Informational outline:

1. Delta Ct and Delta Delta Ct are widespread techniques used for comparing transcript accumulation between experimental groups (Schmittgen and Livak 2008).
   1. Transcript accumulation can be used for a variety of metrics, including gene expression and viral titer (citation needed)
2. “Ct” or “Cq” represents cycle threshold. A cycle threshold is the number of amplification cycles during PCR are necessary to reach an *a priori* concentration of DNA (citation needed)
   1. For example, if a Ct value is 50, this means there was significantly more starting genetic material than a Ct of 10. This is incorrect, Ct is inversely proportional to amplicon material. If there is more material to start, the threshold will be reached in fewer cycles.
3. Ct values are transformed using the formula “2^-delta delta ct” to compare relative transcript accumulation between experimental treatments (citations needed)
   1. The first “delta” is a comparison of the focal gene of interest to an endogenous housekeeping gene (Jain et al. 2008).
      1. This normalizes the ct value since the reaction is done in parallel and accounts for variance in the quality of a sample (citation needed).
   2. The second “delta” is a comparison between a control experimental condition.
      1. The difference between experimental condition and control represents the effect of a treatment on gene expression (citation needed), viral titer (citation needed)
4. The 2^ transformation is supported by mathematical theory surrounding the design of a thermal cycler and how gene expression and cDNA amplification functions (citation needed)
   1. This transformation gives a rough approximation of starting genetic content since the inverse root of a ct of 50 will be lower than a ct of 10
5. Currently how these calculations are completed are problematic, not due to the formula or biology, but with respect to how *variance* is treated
   1. In an experiment and with real-world equipment, CT values are going to randomly vary, even with “perfect” controls
   2. In addition to this, control and housekeeping gene reference will vary as well
6. For example, the formula can be thought of 2^-(((A+e)-(B+e))-((C+e)-(D+e)))
   1. Where A = treatment housekeeping gene
   2. B = treatment gene ct
   3. C= control housekeeping gene
   4. D= control gene ct
   5. e= experimental error
7. At each iteration of the calculation, there is an error term that should be recognized. This error is compounded dramatically at each step of the calculation since an exponent is applied at the end.
   1. Traditionally statistics or comparisons used to evaluate treatment effects occur on the final delta delta ct values (but see Rao et al. 2013).
8. There are some statistical attempts to model delta ct values, including in SAS (Yaun et al. 2006), but this does not deal with error propagation or more sophisticated linear models or multivariate tests
9. The solution is to do analysis on the original CT values
   1. The CT values in our experiments are usually normally distributed, and thus compatible with ANOVA/MANOVA models
   2. The mean and standard error of the ct values can be derived from parameter estimates
      1. These parameter estimates can be transformed using 2^-delta delta ct formula, reducing the potential impact of outliers or extreme observations, as well as reducing the impact of measurement error
10. In this paper we give several examples where this method produces more intuitive results
    1. There are some examples where a “real” difference would be missed (Type I error)
    2. There are other examples where a “false” difference is supported by comparing means and variances calculated at the end of the 2^-delta delta ct formula (Type II error)
11. There are some papers that have shown how to model delta ct (rtPCR data) in SAS
    1. 2011 -
    2. 2009 -
12. We also use a simulation approach to show error rate (e.g. O’Hara and Kotze 2010)
13. Furthermore, an ANOVA/MANOVA approach in a statistical software environment like R is less sensitive to human error by hand calculations in excel
    1. Excel also has other notable issues with rounding and calculations of variance
14. We provide source code, annotated examples used to calculate gene expression and viral titer
    1. Data can be plugged into this code in a relatively straightforward way and does not require highly specialized knowledge of statistical theory or computer programming (but basic R knowledge is necessary)
    2. We have also provided multiple examples of data visualization based on these and highlighted several issues that can arise
15. There are some other packages, but they are mainly for error checking and transformation (Perkins et al. 2012)
    1. The goal was not to change the way gene expression is quantified, but instead to come up with a faster and more accurate way to analyze and visualize transcript accumulation data in R
    2. The most common papers on using delta-delta CT calculate values by hand (e.g. in excel) or use obtuse math.

Preliminary references (stored in zotero under delta ct paper heading)

Jian, B. *et al.* Validation of internal control for gene expression study in soybean by quantitative real-time PCR. *BMC Molecular Biology* **9**, 59 (2008).

Perkins, J. R. *et al.* ReadqPCR and NormqPCR: R packages for the reading, quality checking and normalisation of RT-qPCR quantification cycle (Cq) data. *BMC Genomics* **13**, 296 (2012).

Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3, 1101–1108 (2008).