Reverse Transcriptase Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

SYBR Green Protocol

Genes of interest:

Interleukin 1 beta, *IL1B* (Inflammatory gene)
Beta-actin, *ACTB* (Housekeeping gene)

Requirements/Reagents:

- 384-well PCR plates
- cDNA samples: Full template and NoRT control samples (already prepared from RNA sample using SuperScript First Strand Synthesis Kit; Life Tech, Cat. No. 11904018. See Appendix below.)
- Forward and reverse primers (Primer design discussed in didactic lecture)
- Fast SYBR Green 2X Master Mix (Thermo, Cat. No. 4385610)
- PCR grade water

I. RT-qPCR reaction set-up

1. **Dilute primer stocks:** Each primer is at 100uM stock solution, dilute at 1:10 to achieve working concentration of 10uM.
   Take 10ul of the 100uM stock and add to 90ul of PCR grade water.

2. Prepare Assay Reaction Mixes:
   For **each gene of interest**, prepare a full template sample (run in triplicates) and a "NoRT control" sample (one well) to assess genomic contamination.
   Notes:
   a. To ensure an adequate volume of RM is made, make a plate plan (see next page for an example) and count the number of wells needed per gene

   b. Add an additional 10% volume, to allow for pipetting errors

Assay Reaction mix	1 well	x wells
2X Master mix	5ul	
Forward Primer	0.2ul	
Reverse Primer	0.2ul	
H_20	4.1ul	

3. Plate out 9.5ul of Reaction Mix (prepare in step 2) into appropriate wells for the individual genes

4. Next add 0.5ul of “Full template” and “NoRT” samples to the appropriate wells. Ensure to include water (no template) control.

   See example of plate plan for 2 samples and water control:

   PUT A 96 well plate

5. Cover the plate firmly with an Optical Adhesive film

6. Centrifuge at 2000rpm, 4ºC for 1min

7. Place the plate on ice and proceed to the thermocycler

8. Run the PCR program with the following settings:

   a. Hold Step: 95 ºC for 10mins
   b. PCR step: 95 ºC for 15secs; 60 ºC for 1min; for 50cycles

   Collect melt-curve data

II. Results analysis & discussion

APPENDIX- cDNA First strand synthesis from RNA samples

The first step of a two-step PCR protocol is synthesizing the cDNA from RNA extracted from your samples. *(Due to the unstable nature of RNA, we have already made the cDNA from Cambridge, however the protocol for the cDNA synthesis step is provided below.)*

1. Prepare each RNA sample to reach ~500ng concentration in 5ul of PCR grade water

2. Ensure to make duplicates for each RNA sample, one of the duplicates will be for the full cDNA template with RT enzyme, the other will be a No RT control

3. Next prepare the random primer mix as follows (N is total number of samples)

Primer mix	**x1**	**xN**
10mM dNTP mix	1ul	
Random hexamers	1ul	
H20	3ul	

4. Add 5ul of the primer mix to each 5ul RNA sample prepared in Step 1

5. Incubate at 65^{o}C for 5minutes and then place on ice

6. Within the 5minutes incubation, prepare the RT 2x reaction mix

2x Reaction mix	**x1**	**xN**
10x RT buffer	2ul	
25mM MgCl2	4ul	
0.1M DTT	2ul	
RNase out	1ul	

7. Next Add 9ul of the 2x RM to each sample- mix and collect by brief centrifugation. Incubate at room temperature for 2mins.

8. Add 1ul superscript II RT enzyme to one tube for each sample and 1ul H20 to duplicate (the No RT control) tube for each sample. *NB: Handle the superscript RT enzyme with care- only bring out of the -20^{o}C freezer when ready to use, keep constantly on ice and return to the freezer once done.*

9. Run the synthesis steps as follows:
   Room temperature (approx. 25^{o}C) for 10mins
   42^{o}C for 50mins
   Terminate the reaction at 70^{o}C for 15mins, then chill on ice.

10. Briefly centrifuge tube, add 1ul of RNaseH to each tube and incubate at 37C for 20mins.