# Quantitative PCR (qPCR)

## Introduction

We will amplify the DNA using real-time, or quantitative, PCR. Rather than performing 25-35 cycles of PCR and then analyzing the products at the end, qPCR monitors the production of PCR products in real time. We will be using iTaq Universal SYBR Green Supermix, which contains:

Recombinant *Taq* DNA polymerase
anti-*Taq* DNA polymerase antibody
Mg ++
Nucleotides (dNTPs)
SYBR Green dye

Anti-*Taq* DNA polymerase antibody inhibits polymerase activity. Due to specific binding of the antibody, the Taq DNA polymerase is inactive and is reactivated after incubation at 94°C (called a “hot start”). Antibody-mediated hot starts improve PCR specificity and yield.

Normalization to an Internal Control

In this protocol, we will be looking at the abundance of the RFP gene. Although we will attempt to load the same amount of DNA into each tube, small variations in the amount of template DNA can lead to large changes in the amount of light released. To accurately quantify the DNA, you must use an internal standard. In this case, we will use a second gene that should be present in all of our RNA samples, the ribosomal RNA, 16S (rrsD) gene.

Controls for Contamination
When copying and amplifying nucleic acids, small amounts of contaminants can have a profound effect. To ensure that we do not have any contamination, we include a negative control sample. This sample contains water instead of template DNA and therefore should not produce any PCR product if our experiment is free from contamination.

Due to the controls for normalization and for contamination, you will have four reactions:

1) Template: DNA  and Primers: RFP (gene of interest)
2) Template: Water and Primers: RFP (gene of interest)
3) Template: DNA and Primers: rrsD (internal reference)
4) Template: Water and Primers: rrsD (internal reference)

## Materials

- iTaq qPCR SuperMix
- Primers
  - RFP Primer 1 at 10 uM
  - RFP Primer 2 at 10 uM
  - rrsD Primer 1 at 10 uM
  - rrsD Primer 2 at 10 uM
- template cDNA
- sterilized water

› 96 well plate with clear optical sticker cover

# Procedure

## Prepare primers

1. Dilute primers from 100uM to 10uM

## qPCR procedure

2. For this protocol, we want to start with 100 ng of DNA. Measure the concentration of your DNA using the NanoDrop. Calculate the volume of DNA that corresponds to 100 ng using the formula C1V1= C2V2

   (Concentration of cDNA determined by NanoDrop) (Volume needed) = (100 ng)(1 uL)
   Volume needed = _____ uL

3. Place 96 PCR well plate (or four small 0.2 ml optical PCR tubes) on ice (these are specialized tubes that allow the light produced to be accurately measured).

4. Add the following components to each reaction vessel. Add the template DNA last.

| | |
|---|---|
| iTaq qPCR SuperMix | 10 ul |
| 10 uM Primer 1 (500 nM final concentration) | 1 ul |
| 10 uM Primer 2 (500 nM final concentration) | 1 ul |
| Template DNA or water | _____ ul |
| Water (Add volume to bring total to 20 µl) | _____ ul |

5. Cap reaction vessels, vortex briefly to ensure that all components are mixed. Spin briefly to collect all liquid in the bottom of the tube and then load in qPCR machine.

6. Choose the following settings: 7500, SYBR Green, Comparative, Standard. Fill out the well plate key with targets (RFP and rrsD) and samples (names of promoters)

7. The program for the qPCR begins with one cycle at 95°C for 30 sec to completely denature the template and activate the enzyme. It then performs 35 cycles of PCR (you can use 35-40 cycles) as follows:

   Denature: 95°C for 15 seconds
   Anneal and Extend: 60°C for 30 seconds

8. Finally, the program provides a melt curve analysis by incrementally increasing the temperature from 60-95C. The temperature at which the primer and template dissociate is calculated and is used to verify the specificity of the annealing.