

RT-PCR: a start to finish guide

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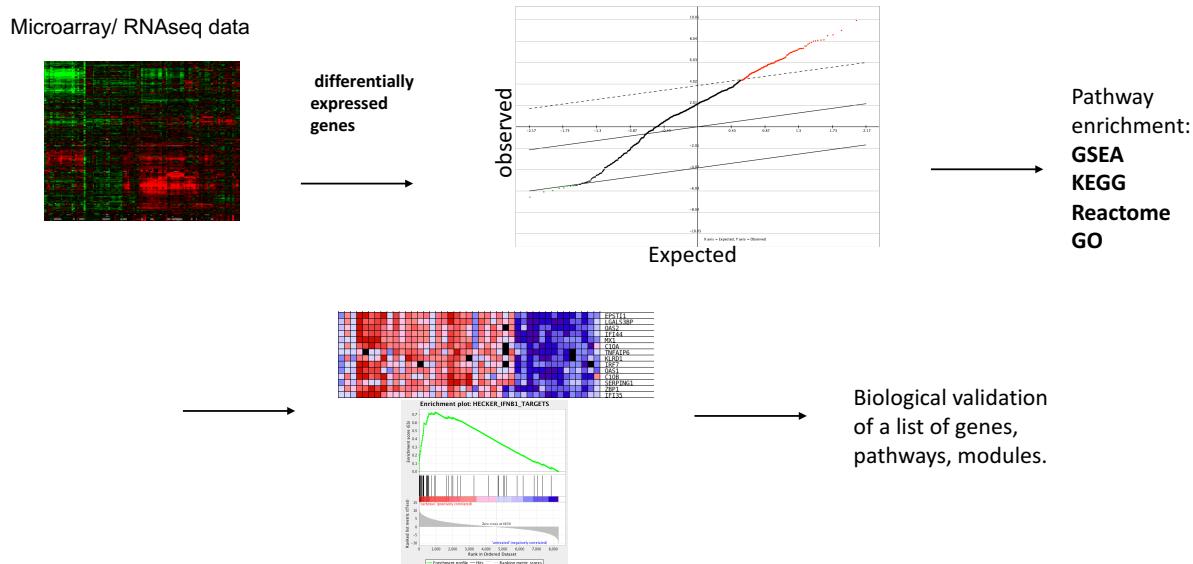
Disclaimer

- I am not affiliated with Qiagen or any company. I'm a graduate student at University of Cambridge/ NIH.
- I'm doing this webinar to spread knowledge and create a resource people can use for free.
- This is an introductory instructional tutorial for biologists who are trying to validate gene expression data.
- Steal this presentation, the analysis code, and all associated files and use/distribute them wherever you want!

Outline

- RT PCR overview
- Designing an assay (**walkthrough**)
- Validating the assay in silico and in the lab (**walkthrough**)
- mRNA extraction and cDNA synthesis important considerations
- Selecting a control gene
- Doing the actual experiment
- data analysis (**walkthrough**) with excel and R.
- **Goal: understand how to design an assay, do a qRT-PCR experiment and analyze the data to validate gene expression data start to finish.**

Typical framework requiring validation



Correlation Structure of genes (sidenote)

OPEN  ACCESS Freely available online

PLOS COMPUTATIONAL BIOLOGY

Most Random Gene Expression Signatures Are Significantly Associated with Breast Cancer Outcome

David Venet¹, Jacques E. Dumont², Vincent Detours^{2,3*}

¹ IRIDIA-CoDE, Université Libre de Bruxelles (U.L.B.), Brussels, Belgium, ² IRIBHM, Université Libre de Bruxelles (U.L.B.), Campus Erasme, Brussels, Belgium, ³ WELBIO, Université Libre de Bruxelles (U.L.B.), Campus Erasme, Brussels, Belgium

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3197658/>

Number of genes dictates experiment design

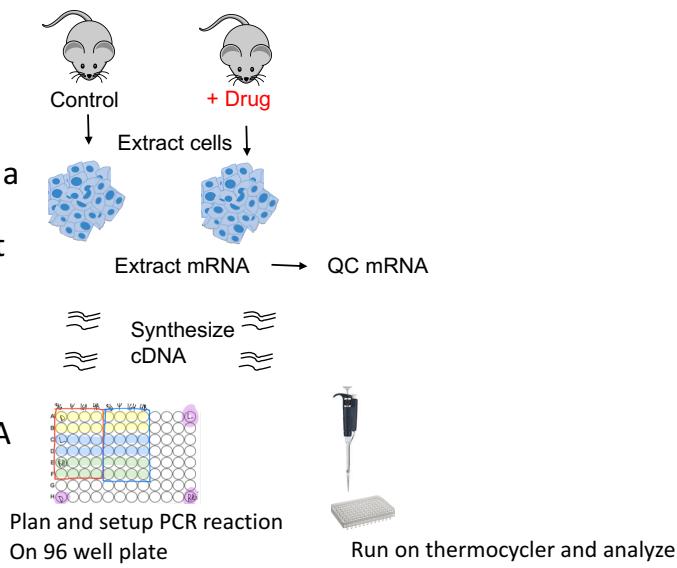
- 5-10 genes:
 - It's feasible to design and validate a few assays yourself.
- 20, 50, 100+ genes:
 - With this many genes to validate, it's likely better to take advantage of a plate or microfluidics platform with pre-designed, wet lab tested and optimized assays.

qRT-PCR Overview

qRT-PCR, RT-qPCR, RQ-PCR, RT-PCR.

High level overview of RT-PCR

- Extract RNA
- Synthesize cDNA
- Detect transcripts of interest with a gene specific primer
- Run PCR on a real time instrument which measures fluorescence accumulation at each cycle.
- Analyze the inverse relationship between cycle number at which fluorescence hits a threshold and the initial starting amount of cDNA specific for your target.



qRT-PCR overview

conceptual model of PCR

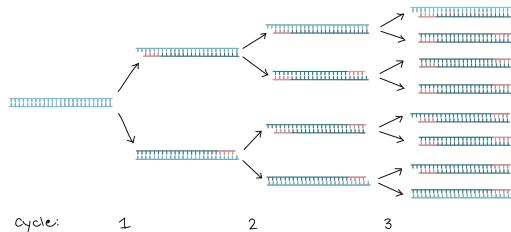


Image from khanacademy.org

[Rutledge and Coté 2003 Nucleic Acids Research](#)

mathematical model of PCR

$$N_c = N_0 * 2^c$$

$$N_C = N_0 \cdot (E + 1)^C$$

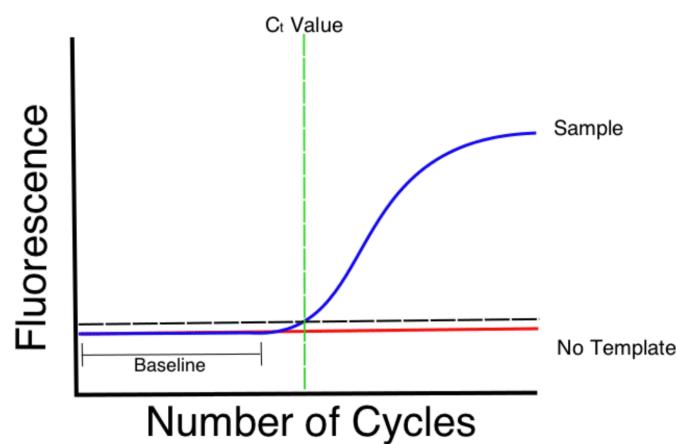
$$N_0 = N_C / (E + 1)^C$$

- C = PCR cycle number
- E = amplification efficiency
- NC = Number of target molecules at cycle number C"
- N0 = Number of molecules when we start the reaction

• 100% Efficiency

- Knowing the cycle at which fluorescence crossed a threshold allows us to know the starting amount. In the examples that follow we will show quantities relative to control samples normalized to housekeeping genes.

Cycle threshold (Ct or Cq)



<https://bitesizebio.com/24581/what-is-a-ct-value/>

Designing an assay

Theory then walkthrough tutorial

Off the shelf vs designing your own assay

JOURNAL OF CLINICAL ONCOLOGY

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PLOS PATHOGENS

From: the Department of Clinical & Biological Sciences, University of Turin, Turin; Department of Hematology, University Federico II, Naples, Italy; Department of Hematology, Biology and Pathology Center, University of Lille Medical School; Cancer Research Institute Jean-Pierre Aubert Research Center, l'Institut National de la Santé et de la Recherche Médicale, U-807, Team 3, Villejuif; Research & Development, Isogen, Marseille, France; Department of Immunology, School of

Real-Time Quantitative Polymerase Chain Reaction Detection of Minimal Residual Disease by Standardized WT1 Assay to Enhance Risk Stratification in Acute Myeloid Leukemia: A European LeukemiaNet Study

glio, and David Grimwade

WT Assay	Assay Location	Assay Reference	Sensitivity to Detect WT in		Phase of the Evaluation	Reason for Exclusion
			Hb6.0 Cell Line Activity	Relative Efficiency		
1	Exon 6-7	Ogawa et al ¹⁰	10 ⁻²	High	II	Lack of sensitivity
2	Exon 7-8	Oborny et al ¹⁰	10 ⁻²	High	II	Reduced efficiency and sensitivity
3	Exon 6-7	Østergaard et al ¹⁰	10 ⁻¹	Medium	II	Location of primers and probe
4	Exon 7-8	Østergaard et al ¹⁰	10 ⁻¹	Medium	II	Location of primers and probe
5	Exon 7-8	Unpublished	10 ⁻³	Medium	II	Reduced efficiency and sensitivity
6	Exon 7-8	Unpublished	10 ⁻¹	Medium	II	Low sensitivity
7	Exon 7-8	Unpublished	10 ⁻¹	Medium	II	Lower efficiency
8	Exon 7-8	Ciloni et al ¹⁰	10 ⁻² –10 ⁻⁴	Medium	II	Inferior level of efficiency
9	Exon 1-2	Van Dick et al ¹⁰	10 ⁻¹	Not excluded	Not applicable	

*All other assays were confirmed to be RNA-specific.

If you want to look at this gene, you don't need to reinvent the wheel.

Identification of a Novel Splice Variant Form of the Influenza A Virus M2 Ion Channel with an Antigenically Distinct Ectodomain

Helen M. Wise^{1,2}, Edward C. Hutchinson^{1*}, Brett W. Jagger^{1,3}, Amanda D. Stuart¹, Zi H. Kang¹, Nicole Robb⁴, Louis M. Schwartzman³, John C. Kash³, Ervin Fodor⁴, Andrew E. Firth¹, Julia R. Gog⁵,

Jeffery K. Taubenberger³, Paul Digard^{1,2*}

1Division of Virology, Department of Pathology, University of Cambridge, Cambridge, United Kingdom, **2**The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian, United Kingdom, **3**Viral Pathogenesis and Evolution Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America, **4**Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom, **5**DAMTP, Centre for Mathematical Sciences, University of Cambridge, Cambridge, United Kingdom

There might not be an off the shelf assay for this specific transcript isoform.

Designing RT-PCR assay (example follows)

- Look up consensus cDNA sequence or your gene of interest.
- Extract the sequence to evaluate primer binding sites
- Use existing software to evaluate and pick primers based on the specifications of your experiment (buffer salt concentration, melt temperature).
- Blast primers against genome to ensure target specificity
- Evaluate the performance of the assay in the lab
- Do the same for your control gene (how to pick a control gene later)
- Do your experiment
- If using a probe based assay, consider an exon junction spanning probe

No shortage of primer design software

- [PerlPrimer](#)
 - [Primer-BLAST](#)
 - [Primer3Plus](#)
 - [PrimerQuest](#)
 - [OligoPerfect](#)
 - [OLIGO](#)
 - [GenScript Real-time PCR](#)
 - [AutoPrime](#)
 - [RExPrimer](#)
 - [Understanding primer annealing thermodynamics :](#)
 - [Helpful link for understanding Primer annealing thermodynamics](#)
- Many of these oligo design tools are extensions of Primer3
 - Let's do an example using Primer3, and blast results against the genome...
 - This has been integrated into a single feature with PrimerBLAST.

Example of designing an assay.

Let's go through an example of how to design an RT PCR assay.

Are you validating RNAseq data?

- Consider MapSplice

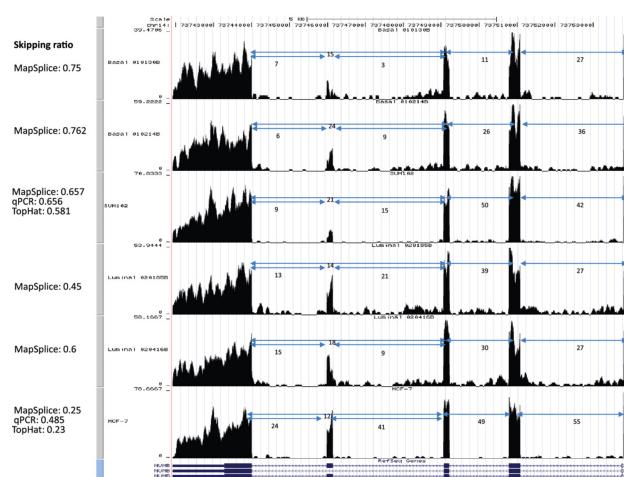
MapSplice: Accurate mapping of RNA-seq reads for splice junction discovery

Kai Wang¹, Darshan Singh², Zheng Zeng¹, Stephen J. Coleman³, Yan Huang¹, Gleb L. Savich⁴, Xiapeng He⁴, Piotr Mieczkowski⁴, Sara A. Grimm⁴, Charles M. Perou⁴, James N. MacLeod³, Derek Y. Chiang⁴, Jan F. Prins² and Jinze Liu^{1,*}

¹Department of Computer Science, University of Kentucky, Lexington, KY 40506, ²Department of Computer Science, University of North Carolina, Chapel Hill, NC 27599-3175, ³Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546-0099 and ⁴Department of Genetics and UNC Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599-7295, USA

Received April 25, 2010; Revised June 21, 2010; Accepted June 28, 2010

> 700 citations, well supported software.



Evaluating the assay

conceptual model of PCR

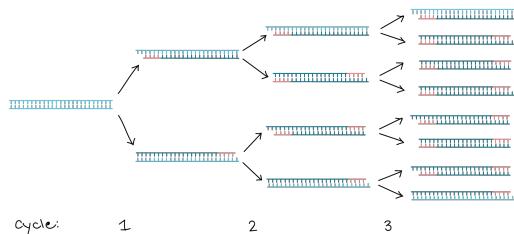


Image from khanacademy.org

[Rutledge and Coté 2003 Nucleic Acids Research](#)

mathematical model of PCR

$$N_C = N_0 \cdot (E + 1)^C$$

- C = PCR cycle number
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- NC = Number of target molecules at cycle number C"
- N0 = Number of molecules when we start the reaction

- 100% Efficiency

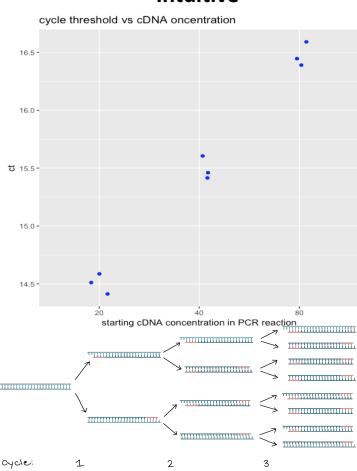
$$N_0 = N_C / (E + 1)^C$$

- Knowing the cycle at which fluorescence crossed a threshold allows us to know the starting amount. In the examples that follow we will show quantities relative to control samples normalized to housekeeping genes.

Evaluating the assay in the lab

- We need to make sure that every cycle of PCR, the concentration of our DNA sequence doubles.

Intuitive



Formally:

$$N_0 = N_t / (E + 1)^{C_t}$$

$$\log(N_0) = \log(N_t) - \log[(E + 1)^{C_t}]$$

$$\log(N_0) = \log(N_t) - \log(E + 1) \cdot C_t$$

$$\log(N_0) = -\log(E + 1) \cdot C_t + \log(N_t)$$

E and Nt are constants – this equation is the equation for a line

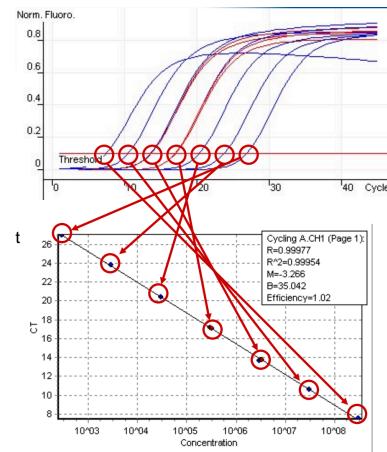
$$\text{Slope} = -\log(E + 1) \rightarrow E_S = 10^{-\text{Slope}} - 1$$

"Es" = efficiency derived from the slope

[Rutledge and Coté 2003 Nucleic Acids Research](#)

Evaluating the assay in the lab cont.

- Serially dilute template
- Plot Ct vs log of concentration of template
- Regress Ct by concentration
- Efficiency of PCR reaction = $10^{(1/\text{slope})} - 1$



Optimize cycling conditions

- Gradient PCR

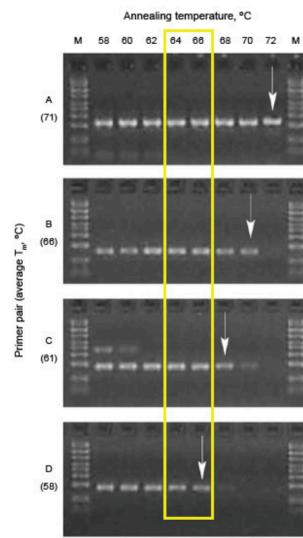
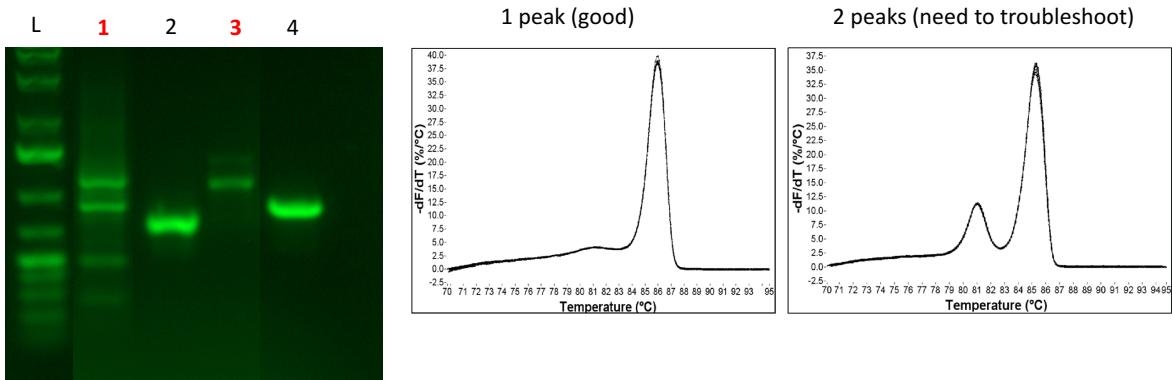


Image from Bio-Rad.com

Evaluate specificity



Run a gel with the same conditions and cycling parameters as your eventual RT-PCR run.

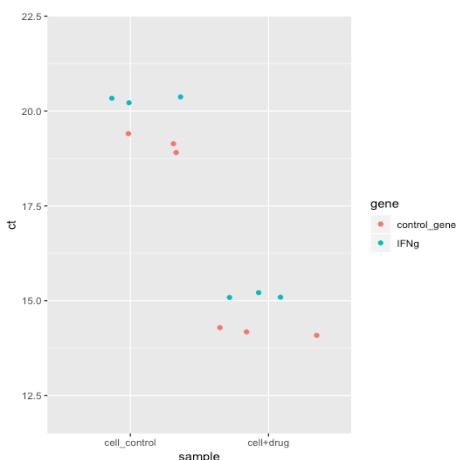
Run a melt curve at the end of your RT PCR experiment run – this is a selection you chose on the instrument and is no additional work.

Block temp is slowly increased at the end of the PCR reaction. Fluorescence monitored throughout. Negative log of the time derivative plotted vs T

[Do I need to do both? -- see this helpful article](#)

Importance of a control gene

- Control gene is for Normalization (normalization for different total amounts of mRNA, total cell numbers etc.)



$$2^{ct(Drug)-ct(CONTROL)} = 35.91$$

Without normalization, this gene appears to have 35-fold increased expression

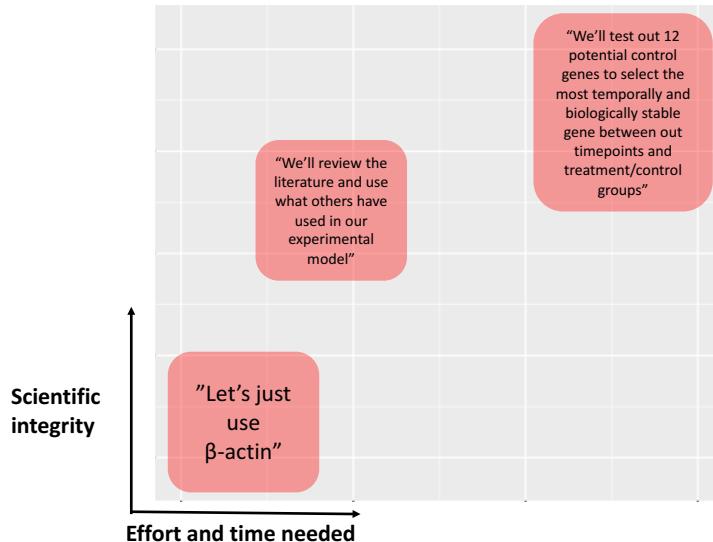
$$2^{\Delta ct(drug)-\Delta ct(CONTROL)} = 0.054$$

After adjusting for the changes in the ct values (expression) of the control gene, we find the fold change value for this gene is ZERO.

How can this happen?

What would happen if our housekeeping gene ct values increased (was less expressed after treatment with drug)?

Selecting a control gene



- [Link to publication: use of multiple normalization genes](#)

Control gene selection

Gene Symbols	Sample 1			Sample 2			Sample 3					
	1	2	3	4	5	6	7	8	9	10	11	12
A	IIS	GAPDH	HPRT1	GUSB	IIS	GAPDH	HPRT1	GUSB	IIS	GAPDH	HPRT1	GUSB
B	ACTB	S2M	GUSB	ITGB3	ACTB	S2M	GUSB	ITGB3	ACTB	S2M	GUSB	ITGB3
C	POK1	RPL20	TPBP	POK1	RPL20	TPBP	POK1	RPL20	TPBP	POK1	RPL20	TPBP
D	UBC	YWHAZ	TPBP	POK1	UBC	YWHAZ	TPBP	YWHAZ	TPBP	YWHAZ	TPBP	YWHAZ
E	CASQ2	SEC14L	SDHB	COL6A3	CASQ2	SEC14L	SDHB	COL6A3	CASQ2	SEC14L	SDHB	COL6A3
F	U2F6	EF1B1	EF1B1	EF1B1	U2F6	EF1B1	EF1B1	EF1B1	U2F6	EF1B1	EF1B1	EF1B1
G	ABLI	RPL11	MPL19	ABLI	RPL11	MPL19	ABLI	RPL11	ABLI	RPL11	MPL19	MPL19
H	PO4	RPL36A	RPL30	RPS17	PO4	RPL36A	RPL30	RPS17	PO4	RPL36A	RPL30	RPS17

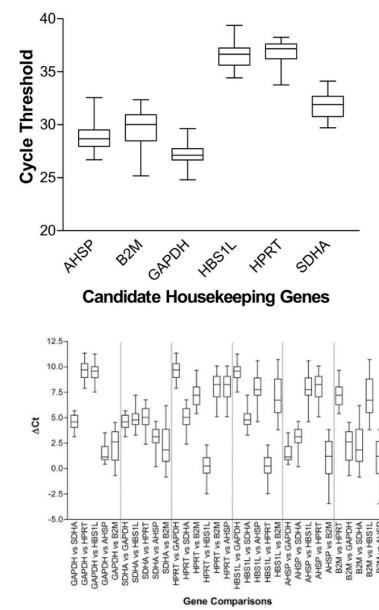
- Note, most of these assays are pre-designed. It is important to select a control assay that is designed to run at the same thermocycling conditions as all other assays.

Great article on the subject:

<https://bmcmolbiol.biomedcentral.com/articles/10.1186/1471-2199-7-33>

Also see: <https://bitesizebio.com/23467/keeping-on-top-of-housekeeping-genes/>

And <https://www.nature.com/articles/srep40290#ref51>



Doing the experiment

Extracting mRNA

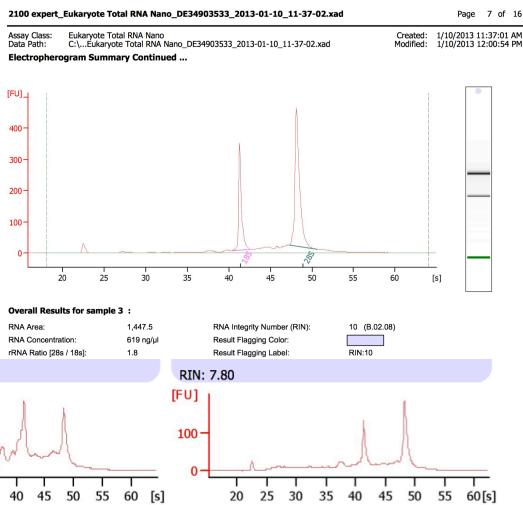
- There are multiple protocols and they will depend on your biological system. In general:
- Work quickly with everything on ice at all times.
- RNases are ubiquitous, work carefully
- Perform QC
- RNase eliminator – use frequently.



Quality control on your RNA

- Optical Density –Why?
 - **Concentration** (essential)
 - Nucleic acids absorb uv light, amount of absorbance (extinction coefficient) inversely correlated with concentration.
 - OD = incident /transmitted
 - A260/280 ratio >2 for RNA
 - Qubit vs Nanodrop...

- Run a RNA gel (Bioanalyzer)



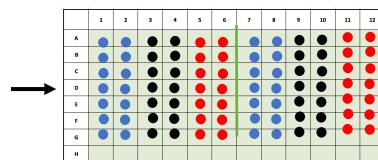
Synthesizing cDNA

- mRNA is a needle in the haystack of ribosomal RNA.
- We are at best capturing 10% of the ~3% of mRNA that is in a given RNA sample.
- Many commercial cDNA synthesis kits are available. If using a plate based high throughput system, it is best to use all reagents recommended by the manufacturer (some chemistries engineer controls at this step, e.g. Qiagen).
- The no RT control: see this article for more information:
<https://bitesizebio.com/37844/control-qrt-rtpcr/>
- External RNA Controls Consortium (ERCC) “Spike-in” controls:
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3091780/>

Setting up your plate + appropriate controls

- -RT control
- Technical replicates or triplicates

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



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

1) + PCR Mastermix to each well

Enzyme
Buffer
dNTP's

How much do I add?

Usually master mixes are formulated as 2X stock concentrations so if we want a 25 μ L reaction volume, add 12.5 μ L.

How much do I add?

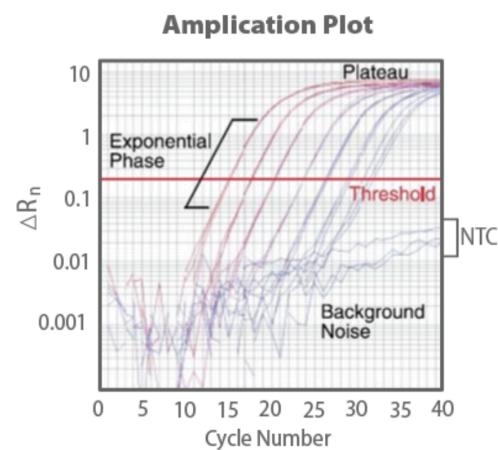
This depends, assay concentrations are variable. usually ~1 μ M.
+2 μ L each oligo
+1 μ L probe*

How much cDNA do I add?

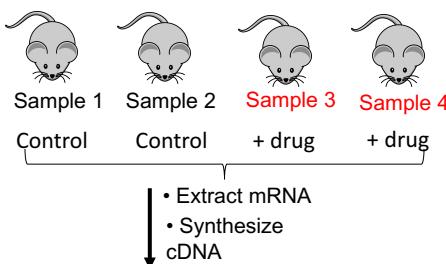
Depends. A good starting point is 50ng cDNA/Rxn
cDNA stock = 1000ng/20 μ L
+ 80 μ L water to all cDNA samples
cDNA stock now = 1000ng/100 μ L (10ng/ μ L)
+5 μ L of cDNA (at 10ng/ μ L) to each well as above

Run the thermocycler

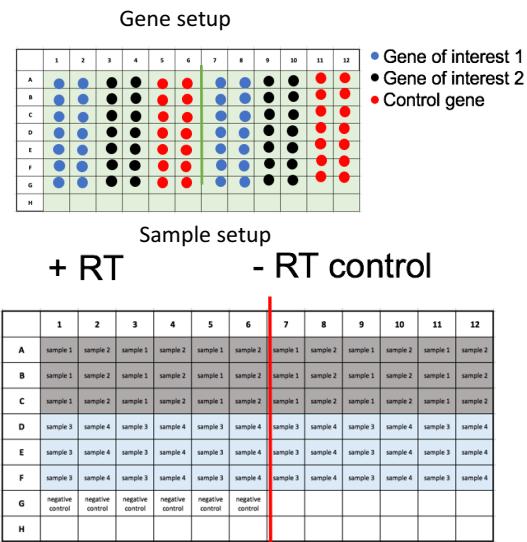
- Look at melt curves for each reaction
- Choosing a threshold*
- Thresholds for multiple runs*



Example Data Analysis – Experiment



Compare transcription of genes of interest in the drug treated vs control groups



►

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

Example Data Analysis

Let's walk through example data in R and Excel.

Recap of this analysis:

Group 1:
healthy controls Group 2:
Disease
vs

$$\text{fold change} = 2^{-(\Delta ct \text{ disease}) - (\Delta ct \text{ healthy controls})}$$

In this example, Δct healthy controls =

$$(Ct(X)^{\text{HD average}} - Ct(\text{ctl})^{\text{HD average}})$$

We then calculated fold change in each treatment (+drug) sample relative to the average expression of the genes in our control samples. In reality you would do this with a larger number of control samples.

Ctl = control gene

X = gene of interest

Note – Analysis could be different in a matched pairs experiment



$$\text{fold change} = 2^{-(\Delta ct \text{ Baseline}) - (\Delta ct \text{ post-drug})}$$

In this case we are directly comparing the Δct at 2 timepoints for each sample

$$(Ct(X)^{\text{baseline}} - Ct(\text{Ctl})^{\text{baseline}}) - (Ct(X)^{\text{post-tx}} - Ct(\text{ctl})^{\text{post-tx}})$$

Ctl = control gene

X = gene of interest

Medium throughput approaches

Advantages

- Great for validating genes predicted to be part of a pathway or module: KEGG, Reactome, GO, GSEA etc.
- The hardest part is done (assay design and validation)
- Get right to the biology

Disadvantages

- Cost
- Usually you have to purchase a minimum quantity of plates/chips.
- Best when you have many samples and > 20 or so genes.

Medium throughput approaches

- Look at up to 370 genes in a single experiment
- Built in controls
- Measure transcription of an entire pathway
- Very simple to set up – add mastermix and cDNA – primers are already in the plate, assay is already validated.

MIQE

- Following these guidelines is a good idea.
- Lays out essential and desirable elements of experiment and analysis

Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

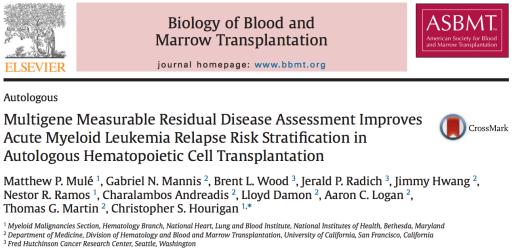
Stephen A. Bustin,^{1*} Vladimir Benes,² Jeremy A. Garson,^{3,4} Jan Helleman,⁵ Jim Huggett,⁶
Mikael Kubista,^{7,8} Reinhold Mueller,⁹ Tania Nolan,¹⁰ Michael W. Pfaffl,¹¹ Gregory L. Shipley,¹²
Jo Vandesompele,⁵ and Carl T. Wittwer^{13,14}

MIQE

Table 1. MIQE checklist for authors, reviewers, and editors.*			
Item to check	Importance	Item to check	Importance
Experimental design	E	qPCR experiments	E
Description of experimental and control groups	E	Power sequences	E
Number within each group	E	RTPrimerID® identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Description	E	Manufacturer identity and model number	D
Volumetric of sample processed	D	Method of synthesis of oligonucleotides	D
Microdissection or nucleic acid extraction	E	Purification method	D
Storage conditions	E	qPCR protocol	E
If frozen, how and how quickly?	E	Complex reaction conditions	E
If fixed, with what and how quickly?	E	Primer (probe), Mg ²⁺ , and dNTP concentrations	E
Sample storage conditions and duration (especially for FFPE samples)	E	Polymerase identity and concentration	E
Sample size, and how determined	E	Reaction identity and conditions	E
Positive control instrumentation	E	Detector identity and conditions of the detector	D
Name of et al and details of any modifications	E	Additives (e.g., Glycerol, DMSO, and so forth)	E
Source of additional reagents used	D	Manufacturer of reagents and catalog number	D
Details of tissue or tissue treatment	E	Complex thermocycling parameters	E
Details of RNA (total, mRNA, rRNA, tRNA, sRNA, or microRNA)	E	Reaction setup (inwell/plate)	D
Nucleic acid quantification	E	Method of qPCR instrument	E
Instrument and method	E	qPCR validation	E
Party (Ag/Rep)	D	Evidence of optimization (from gradient)	D
PCR (qPCR)	D	Slope (not, negative, neutral, or sigmoid)	E
RNA integrity: method/instrument	E	PCR efficiency calculated from slope	E
MRNA or C _t of 3' and 5' transcripts	E	PCR for PCR efficiency or SE	D
Electrophoresis trace	E	PCR for all other purposes	E
Reverse transcriptase (IC, cDNA, spike, or other)	E	Qualification range	E
Reverse transcriptase	E	Validation of LOD	E
Complete reaction conditions	E	Qualification of LOD	D
Amount of RNA and reaction volume	E	Qualification range	D
String oligonucleotide using qSOP and concentration	E	Evidence for LOD	E
Reaction efficiency and concentration	E	Qualification efficiency and LOD of each assay	E
Temperature and time	E	Data analysis	E
Manufacturer of reagents and catalog numbers	D	qPCR analysis program (source, version)	E
C _t with and without reverse transcription	D	Method of C _t determination	E
Qualification of qPCR	D	Qualification range and dispersion	E
qPCR target information	E	Results for NTCS	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Details of normalization	E
Sequence alignment	D	Number and percentage of technical replicates	D
Sequence structure analysis of amplicon	D	Number and stage (reverse transcription or qPCR) of technical replicates	E
Location of primer by exon or intron (if applicable)	E	In silico specificity screen (BLAST, and so on)	E
What splice variants are targeted	E	Inappropriability (mismatches)	E
		Power (mismatches)	D
		Dependability (mismatches)	D
		Sequence alignment	D
		Sequence structure analysis	E
		Location of primer by exon or intron (if applicable)	E
		Software (source, version)	E
		C _t or raw data submitted with ROM	D

* All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from RTPrimerID®, information on qPCR target oligonucleotides, protocols, and validation is available from that source.
† Denatured denaturing gradient gel electrophoresis, nuclease protection assay, and sequencing gel analysis.
‡ Assessing the absence of DNA with a no-reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as DNA free, inclusion of a no-reverse transcription control is desirable but no longer essential.
§ Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it cannot be an essential requirement; use of such assays is discouraged.

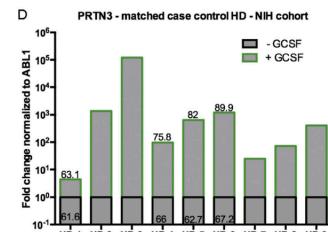
Visualization Examples



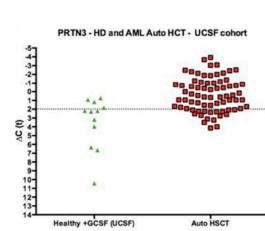
MIQE information included in supplement

See supplement in:

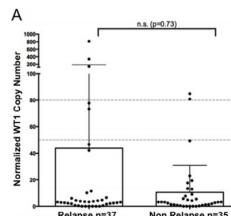
<https://www.ncbi.nlm.nih.gov/pubmed/27544285>



Fold change relative to healthy normalized to control gene.
Matched healthy and treated pairs from the same individual.



ΔCt reverse axis; fold change calculated relative to median Level in 12 healthy.



Interpolated copy number using plasmid standards

Questions?