بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

Topic

Real-time Polymerase Chain Reaction

By:

Dr. Abbas Moridnia

Ph.D of Molecular Medicine

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

It is an easy, cheap, and reliable way to repeatedly replicate a focused segment of DNA, a concept which is applicable to numerous fields in modern biology and related sciences.

Developed in 1983 by Kary Mullis, PCR is now a common and often indispensable technique used in clinical and research laboratories for a broad variety of applications. These include:

- ✓ DNA cloning for sequencing
- ✓ Gene mutagenesis
- ✓ Functional analysis of genes
- ✓ Amplification of ancient DNA
- ✓ Detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases.
- ✓ Gene cloning and manipulation
- ✓ Construction of DNA-based phylogenies
- ✓ Diagnosis and monitoring of hereditary diseases
- ✓ Analysis of genetic fingerprints for DNA profiling (forensic science and parentage testing)

Polymerase Chain Reaction (PCR)=DNA Photocopier

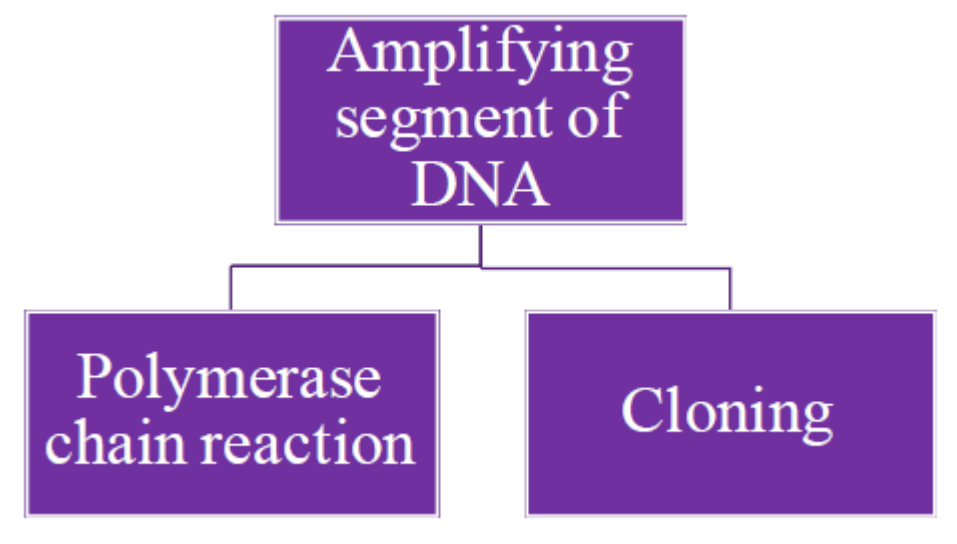
DNA amplification

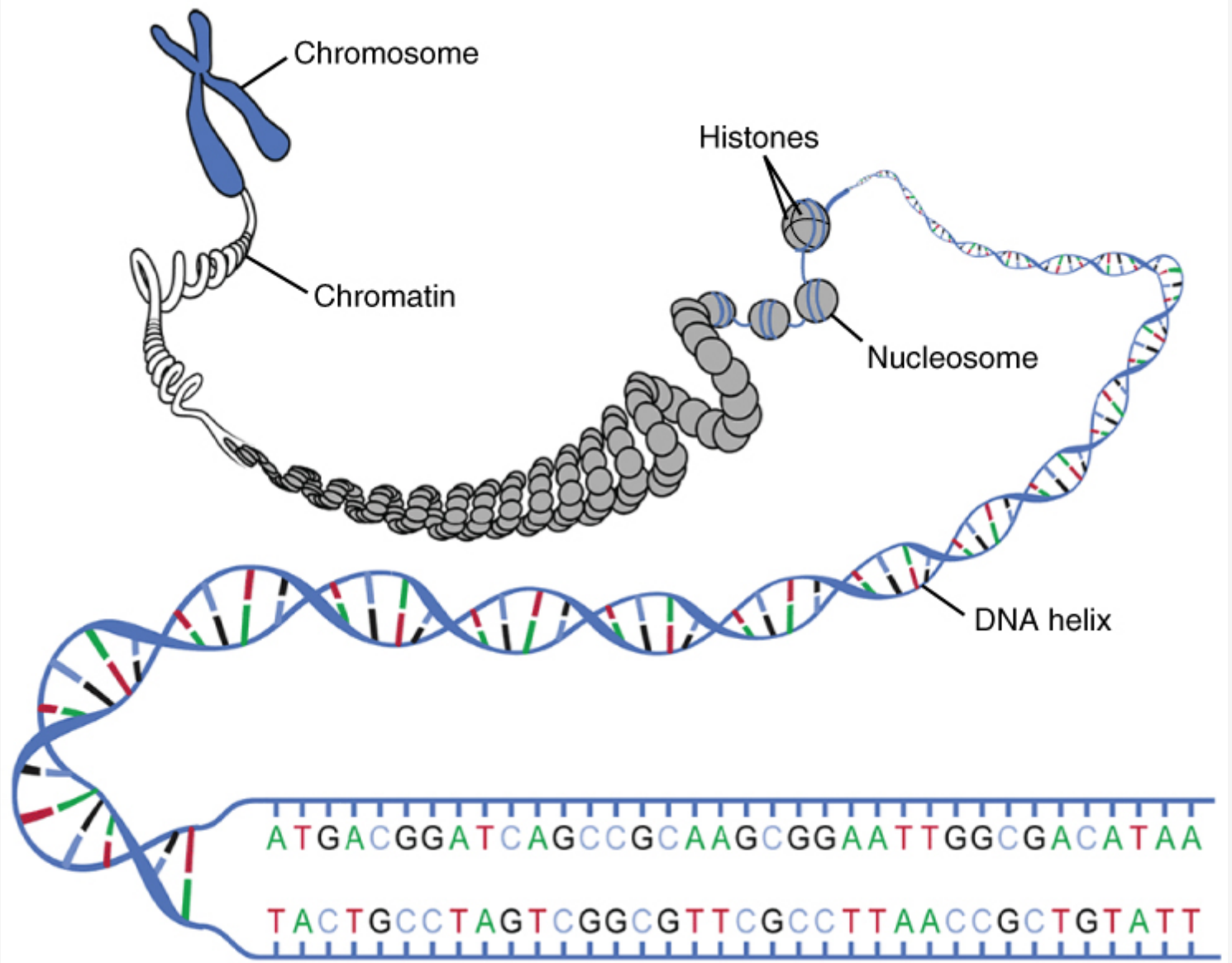
- ✓ In a crime scene, a sample of DNA was found, however **amount of DNA was not enough to** be analyzed.
- ✓ After DNA extraction, the scientist want to study a specific **part of a gene** to do sequencing.

• How scientist solve these problem ?

The solution is to do **amplification of parts of DNA**!!

Mainly there are two methods:




Chromosome
Histones
Chromatin
Nucleosome
DNA helix
ATGACGGATCAGCCGCAAGCGGAATTGGCGACATAA
TACTGCCTAGTCGGCGTTCGCCTTAACCGCTGTATT

Polymerase Chain Reaction (PCR):

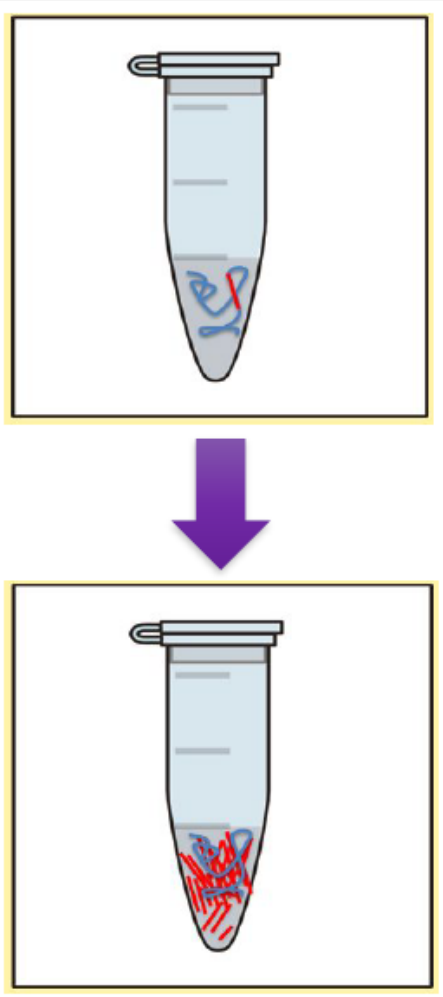
PCR is a means to amplify a particular piece of DNA .

❖ **Amplify=** making numerous copies of a segment of DNA.

• PCR can make billions of copies of a **target sequence of DNA** in short time.

It is a laboratory version of DNA Replication in cells.

❖ The laboratory version is commonly called "in vitro" since it occurs in a test tube while "in vivo" signifies occurring in a living cell.



- So…

✓ How the amplification will be done?

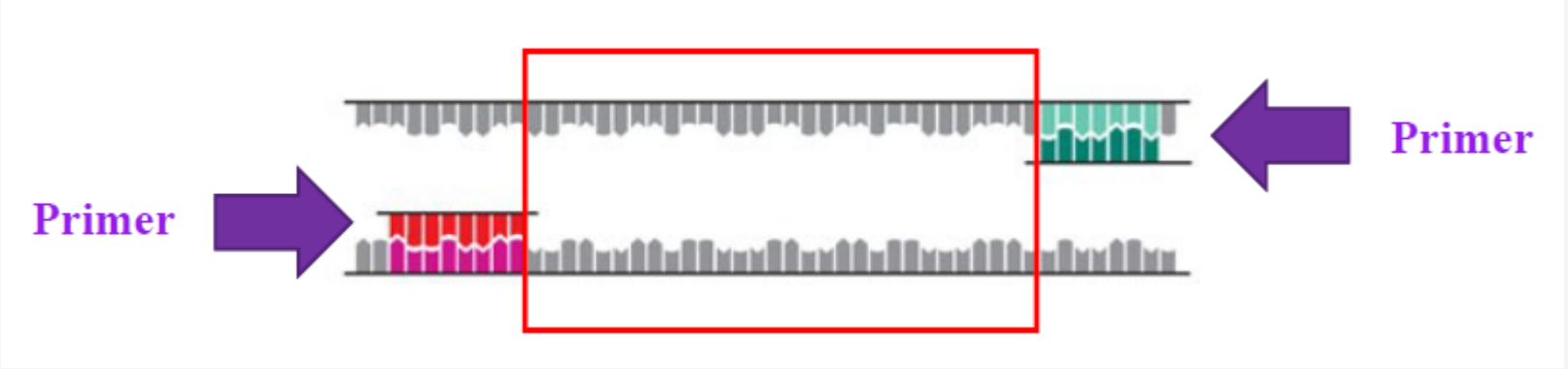
✓ How you will determine your target sequence?

✓ How the amplification will be specific for certain segment?

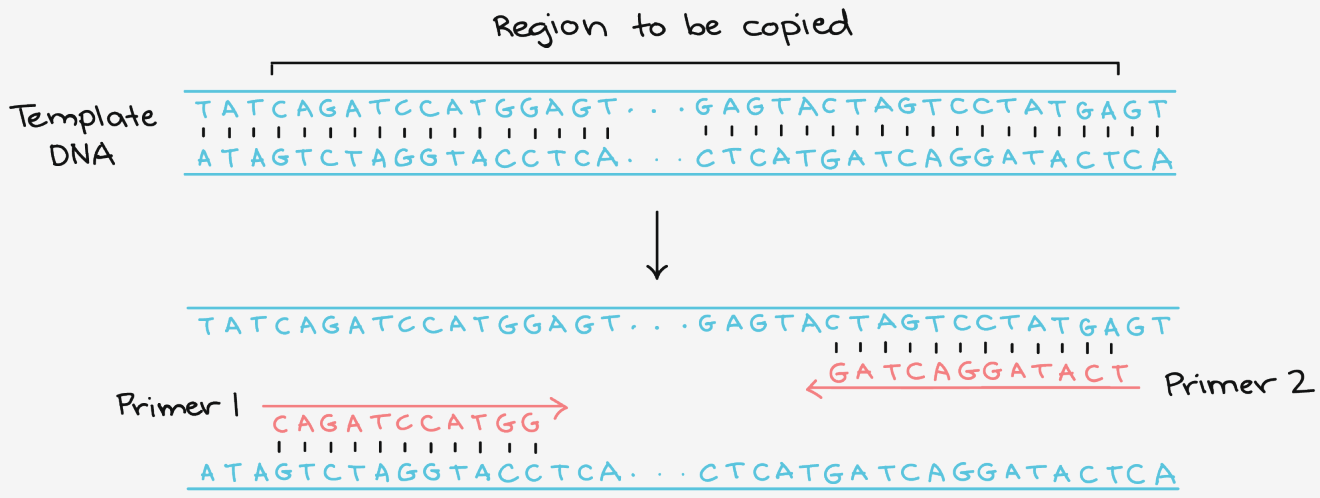
You must to understand these questions

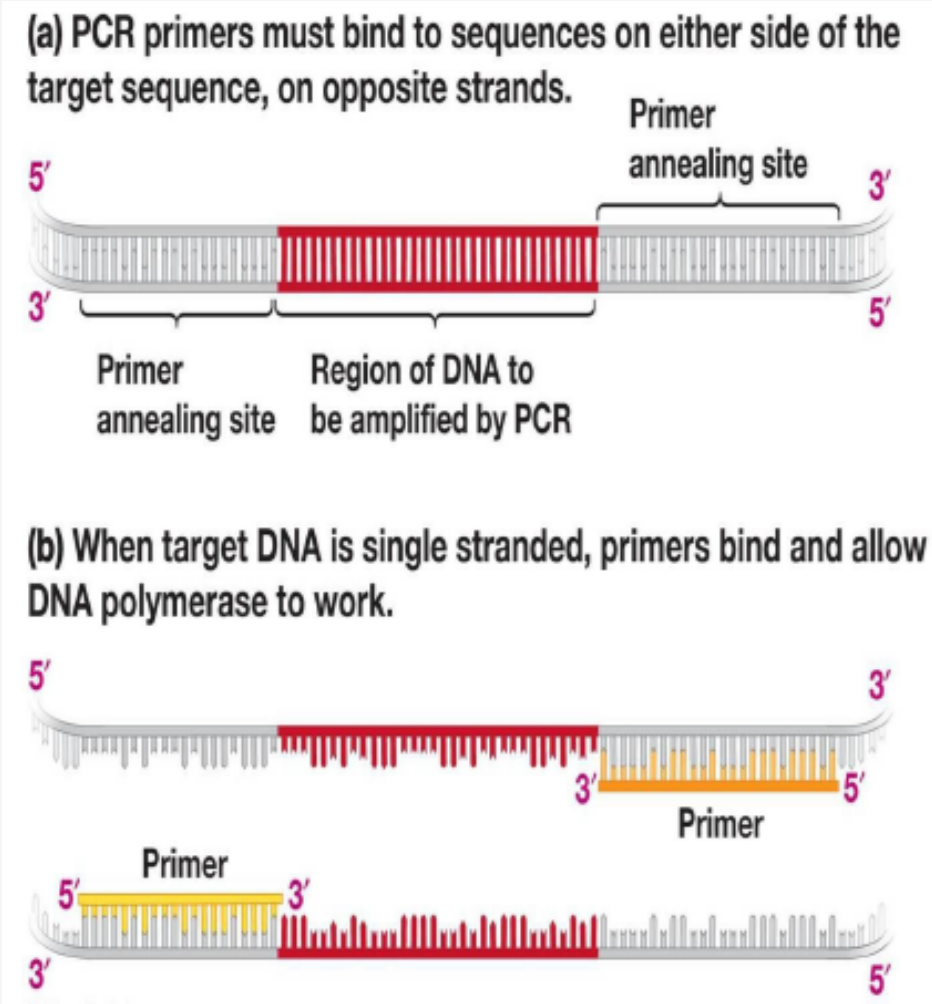
Amplification of a specific target sequence:

- ✓ PCR **does not copy all of the DNA** in the sample. It copies only a very specific sequence of genetic code from a template DNA, targeted by PCR primers.
- ✓ It does require the knowledge of some DNA sequence information which flanks the fragment of DNA to be amplified (**target DNA**).



From this information two synthetic oligonucleotide primers may be chemically synthesized each complementary to a stretch of DNA to the 3' side of the target DNA, one oligonucleotide for each of the two DNA strands (DNA polymerase can add a nucleotide only onto a **preexisting 3'-OH group**).





Principles

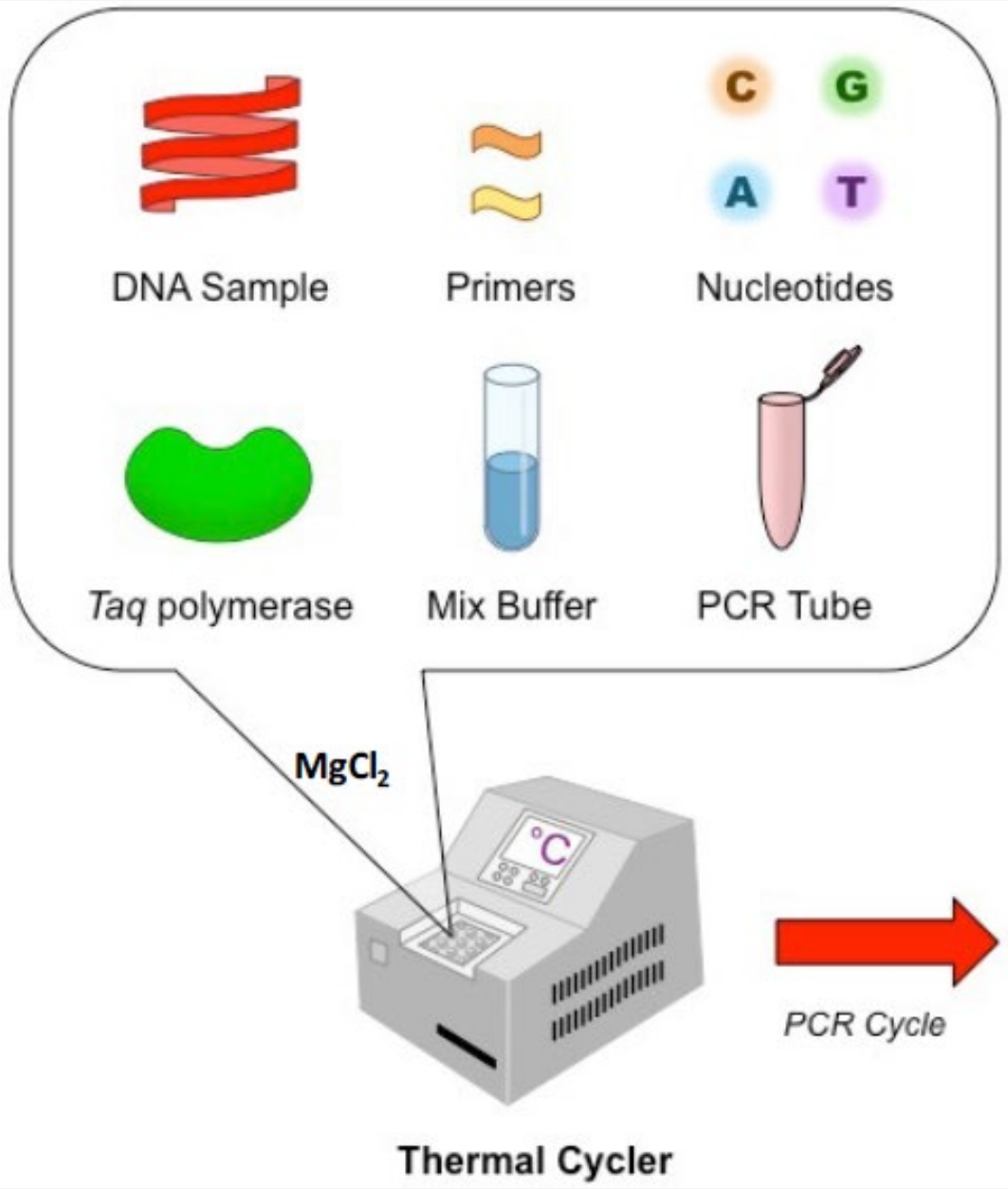
- PCR amplifies a specific region of a DNA strand (the DNA target).

- Most PCR methods amplify DNA fragments of between 0.1 and 10 kbp, although some techniques allow for amplification of fragments up to 40 kbp in size.

- The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses.

Components of PCR

Additional reagents may included



A basic PCR set-up requires several components and reagents, including:

- ✓ A DNA template that contains the DNA target region to amplify.
- ✓ A DNA polymerase, an enzyme that polymerizes new DNA strands; heat-resistant Taq polymerase is especially common.

- ✓ Two DNA primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strands of the DNA target (DNA polymerase can only bind to and elongate from a double-stranded region of DNA; without primers there is no double-stranded initiation site at which the polymerase can bind).

- ✓ Deoxynucleoside triphosphates, or dNTPs, the building blocks from which the DNA polymerase synthesizes a new DNA strand.

- ✓ A buffer solution providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

- ✓ Bivalent cations, typically magnesium (Mg)

Enzyme

✓Usually Taq Polymerase or anyone of the natural or Recombinant thermostable polymerases

✓Stable at T^0 up to 95^0 C

✓High processivity

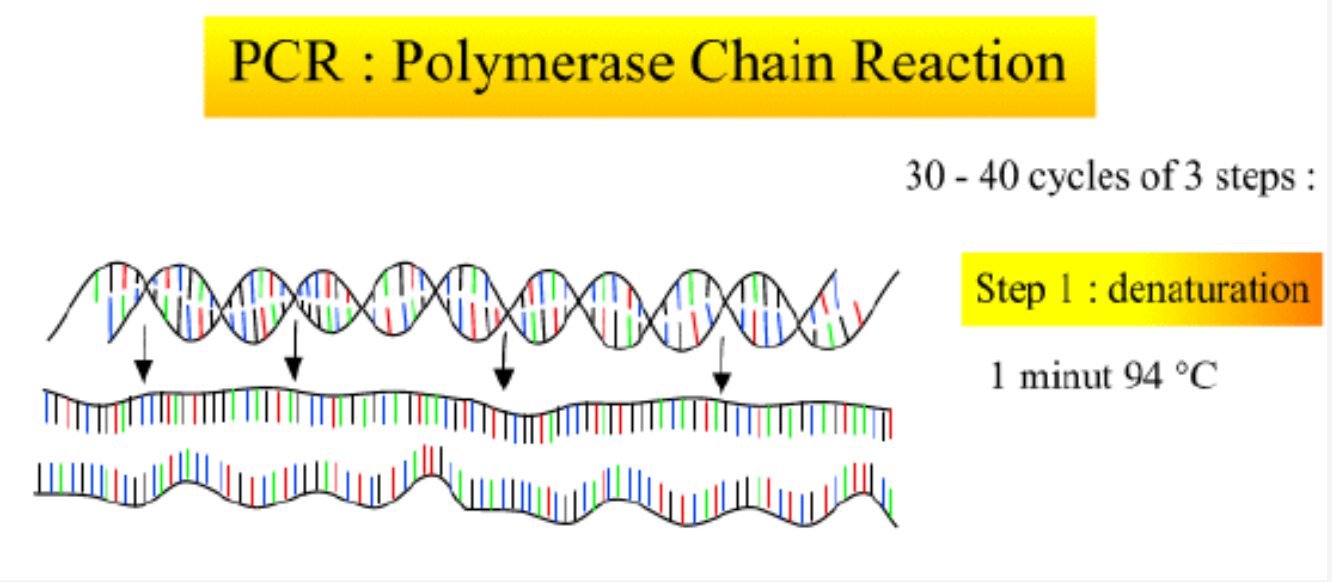
✓Taq Pol has 5'-3' exo only, no proofreading

The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermal cyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

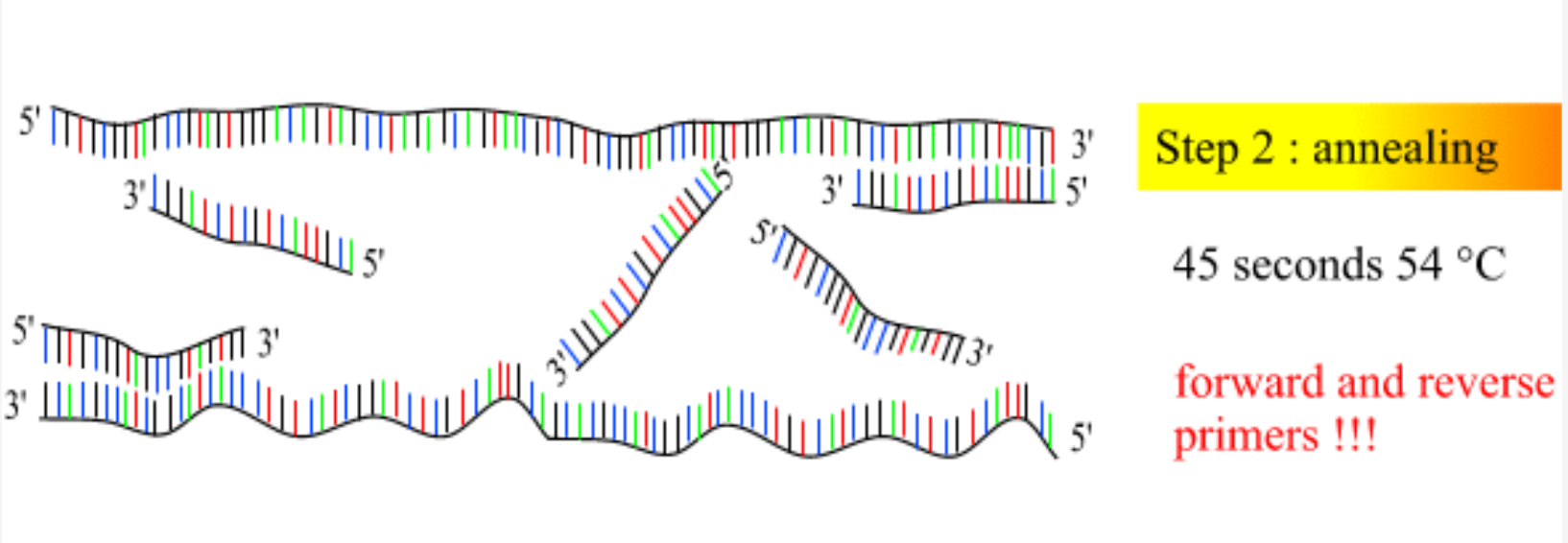
Steps of PCR methods:

- ✓ **Initial Denaturation**

- ✓ **Denaturation**: This step is the first regular cycling event and consists of heating the reaction chamber to 94–95 °C for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.

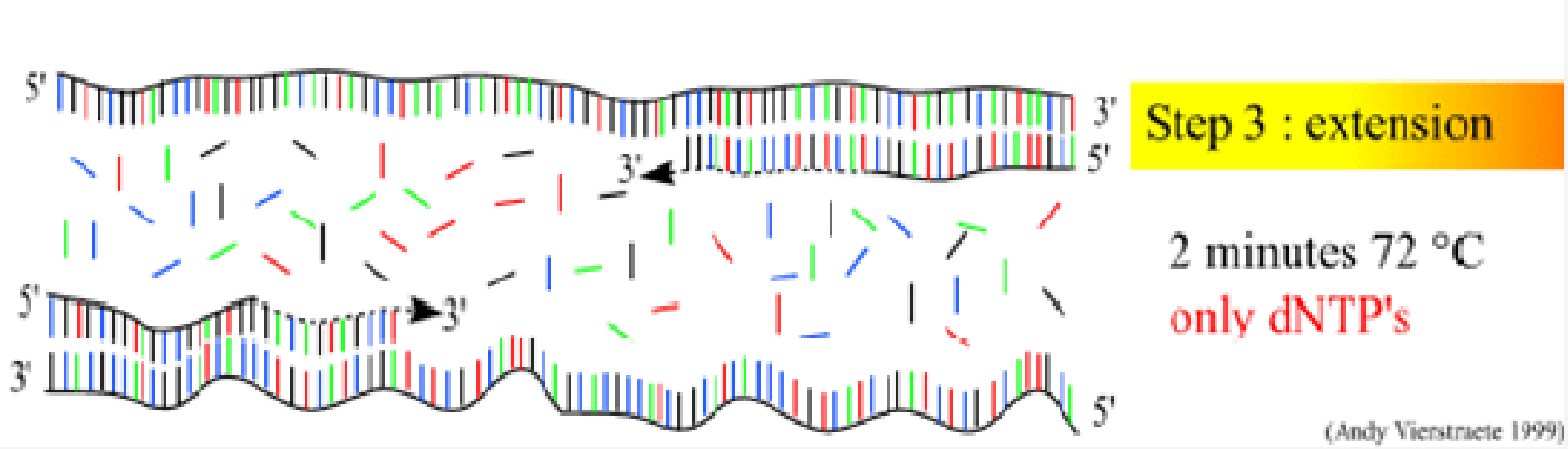


✓ **Annealing**: In the next step, the reaction temperature is lowered to 50–65 °C for 20–40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates.



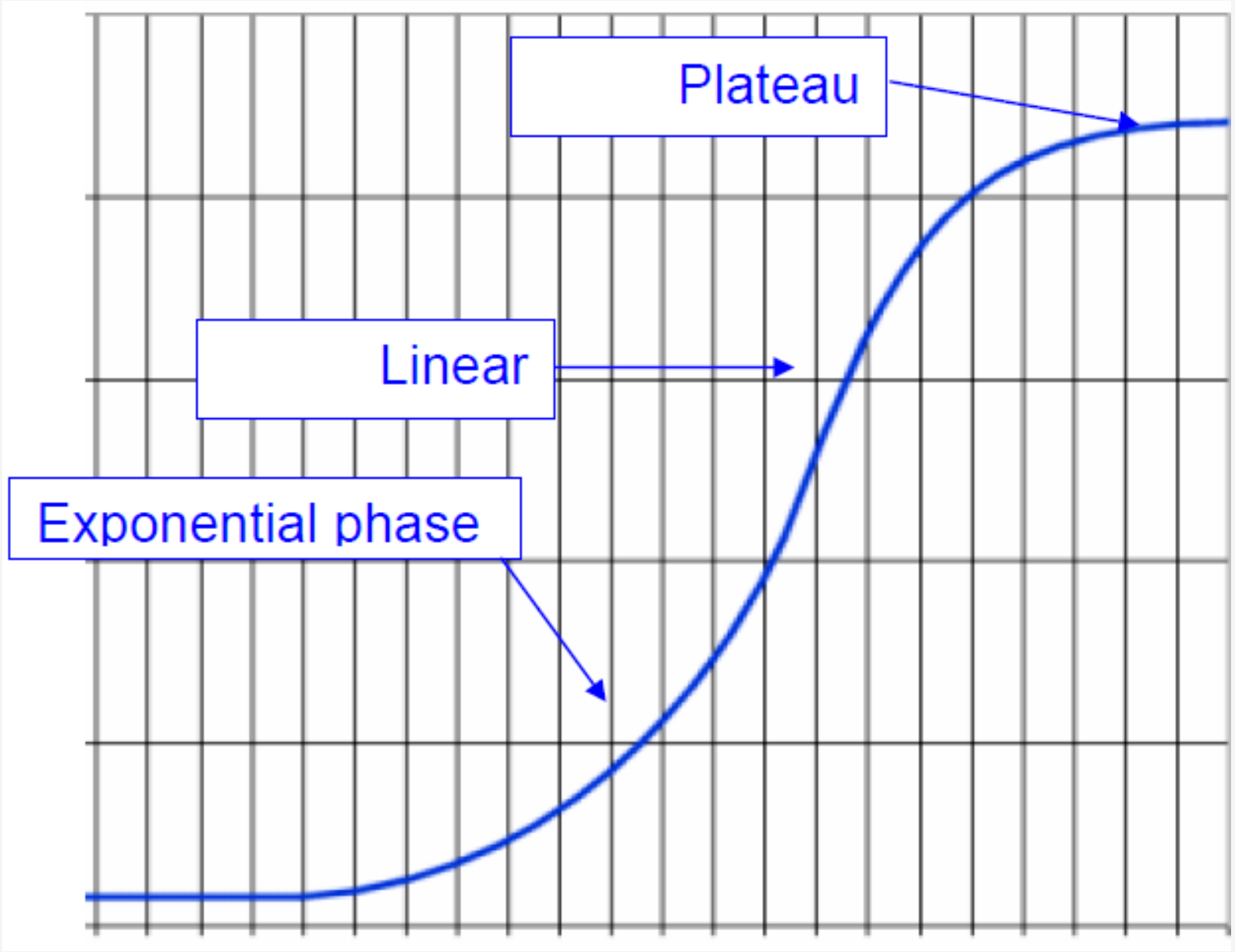
This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should bind only to a perfectly complementary part of the strand, and nowhere else. If the temperature is too low, the primer may bind imperfectly. If it is too high, the primer may not bind at all. A typical annealing temperature is about 3–5 °C below the Tm of the primers used.

✓ **Extension/elongation**: The temperature at this step depends on the DNA polymerase used; the optimum activity temperature for Taq polymerase is approximately 72 °C. In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxy group at the end of the nascent (elongating) DNA strand.

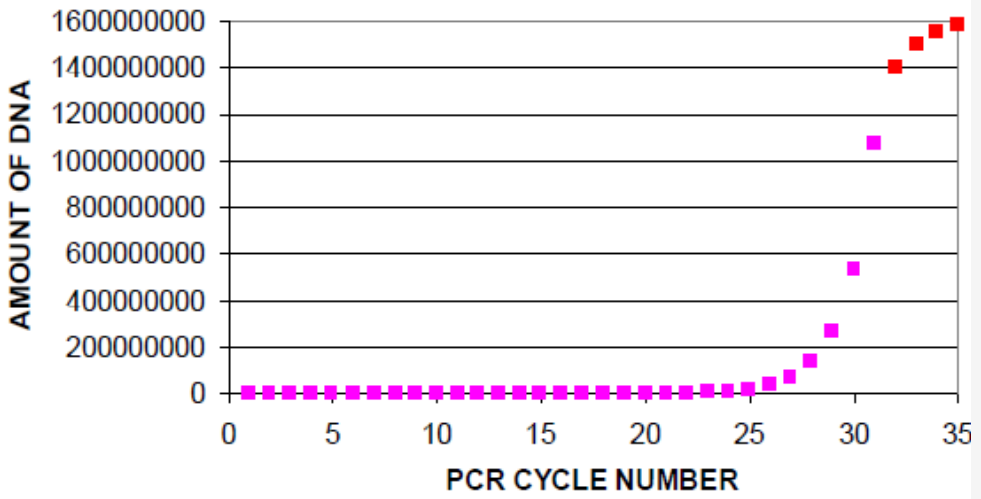


The precise time required for elongation depends both on the DNA polymerase used and on the length of the DNA target region to amplify. As a rule, at their optimal temperature, most DNA polymerases polymerize a thousand bases per minute.

The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies. The formula used to calculate the number of DNA copies formed after a given number of cycles is 2^n, where n is the number of cycles. Thus, a reaction set for 30 cycles results in 2^{30}, or 1073741824, copies of the original double-stranded DNA target region.

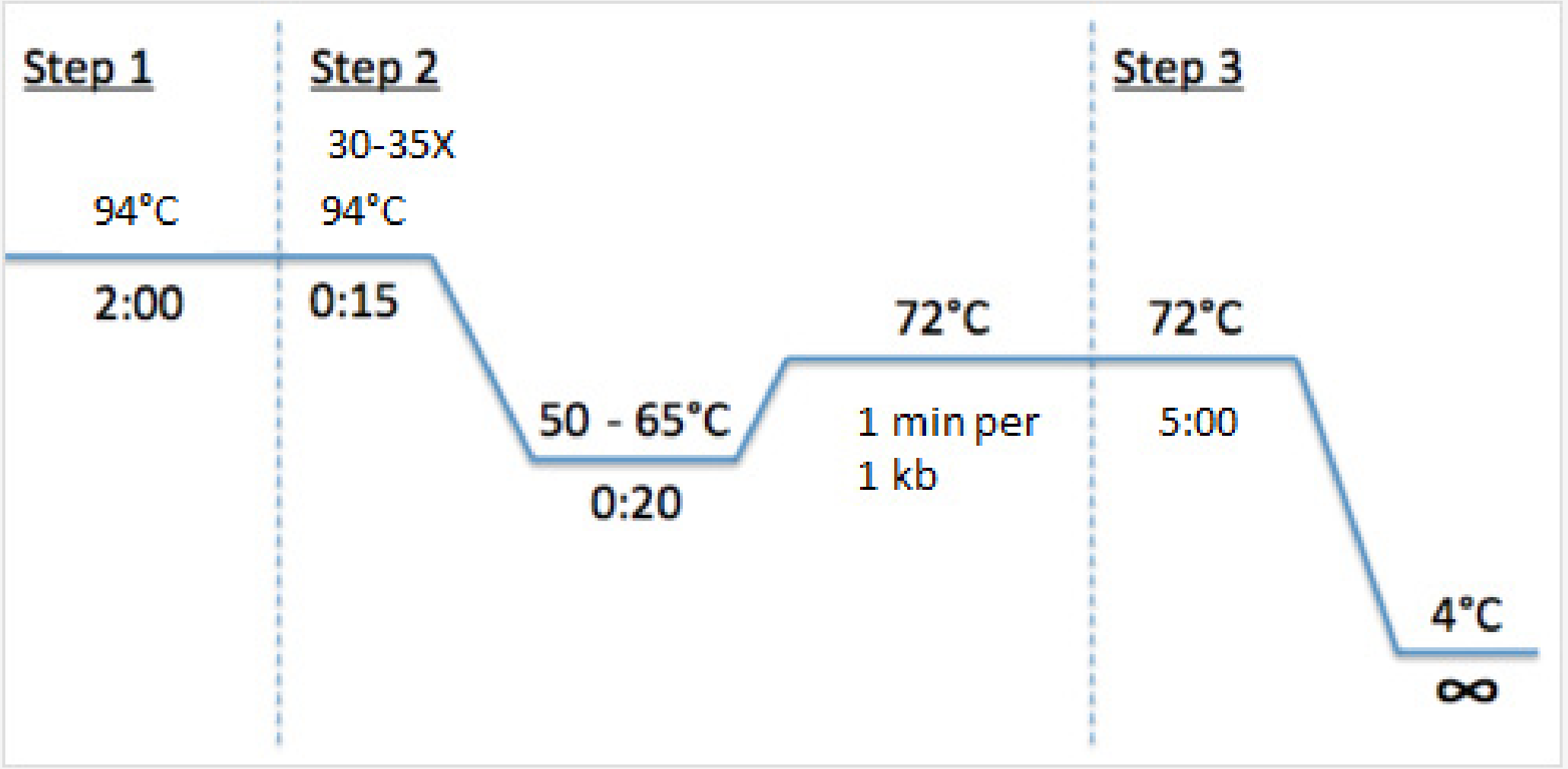

Plateau
Linear
Exponential phase

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



- ✓ **Final elongation**: This single step is optional, but is performed at a temperature of 72 °C for 5 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.

- ✓ **Final hold**: The final step cools the reaction chamber to 4–15 °C for an indefinite time, and may be employed for short-term storage of the PCR products.

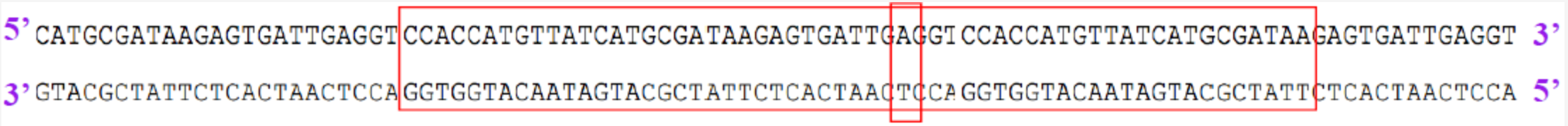

Step 1
Step 2
30-35X
Step 3
94°C
94°C
2:00
0:15
50 - 65°C
0:20
72°C
1 min per
1 kb
72°C
5:00
4°C
∞

Example:

You want to study a mutation in a DLG3 gene and how it relate to memory:

1. Find the sequence of the gene from any website, eg. Ensebmle, NCBI, ...
2. Determine your **target region**.

The segment that you want to amplified is in the red square

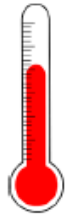


3. Design the primers using primer design tool, eg.Primer3, then send them to any company who will synthesize them.
4. Make sure that the area that you want to study is **between the primers (**the region to be studied should be between the forward and reverse primer**)**.
5. Check primer specificity by BLAST.
6. Optimize your PCR and trouble shooting.
7. Start PCR.

Start your PCR

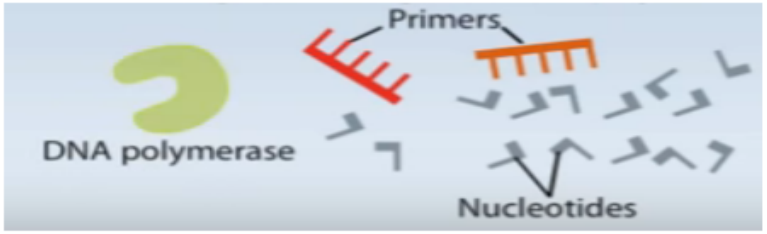
1. Denaturation:

95 °C



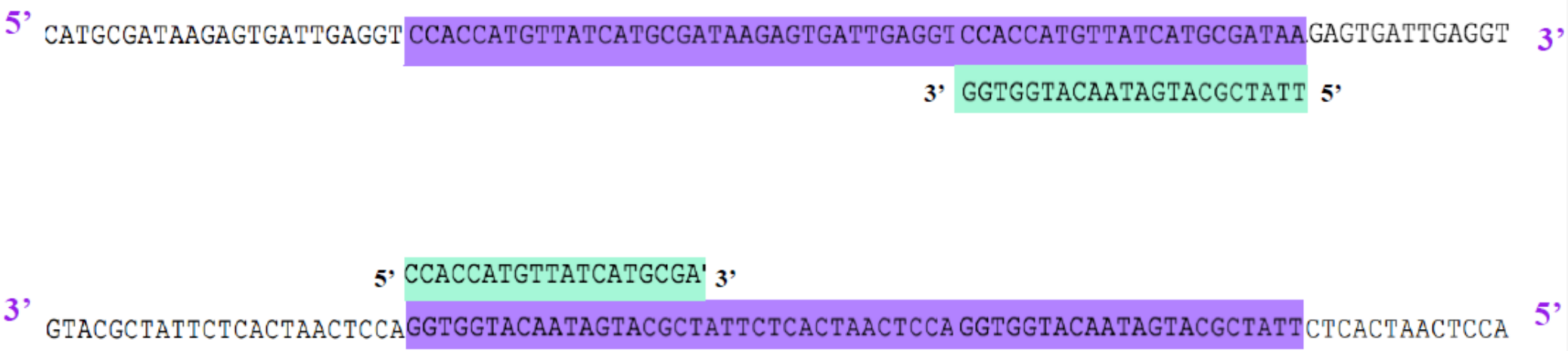
5’ CATGCGATAAGAGTGATTGAGGT CCACCATGTTATCATGCGATAAGAGTGATTGAGGT CCACCATGTTATCATGCGATAA GAGTGATTGAGGT 3’

3’ GTACGCTATTCTCACTAACTCCA GGTGGTACAATAGTACGCTATTCTCACTAACTCCA GGTGGTACAATAGTACGCTATT CTCACTAACTCCA 5’



2. Annealing:

58 °C

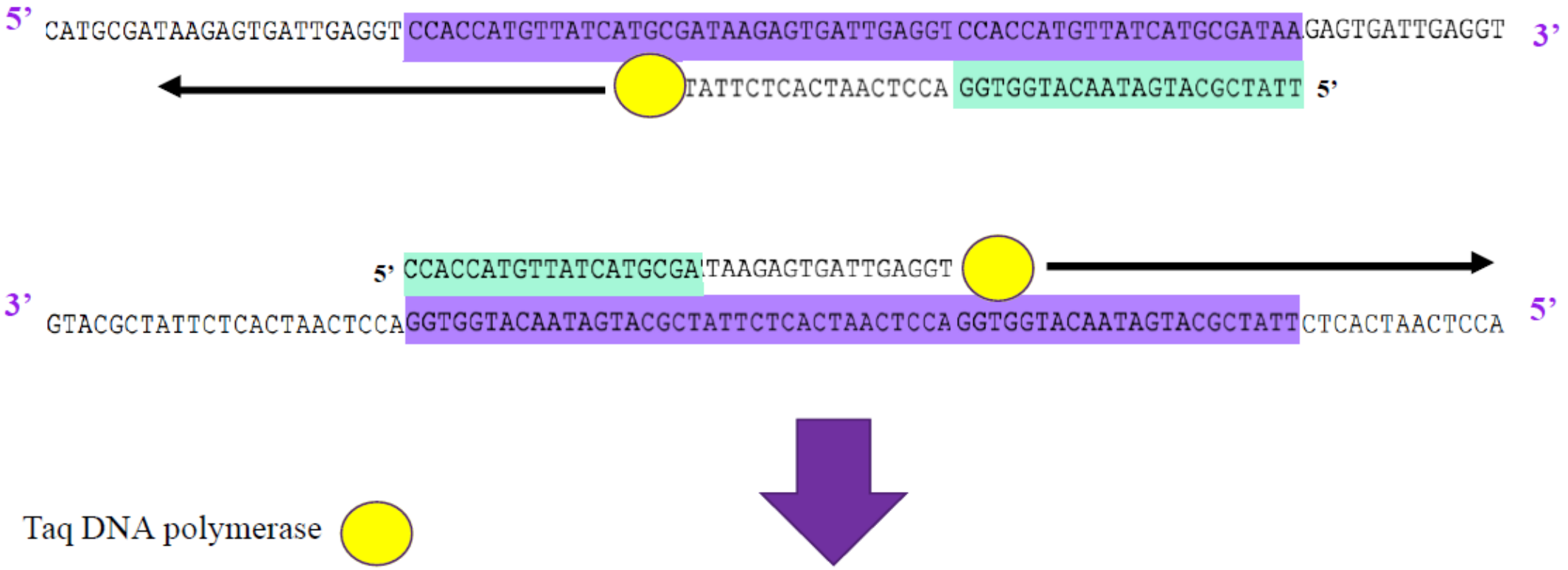


Forward primer: 5’ CCACCATGTTATCATGCGA 3’

Reverse primer: 3’ GGTGGTACAATAGTACGCTATT 5’

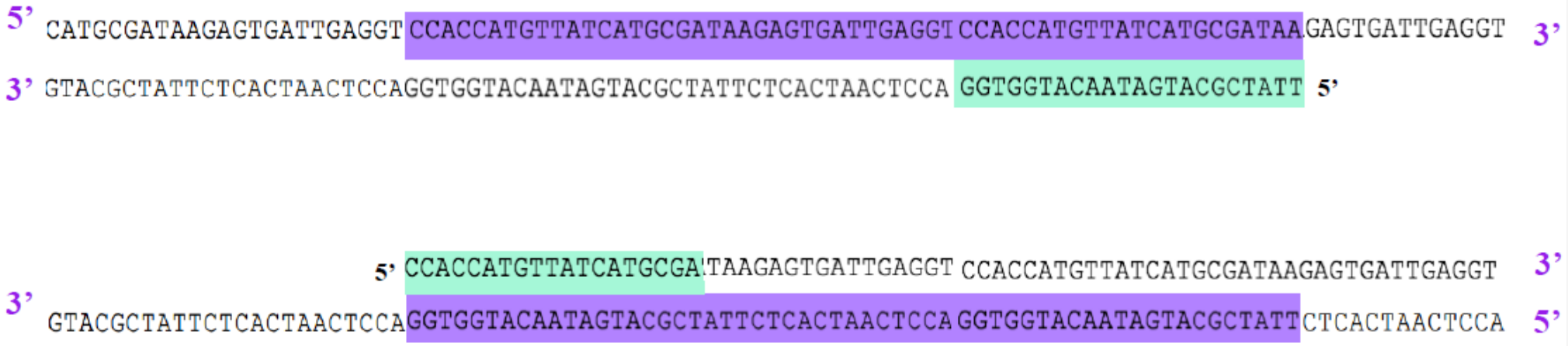
3. Extension:

72 °C

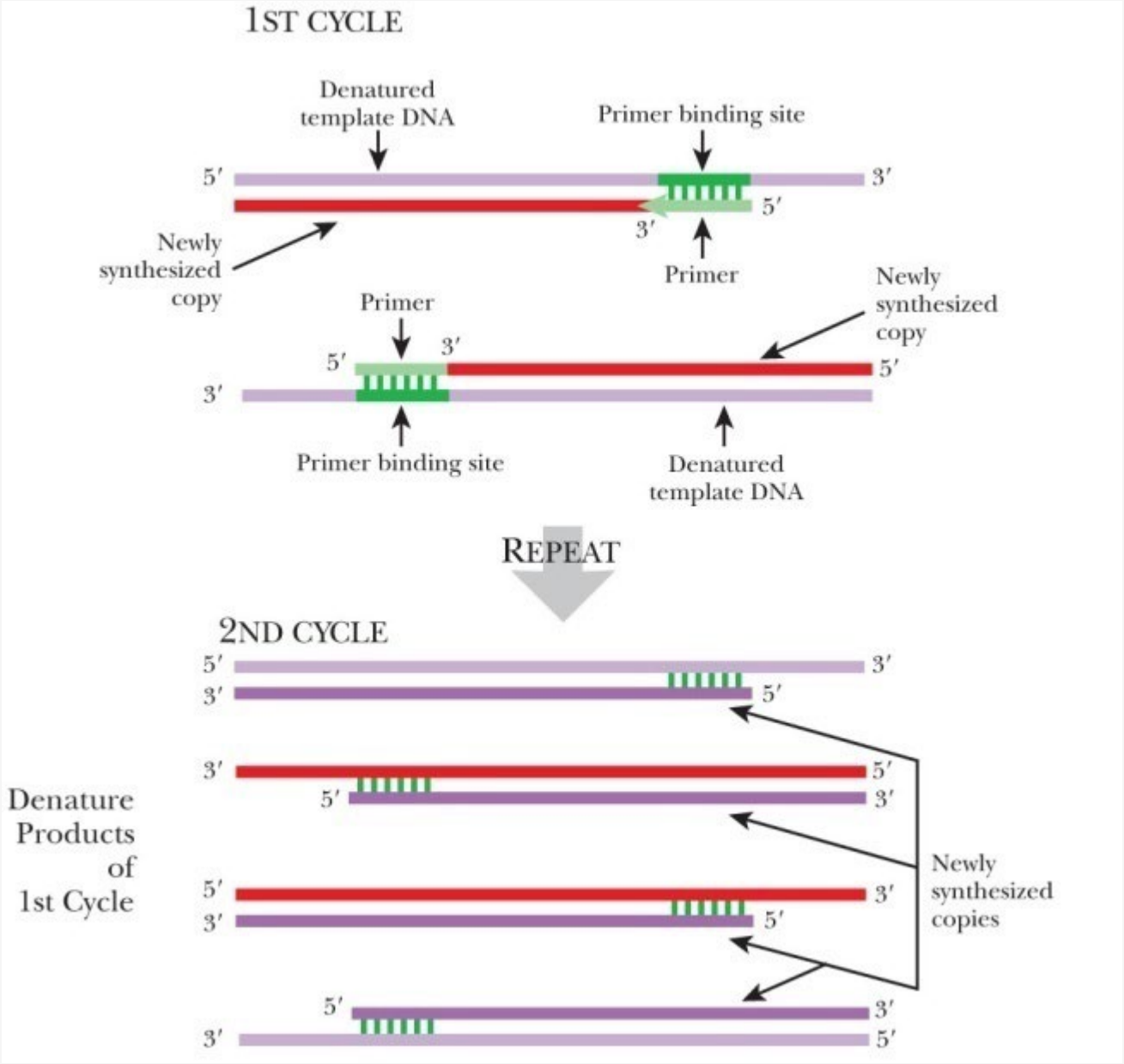


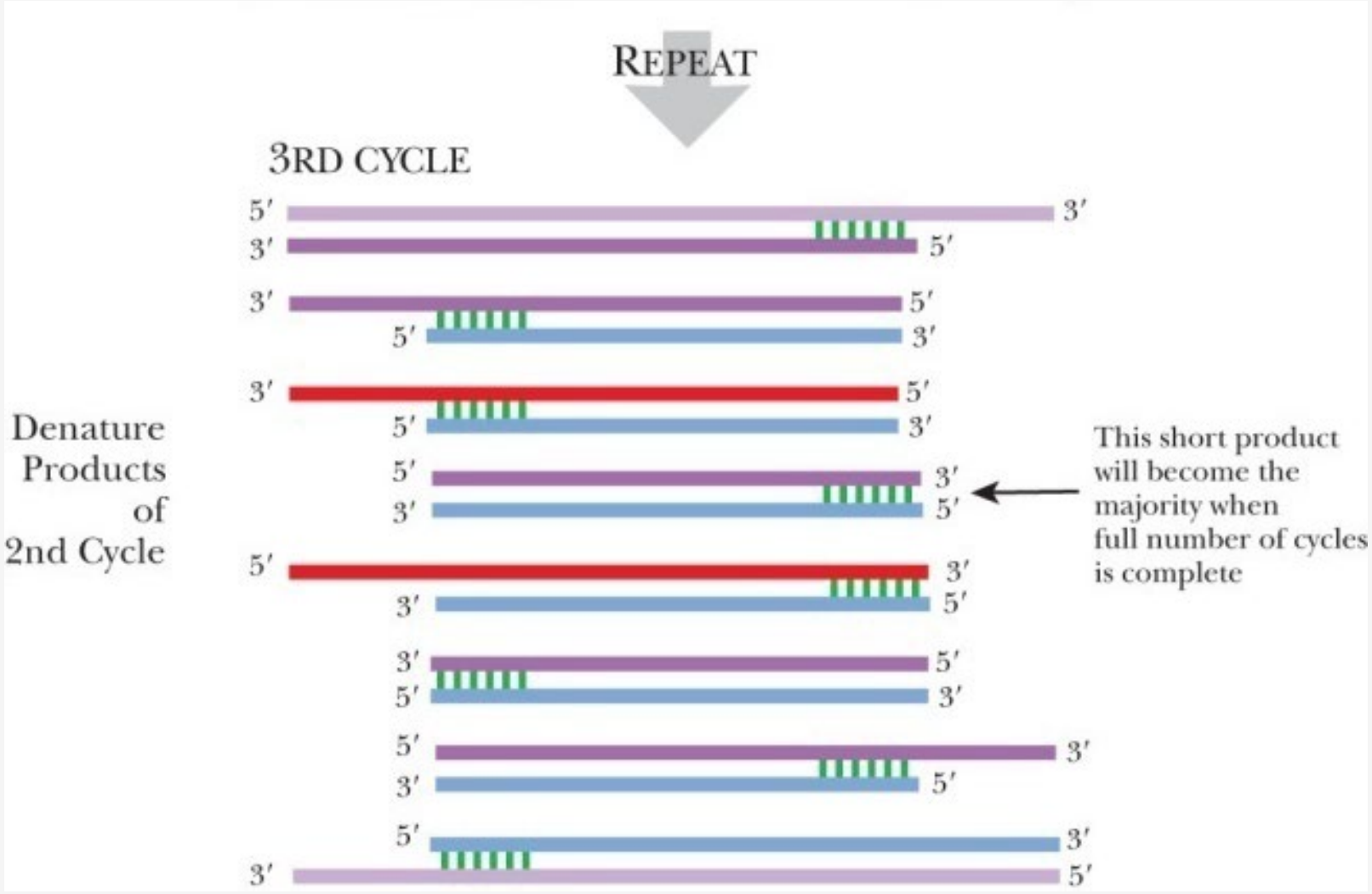
3. Extension:

72 °C



Cycle # 1:
1 DNA amplified to 2 DNA


1ST CYCLE
Denatured template DNA
Primer binding site
5′
3′
5′
3′
Newly synthesized copy
Primer
Primer
Newly synthesized copy
5′
3′
5′
3′
Primer binding site
Denatured template DNA
REPEAT
2ND CYCLE
Denature Products of 1st Cycle
Newly synthesized copies



Target sequence

Appeared after three cycles and start to accumulate

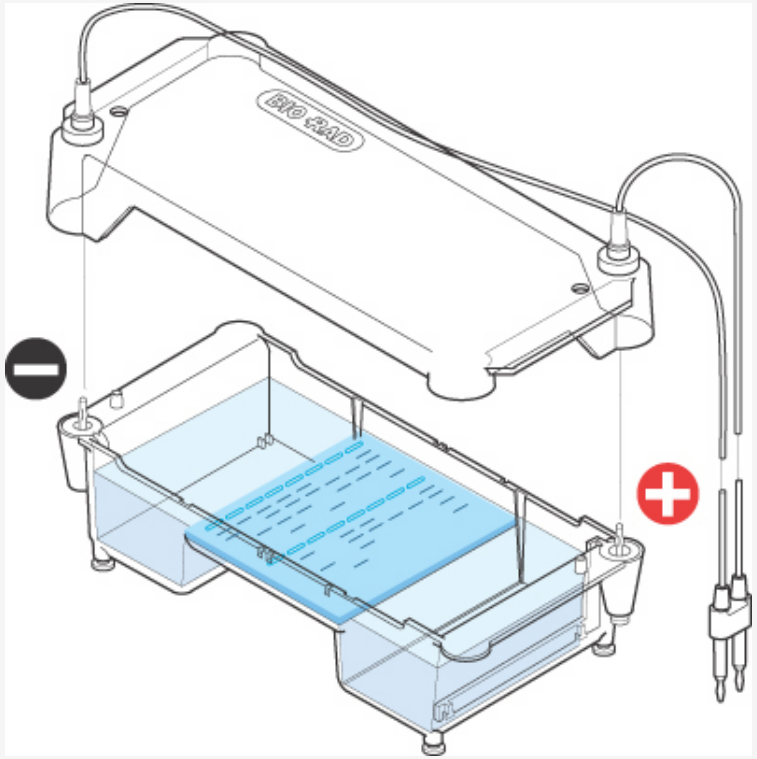
After 30 cycles: 2^{30} copy of target DNA

How you will make sure that your target sequence is amplified?

It is very important to know your product size, why?

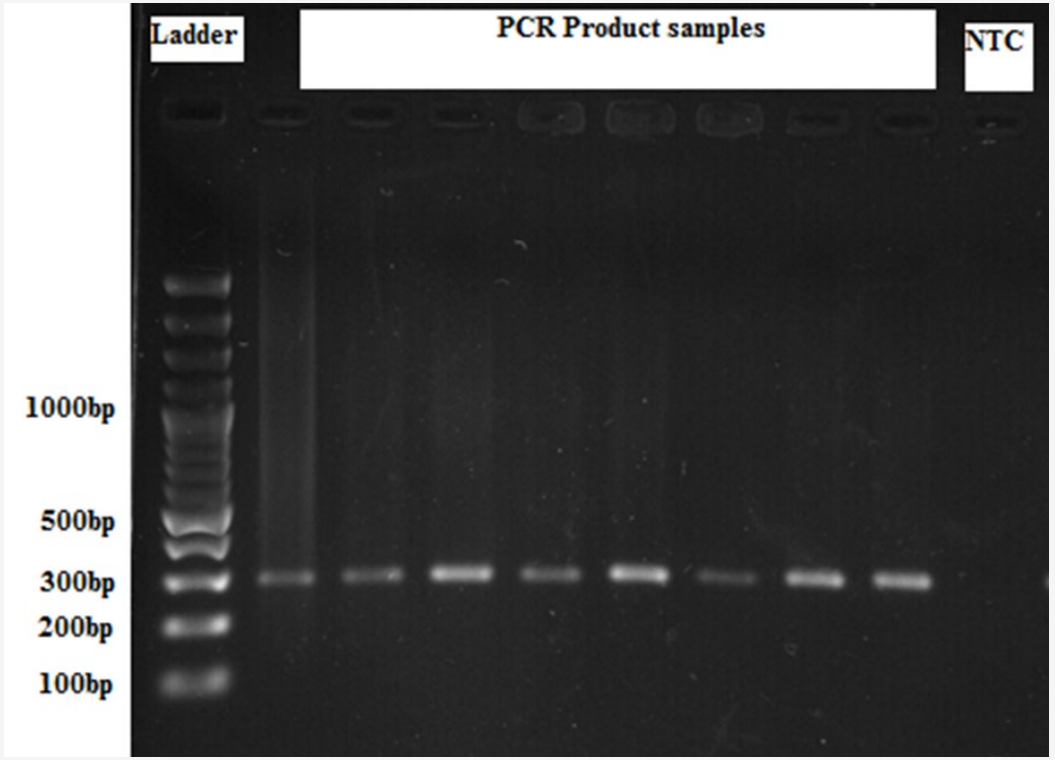
- **Our target sequence size is 300 bp**

To check whether the PCR successfully generated the anticipated DNA target region, agarose gel electrophoresis may be employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder, a molecular weight marker which contains DNA fragments of known size run on the gel alongside the PCR products.



PCR visualizing results

- ✓ After thermal cycling, tubes are taken out of the PCR machine.
- ✓ Contents of tubes are loaded onto an agarose gel.
- ✓ DNA is separated by size using an electric field.
- ✓ DNA is then stained.
- ✓ PCR products are visible as different “bands”.



PCR optimization

In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions. Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants.

Primer-design techniques are important in improving PCR product yield and in avoiding the formation of spurious products, and the usage of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA. Addition of reagents, such as formamide, in buffer systems may increase the specificity and yield of PCR.

PCR Advantages

Important advantages of PCR method as a method of non-cell proliferation of genomic material are high speed and sensitivity and ability the production of significant amounts of genetic material is even from small or inappropriate amounts in the cell genome.

limitations

1- The need for basic information about the base sequence of the desired part

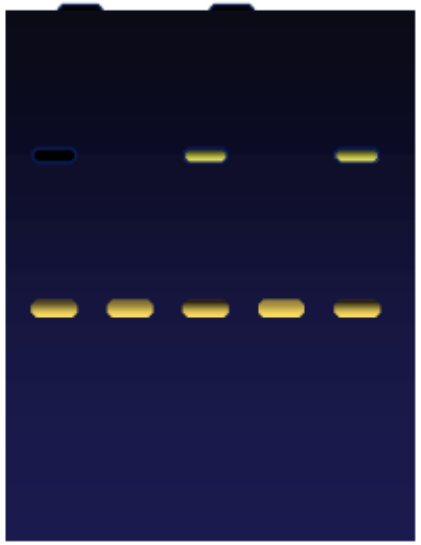
2- Ability to amplify and make small DNA fragments (usually 0.1 to 5 kb fragments)

3- Uncertain replication of the target DNA (reported in rare cases)

4- Need to post-PCR analysis (Agarose gel)

What is Wrong with Agarose Gels?

* Poor precision
* Low sensitivity
* Short dynamic range <2 logs
* Low resolution
* Non automated
* Size based discrimination only
* Results are not expressed as numbers
* Ethidium bromide staining is not very quantitative



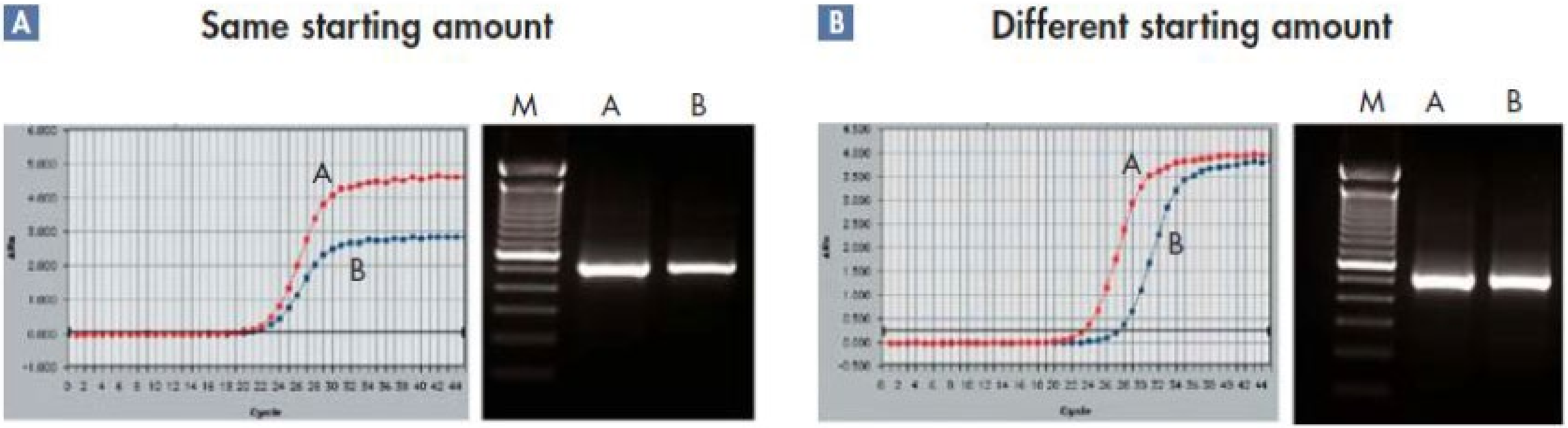


Figure 11. Problems associated with end-point detection. Two-step RT-PCR was carried out with the A same amount and B different amounts of template RNA. The Y-axis is on a linear scale. **M**: markers.

Real-time PCR

Research Plan

- **RNA isolation**
- **RNA electrophoresis and cDNA synthesis**
- **Assessing gene expression**

✓ Northern Blot
✓ RNase protection
✓ Quantitative PCR
✓ Quantitative real time RT PCR

RNases are everywhere!

Control of RNases

- ✓ Wear Gloves and Practice Sterile Technique
- ✓ Use Disposable Plastics or Baked Glassware
- ✓ Use chemicals or reagents that will inactive RNases (DEPC treated water, chaotropic agents, etc)
- ✓ Always Keep RNA on ice or frozen
- ✓ Work quickly and carefully

RNA electrophoresis

RNA is highly susceptible to intra strand H bonding; such secondary structure will affects its migration through an agarose gel unless it is resolved.

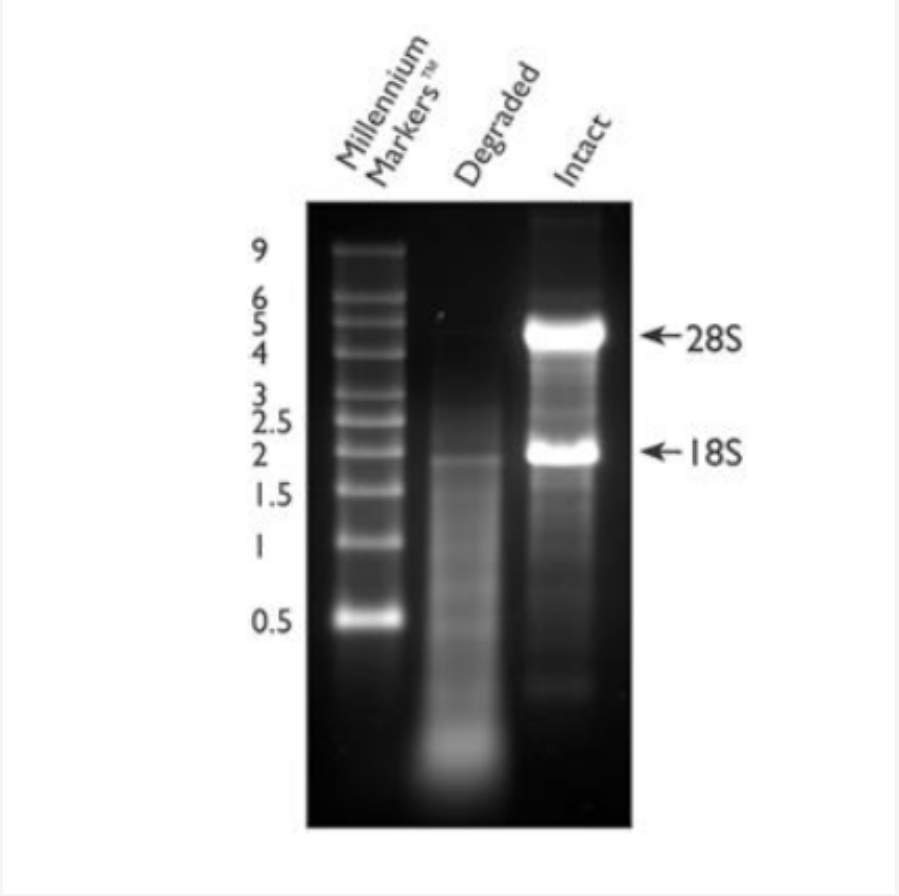
Denaturing Agarose gel electrophoresis

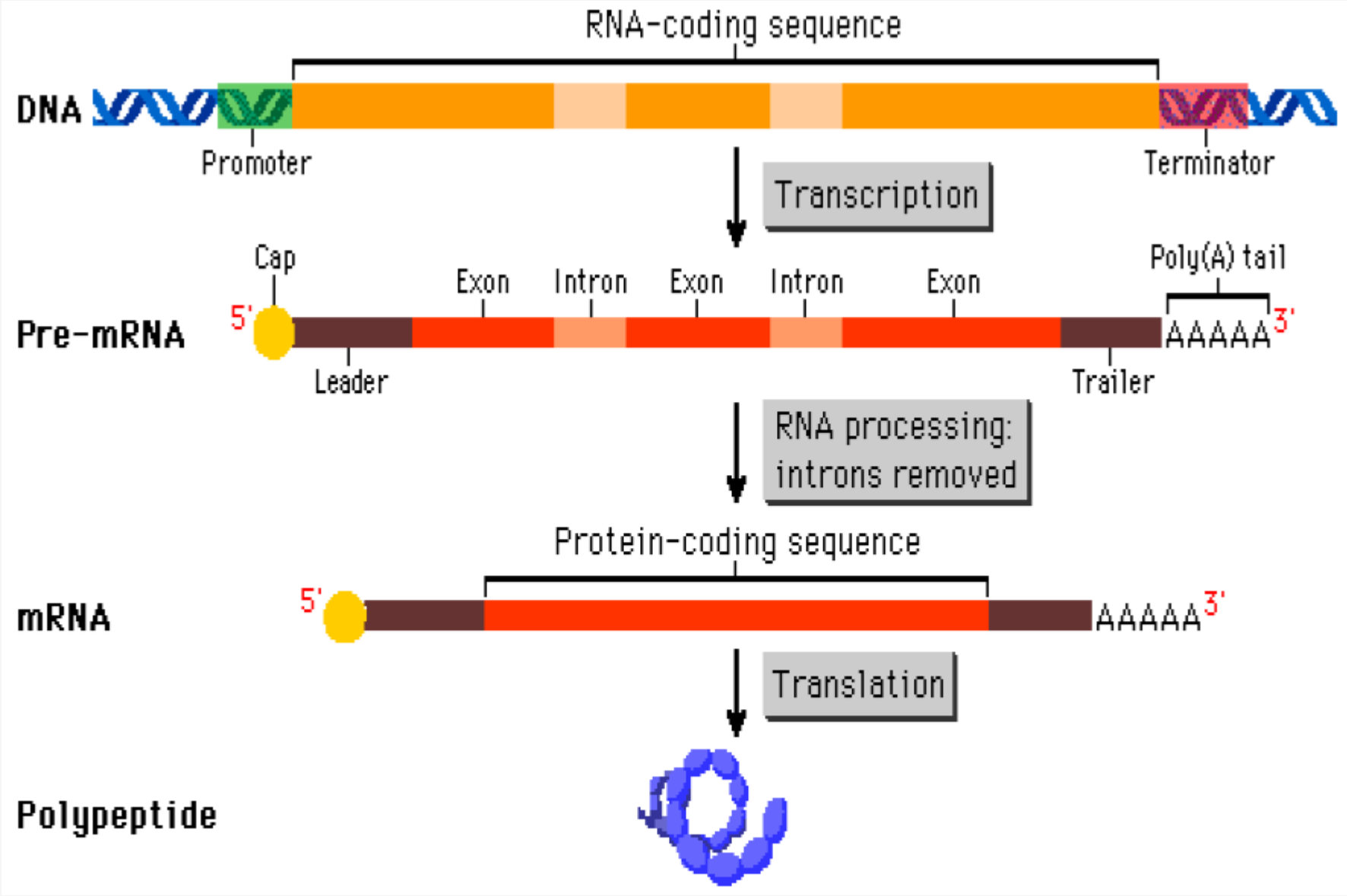
- Two types: 1) Formaldehyde 2) glyoxyl dimethylsulfoxide
- Formamide and formalydehyde are included in loading buffer
- RNA samples heated at 65°C for 5 minutes prior to electrophoresis

Electrophoresis of RNA

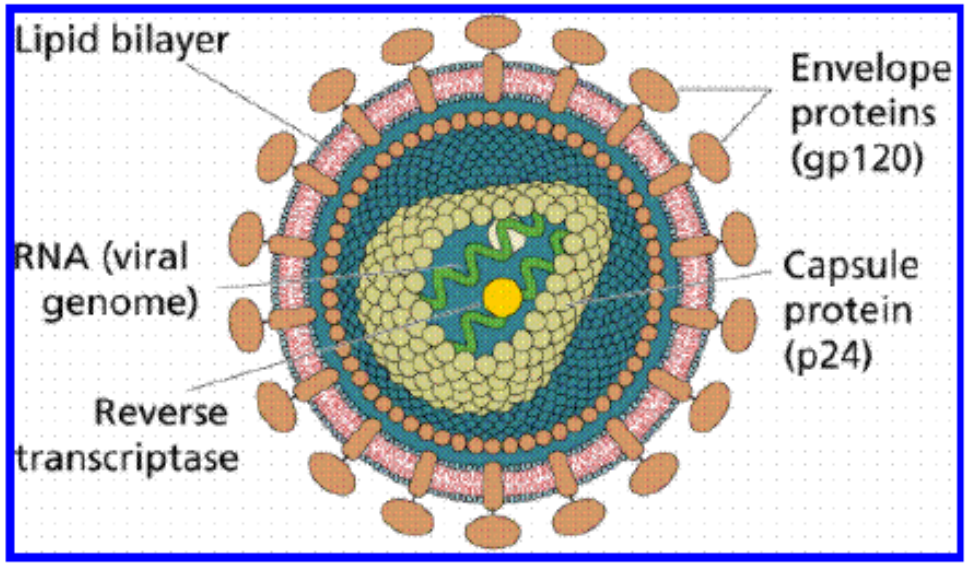
Intact High Quality RNA Characterized by:

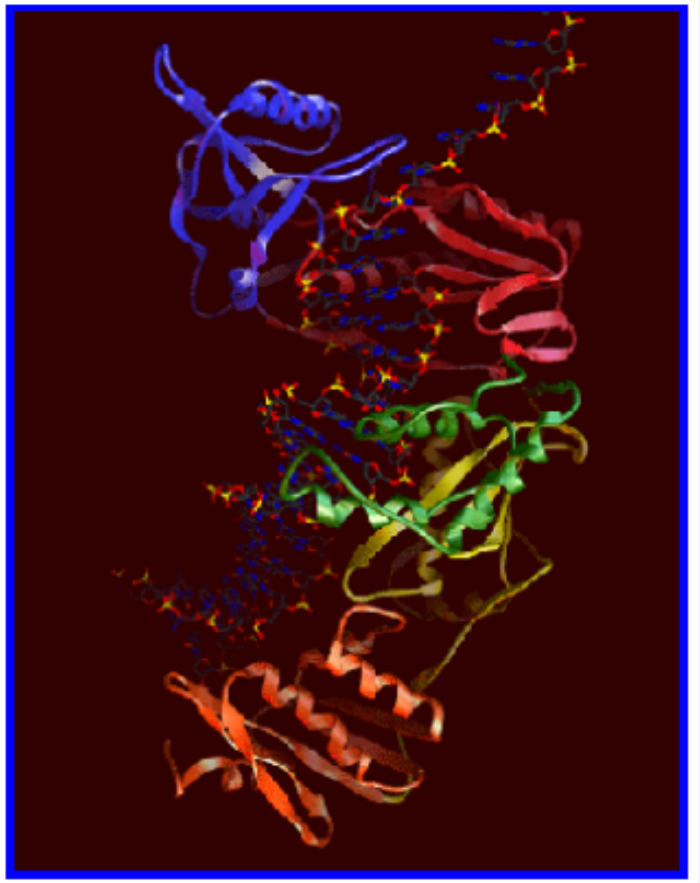
- ✓ Two prominent rRNA Bands
- ✓ Slight smear of various sized mRNA molecules in background




RNA-coding sequence
DNA
Promoter
Terminator
Transcription
Cap
Exon
Intron
Exon
Intron
Exon
Poly(A) tail
Pre-mRNA
5'
AAAAA
3'
Leader
Trailer
RNA processing:
introns removed
Protein-coding sequence
mRNA
5'
AAAAA
3'
Translation
Polypeptide

Reverse Transcriptase

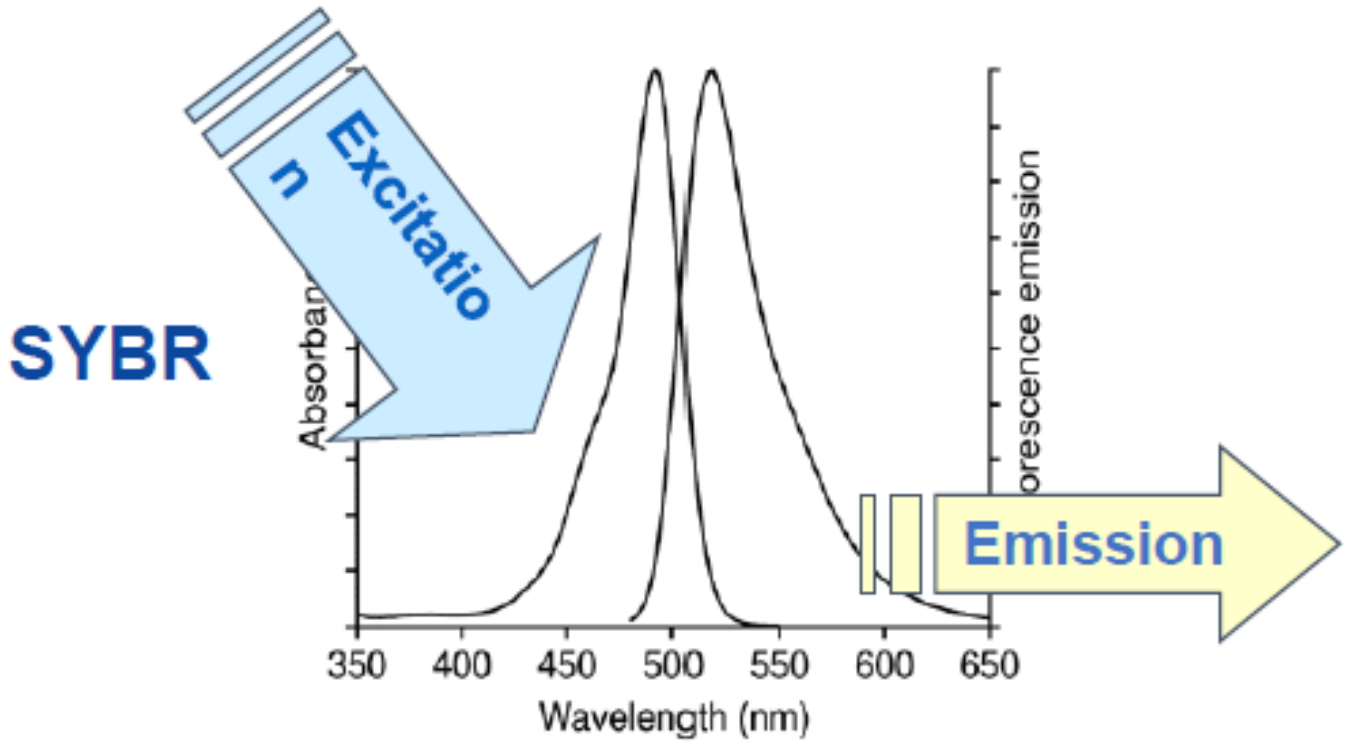




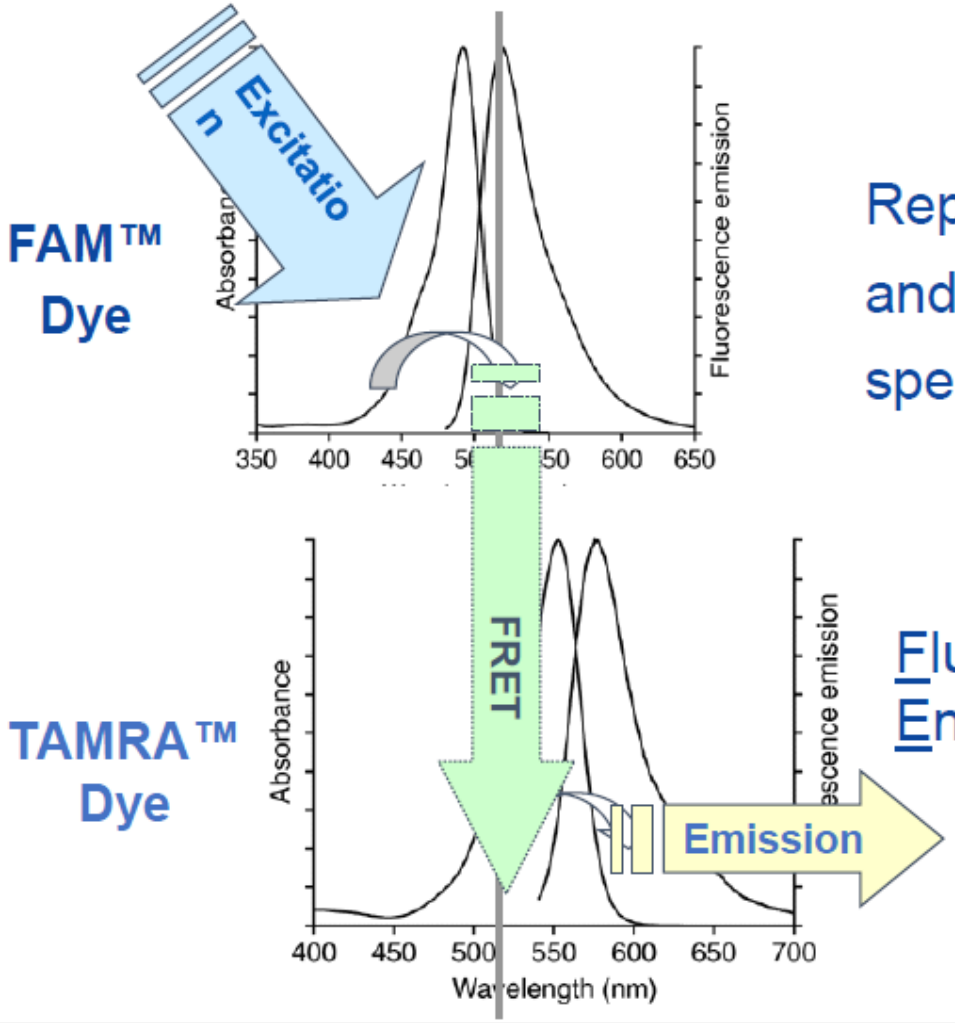
A real time polymerase chain reaction is a laboratory technique of molecular biology based on the polymerase chain reaction (which is used to amplify and simultaneously detect or quantify a targeted DNA molecule).

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.

Fluorescence Process



Quenching Process

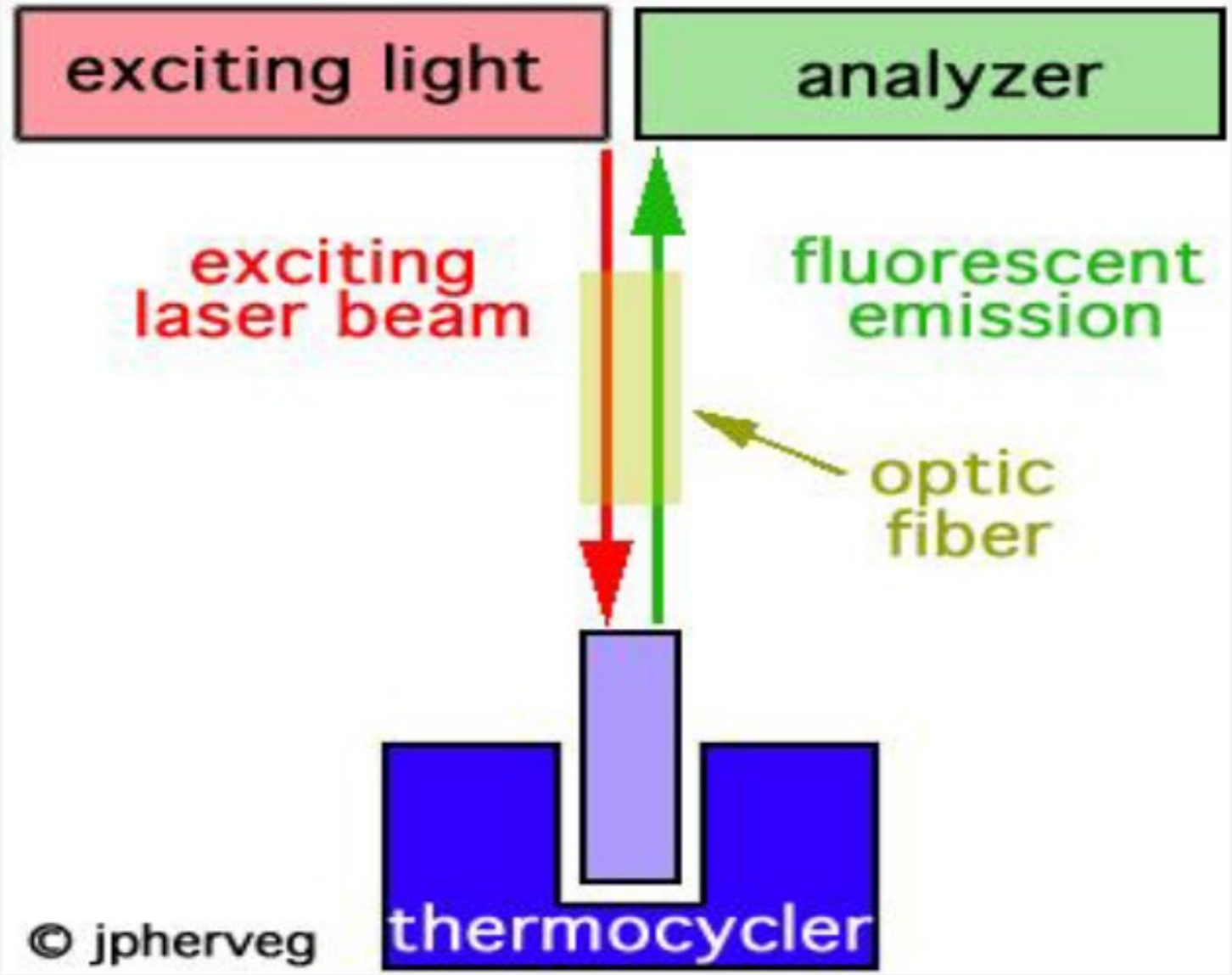


Reporter emission spectra and quencher excitation spectra overlap

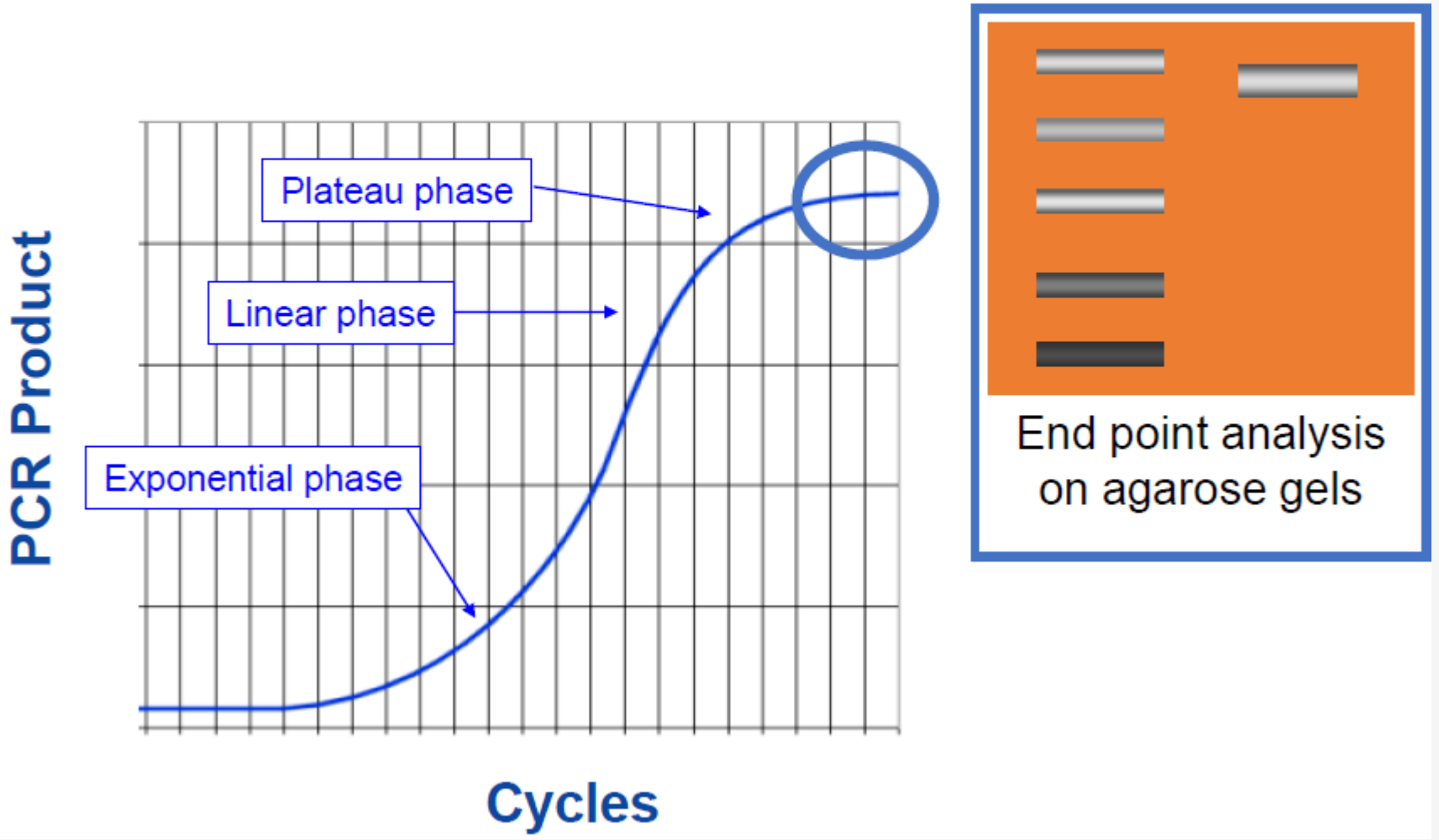
Fluorescence Resonance Energy Transfer

Name	Excitation Maximum (nm)	Emission Maximum (nm)	Compatible Quencher
Reporter Dye			
6-FAM™	494	515	BHQ®-1, TAMRA
JOE™	520	548	BHQ-1, TAMRA
TET™	521	536	BHQ-1, TAMRA
Cal Fluor® Gold 540[a]	522	541	BHQ-1
HEX™[b]	535	555	BHQ-1, TAMRA
Cal Fluor Orange 560[b]	540	561	BHQ-1
TAMRA™	555	576	BHQ-2
Cyanine 3	550	570	BHQ-2
Quasar® 570[c]	548	566	BHQ-2
Cal Fluor Red 590[d]	565	588	BHQ-2
ROX™	573	602	BHQ-2
Texas Red®	583	603	BHQ-2
Cyanine 5	651	674	BHQ-3
Quasar 670[e]	647	667	BHQ-3
Cyanine 5.5	675	694	BHQ-3

[a] JOE/TET alternative
[b] VIC® alternative
[c] Cyanine 3 alternative
[d] TAMRA alternative
[e] Cyanine 5 alternative


exciting light
analyzer
exciting
laser beam
fluorescent
emission
optic
fiber
thermocycler
© jpherveg

Polymerase Chain Reaction



The Basic of Real time PCR

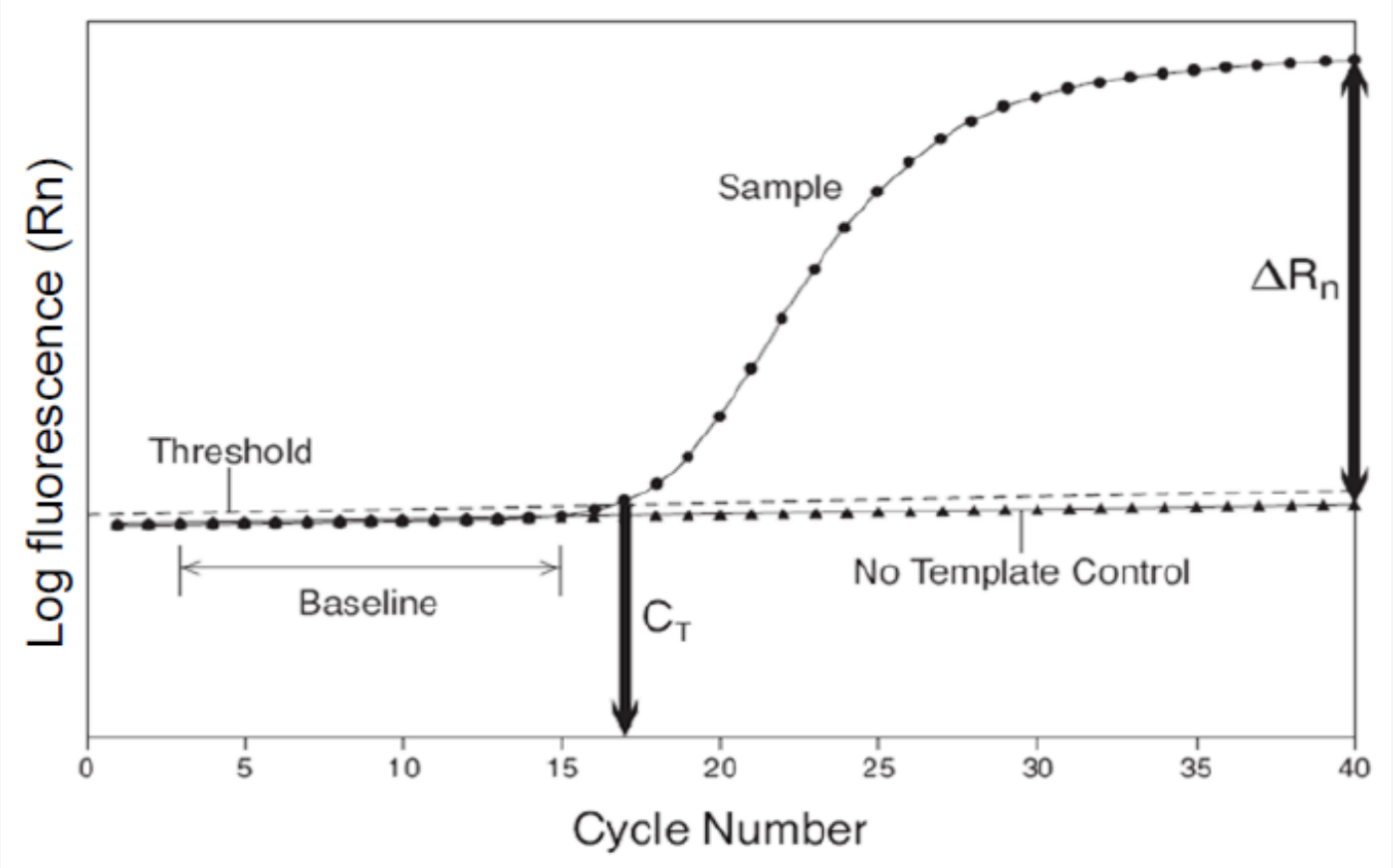
Baseline: The baseline phase contains all the amplification that is below the level of detection of the real time instrument.

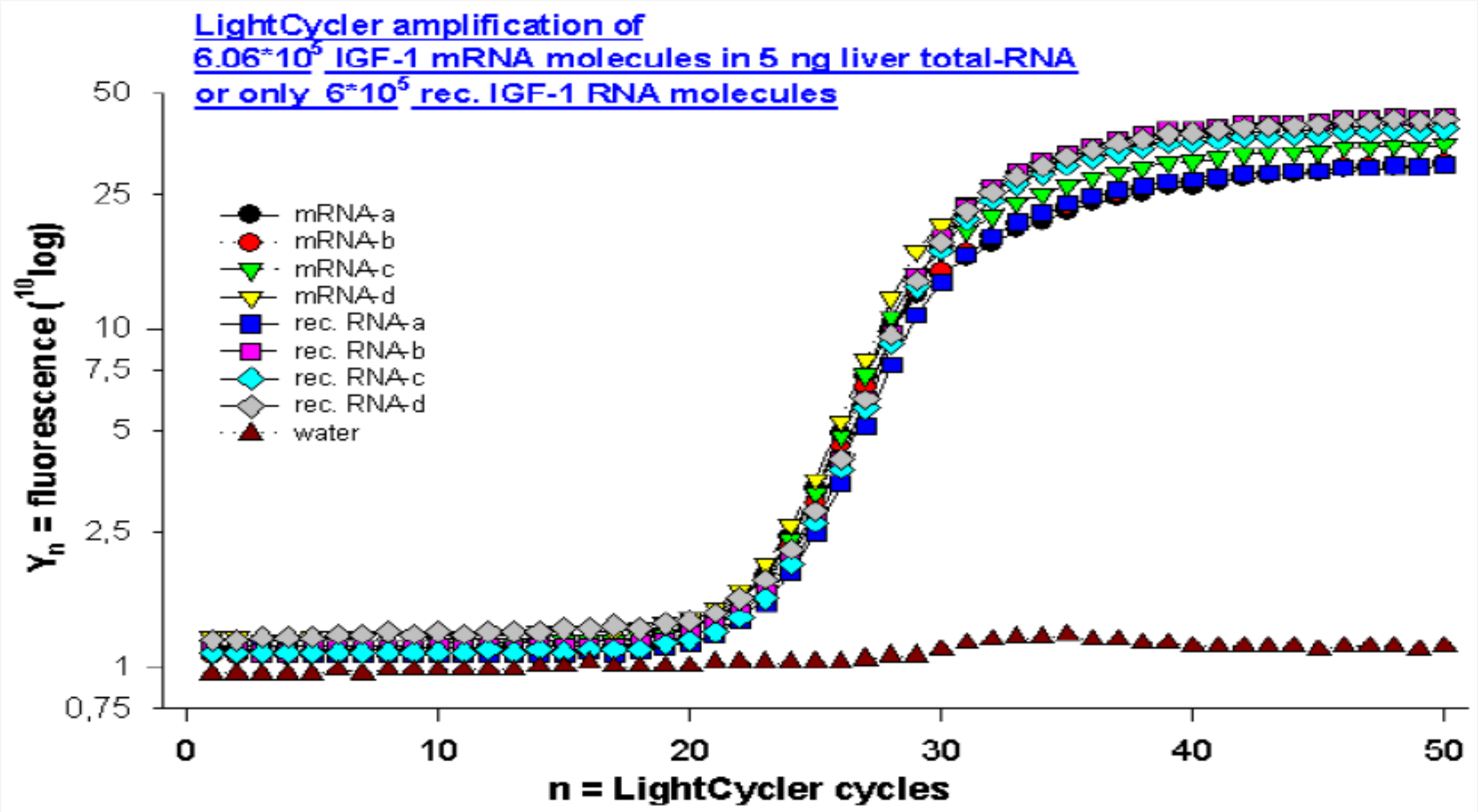
Threshold: where the threshold and the amplification plot intersect defines CT. Can be set manually/automatically

CT: (cycle threshold) the cycle number where the fluorescence passes the threshold

ΔRn: (Rn -baseline)

NTC: no template control



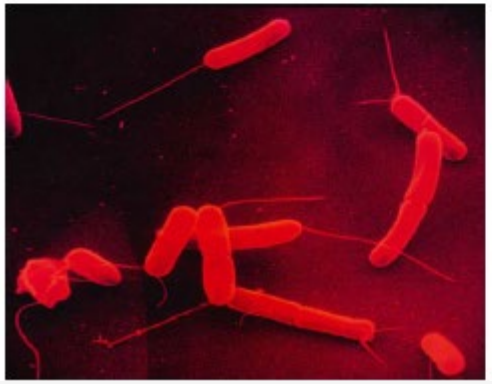

LightCycler amplification of
6.06*10^5 IGF-1 mRNA molecules in 5 ng liver total-RNA
or only 6*10^5 rec. IGF-1 RNA molecules
mRNA-a
mRNA-b
mRNA-c
mRNA-d
rec. RNA-a
rec. RNA-b
rec. RNA-c
rec. RNA-d
water
Y_n = fluorescence (^10log)
50
25
10
7,5
5
2,5
1
0,75
0
10
20
30
40
50
n = LightCycler cycles

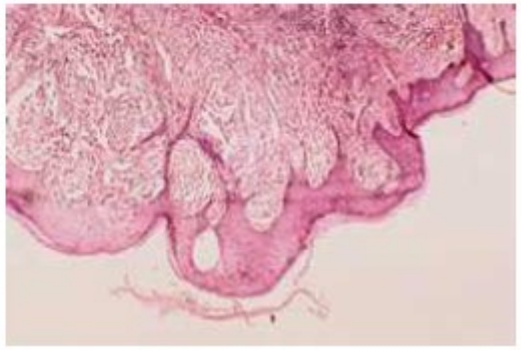
Real time PCR advantages

* not influenced by non specific amplification
* amplification can be monitored real time
* no post PCR processing of products (high throughput, low contamination risk)
* Ultra rapid cycling (30 minutes to 2 hours)
* wider dynamic range of up to 10^{10} −fold
* requirement of 1000 fold less RNA than conventional assays
* detection is capable down to a 2 fold change
* confirmation of specific amplification by melting curve analysis
* most specific, sensitive and reproducible
* not much more expensive than conventional PCR (except equipment cost)

Applications

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





The assay methods

Real Time Principles

Three general methods for the quantitative assays:

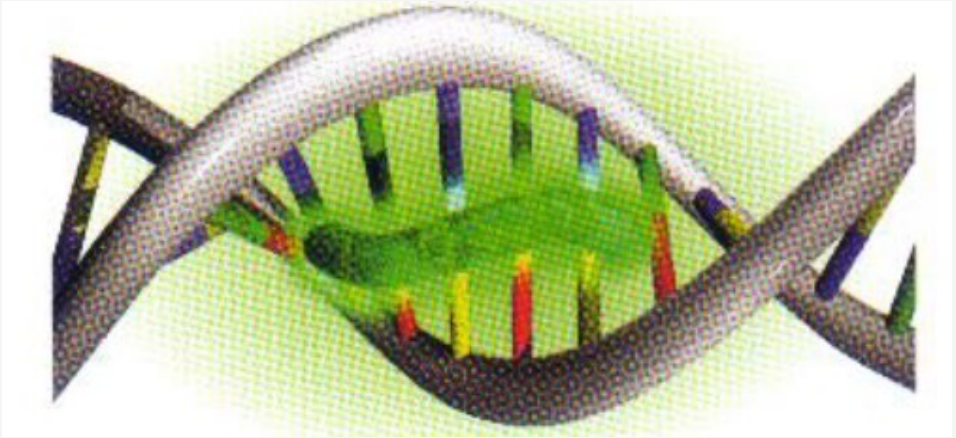
1 . DNA binding agents (SYBR Green)

2 . Hydrolysis probes (TaqMan , Beacons, Scorpions)

3 . Hybridization probes (Light Cycler)

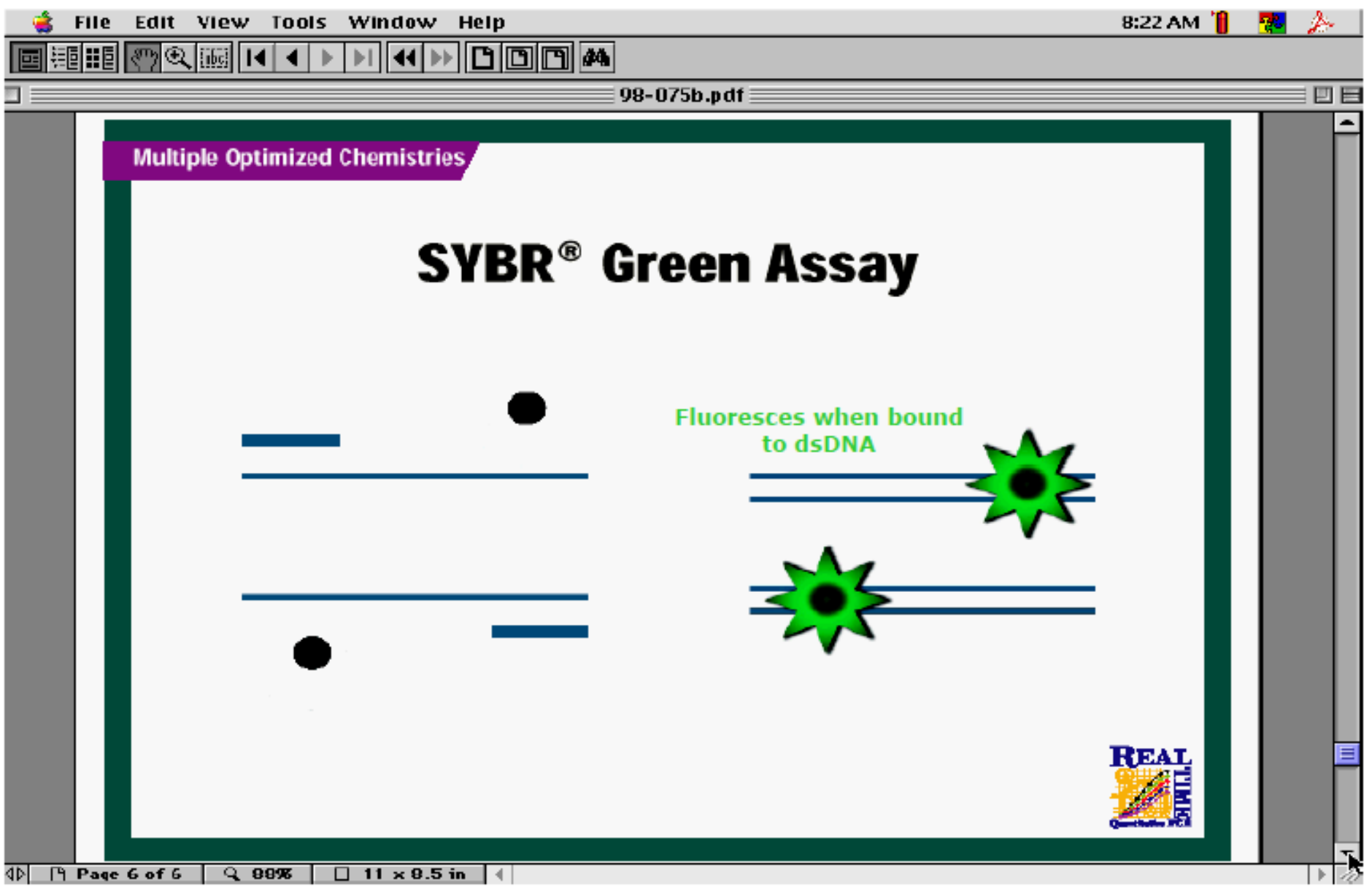
DNA binding agents (SYBR Green)

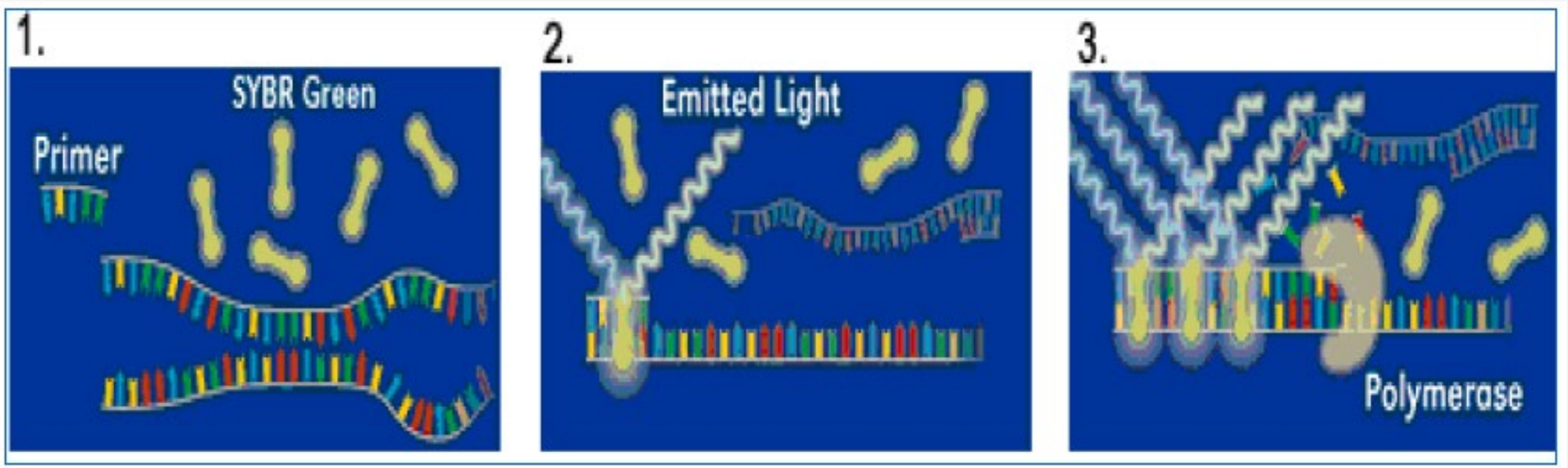
Signal Generation with SYBR ® Green 1 dye



•SYBR ® Green 1 dye binds to the minor groove of ds DNA

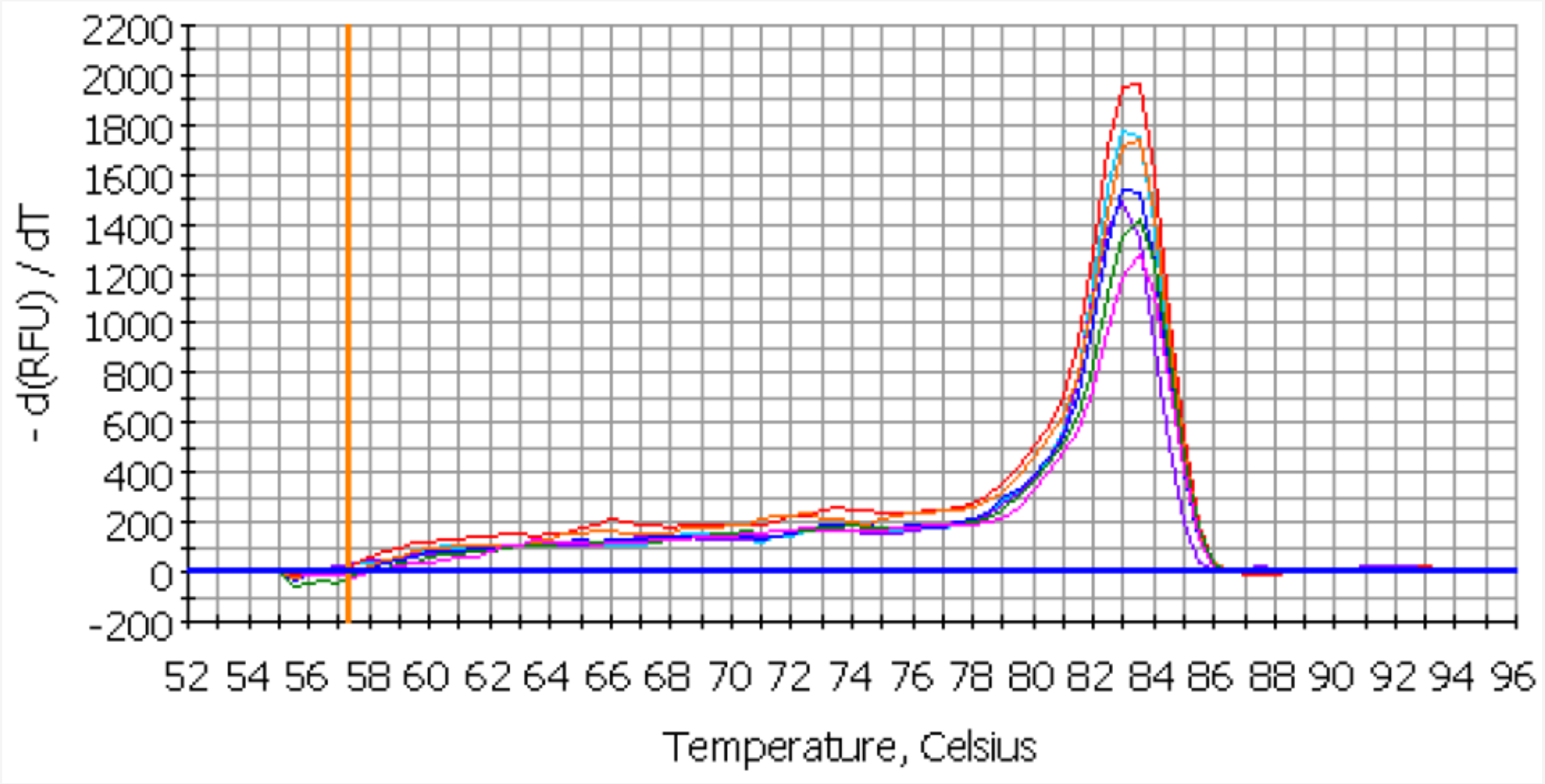
•Detects specific and unspecific products

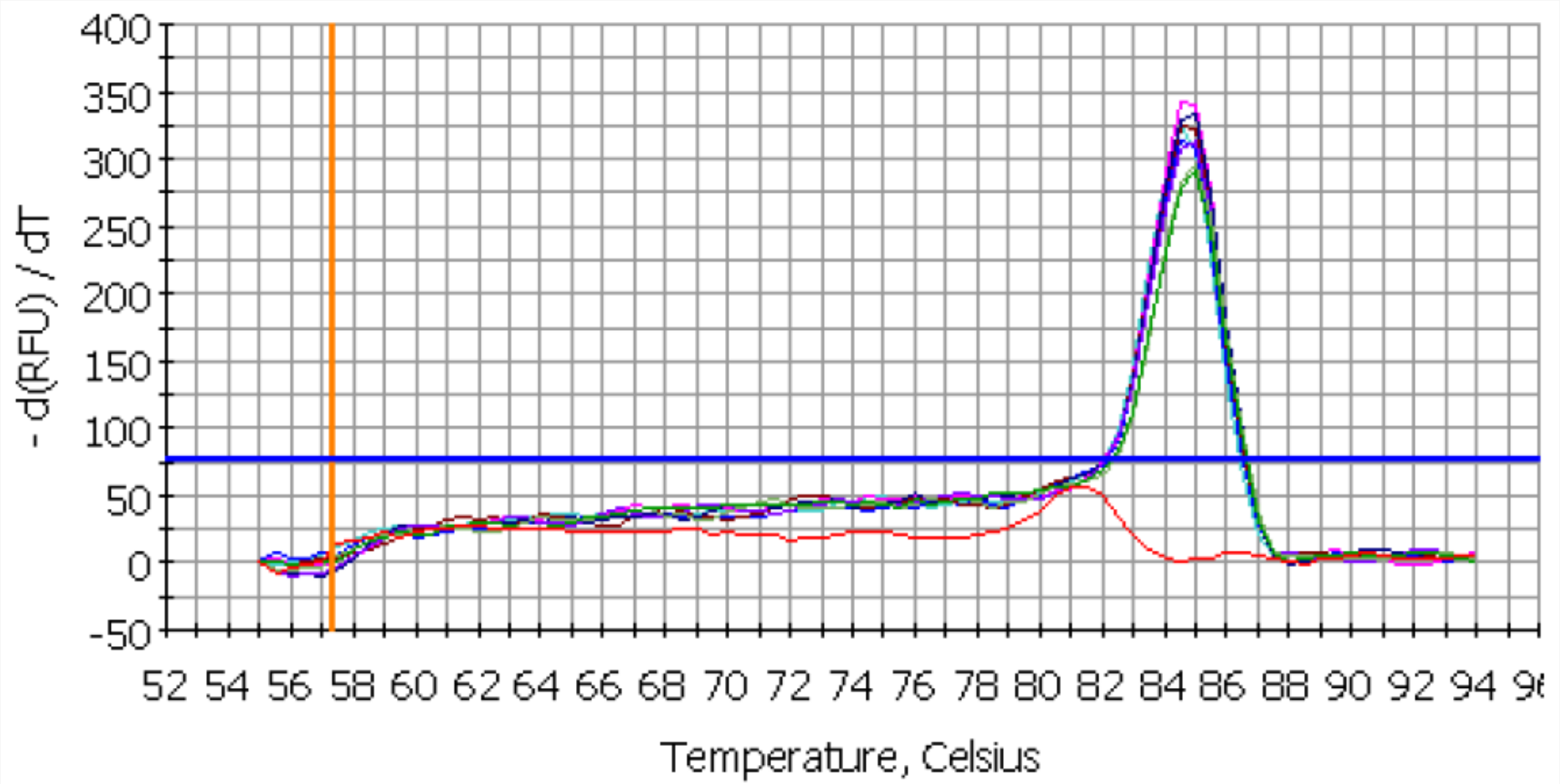

File Edit View Tools Window Help
8:22 AM
98-075b.pdf
Multiple Optimized Chemistries
SYBR® Green Assay
Fluoresces when bound
to dsDNA
REAL TIME
Page 6 of 6
99%
11 x 8.5 in

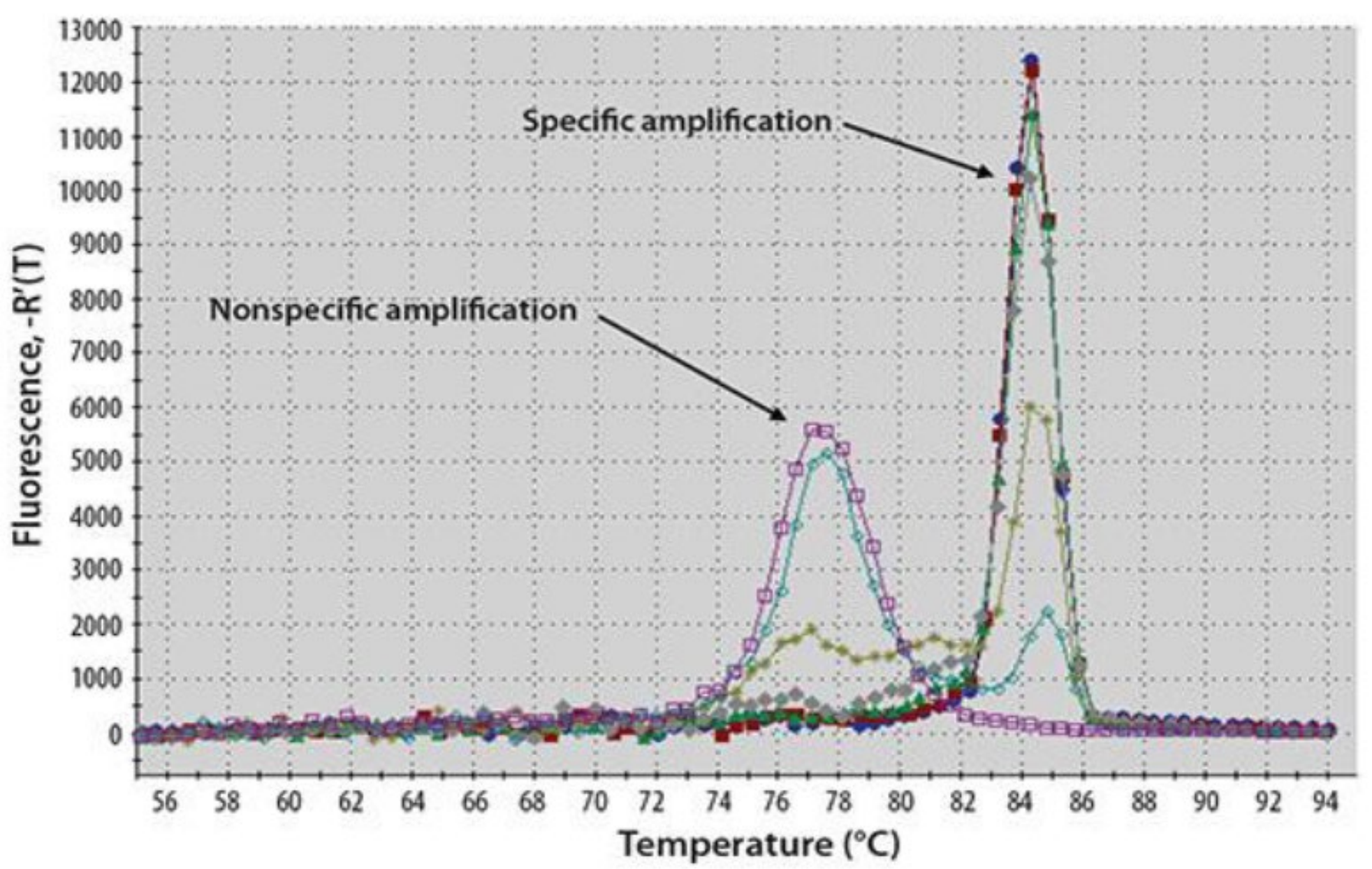


SYBR Green

1) At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis.
2) After annealing of the primers, a few dye molecules can bind to the double strand. DNA binding results in a dramatic increase of the SYBR Green I molecules to emit light upon excitation.
3) During elongation, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.

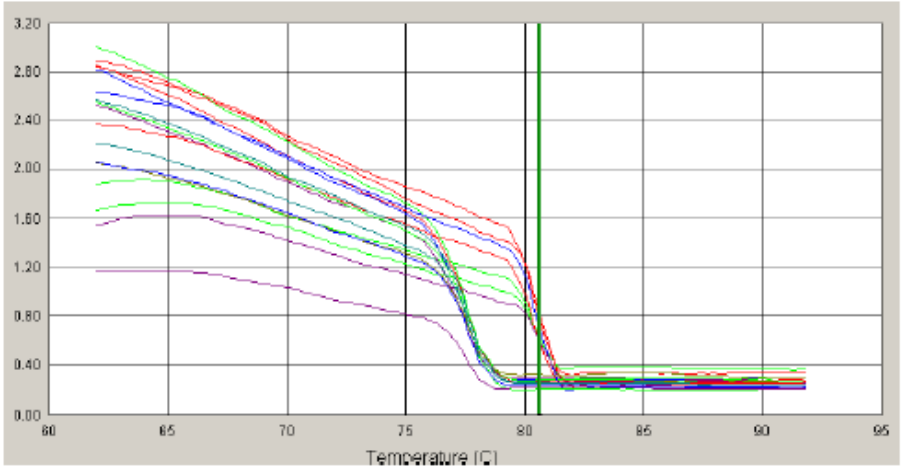

2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
- d(RFU) / dT
52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96
Temperature, Celsius


400
350
300
250
200
150
100
50
0
-50
- d(RFU) / dT
52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94
Temperature, Celsius

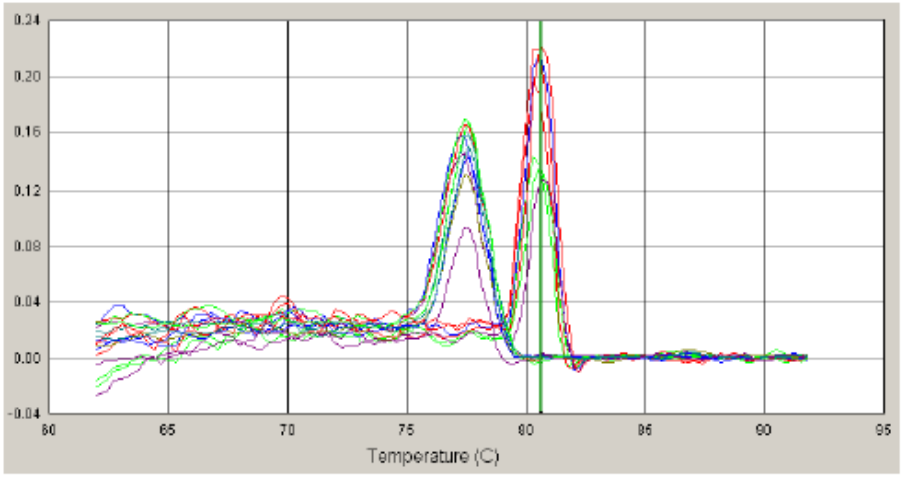

Specific amplification
Nonspecific amplification
Fluorescence, -R'(T)
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94
Temperature (°C)

Dissociation Curve View

SYBR Green Data Analysis



Raw data

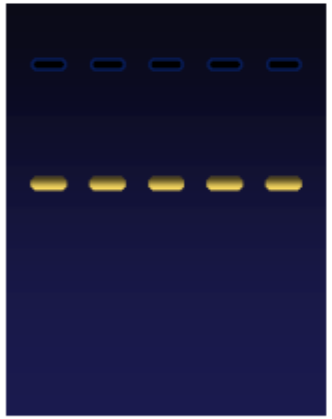


Derivative data

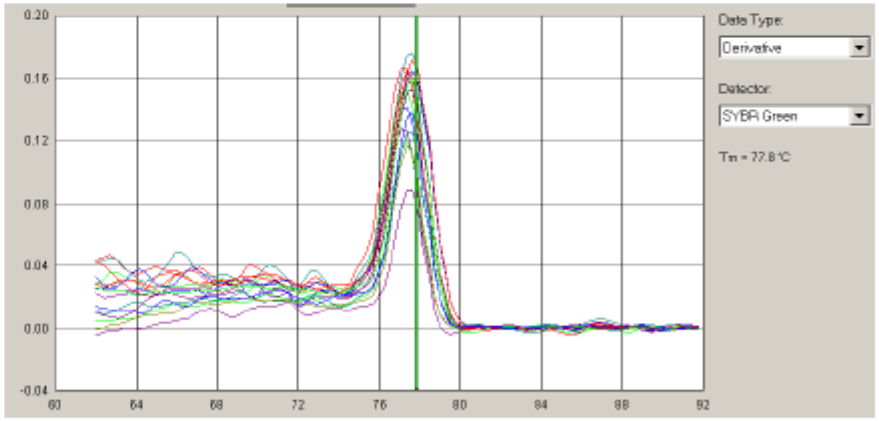
Specificity check

SYBR® Green 1 dye

- When ? Post PCR Analysis
- How ? Gel or Real-Time PCR System



Gels



Real-Time PCR System
Melting curve analysis

SYBR Green (double stranded DNA binding dye)

* Emits a strong fluorescent signal upon binding to double stranded DNA
* Non specific binding is a disadvantage
* Requires extensive optimization
* Requires melting point curve determination
* Longer amplicons create a stronger signal
* May be multiplexed when coupled with melting curve analysis

When to Choose SYBR Green

* Assays that do not require specificity of probe based assays. Detection of 1000s of molecules.

* General screening of transcripts prior to moving to probe based assays.

* When the PCR system is fully optimized no primer dimers or non specific amplicons , e.g. from genomic DNA.

When Not to Choose SYBR Green

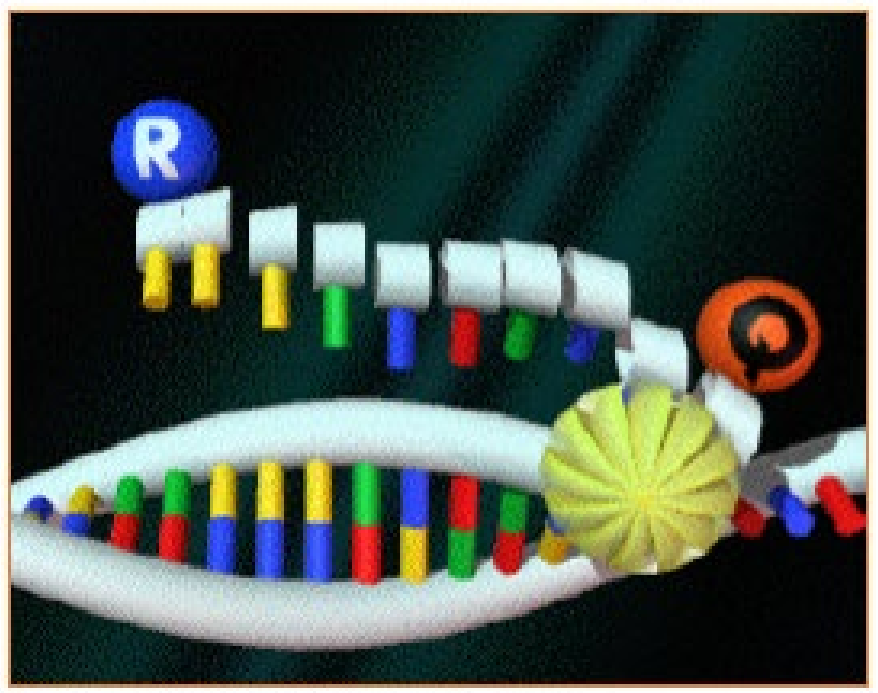
* Allelic discrimination assays
* Multiplex reactions
* Amplification of rare transcripts
* Low level pathogen detection

Signal Generation with a TaqMan ® Probe

The 5’ Nuclease Assay

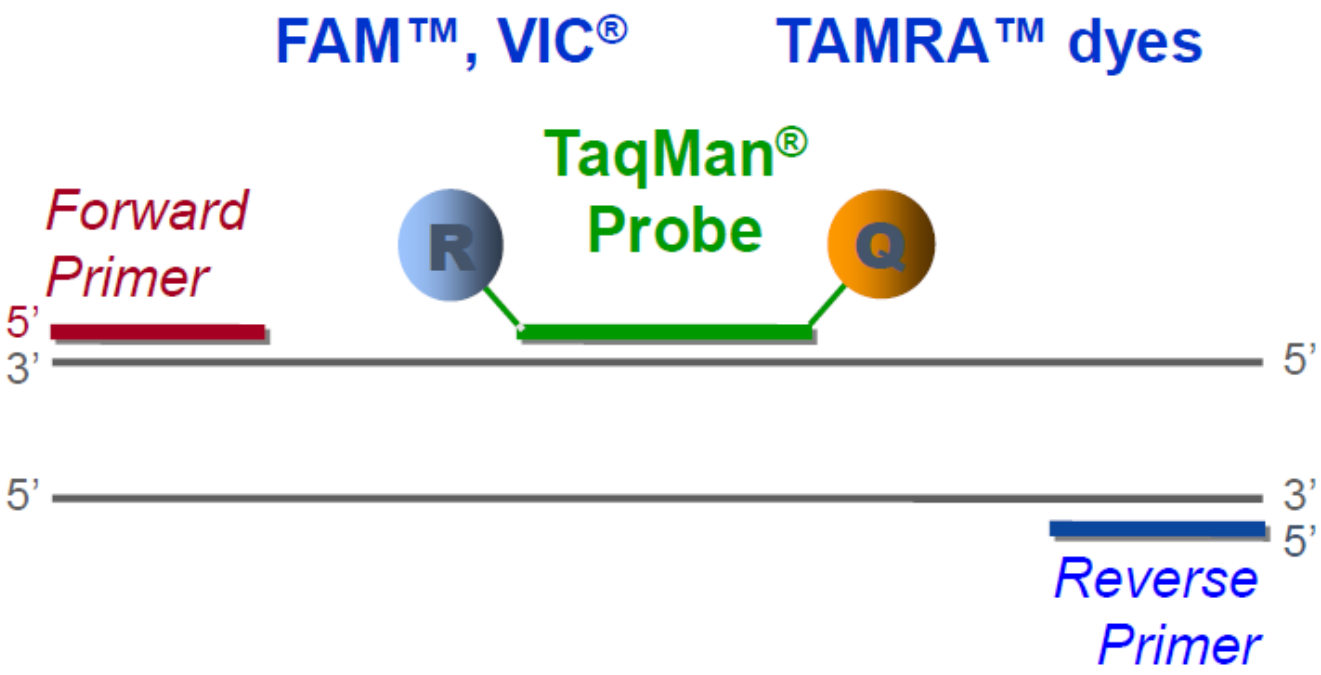
This method uses 2 principles:

- FRET Technology
- 5’ Nuclease Activity of the Taq Polymerase



5’ Nuclease Assay

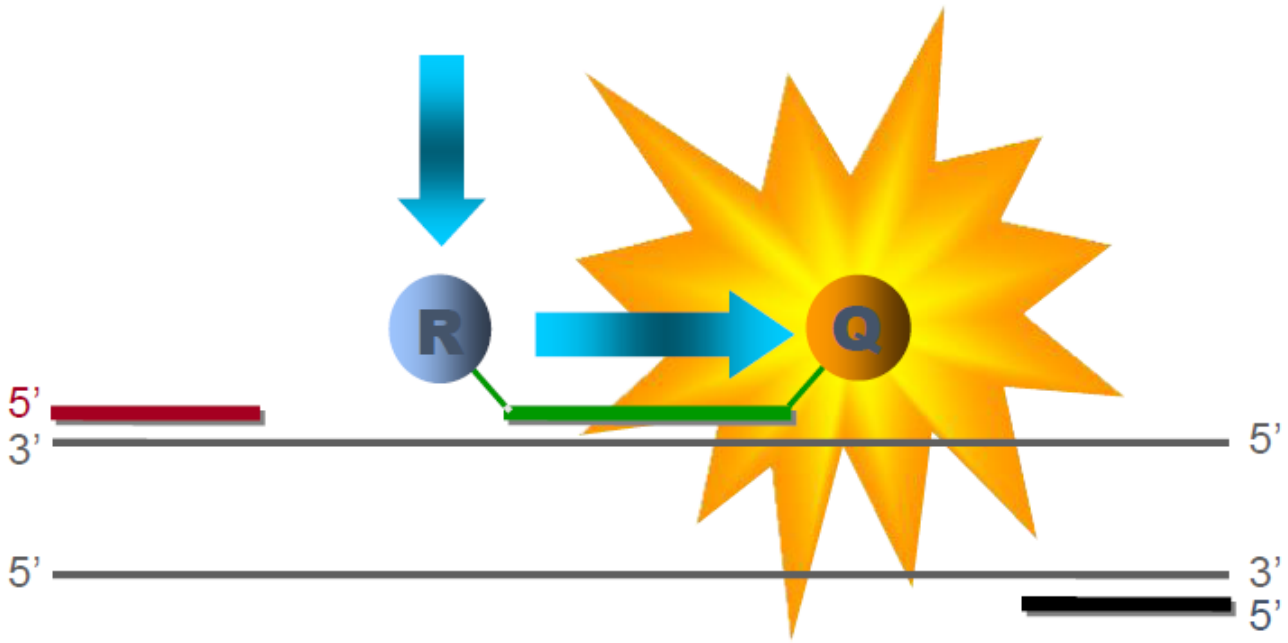
using TaqMan® probes



- PCR specificity (primer)
- Hybridization specificity (probe)

5’ Nuclease Assay

using TaqMan® probes

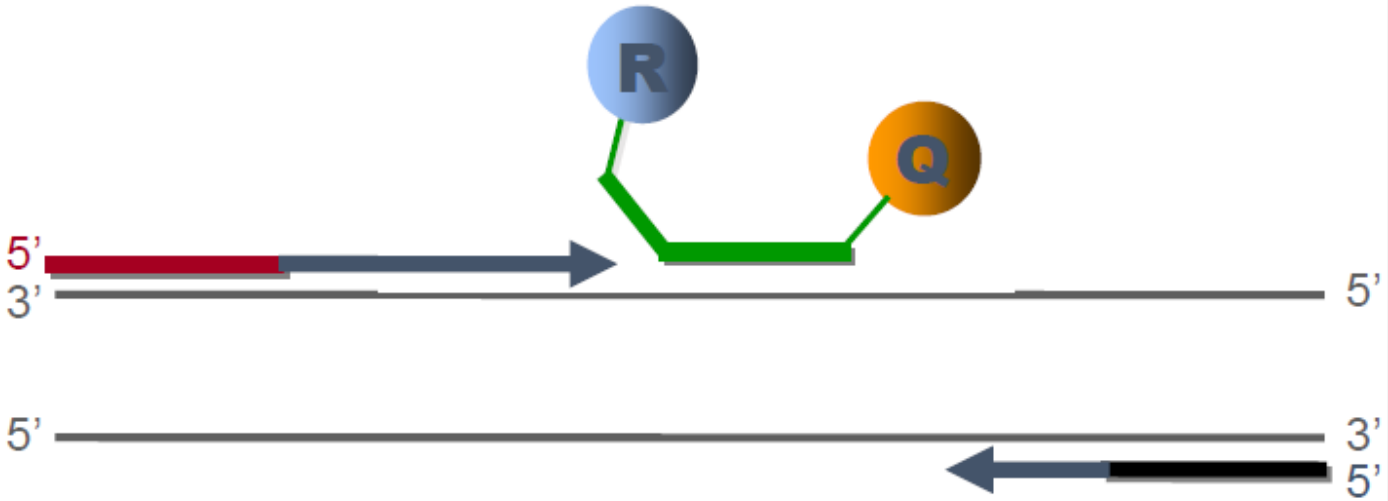


Fluorescence Resonance Energy Transfer (FRET) from high energy to low energy dye

➔ No reporter signal with intact probe

5’ Nuclease Assay

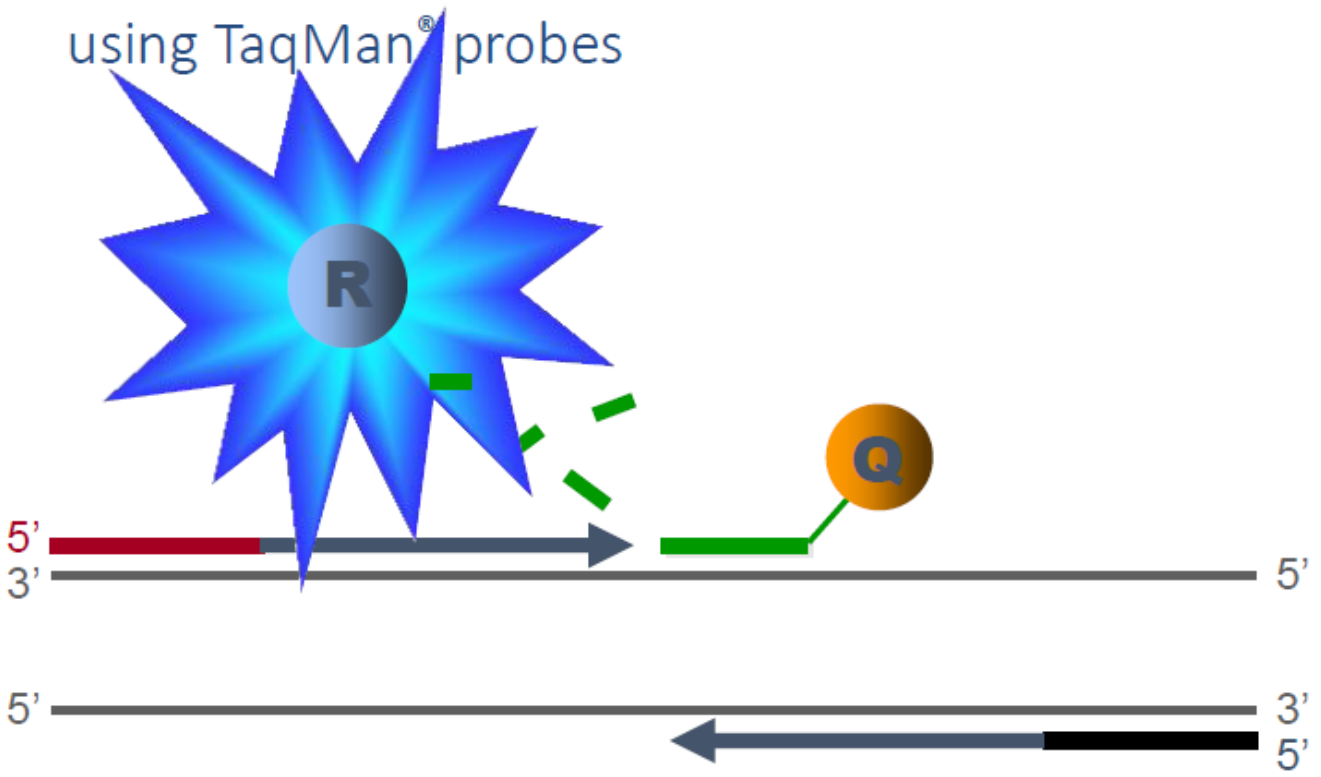
using TaqMan® probes



Displacement of probe by 5‘ nuclease activity of polymerase

5’ Nuclease Assay

using TaqMan® probes

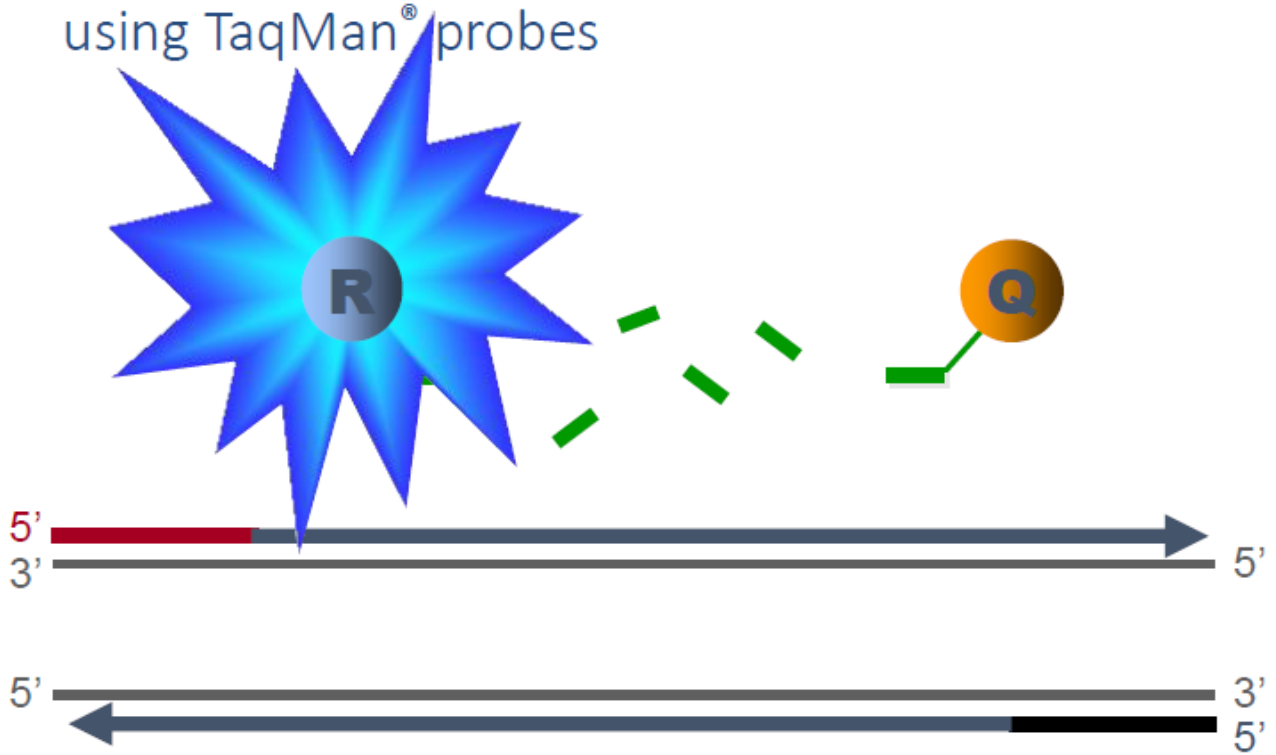


Cleavage of probe by 5‘ nuclease activity of Taq polymerase

➔ FRET disabled, generation of reporter signal

5’ Nuclease Assay

using TaqMan® probes



Polymerization completed

One reporter signal for each new DNA copy

TaqMan® MGB probes

NFQ: Non-Fluorescent Quencher

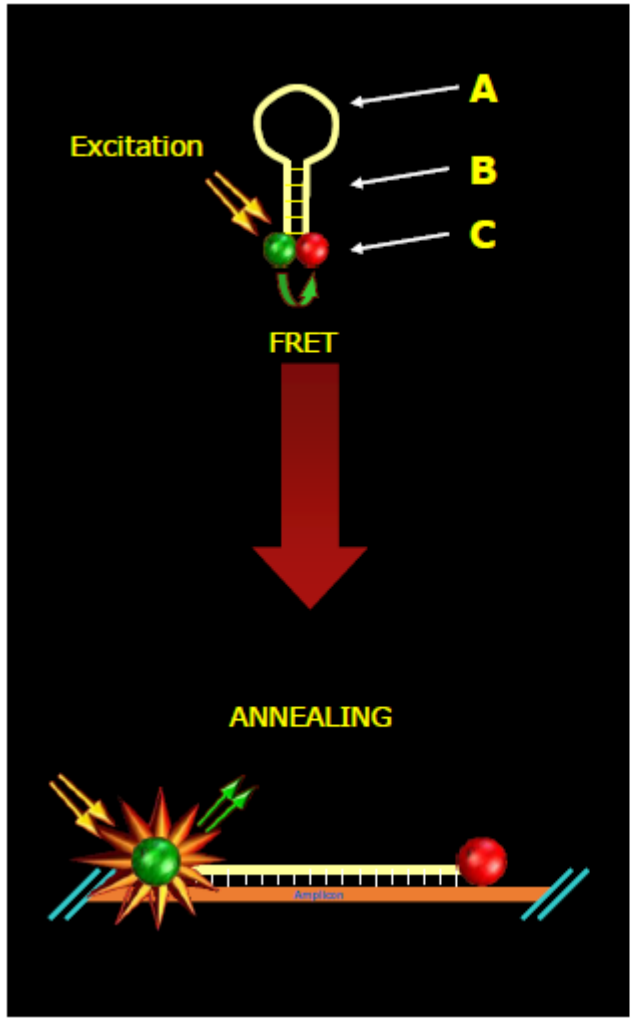
→ Opens up another window for other reporter dyes

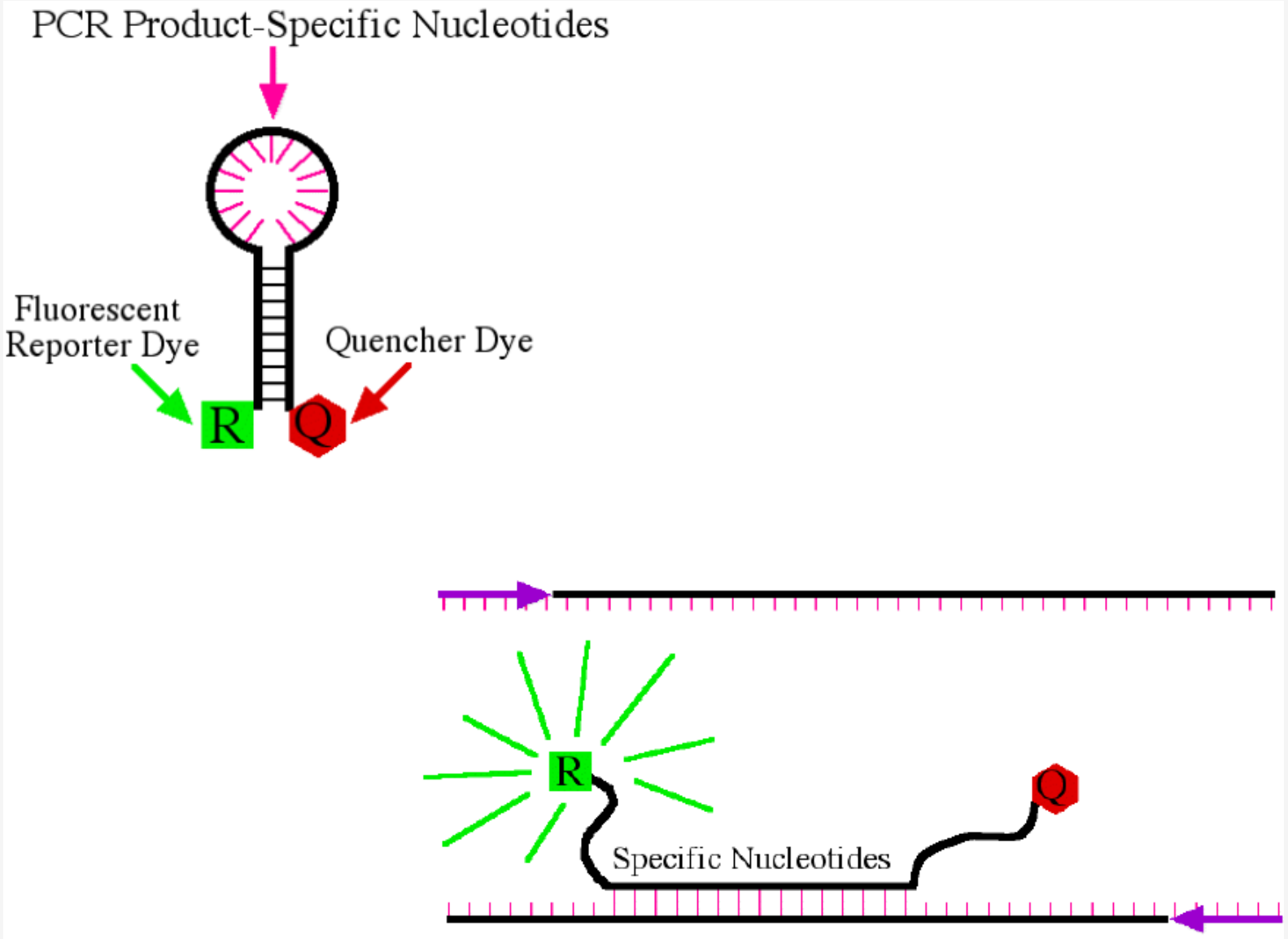
MGB: Minor Groove Binder

→ Stabilizes the last 5-6 bp on 3‘ end

→ **Shorter** and **more specific** probes for a given Tm

Molecular beacons



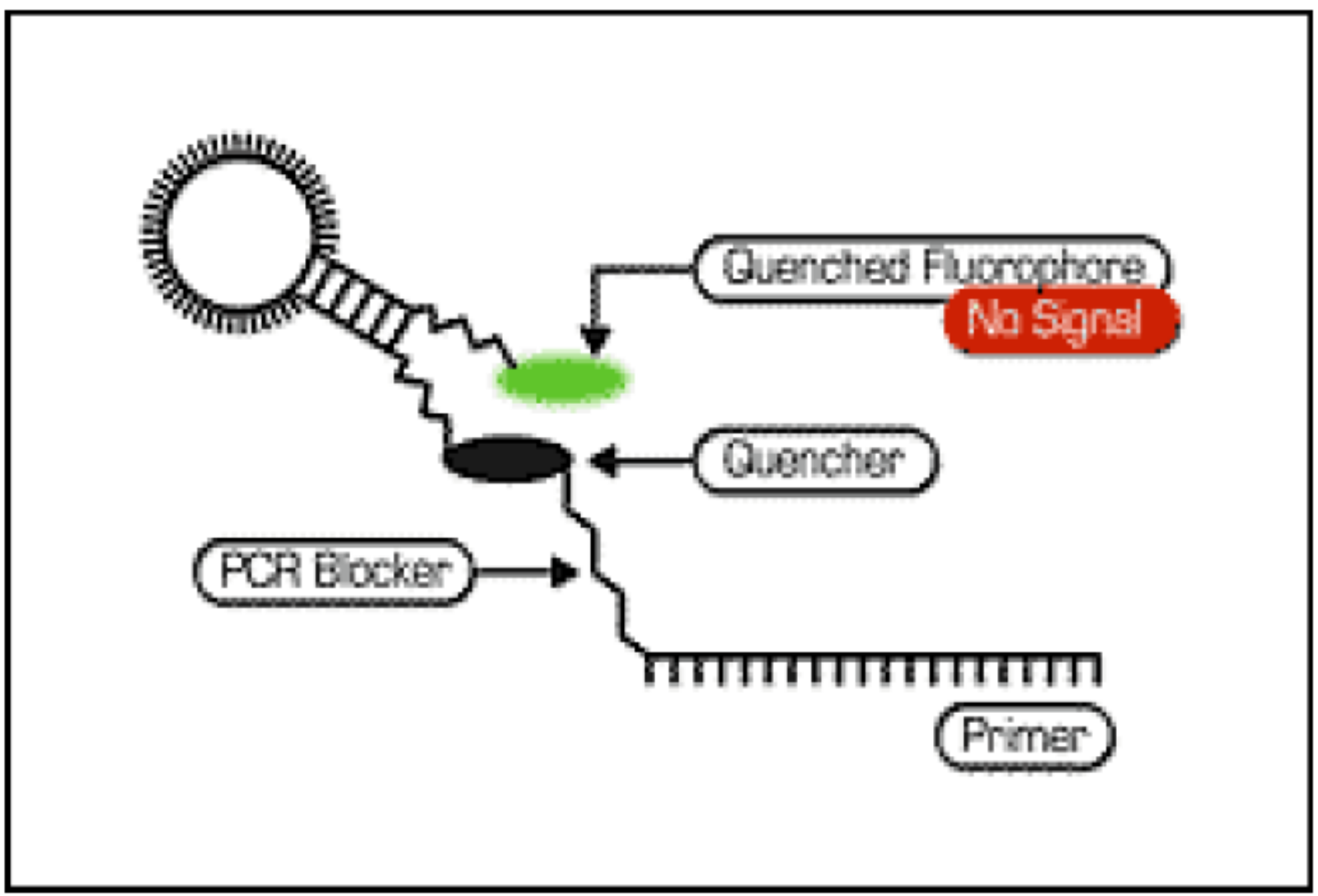

PCR Product-Specific Nucleotides
Fluorescent
Reporter Dye
Quencher Dye
R
Q
R
Q
Specific Nucleotides

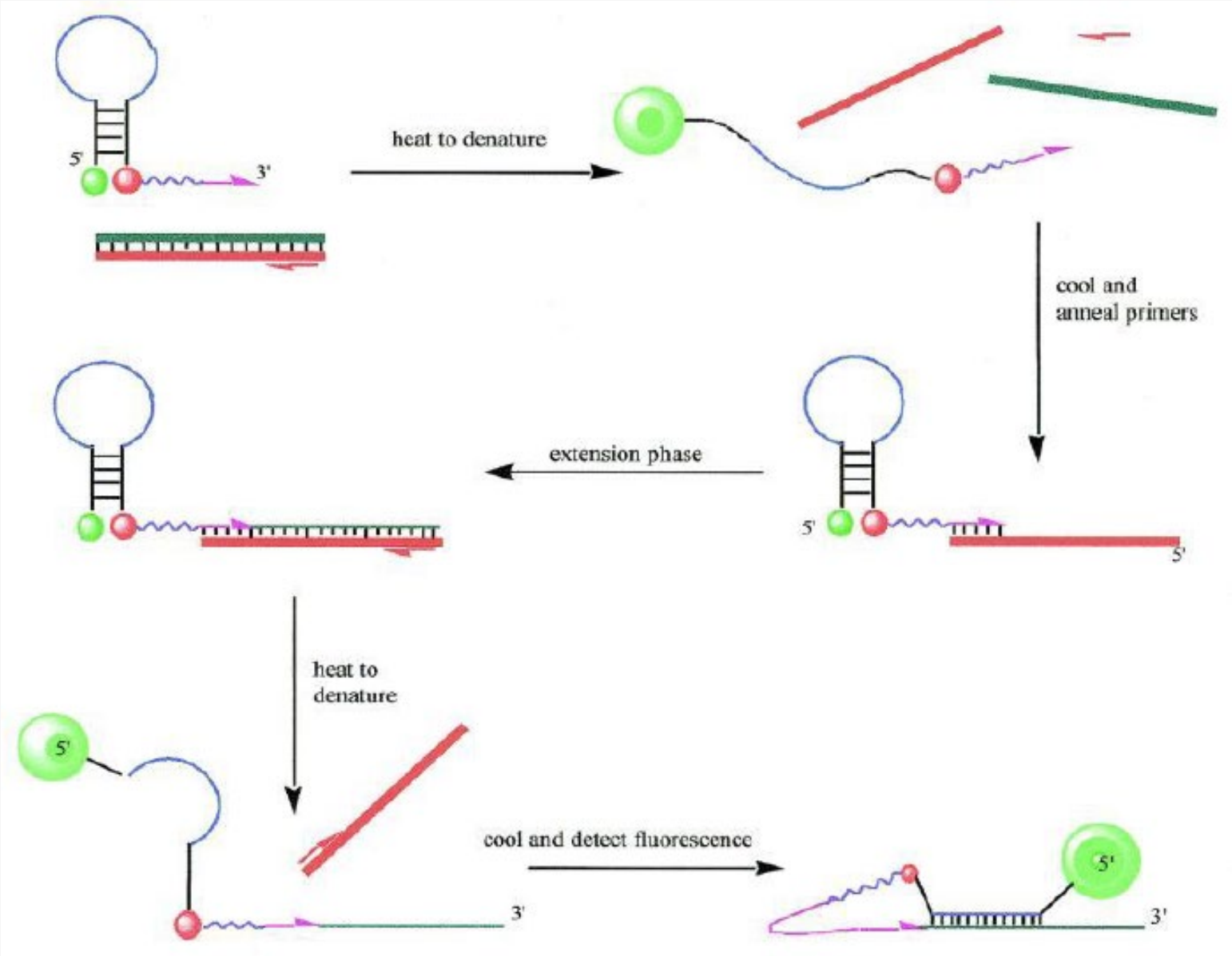
Molecular Beacons

Applications

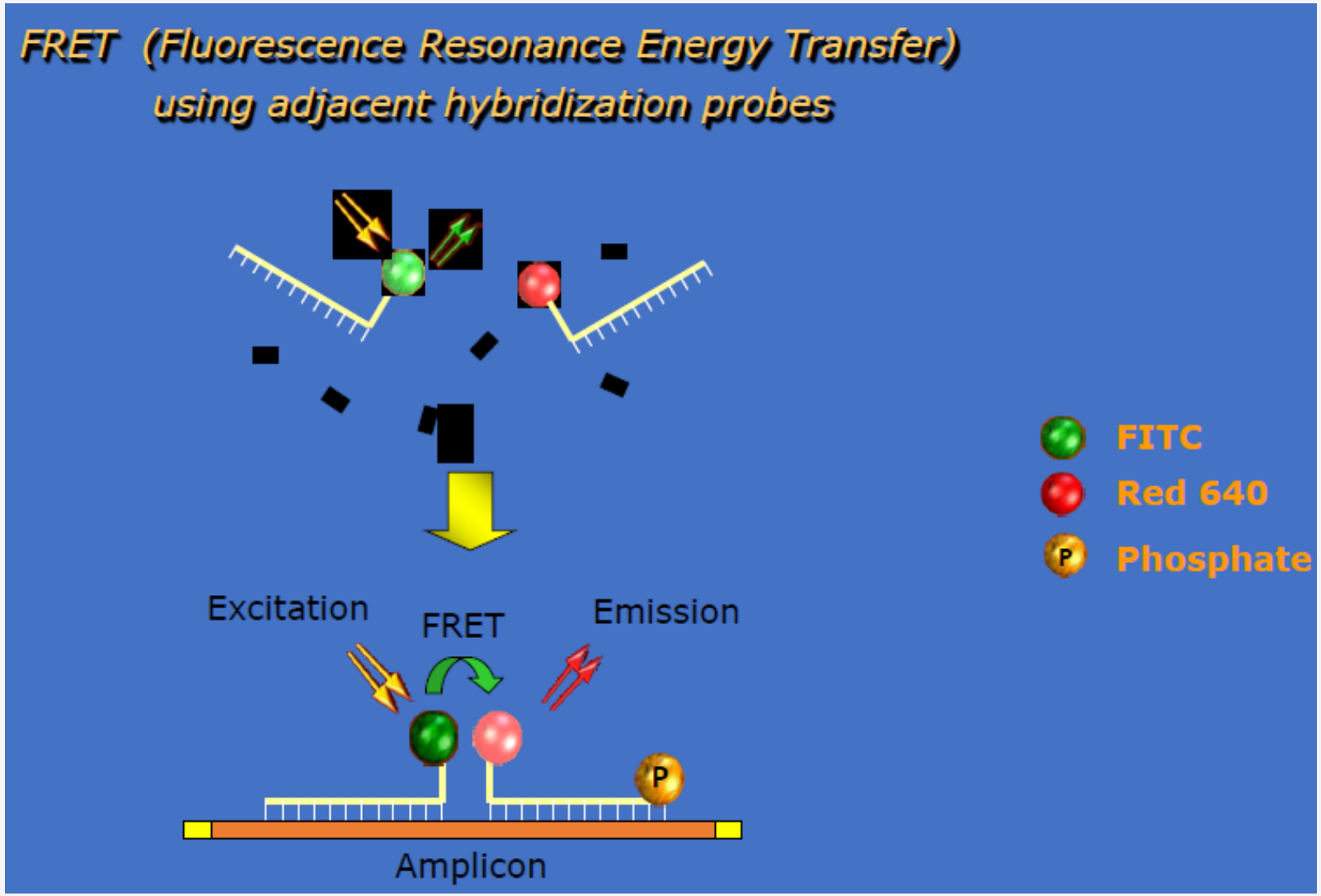
- Detection of amplification products (real time, end point)
- Multicolor beacons detect multiple targets
- Better detection of single point mutation
- Drug resistance analysis
- Non PCR hybridization analysis (in situ labeling)

Scorpions

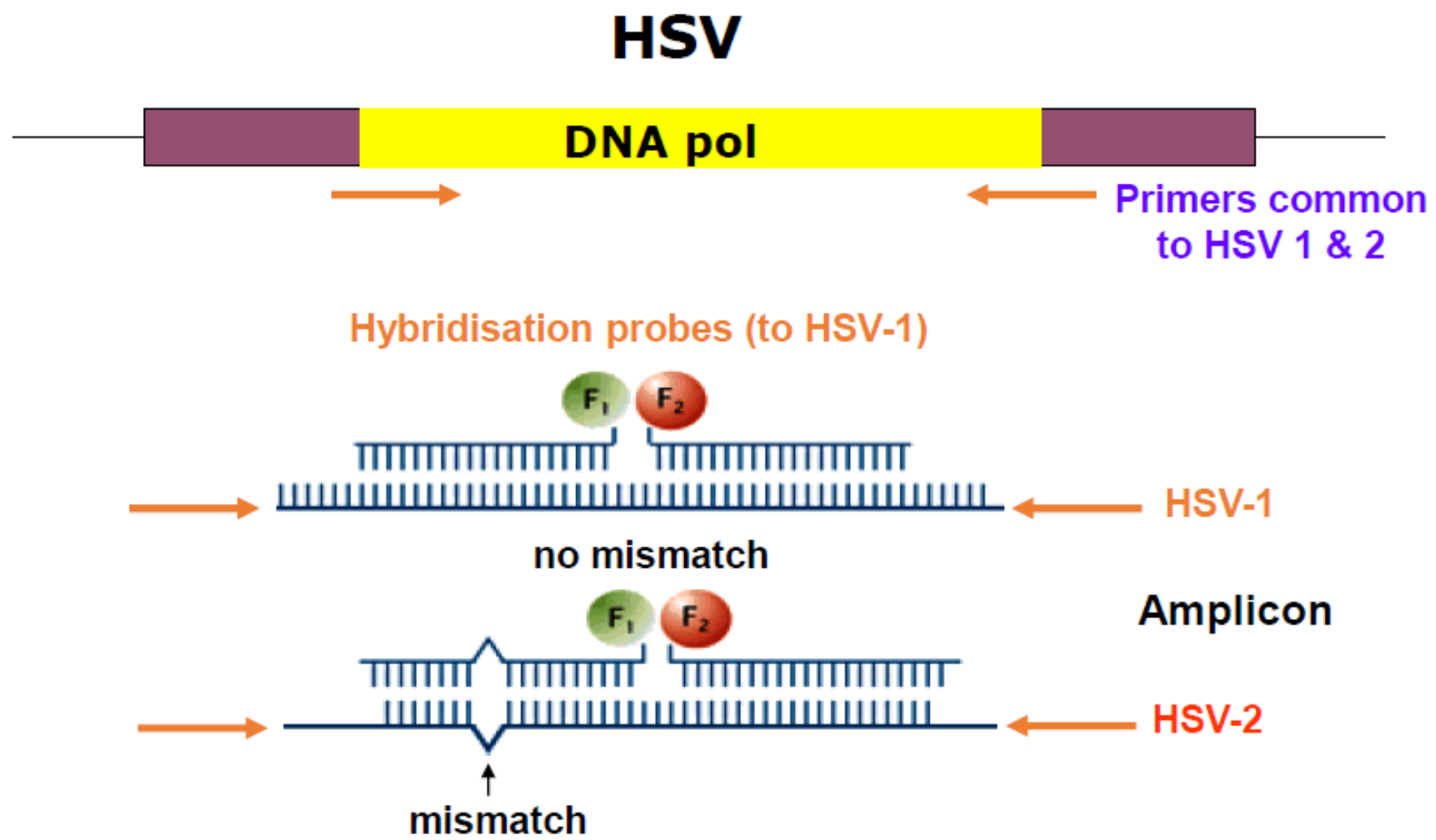

Quenched Fluorophore
No Signal
Quencher
PCR Blocker
Primer

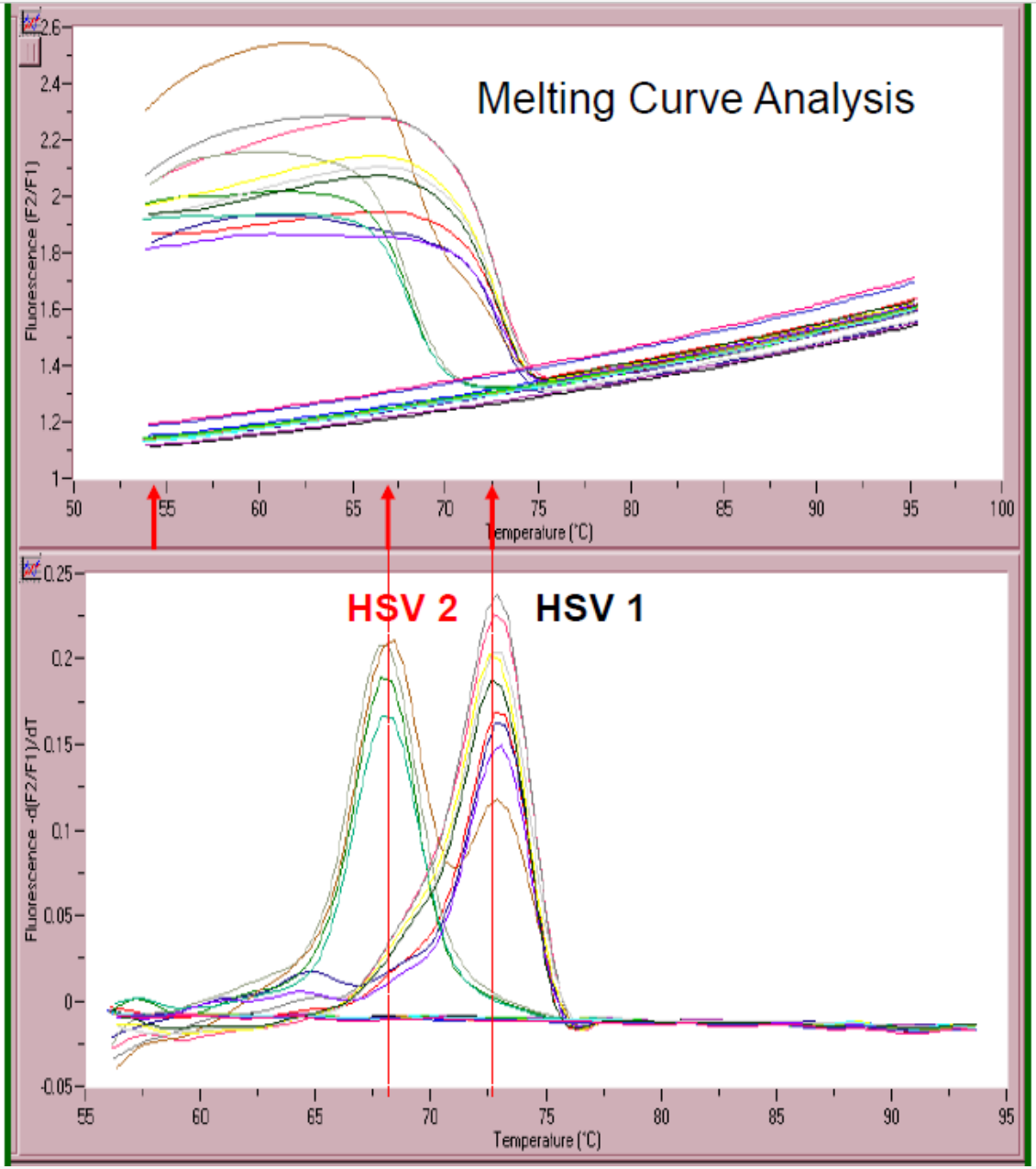

heat to denature
cool and
anneal primers
extension phase
heat to
denature
cool and detect fluorescence
5'
3'

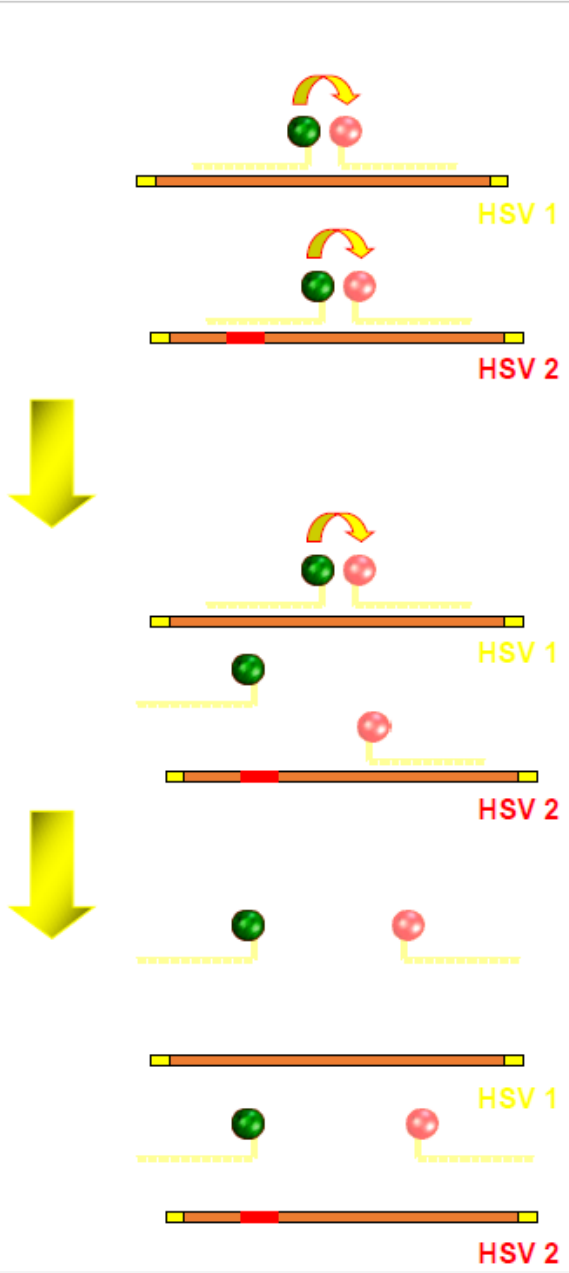
Hybridization probes (Light Cycler)



Characterization of HSV by melting curve




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

Relative Effectiveness of Detection Methods for Common Applications.

Application	SYBR Green I	Dual-Labeled Probes	Molecular Beacons	LightCycler Probes	Scorpions® Probes
Mass Screening	XX				
Microarray Validation	XX	X			
Multiple Target Genes/ Few Samples	X	X			
SNP Detection			X	X	XX
Allelic Discrimination			X	X	XX
Pathogen Detection	X	X	X	X	XX
Multiplexing		XX	XX	X	XX
Viral Load Quantification		X	X	X	XX
Gene Expression Analysis	X	XX	XX	XX	XX
Gene Copy Determination		X	X	X	XX
End-point Genotyping			XX		XX
In vitro Quantification			XX		

Critical concepts and dynamic of reaction

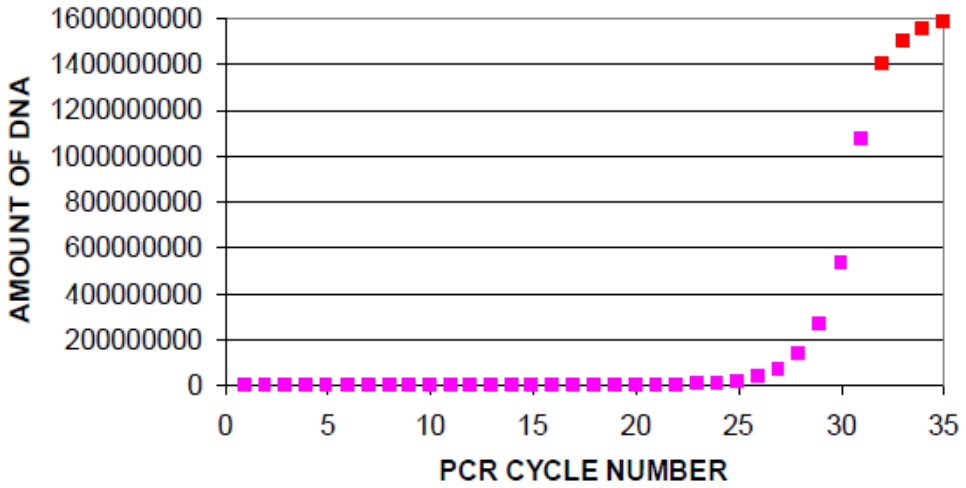
CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29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

PCR kinetics

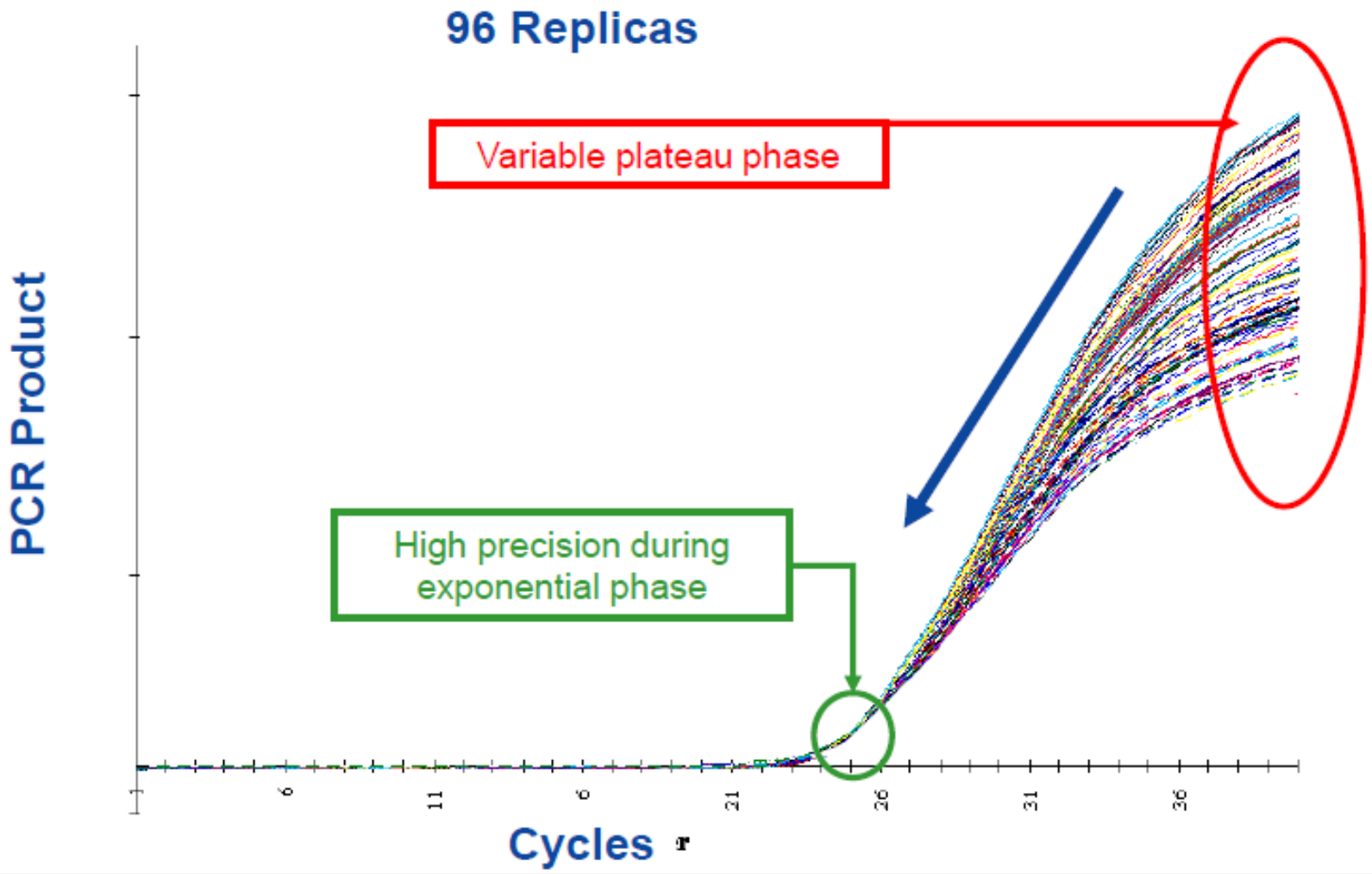
$$N=N_0(2)^n$$

N = Number of DNA molecules
after n cycle of amplification
N_0= Initial number of DNA molecules

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



Time Point of PCR Analysis



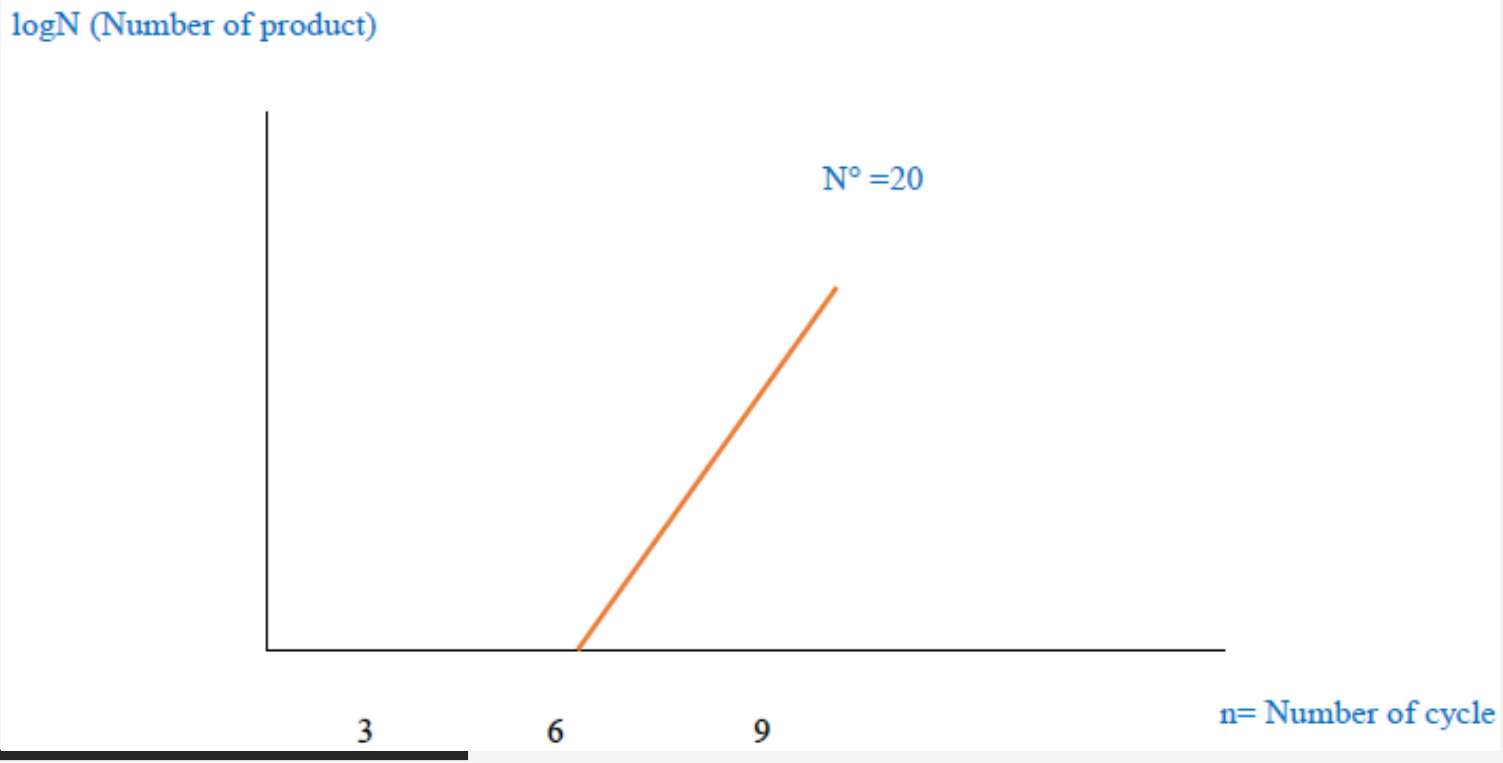
$N = N° \, 2^n$

$\text{Log } N = \text{Log } N° + \text{Log } 2^n$

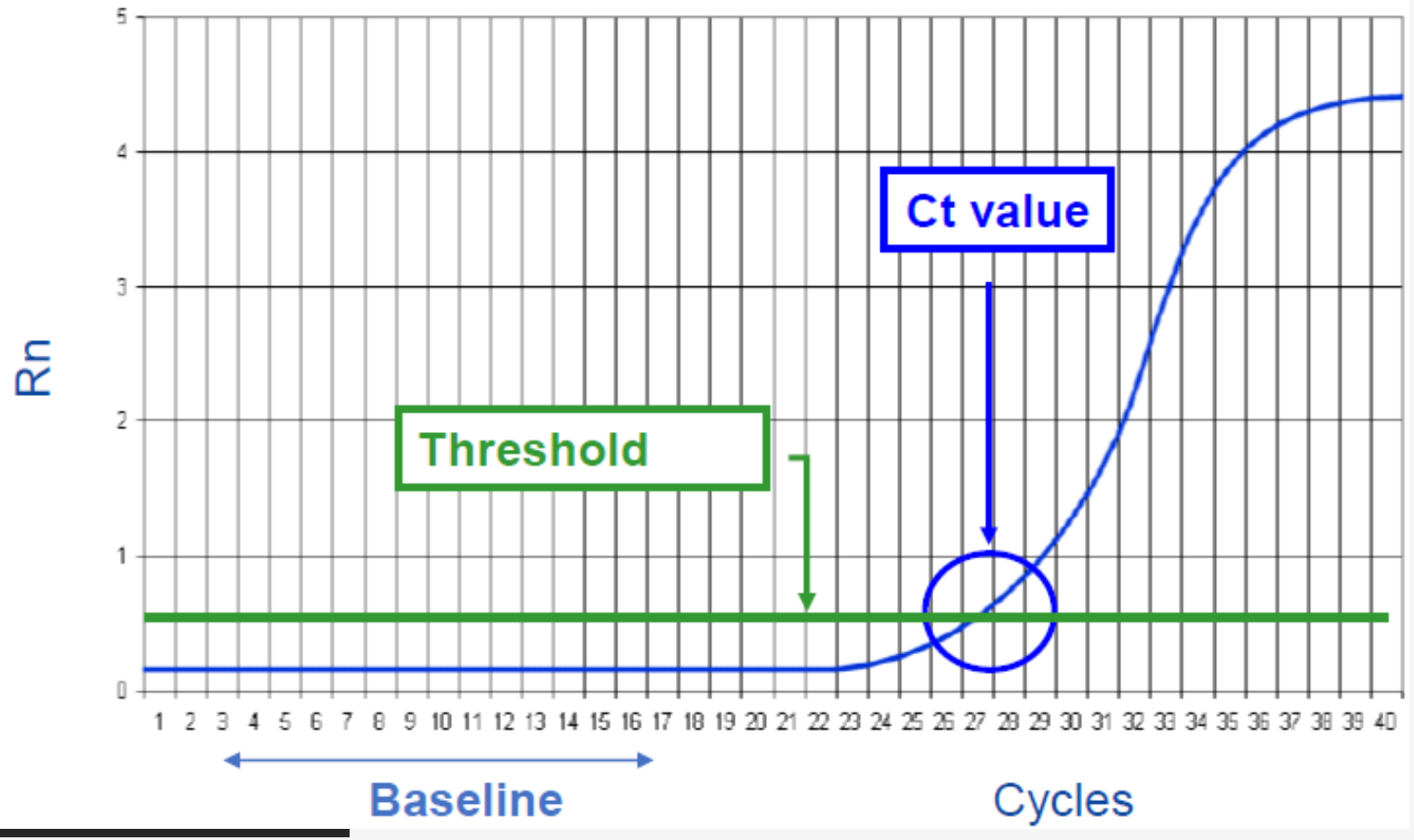
$\text{Log } N = \text{Log } N° + n \text{ Log } 2$

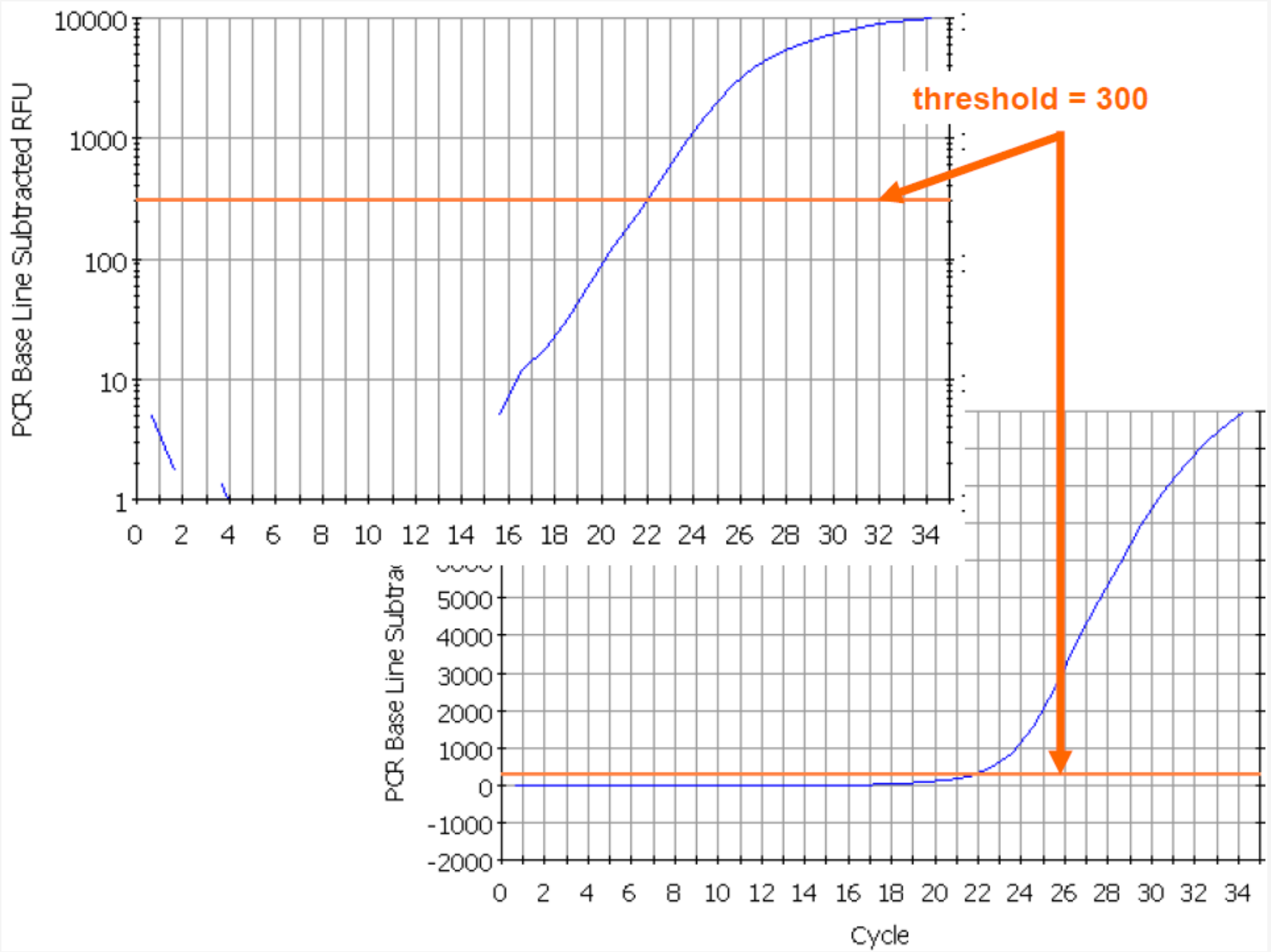
$\text{Log } N = (\text{Log}2)n + \text{Log } N°$

$Y = \quad a X \quad + b$ (For Instance: y= 2x -4)

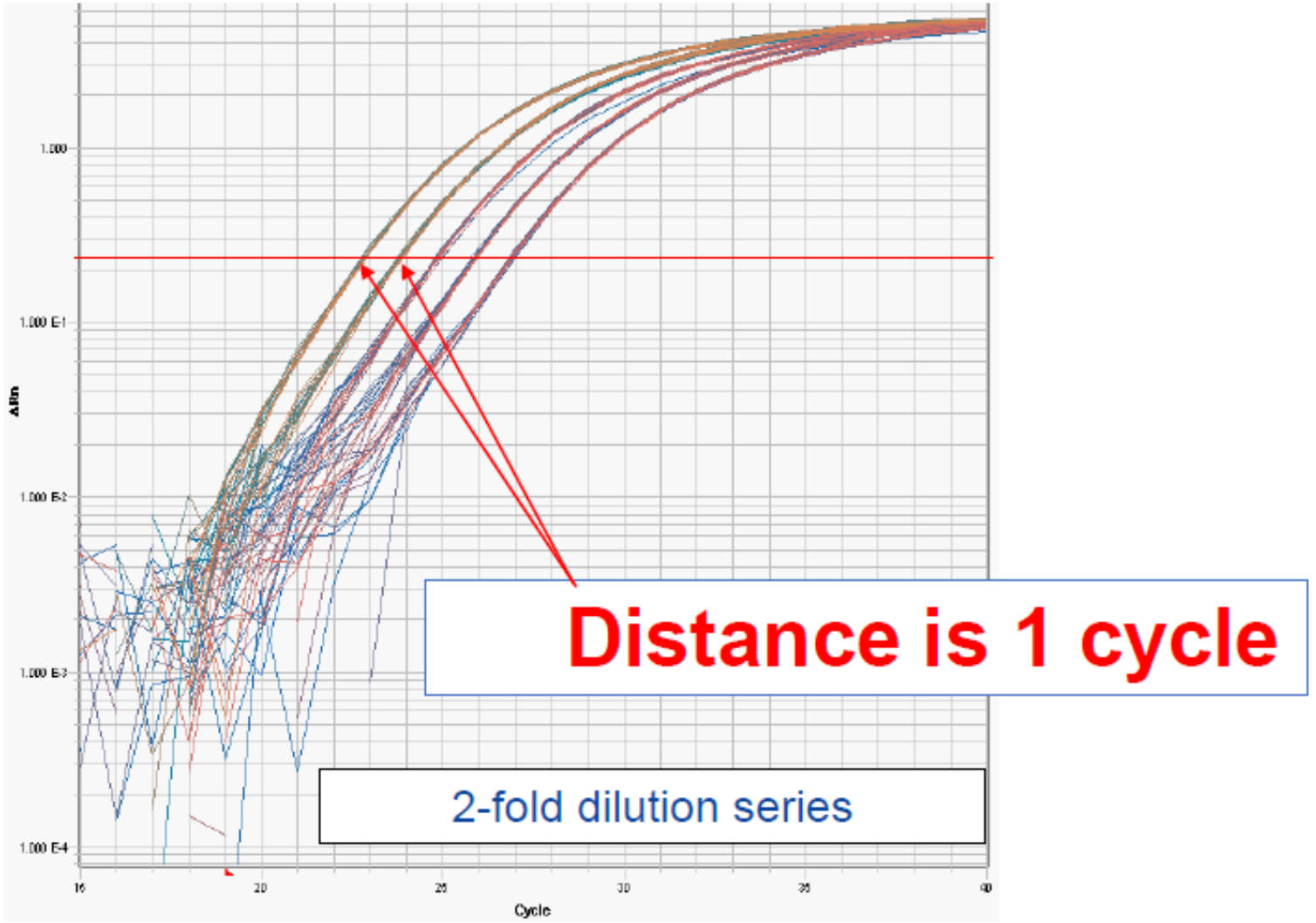


Concept of Threshold and Ct Value




threshold = 300
PCR Base Line Subtracted RFU
10000
1000
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
5000
4000
3000
2000
1000
0
-1000
-2000
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Cycle

Full Efficiency



Effects of efficacy

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

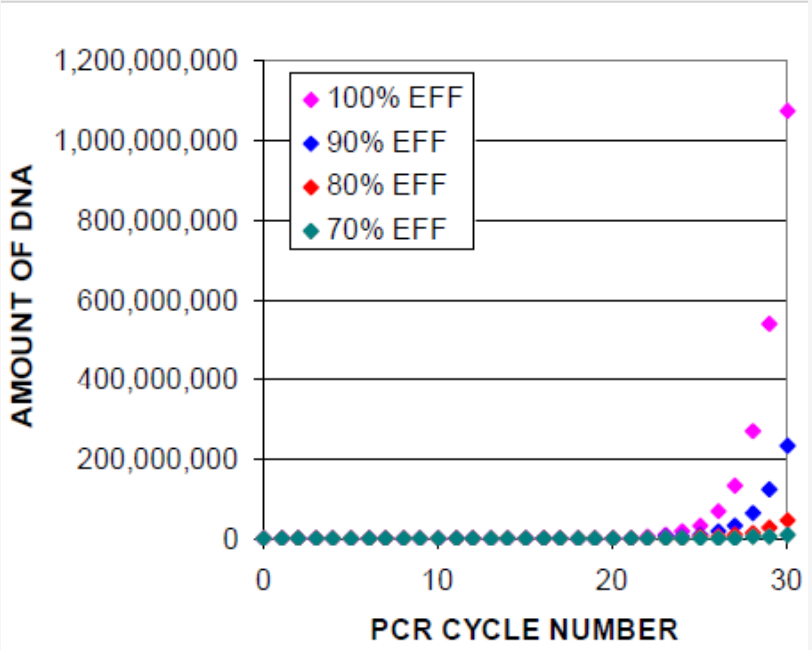
80% = 1.80x

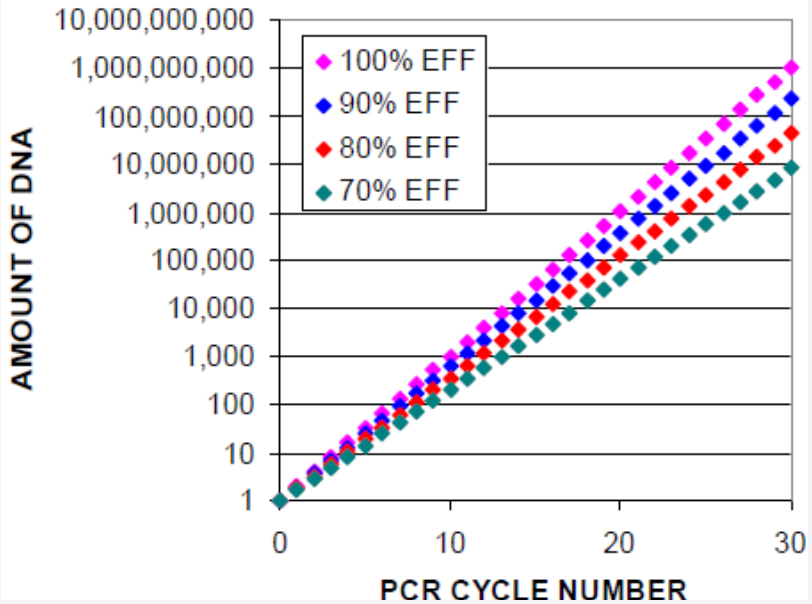
70% = 1.70x

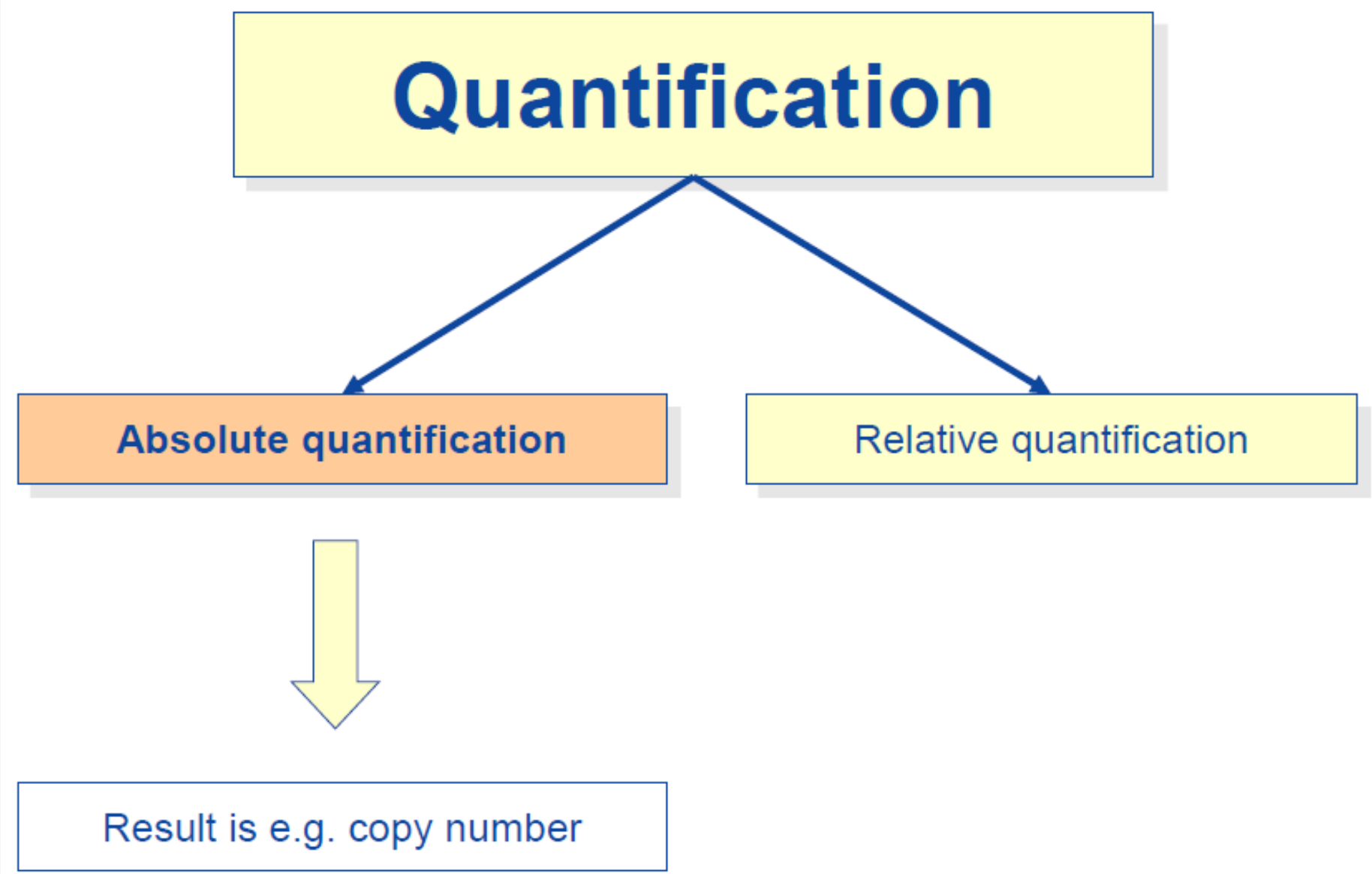
AFTER N CYCLES:

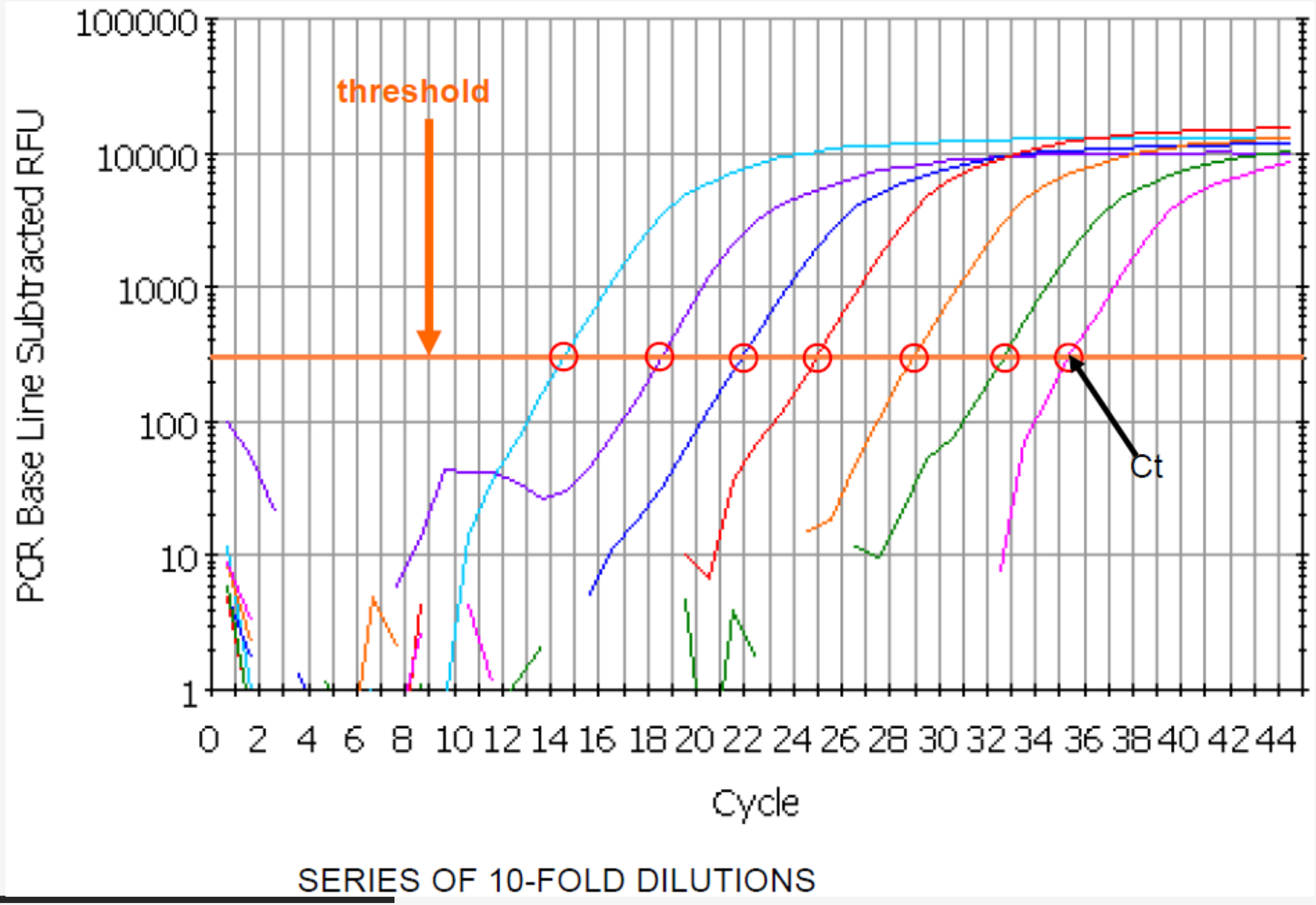
fold increase = (efficiency)^n

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



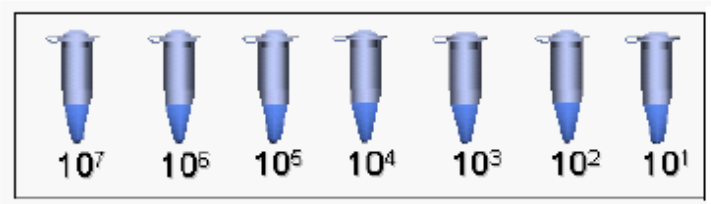


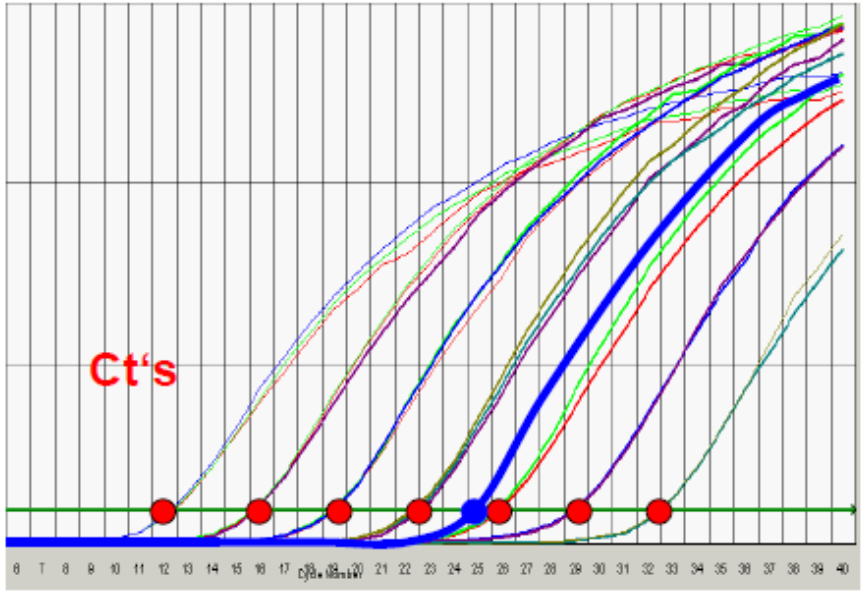

Quantification
Absolute quantification
Relative quantification
Result is e.g. copy number


threshold
PCR Base Line Subtracted RFU
100000
10000
1000
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cycle
Ct
SERIES OF 10-FOLD DILUTIONS

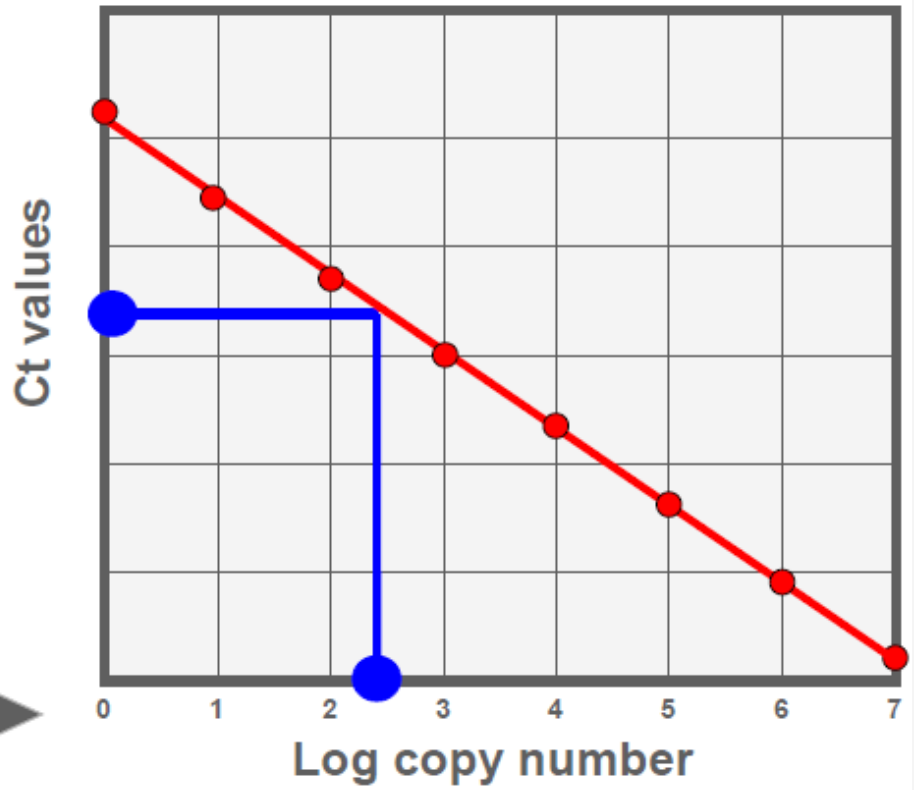
From Fluorescence to Results

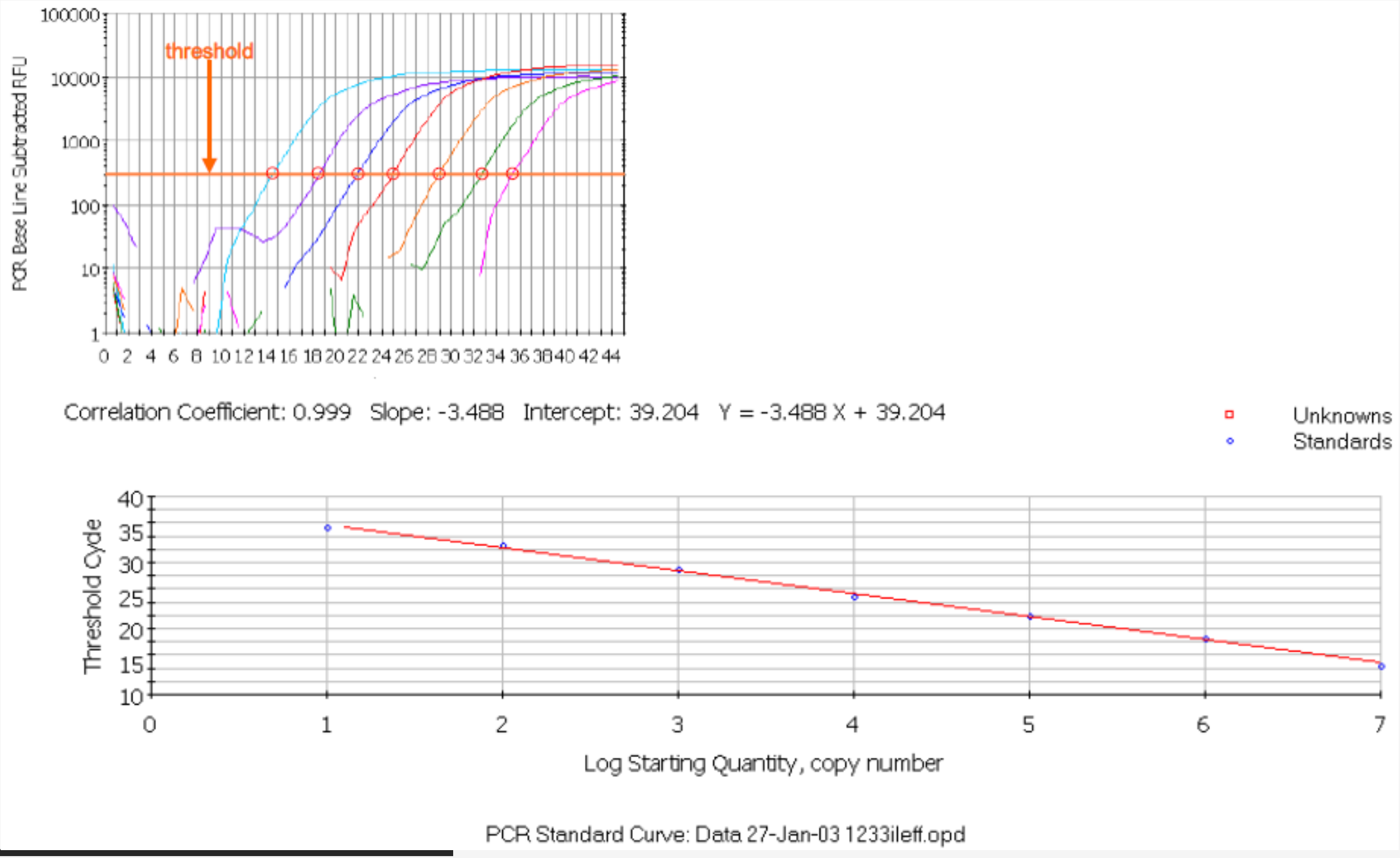
Step 2 „Comparison“ of Ct Values





The well contains
400 target copies




threshold
PCR Base Line Subtracted RFU
100000
10000
1000
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Correlation Coefficient: 0.999 Slope: -3.488 Intercept: 39.204 Y = -3.488 X + 39.204
Unknowns
Standards
Threshold Cycle
40
35
30
25
20
15
10
0 1 2 3 4 5 6 7
Log Starting Quantity, copy number
PCR Standard Curve: Data 27-Jan-03 1233ileff.opd

Linear curve and efficacy

$N = N° \, 2^n$

$\text{Log } N = \text{Log } N° + \text{Log } 2^n$

$\text{Log } N = \text{Log } N° + n \text{ Log } 2$

$(\text{Log}2)n = - \text{Log } N° + \text{Log } N$

$n = (-1/\log 2) \times \text{Log } N° + (1/\log 2) \text{ Log } N$

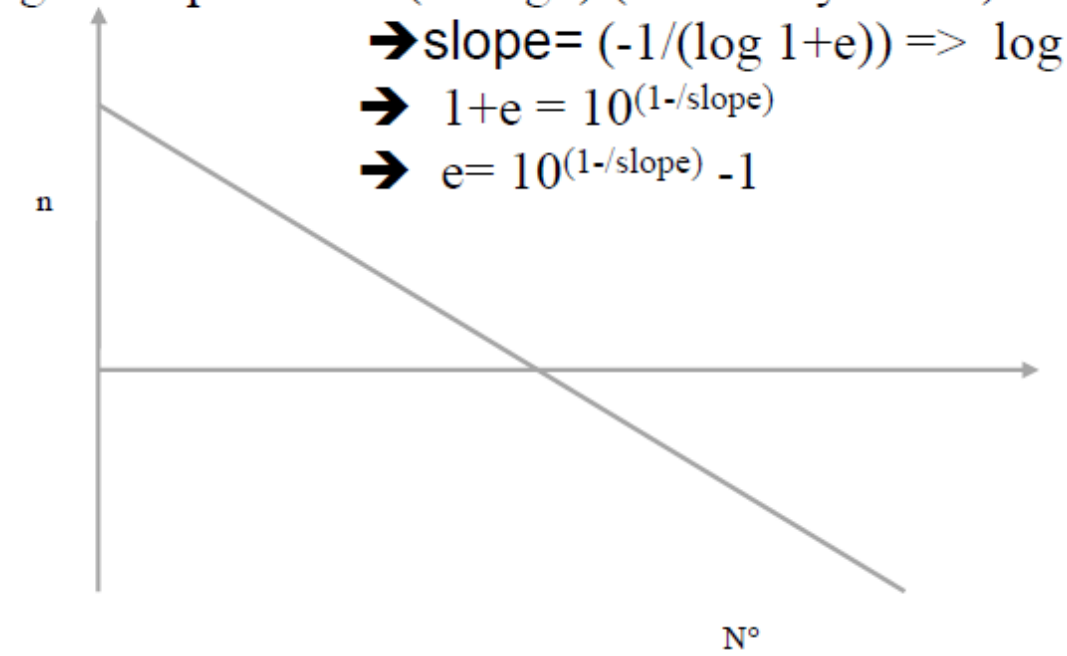
$Y = \quad a X \quad + b$ (For Instance: y= 2x -4)

$tg\alpha = \text{slope} = a = (-1/\log 2)$ (efficiency 100%)

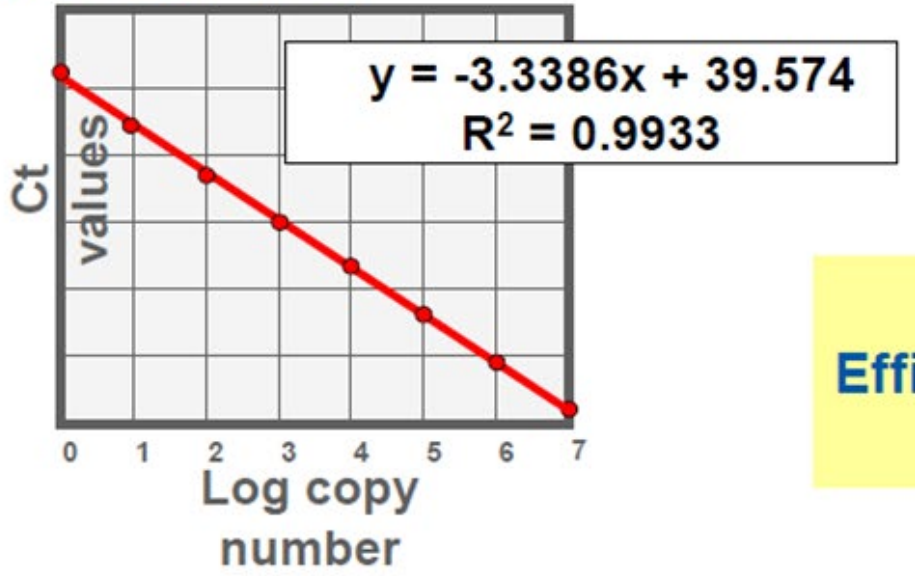
➔ slope= $(-1/(\log 1+e)) \Rightarrow \log (1+e) = -1/\text{slope}$

➔ $1+e = 10^{(1-/\text{slope})}$

➔ $e = 10^{(1-/\text{slope})} - 1$



Standard Curve



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

Example:

Slope = -3.3386

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

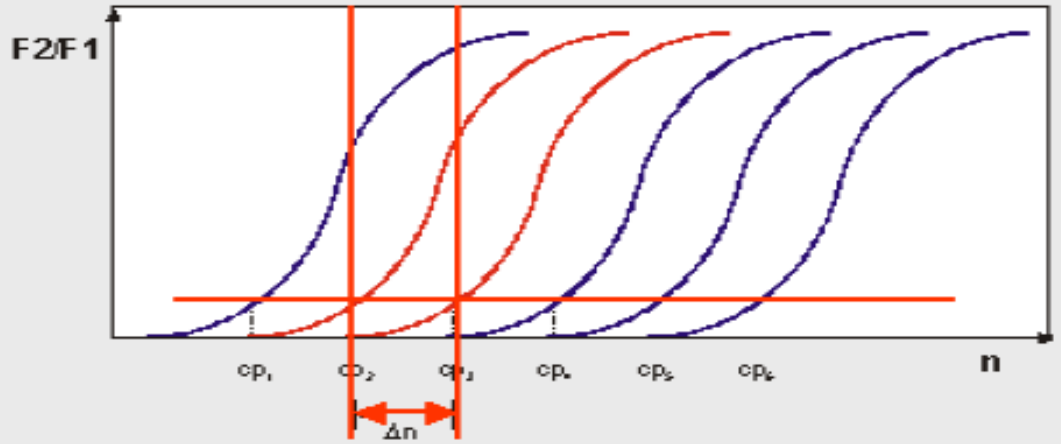
$= 10^{(0.30)} - 1$

$= 1.955 - 1$

$= 0.995$ or 99.5%

Calculation of real-time PCR efficiency

$E = 10^{-1/slope}$ => $E = 10^{-1/-3.337}$ => $E = 10^{0.299}$ => $E = 1.99$



$\Delta CP = \Delta n$

$N_2 = N_{0,2} \times E^{n_2}$ $N_3 = N_{0,3} \times E^{n_3}$

For $N_2 = N_3$:

$N_{0,2} / N_{0,3} = E^{(n_3 - n_2)} = E^{\Delta n}$

For $N_{0,2} = 10 \times N_{0,3}$ (10-fold dilutions):

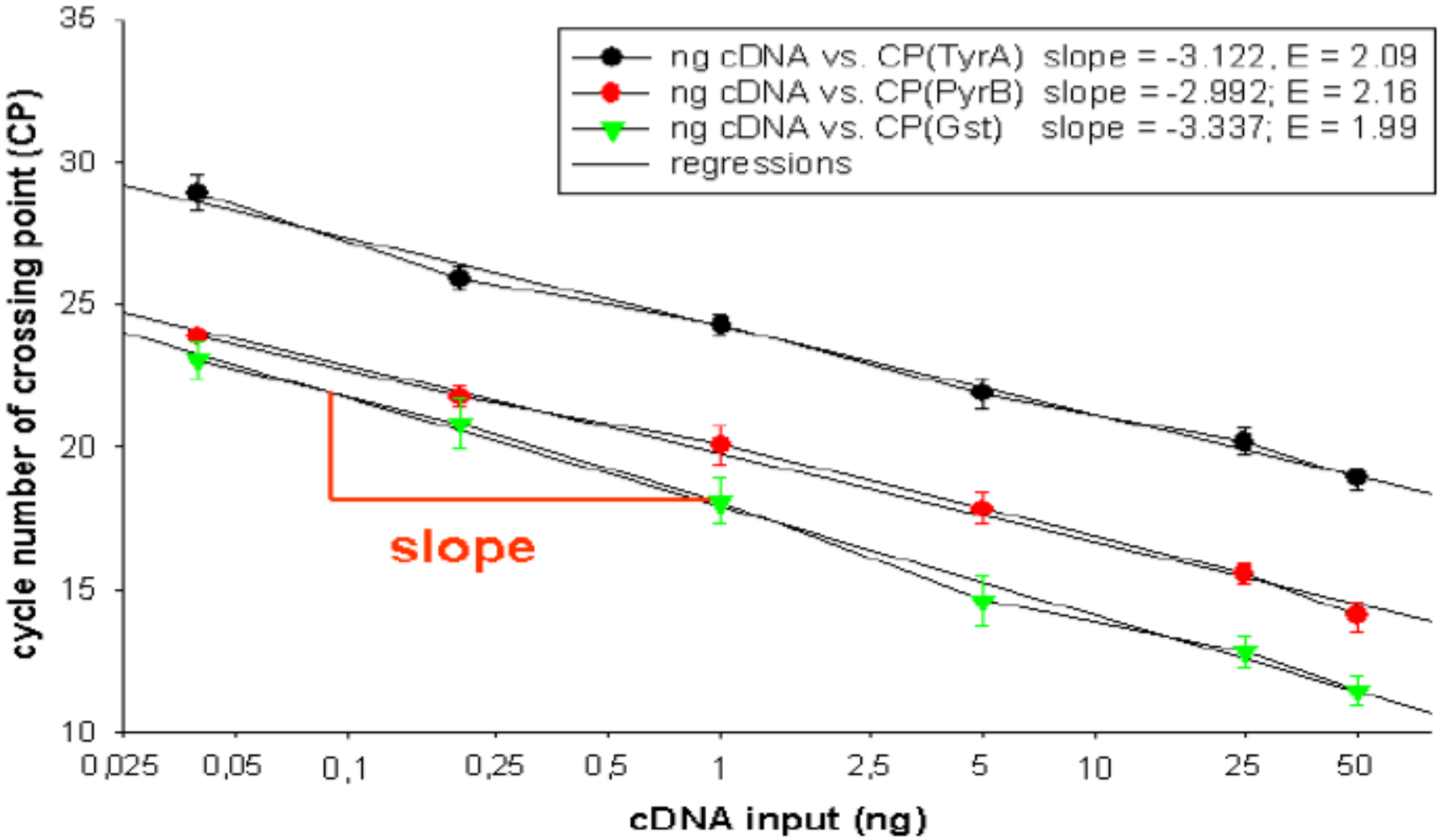
$\Delta n = 1 / \log E$ $E = 10^{-1/slope}$

e.g. $E = 2.0$ $\Delta n = 3.32$
$E = 1.9$ $\Delta n = 3.58$
$E = 1.8$ $\Delta n = 3.91$

Roche Diagnostics, LC rel. Quantification software, March 2001

Rasmussen, R (2001) *Quantification on the LightCycler. In: Meuer, S, Wittwer, C, Nakagawara, K, eds. Rapid Cycle Real-time PCR, Methods and Applications Springer Press, Heidelberg; page 21-34*

Calculation of real-time PCR efficiency



Gene.Quantifiaction@wzw.tum.de © 2002

Full Efficiency

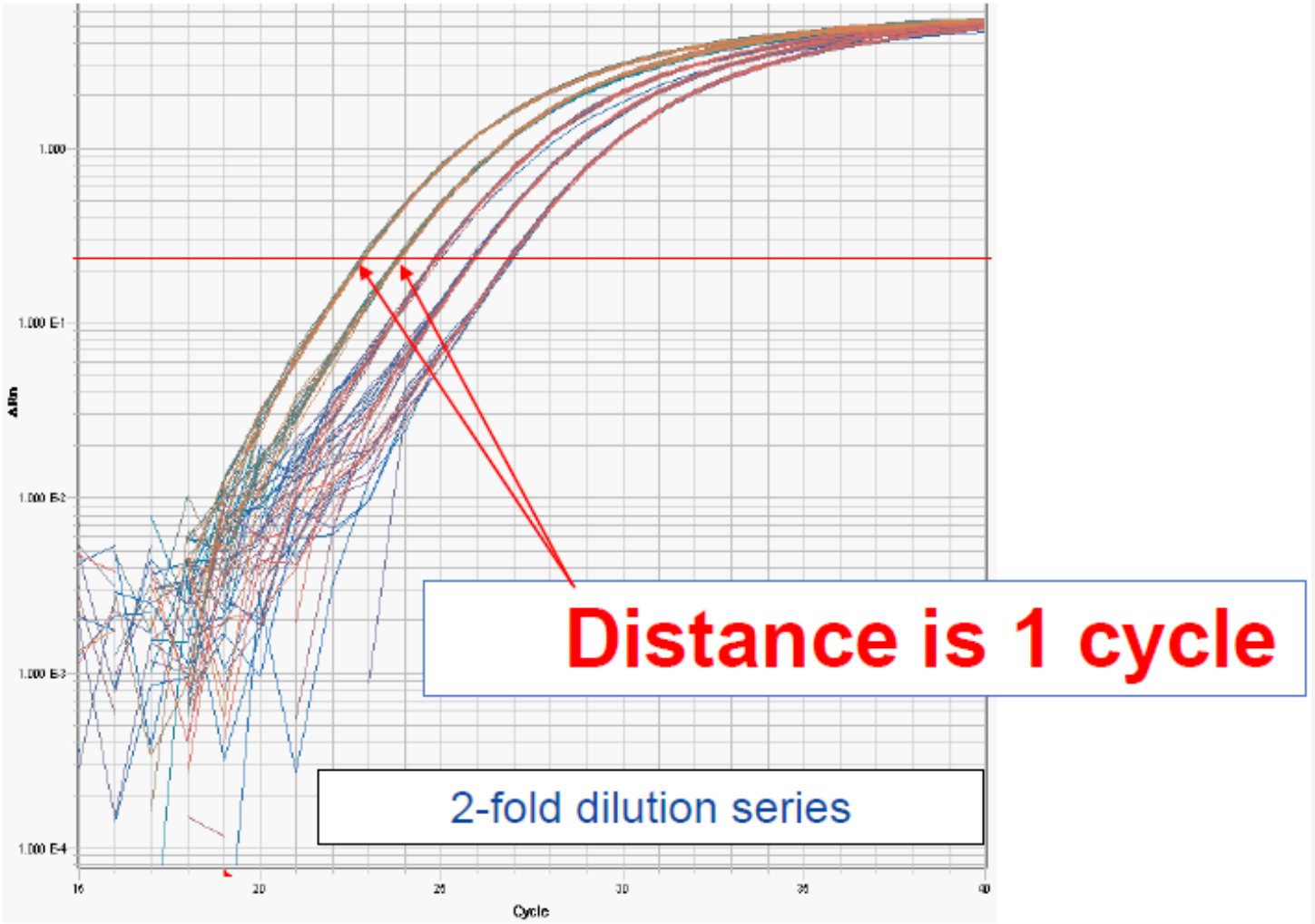
$$y = x\,(1+E)^n$$

If PCR efficiency is 1, the equation becomes

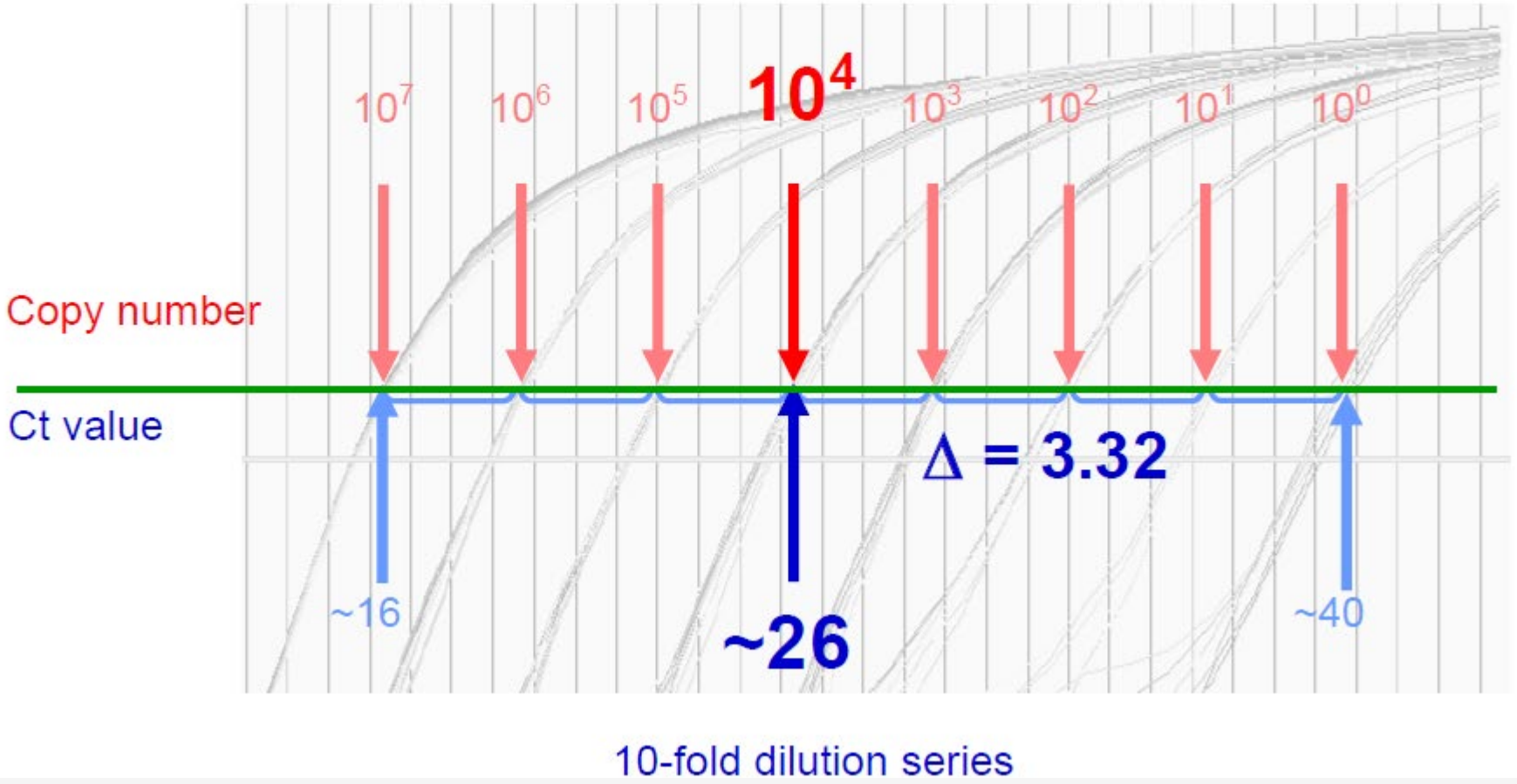
$$y = x\,2^n$$

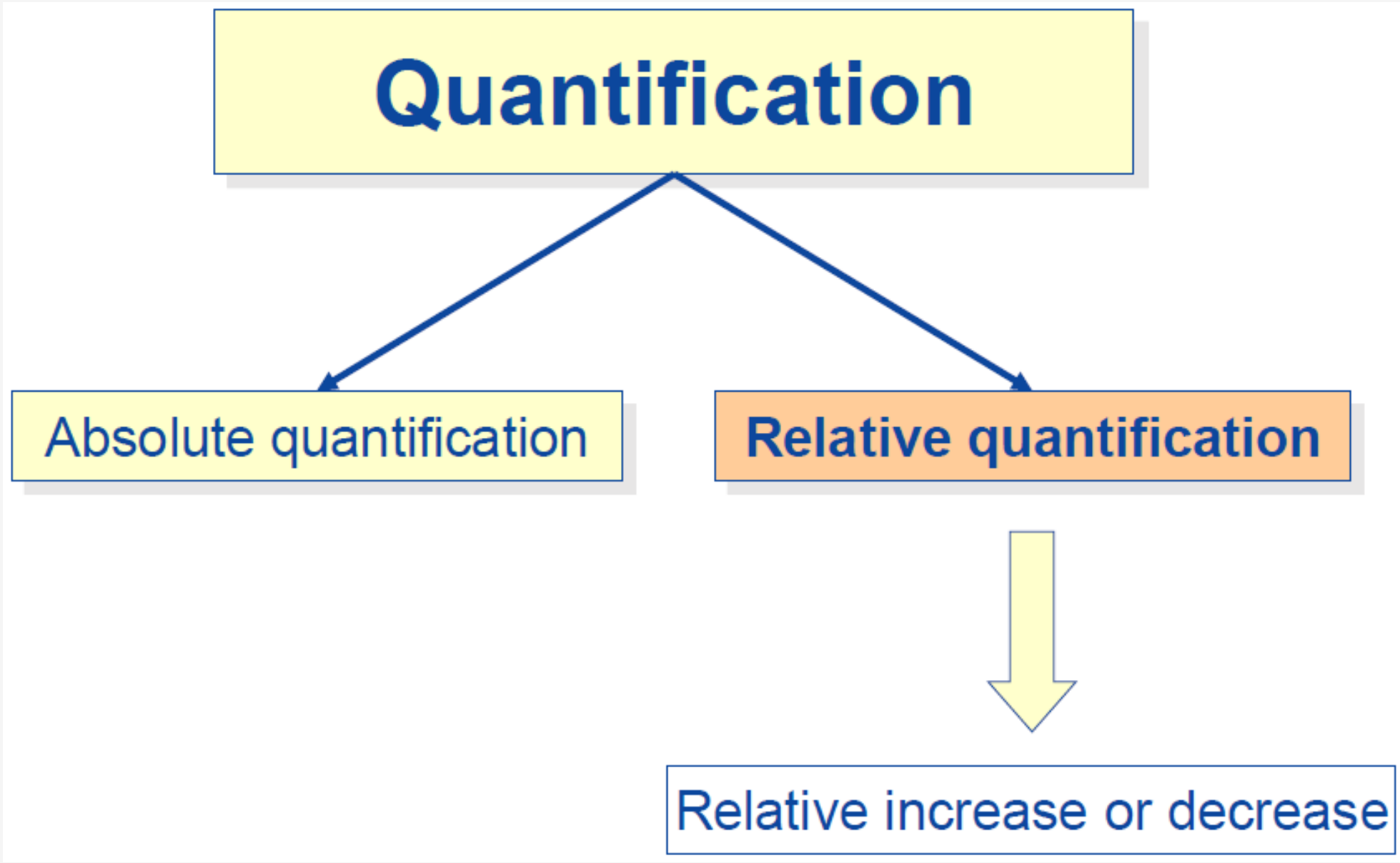
y = Number of PCR products
x = Initial target copy number
E = Efficiency
n = Number of PCR cycles

Full Efficiency



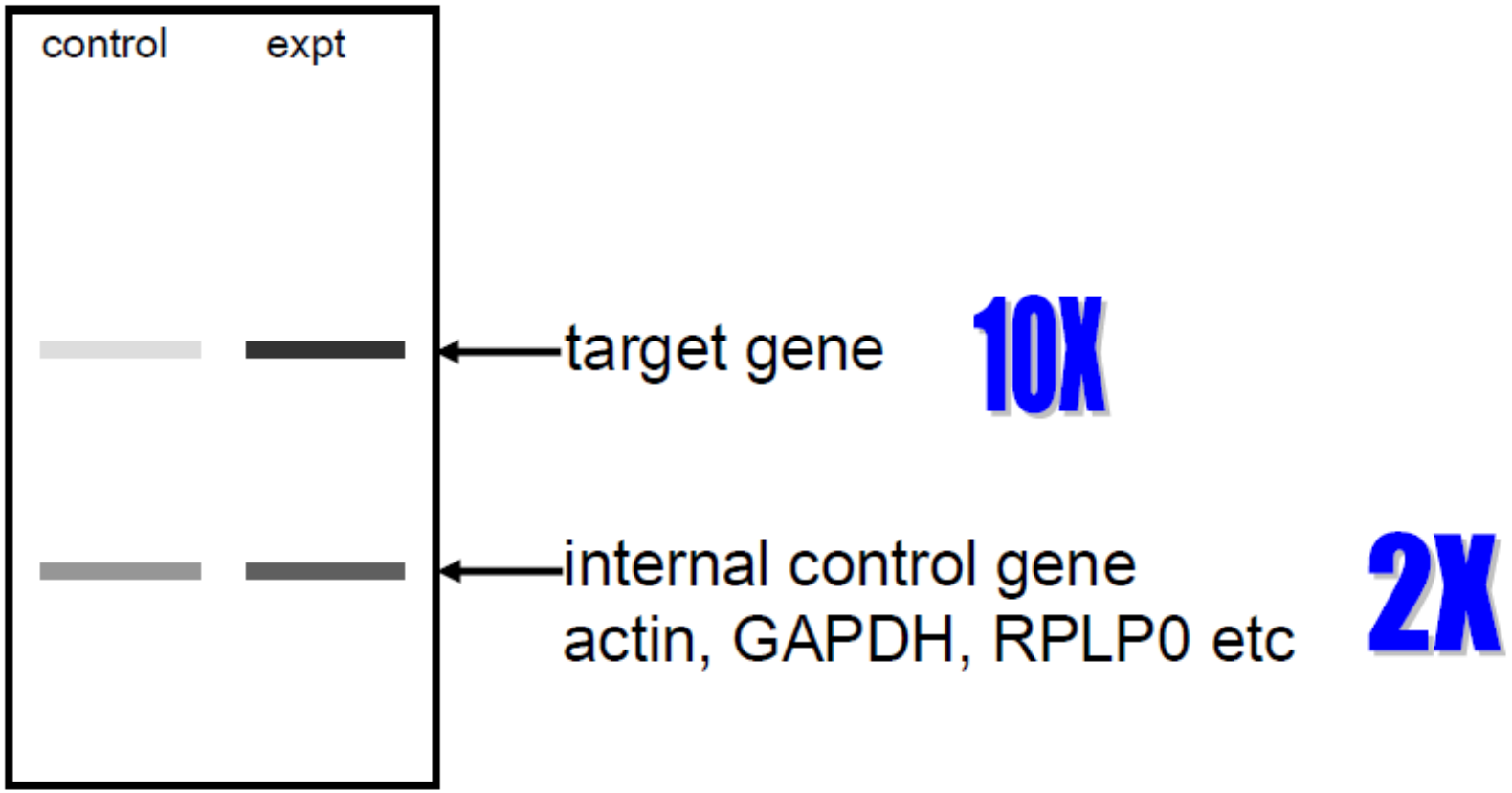
Full Efficiency




Quantification
Absolute quantification
Relative quantification
Relative increase or decrease

- Most sensitive
- Can discriminate closely related mRNAs
- Technically simple
- But difficult to get truly quantitative results using conventional PCR

NORTHERN



Corrected fold increase = 10/2 = 5

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

Table 7. Housekeeping genes commonly used as endogenous references

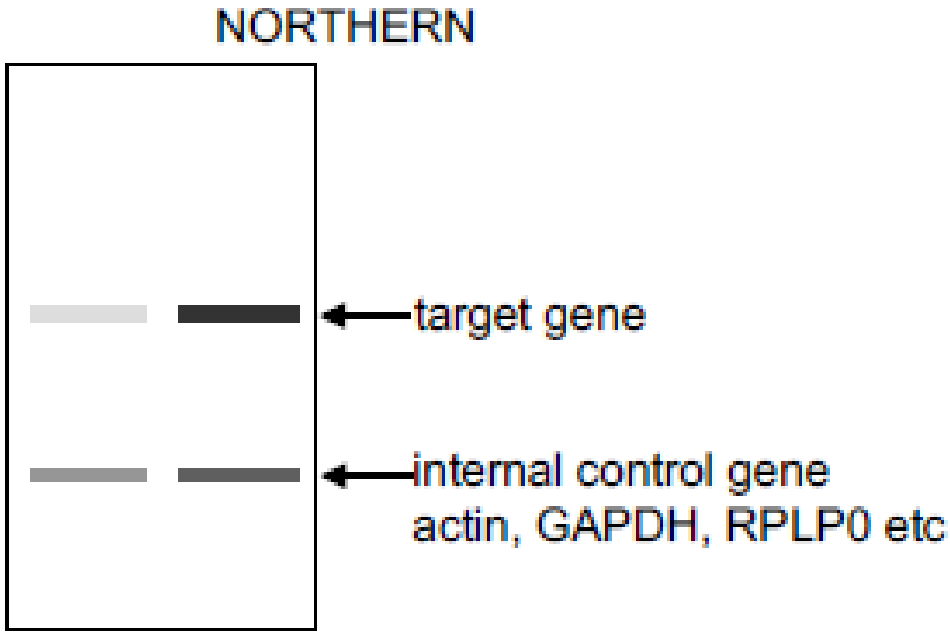
Gene	Gene symbol		Relative expression level*	
	Human	Mouse	Human	Mouse
18S ribosomal RNA	RRN18S	Rn18s	++++	++++
Actin, beta	ACTB	Actb	+++	+++
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Gapdh	+++	+++
Phosphoglycerate kinase 1	PGK1	Pgk1	+++	++
Peptidylprolyl isomerase A	PPIA	Ppia	+++	+++
Ribosomal protein L13a	RPL13A	Rpl13a	+++	+++
Ribosomal protein, large, P0	RPLP0		+++	
Acidic ribosomal phosphoprotein PO		Arbp		+++
Beta-2-microglobulin	B2M	B2m	++ – +++	++ – +++
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	YWHAZ	Ywhaz	++ – +++	+
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	SDHA	Sdha	++	+
Transferrin receptor	TFRC	Tfrc	++	+
Aminolevulinate, delta-, synthase 1	ALAS1	Alas1	+	+
Glucuronidase, beta	GUSB	Gusb	+	+
Hydroxymethylbilane synthase	HMBS	Hmbs	+	++ – +++
Hypoxanthine phosphoribosyltransferase 1	HPRT1	Hprt1	+	+
TATA box binding protein	TBP	Tbp	+	+
Tubulin, beta	TUBB		+	
Tubulin, beta 4		Tubb4		+

* "+" indicates relative abundance of the transcripts.

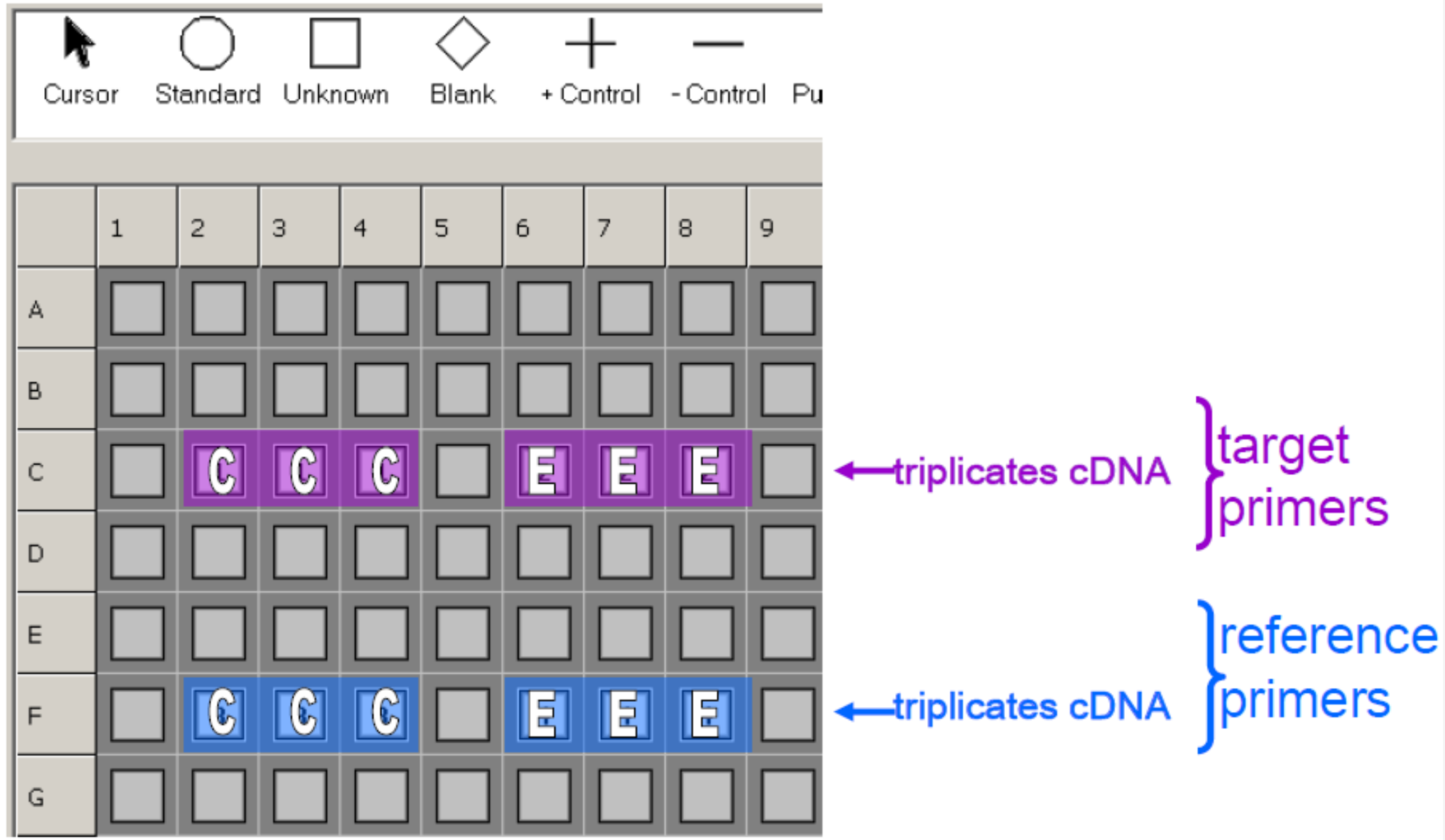
Pfaffl method

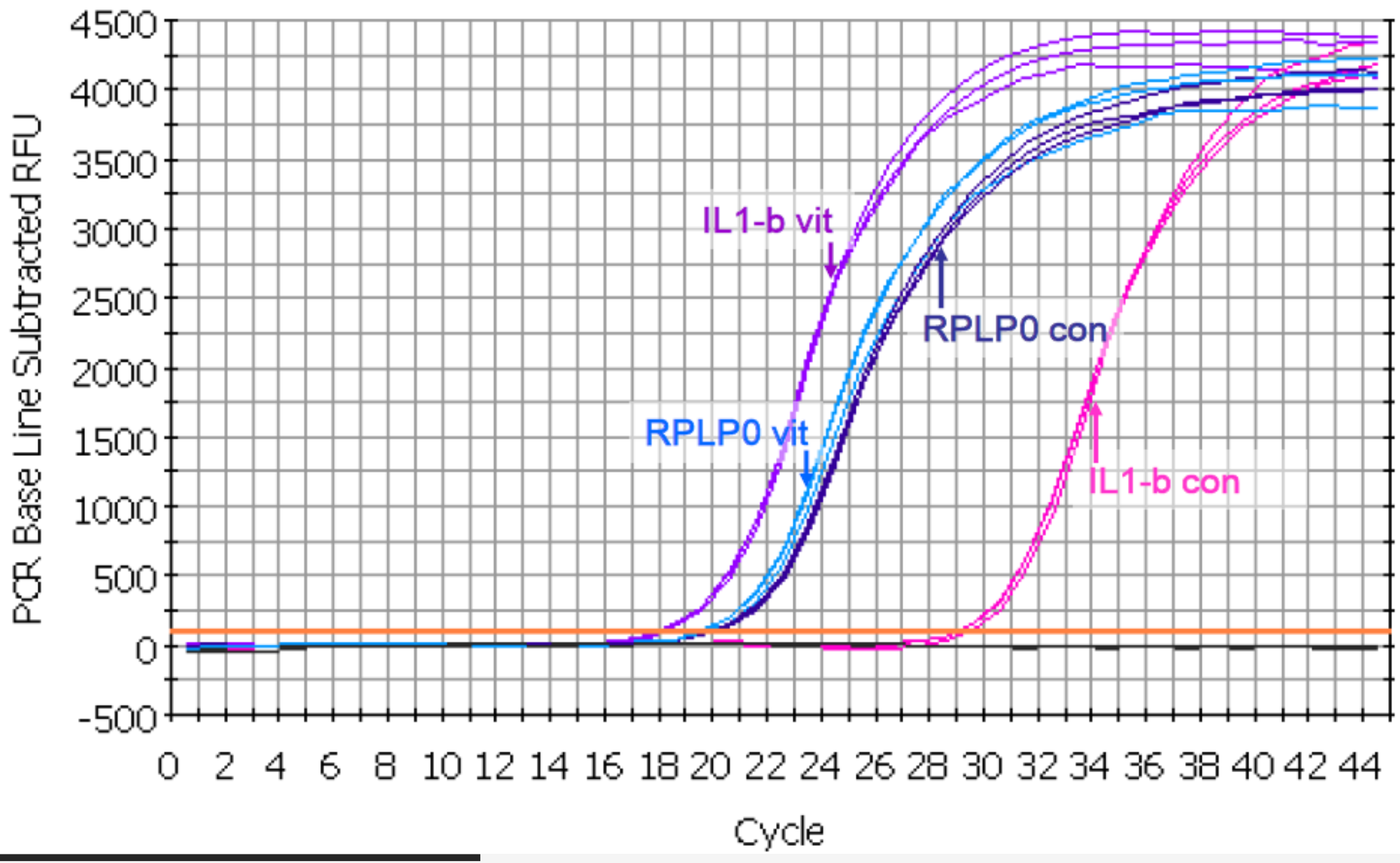
–M.W. Pfaffl, Nucleic Acids Research 2001 29:2002-2007

Pfaffl method



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

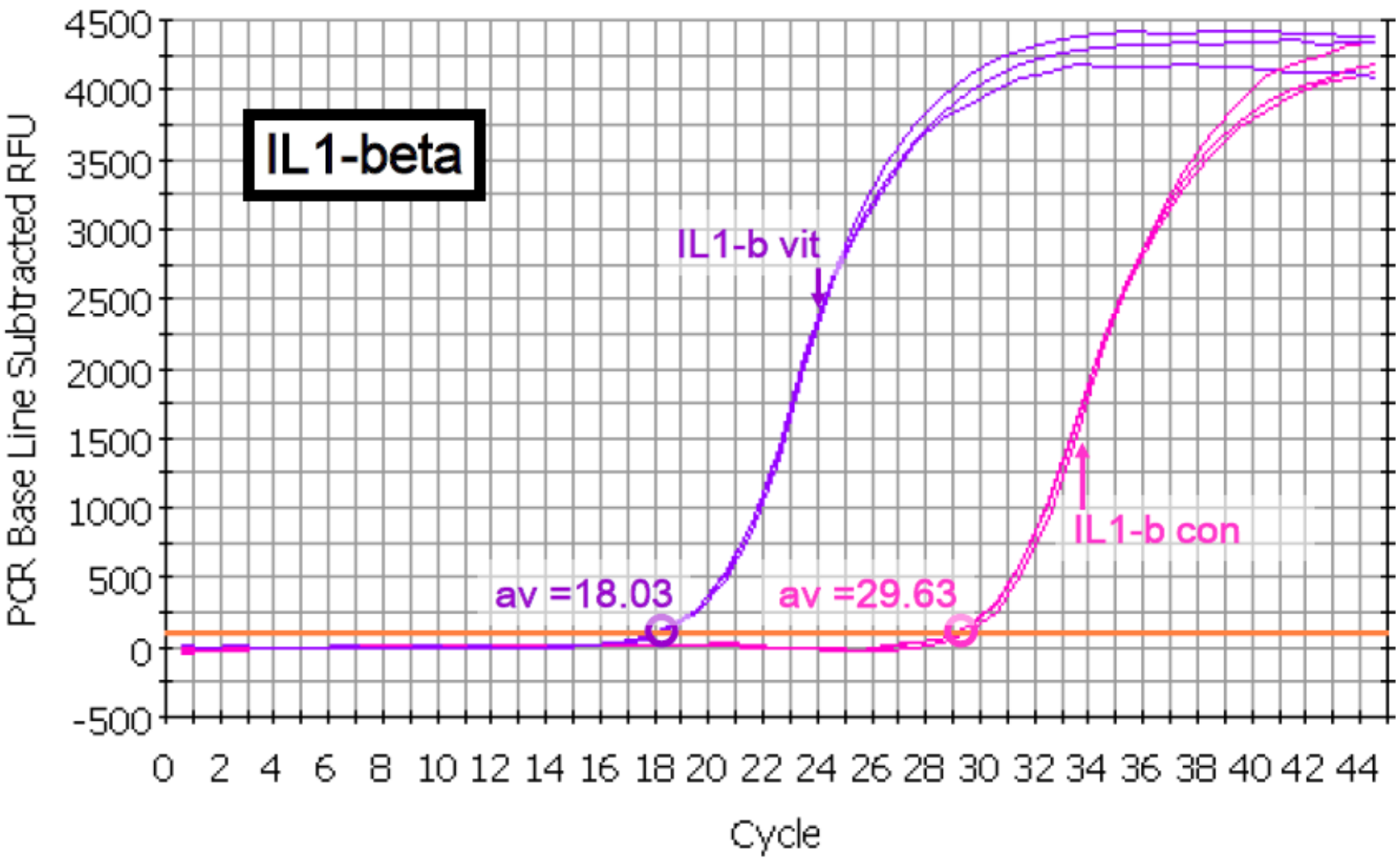

Cursor
Standard
Unknown
Blank
+ Control
- Control
Pu
1
2
3
4
5
6
7
8
9
A
B
C
D
E
F
G
C C C
E E E
C C C
E E E
triplicates cDNA
target primers
triplicates cDNA
reference primers


PCR Base Line Subtracted RFU
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cycle
IL1-b vit
RPLP0 con
RPLP0 vit
IL1-b con

$$N = No\,(2)^{n} \rightarrow N_1 = N_2 \rightarrow No_1(2)^{n1} = No_2\,(2)^{n2}$$

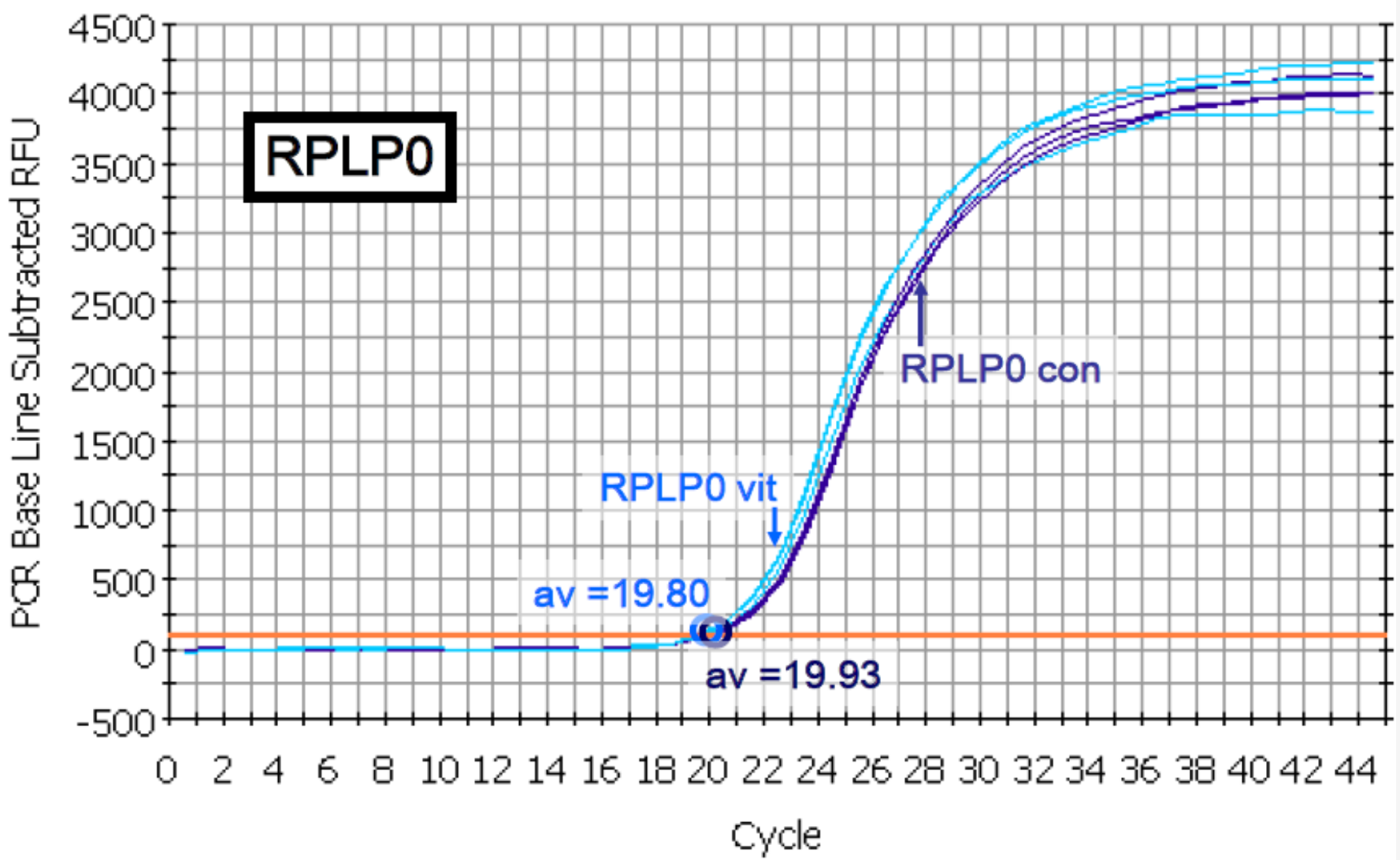
$$\frac{No_1}{No_2} = \frac{(2)^{n2}}{(2)^{n1}} \rightarrow \frac{No_1}{No_2} = (2)^{n2-n1} \rightarrow \frac{No_1}{No_2} = (2)^{\Delta ct}$$

$$\rightarrow \frac{No_1}{No_2} = (1+E)^{\Delta ct}$$



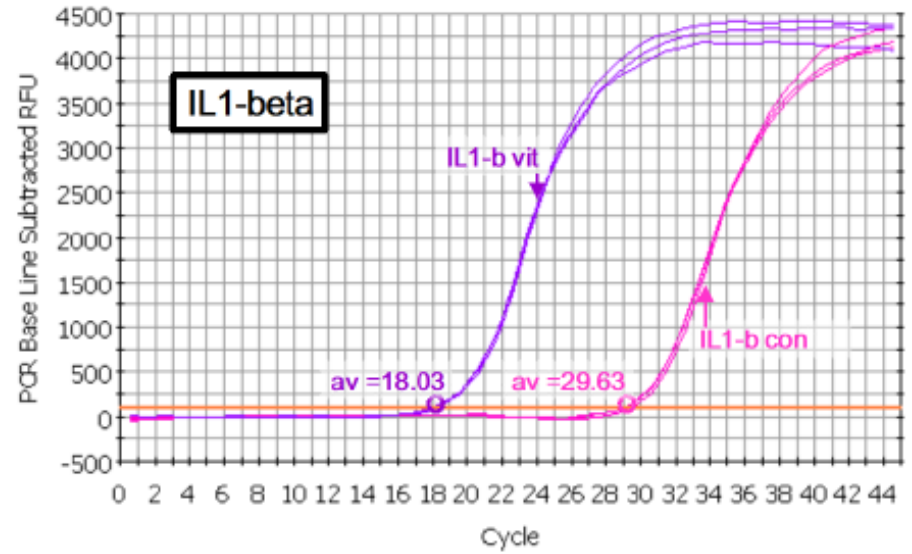
AFTER N CYCLES: change = $(\text{efficiency})^{n}$

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



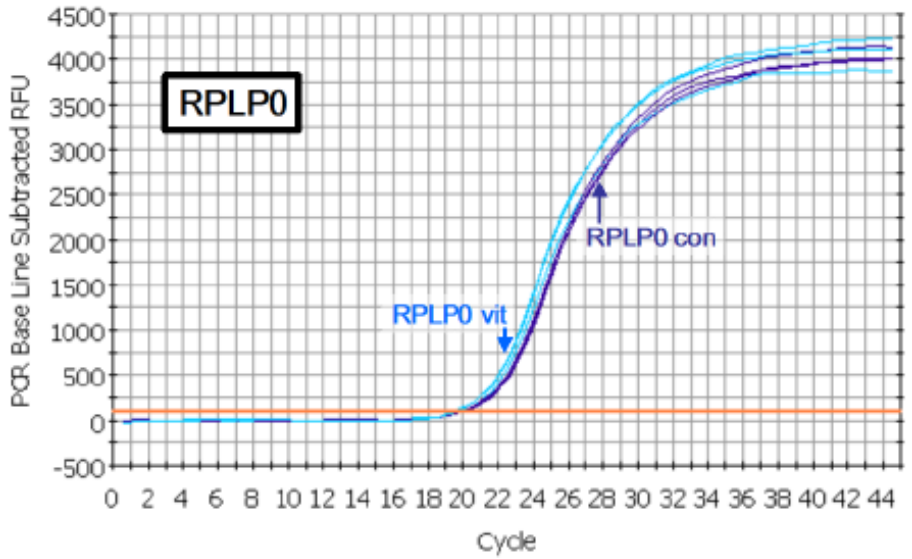
AFTER N CYCLES: change = $(\text{efficiency})^n$

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



AFTER N CYCLES: increase = (efficiency)^n

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



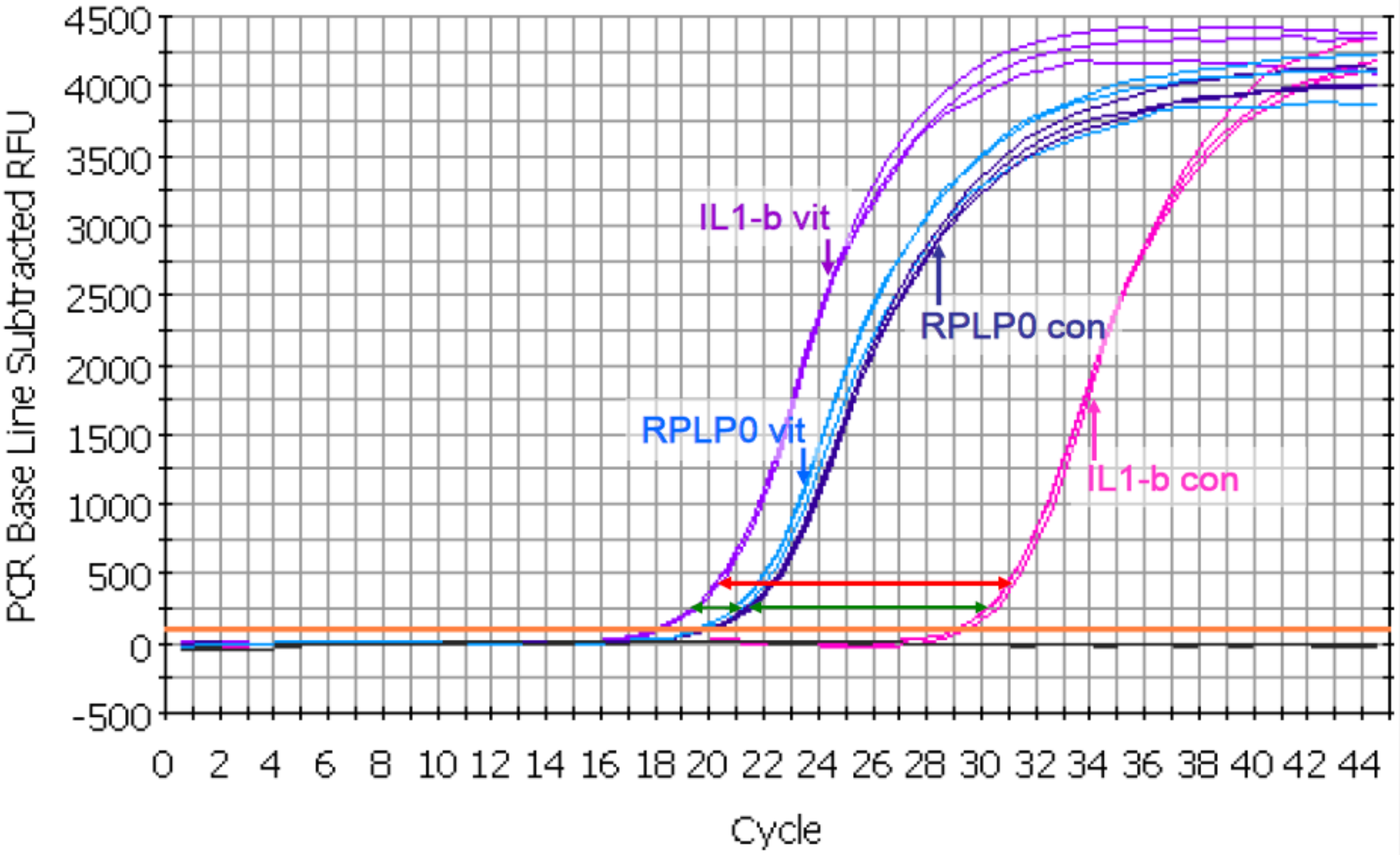
AFTER N CYCLES: increase = (efficiency)^n

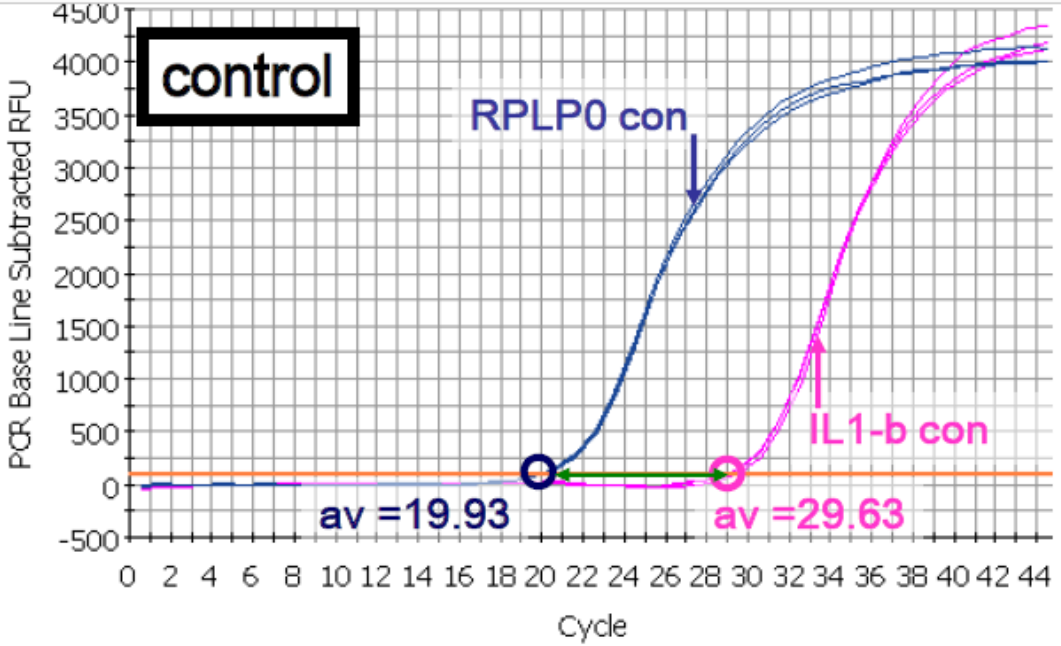
Ratio vit/con = $(1.87)^{19.93-19.80} = 1.87^{0.13} = 1.08$

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

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

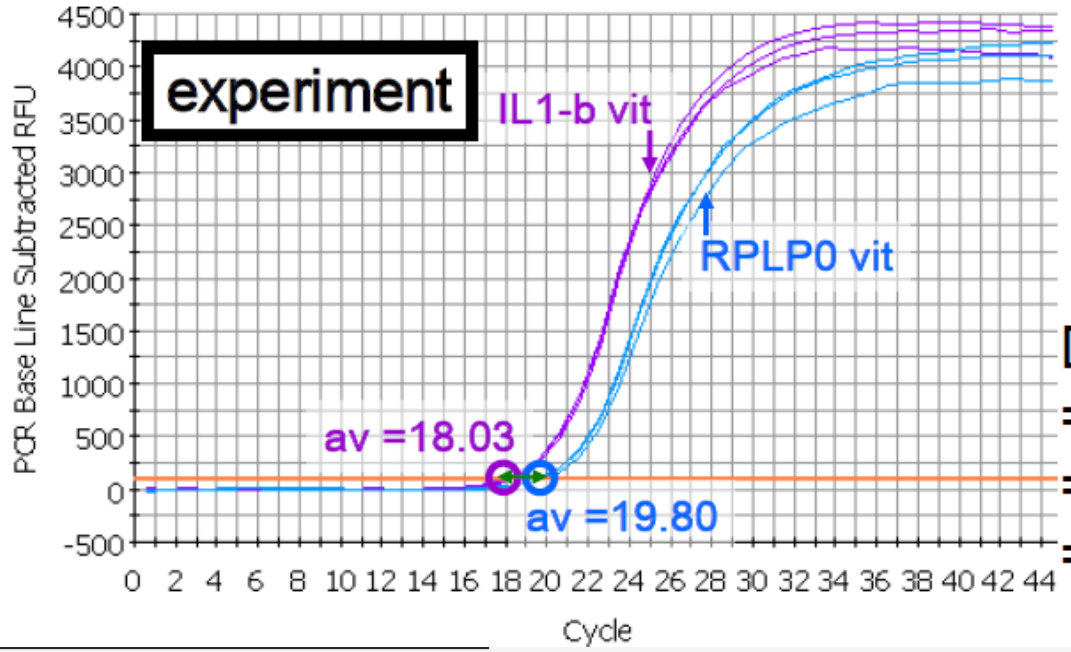
$2^{\Delta\Delta ct}$ efficiency method


PCR Base Line Subtracted RFU
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cycle
IL1-b vit
RPLP0 con
RPLP0 vit
IL1-b con



Δ Ct = target - ref

Δ Ct = 9.70



Δ Ct = target - ref

Δ Ct = -1.7

Difference = ΔCt-ΔCt

= $\Delta\Delta$Ct

= 9.70-(-1.7)

= 11.40

ΔΔCt = 11.40 for IL 1 beta

$2^{\Delta\Delta ct}$ variant: assumes efficiency is 100 % Fold change $= 2^{11/40}$= 2702

But our efficiency for IL 1 beta is 93%

Fold change = $1/93^{11/40}$= 1800

Pfaffl equation corrected for RPLP 0 efficiency

Fold change = 1901

Relative Quantification

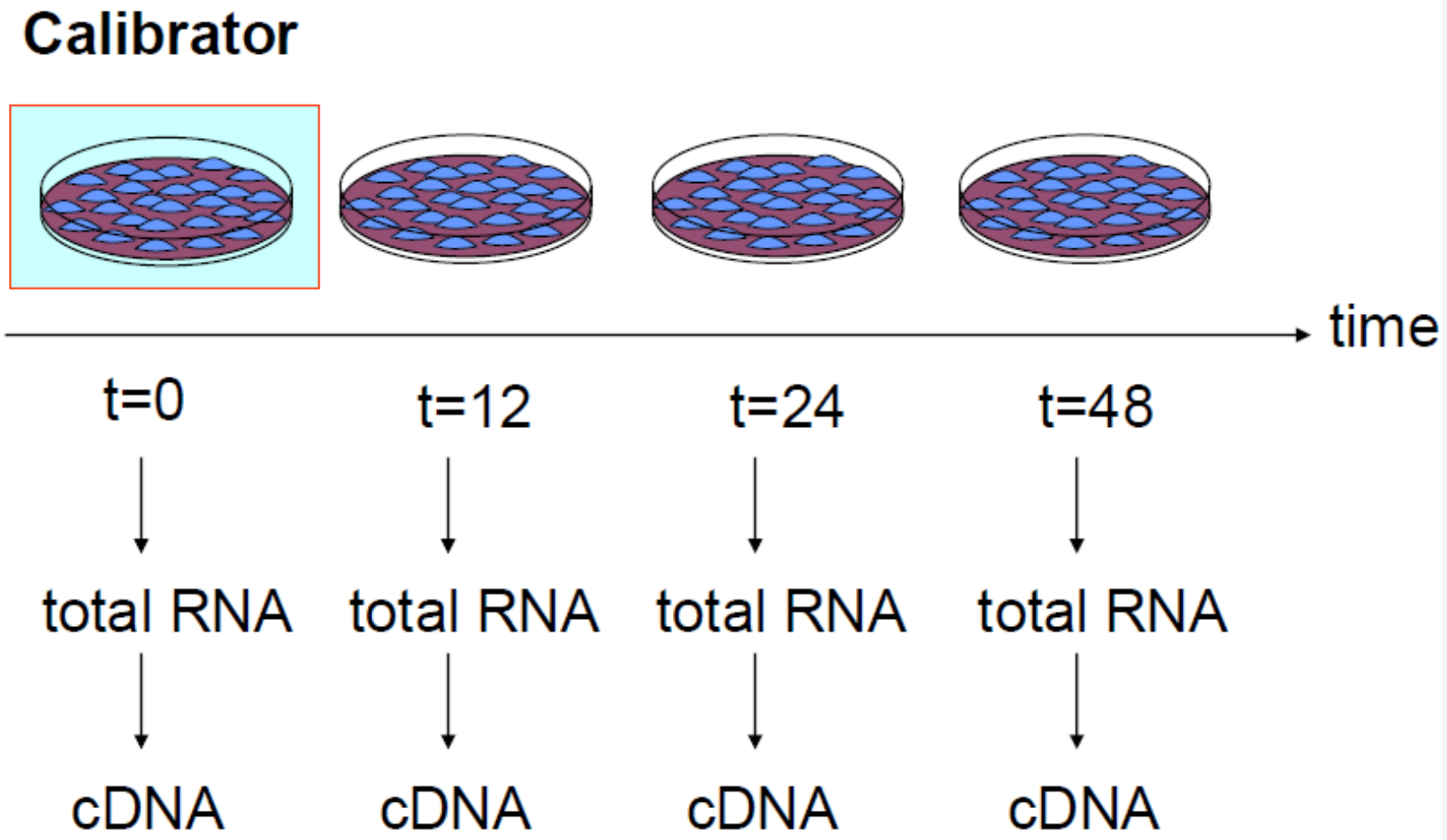
- No standard curve
- Calculation of results by comparison of Ct values "Comparative Ct method"

- Definition of:
- ✓ Endogenous Control
- ✓ Calibrator

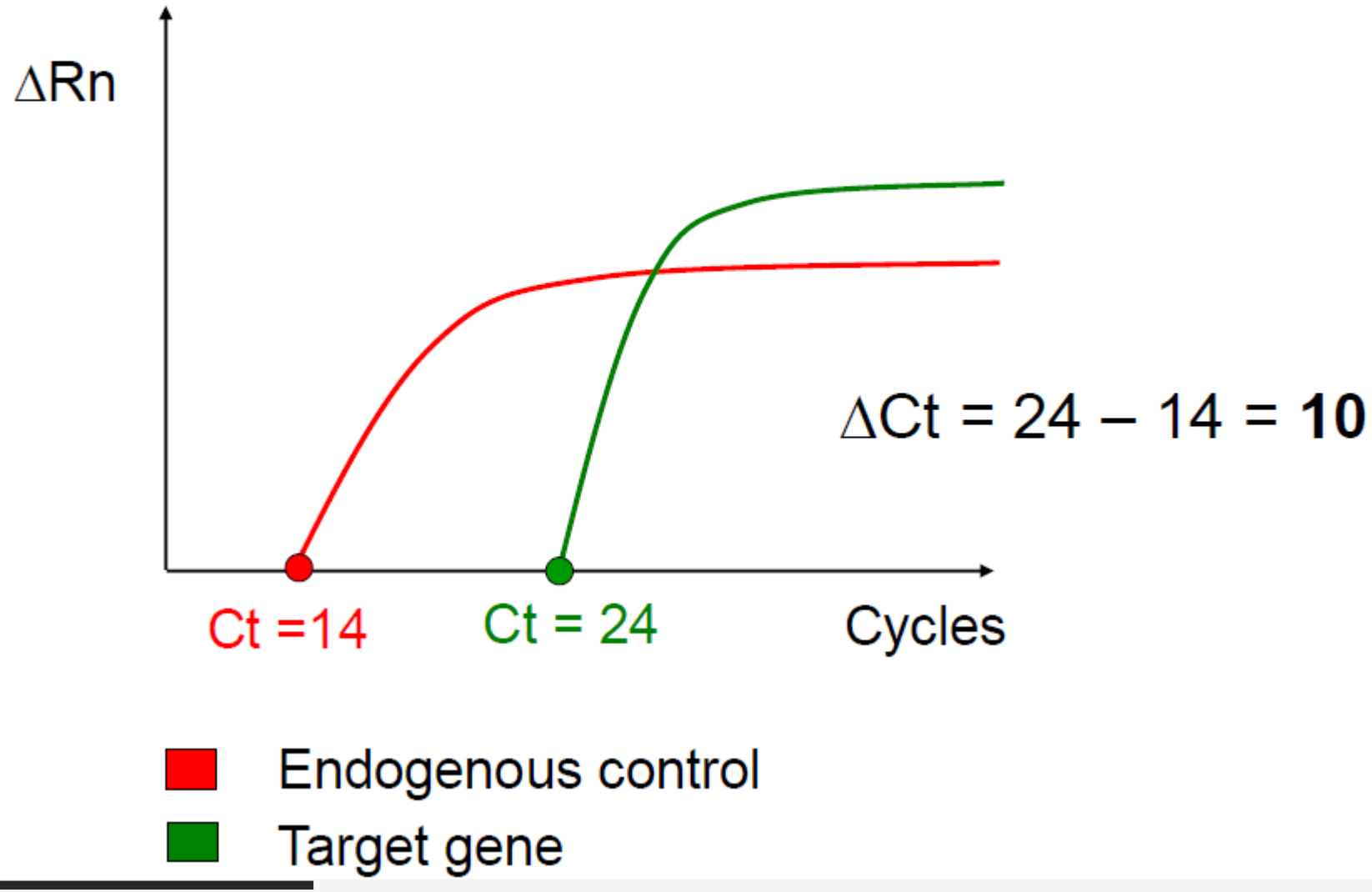
Endogenous Control (EC)

- The perfect EC reflects the amount of cDNA per well
- EC has a constant expression level in all samples which are used in that study
- The EC normalizes for
  - ✓ RNA input measurement errors
  - ✓ RT efficiency variations

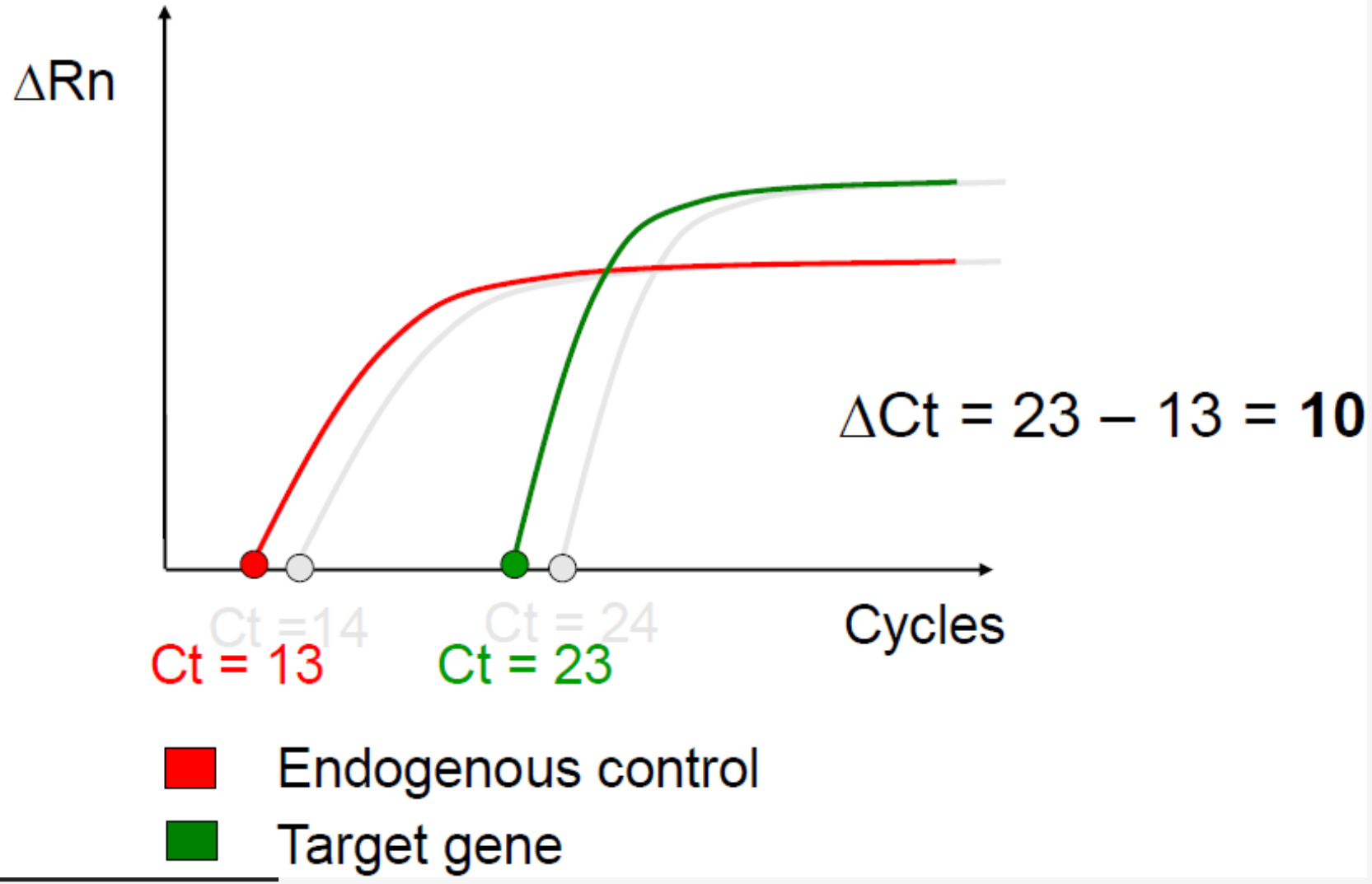
Calibrator


Calibrator
time
t=0
t=12
t=24
t=48
total RNA
total RNA
total RNA
total RNA
cDNA
cDNA
cDNA
cDNA

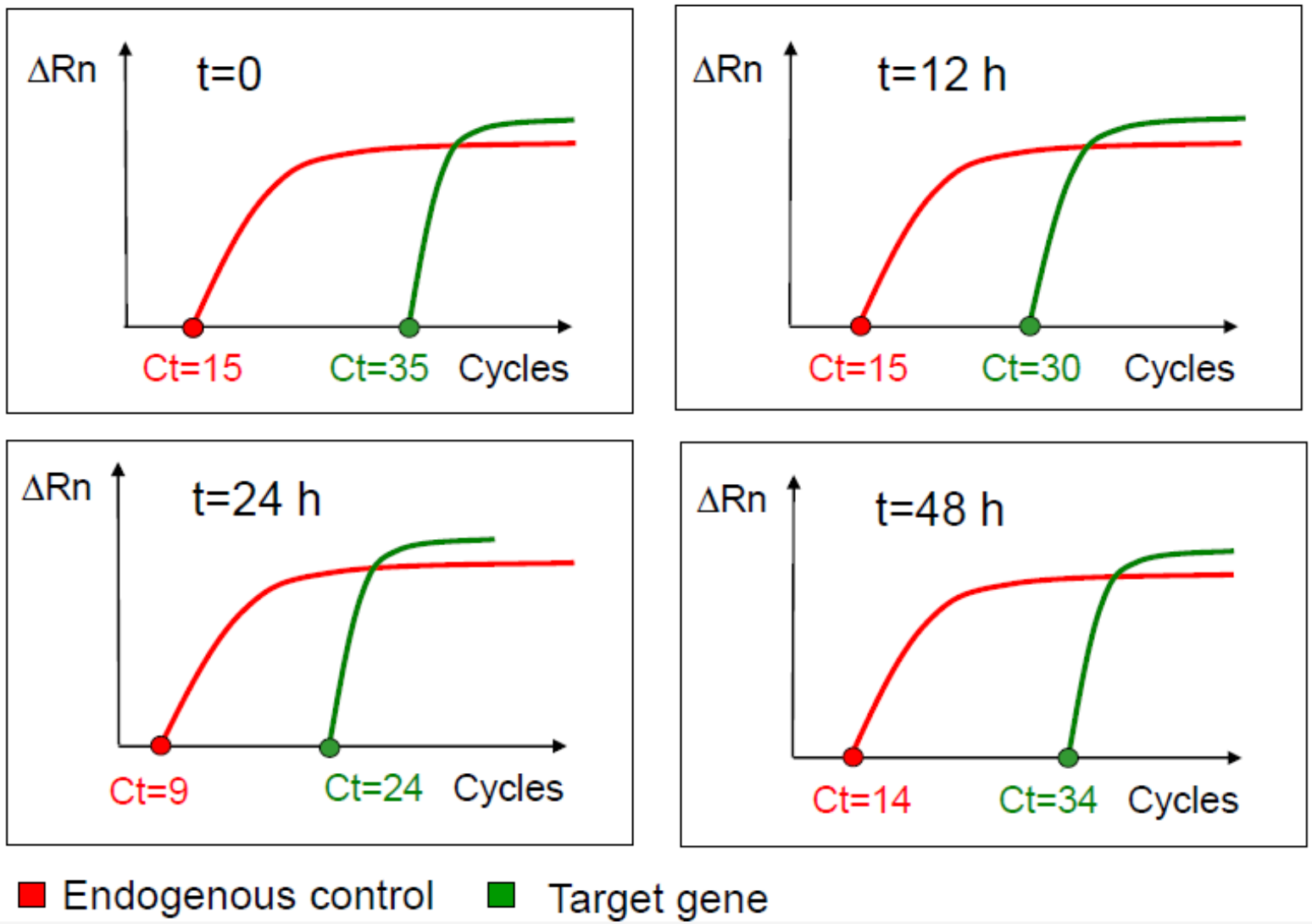
Comparison of Target Gene and Endogenous Control



What if we added the double amount of cDNA?



Comparative Ct Method



Comparative Ct Method

step 1: Normalization to endogenous control

Ct Target gene – Ct Endogenous control = **ΔCt**

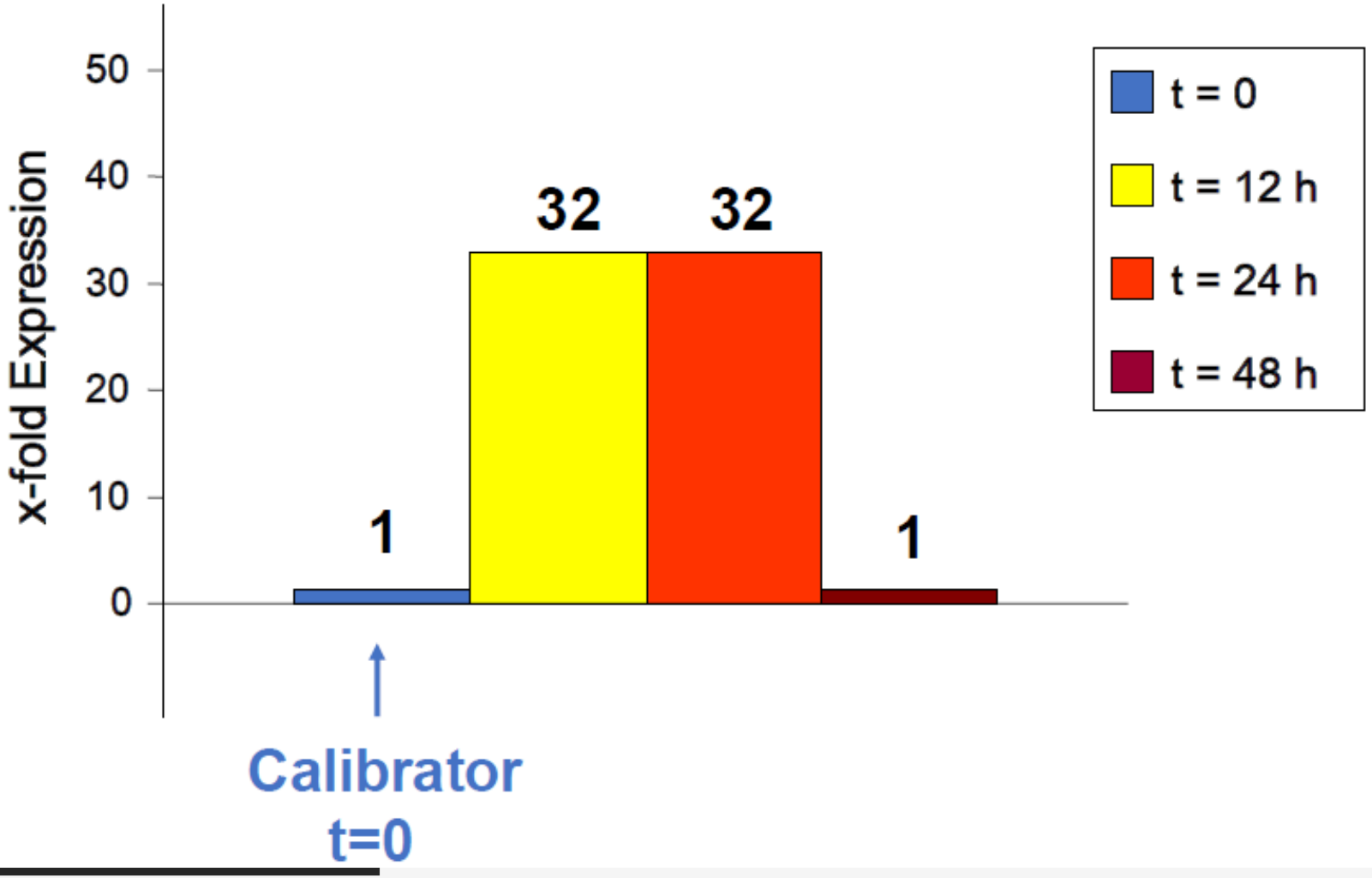
step 2: Normalization to calibrator sample

ΔCt Sample – ΔCt Calibrator = **ΔΔCt**

step 3: use the formula

$$2^{-\Delta\Delta Ct}$$

Relative Quantification Result



Exercise 1

Brain	**18S rRNA**	**Ct 14**
	IL-10	**Ct 32**
Liver	**18S rRNA**	**Ct 16**
	IL-10	**Ct 29**

Which cDNA is higher concentrated, and how many times higher?

Brain, 4 times higher (2^2)

Exercise 2

Brain (Calibrator)	**18S rRNA**	**Ct 14**
	IL-10	**Ct 32**
Liver	**18S rRNA**	**Ct 16**
	IL-10	**Ct 29**

How does the expression of IL-10 differ in Liver and Brain?

ΔCt Brain = 18

ΔCt Liver = 13 → 13 -18 = -5 → 2^5= 32

IL-10 is 32-times higher expressed in Liver!

Definition & terminology

Term	Definition
Baseline	The initial cycles of PCR in which there is little change in fluorescence signal.
Threshold	A level of delta R_n—automatically determined by the software or manually set—used for C_T determination in real-time assays. The level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the Amplification plot defines the C_T.
Threshold cycle (C_T)	The fractional cycle number at which the fluorescence passes the threshold.
No template control (NTC)	A sample that does not contain template. It is used to verify amplification quality.
Nucleic acid target (also called "template")	Nucleotide sequence that you want to detect and quantitate.
Passive reference	A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations caused by changes in concentration or in volume.
Reporter dye	The dye attached to the 5′ end of a TaqMan® probe. The dye provides a fluorescence signal that indicates specific amplification.
Normalized reporter (R_n)	The ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye.
Delta R_n (ΔR_n)	The magnitude of the signal generated by the specified set of PCR conditions. ($\Delta R_n = R_n$ – baseline)
Standard	A sample of known quantity used to construct a standard curve.
Unknown sample	A sample containing an unknown quantity of template that you want to characterize.


Thanks for attachment