Edvo-Kit #330

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

<table>
<thead>
<tr>
<th>Components</th>
<th>Storage</th>
<th>Check (√)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A PCR EdvoBeads™</td>
<td>Room Temp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Each PCR EdvoBead™ contains:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• dNTP Mixture</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Taq DNA Polymerase Buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Taq DNA Polymerase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• MgCl₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Reaction Buffer</td>
<td></td>
</tr>
<tr>
<td>B LyphoPrimer™ Mix</td>
<td>-20° C</td>
<td></td>
</tr>
<tr>
<td>C DNA Standard Marker</td>
<td>-20° C</td>
<td></td>
</tr>
<tr>
<td>D Ultra-pure Water</td>
<td>-20° C</td>
<td></td>
</tr>
<tr>
<td>E LyphoTemplate™</td>
<td>-20° C</td>
<td></td>
</tr>
<tr>
<td>F TE buffer</td>
<td>-20° C</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** Components B and E are supplied in our LyphoPrimer™ and LyphoTemplate™ format. They will require reconstitution before use. Be sure to review Page 23 in the Instructor’s Guide for more details.

## REAGENTS & SUPPLIES

Store all components below at room temperature.

<table>
<thead>
<tr>
<th>Component</th>
<th>Check (√)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• UltraSpec-Agarose™</td>
<td></td>
</tr>
<tr>
<td>• 50X Electrophoresis Buffer</td>
<td></td>
</tr>
<tr>
<td>• 10X Gel Loading Solution</td>
<td></td>
</tr>
<tr>
<td>• FlashBlue™ Stain</td>
<td></td>
</tr>
<tr>
<td>• InstaStain® Ethidium Bromide</td>
<td></td>
</tr>
<tr>
<td>• Microcentrifuge Tubes</td>
<td></td>
</tr>
<tr>
<td>• PCR Tubes</td>
<td></td>
</tr>
<tr>
<td>• Wax beads (for thermal cyclers without heated lid)</td>
<td></td>
</tr>
</tbody>
</table>

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Requirements

- Thermal cycler* (EDVOTEK Cat. # 541 highly recommended)
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Balance
- Microcentrifuge
- UV Transilluminator or UV Photodocumentation system (use if staining with InstaStain® Ethidium Bromide)
- UV safety goggles (use if staining with InstaStain® Ethidium Bromide)
- White light visualization system (use if staining with FlashBlue™)
- Automatic micropipets (5-50 μl) with tips
- Microwave, hot plate or burner
- 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 waterbaths with proper care (EDVOTEK® Cat. #544 highly recommended). However, a thermal cycler assures a significantly higher rate of success. See Appendix C for detailed instructions.
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 groundbreaking 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 x 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
Figure 1:
Polymerase Chain Reaction
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.

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, pipet 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.

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.

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

**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 - 90 min.**

Performing the Polymerase Chain Reaction

**Module II - 30-45 min.**

Analysis of PCR Products by Agarose Gel Electrophoresis

**Module III - 5-30 min.**

Staining Agarose Gels

**Module IV - 15 min.**

Size Determination of the Amplified PCR Products

NOTE: Experimental times are approximate.
Module I: Performing the Polymerase Chain Reaction

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), 15 μl Ultra-pure Water, and 5 μl DNA Template (blue) to the tube labeled "0".

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

4. **TRANSFER** 30 μl of the PCR sample mixture to the labeled 0.2 ml "PCR" tube. **PLACE** the "PCR" tube on ice.

5. **ADD** 5 μl of 10X Gel Loading Solution and 10 μl Ultra-pure Water to the PCR sample mixture remaining in tube "0". **PLACE** tube "0" on ice until ready for electrophoresis.

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

7. **Gently mix** the sample gently. Make sure the PCR EdvoBead™ is completely dissolved.

8. **Centrifuge** to collect the sample at the bottom of the tube.

continued
Module I: Performing the Polymerase Chain Reaction, continued

9. AMPLIFY DNA using PCR guidelines listed below. NOTE: For this experiment, cycling needs to be paused after the 10th, 20th and 30th cycle.
   - 94°C for 45 seconds
   - 45°C for 45 seconds
   - 72°C for 45 seconds
   After the final cycle, the 72°C incubation can be extended to 5 minutes.

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 5 μl of 10X Gel Loading Solution and 10 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 cycle 20 and 30. BE SURE to transfer DNA sample to the appropriately labeled tube.


OPTIONAL STOPPING POINT:
PCR samples may be stored at -20° C for electrophoresis at a later time.
Overview of Agarose Gel Electrophoresis & Staining

1. Prepare agarose gel and casting tray. If staining with InstaStain® Ethidium Bromide, gel preparation procedures begin on page 12. If staining with FlashBlue™, gel preparation procedures begin on page 15.

2. Remove end caps & comb. Place tray into the electrophoresis chamber. Add 1x buffer into the chamber to submerge the gel.

3. Using a variable micropipet, load each sample into consecutive wells.

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

5. After electrophoresis, transfer gel for staining with either InstaStain® Ethidium Bromide (Preferred) or FlashBlue™ Stain.

6. Visualize results on a U.V. Trans-illuminator (InstaStain® Ethidium Bromide) or a white light system (FlashBlue™ Stain).

Gel pattern will vary depending upon experiment.
Module II-A: Separation of PCR Products by Electrophoresis (InstaStain® Ethidium Bromide Staining)

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. **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. Carefully **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

**Table A**

<table>
<thead>
<tr>
<th>Size of Gel Casting Tray</th>
<th>Individual 0.8% UltraSpec-Agarose™ Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentrated Buffer (50X) + Distilled Water + Amt of Agarose = TOTAL Volume</td>
</tr>
<tr>
<td>7 x 7 cm</td>
<td>0.6 ml + 29.4 ml + 0.23 g = 30 ml</td>
</tr>
<tr>
<td>7 x 10 cm</td>
<td>1.0 ml + 49.0 ml + 0.39 g = 50 ml</td>
</tr>
<tr>
<td>7 x 14 cm</td>
<td>1.2 ml + 58.8 ml + 0.46 g = 60 ml</td>
</tr>
</tbody>
</table>

**IMPORTANT:**

7 x 7 cm gels are recommended. Place well-former template (comb) in the first set of notches.

If you are unfamiliar with agarose gel prep and electrophoresis, detailed instructions and helpful resources are available at www.edvotek.com.

Wear gloves and safety goggles.
Module II-A: Separation of PCR Products by Electrophoresis (InstaStain® Ethidium Bromide), continued

8. **PLACE** gel (on the casting tray) into electrophoresis chamber. **POUR** 1X Diluted Chamber Buffer into the electrophoresis chamber (See Table B for recommended volumes). Completely **SUBMERGE** the gel.

9. **LOAD** the entire sample volumes (25 μl) into the wells in consecutive order as indicated by Table 1.

10. **PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the 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). For best results, the orange tracking dye should migrate at least 4 cm from the wells.

12. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber and proceed to **STAINING** the agarose gel with InstaStain® Ethidium Bromide (page 14).

### Table 1

<table>
<thead>
<tr>
<th>Lane</th>
<th>Recommended Sample Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker</td>
</tr>
<tr>
<td>2</td>
<td>DNA Standard Marker</td>
</tr>
<tr>
<td>3</td>
<td>Reaction sample after 0 cycles</td>
</tr>
<tr>
<td>4</td>
<td>Reaction sample after 10 cycles</td>
</tr>
<tr>
<td>5</td>
<td>Reaction sample after 20 cycles</td>
</tr>
<tr>
<td>6</td>
<td>Reaction sample after 30 cycles</td>
</tr>
</tbody>
</table>

### Table B

<table>
<thead>
<tr>
<th>EDVOTek Model #</th>
<th>Total Volume Required</th>
<th>Dilution 50% Conc. Buffer + Distilled Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6+ &amp; M12 (new)</td>
<td>300 ml</td>
<td>6 ml 294 ml</td>
</tr>
<tr>
<td>M12 (classic)</td>
<td>400 ml</td>
<td>8 ml 392 ml</td>
</tr>
<tr>
<td>M36</td>
<td>1000 ml</td>
<td>20 ml 900 ml</td>
</tr>
</tbody>
</table>

### Table C

<table>
<thead>
<tr>
<th>Electrophoresis Model</th>
<th>M6+</th>
<th>M12 (new)</th>
<th>M12 (classic) &amp; M36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vois</td>
<td>Min. / Max.</td>
<td>Min. / Max.</td>
<td>Min. / Max.</td>
</tr>
<tr>
<td>150</td>
<td>15/20 min.</td>
<td>20/30 min.</td>
<td>25 / 35 min.</td>
</tr>
<tr>
<td>125</td>
<td>20/30 min.</td>
<td>30/35 min.</td>
<td>35 / 45 min.</td>
</tr>
<tr>
<td>75</td>
<td>35 / 45 min.</td>
<td>55/70 min.</td>
<td>60 / 90 min.</td>
</tr>
</tbody>
</table>

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

Wear gloves and safety goggles
Module III-A: Staining Agarose Gels with InstaStain® Ethidium Bromide

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 one card to stain a 7 x 7 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 mid-range ultraviolet transilluminator (300 nm). DNA should appear as bright orange bands on a dark background.

**BE SURE TO WEAR UV-PROTECTIVE EYEWEAR!**
Module II-B: Separation of PCR Products by Electrophoresis
(Improved FlashBlue™ Staining)

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. ADD 10x FlashBlue™ Solution to the cooled agarose (see Table A.2 for correct amount). 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. Carefully REMOVE end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

### Table A
<table>
<thead>
<tr>
<th>Size of Gel</th>
<th>Individual 0.8% UltraSpec-Agarose™ Gel</th>
<th>Size of Gel</th>
<th>Ant of FlashBlue™</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casting tray</td>
<td>Concentrated Buffer (50X)</td>
<td>Distilled Water</td>
<td>Amount of Agarose</td>
</tr>
<tr>
<td>7 x 7 cm</td>
<td>0.6 ml</td>
<td>29.4 ml</td>
<td>0.23 g</td>
</tr>
<tr>
<td>7 x 10 cm</td>
<td>1.0 ml</td>
<td>49.0 ml</td>
<td>0.39 g</td>
</tr>
<tr>
<td>7 x 14 cm</td>
<td>1.2 ml</td>
<td>58.8 ml</td>
<td>0.46 g</td>
</tr>
</tbody>
</table>

### Table A.2
<table>
<thead>
<tr>
<th>Size of Gel</th>
<th>Ant of FlashBlue™</th>
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<tbody>
<tr>
<td>7 x 7 cm</td>
<td>10 µl</td>
</tr>
<tr>
<td>7 x 10 cm</td>
<td>16 µl</td>
</tr>
<tr>
<td>7 x 14 cm</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
Module II-B: Separation of PCR Products by Electrophoresis (Improved FlashBlue™ Staining), continued

8. **PLACE** gel (on the casting tray) into electrophoresis chamber. **POUR** the FlashBlue™ Enhanced 1X Diluted Chamber Buffer into the electrophoresis chamber (See Table B.2 for recommended volumes). Completely **SUBMERGE** the gel.

9. **LOAD** the entire sample volumes (25 μl) into the wells in consecutive order as indicated by Table 1.

10. **PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the 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). For best results, the orange tracking dye should migrate at least 4 cm from the wells.

12. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber and proceed to **STAINING** the agarose gel with FlashBlue™ Stain (page 17).

### Table B.2

<table>
<thead>
<tr>
<th>Buffer Name</th>
<th>Total Volume Required</th>
<th>5X Conc. Buffer (ml)</th>
<th>Distilled Water (ml)</th>
<th>10x FlashBlue Solution (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlashBlue™ Enhanced 1x Diluted Chamber Buffer</td>
<td>300 ml</td>
<td>6 ml</td>
<td>294 ml</td>
<td>100 µl</td>
</tr>
<tr>
<td>M6+ &amp; M12 (new)</td>
<td>400 ml</td>
<td>8 ml</td>
<td>392 ml</td>
<td>130 µl</td>
</tr>
<tr>
<td>M36</td>
<td>1000 ml</td>
<td>20 ml</td>
<td>980 ml</td>
<td>330 µl</td>
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### Table C

<table>
<thead>
<tr>
<th>Time and Voltage Guidelines (0.8% Agarose Gel)</th>
</tr>
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<tbody>
<tr>
<td>Electrophoresis Model</td>
</tr>
<tr>
<td>Voltage (V)</td>
</tr>
<tr>
<td>150</td>
</tr>
<tr>
<td>125</td>
</tr>
<tr>
<td>75</td>
</tr>
</tbody>
</table>
Module III-B: Staining Agarose Gels with FlashBlue™ Stain

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 of 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. Frequent changes of the water 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.

**ALTERNATIVE PROTOCOL:**

1. **DILUTE** 1 ml of 10X FlashBlue™ stain with 499 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 IV: 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 log₁₀ 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).

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 log₁₀ 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!
Module IV: 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?
OVERVIEW OF INSTRUCTOR’S PRELAB PREPARATION:

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

<table>
<thead>
<tr>
<th>Preparation For:</th>
<th>What to do:</th>
<th>When:</th>
<th>Time Required:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Module I:</strong> Performing the Polymerase Chain Reaction</td>
<td>Prepare and aliquot various reagents (Primer, DNA template, ladder, etc.)</td>
<td>One day to 30 min. before performing the experiment.</td>
<td>30 min.</td>
</tr>
<tr>
<td></td>
<td>Program Thermal Cycler OR Set up waterbaths (94°C, 45°C and 72°C)</td>
<td>One hour before performing the experiment.</td>
<td>15 min.</td>
</tr>
<tr>
<td><strong>Module II:</strong> Separation of PCR Products by Electrophoresis</td>
<td>Prepare diluted electrophoresis buffer</td>
<td>Up to one day before performing the experiment.</td>
<td>45 min.</td>
</tr>
<tr>
<td></td>
<td>Prepare molten agarose and pour gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Module III:</strong> Staining</td>
<td>Prepare staining components</td>
<td>The class period or overnight after the class period.</td>
<td>10 min.</td>
</tr>
<tr>
<td><strong>Module IV:</strong> Size Determination of Amplified PCR Products</td>
<td>Make copies of graph paper</td>
<td>Any time before the class period.</td>
<td>10 min.</td>
</tr>
</tbody>
</table>
Pre-Lab Preparations

**MODULE I: PERFORMING THE POLYMERASE CHAIN REACTION**

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

**Preparation of the Primer Mix**

1. Thaw the TE buffer (F). Mix well before using.
2. Before preparing the primer mix, make sure the solid material is at the bottom of the LyphoPrimer™ Tube (B). 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 (F). Mix well before using.
2. Before preparing the DNA template, make sure the solid material is at the bottom of the LyphoTemplate™ Tube (E). 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 blue 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.

**Additional Materials**

- Dispense 25 μl of 10 X Gel Loading Solution per tube. Label these 10 tubes “10 X Solution”. Distribute one tube per student group.
- Dispense 60 μl Ultra-pure water per student group.
- Dispense one PCR EdvoBead™ 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 in Module II in the Student’s Experimental Procedure.

- 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.
- For thermal cyclers that do not have a heated lid, it is necessary to place a layer of wax above the PCR reactions in the microcentrifuge tubes to prevent evaporation. See Appendix B for instructions.
Pre-Lab Preparations

MODULE II: ANALYSIS OF PCR PRODUCTS BY ELECTROPHORESIS

Preparation of Agarose Gels

This experiment requires a total of ten 0.8% agarose gels for the class. 7 x 7 cm gels are recommended.

NOTE: The instructor MUST DECIDE whether to use InstaStain® Ethidium Bromide or FlashBlue™ to visualize the gel BEFORE starting the gel preparation. InstaStain® Ethidium Bromide cannot be used to stain DNA in gels that contain FlashBlue™.

Individual Gel Preparation:
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 and agarose powder. If using the Improved FlashBlue™ staining protocol, students will also require concentrated FlashBlue™ solution.

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 (4° C) for up to two weeks.

If using the Improved FlashBlue™ staining protocol, the buffer should contain FlashBlue™. See Table B.2 on page 16 for preparation guidelines.

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
- Dispense 35 μl of DNA Standard Marker (C) per gel.
Pre-Lab Preparations

MODULE III: STAINING AGAROSE GELS

Option A: InstaStain® Ethidium Bromide

InstaStain® Ethidium Bromide provides the sensitivity of ethidium bromide while minimizing the volume of liquid waste generated by staining and destaining a gel. An agarose gel stained with InstaStain® Ethidium Bromide is ready for visualization in as little as 3 minutes! Each InstaStain® card will stain 49 cm$^2$ of gel (7 x 7 cm).

Use a mid-range ultraviolet transilluminator (Cat. #558) to visualize gels stained with InstaStain® Ethidium Bromide. **BE SURE TO WEAR UV-PROTECTIVE EYEWEAR!**

- DNA Standard markers should be visible after staining even if other DNA samples are faint or absent. If bands appear faint, repeat staining with a fresh InstaStain card for an additional 3-5 min. If markers are not visible, troubleshoot for problems with electrophoretic separation.
- Ethidium bromide is a listed mutagen. Wear gloves and protective eyewear when using this product. UV protective eyewear is required for visualization with a UV transilluminator.
- InstaStain® Ethidium Bromide cards and stained gels should be discarded using institutional guidelines for solid chemical waste.

**NOTE:** FlashBlue™ enhanced gels CANNOT be stained with InstaStain® Ethidium Bromide.

Option B: FlashBlue™ Liquid Stain

FlashBlue™ stain is optimized to shorten the time required for both staining and destaining steps. The gels are prestained with FlashBlue™ by adding the concentrate to the gel and running buffer. 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) 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 go 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

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.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA Standard Marker</td>
</tr>
<tr>
<td>2</td>
<td>Reaction sample after 0 cycles</td>
</tr>
<tr>
<td>3</td>
<td>Reaction sample after 10 cycles</td>
</tr>
<tr>
<td>4</td>
<td>Reaction sample after 20 cycles</td>
</tr>
<tr>
<td>5</td>
<td>Reaction sample after 30 cycles</td>
</tr>
</tbody>
</table>

NOTES:

Because waterbaths 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 blue and orange dyes from the LyphoTemplate™ and LyphoPrimer™ will migrate at different positions on the electrophoresis gel. Be sure to use the orange band to determine how far the DNA samples have run.

Includes EDVOTEK’s All-NEW DNA Standard Marker

- Better separation
- Easier band measurements
- No unused bands

NEW DNA Standard ladder sizes:
6751, 3652, 2827, 1568, 1118, 825, 630
Questions and Answers to Study Questions

1. **Why is a thermostable DNA polymerase required for DNA amplification by PCR?**

   Amplification of DNA by PCR requires maintaining the reaction at three different temperatures (94° C, 45° C and 72° C). Unlike non-thermostable DNA polymerases at 94° C, Taq DNA polymerase is stable at high temperatures. It will retain its enzymatic activity through the multiple PCR cycles.

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?**

   Sixteen copies after 4 cycles and 256 copies after 8 cycles.

3. **Why are two different primers required for the PCR reaction?**

   Two primers are required for the two strands of DNA obtained upon melting. The sequences primed will be different since the synthesis in both cases will be in the 5’→ 3’ direction, starting from the 5’ end of each strand.

4. **What are possible reasons for obtaining fainter (usually smaller) bands besides the main PCR product?**

   Possible reasons can include an excess of primer, contaminants in the sample that can be amplified, non-specific primer annealing, or hybridization conditions (primer binding to template) not being optimized.
Appendices

A  EDVOTEK® Troubleshooting Guide
B  Preparation and Handling of PCR Samples With Wax
C  Performing the PCR Experiment Using Three Waterbaths
D  Bulk Preparation of 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

<table>
<thead>
<tr>
<th>PROBLEM:</th>
<th>CAUSE:</th>
<th>ANSWER:</th>
</tr>
</thead>
<tbody>
<tr>
<td>There is very little liquid left in tube after PCR</td>
<td>Sample has evaporated</td>
<td>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 (see Appendix B for details). Make sure students close the lid of the PCR tube properly.</td>
</tr>
<tr>
<td>Pipetting error</td>
<td></td>
<td>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 green in color.</td>
</tr>
<tr>
<td>The ladder, control DNA, and student PCR products are not visible on the gel.</td>
<td>The gel was not prepared properly.</td>
<td>Ensure that the electrophoresis buffer was correctly diluted. Gels of higher concentration (&gt; 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.</td>
</tr>
<tr>
<td>After staining the gel, the DNA bands are faint.</td>
<td>The gel was not stained for a sufficient period of time.</td>
<td>Repeat staining protocol.</td>
</tr>
<tr>
<td>After staining the gel, the gel background is very dark.</td>
<td>The gel needs to be destained longer.</td>
<td>Submerge the gel in distilled or deionized water. Allow the gel to soak for 5 minutes.</td>
</tr>
<tr>
<td>After staining, the ladder is visible on the gel but some student samples are not present.</td>
<td>PCR EdvoBead™ was added to wrong tube.</td>
<td>Be sure to add the PCR EdvoBead™ to the 0.2 ml PCR tube.</td>
</tr>
<tr>
<td></td>
<td>Wrong volumes of DNA and primer added to PCR reaction.</td>
<td>Practice using micropipets. When properly prepared, the PCR sample will appear green in color.</td>
</tr>
<tr>
<td>Low molecular weight band in PCR samples</td>
<td>Primer dimer</td>
<td>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.</td>
</tr>
<tr>
<td>DNA bands were not resolved.</td>
<td>To ensure adequate separation, make sure the tracking dye migrates at least 3.5 cm on 7 x 7 cm gels and 6 cm on 7 x 14 cm gels.</td>
<td>Be sure to run the gel the appropriate distance before staining and visualizing the DNA.</td>
</tr>
<tr>
<td>DNA bands fade when gels are kept at 4°C.</td>
<td>DNA stained with FlashBlue™ may fade with time.</td>
<td>Re-stain the gel with FlashBlue™</td>
</tr>
</tbody>
</table>
Appendix B
Preparation and Handling of PCR Samples with Wax

ONLY For Thermal Cyclers WITHOUT Heated Lids, or Manual PCR Using Three Waterbaths

Using a wax overlay on reaction components prevents evaporation during the PCR process.

How to Prepare a Wax overlay

1. Add PCR components to the 0.2 ml PCR Tube as outlined in Module I.
2. Centrifuge at full speed for five seconds to collect sample at bottom of the tube.
3. Using clean forceps, add one wax bead to the PCR tube.
4. Place samples in PCR machine and proceed with Module I.

Preparing PCR Samples for Electrophoresis

1. After PCR is completed, melt the wax overlay by heating the sample at 94° C for three minutes or until the wax melts.
2. Using a clean pipette, remove as much overlay wax as possible.
3. Allow the remaining wax to solidify.
4. Use a pipette tip to puncture the thin layer of remaining wax. Using a fresh pipette tip, remove the PCR product and transfer to a new tube.
5. Add 5 μl of 10X Gel Loading Buffer to the sample. Proceed to Module II to perform electrophoresis.
Appendix C
Performing the PCR Experiment Using Three Waterbaths

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

We recommend EDVOTEK’s Digital PCR Waterbath (Cat. #544) if you do not have access to a thermal cycler.

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 waterbaths to reach the temperatures specified in Module I (94°C, 45°C, 72°C).
• Cover the waterbaths 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. 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 waterbath float before placing them in the waterbath.

Continue with step 9 (thermal cycling), following the below protocol for 30 cycles.

• 94°C for 1 minute
• 45°C for 1 minute
• 72°C for 1 minute

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

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 pipet tip to gently break through the wax layer, making enough room to fit a clean pipet tip. Using a fresh, clean pipet tip, remove the PCR product and transfer to the appropriate tube.
Appendix D

Bulk Preparation of 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.

*NOTE:* If staining gels with FlashBlue, add 1 ml 10x FlashBlue concentrate to the diluted buffer and mix thoroughly.

**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.

*NOTE:* If staining gels with FlashBlue, add 130 μl 10x FlashBlue concentrate to the cooled agarose and mix thoroughly.

6. 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.*

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.

---

**Table D**

<table>
<thead>
<tr>
<th>Concentrated Buffer (50x)</th>
<th>Distilled Water</th>
<th>TOTAL Volume Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 ml</td>
<td>2,940 ml</td>
<td>3,000 ml (3 L)</td>
</tr>
</tbody>
</table>

**Table E**

<table>
<thead>
<tr>
<th>Ant of Agarose</th>
<th>Concentrated Buffer (50X)</th>
<th>Distilled Water</th>
<th>TOTAL Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 g</td>
<td>7.5 ml</td>
<td>302.5 ml</td>
<td>390 ml</td>
</tr>
</tbody>
</table>

*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.

*NOTE:* QuickGuide instructions and guidelines for casting various agarose gels can be found on our website: [www.edvotek.com/quick-guides](http://www.edvotek.com/quick-guides)