P51™ qPCR Lab

Principles of Quantitative PCR

P51™ qPCR Learning Lab
Principles of Quantitative PCR
Instructor and Student's Guide

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Student's Guide Contents

Background and significance P. 08

Laboratory guide P. 12

Study questions P. 28

Background and significance

Overview

In this lab, you will perform a modified version of a quantitative polymerase chain reaction (qPCR). qPCR monitors the amount of PCR product produced in real-time using fluorescent dyes or probes and a specialized qPCR machine. This lab modifies the reaction so that you can observe the change in fluorescence first hand, using low cost equipment. In this way, this activity serves as an excellent illustrative learning tool for how fluorescence and PCR can be used to quantify nucleic acids while also providing an introduction to the process of PCR in general. Students should have a familiarity with the PCR process before starting this investigation.

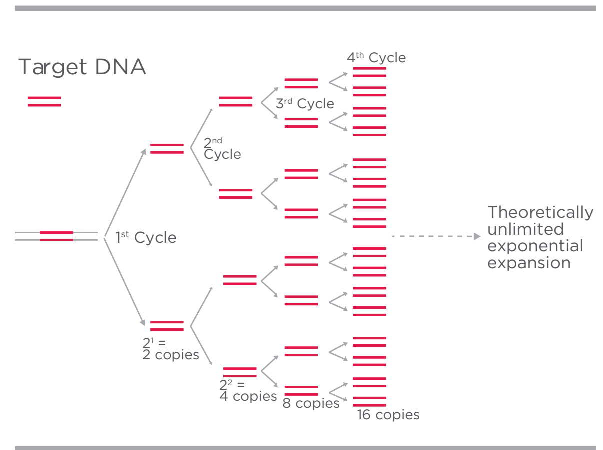
PCR as a measurement

When the polymerase chain reaction, or PCR, was invented in the 1980s, it revolutionized biology and won a Nobel Prize for its inventor, Dr. Kary Mullis. Using a few simple ingredients, scientists could take a complex sample of DNA and make billions of copies of a very specific sequence, all in a few hours. PCR quickly became a mainstay in biology labs around the world. PCR machines soon became automated and protocols became more streamlined, but the basic technique remained the same. Indeed, most PCR protocols performed today are little changed from the original invention.

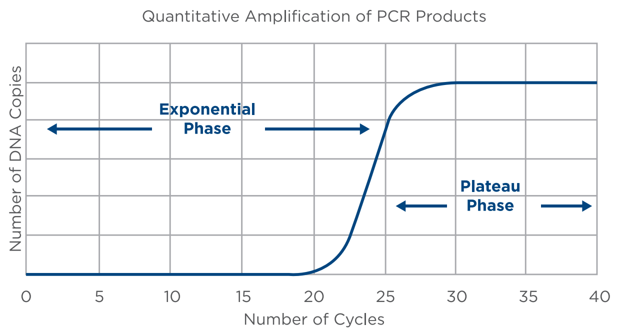
Most PCR is what is known as an end-point assay. Once the reaction starts, we don't observe or use the products until the reaction has finished completely, typically after 30 or more cycles. The copies made in PCR can be used in many other protocols, but during the reaction, the PCR machine is a virtual (and sometimes literal) black box – there is no way to observe what is going on inside the reaction. This changed with the invention of quantitative PCR (qPCR) in the early 1990s.

With qPCR, instead of just looking at the product at the end of the reaction, scientists use light to detect and quantify the reaction product in real time. PCR makes copies of very specific sequences of DNA, known as the target, and in every PCR cycle, the amount of that very specific target is expected to double. qPCR keeps track of how much target is produced by the reaction at every step by combining DNA with a fluorescent dye or probe. The dyes will only fluoresce when bound to double stranded DNA (dsDNA), so the more dsDNA that is produced by the reaction, the brighter the fluorescence.

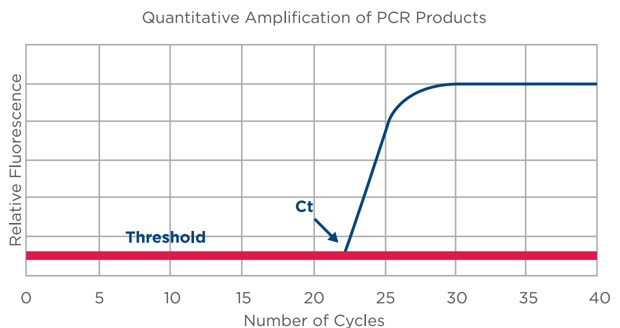
When starting a PCR, the target DNA that you are interested in is present in a complex mix with other DNA. The vast majority of the DNA in the sample is not the target of interest. That very specific sequence is therefore relatively rare. As the PCR progresses, the number of copies of that target sequence will double every cycle, while the rest of the DNA will not. This means that the absolute number of copies of your target DNA is at first small and indistinguishable from the background DNA. As the reaction progresses and the number of copies of your target DNA sequence doubles with each reaction cycle, its abundance will increase faster and faster in what we call exponential growth. The number of copies of the target sequence will eventually plateau when there are not enough reagents left to sustain the reaction.



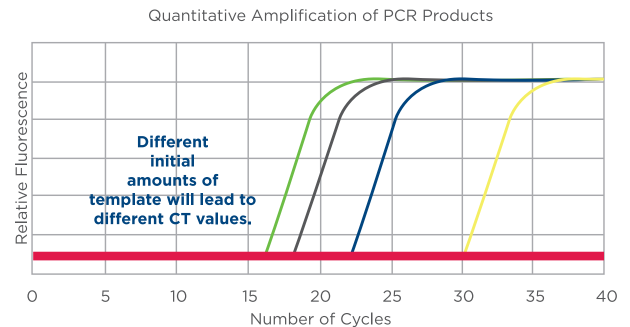
In conventional PCR, we usually try to wait for the reaction to reach this plateau stage to view our results; the goal is to just get as much DNA as we can. In qPCR it is the exponential phase that we are interested in. Exponential growth of our PCR product means that at the scale we are looking at, for a long time, no real change will be observable. Then, the amount of PCR product will seem to grow extremely quickly before leveling off again.



When observing the fluorescent dye in our reaction, this means that for much of the reaction the amount of fluorescence will be relatively small and indistinguishable from the background. Then as the number of new DNA copies grows, it will relatively quickly become significantly larger than the background. At this point a threshold is crossed at which the fluorescence becomes observable. This cycle that fluorescence is first observable above background levels is called Ct, for cycle threshold. As the reaction progresses, the number of target DNA molecules continues to increase, as does the level of fluorescence until the reaction plateaus.



The key to qPCR is that how soon a reaction reaches the threshold is directly related to how much of the target sequence was in the sample to start. The more target DNA there was to begin with, the sooner Ct will be reached. This means that by observing Ct, we can learn about our original DNA concentration. Because the amount of target DNA doubles every cycle, if reaction A reaches Ct one cycle earlier than reaction B, you can estimate that reaction A started with roughly twice as much target sequence as in reaction B. If reaction A reaches Ct two cycles before reaction C, you can estimate that there was a four-fold difference in the original amount of the target sequence, and so on.



The uses of qPCR are many. Any time a researcher wants to quantify the amount of a particular nucleotide sequence, qPCR can be used. For example, in a clinical setting, qPCR can confirm the presence of an infection by amplifying a particular viral sequence, but it can also measure the amount of virus in a person's system, by measuring how relatively common that sequence is in a sample. Probably the most widespread use of qPCR in research has been to quantify RNA. In any one organism, every cell has an identical set of DNA. Yet cells use that DNA very differently and respond to external stimuli by turning up or down how much certain genes are used. In the cell, this is done by transcribing more or less messenger RNA (mRNA), which will then be translated into protein. The invention of qPCR allowed scientists to measure those expression levels on a gene by gene basis more precisely than ever before. Scientists can now collect all the RNA in a sample, perform a reverse transcription step, converting the RNA to DNA, and perform a qPCR on the sample. Comparing how quickly a particular sequence reaches Ct vs. other samples will measure how much that gene is being used in the tissue from which the sample was isolated.

Today's lab

In this lab, we provide you with a sample of DNA of known concentration. Your job is to perform a qPCR on that sample, two other samples, and a negative control. For the two other samples, the first will be a sample that you create by diluting the DNA provided to you to a new concentration of your choice. Your job will then be to predict at what cycle your sample will reach Ct. The second sample will be provided to you by another group. Your challenge with this sample will be to determine its relative concentration compared to your sample by measuring the approximate Ct value. Finally, at the end of your protocol, you will have the option to run your samples on an agarose gel to compare the results of your qPCR with a conventional end-point PCR approach.

The qPCR you perform will amplify a short region of DNA of a known concentration. As the PCR progresses, you will remove samples at set intervals and visually judge the amount of fluorescence produced, using a blue light illuminator. In a qPCR performed in a research lab or medical facility, fluorescence is only ever monitored by an automated fluorometer – people never actually look at the tubes. Though judging fluorescence by sight will have lower precision than an automated machine, fluorescent dyes are easily visible to the naked eye and will give you a first-hand view of the reaction. For this reason, however, the Ct values you obtain in this lab should be considered approximate, and small differences between your expected and observed values should be expected. Despite this, relative differences between two samples of different concentration should readily apparent.

A note on terms

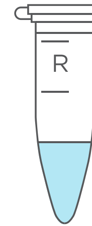
In this lab, we refer to quantitative PCR as "qPCR". qPCR is also sometimes referred to as "real-time PCR", because the progression of the reaction is observed in real-time. We avoid the term "real-time PCR". A common use of qPCR is to quantify RNA copy number. Quantifying RNA requires a first step of copying cellular RNA into DNA, a step known as "reverse transcription". Because "real-time" and "reverse transcription" can both be abbreviated with the letters RT, referring to a reaction as RT-PCR can be ambiguous. We follow the convention of using RT to refer to reverse transcription, and using RT qPCR to refer to quantitatively measuring RNA transcript levels using PCR.

In this lab, we use a non-specific fluorescent DNA dye to measure total DNA levels in the reaction. DNA probes are a separate but related technology. A probe is a short, specific DNA sequence with an attached fluorophore that is complementary to a portion of the PCR product in a qPCR reaction. Probes measure the amount of specific PCR product in solution, whereas dyes measure total DNA in solution. Probes can be useful because they only measure the specific target DNA sequence and are therefore considered more precise. Also, using different colored probes allows a researcher to measure the concentration of multiple target sequences in the same reaction. But probes tend to be much more expensive than DNA dyes. Furthermore, each probe must be designed with a very specific target sequence in mind, whereas DNA dyes can be used generally in any qPCR reaction.

Laboratory guide

A. Experimental planning and making dilutions

You will investigate 3 samples and one negative control using qPCR



1. Your teacher will provide you with a tube labeled R (Reference)

- This contains a known concentration of DNA template: 40 pg/µl. You will use this reference sample as the basis to calculate the relative concentrations of your other samples.

2. Use the "Experimental sample planning" tables below to plan sample E (Experimental)

- To make the E sample, you will mix nuclease-free water and some of your R sample to make a new dilution of DNA.

- Plan to make between 20 and 40 µl of sample E, but do not use more than 10 µl of your R sample to do so.

- Your goal with sample E is to choose a cycle at which you expect your sample to fluoresce relative to R, and create a dilution that will be visibly fluorescent at that cycle.

- Once you have planned this new dilution, your job is to predict at what PCR cycle you will first see fluorescence in your sample. This is based on the fact that, theoretically, the amount of PCR product should double during every cycle of PCR. You get to decide how diluted you want to make your E sample. We recommend using a dilution that will produce fluorescence between 3 and 10 cycles after your original R sample.

Experimental sample planning

My dilution factor for sample E will be:	
The number of additional cycles I expect it will take for sample E to fluoresce compared to R:	
Why I think this:	

3. Use the table below to help you calculate how to make your E sample

	Reference DNA	Dilution	Only use if doing a serial dilution Second tube	Third tube
Concentration	1X (40 pg/μl)			
Volume added from previous tube	-			
Water added	-			

Protective gloves and eyewear should be worn for the entirety of this experiment.

4. Label a new 200 μl tube

- Label your tube E, for Experimental.

5. Make your dilution in tube E

- Use sample R and H_2O[make sure 2 is a subscript] to make your dilution as you described in the table above.
- If your dilution factor is large, you may use more than one tube and make a serial dilution.

6. Label a new 200 μl tube

- Label the tube U for Unknown.
- Using a micropipette, remove at least 10 μl from your E sample and put it in the tube labeled U.
- Be sure that your E tube still contains at least 10 μl.

7. Find another group and trade U tubes

- Do not tell the other group the concentration of your DNA. It is their job to discover it!
- Your job with this tube is to estimate how concentrated the other group made their unknown sample.

8. Plan your experiment

- Before going on to *B. PCR Set up,* fill in the first column (Cycle #) in Table 2 (page 14).
- You get to choose your observation interval—how many PCR cycles to wait between observations. We recommend checking your tubes at most every three to five cycles, but use your dilution factor as a guide.
- Your first observation of fluorescence should be at cycle 10.
- Following this observation, you can make up to 7 additional observations.

This is a potential stopping point. Students may save their samples in the fridge or freezer, or students may proceed to the next step (on the following page).

B. PCR set up

Protective gloves and eyewear should be worn for the entirety of this experiment.

1. Before setting up your PCR you must finish planning your experiment

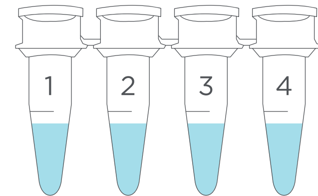
- Fill in which cycles you plan on observing your reaction on Table 2 (page 14).

2. Label your strip of four 200 µl thin-walled PCR tubes on the side, not cap, of the tube

- Label the tubes 1-4.
- Tube 1 will be for your negative control (N).
- Tube 2 will be for your reference DNA (R).
- Tube 3 will be for your experimental sample (E).
- Tube 4 will be for your unknown sample (U).

3. Add PCR reagents to each 200 µl PCR tube in the strip

- Use a micropipette to add each of the reagents.



	Tube 1	Tube 2	Tube 3	Tube 4
2X qGRN Master Mix (qMM)	15 µl	15 µl	15 µl	15 µl
qPCR Primers (Primers)	7.5 µl	7.5 µl	7.5 µl	7.5 µl

4. Add DNA samples to each tube. Use a new tip for each sample

- Add 7.5 µl of your DNA/dilutions. For your negative control (Tube 1) add 7.5 µl H_2O

Remember to change pipette tips each time.

	Tube 1	Tube 2	Tube 3	Tube 4
Template DNA	7.5 µl H_2O	7.5 µl "R"	7.5 µl "E"	7.5 µl "U"

- Pipette up and down three times to mix thoroughly.
- The final volume in all tubes should now be 30 µl.

5. Close the caps on the tubes, pressing down firmly to ensure a proper seal

- Make sure all the liquid volume collects at the bottom of the tube.
- If necessary, shake down with a flick of the wrist or spin briefly in a microcentrifuge.

6. Observe your tubes in P51™ or other blue light illuminator. Record your observations in the Table 1 ("Initial observations table", Page 13) in the row labeled "Room temp."

- Dim ambient lights as needed for proper observation.
- You will make your second observation shortly after starting the PCR program.

7. Place the tubes inside the PCR machine

- Press firmly on the tube caps to ensure a tight fit.
- Close the PCR machine lid and gently tighten the lid.

C. PCR programming (illustrated using miniPCR™ software)

1. Open the miniPCR software app and remain on the "Library" tab

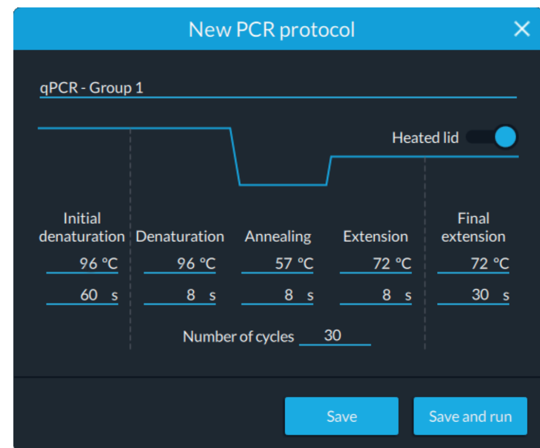
2. Click the ⊕ button on the top right corner

3. Select PCR from the top drop-down menu

4. Enter a name for the Protocol; for example, "qPCR- Group 1"

5. Enter the PCR protocol parameters:

• Initial denaturation	96°C, 60 sec
• Denaturation	96°C, 8 sec
• Annealing	57°C, 8 sec
• Extension	72°C, 8 sec
• Number of cycles	30
• Final extension	72°C, 30 sec
• Heated lid	ON



6. Click "Save" to store the protocol or "Save and run" to start the protocol

7. If prompted, choose the serial number of the miniPCR you are using from the list

- Serial numbers can be found on the white sticker below the power switch.

8. Make sure that the power switch in the back of miniPCR is in the ON position

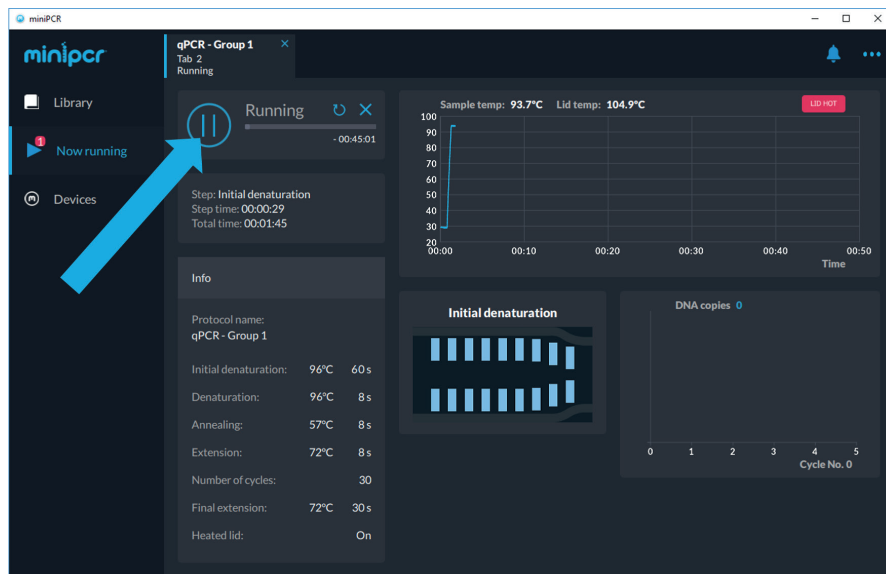
9. To monitor the PCR parameters in real time, choose the "Now running" tab on the left

- If more than one miniPCR is connected to the same device, choose which machine you would like to monitor using the tabs at the top of the window.

D. qPCR monitoring

1. Near the end of the Initial denaturation stage of your PCR, press pause on the miniPCR software

- Initial denaturation is 60 seconds. You may press pause at any point in the initial denaturation, but make sure that tubes have been at 96°C for at least 25 seconds before removing to view.



2. Open the lid and remove your tubes

Be careful opening the miniPCR; lid and heat block will still be hot

3. Immediately place the tubes in the P51™ viewer or other blue light illuminator

- Dim ambient lights as needed for proper observation.
- Record your observations on the INITIAL OBSERVATIONS TABLE (Table 1, Page 13).
- Remember to keep P51™ blue lights off between observations to conserve batteries.

4. Return the tubes to the miniPCR machine and press the Run button

5. Allow the PCR to continue until your first planned observation, the first cycle listed in Table 2, column 1

6. While you wait, complete the questions on page 14 under Table 1 or the "Questions for before experimental set-up" section of the Study Questions, if you have not already done so (page 21)

- It will take approximately 15 minutes to reach cycle 10 of your PCR protocol.
- Keep an eye on the progress of your PCR. You don't want to miss your first observation!

Measuring approximate Ct (starting at cycle 10, see Table 2)

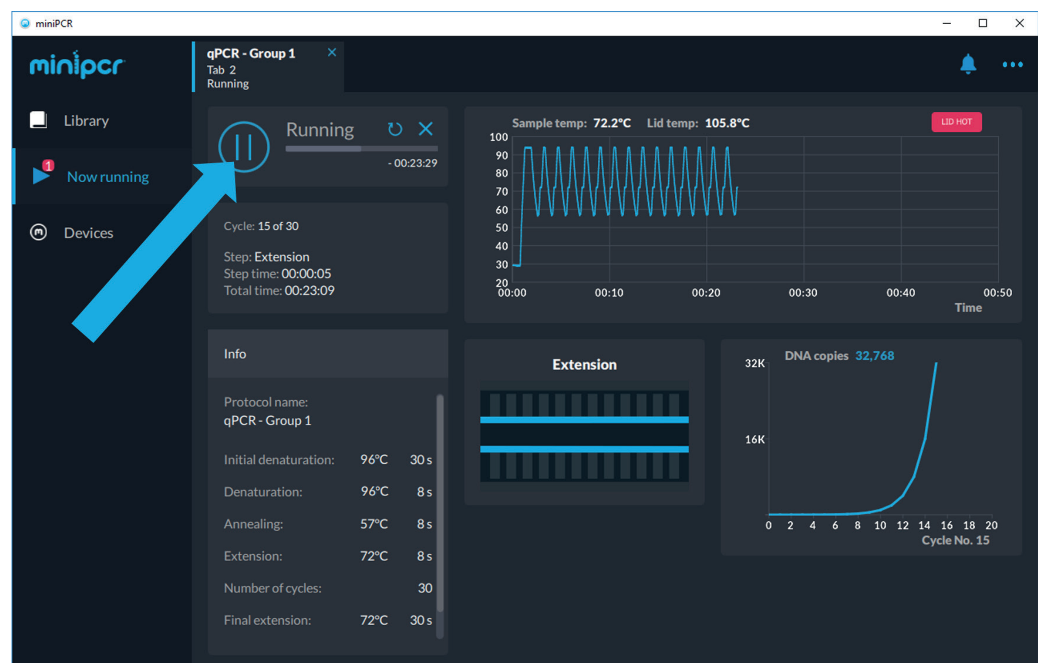
7. To observe fluorescence in your tube, press pause when the machine enters the Extension (72°C) stage

- This will keep the samples at 72°C.
- Allow your samples to remain at 72°C for at least 8 seconds before removing.
- Open the miniPCR machine and remove your samples.

Pressing pause before miniPCR reaches 72°C is OK. If the machine has entered the extension phase, but has not yet reached 72°C, it will continue heating until temperature is reached and will pause at that time.

If you press pause too late in the cycle, the machine will continue on to the denaturation stage and hold at 96°C. If this happens, your samples will denature and no fluorescence will be visible. Skip this cycle and measure at the next extension step.

When opening the miniPCR, be very careful not to touch the metal lid which will still be hot



8. Immediately after removing your samples, place your tubes in the P51™ molecular viewer

- Dim ambient lights as needed for proper observation.
- You may choose to use a camera to record the fluorescence of your samples.
- Record your observations in the qPCR Observation table (Table 2, page 14).

9. Return the tubes to the miniPCR machine and press the Run button

- Remember to turn P51 off between observations to conserve batteries.

10. At each of your scheduled observations, repeat steps 7 through 8

11. When your PCR has finished, remove the tubes from the thermocycler

- PCR product is stable after amplification at room temperature for several days. For long term storage, move tubes to a freezer (up to 1 month).

This is a potential stopping point. Samples may be stored frozen or students may proceed to the next step (optional gel electrophoresis, page 16).

Observation tables

For both Table 1 and Table 2, we recommend recording observations as:
0- no fluorescence / 1- dim fluorescence / 2- moderate fluorescence / 3- full fluorescence
You can expect at least one tube to be at full fluorescence in your original room temp. observation

Table 1 - Initial observations

	Tube 1 (Negative Ctrl)	Tube 2 (Reference DNA)	Tube 3 (Experimental)	Tube 4 (Unknown)
Room temp.				
96°C				

Summarize your observations from the table above in sentence form:

While your PCR advances to your first observation, answer the questions below.

There are three main reagents in your tubes, DNA sample, qGRN Master Mix (which includes a qPCR dye) and Primers. Knowing this, and using your knowledge of DNA and PCR, state whether you think each reagent is important for explaining your initial observations*.

qGRN Master Mix:
(Important Y/N)

What leads you to think this?

Primers:
(Important Y/N)

What leads you to think this?

DNA samples:
(Important Y/N)

What leads you to think this?

*The fluorescent dye used in the experiment will only fluoresce if bound to double stranded DNA.

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Table 2 - qPCR observations

In the table below, record at what cycle you observed your reactions and your observations of each tube. Fill in the cycles in which you will observe your samples before beginning your reaction. You may choose how many observations to take, up to a maximum of seven.

Note: We recommend checking samples every 3-5 cycles. Your exact frequency may depend on your experimental concentration. Because you are using visual detection of fluorescence, more frequent viewing may not result in discernible differences in brightness and will unnecessarily lengthen the overall protocol. We recommend taking your first observation at cycle 10. The table provides room for eight observations. You may make fewer than seven observations if appropriate.

	qPCR observations (at extension temperature, 72˚C)			
Cycle #	**Tube 1 (Negative Control)**	**Tube 2 (Reference DNA)**	**Tube 3 (Experimental)**	**Tube 4 (Unknown)**
10				

Table 3 - Concentration calculations

Record the cycle at which you first observed fluorescence (approximate Ct).

Using the original R sample for reference, calculate the approximate initial concentration of your samples for both E and U samples based on their approximate Ct. Record this number in the column "Concentration based on observed fluorescence". It will be difficult to obtain precise concentrations*, but do your best. You should at least be able to tell which samples were more or less concentrated.

In the column "Concentration based on known values" record the concentration of tube E based on your initial calculations in the Experimental sample planning sheet (pages 4 and 5). After calculating your concentrations based on its approximate Ct, obtain your partner group's calculated concentration for the U sample and record it here.

Sample	Approximate Ct	Concentration based on observed fluorescence	Calculations	Concentration based on known values
Tube 1 (Negative Ctrl)				
Tube 2 (Reference DNA)		—	—	1X
Tube 3 (Experimental)				
Tube 4 (Unknown)				

Concentration calculations

*qPCR machines are able to make precise concentration calculations because they monitor the reaction every cycle and use precise fluorometers to measure very small differences in fluorescence. Using your eye and recording your observations every few cycles, you should expect to be able to easily order your samples in terms of relative (decreasing) concentration and calculate the starting concentration within an order of magnitude.

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D. Gel electrophoresis

We recommend running your products on an electrophoresis gel to compare end-point reactions with your qPCR results. However, this section can be skipped.

Gel electrophoresis – Pouring agarose gels (preparatory activity)

This preparatory activity may be carried out ahead of class by the instructor, or during class by the students.

Gels can be prepared up to three days ahead of time and stored at ambient temperature, covered in air-tight plastic wrap and protected from light.

1. Prepare a clean and dry agarose gel casting tray

- Seal off the ends of the tray as needed for your apparatus.
- Place a well-forming comb at the top of the gel (5 lanes or more).

2. For each lab group, prepare a 2% agarose gel using electrophoresis buffer

- For example, add 0.4 g of agarose to 20 ml of 1X TBE buffer (for blueGel™).
- Adjust volumes and weights according to the size of your gel tray.
- Mix reagents in glass flask or beaker and swirl to mix.

3. Heat the mixture using a microwave or hot plate

- Heat until agarose powder is dissolved and the solution becomes clear.
- Use caution, as the mix tends to bubble over the top and is very hot.

4. Let the agarose solution cool for about 1 minute at room temperature

- Swirl the flask intermittently.

5. Add gel staining dye (e.g. GelGreen™)

- Follow dye manufacturer instructions.
- e.g., 2 µl of 10,000X GelGreen™ per 20 ml agarose gel.

Note: We recommend the use of safe alternatives to ethidium bromide such as GelGreen™ (available at www.miniPCR.com).

6. Pour the agarose solution into the gel-casting tray with comb

7. Allow gel to completely solidify (until firm to the touch) and remove the comb

- Typically, ~10 minutes for blueGel™ gels

8. Place the gel into the electrophoresis chamber and cover it with run buffer

- Add just enough buffer to fill reservoirs on both ends of the gel and to just barely cover the gel.

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Gel electrophoresis – Running the gel

1. Make sure the gel is completely submerged in electrophoresis buffer

• Ensure that there are no air bubbles in the wells (shake the gel gently if bubbles need to be dislodged).
• Fill reservoirs at both ends of the electrophoresis chamber and add just enough buffer to cover the gel and wells.

2. Add loading dye to your sample

• Add 6 µl of 6X Loading Dye to each tube.
• Alternatively, mix 3 µl of Loading Dye with 15 µl of DNA sample on Parafilm or in a separate tube.
• Pipette up and down to mix.

3. Load DNA samples onto the gel in the following sequence

• Lane 1: 7 µl DNA Ladder
• Lane 2: 15 µl PCR product from tube 1
• Lane 3: 15 µl PCR product from tube 2
• Lane 4: 15 µl PCR product from tube 3
• Lane 5: 15 µl PCR product from tube 4

4. Place the cover on the gel electrophoresis box

5. Conduct electrophoresis for ~25 minutes, or until the colored dye has progressed to at least half the length of the gel

• Check that small bubbles are forming near the terminals in the box.
• Longer electrophoresis times will result in better size resolution.

Gel electrophoresis – Visualization

1. Turn on the blueGel™ blue light illuminator

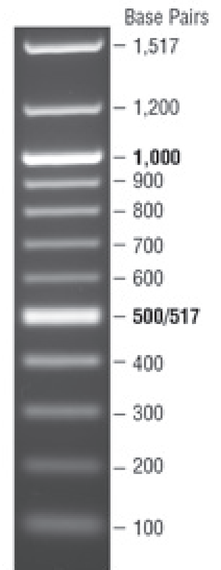
- Or place the gel on a transilluminator if not using blueGel™.

2. Ensure there is sufficient DNA band resolution in the 100-500 bp range of the 100 bp DNA Ladder

- Run the gel longer if needed to increase resolution.

3. Document the PCR amplified DNA fragments by comparing the PCR products to the molecular weight reference marker (100 bp DNA Ladder)

- Capture an image with a smartphone camera or other gel documentation system.



100 bp DNA Ladder visualized by ethidium bromide staining on a 1.3% TAE agarose gel. -Courtesy New England Biolabs

CER Table

Fill in the table based on your results from the lab. Use the rubric on the next page to help your answers.

Question:
Which original sample was more concentrated, the Experimental or Unknown?

Claim Make a clear statement that answers the above question	
Evidence Provide data from the lab that supports your claim	
Reasoning Explain clealry why the data you presented supports your claim. Include the underlying scientific principles that link your evidence to your claim	

Score	4	3	2	1
CLAIM A statement that answers the original question/ problem.	Makes a clear, accurate, and complete claim.	Makes an accurate and complete claim.	Makes an accurate but incomplete or vague claim.	Makes a claim that is inaccurate.
EVIDENCE Data from the experiment that supports the claim. Data must be relevant and sufficient to support the claim.	All of the evidence presented is highly relevant and clearly sufficient to support the claim.	Provides evidence that is relevant and sufficient to support the claim.	Provides relevant but insufficient evidence to support the claim. May include some non-relevant evidence.	Only provides evidence that does not support claim.
REASONING Explain why your evidence supports your claim. This must include scientific principles/ knowledge that you have about the topic to show why the data counts as evidence.	Provides reasoning that clearly links the evidence to the claim. Relevant scientific principles are well integrated in the reasoning.	Provides reasoning that links the evidence to the claim. Relevant scientific principles are discussed.	Provides reasoning that links the evidence to the claim, but does not include relevant scientific principles or uses them incorrectly.	Provides reasoning that does not link the evidence to the claim. Does not include relevant scientific principles or uses them incorrectly.

We recommend that teachers use the following scale when assessing this assignment using the rubric. Teachers should feel free to adjust this scale to their expectations.

Rubric score	3	4	5	6	7	8	9	10	11	12
Equivalent Grade	55	60	65	70	75	80	85	90	95	100

Study questions

Questions for before experimental set-up

1. How is qPCR different from an end-point PCR experiment?

2. Define Ct in your own words.

3. If a DNA sample, F, has a Ct value that is 3 cycles greater than another sample, Q, what is the concentration of your sequence of interest in sample F relative to sample Q?

Explain in words how you got your answer.

4. In what ways is this lab different from how qPCR is normally done in a lab?

Questions for after experiment

1. Did your Experimental tube (E) fluoresce when you expected it to?

2. If it did not, can you explain what sources of error could have led to it fluorescing at a different cycle than you had expected?

3. Did your calculated concentration in your unknown tube match what your partner group reported it should be?

4. If it did not, can you explain what sources of error could have led your calculated concentration to be different from the number they provided?

5. The following two samples were identical, but analyzed by different groups. What was the approximated Ct and calculated starting concentration for both samples?

U sample you tested:
Approximated Ct:
Calculated concentration:

E sample tested by partner group:
Approximated Ct:
Calculated concentration:

6. Do the numbers match? If they do not, can you explain what sources of error could have led your calculated concentration to be different from the number they provided?

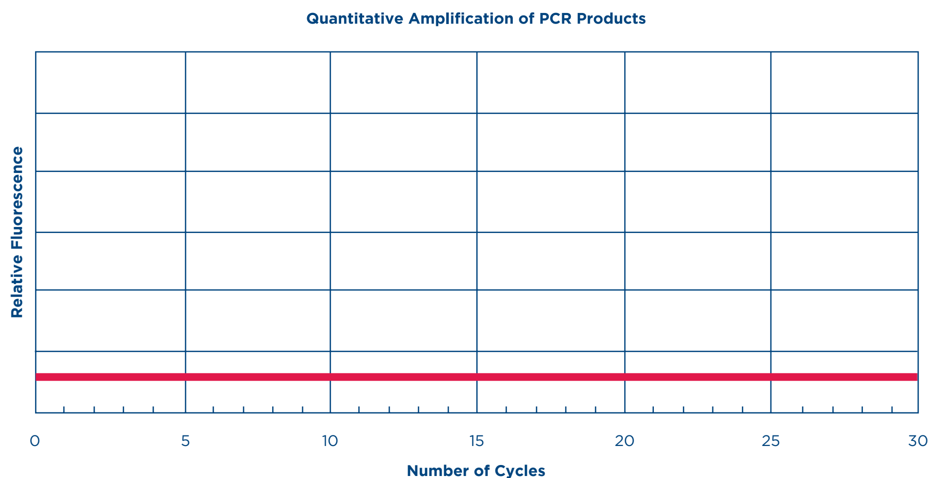
7. The following two samples were also identical, but analyzed by different groups. What was the approximated Ct and calculated starting concentration for both samples?

E sample you tested:
Approximated Ct:
Calculated concentration:

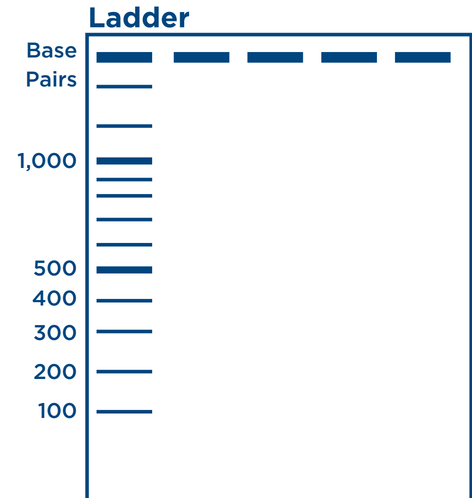
U sample tested by partner group:
Approximated Ct:
Calculated concentration:

8. Do the numbers match? If they do not, can you explain what sources of error could have led your calculated concentration to be different from the number they provided?

9. On the graph below, plot your results. You do not have exact numbers for fluorescence values, but do your best to approximate what you think the graph of your data should look like. Refer to page 2 of the introduction for example graphs and explanations.



Questions for after gel electrophoresis

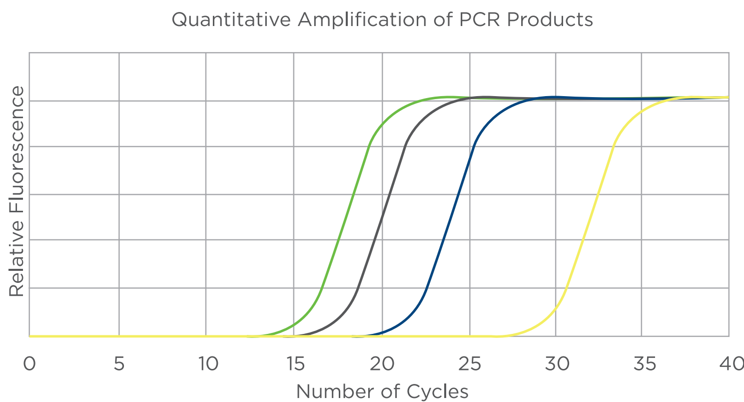


1. Use the image on the right to illustrate what your gel looks like.
Be sure to label each lane with the correct sample (N, R, E, U).

2. Compare the brightness of the bands on your gel to each other. From the gel only, can you determine if any of the samples started with greater or lesser concentration? Explain your answer.

3. If your answer to the previous question was yes, how specific can you be about those concentrations? Explain.

4. Look at the graph below showing qPCR results. Imagine that a PCR was done on these same four samples and that at cycle 25 you stopped the PCR and ran a gel of the four samples. Explain what the four lanes of your gel would look like. Defend your answer with evidence from the graph.



a. Lane 1

b. Lane 2

c. Lane 3

d. Lane 4

5. What information can you get from your gel that you could not get from observing your reaction in a tube?

Additional Student Supports and Extension Activities

Overview P.32

Instructor notes P.33

Student pre-lab worksheets P.35

- Understanding exponential growth P.35
- Calculating dilutions P.37

Overview

The activities in this section are provided as optional activities to do in conjunction with the P51 qPCR lab. These activities are not included in the student guide.

Pre-lab supports

The following optional supports are to help provide additional scaffolding for students who need it.

1. Understanding exponential growth in PCR - We recommend students performing this lab should be familiar with simple exponential equations. Many students have trouble understanding the effect that doubling every cycle has on growth. This is critical for correctly predicting Ct value in this lab. This activity will have students calculate the number of copies of DNA expected at all 30 cycles of a PCR. They will then try altering the starting values to explore how this affects predicted Ct values.

2. Calculating dilutions – This lab asks students to make specific dilutions of DNA to test how different concentrations affect Ct values. Some students struggle knowing how to calculate volumes and concentrations properly when making a dilution. This activity helps students gain confidence calculating dilutions by introducing them to the formula:

Concentration initial **x** Volume initial **=** Concentration final **x** Volume final

Students then practice calculating different volumes and concentrations.

Extension activities

Quantitative Polymerase Chain Reaction: P51™ qPCR lab gives students a hands-on introduction to qPCR. Read more about how qPCR works and its different uses in the laboratory. Link includes article and study questions.

https://dnadots.minipcr.com/dnadots/real-time-polymerase-chain-reaction

Additional Supports

Instructor notes

Understanding exponential growth in PCR

Being able to correctly predict when different reactions will fluoresce requires students to have an understanding of exponential growth.

In PCR, the amount of PCR product will double every PCR cycle. Having students create a table like below can be helpful and can be used as a reference later for when they need to predict the number of cycles needed to see different concentrations of DNA begin to fluoresce. This is expanded to an optional pre-lab worksheet on the following page.

PCR Cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Copies of DNA	2	4	8	16	32										

PCR Cycle	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Copies of DNA															

This table, of course, simply represents 2^n, where n is equal to the PCR cycle. Seeing this relationship written out can be illustrative for students who do not have a quick and firm grasp on exponents. Graphing this relationship can be additionally illustrative for students.

Calculating dilutions

-

Instructor notes

This lab asks students to calculate dilutions and to make such dilutions using micropipettes. This can be challenging for some students – especially those lacking confidence in or needing more support with math skills.

A handy formula to introduce students to is the following:

Concentration initial **x** Volume initial = Concentration final **x** Volume final

For students making dilutions in this lab, the concentration initial is the concentration of DNA supplied to them. The concentration final is the concentration they decide to dilute their sample to. Volume initial is the amount of original DNA sample added to the final volume. Volume final is the total volume of their final dilution including the added initial volume plus water. In this example, both concentrations will be known volumes. Students can either choose a final volume or initial volume and solve for the other algebraically.

For example: Initial concentration = 1x
Final concentration = .1x

If the student wanted to make a final volume of 20 µl, they would solve:

$$1 \times V = 0.1 \times 20\ \mu l$$
$$V = 2\ \mu l$$

The student would add 2 µl of their original DNA concentration to 18 µl H2O to make 20 µl of .1X concentrated DNA.

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Additional Supports

Student pre-lab worksheet no. 1

Understanding exponential growth in PCR

PCR takes a unique sequence of DNA and makes copies of it, doubling the amount of that specific copy every PCR cycle. To understand what this means, look at the table below.

Before filling in the table, make a prediction about how many DNA copies you will have by PCR cycle 30. A thousand? A hundred thousand? A million? More? This is just what you think, don't worry if you are right or wrong.

My prediction: ______________________________

Fill in the table. Under box six, write the number from cycle five, doubled. For cycle seven, take the number for cycle six and double that, and so on.

Cycle one starts with a 2, because, if you start with one (1) copy of DNA, after the first cycle that amount will double and there will be two (2) copies.

PCR Cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Copies of DNA	2	4	8	16	32										

PCR Cycle	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Copies of DNA															

1. By cycle 30, how many copies of DNA do you expect to have for every one of your target sequences you started with?

2. Assume that in a qPCR experiment you first observe your DNA at cycle 18. How many copies of DNA do you have for every original sequence you started with?

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Now imagine that you start with 1/4 the amount of DNA that you started with in the above example. Fill in the table below.

PCR Cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Copies of DNA	0.5														

PCR Cycle	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Copies of DNA															

3. If you started with 1/4 the amount of DNA, at which cycle would you expect the amount of DNA copies to be equal to the amount seen in cycle 18 in the first example?

Now imagine that you start with eight times the amount of DNA that you started with in the first example.

PCR Cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Copies of DNA															

PCR Cycle	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Copies of DNA															

4. If you started with about 8 times the amount of DNA, at which cycle would you expect the amount of DNA copies to be equal to the amount seen in cycle 18 in the first example?

Student pre-lab worksheet no. 2

Calculating dilutions

In this lab you will need to dilute DNA to a specific concentration. In this worksheet you will practice calculating dilutions.

A handy formula for making dilutions is as follows:

Concentration initial **x** Volume initial **=** Concentration final **x** Volume

Concentration initial:	The concentration of the sample you will be diluting. In this lab, that is the original DNA provided to you by your teacher.
Volume initial:	The amount of the original sample that you will be adding to the new tube to make your dilution.
Concentration final:	The concentration you are trying to achieve by making your dilution.
Volume final:	The total volume that you are going to make of your new dilution.

To figure out how much of your original sample to add to a new dilution, fill in as many of the numbers you know and solve for the remaining variables.

Example: Initial concentration = 1x
Final concentration = .1x

If you wanted to make a final volume of 20 µl:

Concentration initial:	1
Volume initial:	V
Concentration final:	.1
Volume final:	20 µl

$$1 \times V = 1 \times 20\ \mu l$$

$$V = \frac{0.1 \times 20\ \mu l}{1}$$

$$V = 2\ \mu l$$

1. If you wanted to make 200 µl of a solution that had a final concentration that was 1/20th the starting concentration, how much of your original solution would you use?

Concentration initial: ______

Volume initial: ______

Concentration final: ______

Volume final: ______

______ **x** ______ **=** ______ **x** ______

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Now instead of using relative concentrations, try using actual concentrations.

2. Your original solution has a concentration 80 ng/µl. If you wanted to make 50 µl of a solution that had final concentration that was 6ng/µl, how much of your original solution would you use?

Concentration initial: ____________ ______ x ______ = ______ x ______

Volume initial: ____________

Concentration final: ____________

Volume final: ____________

To make sure that you have the correct final volume you must add the correct initial volume of your solution and the correct volume of water.

Final Volume **=** Initial Volume **+** H_2O

3. For the problem #2, how much H_2O and how much of your solution should be added to make your final volume?

To make my final concentration I would add:

H_2O: ________ Original Solution: ________

4. You are adding 2 µl of solution that has a concentration of 36 ng/µl to 142 µl of water. What will the final concentration be?

Concentration initial: ____________

Volume initial: ____________

Concentration final: ____________

Volume final: ____________

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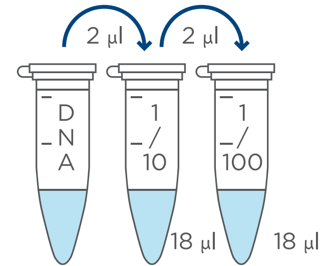
Making serial dilutions

The amount you can dilute a sample is constrained by the volumes you are able to work with. For example, it is very hard to make a 1000-fold dilution of a sample without using large volumes. To get around this problem, when working with larger dilutions, scientists often make a serial dilution. A serial dilution involves diluting your sample multiple times, consecutively.

Say you wanted to make a 100-fold dilution. You could add 1 part sample to 99 parts water, OR you could add 1 part sample to 9 parts water, and then dilute your dilution one more time.



When using a 20 µl pipette, diluting something 10-fold is much easier than 100-fold. If you add 2 µl of your original sample to 18 µl water, you will make 20 µl of 10-fold diluted sample. To get to 100-fold dilution you can repeat the process. Take 2 µl of your 10-fold diluted sample and add that to 18µl water. That will give you 20 µl 100-fold diluted DNA.

In a serial dilution, you will dilute your sample and then dilute your dilution, and possibly even dilute it again. Usually you do the same dilution factor each time, but you don't have to. Each time you dilute your sample, the factors multiply. For example, if you diluted your sample 4-fold and then diluted that new solution 10-fold, you would have a final 40-fold dilution, because 4 **x** 10 = 40.

5. Imagine you want to make a four tube dilution series; each tube is diluted 8-fold compared to the previous tube. What would the final concentration of each tube be?

Please complete

	Original sample	First Dilution	Second Dilution	Third Dilution	Fourth Dilution
Concentration	1X	$\frac{1}{8}$ X			
Volume added from previous tube	-	2	2	2	2
Water added	-	14	14	14	14
Total Volume	-	16	16	16	16

6. Now imagine that you want to make a sample that is 256-fold diluted, how would you go about creating your dilution series? Try to keep all volumes under 20µl.

Use only as many tubes as you would need

	Original sample	First Dilution	Second Dilution	Third Dilution	Fourth Dilution
Concentration	1X				
Volume added from previous tube	-				
Water added	-				
Total Volume	-				

7. Now imagine that you want to make a sample that is 4000-fold diluted, how would you go about creating your dilution series? Try to keep all volumes under 20µl.

Use only as many tubes as you would need

	Original sample	First Dilution	Second Dilution	Third Dilution	Fourth Dilution
Concentration	1X				
Volume added from previous tube	-				
Water added	-				
Total Volume	-				

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