



Real-time PCR principle

- amplification curve
- Cq value determination methods

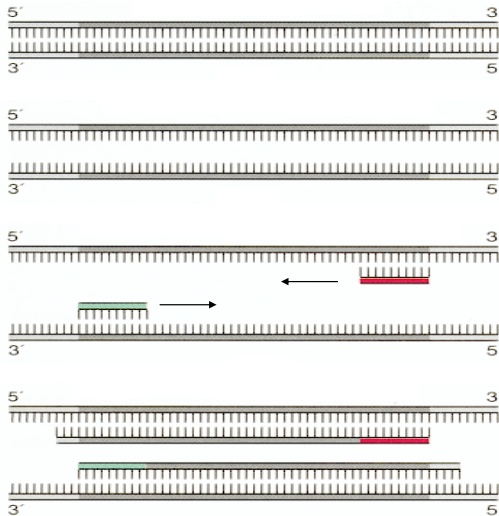
Absolute quantification

Comparative quantification

Efficiency determination

- Dilution series
- Single curve efficiency

Correcting for technical variation

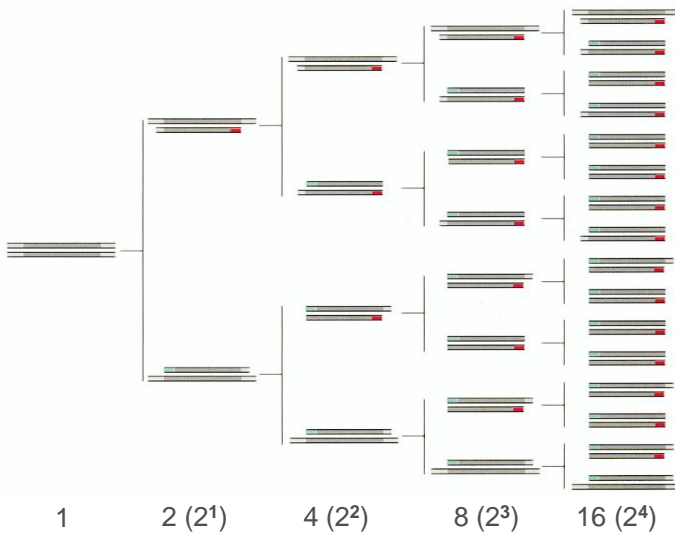


30-45 cycles of a 3-step process

denaturation (95 ° C 15 s)

annealing (50-70 ° C 15 s)
20bp ~1/1000 000 000 000

extension (72 ° C 1 min)



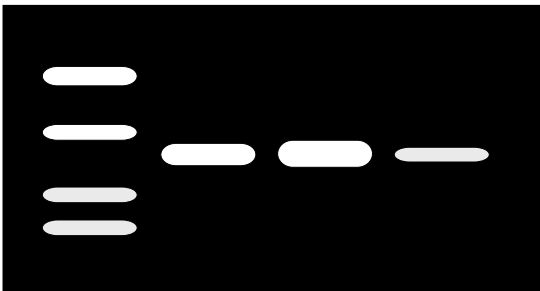
exponential
amplification

..... → 35 cycli

$$2^{35} = 68 \times 10E9$$

Endpoint detection (agarose gel electrophoresis)

- Not really quantitative
- Carry-over contamination
- Laborious



Continuous monitoring of PCR product accumulation

- i.e. measure every cycle the amount of fluorescence

Relationship between the time fluorescence increases above background and the initial amount of template

- i.e. the sooner fluorescence is visible, the more template was present, and vice versa

Kinetic PCR Analysis: Real-time Monitoring of DNA Amplification Reactions

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We describe a simple, quantitative assay for any amplifiable DNA sequence that uses a video camera to monitor multiple polymerase chain reactions (PCRs) simultaneously over the course of thermocycling. The video camera detects the accumulation of double-stranded DNA (dsDNA) in each PCR using the increase in the fluorescence of ethidium bromide (EBr) that results from its binding duplex DNA. The kinetics of fluorescence accumulation during thermocycling are directly related to the starting number of DNA copies. The fewer cycles necessary to produce a detectable fluorescence, the greater the number of target sequences. Results obtained with this approach indicate that a kinetic approach to PCR analysis can quantitate DNA sensitively, selectively and over a large dynamic range. This approach also provides a means of determining the effect of different reaction conditions on the efficacy of the amplification and so can provide insight into fundamental PCR processes.

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To facilitate its automation, any assay intended for large-scale use should be simplified as much as possible. One such simplification, a homogeneous (i.e., "one-tube") approach to sequence-specific DNA detection using a fluorescent, DNA-binding dye directly in a polymerase chain reaction (PCR)* has previously been described¹. This approach depends on the fluorescence enhancement produced when a dye such as ethidium bromide (EBr) binds dsDNA. Since PCR produces dsDNA as the reaction proceeds, the presence of EBr results in a net increase in fluorescence with increasing cycles of amplification. In our previous paper¹, such fluorescence increases were primarily monitored after completion of thermocycling. Such an "endpoint" analysis, as is typically done using PCR, reveals the presence or absence of target DNA but does not provide a good measure of the starting number of DNA targets. In this paper, we show that a kinetic analysis mode, in which the level of amplified DNA is continuously monitored over the course of amplification, can provide this quantitative information as well as provide information about the amplification process itself.

Previously¹, we used a bifurcated optical fiber to bring excitation illumination from a spectrofluorometer to a single PCR and to return fluorescence for measurement. In order to monitor many amplifications simultaneously, we now use a video camera to capture fluorescence images of an array of PCRs in the same block of a thermocycler. This configuration is diagrammed in Figure 1. A computer-controlled, cooled CCD camera, as described in Sutherland et al.², is mounted on a copy stand with its lens focused on the surface of the thermocycler block. UV lights (302 nm) are mounted flanking the camera and directed at the block. To reduce parallax effects, the camera and light assembly is placed as far as is practical from the block. A 600 nm interference filter is placed in front of the lens to limit detection to the desired wavelength. The camera is connected to a "frame-grabber" in a desk-top computer that allows the digitized images to be saved for later manipulation.

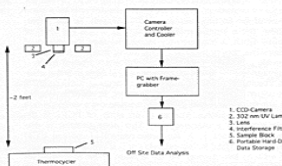


FIGURE 1. Block diagram of video camera system used to monitor amplifications in thermocycler. See Experimental Protocol for more details.

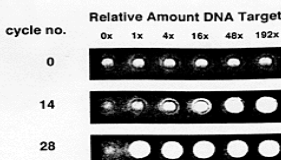


FIGURE 2. Composite of portions of video images taken using the set-up shown in Figure 1. These images are of EBr-containing PCRs in the thermocycler block (Perkin-Elmer Model 480) and are taken looking down through the tube caps under UV (302 nm) illumination. The three images were of the same six PCRs held at the annealing/extension temperature before beginning thermocycling (cycle 0), and during the annealing/extension phase of cycles 14 and 26. Only one row of samples in the image is shown; a full block of 48 samples in a TC 480 or 96 samples in a TC 9600 can be imaged. The PCRs were initiated using a dilution series of target DNA (a 242 bp segment of HLA DQA gene*) at the indicated relative levels ($192 \times = 3 \times 10^6$ target molecules < 8 ng DNA). These images demonstrate the general principle that the higher the starting amount of target DNA, the earlier the cycle at which increased fluorescence is detectable.

cycles. To assess the quantitative performance of this assay, the amplifications were initiated using a dilution series of single-stranded, HIV template DNA (see Experimental Protocol). Starting with 10^6 templates, 10-fold dilutions were made down to 10^1 templates and used with HIV-specific primers to make a 142 bp PCR product. A control amplification with no added template was also monitored. To simulate a PCR-based screen of lymphocyte DNA for integrated HIV genomes³, all reactions contained 300 ng (40,000 cell equivalents) of human genomic DNA. Each video image was taken 20 seconds after the annealing/extension temperature was reached during the 30 second hold. The fluorescence values plotted are the average of the pixel

values in the video image of each amplification tube.

As expected, the fluorescence from each reaction changes little in initial thermocycles and then rises as detectable amounts of PCR product are generated. The more starting template copies in the reaction, the earlier such a rise in fluorescence occurs. There is a considerable variance in the initial fluorescence values obtained from the different amplifications even though it is expected that these initial values should be the same (the varying amounts of single-stranded template DNA are so small that they have a negligible effect on the total fluorescence). The sources of this variation are apparently inhomogeneity of illumination, parallax, and variable attenuation of the fluorescence due to the tube caps. We have found that viewing amplifications without caps but through a vapor barrier of mineral oil or Amplifix™ reduces, but does not eliminate, this variation.

This variation can also be seen in Figure 4, a plot of fluorescence vs. DNA concentration for known amounts of phage λ DNA. The DNA was in PCR buffer with primers and with 4 μg/ml EBr and the measurements were made at 68°C. Each tube was imaged without its cap and through melted Amplifix™. Fluorescence was measured for five replicate samples of each DNA concentration. The standard deviation was, on average, $\pm 4\%$ (as compared to $\pm 11\%$ for the initial values in Fig. 3A). Comparison of Figure 4 with Figure 3A also shows that it takes the fluorescence of ~ 50 ng of dsDNA to exceed the fluctuation in fluorescence during early cycles, suggesting that this amount of amplified DNA is the lower limit of detection of amplified DNA. Also, it is apparent that fluorescence detected in these amplifications is linearly related to the concentration of dsDNA, and that up to 4 μg of dsDNA are being made in the amplifications.

Fluorescence normalization and quantitative analysis. To compensate for measurement variation, a normalization factor for each amplification was derived which is the ratio of the average initial fluorescence of all reactions to the observed initial fluorescence of each reaction. All fluorescence values for each amplification were multiplied by this factor. This normalization (the result of which is shown in Fig. 3B) is derived from the assumption that the source of the image variation attenuates or enhances the fluorescence signal proportionately over the entire range of signal intensities. All reactions now begin at the same fluorescence and most of the reaction profiles have a regular spacing consistent with the dilution series performed. The profiles are very similar in shape and nearly parallel. The early or

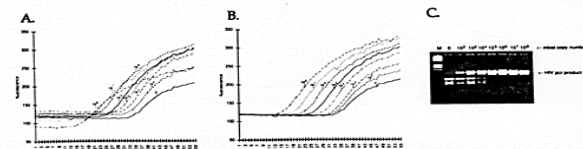
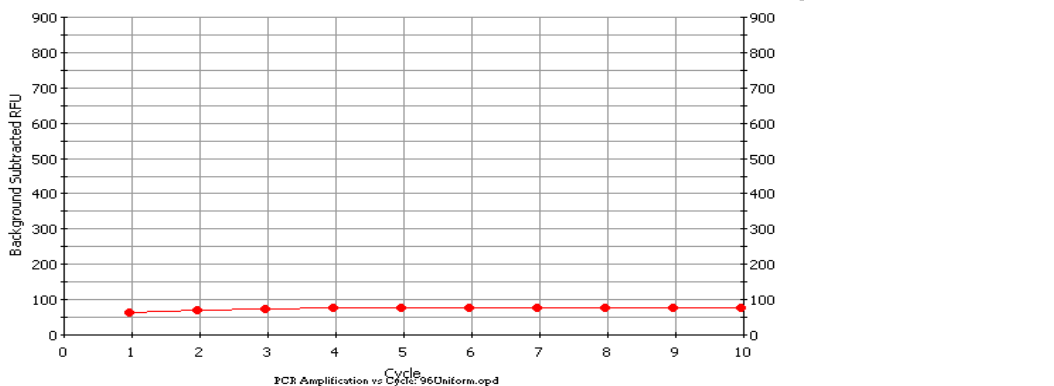
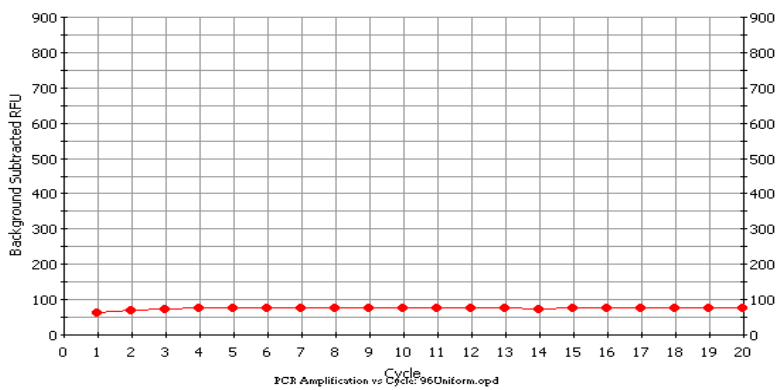
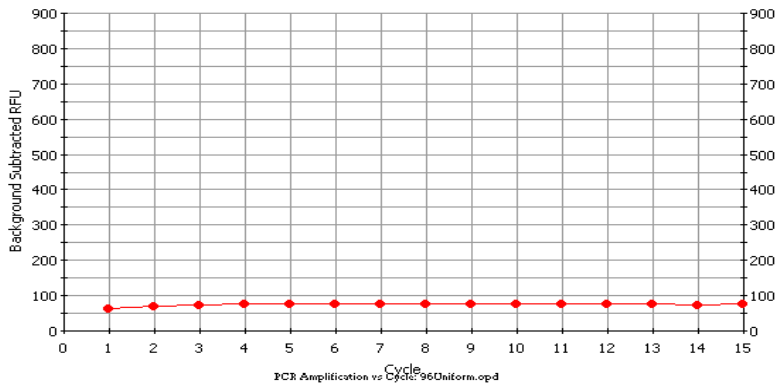
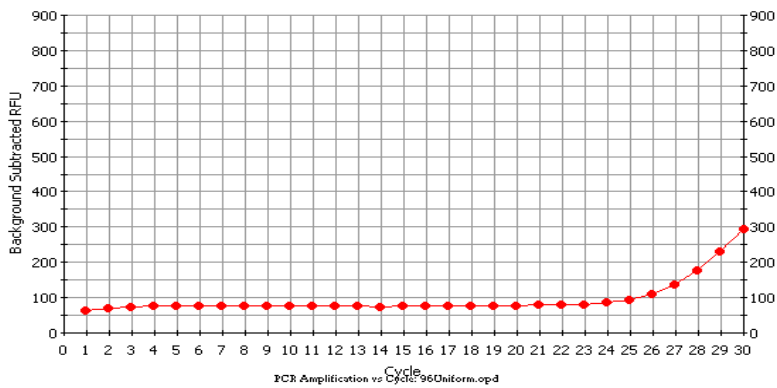
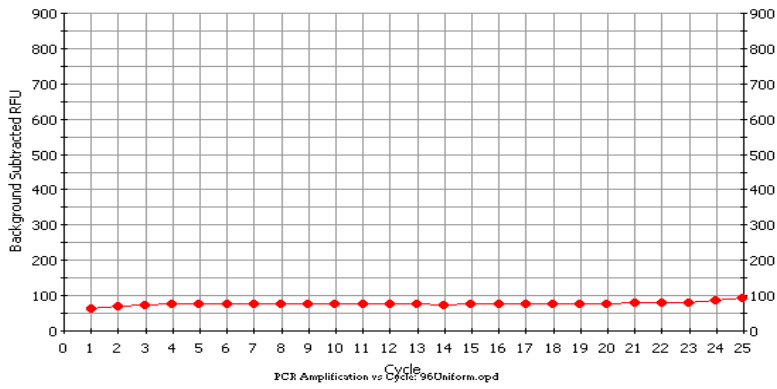
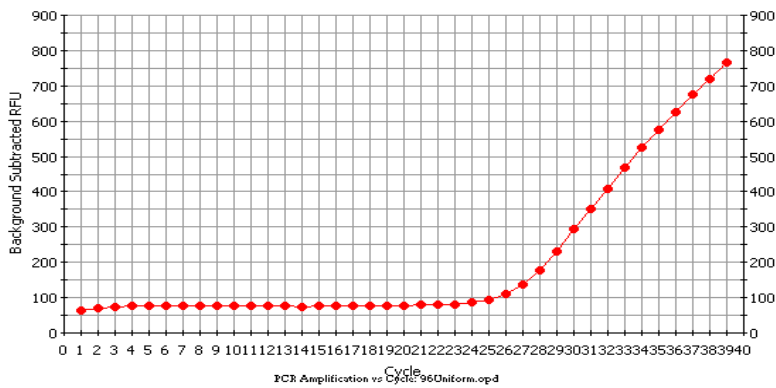
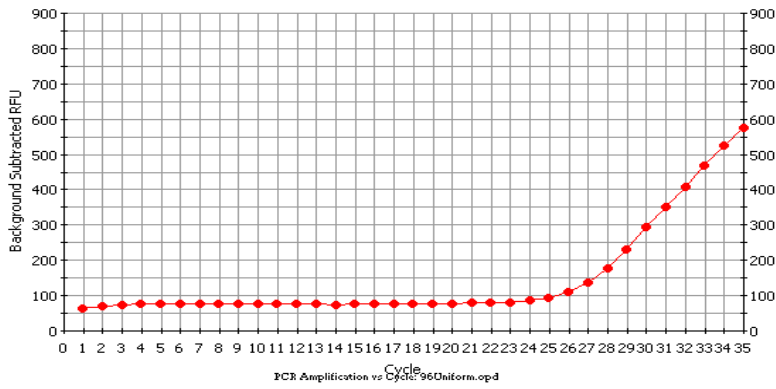


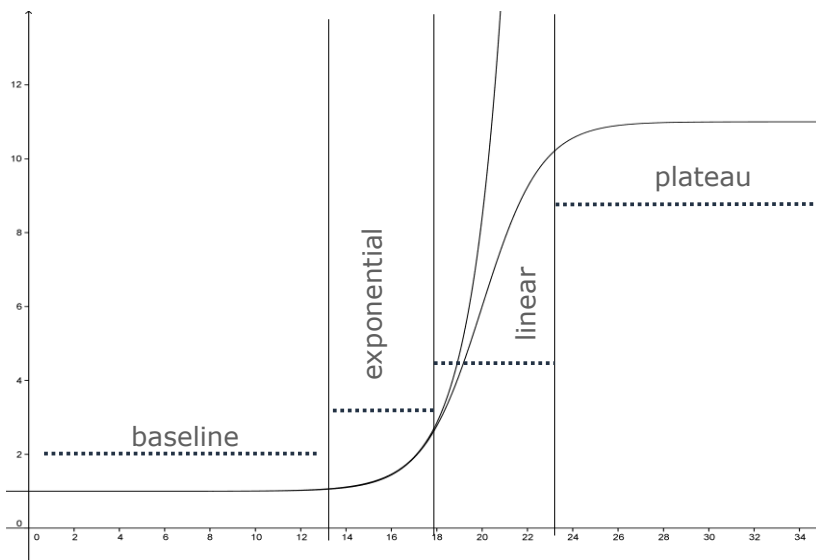
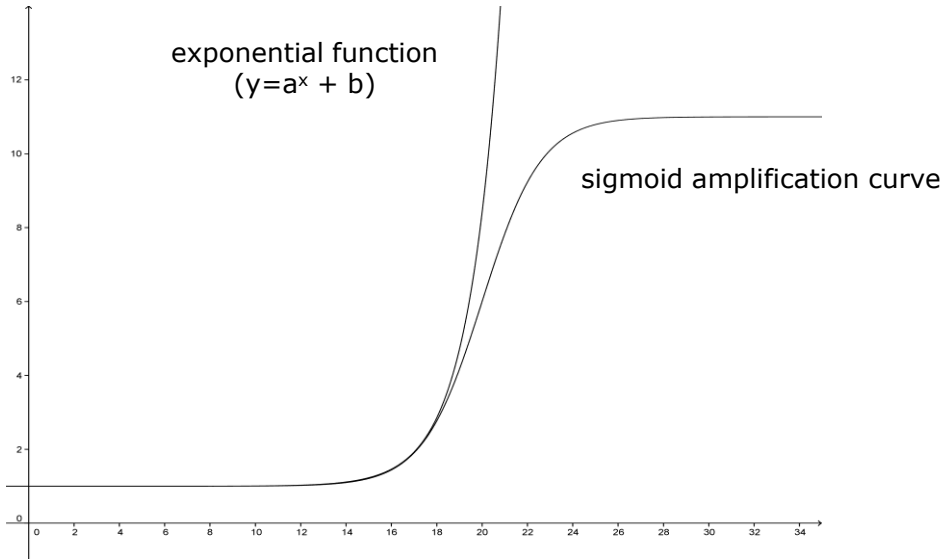
FIGURE 3. (A) "Raw data" fluorescence profiles of eight EBr-containing PCRs that were identical except for the indicated number of HIV template molecules they contained prior to amplification. (B) Normalized fluorescence profiles of the same PCRs shown in A. (C) "Mu-Sieve" agarose gel electrophoresis of the final amplification products of the experiment in (A & B). The expected mobility of the HIV-specific amplification product (142 bp) is indicated. The marker (M) lane contains a Hae III restriction enzyme digest of 0x 174 DNA. The DNA in the gel is stained with EBr and photographed under ultraviolet light. The amount of initial target DNA is indicated for each lane.



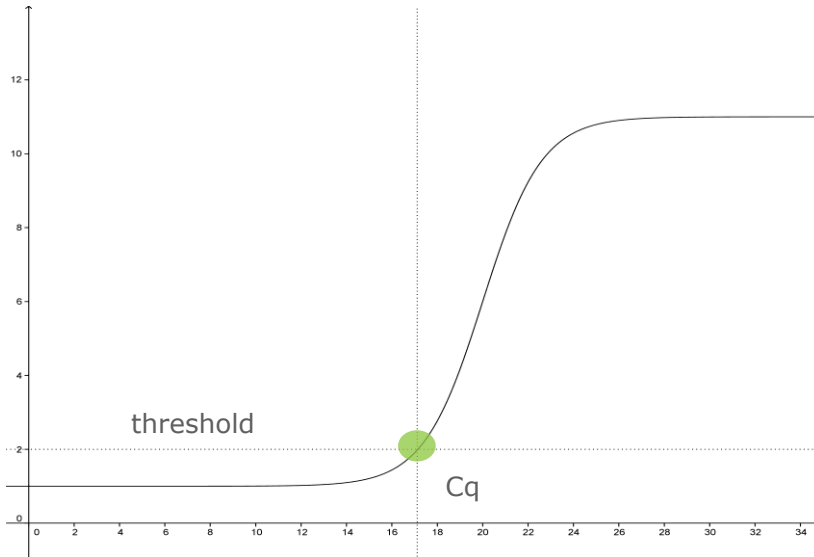




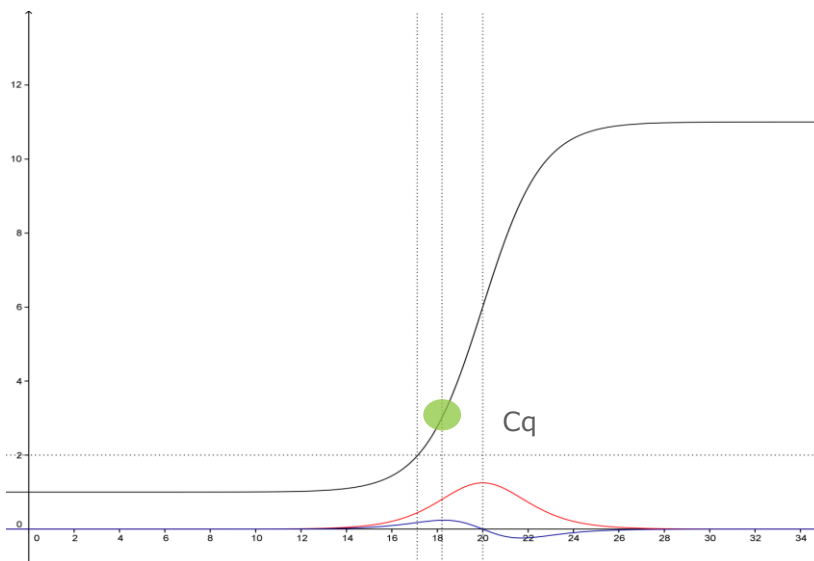


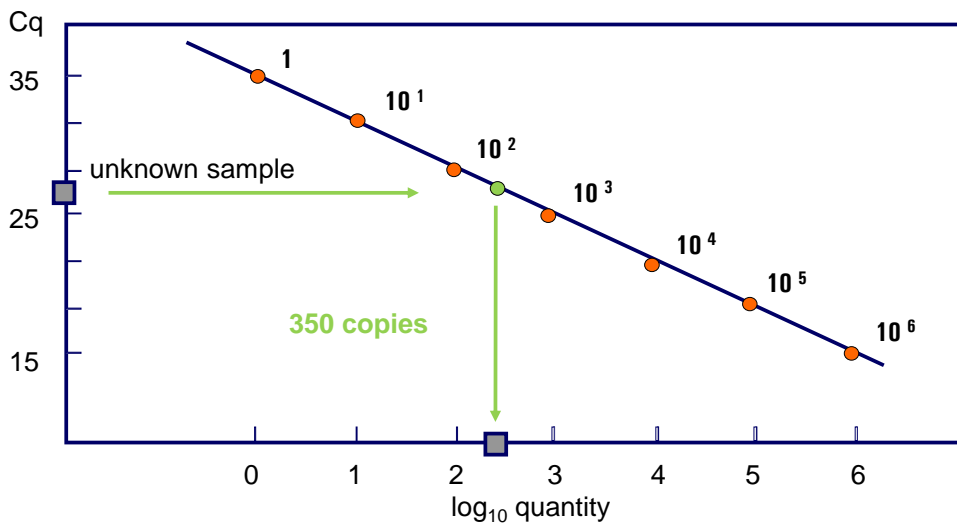
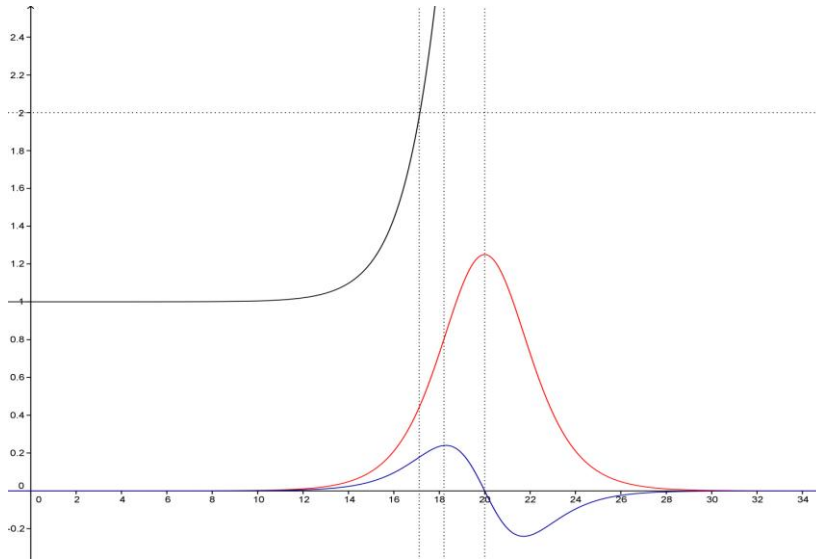


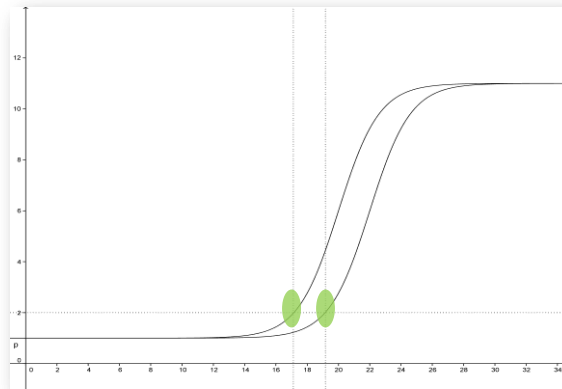
Quantification cycle value: threshold method



Quantification cycle value: 2nd derivative maximum method







- Delta-Cq = $\Delta Cq = 19 - 17 = 2$
- Relative quantity (RQ) = $E^{\Delta Cq} = 2^2 = 4$
 - ▶ E value represents the amount of fold change per cycle per gene
 - ▶ E = 2 represents 100% PCR efficiency

Determine PCR efficiency

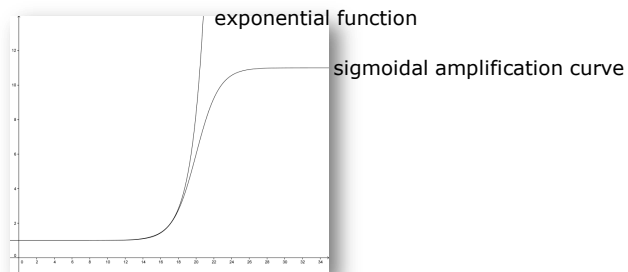
- Standard dilution series
- Single curve efficiency

Standard dilution series → Standard curves

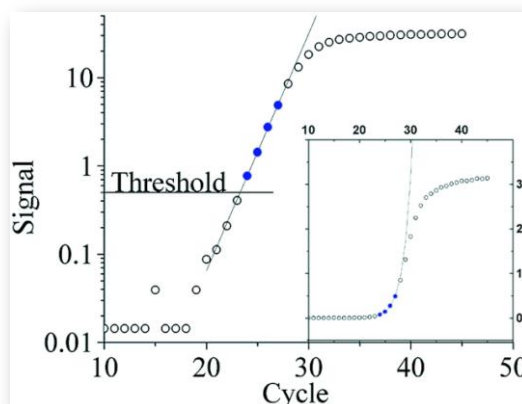
- Gold standard
- Slope (a) of the standard curve ~ efficiency of the PCR
- Efficiency = $10^{(-1/a)} - 1$
- Ideal efficiency
 - ▶ 100 %
 - ▶ 1
 - ▶ slope of -3.322
 - ▶ E = 2 (base exponential amplification)

Single curve efficiency

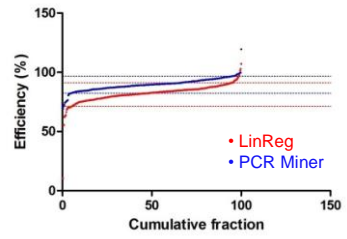
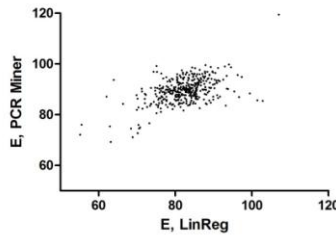
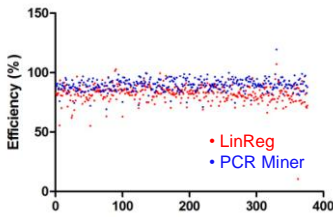
- Calculate the amplification efficiency from the shape of the amplification plot
- Different algorithms
 - ▶ LinReg → Ramakers, Nucleic Acids Res. 2009 Apr;37(6):e45
 - ▶ PCR Miner → Zhao, Comput. Biol. 2005 Oct;12(8):1045-62
 - ▶ LRE (Linear Regression of Efficiency) → Rutledge, BMC Biotechnol. 2008 May 8;8:47
 - ▶ ...



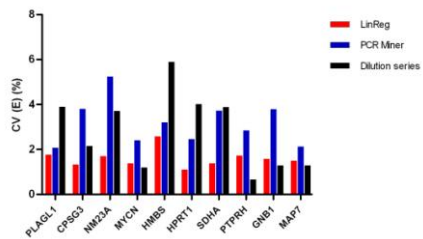
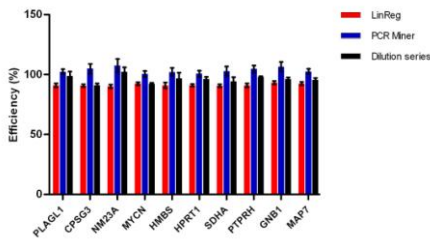
- Estimation of PCR efficiency by exponential fit



- Problem with single curve efficiency determination
 - ▶ Different algorithms give different efficiencies



- Problem with single curve efficiency determination
 - ▶ Different algorithms give different efficiencies
 - ▶ Single curve efficiencies do not correspond to standard curve efficiencies
 - ▶ Imprecise estimates due to noise on limited number of usable data points



- Problem with single curve efficiency determination
 - ▶ Different algorithms give different efficiencies
 - ▶ Single curve efficiencies do not correspond to standard curve efficiencies
 - ▶ Imprecise estimates due to noise on limited number of usable data points

- Individual efficiency values can not be used in comparative Cq quantification model

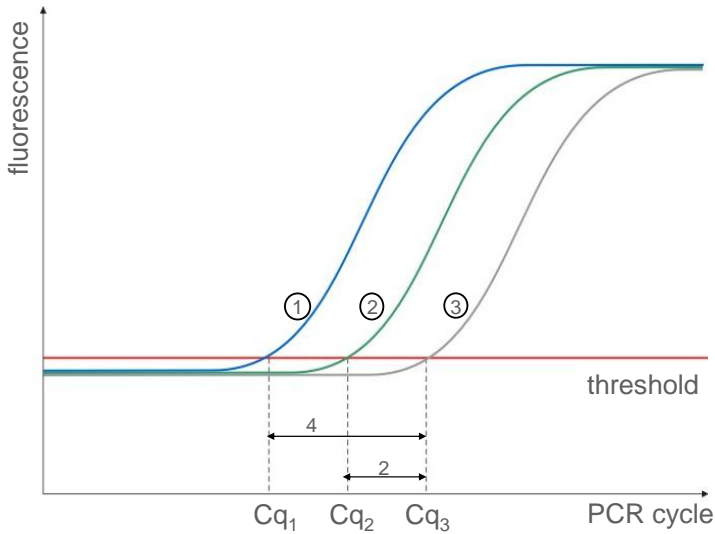
- Individual efficiency values may be averaged across all measurements for a given assay to replace standard curve efficiency estimates

- References
 - ▶ Nordgård et al., Anal Biochem, 2006
 - ▶ Goll et al., BMC Bioinformatics, 2006
 - ▶ Karlen et al., BMC Bioinformatics, 2007

Relative quantification

- One sample relative to another
- One transcript relative to another (e.g. splice isoforms)
Vandenbroucke et al., *Nucleic Acids Research*, 2001

Comparative Cq method / delta-Cq method

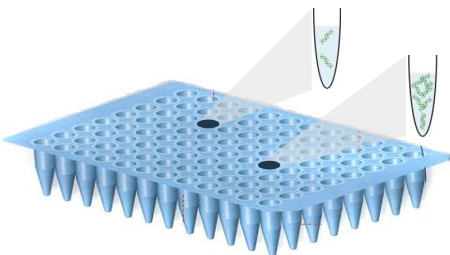


$$RQ = 2^{\Delta Cq}$$

$$RQ_{1/3} = 2^4 = 16$$

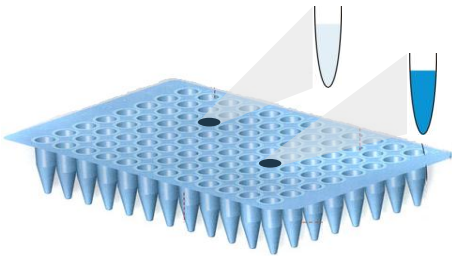
$$RQ_{2/3} = 2^2 = 4$$

$$RQ_{3/3} = 2^0 = 1$$



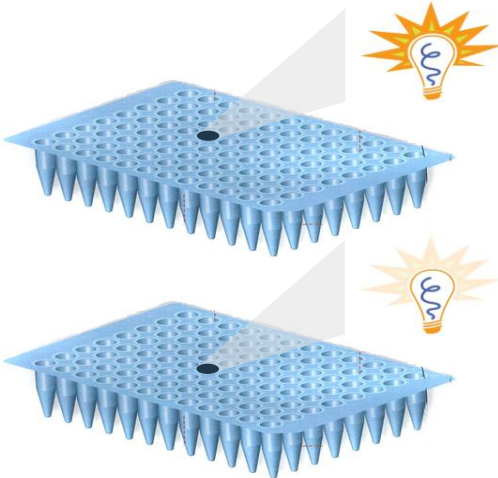
differences in RQ due to

- different gene expression level



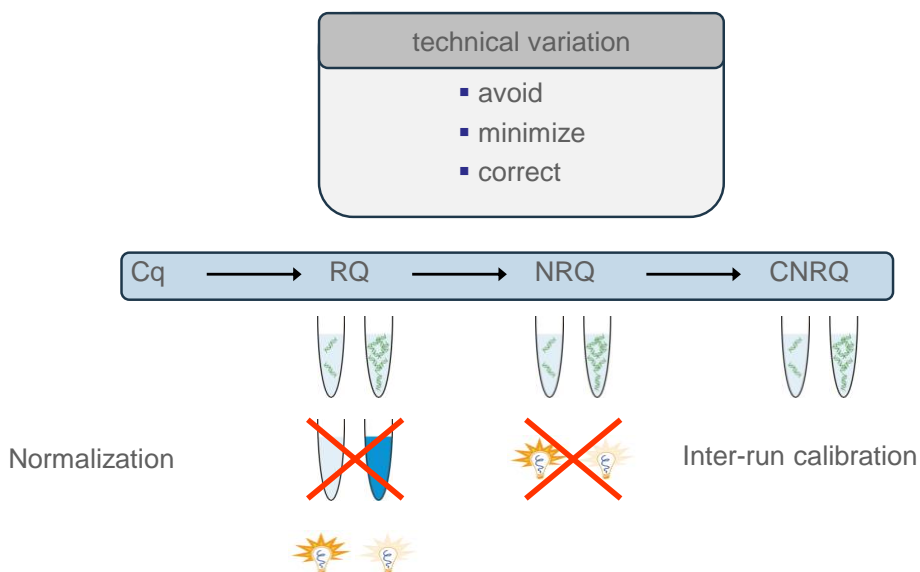
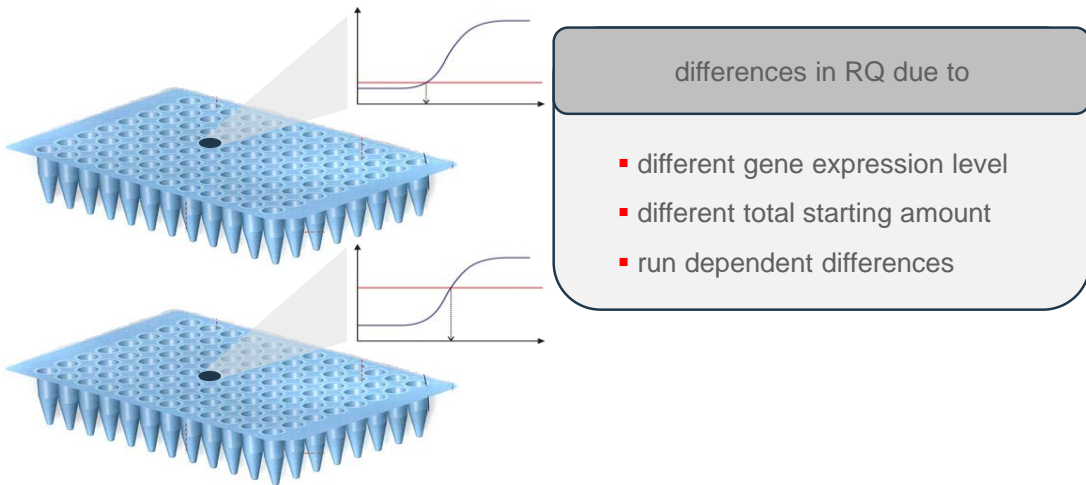
differences in RQ due to

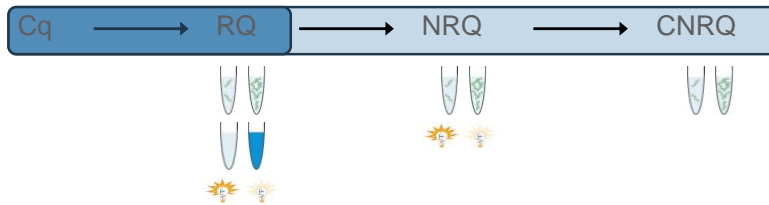
- different gene expression level
- different total starting amount



differences in RQ due to

- different gene expression level
- different total starting amount
- run dependent differences





$$RQ = 2^{\Delta Cq}$$

$$RQ = E^{\Delta Cq}$$

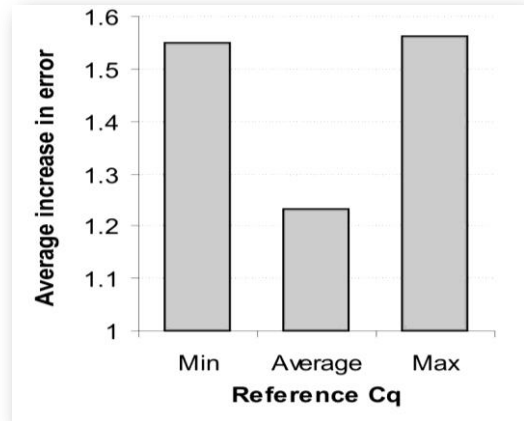
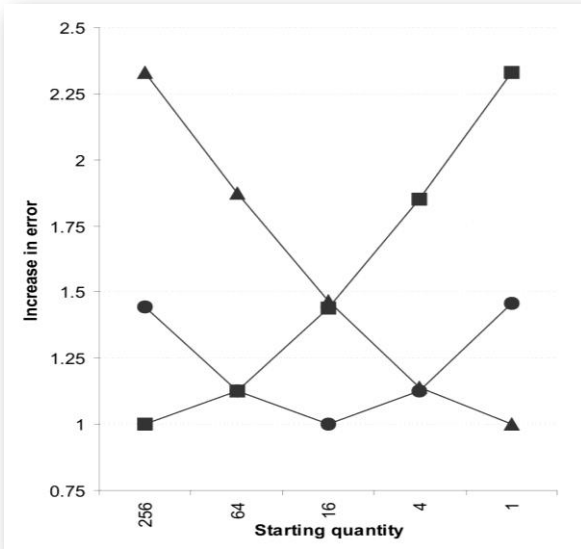


Calculate gene specific amplification efficiency (E) from dilution series

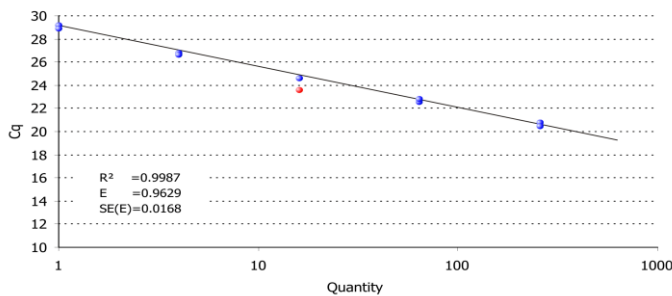
$$RQ = E^{\Delta Cq} \quad SE(RQ) = \sqrt{RQ^2 \left[\left(\frac{\Delta Cq \cdot SD(E)}{E} \right)^2 + (\ln(E) \cdot SD(Cq_{ref}))^2 \right]}$$

$$\Delta Cq = Cq_{ref} - Cq_{soi}$$

- Choice of Cq_{ref} does not affect the RQ ratio between samples
 - ▶ Sample of choice
 - ▶ Sample with lowest expression
 - ▶ Average Cq
 - ▶ Cycle 20
- Choice of Cq_{ref} does affect the error on RQ (if SE(E) is taken into account)
- Minimize error: $Cq_{ref} = \text{average Cq}$
 - ▶ qbase^{PLUS} approach



Hellemans et al., 2007, Genome Biology



$$\text{slope} = \frac{\sum_{a=1}^n (Q_a - \bar{Q})(Cq_a - \bar{Cq})}{\sum_{a=1}^n (Q_a - \bar{Q})^2}$$

$$E = 10^{\left(\frac{-1}{\text{slope}}\right)}$$

$$s_e = \sqrt{\frac{\sum_{a=1}^n (Cq_{a, \text{measured}} - Cq_{a, \text{predicted}})^2}{n - 2}}$$

$$s_x = \sqrt{\frac{1}{n-1} \sum_{a=1}^n (Q_a - \bar{Q})^2}$$

$$SE(\text{slope}) = \frac{s_e}{s_x(n-1)}$$

$$SE(E) = \frac{E \cdot \ln(10) \cdot SE(\text{slope})}{\text{slope}^2}$$

calculate and propagate the error on E
minimize SE(E)

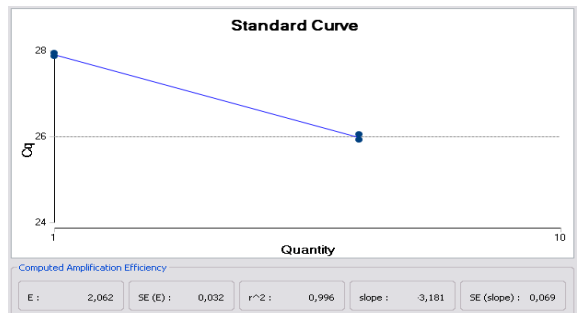
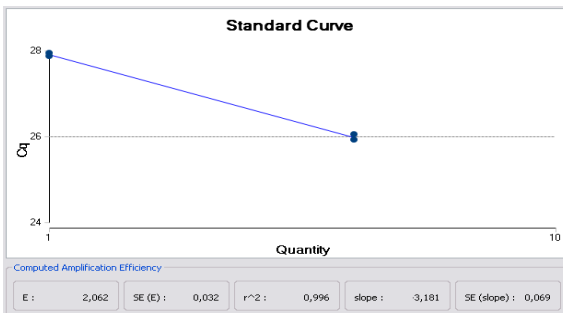
- increase number of dilution points (n)
- increase range of dilution

increase number of points

■ 1-2 SE(E)=0.032

increase range of points

■ 1&2 SE(E)=0.032



increase number of points

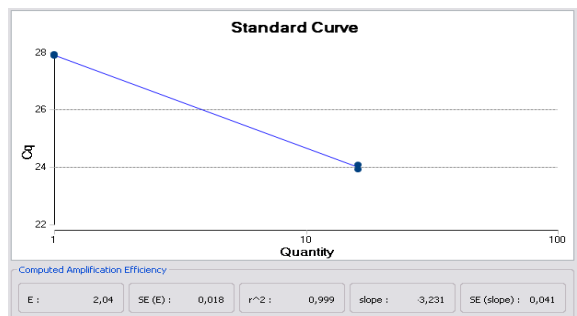
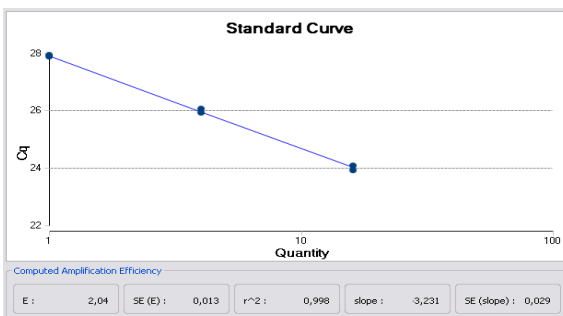
■ 1-2 SE(E)=0.032

■ 1-3 SE(E)=0.013

increase range of points

■ 1&2 SE(E)=0.032

■ 1&3 SE(E)=0.018

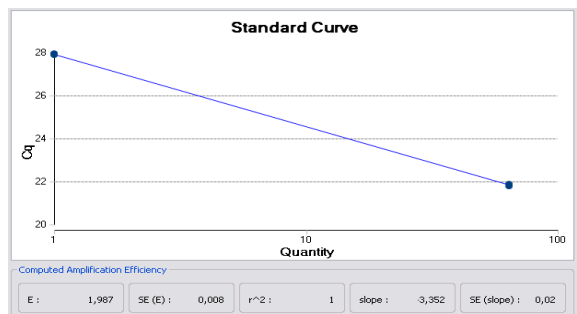
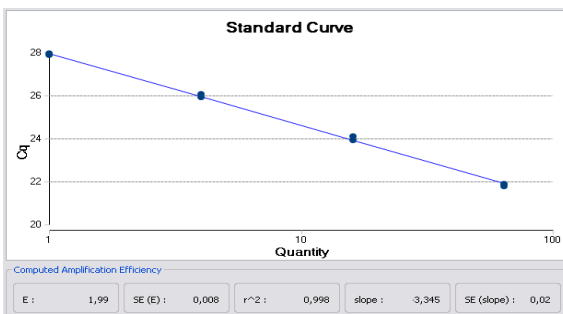


increase number of points

- 1-2 SE(E)=0.032
- 1-3 SE(E)=0.013
- 1-4 SE(E)=0.008

increase range of points

- 1&2 SE(E)=0.032
- 1&3 SE(E)=0.018
- 1&4 SE(E)=0.008

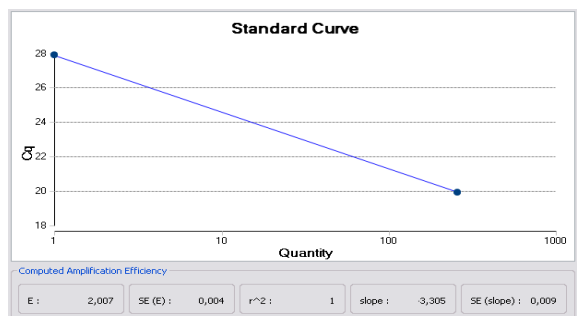
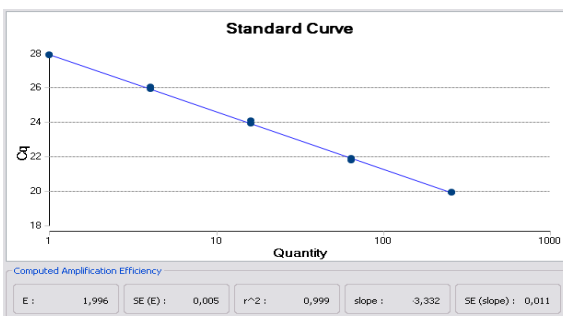


increase number of points

- 1-2 SE(E)=0.032
- 1-3 SE(E)=0.013
- 1-4 SE(E)=0.008
- 1-5 SE(E)=0.005

increase range of points

- 1&2 SE(E)=0.032
- 1&3 SE(E)=0.018
- 1&4 SE(E)=0.008
- 1&5 SE(E)=0.004

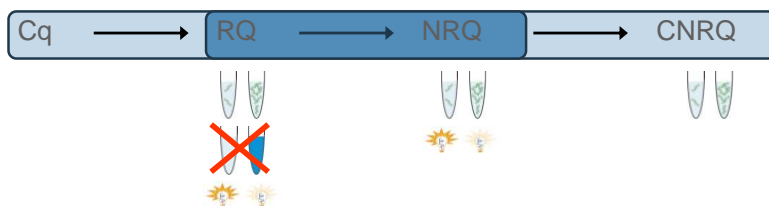
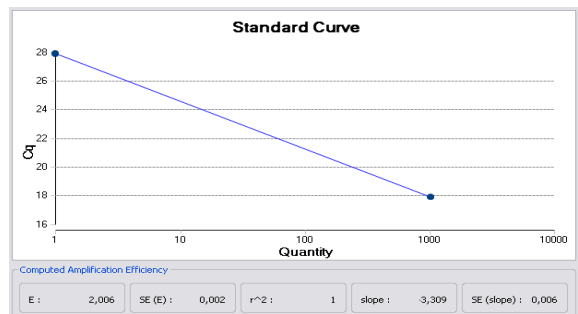
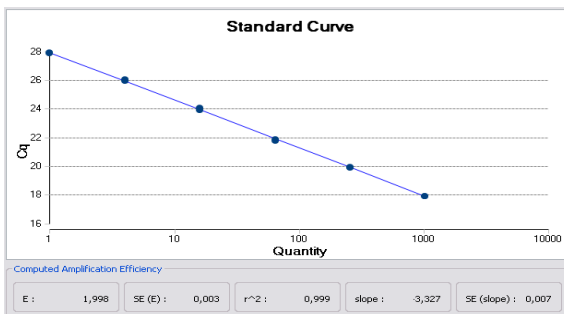


increase number of points

- 1-2 SE(E)=0.032
- 1-3 SE(E)=0.013
- 1-4 SE(E)=0.008
- 1-5 SE(E)=0.005
- 1-6 SE(E)=0.003

increase range of points

- 1&2 SE(E)=0.032
- 1&3 SE(E)=0.018
- 1&4 SE(E)=0.008
- 1&5 SE(E)=0.004
- 1&6 SE(E)=0.002



Minimize technical variation

- sample: size and type
- RNA extraction: quality and quantity
- RNA degradation
- cDNA synthesis

Correct for technical variation

- normalization

Huggett et al., Genes Immun, 2005

Livak and Schmittgen (2001)

- 100% PCR efficiency
- 1 reference gene

$$NRQ = 2^{\Delta\Delta Ct}$$

Pfaffl (2001)

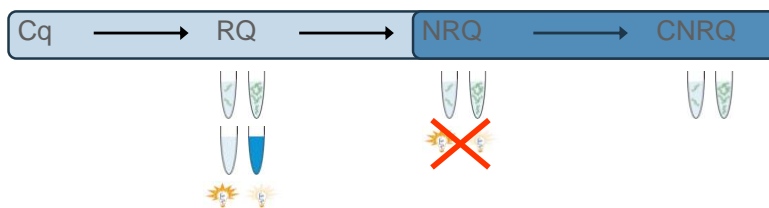
- adjusted PCR efficiency
- 1 reference gene

$$NRQ = \frac{E_{goi}^{\Delta Ct, goi}}{E_{ref}^{\Delta Ct, ref}}$$

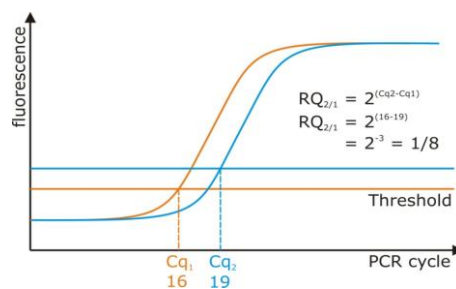
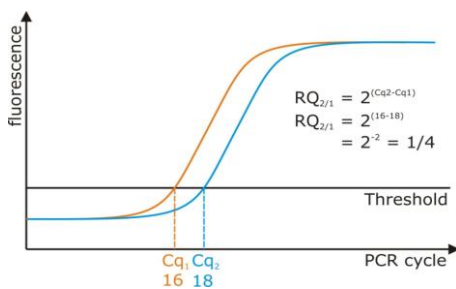
qBase model (2007)

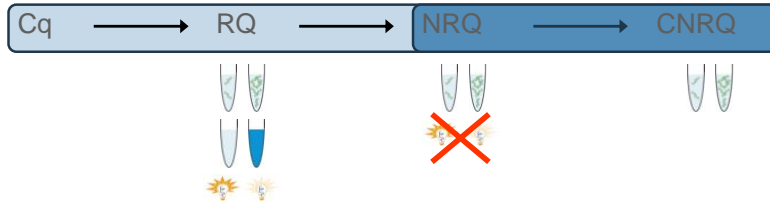
- adjusted PCR efficiency
- multiple reference genes

$$NRQ = \frac{E_{goi}^{\Delta Ct, goi}}{\sqrt[n]{\prod_i E_{ref_i}^{\Delta Ct, ref_i}}}$$



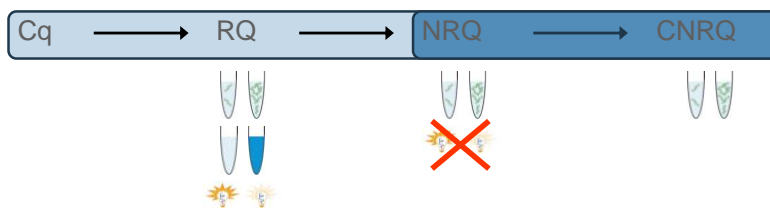
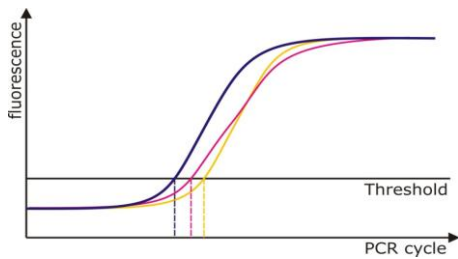
Different analysis settings





Different analysis settings

Instrument, reagents and consumable variation



Avoid inter-run variation

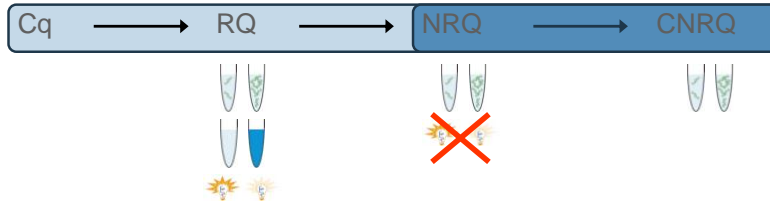
- use sample maximization

Minimize inter-run variation

- use the same instrument, reagents and consumables

Correct for inter-run variation

- inter-run calibration

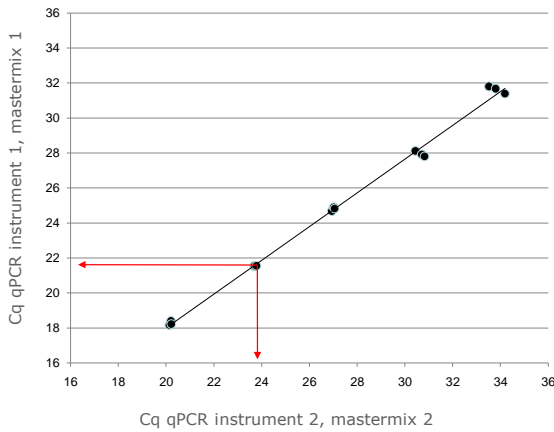


Correct for inter-run variation by including IRC's
IRC

- inter-run calibrator
- identical sample measured for the same gene in different runs



5 standards (triplicates)



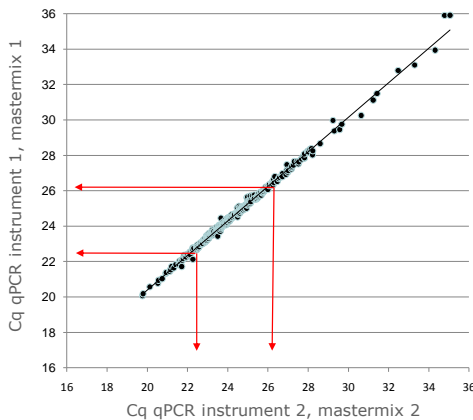
average ΔCq standards



correction Cq samples

ARHGEF7 gene

- 366 samples
- use of 5 standards (triplicates) for correction



$$s_{i,j} = \sqrt{\frac{\sum_{k=1}^n (C_{q_{i,j,k}} - C_{q_{i,j}})^2}{n-2}} \quad (\text{formula 1})$$

$$s_{i,j} = \sqrt{\frac{1}{n-1} \sum_{k=1}^n (C_{q_{i,j,k}} - \bar{C}_{q_{i,j}})^2} \quad (\text{formula 3})$$

$$SE(\text{slope}_j) = \frac{s_{i,j}}{s_{i,j} \sqrt{n-1}} \quad (\text{formula 4})$$

The base for exponential amplification E , and its standard error $SE(E)$ are calculated from these values:

$$E_j = 10^{\left(\frac{1}{\text{slope}_j}\right)} \quad (\text{formula 5})$$

$$SE(E_j) = \frac{E_j \cdot \ln(10) \cdot SE(\text{slope}_j)}{\text{slope}_j^2} \quad (\text{formula 6})$$

Conversion of Cq values into relative quantities

Step 1: Calculation of the average Cq value for all replicates of the same gene/sample combination j within a given run i :

$$\bar{C}_{q_{i,j}} = \frac{\sum_{k=1}^n C_{q_{i,j,k}}}{n} \quad (\text{formula 7})$$

$$SE(\bar{C}_{q_{i,j}}) = \frac{1}{\sqrt{n}} \sqrt{\frac{\sum_{k=1}^n (C_{q_{i,j,k}} - \bar{C}_{q_{i,j}})^2}{n-1}} \quad (\text{formula 8})$$

Step 2: Transformation of mean Cq value into RQ using the gene specific PCR efficiency E_j with minimization of the overall error:

$$C_{q_{i,j}} = \bar{C}_{q_{i,j}} + \frac{SE(\bar{C}_{q_{i,j}})}{E_j} \quad (\text{formula 9})$$

$$\Delta C_{q_{i,j}} = C_{q_{i,j}} - \bar{C}_{q_{i,j}} \quad (\text{formula 10})$$

$$RQ_{i,j} = E_j^{-\Delta C_{q_{i,j}}} \quad (\text{formula 11})$$

$$SE(RQ_{i,j}) = RQ_{i,j} \left[\frac{SE(\bar{C}_{q_{i,j}})}{E_j} + \ln(E_j) \cdot SE(\Delta C_{q_{i,j}}) \right] \quad (\text{formula 12})$$

Normalization: inter-run calibration
The procedures for normalization and inter-run calibration are highly analogous and are therefore described in parallel.

Step 1: Calculation of the normalization factor NF for sample k based on the RQs of the reference genes p .

Step 1': Calculation of the calibration factor CF for gene j in run i based on the NRQs of the IBCs m :

$$NF_k = \prod_{p=1}^P RQ_{k,p} \quad (\text{formula 13})$$

$$CF_j = \prod_{m=1}^M NRQ_{i,m} \quad (\text{formula 13'; for definition of NRQ, see formula 15})$$

$$SE(NF_k) = NF_k \sqrt{\sum_{p=1}^P \left(\frac{SE(RQ_{k,p})}{RQ_{k,p}} \right)^2} \quad (\text{formula 14})$$

$$SE(CF_j) = CF_j \sqrt{\sum_{m=1}^M \left(\frac{SE(NRQ_{i,m})}{NRQ_{i,m}} \right)^2} \quad (\text{formula 14'})$$

Step 2: Conversion of RQs into NRQs.

Step 2': Conversion of NRQs into CNRQs:

$$NRQ_{i,k} = \frac{RQ_{i,k}}{NF_k} \quad (\text{formula 15})$$

$$CNRQ_{i,j} = \frac{NRQ_{i,j}}{CF_j} \quad (\text{formula 15'})$$

$$SE(NRQ_{i,k}) = NRQ_{i,k} \sqrt{\left(\frac{SE(RQ_{i,k})}{RQ_{i,k}} \right)^2 + \left(\frac{SE(NF_k)}{NF_k} \right)^2} \quad (\text{formula 16})$$

$$SE(CNRQ_{i,j}) = CNRQ_{i,j} \sqrt{\left(\frac{SE(NRQ_{i,j})}{NRQ_{i,j}} \right)^2 + \left(\frac{SE(CF_j)}{CF_j} \right)^2} \quad (\text{formula 16'})$$

Coefficient of variation of NRQs of a reference gene

Step 1: Calculation of the mean NRQ for all samples k and a given reference gene p :

$$NRQ_p = \frac{\sum_{k=1}^n NRQ_{k,p}}{n} \quad (\text{formula 17})$$

$$SE(NRQ_p) = \frac{1}{\sqrt{n}} \sqrt{\sum_{k=1}^n (NRQ_{k,p} - NRQ_p)^2} \quad (\text{formula 18})$$

Step 2: Calculation of the coefficient of variation CV of a given reference gene p across all samples k :

$$CV_p = \frac{SE(NRQ_p)}{NRQ_p} \quad (\text{formula 19})$$

Step 3: Calculation of the mean coefficient of variation for all reference genes:

$$CV = \frac{\sum_p CV_p}{P} \quad (\text{formula 20})$$

Reference gene and IRC stability parameter M
Since normalization and inter-run calibration are highly analogous, quality evaluation using the stability parameter M is similar as well. Therefore, both methods are explained in parallel.

Step 1: Calculation of the $s \times 1$ matrix $A^{(s)}$ in which the k^{th} element is the log₂ transformed ratio between the NRQs of two IBCs m and m' for the same gene j within a run i ; matrix $A^{(s)}$ is calculated in an analogous manner.

Step 1': Calculation of the $g \times 1$ matrix $A^{(g)}$ in which the l^{th} element is the log₂ transformed ratio between the relative quantities (not yet normalized) of two reference genes p and p' in sample k ; matrix $A^{(g)}$ is calculated in an analogous manner.

$$(A^{(s)})_{k,l} = \log_2 \left(\frac{NRQ_{i,m}}{NRQ_{i,m'}} \right) \quad (\text{formula 21})$$

$$(A^{(g)})_{k,l} = \log_2 \left(\frac{RQ_{i,p}}{RQ_{i,p'}} \right) \quad (\text{formula 21'})$$

$$(V^{(s)})_{k,l} = \log_2 \left(\frac{SE(NRQ_{i,m})}{NRQ_{i,m}} + \frac{SE(NRQ_{i,m'})}{NRQ_{i,m'}} \right) \quad (\text{formula 22})$$

$$(V^{(g)})_{k,l} = \log_2 \left(\frac{SE(RQ_{i,p})}{RQ_{i,p}} + \frac{SE(RQ_{i,p'})}{RQ_{i,p'}} \right) \quad (\text{formula 22'})$$

Step 2: Calculation of the mean log transformed ratio and the standard deviation $V^{(s)}$ for all runs i and a given reference gene combination (m, m') and a given gene j ; $V^{(s)}$ and $V^{(g)}$ are calculated similarly from $A^{(s)}$ and $A^{(g)}$, respectively.

$$M_j^{(s)} = \frac{\sum_i V^{(s)}_{i,j}}{I} \quad (\text{formula 23})$$

$$M_j^{(g)} = \frac{\sum_i V^{(g)}_{i,j}}{I} \quad (\text{formula 23'})$$

$$A_j^{(s)} = \frac{\sum_i A^{(s)}_{i,j}}{I} \quad (\text{formula 20})$$

$$A_j^{(g)} = \frac{\sum_i A^{(g)}_{i,j}}{I} \quad (\text{formula 20'})$$

$$V_j^{(s)} = SE(A_j^{(s)}) = \frac{1}{\sqrt{I}} \sqrt{\sum_{i=1}^I (A^{(s)}_{i,j} - A_j^{(s)})^2} \quad (\text{formula 21})$$

$$V_j^{(g)} = SE(A_j^{(g)}) = \frac{1}{\sqrt{I}} \sqrt{\sum_{i=1}^I (A^{(g)}_{i,j} - A_j^{(g)})^2} \quad (\text{formula 21'})$$

Step 3: Calculation of the arithmetic mean $M^{(s)}$ of all pairwise variations $V^{(s)}$ of a given reference gene p with all other tested reference genes p' ; $M^{(s)}$ represents the geNorm gene stability measure M for a particular reference gene p .

Step 3': Calculation of the arithmetic mean $M^{(g)}$ of all pairwise variations $V^{(g)}$ of a given IBC m with all the other IBCs m' for the same gene j ; $M^{(g)}$ and $M^{(s)}$ are calculated similarly from $V^{(s)}$ and $V^{(g)}$, respectively.

$$M_j^{(s)} = \frac{\sum_{p=1}^P V_j^{(s)}(p, m)}{P-1} \quad (\text{formula 24})$$

$$M_j^{(g)} = \frac{\sum_{p=1}^P V_j^{(g)}(p, m)}{P-1} \quad (\text{formula 24'})$$

Step 4: Calculation of the mean stability measure for all reference genes.

$$M_j = \frac{\sum_{p=1}^P M_j^{(s)}(p, m)}{P} \quad (\text{formula 25})$$

Step 4': Calculation of the mean stability measure for all IBCs:

$$M_j^{(g)} = \frac{\sum_{m=1}^M M_j^{(g)}(p, m)}{M} \quad (\text{formula 25'})$$

Method

qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data

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Choosing a different reference sample will result in different NRQ values, but differences between samples will be exactly the same

The entire set of NRQ values for a given gene may be rescaled

- Nicer graphs
- Easier to interpret
- Rescale to a reference / normal / calibrator sample to make all data relative to this sample (calibrator = 1 or 100%)

Use the average of multiple reference samples if the absolute value matters

- e.g. for copy number profiling

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