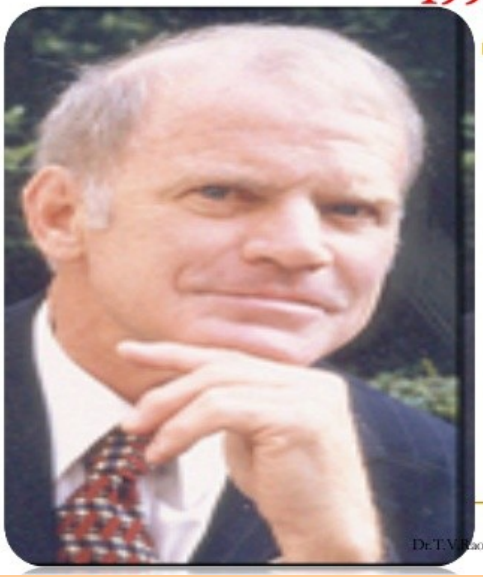
Polymerase Chain Reaction (PCR)

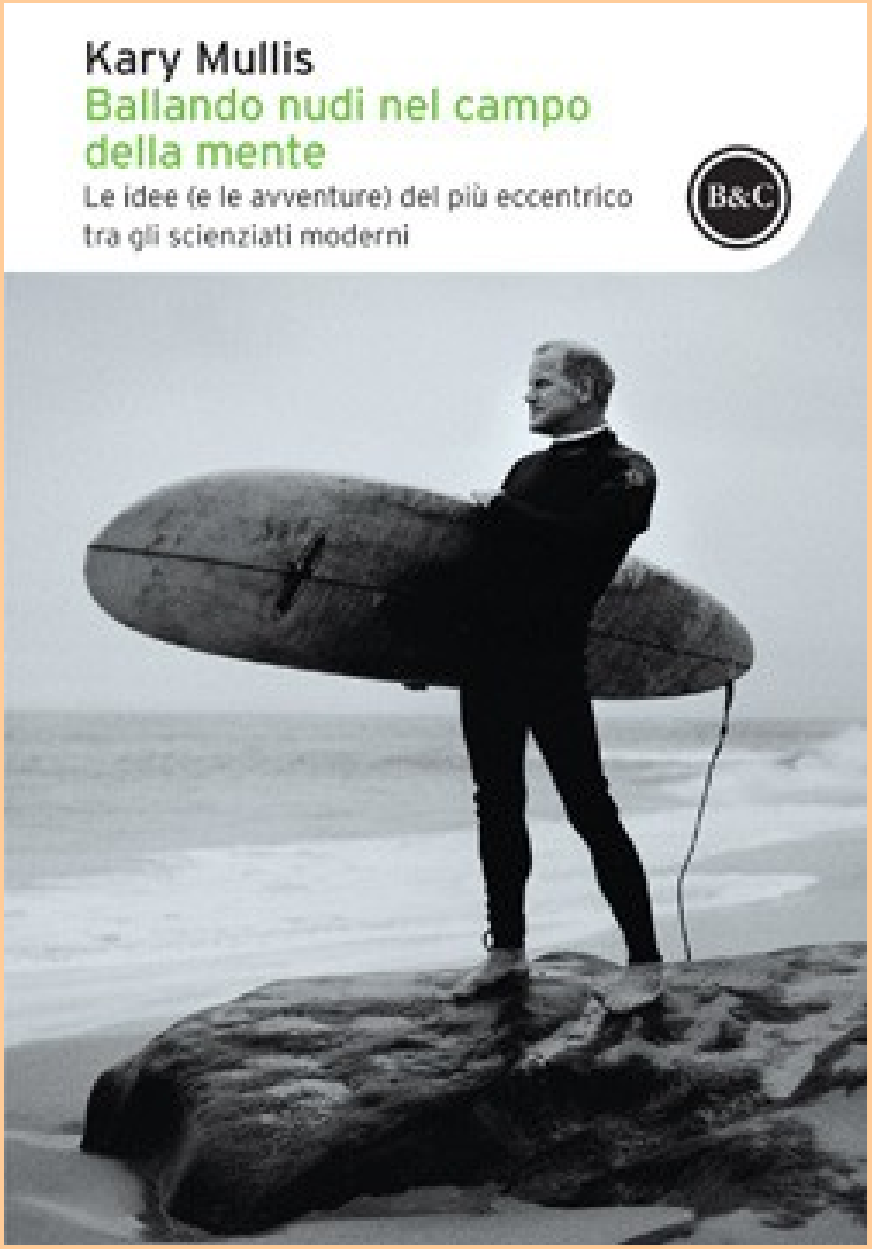
The **polymerase chain reaction** (**PCR**) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. As PCR progresses, the DNA generated is used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. With PCR it is possible to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece. PCR can be extensively modified to perform a wide array of genetic manipulations.

Developed in 1984 by Kary Mullis, PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases. In 1993 Mullis was awarded the Nobel Prize in Chemistry for his work on PCR.

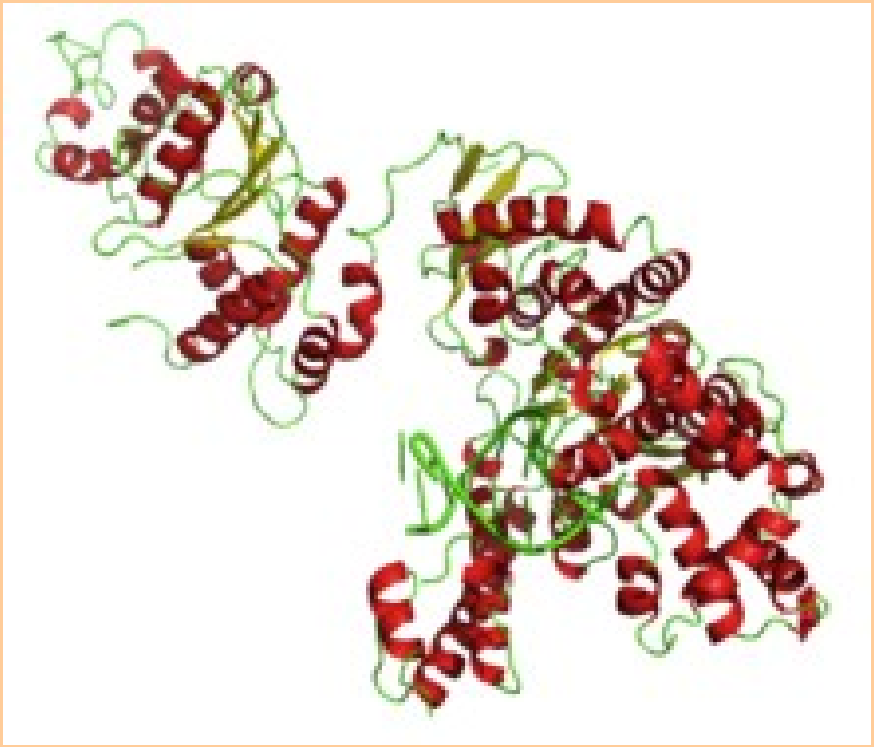
Dr. Kary Mullis, wins Nobel Prize in 1993



- Kary received a Nobel Prize in **chemistry in 1993**, for his invention of the polymerase chain reaction (PCR). The process, which Kary Mullis conceptualized in 1983, is hailed as one of the monumental scientific techniques of the **twentieth century.**

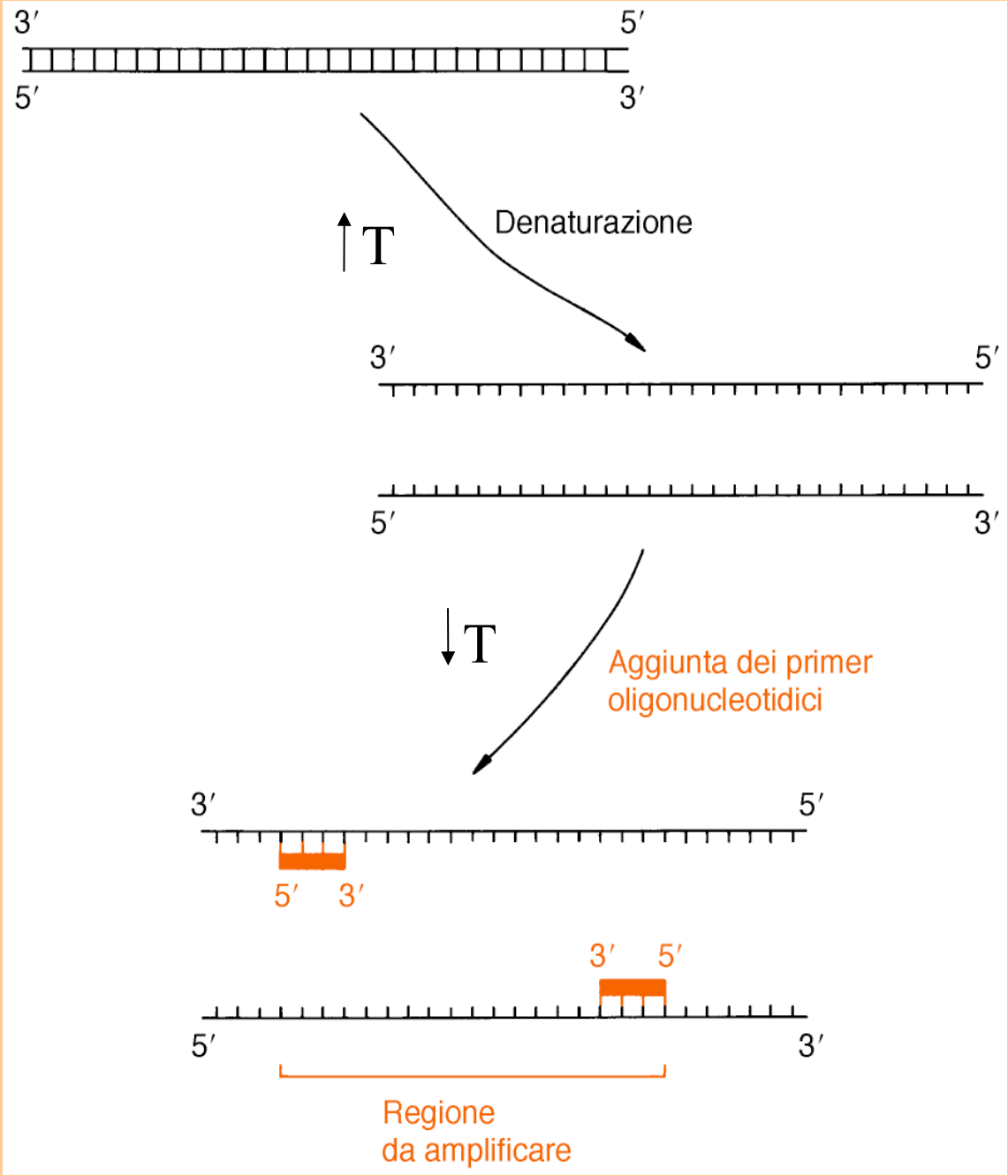


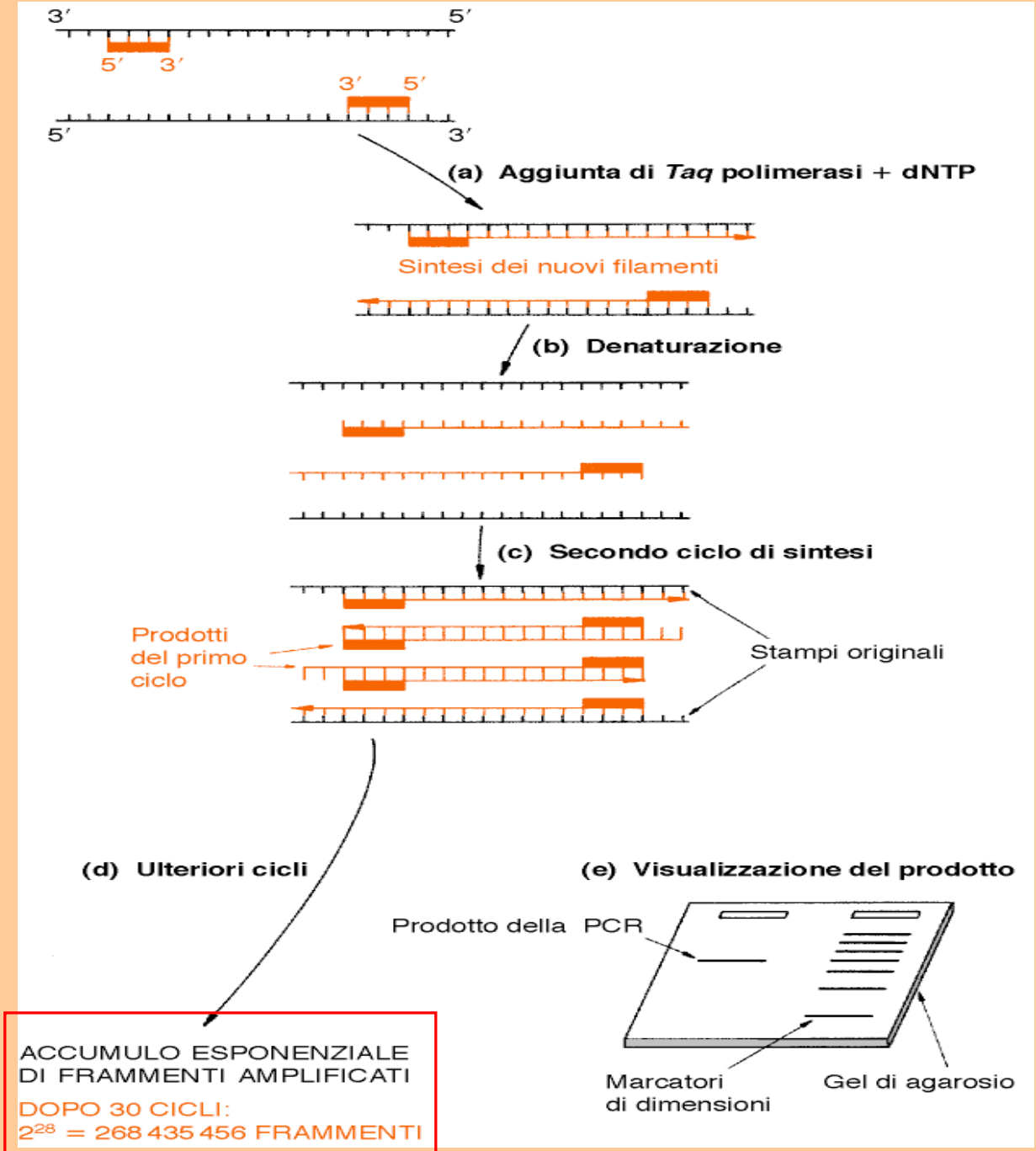
Taq polymerase



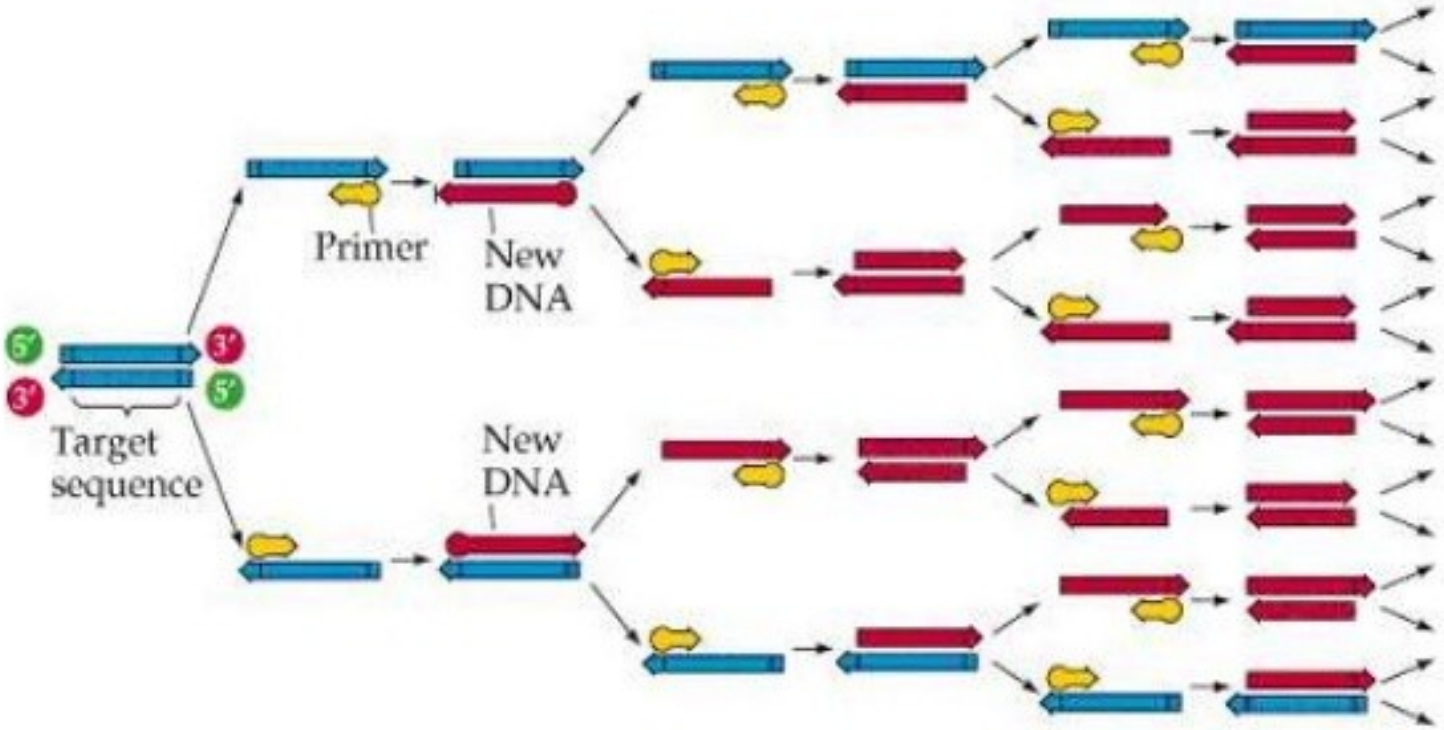
Thermus acquaticus

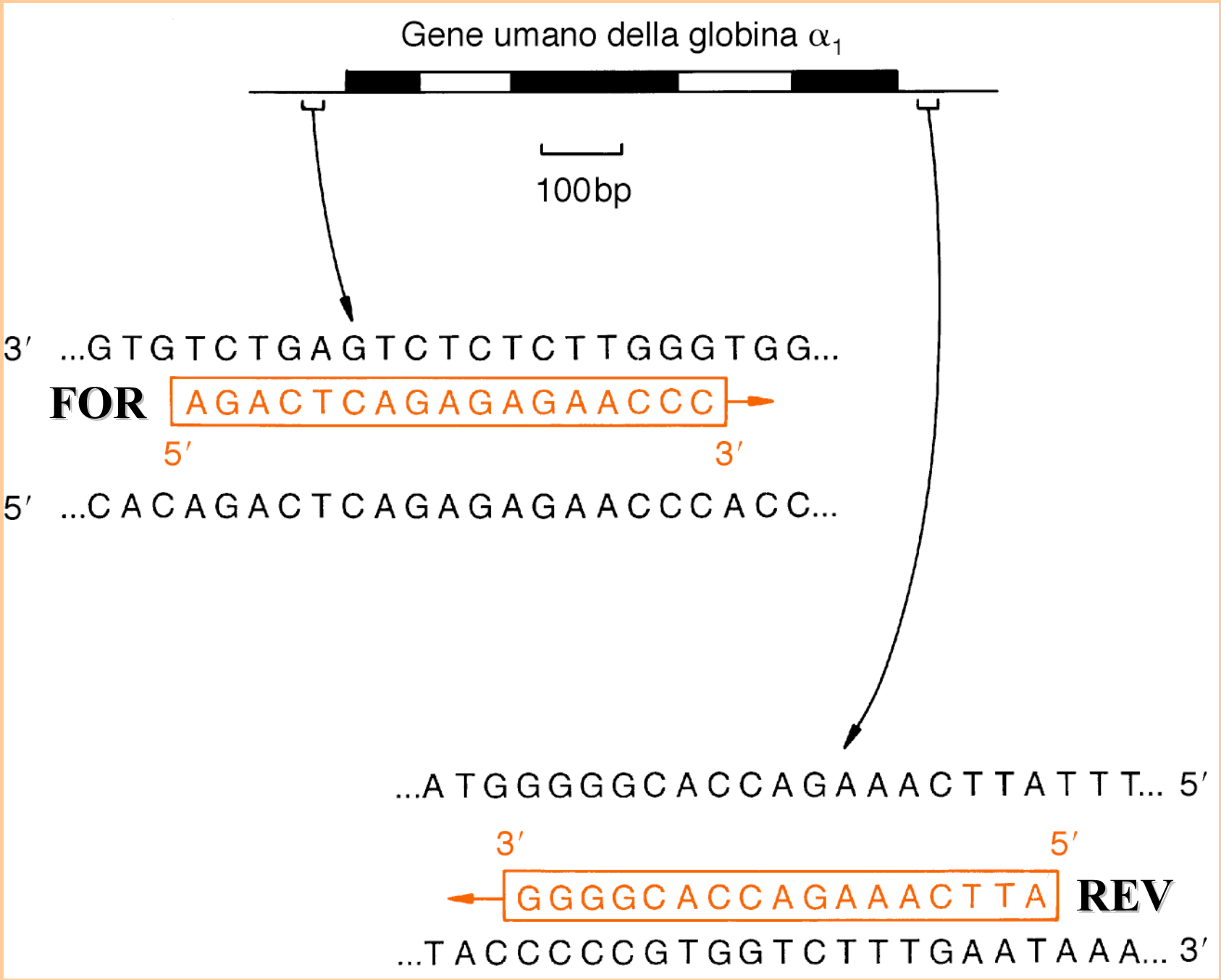
Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*. This DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary to physically separate the strands (at high temperatures) in a DNA double helix (DNA melting) used as the template during DNA synthesis (at lower temperatures) by the DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.


3′
5′
5′
3′
T
Denaturazione
3′
5′
5′
3′
T
Aggiunta dei primer
oligonucleotidici
3′
5′
5′
3′
3′
5′
5′
3′
Regione
da amplificare

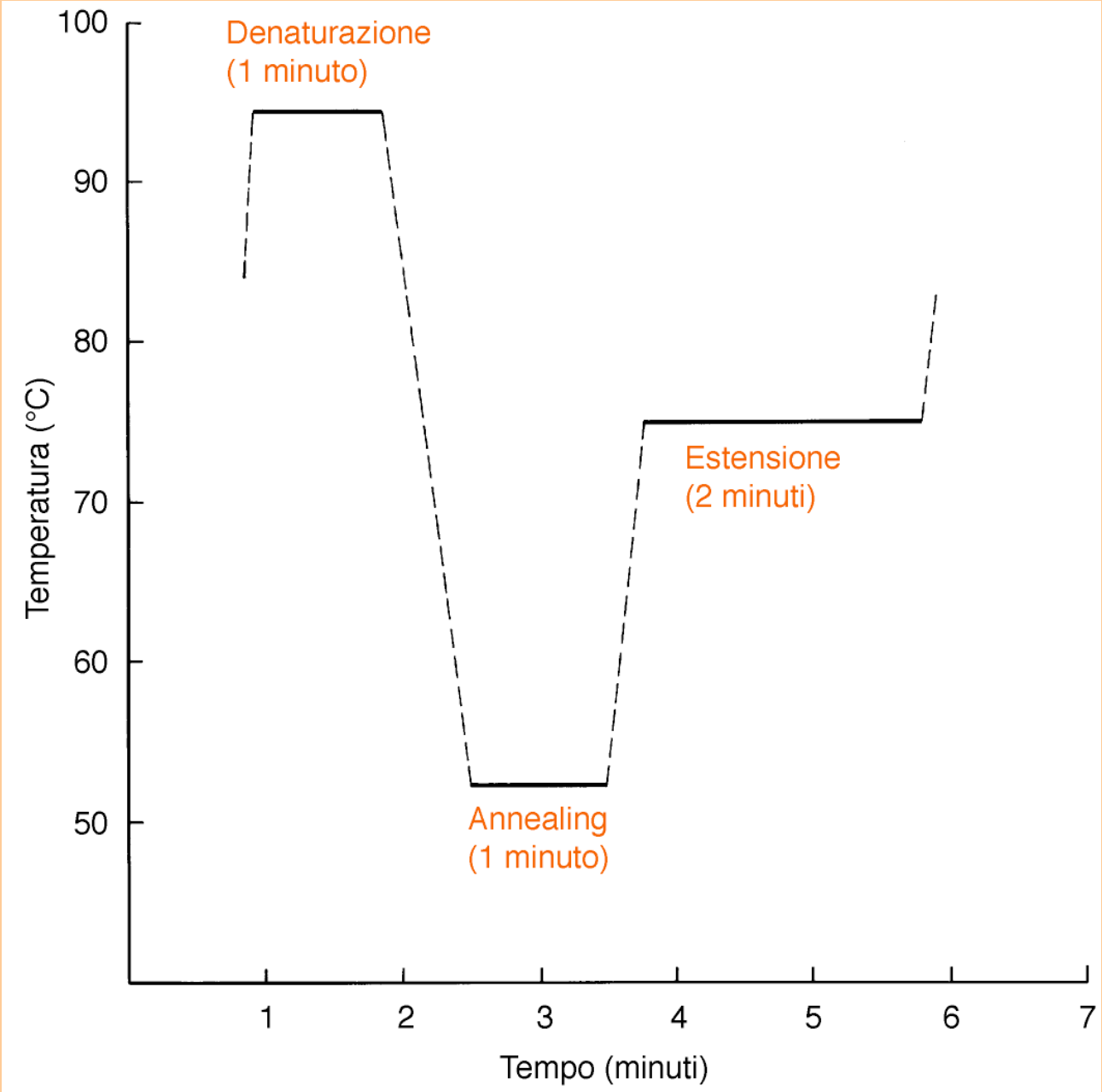

3′
5′
5′
3′
3′
5′
5′
3′
(a) Aggiunta di *Taq* polimerasi + dNTP
Sintesi dei nuovi filamenti
(b) Denaturazione
(c) Secondo ciclo di sintesi
Prodotti del primo ciclo
Stampi originali
(d) Ulteriori cicli
ACCUMULO ESPONENZIALE DI FRAMMENTI AMPLIFICATI
DOPO 30 CICLI:
2^{28} = 268 435 456 FRAMMENTI
(e) Visualizzazione del prodotto
Prodotto della PCR
Marcatori di dimensioni
Gel di agarosio

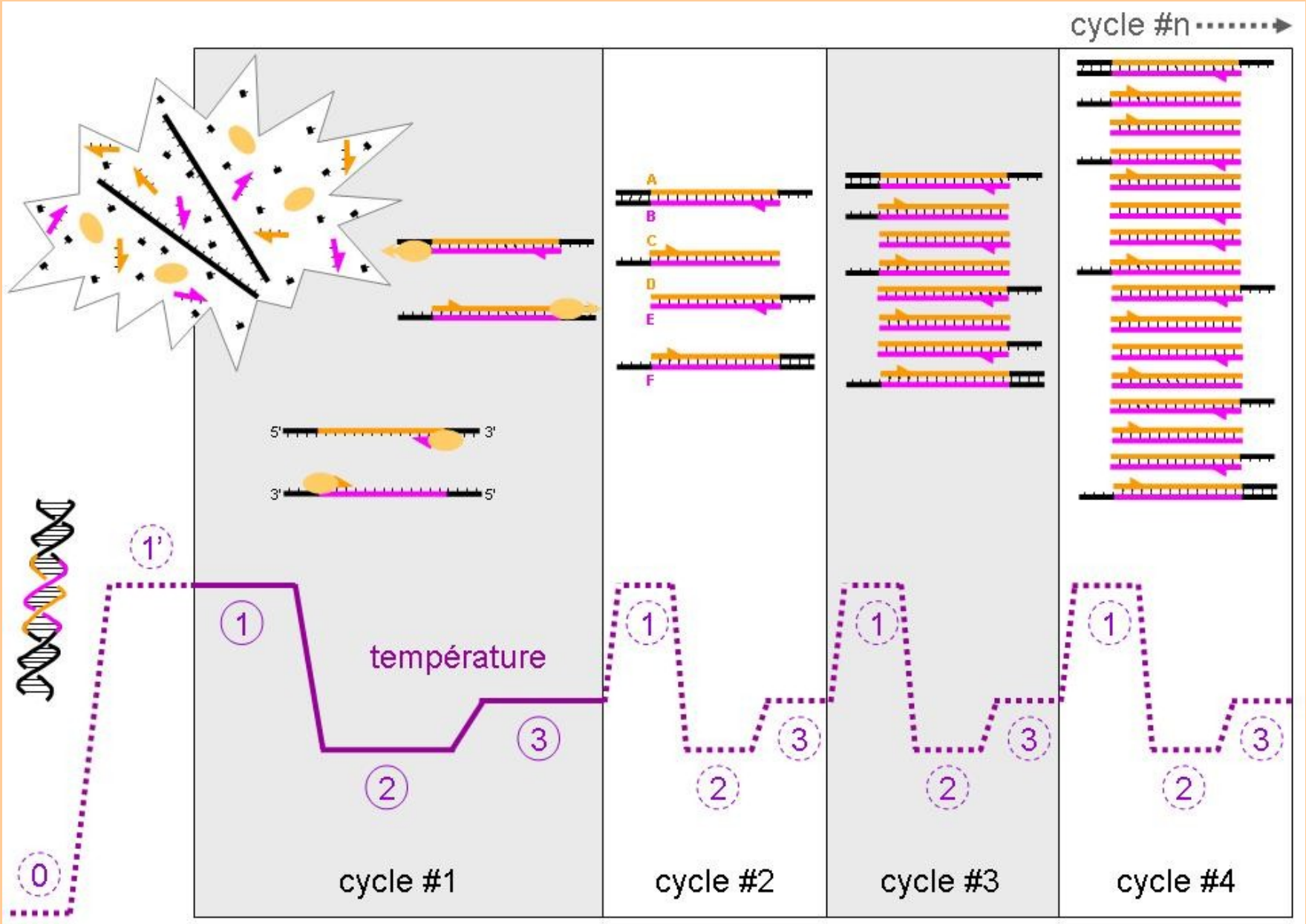
PCR-DNA synthesis cycle



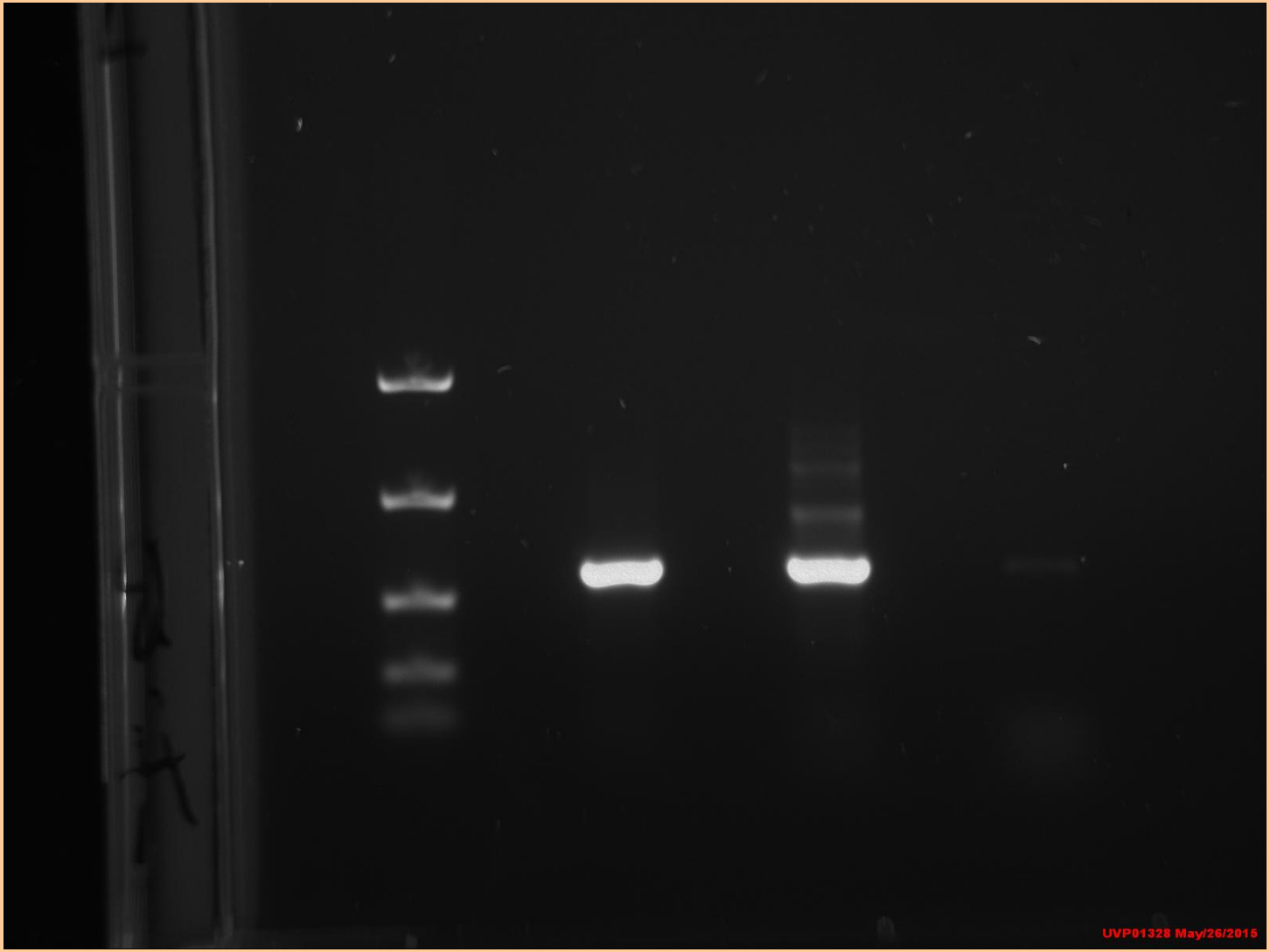

Gene umano della globina α_1
100bp
3′ ...G T G T C T G A G T C T C T C T T G G G T G G...
FOR
A G A C T C A G A G A G A A C C C
5′
3′
5′ ...C A C A G A C T C A G A G A G A A C C C A C C...
...A T G G G G G C A C C A G A A A C T T A T T T... 5′
3′
5′
G G G G C A C C A G A A A C T T A
REV
...T A C C C C C G T G G T C T T T G A A T A A A... 3′

Profilo di temperatura di una PCR




cycle #n
A
B
C
D
E
F
5'
3'
3'
5'
1'
1
2
3
température
0
cycle #1
cycle #2
cycle #3
cycle #4

Risultato di una PCR su gel di agarosio



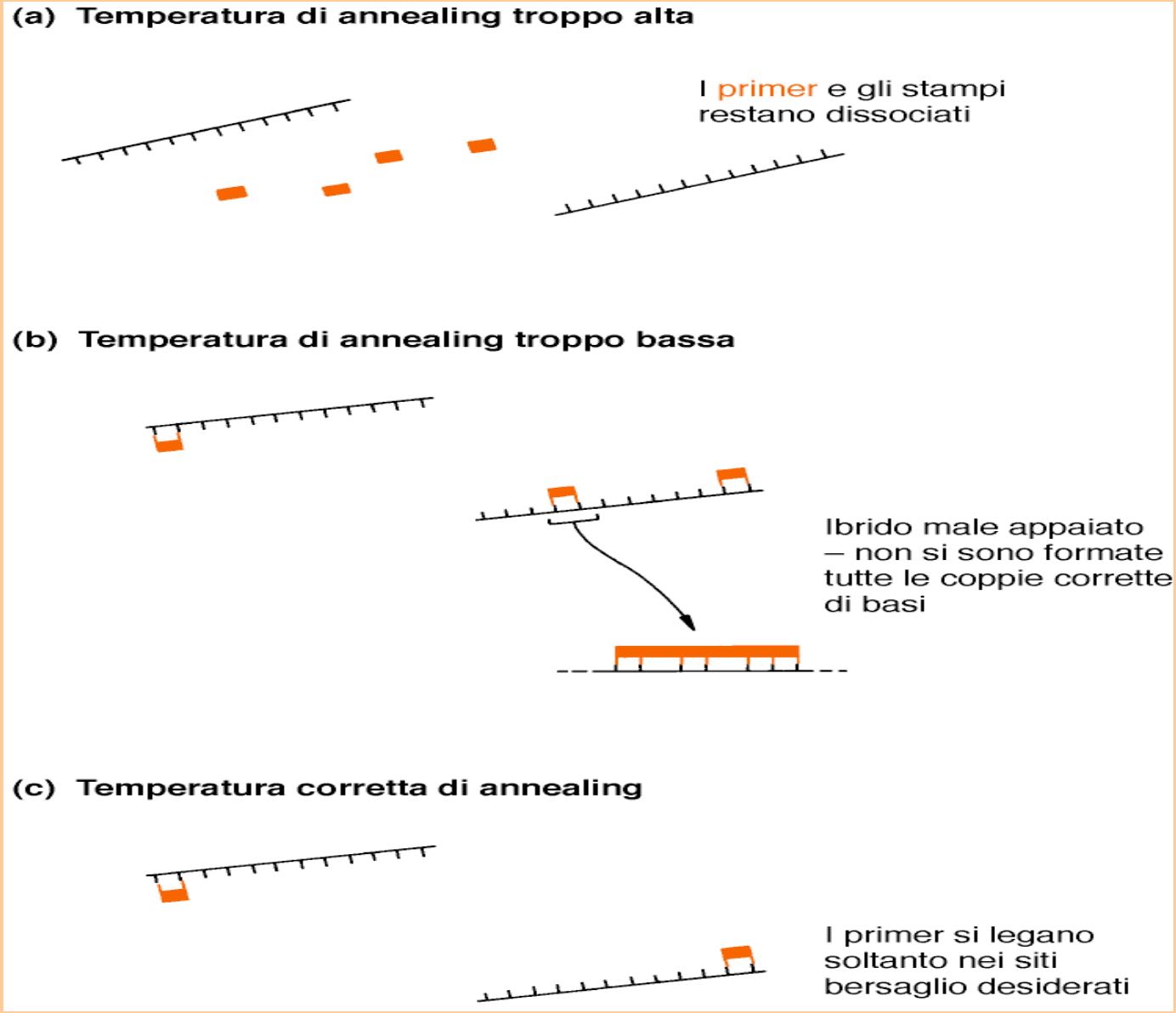
Termociclatore: apparecchio che permette di ottenere i cicli di temperatura necessari per la reazione di PCR. E' composto da una piastra riscaldante con alloggiamenti per i campioni in grado garantire rapidi cambi di temperatura.

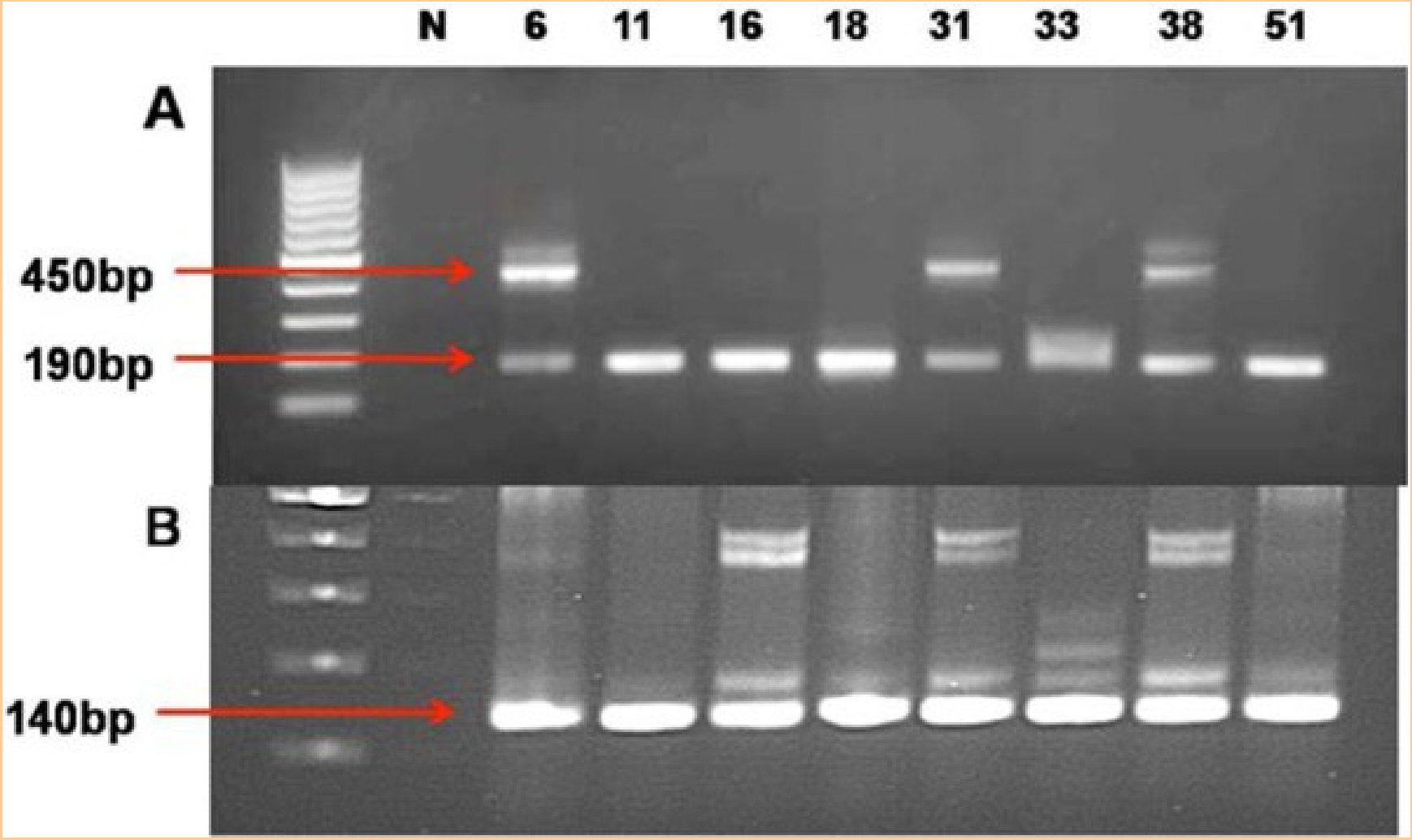
Date le elevate temperature ce si raggiungono durante la fase di denaturazione (95 °C), per evitare l'evaporazione del campione (solitamente 20-50 uL) nei vecchi termociclatori si aggiungeva uno strato di olio minerale nel tubo da pcr.

I termociclatori moderni hanno una coperchio che è possibile scaldare fino ad una temperatura superiore a quella del campione (105 °C) in modo da bloccare l'evaporazione.

Un moderno termociclatore

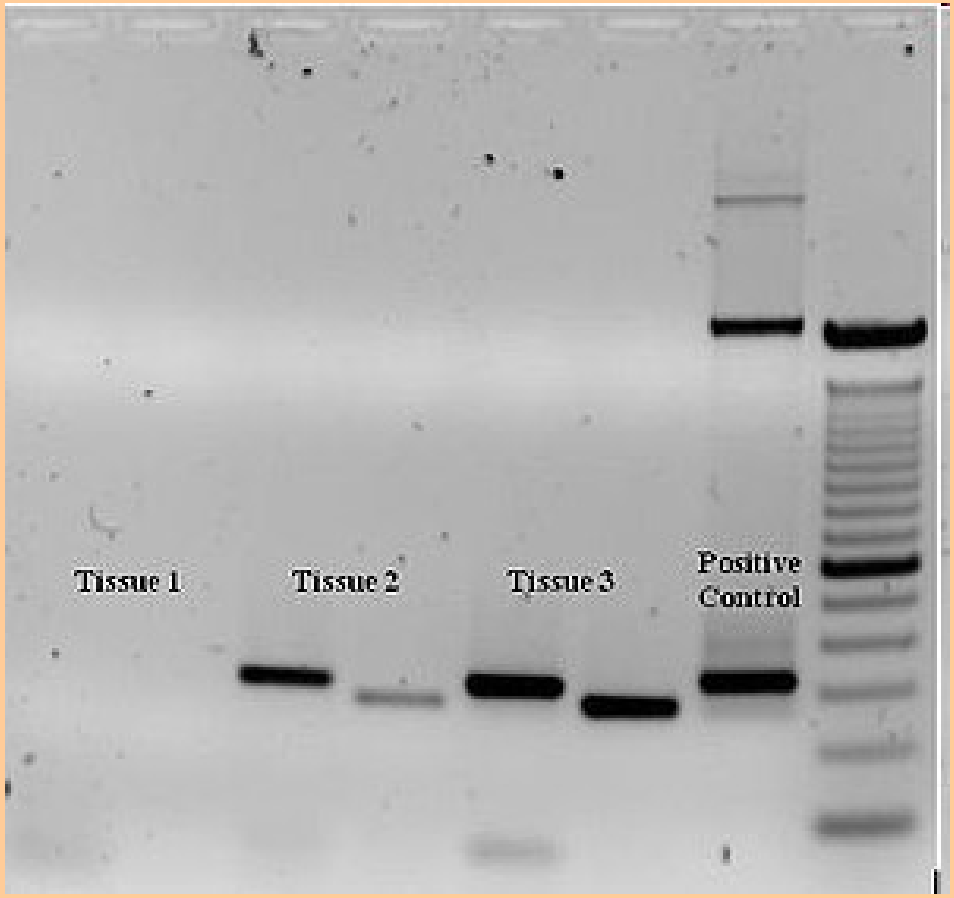
E’ importante la scelta della temperatura corretta




N
6
11
16
18
31
33
38
51
A
450bp
190bp
B
140bp

Thermostable polymerases used in PCR

Polymerase	3'->5' Exonuclease	Source and Properties
Taq	No	From *Thermus aquaticus*. Halflife at 95C is 1.6 hours.
Pfu	Yes	From *Pyrococcus furiosus*. Appears to have the lowest error rate of known thermophilic DNA polymerases.
Vent	Yes	From *Thermococcus litoralis*; also known as Tli polymerase. Halflife at 95 C is approximately 7 hours.



Ethidium bromide-stained PCR products after gel electrophoresis. Two sets of primers were used to amplify a target sequence from three different tissue samples. No amplification is present in sample #1; DNA bands in sample #2 and #3 indicate successful amplification of the target sequence. The gel also shows a positive control, and a DNA ladder containing DNA fragments of defined length for sizing the bands in the experimental PCRs.

Real Time PCR (RT-PCR)

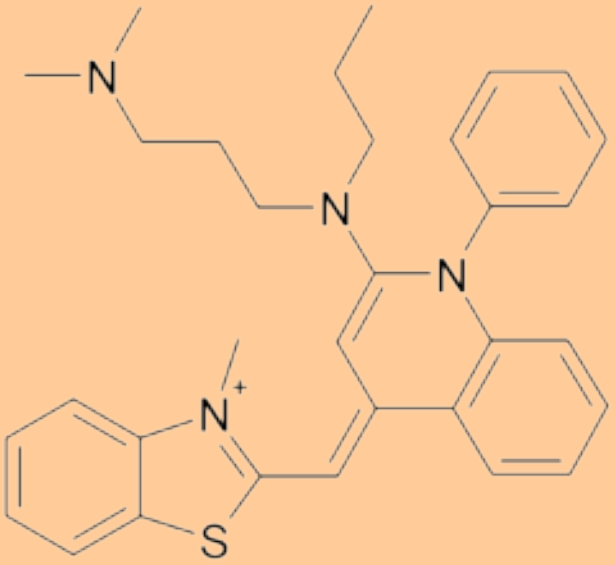
In molecular biology, **real-time polymerase chain reaction**, also called **quantitative real time polymerase chain reaction** (Q-PCR/qPCR) or **kinetic polymerase chain reaction**, is a laboratory technique based on the polymerase chain reaction, which is used to amplify and simultaneously **quantify** a targeted DNA molecule. It enables both **detection and quantification** (**as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes**) of a specific sequence in a DNA sample.

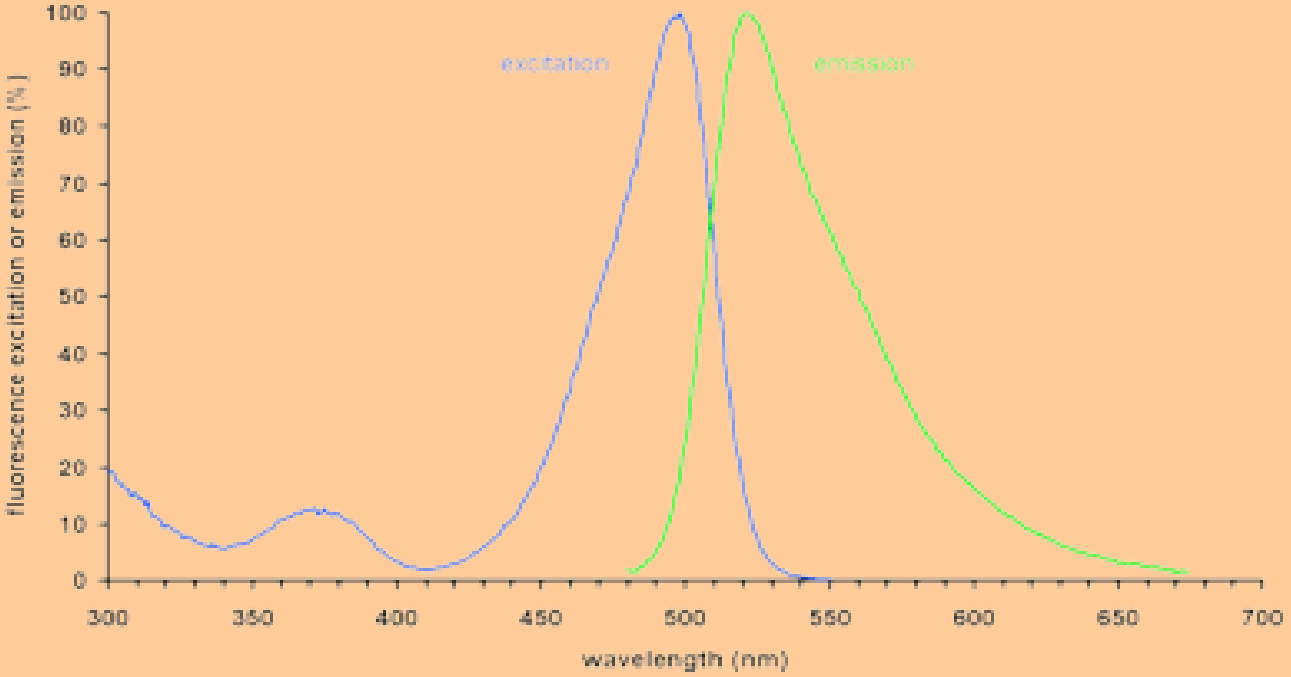
Fluorescent reporter probe method

A **DNA-binding dye** binds to all double-stranded (ds)DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in **fluorescence intensity** and is measured at each cycle, thus allowing DNA concentrations to be quantified. However, dsDNA dyes such as **SYBR Green** will bind to all dsDNA PCR products, including nonspecific PCR products (such as "primer dimers"). This can potentially interfere with or prevent accurate quantification of the intended target sequence.

- The reaction is prepared as usual, with the addition of fluorescent dsDNA dye.
- The reaction is run in a thermocycler, and after each cycle, the levels of fluorescence are measured with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the PCR product). With reference to a standard dilution, the dsDNA concentration in the PCR can be determined.

Syber Green


N
N
N
N+
S

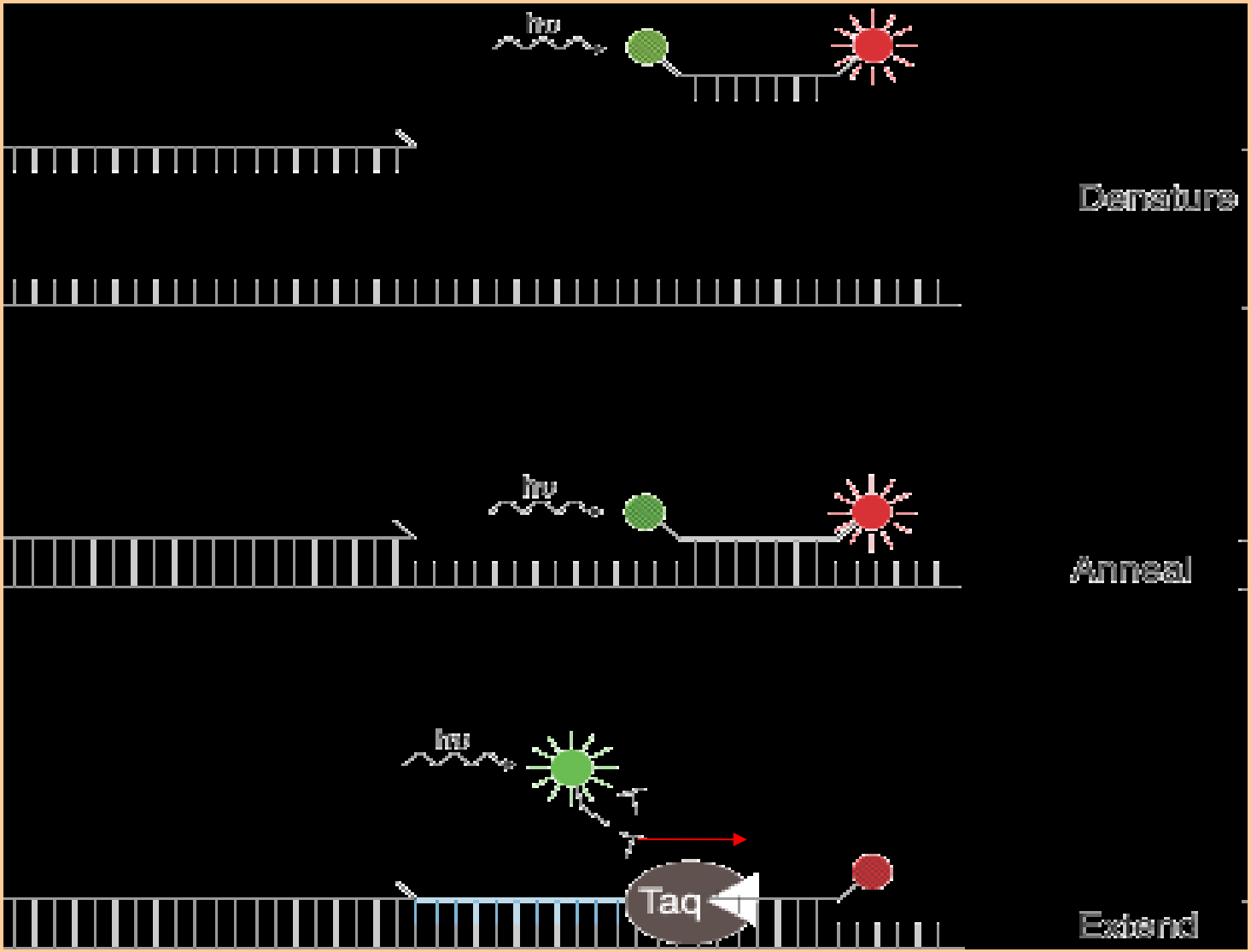

excitation
emission
fluorescence excitation or emission (%)
wavelength (nm)
0
10
20
30
40
50
60
70
80
90
100
300
350
400
450
500
550
600
650
700

Fluorescent reporter probe method

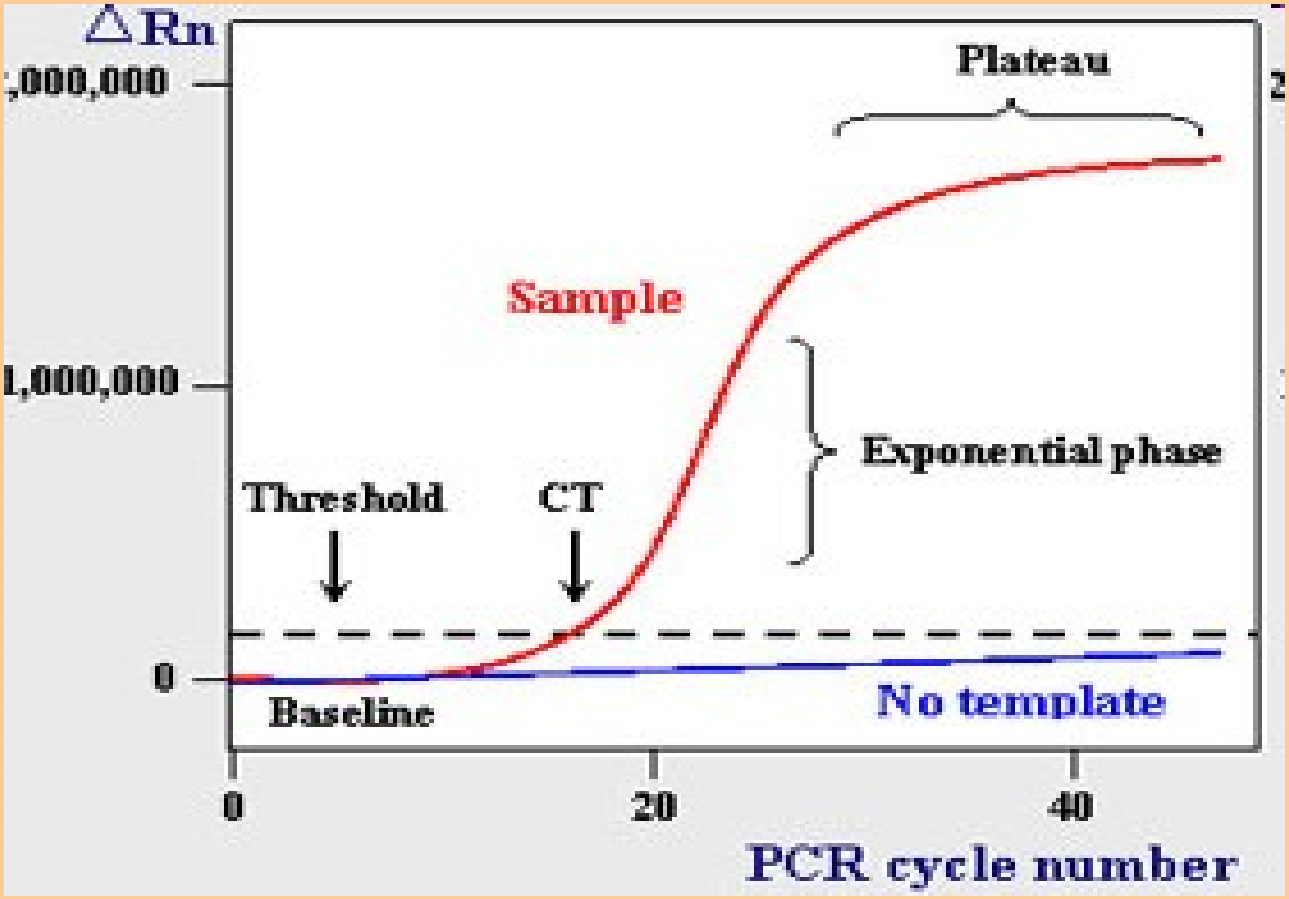
Using **fluorescent reporter probes** is the most accurate and most reliable of the methods, but also the most expensive. It uses a sequence-specific RNA or DNA-based probe to quantify only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and allows quantification even in the presence of some non-specific DNA amplification (Fluorescent dyes reports amplification of any DNA fragment).

It is commonly carried out with an **RNA-based probe with a fluorescent reporter** at one end and a **quencher** of fluorescence at the opposite end of the probe. The close proximity of the reporter to the quencher prevents detection of its fluorescence; **breakdown of the probe** by the 5' to 3' exonuclease activity of the taq polymerase breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter.

Altro esempio di sonda fluoroforo-quencer

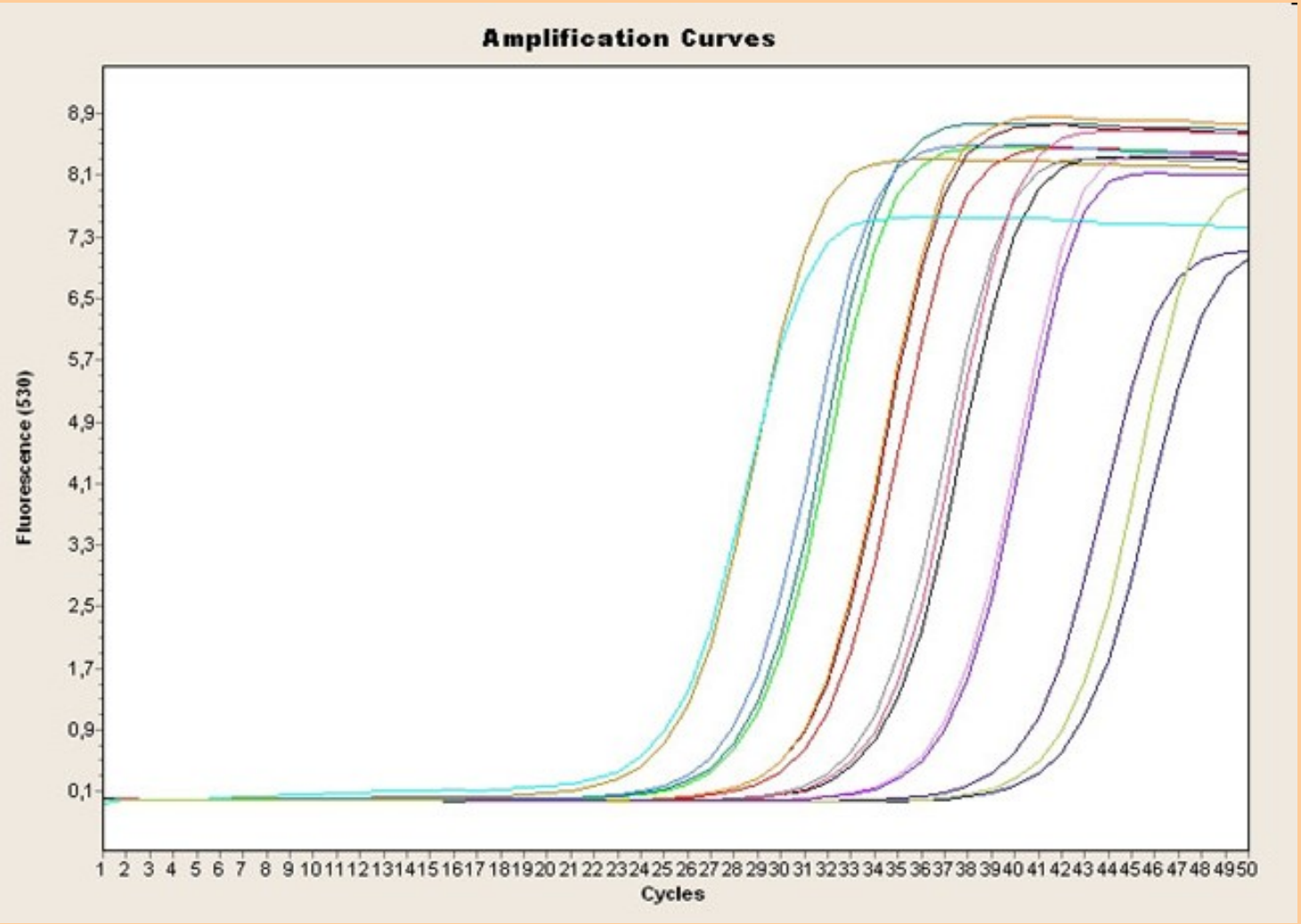


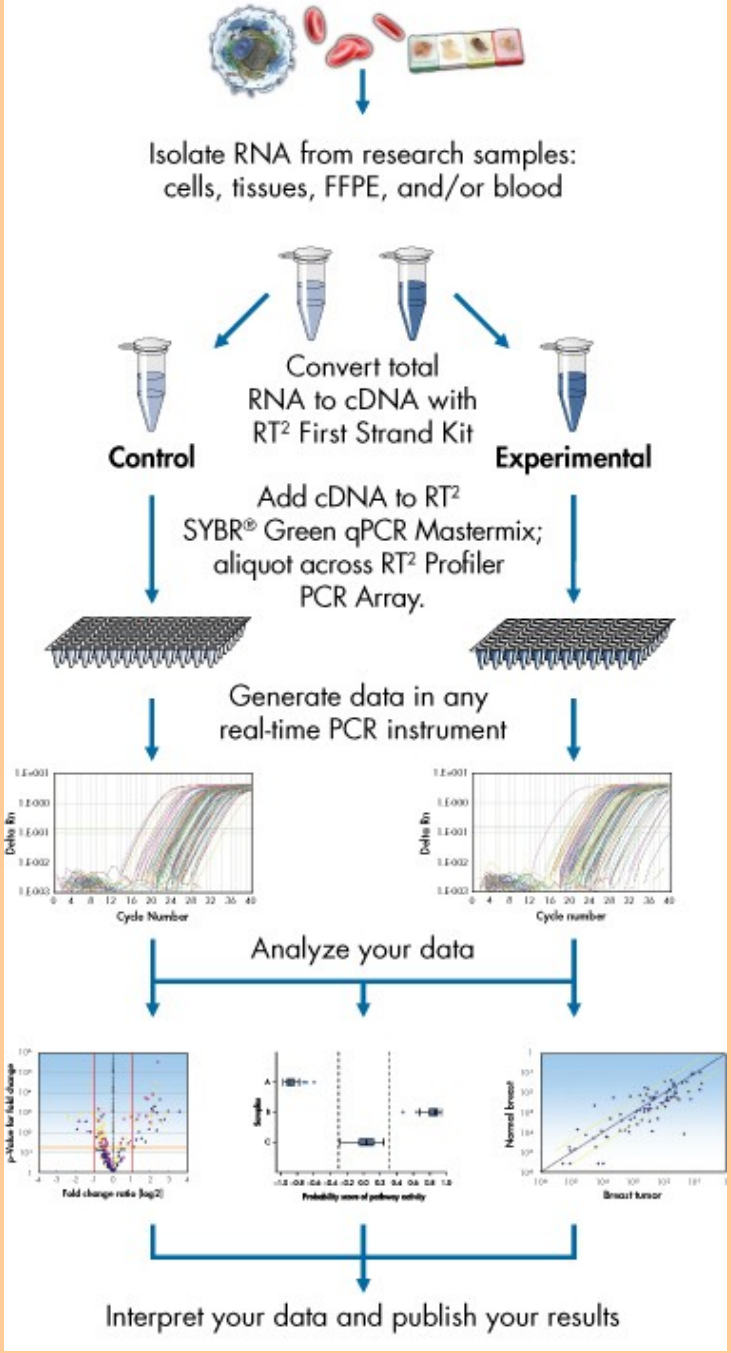
La curva dell'intensità della fluorescenza rispetto al numero di cicli è una sigmoide



Il valore **CT** in corrispondenza del primo flesso è direttamente proporzionale al **numero di copie di templato presenti.**

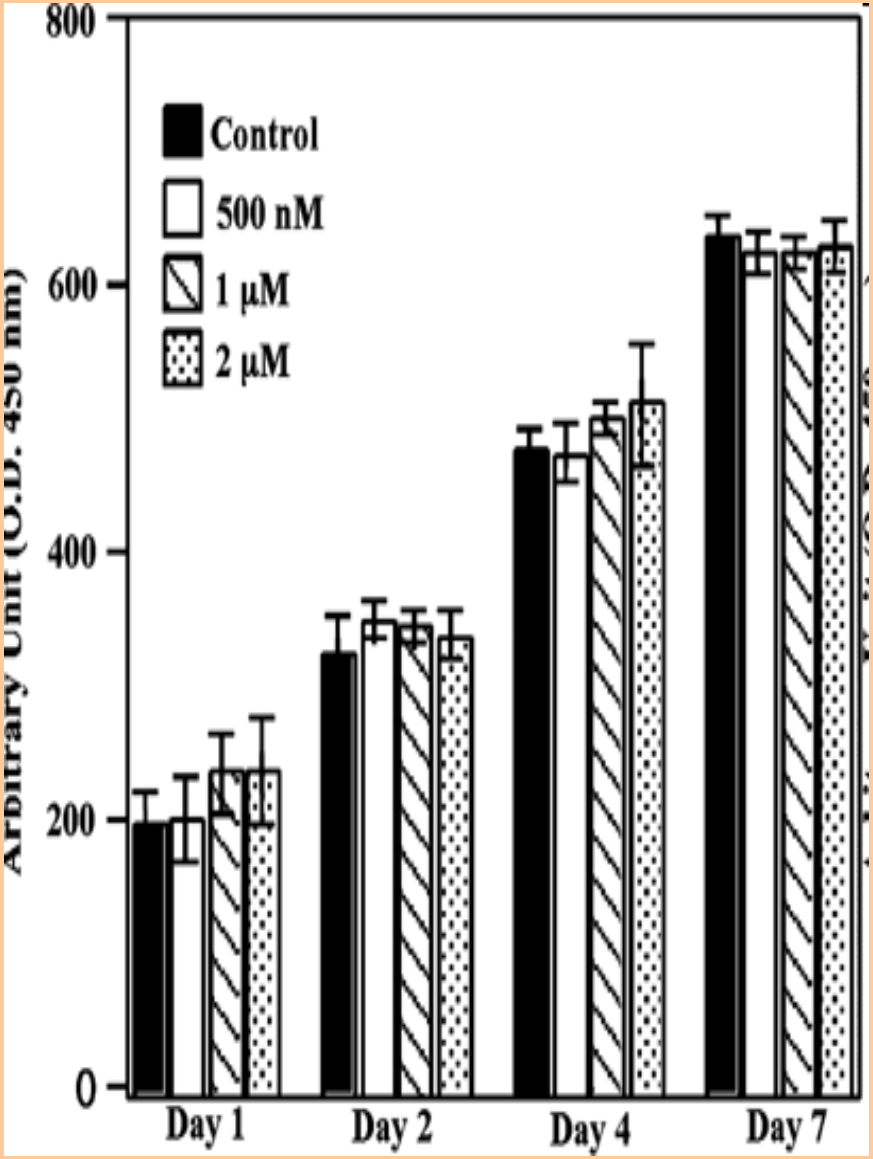
Ad una minore quantità di templato presente corrisponderà una curva spostata verso destra (CT ad un numero di cicli maggiore)

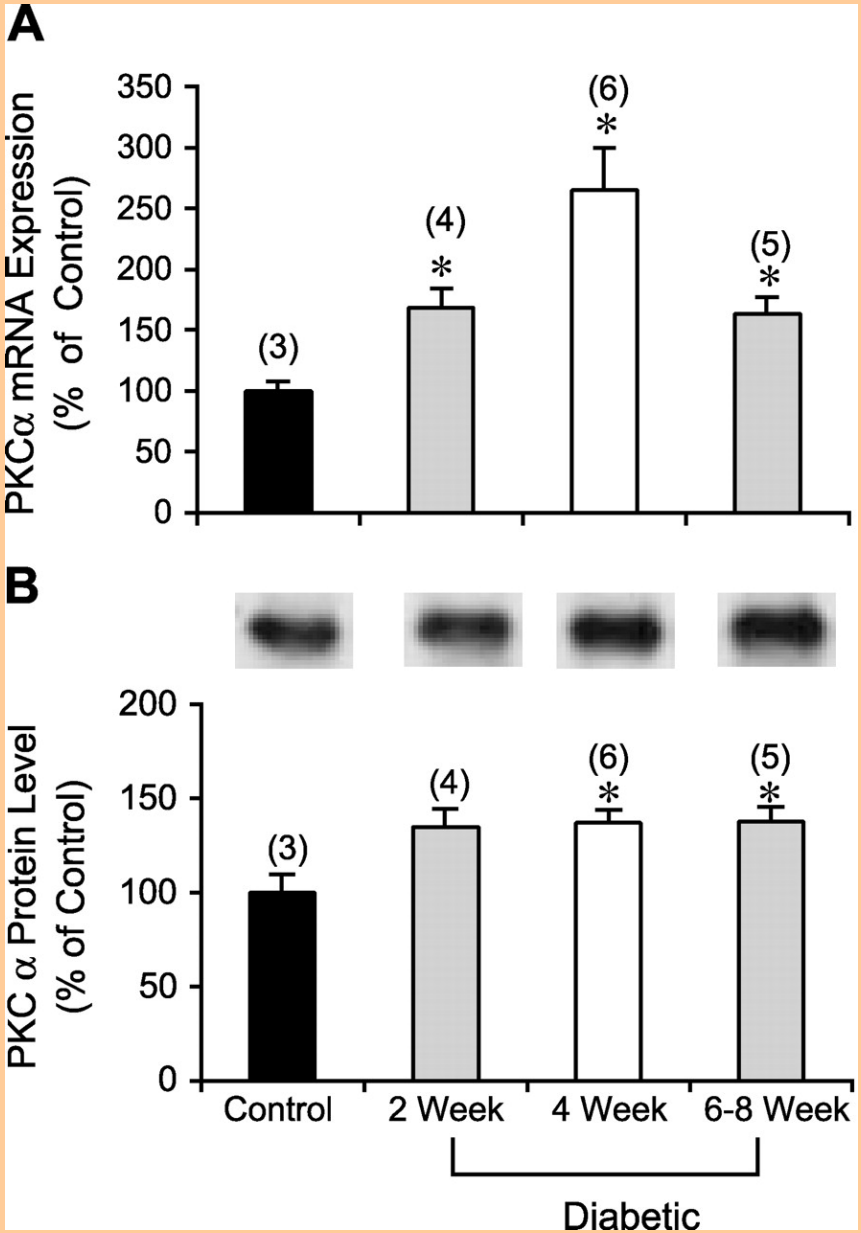



Isolate RNA from research samples:
cells, tissues, FFPE, and/or blood
Convert total
RNA to cDNA with
RT² First Strand Kit
Control
Experimental
Add cDNA to RT²
SYBR® Green qPCR Mastermix;
aliquot across RT² Profiler
PCR Array.
Generate data in any
real-time PCR instrument
Cycle Number
Cycle number
Analyze your data
Interpret your data and publish your results

La RT-PCR viene utilizzata spesso per confrontare l'espressione di un gene di un campione rispetto ad un controllo (per esempio delle cellule sane rispetto a cellule tumorali oppure trattate con una determinata sostanza rispetto a quelle non trattate).

Di solito si utilizza il rapporto con uno standard interno (per esempio l'mRNA di un gene non influenzato dal trattamento) per normalizzare i risultati ed eliminare errori o differenze dovuti al procedimento di preparazione dei campioni.





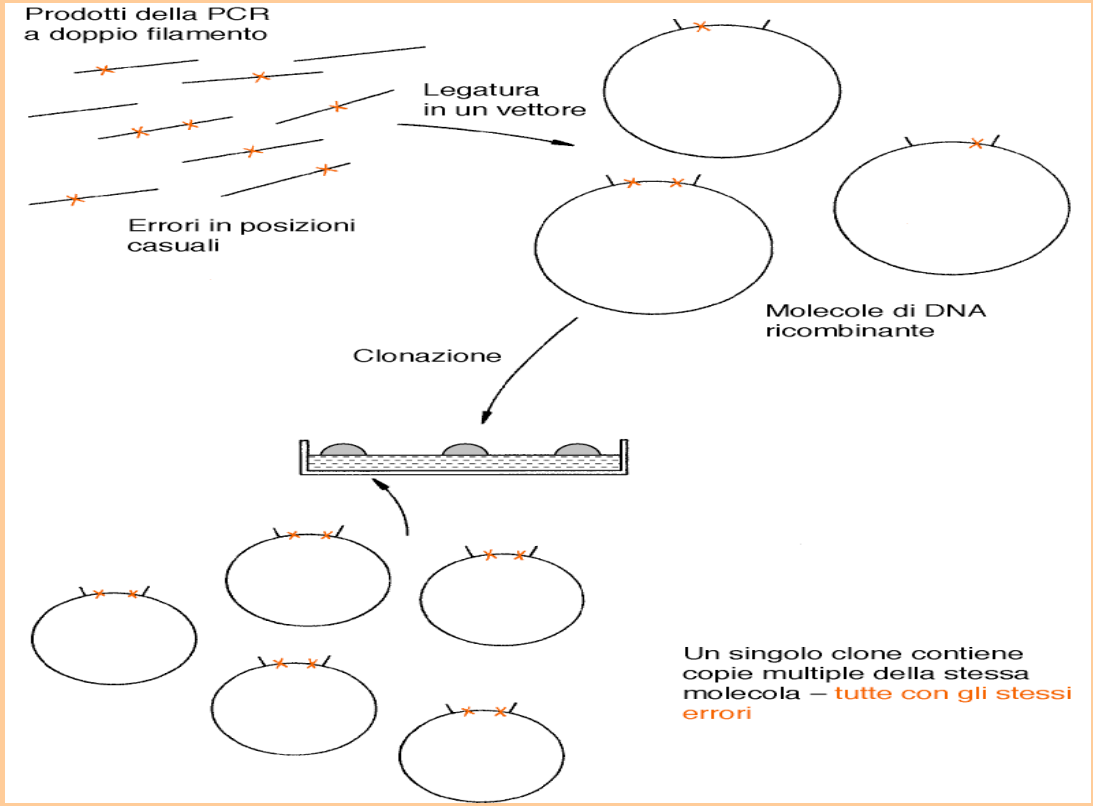
Time-dependent effect of hyperglycemia on mRNA (A) and protein (B) expression of PKCβ1 isozyme in the heart of diabetic pigs. Total RNA was extracted from hearts for real-time RT-PCR quantification, and the relative quantity of **PKCβ1 mRNA was normalized to that of GAPDH mRNA as described in MATERIALS AND METHODS**. The protein content of the PKC isozyme was determined by SDS-PAGE followed by immunoblotting analysis

Errori della polimerasi

Polimerasi termostabili:

-Taq (Termophilus aquaticus polymerase) → non possiede attivita' Proofreading.

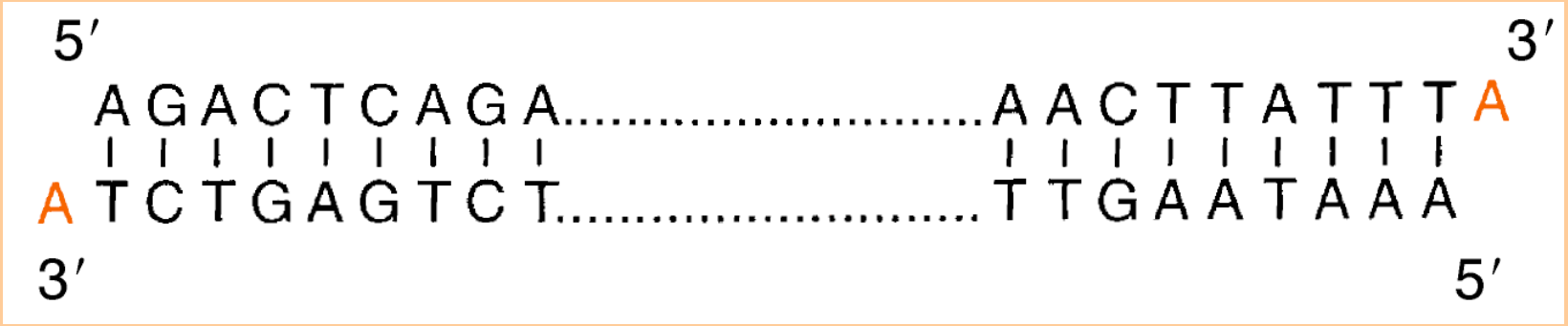
-Pfu (Pirococcus furiosus polymerase) → possiede attivita' Proofreading.

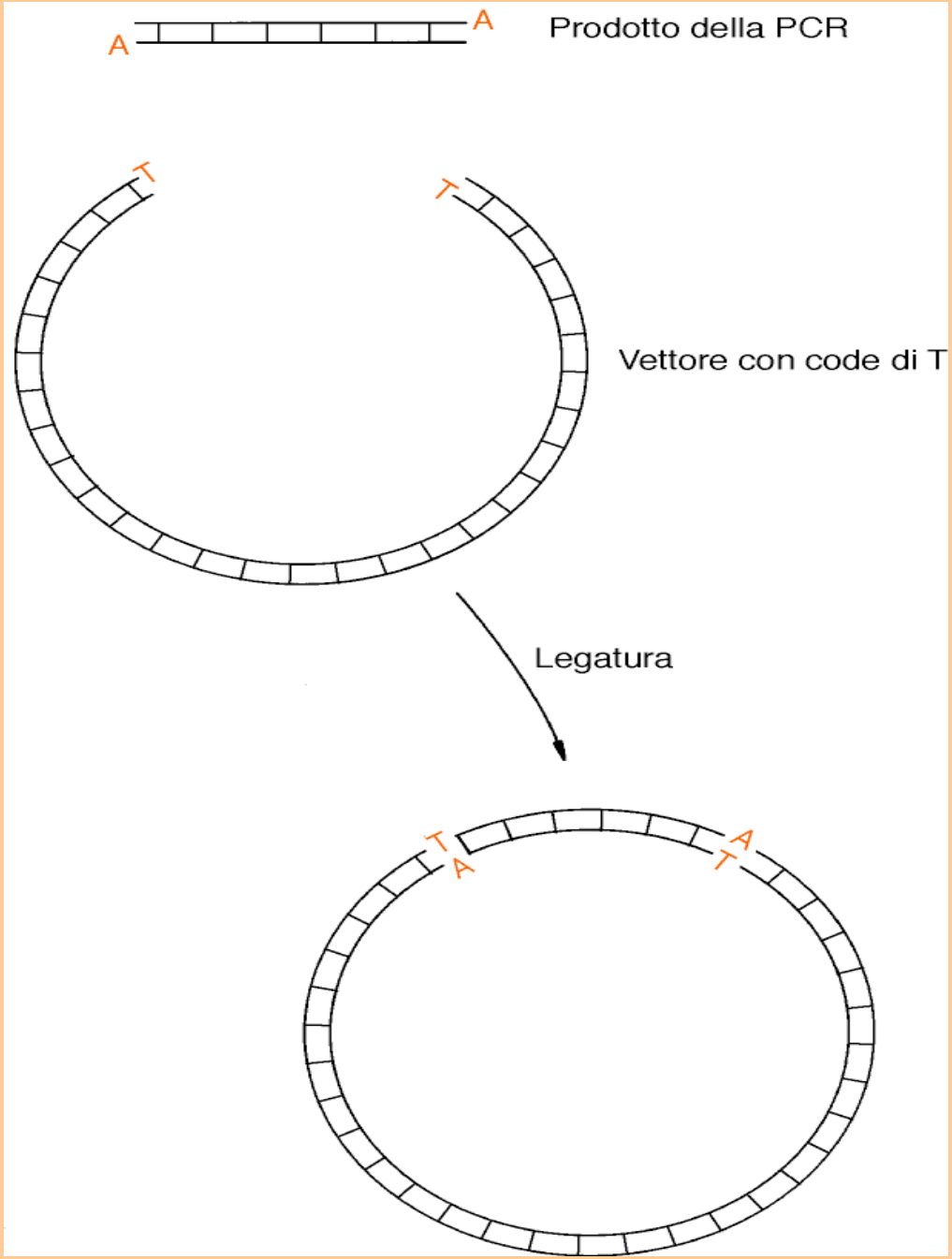


Clonaggio dei prodotti di PCR

TA cloning

I prodotti di PCR amplificati con la TAQ polimerasi presentano una T all'estremita' 3'.




A
A
Prodotto della PCR
T
T
Vettore con code di T
Legatura
T
A
A
T

PCR

In un eppendorf da 250 µl preparare la seguente mix:

12 µl H_20 sterile
2 µl buffer TAQ (10X)
1 µl $MgCl_2$ (50 mM)
1 µl Primer FOR (25 µM)
1 µl Primer REV (25 µM)
1 µl dNTPs (10 mM)
1 µl DNA plasmidico contenente l'inserto da amplificare (GPR3), circa 1000 bp.
1 µl TAQ polimerasi

___
20 µl
! Prestare attenzione a non contaminare!

Programma:

Step	Temperature	Time
Initial denaturation	95°C	3 minutes
Amplification (35 cycles)	95°C (denaturation)	30 sec
	55°C (annealing)	30 sec
	72°C (extension)	60 sec
Final extension	72°C	5 minutes

Preparazione gel di agarosio 0.8% (1 gel per 5 gruppi)

Pesare la quantità necessaria di agarosio per un gel allo 0.8%, aggiungere il TAE 1X e portare a volume.

Ricetta:
0.6 g agarosio
1.6 ml TAE 50X
78.4 ml H_20
---
80 ml

Scaldare nel forno a microonde in una beuta senza fare bollire e mescolare ogni tanto per sciogliere bene l'agarosio. Aspettare qualche minuto e aggiungere **5 µl di SyberSafe;** mescolare e versare nell'apposita vaschetta precedentemente preparata.
Il gel solidificato va posto nella vaschetta di corsa senza il pettinino e coperto con 250 ml di buffer di corsa (TAE 1X).