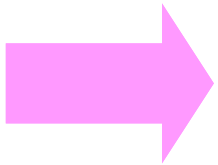
PCR

P olymerase

C hain

R eaction

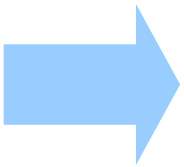


What is it?

A very efficient method for

IN VITRO REPLICATION of DNA

What is for?



Synthesis of several million copies of a specific DNA sequence within a few hours

PCR ingredients

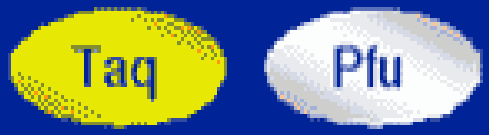
Primers

DNA template

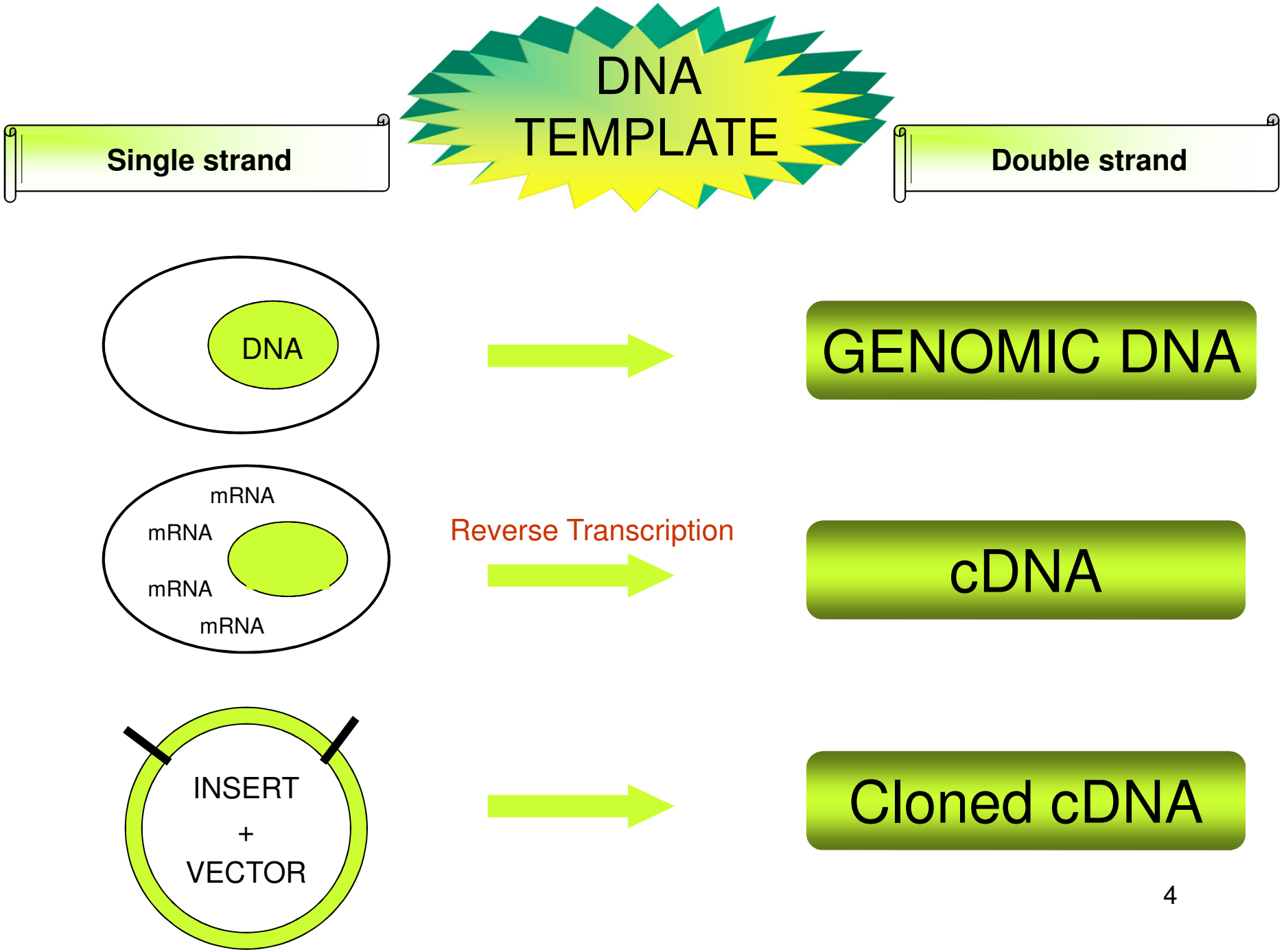
Buffer

dATP
dCTP
dGTP
dTTP

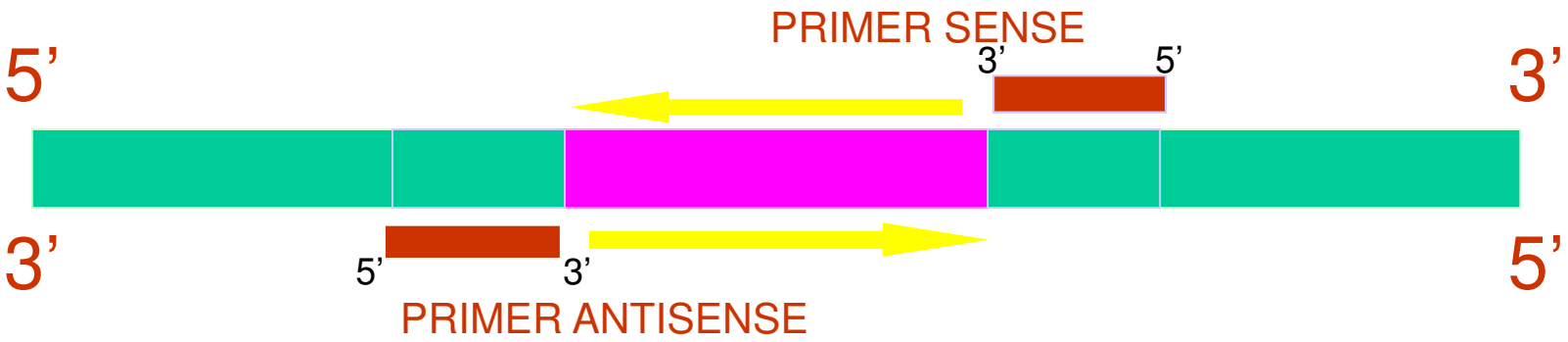
dNTPs



Thermostable DNA polymerase

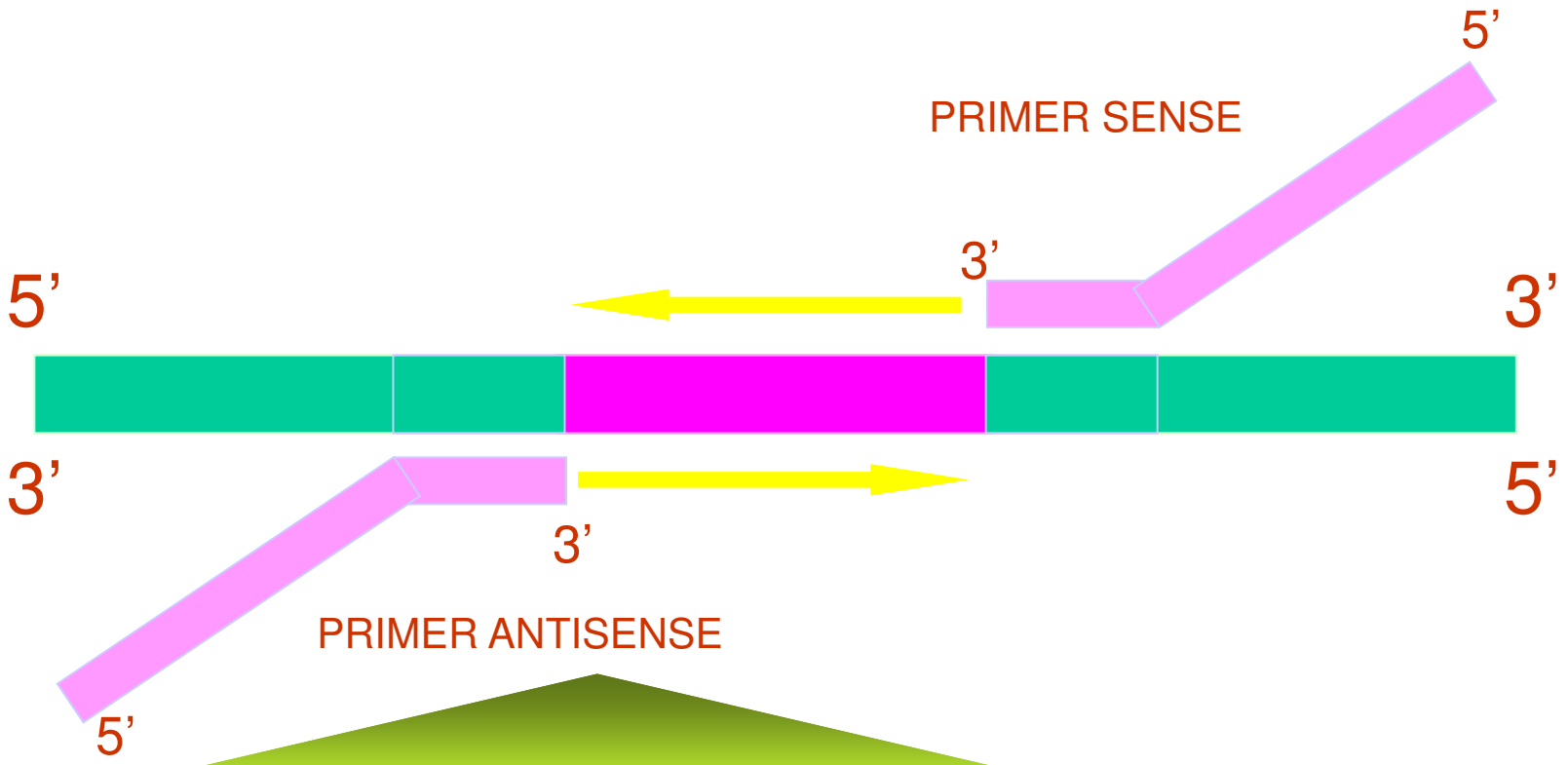

DNA TEMPLATE
Single strand
Double strand
DNA
GENOMIC DNA
mRNA
mRNA
mRNA
mRNA
Reverse Transcription
cDNA
INSERT
+
VECTOR
Cloned cDNA

OLIGONUCLEOTIDE PRIMERS



To amplify a specific DNA sequence

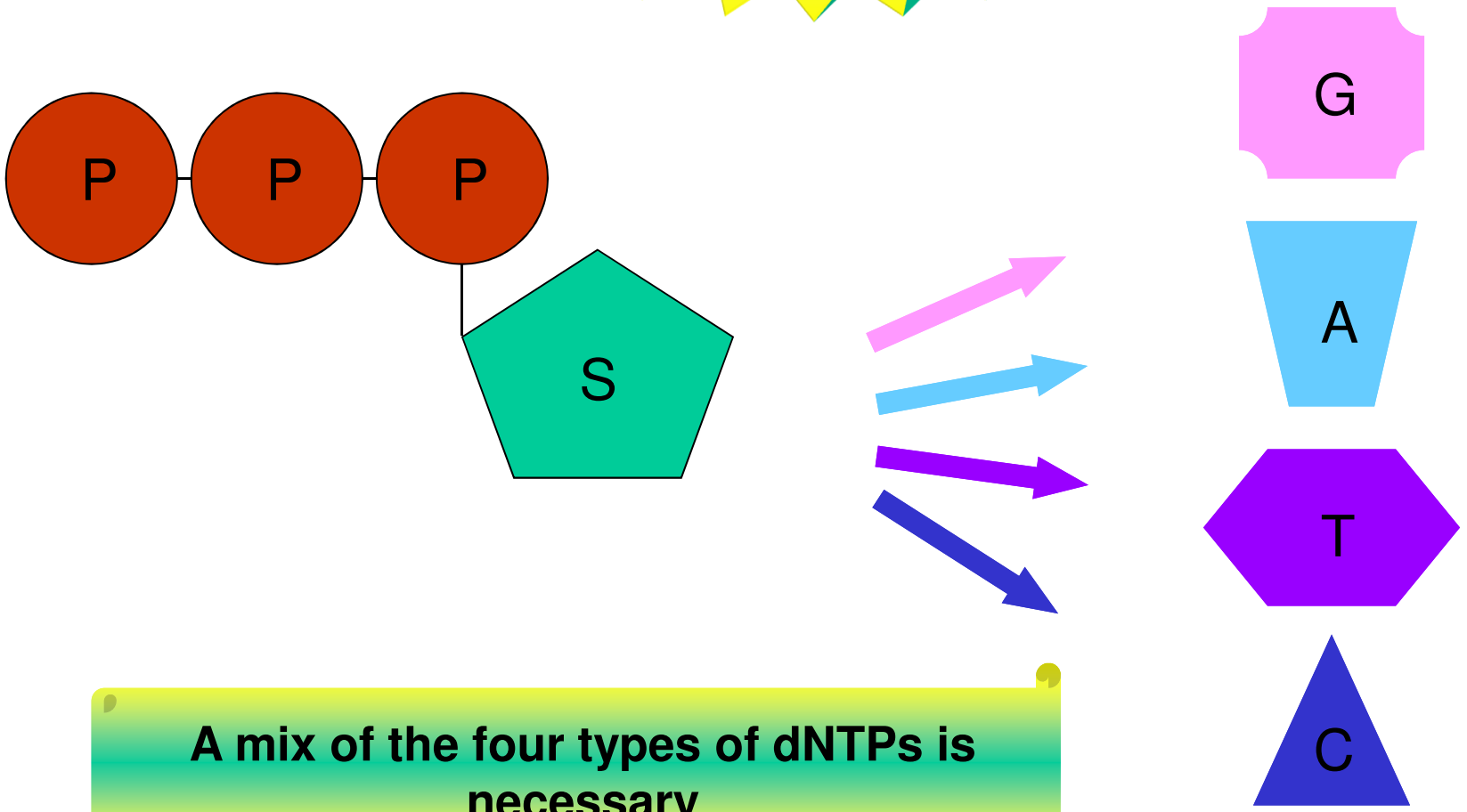
a couple of primers is necessary



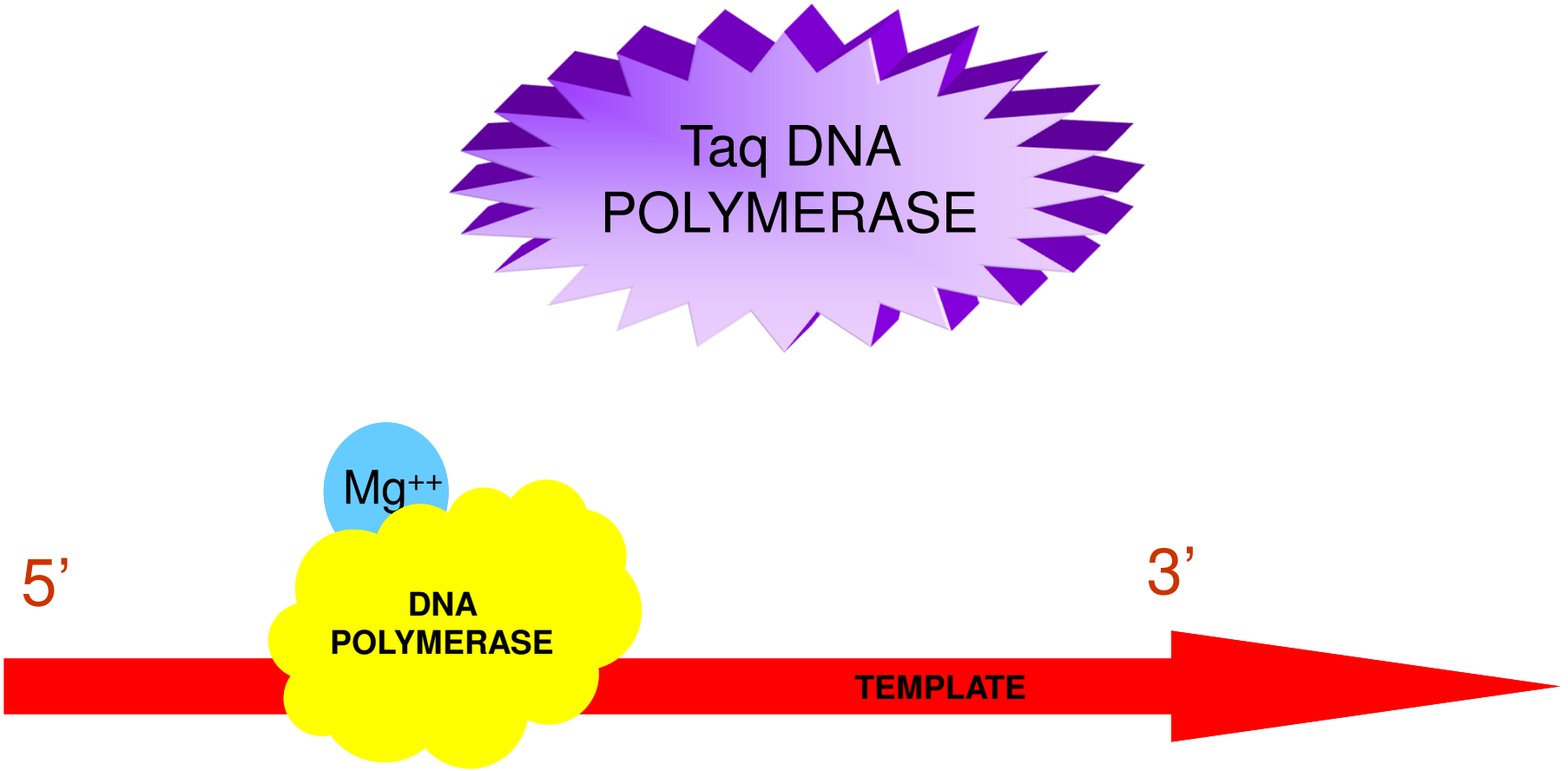
The 3’end of the primer needs to be a good match for the target

The 5’ end of the primer need not be a good match → incorporation of an useful sequence

DEOXYNUCLEOTIDE TRIPHOSPHATES



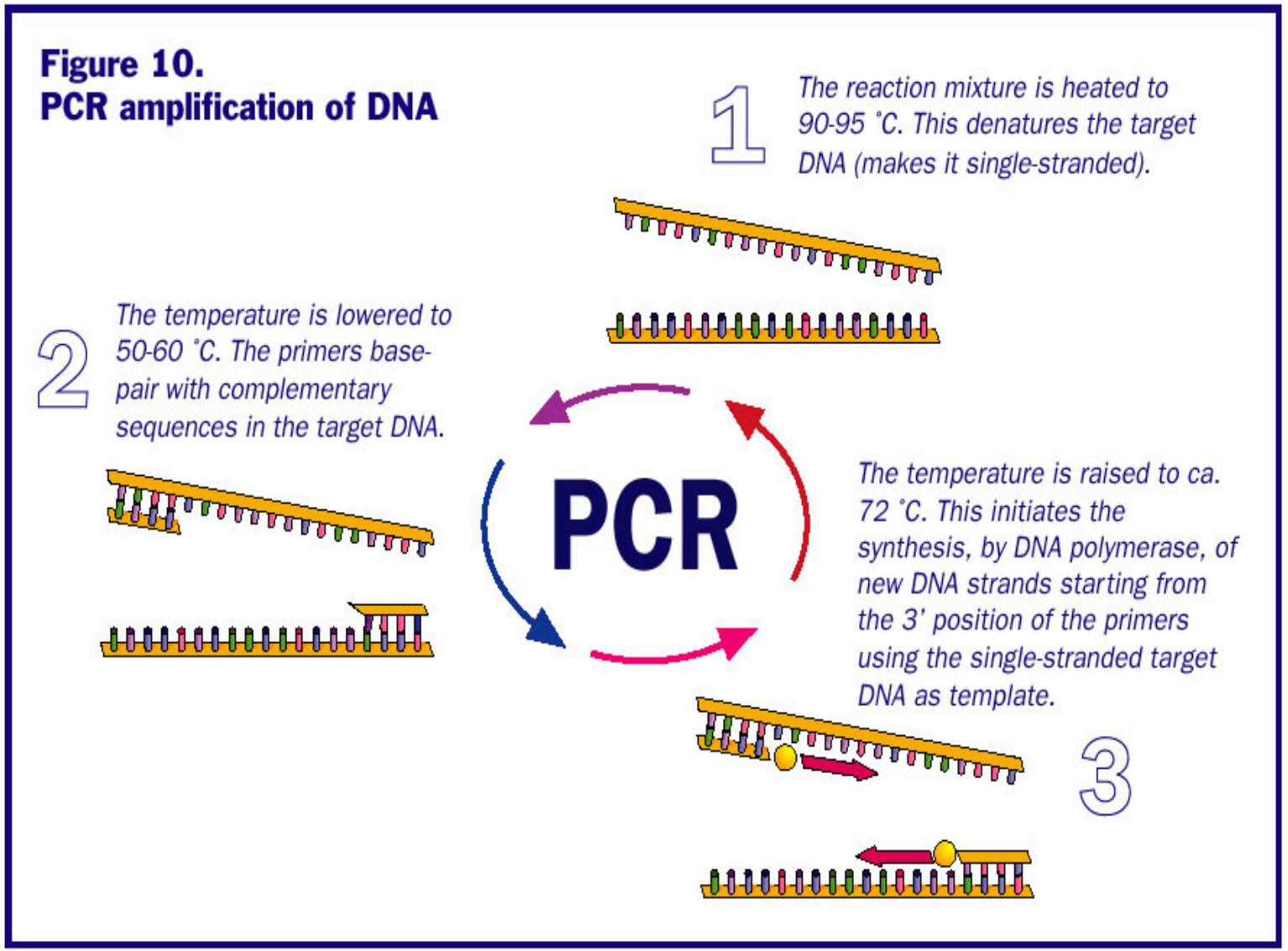
A mix of the four types of dNTPs is necessary



High synthesis rate

High fidelity

Figure 10.
PCR amplification of DNA



DESIGN AND OPTIMIZATION OF THE PCR

PRIMER DESIGN

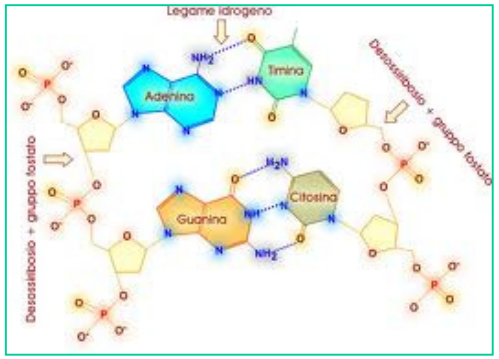
The selection of optimal primers remains somewhat empirical

...BUT

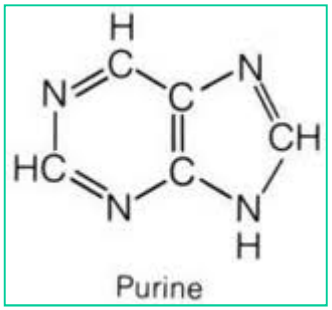
Some guidelines may help in primer selection

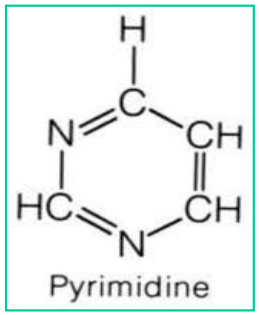
1 Optimal length: 20-30 bases
Optimal concentration: 0.05mM to 0.5mM

2 RANDOM BASE DISTRIBUTION
GC amount = AT amount



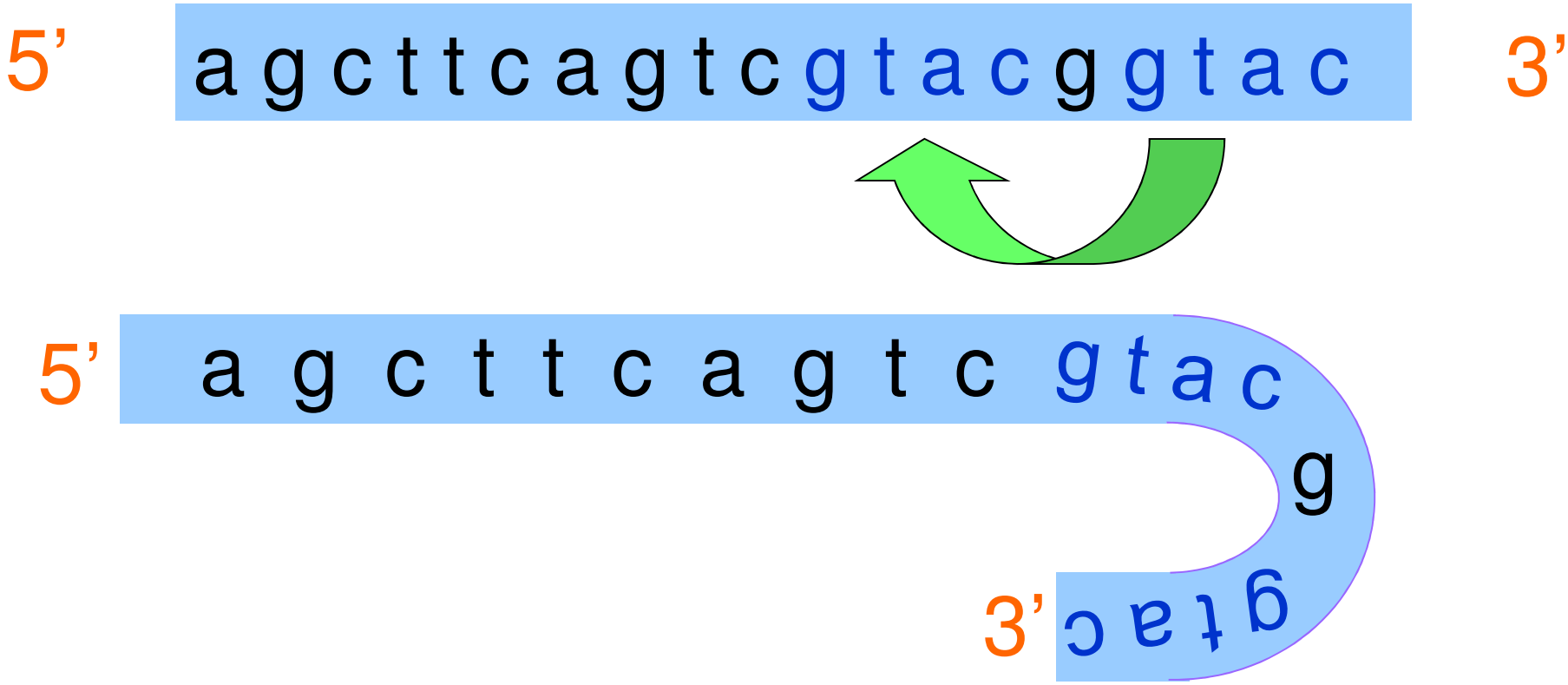
3 Avoid polypurines GA or polypirimidines TC sequences



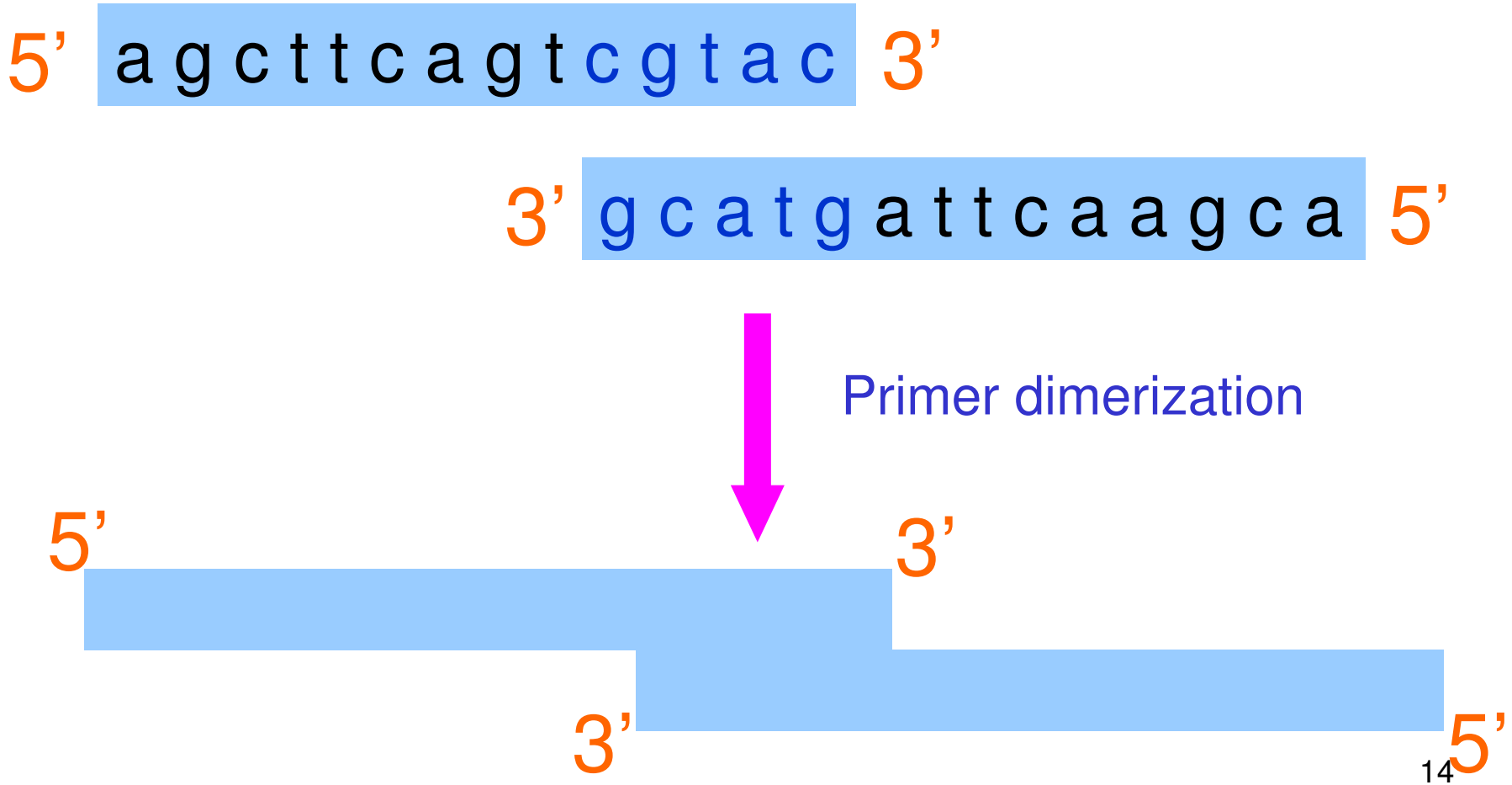


4

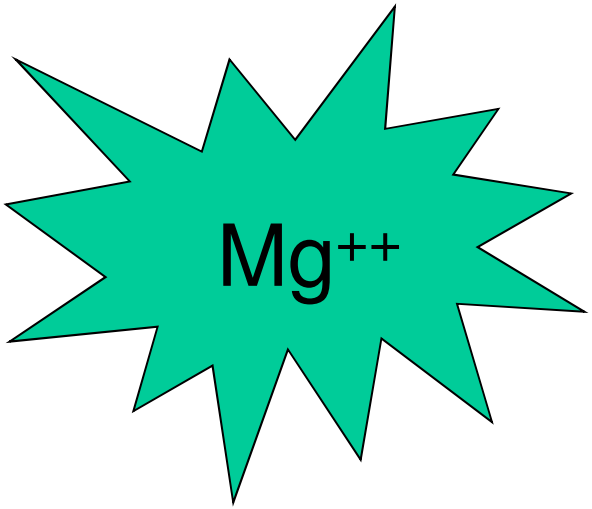
Avoid sequences with significant secondary structure, particularly at the 3'-end of the primer



5 Check the primers against each other for complementarity



PCR BUFFER

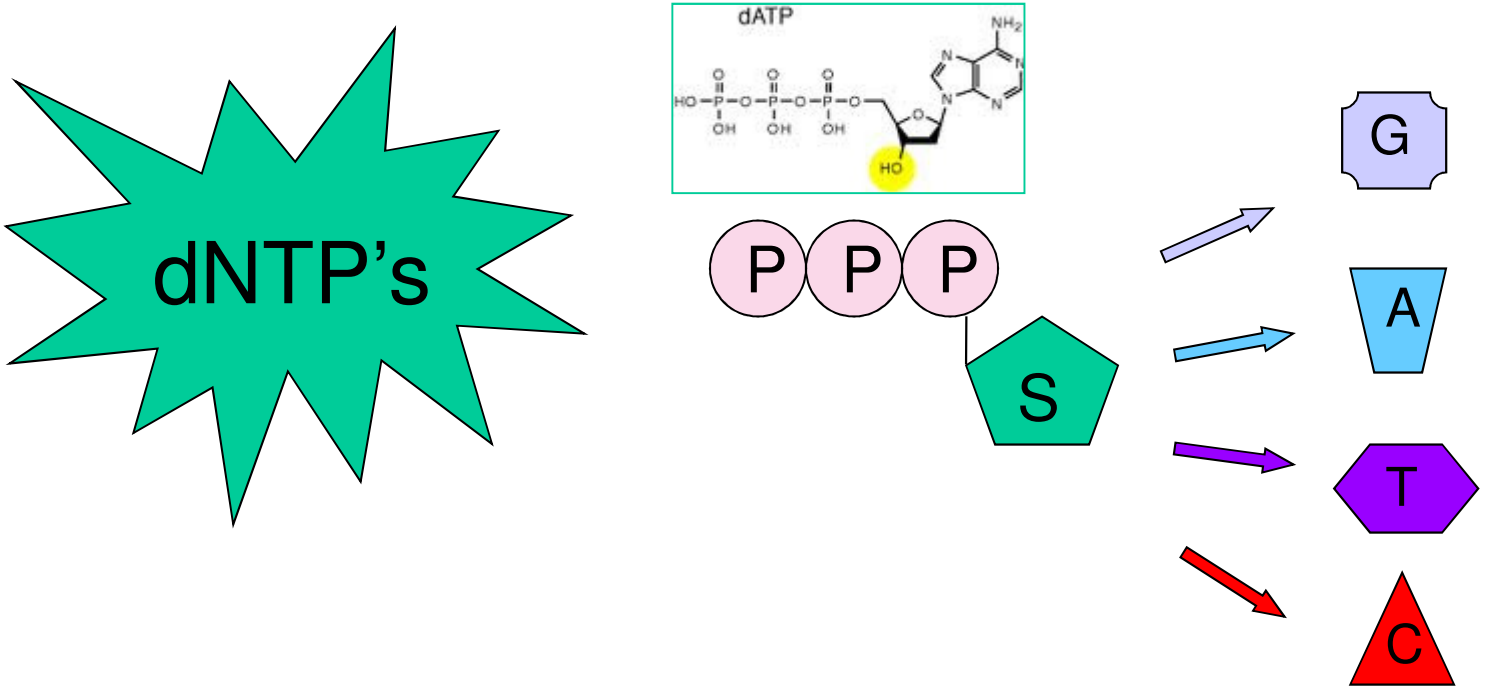


✓Effect on the specificity and yield of an amplification

✓Optimal concentration: 1.5 mM

✓Excess Mg^{++} → accumulation of non-specific amplification products

✓Insufficient Mg^{++} → reduction of the yield



✓Optimum range of concentration:
50 – 200 μM

✓Higher concentrations ➡ misincorporation of the nucleotides by polymerase (thermodynamic infidelity)

CYCLING PARAMETERS

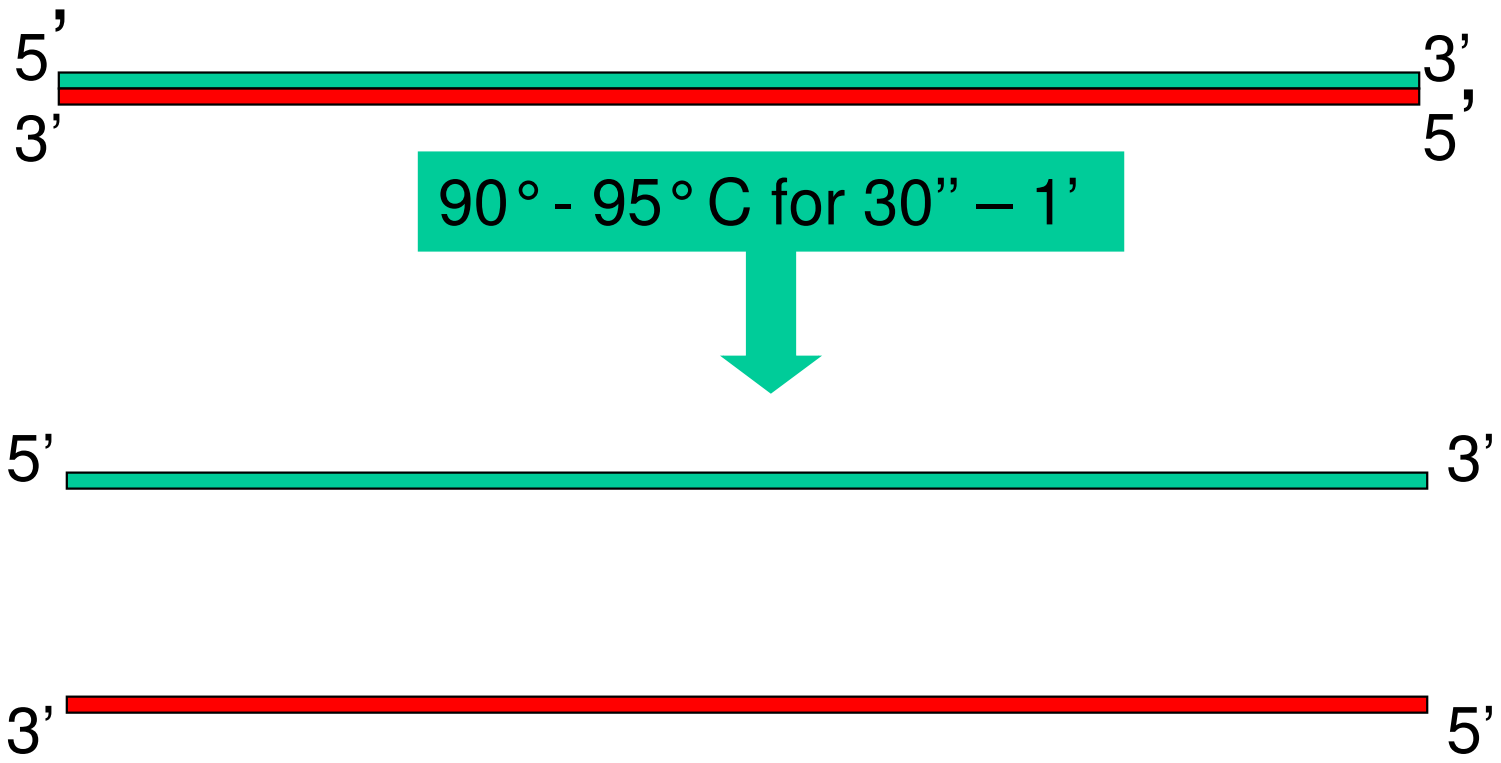
Each PCR cycle consists of three steps of different temperatures

✓**STEP1**: Denaturation of the double-stranded DNA

✓**STEP2**: Annealing of the primers

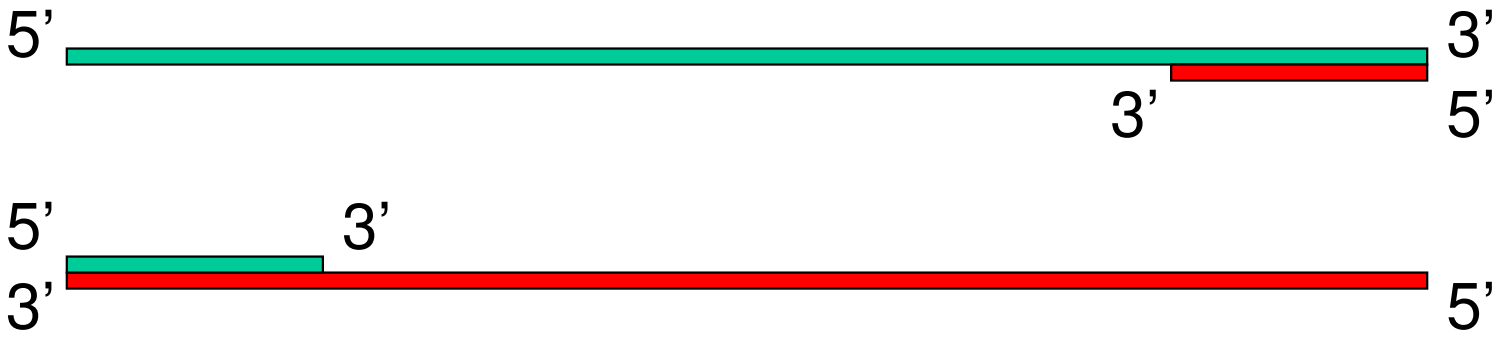
✓**STEP3**: Elongation

STEP1: Denaturation of the double-stranded DNA



Insufficient heating may generate incomplete strand separation

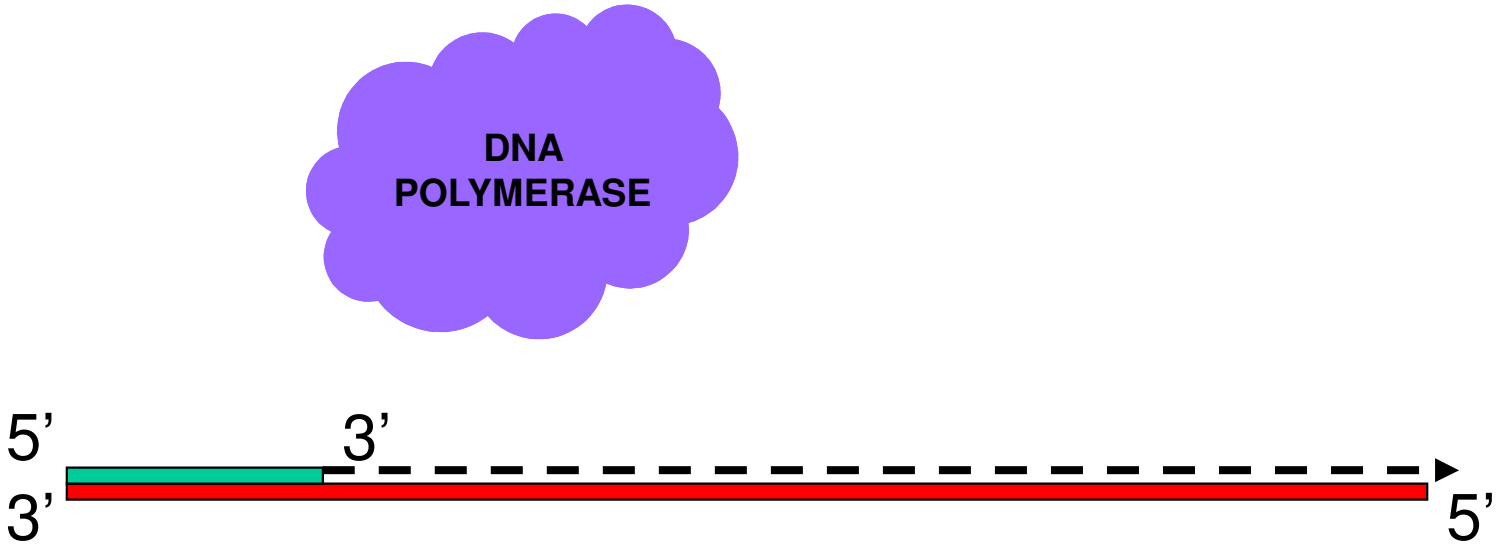
STEP2: Annealing of the primers



The temperature of annealing depends on:

- ✓Length of the primer
- ✓GC content of the primer

STEP3: Elongation

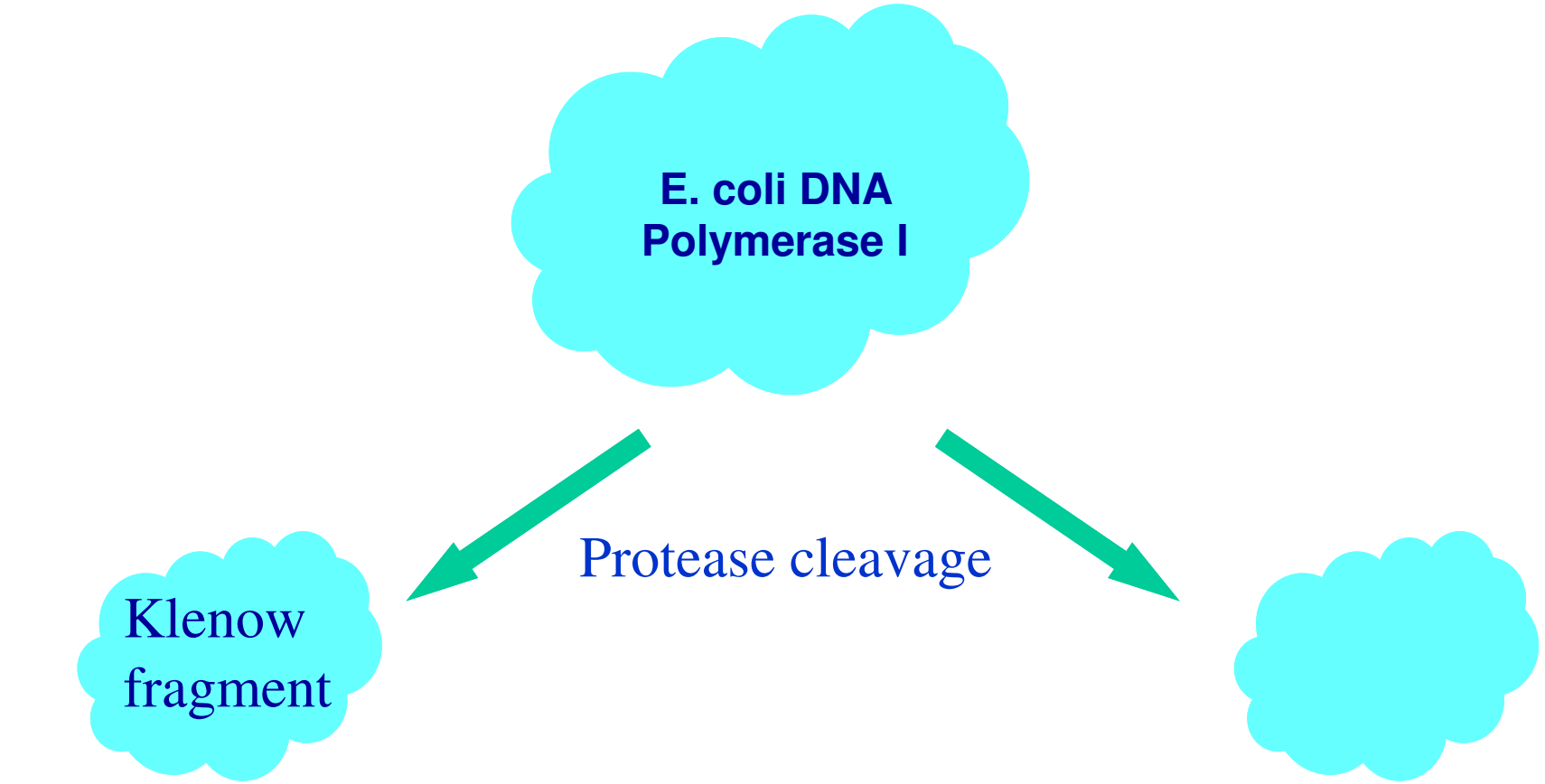


The polymerase extends the annealed primer, using the denatured-DNA as a template

Optimum temperature of elongation: 70°-75°C

The time of elongation varies according to the length of target being amplified: 1 minute for each kilobase of sequence

1st DNA POLYMERASE I used in PCR



DISAVANTAGES:

Thermal lability of the enzyme

The Klenow fragment is irreversibly denatured at each cycle of denaturation

ORIGINAL PCR PROCESS

1st STEP

Denaturation

94°-95°C

2nd STEP

Annealing and elongation

37°C

Addition of Klenow fragment at each cycle

Taq DNA POLYMERASE I

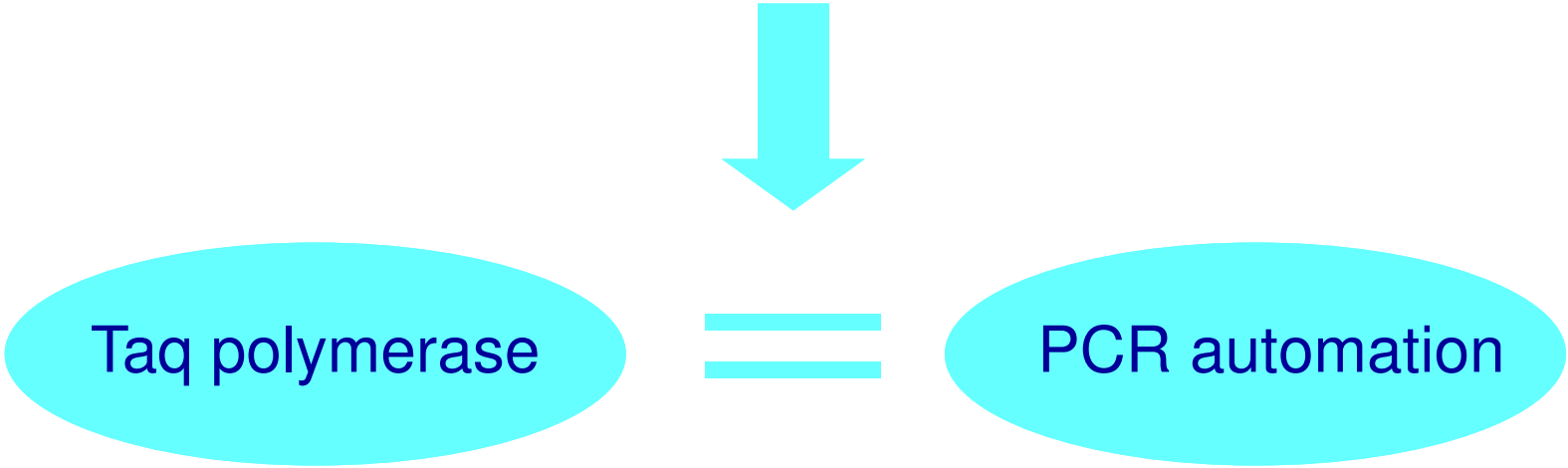
Isolated from Thermus aquaticus,a microorganism capable of growth at 70-75℃

- Stability at high temperatures (>90℃)
- High temperature optimum: 70°-75℃

ADVANTAGES:

Taq polymerase I retains its activity during the denaturation step (90° - 95°C).

It is added once to the sample at the beginning of the procedure, no further addition is required



PCR AUTOMATION

- The sample remains in the same physical position during the process
- A software controls the cyclic temperature changes and incubations

DNA Thermo Cycler:

machine to automatically perform multiple temperature steps

APPLICAZIONE DELLA PCR A SCOPO DIAGNOSTICO

➢DIAGNOSI DI INFEZIONI VIRALI

L'amplificazione di specifici RNAs virali indicano un'infezione in Es: diagnosi di un'infezione da HIV mediante PCR eseguita sulle cellule del sangue del paziente

➢DIAGNOSI DI INFEZIONI BATTERICHE

Amplificazione di geni altamente conservati e specifici *Mycobacterium tuberculosis.*

Sensibilità: 10 microrganismi / 10^6 cellule eucariotiche

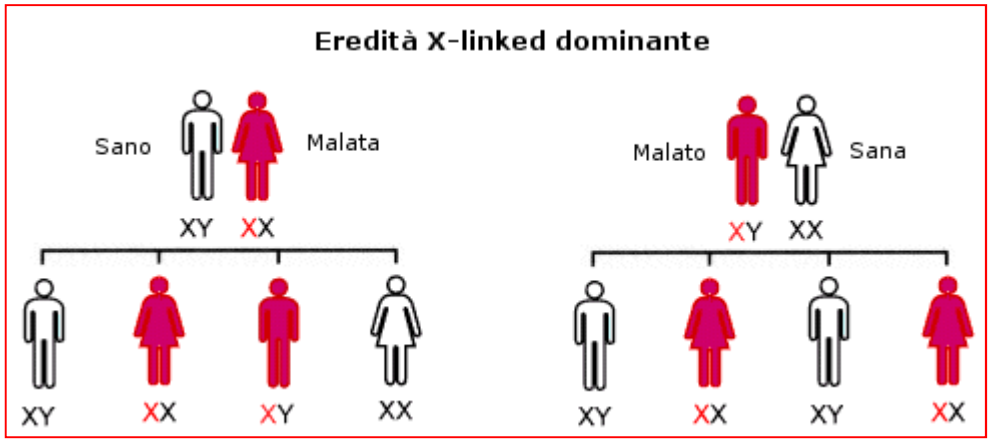
➢DIAGNOSI DEI TUMORI

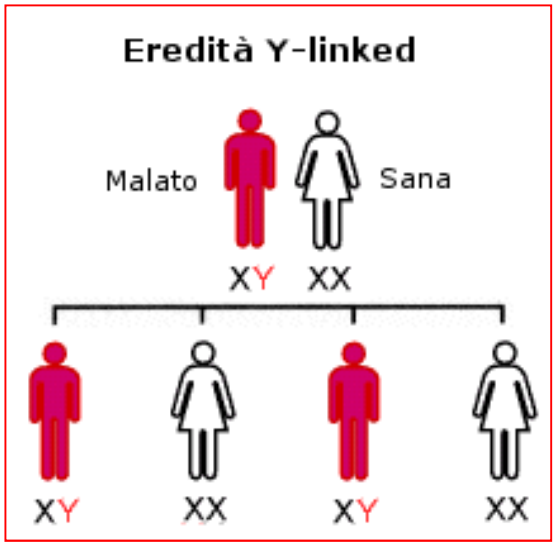
➢Alcuni tipi di leucemia sono caratterizzati da mRNA chimerici (BCR-ABL) che si trovano solo nelle cellule leucemiche del paziente. L'amplificazione della sequenza chimerica è un indicatore del tumore.

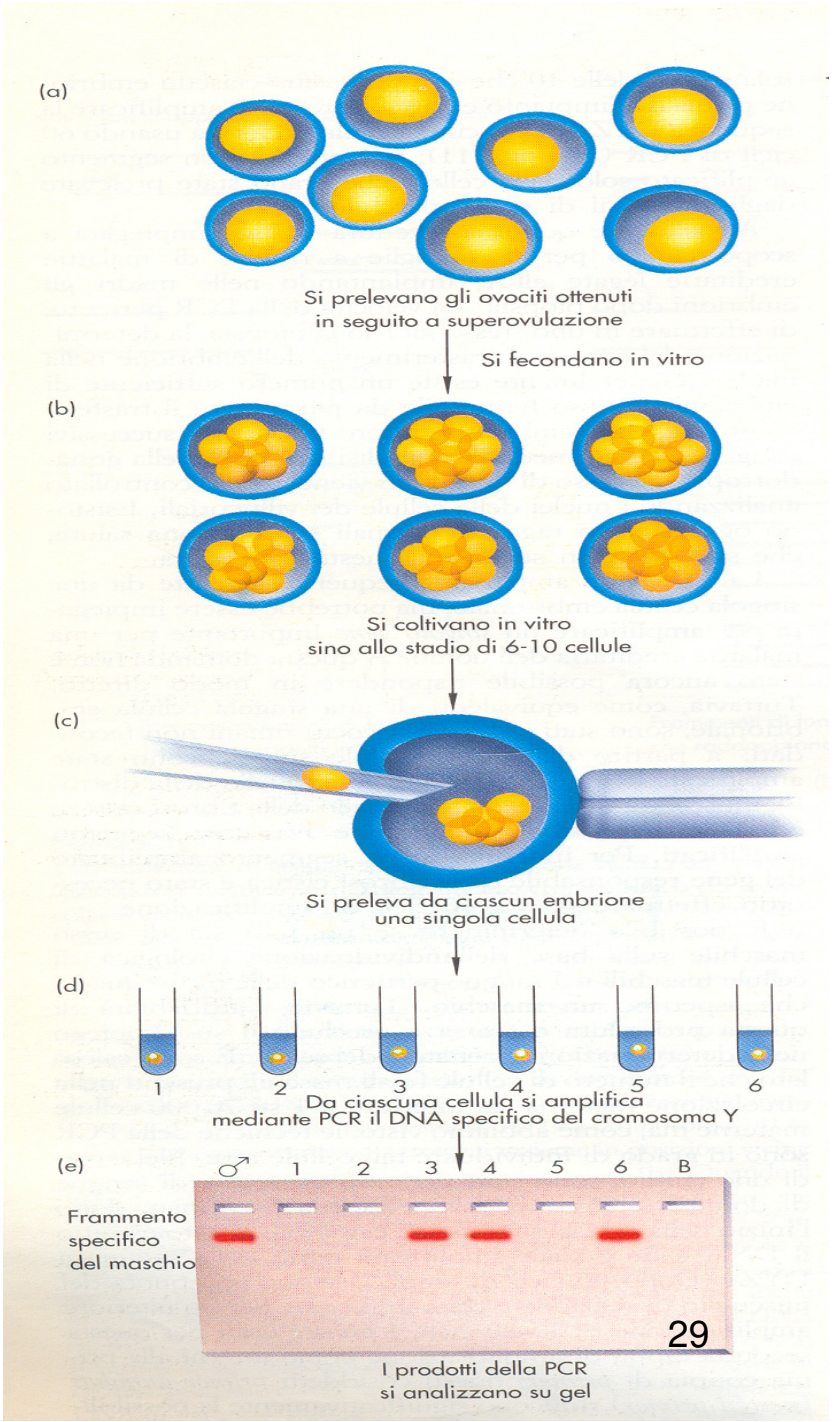
➢L'amplificazione di forme mutate dell'RNAm codificante per la proteina RAS può essere di aiuto nella diagnosi di alcuni tumori.

➢Durante il trattamento chemioterapico della neoplasia, molti tumori diventano farmaco-resistenti. L'amplificazione dei geni responsabili della farmaco-resistenza può dare indicazioni su questo fenomeno.

DETERMINAZIONE DEL SESSO IN CELLULE FETALI





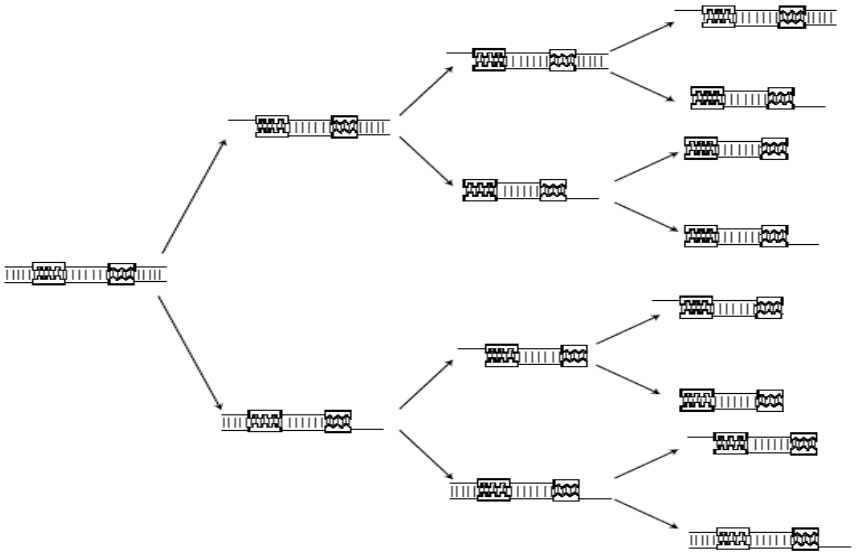


Amplification plateau

The amplification reaction is not infinite. It is constituted of two stages:

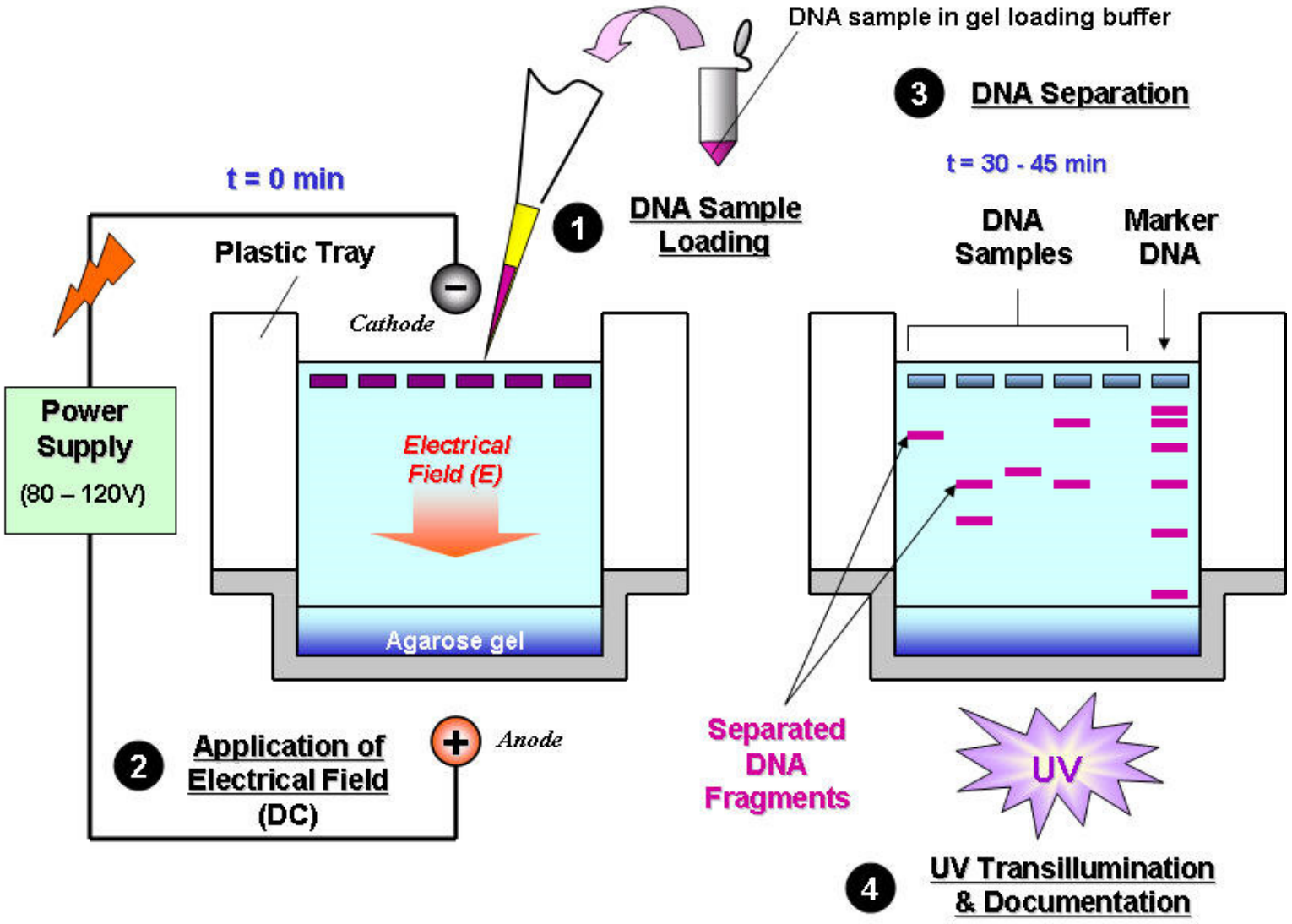
- 1st stage: exponential amplification

Le molecole di DNA sono amplificate esponenzialmente
1→2→4→8→16→32→64→12 8→256…

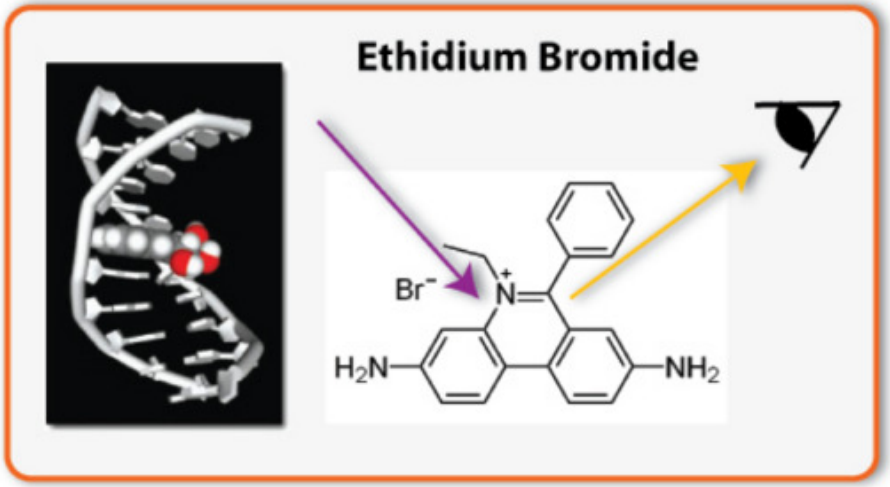


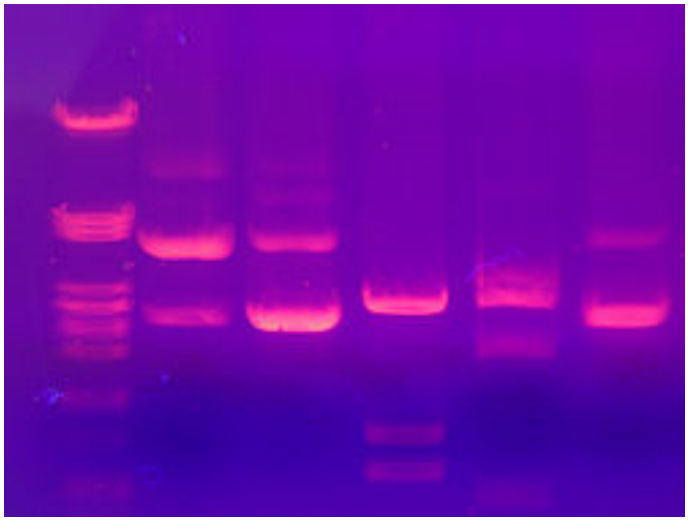
- 2nd stage: amplification plateau

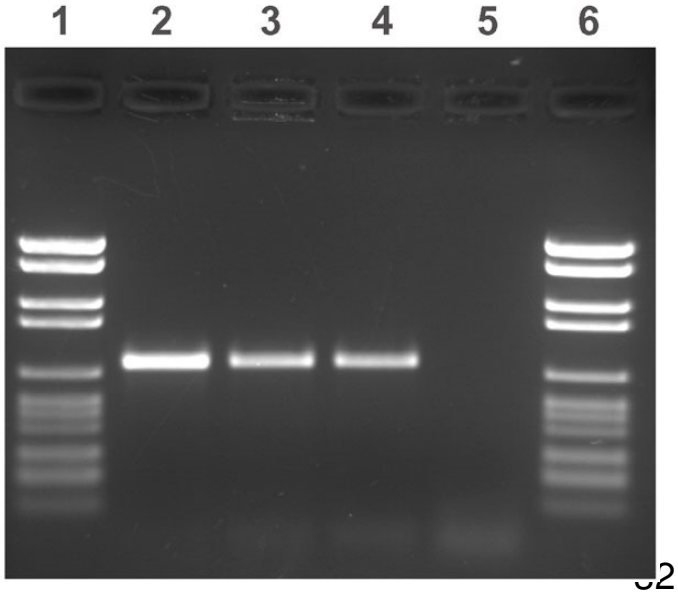
RIVELAZIONE DI UNA END POINT PCR: ELETTROFORESI SU GEL DI AGAROSIO



RIVELAZIONE DI UNA END POINT PCR







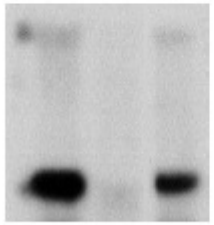
Real-time P C R

Real-Time PCR (Quantitative PCR, qPCR)

Post PCR

Ethidium Bromide

+



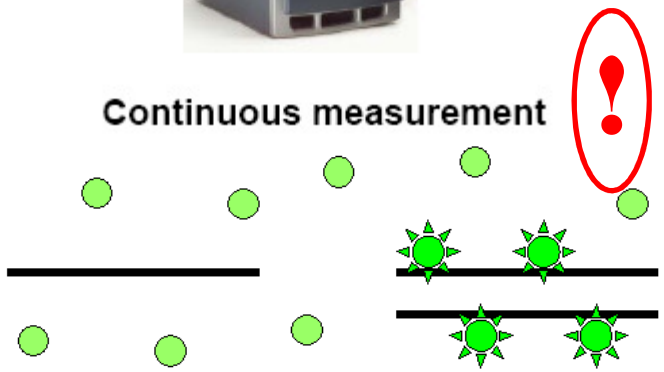
End point measurement

During PCR

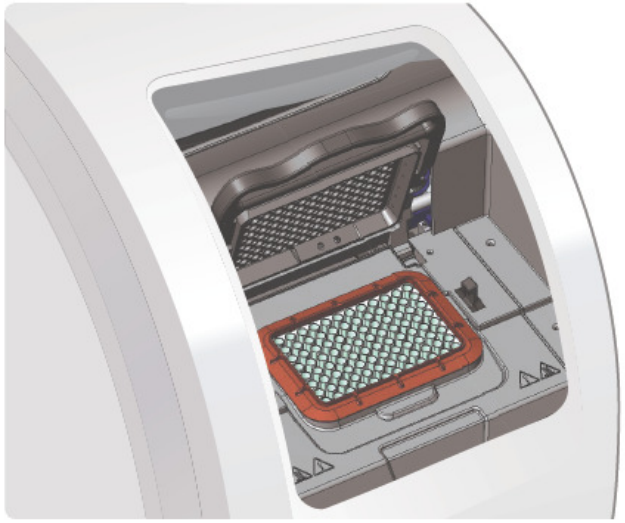
•SYBR Green I
•Fluorogenic Probe

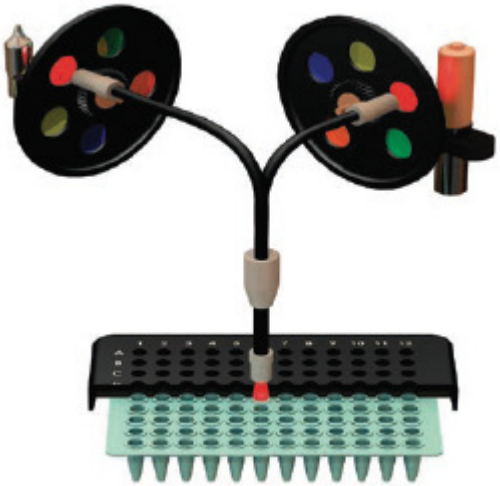
+

Continuous measurement



QPCR instrumentation



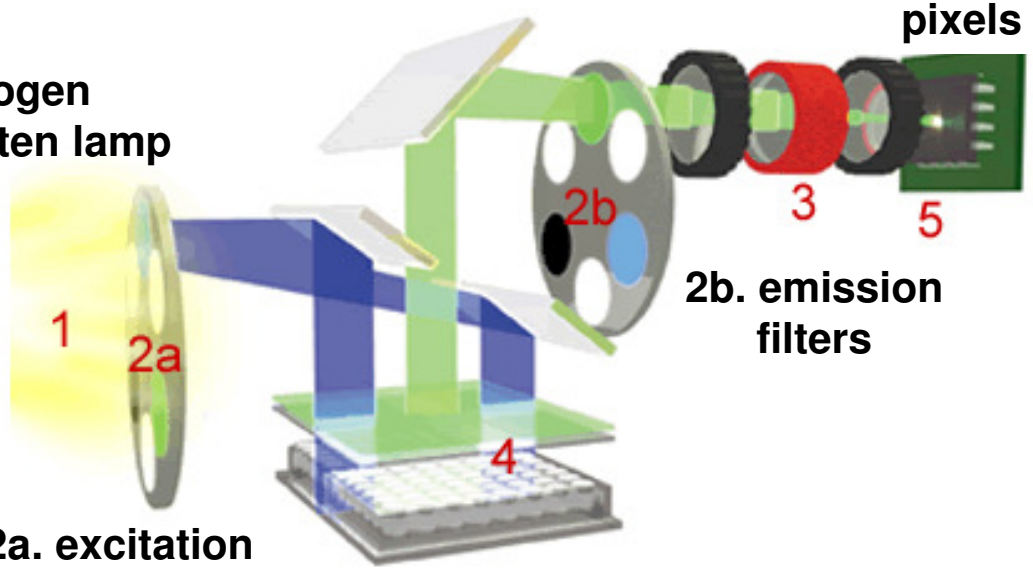


Optical System Design

1. halogen tungsten lamp

3. intensifier

5. ccd detector 350,000 pixels

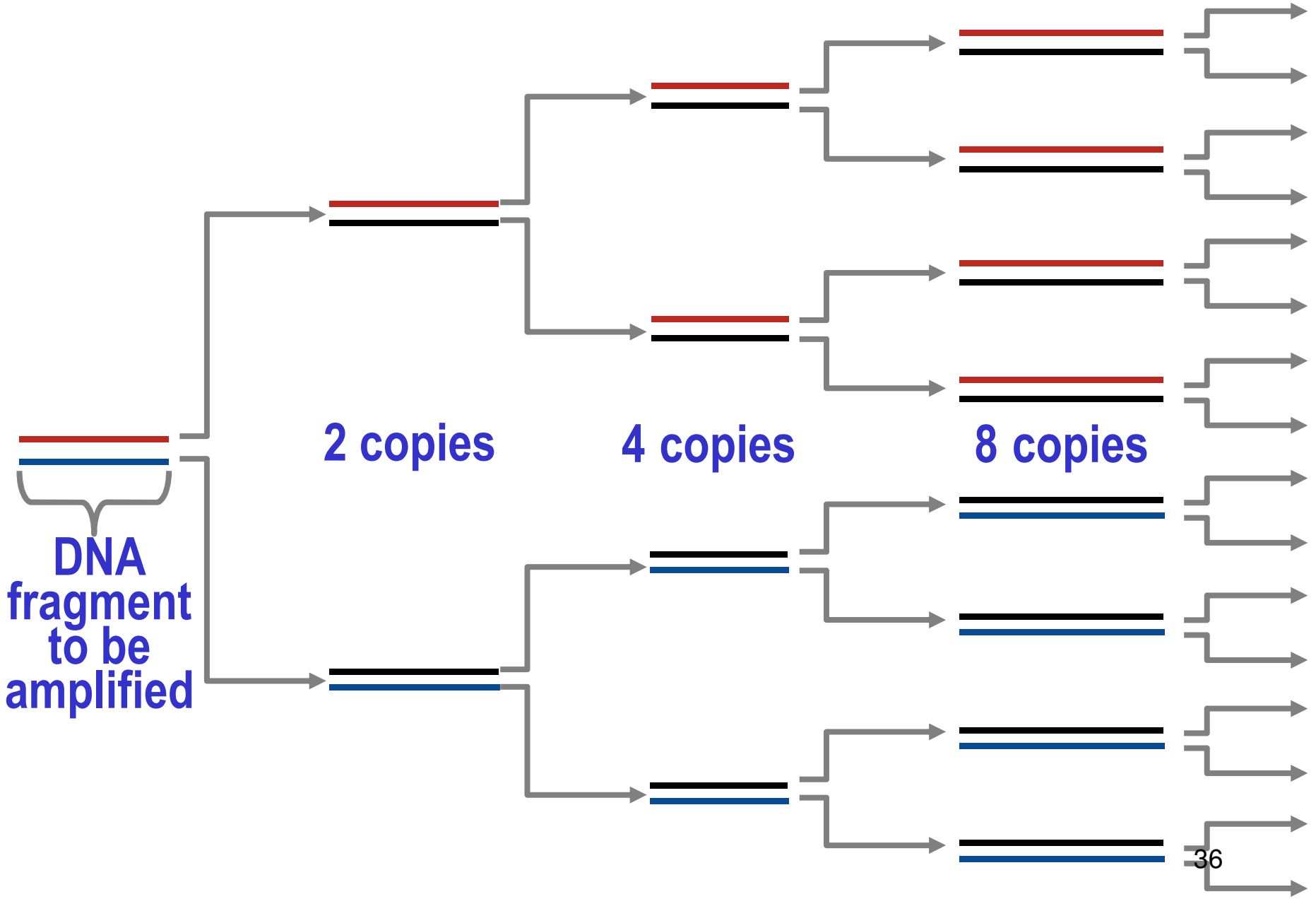


2b. emission filters

2a. excitation filters

4. sample plate

Polymerase Chain Reaction: Multiple PCR Cycles

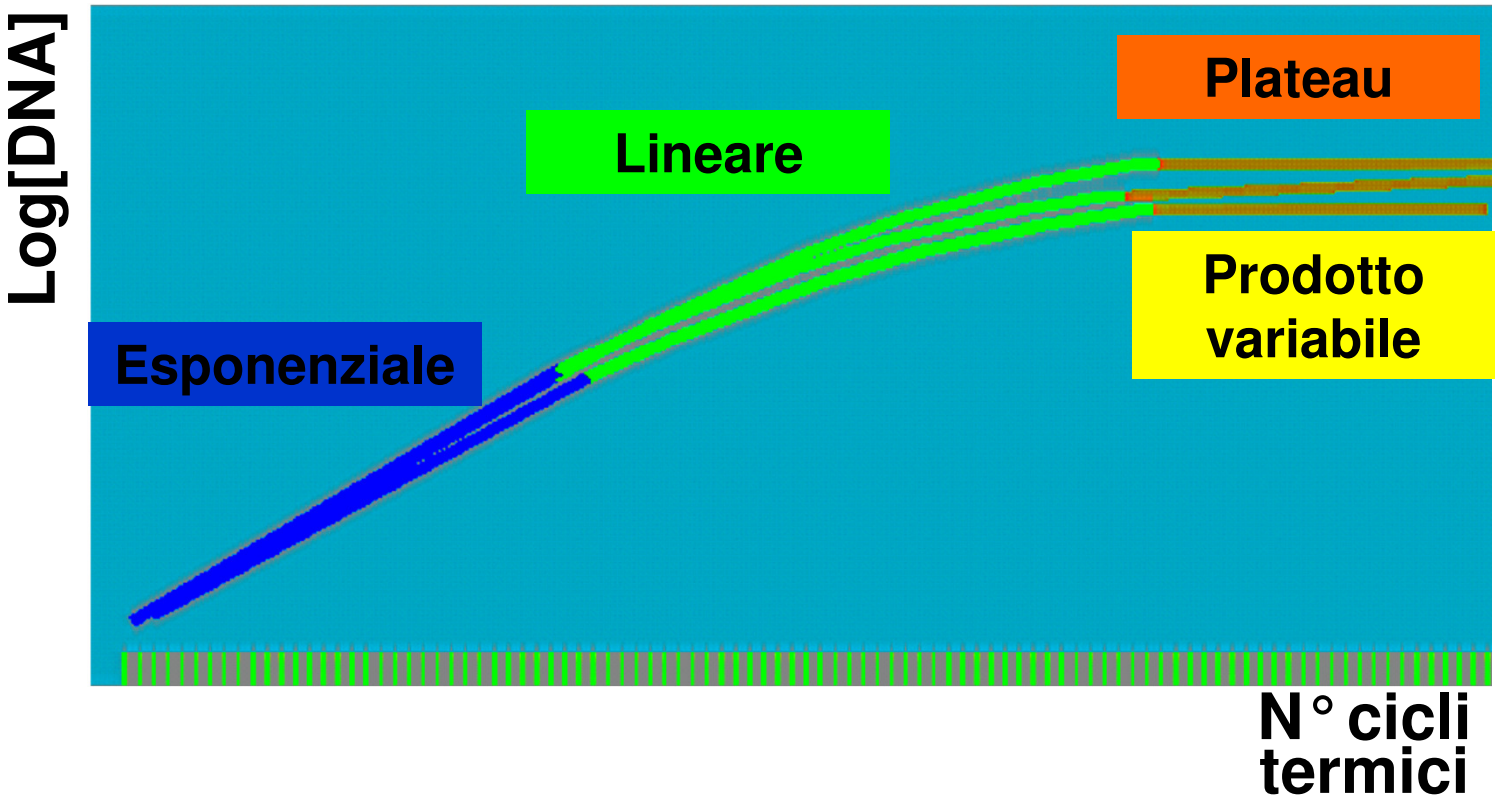


Polymerase Chain Reaction: resa

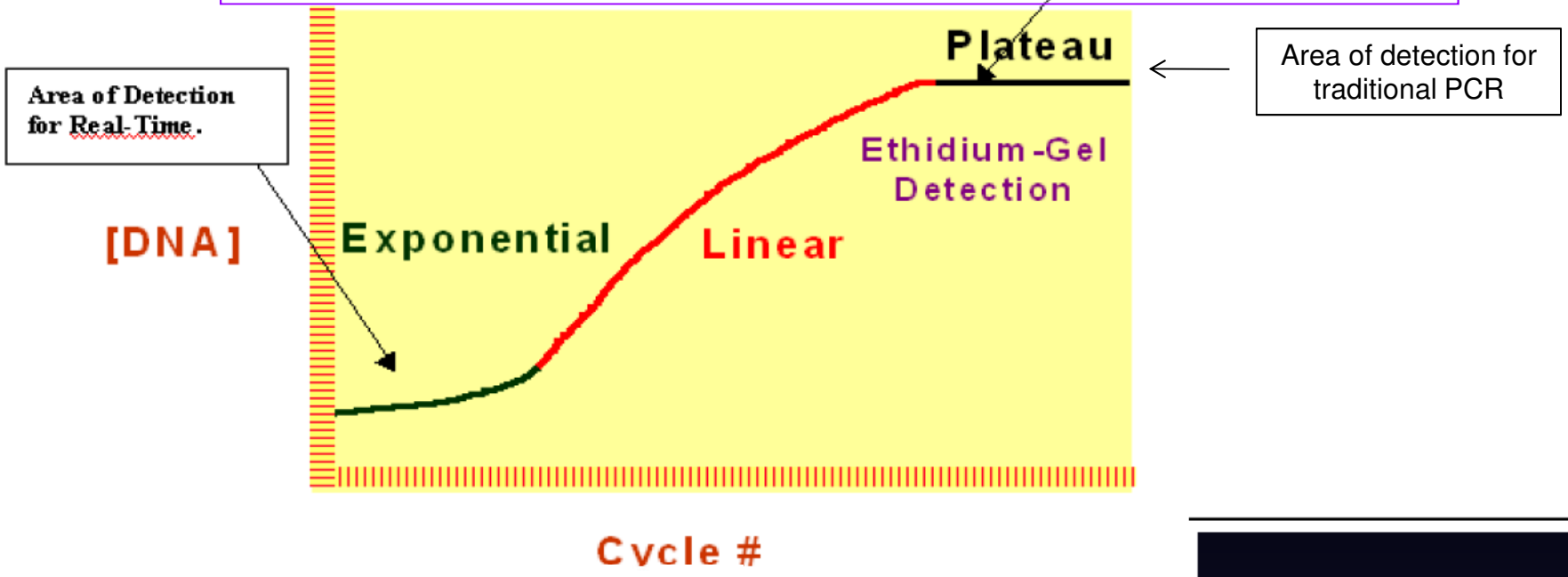
- **Resa teorica: 2^n**

  $P=(2)^n T$ **Il prodotto (P) incrementa esponenzialmente con il numero di cicli di PCR (n)**

  Il prodotto di PCR dipende da T, numero di copie di template di partenza

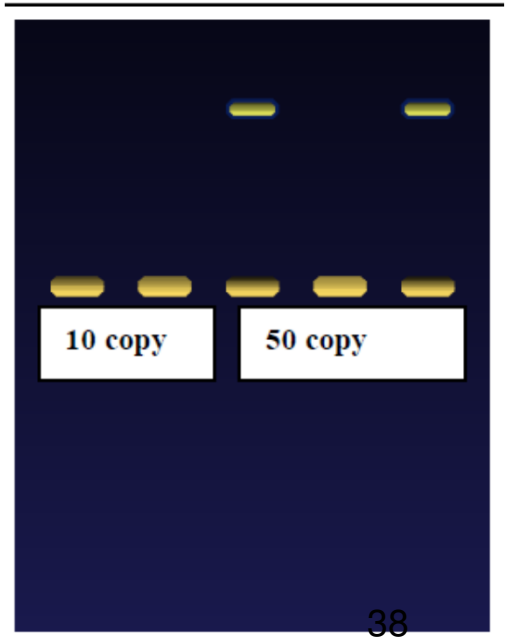


Limitazioni di una end-point PCR



Some of the problems with **End-Point Detection**:

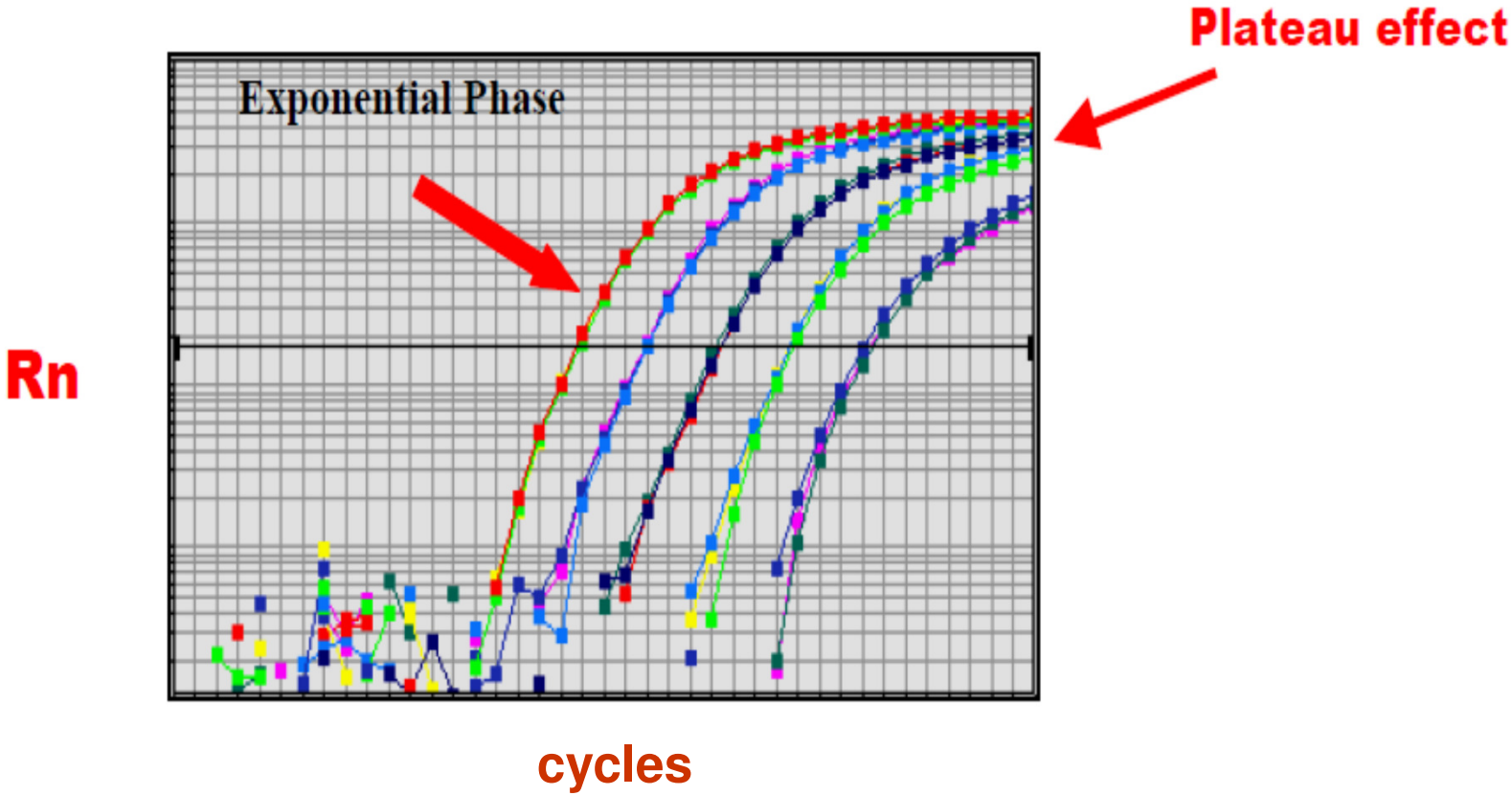
- Poor Precision
- Low sensitivity
- Short dynamic range < 2 logs
- Low resolution
- Non - Automated
- Size-based discrimination only
- Results are not expressed as numbers
- Ethidium bromide for staining is not very quantitative
- Post PCR processing



Ethidium-gel detection

Effetto plateau in una end-point detection

Figure 8: Log view 5-fold dilution series

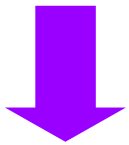


Real-Time PCR vs end-point PCR

Misura l'amplificazione in tempo reale durante la fase esponenziale della PCR, quando cioè l'efficienza di amplificazione è influenzata minimamente dalle variabili di reazione, permettendo di ottenere risultati molto più accurati rispetto alla PCR tradizionale "end point"

Vantaggi di una real-time PCR

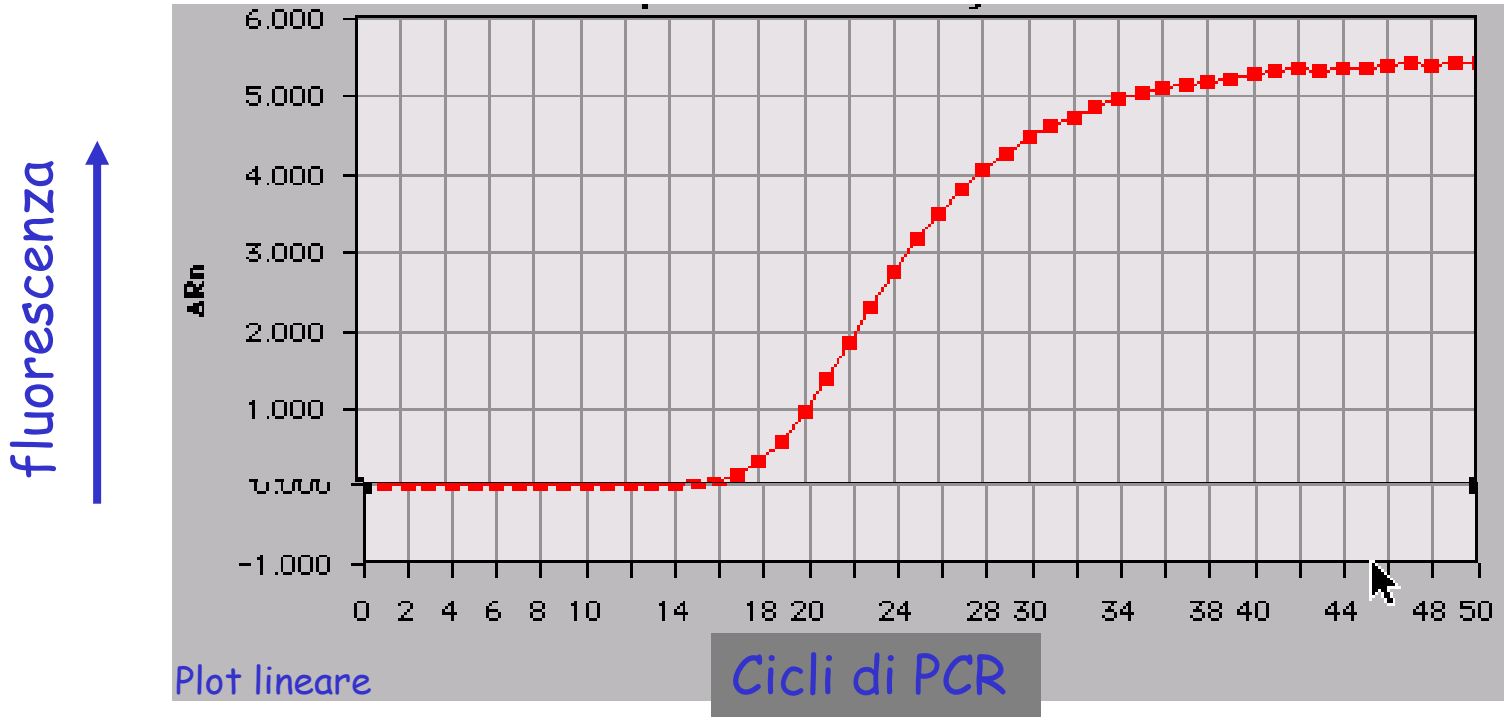
- Risultati più veloci, accurati e precisi
- I dati vengono registrati durante il procedere della reazione (real-time)
- Non richiede operazioni di rivelazione post-PCR



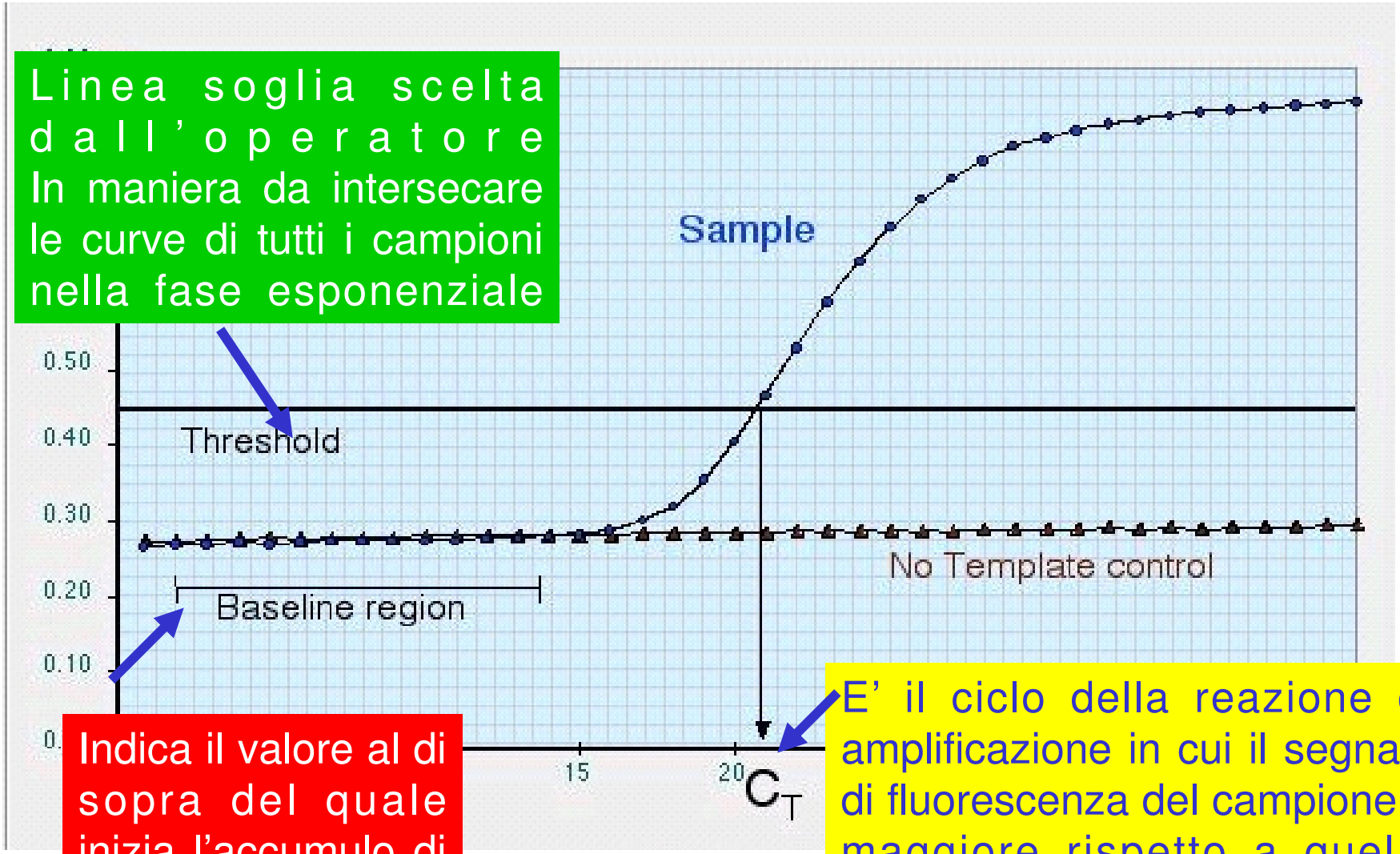
- Metodo più accurato per la quantizzazione del DNA e RNA

Real-time PCR detection

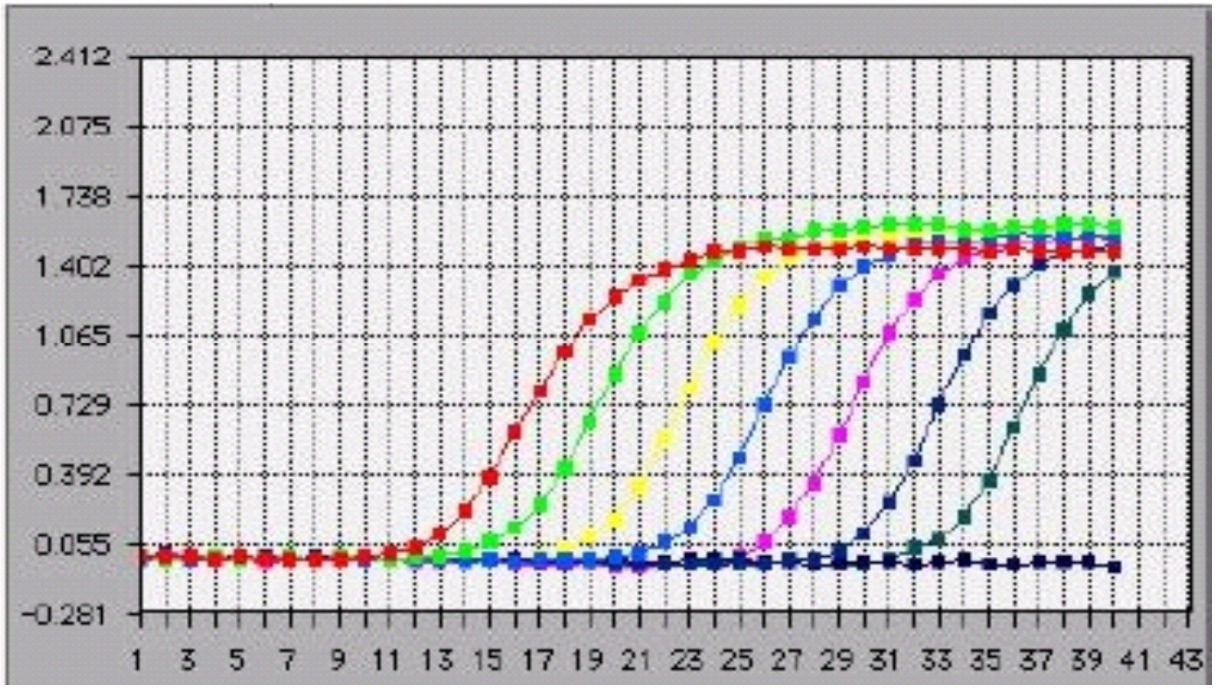
- Rilevamento della fluorescenza associata all'amplificazione
- Il prodotto di PCR non viene analizzato su gel di agarosio
- Analisi del prodotto di fluorescenza tramite computer



Plot di amplificazione



Curve di amplificazione



Plot lineare

Per ogni campione si ottiene una curva di amplificazione il cui C_T(=Threshold Cycle) è inversamente proporzionale alla quantità di template iniziale

Chimiche fluorescenti per PCR Real-Time

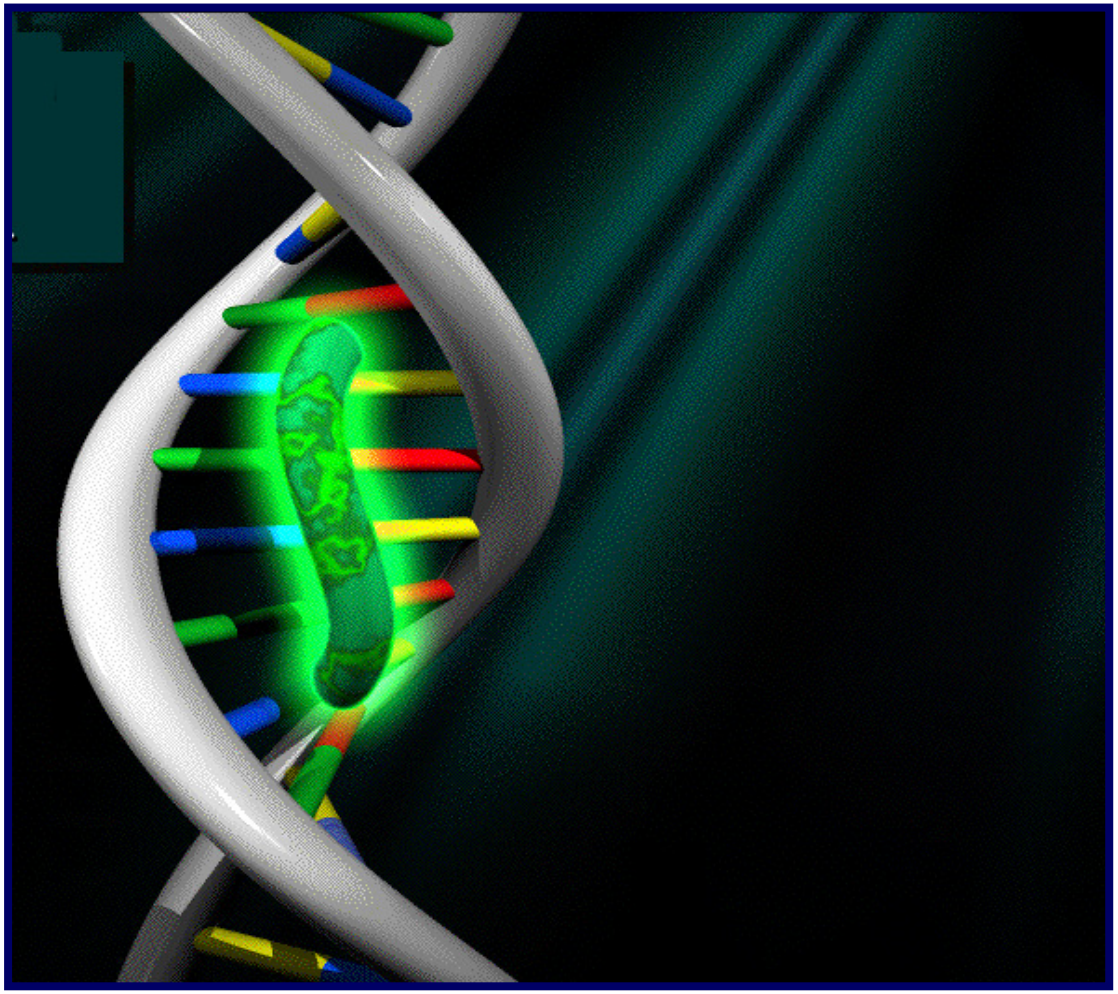
La fluorescenza si genera durante la PCR per effetto di diverse possibili reazioni chimiche

Sybr Green

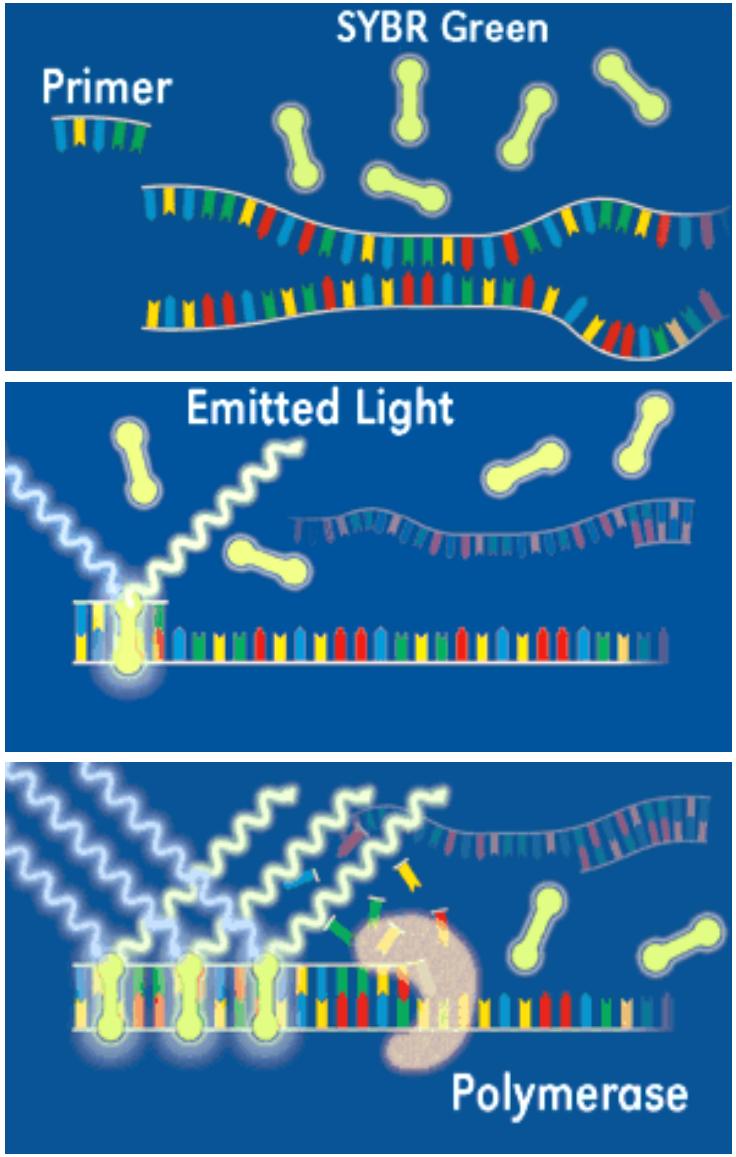
Sonde TaqMan

SYBR Green: principio

Utilizza una molecola fluorescente non specifica che si lega al solco minore del DNA



SYBR Green



INIZIO PROCESSO AMPLIFICAZIONE: la miscela di reazione contiene DNA denaturato, primers e la molecola fluorescente

ANNEALING PRIMERS: poche molecole fluorescenti alla doppia elica

ELONGATION: un aumento di fluorescenza corrisponde ad un aumento del numero di copie dell'amplicone

SYBR Green

- **Metodica semplice**

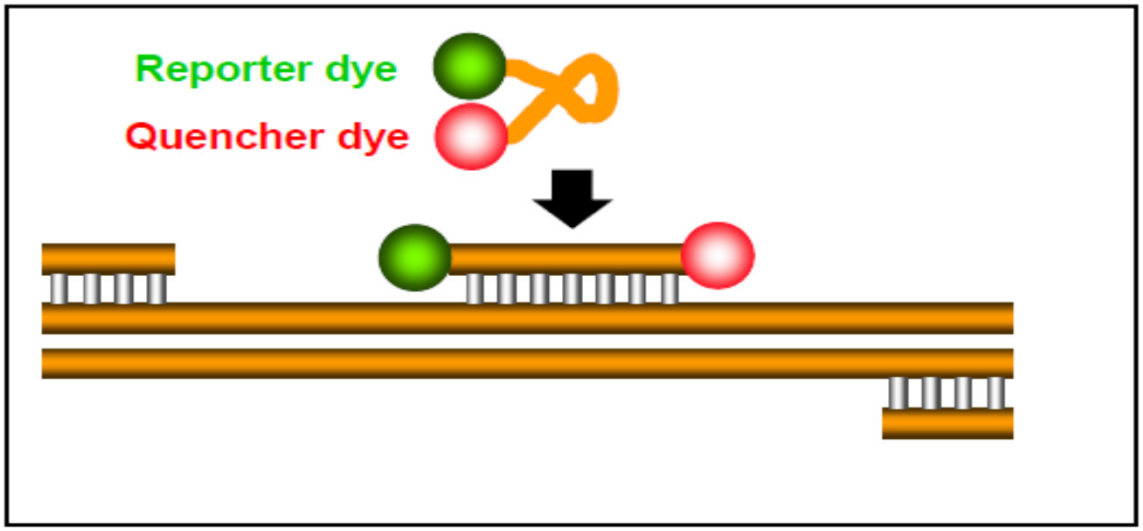
  Possono essere utilizzati primers in uso in qualitativa

- **Non costosa**

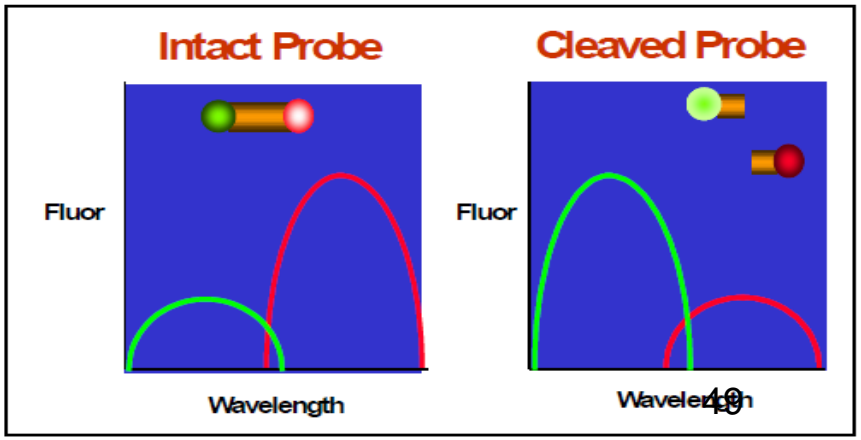
- **Non-specifica**
  - La molecola fluorescente si lega random a tutte le doppie eliche, includendo i dimeri di primers
  - È necessario ottimizzare la metodica per evitare la formazione di prodotti aspecifici

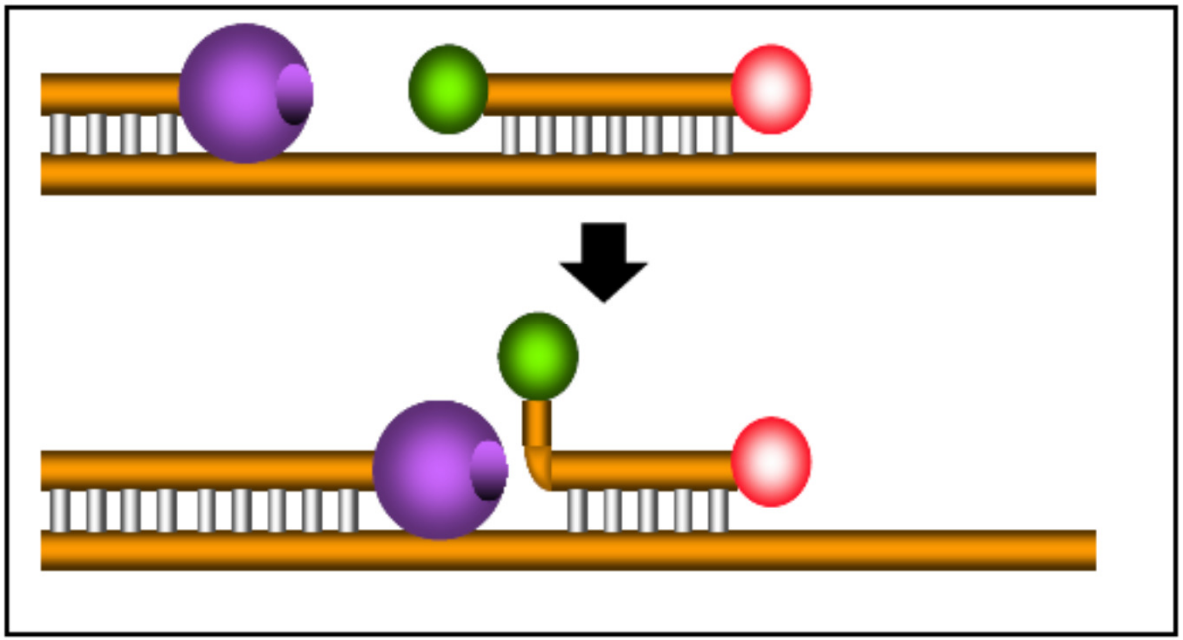
Sonde TaqMan

sonde ad ibridazione, specifiche per il frammento di interesse, marcate con molecole fluorescenti



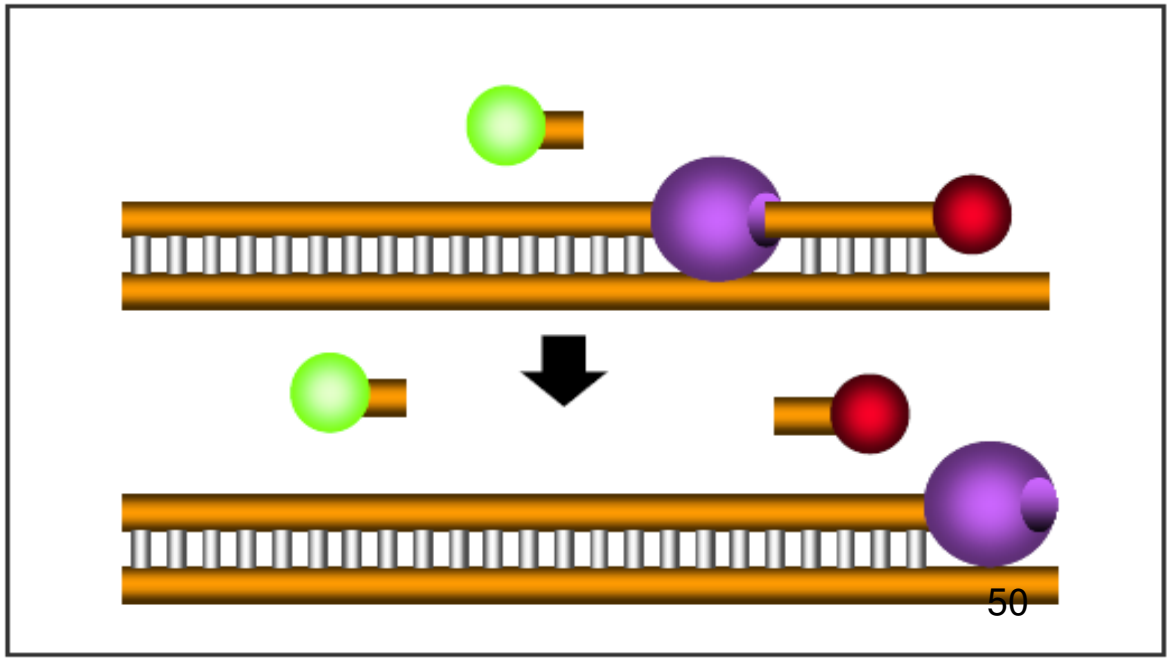
Aumento della fluorescenza in seguito ad idrolisi del probe





L'attività 5'-nucleasica della polimerasi permette l'idrolisi del probe TaqMan.

Allontanamento del quencher dal reporter con conseguente aumento della fluorescenza



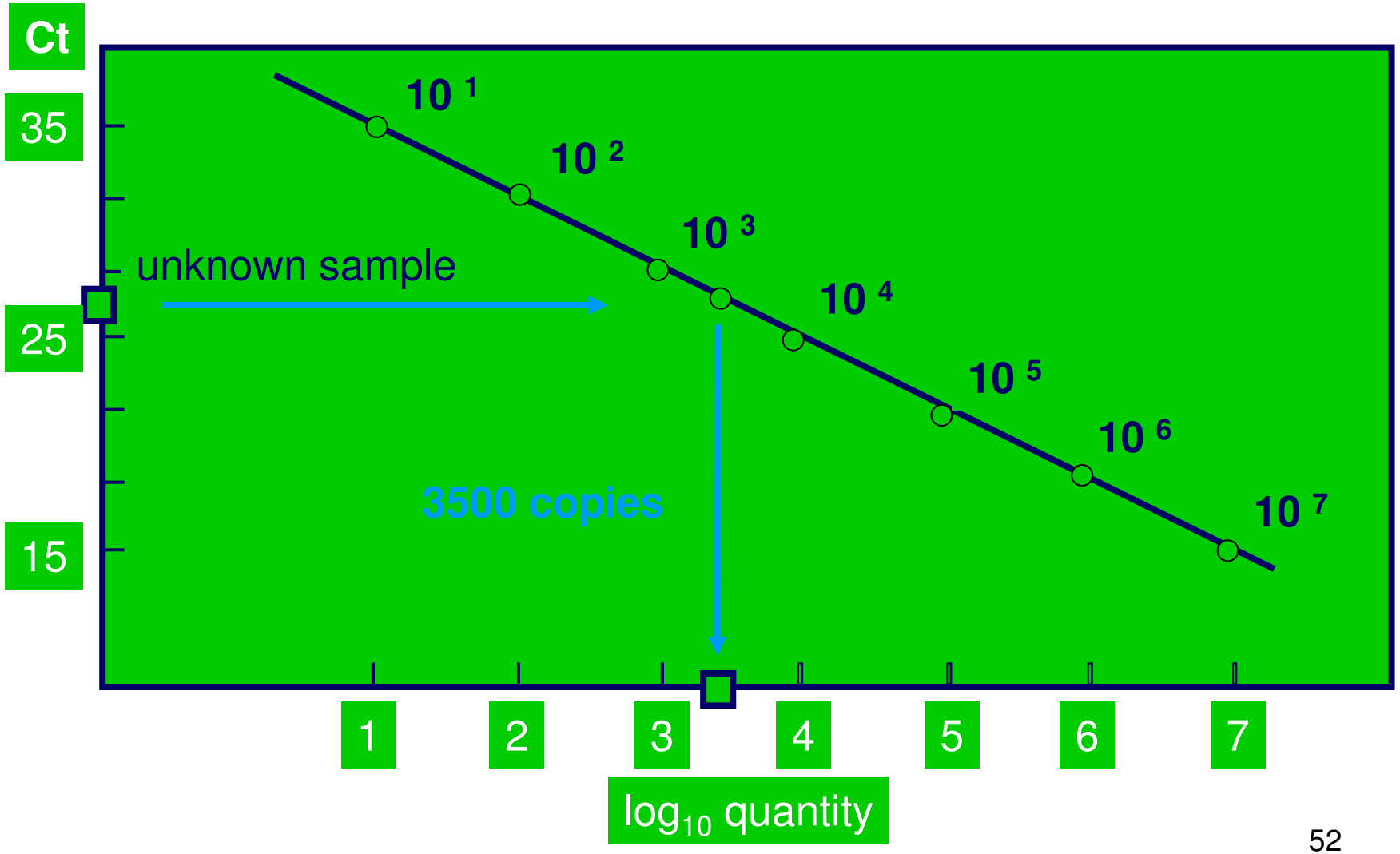
Real-time PCR come strumento di quantificazione dei campioni

QUANTIFICAZIONE ASSOLUTA

I campioni sono quantificati in modo assoluto

- Necessità di DNA standard di cui è nota la quantità assoluta (utilizzo di una standard curve)
- Devono essere saggiate identiche quantità dei campioni incogniti

Quantitativa assoluta



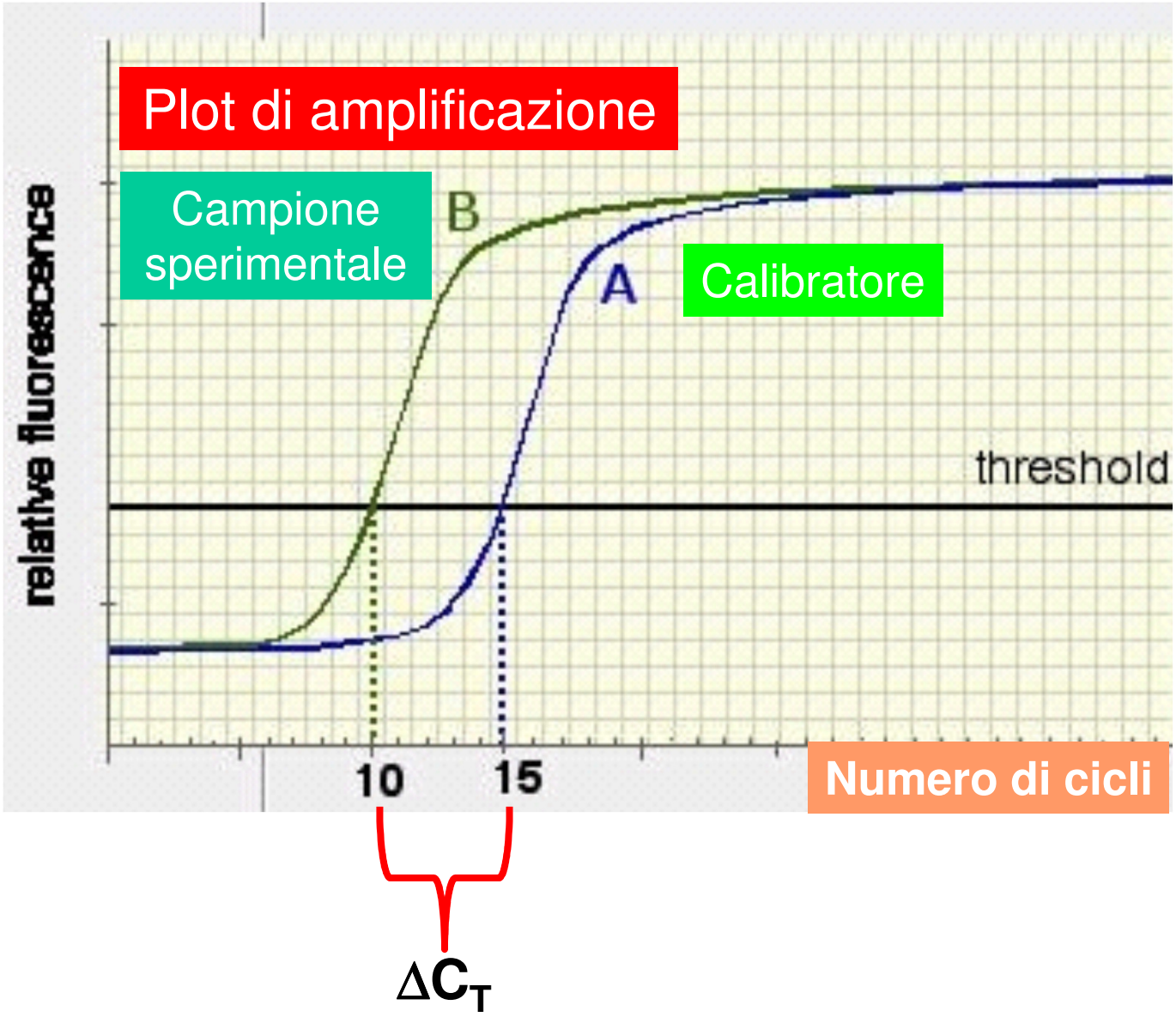
QUANTIFICAZIONE RELATIVA

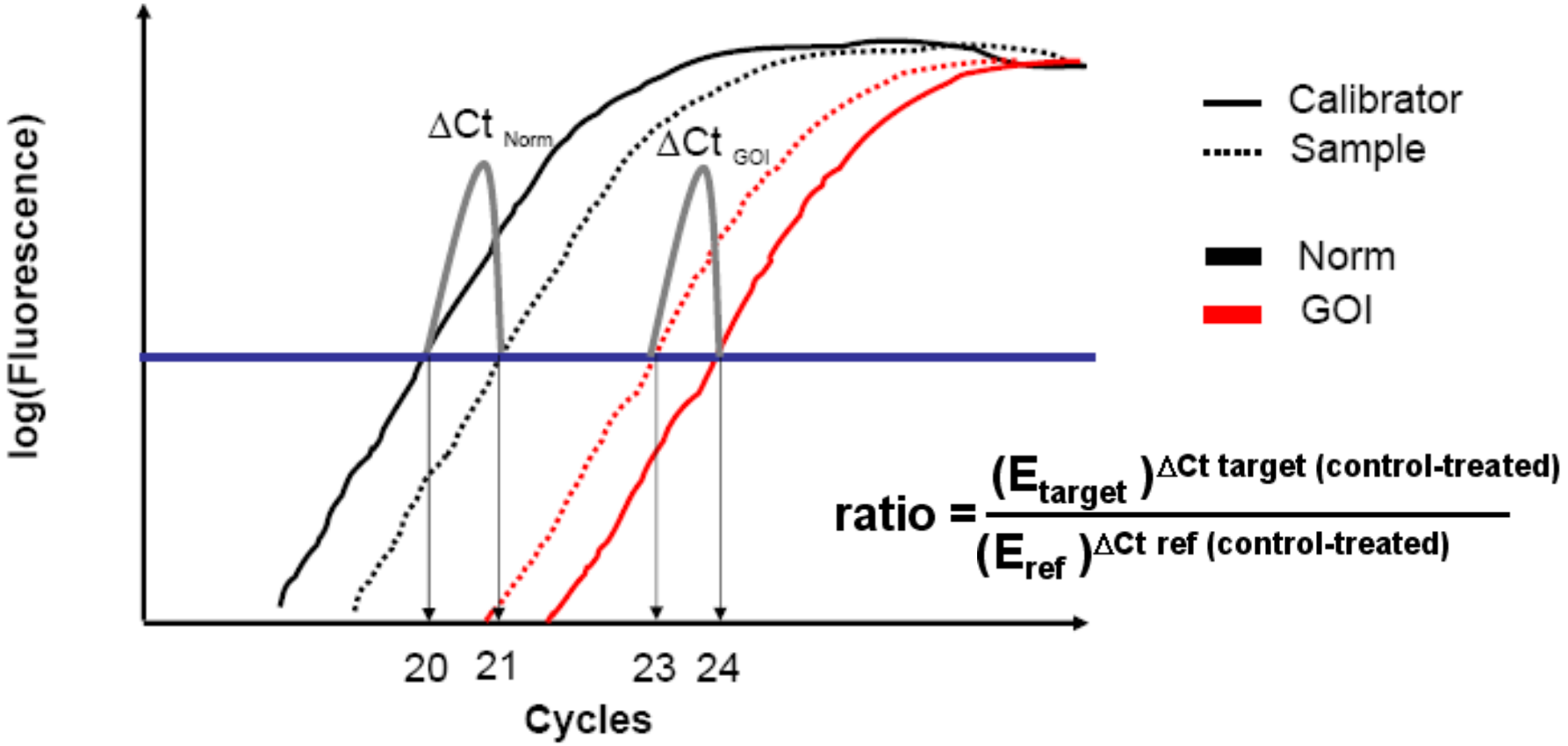
La quantificazione dei campioni viene effettuata paragonando i C_T

- Necessita di controlli endogeni (non si utilizza una standard curve), un housekeeping gene

- I campioni incogniti vengono "quantificati" paragonando il loro ΔC_T con quello del controllo endogeno

Quantitativa relativa





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

$$\text{Fold Variation} = \frac{(1+Eff)^{\Delta Ct\ GOI}}{(1+Eff)^{\Delta Ct\ Norm}} = \frac{(1+1)^{24-23}}{(1+1)^{20-21}} = 4$$

STRATAGENE

Norm: gene di normalizzazione (costitutivo)
GOI: Gene Of Interest (espressione modulabile) DA INDAGARE
Calibrator: campione non trattato, o di riferimento
Sample: campione trattato DA INDAGARE

Digital PCR (dPCR)

- Permette di quantificare in maniera assoluta gli acidi nucleici, senza la necessità di un DNA standard o di un gene di riferimento (normalizzatore).

- Funziona come una real-time PCR, con lo stesso metodo di rivelazione: la FLUORESCENZA

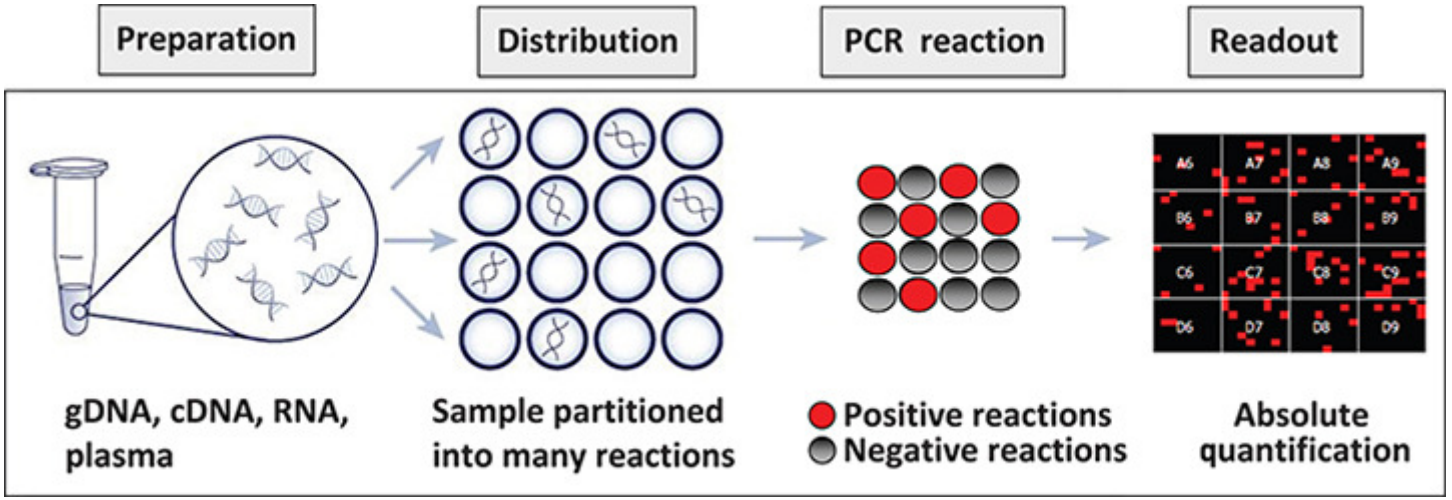
- DIFFERENZA: il campione utilizzato in dPCR viene diluito e ripartito in centinaia di camere di reazione, in maniera tale che in ogni partizione si troverà una sola molecola di acido nucleico o nessuna.

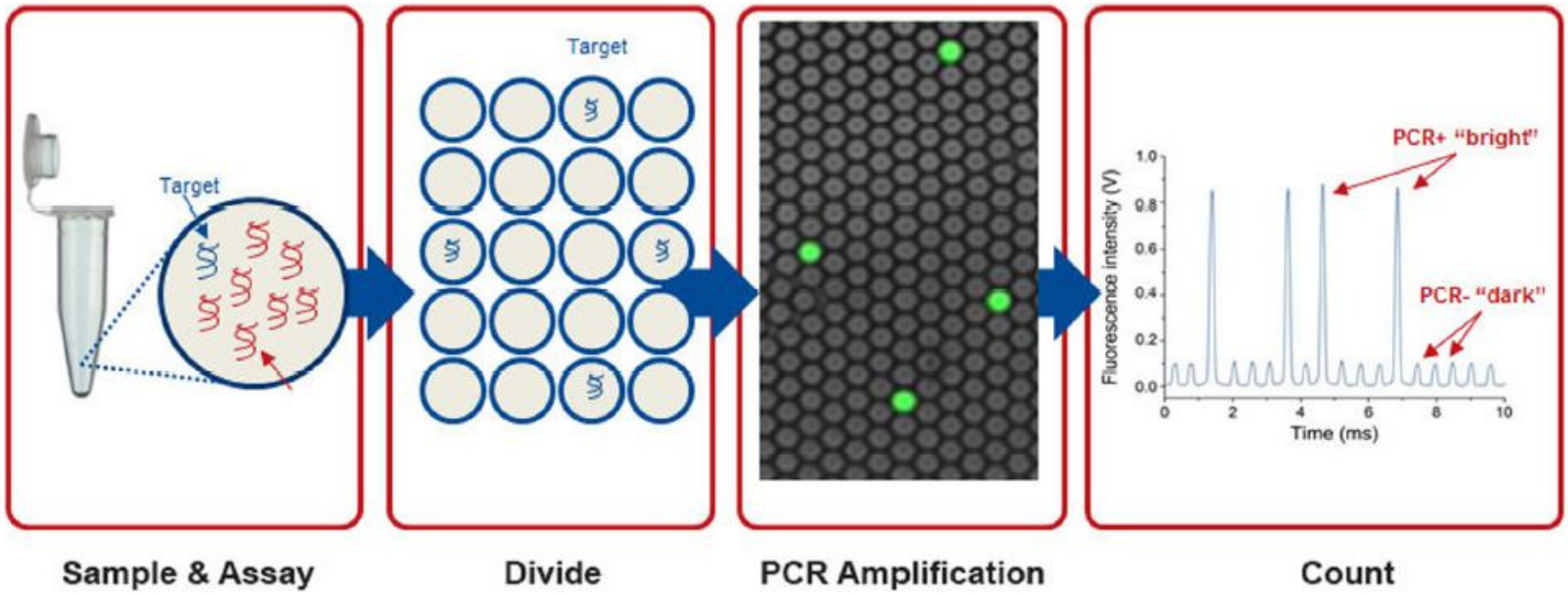
- La partizione del campione diminuisce la quantità di DNA che funge da background e di conseguenza aumenta la sensibilità nei confronti del DNA target.

- Si assegna 1 ai templati che hanno prodotto reazioni positive e 0 alle camere di partizione che non hanno prodotto reazioni positive. Da questo sistema binario di assegnazione deriva il nome ‘digital PCR’.

- Il numero di molecole di templato nel campione originario è calcolato in base alla percentuale di reazioni positive mediante la distribuzione di Poisson

Digital PCR (dPCR)

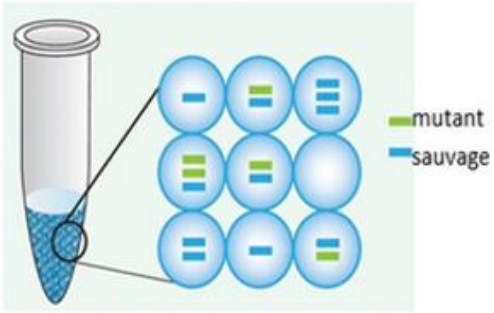




Droplet Digital PCR (ddPCR)

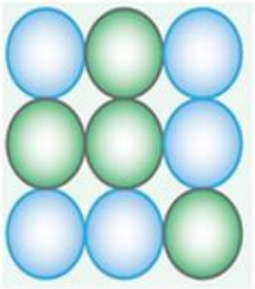
Partition

The mixture of PCR reaction is distributed randomly in ~20000 dropplets.
Each constitutes an independent microreactor



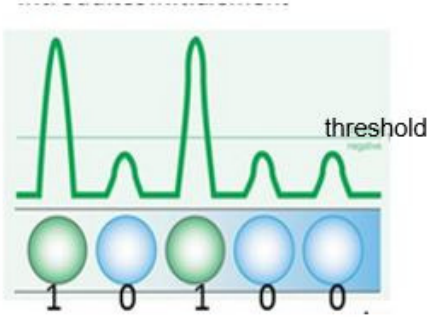
PCR anplification

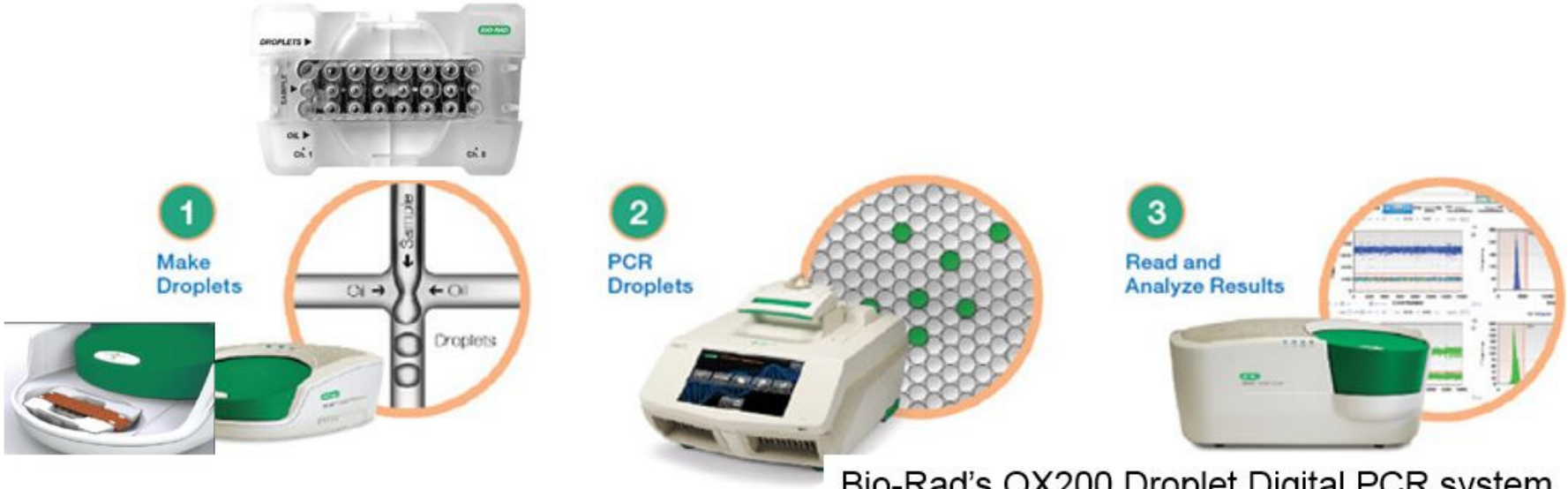
as in standard PCR



Detection

All dropplets were counted. Fluorescence upper the threshold is considered as a positive PCR, whereas under it's negative.



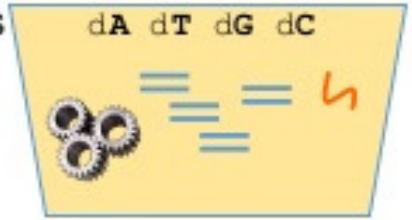


Bio-Rad's QX200 Droplet Digital PCR system

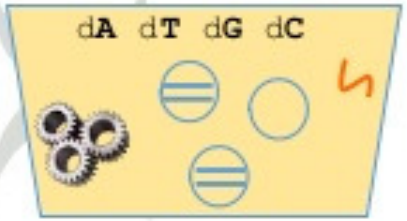
Digital PCR – An evolution of PCR

Conventional PCR → Real-time PCR → Digital PCR

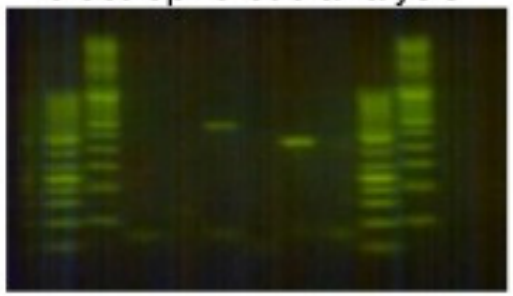
Polymerase + Primers + (Probes) + Nucleotides + Target in a Homogenous system



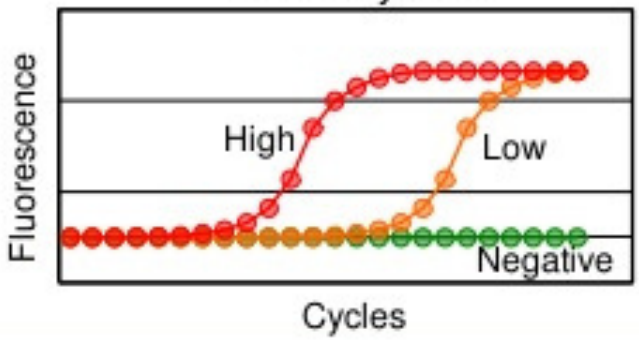
Polymerase + Primers + (Probes) + Nucleotides + Target; Individual Target DNA physically separated



Post PCR gel electrophoretic analysis



Real time detection in closed system



End-point detection of droplets