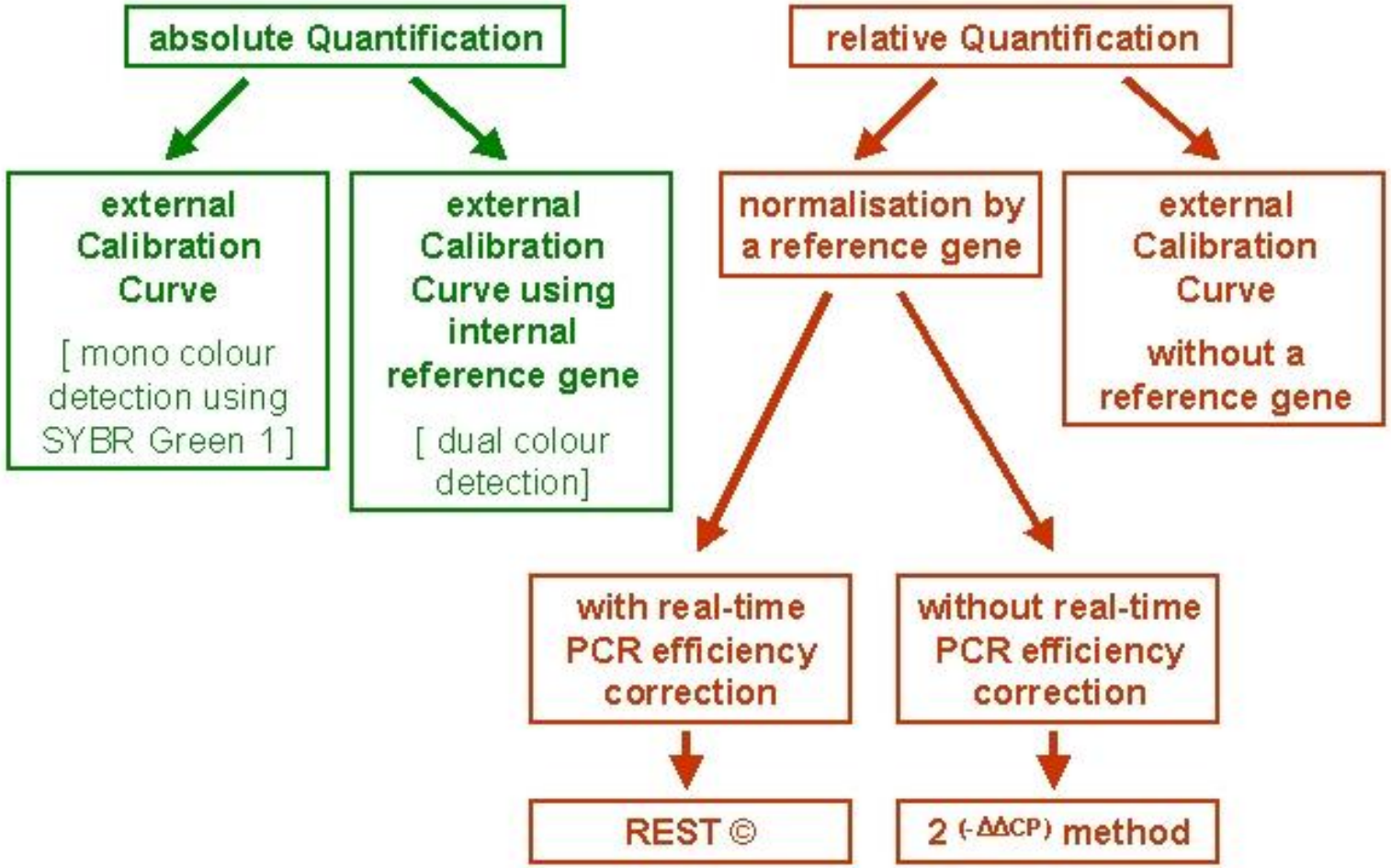
Quantification methods



real time Polymerase Chain Reaction: relative quantification

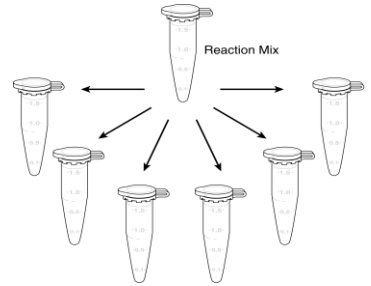
Step Action 1

a. Prepare several dilutions of cDNA (**obtained from control RNA**) within the **range of 80 pg to 50 ng** to perform standard linear equation. Make dilutions with water
b. Prepare one vial for each sample to analyze
c. Include a NTC reaction containing no template, with only water.

(No Template Control, 80 pg/µL, 400 pg/µL, 2 ng/µL, 10 ng/µL, and 50 ng/µL)

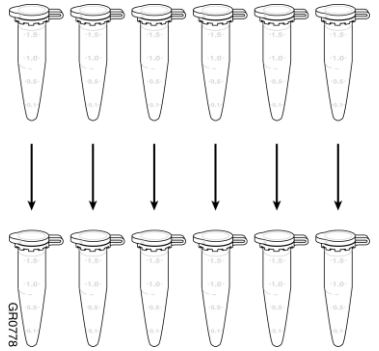
Step Action 2

Prepare Reaction Mix into each 1.5 ml microcentrifuge tube



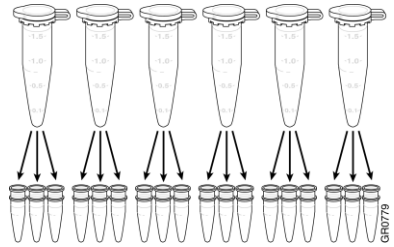
Step Action 3

a. Transfer the volume from each standard dilution and from samples tubes to the new microcentrifuge tube containing the Reaction Mix.
b. Mix the components

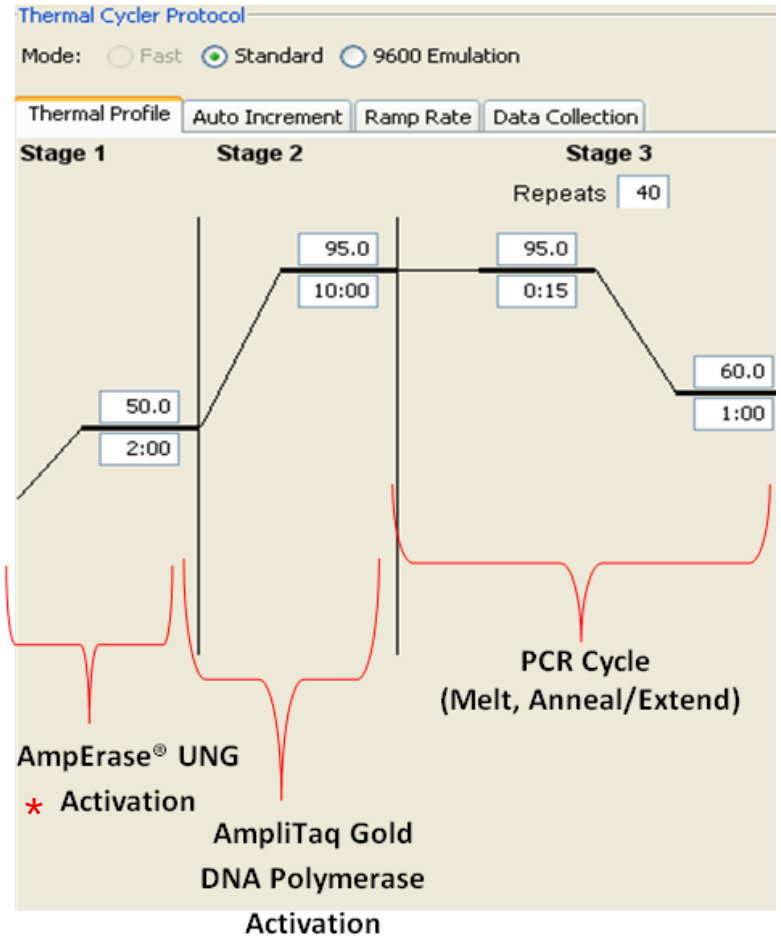


Step Action 4

a. Subdivide each reaction and transfer into the replicate number (**2**, or 3, or 4 aliquots) of a MicroAmp Optical 96-Well Reaction Plate
b. cap the tubes and briefly centrifuge to remove air bubbles and collect the liquid at the bottom of the tube
c. Transfer the plates to the thermal cycler block using the appropriate system for PCR amplification

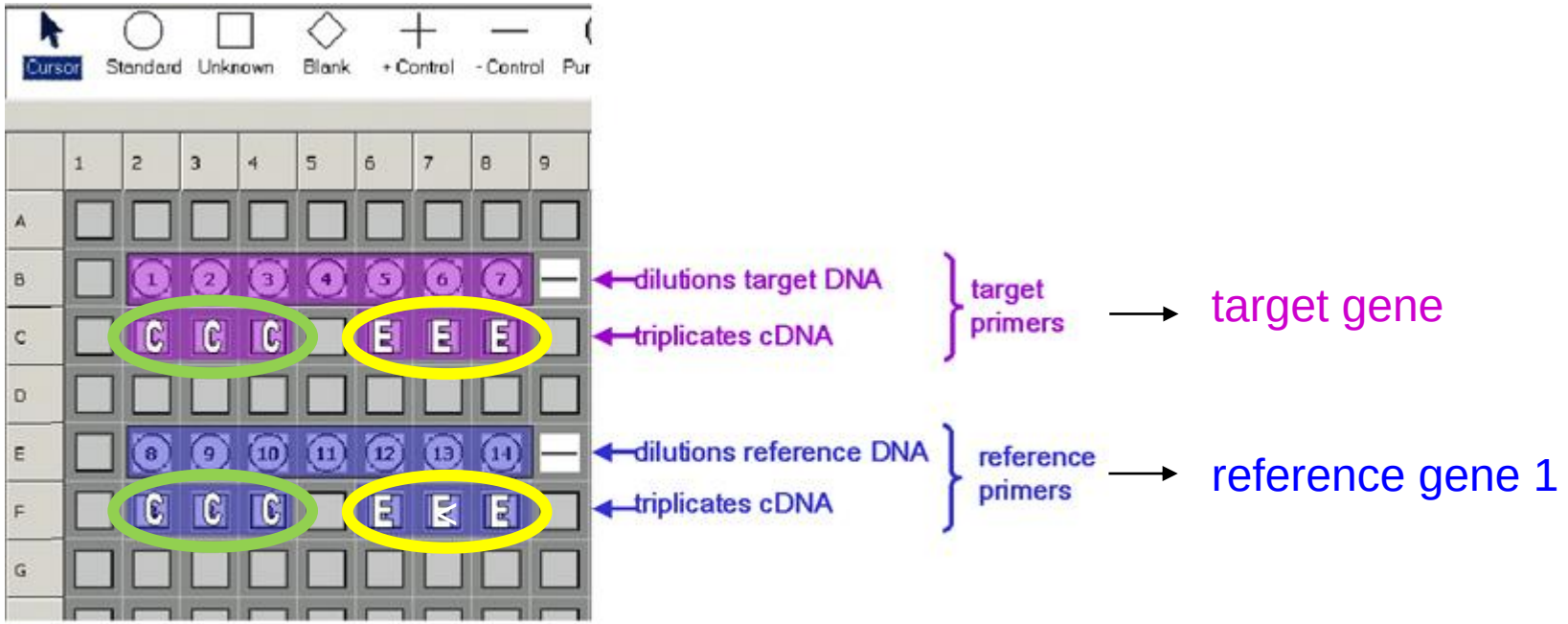


real time Polymerase Chain Reaction: relative quantification

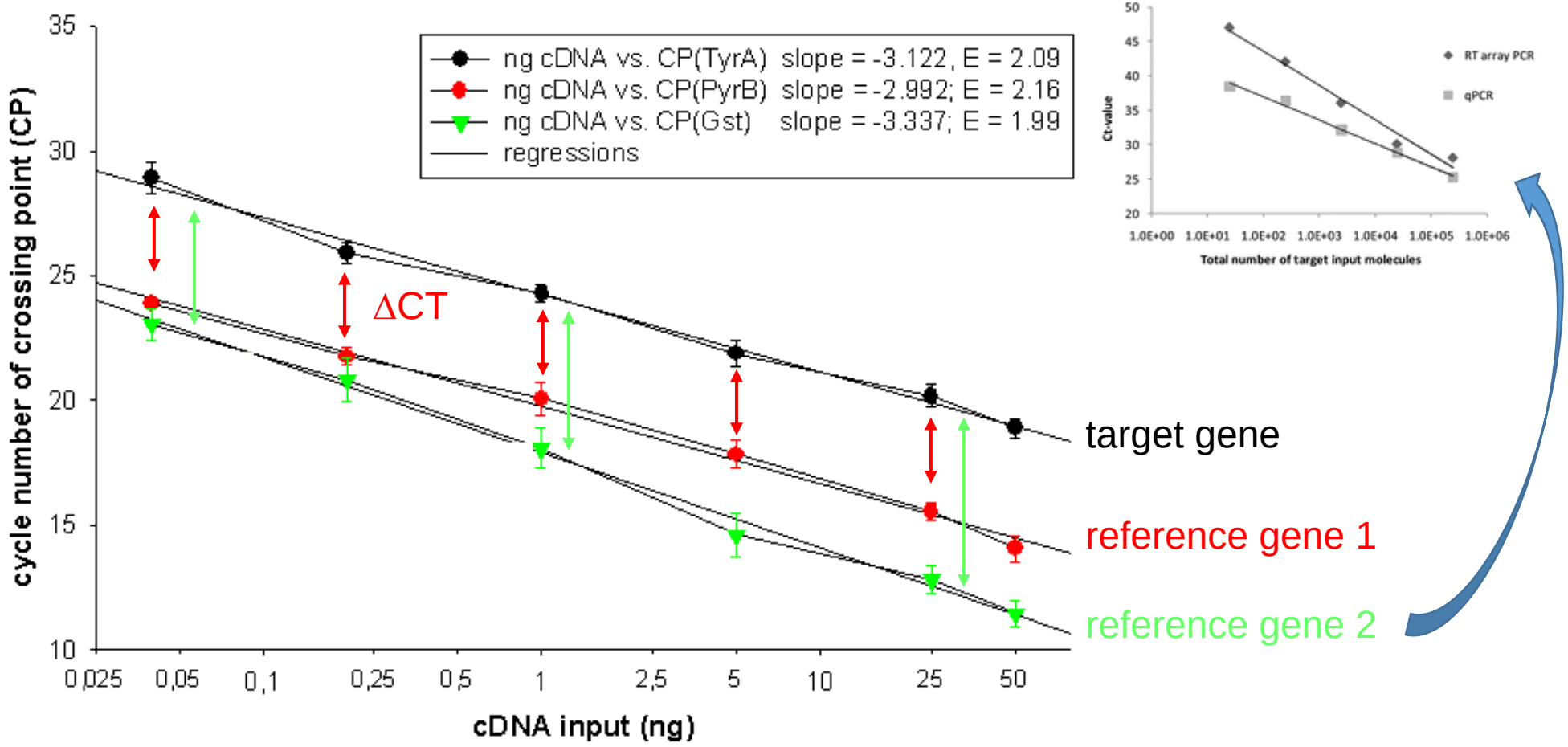


* AmpErase® Uracil N-Glycosylase (UNG) is a 26 kDa, recombinant enzyme encoded by the *E. coli*. The enzymatic reaction specifically degrade PCR products from previous PCR amplifications in which dUTP has been incorporated, without degrading native nucleic acid templates. The method used to render PCR products susceptible to degradation involves substituting dUTP for dTTP in the PCR mixture, and pretreating all subsequent PCR mixtures with AmpErase® Uracil N-glycosylase (UNG) prior to PCR amplification. Products from previous PCR amplifications are eliminated by excising uracil residues using UNG. Although UNG is active on single- and double-stranded dU-containing DNA. AmpliTaq® DNA Polymerase, AmpliTaq Gold® DNA Polymerase, and the other components of the PCR mixture remain intact during UNG treatment, providing PCR amplification free of PCR product carryover.

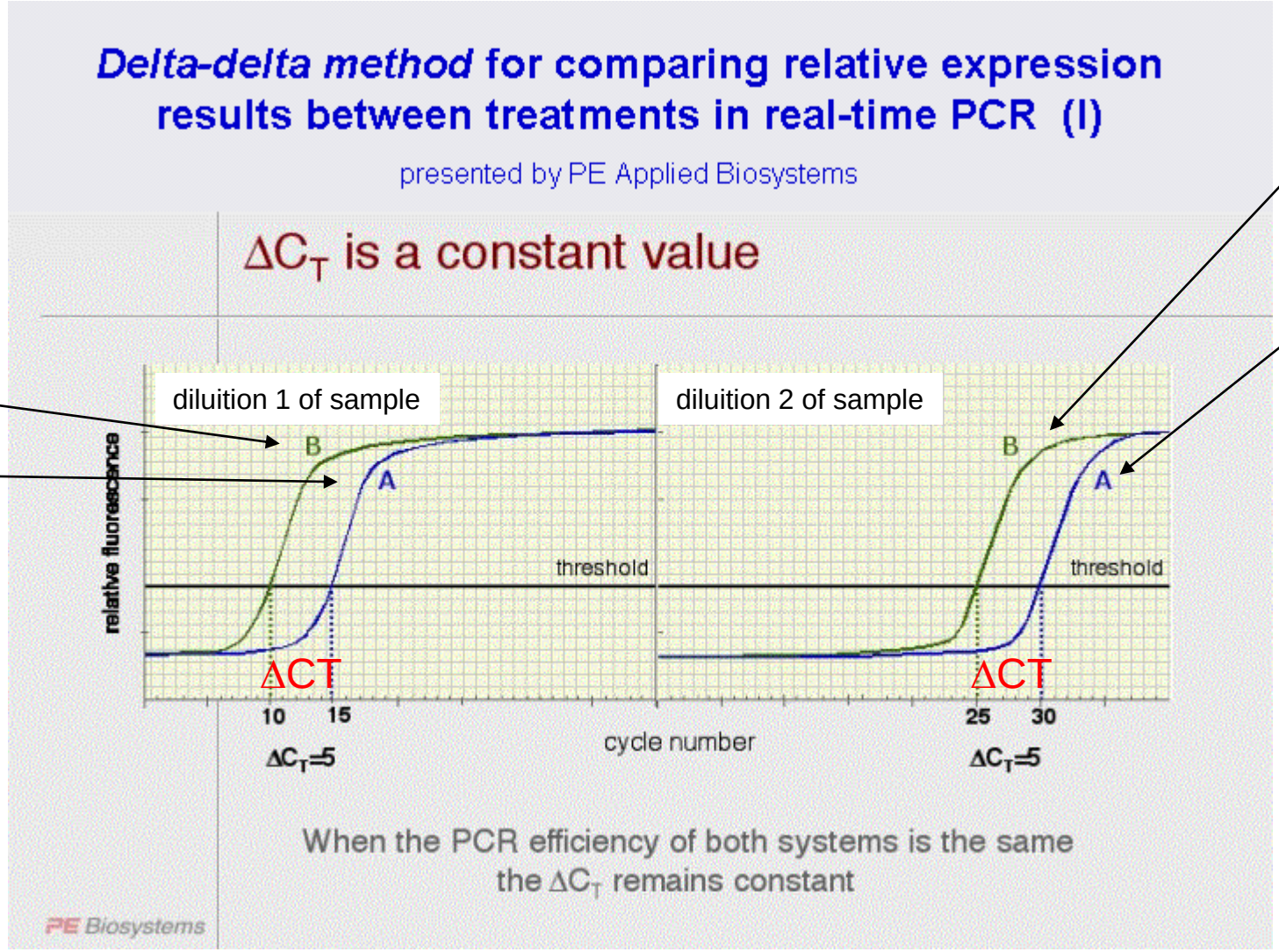
real time Polymerase Chain Reaction: relative quantification



real time Polymerase Chain Reaction: relative quantification



real time Polymerase Chain Reaction: relative quantification



target gene

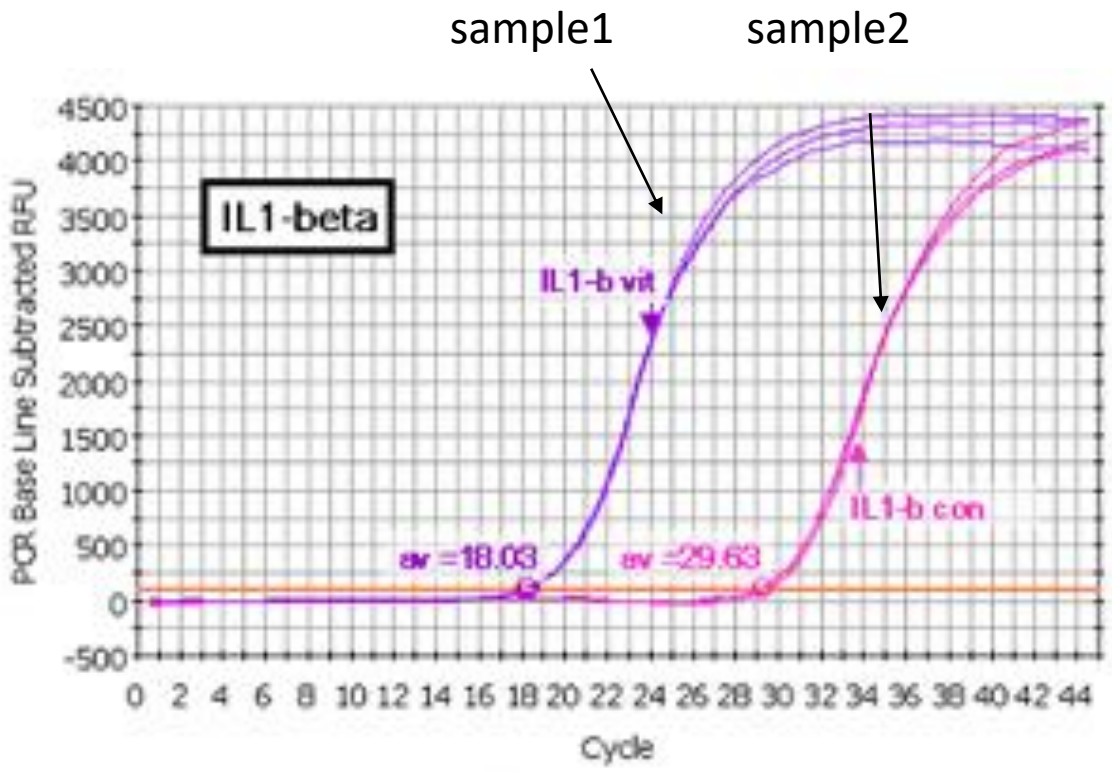
reference gene 1

target gene

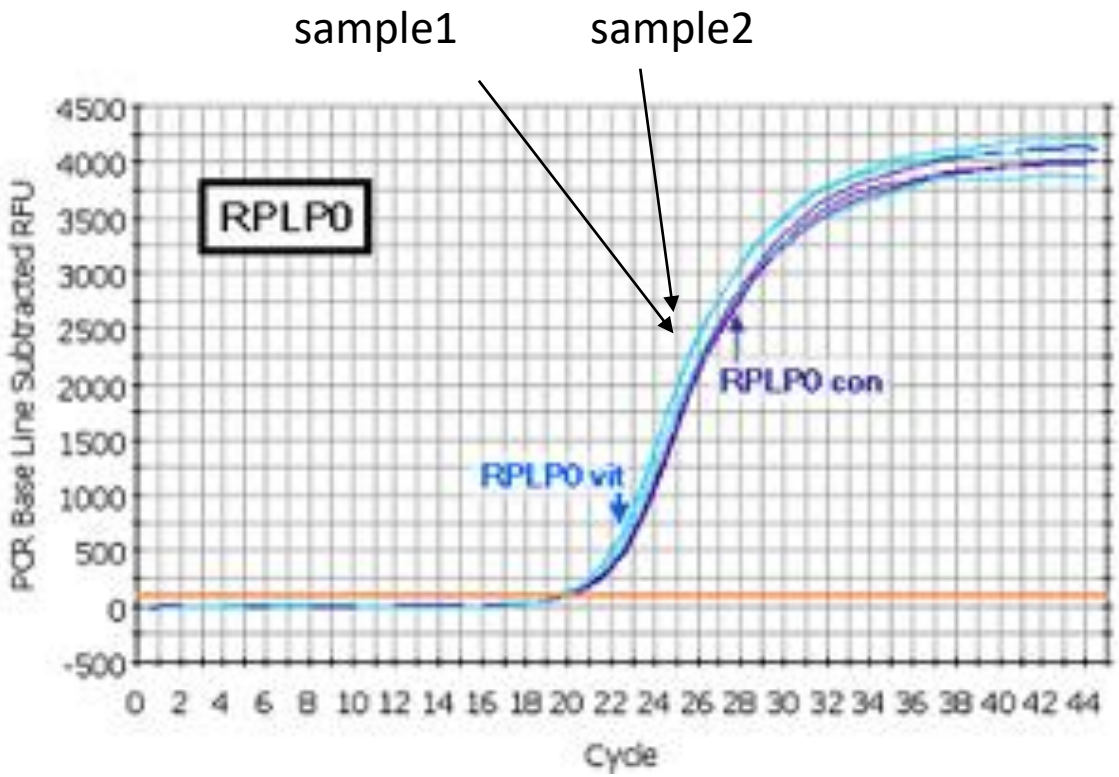
reference gene 1

real time Polymerase Chain Reaction: relative quantification

target gene (gamma-globin gene)



reference gene (18S)



Data Analysis

Vall d'Hebron
Institut de Recerca

RELATIVE QUANTIFICATION: Delta delta Ct (ΔΔCt) method

EXAMPLE:

SAMPLE	GENE	
	Ct p53 (target)	Ct GAPDH (reference)
Control (calibrator)	15.0	16.5
Tumor (test)	12.0	15.9

1) $\Delta Ct_{calibrator} = 15.0 - 16.5 = -1.5$ and $\Delta Ct_{test} = 12.0 - 15.9 = -3.9$

2) $\Delta\Delta Ct = \Delta Ct_{test} - \Delta ct_{calibrator} = -3.9 - (-1.5) = -2.4$

3) $2^{-\Delta\Delta Ct} = 2^{-(-2.4)} = 5.3$

Tumor cells express p53 at a 5.3-fold higher level than control cells

Data Analysis

- **Calcolo dei ΔCT = CT medio gene target - CT medio *housekeeping***

  normalizzare ciascun **campione incognito** sottraendo i CT ottenuti per il gene target a quelli ottenuti per un gene reference endogeno espresso costitutivamente (*housekeeping*).

- **Calcolo dei ΔΔCT = ΔCTi - ΔCTc**

  comparare ciascun ΔCT così ottenuto con il ΔCT di un **campione di riferimento o controllo**, anche detto "calibratore".

- **Calcolare il valore $2^{-\Delta\Delta CT}$**

$$2^{-\Delta\Delta CT} = 2^{-(\Delta CTi - \Delta CTc)}$$

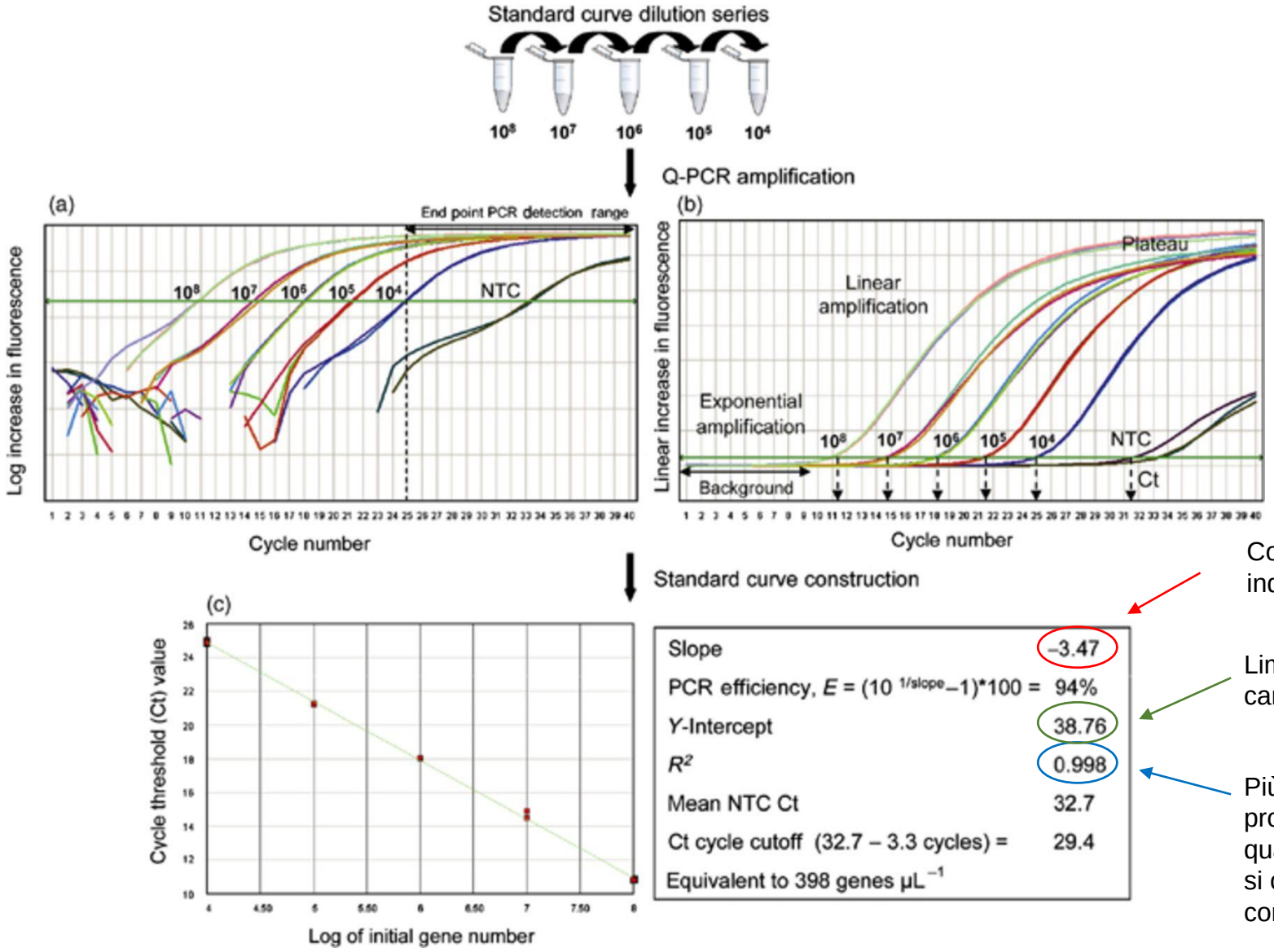
Il valore così ottenuto permette di determinare la concentrazione relativa del gene target nel campione incognito rispetto al campione di controllo

Data Analysis

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

- se $2^{-\Delta\Delta CT} < 0,5$ → il gene target nel campione incognito è ipoespresso rispetto al campione di riferimento
- se $0,5 < 2^{-\Delta\Delta CT} < 2$ → non c'è differenza di espressione
- se $2^{-\Delta\Delta CT} > 2$ → gene target è più espresso rispetto al campione di riferimento

real time Polymerase Chain Reaction: absolute quantification



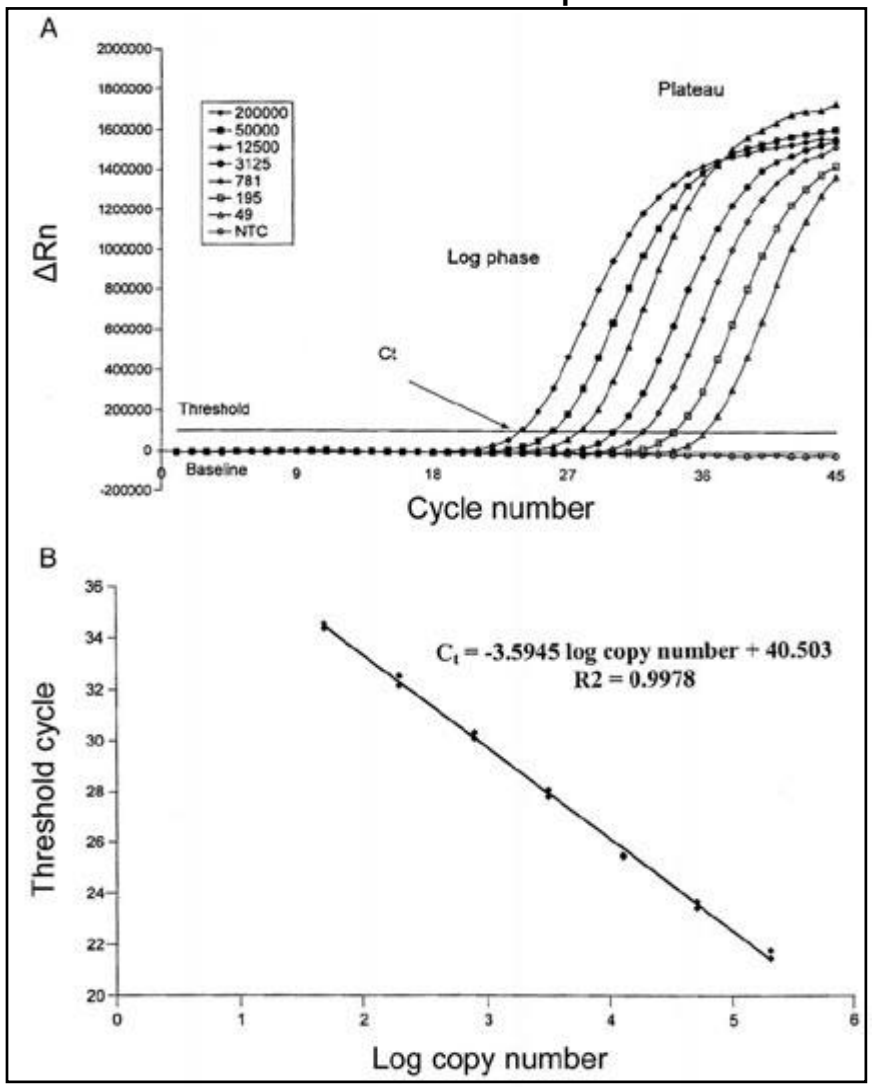
Coefficiente angolare della retta, indica la pendenza

Limite di quantità di campione rilevabile

Più è vicino a 1, più vi è una proporzionalità diretta tra quantità di DNA e CT (al quale si ottiene la fluorescenza scelta come riferimento o soglia)

real time Polymerase Chain Reaction: absolute quantification

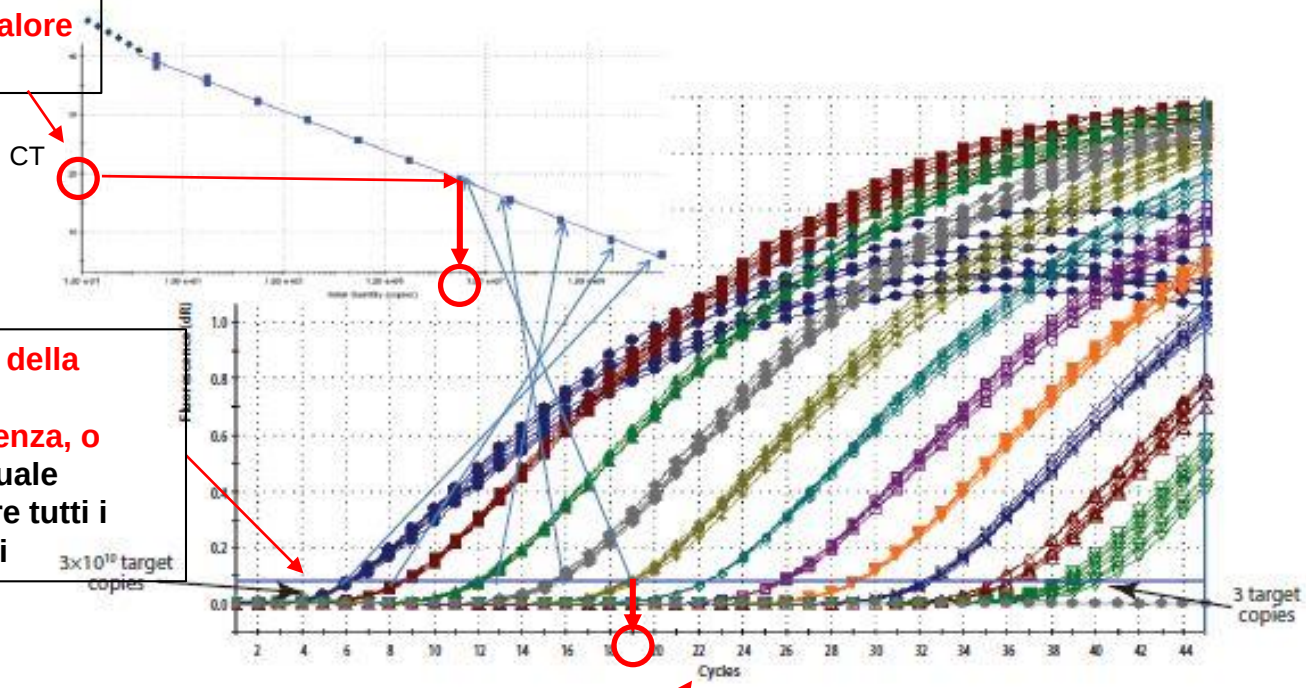
A. Standard template



B. analized samples

3. Si analizza sulla retta standard lo stesso valore di CT

4. si ricava il corrispondente valore di log (ng)



1. Scelta della soglia di fluorescenza, o ΔRu al quale analizzare tutti i campioni

2. Il campione interseca la linea di soglia, si ricava il CT corrispondente a quel valore di ΔRu

real time Polymerase Chain Reaction: absolute quantification

Log ng= y → ng= 10^{y} → gr= ng x 10^{-9} → gr= moli/PM

moli= gr/PM PM= 650xKb

moli= gr/650xKb

N_A = $6{,}023 \cdot 10^{23}$

1 mole= n° molecole x $6{,}023 \times 10^{23}$

gr/650xKb/$6{,}023 \times 10^{23}$=n° molecole di DNA templato

Determination principles of real-time PCR efficiency

Direct methods:

- Dilution series

  (Rasmussen 2001, Peirson et al. 2003, etc.)

- Determination of absolute increase in fluorescence

  (Rasmusen 2001; Peccoud & Jacob 1998; Pfaffl 2001)

Indirect methods: Fit of mathematical models

- Sigmoidal model

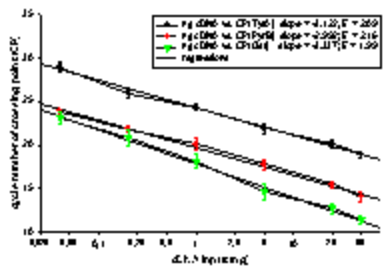
  (Lui & Saint 2002; Tichopad et al. 2002 & 2004)

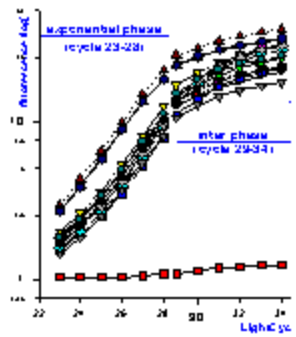
- Logistic model

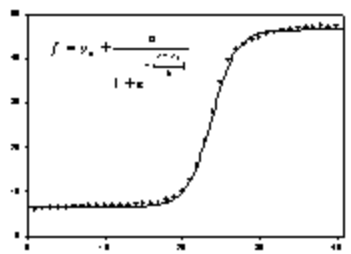
  (Wittwer et al. 2000; Tichopad et al. 2003)

- Comparative Quantitation Analysis

  Rotor-Gene 3000 software

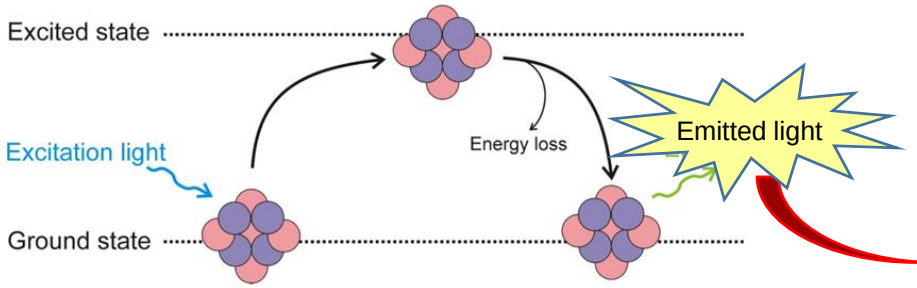


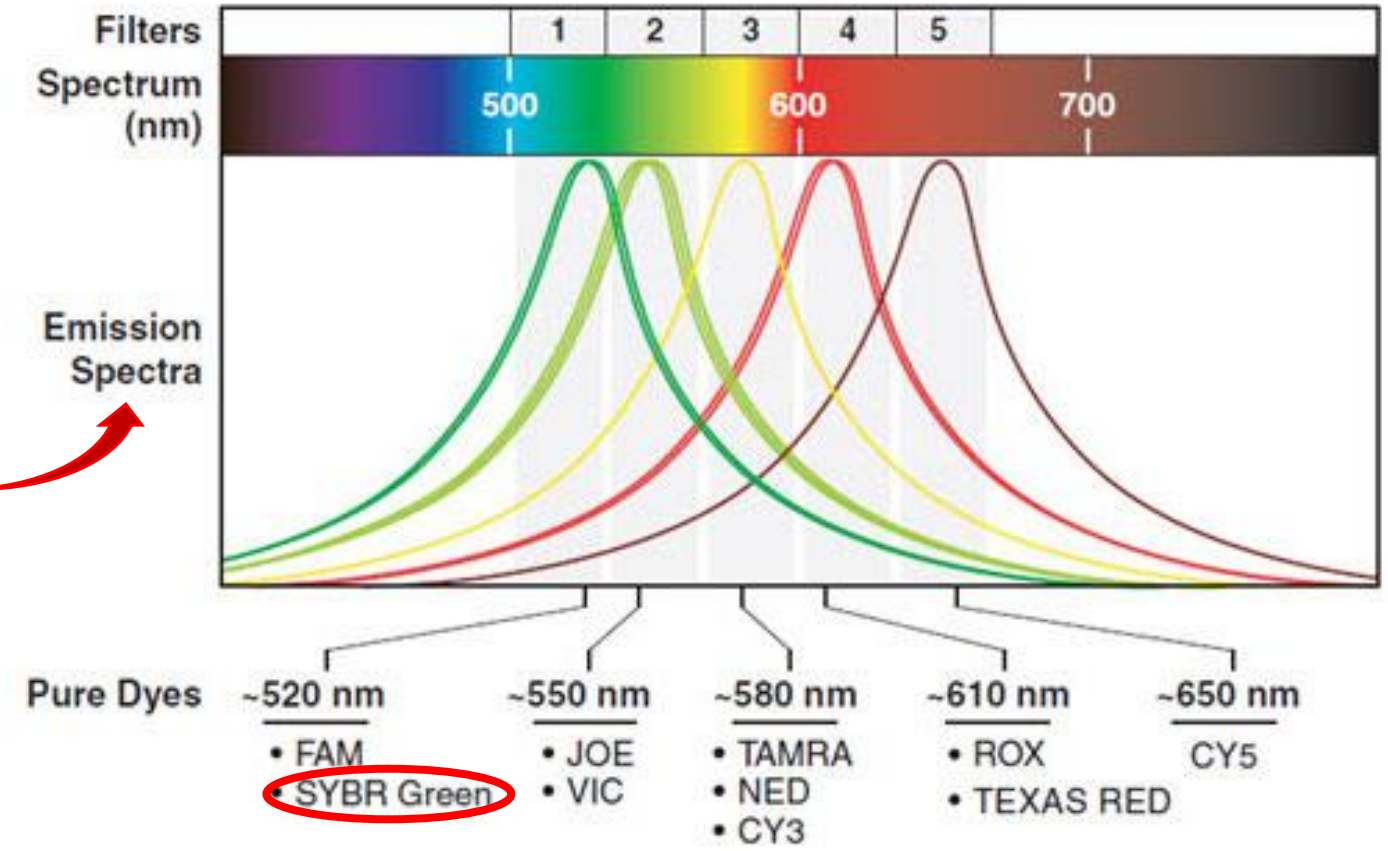




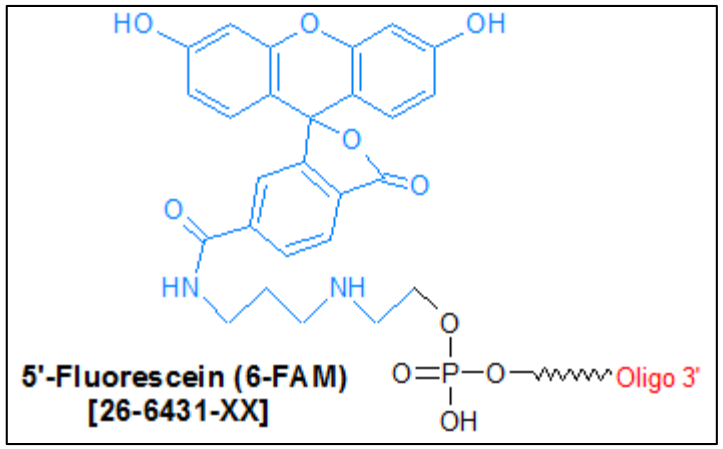
Fluorescent molecules used in quantitative real time PCR

What is fluorescence?

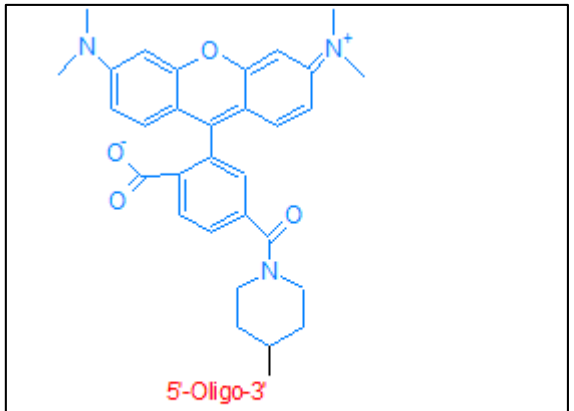




Fluorescent molecules used in quantitative real time PCR



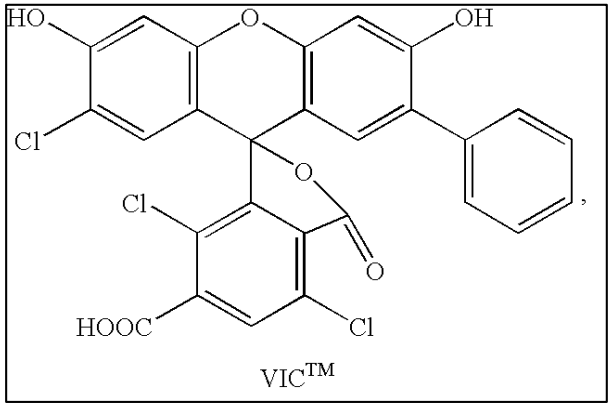
5'-Fluorescein (6-FAM)
[26-6431-XX]



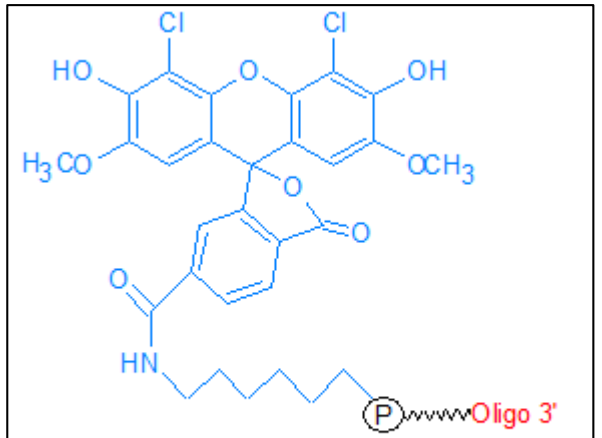
3'-TAMRA (Carboxytetramethylrhodamine)
[26-6451-XX]

5'-reporter, excitation max, emission max			3'-quencher, quenching range (quenching max)					
			BHQ-1®	BHQ-2®	BHQ-3®	ECLIPSE	DABCYL	TAMRA
			480-580 nm (535 nm)	550-650 nm (579 nm)	620-730 nm (672 nm)	390-625 nm (522 nm)	380-550 nm (453 nm)	470-560 nm (544 nm)
LC®Cyan500	450 nm	500 nm	+	-	-	+	+	+
FAM	495 nm	520 nm	+	-	-	+	+	+
TET	521 nm	536 nm	+	-	-	+	+	+
JOE	522 nm	548 nm	+	-	-	+	+	+
Yakima Yellow	530 nm	549 nm	+	-	-	+	+	+
HEX	535 nm	556 nm	+	+	-	+	+	+
Cy3	546 nm	563 nm	-	+	-	-	-	-
TAMRA	564 nm	579 nm	-	+	-	+	-	-
ROX	576 nm	601 nm	-	+	-	+	-	-
Texas Red	586 nm	610 nm	-	+	-	+	-	-
LC®Red610	590 nm	610 nm	-	+	-	+	-	-
LC®Red640	625 nm	640 nm	-	+	+	-	-	-
Cy5	646 nm	662 nm	-	+	+	-	-	-
Cy5.5	683 nm	705 nm	-	+	+	-	-	-
IRD700	685 nm	705 nm	-	+	+	-	-	-

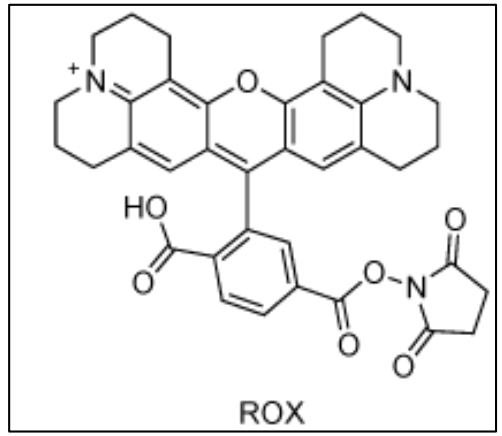
Fluorescent molecules used in quantitative real time PCR



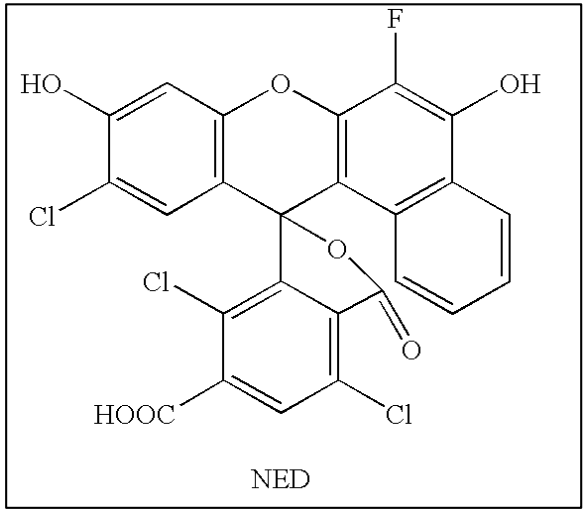
VIC™



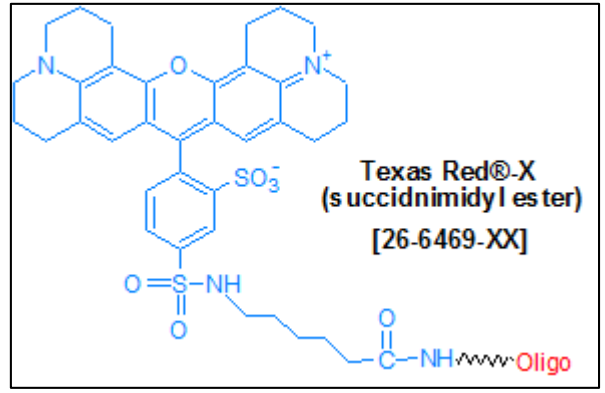
4-5-Dichloro carboxy fluorescein (Joe)
[26-6467-XX]



ROX

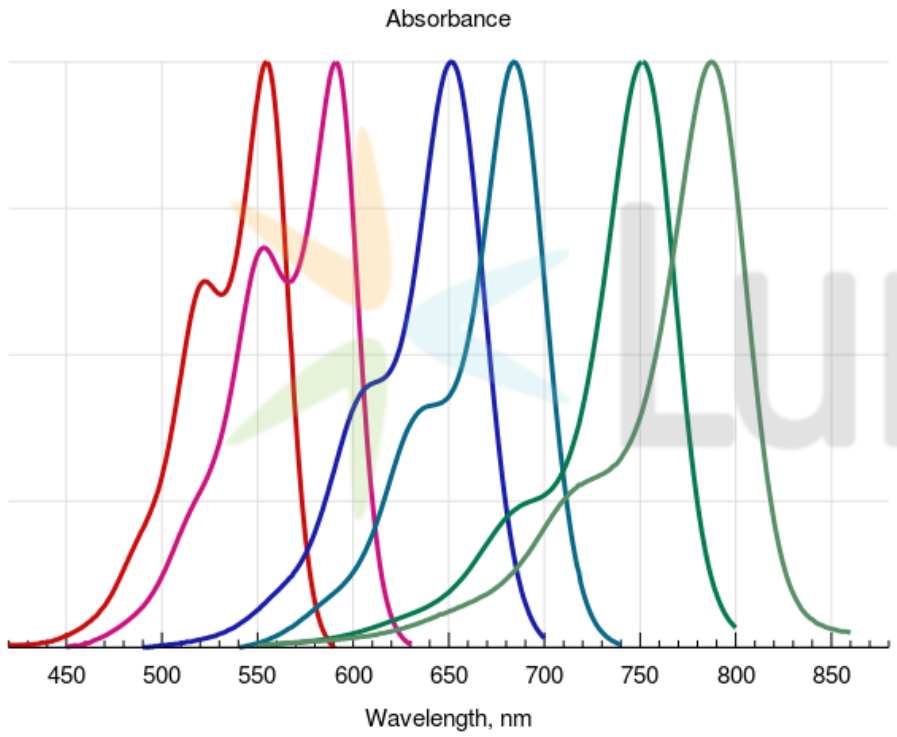


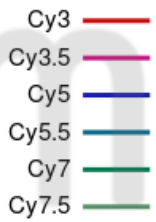
NED

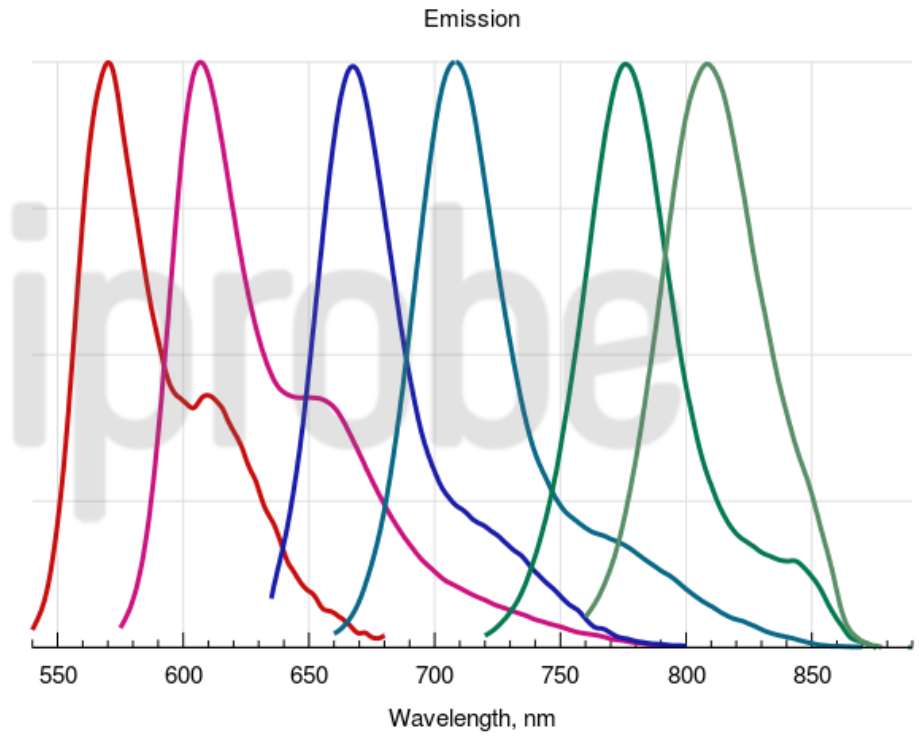


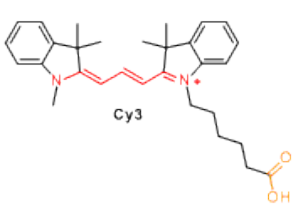
Texas Red®-X
(succidnimidyl ester)
[26-6469-XX]

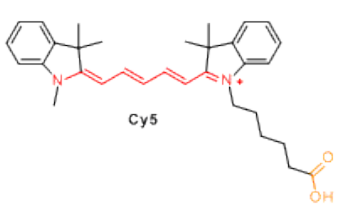
Assorbance and emission spectra of non-sulfonated cyanidine dyes

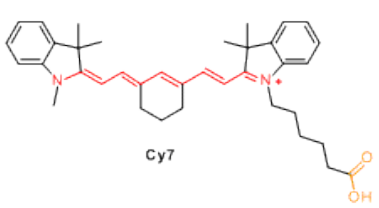


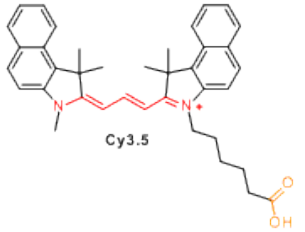


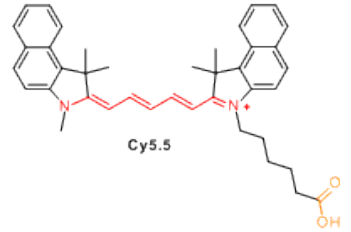


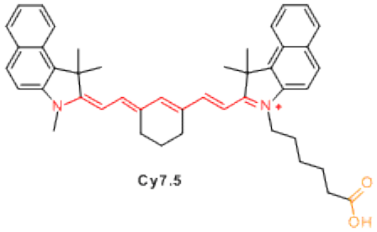








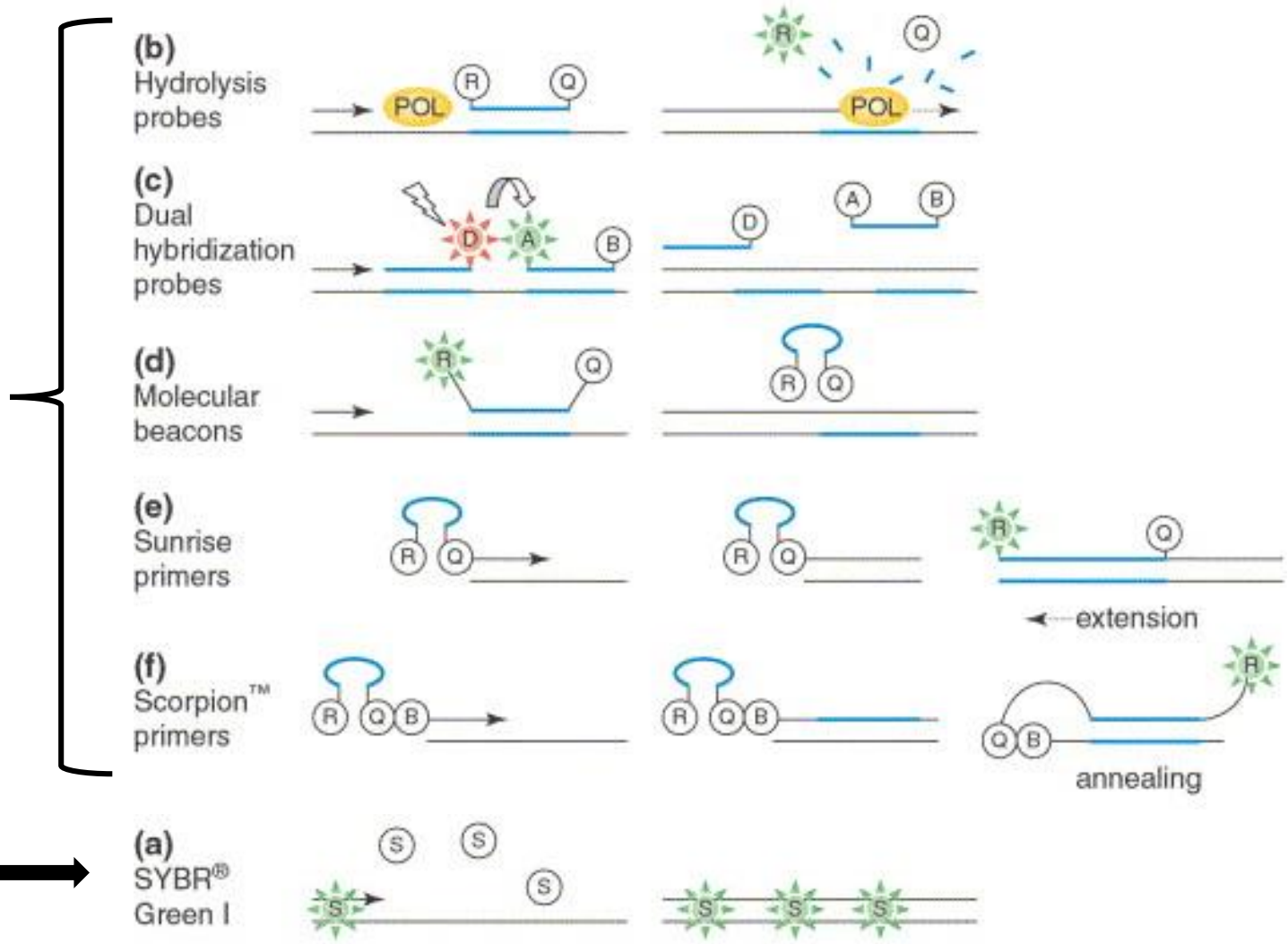




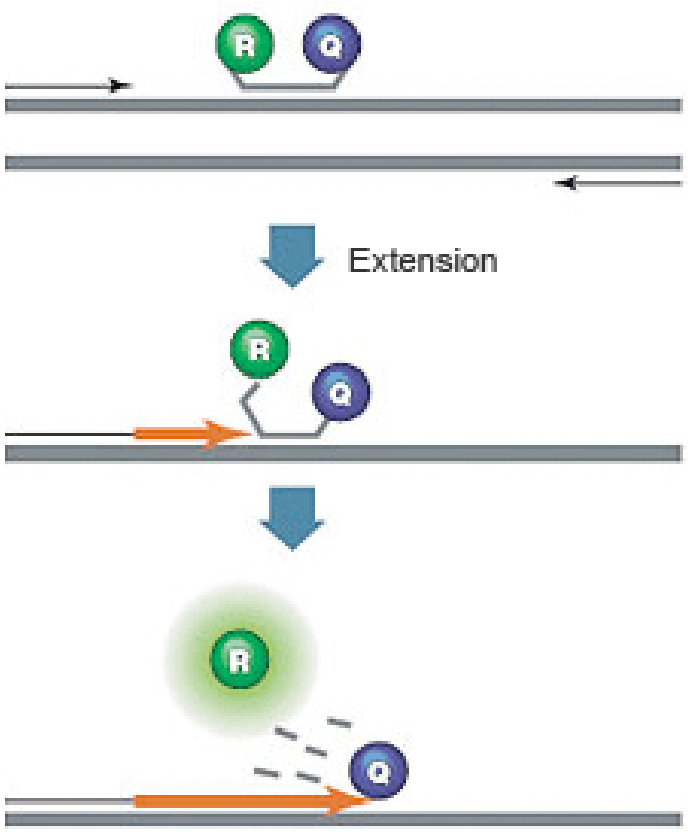
Fluorescent molecules used in quantitative real time PCR

1. Utilizzo di sonde oligonucleotidiche marcate con molecole fluorescenti e leganti specifiche sequenze di DNA bersaglio

2. Utilizzo di molecole fluorescenti che si intercalano in modo aspecifico tra le basi di DNA negli amplificati della reazione di PCR



Hydrolysis probes: use of dual-labeled fluorogenic gene-specific probe called also TaqMan probe



1

2

3

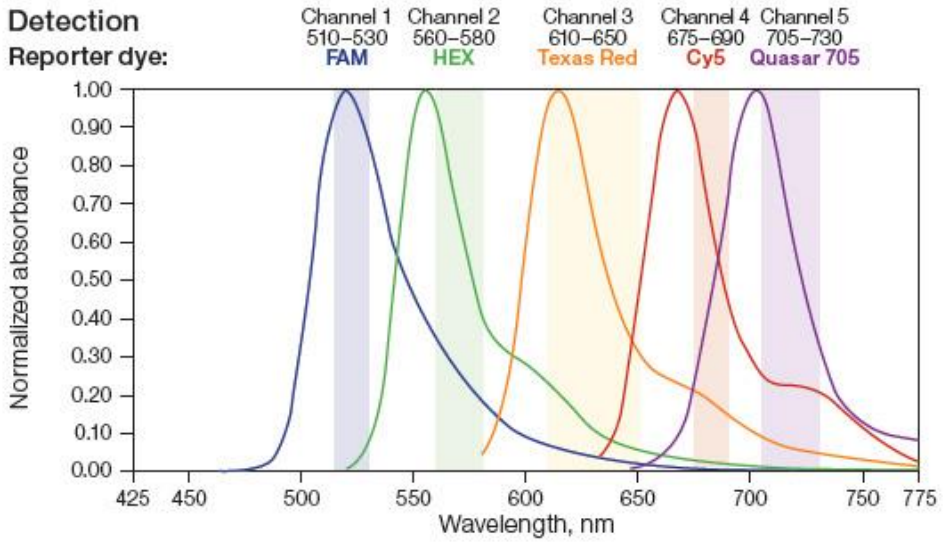
1. This probe is composed of a short (20-25 bases) oligodeoxynucleotide that is labeled with two different fluorescent dyes.

2. On the 5' terminus is a reporter dye and on the 3' terminus is a quenching dye. This oligonucleotide probe sequence is homologous to an internal target sequence present in the PCR amplicon.

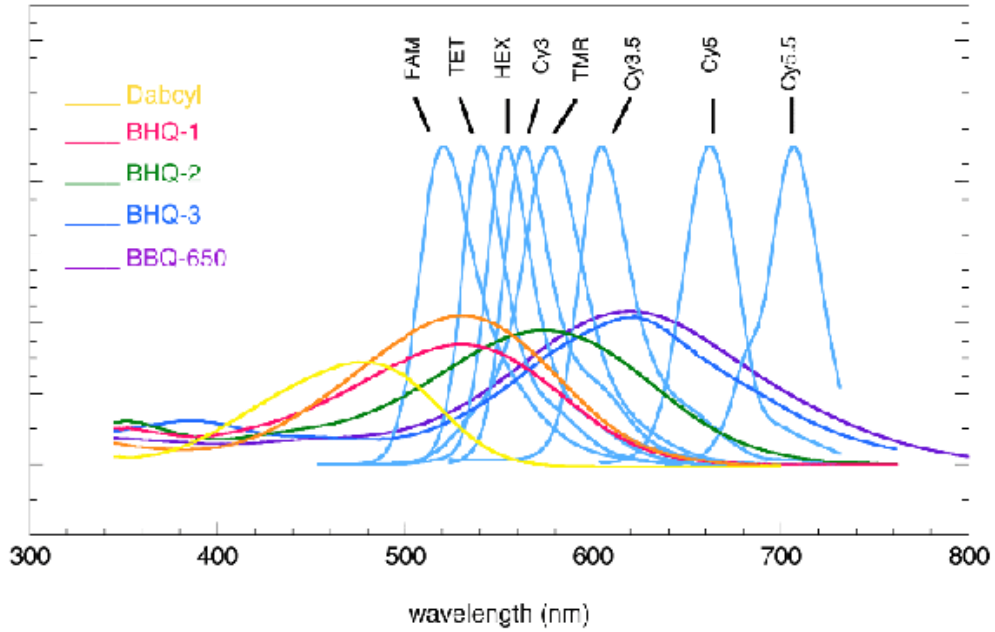
3. When the probe is intact, energy transfer occurs between the two fluorophors and emission from the reporter is quenched by the quencher. During the extention phase of PCR, the probe is cleaved by 5' nuclease activity of Taqpolymerase thereby releasing the reporter from the oligonucleotide-quencher and producing an increase in reporter emission intensity.

Hydrolysis probes: use of dual-labeled fluorogenic gene-specific probe called also TaqMan probe

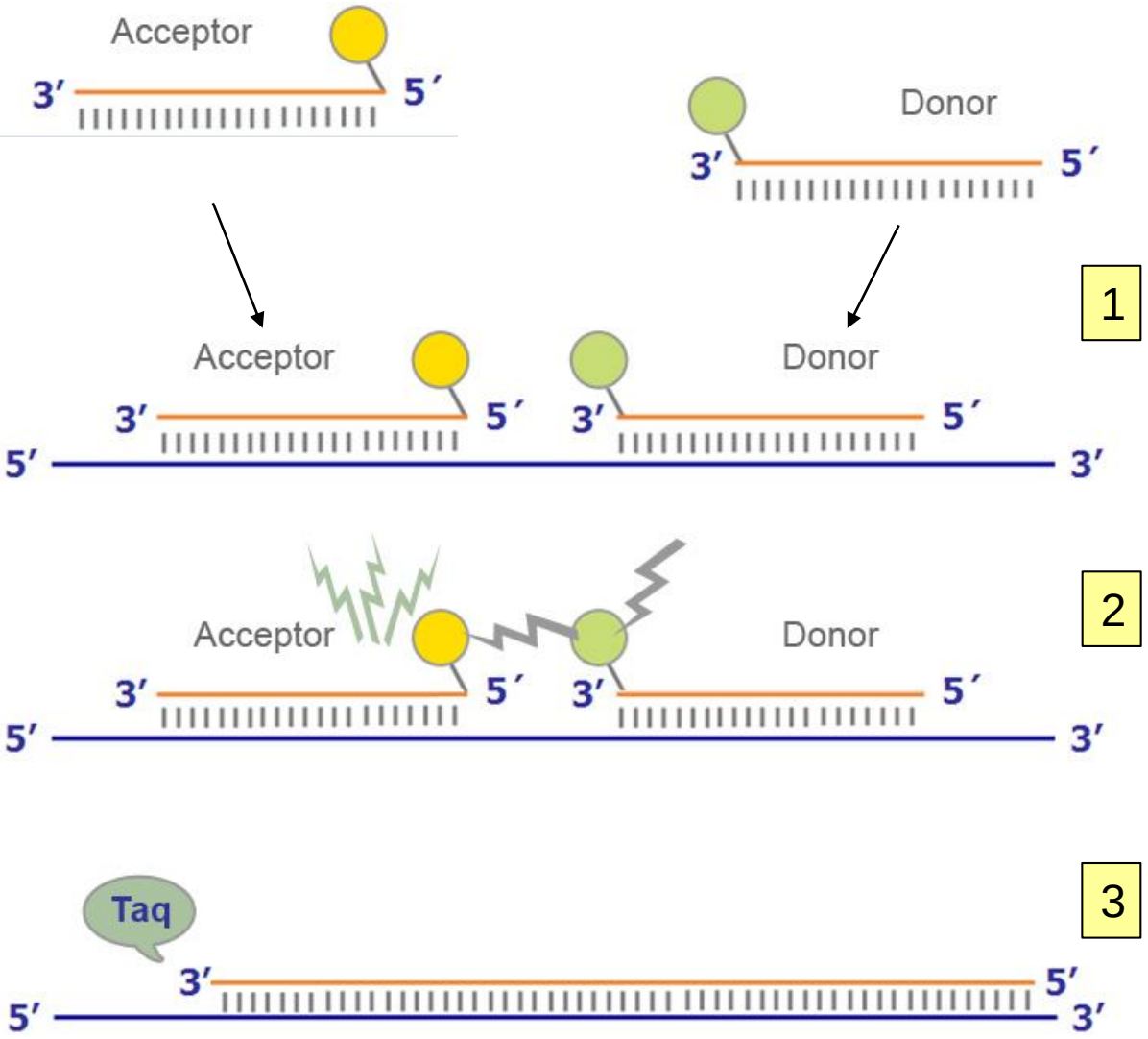
Reporter



Quencer



FRET probe (Fluorescence Resonance Energy Transfer): dual hybridization probes

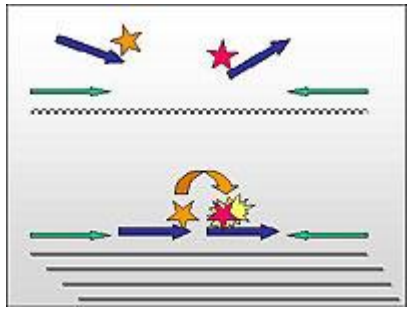


1. Simili alle sonde TaqMan perché si legano al DNA bersaglio e vengono idrolizzate, questo sistema prevede però l'impiego di due sonde ognuna marcata con un solo fluorocromo (accettore e donatore). **Quando le sonde non sono legate alle sequenze target il segnale fluorescente proveniente dall'accettore non è rilevato**

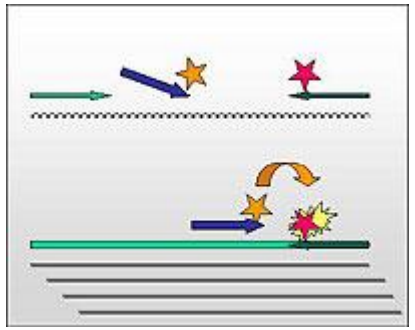
2. **Durante lo *step di annealing***, entrambe le sonde FRET ibridizzano alle sequenze target: ciò avvicina il fluoroforo donatore al fluoroforo accettore permettendo il **trasferimento di energia tra i due fluorofori** e la produzione di un segnale fluorescente da parte dell'accettore che viene così rilevato

3. La polimerasi durante la fase di estensione rimuove le sonde e sintetizza il filamento complementare

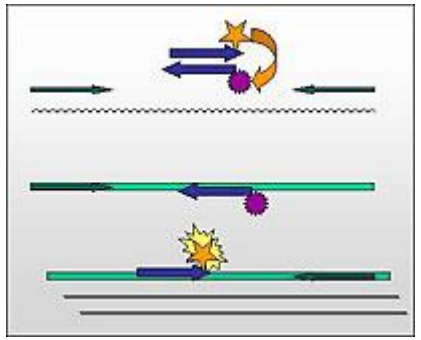
FRET probe (Fluorescence Resonance Energy Transfer): dual hybridization probes



There are various possibilities to allow adjacent hybridization of oligonucleotides. Probably the most popular and successful method is the binding of **two single labeled probes in a head to toe manner**, also known as "**kissing probes**" or HybProbe. The FRET induced fluorescence of the acceptor dye is detected and measured. An advantage over the hydrolysis probes is their modular assembly and for quantitative PCR, their relative robustness towards single base variations; but, probably the most outstanding feature is their excellent suitability **for genotyping**. A disadvantage is the need for a larger sequence area necessary to accommodate two adjacent probes.

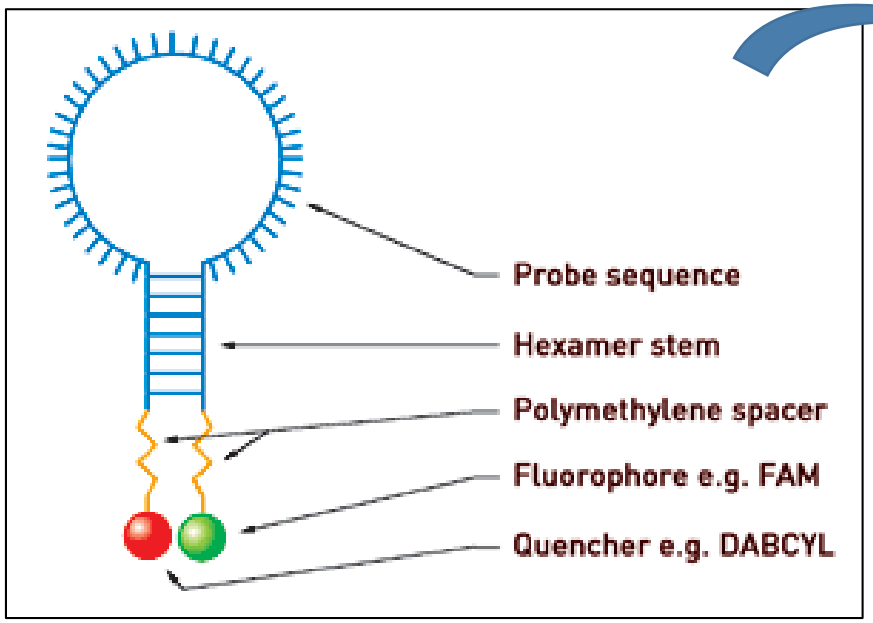


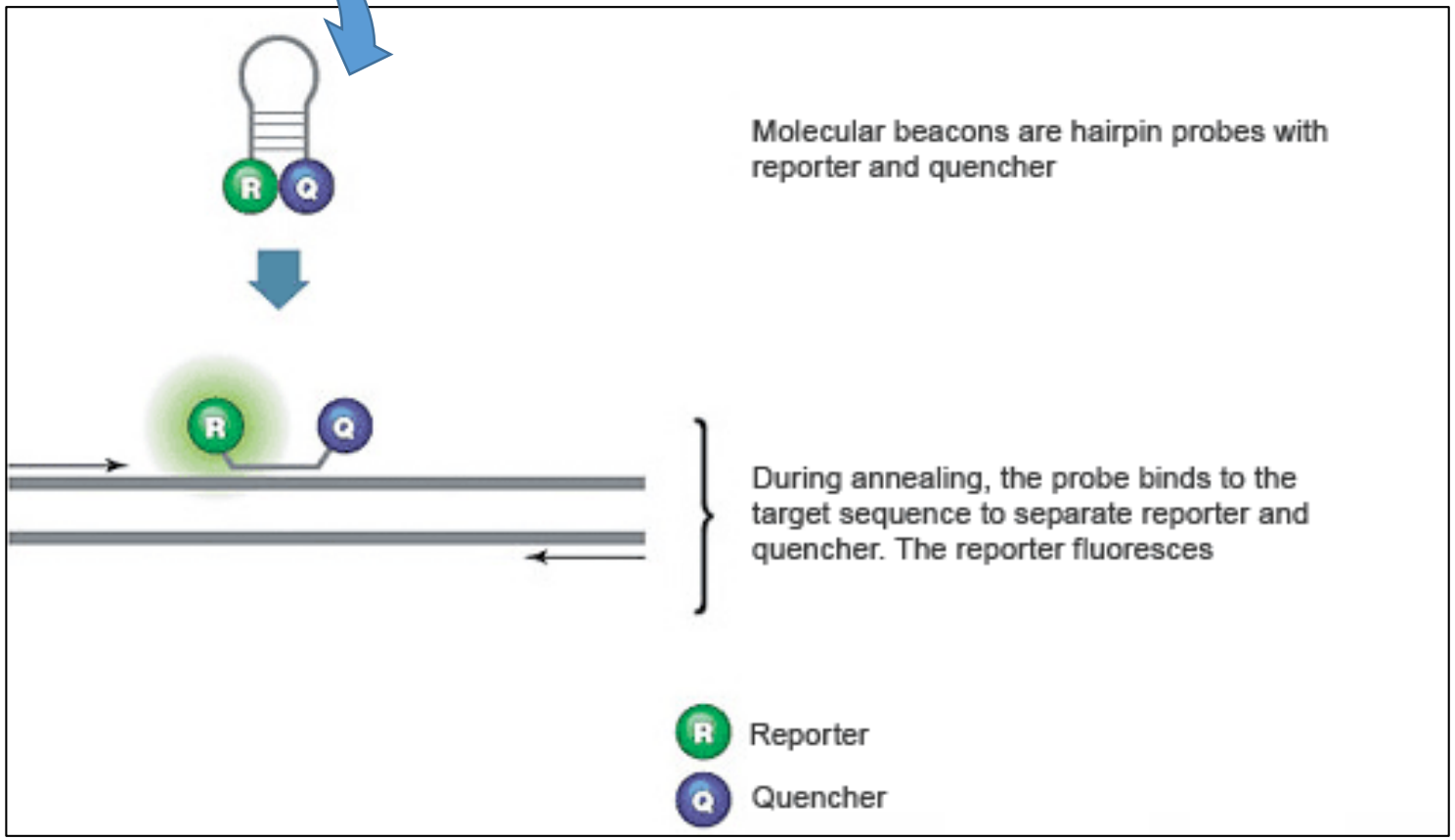
A variation of the preceding format is the replacement of **one of the probes by a labeled amplification primer**. A labeled probe that binds to the strand containing the extended primer provides the FRET reaction necessary for the detection.



Furthermore, **self-complementary oligonucleotides**, one labeled with a fluorophore the other with a quencher have been used **to monitor a PCR reaction**. The increase of target concentration causes a **primer binding equilibrium** that distances the quencher from the reporter.

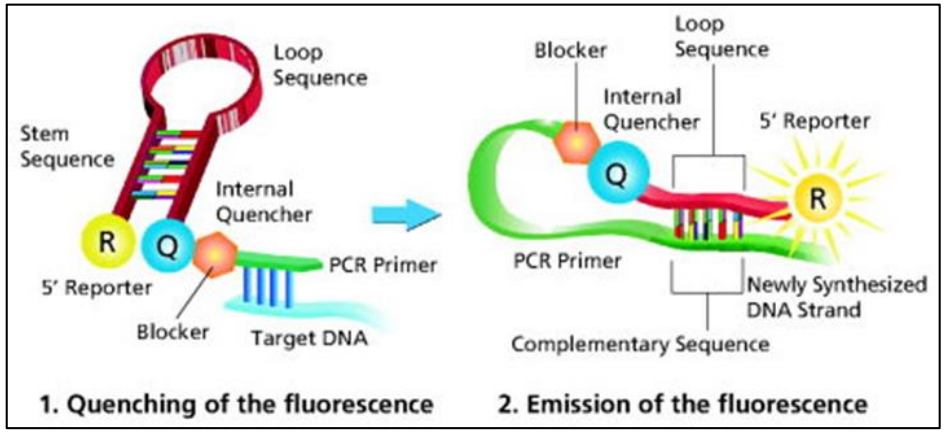
FRET probe (Fluorescence Resonance Energy Transfer): molecular beacons



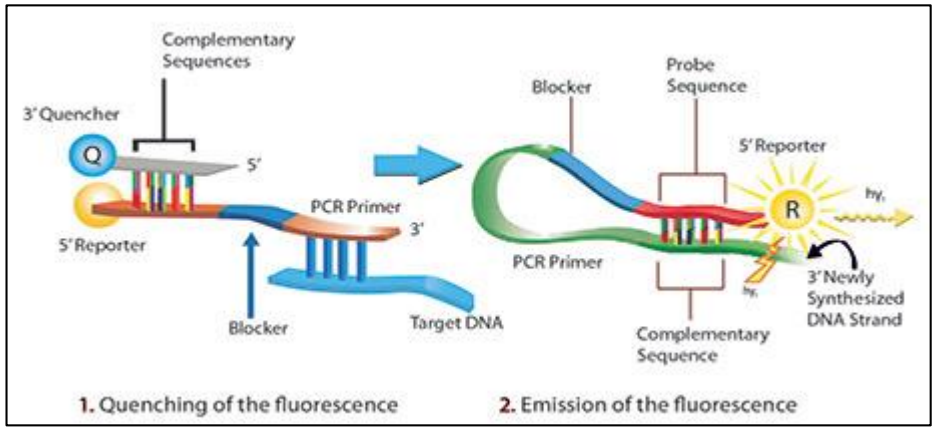


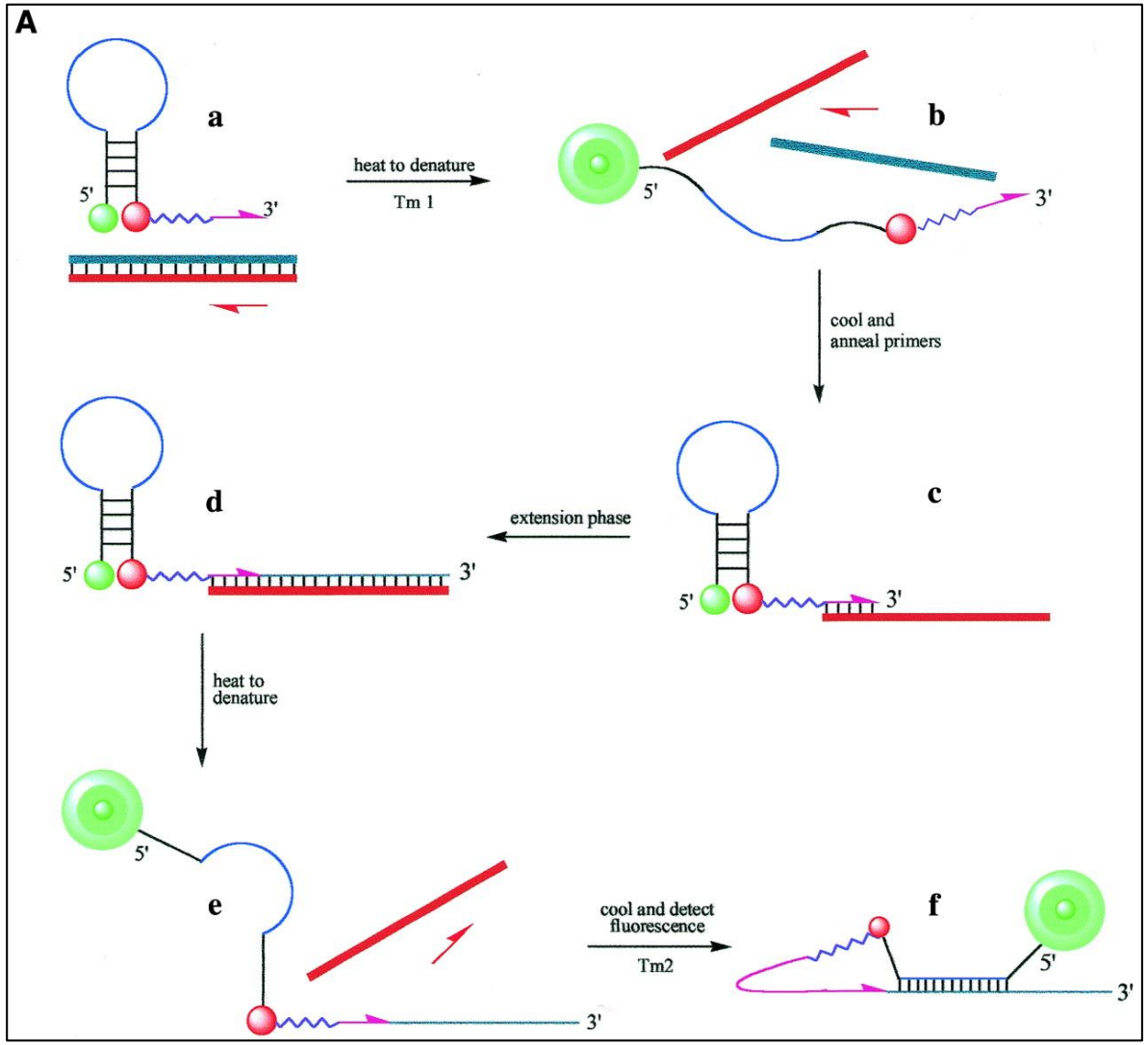
FRET probe (Fluorescence Resonance Energy Transfer): scorpion primers

uni-probe

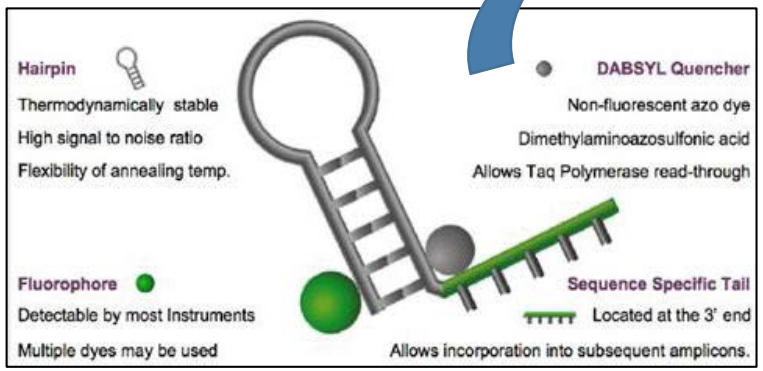


bi-probe

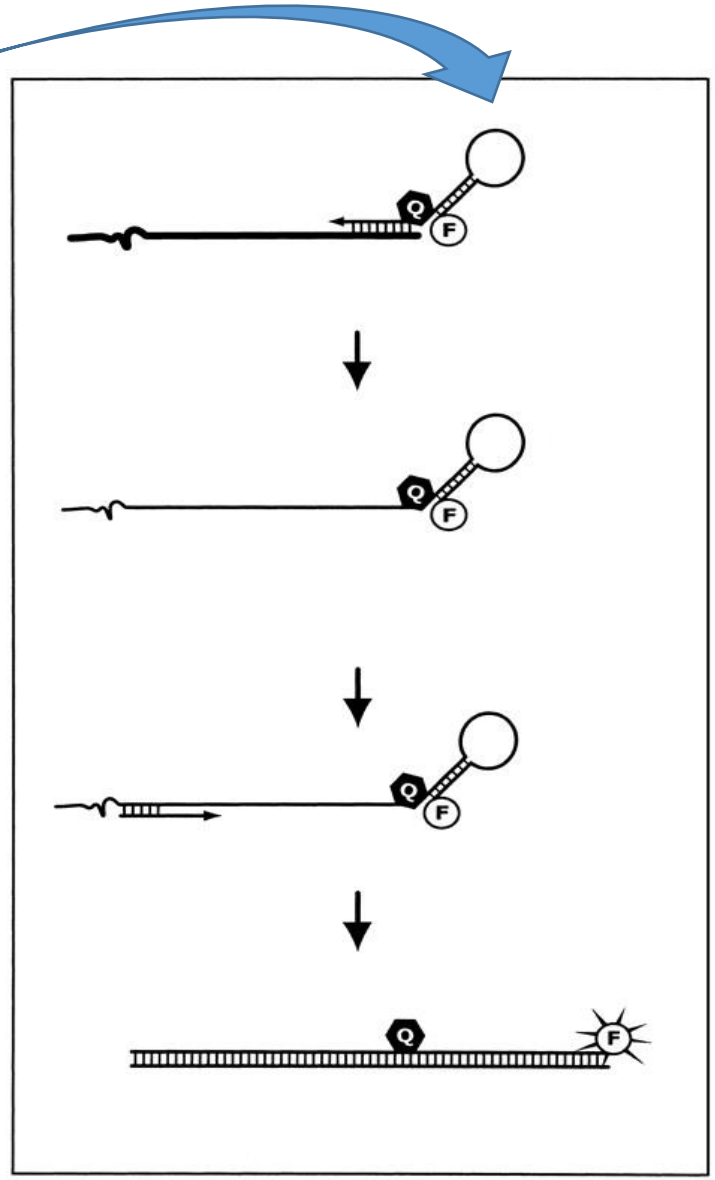




FRET probe (Fluorescence Resonance Energy Transfer): sunrise primers



Sunrise primers are similar to Molecular Beacons and Scorpions primer probes, **which combine in the same molecule both the PCR primer and detection mechanism**



1. The Sunrise primer-probes have reporter dyes attached to 3’ end of the stem and quenchers attached to the 5’ end of the stem

2. These probes consist of a dual-labeled (reporter and quencher fluorophores) hairpin loop on the 5′ end, with the 3′ end acting as the PCR primer. When unbound, the hairpin is intact, causing reporter quenching via FRET

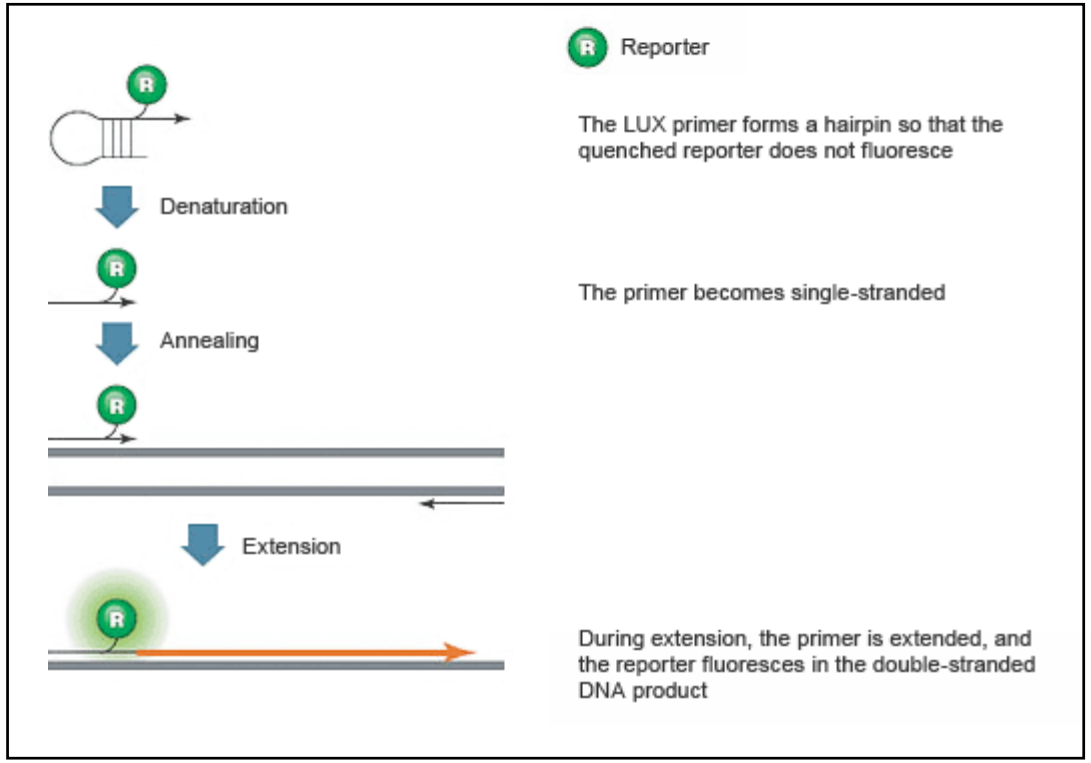
3. They are self-complementary and dissociate through the synthesis of the complementary strand.

4. Upon integration into the newly formed PCR product, the reporter and quencher are held far enough apart to allow reporter emission

FRET probe (Fluorescence Resonance Energy Transfer): LUX™ fluorogenic primers

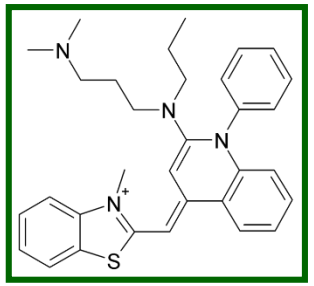
I primer fluorogenici LUX ™ sono primer «spenti» a single-fluoroforo quasi identici ai Sunrise.
Poiché questa chimica non richiede un colorante quencher, è molto meno costoso di sonde marcate con due molecole.

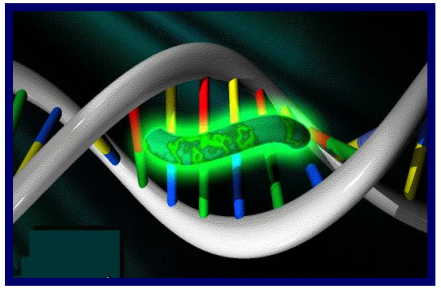
Poichè questo sistema si basa su due oligonucleotidi per la specificità di sequenza, e, a differenza della piattaforma SYBR Green in cui una curva di dissociazione è utilizzata per rilevare l'amplificazione erronea non specifica, non esiste tale rilevamento per la piattaforma LUX, è necessario controllare i prodotti di PCR ottenuti su gel di agarosio.



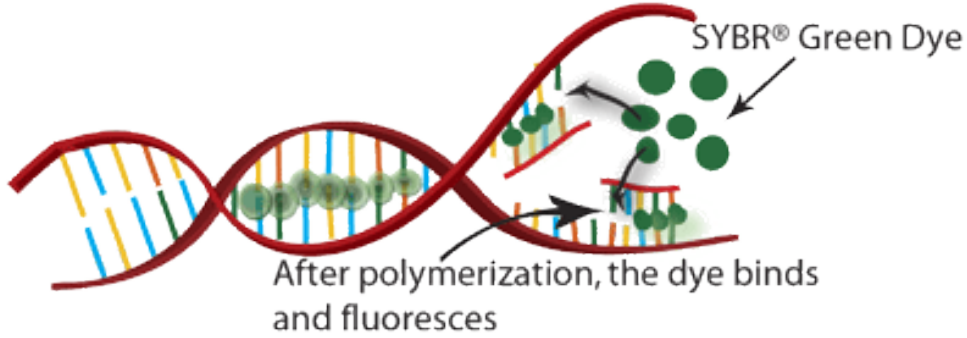
SYBR Green I

1. Il SYBR Green I è una molecola fluorescente

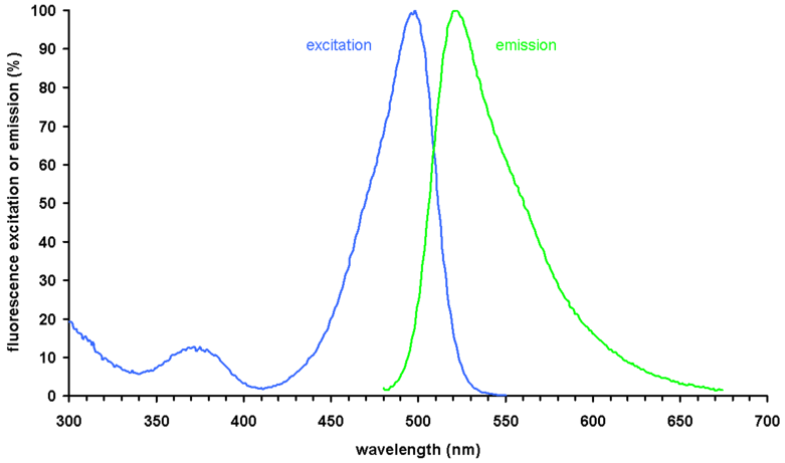




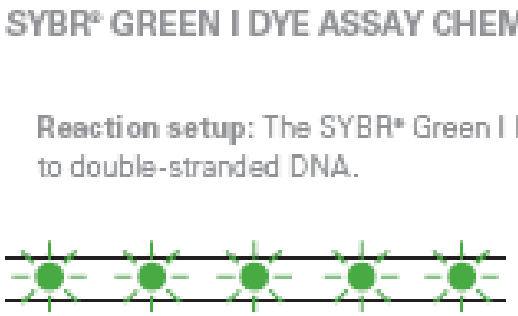
2. agente intercalante e si lega preferenzialmente a DNA a doppio filamento (dsDNA)



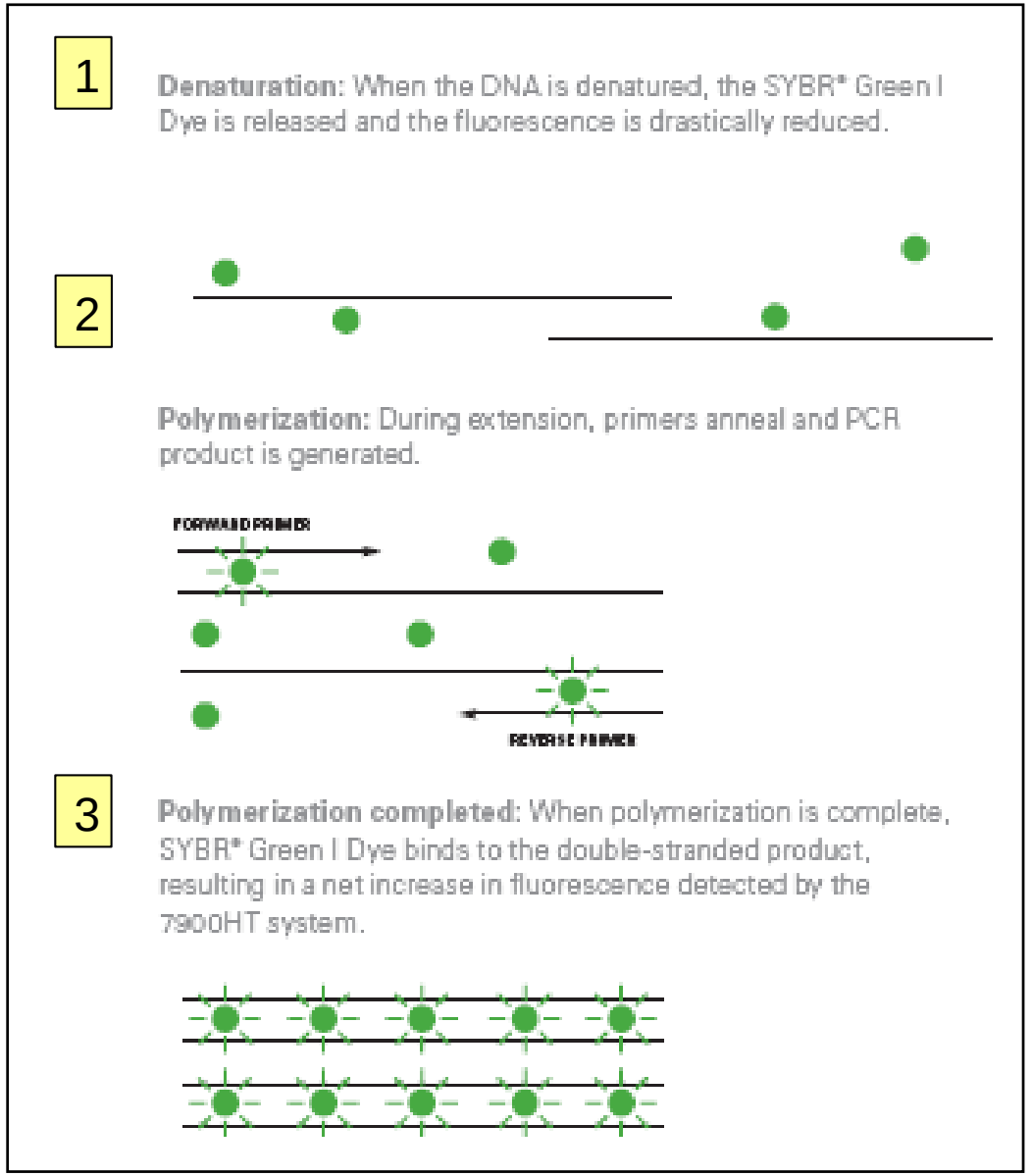
3. formando un complesso DNA-colorante che assorbe luce blu ad una lunghezza d'onda λmax= 488 nm ed emettendo luce verde λmax= 522 nm.



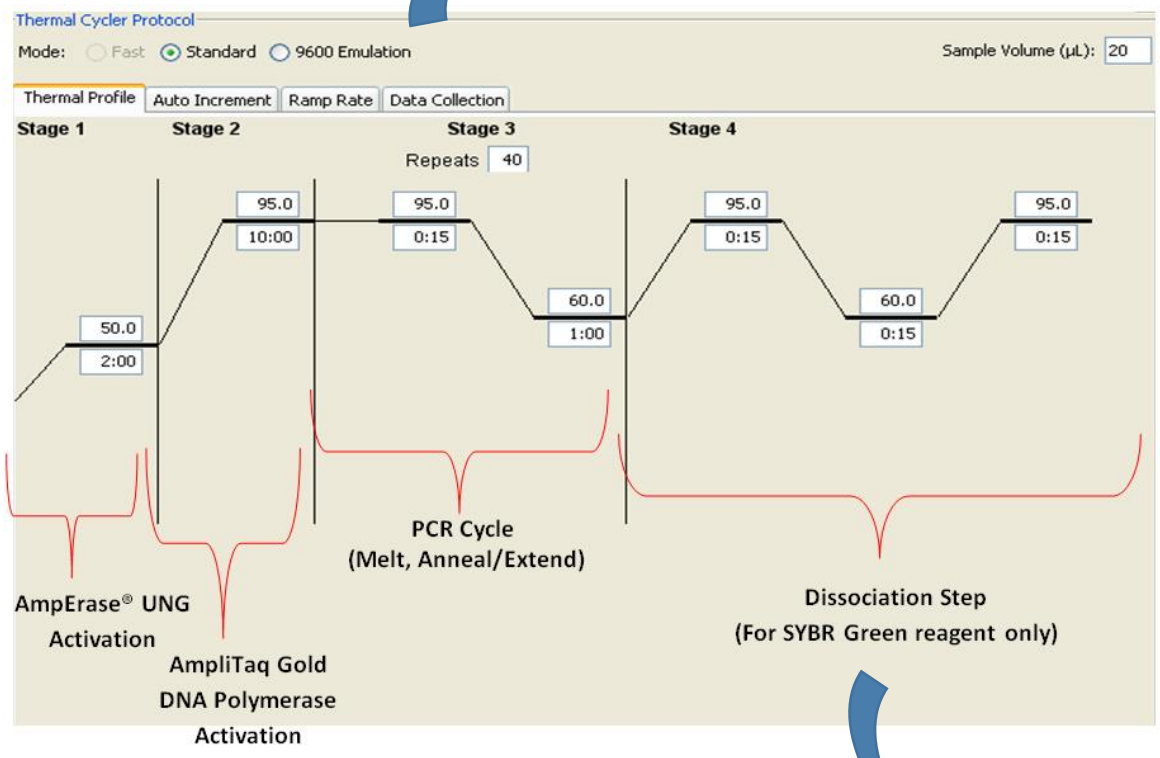
SYBR Green I

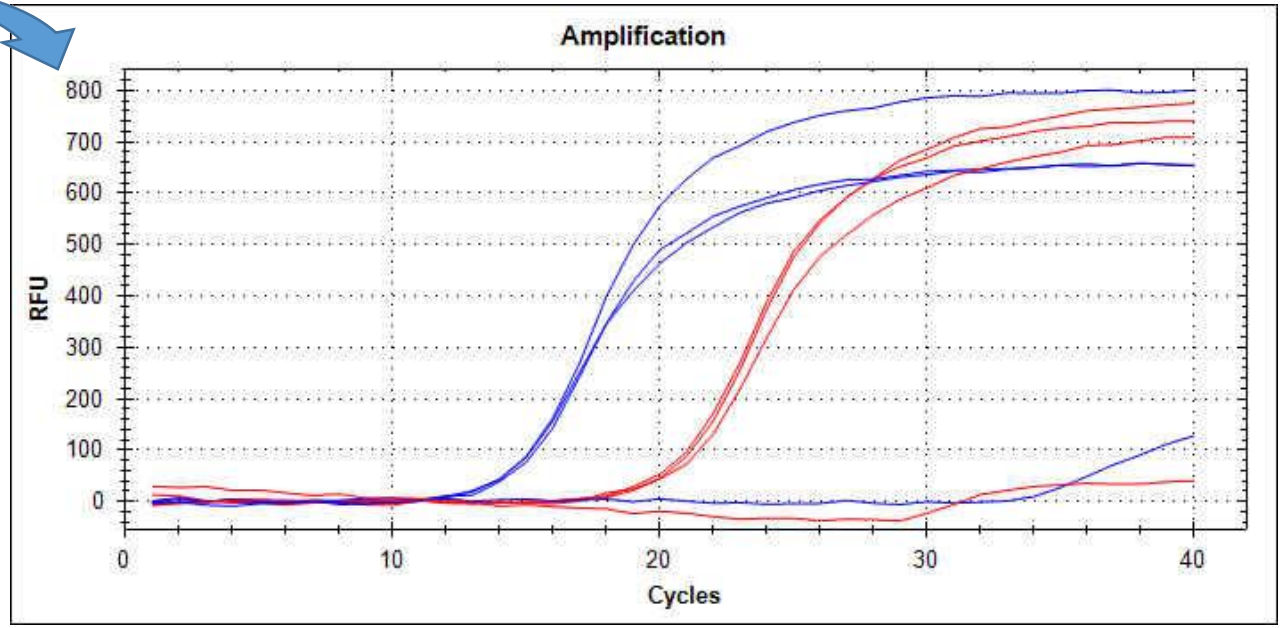


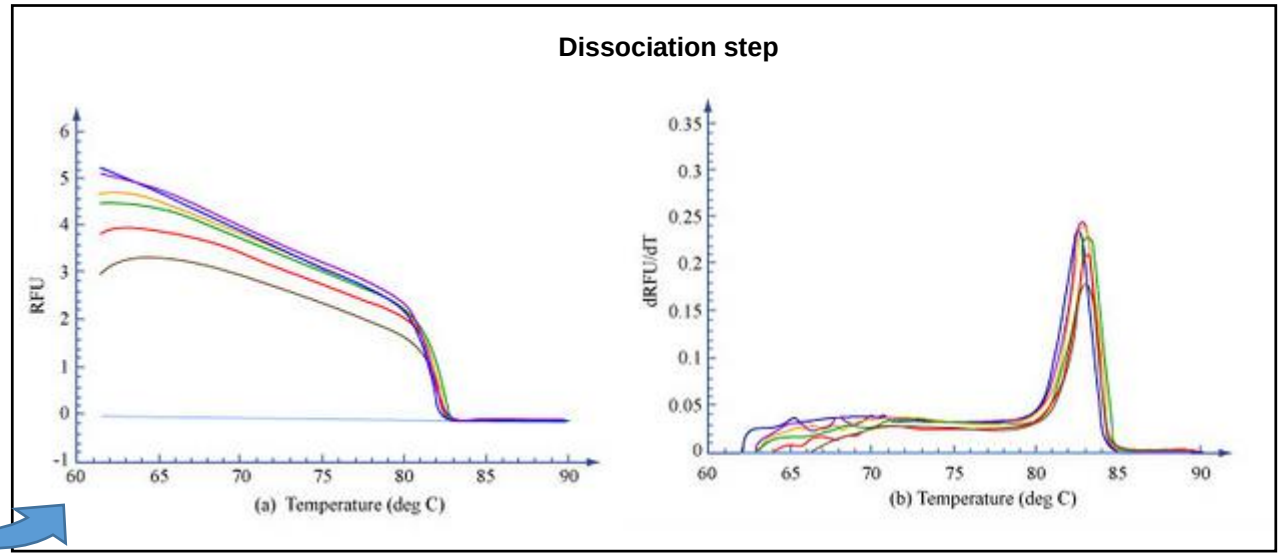
La sua maggiore sensitività (fino a 25 volte) nella rilevazione di acidi nucleici unita alla sua minore pericolosità sta facendo si che il SYBR Green venga utilizzato sempre più spesso come alternativa al meno costoso bromuro di etidio. Infatti il bromuro è un potente mutageno mentre, il SYBR Green viene indicato come non pericoloso. Un'altra caratteristica favorevole deriva dal fatto che la presenza della molecola legata al DNA non impedisce l'attività di numerosi enzimi, tra cui quelli di restrizione, le Ligasi e le **DNA polimerasi**.



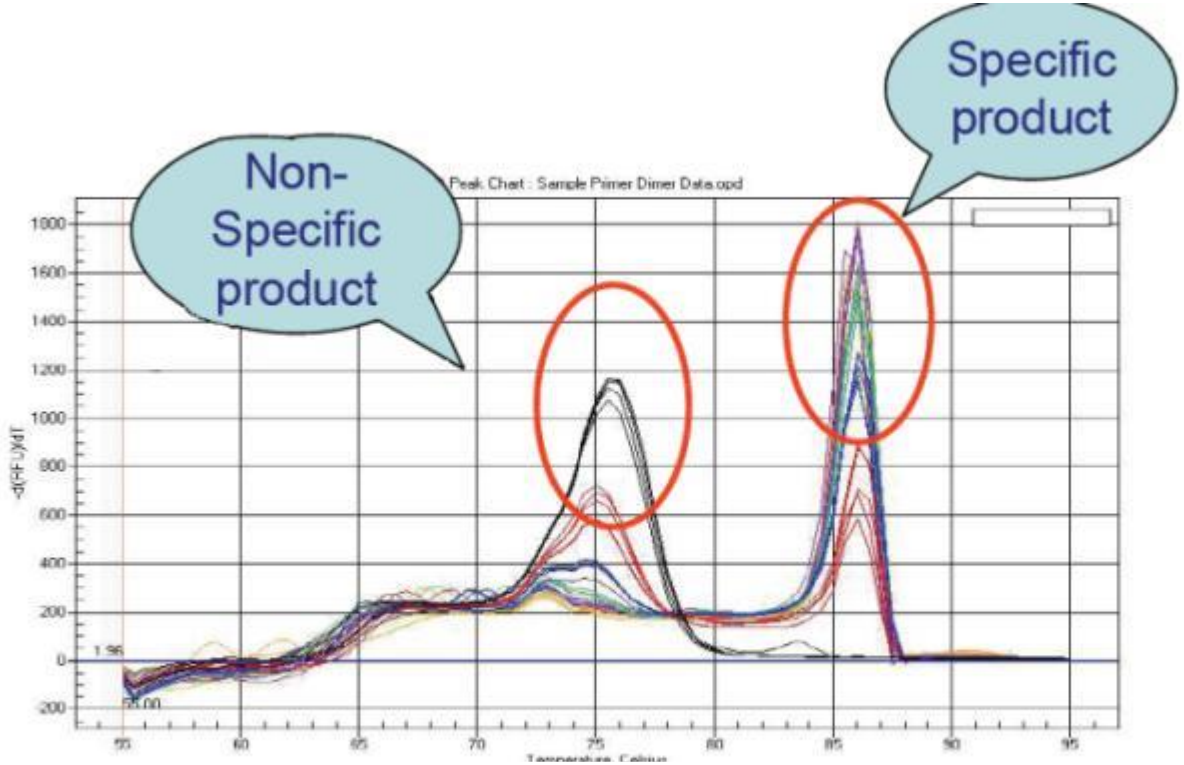
SYBR Green I


Thermal Cycler Protocol
Mode: Fast Standard 9600 Emulation
Sample Volume (µL): 20
Thermal Profile
Auto Increment
Ramp Rate
Data Collection
Stage 1
Stage 2
Stage 3
Stage 4
Repeats 40
50.0
2:00
95.0
10:00
95.0
0:15
60.0
1:00
95.0
0:15
60.0
0:15
95.0
0:15
AmpErase® UNG Activation
AmpliTaq Gold DNA Polymerase Activation
PCR Cycle (Melt, Anneal/Extend)
Dissociation Step (For SYBR Green reagent only)


Amplification
RFU
Cycles
800
700
600
500
400
300
200
100
0
0
10
20
30
40


Dissociation step
RFU
(a) Temperature (deg C)
dRFU/dT
(b) Temperature (deg C)

SYBR Green I



Non-specificità della molecola fluorescente Sybr green, che si lega a tutte le doppie eliche, come prodotti di PCR non specifici, e anche ai dimeri di *primers* (che a volte si formano durante le reazioni di PCR). **È necessario ottimizzare le condizioni di PCR, per evitare la formazione di prodotti aspecifici.**

SYBR Green I

Advantages:

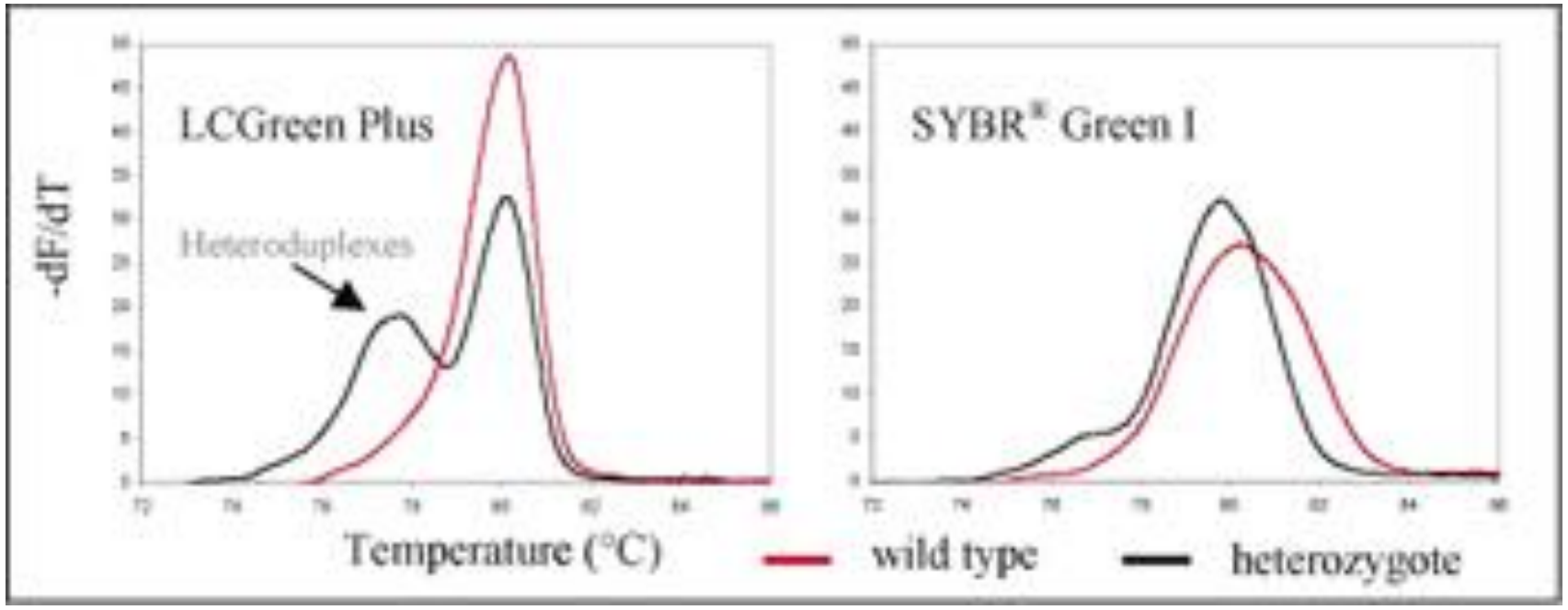
- The simplest technique is the use of a dye that when bound to double stranded DNA will fluoresce (Ethidium bromide, SYBR Green)
- not more expensive
- it's possible to use the same primers employed in normal PCR reactions

Disadvantages:

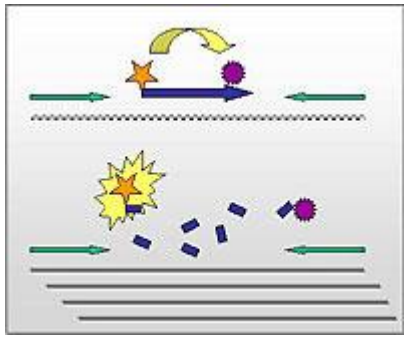
- However, the signal is not truly specific
- These dyes will also detect primer-dimers and false amplicons

LCGreen Plus

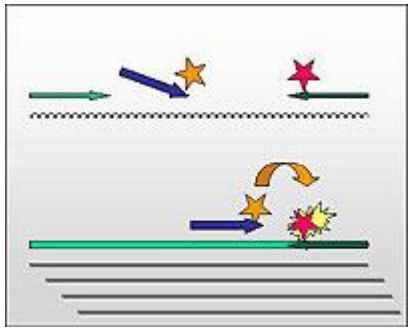
Hi-Res Melting, with the LightScanner system, offers superior performance. Derivative melting curves **illustrate the detection of heteroduplexes** in the heterozygous mutant using **LCGreen Plus**as shown below, **which are not detected using SYBR®**



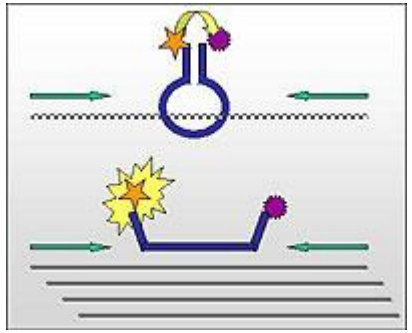
Consideration about probes



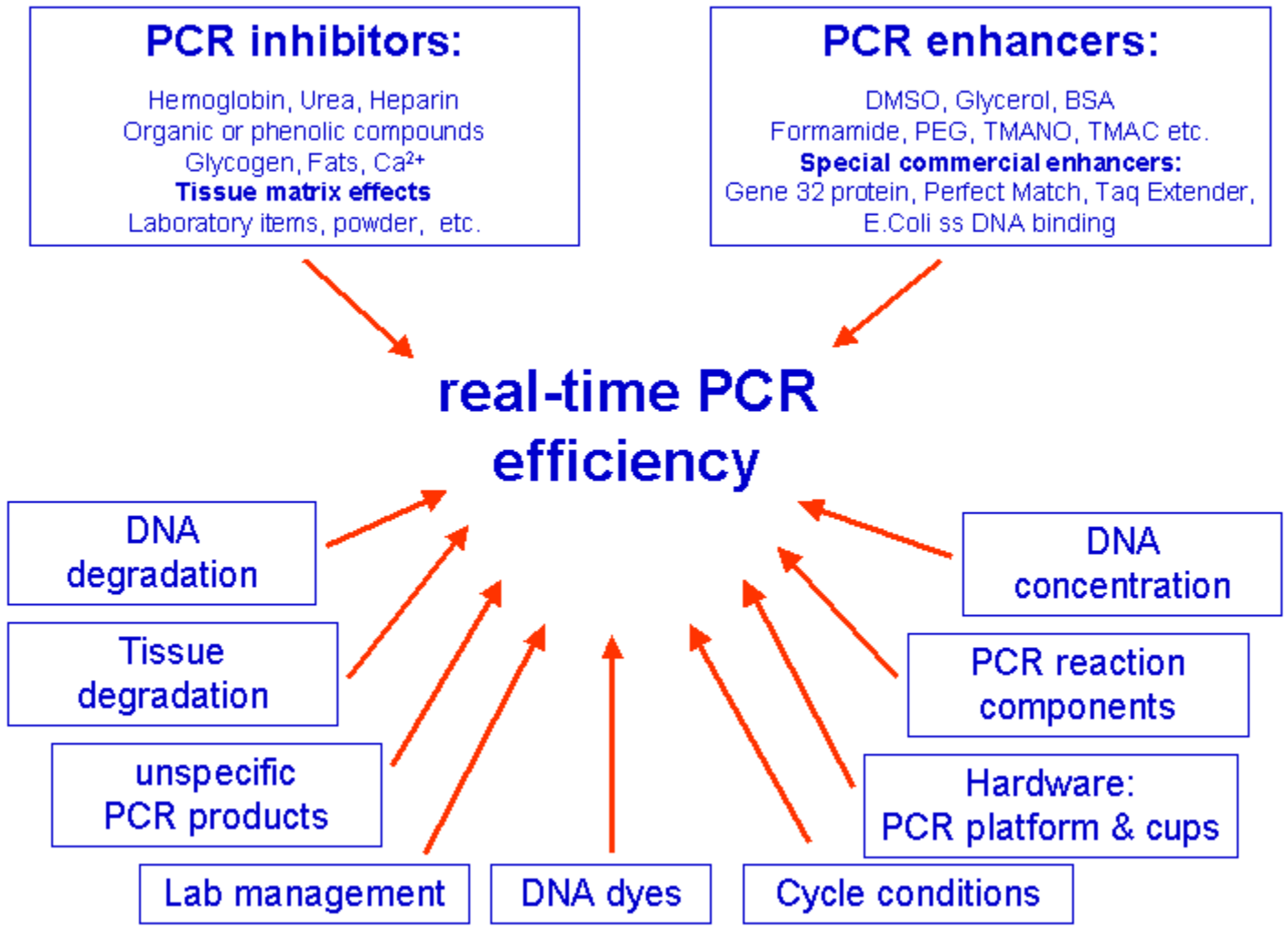
Le sonde TaqMan® sono relativamente **sensibili alle variazioni di base di singoli nucleotidi (mismatch**). Questo potrebbe essere estremamente importante quando si amplificano campioni virologici, dove una variabilità genetica potrebbe essere presente e potrebbe portare ad una mancata amplificazione. Pertanto, un '**non-signal**' dovrà essere associato ad un diverso genotipo non riconosciuto dal probe.



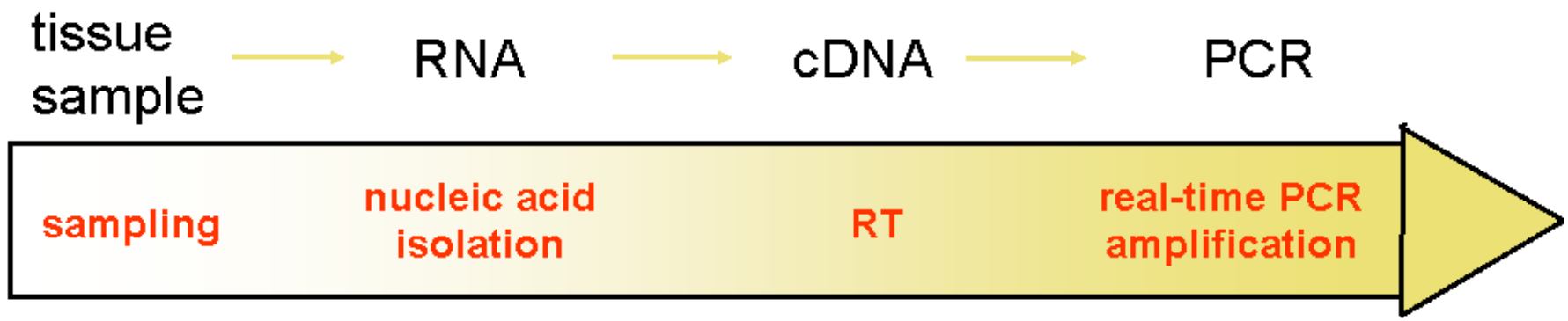
Ci sono varie possibilità per consentire l'ibridazione di oligonucleotidi adiacenti. Probabilmente il metodo più popolare e di successo è il legame di due sonde singole marcate in maniera «testa a punta», noto anche come "sonde baciate" o HybProbe. Un vantaggio rispetto delle sonde che sfruttano la tecnologia FRET rispetto alle sonde di idrolisi è il loro assemblaggio modulare e la loro **alta specificità nel riconoscere le variazioni di singole basi. Uno svantaggio è la necessità di una più lunga sequenza necessaria per ospitare due sonde adiacenti**.



Una variante molto interessante di sonde di ibridazione è Beacons Molecular, sviluppato da Fred R. Kramer. Le estremità delle sonde sono auto-complementari e marcate con una coppia fluoroforo/quencher. In assenza di una sequenza complementare, queste molecole si ripiegano in una struttura stem-loop e la fluorescenza viene cancellata dalla quencher. Una maggiore distanza tra quencher e colorante determina un aumento della fluorescenza rilevabile in seguito all'appaiamento del probe, non è dunque necessario che venga degradato durante la polimerizzazione per avere un segnale rilevabile. **I Beacons molecolari non vengono distrutti durante le reazioni di amplificazione** e possono essere riutilizzati nel ciclo successivo.


PCR inhibitors:
Hemoglobin, Urea, Heparin
Organic or phenolic compounds
Glycogen, Fats, Ca^{2+}
Tissue matrix effects
Laboratory items, powder, etc.
PCR enhancers:
DMSO, Glycerol, BSA
Formamide, PEG, TMANO, TMAC etc.
Special commercial enhancers:
Gene 32 protein, Perfect Match, Taq Extender,
E.Coli ss DNA binding
real-time PCR efficiency
DNA degradation
Tissue degradation
unspecific PCR products
Lab management
DNA dyes
Cycle conditions
Hardware: PCR platform & cups
PCR reaction components
DNA concentration

Steps and variables of a successful mRNA quantification using real-time RT-PCR



Sampling method:

- Biopsy
- Fixed material
- Fresh blood
- Tissue storage
- Liquid Nitrogen
- RNA Later
- 1st extraction buffer
- RNA storage –80°C

  => native RNA

Extraction method:

- total RNA
  - mRNA
  - microRNA
- liquid-liquid
- columns
- Robot vs. hand made
- **RNA integrity:**
  - Bioanalyzer 2100
  - Experion
  - Nano-Drop
  - mFold algorithm

Efficiency of RT:

- RT enzyme type
- RT temperature
- **Primers:**
  - poly-T Primer
  - Random-hexamers
  - Specific primer
  - Primer mixtures
- **one-step qRT-PCR**
- **two-step RT-qPCR**

PCR Efficiency / Specificity:

- Primer design
  - Primer specificity
  - Consensus Primer
- mRNA abundance
- RNA / cDNA input
- Polymerase types
- Polymerase Mixtures
- PCR Inhibitors & Enhancers
- Robot vs. hand made