



The image displays a Real Time PCR (RT-PCR) amplification plot. The y-axis is labeled 'Fluorescence (aR)' and ranges from -10000 to 40000. The x-axis is labeled 'Cycles' and ranges from 2 to 36. The plot shows multiple data series, each represented by a different colored line with unique markers (e.g., triangles, squares, circles, diamonds, crosses). All series exhibit a characteristic sigmoidal amplification curve, starting at a baseline near zero, rising sharply between cycles 18 and 28, and reaching a plateau between cycles 28 and 30. The peak fluorescence for most series is between 30000 and 40000. Two text boxes are overlaid on the graph: a blue box at the top containing the title 'Real Time PCR' and a grey box at the bottom containing the subtitle 'Aspetti Tecnici'.

Real Time PCR

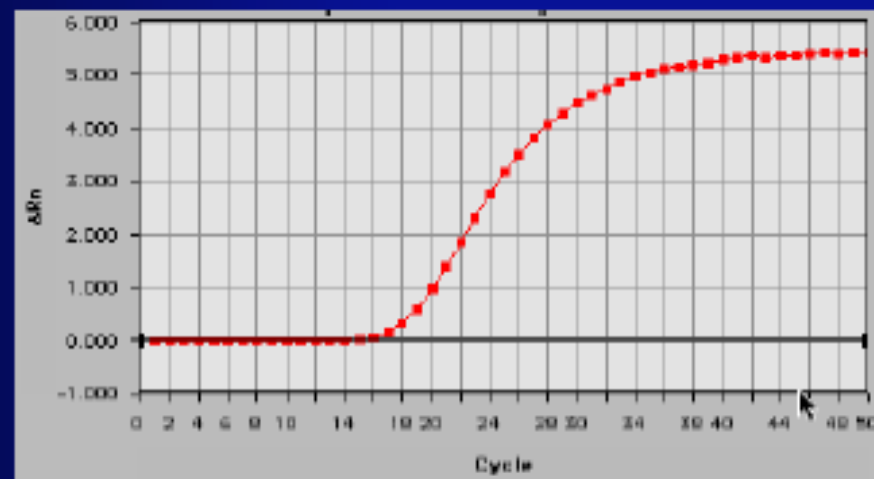
Aspetti Tecnici

Real-time PCR is kinetic

- Detection of “amplification-associated fluorescence” at each cycle during PCR
- No gel-based analysis at the end of the PCR reaction
- Computer based analysis of the cycle-fluorescence time course

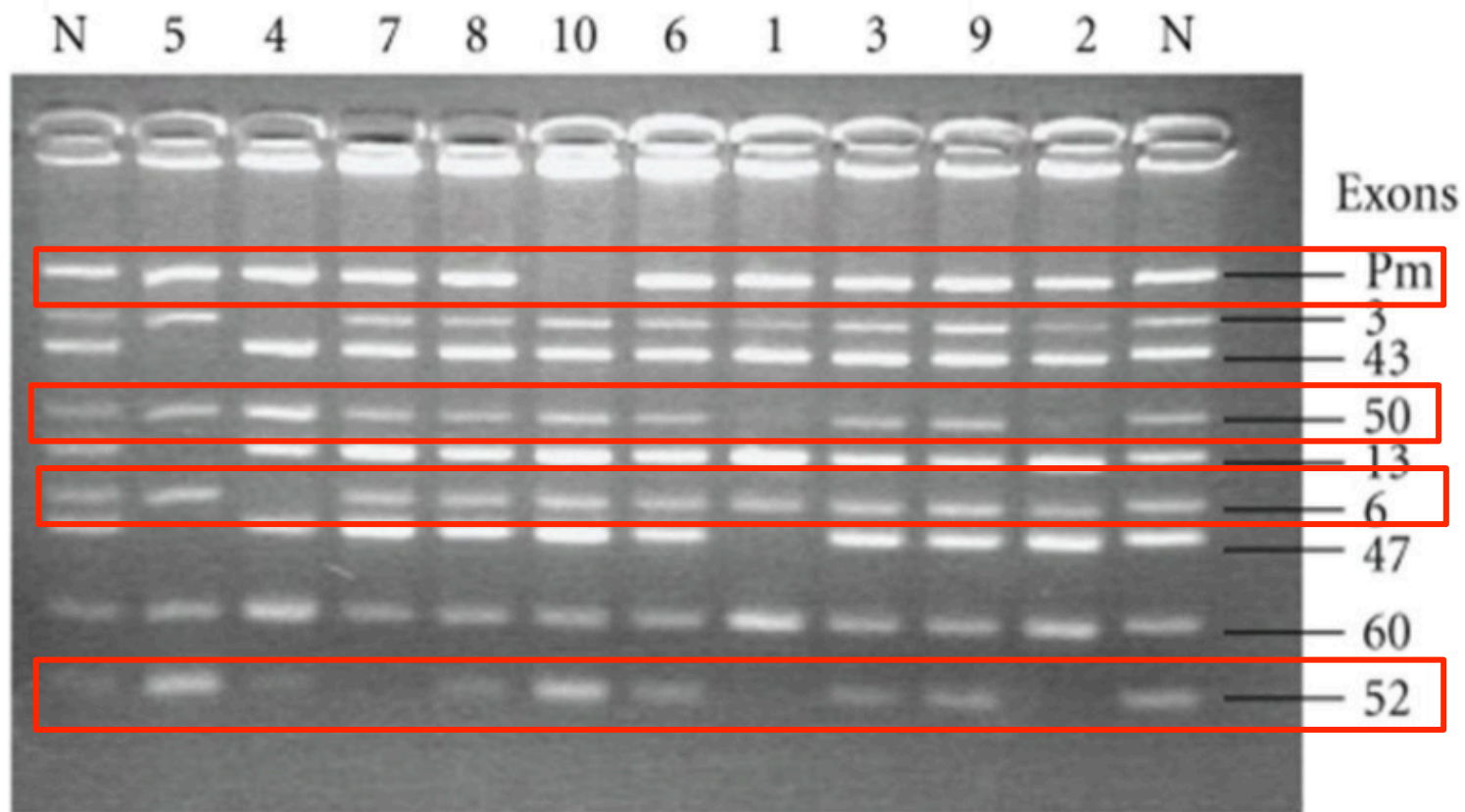
Increasing
fluorescence

Linear plot



PCR cycle

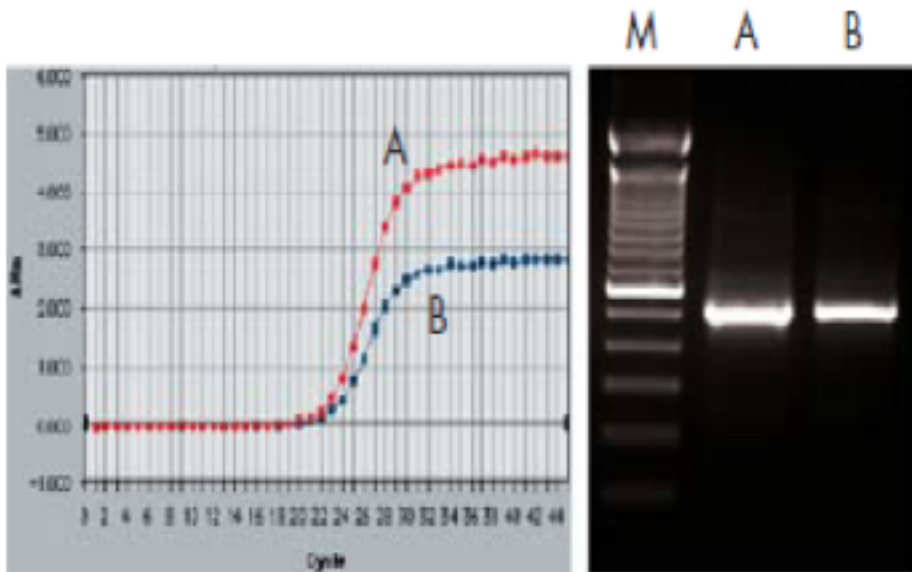
Analisi quantitativa mediante PCR end-point



Analysis of dystrophin gene by multiplex PCR. (The primer sets of Chamberlain et al.). The top numbers correspond to the codes of patients. The numbers at the right indicate the amplified exons. N: Normal control with all exons. *Patient 2* with deletion of exons 50 and 52. *Patient 1* with deletion of exons 50, 47, and 52. *Patient 10* with deletion of promoter Pm. *Patient 7* with deletion of exon 52. *Patient 4* with deletion of exons 3 and 6. *Patient 5* with deletion of exons 43, 13, and 47.

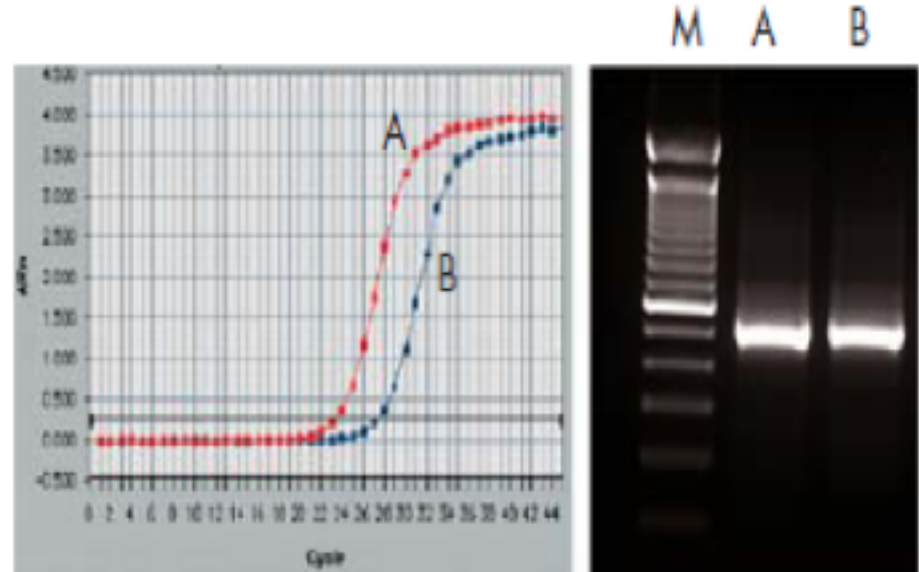
Limiti della PCR end-point per l'analisi quantitativa

A Same starting amount



Look different

B Different starting amount



Look similar

Introduzione

- La Real-time qPCR è la più sensibile ed affidabile tecnica per la quantificazione di acidi nucleici (qPCR, RT-qPCR)
- Si basa sull'identificazione e sulla quantificazione in tempo reale della **fluorescenza** emessa da una molecola reporter
- Questo avviene durante la fase di accumulo del prodotto di PCR e ad ogni ciclo di amplificazione, consentendo così di monitorare l'amplificazione durante la fase iniziale e soprattutto **durante la fase esponenziale** della reazione di PCR

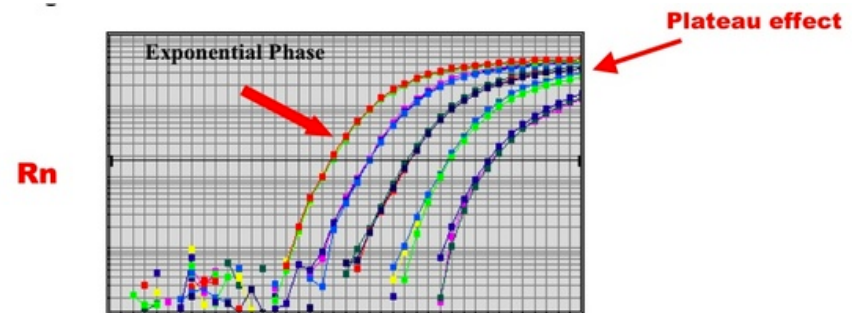
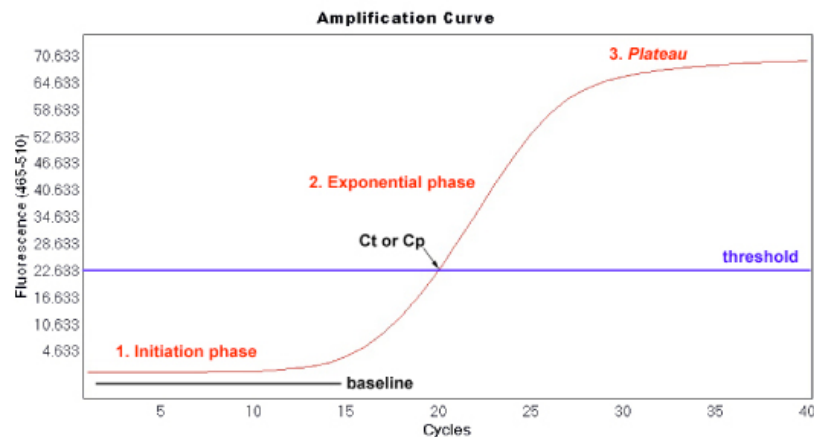
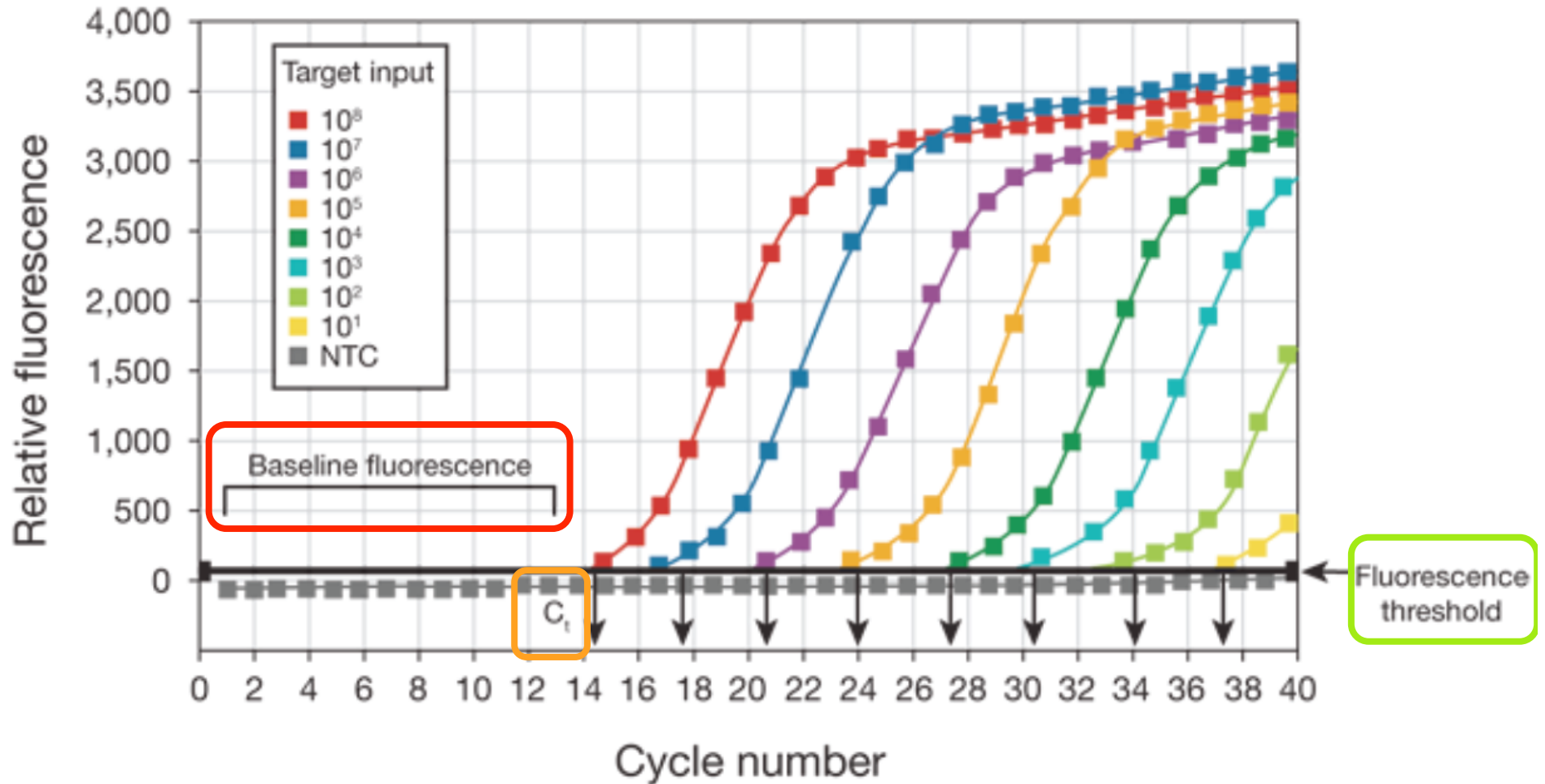


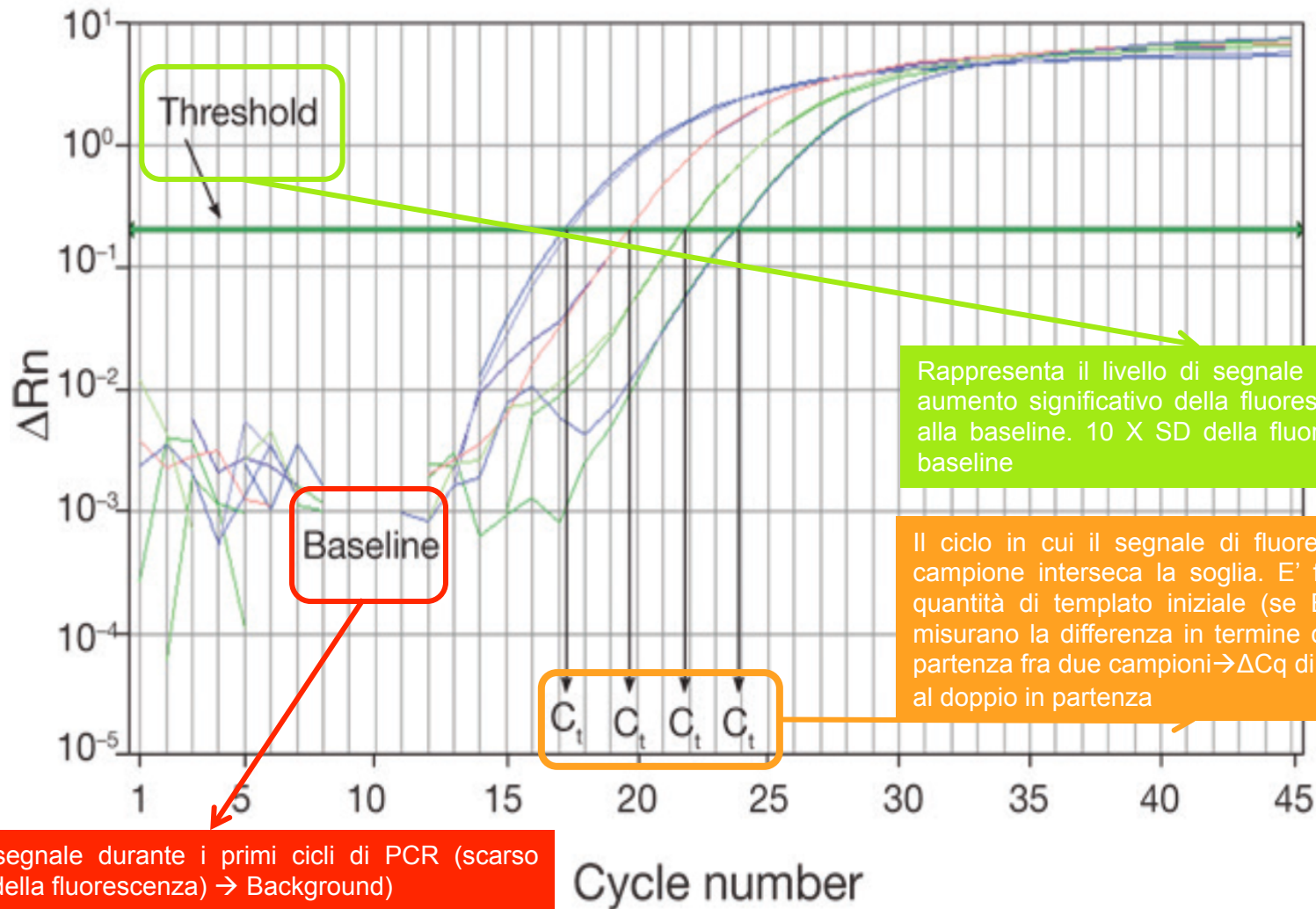
Figure 11. Phases of a PCR amplification curve. Blue: amplification curve of a positive sample. Red: negative control.

Principi: Amplification Plot

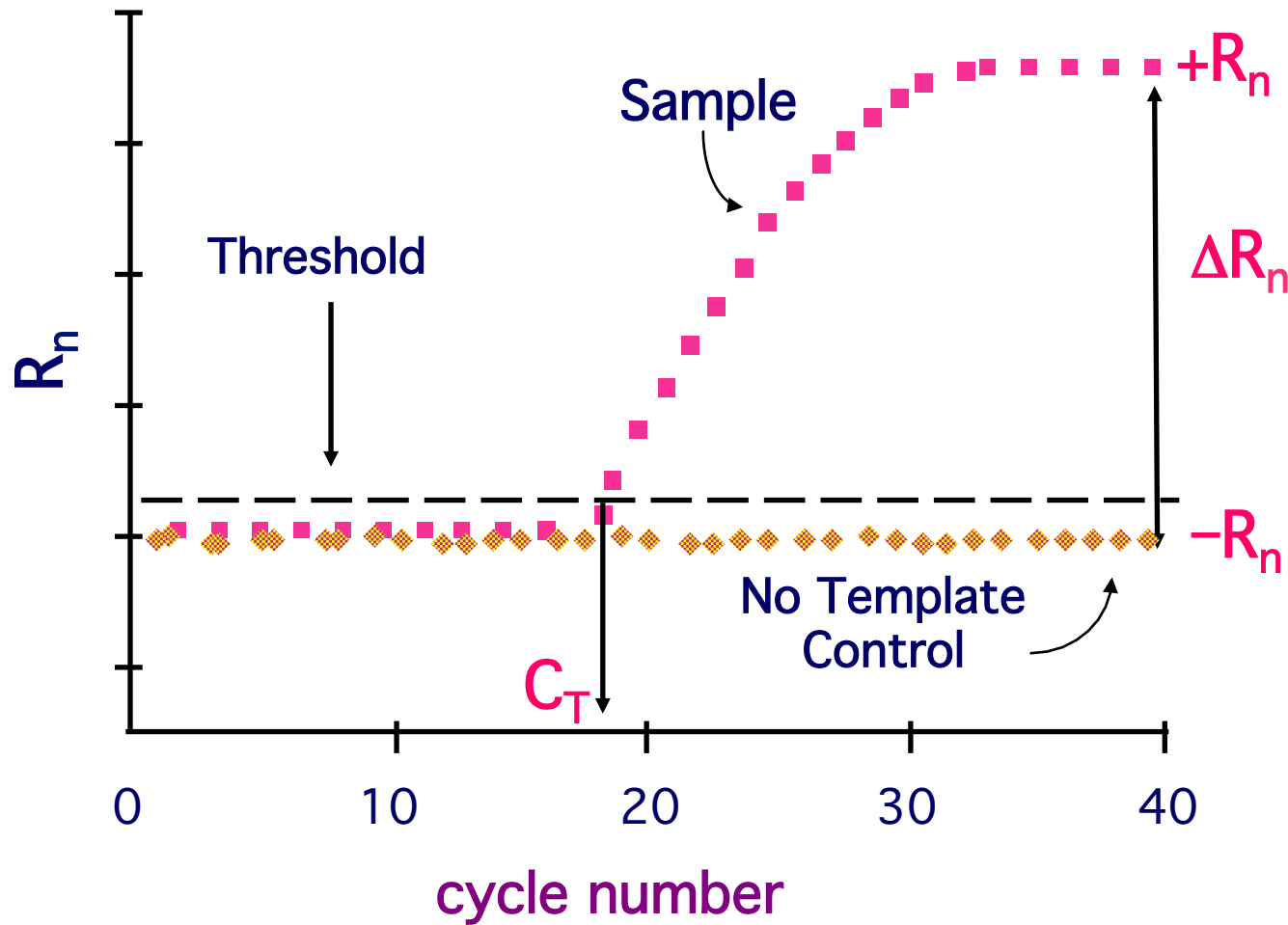


Un **amplification plot** descrive l'accumulo di prodotto durante l'intera durata di un esperimento di real-time PCR

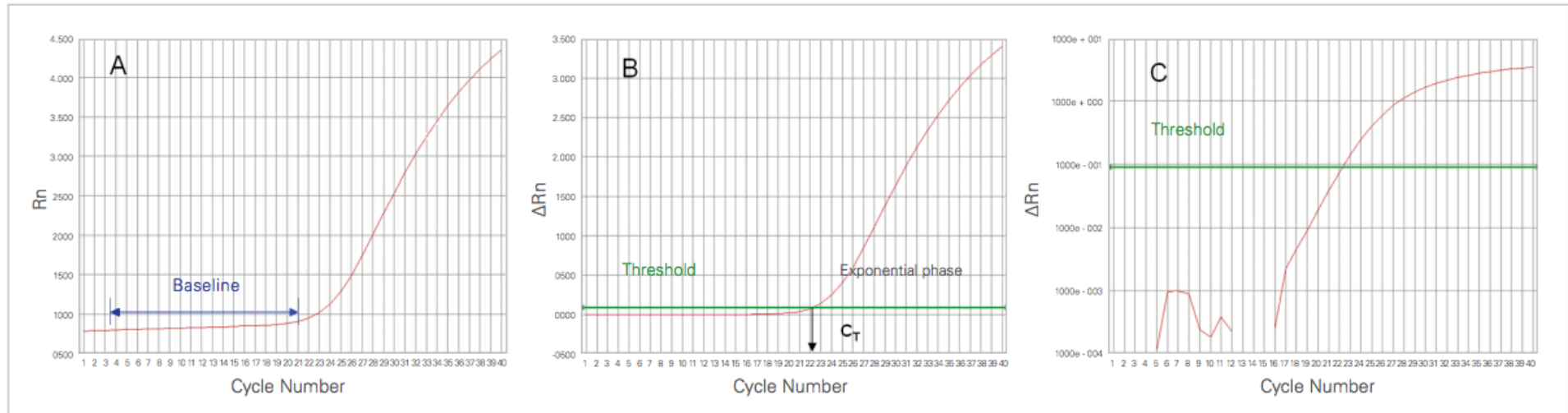
Baseline/Threshold/Threshold cycle



R_n = Normalized reporter \rightarrow The ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye
 ΔR_n = The magnitude of the signal generated by the specified set of PCR conditions.
($\Delta R_n = R_n - \text{baseline}$)



Modalità di rappresentazione dei dati



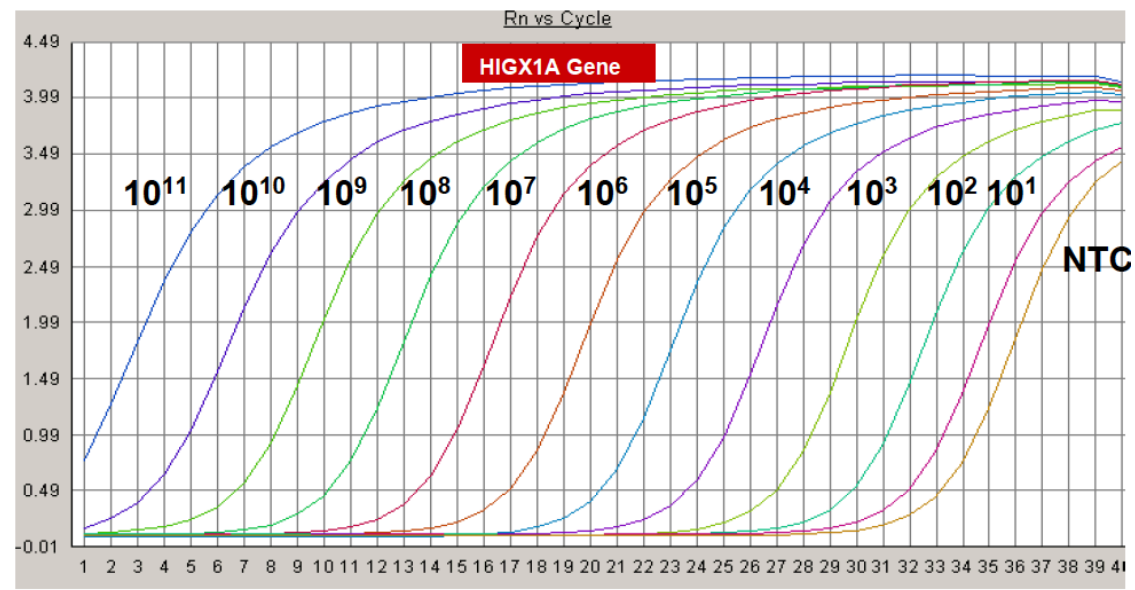
R_n is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. In other words, R_n is the reporter signal normalized to the fluorescence signal of ROX™. In this view, R_n is graphed versus cycle

ΔR_n is R_n minus the baseline, graphed here versus the cycle of PCR.

Amplification plot shows the $\text{Log}(\Delta R_n)$ graphed versus cycle.

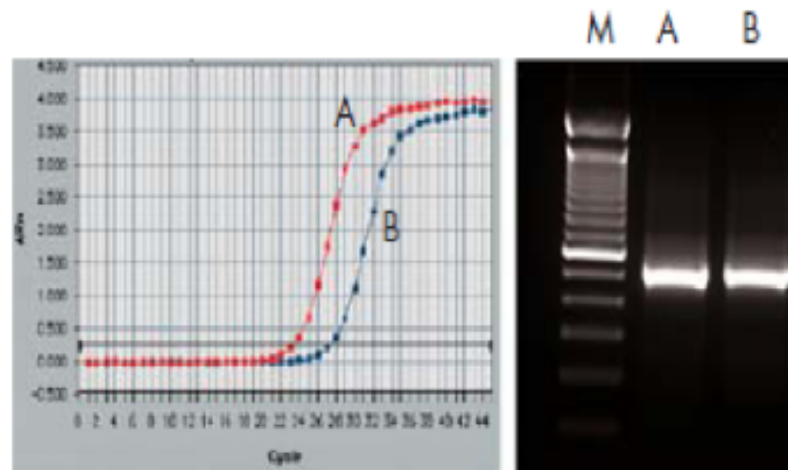
Principali differenze fra Real Time PCR e PCR convenzionale

- Possibilità di monitorare la reazione in corso
- È quantitativa
- Ha un aumentato **Dynamic Detection Range** → ampio range di applicazioni



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- È quantitativa
- Ha un aumentato **Dynamic Detection Range** → ampio range di applicazioni
- Ha una maggiore sensibilità → identifica a partire da differenze di 2 volte



Look similar

Principali differenze fra Real Time PCR e PCR convenzionale

- Possibilità di monitorare la reazione in corso
- È quantitativa
- Ha un aumentato **Dynamic Detection Range** → ampio range di applicazioni
- Ha una maggiore sensibilità → identifica a partire da differenze di 2 volte
- L'amplificazione e la detection avvengono in un unico tubo
 - Si elimina il post processamento (calcolo della melting);
 - Si aumenta il throughput (no gel);
 - Si riduce il rischio di “carry over contaminations” (anche UNG in RT-qPCR)

Componenti della reazione di real time PCR

- DNA polimerasi (enzima hot-start)
- dNTPs
- $MgCl_2$
- Passive reference dye (ROX™)
- Primer ⚠
- Detector fluorescente
- Templato (gDNA, cDNA,...)

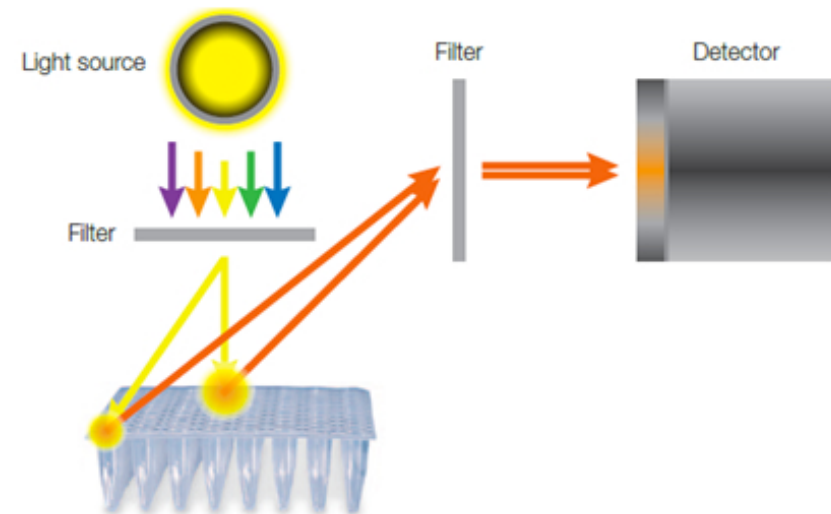
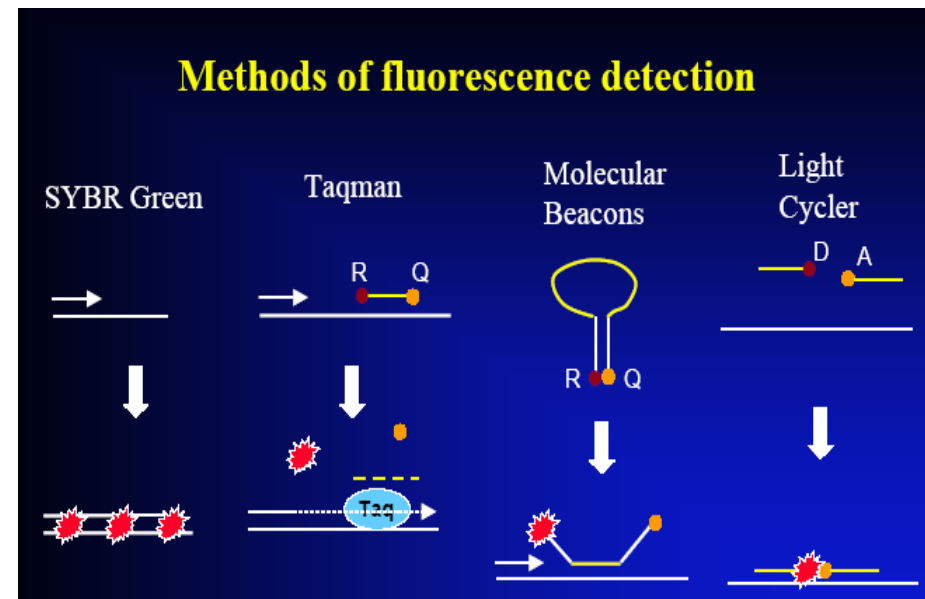


Table 1. Calculation of Rn for two wells at different positions. Fluorescence variation is corrected by normalizing the fluorescent reporter signal to the fluorescent signal of a passive reference dye. RFU, relative fluorescence units.

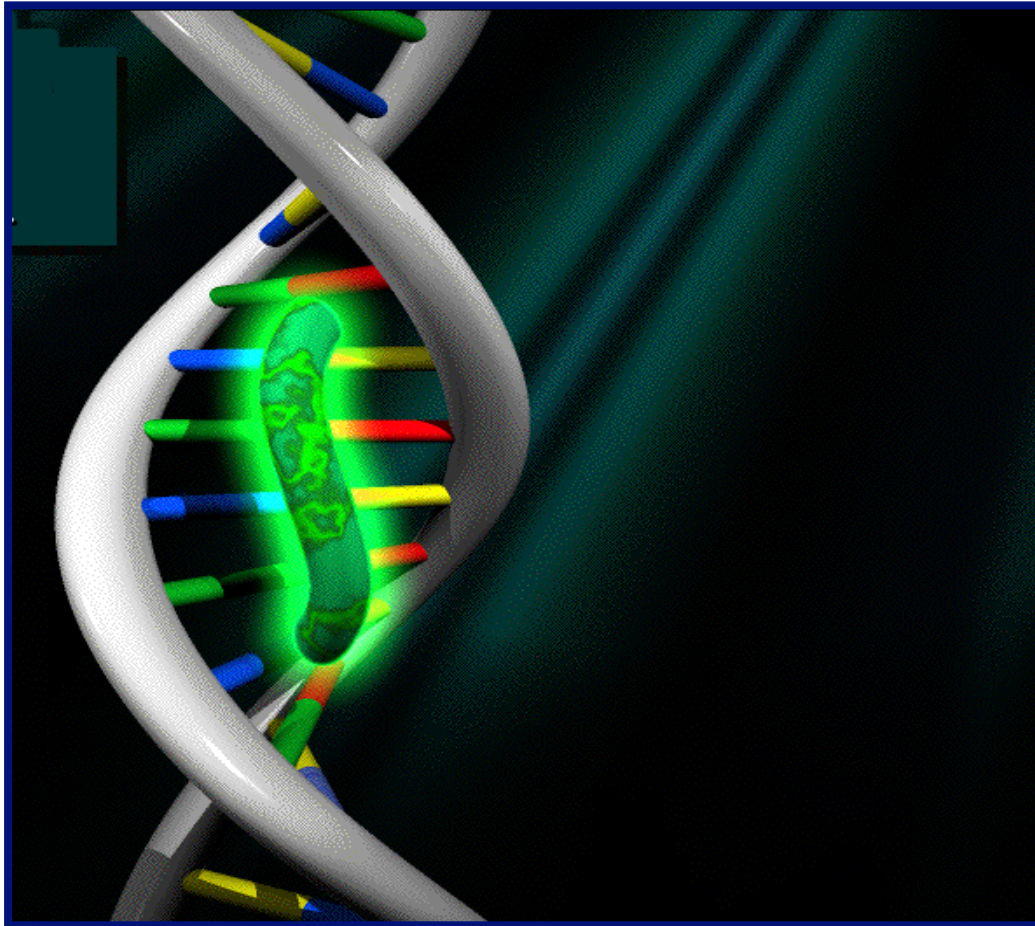
Fluorescence Intensity, RFU			
Well	Reporter	Passive Reference	Rn
Center	1,500	750	2
Perimeter	1,000	500	2

La chimica della Real Time PCR

- DNA binding dyes: SYBR green
- Fluorescent PCR Primer- and Probe-Based Chemistries:
 - Hydrolysis (**TaqMan**) probes o 5' nuclease assay
 - **Molecular beacons**
 - Dual hybridization probes
 - Eclipse probes
 - Amplifluor assays
 - **Scorpions PCR primers**
 - LUX PCR primers
 - QZyme PCR primers

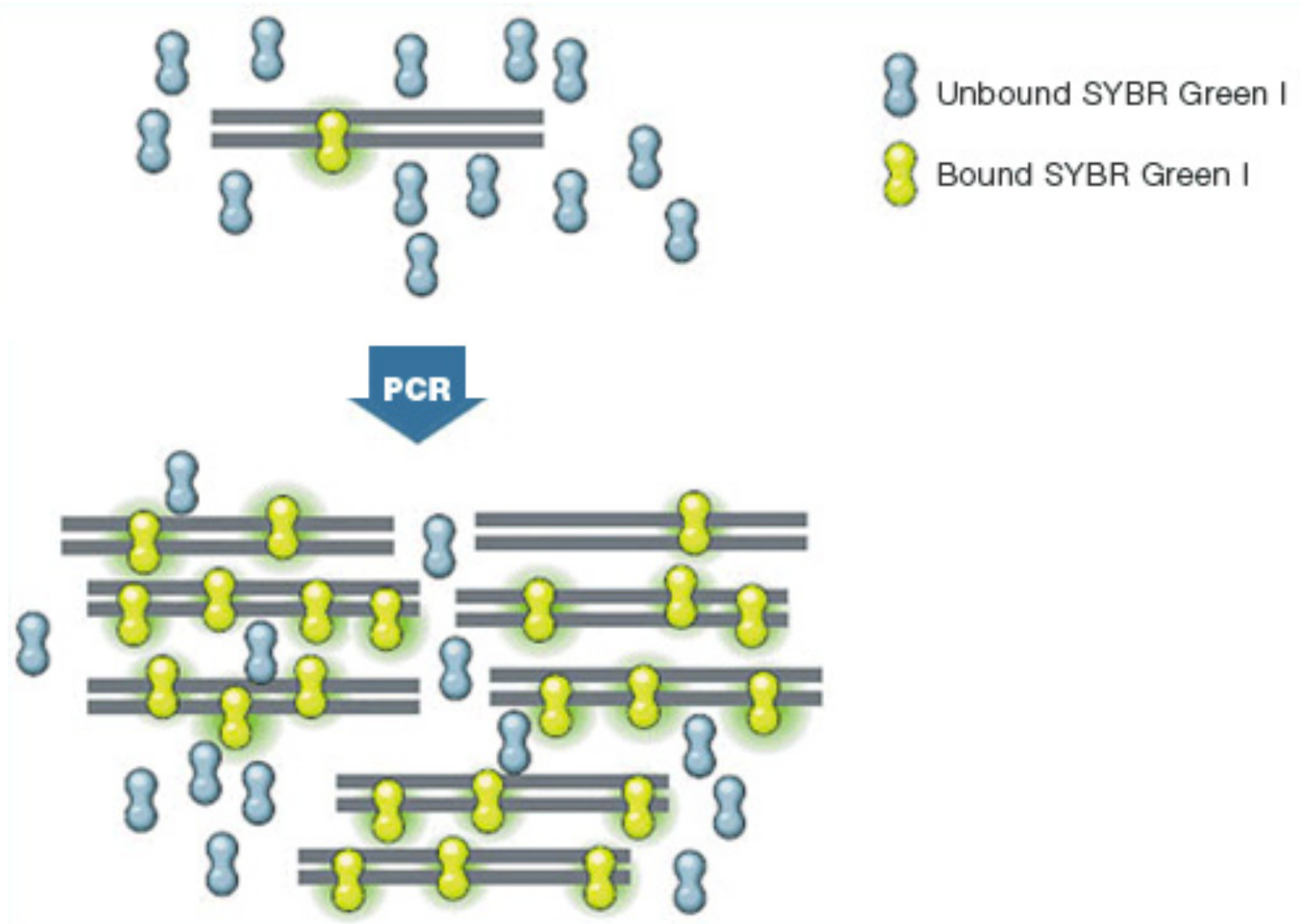


SYBR green

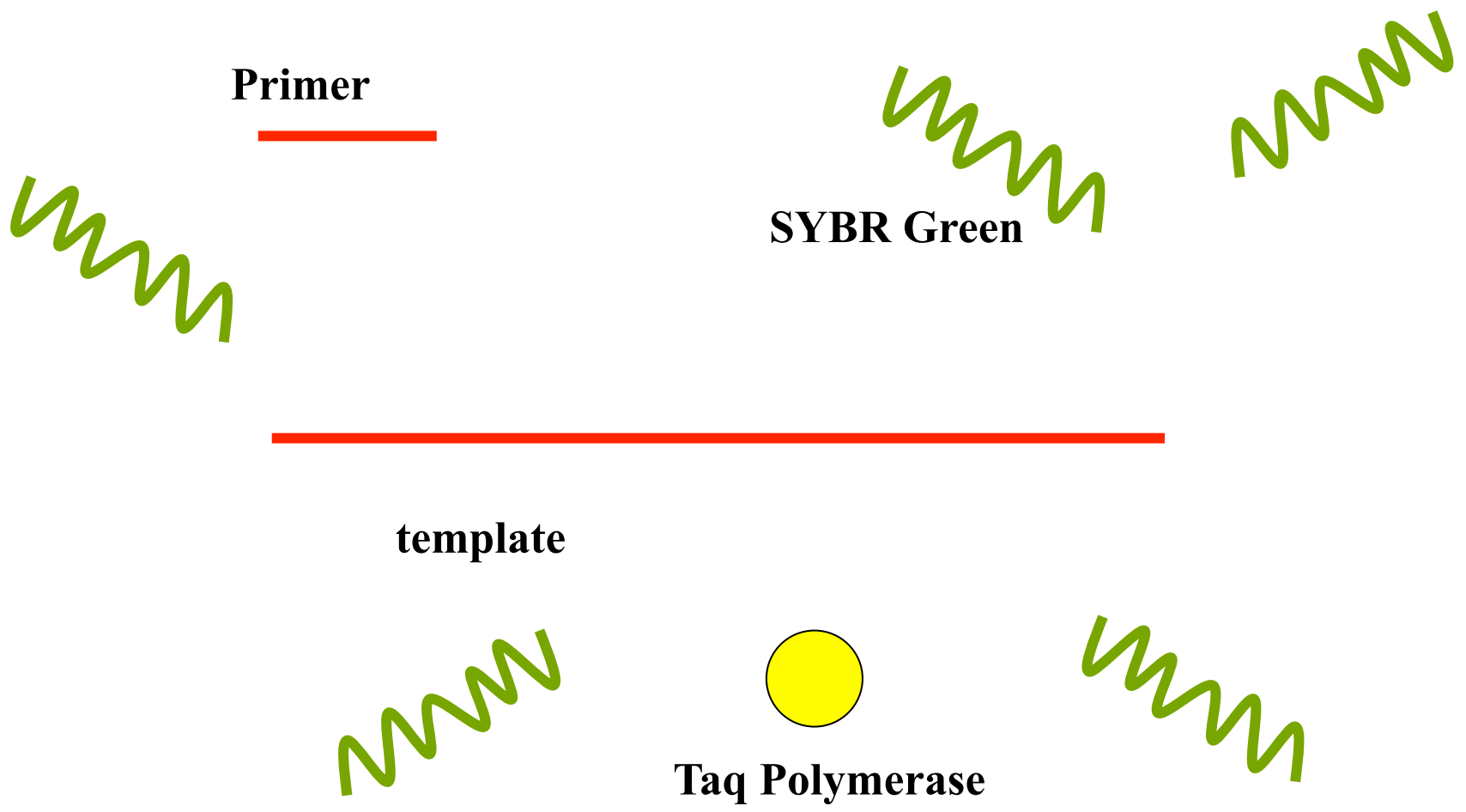


Molecola
fluorescente **non**
specifica che si lega
al solco minore del
DNA a doppio
filamento

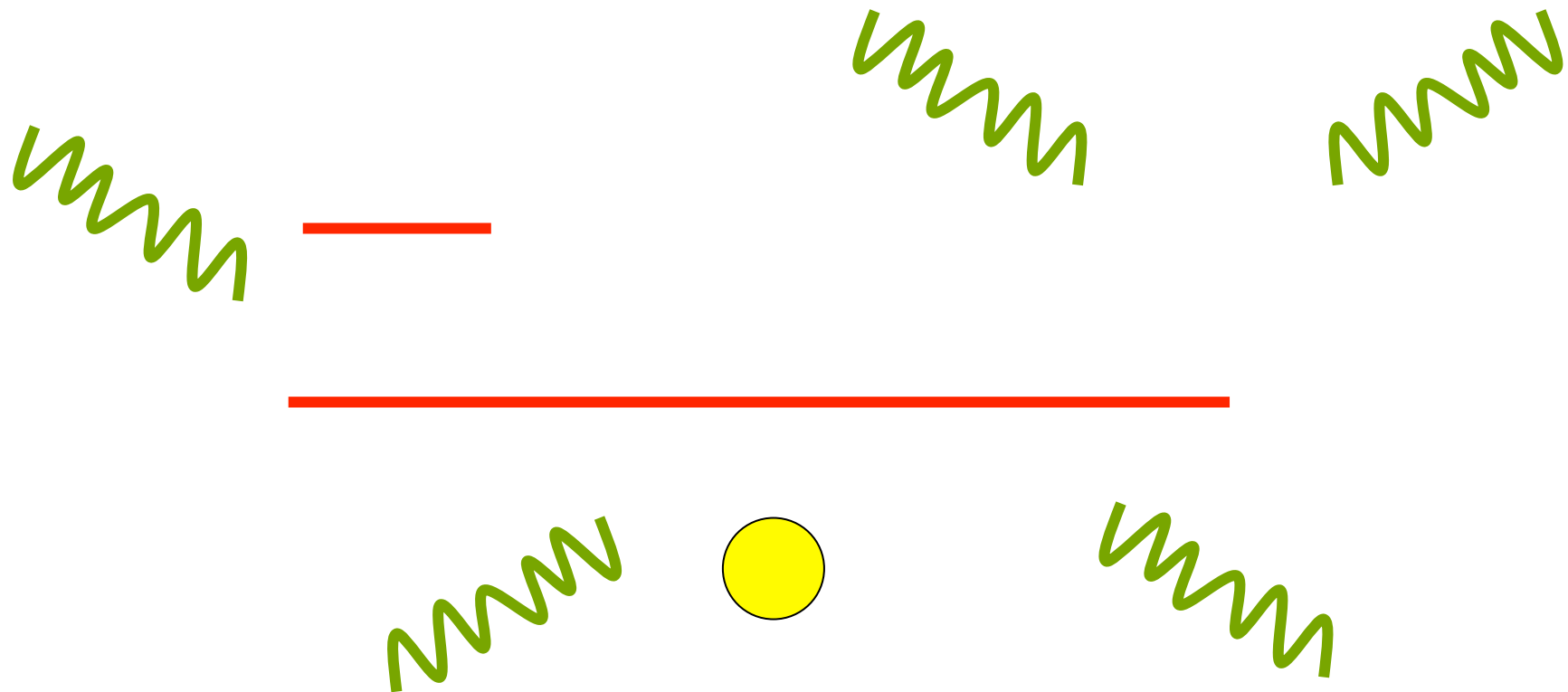
SYBR green



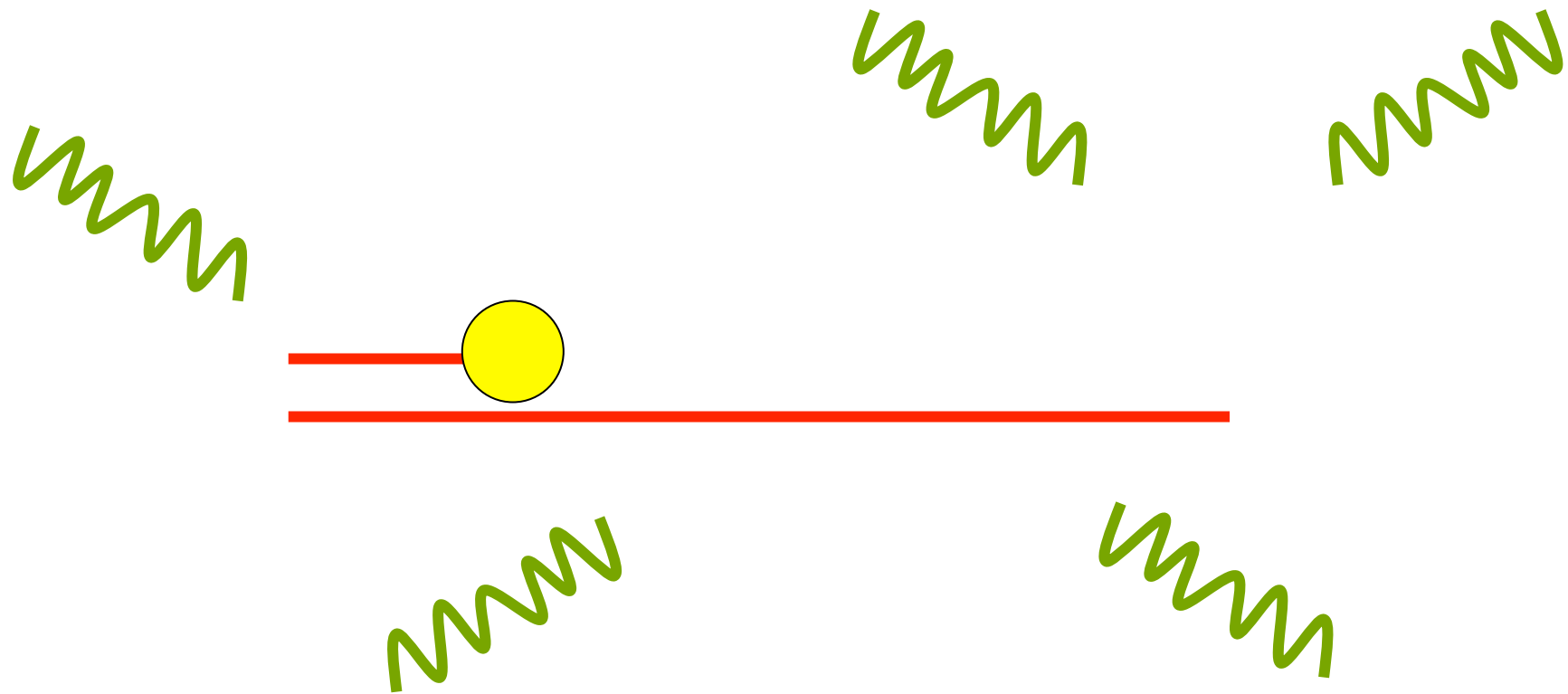
PCR Real-Time
SYBR Green detection



PCR Real-Time
SYBR Green detection

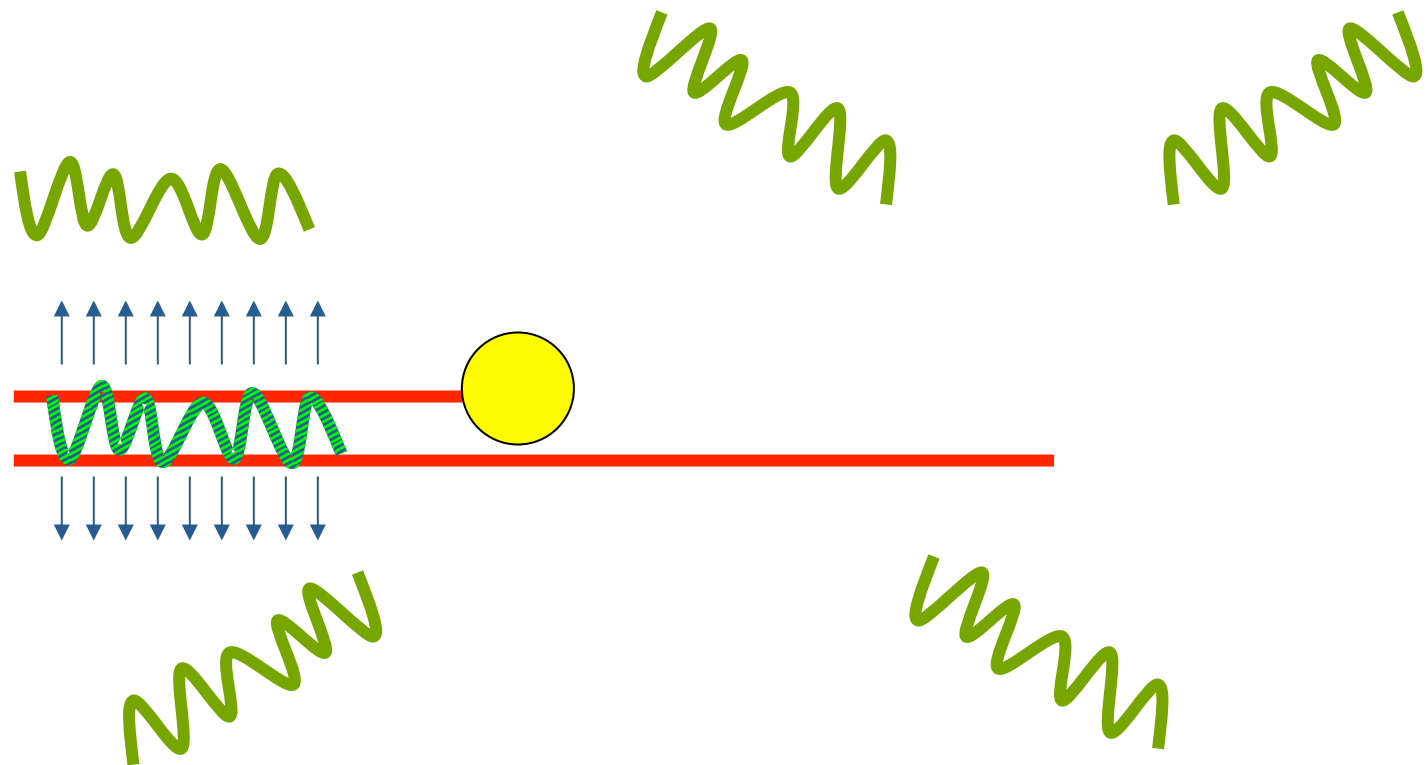


PCR Real-Time
SYBR Green detection

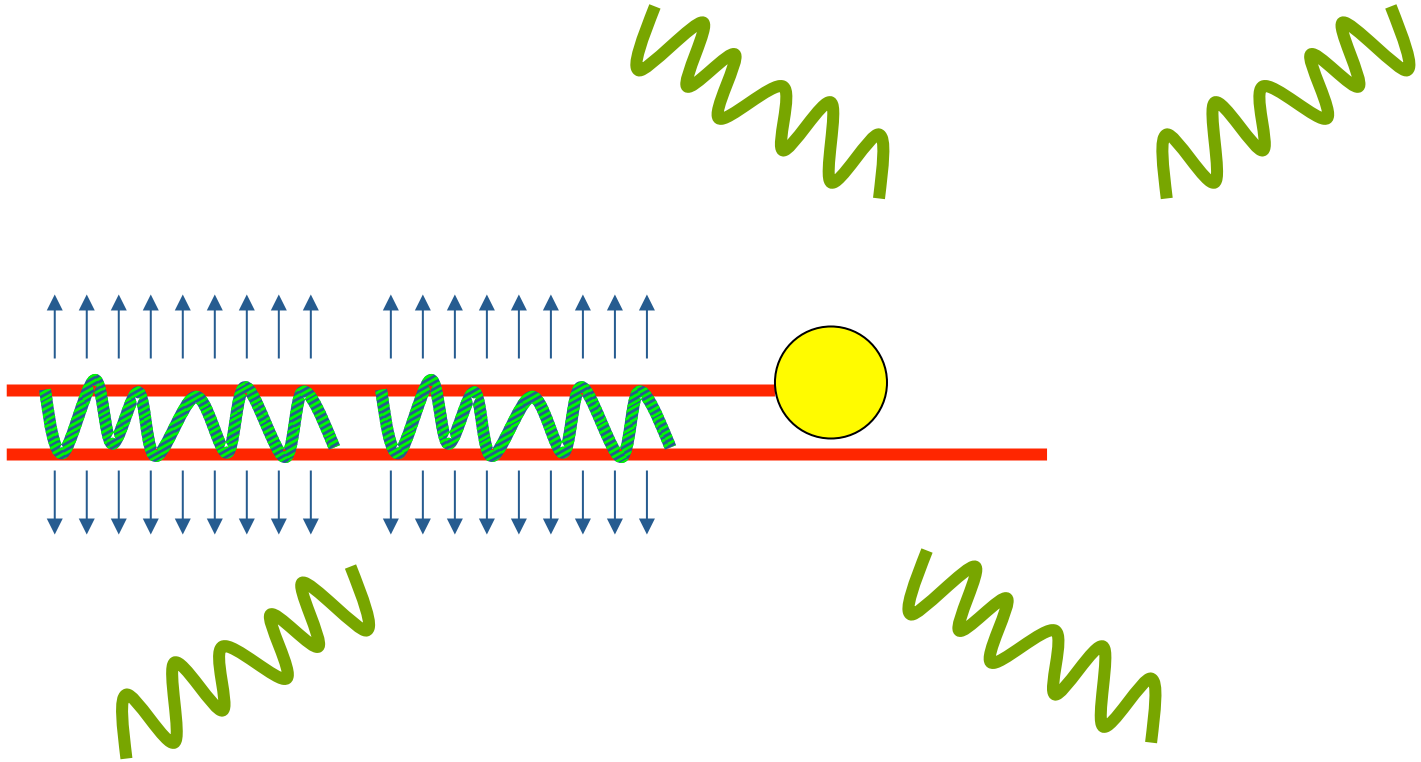


PCR Real-Time

SYBR Green detection

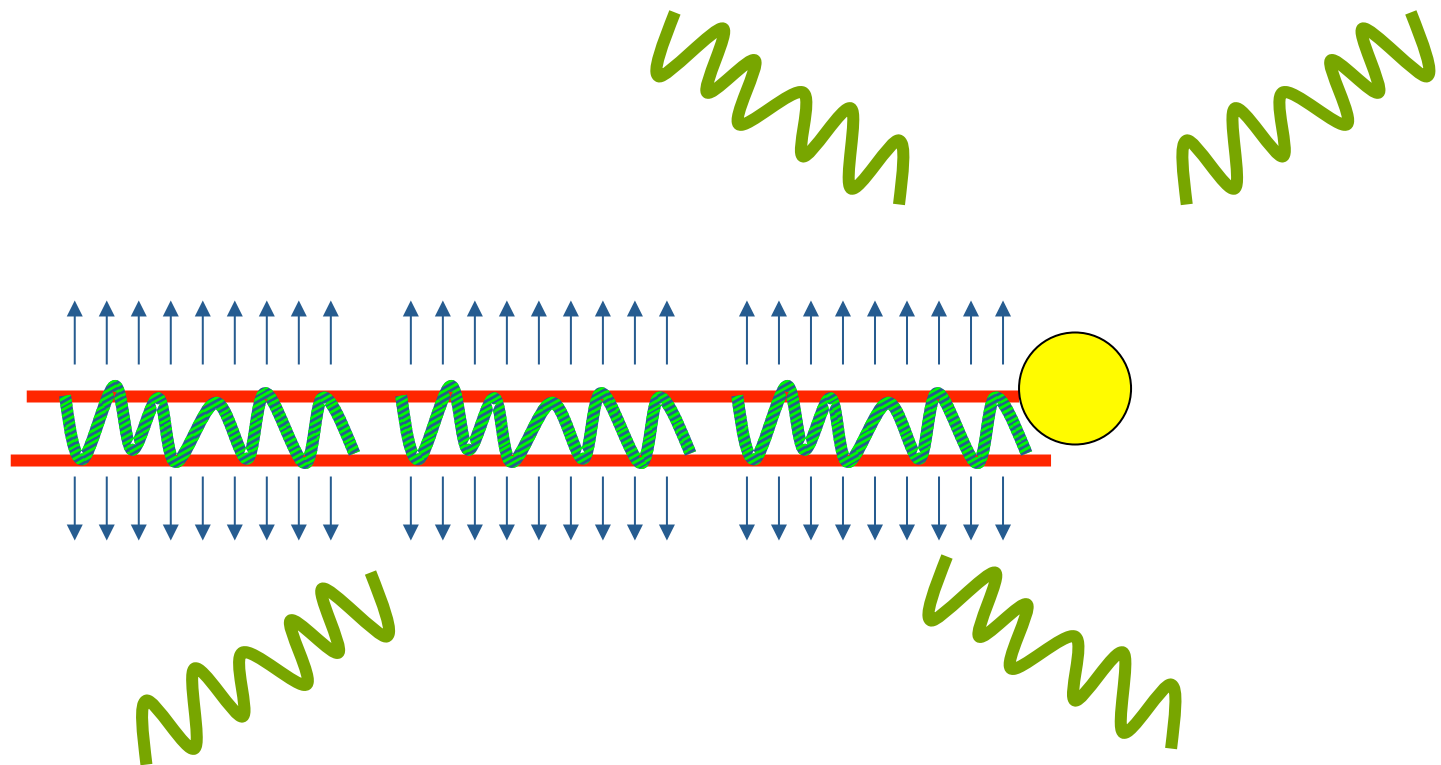


PCR Real-Time
SYBR Green detection

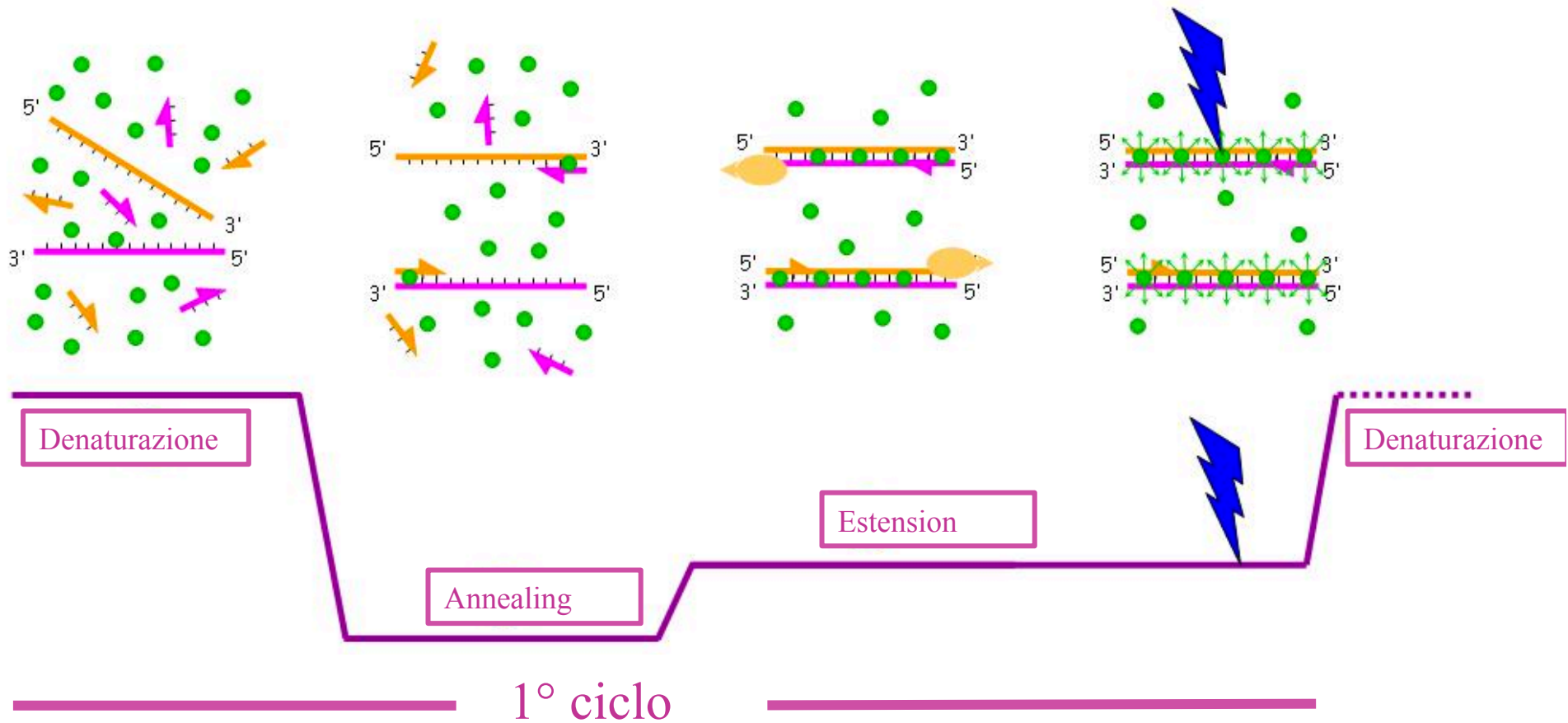


PCR Real-Time

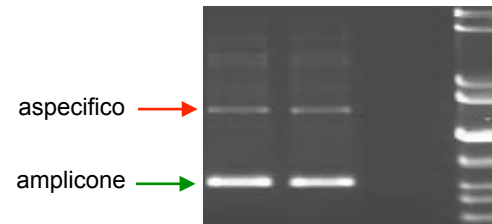
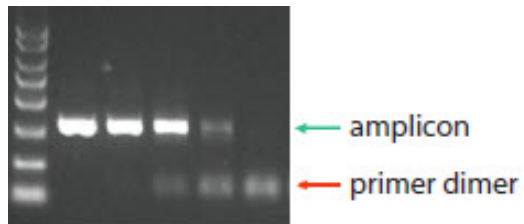
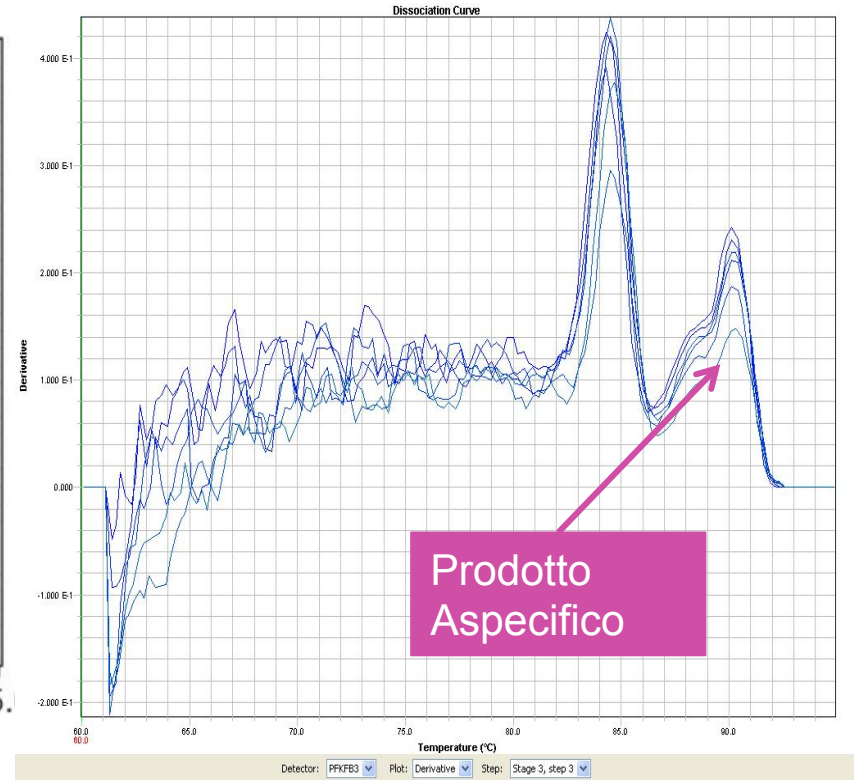
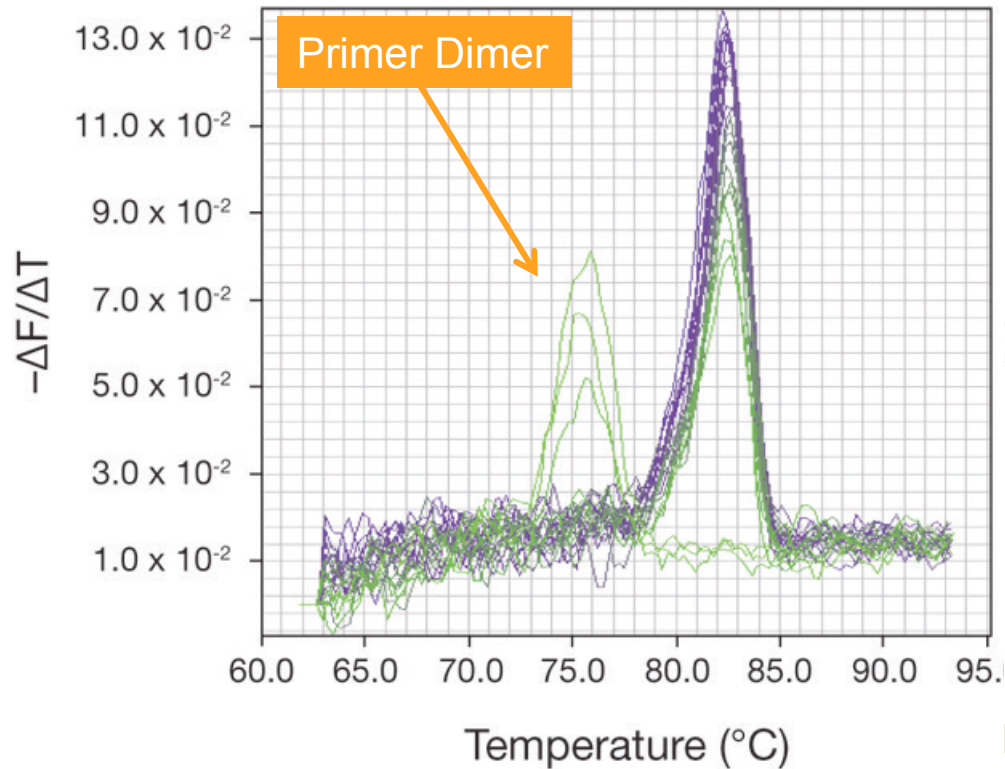
SYBR Green detection



SYBR green

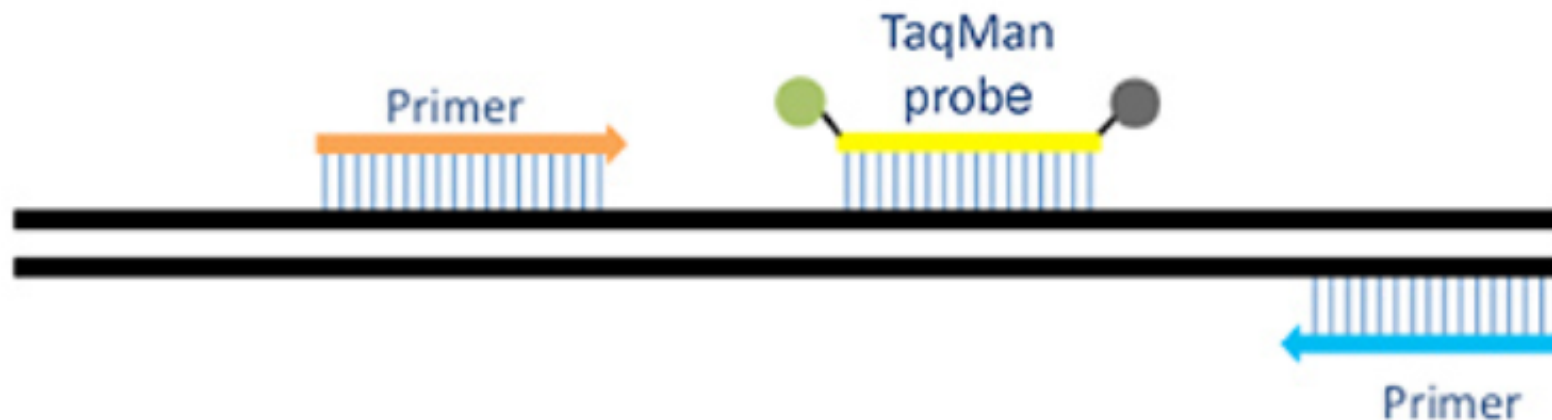


Melting Curve Analysis: misura di specificità



PCR Real Time con sonde Taqman

La sonda di tipo TaqMan è un oligonucleotide che, come i primers della PCR, viene disegnato per essere complementare alla sequenza bersaglio da amplificare

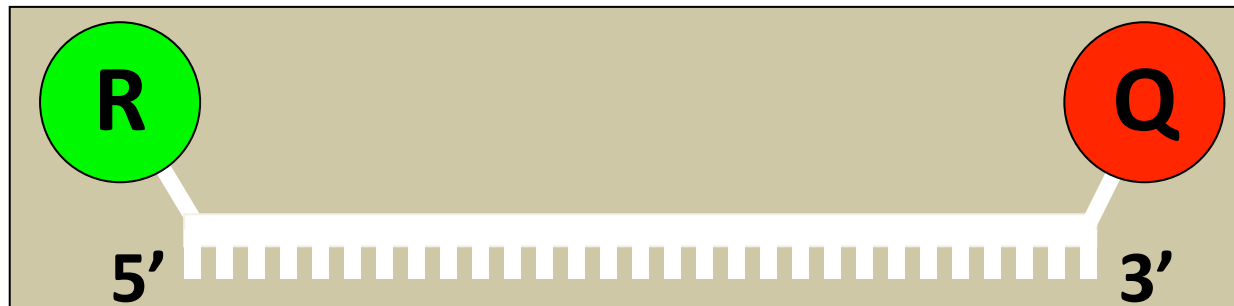


La sonda è disegnata in modo da ibridarsi all'interno del frammento amplificato nella reazione di PCR

Rilevazione del prodotto di PCR mediante saggio Taqman

5' REPORTER (R): fluorocromo ad alta energia che emette fluorescenza

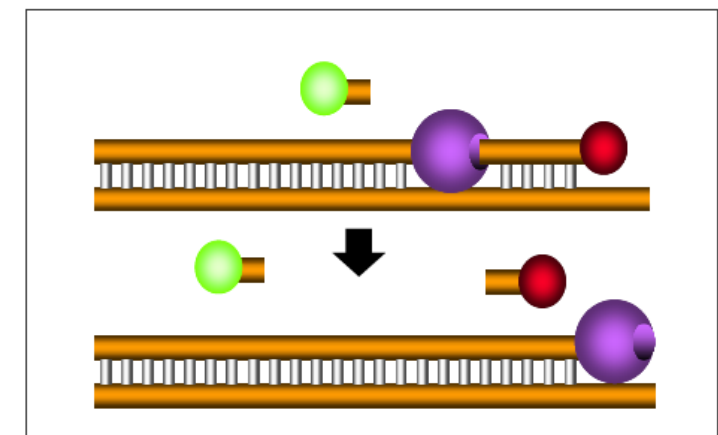
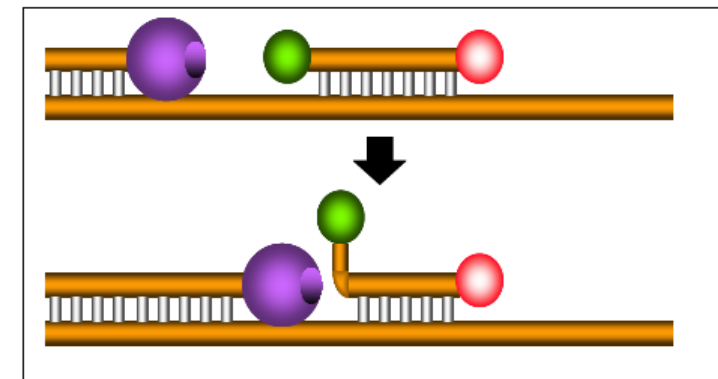
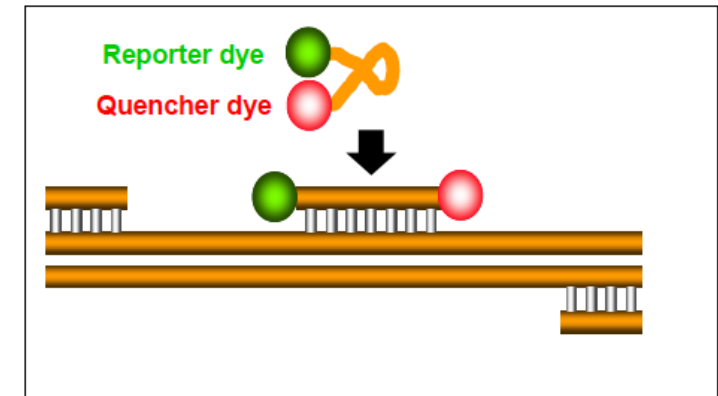
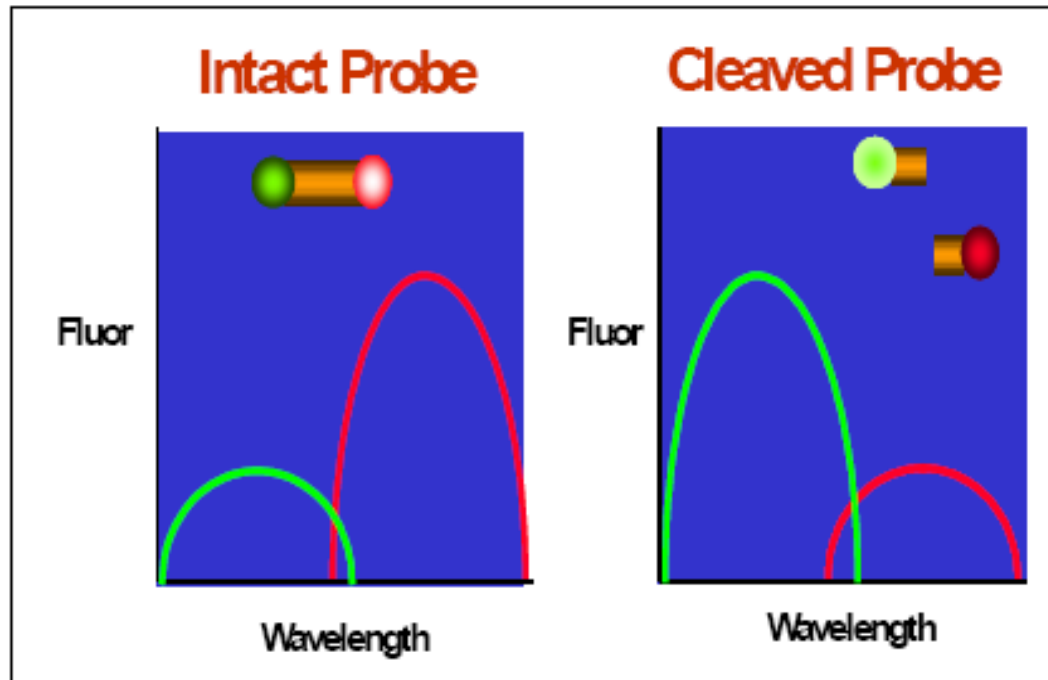
3' QUENCHER (Q): fluorocromo a bassa energia che maschera la fluorescenza del reporter



Se R e Q si trovano vicini, Q spegne l'effetto di R perchè i fotoni di R vengono assorbiti da Q

FRET= Fluorescent (o Förster) Resonance Energy Transfer

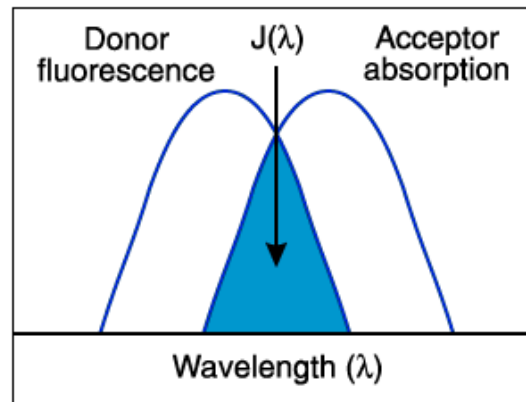
Fenomeno di trasferimento energetico tra fluorofori: Quando un fluorocromo ad alta energia è in prossimità di uno a bassa energia vi è trasferimento dal primo al secondo.



FRET: fluorescence resonance energy transfer

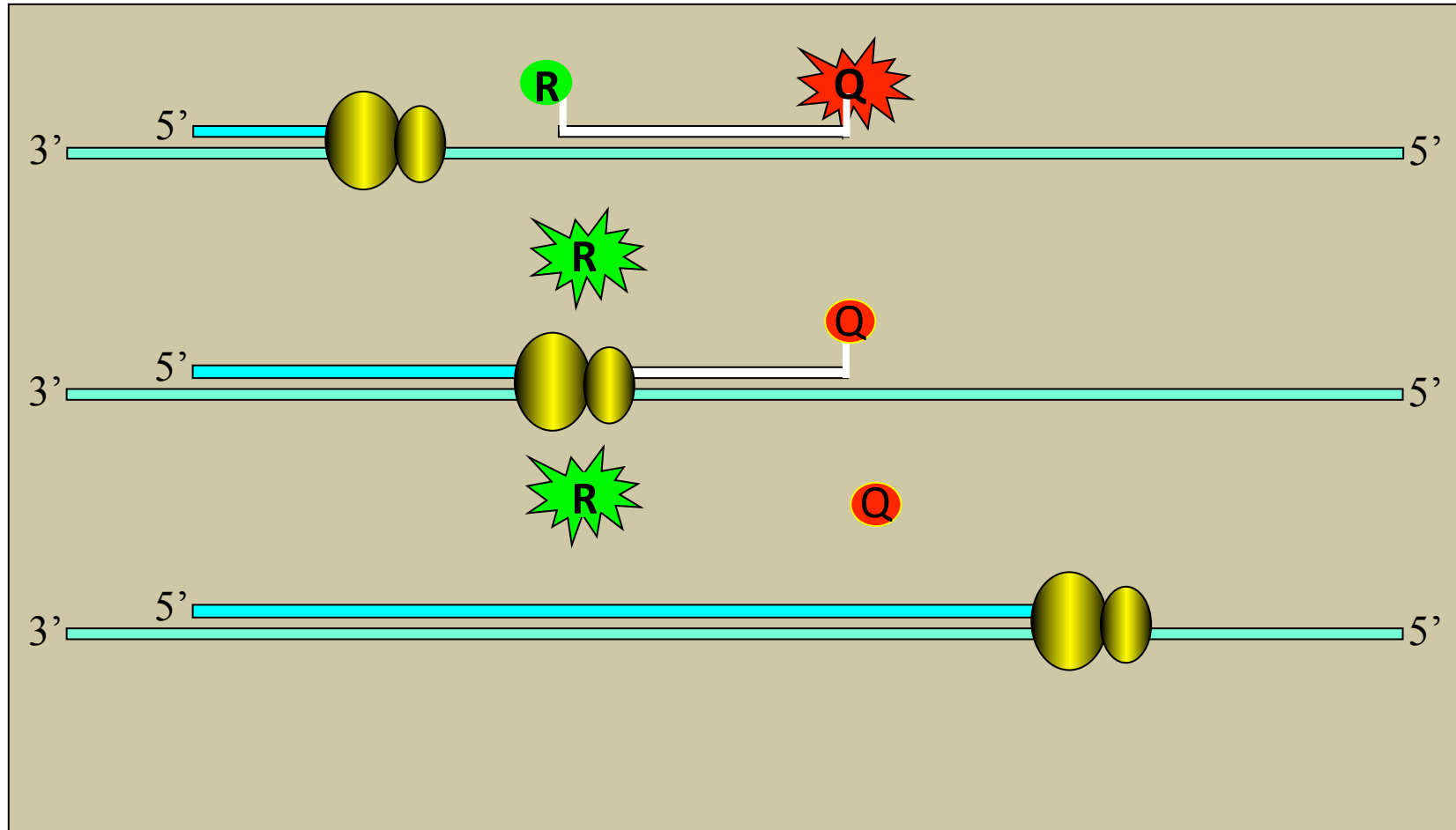
Condizioni essenziali per la FRET

- 1) l'energia persa per il ritorno allo stato basale della molecola donatrice deve essere uguale all'energia richiesta per eccitare l'accettore. In altre parole **lo spettro di assorbimento della molecola accettrice deve sovrapporsi a quello della molecola donatrice.**

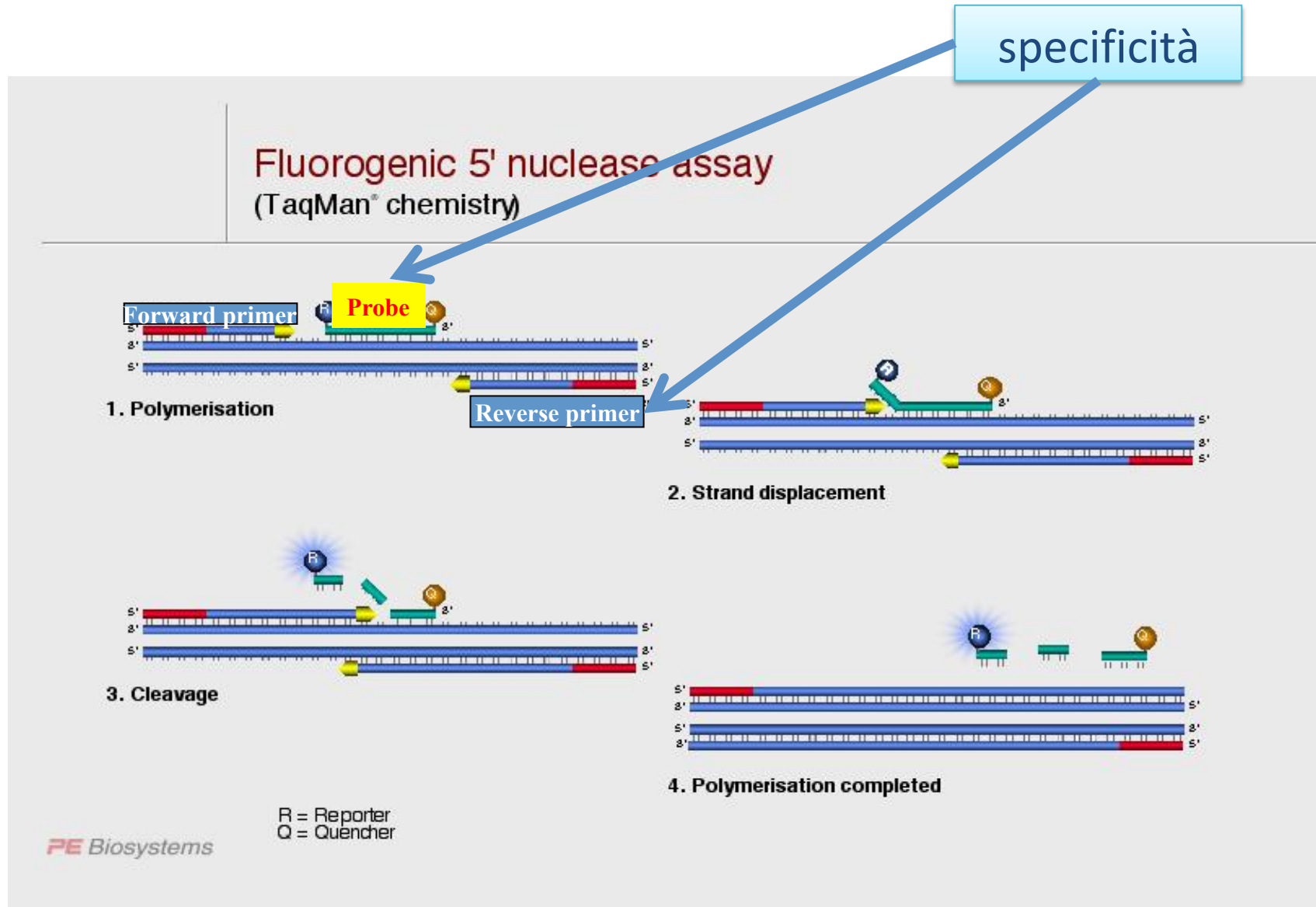


- 2) Donatore and accettore devono essere molto vicini (tipicamente 10-100 Å). La FRET è un **trasferimento di energia distanza-dipendente** tra stati elettronici eccitati di due fluorocromi. La distanza alla quale il trasferimento di energia è efficiente al 50% (il 50% delle molecole donatrici attivate sono deattivate dalla FRET) viene definita R_0 (Förster radius).

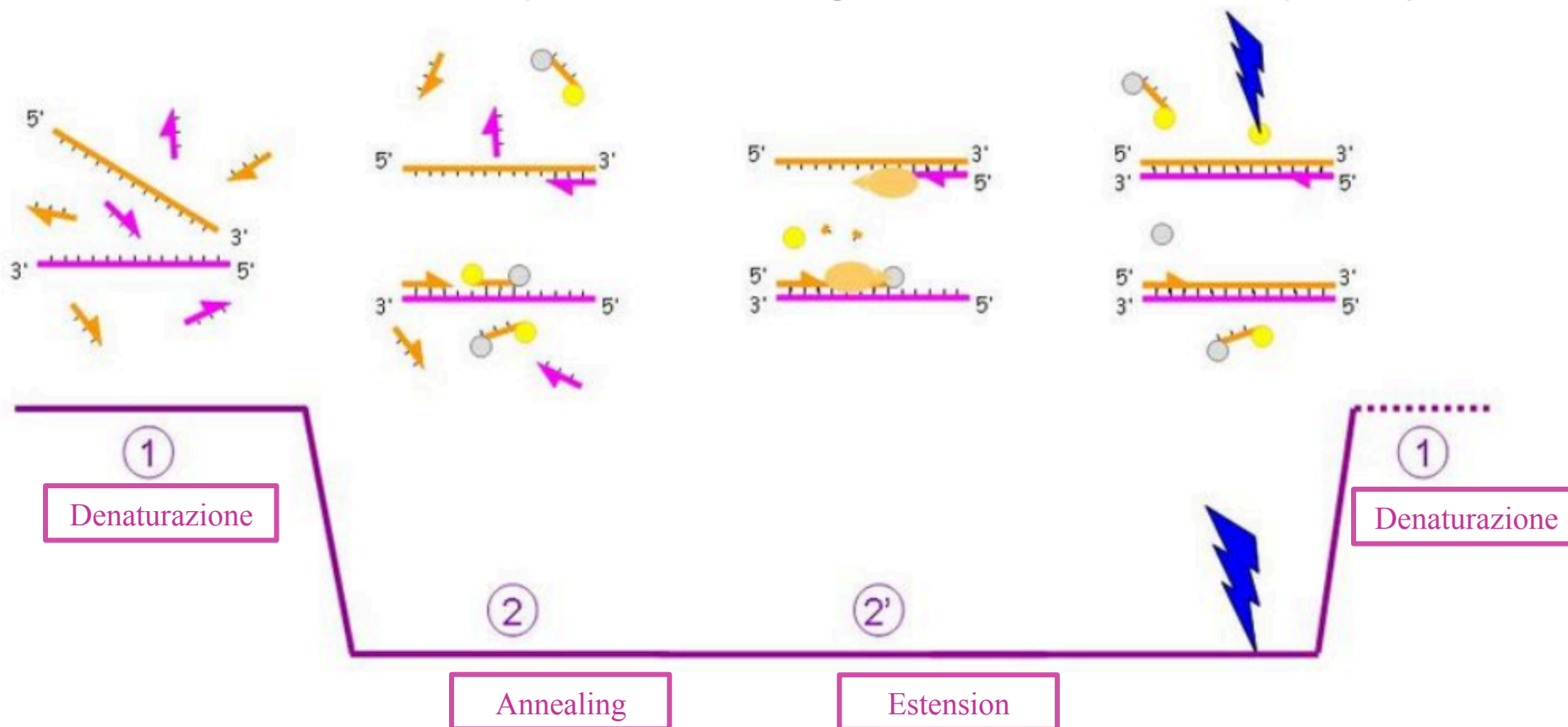
Real-Time PCR: attività 5'>3' esonucleasica



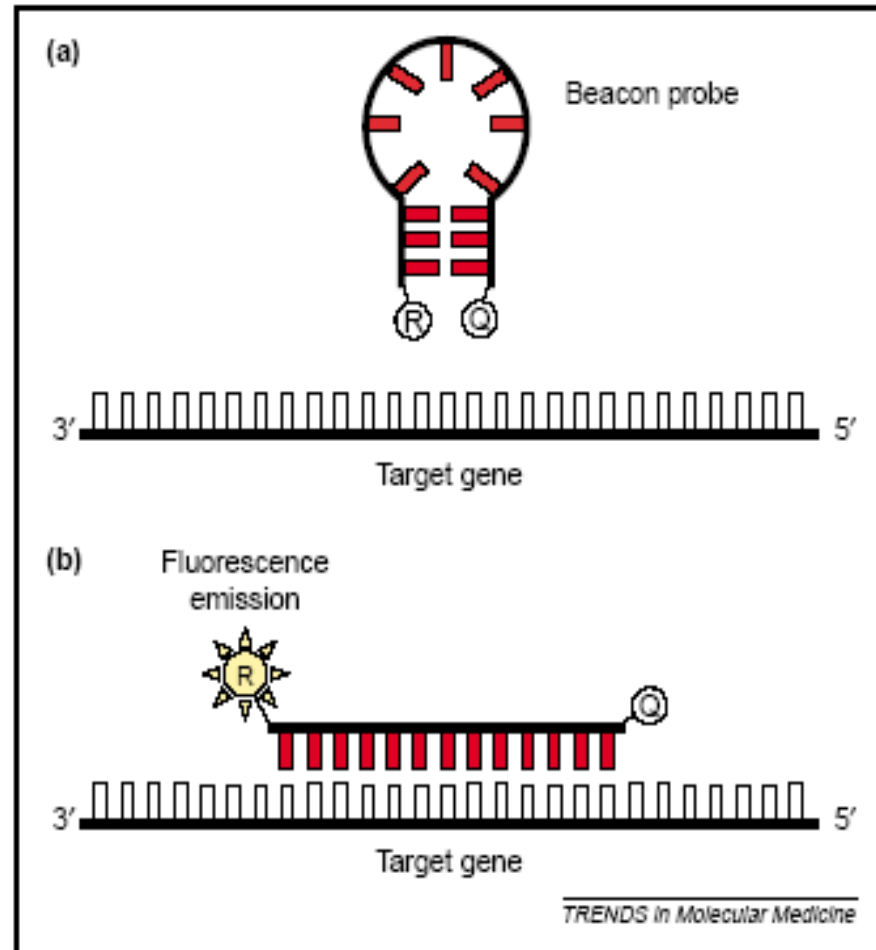
L'aumento di fluorescenza del Reporter è direttamente proporzionale al numero di ampliconi generati



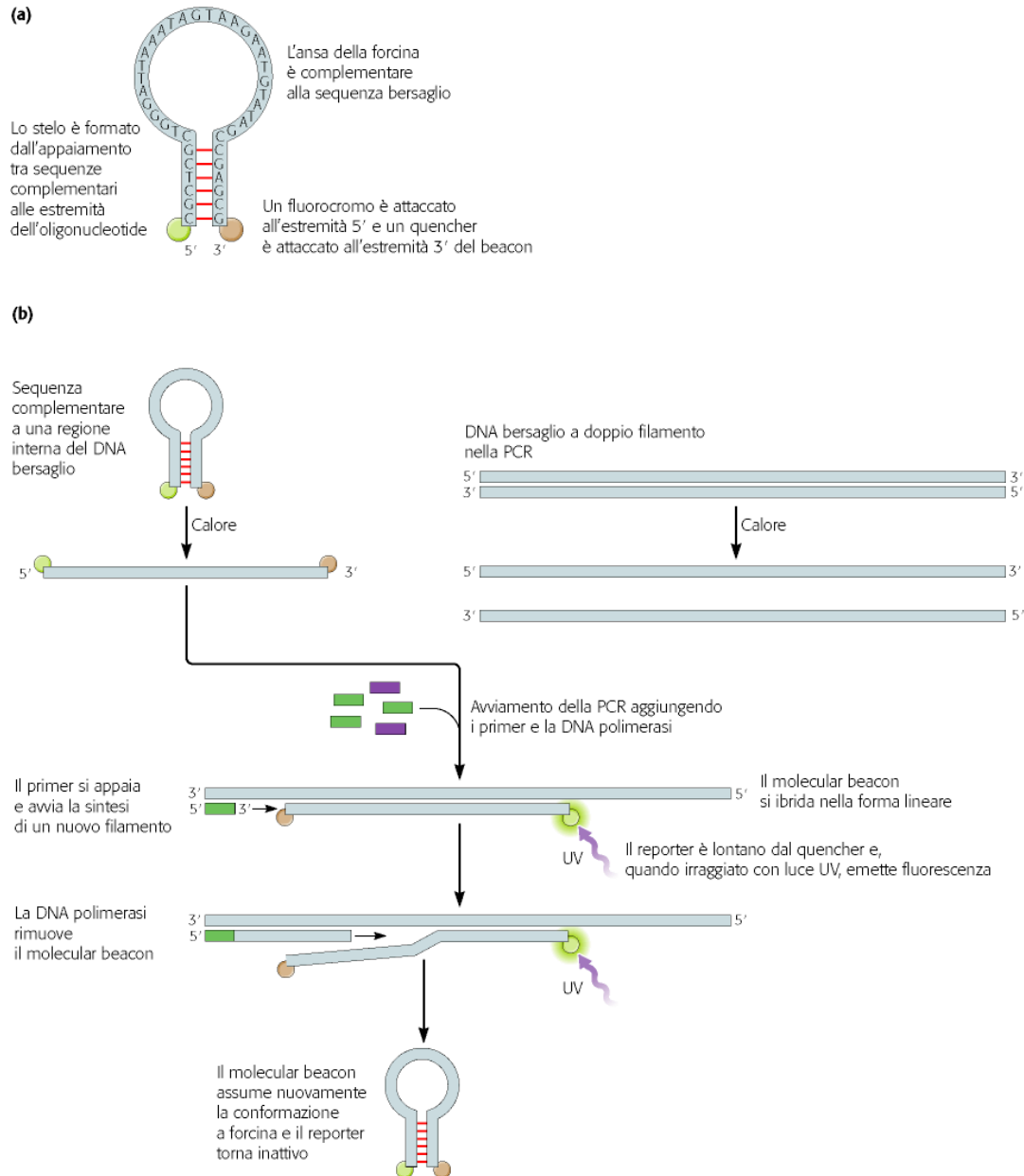
Sonde TaqMan



Molecular Beacons

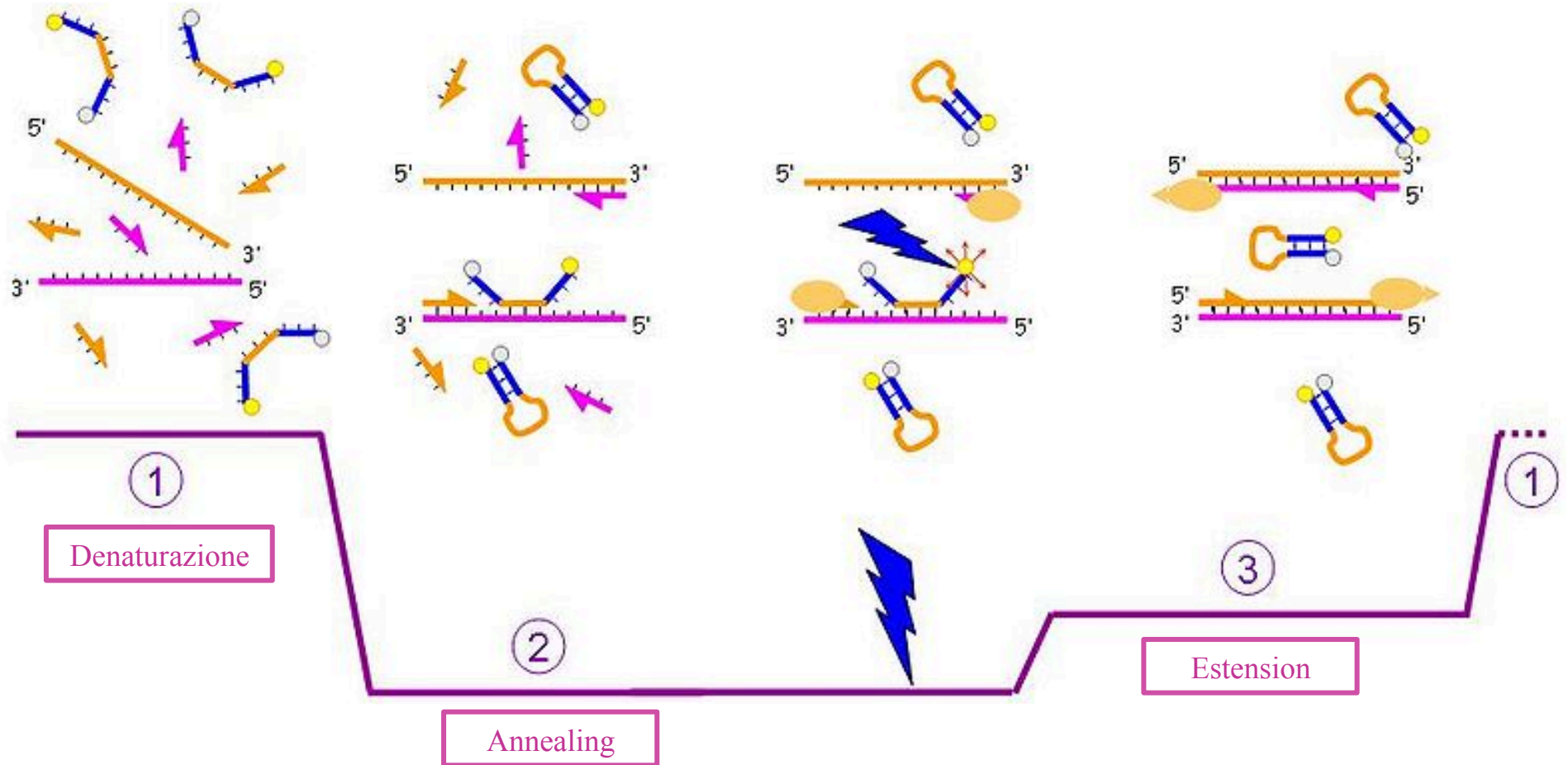


Molecular Beacons

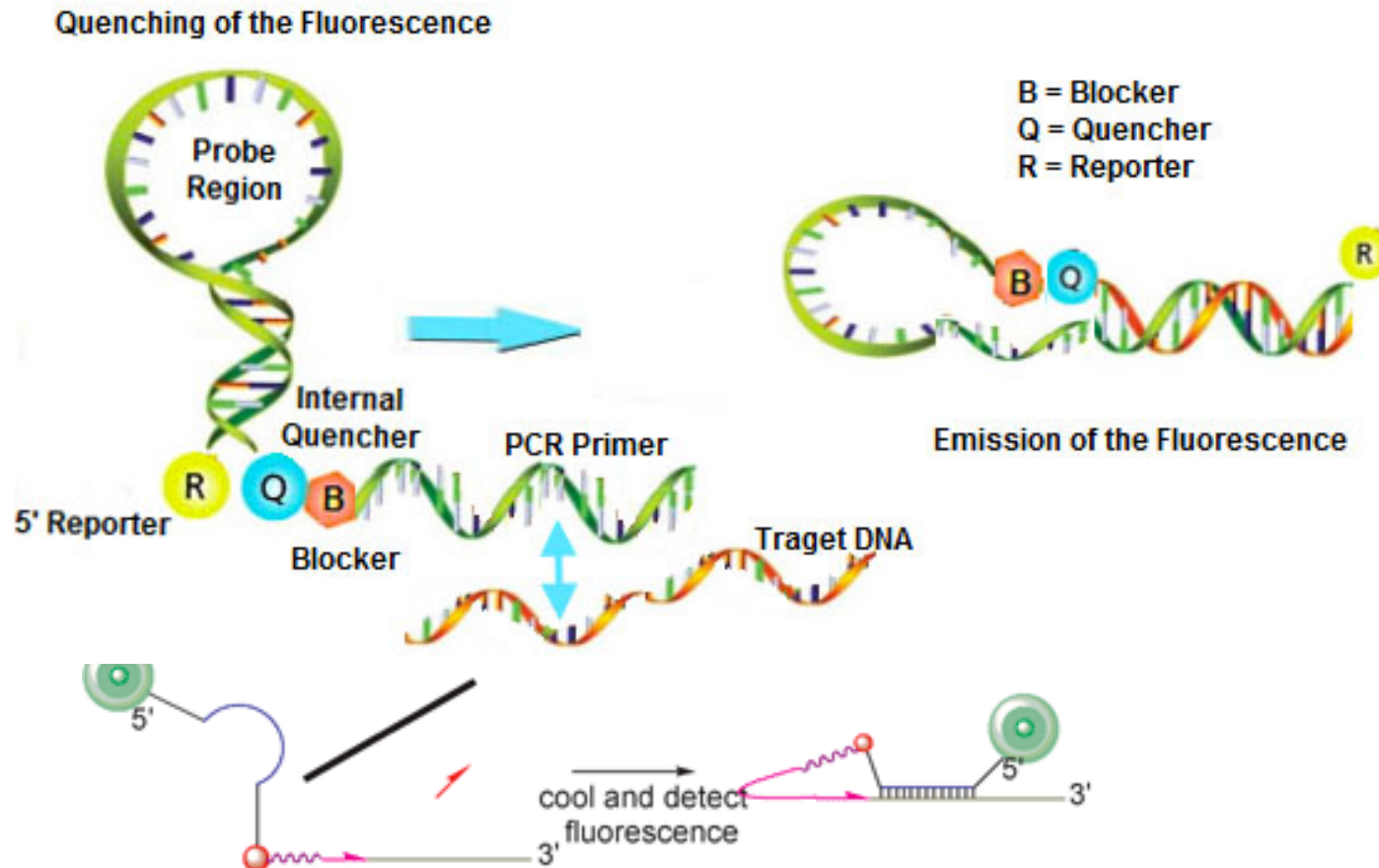


- La sonda è studiata in modo che, formando un uncino, il fluoroforo e il quencher siano vicini.
- In questa configurazione, quando la sonda viene illuminata, l'energia emessa dal fluoroforo viene assorbita dal quencher.
- Con l'appaiamento della sonda alla sequenza di DNA, fluoroforo e quencher si allontanano. L'emissione del reporter quando eccitato può essere rilevata.
- I valori di fluorescenza durante la reazione sono una misura del numero di molecole amplificate.

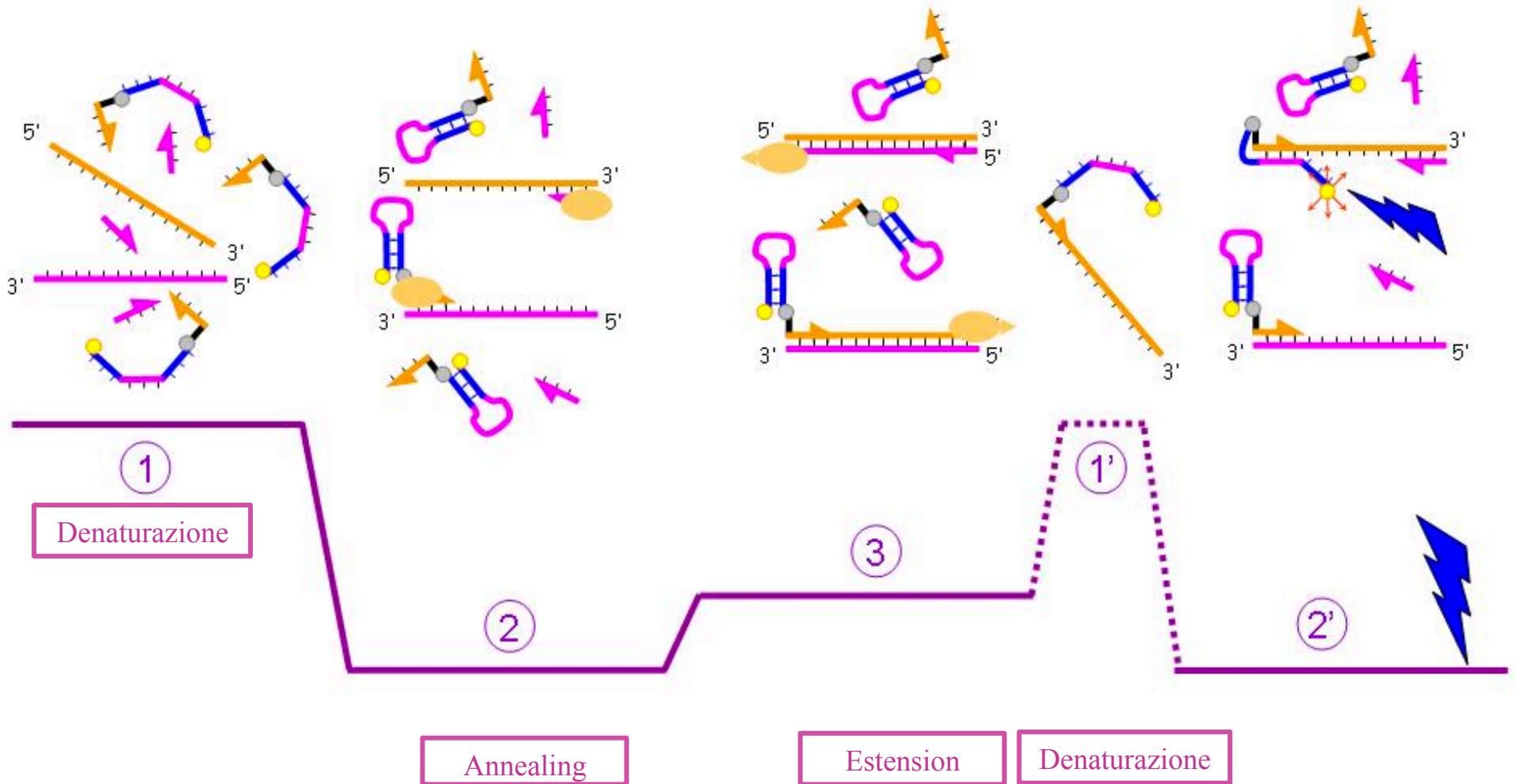
Molecular Beacons



Sonde Scorpions



Sonda Scorpions



SYBR GREEN

PRO:

- Metodica semplice
- Versatilità
- Non costosa

CONTRO:

- 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
- Richiede la costruzione di una retta standard e l'analisi della curva di melting

Sonde

PRO:

- Metodica semplice
- Elevata specificità
- Calcolo attraverso $\Delta\Delta Ct$
- Maggiore facilità nel Multiplexing

CONTRO:

- Costosa
- Non versatile

Cicli di amplificazione

Thermal Cycler Protocol

Mode: Fast Standard 9600 Emulation

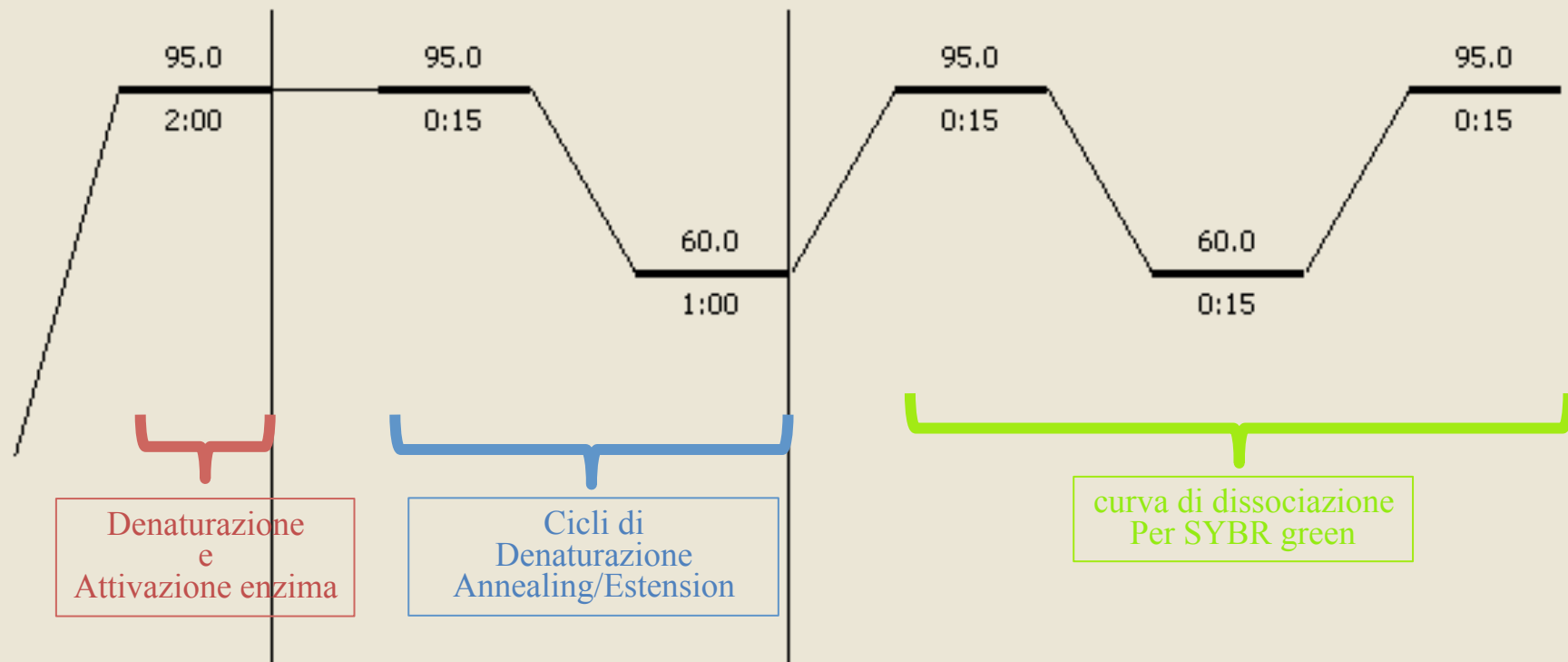
Thermal Profile

Stage 1

Stage 2

Stage 3

Repeats: 40





The image shows a Real Time PCR (RT-PCR) fluorescence plot. The y-axis is labeled 'Fluorescence (dR)' and ranges from -10000 to 40000. The x-axis is labeled 'Cycles' and ranges from 2 to 36. The plot displays multiple colored lines with various markers (triangles, squares, circles, diamonds, etc.) representing different experimental runs. All lines show a similar pattern: a baseline around 0 dR until approximately cycle 10, followed by a sharp increase in fluorescence, peaking between cycles 24 and 28 at approximately 40000 dR, and then a sharp decline back towards 0 dR by cycle 30. The plot is overlaid with a grid of dotted lines.

Real Time PCR

La messa a punto degli
esperimenti

Strategie di quantificazione in Real Time PCR

1. Quantificazione Assoluta

– **Absolute Standard Curve Method** → Richiede standard dei quali si conosce a priori la quantità

- STND_{1/2/.../6}, UNKN, NTC

2. Quantificazione Relativa

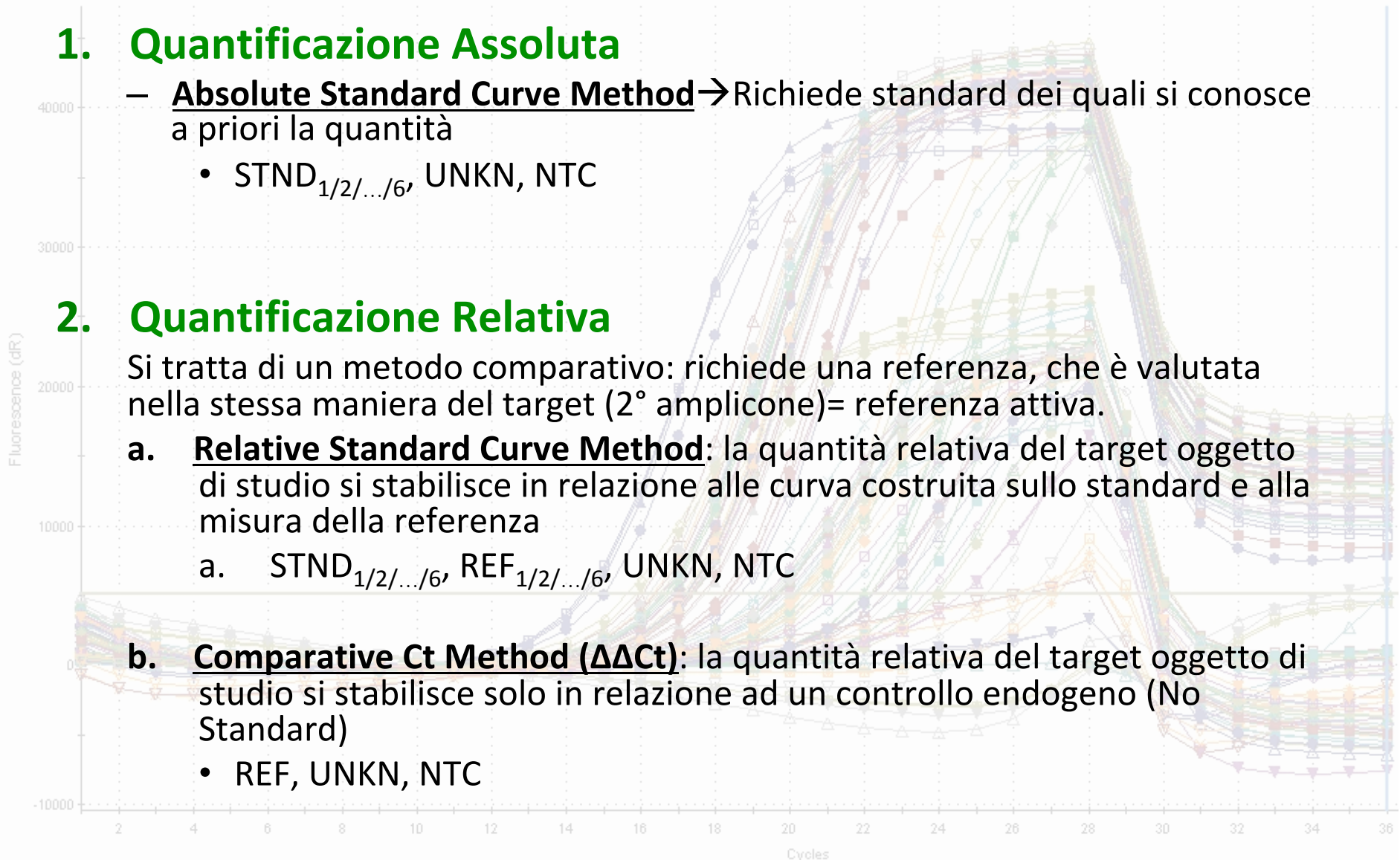
Si tratta di un metodo comparativo: richiede una referenza, che è valutata nella stessa maniera del target (2° amplicone) = referenza attiva.

a. **Relative Standard Curve Method**: la quantità relativa del target oggetto di studio si stabilisce in relazione alle curva costruita sullo standard e alla misura della referenza

- a. STND_{1/2/.../6}, REF_{1/2/.../6}, UNKN, NTC

b. **Comparative Ct Method ($\Delta\Delta Ct$)**: la quantità relativa del target oggetto di studio si stabilisce solo in relazione ad un controllo endogeno (No Standard)

- REF, UNKN, NTC



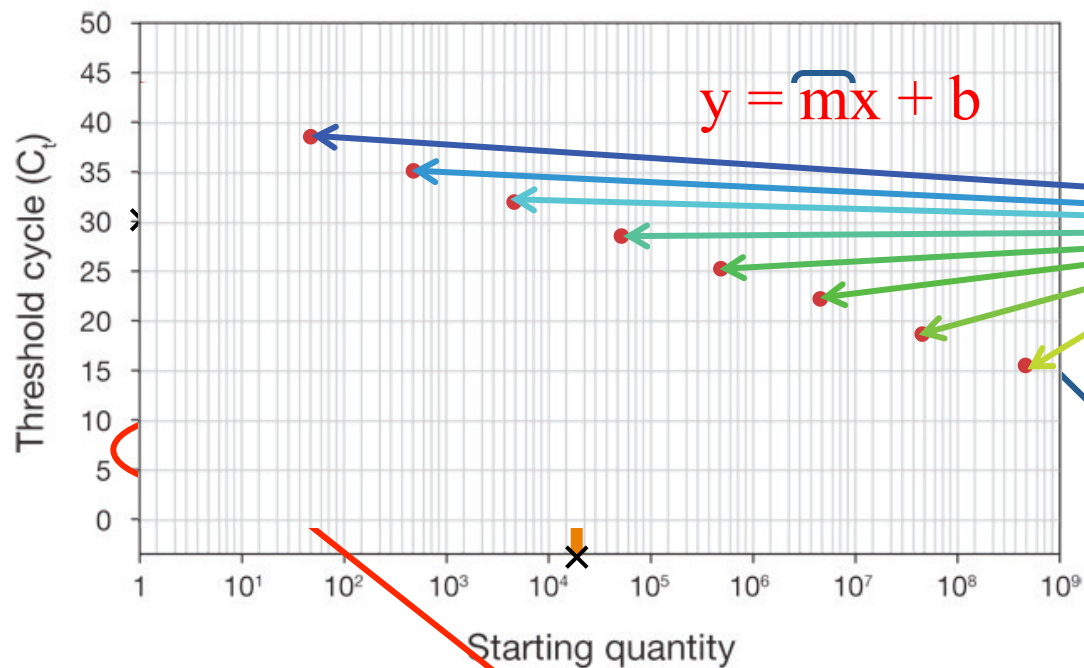
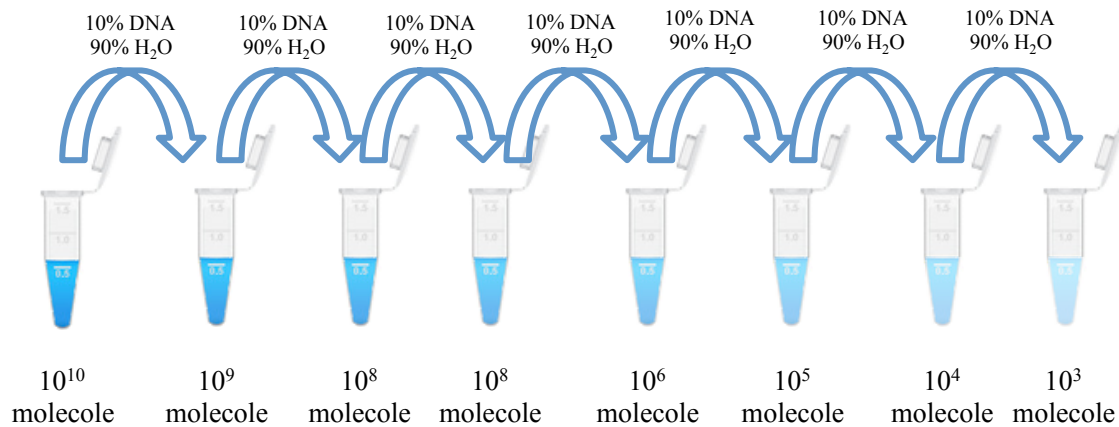
Alcuni concetti

Controls/Terms	Definitions
Standard	A sample of known concentration used to construct a standard curve.

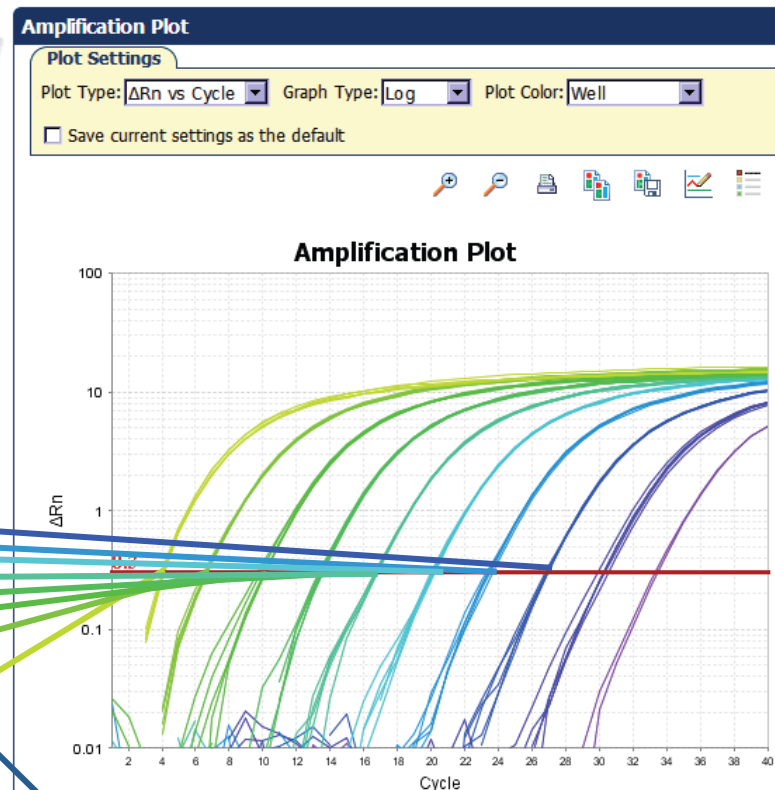
Quantificazione Assoluta (QA)

- Si effettua quando si vuole determinare in maniera precisa il numero effettivo di molecole.
- Quantità note di un campione target (standard) sono amplificate in parallelo ai campioni di studio in modo da costruire una curva standard da cui dedurre il numero di molecole nei campioni d'interesse
- Standards: DNA plasmidico (recDNA), DNA genomico, prodotto di RT-PCR, grandi oligonucleotidi sintetizzati
- La quantità assoluta dello standard deve essere determinata con tecniche indipendenti

QA

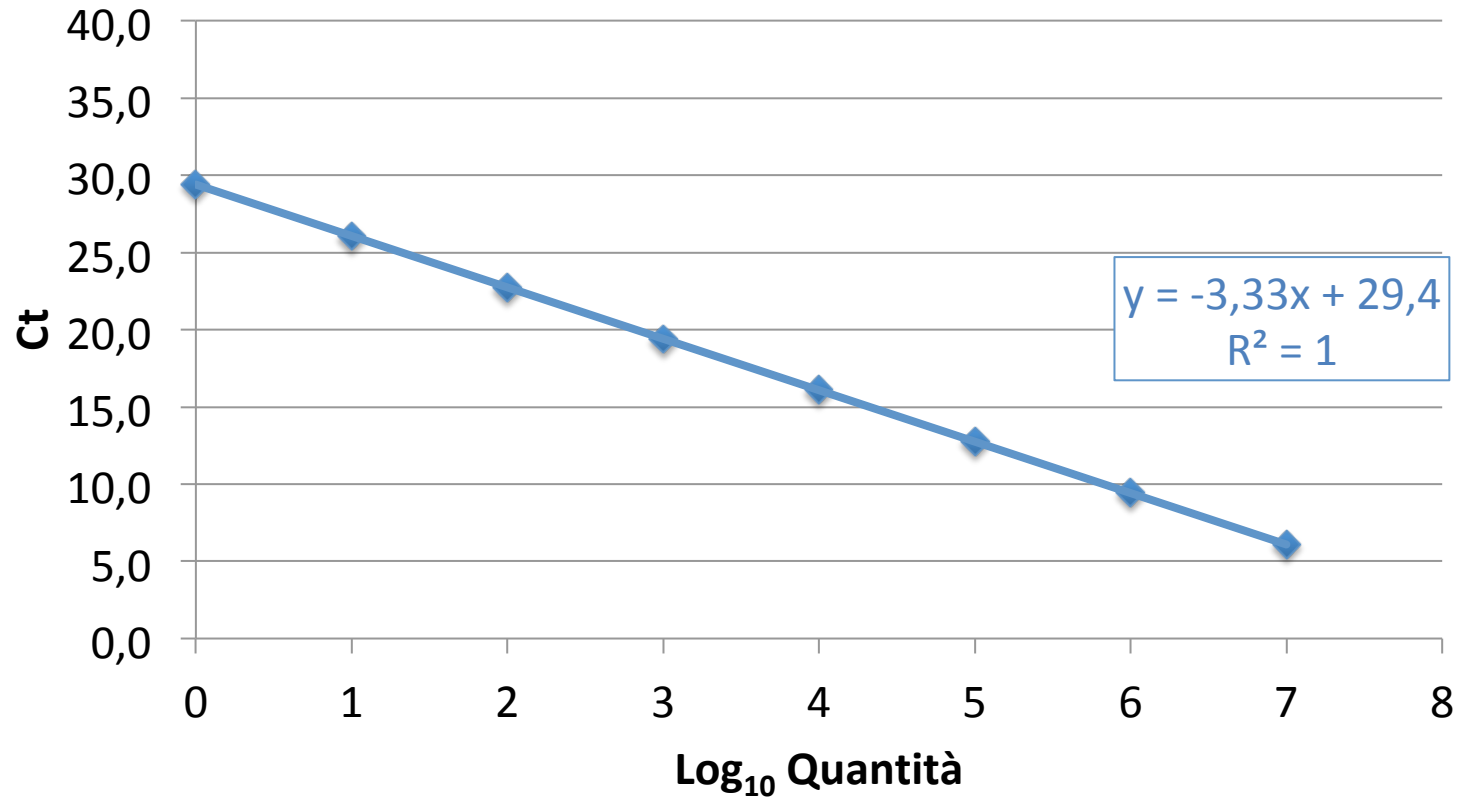


Il **coefficiente di correlazione** è una misura di quanto bene i dati si posizionano sulla curva standard. Il valore R² riflette la linearità della curva standard.



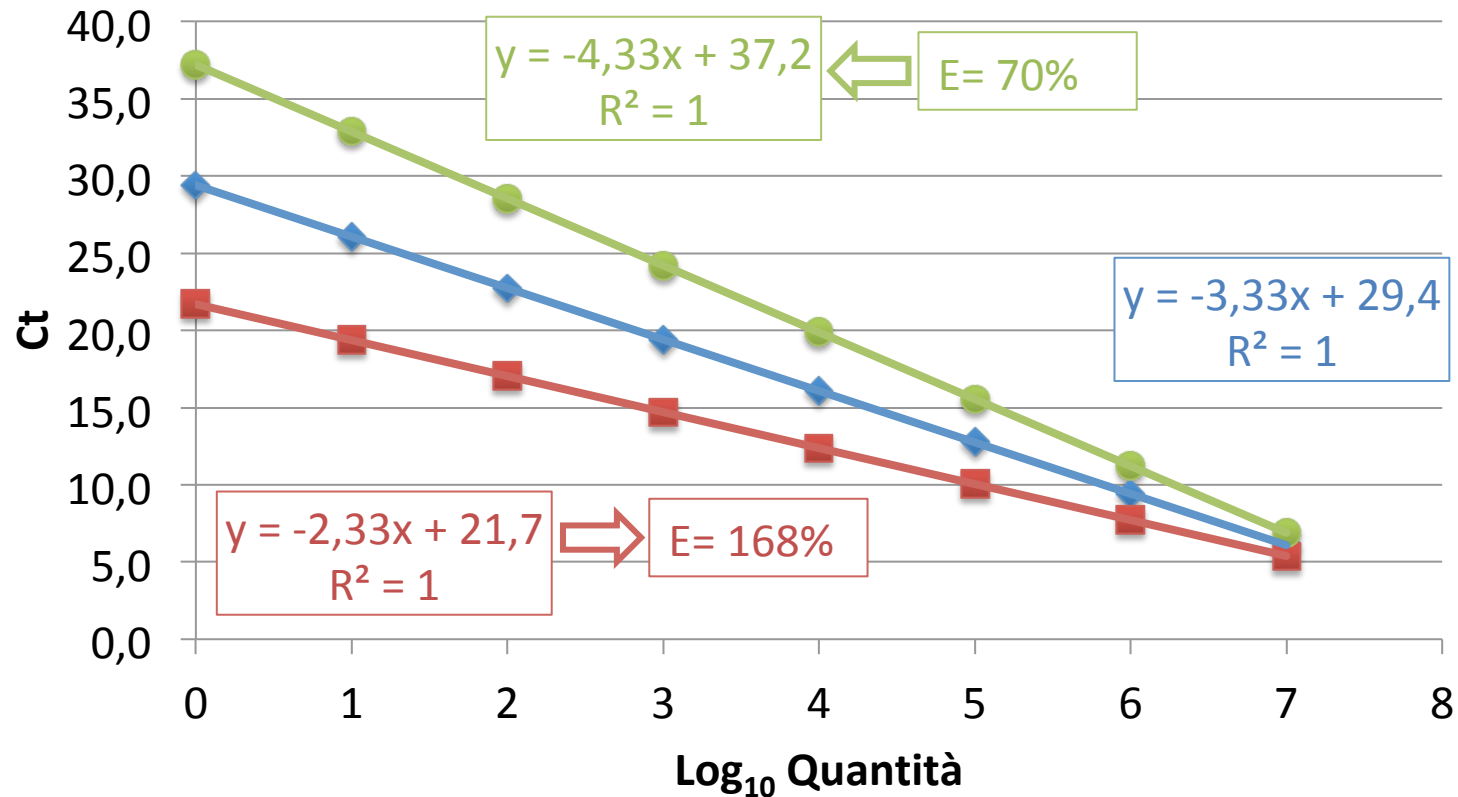
La **pendenza della retta** è una misura dell'efficienza della reazione. Risultati accurati e riproducibili

QA



Efficienza = $10^{(-1/\text{slope})-1} \longrightarrow 10^{(-1/-3,33)-1} = 100\%$

QA

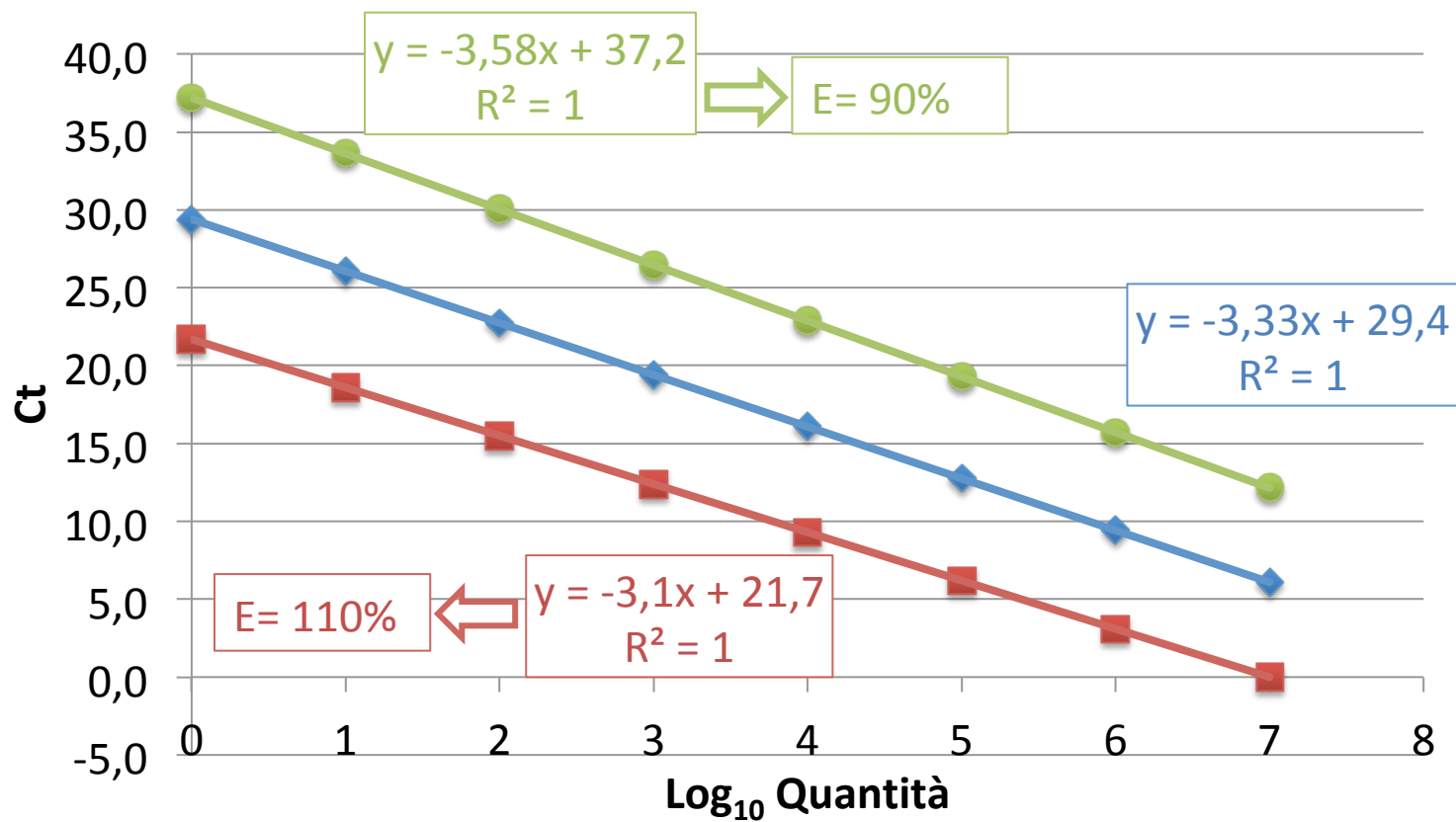


Fattori che influenzano E:

- Lunghezza amplicone
- Strutture secondarie
- Contenuto in GC

- Concentrazioni non ottimali dei reagenti ($E < 1$)
- Presenza di inibitori
- ...

Quantificazione Assoluta: limiti di validità del saggio



E = Efficienza

Calculation

The slope of the standard curve can be used to determine the exponential amplification and efficiency of the PCR reaction by the following equations:

$$\text{Exponential Amplification} = 10^{(-1/\text{slope})}$$
$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

The following table shows the amplification and efficiency for various values of the slope:

Slope	Amplification	Efficiency
-3.60	1.8957	0.8957
-3.55	1.9129	0.9129
-3.50	1.9307	0.9307
-3.45	1.9492	0.9492
-3.40	1.9684	0.9684
-3.35	1.9884	0.9884
-3.30	2.0092	1.0092
-3.25	2.0309	1.0309
-3.20	2.0535	1.0535
-3.15	2.0771	1.0771
-3.10	2.1017	1.1017

As the table illustrates, optimal PCR efficiency is indicated by a slope of -3.3 .

Effetti dell'efficienza di amplificazione

$$X_n = X_0(1+E)^n$$

Case 1: $E = 0.9$

$$X_n = 100 (1+0.9)^{30}$$

$$X_n = 2.3 \times 10^{10}$$

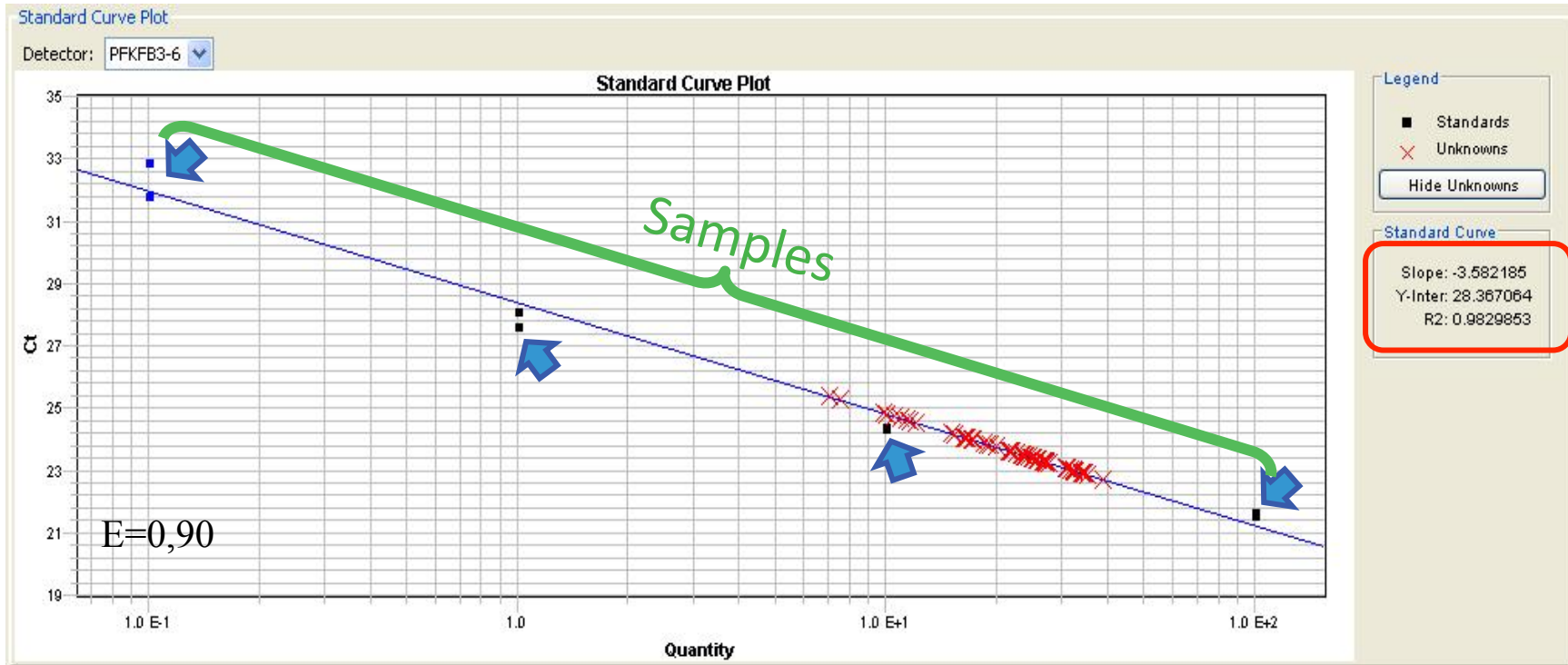
Case 2: $E = 0.8$

$$X_n = 100 (1+0.8)^{30}$$

$$X_n = 4.6 \times 10^9$$

Una differenza di 0.1 in termini di efficienza di amplificazione crea una differenza di 5 volte nella quantità finale del prodotto di PCR dopo 30 cicli

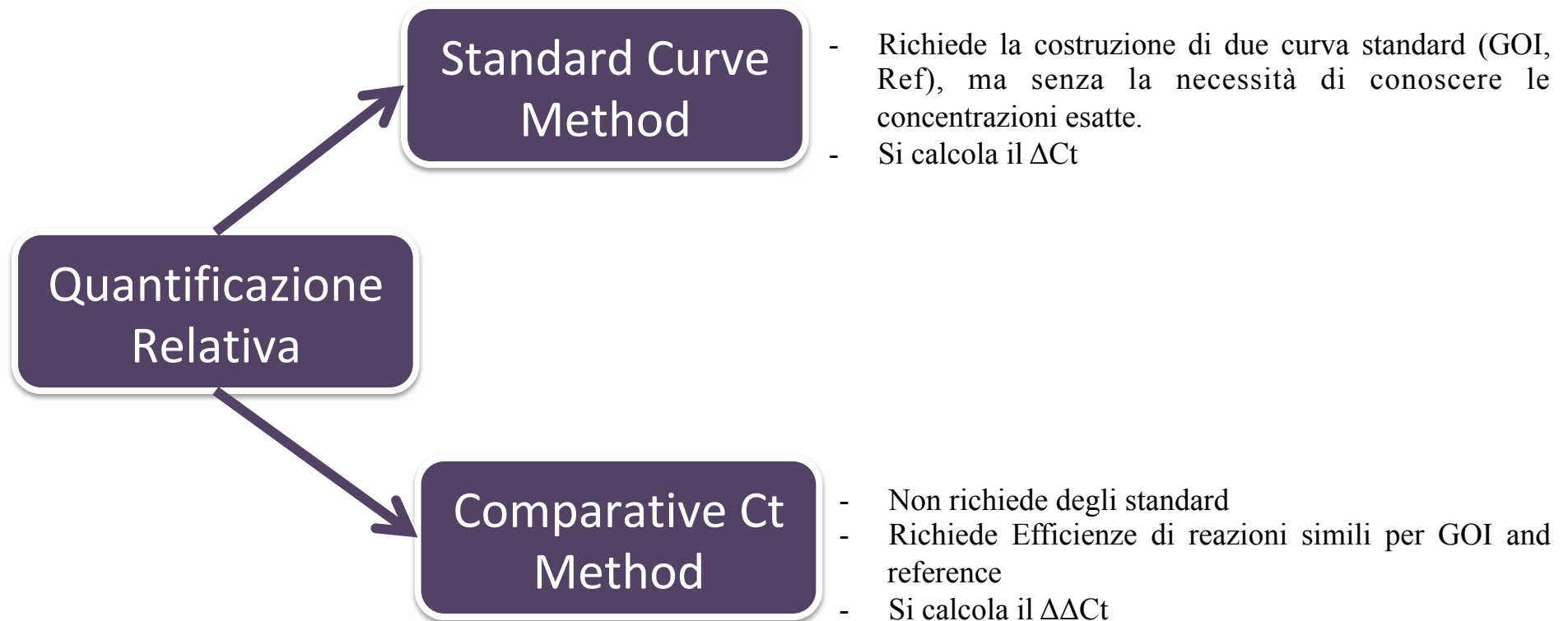
PCR quantitativa: altri concetti



- 1) Templato e campioni simili per natura, trattamento, ecc.
- 2) Analisi di doppietti/tripletti
- 3) Tutti i punti devono cadere all'interno della retta

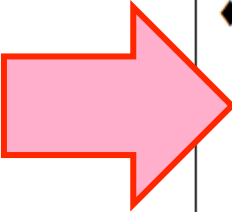
Quantificazione Relativa (QR)

La quantificazione relativa determina i cambiamenti nei livelli di mRNA di un gene su più campioni ed è espressa in relazione ai livelli di un RNA di controllo interno → un gene housekeeping



Altri concetti sulla Real Time PCR

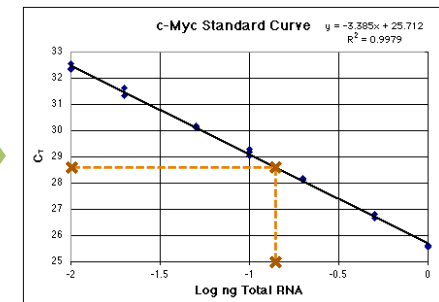
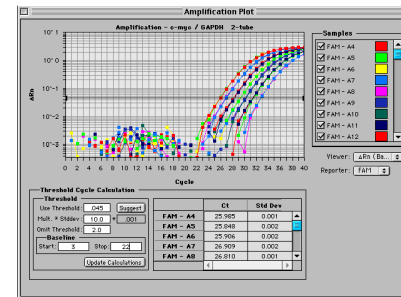
Controls/Terms	Definitions
Standard	A sample of known concentration used to construct a standard curve.
Calibrator	A sample used as the basis for comparative results.
Reference	<p>A passive or active signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. Active reference means the signal is generated as the result of PCR amplification. The active reference has its own set of primers and probe.</p> <ul style="list-style-type: none">◆ Endogenous control – This is an RNA or DNA that is present in each experimental sample as isolated. By using an endogenous control as an active reference, you can normalize quantitation of a messenger RNA (mRNA) target for differences in the amount of total RNA added to each reaction.◆ Exogenous control – This is a characterized RNA or DNA spiked into each sample at a known concentration. An exogenous active reference is usually an <i>in vitro</i> construct that can be used as an internal positive control (IPC) to distinguish true target negatives from PCR inhibition. An exogenous reference can also be used to normalize for differences in efficiency of sample extraction or complementary DNA (cDNA) synthesis by reverse transcriptase. <p>Whether or not an active reference is used, it is important to use a passive reference containing the dye ROX in order to normalize for non-PCR-related fluctuations in fluorescence signal.</p>



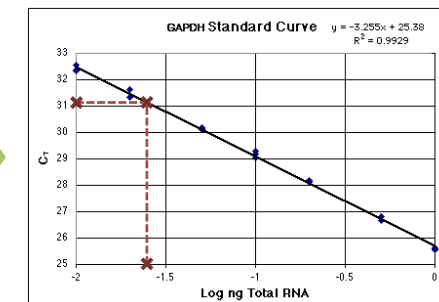
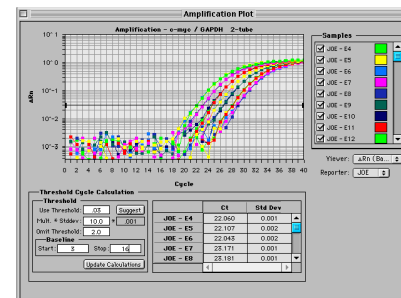
QR Standard Curve Method: Esempio 1

- Due diluizioni Seriali: uno per Myc (GOI) e una per GAPDH (EnCon)
- Studio dell'espressione di Myc in Brain, Kidney, Liver, Lung

Show Analysis		Dye Layer: FAM											
	1	2	3	4	5	6	7	8	9	10	11	12	
A	NTC1 A1	NTC1 A2	NTC1 A3	STND 1000 pg	STND 1000 pg	STND 1000 pg	STND 500 pg	STND 500 pg	STND 500 pg	STND 200 pg	STND 200 pg	STND 200 pg	
B	STND 100 pg	STND 100 pg	STND 100 pg	STND 50 pg	STND 50 pg	STND 50 pg	STND 20 pg	STND 20 pg	STND 20 pg	STND 10 pg	STND 10 pg	STND 10 pg	
C	cMYC Brain	cMYC Brain	cMYC Brain	cMYC Brain	cMYC Brain	cMYC Brain	cMYC Kidney	cMYC Kidney	cMYC Kidney	cMYC Kidney	cMYC Kidney	cMYC Kidney	
D	cMYC Liver	cMYC Liver	cMYC Liver	cMYC Liver	cMYC Liver	cMYC Liver	cMYC Lung	cMYC Lung	cMYC Lung	cMYC Lung	cMYC Lung	cMYC Lung	



E	NTC2 E1	NTC2 E2	NTC2 E3	STD2 1000 pg	STD2 1000 pg	STD2 1000 pg	STD2 500 pg	STD2 500 pg	STD2 500 pg	STD2 200 pg	STD2 200 pg	STD2 200 pg
F	STD2 100 pg	STD2 100 pg	STD2 100 pg	STD2 50 pg	STD2 50 pg	STD2 50 pg	STD2 20 pg	STD2 20 pg	STD2 20 pg	STD2 10 pg	STD2 10 pg	STD2 10 pg
G	GAPDH Brain	GAPDH Brain	GAPDH Brain	GAPDH Brain	GAPDH Brain	GAPDH Brain	GAPDH Kidney	GAPDH Kidney	GAPDH Kidney	GAPDH Kidney	GAPDH Kidney	GAPDH Kidney
H	GAPDH Liver	GAPDH Liver	GAPDH Liver	GAPDH Liver	GAPDH Liver	GAPDH Liver	GAPDH Lung	GAPDH Lung	GAPDH Lung	GAPDH Lung	GAPDH Lung	GAPDH Lung



QR Standard Curve Method: Esempio 1

- La media di replicati tecnici del GOI viene divisa per la media del geni di riferimento (GAPDH)
- Ad esempio:

Tissue	c-myc ng Total Raji RNA	GAPDH ng Total Raji RNA	c-myc _N Norm. to GAPDH ^a	c-myc _N Rel. to Brain ^b
Brain	0.033	0.51		
	0.043	0.56		
	0.036	0.59		
	0.043	0.53		
	0.039	0.51		
	0.040	0.52		
Average	0.039±0.004	0.54±0.034	0.07±0.008	1.0±0.12
Kidney	0.40	0.96		
	0.41	1.06		
	0.41	1.05		
	0.39	1.07		
	0.42	1.06		
	0.43	0.96		
Average	0.41±0.016	1.02±0.052	0.40±0.025	5.5±0.35
Liver	0.67	0.29		
	0.66	0.28		
	0.70	0.28		
	0.76	0.29		
	0.70	0.26		
	0.68	0.27		
Average	0.70±0.036	0.28±0.013	2.49±0.173	34.2±2.37

Calibratore: Brain

QR Standard Curve Method: Esempio 1

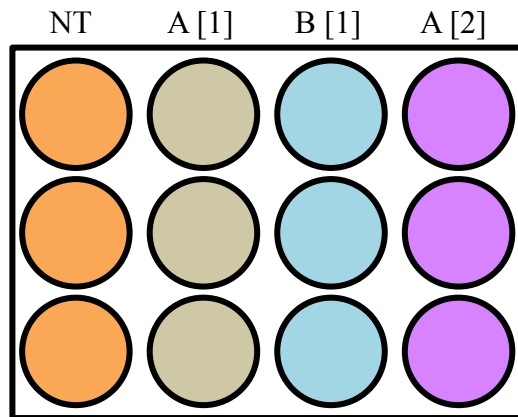
- Cosa accadrebbe se non usassimo il controllo endogeno:

Calibratore: Brain

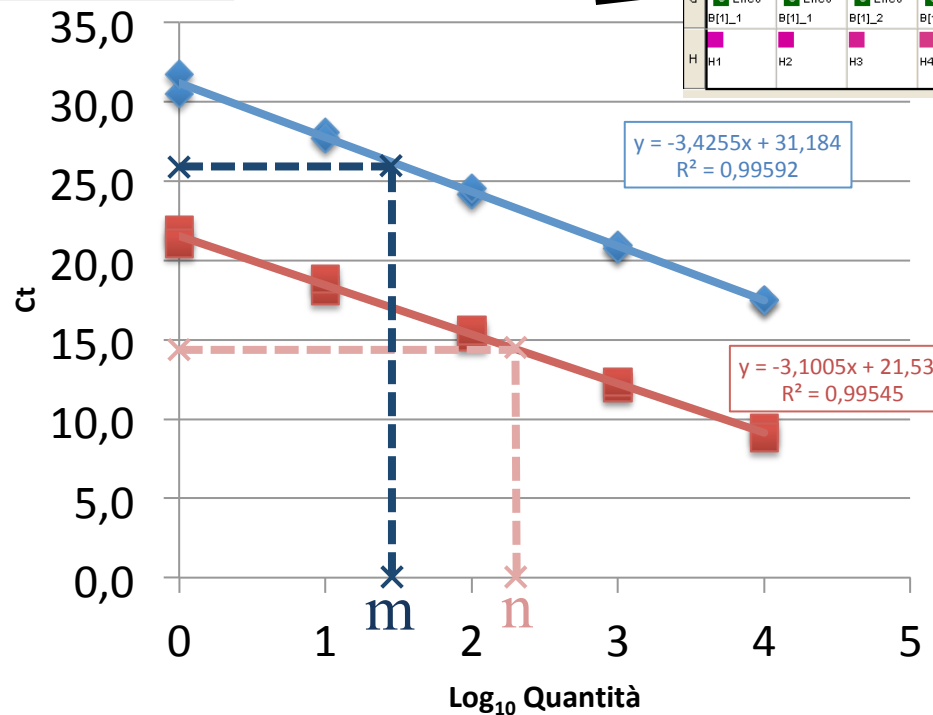
Tissue	c-myc ng Total Raji RNA	c-myc _N Rel. to Brain ^b	c-myc _N Rel. to Brain ^b
Brain	0.033		
	0.043		
	0.036		
	0.043		
	0.039		
	0.040		
Average	0.039±0.004	1.0	1.0±0.12
Kidney	0.40		
	0.41		
	0.41		
	0.39		
	0.42		
	0.43		
Average	0.41±0.016	10.5	5.5±0.35
Liver	0.67		
	0.66		
	0.70		
	0.76		
	0.70		
	0.68		
Average	0.70±0.036	17.9	34.2±2.37

QR Standard Curve Method: Esempio 2

Effetto dei farmaci A e B sull'espressione del gene *GOI*



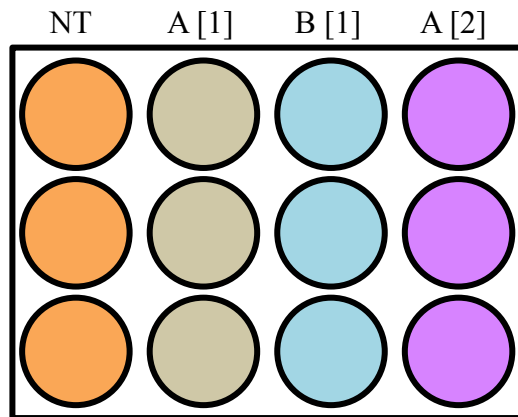
Untitled 2 - Absolute Quantification												
	1	2	3	4	5	6	7	8	9	10	11	12
A	S φOI 1000 pg	S φOI 1000 pg	S φOI 100 pg	S φOI 100 pg	S φOI 10 pg	S φOI 10 pg	S φOI 1 pg	S φOI 1 pg	S φOI 0.1 pg	S φOI 0.1 pg	N φOI NTC	N φOI NTC
B	U φOI NTC1	U φOI NTC1	U φOI NTC2	U φOI NTC2	U φOI NTC3	U φOI NTC3	U φOI A[1]_1	U φOI A[1]_1	U φOI A[1]_2	U φOI A[1]_2	U φOI A[1]_3	U φOI A[1]_3
C	U φOI B[1]_1	U φOI B[1]_1	U φOI B[1]_2	U φOI B[1]_2	U φOI B[1]_3	U φOI B[1]_3	U φOI A[2]_1	U φOI A[2]_1	U φOI A[2]_2	U φOI A[2]_2	U φOI A[2]_3	U φOI A[2]_3
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	S EnCo 1000 ng	S EnCo 1000 ng	S EnCo 100 pg	S EnCo 100 pg	S EnCo 10 pg	S EnCo 10 pg	S EnCo 1 pg	S EnCo 1 pg	S EnCo 0.1 pg	S EnCo 0.1 pg	N EnCo NTC	N EnCo NTC
F	U EnCo NTC1	U EnCo NTC1	U EnCo NTC2	U EnCo NTC2	U EnCo NTC3	U EnCo NTC3	U EnCo A[1]_1	U EnCo A[1]_1	U EnCo A[1]_2	U EnCo A[1]_2	U EnCo A[1]_3	U EnCo A[1]_3
G	U EnCo B[1]_1	U EnCo B[1]_1	U EnCo B[1]_2	U EnCo B[1]_2	U EnCo B[1]_3	U EnCo B[1]_3	U EnCo A[2]_1	U EnCo A[2]_1	U EnCo A[2]_2	U EnCo A[2]_2	U EnCo A[2]_3	U EnCo A[2]_3
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12



$$Q_{NT} = m/n$$

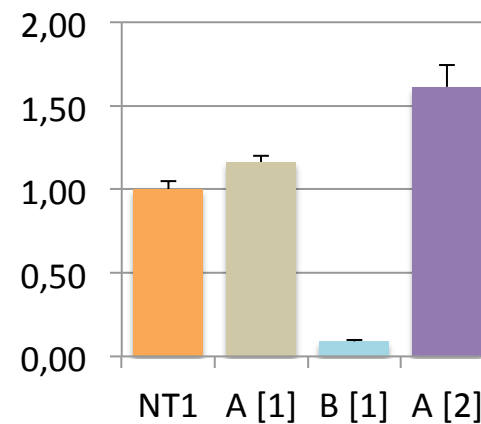
QR Standard Curve Method: Esempio 2

Effetto dei farmaci A e B sull'espressione del gene *GOI*

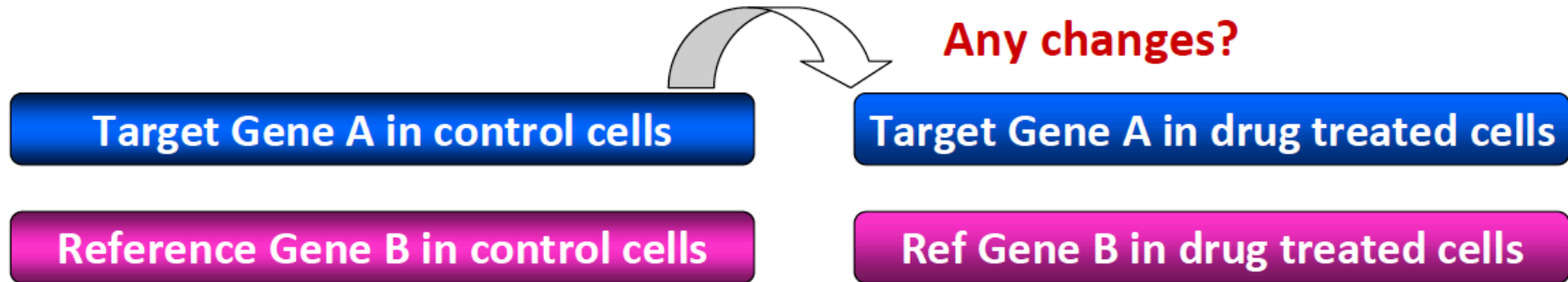


Name	GOI			EnCo			GOI _n /EnCo _n	Dev.St.
	Quantità ng	Media	Coeff. Var	Quantità ng	Media	Coeff. Var		
NT1	20,96	20,41	0,04	21,1	21,63	0,03	0,94	0,05
	19,85			22,1				
A [1]	24,03	23,44	0,04	21,4	21,38	0,00	1,10	0,04
	22,86			21,4				
B [1]	2,83	2,67	0,08	30,6	31,46	0,04	0,08	0,01
	2,51			32,3				
A [2]	38,85	36,73	0,08	24,3	24,17	0,01	1,52	0,12
	34,62			24,1				

- La molecola A non ha alcun effetto su GOI
- La molecola B induce una significativa riduzione dell'espressione di GOI (oncogene)



QR Comparative Ct Method

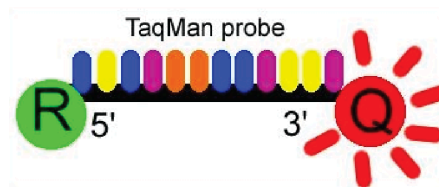


→ $\Delta Ct1 = Ct (\text{Target A -treated}) - Ct (\text{Ref B-treated})$

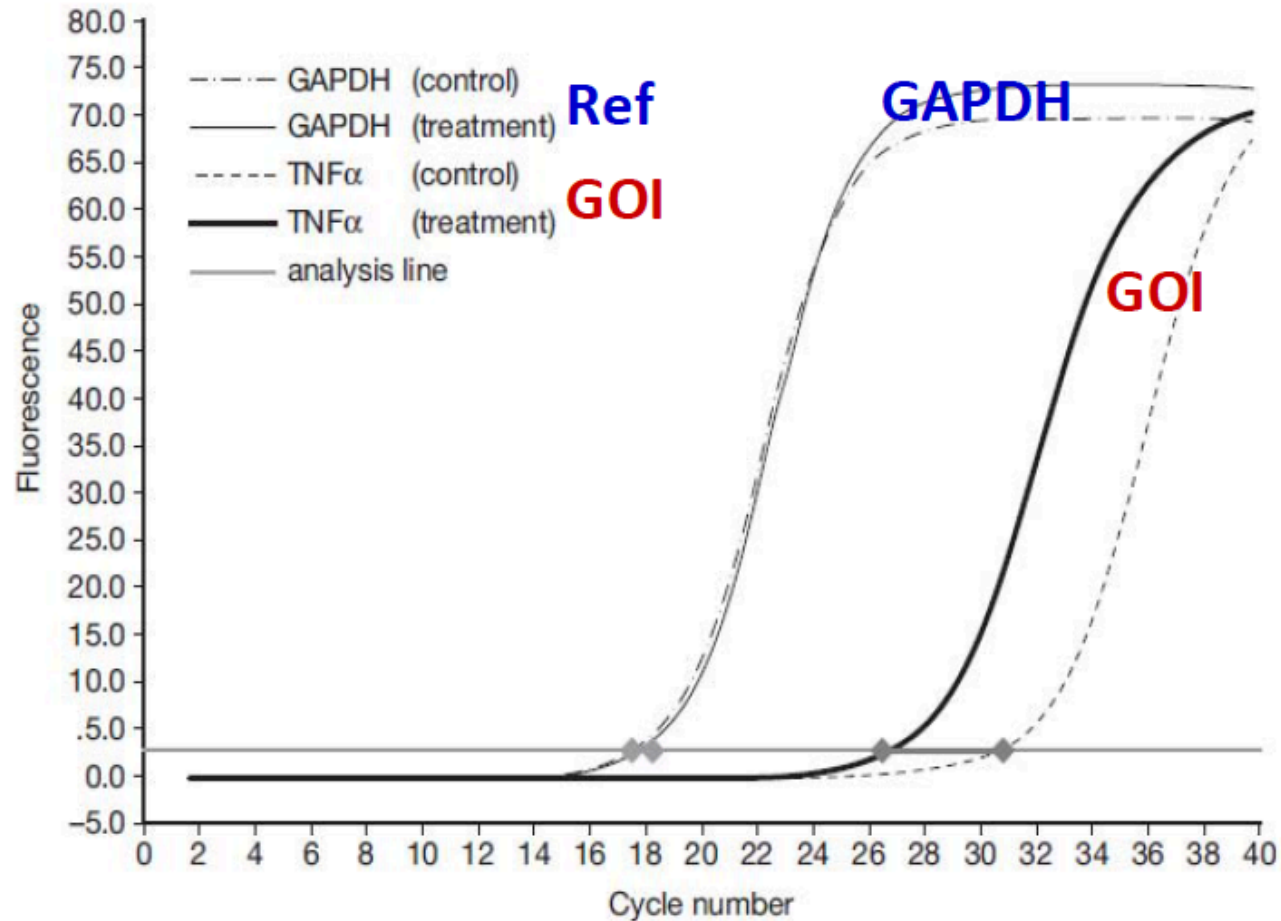
→ $\Delta Ct2 = Ct (\text{Target A-control}) - Ct (\text{Ref B-control})$

→ $\Delta \Delta Ct = \Delta Ct1 (\text{treated}) - \Delta Ct2 (\text{control})$

Normalized target gene expression level = $2^{\Delta \Delta Ct}$



QR Comparative Ct Method



$$\Delta\Delta Ct = \Delta Ct (\text{TNF}\alpha_{\text{treat}} - \text{GAPDH}_{\text{treat}}) - \Delta Ct (\text{TNF}\alpha_{\text{control}} - \text{GAPDH}_{\text{control}})$$

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

Comparative vs Standard Curve method

Tissue	c-myc Average C_T	GAPDH Average C_T	ΔC_T c-myc-GAPDH ^a	$\Delta \Delta C_T$ $\Delta C_T - \Delta C_{T, \text{Brain}}$ ^b	c-myc _N Rel. to Brain ^c	St curve method
Brain	30.49±0.15	23.63±0.09	6.86±0.17	0.00±0.17	1.0 (0.9–1.1)	1.0±0.12
Kidney	27.03±0.06	22.66±0.08	4.37±0.10	-2.50±0.10	5.6 (5.3–6.0)	5.5±0.35
Liver	26.25±0.07	24.60±0.07	1.65±0.10	-5.21±0.10	37.0 (34.5–39.7)	34.2±2.37

Il problema della normalizzazione

Fonti di variabilità sperimentale

Natura e quantità
del materiale di
partenza

Metodi di
estrazione

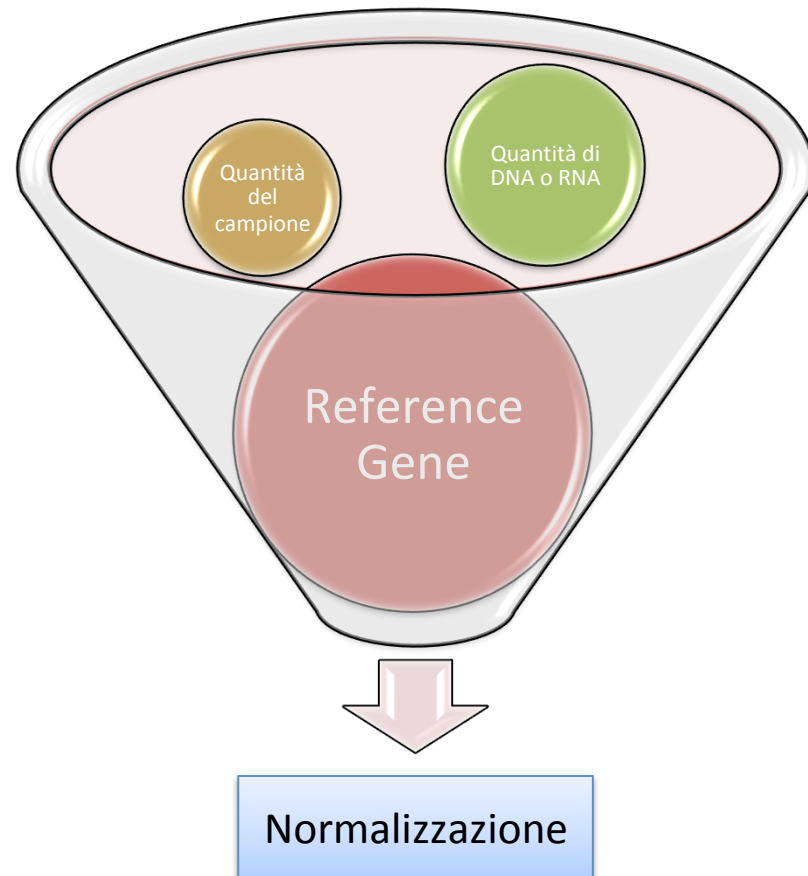
Retrotrascrizione
(RNA)

Amplificazione

Caricamento del
campione

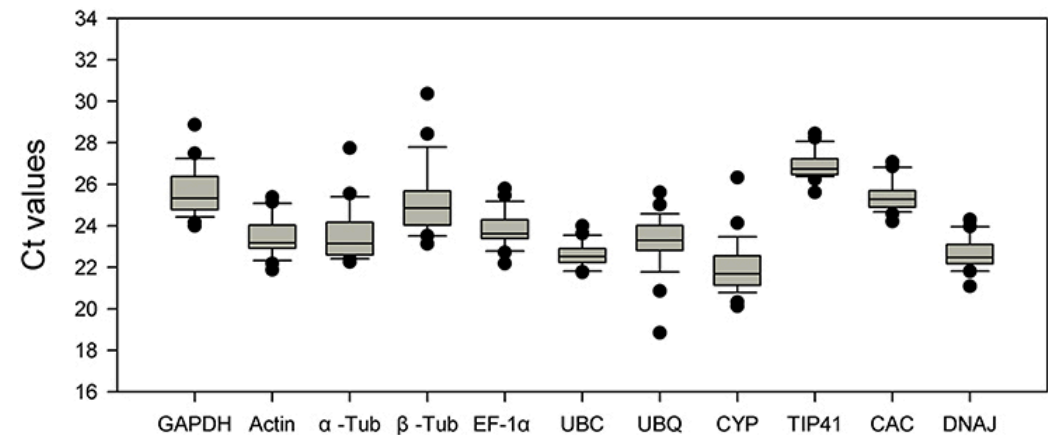
La normalizzazione è il processo volto a neutralizzare gli effetti della variabilità derivante da queste fonti.

Normalizzazione e Reference genes



Requisiti di un buon controllo endogeno

- Livelli di espressione simili a quelli del gene di studio → competizione
- Simile efficienza di amplificazione
- Espressione stabile (GeNorm, NormFinder, BestKeeper)



Comuni normalizzatori endogeni

- **β -actin (ACTB)**: cytoskeletal gene
- **18S ribosomal RNA (rRNA)**: ribosomal subunit
- **Cyclophilin A (PPIA)**: serine-threonine phosphatase inhibitor
- **GAPDH**: glycolysis pathway
- **β -2-microglobulin (B2M)**: major histocompatibility complex
- **β -glucuronidase (GUSB)**: exoglycosidase in lysosomes
- **Hypoxanthine ribosyltransferase (HPRT1)**: purine salvage pathway
- **TATA-Box binding protein (TBP)**: RNA transcription

- Vanno validati per ogni tessuto/condizione sperimentale
- Possono essere usati in combinazione \rightarrow riduzione delle fluttuazioni (media geometrica)

What is Real-Time PCR used for?

Real-Time PCR Applications I

- * quantitation of gene expression**
 - * array verification**
- * quality control and assay validation**
- * biosafety and genetic stability testing**
- * drug therapy efficacy / drug monitoring**
 - * viral quantitation**
 - * pathogen detection**

What is Real-Time PCR used for?

Real-Time PCR Applications II

- * **DNA damage (microsatellite instability) measurement**
 - * **radiation exposure assessment**
 - * **in vivo imaging of cellular processes**
 - * **mitochondrial DNA studies**
 - * **methylation detection**
- * **detection of inactivation at X-chromosome**
- * **linear-after-the-exponential (LATE)-PCR: a new method for real-time quantitative analysis of target numbers in small samples, which is adaptable to high throughput applications in clinical diagnostics, biodefense, forensics, and DNA sequencing**

What is Real-Time PCR used for?

Real-Time PCR Applications III

- * **Determination of identity at highly polymorphic HLA loci**
- * **Monitoring post transplant solid organ graft outcome**
- * **Monitoring chimerism after HSCT**
- * **Monitoring minimal residual disease after HSCT**
- * **Genotyping (Allelic discrimination)**
 - **Trisomies and single-gene copy numbers**
 - **Microdeletion genotypes**
 - **Haplotyping**
 - **Quantitative microsatellite analysis**
 - **Prenatal diagnosis from fetal cells in maternal blood**
 - **Intraoperative cancer diagnostics**

What is Real-Time PCR used for?

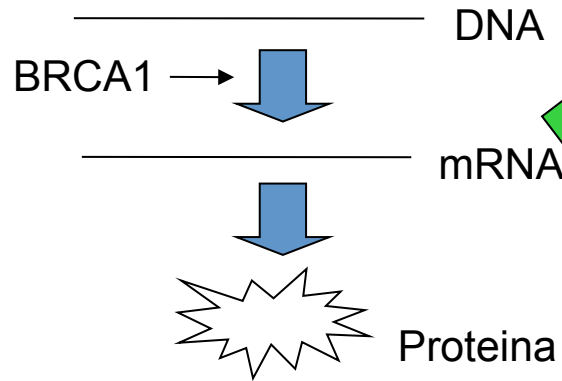
Real-Time PCR has become a cornerstone of molecular biology. Just some of the uses include:

- **Gene expression analysis**
 - Cancer and Drug research
- **Disease diagnosis and management**
 - Viral quantification
- **Food testing**
 - Percent GMO food
- **Animal and plant breeding**
 - Gene copy number
- **Forensics**
 - Sample identification and quantification

Real-Time PCR in Gene Expression Analysis

Esempio: Profilo di Espressione di BRCA1

BRCA1 è un oncosoppressore e controlla l'espressione di altri geni.

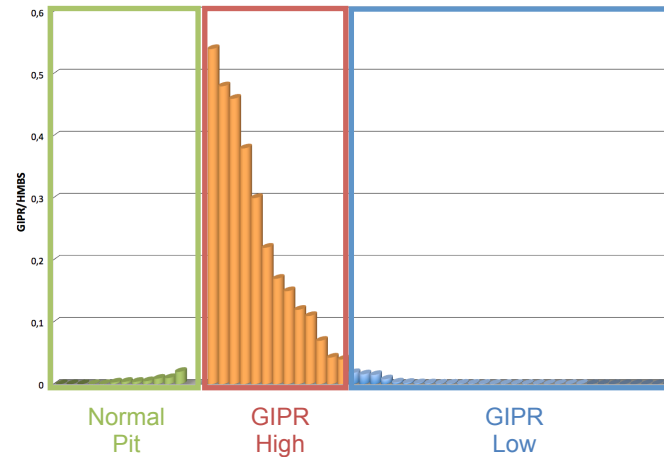
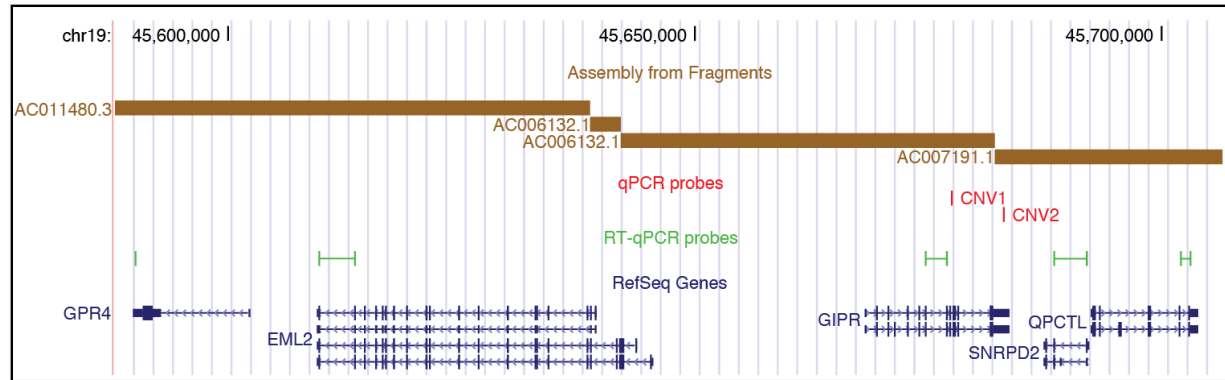
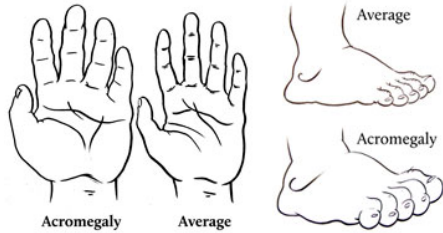


Real-Time PCR

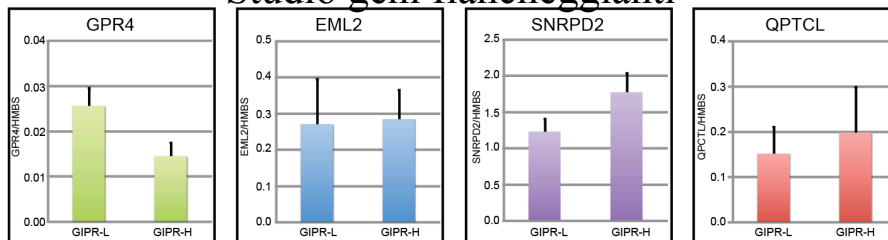
Misura di gene
expression



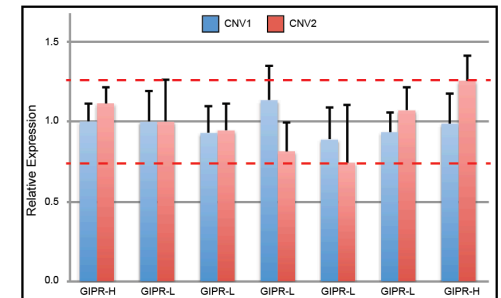
GIPR nell'acromegalia



Studio geni fiancheggianti



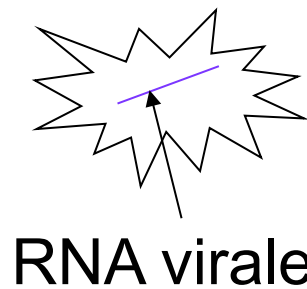
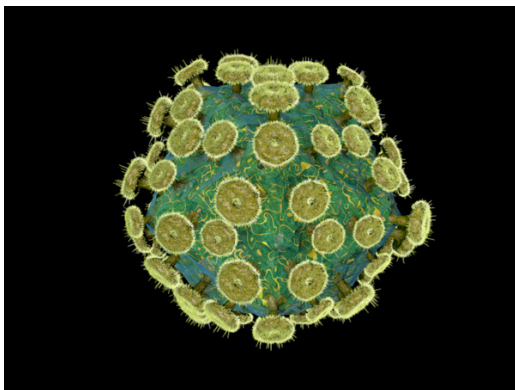
Studio Copy number variation



Real-Time PCR nella terapia farmacologica

Esempio: Trattamento dell'HIV

Il trattamento farmacologico dell'HIV dipende spesso dai livelli di carica virale.



RNA virale

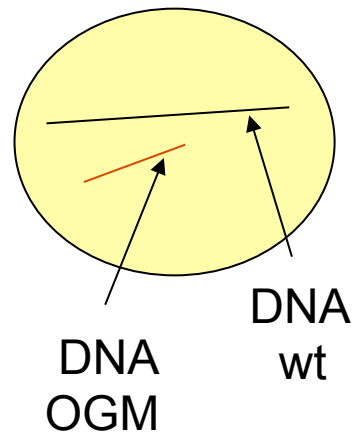


Misurare la
quantità di virus.
Consente di
adattare la
terapia

Real-Time PCR in Food Testing

Esempio: Determinare la percentuale di
OGM

La determinazione della percentuale del contenuto di
OGM nel cibo può essere estremamente importante nei
processi di import / export



Real-Time PCR



Le spedizioni
internazionali possono
dipendere dai risultati!

Real-Time PCR in Forensics

STAIN IDENTIFICATION

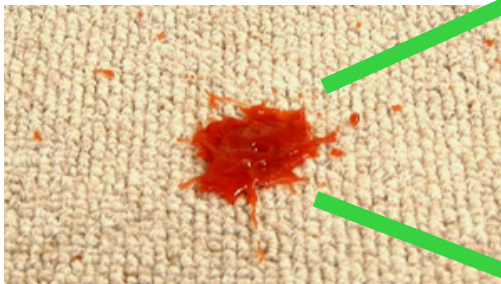
The Use of Real-Time PCR for
Forensic Stain Identification

By Trisha L. Noreault-Conti, Ph.D., and Eric Buel, Ph.D.
Vermont Forensic Laboratory, Department of Public Safety

Stain Identification:

La Real Time PCR può essere utilizzata per l'identificazione della composizione di macchie "ignote" con maggiore accuratezza rispetto ai tradizionali test colorimetrici

Cos'è??



Abbastanza DNA??



DNA Quantification:

Poiché la genotipizzazione degli STR in genetica forense richiede specifiche quantità di DNA, la Real-Time PCR può essere usata per quantificare accuratamente la quantità di DNA in un campione ignoto

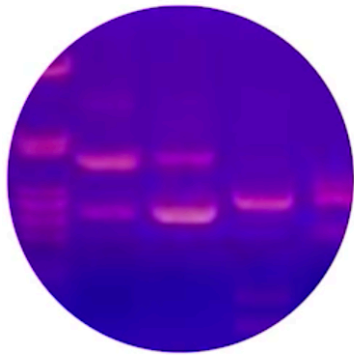
Popular Real-Time PCR Systems



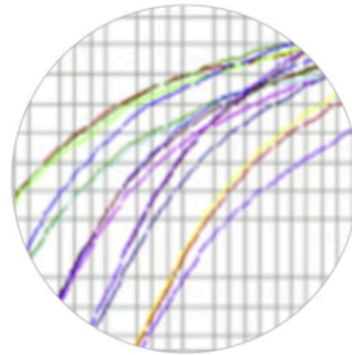
Link utili

- <http://www.gene-quantification.info/>
- http://www6.appliedbiosystems.com/support/tutorials/pdf/rtpcr_vs_tradpcr.pdf
- <http://www.gene-quantification.com/real-time-pcr-handbook-life-technologies-update-flr.pdf>
- http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041019.pdf
- http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf

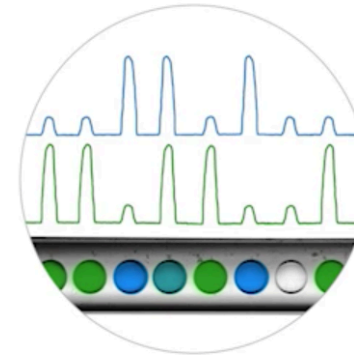
Digital PCR: l'evoluzione della PCR



PCR
Qualitative



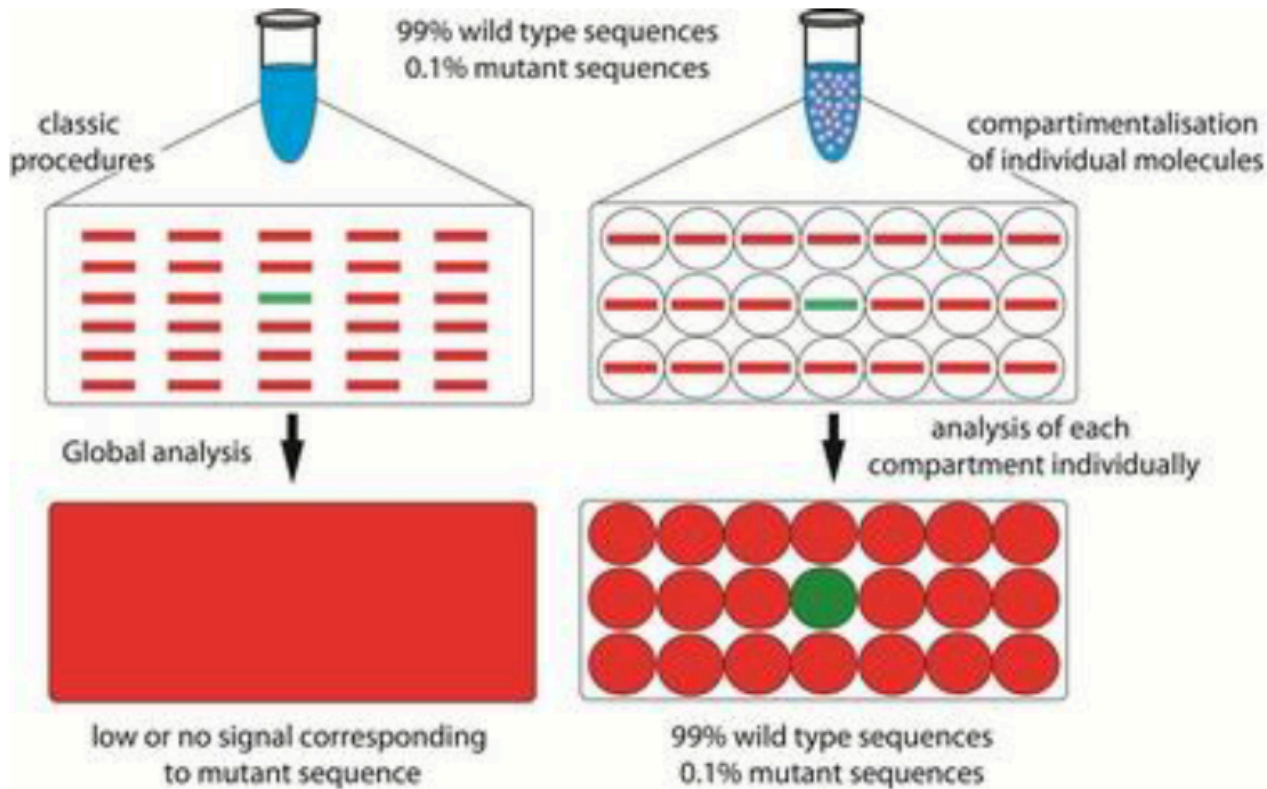
Real-Time PCR
Relative Quantification



Droplet Digital PCR
Absolute Quantification

<https://www.youtube.com/watch?v=WU3qKhIUc54>

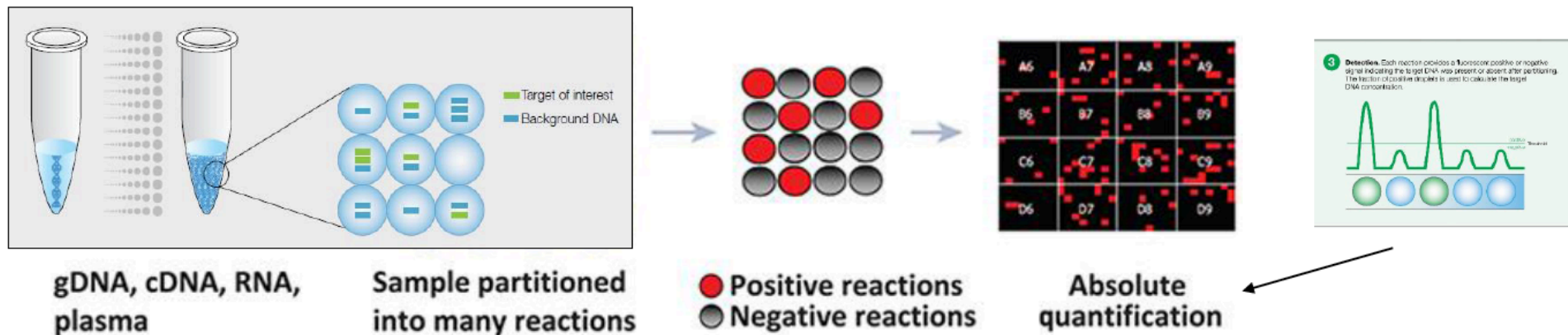
Digital PCR: l'evoluzione della PCR



I target rari (una mutazione a mosaico) non possono essere amplificati mediante PCR a causa della competizione operata dal DNA più rappresentato per i reagenti (primers, etc,...)

Droplet digital PCR

Droplet digital PCR is an analytical technique for quantification of nucleic acid samples based on **PCR amplification of single template molecules**



- **Partition reagents and sample into droplets**
- Perform PCR on thermal cycler
- **Count droplets** with a **positive** PCR product (fluorescent) **and a negative** PCR product
- Digital readout provides **concentration of target DNA**

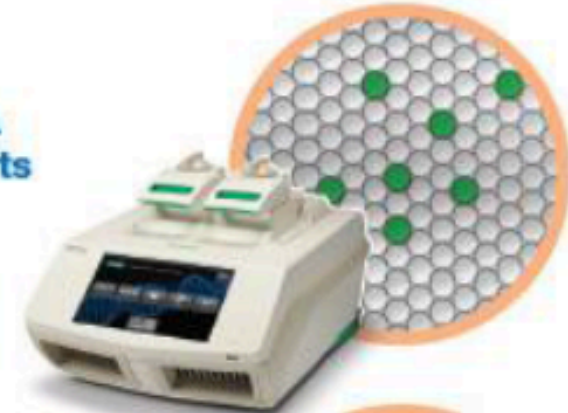
Droplet digital PCR system



1 Make droplets



2 PCR DNA in droplets



3 Read and analyze results



Droplet digital PCR system

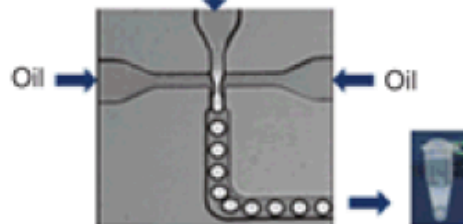
1 Make droplets

Source



Disposable chip

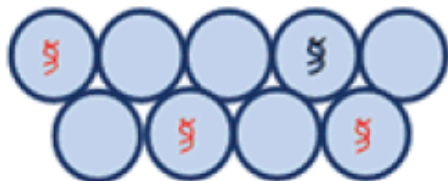
cDNA sample + assays



10 million droplets per 50 μ l
(5 pl droplet volume)

Make droplets

Target molecules: \S IDH1 WT \S IDH1 R132H



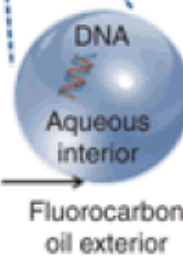
2 PCR amplification

Closed tubes from source



Thermal
cycler

Droplet
schematic



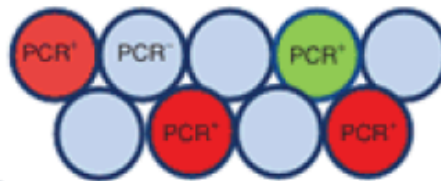
Surfactant
interface

Fluorocarbon
oil exterior

Digital fluorescent droplets

VIC probe IDH1 WT

FAM probe IDH1 R132H



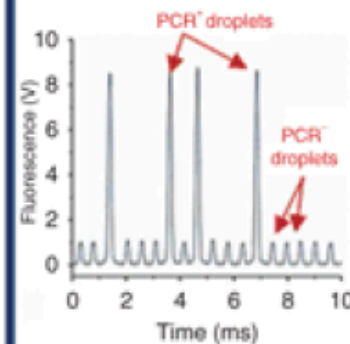
3 Count droplets

Sense

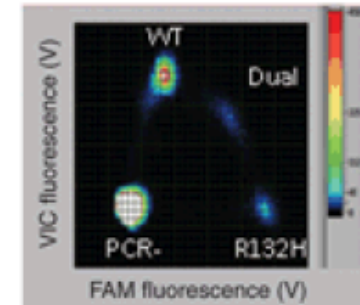


Disposable chip

Raw data

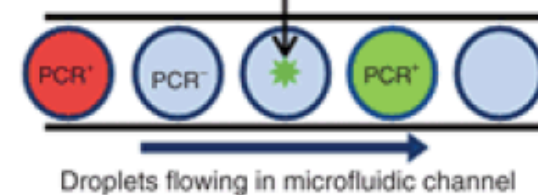


Processed data

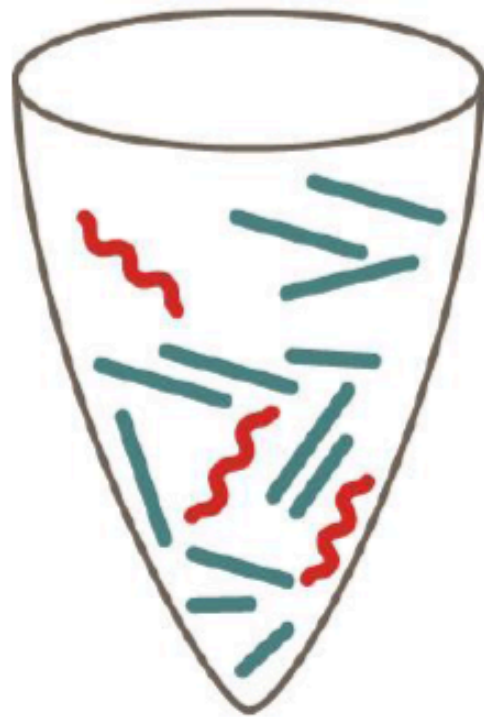


Count droplets

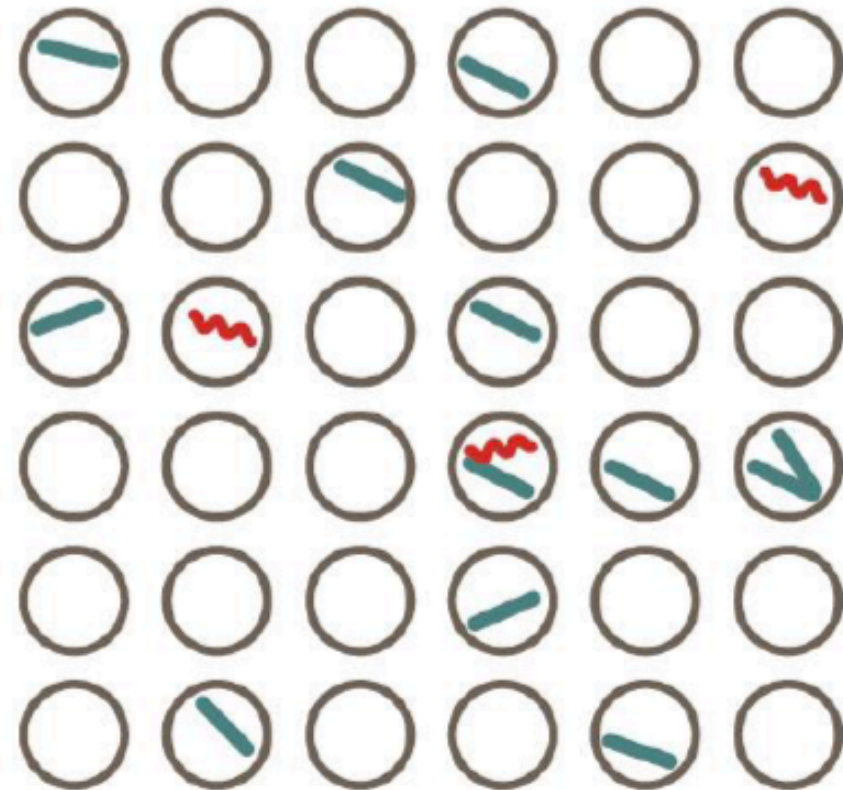
Laser spot



Droplet digital PCR: emulsione



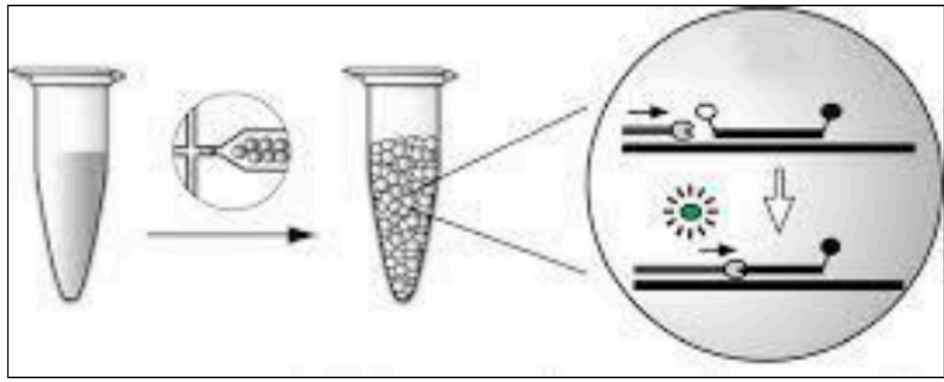
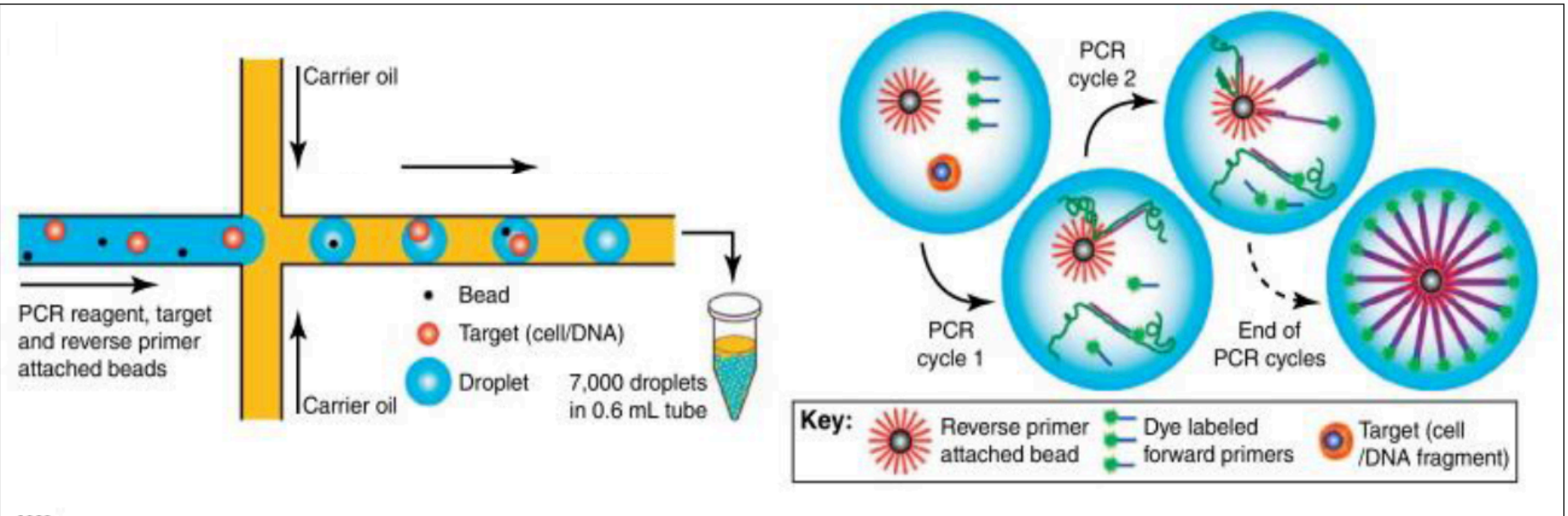
— Wild type
~ Mutant



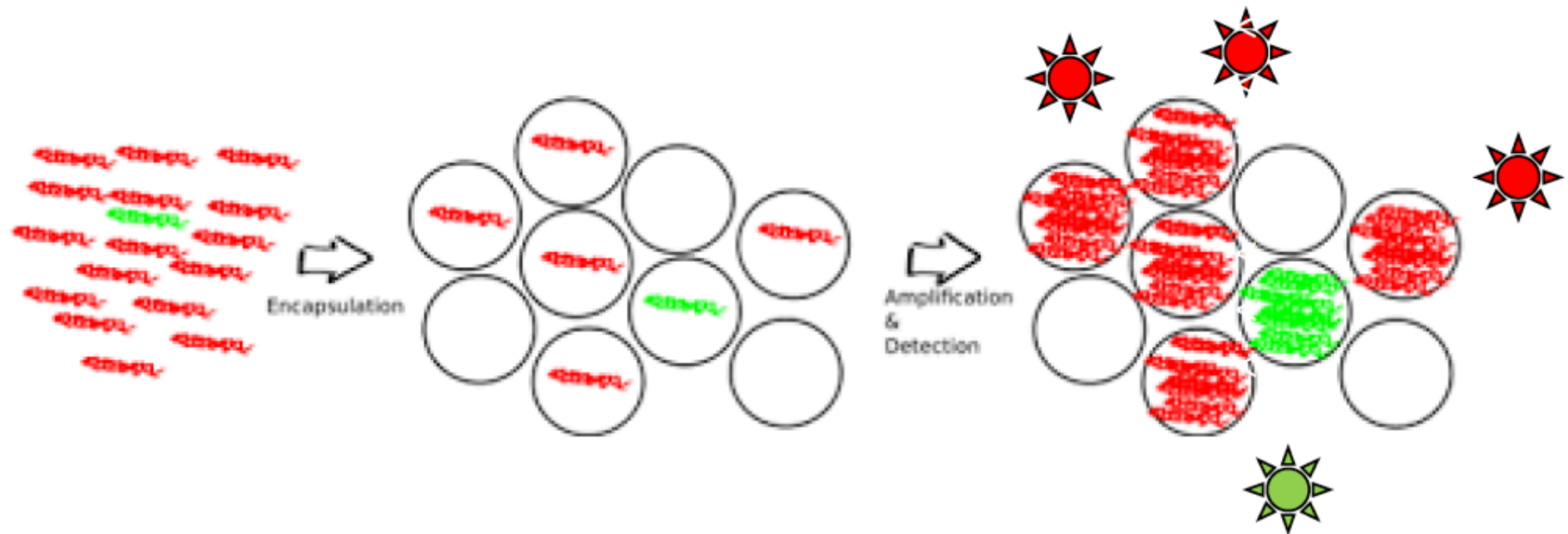
Source: (Sedlaka & Keith, 2013).¹⁰

Partition reagents and sample into **20.000 droplets**

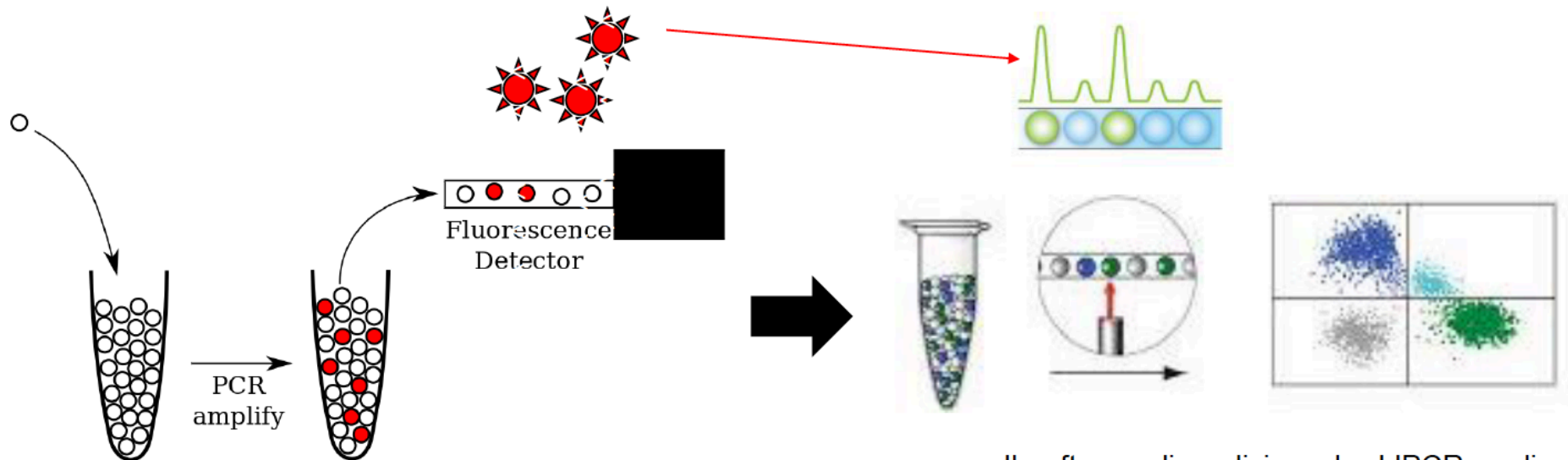
Fare le droplets



qPCR nelle droplets



Lettura delle droplets



Il software di analisi per la ddPCR applica al dato ottenuto il calcolo statistico della distribuzione di Poisson ed esprime il risultato in **numero di copie di template per uL di reazione** (copie/uL), considerando che il volume di reazione che è pari a 20 uL.

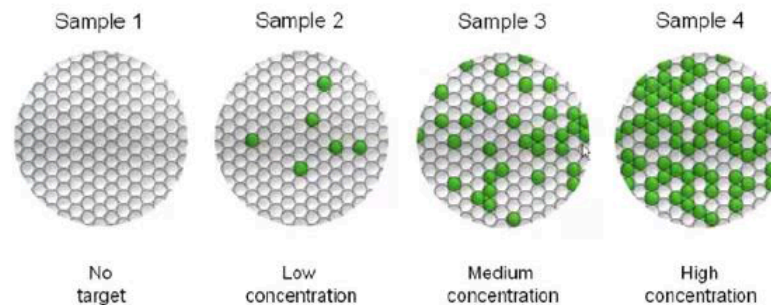
Stima della concentrazione del DNA d'interesse

Vi è una distribuzione casuale (di eventi indipendenti) delle molecole di DNA (da analizzare) nelle gocce, queste vengono ripartite casualmente durante l'emulsione del campione. **Producendo un numero sufficientemente elevato di gocce avremo la probabilità queste conterranno una sola molecola di DNA.**

Il numero di gocce contenenti una molecola di DNA dipenderà dalla **concentrazione di DNA** del campione



Siméon Denis Poisson
(1781-1840)



Poisson equation:

$$-\ln\left(1 - \frac{\text{positives}}{\text{total counted}}\right)$$

(positives + negatives)

Legge dei piccoli numeri o degli eventi rari