Biotechnology Project Lab

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The lecture of November 26th 2021 is about to begin….

- Quantitative real time PCR (qRT-PCR)
- Example of qRT-PCR data analysis

- PCR reaction
- conventional versus real time PCR
- real time PCR principles
- threshold cycle C_T
- efficiency
- relative quantification
- reference genes
- primers
- detection chemistry
- GLP in real time PCR

quantitative real time PCR (qRT-PCR)

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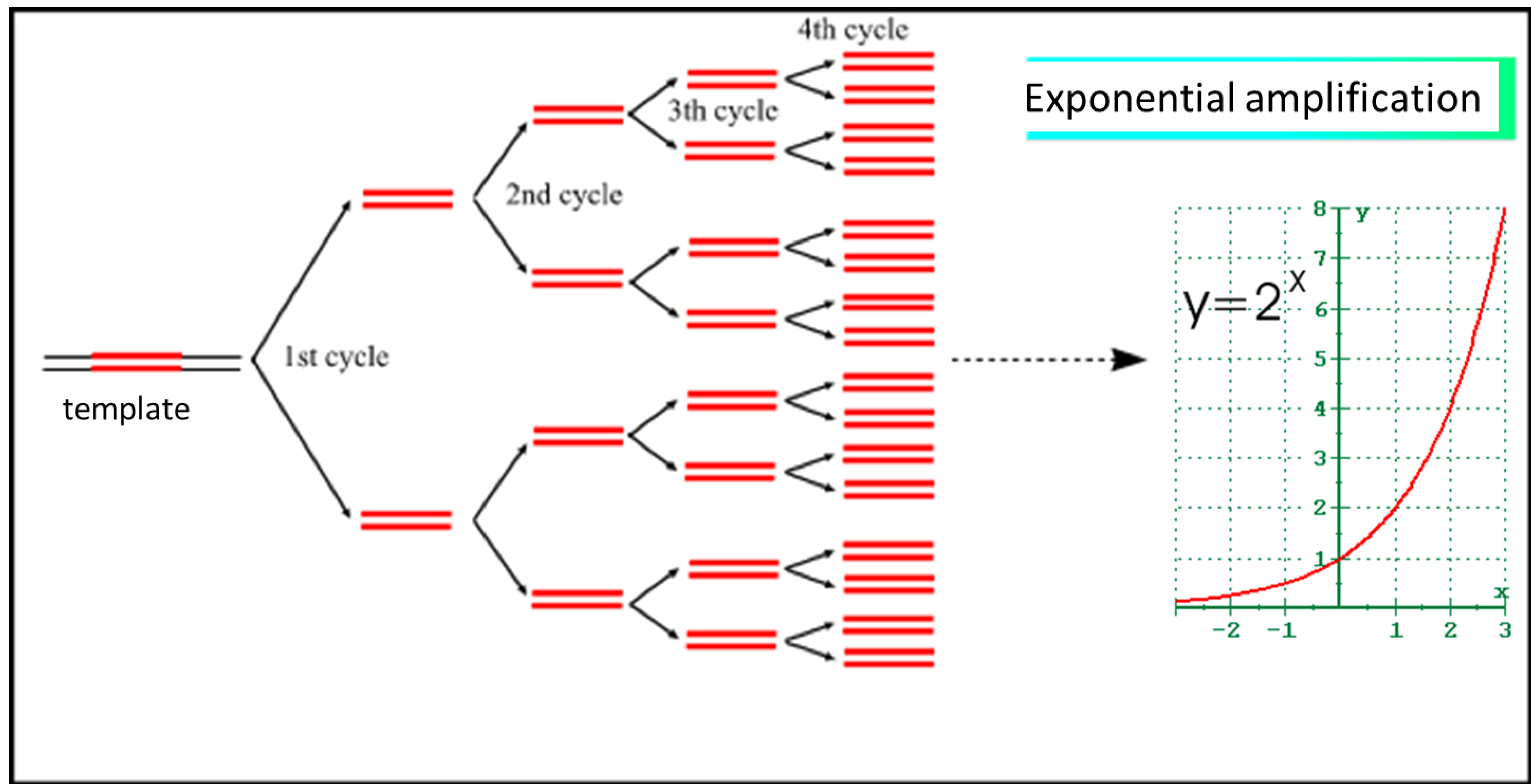
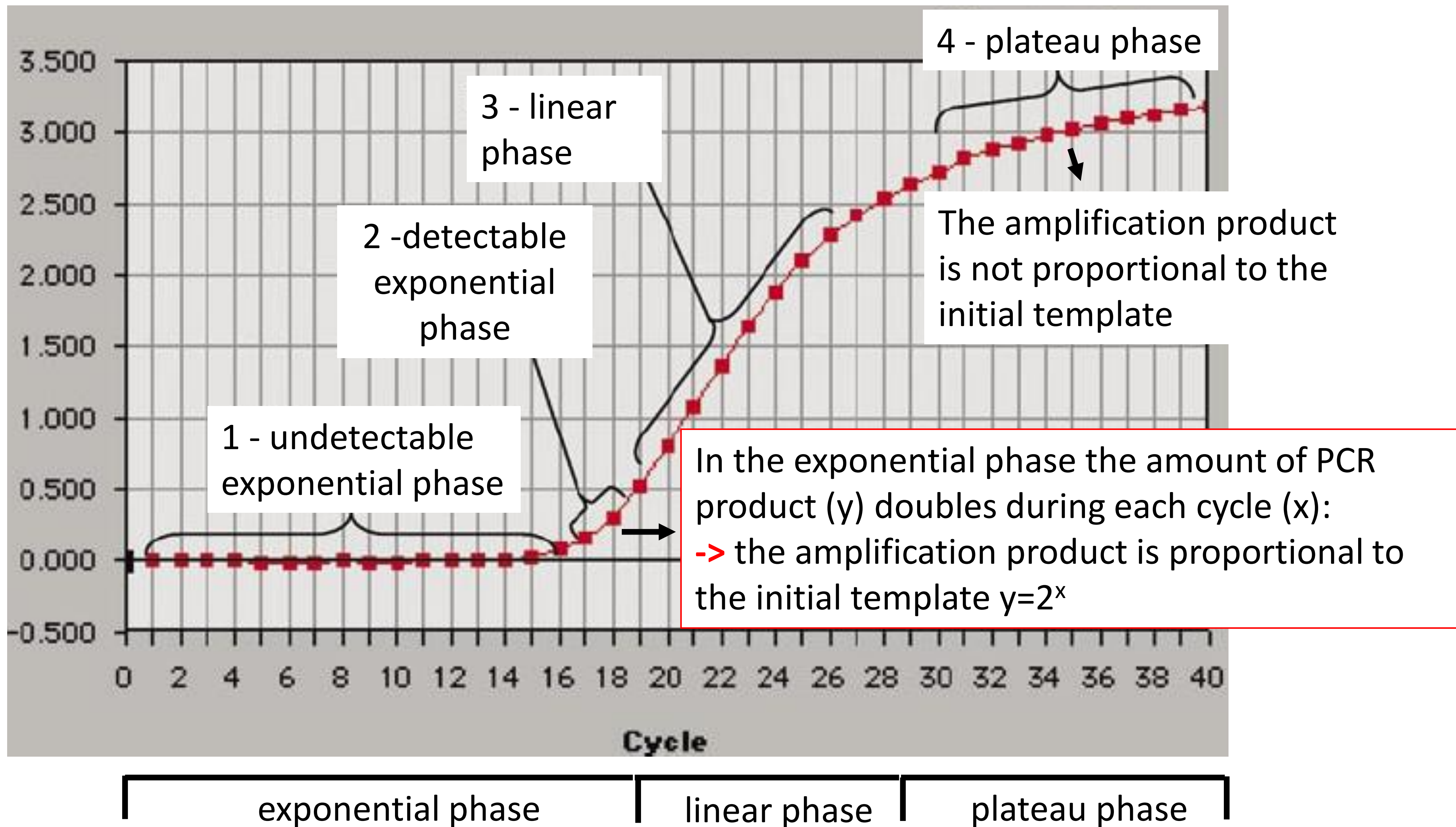
DNA polymerase chain reaction (PCR)





As the reaction proceeds, reaction components are consumed and one or more of the components becomes limiting.

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4 - plateau phase

3 - linear phase

2 -detectable exponential phase

1 - undetectable exponential phase

conventional PCR

real-time PCR

fluorescence

3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
-0.500

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40

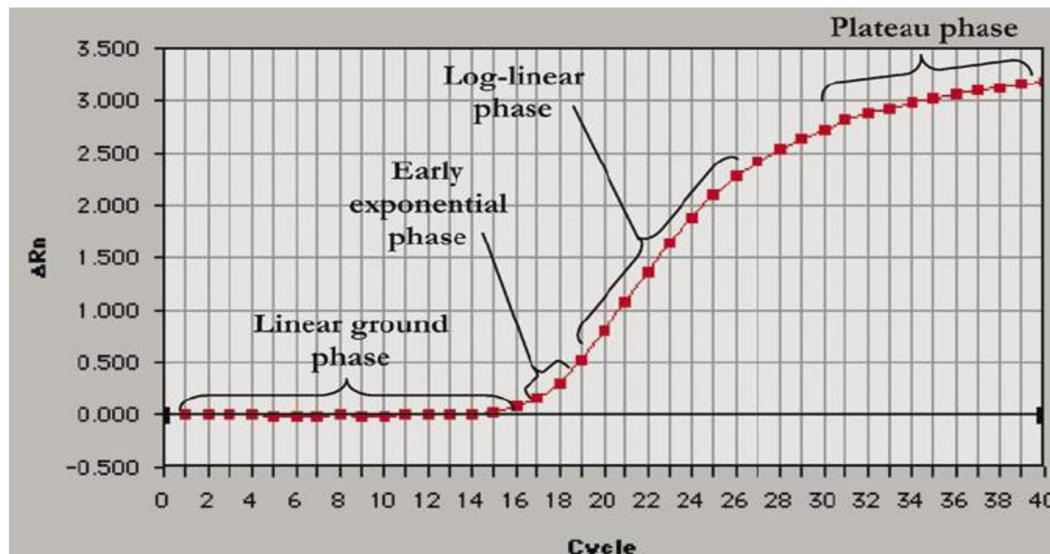
Cycle

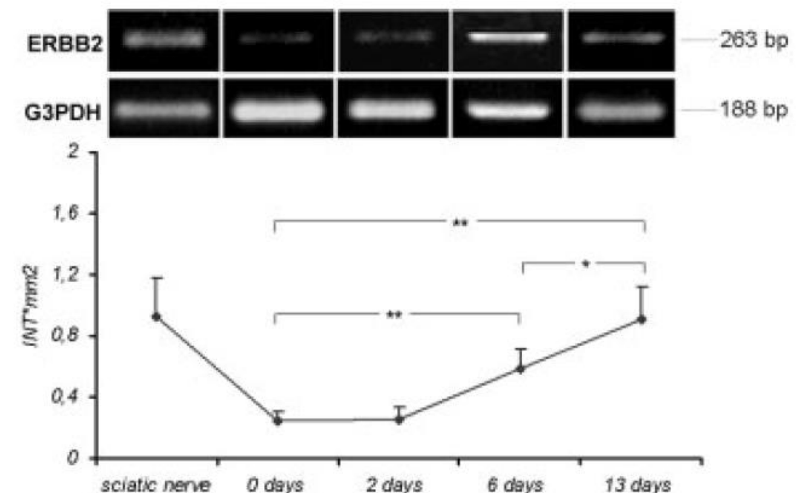
Only for teaching purposes - not for reproduction or sale

Conventional PCR

In conventional PCR, the amplified product, or amplicon, is detected by an **end-point analysis**, by running DNA on an agarose gel after the reaction has finished.

- different initial amounts of template can produce same end-point results
- poor precision
- low sensitivity
- not very quantitative
- BUT cheap

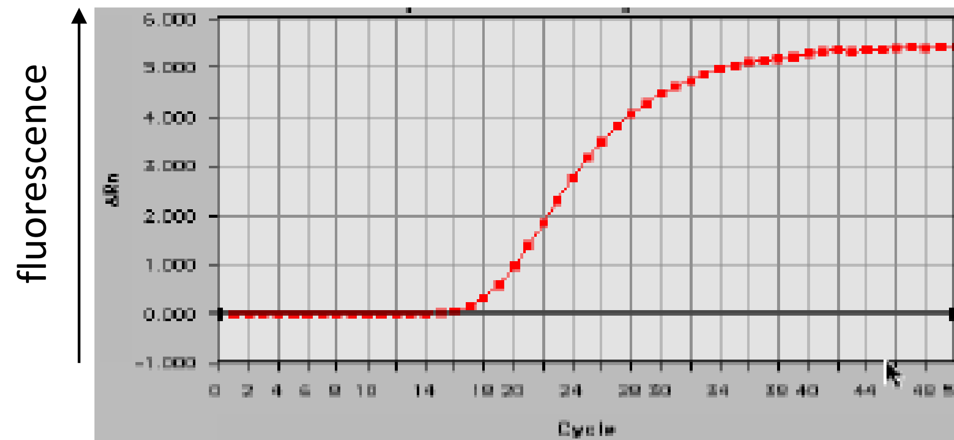




“Real-Time” PCR

- Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal.
- Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (“Real time” detection) as opposed to the “endpoint detection” of the conventional PCR.
- The measured fluorescence reflects the amount of amplified product in each cycle.

Real time PCR is kinetic:
“amplification associated fluorescence” is detected at each cycle



- amplification can be monitored in “**real-time**”
- no post-PCR processing of products (low contamination risk)
- wider dynamic range
- requirement of 1000-fold less RNA than conventional assays
- detection is capable down to a two-fold change
- confirmation of specific amplification by melting curve analysis
- most specific, sensitive and reproducible

- setting up requires high technical skill and support
- high equipment cost

Real time instrument

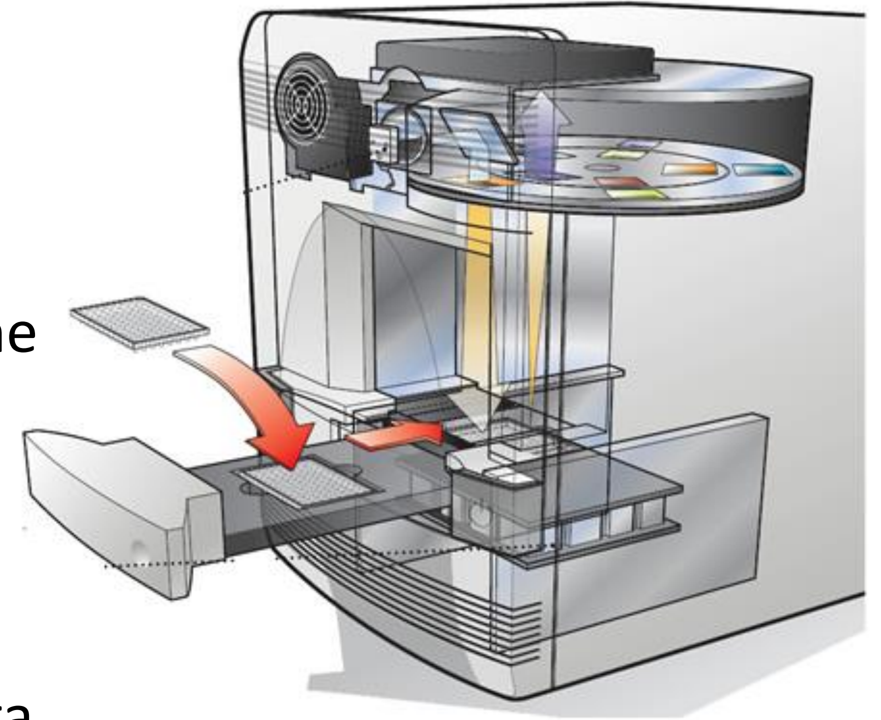
The instrument is equipped with :

- a thermal cycler for amplification

- a light source for excitation of fluorescent reagents or probes (can be a simple halogen lamp) shining through one of different excitation filters

- a CCD camera positioned above the samples recording fluorescence from behind one of different emission filters

- a computer controlling the instrument and recording data



Plateau phase

Log-linear phase

Early exponential phase

Linear ground phase

3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
-0.500

fluorescence

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40

Cycle

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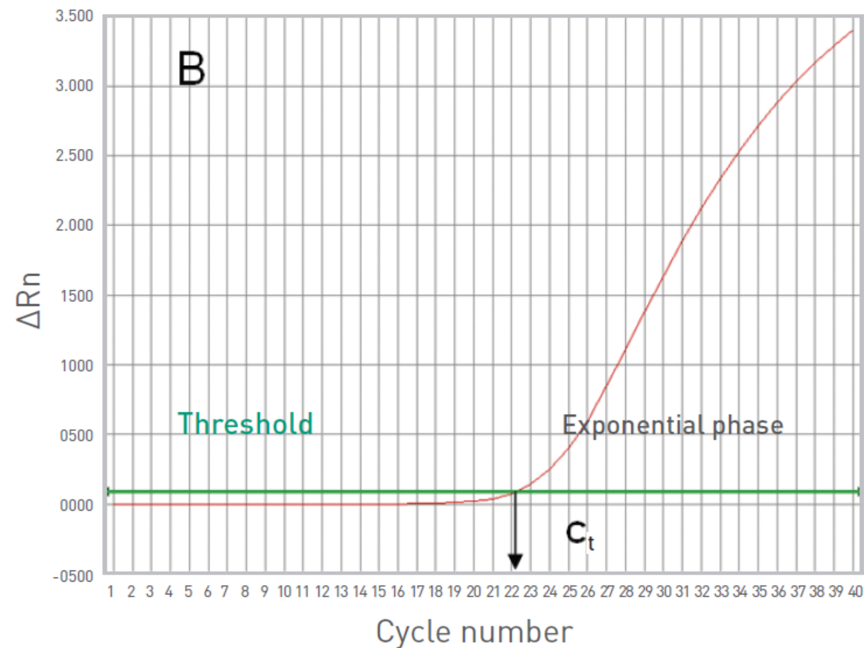
C_T - Threshold Cycle

❅ threshold cycle (C_T value) is the cycle at which a sample crosses the threshold, which usually corresponds to the level of fluorescence which is detectable by the instrument

❅ Ct is the parameter used for quantitation

❅ Ct correlates with the initial amount of template, since its value is measured in the exponential phase, when reagents are not limited

linear view



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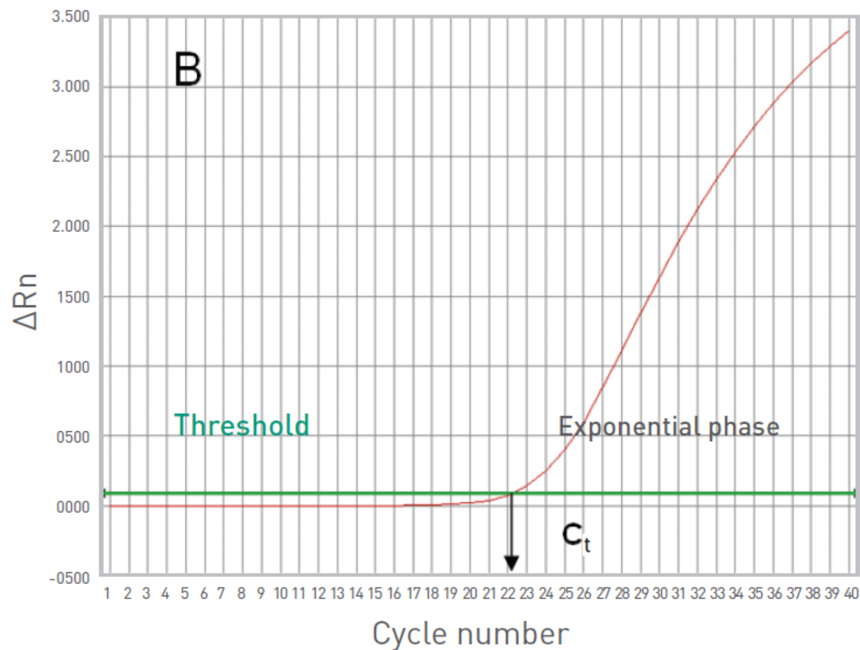
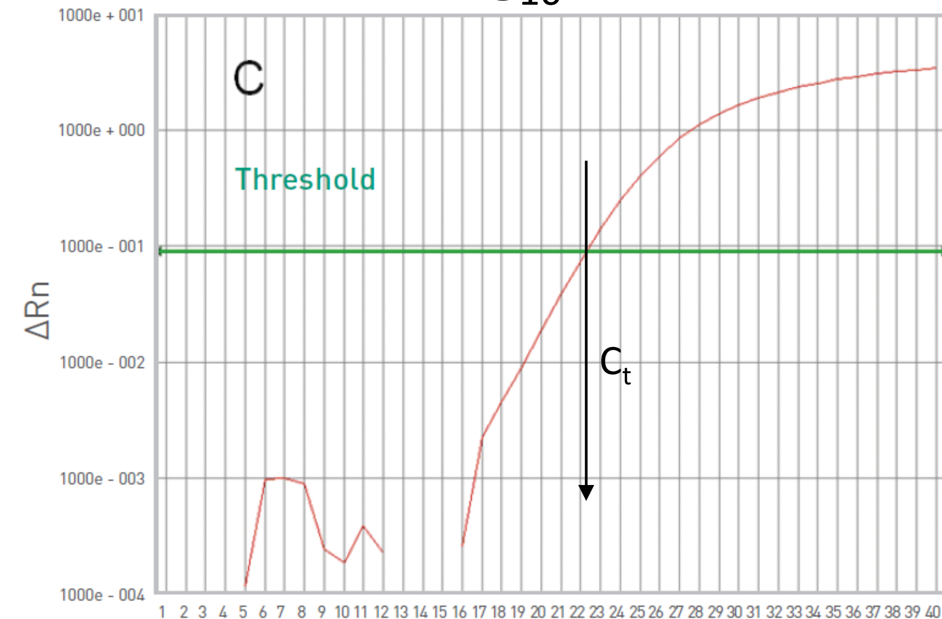
linear view

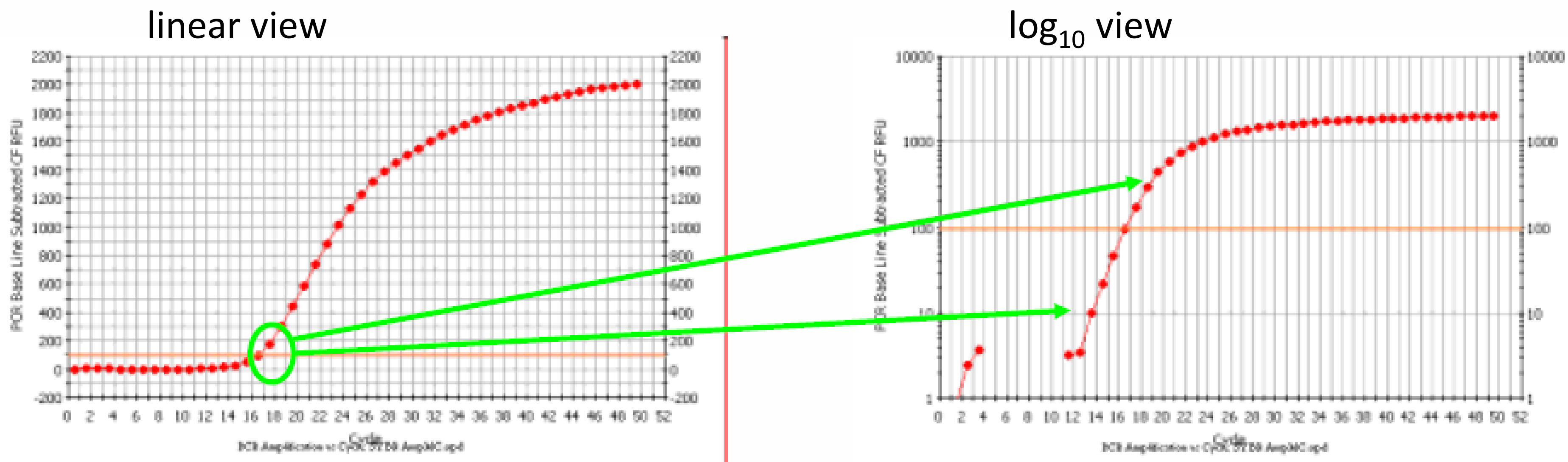


log_{10} view



Real-time PCR Principles

Linear vs Log View



$\log_{10} 1=$

$\log_{10} 10=$

$\log_{10} 100=$

$\log_{10} 1000=$

$\log_{10} 10000=$

LOGARITHM

The **logarithm** is the inverse operation to **exponentiation**.

The logarithm of a number is the exponent to which another number, the base, must be raised to produce that number.

If $\mathbf{b^y = x}$, then the logarithm of *x* to *base b*, denoted $\mathbf{\log_b(x) = y}$

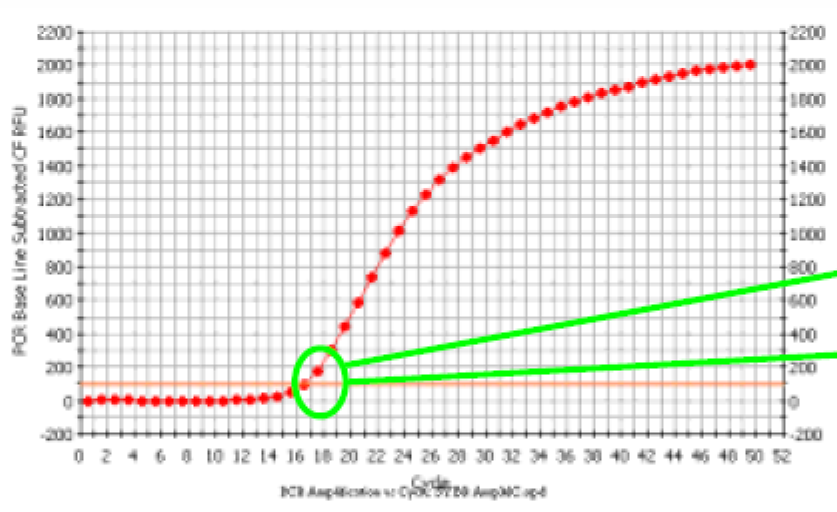
For example:
the base 10 logarithm of 1000 is 3, as 10 to the power 3 is 1000 ($1000=10\times10\times10=10^3$); the multiplication is repeated three times.
More generally, exponentiation allows any positive real number to be raised to any real power, always producing a positive result, so the logarithm can be calculated for any two positive real numbers *b* and *x* where *b* is not equal to 1.

For example:
The base 2 logarithm of 64 is 6, as $64 = 2^6$ (64=2x2x2x2x2x2), then: $\log_2(64) = 6$

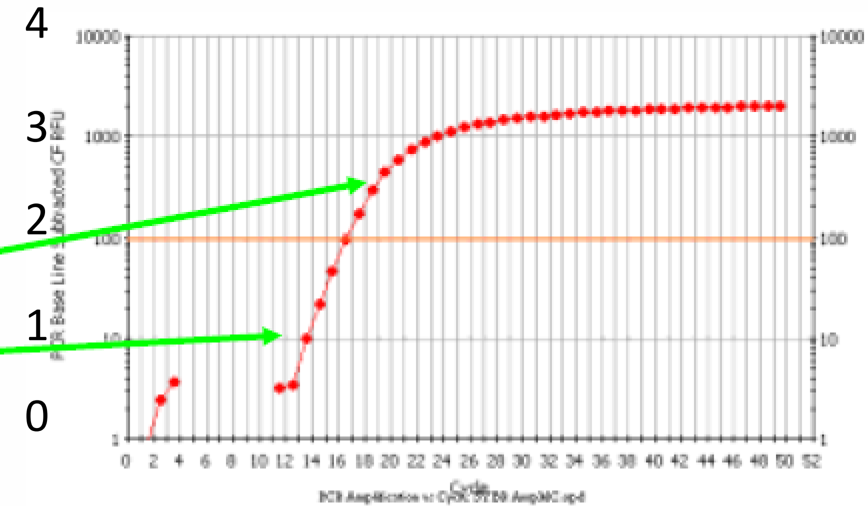
Real-time PCR Principles

Linear vs Log View

linear view



$\log_{10}$ view



$\log_{10} 1=0$

$\log_{10} 10=1$

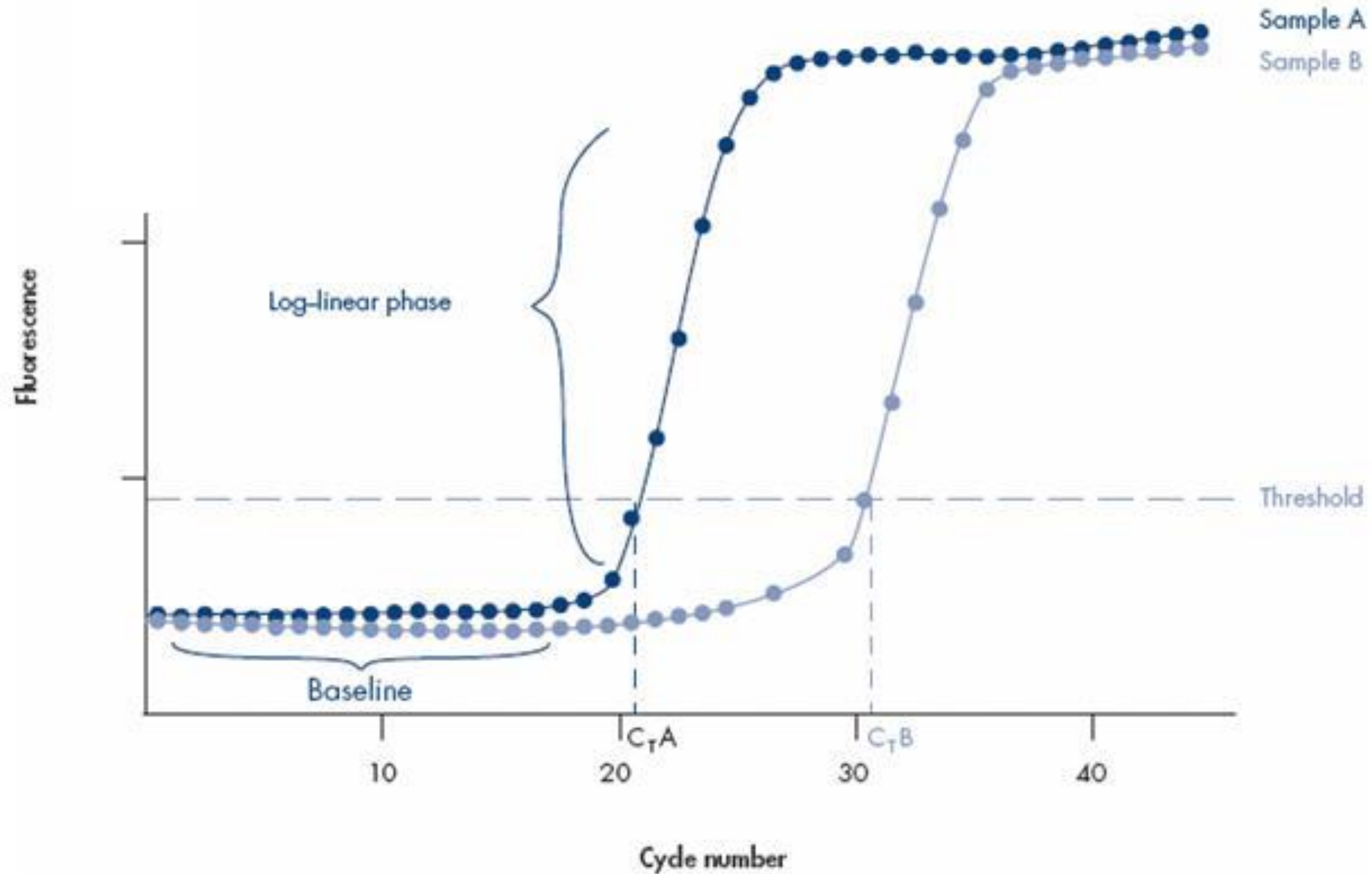
$\log_{10} 100=2$

$\log_{10} 1000=3$

$\log_{10} 10000=4$

The logarithm of a number is the exponent to which another number, the base, must be raised to produce that number.

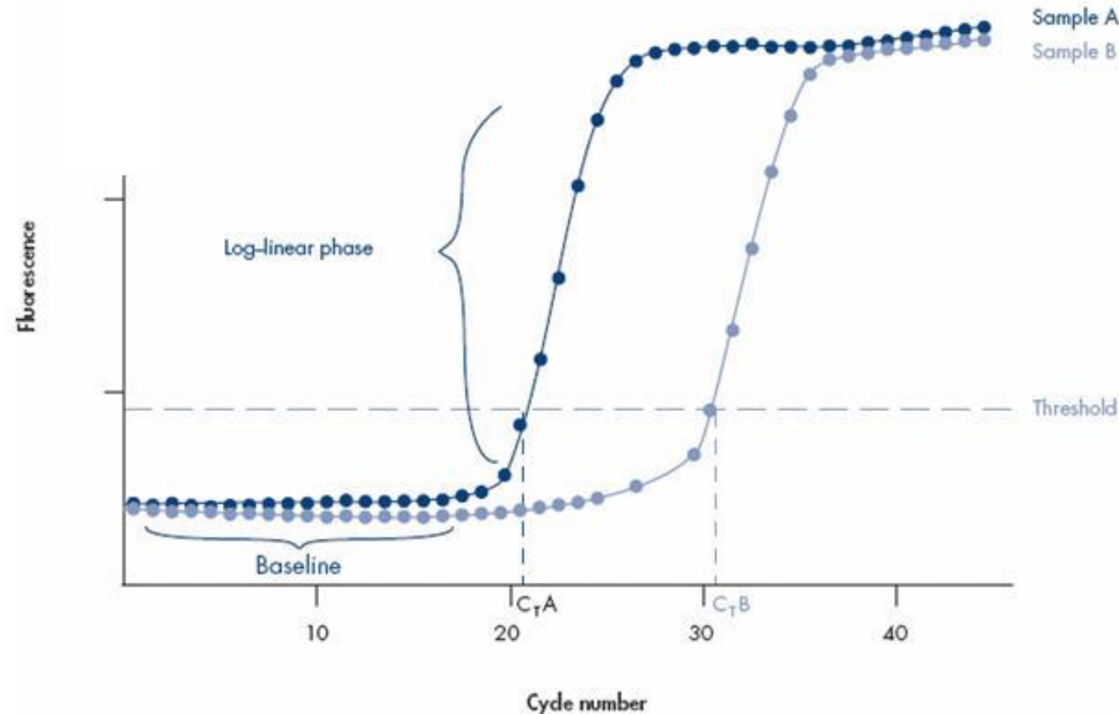
The C_T is determined mainly by the amount of template present at the start of the amplification reaction.

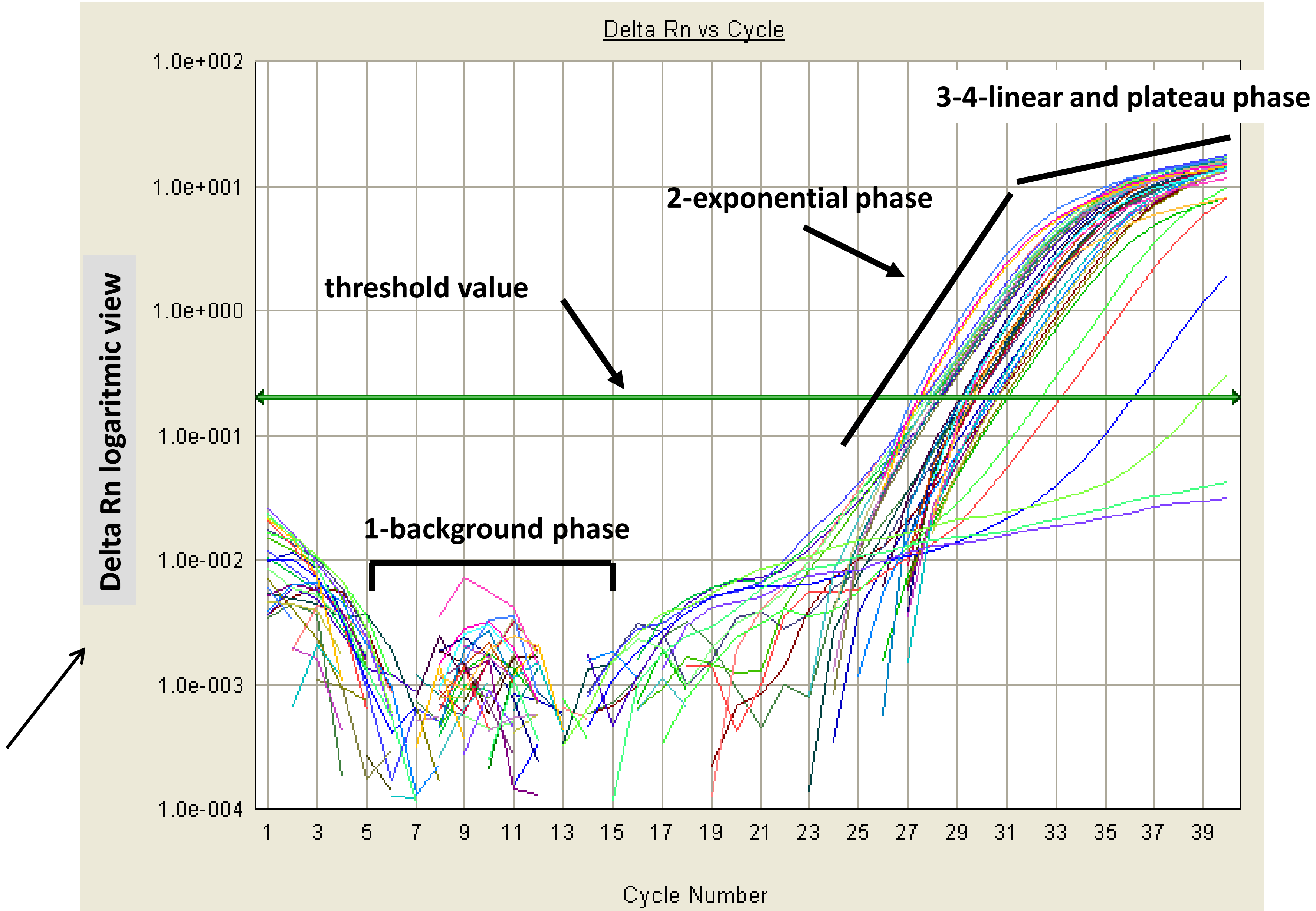


The C_T is determined mainly by the amount of template present at the start of the amplification reaction.

If a large amount of template is present at the start of the reaction, few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background -> low, or early, C_T (sample A).

If a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background -> high, or late, C_T (sample B).



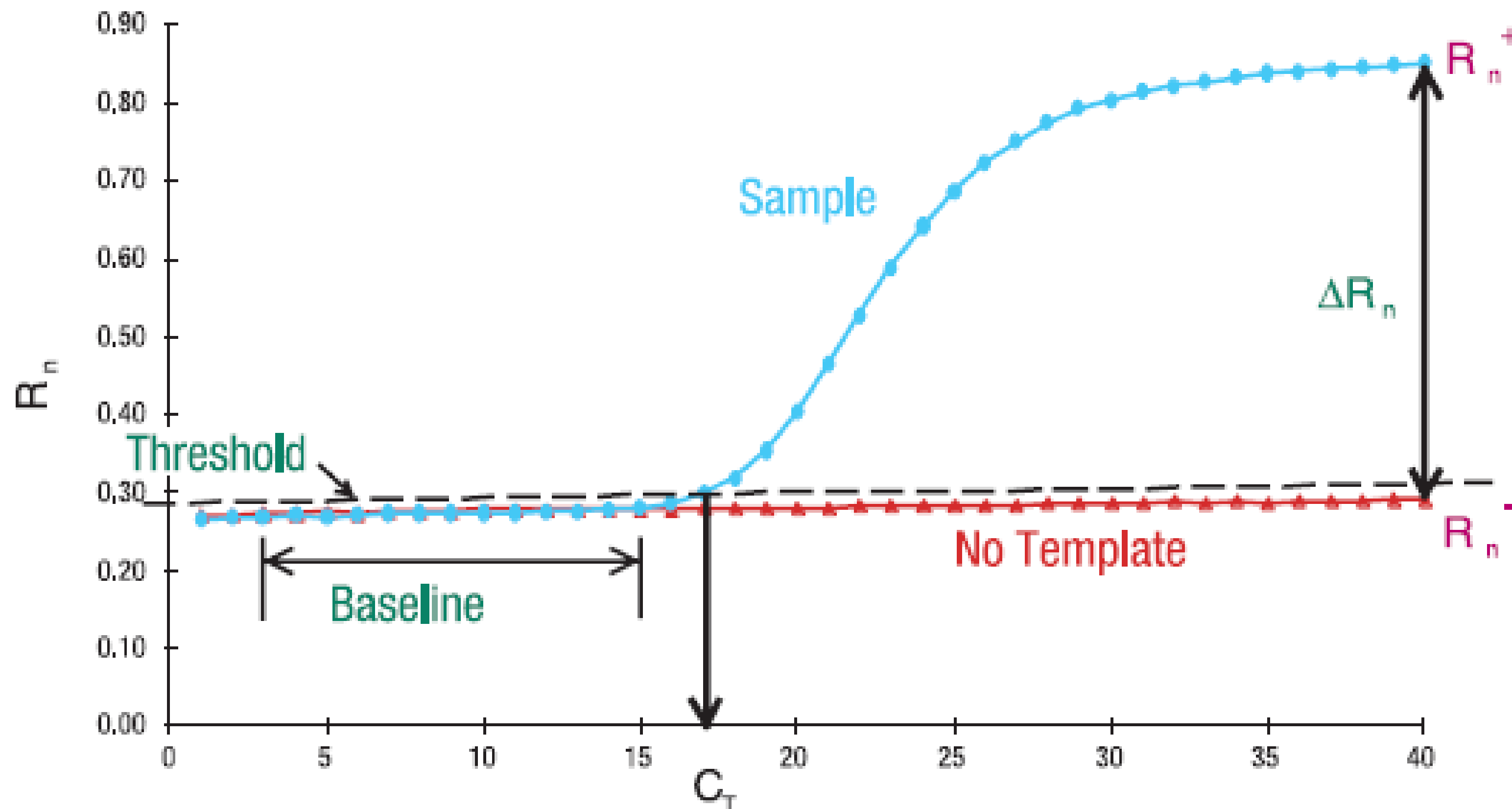


Delta Rn vs Cycle
3-4-linear and plateau phase
2-exponential phase
threshold value
1-background phase
Delta Rn logaritmic view
Cycle Number
1.0e+002
1.0e+001
1.0e+000
1.0e-001
1.0e-002
1.0e-003
1.0e-004
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39

Rn = normalized reporter value

The **Rn value**, or **normalized reporter value**, is the **fluorescent signal** from SYBR Green normalized to (divided by) the signal of the passive reference dye (ROX) for a given reaction.

The **ΔRn value** is the Rn value of an experimental reaction minus the Rn value of the baseline signal generated by the instrument.



In the exponential phase, ideally (: if the amplification efficiency is 100%), the amount of PCR product will perfectly double during each cycle.

Cycle	DNA relative quantity Sample A (calibrator)	DNA relative quantity Sample B	DNA relative quantity Sample C
0	2	4	8

- if the detectable amount of DNA is **128** **(128=threshold)**

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In the exponential phase, ideally (: if the amplification efficiency is 100%), the amount of PCR product will perfectly double during each cycle.

Cycle	DNA relative quantity Sample A (calibrator)	DNA relative quantity Sample B	DNA relative quantity Sample C
0	**2**	**4**	**8**
1	4	8	16

- if the detectable amount of DNA is **128** **(128=threshold)**

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In the exponential phase, ideally (: if the amplification efficiency is 100%), the amount of PCR product will perfectly double during each cycle.

Cycle	DNA relative quantity Sample A (calibrator)	DNA relative quantity Sample B	DNA relative quantity Sample C
0	**2**	**4**	**8**
1	4	8	16
2	8	16	32

- if the detectable amount of DNA is **128** **(128=threshold)**

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Cycle	DNA relative quantity Sample A (calibrator)	DNA relative quantity Sample B	DNA relative quantity Sample C
0	**2**	**4**	**8**
1	4	8	16
2	8	16	32
3	16	32	64

- if the detectable amount of DNA is **128** **(128=threshold)**

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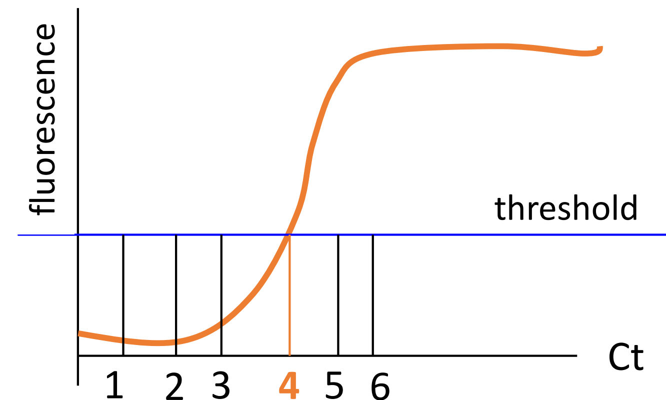
In the exponential phase, ideally (: if the amplification efficiency is 100%), the amount of PCR product will perfectly double during each cycle.

Cycle	DNA relative quantity Sample A (calibrator)	DNA relative quantity Sample B	DNA relative quantity Sample C
0	2	4	8
1	4	8	16
2	8	16	32
3	16	32	64
4	32	64	128

- if the detectable amount of DNA is **128** **(128=threshold)**

- **the Ct value for sample C will be 4**



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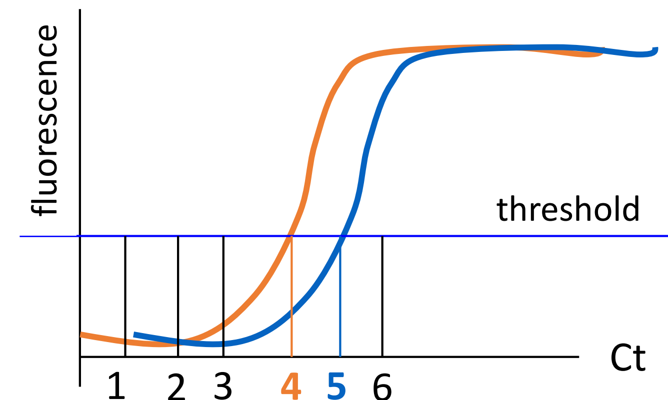
Cycle	DNA relative quantity Sample A (calibrator)	DNA relative quantity Sample B	DNA relative quantity Sample C
0	**2**	**4**	**8**
1	4	8	16
2	8	16	32
3	16	32	64
4	32	64	**128**
5	64	**128**	256

- if the detectable amount of DNA is **128** **(128=threshold)**

- **the Ct value for sample B will be 5,**
- **the Ct value for sample C will be 4**

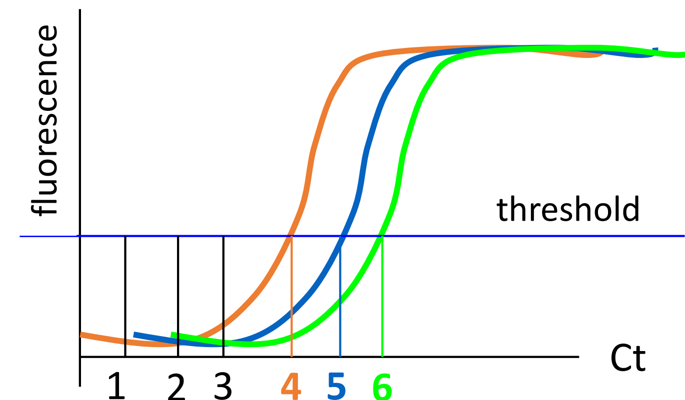


In the exponential phase, ideally (: if the amplification efficiency is 100%), the amount of PCR product will perfectly double during each cycle.

Cycle	DNA relative quantity Sample A (calibrator)	DNA relative quantity Sample B	DNA relative quantity Sample C
0	2	4	8
1	4	8	16
2	8	16	32
3	16	32	64
4	32	64	128
5	64	128	256
6	128	256	512

- if the detectable amount of DNA is **128** **(128=threshold)**

- **the Ct value for sample A will be 6,**
- **the Ct value for sample B will be 5,**
- **the Ct value for sample C will be 4**



In the exponential phase, ideally (: if the amplification efficiency is 100%), the amount of PCR product will perfectly double during each cycle.

Cycle	DNA relative quantity Sample A (calibrator)	DNA relative quantity Sample B	DNA relative quantity Sample C
0	2	4	8
1	4	8	16
2	8	16	32
3	16	32	64
4	32	64	128
5	64	128	256
6	128	256	512
7	256	512	1024
8	512	1024	2048

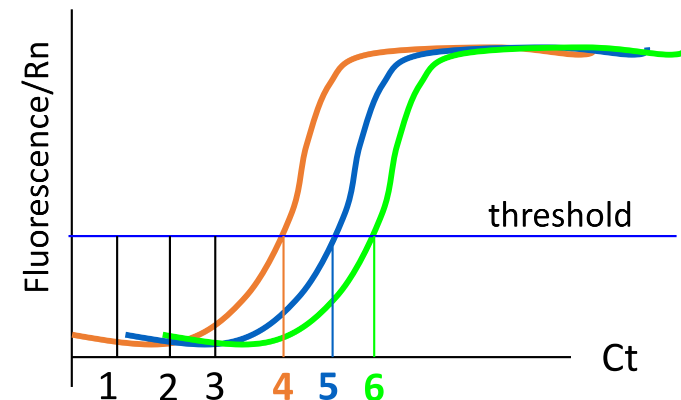
- the initial template in sample C is the double of sample B
- the initial template in sample B is the double of sample A

-If the detectable amount of DNA is **128**

-the Ct value for sample A will be 6,

-the Ct value for sample B will be 5,

-the Ct value for sample C will be 4



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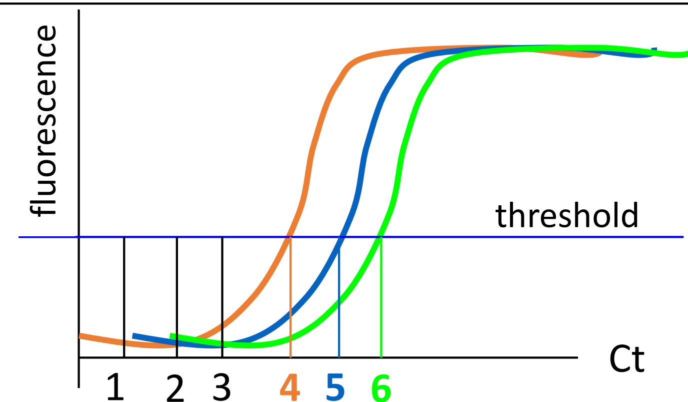
Cycle	DNA quantity Sample A (calibrator)	DNA quantity Sample B	DNA quantity Sample C
0	2	4	8
1	4	8	16
2	8	16	32
3	16	32	64
4	32	64	128
5	64	128	256
6	128	256	512
7	256	512	1024
8	512	1024	2048

- If the detectable amount of DNA is **128**

-the Ct value for sample A will be 6,

-the Ct value for sample B will be 5,

-the Ct value for sample C will be 4



The logarithm of a number is the exponent to which another number, the base, must be raised to produce that number.

At each cycle, during the exponential phase, the DNA doubles

Ct represents the number of amplification cycles necessary to obtain a detectable fixed amount of DNA -> **Ct are base 2 logarithms**.

- if the starting material is low, you will need many amplification cycles (Ct high),
- if the starting material is high, you will need less amplification cycles (Ct low).

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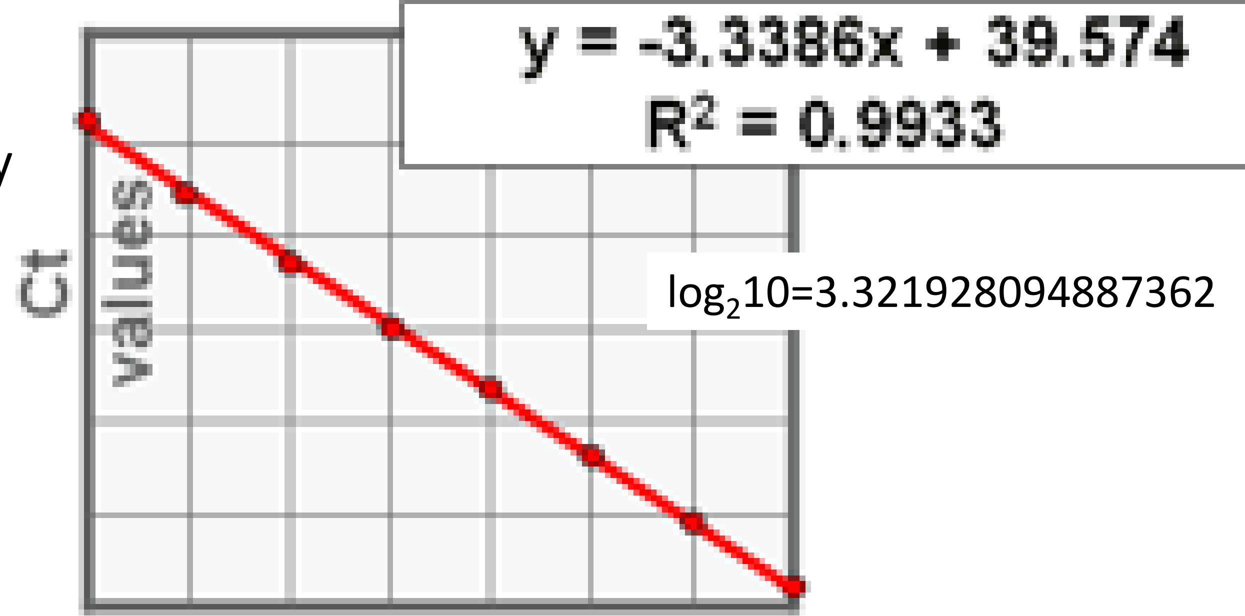
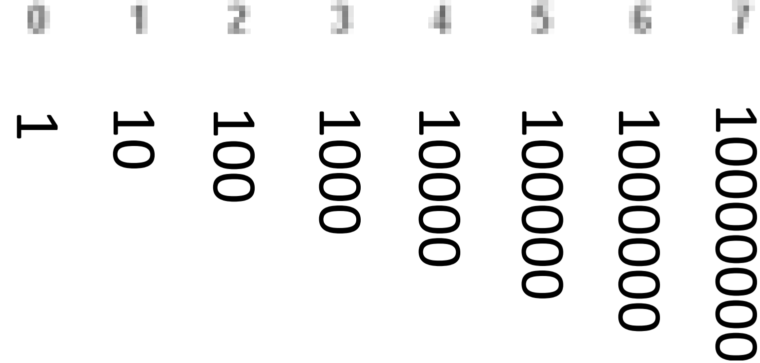
You have to prepare serial template DNA dilutions and then you have to carry out qRT-PCR on the diluted samples to obtain their Ct.

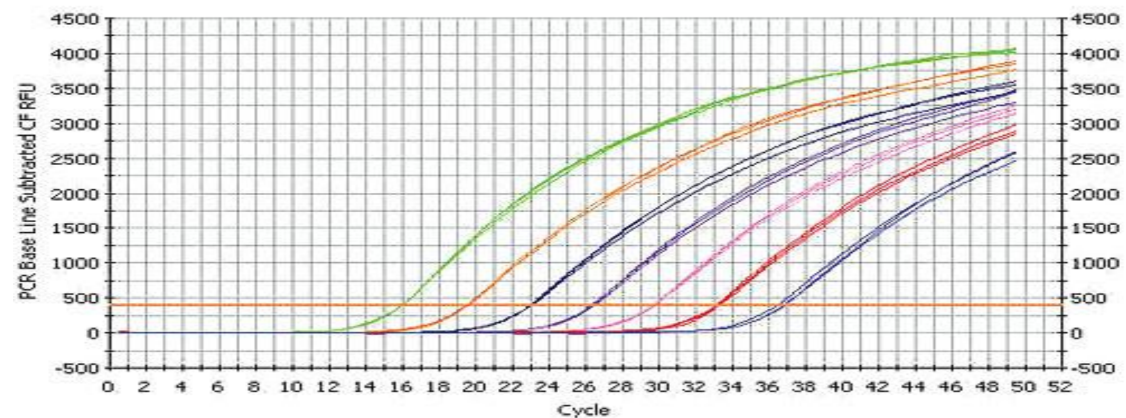
If you know the number of template molecules you have in your reaction



$\log_{10}$(copy number) ⟶

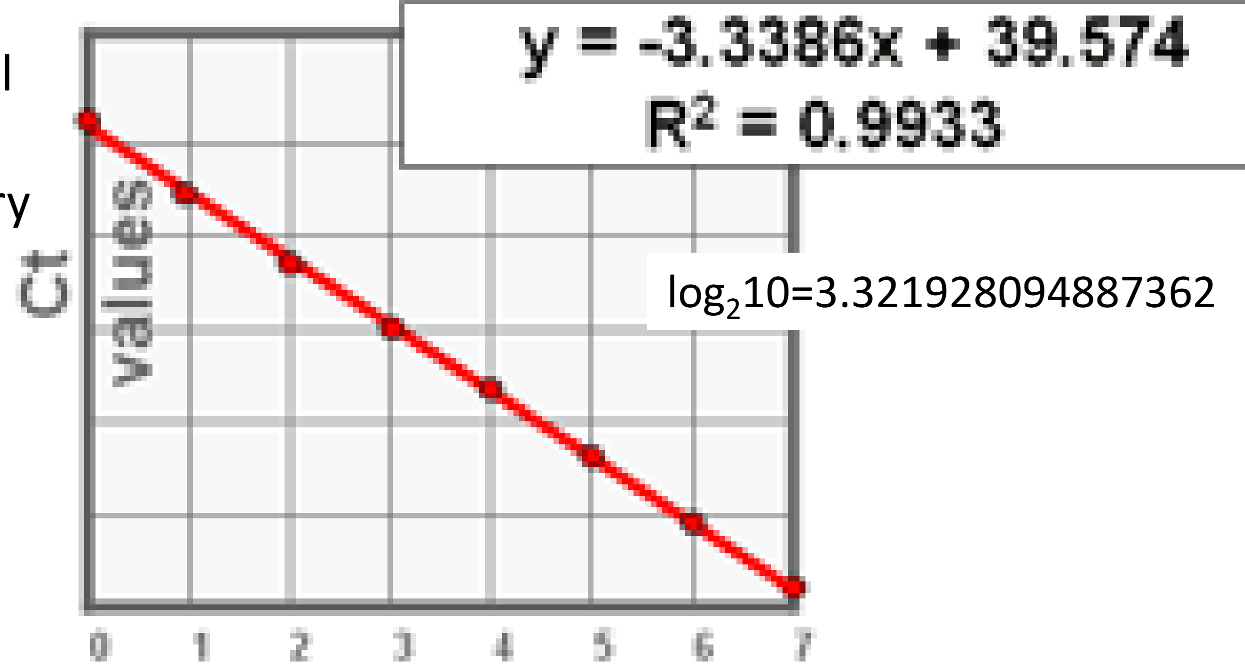
copy number ⟶



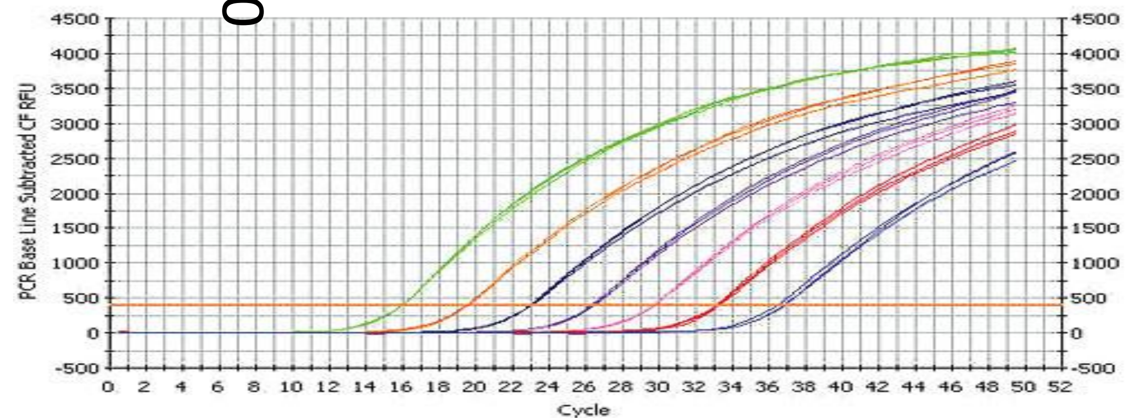


You have to prepare serial template DNA dilutions and then you have to carry out qRT-PCR on the diluted samples to obtain their Ct.

If you don't know the number of template molecules you have in your reaction

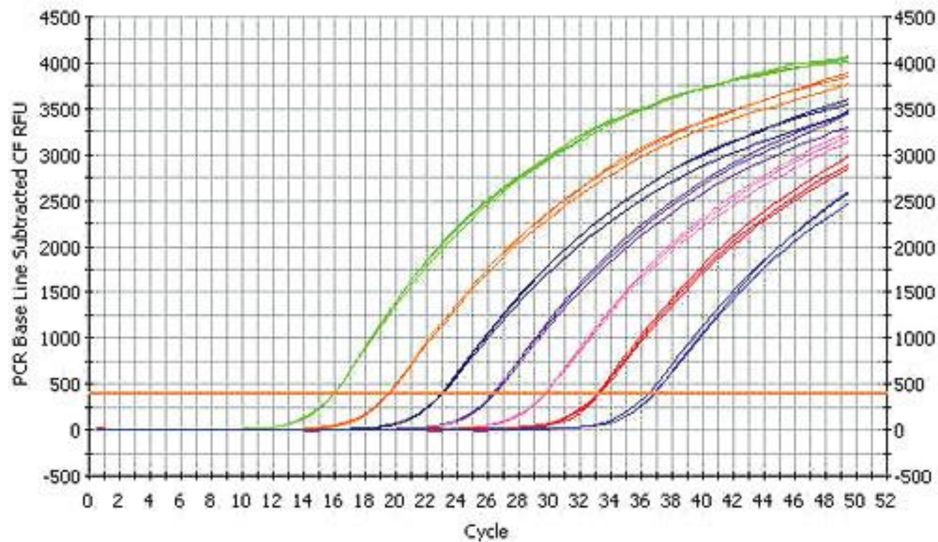


1:10000000
1:1000000
1:100000
1:10000
1:1000
1:100
1:10
1

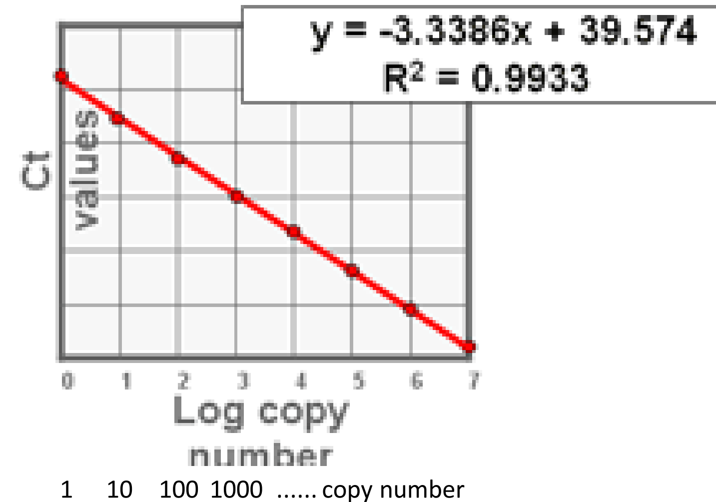


Efficiency (expected: 90–105%)

- analyse serial dilutions of a template
- the dilution series will produce amplification curves that are evenly spaced

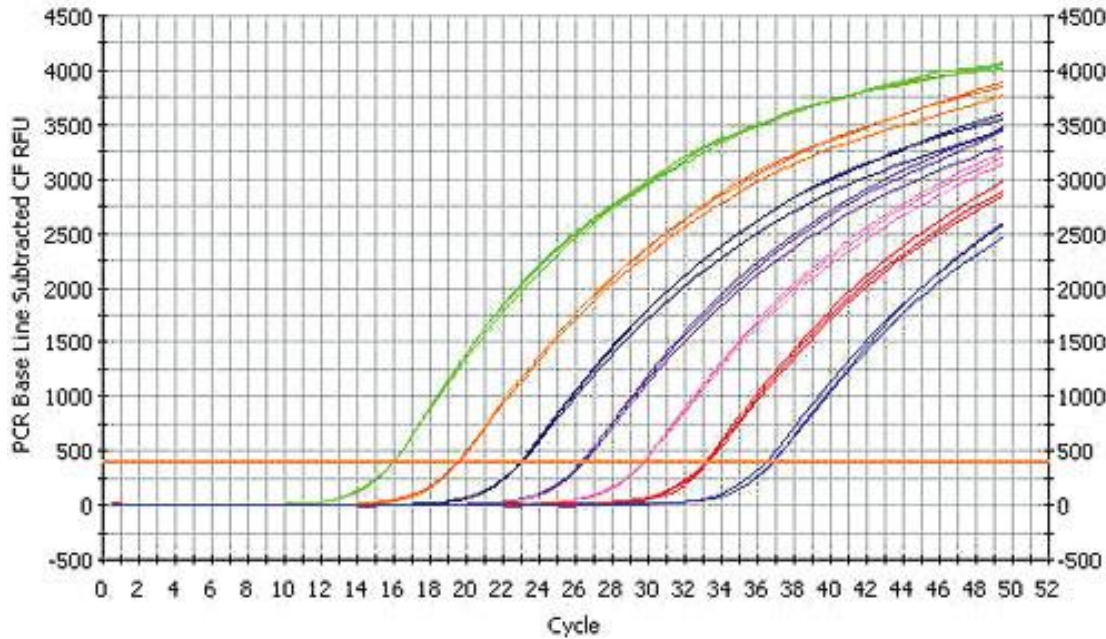


Standard Curve

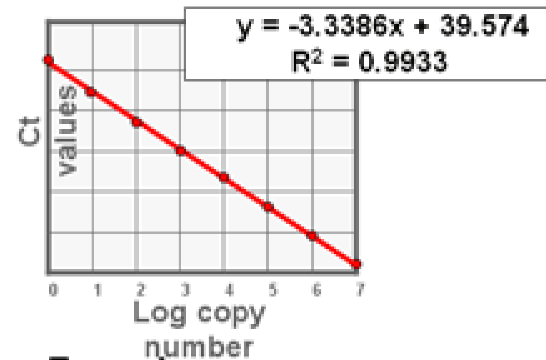


• by plotting the $\log_{10}$ of the starting quantity of template (or the dilution factor, for unknown quantities) against the Ct value obtained during amplification of each dilution, a standard curve is constructed.

- the coefficient of determination (R^2) shows how linear the data are and linearity gives a measure of whether the amplification efficiency is the same for different starting template copy numbers (R^2 value must be >0.980).



$log_2 10 = 3.321928094887362$



$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

Example:

Slope = -3.3386

$E = 10^{(-1/-3.3)} - 1$

$= 10^{(0.30)} - 1$

$= 1.995 - 1$

= 0.995 or 99.5%

If slope = -3.32

efficiency becomes 1

Slope	Efficiency
-3.32	100%
-3.5	93%
-3.6	90%
-3.8	83%
-4.0	78%

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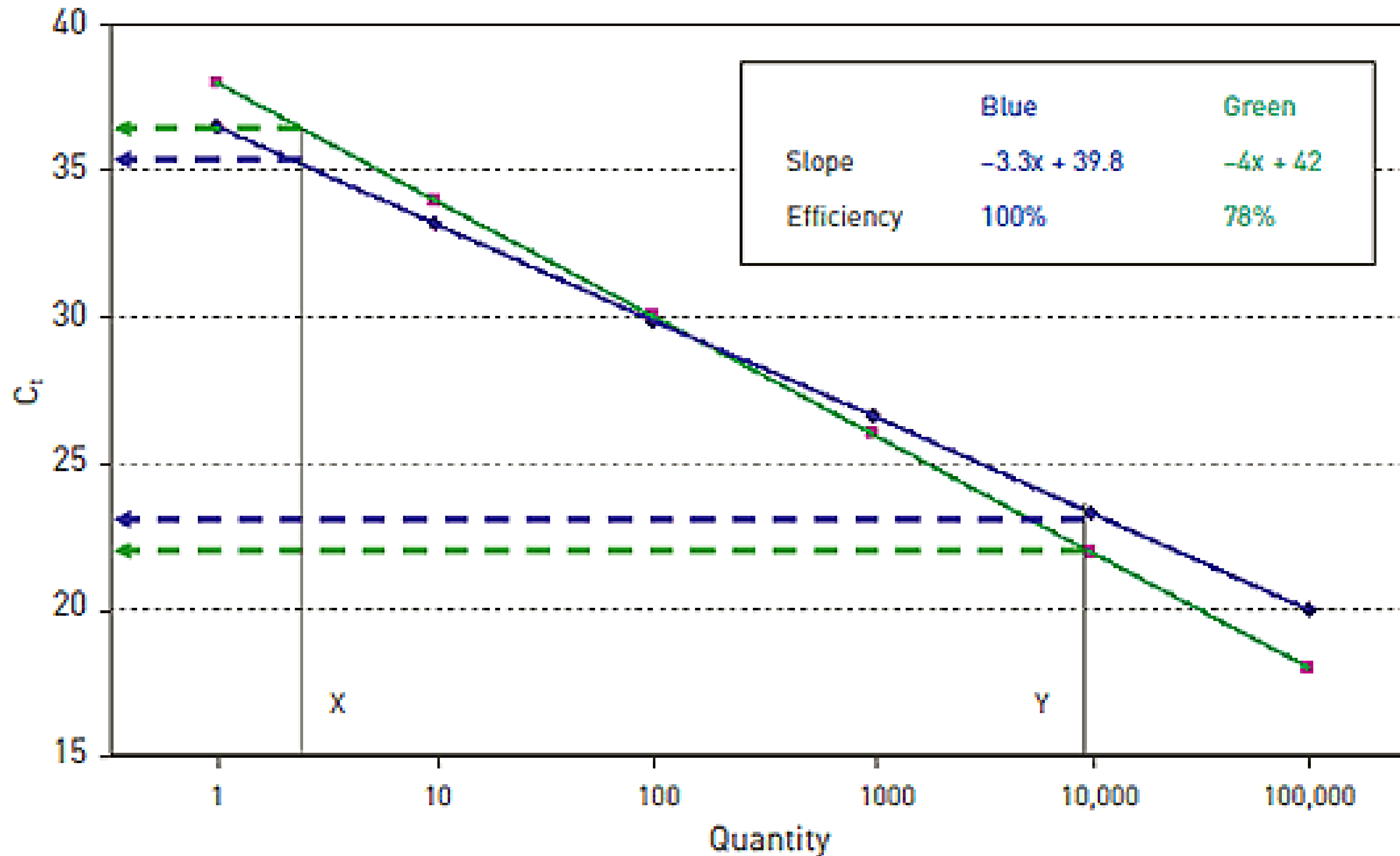
Variation of C_t with PCR efficiency.



The **blue standard curve has an efficiency of 100% (the slope is –3.3). The green standard curve has an efficiency of 78% (the slope is –4).** Amplification of quantity Y gives an earlier C_t under low efficiency conditions (green) compared to the high efficiency condition (blue). With a lower quantity (X) there is an inversion and the low efficiency condition (green) gives a later C_t than the high efficiency condition (blue).

* the slope of the $\log_{10}$-linear phase is a reflection of the amplification efficiency

* the efficiency of the reaction can be calculated by the following equation:

* $Eff=10^{(-1/slope)} - 1$.

* the efficiency of the PCR should be 90-105% (ideal slope = 3.32)

* a number of variables can affect the efficiency of the PCR. These factors can include length of the amplicon, secondary structure and primer design

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Relative quantification versus absolute quantification

Relative quantification

- calibrator sample is used as 1x standard
- for gene expression studies
- to verify trends and compare different samples

Absolute quantification

- used to obtain the absolute copy number of initial template
- the preparation of a standard curve is **necessary**
- cDNAs of target and reference genes can be subcloned into a vector to obtain the standard curve dilutions
- standards must be accurately quantified

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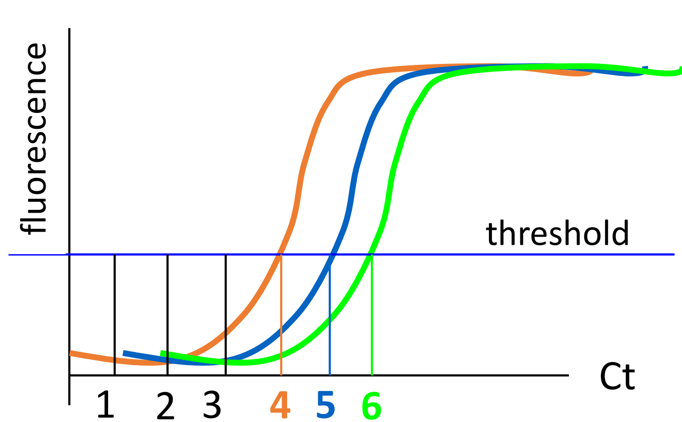
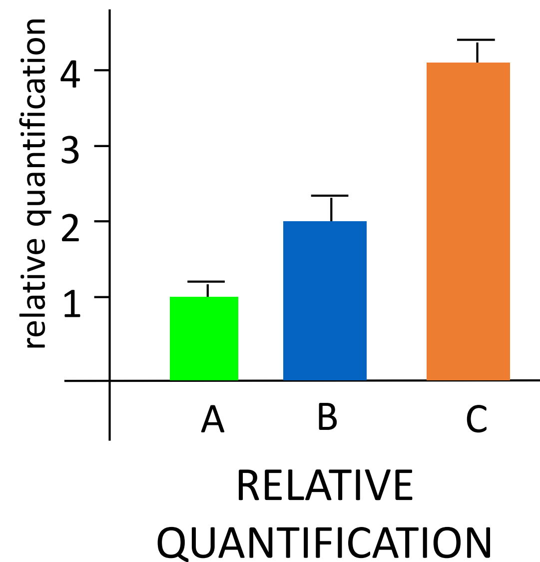
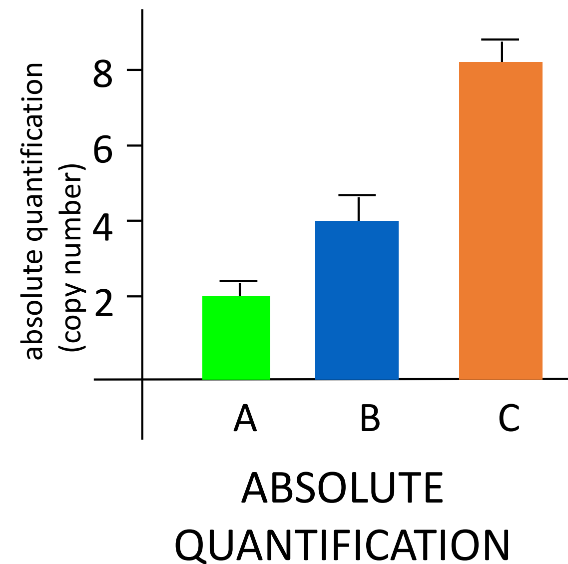
0	2	4	8
1	4	8	16
2	8	16	32
3	16	32	64
4	32	64	128
5	64	128	256
6	128	256	512
7	256	512	1024
8	512	1024	2048
9	1024	2048	4096





RELATIVE QUANTIFICATION



ABSOLUTE QUANTIFICATION

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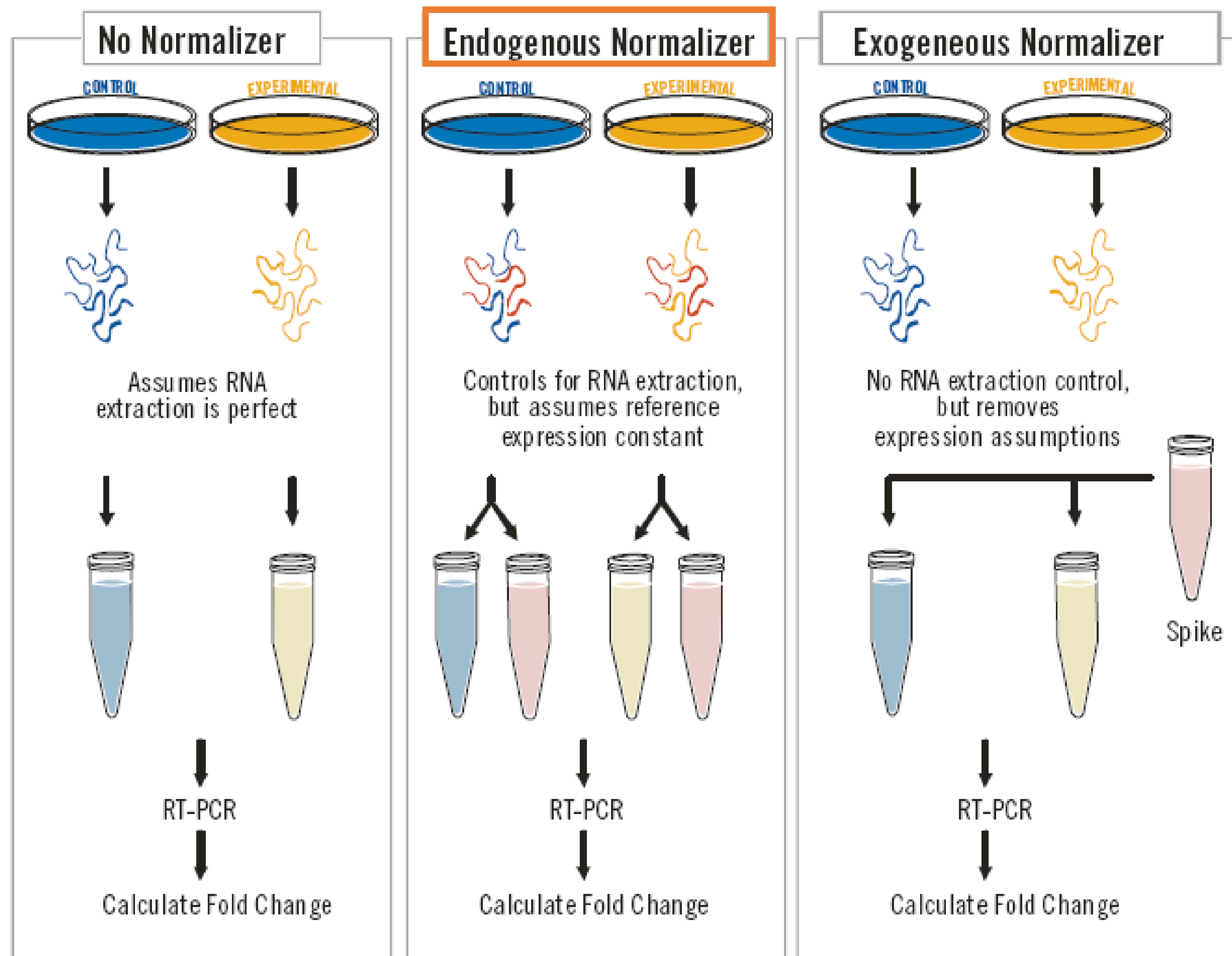
No Normalizer
CONTROL
EXPERIMENTAL
Assumes RNA extraction is perfect
RT-PCR
Calculate Fold Change
Endogenous Normalizer
CONTROL
EXPERIMENTAL
Controls for RNA extraction, but assumes reference expression constant
RT-PCR
Calculate Fold Change
Exogeneous Normalizer
CONTROL
EXPERIMENTAL
No RNA extraction control, but removes expression assumptions
Spike
RT-PCR
Calculate Fold Change

Relative Quantification Normalized to a Reference Gene

- the advantage of using a reference gene is that this method circumvents the need for accurate quantification and loading of the starting material.

- this is especially convenient when performing relative gene expression experiments where starting material is frequently limited.

- the drawback is that this method requires the availability of a known reference gene or genes with constant expression in all samples tested and whose expression is not changed by the treatment under study.

- the identification of such a reference gene is not trivial, and recently it has been proposed that in most cases, the use of multiple reference genes may be necessary for accurate quantification.

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When comparing multiple samples using relative quantification, one of the samples is usually chosen as the **calibrator**, and the expression of the **target** gene in all other samples is expressed as an increase or decrease relative to the calibrator. Usually, the untreated or baseline sample is chosen as the calibrator.

	Ct-target gene	Ct-Reference gene
Calibrator	$Ct_{(target)C}$	$Ct_{(reference)C}$
Sample 1	$Ct_{(target)1}$	$Ct_{(reference)1}$
Sample 2	$Ct_{(target)2}$	$Ct_{(reference)2}$
Sample 3	$Ct_{(target)3}$	$Ct_{(reference)3}$

Different methods can be used to determine the expression level of the target gene in the test samples relative to the calibrator sample. The most used are:

1) the Livak method, also known as the "$2^{-\Delta\Delta CT}$ method" using a reference gene
2) the Pfaffl method.

Each method has advantages and disadvantages, as well as assumptions that must be satisfied for the results of the analysis to be valid.

The $2^{-\Delta\Delta CT}$ (Livak) Method

- the $2^{-\Delta\Delta CT}$ method is widely used and easy to perform.

- this method assumes that both **target** and **reference** genes are amplified with efficiencies near 100% and within 5% of each other.

- before using the $2^{-\Delta\Delta CT}$ method, it is essential to verify the assumptions by determining the amplification efficiencies of the target and the reference genes.

- if the target and the reference genes have identical amplification efficiency, but the efficiency is not equal to 2, a modified form of the $2^{-\Delta\Delta CT}$ method may be used by replacing the 2 in the equation by the actual amplification efficiency.

Normalized expression ratio = $E^{-\Delta\Delta CT}$ (E=efficiency)

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1 - copy the Ct of the target gene and of the reference gene for calibrator and samples

	Ct-target gene	**Ct-Reference gene**
Calibrator	$Ct_{(target)C}$	$Ct_{(reference)C}$
Sample 1	$Ct_{(target)1}$	$Ct_{(reference)1}$
Sample 2	$Ct_{(target)2}$	$Ct_{(reference)2}$
Sample 3	$Ct_{(target)3}$	$Ct_{(reference)3}$

2 - normalize the Ct of the target gene to that of the reference gene for calibrator and samples, calculating the ΔCt

1 - copy the Ct of the target gene and of the reference gene for calibrator and samples

	Ct-target gene	Ct-Reference gene
Calibrator	$Ct_{(target)C}$	$Ct_{(reference)C}$
Sample 1	$Ct_{(target)1}$	$Ct_{(reference)1}$
Sample 2	$Ct_{(target)2}$	$Ct_{(reference)2}$
Sample 3	$Ct_{(target)3}$	$Ct_{(reference)3}$

2 - normalize the Ct of the target gene to that of the reference gene for calibrator and samples, calculating the ΔCt

We have seen that **the logarithm of a number is the exponent to which another number, the base, must be raised to produce that number.**

At each cycle, during the exponential phase, the DNA double
-> Ct are base 2 logarithms, because represent the number of amplification cycles necessary to obtain a detectable fixed amount of DNA.

When you divide two values with the same base you have to subtract the exponents:

$$2^{10}/2^{4}=2^{(10-4)}=2^{6}$$

the Ct represents a logaritm -> it represents an exponent
-> the ratio of 2 exponents is a subtraction

-> Ct normalized = Ct target – Ct reference

1 - copy the Ct of the target gene and of the reference gene for calibrator and samples

	Ct-target gene	**Ct-Reference gene**
Calibrator	$Ct_{(target)C}$	$Ct_{(reference)C}$
Sample 1	$Ct_{(target)1}$	$Ct_{(reference)1}$
Sample 2	$Ct_{(target)2}$	$Ct_{(reference)2}$
Sample 3	$Ct_{(target)3}$	$Ct_{(reference)3}$

2 - normalize the Ct of the target gene to that of the reference gene for calibrator and samples, calculating the ΔCt

	Ct-target gene	**Ct-Reference gene**	**ΔCt**
Calibrator	$Ct_{(target)C}$	$Ct_{(reference)C}$	$\Delta Ct_C = Ct_{(target)C} - Ct_{(reference)C}$
Sample 1	$Ct_{(target)1}$	$Ct_{(reference)1}$	$\Delta Ct_1 = Ct_{(target)1} - Ct_{(reference)1}$
Sample 2	$Ct_{(target)2}$	$Ct_{(reference)2}$	$\Delta Ct_2 = Ct_{(target)2} - Ct_{(reference)2}$
Sample 3	$Ct_{(target)3}$	$Ct_{(reference)3}$	$\Delta Ct_3 = Ct_{(target)3} - Ct_{(reference)3}$

3 - normalize the ΔCt of the samples to the ΔCt of calibrator, calculating the ΔΔCt

	ΔΔCt	**NRQ**
Calibrator	$\Delta\Delta Ct_C = \Delta Ct_C - \Delta Ct_C = 0$	$2^{-\Delta\Delta Ct_c} = 1$
Sample 1	$\Delta\Delta Ct_1 = \Delta Ct_1 - \Delta Ct_C$	$2^{-\Delta\Delta Ct_1}$
Sample 2	$\Delta\Delta Ct_2 = \Delta Ct_2 - \Delta Ct_C$	$2^{-\Delta\Delta Ct_2}$
Sample 3	$\Delta\Delta Ct_3 = \Delta Ct_3 - \Delta Ct_C$	$2^{-\Delta\Delta Ct_3}$

4 - finally, calculate the normalized relative quantification= $2^{-\Delta\Delta CT}$

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1 - copy the Ct of the target gene and of the reference gene for calibrator and samples

	Ct-target gene	Ct-Reference gene
Calibrator	$Ct_{(target)C} = 25$	$Ct_{(reference)C} = 24$
Sample 1	$Ct_{(target)1} = 22$	$Ct_{(reference)1} = 23$
Sample 2	$Ct_{(target)2} = 23$	$Ct_{(reference)2} = 24$
Sample 3	$Ct_{(target)3} = 27$	$Ct_{(reference)3} = 23$

2 - normalize the Ct of the target gene to that of the reference gene for calibrator and samples, calculating the ΔCt

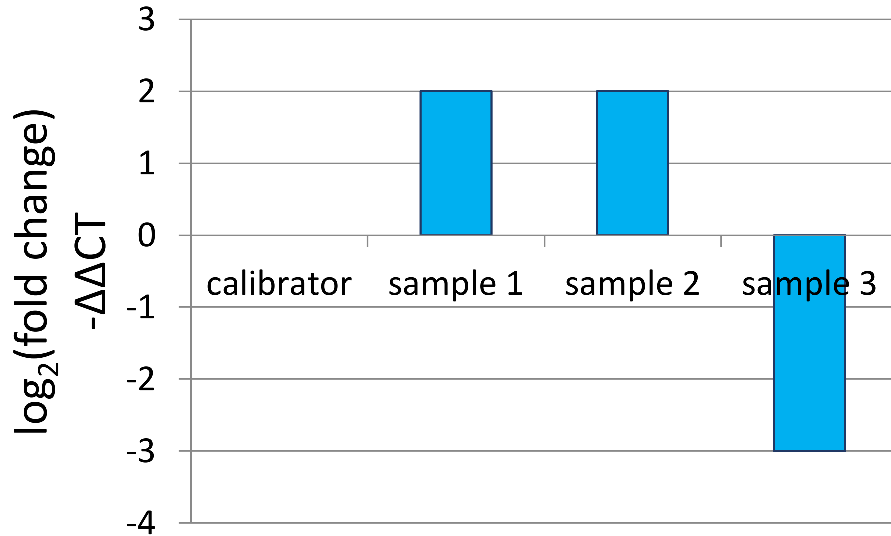
	Ct-target gene	Ct-Reference gene	ΔCt
Calibrator	$Ct_{(target)C} = 25$	$Ct_{(reference)C} = 24$	$\Delta Ct_C = Ct_{(target)C} - Ct_{(reference)C} = 1$
Sample 1	$Ct_{(target)1} = 22$	$Ct_{(reference)1} = 23$	$\Delta Ct_1 = Ct_{(target)1} - Ct_{(reference)1} = -1$
Sample 2	$Ct_{(target)2} = 23$	$Ct_{(reference)2} = 24$	$\Delta Ct_2 = Ct_{(target)2} - Ct_{(reference)2} = -1$
Sample 3	$Ct_{(target)3} = 27$	$Ct_{(reference)3} = 23$	$\Delta Ct_3 = Ct_{(target)3} - Ct_{(reference)3} = 4$

3 - normalize the ΔCt of the samples to the ΔCt of calibrator, calculating the ΔΔCt

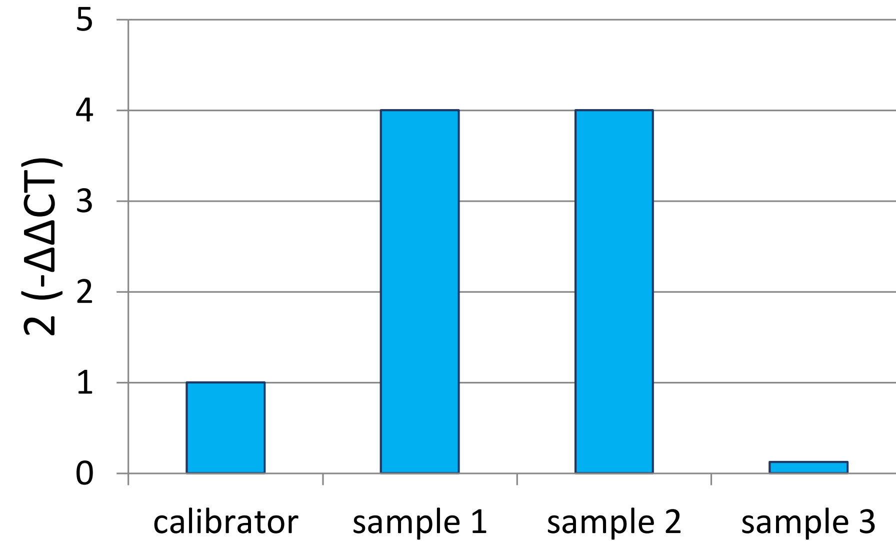
	ΔΔCt	-ΔΔCt	$2^{-\Delta\Delta Ct}$
Calibrator	$\Delta\Delta Ct_C = \Delta Ct_C - \Delta Ct_C = 1-1 = 0$	0	$2^{-\Delta\Delta Ct_C} = 2^0 = 1$
Sample 1	$\Delta\Delta Ct_1 = \Delta Ct_1 - \Delta Ct_C = -1-1 = -2$	2	$2^{-\Delta\Delta Ct_1} = 2^2 = 4$
Sample 2	$\Delta\Delta Ct_2 = \Delta Ct_2 - \Delta Ct_C = -1-1 = -2$	2	$2^{-\Delta\Delta Ct_2} = 2^2 = 4$
Sample 3	$\Delta\Delta Ct_3 = \Delta Ct_3 - \Delta Ct_C = 4-1 = 3$	-3	$2^{-\Delta\Delta Ct_3} = 2^{-3} = 0{,}125$

4 - finally, calculate the normalized relative quantification= $2^{-\Delta\Delta CT}$

$\log_2(\text{fold change}) = \log_2 (2^{-\Delta\Delta Ct}) =$ **$-\Delta\Delta Ct$**



fold change = **$2^{-\Delta\Delta Ct}$**

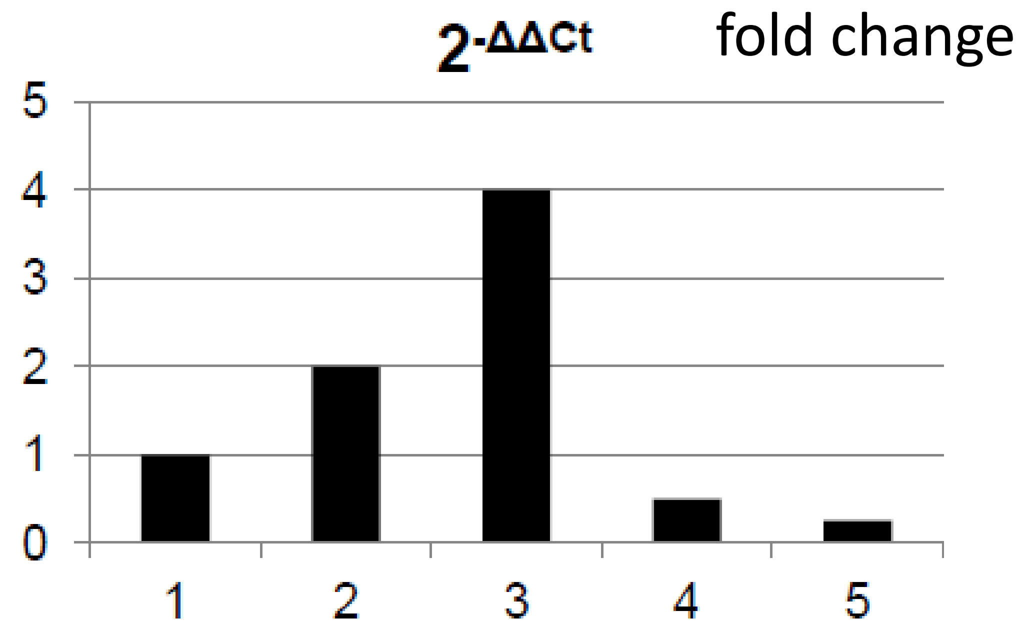


	ΔΔCt	**-ΔΔCt** log₂fold change	**$2^{-\Delta\Delta Ct}$** fold change
Calibrator	$\Delta\Delta Ct_C = \Delta Ct_C - \Delta Ct_C =$ 1-1 = 0	0	$2^{-\Delta\Delta Ct_c} = 2^0 = 1$
Sample 1	$\Delta\Delta Ct_1 = \Delta Ct_1 - \Delta Ct_C =$ -1-1 = -2	2	$2^{-\Delta\Delta Ct_1} = 2^2 = 4$
Sample 2	$\Delta\Delta Ct_2 = \Delta Ct_2 - \Delta Ct_C =$ -1-1 = -2	2	$2^{-\Delta\Delta Ct_2} = 2^2 = 4$
Sample 3	$\Delta\Delta Ct_3 = \Delta Ct_3 - \Delta Ct_C =$ 4-1 = 3	-3	$2^{-\Delta\Delta Ct_3} = 2^{-3} = 0{,}125$

$2^{-\Delta\Delta Ct}$ versus $-\Delta\Delta Ct$

#	fold change $2^{-\Delta\Delta Ct}$	$\log_2$fold change $-\Delta\Delta Ct$
1	1	0
2	2	0,30103
3	4	0,60206
4	0,5	-0,30103
5	0,25	-0,60206

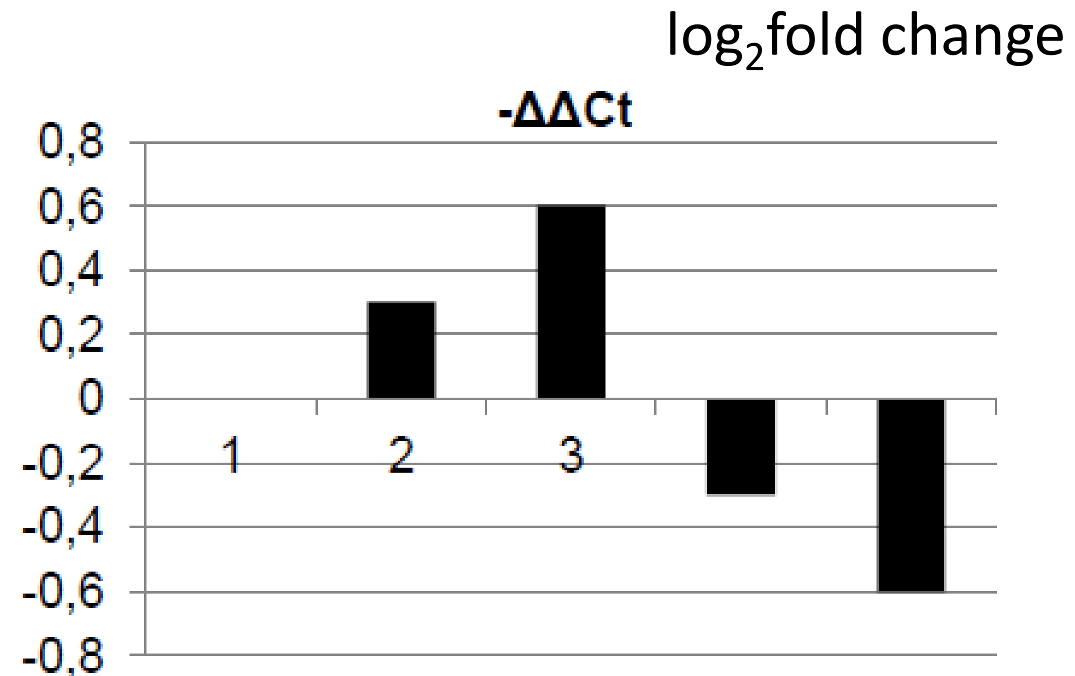
fold change



- with $2^{-\Delta\Delta Ct}$ you can appreciate the fold induction, but the down-regulation is grafically understimated when compared to up-regulation (instead of -2 and -4 you see 0,5 and 0,25).

- with $-\Delta\Delta Ct$ up-regulation and down-regulation are shown with the same intensity.

$\log_2$fold change



	Ct target	Ct reference	Δ Ct	ΔΔ Ct	Fold change
sample	Gene of interest	housekeeping gene	=Ct target - Ct reference	=ΔCT – ΔCT calibrator	$2^{-\Delta\Delta Ctc}$
1=calibrator	30,57	20,74	9,83	0,00	1,00
2	28,10	20,02	8,08	-1,75	3,37
3	28,23	18,36	9,86	0,03	0,98
4	27,25	16,01	11,24	1,41	0,38
5	29,16	17,50	11,66	1,82	0,28
6	29,36	17,56	11,80	1,97	0,26
7	30,58	18,45	12,13	2,29	0,20
8	29,49	18,93	10,55	0,72	0,61
9	27,65	19,69	7,96	-1,88	3,67
10	27,97	18,15	9,82	-0,02	1,01
11	29,26	16,81	12,45	2,62	0,16
12	29,26	17,67	11,59	1,76	0,30
13	32,73	21,26	11,46	1,63	0,32
14	30,32	18,15	12,17	2,33	0,20

Reference Genes to Normalize Relative Quantification

- the advantage of using a reference gene (such as GAPDH, β-actin, etc.) is that this method circumvents the need for accurate quantification and loading of the starting material.

- this is especially convenient when performing relative gene expression experiments where starting material is frequently limited.

- the drawback is that this method requires the availability of a known reference gene or genes with constant expression in all samples tested and whose expression is not changed by the treatment under study.

- the identification of such a reference gene is not trivial, and it has been proposed that in most cases, the use of multiple reference genes may be necessary for accurate quantification.

Reference Gene/s
(Normalization)

* usually an abundantly and constantly expressed (housekeeping) gene
* most commonly used ones are the least reliable ones
* best to run a validity test for the selected endogenous control
* **combination may/should be used**

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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

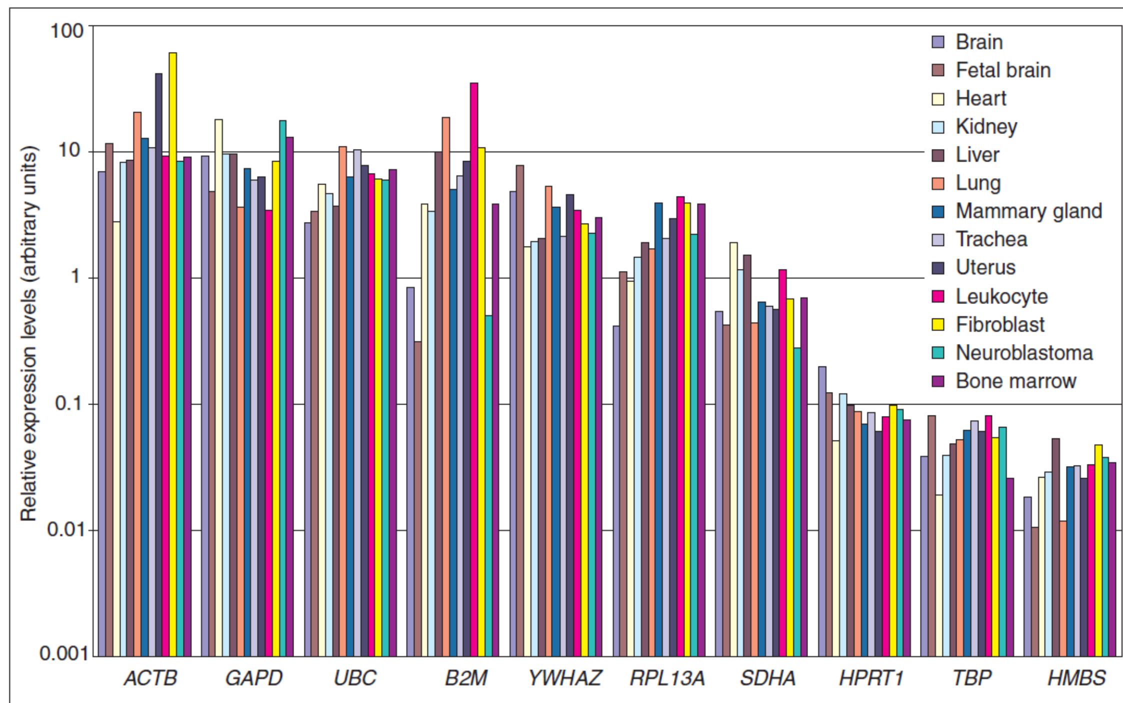

Figure 5
Logarithmic histogram of the expression levels of 10 internal control genes determined in 13 different human tissues, normalized to the geometric mean of 6 control genes (*GAPD*, *HPRT1*, *SDHA*, *TBP*, *UBC*, *YWHAZ*). An approximately 400-fold expression difference is apparent between the most and least abundantly expressed gene, as well as tissue-specific differences in expression levels for particular genes (for example, *B2M*).

Table 3

Control genes ranked in order of their expression stability*

Neuro-blastoma	Fibroblast	Leukocyte	Bone marrow	Normal pool
B2M	*HMBS*	*ACTB*	*ACTB*	*B2M*
RPL13A	*B2M*	*HMBS*	*B2M*	*ACTB*
ACTB	*RPL13A*	*HPRT1*	*HMBS*	*YWHAZ*
TBP	*SDHA*	*SDHA*	*TBP*	*RPL13A*
YWHAZ	*TBP*	*TBP*	*SDHA*	*UBC*
HMBS	*ACTB*	*RPL13A*	*GAPD*	*TBP*
UBC	*UBC*	*GAPD*	*HPRT1*	*HPRT1*
SDHA	*YWHAZ*	*B2M*	*YWHAZ*	*HMBS*
HPRT1 - GAPD	*HPRT1 - GAPD*	*UBC - YWHAZ*	*UBC - RPL13A*	*SDHA - GAPD*

*Increasing from top to bottom; the two most stable control genes in each cell type, for example *HPRT1* and *GAPD* in fibroblasts, cannot be ranked in order because of the required use of gene ratios for gene-stability measurements.

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Commonly used housekeeping genes are on a single exon or have pseudogenes

Symbol	Accession number	Name	Function	Localization	Pseudo-gene*	Primers†	Alias	IMAGE‡
ACTB	NM_001101	Beta actin	Cytoskeletal structural protein	7p15-p12	+	S		510455
B2M	NM_004048	Beta-2-microglobulin	Beta-chain of major histocompatibility complex class I molecules	15q21-q22	-	S		51940
GAPD	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis	12p13	+	D		510510
HMBS	NM_000190	Hydroxymethyl-bilane synthase	Heme synthesis, porphyrin metabolism	11q23	-	D	Porphobilinogen deaminase	245564
HPRT1	NM_000194	Hypoxanthine phosphoribosyl-transferase I	Purine synthesis in salvage pathway	Xq26	+	D		345845
RPL13A	NM_012423	Ribosomal protein L13a	Structural component of the large 60S ribosomal subunit	19q13	+	D	23 kDa highly basic protein	-
SDHA	NM_004168	Succinate dehydrogenase complex, subunit A	Electron transporter in the TCA cycle and respiratory chain	5p15	+	D		375812
TBP	NM_003194	TATA box binding protein	General RNA polymerase II transcription factor	6q27	-	D		280735
UBC	M26880	Ubiquitin C	Protein degradation	12q24	+	D		510582
YWHAZ	NM_003406	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide	Signal transduction by binding to phosphorylated serine residues on a variety of signaling molecules	2p25	+	S§	Phospholipase A2	416026

*Presence (+) or absence (-) of a retropseudogene in the genome determined by BLAST analysis of the mRNA sequence using the high-throughput genomic sequences database (htgs) or human genome as database. †Localization of forward and reverse primer in different exons (D) or the same exon (S). ‡IMAGE cDNA clone number according to [14]. §A single-exon gene.

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GAPD	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis	12p13	+	D		510510
HMBS	NM_000190	Hydroxymethyl-bilane synthase	Heme synthesis, porphyrin metabolism	11q23	-	D	Porphobilinogen deaminase	245564
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Quantification of Gene Expression after Painful Nerve Injury: Validation of Optimal Reference Genes

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Madhavi Latha Yadav Bangaru · Frank Park ·
Andy Hudmon · J. Bruce McCallum · Quinn H. Hogan

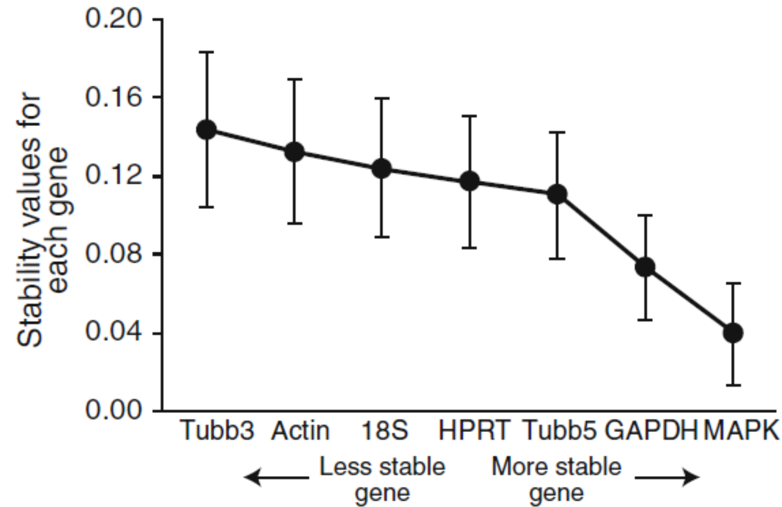

Fig. 3 NormFinder analysis for identifying the most stable HKGs, showing the seven HKGs in the sequence of their stability

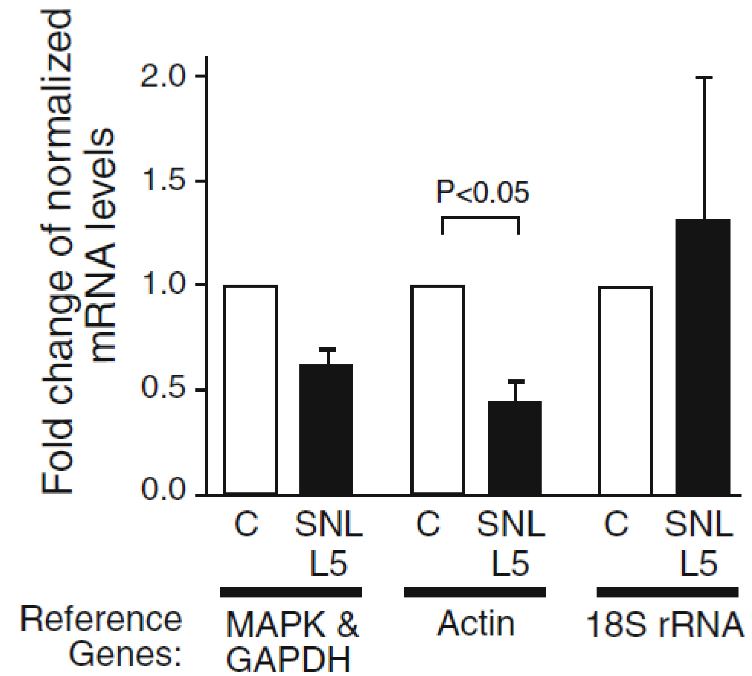

Fig. 5 Effect of reference genes on stromal interaction molecule-1 (STIM1) gene expression. The expression level of STIM1 was measured at day 7, and the fold difference was calculated between injured versus control DRG using various normalizing genes. The data represent mean ± SEM. *Brackets* represent significant differences by Mann–Whitney *U* test

MAPK6 and GAPDH are the most stable reference genes for use in normalizing transcript level of a target gene in the context of nerve injury determined by the geNorm and NormFinder program analysis.

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Reference genes

		CT	CT	CT	CT	CT	CT		
Days	**#**	**TBP**	**UbC**	**MAPK6**	**GAPDH**	**HPRT**	**18S**		**geometric mean**
0	**4,3**	27,56	24,63	26,483	21,124	26,66	7,854		20,74
1	**4.17**	27,03	24,63	25,491	20,107	25,186	7,498		20,02
2	**4.29**	25,92	24,16	24,303	19,162	24,074	5,456		18,36
7	**4.41**	23,10	23,55	21,787	17,356	21,769	3,757		16,01
14	**4.53**	24,96	24,40	24,168	19,692	23,969	4,133		17,50
21	**4,65**	24,49	23,65	24,156	19,462	23,436	4,59		17,56
28	**4,77**	25,36	23,74	25,11	18,579	24,1	5,827		18,45
0	**4.10**	25,92	23,05	25,229	19,58	24,846	6,281		18,93
1	**4.23**	26,70	24,18	24,781	19,591	24,737	7,513		19,69
2	**4.35**	25,86	23,90	23,892	18,846	23,659	5,425		18,15
7	**4.47**	24,28	23,75	23,105	18,648	22,643	4,005		16,81
14	**4.59**	24,85	24,92	24,389	17,92	23,82	4,719		17,67
21	**4,71**	27,43	25,88	27,317	22,032	26,442	8,185		21,26
28	**4,83**	24,88	24,08	24,742	19,407	23,934	5,2		18,15

$$C_t\text{-geometric mean} = \sqrt[n]{Ct_1 \times Ct_2 \times Ct_3 \times \ldots\ Ct_n}$$

Solo per uso didattico - vietata la riproduzione o la vendita

- PCR reaction
- conventional versus real time PCR
- real time PCR principles
- threshold cycle C_T
- efficiency
- relative quantification
- reference genes
- **primers**
- detection chemistry
- GLP in real time PCR

Primers

- equal Tm (58 – 60°C)
- 18 - 30 bases in length
- G-C content > 50%
- G or C at the 3’ end
- amplicon size 50 - 120 bp
- span exon-exon junctions in cDNA

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* Use standard assay conditions: 300 nM primers (or 600nM)

* Choose the primer pair that gives the highest ΔRn and the lowest C_T

* Make at least three (1:10) dilutions of a template (in triplicates) for a standard curve

* An ideal assay will have a slope of **-3.32**, **R^2 >0.99**

* If the slope is higher than -3.6, change primers

* Target and reference standard curves should be parallel
(same slope = same efficiency)

- PCR reaction
- conventional versus real time PCR
- real time PCR principles
- threshold cycle C_T
- efficiency
- relative quantification
- reference genes
- primers
- **detection chemistry**
- GLP in real time PCR

Real-Time PCR Detection Chemistry

1. DNA-binding (intercalating) agents (SYBR Green I, Eva Green, LC Green)
2. Hydrolysis probes (TaqMan)
3. Hybridization (Beacons, Scorpions, Pleiades)
4. Hybridization with FRET probes (Light Cycler)

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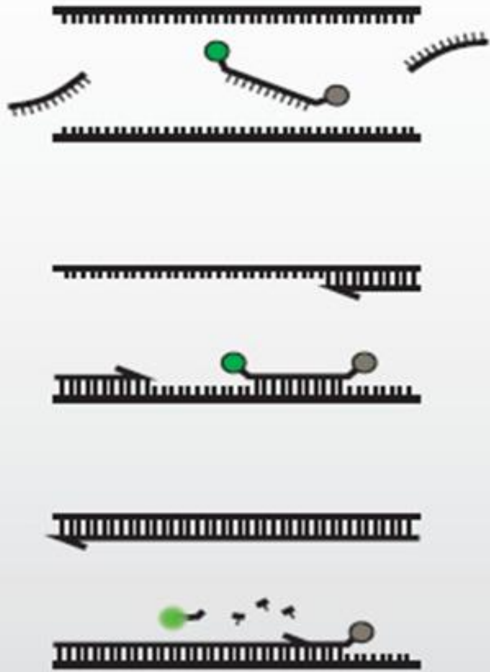
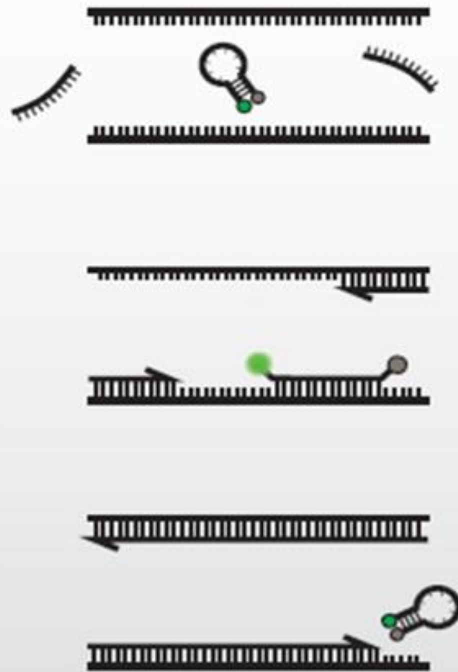
hydrolysis



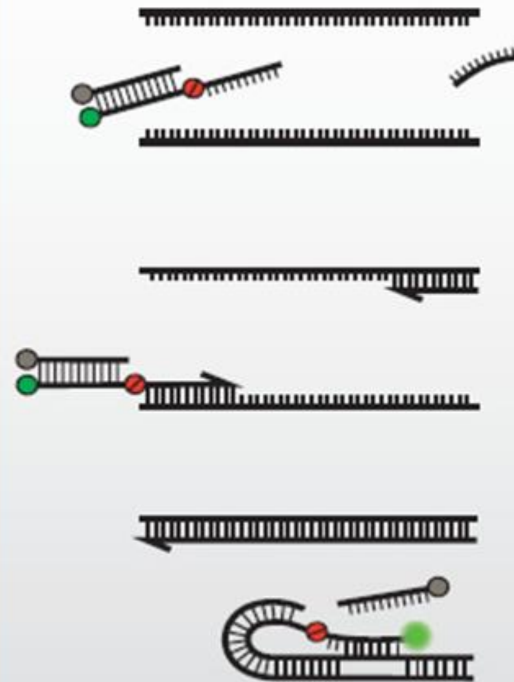
TaqMan

hybridization



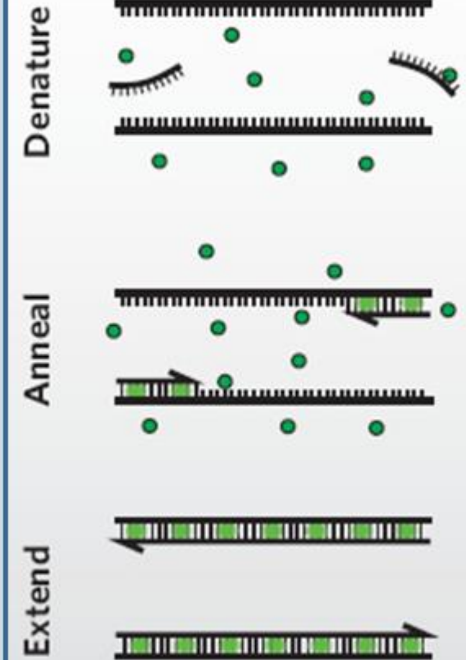
Molecular Beacon

hybridization



Scorpions

DNA binding



Sybr Green I