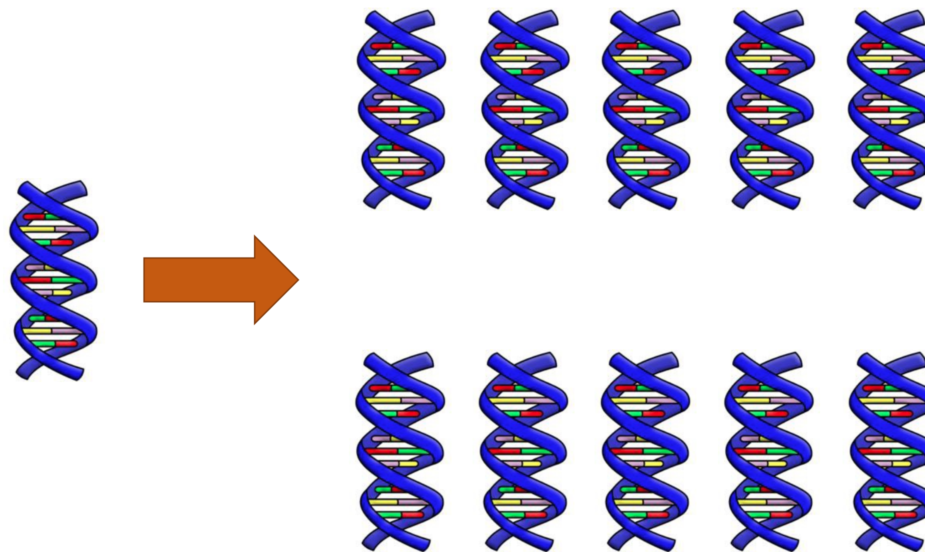
Polymerase Chain Reaction (PCR)

Enzymatic reaction allowing to amplify a specific region of DNA

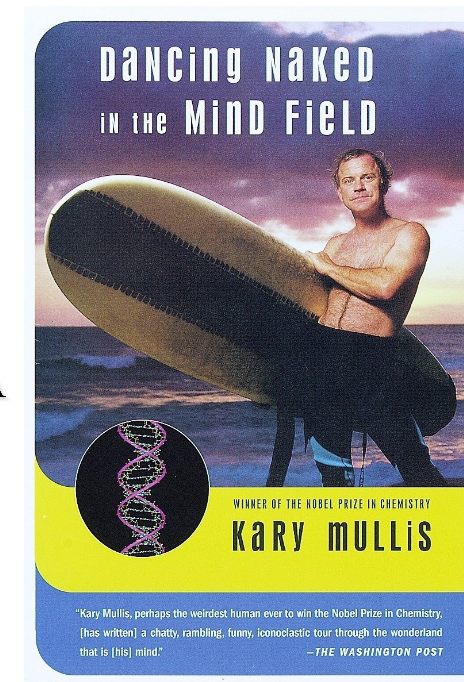


PCR can make billions of copies of a target sequence in a few hours

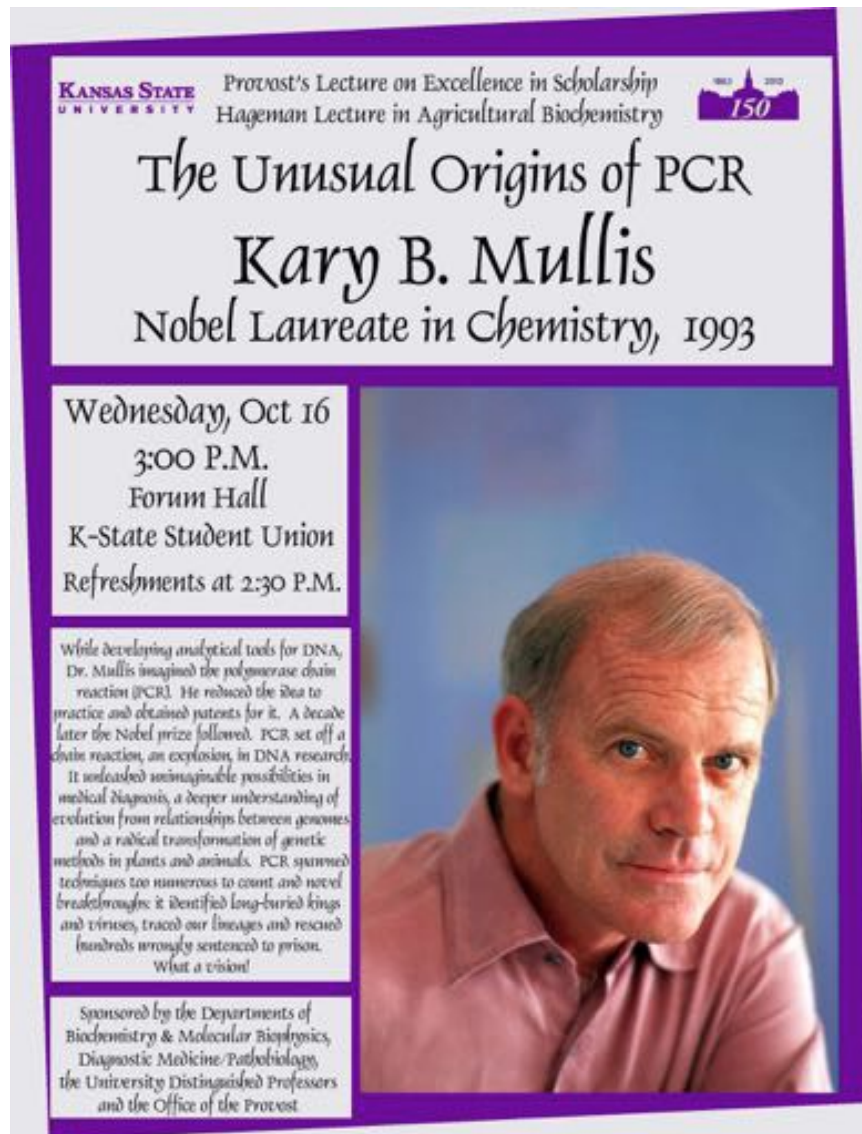
PCR is highly sensitive and can start from minimal quantities of DNA

Invented in 1983 by Kary Mullis

(Nobel Prize in 1993)



1983, Kary Mullis defined the concept of PCR (Nobel Prize in 1993)

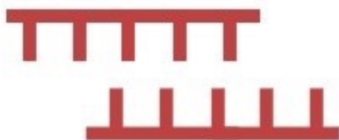


"In a famous interview, Mullis provocatively wondered whether he would have ever discovered PCR if he had not taken LSD, concluding that he seriously doubted it, as he could literally see the individual polymers at work and admitted that he had learned a great deal from that experience."

PCR Ingredients

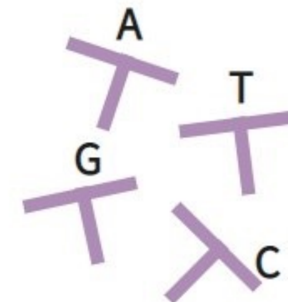
Template DNA

Primers



DNA polymerase
And its buffer

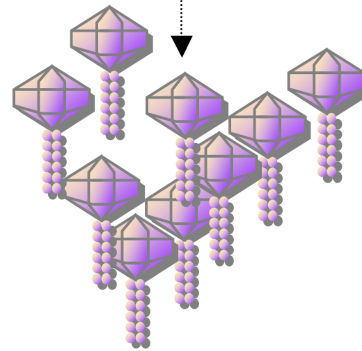
dNTPs

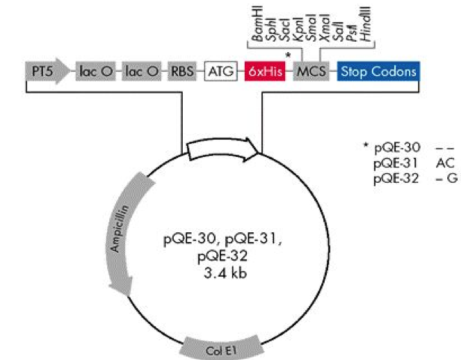


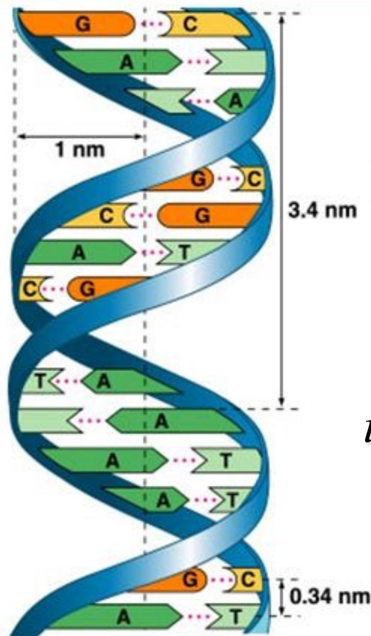
Template DNA

genomic, viral, plasmid DNA

DNA molecule







PCR is based on **base complementarity**, which was first hypothesized when the DNA double helix was discovered

"It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible ***copying mechanism*** *for the genetic material"*

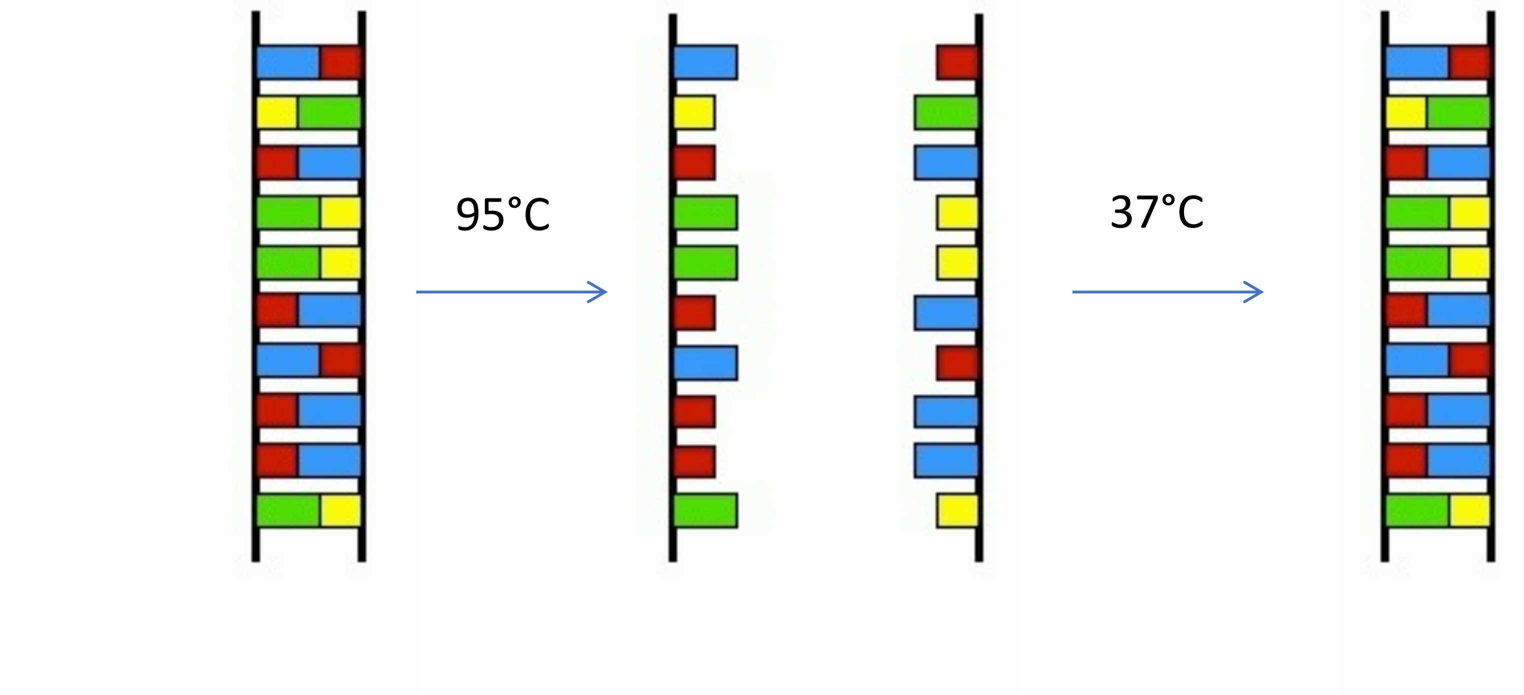
***Watson and Crick**, 1953*

The reaction

Everything is based on:

Base complementarity

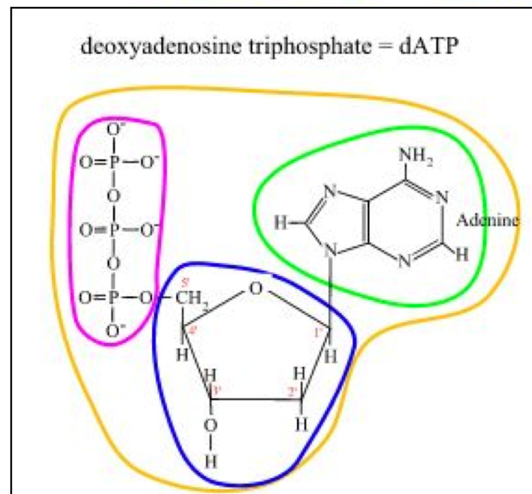
DNA ability to renaturate after being denaturated

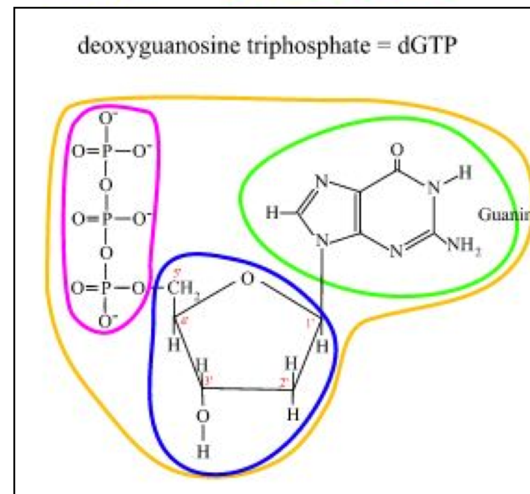


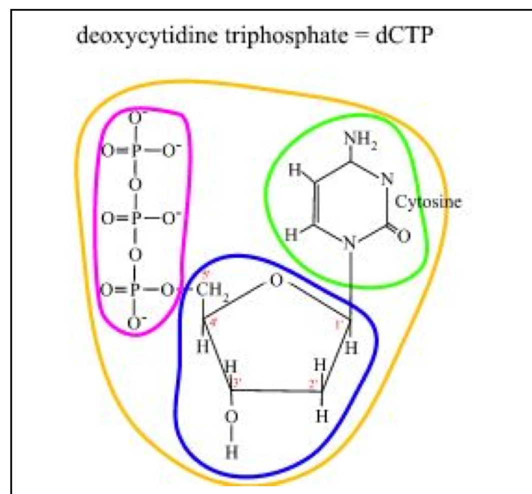
dNTPs

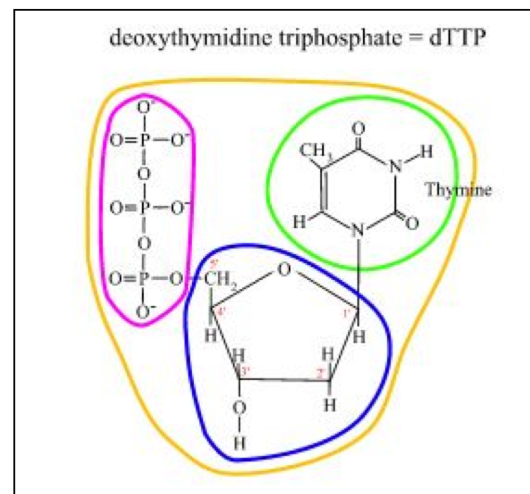
dNTP= deoxynucleotide triphosphate

Nucleotide = base + sugar + phosphate





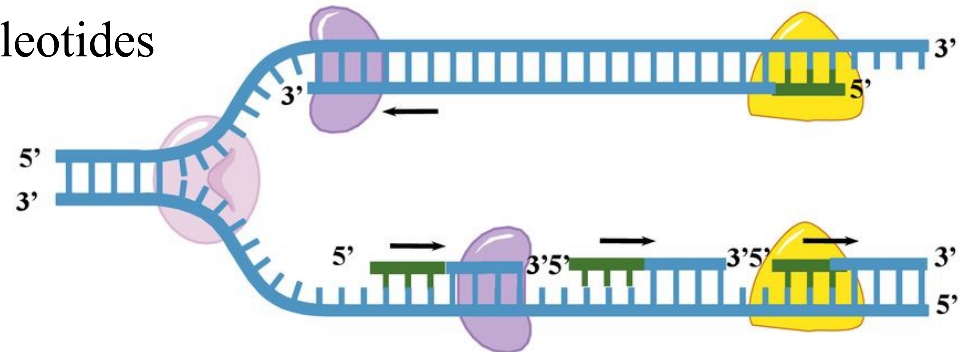




Primers

Primers are short single-stranded nucleic acids necessary for the initiation of DNA synthesis.

- DNA polymerase is only able to add nucleotides to the 3'-end of an existing fragment
- RNA primers are used by living cells during DNA replication



You need a **forward and a reverse primer** for your PCR

5'-ATGTCAAAGGAGGATTTCGTTATTAAGCCTGAAGCTGCAGGTGCTTCCACTGACACTA-3'
3'-TACAGTTTCCTCCTAAAGCAATAATTCGGACTTCGACGTCCACGAAGGTGACTGTGAT-5' dsDNA

CACGAAGGTGACTGTGAT
5'-ATGTCAAAGGAGGATTTCGTTATTAAGCCTGAAGCTGCAGGTGCTTCCACTGACACTA-3'

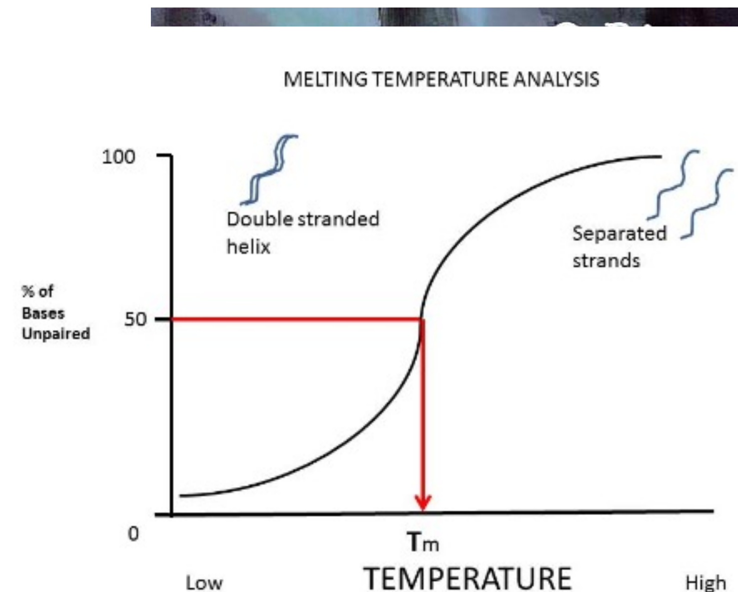
ATGTCAAAGGAGGATTTC
3'-TACAGTTTCCTCCTAAAGCAATAATTCGGACTTCGACGTCCACGAAGGTGACTGTGAT-5'

1971, Khorana

Primer design

You need good primers in order to have a successful PCR

- Length of 18-24 bases
- 40-60% G/C content
- 1-2 G/C in the last 5 bases (GC clamp)
- Melting temperature (Tm) of 50-60°C
  $$Tm = 2(A + T) + 4(G + C)$$
- Tm difference between primers < 5°C
- Primers have to be specific (use BLAST)
- Primer pairs should not be self-complementary



You can use online tools !

The reaction

PCR is based on base complementarity and the DNA ability to renaturate after being denaturated

primers

Primers anneal

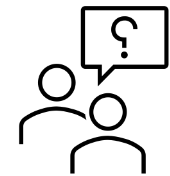
95°C

55°C

72°C

primers Tm -5°C

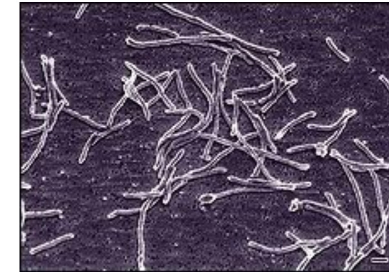
Do proteins resist to high temperature?



Thermus aquaticus DNA polymerase (Taq)

Taq polymerase is a thermostable DNA polymerase I named after the thermophilic eubacterial microorganism *Thermus aquaticus that lives in hot springs*

1976, *Chien*



Taq polymerase features:

- Stable (even if inactive) at T > 90°C
  = no need to add new enzyme to each round

- Optimal activity at 75-80°C
  = can replicate 1000 bp in < 10 seconds at 72°C

Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase

Randall K. Saiki, David H. Gelfand, Susanne Stoffel, Stephen J. Scharf, Russell Higuchi, Glenn T. Horn, Kary B. Mullis,* Henry A. Erlich

A thermostable DNA polymerase was used in an in vitro DNA amplification procedure, the polymerase chain reaction. The enzyme, isolated from *Thermus aquaticus*, greatly simplifies the procedure and, by enabling the amplification reaction to be performed at higher temperatures, significantly improves the specificity, yield, sensitivity, and length of products that can be amplified. Single-copy genomic sequences were amplified by a factor of more than 10 million with very high specificity, and DNA segments up to 2000 base pairs were readily amplified. In addition, the method was used to amplify and detect a target DNA molecule present only once in a sample of 10^5 cells.

Not all the polymerases are the same

DNA polymerase	Error rate	Activities	Extension times
PfuTurbo	1.3×10^{-6}	5'-3' polymerase activity 3'-5' proofreading activity	1kb/min
Taq	8×10^{-6}	5'-3' polymerase activity	1kb/min

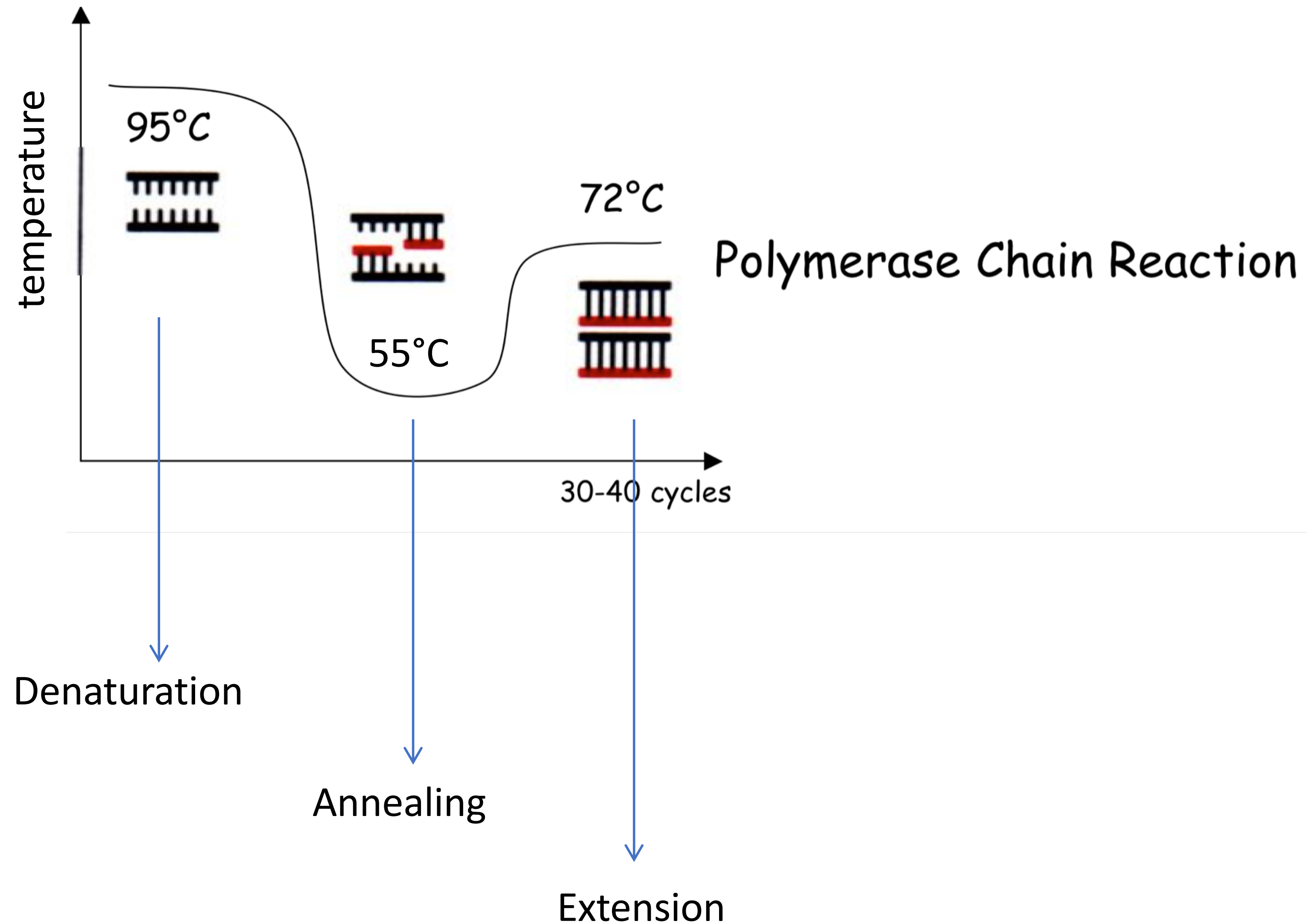
The enzyme to be used must be selected based on the type of experiment.

- proof-reading

If you need precision in the copy (es. diagnosis,cloning)

- No proof reading (es. colony PCR, contamination check, gene expression)

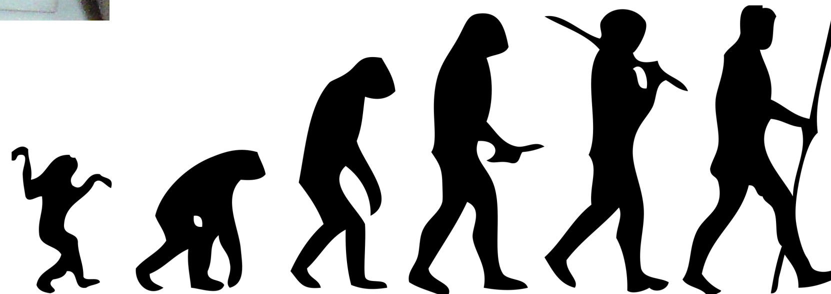
PCR Steps



The evolution of the PCR Thermocycler

Manual trasfer of the sample between three water baths at different temperatures

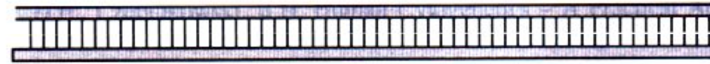
Metal block programmed to adjust the sample to specific temperatures for a defined time and n of cyckes



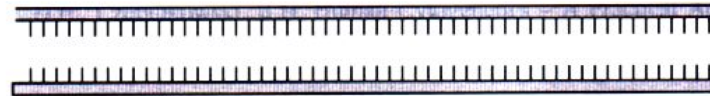
Baking time

template	y (ng)
primer F (10mM)	1mM (10 μl)
primer R (10mM)	1mM (10 μl)
dNTP 2.5mM	0,25 mM (10 μl)
Polymerase	x
Buffer 10X	1X (10 μl)
$MgCl_2$ 50mM	≅2mM (2 μl)
H_20	up to final volume (100 μl)

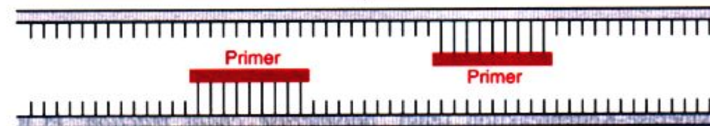
I cycle...



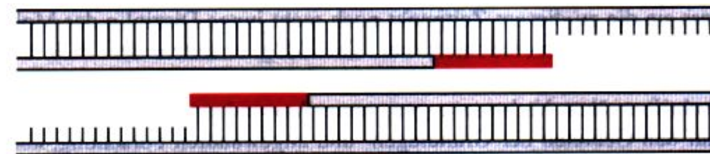
dsDNA



DENATURATION

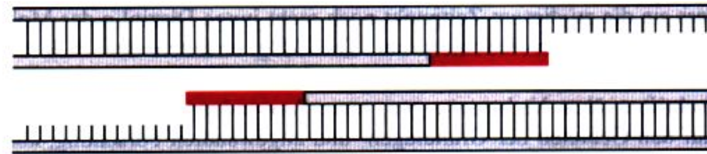


ANNEALING

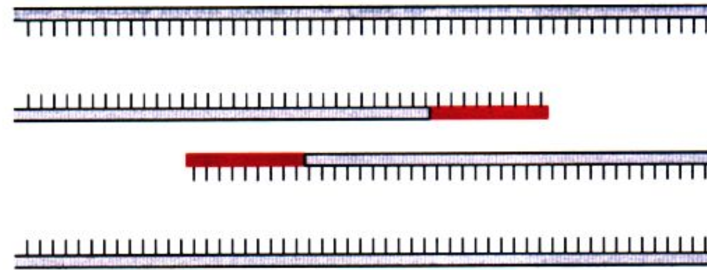


SYNTHESIS

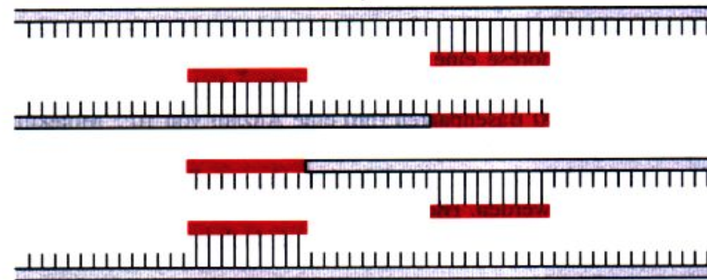
..II cycle



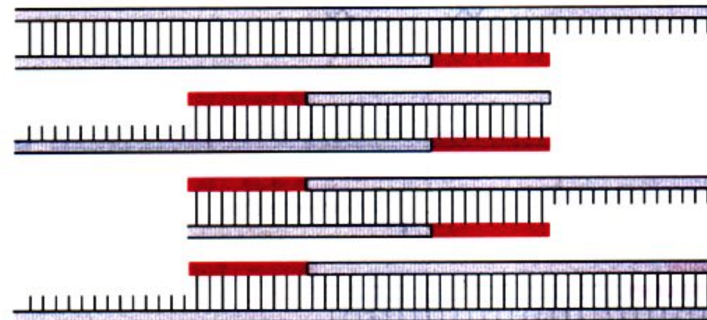
dsDNA



DENATURATION

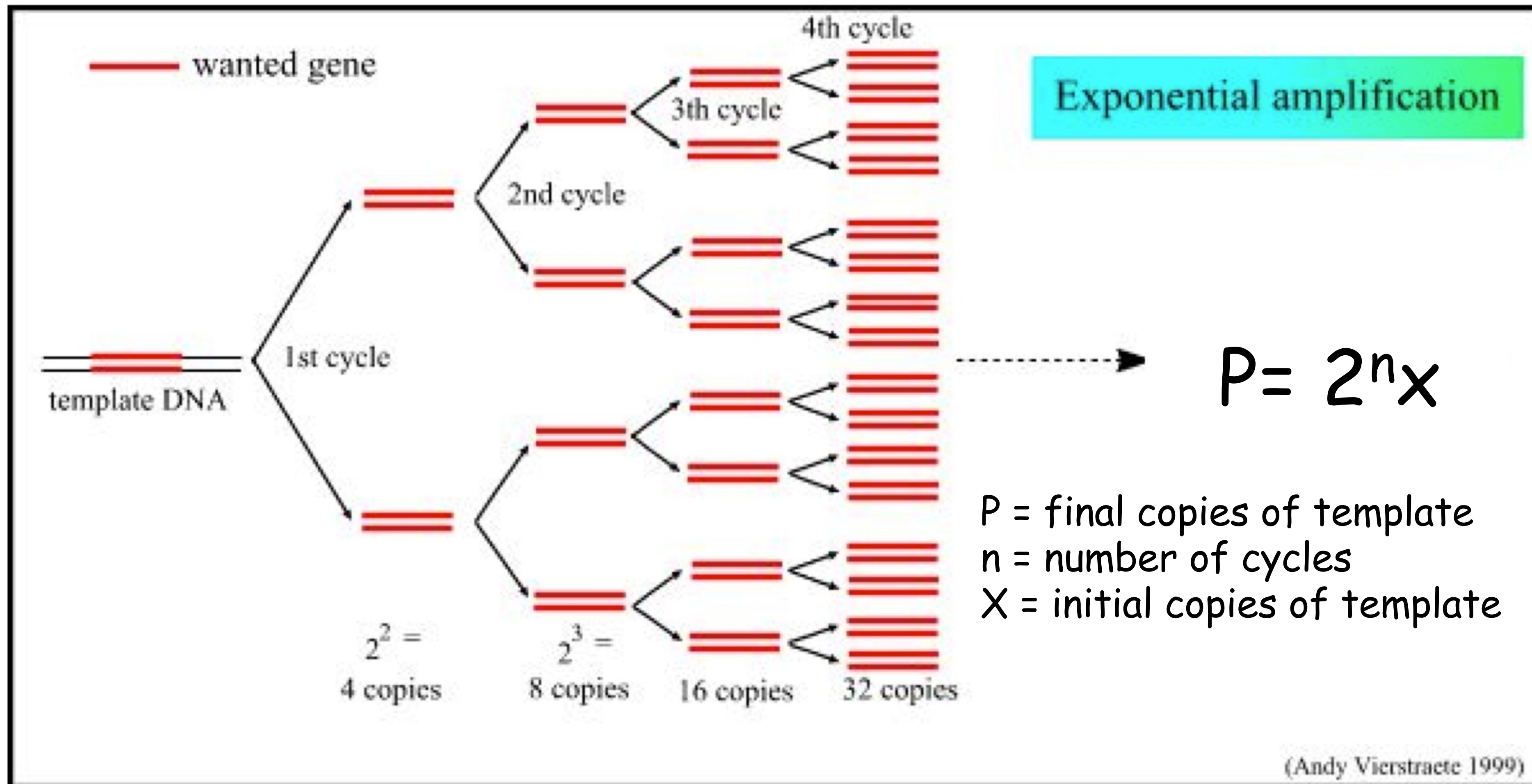


ANNEALING

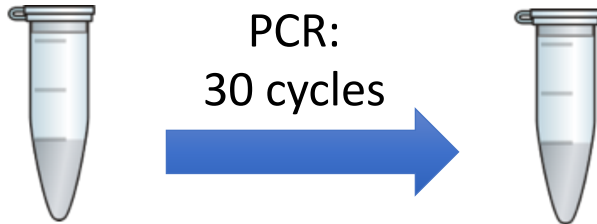


SYNTHESIS

....n cycles

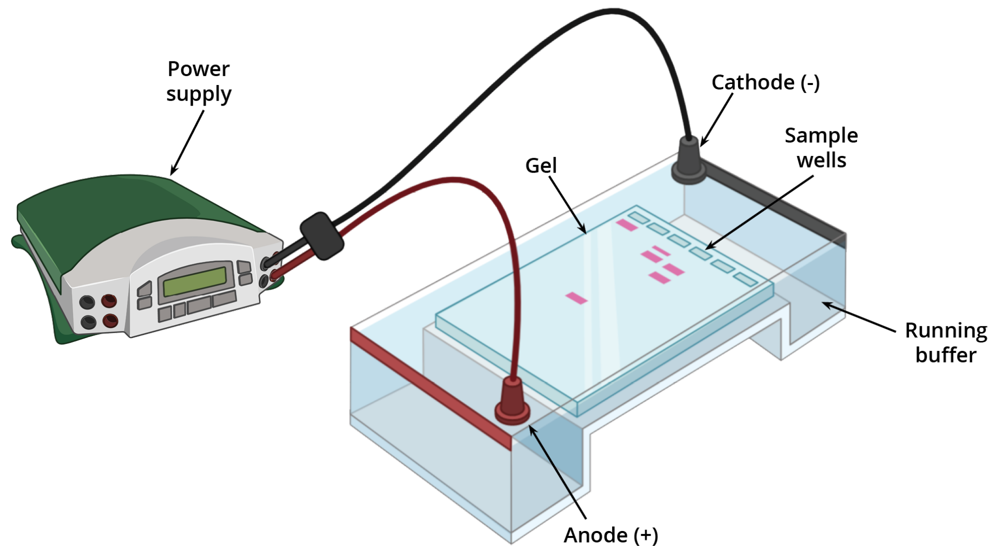


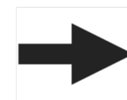
PCR CHECK: Agarose gel electrophoresis

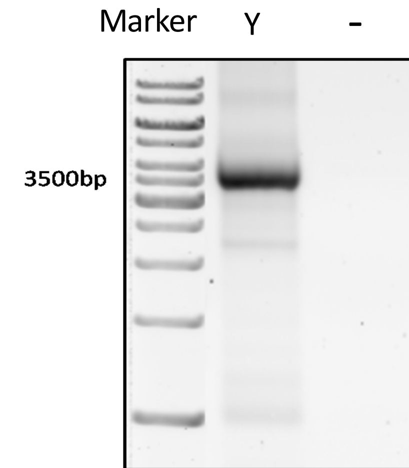


Many DNA molecules ($P=2^nX$, n=30)
How to check if the desired amplicon is present?

You know your fragment lenght !







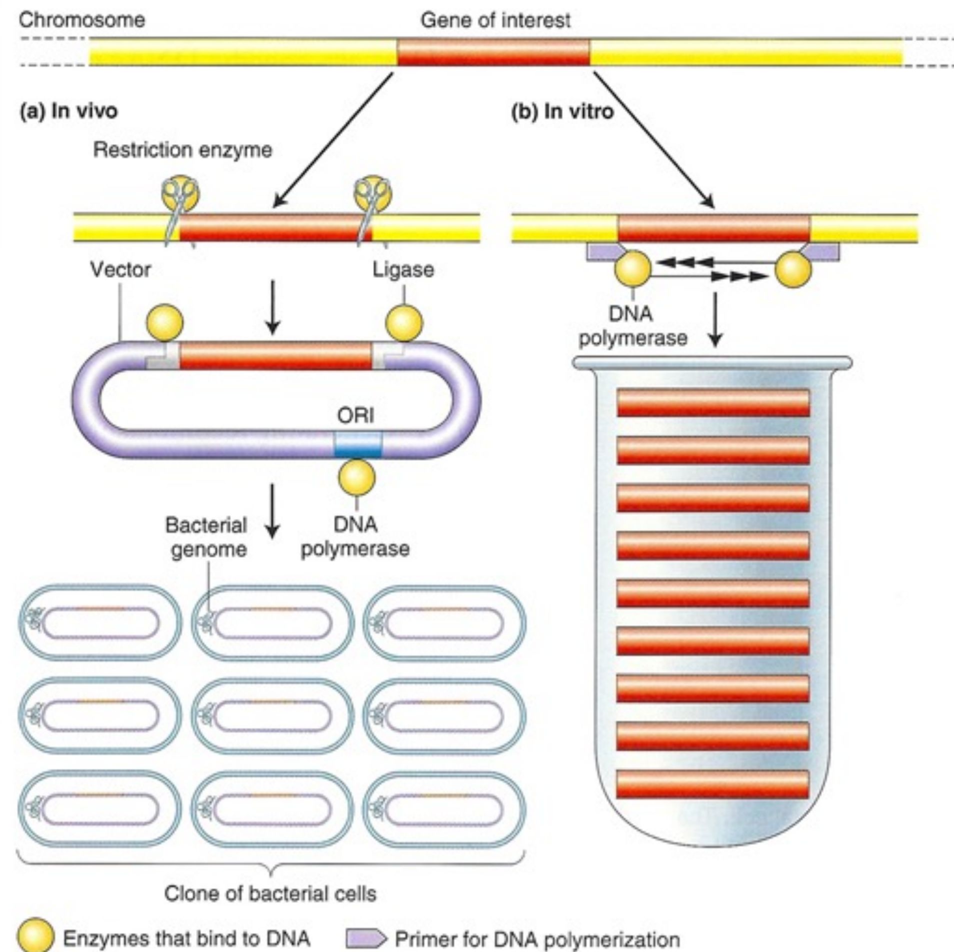
Agarose concentration	Resolution
0,8%-1%	0.5 - 20 Kb
1,5%-2,5%	0.1 - 3kb

PCR Applications

- **Molecular Cloning and mutagenesis**
- **Genotyping**
- **Forensic analysis** from small DNA samples on crime scenes
- **Diagnostic tests** for genetic deseases, bacterial or viral infections
- **Gene expression analyses**

Alternative to Molecular cloning

PCR is an alternative to classic cloning strategies



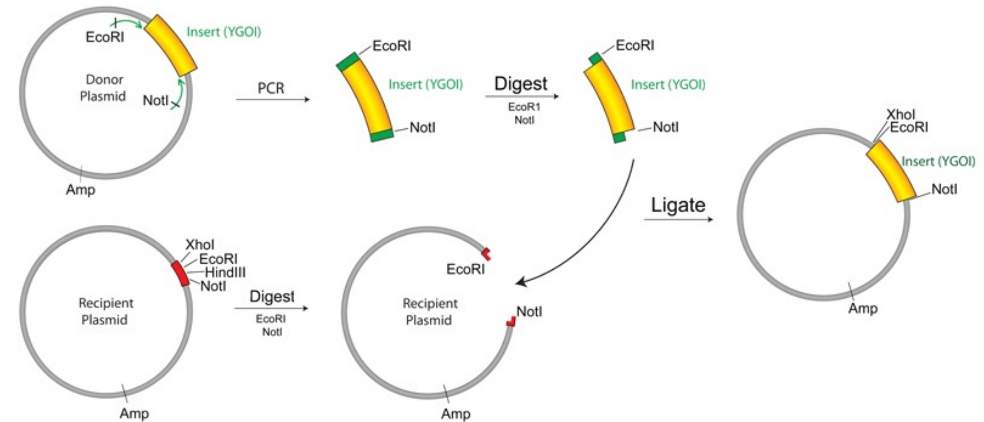
Molecular cloning strategies

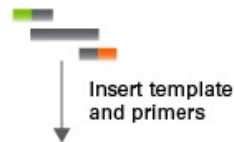
Direct PCR cloning

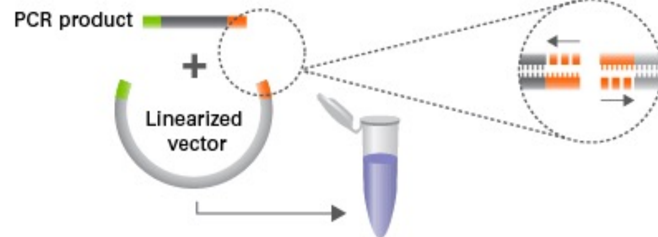
1) Amplification of the target sequence in a region containing sites for restriction enzymes

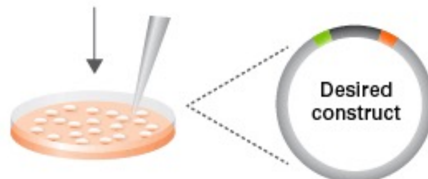
2) Digestion of the amplified product and of the recipient plasmid with the same restriction enzymes

3) Ligation









Indirect PCR cloning (Gibson Assembly, In-Fusion)

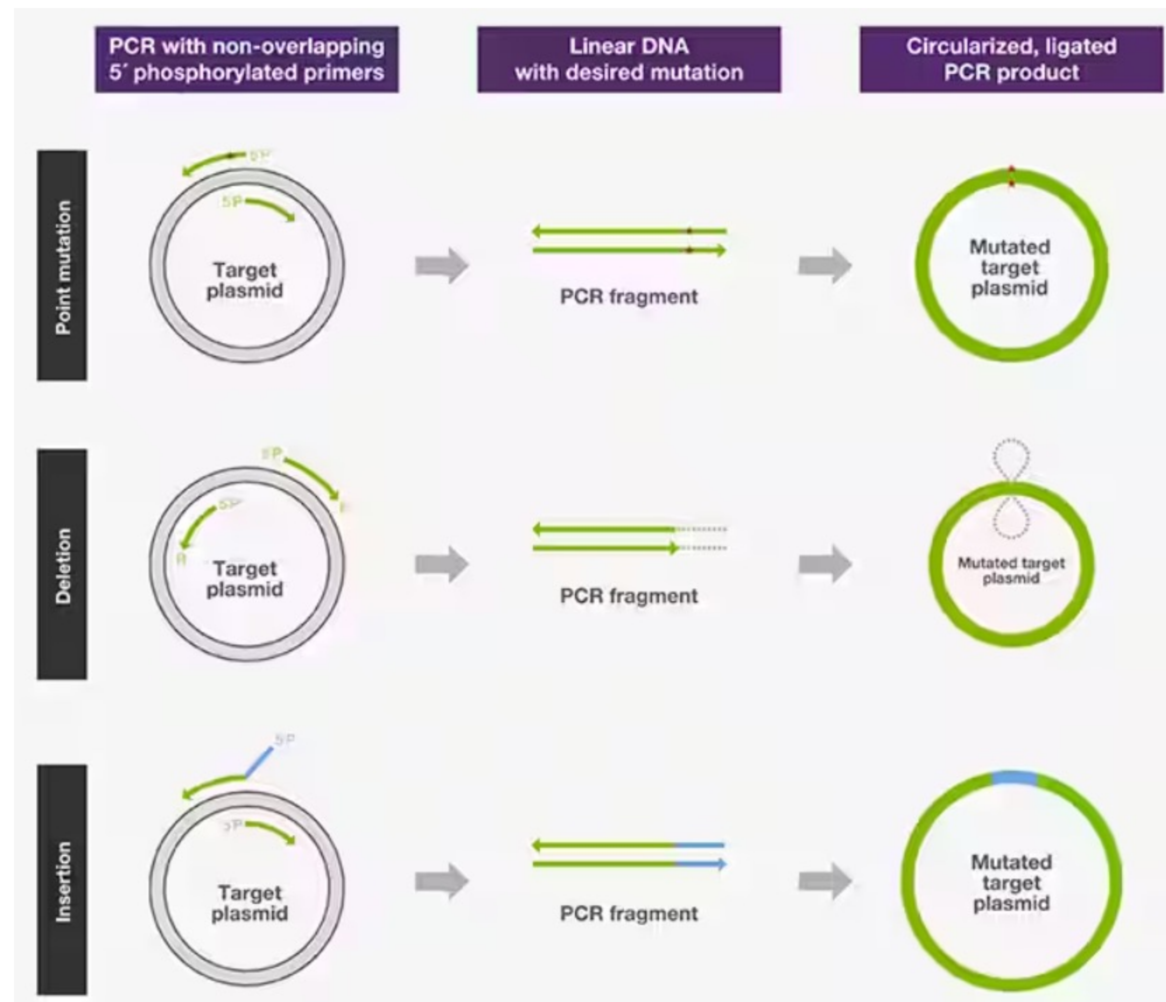
1) Amplification of the target sequence with primers starting with 15bp sequences complementary to the vector ends

2) The PCR product and the linearized vector are combined in a mix containing a 5' exonuclease that creates 15bp overhangs

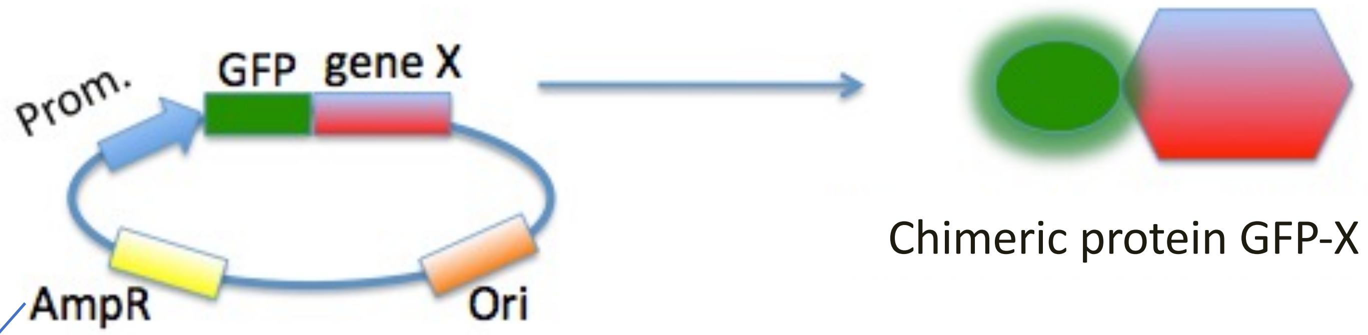
3) Competent cells are transformed with the reaction, so that bacterial ligase can produce the final construct

Mutagenesis

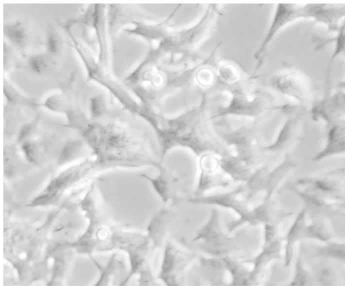
PCR can be exploited to introduce in-site mutations within the sequence of a plasmid by designing primers that introduce base substitutions, deletions or insertions



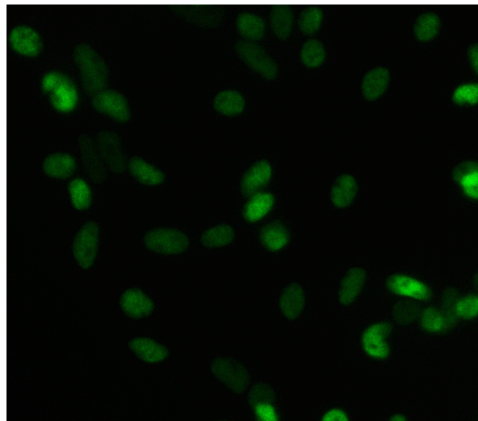
Generation of fusion protein

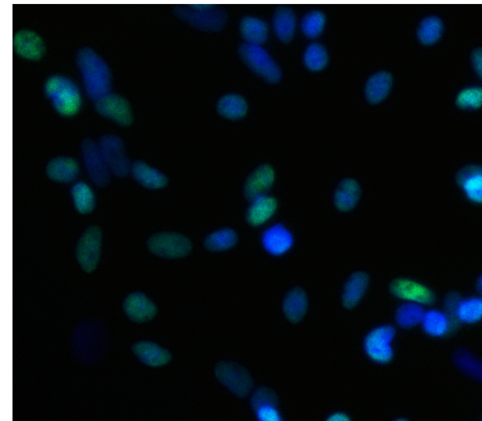


SK-N-BE cells

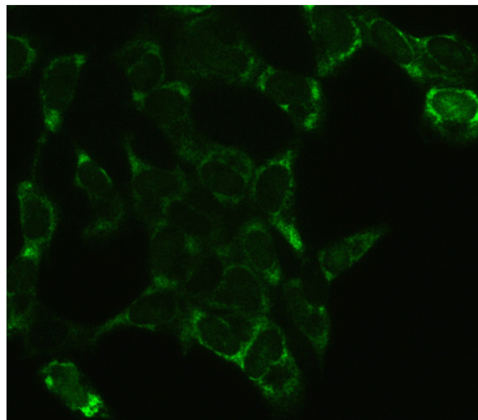


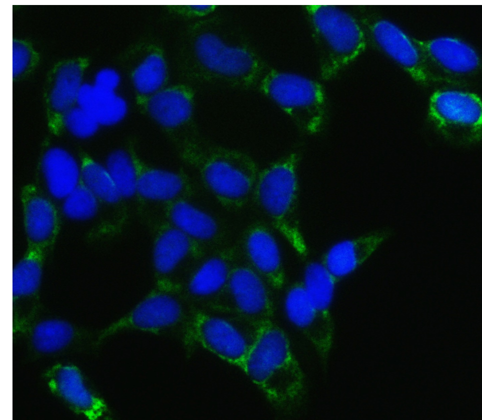
FUS WT



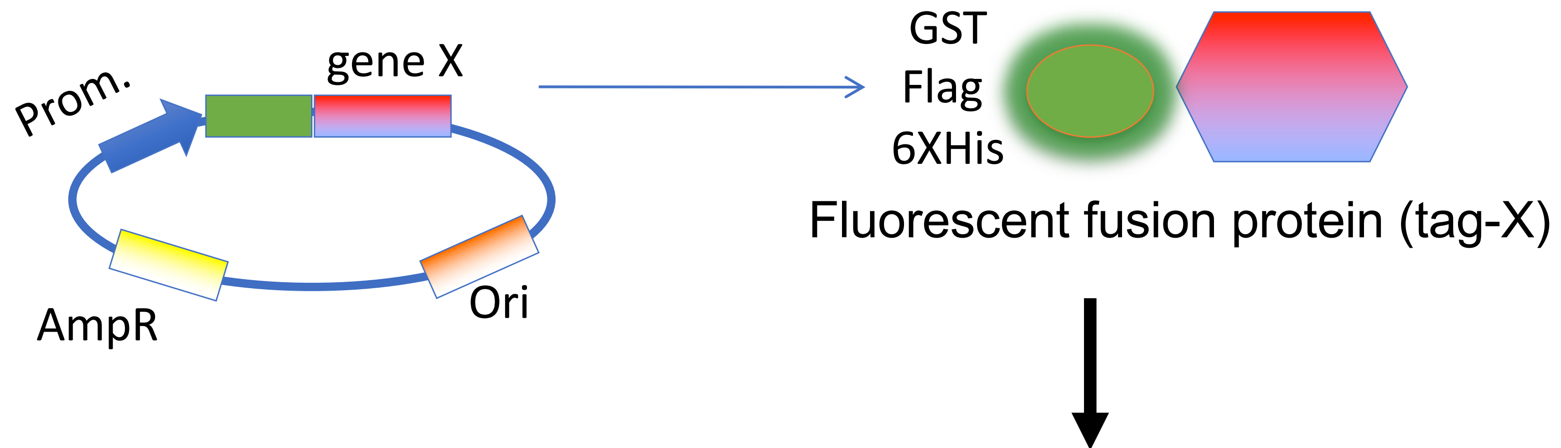


FUS mut





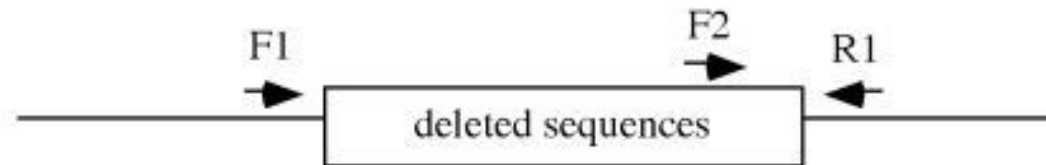
Generation of fusion protein



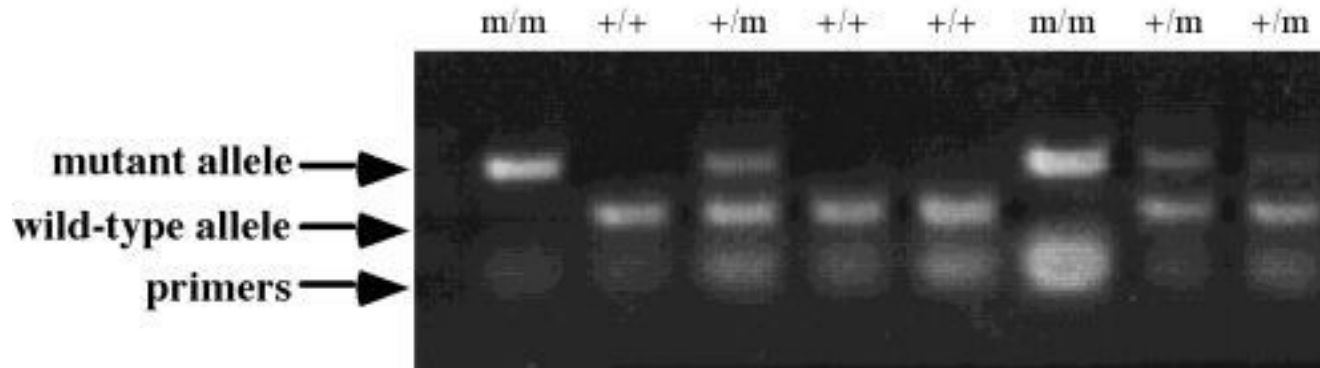
The fusion protein can be purified from the cell extract using chromatographic columns capable of binding the tag.

Genotyping

PCR can be coupled to genome editing approaches
to identify clones that contain the desired mutation



Placement of duplex PCR primers: F1+R1 give a ~350 bp mutant-specific product (their product from the wild type is too large to be significantly amplified). F2+R1 give a ~250 bp wild-type specific product.

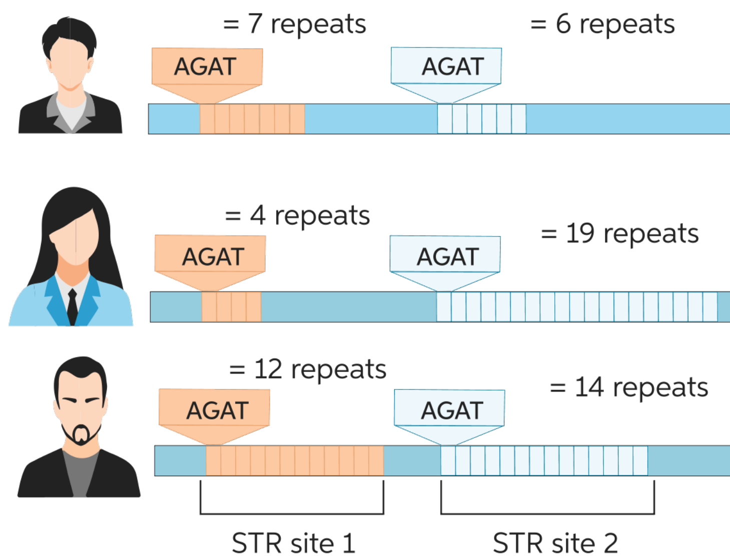


Forensic analysis

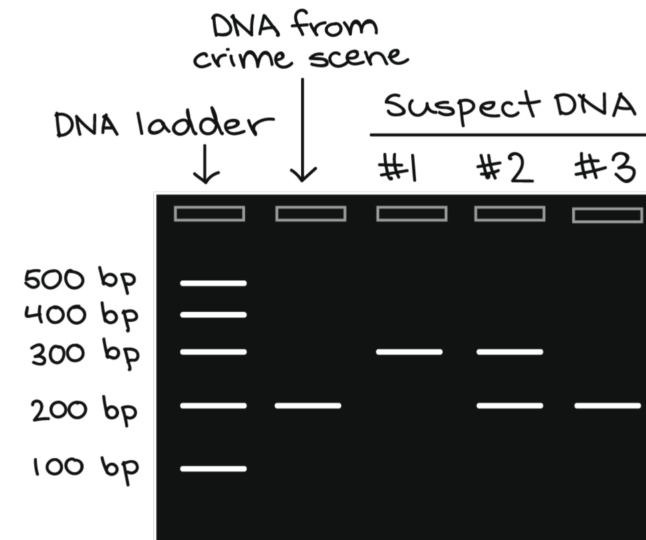
PCR can be exploited to match the sample from a crime scene to a suspect.

DNA fingerprinting through **Short Tandem Repeat (STR) analysis**

STRs are highly polymorphic regions of the genome containing a number of repeat units that differ from individual to individual



The evaluation of multiple STR sites can identify an individual with high confidence



PCR Application

- **Diagnostic tests** for genetic deseases, bacterial or viral infections

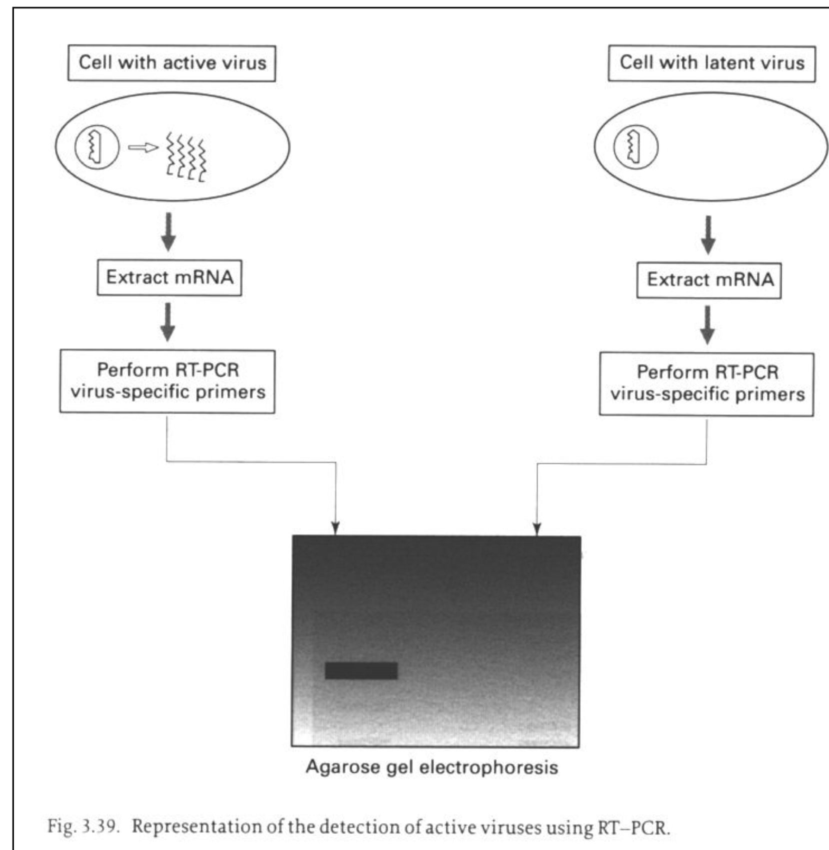


Fig. 3.39. Representation of the detection of active viruses using RT–PCR.

PCR application: Should we talk about COVID-19?
Then let's talk about «RT» first

PCR Applications involving the RNA

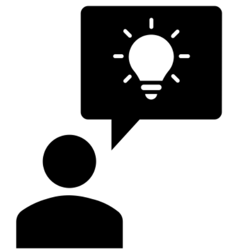
- **Diagnostic tests** for genetic deseases, bacterial or viral infections
- **Gene expression analyses**

Virus can have either *DNA* or *RNA* genomes.

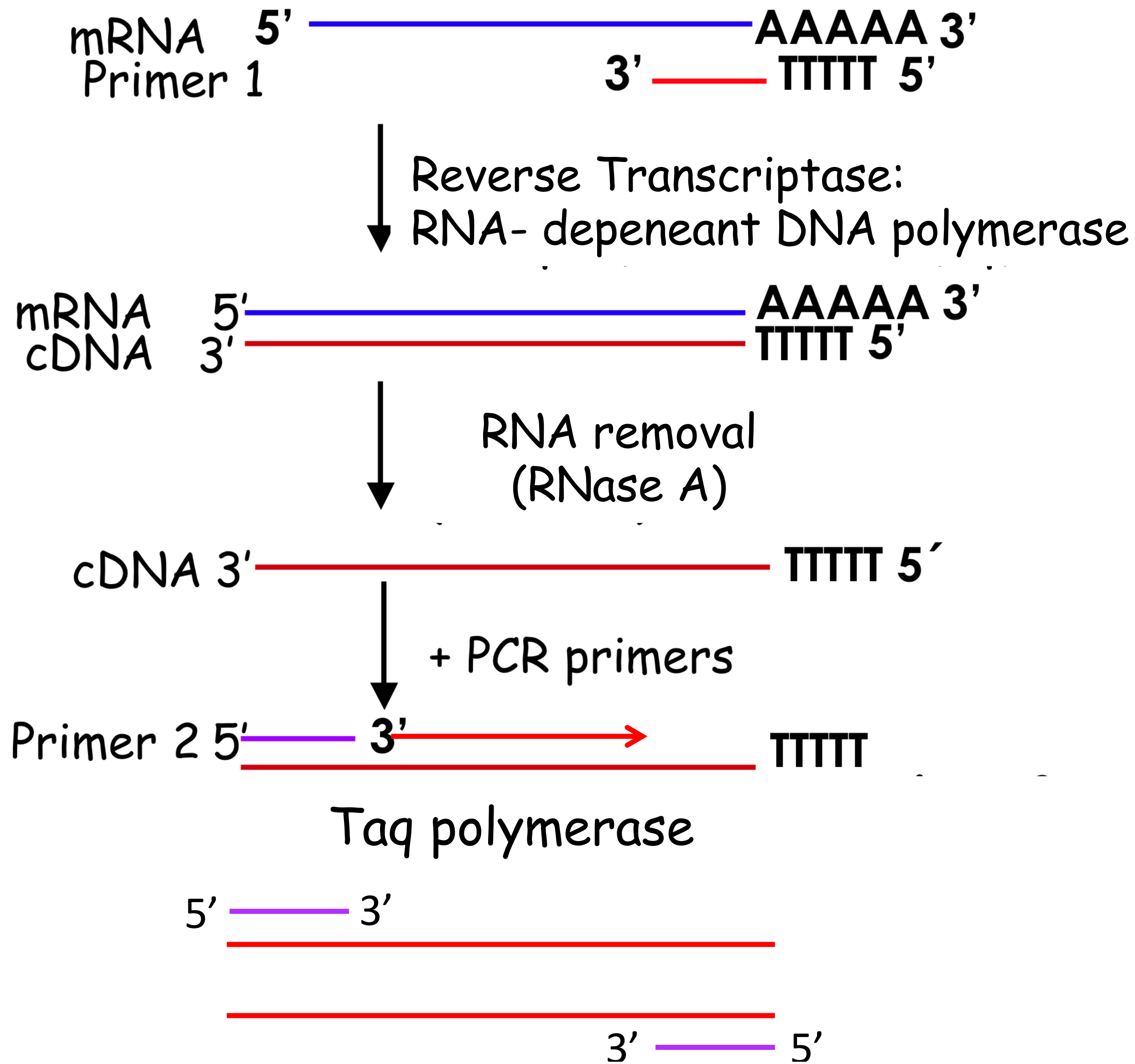
Retroviruses can replicate in a cell through **reverse transcription**

reverse transcriptases (RT) can make a DNA strand from a RNA template

With RT-PCR you can test the target RNA presence in your sample

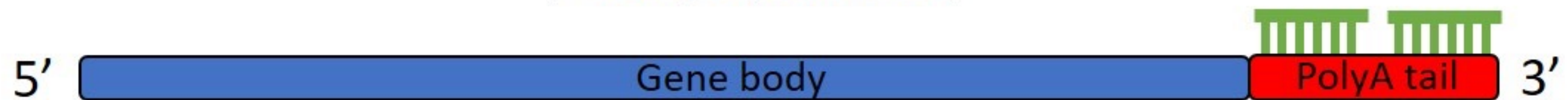


The RT-PCR



Many RT primers

Oligo(dT) primers



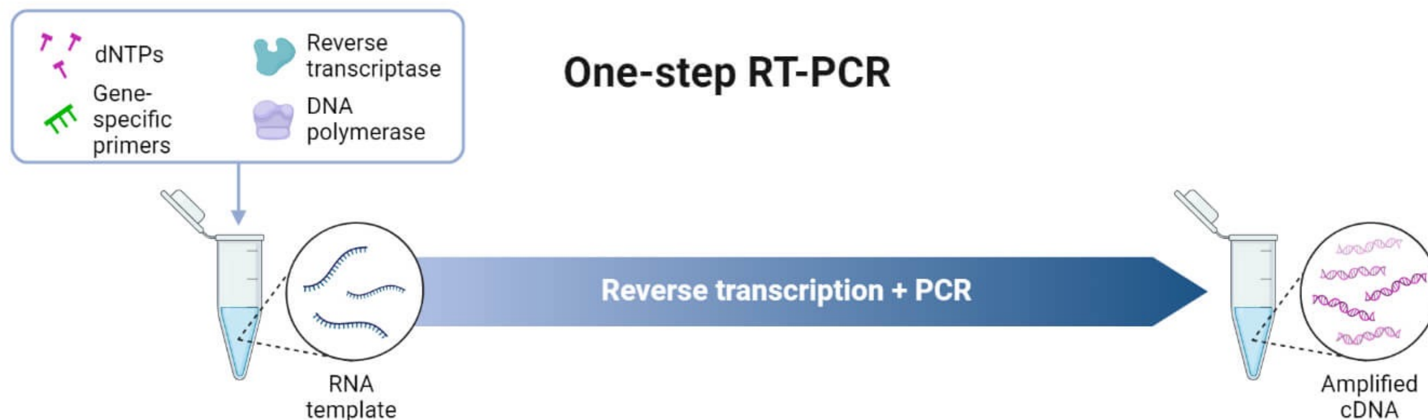
Random hexamers

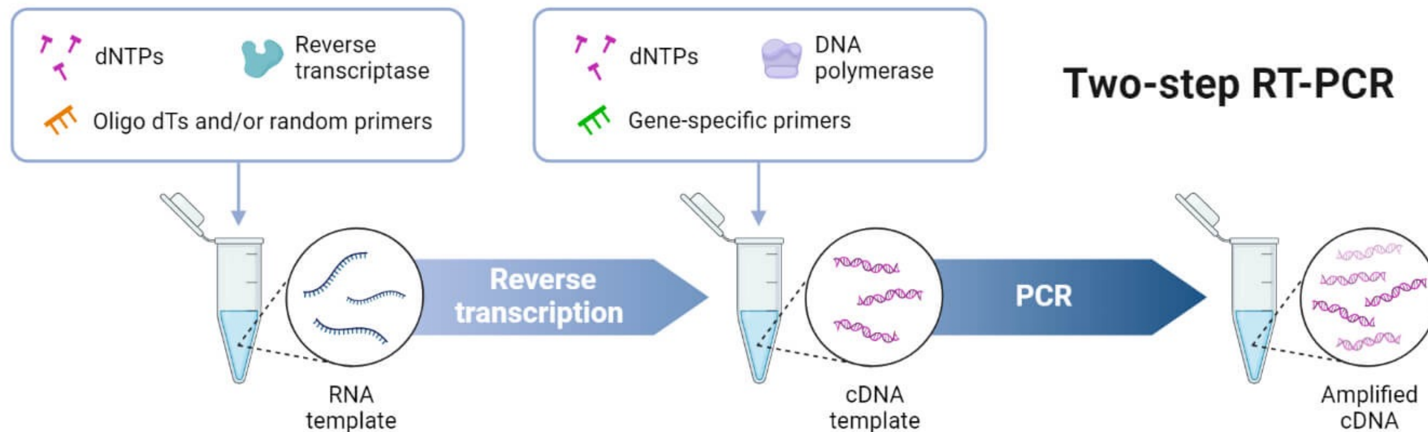
Region-specific primers

One-step vs Two-step RT-PCR

RT-PCR proceeds with two steps:

1. cDNA synthesis (to make cDNA from RNA)
2. PCR (to amplify cDNA)



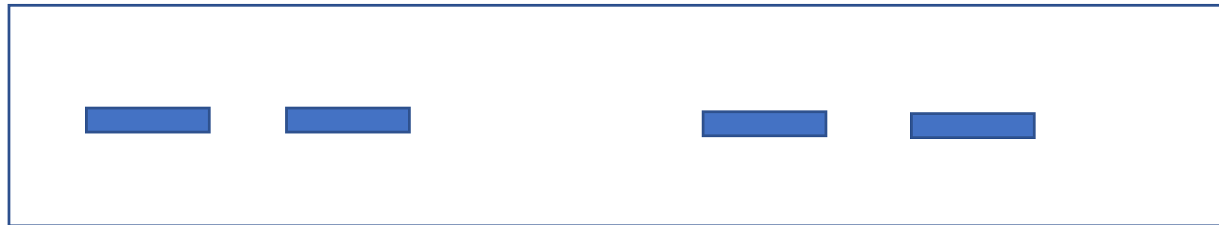


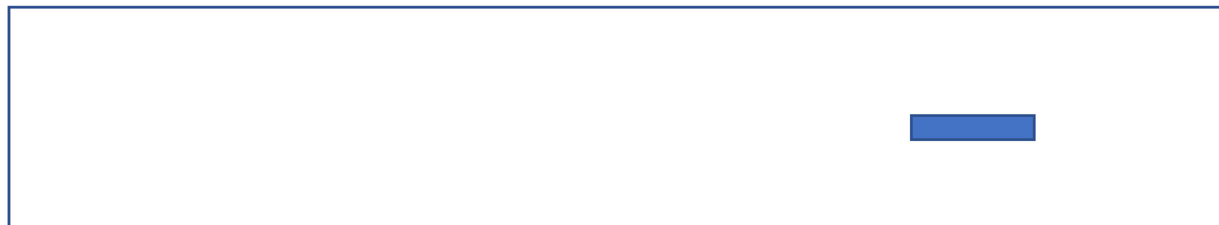
The kit is intended for the *in vitro* detection and quantification of COVID-19 RNA in human nasopharyngeal and oropharyngeal swab or sputum specimens utilizing **Reverse Transcriptase-Polymerase Chain Reaction (PCR)**

	Reagent	Manufacturer	Catalog #
	DNA and Viral Small Volume Kit (3x192 purifications)	Roche	06543588001
	TaqPath 1-Step RT-PCR Master Mix, GC (2000 reactions)	ThermoFisher	A15300
N1	COVID-19_N1-F Primer	IDT	Custom
N1	COVID-19_N1-R Primer	IDT	Custom
	COVID-19_N1-P Probe	IDT	Custom
N2	COVID-19_N2-F Primer	IDT	Custom
N2	COVID-19_N2-R Primer	IDT	Custom
	COVID-19_N2-P Probe	IDT	Custom
N3	COVID-19_N3-F Primer	IDT	Custom
N3	COVID-19_N3-R Primer	IDT	Custom
	COVID-19_N3-P Probe	IDT	Custom
RNase P	RP-F Primer	IDT	Custom
RNase P	RP-R Primer	IDT	Custom
	RP-P Probe	IDT	Custom
+	COVID-19_N_Positive Control	IDT	Custom
	Hs_RPP30_Internal Extraction Control	IDT	Custom

+ In vitro produced viral RNA

N1 N2 N3 RP + -





N1 N2 N3 RP + -

POSITIVE

POSITIVE

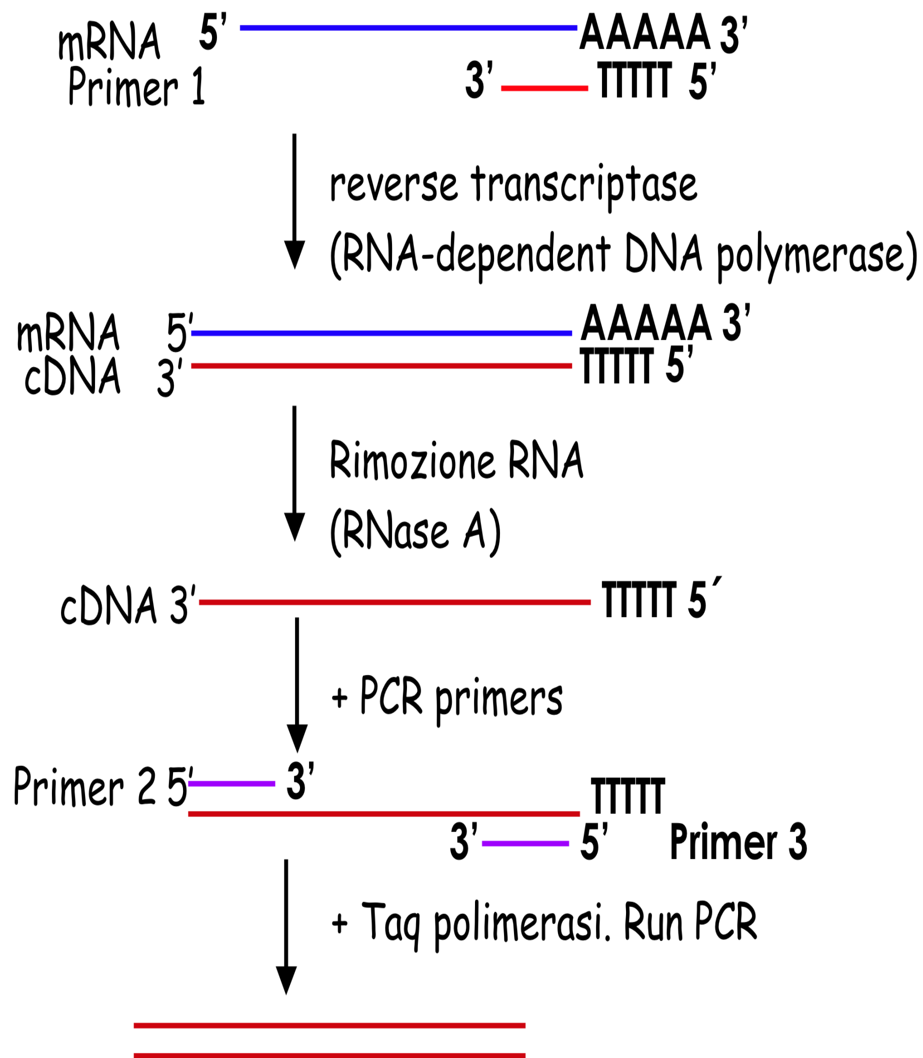
?

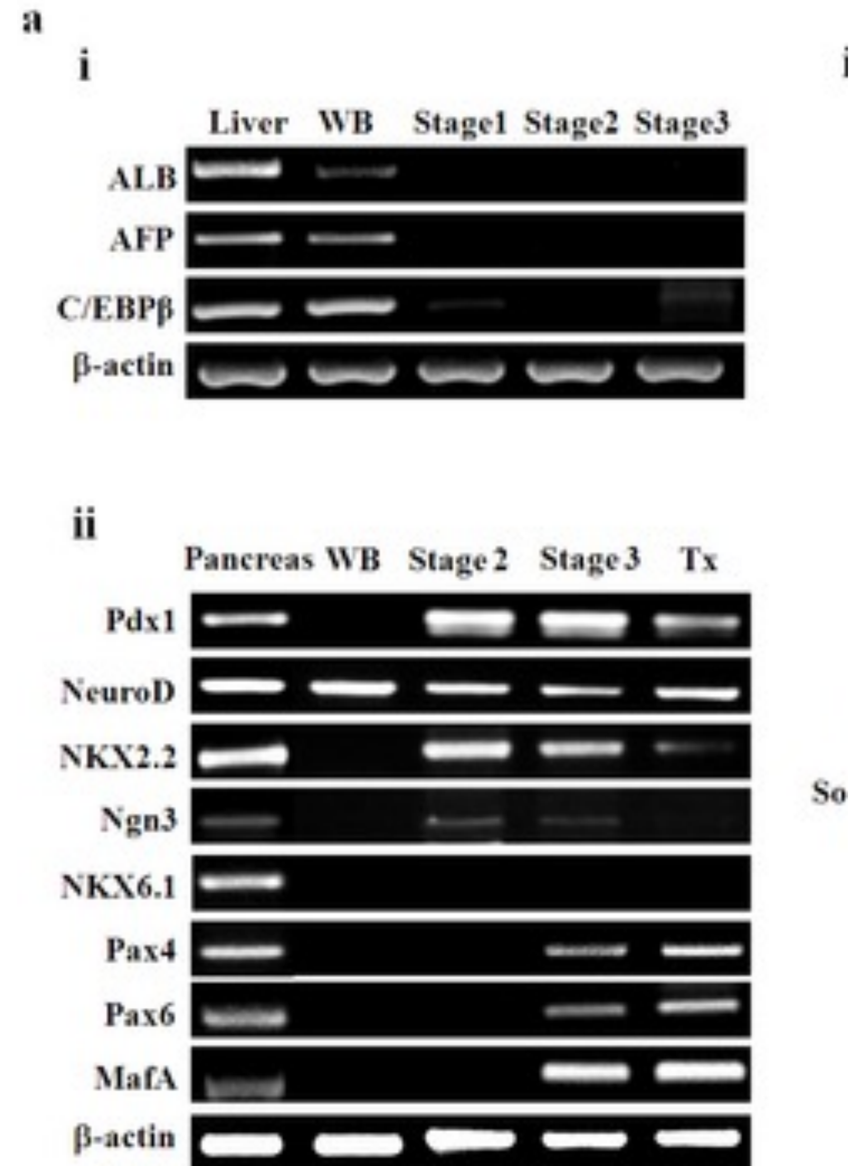
NEGATIVE

invalid

invalid

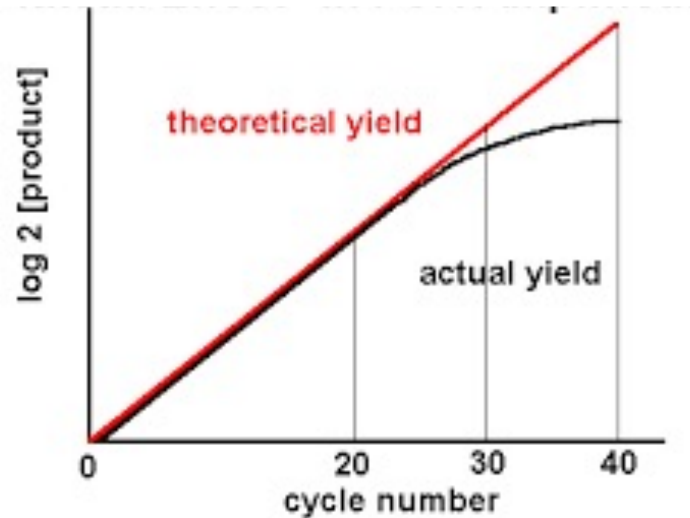
RT-qPCR to study gene expression

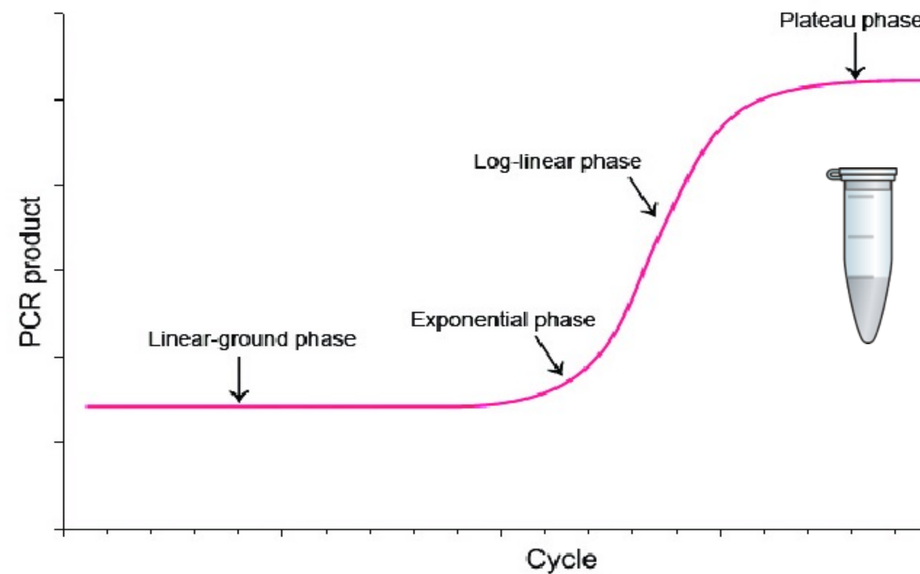




The plateau effect

PCR doesn't go forever

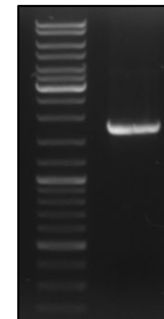




"PLATEAU" effect

- Reagents are going over
- Taq polymerase activity decreases
- DNA does not increase any more

End-point analysis



How can we obtain data from the exponential phase?

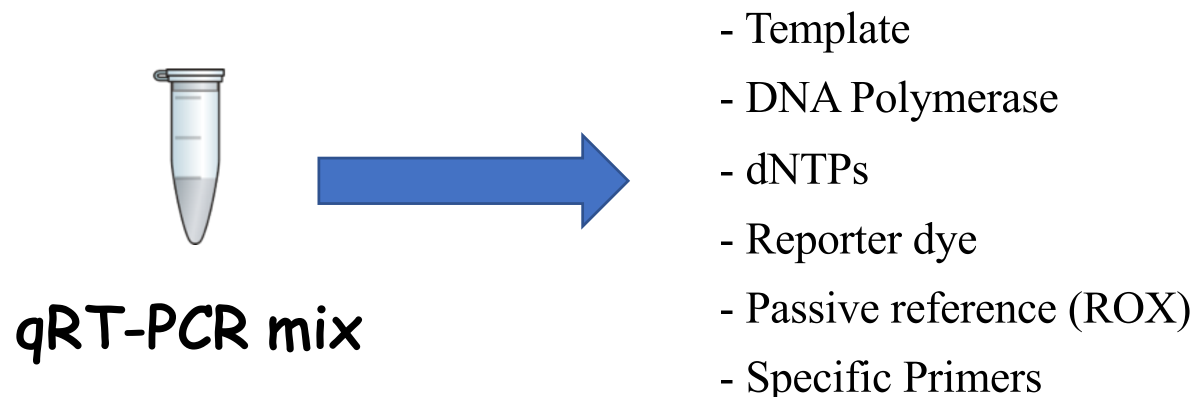
How could we **quantify DNA** in the reaction in *real-time*?

qRT-PCR: The PCR that "quantifies"

REAL TIME PCR or qRT-PCR allows to quantify the synthesis of PCR product <u>at every stage of amplification</u> *in real-time*

Thanks to **fluorescent reporter dyes that bind DNA**

Quantitative measurment: **the fluorescence signal is proportional to the number of target DNA copies**



Reporter dyes

Reporter DYE:

NON – SPECIFIC

Fluorescent intercalant

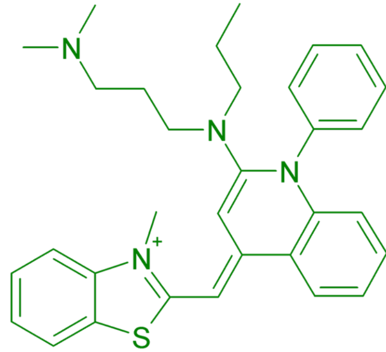
(SYBER GREEN)

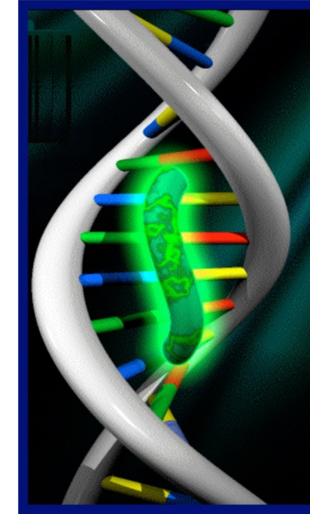
SPECIFIC

Molecular probes marked with fluorescent molecules

(TAQ-MAN, Molecular beacon, etc.)

Syber green



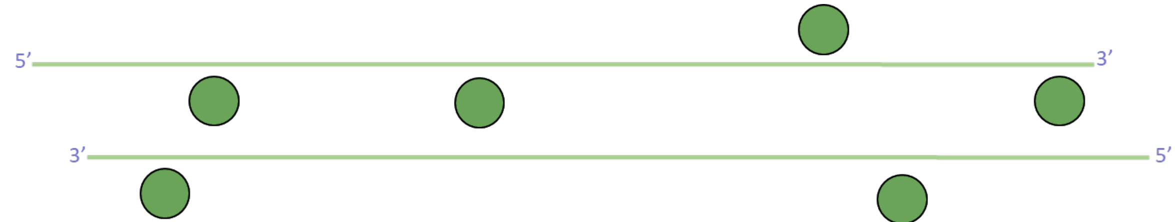


Syber green is not fluorescent in solution, it is when it's inside the minor groove of DNA

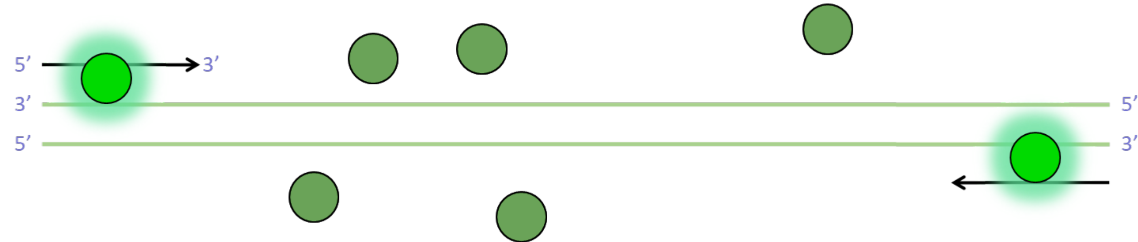
In dsDNA, Syber green absorbs blue light and emits green light

Syber green

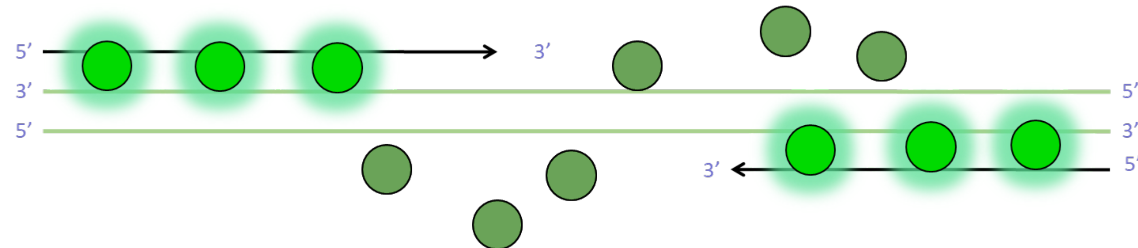
1) Denaturation



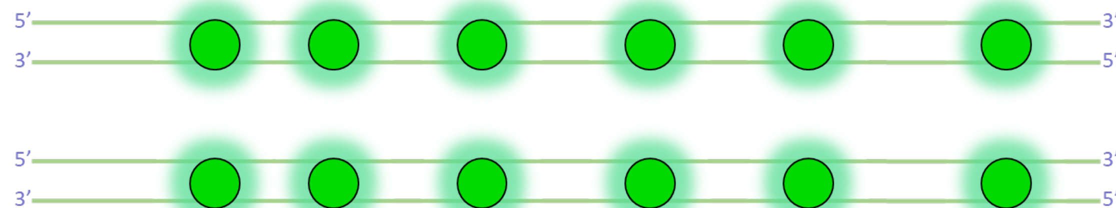
2) Annealing



3) Extension



4) End of first cycle



Taq-man probe

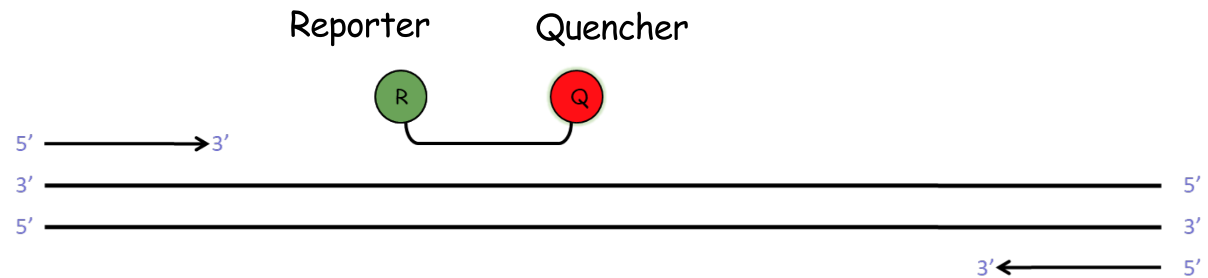
The TaqMan probe is a oligonucleotide complementary to a region in the target sequence.

It contains a «Reporter» fluorophore at the 5′ and a «Quencher» molecule at the 3′.

1) Denaturation / Annealing

Photons emitted by R are absorbed

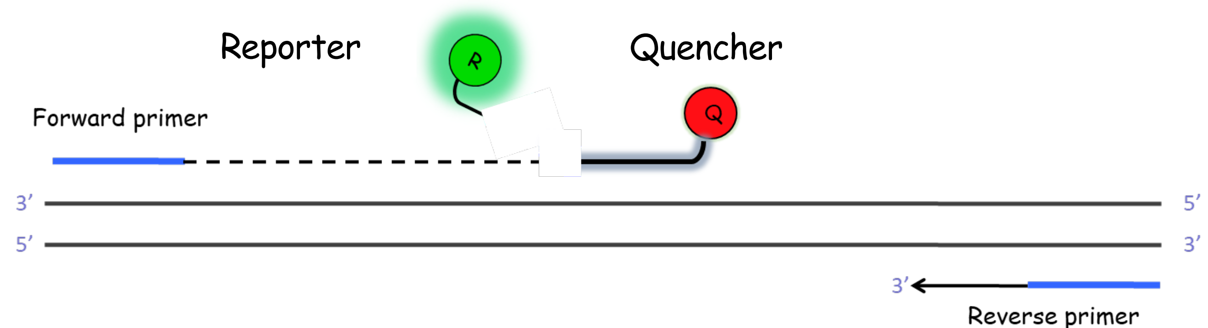
by the Q = no fluorescence



2) Extension

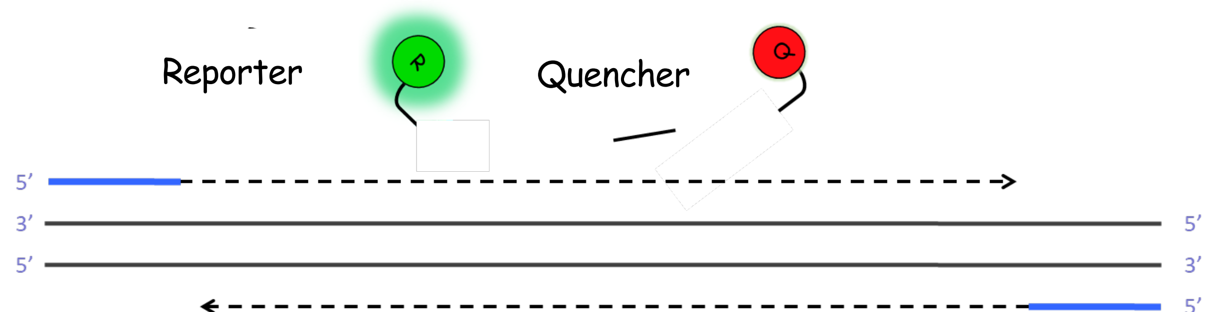
DNA polymerase 5’>3’

exonuclease activity cuts off the R



Once far from the Q,

the R can fluoresce

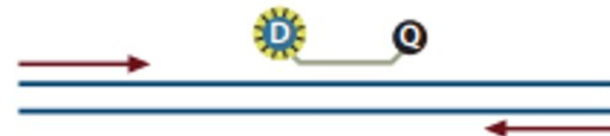


Molecular Beacon

Molecular beacons contain a fluorophore and a quencher at opposite ends of an oligonucleotide which contains a stem and a loop embedding a sequence complementary to the target

1) Denaturation

2) Annealing



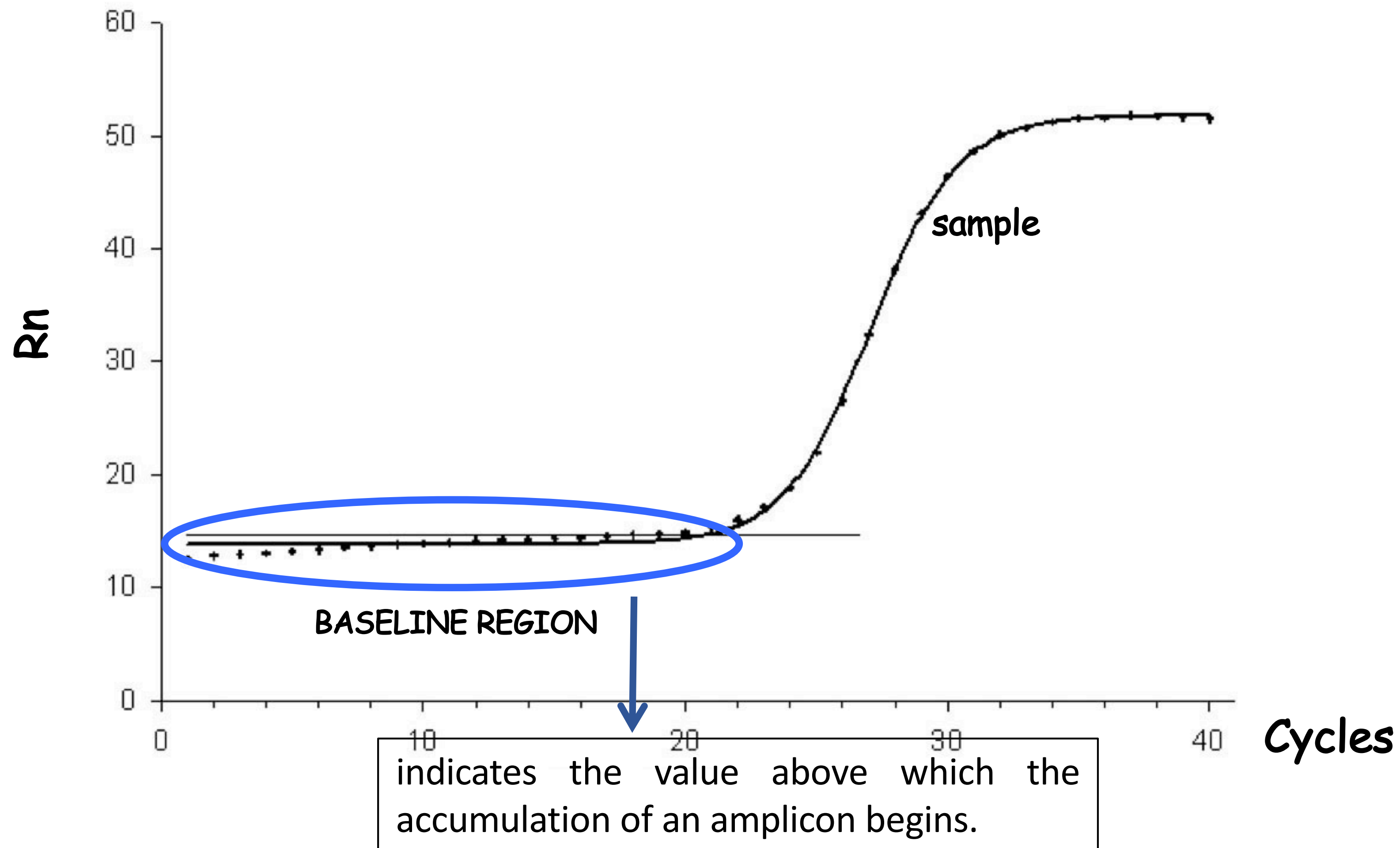
3) Extension

4) End of first cycle

Molecular beacons are not destroyed during the extension and can therefore rehybridize during the next PCR cycle

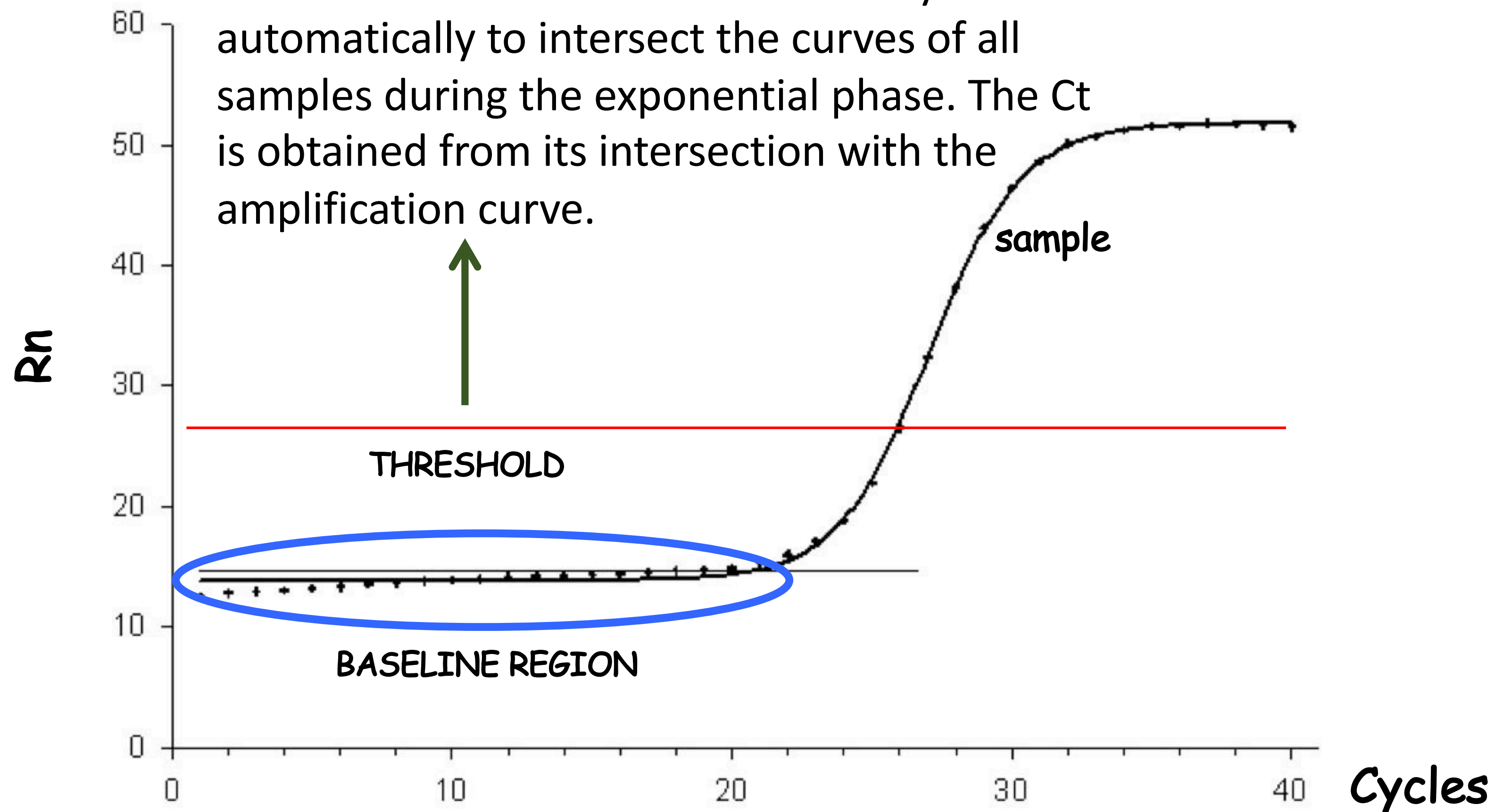
Amplification plot

The fluorescence (Rn) is recorded at every cycle



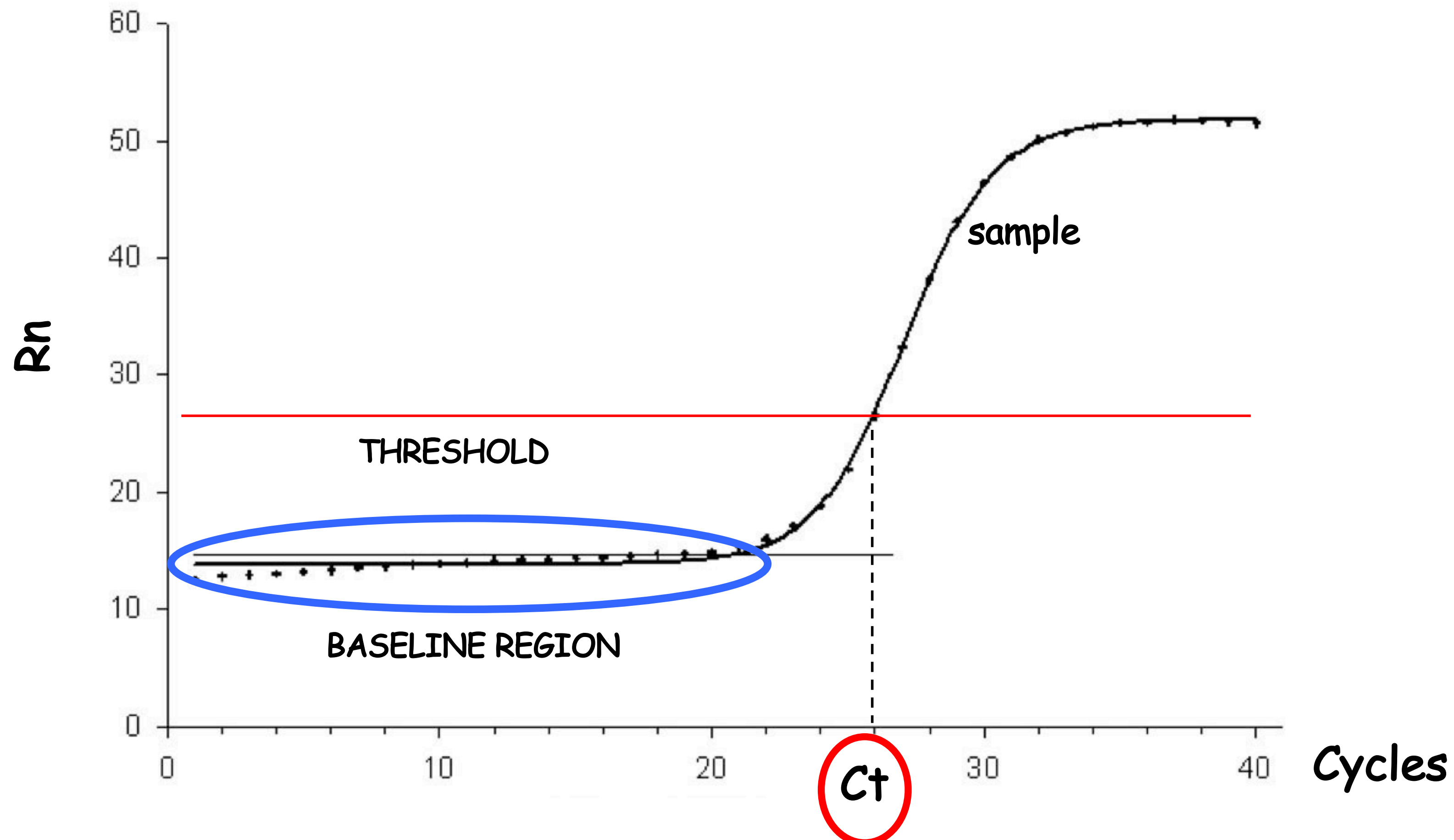
Amplification plot

The fluorescence (Rn) is recorded at every cycle



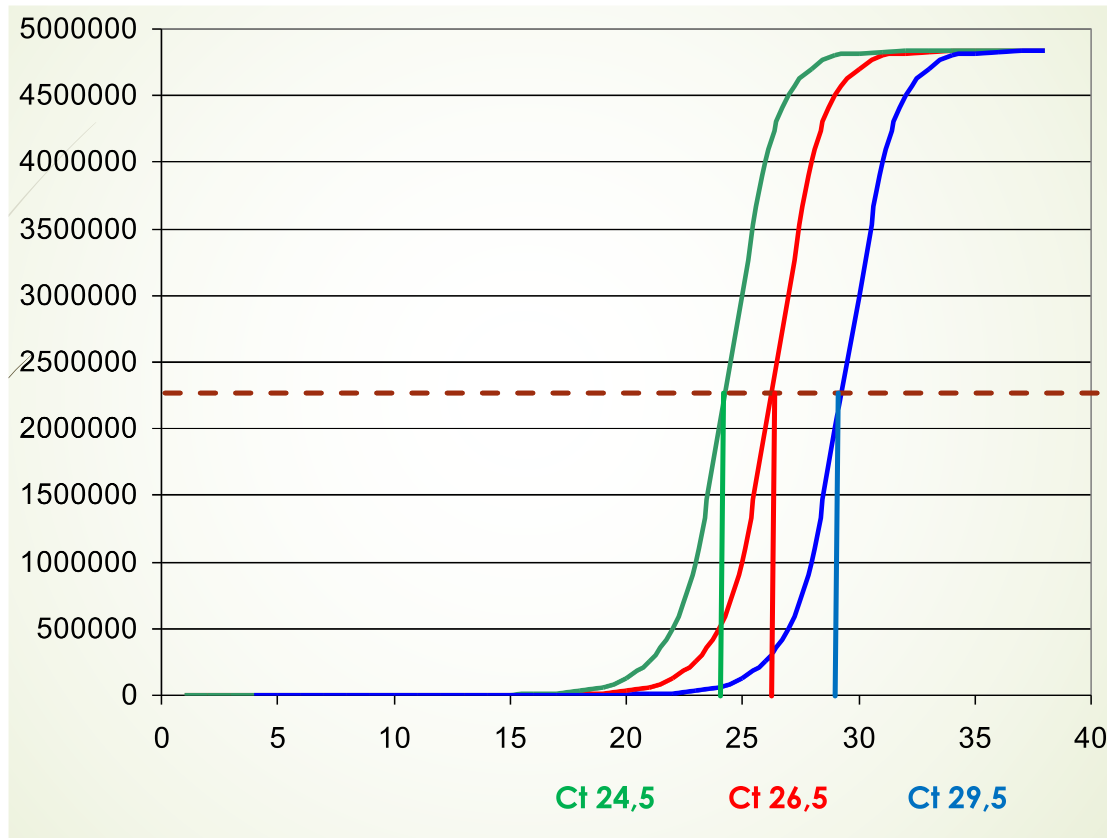
Amplification plot

The fluorescence (Rn) is recorded at every cycle



It is the cycle of the amplification reaction in which the fluorescence signal of the sample exceeds the threshold level.

Amplification plot



Target quantity in each sample:

A: 1 000 copies

B: 8 000 copies

C: 32 0000 copies

THRESHOLD

Cts are the measure we need!

← More target in the sample

Less target in the sample →

For a given target, each target in each sample has its own Ct

Ct values are inversely proportional to the initial amount of the target in the sample

Quantification

ABSOLUTE

The samples are quantified in absolute terms:

•Requires standards with known concentrations (ng/ml) to create a line or "standard curve.»

•For all "unknowns," identical amounts of samples must be tested.

RELATIVE

Quantification is performed by comparing CT values:

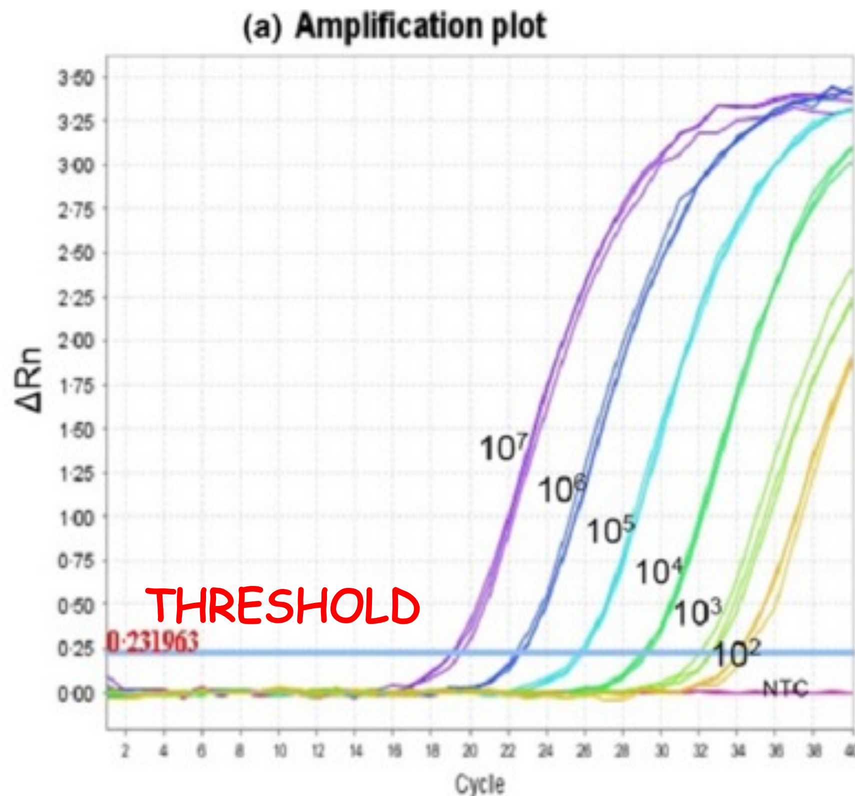
•Requires endogenous controls (a standard curve is not used).

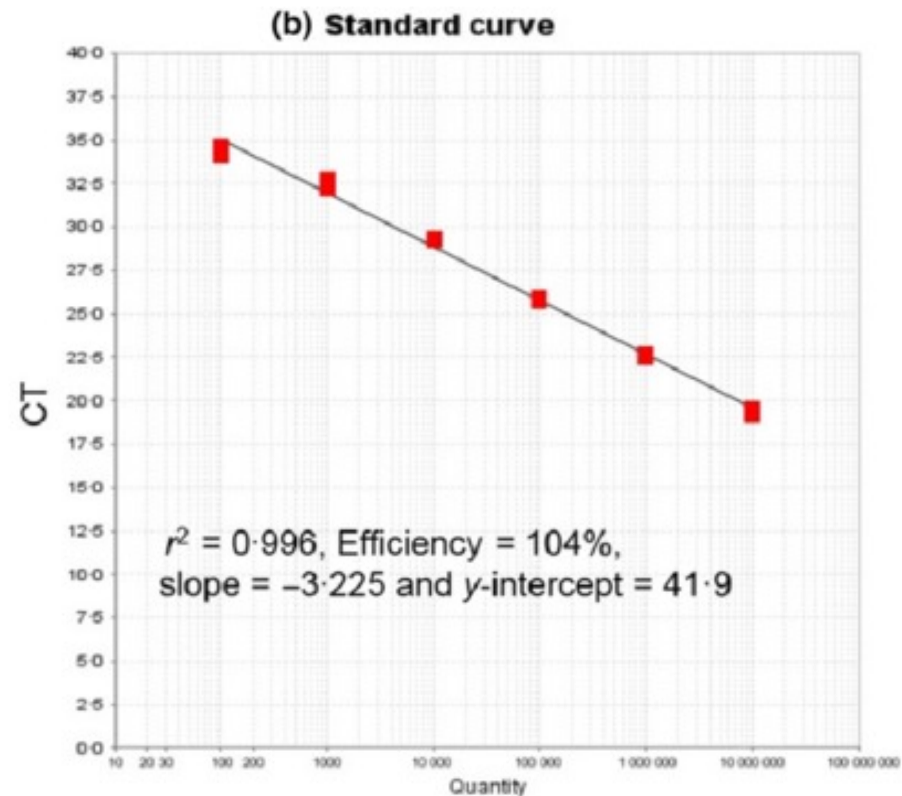
•The "unknowns" are "quantified" by comparing their ΔCT with that of the endogenous control.

Absolute quantification

We need do construct a standard curve.

$y = ax + b$



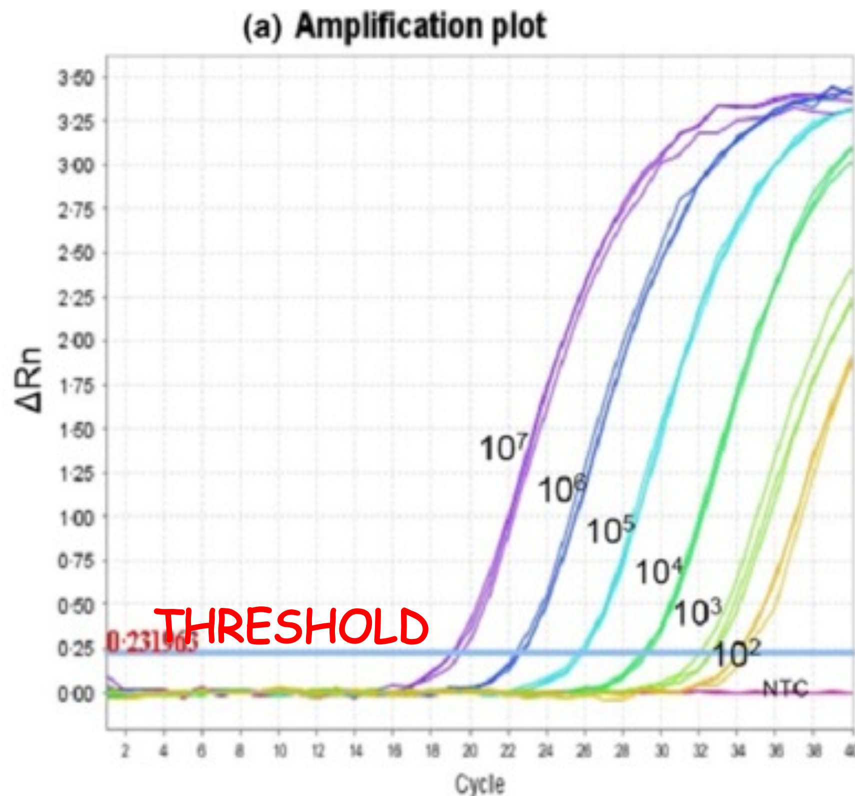


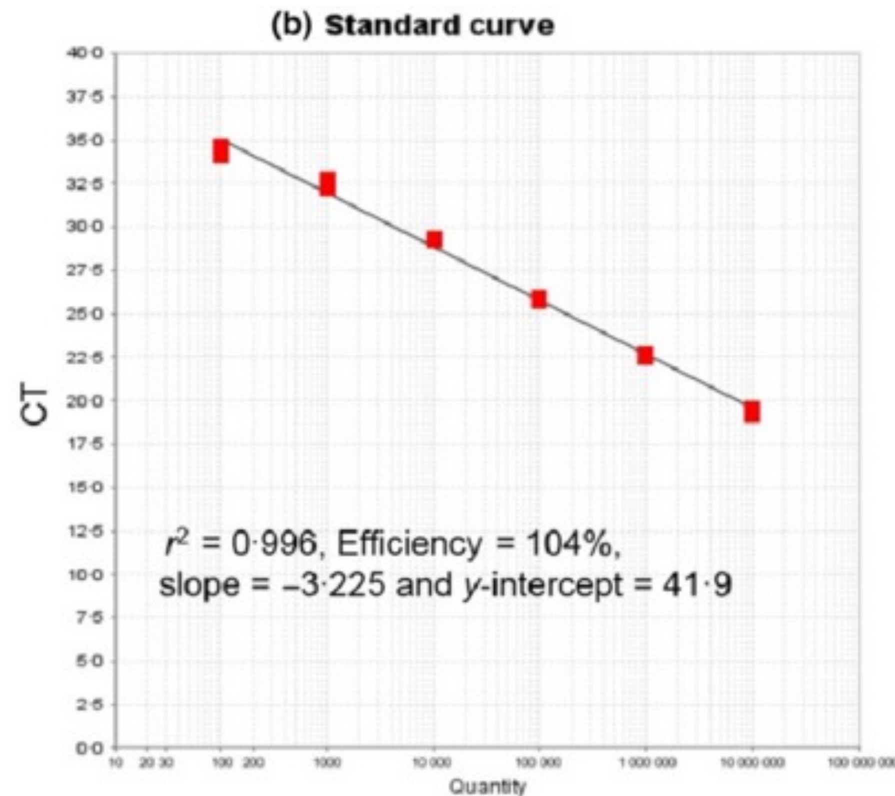
amplification EFFICIENCY of the oligo is associated with the R^2 value, or the coefficient of determination, which represents the proportion between data variability and the accuracy of the statistical model used. An $R^2 \geq 0.98$ indicates an excellent correlation between the obtained data and the calibration line.

Absolute quantification

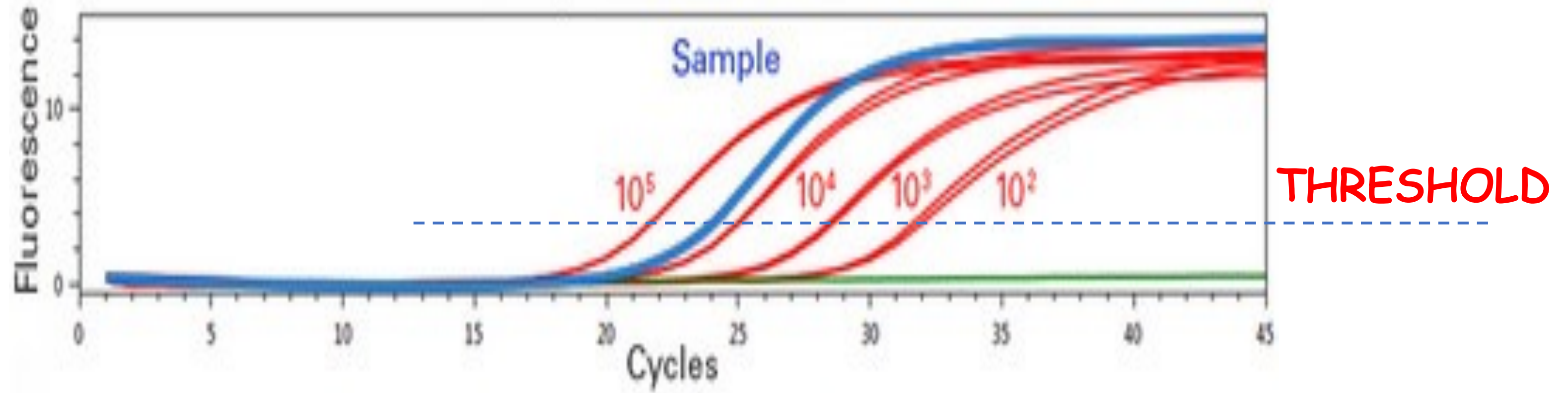
We need do construct a standard curve.

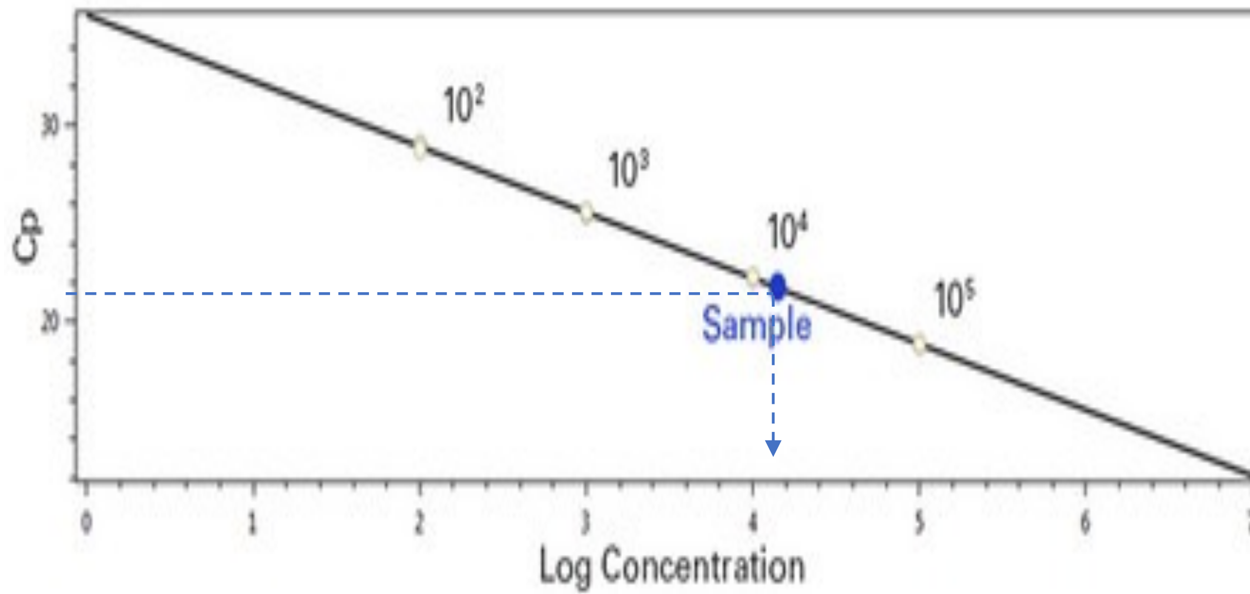
$y = ax + b$





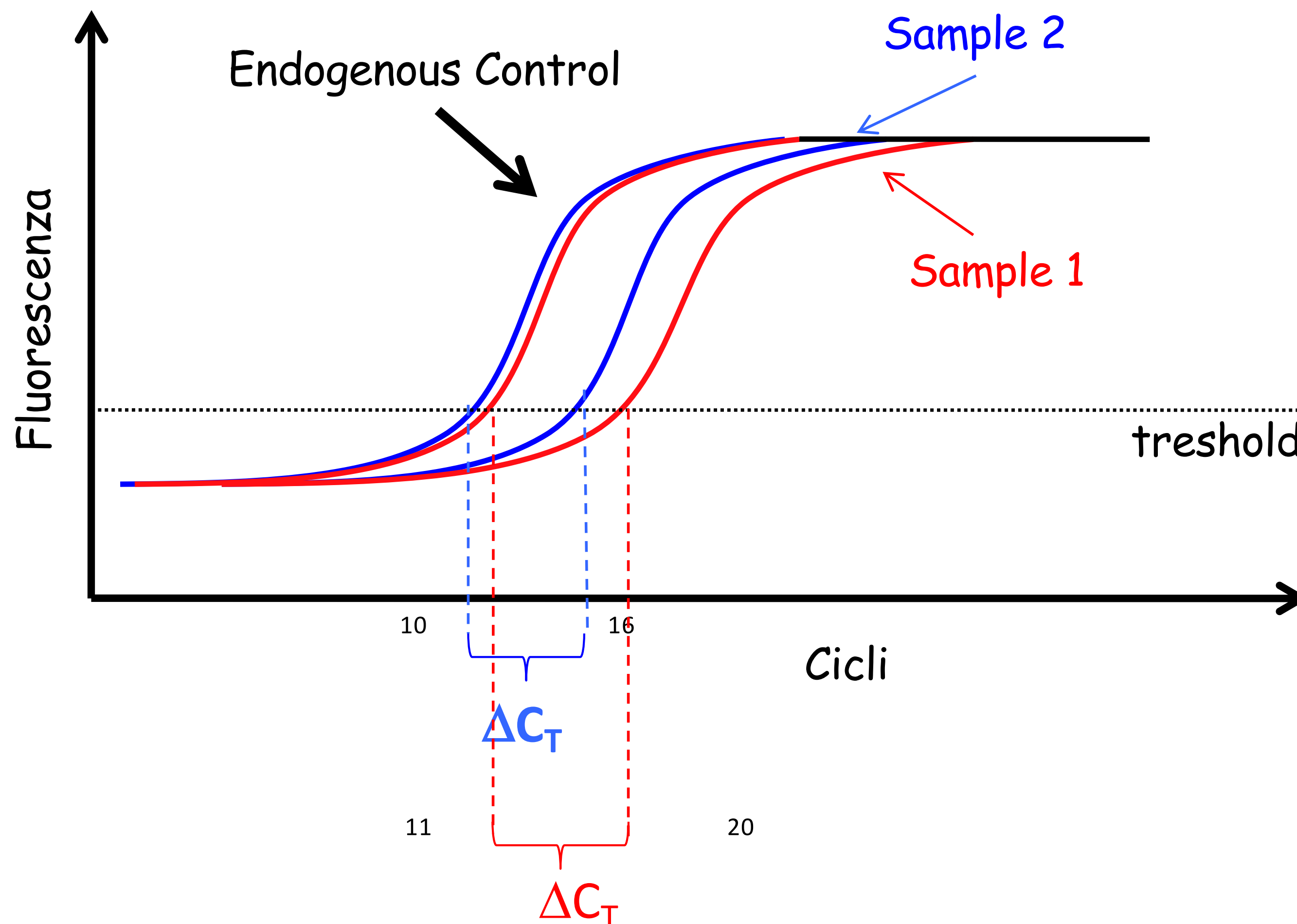
The slope of the calibration curve is the average of the slope values obtained from three independent calibration lines. The acceptability criterion is met when -3.1 ≥ slope ≥ -3.6. A PCR reaction with 100% efficiency will have a slope of -3.32.


Amplification Curves
Sample
10⁵
10⁴
10³
10²
THRESHOLD
Fluorescence
Cycles


Standard Curve
10²
10³
10⁴
10⁵
Sample
Cp
Log Concentration

Relative quantification

You can compare Ct of different samples once enstablished the endogenous control



Relative quantification

$$\Delta Ct_{sample} = Ct_{sample} - Ct_{reference\ RP}$$

$$\text{fold change} = 2^{-Ct\ sample} / 2^{-Ct\ reference} = 2^{-\Delta Ct\ sample}$$

Relative quantification

Normalization:

$$\Delta Ct_{sample} = Ct_{sample} - Ct_{reference\ RP}$$

$$\text{fold change}_c = 2^{-Ct\ sample} / 2^{-Ct\ reference} = 2^{-\Delta Ct\ sample}$$

Is there difference between my samples?

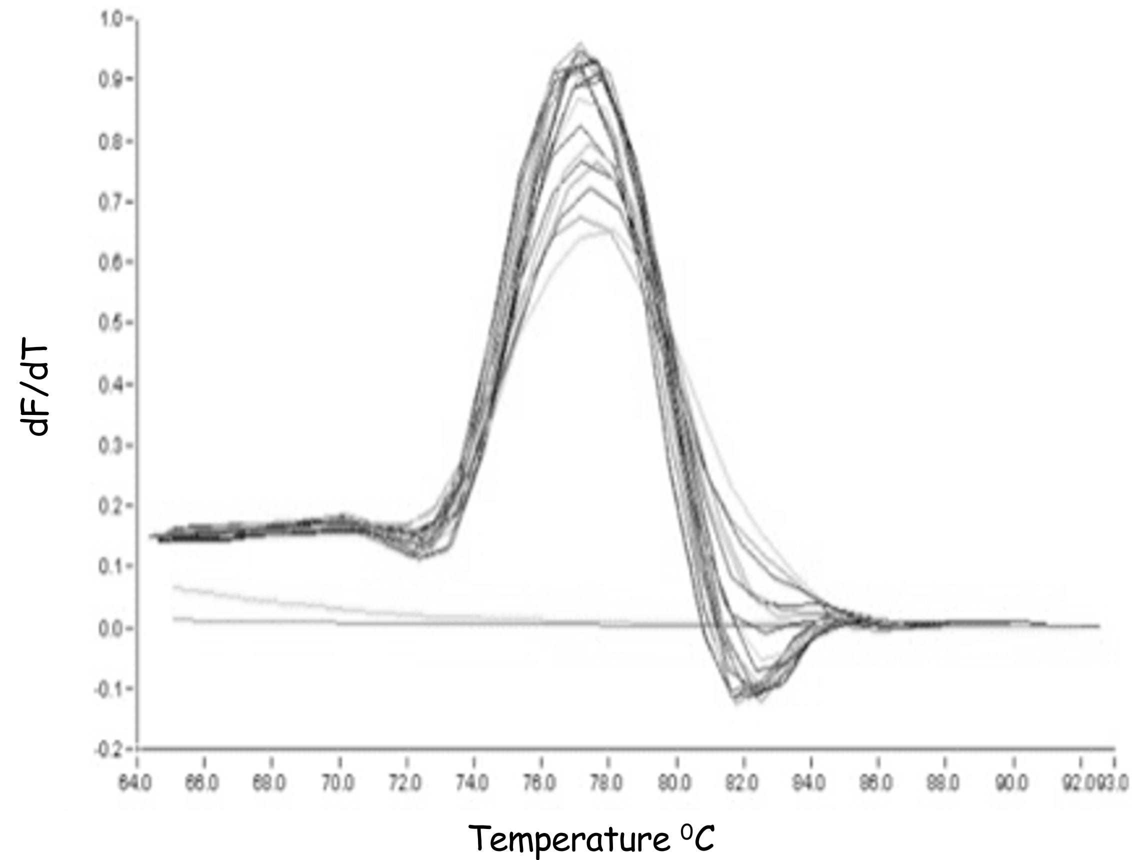
$$\text{fold change}_{c1} = 2^{-Ct\ sample} / 2^{-Ct\ reference} = 2^{-\Delta Ct\ sample1}$$

$$\text{fold change}_{c2} = 2^{-Ct\ sample} / 2^{-Ct\ reference} = 2^{-\Delta Ct\ sample2}$$

$$\text{fold change} = \text{fold change}_{c1} / \text{fold change}_{c2}$$

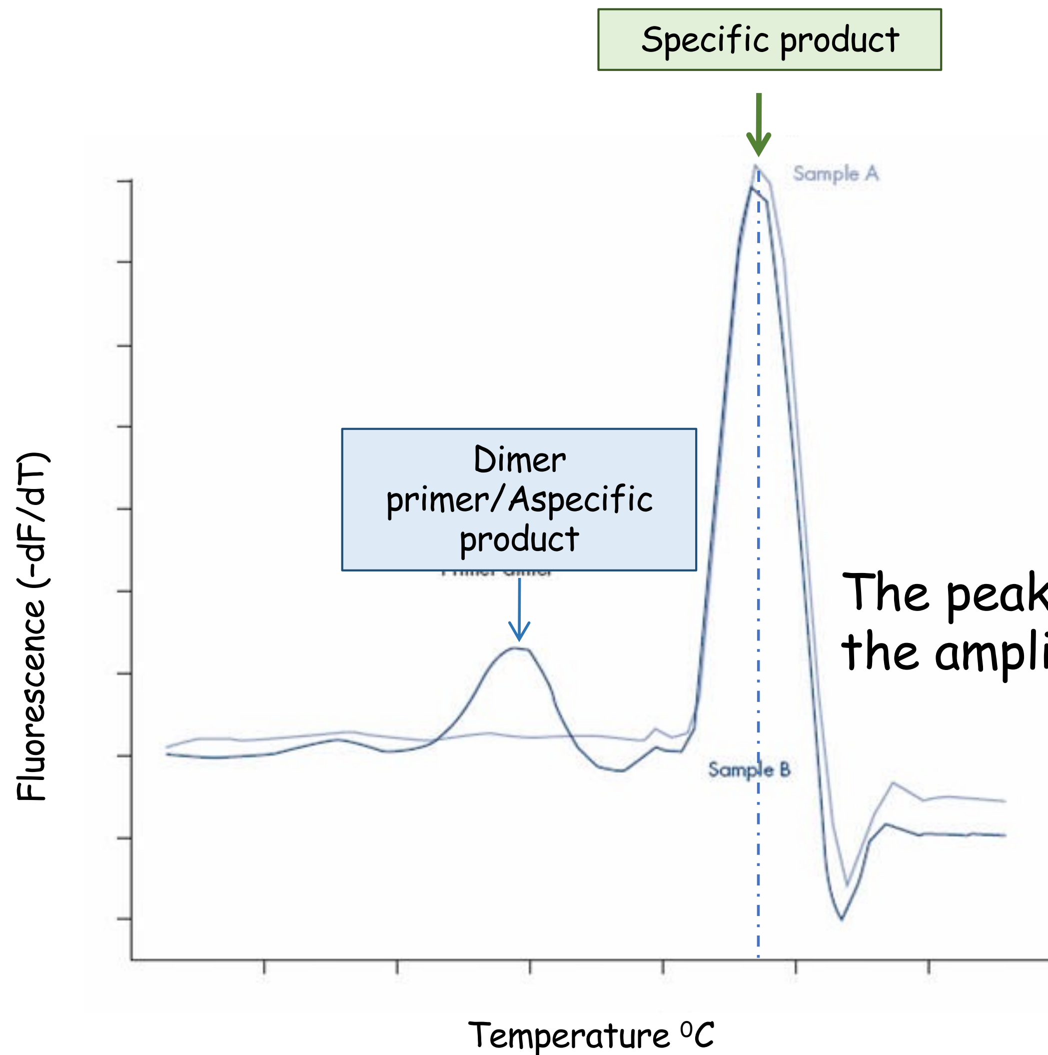
$$\text{fold change} = 2^{-\Delta\Delta Ct\ sample}$$

The Melting curve



The evaluation of the Tm of our amplicons

The Melting curve analysis



Syber Green cannot distinguish between different products but the Tm does

Sybr Green will bind to all double-stranded DNA molecules, and dissociation peaks different from that of the specific product will be observed.

The peak heightis proporzional to the amplification of the product

The Melting curve analysis