

## A guide to Real Time PCR at IRIC (qPCR)

The genomic platform offers a complete service for real time PCR including the analysis of your RNA, preparation of your cDNA, design of assays, validation and analysis of your results. On the other hand, you can prepare the reactions yourself and then use our Via7 instrument from Life Technologies. This document is intended to guide you through the steps involved in a qPCR experiment.

If you have questions on the service, or need help setting up your experience, you can contact Raphaëlle Lambert at [raphaelle.lambert@umontreal.ca](mailto:raphaelle.lambert@umontreal.ca) or visit [www.genomique.irc.ca](http://www.genomique.irc.ca).

### **1- Before getting started...**

There are a lot of different details to consider in a qPCR experiment and it's important to plan ahead before starting the project. Here are a few details to consider:

#### **Endogenous controls**

First of all, you will need endogenous controls. They are essential because the whole analysis is based on them. They eliminate the variation between samples (technical variation, RNA quantity). The expression of the endogenous control should not vary between the samples tested, no matter which treatment is used. Here are a few examples that we have at the platform, but there are a lot more that you can use: GAPDH, ACTB, TBP, HPRT, PPIA, YWHAZ. It is recommended to use an endogenous control with an expression level similar to the genes tested, and ideally use a few controls in your experiment. We don't recommend using 18S, because it's much more abundant than regular genes, and it's not an mRNA.

If you have no idea which control to use, we suggest testing a few of them and selecting the ones with the least variation between the samples. As always, it's important to use exactly the same amount of RNA in all RT reactions. Ideally, the variation should be less than 2 cycles between all samples. Once you have tested the potential endogenous controls, you can use GeNorm to determine which ones to use (<http://medgen.ugent.be/~jvdesomp/genorm>). Usually, in the end, only one control is used in the analysis. It's also possible for you to calculate your results based on more than one control, which improves the accuracy of the results.

#### **Calibrator sample**

A calibrator sample is needed so that you obtain a relative expression value (i.e : your gene X is two times more present in your sample Z than in your calibrator sample). This sample is the control sample, without treatment or time zero, and the fold changes will be compared to the calibrator. It needs to be as much as possible alike the samples you are testing.

#### **Biological replicates**

When you're designing your experiment, always keep in mind that it's a good idea to have biological replicates for each of your RNA samples (at least three). When you do your analysis at the end, it will help you identify if your results are statistically relevant, or in other words, if what you see is real. And it's important that all those replicates be treated together. For example, RNA extracted at the same time, RT reaction done at the same time. As much as possible, they need to be treated the same way from the beginning to the end. You want to eliminate as much experimental variations as you can, so what you measure is the real biological variation.

If you plan on doing statistics, use biological replicates and not qPCR technical replicates, as only technical variation is measured. If you want to bring together a set of results, don't do 3 experiences during 3 different weeks. Results will still be good and significant, but Fold changes will not be exactly the same. You won't be able to bring them together in a figure.

Here is an interesting article about experimental design in qPCR:

### The MIQE guidelines

The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. Clin Chem. 2009 Apr;55(4):611-22. Epub 2009 Feb 26

## **RNA preparation**

You have to perform your own total RNA extraction. When extracting RNA from cells or tissue, an RNA extraction protocol using Trizol or using a column usually works well. RNA extraction from some tissues can be tricky, a special column might be needed and sometimes Trizol extraction should be followed by a column. Find out if your tissue requires specific handling.

### **No inhibition**

If you're extracting RNA with Trizol, you have to be very careful as to not carry any solvent over when transferring the supernatant after centrifugation. Solvents can inhibit the Reverse Transcription reaction and the PCR reaction. Always take less and leave some behind. When quantifying your RNA with the spectro, make sure you have a good OD 260/280 ratio between 1.8 and 2 and OD 260/230 ratio of 1.8 or greater. Nucleic acid is detected at 260nm, whereas protein, salts and solvents are detected at 230 and 280nm.

### **No degradation**

It is absolutely essential that your RNA be of good quality. No degradation is acceptable. If your RNA is degraded, it will be in small pieces. The PCR may still amplify because the qPCR assays are small (60-150pb), but degradation introduces bias that are not acceptable.

We recommend migrating your RNA samples on gel (RNase free) or analysing them on our Bioanalyzer (fig 1). You need to see two bands: 28S and 18S subunits (and no other band).

### **Good quantity**

Always quantify your RNA (spectro, nanodrop) before bringing it to the platform or perform your RT reaction. The quantity of RNA needed is approx. 25ng for each individual PCR reaction.

For example: 5 samples to test with 10 genes + 2 endogenous controls:

12 genes x triplicata = 36 reactions  
36 reactions x 25ng = 900 ng of RNA per sample

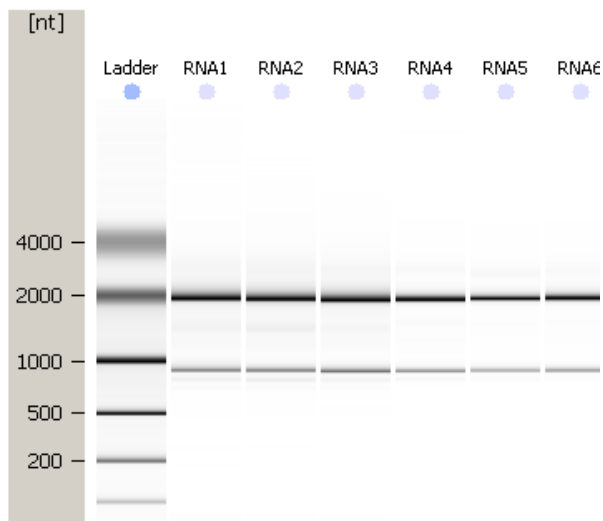


Fig 1. Migration of RNA samples on a Bioanalyzer from Agilent

## 2- cDNA synthesis from total RNA

Before doing the qPCR itself, you need to transcribe the RNA into cDNA. It's an enzymatic reaction, done with a reverse transcriptase.

We are using the « High Capacity cDNA Reverse Transcription Kit » from Applied Biosystems, but most known companies have good kits. The RT reaction needs to be done with random primers, which are degenerate primers that will bind everywhere. RT reaction using an oligo dT is not recommended since assays near the polyA tail of the mRNA will be favoured and it could introduce bias. A kit that uses both oligo dT and random primers is good.

We recommend doing a noRT sample (no Reverse Transcriptase). This sample will contain RNA and all ingredients except the enzyme. This sample should be negative/no amplification or a very late amplification (>10 cycles after regular samples). But if it's contaminated with genomic DNA, it will amplify (for the assays that are not inter-exon). Ideally, all assays are made so as to not detect genomic DNA (inter-exon), but sometimes it's impossible to do a inter-exon assay, and the presence of DNA is not wanted anyway. You can treat your RNA with DNase if it's contaminated.

If you're over-expressing a gene in cells, don't forget the presence of the vector in your total RNA. If the vector contains the coding sequence of the gene, you will amplify this sequence with your qPCR assay and the signal will affect your results. Do a DNase treatment and a noRT control, and note that sometimes it's difficult to get rid of a vector.

Prepare a RT reaction for what you need. With 2ug of RNA you can test up to 20 genes, and with 5ug you can test up to 60 genes. If you don't have enough RNA, put the maximum you can in the RT.

Protocol for the RT according to the quantity of RNA needed :

<b>Composantes</b>	<b>2ug</b>	<b>5ug</b>
10X Reverse Transcript Buffer	2 µl	5 µl
10X Random primer	2 µl	5 µl
25X dNTPs	0.8 µl	2 µl
MultiScribe Reverse Transcriptase enzyme (50U/µl)	1 µl	2.5 µl
H <sub>2</sub> O Nuclease free	4.2 µl	10,5 µl
RNA	10 µl	25 µl
<b>Total</b>	<b>20 µl</b>	<b>50 µl</b>

Incubate your samples 10 minutes at room temperature and then 2 hours at 37°C. When the reaction is over, you can keep your samples at -20°C.

The only way of verifying your RT reaction is to amplify it by qPCR. A first test could be for example with a few endogenous controls. Curves should come out at a known Ct for those genes, and all curves should be grouped together (if you put the same amount of RNA in all reaction).

### 3- Design of qPCR assay

You will need a qPCR assay for each gene that you want to test. There are two technologies commonly used: SYBR green and Taqman. SYBR Green is a DNA intercalant, so it will detect everything double stranded, while Taqman works with a gene specific fluorescent probe. We prefer using Taqman because it requires less fine tuning when you setup an assay, and it's more specific. But both technologies are good.

**Never trust qPCR oligos coming from a publication.** We recommend ordering your own oligos from the start. If you absolutely need to use oligos from a publication, always verify that they target the gene they are supposed to, that the size of the amplicon is normal, and always validate the oligos before using them. Do a standard curve plus a melt curve if the assay is SYBR.

At the Genomic Platform, we have the complete library of UPL probes (Universal probe library from Roche) for human and mouse (#1 to #110). It's possible to use these probes for assays on other species. This technique is based on the TaqMan principle except that the length of the probe is limited to 8 nucleotides (some chemical additives are present to increase the T<sub>m</sub> of the probe). Therefore, these small probes can hybridized at many places on the genome but when the assay is combined with specific oligonucleotides, the assay becomes very sensitive and specific. With this library, we can detect 99% of the genome (human and mouse).

#### Doing your own qPCR

If you are at IRIC and you want to do your assays yourself, you can use our library of UPL probes, and pay by the reaction. If you are not at IRIC and you want to do your own assays, we suggest using SYBR green because the price of an individual gene specific Taqman probe is quite high (around 125\$ per gene). Designing a SYBR green assay follows the same recommendations as a Taqman assay, except there is no probe, and the qPCR mix to use in the final reaction needs to contain SYBR Green.

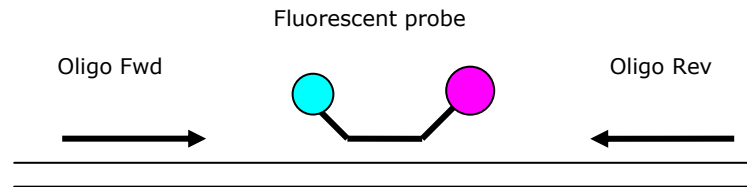


Fig2. Taqman qPCR assay

A Taqman qPCR uses two gene specific oligonucleotides and a specific fluorescent probe. During amplification of the amplicon, the probe is degraded by the Taq polymerase and fluorescence is emitted. Without amplification, the fluorescence (pink) is quenched by the quencher (blue) and is not emitted.

To design your qPCR assay with the UPL library, follow this link:

[www.universalprobelibrary.com](http://www.universalprobelibrary.com)

Here are a few recommendations:

- Length between 60 and 125 bp
- Select an assay that is located between two exons. The intron has to be 500 bp or more to avoid its amplification if your sample contains genomic DNA. The program indicates the position of the introns with a triangle.
- Note the sequence of your primers and the probe number (#1 to #110)

## **4- Standard Curve to validate the assays**

For each assay, you need to do a standard curve to verify three things:

- 1- Is the assay working?
- 2- Is the gene present in my samples?
- 3- Is the efficiency of the PCR at 100%?

First, you can resuspend your oligos at a stock concentration of 100  $\mu$ M. Then, mix these two primers to obtain a final concentration of 50  $\mu$ M (for example: take 25  $\mu$ l of primer A and 25  $\mu$ l of primer B).

To make a standard curve, you need to do 4 serial dilutions: 1:5, 1:25, 1:125 and 1:625. This experience can be done in technical duplicates. It's recommended to do the dilutions with cDNA (or a mixture of many cDNA) similar to the ones that will be used in your real experiment. If it's not possible, you can use a universal cDNA (including several tissues of the same specie). It's important that you always use a diluted cDNA (no undiluted for the first point of the curve). The RT solution contains PCR inhibitors and when they are present at a higher concentration they inhibit the qPCR reaction.

If the assay is SYBR green, you need to do a Melt Curve to make sure you amplify a single product.

### **How do I know if my assay is good?**

#### **1- General aspect of the PCR curves**

Your first dilution 1:5 should come out at a respectable cycle (generally around 20-30). You need to see at least 3 points of your curve, the duplicates need to be nice and together and the curves need to be clean and not frizzled. If your gene comes out at a very late cycle (30-35), there is more chance that your results will be of bad quality in your real experience. The later the curves come out, the more variations you will observe between replicates.

#### **2- Slope of the standard curve**

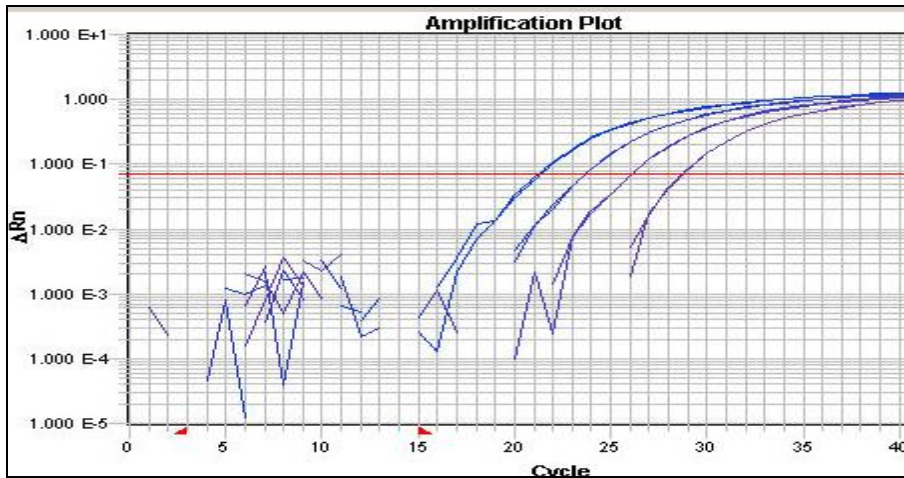
The slope of the standard curve should ideally be -3.33 (which corresponds to an efficiency of 100%). In practice, we accept a slope between -3.1 and -3.6 (90 to 110%).

What you need is a similar efficiency for all genes you're testing. For example, your test gene and your endogenous gene need to have approximately the same efficiency because you will compare those genes together. If an assay's efficiency is too low, the curve will come out a little bit later than the others and it can make a difference in your final results. A difference of 1 cycle already equals a fold change of 2.

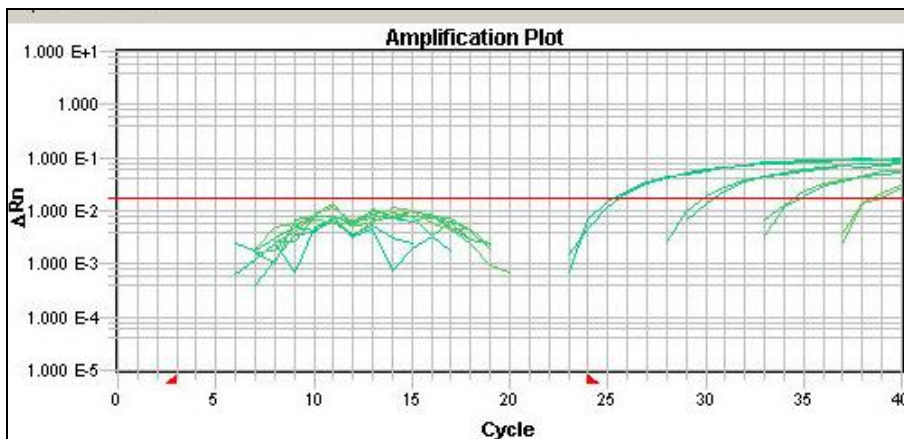
If the slope of the standard curve does not fall within an acceptable interval, it is suggested to do the curve a second time and if the results remain the same, a new assay has to be designed and ordered. If you only have 1 or 2 genes, you can order 2 assays the first time to make sure one of them is good and save time.

Remember that each qPCR assay has its own personality. Two assays targeting the same mRNA will not come out at the same cycle and will not have the same efficiency. And all this is dependent on the samples you are testing. Finally, don't forget that sometimes, it's possible that a gene will not be expressed in your samples.

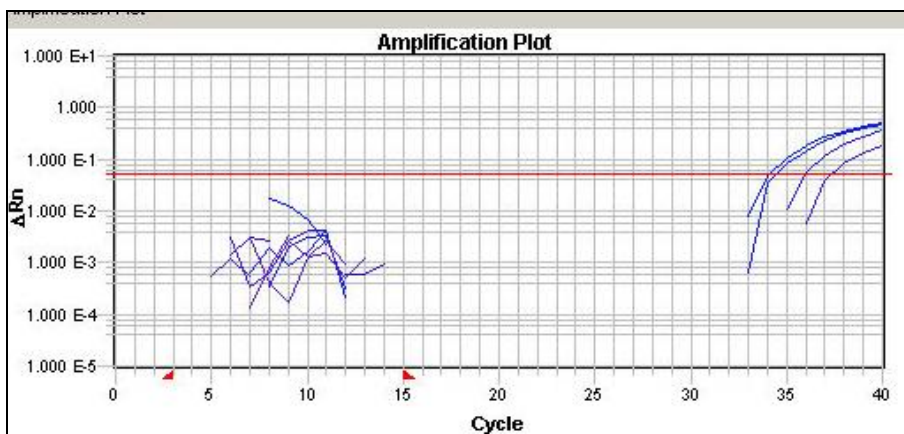
### Examples of Standard Curve runs



Perfect! Slope of de -3.3.



Very low efficiency, around 70%.



Here, the curves are coming out too late. The curves are flat and the duplicates are not together. Either the oligos are not working well, or the gene is not expressed at a sufficient level.

Range of acceptable efficiencies when testing a new qPCR assay.

<b>Slope</b>	<b>Amplification</b>	<b>Efficiency (%)</b>
-3.60	1.8957	89.57
-3.55	1.9129	91.29
-3.50	1.9307	93.07
-3.45	1.9492	94.92
-3.40	1.9684	96.84
-3.35	1.9884	98.84
-3.30	2.0092	100.92
-3.25	2.0309	103.09
-3.20	2.0535	105.35
-3.15	2.0771	107.71
-3.10	2.1017	110.17

$$\text{Amplification} = 10^{(-1/\text{slope})}$$

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$



## 5- How to do a qPCR reaction

All qPCR reactions should be done in triplicate for each sample and all genes. If you have biological triplicates, you can do technical duplicates.

Do not forget to add the noRT control and the NTC control (no template control : replacing cDNA with water).

It is important to dilute the RT reaction (min 1:2, max 1:10) since RT reagents can inhibit qPCR reaction.

We use 384 wells plate and a final volume of 10 ul. The Master Mix we use for qPCR is Taqman Fast qPCR MasterMix from Applied Biosystems (Roche, Qiagen and Invitrogen are also recommended). The qPCR mix « fast » allows us to use a faster qPCR program which takes 1h instead of 1h30 (40 cycles).

Protocol for Roche assays with UPL probes:

Reactives	1 reaction	initial [ ]	final [ ]
2X PCR Master Mix	5 µl	2X	1X
Primers (F and R mixed)	0,05 µl	50 µM	250 nM
UPL probe (diluted 1/10)	1 µl	1 µM	100 nM
cDNA (diluted 1/5)	1.5 µl	---	---
H <sub>2</sub> O	2.5 µl	---	---
Total	10 µl		

Protocol for *Taqman gene expression assays*

Reactives	1 reaction
2X PCR Master mix	5 µl
TaqMan assay (20X)	0.5 µl
cDNA (diluted 1/5)	1.5 µl
H <sub>2</sub> O	3 µl
Total	10 µl

FAST and regular qPCR programs

	95°C	40 cycles	
	95°C	60°C	
Fast	3 min	5 sec	30 sec
Regular	10 min	15 sec	1 min

For results analysis, see document "How to deal with qPCR results".