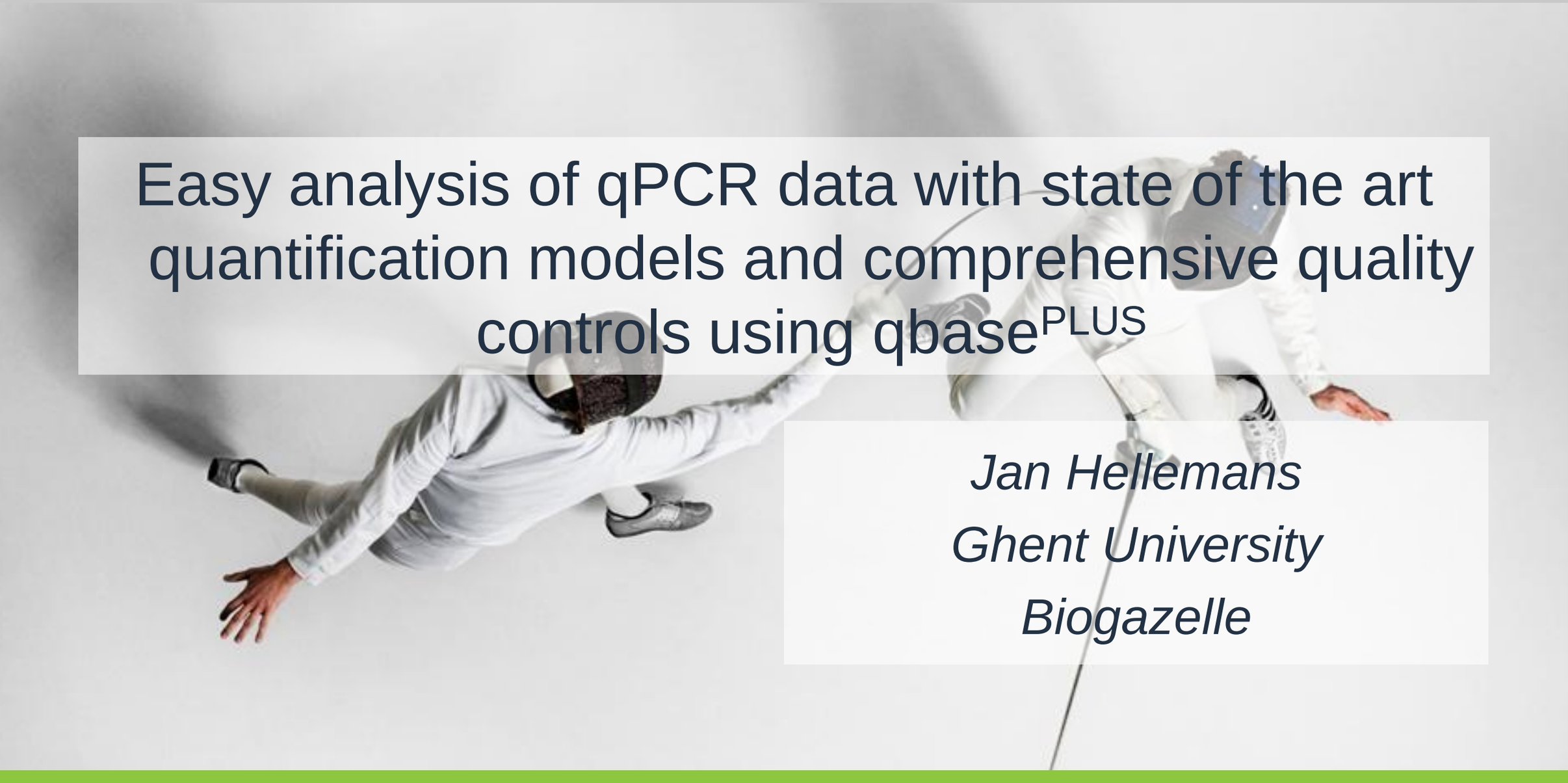
qbase PLUS

accelerating your analysis



Easy analysis of qPCR data with state of the art quantification models and comprehensive quality controls using qbase^{PLUS}

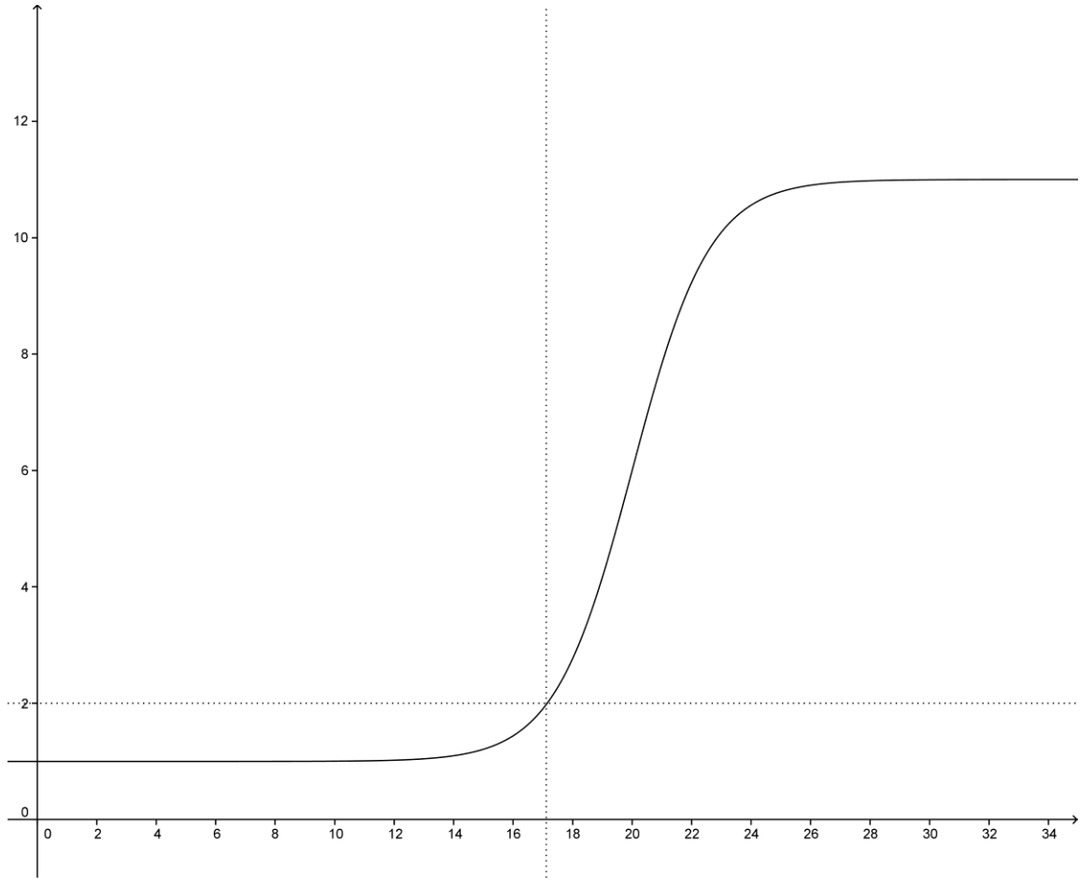
Jan Hellemans

Ghent University

Biogazelle

qPCR - the simple way

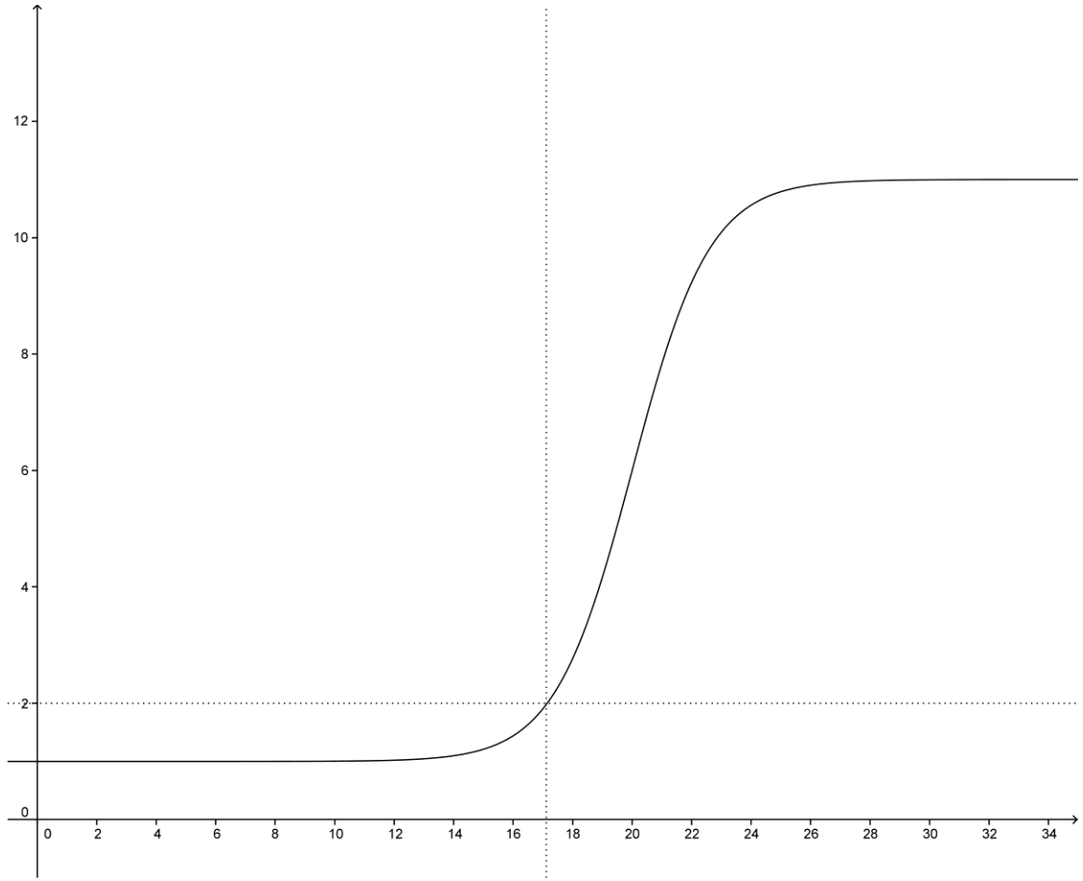
accelerating your analysis



→ ΔΔCt → paper

qPCR - the simple way

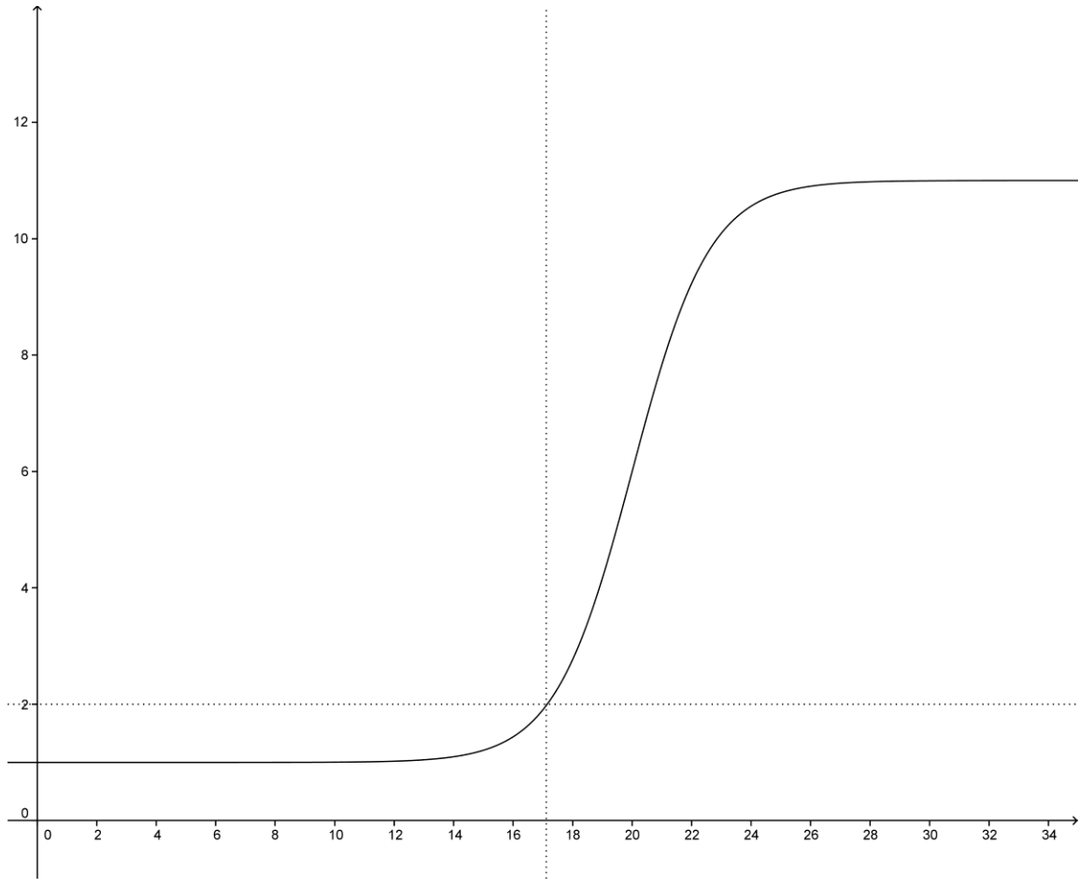
accelerating your analysis



→ ΔΔCq → paper

qPCR - the appropriate way

accelerating your analysis

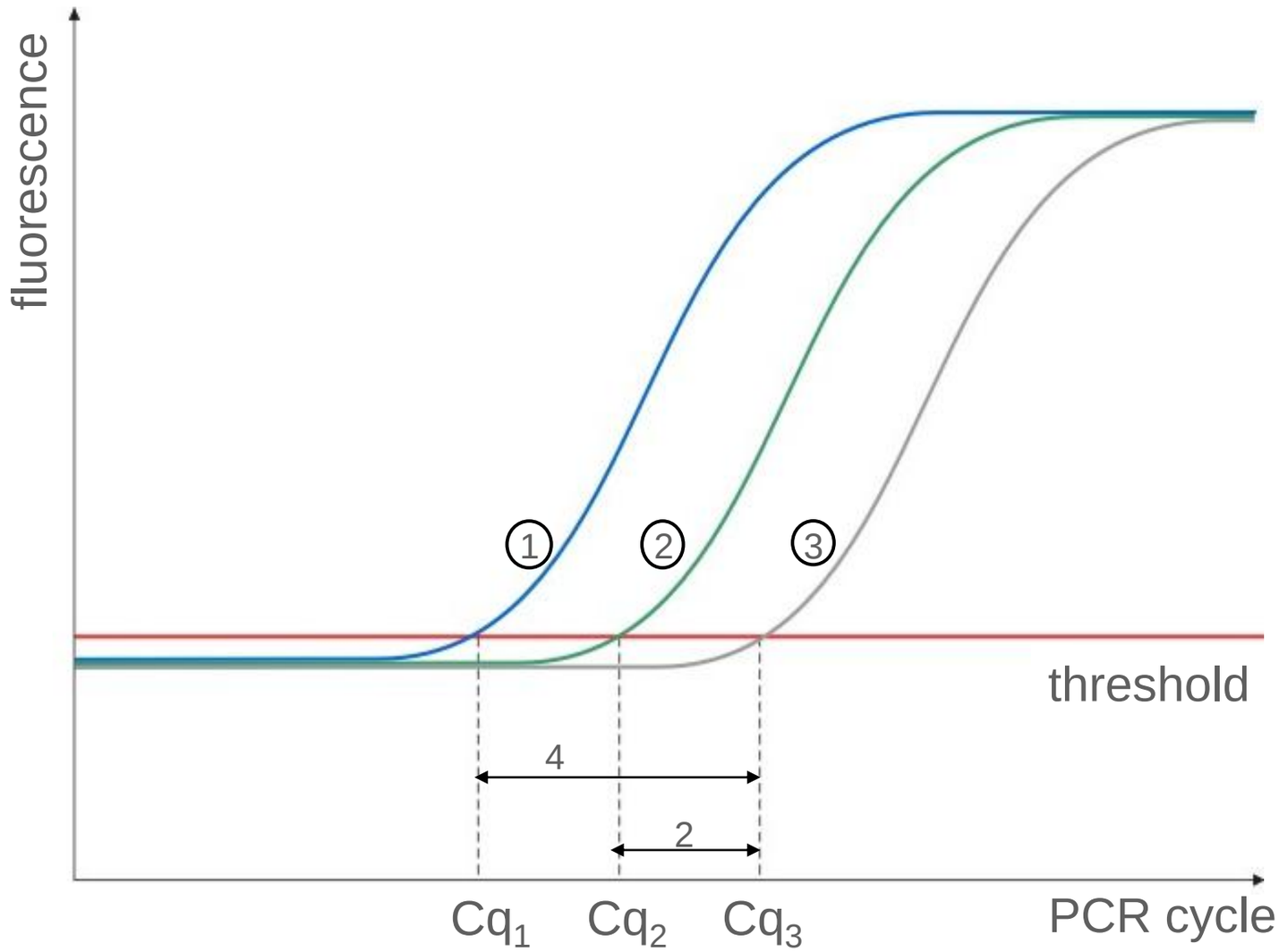


- advanced quantification models
- quality control

statistics

paper

relative quantities (RQ)



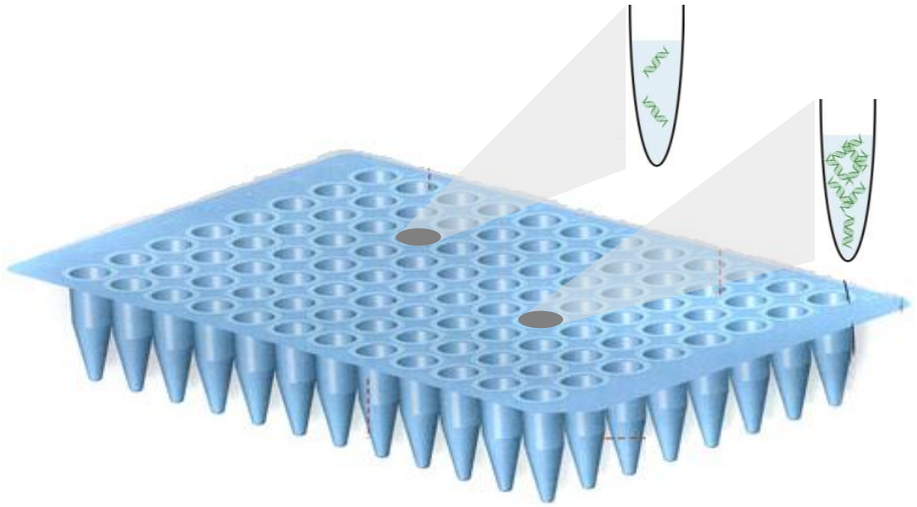
$$RQ = 2^{\Delta Cq}$$

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

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

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

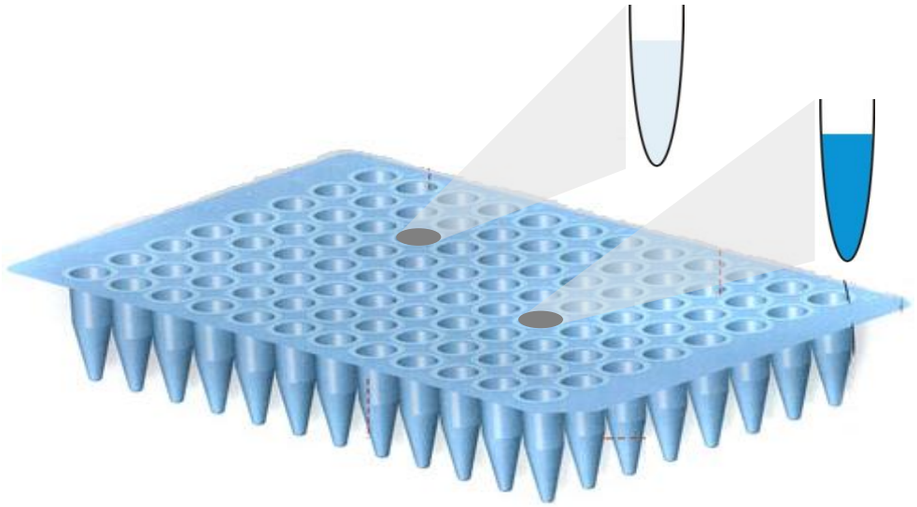
relative quantities (RQ)



differences in RQ due to

- different gene expression level

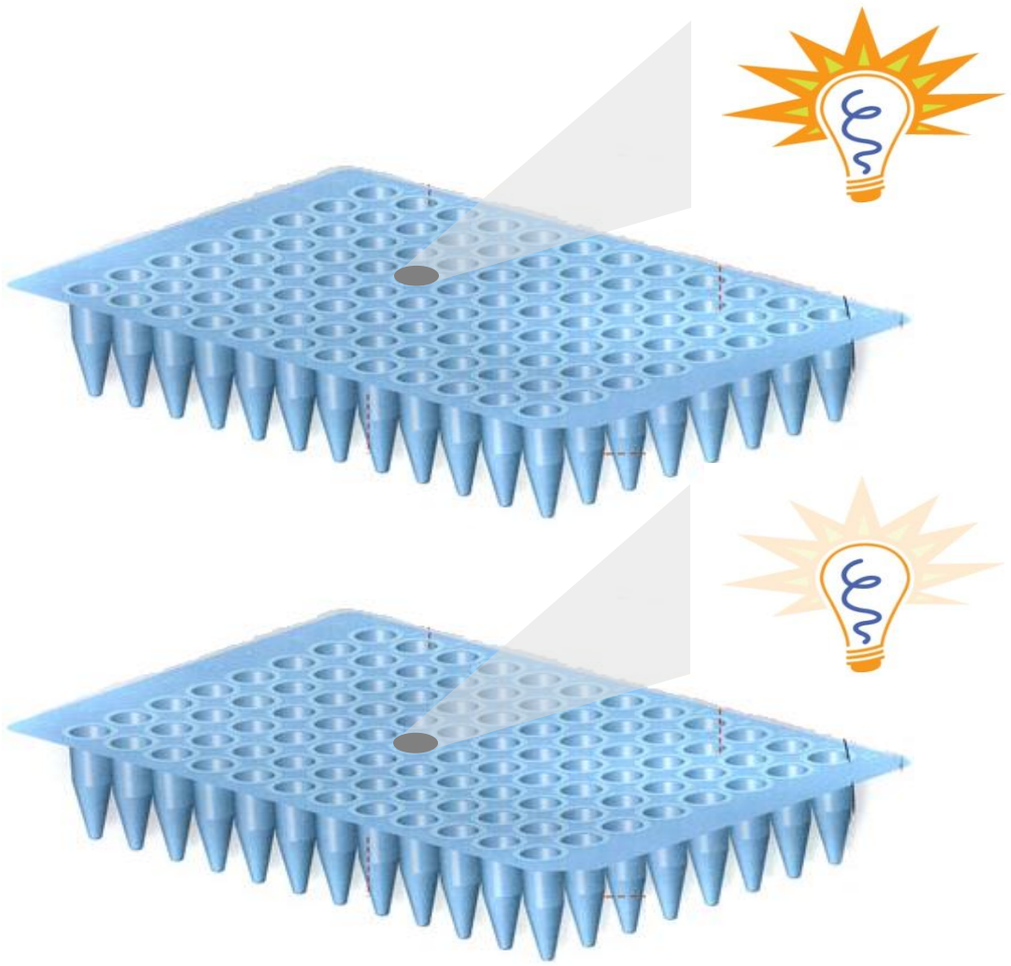
relative quantities (RQ)



differences in RQ due to

- different gene expression level
- different total starting amount

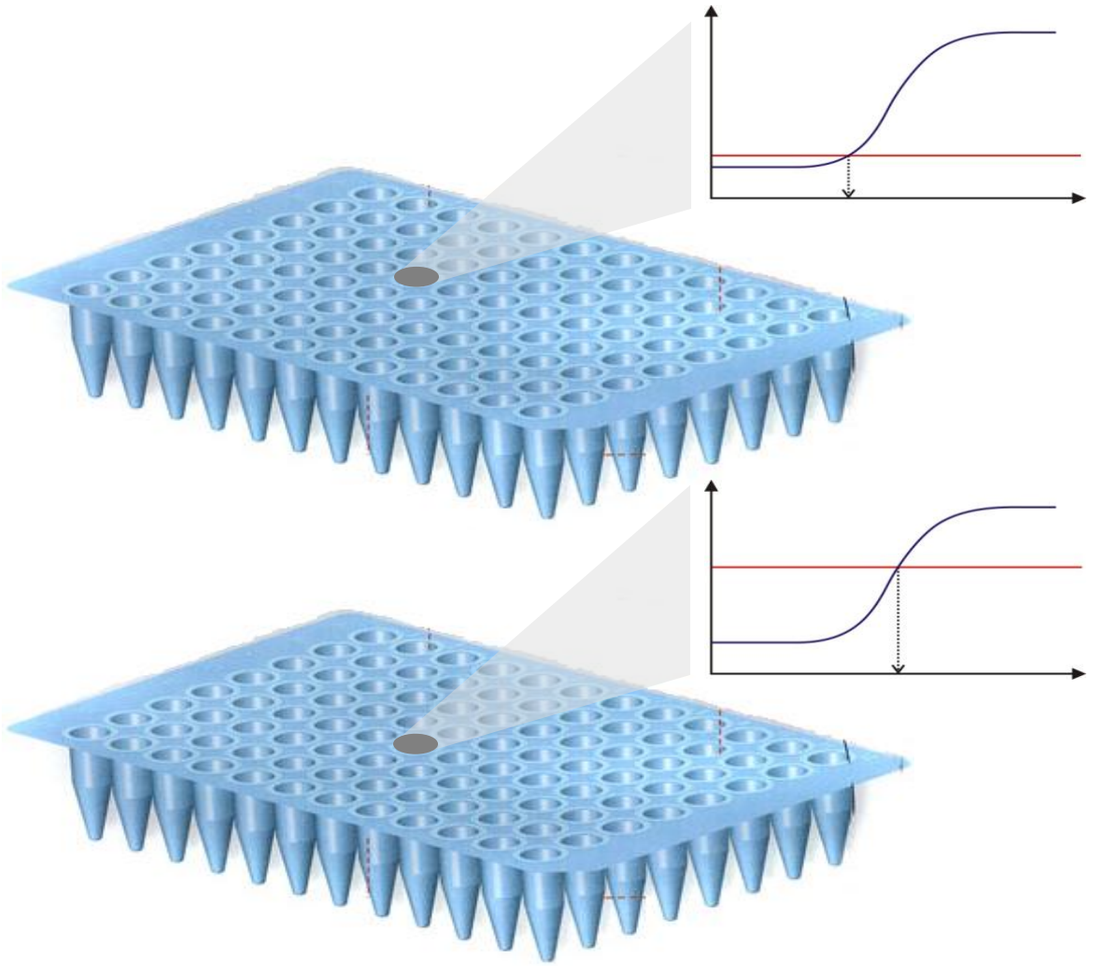
relative quantities (RQ)



differences in RQ due to

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

relative quantities (RQ)



differences in RQ due to

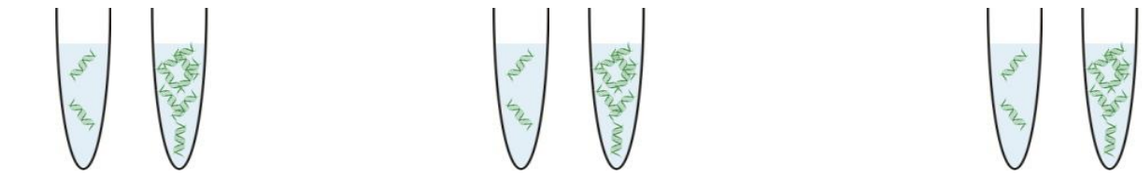
- different gene expression level
- different total starting amount
- run dependent differences

correcting unwanted variation

technical variation

- avoid
- minimize
- correct

Cq → RQ → NRQ → CNRQ



Normalization

Inter-run calibration

3 generations of quantification models

Livak and Schmittgen (2001)

- 100% PCR efficiency
- 1 reference gene

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

Pfaffl (2001)

- adjusted PCR efficiency
- 1 reference gene

$$NRQ = \frac{E_{toi}^{\Delta Cq,toi}}{E_{ref}^{\Delta Cq,ref}}$$

Hellemans et al. (2007)

- adjusted PCR efficiency
- multiple reference genes
- qBase model

$$NRQ = \frac{E_{toi}^{\Delta Cq,toi}}{\sqrt[n]{\prod_{i}^{n} E_{ref_i}^{\Delta Cq,ref_i}}}$$

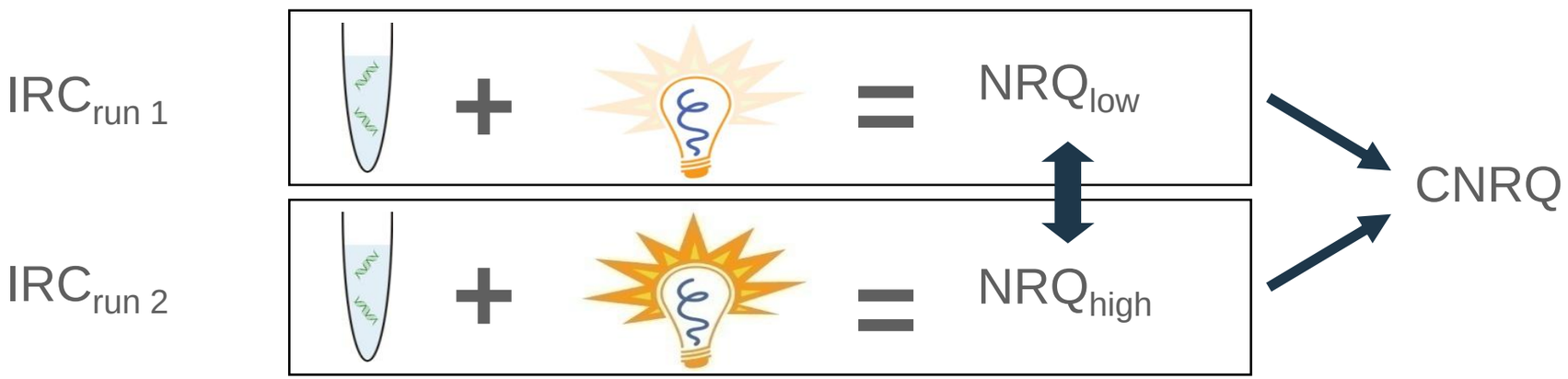
most flexible & most accurate

inter-run calibration

correct for inter-run variation by including IRCs

IRC:

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



the error

take all sources of error into account

- repeated measurements
- PCR efficiency
- normalization factor (based on multiple genes)
- calibration factor (based on multiple IRCs)

propagate the error over all calculations

true confidence in your results

proven technology - geNorm

geNorm normalization

- >1250 citations in PubMed
- >8000 downloads

http://genomebiology.com/2002/3/7/research/0034.1

Research

Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes

Jo Vandesompele, Katleen De Preter, Filip Pattyn, Bruce Poppe, Nadine Van Roy, Anne De Paepe and Frank Speleman

Address: Center for Medical Genetics, Ghent University Hospital 1K5, De Pintelaan 185, B-9000 Ghent, Belgium.

$$s_{e,jl} = \sqrt{\frac{\sum_{q=1}^{h}\left(Cq_{qjl,measured} - Cq_{qjl,predicted}\right)^2}{h-2}} \quad \text{(formula 2)}$$

$$s_{x,jl} = \sqrt{\frac{1}{h-1}\sum_{q=1}^{h}\left(Q_{qjl} - \overline{Q_{jl}}\right)^2} \quad \text{(formula 3)}$$

$$SE\left(slope_{jl}\right) = \frac{s_{e,jl}}{s_{x,jl}(h-1)} \quad \text{(formula 4)}$$

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

$$E_{jl} = 10^{\left(\frac{1}{slope_{jl}}\right)} \quad \text{(formula 5)}$$

$$SE\left(E_{jl}\right) = \frac{E_{jl}\cdot\ln(10)\cdot SE\left(slope_{jl}\right)}{slope_j^2} \quad \text{(formula 6)}$$

Conversion of Cq values into relative quantities

Step 1

Calculation of the average Cq value for all replicates of the same gene/sample combination jk within a given run l:

$$\overline{Cq_{jkl}} = \frac{\sum_{i=1}^{n} Cq_{ijkl}}{n} \quad \text{(formula 7)}$$

$$SE\left(Cq_{jkl}\right) = \sqrt{\frac{1}{n(n-1)}\sum_{i=1}^{n}\left(Cq_{ijkl} - \overline{Cq_{jkl}}\right)^2} \quad \text{(formula 8)}$$

Step 2

Transformation of mean Cq value into RQ using the gene specific PCR efficiency E_{jl}, with minimization of the overall error:

$$Cq_{refernce,jl} = \overline{Cq_{jl}} = \frac{\sum_{k=1}^{s} Cq_{jkl}}{s} \quad \text{(formula 9)}$$

$$\Delta Cq_{jkl} = Cq_{reference,jl} - Cq_{jkl} \quad \text{(formula 10)}$$

$$RQ_{jkl} = E_{jl}^{\Delta Cq_{jkl}} \quad \text{(formula 11)}$$

$$SE\left(RQ_{jkl}\right) = \sqrt{RQ_{jkl}^2\left[\left(\frac{\Delta Cq_{jkl}\cdot SD\left(E_{jl}\right)}{E_{jl}}\right)^2 + \left(\ln\left(E_{jl}\right)\cdot SD\left(\overline{Cq_{jkl}}\right)\right)^2\right]} \quad \text{(formula 12)}$$

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 l based on the NRQs of the IRCs m:

$$NF_k = \sqrt[f]{\prod_{p=1}^{f} RQ_{pk}} \quad \text{(formula 13)}$$

$$CF_{jl} = \sqrt[c]{\prod_{m=1}^{c} NRQ_{jlm}} \quad \text{(formula 13'; for definition of } NRQ\text{, see formula 15)}$$

$$SE\left(NF_k\right) = NF_k\sqrt{\sum_{p=1}^{f}\left(\frac{SE\left(RQ_{pk}\right)}{f\cdot RQ_{pk}}\right)^2} \quad \text{(formula 14)}$$

$$SE\left(CF_{jl}\right) = CF_{jl}\sqrt{\sum_{m=1}^{c}\left(\frac{SE\left(NRQ_{jlm}\right)}{c\cdot NRQ_{jlm}}\right)^2} \quad \text{(formula 14')}$$

Step 2

Conversion of RQs into NRQs.

Step 2'

Conversion of NRQs into CNRQs:

$$NRQ_{jk} = \frac{RQ_{jk}}{NF_k} \quad \text{(formula 15)}$$

$$CNRQ_{jkl} = \frac{NRQ_{jkl}}{CF_{jl}} \quad \text{(formula 15')}$$

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

$$SE\left(CNRQ_{jkl}\right) = CNRQ_{jkl}\sqrt{\left(\frac{SE\left(CF_{jl}\right)}{CF_{jl}}\right)^2 + \left(\frac{SE\left(NRQ_{jkl}\right)}{NRQ_{jkl}}\right)^2} \quad \text{(formula 16')}$$

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:

$$\overline{NRQ_p} = \frac{\sum_{k=1}^{s} NRQ_{pk}}{s} \quad \text{(formula 17)}$$

$$SE\left(NRQ_p\right) = \sqrt{\frac{1}{s-1}\sum_{k=1}^{s}\left(NRQ_{pk} - \overline{NRQ_p}\right)^2} \quad \text{(formula 18)}$$

Step 2

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

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

Step 3

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

$$\overline{CV} = \frac{\sum_{p=1}^{f} CV_p}{f} \quad \text{(formula 20)}$$

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 × 1 matrix A^{gene} in which the kth element is the $\log_2$ transformed ratio between the relative quantities (not yet normalized) of two reference genes p and p' in sample k; matrix A^{sample} is calculated in an analogous manner.

Step 1'

Calculation of the g × 1 matrix A^{irc} in which the jth element is the $\log_2$ transformed ratio between the NRQs of two IRCs m and m' for the same gene j within a run l; matrix A^{run} is calculated in an analogous manner:

$$\left(\forall p,p' \in [1,f], p \neq p'\right): A_{pp'k}^{gene} = \log_2\left(\frac{RQ_{kp}}{RQ_{kp'}}\right) \quad \text{(formula 21)}$$

$$\left(\forall m,m' \in [1,c], m \neq m'\right): A_{mm'jl}^{irc} = \log_2\left(\frac{NRQ_{mjl}}{NRQ_{m'jl}}\right) \quad \text{(formula 21')}$$

Step 2

Calculation of the mean log transformed ratio and the standard deviation V^{gene} for all samples k and a given reference gene combination (p, p'). V^{gene} is the geNorm pairwise variation V for two reference genes.

Step 2'

Calculation of the mean log transformed ratio and the standard deviation V^{irc} for all runs l and a given IRC combination (m, m') and a given gene j. V^{sample} and V^{run} are calculated similarly from A^{sample} and A^{run}, respectively:

$$A_{pp'}^{gene} = \frac{\sum_{k=1}^{s} A_{pp'k}^{gene}}{s} \quad \text{(formula 22)}$$

$$A_{mm'j}^{irc} = \frac{\sum_{l=1}^{r} A_{mm'jl}^{irc}}{r} \quad \text{(formula 22')}$$

$$V_{pp'}^{gene} = SD\left(A_{pp'}^{gene}\right) = \sqrt{\frac{1}{s-1}\sum_{k=1}^{s}\left(A_{pp'k}^{gene} - \overline{A_{pp'}^{gene}}\right)^2} \quad \text{(formula 23)}$$

$$V_{mm'j}^{irc} = SD\left(A_{mm'j}^{irc}\right) = \sqrt{\frac{1}{r-1}\sum_{l=1}^{r}\left(A_{mm'jl}^{irc} - \overline{A_{mm'j}^{irc}}\right)^2} \quad \text{(formula 23')}$$

Step 3

Calculation of the arithmetic mean M^{gene} of all pairwise variations V^{gene} of a given reference gene p with all other tested reference genes p'. M^{gene} represents the geNorm gene stability measure M for a particular reference gene p.

Step 3'

Calculation of the arithmetic mean M^{irc} of all pair wise variations V^{irc} of a given IRC m with all the other IRCs m', for the same gene j. M^{sample} and M^{run} are calculated similarly from V^{sample} and V^{run}, respectively:

$$M_p^{gene} = \frac{\sum_{p'=1}^{f} V_{pp'}^{gene}}{f-1} \quad \text{(formula 24)}$$

$$M_{mj}^{irc} = \frac{\sum_{m'=1}^{c} V_{mm'j}^{irc}}{c-1} \quad \text{(formula 24')}$$

Step 4

Calculation of the mean stability measure for all reference genes.

Step 4'

Calculation of the mean stability measure for all IRCs:

$$\overline{M^{gene}} = \frac{\sum_{p=1}^{f} M_p^{gene}}{f} \quad \text{(formula 25)}$$

$$\overline{M_j^{irc}} = \frac{\sum_{m=1}^{f} M_{mj}^{irc}}{f} \quad \text{(formula 25')}$$

Open Access

Method

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

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qbase^{PLUS} – Easy. Fast. Reliable.

a simple workflow

- import Cq values from your favorite qPCR machine
- select one or more reference genes
- take a look at the results

calculations on the fly

- immediately see the impact of any change in
  - data - reference genes – settings - rescaling

based on proven geNorm and qBase quantification models

- efficiency correction
- multi-gene normalization
- inter-run calibration
- proper error propagation

qbase^{PLUS} – Easy. Fast. Reliable.

accelerating your analysis

qbase^{PLUS} – Easy. Fast. Reliable.

Analysis of more than 200 samples in triplicate for 13 genes in total took only 35 minutes in qbase^{PLUS} (including preparation of the instrument data export files). As such a job used to to take 2 days of time in Excel, qBasePlus makes our lives much more comfortable.

qbase^{PLUS} – Easy. Fast. Reliable.

accelerating your analysis

Streamlined TaqMan analysis

- more than 10 plates
- automated analysis
- no manual interaction required

quality control

technical replicates

- ΔCq < threshold?

negative controls

- no Cq value?
- ΔCq (toi – ctrl) < threshold?

positive controls

- Cq value?

dilution series

- E close to 2 (100% efficiency)?
- linear over full range & no outliers?
- small SE(E)?

quality control

stability of reference genes

- CV of NRQs for reference genes?
- geNorm M value?

normalization factors

- no unexpected high variation?

qbase^{PLUS} – Easy. Fast. Reliable.

unique set of built in quality controls

- based on user defined thresholds
- highlighting or filtering of low quality results

exclusion of bad data points

- data is not deleted
- immediate improvement of results

tutorial

get more info

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- courses
- other services
- room S2 – booth 29

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buy now with discount

- ask promotion code at booth

acknowledgements

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