Setting up a qPCR Primer Standard Curve Assay
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Objectives:

• Overview of qPCR primer efficiency and standard curve analysis
• Assess the doubling efficiency of 36 primers sets for 18 genes selected for follow up analysis from the chicken retina/cornea RNA-Seq data set
• KEGG Pathway Analysis of selected candidate genes

I. Standard Curve analysis of qPCR primer efficiency

Prior to break you each selected a single differentially expressed gene from the RNA-Seq data in the phototransduction or notch signaling pathway for follow up gene-specific analysis. For each gene selected you designed 2 sets of forward & reverse primers. In order to conduct an optimal quantitative PCR (qPCR) experiment next week, we will 1st measure the doubling efficiency of each primer set.

A. What is a primer’s doubling efficiency?

Unlike conventional PCR where we only care about the end product, qPCR gives us a readout of the amount of starting material (i.e. cDNA) from one sample to another by measuring how much PCR product has accumulated after each cycle. This quantitative readout is absolutely depends on the assumption that the PCR product is doubling every cycle. If this assumption holds true, then samples with more starting material will reach a threshold amount of PCR product in fewer cycles than a sample with less starting material.

This is illustrated in the chart to the left where sample 1 contains more cDNA copies for the gene being assayed than sample #2. This results in accumulation of x amount of PCR product in less cycles of replication in sample #1 relative to sample #2. The cycle in which the threshold of PCR product is achieved is referred to as the Quantification cycle (Cq) or sometimes the Crossing Threshold (Ct). We will use the Cq naming convention in this lab.
PCR product is quantified after each cycle by virtue of a fluorescent double stranded DNA binding molecule called SYBR Green. At the conclusion of each cycle, PCR reactions are pulsed with fluorescent light and SYBR Green emission is measured by an imager in the lid of the cycler.

The qPCR method only works if the primers we designed have perfect or near perfect doubling efficiency (i.e. the PCR product doubles every cycle). We will assay each primer set individually by amplifying a set of 10 fold dilutions of E8 or E18 chicken retina cDNAs. Since we know the input amount of cDNA for the set of samples, we should generate a standard curve if there’s perfect doubling that can be plotted using the equation of the line.

**B. Standard Curve PCR Set Up** (*clean bench; wear gloves; pre PCR pipettes; filter tips*)

As a class we’ve designed 36 primer sets to analyze the expression of 18 genes. We will set **10 uL PCR reactions** and run 4 cDNA dilutions in duplicate for each primer set. Before beginning your set up,
refer to the primer [GoogleSheet](tinyurl.com/jxz8nmj) to determine which primers and cDNA you are using for your experiment. Please note that I changed genes for 7 of you for various reasons. You will use cDNA from the sample that had higher expression of your gene of interest for your standard curve experiment. **If your gene is up regulated in E18 retina then you will use the E18 cDNA serial dilutions for your experiment. If your gene is down regulated in E18 retina then you will use the E8 cDNA serial dilutions for your experiment.**

**SYBR Green PCR master mixes (mix and store on ice)**

Each student will set up 2 PCR master mixes, 1 for each primer set. Each mix will be enough for 4 cDNA dilutions in duplicate (i.e. 8 samples). Therefore we will calculate our mixes for 10 samples. Add H2O, 2X SYBR mix and premixed F & R primers to the master mix using this recipe (2X SYBR Green reaction mix contains DNA polymerase, pol buffer, dNTPs, and SYBR Green protein):

<table>
<thead>
<tr>
<th>per sample</th>
<th>master mix</th>
<th>X 10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>dH2O</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>2X SYBR mix</td>
<td>50</td>
</tr>
<tr>
<td>0.5</td>
<td>F/R primer (premixed; 10 μM)</td>
<td>5</td>
</tr>
</tbody>
</table>

*careful pipetting is critical!*

PCR reactions will be set up in 96 well plates with each primer set taking up 1 row of 8. Plates have 12 rows of 8 meaning that each student will use 2 rows to assay their 2 primer sets. Each table will set up a plate of their own (6 students * 2 primers each = 12 rows of 8). Label your plate/rows with each individual's initials and primer info. Keep your plate on a cold block and add 8 μL of master mix to each row. The loading orientation of the plate absolutely matters so please use the following loading convention keeping track in your notebook of who loaded what in which row:

**Example: Plate #1 (groups 1-2)**

- label your rows with initial & primer info
- Start at the bottom of the plate (A1) and work up to top of plate (A12)
- Add 8 μL of each primer master mix to each row of 8 with regular pipette
- Add 2 μL of E8 or E18 retina cDNA dilution series to each row using multichannel pipette
- Extreme pipetting accuracy is required!
- Keep plates on cold plate until ready to cycle
- Seal and spin down plate in centrifuge prior to loading
I have prepped cDNA dilutions series for E8 retina and E18 retina in PCR strip tubes. Add 2 µL of each of the 8 sample to each row of your plate using a multichannel pipette. This will help to improve the accuracy of the pipetting. Extreme accuracy is required for this experiment:

<table>
<thead>
<tr>
<th>Tube #</th>
<th>cDNA</th>
<th>Dilution</th>
<th>Or</th>
<th>Tube #</th>
<th>cDNA</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E8 retina</td>
<td>1:10</td>
<td>1</td>
<td>E8 retina</td>
<td>1:10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>E8 retina</td>
<td>1:100</td>
<td>2</td>
<td>E18 retina</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>E8 retina</td>
<td>1:1000</td>
<td>3</td>
<td>E18 retina</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>E8 retina</td>
<td>1:10K</td>
<td>4</td>
<td>E18 retina</td>
<td>1:10K</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>E8 retina</td>
<td>1:10</td>
<td>5</td>
<td>E18 retina</td>
<td>1:10</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>E8 retina</td>
<td>1:100</td>
<td>6</td>
<td>E18 retina</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>E8 retina</td>
<td>1:1000</td>
<td>7</td>
<td>E18 retina</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>E8 retina</td>
<td>1:10K</td>
<td>8</td>
<td>E18 retina</td>
<td>1:10K</td>
<td></td>
</tr>
</tbody>
</table>

- Label your designated rows with gene name
- pipette 8 µL PCR master mix into each of 8 wells for each primer
- With multichannel pipette, add 2 µL of each cDNA sample to each rows
- Change tips after each loading and use careful pipetting.
- Give the plate to the rest of your table mates to load.

Once the plate is fully loaded:

- cover plate with an adhesive plate seal making sure all wells are airtight
- spin down plate in swinging bucket plate holder rotor centrifuge
- load plates into Bio-Rad CFX96 thermal cycler and run “2-step 60C program”

When the run is finished (~1 hour 10 min), I will save the data to a jump drive and store the amplified cDNA plate at -20°C. We will analyze the qPCR standard curve data next week.

FYI, the “2-step 60C” PCR program will run the following thermal cycle:
1. 95°C for 30 sec (initial denaturation and heat activation of taq polymerase)
2. 95°C for 5 sec (denaturation)
3. 60°C for 30 sec (primer annealing and pol extension)
4. steps #2-3 39X more times
5. 60°C - 95°C in 0.5°C increments (melt curve)

After setting up your PCRs do some research on your gene and figure out where your gene orthologs lies in the human Notch or phototransduction pathway. Use the KEGG pathway analysis websites for phototransduction (tinyurl.com/zxfhefu) and notch signaling pathway (tinyurl.com/hxflh43) for this analysis.