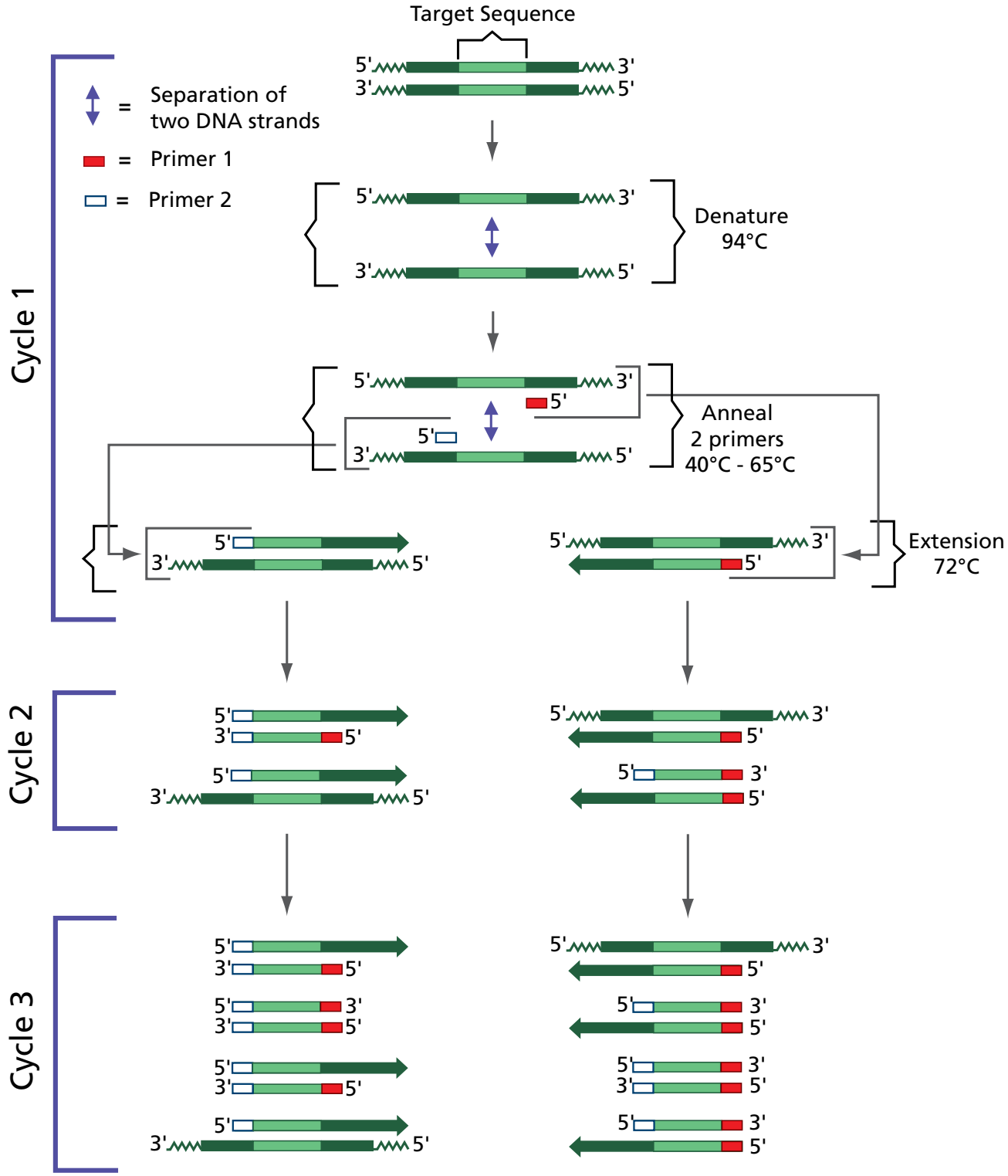
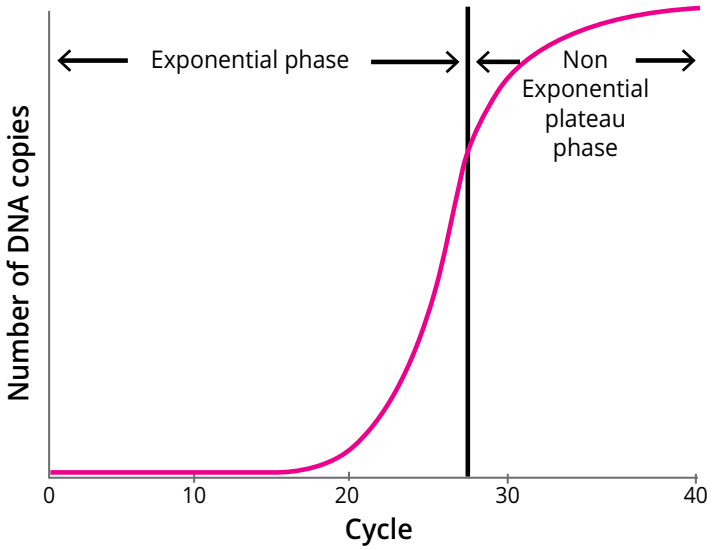


Figure 1:
Polymerase Chain Reaction

The exact temperature and incubation time required for each step depends on several factors, including the length of the target DNA and GC content of the primer/template. In some cases, the annealing and extension steps may be combined resulting in a two step PCR experiment.

One common problem that occurs during PCR is unwanted amplification products. These are due to contamination of the sample or nonspecific annealing of the primers. To reduce contamination, autoclaved tubes, pipette tips, and sterile water should be used. Gloves should always be worn when performing PCR. To minimize unwanted PCR products due to nonspecific primer annealing, the primer concentration should be minimized, if possible. Another common technique is "hot start", in which the components of the PCR reaction are fully mixed only after the DNA is fully denatured above 94°C.

Figure 2: Graph showing the exponential phase and plateau phase of PCR.



Because of its ease of use and its ability to rapidly amplify DNA, PCR has become indispensable in medical and life sciences labs, replacing as the method of choice more time- and labor-intensive methods such as Southern blotting. For example, today's research laboratories can quickly create copies of a specific region of DNA for cloning applications. Medical diagnostics use PCR to identify genetic mutations and infectious agents. In addition, because amplification by PCR requires very little starting material, it is ideal for forensic analysis of biological samples or determination of paternity.

Experiment Overview

EXPERIMENT OBJECTIVE:

The objective of this experiment is for students to gain hands-on experience of the principles and practice of Polymerase Chain Reaction (PCR). The PCR products are then analyzed by agarose gel electrophoresis.

Wear gloves and safety goggles

LABORATORY SAFETY:

Be sure to READ and UNDERSTAND the instructions completely BEFORE starting the experiment. If you are unsure of something, ASK YOUR INSTRUCTOR!

- Wear gloves and goggles while working in the laboratory.
- Exercise caution when working in the laboratory – you will be using equipment that can be dangerous if used incorrectly.
- Wear protective gloves when working with hot reagents like boiling water and melted agarose.
- DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
- Always wash hands thoroughly with soap and water after working 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

Performing the Polymerase Chain Reaction - 90 min.

↓

MODULE II

Analysis of PCR Products by Electrophoresis - 30 to 45 min.

Staining Agarose Gels - 5 to 30 min.

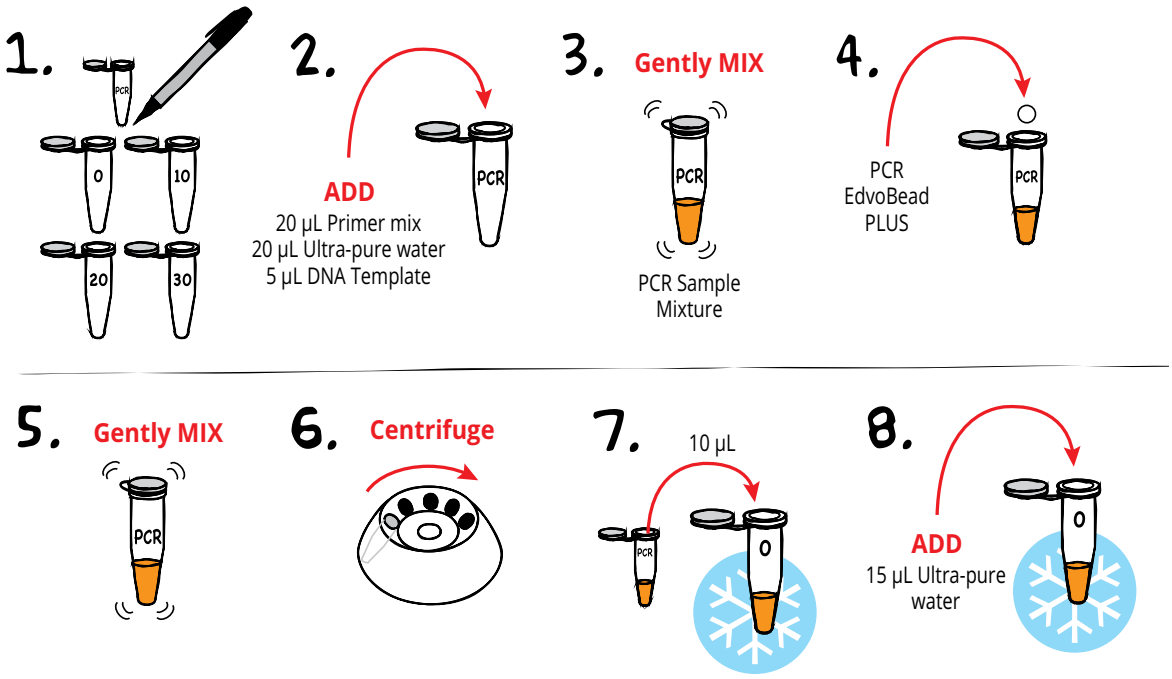
↓

MODULE III

Size Determination of the Amplified PCR Products - 15 min.

NOTE: Experimental times are approximate.

Module I: Performing the Polymerase Chain Reaction



NOTE: Check that your PCR program matches the protocol found in the student and instructor's guides before starting the experiment!

Wear gloves and safety goggles

1. **LABEL** one 0.2 mL PCR tube with "PCR" and your group name or initials. **LABEL** four 0.5 mL tubes with "0", "10", "20", or "30" AND your group name or initials.

2. **ADD** 20 µL Primer Mix (orange), 20 µL Ultrapure water, and 5 µL DNA template (red) to the 0.2 mL "PCR" tube with your initials.

3. **MIX** the sample by gently flicking the tube. This is the PCR sample mixture. The solution should be orange in color. ***NOTE: If the solution is not orange, the PCR sample has not been correctly assembled.***

4. **TRANSFER** one PCR EdvoBead™ PLUS to the labeled 0.2 mL "PCR" tube. Gently **TAP** the tube on the bench top to move the PCR EdvoBead™ PLUS into the liquid at the bottom of the tube.

5. **MIX** the sample gently. Make sure the PCR EdvoBead™ PLUS is completely dissolved.

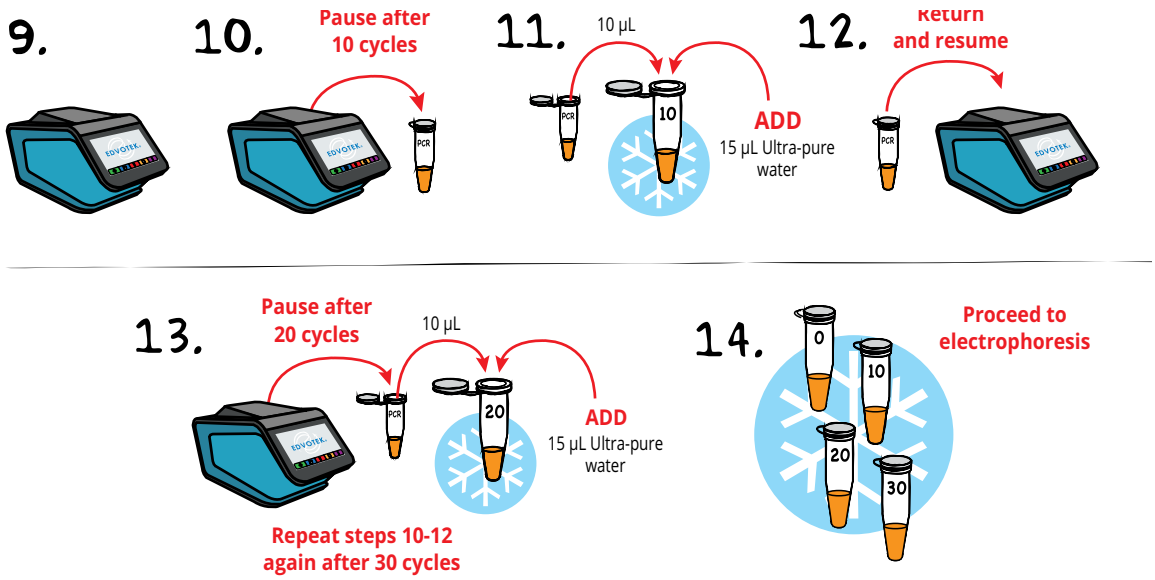
6. Quickly **CENTRIFUGE** to collect the sample at the bottom of the tube.

7. **TRANSFER** 10 µL of the PCR sample mixture to tube "0". **PLACE** the "PCR" tube on ice.

8. **ADD** 15 µL Ultra-pure Water to the PCR sample mixture in tube "0". **PLACE** tube "0" on ice until ready for electrophoresis.

continued

Module I: Performing the Polymerase Chain Reaction, continued



9. **AMPLIFY** DNA using PCR guidelines listed below.

For this experiment, cycling needs to be paused after the 10th, 20th and 30th cycle.

- 94°C for 15 seconds
- 45°C for 15 seconds } 30 cycles
- 72°C for 30 seconds
- Final extension at 72°C for 60 seconds.

NOTE: If you do not have a thermal cycler, this experiment can be conducted using three water baths with proper care. ***However, a thermal cycler assures a significantly higher rate of success.***

See Appendix B for detailed instructions.

10. **PAUSE** the thermal cycler after the 10th cycle. **REMOVE** your PCR sample from the thermal cycler. Take care when removing the sample because the heat block and heated lid may be hot.

11. **TRANSFER** 10 µL of the PCR mixture from your "PCR" tube to the tube labeled "10". **ADD** 15 µL Ultra-pure Water to the tube labeled "10". **PLACE** tube "10" on ice until ready for electrophoresis.

12. **RETURN** the "PCR" tube to the thermal cycler and resume cycling.

13. **REPEAT** steps 10-12 when the PCR program reaches both cycle 20 and cycle 30. BE SURE to transfer DNA sample to the appropriately labeled tube.

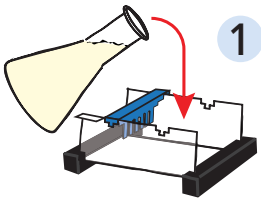
14. After PCR, **PROCEED** to Module II: Separation of PCR Products by Electrophoresis.

OPTIONAL STOPPING POINT:
PCR samples may be stored at -20°C for electrophoresis at a later time.

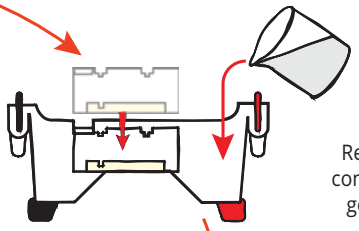
OVERVIEW

MODULE II-A: Agarose Gel Electrophoresis

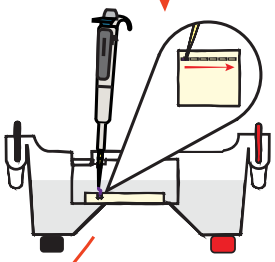
Time required: See Table C



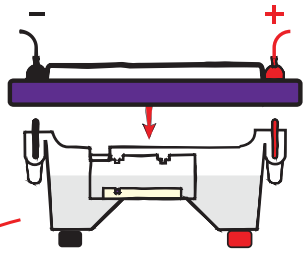
1 Prepare agarose gel in casting tray.



2 Remove end caps & comb, then submerge gel under buffer in electrophoresis chamber.



3 Load each sample in consecutive wells

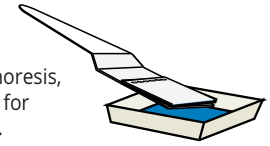


4 Attach safety cover & connect leads to power source to conduct electrophoresis.

(OPTIONAL) MODULE II-B: Staining Using FlashBlue™

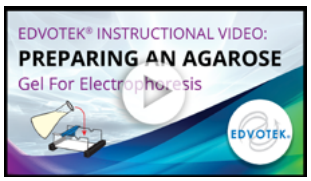
Time required: 30 min.

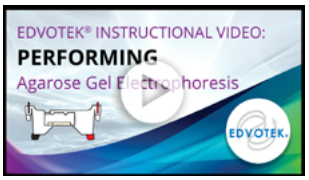
5 After electrophoresis, transfer gel for staining.

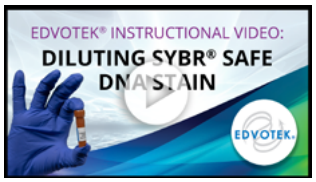


6 Analysis on white light source.

Related EDVOTEK® Instructional Videos

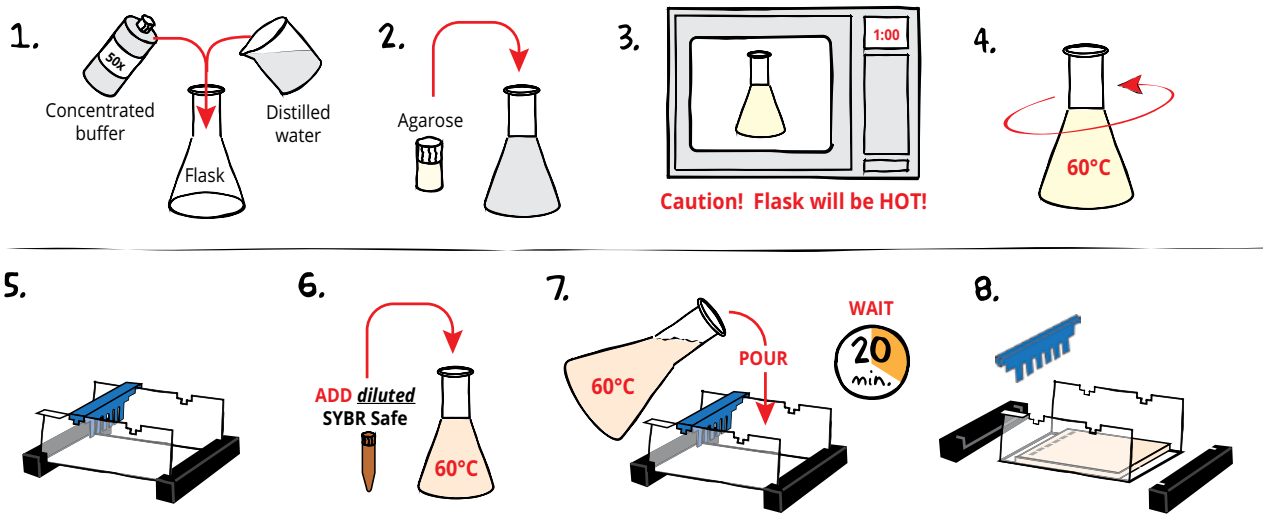






www.youtube.com/edvotekinc

Module II-A: Separation of PCR Products by Electrophoresis



CASTING THE AGAROSE GEL

1. **DILUTE** concentrated (50X) buffer with distilled water to create 1X buffer (see Table A).
2. **MIX** agarose powder with 1X buffer in a 250 mL flask (see 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. Before casting the gel, **ADD *diluted* SYBR® Safe** to the molten agarose and swirl to mix (see Table A).
7. **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.
8. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

Wear gloves and safety goggles

IMPORTANT:

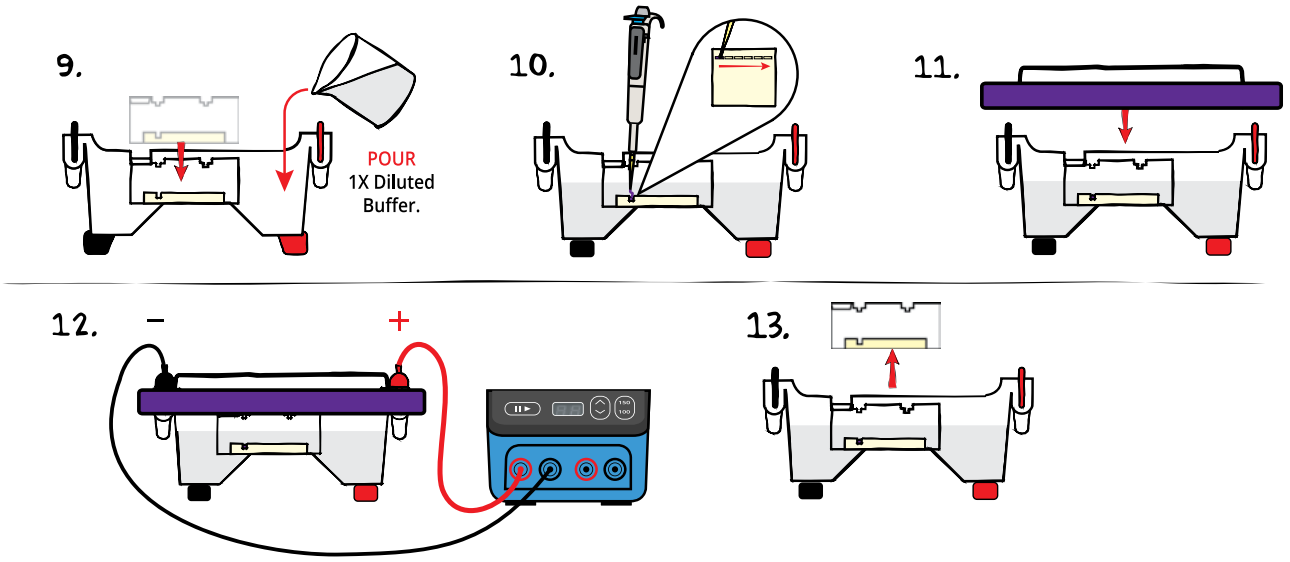
This experiment requires 0.8% agarose gels cast with 6 wells. 7 x 7 cm gels are recommended. Each gel can be shared by one student group.

Table A — **Individual 0.8% UltraSpec-Agarose™ with SYBR® Stain**

Size of Gel Casting tray	Concentrated Buffer (50x)	+ Distilled Water	+ Amt of Agarose	= TOTAL Volume	Diluted SYBR® (Step 6)
7 x 7 cm	0.6 mL	29.4 mL	0.24 g	30 mL	30 µL
10 x 7 cm*	0.9 mL	44.1 mL	0.36 g	45 mL	45 µL
14 x 7 cm	1.2 mL	58.8 mL	0.48 g	60 mL	60 µL

** Recommended gel volume for the EDGE™ Integrated Electrophoresis System.*

Module II-A: Separation of PCR Products by Electrophoresis, continued



RUNNING THE GEL

9. **PLACE** gel (on the tray) into electrophoresis chamber. **COVER** the gel with 1X electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.
10. Using Table 1 as a guide, **LOAD** the entire sample (25 µL) into the wells in consecutive order.
11. **PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
12. **CONNECT** leads to the power source and **PERFORM** electrophoresis. (See Table C for time and voltage guidelines.)
13. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber.

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

TABLE 1: GEL LOADING

Lane	Recommended	Sample Name
1	Marker	DNA Standard Marker
2	0	Reaction sample after 0 cycles
3	10	Reaction sample after 10 cycles
4	20	Reaction sample after 20 cycles
5	30	Reaction sample after 30 cycles

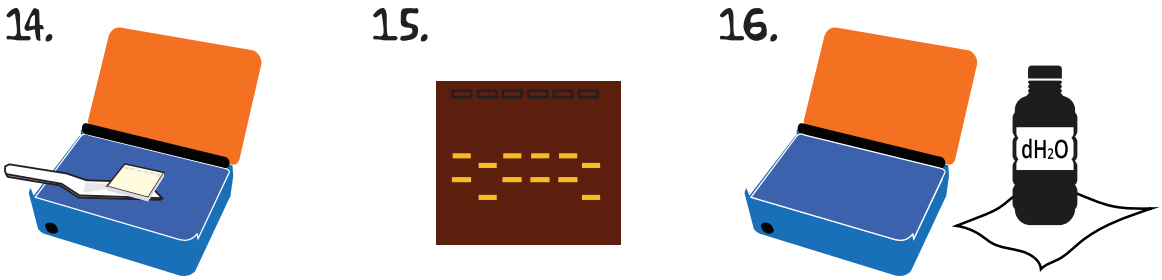
Table B — **1x Electrophoresis Buffer (Chamber Buffer)**

EDVOTEK Model #	Total Volume Required	Dilution: 50x Conc. Buffer	+ 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 (0.8% Agarose Gel)**

	Electrophoresis Model: EDGE™	M12 & M36
Volts	Min/Max (minutes)	Min/Max (minutes)
150	10/20	20/35
125	N/A	30/45
100	15/25	40/60

Module II-A: Separation of PCR Products by Electrophoresis, continued



VISUALIZING THE SYBR® GEL

14. **SLIDE** gel off the casting tray onto the viewing surface of the transilluminator.

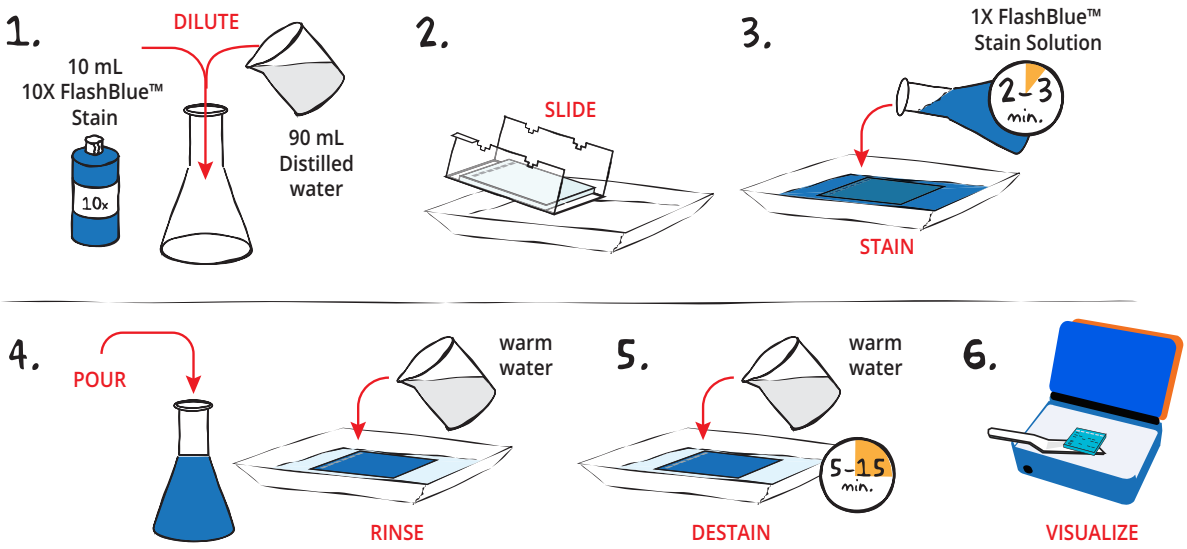
15. **TURN** the unit on. DNA should appear as bright green bands on a dark background. **PHOTOGRAPH** results.

16. **TURN** the unit off. **REMOVE** and **DISPOSE** of the gel and **CLEAN** the transilluminator surfaces with distilled water

Be sure to wear UV goggles if using a UV transilluminator.

Module II-B: Staining Using FlashBlue™ (OPTIONAL)

FlashBlue™ Stain is a simple and effective visible DNA stain that can be used as an alternative, or in addition to, UV-reactive DNA stains like SYBR® Safe. ***IF staining with both SYBR® Safe and FlashBlue™, you must examine and record the SYBR® Safe bands before beginning the FlashBlue™ Staining.***



1. **DILUTE** 10 mL of 10X concentrated FlashBlue™ with 90 mL of distilled water in a flask. **MIX** well.
2. **REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off the casting tray into a small, clean gel-staining tray.
3. **COVER** the gel with the 1X FlashBlue™ stain solution. **STAIN** the gel for 2-3 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 3 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**
4. **POUR** the 1X FlashBlue™ back into the flask (the stain can be reused). **COVER** the gel with warm water (40-45 °C). Gently **RINSE** the gel for 20-30 seconds. **POUR** off the water.
5. **COVER** the gel with clean, warm water (40-45 °C). **DESTAIN** for 5-15 minutes with gentle shaking (longer periods will yield better results). DNA bands will start to appear after 5 minutes of destaining. Changing the water frequently will accelerate destaining.
6. Carefully **REMOVE** the gel from the destaining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.


Wear gloves and safety goggles

ALTERNATIVE FLASHBLUE™ STAINING PROTOCOL:

1. **DILUTE** 1 mL of 10X FlashBlue™ stain with 149 mL distilled water.
2. **COVER** the gel with diluted FlashBlue™ stain.
3. **SOAK** the gel in the staining liquid for at least three hours. For best results, stain gels overnight.
4. Carefully **REMOVE** the gel from the staining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

Module III: Size Determination of Amplified PCR Products

Agarose gel electrophoresis separates DNA molecules into discrete bands, each comprising molecules of the same size. How can these results be used to determine the lengths of different fragments? Remember, as the length of a DNA molecule increases, the distance to which the molecule can migrate decreases because large molecules cannot pass through the channels in the gel with ease. Therefore, the migration rate is inversely proportional to the length of the molecules—more specifically, to the $\log_{10}$ of molecule's length. To illustrate this, we ran a sample that contains bands of known lengths called a "standard". We will measure the distance that each of these bands traveled to create a graph, known as a "standard curve", which can then be used to extrapolate the size of unknown molecule(s).

Figure 3: Measure distance migrated from the lower edge of the well to the lower edge of each band.

1. Measure and Record Migration Distances

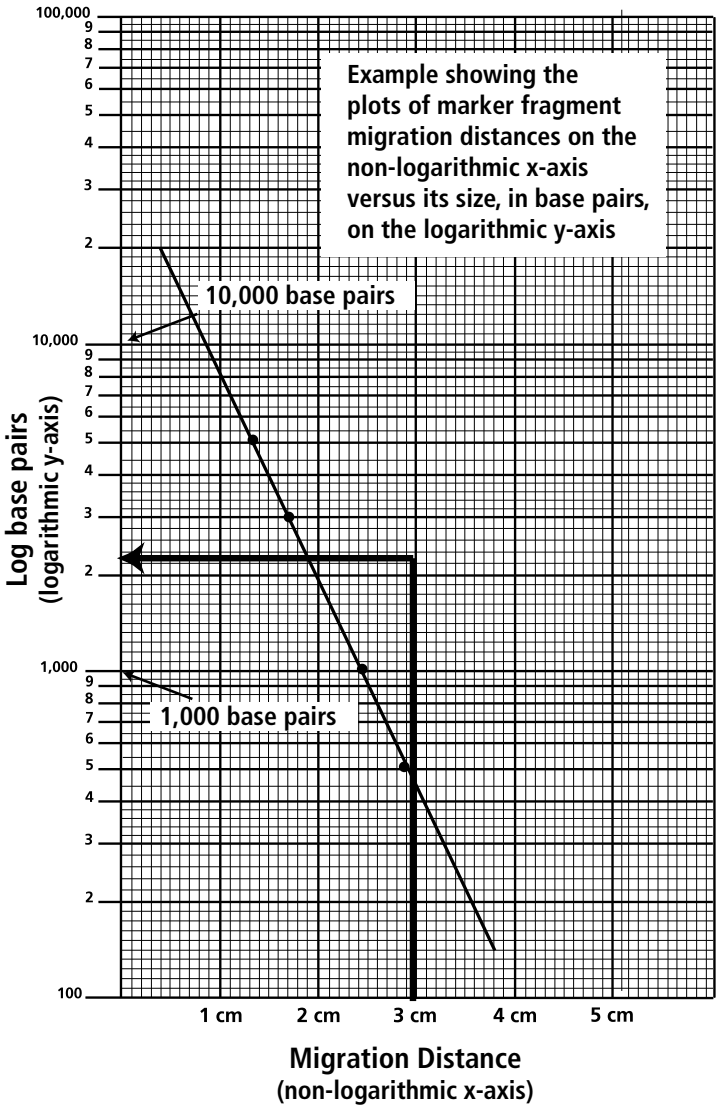
Measure the distance traveled by each Standard DNA Fragment from the lower edge of the sample well to the lower end of each band (Figure 3). Record the distance in centimeters (to the nearest millimeter) in your notebook. Repeat this for each DNA fragment in the standard.

Measure and record the migration distances of each of the fragments in the unknown samples in the same way you measured the standard bands.

2. Generate a Standard Curve.

Because migration rate is inversely proportional to the $\log_{10}$ of band length, plotting the data as a semi-log plot will produce a straight line and allow us to analyze an exponential range of fragment sizes. You will notice that the vertical axis of the semi-log plot appears atypical at first; the distance between numbers shrinks as the axis progresses from 1 to 9. This is because the axis represents a logarithmic scale. The first cycle on the y-axis corresponds to lengths from 100-1,000 base pairs, the second cycle measures 1,000-10,000 base pairs, and so on. To create a standard curve on the semi-log paper, plot the distance each Standard DNA fragment migrated on the x-axis (in mm) versus its size on the y-axis (in base pairs). Be sure to label the axes!

Figure 4: Semilog graph example.



Module III: Size Determination of Amplified PCR Products, continued

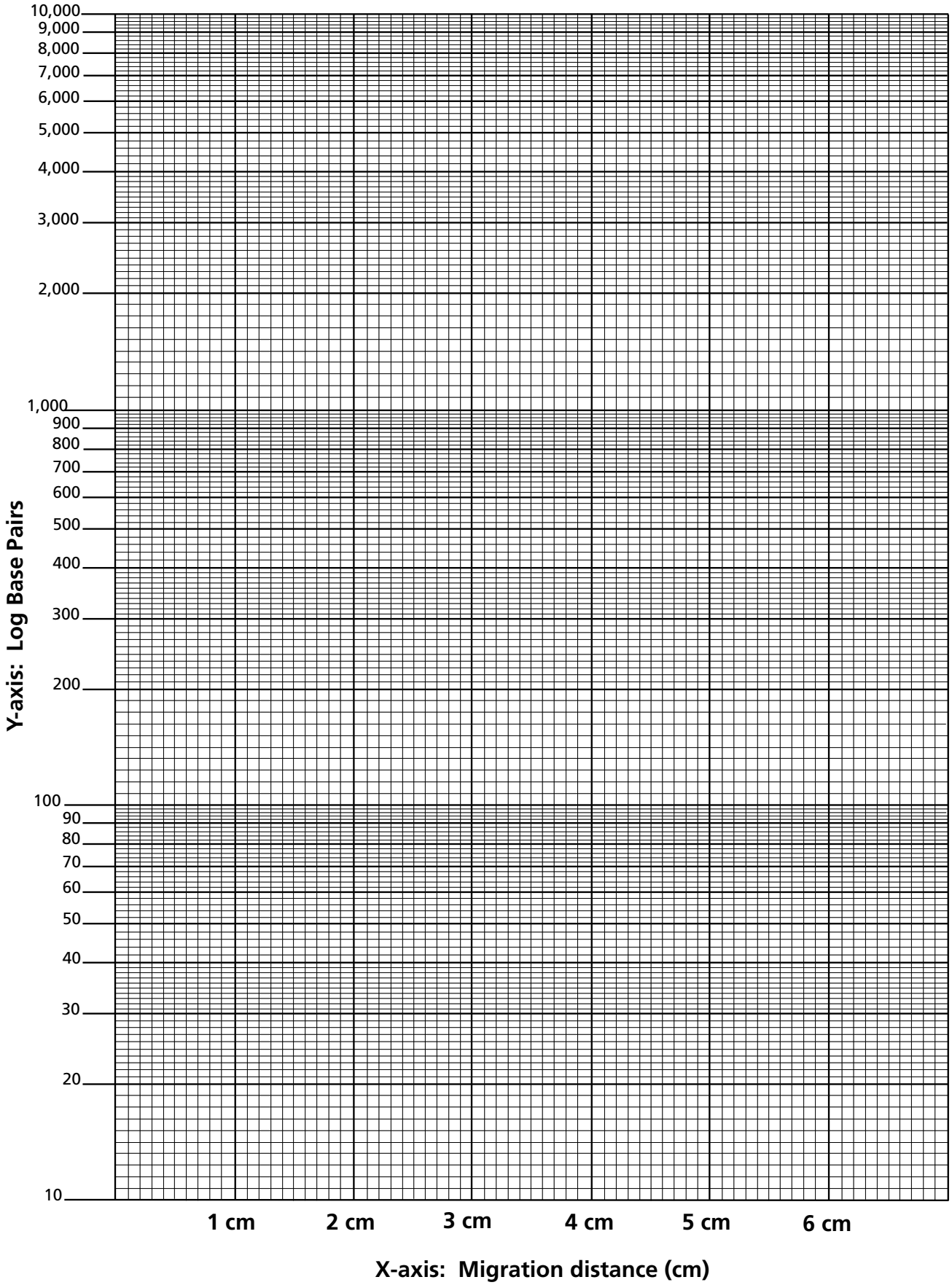
After all the points have been plotted, use a ruler or a straight edge to draw the best straight line possible through the points. The line should have approximately equal numbers of points scattered on each side of the line. It is okay if the line runs through some points (see Figure 4 for an example).

3. Determine the length of each unknown fragment.

a. Locate the migration distance of the unknown fragment on the x-axis of your semi-log graph. Draw a vertical line extending from that point until it intersects the line of your standard curve.

b. From the point of intersection, draw a second line, this time horizontally, toward the y-axis. The value at which this line intersects the y-axis represents the approximate size of the fragment in base pairs (refer to Figure 4 for an example). Make note of this in your lab notebook.

c. Repeat for each fragment in your unknown sample.



Study Questions

1. Why is a thermostable DNA polymerase required for DNA amplification by PCR?
2. If starting with one copy of the DNA template, how many copies of the DNA template have been produced after four complete cycles of PCR? After 8 cycles?
3. Why are two different primers required for the PCR reaction?
4. What are possible reasons for obtaining fainter (usually smaller) bands besides the main PCR product?

Instructor's Guide

OVERVIEW OF INSTRUCTOR'S PRELAB PREPARATION:

This section outlines the recommended prelab preparations and approximate time requirement to complete each prelab activity.

Preparation For:	What to do:	When:	Time Required:
Module I: Performing the Polymerase Chain Reaction	Prepare and aliquot various reagents (Primer, DNA template, ladder, etc.)	One day to 30 min. before performing the experiment.	30 min.
	Program Thermal Cycler OR Set up waterbaths (94° C, 45° C and 72° C)	Any time before performing the experiment.	15 min.
Module II: Separation of PCR Products by Electrophoresis	Prepare diluted electrophoresis buffer	Up to one week before performing the experiment.	45 min.
	Prepare molten agarose and pour batch gels (OPTIONAL)		
Module II-A: SYBR® Safe Stain	Prepare SYBR® Safe Stain	Up to 24 hours before performing the experiment.	10 min.
Module II-B: FlashBlue™ Staining	Prepare staining components	The class period or overnight after the class period.	10 min.
Module III: Size Determination of Amplified PCR Products	Make copies of graph paper	Any time before the class period.	10 min.

Red = Prepare immediately before module. Yellow = Prepare shortly before module. Green = Flexible / prepare up to a week before the module.

IMPORTANT NOTE: Before running the experiment, confirm that your PCR program matches the settings below.

For this experiment, cycling needs to be paused after the 10th, 20th and 30th cycle.

- 94°C for 15 seconds
- 45°C for 15 seconds
- 72°C for 30 seconds

} 30 cycles

- Final extension at 72°C for 60 seconds.

Pre-Lab Preparations - Module I: Performing the Polymerase Chain Reaction

This kit features EDVOTEK® LyphoTemplate™ and LyphoPrimer™. The reagents are also color coded so that a correctly assembled PCR reaction should appear orange in color. These innovations will help ensure experimental success.

FOR MODULE I
Each Group should receive:
- One PCR tube and one PCR EdvoBead™ PLUS
- 25 µL Diluted Primer Mix
- 6 µL Diluted DNA Template
- 100 µL Ultra-pure water
- Four Microcentrifuge tubes

Preparation of the Primer Mix

1. Thaw the TE buffer (A). Mix well before using.
2. Before preparing the primer mix, make sure the solid material is at the bottom of the LyphoPrimer™ Tube (C). If not, centrifuge the tube at full speed for 20 seconds or tap the tube on the lab bench.
3. Dilute the LyphoPrimer™ by adding 1 mL of TE Buffer to the tube. Cap and mix well and place on ice. The solution should be clear and light orange in color, and no solid pieces should remain.
4. Dispense 25 µL of the diluted primer per tube. Label these 10 tubes "Primer Mix". Distribute one tube per student group.

Preparation of the DNA Template

1. Thaw the TE buffer (A). Mix well before using.
2. Before preparing the DNA template, make sure the solid material is at the bottom of the LyphoTemplate™ Tube (D). If not, centrifuge the tube at full speed for 20 seconds or tap the tube on the lab bench.
3. Dilute the LyphoTemplate™ by adding 70 µL of TE Buffer to the tube. Cap and mix well and place on ice. The solution should be clear and light red in color, and no solid pieces should remain.
4. Dispense 6 µL of the diluted DNA template per tube. Label these 10 tubes "DNA Template". Distribute one tube per student group.

IMPORTANT:
The PCR EdvoBeads™ absorb moisture if left open, even for a short period of time. Only open the vial when you are ready to use the beads. If you are not using all of the beads in one session, quickly remove the required number and promptly secure the gray stopper back into the vial. Store the vial of unused beads at -20°C (desiccated) until needed.

PCR EdvoBeads™

NOTE: The beads are vacuum-sealed and may move around once the stopper is removed. Only open the vial when you are ready to use the beads and quickly recap the vial.

Dispense the PCR EdvoBeads™ just prior to the students performing their PCR reactions. Alternatively, student groups can dispense PCR EdvoBeads™ during Module I. Each group will require 1 bead and 1 tube.

1. Open the vial of PCR EdvoBeads™ (carefully remove metal crimp) and gently/slowly remove the gray rubber stopper.
2. With gloved hand, forceps or sterile loop, remove one single bead and transfer to a PCR tube. Do this for the number of reactions you wish to perform. Quickly cap each of the tubes and distribute to the student groups.

Additional Materials

- Dispense 100 µL Ultra-pure water (B) per student group.
- Each student group receives one 0.2 mL PCR tube and four microcentrifuge tubes.

PCR Amplification

The Thermal cycler should be programmed as outlined on page 20 and in Module I in the Student's Guide.

- Accurate temperatures and cycle times are critical. A pre-run for one cycle (takes approximately 3 to 5 min.) is recommended to check that the thermal cycler is properly programmed.

Pre-Lab Preparations - Module II-A

ANALYSIS OF PCR PRODUCTS BY ELECTROPHORESIS

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

Prepare SYBR® Safe Stain:

1. Following the instructions in Appendix C, prepare 1x Electrophoresis Buffer by combining 10 µL of 50X Concentrated Buffer with 490 µL of distilled water.

2. Add 390 µL of the 1X buffer from step 1 to the tube of SYBR® Safe and mix by tapping the tube several times. The diluted SYBR® Safe Stain is now ready to be used during agarose gel preparation.

FOR MODULE II-A
Each Group should receive:
- 50X electrophoresis buffer
- Distilled Water
- UltraSpec-Agarose™ Powder
- Diluted SYBR® Safe
- 35 µL DNA Standard Marker

Individual Gel Preparation:
This experiment requires a total of ten 0.8% agarose gels for the class. 7 x 7 cm gels are recommended. Each student group can be responsible for casting their own individual gel prior to conducting the experiment. See Module II in the Student's Experimental Procedure. Students will need 50X electrophoresis buffer, distilled water, agarose powder, and ***diluted*** SYBR® Safe Stain.

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 C.

Preparing Gels in Advance:
Gels may be prepared ahead and stored for later use. Solidified gels can be store in the refrigerator for up to 2 weeks. Place 1-2 mL of electrophoresis buffer in a sealable bag with the gels to prevent them from drying out. Excessive buffer will cause SYBR® Safe to diffuse out of the gels.

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.

Additional Materials:
Each 0.8% gel should be loaded with the DNA Standard Marker. Aliquot 35 µL of DNA Standard Marker (E) into labeled microcentrifuge tubes and distribute one tube per gel.

Visualizing SYBR® Safe-Stained Gels:
SYBR® Safe is a DNA stain that fluoresces when bound to double-stranded DNA, allowing us to visualize our samples. This DNA stain is compatible with both UV and blue-light transilluminators. For best results, we recommend the TruBlu™ 2 Blue Light Transilluminator (**Cat #557**).

Pre-Lab Preparations - Module II-B

OPTIONAL STAINING USING FLASHBLUE™

MODULE II-B (OPTIONAL)
Each Group should receive:
- 10 mL 10X concentrated FlashBlue™
- Small plastic tray or weight boat
- Distilled or deionized water

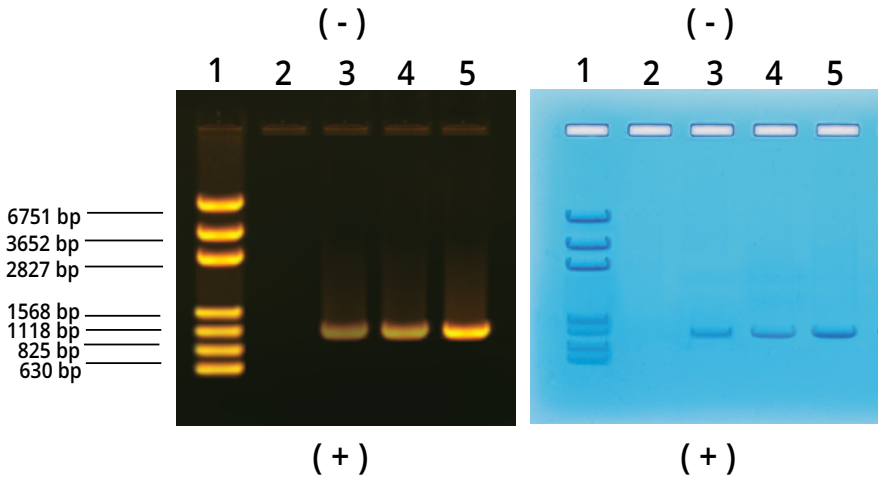
FlashBlue™ stain is optimized to shorten the time required for both staining and destaining steps. Agarose gels can be stained with diluted FlashBlue™ for 5 minutes and destained for only 20 minutes. For the best results, leave the gel in liquid overnight. This will allow the stained gel to "equilibrate" in the destaining solution, resulting in dark blue DNA bands contrasting against a uniformly light blue background. A white light box (**Cat. #552**) or the white light feature of the TruBlu™ 2 (**Cat #557**) is recommended for visualizing gels stained with FlashBlue™.

- Stained gels may be stored in destaining liquid for several weeks with refrigeration, although the bands may fade with time. If this happens, re-stain the gel.
- Destained gels can be discarded in solid waste disposal. Destaining solutions can be disposed of down the drain.

PHOTODOCUMENTATION OF DNA (OPTIONAL)

Once gels are stained, you may wish to photograph your results. There are many different photodocumentation systems available, including digital systems that are interfaced directly with computers. Specific instructions will vary depending upon the type of photodocumentation system you are using.

Experiment Results and Analysis



Lane	Sample Name
1	DNA Standard Marker
2	Reaction sample after 0 cycles
3	Reaction sample after 10 cycles
4	Reaction sample after 20 cycles
5	Reaction sample after 30 cycles

The results photo shows the approximate intensity of the PCR amplified band after various cycles. Smaller fragments will stain less efficiently and will appear as fainter bands.

This PCR experiment produces a single DNA fragment of 1000 base pairs that increases in intensity as the number of cycles increases. In some experiments, a faint band above the PCR amplified DNA band may also be visible (not shown in idealized schematic), which is the DNA template. The predominant band is the PCR product. Likewise, smaller bands may be faintly visible at the bottom of the gel due to partial PCR amplification products or primer.

NOTES:

Because water baths do not maintain temperatures as precisely as a thermal cycler, additional faint template artifact bands may possibly be observed in the control and PCR lanes in some experiments.

The red and orange dyes from the LyphoTemplate™ and LyphoPrimer™ will migrate at a similar position on the electrophoresis gel. Be sure to use the bands to determine how far the DNA samples have run.

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

Appendices

A EDVOTEK® Troubleshooting Guide
B Performing the PCR Experiment Using Three Water Baths
C Bulk Preparation of Electrophoresis Buffer and Agarose Gels

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

Appendix A
EDVOTEK® Troubleshooting Guides

PCR AND ELECTROPHORESIS

PROBLEM:	CAUSE:	ANSWER:
There is very little liquid left in tube after PCR.	Sample has evaporated.	Make sure the heated lid reaches the appropriate temperature.
		If your thermal cycler does not have a heated lid, overlay the PCR reaction with wax (visit www.edvotek.com for details).
		Make sure students close the lid of the PCR tube properly.
	Pipetting error.	Make sure students pipet 20 µl Primer Mix, 15 µl Ultra-pure water, and 5 uL DNA Template into the appropriate tube. When properly prepared, the PCR sample will appear orange in color.
The ladder and student PCR products are not visible on the gel.	The gel was not prepared properly.	Ensure that the electrophoresis buffer was correctly diluted.
		Gels of higher concentration (> 0.8%) require special attention when melting the agarose. Make sure that the solution is completely clear of "clumps" and glassy granules before pouring gels.
		The proper buffer was not used for gel preparation. Make sure to use 1x Electrophoresis Buffer.
	The gel was not stained properly.	Ensure that stain was added to the gel. Repeat staining.
	Malfunctioning electrophoresis unit or power source.	Contact the manufacturer of the electrophoresis unit or power source.
After staining the gel with FlashBlue™, the DNA bands are faint.	The gel was not stained for a sufficient period of time.	Repeat staining protocol.
After staining the gel with FlashBlue™, the gel background is very dark.	The gel needs to be destained longer.	Submerge the gel in distilled or deionized water. Allow the gel to soak for 5 minutes.
After staining, the ladder is visible on the gel but some student samples are not present.	PCR EdvoBead™ PLUS was added to the wrong tube.	Be sure to add the PCR EdvoBead™ PLUS to the 0.2 ml PCR tube.
	Wrong volumes of DNA and primer added to PCR reaction.	Practice using micropipets. When properly prepared, the PCR sample will appear green in color.
Low molecular weight band in PCR samples.	Primer dimer.	Low concentration of DNA in PCR sample as a result of pipetting error. Be sure student pipets 5 µl DNA template into the appropriate tube.
DNA bands were not resolved.	To ensure adequate separation, make sure the blue tracking dye migrates at least 3.5 cm on 7x7 cm gels and 6 cm on 7x14 cm gels.	Be sure to run the gel the appropriate distance before staining and visualizing the DNA.
DNA bands fade when gels are kept at 4°C.	DNA stained with FlashBlue™ may fade with time.	Re-stain the gel with FlashBlue™.

Appendix B
Performing the PCR Experiment Using Three Water Baths

This experiment can be modified to use three water baths in place of a thermal cycler. In this method, the PCR samples are cycled through three water baths, each maintained at a different temperature, for a specified period. The sequential placement of the reaction sample through the three water baths constitutes one PCR cycle. Please note that results obtained when using three water baths are often variable. **A thermal cycler assures a significantly higher rate of success.**

Set up the samples as outlined in Module I steps 1-8. Before cycling the samples, be sure to:

- Allow at least 15 minutes for the water baths to reach the temperatures specified in Module I (94°C, 45°C, 72°C).
- Cover the water baths when not in use to maintain the proper temperature and to prevent water evaporation.
- The volume of the PCR sample is small and can evaporate easily. To prevent this, transfer one wax bead to each PCR sample. *(Wax beads are not provided.)* The melted wax bead forms a barrier over the PCR sample to prevent its evaporation during heating.
- Make sure that the sample remains undisturbed at the bottom of the tube. If necessary, centrifuge or shake the tube to get the sample to the bottom of the tube.
- Place the PCR samples in a water bath float before placing them in the water bath.

Continue with step 9 (thermal cycling), following the below protocol for 30 cycles. **NOTE: For this experiment, cycling needs to be paused after the 10th, 20th and 30th cycle.** Refer to the protocol in Module I for detailed instructions.

- 94°C for 15 seconds
- 45°C for 15 seconds
- 72°C for 30 seconds

Handle the samples carefully when shifting between water baths. Use forceps to carefully raise/lower the float into the water baths.

Remove samples at time points specified by Module I. **Take care to avoid the liquid wax layer when removing the sample.** We recommend placing the tube on ice for a few seconds to solidify the wax. Use a clean pipette tip to gently break through the wax layer, making enough room to fit a clean pipette tip. Using a fresh, clean pipette tip, remove the PCR product and transfer to the appropriate tube.

- Final extension at 72°C for 60 seconds.

Appendix C
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

Concentrated Buffer (50x)	+	Distilled Water	=	TOTAL Volume Required
60 mL		2,940 mL		3000 mL (3 L)

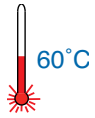
Batch Agarose Gels (0.8%)

To prepare a large batch of agarose for the entire classroom, see **Table E**.

Table E Batch Prep of 0.8% UltraSpec-Agarose™

Amt of Agarose	+	Concentrated Buffer (50X)	+	Distilled Water	=	TOTAL Volume	Cool, then add SYBR® Safe
3.0 g		7.5 mL		382.5 mL		390 mL	Entire Tube

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. Add the entire volume of ***diluted*** **SYBR® Safe** (from page 22) to the cooled agarose and mix thoroughly.
7. Dispense the required volume of cooled agarose solution for casting each gel. Measure 30 mL for a 7 x 7 cm tray, 50 mL for a 7 x 10 cm tray, and 60 mL for a 7 x 14 cm tray. ***For this experiment, 7 x 7 cm gels are recommended.***
8. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Gels can be used immediately or stored in a small amount of buffer in the refrigerator for several days.

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.