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Real-Time PCR

August 2014

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Lectoraat IMD

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Real-Time PCR

AN INTRODUCTION

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qPCR cyclers

Roche

Lightcycler

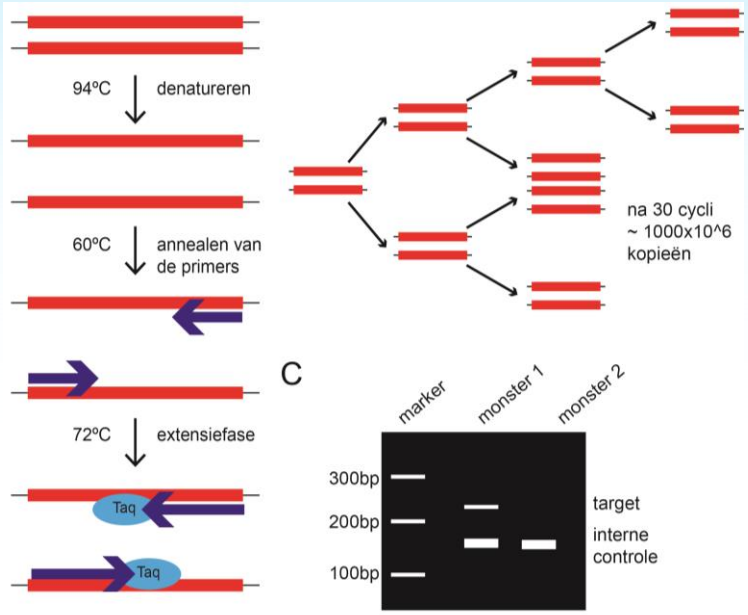
BioRad IQ5 en CFX

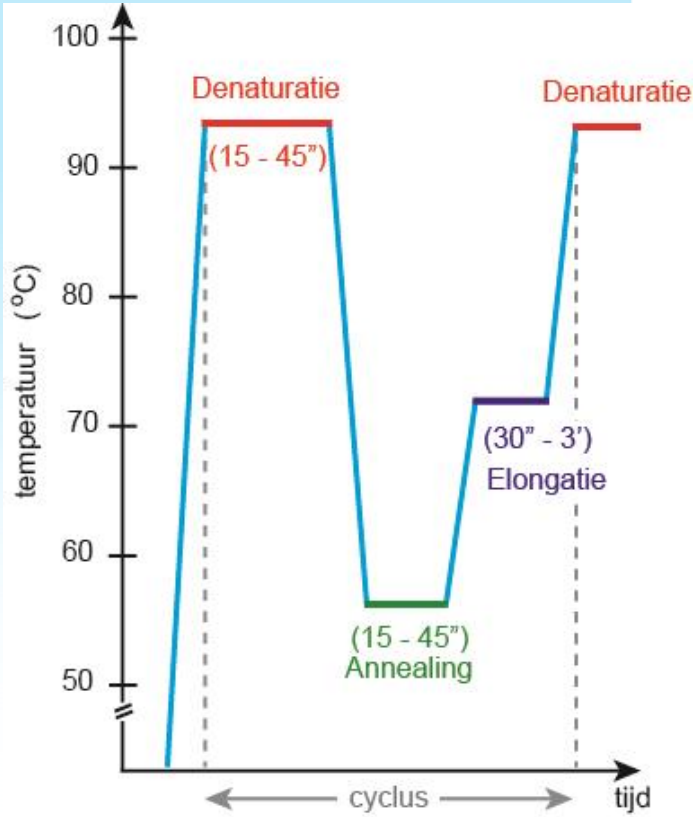
Applied Biosystems Realtime PCR system

Eurogentec Smartcycler

PCR amplification

- PCR cycle: denaturation, annealing, elongation
- Number of cycles: 25-40
- PCR reagent: template, dNTPs, buffer, primers (probe), Taq NA polymerase,





C.C.Orelio en M.J.Plug; Basisprincipes van de PCR 2013

Conventional PCR procedure

DNA isolation

PCR amplification

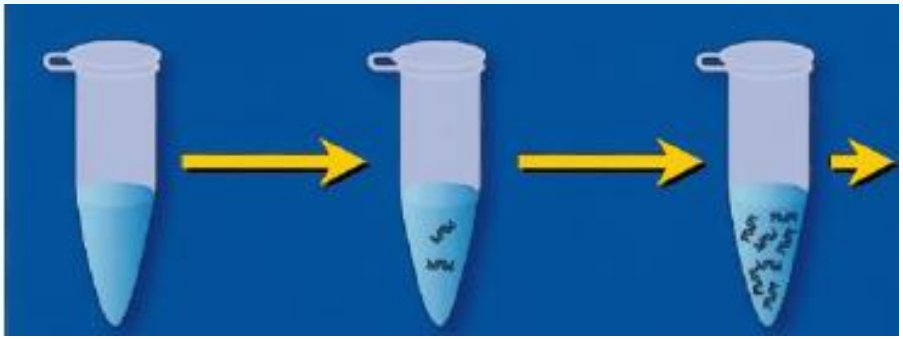
Gel elektrophoresis + Southern blotting

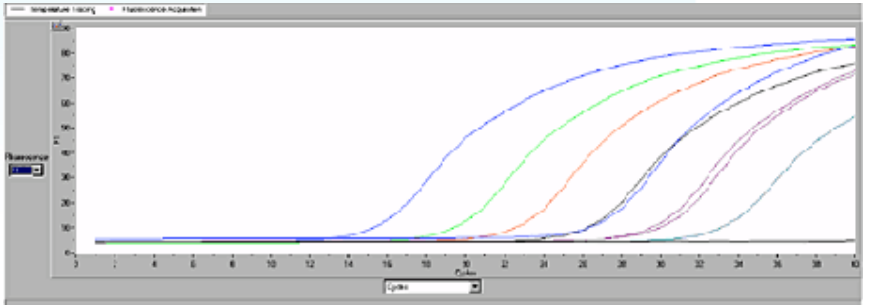
Cockerill FR III. Arch Pathol Lab Med. 2003;127:1112

Real-Time PCR (qPCR)

- Detection: measure fluorescence in each cycle
- Follow PCR cycles in real time

- Simultaneous detection of products in several reactions in 4-5 channels (multiplex PCR)





Conventional PCR versus qPCR

Conventional PCR:

- endpoint measurement of synthesized PCR product
- qualitative
- post-PCR analysis using agarose gel, southern blot,….
- contamination (open system)
- Primers

qPCR:

- measurement of fluorescence intencity in every cycle (= PCR product)
- qualitative and quantitative
- Post-PCR analyse m.b.v. Computer
- low risk on contamination (closed system)
- primers and fluorescent probe or -dye

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Detection

non specific or specific

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Real-Time PCR detection methods

Direct detection with non-specific DNA intercalating dyes:

- SYBR®Green I
- BEBO, SYTO9, Chromofy
- LCGreen(Plus+), EvaGreen
- PicoGreen®

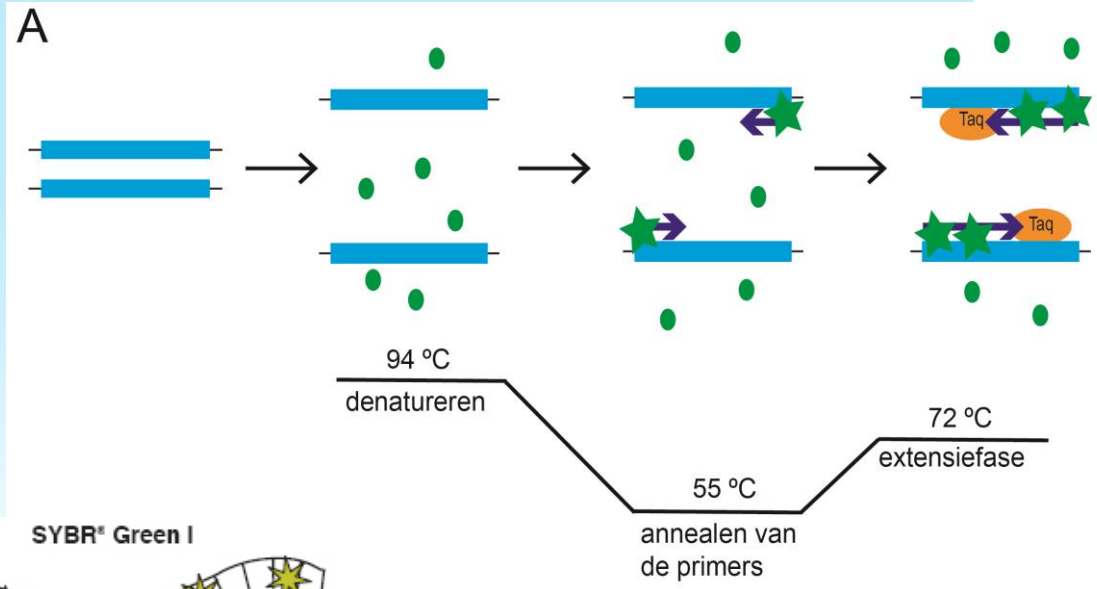
Indirect detection with specific hybridization:

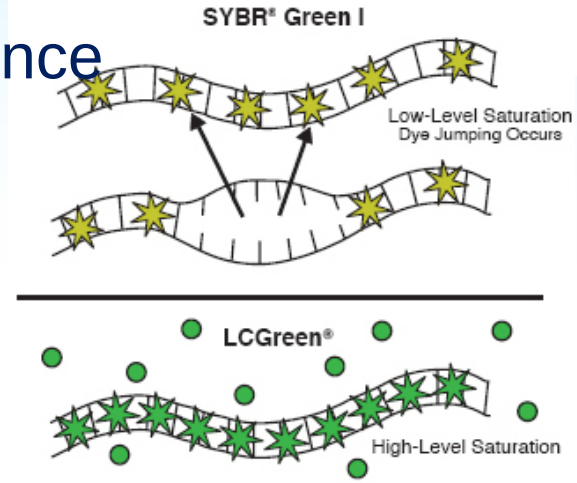
- Hydrolization (TaqMan) probe
- Molecular beacons
- FRET/ hybridisation probes
- …..

dyes

Non specific chemistry

- Intercalation dsDNA
- Eva Green> SYBr Green
- Only fluorescence in dsDNA

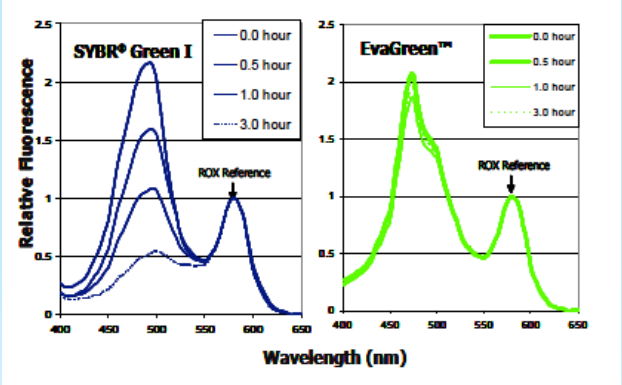




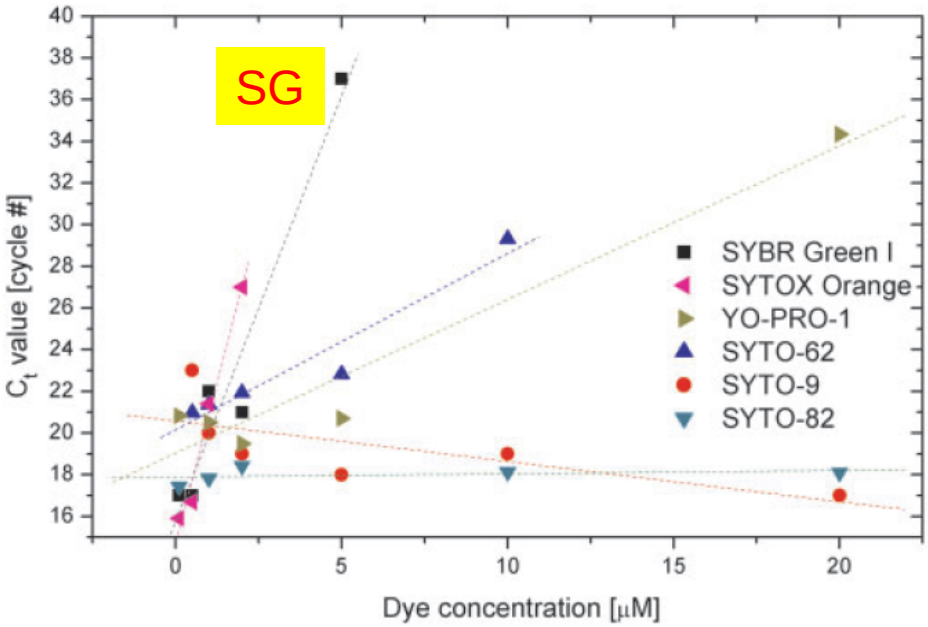
C.C.Orelio en M.J.Plug; Basisprincipes van de PCR 2013

Fluorescence intensity: differs per method/fluorochrome

- Heat-lability (99 ° C)
  - More background
    - SYBr Green > LC Green
- ‘inhibition’ PCR
  - increase T_m
  - SYBR Green:
    - Not too much
    - <1 uM
  - Decrease of efficiency
    - New titration



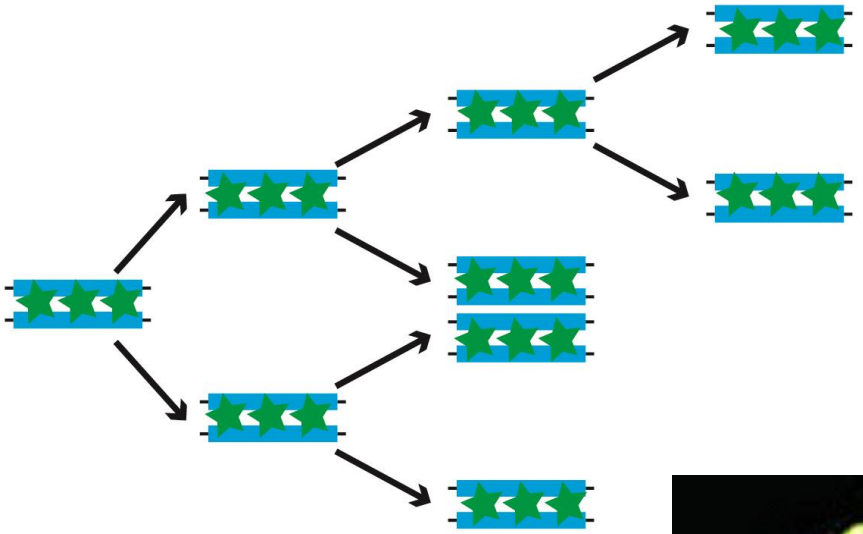
Biotium product information

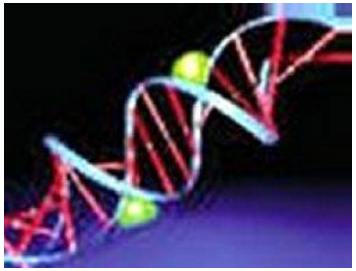


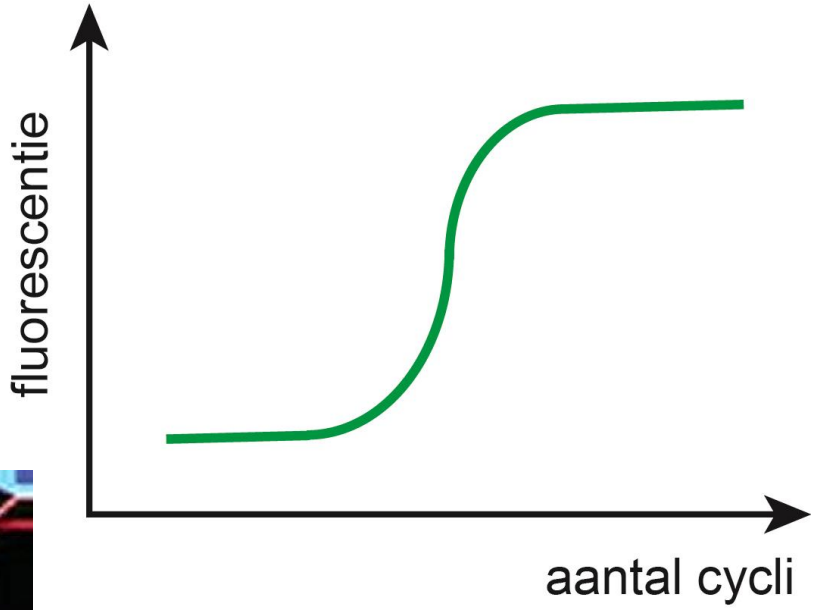
Monis 2005

SYBR green

- non specific
- dsDNA ⇧ -> fluorescence intensity ⇧





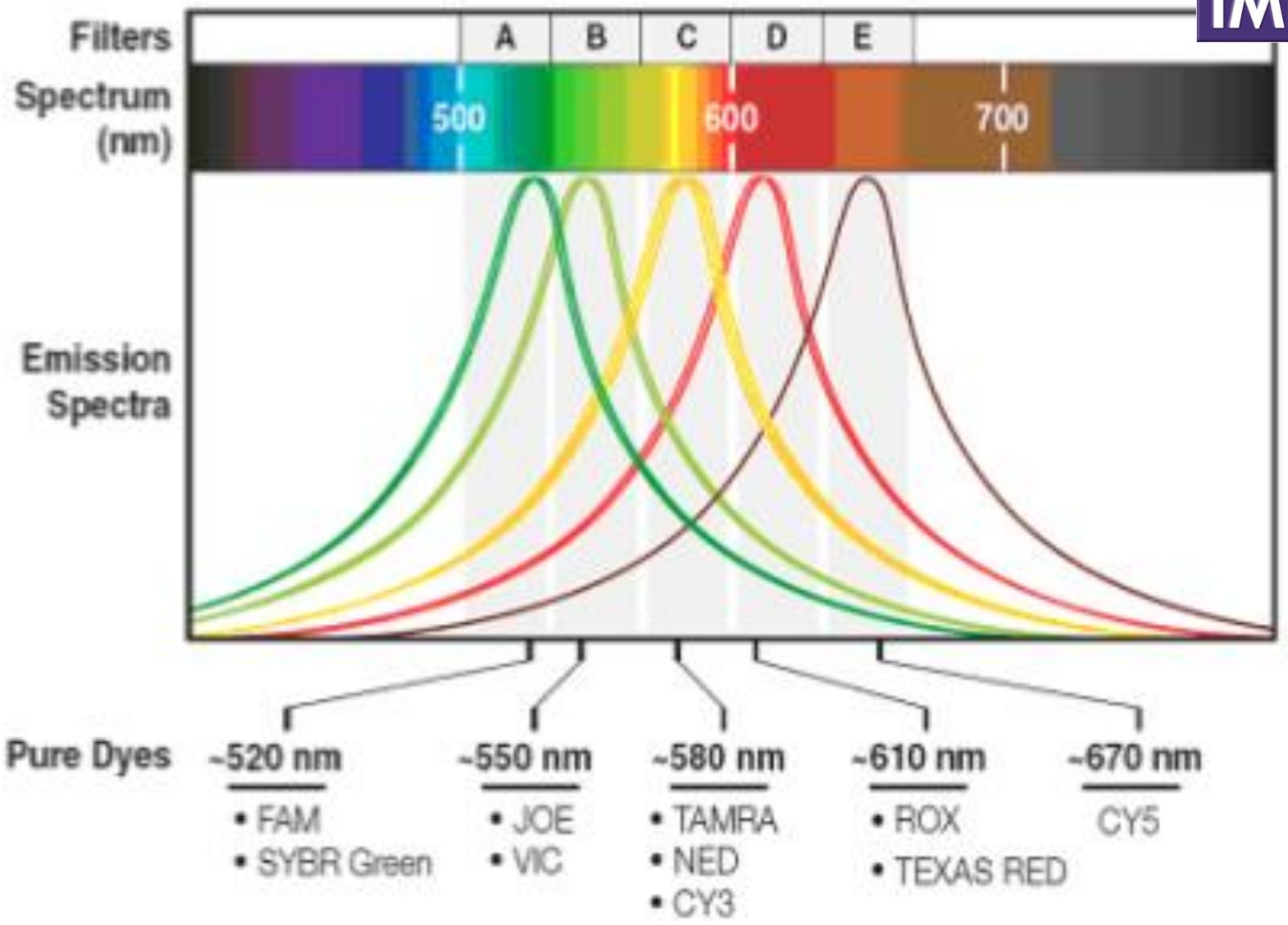


C.C.Orelio en M.J.Plug; Basisprincipes van de PCR 2013

Probes

- hybridize specifically to DNA sequence in PCR product
- Fluorescent dye (reporter) linked to 5’- hydrolization probe
- Quencher linked to 3’- end of hydrolization probe
- Quencher absorbs energy and quenched fluorescent signal

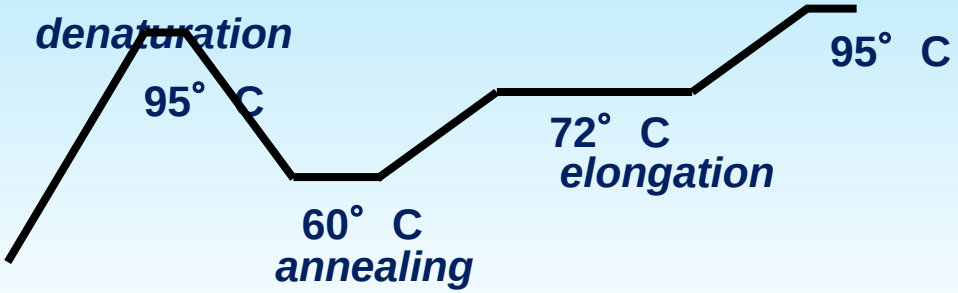
Different fluorochromes



http://www3.appliedbiosystems.com/AB_Home/index.htm

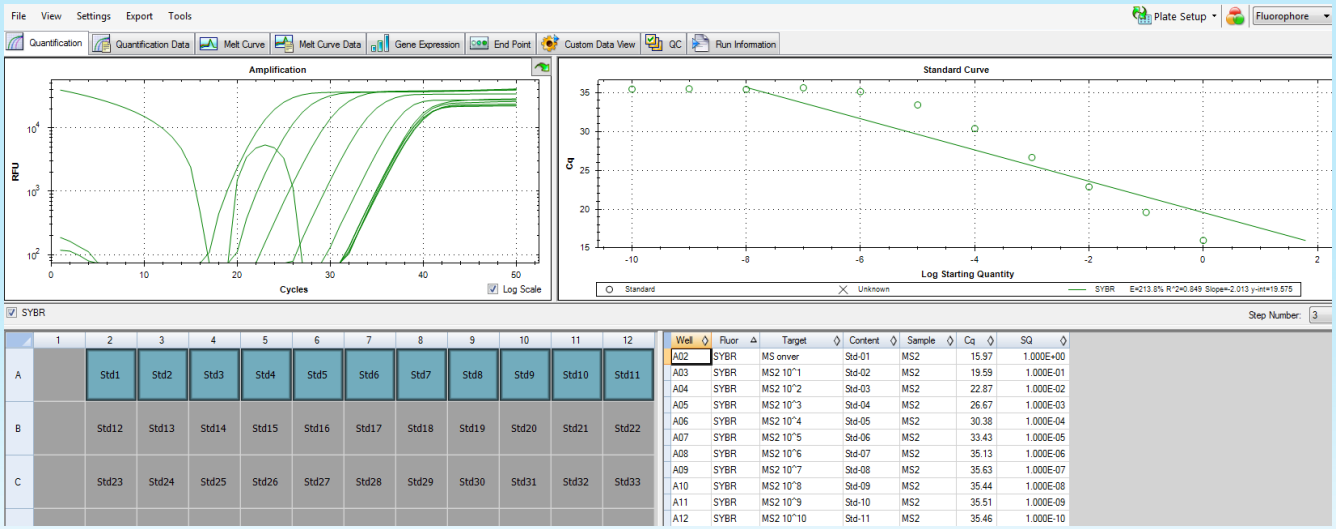
On what moment will the signal be measured?

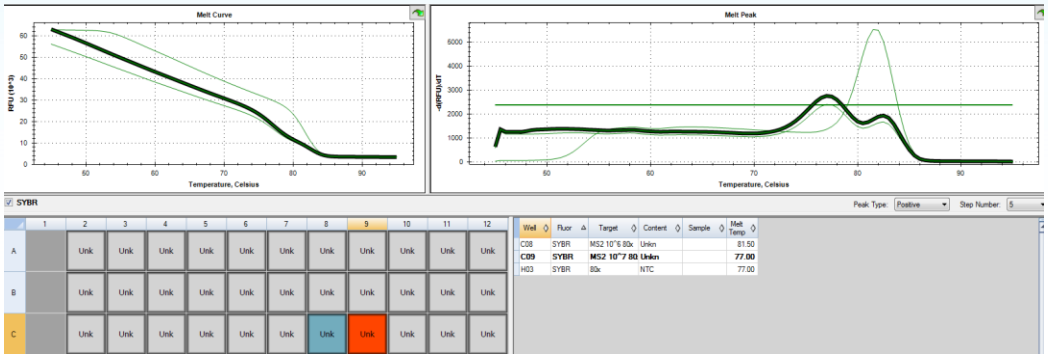
- Same moment?
- Two or three-step protocol?
- Same fluorescence intensity?

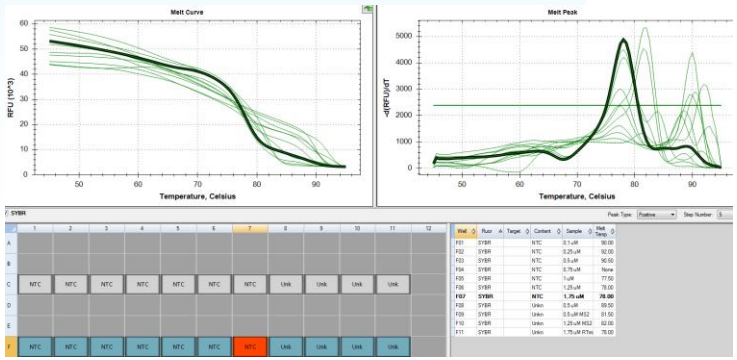


  - DNA binding dyes (end elongation; 2/3 step)
  - Hydrolyse probe (end elongation; 2/3 staps)
  - Hybridization/Dual probe (before elongation only in 3-step protocol)

In a PCR validation it is better to use a non-specific detection (detection of by-products, influencing Cq)







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The amplification curve in more detail

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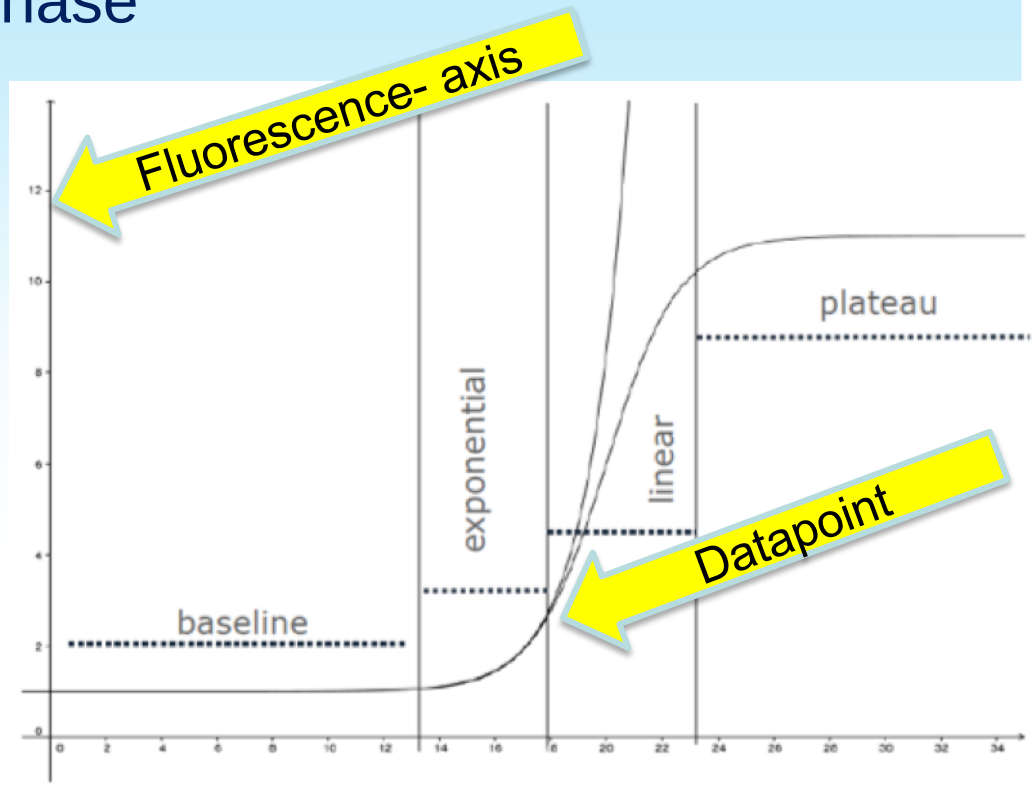
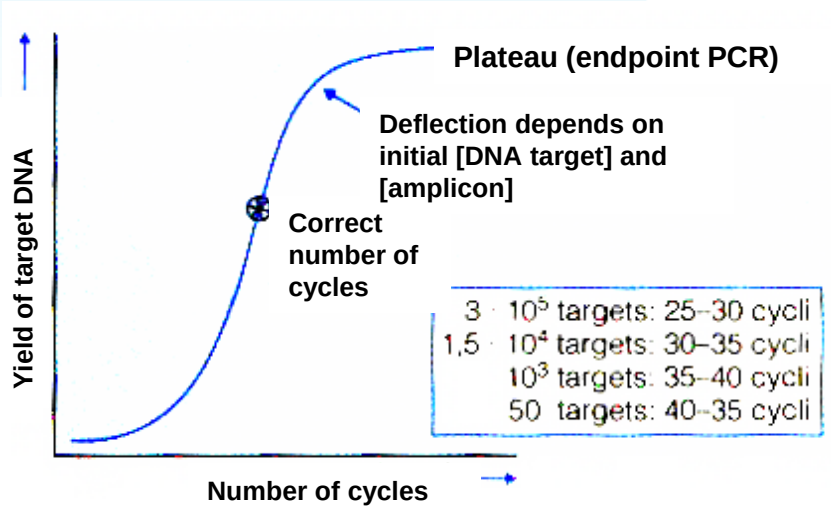
Most frequent used display of amplification curve

- Start-up phase
- early exponential phase
- Late exponential phase
- Linear phase
- Plateau phase



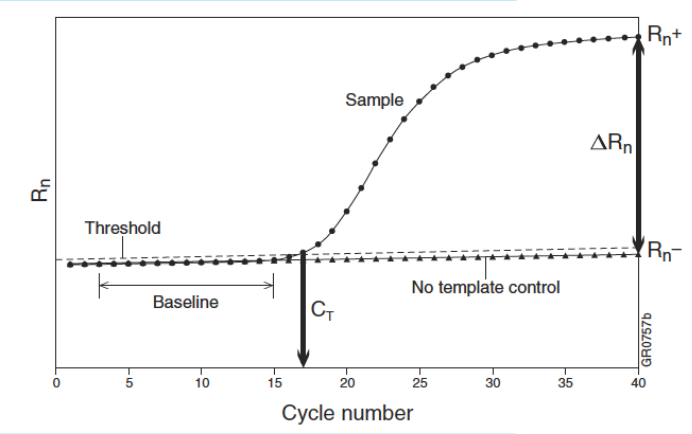
Development in amplicon synthesis

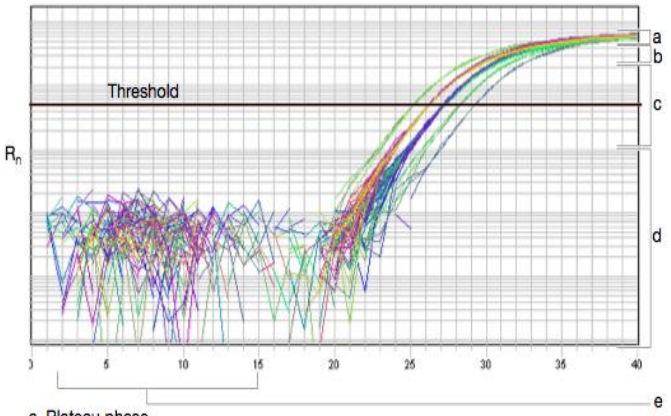
- DNA synthesis of PCR product via S curve
- Length start-up phase
  - Depends on input DNA [conc. target]
- Most effective at exponential increase:
- Effectivity decreases on a certain moment
  - Synthesis of amplicons may reduced rather than by-products
- Synthesis stops
  - Reaction componentshave been used
  - Plateau phase



‘Threshold setting’

- linear or log-linear display
  - Watch the differences!
- ‘Threshold setting’
  - Automatic (average I_{fluor} within cycle 3 and 15)
  - Manual (subjective) in middle of log-linear curve





MIQE guidelines and nomenclature

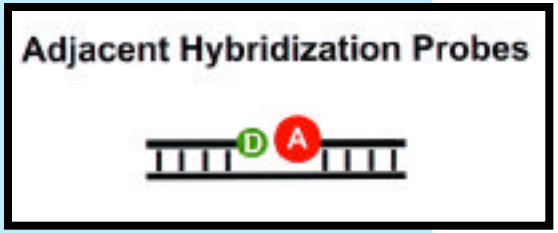
- *Minimum Information for publication of Quantitative Real-Time PCR Experiments*

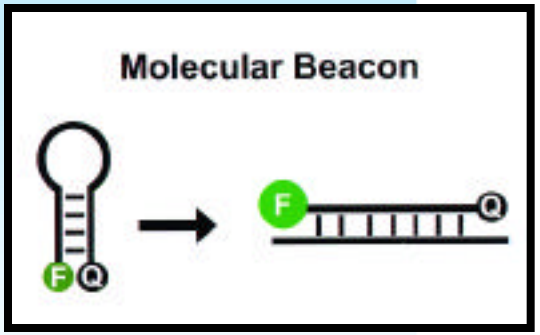
- Uniformity in terminology (publication)
  - qPCR (quantitative real-time PCR)
  - RT-qPCR (reverse transcription qPCR)
  - Cq, quantification cycle, not:
    - threshold cycle (Ct),
    - crossing point (Cp),
    - take-off point (TOP), etc

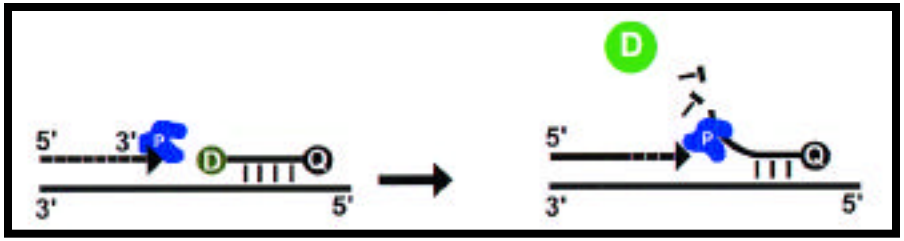
Clinical Chemistry 55:4,611–622 (2009); Bustin et al

Probe classification

- labelde probes ➔ only signal through hybridization to specific DNA target
  - ‘molecular beacon’, ‘dual hybridisation’ probe
    - Meltcurve analysis possible
  - hydrolyzion probe (former: TaqMan probe)
    - MCA not possible







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Detection limit, reliability and efficiency of PCR

TopLab

PCR reliability

- Check specificity
  - The size of expected product?
  - By-products (non-specific)
  - No product in negative control or in negative samples

PCR reliability: choice of the enzyme

Reliability depends on.:

- Quality and quantity of enzyme
- Type Taq
  - proofreading
  - hot-start
- Template quantity
- Primers
  - concentration
  - Primer design
  - Tm

- dNTP quantity
- Mg2+ concentration

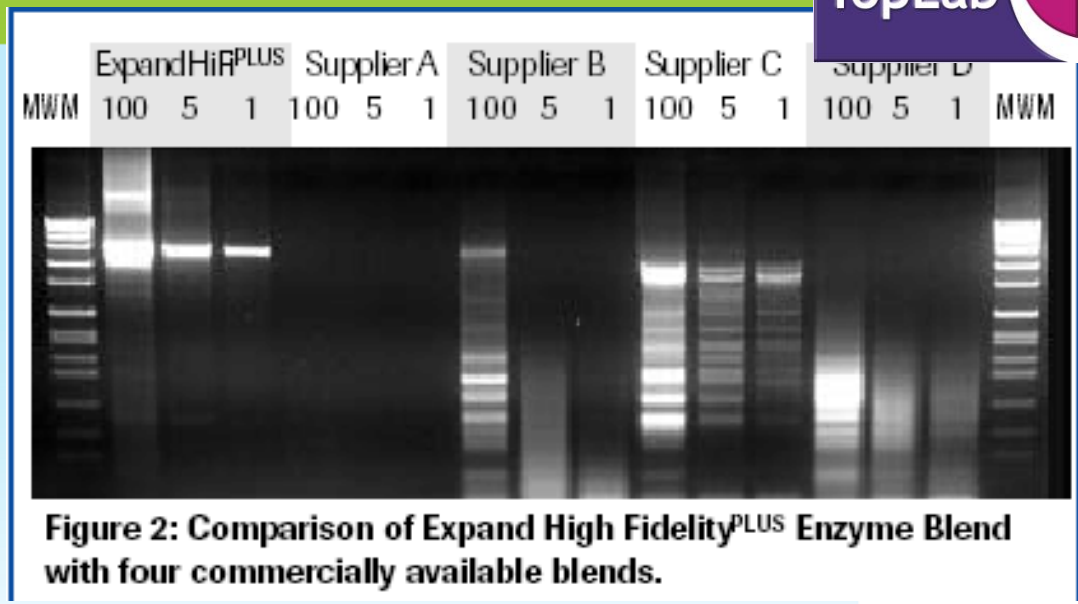
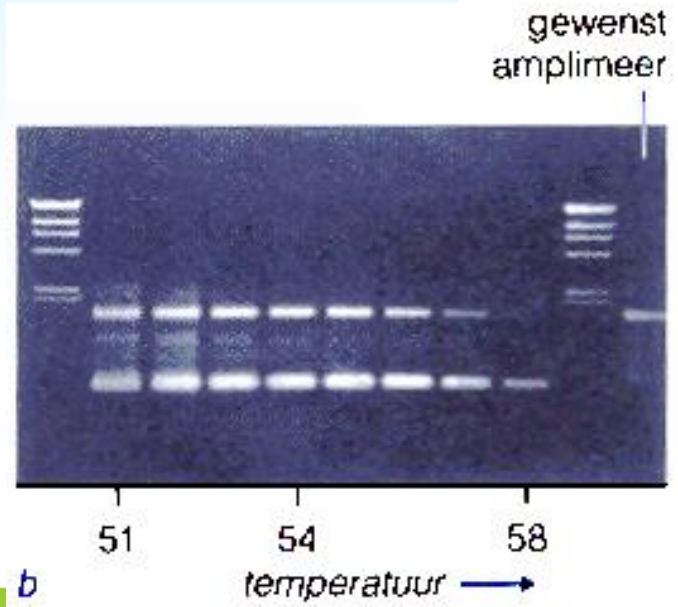
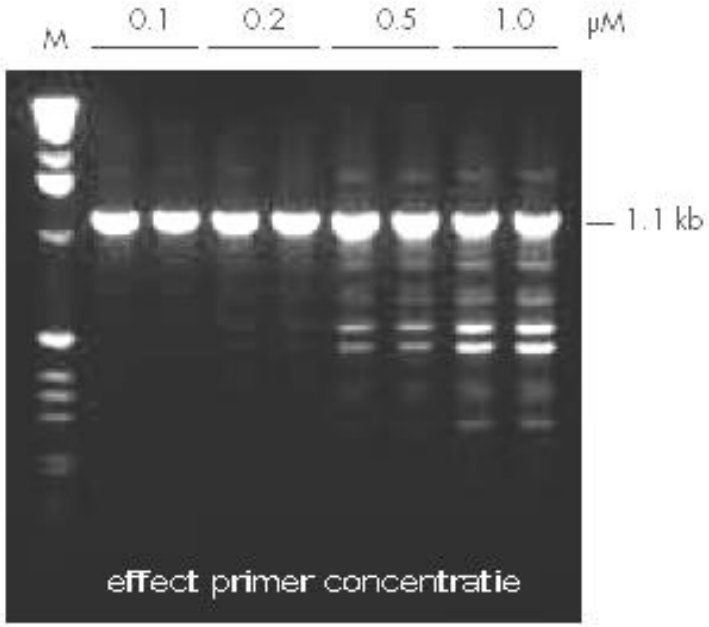


Figure 2: Comparison of Expand High Fidelity^{PLUS} Enzyme Blend with four commercially available blends.

Illustratie: Roche

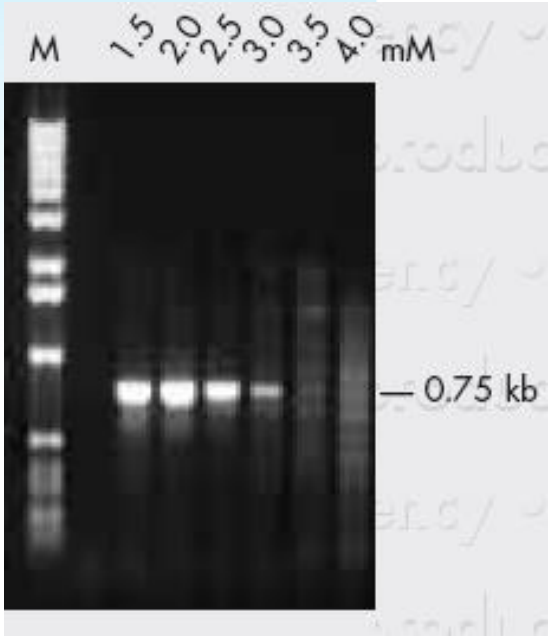


PCR reliability: primer concentration



primer concentration too high

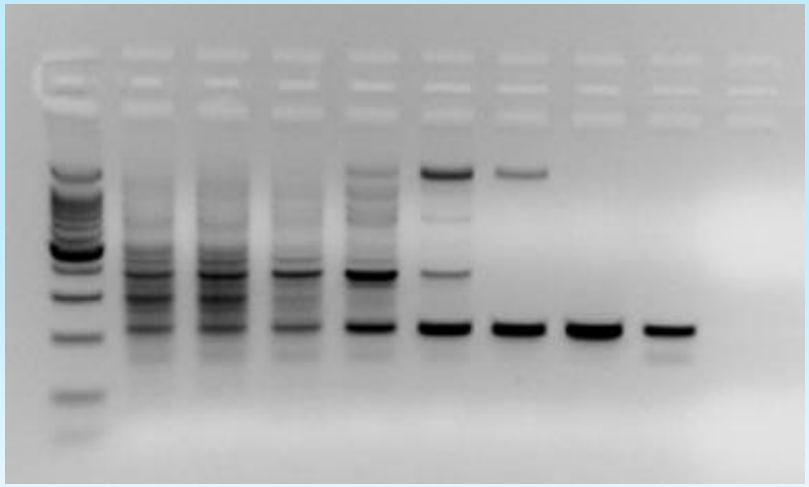
=> undesirable products



Mg concentration to high=> inhibition, undesirable products

Mg concentration too low=> Taq polymerase not active

PCR reliability: optimization Tm



Factor V Leiden
35 tot 75˚ gradiënt

- less mispriming/maximum yield
- Correct T_m-calculation
- Thermodynamic approachj
- Normal PCR
  - T_m= -5 ˚ C
  - "degenerate primers"; T_m ↓(15-20 ˚ C)

PCR sensitivity

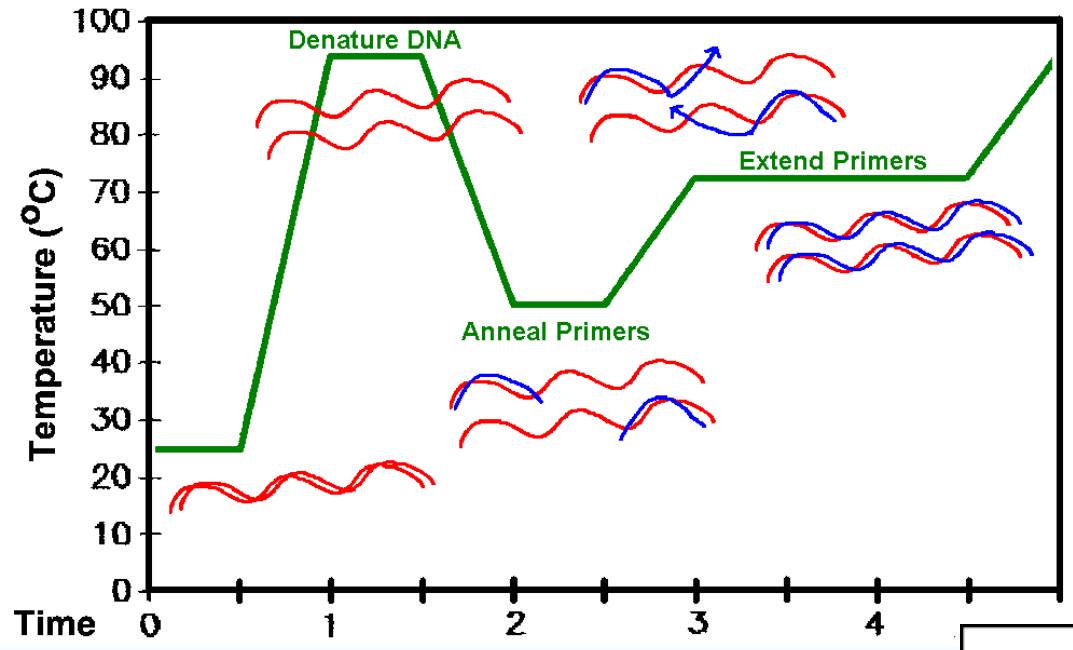
- What is the detection limit?
  - Theoretically 1 molecule may be detected
  - after 30 cycles, 1 DNA molecule has been increased to 530 millon copies
  - Titrate input DNA to determine detection limit or PCR test sensitivity

PCR inhibition

- Inhibitors in the sample
  - Hemoglobin
  - heparin
- Inhibitors in DNA isolation/ preparation protocol
  - Ethanol
  - EDTA
- In PCR reaction
  - High target DNA concentration

stof	concentratie
fenol	>2% vv
ethanol	>1% vv
isopropanol	>1% vv
NaAc	>5mM
NaCl	>25mM
EDTA	>0/5mM
CTAB	0,05
SDS	0,05
hemoglobine	>1 mg/ml
heparine	>0.15U/ml
RT-mix	>15%

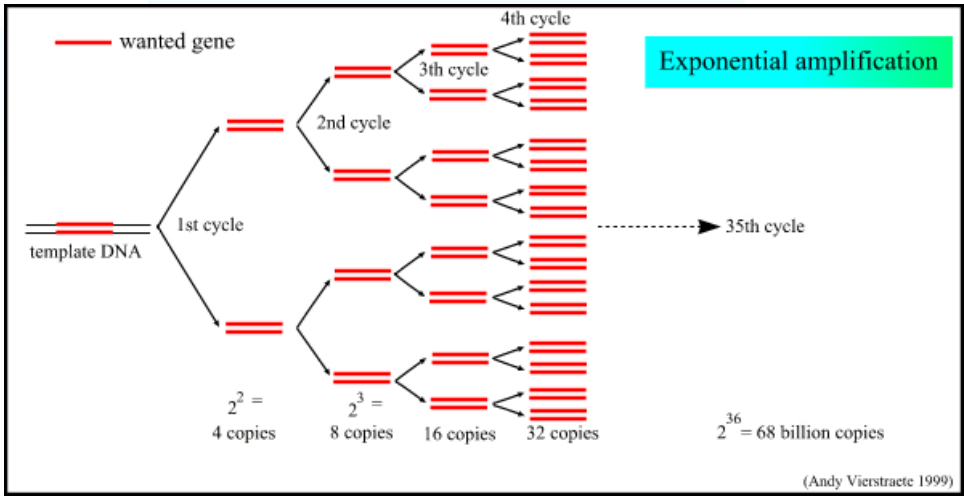
PCR efficiency



http://users.ugent.be/~avierstr/principles/pcr.html

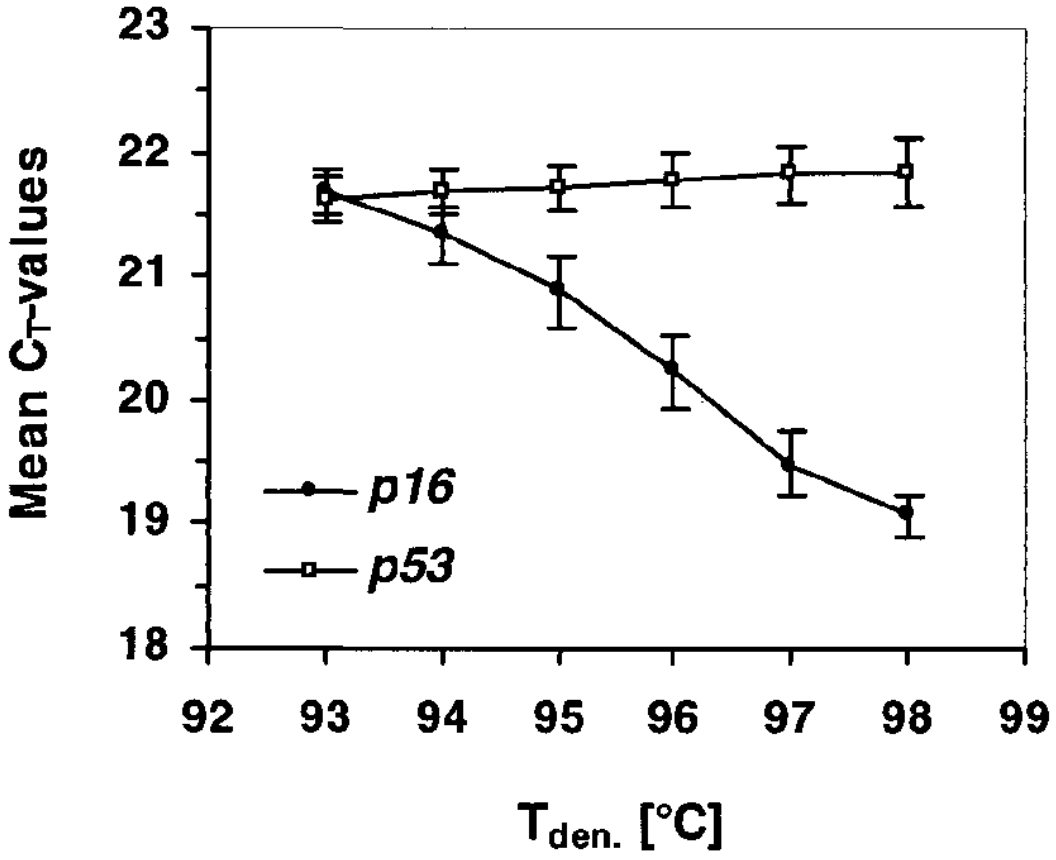
What is meant with100% PCR efficiency?

What can affect PCR efficiency?

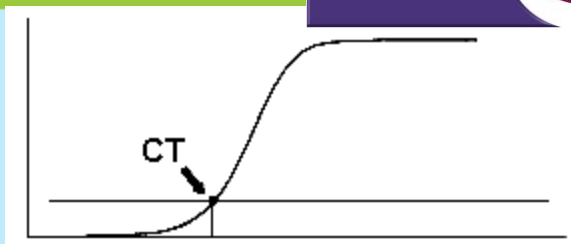


http://users.ugent.be/~avierstr/principles/pcr.html

PCR efficiency: denaturation effect



Wilhelm et al 2000
Clinical Chemistry



Real-time PCR:
C_q low: high yield initial target

Melting of genomic DNA
is important for PCR efficiency in the first few cycles

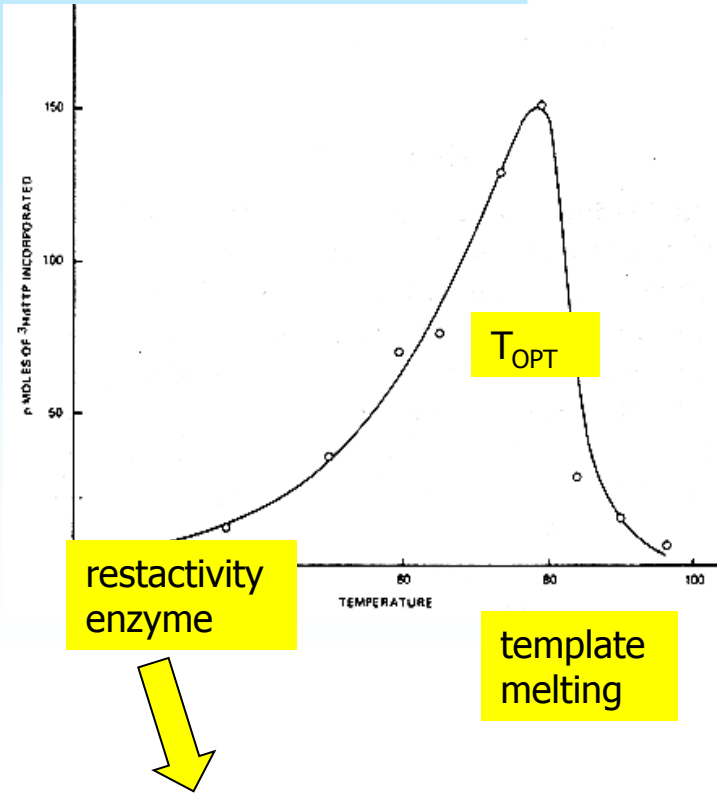
PCR efficiency: denaturation and mispriming

- Primers bind to DNA target before PCR has started
- Taq polymerase: dsDNA + 3'-OH = start DNA synthesis

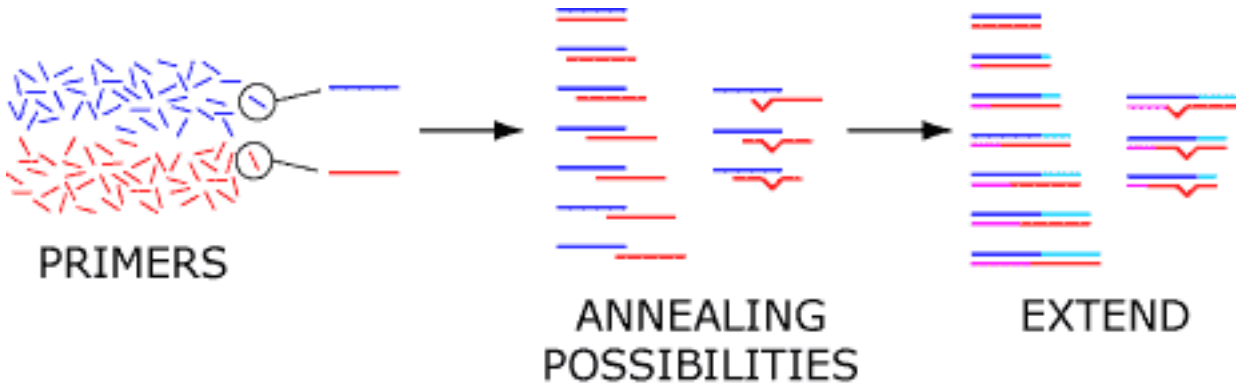
=>rest-activity Taq polymerase starts DNA synthesis

- At insufficient stringency
  - Cross hybridization/ undesirable hybridization

- reasons:
  - Bad primer design (primer-dimers)
  - Too much Mg conc (increases stability primer-DNA)



PCR efficiency: mispriming and primer-dimers



http://www.uq.edu.au/vdu/PDU_PCRPrimers.htm

avoid

- complementarity of primers at 3' end
- long single nucleotide repeat stretches
- high CG% level at 3' end of primers

PCR efficiency: decrease mispriming

- T_m optimalise (use gradient PCR)
- Hot start; time release enzymes
- Increase T_m ↑ primers (long primers)
- Competitor primers
- Short synthesis/elongation time
- lower concentration of
  - dNTP
  - primers
  - Taq DNApolymerase

PCR efficiency

- Can be influenced by reaction ingredients
  - Buffer
  - Concentration Mg
  - Concentration dNTPs, primers
  - …..
- Can also be influenced by
  - Quantity input target
  - Secundary structures in amplicon
  - inhibitors in matrix (sample)

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

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Na 1 cyclus

100% = 2.00x

90% = 1.90x

80% = 1.80x

70% =

De toename per cyclus:

2^n, $1{,}9^n$, $1{,}8^n$, $1{,}7^n$

How to measure PCR efficiency?

- Efficiency real-time PCR
  - 100% efficient: 2^n function
  - 90% efficient: 1.9^n
- Derived from C_q 's reference series
  - Different techniques (**threshold**, noise band, fit point, LinReg, SDM, curve fit)
- Efficiency analysis
  - slope individual amplification curve (tangent line)
  - Slope reference curve (**workshop ref curve**)

CYCLE	AMOUNT OF DNA	AMOUNT OF DNA	AMOUNT OF DNA	AMOUNT OF DNA
	100% EFFICIENCY	90% EFFICIENCY	80% EFFICIENCY	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
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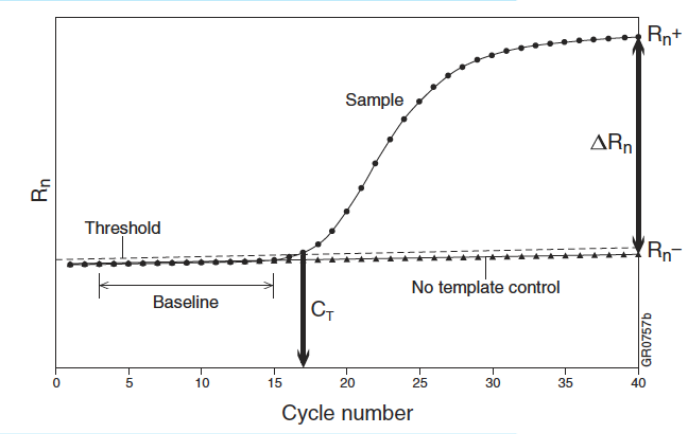
25	33,554,432	9,307,650	2,408,866	577,063
26	67,108,864	17,684,534	4,335,959	981,007
27	134,217,728	33,600,615	7,804,726	1,667,711
28	268,435,456	63,841,168	14,048,506	2,835,109
29	536,870,912	121,298,220	25,287,311	4,819,686
30	1,073,741,824	230,466,618	45,517,160	8,193,466

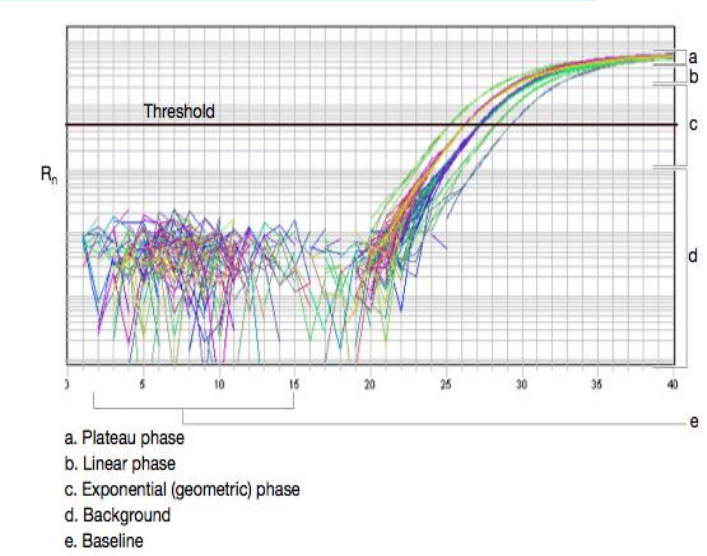
AFTER 1 CYCLE
100% = 2.00x
90% = 1.90x
80% = 1.80x
70% = 1.70x

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

C_q: threshold setting method

- 'Threshold setting'
  - Automatic (software PCR processor)
  - Manual setting: middle l**og-linear** S-curve
- In what way determined?
  - Baseline/background (= average I_{fluor} between cycle 3 and 15)



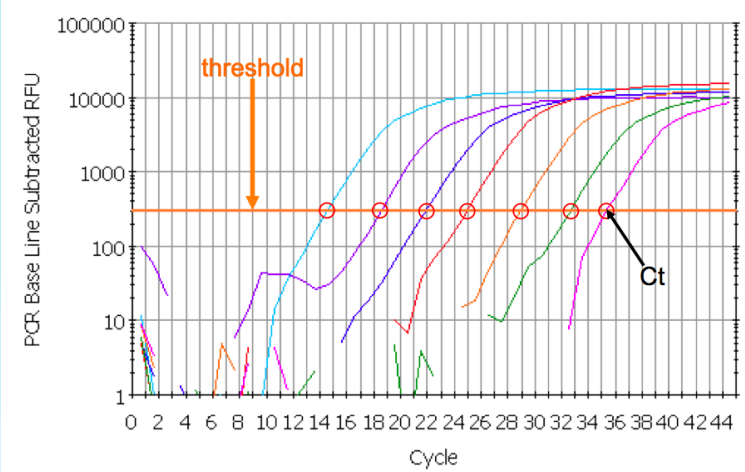


Efficiency assay

- why?
- How?
- Derived from exponential (log-linear) part and C_q s from reference curve
  - 100% efficient: 2^n function
  - 90% efficient: 1.9^n
- many methods: most frequently used
- Slope reference- curve
  - If not possible: individual curve
    - Tangent line on inflection point(1^{st} derivative): slope
    - EFFICIENCY: $E= 10^{slope}$

Quality criteria for amplification curve

- slope
  - Equal at different dilutions (1,8-2,0)
    - Too steep: by-products
    - Too flat: inhibition
- Distance between consecutive curves
  - equal
  - Approx 3,3 units (efficiency 100%)
- ΔRn of individual curves
  - equal

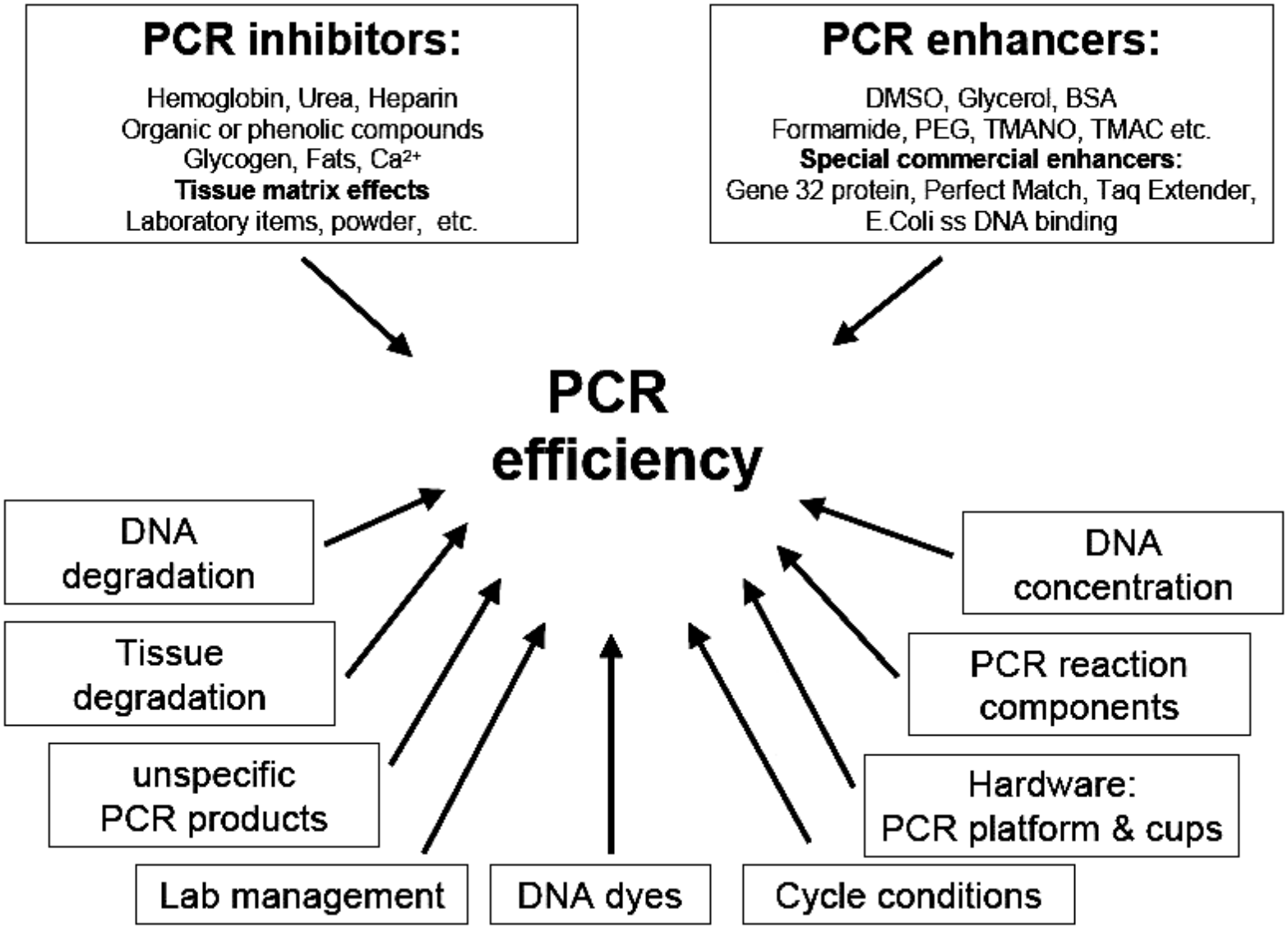


$E=10^{slope}\ (10^{\alpha(i)})$

Causes plateau phase; efficiency DNA synthesis reduces

- dNTP and primers lacking
  - usage, damage
- Inhibition by synthesized PCR products
- Competition
  - primers ⇔ complementary amplicons (renaturation)
  - Taq DNA polymerase: amplicons.
- Limited enzyme capacity
  - Many PCR products have to be synthesized every cycle
  - Enzyme activity decreases in time (temperature)

In conclusion: PCR efficiency



hogeschool Leiden

finally

Bep van Pelt-Verkuil

Lectoraat IMD

In your laboratory

- For what purposes is (q/RT) PCR used?
- Which nomenclature?
- How do you interpret data/results?