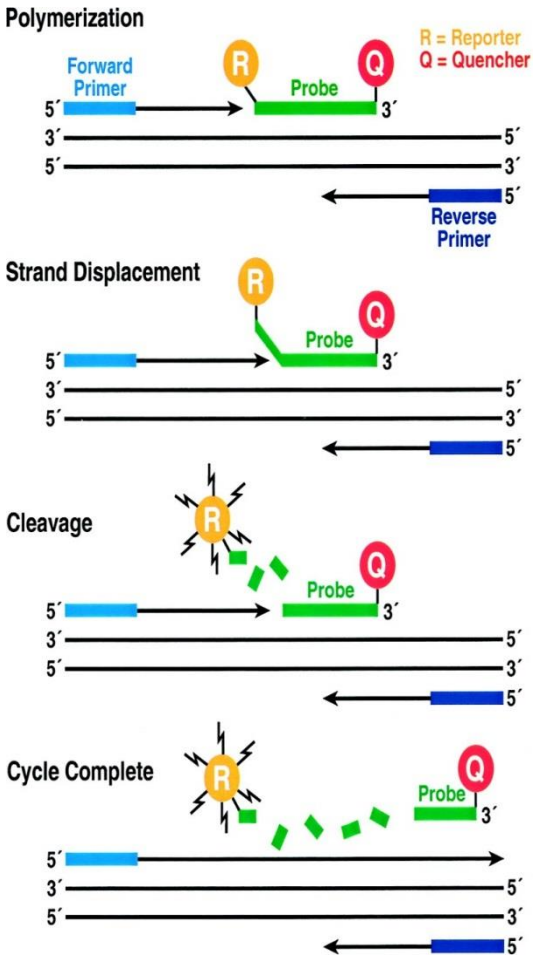
Quantitative *real time* Polymerase Chain Reaction

sonde TaqMan

attività 5'→ 3' esonucleasica della DNA polimerasi

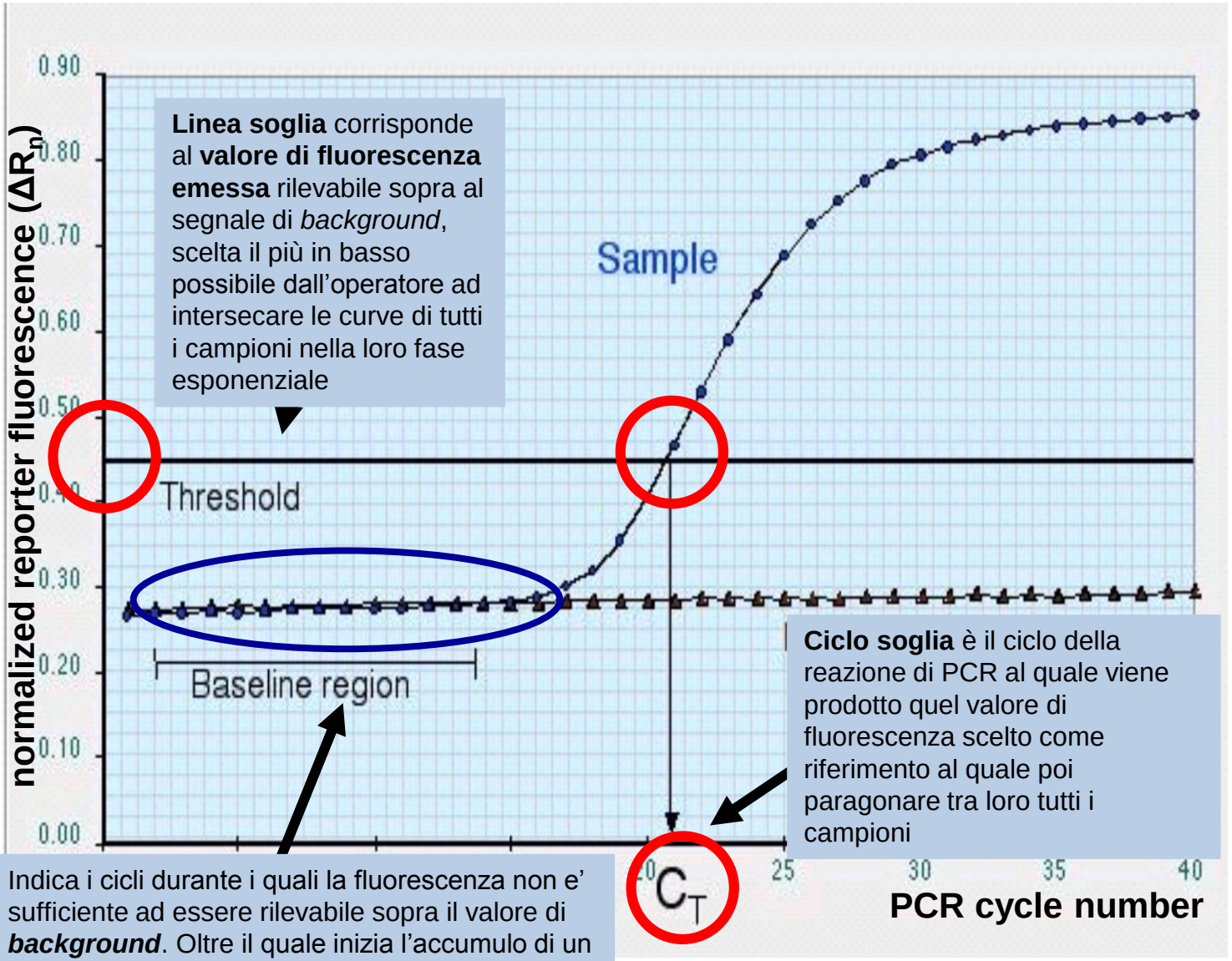
Un filamento di DNA polimerizzato = 1 emissione di fluorescenza



Permette di vedere cosa succede prima del plateau:

Stuart N. Peirson, Jason N. Butler, Circadian Rhythms, Methods in Molecular Biology™ Volume 362, 2007, pp 349-362

Plot di amplificazione lineare

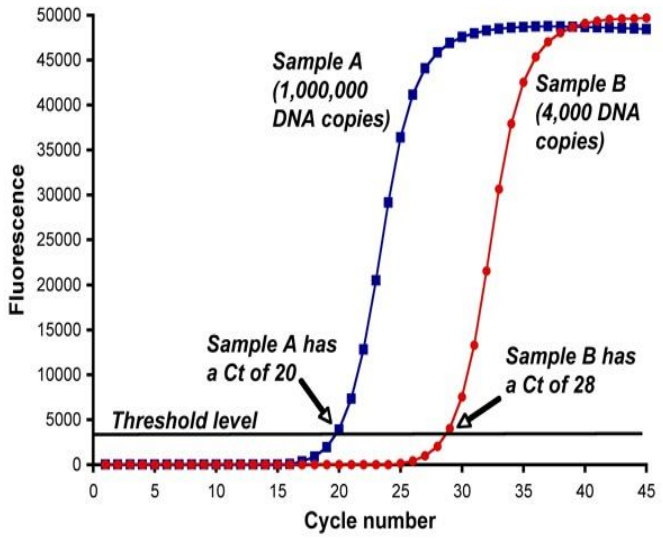


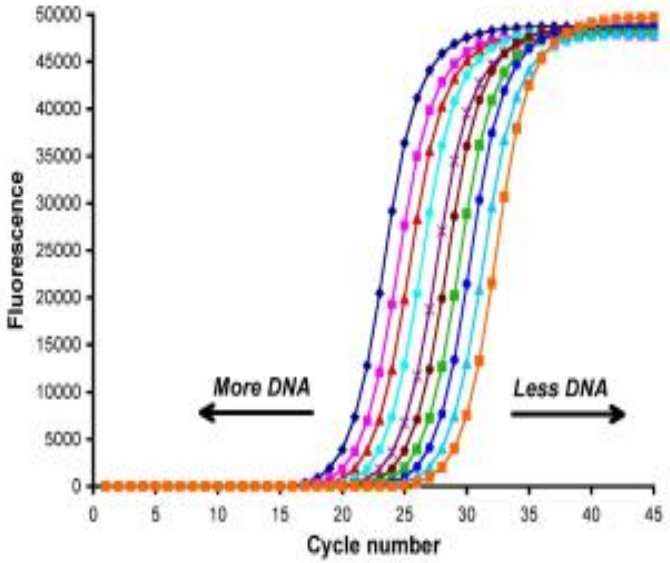
Linea soglia corrisponde al **valore di fluorescenza emessa** rilevabile sopra al segnale di *background*, scelta il più in basso possibile dall'operatore ad intersecare le curve di tutti i campioni nella loro fase esponenziale

Ciclo soglia è il ciclo della reazione di PCR al quale viene prodotto quel valore di fluorescenza scelto come riferimento al quale poi paragonare tra loro tutti i campioni

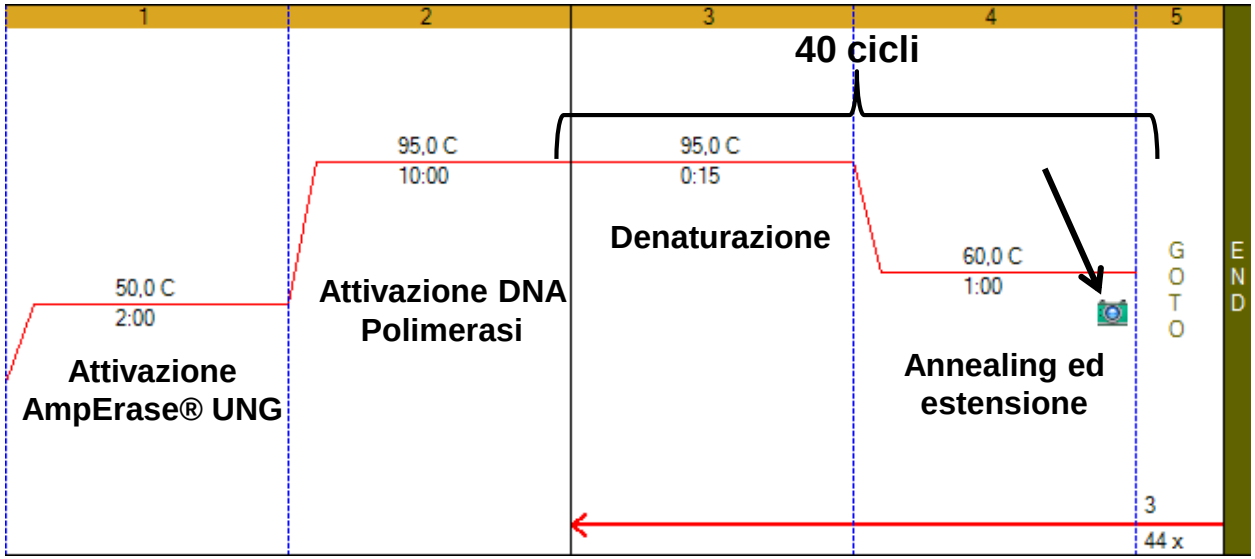
Indica i cicli durante i quali la fluorescenza non e' sufficiente ad essere rilevabile sopra il valore di ***background***. Oltre il quale inizia l'accumulo di un amplificato e la fluorescenza emessa dal probe viene registrata diventando statisticamente significativa

Quantitative *real time* Polymerase Chain Reaction





Per ogni campione si ottiene una curva di amplificazione e (definita una *threshold*) il relativo CT (*Threshold Cycle*) è inversamente proporzionale alla quantità di DNA stampo iniziale contenuto nel campione



1.Attivazione AmpErase® UNG

Urancil-N-Glycosylase è un enzima che elimona contaminanti contenenti dU.

2.Attivazione DNA Polimerasi

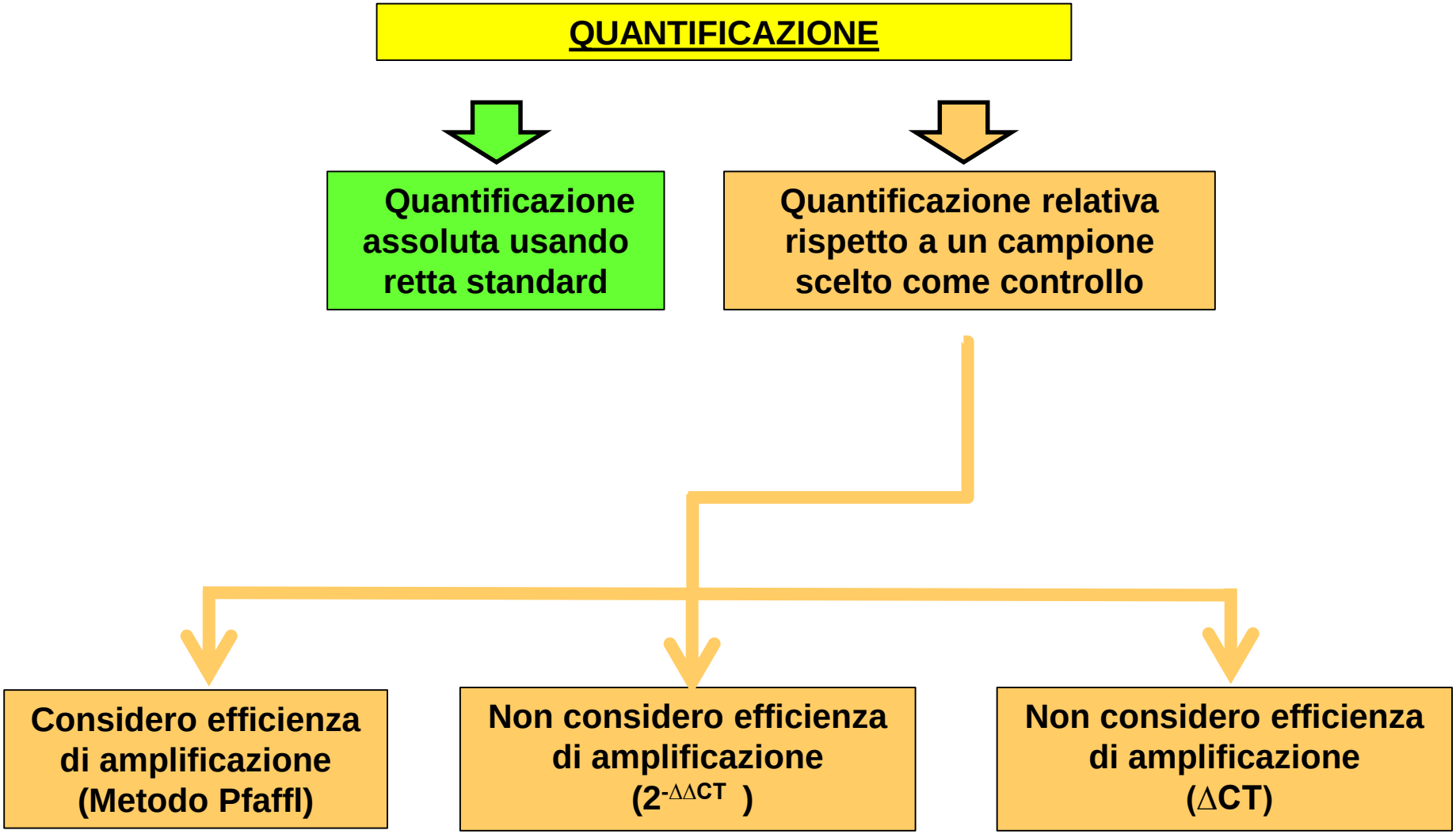
Per attivare l'enzima **AmpliTaq Gold ® DNA Polymerase** è necessaria un'incubazione a 95°C.

3.Denaturazione del DNA stampo

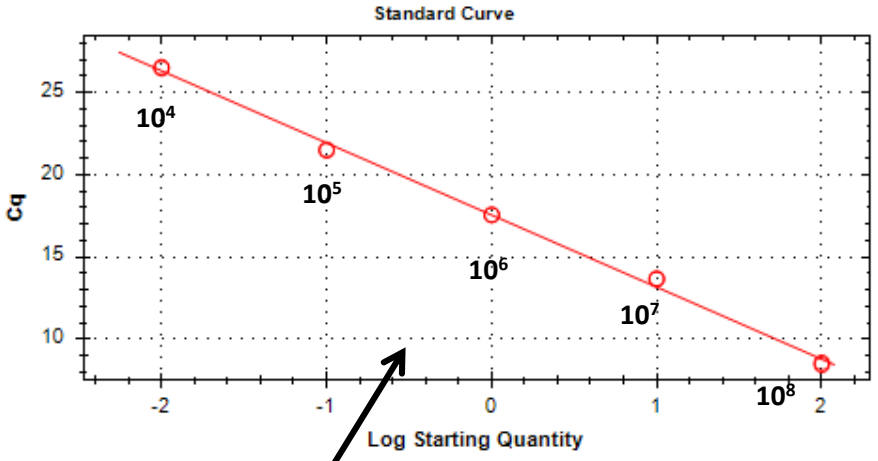
Il riscaldamento a 95°C consente di rompere i legami idrogeno che uniscono i singoli filamenti.

4.Annealing ed estensione

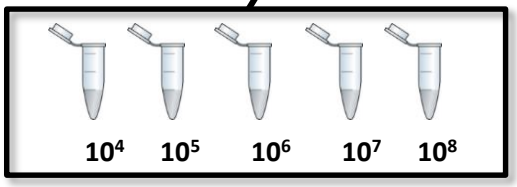
I primers si legano alla regione complementare presente nel templato e successivamente la polimerasi va a formare il nuovo filamento.


QUANTIFICAZIONE
Quantificazione assoluta usando retta standard
Quantificazione relativa rispetto a un campione scelto come controllo
Considero efficienza di amplificazione (Metodo Pfaffl)
Non considero efficienza di amplificazione ($2^{-\Delta\Delta CT}$)
Non considero efficienza di amplificazione (ΔCT)

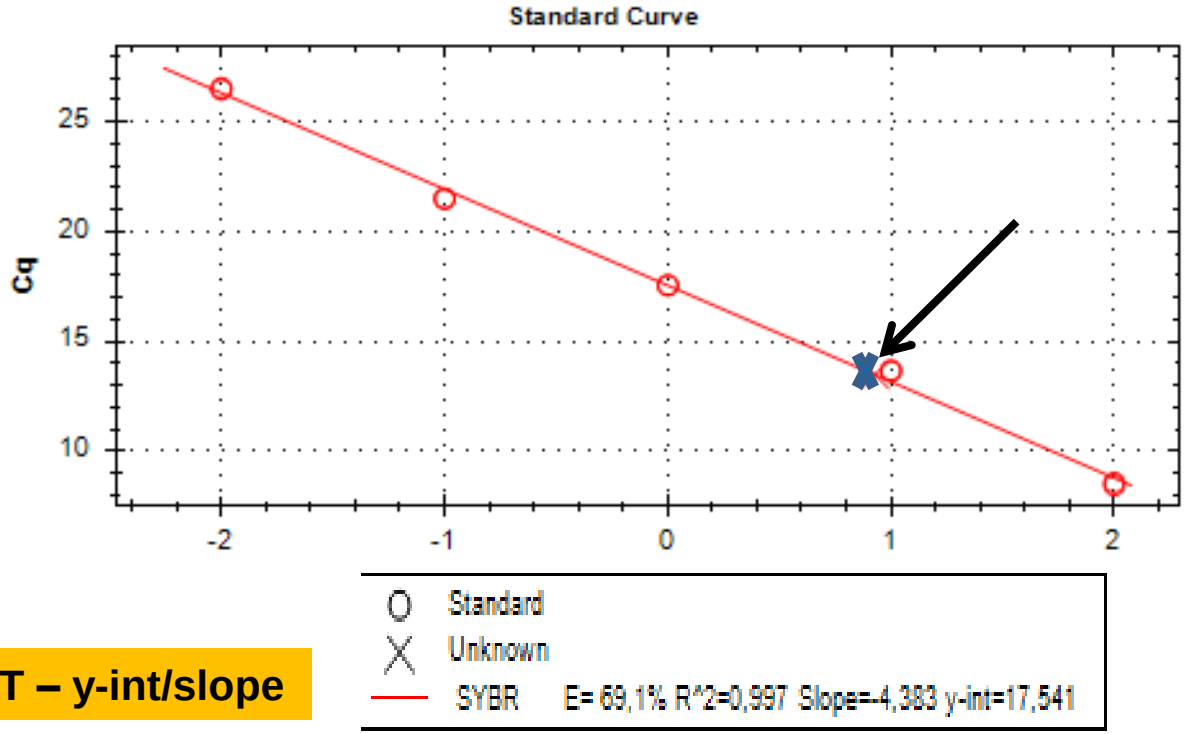
QUANTIFICAZIONE ASSOLUTA



La concentrazione del campione incognito (unknown) si ricava per interpolazione da **retta di taratura** creata amplificando campioni a concentrazione nota**.**



✓Prodotti di PCR
✓Oligonucleotidi sintetici
✓Plasmidi
✓DNA genomico

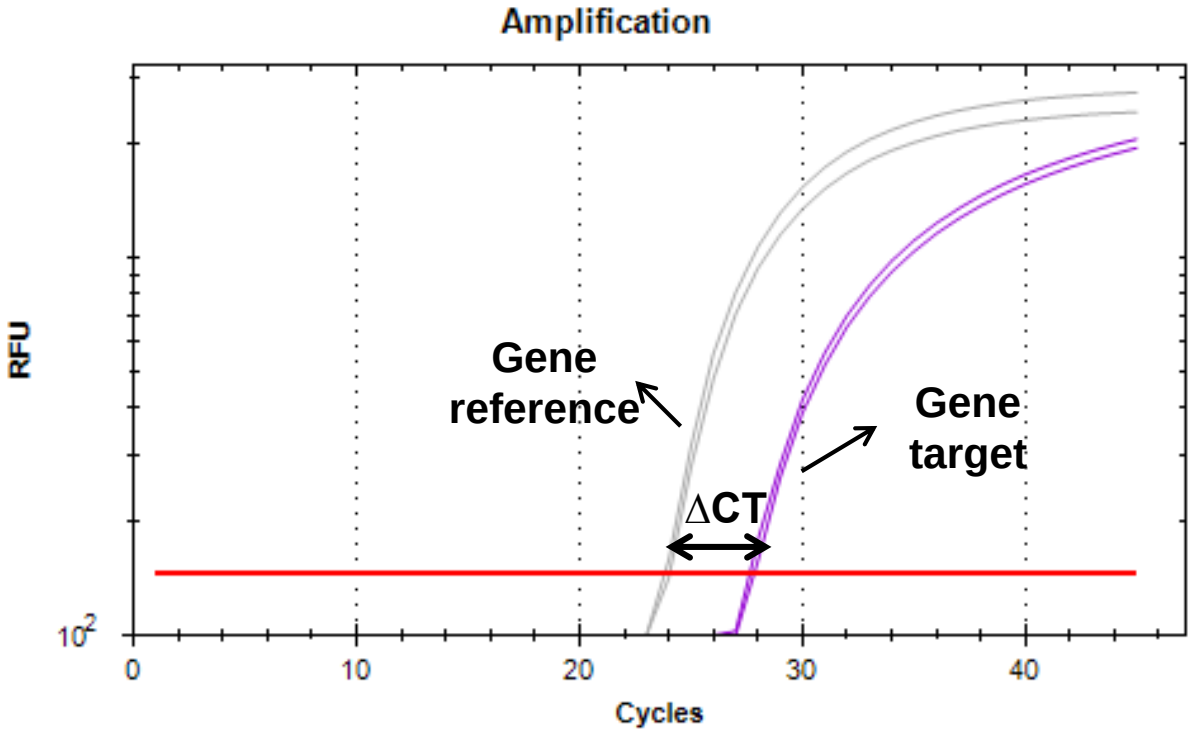


Log $_{10}$ quantità campione = CT – y-int/slope

QUANTIFICAZIONE RELATIVA

La quantificazione si esegue attraverso il confronto tra CT, esprime una variazione di concentrazione di un campione incognito rispetto ad un campione scelto come controllo.

✓Metodo ΔCT
✓**Metodo ΔΔCT (Livak)**
✓Metodo Pfaffl



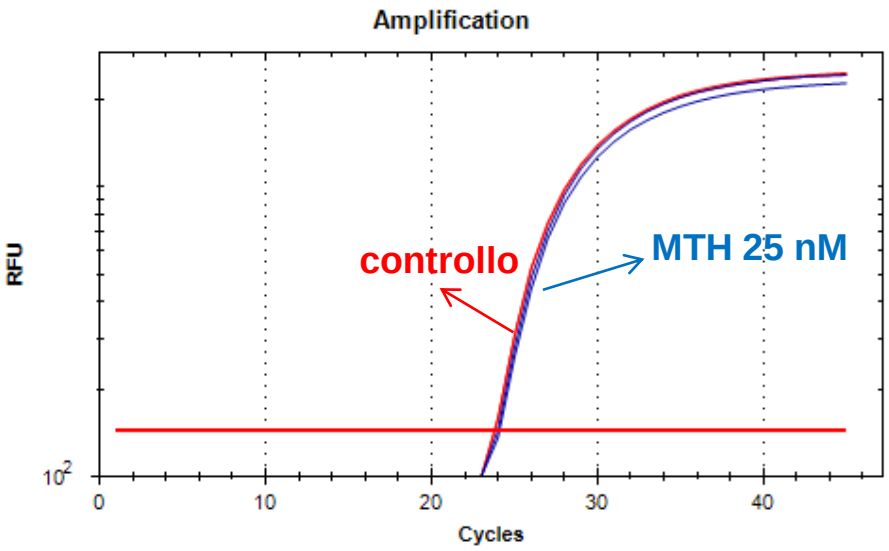
GENE REFERENCE (gene housekeeping)

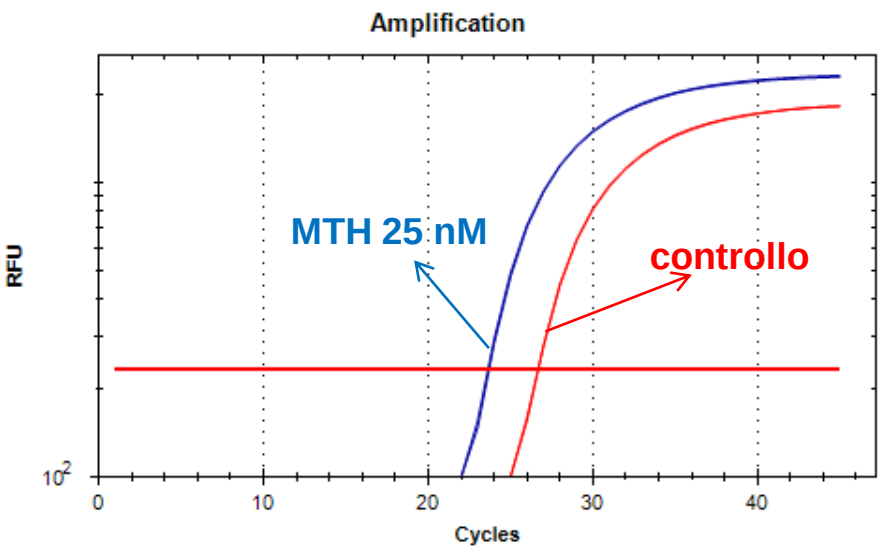
Gene costitutivamente espresso, la cui espressione non varia nei campioni trattati rispetto a quelli di controllo. Serve a ridurre l'errore sperimentale.

✓La sua espressione non deve essere alterata dal trattamento o dalla condizione patologica

✓Molto spesso è utile usare più reference

✓geNorm http://medgen.ugen.be/~jvdesomp/genorm/



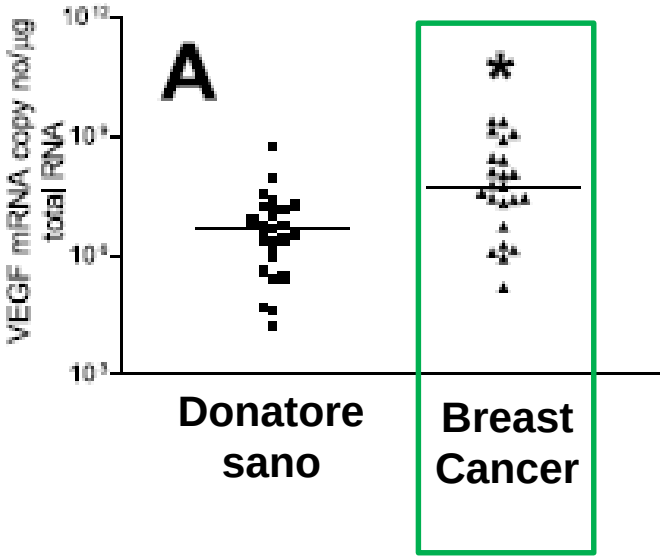


Housekeeping analizzati

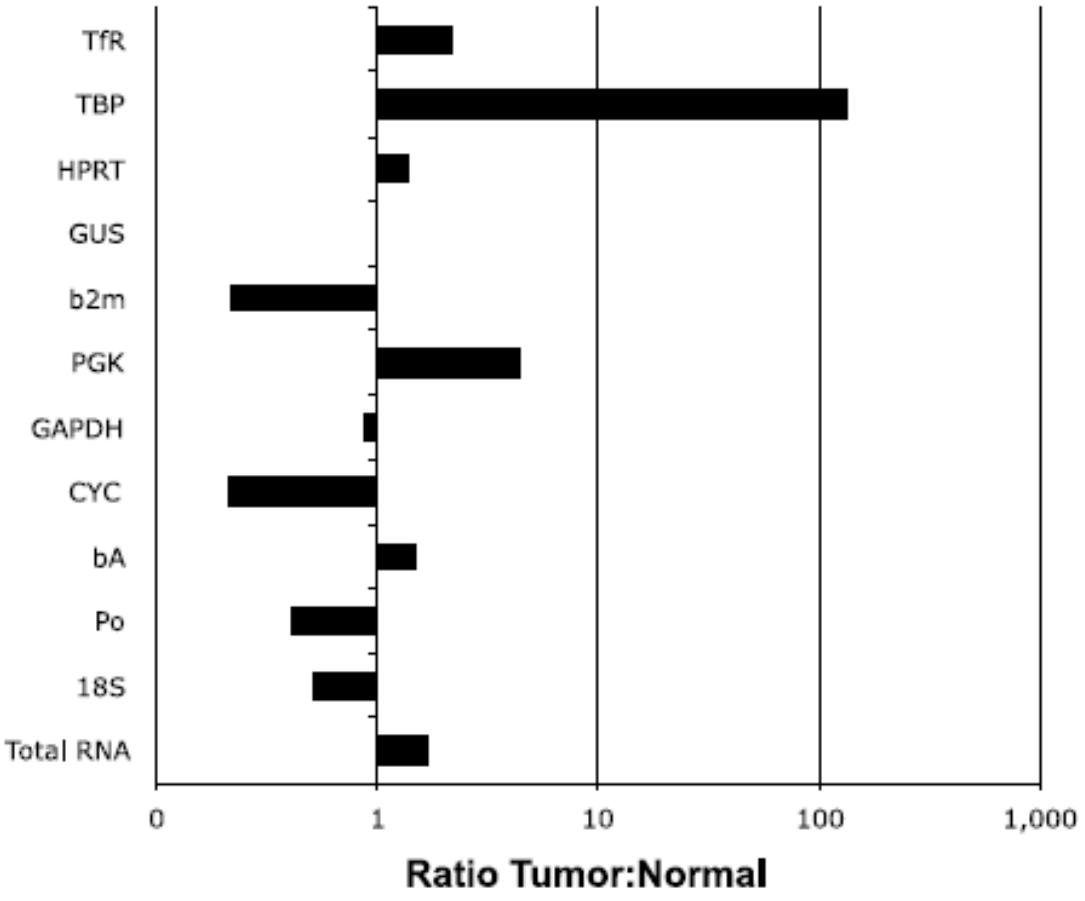
Endogenous control gene	Abbreviation
18S rRNA	18S
Acidic ribosomal protein	PO
β-actin	βa
Cyclophilin	CYC
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Phosphoglycerokinase	PGK
β_2-Microglobulin	β2m
β-Glucuronidase	GUS
Hypoxanthine ribosyl transferase	HPRT
TATA-binding protein	TBP
Transferrin receptor	TfR

Tricarico et al., Analytical Biochemistry (2002).

Quantificazione assoluta VEGF

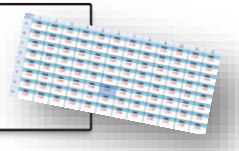


Quantificazione relativa VEGF

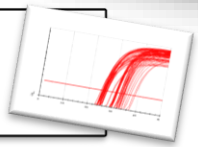


ANALISI DEI DATI

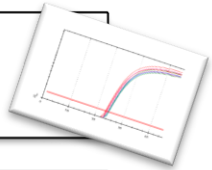
1. Configurare le piastra



2. Posizionare la threshold

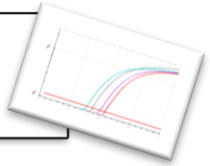


3. Verificare i controlli

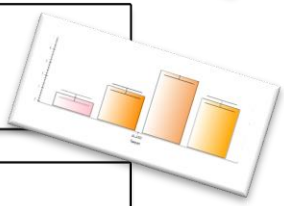


4. Verificare i replicati

5. Verificare l’andamento del gene reference

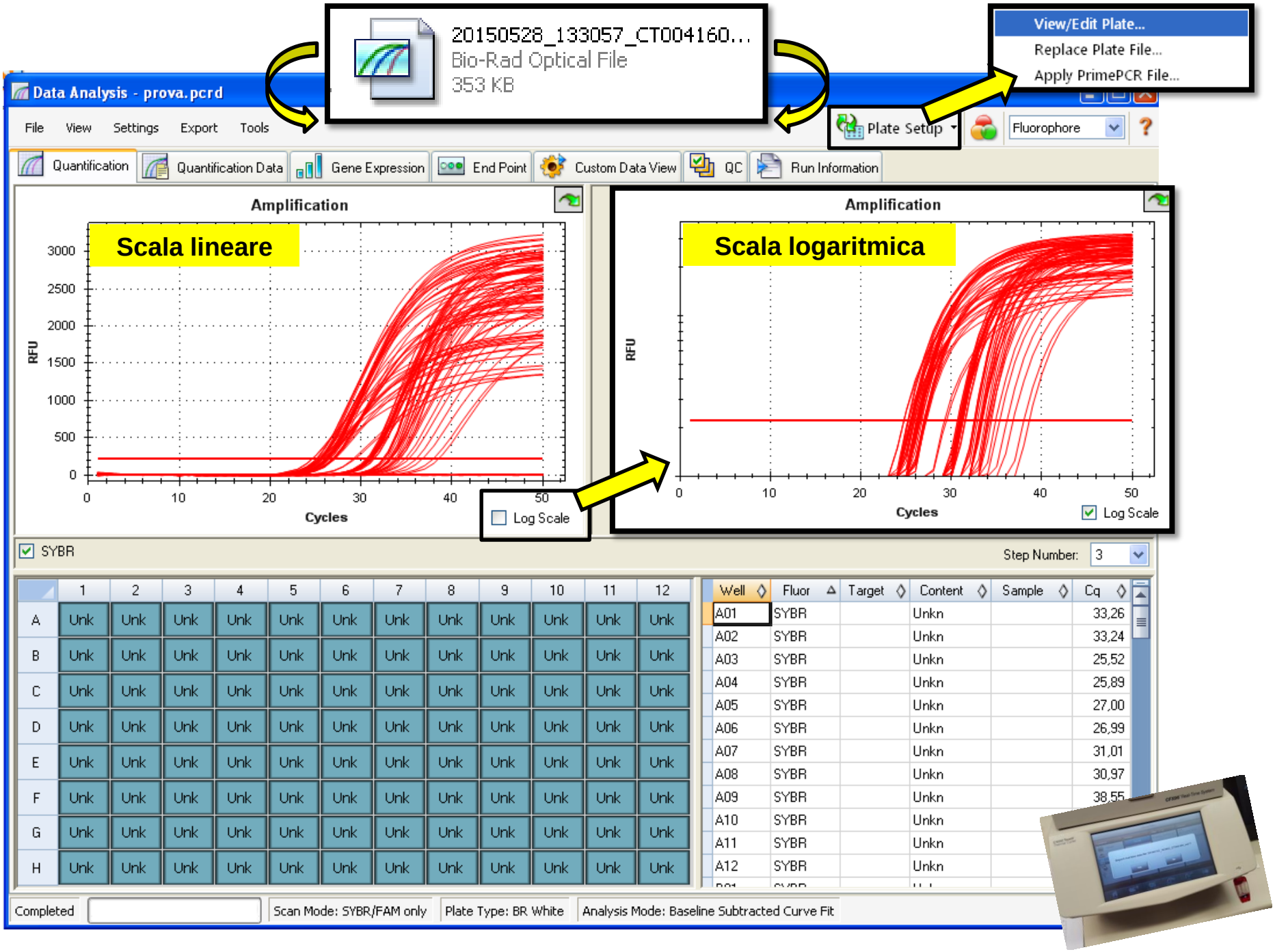


6. Verificare l’andamento del gene target

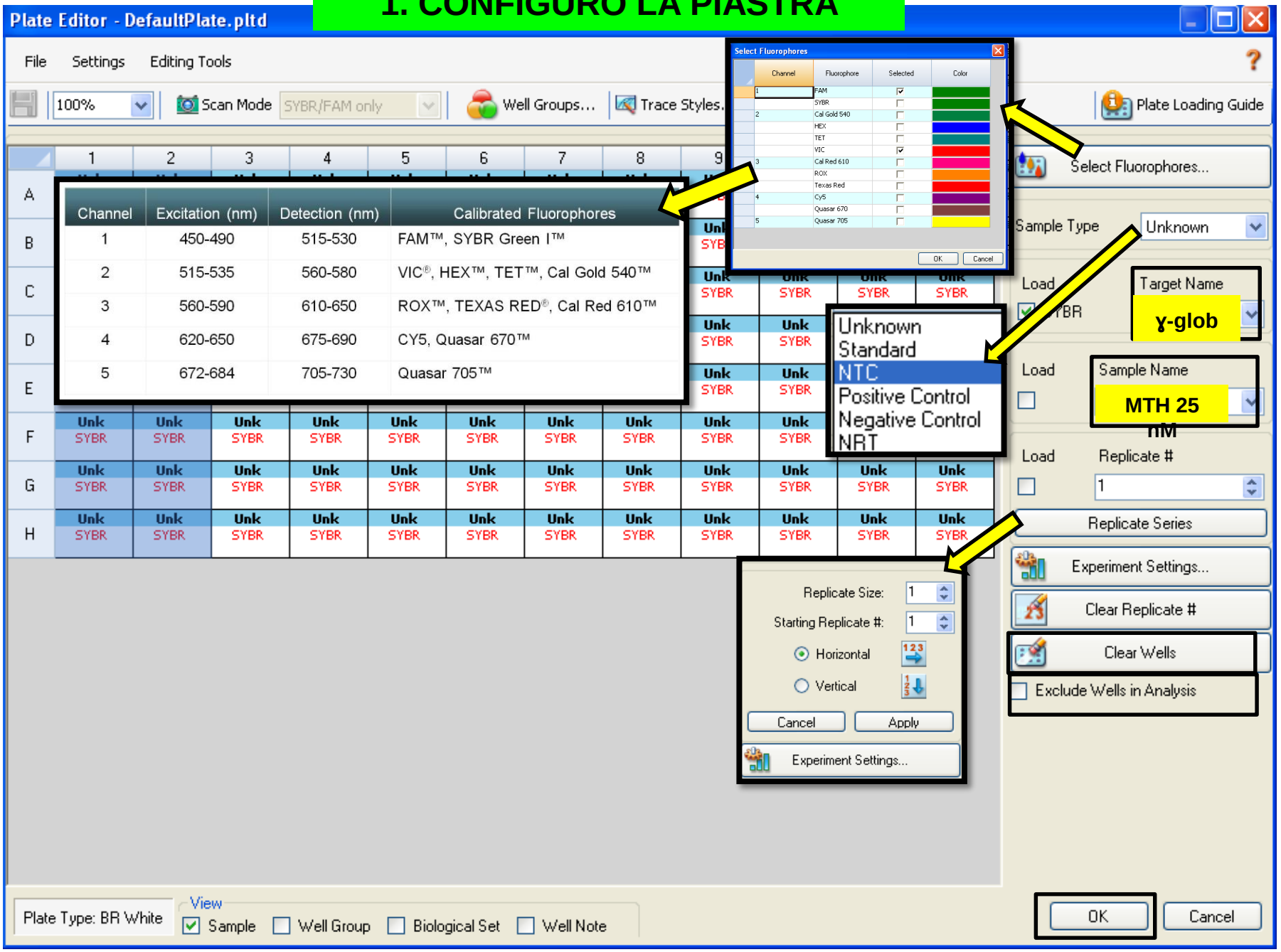


7. Eseguire la quantificazione relativa del gene target

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

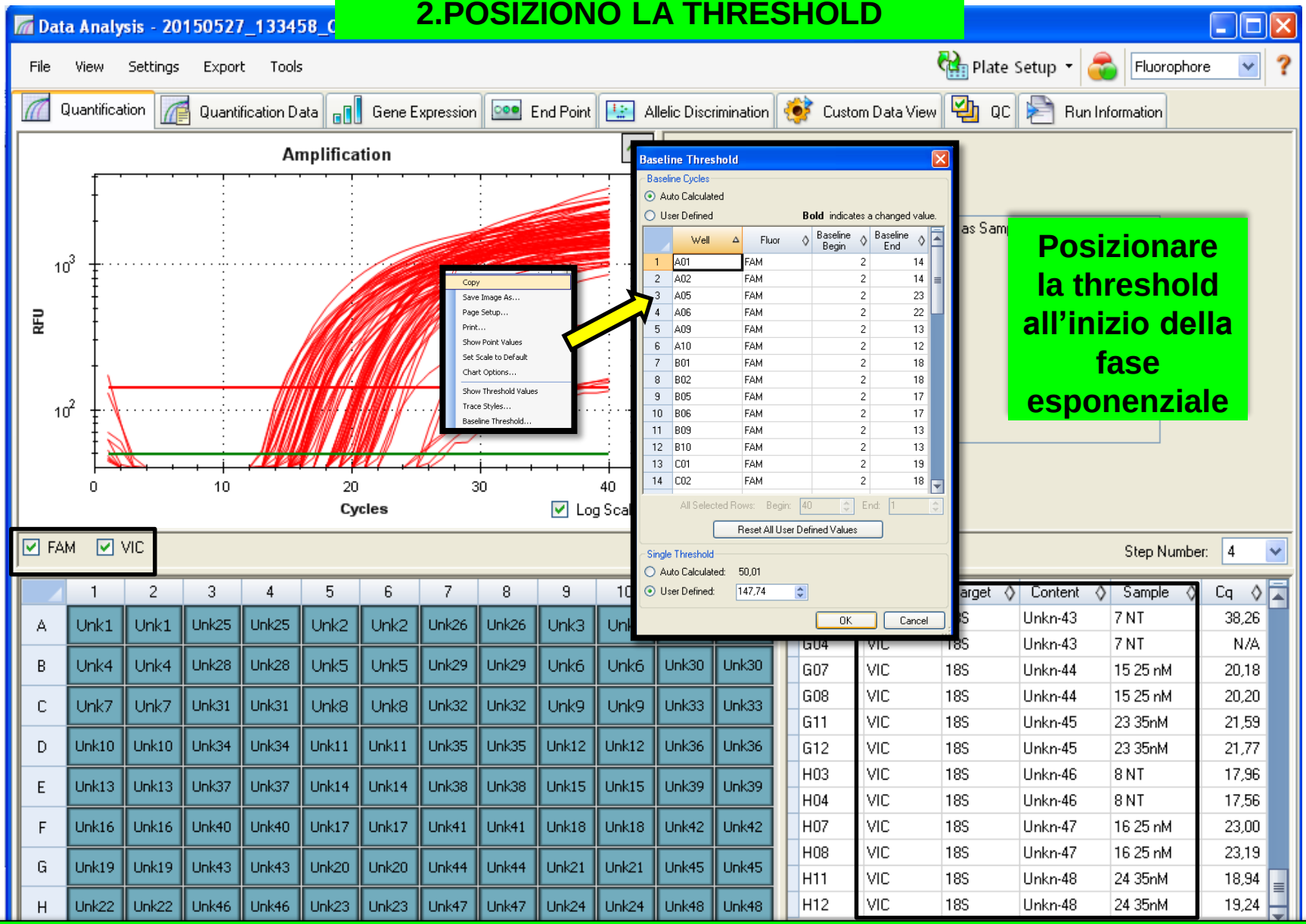

20150528_133057_CT004160...
Bio-Rad Optical File
353 KB
View/Edit Plate...
Replace Plate File...
Apply PrimePCR File...
Data Analysis - prova.pcrd
File
View
Settings
Export
Tools
Plate Setup
Fluorophore
Quantification
Quantification Data
Gene Expression
End Point
Custom Data View
QC
Run Information
Amplification
Scala lineare
RFU
Cycles
Log Scale
Amplification
Scala logaritmica
RFU
Cycles
Log Scale
SYBR
Step Number:
3
Well
Fluor
Target
Content
Sample
Cq
A01 SYBR Unkn 33,26
A02 SYBR Unkn 33,24
A03 SYBR Unkn 25,52
A04 SYBR Unkn 25,89
A05 SYBR Unkn 27,00
A06 SYBR Unkn 26,99
A07 SYBR Unkn 31,01
A08 SYBR Unkn 30,97
A09 SYBR Unkn 38,55
A10 SYBR Unkn
A11 SYBR Unkn
A12 SYBR Unkn
Completed
Scan Mode: SYBR/FAM only
Plate Type: BR White
Analysis Mode: Baseline Subtracted Curve Fit

1. CONFIGURO LA PIASTRA



Channel	Excitation (nm)	Detection (nm)	Calibrated Fluorophores
1	450-490	515-530	FAM™, SYBR Green I™
2	515-535	560-580	VIC®, HEX™, TET™, Cal Gold 540™
3	560-590	610-650	ROX™, TEXAS RED®, Cal Red 610™
4	620-650	675-690	CY5, Quasar 670™
5	672-684	705-730	Quasar 705™

2.POSIZIONO LA THRESHOLD

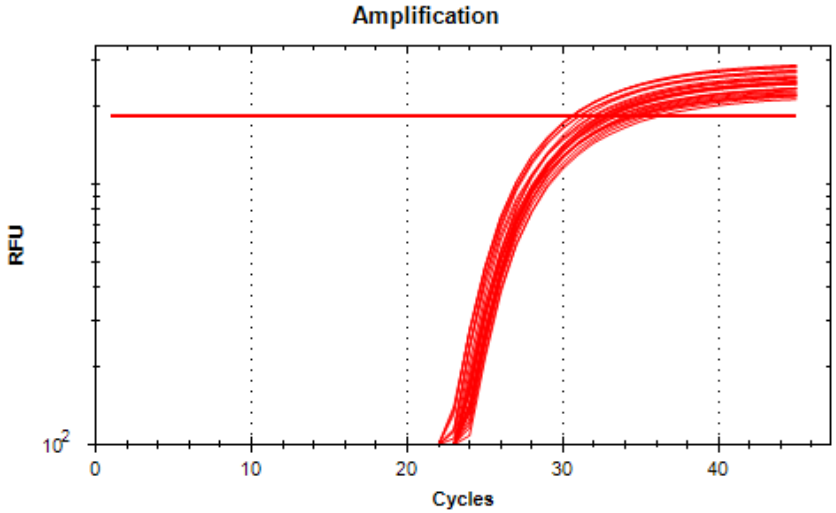


	1	2	3	4	5	6	7	8	9	10		
A	Unk1	Unk1	Unk25	Unk25	Unk2	Unk2	Unk26	Unk26	Unk3	Unk3		
B	Unk4	Unk4	Unk28	Unk28	Unk5	Unk5	Unk29	Unk29	Unk6	Unk6	Unk30	Unk30
C	Unk7	Unk7	Unk31	Unk31	Unk8	Unk8	Unk32	Unk32	Unk9	Unk9	Unk33	Unk33
D	Unk10	Unk10	Unk34	Unk34	Unk11	Unk11	Unk35	Unk35	Unk12	Unk12	Unk36	Unk36
E	Unk13	Unk13	Unk37	Unk37	Unk14	Unk14	Unk38	Unk38	Unk15	Unk15	Unk39	Unk39
F	Unk16	Unk16	Unk40	Unk40	Unk17	Unk17	Unk41	Unk41	Unk18	Unk18	Unk42	Unk42
G	Unk19	Unk19	Unk43	Unk43	Unk20	Unk20	Unk44	Unk44	Unk21	Unk21	Unk45	Unk45
H	Unk22	Unk22	Unk46	Unk46	Unk23	Unk23	Unk47	Unk47	Unk24	Unk24	Unk48	Unk48

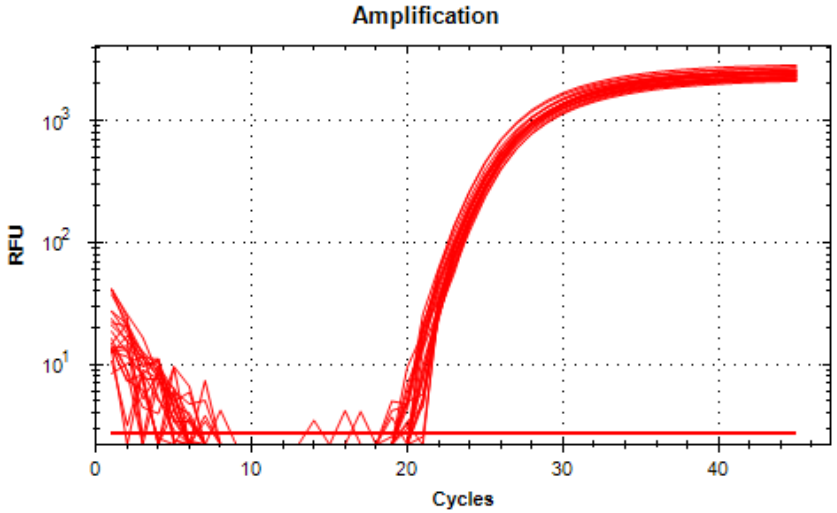
			Content	Sample	Cq
			Unkn-43	7 NT	38,26
			Unkn-43	7 NT	N/A
G07	VIC	18S	Unkn-44	15 25 nM	20,18
G08	VIC	18S	Unkn-44	15 25 nM	20,20
G11	VIC	18S	Unkn-45	23 35nM	21,59
G12	VIC	18S	Unkn-45	23 35nM	21,77
H03	VIC	18S	Unkn-46	8 NT	17,96
H04	VIC	18S	Unkn-46	8 NT	17,56
H07	VIC	18S	Unkn-47	16 25 nM	23,00
H08	VIC	18S	Unkn-47	16 25 nM	23,19
H11	VIC	18S	Unkn-48	24 35nM	18,94
H12	VIC	18S	Unkn-48	24 35nM	19,24

Threshold: valore numerico, assegnato per ogni corsa, che indica un valore di fluorescenza superiore rispetto al background.

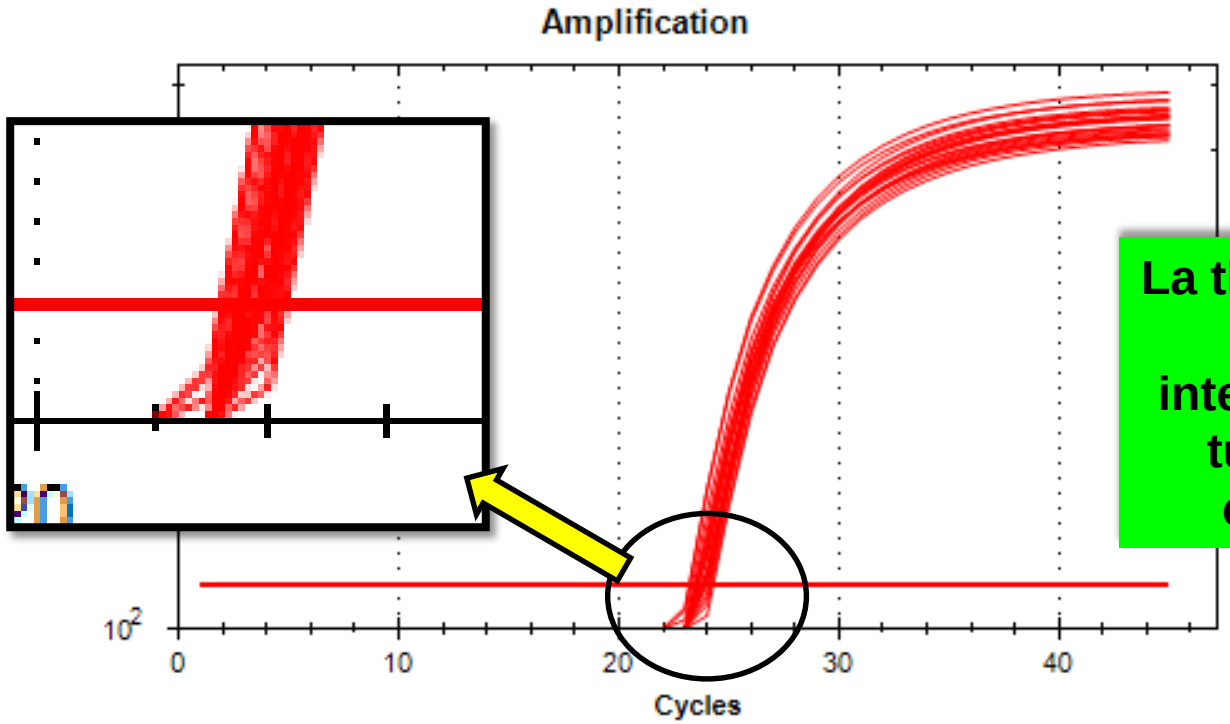
2.POSIZIONO LA THRESHOLD



Threshold oltre la fase esponenziale



Threshold sotto la fase esponenziale



La threshold deve intersecare tutte le curve

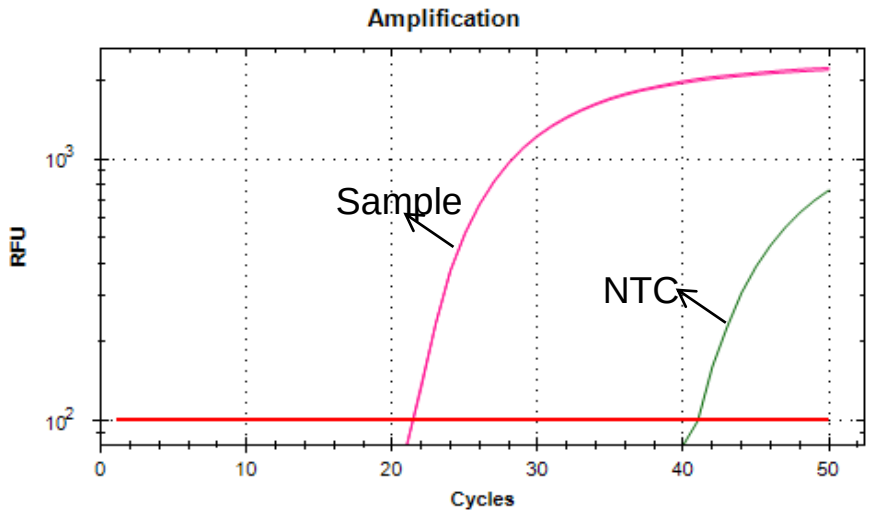
3.VERIFICA DEI CONTROLLI

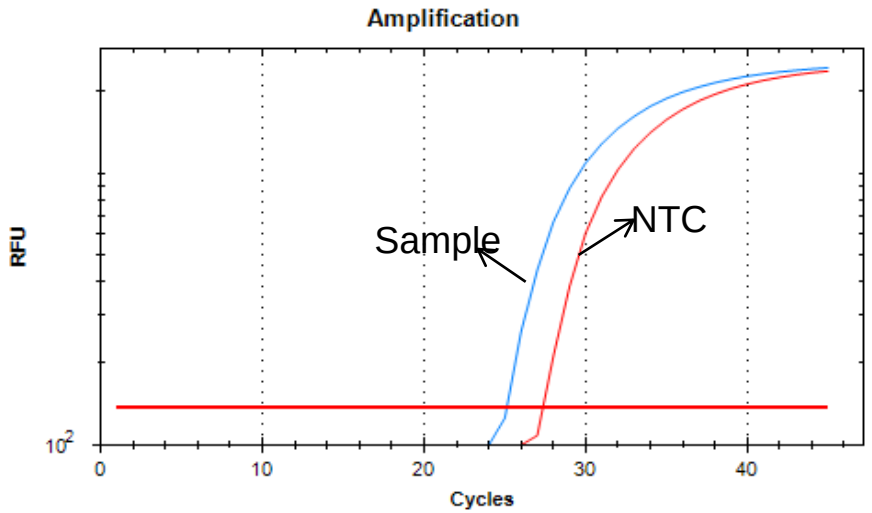
✓ **NTC** (No Template Control): contiene Master mix, sonda, primers ma non il templato.

✓Reagenti contaminati

✓Contaminazione in fase di piastratura

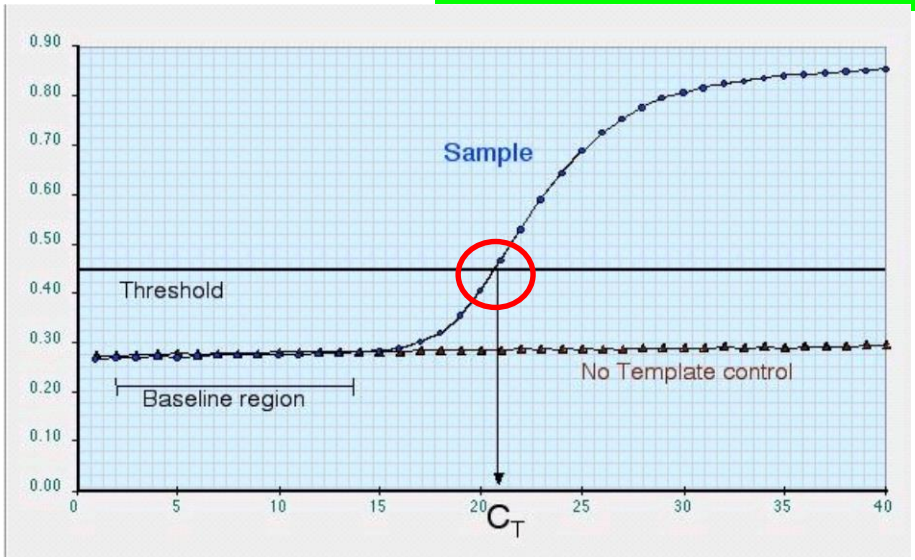
	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk1	Unk1	Unk2	Unk2	Unk3	Unk3	Unk4	Unk4	Unk5	Unk5	Unk6	Unk6
B	Unk7	Unk7	Unk8	Unk8	Unk9	Unk9	Unk10	Unk10	Unk11	Unk11	NTC1	NTC1
C	Unk12	Unk12	Unk13	Unk13	Unk14	Unk14	Unk15	Unk15	Unk16	Unk16		
D	Unk17	Unk17	Unk18	Unk18	Unk19	Unk19	NTC2	NTC2	Unk20	Unk20		
E	Unk21	Unk21	Unk22	Unk22	Unk23	Unk23	Unk24	Unk24	Unk25	Unk25		
F	Unk26	Unk26	NTC3	NTC3	Unk27	Unk27	Unk28	Unk28	Unk29	Unk29		
G	Unk30	Unk30	Unk31	Unk31	Unk32	Unk32	Unk33	Unk33	Unk34	Unk34		
H	Unk35	Unk35	Unk36	Unk36	Unk37	Unk37	Unk38	Unk38	Unk39	Unk39		





✓**Controllo Positivo**: campione che sicuramente esprime il gene di interesse.

4.VERIFICA DEI REPLICATI



CT (ciclo soglia): corrisponde al ciclo della reazione di PCR in cui la fluorescenza emessa supera la soglia (threshold).

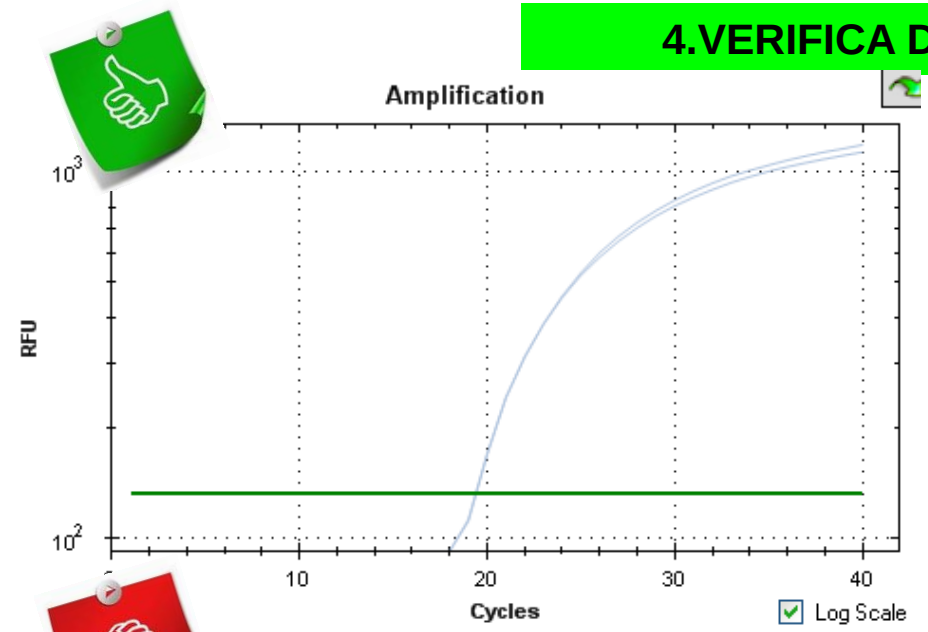
	1	2	3	4	5	6
A	1 NT	1 NT	1 NT	1 NT	9 NT	9 NT
B	2 NT	2 NT	2 NT	2 NT	10 NT	10 NT
C	3 NT	3 NT	3 NT	3 NT	11 25nM	11 25nM
D	4NT	4NT	4NT	4NT	12 25 nM	12 25 nM
E	5 NT	5 NT	5 NT	5 NT	13 25 nM	13 25 nM
F	6NT	6NT	6NT	6NT	14 25 nM	14 25 nM
G	7 NT	7 NT	7 NT	7 NT	15 25 nM	15 25 nM
H	8 NT	8 NT	8 NT	8 NT	16 25 nM	16 25 nM

Nel nostro caso

	1	2	3	4	5	6
A	1 NT	1 NT	1 NT	9 NT	9 NT	9 NT
B	2 NT	2 NT	2 NT	10 NT	10 NT	10 NT
C	3 NT	3 NT	3 NT	11 25nM	11 25nM	11 25nM
D	4NT	4NT	4NT	12 25 nM	12 25 nM	12 25 nM
E	5 NT	5 NT	5 NT	13 25 nM	13 25 nM	13 25 nM
F	6NT	6NT	6NT	14 25 nM	14 25 nM	14 25 nM
G	7 NT	7 NT	7 NT	15 25 nM	15 25 nM	15 25 nM
H	8 NT	8 NT	8 NT	16 25 nM	16 25 nM	16 25 nM

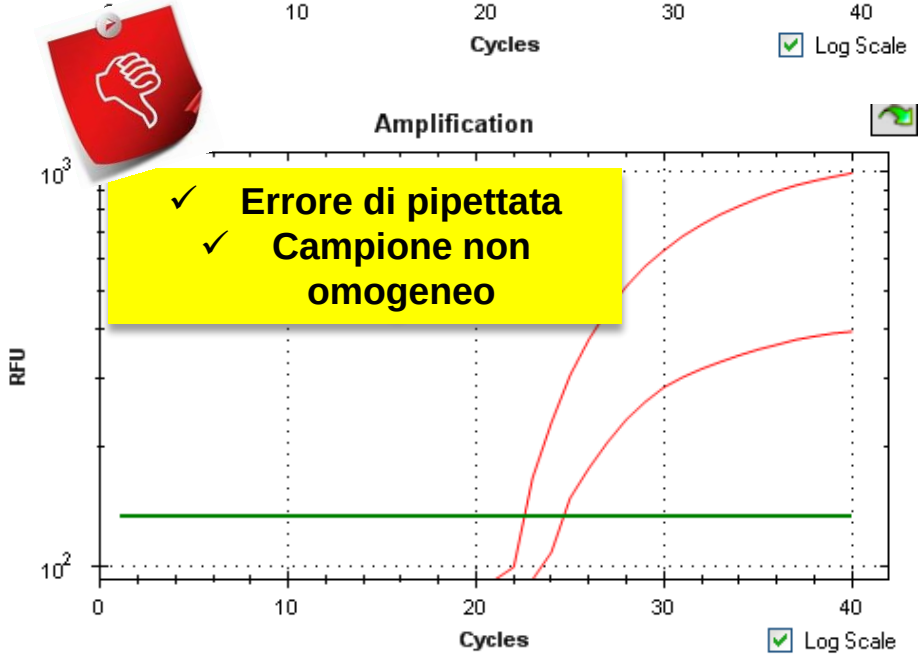
Statisticamente corretto

4.VERIFICA DEI REPLICATI



Well	Fluor	Target	Content	Sample	Cq
A01	FAM	Gamma glob	Unkn-01	1 NT	19,37
A02	FAM	Gamma glob	Unkn-01	1 NT	19,35

Δ(A01-A02)= 19.37-19.35= **0.02**



- ✓ **Errore di pipettata**
- ✓ **Campione non omogeneo**

Well	Fluor	Target	Content	Sample	Cq
F01	FAM	Gamma glob	Unkn-16	6NT	24,61
F02	FAM	Gamma glob	Unkn-16	6NT	22,50

Δ(F01-F02)= 24.61-22.50= **2.11**

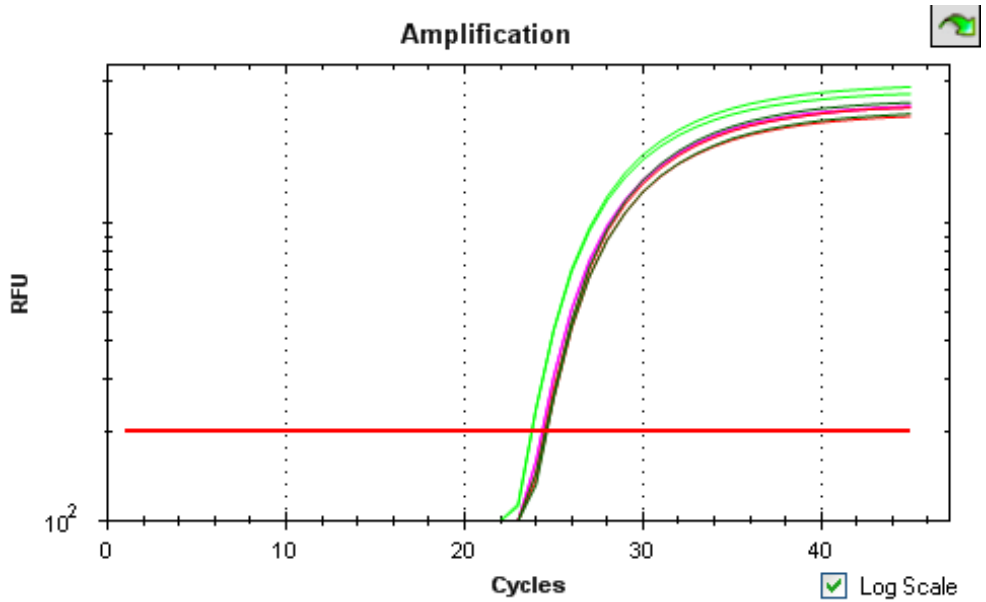
La differenza di CT tra i replicati dello stesso campione non deve superare 0.5 CT

NB: Questa verifica deve essere eseguita su tutti i campioni caricati nella piastra

4.VERIFICO L'ANDAMENTO DEL GENERE REFERENCE

✓**Stabile**

✓**La sua espressione non deve essere alterata dal trattamento o dalla condizione patologica**

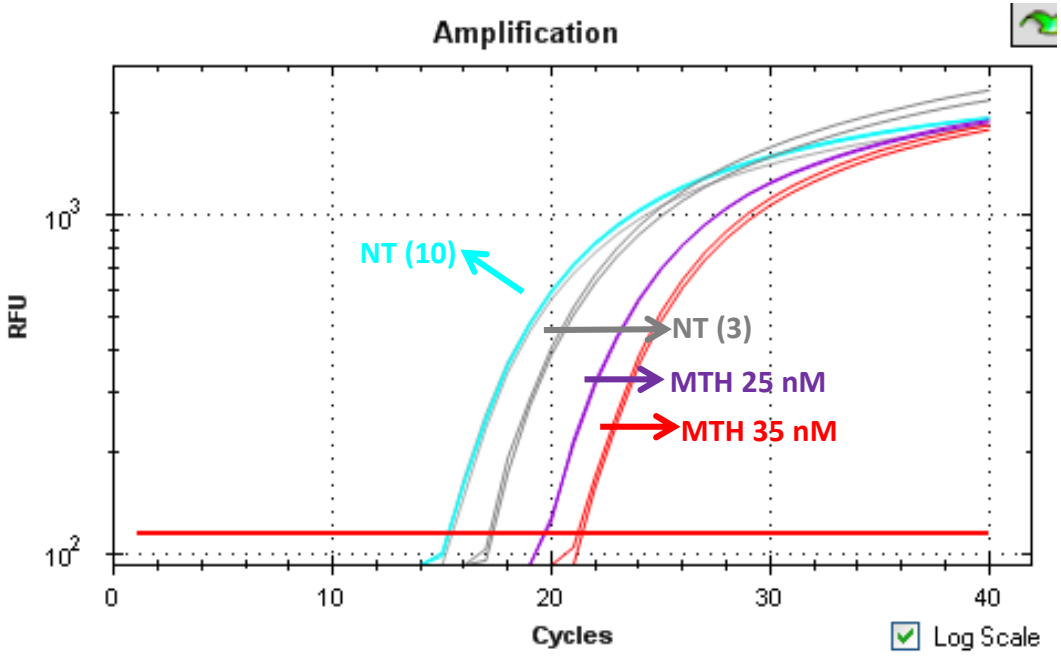


Situazione ottimale ΔCT < 0.5

Well	Fluor	Target	Content	Sample	Cq
E09	SYBR	RPL13A	Unkn-29	K562(-)5	24,28
E10	SYBR	RPL13A	Unkn-29	K562(-)5	24,28
F09	SYBR	RPL13A	Unkn-35	K562 20nM5	23,69
F10	SYBR	RPL13A	Unkn-35	K562 20nM5	23,71
G09	SYBR	RPL13A		25nM 5	24,41
G10	SYBR	RPL13A		25nM 5	24,52
H09	SYBR	RPL13A	Unkn-47	K562 30nM 5	24,47
H10	SYBR	RPL13A	Unkn-47	K562 30nM 5	24,54

ΔCT= 0.85

Non sempre è così…



B07	VIC	18S	Unkn-29	10 NT	15,24
B08	VIC	18S	Unkn-29	10 NT	15,27
C03	VIC	18S	Unkn-31	3 NT	17,14
C04	VIC	18S	Unkn-31	3 NT	17,26
G07	VIC	18S	Unkn-44	15 25 nM	19,78
G08	VIC	18S	Unkn-44	15 25 nM	19,82
G11	VIC	18S	Unkn-45	23 35nM	21,17
G12	VIC	18S	Unkn-45	23 35nM	21,36

ΔCT= 6.12

6.QUANTIFICAZIONE RELATIVA DEL GENE TARGET

Quantificazione secondo il metodo del ΔΔCT (Delta-Delta CT)

Determino la concentrazione relativa del gene target nel campione incognito rispetto al campione di controllo

Fold expression = $2^{-\Delta\Delta Ct}$

Modello matematico

1. Calcolo il ΔCT

ΔCT = CT medio GENE TARGET – CT medio GENE HOUSEKEEPING

Questo step serve a normalizzare il campione, cioè escludere variazioni dovute al caricamento di quantità diverse tra un campione e l'altro.

2. Calcolo il ΔΔCT

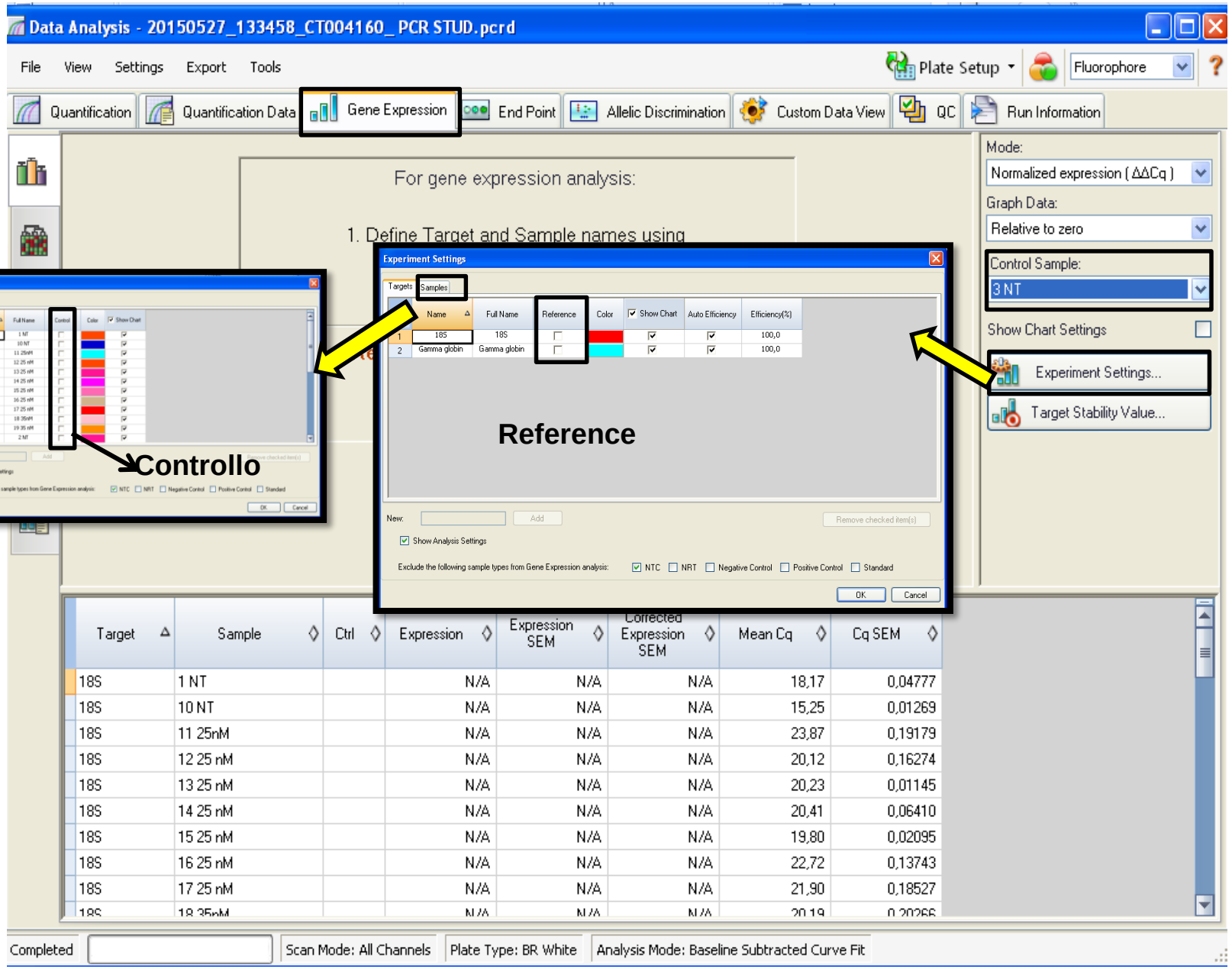
ΔΔCT = ΔCT campione INCOGNITO – ΔCT campione scelto come CONTROLLO

Confronto i CT dei campioni incogniti rispetto a un campione scelto dall'operatore come controllo (ad es. trattato vs non trattato, oppure patologia vs sano etc…)

3. Calcolo il FOLD change

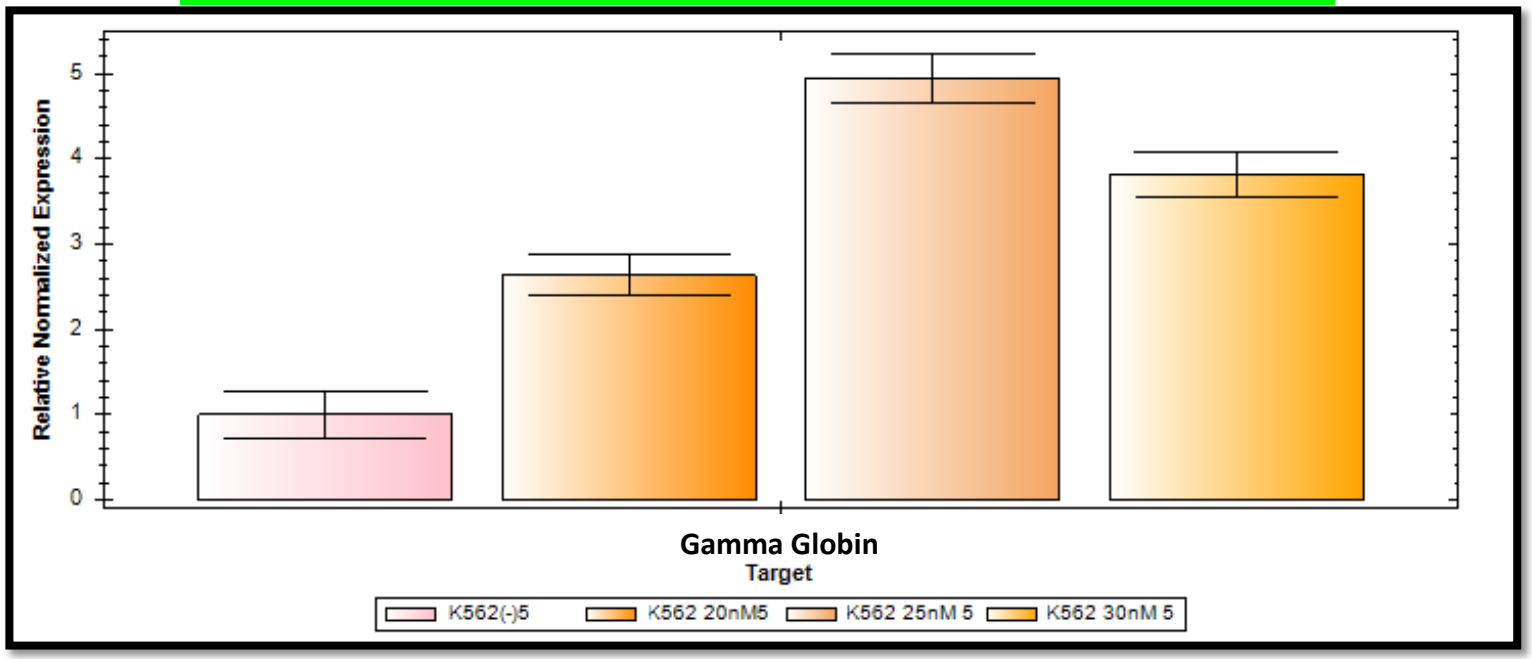
Fold change = $2^{-\Delta\Delta Ct}$

6.QUANTIFICAZIONE RELATIVA DEL GENE TARGET



Target	Sample	Ctrl	Expression	Expression SEM	Corrected Expression SEM	Mean Cq	Cq SEM
18S	1 NT		N/A	N/A	N/A	18,17	0,04777
18S	10 NT		N/A	N/A	N/A	15,25	0,01269
18S	11 25nM		N/A	N/A	N/A	23,87	0,19179
18S	12 25 nM		N/A	N/A	N/A	20,12	0,16274
18S	13 25 nM		N/A	N/A	N/A	20,23	0,01145
18S	14 25 nM		N/A	N/A	N/A	20,41	0,06410
18S	15 25 nM		N/A	N/A	N/A	19,80	0,02095
18S	16 25 nM		N/A	N/A	N/A	22,72	0,13743
18S	17 25 nM		N/A	N/A	N/A	21,90	0,18527
18S	18 35nM		N/A	N/A	N/A	20,19	0,20266

6.QUANTIFICAZIONE RELATIVA DEL GENE TARGET



Sample	Ctrl	Fold Expression	Errore Standard Expression SEM	Corrected Expression SEM	CT medio Mean Cq	Cq SEM
K562 20nM5		2,63328	0,24131	0,24131	26,12	0,12941
K562 25nM 4		3,35843	0,23284	0,23284	25,04	0,09980
K562 25nM 5		4,94617	0,29011	0,29011	25,66	0,07956
K562 30nM 4		3,22014	0,28171	0,28171	24,83	0,11791
K562 30nM 5		3,81577	0,26504	0,26504	26,16	0,09736
K562(-)5	*	1,00000	0,27098	0,27098	32,32	0,20024

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

✓ $2^{-\Delta\Delta CT} > 2$

Il gene target, nel campione in analisi, è più espresso rispetto al campione scelto come riferimento (controllo).

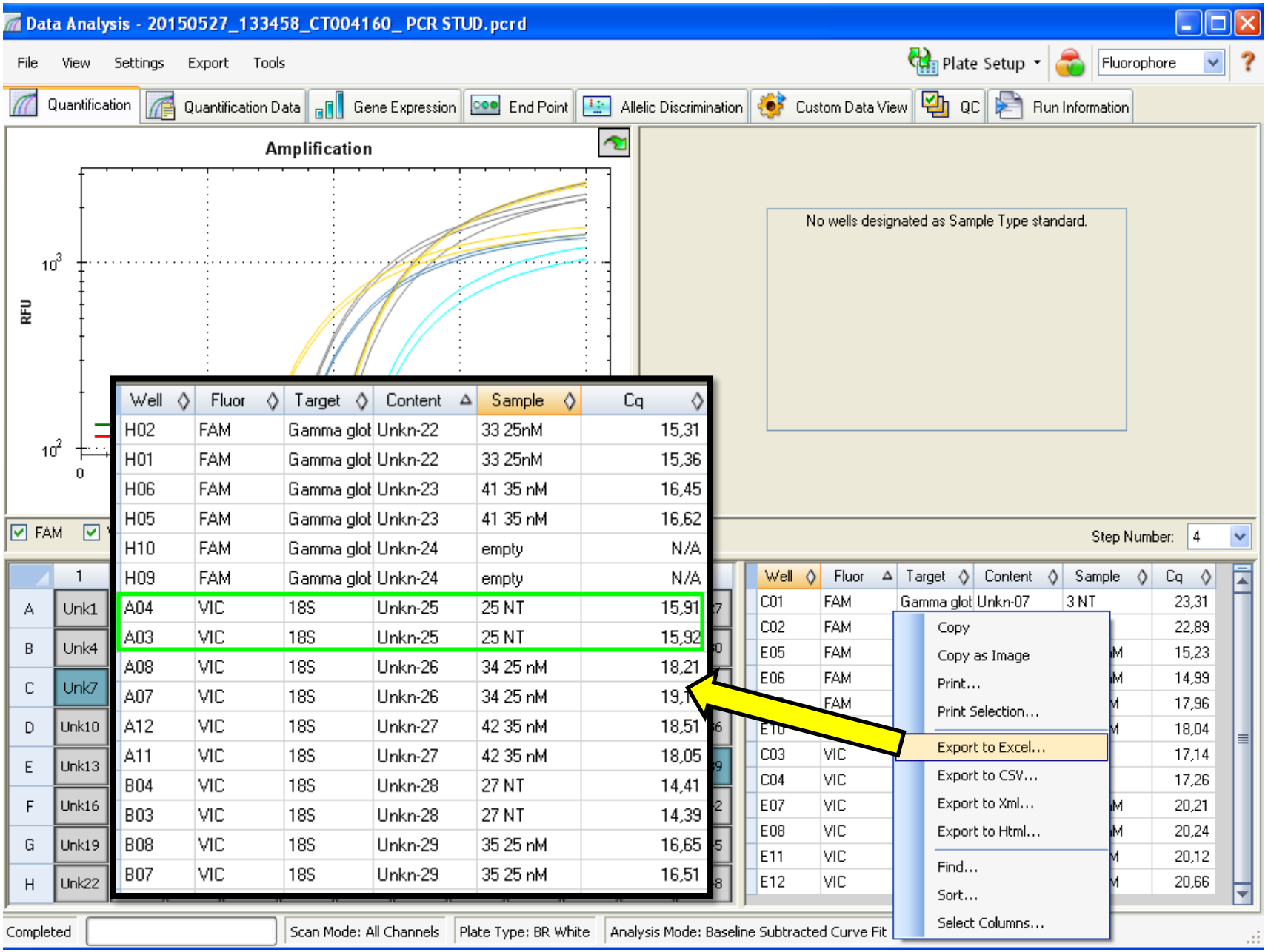
✓ $2^{-\Delta\Delta CT} < 0.5$

Il gene target, nel campione in analisi, è meno espresso rispetto al campione scelto come riferimento (controllo).

✓ $0,5 < 2^{-\Delta\Delta CT} > 2$

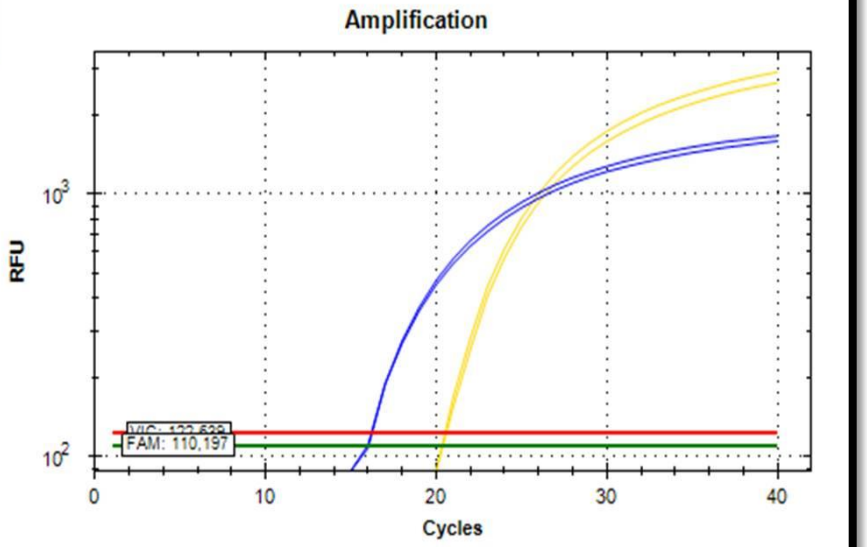
La variazione di espressione del gene target nel campione in analisi rispetto al campione scelto come controllo non è significativa.

6.QUANTIFICAZIONE RELATIVA DEL GENE TARGET



Well	Fluor	Target	Content	Sample	Cq
H02	FAM	Gamma glot	Unkn-22	33 25nM	15,31
H01	FAM	Gamma glot	Unkn-22	33 25nM	15,36
H06	FAM	Gamma glot	Unkn-23	41 35 nM	16,45
H05	FAM	Gamma glot	Unkn-23	41 35 nM	16,62
H10	FAM	Gamma glot	Unkn-24	empty	N/A
H09	FAM	Gamma glot	Unkn-24	empty	N/A
A04	VIC	18S	Unkn-25	25 NT	15,91
A03	VIC	18S	Unkn-25	25 NT	15,92
A08	VIC	18S	Unkn-26	34 25 nM	18,21
A07	VIC	18S	Unkn-26	34 25 nM	19,1
A12	VIC	18S	Unkn-27	42 35 nM	18,51
A11	VIC	18S	Unkn-27	42 35 nM	18,05
B04	VIC	18S	Unkn-28	27 NT	14,41
B03	VIC	18S	Unkn-28	27 NT	14,39
B08	VIC	18S	Unkn-29	35 25 nM	16,65
B07	VIC	18S	Unkn-29	35 25 nM	16,51

14 MTH
25nM



Well	Fluor	Target	Content	Sample	Cq
F05	FAM	Gamma glob	Unkn-17	14 25 nM	15,99
F06	FAM	Gamma glob	Unkn-17	14 25 nM	16,03
F07	VIC	18S	Unkn-41	14 25 nM	20,57
F08	VIC	18S	Unkn-41	14 25 nM	20,43

1. Calcolare la media dei replicati per ogni gene

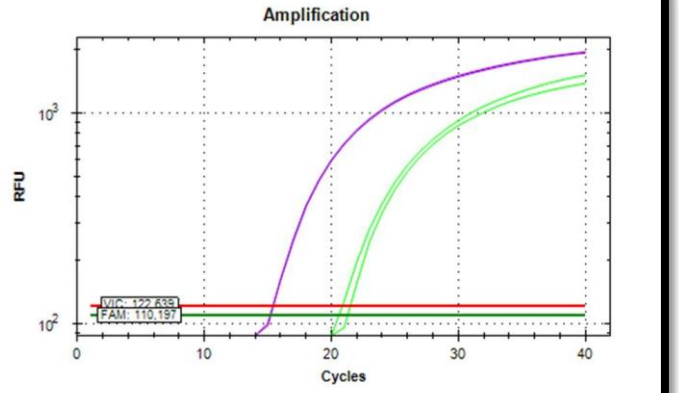
Gamma Globina-> (15.99+16.03)/2 = 16.01
18 s-> (20.57+20.43)/2= 20.5

2. Calcolare il ΔCT

ΔCT= CT gene target (gamma globin) – CT gene reference (18S)

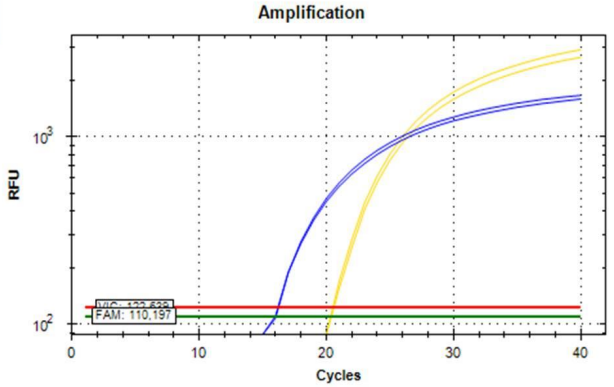
ΔCT= 16.01-20.5 = -4.49

10 NT



Well	Fluor	Target	Content	Sample	Cq
B05	FAM	Gamma glob	Unkn-05	10 NT	21,23
B06	FAM	Gamma glob	Unkn-05	10 NT	20,70
B07	VIC	18S	Unkn-29	10 NT	15,35
B08	VIC	18S	Unkn-29	10 NT	15,37

14 MTH
25nM



Well	Fluor	Target	Content	Sample	Cq
F05	FAM	Gamma glob	Unkn-17	14 25 nM	15,99
F06	FAM	Gamma glob	Unkn-17	14 25 nM	16,03
F07	VIC	18S	Unkn-41	14 25 nM	20,57
F08	VIC	18S	Unkn-41	14 25 nM	20,43

3. Calcolare il ΔΔCT

ΔΔCT= ΔCT trattato MTH - ΔCT del campione NT

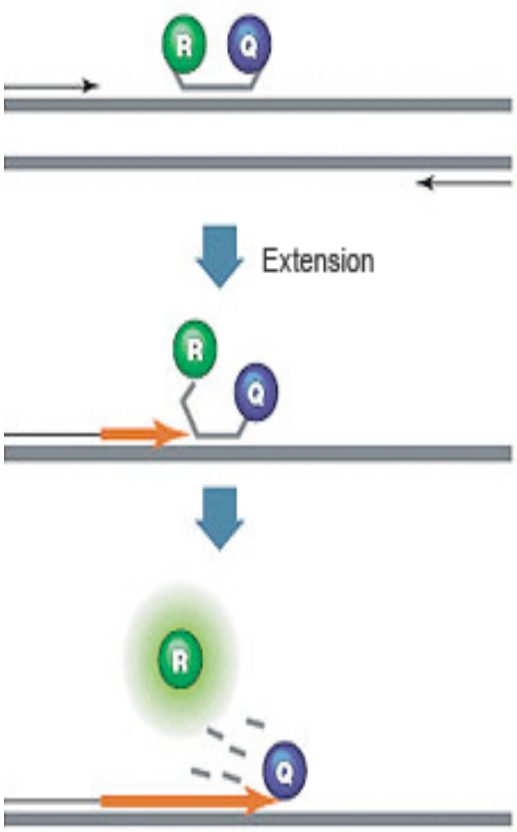
ΔΔCT= -4.49-5.6=-10.09

4. Calcolare il fold change

Fold change = $2^{-\Delta\Delta Ct}$

Fold change = 1089

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



1

2

3

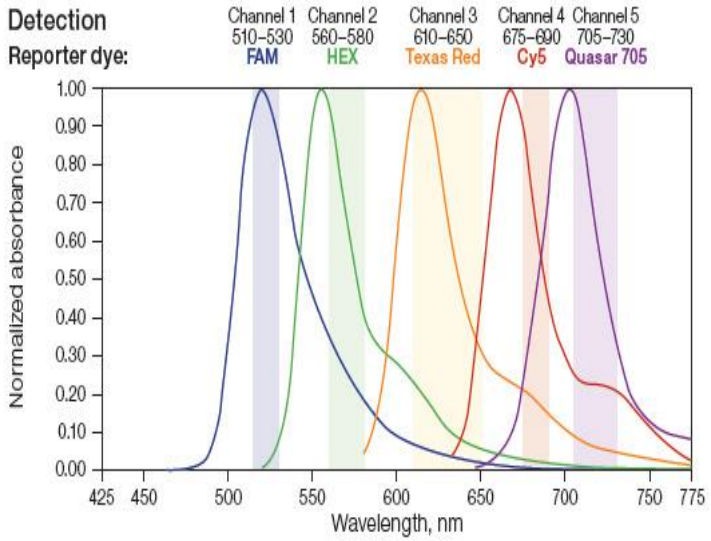
1. This probe is composed of a short (20-25 bases) oligodeoxynucleotide that is labeled with two different fluorescent dyes.

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.

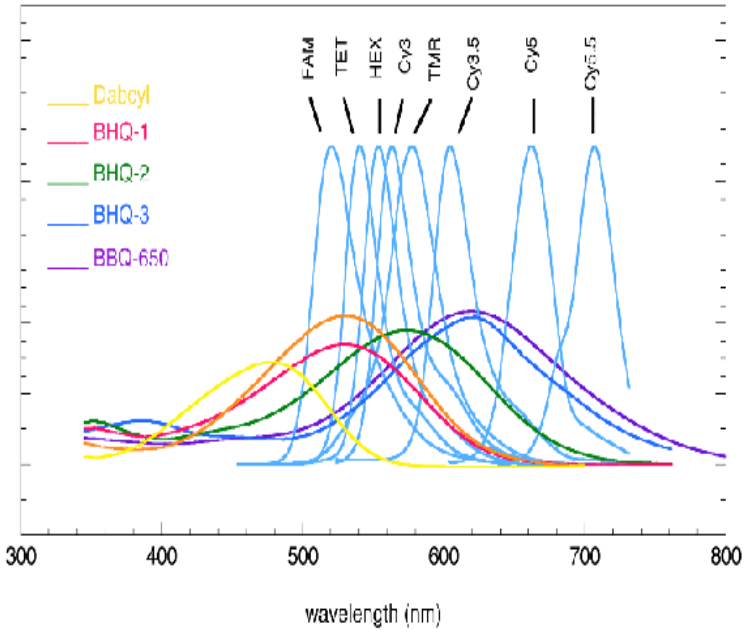
3. When the probe is intact, energy transfer occurs between the two fluorophors and emission from the reporter is quenched by the quencher. During the extention phase of PCR, the probe is cleaved by 5' nuclease activity of Taqpolymerase thereby releasing the reporter from the oligonucleotide-quencher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

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

Reporter



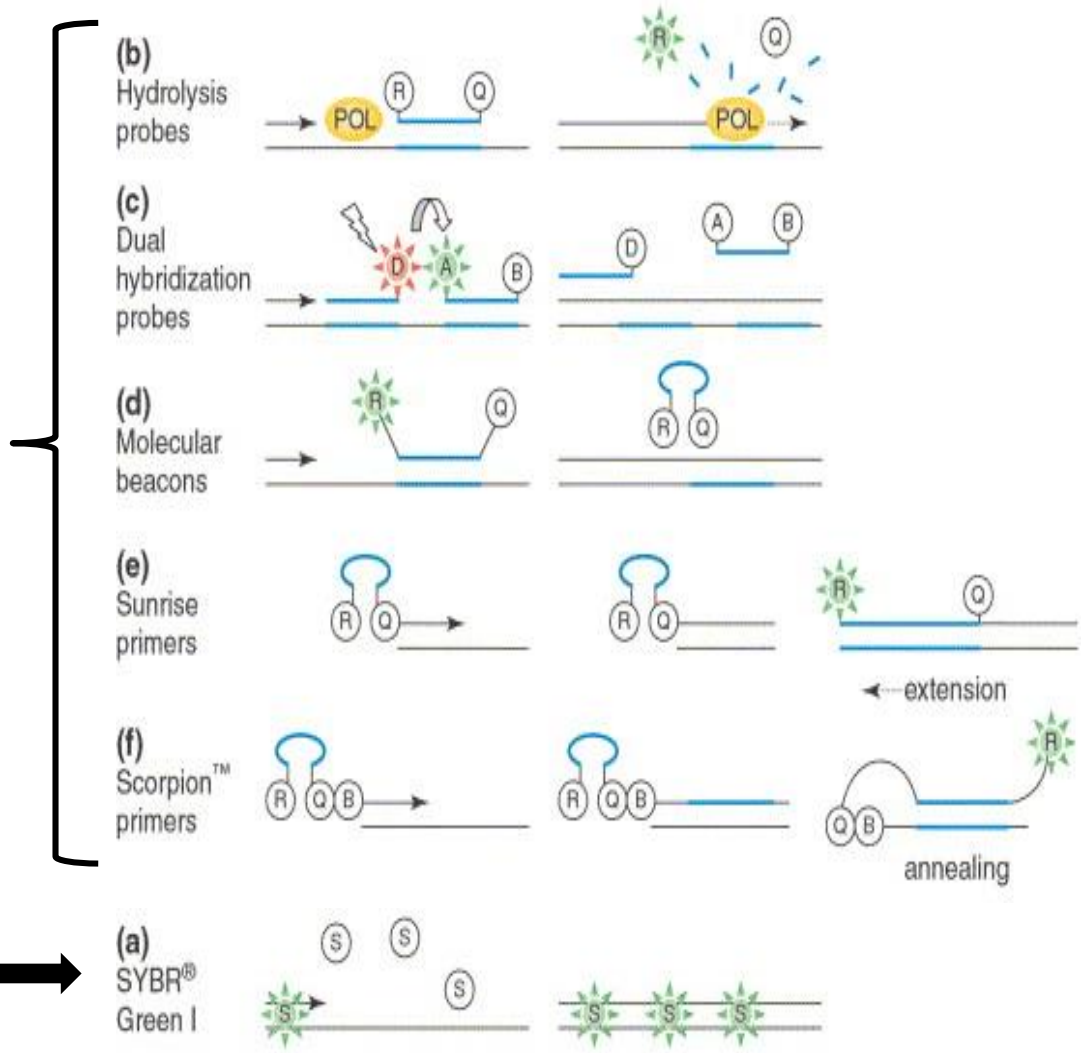
Quencer



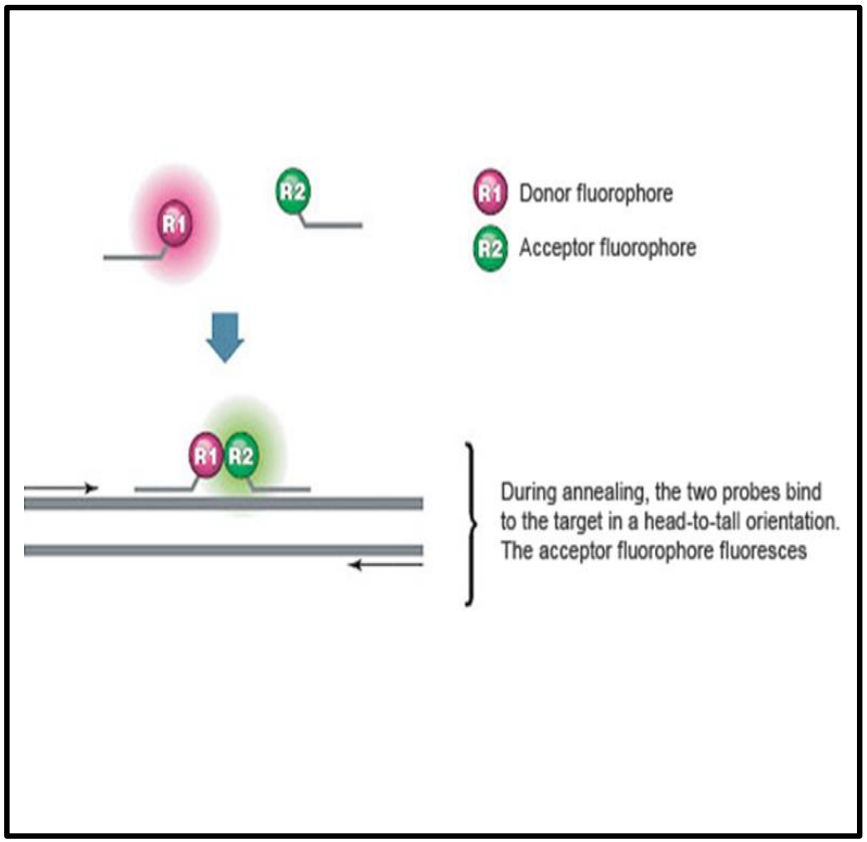
Fluorescent molecules used in quantitative real time PCR

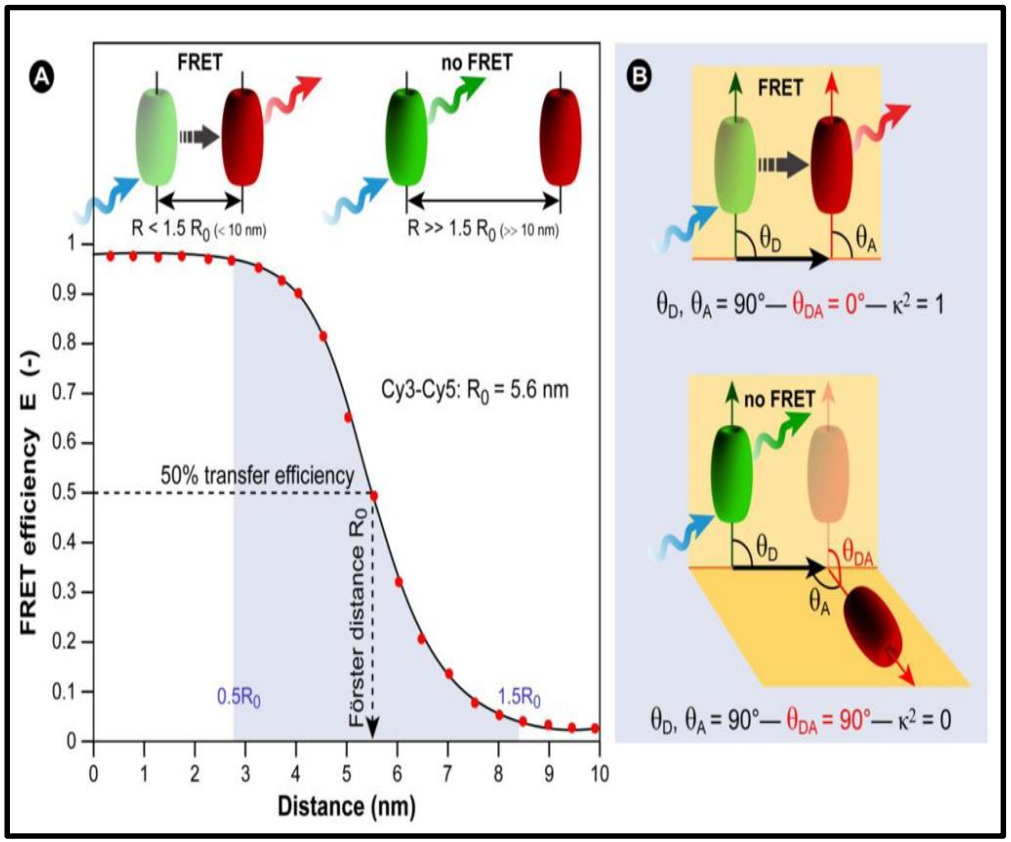
1. Utilizzo di sonde oligonucleotidiche marcate con molecole fluorescenti e leganti specifiche sequenze di DNA bersaglio

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

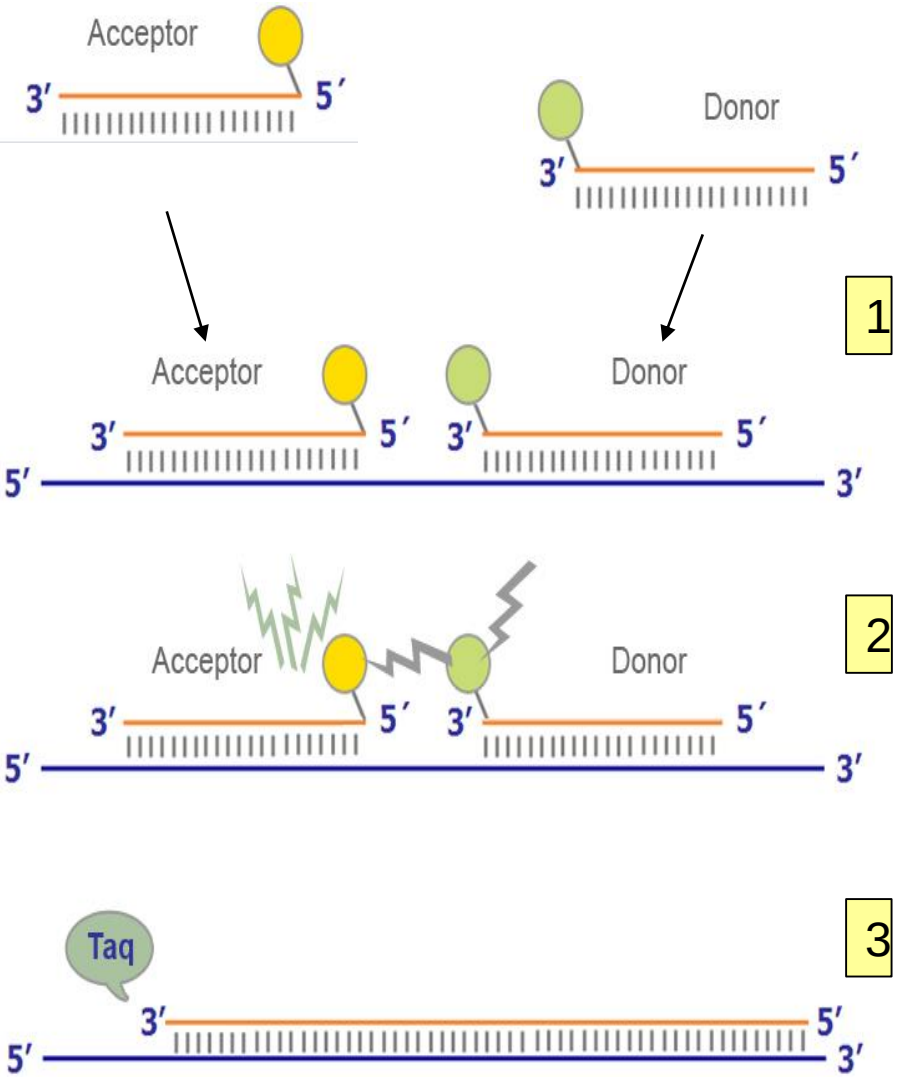


FRET probe (Fluorescence Resonance Energy Transfer): dual hybridization probes





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

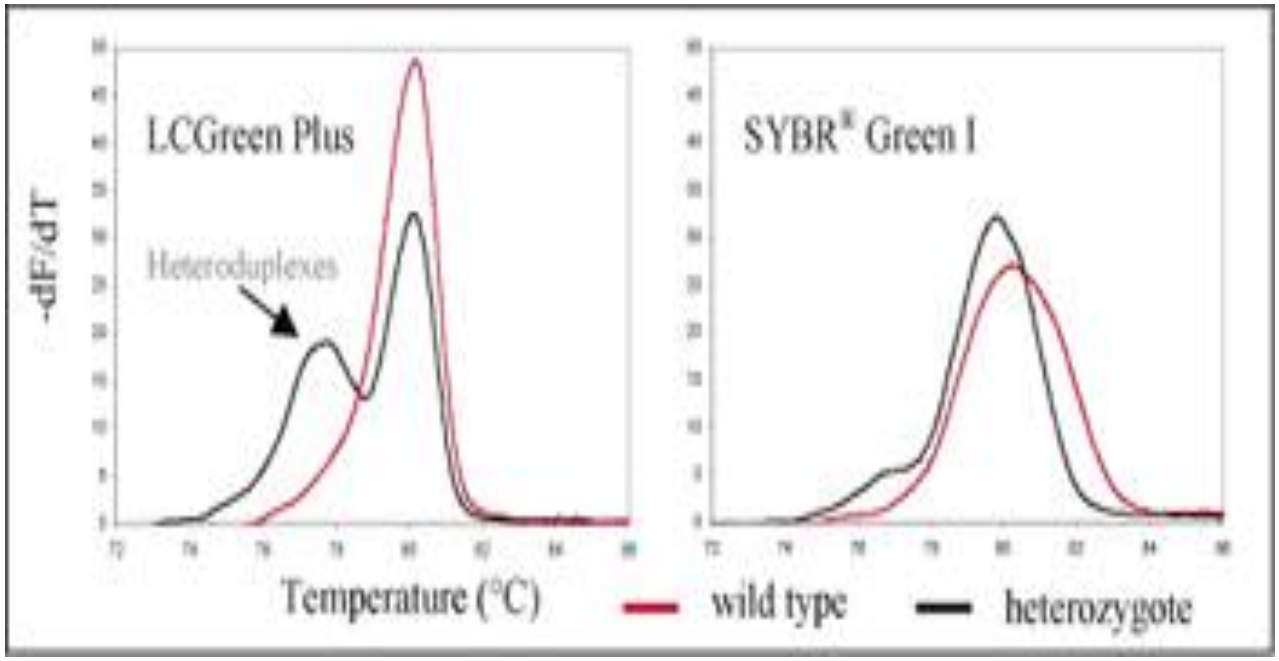


1. Simili alle sonde TaqMan perché si legano al DNA bersaglio e vengono idrolizzate, questo sistema prevede però l'impiego di due sonde ognuna marcata con un solo fluorocromo (accettore e donatore). **Quando le sonde non sono legate alle sequenze target il segnale fluorescente proveniente dall'accettore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

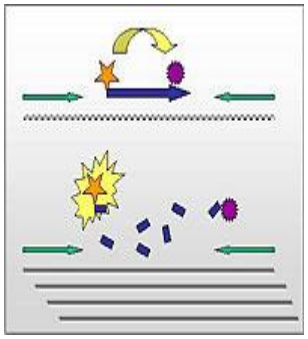
2. **Durante lo *step di annealing***, entrambe le sonde FRET ibridizzano alle sequenze target: ciò avvicina il fluoroforo donatore al fluoroforo accettore permettendo il **trasferimento di energia tra i due fluorofori** e la produzione di un segnale fluorescente da parte dell'accettore che viene così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

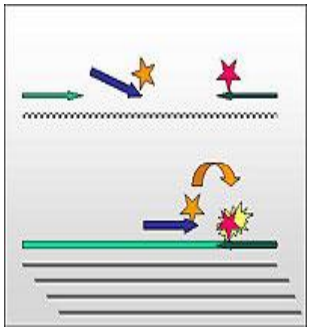
LCGreen Plus

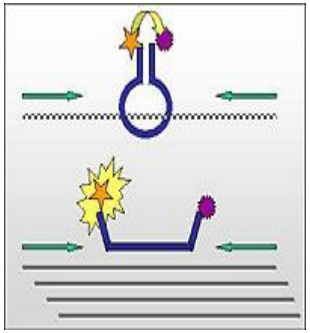
Hi-Res Melting, with the LightScanner system, offers superior performance. Derivative melting curves **illustrate the detection of heteroduplexes** in the heterozygous mutant using **LCGreen Plus** as shown below, **which are not detected using SYBR®**



Consideration about probes



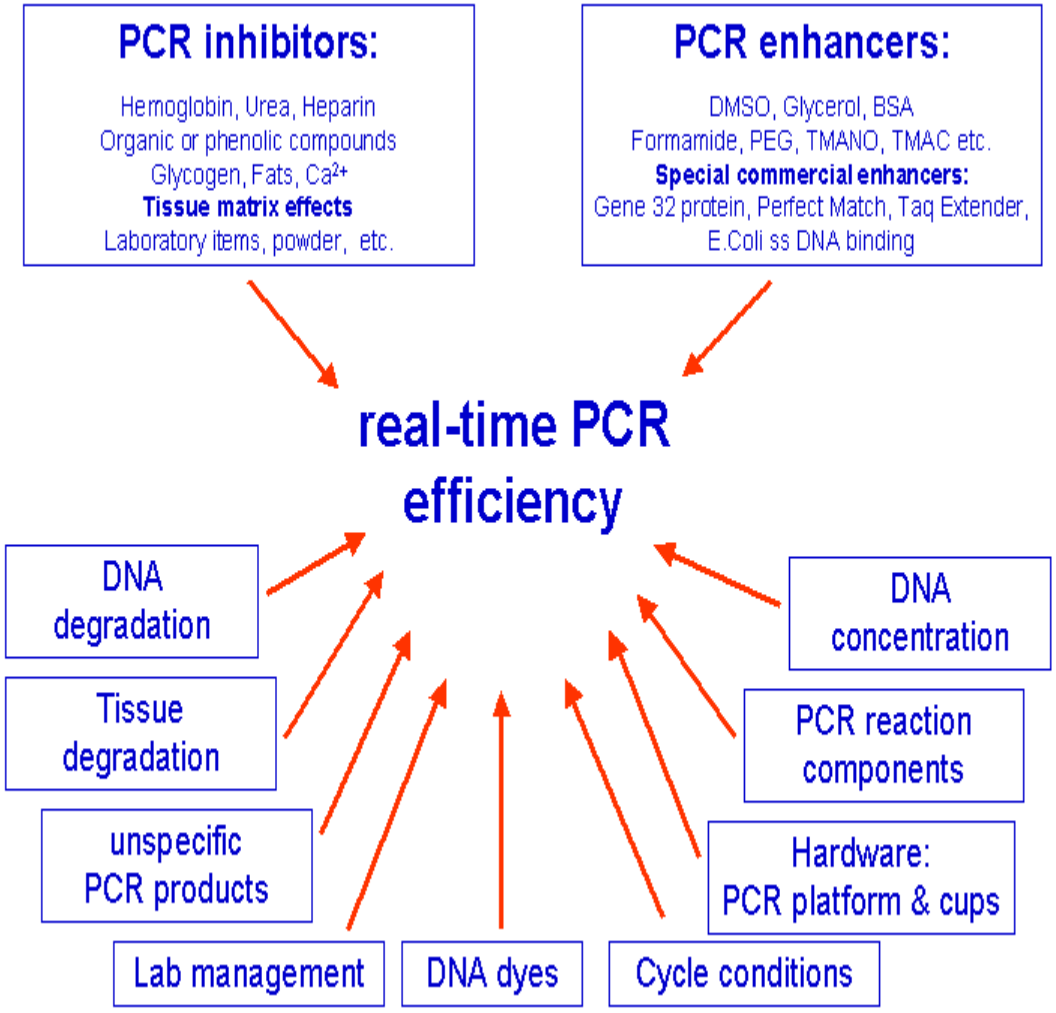




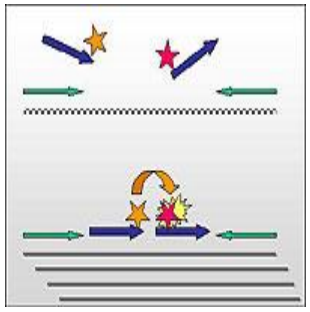
TaqMan® probes are more sensitive to single base variations (**mismatch**) than other probes. This could be extremely important when amplifying virological samples, where such a **genetic variability** could be present that a successful amplification may fail to result in a positive signal. Unfortunately, **this sensitivity may render TaqMan® probes inappropriate for genotyping**, **since a 'non-signal' will have to be attributed to a genotype.**

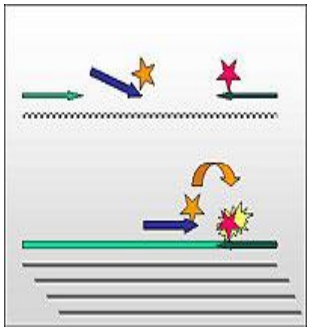
There are various possibilities to allow adjacent hybridization of oligonucleotides. Probably the most popular and successful method is the binding of two single labeled probes in a head to toe manner, also known as "kissing probes" or HybProbe. **The FRET** induced fluorescence of the acceptor dye is 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.

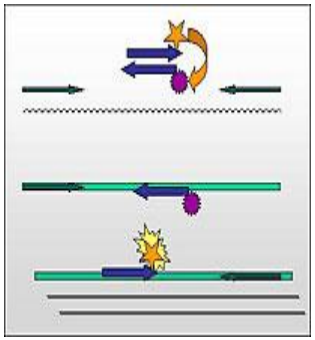
A very interesting variation of hybridization probes is **Molecular Beacons**, developed by *Fred R. Kramer*. The ends of the probes are self-complementary and labeled with a fluorophore/quencher pair. In absence of a complementary sequence, these molecules fold into a stem-loop structure and the fluorescence is extinguished by the quencher. When bound to a target the increased distance between quencher and dye results in an increase of detectable fluorescence. **Molecular beacons are sensitive to mismatches.** Unlike **TaqMan probes, the molecular beacons are not destroyed during the amplification reactions so they can be used again during the next cycle**.


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

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





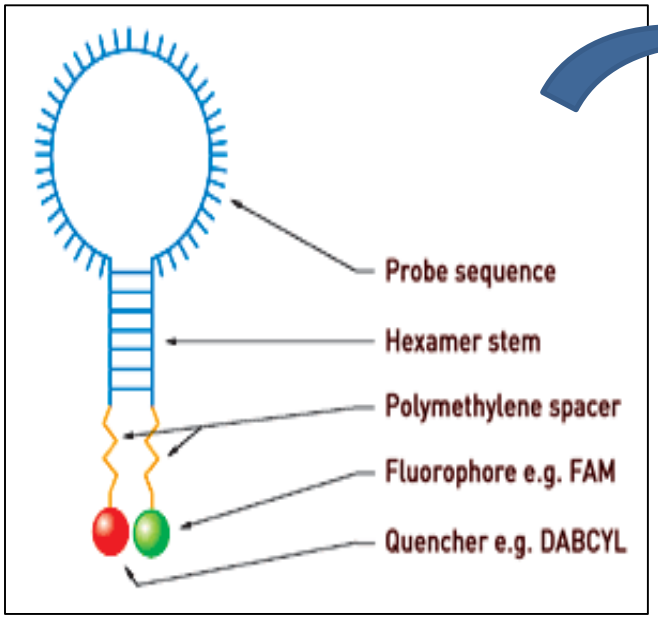


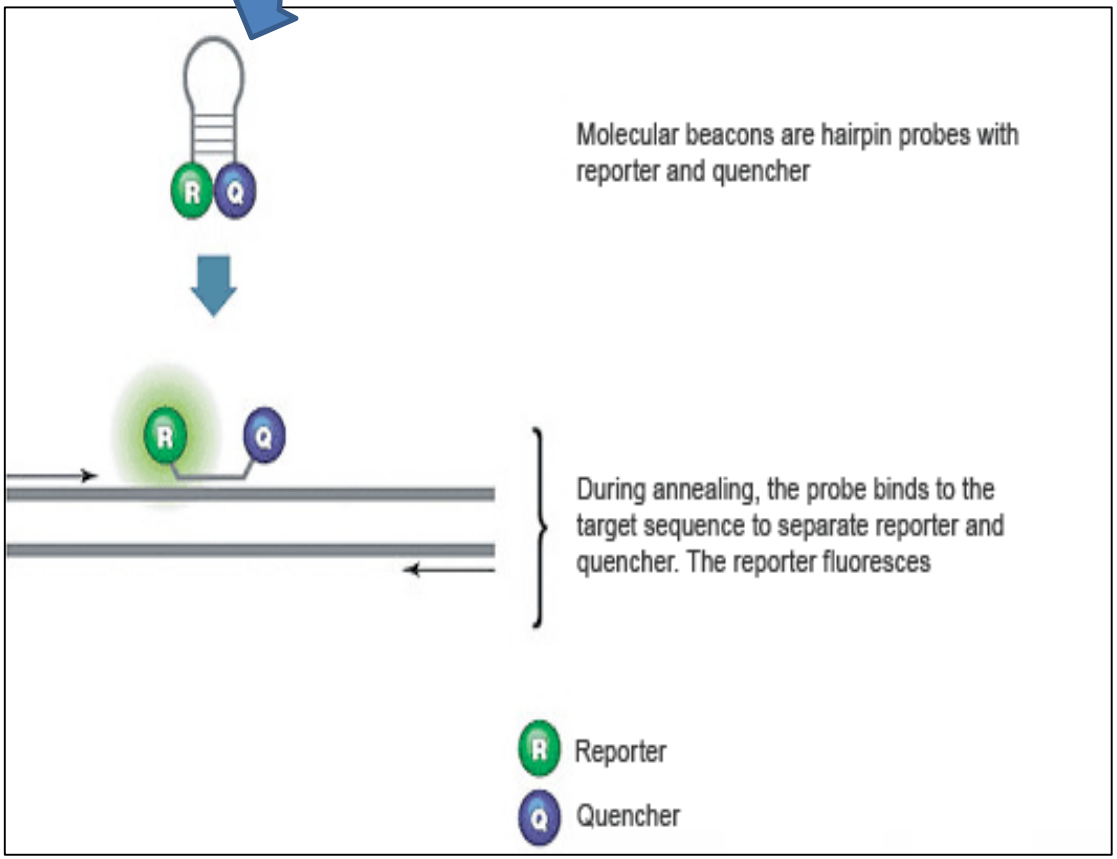
There are various possibilities to allow adjacent hybridization of oligonucleotides. Probably the most popular and successful method is the binding of **two single labeled probes in a head to toe manner**, also known as "**kissing probes**" or HybProbe. The FRET induced fluorescence of the acceptor dye 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.

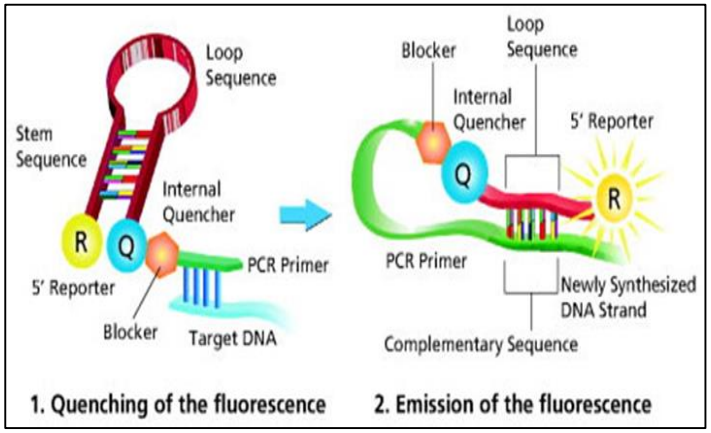
FRET probe (Fluorescence Resonance Energy Transfer): molecular beacons



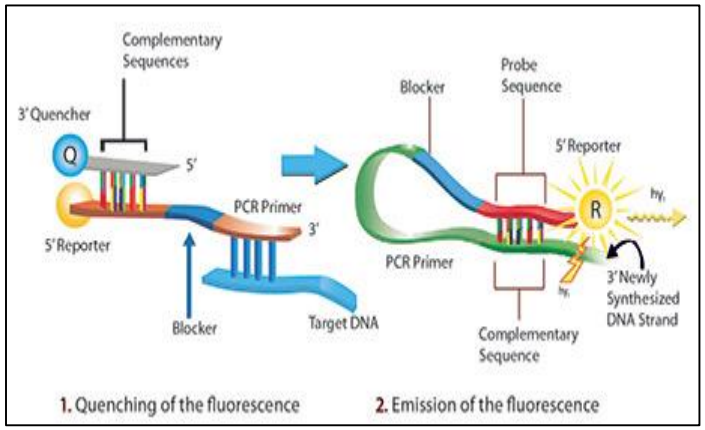


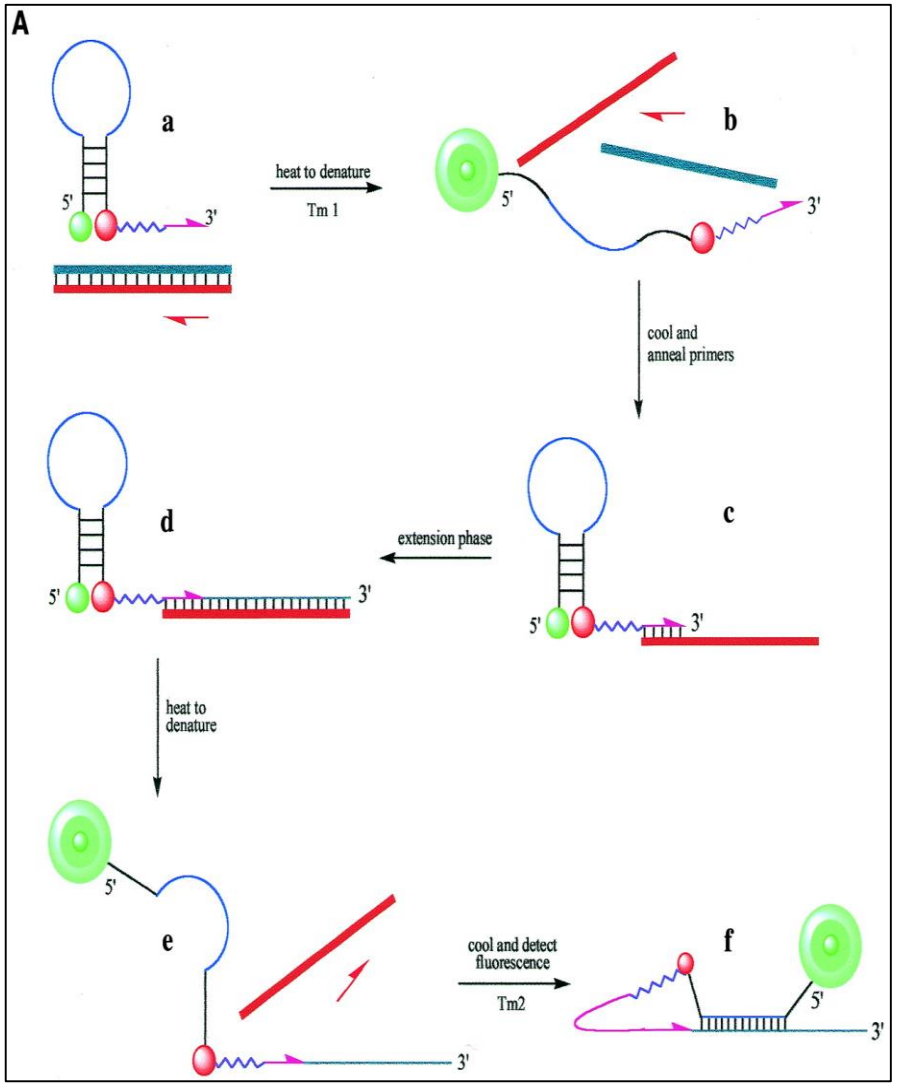
FRET probe (Fluorescence Resonance Energy Transfer): scorpion primers

uni-probe

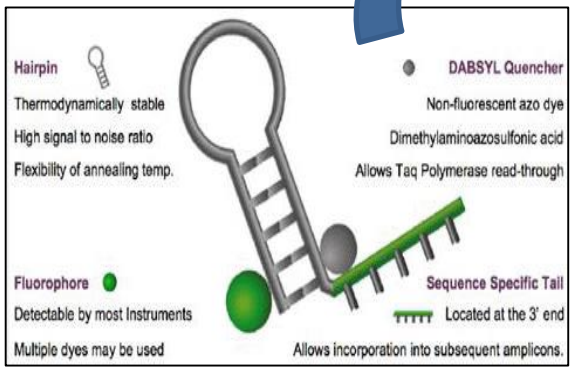


bi-probe

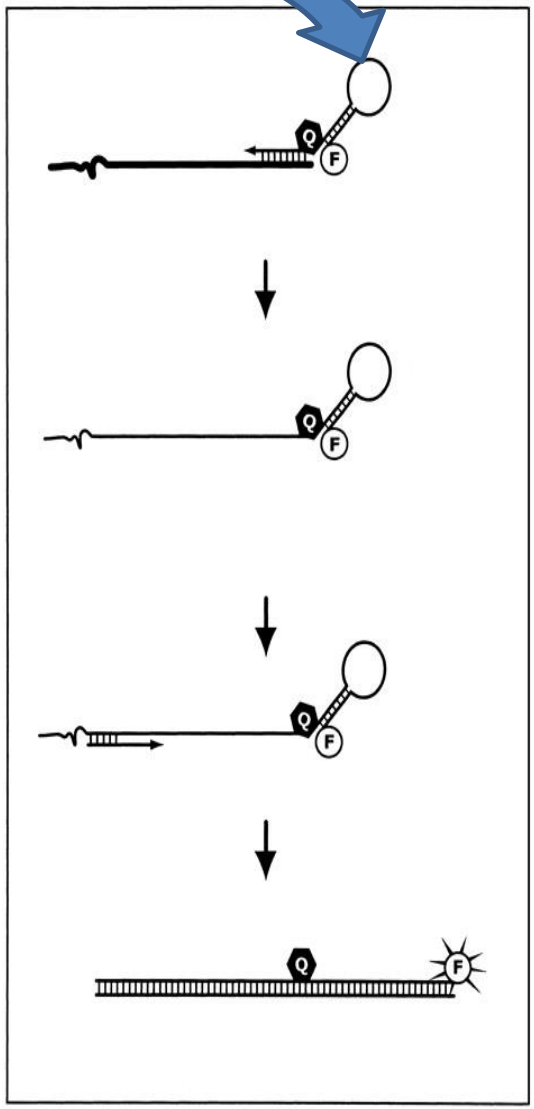




FRET probe (Fluorescence Resonance Energy Transfer): sunrise primers



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

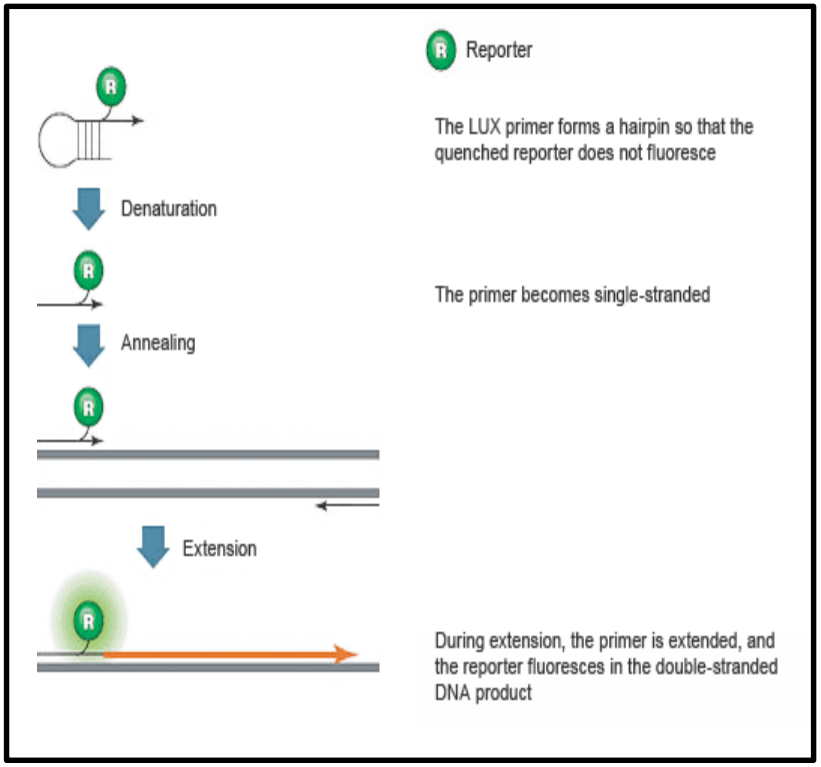


1. The Sunrise primer-probes have reporter dyes attached to 3' end of the stem and quenchers attached to the 5' end of the stem
2. These probes consist of a dual-labeled (reporter and quencher fluorophores) hairpin loop on the 5′ end, with the 3′ end acting as the PCR primer. When unbound, the hairpin 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

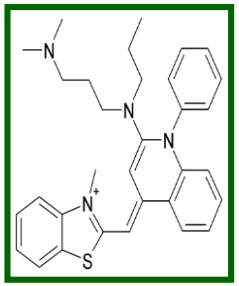
LUX™ fluorogenic primers are self-quenched single-fluorophore labeled primers almost identical to Sunrise primers.
However, rather than using a quencher fluorophore**, the secondary structure of the 3′ end reduces initial fluorescence to a minimal amount**. Because this chemistry **does not require a quencher dye**, it is much less expensive than dual-labeled probes.

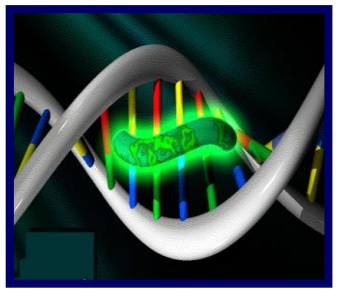
While this system relies on only two oligonucleotides for specificity, unlike the SYBR Green I platform in which a dissociation curve is used to detect erroneous amplification, no such convenient detection exists for the LUX platform. Agarose gels must be run to ensure the presence of a single PCR product, a step that is extremely important not only for the LUX primers, but also for the Sunrise primers and scorpions because PCR priming and probe binding are not independent in these chemistries.



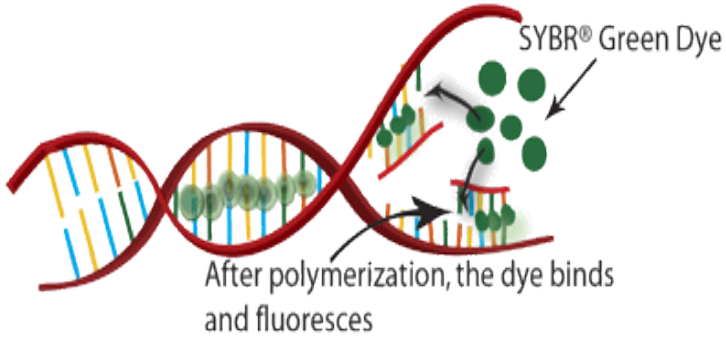
SYBR Green I

1. Il SYBR Green I è una molecola fluorescente

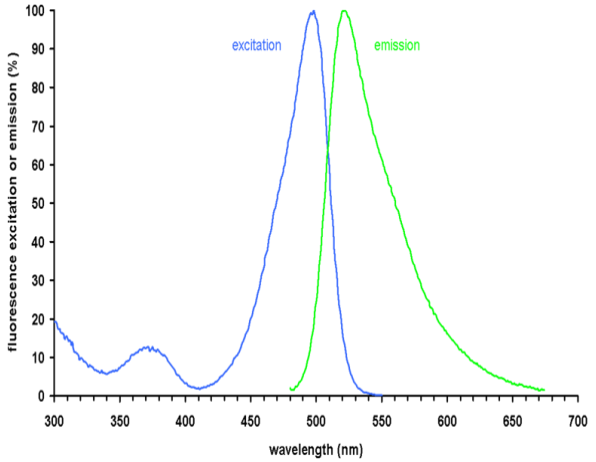




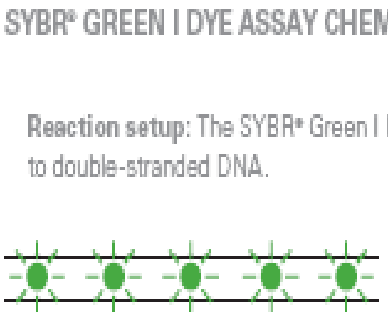
2. agente intercalante e si lega preferenzialmente a DNA a doppio filamento (dsDNA)



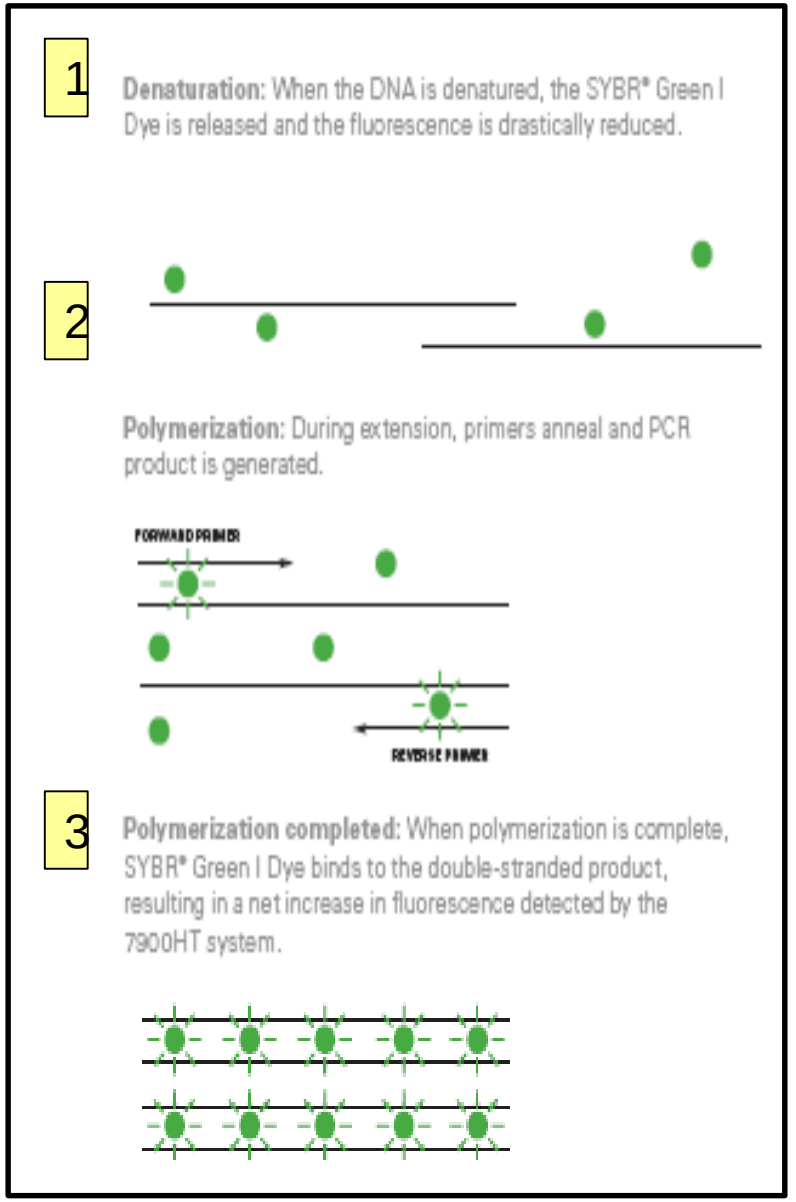
3. formando un complesso DNA-colorante che assorbe luce blu ad una lunghezza d'onda λmax= 488 nm ed emettendo luce verde λmax= 522 nm.



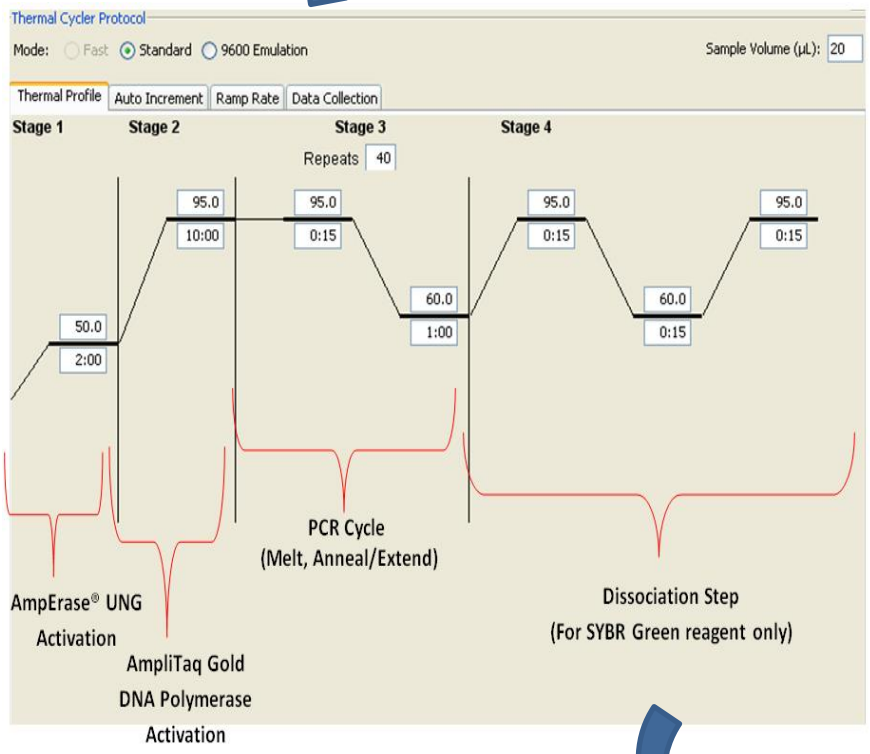
SYBR Green I

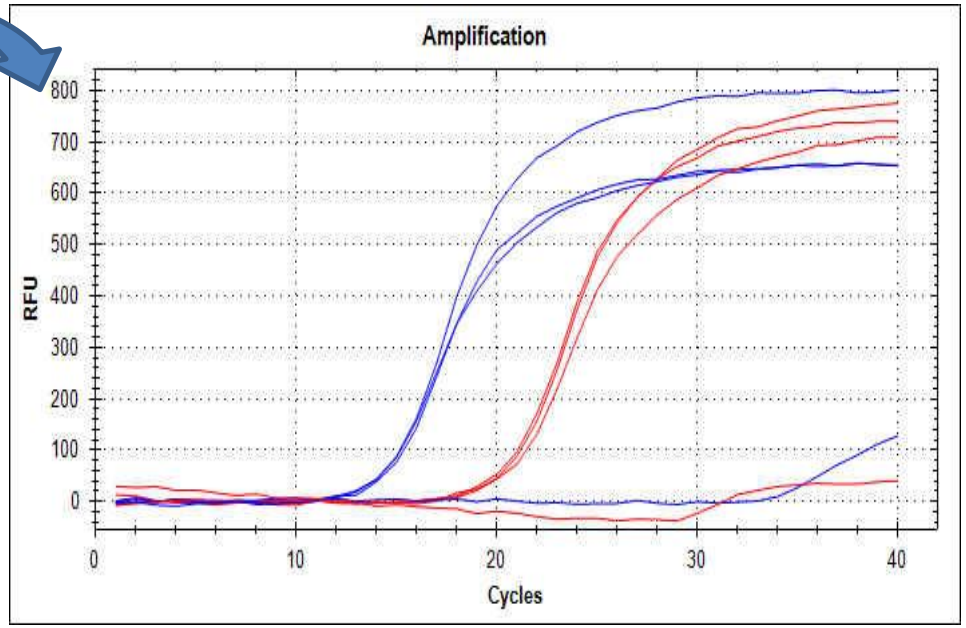


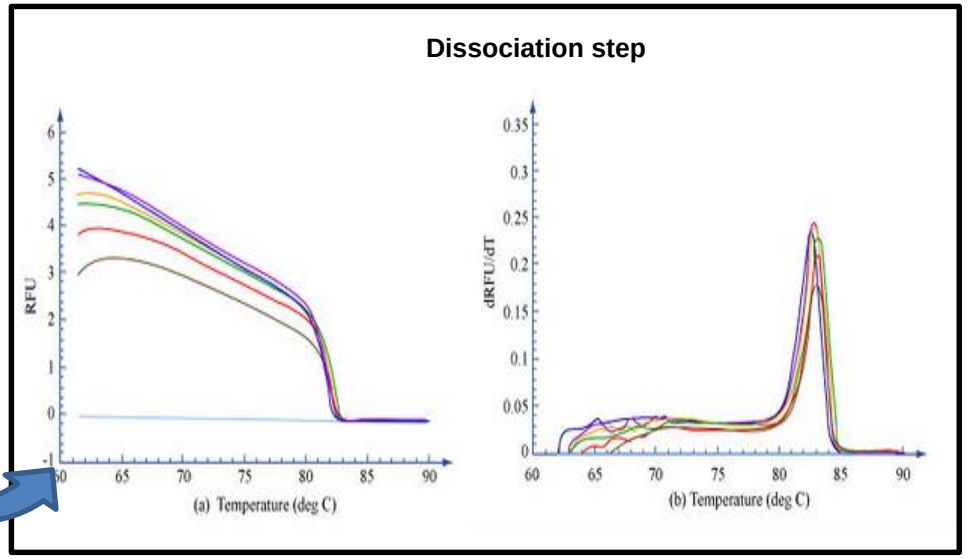
La sua maggiore sensitività (fino a 25 volte) nella rilevazione di acidi nucleici unita alla sua minore pericolosità sta facendo si che il SYBR Green venga utilizzato sempre più spesso come alternativa al meno costoso bromuro di etidio. Infatti il bromuro è un potente mutageno mentre, il SYBR Green viene 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**.



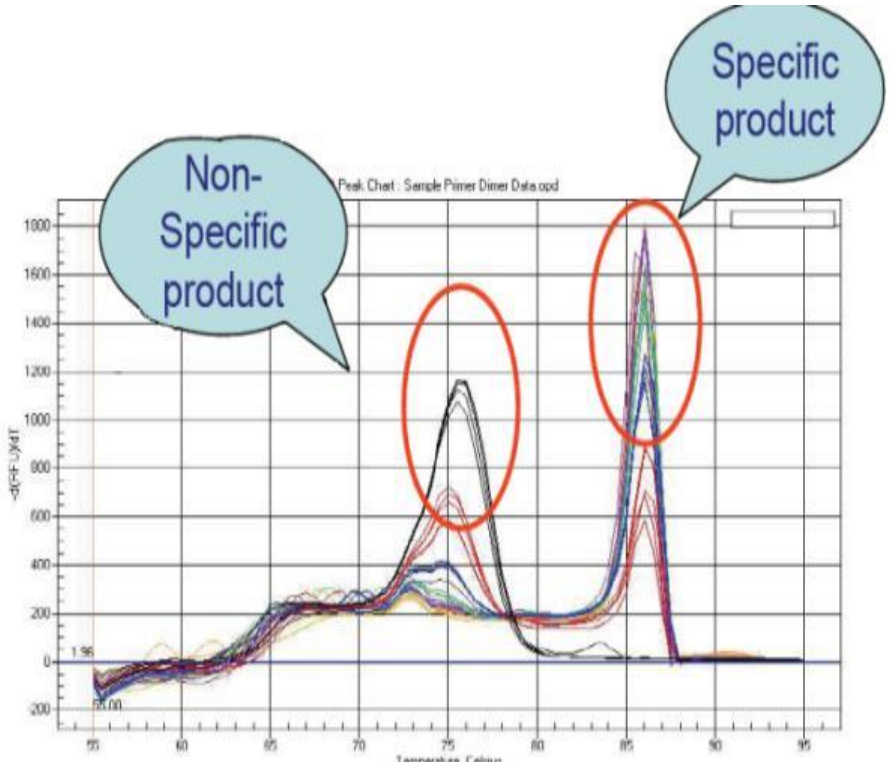
SYBR Green I


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
PCR Cycle
(Melt, Anneal/Extend)
AmpErase® UNG
Activation
AmpliTaq Gold
DNA Polymerase
Activation
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