Quantitative Real time PCR

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- 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

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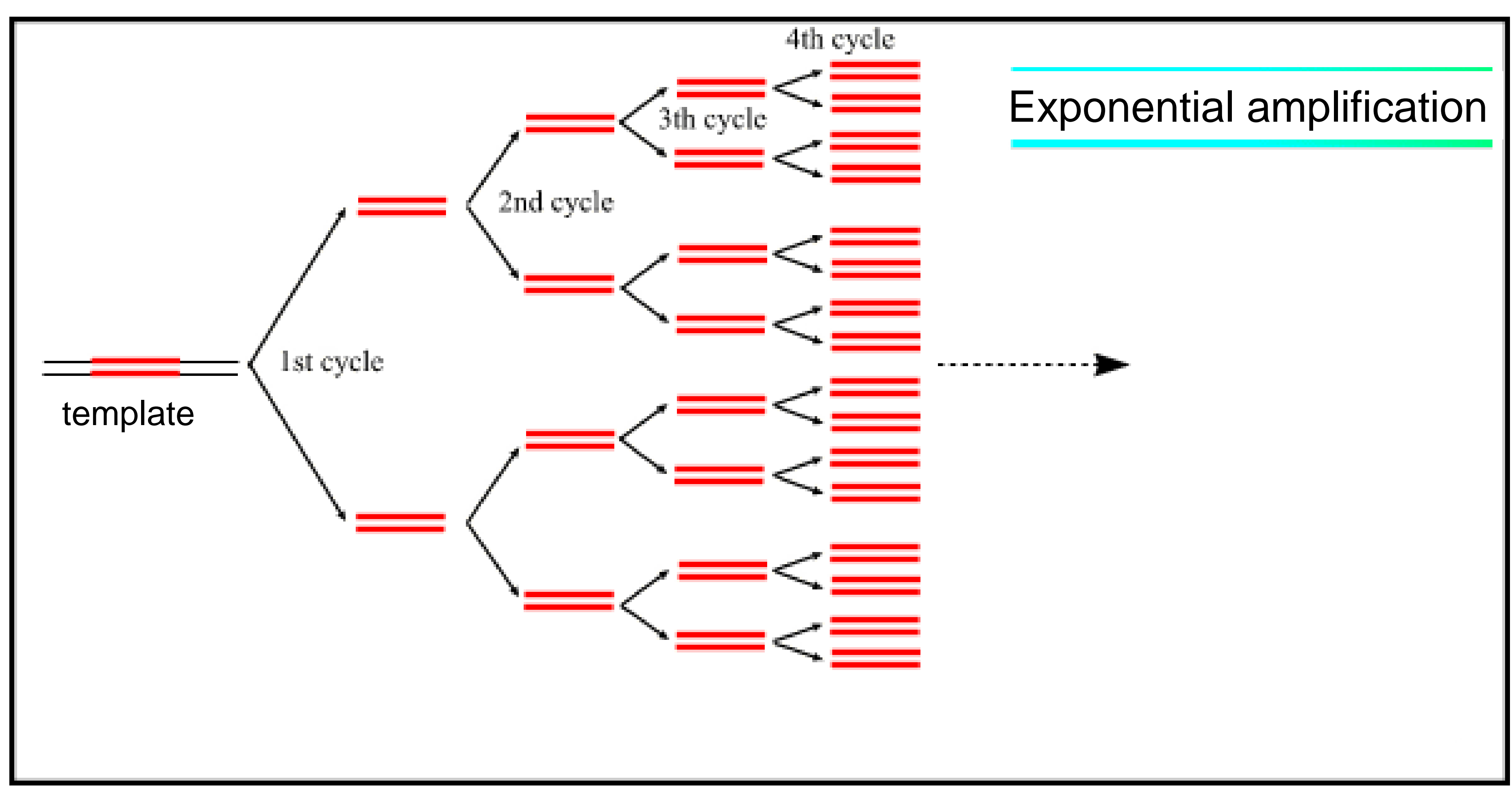
DNA polymerase chain reaction (PCR)



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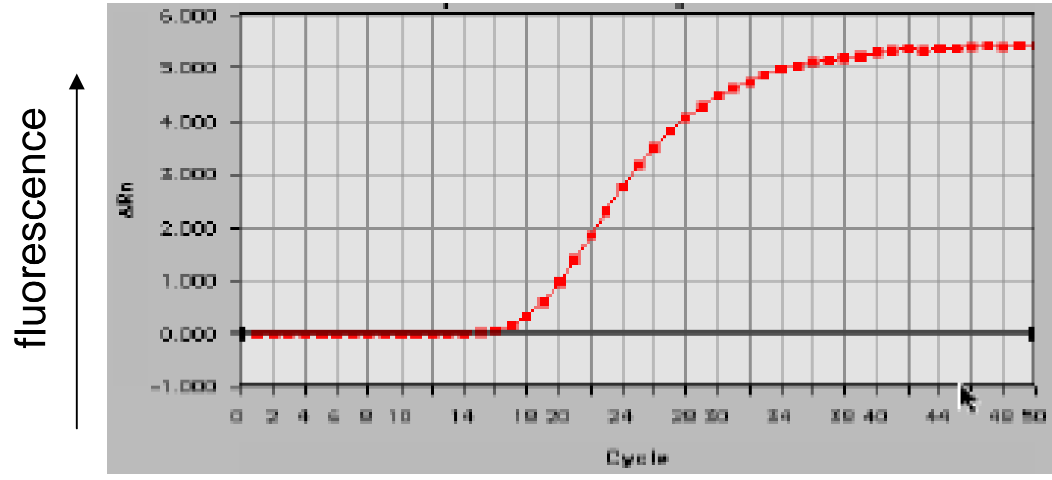
“Real-Time” PCR

* 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



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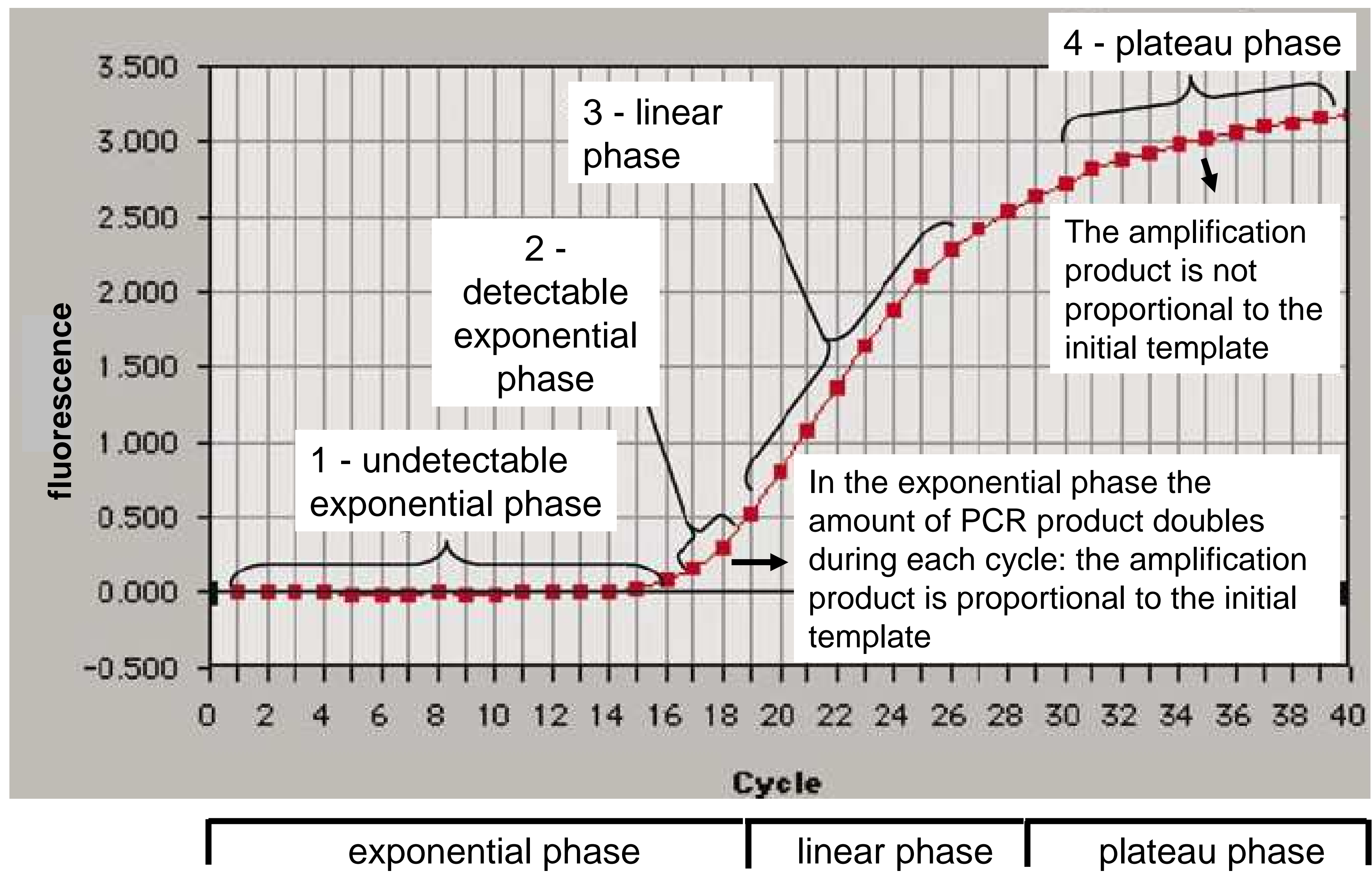

As the reaction proceeds, reaction components are consumed and one or more of the components becomes limiting.

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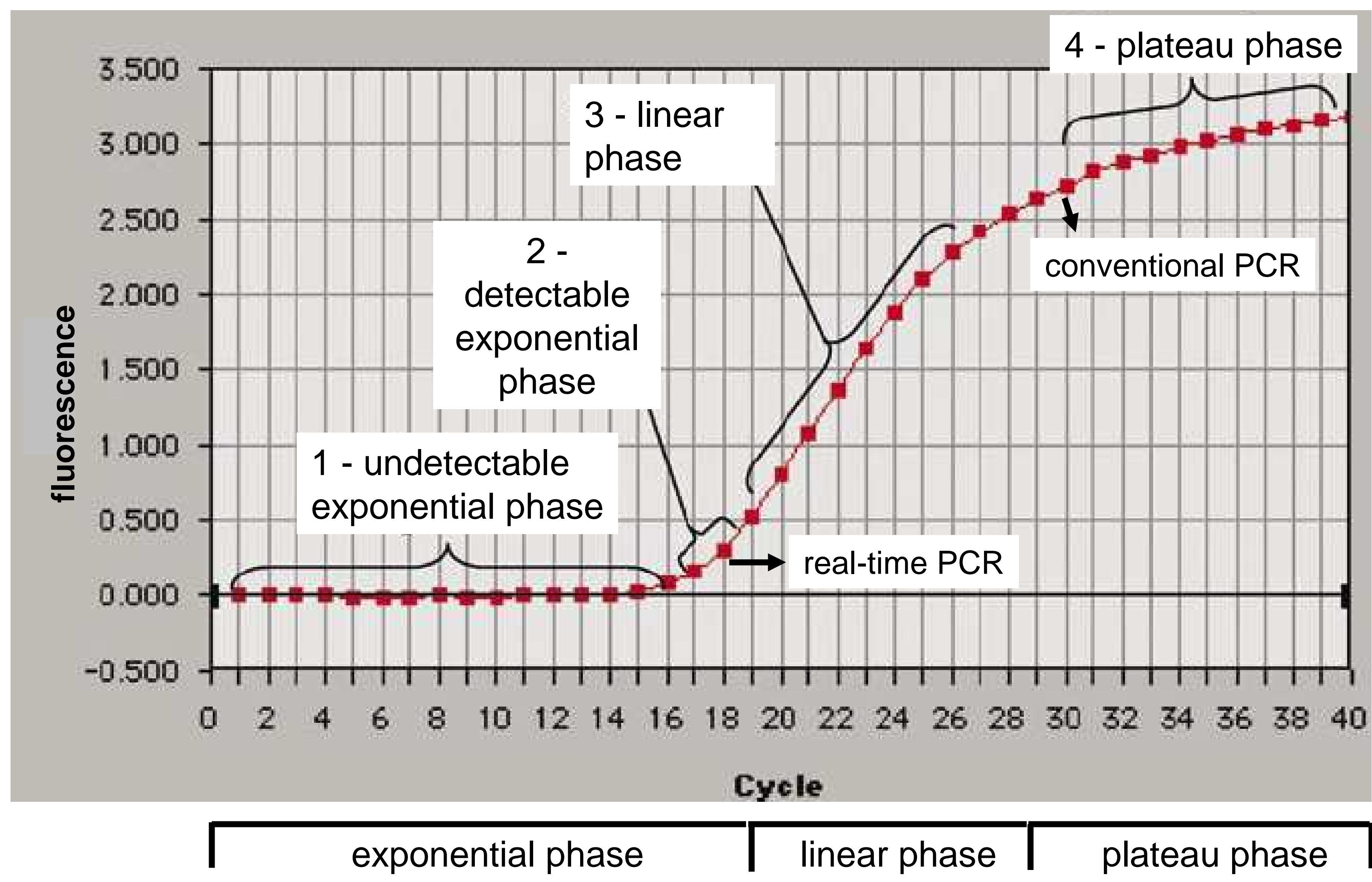

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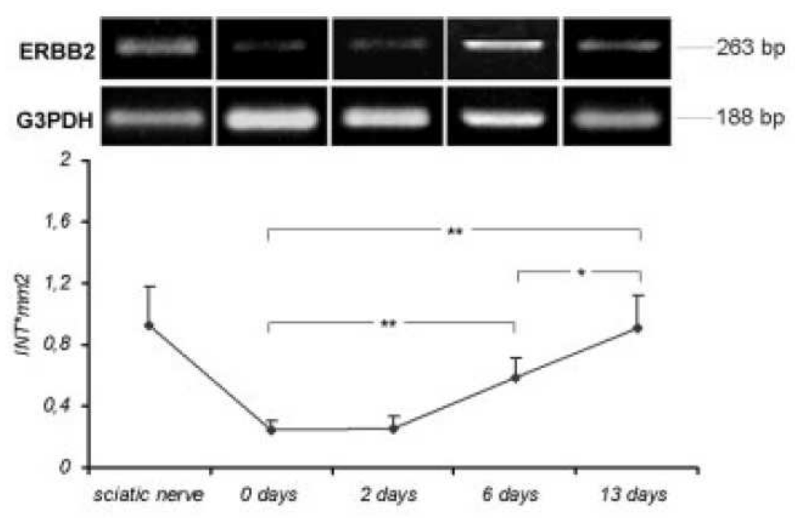
Conventional PCR

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



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Real-time PCR advantages

* 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

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Real-time PCR disadvantages

* not ideal for multiplexing (it is possible to multiplex though)
* setting up requires high technical skill and support
* high equipment cost

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Real time instrument

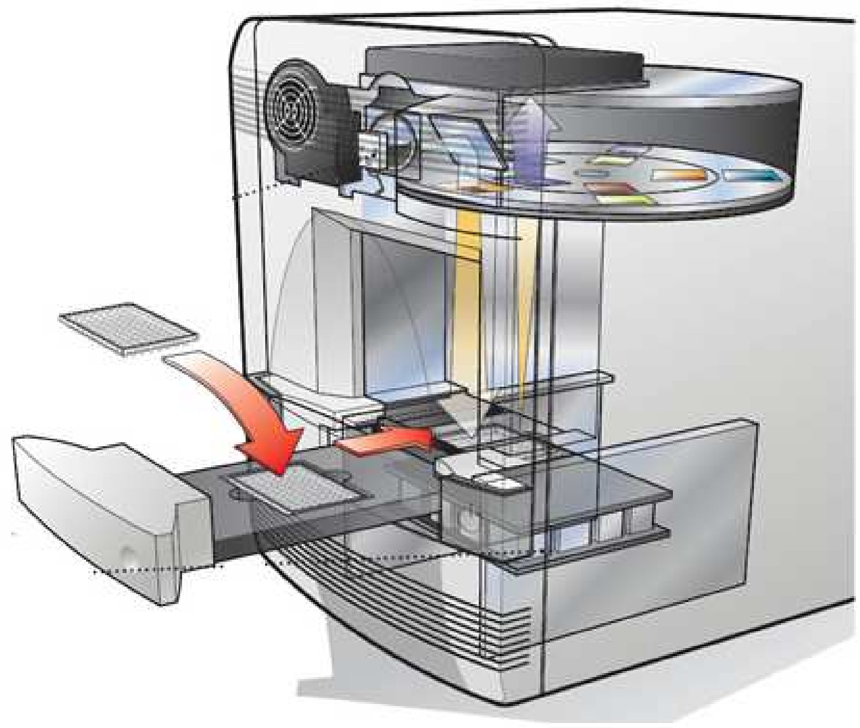
The instrument is equipped with :

- a thermal cycler for amplification

- a light source for excitation of fluorescent reagents or probes (can be a simple alogen 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



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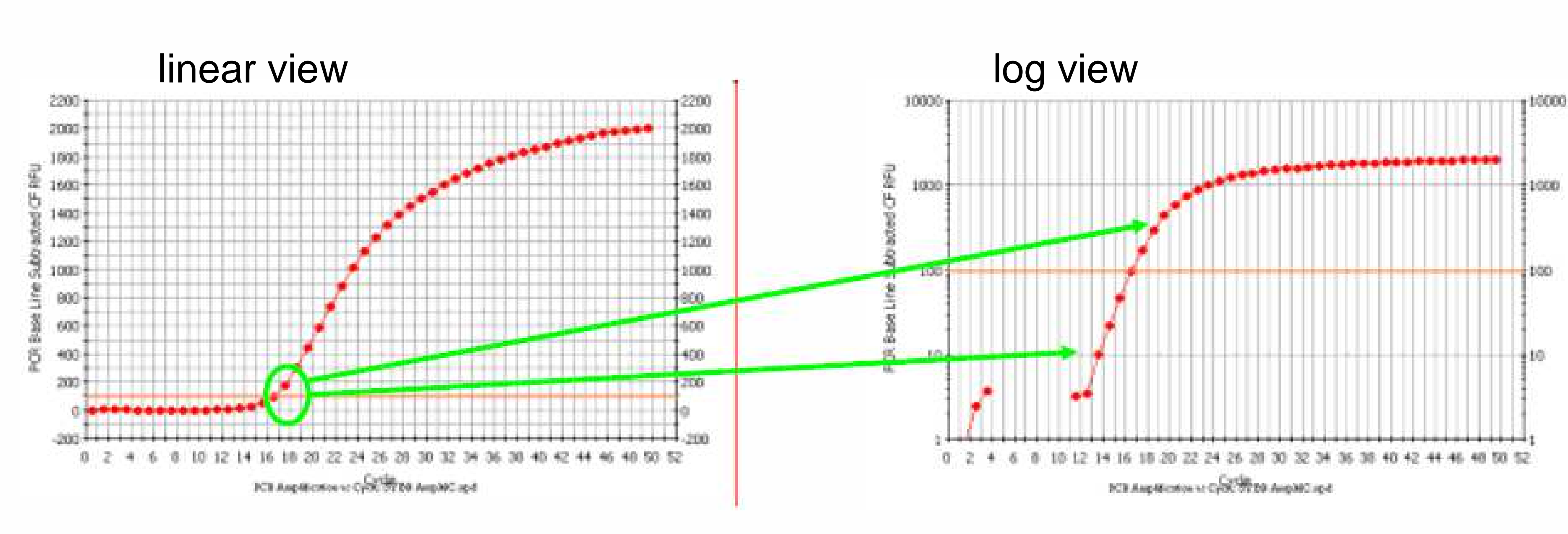
Real-time PCR Principles

Linear vs Log View



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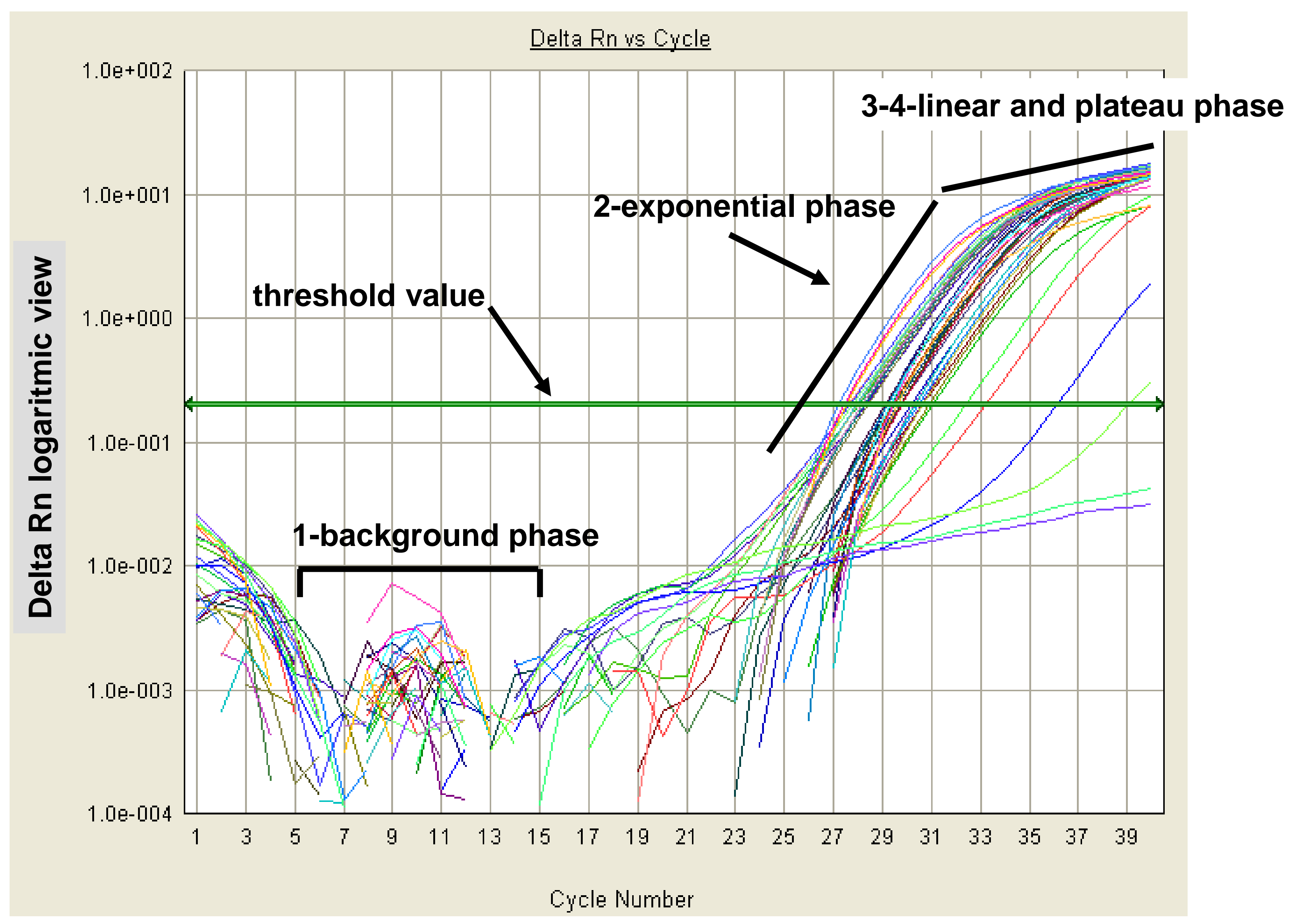

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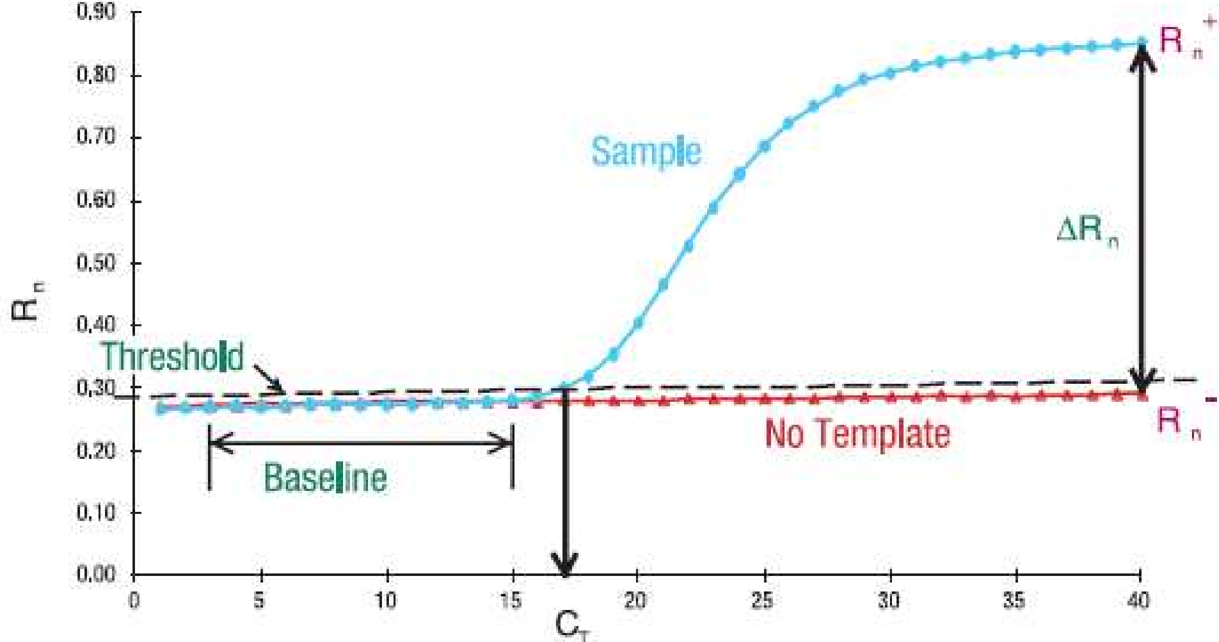
ΔRn

* Rn^+ is the Rn value of a reaction containing all components

* Rn^- is the Rn value detected in NTC (baseline value)

* ΔRn is the difference between Rn^+ and Rn^-. It is an indicator of the magnitude of the signal generated by the PCR

* ΔRn is plotted against cycle numbers to produce the amplification curves and to estimate the C_T values



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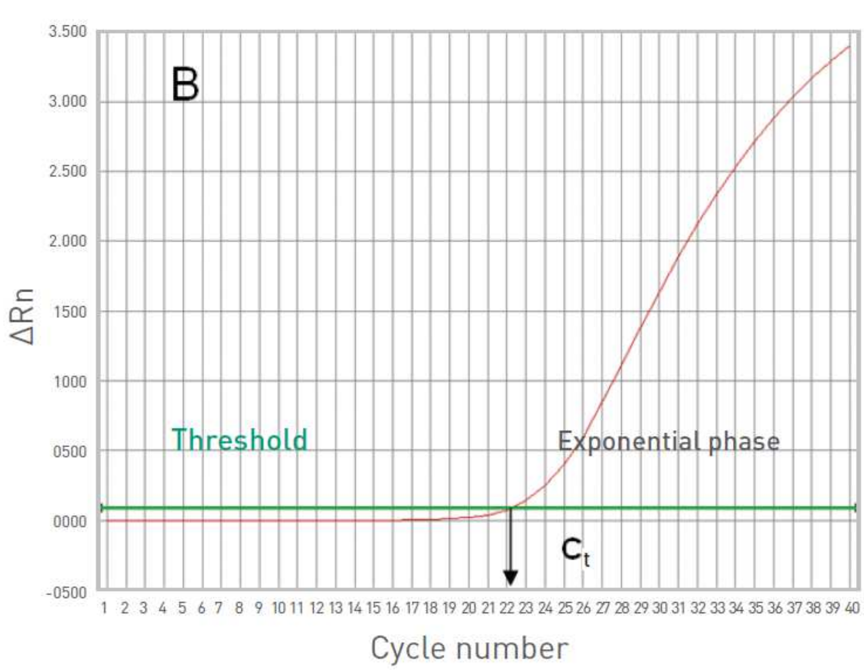
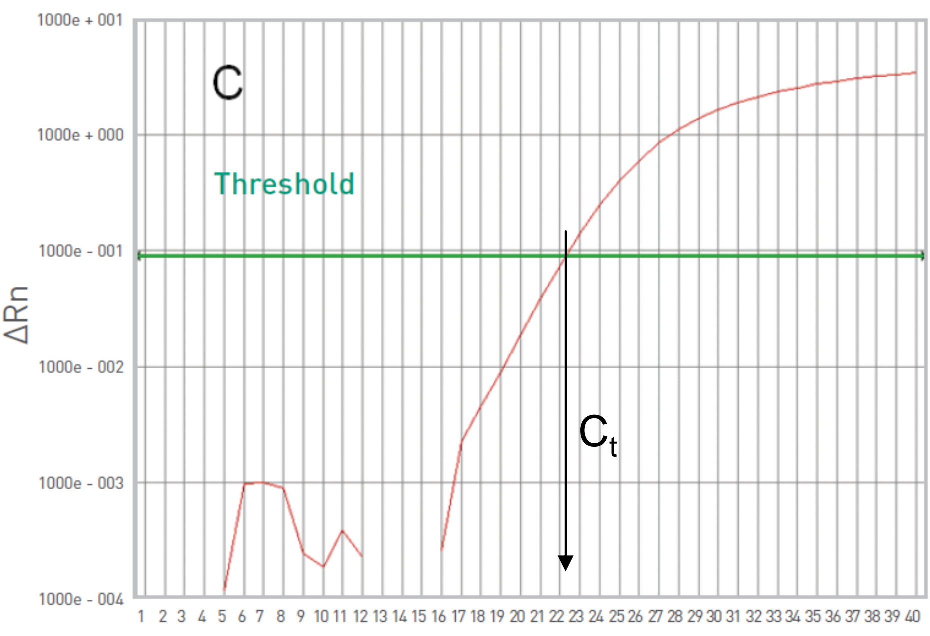
C_T - Threshold Cycle

* is the parameter used for quantitation, correlates with the initial amount of template, since the C_T value is measured in the exponential phase, when reagents are not limited

* threshold cycle (C_T value) is the cycle at which sample crosses threshold

* C_T value of 40 or more means no amplification





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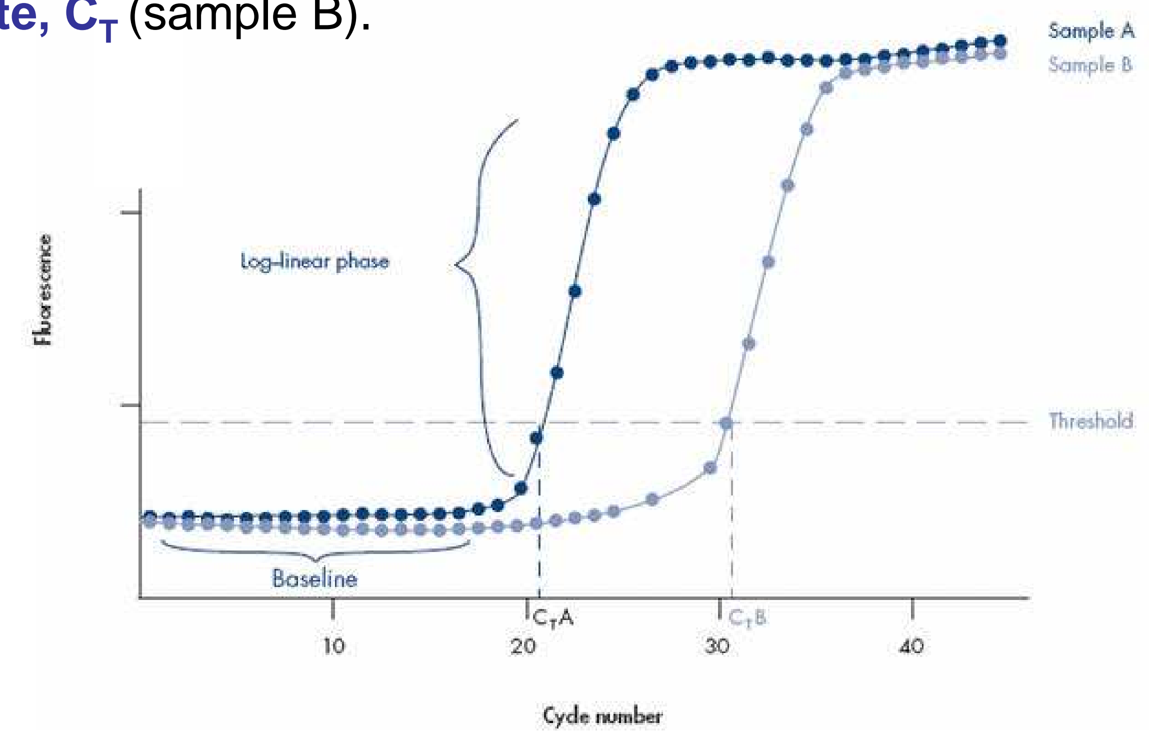
The C_T is determined mainly by the amount of template present at the start of the amplification reaction.

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).



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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

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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**
5	64	**128**	256
6	**128**	256	512
7	256	512	1024
8	512	1024	2048
9	1024	2048	4096

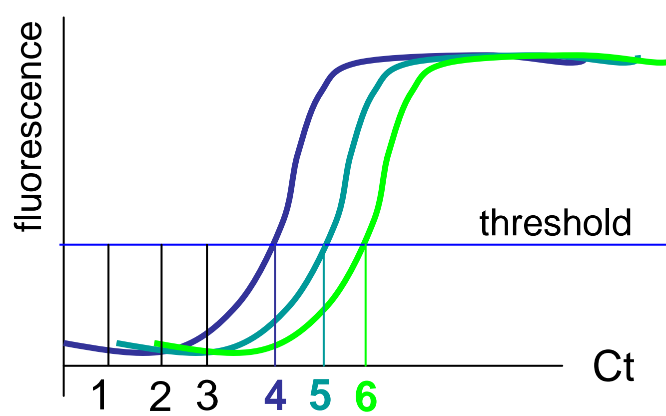
- the initial template in sample C is the double of sample B
- the initial template in sample B is the double of sample A

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

-the Ct value for sample 1 will be 6,

-the Ct value for sample 2 will be 5,

-the Ct value for sample 3 will be 4



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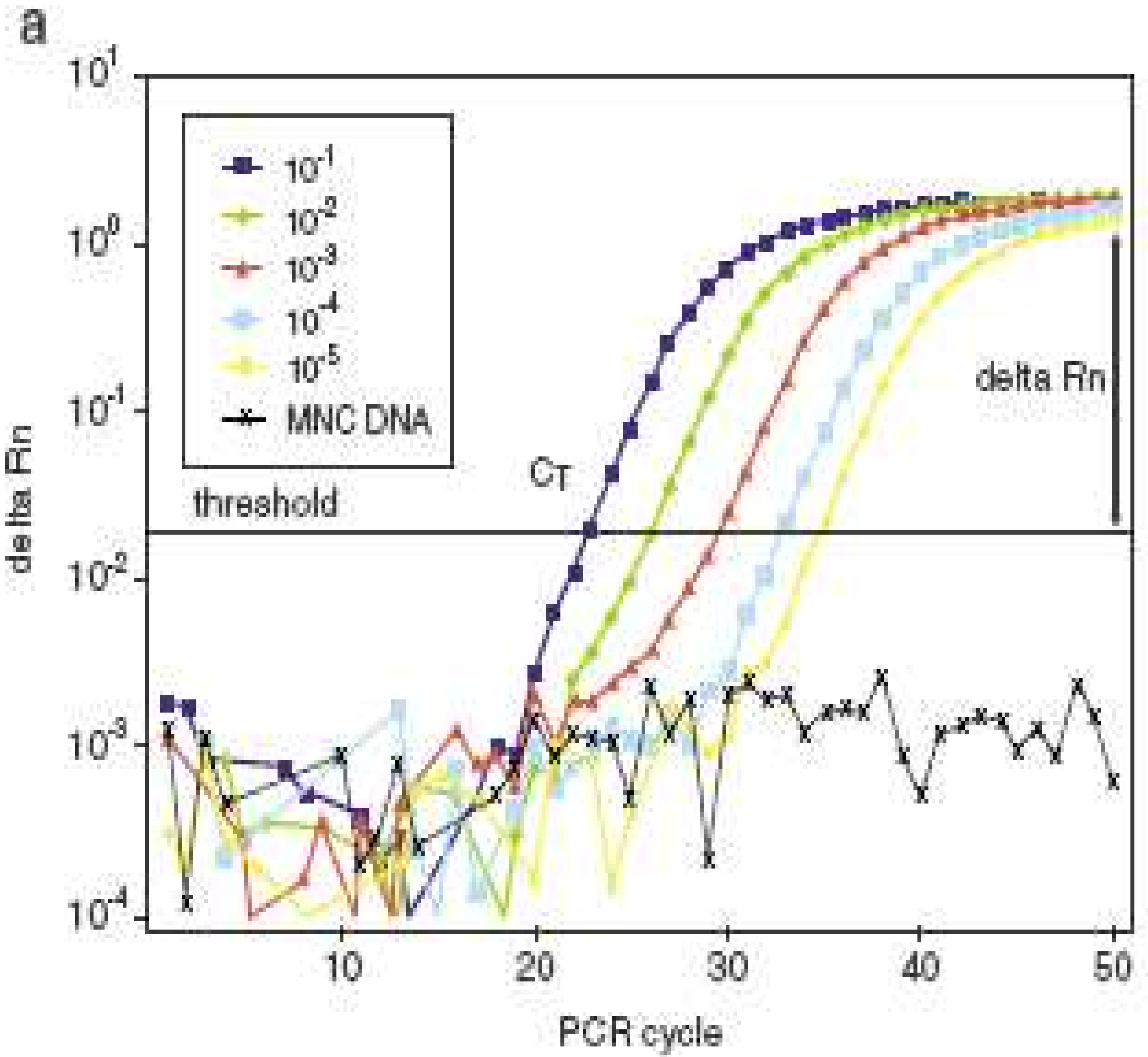

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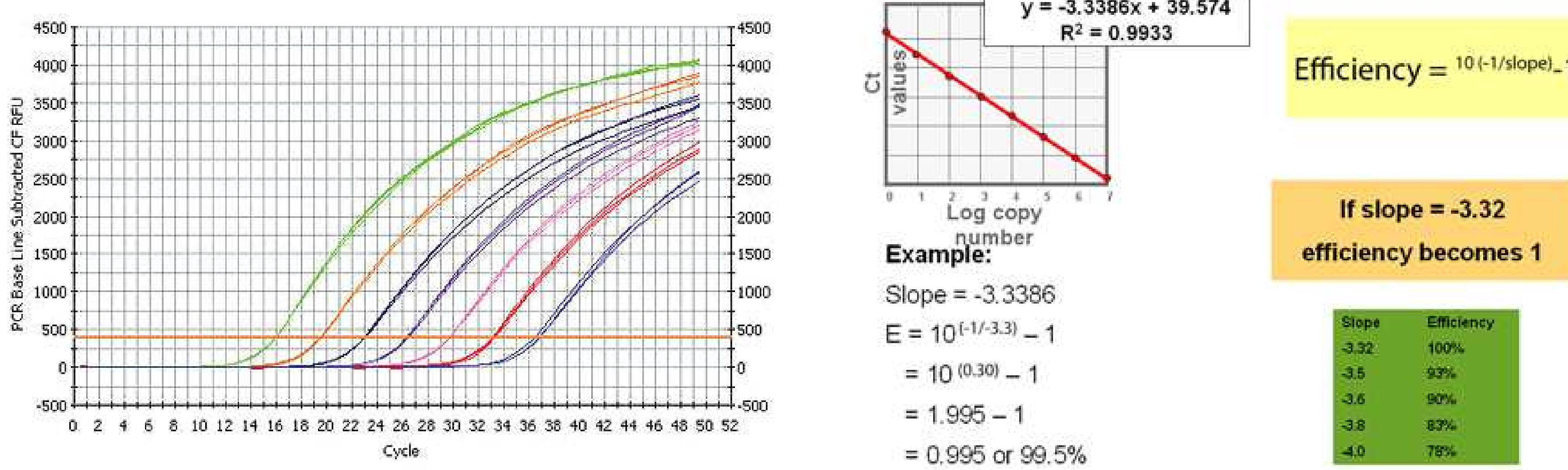
Efficiency (expected: 90–105%)

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

-by plotting the log 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).

Standard Curve



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Efficiency

* the slope of the log-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, to name a few

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Calculation

The slope of the standard curve can be used to determine the exponential amplification and efficiency of the PCR reaction by the following equations:

$$\text{Exponential Amplification} = 10^{(-1/\text{slope})}$$
$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

The following table shows the amplification and efficiency for various values of the slope:

Slope	Amplification	Efficiency
-3.60	1.8957	0.8957
-3.55	1.9129	0.9129
-3.50	1.9307	0.9307
-3.45	1.9492	0.9492
-3.40	1.9684	0.9684
-3.35	1.9884	0.9884
-3.30	**2.0092**	**1.0092**
-3.25	2.0309	1.0309
-3.20	2.0535	1.0535
-3.15	2.0771	1.0771
-3.10	2.1017	1.1017

As the table illustrates, optimal PCR efficiency is indicated by a slope of –3.3.

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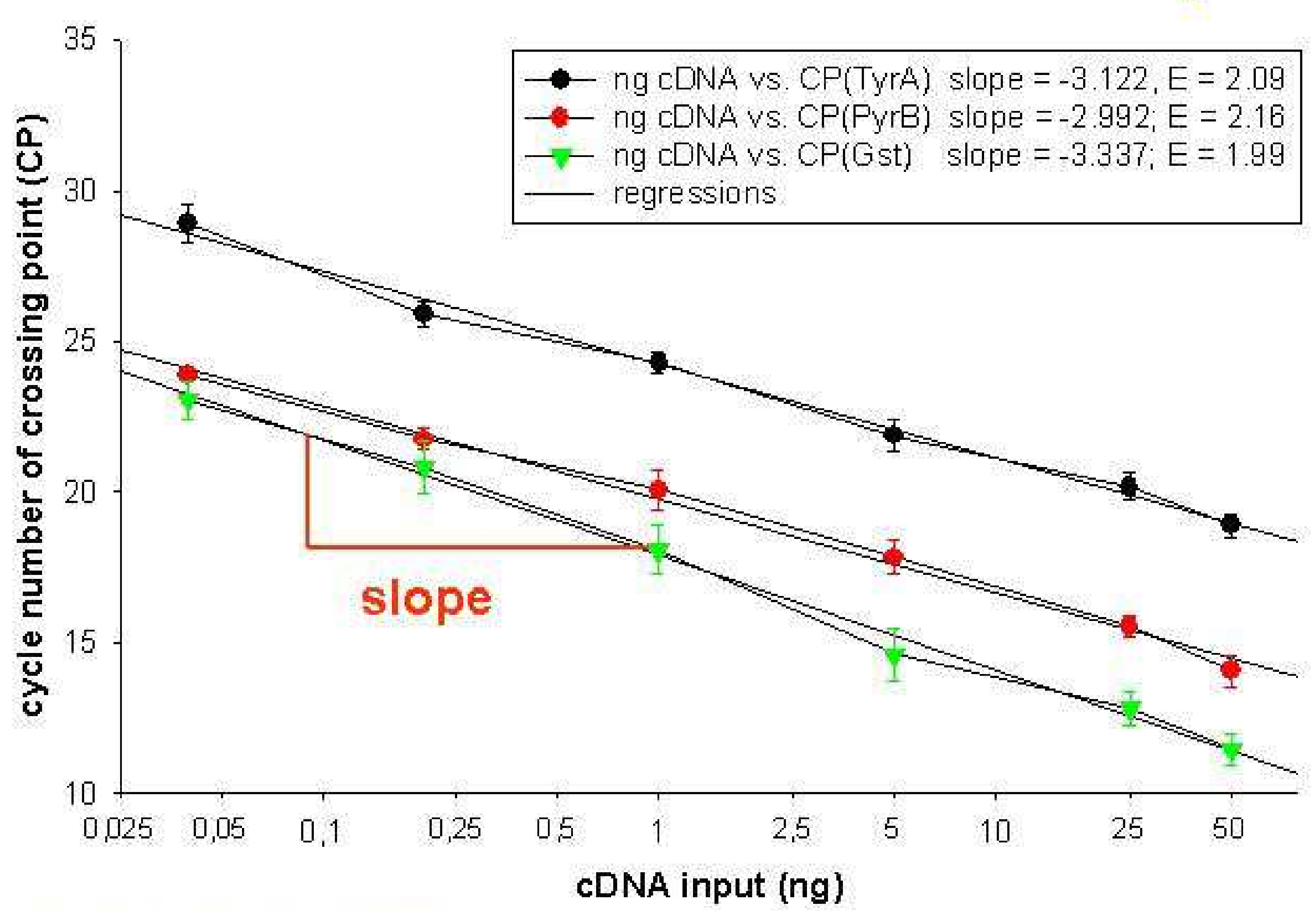
Calculation of real-time PCR efficiency



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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 must be subcloned in a vector to obtain the standard curve dilutions
- standards must be accurately quantified

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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
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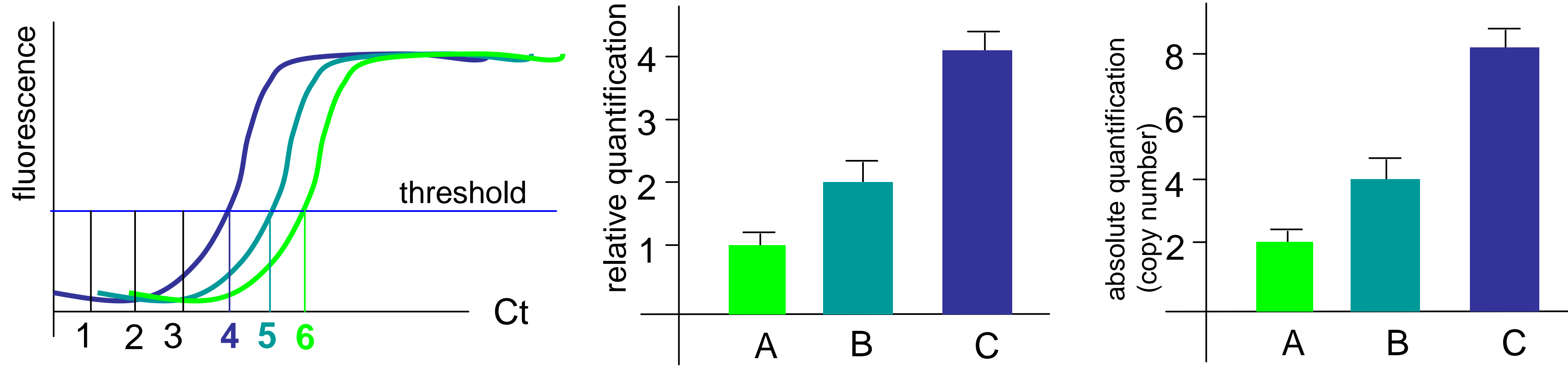

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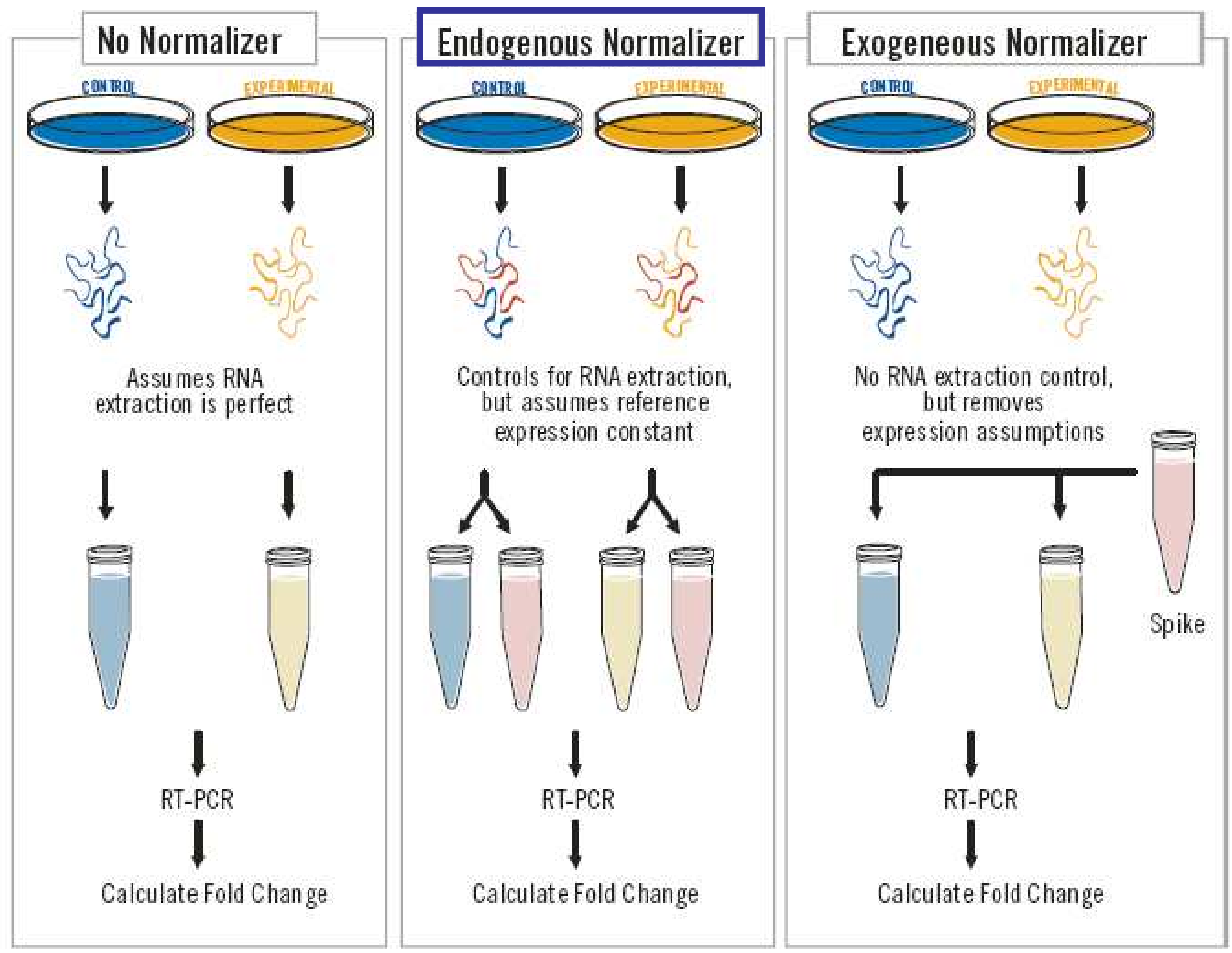

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Relative Quantification Normalized to a Reference Gene

- 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.

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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 do not have similar amplification efficiencies the Pfaffl method can be used

- 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

	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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	NRG-I/II		CT target	CT reference	Δ CT	ΔΔ CT	NRQ
	Days		NRG-I/II	HKG	CT targ - CT ref	ΔCT – ΔCT cal	$2^{-\Delta\Delta Ctc}$
4,3	**0**	**A**	30,57	20,74	9,83	0,00	1,00
4.17	**1**	**A**	28,10	20,02	8,08	-1,75	3,37
4.29	**2**	**A**	28,23	18,36	9,86	0,03	0,98
4.41	**7**	**A**	27,25	16,01	11,24	1,41	0,38
4.53	**14**	**A**	29,16	17,50	11,66	1,82	0,28
4,65	**21**	**A**	29,36	17,56	11,80	1,97	0,26
4,77	**28**	**A**	30,58	18,45	12,13	2,29	0,20
4.10	**0**	**B**	29,49	18,93	10,55	0,72	0,61
4.23	**1**	**B**	27,65	19,69	7,96	-1,88	3,67
4.35	**2**	**B**	27,97	18,15	9,82	-0,02	1,01
4.47	**7**	**B**	29,26	16,81	12,45	2,62	0,16
4.59	**14**	**B**	29,26	17,67	11,59	1,76	0,30
4,71	**21**	**B**	32,73	21,26	11,46	1,63	0,32
4,83	**28**	**B**	30,32	18,15	12,17	2,33	0,20

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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.

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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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Research

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

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

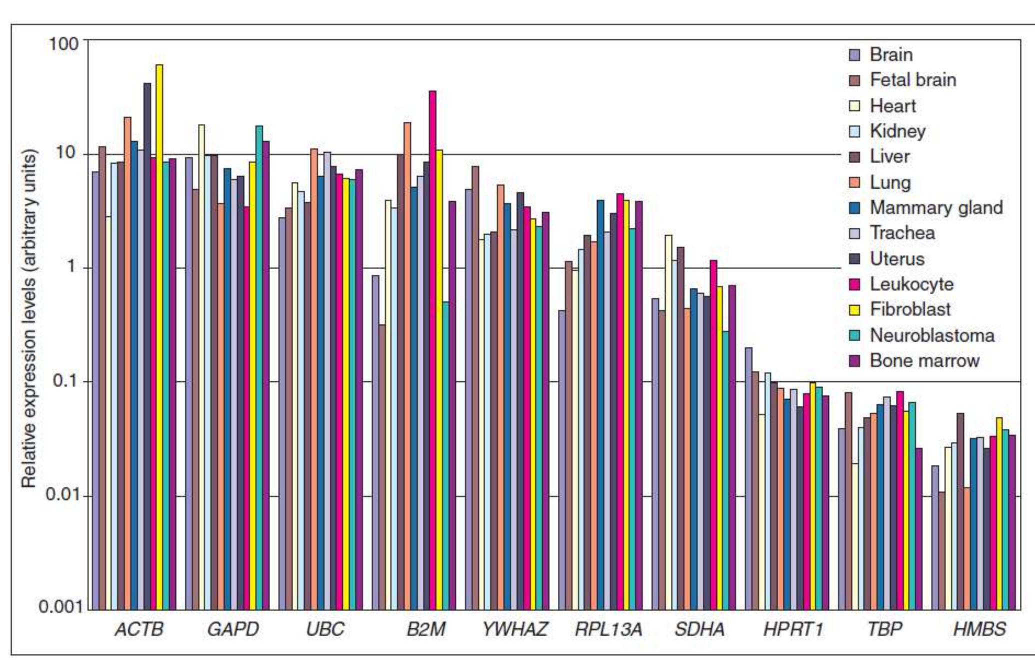

Table 3

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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Internal control genes evaluated in this study

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

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Quantification of Gene Expression after Painful Nerve Injury: Validation of Optimal Reference Genes

J Mol Neurosci
DOI 10.1007/s12031-011-9628-x

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

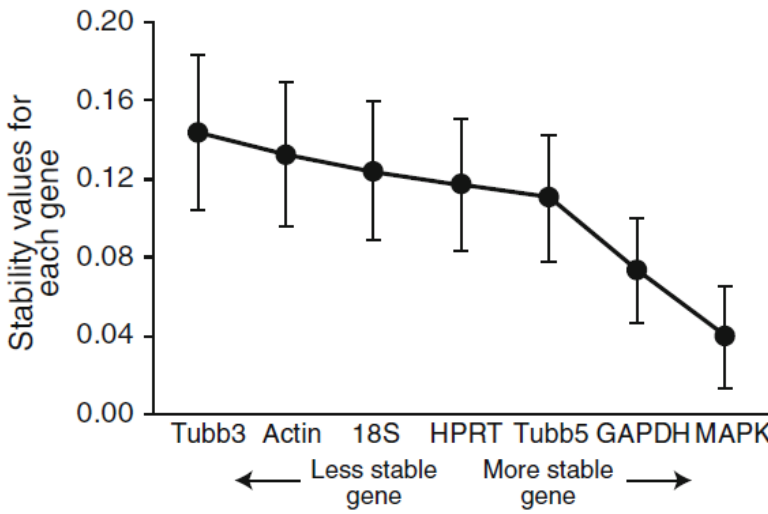

Fig. 3 NormFinder analysis for identifying the most stable HKGs, showing the seven HKGs in the sequence of their stability

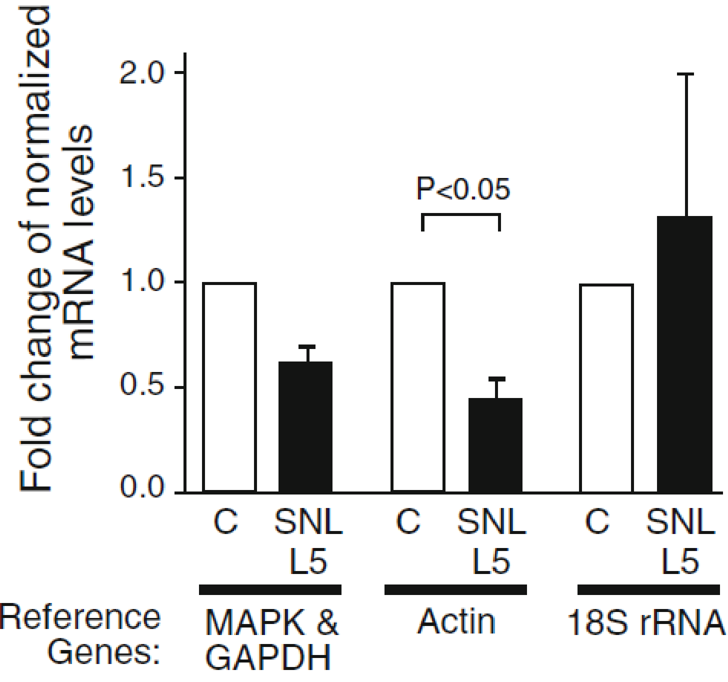

Fig. 5 Effect of reference genes on stromal interaction molecule-1 (STIM1) gene expression. The expression level of STIM1 was measured at day 7, and the fold difference was calculated between injured versus control DRG using various normalizing genes. The data represent mean ± SEM. *Brackets* represent 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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Internal control genes evaluated in this study

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 1	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

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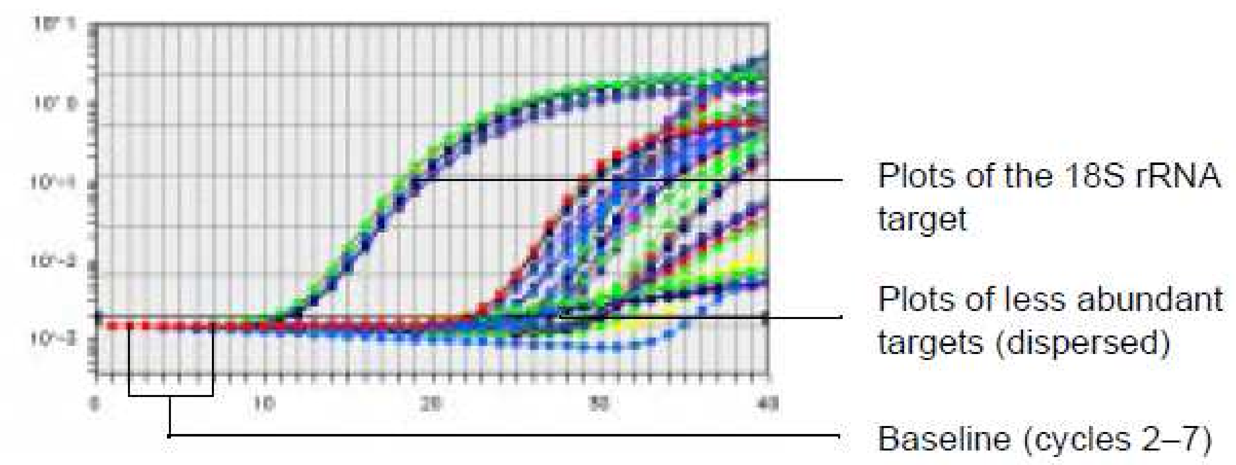
18S rRNA as a normalizer



- most abundant RNA: may need singleplex runs using diluted samples
- forces separate baseline settings in some instruments
- not mRNA
- does not have 3’ poly-A tail
- Ct value should be smaller than 22 for valid results

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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 - 150 bp

* span exon-exon junctions in cDNA

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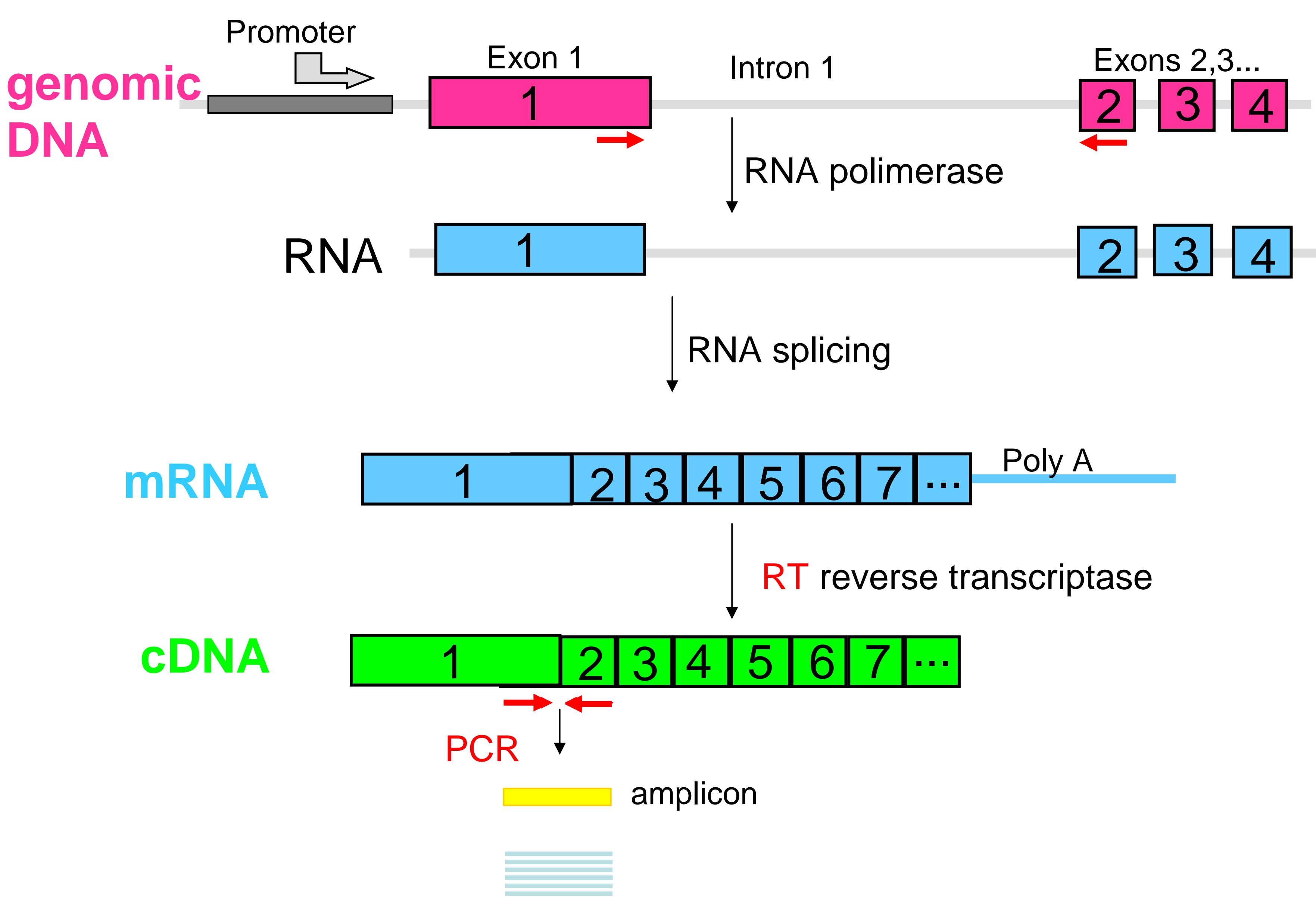

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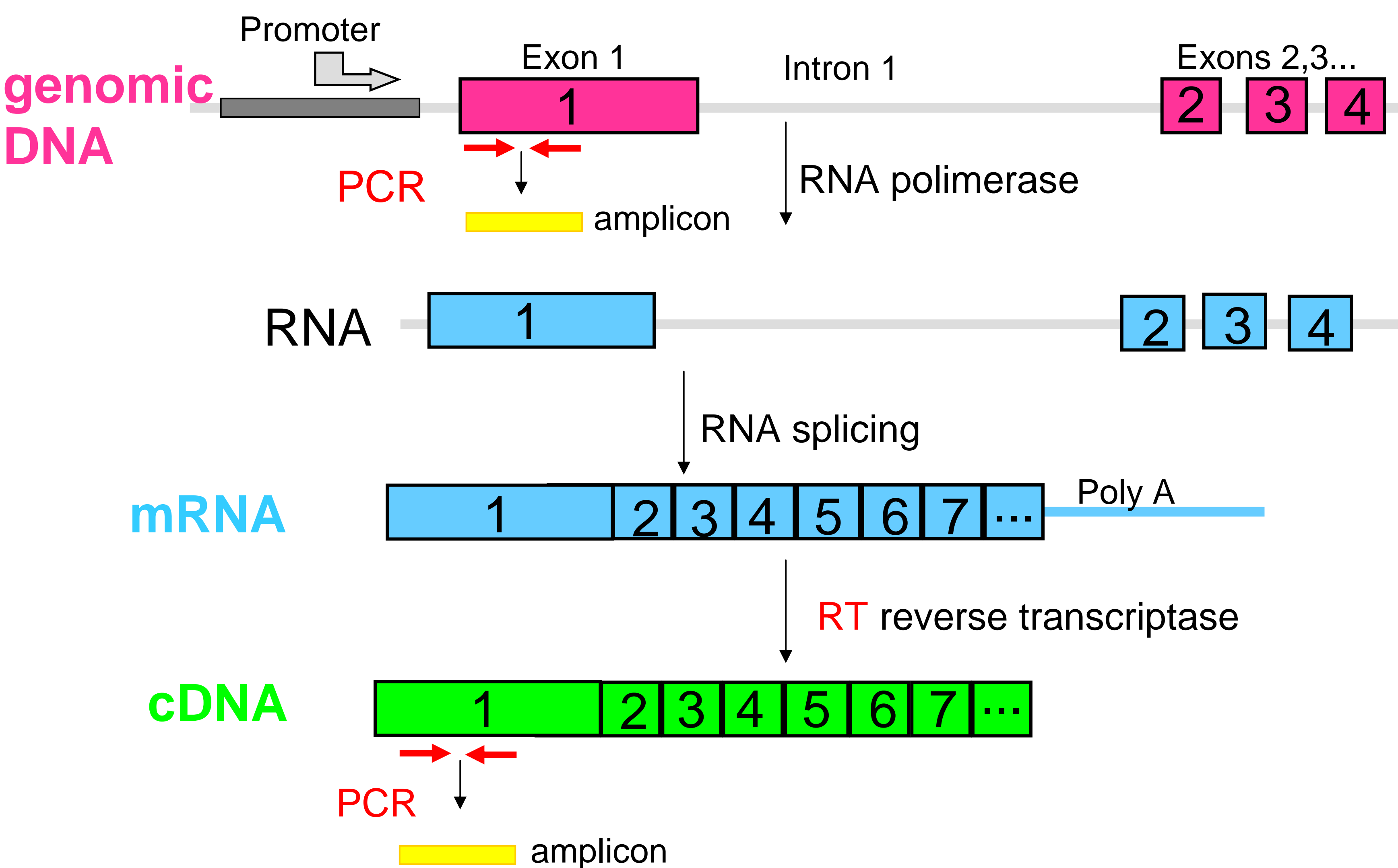

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EXON-INTRON MAP

- go to the ncbi gene bank to identify the complete coding sequence of the gene of interest

- compare the coding sequences with the genomic sequences: sequences that will anneal are exons

- reorder the annealead sequences from the first to the last exon

- calculate on the genomic sequences the intron dimensions

- choose two exons separated by an intron larger than 1000 bp

- design the primers

- check for primer dimers or primer loop

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Assay Validation

* 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** (coefficient of determination) **>0.99**,

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

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

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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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How it Works: Real Time PCR

Brendan Maher

hydrolysis	hybridization	hybridization	DNA binding
TaqMan requires a sequence-specific probe that connects fluorophore and quencher.	Molecular Beacons uses sequence specific probes that take on a hairpin structure.	Scorpions chemistry combines probe and primer. A polyermase blocker prevents unwanted replication.	SYBR Green I fluoresces only when bound to dsDNA.
* Pros: specificity, different colors can be used in multiplex assays * Cons: some background noise due to irreversibility of the reaction	* Pros: greater specificity, reversible fluorescence means lower background * Cons: some non-specific interactions of the hairpins can lead to false positives	* Pros: high specificity, faster cycling * Cons: probe/primer design is involved and pricey	* Pros: relatively cheap, doesn't require probe design * Cons: nonspecificity can lead to false positives, not attuned for complex protocols
TaqMan	Molecular Beacon	Scorpions	Sybr Green I

(www)

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Principles of Real-Time Detection Chemistry

(a) SYBR Green technique: SYBR Green fluorescence is enormously increased upon binding to double-stranded DNA. During the extension phase, more and more SYBR Green will bind to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal will be detected.

(b) Hydrolysis probe technique: The hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the Taq polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.

(c) Hybridization probes technique: In this technique one probe is labelled with a donor fluorochrome at the 3′ end and a second –adjacent- probe is labelled with an acceptor fluorochrome. When the two fluorochromes are in close vicinity (1–5 nucleotides apart), the emitted light of the donor fluorochrome will excite the acceptor fluorochrome (FRET). This results in the emission of fluorescence, which subsequently can be detected during the annealing phase and first part of the extension phase of the PCR reaction. After each subsequent PCR cycle more hybridization probes can anneal, resulting in higher fluorescence signals.

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SYBR Green
(double-stranded DNA binding dye)

* emits a strong fluorescent signal upon binding to double-stranded DNA

* non-specific binding is a disadvantage

* requires extensive optimization

* requires melting curve analysis to ensure specificity

* longer amplicons create a stronger signal

* may be multiplexed when coupled with melting curve analysis

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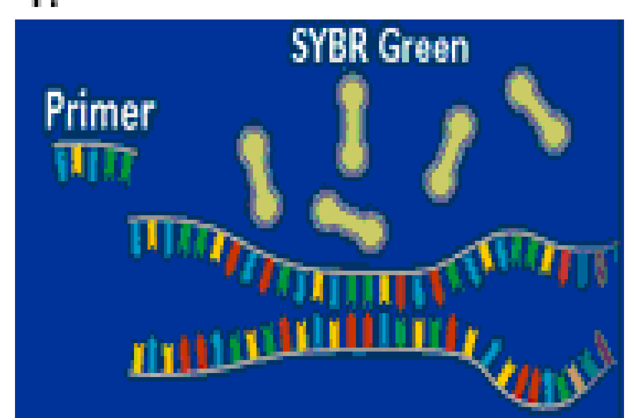

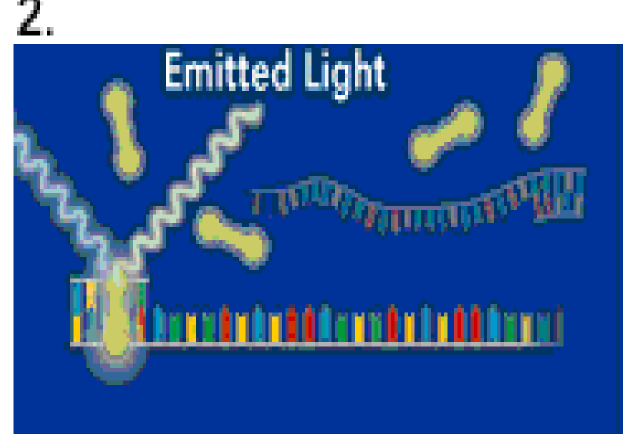

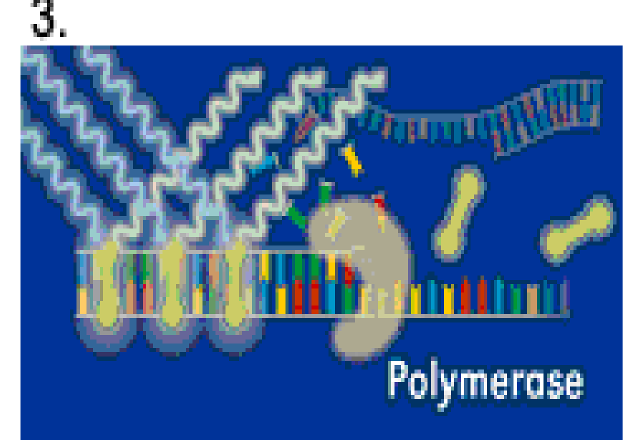

1- at the beginning of amplification, the reaction mixture contains the denatured DNA, the primers and the SYBR Green. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis.

2- after annealing of the primers, a few dye molecules can bind to the double strand. DNA binding results in a dramatic increase of the SYBR Green molecules to emit light upon excitation.

3- during elongation, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.

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When to Choose SYBR Green

* assays that do not require specificity of probe based assays.

* detection of 1000s of molecules

* general screening of transcripts prior to moving to probe based assays

* when the PCR system is fully optimized

* no primer dimers or non-specific amplicons

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When Not to Choose SYBR Green

* allelic discrimination assays

* multiplex reactions

* amplification of rare transcripts

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The highest risk in PCR is the risk of contamination

- it is important to have two separate laboratory rooms for real time and DNA manipulation
- use pipettes and reagents dedicated only to PCR
- change gloves when you go to the PCR room
- check everytime for contamination by introducing negative controls in your reactions
- after the PCR reaction, discard the plate in a different room
- run the agarose gel in a different room (in the conventional PCR)
- never introduce plasmidic DNA in the PCR room!!!!

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