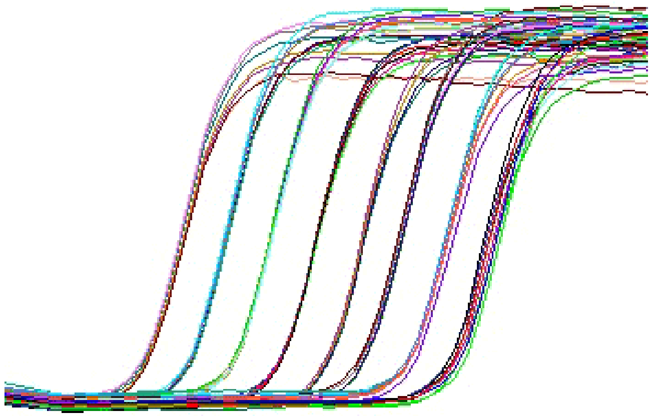
Real Time PCR

=

Quantitative PCR (qPCR)

(RT-qPCR = reverse-transcription – quantitative PCR)

Michał Koper, IGiB UW



Pioneering work on qPCR
(also known as kinetic PCR)

- **Higuchi R., Dollinger G., Walsh P.S., Griffith R. (1992). Simultaneous amplification and detection of specific DNA sequences. Biotechnology 10:413-7.**

- **Higuchi R., Fockler C., Dollinger G., Watson R. (1993). Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 11:1026 -30.**

Applications of qPCR:

- **Gene expression analysis (RT-qPCR)**
- **Detection of nucleic acids of pathogens: detecting the presence and determining the concentration of viruses and bacteria in serum**
- **Genotyping: SNP or HRM analyses**
- **Detection of GMOs in feed and food**

qPCR is based on detection of PCR product increment over time

- **Method is based on the determination of the DNA concentration for each reaction cycle, using fluorescence**

- **Fluorescent dyes binds dsDNA directly (nonspecific detection) or dayes are coupled with hybridization probes (specific detection - targeted to a selected strand of the amplicon under study)**

amplicon = PCR product, PCR fragmnet

- **Requires excitation system: halogen lamp, LED system or laser**

- **Requires detection system: separate CCD elements, CCD array or photomultiplier circuits**

Examples of qPCR cyclers

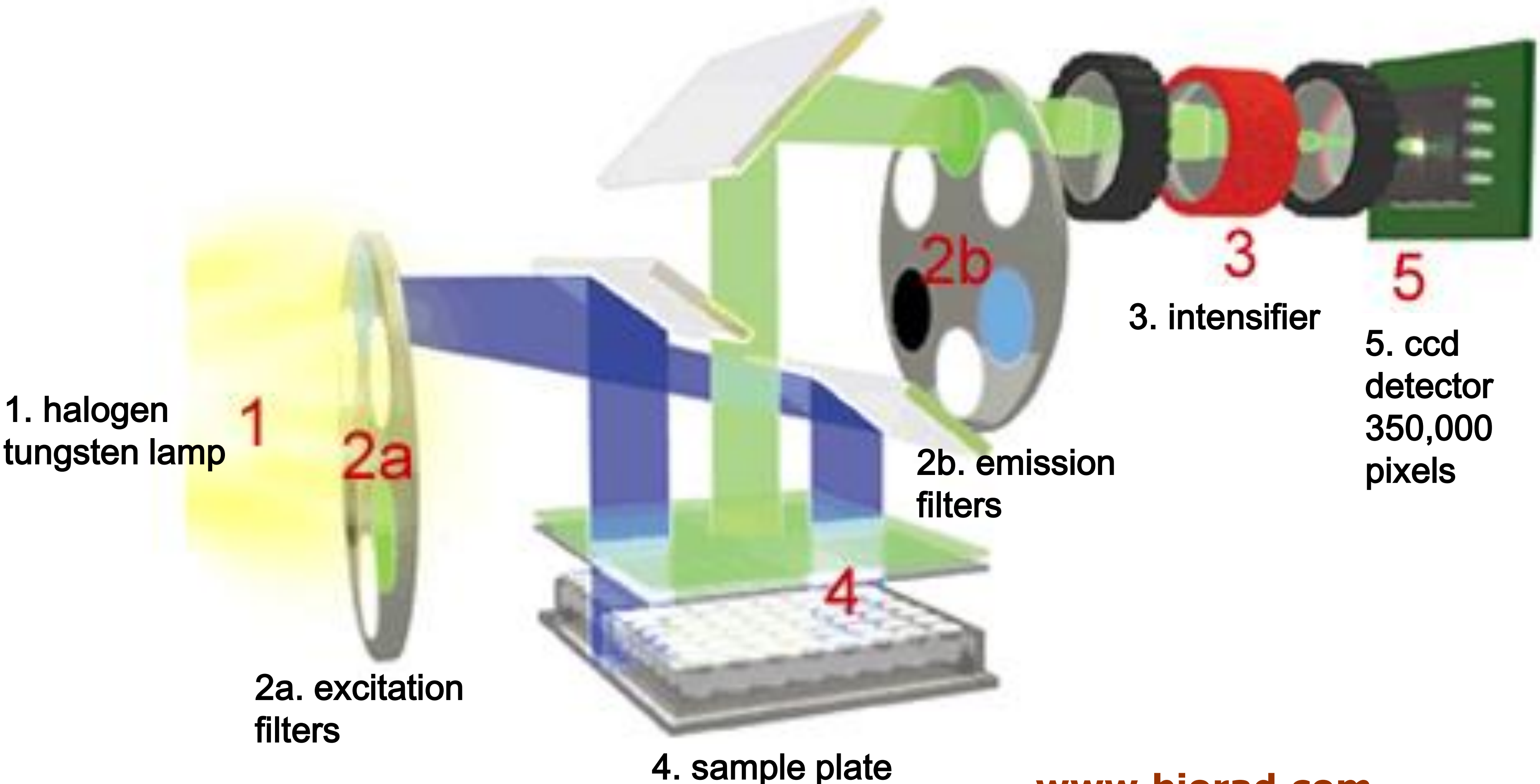
Roche LightCycler 480

QIAGEN Rotor-Gene Q

BioRad CFX96/ CFX384

Appliedbiosystems 7900HT

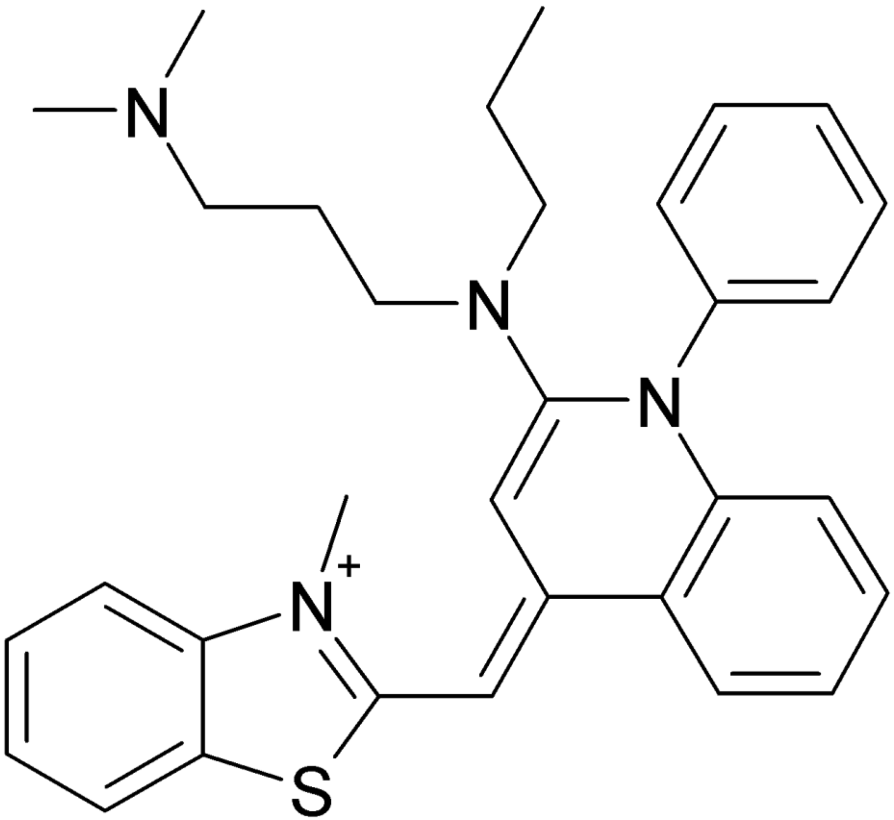
qPCR cycler scheme



www.biorad.com

How to detect DNA products in qPCR?

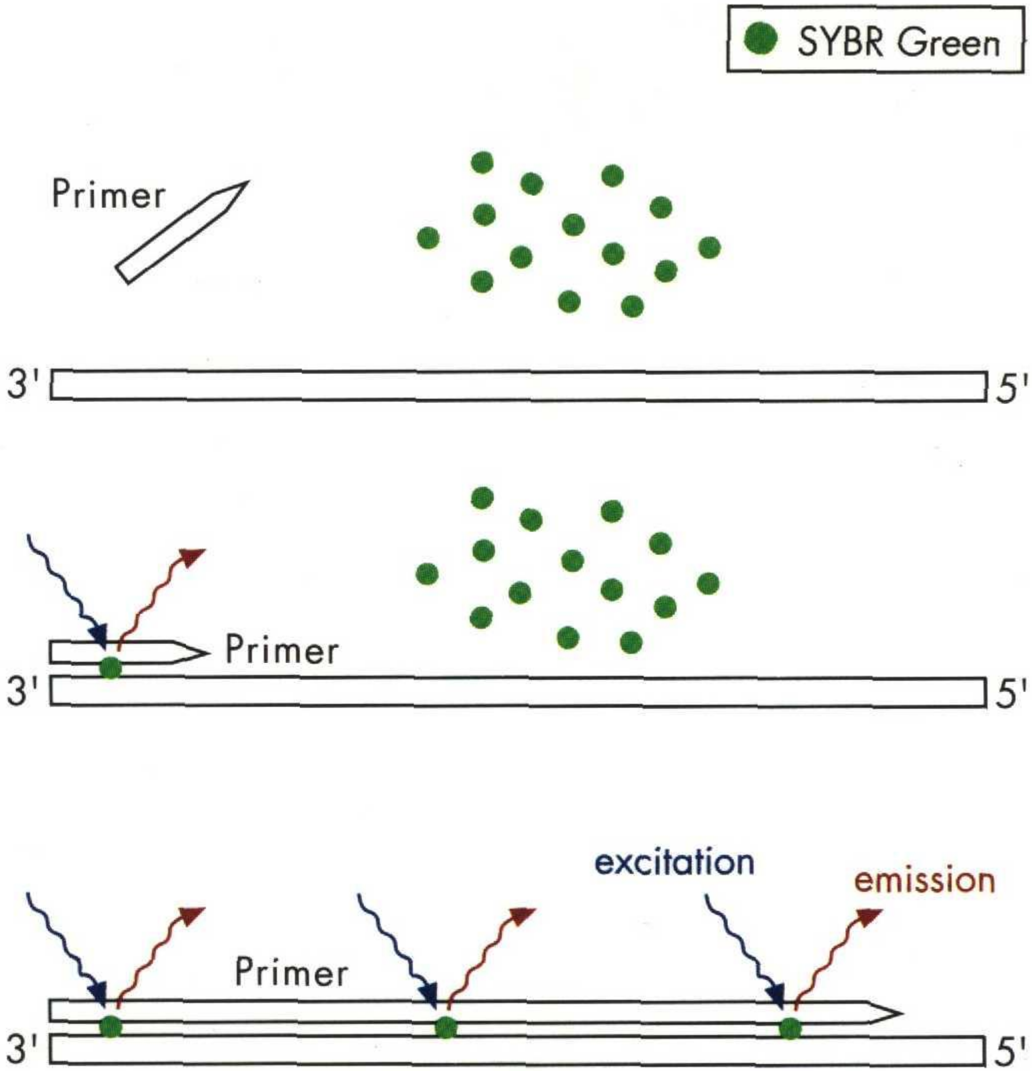
DNA-binding dyes: SYBR Green



SYBR Green I, Wikipedia

- **Belongs to cyanine dyes (containing heterocyclic rings with -C= bonds)**
- **Binds dsDNA, weakly ssDNA**
- **Excited by blue light (λmax = 488 nm)**
- **Emits green light (λmax = 522 nm)**

SYBR Green



- Detects any **dsDNA**
- Detection at the **ELONGATION** stage
- Primers cannot form **PRIMER-DIMERS**
- Product size preferably in the range of **100-200 bp**
- The size of the detected products for different genes must be **VERY CLOSE**
- Enables the analysis of **MELTING CURVES**
- **THE CHEAPEST** method

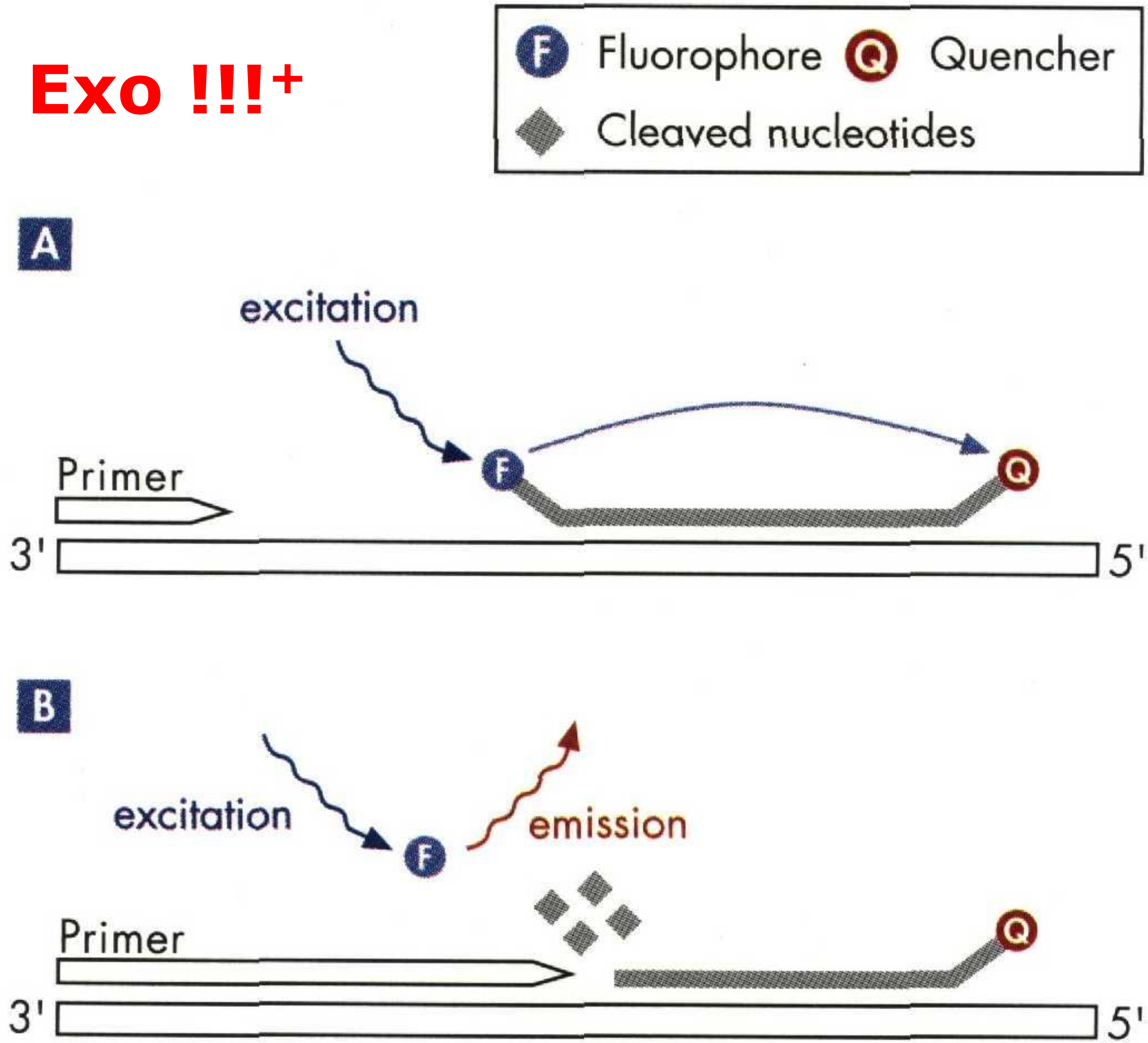
qPCR primers design

- **Length of the amplicon preferably less than 150 bp**
- **Avoid sequence complementarity within a primer or between primers = "primer dimer"**
- **Avoid unpaired bases**
- **Preferably G or C at the 3' end, never T**
- **Length of primers 18-30 nt**
- **GC content 40-60%**
- **Tm = (A+T)x2°C + (G+C)x4°C**
- **Use at least 2 programs and compare results!!! Different software = different algorithms (not always…)!**

Hybridization probes

TaqMan – hydrolysis probes

DNA Pol Exo !!!$^{+}$

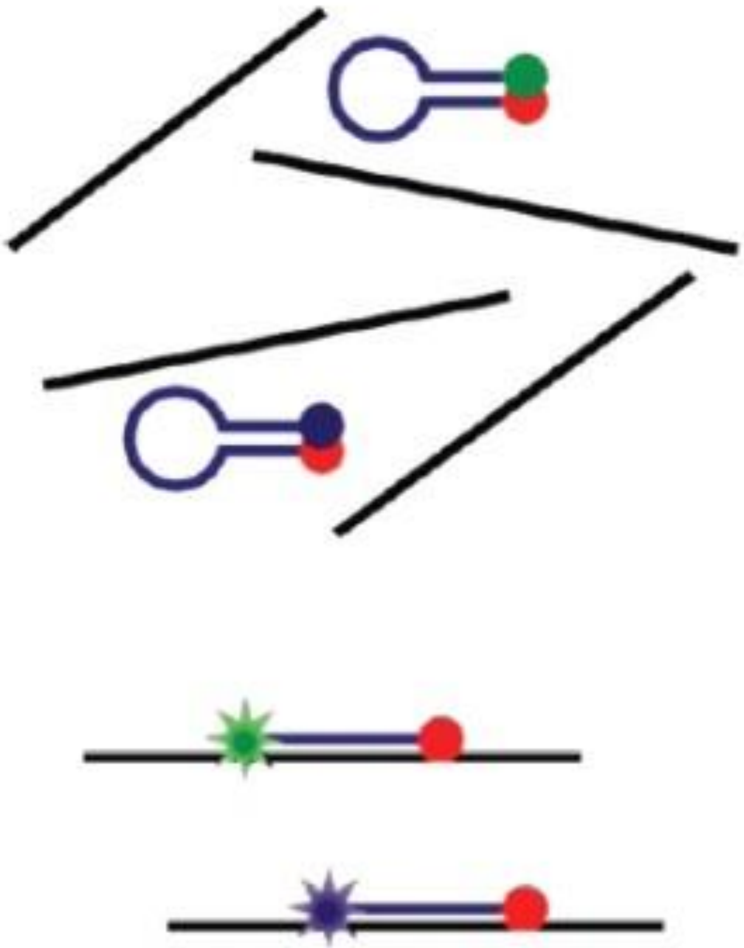


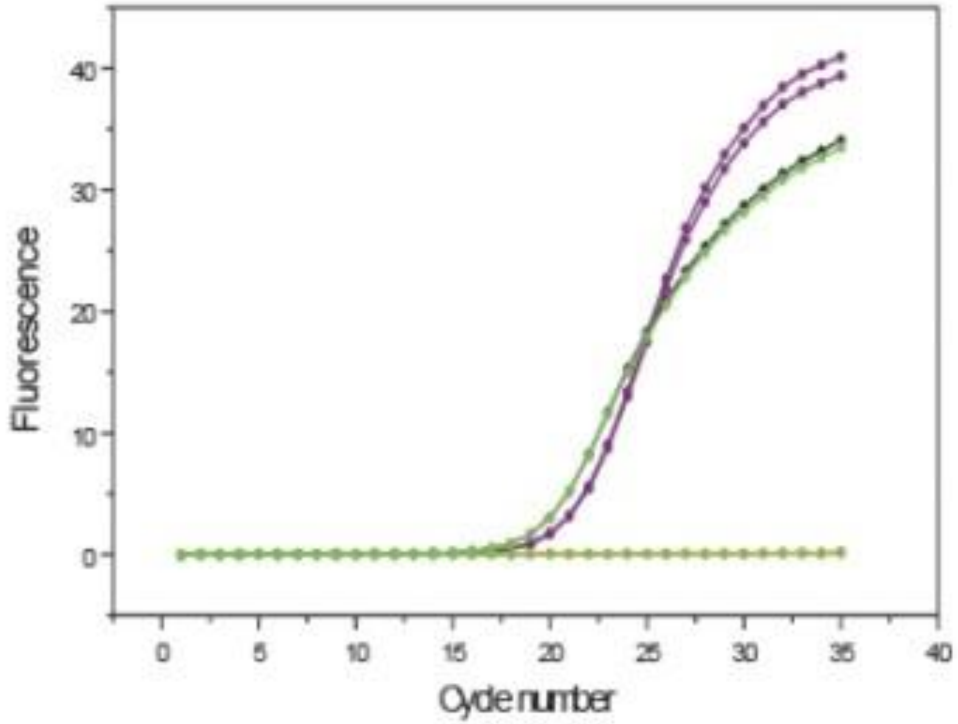
ABI, Roche (UPL)

TaqMan probe – design principles

- Short amplicons: 70-150 bp
- Tm = 68-70°C
- GC content 30-60%
- Never G at the 5' end (G is natural quencher)
- Probe length max. 30 nt
- Avoid strings of identical bases
- Avoid secondary structures
- Avoid complementarity with primers
- Choose a strand with a higher C content

Multiplex qPCR



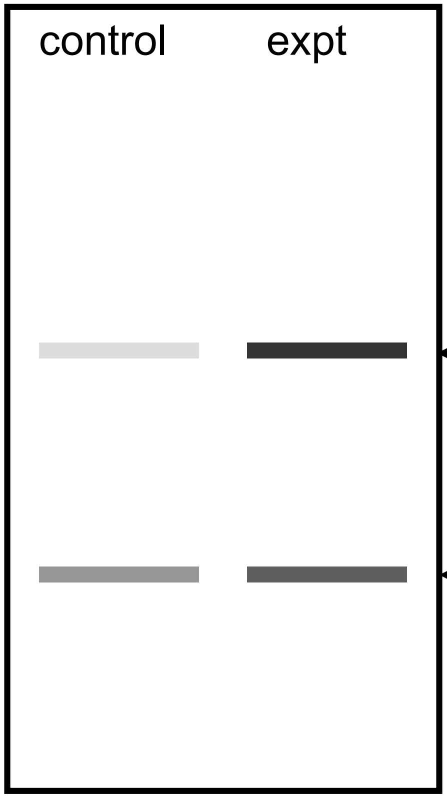


Different detection formats and different dyes allow detection of 2 or more products in a single reaction

(not possible with SYBR Green!!!)

Determination of the RNA levels

Classic northern-blot technique



target gene **10X**

internal control gene
actin, GAPDH, RPLP0 etc. **2X**

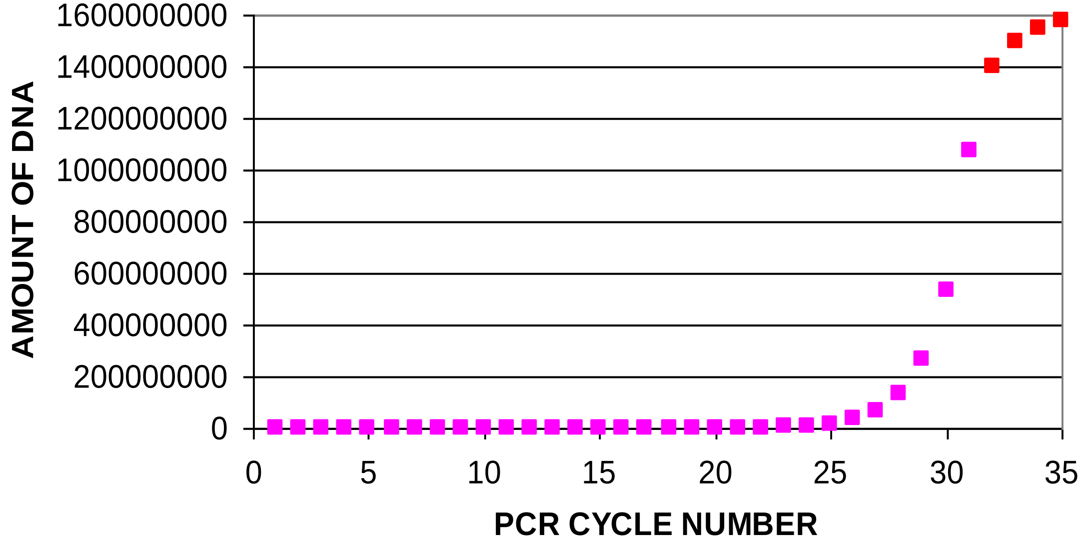
Corrected fold increase = 10/2 = 5

$$\text{Ratio target gene in experimental/control} = \frac{\text{fold change in target gene}}{\text{fold change in reference gene}}$$

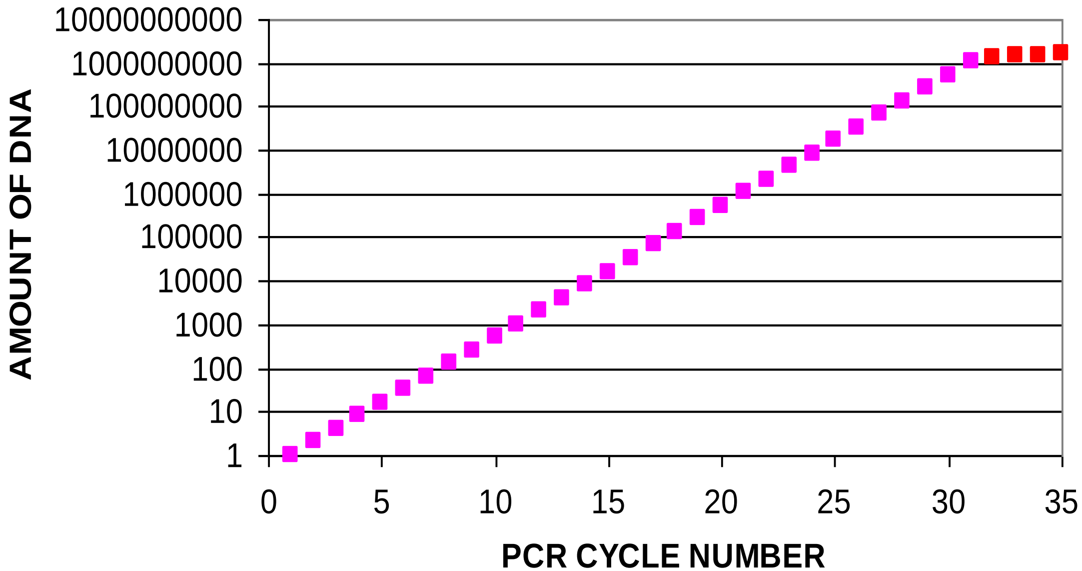
Theoretical basis of qPCR

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000

Theoretical curve



Transformed on a logarithmic scale

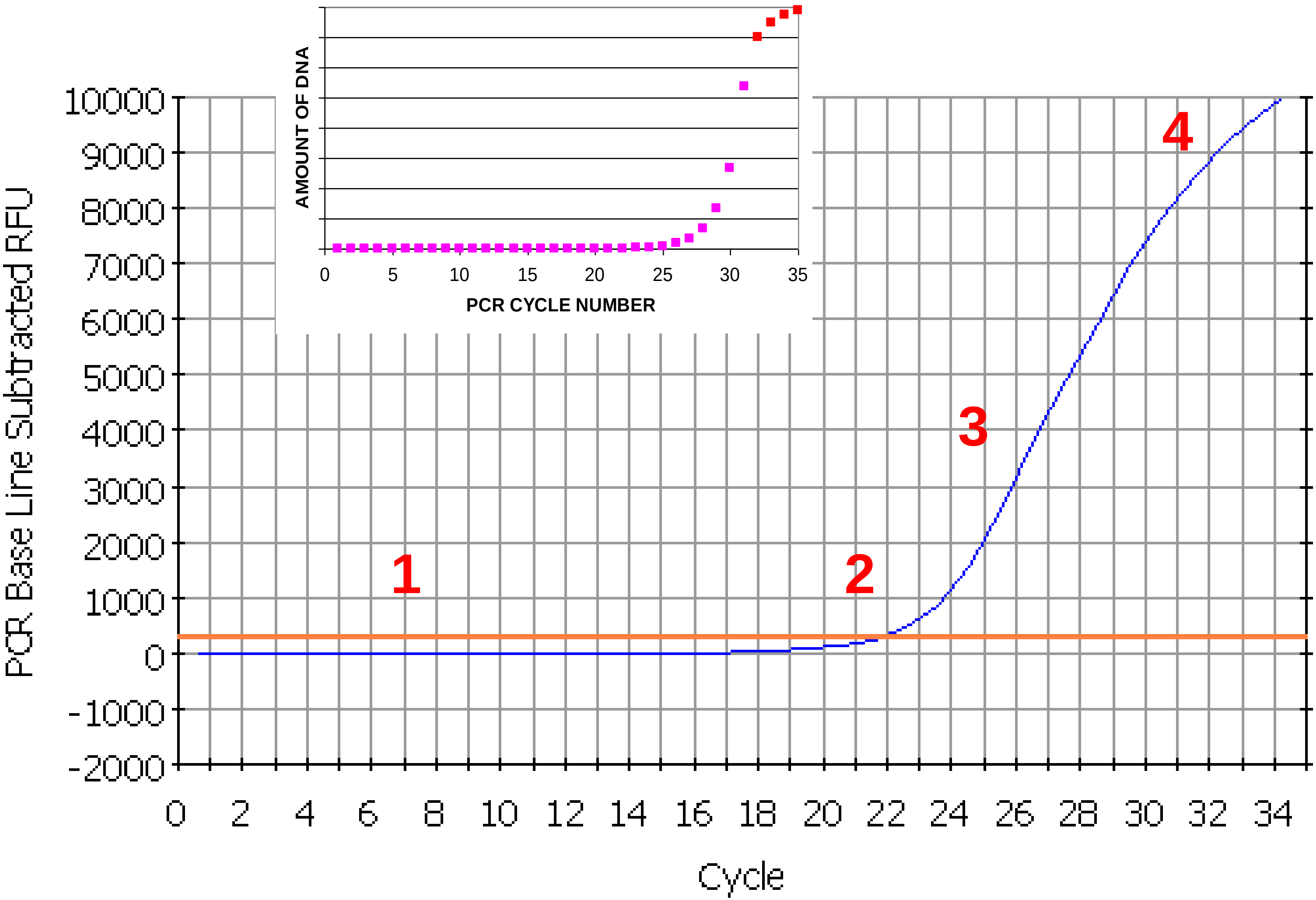


Microbiology and Immunology On-line; University of South Carolina School of Medicine

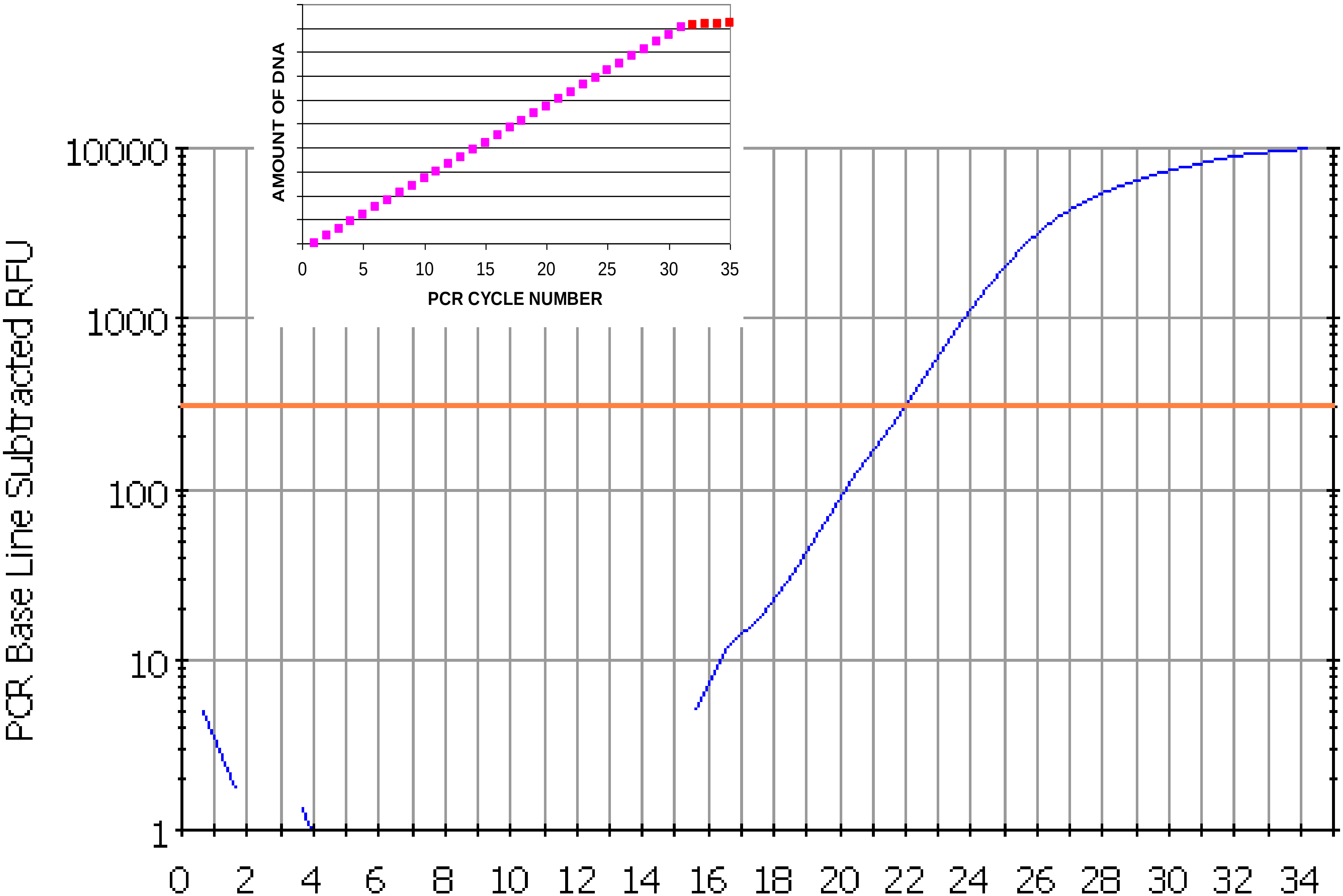
4 phases of the qPCR reaction

- **Phase 1 : fluorescence at background level, reaction occurs exponentially but detection is not possible**
- **Phase 2: exponential growth detectable, fluorescence above background**
- **Phase 3: a "steep" increase in fluorescence allows detection that the reaction is occurring linearly**
- **Phase 4 - plateau: breakdown and saturation of the reaction**

M. W. Pfaffl: *Quantification strategies in real-time PCR* in *A-Z of quantitative PCR* (Editor: S.A. Bustin)

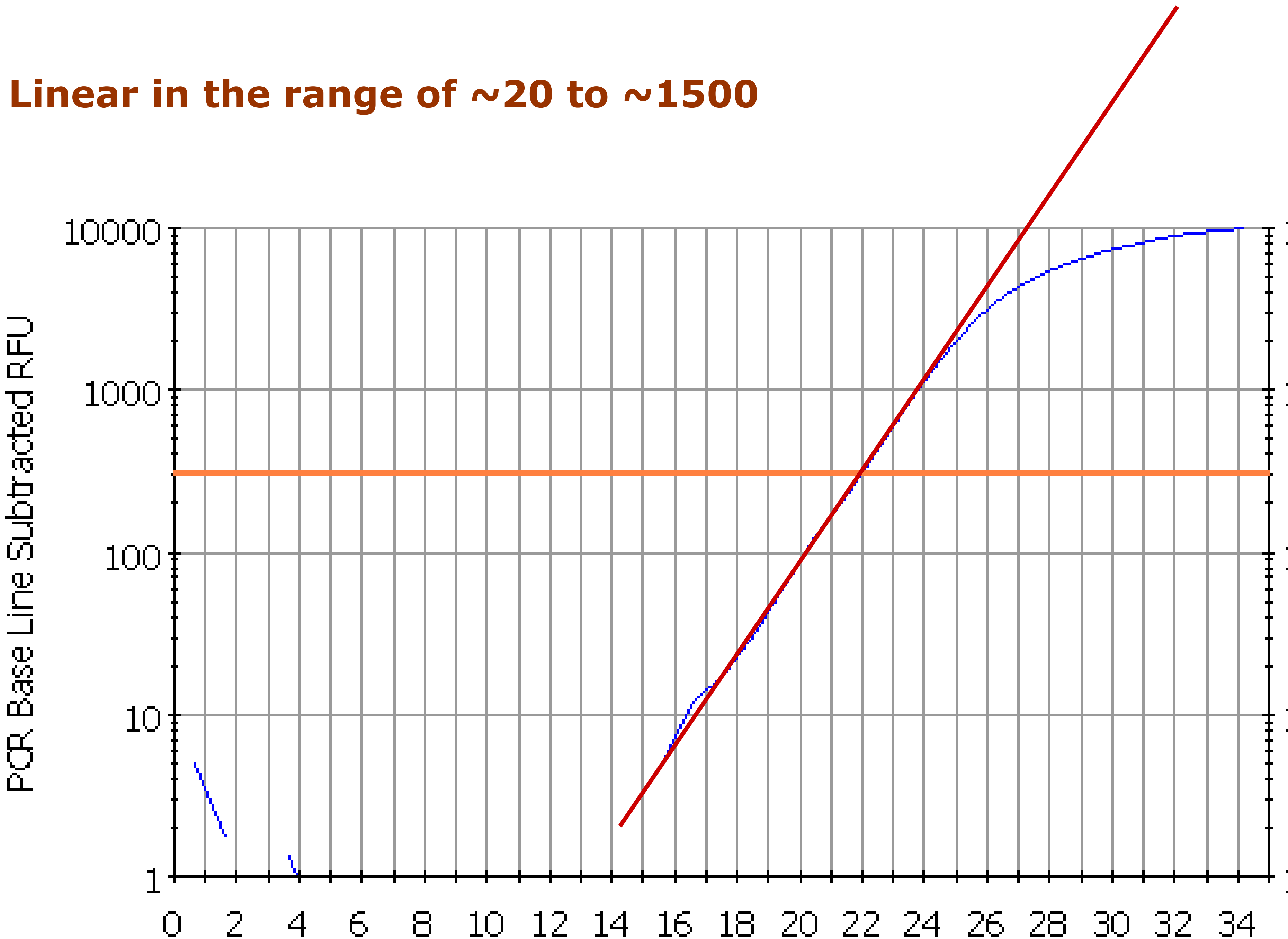


Microbiology and Immunology On-line; University of South Carolina School of Medicine



Microbiology and Immunology On-line; University of South Carolina School of Medicine

Linear in the range of ~20 to ~1500



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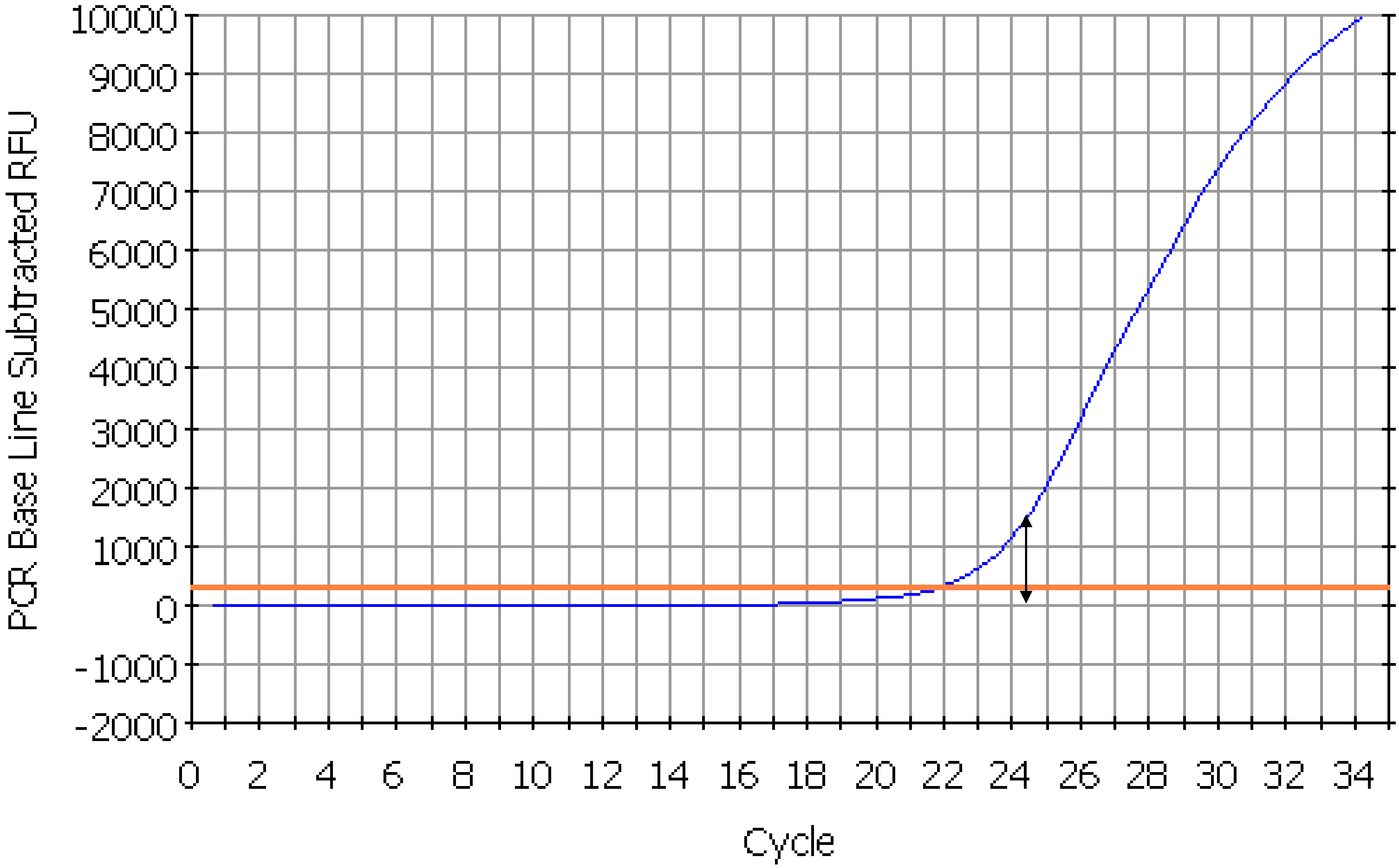
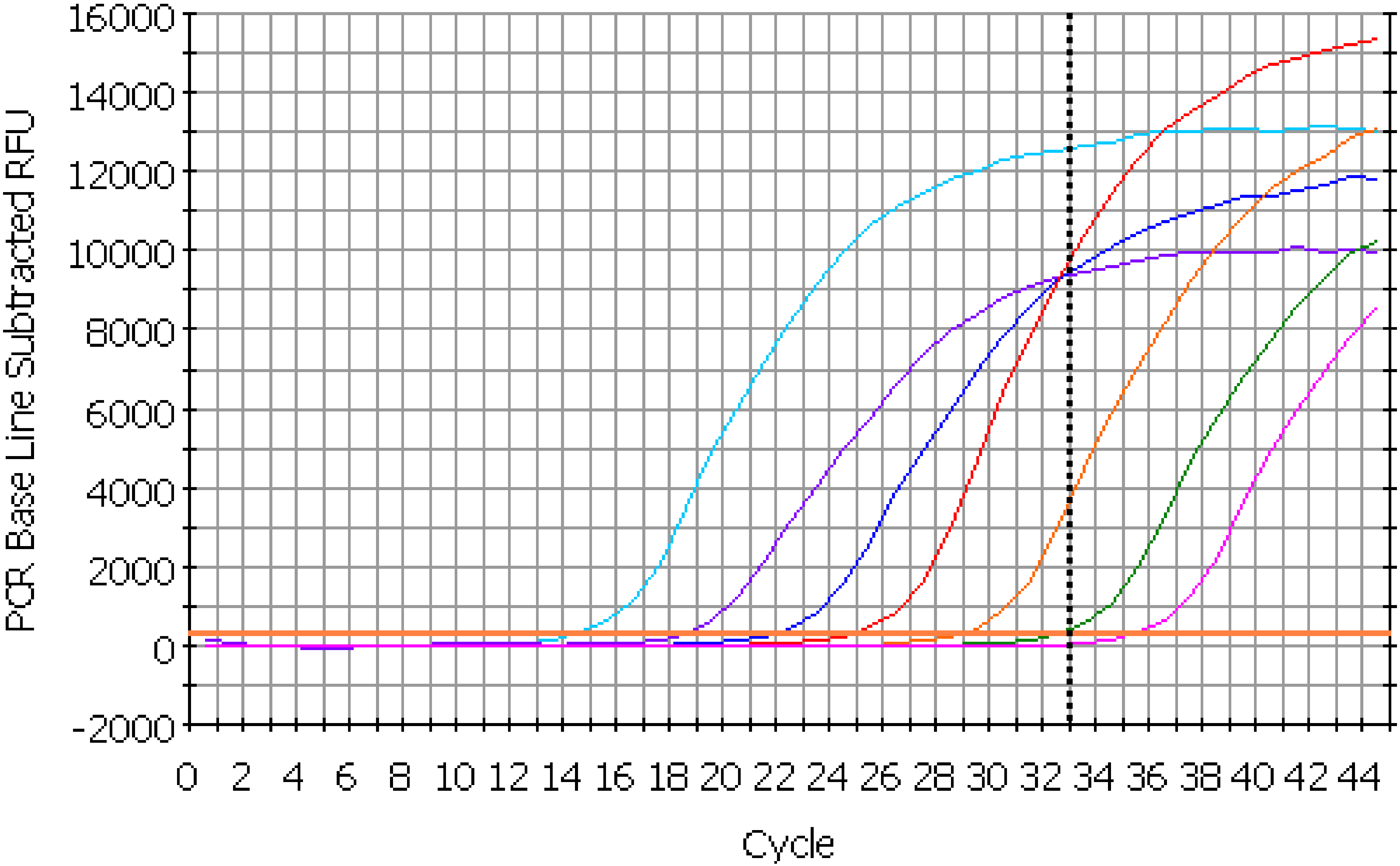
Linear in the range of ~20 to ~1500

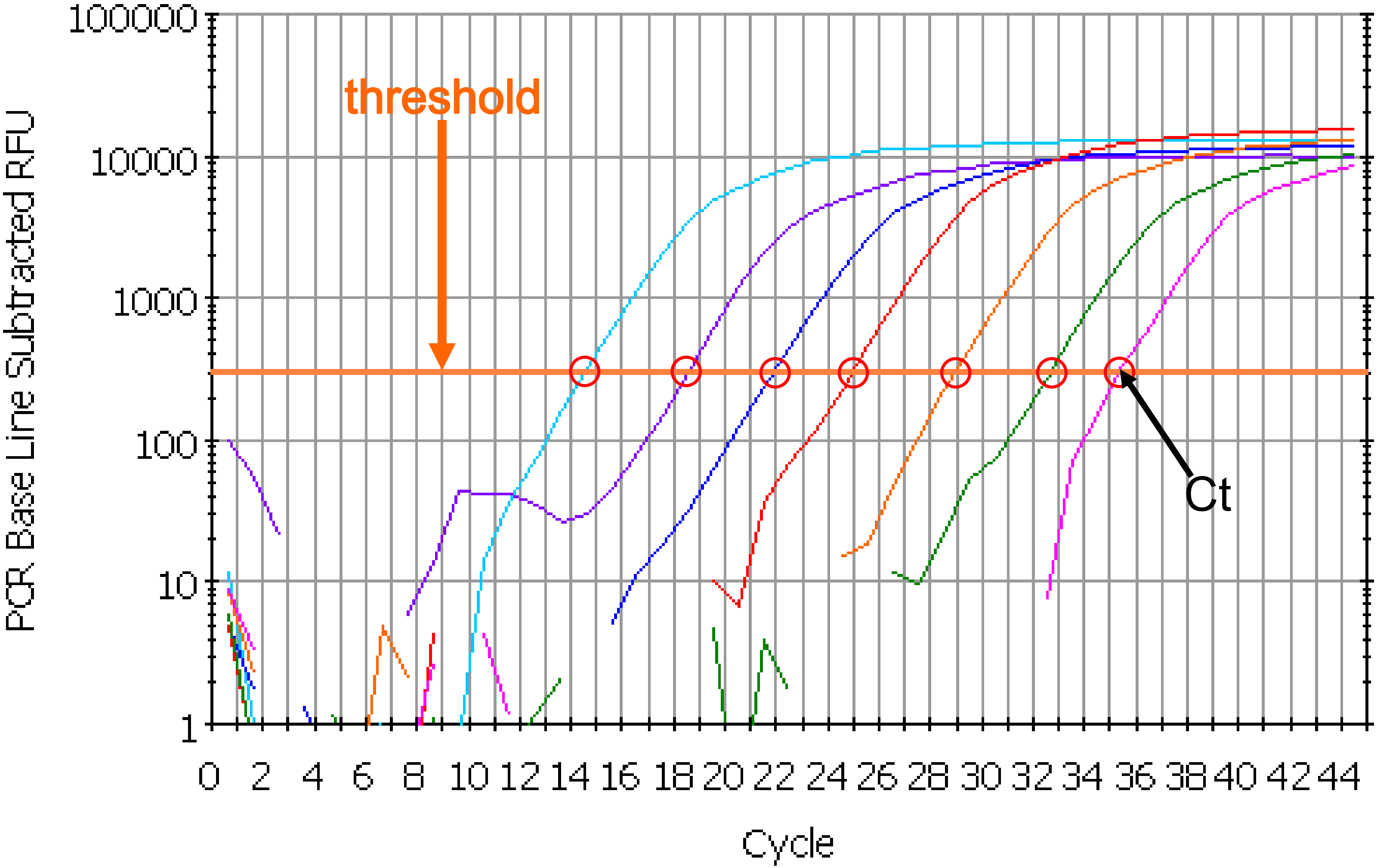


Microbiology and Immunology On-line; University of South Carolina School of Medicine



SERIES OF 10-FOLD DILUTIONS

Microbiology and Immunology On-line; University of South Carolina School of Medicine



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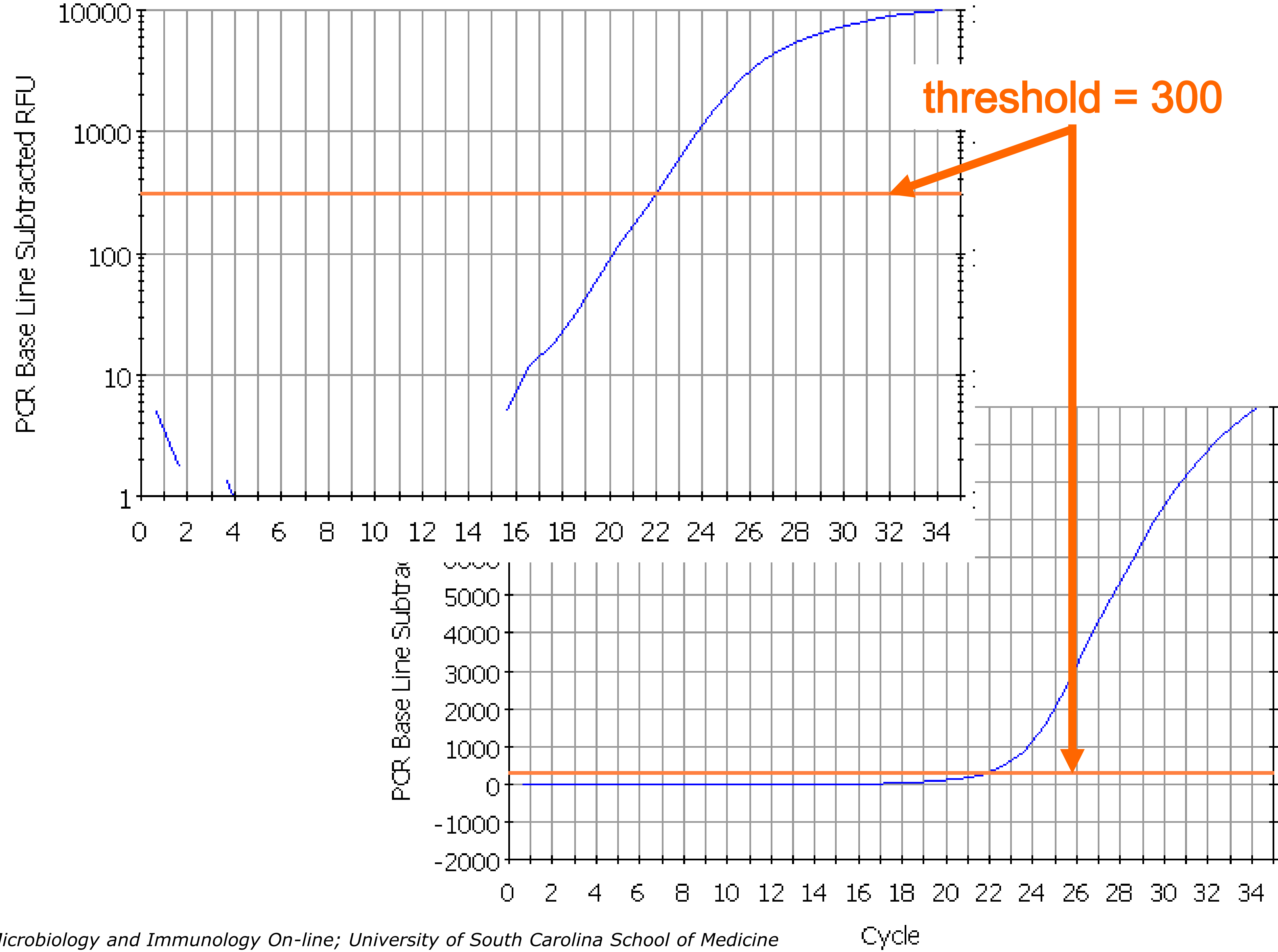
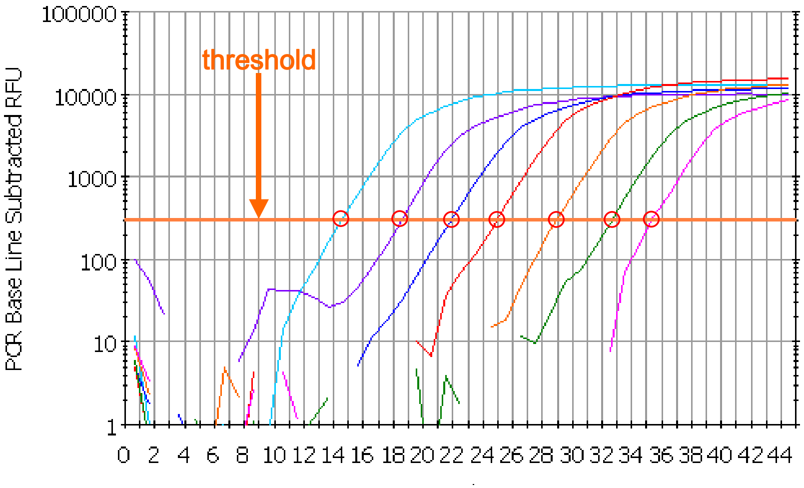
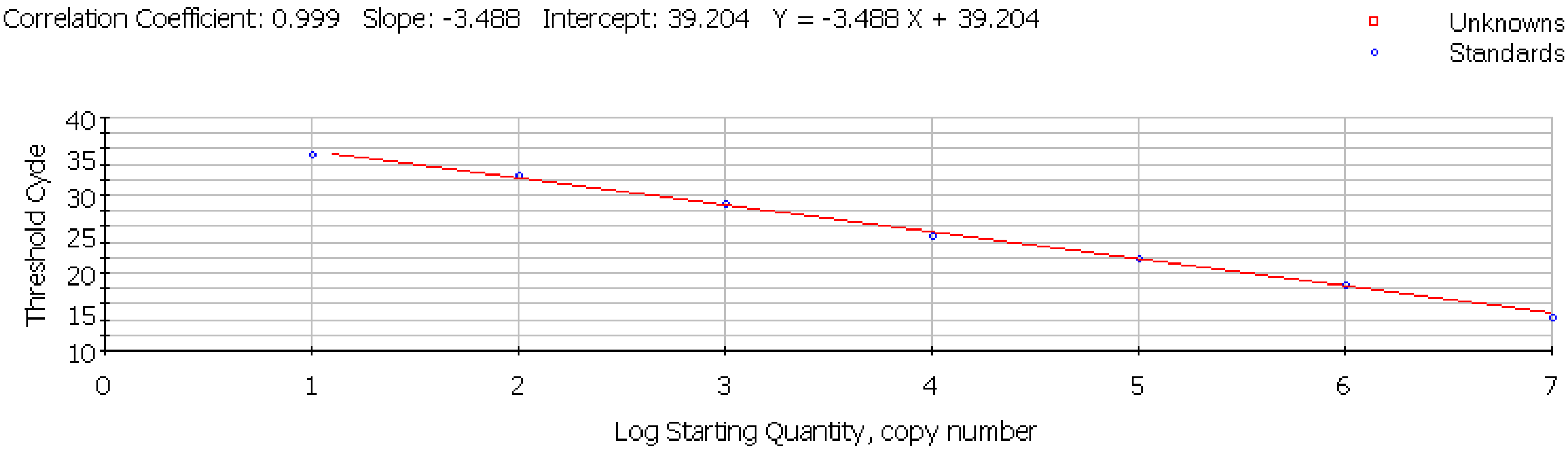


Microbiology and Immunology On-line; University of South Carolina School of Medicine



Correlation Coefficient: 0.999 Slope: -3.488 Intercept: 39.204 Y = -3.488 X + 39.204



PCR Standard Curve: Data 27-Jan-03 1233ileff.opd

Sensitivity and reproducibility: qPCR vs semi-qPCR

- **Dynamic range: up to 9 orders of magnitude (10^9) in qPCR and at most 2 (10^2) in semi-quantitative-PCR**
- **Variations inside the qPCR experiment - up to a few % and in semi-qPCR up to 30-40%**
- **Variance between qPCR experiments - up to a dozen % and in semi-qPCR up to 50-70%**
- **Detection level in qPCR: about 10 molecules at 50% reproducibility and about 100 molecules at 100% reproducibility.**

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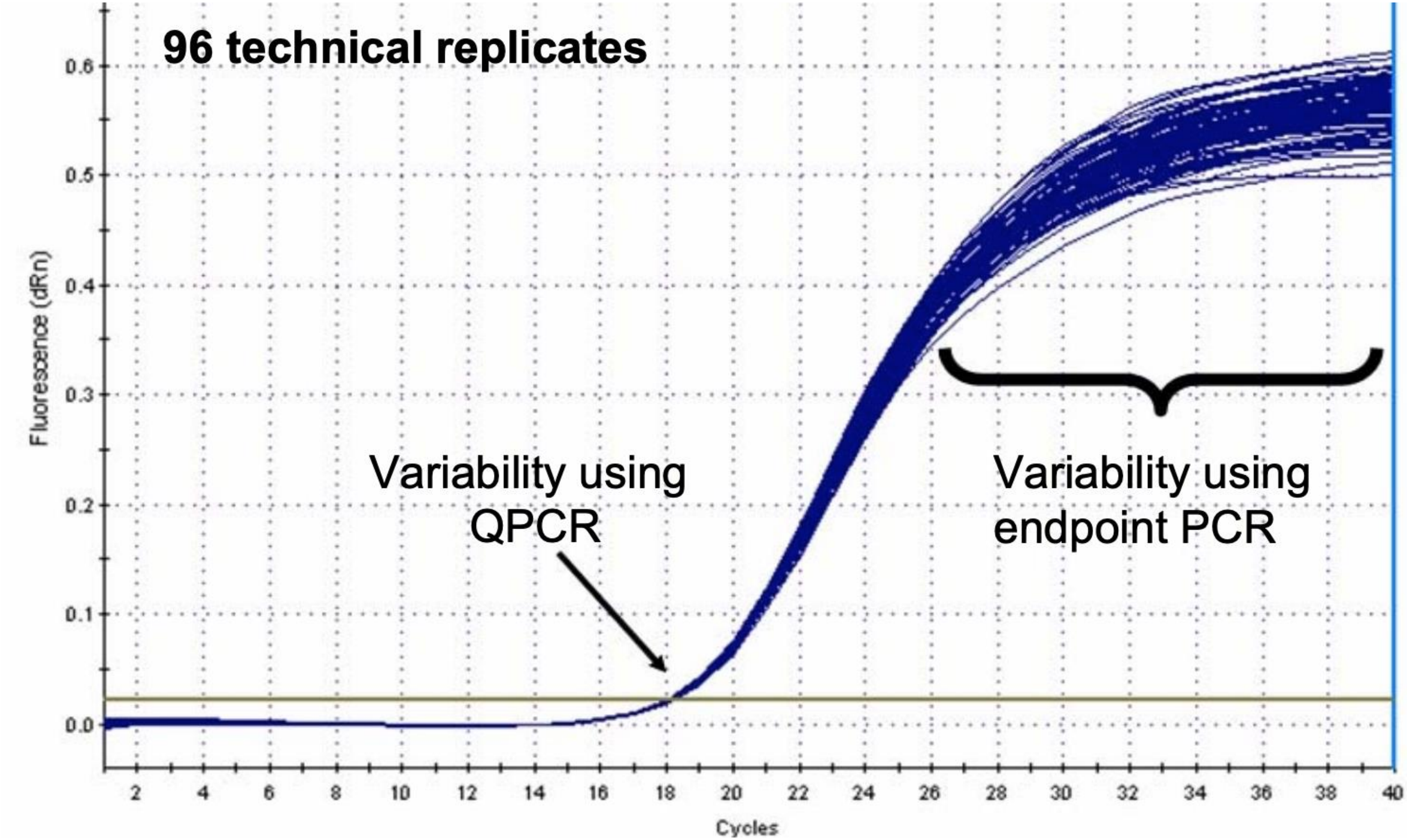
Sensitivity and reproducibility: qPCR vs semi-qPCR



https://help.medicinalgenomics.com/qpcr-vs-end-point-pcr#

Different algorithms for determining the value of the threshold cycle

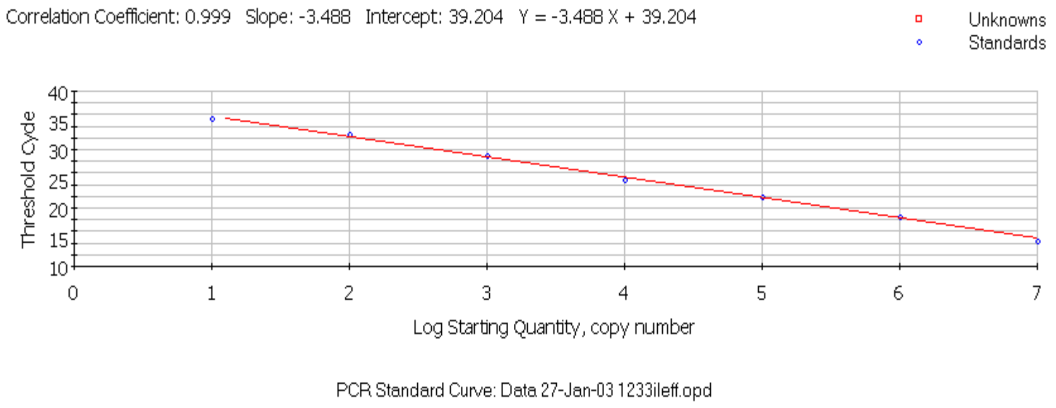
Ct = Cp = Cq

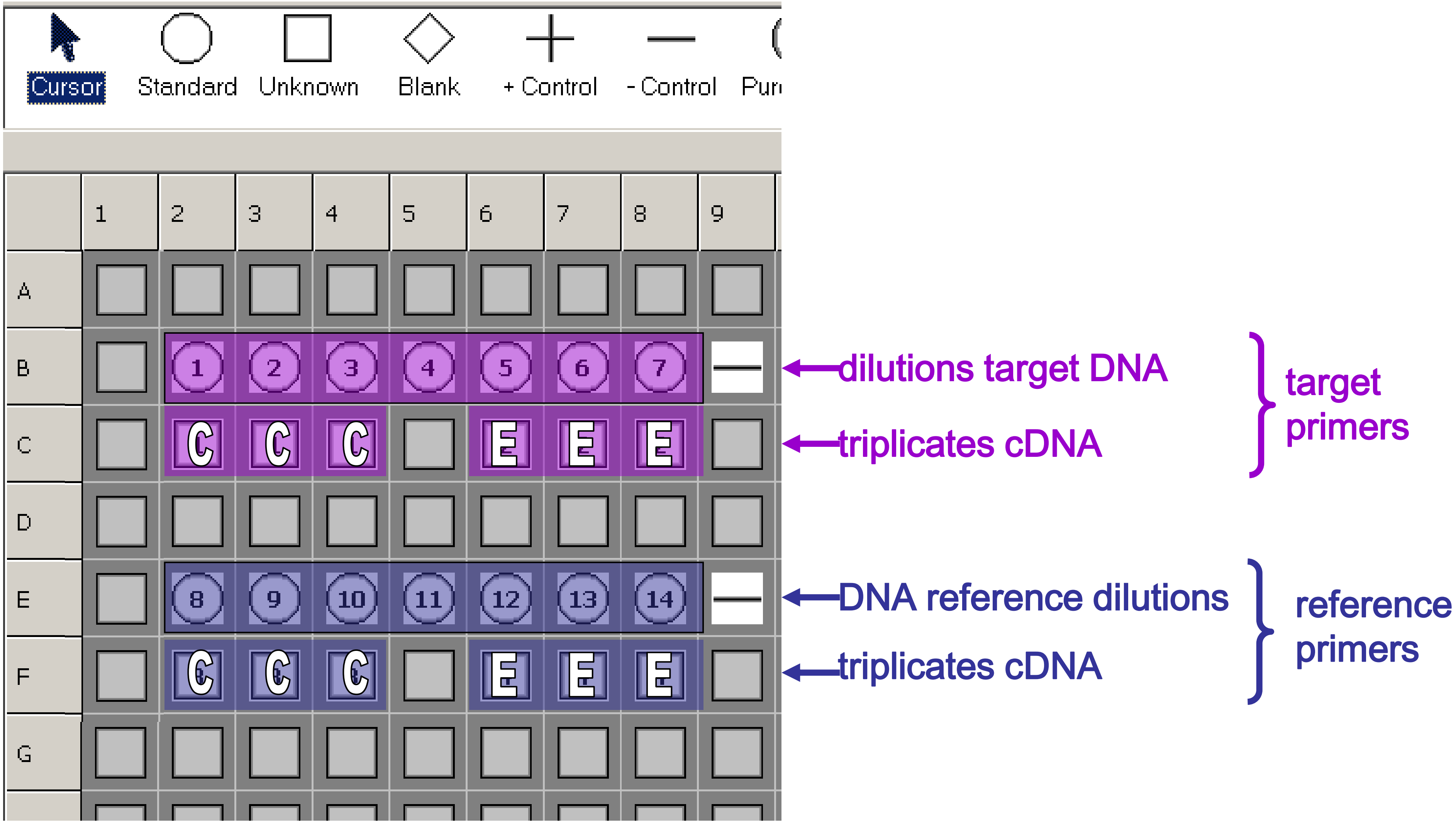
Threshold cycle	Maximum of the 2nd derivative (Roche)	Quantification cycle

Two methods of quantification

- **"ABSOLUT QUANTIFICATION" or the method of standard curves.**
- **"RELATIVE QUANTIFICATION"**
- **Both methods are in fact proportional!**

The standard curve method





Cursor
Standard
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Blank
+ Control
- Control
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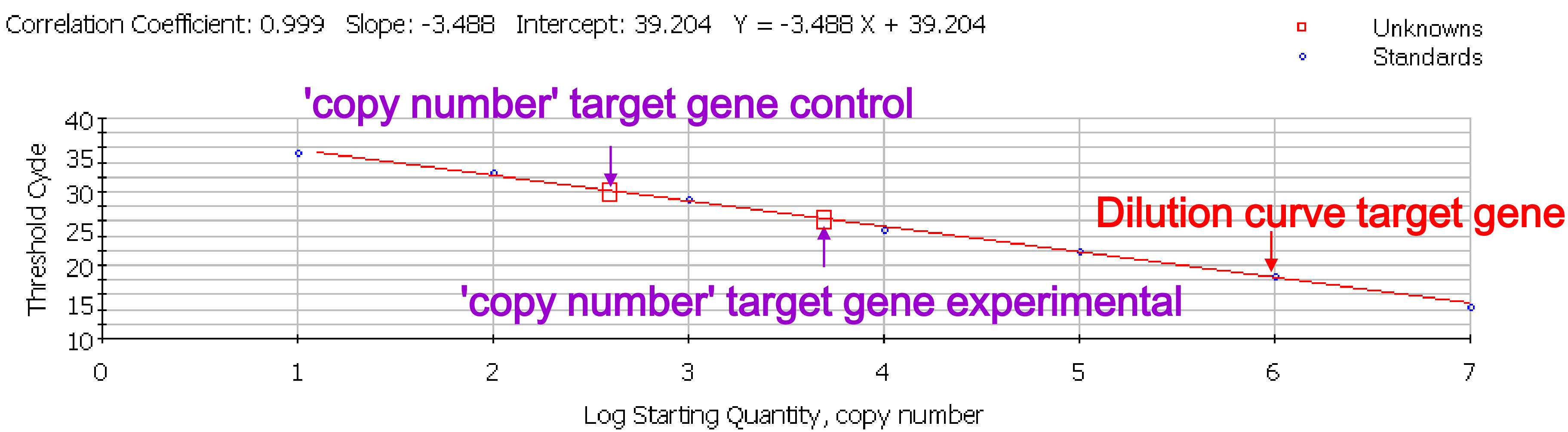
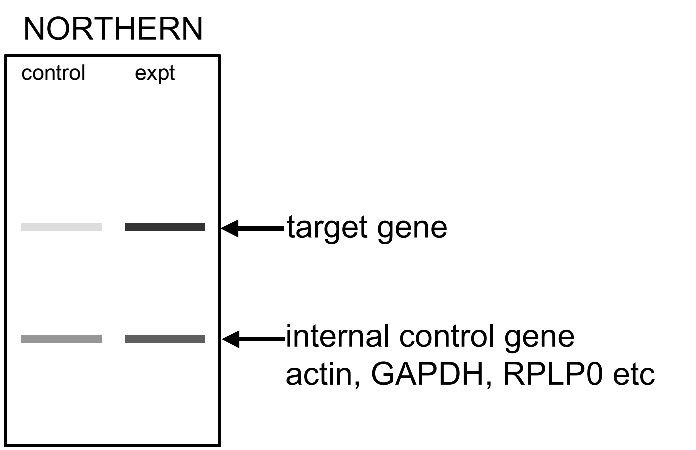
dilutions target DNA
triplicates cDNA
target primers
DNA reference dilutions
triplicates cDNA
reference primers

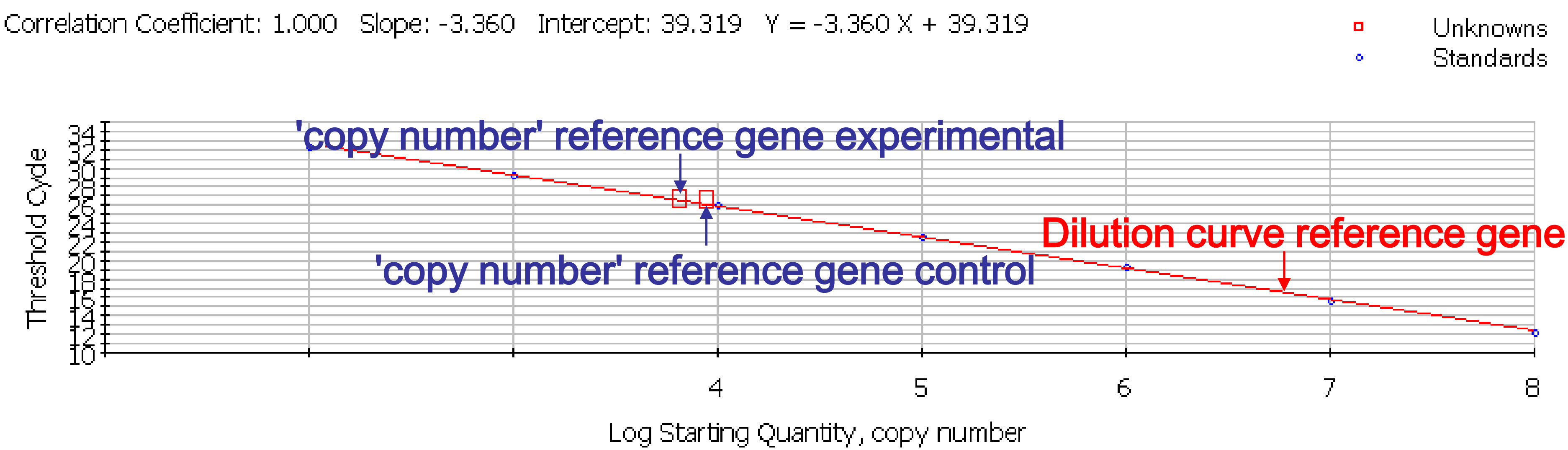


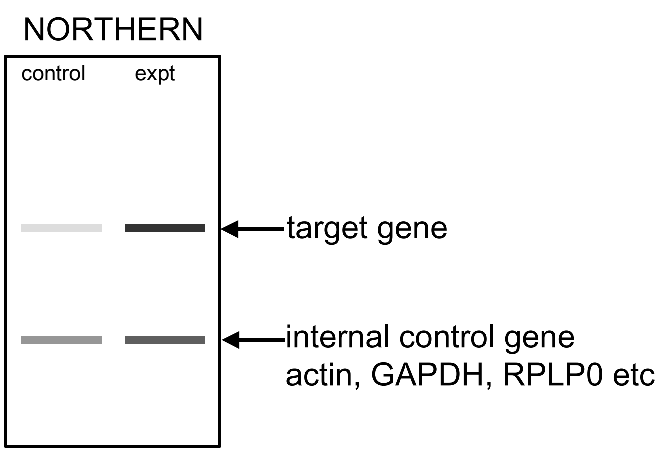


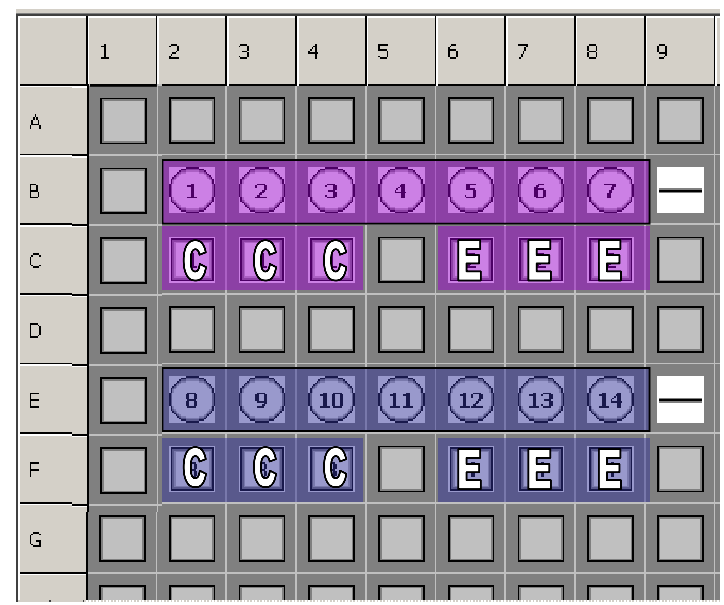
fold change in target gene=

$$\frac{\text{copy number experimental}}{\text{copy number control}}$$

Ratio experimental/control = $\dfrac{\text{fold change in target gene}}{\text{fold change in reference gene}}$







Ratio experimental/control = fold change in target gene / fold change in reference gene

Efficiency is a key factor of the qPCR reaction

CYCLE	AMOUNT OF DNA 100% EFFICIENCY	AMOUNT OF DNA 90% EFFICIENCY	AMOUNT OF DNA 80% EFFICIENCY	AMOUNT OF DNA 70% EFFICIENCY
0	1	1	1	1
1	2	2	2	2
2	4	4	3	3
3	8	7	6	5
4	16	13	10	8
5	32	25	19	14
6	64	47	34	24
7	128	89	61	41
8	256	170	110	70
9	512	323	198	119
10	1,024	613	357	202
11	2,048	1,165	643	343
12	4,096	2,213	1,157	583
13	8,192	4,205	2,082	990
14	16,384	7,990	3,748	1,684
15	32,768	15,181	6,747	2,862
16	65,536	28,844	12,144	4,866
17	131,072	54,804	21,859	8,272
18	262,144	104,127	39,346	14,063
19	524,288	197,842	70,824	23,907
20	1,048,576	375,900	127,482	40,642
21	2,097,152	714,209	229,468	69,092
22	4,194,304	1,356,998	413,043	117,456
23	8,388,608	2,578,296	743,477	199,676
24	16,777,216	4,898,763	1,338,259	339,449
25	33,554,432	9,307,650	2,408,866	577,063
26	67,108,864	17,684,534	4,335,959	981,007
27	134,217,728	33,600,615	7,804,726	1,667,711
28	268,435,456	63,841,168	14,048,506	2,835,109
29	536,870,912	121,298,220	25,287,311	4,819,686
30	1,073,741,824	230,466,618	45,517,160	8,193,466

AFTER 1 CYCLE

100% = 2.00x

90% = 1.90x

80% = 1.80x

70% = 1.70x

Microbiology and Immunology On-line; University of South Carolina School of Medicine

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AFTER 1 CYCLE

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80% = 1.80x

70% = 1.70x

AFTER N CYCLES:

fold increase = $(\text{efficiency})^n$

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2	4	4	3	3
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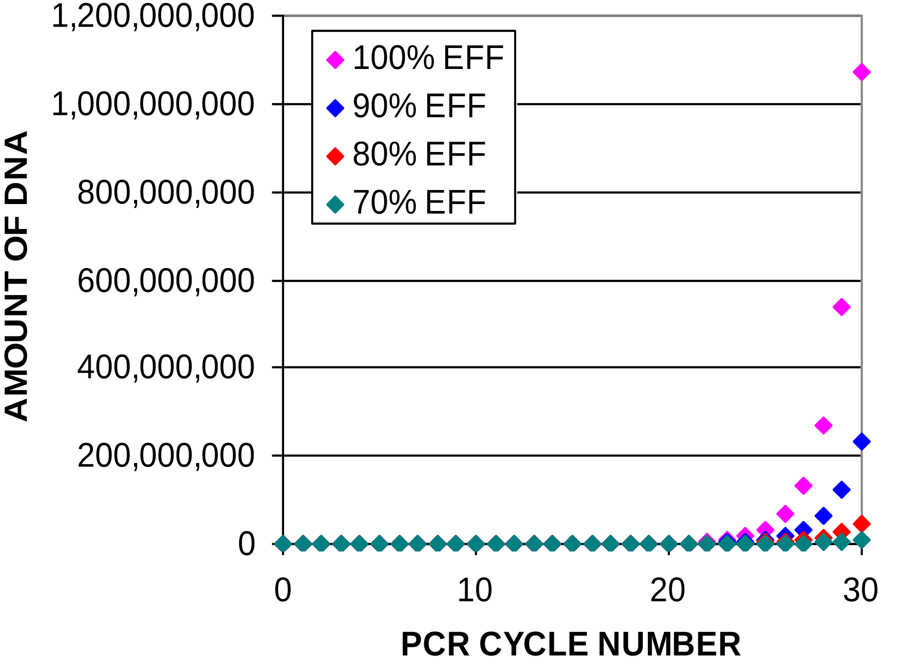

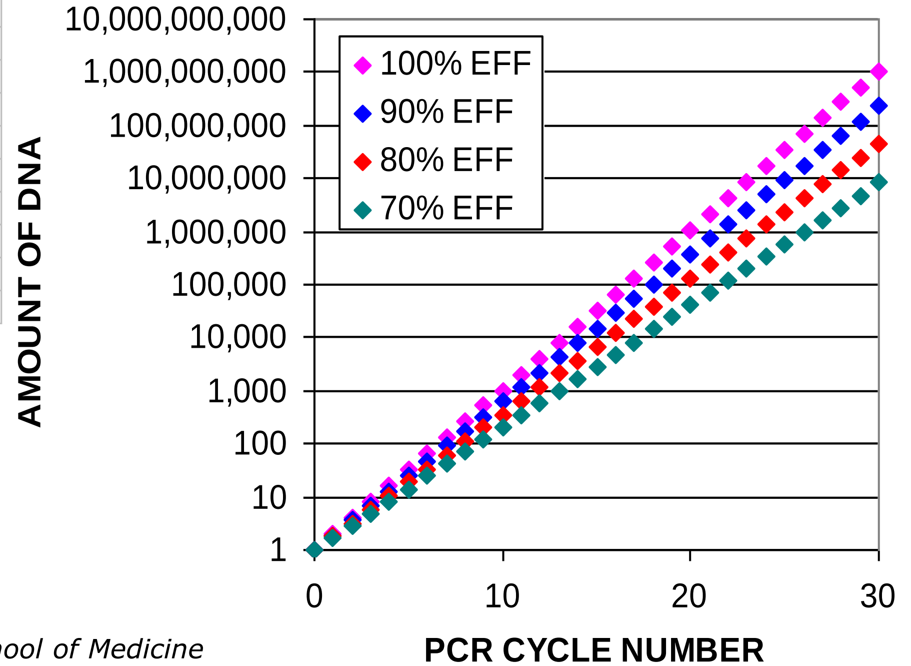

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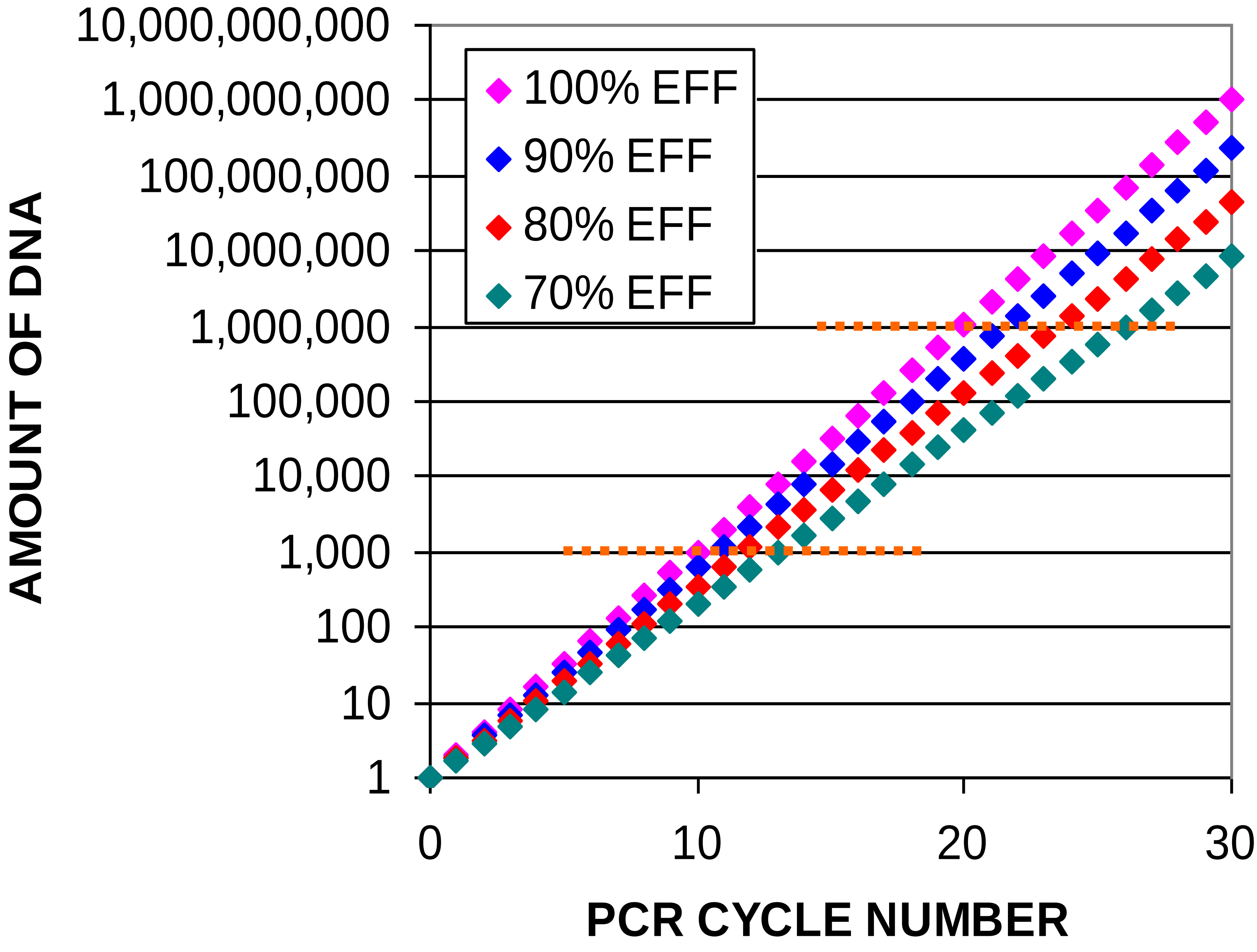

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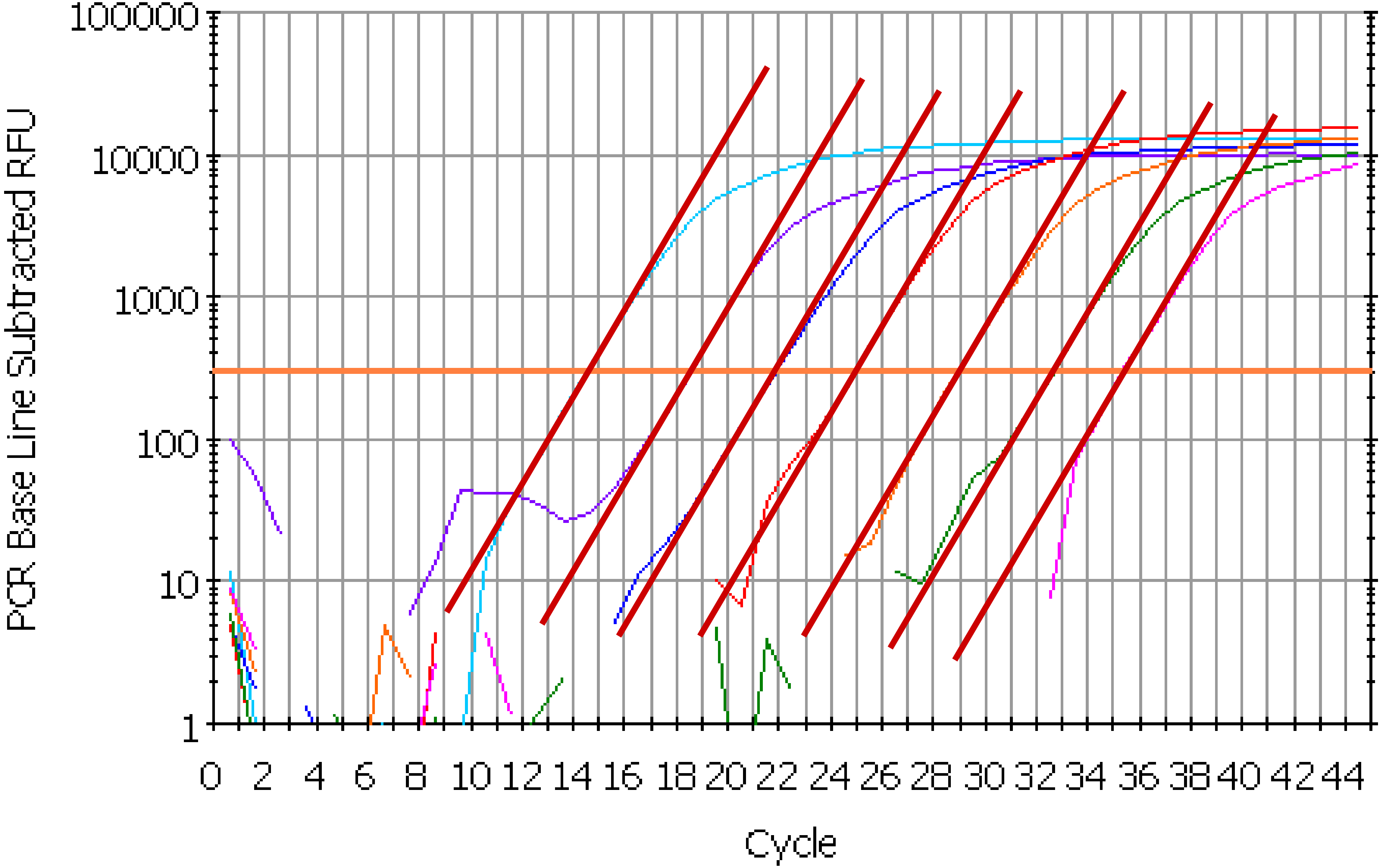

SERIES OF 10-FOLD DILUTIONS

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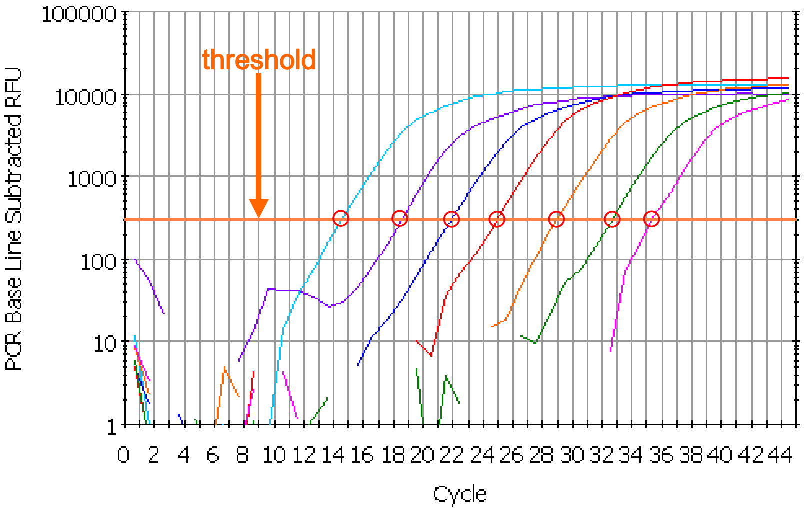
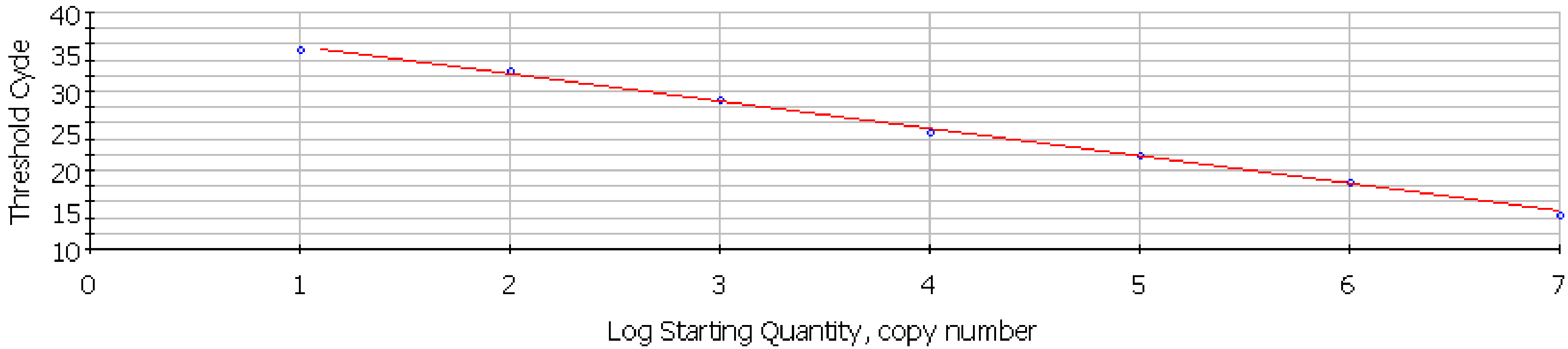


Correlation Coefficient: 0.999 Slope: -3.488 Intercept: 39.204 Y = -3.488 X + 39.204
PCR Efficiency: 93.5 %

Unknowns
Standards



PCR Standard Curve: Data 27-Jan-03 1233ileff.opd

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Determination of the qPCR reaction efficiency

4 methods for determining E of the qPCR:

1. **Calculated from the slope of the standard (calibration) curve - often E overestimated**

Possible for each individual reaction:

2. **Calculated from fluorescence growth history using linear regression - "manual" method - often E underestimated**
3. **Fitting the curve according to the assumed model to the fluorescence values from the 1st to the last cycle - often E underestimated**
4. **Calculated from polynomial curve fitting to fluorescence data from phase 2 only - intermediate values**

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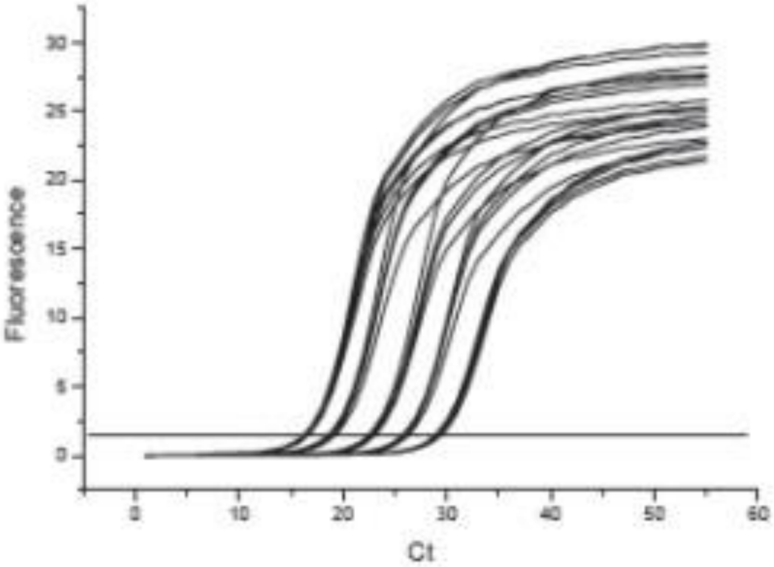
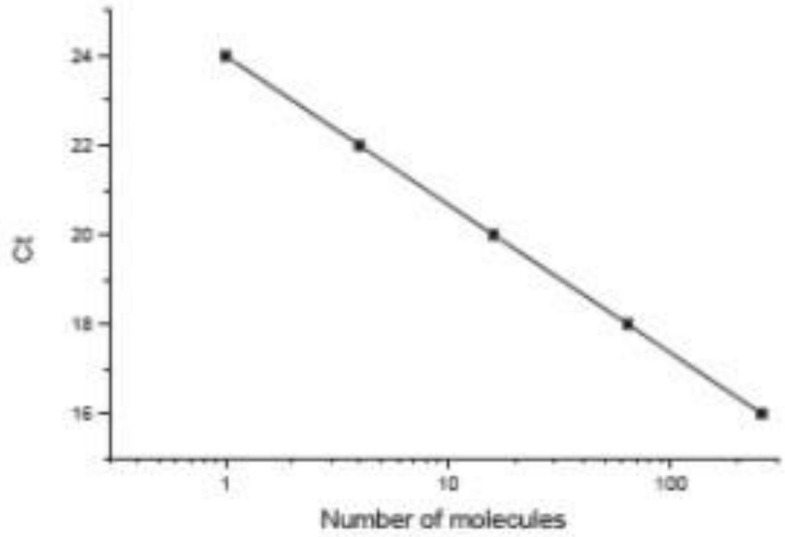
Determination of the qPCR reaction efficiency based on standard curves





Serial dilutions, min. 5, better 6 to 8

$$E = 10^{-1/slope} - 1$$

TATA Biocenter

qPCR reaction equation

$$N_{Ct} = N_0\,(1+E)^{Ct}$$

N_{Ct}: number of molecules after Ct cycles of amplification

N_0: initial number of molecules

E: PCR reaction efficiency

Ct: the value of the threshold cycle

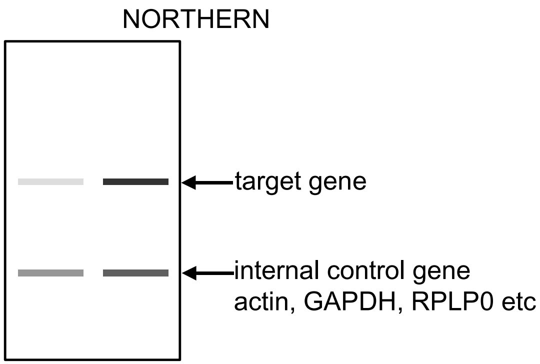
The PFAFFL method or the Realtive Quantification Method

Michael W. Pfaffl

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

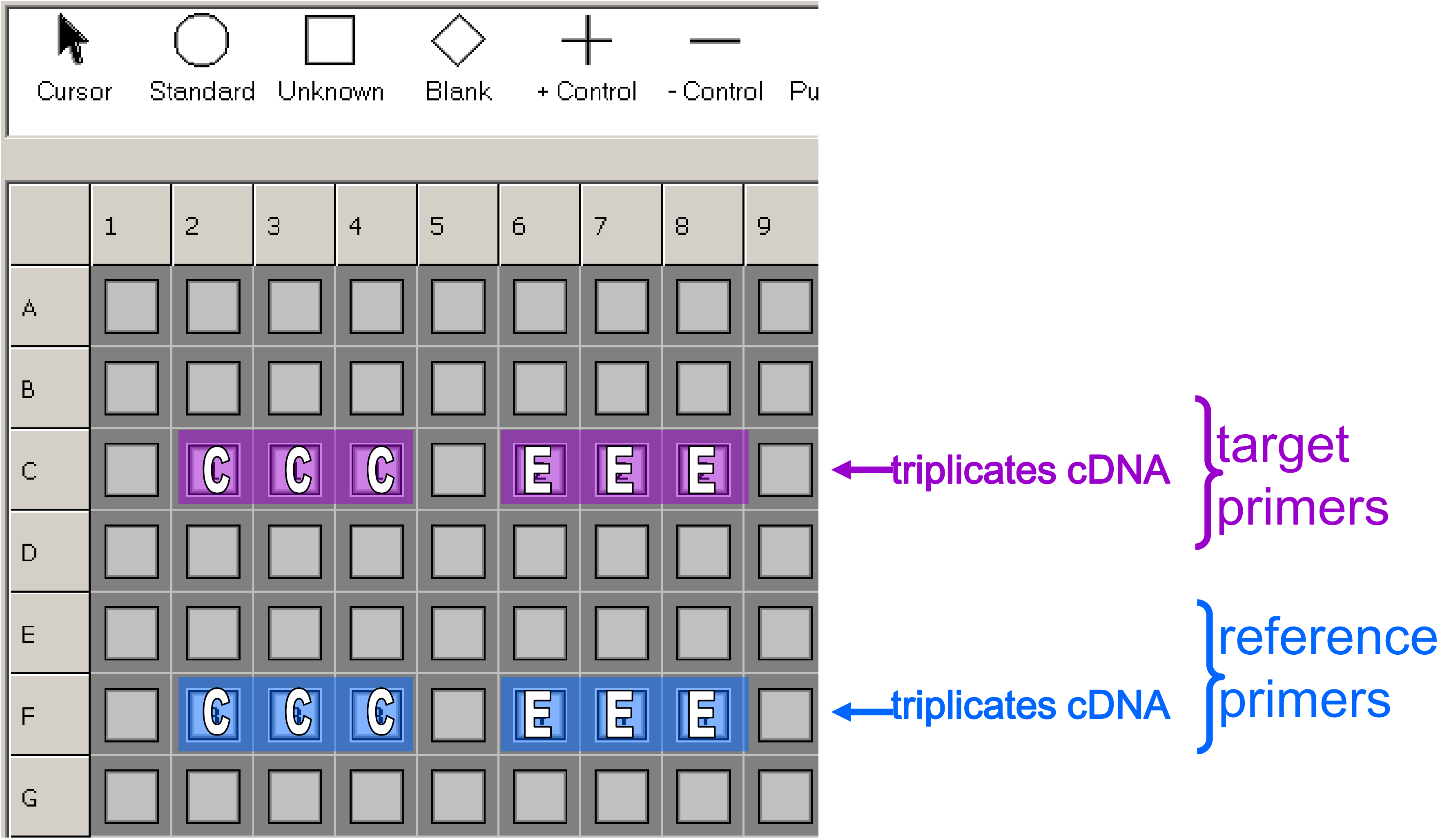
Nucleic Acids Research, 2001, 29:2002-2007

https://doi.org/10.1093%2Fnar%2F29.9.e45



$$\text{ratio} = \frac{\text{fold increase in target gene}}{\text{fold increase in reference gene}}$$

Microbiology and Immunology On-line; University of South Carolina School of Medicine


Cursor
Standard
Unknown
Blank
+ Control
- Control
Pu
1
2
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4
5
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A
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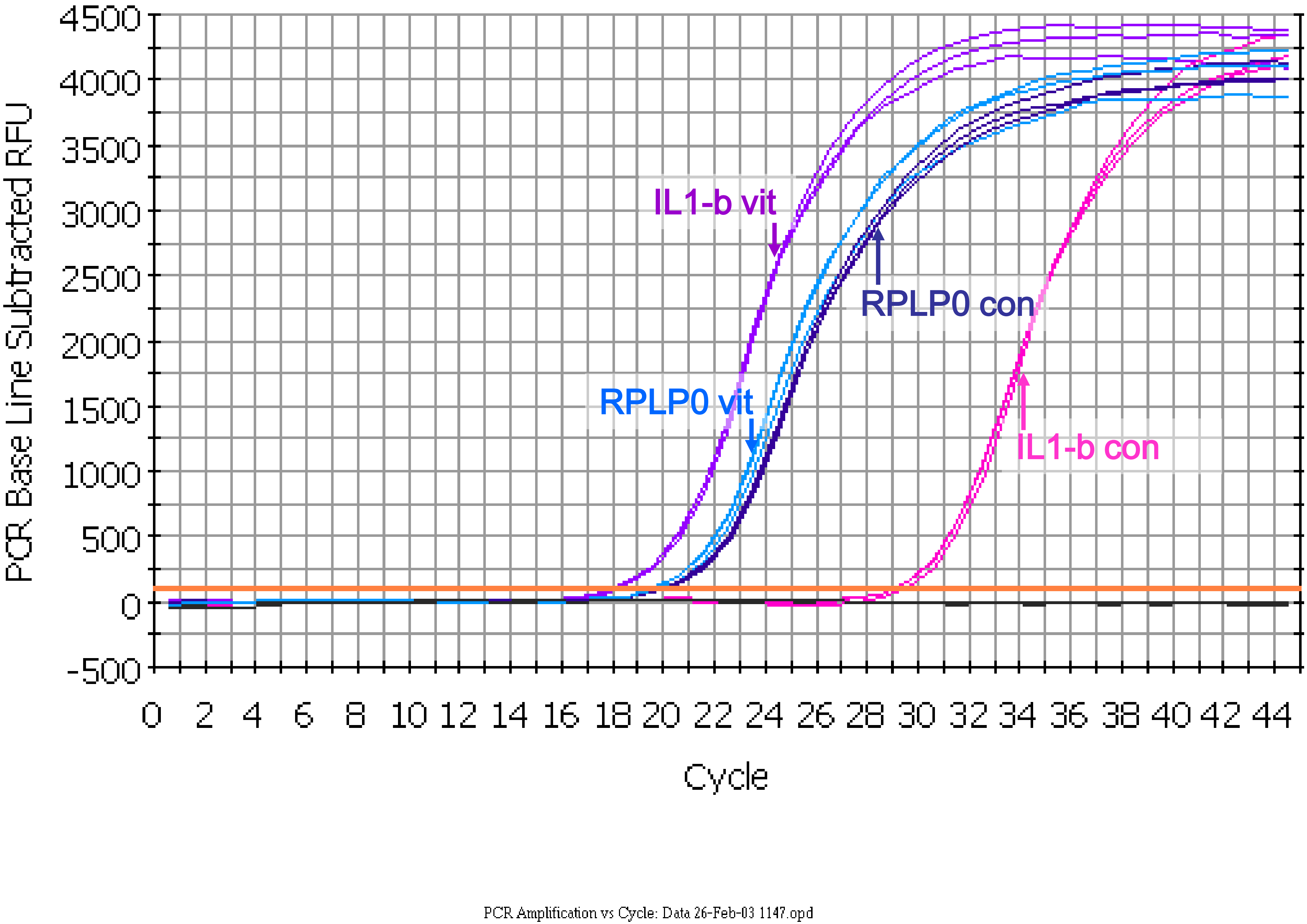
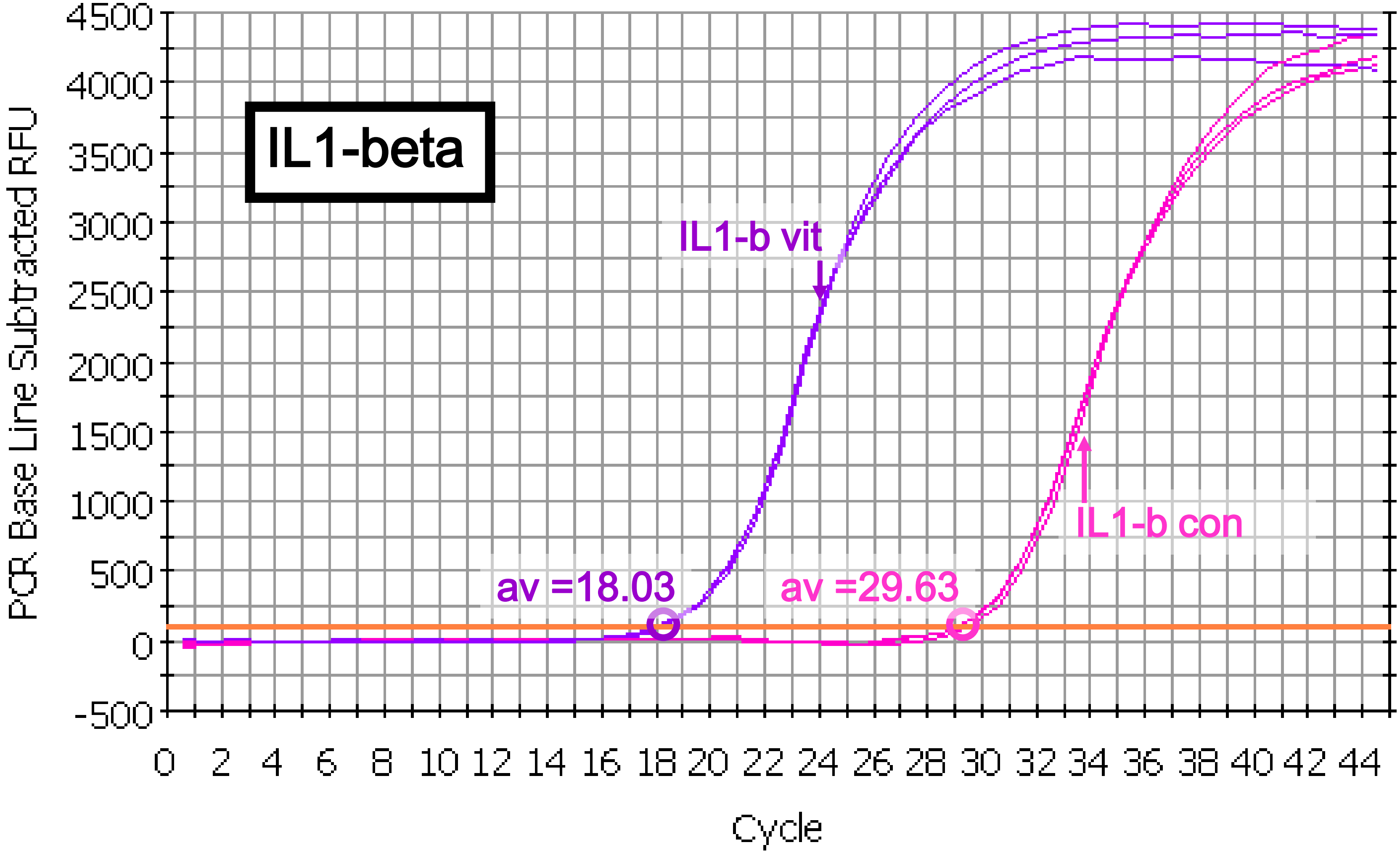
E
triplicates cDNA
target primers
triplicates cDNA
reference primers



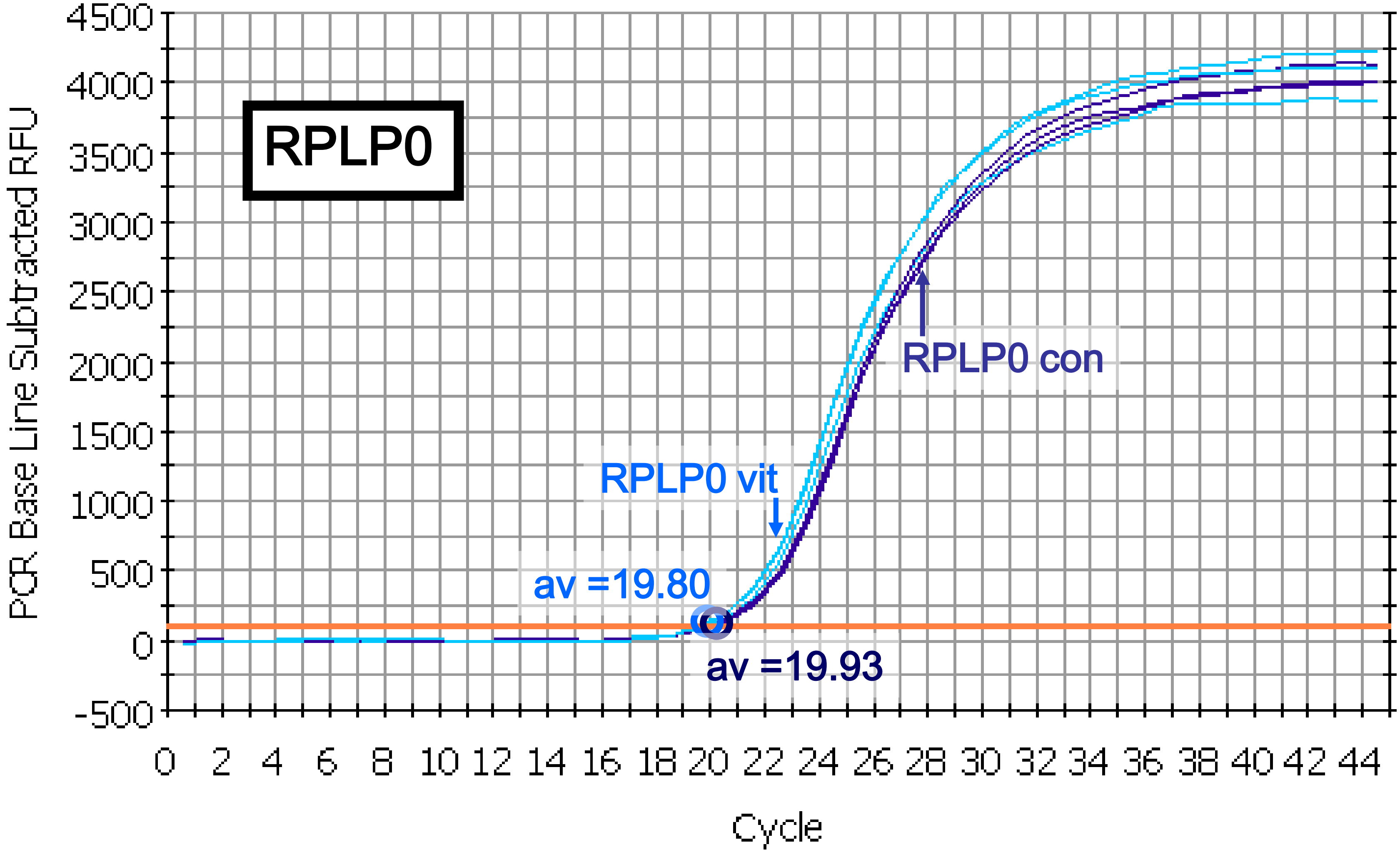
Microbiology and Immunology On-line; University of South Carolina School of Medicine



AFTER N CYCLES: change = (efficiency)^n

AFTER N CYCLES: ratio vit/con = $(1.93)^{29.63-18.03}$ = $1.93^{11.60}$ = 2053

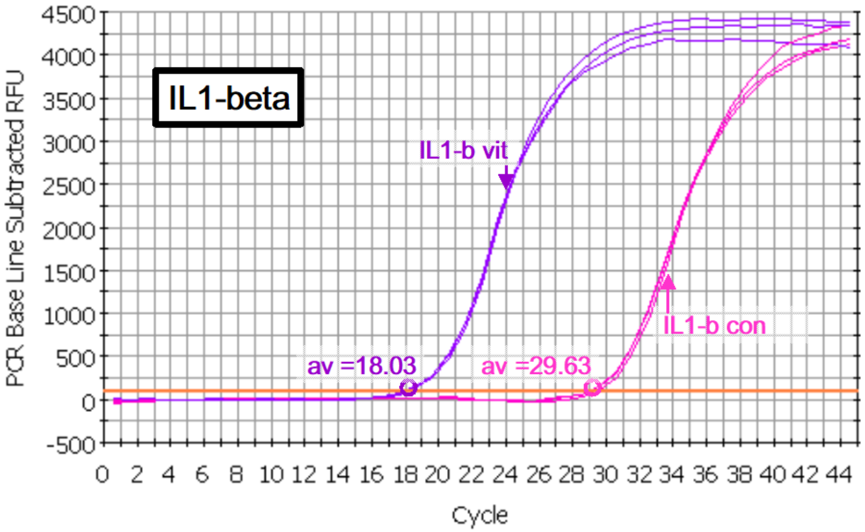
PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd



AFTER N CYCLES: change = (efficiency)^n

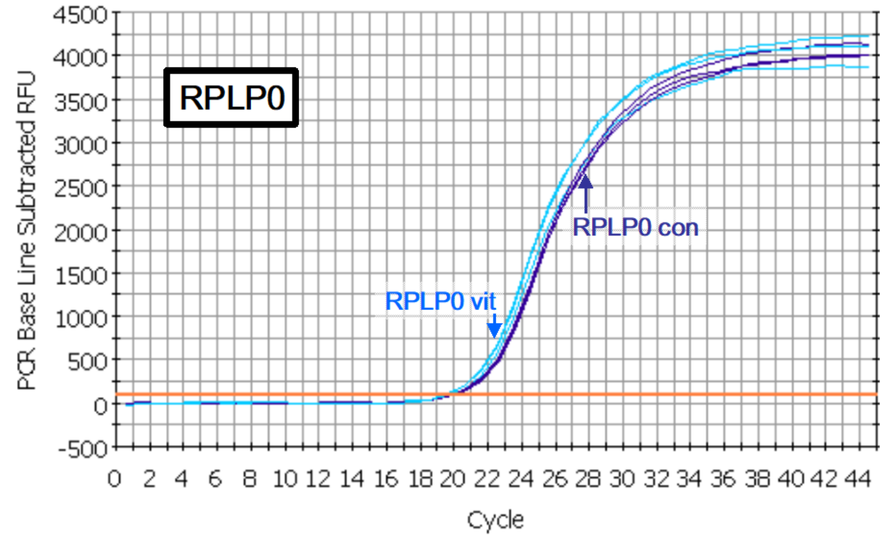
AFTER N CYCLES: ratio vit/con = $(1.87)^{19.93-19.80} = 1.87^{0.13} = 1.08$

PCR Amplification vs Cycle: Data 26-Feb-03 1147.opd



AFTER N CYCLES: increase = $(\text{efficiency})^n$

Ratio vit/con = $(1.93)^{29.63-18.03} = 1.93^{11.60} = 2053$



AFTER N CYCLES: increase = $(\text{efficiency})^n$

Ratio vit/con = $(1.87)^{19.93-19.80} = 1.87^{0.13} = 1.08$

$$\text{ratio} = \frac{\text{change in IL1-B}}{\text{change in RPLP0}} = 2053/1.08 = 1901$$

$$\text{ratio} = \frac{(E_{target})^{\Delta \text{ Ct target (control-treated)}}}{(E_{ref})^{\Delta \text{Ct ref (control-treated)}}}$$

Determination of reaction efficiency is essential!

Given: slope: -3.9 from a standard curve

Ct(sample A) = 23.5

Ct(sample B) = 26.5

Calculate: (A) PCR efficiency

$$E = 10^{-1/slope} - 1 = 80\ \%$$

(B) Relative expression between sample A and B:

$$(1+E)^{\Delta Ct} = 1.80^{(26.5-23.5)} = 5.8$$

(C) Interpretation:

Sample A contains 5.8 times more target than sample B

TATAA Biocenter

Determination of reaction efficiency is essential!

Given: slope: -3.5 from a standard curve

Ct(sample A) = 23.5

Ct(sample B) = 26.5

Calculate the relative expression:

$E = 10^{-1/slope} - 1 = 93\ \%$

$(1+E)^{\Delta Ct} = 1.93^{(26.5-23.5)} = 7.2$

Interpretation:

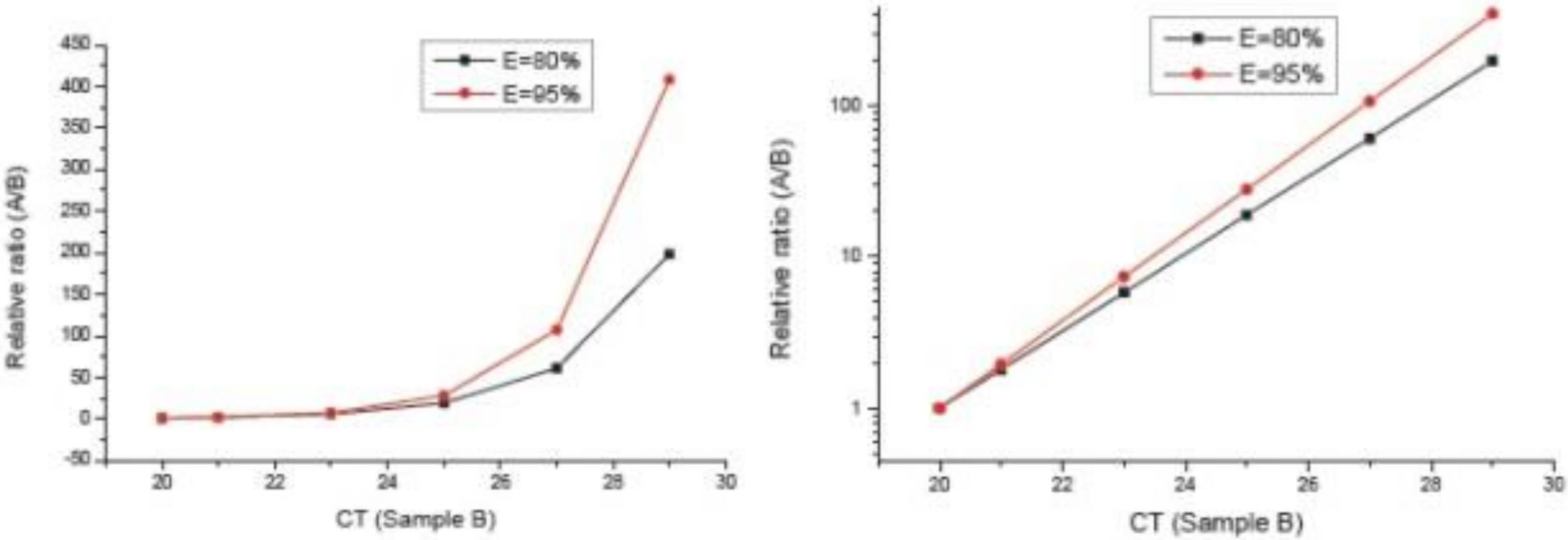
The relative expression increased from 5.8 to 7.2 when the PCR efficiency was 13% higher.

TATAA Biocenter

Determination of reaction efficiency is essential!

E = 80 %	$Ct_A = 20$	E = 95%
1.8	$Ct_B = 21$	1.95
5.8	$Ct_B = 23$	7.4
19	$Ct_B = 25$	28
61	$Ct_B = 27$	107
198	$Ct_B = 29$	408

Error in qPCR reaction accumulates exponentially!



The mRNA equation

$$\frac{N_A}{N_B} = K_{RS}\frac{\eta_B(1+E_B)^{CT_B-1}}{\eta_A(1+E_A)^{CT_A-1}}$$

η - RT efficiency CT - value (cycle) Ct

E - PCR yield K_{RS} - relative qPCR sensitivity

TATAA Biocenter

Quantification: 2 samples, 2 genes

$$\frac{\text{Sample A}}{\text{Sample B}} = \frac{\left[\dfrac{N0_{tar}}{N0_{ref}}\right]_{\text{SampleA}}}{\left[\dfrac{N0_{tar}}{N0_{ref}}\right]_{\text{Sample B}}} = \frac{\left[\dfrac{(1+E_{ref})^{Ct_{ref}}}{(1+E_{tar})^{Ct_{tar}}}\right]_{\text{SampleA}}}{\left[\dfrac{(1+E_{ref})^{Ct_{ref}}}{(1+E_{tar})^{Ct_{tar}}}\right]_{\text{Sample B}}}$$

$$\frac{\text{Sample A}}{\text{Sample B}} = \frac{(1+E_{ref})^{CtA_{ref}-CtB_{ref}}}{(1+E_{tar})^{CtA_{tar}-CtB_{tar}}}$$

"garbage in, garbage out"

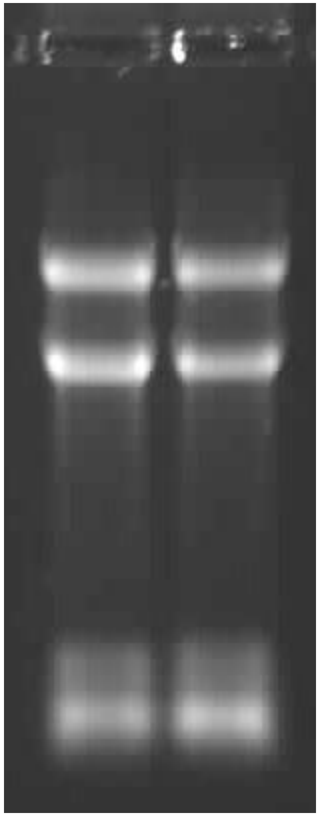
"Real-time quantitative RT-PCR is a wonderful method for fast, accurate, sensitive and cost-effective gene expression analysis. However, the simplicity of the technology itself makes it vulnerable for abuse in experiments in which the operator does not perform the required quality control throughout the entire procedure."

Derveaux S. et al, Methods 50 (2010) 227-230

RT-qPCR in practice

RNA quality is a key factor!!!

DNase treatment
1h, 37° C
\- +



RNA quality = purity + integrity

Purity – determined spectrophotometrically

($OD_{260/230}$ >2.0; $OD_{260/280}$ >1.8)

RNA quality control after isolation is always necessary!

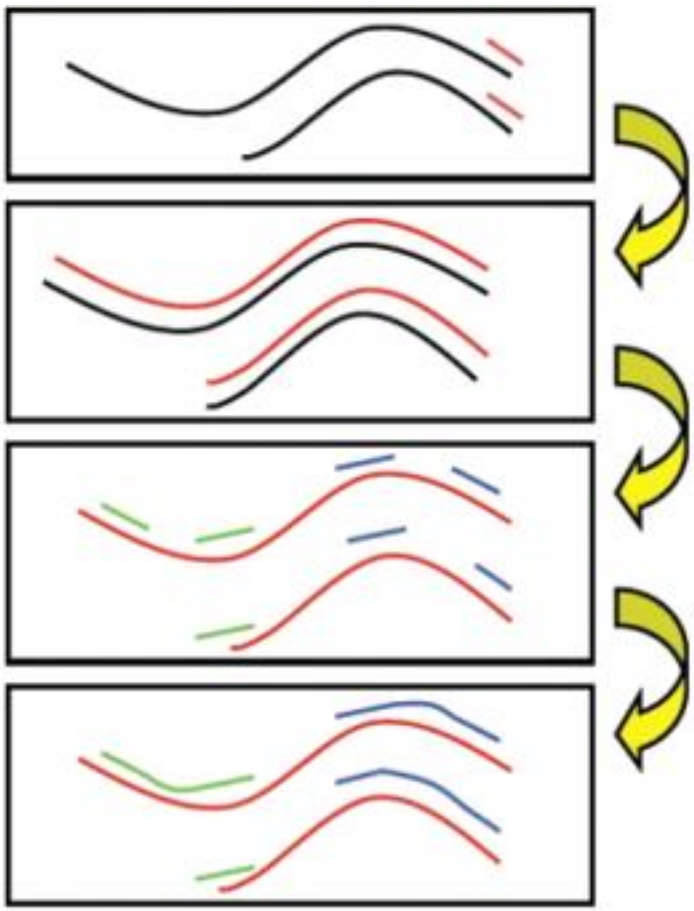
DNase treatment is always necessary!

20% of human genes have 1 exon, or there are expressed retropseudogenes or copies lacking introns!!!

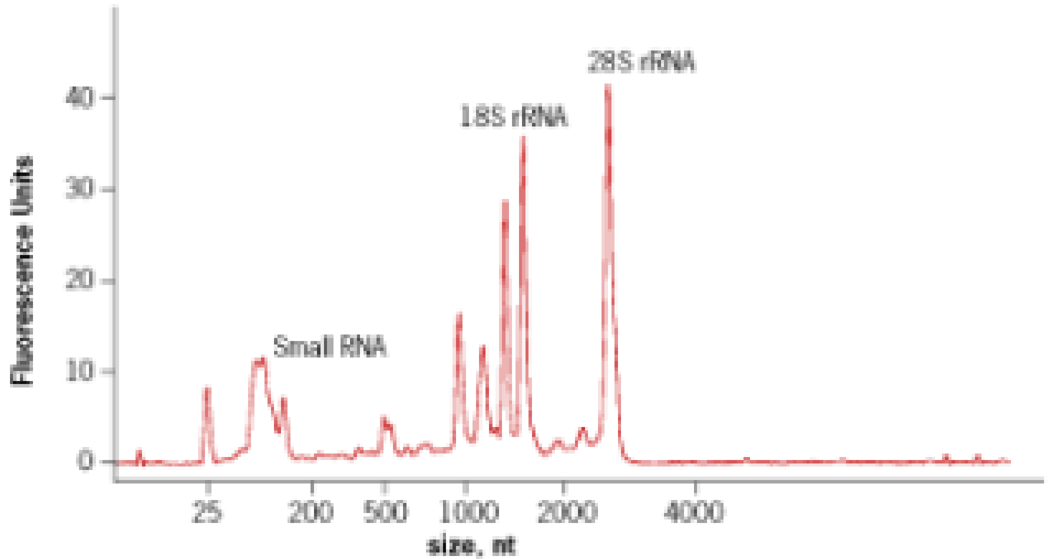
Recommended use of RNase inhibitors at RT!

RNA quality assays

5'-3' RNA ends integrity test

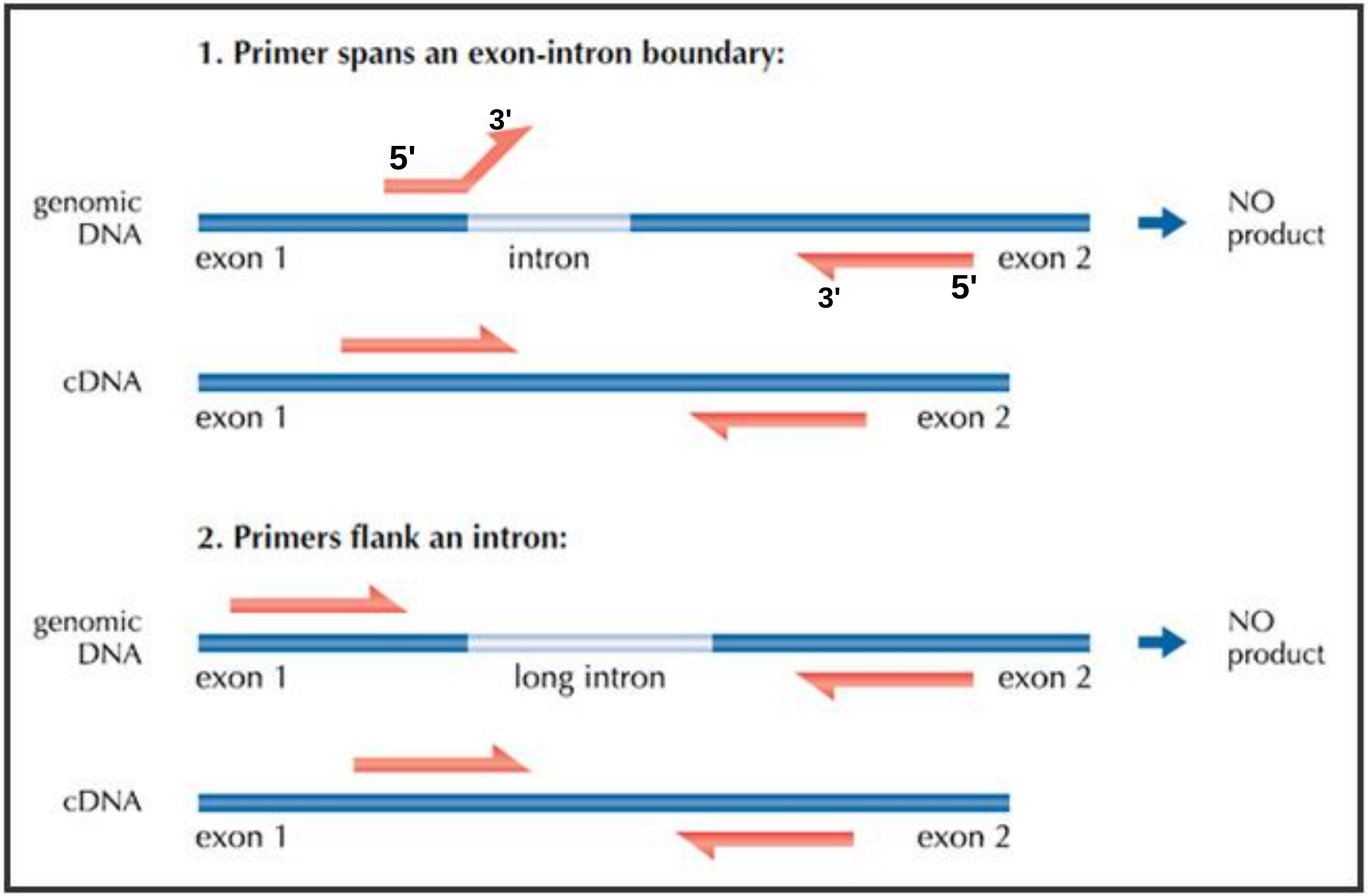


Microfluidic Analyzers



- **Agilent: Bioanalyzer, Tapestation**
- **Biorad: Experion**
- **Capillary electrophoresis**

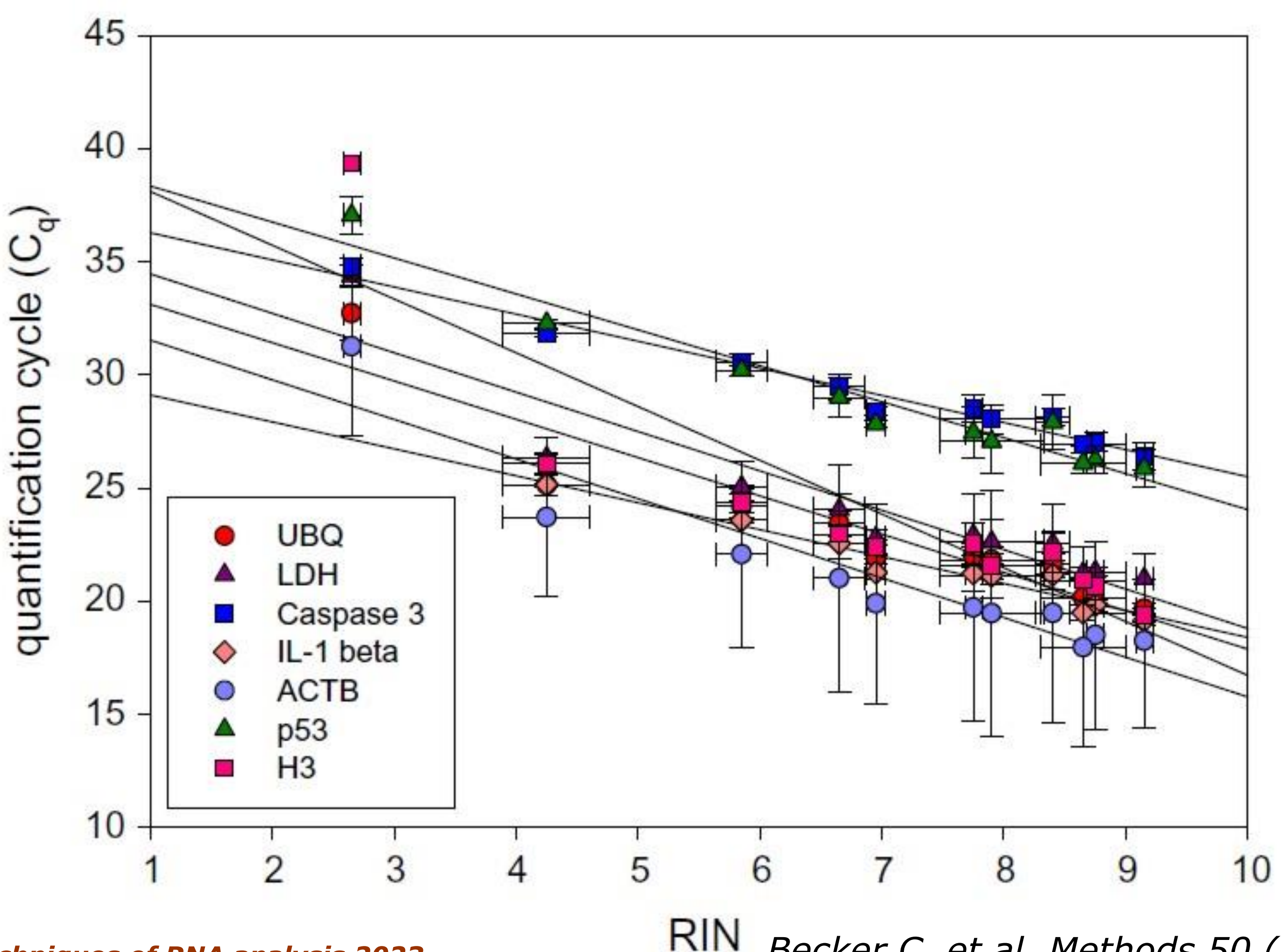
Primer design strategies to minimize signals for gDNA contamination



https://www.thermofisher.com/pl/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/basic-principles-rt-qpcr.html

Quality affects results!!!

RIN: "RNA Integrity Number", min. 7
(according to Bioanalyzer, Agilent Technologies).



Becker C. et al, Methods 50 (2010) 237-243

The most crucial step of RT-qPCR is the

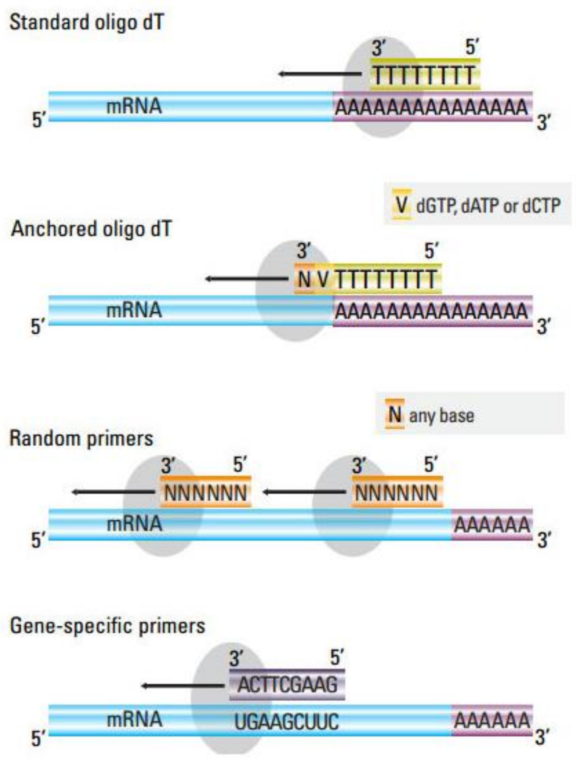
reverse transcription reaction!!!

Efficiency and specificity of RT

Efficiency and specificity of reverse transcription reaction STRONGLY depends on:

- **Methods of priming**
- **Type of reverse transcriptase (features of the enzyme)**
- **The sequence of a particular RNA molecule – it's tendency to form complex secondary structures**
- **Efficiency differences can be as high as 100X!!!**
- **The optimal RT conditions should always be determined experimentally**

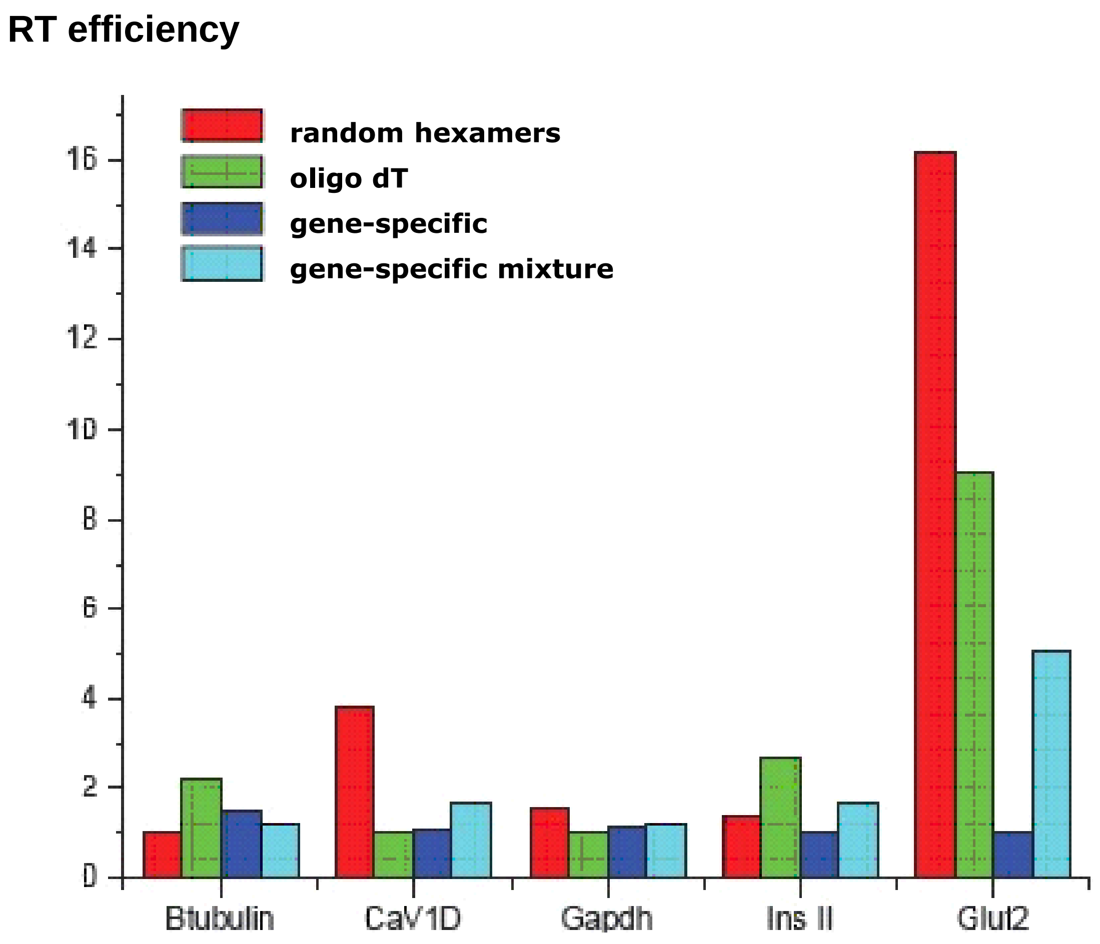
RT priming methods – advantages and disadvantages



Primer Options	Structure and Function	Advantages	Disadvantages
Oligo(dT)s (or anchored oligo(dT)s)	Stretch of thymine residues that anneal to poly(A) tail of mRNA; anchored oligo(dT)s contain one G, C, or A (the anchor) residue at the 3' end	•Generation of full length cDNA from poly(A)-tailed mRNA •Good to use if little starting material is available •Anchor ensures that the oligo(dT) primer binds at the 5' end of the poly(A) tail of mRNA	•Only amplify gene with a poly(A) tail •Truncated cDNA from priming internal poly(A) sites*2 •Bias towards 3' end*. *Minimized if anchored oligo(dT)s are used.
Random Primers	Six to nine bases long, they anneal at multiple points along RNA transcript	•Anneal to all RNA (tRNA, rRNA, and mRNA) •Good to use for transcripts with significant secondary structures, or if little starting material is available •High cDNA yield	•cDNA is made from all RNAs which is not always desirable and can dilute mRNA signaling •Truncated cDNA
Sequence Specific Primers	Custom made primers that target specific mRNA sequence	•Specifc cDNA pool •Increased sensitivity •Use reverse qPCR primer	•Synthesis is limited to one gene of interest

https://www.thermofisher.com/pl/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/basic-principles-rt-qpcr.html

Effect of the priming method on RT efficiency



Sthalberg et al, 2004, Comparison of reverse transcriptases In gene expression analysis, Clin Chem. 50(9):1678-80.

Specificity of priming methods

RT priming[a]	β-tubulin[b]	CaV1D[b]	GAPDH[b]	Insulin II[b]	Glut2[b]
hexamers	19.5	26.5	15.8	16.9	27.5
oligo(dT)	18.1	28.8	16.6	15.9	28.4
specific	18.8	28.7	16.4	17.4	31.8
mix	19.1	27.9	16.2	16.6	29.3
maxΔCt[c]	1.4	2.3	0.8	1.5	4.4

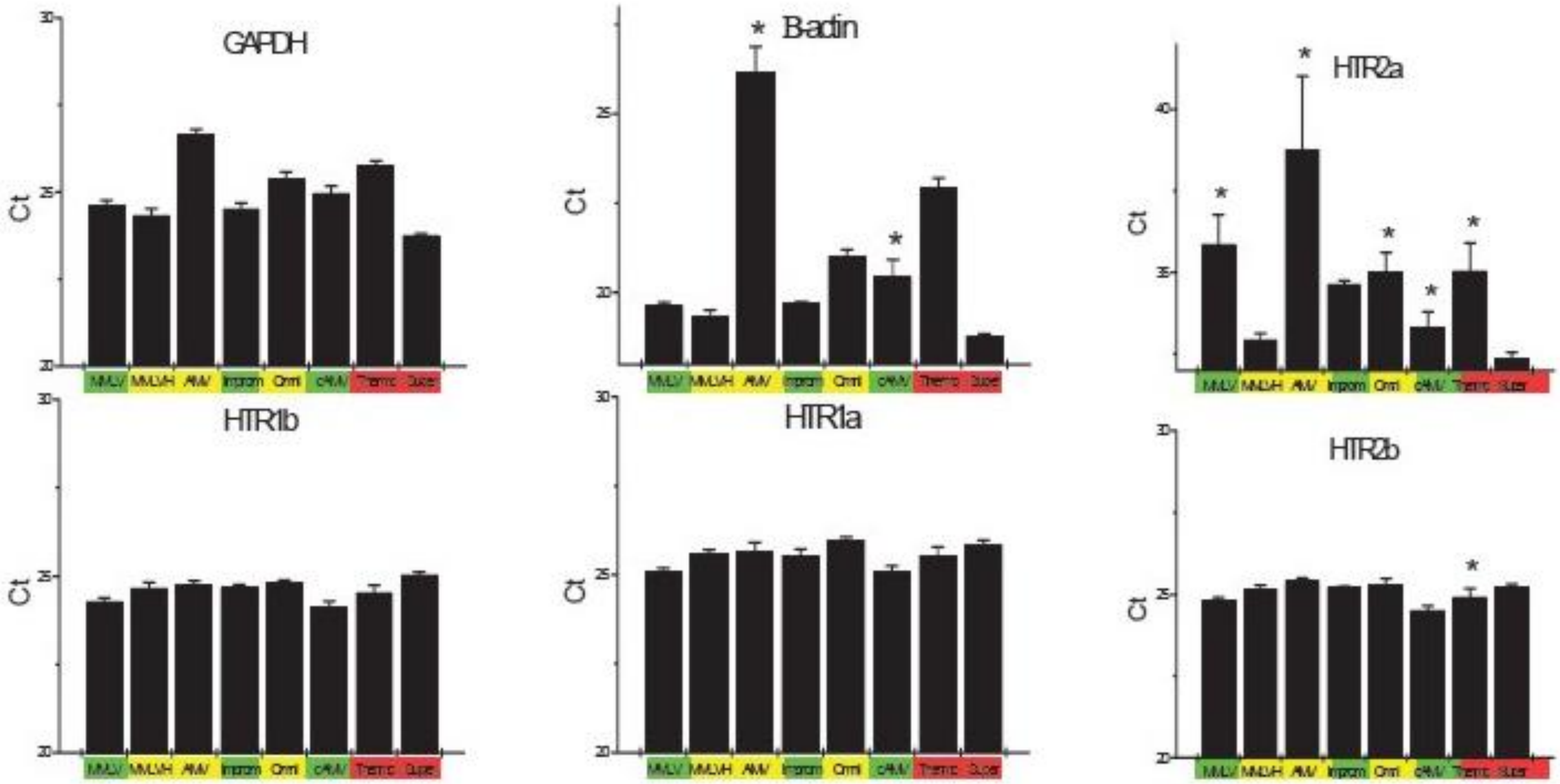
Stahlberg et al, 2004, Comparison of reverse transcriptases In gene expression analysis, Clin Chem. 50(9):1678-80.

Effect of the priming method on RT efficiency

RT priming	β-tubulin[a]	CaV1D[a]	GAPDH[a]	Insulin II[a]	Glut2[a]
β-tubulin	18.8	28.7	19.0	18.8	30.6
CaV1D	27.0	28.7	19.9	22.8	b
GAPDH	23.4	30.1	16.4	20.1	29.7
Insulin2	23.5	31.6	20.0	17.4	31.0
Glut2	25.8	31.9	22.7	22.7	31.8
no primer	27.6	33.7	23.6	23.1	32.6

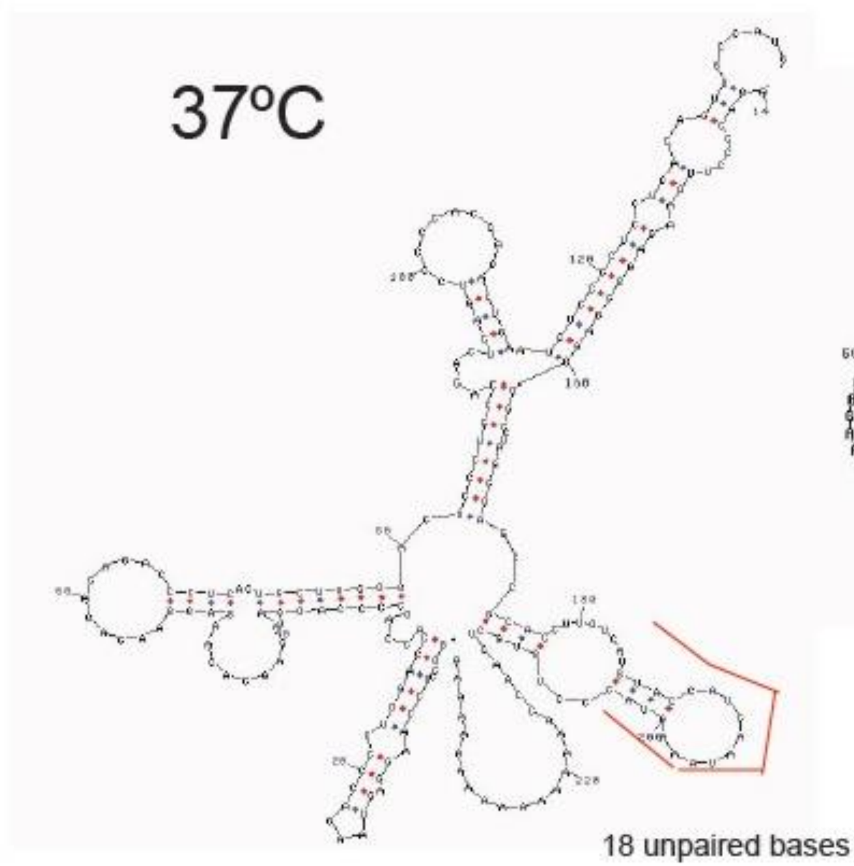
Stahlberg et al, 2004, Comparison of reverse transcriptases In gene expression analysis, Clin Chem. 50(9):1678-80.

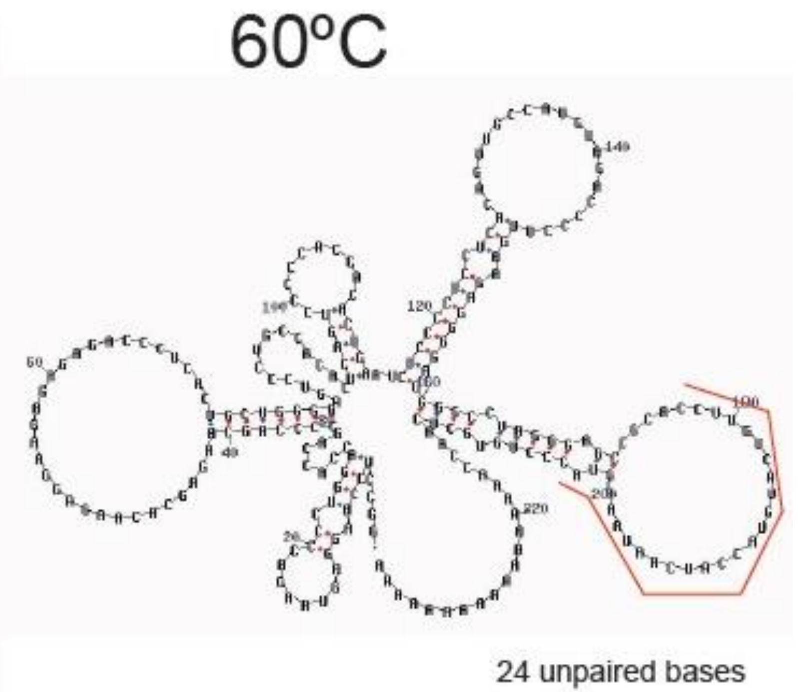
Differences in RT reaction efficiency can reach 100X!



Stahlberg et al, 2004, Comparison of reverse transcriptases
In gene expression analysis, Clin Chem. 50(9):1678-80.

RT optimization





Behind the TATA Biocenter

Always CONTROLS!!!

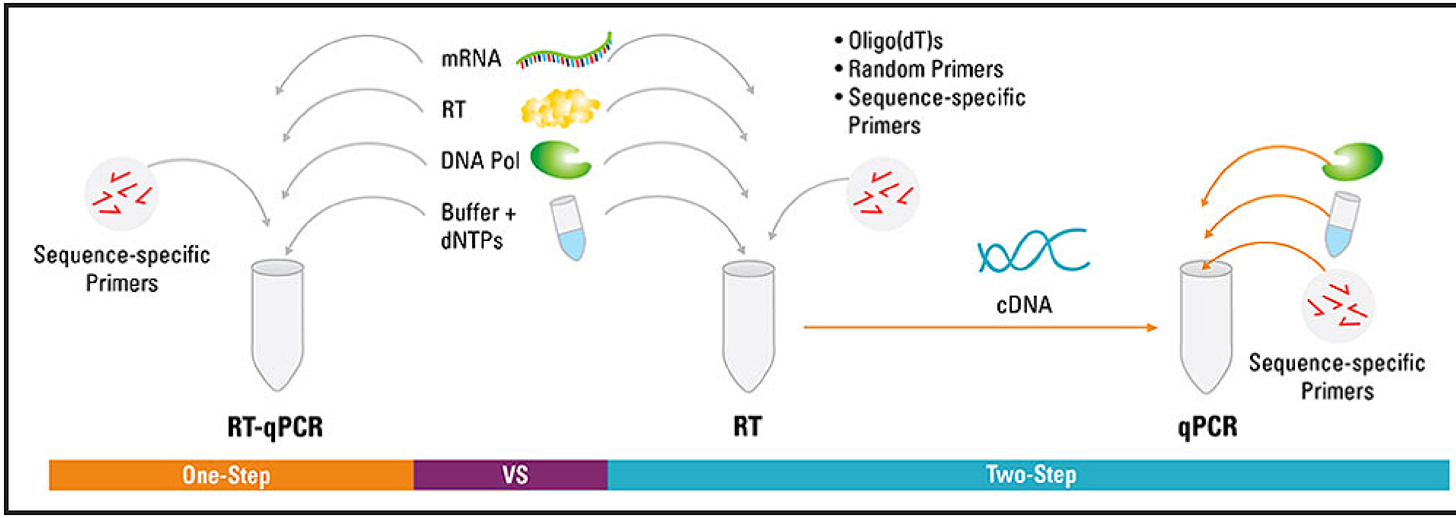
Negative Controls:

- -RT: control of genomic DNA contamination, necessary!
- NTC: "no template controle": reagent purity control!!!

Positive control:

- min. 2-3 samples, e.g., from the standard curve - control of reaction performance! (**IRC** = inter-run calibrators)

Choice between 1-step or 2-step RT-qPCR reaction



	Advantages	Disadvantages
1-step	•Less experimental variation since both reactions take place in the same tube •Fewer pipetting steps reduces risk of contamination •Suitable for high throughput amplification/screening Fast and highly reproducible	•Impossible to optimize the two reactions separately •Less sensitive than two-step because the reaction conditions are a compromise between the two combined reactions •Detection of fewer targets per sample
2-step	•A stable cDNA pool is generated that can be stored for long periods of time and used for multiple reactions •The target and reference genes can be amplified from the same cDNA pool without multiplexing •Optimized reaction buffers and reaction conditions can be used for each individual reaction •Flexible priming options	•The use of several tubes and pipetting steps exposes the reaction to a greater risk of DNA contamination Time consuming •Requires more optimization than one-step

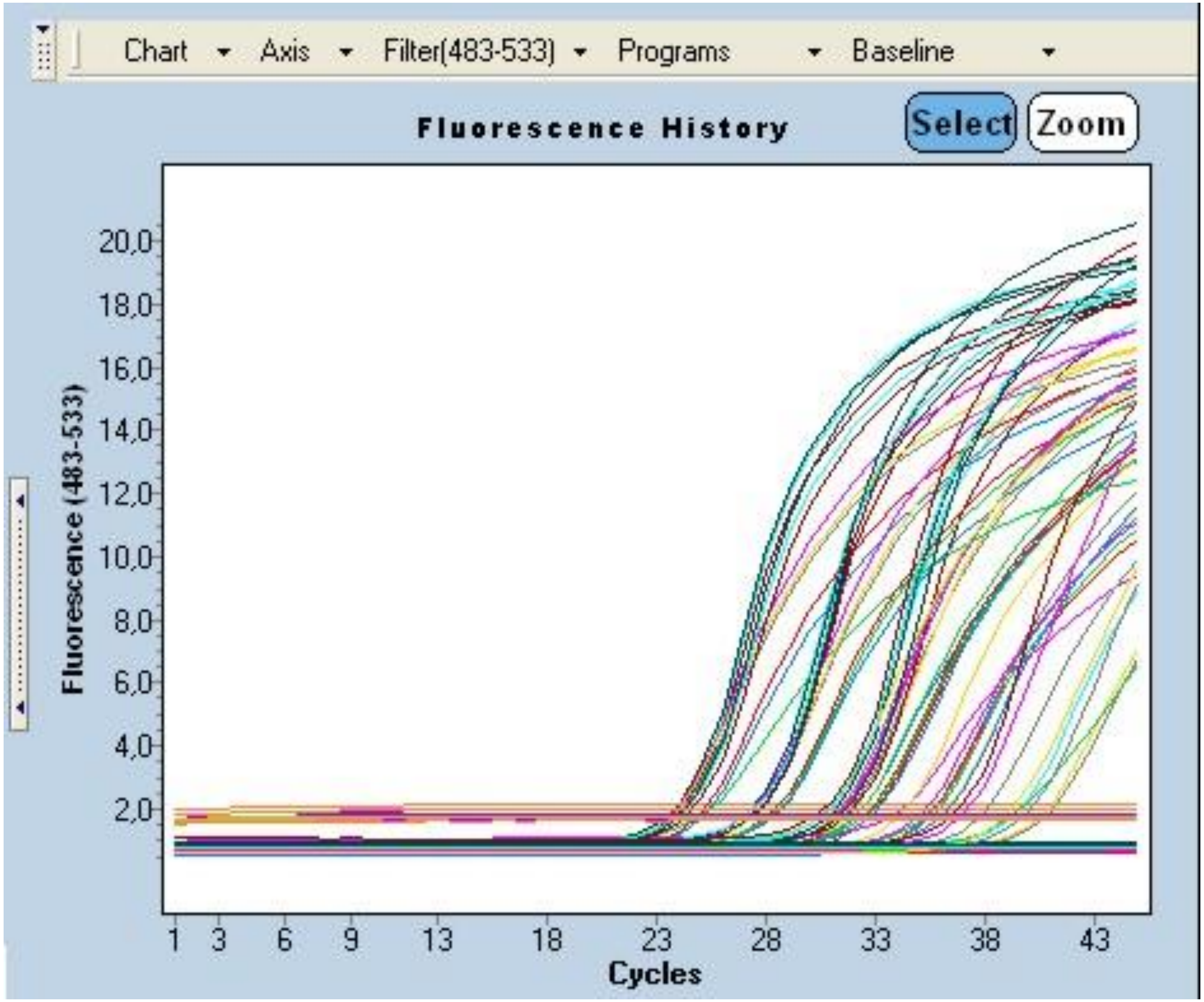
https://www.thermofisher.com/pl/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/basic-principles-rt-qpcr.html

Good laboratory practice
IT'S BASIC!!!

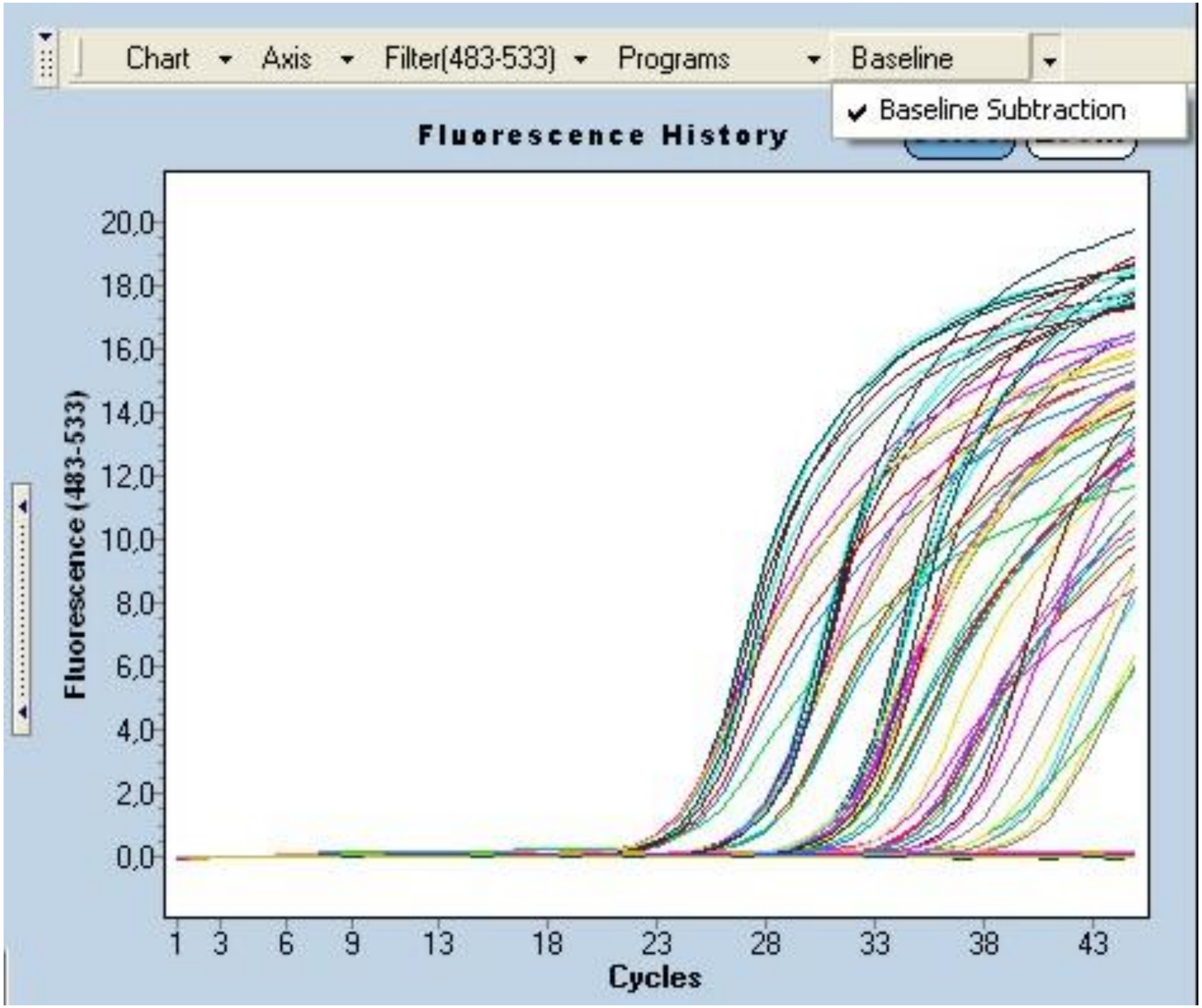
- Do not pipette less than 2µl at a time by hand!
- Separate place for preparing the reaction without templat and separate for adding DNA!
- Powder-free gloves!
- Prepare reactions in "MIX" not separate!
- Do not open tubes/plates after reaction in PCR rooms!
- Laminar flow chambers!

Basic analysis of qPCR reactions

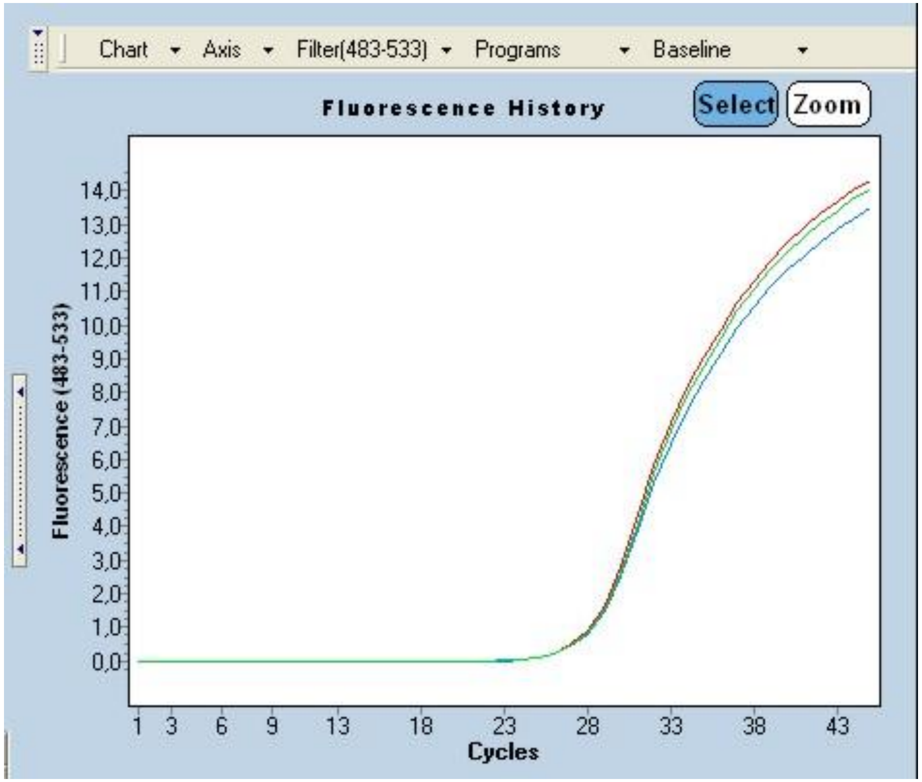
Amplification curves - raw data

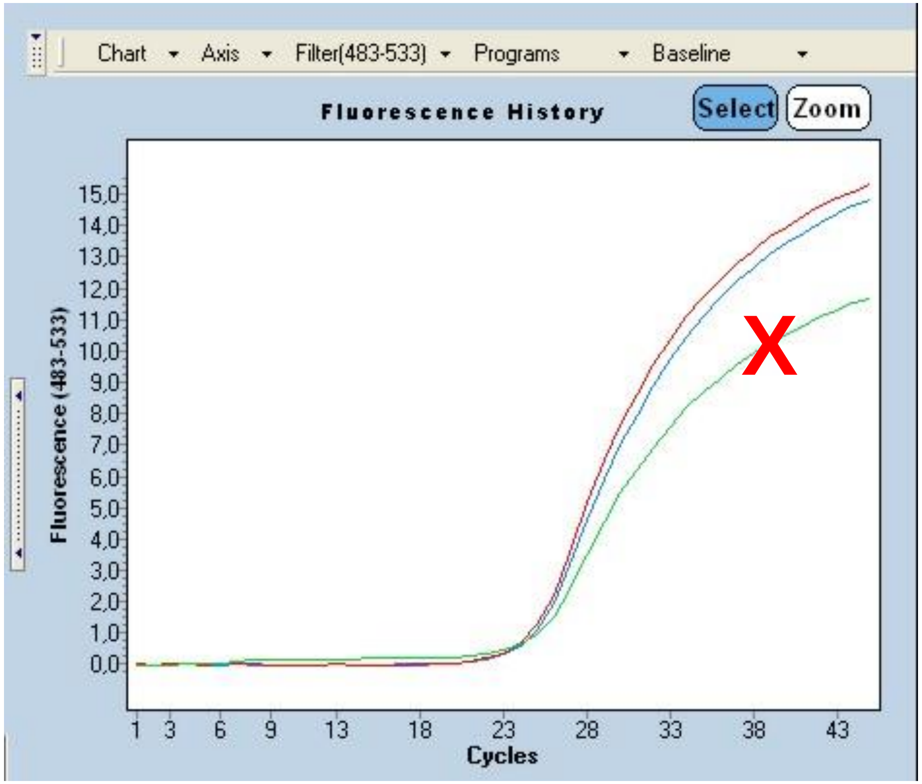


Baseline subtraction



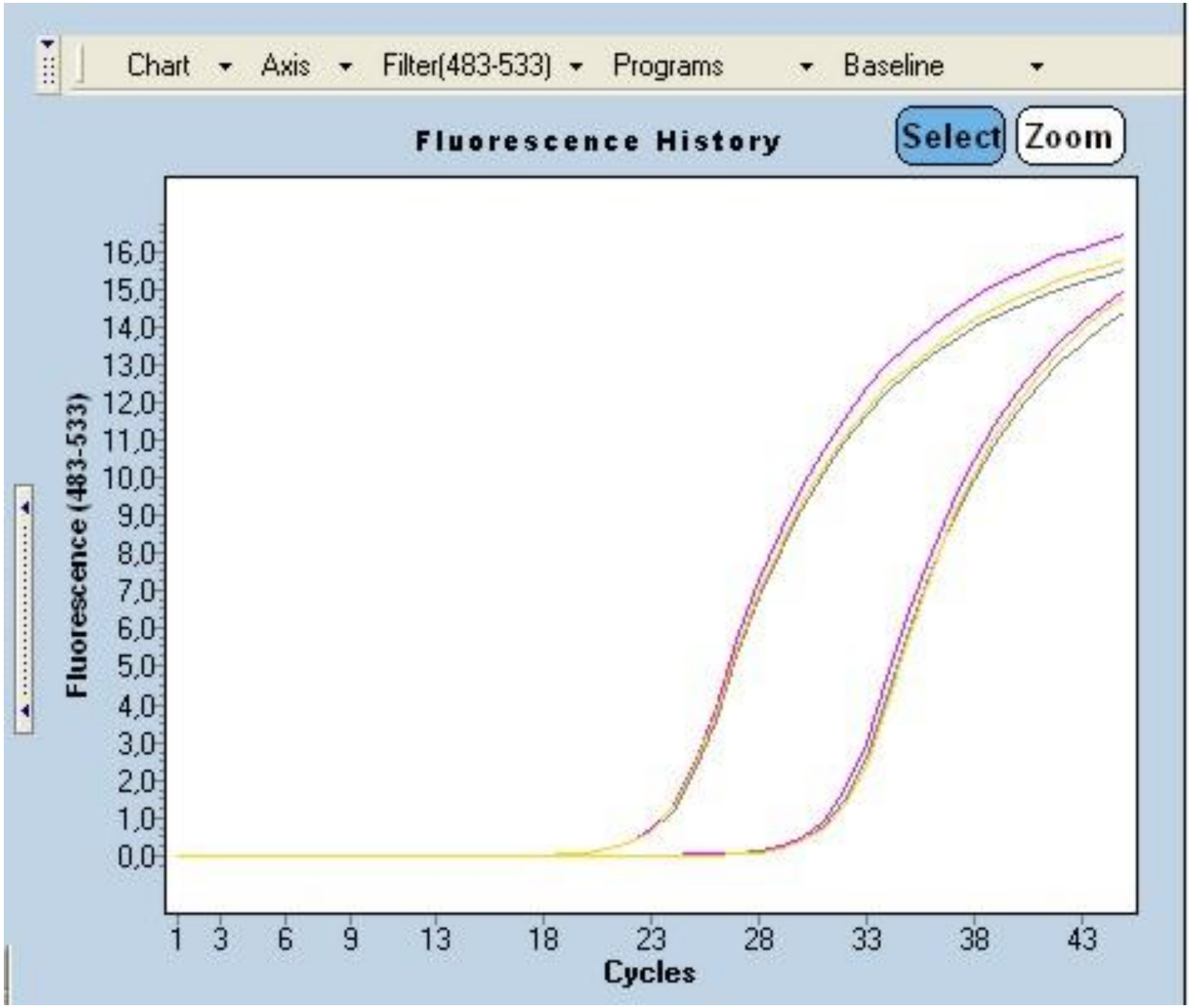
1 qPCR = 3 technical replicas!!!





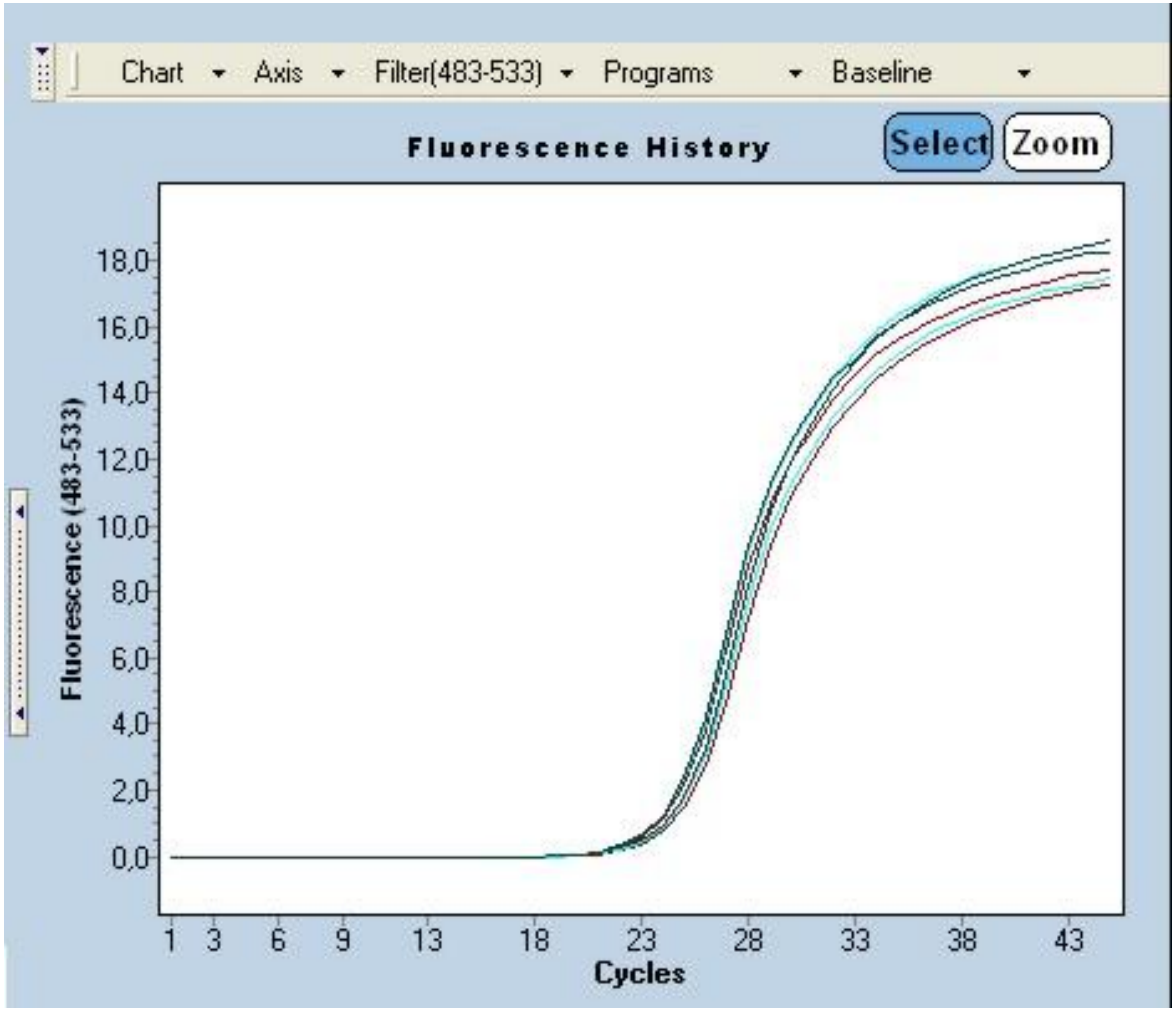
Inspection of amplification curves

Gene tested



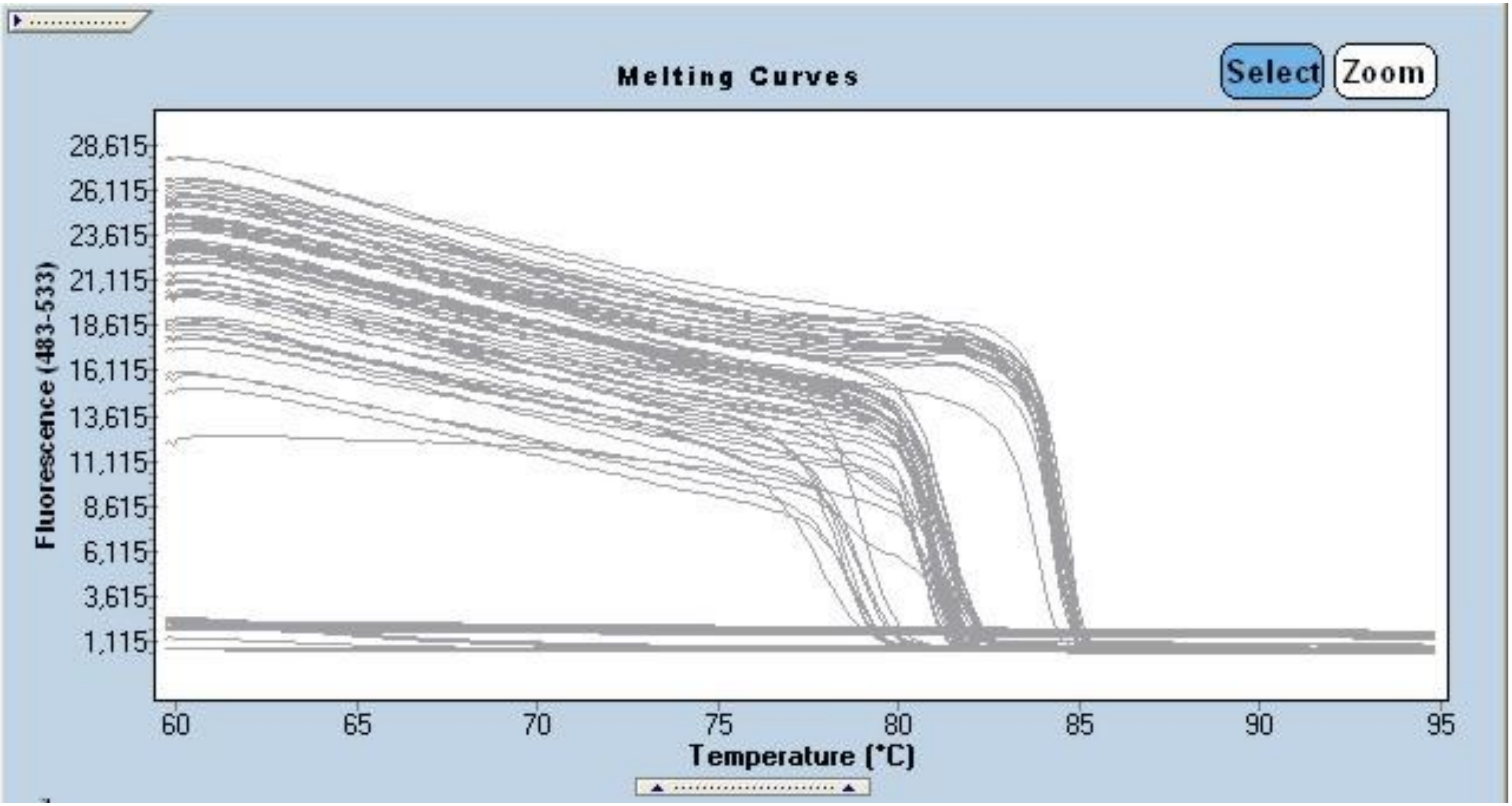
Inspection of amplification curves

Reference gene



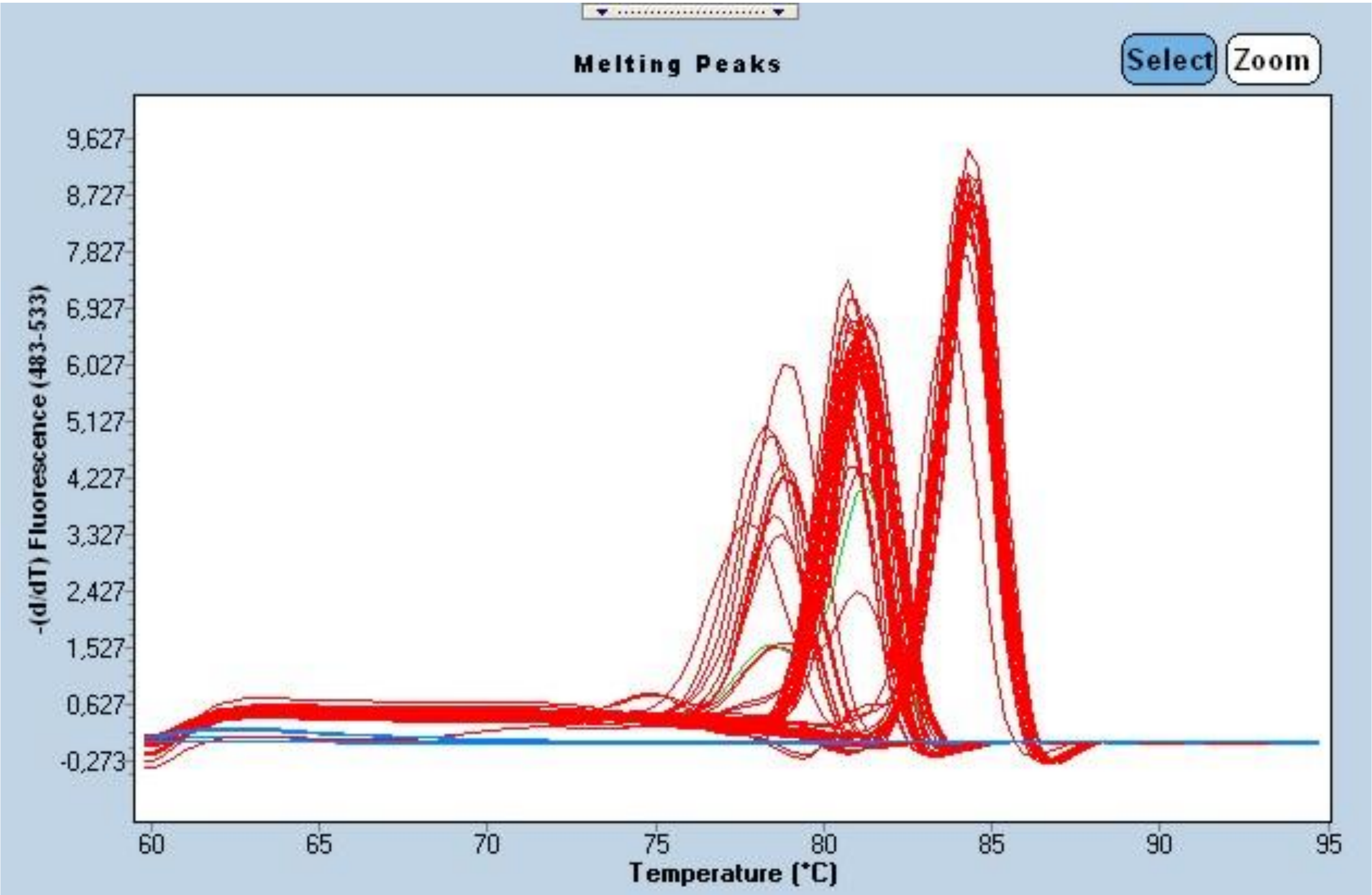
Analysis of melting curves

Possible mainly for SYBR Green



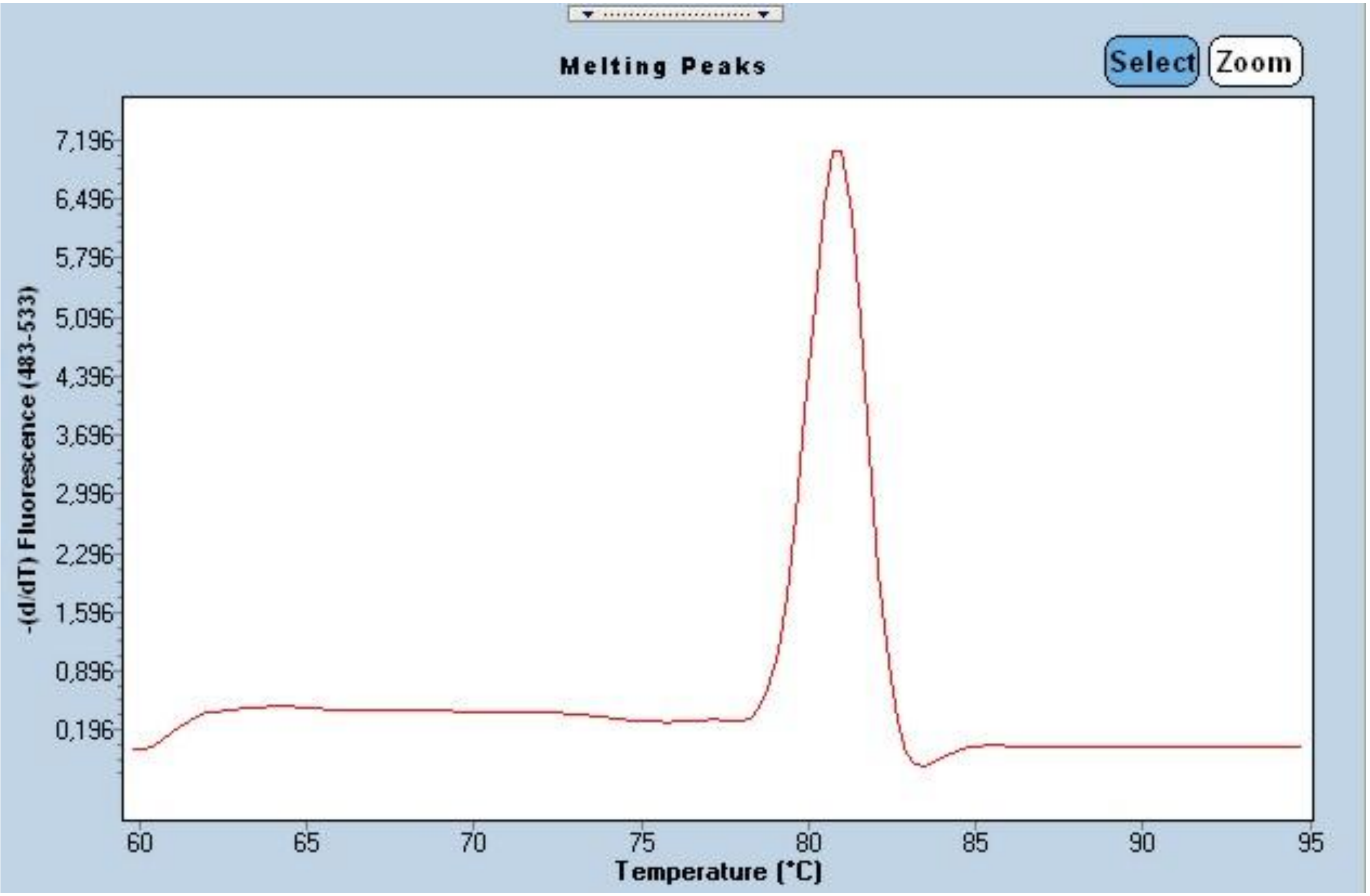
Analysis of melting curves

Tm – depends on the length and GC/AT content



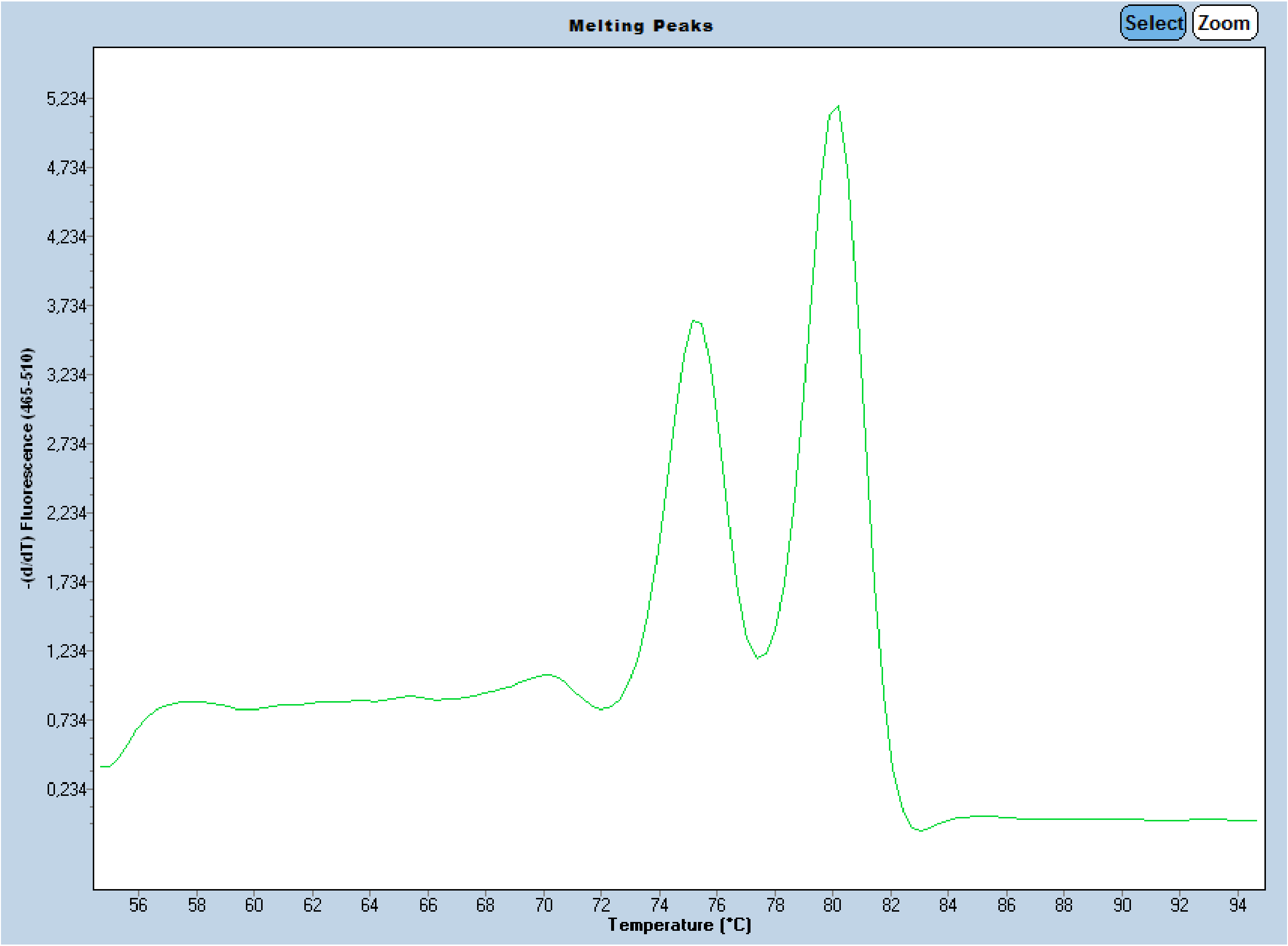
Analysis of melting curves

Pure, specific qPCR product



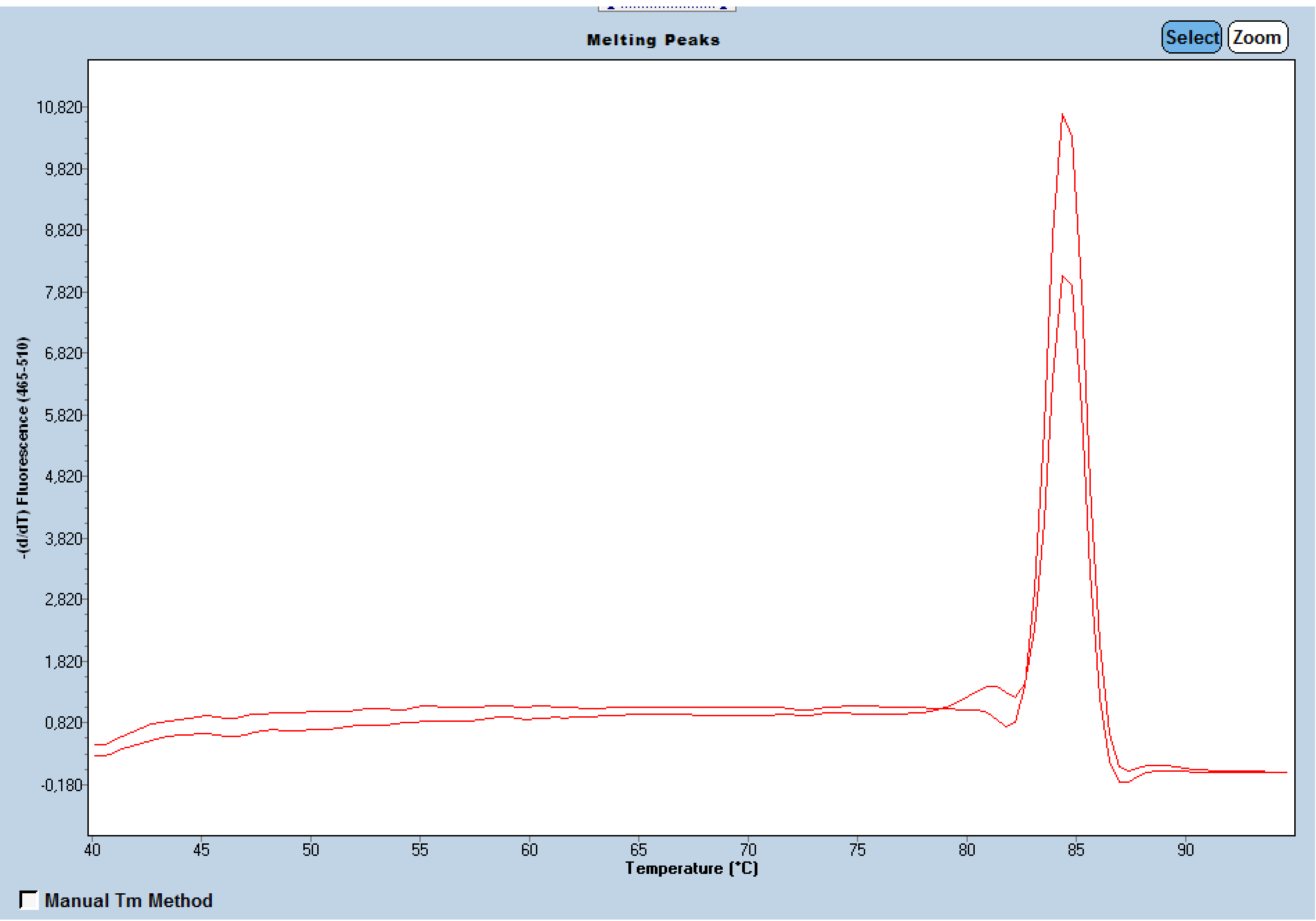
Analysis of melting curves

Non-specific products



Analysis of melting curves

The primer-dimer problem



Analysis of melting curves

Do not blindly believe the melting curves!!!

A longer AT-rich product can have the same Tm as a shorter GC-rich one!!!

Always check the products for each new primer pair on a high-resolution gel! At least once, at the "reaction setup" stage.

Standardization

Reference gene selection

THERE IS NO "PERFECT" REFERENCE GENE!!!

- **Equal number of RNA copies in all cells**
- **Expressed in all cells**
- **Expressed at a medium level**

- **At least 2 different reference genes should be used! Better 3 to even 5!!!**
- **Standardize against the geometric mean for the reference.**
- **Genes should be selected EXPERIMENTALLY from a larger group!!!**

Vandesompele et al, Genome Biology, 2002,

Statistical analysis of Cq value variation for reference genes

geNorm

Change Data	GAPD	ACTB	HPRT1	UBC	YHWAZ	Normalisation
FIB1	0,516257	0,499303	0,482906	0,447207	0,572560	0,5028
FIB2	0,287796	0,238713	0,313899	0,221805	0,351638	0,2787
FIB3	0,160974	0,262108	0,147588	0,177935	0,306647	0,2024
FIB4	0,462392	0,151078	0,284928	0,372176	0,221805	0,2774
FIB5	0,694914	0,678860	0,765572	0,572560	0,851906	0,7066
FIB6	0,001146	0,000160	0,000765	0,000377	0,000690	0,0005
FIB7	0,487767	0,574475	0,512821	0,418316	0,685694	0,5285
FIB8	0,192781	0,183976	0,151584	0,181536	0,169808	0,1753
FIB9	0,393914	0,281148	0,386101	0,411390	0,342369	0,3597
FIB10	0,011902	0,005503	0,009390	0,010310	0,012347	0,0095
FIB11	0,016844	0,008107	0,013740	0,022522	0,023837	0,0159
FIB12	0,011059	0,014301	0,011902	0,017709	0,015443	0,0139
FIB13	0,008438	0,007141	0,009676	0,010589	0,014206	0,0097
FIB14	0,593982	0,697238	0,624490	0,550071	0,708977	0,6320
FIB15	0,283978	0,196683	0,218133	0,445716	0,234760	0,2637
FIB16	0,572560	0,423941	0,544589	0,414147	0,528466	0,4923
FIB17	0,720913	0,990033	0,877896	0,880833	0,983443	0,8850
FIB18	0,514536	0,504330	0,533786	0,590028	0,467047	0,5204
FIB19	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,00E+00	1,0000
FIB20	0,399211	0,316002	0,283978	0,441273	0,349298	0,3535
M < 1.5	0.513	0.664	0.432	0.523	0.475	

https://genorm.cmgg.be/

Vandesompele et al., Genome Biology, 2002,

Nowadays geNorm is part of Biogazelle's qbase+ software for quantitative PCR data-analysis

https://www.qbaseplus.com/

Reference gene selection

Alternatively…

- **Trust published data, e.g., for human cells: GAPDH, albumin, actin, tubulin, cyclophilin, microglobulin, 18S or 28S rRNA… though risky**

- **e.g. https://www.gene-quantification.de/hkg.html#refgenes**

- **Or use commercial primer panels (only possible for some model organisms)**

Layout design of the experiment

RT error accumulation

$$SD^2_{mRNA} = SD^2_{RT} + SD^2_{QPCR}$$

2 experimental designs

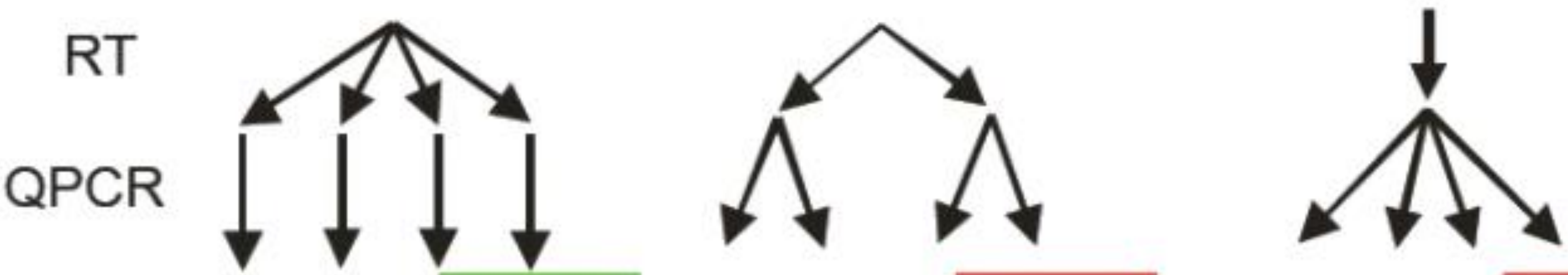
- **The "sample maximization" method: as many different samples as possible analyzed in a single run of the instrument. That is, different genes analyzed in different reactions (preferred in routine research work - many culture conditions, many mutants, etc.).**

- **The "gene maximization" method: as many different primer pairs as possible during a single run of the device (preferred in commercial diagnostic kits: one patient - one reaction plate for multiple genes)**

- **Whichever method you choose, use IRC**

Hellemans et al, Genome Biology, 2007,

How many biological repeats, RT, qPCR?



$$e=t\left(\frac{SD_{RT}^{2}}{4}+\frac{SD_{QPCR}^{2}}{4}\right)\approx t\frac{26SD_{QPCR}}{4}$$

$$e=t\left(\frac{SD_{RT}^{2}}{2}+\frac{SD_{QPCR}^{2}}{4}\right)\approx t\frac{51SD_{QPCR}}{4}$$

$$e=t\left(\frac{SD_{RT}^{2}}{1}+\frac{SD_{QPCR}^{2}}{4}\right)\approx t\frac{101SD_{QPCR}}{4}$$

Ref: Ståhlberg *et.al.* Properties of the reverse transcription reaction in mRNA quantification Clin Chem 2004;50:3

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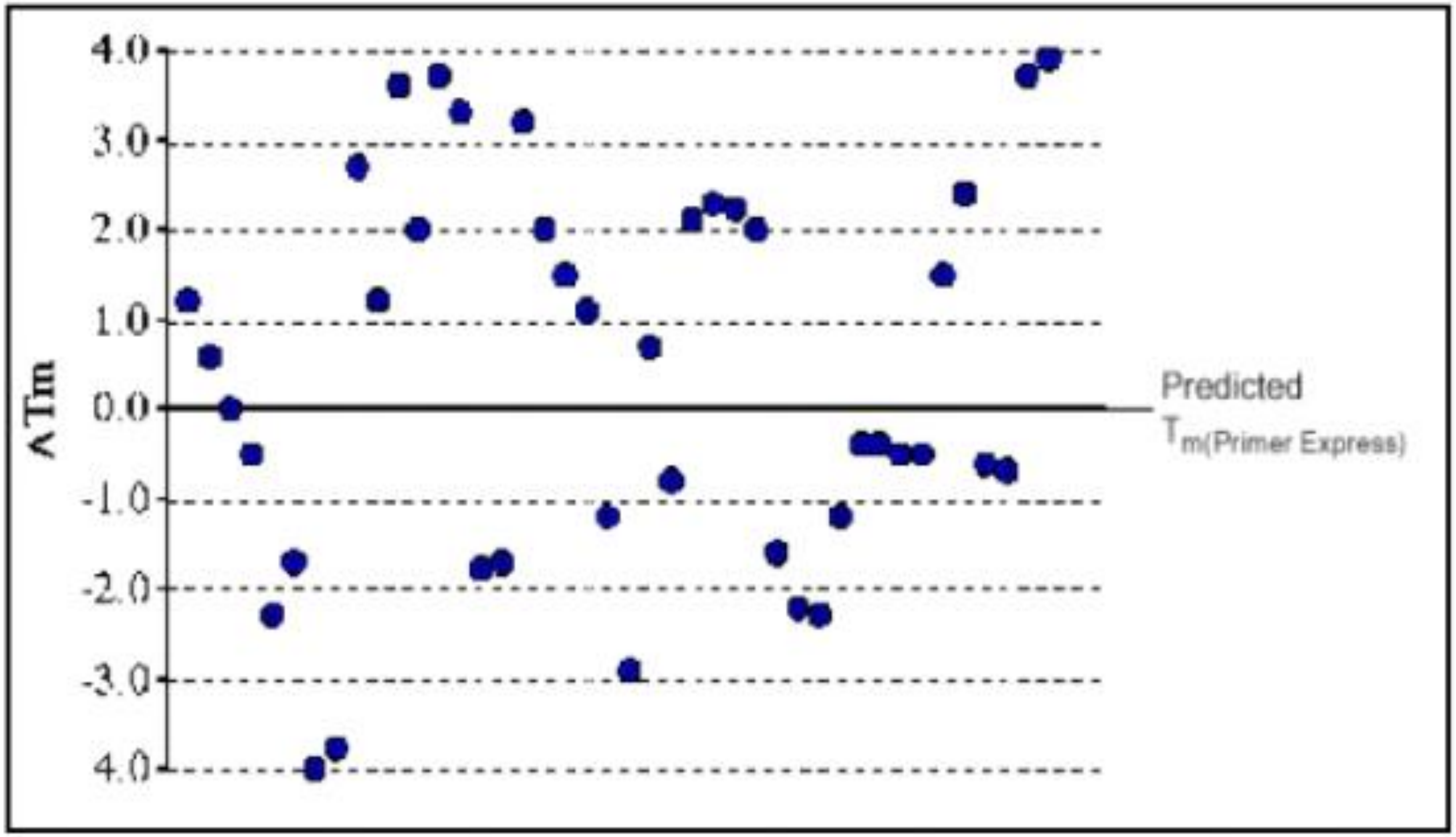
Optimization of qPCR reactions

Optimization of qPCR reactions

- **Optimization of reaction conditions: concentrations of dNTPs, Mg^{2+} , primers, SYBR Green - currently difficult because most "mixes" are "ready to use" and 2X concentrated.**
- **Program optimization: temperature and time of primers annealing and synthesis.**
- **4-step PCR (SYBR Green fluorescence measurement above Tm for product) or 2-step PCR (95 and 72°C only – common temperature of primer annealing and DNA synthesis)**
- **"touch-down" PCR, "hot-start" DNA polymerase**
- **The fastest way to optimize is to redesigning oligonucletodes**

PRIMERS Tm: PREDICTED vs DETERMINED EXPERIMENTALLY



Source: ABI User Bulletin 6 ABI PRISM® Sequence Detection System

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Take home message!

Alwayes keep standards!!!

Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

http://www.gene-quantification.de/miqe.html

Methods 50 (2010) S1–S5

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

ELSEVIER

METHODS

Mini-review

A practical approach to RT-qPCR—Publishing data that conform to the MIQE guidelines ☆,☆☆

Sean Taylor, Michael Wakem, Greg Dijkman, Marwan Alsarraj, Marie Nguyen *

Bio-Rad Laboratories, Inc., Hercules, CA 94547, USA

www.Gene-Quantification.info

DESIGN, DEVELOPMENT, GROWTH
ORIGIN DOES MATTER
BIORON

10th Gene Quantification Event
qPCR dPCR & NGS 2023
Sars-Cov-2, Spatial-Transcriptomics, Liquid Biopsy & CNA, Multi-Omics Biomarkers
20-24 March 2023
@ TUM School of Life Sciences, Freising-Weihenstephan, Germany
#GQ2023

www.Gene-Quantification.info ***21st Anniversary!***

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Quantification Strategy	Quantification strategies: absolute Quantification & relative Quantification / qPCR Evolution
Normalisation	RT-qPCR normalisation strategies & microRNA / Genorm / BestKeeper / RefGenes
qPCR & Optimisation	real-time PCR / interesting reviews / MIQE & Apps & iBook / Primer-Recources & Algorithms
Bioinformatics & Statistics	Big Data in Biology / data analysis / Regulatory Networks / data clustering / Integrated Analysis
Software Downloads	REST (main page) / REST-2009 / RDML / RefGenes / GenEx 7.0 ... FREE DOWNLOAD
RNAi -- RNA interference	siRNA / saRNA / microRNA & microRNA normalisation / mirtron / qPCR statistics
Gene Quantification Events	impressions GQ2023 / qPCR dPCR NGS 2023 / MicroGenomics 2023 / eConferences / previous Events
digital PCR & qPCR Array	digital-PCR ... UPDATE / MIQE & dPCR / qPCR array / normalisation and analysis software
Cyclers & Robotics	real-time PCR hardware: overview of qPCR cyclers / qPCR robotics / page statistics
RNA & RT & RNA QC	reverse transcription / mRNA transcript analysis ... UPDATE / RNA QC & RNA / DNA integrity
Dyes & HRM dyes	detection dyes, probes, and chemistries in real-time PCR / HRM dyes / Chips & Lab-on-Chip
Liquid Biopsy & Biomarkers	Exosomes / EV & exRNA ... UPDATE / CNA / microRNA / Transcriptional Biomarkers
Molecular Physiology	CNA / HRM / CNV / microRNA / siRNA / saRNA / RNAi / digital-PCR / single-cell qPCR
qPCR Efficiency	determination of real-time qPCR efficiency by various methods ... new efficiency papers!
qPCR & dPCR applications	EVs & Exosomes / circulating nucleic acids / digital-PCR / single-cell handling / single-cell qPCR
GQ info portal	microRNA / CNV / microRNA transfer / HRM / RNAi / siRNA / saRNA / Liquid Biopsy

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10th Gene Quantification Event
qPCR dPCR & NGS 2023

qPCR NEWS
The reference in qPCR
powered by www.Gene-Quantification.info

Gene Quantification Newsletter is sponsored by GenEx

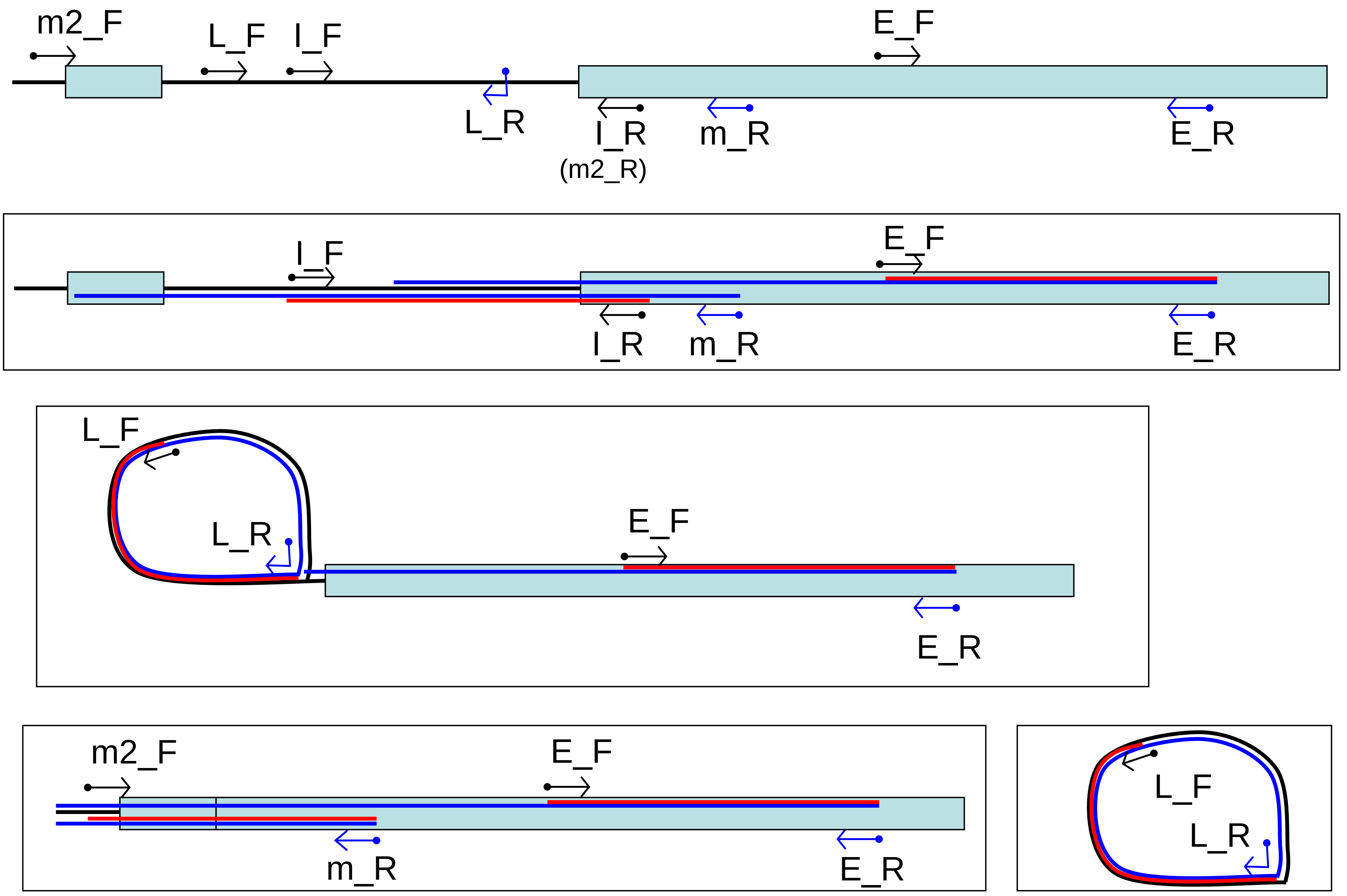
SUMMARY

- **qPCR is a highly efficient, rapid and accurate method for nucleic acid quantification**
- **Enables low and medium throughput gene expression studies**
- **qPCR is based on real-time detection of DNA using fluorescent dyes**
- **$(1+E)^n = 2^n$ if E=100% ($2^{\Delta\Delta Ct}$)**
- **Quality control and correct design of the experiment are crucial!!!**

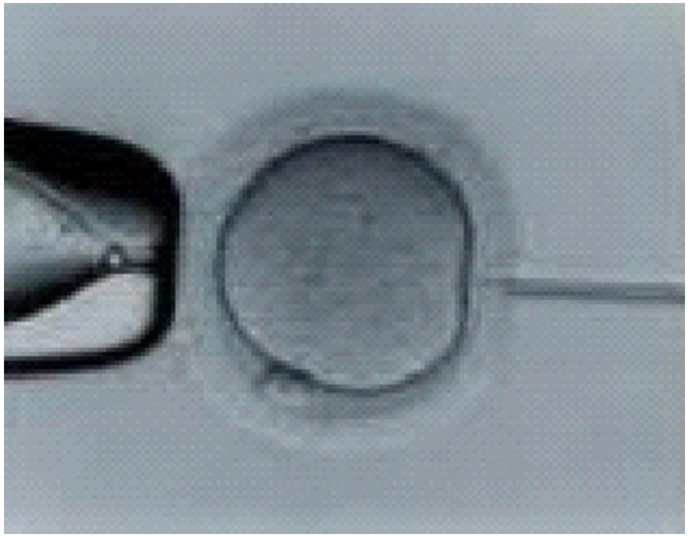
Thank you for your attention!

Supplement 1- examples of qPCR application

Actin mRNA precursor qPCR *By David Barras*



Single cel qPCR



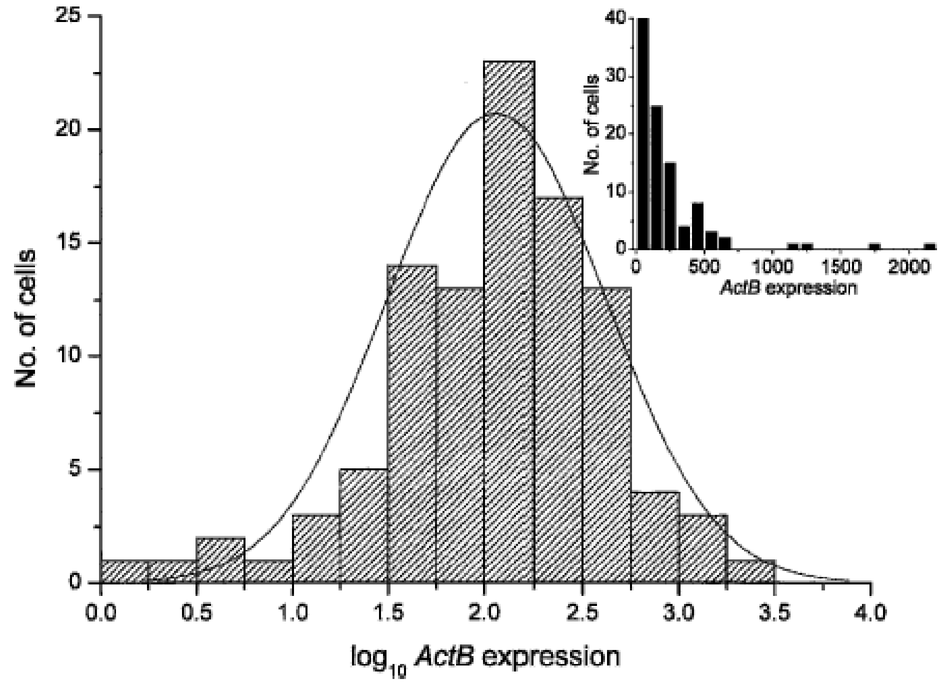
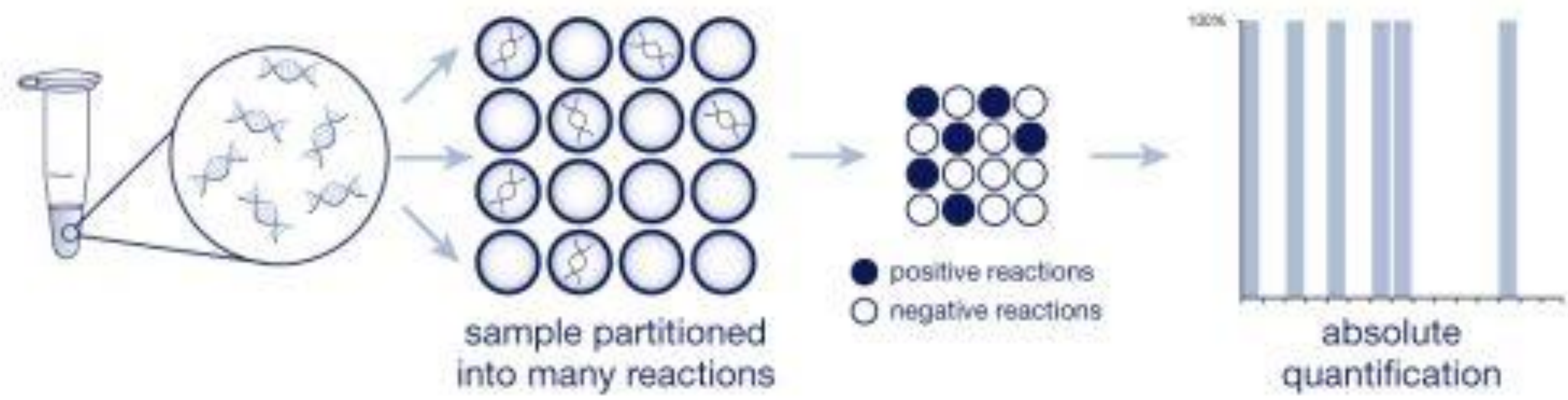


Figure 1. Histograms showing the expression levels of 96 cells expressing *ActB* in logarithmic and linear scale (*inset*). Logarithms of transcript levels are mean-centered for the two glucose concentrations. Solid line describes lognormal distribution centered on the geometric mean (2.06) of the *ActB* expression levels. *Inset* shows histogram of the expression levels in linear scale.

Bengtsson M, Stahlberg A, Rorsman P, Kubista M.
Genome Res. 2005 Oct;15(10):1388-92.

Digital PCR - dPCR



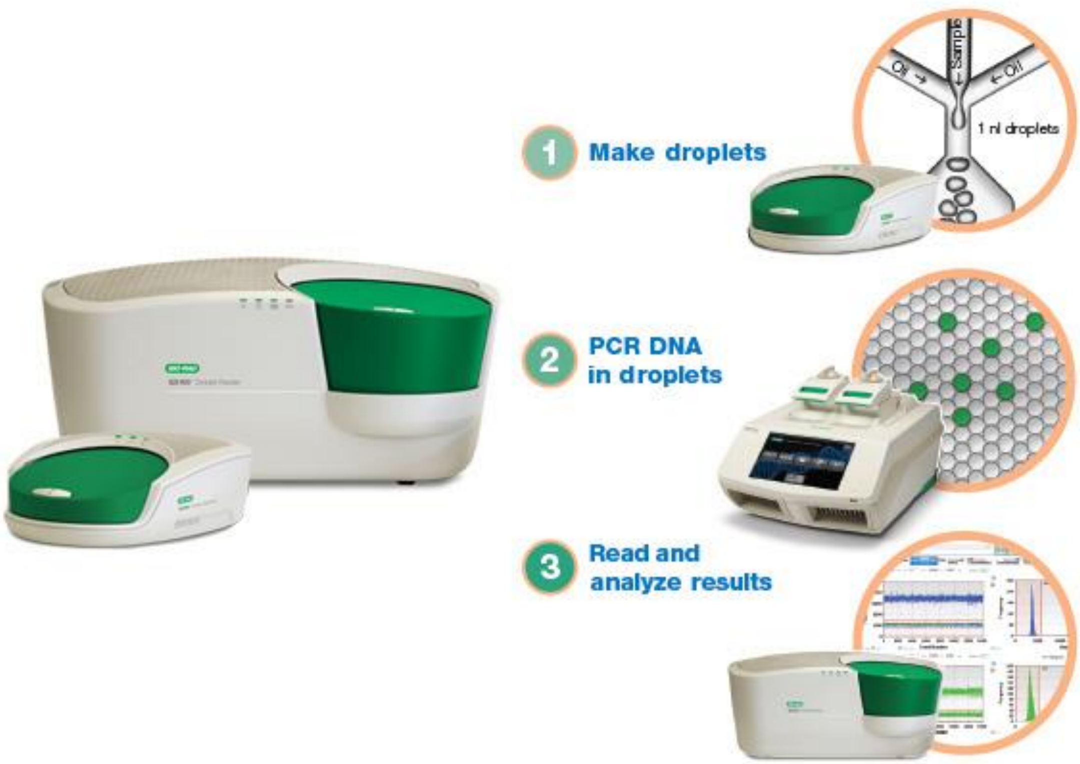
	Absolute Quantification (Digital PCR Method)	Absolute Quantification (Standard Curve Method)
Overview	In absolute quantification using Digital PCR, no known standards are needed. The target of interest can be directly quantified with precision determined by number of digital PCR replicates.	In absolute quantification using the Standard Curve Method, you quantitate unknowns based on a known quantity. First you create a standard curve; then you compare unknowns to the standard curve and extrapolate a value.
Example	Quantify copies of rare allele present in heterogenous mixtures. Count the number of cell equivalents in sample by targeting genomic DNA. Determine absolute number of viral copies present in a given sample without reference to a standard.	Correlating **viral copy number** with a disease state.

http://digital-pcr.gene-quantification.info/

dPCR - examples of available platforms

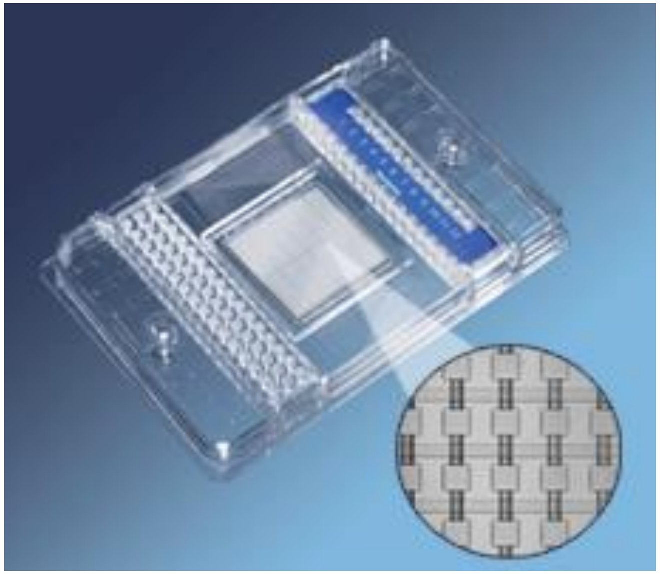
Biorad QX200

Possible preparation of 1-8 samples simultaneously
Up to 20,000 reactions / sample
Up to 96 samples can be read simultaneously



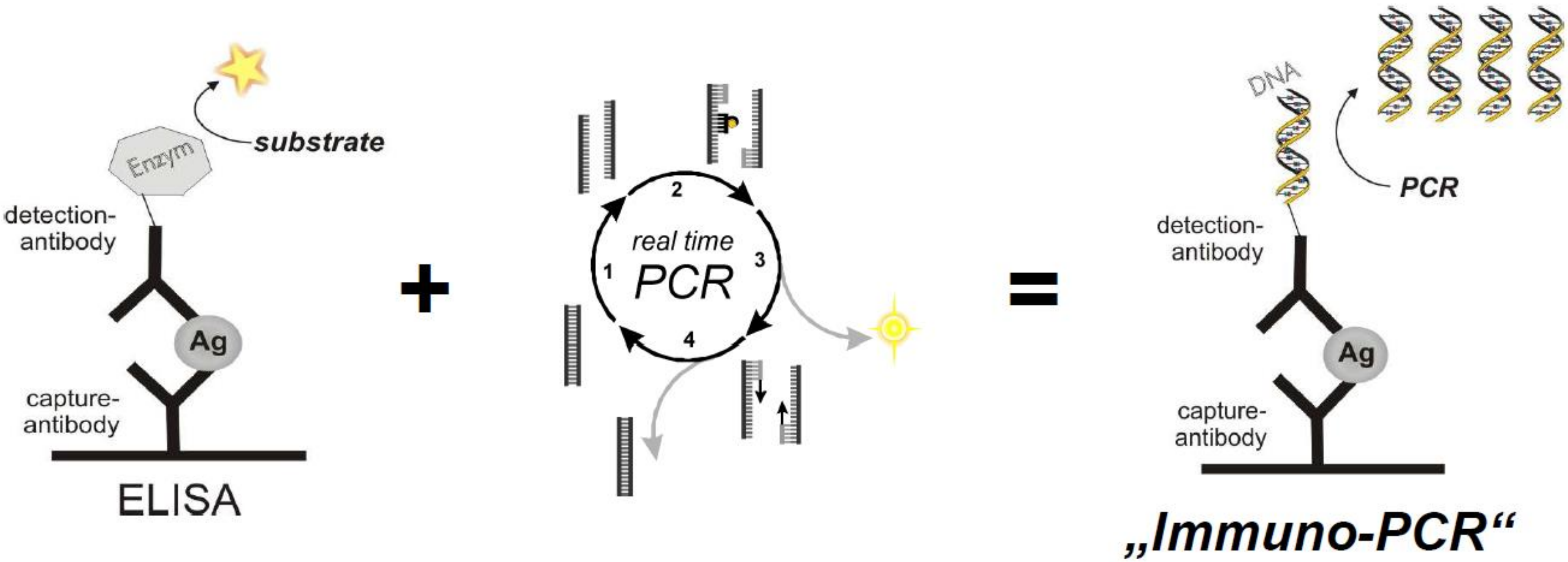
Fluidigm Biomark HD

(also mass qPCR!)
From 2,304 to 36,960 independent reactions
(e.g., 48x48, 192x24, 48x770 layouts)



http://digital-pcr.gene-quantification.info/

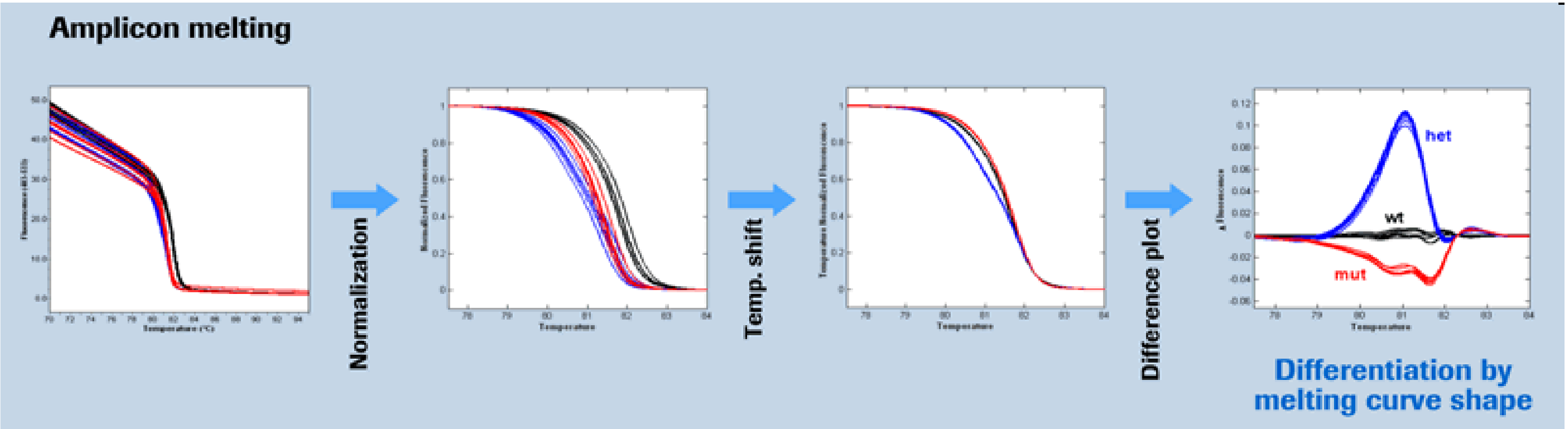
Immuno-qPCR



T. Sano, C.L. Smith, C.R. Cantor, *Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates*, Science 258 (1992), 120-122

M. Niemeyer; qPCR 2007

Analysis of High-Resolution Melting curves

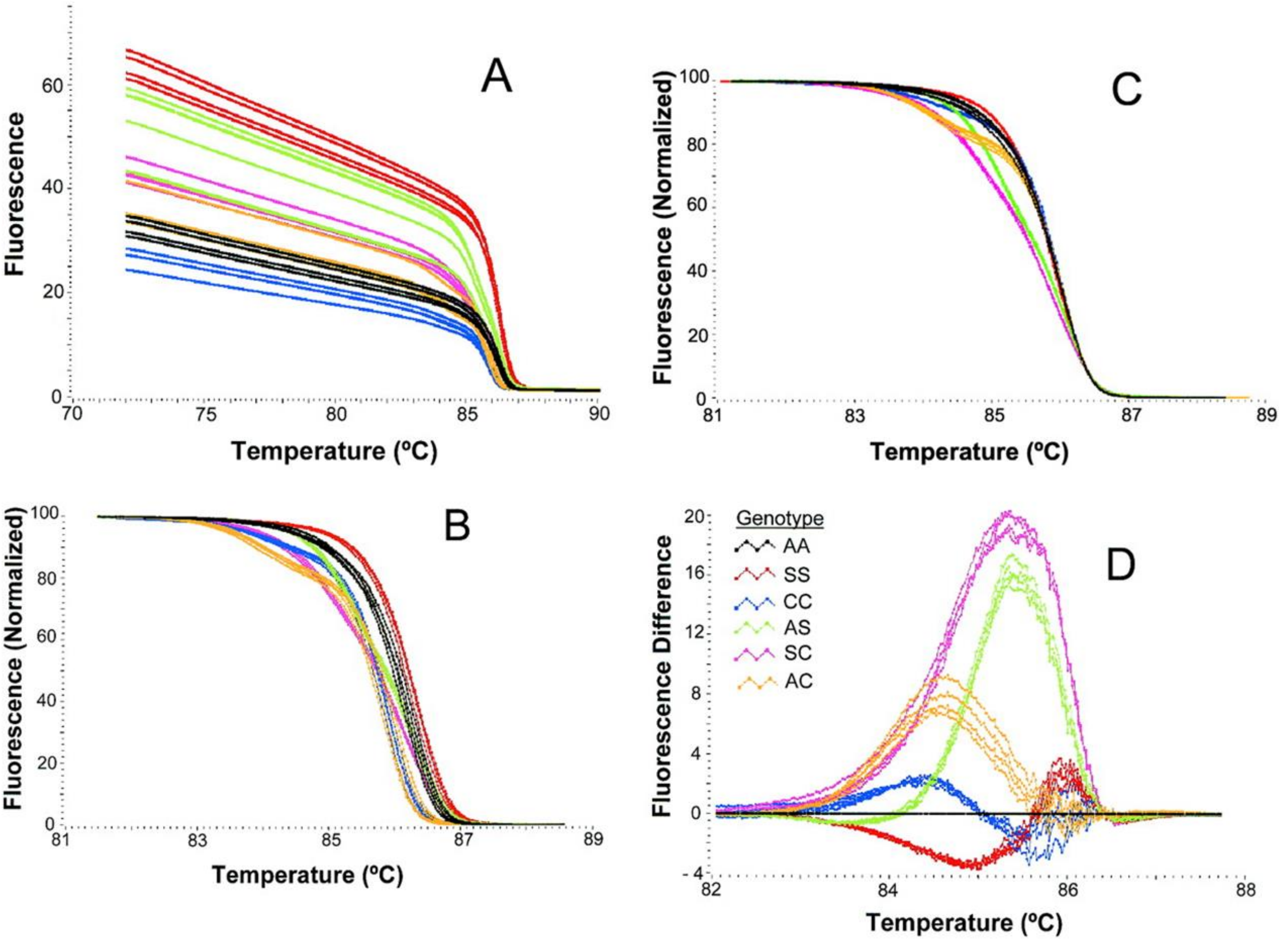


Roche

HRM = High Resolution Melting

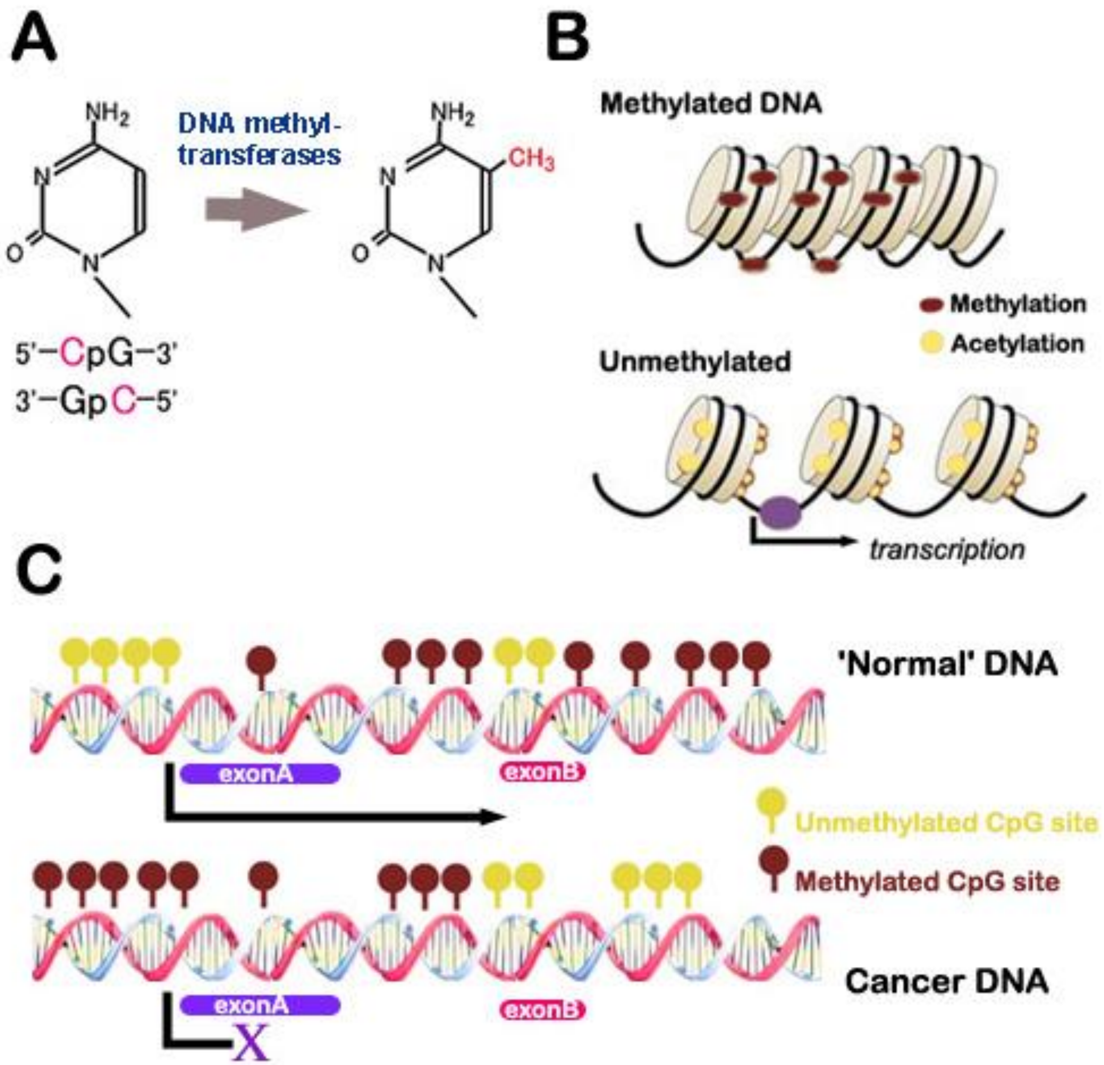
- **DNA SATURATING dyes instead of SYBR Green!!!**
- **Resolution up to 50 florescence measurements / 1ºC**
- **Amplicons of different lengths**
- **Efficiency doesn't matter: end-point analysis!**
- **Standardization of the template concentration less important**

HRM = High Resolution Melting



Supplement 2 - using qPCR to analyze chromatin methylation status

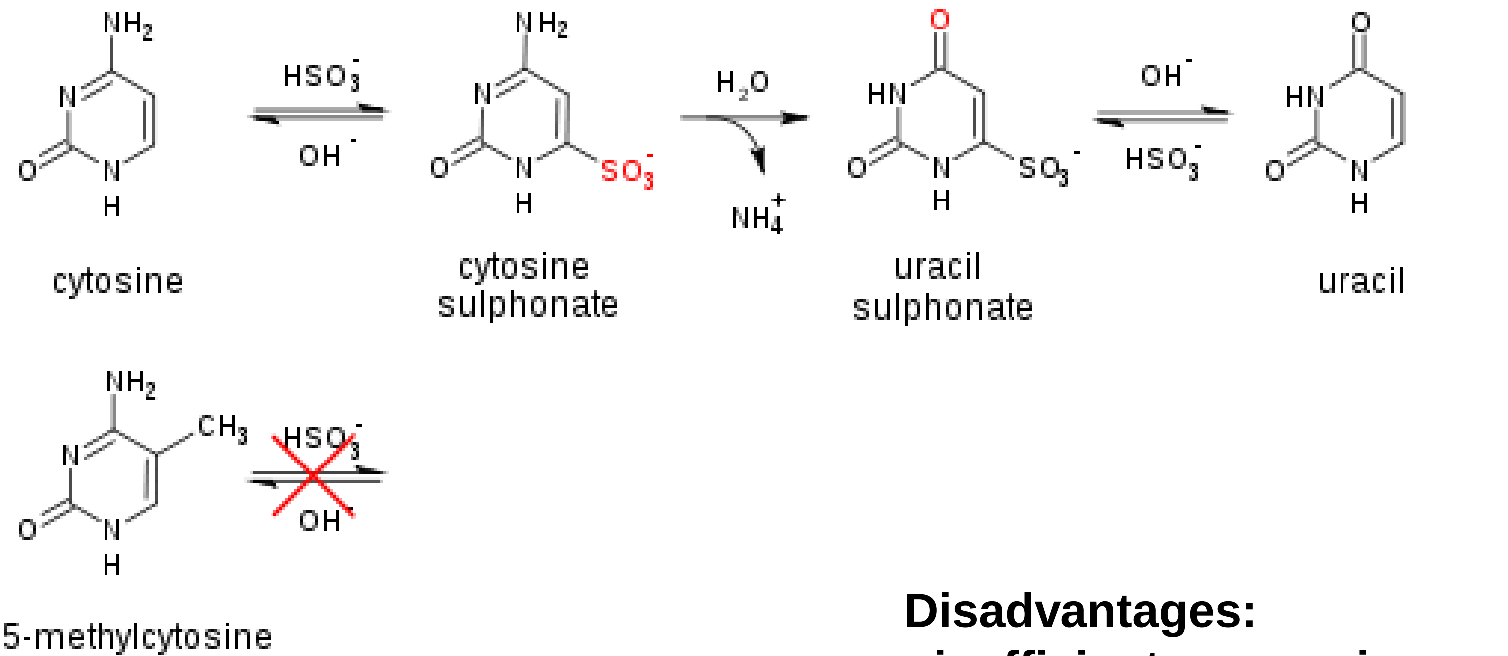
DNA methylation silences transcription



http://philos.biol.mun.ca

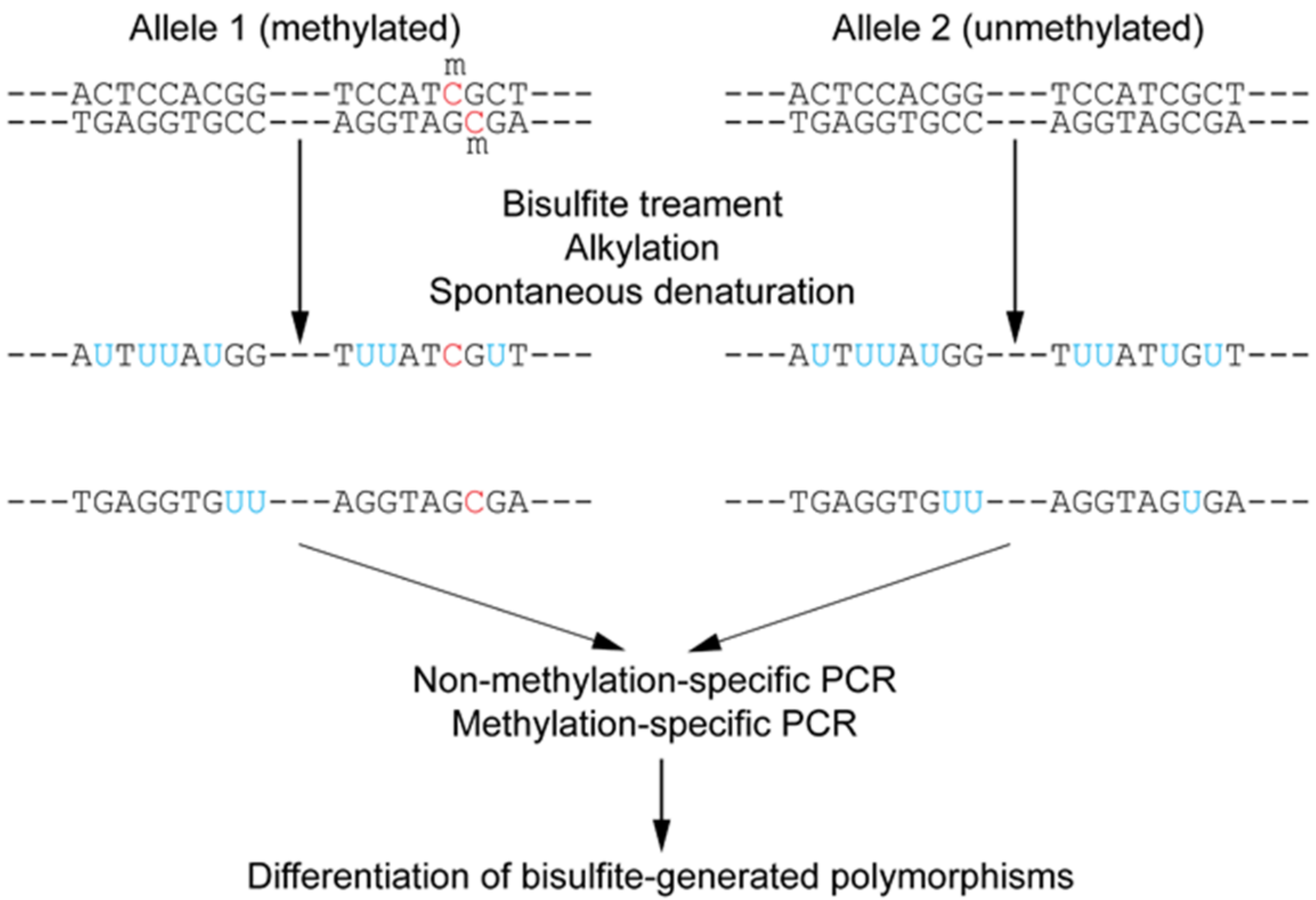
DNA bisulfite conversions

- **Sodium bisulfie converts CYTOSINE into URACYL**
- **Does not modify methylated CYTOSINE**



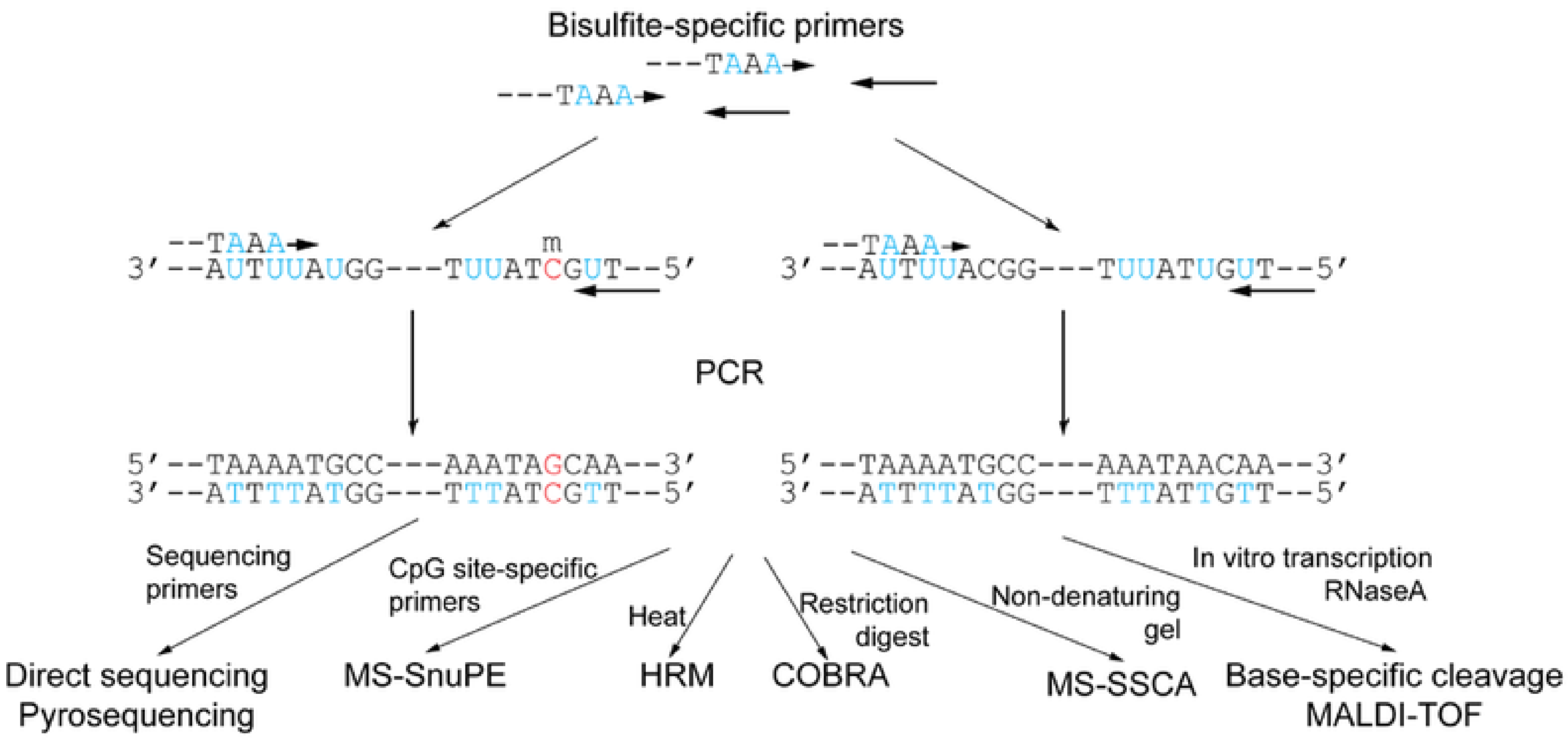
Disadvantages:
- inefficient conversion
- DNA degradation

DNA bisulfite conversions



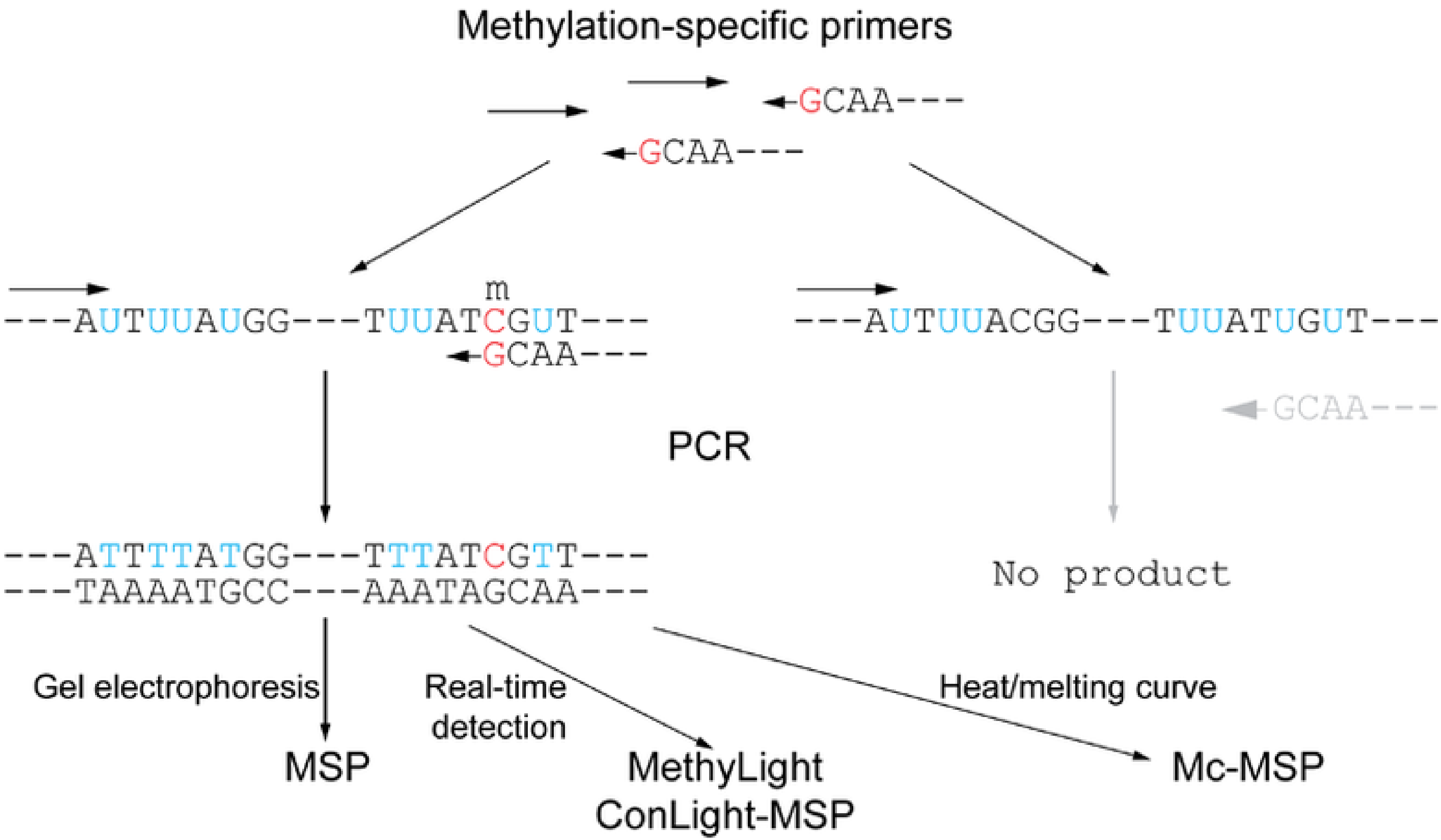
Wikipedia, *Bisulfite sequencing*

Met-DNA analyses



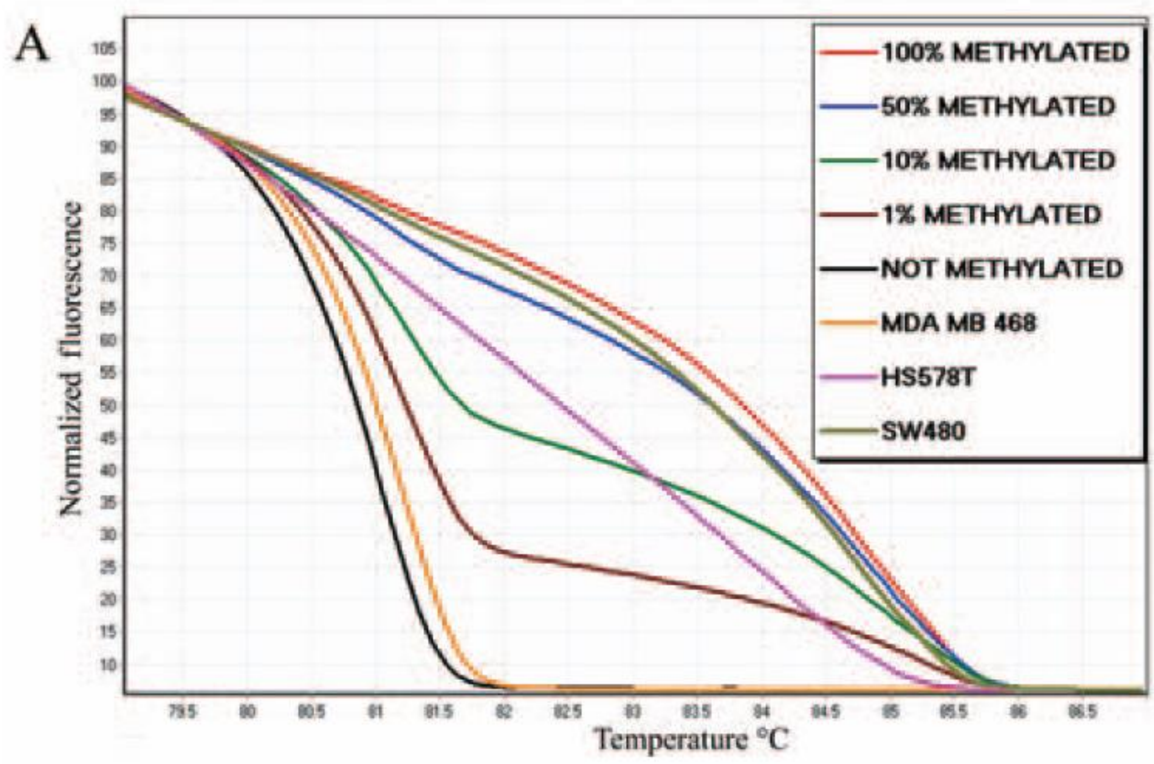
Wikipedia, *Bisulfite sequencing*

Met-DNA analyses



Wikipedia, *Bisulfite sequencing*

Analysis of methylation status using high-resolution melting curves: MS-HRM



Published online 8 February 2007 Nucleic Acids Research, 2007, Vol. 35, No. 6 e41
doi:10.1093/nar/gkm013

Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation

Tomasz K. Wojdacz[1,2] and Alexander Dobrovic[1,3,*]

PROTOCOL

Methylation-sensitive high-resolution melting

Tomasz K Wojdacz[1,2], Alexander Dobrovic[2] & Lise Lotte Hansen[1]

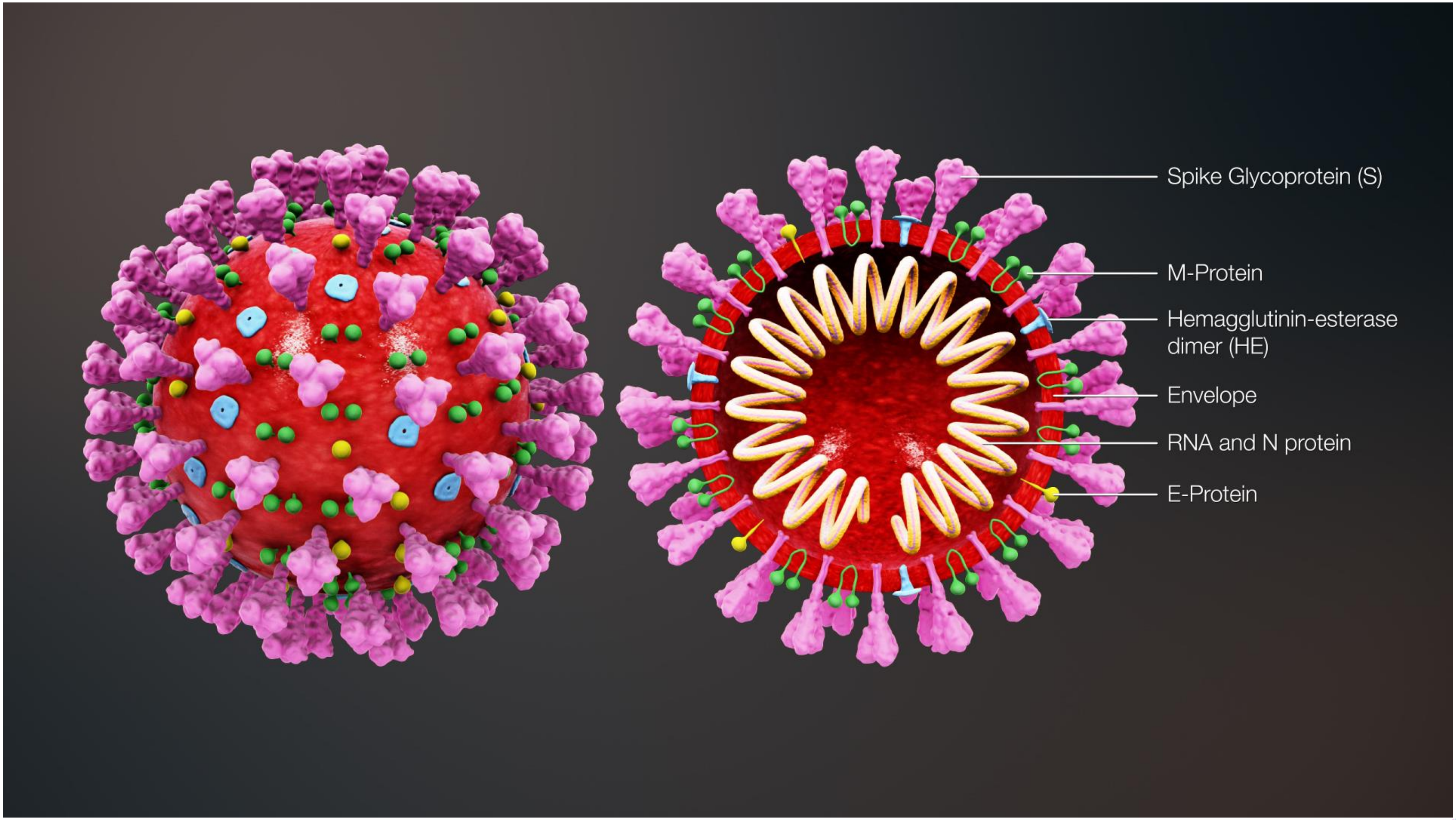
[1]Institute of Human Genetics, University of Aarhus, The Bartholin Building, Wilhelm Meyers Allé, Bygn. 1242, DK-8000 Aarhus C, Denmark. [2]Department of Pathology, Peter MacCallum Cancer Centre, Locked Bag 1, A'Becket Street, Victoria 8006, Australia. Correspondence should be addressed to T.K.W. (wojdacz@humgen.au.dk).

NATURE PROTOCOLS | VOL.3 NO.12 | 2008 | 1903

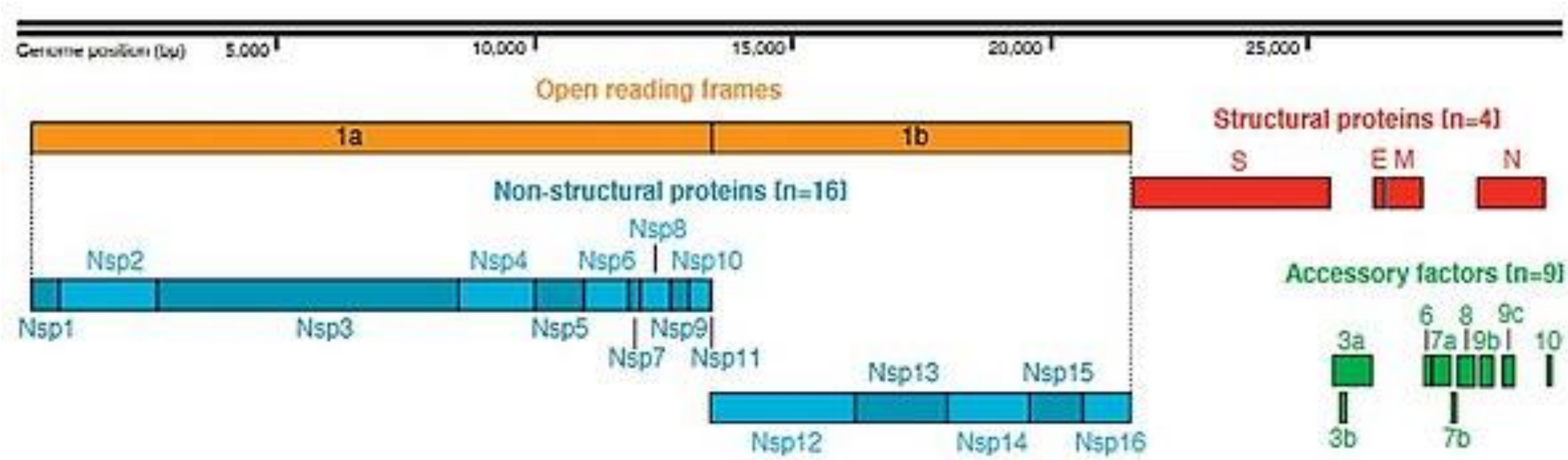
Supplement 3 - use of RT-qPCR in the diagnosis of coronavirus infections

SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) - virus causing COVID-19 (Coronavirus Disease 2019)

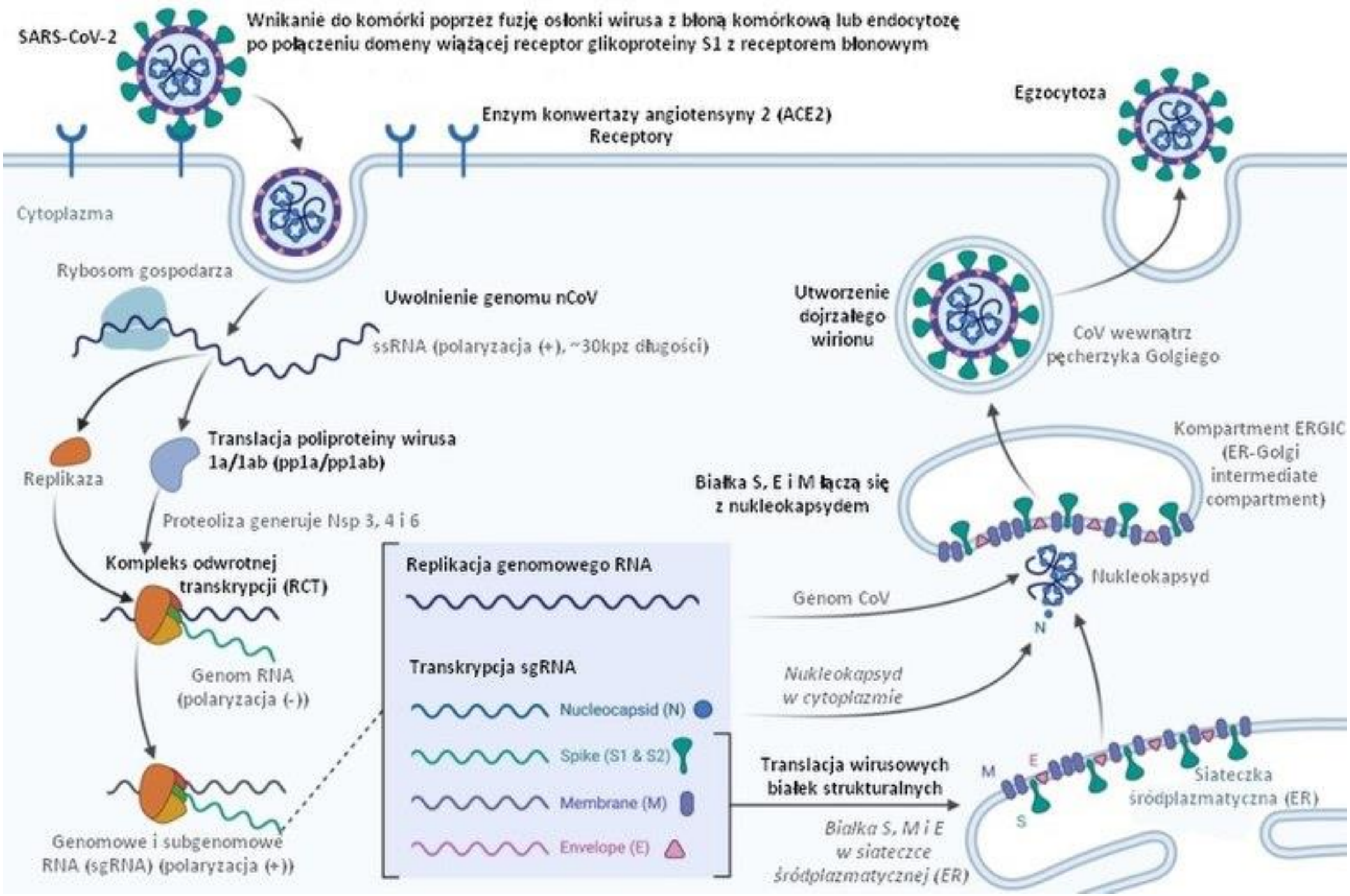


SARS-CoV-2: a beta-coronavirus with a genome in the form of a single RNA molecule of approx. 30,000 nt in length, and with (+) polarity



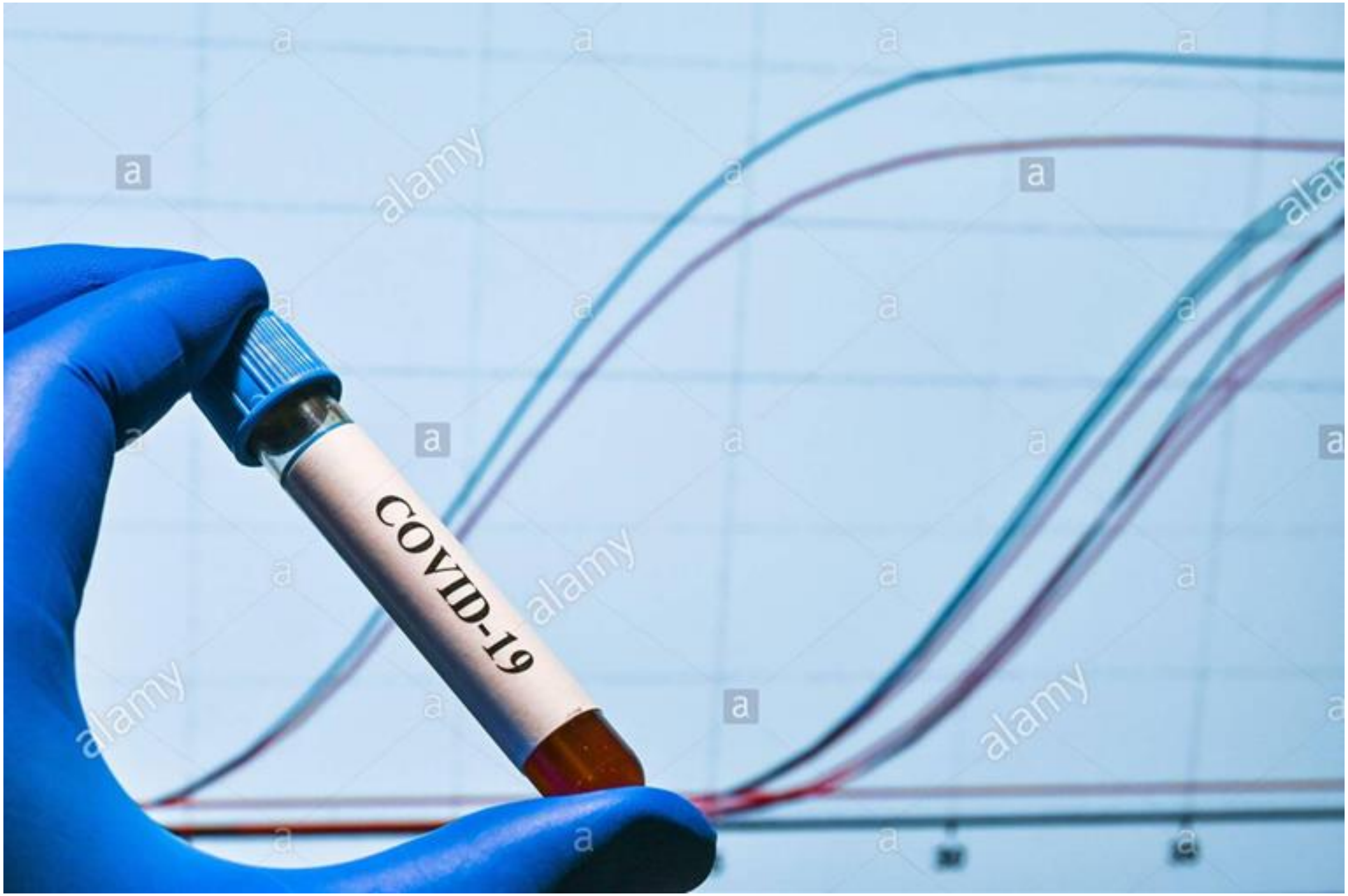
Wikipedia

SARS-CoV-2 replication cycle



Wikipedia

How can SARS-CoV-2 be detected?



Types of diagnostic tests used

Immunological tests

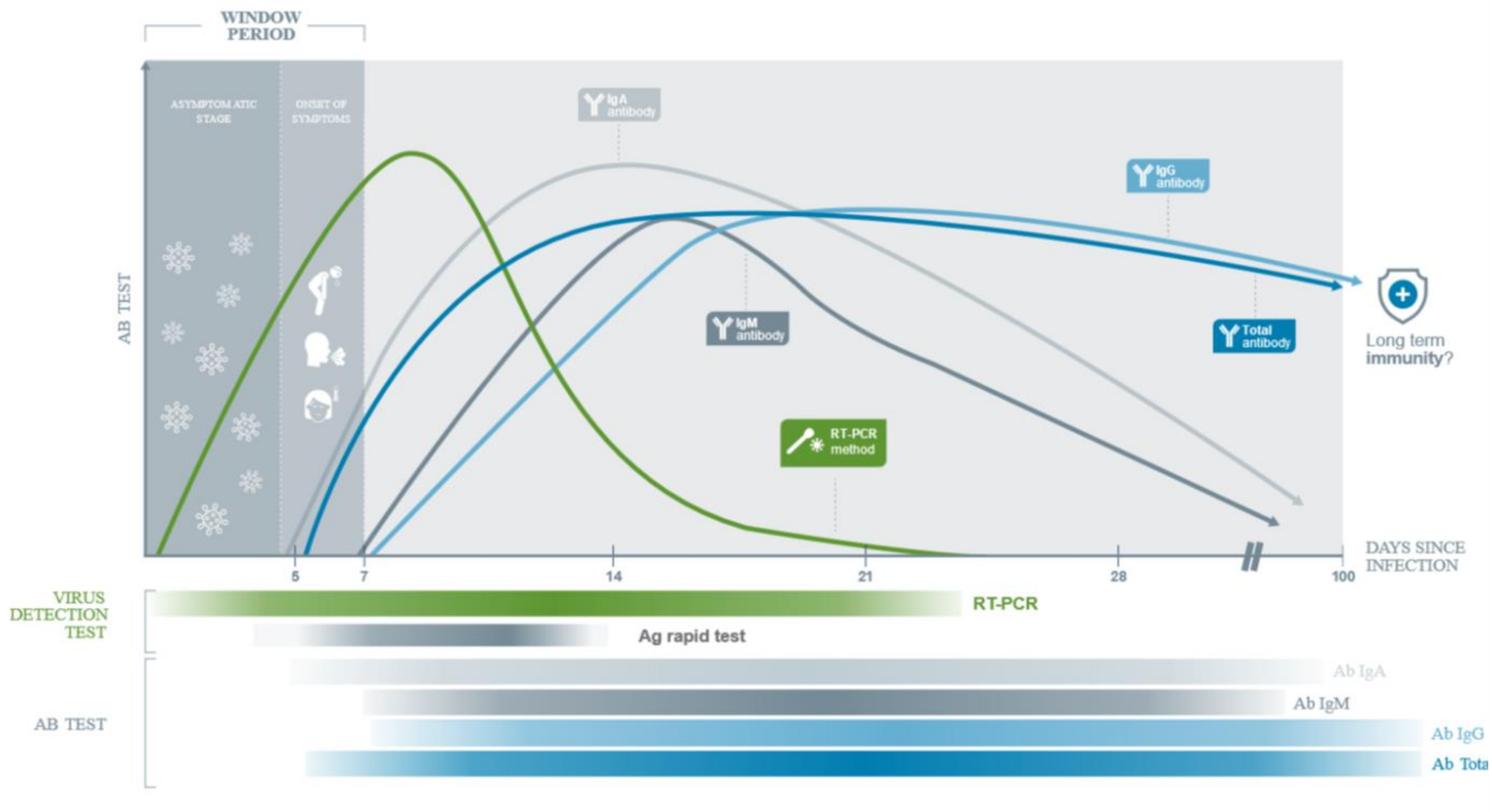
Detecting (using antibodies) anti-virus antibodies (does not necessarily detect the presence but the fact of passing infection): cassette, ELISA.

Detecting (using antibodies) the antigens of the virus (directly detecting its presence in the material)

Molecular tests

Detecting the genetic material of the virus (RNA) using RT-qPCR.

"Windows" of sensitivity of diagnostic tests



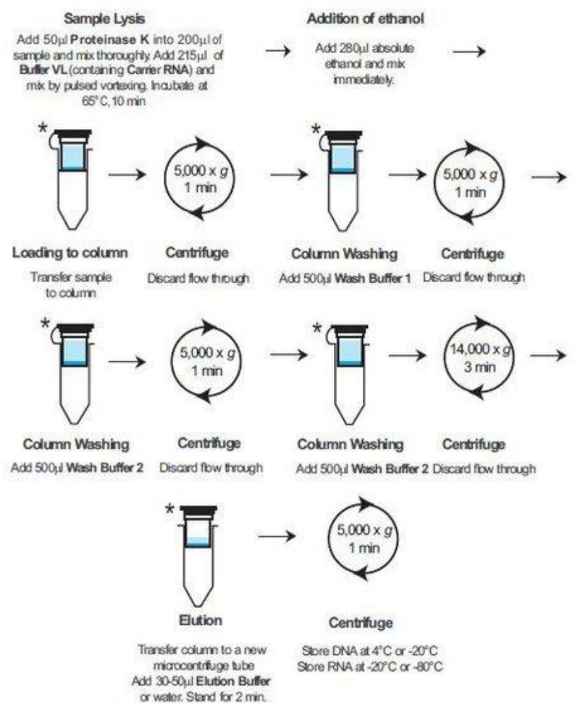
https://www.synlab.com/news-publications/sars-cov-2/antigen-tests-for-sars-cov-2-detection

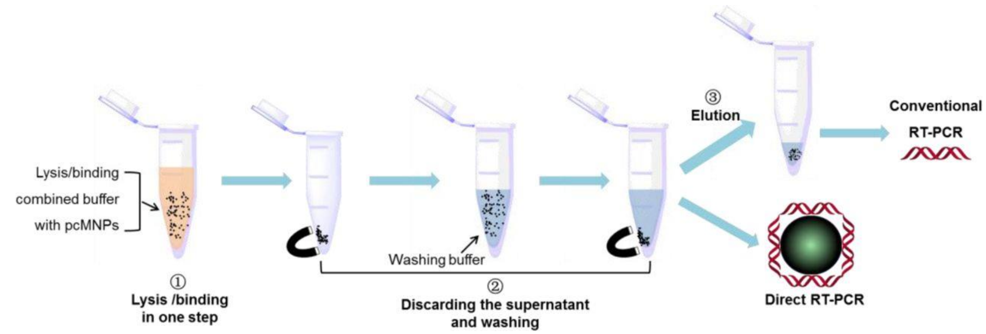
How to isolate viral RNA?

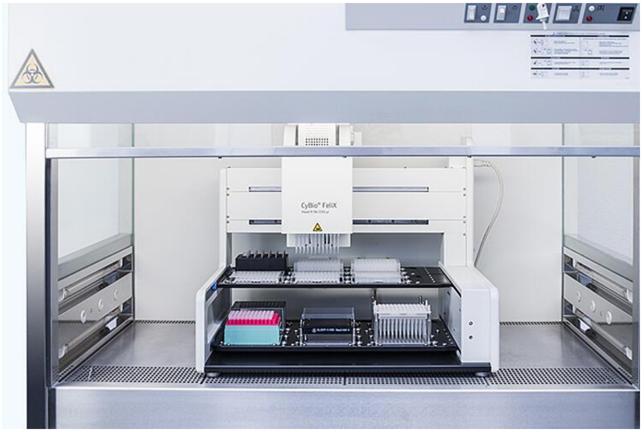
First, the collection of material - preferably a nasopharyngeal swab.

Then lysis of the material: enzymatic (proteinase K digestion) or chemical (chaotropic salts such as GTC).

RNA recovery based on the affinity of nucleic acids for silica resin ("columns") or ferromagnetic beads ("beads").







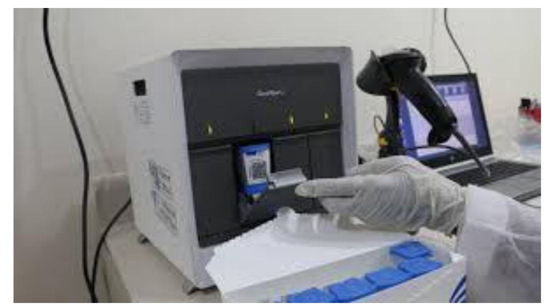
Open vs closed systems

Open systems

- Manual work (danger of contamination, not very efficient)
- open robots (you can choose different suppliers of plastics or reagents)

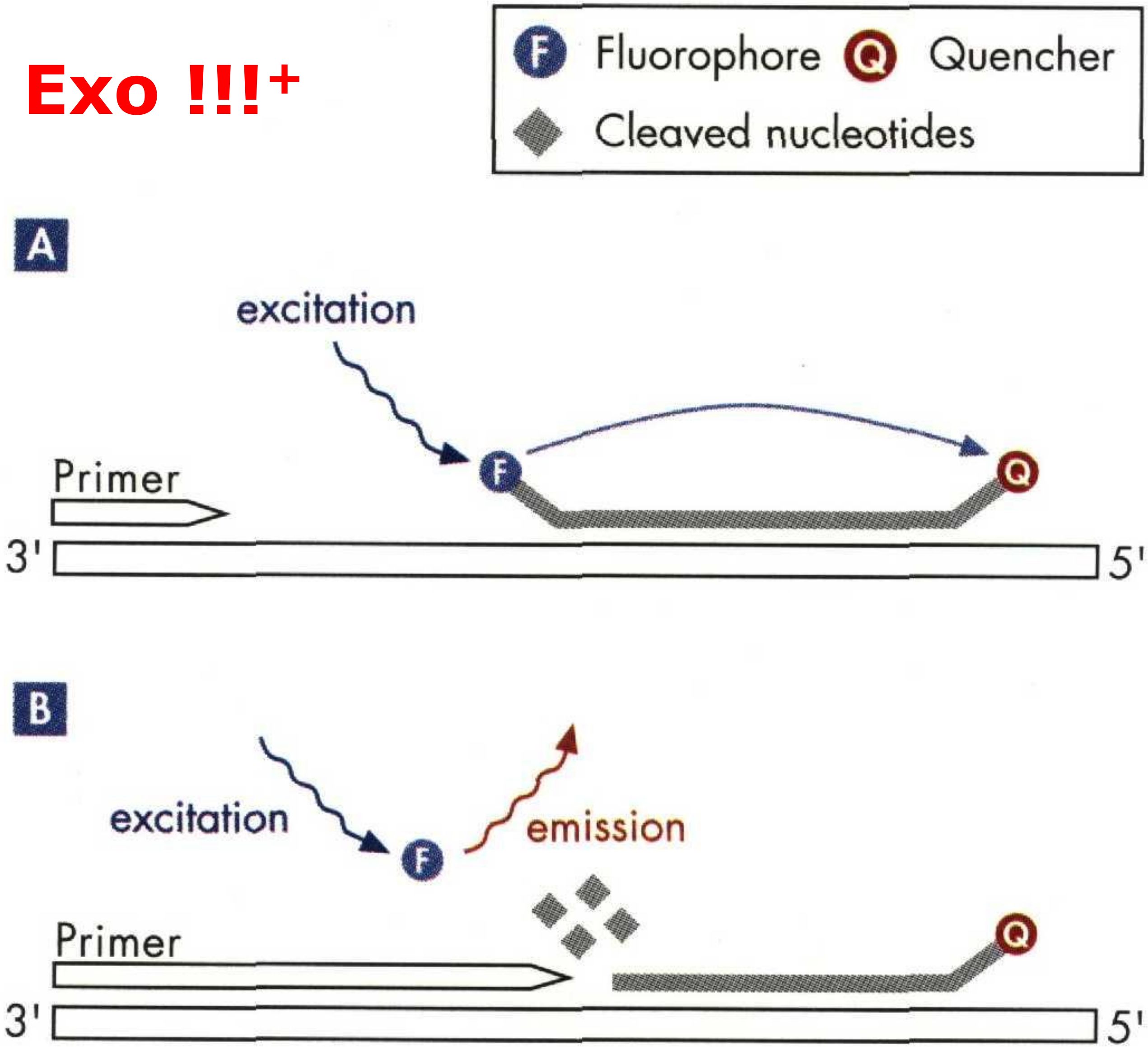
Closed systems

- high cost but better standardization
- "All-in-one" (isolation+RT-qPCR) or only isolation
- simplified operation
- „personal devices”
- problems with reagent availability
- Isolation + RTqPCR even in a few tens of minutes



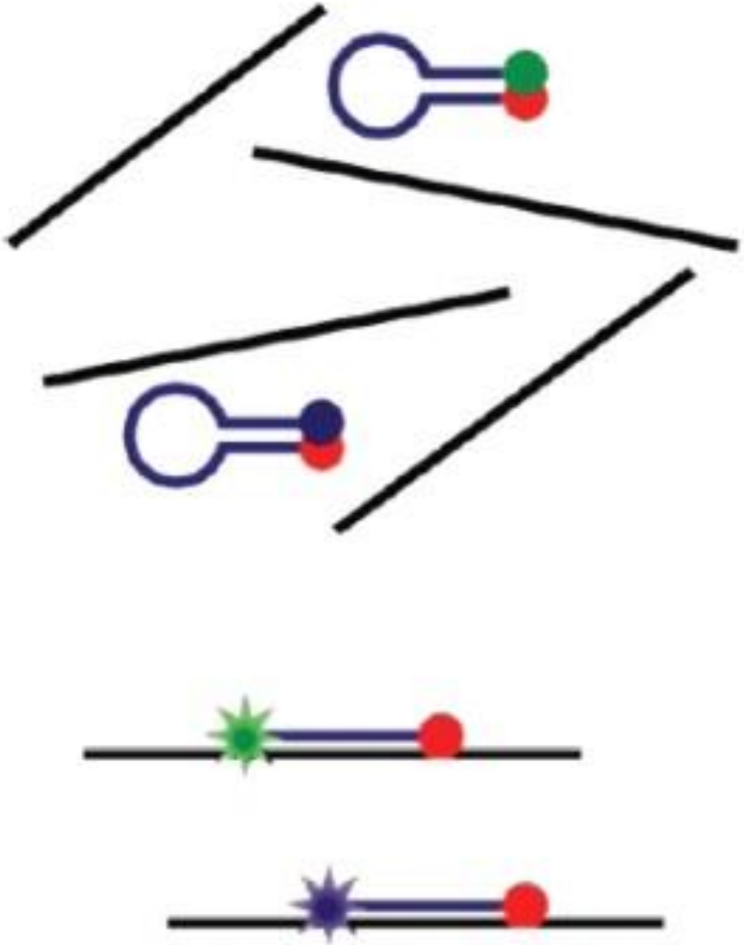
TaqMan – hydrolysis probes

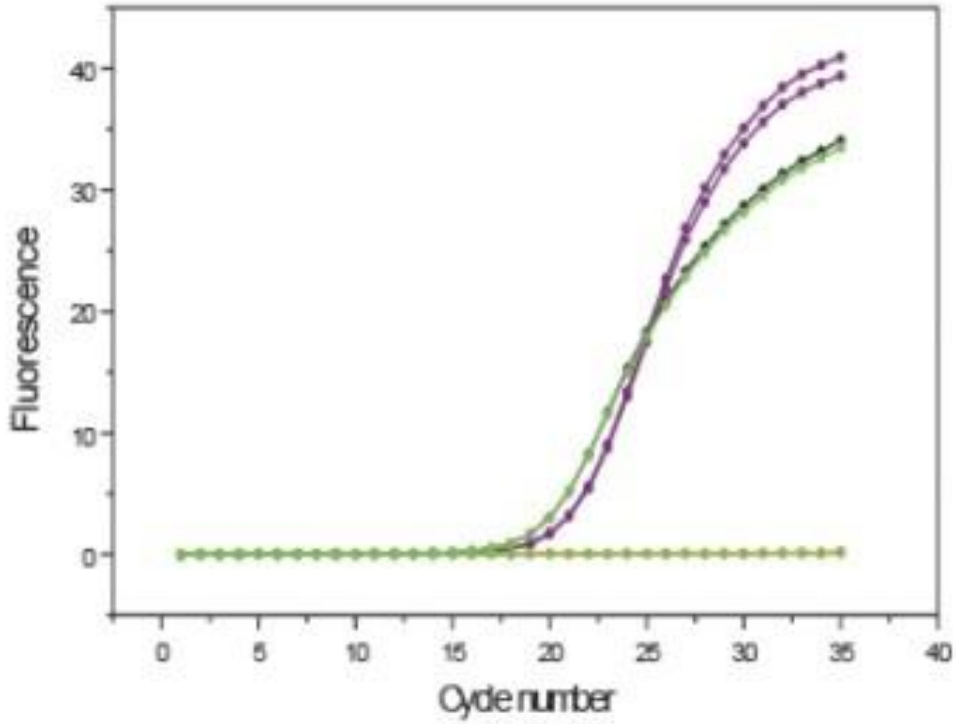
DNA Pol Exo !!!+



ABI, Roche (UPL)

Multiplex qPCR





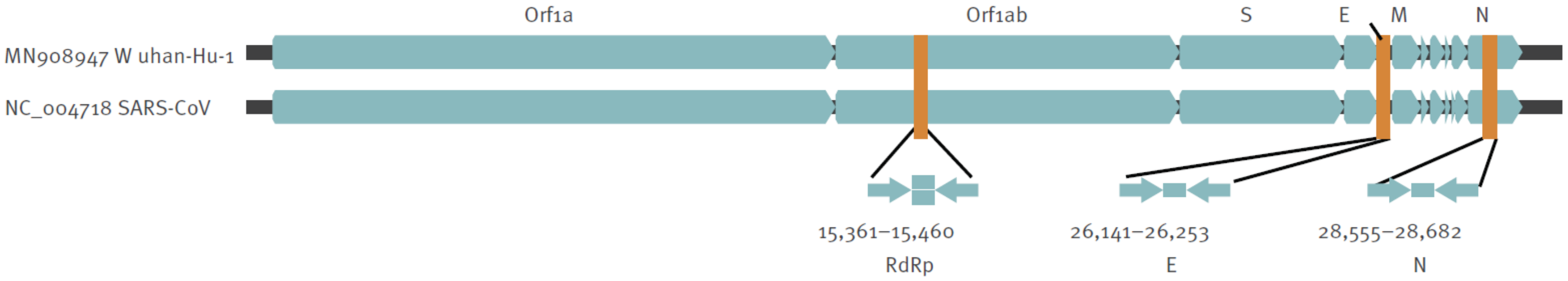
Different detection formats and different dyes allow detection of 2 or more products in a single reaction

(not possible with SYBR Green!!!)

What amplicons should be used in RT-qPCR ?

FIGURE 1

Relative positions of amplicon targets on the SARS coronavirus and the 2019 novel coronavirus genome



E: envelope protein gene; M: membrane protein gene; N: nucleocapsid protein gene; ORF: open reading frame; RdRp: RNA-dependent RNA polymerase gene; S: spike protein gene.

Numbers below amplicons are genome positions according to SARS-CoV, GenBank NC_004718.

Corman et al, Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020 Jan;25(3)

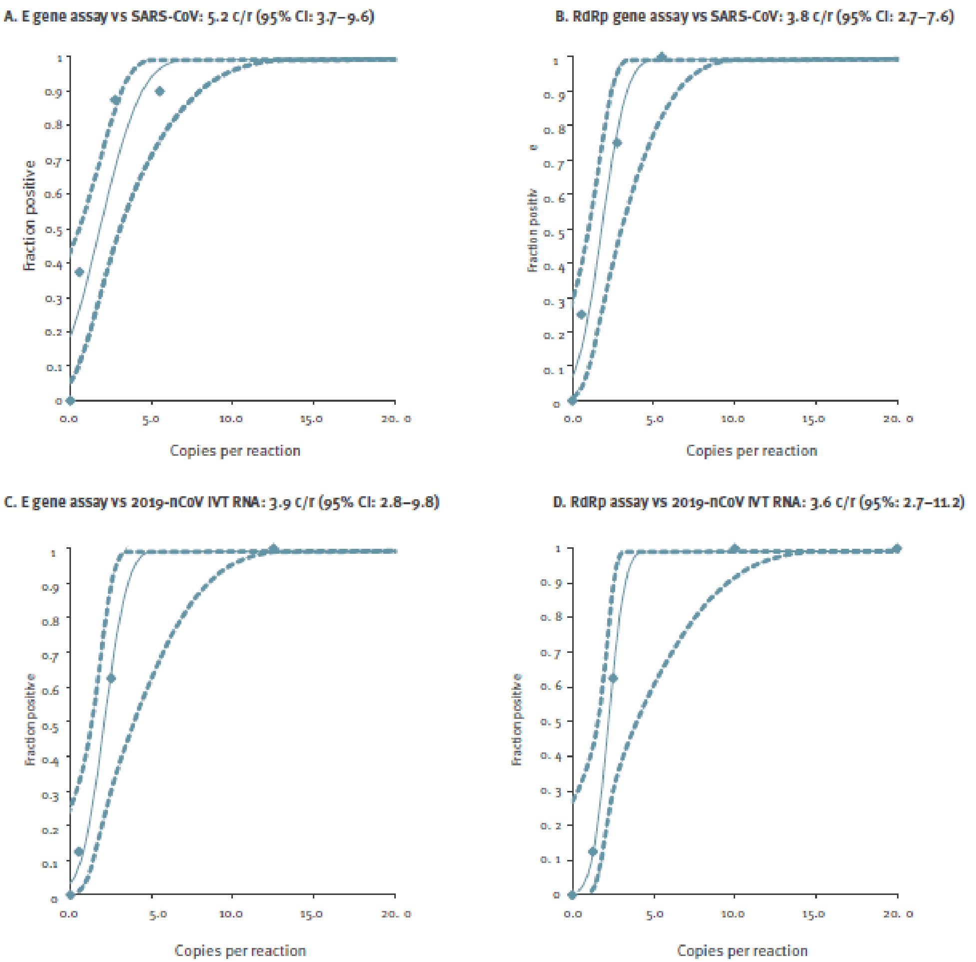
In addition, control amplicons (not always all used at the same time):

- **SPIKE-IN RNA (synthetic - isolation control only)**
- **Internal control (human mRNA - isolation and collection control!)**

Sensitivity of the RT-qPCR method

FIGURE 3

Determination of limits of detection based on SARS coronavirus genomic RNA and 2019 novel coronavirus-specific in vitro transcribed RNA



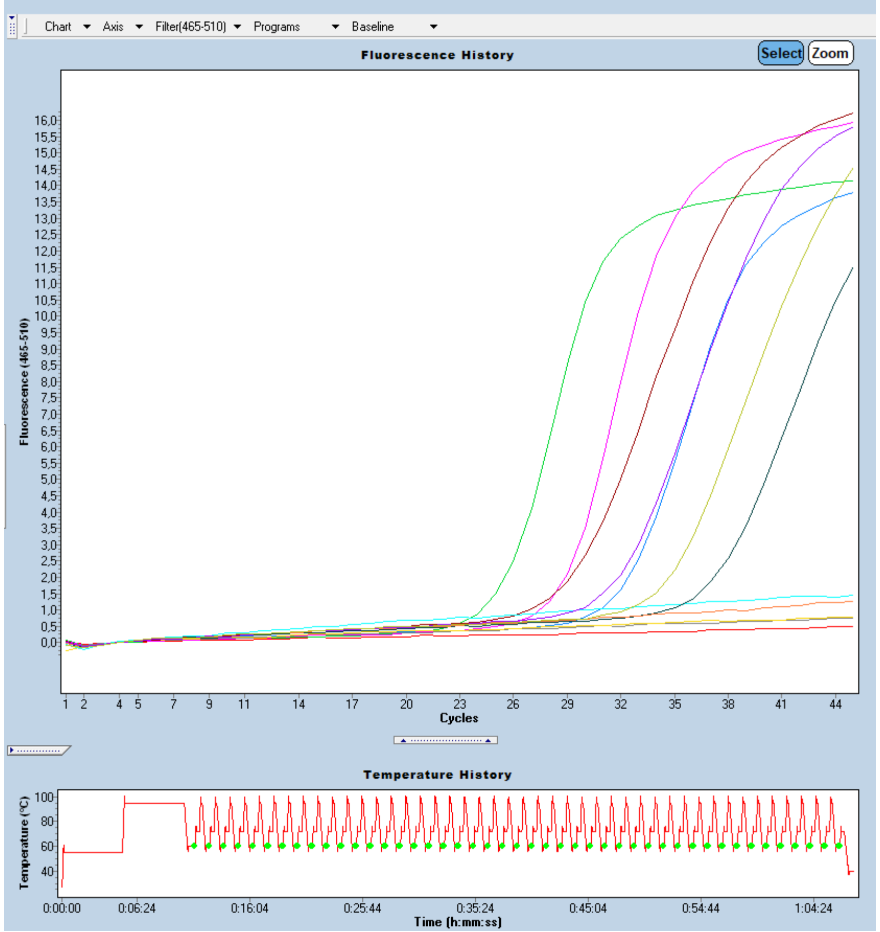
CI: confidence intervals; c/r: copies per reaction; IVT: in vitro-transcribed RNA.

Corman et al, Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020 Jan;25(3)

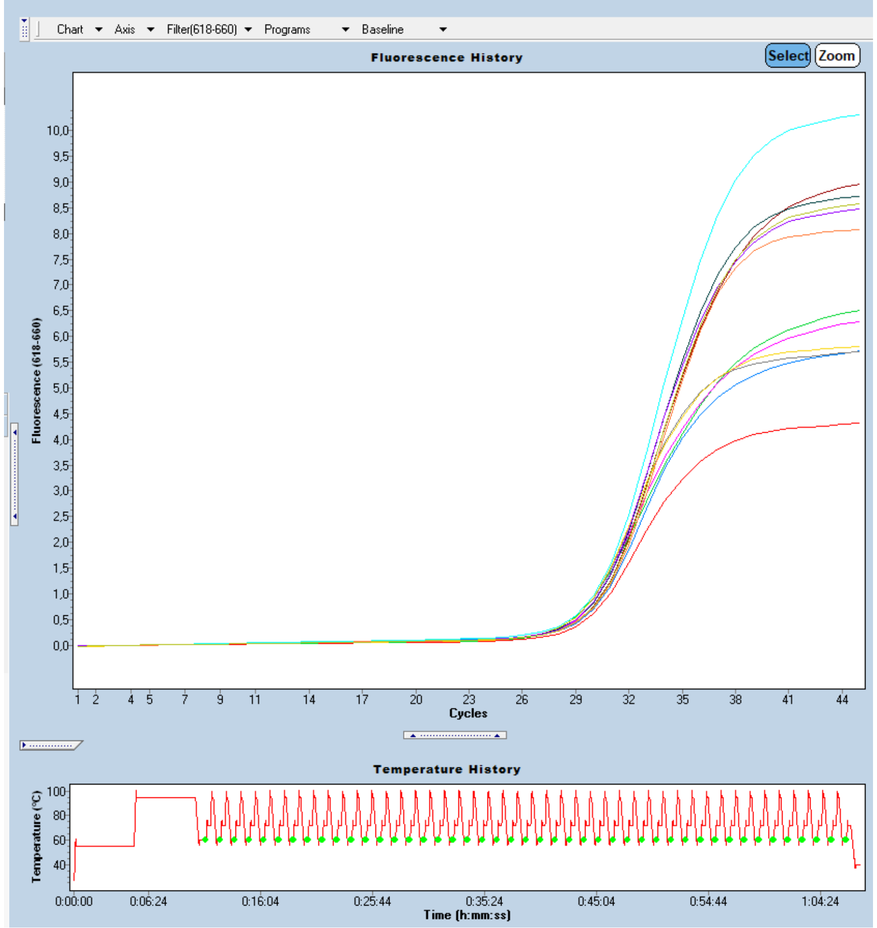
Safety first!!!

Example result

Viral amplicons



Control amplicons



For more watch on YT: https://www.youtube.com/watch?v=SNvGQJlcQfQ (in polish)