

REAL TIME PCR SIMPLIFIED

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$$X = X_0 (1 + E)^n$$

REAL TIME PCR DATA ?

NUMERICAL DATA !

→ ANALYTICAL EVALUATION
(precision, limit of detection ...)

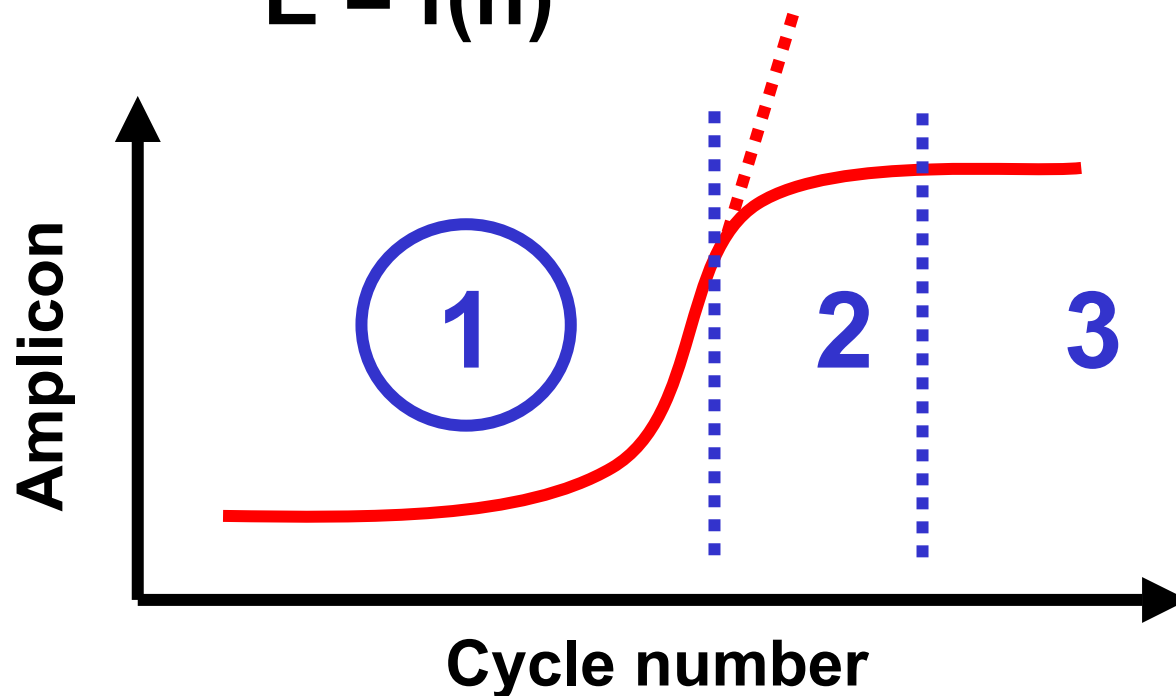
OVERVIEW ...

REAL TIME PCR = PCR

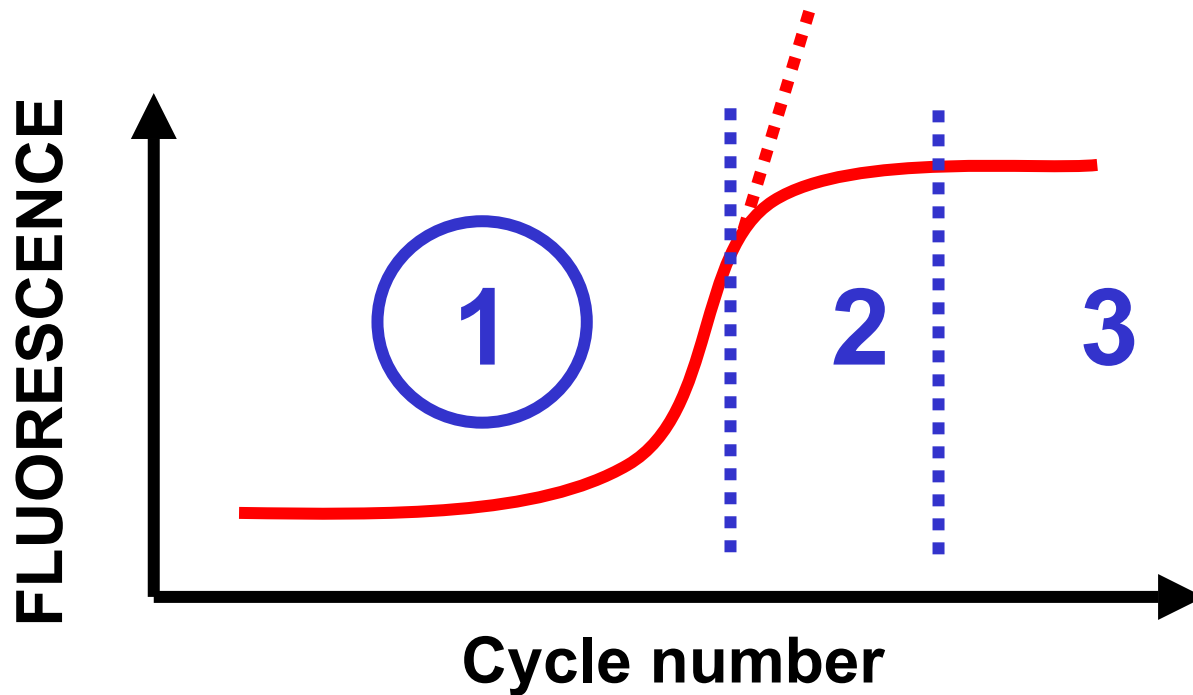
$$X = X_0 (1 + E)^n$$

$$0 < E < 1$$

$$E = f(n)$$

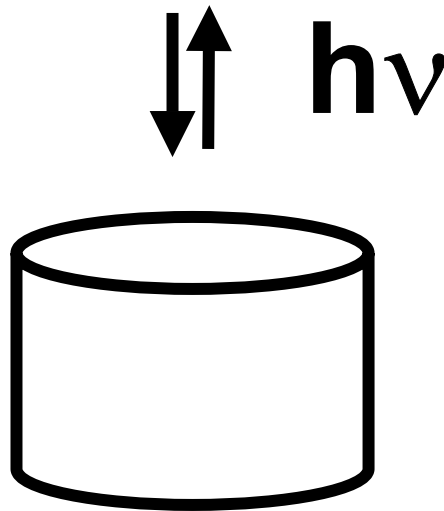


**REAL TIME PCR =
PCR + FLUORESCENCE MEASUREMENT**



FLUORESCENCE = k [AMPLICON]

**REAL TIME PCR =
PCR WITHOUT POST-PCR**

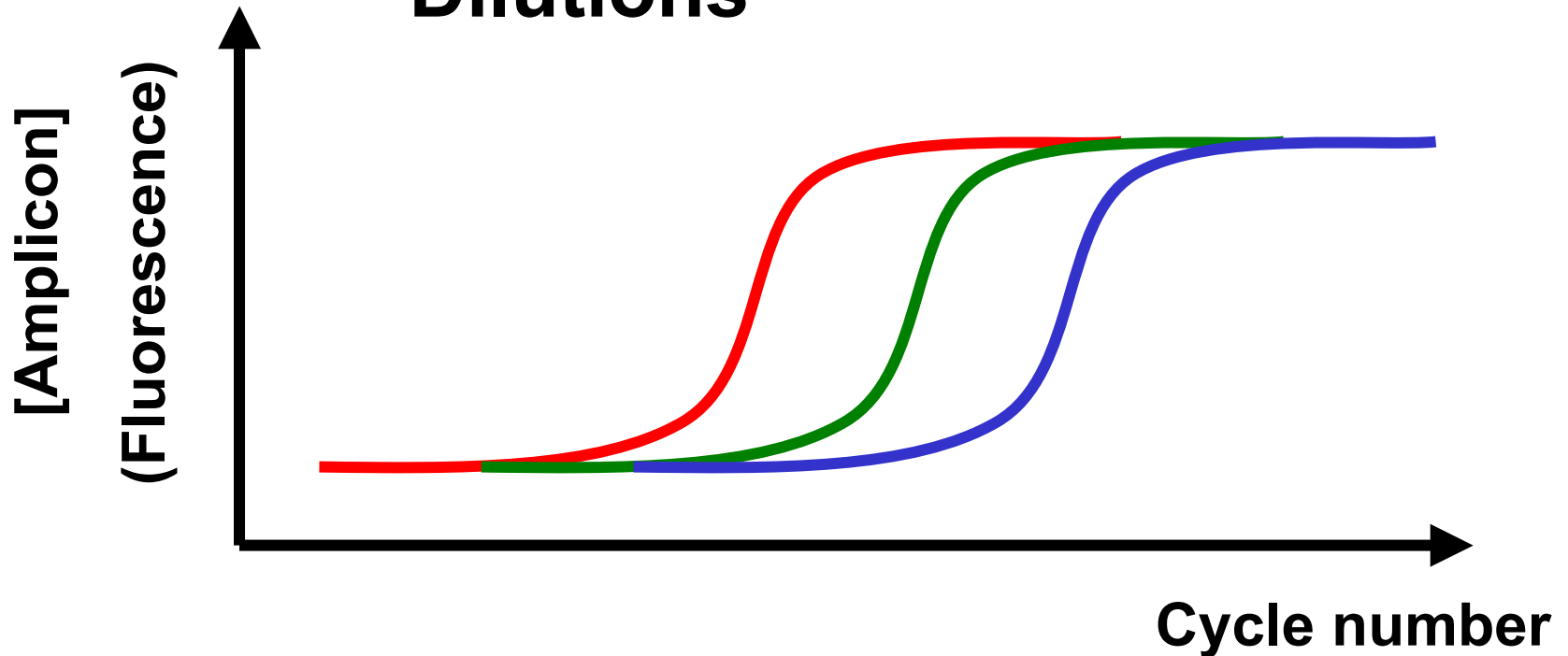


AUTOMATION

REAL TIME PCR = QUANTITATIVE PCR

[DNA]₁ → **[DNA]₂** → **[DNA]₃**

Dilutions



COMPARISON

PCR

1st ORDER KINETICS

FIRST ORDER KINETICS :

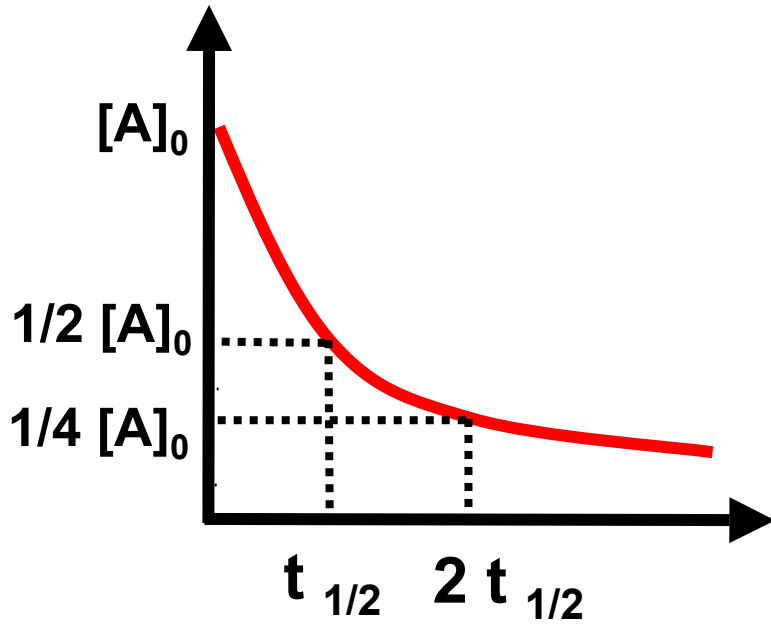


$$d[A] / dt = - k [A]$$

$$\ln ([A]_t / [A]_0) = - k t$$

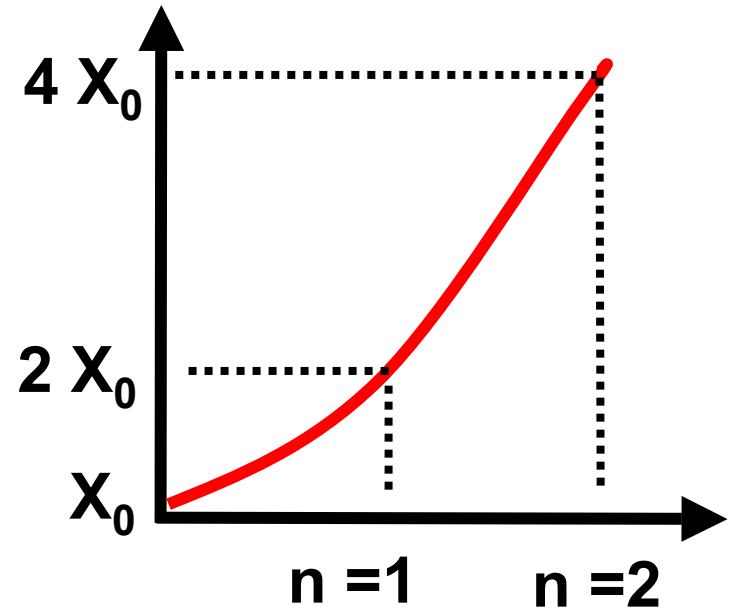
$$[A]_t = [A]_0 e^{-k t}$$

$$X = X_0 (1 + E)^n$$



$$[A]_t = [A]_0 e^{-k t}$$

$$t_{1/2} = 0.6932 / k$$



$$X = X_0 (1 + E)^n$$

$$n_2 = 0.3010 / \log (1 + E)$$

$$n_2 = 0.3010 / \log (1 + E)$$

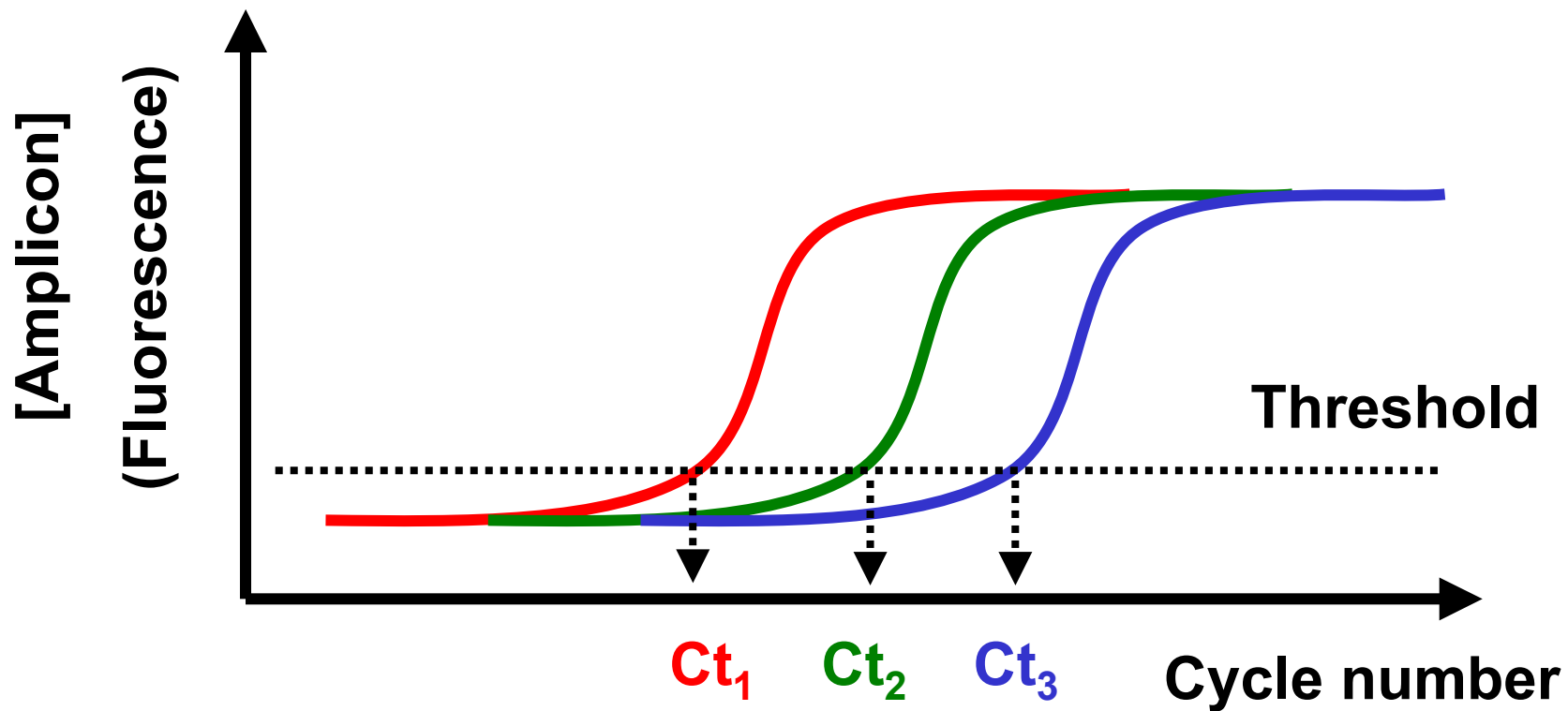
E	n₂
1.0	1.0
0.9	1.1
0.8	1.2

PCR EFFICIENCIES ?

Ct REVOLUTION

[DNA]₁ → **[DNA]₂** → **[DNA]₃**

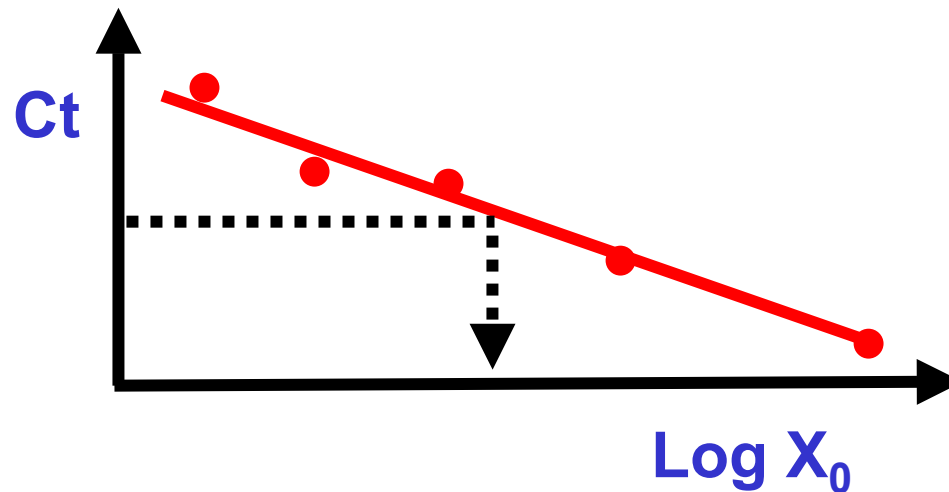
Dilutions



$$X = X_0 (1 + E)^n$$

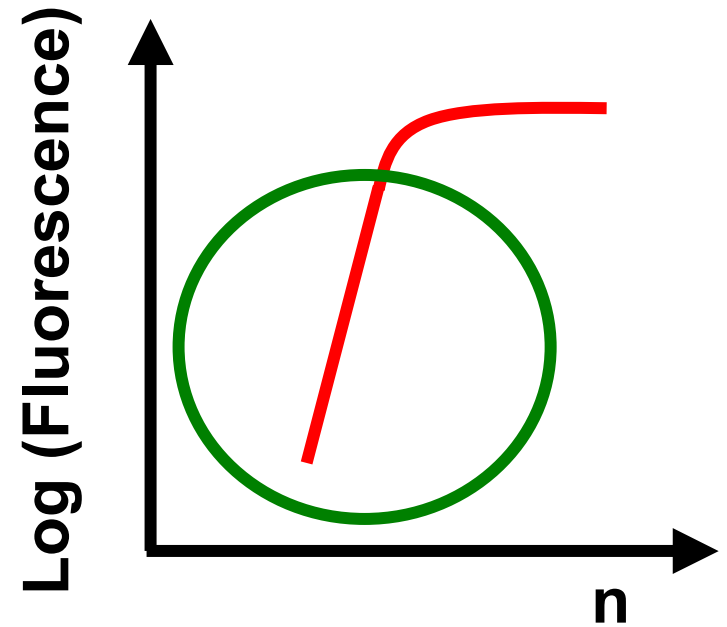
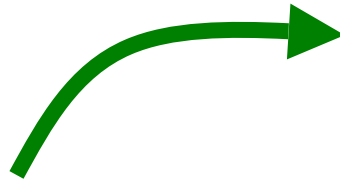
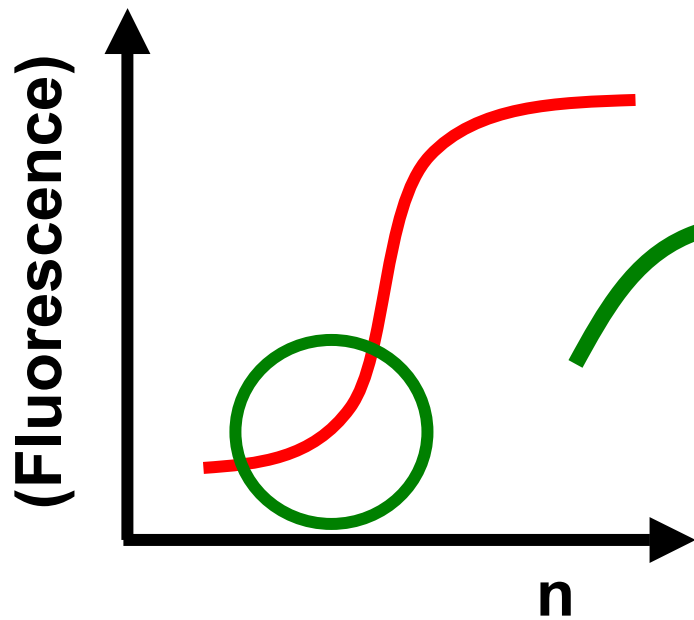
IF $X = \text{THRESHOLD}$, $n = C_t$

$$C_t = \text{Log } X / \text{Log } (1+E) - \text{Log } X_0 / \text{Log } (1+E)$$



ASSUMPTION :

PCR EFFICIENCY = CONSTANT



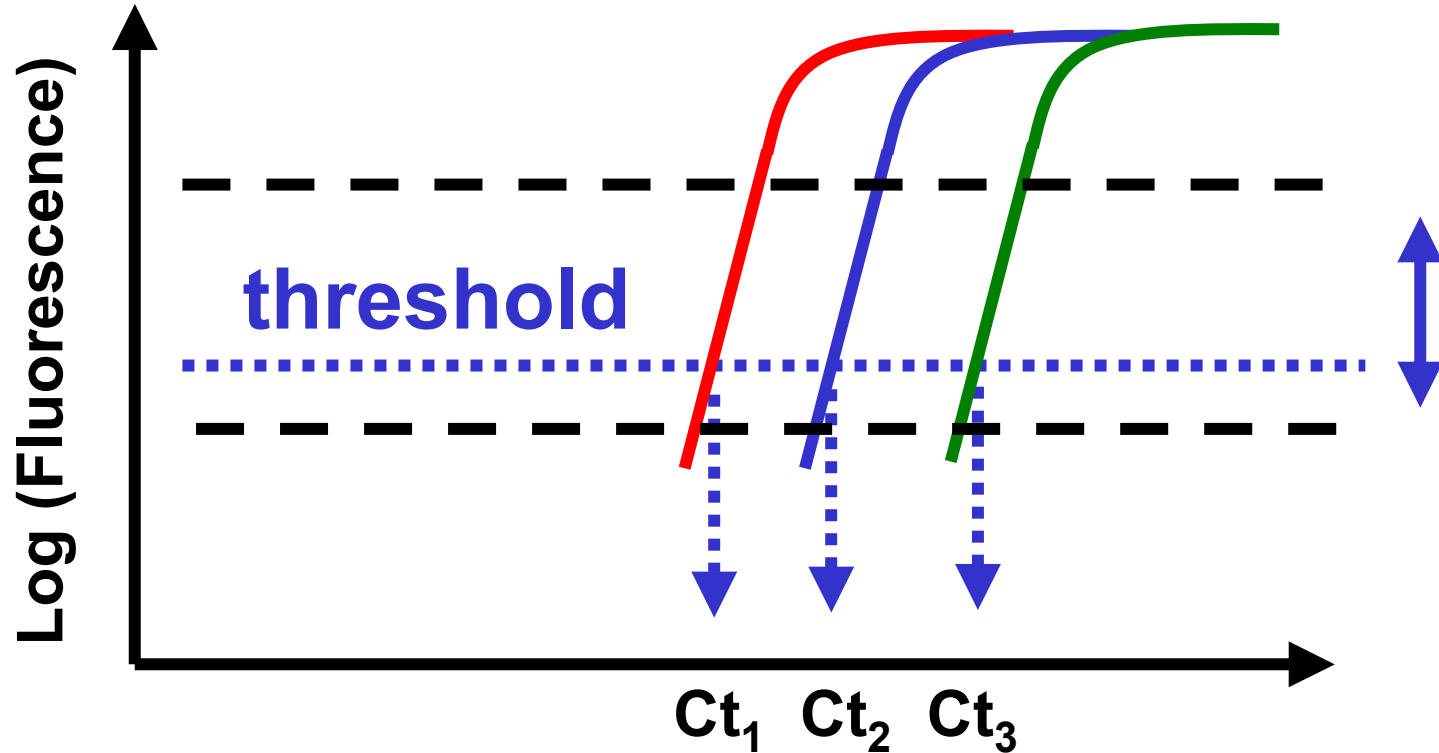
$$X = X_0 (1 + E)^n$$

$$\text{Log } X = \text{Log } X_0 + n \text{ Log } (1 + E)$$

Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions.

R. Higuchi *et al.*

Biotechnology (1993) 11, 1026-1030



Ct1 = 26.66

Ct2 = 27.07

Ct3 = 26.90

PCR IN TRIPLICATE ...

→ $\bar{Ct} = 26.88 \pm 0.21$

$\bar{Ct} = 26.9 \pm 0.2$ ($\pm S, n = 3$)

PRECISION ON Ct ?

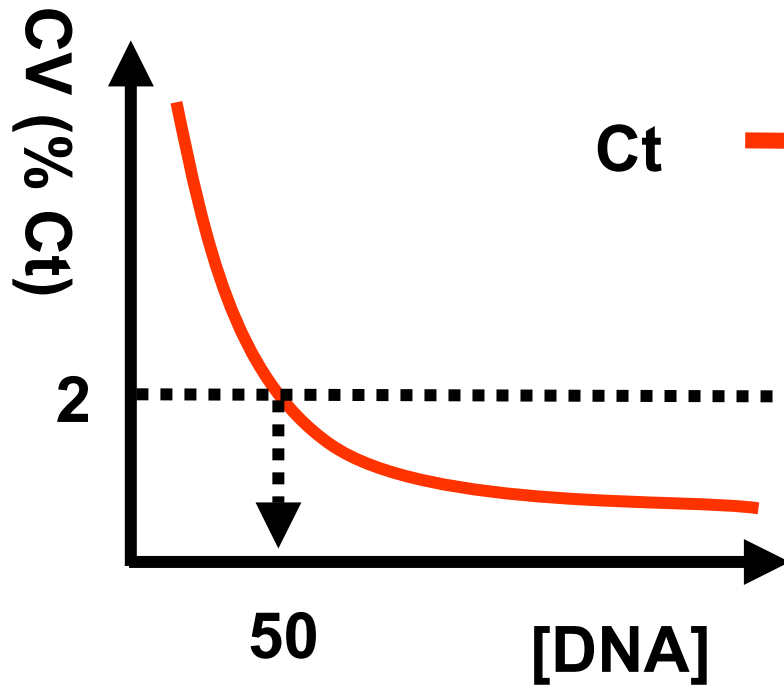
$$\text{Confidence interval} = \bar{Ct} \pm \frac{t S}{\sqrt{n}}$$

$$26.9 \pm 0.5 \text{ (95\%)}$$

$$\text{Confidence interval} = \bar{Ct} \pm \frac{Z \sigma}{\sqrt{n}}$$

$$26.9 \pm 0.2 \text{ (95\%)}$$

PRECISION

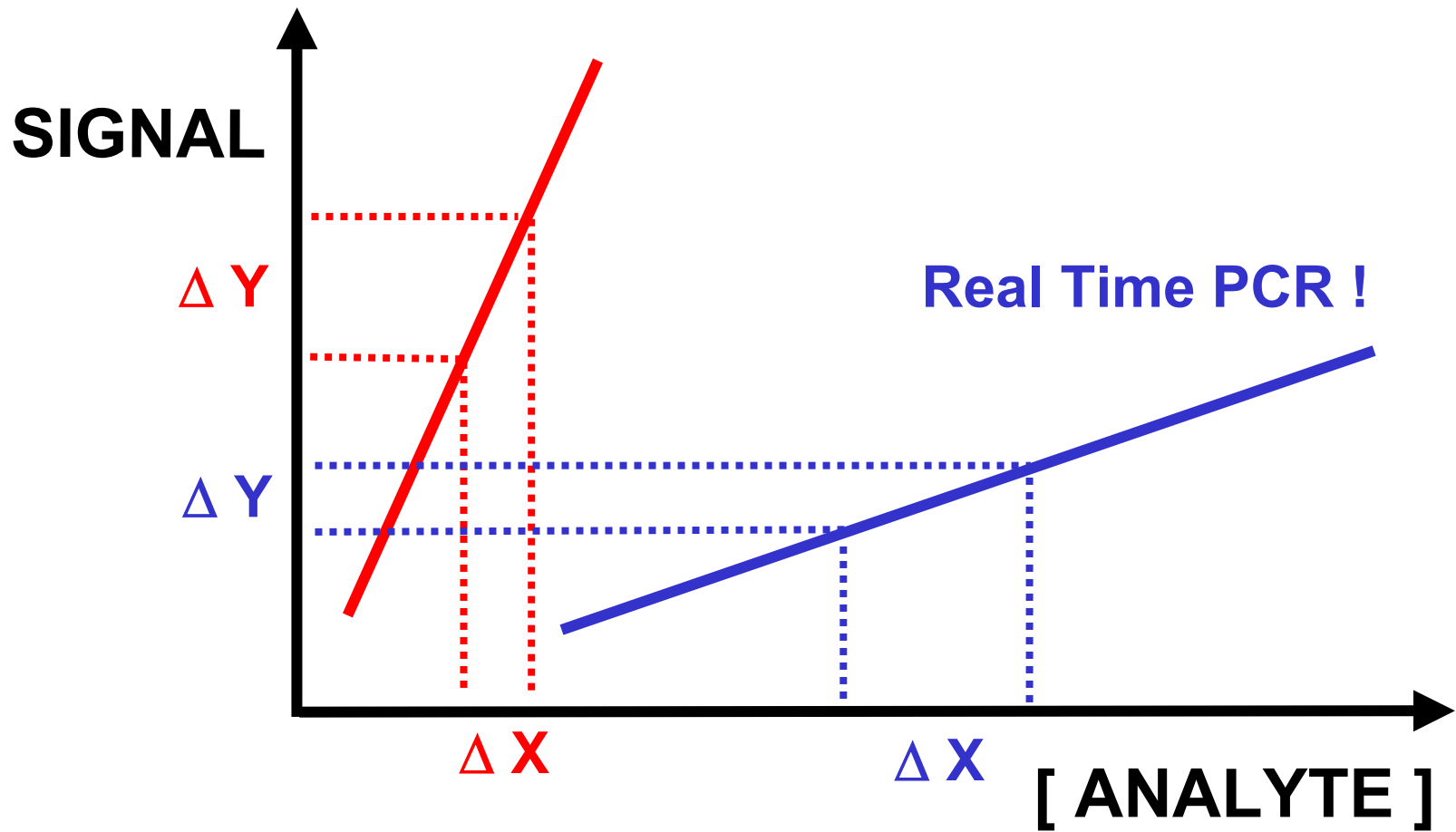


Ct → COPY NUMBER

$$Ct = a \text{ Log } [ADN] + b$$

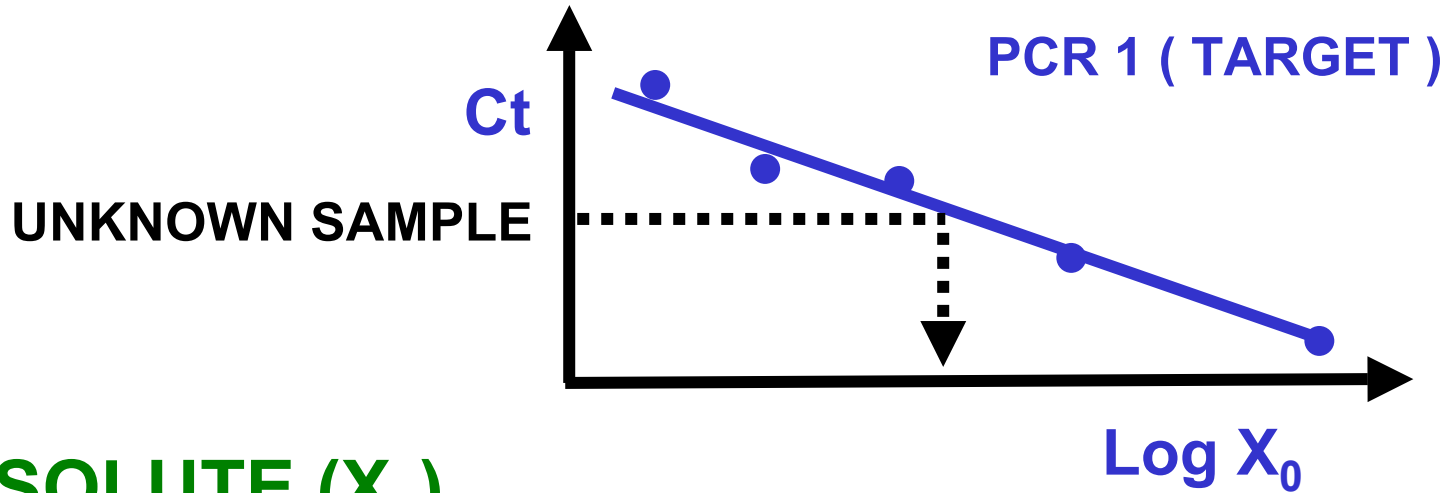
(Copy Number)

Analytical sensitivity $\frac{\Delta Y}{\Delta X}$



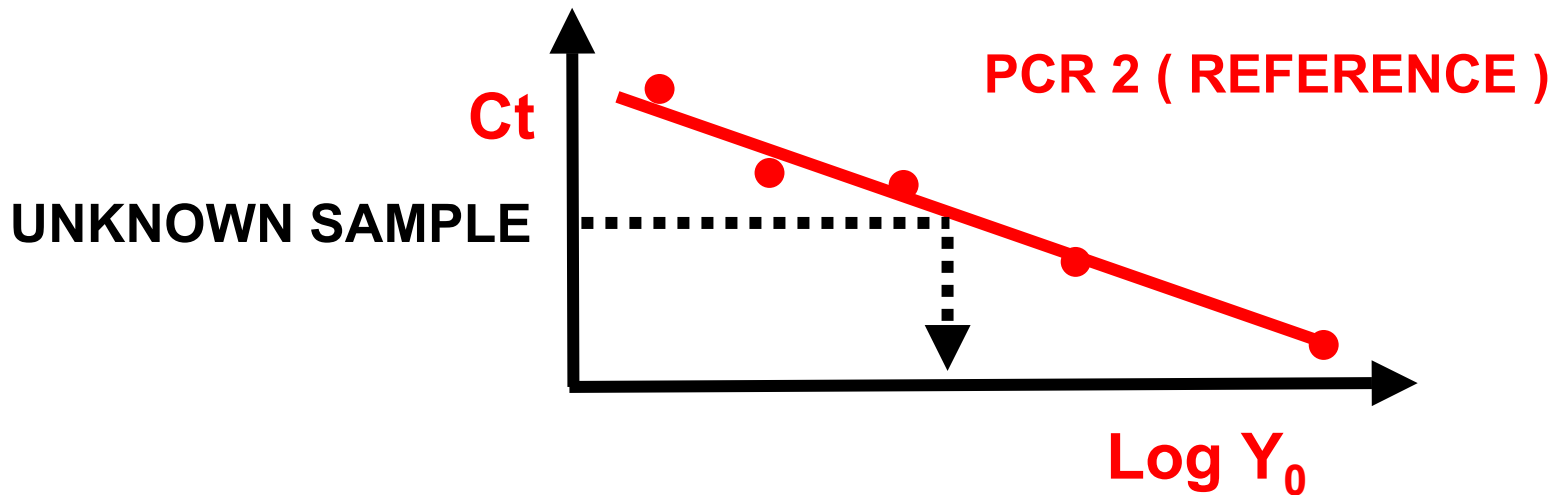
« **TWOFOLD** »

QUANTIFICATION



ABSOLUTE (X₀)

RELATIVE (X₀ / Y₀)



ALL QANTIFICATION IS RELATIVE

ABSOLUTE QUANTIFICATION ?



Accuracy of external standards

Calibration of the standards ?

QUALITY OF QUANTITATIVE DATA ?

not better than the quality of the denominator



ARTEFACTUAL CHANGES !

CAREFUL USE OF CONTROLS !

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

J. Vandesompele *et al.*

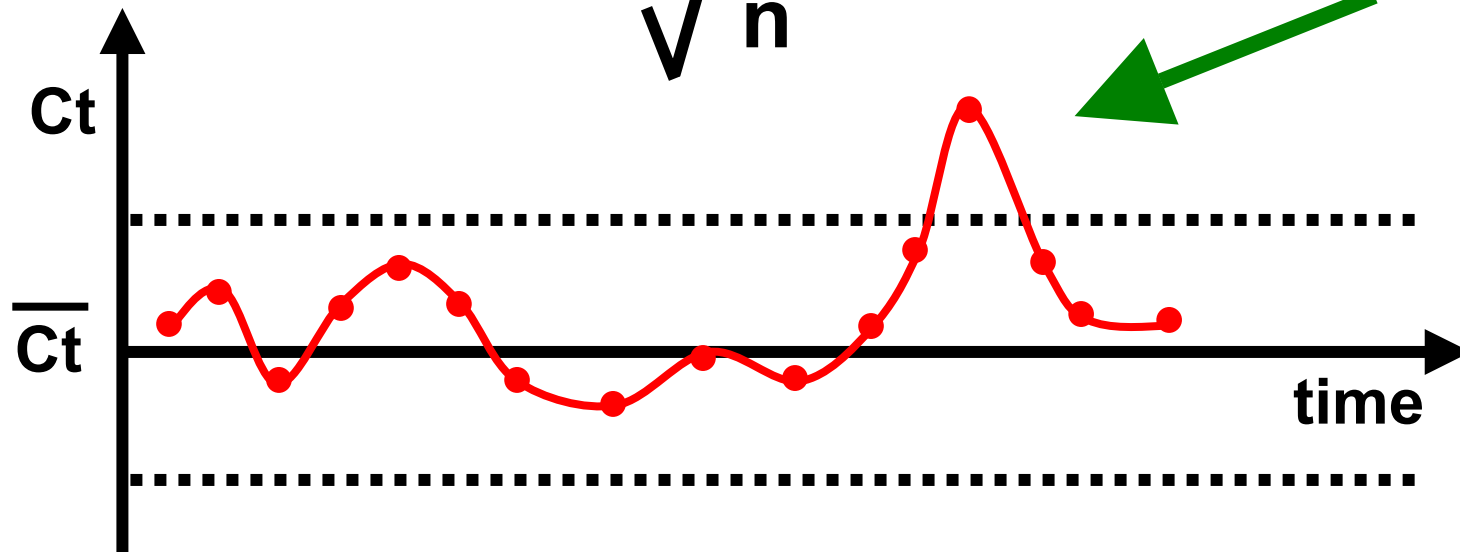
Genome Biol. (2002) 3, 0034.1-0034.11

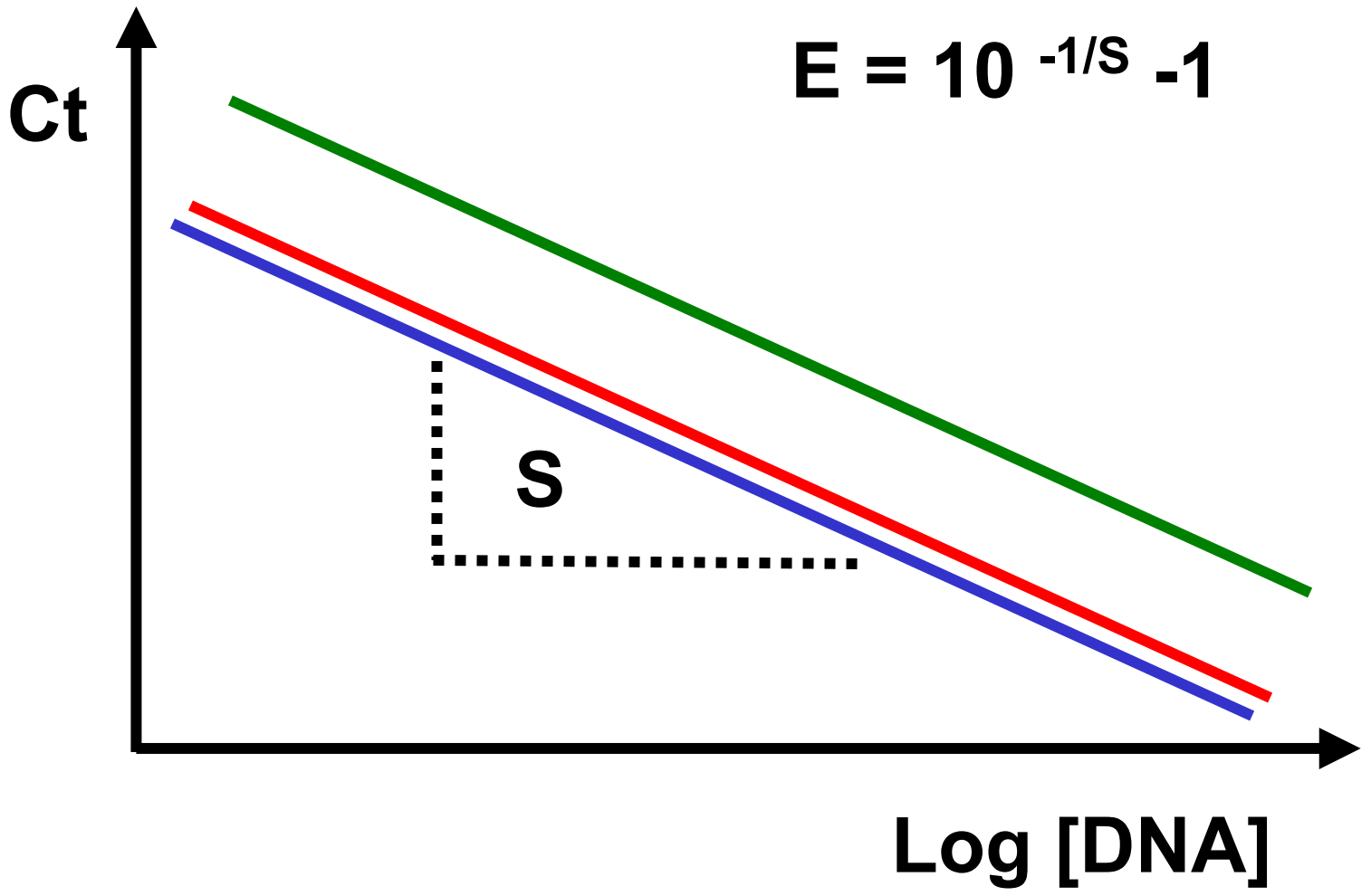
CALIBRATION CURVES ?

SINGLE + CONTROL

(CONTROL CHART)

$$\bar{C}_t \pm \frac{Z \sigma}{\sqrt{n}}$$





$$Ct = -3.6 \text{ Log } (X_0) + 37.1$$

Precision in slope (relative deviation) = 2 - 3 %

Derived result : $E = 10^{-1 / \text{slope} - 1}$

Slope = - 3.5  $E = 0.93$

Precision in E (relative deviation) = 3 %

 $\Delta E > 0.1 (> 10\%)$

Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C_t}$ Method

K.J. Livak *et al.*

Methods 25, 402-408 (2001)

Unknown

target X_0 reference R_0

$$X_0 / R_0 = K (1 + E)^{-\Delta C_t}$$

calibrator

target X_0 reference R_0

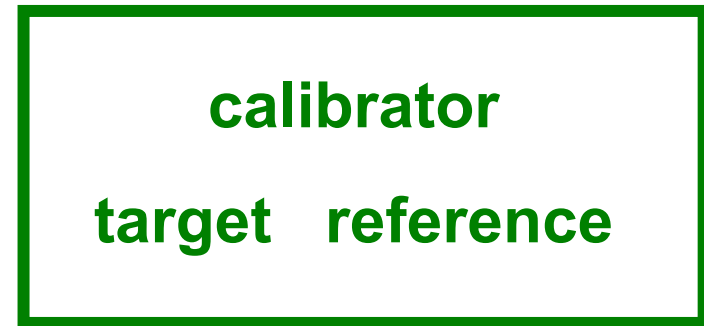
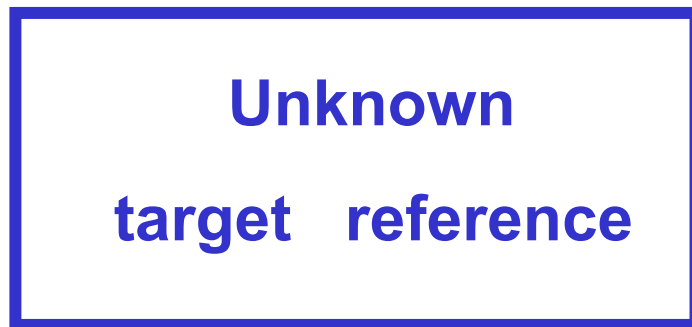
$$X_0 / R_0 = K (1 + E)^{-\Delta C_t}$$

Relative to calibrator : $N = 2^{-\Delta\Delta C_t}$

A new mathematical model for relative quantification in real-time RT-PCR

M. W. Pfaffl

Nucleic Acids Research, 2001, 29, 9



$$N = \frac{(1 + E_t)^{\Delta Ct}}{(1 + E_r)^{\Delta Ct}}$$

$$\Delta Ct = (Ct \text{ calibrator} - Ct \text{ unknown})$$

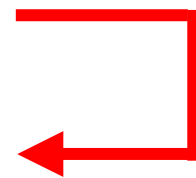
ΔE

E	X
0.5	2.5E+7
0.6	1.3E+8
0.7	5.8E+8
0.8	2.4E+9
0.9	9.3E+9
1.0	3.4E+10

IF n = 25

AND

$X_0 = 1000$



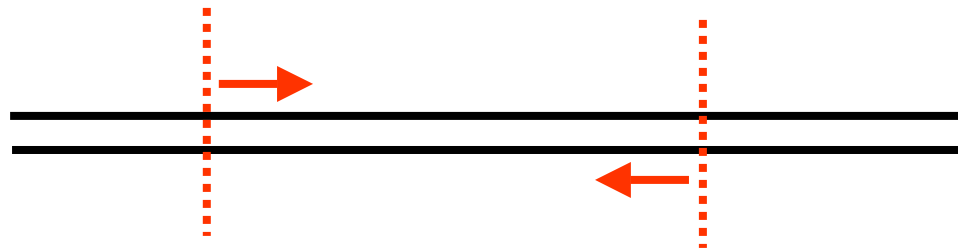
4 X

ANALYTICAL VALIDATION

SPECIFICITY

PCR SPECIFICITY = CRICK - WATSON

PRIMER DESIGN



OPTIONS

SYBR Green I

SPECIFIC FLUORESCENT PROBES

SYBR Green I

ADVANTAGE = BINDS TO ANY dsDNA

DISAVANTAGE = BINDS TO ANY dsDNA

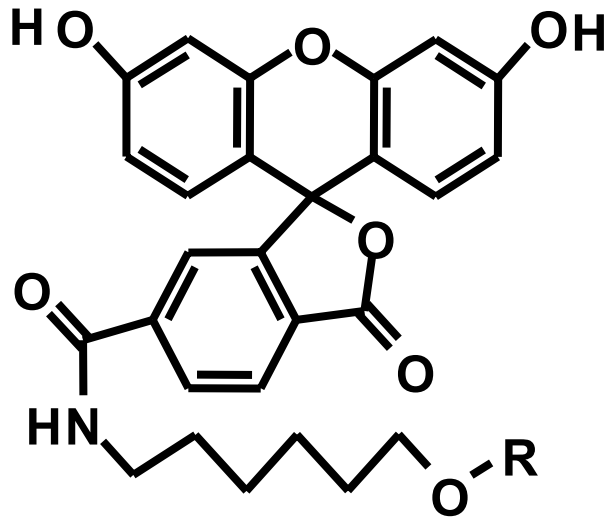


PRIMER DIMERS

Careful optimization

Melting curve analysis

SEQUENCE SPECIFIC RECOGNITION



5' - OLIGONUCLEOTIDE PROBE - 3'

CHEMICAL SYNTHESIS, PURIFICATION (HPLC)

AS FAR AS THE EYE CAN SEE ...

REAL TIME PCR :

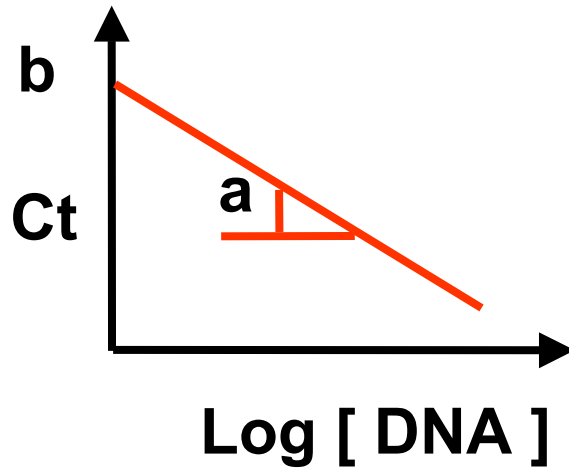
ALL THE SAME ?

ALL DIFFERENT ?

UNIVERSAL MASTER MIX ?

UNIVERSAL CYCLING CONDITIONS ?

LINEARITY



FROM 1 TO $1 \cdot 10^6$
USEFULNESS ?

DEVIATION FROM LINEARITY :

[DNA] \searrow (< 50 COPIES)

[DNA] \nearrow (INHIBITORS)

INHIBITIONS



$$\Delta Ct = 3.3$$

Target cDNA	ABL	MPO
Ct (25 ng)	25.9	22.7
Ct (2.5 ng)	25.1	25.0
ΔCt	- 0.9	2.3

ACCURACY

Absolute error

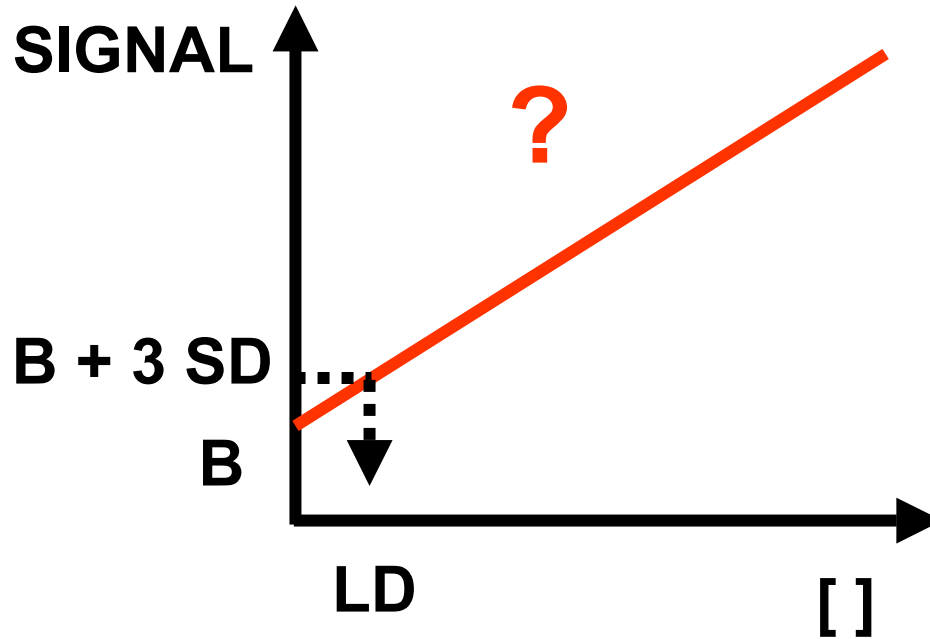
Relative error

STANDARDS !

Synthesis of standards

Calibration of the standards

LOD

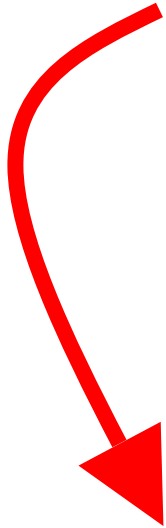
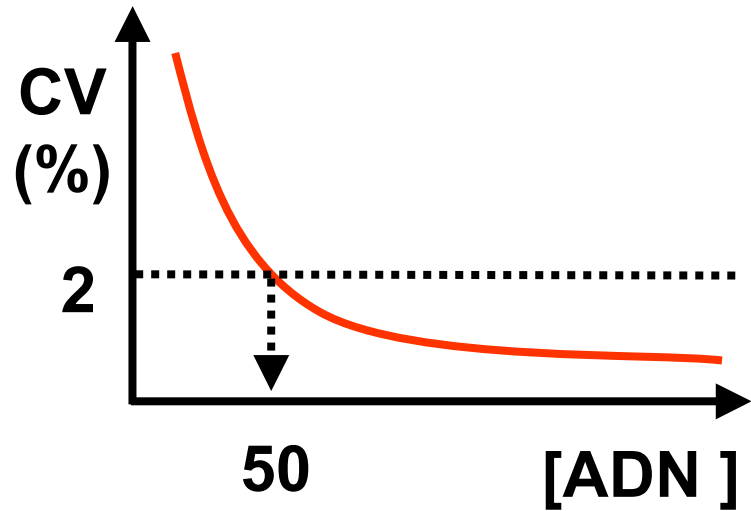
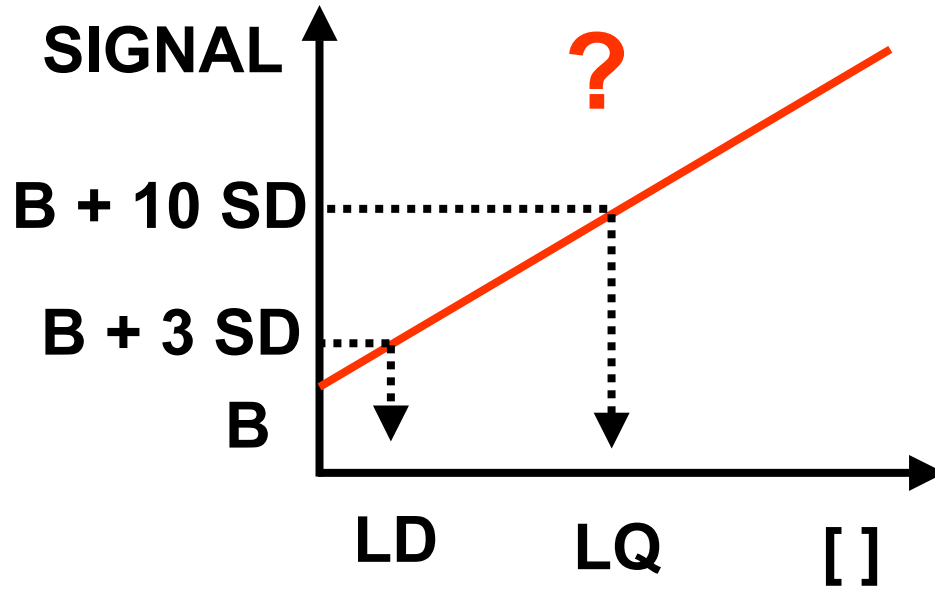


NO NOISE !

1 (10) MOLECULE(S) = Amplification

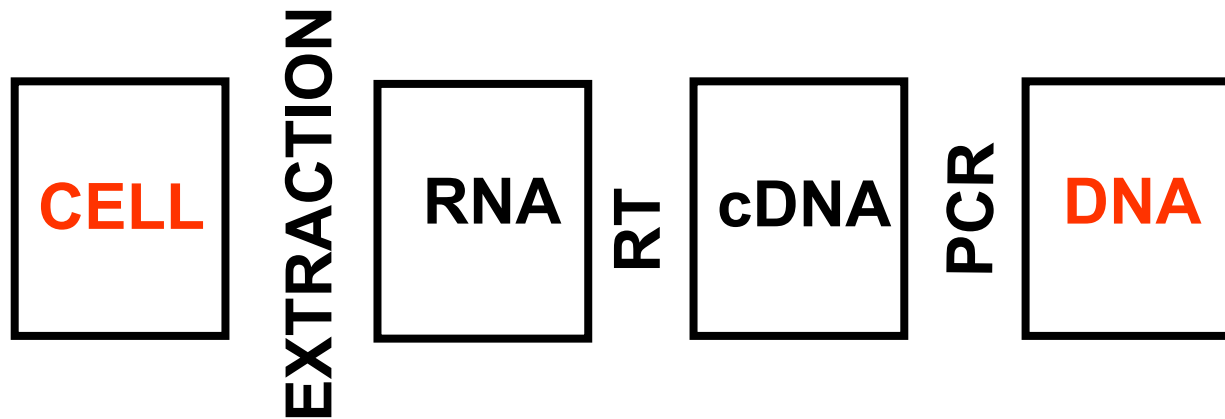
0 MOLECULE = No amplification

LOQ

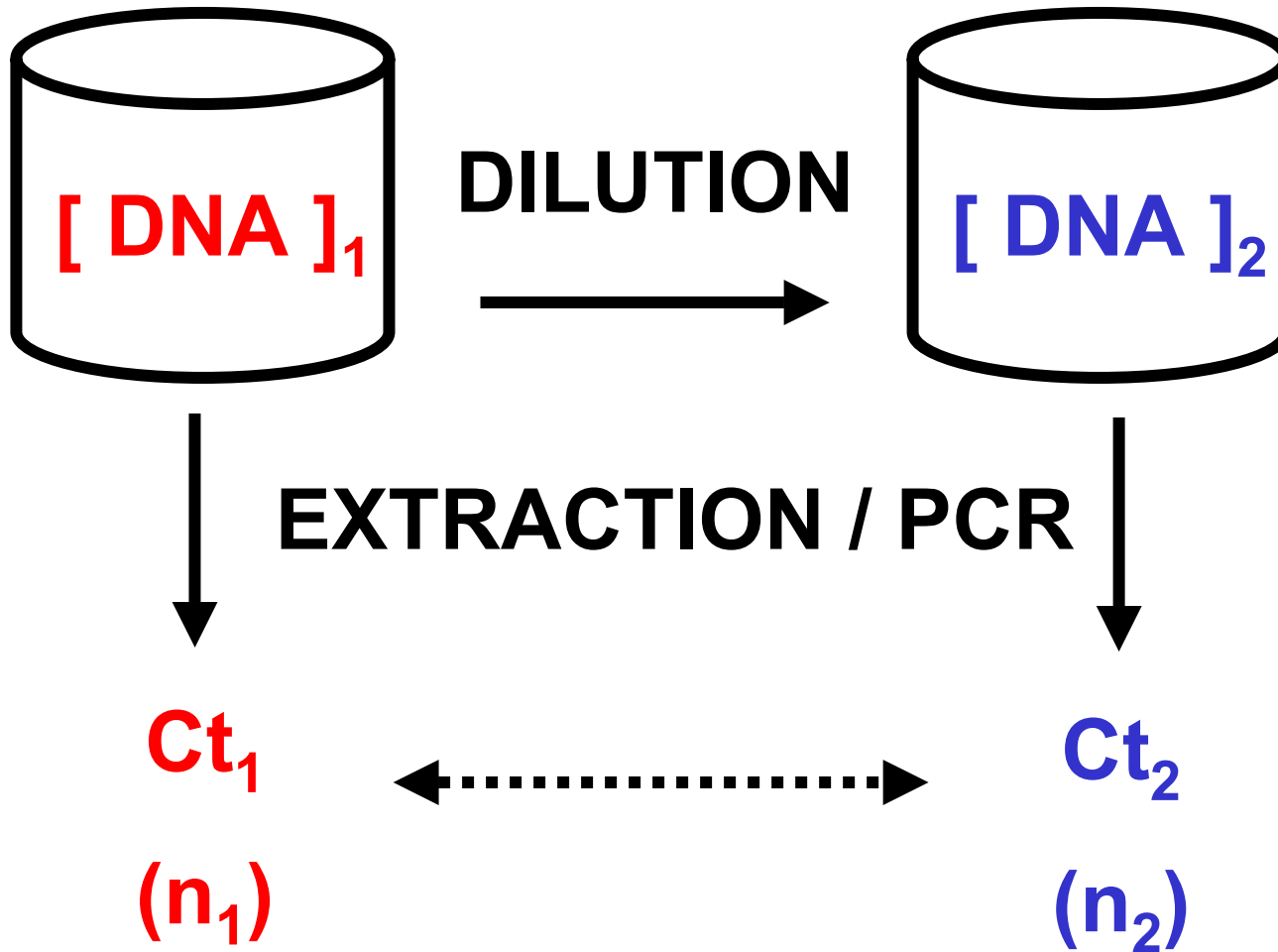


ANALYTICAL PROCEDURE :

RT - RTQ PCR



HOW TO DESIGN RTQ PCR EXPERIMENTS ?



t - TEST METHOD

IF [DNA]₁ < > [DNA]₂

THEN $|\bar{C}t_2 - \bar{C}t_1| > t S_g \sqrt{\frac{n_1 + n_2}{n_1 n_2}}$

CONFIDENCE INTERVAL

$$IC_1 = \bar{Ct}_1 \pm \frac{Z \sigma}{\sqrt{n_1}} \quad IC_2 = \bar{Ct}_2 \pm \frac{Z \sigma}{\sqrt{n_2}}$$

IF $[ADN]_1 < > [ADN]_2$

THEN NO OVERLAP OF IC_1 AND IC_2

PCR

IN DUPLICATE

Replicate	Dilution 1	Dilution 2
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1	26.1	27.9
2	25.9	28.0
3	26.1	28.1

1

Combination	Mean	Dilution 1	Dilution 2
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1	1+2	26.0	28.0
2	1+3	26.1	28.0
3	2+3	26.0	28.0

2

All Δ Ct Combinations (9)

3



Real Time PCR

2X differences (< 100 copies/PCR)

3X PCR

10X differences (< 100 copies/PCR)

1X PCR



Extraction and Real Time PCR

2X differences

4X extraction + 3X PCR

8X differences

1X extraction + 3X PCR

$$X = X_0 (1 + E)^n$$