REAL TIME PCR (QUANTITATIVE PCR OR QPCR) ANALYSIS (BASIC VERSION)

Purpose: To determine **quantitatively** prevalent levels of messenger RNA of the gene of interest in various organs.

Reference:

- BioRad - The iCycler iQ Real-Time Detection System Resource Guide.
- BioRad Technote 2567 - Real-Time PCR Using the iCycler iQ Detection System and Intercalation Dyes.
- BioRad Technote 2593 - Real-Time PCR: General Considerations.
- BioRad - Designing Primers/Probes for Real-Time PCR.

Recommendations for Optimal Results by Bio-Rad:

Due to the **sensitivity** of **quantitative PCR**, results can be easily affected by pipetting errors. Therefore, **follow the following practices**:

- Always prepare a master mix of iQ SYBR Green Supermix containing the primers and probe.
- Add the template DNA solution to aliquots of the master mix for optimal reproducibility of replicate samples.
- Set up at least two duplicates for each DNA solution (Recommended triplicates).

Note:

1. Different **Taq DNA polymerases require** different length of **HOT START period** (or denaturation step at 95 ºC) to **be activated**. **Improper activation** of Taq DNA polymerase WILL affect QPCR results. For example, iTaq in the BioRad SYBR green supermix is activated after 3 minutes.
2. In this version**, qPCR reaction volume** is **50 µL.** This volume is suggested when this is your first time to perform qPCR analysis because accuracy of pipetting is crucial. Once you are confident with your pipetting technique, you can set up **25-µL** reactions so you can perform triplicate reactions instead of replicate ones.
3. It is recommended to use a **96-well plate**, instead of individual 0.2-mL PCR tubes, to perform qPCR because of the convenience.

Materials and Reagents Needed:

- 12 μM Gene-of-interest Fw primer solution
- 12 μM Gene-of-interest Rv primer solution
- 12 μM Control (PcL1L) Fw primer solution (used in standard reactions)
- 12 μM Control (PcL1L) Rv primer solution (used in standard reactions)
- 10-fold serial Dilutions of plasmid DNA of pPcL1L starting at 1 ng/μL (such as,1 ng/μL, 0.1 ng/μL, 0.01 ng/μL, 0.001 ng/μL, 0.0001 ng/μL, 0.00001 ng/μL, 0.000001 ng/μL)
- Sterile water
- Ice bucket and ice
- 2X SYBR Green Supermix (BioRad, cat.# 170-8882) is composed of
  100 mM KCl,
  40 mM Tris-HCl, pH 8.4
  0.4 mM of each dNTP (dATP, dCTP, dGTP, dTTP)
  iTaq DNA polymerase (50 Units/mL)
  6 mM MgCl2
  SYBR Green I (DNA intercalator is >25X more sensitive than ethidium bromide),
  20 nM Fluoresein,
  Stabilizers
- Aerosol-barrier (PCR) pipet tips
- A pipetman set (P-10, P-20, P-100, P-200, P-1000)
- Vortex mixer
- **Optical** 96-well PCR plates or 0.2-mL PCR tubes in strips of 8 with individual caps
- 1.5 mL sterile microcentrifuge tubes
- Rack for 1.5 mL sterile microcentrifuge tubes
- PCR rack for Optical 96-well plates or 0.2-mL PCR tubes
- Real-Time PCR detection system (iCycler)

PROCEDURE

1. Get ice from the icemaker.
2. Thaw out 12-μM primer solutions and 2X SYBR Green Supermix (BioRad). Keep the thawed primer solutions and 2X SYBR supermix **on ice**.
3. Prepare solution mixes for Standard curve (See the **Table 1** below).
   Note: For **each dilution** of **DNA template** (for example, 1 ng/μL), we **need** to have a **solution mix** for **2 reactions**. Therefore, we **prepare** a **tube of solution mix** (example, Mix A) for **2.5 reactions** (including **extra)**.
   a. Label 1.5-mL microcentrifuge tubes **A** to **H** and put them on a microfuge rack.
   b. Pipet **122.5 μL** of **Std mix** (see **Table 3**) to each tube (A to H).
   c. Add **2.5 μL** of **serial diluted DNA** solutions or sterile **water** into appropriate tubes. Vortex for 5 seconds to mix the contents.
   d. Spin tubes in a microcentrifuge for 10 seconds. Put tubes on ice.

Table 1: Preparation of solution mixes for standard curve

Solution Mix		**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Components	**One** Reaction	**1 ng/μL**	**0.1 ng/μL**	**0.01 ng/μL**	**0.001 ng/μL**	**0.0001 ng/μL**	**0.00001 ng/μL**	**0.000001 ng/μL**	**No DNA**
pPcL1L DNA	1 μL	2.5 μL	2.5 μL	2.5 μL	2.5 μL	2.5 μL	2.5 μL	2.5 μL	*
Sterile water	22 μL	**122.5** μL **Std Mix**	**122.5** μL **Std Mix**	**122.5** μL **Std Mix**	**122.5** μL **Std Mix**	**122.5** μL **Std Mix**	**122.5** μL **Std Mix**	**122.5** μL **Std Mix**	**122.5** μL **Std Mix**
12 μM **PcL1L-Fw** primer	1 μL								
12 μ**M PcL1L-Rv** primer	1 μL								
2X SYBR supermix	25 μL								
Total Volume	**50 μL**								

*: Add **2.5 μL** of sterile **water. Standard (Std) mix** is prepared in **Table 3** below

4. Prepare Experimental reactions (see the example in **Table 2** below).
   a. Label 1.5-mL microcentrifuge tubes **1** to **5** and put them on a microfuge rack.
   b. Pipet **122.5 µL** of **Exp'tal mix** (see **Table 3**) to each tube
   c. Add **2.5 µL** of **c DNA** solutions or sterile **water** into appropriate tubes. Vortex tubes for 5 seconds to mix the contents.
   d. Spin tubes in a microcentrifuge for 10 seconds. Put tubes **on ice**.

Table 2: Preparation of Solution Mixes for Experimental Reactions

		Leaf		**14-DAP Embryo**		**- Control**
Components	**One** Reaction	**+RT Mix (1)**	**-RT Mix (2)**	**+RT Mix (3)**	**-RT Mix (4)**	**No** DNA **(5)**
cDNA	1 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	* 2.5 µL
Sterile water	22 µL	**122.5** µL **Exp'tal Mix**	**122.5** µL **Exp'tal Mix**	**122.5** µL **Exp'tal Mix**	**122.5** µL **Exp'tal Mix**	**122.5** µL **Exp'tal Mix**
12 µM **Gene**-specific Fw primer	1 µL					
12 µM **Gene**-specific Rv primer	1 µL					
2X SYBR supermix	25 µL					
Total Volume	**50 µL**					

*: Add **2.5 µL** of sterile **water. Experimental (Exp'tal) mix** is prepared in **Table 3** below

5. Prepare master mixes (see the example **Table 3** below).
   a. Label 1.5-mL microcentrifuge tubes as **Std. Mix** and **Exp. Mix**. Keep tubes **on ice**.
   b. Pipet **components** starting from top (water) down into appropriate tubes.
   c. Vortex the tubes for 5 seconds to mix the contents.
   d. Spin tubes in a microcentrifuge for 10 seconds. Put tubes **on ice**.

Table 3: Preparation of standard (Std) and experimental (Exp'tal) mixes

Components	**Std** Mix (x **25 rxns** = 20 rxns + extra)	**Exp'tal** Mix (x **15 rxns** = 10 rxns + 2.5 rxns w/ No DNA + extra)
Sterile water	550 µL	330 µL
12 µM **PcL1L** - Fw primer	25 µL	--------
12 µM **PcL1L** - Rv primer	25 µL	--------
12 µM **Gene** - Fw primer	-------	15 µL
12 µM **Gene** - Rv primer	-------	15 µL
2X SYBR supermix	625 µL	375 µL
Total Volume	**1225 µL**	**735 µL**

6. Label on the side of **every two** 0.2-mL PCR **tubes** in strips of 8 with individual caps as following:

   SA, SB, SC, SD, SE, SF, SG, SH (for **Standard**)

   1, 2, 3, 4, 5 (for **Experimental**)

   Caution: Do **NOT** write on the **caps** of the tubes because during PCR cycles, a **laser beam** goes in the tubes through the cap into the solution for measuring the amount of DNA being intercalated by SYBR green dye. Also, make sure that the caps are clean.

   or using a **96-well plate** (preferable):

	1-2	**3-4**	**5-6**	**7-8**	**9-10**	**11-12**
A						
B						
C						
D						
E						
F						
G						
H						

7. Pipet 50 μL of solution mixes (**A - H** in **Table 1**) and experimental mixes (**1 - 5** in **Table 2**) into appropriate labeled 0.2 mL PCR tubes or wells.
8. Turn on the BioRad iCycler and its OPTICAL System power source on the right side of the iCycler. Allow the lamp to warm up for at least 10 minutes.
9. Turn on the Toshiba laptop that runs the iCycler.
10. Open the iCycler program.
11. Organize the tubes into wells of the heat block of the BioRad iCycler starting from the **top row down** and from **left column to right** (see the chart below). This step is very important because name of samples will be entered into a file so that during PCR cycles, you can follow at which cycles (C_T = Threshold cycle) samples A and B are.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

12. Enter all information into an iCycler file by following the instruction for operating the iCycler. (TAs will help entering information).
13. Run the iCycler with a protocol " **3 Step Amp + Melt.tmo**" for an amplification and melt-curve protocol that has a following profile: 95^{o}C, 3 min → 40 cycles of 95^{o}C, 10 sec./ 60^{o}C, 30 sec./ 72^{o}C, 30 sec → 80 Repeats of Melt-Curve.
14. It would take about 2-3 hours to get the results, which are in the **rich-text format** so the data can be opened with a Microsoft WORD program.