EDVOTEK®

THE **BIOTECHNOLOGY**
EDUCATION COMPANY®

Edvo-Kit #
380

Edvo-Kit #380

Discovering Quantitative PCR Amplification & Analysis

Experiment Objective:

The objective of this experiment is for students to gain hands-on experience with the principles and practice of qPCR. Students will quantify the DNA concentration of experimental samples using a standard curve approach and confirm specificity using both melt curve analysis and gel electrophoresis.

See page 3 for storage instructions.

1.800.EDVOTEK • fax 202.370.1501 • www.edvotek.com • info@edvotek.com

Table of Contents

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

Experiment Components

Component		Storage	Check (✓)
A	qPCR Master Mix	-20° C Freezer (Dark)	❑
	Contains: dNTP, Taq, $MgCl_2$, Reaction Buffer, Chia Green Fluorescent Dye		
B	LyphoPrimer™ Mix	-20° C Freezer	❑
C	LyphoTemplate™ DNA	-20° C Freezer	❑
D	Ultra-pure Water	-20° C Freezer	❑
E	DNA Standard Marker	-20° C Freezer	❑

Components B and C are supplied in concentrated form.

This experiment is designed for 4 lab groups.

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 components below at room temperature.

Component	Check (✓)
• Microcentrifuge Tubes	❑
• qPCR Tubes	❑
• UltraSpec-Agarose™	❑
• 50x Electrophoresis Buffer	❑
• 10x Gel Loading Solution	❑
• SYBR® Safe Stain	❑

Requirements

- qPCR Themal Cycler (Edvotek Cat #543 highly recommended)*
- Microcentrifuge
- Adjustable micropipettes (5-50 µL) with tips (filtered tips preferred)
- Disposable vinyl or latex laboratory gloves
- Ice buckets and ice
- Electronic Devises with web access and graphing software (computers, tablets, or smart phones)
- Horizontal gel electrophoresis apparatus
- DC power supply
- Balance
- Blue Light Transilluminator or UV Transilluminator compatible with SYBR® Safe stain (Cat #557 recommended)
- Microwave, hot plate or burner
- Pipet Pump
- 250 mL flasks or beakers
- Hot gloves
- Distilled or deionized water

* *Reagents provided in this kit are compatible with all instruments that support either SYBR Green I or FAM fluorescence. However, some qPCR Thermal Cycle may require specialized tubes or trays not supplied in this kit. Check the manufacturer's instructions.*

Background Information

THE POLYMERASE CHAIN REACTION (PCR)

In 1984, Dr. Kary Mullis revolutionized the field of molecular biology when he devised a simple and elegant method to copy specific pieces of DNA. Recognizing that an initial step in DNA replication is the binding of RNA primers, Mullis discovered that he could replicate DNA in vitro using short, synthetic DNA primers and DNA polymerase I. Furthermore, because the primer's sequence is specific, this method allowed for the precise amplification of a preselected DNA sequence. For the development of this technique, known today as the Polymerase Chain Reaction (or PCR), Mullis was awarded the Nobel Prize in Chemistry in 1993.

Modern PCR differs only slightly from the early versions. Researchers mix purified double-stranded DNA with short DNA primers, a thermostable DNA polymerase (*Taq*) and nucleotides. The mixture is heated to 94°C to "denature" (i.e., unzip into single strands by breaking hydrogen bonds) the DNA duplex. Next, the sample is cooled to 45°C-65°C, allowing the primers to pair with their target DNA sequences (a step known as "annealing"). Lastly, the temperature is raised again, to between 68° C and 72°C, the optimal temperature at which *Taq* polymerase will "extend" the primer to synthesize a new strand of DNA. Each cycle (denaturation, annealing, extension) doubles the amount of target DNA (Figure 1). Today, a specialized machine - called a "thermal cycler" or "PCR machine" - is used to rapidly heat and cool the samples. As a result, a PCR cycle can be completed in less than 5 minutes; 20-40 cycles produce sufficient DNA for analysis.

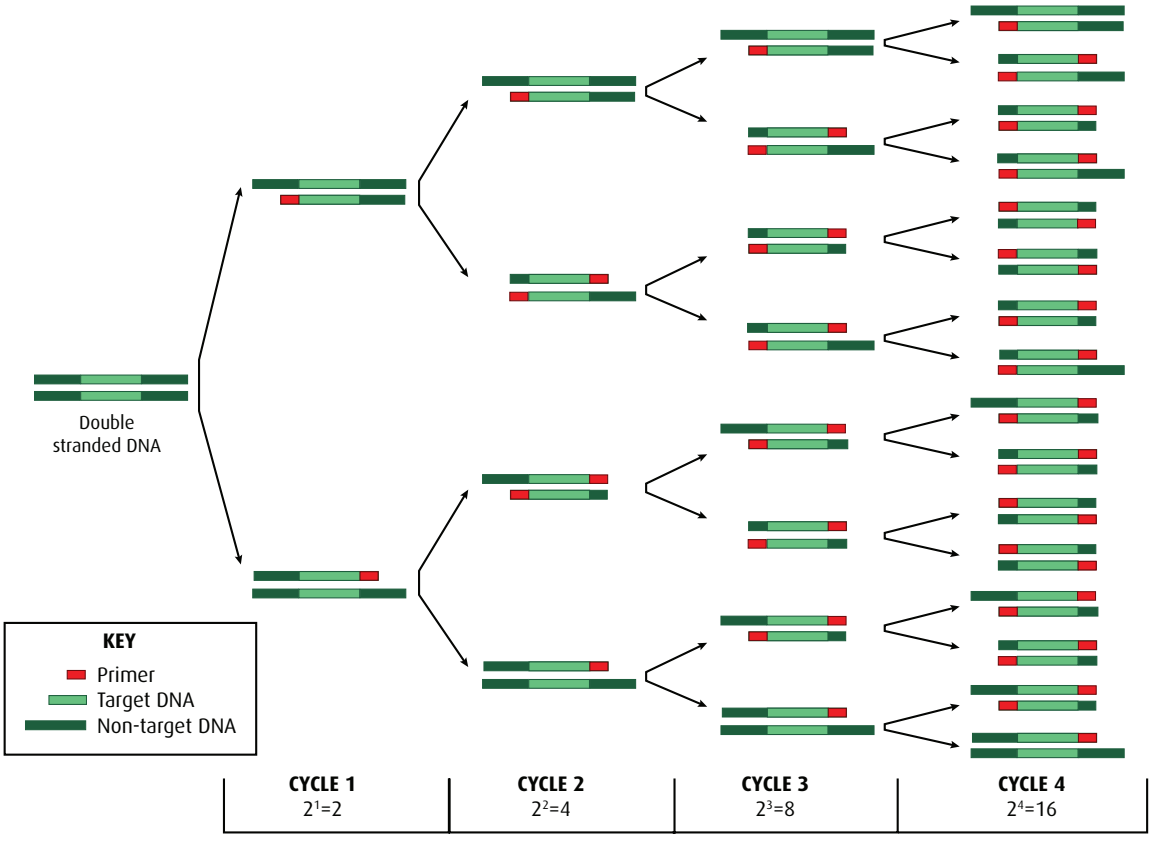


Figure 1: The Polymerase Chain Reaction

The products of conventional PCR are most often analyzed by agarose gel electrophoresis. If the target DNA sequence was present in the starting material and was amplified successfully, the resulting copies of DNA will create a distinct band when the gel is stained (Figure 2). This band can be used to confirm the presence of the DNA region and to describe its nucleotide length.

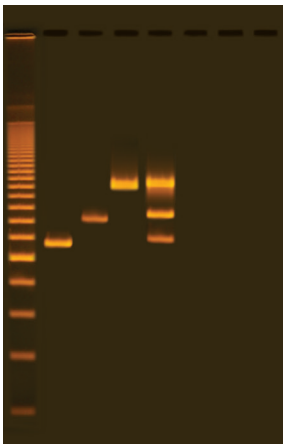


Figure 2: PCR Products after electrophoresis.

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

PRINCIPLES OF QUANTITATIVE PCR

In quantitative PCR (qPCR, also known as "real-time" PCR) the amplified DNA is observed and measured *during* the PCR experiment. Scientists then use this data to calculate the starting amount of DNA in a sample. Quantitative PCR can also be combined with reverse transcription to measure the amount of starting RNA in a sample. Both qPCR and reverse transcription with qPCR are used in a range of diagnostic and research settings where the levels of gene transcription, DNA damage, or the number of organisms present in samples are key pieces of information. In addition, qPCR is chosen for experiments where there is a high risk of contamination or where efficiency and automation are paramount. This is because the entire reaction occurs in a single tube and does not require any downstream manipulation, such as gel loading.

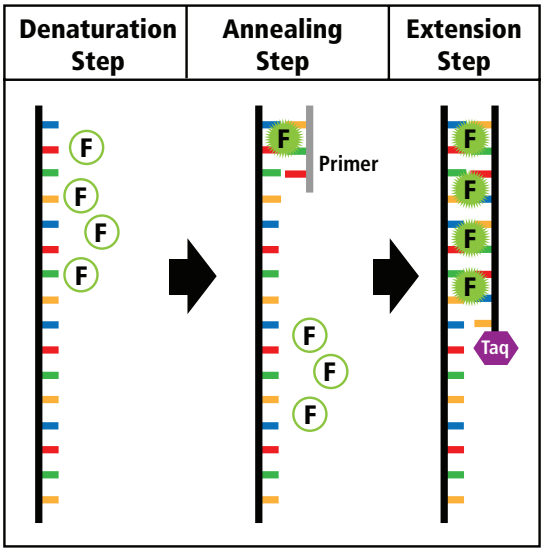


Figure 3: qPCR Using Fluorescent Intercalating Dyes.

In qPCR, the amount of DNA created during each PCR cycle is measured as a fluorescent signal. This signal is produced by using either intercalating dyes that fluoresce when positioned between DNA base pairs (Figure 3) or fluorescently labeled probes that attach to specific DNA sequences during annealing and then release a signal following extension. Both dyes and DNA probes have advantages and disadvantages. Fluorescent dyes are simple to use and versatile. However, because they are non-specific they will report the presence of any double-stranded DNA in the PCR sample. In contrast, fluorescently labeled DNA probes only produce a signal when the specific sequence of interest is present. However, they also require extensive research and development before each experiment.

Quantitative PCR is performed in a thermal cycler that has been equipped with a laser and a detector. This allows the machine to excite the fluorescent molecules and then detect the signal that dyes or probes produce during PCR. An increase in fluorescence directly relates to an increase in the amount of amplified DNA in the sample. In early cycles of PCR, the fluorescence signal is faint because only a small amount of DNA is present. This signal can be hard to distinguish from background noise. However, as the number of cycles increases and the PCR product accumulates the signal becomes clear and displays a predictable amplification curve (Figure 4).

Similar to conventional PCR, each cycle of qPCR ideally doubles the amount of the amplified DNA. Mathematically, this doubling 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, if we start with one copy of our target, we will have two copies after the first PCR cycle (or $1x2^1$), four after the second PCR cycle (or $1x2^2$), eight after the third PCR cycle (or $1x2^3$), and so on (Figure 1). Such a reaction would have PCR efficiency of 100%.

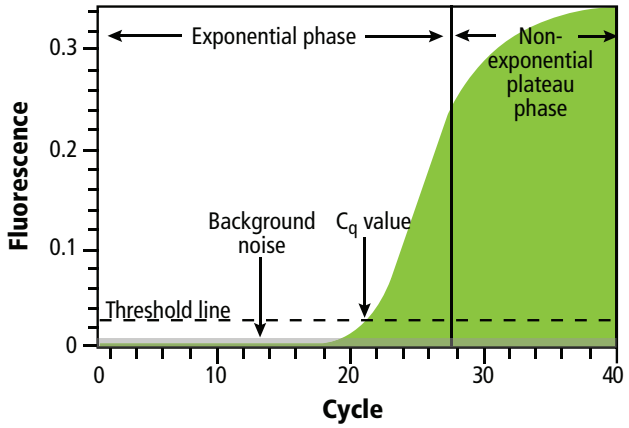


Figure 4: qPCR Amplification Curve.

However, in reality, PCR efficiency is rarely 100% and never constant. Sub-optimal amplification can occur as a result of enzyme inhibiting agents that are either present in the biological sample or introduced during extraction. Time is also an inhibitor – as a PCR experiment progresses, components like nucleotides and primers become depleted and Taq polymerase loses its full potency. This causes an eventual leveling off of the product after a certain number of cycles, which can be observed as a plateau when the fluorescent signal is graphed (Figure 4). The phase before this leveling off is often called the exponential amplification phase.

APPLICATIONS OF QPCR TECHNOLOGY

qPCR is a commonly used technique in both the research and diagnostic laboratories because it is fast, sensitive, and quantitative. Unlike Northern and Southern blotting, qPCR requires only a small amount of starting DNA template. Moreover, this starting template can be either genomic DNA or complementary DNA meaning that either the starting DNA or RNA content of a sample can be examined.

In research laboratories, qPCR is used to study changes in gene expression, as it is easier and less expensive than microarrays. For example, qPCR may be used to study gene expression in a cell line that has been treated with experimental drugs. (In these experiments, RNA is a much better indicator of gene expression levels and so an initial reverse transcription step is added.) Medical labs also use qPCR to test for diseases - particularly when the disease is caused by a fast evolving viral strain such as the influenza virus. In addition, qPCR is used to identify and quantify microorganisms in food and water samples and to monitor transgene insertions in GMOs.

QUANTIFICATION IN qPCR

The technology of qPCR allows users to observe a fluorescent signal from DNA as it is amplified during the cyclic heating/cooling process. However, scientists using qPCR are interested in the amount of DNA in a sample before amplification. To gain this information, researchers must derive the beginning amount of template DNA from the amount of DNA measured later in the experiment when conditions are better for observing the fluorescent signal. The relationship between DNA at these two time points can be described by the equation $y=y_0E^x$ where y is the measured DNA amount, y_0 is the starting DNA amount, E is the amplification efficiency, and x is the number of cycles that have occurred. In addition, researchers must convert the intensity of the fluorescent signal during an experiment into a value that directly describes the DNA amount and that can be compared to other studies. Strategies to accomplish these two translations are continually being invented and refined.

One set of analyses - known as relative quantifications - compare the signal from a DNA region of interest to the signal from an internal reference gene. This internal reference gene is ideally expressed in all the cells of an organism (or multiple organisms) and expressed at a constant rate regardless of location and experimental conditions.

Good candidates for this are housekeeping genes – genes that code for proteins involved in basic cellular function. Following the qPCR amplification of both regions, the amount of target DNA in a sample is described in terms of how much stronger or weaker its signal was in comparison to the housekeeping gene.

Another approach is to use several control reactions to create a standard curve that characterizes the relationship between signal and DNA concentration. This is known as absolute quantification. In these experiments, a DNA template of known concentration is diluted over several orders of magnitude and amplified alongside samples of interest. Researchers graph the fluorescent levels of the serially diluted samples versus their DNA concentrations and then calculate a best-fit linear regression equation from this graph. The fluorescent results of the unknown samples are then entered into the equation in order to determine the DNA concentrations of the unknown samples.

Each sample in a qPCR experiment generates a continuous feed of fluorescent intensities, but most relative and absolute comparison methods require only one fluorescent value per sample. Consequently, another analytical choice is how to best summarize amplifying samples. One popular method is to use the quantification cycle (Cq). This is the cycle number where a sample's fluorescence hits a pre-set value (Figure 4). This value - known as the threshold – can be arbitrarily chosen provided that it exceeds the background noise and that it occurs during the exponential amplification of all samples. If the DNA target is present in a sample in low levels it will take many cycles before the fluorescence equals the threshold and the target will have a high Cq. Conversely, if the target DNA is abundant in a sample the fluorescence will quickly reach the threshold and the target will have a low Cq.

The Cq method assumes identical amplification efficiencies between samples. However, samples with different starting DNA concentrations, extraction preparations, source DNA, and amplification targets do differ in efficiency. These differences can be inflated by quantification calculations and result in poor DNA concentration estimates. One way to overcome this is to use additional data from each sample's amplification curve to select a more curated Cq point. For example, the Cy^0 method is similar to the Cq method but focuses on the inflection point in a sample's modified amplification curve rather than a universal threshold line in order to select fluorescent quantification cycles that are more sensitive and robust.

ADDITIONAL ANALYSIS

Post run analyses can also provide specific information about the precision of the amplification, and how to accurately interpret the final data. One value to look at if running an absolute quantification method is the R^2 value of the linear regression equation. The R^2 value describes how well the linear model fits the data and indicates if the assay has been optimized. An optimized qPCR experiment and a high-quality standard curve are also essential to accurately determining the initial DNA concentration. The R^2 value ranges from 0 (no fit) to 1 (perfect fit). Efficient qPCR reactions will have an R^2 value ≥ 0.98.

If using intercalating fluorescent dyes for standard curve analysis, a researcher must also analyze the sample after the experiment because the dyes will bind to any double-stranded DNA present in the qPCR sample. For example, a common artifact is produced when primers hybridize with one another instead of the DNA template during PCR. These "primer-dimers" are amplified by the Taq polymerase, forming small non-specific bands. Primer-dimers present a

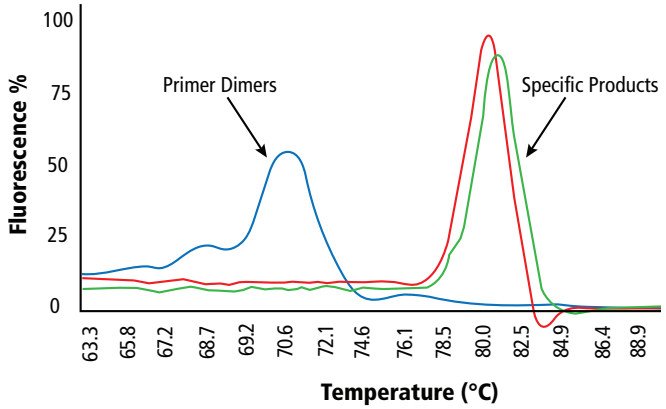


Figure 5: Melt Curve Analysis

problem for qPCR analysis because they compete with the longer amplicon for reagents. Furthermore, the double-stranded DNA and dye increase the total level of fluorescence in a sample and skews the results.

A technique called melting curve analysis allows us to determine whether multiple amplicons were produced during the qPCR experiment (Figure 5). Following the last cycle of the qPCR experiment, the temperature of a sample is slowly increased. As the hydrogen bonds between the two strands of DNA begin to dissociate, the dye is released from the DNA and the total fluorescence of the sample decreases significantly. Longer double-stranded DNA molecules require more heat to dissociate than shorter, non-specific DNA molecules, meaning that the short primer-dimers will dissociate at lower temperatures than longer amplicons. If only one PCR product exists, there will only be one change in fluorescence in a melt cure graph and it will occur at a high temperature. If two or more amplicons are present, multiple changes in fluorescence will be observed.

In this experiment, you will prepare a serial dilution in order to create a standard curve for absolute quantification. Next, you will amplify these standard samples as well as experimental samples with unknown DNA concentrations using qPCR. Following qPCR, you will calculate the starting levels of DNA in each experimental sample and assess the validity of your experimental data. In addition, you will confirm single target amplification using both a melt curve experiment and gel electrophoresis.

Experiment Overview

EXPERIMENT OBJECTIVE

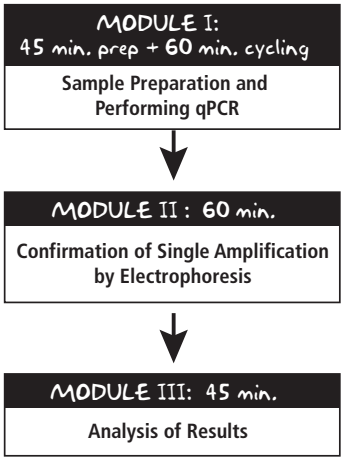
The objective of this experiment is for students to gain hands-on experience with the principles and practice of qPCR. Students will quantify the DNA concentration of experimental samples using a standard curve approach and confirm specificity using both melt curve analysis and gel electrophoresis.

PRELAB QUESTIONS

1. List at least three possible sources of experimental error during the experiment. With your group, brainstorm ways to prevent these from occurring.

2. Choose and describe at least two ways to assess the accuracy of your qPCR experiment.

3. (Optional) Explore an area of research where qPCR is commonly used such as gene expression analysis, biomedical science, environmental monitoring, and pathogen detection. Next, invent a possible experimental scenario by naming and giving a background to each of the four experimental samples.



NOTE: Experimental times are approximate.

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.

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 safety goggles

- 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.
- Always wash hands thoroughly with soap and water after working in the laboratory.

Module I-A: Sample Preparation for qPCR

Preparation of the Standard Curve Samples

NOTE:
Accurate pipetting is **essential** during Module I. If you are unfamiliar with this technique, review and practice before starting this experiment.

1. **COLLECT** the standard curve starting sample from your teacher. The DNA concentration of this sample is 2 ng/µL.
2. **LABEL** three microcentrifuge tubes "SC 1:10", "SC 1:100", and "SC 1:1000" (Figure 6, below).

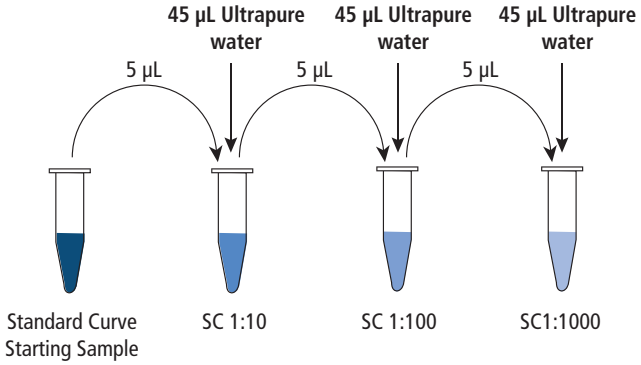


Figure 6

3. **ADD** 45 µL of Ultrepure water to these three tubes.
4. **CHANGE** tip and **ADD** 5 µL of the starting sample to the 45 µL of water in tube "SC 1:10". **MIX** thoroughly by pipetting up and down or by tapping the tube on your lab bench 4-5 times.
5. **CHANGE** tip and **ADD** 5 µL of the "SC 1:10" sample to the 45 µL of water in tube "SC 1:100". **MIX** thoroughly by pipetting up and down or by tapping the tube on your lab bench 4-5 times.
6. **CHANGE** tip and **ADD** 5 µL of the "SC 1:100" sample to the 45 µL of water in tube "SC 1:1000". **MIX** thoroughly by pipetting up and down or tapping the tube on your lab bench 4-5 times.
7. **KEEP** all four standard sample tubes on ice.
8. **CALCULATE** the DNA concentrations of these three samples and **RECORD** your results in your lab book or in the provided student worksheet (Appendix C). Use the equation:

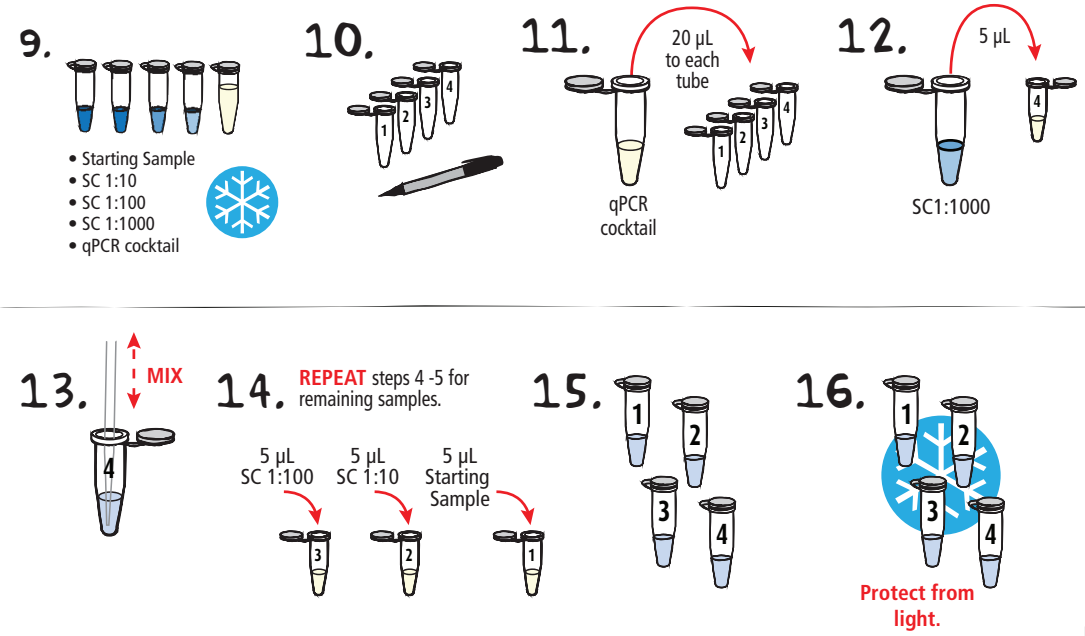
$$\text{Concentration}_1 \times \text{Volume}_1 = \text{Concentration}_2 \times \text{Volume}_2$$

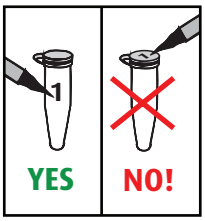
In this equation, Volume_1 is the amount of sample added (5 µL), Concentration_1 is the DNA concentration of the added sample, Volume_2 is the final volume in each tube (50 µL), and Concentration_2 is the DNA concentration in the newly created sample.

OPTIONAL STOPPING POINT:
The prepared standard samples can be stored in the fridge for several hours or stored in the freezer for several days.

Module I-A: Sample Preparation for qPCR, continued

Preparation of Standard Curve Reactions for qPCR





9. **COLLECT** your four standard curve samples and the qPCR cocktail. **KEEP** on ice.
10. Properly **LABEL** four qPCR tubes* with the numbers 1 through 4.
    NOTE: Incorrect markings can distort the signal read between a machine and your samples. Check with your instructors that the qPCR machine can accommodate labels and where those labels should be. If you are using the OpenPCR machine, the provided PCR tubes can be labeled on the side. However, DO NOT mark tubes on their top.
11. Slowly **ADD** 20 µL of qPCR cocktail to each qPCR tube. This cocktail contains *Taq* polymerase, additional PCR reagents, primers, and fluorescent dye. Limit light exposure of this solution to under an hour and keep on ice.
12. With a new pipette tip, **ADD** 5 µL of the "SC 1:1000" sample to qPCR tube 4.
13. Gently **MIX** the sample by pipetting up and down several times.
    NOTE: Bubbles introduced during mixing can also interfere with the machine's detection ability. Avoid bubbles by gentle mixing and careful handling. If bubbles do form consider briefly centrifuging the sample or gently tapping the tube on your lab bench.
14. **REPEAT** steps 12 and 13 for the remaining qPCR standard curve samples according to Table 1.
15. **SEAL** the tubes as specified by the manufacturer. Wear gloves during this step as fingerprints can sometimes interfere with the fluorescent signal.
16. **STORE** the tubes on ice and protected from light until ready to run the qPCR program.

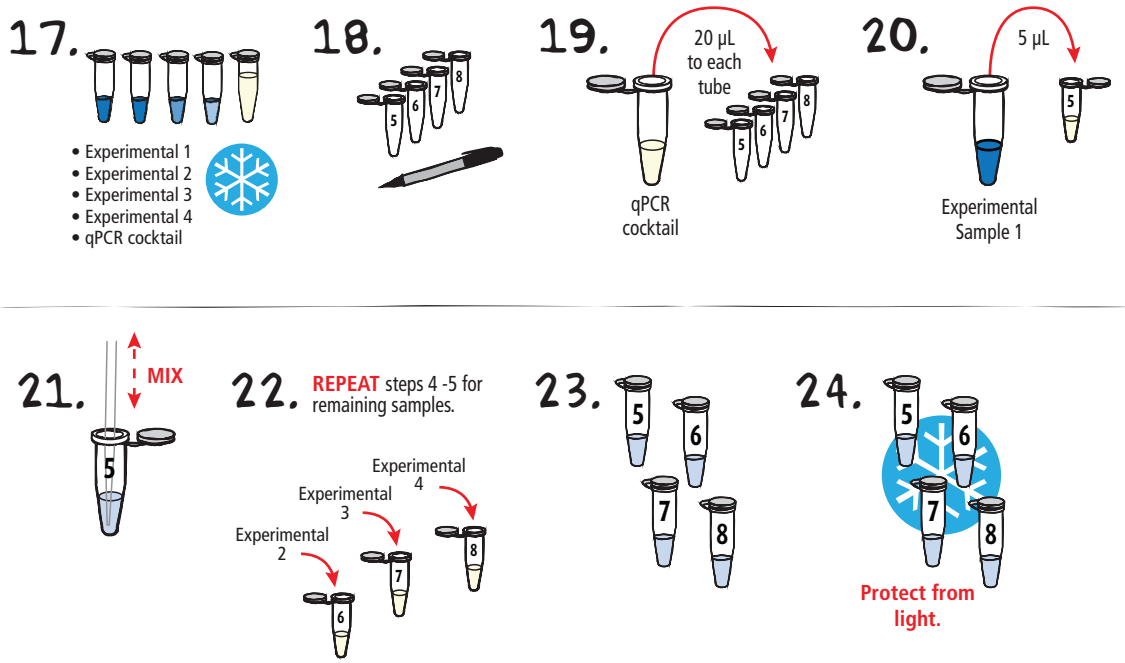
**Some qPCR systems may require using a plate with marked wells rather than individual tubes.*

Tube Label	Cocktail Volume	Template Volume	Template	Prep Order
1	20 µL	5 µL	Start sample	4th
2	20 µL	5 µL	SC 1:10	3rd
3	20 µL	5 µL	SC 1:100	2nd
4	20 µL	5 µL	SC 1:1000	1st

TABLE 1: Standard Curve qPCR Reactions

Module I-A: Sample Preparation for qPCR, continued

Preparation of Experimental Sample Reactions for qPCR



17. **COLLECT** the four experimental samples from your teacher.
18. Properly **LABEL** four qPCR tubes with the numbers 5 through 8. See notes on page 12.
19. Slowly **ADD** 20 µL qPCR cocktail to each tube.
20. With a new pipette tip, **ADD** 5 µL of experimental sample 1 to qPCR tube 5.
21. Gently **MIX** the samples by pipetting up and down several times. See notes on page 12.
22. **REPEAT** steps 20 and 21 for the remaining experimental samples according to Table 2.
23. **SEAL** the tubes as specified by the manufacturer.
24. **STORE** the tubes on ice and protected from light until ready to run the qPCR program.

OPTIONAL STOPPING POINT:
The prepared qPCR samples can be stored in the fridge for several hours or stored in the freezer for several days. Make sure samples are kept dark or are covered.

NOTE: The Open qPCR machine has a capacity of 16 samples. This means that in this experiment two student groups can run qPCR simultaneously. Work with your classmates to determine when each group will run its samples.

Tube Label	Cocktail Volume	Template Volume	Template	Prep Order
5	20 µL	5 µL	Experimental 1	1st
6	20 µL	5 µL	Experimental 2	2nd
7	20 µL	5 µL	Experimental 3	3rd
8	20 µL	5 µL	Experimental 4	4th

TABLE 2: Experimental Samples qPCR Reactions

Module I-B: Performing qPCR

RUNNING qPCR

1. **START** your qPCR machine and **SELECT** the correct thermal cycle program. The time and temperature conditions for this experiment are listed below. See Appendix A (or your thermal cycle's operational manual) for specific instructions.

PCR cycling conditions:
Initial denaturation 94° C for 3 minutes
94° C for 30 sec.
60° C for 30 sec.* } 30 cycles
68° C for 30 sec.*
Final extension 68° C for 5 min.

Melting curve conditions:
90° C for 30 sec.
50° C for 1 min.
From 50° C to 95° C with a ramp speed of 0.09° C/s and a hold duration of 30 seconds.*
Hold at 4° C forever.

** Data gathering should be switched on at these three parts of the experiment.*

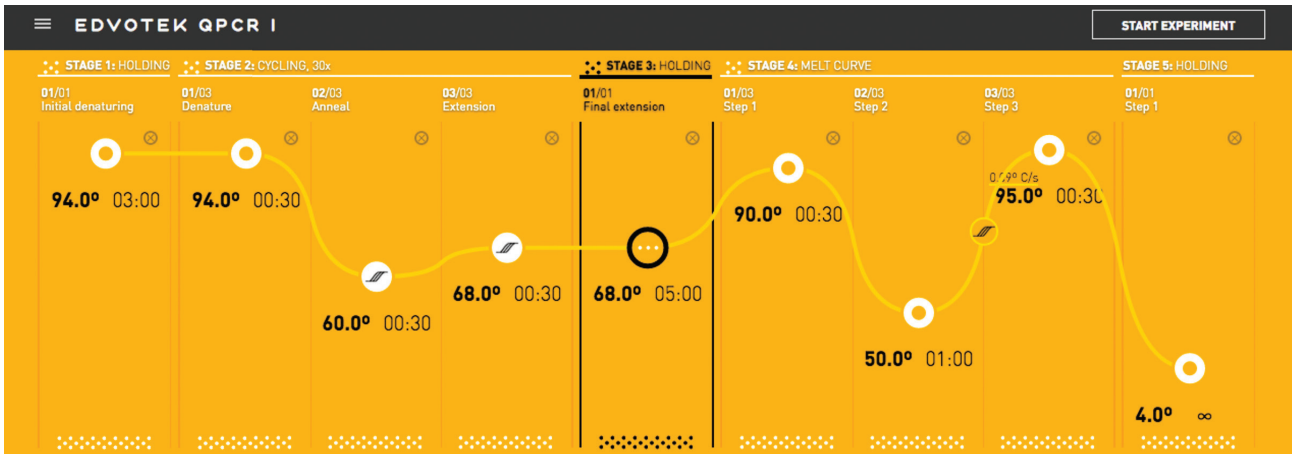


Figure 7: The 380 qPCR Program on the Open QPCR user interface.

2. **DECIDE** on the locations of your 8 samples within the qPCR machine. **RECORD** these locations in your lab book or in the provided student worksheet (Appendix C).
3. Briefly, **CENTRIFUGE** all eight PCR tubes until samples are in the bottom and until no bubbles are visible.

Module 1-B: Performing qPCR, continued

4. **ADD** all qPCR samples to the thermal cycler and **START** the program. The thermal cycler will amplify the DNA and perform a melt curve experiment. While the experiment is running, **REVIEW** the experimental results in real time.

   NOTE: Switching between log and linear views will to highlight different aspects of your experiment. Log view (Figure 8a) best depicts early amplification particularly in the highest concentration samples. Linear view (Figure 8b) provides a powerful overview of all the cycles.

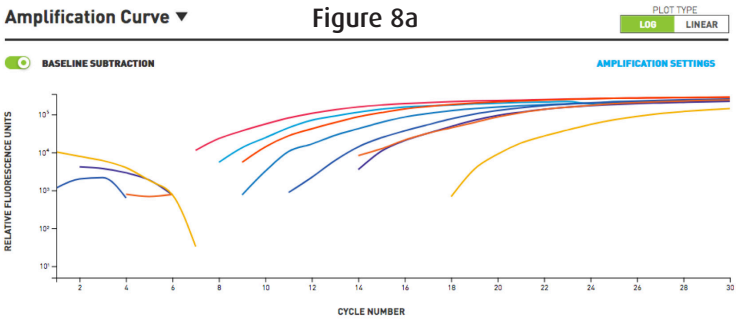


Figure 8a

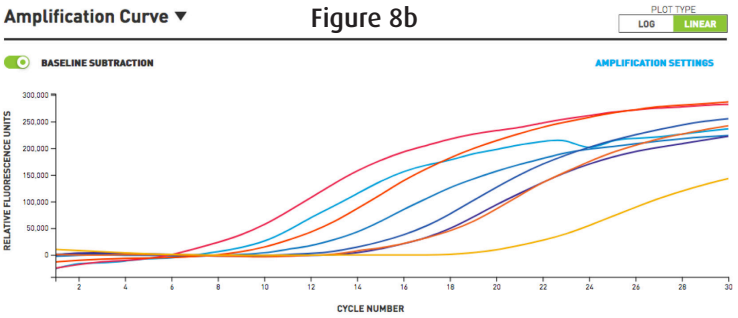


Figure 8b

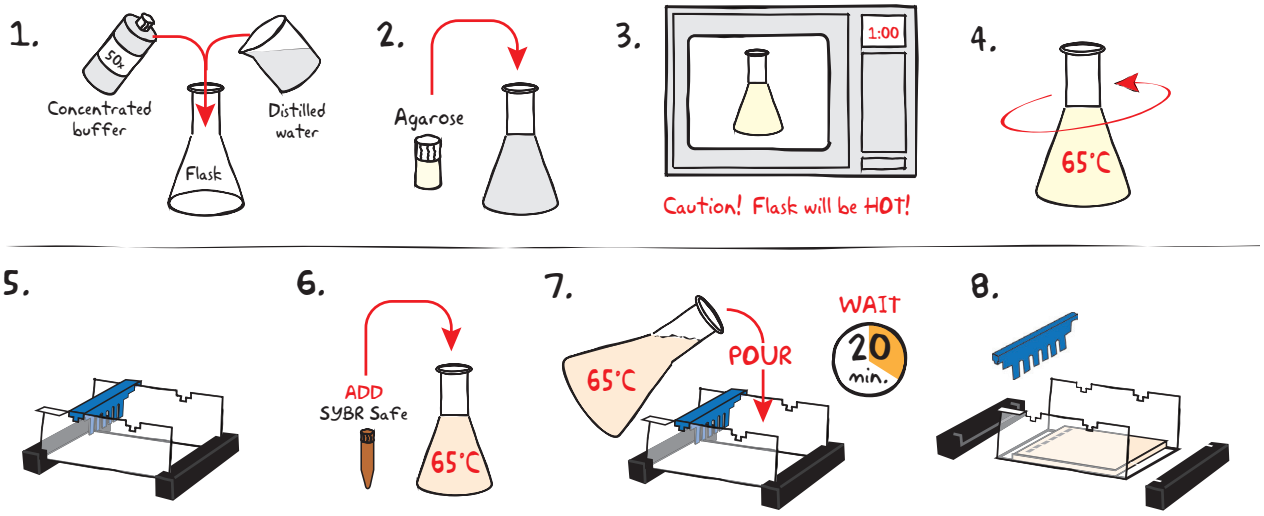
OPTIONAL STOPPING POINT:
The qPCR machine can be left to finish the programs and hold samples at 4° C until needed.

5. Once the thermal cycler program has finished, **SAVE** the data for future analysis. Many platforms do this automatically. However, exporting data now as backup will guarantee access to the data even when the network or machine is not accessible.
6. **RECORD** the Cq values for each of your 8 qPCR reactions in your lab book or in the provided student worksheet.
7. **REMOVE** your samples and **ADD** 5 µL of 10x gel loading solution to each sample and continue to Module II and III.

OPTIONAL STOPPING POINT:
Samples can be stored for multiple days at -20° C.

Module II: Confirmation of Specific Amplification by Electrophoresis

Module II and Module III can be performed interchangeably or concurrently as the latter is entirely paper and computer-based.



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 65° C with careful swirling to promote even dissipation of heat.
5. While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the well template (comb) in the appropriate notch.
6. Before casting the gel, **ADD** SYBR® Safe concentrate to the molten agarose and swirl to mix (see Table A).
7. **POUR** the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
8. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

NOTES:

7 x 14 cm gels are recommended. Place well-former template (comb) in the first and third set of notches.

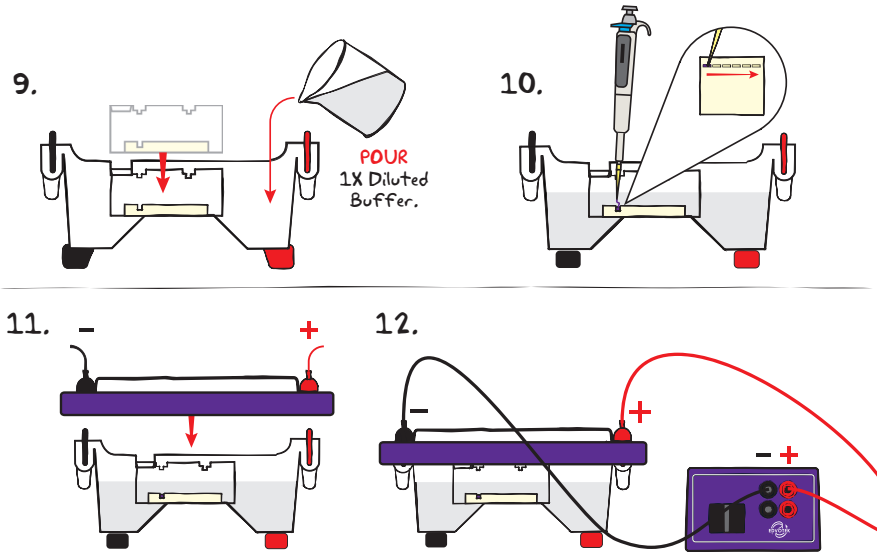
SYBR® Safe can be used as an in-gel stain or as a post-electrophoresis stain. For post-electrophoresis instructions, download our SYBR® Quick Guide at **www.edvotek.com**.

OPTIONAL STOPPING POINT:
Gels can be stored overnight submerged in electrophoresis buffer, in the fridge, and protected from light.

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

Size of Gel Casting tray	Concentrated Buffer (50x)	+ Distilled Water	+ Amt of Agarose	= TOTAL Volume	ADD SYBR® (Step 6)
7 x 7 cm	0.6 mL	29.4 mL	0.23 g	30 mL	30 µL
7 x 10 cm	1.0 mL	49.0 mL	0.39 g	50 mL	50 µL
7 x 14 cm	1.2 mL	58.8 mL	0.46 g	60 mL	60 µL

Module II: Confirmation of Specific Amplification by Electrophoresis



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

Wear gloves and safety goggles

9. **PLACE** gel (on the tray) into electrophoresis chamber. **COVER** the gel with 1X electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.
10. Using Table 3 as a guide, **LOAD** the entire sample (25 µL) into the well.
11. **PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
12. **CONNECT** leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines).
13. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber.

OPTIONAL STOPPING POINT:
Gels can be stored for several days. Protect from light, refrigerate, and keep hydrated by storing each gel in a watertight plastic bag with a small amount of electrophoresis buffer.

Row #	Lane #	Sample
1	1	Standard DNA Marker
1	2	Standard Curve 1
1	3	Standard Curve 1:10
1	4	Standard Curve 1:100
1	5	Standard Curve 1:1000
1	6	
2	1	Standard DNA Marker
2	2	Experimental Sample 1
2	3	Experimental Sample 2
2	4	Experimental Sample 3
2	5	Experimental Sample 4
2	6	

TABLE 3: Gel Loading Table

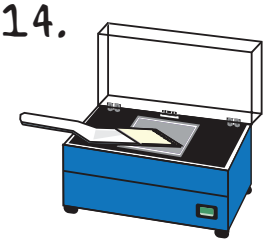
Table B — 1x Electrophoresis Buffer (Chamber Buffer)

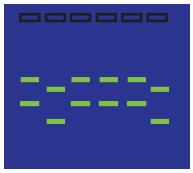
EDVOTEK Model #	Total Volume Required	Dilution: 50x Conc. Buffer	+ Distilled Water
M6+ & M12 (new)	300 mL	6 mL	294 mL
M12 (classic)	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

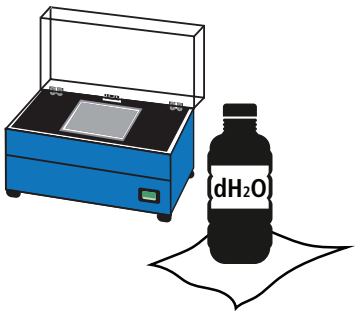
Table C — Time & Voltage Guidelines (0.8% Agarose Gel)

Volts	Electrophoresis Model: M6+ Min. / Max.	M12 (new) Min. / Max.	M12 (classic) & M36 Min. / Max.
150	15/20 min.	20/30 min.	25 / 35 min.
125	20/30 min.	30/35 min.	35 / 45 min.
75	35 / 45 min.	55/70 min.	60 / 90 min.

Module II: Confirmation of Specific Amplification by Electrophoresis

14. 

15. 

16. 

14. **SLIDE** 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** results.

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

Module III: Analysis of Results

CREATING A STANDARD CURVE AND EQUATION

1. **CALCULATE** the amount of DNA in each of the four standard curve qPCR reactions. **RECORD** your results in your lab book or in the provided student worksheet (Appendix C).

2. **CALCULATE** the log amount of DNA from step 1 and **RECORD** these values in your lab book or in the provided student worksheet (Appendix C).

3. **OPEN** a graphing program (Microsoft® Excel or similar application) and **ENTER** the Cq value and log DNA value of each standard sample as a new table.

4. **CREATE** a standard curve by plotting the log (DNA) on the y-axis versus the corresponding Cq value on the x-axis.

   a. **HIGHLIGHT** the Log and Cq columns.

   b. **CHOOSE** a marked scatter graph.

   c. **CONFIRM** that the correct x and y values are selected.

   d. **LABEL** the x and y axis and add a graph title.

5. **ADD** a linear trendline to the graph from step 4.

6. **DISPLAY** the linear equation (e.g. y=ax+b) and the R^2 value of the equation on the graph.

7. **RECORD** the equation and R^2 value in your lab book or in the provided student worksheet.

8. **EXAMINE** the R^2 value for your experiment and describe your confidence in calculating the DNA amount in a sample based on its Cq value.

CALCULATE EXPERIMENTAL SAMPLES' DNA CONCENTRATIONS

9. Prepare the standard curve equation from step 6 to solve for y.

10. Use this equation (Step 9) and the Cq values of your experimental qPCR reactions to **CALCULATE** the log of the amount of DNA in these reactions. **RECORD** these values in your lab book or in the provided student worksheet.

11. **CALCULATE** the amount of DNA in each of the experimental qPCR reactions using Equation 1. **RECORD** your results in your lab book or in the provided student worksheet (Appendix C).

    Equation 1: Amount of DNA (ng) = $10^{\text{Log (DNA amount)}}$

    In this equation, Log (DNA amount) is the calculated value from step 10.

Module III: Analysis of Results, continued

12. (Optional) **CALCULATE** the starting DNA concentration for your original experimental samples using the concentration and volume equation given in step 1. In this case $Volume_1$ is the final volume of the qPCR reaction (25 µL), $Concentration_1$ is the calculated DNA concentration from step 11, $Volume_2$ is the amount of sample you added to each reaction (5 µL), and $Concentration_2$ is the final concentration of DNA in the experimental sample. **RECORD** these values in your lab book or in the provided student worksheet.

13. (Optional) **SHARE** your results with the class.

14. (Optional) **INVESTIGATE** additional ways to evaluate your experimental results using Appendix D as a springboard.

Study Questions

1. What are the three steps in a polymerase chain reaction and what does each accomplish?
2. How much does target DNA increase during each PCR cycle under ideal conditions? What are some factors that can dampen this rate of increase?
3. How is amplified DNA visualized in regular PCR? How is it visualized in qPCR? What information can be gained from each approach?
4. Compare and contrast relative quantification and absolute quantification in the context of qPCR.
5. What occurs to DNA during a melting curve program? And what do the results say about the qPCR reaction?
6. Why might a researcher decide to use qPCR over traditional PCR?

Instructor's Guide

OVERVIEW OF INSTRUCTOR'S PRELAB PREPARATION

This section outlines the recommended prelab preparations and approximate time requirements to complete each prelab activity. Depending on your schedule, you may choose to perform all or part of Module III (a dry lab) after, before, or during Module II.

Preparation For:	What to do:	When:	Time Required:
Module I: Sample Preparation and Performing qPCR	Set up and Program qPCR machine*	Anytime before Module I	30 min.
	Prepare and aliquot reagents	Day of Module I	30 min.
Module II: Confirmation of Single Amplification by Electrophoresis	Prepare SYBR® Safe Stain	Anytime before Module II	10 min.
	(Optional) Bulk prepare diluted electrophoresis buffer	Up to one week before performing the experiment.	10 min.
	(Optional) Bulk prepare molten agarose and pour gels	Up to one day before performing the experiment.	35 min.
Module III: Analysis of Results	Procure access to graphing software	Anytime before Module III	Varies

** The Open qPCR machine has a capacity of 16 samples. This means that in this experiment two student groups can run qPCR simultaneously. Determine before hand when each student group will perform their qPCR program (see page 23).*

General Tips and Tricks

- QPCR experiments are powerfully sensitive and can detect even small amounts of DNA. To avoid cross contamination during the experiment change pipette tips frequently and, when possible, use filtered tips.
- Other strategies to avoid contamination are to prepare the standard curve and experimental PCR samples separately, move from lower concentration to higher concentration samples, and to prepare the qPCR cocktail before the DNA samples and even in a separate room.
- These experiments are also sensitive to slight differences in reagent concentrations. Invert tubes several times or vortex before using in order to disturb any type of concentration gradient that may have formed.
- Make sure everyone involved is comfortable and practiced in pipetting small volumes. For example, small droplets that remain outside of a pipette tip can affect DNA and master mixture concentrations. Use both the soft and hard stops when pipetting mixtures and immerse the top of the tip in the mixture when possible.
- Consider re-calibrating pipettes before the experiment.
- To maintain the integrality of reagents, keep perishables in the fridge/freezer until needed and avoid unnecessary freeze-thaw cycles. Keep all thawed reagents on ice. Also protect the qPCR master mix from prolonged light exposure by wrapping tubes in tinfoil or by keeping them shaded.

Pre-Lab Prep for Module I: Sample Preparation and Performing qPCR

EQUIPMENT PREPARATIONS

1. Reserve and set-up your qPCR machine. Detailed instructions for the Open qPCR Machine are given in Appendices A and B.

2. Preprogram the qPCR time and temperature conditions for this experiment (listed below) and save the program as "qPCR I EVT 380" or similar for quick student access.

PCR cycling conditions:
Initial denaturation 94°C for 3 minutes
94° C for 30 sec.
60° C for 30 sec.*
68° C for 30 sec.*
} 30 cycles
Final extension 68° C for 5 min.

Melting curve conditions:
90° C for 30 sec.
50° C for 1 min.
From 50° C to 95° C with a ramp speed of 0.09° C/s and a hold duration of 30 seconds.*
Hold at 4° C forever.

** Data gathering should be on at these three parts of the experiment.*

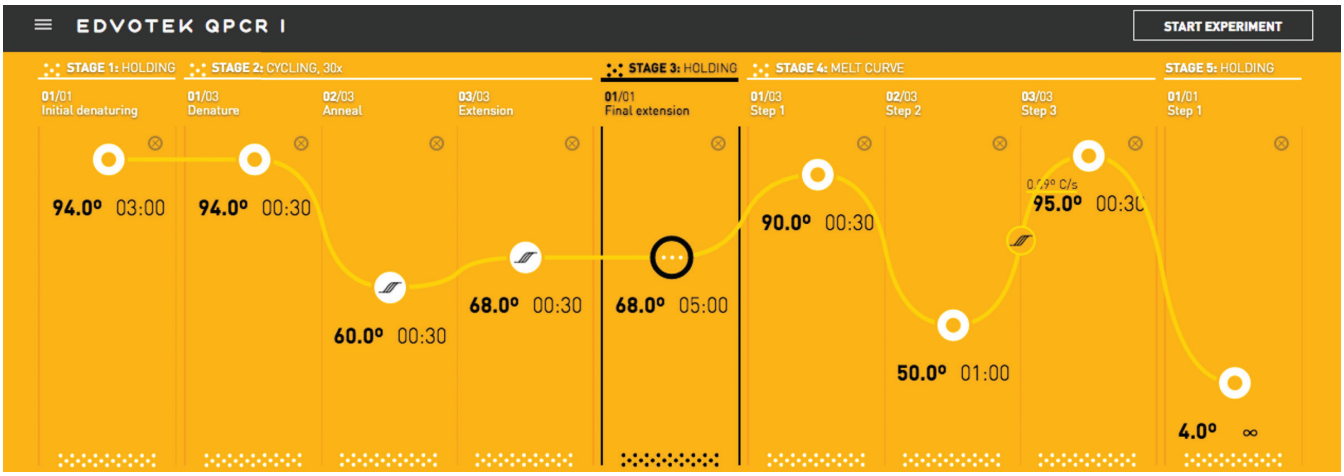


Figure 9: The 380 qPCR Program on the Open QPCR user interface.

PLAN FOR QPCR RUN TIMES

The Open qPCR machine has a capacity of 16 samples. This means that in this experiment two student groups can run their qPCR experiments simultaneously. The qPCR program should take ~1 hour. Additional groups can store their PCR samples in a dark fridge while the first set of samples are running or stagger their sample preparation. Groups can also store their PCR samples in the freezer and run on different days.

Pre-Lab Prep for Module I: Sample Preparation and Performing qPCR, continued

PREPARE AND ALIQUOT EXPERIMENTAL REAGENTS

These reagents should be prepared shortly before performing qPCR and kept on ice. It is possible to pre-aliquot reagents and then freeze them. However, the reagent stability can be compromised by multiple freeze-thaw cycles.

1. Prepare the primer mix.
   a. Make sure the solid material is at the bottom of the LyphoPrimer™ mix (B). If not, centrifuge the tube at full speed for 20 seconds or tap the tube on the lab bench.
   b. Dilute the LyphoPrimer™ by adding 250 µL of Ultrapure water (H) to the tube. Cap, mix well and place on ice. The solution should be mixed with no solid pieces remaining.
2. Prepare the standard curve starting sample and experimental samples.
   a. Make sure the solid material is at the bottom of the LyphoTemplate™ DNA (C). If not, centrifuge the tube at full speed for 20 seconds or tap the tube on the lab bench.
   b. Dilute the LyphoTemplate™ DNA (C) by adding 200 µL of Ultrapure water (H) to the tube. Cap, mix well and place on ice. The solution should be clear with no solid pieces remaining.
   c. Aliquot 14 µL of the rehydrated Template DNA to 4 snap-top tubes labeled "SC Starting Sample".
   d. Label four 1.5 mL tubes as ES1, ES2, ES3, and ES4.
   e. Add 90 µL of Ultrapure water to ES1, 90 µL of Ultrapure water to ES2, 90 µL of Ultrapure water to ES3, and 50 µL of Ultrapure water to ES4.
   f. Add 50 µL of rehydrated Template DNA (prepared in step 2b) to the ES1 tube and mix well.
   g. Add 10 µL of ES1 solution (prepared in step 2f) to the ES2 tube and mix well.
   h. Add 10 µL of ES2 solution (prepared in step 2g) to the ES3 tube and mix well.
   i. Add 10 µL of ES3 solution (prepared in step 2h) to the ES4 tube and mix well.
   j. Invert the ES1 tube and aliquot 7 µL to 4 snap-top tubes labeled "Experimental Sample 1". Repeat for Experimental Samples 2, 3, and 4.
3. Add 240 µL of the prepared primer mix to the qPCR Master Mix (A) to create a qPCR Cocktail and mix well.
4. Invert the "cocktail" tube and aliquot 180 µL to 4 snap-top tubes labeled "qPCR Cocktail".
5. Aliquot the 150 µL of Ultrapure water (H) to 4 snap-top tubes.
6. Aliquot the 50 µL of 10x Gel Loading Solution to 4 snap-top tubes.
7. Students will also need three 1.5 mL snap-top tubes, eight qPCR tubes*, pipettes, and pipette tips.

NOTE:
The fluorescent dye in the master mix is sensitive to long term light exposure. Consider using tinfoil to protect the cocktail from light if the samples will be exposed for more than an hour.

**Check your machine's instructions to confirm that the correct plates/tubes are being used.*

Pre-Lab Prep for Module II: Confirmation of Single Amplification by Electrophoresis

Prepare SYBR® Safe Stain:

1. Prepare 1x Electrophoresis Buffer by following the instructions in Appendix F or by combining 5 µL of the 50x Concentrate Buffer and 250 µL of distilled water.
2. Add 220 µL of the diluted buffer from step 1 to the tube of SYBR® Safe and mix by tapping the tube several times.

Individual Gel Preparation:

Each student group can be responsible for casting their own individual gel prior to conducting the experiment. Allow approximately 30-40 minutes for this procedure. See Module II in the Student's Experimental Procedure. Students will need 50x electrophoresis buffer (for gel preparation and for running the gel).

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 F.

Preparing Gels in Advance:

Gels may be prepared ahead and stored for later use. Solidified gels can be stored overnight under buffer.

All gels should be covered and kept in the dark as SYBR® safe will begin to degrade if exposed to light for >1 hour.

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.

FOR MODULE II
Each Group will require:
- 50x concentrated buffer
- Distilled Water
- UltraSpec-Agarose™ Powder
- DNA Standard Marker (55 µL)
- 55 µL SYBR® Safe Stain (if students are casting their own gels)

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

Additional Materials:

- Each gel should be loaded with the Standard DNA marker. Aliquot 55 µL of the DNA Standard Marker (I) into labeled microcentrifuge tubes and distribute one tube of DNA Standard Marker per group.
- If students are casting their own individual gels, aliquot 55 µL of SYBR® Safe Stain into labeled microcentrifuge tubes and distribute one tube of stain per group.

Pre-Lab Prep for Module III: Analysis of Results

In this section, students will use the data generated in Module I to graph a standard curve, generate a linear regression equation, and determine a R^2 value. Before Module III, determine which graphing software(s) students will use and ensure they have access to this. You may also want to decide whether each group will analyze their own data or whether the class will pool its collective results to create classroom "replicate" samples.

Experiment Results and Analysis

MODULE I

These are representative results using the Open qPCR Platform. The starting amount of DNA of each of the standard curve samples should match those given below. However, your amplification curves and Cq values will vary.

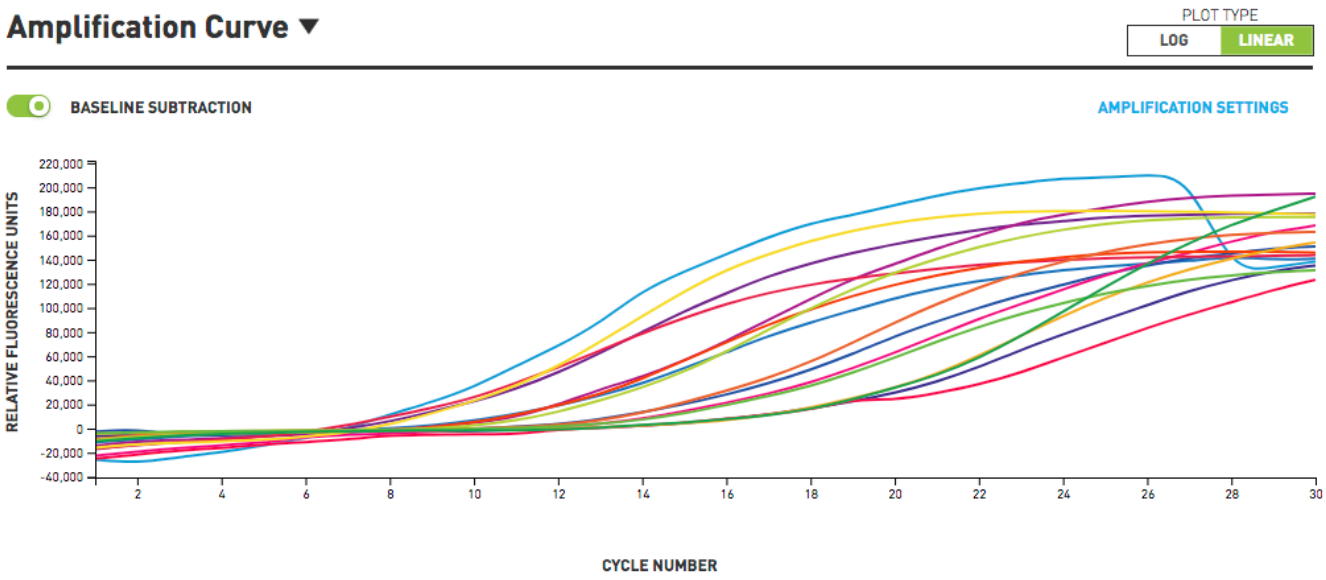
Amount of DNA in Standard Curve Samples

Sample Name	DNA Concentration
Standard Curve Starting Sample	10 ng
Standard Curve 1:10 Sample	1 ng
Standard Curve 1:100 Sample	0.1 ng
Standard Curve 1:1000 Sample	0.01 ng

Well Locations of qPCR Samples and Experimental Results

Tube	Row, Column	Sample Description	Cq Value
A1	A,1	Standard Curve Sample 1	9.22
A2	A,2	Standard Curve Sample 2	11.13
A3	A,3	Standard Curve Sample 3	14.15
A4	A,4	Standard Curve Sample 4	17.92
A5	A,5	Experimental Sample 1	9.23
A6	A,6	Experimental Sample 2	11.7
A7	A,7	Experimental Sample 3	15.19
A8	A,8	Experimental Sample 4	19.84
B1	B,1	Standard Curve Sample 1	8.38
B2	B,2	Standard Curve Sample 2	11.34
B3	B,3	Standard Curve Sample 3	14.46
B4	B,4	Standard Curve Sample 4	18.09
B5	B,5	Experimental Sample 1	9.63
B6	B,6	Experimental Sample 2	12.39
B7	B,7	Experimental Sample 3	15.19
B8	B,8	Experimental Sample 4	18.95

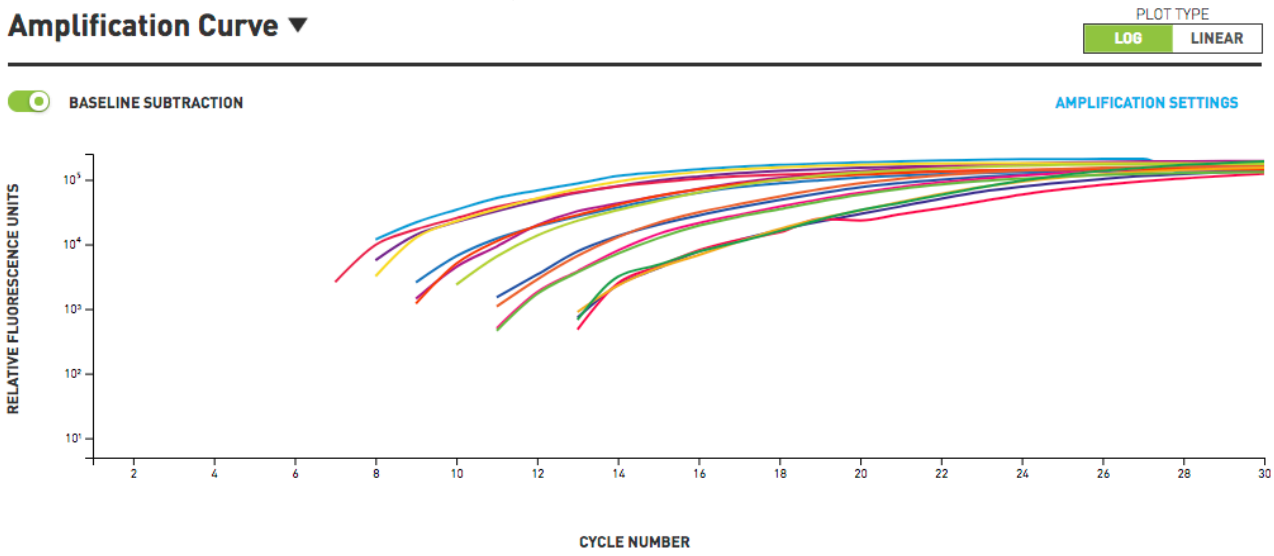
Linear Amplification Plot



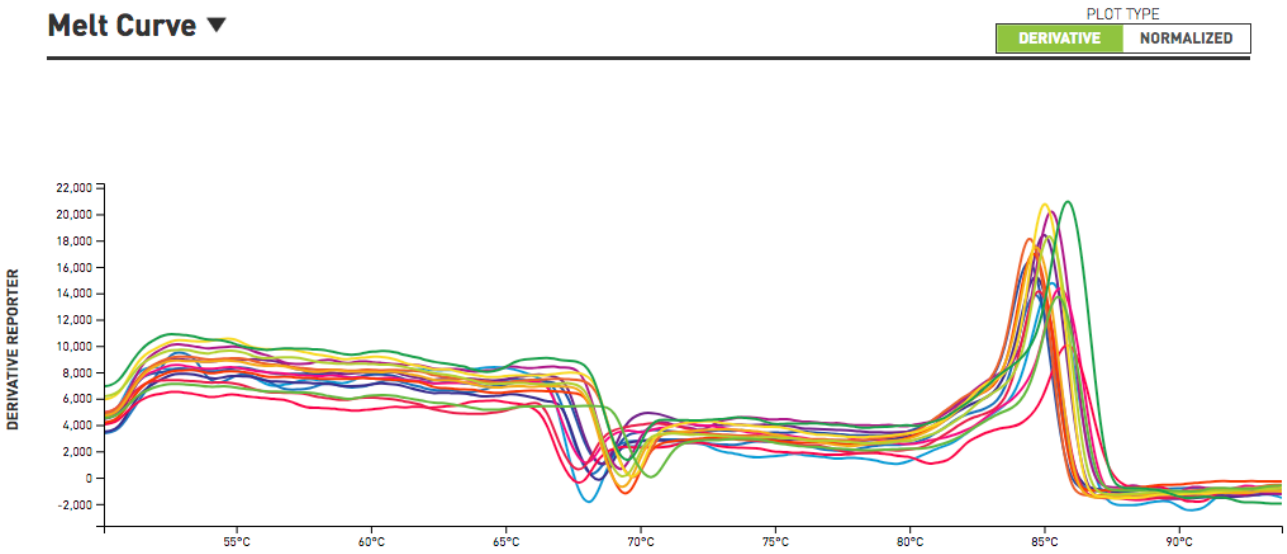
Experiment Results and Analysis, continued

MODULE I, CONTINUED

Log Amplification Plot



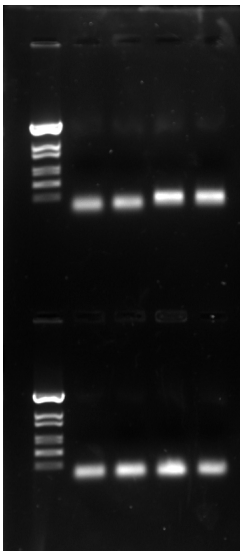
In the melt curve, all of the qPCR samples show a single amplification peak around 85° C, indicative of one specific product.



Experiment Results and Analysis, continued

MODULE II

Electrophoresis show only single amplification products for both the standard curve samples and the experimental samples during this particular qPCR experiment.



MODULE III

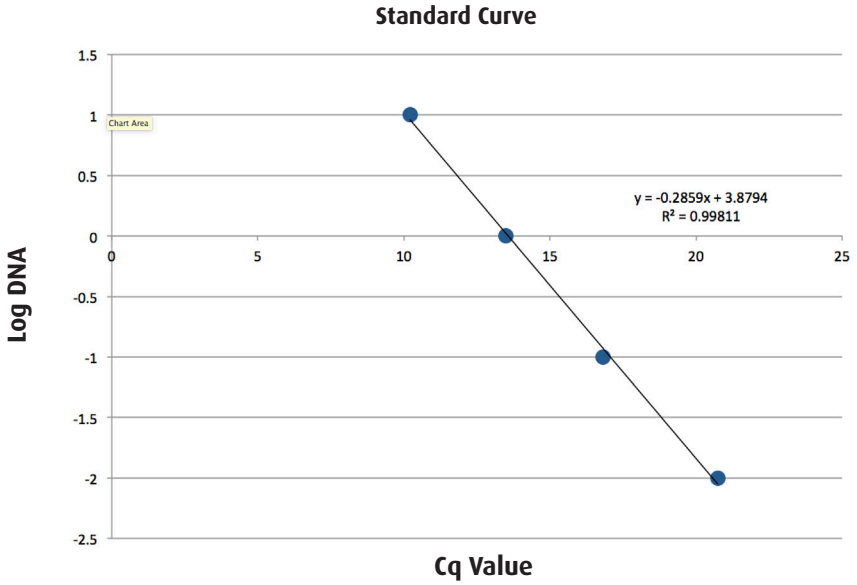
The amount of DNA (ng) in each Standard Curve qPCR reactions and its log value should match exactly the table below. However, because the Cq values vary depending on experimental conditions, your standard curve equation will be unique to your qPCR experiment. Likewise, the amount of DNA in your experimental sample qPCR reactions will also vary from the representative values given below. The final DNA concentrations of the four experimental samples should be very close to those listed at the end of this section.

DNA Concentration of Standard Curve qPCR Samples:

	DNA in qPCR Reaction (step 1)	Log Value of DNA in qPCR Reaction (step 2)
Standard Curve Starting Sample (qPCR Tube 1)	10 ng	1 ng
Standard Curve 1:10 Sample (qPCR Tube 2)	1 ng	0 ng
Standard Curve 1:100 Sample (qPCR Tube 3)	0.1 ng	-1 ng
Standard Curve 1:1000 Sample (qPCR Tube 4)	0.01 ng	-2 ng

Experiment Results and Analysis, continued

MODULE III, CONTINUED



The four standard curve reactions in this example experiment created a standard curve with a linear equation of **y = -0.2859x + 3.8794** and an R^2 value of **0.99811**.

The starting amount of DNA in the four experimental qPCR reactions can then be calculated based on this equation. This is outlined below using Experimental Sample 1 (Cq value of 9.23) as an example. Values for the three other experimental samples are given in the table at the end.

$y = x(-0.2859) + 3.8794$
$y = 9.23(-0.2859) + 3.8794$
$y = -2.638857 + 38794$
$y = 1.24$

$10^{1.24} = 17.38$ ng

{ (1) Substitute the Cq value of the sample for x and solve for y. This is the Log Value of DNA Concentration in the qPCR Reaction.

{ (2) Remove the log transformation by raising 10 to the x value. This is the amount of DNA in the qPCR reaction.

Total DNA in Experimental Samples:

Sample Description	Starting amount of DNA
Experimental Sample 1	87.5 ng/µL
Experimental Sample 2	24.9 ng/µL
Experimental Sample 3	3.41 ng/µL
Experimental Sample 4	0.28 ng/µL
Experimental Sample 1	152 ng/µL
Experimental Sample 2	21.6 ng/µL
Experimental Sample 3	2.78 ng/µL
Experimental Sample 4	0.25 ng/µL

PreLab Questions and Answers

1. **List at least three possible sources of experimental error during the experiment. With your group brainstorm ways to prevent these from occurring.**

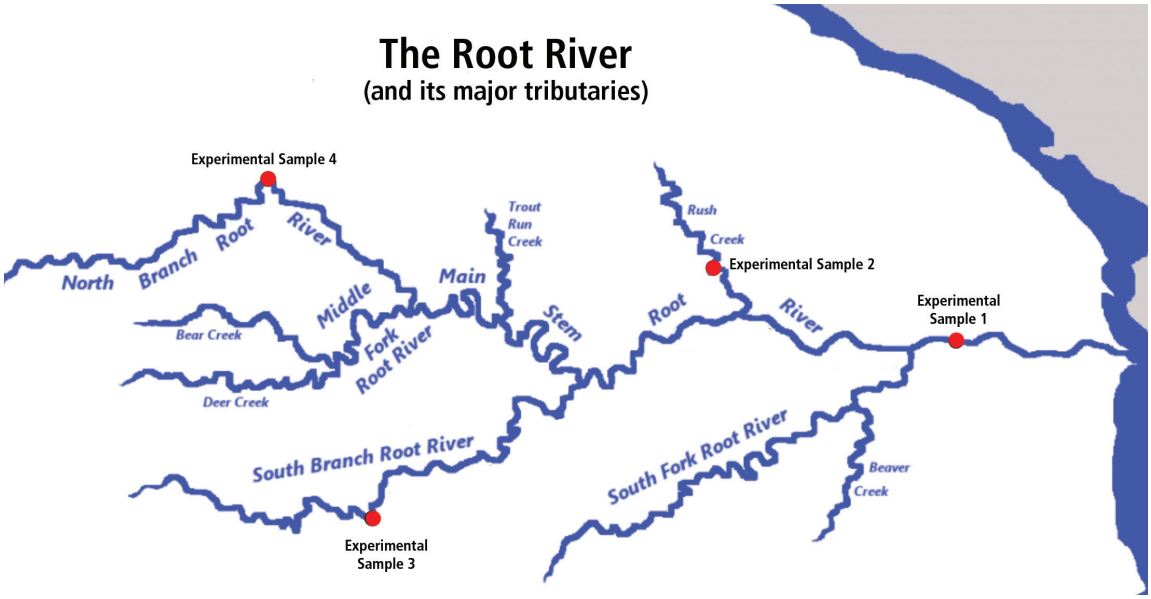
   Major sources of experimental error in this experiment are contamination, variability in sample reagent volumes/concentrations, reagent degradation (particularly of fluorescent dyes), and detection errors. Students may brainstorm and discuss a number of proactive measures to limit these experimental errors. These include proper pipetting, frequently changing pipette tips, using filter pipettes, starting with the lowest concentration samples, preparing standard samples and control samples separately, thoroughly mixing solutions, keeping reagents on ice, limiting long-term light exposure, checking for bubbles in solutions, properly labeling tubes, and properly programming the qPCR machine.

2. **Choose and describe at least two ways to assess the accuracy of your qPCR experiment.**

   Students should describe key assessment methods including melt curve analysis, gel electrophoresis, and assessment of the slope and R^2 value of the standard curve equation. In addition, they could also mention examining amplification curve shapes and determining the variance between classroom replicates.

3. **(Optional) Explore an area of research where qPCR is commonly used such as gene expression analysis, biomedical science, and pathogen detection. Next, invent a possible experimental scenario by naming and giving a background to each of your experimental samples.**

   Answers will vary. Here is an example: qPCR has been used in environmental studies to detect aquatic invasive species using local water samples. The four experimental samples are from four locations in a river where Asian carp is thought to be invading but has not yet been observed.



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

Appendices

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

Appendix A
Setting Up the Open qPCR Machine

EQUIPMENT AND SOFTWARE SETUP FOR THE OPEN qPCR MACHINE

(If using a different qPCR machine consult the manufacturers instructions.)

Figure 10a

Open qPCR uses an IP address to interface between the machine and you the user. Follow the instructions below to establish this connection. You may need to involve your network administrator in the process.

Figure 10b

1. Decide on and connect to a network. For a Local Area Network (LAN) connection, you will need to insert an Ethernet cable (Figure 10a) and for a Wireless Local Area Network (WLAN) connection you will need to insert a USB adaptor (Figure 10b).
2. Turn on the Open qPCR machine.
3. While the machine starts up, confirm that your electric devices (computers, tablets, smartphones etc.) are also connected to the selected network.
4. Once the opening screen has loaded (Figure 12, page 35), find and record the IP address displayed in the lower left corner of the screen. (During the lab, multiple groups will be able to simultaneously use this address).
5. Open a browser, enter the IP address in the navigation window and press enter. Wait until the home page loads (Figure 13, page 35).
6. If you are setting up the devise for the first time, follow screen prompts to set up a new username and password. Otherwise, enter your username and password.

NOTE: Multiple users may access the same machine. To set up additional users login to your original account and then go to "Settings">"Manage Users">"Add User".

PREPROGRAM qPCR CONDITIONS

The following are detailed instructions. However, Open qPCR software is highly intuitive so feel free to play and find the fastest way to program qPCR conditions for your classroom. Final conditions should be:

PCR cycling conditions:
Initial denaturation 94°C for 3 minutes
94° C for 30 sec.
60° C for 30 sec.* } 30 cycles
68° C for 30 sec.*
Final extension 68° C for 5 min.

Melting curve conditions:
90° C for 30 sec.
50° C for 1 min.
From 50° C to 95° C with a ramp speed of 0.09° C/s and a hold duration of 30 seconds.*
Hold at 4° C forever.

* *Data gathering should be switched on at these three parts of the experiment as indicated by the graph icon ( ).*

1. Select “Create a New Experiment”.
2. When prompted enter "qPCR I (EVT 380)" or similar name for your experiment and then select “Create Experiment”.
3. Set initial denaturation conditions.
   a. Use the left and right triangles on the screen to navigate to “Stage 1: Holding” and the “01/01 Initial denaturing”. Alternatively, select this stage by clicking on the first circle.
   b. Modify time and temperature in this stage to match the initial denaturing conditions of 94°C for 3 minutes. This can be done by changing number values at the top (orange) or bottom (white) screen section or by dragging the circle marker up or down.

Appendix A: Setting Up the Open qPCR Machine, continued

4. Set cycling conditions.
   a. Navigate to "Stage 2: Cycling"
   b. Select "01/02 Denature". Modify time and temperature in this stage to match the cycle denaturing conditions of 94° C for 30 seconds.
   c. Select "02/02 Anneal". Modify time and temperature in this stage to match the cycle annealing conditions of 60° C for 30 seconds.
   d. Click on "Add Step".
   e. Select "03/03". Modify time and temperature in this stage to match cycle extension conditions of 68° C for 30 seconds.
   f. Rename step 3 "Extension" and toggle the "Gather Data" switch to on.
   g. On the top line of the white screen section click on "40x" and modify to match the experiment cycle number of 30.

5. Set final denature conditions.
   a. Click on "Add Stage" and select "Hold".
   b. Rename the "01/01 Step" "Final Extension" and modify time and temperature to match the experimental conditions of 68° C for 5 minutes.

6. Set melt curve conditions.
   a. Click on "Add Stage" and select "Melt Curve".
   b. Select "01/03 Step 1". Modify temperature and time to 90° C and 30 seconds.
   c. Select "02/03 Step 2". Modify temperature to starting melt curve temperature of 50° C and set time to 1 minute.
   d. Select "03/03". Modify temperature to the end melt curve temperature of 95° C for 30 seconds and ramp speed of (0.09° C/second).
   e. Confirm that the data gather symbol ( ) is present between steps 2 and 3.

7. Select low temperature hold.
   a. Click on "Add Stage" and select "Hold".
   b. Set temperature to 4° C and duration to "00:00". This should prompt the program to add an infinite hold.

8. Navigate to the Experiment Menu by clicking on the striped box in the upper left hand corner ( ) and select "View Protocol" and confirm that all modifications and changes have been saved and are correct. The program should match the image below. Check times, temperatures, cycle numbers, and the gather data symbol ( ).

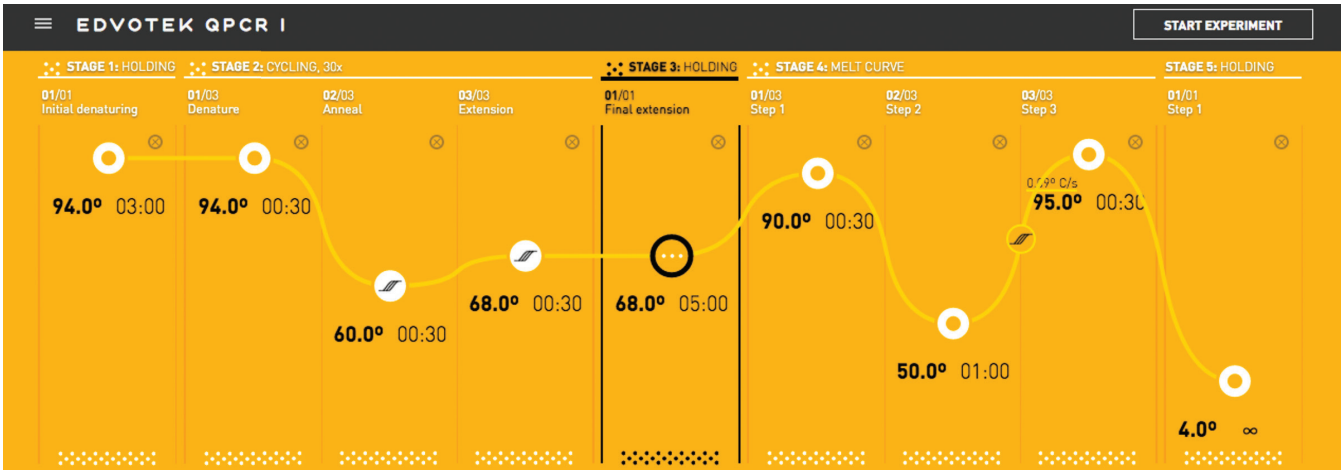


Figure 11: The 380 qPCR Program on the Open QPCR user interface.

Appendix B
Performing qPCR Using the Open qPCR Machine

STARTING THE OPEN qPCR MACHINE

Figure 12

Figure 13

NOTE: The Open qPCR machine has well room for 2 groups to simultaneously run a qPCR experiment - coordinate with your class to determine run times.

1. **TURN ON** the machine. **RECORD** the IP address on the Open qPCR screen (Figure 12) and **RE-ENTER** the address into a browser. When the Welcome Screen loads (Figure 13), type in your class's user name and password.
2. **SELECT** the correct qPCR experiment. **NAVIGATE** to the "Experiments" list on the right portion of the screen and **CLICK ON** "qPCR I (EVT 380)". If the orange and white protocol screen does not appear (Figure 14) **CLICK ON** the menu bar in the upper left hand corner (☰) and **SELECT** "View Protocol".
3. **CONFIRM** that the PCR cycling and melt curve conditions match the temperatures, times, and cycle numbers listed below.

PCR cycling conditions:
Initial denaturation 94°C for 3 minutes
94° C for 30 sec.
60° C for 30 sec.* } 30 cycles
68° C for 30 sec.*
Final extension 68° C for 5 min.

Melting curve conditions:
90° C for 30 sec.
50° C for 1 min.
From 50° C to 95° C with a ramp speed of 0.09° C/s and a hold duration of 30 seconds.*
Hold at 4° C forever.

** Data gathering should be switched on at these three parts of the experiment as indicated by the graph icon ( ).*

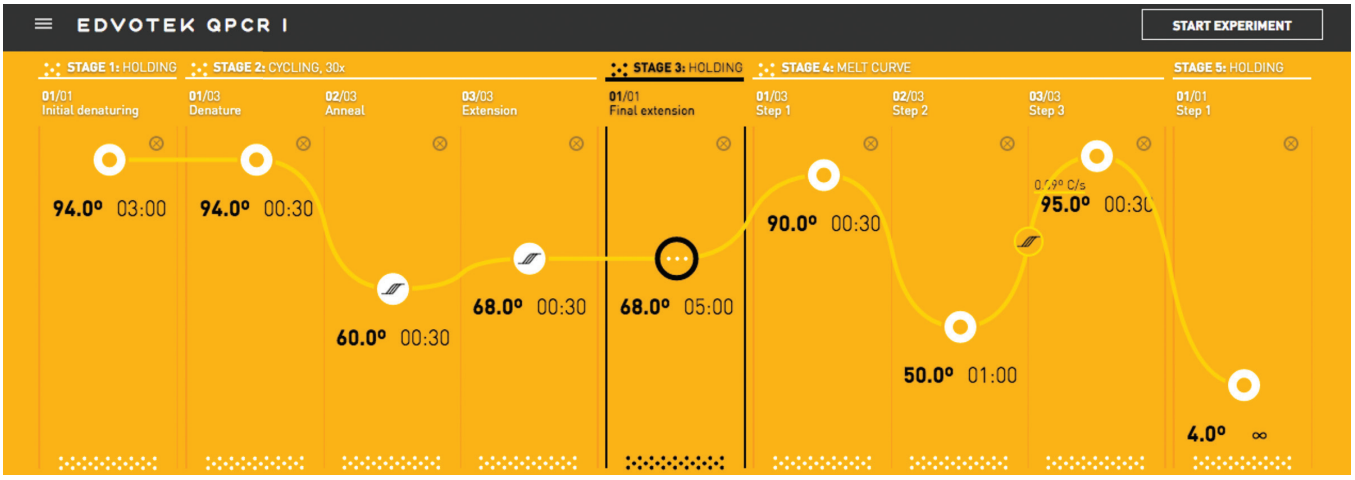


Figure 14: The 380 qPCR Program on the Open QPCR user interface.

Appendix B: Performing qPCR Using the Open qPCR Machine, continued

RUNNING THE OPEN qPCR MACHINE

4. **SELECT** the "Duplicate Experiment" box (located in the top left hand part of the screen). When prompted, **ENTER** a new name.
5. **DECIDE** on the locations of your 8 samples within the Open qPCR Machine. **RECORD** these locations in your lab book or in the provided student worksheet (Appendix C). With the Open qPCR platform, sample names and positions can also be saved as part of the experimental record. To do this, **ENTER** the sample names into the right hand table of the screen either before the experiment or while it is running.
6. **CLICK** on the menu icon in the upper left hand corner ( ) and then **SELECT** "Run Experiment".
7. The thermal cycler will amplify the DNA and perform a melt curve experiment. While the experiment is running, **REVIEW** the experimental results in real time by toggling between log and linear views.

OPTIONAL STOPPING POINT:
The qPCR machine can be left to finish the programs and hold samples at 4° C until needed.

8. Once the thermal cycler program has finished, **SAVE** the data for future analysis. **CLICK** on the menu icon ( ) and then **SELECT** "Export".

Appendix C
Student Worksheets

MODULE I

1 Snap-top microcentrifuge tube containing 170 µL qPCR Cocktail
1 Snap-top microcentrifuge tube containing 14 µL Starting Sample
1 Snap-top microcentrifuge tube containing 7 µL Experimental Sample 1
1 Snap-top microcentrifuge tube containing 7 µL Experimental Sample 2
1 Snap-top microcentrifuge tube containing 7 µL Experimental Sample 3
1 Snap-top microcentrifuge tube containing 7 µL Experimental Sample 4
1 Snap-top microcentrifuge tube containing 150 µL Ultrapure Water
3 1.5 mL Conical Tubes
8 qPCR Tubes
• Adjustable Pipets and Pipet Tips

Amount of DNA in Standard Curve Samples

Sample Name	DNA Concentration
Standard Curve Starting Sample	10 ng
Standard Curve 1:10 Sample	
Standard Curve 1:100 Sample	
Standard Curve 1:1000 Sample	

Well Locations of qPCR Samples and Experimental Results

Tube	Row, Column	Sample Description	Cq Value
A1			
A2			
A3			
A4			
A5			
A6			
A7			
A8			
B1			
B2			
B3			
B4			
B5			
B6			
B7			
B8			

MODULE II

1 Snap-top microcentrifuge tube containing 1.2 mL of Concentration Buffer
1 Beaker containing 58.8 mL of Distilled Water
1 Snap-top microcentrifuge tube containing 0.46 g of Ultra-Spec Agarose™ Power
1 Snap-top microcentrifuge tube containing DNA Standard Marker
• Gel Casting Tray(s), Seals, and Combs
• SYBR® Safe Stain

Appendix C
Student Worksheets

MODULE III

DNA Concentration in Standard Curve qPCR Samples

	DNA in qPCR Reaction (Step 1)	Log Value of DNA in qPCR Reaction (Step 2)
Standard Curve Starting Sample (qPCR Tube 1)		
Standard Curve 1:10 Sample (qPCR Tube 2)		
Standard Curve 1:100 Sample (qPCR Tube 3)		
Standard Curve 1:1000 Sample (qPCR Tube 4)		

Linear Regression Equation: _____

R^2 value: _____

Rewritten Linear Regression Equation: _____

Total DNA in Experimental Samples

Sample Name	DNA Concentration
Experimental Sample 1	
Experimental Sample 2	
Experimental Sample 3	
Experimental Sample 4	
Experimental Sample 1	
Experimental Sample 2	
Experimental Sample 3	
Experimental Sample 4	

Appendix D
In-depth qPCR Analysis

When a researcher obtains a qPCR data point, they need to provide evidence that their amplification results are valid, reliable and meaningful. Understanding of the underlying biology behind qPCR is essential to identifying potential errors and determining the validity of experimental data. In addition, there are several ways to critically examine the generated data. Below is a rough outline of key examination points.

1. **Examining amplification curves.**

   Much of your data analysis will focus on a single cycle point where the amplification curve and threshold line intersect. However, examining the amplification curve gives you an overview of the complete amplification process. Check that each sample's amplification curve has clear baseline, exponential, and plateau regions. Amplification curves should also be roughly parallel and classroom replicates should be clustered.

2. **Examine the melt curve.**

   A melt curve with a single DNA peak strongly suggests that qPCR amplified only the target product. The presence of multiple peaks is more ambiguous. Additional peaks can indicate nonspecific amplification (primer dimer, DNA contamination, multiple target sites). However, additional peaks may also be the result of uneven melting in AT rich regions of the amplified DNA. The location of the peaks can help distinguish between these two scenarios. For example, primer dimers form product peaks usually around 70° C. The clearest test for primer dimer and non-specific amplification is running the final sample on an agarose gel.

3. **Consider the slope of the standard curve**

   The standard curve slope can provide information about a PCR assay's efficiency. Generally, a slope of -3.32 indicates 100% efficiency. Your experimental qPCR experiment will ideally have a slope with a value between -2.5 and -5. For a specific estimate of efficiency calculate the efficiency value using these two equations: (1) Exponential amplification = $10^{-1/slope}$ and (2) Efficiency = (Exponential amplification – 1) x 100.

4. **Look at each samples average Cq.**

   This value depends on the starting amount of target DNA in each sample and experimental conditions so it will vary between different samples. In general, the Cq values of experimental samples should fall within the range of the standard curve. Another general rule is to flag any samples with a Cq higher than 40 as these often indicate that you are measuring less than a molecule of starting DNA.

5. **Look at replicates.**

   This experiment is designed to generate classroom data with four replicates of each sample. (Most published qPCR data sets have at least 3 replicate samples.) Large differences between replicates indicate a lack of reproducibility either due to experimental error or biological variance. One way to detect this variance and identify outliers is to calculate the standard deviation (square root of the variance) between replicate samples or use qualitative method such as a boxplot or histogram. A more general rule of thumb is that replicates value should differ by no more that 0.5 Cq. Remember that certain replicates can be more sensitive than others. For example low concentration replicates will often show more dispersion than higher concentration replicates. Because you are using your classmates' data points as replicates, the difference between these points may be slightly higher due to the differences in experimental technique.

Appendix E
qPCR Troubleshooting Guide

PROBLEM:	CAUSE:	ANSWER:
No amplification detected.	Amplification not occurring.	Run samples on gel. If no bands are visible it is likely a PCR error. Possible causes are pipetting error or a missing reagent. Lack of amplification can also be caused by sample or enzyme degradation.
	No signal / No fluorescent detection.	Run samples on gel. If bands are visible it is likely a detection error. Make sure that the correct PCR tubes are being used and that the instrument's setting is optimized for a SYBR Green probe. Also confirm that the fluorescent dye is not degraded from extended light exposure or extreme temperatures.
Abnormal amplification observed (curves are sigmoidal or negative, lines are wavy or erratic).	High background signal.	Recalibrate the machine or manually adjust the baseline setting to a higher value.
	Samples were not adequately mixed.	Briefly centrifuge samples before adding to to machine and carefully pipet small volumes.
Amplification begins very late in the program.	Poor template quality in certain samples.	Keep samples on ice and carefully pipet small volumes.
Amplification begins very early in the program.	Too much template is present in certain samples.	The instrument is having trouble distinguishing between noise and true amplification. Manually reset the baseline or further dilute high concentration samples.
Low PCR efficiency (<80%)	Incorrect DNA dilutions causing error in the standard curve	Pipetting error during the serial dilution. Use a classmate's standard curve.
	To little dye or probe in sample.	Make sure instrument setting for dye matches SYBR. Confirm fluorescence is not degraded from extended light exposure or extreme temperatures. Thoroughly mix and carefully pipet master mix.
	Sample inhibition.	Provided samples have been purified and optimized for this reaction. If using outside samples the DNA concentration may be too low or there may be inhibitors.
High PCR efficiency (>110%)	Incorrect DNA dilutions causing error in the standard curve	Pipetting error during the serial dilution. Use a classmate's standard curve.
	Contamination	Change tips between samples. Prepare serial dilution and samples separately. Always start with the lowest concentration standard dilution sample.
	Too much dye or probe in sample.	Invert the master mix several times to avoid a concentration gradient and pipet accurately. Also check that tube/well seals are secure and no evaporation is occurring.
Replicates show high variability.	Evaporation	Make sure caps or other covers are securely fitted before adding samples.
	Concentration differences	Thoroughly mix and accurately pipet samples.
Non-specific peaks in the melt curve.	High primer concentration	Thoroughly mix and accurately pipet samples.
	Contamination	Keep tubes sealed when possible.

Appendix E
qPCR Troubleshooting Guide

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.
		Make sure students close the lid of the PCR tube properly.
	Pipetting error.	Make sure students pipet 20 µL qPCR cocktail and 5 µL extracted DNA into the 0.2 mL tube.
The ladder and PCR products are not visible on the gel.	The gel was not prepared properly.	Ensure that the electrophoresis buffer was correctly diluted.
		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 for a sufficient period of time.	Re-stain using post-electrophoresis SYBR® staining instructions.
After staining the gel, the gel background is very dark.	The gel needs to be destained.	Submerge the gel in distilled or deionized water. Allow the gel to soak for 5 minutes.
Some samples have more/less amplification than others.	Concentration of DNA or qPCR cocktail varies by sample.	Check pipeting accuracy and make sure reagents are well mixed.
Low molecular weight band in PCR samples	Primer dimer.	Low concentration of DNA in qPCR reaction.
DNA bands fade when gels are kept at 4°C.	DNA stained with SYBR® Safe Stain may fade with time.	Re-stain the gel with SYBR® Safe Stain.

Appendix F
Bulk Preparation of Electrophoresis Buffer and Agarose Gels

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities for sharing by the class. Unused diluted buffer can be used at a later time and solidified agarose gel solution can be remelted.

Bulk Electrophoresis Buffer

Quantity (bulk) preparation for 3 liters of 1x electrophoresis buffer is outlined in **Table D**.

Table D — Bulk Preparation of Electrophoresis Buffer

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

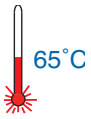
BATCH AGAROSE GELS (0.8%)

Bulk preparation of 0.8% agarose gel is outlined in Table E.

Table E — Batch Prep of 0.8% UltraSpec-Agarose™ with Sybr®

Amt of Agarose (g)	+	Concentrated Buffer (50X) (mL)	+	Distilled Water (mL)	=	Total Volume (mL)	ADD SYBR® (Before casting)
1.9 g		4.8 mL		245.2 mL		250 mL	220 µL

1. Use a 500 mL flask to prepare the diluted gel buffer
2. Pour the appropriate amount 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 65° 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. While agarose is cooling, seal the end of the gel-casting trays with the rubber end caps and place the well templates (combs) in the appropriate notches.
7. Before casting the gel, add 220 µL of SYBR® Safe to the molten agarose and swirl to mix. The final agarose solution may appear pale orange in color.
8. Pour the required volume of agarose solution into the prepared gel casting trays. The volume required is dependent upon the size of the gel bed.
9. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Proceed with electrophoresis (Module II) or store the gels overnight at 4° C under buffer and in the dark.

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