Analyzing your QRT-PCR Data

The Comparative C_T Method ($\Delta\Delta C_T$ Method): Data Analysis Example

The following table presents data from an experiment where the expression levels of a target (c-*myc*) and an endogenous control (GAPDH) are evaluated. The levels of these amplicons in a series of drug-treated samples are compared to an untreated calibrator sample.

The number of experimental replicates run in a study directly affects the downstream data analysis (i.e. are the observed fold-differences in nucleic acid statistically significant?). Careful consideration must be exercised when determining the number of experimental replicates that will be tested in a relative quantitation study. Mean C_T values and standard deviations are used in the $\Delta\Delta C_T$ calculations. In this example, each sample type was run in triplicate. Each sample C_T mean was calculated and standard deviations were calculated for each mean C_T value.

Table 11: Fold change expression of c-myc after treatment, calculated by $\Delta\Delta C_T$ method

Sample	**c-*myc* Average C_T**	**GAPDH Average C_T**	**ΔC_T c-*myc*-GAPDH**	**$\Delta\Delta C_T$** **ΔC_T treated -ΔC_T untreated**	**Fold difference in c-*myc* relative to untreated = $2^{-\Delta\Delta CT}$**
untreated	30.49±0.15	23.63±0.09	6.86±0.19	0.00±0.19	1 (0.9-1.1)
Drug treatment A	27.03±0.06	22.66±0.08	4.37±0.10	−2.4±0.10	5.6 (5.3-6.0)
Drug treatment B	26.25±0.07	24.60±0.07	1.65±0.10	−5.11±0.10	37 (34.5-39.7)
Drug treatment C	25.83±0.07	23.01±0.07	2.81±0.10	−3.95±0.10	16.5 (15.4-17.7)

Calculate the ΔC_T value.

Open data up in an excel file:

Based on consistency of amplification, choose either 18S or GAPDH as your endogenous control.

Calculate the average CT for your endogenous control and each experimental gene as follows: =AVG(select boxes with values of interest)

The ΔC_T value is calculated by:

For example, subtraction of the average GAPDH C_T value from the average c-*myc* C_T value of the untreated sample yields a value of 6.86.

ΔC_T untreated = 30.49 – 23.63 = 6.86

Calculate the standard deviation of C_T values and variance of the ΔC_T value.

The variance of the ΔC_T is calculated from the standard deviations of the target and reference values using the formula:

$s = (s_1^2 + s_2^2)^{1/2}$; where $X^{1/2}$ is the square root of X

and s= standard deviation. For example, to calculate the standard deviation of the untreated sample ΔC_T value:

$s_1 = 0.15$ and $s_1^2 = 0.022$ [in excel for s_1 simply =STD(select boxes with values of interest)]

$\Delta C_T = C_T$ target $- C_T$ reference

$s_2 = 0.09$ and $s_2^2 = 0.008$ $s = (0.022 + 0.008)^{1/2} = 0.17$

Therefore, ΔC_T untreated = (30.49 ±0.15) – (23.63 ±0.09) = 6.86 ±0.17

Calculate the $\Delta\Delta C_T$ value.

The $\Delta\Delta C_T$ is calculated by:

$\Delta\Delta C_T = \Delta C_T$ test sample – ΔC_T calibrator sample

For example, subtracting the ΔC_T of the untreated from the ΔC_T of Drug Treatment A yields a value of –2.5.

$\Delta\Delta C_T = 4.37 - 6.86 = -2.5$

Calculate the standard deviation of the $\Delta\Delta C_T$ value.

The calculation of $\Delta\Delta C_T$ involves subtraction of the ΔC_T calibrator value. This is subtraction of an arbitrary constant, so the standard deviation of the $\Delta\Delta C_T$ value is the same as the standard

deviation of the ΔC_T value.

Therefore, $\Delta\Delta C_T$ Drug Treatment A sample = $\Delta\Delta C_T = 4.37 \pm 0.10 - 6.86 \pm 0.17 = -2.5 \pm 0.10$

Standard deviation of the $\Delta\Delta C_T$ value is the same as the standard deviation of the ΔC_T value

Incorporating the standard deviation of the $\Delta\Delta C_T$ values into the fold- difference.

Fold-differences calculated using the $\Delta\Delta C_T$ method are usually expressed as a range, which is a result of incorporating the standard deviation of the $\Delta\Delta C_T$ value into the fold- difference calculation.

The range for $target_N$, relative to a calibrator sample, is calculated by: $2^{-\Delta\Delta Ct}$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s is the standard deviation of the $\Delta\Delta C_T$ value.

For example, the drug-treatment A sample has a 5.3 to 6.0-fold difference in expression of the $target_N$ relative to the untreated (calibrator) as indicated below.

$\Delta\Delta C_T + s = -2.5 + 0.1 = -2.4$

$2^{-\Delta\Delta Ct} = 2^{-(-2.4)} = 5.3$

and

$\Delta\Delta C_T + s = -2.5 - 0.1 = -2.6$

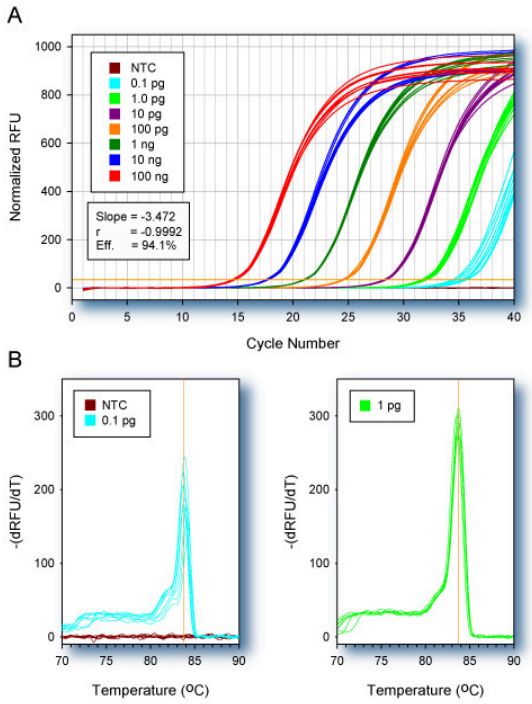
$2^{-\Delta\Delta Ct} = 2^{-(-2.6)} = 6.0$

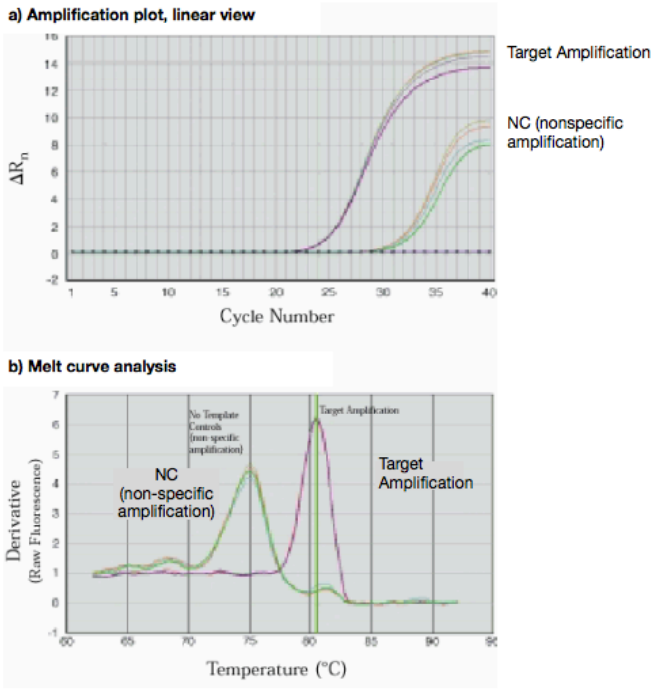
At this point to get the true fold change, we take the log base 2 of this value to even out the scales of up regulated and down regulated genes. Otherwise upregulated has a scale of 1-infinity while down regulated has a scale of 0-1.

Once you have your fold changes, you can then look into the genes that seem the most interesting based on this data. There are hundreds of resources online that will tell you what the gene does, what pathways it is involved in, etc. We will start by going to the website created by the Barres Lab team from Stanford that wrote the RNA Seq paper on the various isolated cells in the NS.

Find the link below: Works better in Safari
http://web.stanford.edu/group/barres_lab/brain_rnaseq.html

QRT-PCR – Analyzing your Data – Further Notes for consideration and questions for discussion.





Based on your amplification plots, the computer will determine the best threshold to set whereby the most amplification plots are in a linear growth phase. Once the threshold is set, the cycle at which each amplification curve crossed that threshold is determined and assigned as the CT for that sample. With this data, you will work in groups and proceed to calculate the change in expression values for each gene in liver and brain tissue.

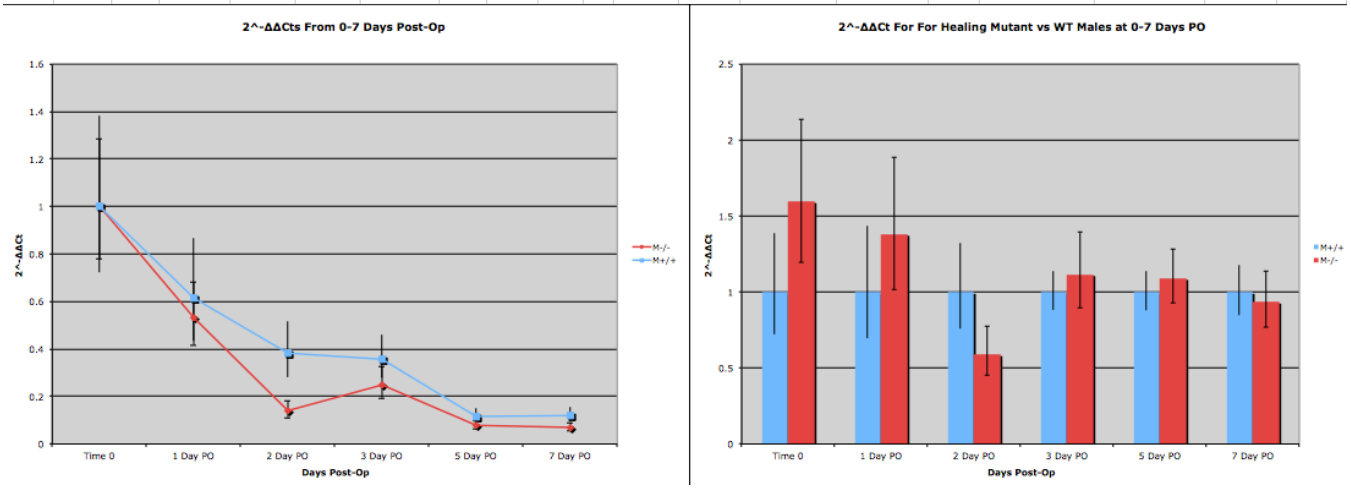
The CT data is used to determine the amount of each gene/mRNA present relative to each sample. The table below shows the average CT results for the expression of VEGF in healing Achilles tendons in mice immediately post-op and 1 day post-op, and how these CTs are manipulated to determine ΔCT, ΔΔCT, and the relative amount of VEGF mRNA in terms of fold change. ΔCT is calculated by subtracting the CT for VEGF for the sample from the CT for the endogenous control (in this case 18S). The calculation of ΔΔCT involves subtraction by the ΔCT reference sample value (in this case from the wild type for one calculation and from day 0 for a second calculation). The range given for VEGF in wild type mice relative mutant mice is determined by evaluating the expression: $2^{-\Delta\Delta CT}$

Data can be graphed in a variety of ways, once expression has been determined, for easier visualization. Below are examples of how the data in the table may look. Only the Day0 and Day1 points are shown in the table while the graphs show the data through Day7 post-op. The scatter plot displays the difference in expression of VEGF in both the wild type and mutant mice using the Day 0 data for each mouse type as the reference. Notice that overall expression decreases for both mice types as healing progresses, though the decrease is greater for the mutants. The bar graph shows the difference in expression of VEGF in the wild type and mutant mice at each day post-op using the wild type for that day as the reference. Notice that the wild type mice have the lower expression on each day except day 2 and day 7, when their expression is higher than the mutants.

Healing Female QRT VEGF

Sample #	Time 0		Avg Time0 Ct	Avg 18S Time	ΔCt per smp	Avg ΔCt per gr.	Std Dev.	Variance	SE^2 (var/n)	ΔΔCt	SSE^2 (sum var/n)	SQRTSSE^2	ΔΔCt+SQRTSSE^2	ΔΔCt-SQRTSSE^2	2^-ΔΔCt	2^-ΔΔCt+SQRTSSE^2	2^-ΔΔCt-SQRTSSE^2	Neg Error Bar	Pos Error Bar
AT	Female -/-	Duplicate																	
3265	30.43	30.38	30.405	24.365	6.04	6.075	0.804	0.64642	0.0646	-0.7	0.17473081	0.4180081	-0.257491859	-1.093508141	1.59715	1.195398684	2.133923032	0.401751523	0.536772825
3419	29.75	30.07	29.91	24.97	4.94					0	0.12928444	0.3595615	0.359561461	-0.359561461	1	0.77940146	1.283035831	0.22059854	0.283035831
3424	30.62	30.35	30.485	25.2	5.285														
RF3486	30.33	30.43	30.38	23.855	6.525														
3526	30.61	30.87	30.74	25.03	5.71														
3582	30.99	30.77	30.88	25.275	5.605														
3671	30.47	30.74	30.605	23.27	7.335														
3739	29.91	30.3	30.105	24.62	5.485														
3748	30.36	30.37	30.365	23.52	6.845														
4202	32.1	31.54	31.82	24.84	6.98														
	Female +/+	Duplicate																	
2909	29.96	30.06	30.01	24.335	5.675	6.7505	1.04923	1.10089	0.1101	0	0.22017717	0.4692304	0.469230398	-0.469230398	1	0.722349831	1.384370782	0.277650169	0.384370782
2930	30.99	30.65	30.82	25.145	5.675														
2935	31.04	30.82	30.93	25.49	5.44														
2962	30.66	31.12	30.89	23.98	6.91														
2997	30.4	30.49	30.445	23.105	7.34														
3023	30.25	30.32	30.285	23.535	6.75														
3067	31.95	31.98	31.965	24.345	7.62														
3068	29.74	30.29	30.015	23.76	6.255														
3096	32.23	32.69	32.46	23.6	8.86														
3179	31.48	31.26	31.37	24.39	6.98														

Sample #	1 Day PO		Avg d1 Ct	Avg 18S d1 Ct	ΔCt per smp	Avg ΔCt per gr.	Std Dev.	Variance	SE^2 (var/n)	ΔΔCt	SSE^2 (sum var/n)	SQRTSSE^2	ΔΔCt+SQRTSSE^2	ΔΔCt-SQRTSSE^2	2^-ΔΔCt	2^-ΔΔCt+SQRTSSE^2	2^-ΔΔCt-SQRTSSE^2	Neg Error Bar	Pos Error Bar
AT	Female -/-	Duplicate																	
M2287	31.24	31.5	31.37	24.425	6.945	6.987	0.78676	0.619	0.0619	-0.5	0.19891181	0.4459953	-0.020504702	-0.912495298	1.38175	1.014314258	1.882298324	0.367438986	0.500545079
2303	29.27	29.52	29.395	23.115	6.28					0.91	0.12654178	0.3557271	1.267727111	0.556272889	0.53145	0.415313562	0.68005678	0.116134275	0.148608943
**2996	31.23	31.26	31.245	24.62	6.625														
3083	30.76	31.01	30.885	22.665	8.22														
3640	29.37	29.66	29.515	23.62	5.895														
3658	29	29.38	29.19	22.43	6.76														
3693	28.39	28.64	28.515	21.6	6.915														
3701	30.25	30.2	30.225	22.86	7.365														
3801	31.17	31.42	31.295	22.96	8.335														
3818	31.01	30.42	30.715	24.185	6.53														
	Female +/+	Duplicate																	
2174	29.32	29.5	29.41	23.03	6.38	7.4535	1.17052	1.37012	0.137	0	0.2740245	0.5234735	0.523473495	-0.523473495	1	0.69569483	1.43741186	0.30430517	0.43741186
2175	30.62	31.48	31.05	23.18	7.87					0.7	0.24710083	0.4970924	1.200092379	0.205907621	0.61429	0.435247411	0.866993077	0.179046079	0.252699587
2195	29.78	30.02	29.9	22.965	6.935														
2196	28.7	28.88	28.79	22.845	5.945														
2212	29.29	29.4	29.345	23.515	5.83														
2226	31.27	31.62	31.445	23.62	7.825														
2231	32.39	32.02	32.205	22.88	9.325														
2276	32.15	31.84	31.995	23.39	8.605														
2279	31.17	31.25	31.21	22.83	8.38														
2283	31.28	31.02	31.15	23.71	7.44														



Once calculations are done, you can further investigate the genes that you are still interested in by going online and finding databases that help you determine gene function and rolls in pathways. There are many tools available free online - Gene Expression Omnibus (GEO), Online Mendelian Inheritance in Man (OMIM), and Biocyc, just to name a few. We will investigate this a little bit together if time today and finish up on the last day.

Questions for Discussion

1. Which genes were most were more highly expressed in the brain?

2. Which genes were more highly expressed in the liver?

3. Based on the functions of these genes, does it make sense that they are differentially expressed in these two organs? Use two of the genes to help explain why or why not.