



## SOP B8 : Quantitative PCR

### 1.0 Materials

2x TaqMan Fast Universal PCR Master Mix (Applied Biosystems, 4364103)  
MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems, 4346906)  
MicroAmp Optical Adhesive Film (Applied Biosystems, 4311971)  
20x TaqMan Gene Expression Assay : see index BeNe  
QuantStudio 12K Flex (Applied Biosystems)

### 2.0 Procedure

#### Plate setup recommendations

It is recommended to use at least two replicate reactions for every sample. In addition, a positive control and (a) reference gene(s) must be amplified.

Run No Template Controls (NTC's) (nuclease-free water instead of cDNA sample) for each Gene Expression Assay used.

If possible, use exon-spanning primers.

#### Preparing the plate

All steps nuclease-free !

Keep the plate at 4°C during preparation (use plate cooler).

The following table lists the universal assay conditions (volume and final concentration) for use with the TaqMan Fast Universal PCR Master Mix.

Reaction Component	µL/ sample	Final concentration
TaqMan Fast Universal PCR Master Mix (2x)	10	1x
TaqMan Gene Expression Assay (20x)	1	1x
cDNA sample	5*	5.7 ng**
Nuclease-free water	4	-
Total	20	-

\* can be increased to 9 µL (by leaving out the nuclease-free water) in case duplicate Ct's are high and variable (>35)

\*\* assuming a 100% efficient reverse transcription reaction

Add 20 µL of the appropriate PCR reaction mix per well.

Seal the reaction plate with Optical Adhesive Film.

**Important** : centrifuge the plate during 1 minute at 900 x g to collect all reagents at the bottom of the well and to remove air bubbles.

Keep the reaction plate at 4°C until the plate can be loaded onto the QuantStudio.

Note : A plate can be prepared the day before the run and kept at 4°C overnight.

## Running the plate

Switch PCR machine on using the button at the back of the machine.

Open the program Quantstudio 12K Flex Software.

Pop-up window: Experiment: Create: setup menu.

### 1) Experiment properties

Which block?	Fast 96-well (0,1 mL)
Type of experiment?	Comparative Ct
Which reagents?	TaqMan Reagents
Properties for instrument run?	Fast

### 2) Define

#### *Targets:*

Fill in target names or import them from the library. Click "new" for each target.

#### *Samples:*

Fill in sample name or import them from the library. Click "new" for each sample.

### 3) Assign

Click on a well (or group of wells) to select it, select the appropriate detector for the selected well(s).

Click on a well (or group of wells) to select it, enter a sample identification number.

*File > Save As ...*

Note : save the file in D:\Applied Biosystems\Quantstudio 12K Flex software\user files\experiments\...

Open drawer using the touch screen on the PCR machine. Load plate into the instrument tray. **Important** : orient the correct A1 position !

Click "Run", then the green "Start run" button. Finally click on the number that appears under the green "Start run" button.

PCR run will start. Each run takes about 35 minutes.

After the run, save data:

Click on the green "Analyze" button.

Export data to an Excel file.

Click 2x on "Results".

Select "Load export set" Veerle Laurysens.

Click "Browse" and open your file.

Fill in the appropriate file name in "Export file name".

File type: \*.xlsx.

Click "Start export", results appear in an Excel file.

For data quality control and analysis, the use of "qBase+" software is recommended.

**Relative Quantitation** (RQ) using Comparative Ct ( $\Delta\Delta Ct$ ) is used to determine the change in gene expression between samples (see example below and Schmittgen TD, Nat Protocol 2008).

Note :

Experimental error may cause some wells to be amplified insufficiently or not at all. These wells typically produce Ct values that differ significantly from the associated replicate well(s). If included in the calculations, these outlying data (outliers) can result in erroneous measurements. To ensure precision, carefully examine replicates for outliers. **Replicate Ct values should lie within 0.50 from each other !**

**No template control should have Ct = 40.00.**

Example : Relative quantification using the comparative Ct method :

Sample	Average Ct		Average $\Delta Ct$ <sup>1</sup>	- $\Delta\Delta Ct$ <sup>2</sup>	Fold change <sup>3</sup>
	Ngn3	Cyclo A			
Duodenum	27.58	22.20	5.38	0.00	1.00
PDL-head	35.23	25.90	9.33	3.95	0.06
PDL-tail	35.14	29.20	5.94	0.56	0.68
No template	40.00	40.00			

<sup>1</sup> : Average  $\Delta Ct$  = average Ngn3 Ct - average Cyclo A Ct

<sup>2</sup> :  $-\Delta\Delta Ct$  =  $\Delta Ct$  target -  $\Delta Ct$  calibrator

For example :  $-\Delta\Delta Ct$  PDL-head =  $-(\Delta Ct$  PDL-Head -  $\Delta Ct$  Duodenum)

<sup>3</sup> : Fold change Ngn3 relative to duodenum =  $2^{-(\Delta\Delta Ct)}$

For example : fold change Ngn3 PDL-head relative to duodenum =  $2^{-(-3.95)} =$

### 3.0 Remarks

Do not use other software while data are collected.

Do not use screensavers on the computer that is connected to the qPCR machine.