Real time and Quantitative (RTAQ) PCR

or….. for this audience…

“ so I have an outlier and I want to see if it really is changed”

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I’ve got 30 minutes- what am I going to say?

- What I am going to tell you
  - Overview of the technology
  - Overview of Software based quantitation
  - Assay Designs
  - Types of Quantitative analysis
- What I am not going to tell you
  - How to design primers
  - How to use different types of PCR machines
  - All there is to know- this is just a brief intro

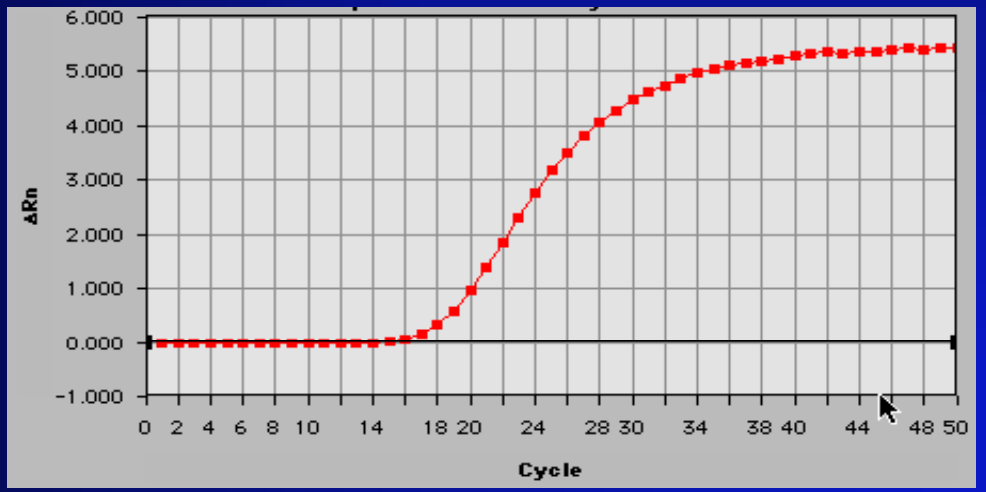
Conventional RT-PCR

- Reverse transcription (RT) of cDNA from RNA
  - Oligo d(T)
  - Random Hexamer
  - Gene specific primer
- PCR amplification of a defined DNA sequence from cDNA
  - Traditionally a 3-phase multi-cycle reaction
  - Denaturation, annealing, primer extension
- Electrophoretic separation of PCR products (amplicons)
  - PCR for specific number of cycles
  - Run products on an agarose gel

Real-time PCR is kinetic

- Detection of “amplification-associated fluorescence” at each cycle during PCR
- No gel-based analysis at the end of the PCR reaction
- Computer based analysis of the cycle-fluorescence time course

Increasing fluorescence



Linear plot

PCR cycle

Specialized Instrumentation is needed

- 96-well format
  - ABI SDS 7700
  - ABI 7000
  - ABI 5700
  - BioRad Icycler
  - Stratagene Mx4000

- Capillary tube format-
  - Roche Light cycler

- 384-well format HT systems
  - ABI SDS 7900

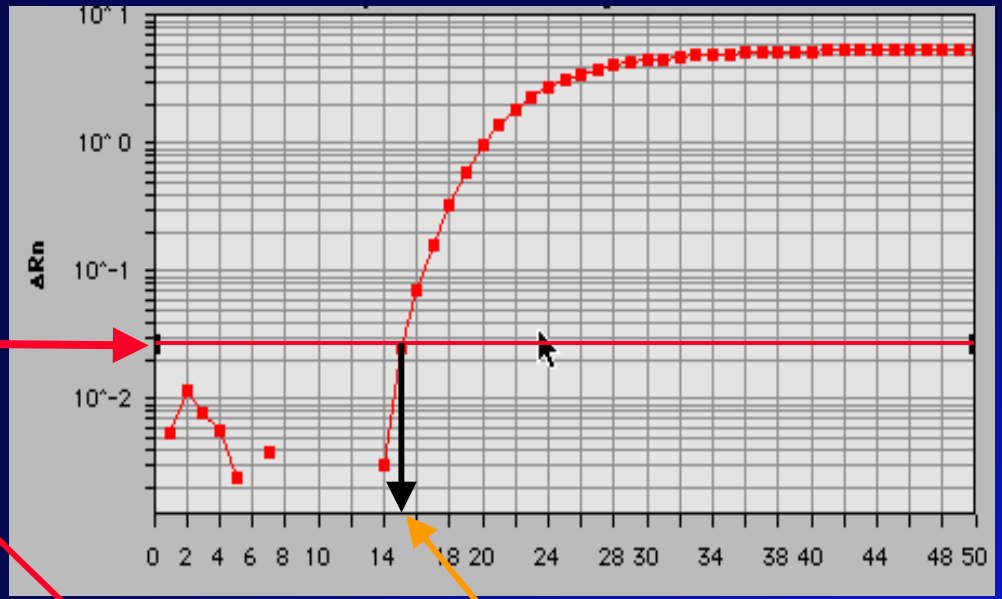
Software-based analysis

- Data acquisition
  - Fluorescence in each well at all cycles.
  - Software-based curve fit of fluorescence vs cycle #
- Threshold
  - Fluorescence level that is significantly greater than the baseline.
  - Automatically determined/User controlled
- C_T (Cycle threshold)
  - Cycle at which fluorescence for a given sample reaches the threshold.
  - C_T correlates, inversely, with the starting concentration of the target.
  - Varies with threshold- not transferable across different plates

Software-based analysis

Increasing fluorescence

Threshold (10sd)

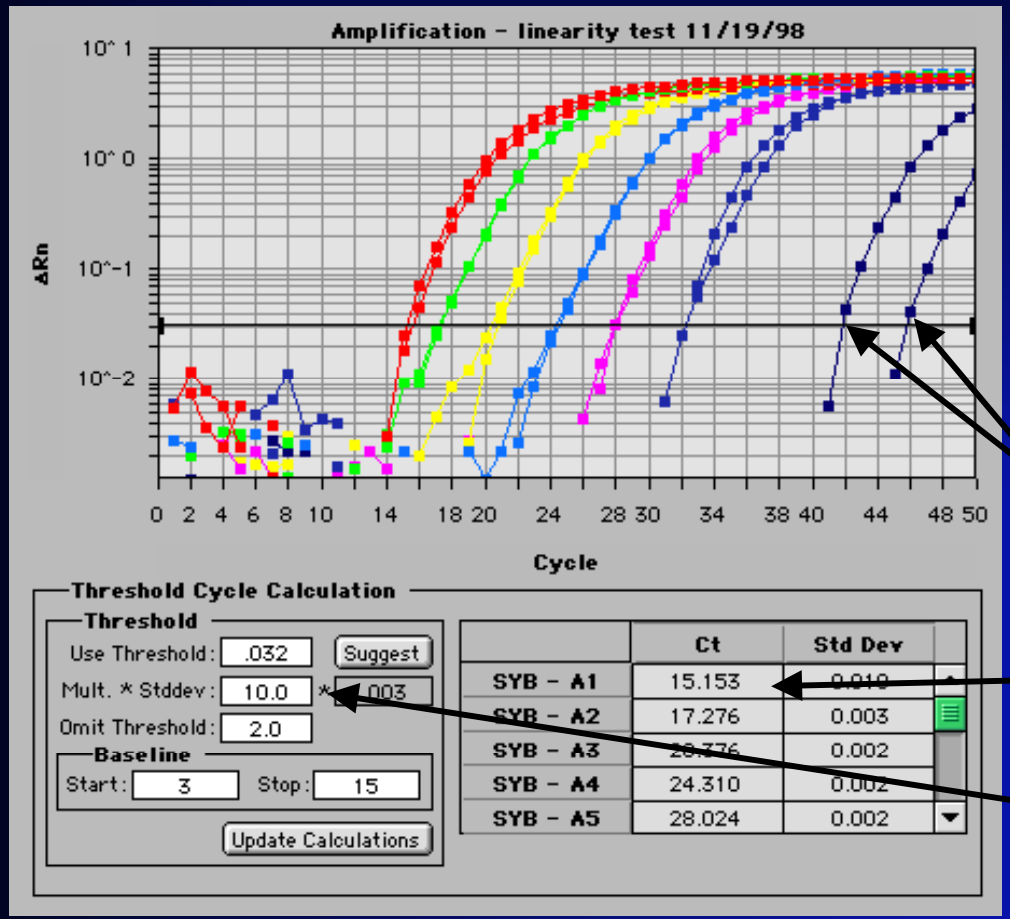


Baseline

C_T (cycle threshold)

Log plot

Example analysis of CYP1A1



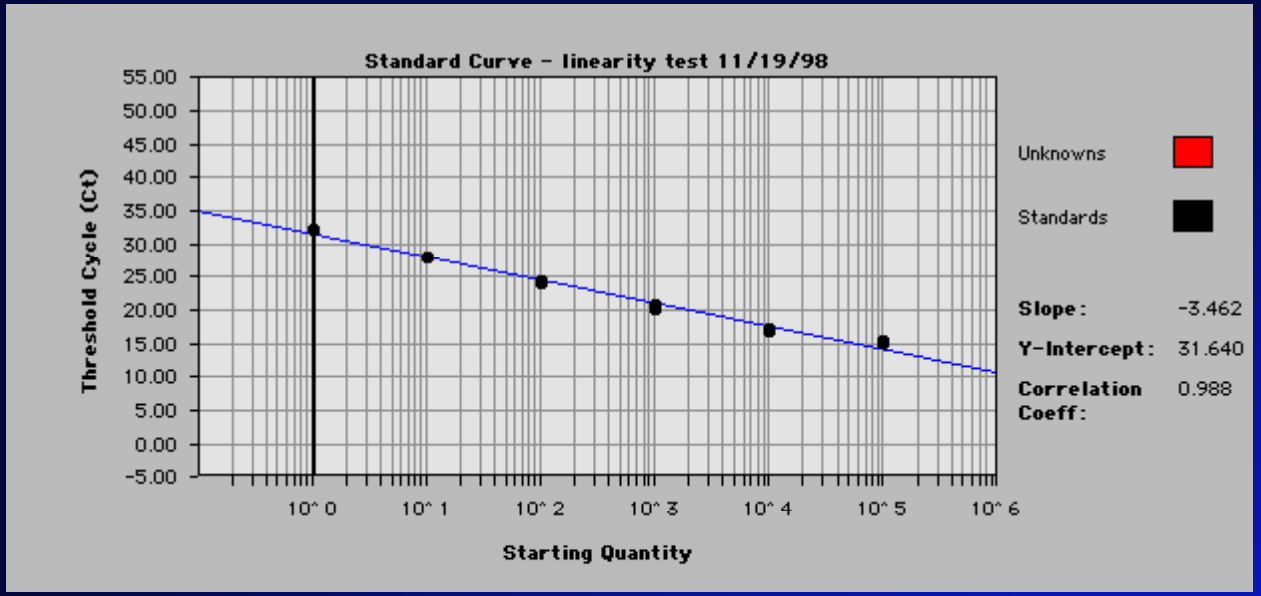
- SYBR Green detection
- 10-fold dilution series

No RNA controls

C_T values

Threshold

Linear range for CYP1A1 by RTAQ-PCR



95% E

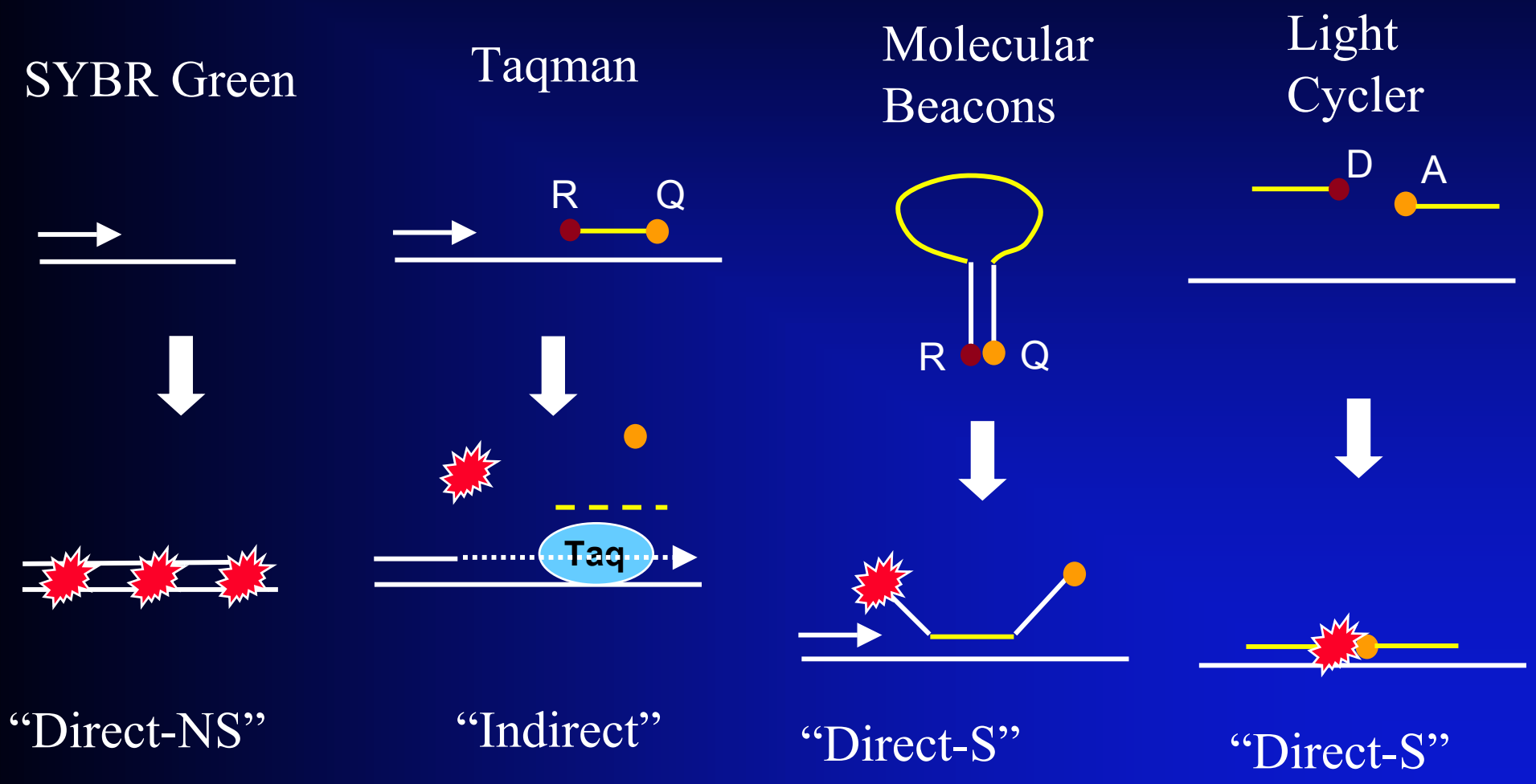
1pg-100ng Total RNA

- C_T correlates, inversely, with the starting concentration of the target.

Amplification-associated fluorescence

- Fluorescent dye
  - Detects accumulation of DNA (SYBR green)
- FRET (Fluorescent Resonance Energy Transfer) based.
  - Detect accumulation of a fluorescent molecule (Taqman)
  - Detect accumulation of specific DNA product-(Molecular Beacons, LC)

Methods of fluorescence detection

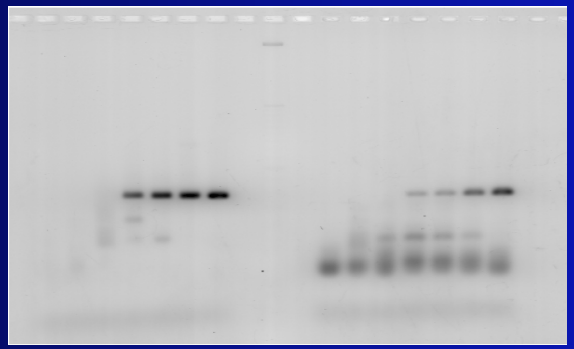


Dye-based

- Upside
  - Quick
  - No primer/probe optimization
- Downside
  - Non-specific
- Application
  - Many genes few samples
  - "That sounds like just the ticket for checking my microarray data"

FRET-based

- Upside
  - Specific
- Downside
  - Primer/probe optimization
  - More costly
- Application
  - Many samples few genes

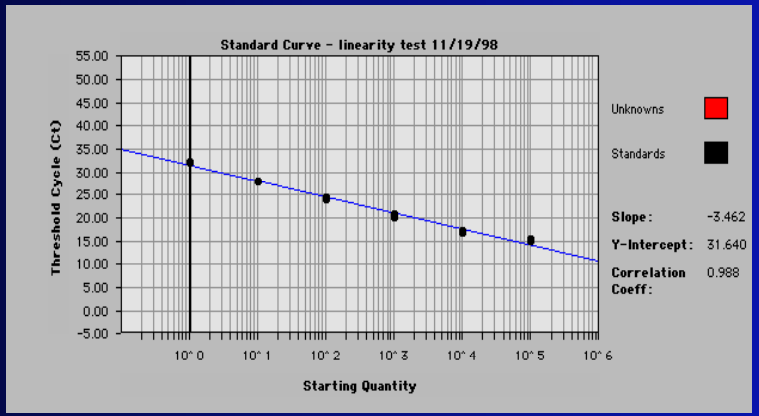


Primer sets

- There are no large databases of primer sets
  - We attempted to get a database going early on with little success.
- Commercial pre-developed assays are available
- Dye-based
  - Existing primers could be adapted but may not be the best
- Probe-based
  - Unlikely that existing PCR primer pairs will be suitable
  - Primers and probes designed to match specific reaction conditions.
- Primer design
  - Primer Express™ software facilitates design (for ABI system) (SCL copies)
  - No optimization of primer annealing temperature
  - Multiple primer sets can use same default cycling conditions on SDS7700

Quantitation

- Absolute quantitation (eg. copies/ug RNA)
  - Interpolate C_T vs standard curve of known copies of nucleic acid
  - Total RNA, in vitro transcribed RNA, DNA
- Unit-less quantitation (arbitrary values/ug RNA)
  - Interpolate C_T vs dilution curve of a “quantitator standard RNA”



Relative quantitation

- ΔC_T between "control" and "treated" RNAs on a single plate
  - Fold-difference
  - Cannot compare Ct between samples on different plates
- ΔC_T between "calibrator" RNA sample and unknown RNA
  - Same calibrator RNA can be on multiple plates
- $\Delta\Delta C_T$ between "control" and "treated"
  - Fold change-normalized to a separate reference gene/sample

Relative fold change

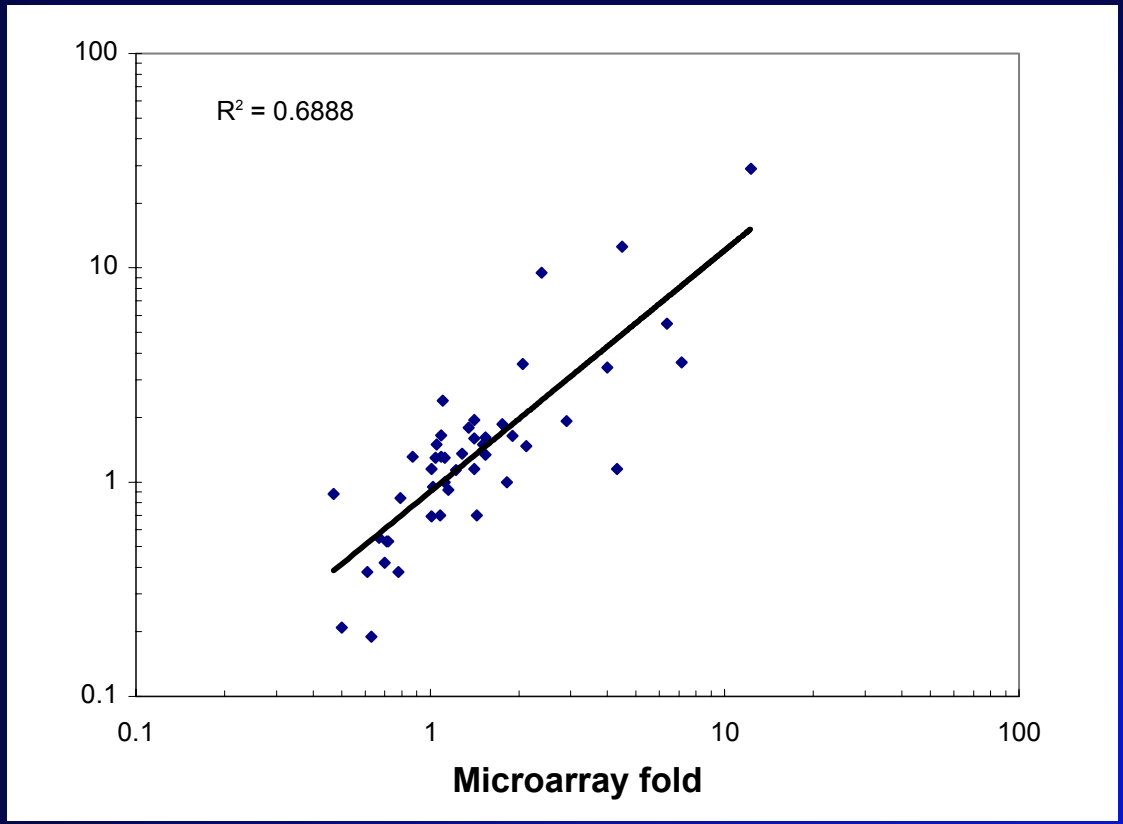
- C_T inversely correlated with starting copies
- Each cycle there is a “doubling” of amplicons (assuming 100% efficiency)
- Difference in 1 cycle therefore a 2=fold difference in copies

Fold change = $2^{\Delta CT}$

$\Delta Ct = 3.31$

Fold difference in starting copy number = $2^{3.31} = 9.9$

Correlation of real-time and microarray



Data from Martinez et al (submitted), SYBR, $2^{\Delta Ct}$

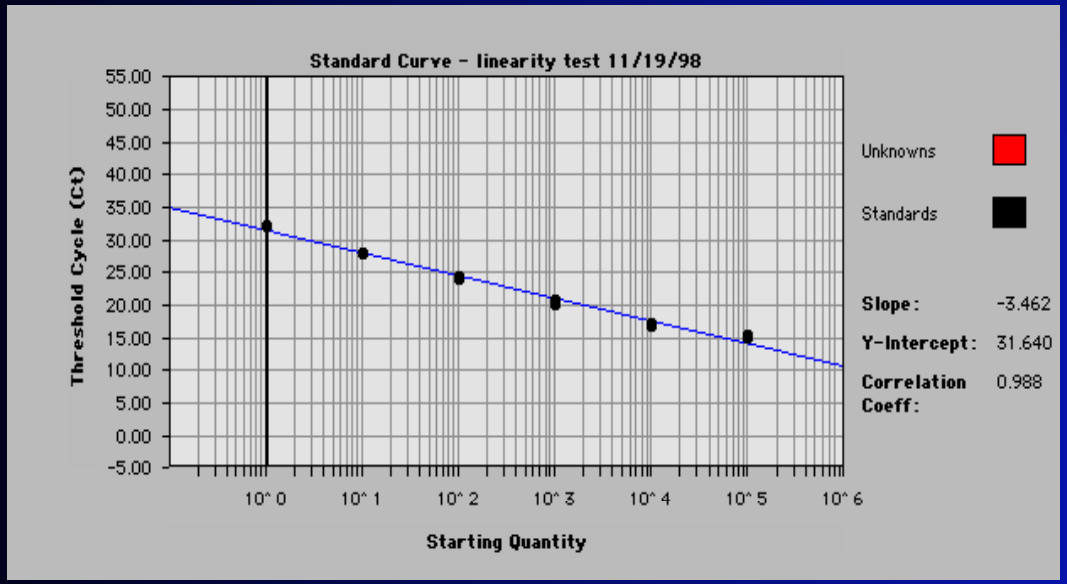
Efficiency adjustment

For a ΔC_t=1, Fold change = efficiency

- $2^{\Delta CT}$ assumes a 100% efficient amplification
- For single gene efficiency adjustment use
  - Fold change = $e^{\Delta Ct}$
  - Where efficiency(e%) = $10^{(-1/slope)}$
- For a ΔCt=1, Fold change = efficiency

Calculation of Efficiency

- Based on a linear plot of C_T vs. log copies:
- Efficiency(e%) = 10 $^{(-1/slope)}$
- 100% efficiency (2 copies each cycle) slope of –3.3219.



Slope = -3.462

e = 10 $^{(-1/3.462)}$ = 1.95

1.95 copies per cycle

ΔCt = 3.3

Fold= (1.95) $^{3.31}$ = 9.1 fold

Efficiency adjusted Normalization

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(control - sample)}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}(control - sample)}}$$

- Fold-change can be “normalized” relative to a “reference gene”
- Reference can be a separate sample on the plate
- Beware of the interpretation of a normalized fold change
  - Assumption that the reference gene is “unaffected” by treatment

Can I really detect <2X changes?

- How can you be sure a difference is real?
  - T-test on triplicates C_Ts
- Sensitivity of discrimination is dependent upon..
  - Efficiency
  - Assay variability
  - Number of Replicates

Variability impact on ΔCt

- Taqman or SYBR
- Standard deviations (n = 9)
  - 0.2-0.5 cycles
  - CV, 1-2% on C_T values
- Power calculation for ΔCt =1, 90%, $p<0.05$ (T-test)
  - sd=0.25, n = 3
  - sd =0.33, n = 4
  - sd= 0.5. n = 7

Issues of assay design

- RNA specific sets -ie Primers spanning intron location
  - If you know the gene and have the time go for it.
  - Not all genes in database and annotated esp. rat
- Do you need RNA specific sets?
  - RNA expression 10^3-10^8 copies/100ng total RNA
  - 100 ng RNA approx = 100 single gene copies (assuming 1% DNA contam)
- Reverse transcription
  - Gene specific primer is best especially if using a synthetic RNA standard
  - Oligo d(T)-may not be good for 5' end targets
  - Random hexamers - poor for synthetic RNA standard

Some Take-home advice

- You're not in Kansas anymore, so do your homework first
  - Learn the concepts before you do the assay
- Specialized machines
  - Make contact with someone who will let you use their machine
- The devil is in the details.
  - You can get the same CT from very different curves of different quality
- You still need gels
  - While quantitation is gel-free, a picture tells a thousand words
- Replicate
  - You can detect a 2X change with duplicates but is it for real?

Acknowledgements/Information sources

- Chris Miller- now the ABI Field Application Specialist!

- NIEHS Real-time PCR webpage
  - http://dir.niehs.nih.gov/pcr
- Applied Biosystems
  - http://www.appliedbiosystems.com/apps
- BioRad ICycler
  - http://www.bio-rad.com/iCycler/
- Stratagene
  - http://www.stratagene.com/q_pcr/index.htm
- Light Cycler
  - http://biochem.roche.com/lightcycler/lc_sys/lc_sys.htm

Additional Reference

Michael W. Pfaffl. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**(9): 2005-2007, 2001.