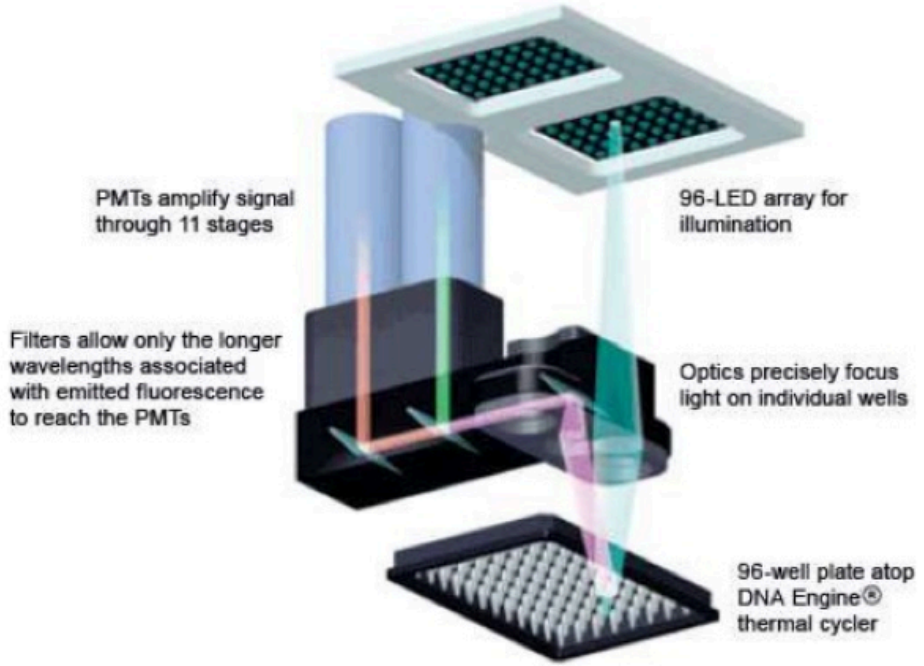
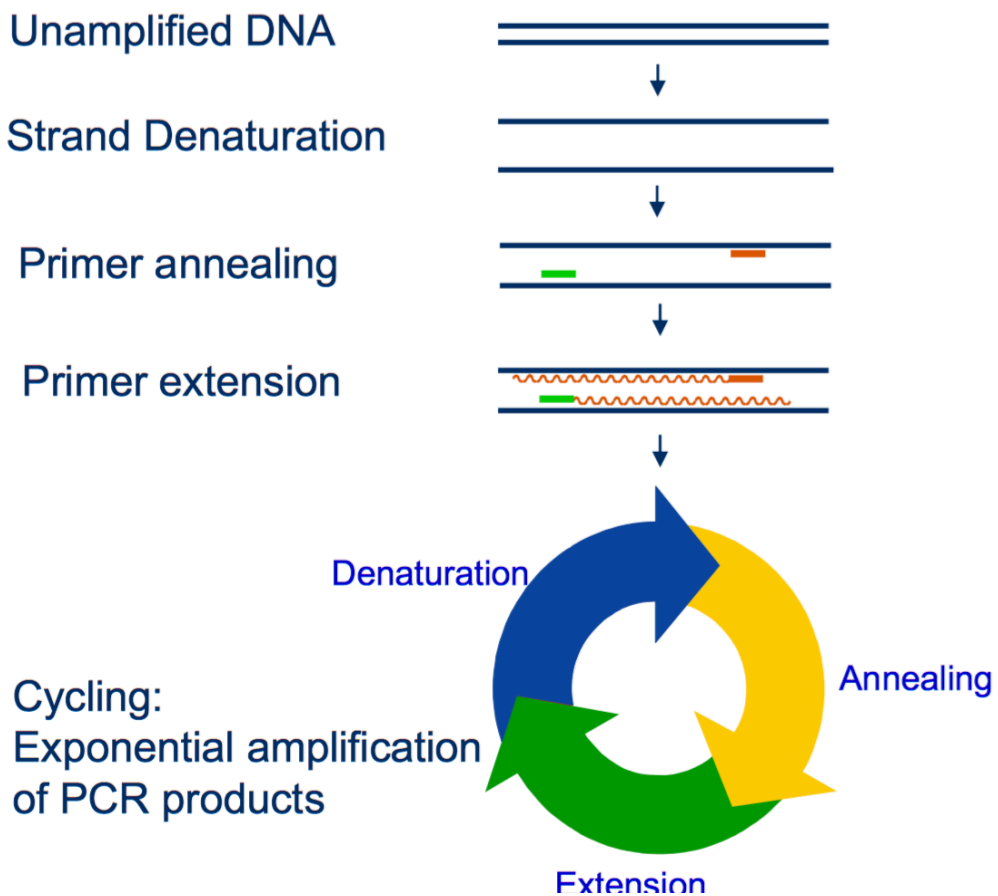
Real time PCR



Classic polymerase chain reaction

Traditional End-Point PCR



- Low sensitivity
- Poor precision
- Results are not expressed as numbers
- Ethidium bromide staining is not quantitative

•Post-PCR processing required

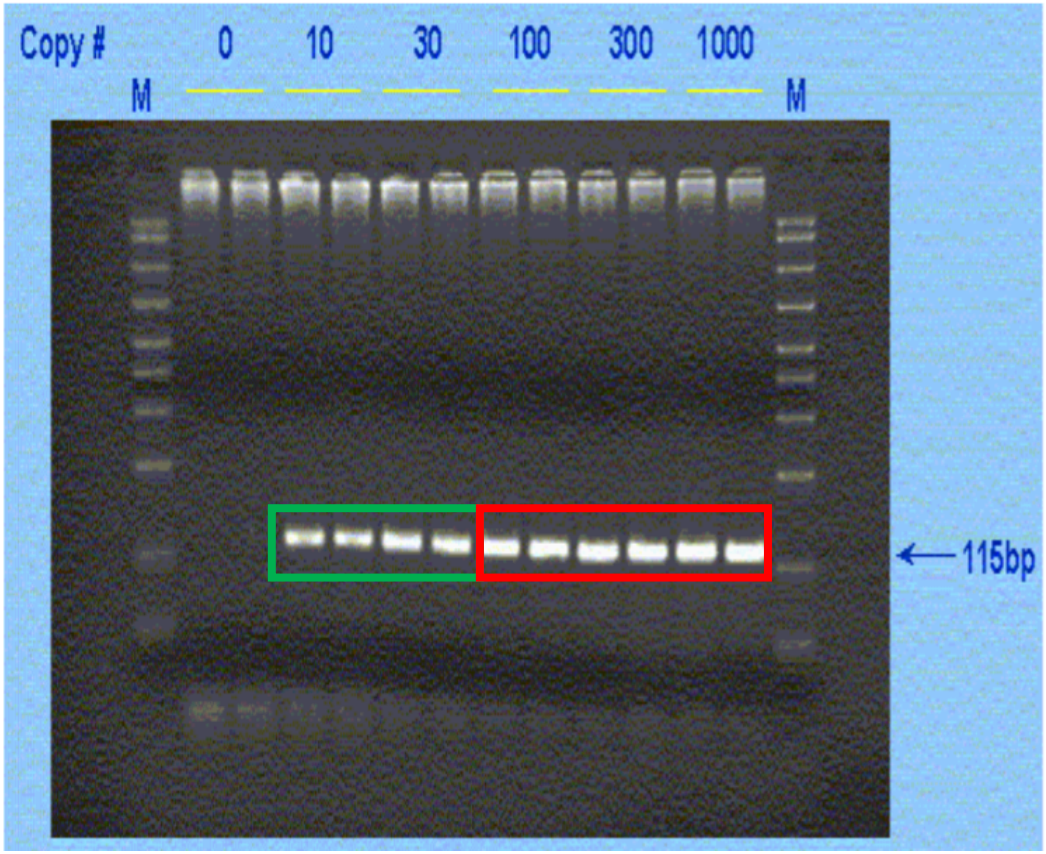
•Narrow dynamic range (<2 logs)

Classic polymerase chain reaction

Traditional End-Point PCR

- has a narrow dynamic range (<2 logs) → **SEMI-QUANTITATIVE**

Template copy numbers



Copy number of PCR template

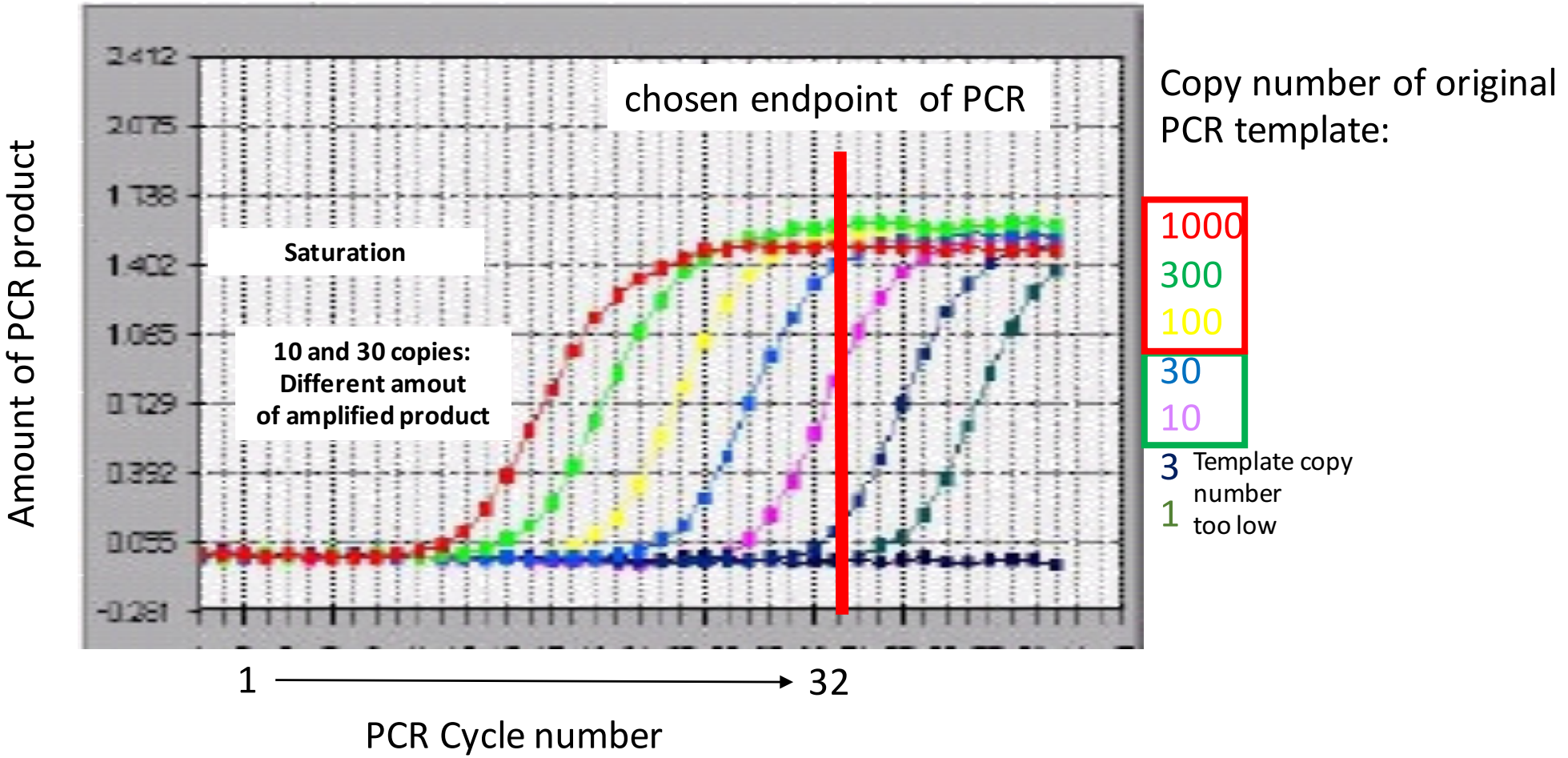
PCR conditions: 32 cycles

Amplification in dynamic range: increase in template number results an increased amount of PCR product, visibile by gel electrophoresis → semi-quantitative information

Amplification out of dynamic range: increase in template number (or cycle number – see before!!)does not result an increased amount of PCR product, visibile by gel electrophoresis –
→ End point arrived already with 100 template-copies
→ No quantitative information

Background of end point PCR

Traditional End-Point PCR



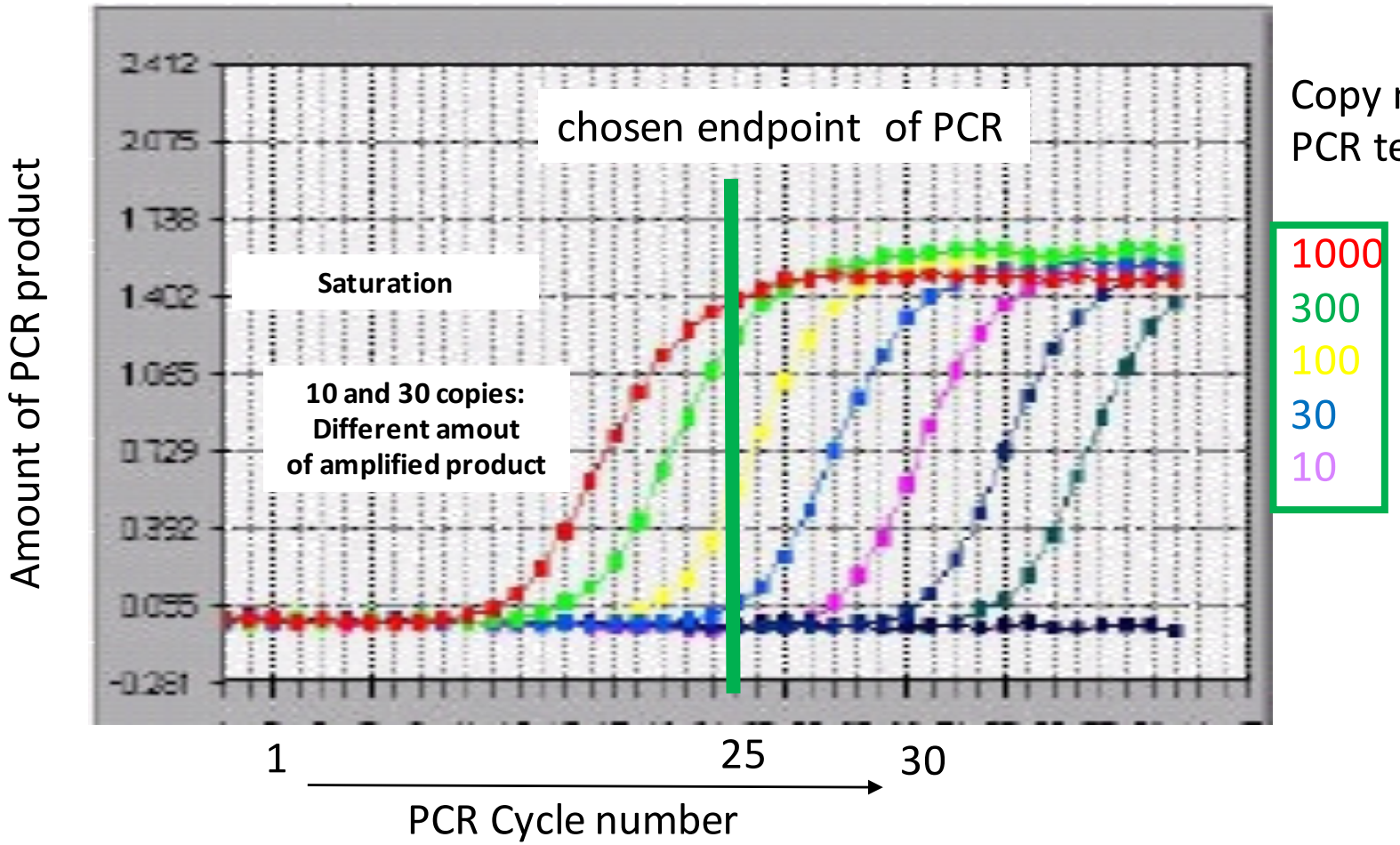
End-point PCR:

→ ideal to give qualitative information (for exampe amplification of a mutation using specific primers)

→ Only limited quantitative information possible

Optimizing semi-quantitative information from classic PCR

Adjust ideal number of cycles



Get better quantitative information from classic PCR:

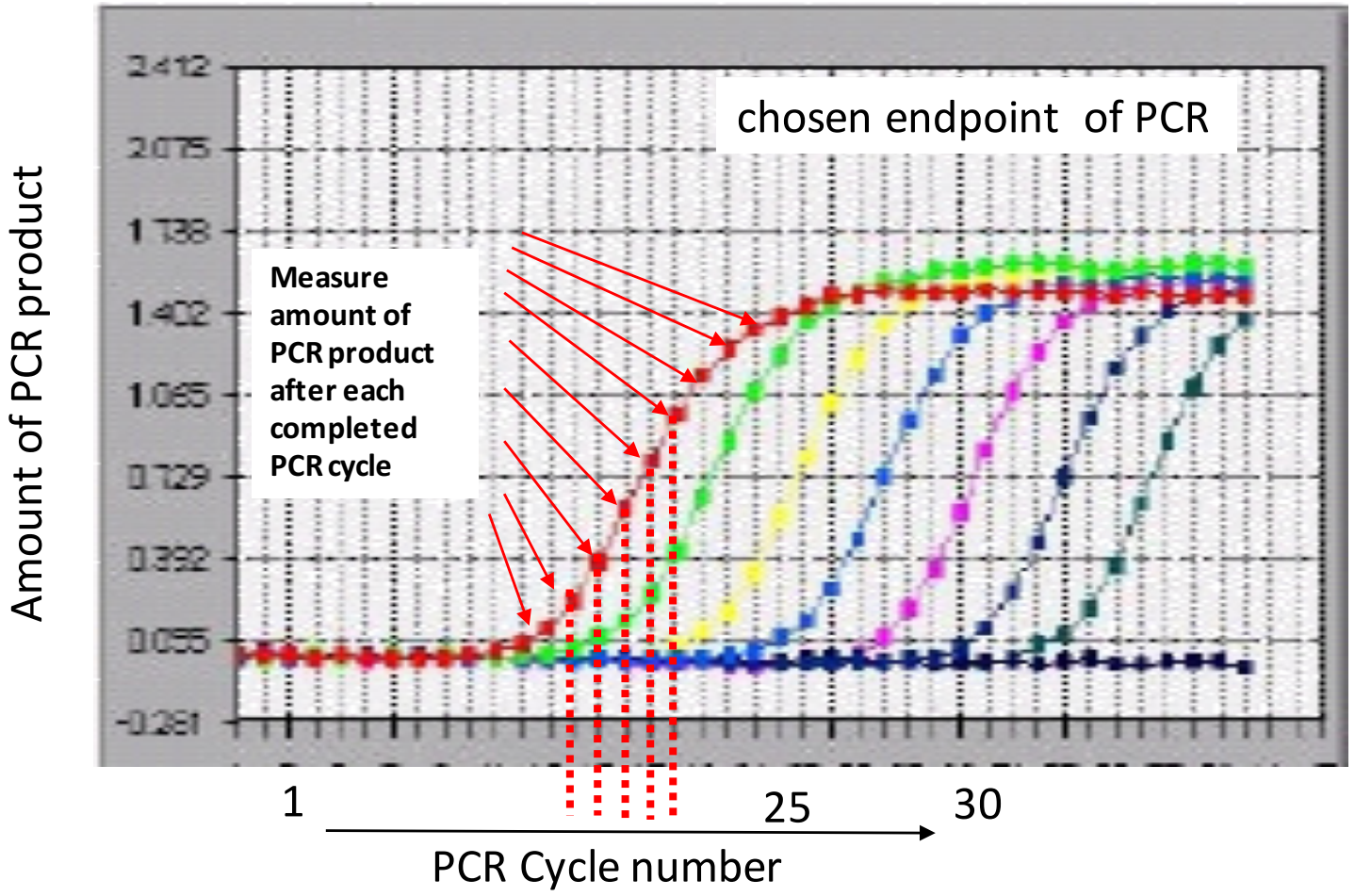
- Optimize PCR conditions:
  - A. Test for ideal end-point of PCR (example 25)
  - B. Optimize the amount of original template used for PCR

Time
Waste of primary material
Costs
Variability

Real-time PCR

Follow PCR product amplification in real-time (RT-PCR)

Obtaining QUANTITATIVE information from PCR



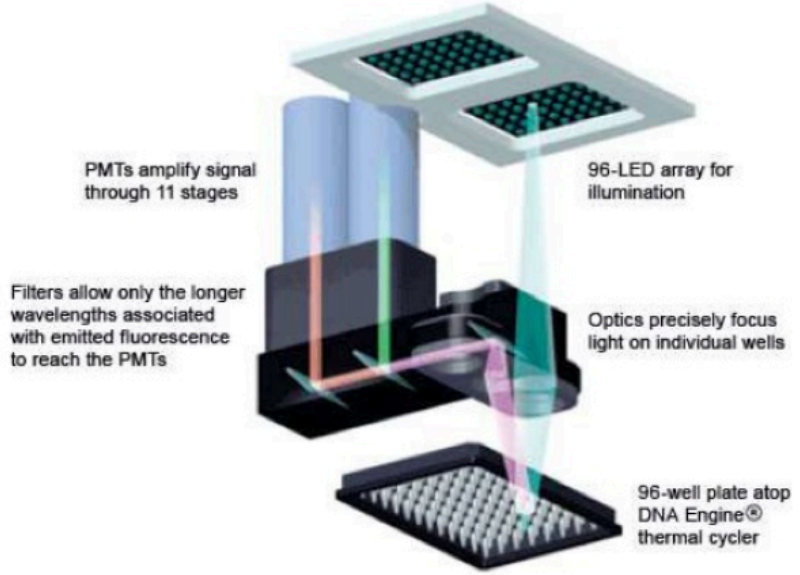
Copy number of original PCR template:

1000

Follow the amplification of PCR amplicons in "REAL-TIME" = REAL TIME PCR

Real-time PCR

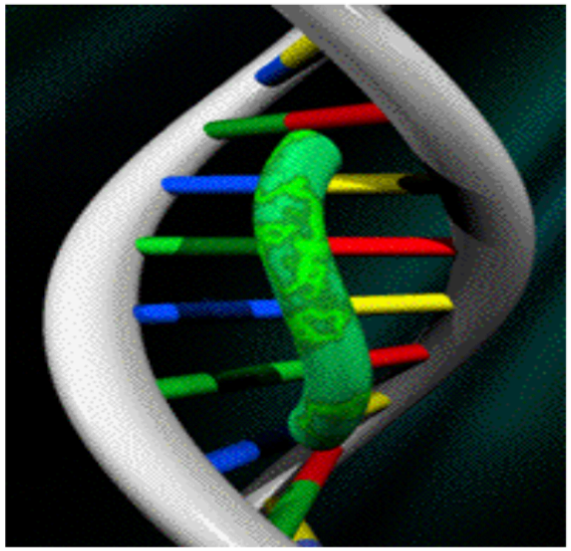
- ♦Eliminate use of gel electrophoresis
- ♦Increase reproducibility
- ♦Enable use of internal controls/standards
- ♦Reduce turnaround time
- ♦Increase throughput
- ♦Reduce sample amount usage
- ♦Results expressed as numbers



Real-Time PCR Chemistries

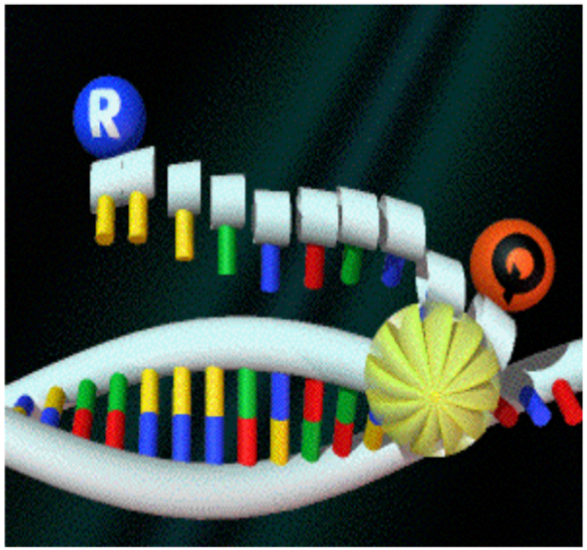
Strategies to follow PCR product generation

SYBR® Green I dye



Binds double stranded DNA

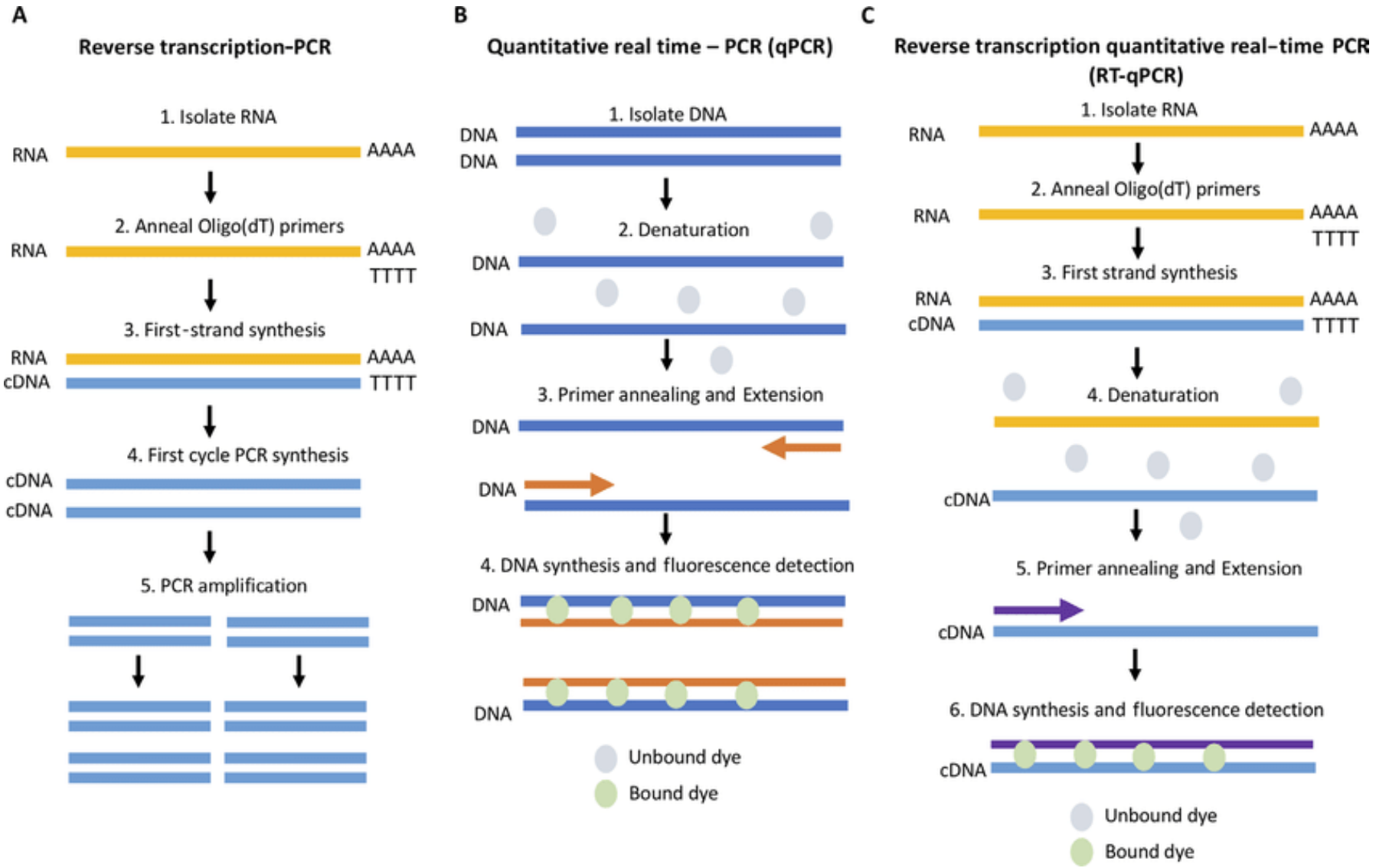
Fluorogenic 5' Nuclease Assay



Uses a TaqMan® probe

Real-Time PCR

Determine gene expression of different samples



Schematic comparing RT-PCR, qPCR and RT-qPCR. (A) RT-PCR workflow. RNA is isolated and cDNA is generated via reverse transcription (RT); PCR is then carried out to amplify areas of interest. (B) qPCR schematic. DNA is isolated and amplified; amplification is quantitated using a probe which fluoresces upon intercalation with double-stranded DNA. (C) RTqPCR procedure. RNA is isolated and cDNA generated before commencing a qPCR procedure.

What is Real-Time PCR used for?

Real-Time PCR has become a cornerstone of molecular biology:

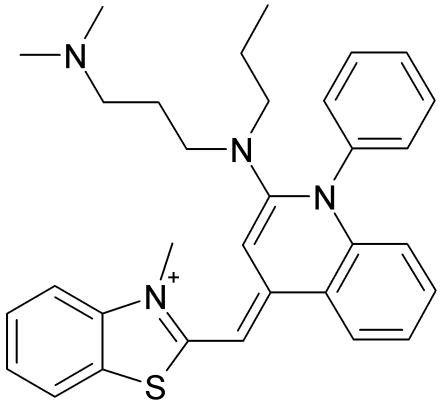
- **Gene expression analysis**
  - Cancer research
  - Drug research
- **Disease diagnosis and management**
  - Viral quantification
- **Food testing**
  - Percent GMO food
- **Animal and plant breeding**
  - Gene copy number

For all applications that require the quantification of RNA/DNA sequences

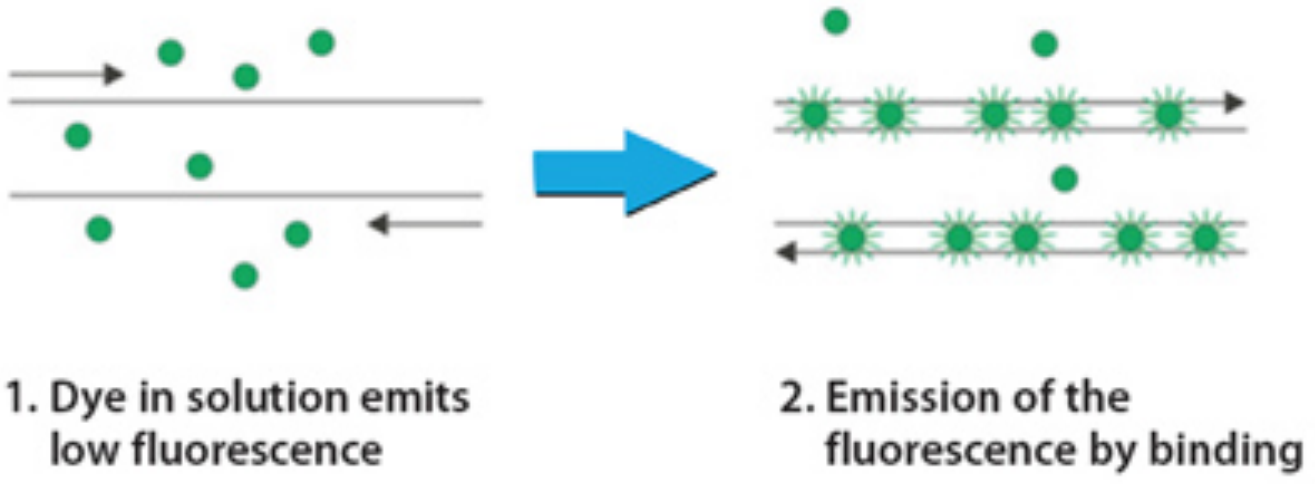
1. SYBR® Green I Dye Assay Chemistry

Classic PCR setup with addition of SYBR Green:

SYBR Green is a green fluorescent cyanine dye that has high affinity for double-stranded DNA. The mode of binding is believed to be a combination of DNA intercalation and external binding. When bound, SYBR absorbs at a wavelength around 497 nm and emits fluorescence around 520 nm.



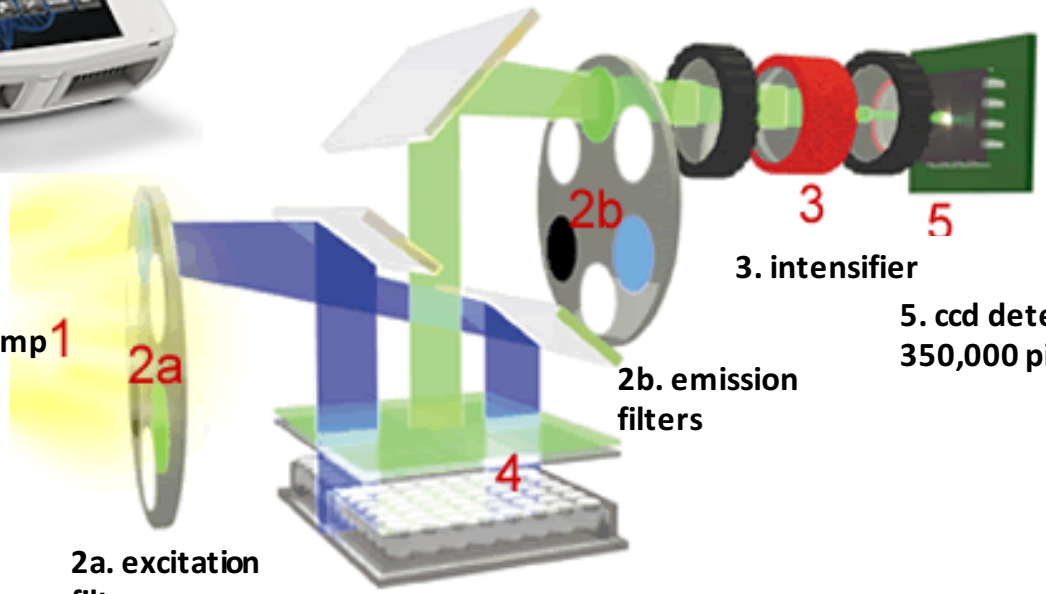
Denaturation
Annealing



DNA synthesis
Detection of emission of fluorescence

Fluorescence emmission is increasing with increasing of PCR cycles

Basics of real-time PCR measurements



1. halogen tungsten lamp

2a. excitation filters

2b. emission filters

3. intensifier

4. sample plate

5. ccd detector 350,000 pixels

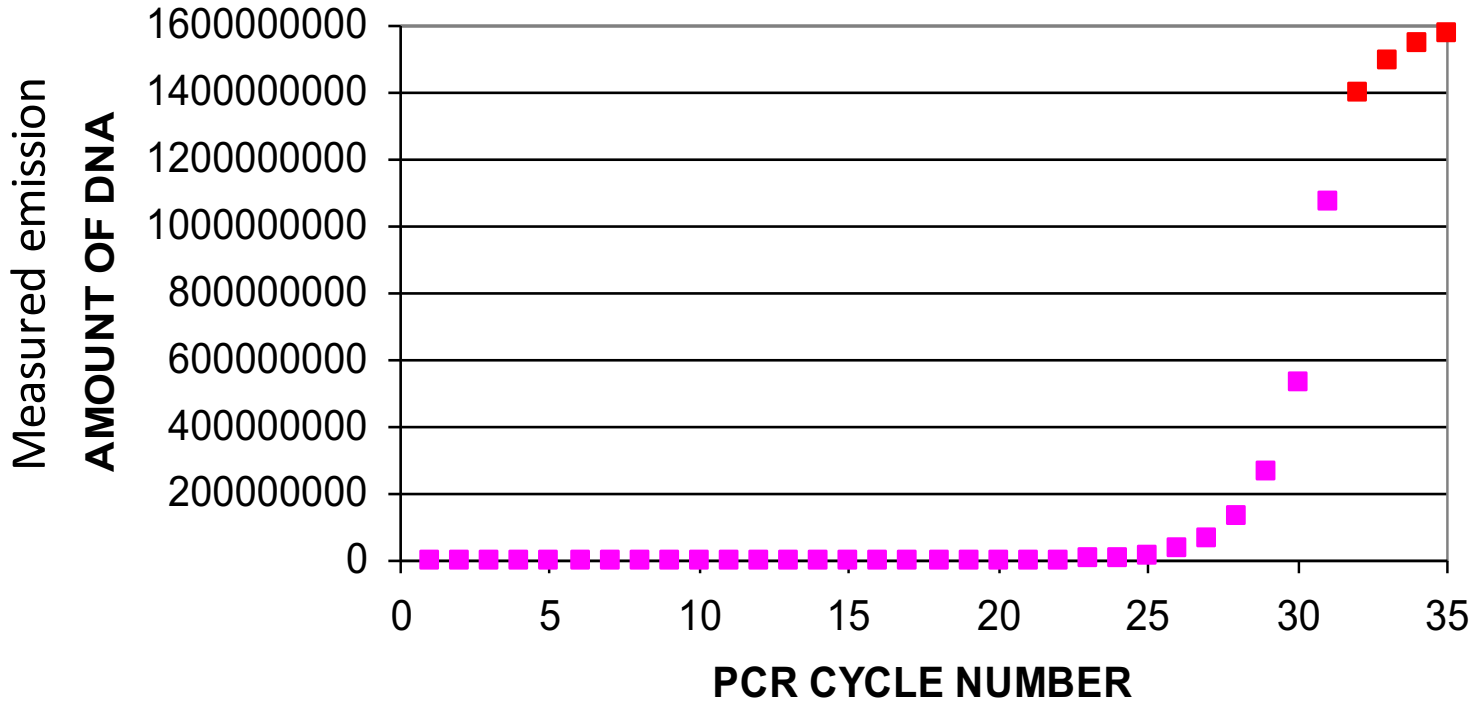
Fig. 1.2. Representation of Optical Detection System layout.

Every PCR cycle: Exitation of SYBR green (497nm) + measurment of emission from SYBR green (520nm)

www.biorad.com

Basics of real-time PCR measurements

AMPLIFICATION BLOT



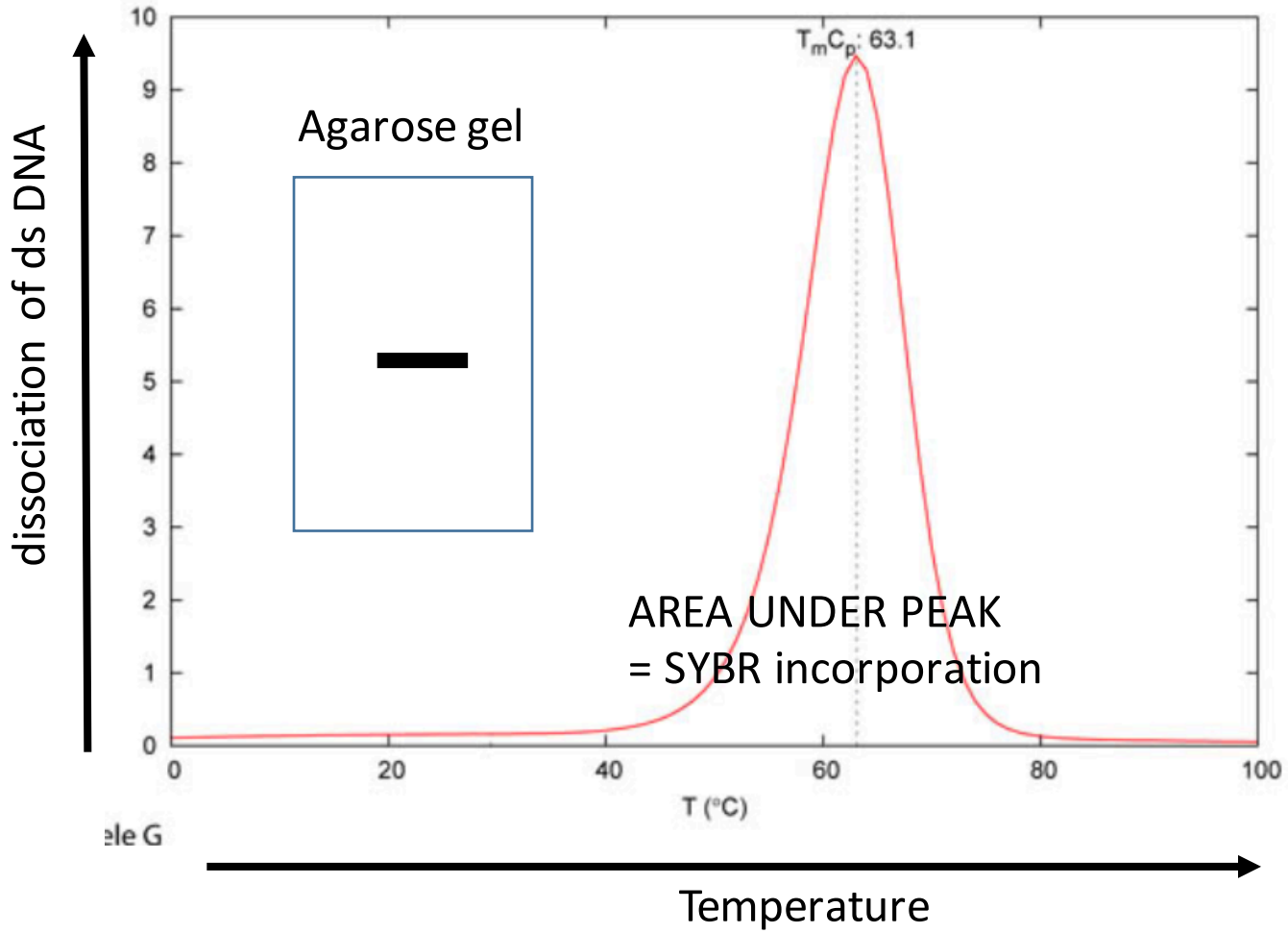
Quantitative information

Basics of real-time PCR measurements

METLTING CURVE ANALYSIS

The temperature-dependent dissociation between two DNA-strands can be measured using a DNA-intercalating fluorophore such as SYBR green,or fluorophore-labelled DNA probes. In the case of SYBR green (which fluoresces 1000-fold more intensely while intercalated in the minor groove of two strands of DNA), the dissociation of the DNA during heating is measurable **by the large reduction in fluorescence that results.**

The temperature at which 50% of DNA is denatured is known as the melting temperature.



Melting curve is determined after the last cycle of PCR:

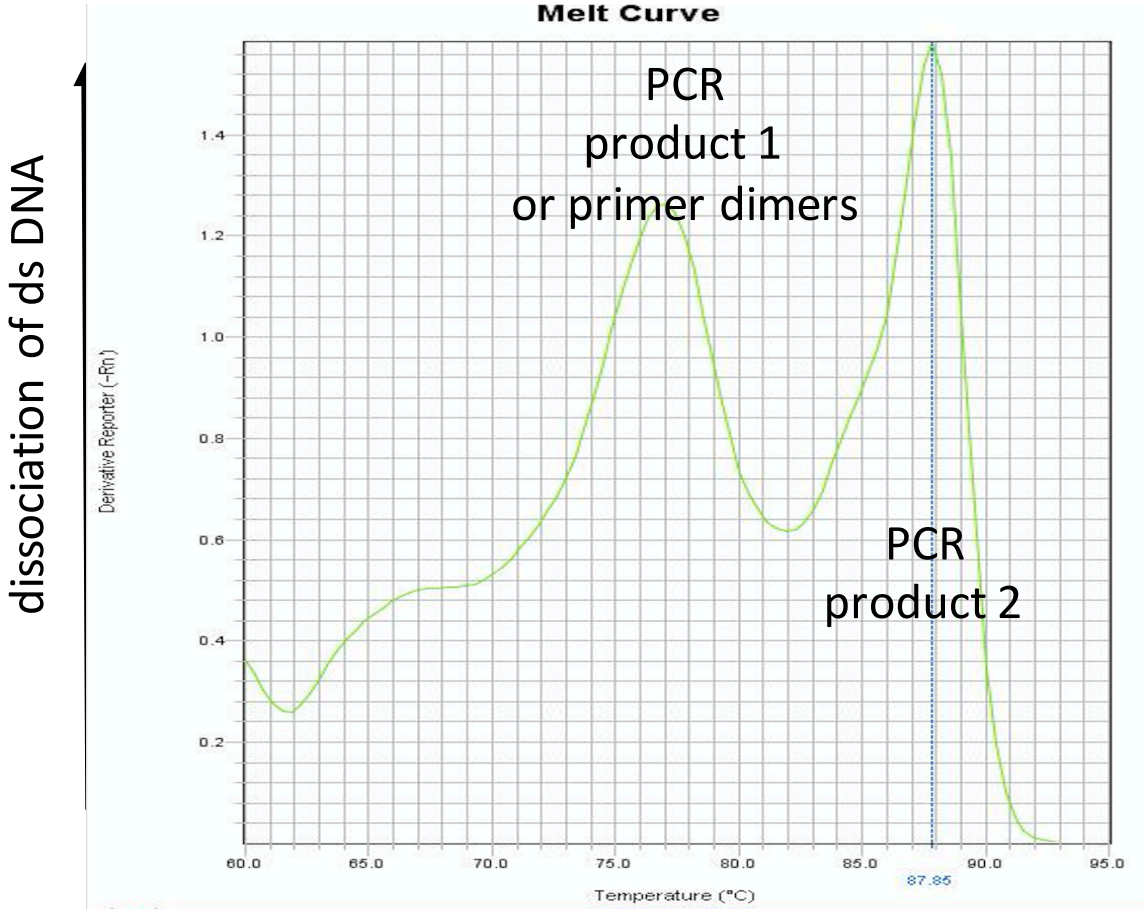
- → **PCR machine heats uo PCR products from 0°C to 100°C**
- → **Dissociation of DNA filaments is measured**
- → **IF PCR HAS AMPLIFIED SPECIFICALLY AMPLIFIED A SPECIFIC REGION ALL DNA MOLECULES WILL MELT AT A SPECIFIC TEMPERATURE → melting temperature is determined by DNA sequence!!!**
- → **IF YOU RUN PCR PRODUCT ON AGAROSE GEL, ONLY ONE BAND WILL BE VISIBLE**

Basics of real-time PCR measurements

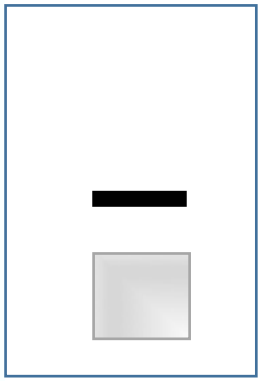
METLTING CURVE ANALYSIS

Melting curve is determined after the last cycle of PCR:

- → **PCR machine heats uo PCR products from 0°C to 100°C**
- → **Dissociation of DNA filaments is measured**
- → **IF PCR HAS AMPLIFIED MULTIPLE FRAGMENTS IN A NON_SPECIFIC MANNER THE MELTING CURVE ANALYSIS WILL IDETIFY MORE THEN ONE PEAK (PCR primers are not sepcific!!)**
- → **Example: IF YOU RUN PCR PRODUCT ON AGAROSE GEL, FIVE BANDs WILL BE VISIBLE**



Agarose gel



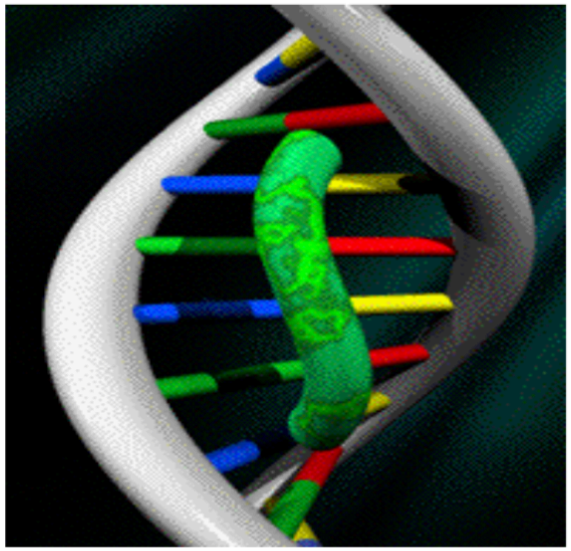
AREA UNDER PEAKs
= SYBR incorporation
Here: signals from different types of dsRNA
(not only target amplicon)

METLTING CURVE ANALYSIS GIVE QUALITATIVE INFORMATION OF THE REAL-TIME PCR REACTION (without necessarily requiring an agarose gel run)

Real-Time PCR Chemistries

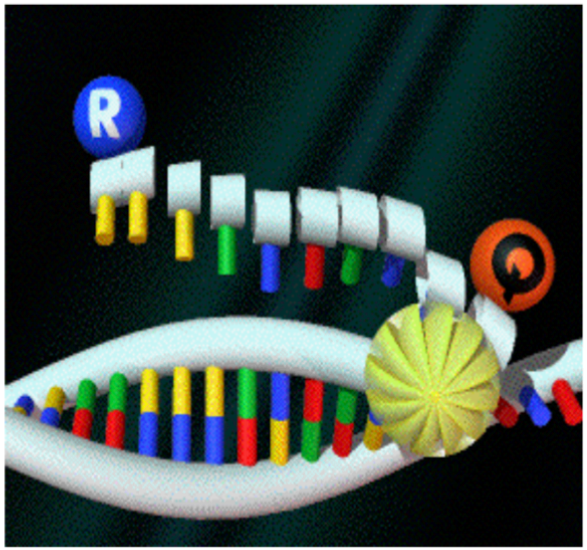
Strategies to follow PCR product generation

SYBR® Green I dye



Binds double stranded DNA

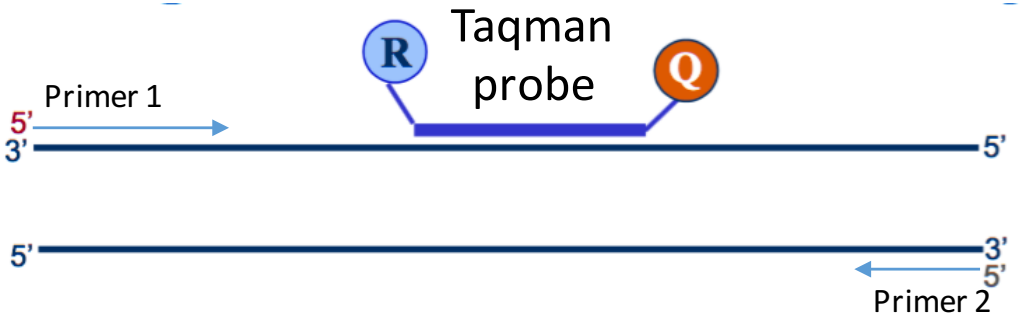
Fluorogenic 5' Nuclease Assay



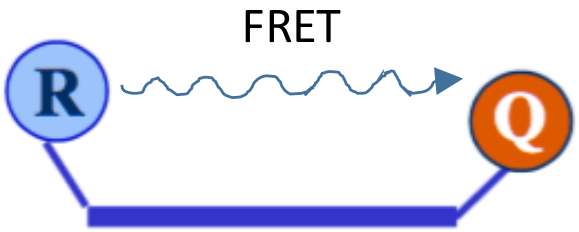
Uses a TaqMan® probe

2. Real-Time PCR Chemistries based on Fluorogenic 5' Nuclease assay

Classic PCR setup with addition of amplicon-specific, modified ssDNA oligonucleotide



PCR target region amplified using primer 1 and 2



FRET:
Fluorescence Resonance Energy Transfer

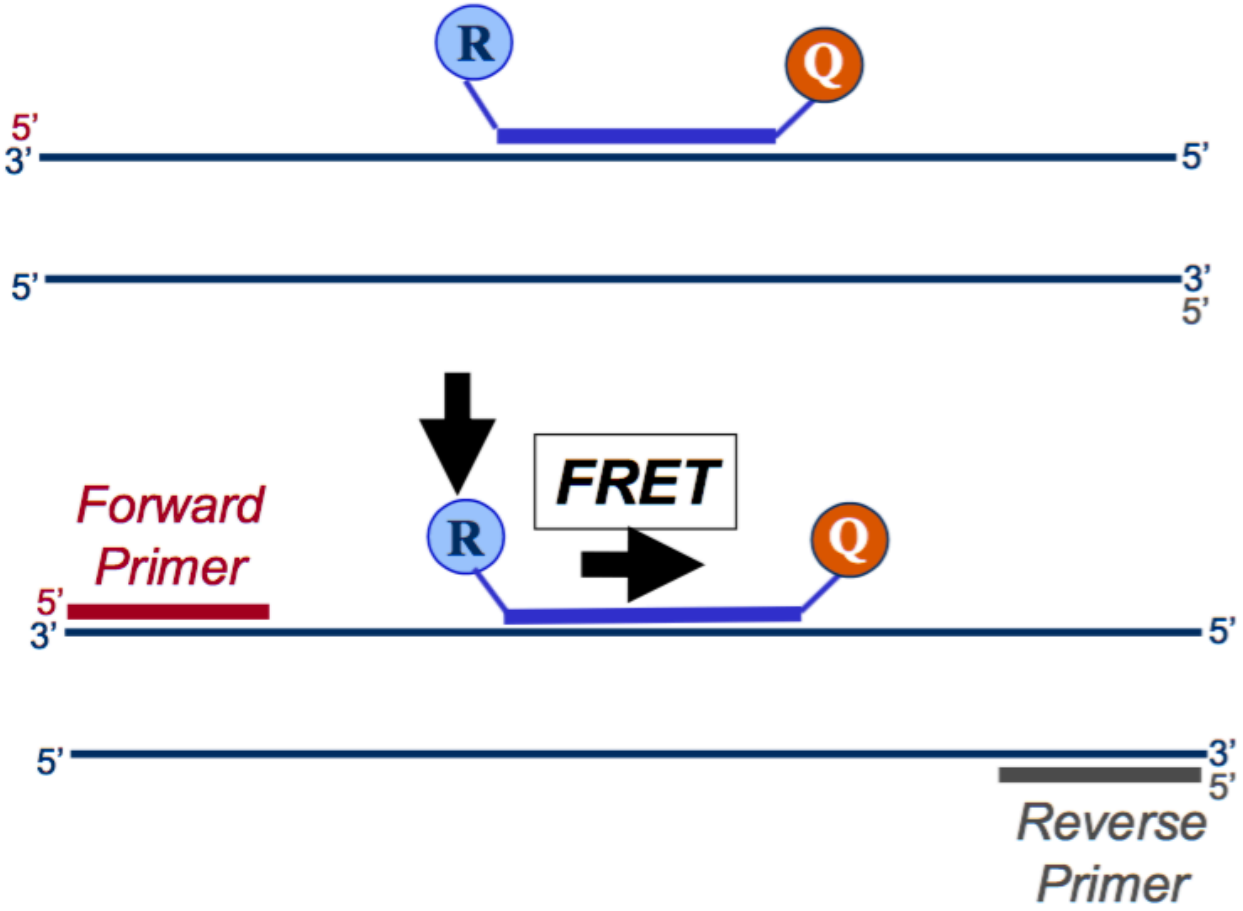
Taqman probe:

- Oligonucleotide
- PCR amplicon site specific
- Hybridizes with one strand of the PCR product
- Carries a fluorophor (R)
- Carries a Quencher that absorbes light emited from fluorophor ="FRET"

Important: FRET only works when Q is in close proximity to R

2. Real-Time PCR Chemistries based on Fluorogenic 5’ Nuclease assay

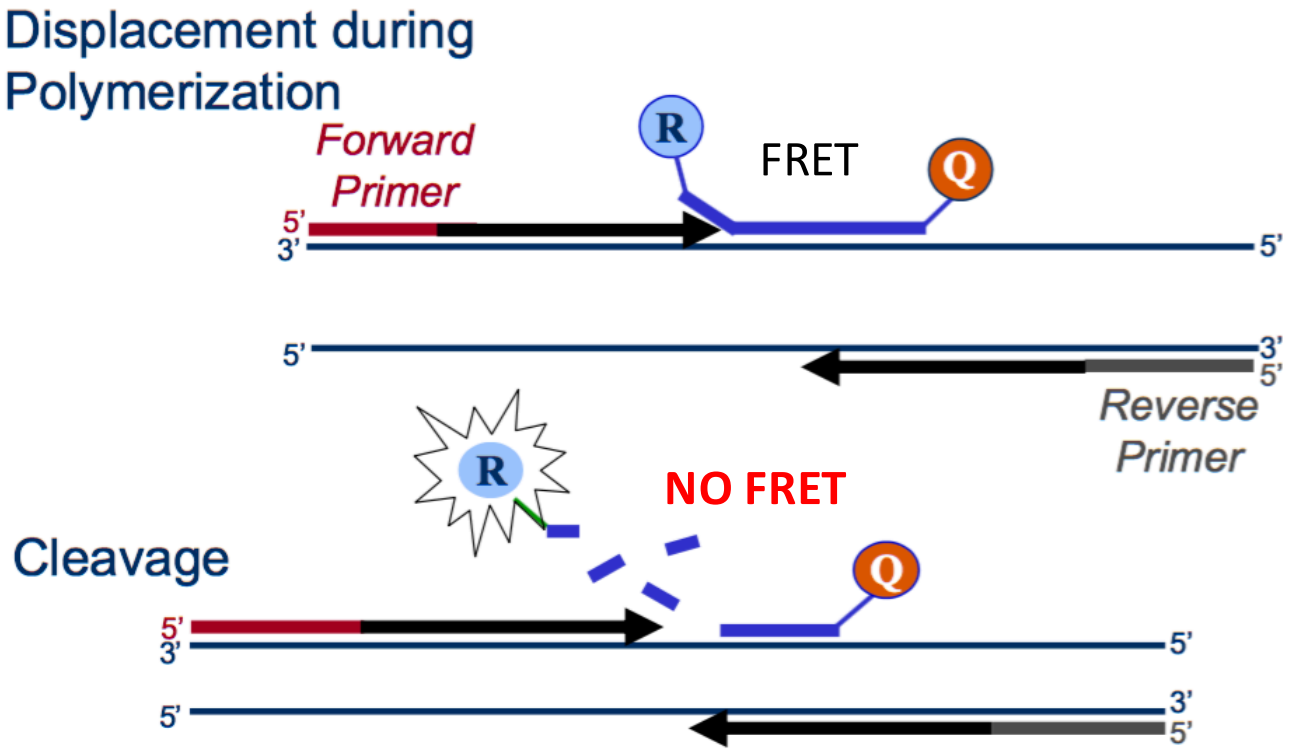
For example:
Cycle 5 during PCR



- Denaturation at 95°C
- Annealing of PCR primers and Taqman probe

***FRET= Fluorescence Resonance Energy Transfer**

2. Real-Time PCR Chemistries based on Fluorogenic 5’ Nuclease assay

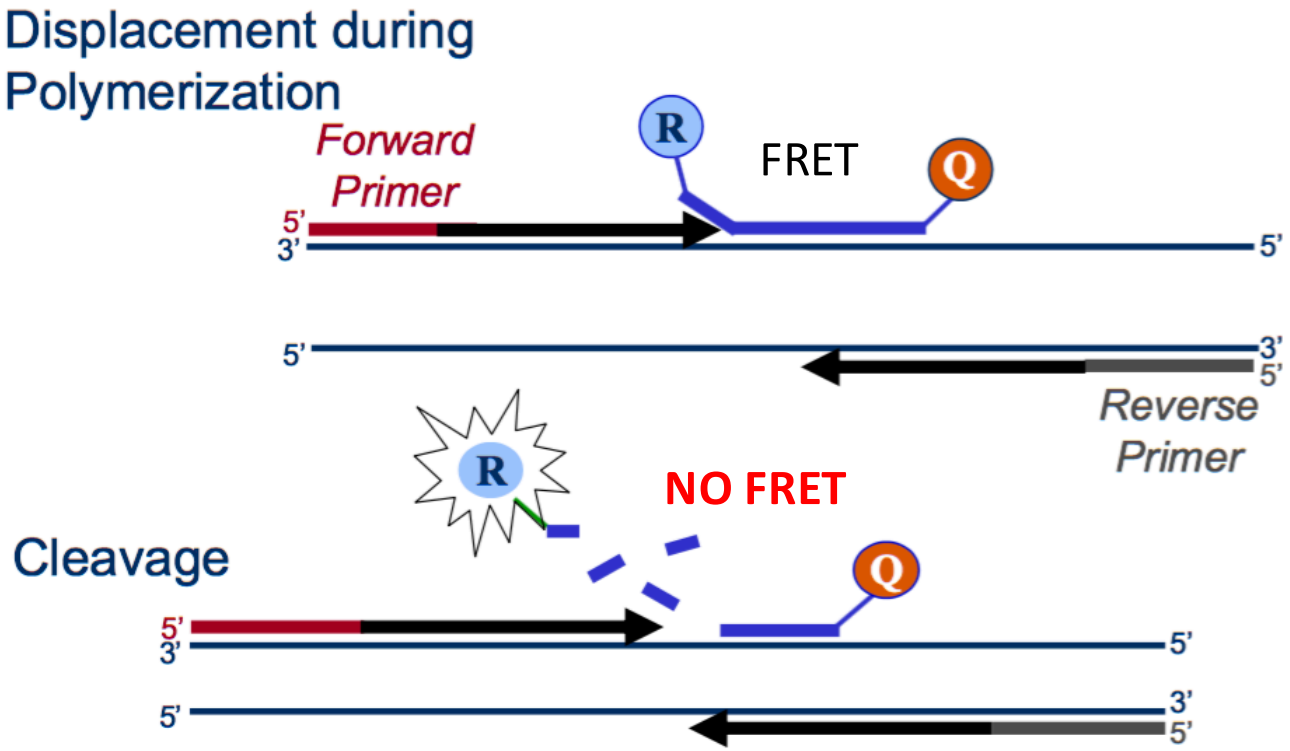


- DNA SYNTHESIS BY Taq polymerase

Taq has 5’→ 3’ exonuclease activity:
Taqman probe es degraded

Loss of FRET: light from R is not cheched and can be detected in “real-time” during PCR
Fluoresence increases with every cycle of PCR until reaching saturation in PCR plateau phase

2. Real-Time PCR Chemistries based on Fluorogenic 5’ Nuclease assay



- DNA SYNTHESIS BY Taq polymerase

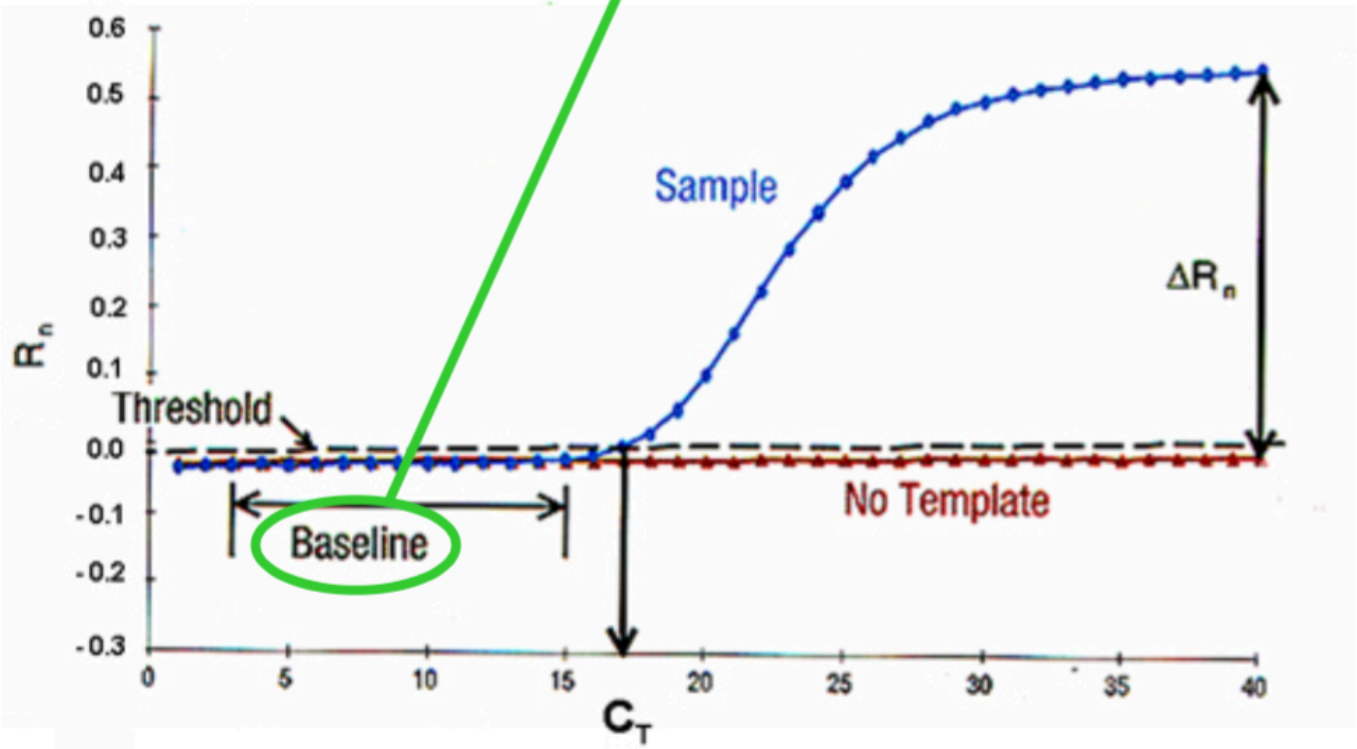
Taq has 5’→ 3’ exonuclease activity:
Taqman probe es degraded

ADVANTAGE:
HIGHLY SPECIFIC DETECTION OF AMPLIFIED DNA REGIONs:
1. Seqeunce specific PCR primers for PCR
2. Amplicon specific DNA probe enables selective detection of region of interest!!!

Terminology of amplification blots

Terminology Baseline:

The initial cycles prior to any detectable amplification, in which there is little change in fluorescent signal.

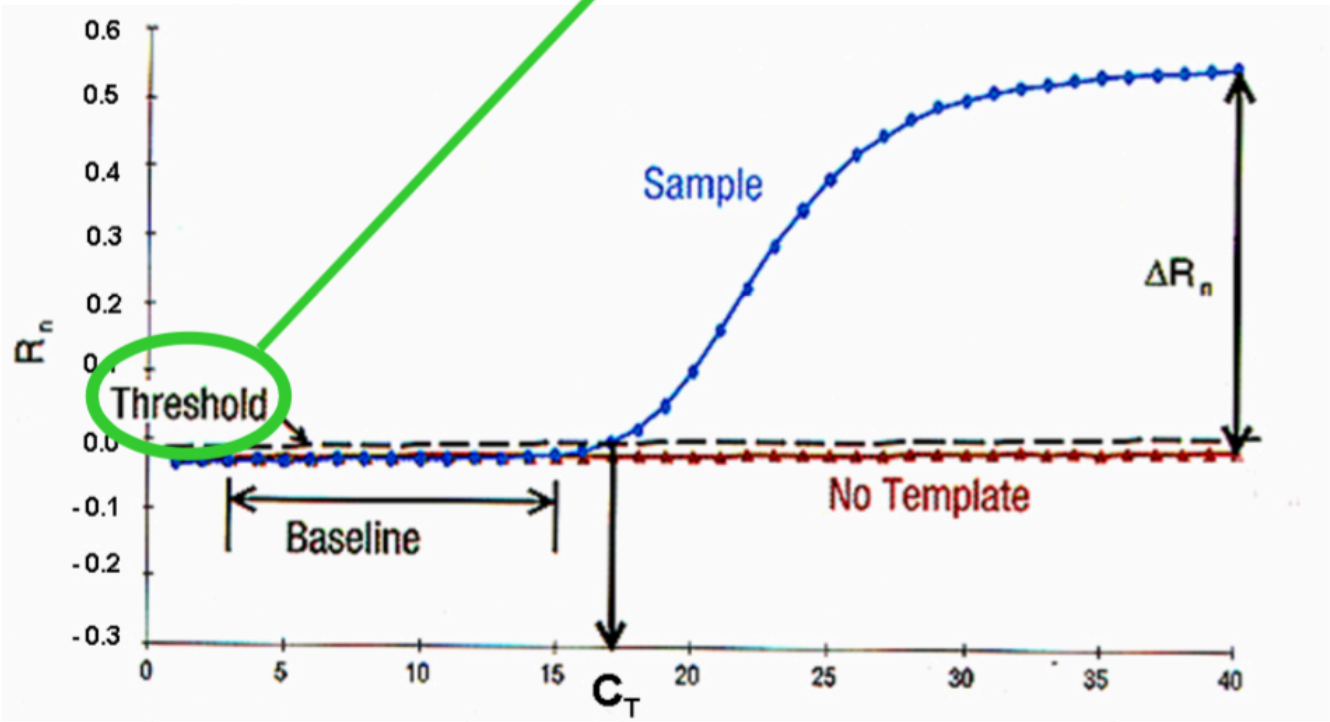


2 parallel PCR reacations shown in blot:
BLUE: PCR with template
RED: PCR without template (negative control)

Rn: reporter signal obtained from detector

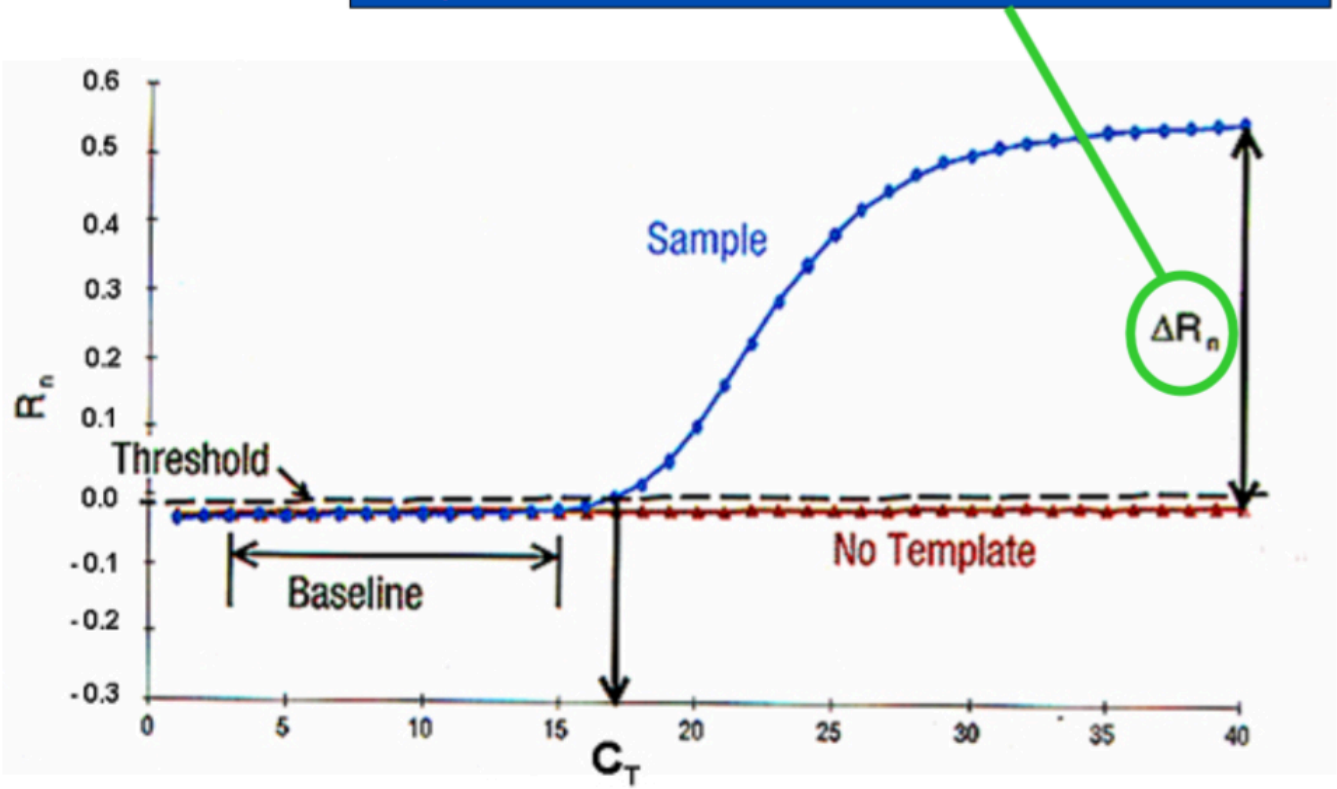
Terminology of amplification blots

Threshold: Level at which fluorescence is detected in reactions during the exponential phase of PCR



Terminology of amplification blots

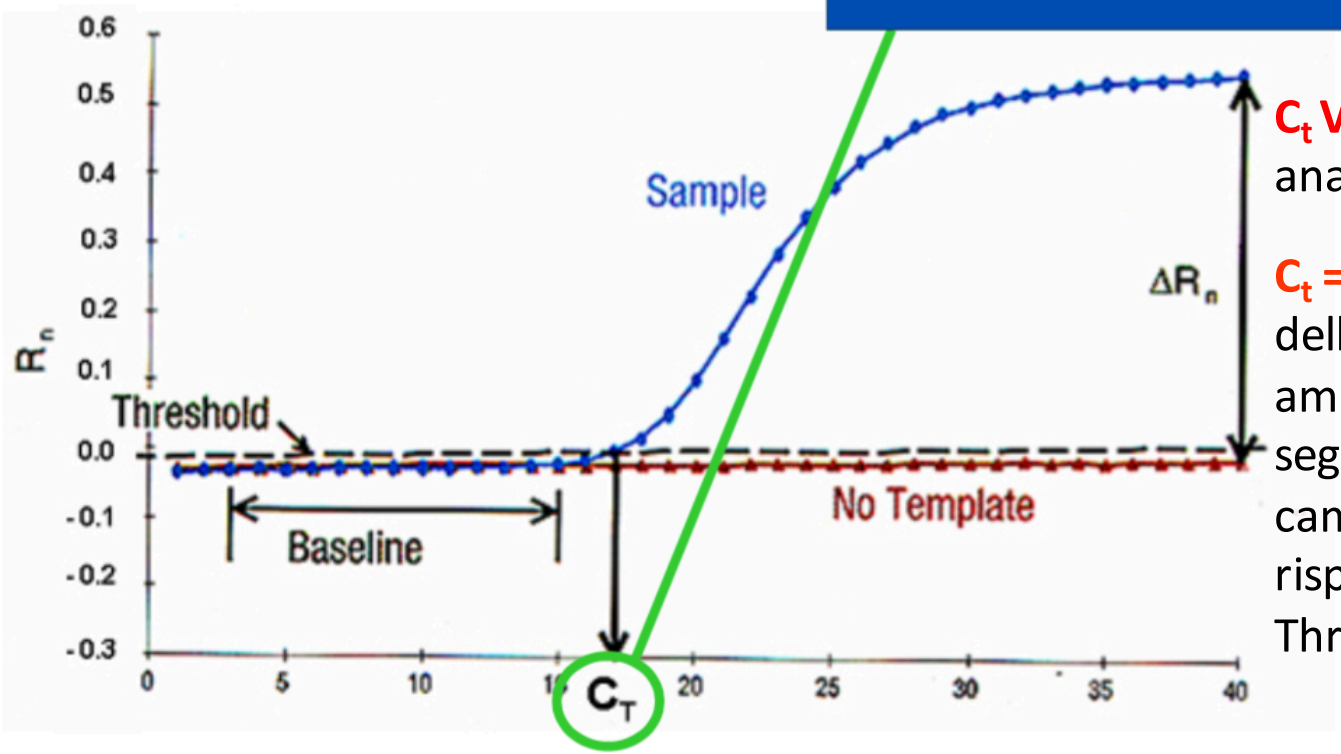
ΔR_n: **Normalized reporter signal minus background (baseline level).**



Terminology of amplification blots

Cycle Threshold (C_T):

The cycle (point in time) at which the PCR product crosses the threshold of detection.



C_t VALUE: Most important value for the analysis of real-time PCR data

C_t = threshold cycle: è il ciclo della reazione di amplificazione in cui il segnale di fluorescenza del campione è maggiore rispetto a quello della Threshold

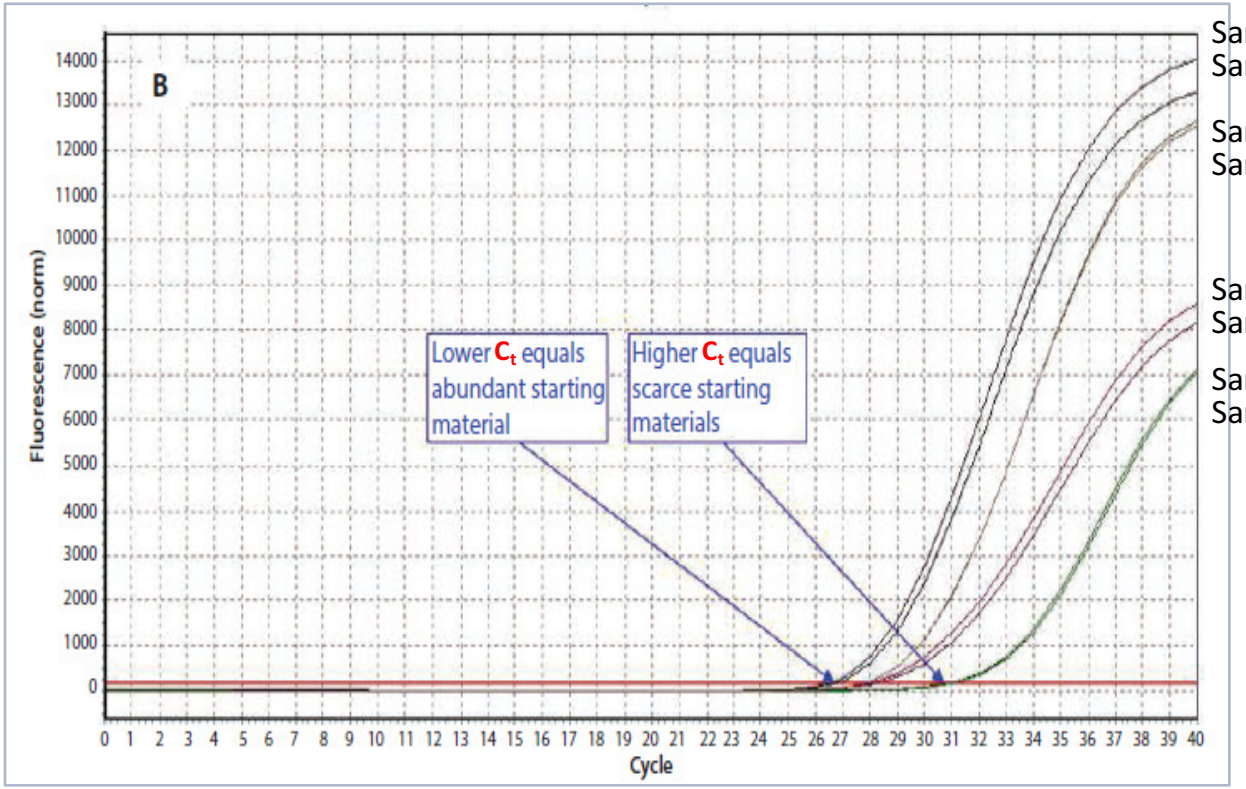
Terminology of amplification blots

WITH EVERY CYCLE OF PCR, THE AMOUNT OF AMPLIFIED DNA DOUBLES

$$Y = N(1+E)^n$$

Y = resa di amplificazione/amount amplified
N = numero di molecole di DNA di partenza (number of starting DNA molecules)
E = efficienza di reazione (efficieny of reaction)
n = numero di cicli di amplificazione (number of PCR cycles)

The amount of initial DNA is reverse proportional to the number of cycles required to overcome the threshold (arrive at Ct)



Sample 1 (dublicate 1)
Sample 1 (dublicate 2) C_t=26,2

Sample 2 (dublicate 1)
Sample 2 (dublicate 2) C_t=28,0

Sample 3 (dublicate 1)
Sample 3 (dublicate 2) C_t=28,5

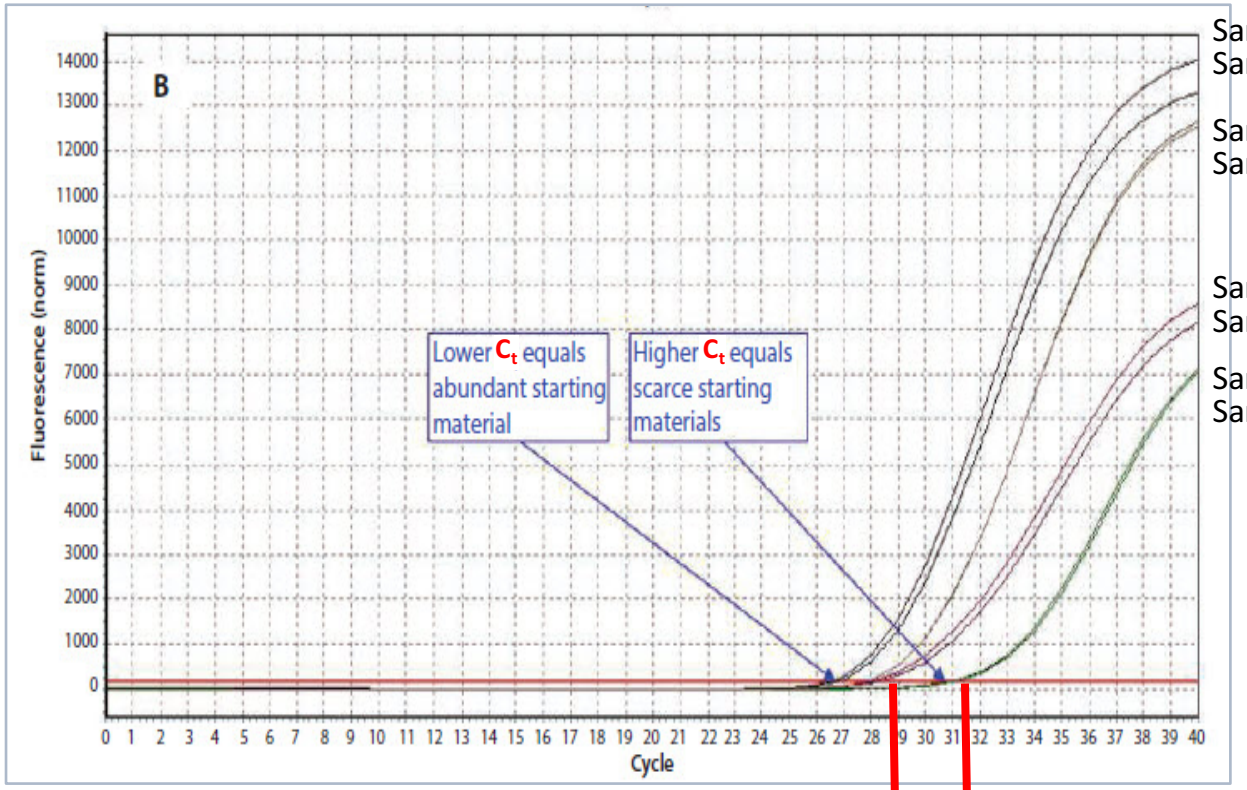
Sample 4 (dublicate 1)
Sample 4 (dublicate 2) C_t=30,5

Threshold

Amount of DNA of interest in different samples

Basics for the analysis of real-time PCR data: C_t and ΔC_t

WITH EVERY CYCLE OF PCR, THE AMOUNT OF AMPLIFIED DNA DOUBLES



Sample 1 (dublicate 1)
Sample 1 (dublicate 2) C_t=26,2

Sample 2 (dublicate 1)
Sample 2 (dublicate 2) C_t=28,0

Sample 3 (dublicate 1)
Sample 3 (dublicate 2) C_t=28,5

Sample 4 (dublicate 1)
Sample 4 (dublicate 2) C_t=30,5

Amount of DNA of interest in different samples

C_t=28,5 C_t=30,5

ΔC_t=2

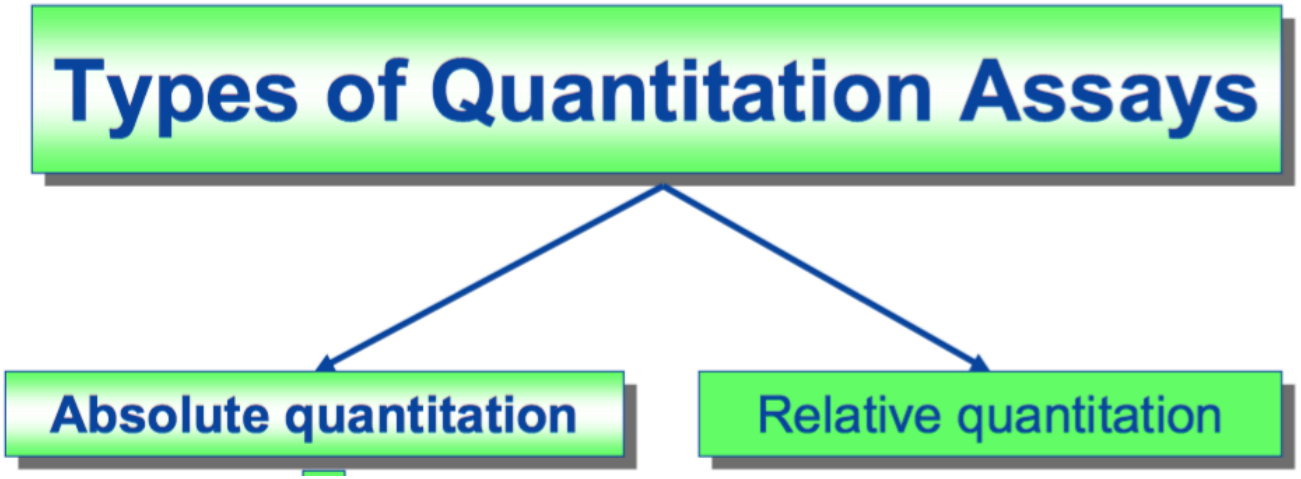
Fold change sample 3 to sample 4 =

$2^{\Delta Ct}$

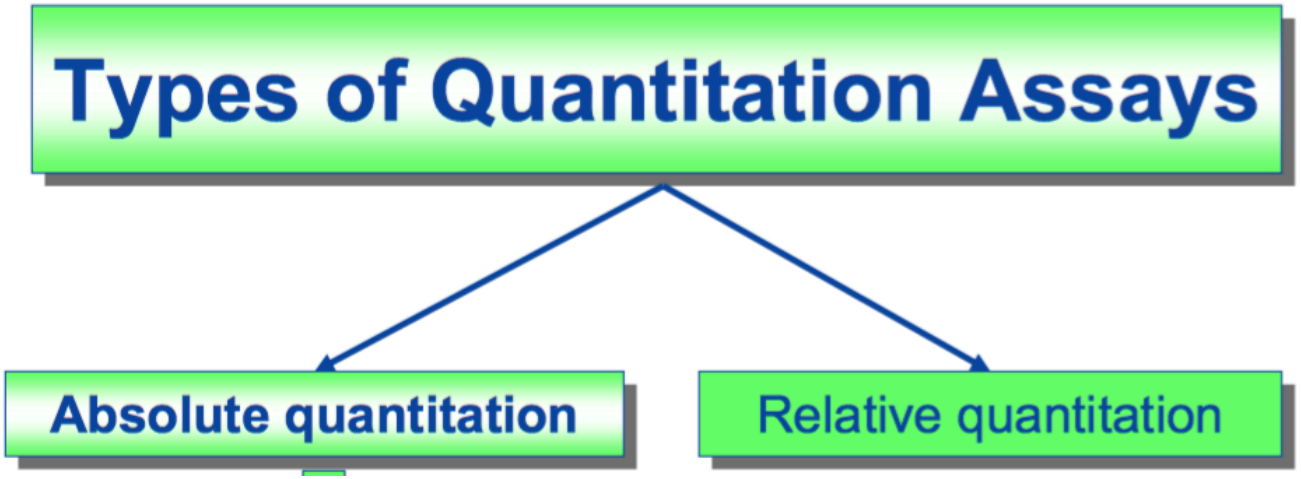
2^2=4

C_t (sample 4) appears 2 cycles later that C_t (sample3) → note: in every cycle of PCR the amout of amplified DNA doubles → 2 cycles difference = concentration of target DNA is 4 times lower in sample 4 compared to sample 3

Analysis of real-time PCR data: C_t and ΔC_t



Analysis of real-time PCR data: C_t and ΔC_t



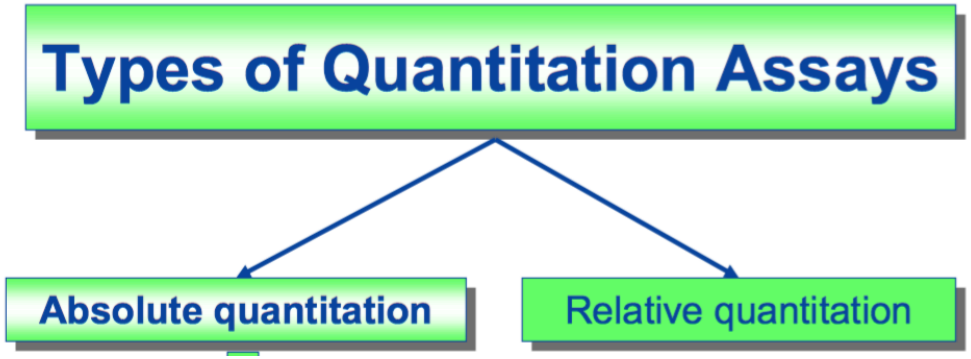
Provides absolute measurement of starting copy number

–**Requires standards of known quantity (Mol or ng)**

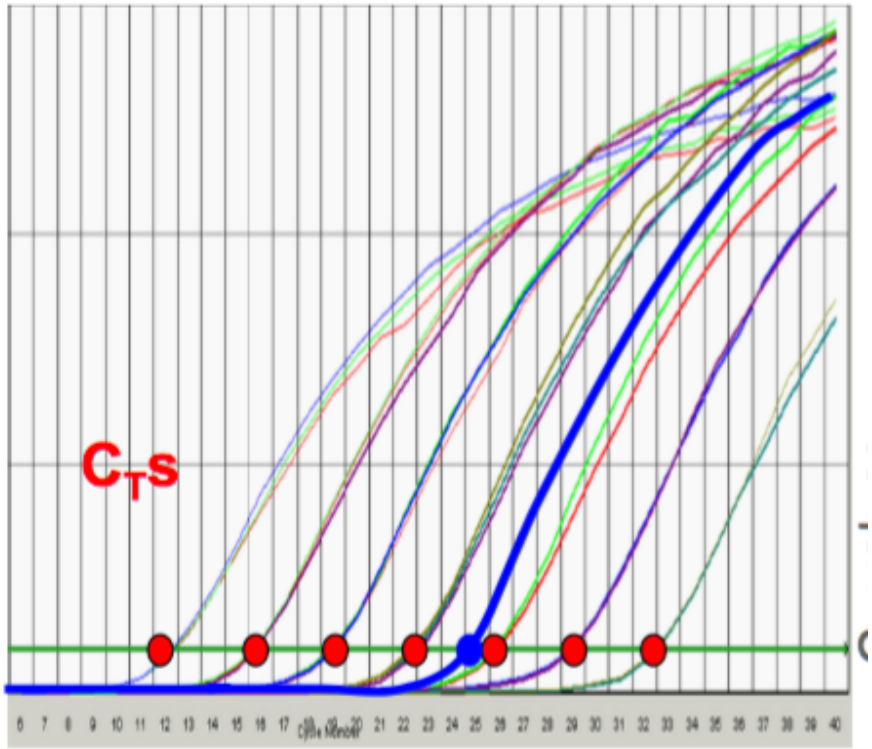
–e.g. Forensic science: Is there DNA and how much DNA (copy number) is there for forensics purposes

- e.g. Diagnostics: Virus titer in blood: is there virus DNA and how much is there?

Basics for the analysis of real-time PCR data: C_t and ΔC_t



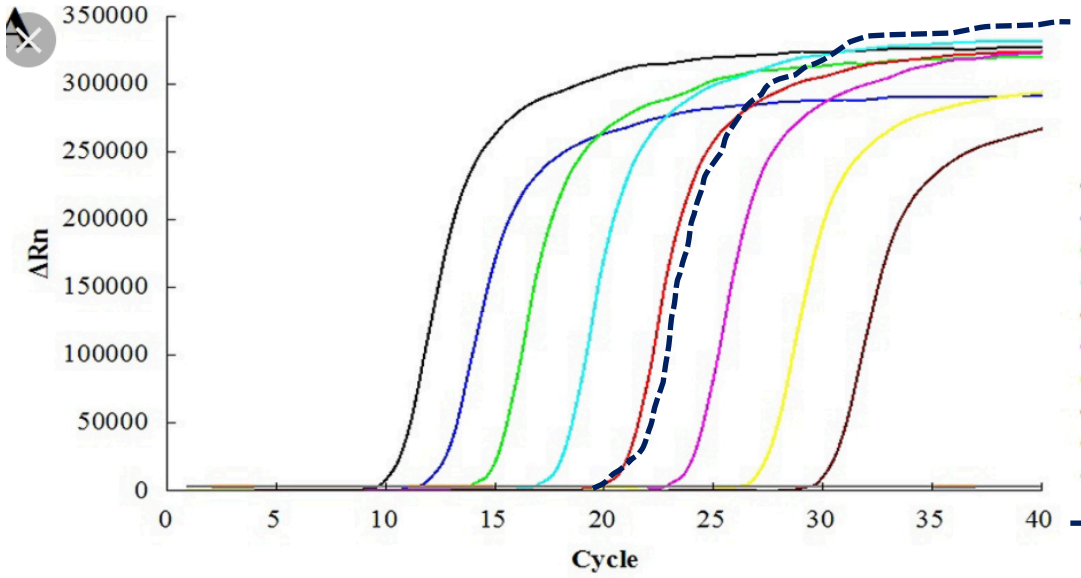
ABSOLUTE QUANTITATION



- C_ts derived from real-time PCR using and increased copy number of target site:
  PCR TARGET REGION MUST BE AVAILABLE
  (for example cloned into a plasmid)
  DIFFERENT DILLUTIONS ARE USED FOR PCR TO GENERATE A STANDARD CURVE

- Biological sample with unknown copynumber of PCR target site

Basics for the analysis of real-time PCR data: C_t and ΔC_t

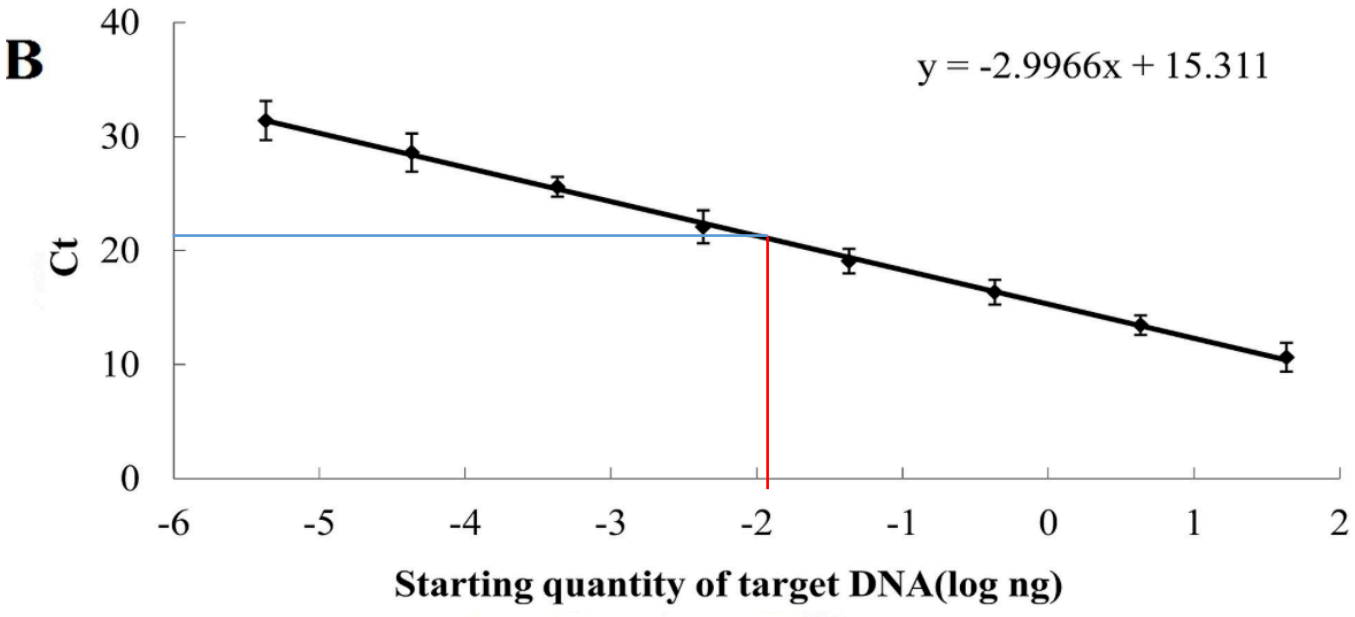


ABSOLUTE QUANTITATION

Defined amounts of target site used for PCR (standards)

Defined amounts of target site used for PCR (standards)

Biological sample C_t=21



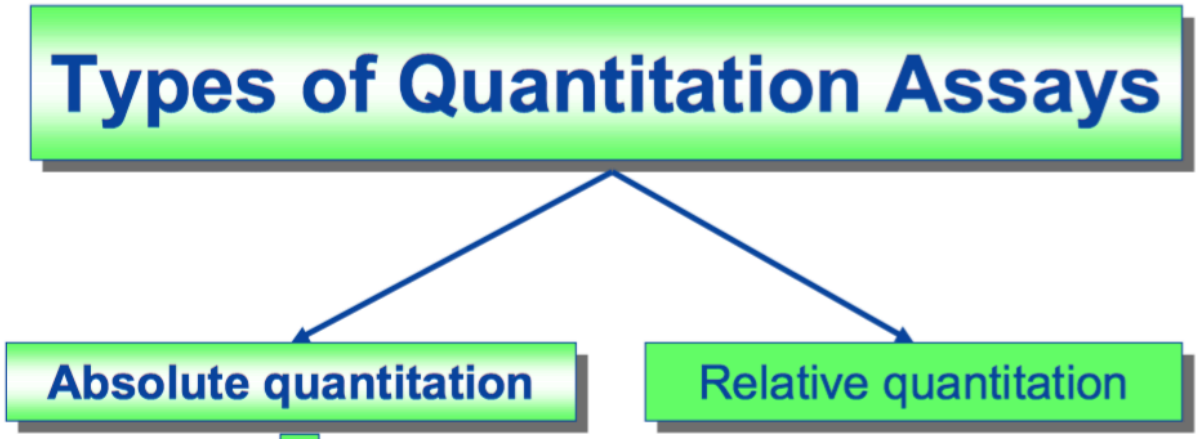
C_t of all standards are used to generate a standard curve: y=-2,9966x+15.311

C_t of Biological sample = 21

21=-2,9966x+15.311

x=-1,89848 log ng

Basics for the analysis of real-time PCR data: C_t and ΔC_t



RELATIVE QUANTITATION

Provides accurate discrimination between relative amounts of starting material

–e.g. Comparing expression levels of wildtype vs. mutated alleles

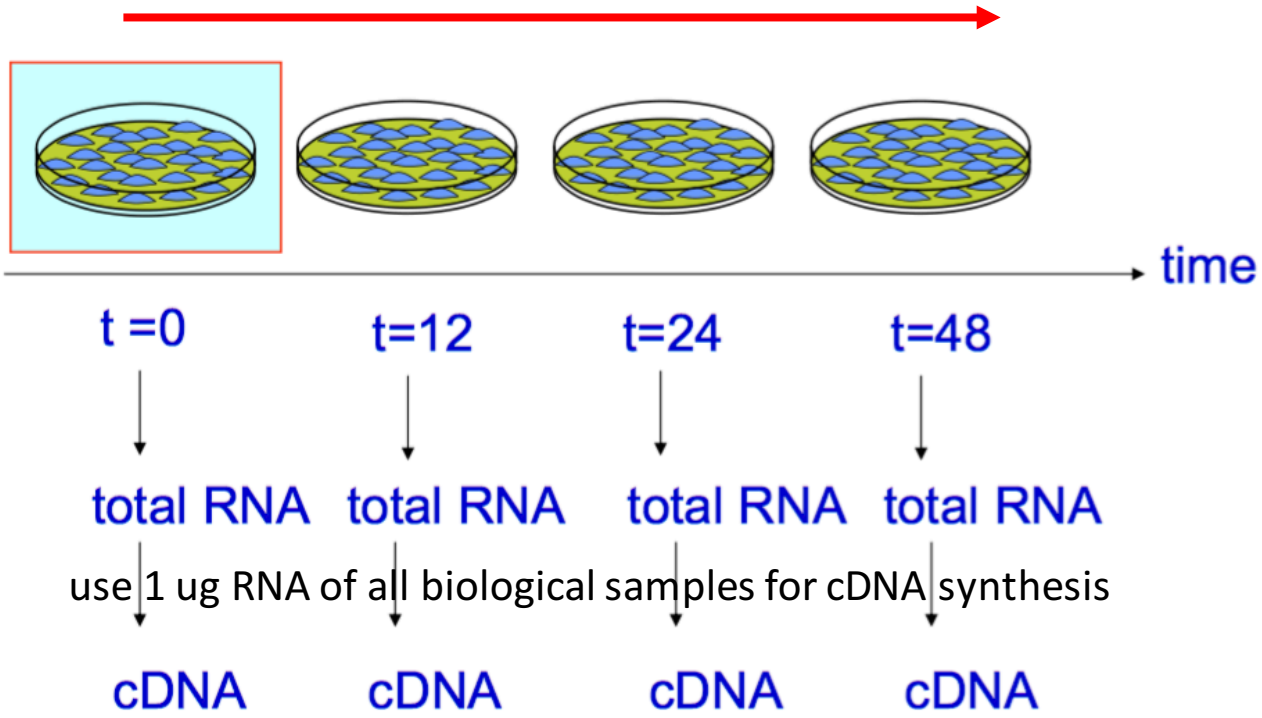
–e.g. **Comparing expression levels of a gene across different tissues or between different biological conditions**

–e.g. Validating array results

Basics for the analysis of real-time PCR data: relative quantitation

RELATIVE QUANTITATION

Cells stimulated for several hours (0-48h) with retinoic acid
(retinoic acid binds a trasncription factor that targets gene promoters)



QUESTION:
How are genes of interest (for example Hox A gene) regualted during this time

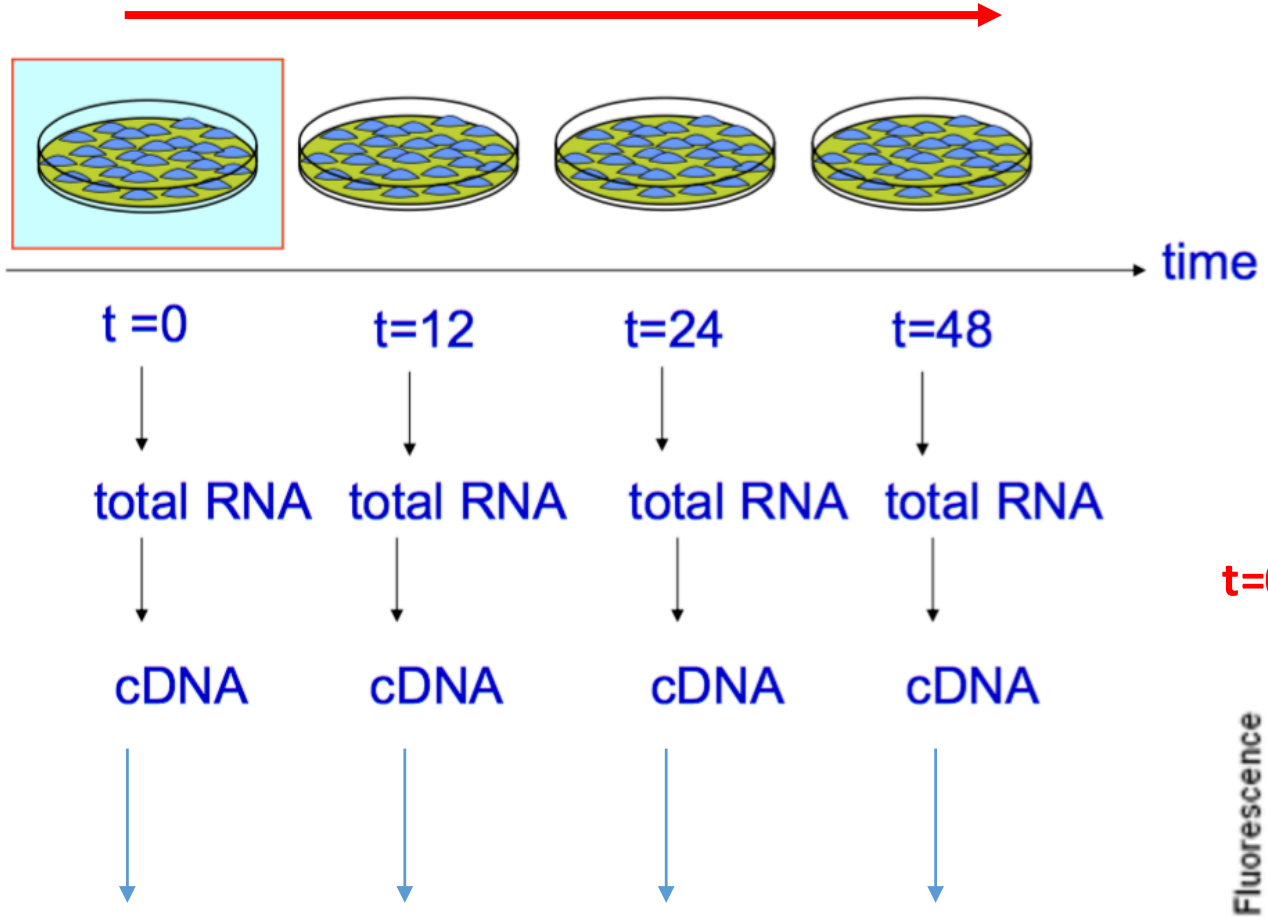
Used prepared cDNA for real-time PCR to determine the levels of mRNAs of interest in differnet experimental samples. Note that the mRNAs composition in biological difference changes, due to the retinoic acid treatment

Hox genes

REFERENCE GENE THAT IS NOT AFFECTED BY RETINOIC ACID TREATMENT

Basics for the analysis of real-time PCR data: relative quantitation

Cells stimulated for several hours (0-48h) with retinoic acid



REAL TIME PCR: performed in parallel (t=0 – 48) at the same time.
Very important: precise pipetting!

RELATIVE QUANTITATION

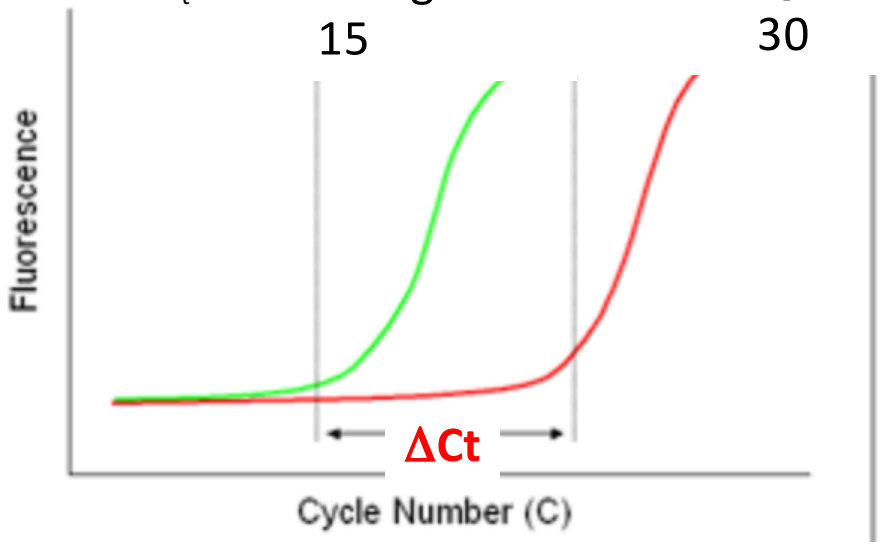
REFERENCE GENE:

- **no altered expression in relevant biological**
- **normally expressed at high levels**
- **Serves to control of sample quantity**
- **Serves to control for pipetting errors**
- **Examples:18S rRNA, GAPDH, β-actin, tubilin, RNA polymerase II, histone H3**

t=0

C_t reference gene= 15

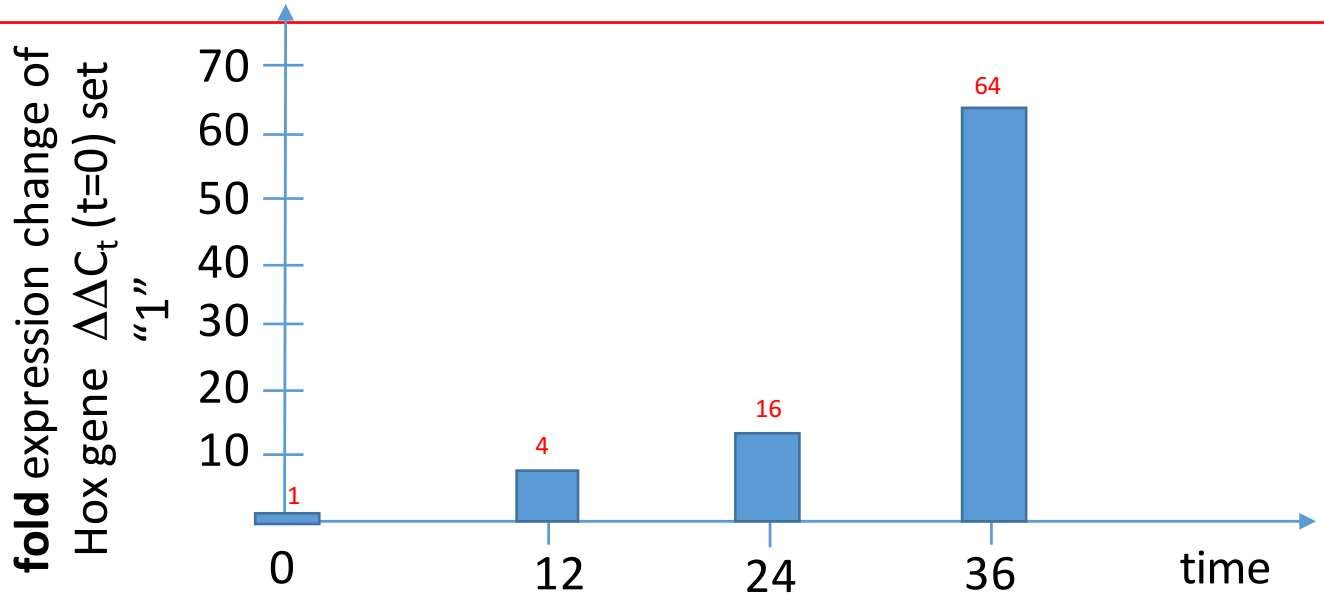
C_t Hox A gene= 30



Basics for the analysis of real-time PCR data: C_t and ΔC_t and $\Delta\Delta C_t$

Gene	t=0	t=12	t=24	t=36
C_t Reference	15	15	15	15
C_t Hox gene	30	28	26	24
ΔC_t	15	13	11	9

$\Delta\Delta C_t$	15-13=2 (cicli)	15-11= 4	15-9=6
$2^{\Delta\Delta Ct}$ 1	2^2=4	2^4=16	2^6=64



Basics for the analysis of real-time PCR data: C_t and ΔC_t and $\Delta\Delta C_t$

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C_t Reference	15	15	**17**	15
C_t Hox gene	30	28	**28**	24
ΔC_t	15	13	**11**	9

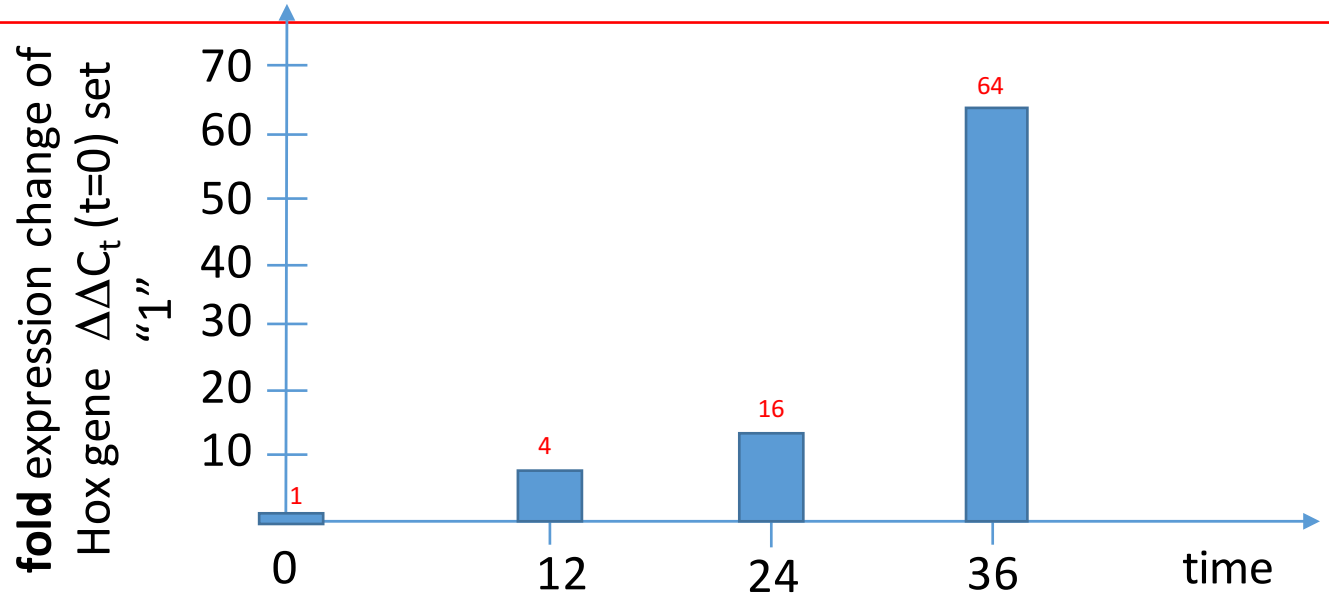
Reduced cDNA level, when compared to t=0, 12,36

→ Ligher Ct for Hox and reference gene

→ Delta Ct remains unchanged

→ REFERENCE GENE SERVES TO COMPENSATE DIFFERENT EFFICIENCY OF UPSTREAM STEPs

$\Delta\Delta C_t$		15-13=2 (cicli)	15-11= 4	15-9=6
$2^{\Delta\Delta Ct}$	1	2^2=4	2^4=16	2^6=64



What is Real-Time PCR used for?

Real-Time PCR has become a cornerstone of molecular biology:

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

For all applications that require the quantification of RNA/DNA sequences