Real-Time Quantitative PCR Assay Data Analysis, Evaluation and Optimization

A Tutorial

on

Quantification Assay Analysis and Evaluation

and

Trouble-Shooting Sub-Optimal Real-Time QPCR Experiments

by

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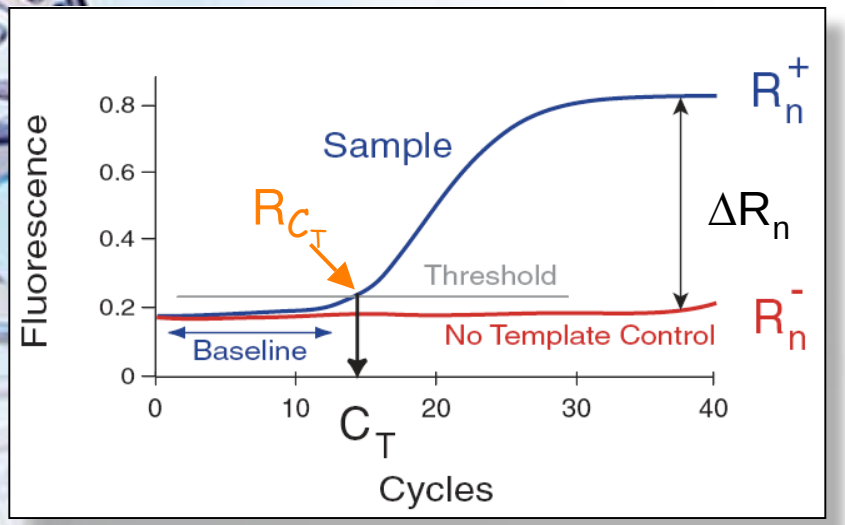
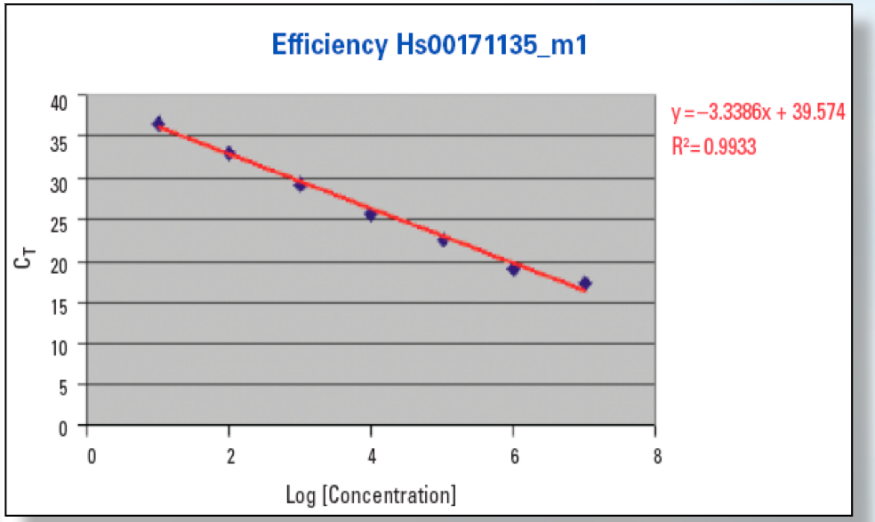
Content IV: A&E Class

- Introduction:
  - Real-time QPCR & Amplification Efficiency,
  - Mathematics of QPCR
- Data Analysis and Evaluation:
  - Quantification Strategies in QPCR
    - Absolute Quantification
    - Relative Quantification:
      - Standard curve method
      - Comparative CT method
  - Fidelity in QPCR
    - Specificity, Sensitivity, Accuracy, Reproducibility
    - Experimental Variations, Replicates,
    - Standard Deviation Calculations
- Optimizing QPCR experiments
  - Primer and probe optimization
  - Multiplex assay optimization

Essentials - One More Time

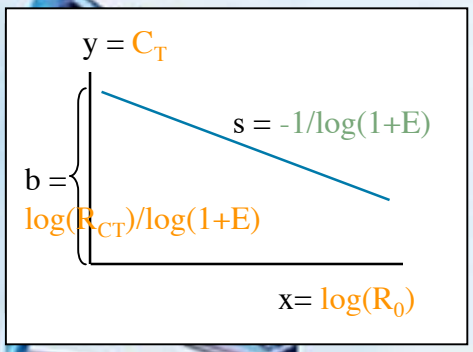
- Target Reporter Fluorescence…
  - is determined from the fractional cycle at which a threshold amount of amplicon DNA is reached:
    - $R_{CT} = R_0 \cdot (1+E_T)^{CT}$
  - Amplification Efficiency (@ threshold T): $E_T = 10^{(-1/s)} - 1$
    - slope (s) of linear regression of C_T values vs. log[cDNA]
  - Fluorescence increase I is proportional to the amount of target DNA: $I = k \cdot R_{CT}$





Mathematics of QPCR

- Basic Equations:
  - $R_{CT} = R_0 \cdot (1+E_T)^{CT}$
    - Taking the logarithm yields: $\log(R_{CT}) = \log(R_0) + \log(1+E) \cdot C_T$
    - rearrangement: $C_T = \log(R_{CT})/\log(1+E) - \log(R_o)/\log(1+E)$, or:
      $C_T = -1/\log(1+E) \cdot \log(R_o) + \log(R_{CT})/\log(1+E)$
    - Comparison with $y = sx + b$ indicates that plotting C_T versus $\log(R_0)$ produces a line with the slope s, therefore:
      $s = -1/\log(1+E)$, or: $\log(1+E) = -1/s$
    - Solving the logarithm then yields the amplification efficiency:
      $1+E = 10^{-1/s}$, $E = 10^{(-1/s)} - 1$
      [for E=1: $2 = 10^{-1/s}$, or $\log 2 = -1/s$, or: $s = -1/\log 2 = -3.32$]



  - Because we aim at obtaining the initial numbers of target molecules, it is appropriate to now substitute reporter fluorescence R with numbers N:
    - $N_0 = N_{CT}/(1+E)^{CT}$ (I) and $I = k\, N_{CT}$

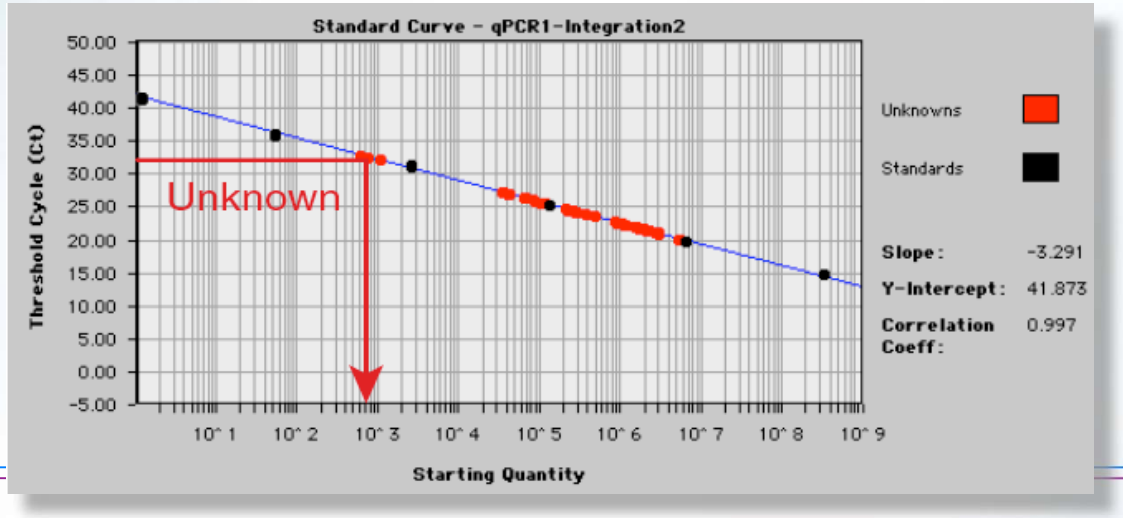
Quantification Strategies in QPCR

- Absolute Quantification
  - Absolute Standard Curve Method > requires standards of known quantities
    - $STND_{1/2/\ldots/6}$, UNKN, NTC
- Relative Quantification

  A comparative method: requires a reference, which is also a target (2nd amlicon), = active reference.
  - Relative Standard Curve Method: relative target quantity in relation to standard curves of standard and reference
    - $STND_{1, 2, \ldots, 6}$, $REF_{1, 2, \ldots, 6}$, UNKN, NTC
  - Comparative C_T Method ($\Delta\Delta C_T$): relative target quantity in relation to a endogenous control only (no standards)
    - REF, UNKN, NTC

Absolute Quantification: AQ

- A Calibration Curve Method
  - Known amounts of external targets are amplified in a parallel group of reactions run under identical conditions to that of the unknown samples.
  - Standards: recRNA, recDNA, gDNA
  - The absolute quantities of the standards must first be determined by some other independent means.
  - SDS determines N_0 for each Unknown based on linear regression calculations of the standards.



AQ … continued

- No Data Munching
  - Quantities exported
    - to Excel
    - to text only
  - calculated on the basis of a calibration curve (standard curve).

- Easy, but …
  - Standards
    - DNA: appropriate?
    - RNA: different RT
  - Expensive
  - Least accurate method
    - quantitative accuracy = f(standards, RT, standard curve)

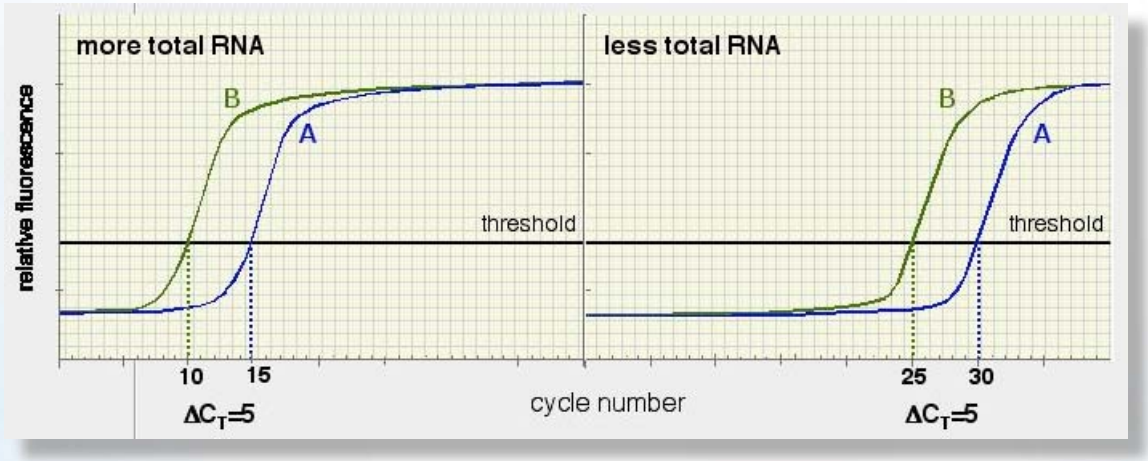
20	Standard Curve								
21	-3.36	18.646	0.997	0.11	(3 , 14)				
22									
23	Sample Information								
24	Well	Type	Sample	Replicate	Ct	Quantity	Std. Dev.	Mean	
25	A4	UNKN	A4	1	18.89	8.50E-01	0.06	0.88	
26	A5	UNKN	A5	1	18.9	8.40E-01	0.06	0.88	
27	A6	UNKN	A6	1	18.72	9.50E-01	0.06	0.88	
28	C7	UNKN	C7	10	21.19	1.80E-01	0.01	0.19	
29	C8	UNKN	C8	10	21.1	1.90E-01	0.01	0.19	
30	C9	UNKN	C9	10	21.04	1.90E-01	0.01	0.19	
31	C10	UNKN	C10	11	24.99	1.30E-02	0	0.01	
32	C11	UNKN	C11	11	25.06	1.20E-02	0	0.01	
33	C12	UNKN	C12	11	25	1.30E-02	0	0.01	
34	A7	UNKN	A7	2	22.57	6.80E-02	0.01	0.06	
35	A8	UNKN	A8	2	22.82	5.70E-02	0.01	0.06	
36	A9	UNKN	A9	2	22.62	6.50E-02	0.01	0.06	
37	A10	UNKN	A10	3	19.49	5.60E-01	0.03	0.57	
38	A11	UNKN	A11	3	19.54	5.40E-01	0.03	0.57	
39	A12	UNKN	A12	3	19.39	6.00E-01	0.03	0.57	
40	B1	UNKN	B1	4	19.45	5.80E-01	0.04	0.57	
41	B2	UNKN	B2	4	19.58	5.30E-01	0.04	0.57	
42	B3	UNKN	B3	4	19.38	6.00E-01	0.04	0.57	
43	B4	UNKN	B4	5	23.02	5.00E-02	0	0.05	
44	B5	UNKN	B5	5	23.03	4.90E-02	0	0.05	
45	B6	UNKN	B6	5	23.03	5.00E-02	0	0.05	
46	B7	UNKN	B7	6	20.99	2.00E-01	0.01	0.22	
47	B8	UNKN	B8	6	20.82	2.20E-01	0.01	0.22	
48	B9	UNKN	B9	6	20.83	2.20E-01	0.01	0.22	
49	B10	UNKN	B10	7	20.83	2.20E-01	0.02	0.23	
50	B11	UNKN	B11	7	20.86	2.20E-01	0.02	0.23	
51	B12	UNKN	B12	7	20.65	2.50E-01	0.02	0.23	
52	C1	UNKN	C1	8	25.06	1.20E-02	0	0.01	
53	C2	UNKN	C2	8	24.89	1.40E-02	0	0.01	
54	C3	UNKN	C3	8	24.83	1.40E-02	0	0.01	
55	C4	UNKN	C4	9	21.15	1.80E-01	0.01	0.18	
56	C5	UNKN	C5	9	21.03	2.00E-01	0.01	0.18	
57	C6	UNKN	C6	9	21.17	1.80E-01	0.01	0.18	
58	D10	NTC	D10	NTC	37.81		0	0	
59	A1	STND	A1	s1	18.43	1.00E+00	0	1	
60	A2	STND	A2	s1	18.86	1.00E+00	0	1	
61	A3	STND	A3	s1	18.85	1.00E+00	0	1	
62	D1	STND	D1	s2	22.07	1.00E-01	0	0.1	
63	D2	STND	D2	s2	22.05	1.00E-01	0	0.1	
64	D3	STND	D3	s2	21.73	1.00E-01	0	0.1	
65	D4	STND	D4	s3	25.19	1.00E-02	0	0.01	
66	D5	STND	D5	s3	25.23	1.00E-02	0	0.01	
67	D6	STND	D6	s3	25.39	1.00E-02	0	0.01	
68	D7	STND	D7	s4	28.4	1.00E-03	0	0	
69	D8	STND	D8	s4	29	1.00E-03	0	0	
70	D9	STND	D9	s4	29.01	1.00E-03	0	0	
71									

Relative Quantification: RQ

- An Active Reference
  - …is used to determine changes in the amount of a given sample *relative* to another -internal - control sample.
    - a different amplicon in the same PCR reaction as the amplification of the amplicon for the GOI
  - Does not require standards with known concentrations
- Calculation Methods for Relative Quantitations
  - Standard Curve method (ΔC_T)
    - Two 'standard' curves (relative control & GOI)
    - May include a 2nd normalization with an arbitrarily chosen calibrator
  - Comparative C_T method ($\Delta\Delta C_T$)
    - no standards, but with amplification of a reference
    - contingent upon similar amplification efficiencies of the amplicons for GOI and reference
    - Always relative to a calibrator sample

RQ: Intuitively

- ΔC_T = const because E = const (note: $E_A \neq E_b$ is allowed)



- Same amplicon:
  - $E_A = E_B \Rightarrow N_A/N_B = 2^{-\Delta CT}$

    For example: if ΔC_T between A and B is 5 cycles, then there is $2^{-5} = 1/32$ as much A than B.
- Different amplicons:

  For example: GOI (x) and endogenous control (c):
  - $E_X \neq E_C \Rightarrow N_x/N_c = K\,(1+E_c)^{CTc} / (1+E_x)^{CTx}$

RQ: Mathematically

- $N_{CT} = N_0 (1+E)^{CT}$ and $I = k N_{CT}$
- The relative Intensities of samples A and B is:
  - $I_A = k_A \cdot N_{CTA} = k_A \cdot N_{0A} (1+E_A)^{CTA}$ and
  - $I_B = k_B \cdot N_{CTB} = k_B \cdot N_{0B} (1+E_B)^{CTB}$
- at threshold: $I_A = I_B$ thus: $k_A \cdot N_{CTA} = k_B \cdot N_{CTB}$
- Solving for constants yields: $K = k_B/k_A = N_{CTA}/ N_{CTB}$,
  - inserting $N_{CTA} = N_{0A} (1+E_A)^{CTA}$ and $N_{CTB} = N_{0B} (1+E_B)^{CTB}$ and rearranging we get:
- $N_{0A}/N_{0B} = K \cdot (1+E_B)^{CTB} / (1+E_A)^{CTA}$ (II)
  - The fractions of A and B expressed as percentages are:

$$A = 100 \cdot [K \cdot (1+E_B)^{CTB}/(1+E_A)^{CTA}] / 1+K \cdot [(1+E_B)^{CTB}/(1+E_A)^{CTA}]$$

$$B = 100 \cdot [1] / 1+K \cdot [(1+E_B)^{CTB}/(1+E_A)^{CTA}]$$

- Relative Standards:
  - For example: the ratio of treatment (t) vs. control (c):

$$\frac{(N_A/N_B)_t}{(N_A/N_B)_c} = K \frac{(1+E_{Bt})^{CTBt} / (1+E_{At})^{CTAt}}{(1+E_{Bc})^{CTBc} / (1+E_{Ac})^{CTAc}}$$

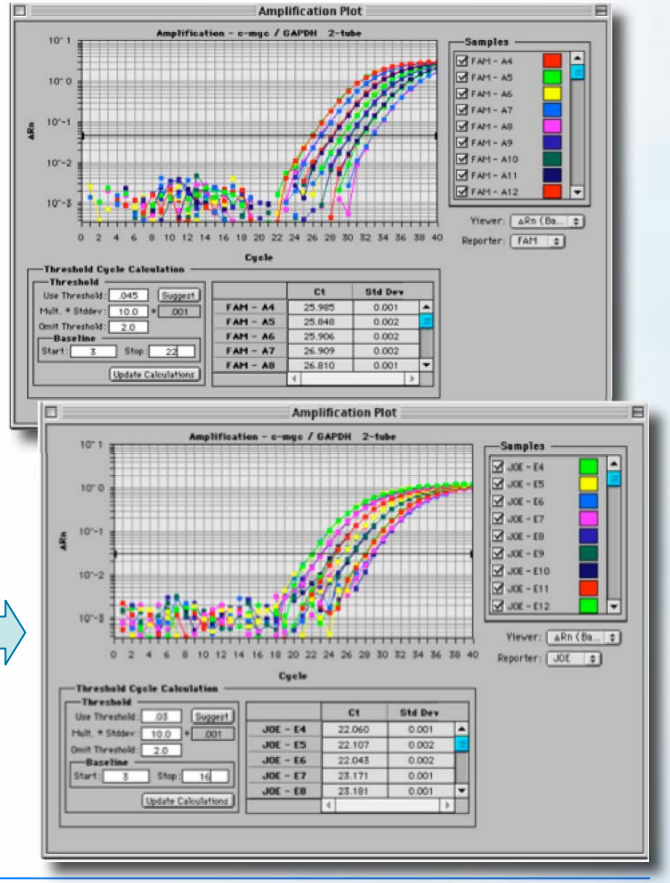
Relative Standard Method, Example A

- Two serial dilutions: one for GOI (c-myc), another one for the endogenous control (GAPDH)
- Expression profiling in brain, kidney, liver, lung

Show Analysis | Dye Layer : FAM

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC1 A1	NTC1 A2	NTC1 A3	STND 1000 pg	STND 1000 pg	STND 1000 pg	STND 500 pg	STND 500 pg	STND 500 pg	STND 200 pg	STND 200 pg	STND 200 pg
B	STND 100 pg	STND 100 pg	STND 100 pg	STND 50 pg	STND 50 pg	STND 50 pg	STND 20 pg	STND 20 pg	STND 20 pg	STND 10 pg	STND 10 pg	STND 10 pg
C	cMYC Brain	cMYC Brain	cMYC Brain	cMYC Brain	cMYC Brain	cMYC Brain	cMYC Kidney	cMYC Kidney	cMYC Kidney	cMYC Kidney	cMYC Kidney	cMYC Kidney
D	cMYC Liver	cMYC Liver	cMYC Liver	cMYC Liver	cMYC Liver	cMYC Liver	cMYC Lung	cMYC Lung	cMYC Lung	cMYC Lung	cMYC Lung	cMYC Lung
E												

	1	2	3	4	5	6	7	8	9	10	11	12
E	NTC2 E1	NTC2 E2	NTC2 E3	STD2 1000 pg	STD2 1000 pg	STD2 1000 pg	STD2 500 pg	STD2 500 pg	STD2 500 pg	STD2 200 pg	STD2 200 pg	STD2 200 pg
F	STD2 100 pg	STD2 100 pg	STD2 100 pg	STD2 50 pg	STD2 50 pg	STD2 50 pg	STD2 20 pg	STD2 20 pg	STD2 20 pg	STD2 10 pg	STD2 10 pg	STD2 10 pg
G	GAPDH Brain	GAPDH Brain	GAPDH Brain	GAPDH Brain	GAPDH Brain	GAPDH Brain	GAPDH Kidney	GAPDH Kidney	GAPDH Kidney	GAPDH Kidney	GAPDH Kidney	GAPDH Kidney
H	GAPDH Liver	GAPDH Liver	GAPDH Liver	GAPDH Liver	GAPDH Liver	GAPDH Liver	GAPDH Lung	GAPDH Lung	GAPDH Lung	GAPDH Lung	GAPDH Lung	GAPDH Lung



Applied Biosystems User Bulletin #2 (PN 4303859)

RQ: Data Munching in Excel

- Average replicates, then divide the average c-myc (GOI) value by the average GAPDH reference value of the corresponding samples.
- For example:

$$\frac{\langle GOI \rangle}{\langle Ref \rangle}$$

2nd normalization:
Calibrator = Brain

see slide 30 for error handling

Table 1. Amounts of c-myc and GAPDH in Human Brain, Kidney, Liver, and Lung

Tissue	c-myc ng Total Raji RNA	GAPDH ng Total Raji RNA	$c\text{-myc}_N$ Norm. to GAPDH[a]	$c\text{-myc}_N$ Rel. to Brain[b]
Brain	0.033	0.51		
	0.043	0.56		
	0.036	0.59		
	0.043	0.53		
	0.039	0.51		
	0.040	0.52		
Average	0.039±0.004	0.54±0.034	0.07±0.008	1.0±0.12
Kidney	0.40	0.96		
	0.41	1.06		
	0.41	1.05		
	0.39	1.07		
	0.42	1.06		
	0.43	0.96		
Average	0.41±0.016	1.02±0.052	0.40±0.025	5.5±0.35
Liver	0.67	0.29		
	0.66	0.28		
	0.70	0.28		
	0.76	0.29		
	0.70	0.26		
	0.68	0.27		
Average	0.70±0.036	0.28±0.013	2.49±0.173	34.2±2.37

Applied Biosystems User Bulletin #2 (PN 4303859)

… continued

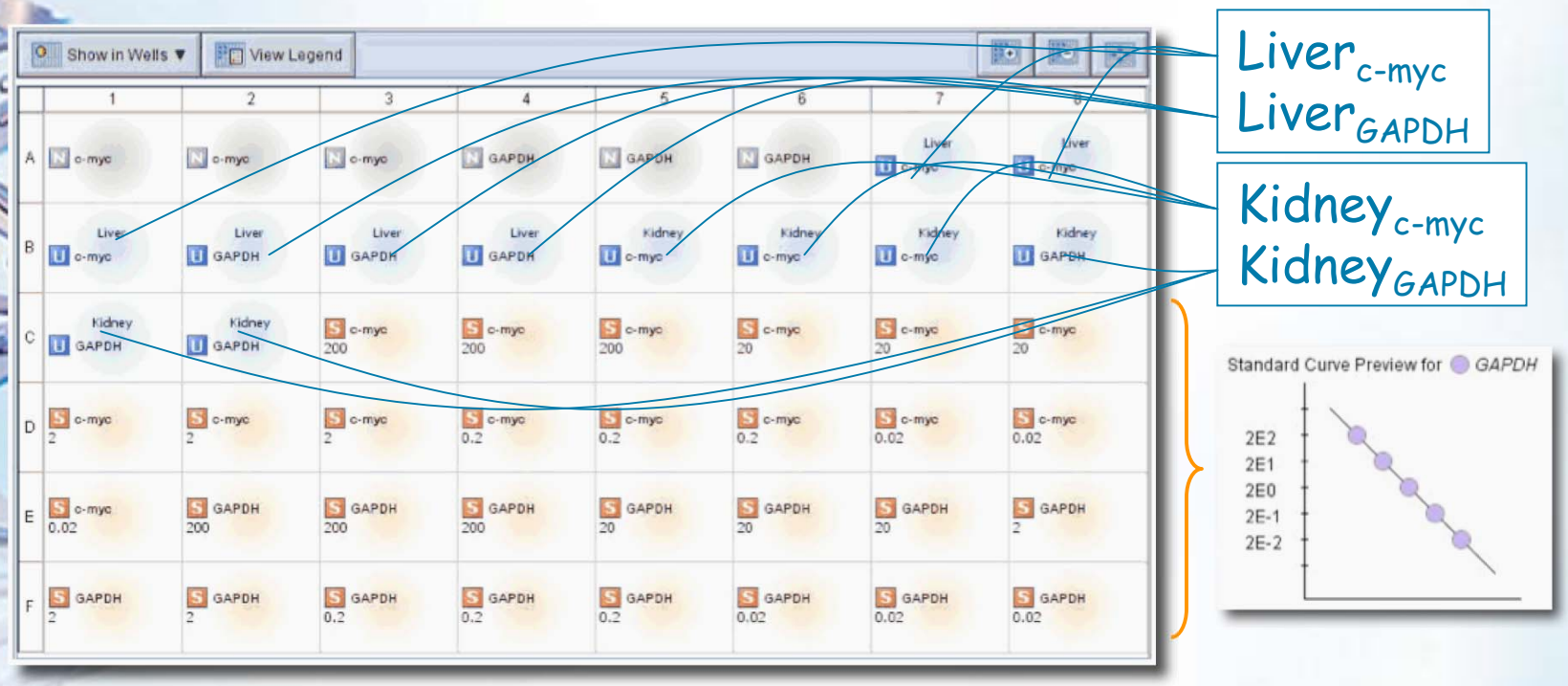
- Relative Quantification with Absolute Values: involves the division by a *calibrator* value:
  - normalize using an endogenous control, then
  - divide the normalized values by an arbitrarily chosen calibrator value (e.g. kidney in this example)

	GOI raw	18S raw	Normalized GOI/18S	Relative Value
kidney	82	3592	0.023	1.0
liver	18351	8996	2.05	90
ovary	44	1669	0.03	1.3
spleen	1	8	0.13	5.6

- Quality of quantification using the relative standard curve method:
  - quantitative accuracy = f (standard curve)
  - More accurate than the absolute standard method

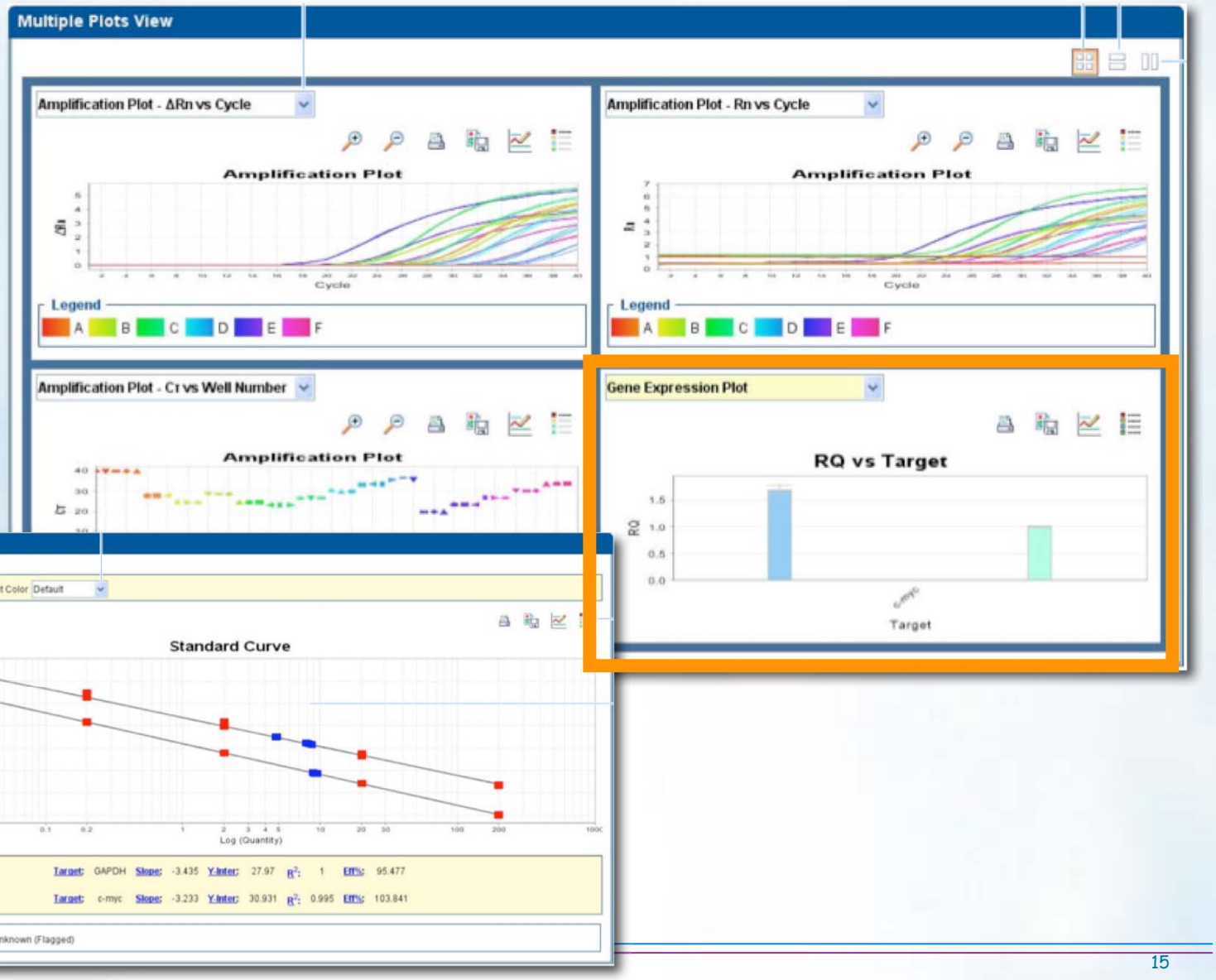
Relative Standard Method, Example B

- e.g. c-myc Expression Analysis in Liver, Kidney Tissues
  - GOI is c-myc, endogenous control is GAPDH,
  - reference sample is RNA isolated from lung tissue
  - 2 Standard curves: serial dilutions of a cDNA sample generated from lung tissue tRNA - one series is analyzed for c-myc, the other for GAPDH.



From: Applied Biosystems Documentation PN 4376785 Rev D

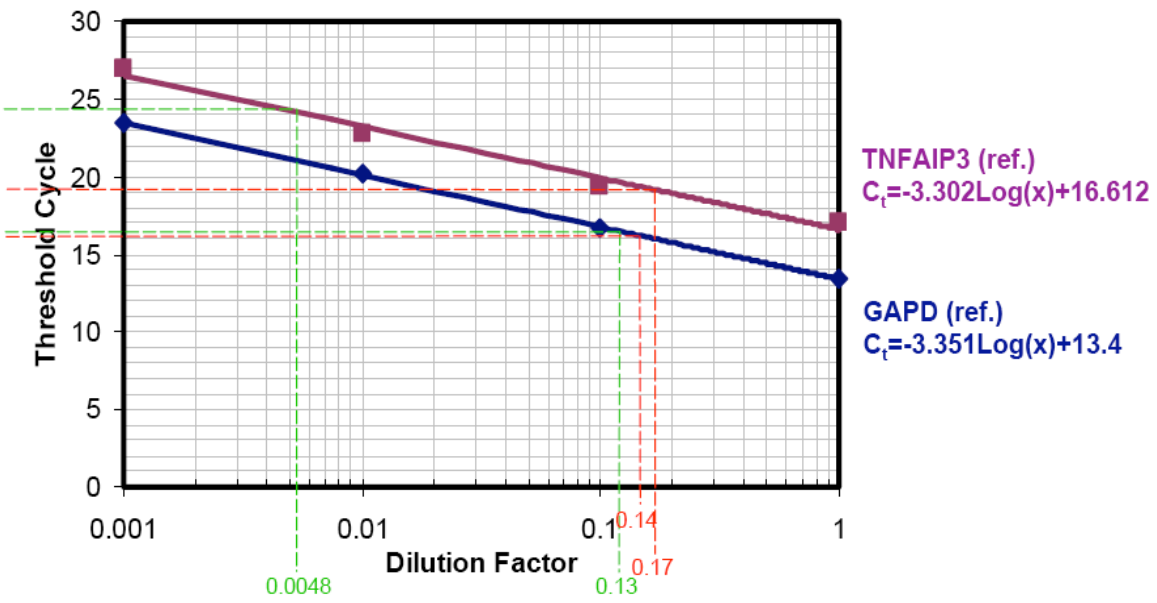
SDSv2 Does the Analysis For You



Relative Standard Method, Example C

- Relative to endogenous control AND treatment(s)
- For example: +/- TNFa induced $TNFAIP_3$ and GAPDH

$$\frac{(N_A/N_B)_t}{(N_A/N_B)_c} = K \frac{(1+E_{Bt})^{CTBt}/(1+E_{At})^{CTAt}}{(1+E_{Bc})^{CTBc}/(1+E_{Ac})^{CTAc}}$$



TNFa untreated: C_t(TNFAIP3)=24.25 C_t(GAPD)=16.49
TNFa treated: C_t(TNFAIP3)=19.17 C_t(GAPD)=16.36

$$\frac{(TNFAIP3/GAPD)_{treated.}}{(TNFAIP3/GAPD)_{untreated}} = \frac{0.17/0.14}{0.0048/0.13} = 32.9$$

SuperArray Bioscience Corporation Newsletter 1

The Comparative C_T Method

- Derivation of the $\Delta\Delta C_T$ Method
  - Targets at threshold cycle C_T: $\Rightarrow N_{CT} = N_0 \cdot (1+E)^{CT}$
    - For X_T: number of target GOI molecules at threshold
    - and R_T: number of reference molecules at threshold
    - $X_T/R_T = X_0 \cdot (1+E_x)^{CTX} / R_0 \cdot (1+E_R)^{CTR} = K_x/K_R = K$
  - If $E_X \approx E_R =: E \Rightarrow K = X_0/R_0 \cdot (1+E)^{CTX-CTR} = X_N \cdot (1+E)^{\Delta CT}$

    Whereby $\Delta C_T = CT_X - CT_R$, and $X_N = X_0/R_0$

    Rearranged: $X_N = K/(1+E)^{\Delta CT}$, or $X_N = K \cdot (1+E)^{-\Delta CT}$ (III)
  - Another normalization of each normalized sample X_N by the X_N of a calibrator (cb) yields:

    $X_{N,}/X_{N,cb} = K\,(1+E)^{-\Delta CT} / K\,(1+E)^{-\Delta CT,cb} = (1+E)^{-\Delta\Delta CT}$
  - E = const., and with $N = X_N/X_{N,cb}$: $N = 2^{-\Delta\Delta CT}$ (IV)
  - Quality of quantification:
    - quantitative accuracy = f(amplification efficiency)
    - Accurate and most efficient QPCR data analysis method.
    - (don't use the ΔΔCT method if CV > 4%, see later)

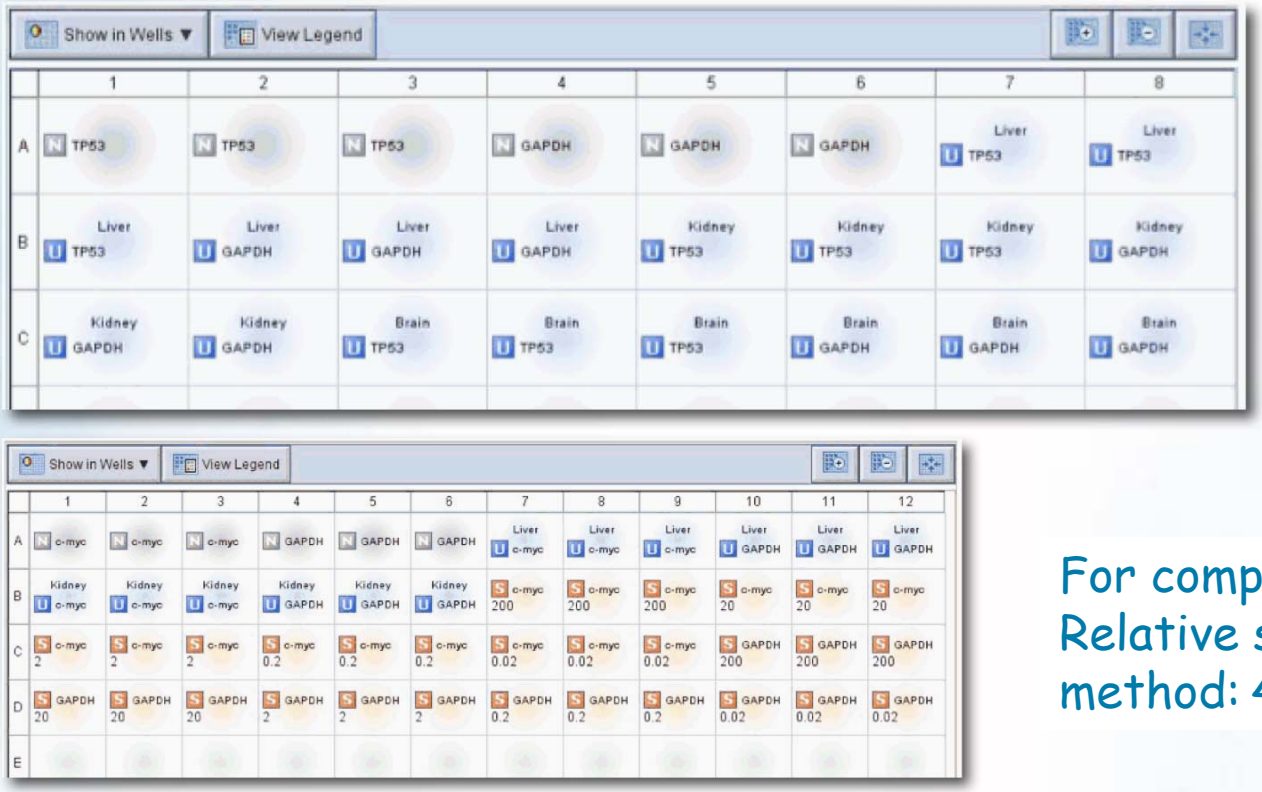
$\Delta\Delta C_T$ Method continued

- SDS v2 does it for you! Otherwise, use Excel
- Normalize GOI signals to signals of an endogenous reference (e.g. 18S): $CT_{GOI} - CT_{18S} \Rightarrow \Delta CT_r$
- Normalize each ΔCT_r value to a particular ΔCT_c value of an assay calibrator (cb): $\Delta CT_r - \$\Delta CT_{cb}\$ \Rightarrow \Delta\Delta CT_r$ and one $\Delta\Delta CT_{cb}$.
  - This is a second subtraction, and $\Delta\Delta CT_{cb} = 0$
  - Calibrator cb may be a control treatment, or the sample with the *highest* $\Delta C_T r$ value
- The relative target number N then is $2^{-\Delta\Delta CT}$

	GOI CT	18S CT	Norm. I ΔCT	Norm. II ΔΔCT	N
E	24	14	10	-1	2
P	20	11	9	-2	4
E+P	21	11	10	-1	2
DMSO	27	16	11	0	1

Comparative C_T Method ($\Delta\Delta C_T$) Example B

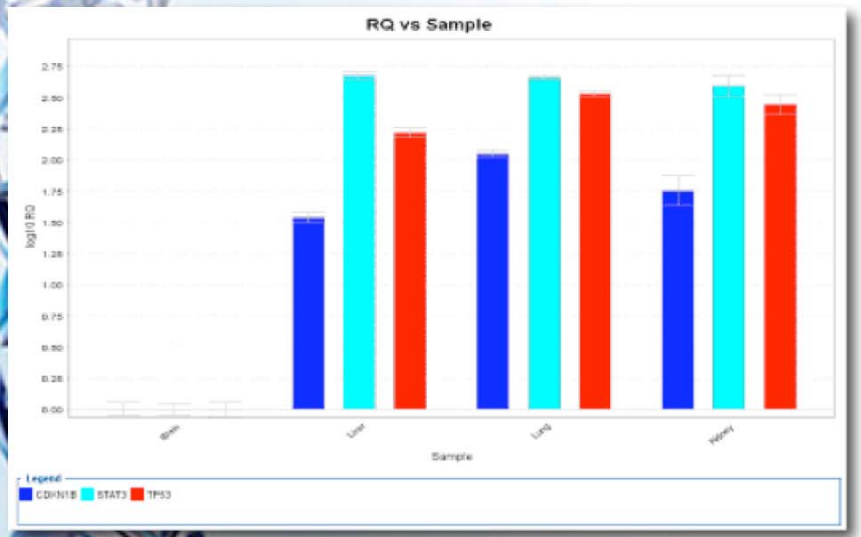
- e.g. p53 Expression in Liver, Kidney, Brain Tissues
  - GOI is TP53, endogenous control is GAPDH
  - Assumption: similar amplification efficiencies ($E_{TP53} = E_{GAPDH}$) ($\Delta\Delta C_T$ validation experiment, see later)

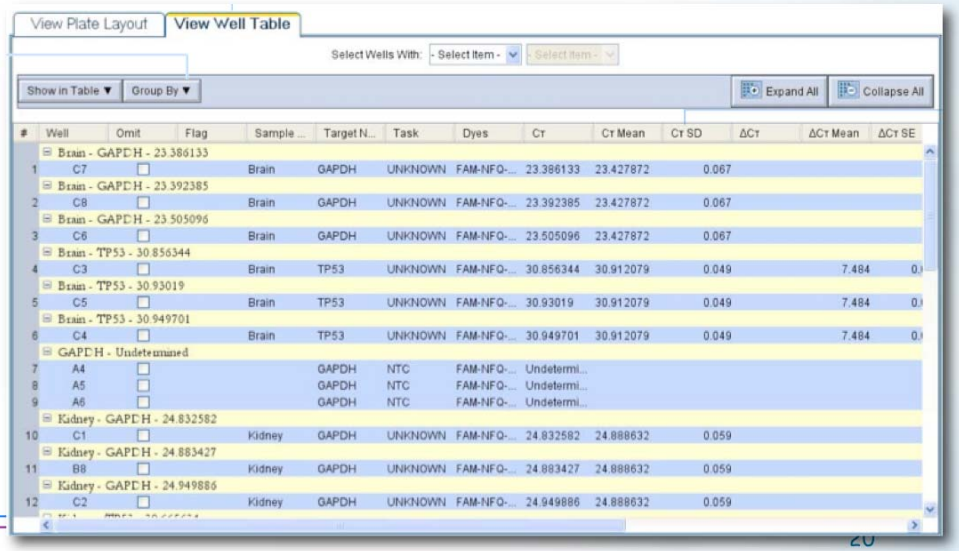


For comparison: Relative standard method: 48 wells

From: Applied Biosystems Documentation PN 4376785 Rev D

SDSv2 Does the Analysis For You





$\Delta\Delta C_T$ Method, Example C

- siRNA Transfection
  - Quantitation of % Knock-down and remaining gene expression:

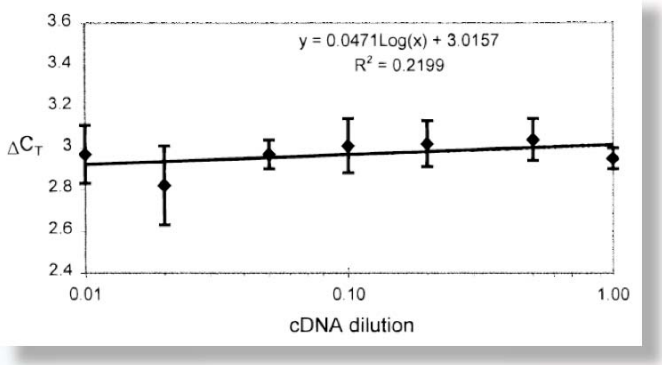
Sample	Amplicon	CT	ΔCT	ΔΔCT
siRNA Target	Primer/Probe Target	CT	CT(GOI) - CT(control)	ΔCT(GOI) - ΔCT(NC)
GOI	GOI	26.98	15.23	4.89
GOI	18S rRNA	11.75		
NC	GOI	22.87	10.34	
NC	18S rRNA	12.53		
Percent remaining gene expression:			2exp-ΔΔCT	$2^{-4.89}$ = 3.37%
Percent knockdown:			100 - 3.37%	**96.63%**

Validation Experiment

- $\Delta\Delta C_T$ Method is contingent upon $E_{GOI} \approx E_{Ref}$
  - The absolute value (|s|) of the slope s of log input amount (or dilutions) vs. ΔC_T should be less than 0.1

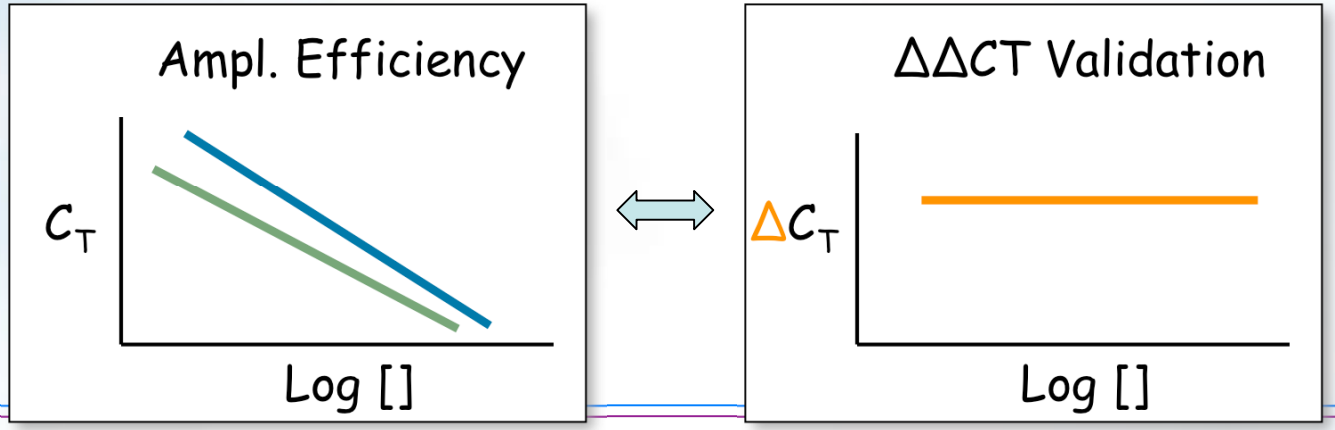
E_X vs. E_R Efficiencies: |s| < 0.1

E max. amplification efficiency: s = -3.32



Livak and Schmittgen, 2001, Methods 25, 402-408

- Comparing important linear regression plots for QPCR:



What If $E_{GOI} \neq E_{ref}$?

- Use Efficiency Correction
  - Note: Rainer does NOT recommend this method of QPCR data analysis (if you had followed all the recommendations thus far, you most likely would not have this problem now)

$$\text{Relative N} = \frac{(E_x)^{\Delta CT\, x(\text{control-sample})}}{(E_R)^{\Delta CT\, R(\text{control-sample})}}$$

$$= \frac{(E_R)^{CT\,\text{sample}}}{(E_X)^{CT\,\text{sample}}} \div \frac{(E_R)^{CT\,\text{calibrator}}}{(E_X)^{CT\,\text{calibrator}}}$$

- Use REST Software
  - REST© (Relative Expression Software Tool)
    - Pfaffl et al. 2002. Nucl. Acids Res; 30(9): E36
    - http://www.gene-quantification.info/ then go to 'Data Analysis', 'qPCR software applications', 'REST versions', then scroll down to 'New REST software application are available:'

Fidelity in QPCR

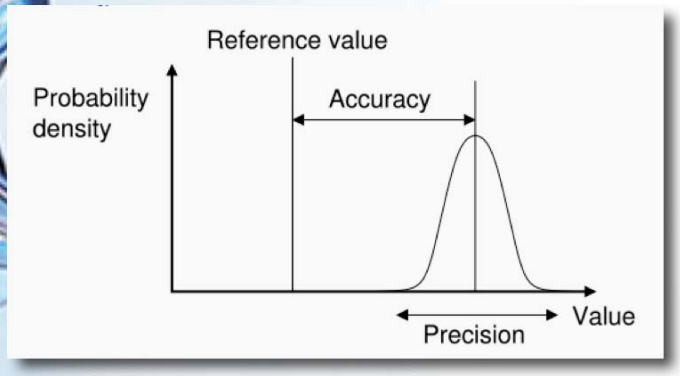
- ✓ Specificity
  - Assay design and project integration: *a prerequisite*
  - Determining the amplification efficiency: *a prerequisite*
  - Melting curve analysis: *maybe* (for spotting primer-dimers)
- ✓ Sensitivity
  - TaqMan® or SYBR®: comparable dynamic range, sensitivity
- ✓ Efficiency
  - $E_{exp} = 10^{(-1/s)} - 1$ over a wide range of input material
  - Pearson correlation coefficient $r \geq 0.95$
- Accuracy and Reproducibility
  - Replicates for intra-assay precision
  - Strategy: RT = main source of variability ⇒ single cDNA pool, RT assay optimization
  - Repetitions for inter-assay precision (Reproducibility)
    - not necessary (≠ peer reviewer's thinking)
    - Use a calibrator for inter-plate-normalizations
  - Optimizing sub-optimal experiments: always E, RT rxn

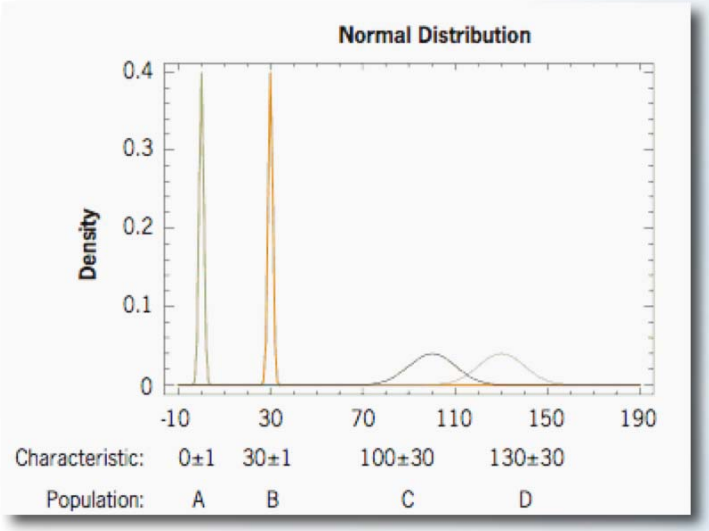
Experimental Variation

- Biological Variations
  - = f{population being studied},
  - Large CV (e.g. gene expression: CV 20 to 100%)
- Process Variations
  - Random variations: common-cause errors, not affecting all samples, = f{accuracy, standard operating procedure}
    - e.g. pipetting errors
  - Systemic variations: biasing all samples, = f{calibration, standard operating procedure}
    - e.g. software settings in sequence detection systems
- System Variations
  - System constant, affecting all samples equally, = f{instrument accuracy}
    - Fluorescence increase I is proportional to the amount of target DNA: $I = k \cdot R_{CT}$

Accuracy versus Precision

- Accuracy
  - How close a measurement is to the true or actual value
- Precision
  - How close the measured values are to each other,
  - = f{variability of the data}





AppliedBiosystems TechNotes 14-4

- Example: 4 Populations
  - A, B: small system and population variability, large fold difference between the means (30-fold, ~3% CV)
  - C, D: larger dispersion around the means, small fold difference between the means (1.3-fold, ~30% CV)

Replicates

- Biological Replicates
  - Separate biological samples, same treatment, > variability of the biology + variability of the quantitation process
    - e.g. different RNA extractions from multiple animals, …
- Technical (Systematic) Replicates
  - Aliquots from the same source run through the quantitation process independently, > variability of the process
    - e.g. triplicates for PCR from cDNA from one RT reaction
- How Many Replicates?
  - The greater the fold changes between the means of different populations, the fewer replicates are needed.
  - The more dispersed the population variability, the more biological replicates are needed:

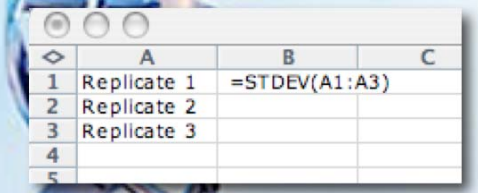
Fold Difference	**Experimental Variation (%CV)** [STDEV/MEAN x100]				
	10%	*25%*	*50%*	*75%*	*100%*
1.5	4	7	18	38	136
2	3	4	9	16	28
3	3	3	6	10	15
5	3	3	5	7	11
10	3	3	4	6	9
	one-tailed t-test				

PCR Reproducibility

- Standard Deviation and Coefficient of Variation
  - Expressed as the Standard Deviation (SD) in C_T, as the square root of the variance. The variance is

$$SD^2 = \frac{\sum_{i=1}^{n} (C_{Ti} - \langle C_T \rangle)^2}{n - 1}$$

where $\langle C_T \rangle$ is the mean of the measured C_T



	A	B	C
1	Replicate 1	=STDEV(A1:A3)	
2	Replicate 2		
3	Replicate 3		
4			
5			

    - Use "=STDEV(number1, number2, number3, ...)" in Excel
  - The relative uncertainty in the number of DNA molecules is expressed by the CV, the Coefficient of Variation, which is the ratio of the standard deviation of a distribution to its arithmetic mean ($\langle X \rangle$):
    $CV = SD/\langle X \rangle$, or for QPCR: $CV = SD/\langle C_T \rangle$, or in %:

$$CV_{\%} = 100 \frac{SD}{\langle (1+E)^{-CT} \rangle}$$

where $\langle (1+E)^{-CT} \rangle$ is the mean of $(1+E)^{-CT}$

Coefficient of Variation: Example

$$CV_{\%} = 100 \frac{SD}{\langle (1+E)^{-CT} \rangle}$$

0.039 / 14.561 x 100 = 0.267%

Sample Name	Detector	Reporter	Task	Ct	Ct mean	St dev	CV on Ct (%)
Dil. 1:10	18S	VIC	Std	14.589	14.561	0.039	0.267
Dil. 1:10	18S	VIC	Std	14.577			
Dil. 1:10	18S	VIC	Std	14.517			
Dil. 1:100	18S	VIC	Std	18.115	18.148	0.092	0.508
Dil. 1:100	18S	VIC	Std	18.252			
Dil. 1:100	18S	VIC	Std	18.077			
Dil. 1:1000	18S	VIC	Std	22.051	21.973	0.085	0.387
Dil. 1:1000	18S	VIC	Std	21.882			
Dil. 1:1000	18S	VIC	Std	21.882			
Dil. 1:10000	18S	VIC	Std	25.462	25.365	0.088	0.348
Dil. 1:10000	18S	VIC	Std	25.291			
Dil. 1:10000	18S	VIC	Std	25.341			
Dil. 1:100000	18S	VIC	Std	29.261	29.244	0.024	0.083
Dil. 1:100000	18S	VIC	Std	29.216			
Dil. 1:100000	18S	VIC	Std	29.255			

Calculating Standard Deviations

- SD = f{QPCR Data Analysis Method}
- For the Standard Curve Method:
  - The SD_Q for the normalized (GOI/Ref) quotient Q is calculated using: $SD_Q = CV_Q \cdot \langle X \rangle$, with
    $$CV_Q = (CV_{GOI}^2 + CV_{Ref}^2)^{1/2}$$
- For the Comparative Method:
  - The SD_S for the difference (of ΔC_T values) is based on the SD of the GOI AND SD of the reference values: $SD_S = (SD_{GOI}^2 + SD_{Ref}^2)^{1/2}$
  - The SD of the $\Delta\Delta CT_r$ is the same as the SD_S.

OK, now let's put everything together - Error Handling for the relative quantification in practice:

a) Standard curve method, b) Comparative method

a) Error Handling for the Standard Curve Method

- $N = (N_{GOI}/N_{Ref}) \times (CV_{GOI}^2 + CV_{Ref}^2)^{1/2}$
  - The average values of the GOI replicates is divided by the average values of the reference samples (N_{GOI}/N_{Ref} =:Q). The SD_Q of the quotient is calculated using:

    $CV_Q = SD_Q/\langle X\rangle = (CV_{GOI}^2 + CV_{Ref}^2)^{1/2}$ (V)

    i.e., calculate the SDs for the replicates of GOI and Ref first, then their individual CVs. Use these CVs to calculate the CV for the normalized (GOI/Ref) using (V). Obtain the SD_Q of the quotient using $SD_Q = CV_Q \cdot \langle X\rangle$

	GOI mean	GOI SD	GOI CV	Ref mean	Ref SD	Ref CV	GOI/ Ref	CV_Q	SD_Q
Brain[&]	0.039	0.004	0.004/ 0.039= 0.1026	0.54	0.034	0.034/ 0.54= 0.063	0.039/ 0.54 = 0.072	0.12*	0.12 · 0.072= 0.009
Kidney[&]	0.41	0.016	0.016/ 0.41= 0.039	1.02	0.052	0.052/ 1.02= 0.051	0.41/ 1.02 = 0.402	0.06#	0.06 · 0.402= 0.026

&: samples from Table 1, slide 13

*: SQRT[$0.1026^2 + 0.063^2$] = 0.12 SD = CV $\langle X\rangle$ = 0.12 x 0.072 = 0.0087

#: SQRT[$0.039^2 + 0.051^2$] = 0.06 SD = CV $\langle X\rangle$ = 0.06 x 0.402 = 0.0258

b) Error Handling for the Comparative C_T Method

- $N = 2^{-\Delta\Delta CT}\,(2^{-\Delta\Delta CT-SDs} - 2^{-\Delta\Delta CT+SDs})$
  - Calculate mean, SD and CV for replicate $C_T$values of GOI and Ref, reject >4%CV.
  - Determine $\Delta CT_r = \langle CT_{GOI}\rangle - \langle CT_{18S}\rangle$. The SD of the difference (SD_S) is based on the SD of the GOI and the SD of the reference values: $SD_S = (SD_{GOI}^2 + SD_{Ref}^2)^{1/2}$
  - Normalize each ΔCT_r value to a particular ΔCT_c value of an assay calibrator (cb): $\Delta\Delta CT_r = \Delta CT_r - \Delta CT_{cb.}$ The SD of the $\Delta\Delta CT_r$ is the <u>same</u> as the SD_S ($SD_{\Delta\Delta CTr} = SD_{\Delta CTr}$).
  - The final relative values (fold induction) are $2^{-\Delta\Delta CT}$ with $\Delta\Delta CT_r - SD_S$ and $\Delta\Delta CT_r + SD_S$

Table 3. Relative Quantitation Using the Comparative C_T Method

Tissue	c-myc Average C_T	GAPDH Average C_T	ΔC_T c-myc–GAPDH[a]	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_{T,\,Brain}$[b]	c-myc$_N$ Rel. to Brain[c]
Brain	30.49±0.15	23.63±0.09	6.86±0.17	0.00±0.17	1.0 (0.9–1.1)
Kidney	27.03±0.06	22.66±0.08	4.37±0.10	–2.50±0.10	5.6 (5.3–6.0)

a, b: $SQRT[0.15^2 + 0.09^2] = 0.175$, c: $2^{0.0+0.175} = 1.1$, $2^{0.0-0.175} = 0.88$

a, b: $SQRT[0.06^2 + 0.08^2] = 0.100$, c: $2^{2.5+0.100} = 6.06$, $2^{2.5-0.100} = 5.28$

Remarks to Quantitative Precision

$C_T < 36$

Baseline Threshold

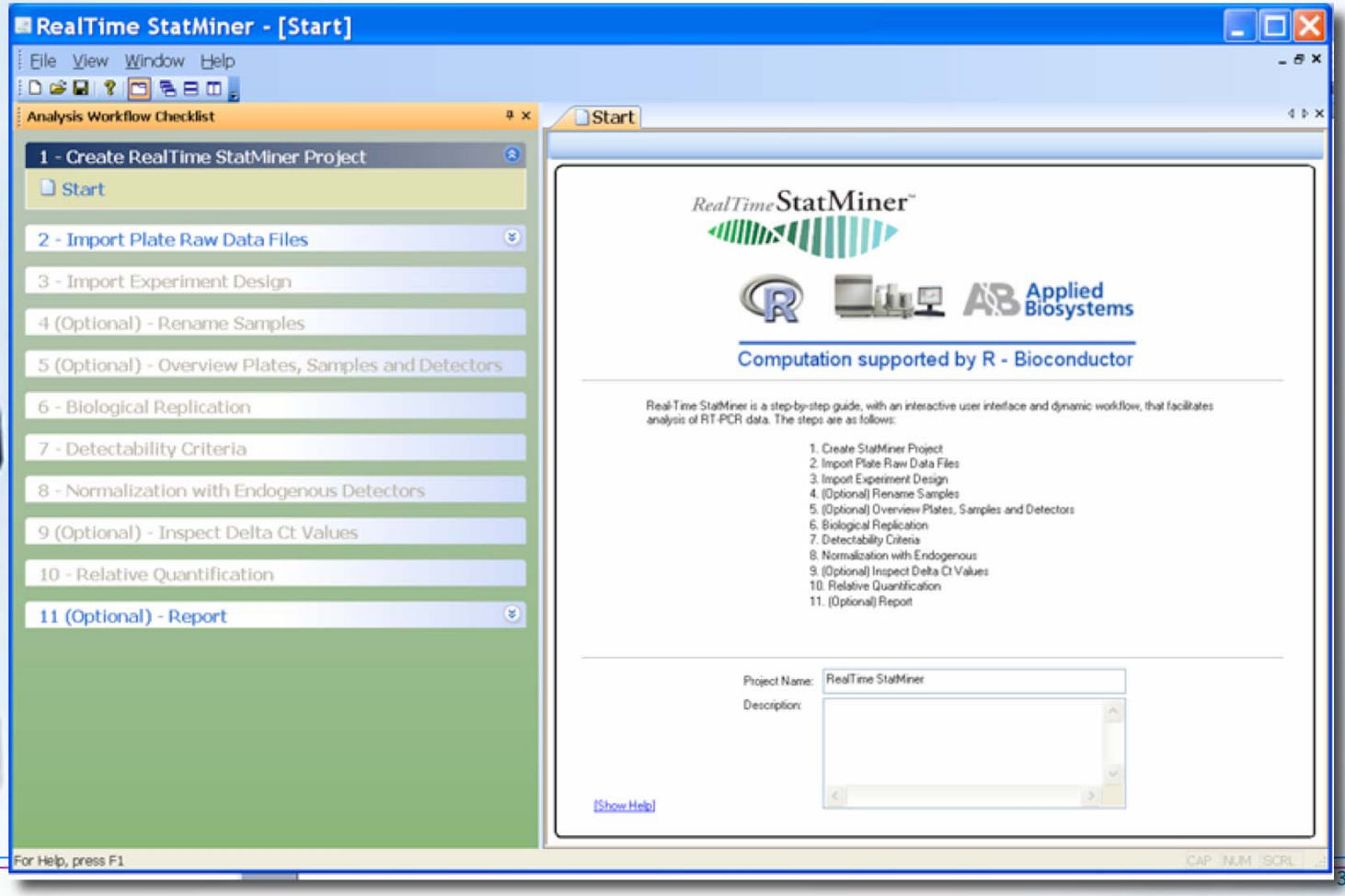
- Implications
  - The calculations of precision given above have been questioned in some peer-reviewed publications.
  - Replicate standard curves may produce potentially large inter-curve variations.
  - In general, the intra-assay variation of 10-20% and a mean inter-assay variation of 15-30% on molecule basis is realistic over the wide dynamic range (of over a billion fold range).
  - Variability is highest at $>10^7$ and $<10^2$ template copy ranges
    - Cut-off value: cycle 35, i.e. disregard C_T values for cycle numbers 36 and higher.
  - For the threshold methods, the precision is dependent on the proper setting of the threshold, which itself is dependent on proper base line settings.

A Recent User Submission

A	B	C	D	E	F	G	H	I	J	K	L	M
GOI	**REF**	AV GOI	AV Ref	STDEV GOI	STDEV REF	CV on CT GOI	CV on CT ref	∂CT	SD ∂CT	∂∂CT	SD ∂∂CT	**Result**
21.82	6.89											
23.62	8.13											
21.47	7.35											
23.14	8.53	22.51	7.73	1.03	0.74	4.58	9.60	14.79	1.27	3.51	1.27	**4.71**
												27.37
22.42	7.81											
23.01	7.79											
23.21	8											
22.41	7.05	22.76	7.66	0.41	0.42	1.80	5.47	15.10	0.586	3.82	0.59	**9.39**
												21.16
22.48	8.03											
20.7	7.36											
20.56	7.63											
20.66	7.58	21.10	7.65	0.92	0.28	4.37	3.65	13.45	0.963	2.17	0.96	**2.30**
												8.76
19.3	7.92											
19.11	7.97											
18.94	7.89											
19.42	7.86	19.19	7.91	0.21	0.05	1.10	0.59	11.28	0.216	0.00	0.22	0.86
												1.16

Integrated Genomics - The Future?

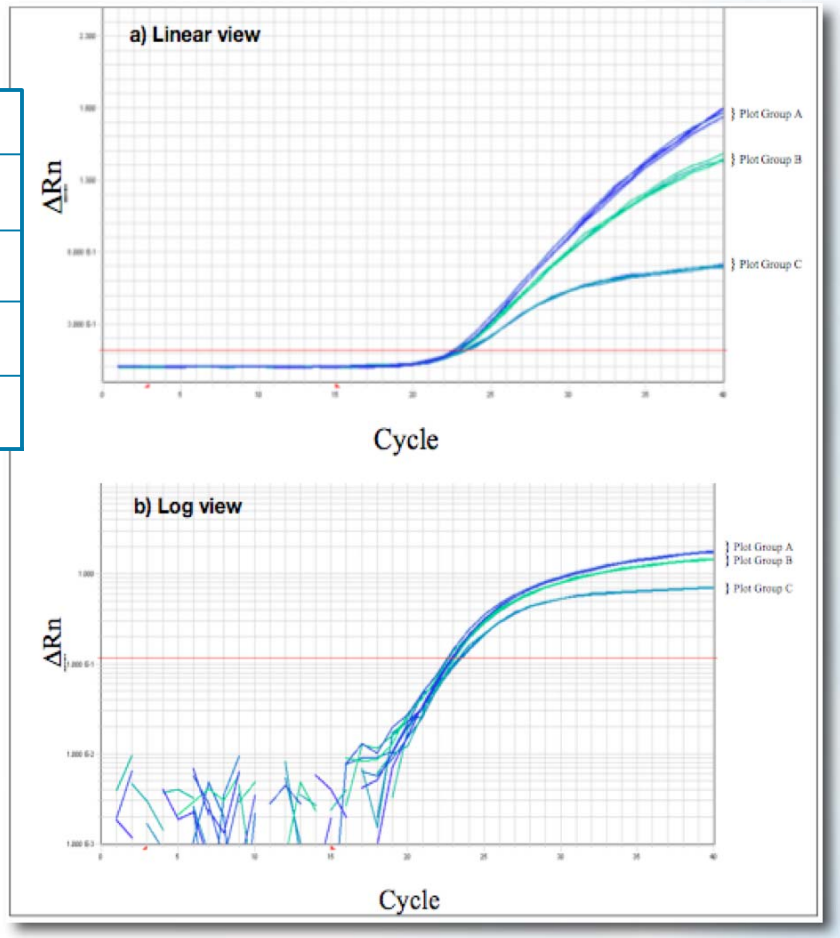
- Real-Time StatMiner™
  - http://www.integromics.com/StatMiner.php



Optimizing Primer Concentrations

- Primer Optimization Matrix
  - Maximize ΔRn :

Reverse Primer [nM]	Forward Primer [nM]		
	50	300	900
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900



  - Suggested conc.:
    - 900nM for TaqMan
    - 50nM for SYBR Green

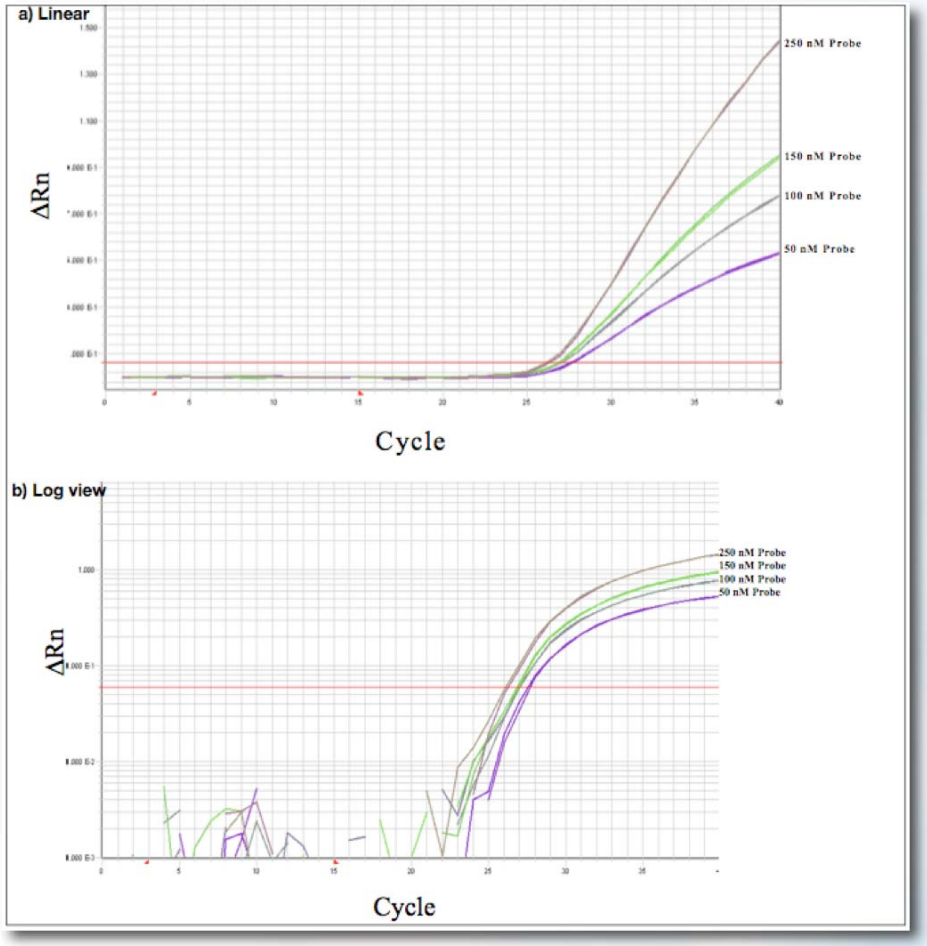
Applied Biosystems SDS Chemistry Guide (PN 4348358)

Optimizing Probe Concentrations

- Secondary to Primer Optimization
  - Maximize ΔRn :

Primer [nM]	Probe [nM]
100/900	50
100/900	125
100/900	250
100/900	500

  - Suggested conc.:
    - 250nM



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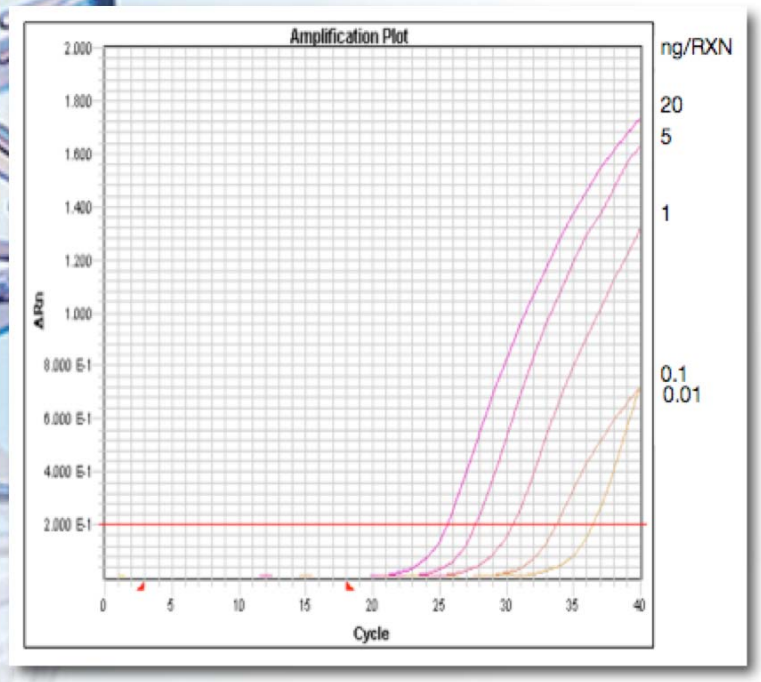
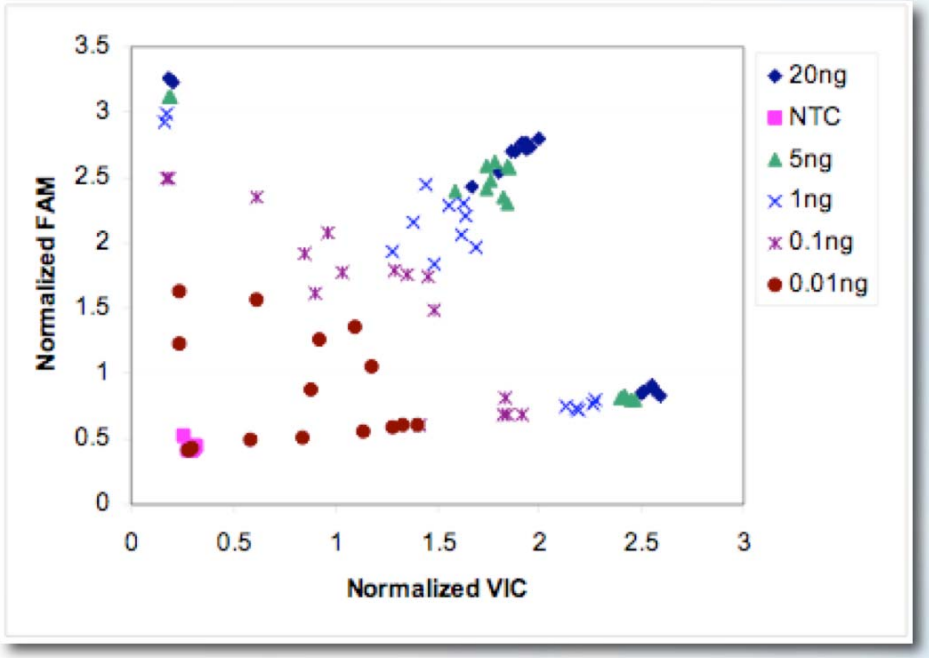
Optimizing Genotyping Experiments

- Scattering of Data Points / Diffuse Clusters
  - Low DNA concentrations
  - Suggested: > 1ng (relatively high)

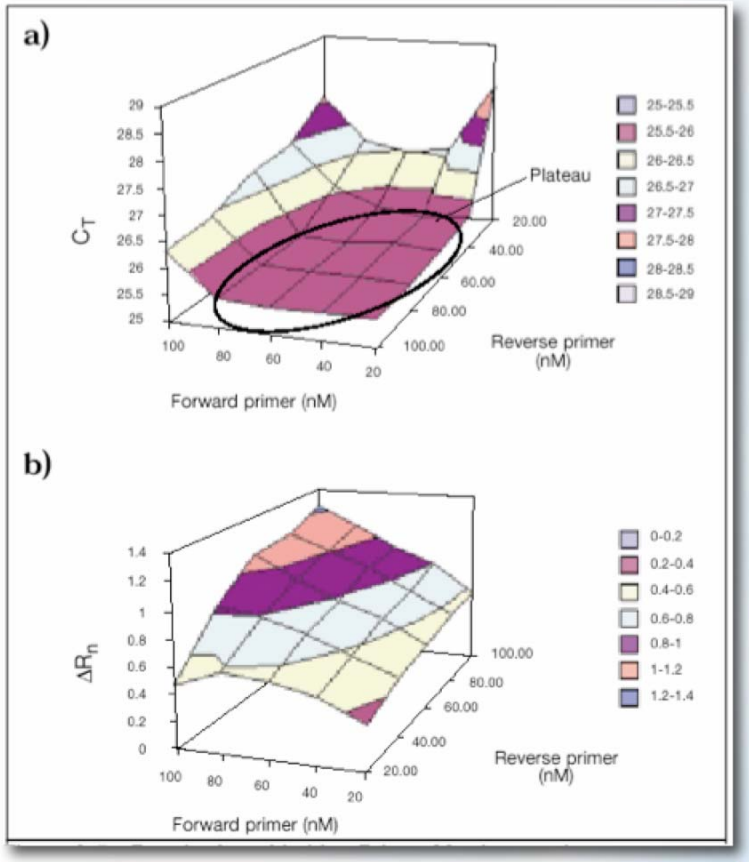




Applied Biosystems SDS Chemistry Guide (PN 4348358)

Multiplexing

- Primer-Limited Assays
  - ABI Vic® reporter dyes are primer limited, allowing multiplexing of TaqMan® endogenous controls with GOI quantitation.
  - Extensive assay optimization
  - Normal probe levels: 250nM
  - Suggested primer conc.:
    - 50nM or less
  - Determine plateau region:
    - CT values are constant



Applied Biosystems SDS Chemistry Guide (PN 4348358)

Revisiting the Goals

- Questions a PI should ask when presented with QPCR data:
  - How does this assay integrate with the project?
    - 1 primer pair per question! (1pppq)
  - Did you use a 'One-step' kit?
    - If "Yes" -> deny the assay!
  - What assay was used? commercial or custom design?
  - What chemistry was used? Why?
    - If TaqMan: MGB or conventional probe?
  - What is the amplification efficiency (E) for this amplicon?
    - Show me the 'Primer validation' experiment!
  - How do the amplification plots look like?
    - How did you adjust the baseline, the threshold?
  - How many times did you measure this result? How many runs were necessary to get to this result?
  - What method of data evaluation did you use?
    - If $\Delta\Delta C_T$: show me the validation experiment.
  - How many replicates were used for the measurements?
  - Are any C_T values larger than 35?
  - What did you do for error handling?

Selected References

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