EDVOTEK®

THE **BIOTECHNOLOGY EDUCATION** COMPANY®

Edvo-Kit #
363

Edvo-Kit #363

Detecting COVID-19 Using Reverse-Transcription PCR (RT-PCR)

Experiment Objective:

Reverse-transcription PCR (RT-PCR) is the gold standard for the detection of SARS-CoV-2 due to the sensitivity and specificity of the test. In this simulated RT-PCR experiment, students will explore the diagnostic test used worldwide to diagnose and monitor the spread of COVID-19. This experiment requires a PCR thermal cycler.

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

	Components	Storage	Check (√)
A	LyphoPrimer™ Mix	-20 °C, desiccated	❑
B	EdvoQuick™ DNA Ladder	-20 °C	❑
C	Negative Control	-20 °C, desiccated	❑
D	Positive Control	-20 °C, desiccated	❑
E	Patient #1 Sample	-20 °C, desiccated	❑
F	Patient #2 Sample	-20 °C, desiccated	❑
G	Patient #3 Sample	-20 °C, desiccated	❑
H	TE Buffer	-20 °C	❑
•	PCR EdvoBeads™ PLUS	Room Temp.	❑

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

NOTE: Components A and C-G are supplied in concentrated and lyophilized form.

Experiment #363 contains enough reagents to amplify 25 DNA samples using the Polymerase Chain Reaction. This represents five complete sets of reactions.

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 *(Included with this experiment)*

Store all components below at room temperature.

Component	Check (√)
• UltraSpec-Agarose™	❑
• Electrophoresis Buffer (50x)	❑
• SYBR® Safe Stain	❑
• FlashBlue™ Liquid Stain	❑
• Microcentrifuge Tubes	❑
• 0.2 mL PCR tubes	❑

Requirements *(NOT included with this experiment)*

- Thermal cycler (EDVOTEK Cat. #541-542 or Cat. #540 highly recommended)
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Microcentrifuge
- UV Transilluminator or Blue Light visualization (EDVOTEK Cat. #558 or Cat. #557 highly recommended)
- White light visualization system (OPTIONAL - use if staining with FlashBlue™) (EDVOTEK Cat. #557 or Cat. #552 highly recommended)
- UV safety goggles
- Automatic micropipettes (5-50 µL) with tips (EDVOTEK Cat. #590 recommended)
- Microwave
- 250 mL flasks or beakers
- Hot gloves
- Disposable laboratory gloves
- Blue and black pens
- Tape
- Scissors

Background Information

The Polymerase Chain Reaction (PCR) is a powerful diagnostic tool in medicine. This technology rapidly and reliably amplifies specific sections of DNA. This allows doctors and clinicians to observe mutations in a person's genome and confirm a genetic condition or cancer. It also allows them to detect and identify other organisms in a patient's sample such as bacteria and viruses and diagnose an infection. But what happens when the organism causing the infection lacks DNA? While, pathogenic bacteria, fungi, protozoa, and worms all have DNA, many viruses do not. Instead, these viruses use ribonucleic acid as their genetic material. Several human diseases are caused by RNA viruses including Dengue Virus, hepatitis C & E, West Nile fever, Ebola virus disease, rabies, polio, measles, and COVID-19. Luckily a modified PCR known as reverse transcription PCR allows doctors to first convert RNA to DNA and then amplify any copies of the viral genome that are present.

THE POLYMERASE CHAIN REACTION

PCR was invented by Dr. Kary Mullis in 1984. Mullis recognized that he could replicate DNA *in vitro* using short, synthetic DNA oligonucleotides (known as primers) and DNA Polymerase I in a process similar to DNA replication in a cell's nucleus. Furthermore, because researchers can customize the primers to target a specific gene, this method would allow for the rapid amplification of a selected DNA sequence. This technology was then supercharged by Dr. Randy Saiki who began using a thermostable DNA polymerase from the bacteria *Thermus aquaticus* called *Taq* in PCR reactions. This enzyme, which could withstand the large temperature changes involved in the process, allowed PCR to be automated and made it far more accessible and affordable.

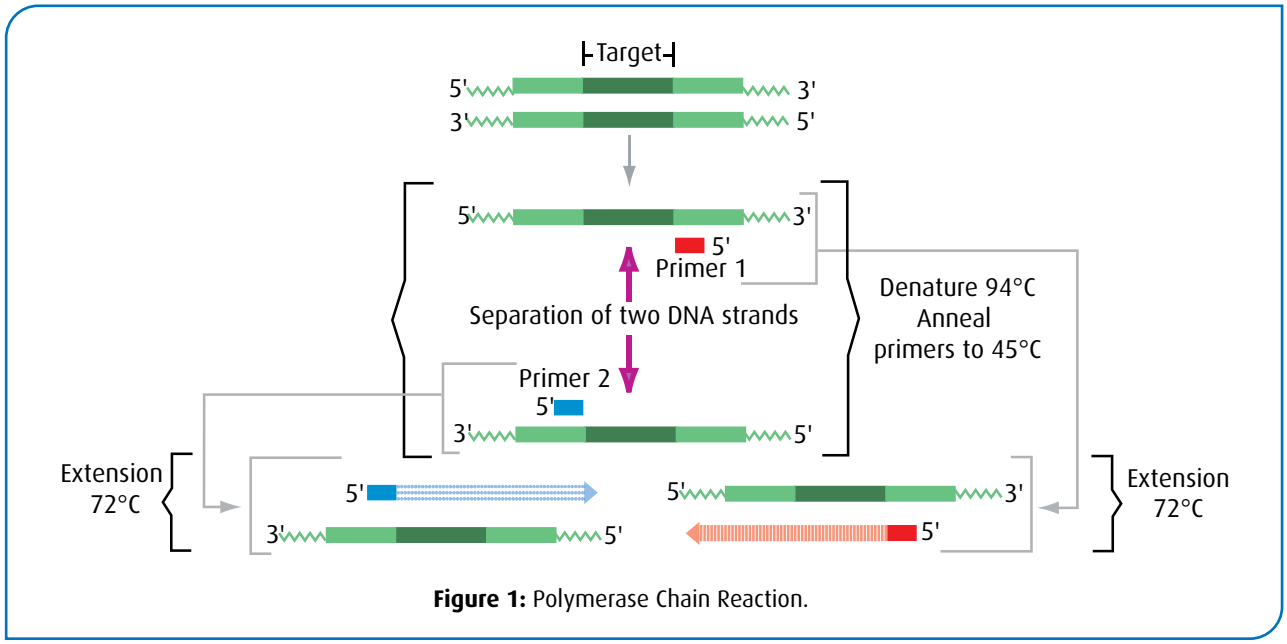


Figure 1: Polymerase Chain Reaction.

To perform PCR, purified double-stranded DNA is mixed with primers, *Taq* polymerase, and nucleotides. Then, the mixture is heated to 94°C to denature the DNA duplex (i.e., unzip it into single strands). Next, the sample is then cooled to 45°C-60°C, allowing the primers to base pair with the target DNA sequence (called "annealing"). Lastly, the temperature is raised to 72°C, the optimal temperature at which *Taq* polymerase will extend the primer to synthesize a new strand of DNA. Each "PCR cycle" (denaturation, annealing, extension) doubles the amount of the target DNA in less than five minutes (summarized in Figure 1). 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.

In diagnostic PCR multiple primers are often used. When these primers are combined into a single reaction the PCR is called a multiplex reaction. By amplifying two or more regions of the organism's genome, multiplex tests increase the chance that the pathogen will be detected if present. They also allow an internal control to be amplified in every single reaction. The control is often a common DNA sequence found in humans, called a "housekeeping gene", that indicates whether or not the experiment was successful. As a result, multiplex reactions allow diagnostic tests to increase their sensitivity and specificity (Box 1).

In order to analyze amplified DNA, scientists may use a technique called agarose gel electrophoresis. In electrophoresis, mixtures of amplified DNA molecules are added into depressions (or "wells") within a gel, and then an electrical current is passed through the gel. The current drives the negatively charged DNA molecules through a network of microscopic channels in the gel and towards the positive electrode. Small DNA fragments move through these channels easily, but large DNA fragments have a more difficult time. As a result, molecules of different size become separated and form discrete "bands" within the gel (Figure 2). A test is positive when the expected band(s) are present and negative when the expected band(s) are absent.

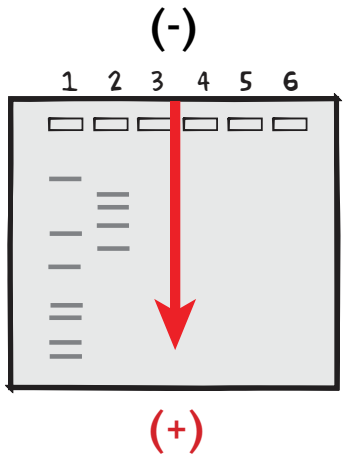


Figure 2: DNA fragments move through the agarose gel.

Alternatively, scientists can visualize PCR results in real-time by using fluorescent dyes. These dyes are activated whenever DNA amplifies. As a result, the PCR reaction emits a fluorescence whose intensity is proportional to the number of DNA copies created during each cycle. Such tests can quickly provide a positive or negative result. This assay, called quantitative pcr (or qPCR), can also be used to estimate how much genetic material was originally present.

REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTIONS

Reverse Transcription (RT) PCR is used when the genetic material of interest is RNA rather than DNA. This technique involves the same PCR process outlined above but with an added precursory step that first converts RNA into DNA. This step is necessary because *Taq* polymerase only works on DNA. In nature genetic information generally flows in a single direction from DNA to RNA (and then eventually to proteins). However, another enzyme called reverse transcriptase allows for the opposite to occur: it can construct DNA from an RNA template. In RT-PCR this enzyme is used to first create DNA that is then amplified by traditional PCR methods. This synthesized DNA is often called complementary DNA or simply cDNA.

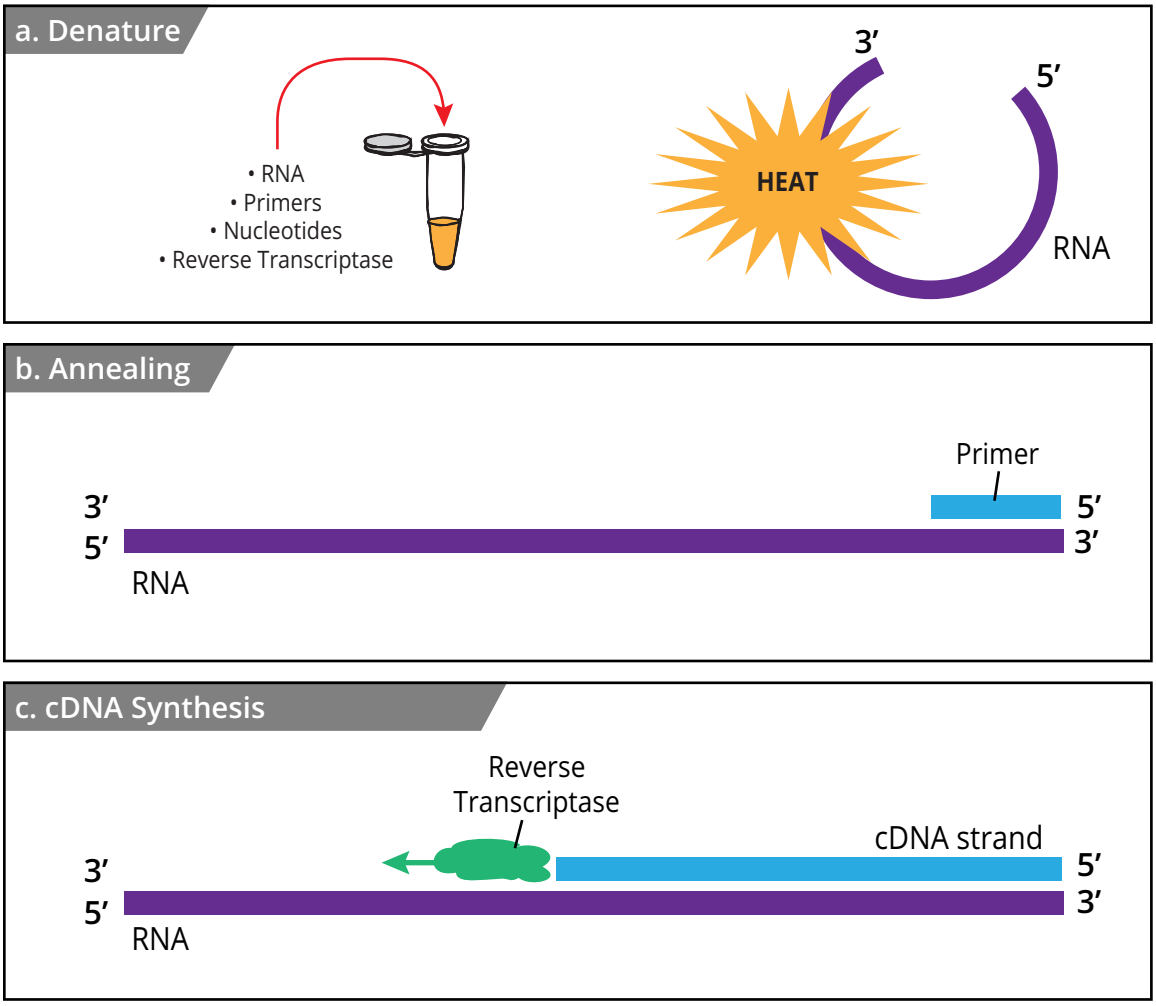
BOX 1:

Sensitivity The ability to test to correctly identify an individual with a disease as positive. A highly sensitive test will have a low rate of false negatives. This means there will be fewer cases where the diseased is missed.

Specificity The ability of a test to correctly identify an individual without the disease as negative. A highly specific test will have a low rate of false positives. This means there will be fewer cases where someone without the disease tests positive and receives unnecessary treatment or takes unnecessary precautions.

During RT-PCR, a sample containing RNA, primers, nucleotides, and reverse transcriptase is first heated to denature the secondary structure of the RNA (Figure 3a) and then cooled to allow the primers to anneal to the RNA (Figure 3b). Next, the solution is slightly heated to activate the reverse transcriptase which creates new DNA using the RNA molecule as a template. The reaction then transitions to traditional PCR.

Figure 3: Overview of Reverse Transcriptase Reaction.



RT-PCR can be performed as either a one-step or two-step reaction. In the former, the reverse transcriptase enzymes and *Taq* are premixed and the entire reaction occurs in a single tube. This reduces the risk of contamination, streamlines the process, and makes it more specific, but can also make the test less sensitive. In the latter, cDNA is first created in a separate reverse transcriptase reaction and then transferred to a new tube for PCR. In addition to being more sensitive, this two-step approach allows for a broader range of amplifications but risks contamination and takes longer to both setup and carry out.

DIAGNOSING COVID-19 WITH RT-PCR

SARS-CoV-2 is a novel coronavirus that has caused a worldwide outbreak of the respiratory disease COVID-19. This virus consists of a single-stranded RNA genome wrapped in a helical capsid that is covered by a host-derived and protein-studded envelope (Box 2). Symptoms of COVID-19 may include fever, cough, and shortness of breath. In severe cases, patients may have pneumonia, respiratory distress, acute kidney injuries, and long term neurological and cardiovascular damage (Figure 4). Sadly, this infection can be fatal.

Combatting the global pandemic of COVID-19 is essential. This fight has several fronts. Treatment for COVID-19 includes rest, fluids, and over-the-counter cold medications for mild cases and antiviral medications, steroids, and oxygenation for more severe cases. However, prevention is the best treatment. Researchers and governments worldwide are working rapidly to develop and deploy vaccines. Because the virus is transmitted from person-to-person primarily through liquid droplets, social distancing, mask wearing, and frequent hand washing can also dramatically lower infection risk.

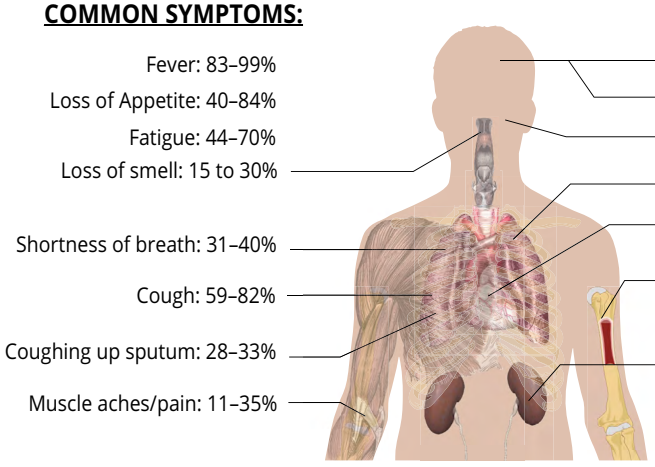
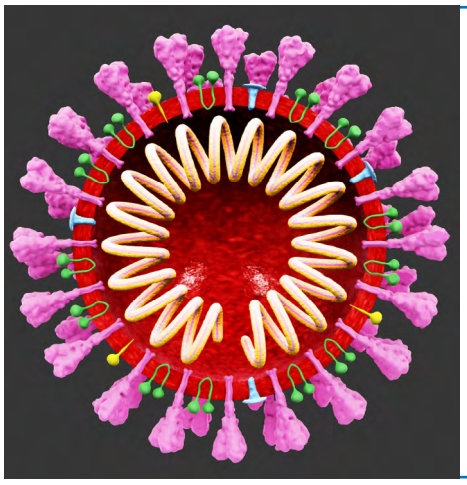


Figure 4: Symptoms of COVID-19.

Perhaps the most important public health tool has been isolating individuals who test positive for COVID-19. There are several diagnostic tests to confirm COVID-19 infection. The two most common are RT-PCR and Enzyme-Linked Immunosorbent Assay (ELISA). In the hospital these tests allow doctors to most effectively treat a patient and to take necessary precautions that protect themselves and others from infection. Because SARS-CoV-2 can also be spread by infected individuals with mild or no symptoms these test are ideally part of a rapid and widespread population testing program. Such programs let individuals repeatedly screen themselves and then quarantine and alert others if they test positive for the virus. They also allow participating individuals to more confidently engage in higher-risk activities like traveling or attending a class. Consequently, regular screenings are seen as key



BOX 2:

Like all coronaviruses, SARS-CoV-2 has four main structural proteins. Monomers of the nucleocapsid or N protein (cream) link together to form a helical capsid which wraps around and protects the RNA genome. Embedded in the membrane are several viral proteins: the spike or S protein (pink), the envelope or E protein (red) and the membrane or M protein (green). The spike protein binds with human cell surface proteins, allowing the virus to inject its genetic material into its host cells. The membrane protein coordinates interactions between the other viral proteins and the host cell factors, turning cells into virus factories. As a viroporin, the envelope protein binds to itself to form channels that facilitate viral release.

to restoring a level of normalcy in daily life - particularly when combined with contact tracing, mask wearing, distancing, and hand washing.

The RT-PCR test is regarded as the gold standard for identifying active infections because of its high level of sensitivity and specificity. Most COVID-19 RT-PCR tests combine three primer sets. The first two sets target regions in the SARS-CoV-2 N protein while the third set amplifies the human housekeeping gene RNase P (RP). Another strength of the test is that it's able to identify infections at an earlier stage than most antibody-based tests. However, the SARS-CoV-2 genome does not integrate into the human genome, like the human immunodeficiency virus (HIV) and other retroviruses. As a result, RT-PCR tests for SARS-CoV-2 cannot identify past infections like the ELISA antibody test can. RT-PCR tests can also take longer (2-4 hours) and require special equipment.

During a typical RT-PCR test the following occurs (Figure 5):

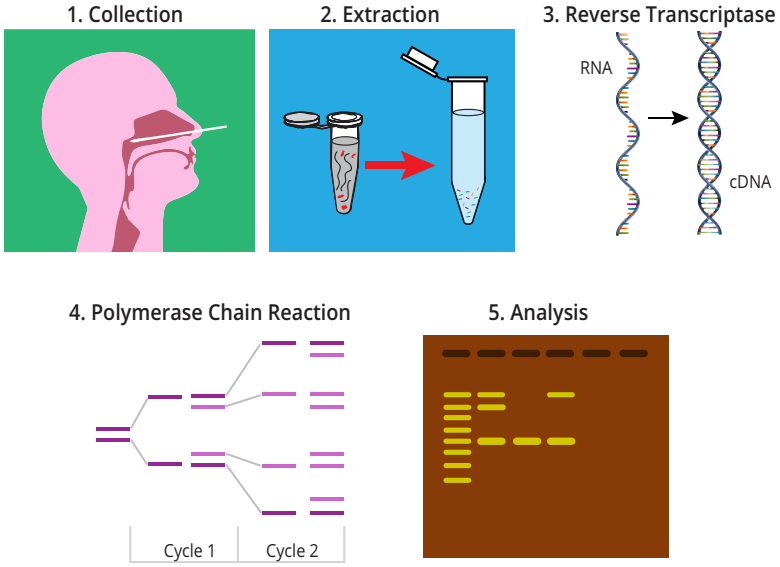


Figure 5: RT-PCR testing for COVID-19.

1. Collection: A sample is collected from the patient, ideally from a place where there's a high concentration of viral particles. Often this is from the upper respiratory tract via a nasopharyngeal or pharyngeal swab although saliva samples can also be used.

2. Extraction: Cells in the solution are broken open (lysed) and the RNA is extracted. This can be challenging as RNA quickly degrades in the presence of omnipresent RNA eating enzymes called RNases. Following successful extraction, the sample will contain a diverse mixture of different RNA molecules from both the virus and the human host.

3. Reverse Transcriptase: RNA molecules are converted to complementary DNA. Depending on which primers are used, this step can target a subtype of RNA called messenger RNA, a specific RNA sequence, or all the RNA present in the sample.

4. Polymerase Chain Reaction: Region-specific primers and *Taq* polymerase are used to create millions of copies of the targeted cDNA regions. This step can be further broken down into a) an initial denaturation, b) multiple cycles of denaturation, annealing, and elongation, and c) a final elongation cycle.

5. Analysis: Results are observed and analyzed. The patient is given a positive, negative, or indeterminate test result and, in some cases, an estimated viral load number.

A positive test indicates the presence of SARS-CoV-2 RNA. But it does not mean that a patient will become seriously ill. It also does not rule out the possibility of other co-infections. It does however mean that a person should isolate themselves, alert others they may have come in contact with, and seek medical help if symptoms worsen. A negative test indicates the absence of SARS-CoV-2 RNA but should be interpreted carefully. RT-PCR results are only as good as their starting samples. Patient samples that were improperly collected, stored wrong, or collected before an individual's infection level reached a

certain point can all lead to false negatives. This is why the timing of the test and repeated testing is important. An indeterminate or inconclusive test occurs when only one of the two SARS-CoV-2 targets amplify. This is often considered a presumptive positive test which can be confirmed with a repeat test.

In this experiment, you will first simulate the synthesis of cDNA from RNA in Module I. In Module II you will then perform PCR on five cDNA samples – three from patients who came in contact with a friend who is now being treated for COVID-19 and two controls. Finally, you will separate the PCR products by electrophoresis and analyze the resulting banding patterns in order to diagnose each patient.

IMAGE CREDITS:
BOX 2 image by https://www.scientificanimations.com/wiki-images/, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=86436446
Figure 4: https://en.wikipedia.org/wiki/Coronavirus_disease_2019#/media/File:Symptoms_of_coronavirus_disease_2019_3.0.svg

Experiment Overview

EXPERIMENT OBJECTIVE:

Reverse-transcription PCR (RT-PCR) is the gold standard for the detection of SARS-CoV-2 due to the sensitivity and specificity of the test. In this simulated RT-PCR experiment, students will explore the diagnostic test used worldwide to diagnose and monitor the spread of COVID-19. This experiment requires a PCR thermal cycler.

LABORATORY SAFETY

Wear gloves and safety goggles

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

LABORATORY NOTEBOOKS:

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

Before starting the Experiment:

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

During the Experiment:

- Record your observations.

After the Experiment:

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

Module I: 45 min.
Paper Simulation of Reverse Transcription

↓

Module II: 15 min. + 90 min.
Prepare and Run PCR Amplification

↓

Module III: 50-70 min.
Separation of PCR Products by Electrophoresis

↓

Module IV: 10-30 min.
Staining with FlashBlue™ (OPTIONAL)

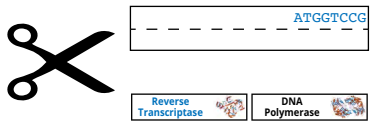
NOTE: Experimental times are approximate.

Module I: Paper Simulation of Reverse Transcription

This exercise simulates reverse transcription using sequence-specific primers for SARS-CoV-2 N proteins (*N1 primer or N2 primer*) and for the human housekeeping gene RNase P (*RP primer*). The simulation begins after a patient's sample has been collected and the RNA has been extracted and denatured.

1. **SELECT** a primer. **CIRCLE** the primer you've selected here: N1 N2 RP

2. On page 15, **CUT OUT** the strip associated with your primer as well as both enzyme tags.

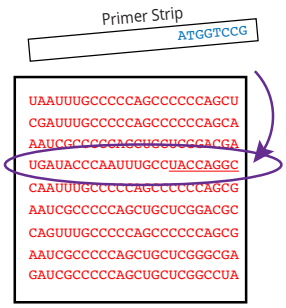


3. **PREPARE** the primer strip by folding it once along the dotted black line so that the boxes and primer sequences are on the outside.

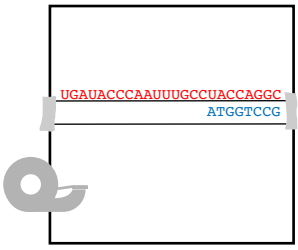
4. **PREPARE** the enzymes by attaching a "reverse transcriptase" flag to a blue pen and a "DNA polymerase" flag to a black pen.

ANNEAL YOUR PRIMER

6. **FIND** the complementary RNA sequence on page 14 that matches your primer sequence - use Table 1a as a guide for base pairing between DNA and RNA.

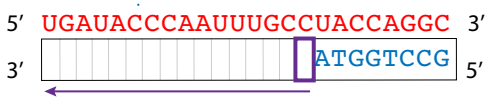


7. Use these paired bases to properly **ALIGN** the primer strip with this RNA molecule. Apply a small amount of tape to **STABILIZE** the strip.

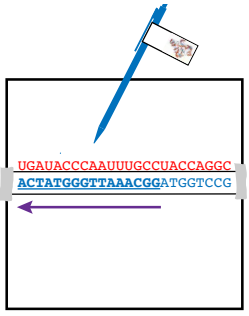


SYNTHESIZE COMPLEMENTARY DNA

8. **FIND** the first blank box immediately to the left of the primer sequence. (Like DNA polymerase, reverse transcriptase builds DNA by attaching nucleotides to the 3′ end of the primer or cDNA strand.)



9. **FIND** your reverse transcriptase (blue) pen. Use the RNA sequence above this blank box and complementary base pairing rules (Table 1b) to **WRITE IN** a DNA nucleotide. **CONTINUE** pairing and filling in the boxes until you reach the end of the RNA molecule.



10. Carefully **REMOVE** the tape and then the strip. This strip is the cDNA that you will be amplifying in Module II.

Module I: Paper Simulation of Reverse Transcription, continued

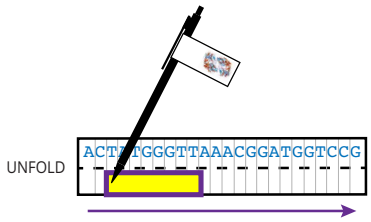
MODEL PART OF THE FIRST PCR CYCLE

11. Use Table 2 to **IDENTIFY** the appropriate forward primer. **FIND** the target sequence that *complements* this primer on your strip. (Remember to use complementary base pair rules! See Table 1C.)

ACTATGGGTTAAACGGATGGTCCG

Primer Strip

12. **UNFOLD** your primer strip. **WRITE** the forward primer sequence in the boxes below this region. To help visualize this primer you may want to highlight these boxes.



13. Using your DNA polymerase (black) pen, continue to **WRITE** complementary base pairs in the empty boxes to the right of the forward primer until you reach the end of the strip. (There may be empty boxes to the left of the forward primer on your final cDNA strip. This is all right as cDNA can contain extra bases beyond the region that will be amplified during subsequent PCR steps.)

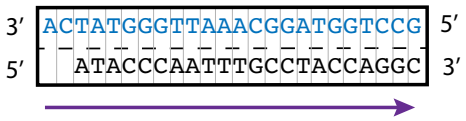


TABLE 1

a. DNA to RNA	b. RNA to DNA	c. DNA to DNA
C→G	G→C	C→G
G→C	C→G	G→C
T→A	A→T	T→A
A→U	U→A	A→T

TABLE 2

Name	Forward Primer
N1	5' GACCCCAAAATCAGCGAAA 3'
N2	5' CAAACATTGGCCGCAAA 3'
RP	5' TGCGAGCGGGTTCTGACC 3'

RNA EXTRACTION

5′ GAGCAGAGGCGGCACUCUUCUCGUUCCUCUUCU 3′

5′ UGGACCCCAAAAUCAGCGAAAUGCACCCCGCAUUACGUUUGGUGGACCCUCAGAUUCAACUGGCAGUAACCAGA 3′

5′ ACAUUGCUCCAAUUUUUCUUAUGACAAAAGUGCCAACACAGCAGAAGUCUGCU 3′

5′ GAGGAAGUUCAAGAACUUUACUCUCCAAUUUUUCUU 3′

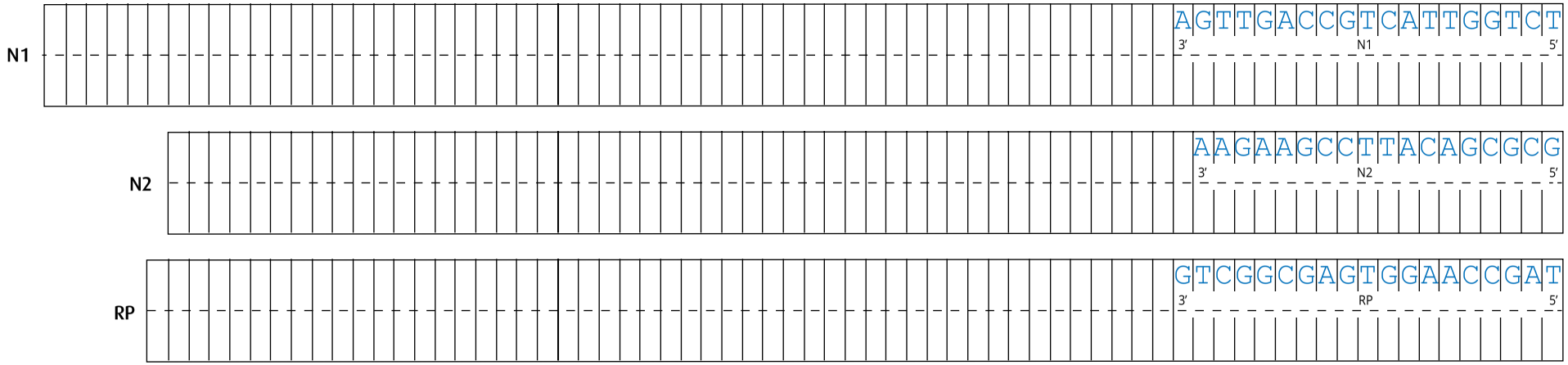
5′ GGACCUGCGAGCGGGUUCUGACCUGAAGGCUCUGCGCGGACUUGUGGAGACAGCCGCUCACCUUGGCUAUUCAGU 3′

5′ AGGACAAACAUUGGCCGCAAAUUGCACAAUUUGCCCCCAGCGCUUCAGCGUUCUUCGGAAUGUCGCGCAU 3′

5′ CUAUUCAGUUGUUGCUAUCACUCUUCUCGA 3′

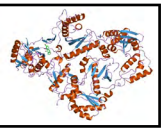
PRIMER STRIPS

Prepare each primer strip by **CUTTING** along the outer solid line and then **FOLDING** along the dotted line.

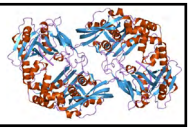


PEN TAGS

Reverse Transcriptase



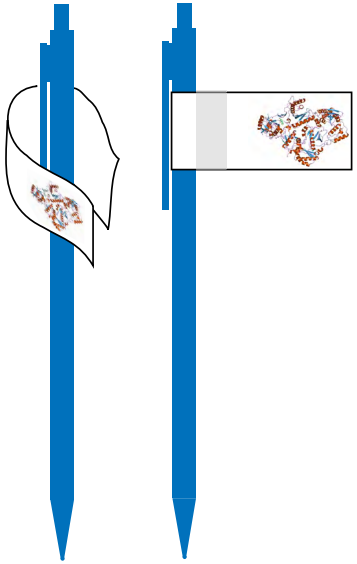
DNA Polymerase



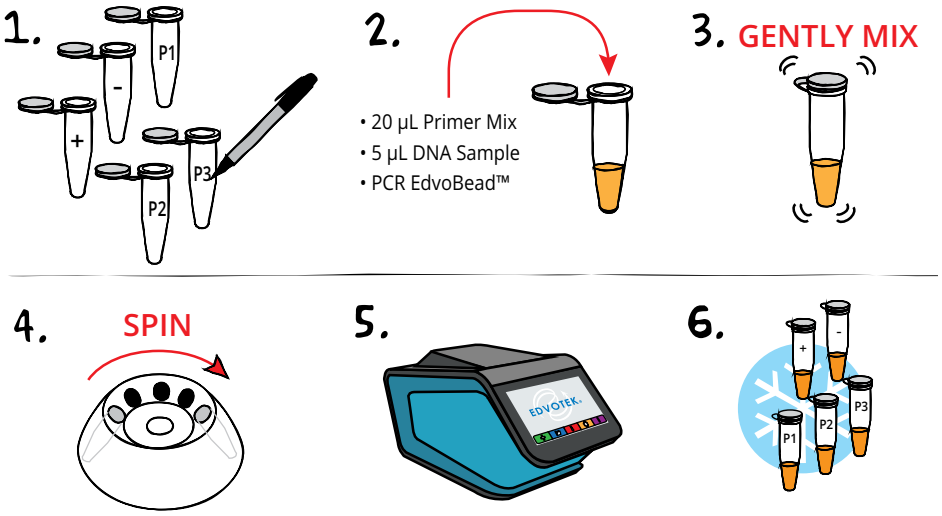
1. **CUT OUT** pen tags along the solid lines.
2. **WRAP** tags around corresponding colored pens and **TAPE** to secure.

   Blue pen - Reverse Transcriptase

   Black pen - DNA Polymerase



Module II: PCR Amplification



*The primer mix in this experiment contains six primers: N1 forward, N1 reverse, N2 forward, N2 reverse, RP forward, RP reverse. These will create three PCR products/bands in a positive sample and one PCR product/band in a negative sample.

1. **LABEL** 5 PCR tubes for: –, +, P1, P2, and P3. Put your initials or group number on the tubes.
2. All PCR reactions should be prepared as follows: **ADD** 20 µL primer mix* (yellow), 5 µL cDNA sample (–, +, P1, P2, or P3) (red) and one PCR EdvoBead™ PLUS to the appropriately labeled 0.2 mL PCR tube.
3. **MIX** each PCR sample. Make sure the PCR EdvoBeads™ PLUS are completely dissolved. ***NOTE: Double-check that both the primer mix and cDNA have been added by looking at the color of the mixture in the PCR tube. The mixture should be orange with the primers and cDNA mixed together.***
4. **CENTRIFUGE** the samples for a few seconds to collect the sample at the bottom of the tubes.
5. **AMPLIFY** the DNA using PCR:

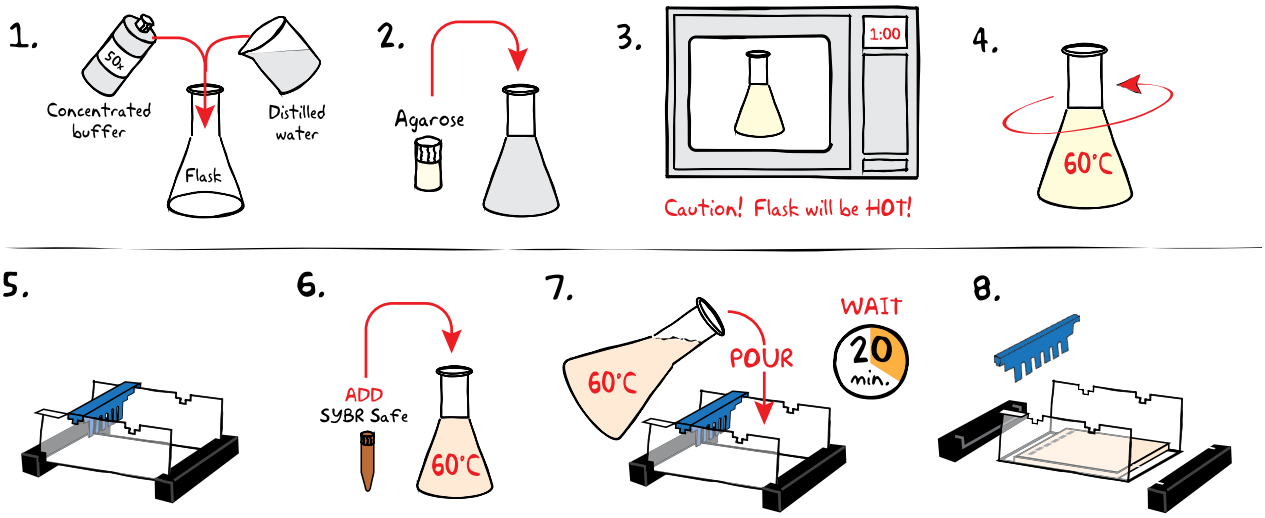
   PCR cycling conditions:
   Initial denaturation 94°C for 3 minutes
   94° C for 30 seconds
   55° C for 30 seconds } 30 cycles
   72° C for 65 seconds
   Final Extension 72° C for 4 minutes

6. After PCR, **PLACE** the tubes on ice. **PROCEED** to Module III: Separation of PCR Products by Electrophoresis.

OPTIONAL STOPPING POINT

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

Module III: Separation of PCR Products by Electrophoresis



PREPARING THE AGAROSE GEL WITH SYBR® SAFE STAIN

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** 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** diluted SYBR® Safe stain to the cooled molten agarose and swirl to mix (see Table A). ***IMPORTANT: Make sure that you or your instructor has diluted the SYBR® Safe before adding it to your agarose solution.***
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 7 cm gels are recommended. Place the comb in the first set of notches.

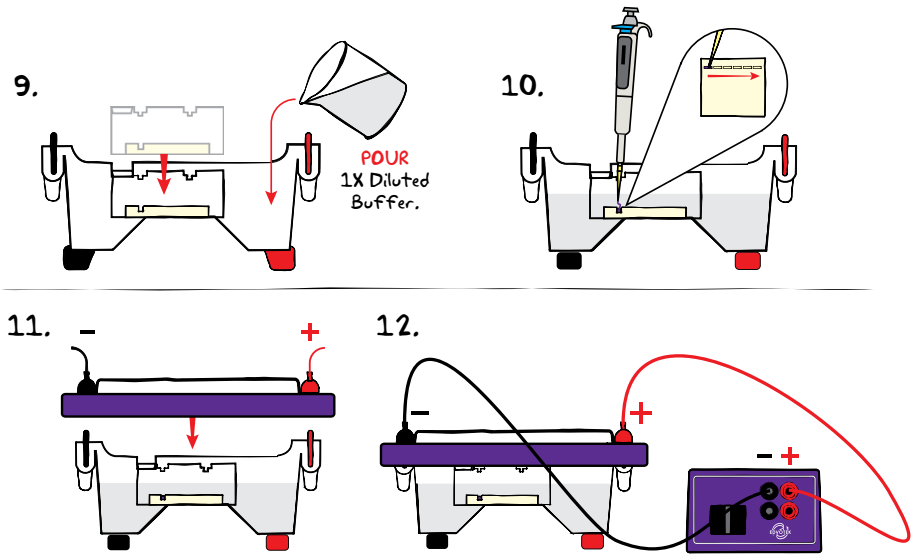
If you are unfamiliar with agarose gel prep and electrophoresis, detailed instructions and helpful resources are available at **www.edvotek.com**

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

Module III: Separation of PCR Products by Electrophoresis, continued



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 3, 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 3: Gel Loading

Lane 1	EdvoQuick™ DNA Ladder
2	Negative Control
3	Positive Control
4	Patient #1 Sample
5	Patient #2 Sample
6	Patient #3 Sample

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. Limit light exposure.

Table B 1x Electrophoresis Buffer (Chamber Buffer)

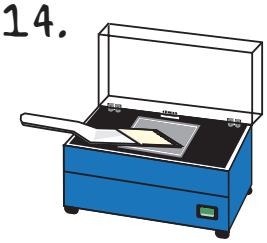
EDVOTEK Model #	Total Volume Required	Dilution: 50x Conc. Buffer	+ Distilled Water
M6+	300 mL	6 mL	294 mL
M12	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

Table C Time and Voltage Guidelines (1.0% – 7 x 7 cm Agarose Gel)

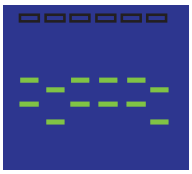
Volts	Recommended Time: Minimum	Maximum
150	15 min.	20 min.
125	20 min.	35 min.
70	35 min.	1 hour

Module III: Separation of PCR Products by Electrophoresis, continued

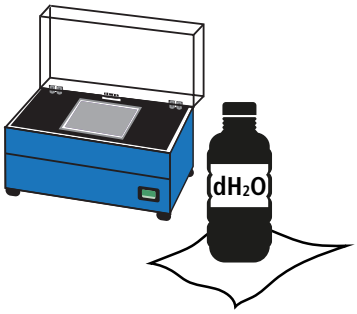
14.



15.



16.



VISUALIZING THE SYBR® GEL

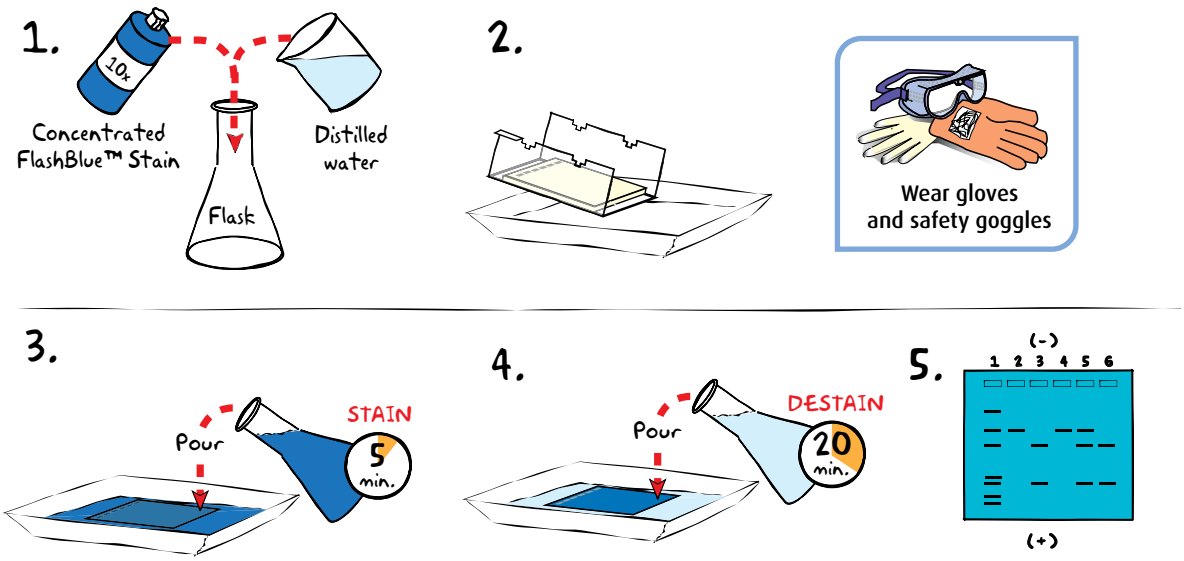
14. **SLIDE** the gel off the casting tray onto the viewing surface of the transilluminator and turn the unit on. **ADJUST** the brightness to the desired level to maximize band visualization. DNA should appear as bright green bands on a dark background.

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 IV: Staining with FlashBlue™ (OPTIONAL)



1. **DILUTE** 10 mL of 10x concentrated FlashBlue™ with 90 mL of water in a flask and **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 5 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 5 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**
4. **TRANSFER** the gel to a second small tray. **COVER** the gel with water. **DESTAIN** for at least 20 minutes with gentle shaking (longer periods will yield better results). Frequent changes of the water will accelerate destaining.
5. 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.

Alternate Protocol:

1. **DILUTE** one mL of concentrated FlashBlue™ stain with 149 mL dH_2O.
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.

Study Questions

1. What are the three steps in a PCR cycle and what does each step accomplish?

2. What is RT-PCR? How does it differ from traditional PCR? How is it the same?

3. Name two popular tests used to identify COVID-19? What are their advantages and disadvantages?

4. What is the difference between sensitivity and specificity? Use COVID-19 diagnostic tests as an example to describe why both are important. Do you think that one is more important than the other?

Instructor's Guide

ADVANCE PREPARATION:

Preparation For:	What to do:	When:	Time Required:
Module I: Paper Simulation of Reverse Transcription	Print copies for each student and assemble required materials.	Any time before performing the experiment.	15 min.
Module II: PCR Amplification	Prepare and aliquot various reagents (Primer mix, cDNA template)	Up to 2 hours before the experiment.	30 min.
	Program Thermal Cycler	Any time before performing the experiment.	15 min.
Module III: Separation by Electrophoresis	Prepare 1X Electrophoresis Buffer and dilute SYBR® Safe Stain. Aliquot ladder.	Up to one day before performing the experiment.	45 min.
	Prepare molten agarose and pour gel (optional)		
Module IV: Staining with FlashBlue™ (OPTIONAL)	Prepare staining components	Any time before the class period.	10 min.

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

Pre-Lab Preparations

MODULE I

1. Each student will require a separate page 15 for cutting out primer strips and pen tags.
2. Instructors should also provide students scissors, tape, and blue and black pens.

FOR MODULE I
Each student will need:

- One copy of page 15 for cut outs
- Colored pens (blue and black)
- Scissors and tape

Pre-Lab Preparations

MODULE II

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

There are five different cDNA samples provided.

Preparation of the DNA Template

1. Add 75 µL TE Buffer (H) to each LyphoTemplate™ (C-G) and mix to dissolve.
2. Label twenty-five 0.5 mL snap-top microcentrifuge tubes as follows:
   5 - Negative Control
   5 - Positive Control
   5 - Patient #1 Sample
   5 - Patient #2 Sample
   5 - Patient #3 Sample
3. Dispense 10 µL of the cDNA template into the appropriately labeled tube.

NOTE:
There is enough material to perform 25 PCR reactions and 5 gels. Students can be divided into groups of five students per group and samples from each group can be run on a gel. Each student will be responsible for one PCR reaction. Groups will be responsible for making, running, and analyzing the gel.

Preparation of the Primer Mix

1. Add 1 mL of TE Buffer (H) to the tube of LyphoPrimer™ Mix (A). Cap tube and mix. The solution should be light yellow and no solid pieces should remain.
2. Pipette 150 µL of the diluted Primer Mix into five labeled snap-top microcentrifuge tubes.
3. Distribute one tube of diluted Primer Mix to each student group.

FOR MODULE II
Each group should receive:
- 5 PCR tubes
- 5 PCR EdvoBeads™ PLUS
- 10 µL Negative Control
- 10 µL Positive Control
- 10 µL Patient #1 Sample
- 10 µL Patient #2 Sample
- 10 µL Patient #3 Sample
- 150 µL Diluted Primer Mix

Programming the Thermal Cycler

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 or mineral oil above the PCR reactions in the microcentrifuge tubes to prevent evaporation. Visit **www.edvotek.com** for more information.

Pre-Lab Preparations

MODULE III: SEPARATION BY ELECTROPHORESIS

Preparation of Agarose Gels:

This experiment requires one 1.0% agarose gel per student group. For best results, we recommend using 7 x 7 cm gels. 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 their own individual gel prior to conducting the experiment. See Module III in the Student's Experimental Procedure. Students will need 50x concentrated buffer, distilled water and agarose powder.

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

SYBR® Safe Stain Preparation
Prepare *diluted* SYBR® Safe by adding 250 µL of 1X TAE 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 30 µL of the diluted SYBR® Safe for a 7 x 7 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 (B) 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.

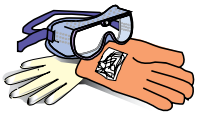
FOR MODULE III
Each group should receive:
- 50x concentrated buffer
- Distilled Water
- UltraSpec-Agarose™ Powder
- 30 µL *diluted* SYBR® Safe Stain
- 30 µL EdvoQuick DNA ladder
- PCR Samples from Module I

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

Pre-Lab Preparations: Module III

MODULE IV: STAINING WITH FLASHBLUE™ (OPTIONAL)

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) is recommended for visualizing gels stained with FlashBlue™.



Wear gloves and safety goggles

- 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.

FOR MODULE IV
Each group should receive:
- 10 mL 10X concentrated FlashBlue OR 100 mL 1x diluted FlashBlue
- Small plastic tray or weight boat
- Distilled or deionized water

Photodocumentation of DNA

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.

MODULE I: PAPER SIMULATION OF REVERSE TRANSCRIPTION

5′ GAGCAGAGGCGGCACUCUUCUCGUUCCUCUUCU 3′

5′ UGGACCCCAAAAUCAGCGAAAUGCACCCCGCAUUACGUUUGGUGGACCCUCAGAUUCAACUGGCAGUAACCAGA 3′
N1

5′ ACAUUGCUCCAAUUUUUCUUAUGACAAAAGUGCCAACACAGCAGAAGUCUGCU 3′

5′ GAGGAAGUUCAAGAACUUUACUCUCCAAUUUUUCUU 3′

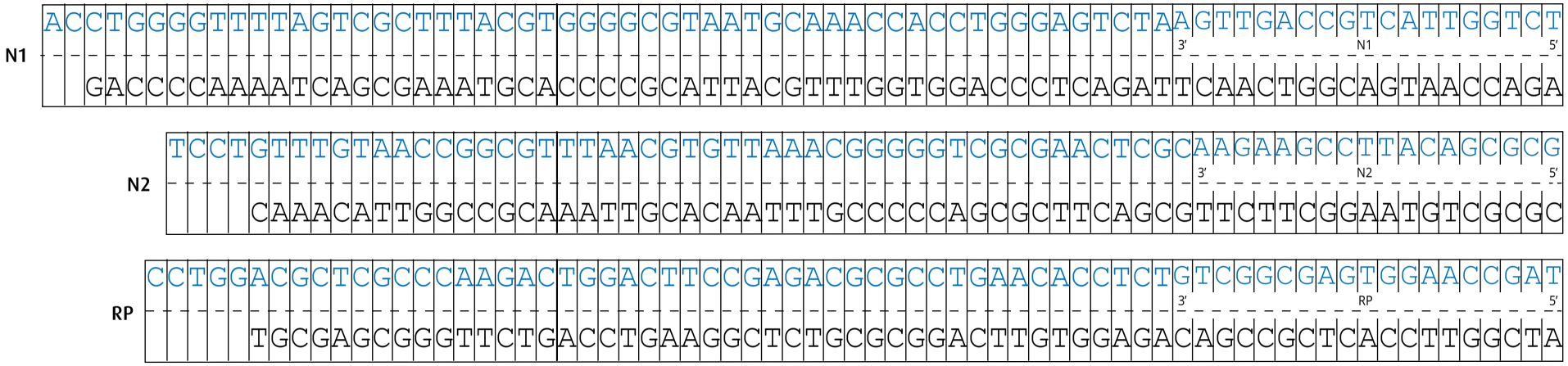
5′ GGACCUGCGAGCGGGUUCUGACCUGAAGGCUCUGCGCGGACUUGUGGAGACAGCCGCUCACCUUGGCUAUUCAGU 3′
RP

5′ AGGACAAACAUUGGCCGCAAAUUGCACAAUUUGCCCCCAGCGCUUCAGCGUUCUUCGGAAUGUCGCGCAU 3′
N2

5′ CUAUUCAGUUGUUGCUAUCACUCUUCUCGA 3′

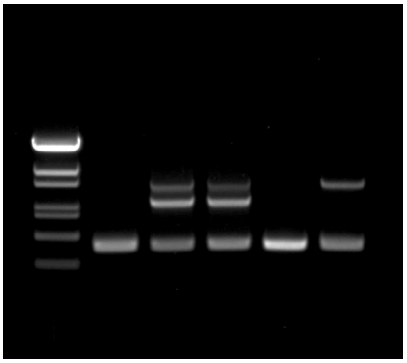
Experiment Results and Analysis

MODULE I: PAPER SIMULATION OF REVERSE TRANSCRIPTION



Experiment Results and Analysis

MODULE III: SEPARATION OF PCR PRODUCTS BY ELECTROPHORESIS



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

The EdvoQuick™ DNA Ladder in lane 1 makes it possible to measure the size of the amplicons produced by the PCR reactions in lanes 2-6.

Lane	Sample	Sizes	Results
1	EdvoQuick™ DNA Ladder	2640, 1400, 1100, 700, 600, 400, 200	---
2	Negative Control	290	Negative (human control only)
3	Positive Control	290, 715, 1000	Positive (human control and viral proteins)
4	Patient #1 Sample	290, 715, 1000	Positive for SARS-CoV-2
5	Patient #2 Sample	290	Negative for SARS CoV-2
6	Patient #3 Sample	290, 1000	Indeterminate

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

Appendices

A EDVOTEK® Troubleshooting Guide

B Bulk Preparation of Electrophoresis Buffer and Agarose Gels

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

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 (see www.edvotek.com for more info).
		Make sure students close the lid of the PCR tube properly.
	Pipetting error.	Make sure students pipet 20 µL primer mix and 5 µL DNA into the 0.2 mL tubes.
The ladder, control DNA, and 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 gel was not stained properly.	Re-stain the gel with SYBR® Safe or FlashBlue™.
	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 for a sufficient period of time.	Re-stain the gel with SYBR® Safe or FlashBlue™.
After staining, the ladder is visible but no PCR products are present.	PCR amplification was unsuccessful.	Repeat PCR with fresh PCR EdvoBeads™ PLUS and primers.
		Ensure that the thermal cycler has been properly programmed.
DNA bands were not resolved.	Blue tracking dye **in ladder** should migrate at least 3.5 cm (if using a 7x7 cm tray), and at least 6 cm (if using a 7x14 cm tray) from the wells to ensure adequate separation.	Be sure to run the gel at least 3.5 cm (7x7 cm tray) or 6 cm (7x14 cm tray) before staining and visualizing the DNA. (See Table C for time and voltage guidelines.)
DNA bands fade when gels are kept at 4°C.	DNA stained with SYBR® Safe or FlashBlue™ may fade with time.	Re-stain the gel with SYBR® Safe or FlashBlue™.

Appendix B
Bulk Preparation of Electrophoresis Buffer and Agarose Gels

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities 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**.

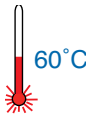
Table D Bulk Preparation of 1X Electrophoresis Buffer

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**.

1. Use a 500 mL flask to prepare the 1X Diluted gel 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 24, to the cooled agarose. This concentration of SYBR® Safe will work for either 300 mL or 400 mL preparations of agarose gels.
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 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. 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.

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
8.0 ml		392 ml		4.0 g		400 ml

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/guides-lesson-plans