

Real-time PCR machine

BE/EE/MedE 189 a

1 Background

Polymerase Chain Reaction (PCR) is a ubiquitous technique in molecular biology to boost the copy number of DNA strands in vitro. Imagine you have a few strands of DNA in a solution. You may wish to sequence the DNA, further characterize it, or introduce it into another organism. In any case, you need more copies of the molecule. Cells have molecular machines called DNA polymerases that make new copies of DNA strands, provided they are supplied with the nucleotide building blocks. PCR uses these biological inventions to repeatedly copy, or amplify, the original template DNA.

The basic idea of PCR is shown in Fig. 1. The template strand is present as double stranded DNA. The solution is heated and the template strand denatures, splitting into two single strands. The reaction mixture is cooled enough so that short segments of single stranded DNA called oligonucleotide primers can bind to the separated single strands. These primers have the correct sequence complementarity to bind to the appropriate regions of the denatured template DNA. Next, when held at the elongation temperature, the DNA polymerase adds nucleotides to build a new double-stranded copy of the template strand. This constitutes one cycle of PCR. When the cycle repeats, all double strands denature, have primers bind, and get nucleotides added. In this way, each cycle results in (ideally) a doubling of the copy number of the original double stranded DNA.

Since its invention in the early 1980s, PCR has been used extensively and new variations have been developed. Because PCR can be seen as the amplification of a weak signal (the small copy number of the template DNA), it can be used to quantify the amount of double stranded DNA originally present in the solution. The amount of DNA present in solution is monitored for each PCR cycle, resulting in an amplification curve. Based on this curve, the original amount of starting material can be inferred. The amount of double stranded DNA present is typically monitored using fluorescent probes. The technique gets its name because the progress of the amplification is monitored for each cycle (in real time), and is also called quantitative PCR (qPCR) because it is used to quantify amounts of DNA.

Polymerase chain reaction - PCR

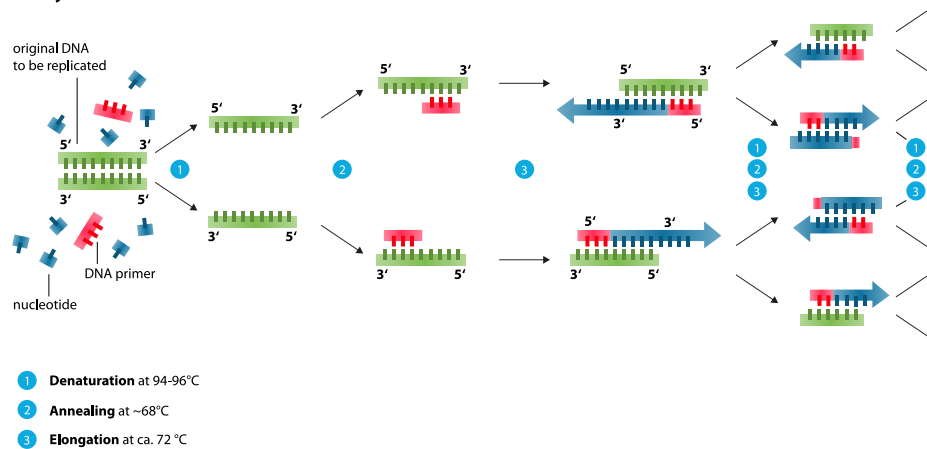


Figure 1: Schematic of PCR. Copyright Enzoklop, licensed under [CC-BY-SA-3.0](#)

2 Specification

Your task is to construct a real-time PRC machine. The machine consists of a sample holder that can hold a standard PCR tube in thermal contact with a heat source/sink to enable thermal cycling. The fluorescence intensity of a reporter fluorophore is measured in real time by an optical system. You do not need to worry about mixing the reaction solution; thermal diffusion should be sufficient.

Your LabVIEW interface should also provide/allow for the following.

- Storing of data for later processing, including specification of output file names.
- Interface for setting rt-PCR parameters. These include
 - Temperature and time for initial denaturation of template DNA;
 - Number of cycles;
 - Cycling parameters: denaturation, annealing, and extension temperatures and hold times;
 - Final cool down and hold temperature;
 - Tolerances for temperatures.
- Display of temperature, time, cycle count, and fluorescence intensity, in real time.
- Ability to stop, start, and pause the reaction with automatic shutdown of the heating element when appropriate.

Generally, whenever you shut down the device, the heat and light sources should be automatically powered down (this is also for safety reasons).

You are advised to first establish the thermal cycling functionality and then build out the optical components of the device.

2.1 Heating and cooling using a thermal electric cooler (TEC)

A thermoelectric cooler (TEC) is the central part in the thermal cycling subsystem. A TEC uses the Peltier effect to work as a solid state heat pump. It ideally absorbs heat through one side (thus making that side cool) and releases it on the other side (thus making that side hot), creating a temperature differential. The direction it pumps heat is dependent on the direction of the current flowing through it.

For purely heating applications, a 12 V DC power supply is used to drive the TEC. Use thick wires for this 12 V power, and separate all thick wires from the ELVIS II board. The TEC can be turned on and off using a 5 V signal through the use of a relay. In Fig. 2, we show a diagram for the TEC subsystem.

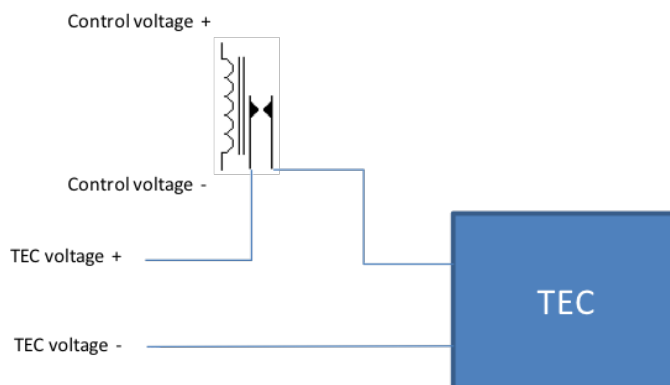


Figure 2: Schematic of TEC heating and cooling system.

When the heating subsystem is turned off, the TEC will cool down to room temperature naturally, but far more slowly than we need for a PCR reaction. We can cool the system more rapidly by drawing heat away from the surface using a heatsink and fan. However, even this process can be too slow for a PCR application. A more effective heating and cooling system must incorporate heat sinks and sources creatively and utilize the fact that the TEC can both heat and cool based on the direction of the current flow.

Picture a TEC that is not directly connected to a fan or heat sink. As usual, when current flows through the device, the TEC creates a temperature differential. At some point, there is no more heat (locally) to absorb from the cold side, and the hot

side (locally) cannot transfer the heat fast enough to the air. Eventually, the device can overheat and fail.

Now imagine that a TEC has a heat sink attached to both sides. Imagine that each sink has a fan that aids in convective heat transfer between the heat sink and the surrounding air. Fig. 3 is a visual example of this. We will refer to the bottom side of the TEC as side A, and the top side as side B. Imagine current is flowing in one direction through the TEC, which pumps heat from side A to side B. The heat sink on side B will act like a true sink and draw the heat away from side B and dump it into the air. This prevents side B from overheating and keeps the temperature gradient across the TEC stable. When the current direction is reversed, this whole process happens again, only in the opposite direction. This time, heat is pumped from side B to side A and the heat sink on side A draws the heat away from the TEC.

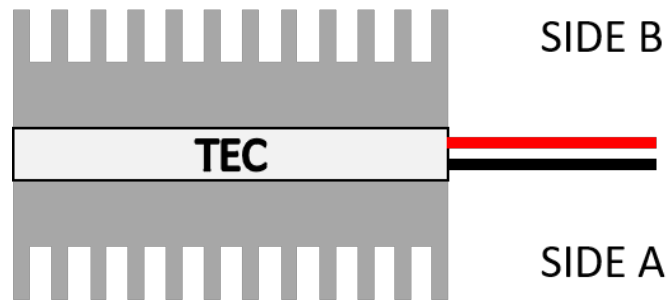


Figure 3: Schematic of TEC with heat sinks on both sides.

Now imagine this scenario for the purposes of setting up a rt-PCR device. Instead of a heat sink on side B, we have a sample holder on that side, as shown in Fig. 4. When current flows in one direction through the TEC, the sample holder draws away heat from side B. Since the sample holder does not transfer the heat to the air all that well, the heat is directed towards the sample and is heated. When current flows in the opposite direction through the TEC, the sample holder is now supplying side B with heat to pump across to side A. Heat is drawn from the sample, thereby cooling it down.

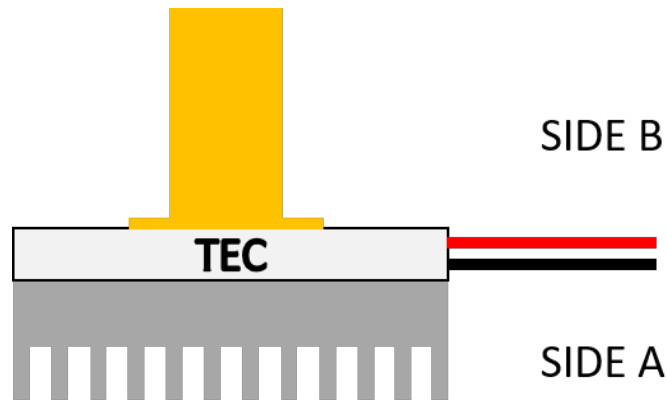


Figure 4: Schematic of TEC with heat sink on one side and a sample holder on the other.

You will receive a fan/heatsink cooler, a TEC, and sample holder. You will be given thermal paste to bond the TEC to the cooler, and then the TEC to the sample holder. The fan/heatsink cooler must **always** be powered on when the TEC is in use, lest it overheat and be destroyed. (It is best practice to just always leave the fan on; there is no reason to control it.) Refer to the 4 pin pinout in Fig. 5. To supply the 12 V for the fan, use the variable power supply function on the ELVIS II instrument launcher.

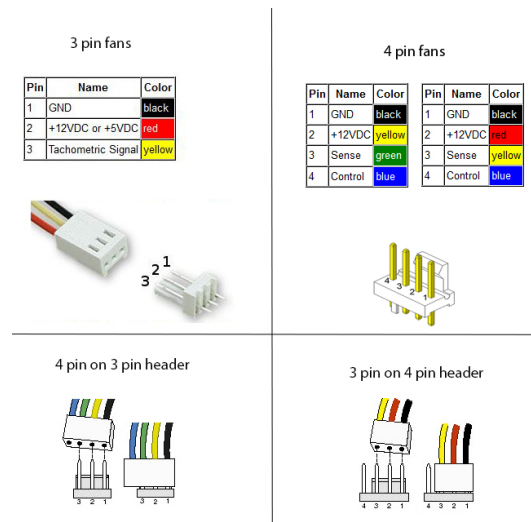


Figure 5: Pinout diagram for three and four pin fans.

There are several ways you can control the current going through the TEC. One example given below in Fig. 6 is an H-bridge circuit that has been implemented using four relay modules. (We provide you with Omron G5LE PCB relays.) Use a separate power supply to power the TEC with 12 V. The relay module can be powered directly

from the ELVIS II board 5 V and ground line, and the input signals on the relay can be supplied by the DIO pins. *Important note:* The Omron G5LE PCB relays are active low. This means that sending a `True` to your DAQ assistant will not trigger the relay; the switch will stay toward the NC side, as in the given circuit diagram (indicated by the corresponding LED not lighting up). Sending a `False` will trigger the relay, (switch changes sides/ LED lights up). If you trigger all the relays (send 4 `False`s), you may cause a short circuit that can burn wires. On the other hand, sending `True` to IN1-IN4 will cause no current to flow through the relay module.

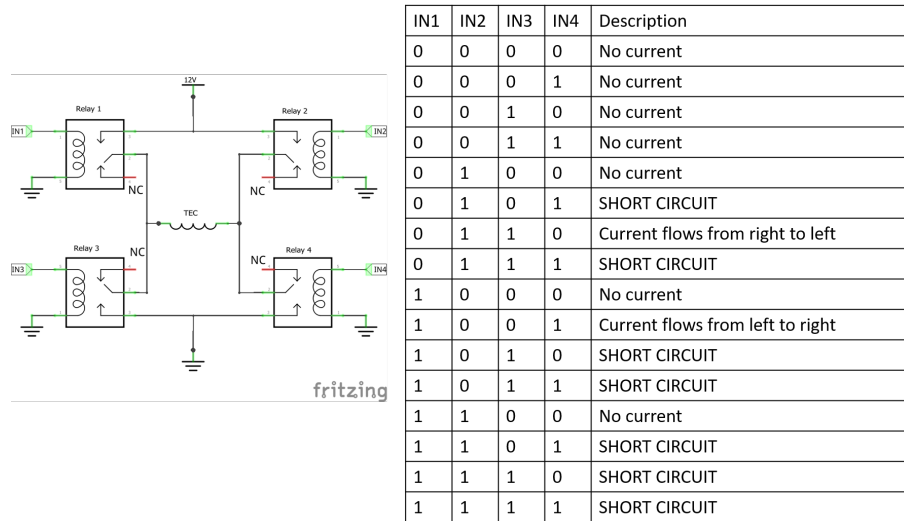


Figure 6: Example H-bridge setup using a relay module.

Your system should be able to heat and cool at a rate of one to two degrees Celsius per second. You should be care not to switch the TEC on and off too often, as this can damage it.

2.2 Temperature sensing with the LM35 thermistor

Use the LM35 temperature sensor for measuring the temperature. Refer to its data sheet for detailed information. You are advised to attach the LM35 to the surface of the sample holder for temperature measurement (you cannot put it in the sample). That said, you will need to calibrate the voltage readings from the LM35 attached to the sample holder with the actual temperature values in the PCR mix measured with a probe thermometer. You will likely need to develop a calibration curve and implement it in your LabVIEW VIs to accurately measure the temperature.

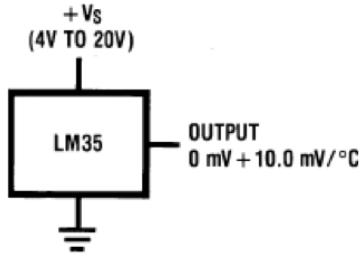


Figure 7: Diagram for the LM35 thermistor.

To set up the thermistor, you can use the basic diagram as shown Fig. 7. The first pin can be connected to either 5 or 15 V for power (in fact, it can flexibly take 4 V to 30 V as input.) Pin 3 should be connected to ground. Pin 2 provide a voltage output that scales as 0.01 V/°C.

2.3 Temperature control

You may choose any means of temperature control you like. However, for a first prototype, on-off (a.k.a. bang-bang) is a good choice. That is, you control the temperature only by turning the TEC on and off. More sophisticated control strategies, such as PID controllers, are possible, but may be better implemented in later generation prototypes.

2.4 Thermal cycling protocol

While the time and temperature of the thermal cycling protocol need to be adjustable in the front panel of your LabVIEW VI, the protocol below is suggested for the provided polymerase and should be the defaults.

Temperature (°C)	Time	Action
94	2 min	Initial denaturation of DNA
94	30 sec	Denaturation
52	30 sec	Annealing of probe to DNA template strand
72	1 min	Extension
Repeat preceding 3 steps for specified number of cycles		
10	∞	Final cool down and hold until user stops program

2.5 Optical detection

The fluorophore you will use is excited by blue light and emits green. You therefore need to provide blue light for excitation and then filter the emission to detect only green light. Be sure to shield the sample from light except when you are acquiring images because the fluorophores will photobleach. The optical setup is shown in Fig. 8. The optical components are provided for you.

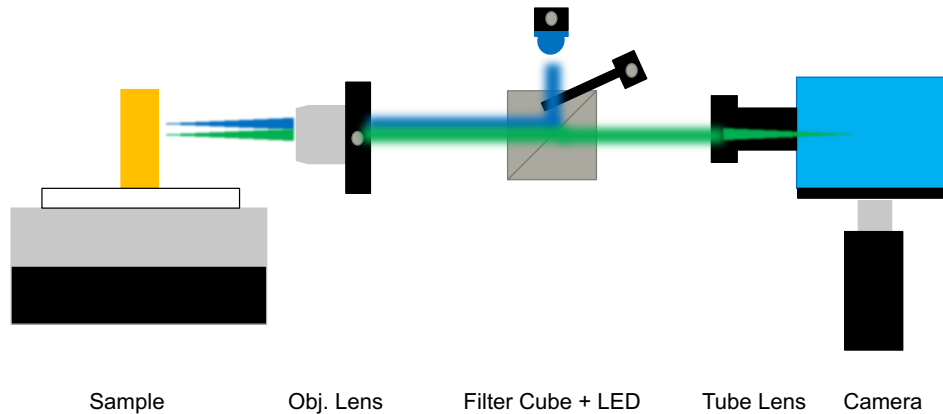


Figure 8: Setup for the optical detection system.

Tips

- Images from the camera are most easily read through LabVIEW's Matlab interface. To initialize, access, and change the camera's settings, type `imaqtool` in the Matlab window, and copy the lines of code displayed in `Session Log` according to your desired settings.
- To capture images from the camera, type `var = getsnapshot(vid)` in the Matlab Script VI after initialization of the camera, where `var` is the name of the variable to which you assign the captured image.
- To focus the camera on the tube, visible through the hole in the copper holder, you can place a small piece of paper with printed letters in the copper holder, shine a flashlight on the paper, and adjust the objective and camera positions so that you can read the letters on your LabVIEW display.
- You can use provided black tarps and/or cardboard boxes, or anything else you think might be useful, to secure light isolation. The lab will have designated "lights out" hours while you are working on this project so you can acquire data.

2.6 System test

To test your system, a tube of complete reaction mix (containing template DNA, DNA primers, polymerase, dNTPs, SYBR Green, etc.) will be provided. You will use primers from a the BioRad GMO investigator kit. The kit is designed to test the 35S promoter of the cauliflower mosaic virus and the terminator of the nopaline synthase gene of *Agrobacterium tumefaciens*. These sequences are present in most genetically modified crops.

Obtain an amplification plot for this system and determine the initial quantity of template DNA in your sample. While the fluorescence versus cycle number curve should be displayed in real time on your LabVIEW VI, you may output the acquired data as a text file and do subsequent analysis using your preferred software.

3 Prelab questions

1. Two of the more common probes are TaqMan and SYBR Green. The understanding of the working principles of these two probes will equip you with understanding of other qPCR probes available, should you be interested to find out more. A TaqMan probe is an example of a sequence specific probe. It is a short strand of DNA that has a fluorophore conjugated to its 5' end and a fluorescence quencher on its 3' end. Thus, in its native state, the probe does not fluoresce under the excitation light (the quencher quenches the fluorophore). These probes are usually specifically designed to be complementary to a DNA sequence in the internal region of the template DNA. DNA polymerase, the enzyme used to extend a growing DNA strand, has 5'-nuclease activity. This means that when the DNA polymerase arrives at the internal region of the target template, as it grows the DNA strand, it cleaves the TaqMan probe and allows the fluorophore to be separated from its quencher. Fluorescence, which is related to the amount of DNA copies made, thus accumulates as the PCR reaction proceeds.

The principle of SYBR Green is relatively simple. SYBR Green simply binds indiscriminately to double-stranded DNA. SYBR Green fluoresces quite minimally in the unbound state. Once bound to a dsDNA strand, its fluorescence dramatically increases (up to 1000 fold).

What are the advantages and disadvantages of these two qPCR probes?

2. Sketch a typical amplification curve. What region of this curve is most useful for quantifying how much of the template strand was originally present?
3. We said in the more detailed specification that you should not be continuously

monitoring the signal of the fluorophore because of photobleaching. What is photobleaching? At what part of the thermal cycling should signal acquisition be done?

4. Remember the importance of sketching your user interface on paper. Provide a sketch of the front panel for the device. This need not be the final interface, but your first pass on how it will be laid out and what features it will have.
5. The objective lens you will use is infinity corrected. What does this mean, and why is it useful?
6. The tube lens is a plano-convex lens, which means that one side of the lens is curved (convex) and the other is flat (planar). Should the flat surface face the objective lens or the camera? Why? *Hint*: Think about which orientation is useful for focusing light to a point.
7. In setting up your optics, is it better to focus the tube lens first or last? Why?
8. If you have limited experience with PCR and fluorescence, you should read up on those concepts. The [Wikipedia page on PCR](#) is quite good. ThermoFisher has a [nice tutorial on fluorescence](#) that is worth reading. You do not need to submit an answer for this prelab question, but please make sure you are up to speed on these concepts.

4 Postlab questions

1. What is the focal length of the tube lens? How did you find that out (without referring to its part number)?
2. Provide a calibration of the magnification of the microscope system in the setup. Be sure to discuss how you did this.
3. “On-chip” rt-PCR devices use microfluidics to perform the PCR reactions with low volumes. What are some advantages of an on-chip rt-PCR device over the kind of device you have built?
4. Without consulting literature, provide a concise block diagram (a complete blueprint is not required) of what an on-chip rt-PCR device would look like. You want a device that can start with an intact tissue or cell culture sample. The goal is detection and quantification of a specific DNA sequence. You should have fun with this question; do not sweat the details too much.
5. Look at some recent papers about on-chip PCR. What features does the current state of the art share with your imaginary design (provide citations)?
6. You do not have to answer this question directly; it is a friendly reminder of a few things to be sure to include in your report.

- You should check your amplification curve you got in your system test and use it to infer, up to a multiplicative constant, the copy number of template in your original reaction mixture. Include descriptions of your calculation and any code you used to make it beyond your LabVIEW VIs.
- Comment on the amplification curve, including possible sources of noise and design inadequacies, and what you can do to mitigate those issues.
- Comment on how you determine the background signal and how you mitigated it and corrected for what you could not eliminate. How did you go about maximizing your signal-to-noise ratio?