The Application of Real-Time PCR in the Diagnosis of Infectious Disease

By: Dr. Amin Marashi

Science 1988 Jan 29;239(4839):487-91

Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA

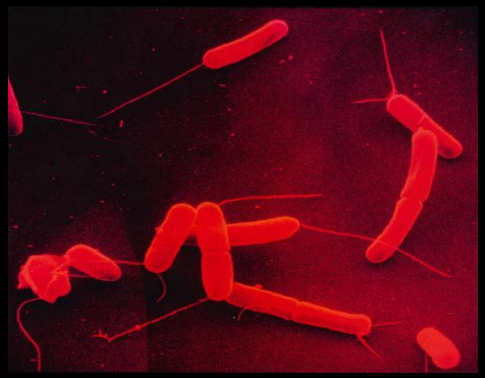
Cetus Corporation, Department of Human Genetics, Emeryville, CA 94608.

Some facts about PCR

- 300,000,000 US$ were paid for the patent (1991)
- Nobel Prize 1993
- Included in the title or abstract of more than 100,000 (!) scientific publications.
- Real-time PCR is becoming the most common use of PCR

Applications relevant to drug development

- Gene expression analysis
  - Splicing variants
- Diagnosis / viral load
- Mutation analysis



Aim

Real-time PCR is technically easy technique with difficulties in the setting and analysis.

The aim of this meeting is to prepare you towards proper use of real-time

Objectives: By the end of this meeting you should be able to:

- Create variations on PCR
- (Theoretically) quantify DNA with real-time PCR
- Recognize the problems associated with quantification of gene expression by real-time PCR and rank their influence on the quantification.
- Select a solution

Why should we use PCR?

- Very sensitive (1 copy – 10 copies of DNA)
- Can detect organisms that cannot be isolated
- Rapid (< 24 hrs)

Disadvantages of PCR

- Technically demanding
- Can be expensive
- Risk of contamination
- Need rigid QC

ADVANTAGES OF REAL-TIME PCR

- ✓ Rapid cycling times (1 hour)
- ✓ High sample throughput (~200 samples/day)
- ✓ Low contamination risk (sealed reactions)
- ✓ Very sensitive (3pg or 1 genome eq of DNA)
- ✓ Broad dynamic range (10 - 10^{10} copies)
- ✓ Reproducible (CV < 2.0 %)
- ✓ Allows for quantitation of results
- ✓ Software driven operation
- ✓ No more expensive than “in house” PCR ($15/test)

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

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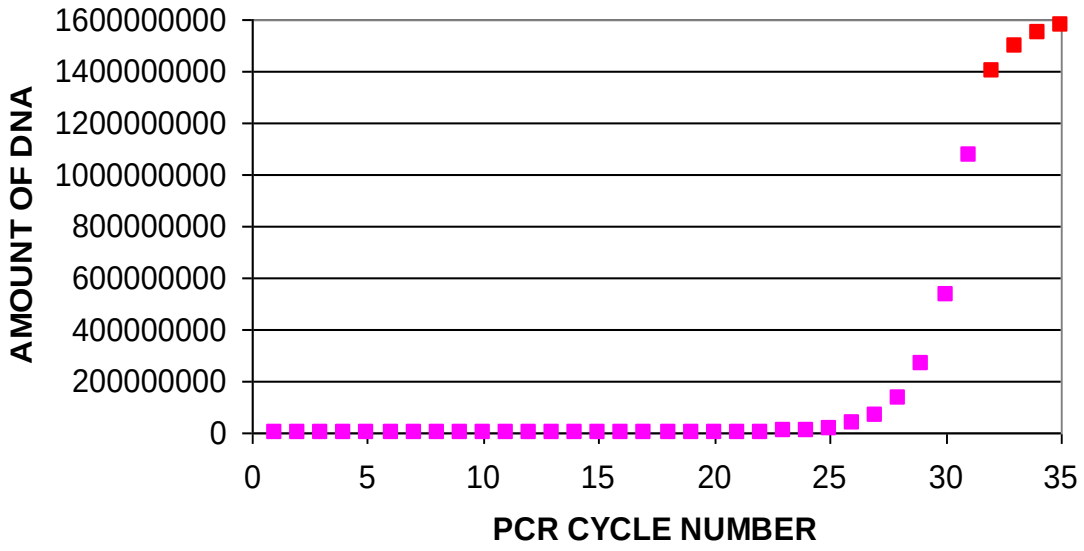
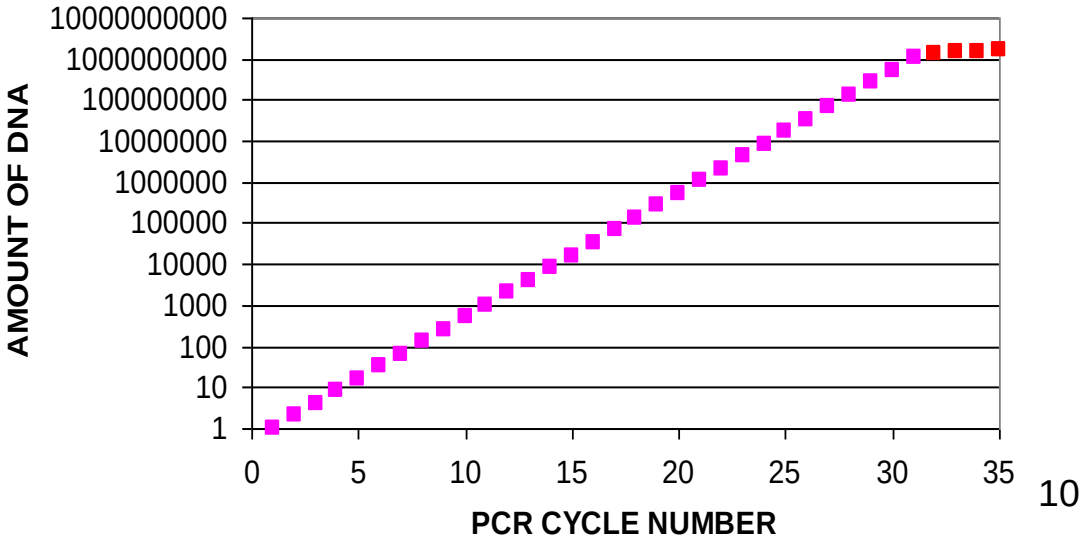
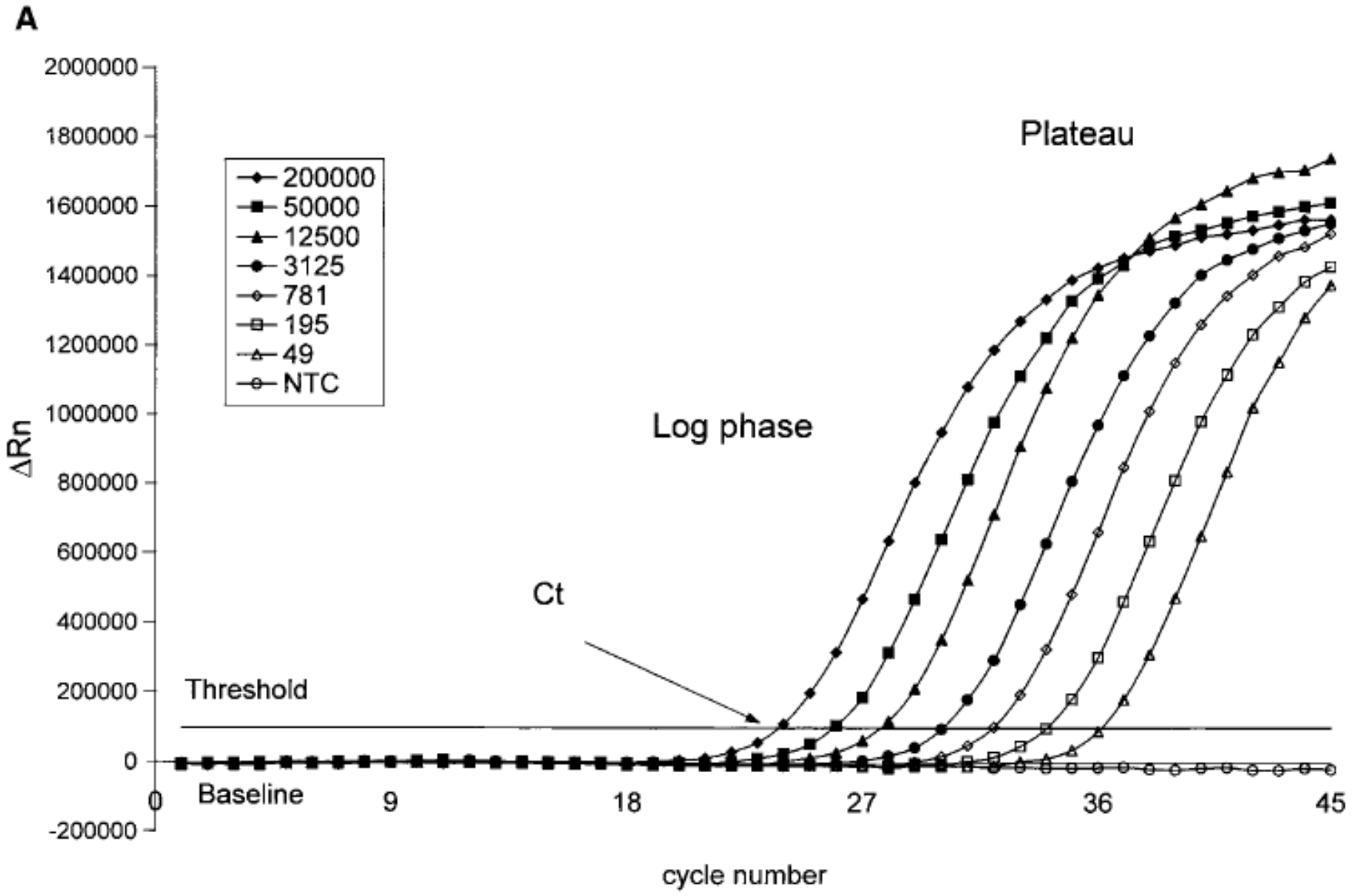
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000






A
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1800000
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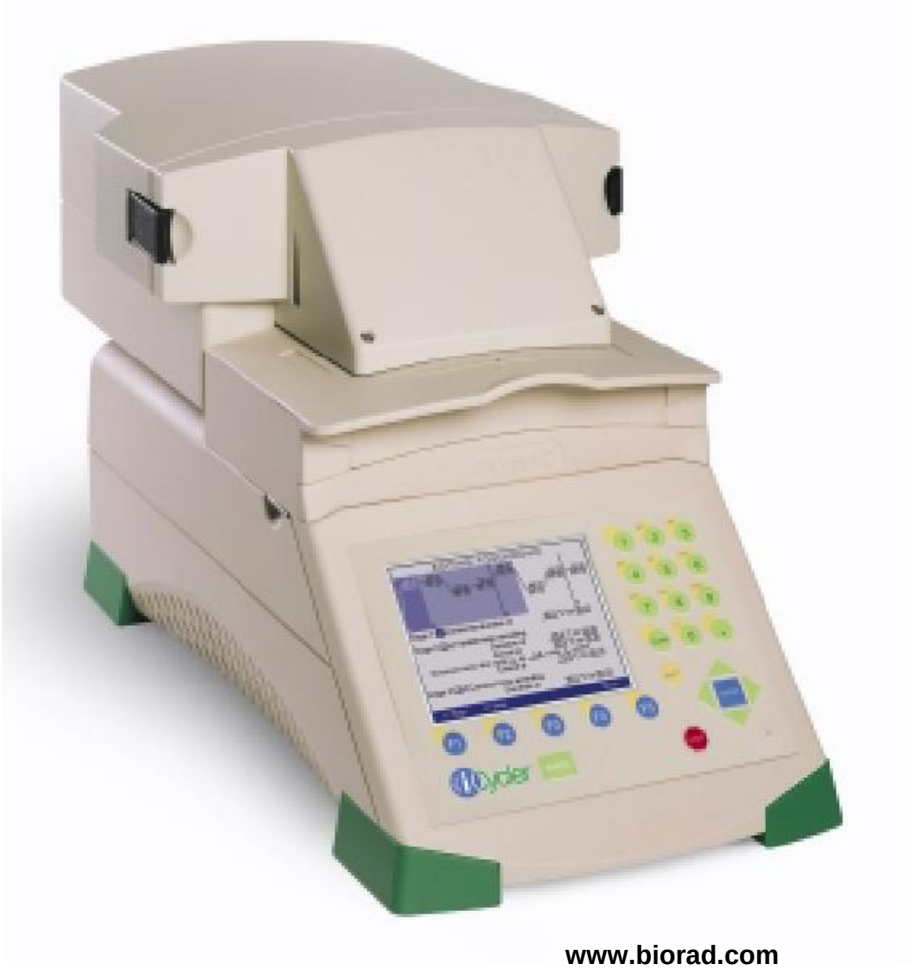
1400000
1200000
1000000
800000
600000
400000
200000
0
-200000
ΔRn
200000
50000
12500
3125
781
195
49
NTC
Plateau
Log phase
Ct
Threshold
Baseline
0
9
18
27
36
45
cycle number

REAL TIME PCR

- kinetic approach
- early stages
- while still linear



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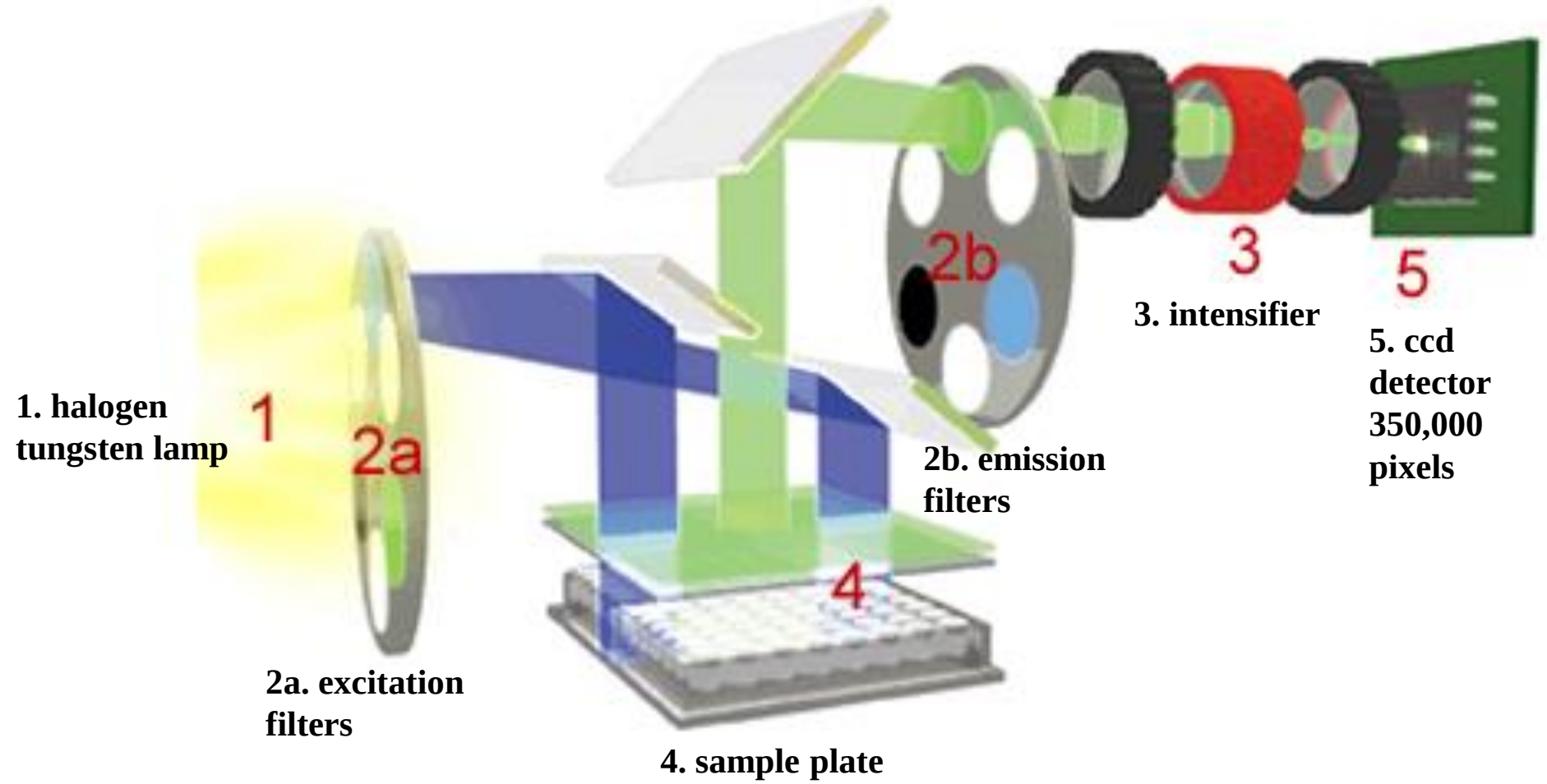

Fig. 1.2. Representation of Optical Detection System layout.

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Real Time PCR :

- Definition: amplification and detection occur in one time as real-time
- More rapid than traditional methods of amplicon detection
- Reduction in chance of amplicon contamination
- Dose not need to any instrument for amplicon detection

DNA quantification by Real-time PCR

Real-Time PCR

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection

Real-time Principles

Three general methods for the quantitative detection:

1. Hydrolysis probes

(TaqMan, Beacons, Scorpions)

2. Hybridisation probes

3. DNA-binding agents

(SYBR Green, Eva Green, LC Green)

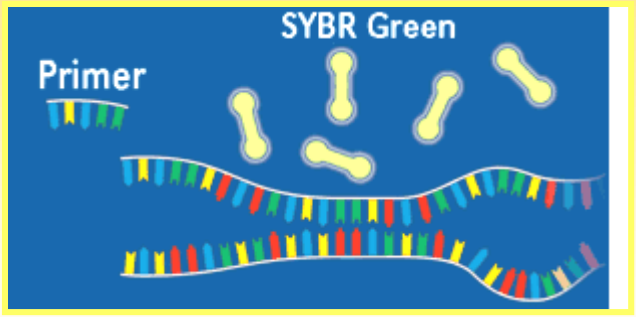
The first two methods are specific formats.

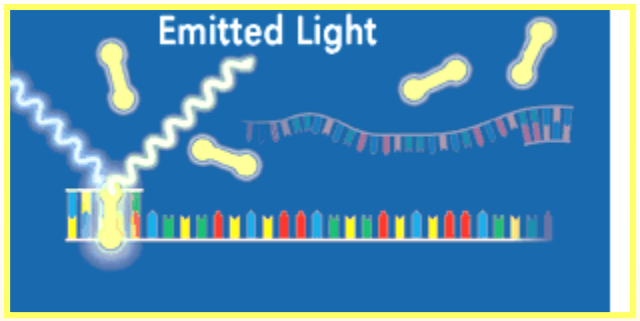
SYBR Green I technique

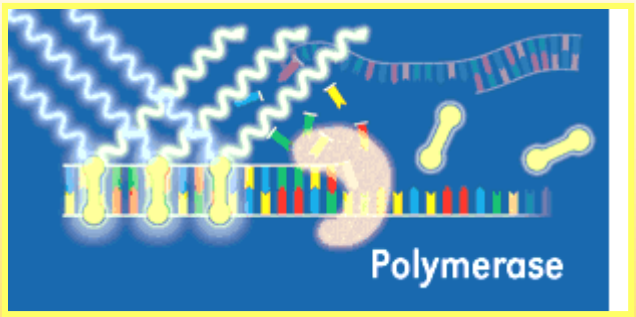
Methods of detecting the products of Real Time amplification

- SYBR green:
  - Is a dye that binds to the minor groove dsDNA
  - Generates more fluorescence when bound to DNA
  - This dye is much less expensive
  - Will bind to any dsDNA
  - Lacks any specificity for diagnosis protocol

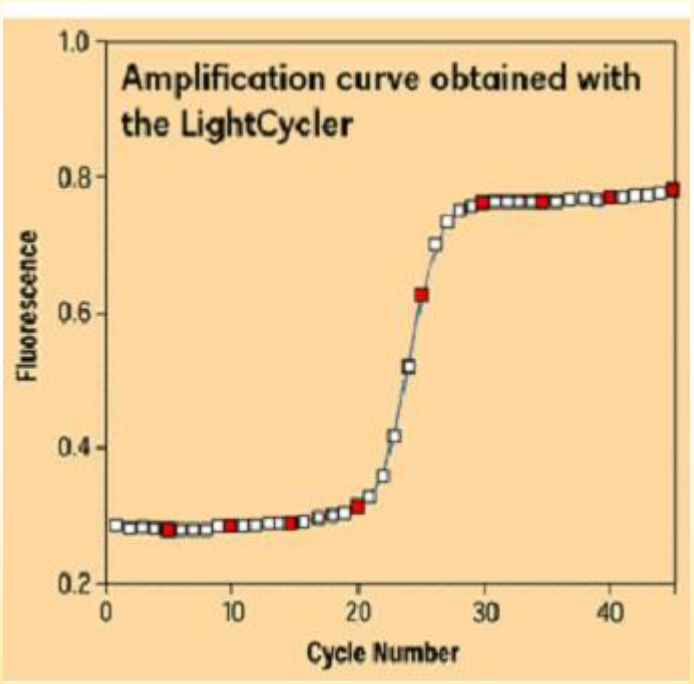
Quantitative real-time PCR with SYBR ®Green





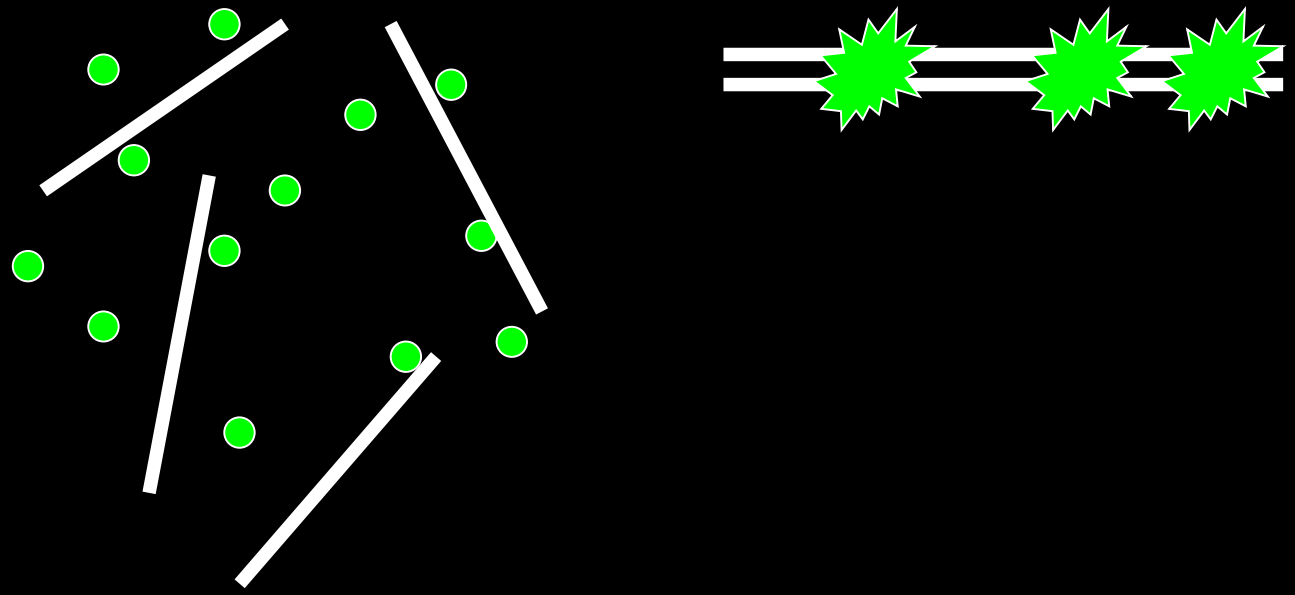


Fluorescence detection at elongation step



Detection methods

Unspecific dye – fluoresces upon binding to double strand DNA



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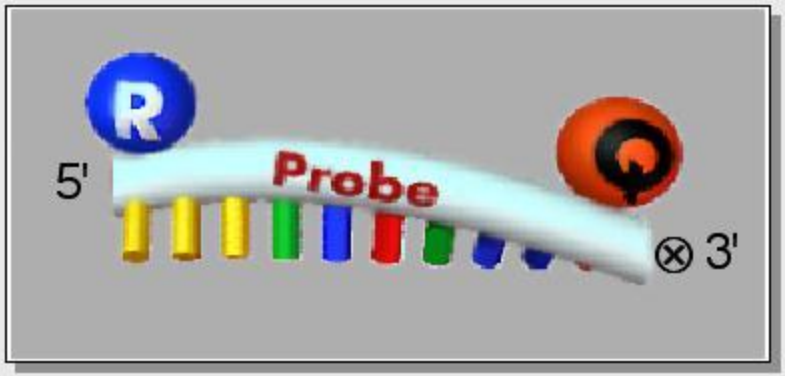
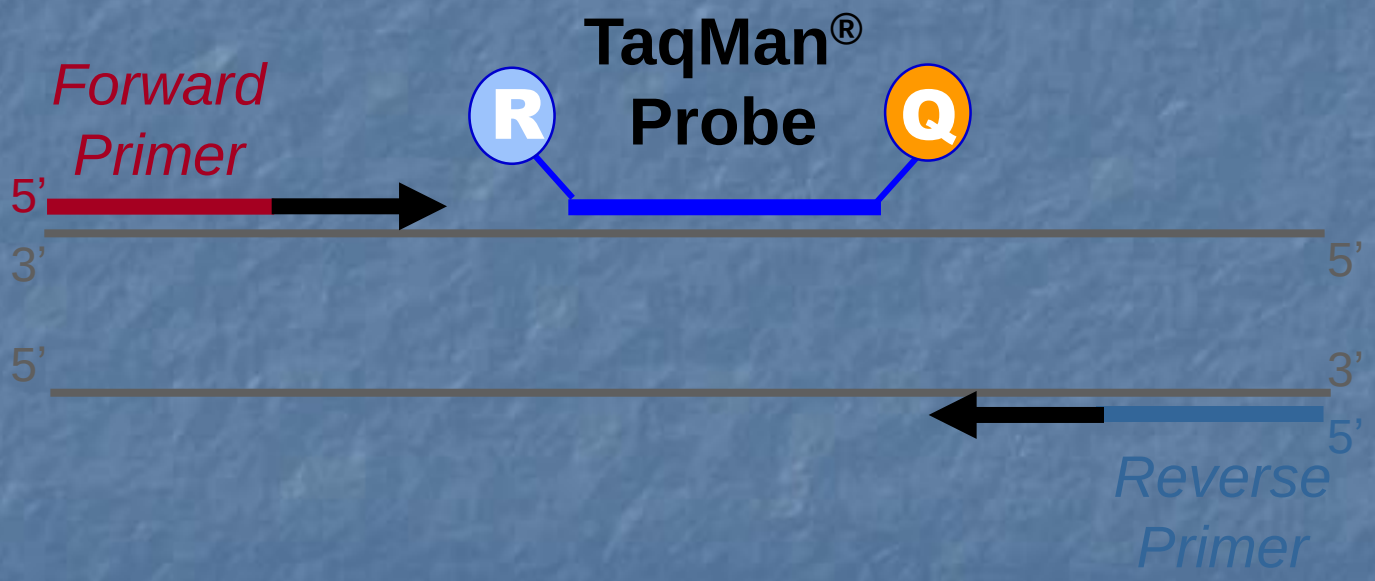
TaqMan probe



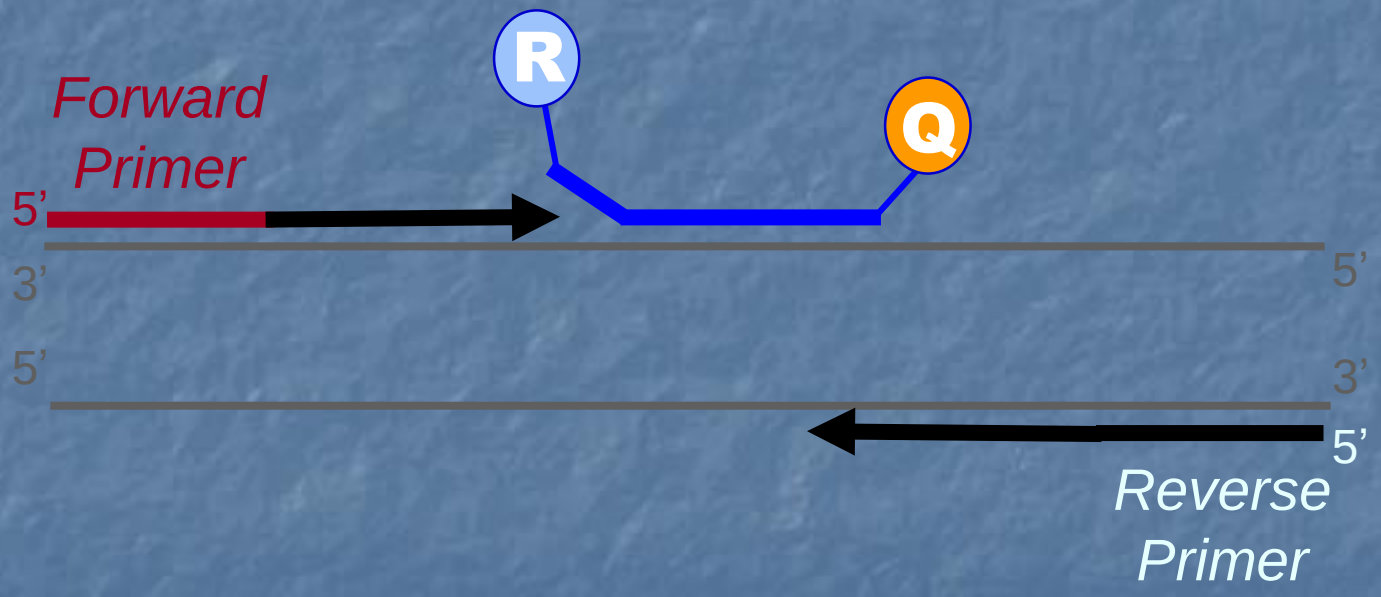
- Hybridises with the target amplicon
- Is 3' terminally blocked (cannot be extended by the polymerase)
- Has two fluorescent dyes attatched:
  1. Reporter (R)
  2. Quencher (Q)

Taqman probes are “hydrolysis” probes

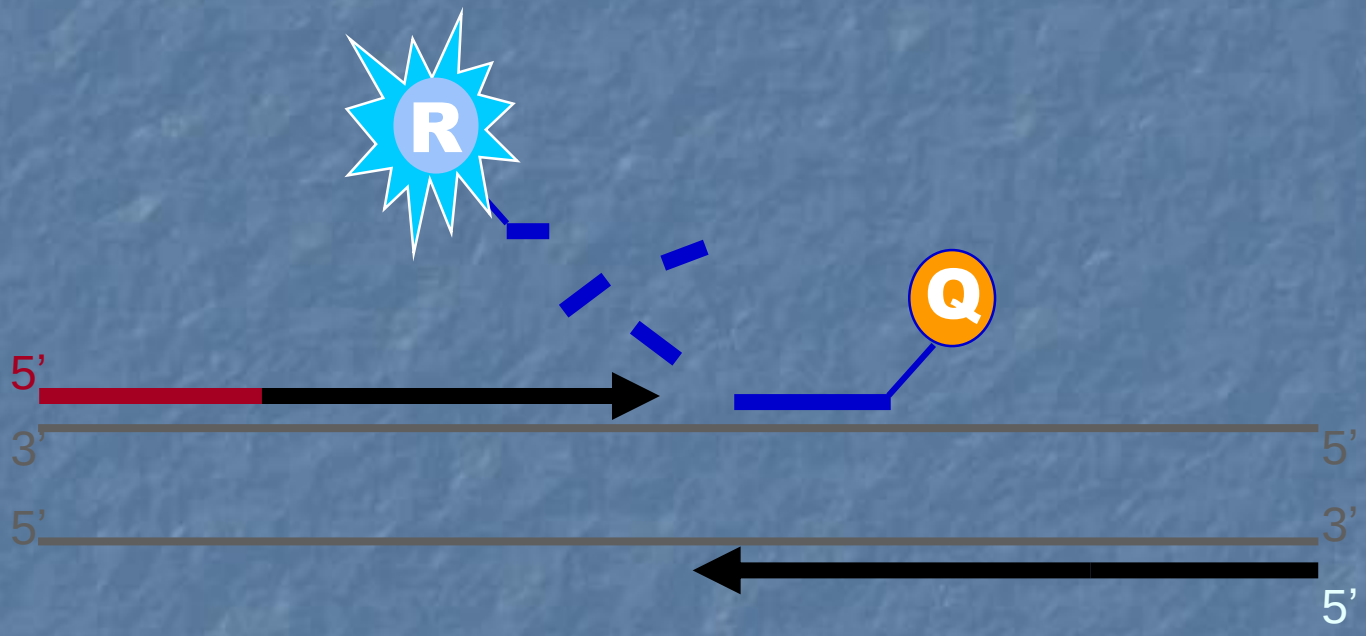
Polymerization



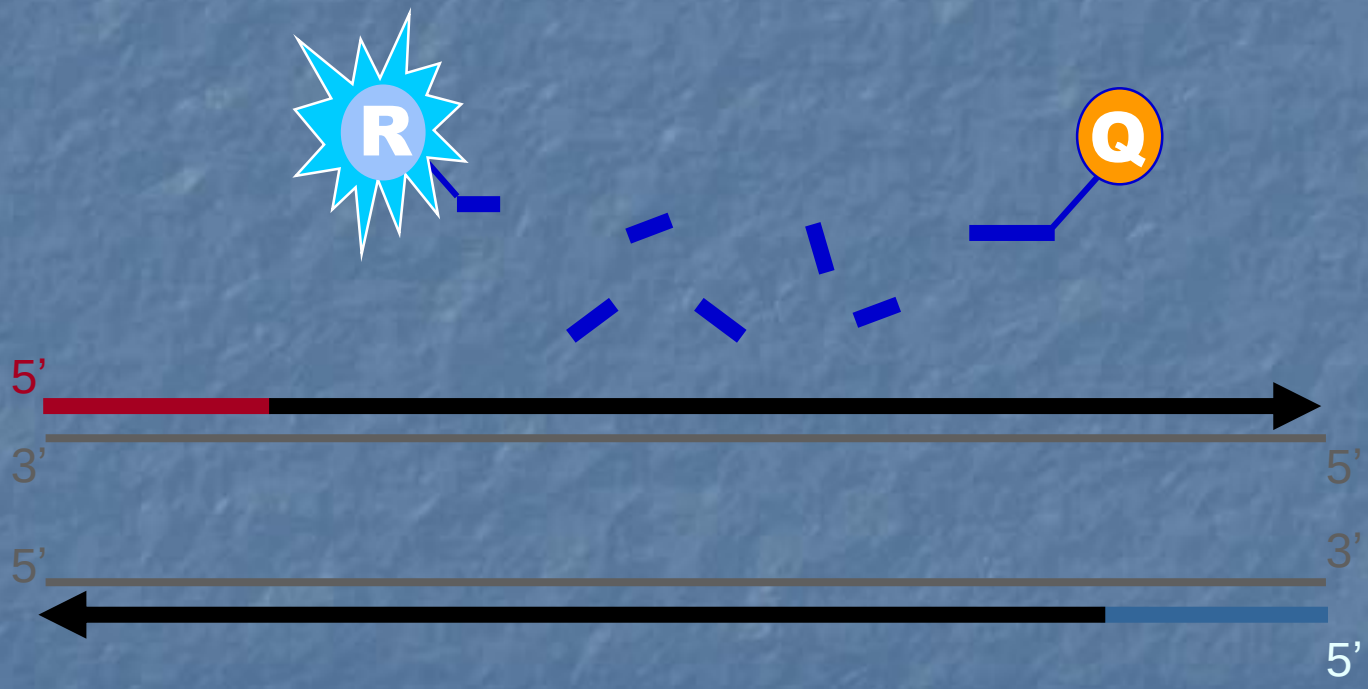
Displacement

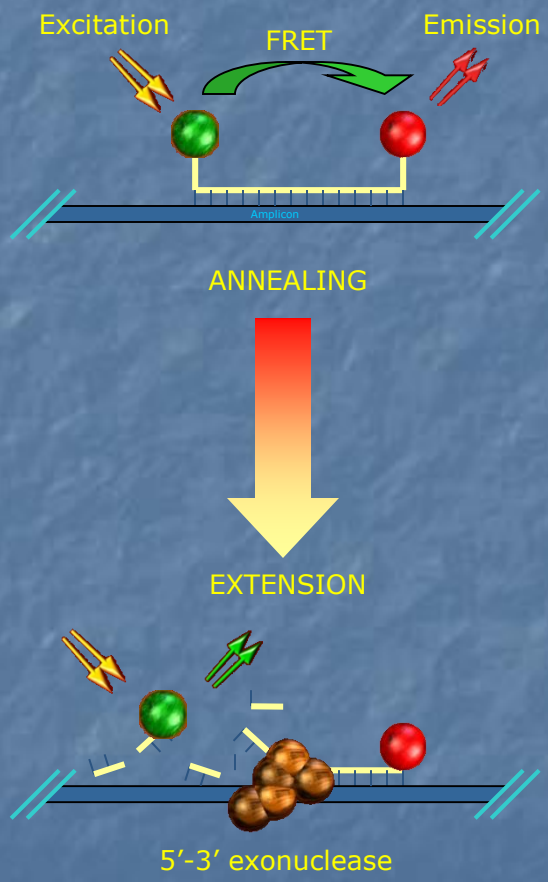


Hydrolysis



Polymerization Completed





Reporter

Quencher

Real-time Principles

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(TaqMan, Beacons, Scorpions)

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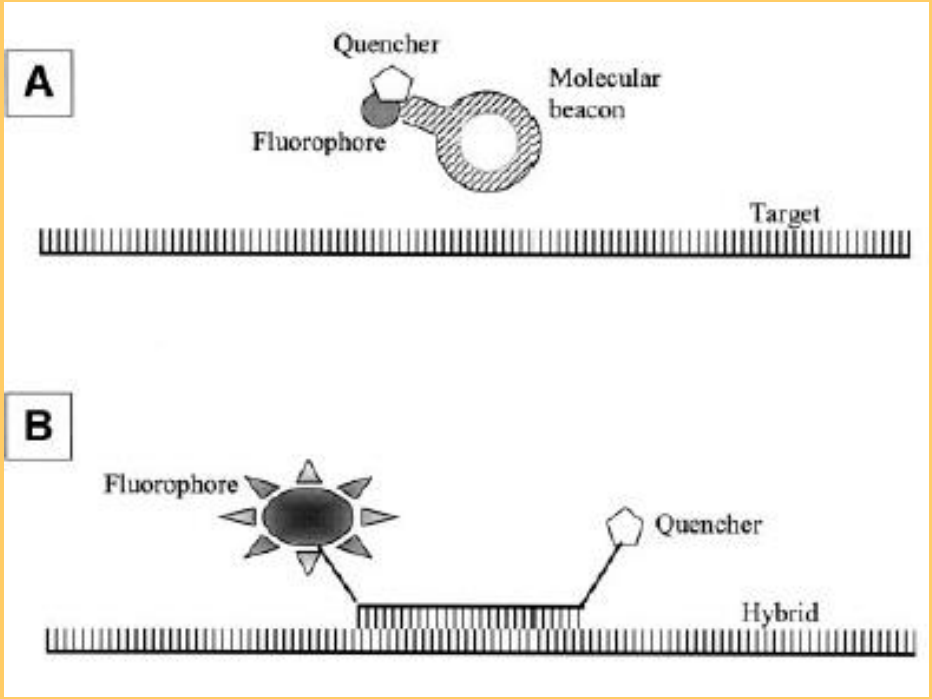
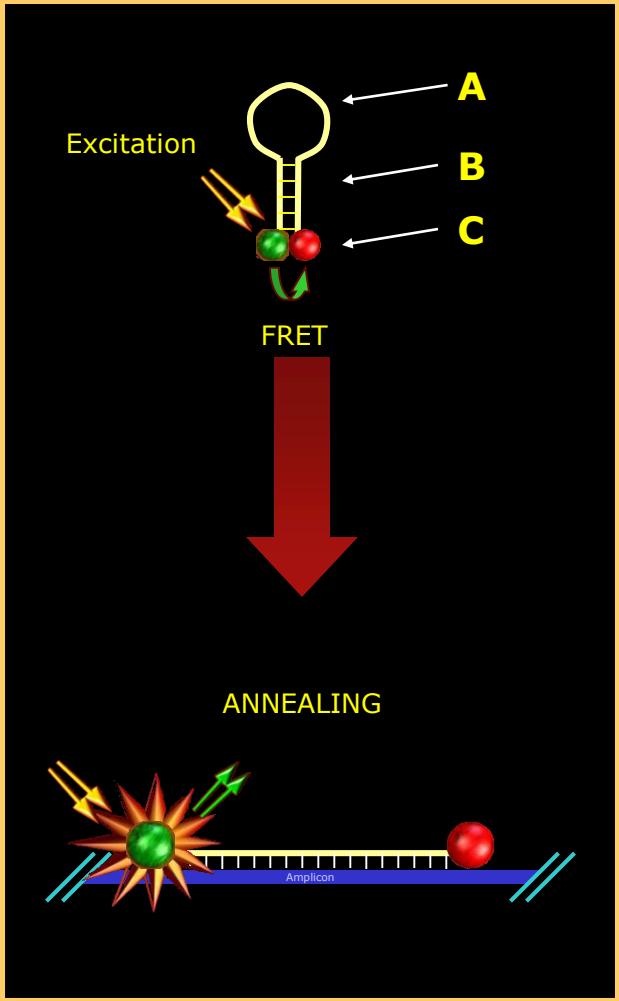
(SYBR Green)

The first two methods are specific formats.

Molecular beacons:



molecular beacons



Reporter

Non-fluorescent Quencher

Scorpions

The Scorpions primer acts as a probe.
The intact primer forms a hairpin so that the quenched reporter does not fluoresce

During annealing, the hairpin primer binds to the template, and is then extended

During subsequent denaturation, the reporter separates from the quencher, and the loop sequence binds to the internal target sequence. The reporter on the extended Scorpions primer fluoresces

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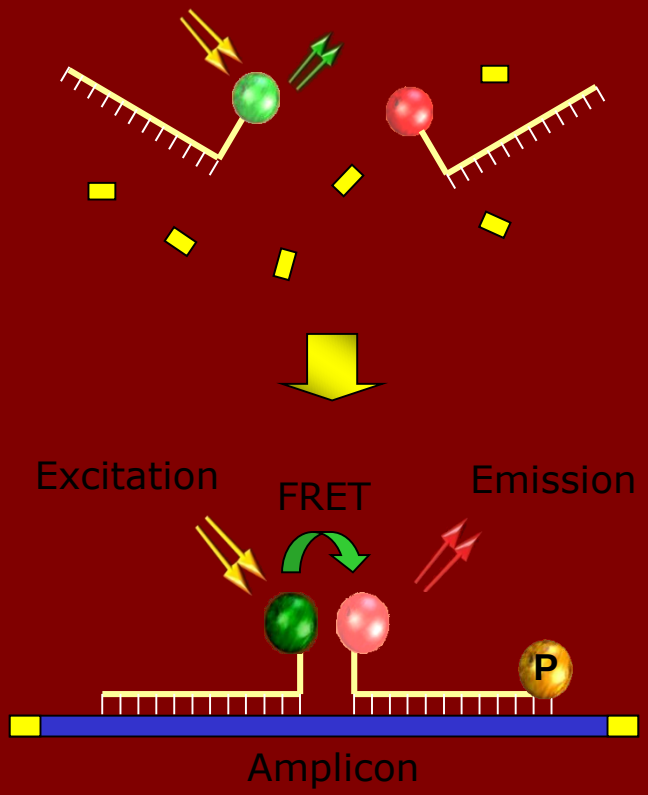
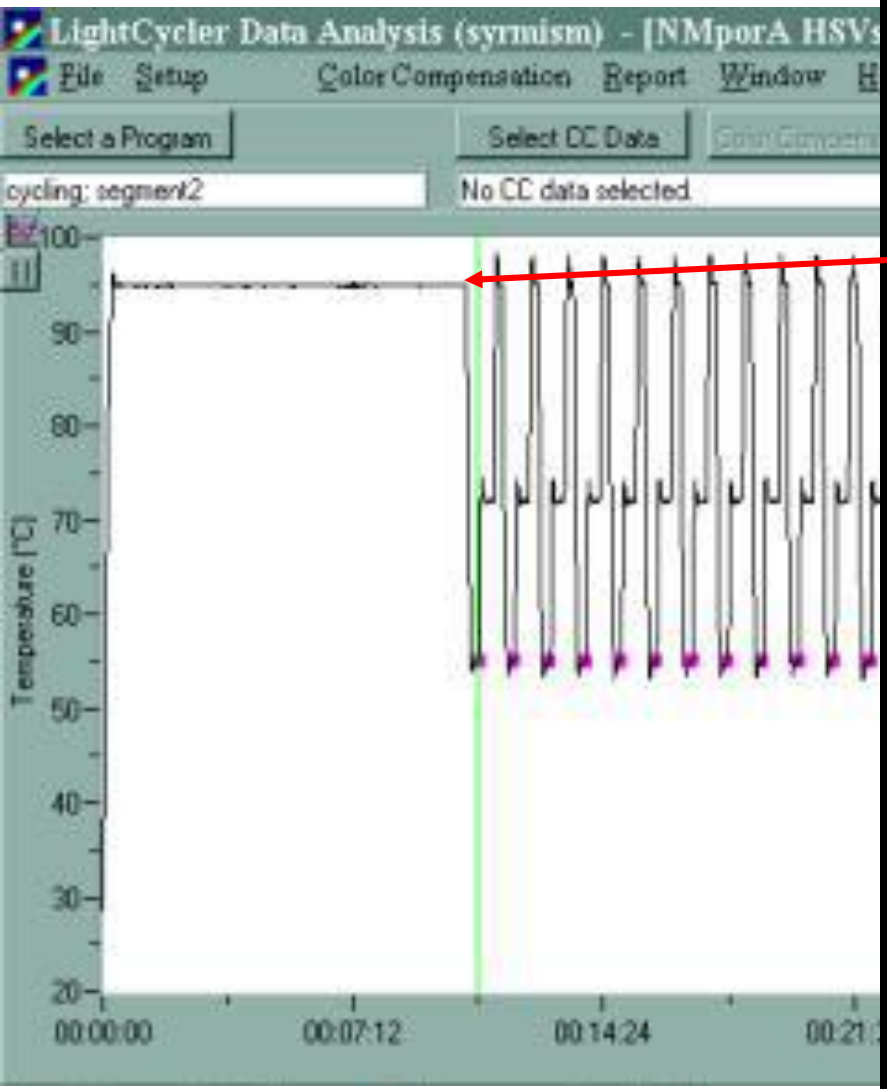
Methods of detecting the products of Real Time amplification

- HYBRIDIZATION PROBES:
  - Fluorescence Resonance Energy Transfer (FRET) Probes
    - FRET probes function through the transfer of energy
    - Tow detector probes used
    - Hybridize in close proximity to one another on the amplicon
    - Probe on upsream side of the pair has a fluorescein molecule attached to 3 end of the probe (donor)
    - The second probe is situated downstream in relation to the first probe (acceptor)

FRET (Fluorescence Resonance Energy Transfer)
using adjacent hybridization probes



FITC
Red 640
P Phosphate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Select a Program
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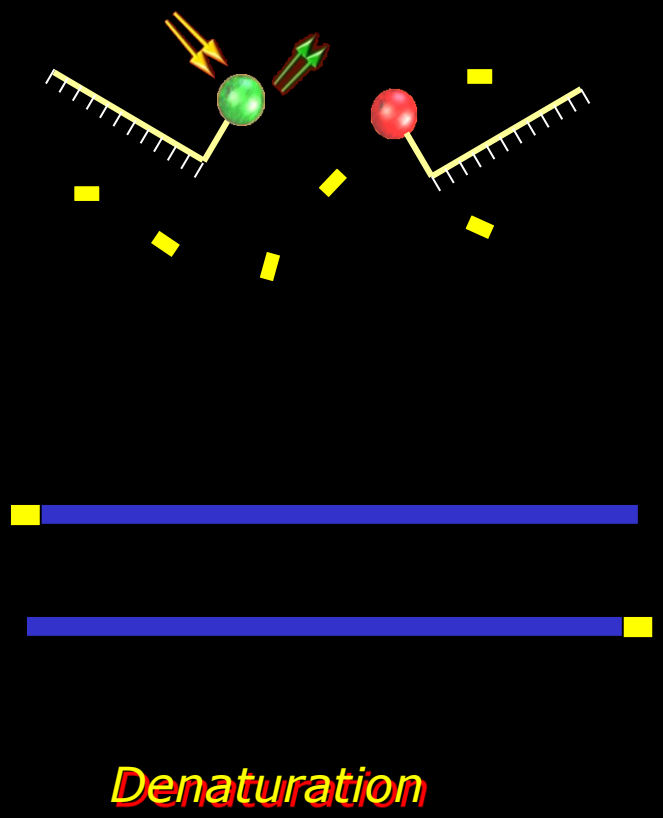
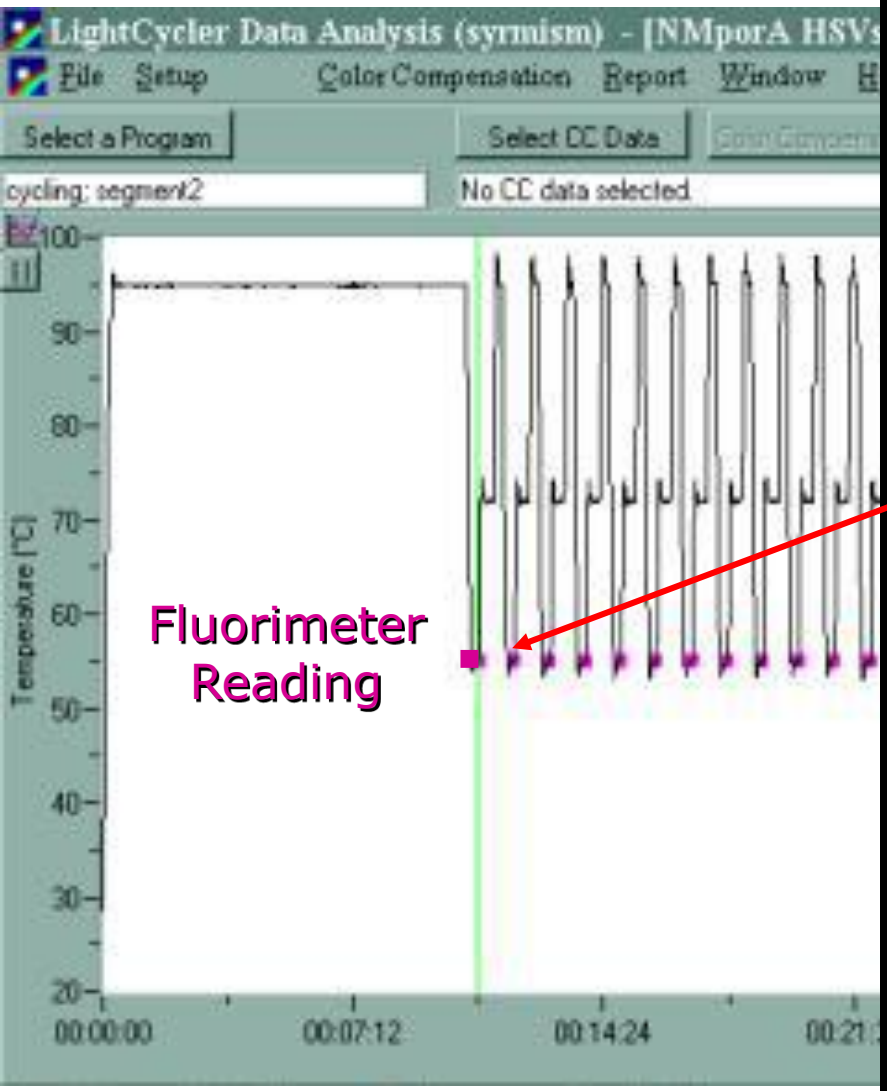
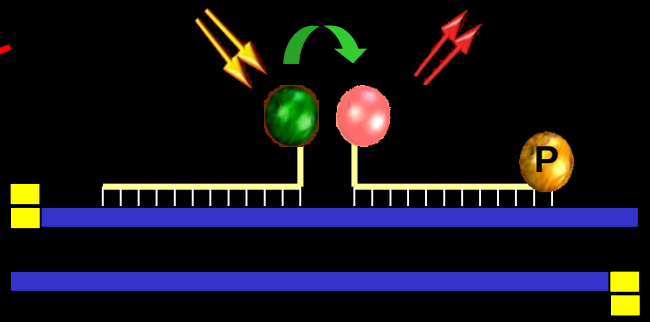
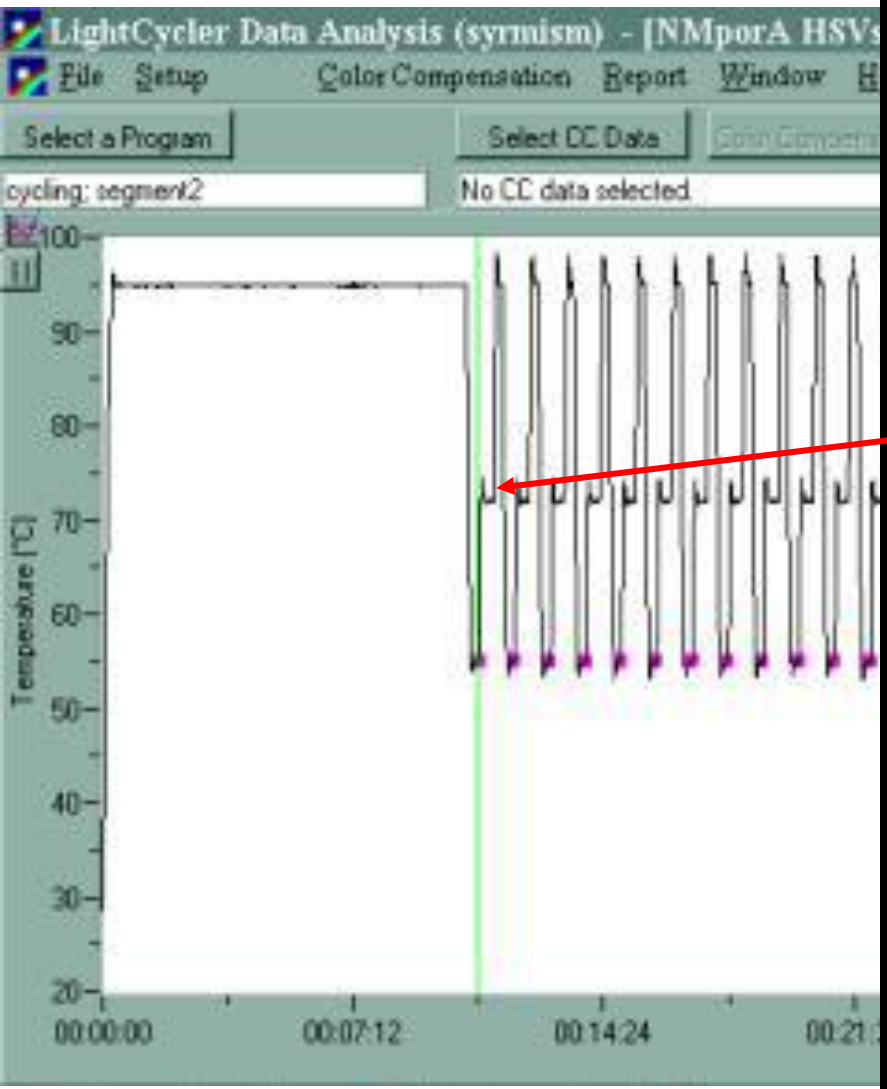
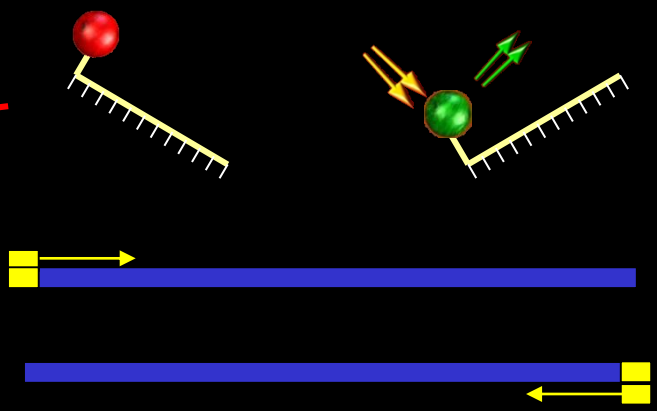

Denaturation



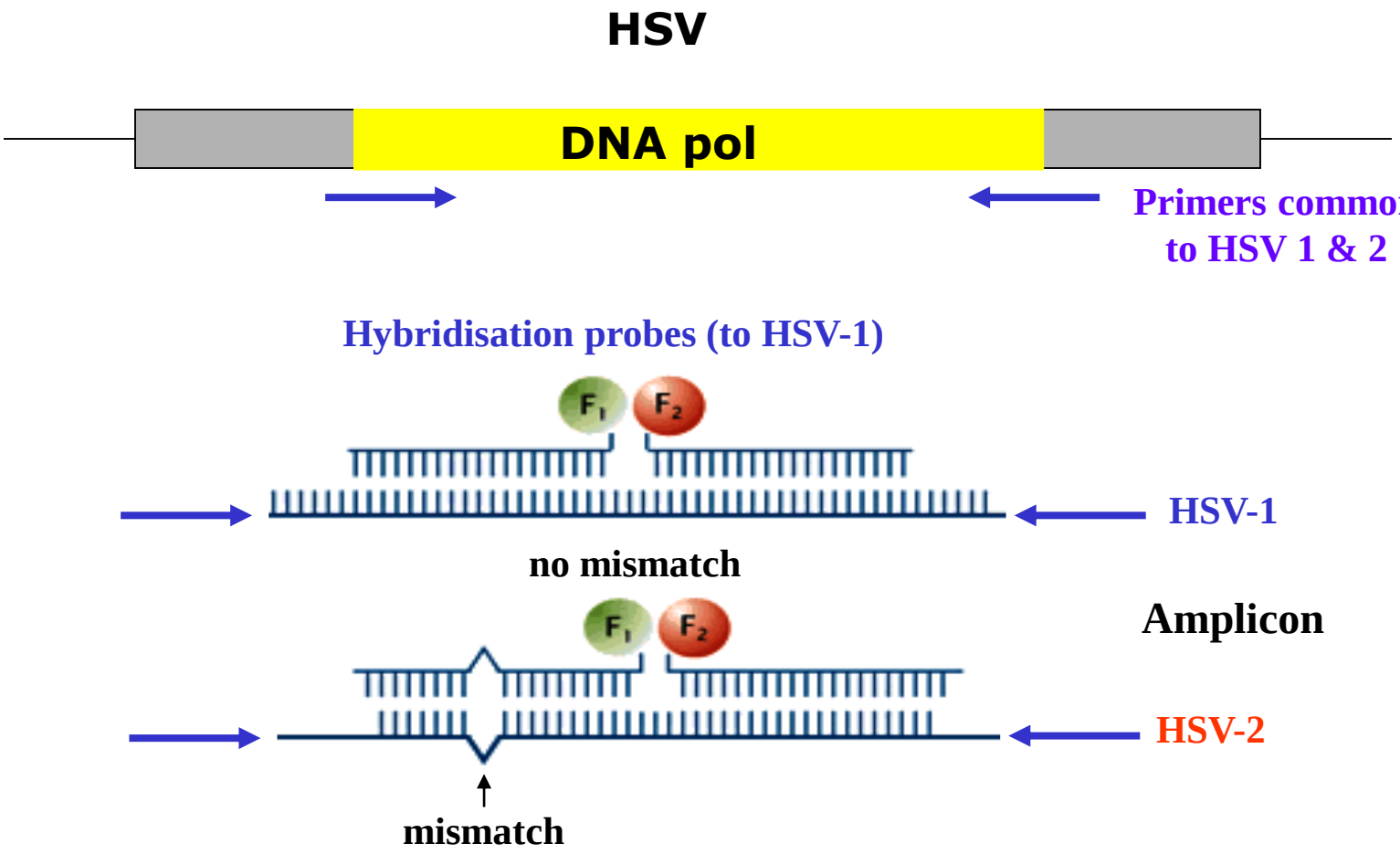


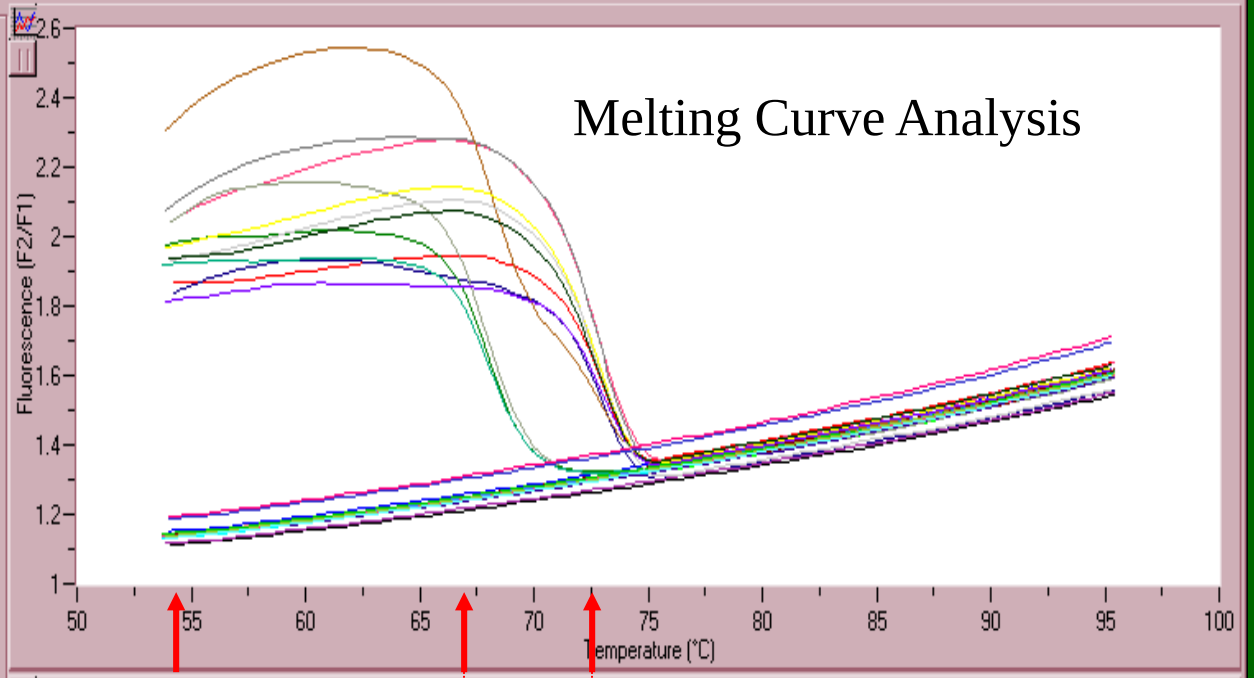
Primer/Probe Annealing

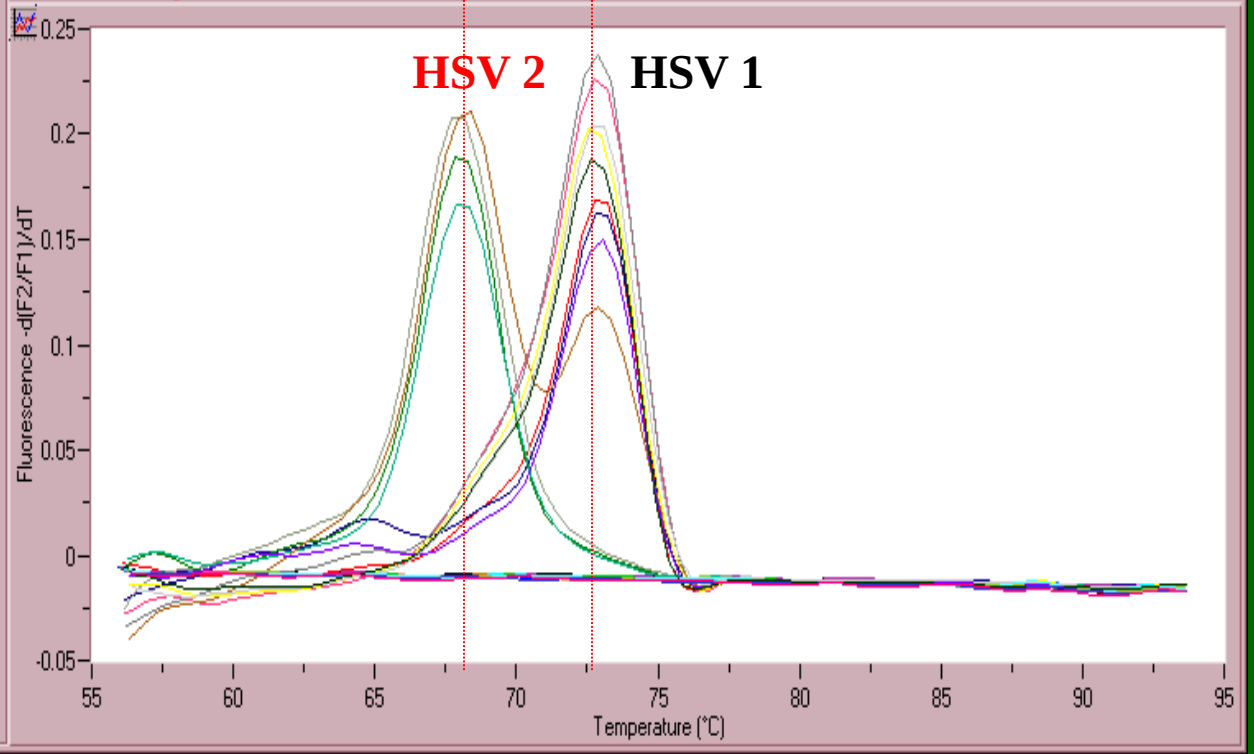


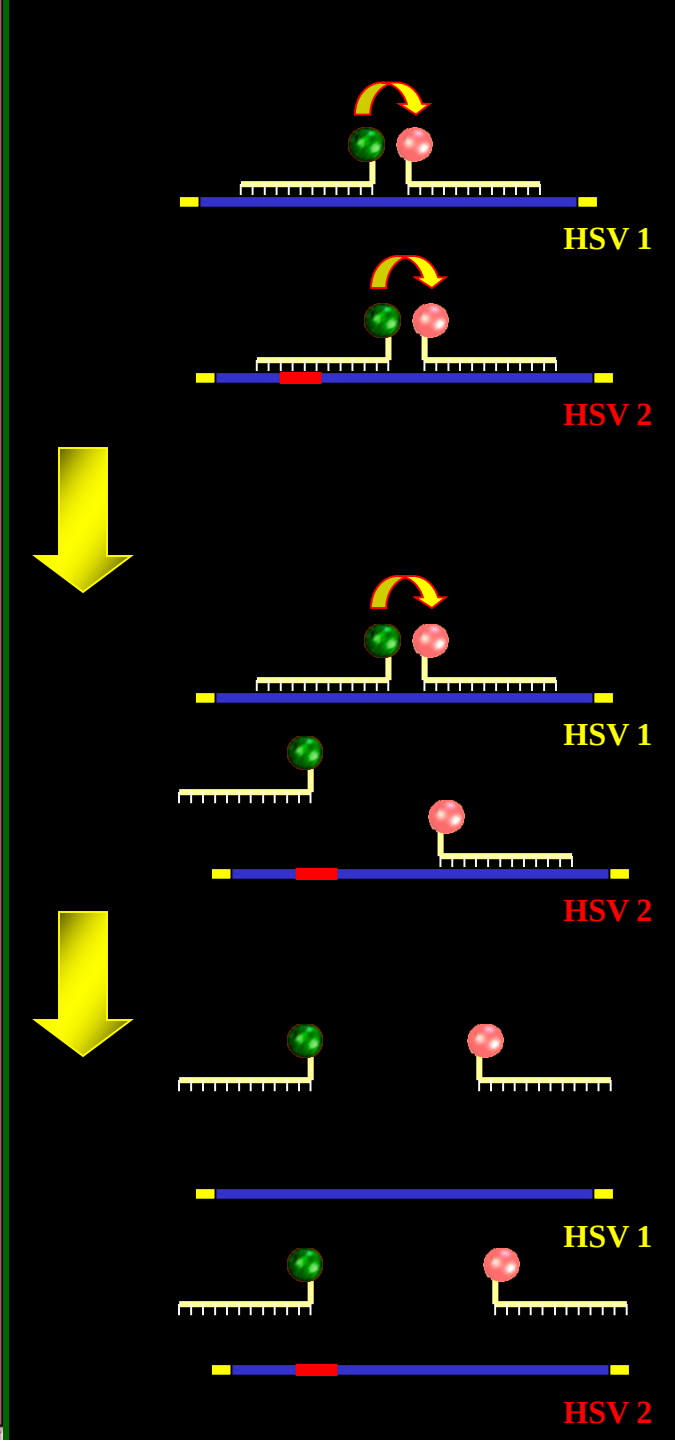


Primer Extension


HSV
DNA pol
Primers common
to HSV 1 & 2
Hybridisation probes (to HSV-1)
F1
F2
HSV-1
no mismatch
Amplicon
F1
F2
HSV-2
mismatch

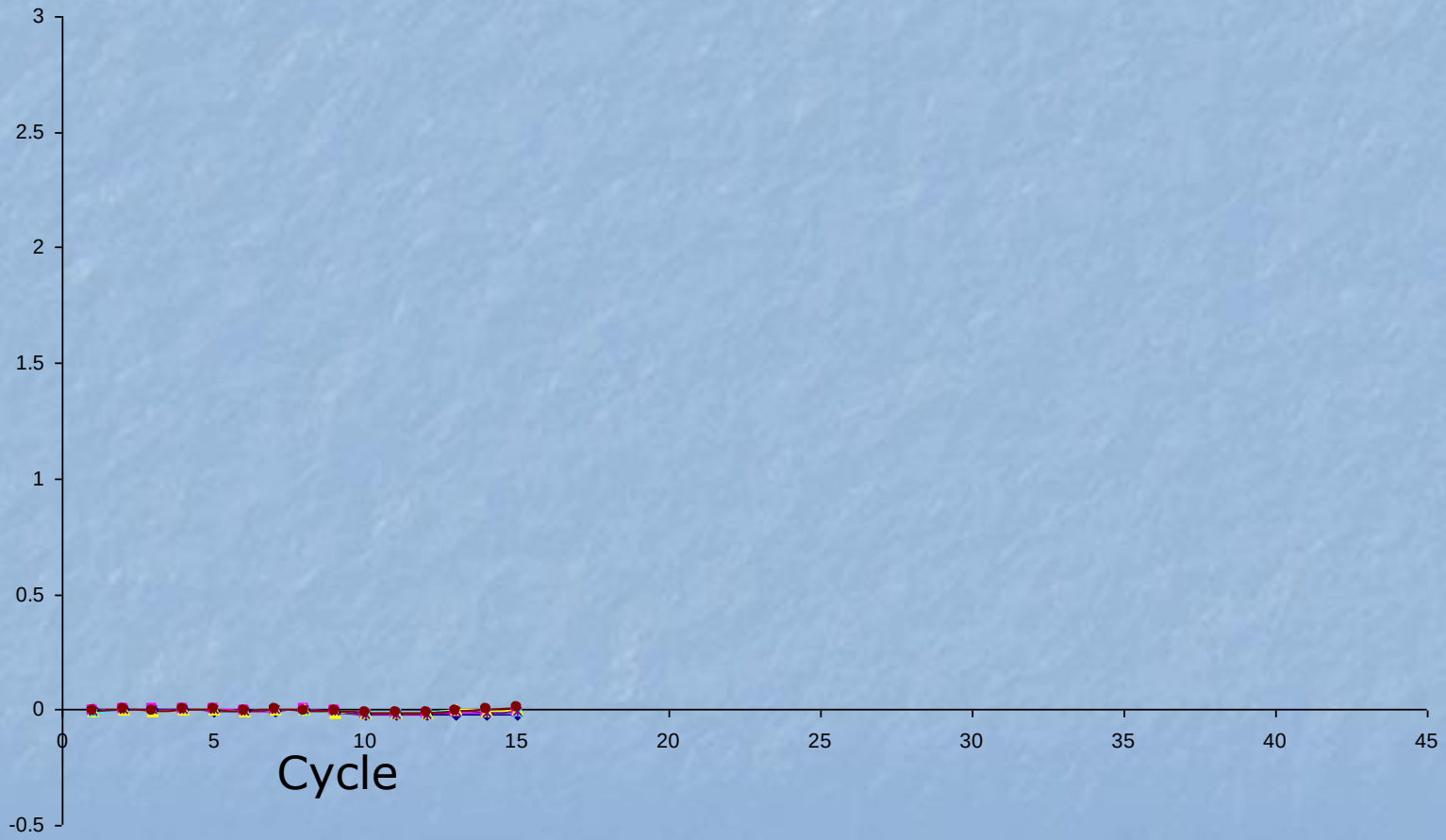

Melting Curve Analysis
Fluorescence (F2/F1)
Temperature (°C)


HSV 2
HSV 1
Fluorescence -d(F2/F1)/dT
Temperature (°C)


HSV 1
HSV 2
HSV 1
HSV 2
HSV 1
HSV 2

Real-Time PCR

Fluoresens



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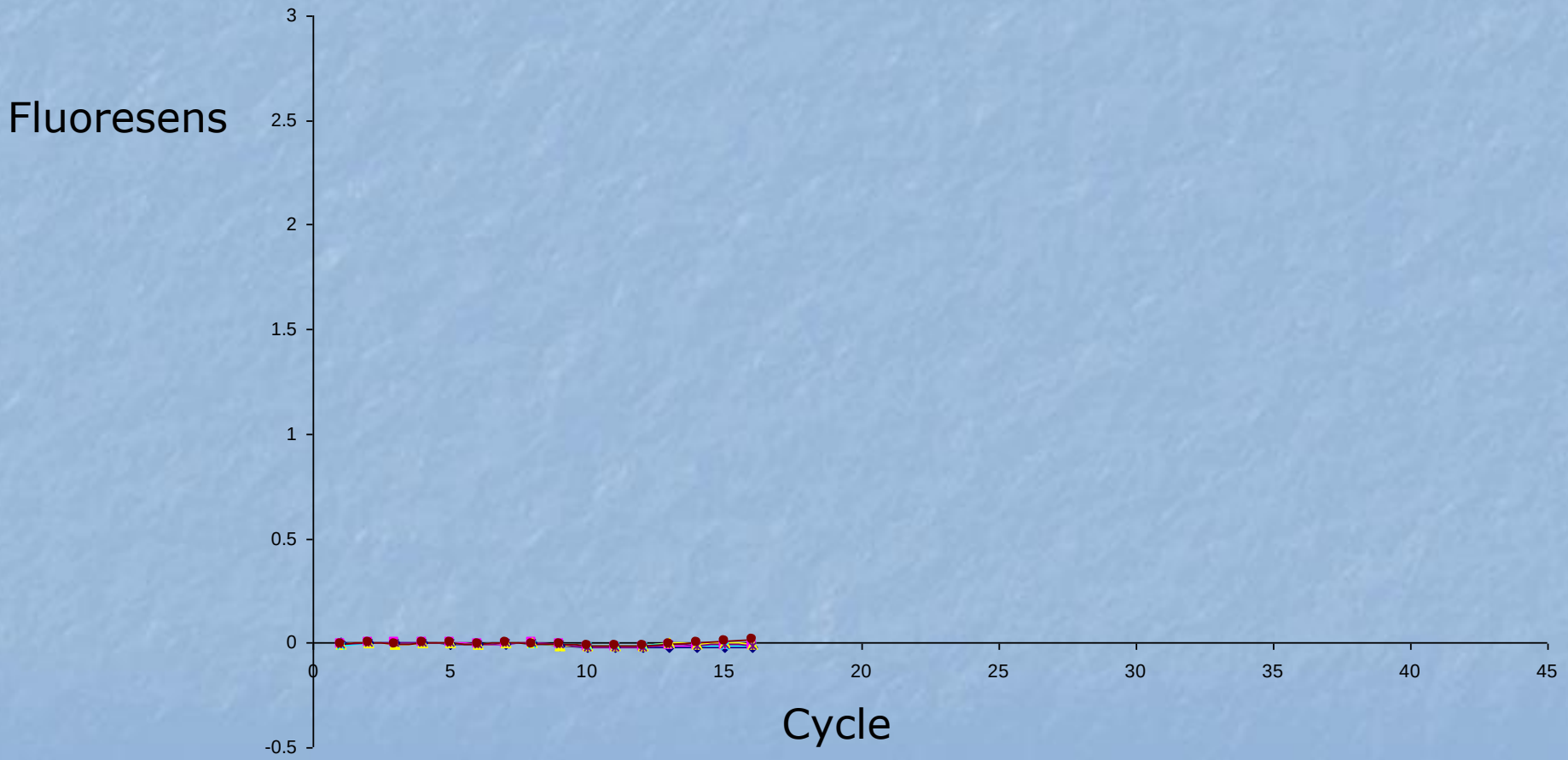
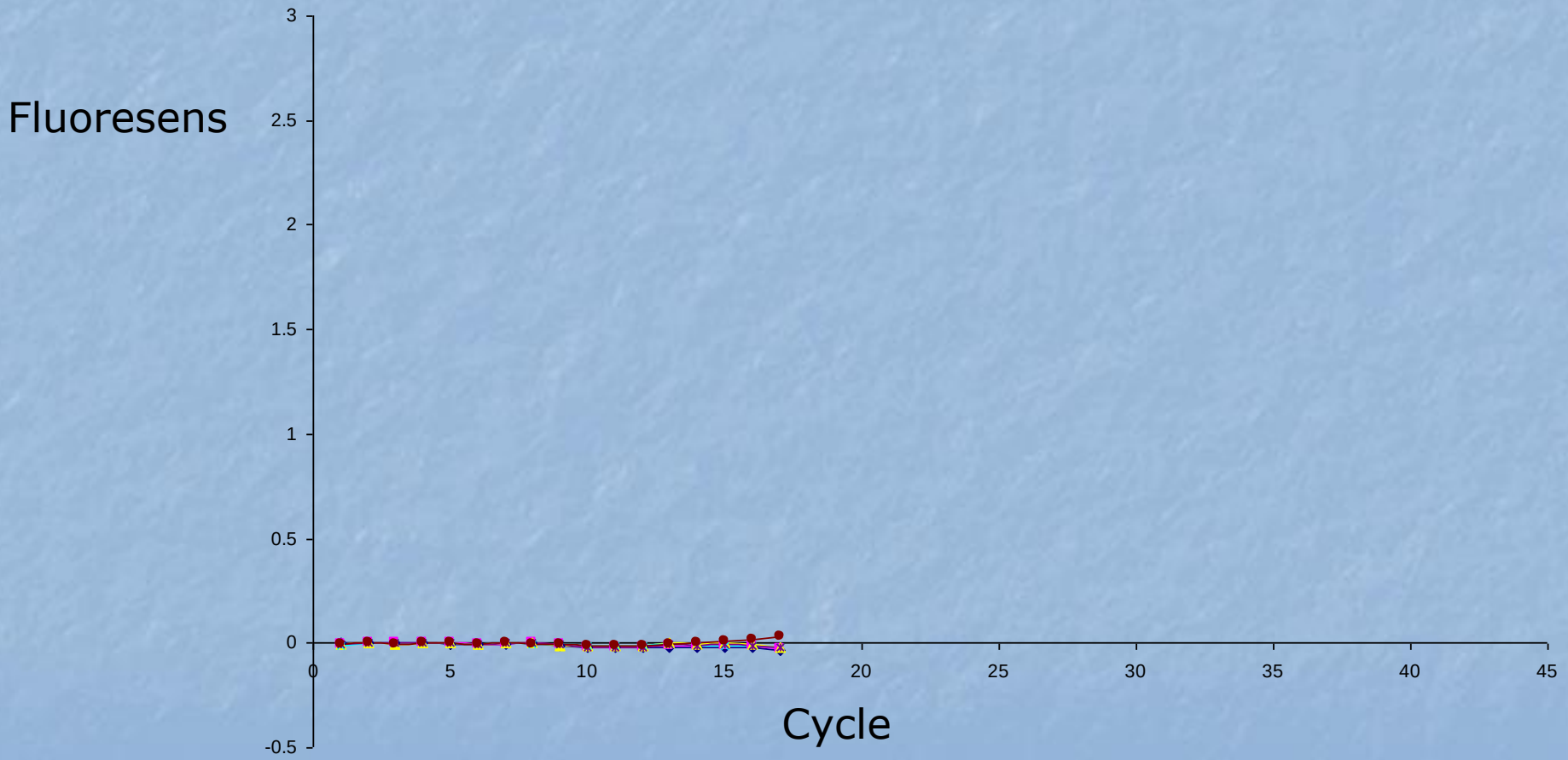
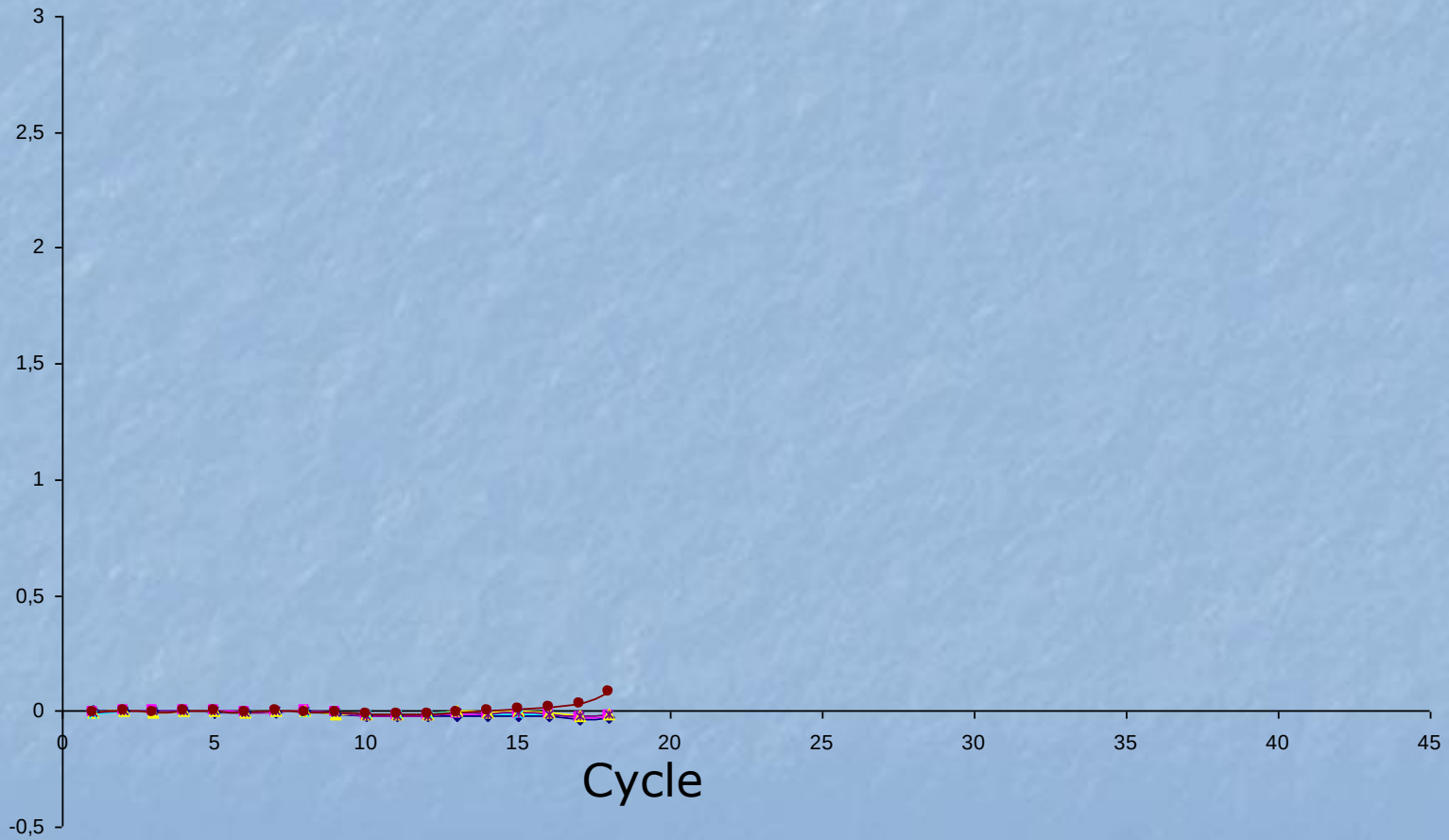


Real-Time PCR

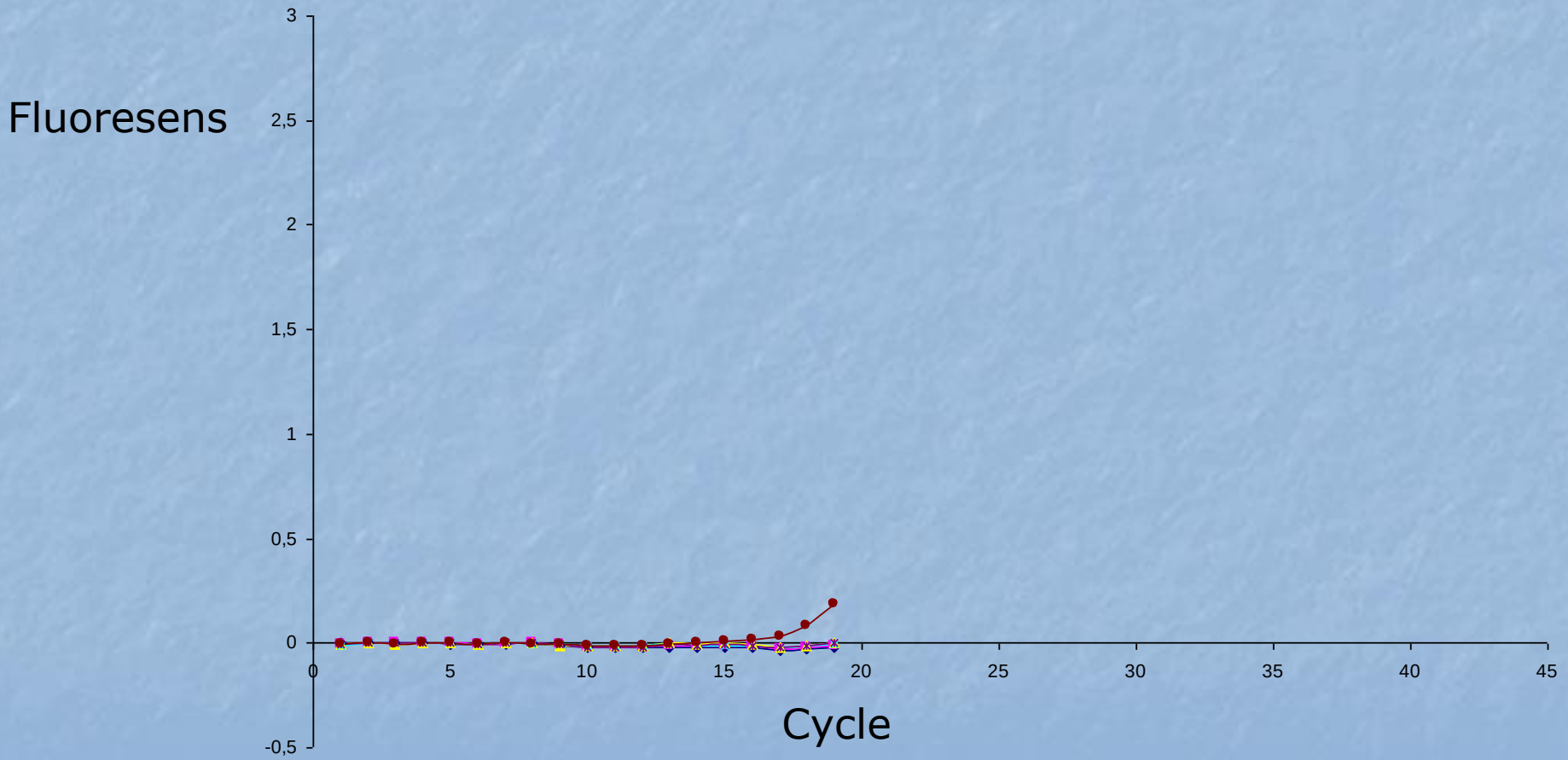


Real-Time PCR

Fluoresens



Real-Time PCR



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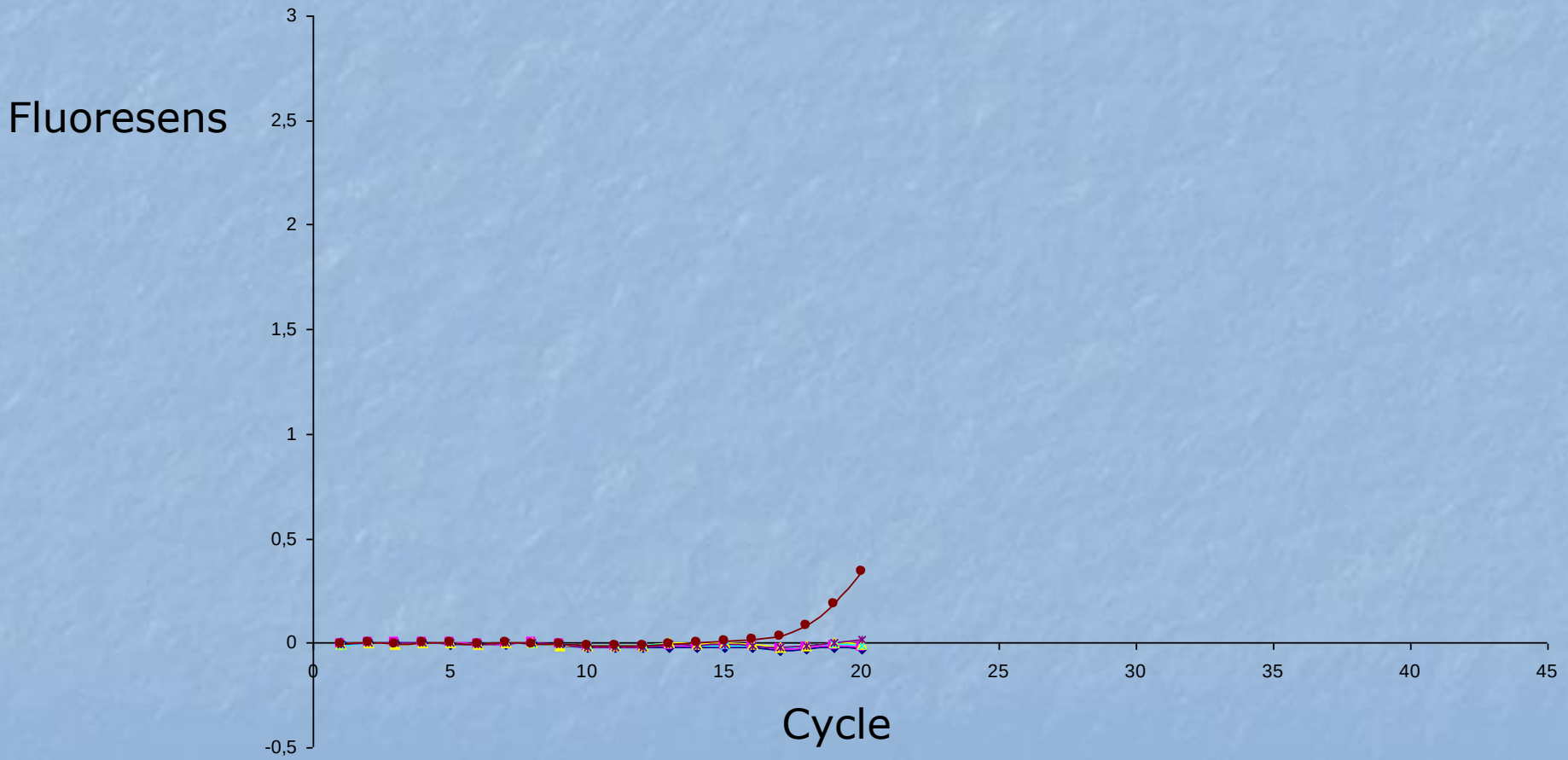

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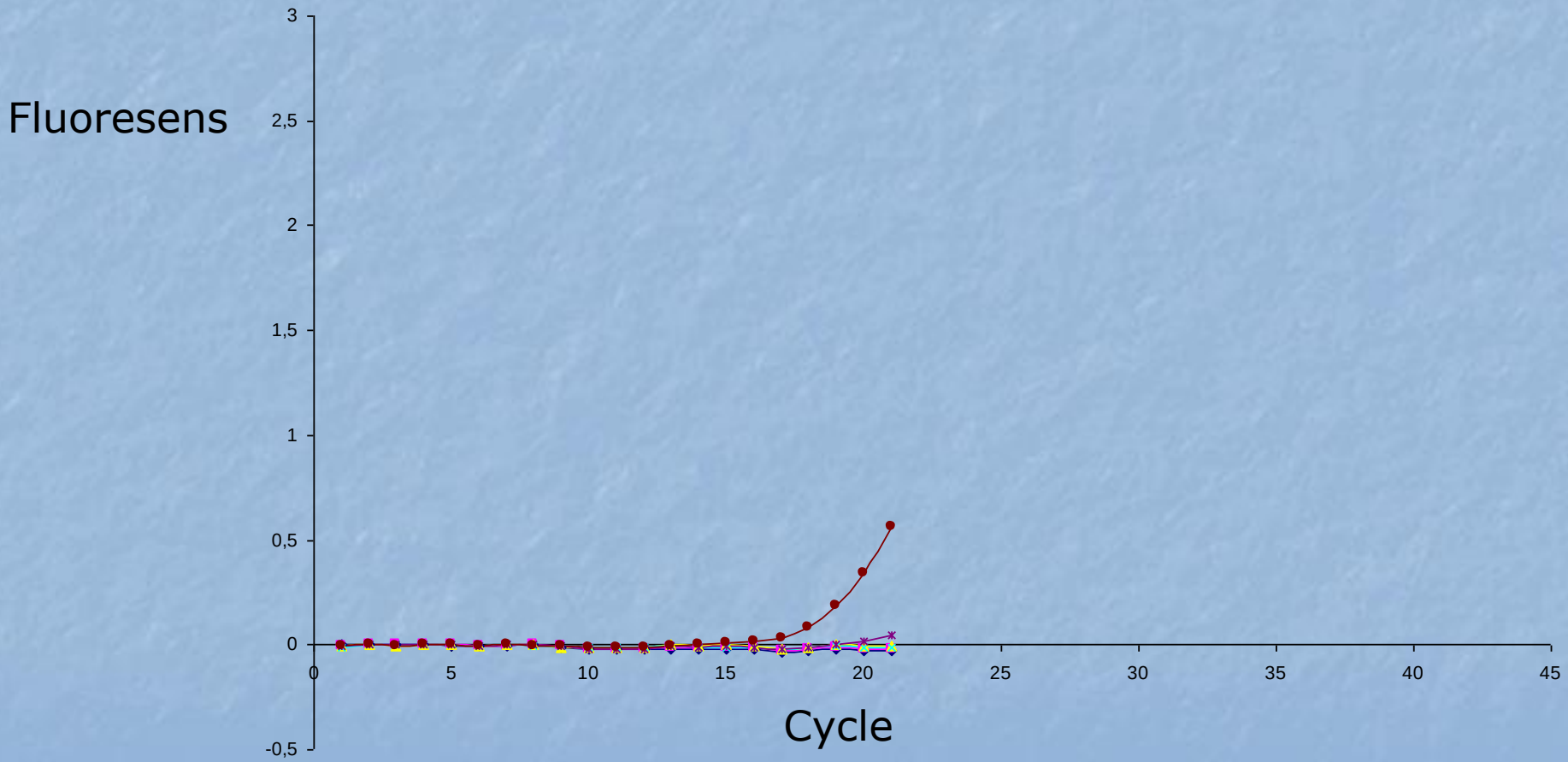

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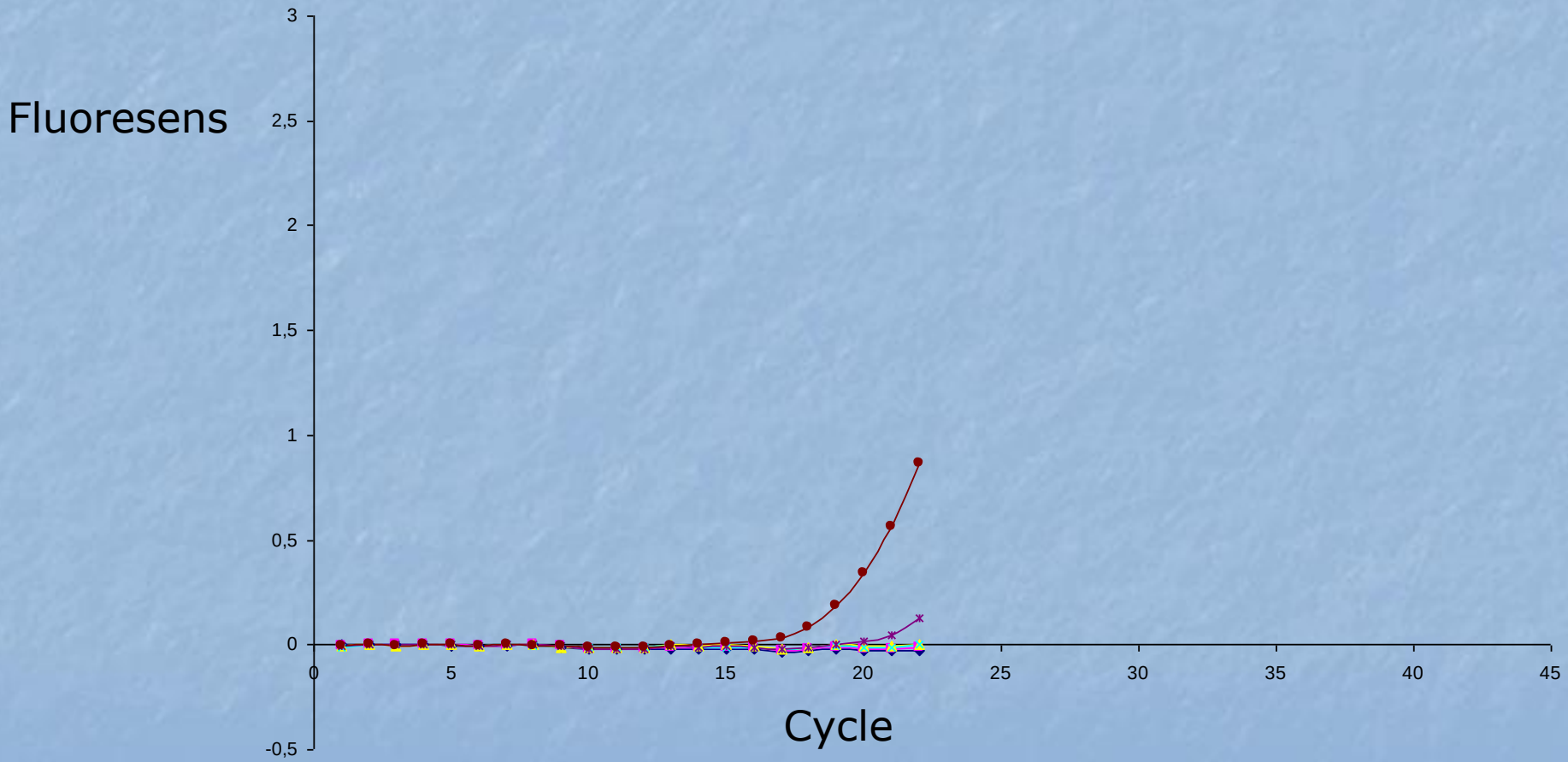

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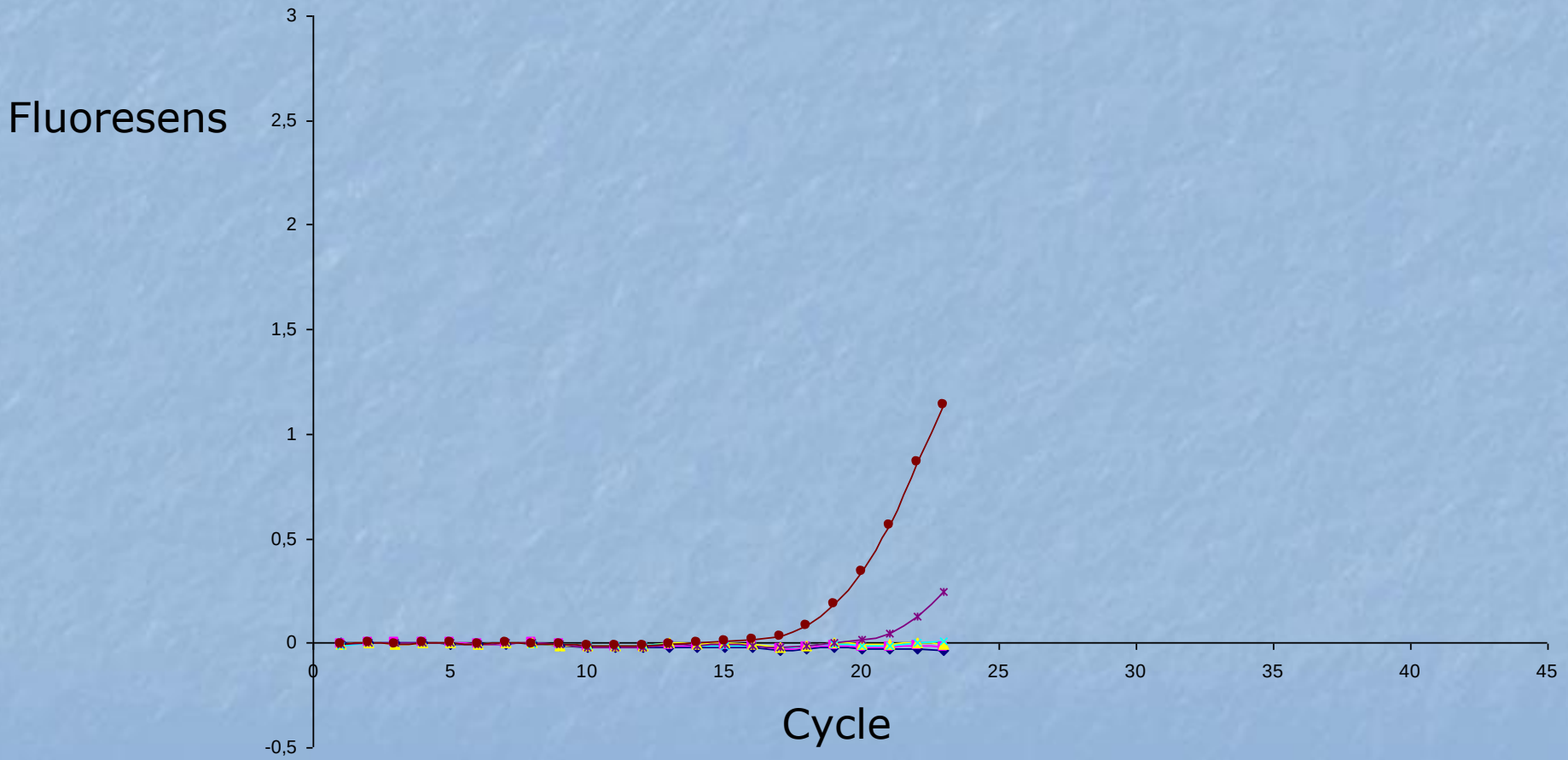

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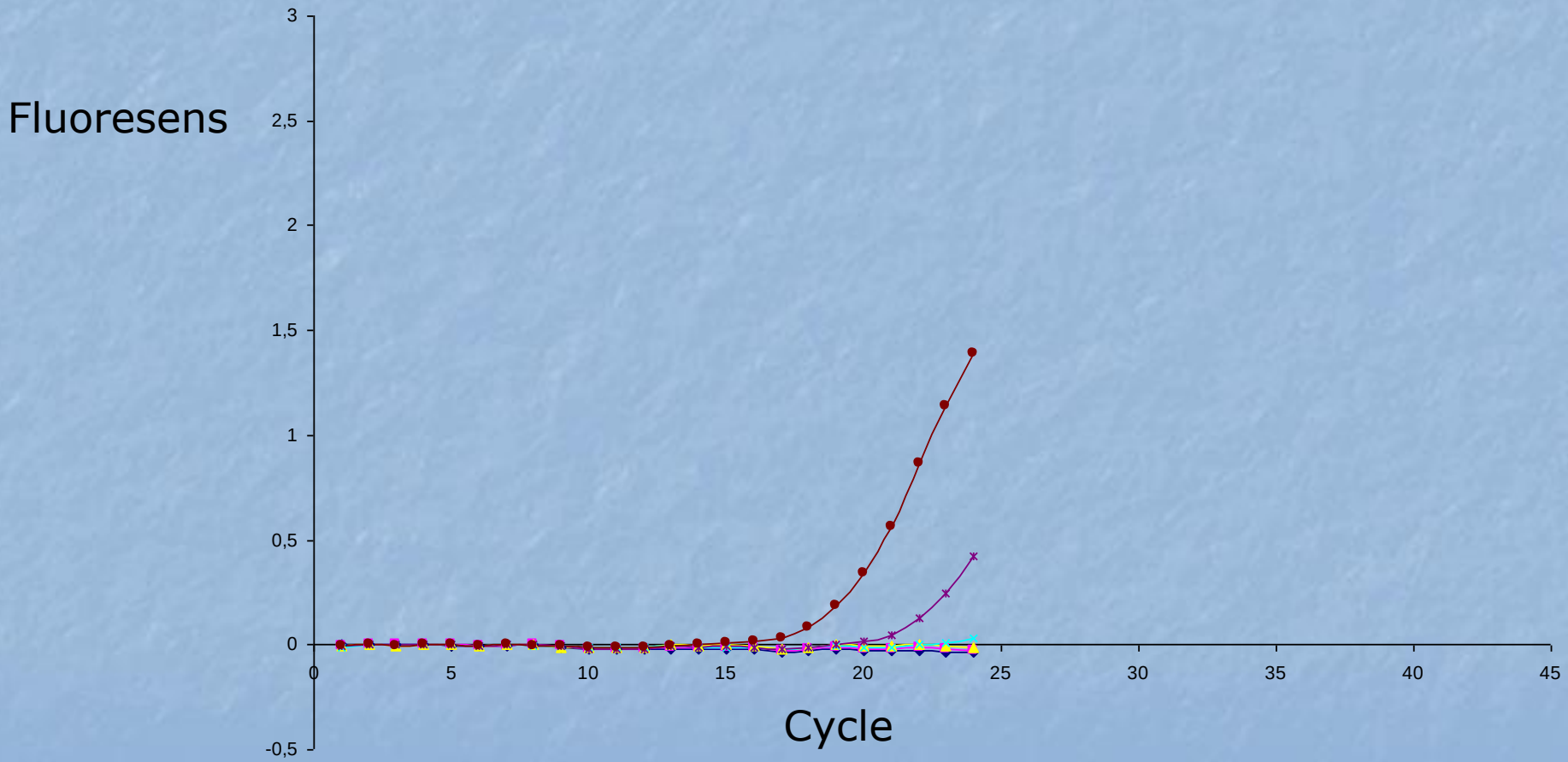
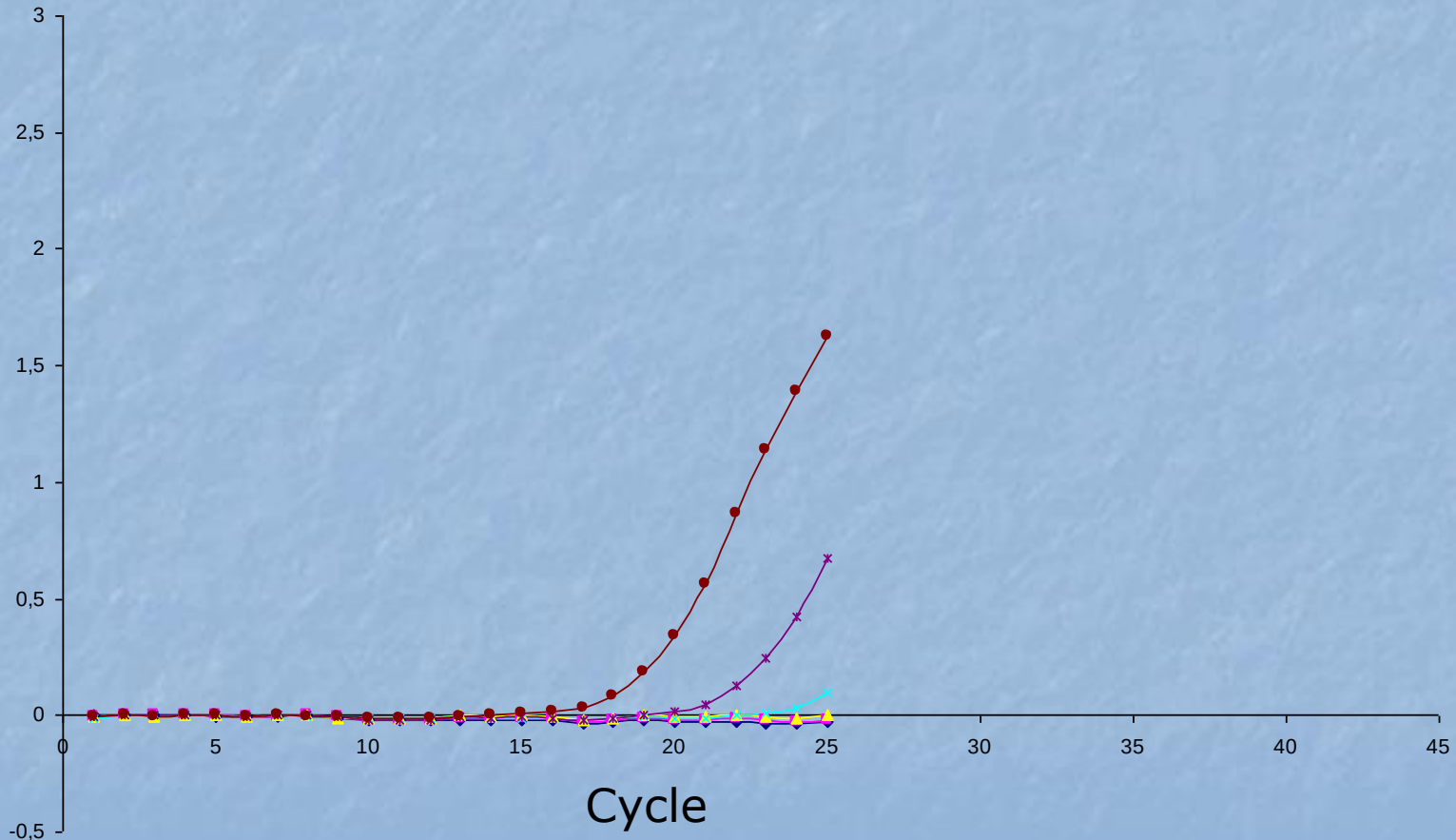


Real-Time PCR

Fluoresens



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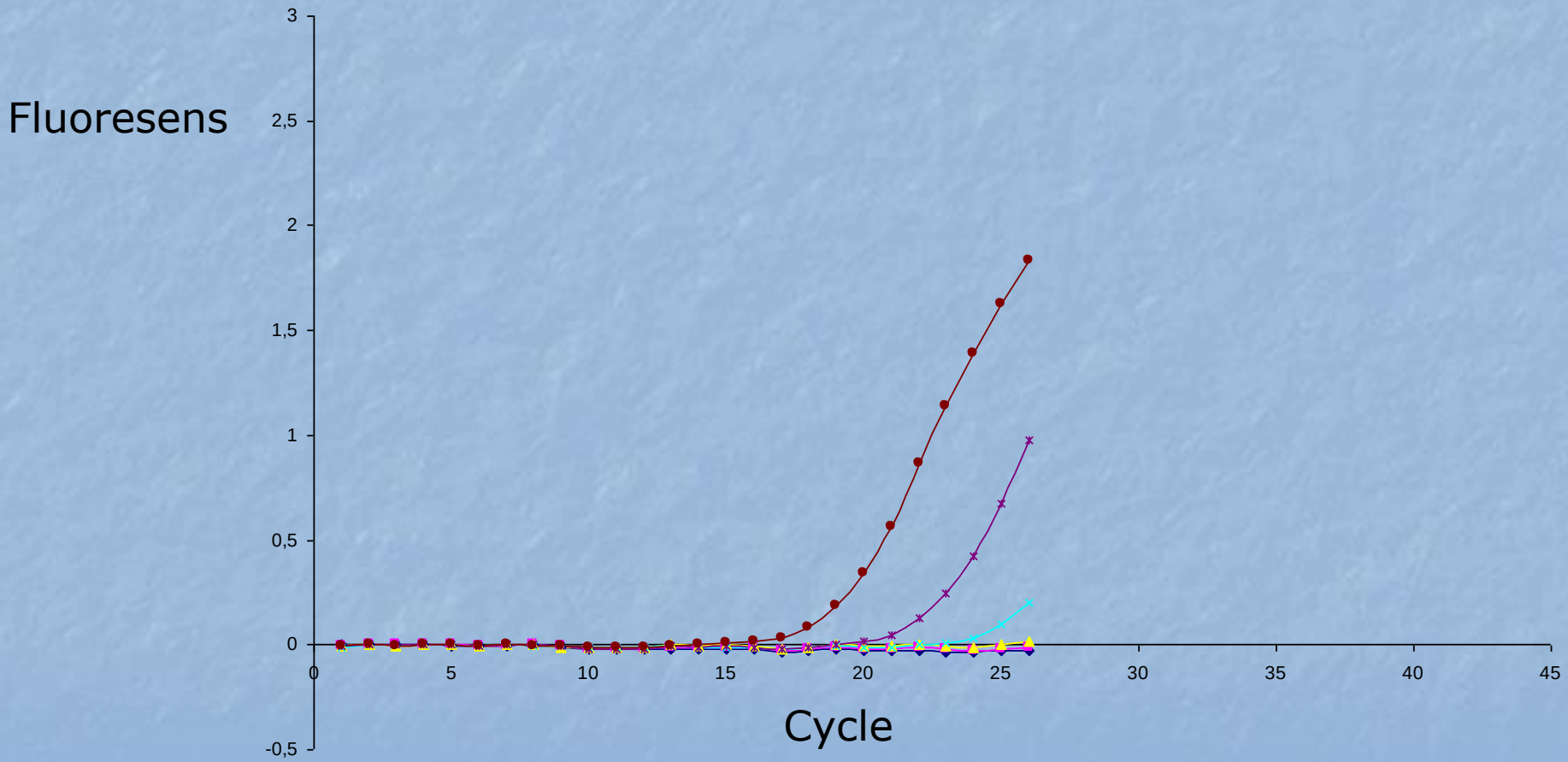
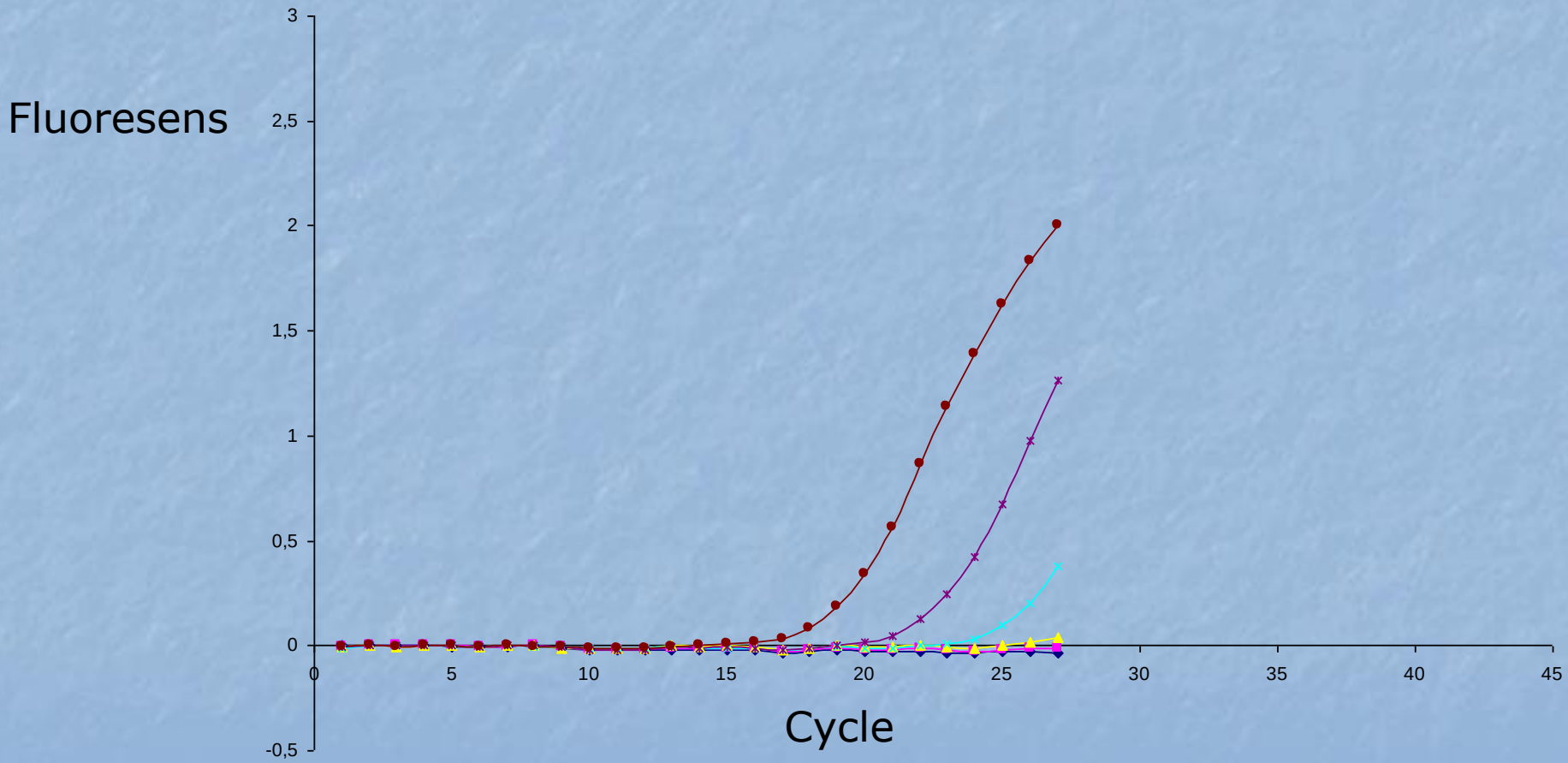
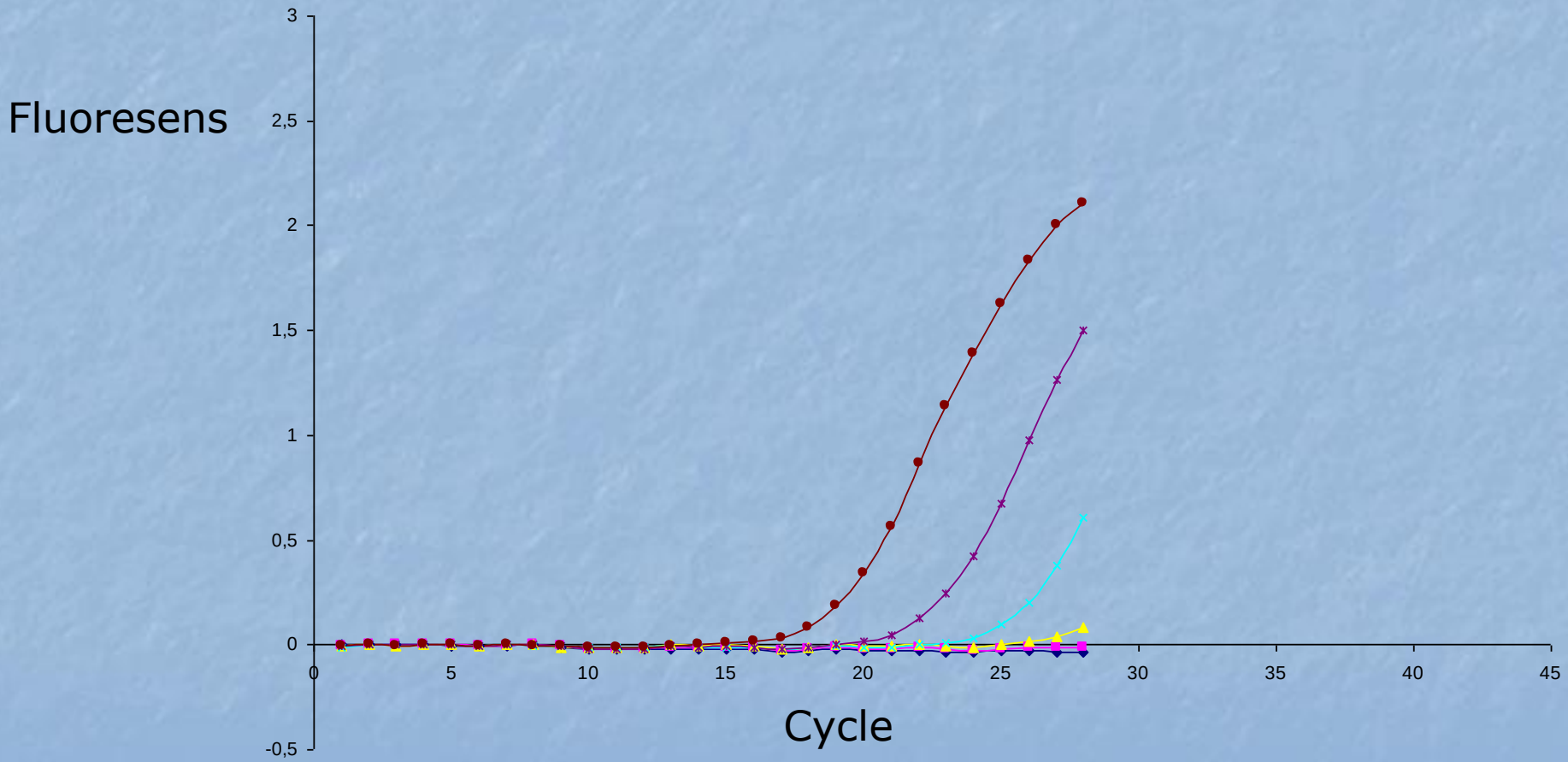


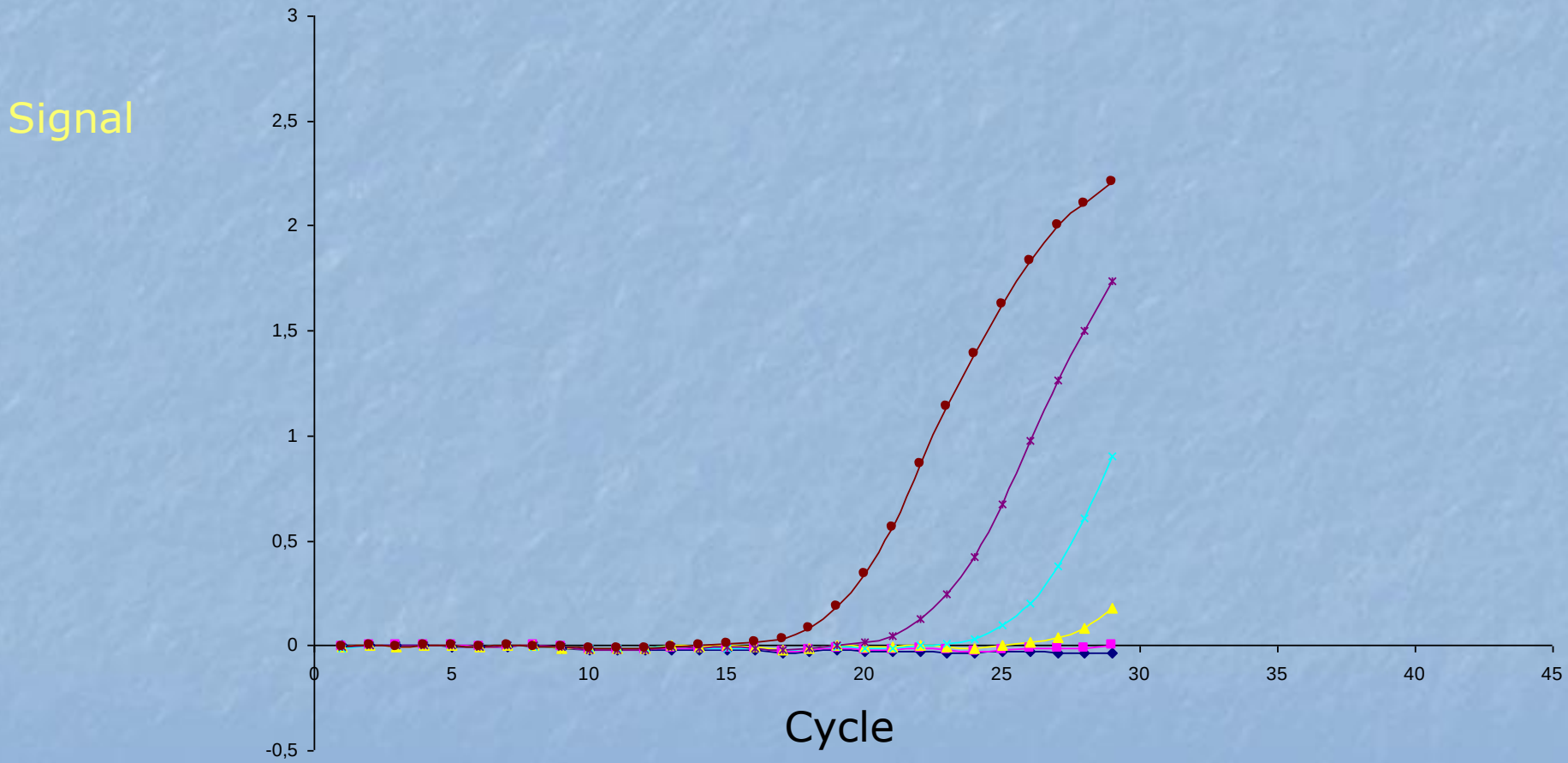
Real-Time PCR



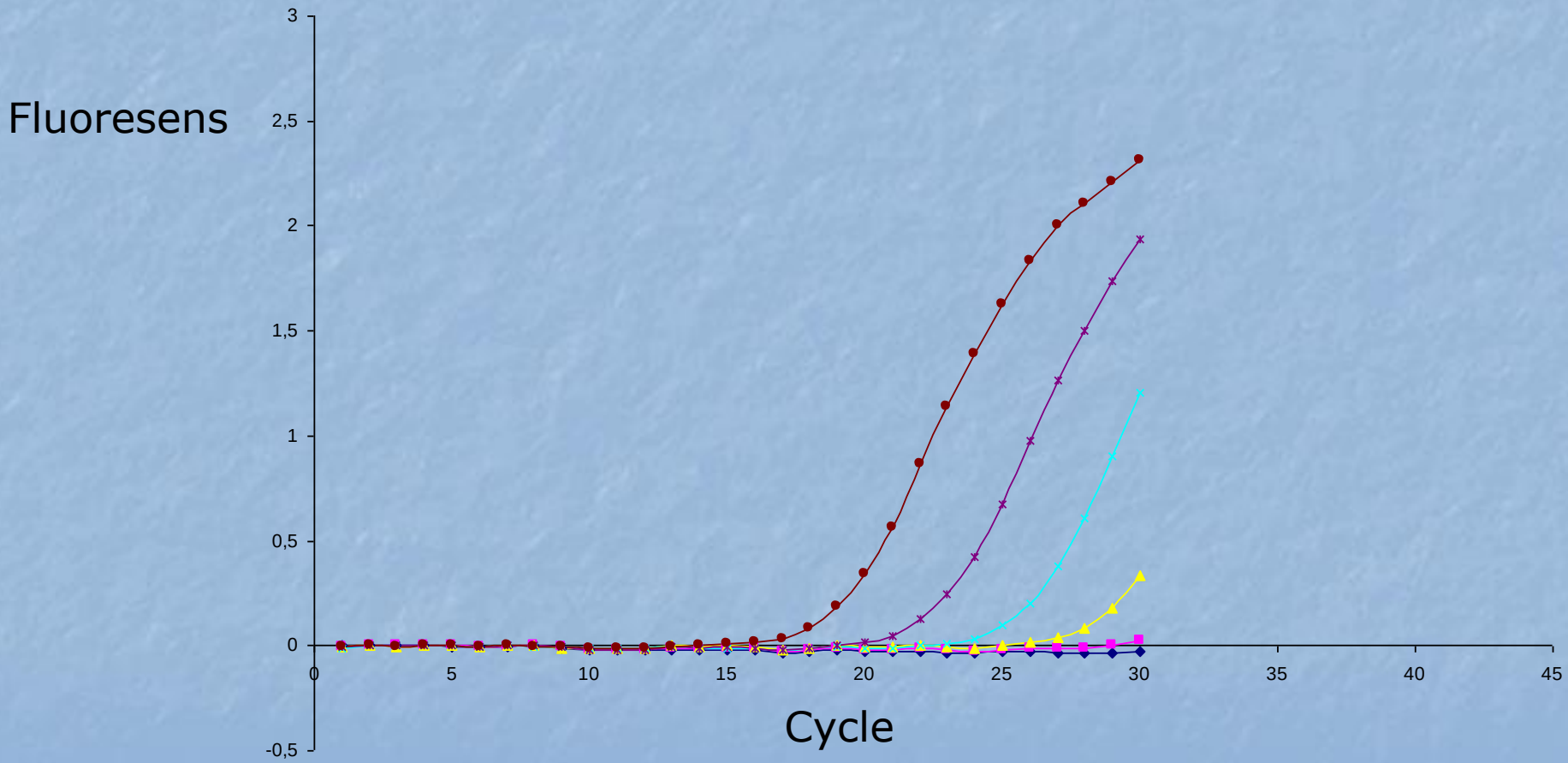
Real-Time PCR



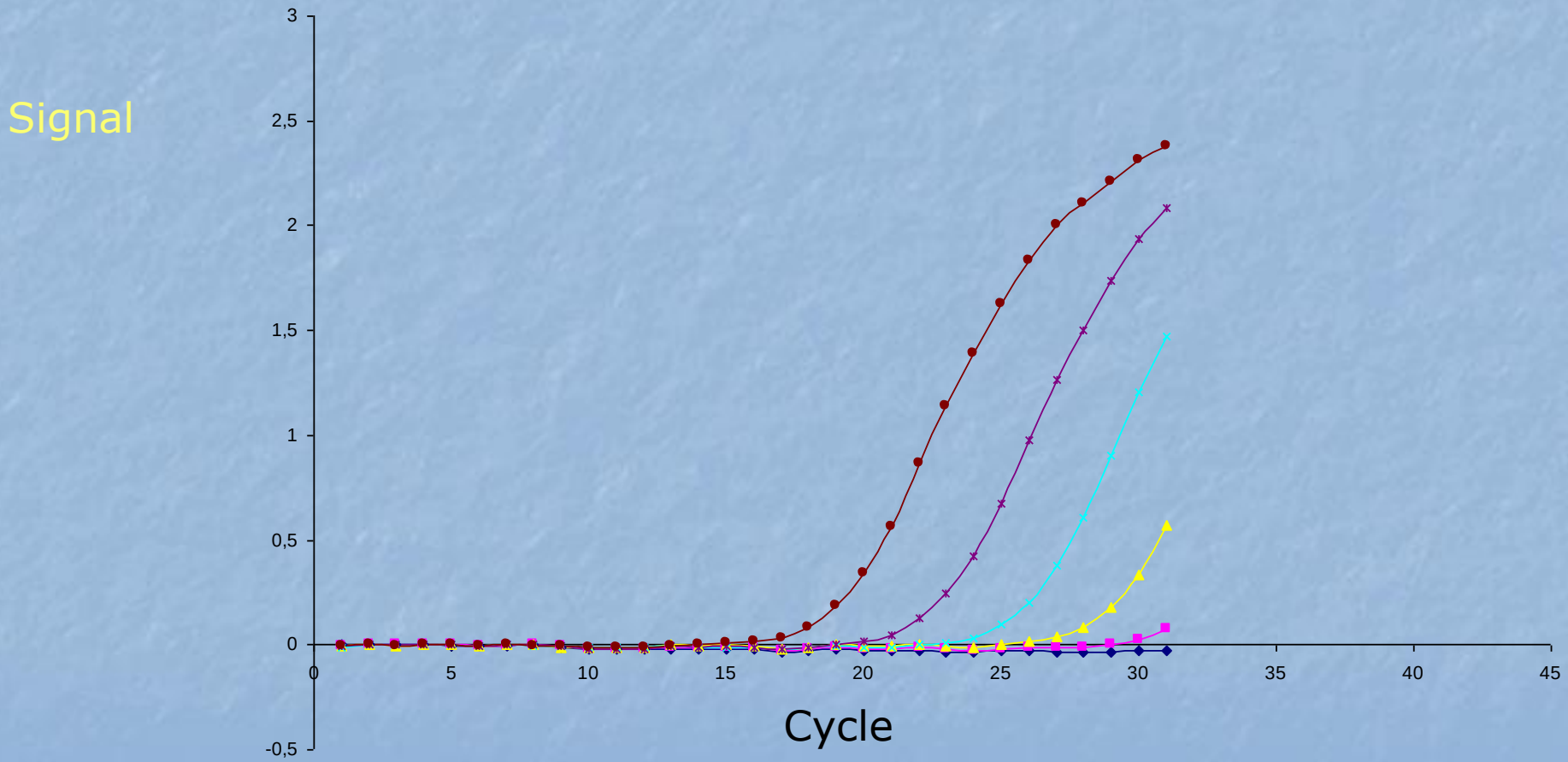
Real-Time PCR



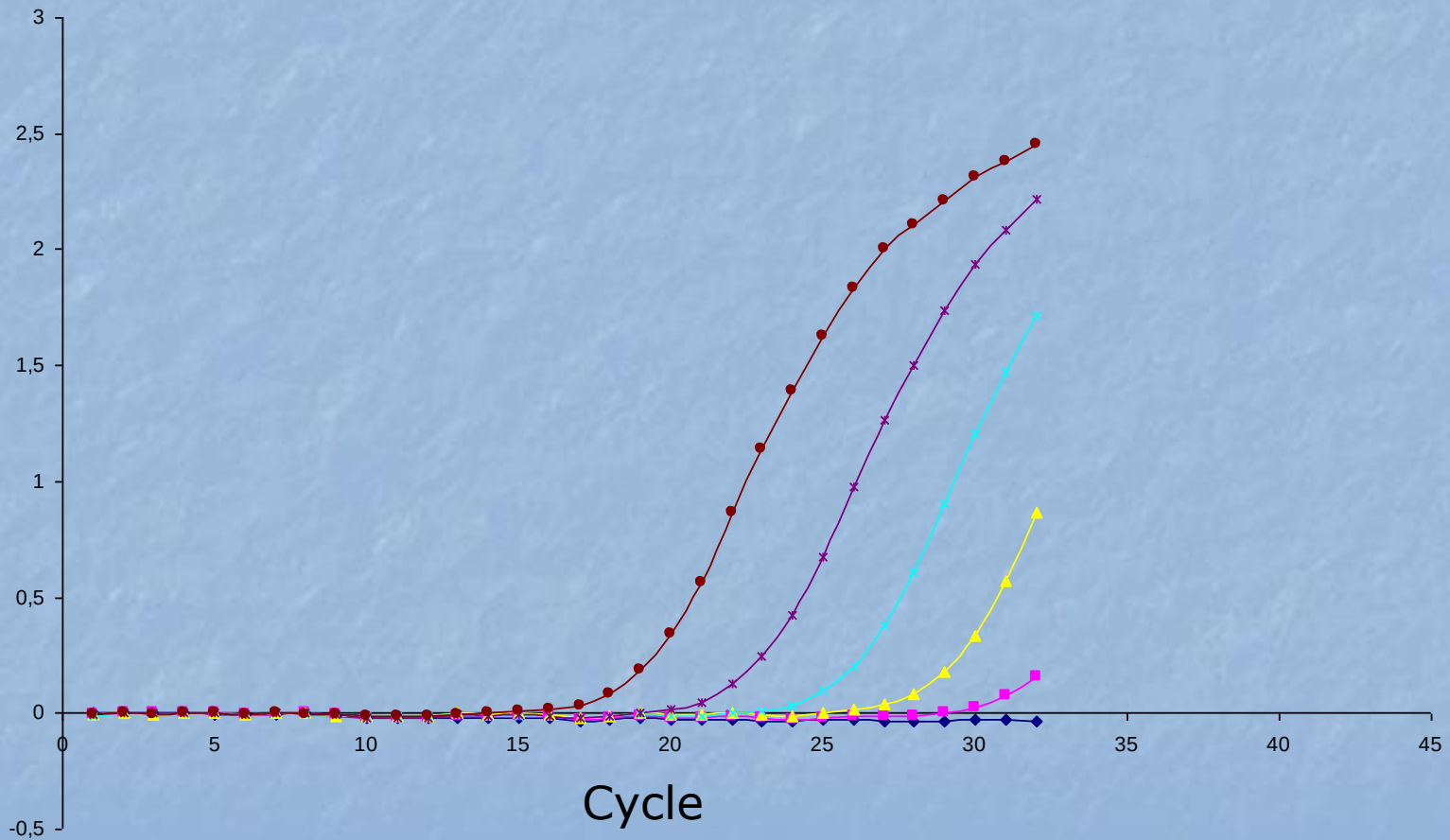
Real-Time PCR



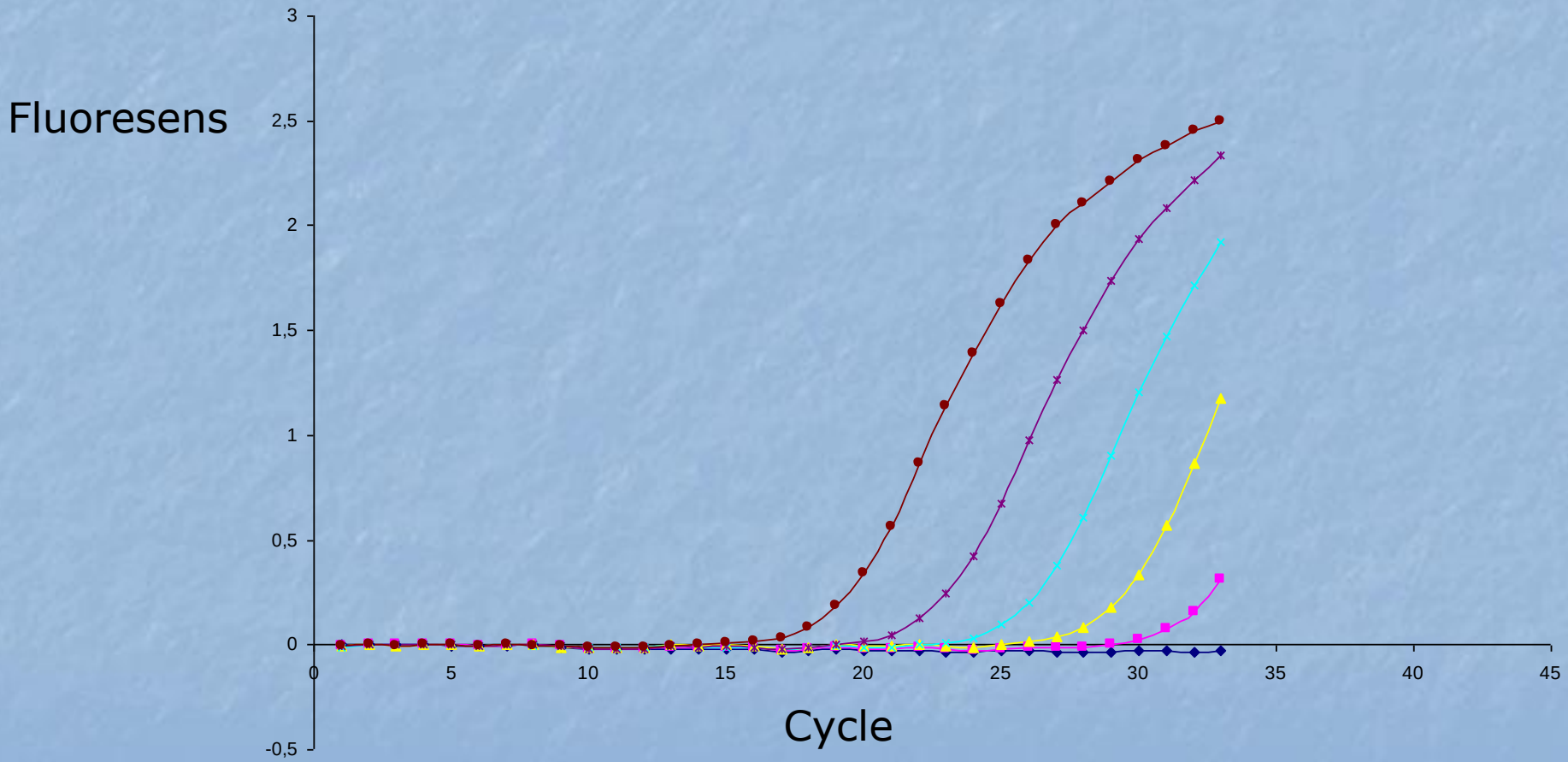
Real-Time PCR



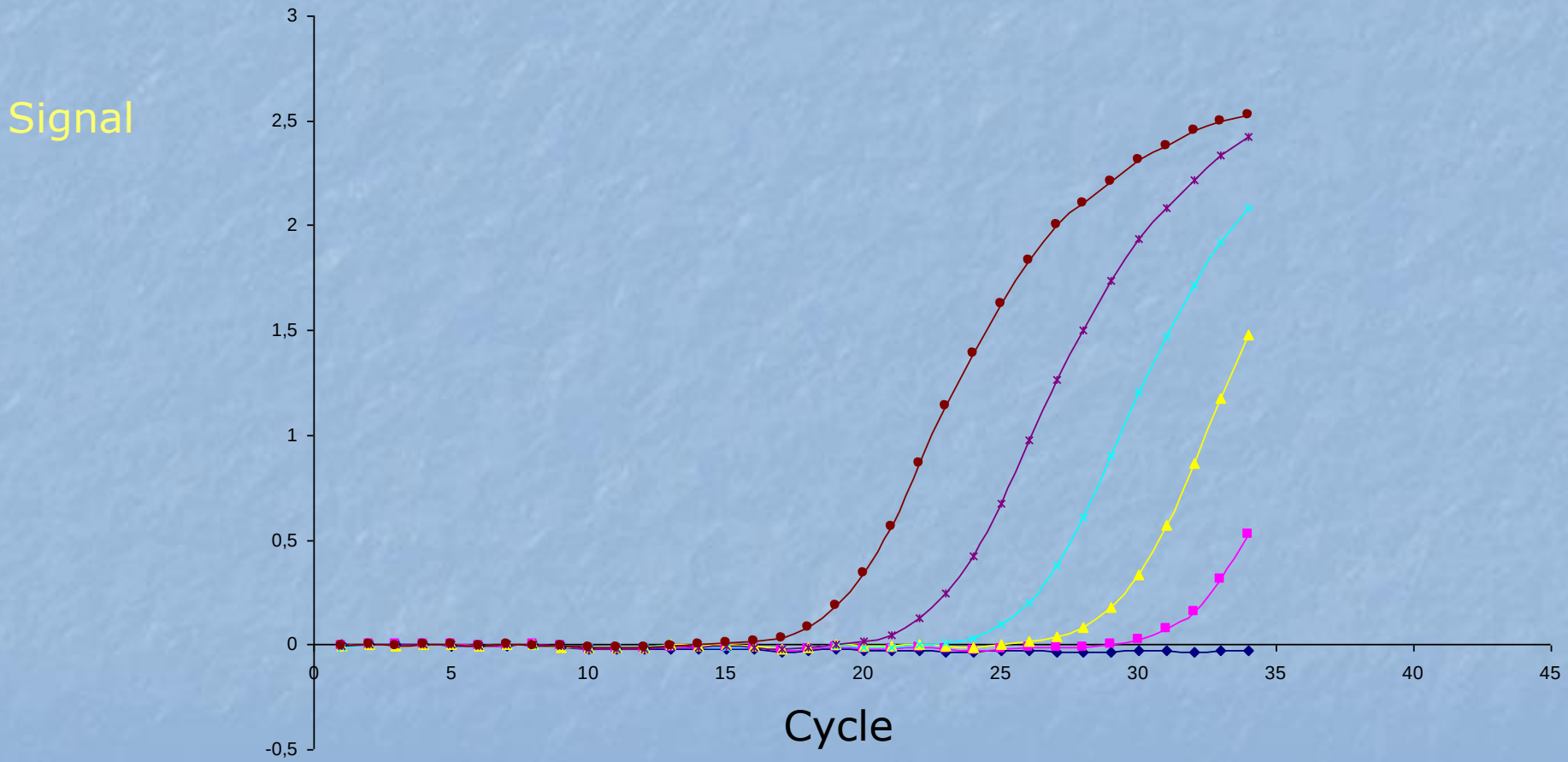
Real-Time PCR



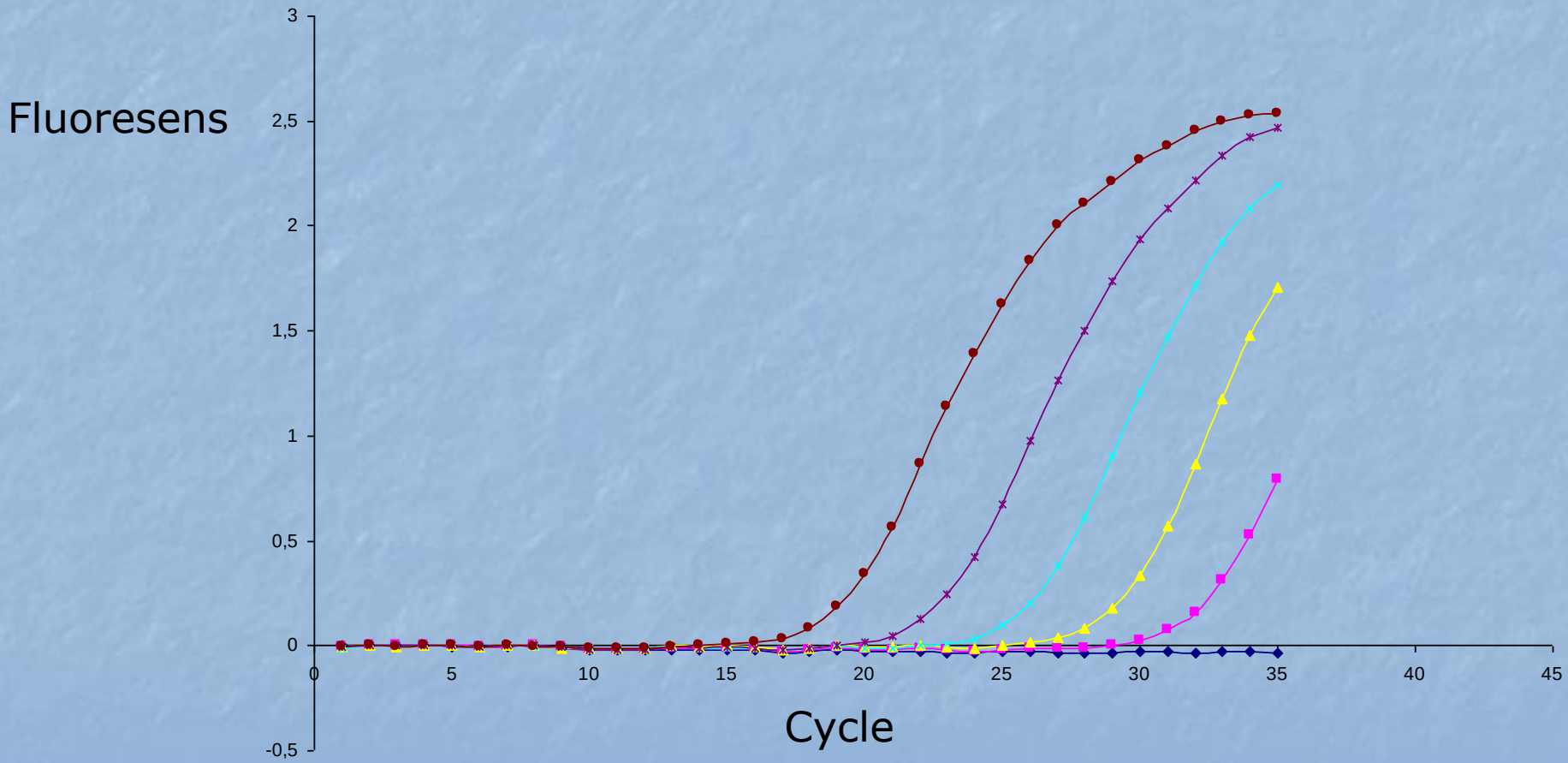
Real-Time PCR



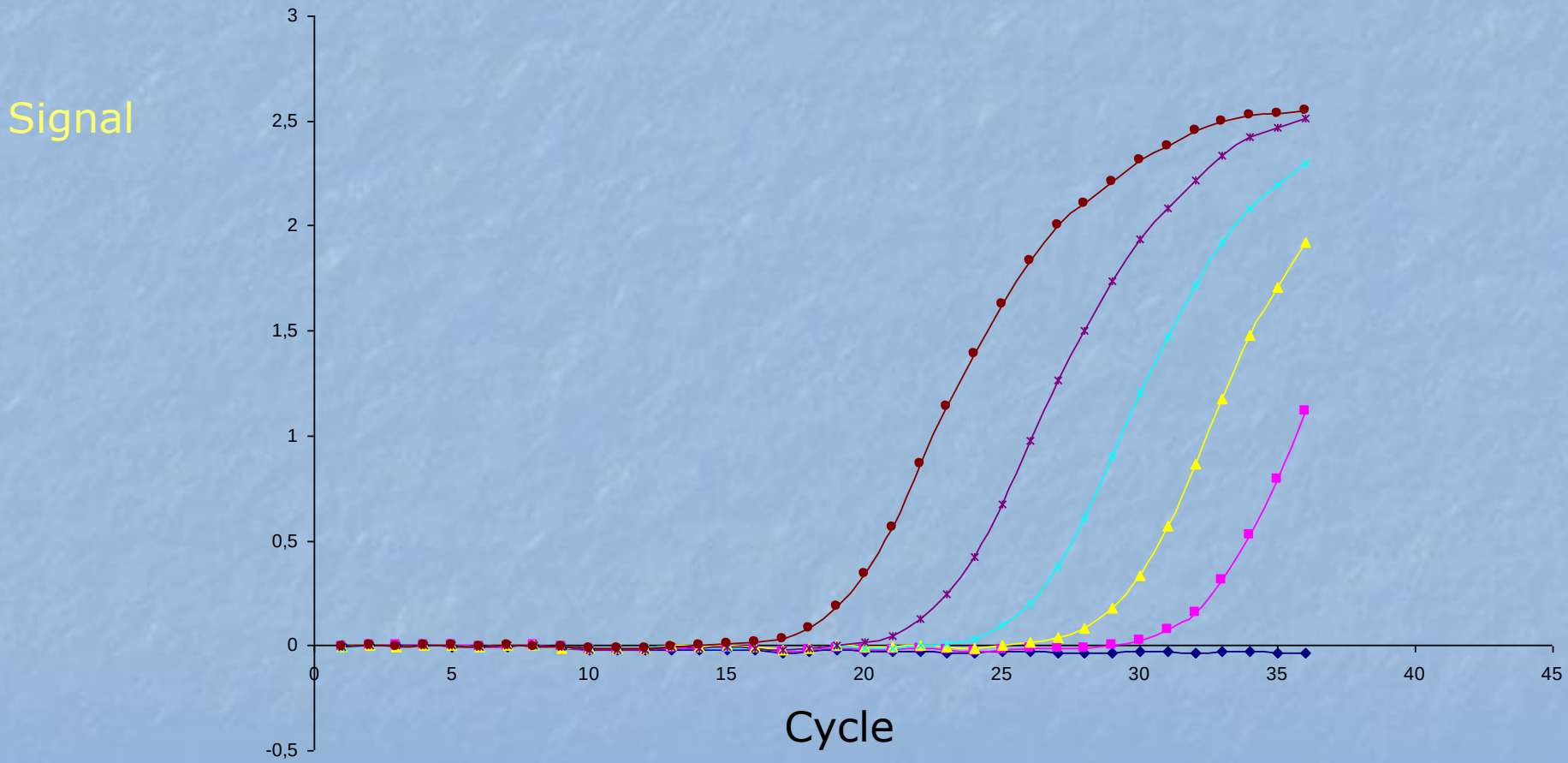
Real-Time PCR



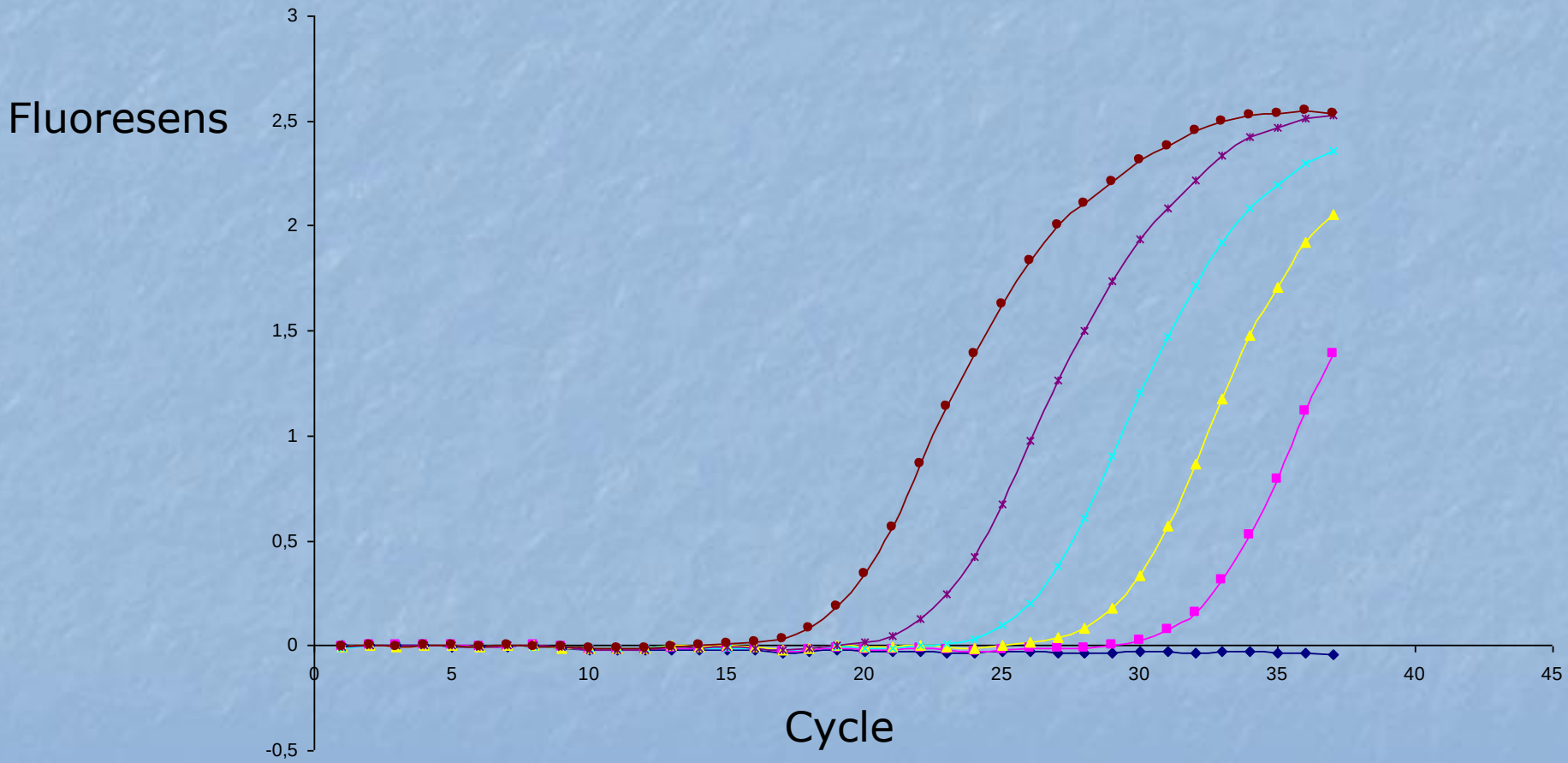
Real-Time PCR



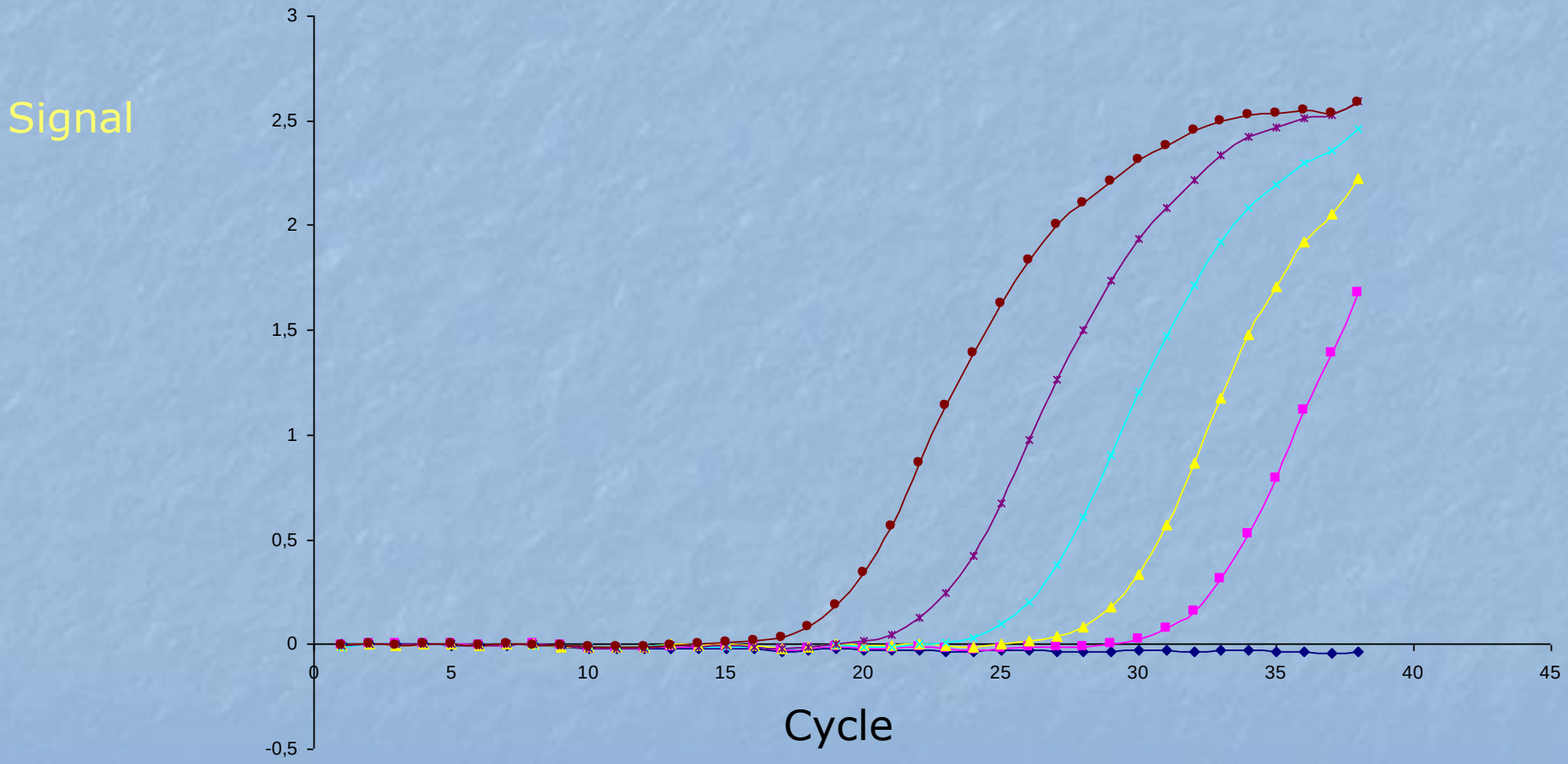
Real-Time PCR



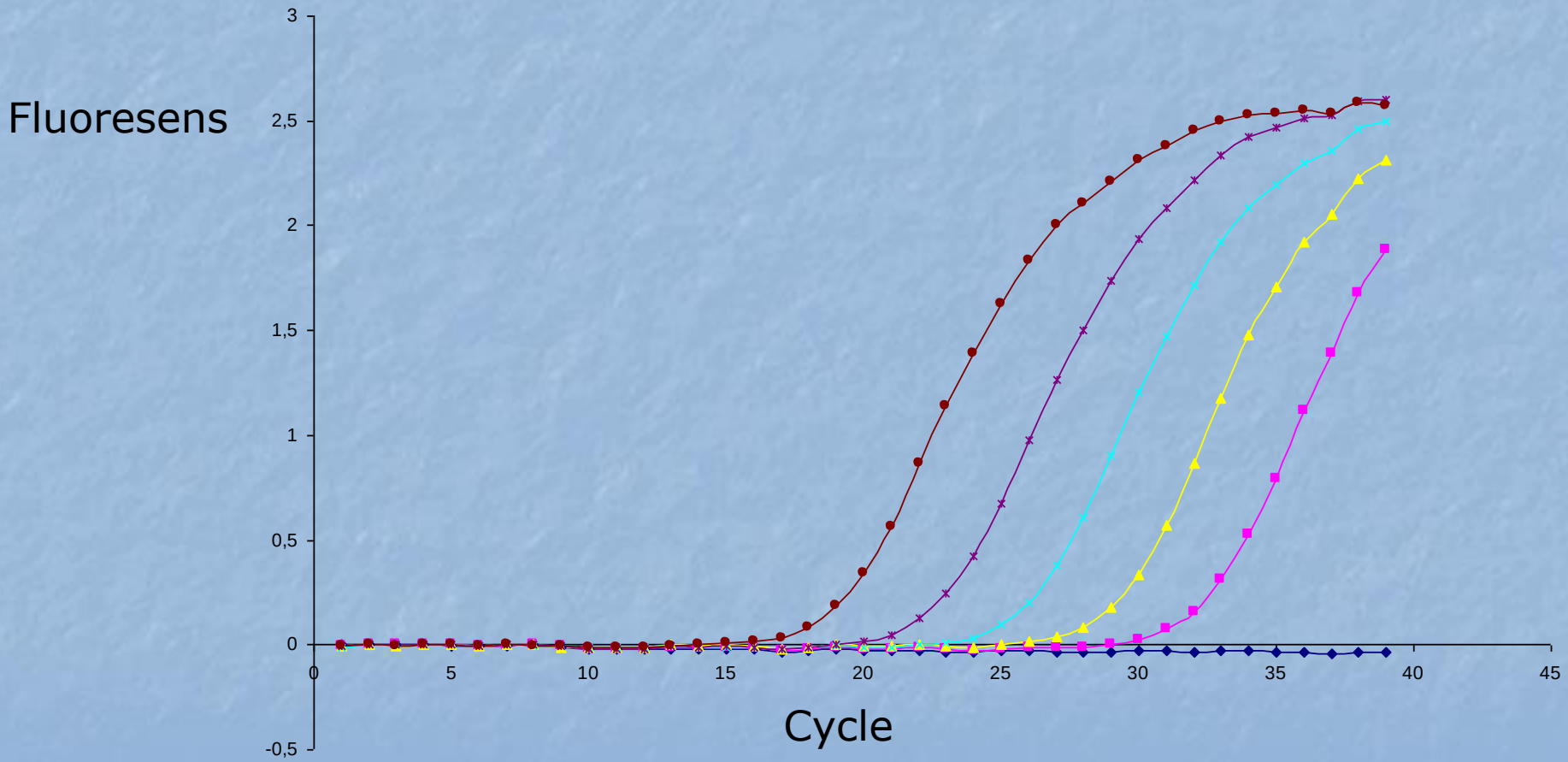
Real-Time PCR



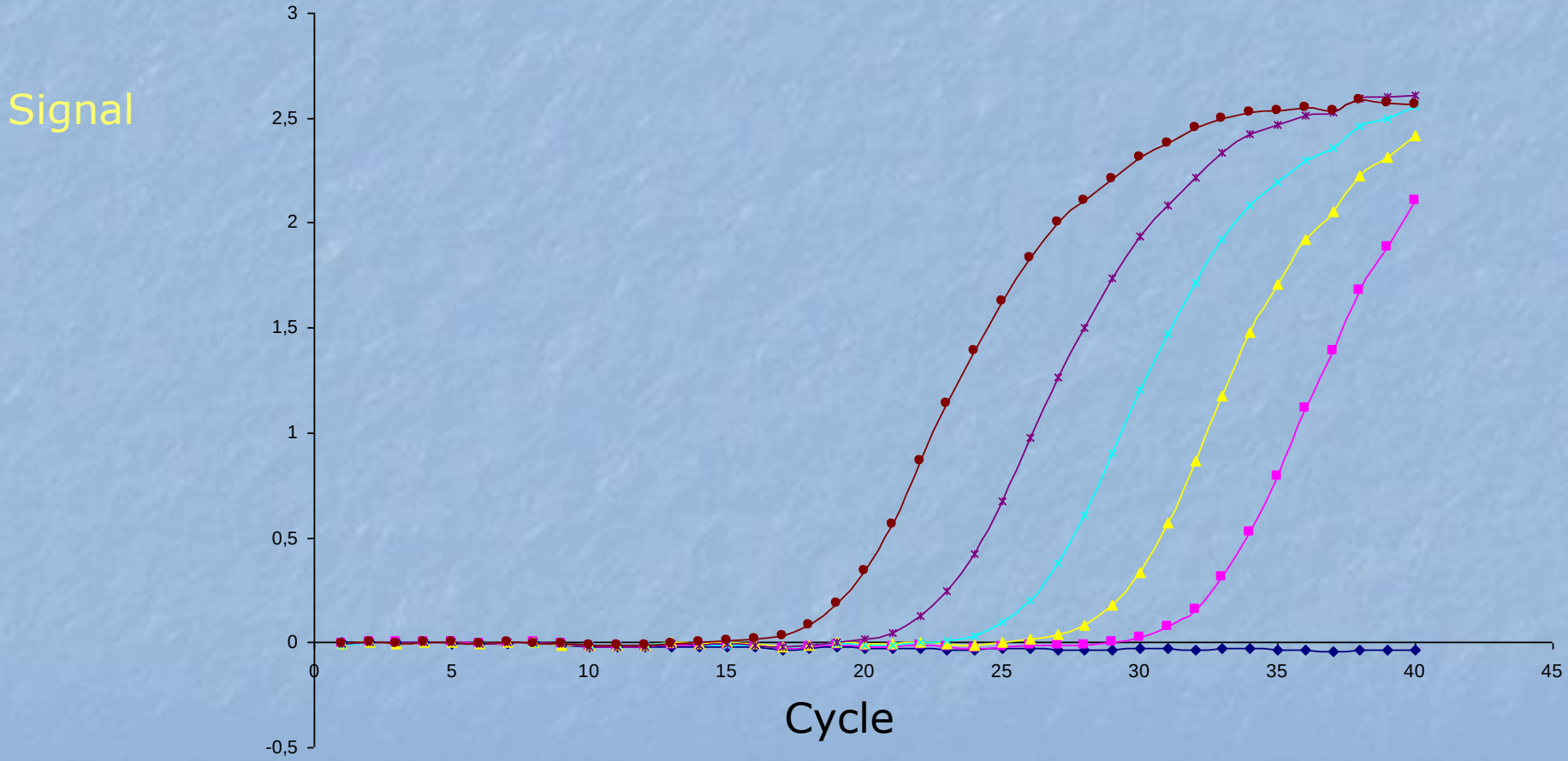
Real-Time PCR



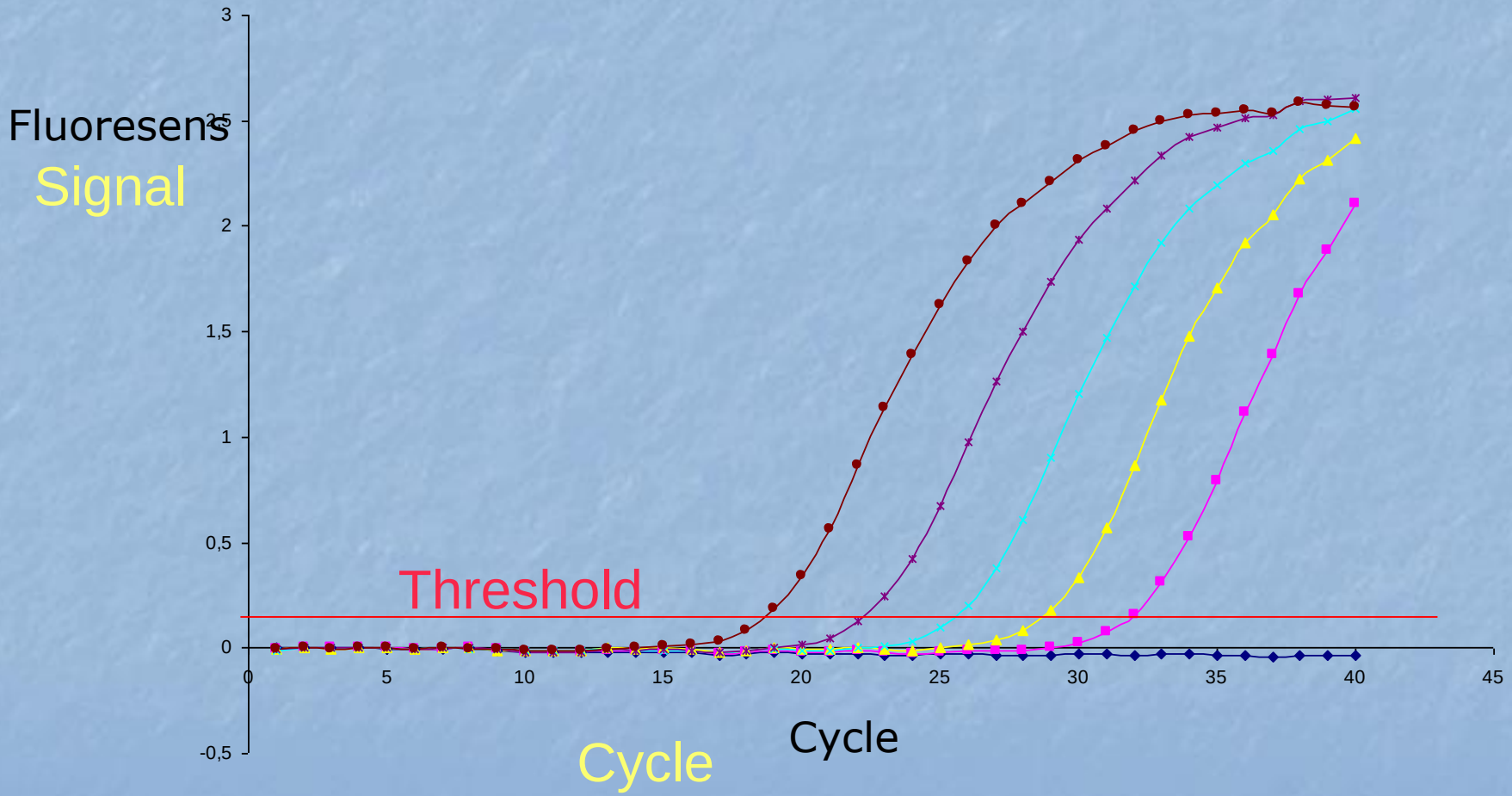
Real-Time PCR

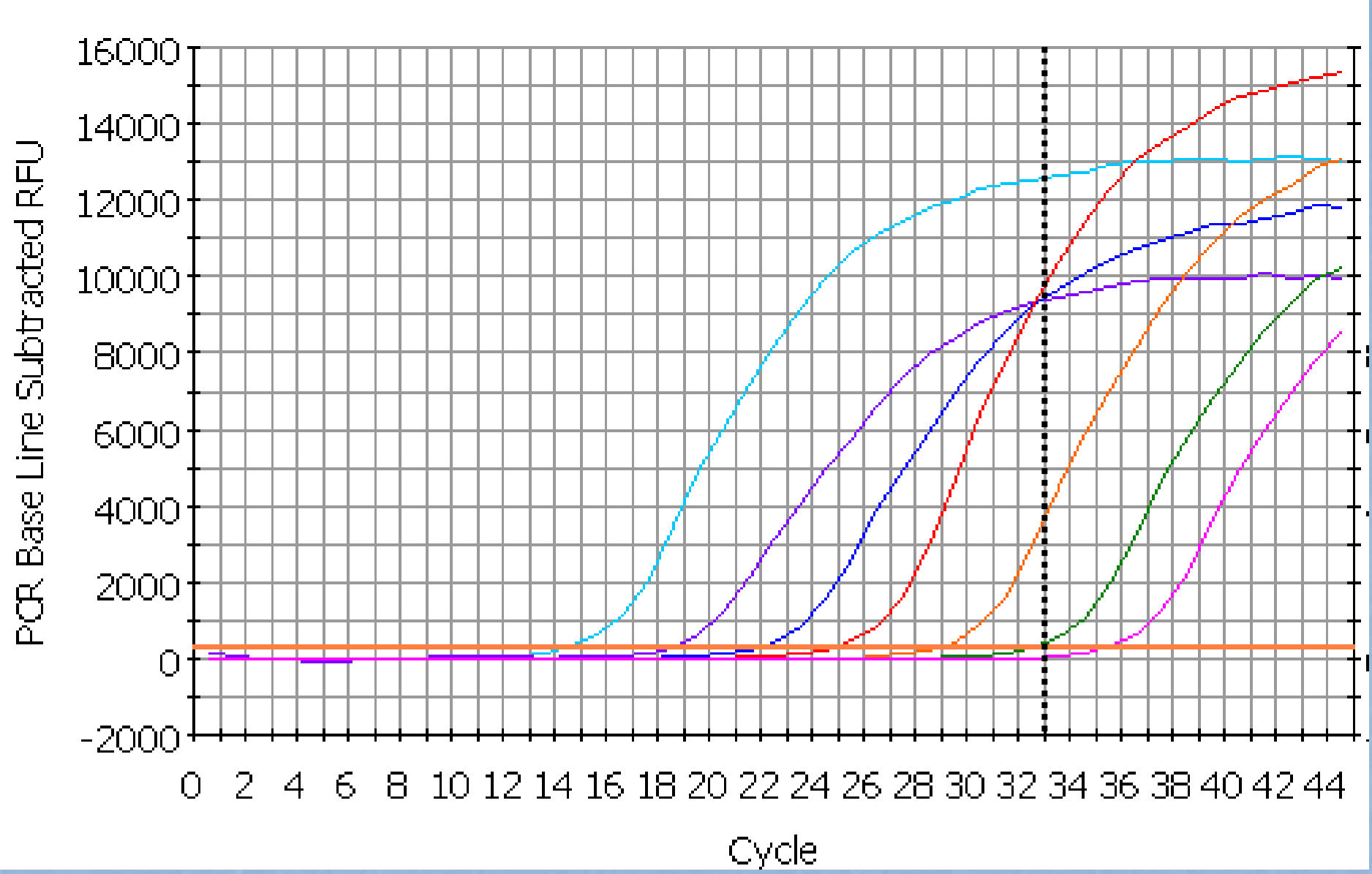


Real-Time PCR



Threshold setting

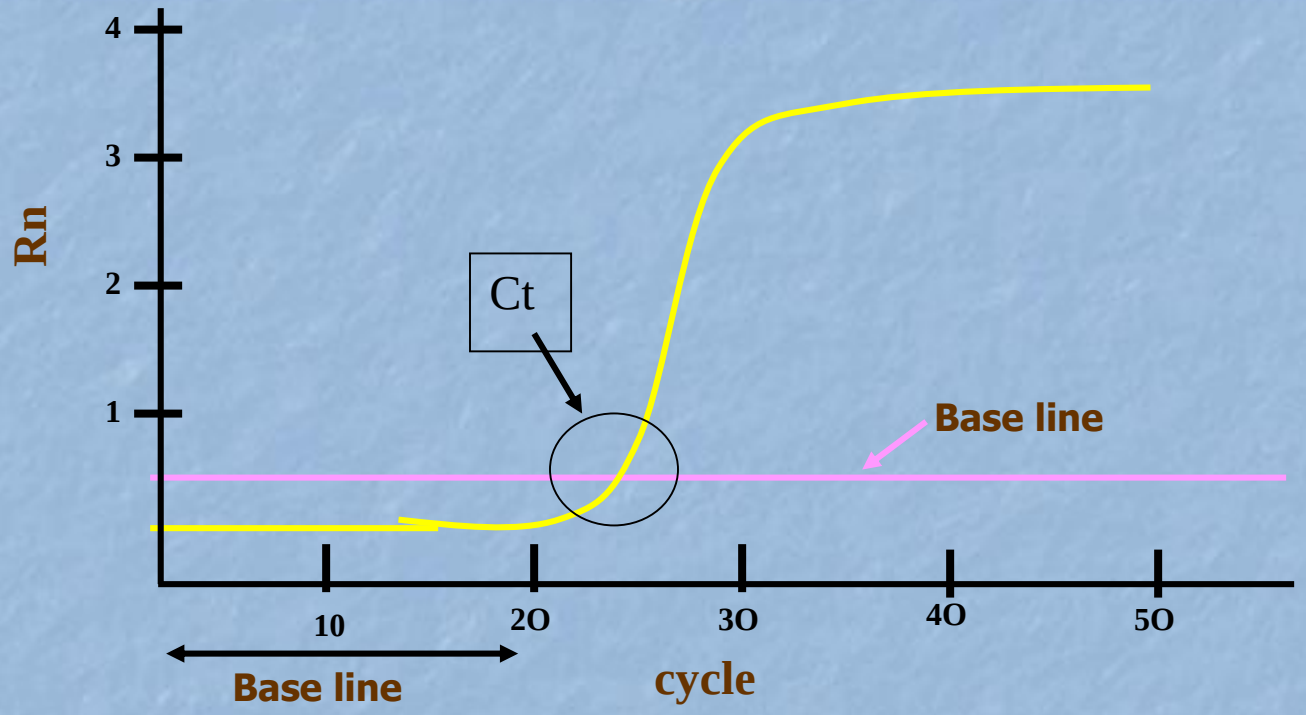




SERIES OF 10-FOLD DILUTIONS

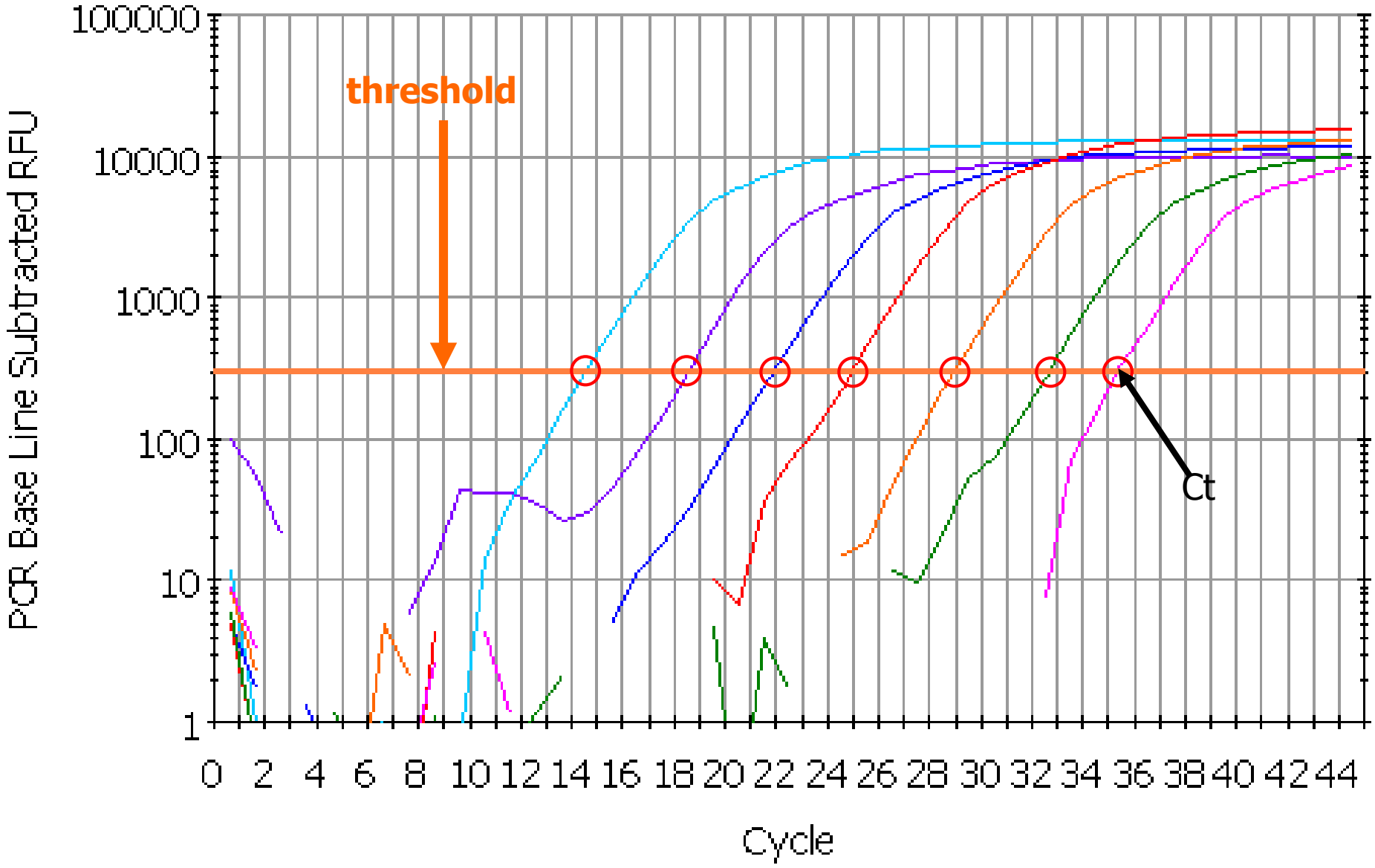
Data Analysis

Ct determination (crossing threshold)



The value of the Ct is determined during the exponential phase, at the intersection of the base line with the fluorescence curve

The number of cycles necessary to reach a certain fluorescence is function of the number of target DNA initially present



SERIES OF 10-FOLD DILUTIONS

Standard curves

- a plasmid containing a relevant portion of the gene of interest, PCR product, synthetic oligonucleotide or transcribed RNA to perform a standard curve.

- For double-stranded templates, use 660 gm/mole/base.

$$\frac{\text{Mass (in grams)} \times \text{Avogadro's Number}}{\text{Average mol. wt. of a base} \times \text{template length}} = \text{molecules of DNA}$$

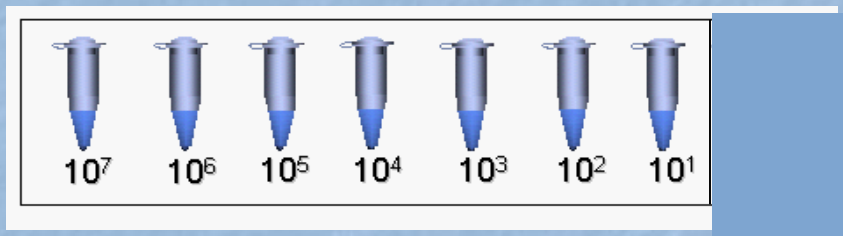
For example, if a synthetic 75-mer oligonucleotide (single-stranded DNA) is used as the template, 0.8 pg would be equal to 2 × 107 molecules:

$$\frac{0.8 \times 10^{-12} \text{ gm} \times 6.023 \times 10^{23} \text{ molecules/mole}}{330 \text{ gm/mole/base} \times 75 \text{ bases}}$$

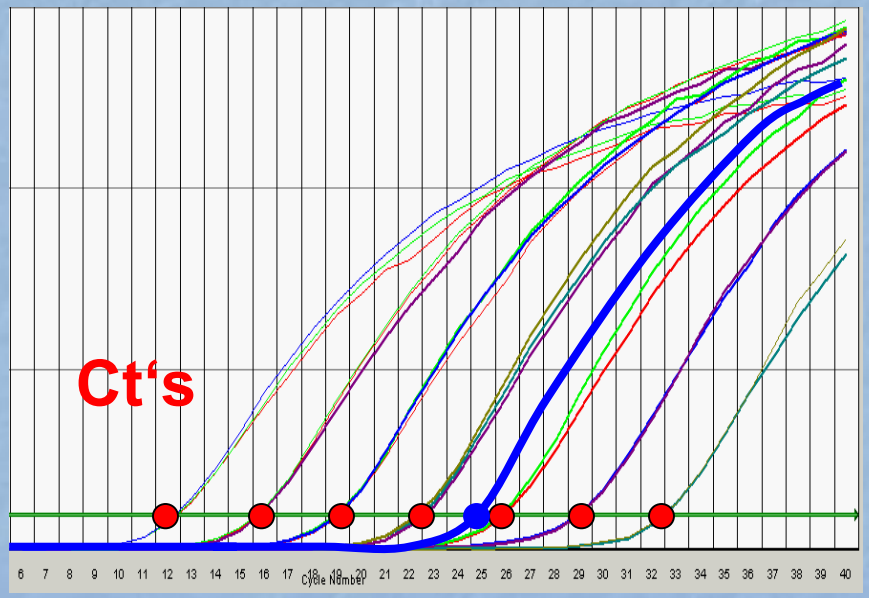
$$= 2.0 \times 10^{7} \text{ molecules (copies) of DNA}$$

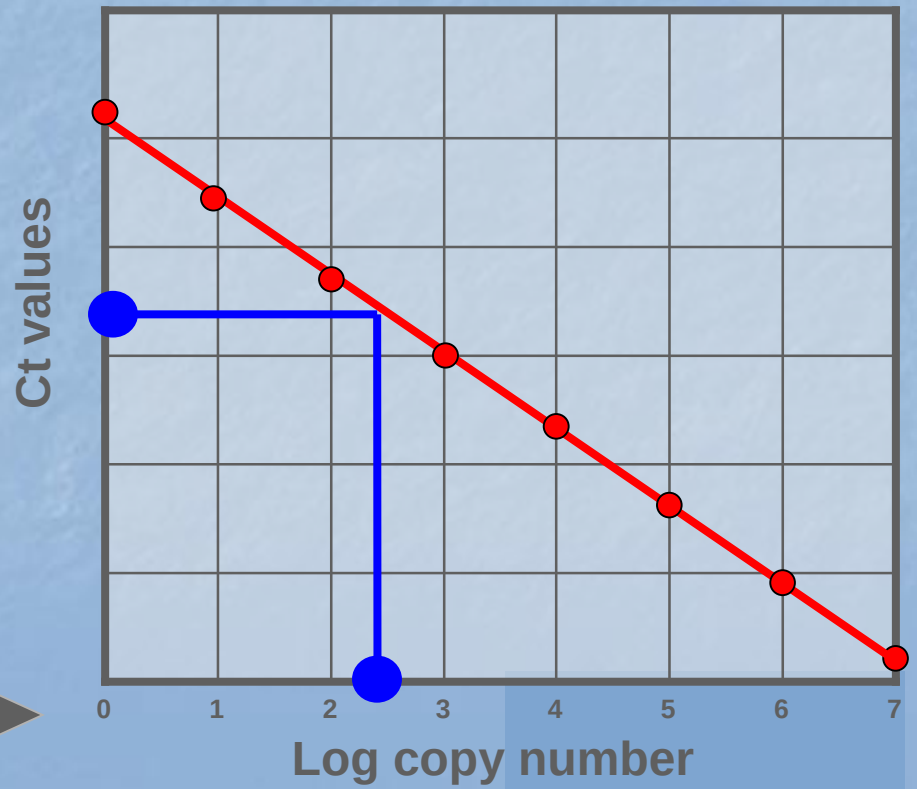
From Fluorescence to Results

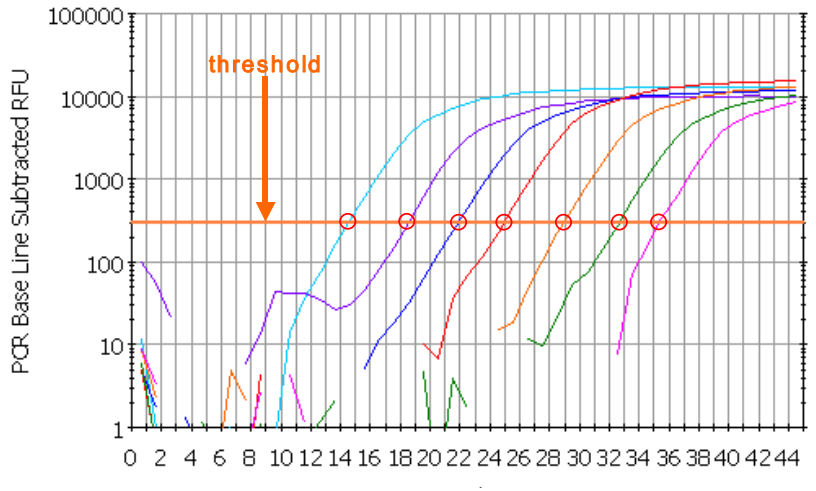
Step 2 „Comparison“ of Ct Values



The well contains
400 target copies







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

EFFICIENCY OF PCR

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
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

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
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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

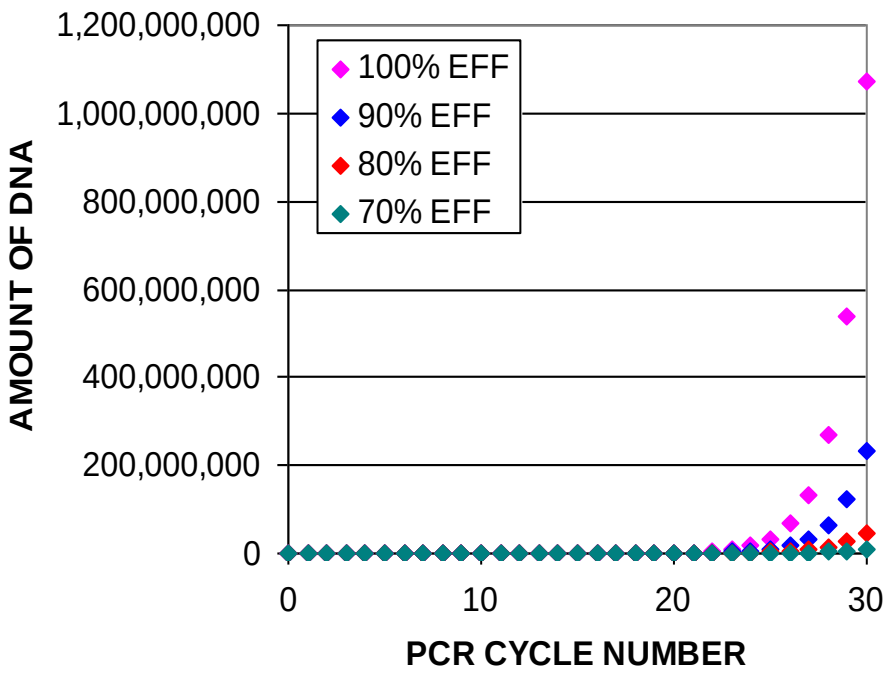
90% = 1.90x

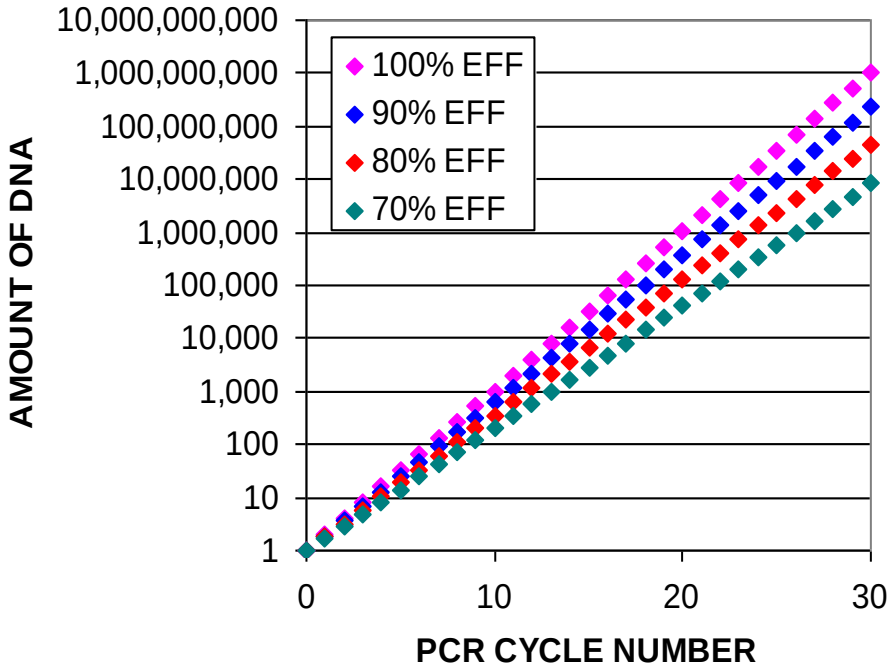
80% = 1.80x

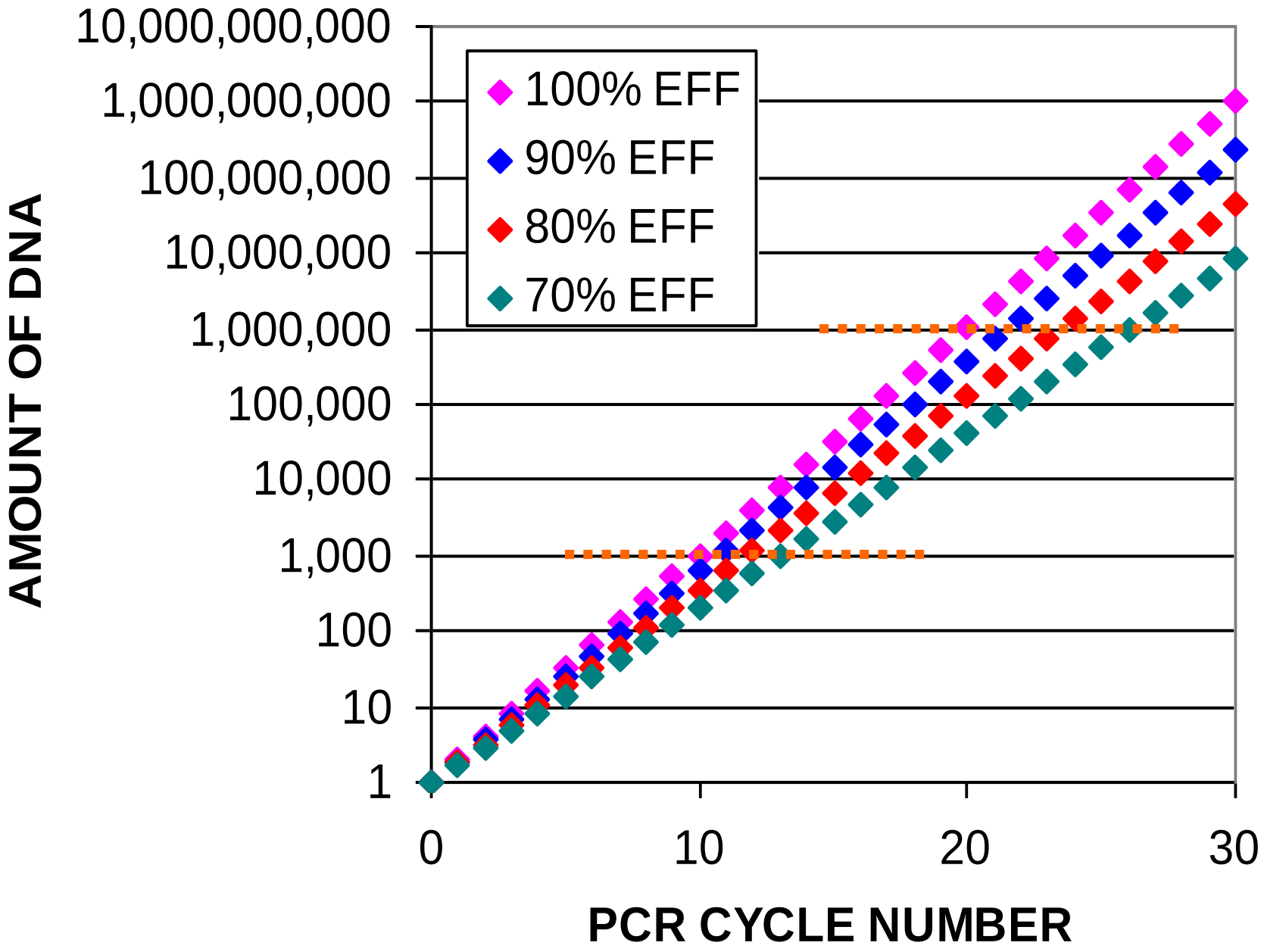
70% = 1.70x

AFTER N CYCLES:
fold increase = (efficiency)^n

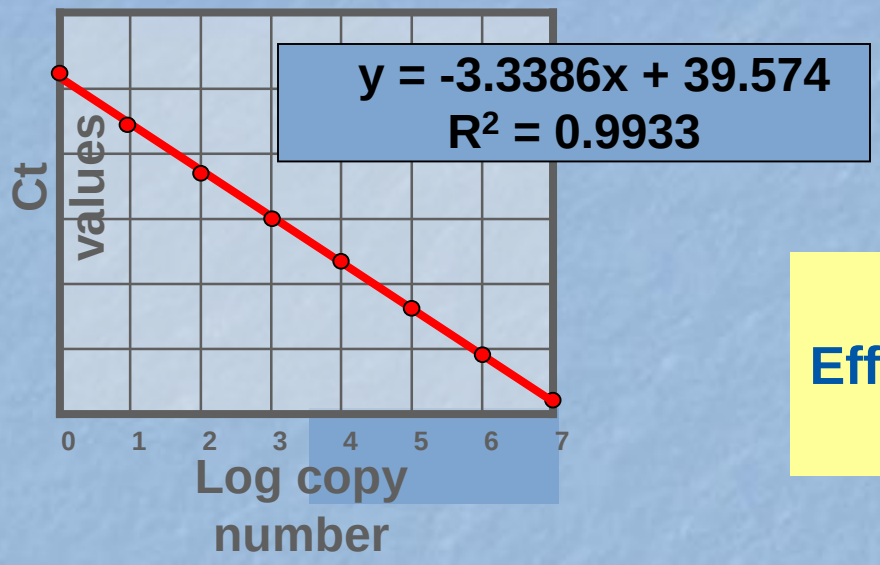
CYCLE	AMOUNT OF DNA	AMOUNT OF DNA	AMOUNT OF DNA	AMOUNT OF DNA
	100% EFFICIENCY	90% EFFICIENCY	80% EFFICIENCY	70% EFFICIENCY
0	1	1	1	1
1	2	2	2	2
2	4	4	3	3
3	8	7	6	5
4	16	13	10	8
5	32	25	19	14
6	64	47	34	24
7	128	89	61	41
8	256	170	110	70
9	512	323	198	119
10	1,024	613	357	202
11	2,048	1,165	643	343
12	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
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






AMOUNT OF DNA
10,000,000,000
1,000,000,000
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
100% EFF
90% EFF
80% EFF
70% EFF
0
10
20
30
PCR CYCLE NUMBER

Standard Curve



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

Example:

Slope = -3.3386

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

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

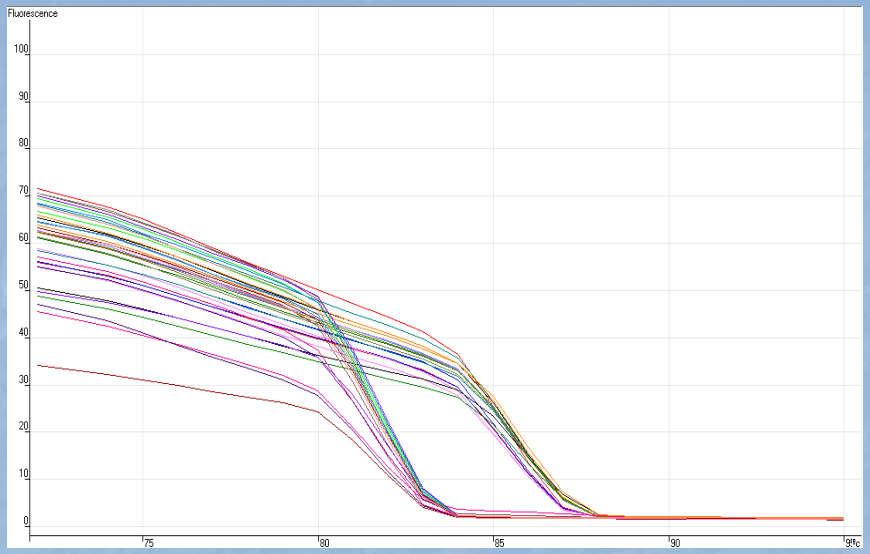
= 1.955 – 1

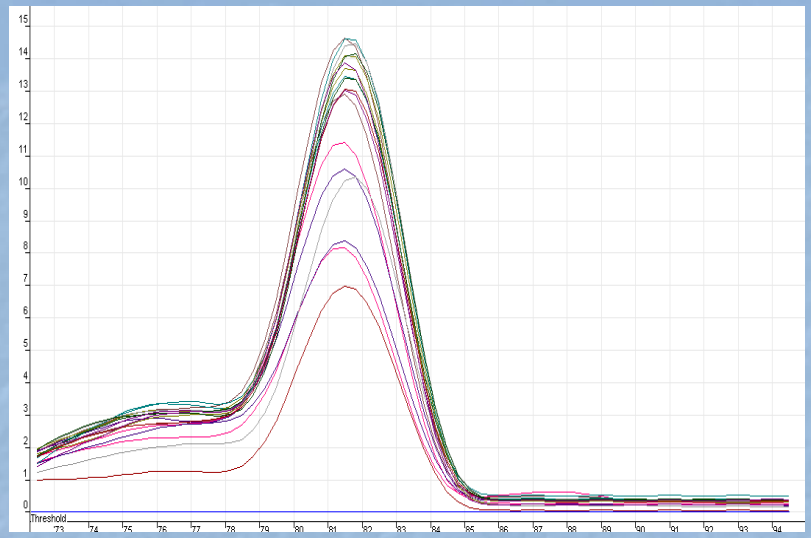
= 0.995 or 99.5%

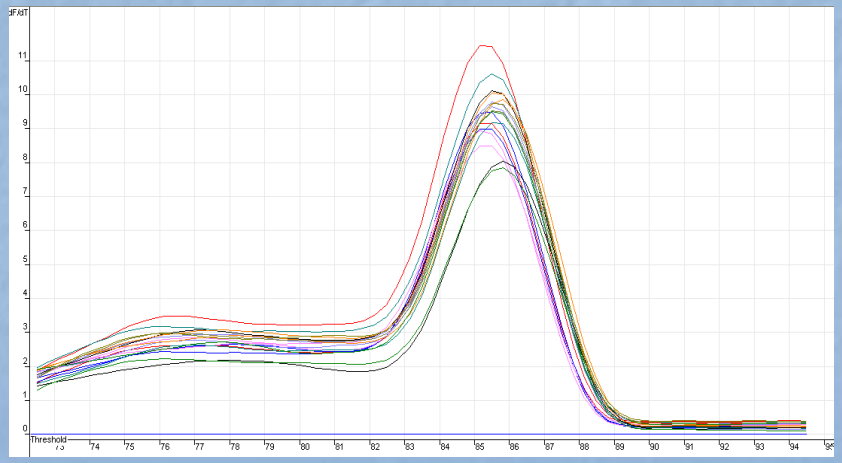
If slope = -3.32

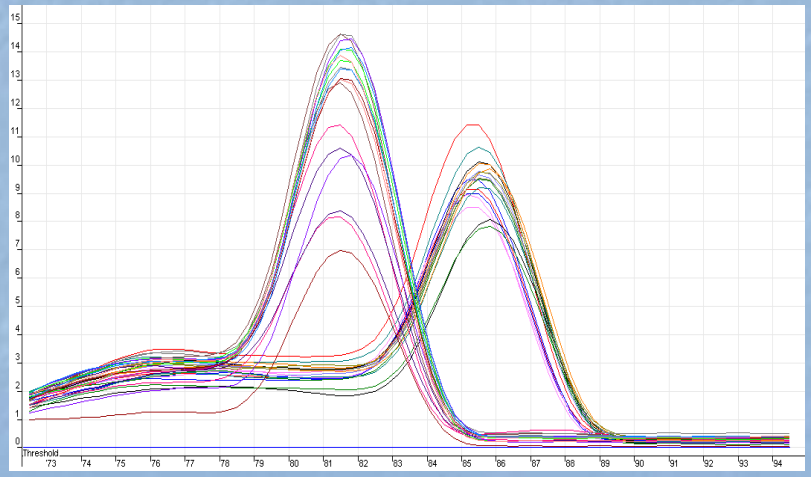
efficiency becomes 1

Melting Curve for ctxAB & recA

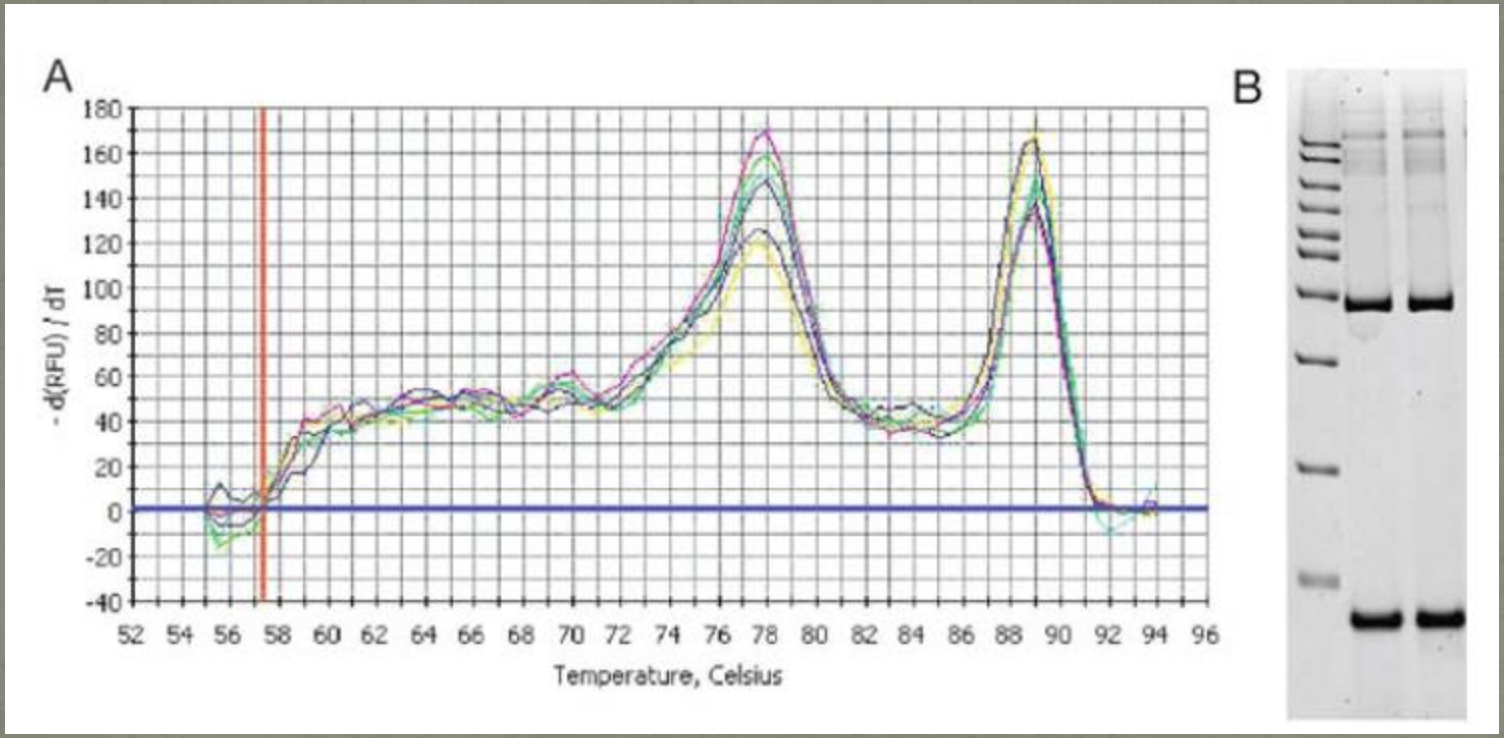


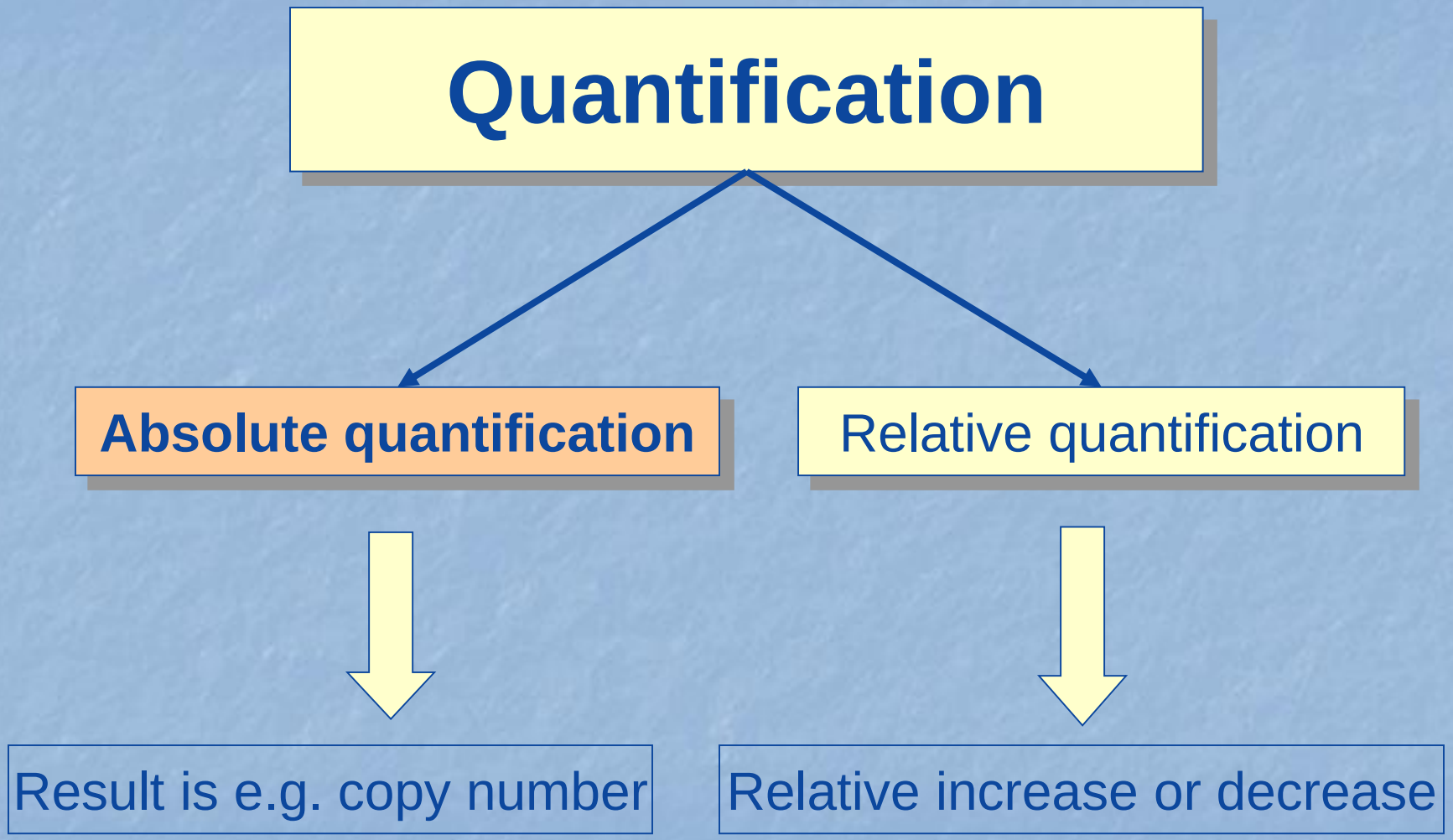






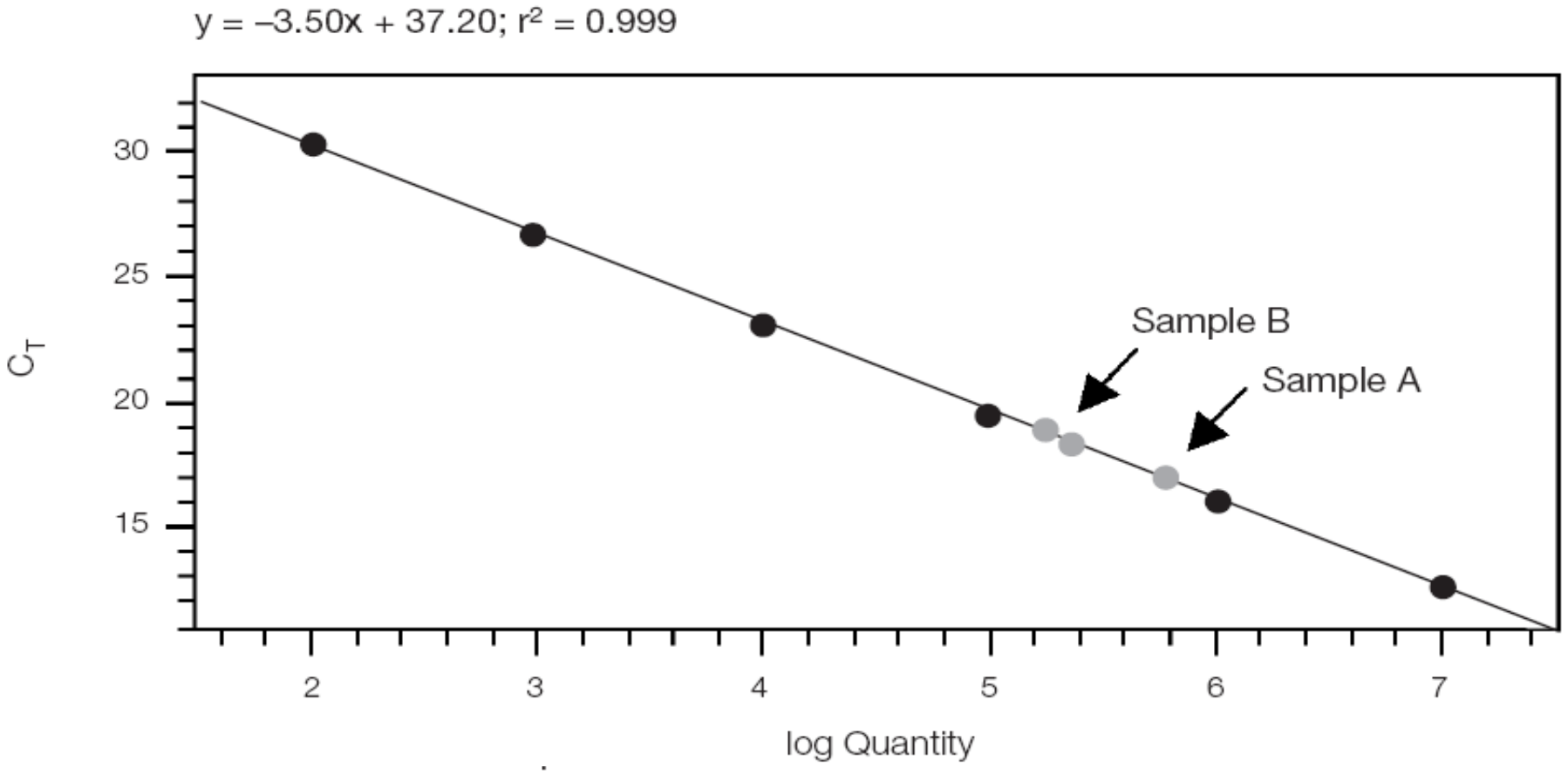
Melting curve analysis




Quantification
Absolute quantification
Relative quantification
Result is e.g. copy number
Relative increase or decrease

recA	Sam/stn	Con.	Ct	ctxAB	Sam/stn	Con.	Ct
1	Stx1	10	21.14	19	Stx1	10	14.39
2	Stx1	10	20.95	20	Stx1	10	15.25
3	Stx2	1000	27.01	21	Stx2	1000	20.92
4	Stx2	1000	27.18	22	Stx2	1000	20.43
5	Stx3	10000	30.54	23	Stx3	10000	22.39
6	Stx3	10000	30.60	24	Stx3	10000	22.99
7	9	unknown	21.89	25	9	unknown	17.20
8	9	unknown	21.85	26	9	unknown	15.01
9	34	unknown	20.89	27	34	unknown	15.38
10	34	unknown	20.95	28	34	unknown	15.52
11	95	unknown	20.93	29	95	unknown	13.21
12	95	unknown	21.02	30	95	unknown	12.90
13	97	unknown	21.61	31	97	unknown	13.91
14	97	unknown	21.35	32	97	unknown	14.23
15	116	unknown	20.87	33	116	unknown	15.27
16	116	unknown	20.80	34	116	unknown	16.03
17	2t	unknown	21.97	35	2t	unknown	16.87
18	2t	unknown	21.94	36	2t	unknown	17.20

Absolute quantification



Sample	Replicate	C_T	Copies
A	1	18.61	204,577
A	2	18.41	234,115
A	3	18.87	172,300
Average			**203,664 ± 30,917**
B	1	17.06	569,789
B	2	17.07	563,823
B	3	17.00	591,173
Average			**574,928 ± 14,381**

Relative quantification

The Pfaffl Method

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_T,\ \text{target (calibrator – test)}}}{(E_{\text{ref}})^{\Delta C_T,\ \text{ref (calibrator – test)}}}$$

The Livak Method

$$\Delta C_{T(test)} = C_{T(target,\ test)} - C_{T(ref,\ test)}$$

$$\Delta C_{T(calibrator)} = C_{T(target,\ calibrator)} - C_{T(ref,\ calibrator)}$$

$$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)}$$

$$2^{-\Delta\Delta C_T} = \text{Normalized expression ratio}$$

Relative Quant. Analysis

Delta Delta CT

Experiment Information

Run Name	1388.8.17
Run Start	11/8/2009 11:31:44 AM
Run Finish	11/8/2009 12:46:00 PM
Operator	
Notes	
Run On Software Version	Rotor-Gene 6.0.38
Run Signature	The Run Signature is valid.
Gain FAM/Sybr	9.33

Colour	Replicate Name	GOI CT	GOI Count	Norm. CT	Norm. Count	Delta CT	Delta Delta CT	Relative Conc.	Calibrator
	stx 1	18.15	2	21.30	2	-3.15	0.00	1.00	Yes
	stx 2	23.54	2	27.33	2	-3.79	-0.64	1.56	
	stx 3	24.02	2	30.80	2	-6.79	-3.63	12.40	
	sample 9	17.98	2	22.12	2	-4.13	-0.98	1.97	
	sample 34	16.84	2	18.19	2	-1.34	1.81	0.29	
	sample 95	14.31	2	18.23	2	-3.92	-0.77	1.70	
	sample 97	15.73	2	21.72	2	-5.99	-2.83	7.13	
	sample 116	16.72	1	21.08	2	-4.36	-1.21	2.31	
	sample 2t	18.47	2	23.20	2	-4.73	-1.58	2.98	

A riddle:

- What infectious disease can't be detected by qPCR?