TaqMan Preamplification

Reverse Transcription and Preamplification

First RNA is converted to single-stranded cDNA by a reverse transcription enzyme. The preamplification process selectively amplifies a cohort of genes in a linear manner so that an expression profile for the selected genes may be determined from a small sample of cDNA.

<u>Reverse Transcription Reaction</u>

1. Thaw High-Capacity cDNA Reverse Transcription kit reagents on ice
2. Prepare a 2X master mix using the following quantites of reagent per reaction: 2 ul 10X RT Buffer, 0.8 ul 25X dNTP Mix (100 mM), 2 ul 10X RT random primers, 1 ul MultiScribe™ Reverse Transcriptase, 1 ul RNase Inhibitor and 3.2 ul nuclease-free H_2O.
   To allow for reagent that is lost during pipetting the master mix should include at least one more reaction than you have samples, 1 additional reaction for every 10 samples is usually sufficient. Mix gently and maintain on ice.
3. Pipette 10 ul RNA into a PCR tube and add 10 ul master mix to the sample, mix by gentle pipetting and seal the tube. If necessary briefly centrifuge the samples to collect all liquid to the bottom of the PCR tubes, maintain on ice until ready to transfer to the thermal cycler.
4. Create the following program in the thermal cycler you will use: Step1: 25°C for 10 minutes, Step2: 37°C for 120 minutes, Step3: 85°C for 5 seconds, Step4: 4°C hold.
5. Load the samples into the thermal cycler and begin the program.
6. Once the cDNA samples have cooled remove them from the thermal cycler. The cDNA may be transferred to a RNase-free microfuge tube and stored at -20°C until required or used immediatley.

<u>Preamplification reaction</u>

1. Prepare a pooled assay mix of the TaqMan gene expression assays for the genes of interest, a maximum of 100 genes can be preamplified. Thaw the 20X assays on ice and pipette 5 ul of each assay into a nuclease-free microfuge tube. Add 1X TE buffer to a final volume of 500 ul.
   For example, if the pooled assay mix contains 5 ul each of 10 assays add 450 ul TE buffer for a final volume of 500 ul.

Each assay is present at a final concentration of 0.2X. The pooled assay mix can be frozen at -20°C for storage or used immediatley.

2. Pipette 1-250 ng cDNA sample into a PCR tube, aim to use a consistent amount for all samples, and adjust the volume with nuclease-free H2O to 10 ul. Add 10 ul pooled assay mix and 20 ul TaqMan® PreAmp Master Mix to each sample, mix gently by pipetting and maintain on ice until ready to transfer to the thermal cycler.
3. Create the following program in the thermal cycler:
   Step1: 95°C for 10 minutes, Step2: 10 cycles of 95°C for 15 seconds, 60°C for 4 minutes.
4. Load the samples into the thermal cycler and begin the program. Once cycling is complete it is important to immediatley transfer the samples from the cycler onto ice to halt the reaction.
5. Transfer the 40 ul preamplified sample to a nuclease-free microfuge tube and dilute with 160 ul 1X TE buffer. The preamplified cDNA can be stored at -20°C or used immediatley in a PCR reaction.

Quantitative Real-Time PCR

1. Thaw cDNA samples and TaqMan Gene Expression Assays for genes of interest and the endogenous control on ice.
2. Calculate the number of reactions needed for each assay; each sample should analysed in duplicate wells, appropriate positive and negative controls should be included and an additional reaction for every 10 to allow for reagent volume lost through pipetting. Also prepare a template indicating which sample and gene will be analyzed in each well.
3. Prepare a master mix for each TaqMan assay in a microfuge tube containing 10 ul TaqMan Gene Expression Master Mix, 1 ul TaqMan Gene Expression Assay, 4 ul nuclease-free H_2O per reaction . Cap the tube and mix contents by inverting multiple times, briefly centrifuge to bring contents to the bottom of the tube. Maintain on ice until required.
4. Pipette 5 ul preamplified cDNA into the wells of a reaction plate.
5. To the appropriate wells add 15 ul of TaqMan assay master mix, change tips as frequently as required to avoid cross-contamination between wells.
6. Seal plate with adhesive film and centrifuge plate briefly to bring reagents to the bottom of the wells.
7. Load plate into the real-time PCR instrument.

Checking Preamplification uniformity of TaqMan Assays

- Pool the required TaqMan assays along with assays for 2 house-keeping genes: Recommended PPIA (Hs99999904 m1) and CDKN1B (Hs00153277 m1). Dilute the pooled assay mix with TE buffer to 0.2x concentration

For example if you are using 5ul of each assay the final volume of the pooled assay mix should be 500 ul.

- Prepare preamplified cDNA as described above

- Dilute unamplified cDNA to approximately 1 ng / ul.

Prepare 2 master mixes per TaqMan Assay; one containing diluted cDNA and the other containing preamplified cDNA:

TaqMan Assay	5.0 ul
cDNA (dil. or preamp)	25.0 ul
TaqMan GEMM	50.0 ul
H_2O (nuclease-free)	20.0 ul

Add 20ul of the master mix to 4 wells, for example:

1 2	3 4	5 6	7 8	9 10	11 12
PPIA	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5
CDKN1B	Assay 6	Assay 7	Assay 8	Assay 9	Assay 10
PPIA	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5
CDKN1B	Assay 6	Assay 7	Assay 8	Assay 9	Assay 10

Run the real-time PCR using the standard cycling protocol.

<u>Calculate the average Ct values for each assay</u>

- Calculate the dCt by subtracting the Ct value for PPIA from the Ct values for each of the other assays.
  Keep undiluted cDNA and preamplified cDNA values seperate in this step.
- Calculate ddCt by subtracting the dCt of the diluted cDNA from the dCt of the preamplified cDNA for each gene. A ddCt value close to zero indicates uniform preamplification. A ddCt value within +/- 1.5 is considered acceptable, 90% of TaqMan probes should be within the acceptable limit.