REAL TIME POLYMERASE CHAIN REACTION (RT-PCR)

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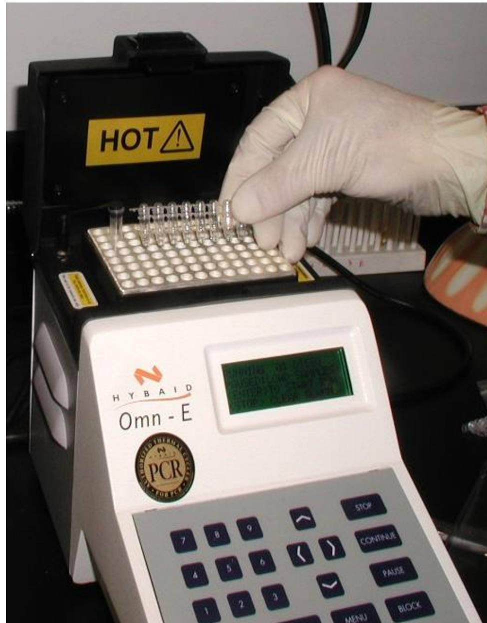
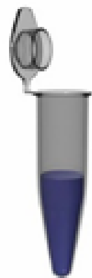
Detection and measurement of DNA synthesis



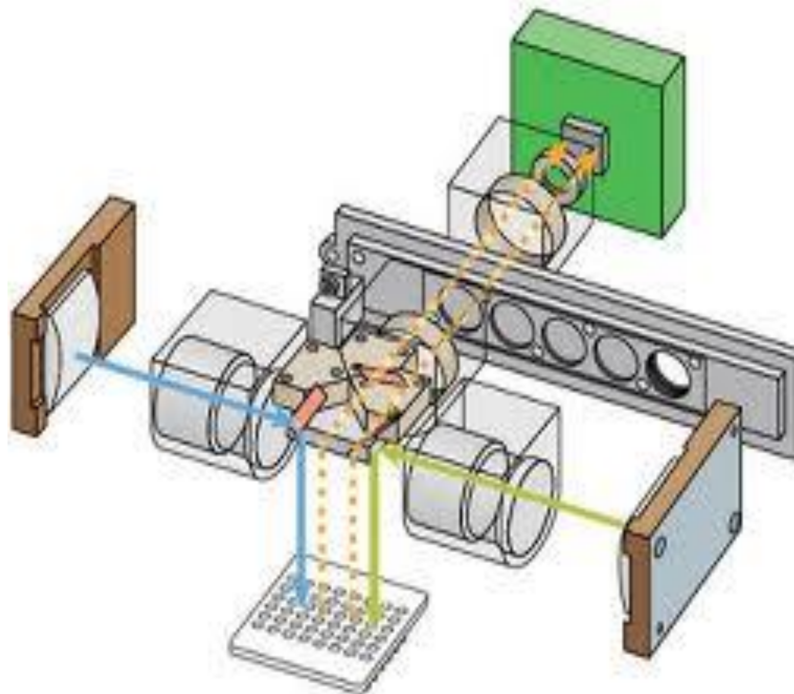


Fluorescent reporter molecules

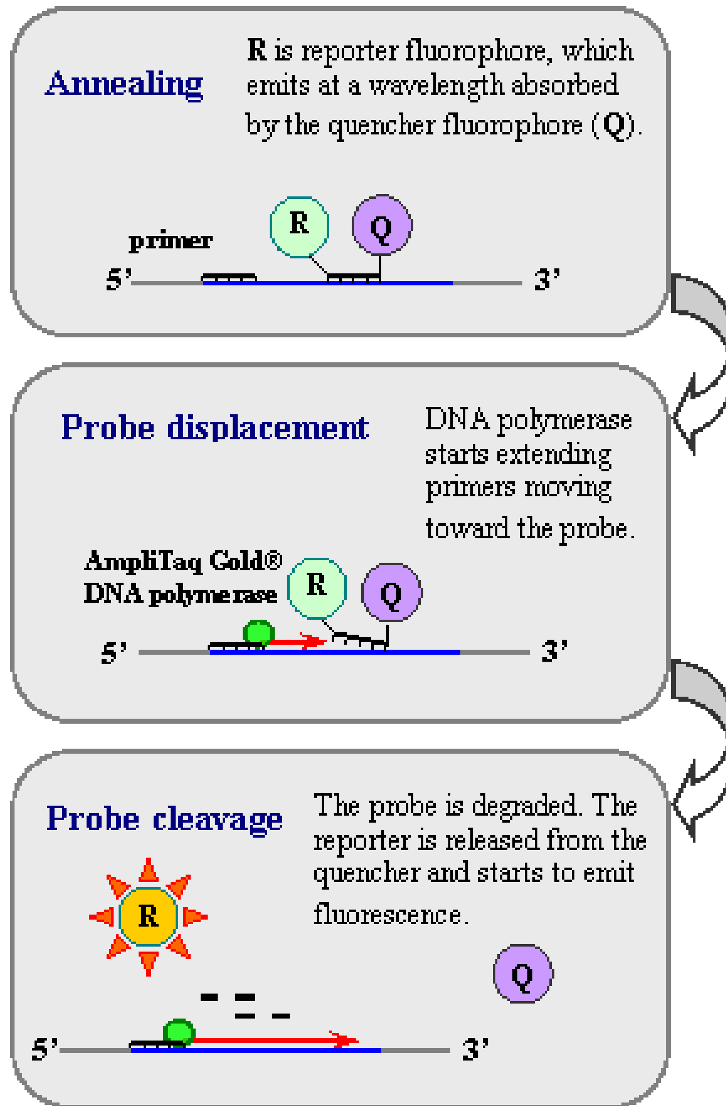
Reporter molecules;

Fluorescent chemistry & detection;

- **Non-specific detection** : DNA binding dyes
- **Specific detection** : Target Specific Probes



RT-PCR; Specific detection: Target Specific Probes



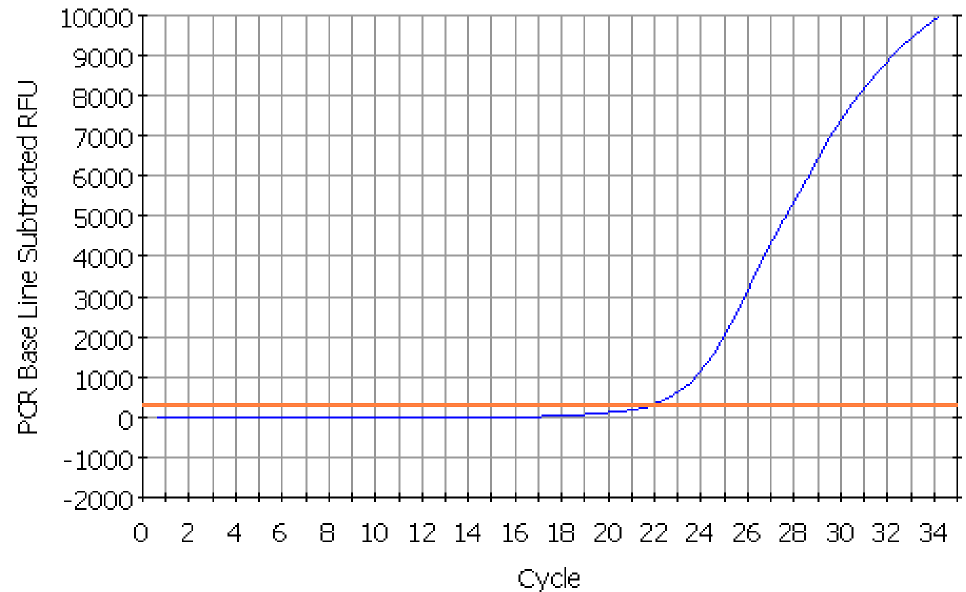
Molecular Beacons

TaqMan® Probes

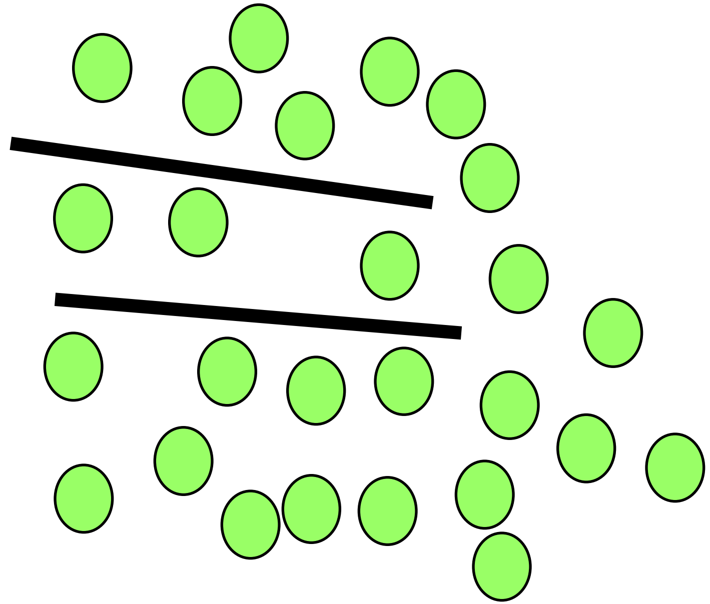
- .FRET
- .MGB

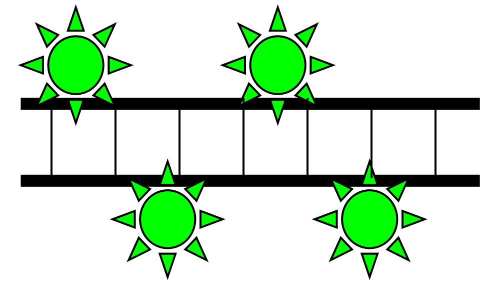
Scorpion® Primers

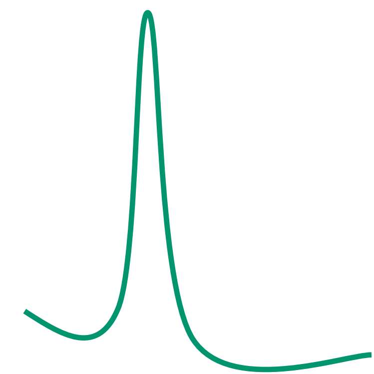
……

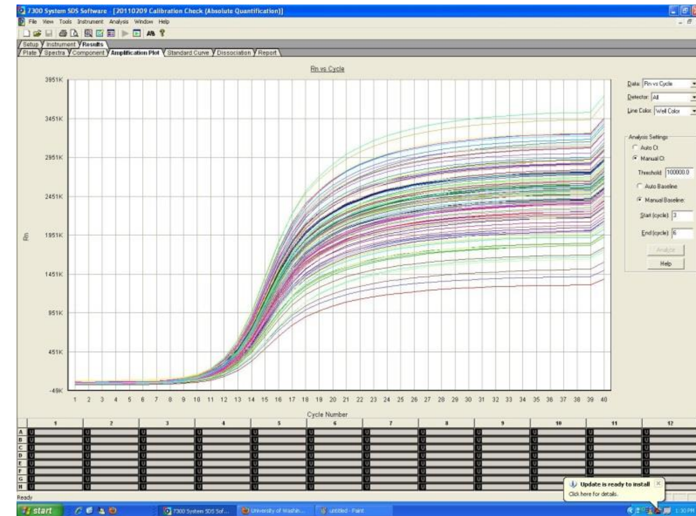


RT-PCR; Non-specific detection, DNA binding dyes







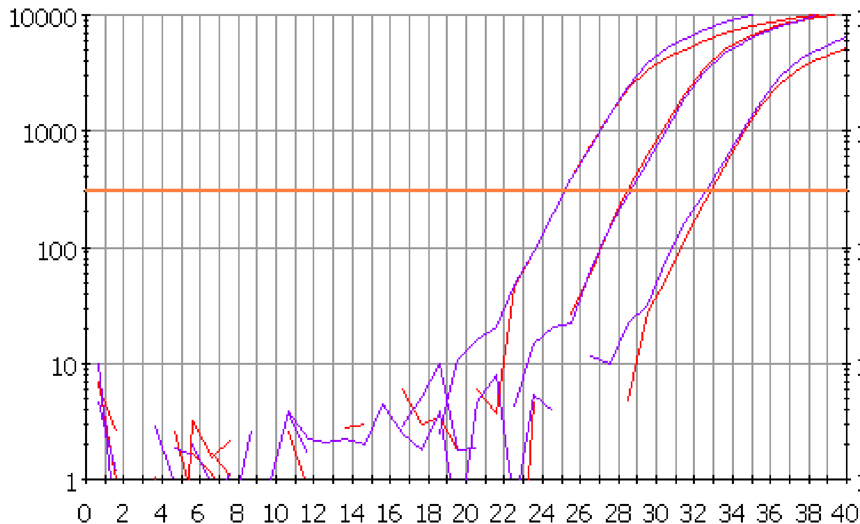


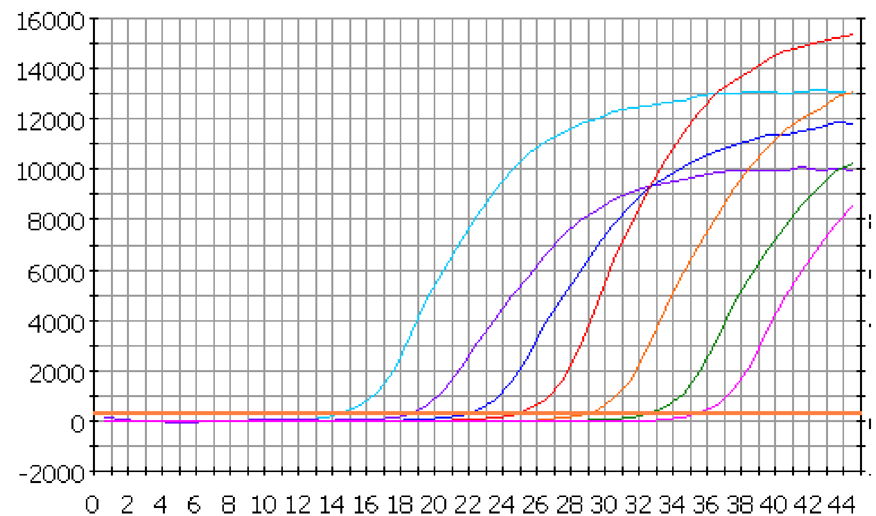
- Monitor the progress of the PCR as it occurs in real time.
- Precisely measure the amount of amplicon at each cycle, which allows highly accurate quantification.
- Amplification and detection occurs in a single tube, eliminating post-PCR manipulations.

Be careful ! *

- Check the efficiency & consistency:
  - RT-PCR system, reporter molecules, reaction mixtures, ….
- Optimize the initial starting amount of target template:
  - Determine the initial starting amount of DNA/cDNA and concentration of target sequence in it.
- Identify and assign the amplification reaction specifications:
  - Outline target specific amplification related fluorescent signal
  - Clear nonspecific background (the noise)
  - State significant amplification starting point over background fluorescent signal and determine the plateau effect starting point to assign a precise exponential phase essential for data acquisition and calculations.
  - Prove that amplification reaction has an acceptable and uniform efficiency.
- Check the uniformity and specificity of amplicon:

	Qualitative	Quantitative
Target :	cDNA / DNA	cDNA
Result :	Present / Absent More/Less; arbitrary	10^3 copies Fold changes





HPV screening

L1-CP: GP 5/6

Reaction mix prep.

Protocol: dsDNA specific dye: SYBR® Green

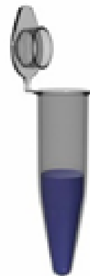
Forward primer

Reverse primer

Optimized reaction mix: DNA polymerase, $MgCl_2$, dNTP, SybrGreen

Template DNA

Final volume: 20 µ



HPV screening

Test

negative control

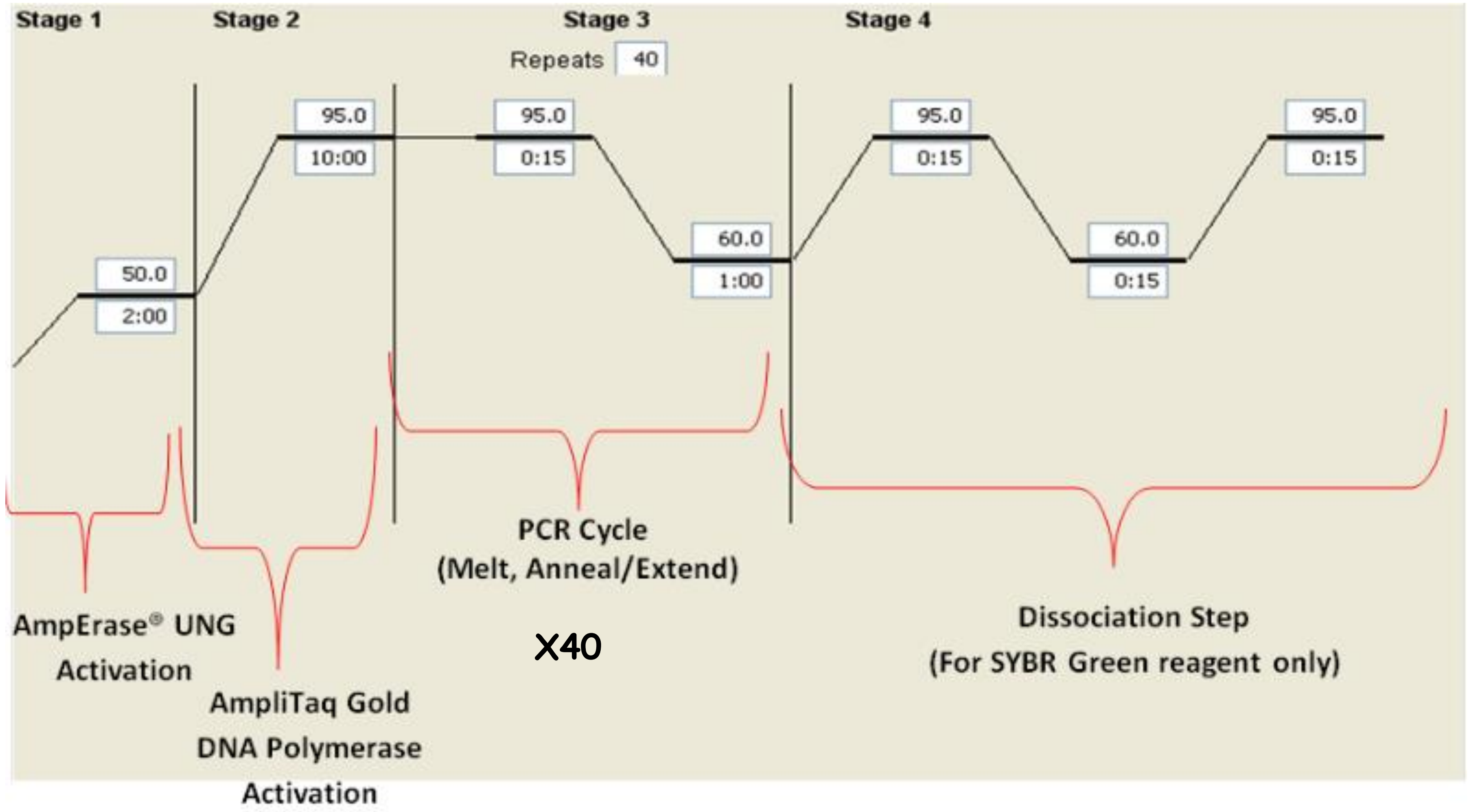
positive controls

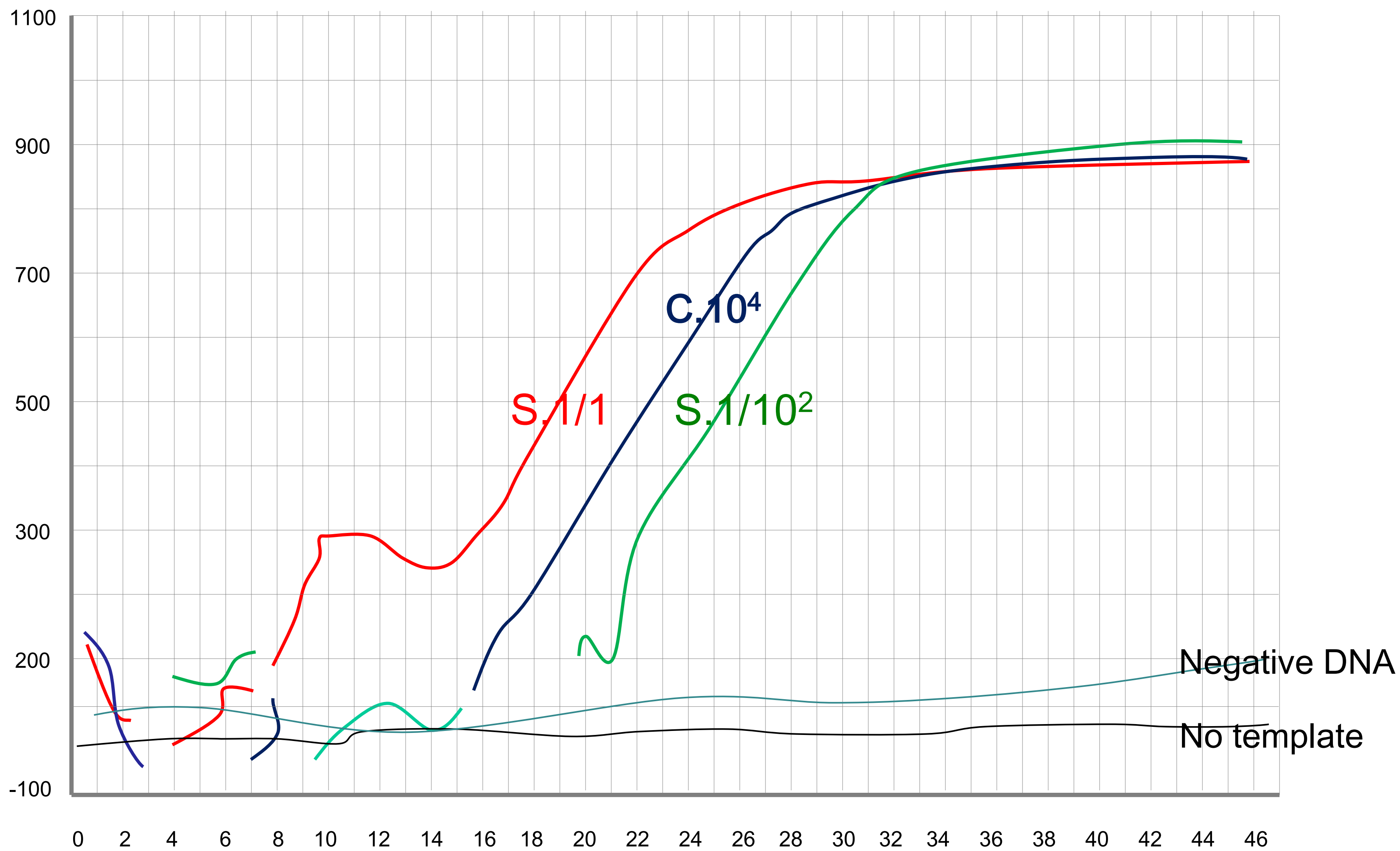
2 reactions

2 reactions

1 reactions

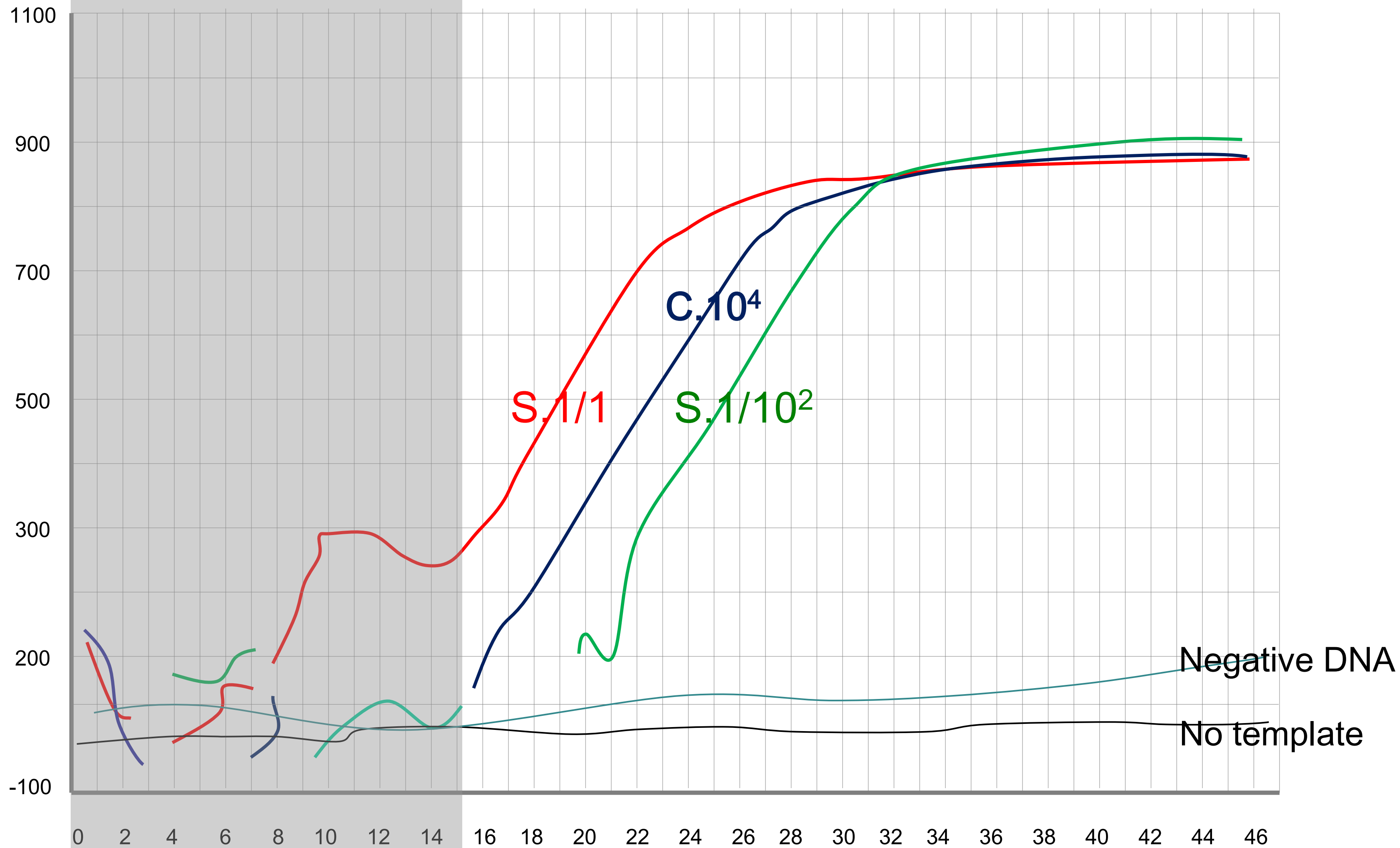
Total: 3-6 reactions


Stage 1
Stage 2
Stage 3
Repeats 40
Stage 4
50.0
2:00
95.0
10:00
95.0
0:15
60.0
1:00
95.0
0:15
60.0
0:15
95.0
0:15
AmpErase® UNG
Activation
AmpliTaq Gold
DNA Polymerase
Activation
PCR Cycle
(Melt, Anneal/Extend)
X40
Dissociation Step
(For SYBR Green reagent only)

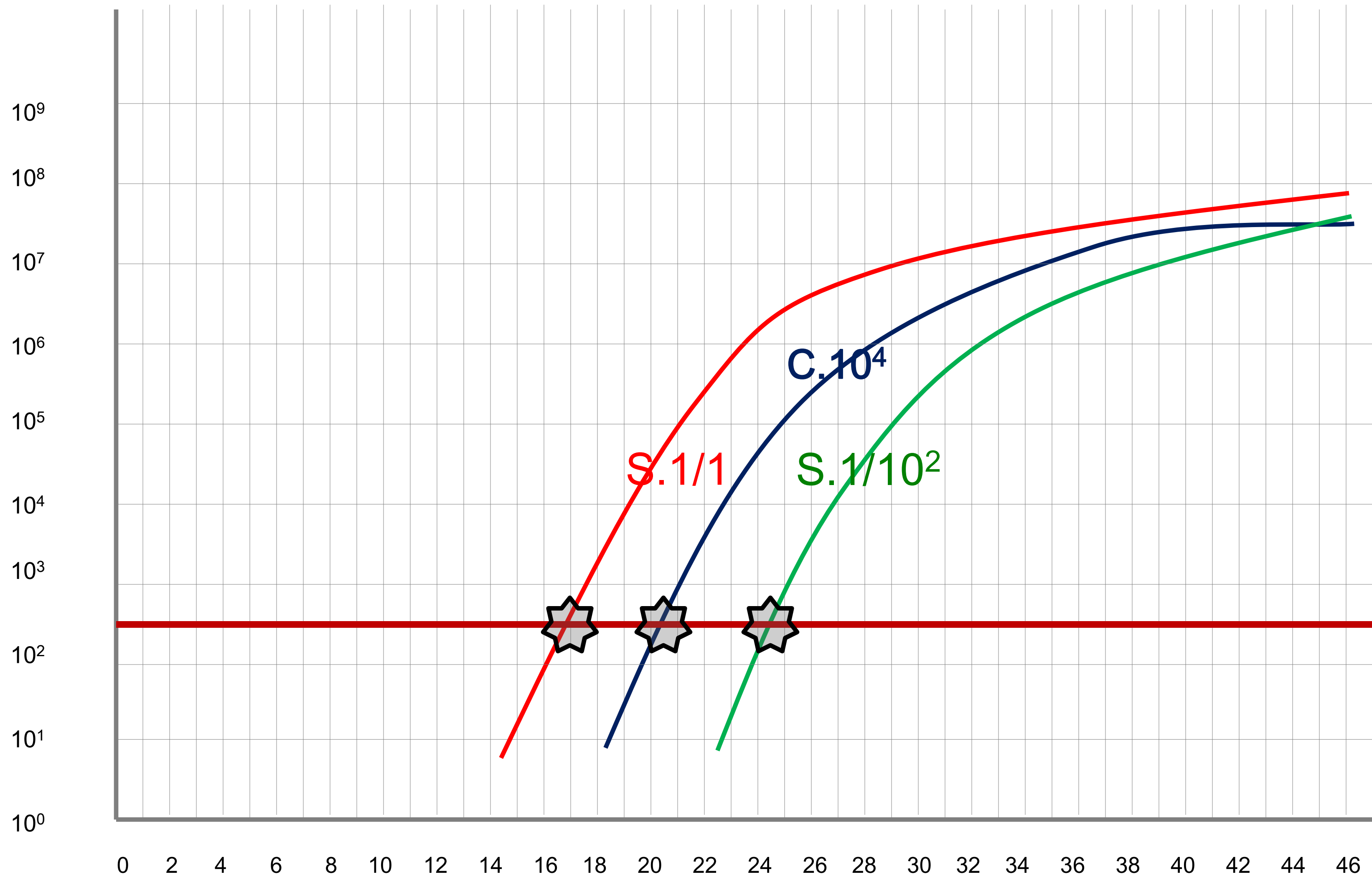


1100
900
700
500
300
200
-100
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
C.10⁴
S.1/1
S.1/10²
Negative DNA
No template

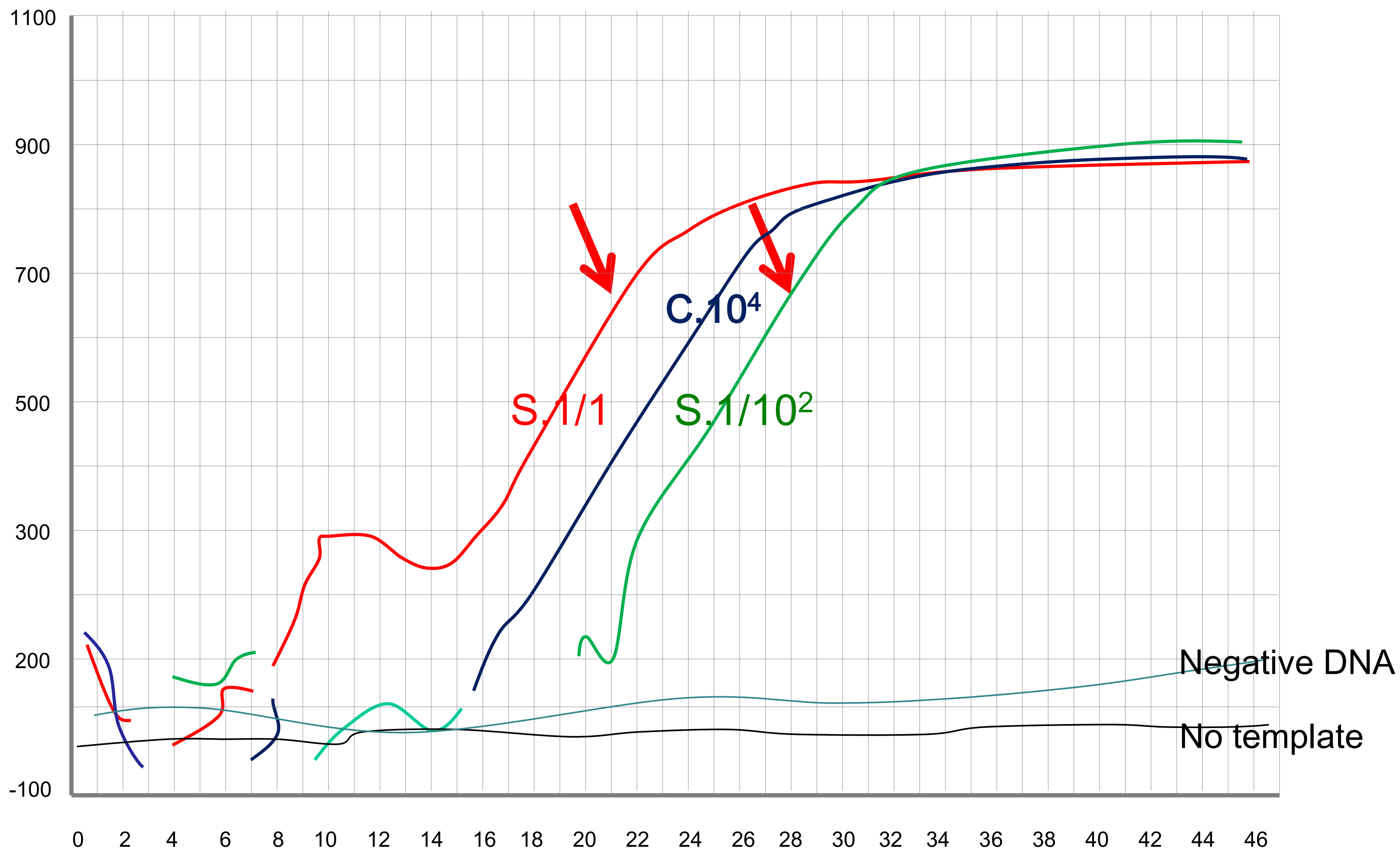
Baseline substraction

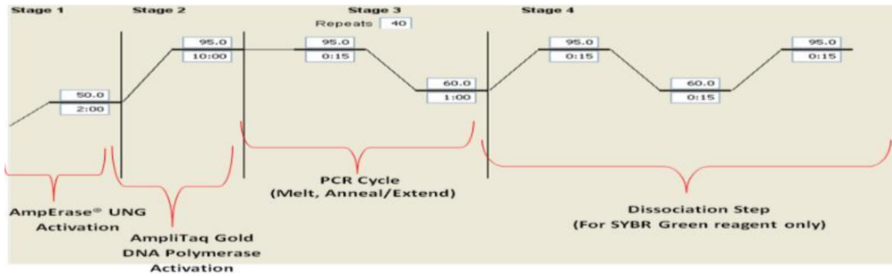


Threshold set



Is that amplicon uniform / specific ?





Melting curve: - ΔF / ΔT

- ΔF

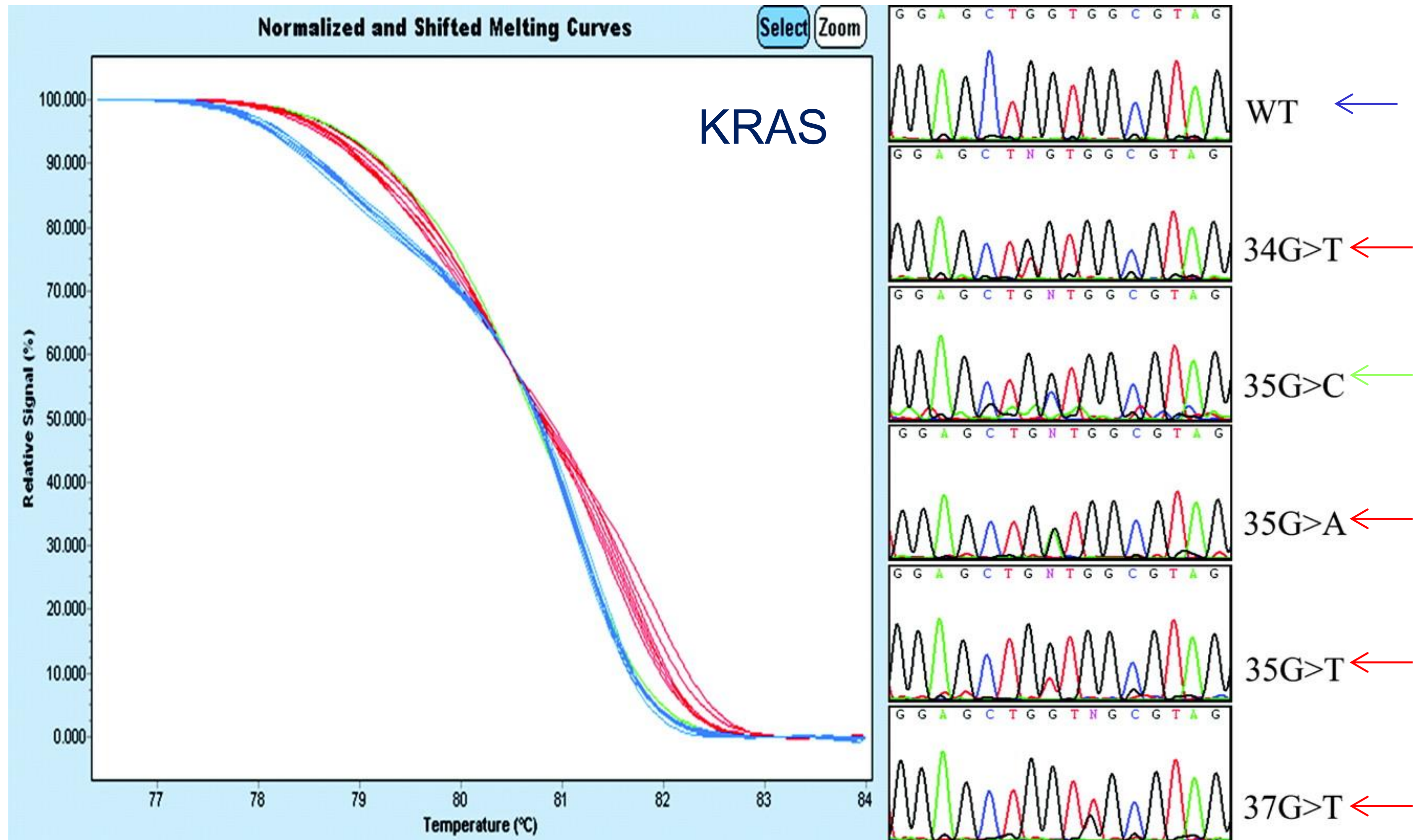
350
300
250
200
150
100
50
0
-50

95 90 85 80 75 70 65 60

ΔT (ºC)

How sensitive and specific the melting curve analysis is to discriminate similar DNA sequences?

- MCA can discriminate wild type allele
- Limitation: Same melting curve, different sequence



J Clin Pathol 2009;62:1096-1102

Quantitative RT-PCR assay types (**qRT-PCR**)

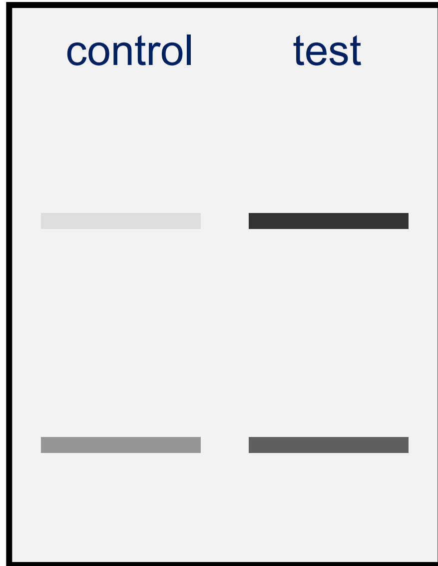
- Gene expression profiling
- Genomic profiling
- Allelic discrimination
- Copy number determination

Quantitation / Comparison

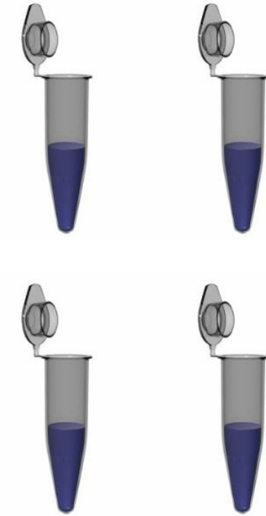
- Northern blot
- Insitu hybridization
- PCR
- RT-PCR

NORTHERN

RT-PCR



target
EGFR



internal control
RPLP0

No amplification

PCR amplification

direct

change

Indirect:
* Amplification factor

Hypothesis : EGFR has an important role in nasal polyp development

Experimental design:

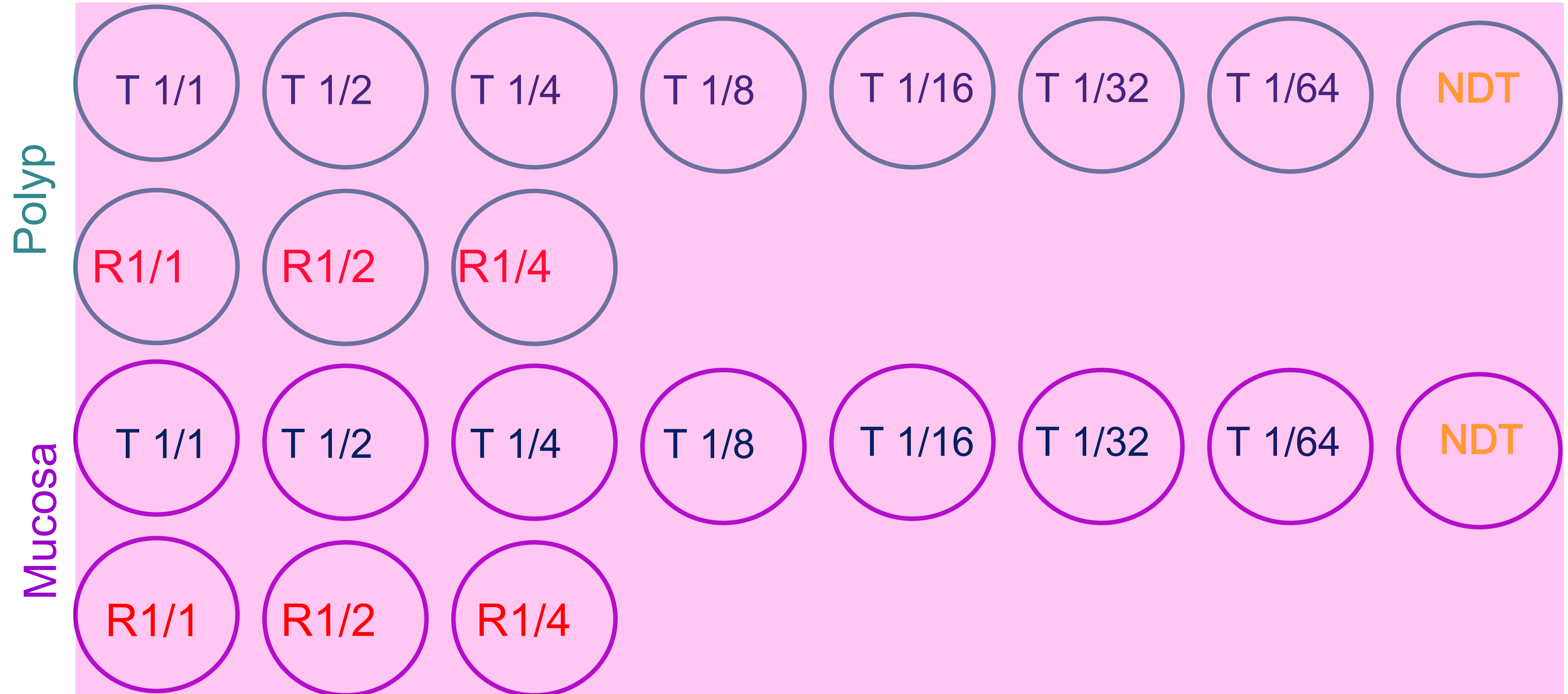
1. Nasal polyp sample
2. Normal mucosal sample

Steps;

1. mRNA extraction *
2. qRT-PCR set up
3. Result

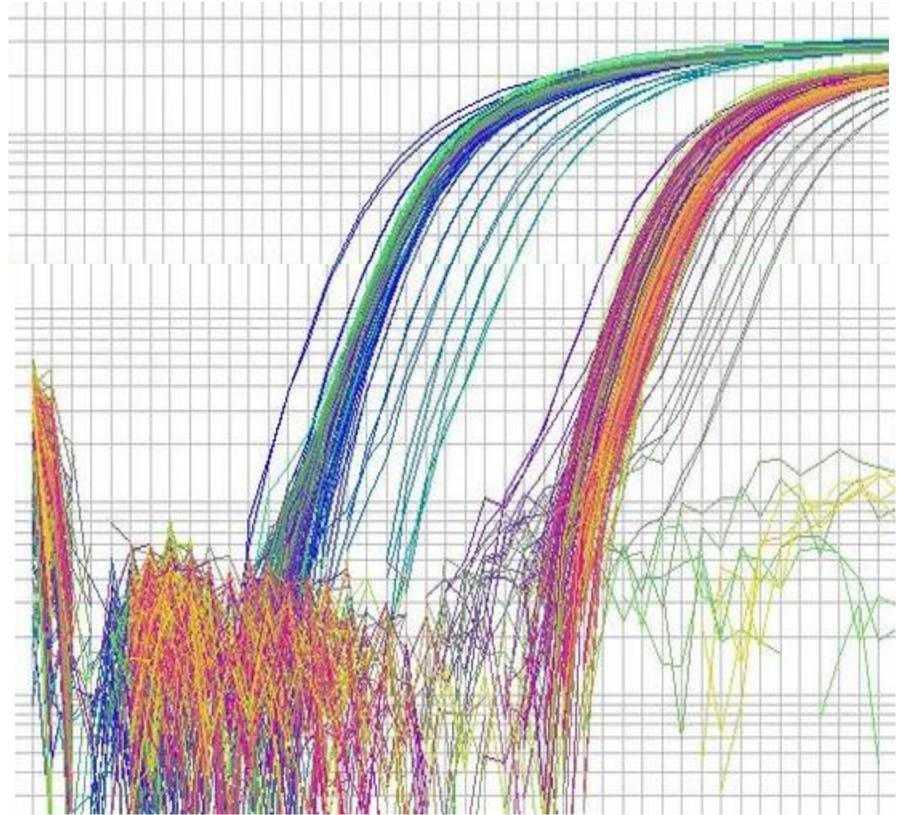
Target gene : EGFR

Reference gene : RPLP0



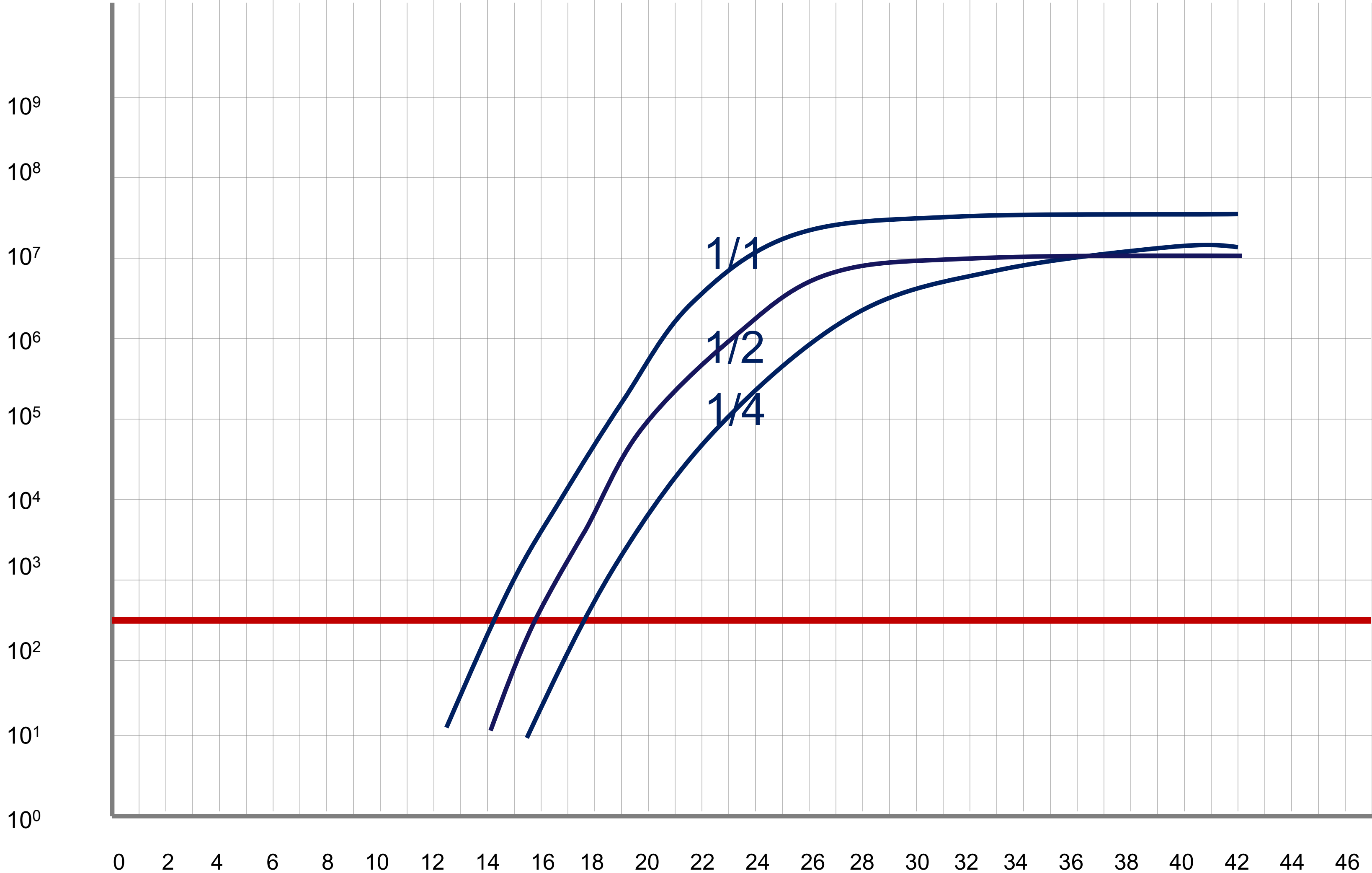
QC Fluorescence Absorbance curves

- Baseline subtraction
- Threshold setting
- Efficiency > 0.99



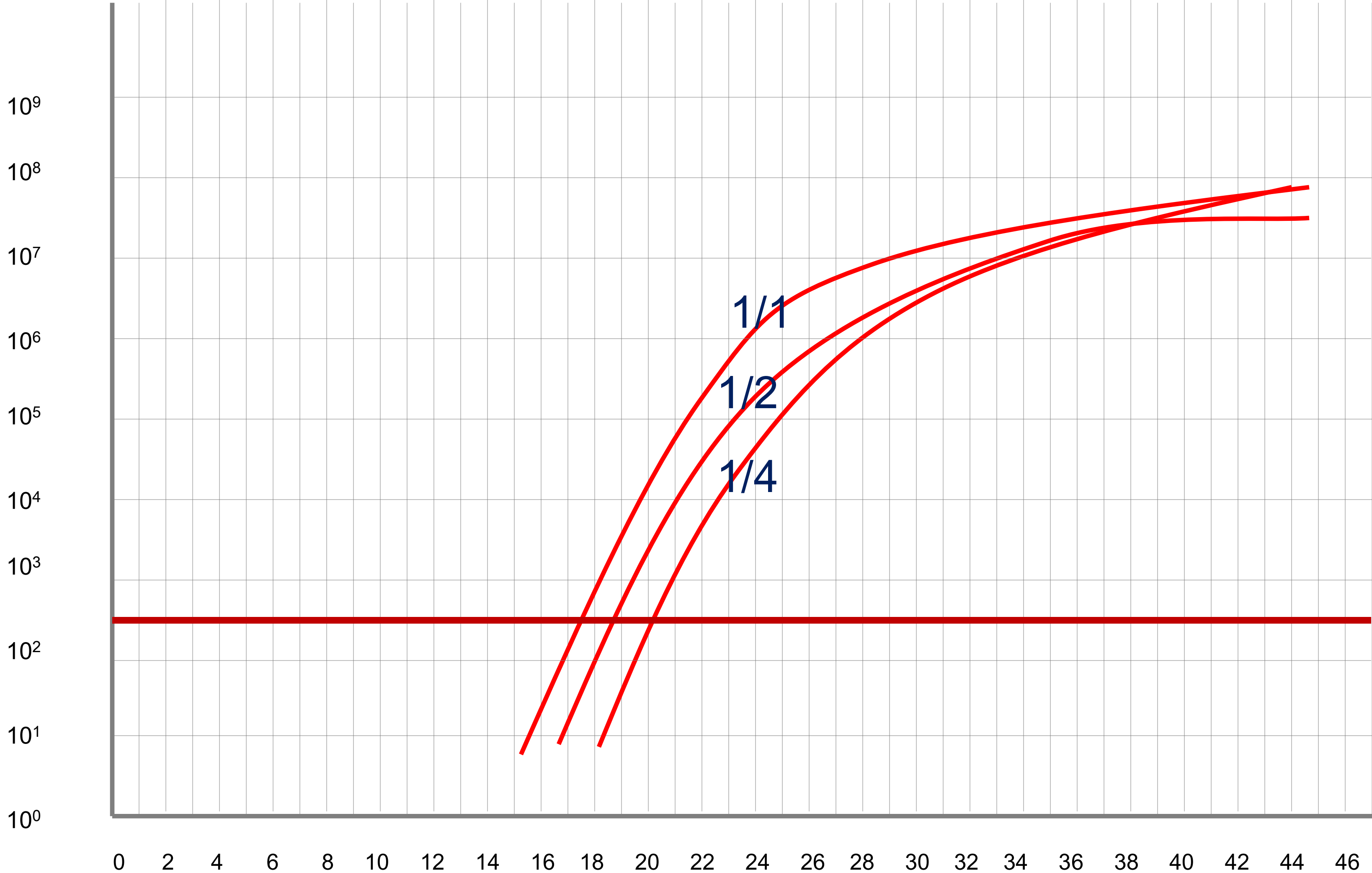
Reference: RPLP0

Polyp normal mucosa



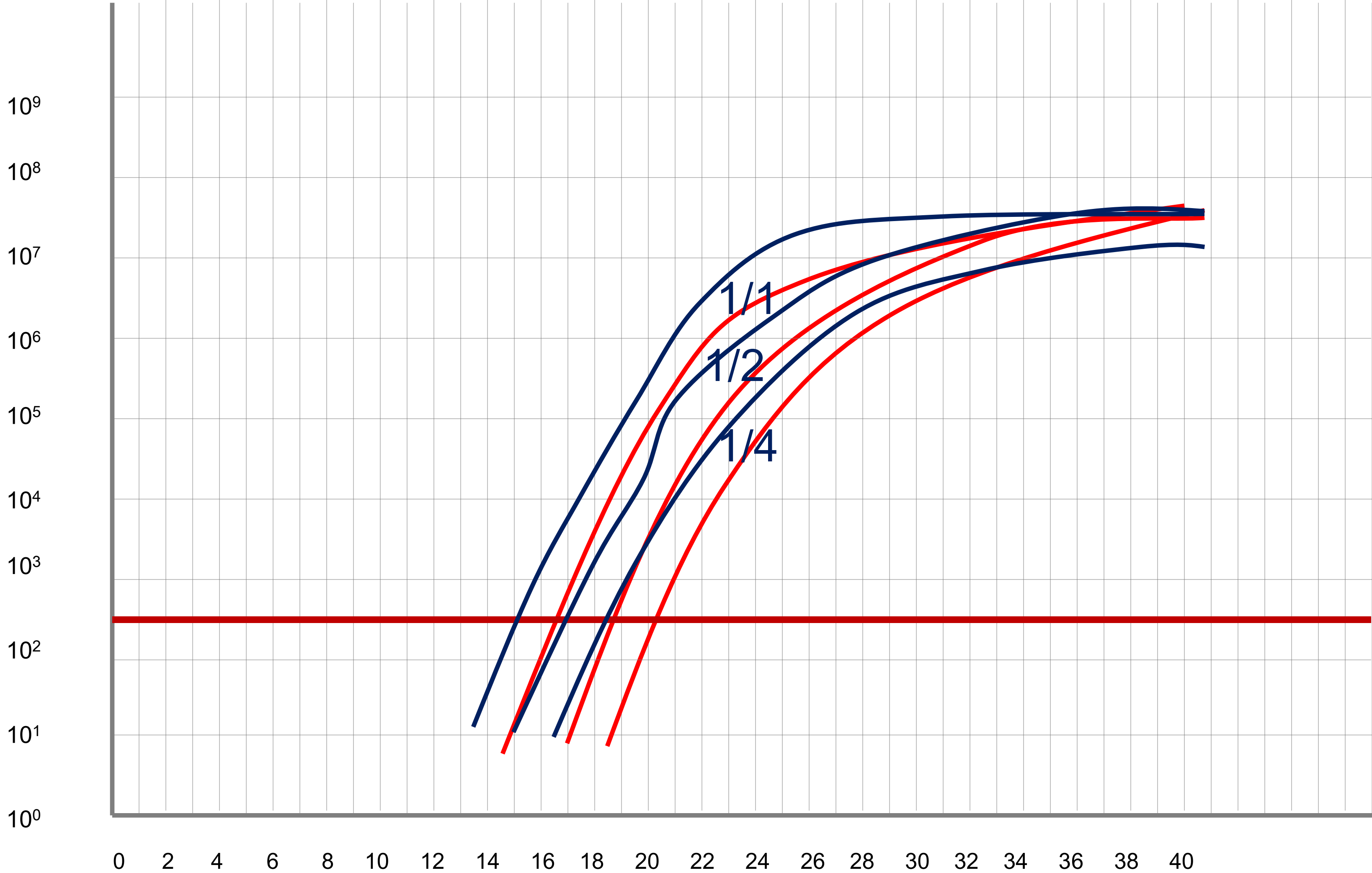
Reference: RPLP0

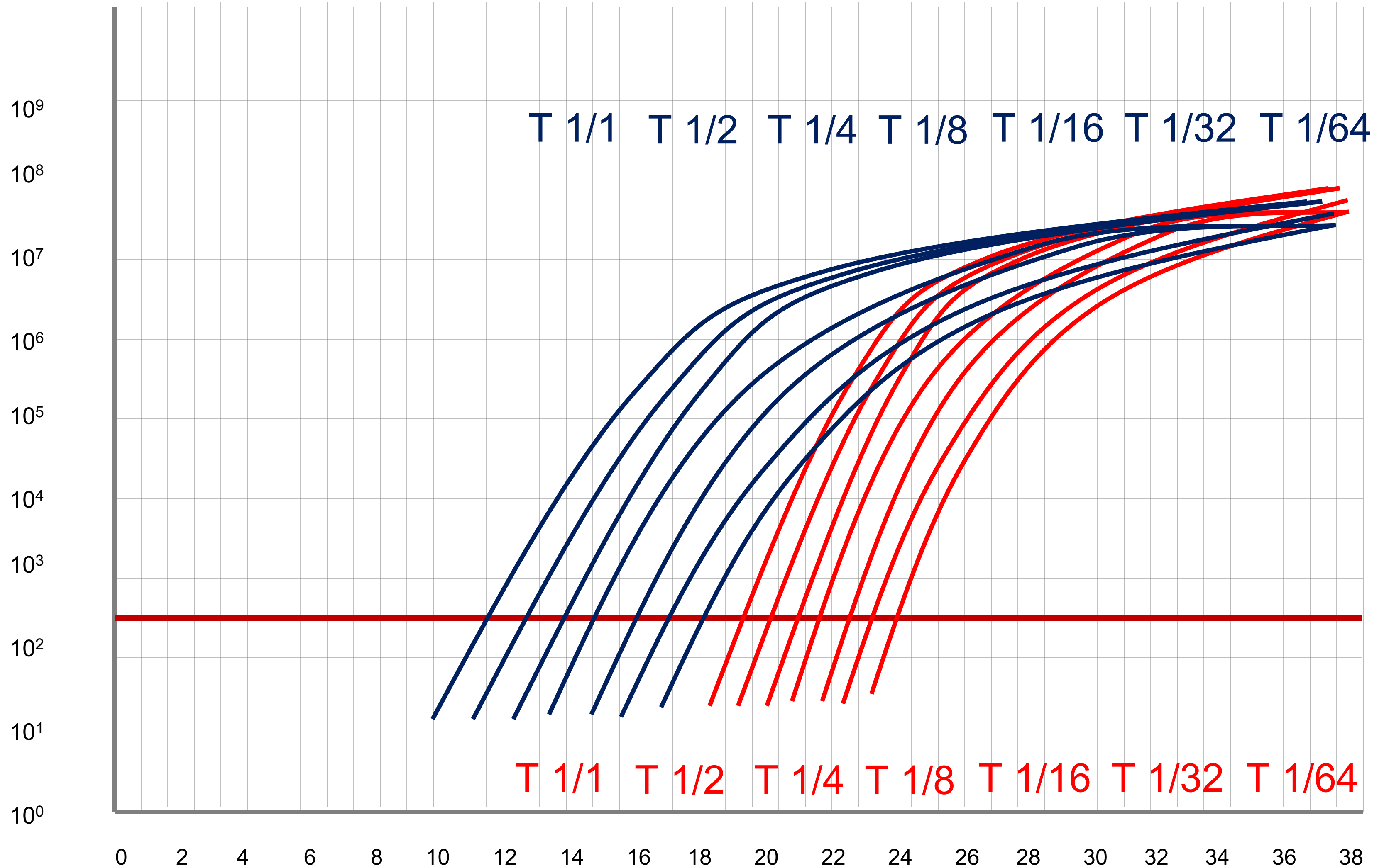
Polyp normal mucosa



Reference: RPLP0

Polyp normal mucosa



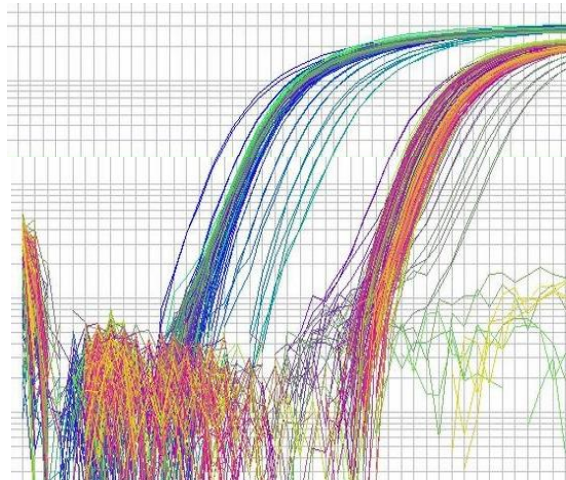


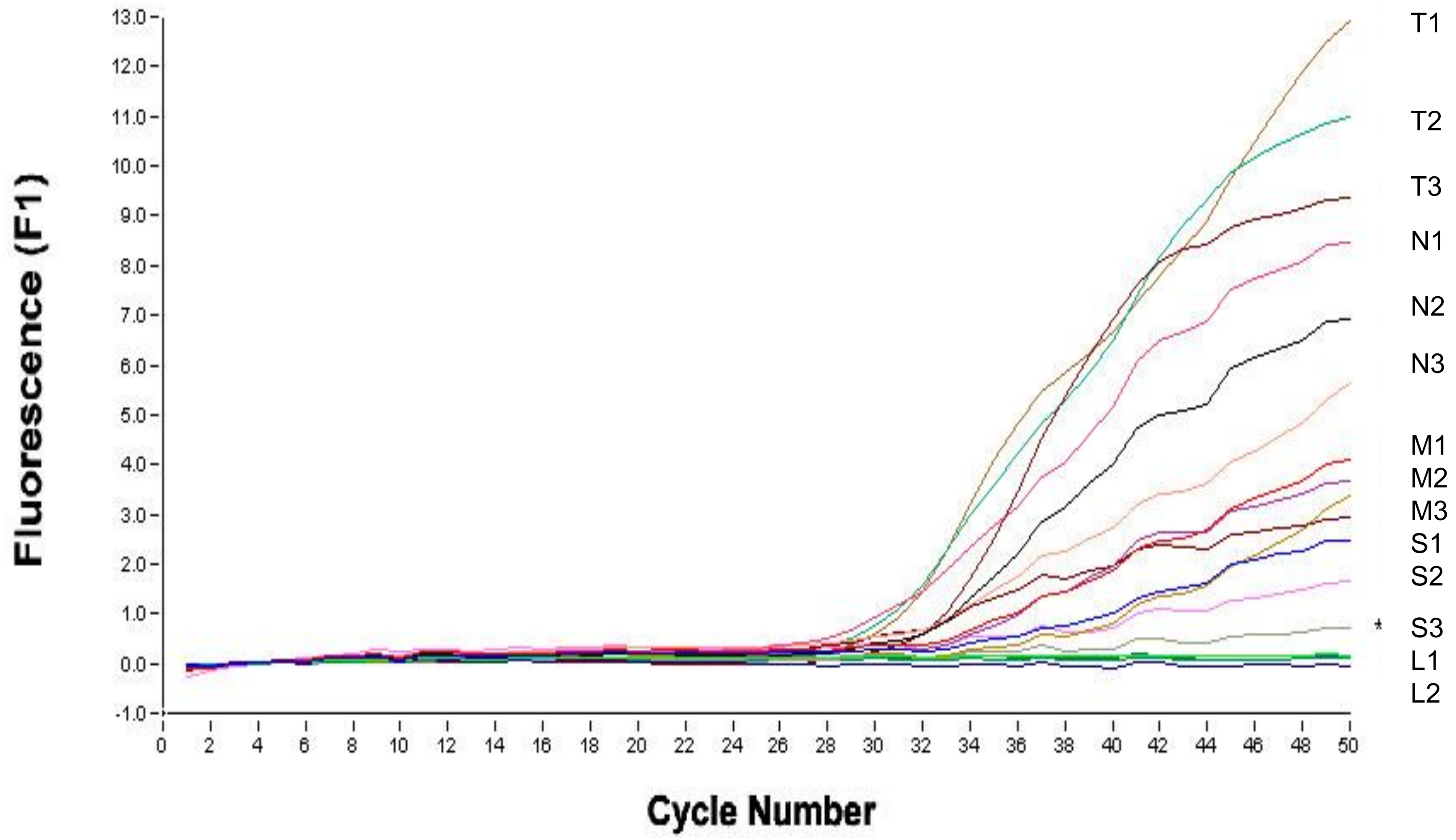
Target: EGFR
Polyp normal mucosa
T 1/1 T 1/2 T 1/4 T 1/8 T 1/16 T 1/32 T 1/64
T 1/1 T 1/2 T 1/4 T 1/8 T 1/16 T 1/32 T 1/64
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38

$$\text{Ratio target gene in experimental/control} = \frac{\text{Corrected flourescence of target gene}}{\text{Corrected flourescence of reference gene}}$$

$$\text{Ratio target gene in experimental/control} = \frac{15.121\ \text{RFU}}{301\ \text{RFU}}$$

Corrected increment = 5 fold




Fluorescence (F1)
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50
Cycle Number
T1
T2
T3
N1
N2
N3
M1
M2
M3
S1
S2
*
S3
L1
L2