EDVOTEK®

THE **BIOTECHNOLOGY**
EDUCATION COMPANY®

REVISED & UPDATED

Edvo-Kit #
335

Edvo-Kit #335

Reverse Transcription PCR (RT-PCR): The Molecular Biology of HIV Replication

Experiment Objective:

The objective of this experiment is for students to gain an understanding of the principles and practice of RT-PCR and to relate these reactions to HIV replication.

See page 3 for storage instructions.

LyphoPrimer™

LyphoTemplate™

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

Experiment Components

Component		Storage	Check (✓)
A	RNA LyphoTemplate™	-20° C Freezer	❑
B	LyphoPrimer™ mix	-20° C Freezer	❑
C	EdvoQuick™ DNA ladder	-20° C Freezer	❑
D	RNAse-free water	-20° C Freezer	❑
•	Tubes with RT-PCR EdvoBeads™	Room Temp., desiccated	❑

Each RT-PCR EdvoBead™ contains:

- *dNTP Mixture*
- *Taq DNA Polymerase Buffer*
- *Taq DNA Polymerase*
- *$MgCl_2$*
- *Reverse Transcriptase*
- *RNAse Inhibitor*

NOTE: Use RT-PCR EdvoBeads™ within two weeks of receipt.

NOTE: Components A and B are now supplied in our LyphoPrimer™ and LyphoTemplate™ format. They will require reconsitiution prior to setting up RT-PCR reactions.

Experiment # 335 contains material for up to six sets of RT-PCR reactions.

Sample volumes are very small. It is important to quick spin the tube contents in a microcentrifuge to obtain sufficient volume for pipetting. Spin samples for 10-20 seconds at maximum speed.

This experiment does not contain HIV virus or its components. None of the components have been prepared from human sources.

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

REAGENTS & SUPPLIES

Store all components below at room temperature.

Component	Check (✓)
• UltraSpec-Agarose™	❑
• 50X Electrophoresis Buffer	❑
• 10x Gel Loading Solution	❑
• SYBR® Safe Stain	❑
• Microcentrifuge tubes	❑
• PCR tubes (0.2 mL)	❑

CAUTION!
Wear gloves when handling all tubes for this experiment.
RNA from your fingers will interfere with the experiment results.

Requirements

- Thermal cycler (EDVOTEK Cat. # 541 highly recommended) or three waterbaths*
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Balance
- Microcentrifuge
- UV transilluminator or blue light visualization (EDVOTEK Cat. #557 or #558 highly recommended)
- UV safety goggles
- Automatic micropipettes (5-50 µL) with tips
- Microwave or hot plate
- 250 mL flasks or beakers
- Hot gloves
- Disposable laboratory gloves
- Distilled or deionized water
- Ice buckets and ice

*If you do not have a thermal cycler, PCR experiments can be conducted, with proper care, using three waterbaths. However, a thermal cycler assures a significantly higher rate of success.

Background Information

HUMAN IMMUNODEFICIENCY VIRUS

Acquired immune deficiency syndrome (AIDS) is a disease characterized by the progressive deterioration of a patient's immune system. This immunological impairment allows infectious agents such as viruses, bacteria, fungi and parasites to invade the body and propagate. The incidence of certain cancers dramatically increases in these patients because of their compromised immune system. AIDS is a serious threat to human health and is a global problem. Intensive research is being done to advance methods of detection, clinical treatment and prevention.

Retroviruses

The AIDS etiologic agent is the human immunodeficiency virus type 1 (HIV-1), a retrovirus. Retroviruses contain an RNA genome and the RNA-dependent DNA polymerase also termed reverse transcriptase. Members of the retrovirus family are involved in the pathogenesis of certain types of leukemias and other sarcomas in animals. The structure and replication mechanism of HIV is very similar to other retroviruses. HIV is unique in some of its properties since it specifically targets the immune system, is very immunoevasive, forms significant amounts of progeny virus *in vivo* during the later stages of the disease and can also be transmitted during sexual activity.

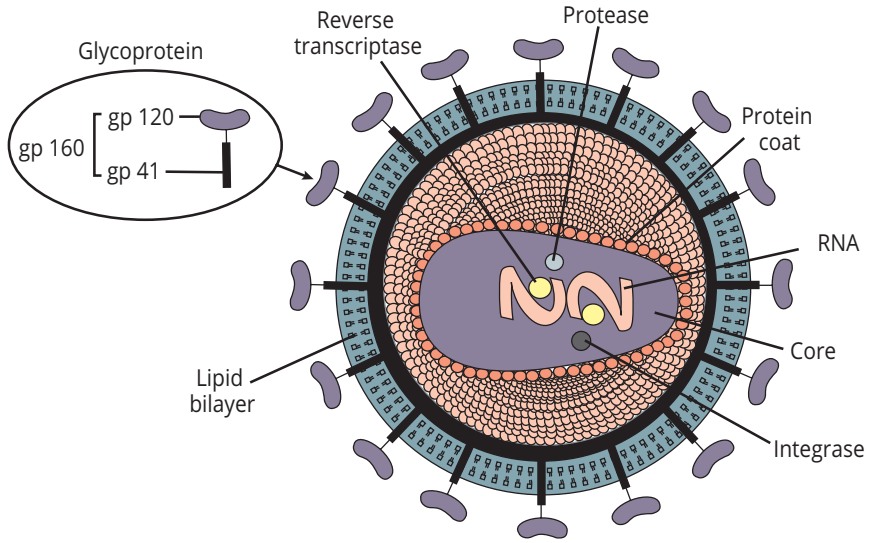


Figure 1: Overview of HIV structural components

The HIV viral particle is surrounded by a lipid bilayer derived from the host cell membrane during budding (Figure 1). The viral proteins are identified by the prefix gp (glycoprotein) or p (protein) followed by a number indicating the approximate molecular weight in kilodaltons. The lipid bilayer is studded with a protein complex called gp160, a protein complex made from gp120 and gp41. The gp 41 anchors gp 120 in the bilayer.

Beneath the bilayer is a capsid consisting of p17 and p18. Within this shell is the viral core. The walls of the core consists of p24 and p25. Within the core are two identical RNA molecules 9000 nucleotides in length. Hydrogen bonded to each genomic RNA is a cellular tRNA molecule. The core also contains reverse transcriptase. Protein products obtained from the HIV genome are displayed in Figure 2.

Large quantities of virus can be grown in tissue culture for diagnostic and research purposes. Several of the viral proteins have been cloned and expressed in relatively large quantities.

Biology of HIV Infection

HIV only infects cells which have a CD4+ receptor on their surface. Receptors are used by cells to communicate. They let information in and out of the cell, and different types of cells have different receptors. Two kinds of immune system cells have CD4+ receptors and can be infected by HIV: macrophages (white blood cells called "macs") and CD4+ lymphocytes (also called "TH", "T4 cells" or "CD4+ cells"). In the case of HIV, the viral envelope has protein "spikes" on it, called gp120. These spikes fit the CD4+ receptor on a cell's surface. When the gp120 spike fits onto the host's receptor, it unlocks the receptor and allows HIV to enter the cell. Figure 3 shows HIV binding to a CD4+ cell.

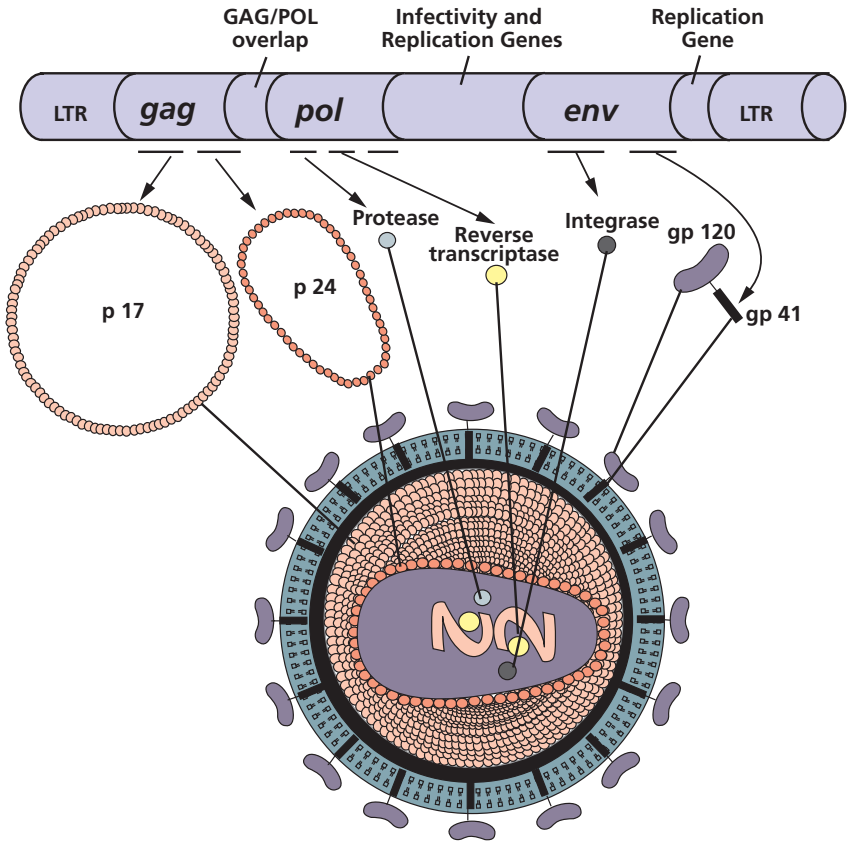


Figure 2: Protein products obtained from the HIV genome.

Even after HIV binds firmly to the CD4+ receptor, a second molecule, a protein called fusin, is necessary for certain strains of HIV to fuse with the cell membrane and penetrate it. After HIV enters the cell, the process of replication begins with the help of the virus' own enzymes. As described below, reverse transcriptase transcribes the RNA genome of the virus to DNA (provirus). The provirus enters the nucleus of the cell where the viral enzyme integrase inserts it into the host cell's DNA and can synthesize new viral RNA.

Some of this new RNA will become the genetic material contained in new viruses. Some will make the proteins which will coat the new virus core. The HIV proteins will cut the polyproteins into functional sizes. Finally, the viral proteins and the viral RNA are assembled into new HIV and bud off the host cell's surface. The HIV life cycle is illustrated in Figure 4.

It is important to understand the viral life cycle in order to know how to combat the progression of HIV disease. For example, as researchers learn more about HIV, it is easier to

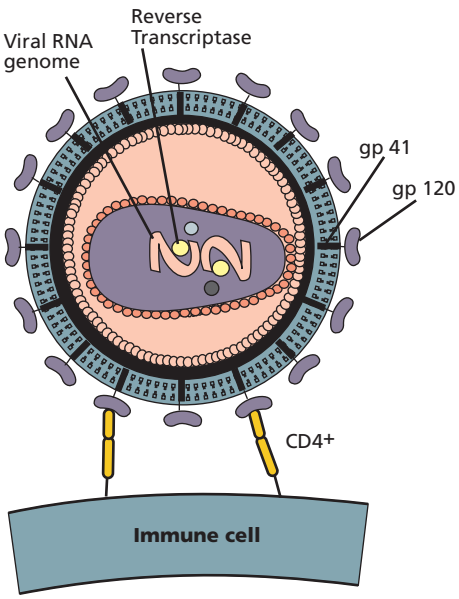


Figure 3: HIV binding to an immune cell.

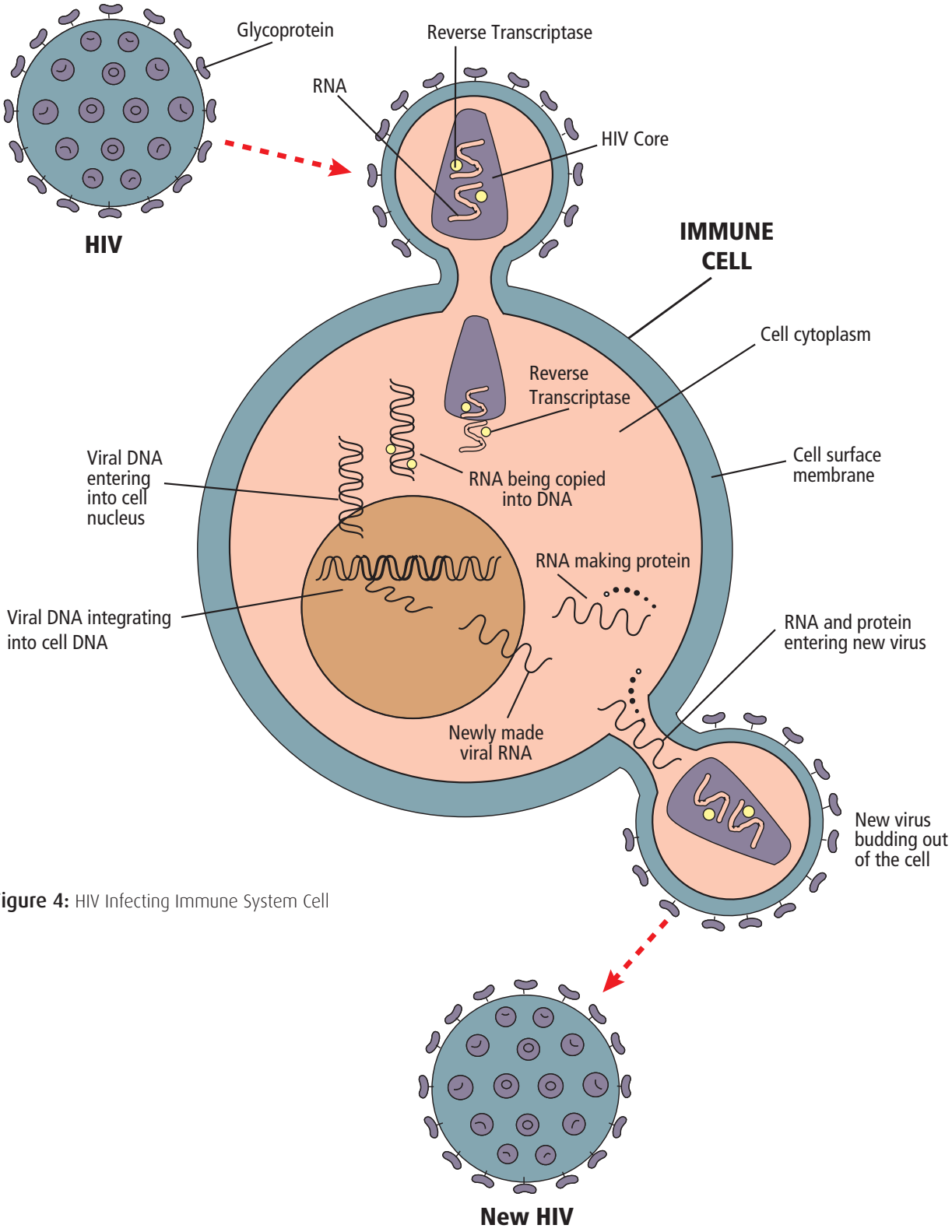


Figure 4: HIV Infecting Immune System Cell

see why treatments with a single drug have failed. HIV is different from other viruses that mutate rapidly because it stays in the body for years, replicating again and again. This large number of rounds of replication multiplied by the high mutation rate makes HIV uniquely dangerous to the host. Researchers continue to examine each stage of the life cycle as well as to note the various ways the immune system fights HIV at different stages. Many drugs called "antiretrovirals" that fight HIV infection are based on different aspects of the structure and function of the HIV molecule.

HIV Replication and Transcription

Through a complex mechanism involving several events, reverse transcriptase synthesizes a double stranded DNA copy of the genomic RNA template. Transfer RNA acts as the primer for the first DNA strand synthesis, resulting in an RNA-DNA hybrid. RNAse H degrades the RNA strand of the RNA-DNA duplex and the polymerase activity synthesizes a complementary DNA strand.

Unlike other cellular DNA polymerases, HIV DNA polymerase (reverse transcriptase) has a high error rate (1 in 10^4). The frequent mutations change the viral protein epitopes. This is believed to be the main mechanism of HIV immunoevasion. The double stranded DNA (dsDNA) migrate into the cell nucleus where they become covalently integrated into the cellular genomic DNA. This integration is catalyzed by HIV integrase.

The viral DNA integrates via specific, self-complimentary sequences at both ends called long terminal repeats, or LTRs (see Figure 2). The integrated DNA is called proviral DNA or the provirus. The provirus enters a period of latency that can last for several years. The proviral DNA is replicated along with the cellular DNA and can be inherited through many generations.

The HIV proviral DNA contains the major genes common to all non-transducing retroviruses. These genes are *gag*, *pol* and *env* (see Figure 2). HIV also contains five or six other genes that are much smaller. Retroviral transcription is a complex process producing a variety of RNAs. Production of transcripts is controlled in the LTR and transcriptional termination signals are located in each major gene. The RNA transcripts that remain unspliced become packaged in the new viral particles.

The *gag* gene is translated into a polypeptide that is cleaved by a viral protease into four proteins that form the inner shells. Specific protease inhibitors are clinically being used to inhibit protein processing and control the further spread of the HIV virus in patients suffering from AIDS. The *pol* gene encodes the reverse transcriptase and the integrase which is responsible for the genomic incorporation of copy DNA. The *env* gene encodes the surface glycoproteins the viral particles acquire as they bud from the cells.

Experiment Overview

EXPERIMENT OBJECTIVE:

The objective of this experiment is for students to gain an understanding of the principles and practice of RT-PCR and to relate these reactions to HIV replication.

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.

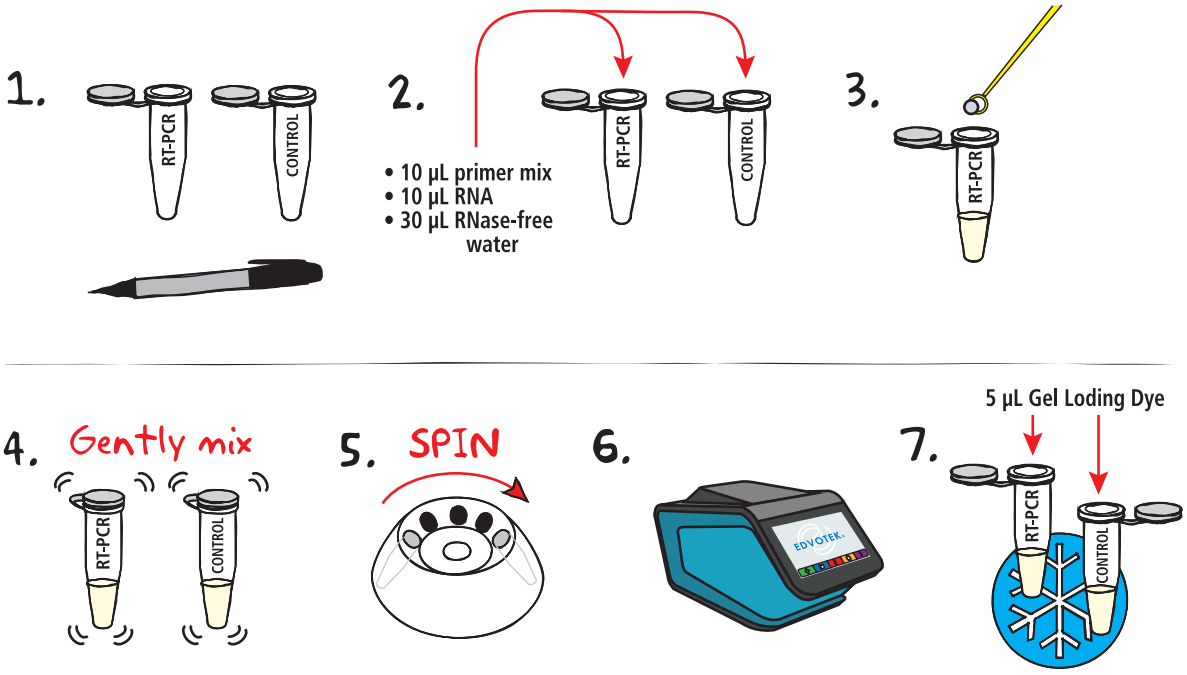
During the Experiment:

- 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: RT-PCR Reaction



1. **LABEL** two PCR tubes with "RT-PCR" or "Control" plus your group name or number.
2. To each tube, **ADD** 10 µL RT-PCR primer mix, 10 µL RNA, and 30 µL RNase-free water.
3. **ADD** the RT-PCR EdvoBead™ to the tube labeled "RT-PCR".
4. **MIX** the samples gently. Make sure the RT-PCR EdvoBead™ is completely dissolved.
5. **CENTRIFUGE** to collect the sample at the bottom of the tube.
6. **AMPLIFY** sample using RT-PCR:

   Reverse Transcription:

   65° C for 40 minutes

   PCR cycling conditions:

   Initial Denaturation 94°C for 5 minutes

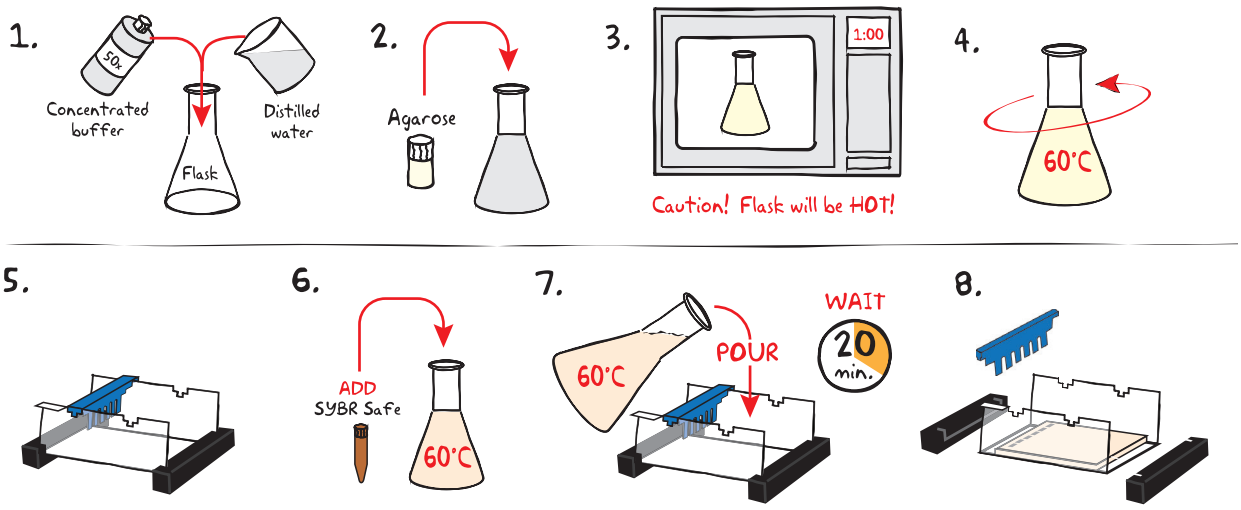
   94° C for 30 seconds
   50° C for 30 seconds } 35 cycles
   72° C for 30 seconds

   Final Extension 72° C for 5 minutes
7. After RT-PCR, **ADD** 5 µL 10X Gel Loading Dye to the samples. **PLACE** tubes on ice. **PROCEED** to Module II: Agarose Gel Electrophoresis.

OPTIONAL STOPPING POINT

The RT-PCR samples may be stored at -20°C for electrophoresis at a later time.

Module II: Agarose Gel Electrophoresis



PREPARING THE AGAROSE GEL WITH SYBR® SAFE STAIN

1. **DILUTE** the concentrated (50X) electrophoresis buffer with distilled water to create 1X buffer (see Table A).
2. **MIX** the agarose powder with 1X buffer in a 250 mL flask (see Table A).
3. **DISSOLVE** the 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** the agarose to 60° C by carefully swirling the flask to promote even dissipation of heat.
5. While the agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the comb in the appropriate notch.
6. Before casting the gel, **ADD** the diluted SYBR® Safe stain to the cooled 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** the end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

Wear gloves and safety goggles

IMPORTANT:

7 x 14 cm gels are recommended. Place the comb in the first set of notches.

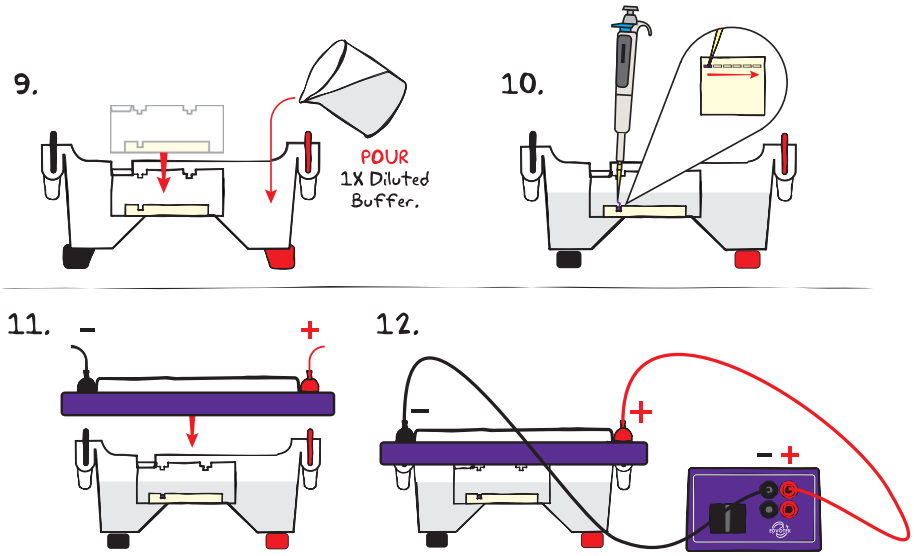
If you are unfamiliar with agarose gel prep and electrophoresis, detailed instructions and helpful resources are available at **www.edvotek.com**

Table A — Individual 1.0% UltraSpec-Agarose™ Gel with Diluted SYBR® Safe Stain

Size of Gel Casting tray	Concentrated Buffer (50x)	+ Distilled Water	+ Amt of Agarose	= TOTAL Volume	Add DILUTED SYBR® (Step 6)
7 x 7 cm	0.5 mL	24.5 mL	0.25g	25 mL	25 µL
7 x 14 cm	1.0 mL	49.0 mL	0.50 g	50 mL	50 µL

NOTE:
The listed volumes of SYBR® Safe are for stain that has already been diluted by the instructor.

Module II: Agarose Gel Electrophoresis



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

Wear gloves and safety goggles

RUNNING THE GEL

9. **PLACE** the gel (on the tray) into the electrophoresis chamber. **COVER** the gel with 1X electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.
10. **LOAD** the entire volume (25 µL) into the well in the order indicated by Table 1, right.
11. **CHECK** that the gel is properly oriented, then **PLACE** the safety cover onto the chamber. Remember, the DNA samples will migrate toward the positive (red) electrode.
12. **CONNECT** the 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.

Table 1

Lane	Recommended
1	EdvoQuick™ DNA Ladder
2	Control Sample Group 1
3	RT-PCR Sample Group 1
4	Control Sample Group 2
5	RT-PCR Sample Group 2

OPTIONAL STOPPING POINT:
Gels can be stored for several days. Place gel in a watertight plastic bag with 2 mL of electrophoresis buffer and store in the refrigerator.

Table B — 1x Electrophoresis Buffer (Chamber Buffer)

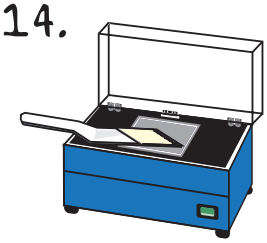
EDVOTEK Model #	Total Volume Required	Dilution: 50x Conc. Buffer	+ Distilled Water
M6+	300 mL	6 mL	294 mL
M12	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

Table C — Time and Voltage Guidelines (1.0% - 7 x 14 cm Agarose Gel)

Volts	Recommended Time: Minimum	Maximum
150	40 min.	55 min.
125	55 min.	1 hour 15 min.
70	2 hours 15 min.	3 hours

Module II: Agarose Gel Electrophoresis

14.



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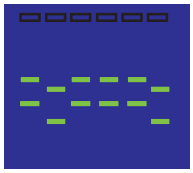

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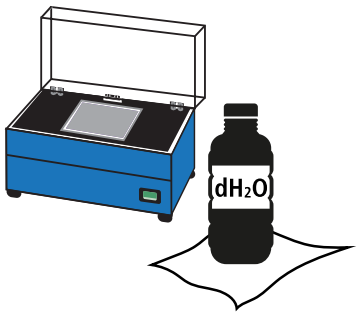


VISUALIZING THE SYBR® GEL

14. **SLIDE** the gel off the casting tray onto the viewing surface of the transilluminator and turn the unit on. **ADJUST** the brightness to the desired level to maximize band visualization. DNA should appear as bright green bands on a dark background.
15. **PHOTOGRAPH** the results.
16. **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 III: Size Determination of RT-PCR Amplified DNA Fragment

Agarose gel electrophoresis separates DNA 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 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 5: 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. 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!

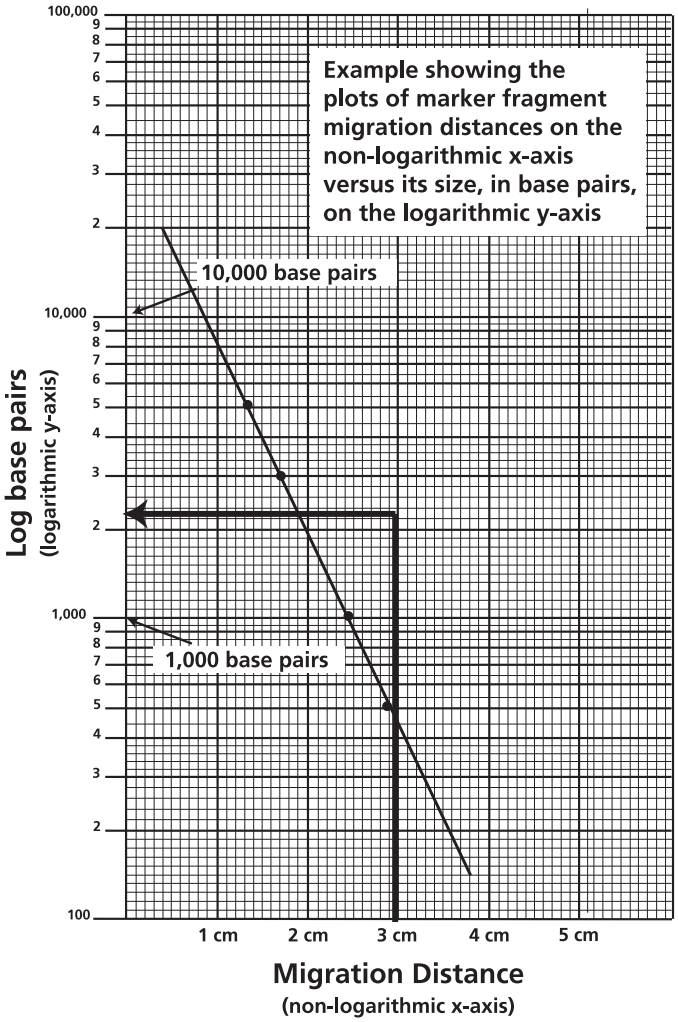


Figure 6: Semilog graph example

Module III: Size Determination of RT-PCR Amplified DNA Fragment

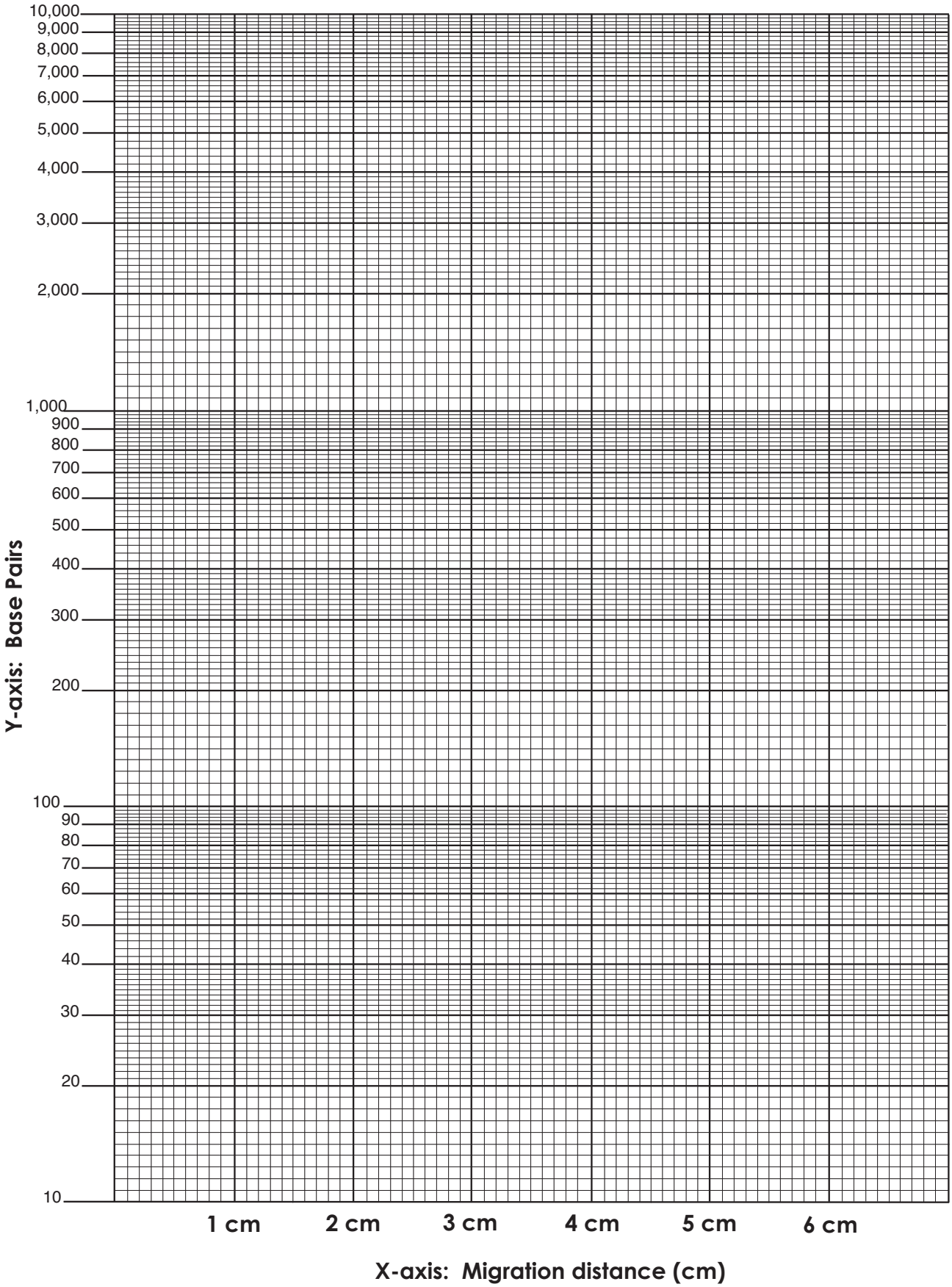
After all the points have been plotted, use a ruler or a straight edge to draw the best straight line possible through the points. The line should have approximately equal numbers of points scattered on each side of the line. It is okay if the line runs through some points (see Figure 6 for an example).

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 6 for an example). Make note of this in your lab notebook.

c. Repeat for each fragment in your unknown sample.



Study Questions

1. Why can the onset of AIDS take several years?
2. Why are there so many immunological variants of HIV?
3. What is the source of the four dXTPs required for DNA synthesis?
4. What is the source of the tRNA primer and what is its function in the viral replication?

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: RT-PCR 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.	Any time before performing the experiment.	15 min.
Module II: Agarose Gel Electrophoresis	Prepare diluted electrophoresis buffer & dilute SYBR® Safe	Up to one week before performing the experiment.	45 min.
	Prepare molten agarose and pour batch gels (OPTIONAL)		
Module III: Size Determination of PCR Amplified DNA Fragment	Print semi-log paper	Any time before the class.	5 min.

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

NOTE:
The PCR cycling conditions may have changed. Before running the experiment, confirm that the program matches the settings below:

Reverse Transcription:
65° C for 40 minutes

PCR cycling conditions:
Initial Denaturation 94°C for 5 minutes
94° C for 30 seconds
50° C for 30 seconds } 35 cycles
72° C for 30 seconds
Final Extension 72° C for 5 minutes

Pre-Lab Preparations - Module I

RT-PCR REACTION

NOTE: Wear gloves when handling all tubes for this experiment. RNA from your fingers will interfere with the experimental results.

This kit features the NEW EDVOTEK® LyphoTemplate™ and LyphoPrimer™. The reagents must be reconstituted before use.

PREPARATION OF PRIMER MIX

1. Thaw the RNase-free water (D).
2. Before preparing the primer mix, make sure the solid material is at the bottom of the LyphoPrimer™ tube. If not, centrifuge the tube at full speed for 20 seconds or tap the tube on lab bench.
3. Add 500 µL of RNase-free water (D) to the LyphoPrimer™ tube (B). Mix well by pipetting up and down, then place on ice. Be sure that the white pellet is completely dissolved.
4. Aliquot 25 µL of the diluted primer mix into appropriately labeled microcentrifuge tubes. Place the tubes on ice until they are needed.
5. Distribute tubes of diluted primer to each group.

PREPARATION OF RNA TEMPLATE

1. Before preparing the RNA template, make sure the solid material is at the bottom of the LyphoTemplate™ tube. If not, centrifuge the tube at full speed for 20 seconds or tap the tube on lab bench.
2. Add 150 µL oof RNase-free water (D) to the tube containing RNA LyphoTemplate™ (A). Mix well by pipetting up and down, then place on ice. Be sure that the white pellet is completely dissolved.
3. Aliquot 25 µL of the diluted RNA control into appropriately labeled microcentrifuge tubes. Place the tubes on ice until they are needed.
4. Distribute tubes of diluted RNA.

ADDITIONAL MATERIALS

- Dispense 50 µL of 10X Gel Loading Solution per tube. Label these 6 tubes "10x Solution". Distribute one tube per student group.
- Dispense 100 µL of RNAse-free water (D) per tube. Label these tubes "Water". Distribute one tube per student group.
- Each group will also receive two PCR tubes and one RT-PCR EdvoBead™. For best results, use RT-PCR EdvoBeads™ within two weeks of receipt. The RT-PCR EdvoBEads™ should arrive as small, white, spherical pellets in a plastic tube. If the bead appears to be shriveled or melted, please contact EDVOTEK® customer service before performing the experiment.

Notes and Reminders:

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

For thermal cyclers which do not have a top heating plate, it is necessary to place a layer of wax above the PCR reactions in the microcentrifuge tubes to prevent evaporation. Visit **www.edvotek.com** for more information.

FOR MODULE I
Each Group should receive:

- 25 µL RNA Template
- 25 µL Primer Mix
- 100 µL RNA-free Water
- 50 µL 10x Gel Loading Solution
- One RT-PCR EdvoBead™ in tube
- Two PCR tubes

Pre-Lab Preparations - Module II

AGAROSE GEL ELECTROPHORESIS

Preparation of Agarose Gels:

This experiment requires one 1.0% agarose gel per student group. For best results, we recommend using a 7 x 14 cm gel. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow approximately 30-40 minutes for this procedure.

Individual Gel Preparation
Each student group can be responsible for casting its own individual gel prior to conducting the experiment (see Module III in the Student's Experiment Procedures). Students will need 50X concentrated buffer, distilled water and agarose powder.

Batch Gel Preparation
To save time, a larger quantity of agarose solution can be prepared for sharing by the class (see Appendix B).

SYBR® Safe Stain Preparation
Prepare diluted SYBR® Safe by adding 300 µL of 1X electrophoresis buffer to the tube of concentrated SYBR® Safe and tapping the tube several times to mix. For individual gel preparation, each group will need 50 µL of the diluted SYBR® Safe for a 7 x 14 cm gel. For Batch Gel Preparation, you will use the entire tube of SYBR® Safe (see Appendix B).

Preparing Gels in Advance
Gels may be prepared ahead and stored for later use. Solidified gels can be stored for up to a week in the refrigerator in plastic bags containing a small amount of buffer to prevent drying. We recommend adding only 2 mL of buffer to the bag to prevent SYBR® Safe Stain from diffusing out of the gel.

Do not store gels at -20° C because freezing will destroy them.

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 1.0% gel should be loaded with the EdvoQuick™ DNA ladder and PCR reactions from one student group.

- Pipette 30 µL of the EdvoQuick™ DNA ladder (E) into labeled microcentrifuge tubes and distribute one tube of EdvoQuick™ DNA ladder per gel.

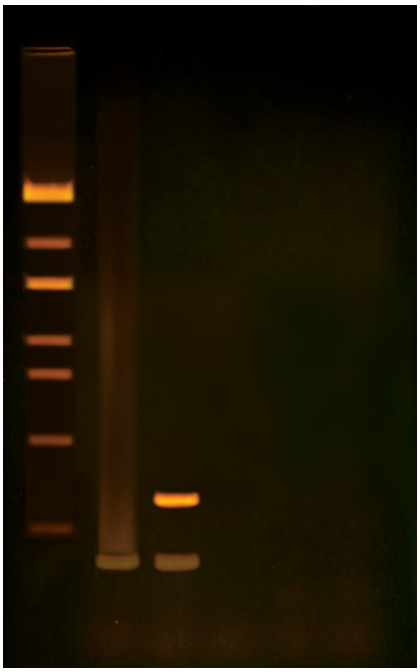
NOTE:
Accurate pipetting is critical for good experiment results. This experiment is designed for students who have had previous experience with micropipetting techniques and agarose gel electrophoresis.

If students do not know how to use micropipettes, we recommended performing Cat. #S-44, Micropipetting Basics or Cat. #S-43, DNA DuraGel™ prior to conducting this advanced level experiment.

FOR MODULE II
Each Group should receive:
- 50X concentrated buffer
- Distilled Water
- UltraSpec-Agarose™ Powder
- Diluted SYBR® Safe Stain (25 µL)
- EdvoQuick DNA ladder (30 µL)

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

Experiment Results and Analysis



Lane	Recommended
1	EdvoQuick™ DNA Ladder
2	Control Sample
3	RT-PCR Sample

The gel photo example shows the approximate relative amplifications of the PCR amplified band. Smaller fragments stain less efficiently and will appear as fainter bands. A 300 bp amplicon will be observed in the samples that contain the RT-PCR EdvoBead™.

Includes EDVOTEK's All-NEW
EdvoQuick™ DNA Ladder

- Better separation
- Easier band measurements
- No unused bands

EdvoQuick™ DNA ladder sizes:
2640, 1400, 1100, 700, 600, 400, 200

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

Appendices

A EDVOTEK® Troubleshooting Guide

B Bulk Preparation of Agarose Gels

Safety Data Sheets:

Now available for your convenient download on **www.edvotek.com/Safety-Data-Sheets**

Appendix A
EDVOTEK® Troubleshooting Guides

RT-PCR AND ELECTROPHORESIS

PROBLEM:	CAUSE:	ANSWER:
There is very little liquid left in tube after RT-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 RT-PCR reaction with wax (see Appendix B for details)
		Make sure students close the lid of the RT-PCR tube properly.
	Pipetting error.	Make sure students pipet 20 µL primer mix and 5 µL extracted DNA into the 0.2 mL tube.
The ladder, control DNA, and student RT-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.	Repeat staining.
	Malfunctioning electrophoresis unit or power source.	Contact the manufacturer of the electrophoresis unit or power source.
After staining the gel, the DNA bands are faint.	The gel was not stained properly.	Ensure that the SYBR® Safe Staining protocol was followed properly.
After staining the gel, 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 and control RT-PCR products are visible on the gel but some student samples are not present.	Wrong volumes of DNA and primer added to RT-PCR reaction.	Practice using micropipets
	RT-PCR EdvoBead™ was shriveled or melted.	RT-PCR EdvoBead™ had expired. Please contact customer support.
Low molecular weight band in RT-PCR samples	Pipetting error-low amount of RNA template in RT-PCR sample.	Practice using micropipets.
DNA bands were not resolved.	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.	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 SYBR® Safe may fade with time.	Use only a minimal amount of buffer when storing gels at 4°C. Too much buffer will lead to diffusion of the SYBR® Safe Stain.

Appendix B
Bulk Preparation of Agarose Gels

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities that the whole class can share. Leftover 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 1X Electrophoresis Buffer

50x Conc. Buffer	+	Distilled Water	Total Volume 1X Buffer
60 mL		2,940 mL	3000 mL (3 L)

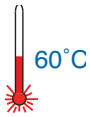
BATCH AGAROSE GELS (1.0%)

For quantity (batch) preparation of 1.0% agarose gels, reference **Table E**.

Table E — Batch Preparation of 1.0% UltraSpec-Agarose™

50x Conc. Buffer	+	Distilled Water	+	Amt of Agarose	=	Total Volume
6.0 mL		294 mL		3.0 g		300 mL

1. Use a 500 mL flask to prepare the 1X electrophoresis buffer.
2. Pour the measured UltraSpec-Agarose™ into the prepared buffer. Refer to **Table E** for the mass. 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. If staining with SYBR® Safe, add the entire volume of diluted SYBR® Safe, prepared on page 20, to the cooled agarose.
7. Dispense the required volume of cooled agarose solution for casting each gel. Measure 25 mL for each 7 x 7 cm gel, 50 mL for each 7 x 14 cm gel. ***For this experiment, 7 x 14 cm gels are recommended.***
8. 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. Alternatively, gels can be stored in water-tight plastic bags with 2 mL of 1X electrophoresis buffer for up to 1 week in the refrigerator.

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 our website. **www.edvotek.com/quick-guides**