The Fleischman Lab

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Title	**Analysis of Gene Expression via qPCR (Pfaffl Method)**	

Introduction	Quantitative PCR (qPCR) is a variation of traditional PCR using fluorescent dyes (e.g. SYBR) or probes (e.g. TaqMan) to allow detection and quantification of DNA after each individual cycle. qPCR may be paired with reverse transcription PCR (RT-PCR) to enable the quantification of mRNAs for gene expression analysis. This variation is called quantitative reverse transcription PCR (qRT-PCR). Genes of interest, called target genes, are normalized to reference genes whose expression levels are not changed across conditions using the Pfaffl method. The Pfaffl method is a hybrid of the standard curve and $\Delta\Delta C_P$ methods. It uses a standard curve to calculate the primer efficiency – that is, what percentage of the DNA is actually replicated with each cycle – and takes the differences in primer efficiencies into account during the fold change calculation. This reduces the workload of the standard curve method as knowing the efficiency negates the need for a standard curve with each plate, and it is more quantitative than $\Delta\Delta C_P$ as the primers are not assumed to have identical efficiencies. For more information, please see the following resources: www.gene-quantification.com www.microbiologybook.org/pcr/realtime-home.htm
Materials	1. cDNA (see our RNA Extraction and cDNA Synthesis protocol) 2. Primers for target and reference genes (common reference genes include 18S, GAPDH, and β-actin). Primers should be designed to span exon-exon junctions to prevent gDNA contamination. 3. Nuclease-free water 4. Maxima SYBR Green qPCR Master Mix (2x), ROX provided in separate tube 5. 96 well plates, white, for the LightCycler 480 (we use USA Scientific cat# 1402-9990) 6. Optically-clear sealing film (we use Bio-Rad Microseal B, cat# MSB1001) 7. Roche LightCycler 480 8. REST 2009 software (available on the LightCycler computer and the lab desktop) 9. 2% DNA gel and low-range DNA ladder (25-700bp) 10. Micropipettes and barrier tips 11. Plate spinner

Protocol	**A. Determine primer efficiencies**	*Notes*
1.	Prepare 1:5 serial dilutions of cDNA:	*Mix each tube well before preparing the next dilution.*

Tube #	cDNA	H₂O	Conc.
1	75 µL cDNA (100 ng/µl)	--	1
2	15 µl tube 1	60 µl	0.2
3	15 µl tube 2	60 µl	0.04
4	15 µl tube 3	60 µl	0.008
5	15 µl tube 4	60 µl	0.0016
6	15 µl tube 5	60 µl	0.00032
7	15 µl tube 6	60 µl	0.000064
8	--	60 µl	0

In the LightCycler software, denote the wells as standards and input the corresponding concentrations. Label the 0 concentration wells as negative controls.

2.	Prepare master mix as follows:			It may be necessary to use 4 µl (400ng) of cDNA per reaction for reference genes or low-expression genes. If so, adjust the volume of water to 7 µl per well and transfer 21µl of master mix per well.
	Reagent	**1 well**	**26 wells**	
	2x SYBR Green qPCR master mix	12.5 µl	325 µl	
	F primer (10 mM)	0.75 µl	19.5 µl	
	R primer (10 mM)	0.75 µl	19.5 µl	
	Nuclease-free H_2O	9 µl	234 µl	
	cDNA standards	2 µl	--	
3.	Transfer 23µl master mix to each well. Add 2µl of standard per well. Seal plate well and briefly centrifuge in plate spinner.			*Each standard or control should be prepared in triplicate.*
4.	Place plate into the LightCycler. In the LightCycler software, select "New Experiment from Template" and choose "SYBR Green I 96-II." Adjust the sample volume as needed and set the cycling parameters as follows: 1x — 95°C — 10 min 50x — 95°C — 15sec — 60°C — 30 sec — 72°C — 30 sec			*The "SYBR Green I 96-II" template is set to read after each extension and includes a melt-curve analysis cycle at the end.* *Adjust the annealing temperature and extension time as needed.*
5.	Perform the following quality control steps before calculating the efficiency: 1. Check melting curves to make sure that only one product is amplified. 2. Remove negative wells from the sample subset. 3. Remove samples detected before cycle 10. 4. Remove samples if multiple dilutions have the same C_p (usually the more dilute concentrations). 5. Slopes should be parallel in log view. 6. Ensure that there are at least 5 concentrations in duplicate or triplicate that pass the above criteria.			*Melting curve analysis, coupled with designing primers to span exon-exon junctions, ensures the absence of gDNA contamination.*
6.	Under the Analysis tab, select the standards subset and click "calculate" to generate a standard curve and calculate the efficiency.			*The correlation coefficient should be 0.99 or above.*
7.	Record the efficiency on the primer spec sheet and store it in the primer binder.			*A "good" efficiency is between 1.90-2.00.*
8.	Run the product on a 2% agarose gel to confirm the product size is correct.			
	B. qPCR			
1.	Prepare master mix as follows:			*Be sure to make enough master mix for 3 negative control wells and at least 1 additional well. For larger volumes, make enough for 5-10 extra wells.*
	Reagent	**1 well**		
	2x SYBR Green qPCR master mix	12.5 µl		
	F primer (10 mM)	0.75 µl		
	R primer (10 mM)	0.75 µl		
	Nuclease-free H_2O	9 µl		
	cDNA (100 ng/µl)	2 µl		

	2.	Transfer 23µl master mix to each well. Add 2µl of cDNA (100 ng/µl) to sample wells. Add 2µl of nuclease-free H$_2$O to negative control wells. Seal plate well and briefly centrifuge in plate spinner.	*Each sample or control should be prepared in triplicate.*
	3.	Place plate into the LightCycler. In the LightCycler software, select "New Experiment from Template" and choose "SYBR Green I 96-II." Adjust the sample volume as needed and set the cycling parameters as follows: <table><tr><td>1x</td><td>95°C</td><td>10 min</td></tr><tr><td>45x</td><td>95°C</td><td>15sec</td></tr><tr><td></td><td>60°C</td><td>30 sec</td></tr><tr><td></td><td>72°C</td><td>30 sec</td></tr></table>	*The "SYBR Green I 96-II" template is set to read after each extension and includes a melt-curve analysis cycle at the end.* *Adjust the annealing temperature and extension time as needed.*
	4.	Set up the plate layout using the "Sample Editor" tool: • Input the gene name, primer efficiency, and target/reference status for each sample. • Label the negative control wells for each primer set. • Utilize the replicates feature to simplify analysis.	
	5.	Return to the "Experiment" tab and click "Start Run."	
	C. Analysis		
	1.	Under the "Analysis" tab, choose the "Advanced Relative Quantification" method. Do not make any changes to the default settings.	*Advanced relative quantification calculates fold changes via the efficiency (Pfaffl) method. Basic relative quantification uses ΔΔC$_P$.*
	2.	Use the following equation to calculate the fold change in target gene expression relative to controls: $$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_{P\,\text{target}}\,(\text{control}-\text{sample})}}{(E_{\text{Ref}})^{\Delta C_{P\,\text{Ref}}\,(\text{control}-\text{sample})}}$$	
	3.	Alternatively, the REST 2009 software may be used. REST 2009 is especially helpful when using multiple reference genes.	*For more information, visit www.gene-quantification.de/rest-2009.html*