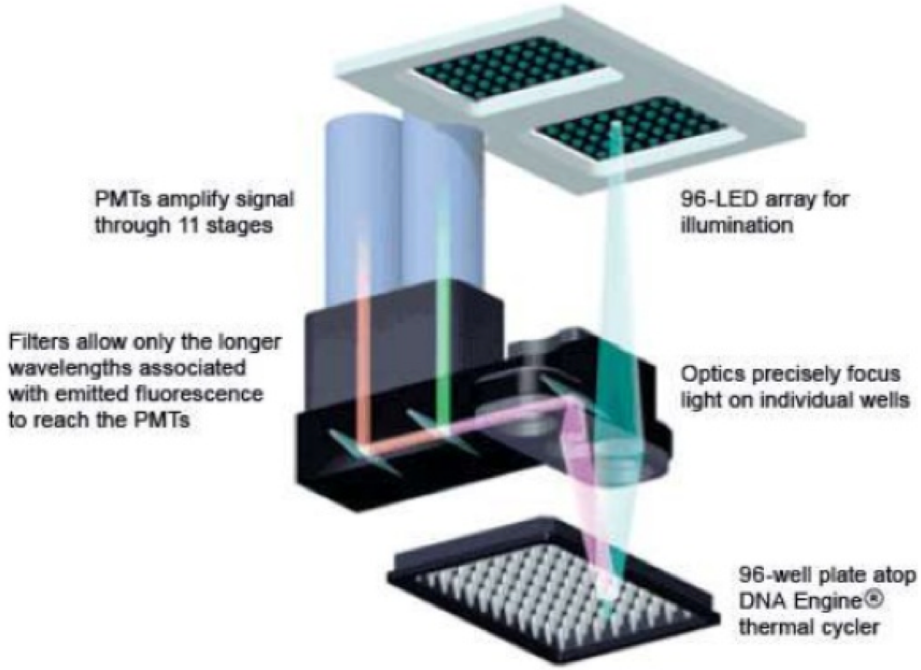
We appreciate your registration through the QR code

Real time PCR



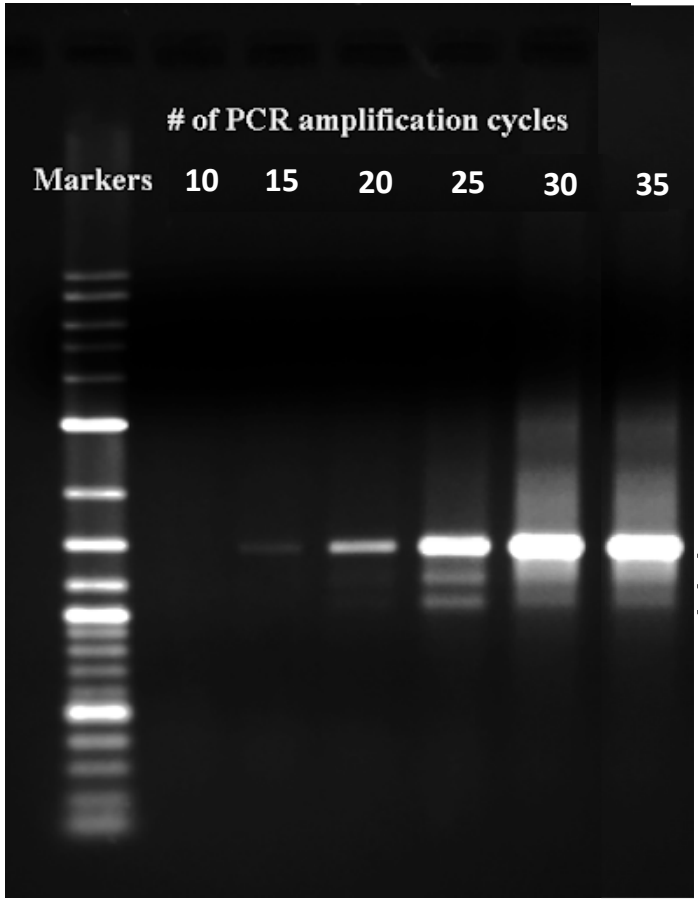
…follow the accumulation of PCR products during increasing cycle numbers in "real-time" using a detection system (gel electrophoresis non necessary)

Let’s compare semiqantitative PCR and quantitative PCR

Example: gene expression analysis

- **Semi-quantitative PCR**
- **Technical details on quantitative PCR**
- **Quantitative PCR and quantitative gene expression analysis**

Visualization of PCR products by agarose gel electrophoresis:



"End point PCR":

PCR performed with increasing cycle numbers that bring PCR to plateau phase.

Example: results indicate that plateau phase is reached at cycle number 30 = end point

Note: we do not have information on amplification between cycle number 26 and 29. It might be that plateau phase is reached at cycle number 26, 27, 28 or 29.

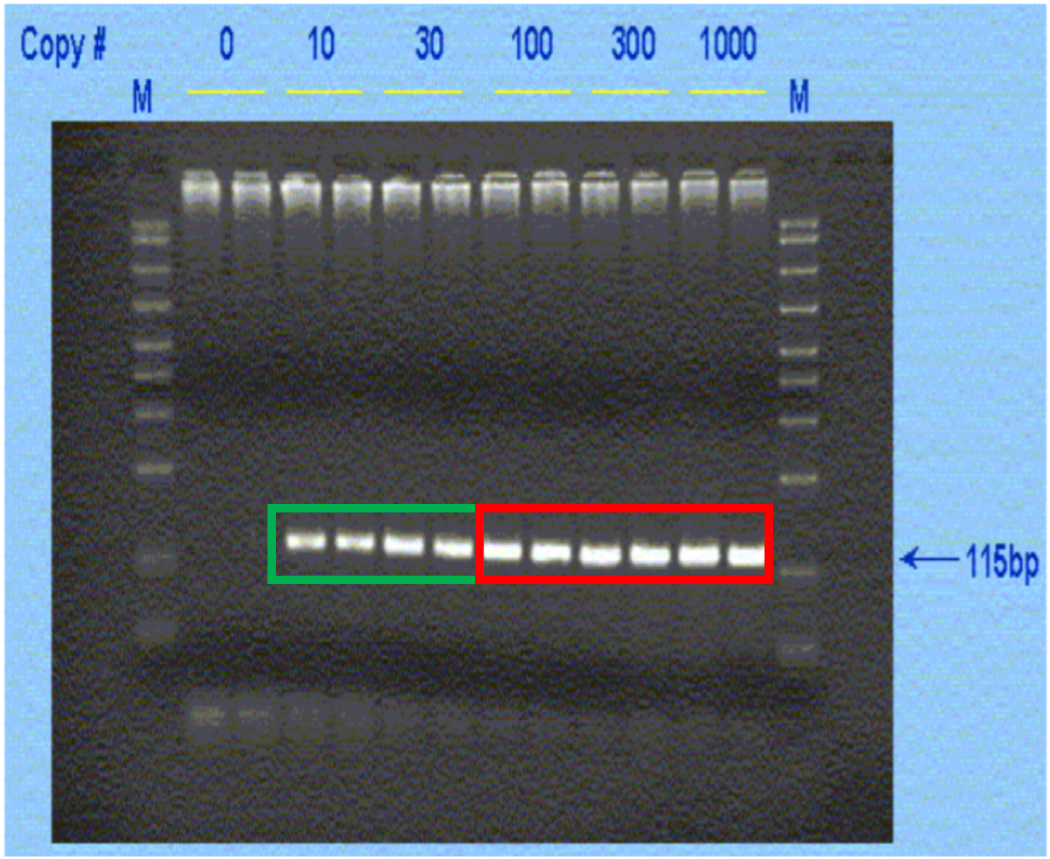
Semi-quantitative PCR and gene expression analysis

Traditional End-Point PCR = Researcher decides cycle number

- has a narrow dynamic range (<2 logs) → **SEMI-QUANTITATIVE**
- when multiple samples are used to perform PCR, template number can be different in different samples → a fixed cycle number suitable for all samples is are difficult to determine

Template copy numbers

Negative control



Copy number of PCR template

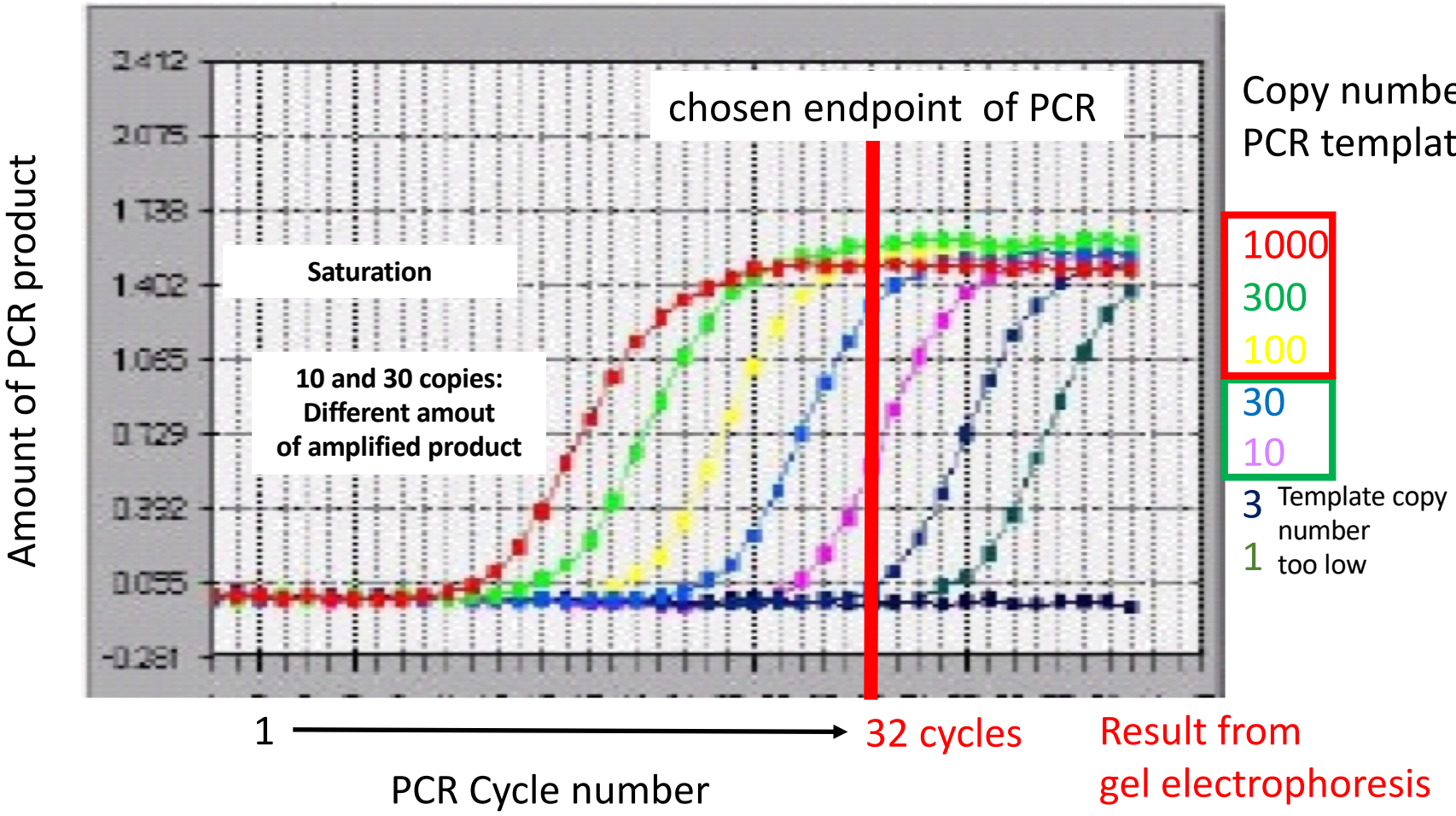
PCR conditions: **32 cycles**

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 – → max. amplification already reaached with 100 template-copies
→ No quantitative information between 100 and 300 copies as template

Semi-quantitative PCR and gene expression analysis

Traditional End-Point PCR



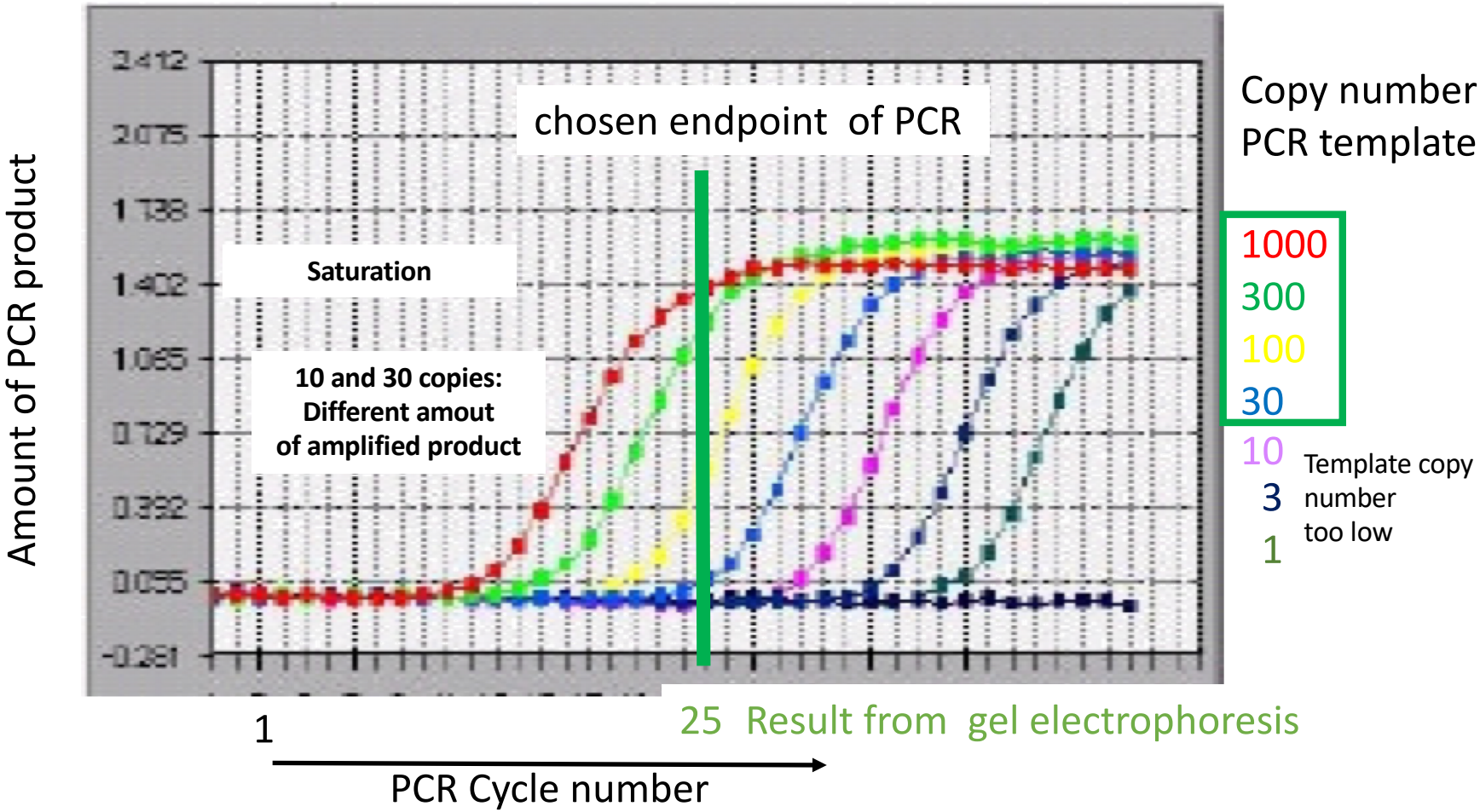
End-point PCR:

→ ideal to give qualitative information (for example amplification of a mutation using specific primers or presence of pathogen)

→ Only limited quantitative information possible

Semi-quantitative PCR and gene expression analysis

Adjust ideal number of cycles – can only be experimentally defined (laborious)



Get better quantitative information from classic PCR:

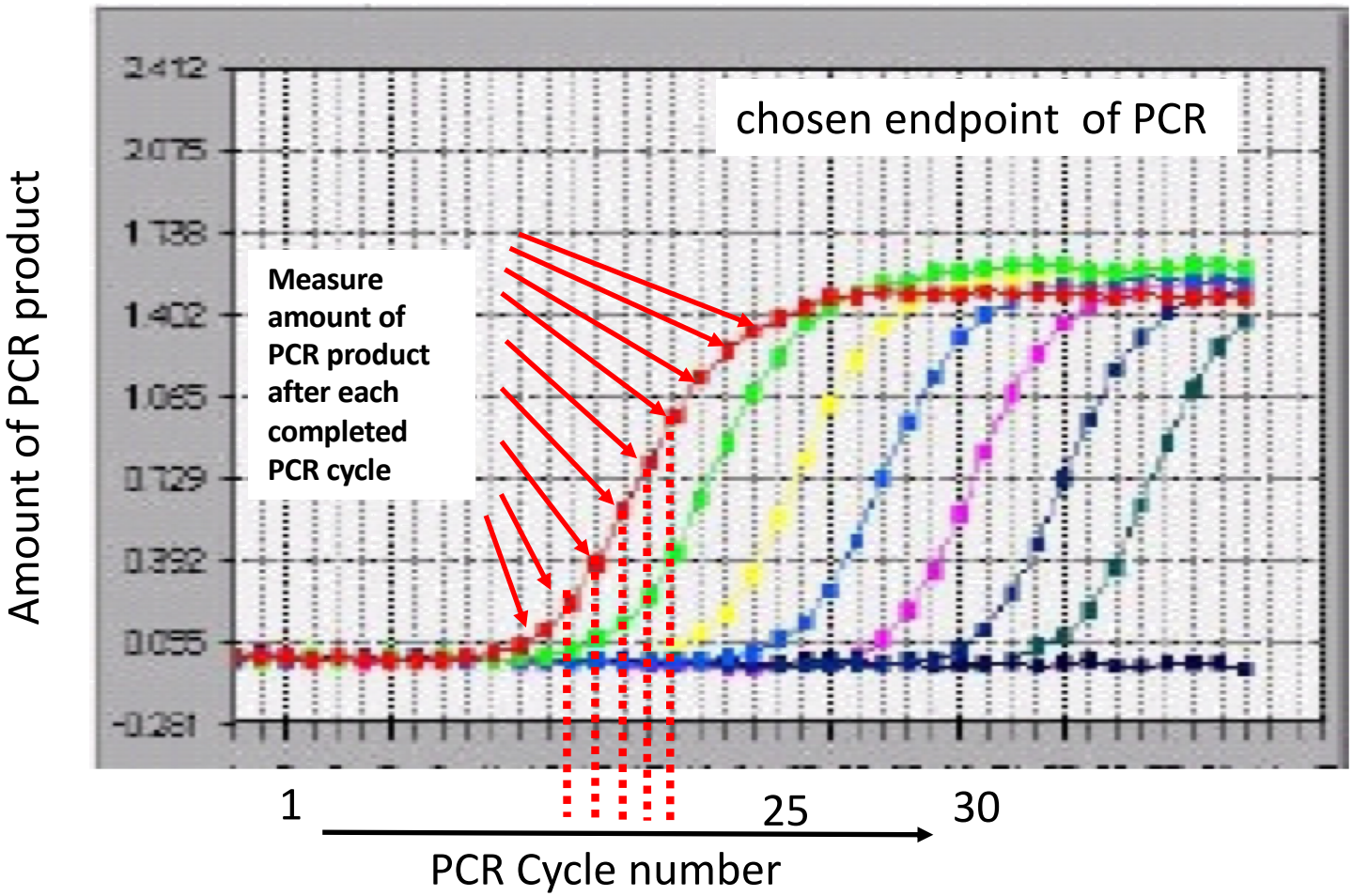
- Optimize PCR conditions:
  - A. Test for ideal end-point of PCR (example 25)
  - B. Optimize the amount of original template used for PCR

Time
Waste of primary material
Costs
Variability

Technical details on quantitative 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" in all biological samples analyzed = REAL TIME PCR

Technical details on quantitative PCR

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

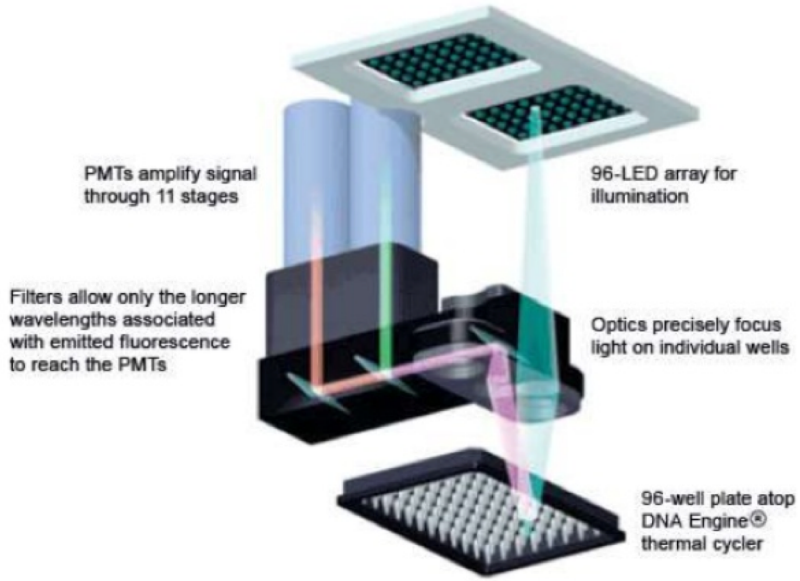
- **Gene expression analysis**
  - Cancer research
  - Drug research
- **Disease diagnosis and management**
  - Viral quantification
- **Food testing**
  - Percent GMO food
- **Animal and plant breeding**
  - Gene copy number

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

Technical details on quantitative PCR

Real-time PCR

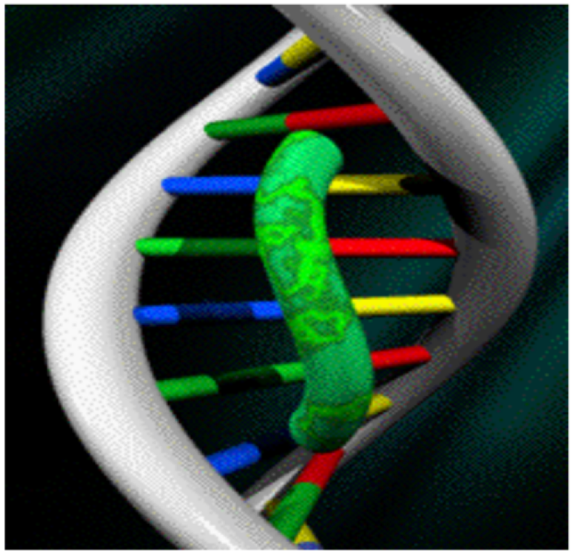
Eliminate use of gel electrophoresis
Increase reproducibility
Enable use of internal controls/standards
Reduce turnaround time
Increase throughput; 96 well format, 384 well format
Reduce sample amount usage
Results expressed as numbers



Real-Time PCR Chemistry

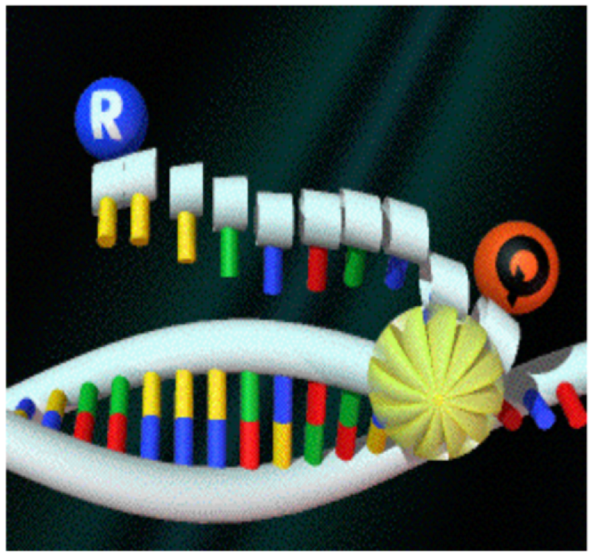
Strategies to follow PCR product generation

SYBR® Green I dye



Binds double stranded DNA

Fluorogenic 5' Nuclease Assay

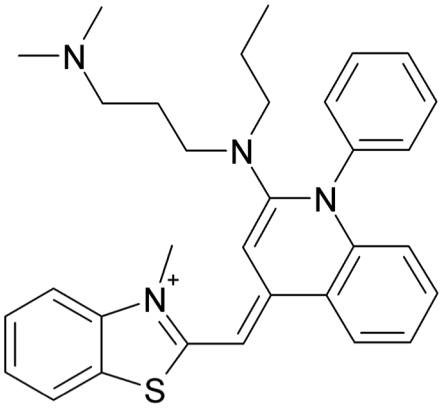


Uses a TaqMan® probe

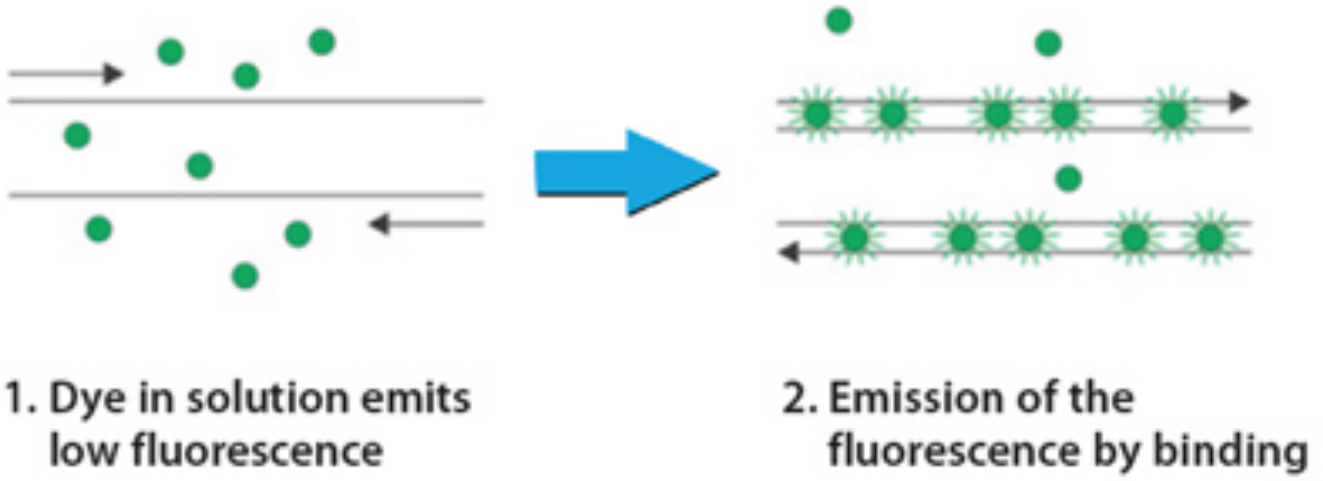
1. SYBR® Green I Dye Assay Chemistry

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.



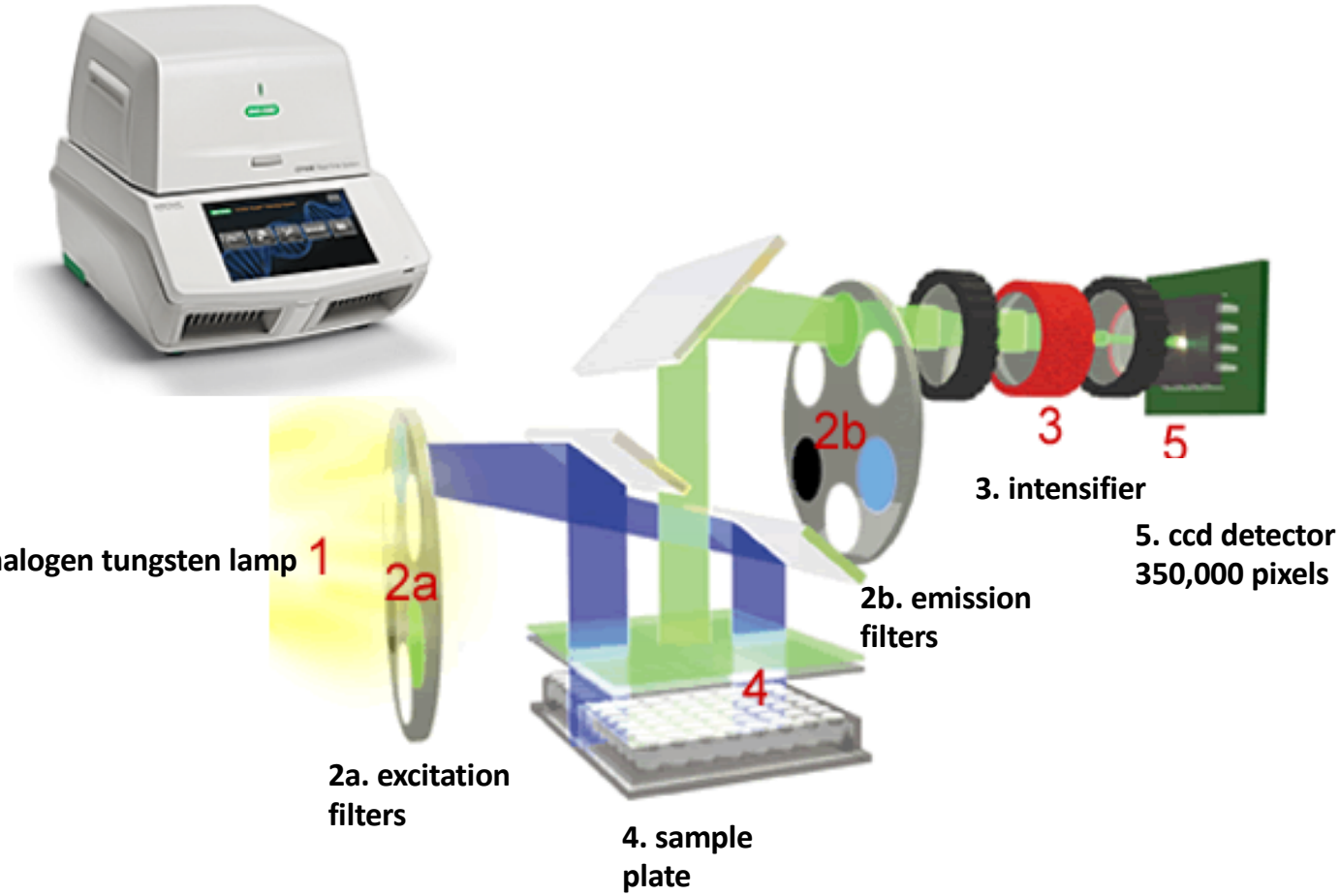
Denaturation
Annealing



DNA synthesis
Detection of emission of fluorescence

Fluorescence emmission is increasing with increasing of PCR cycles

Basics of real-time PCR measurements



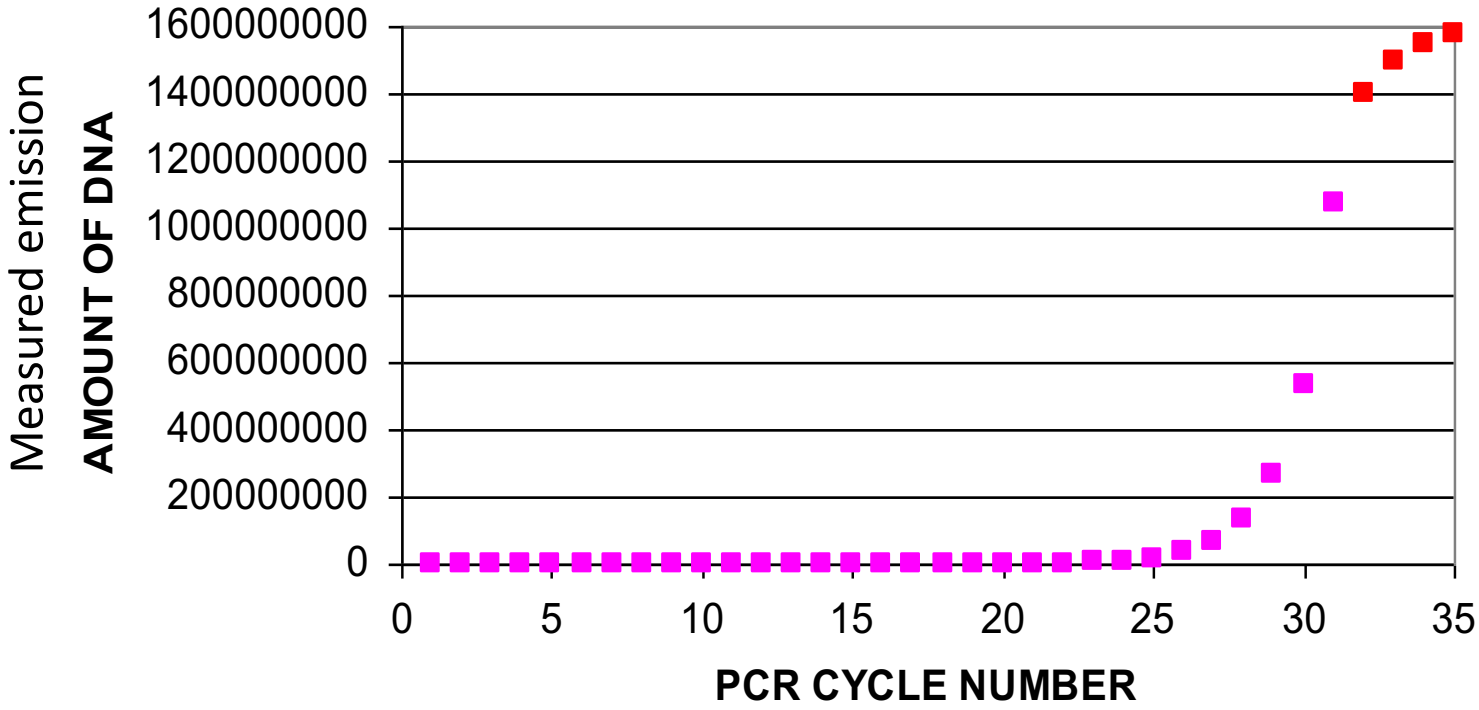
Every PCR cycle: Exitation of SYBR green (497nm) + measurment of emission from SYBR green (520nm)

Fig. 1.2. Representation of Optical Detection System layout.

www.biorad.com

Basics of real-time PCR measurements

AMPLIFICATION BLOT



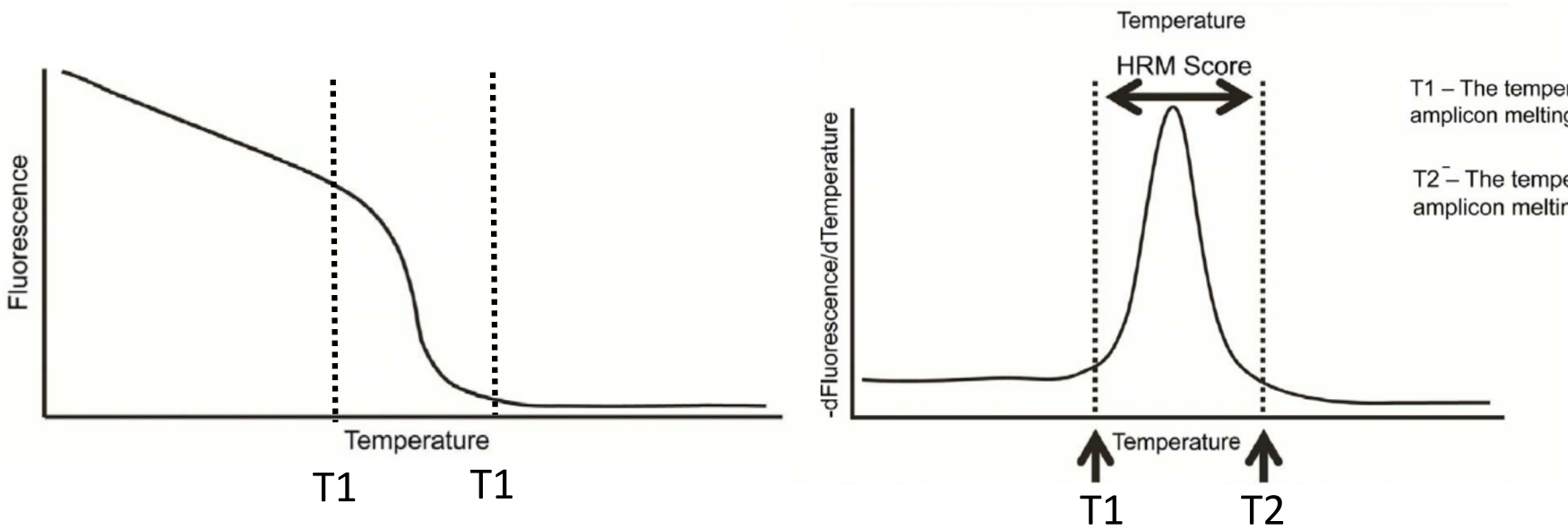
Quantitative information

Basics of real-time PCR measurements – Melting curve

METLTING CURVE ANALYSIS (HRM = high resolution melting score)

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 (emmitting fluorescense 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.



Agarose gel

Single band

Generation of melting curves, melting peaks, and HRM scores. Melting curves (top panel) are generated by graphing Fluorescence against Temperature. Fluorescence declines as the DNA melts. DNA melting is visualized through the use of a saturating duplex-dependent DNA intercalating dye. As the DNA melts, the dye is released; unbound dye does not fluoresce. Melting peaks (bottom panel) are generated by taking the negative derivative of Fluorescence with respect to Temperature and graphing these values against Temperature (2 dF/dT vs T).

Melting curve is determined after the last cycle of PCR:

- → **PCR machine heats up PCR products from 0°C to 100°C**
- → **Dissociation of SYBR from dsDNA filaments is measured**
- → **IF PCR HAS AMPLIFIED SPECIFICALLY 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**

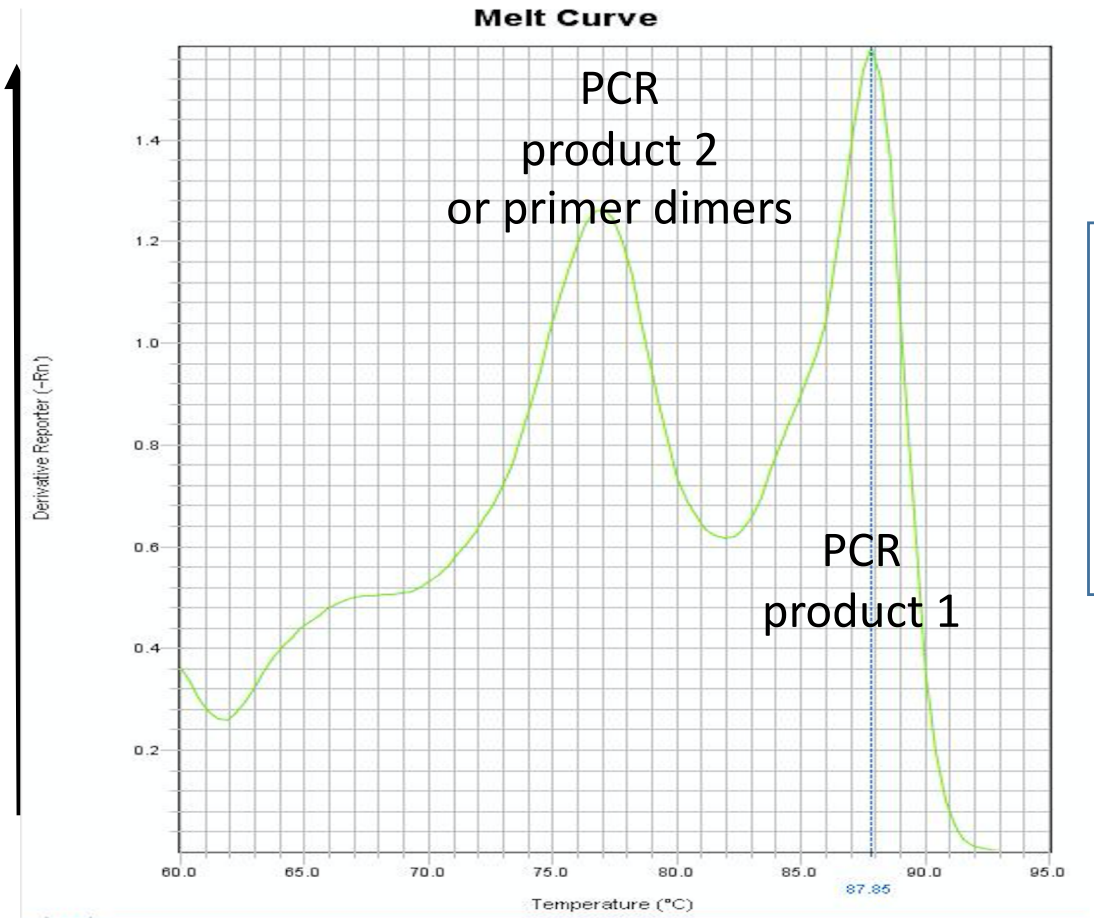
Basics of real-time PCR measurements

METLTING CURVE ANALYSIS

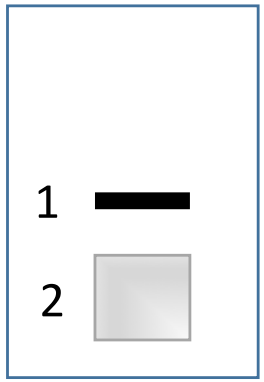
Melting curve is determined after the last cycle of PCR:

- → **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 specific!!)**
- → **Example: IF YOU RUN PCR PRODUCT ON AGAROSE GEL, MORE THAN ONE BAND WILL BE VISIBLE**

dissociation of ds DNA



Agarose gel



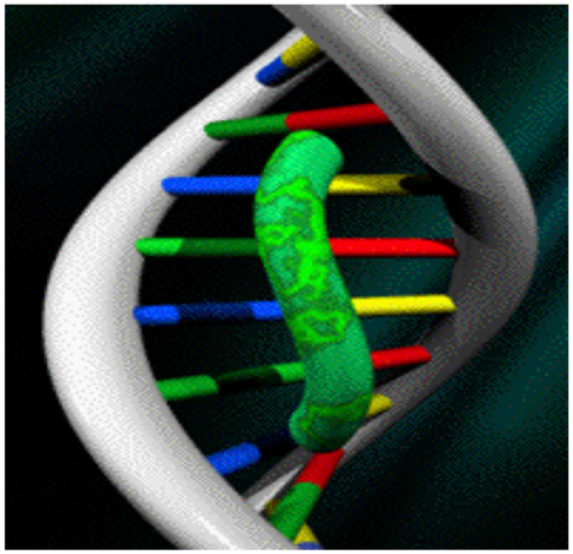
AREA UNDER PEAKs
= SYBR incorporation
Here: signals from different types of dsRNA
(not only target amplicon)

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

Real-Time PCR Chemistries

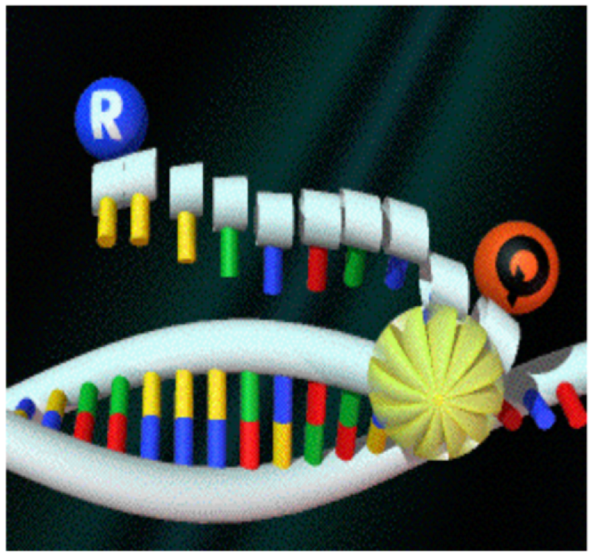
Strategies to follow PCR product generation

SYBR® Green I dye



Binds double stranded DNA

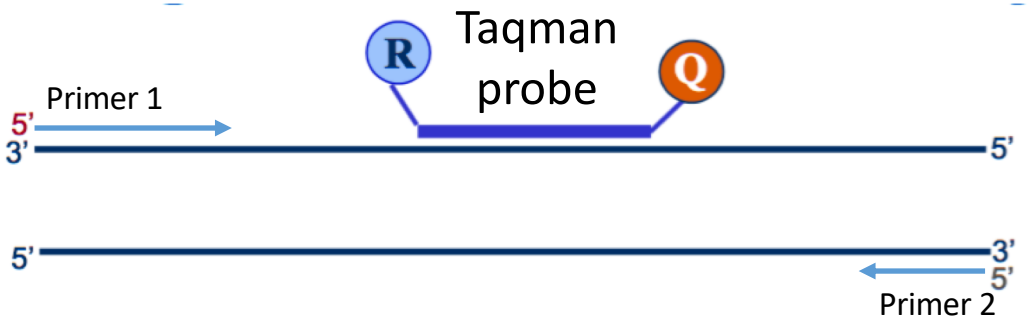
Fluorogenic 5' Nuclease Assay



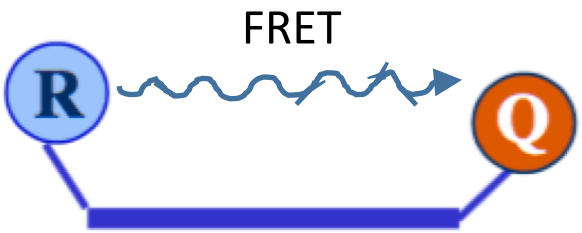
Uses a TaqMan® probe

2. Real-Time PCR chemistry 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

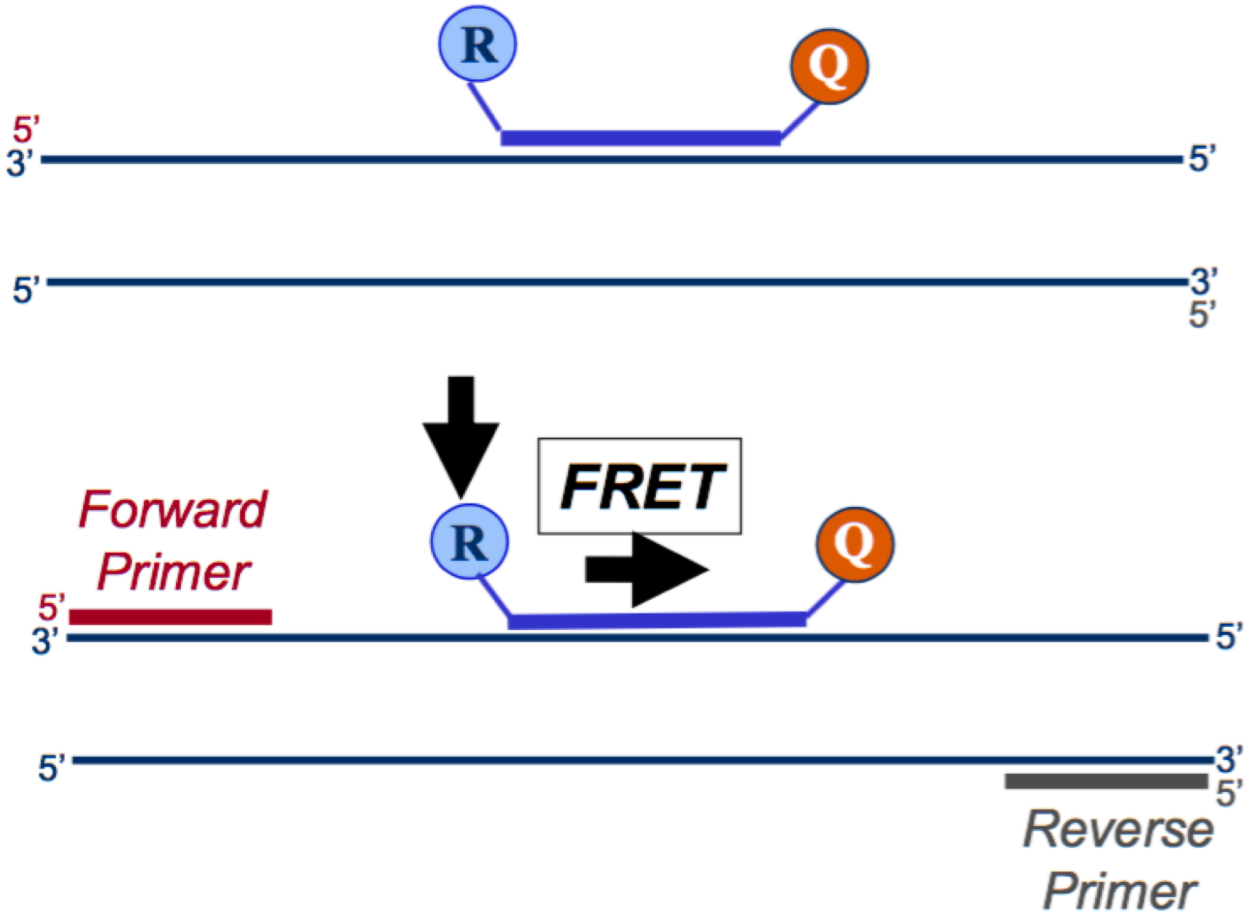
Taqman probe:

- 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

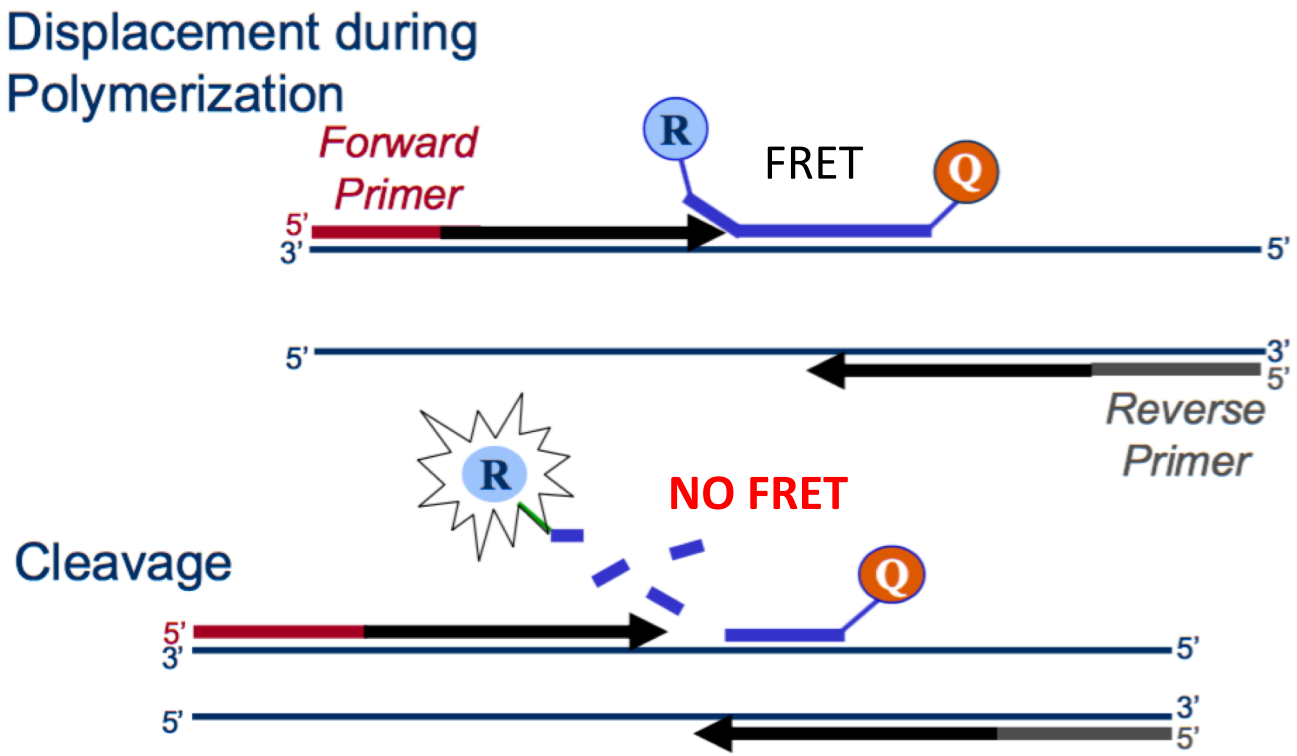
For example:
Cycle 5 during PCR



- 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

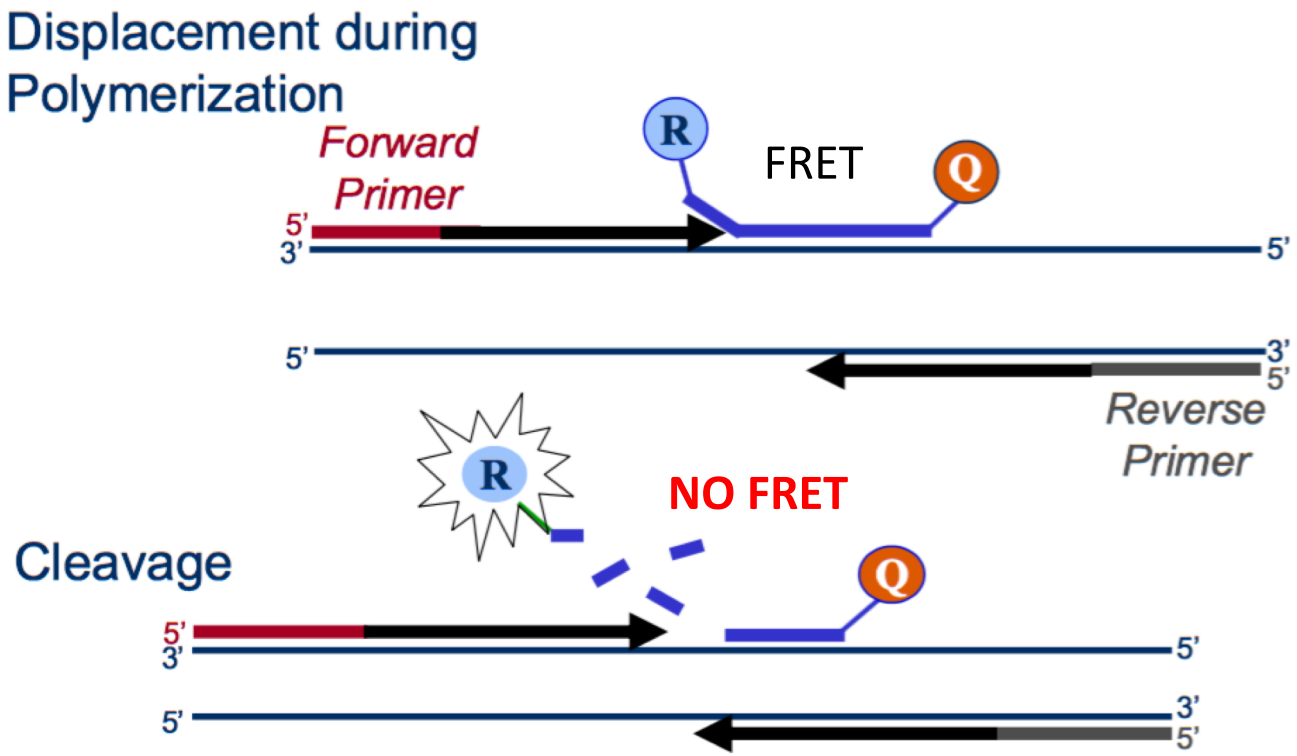


- DNA SYNTHESIS BY Taq polymerase

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



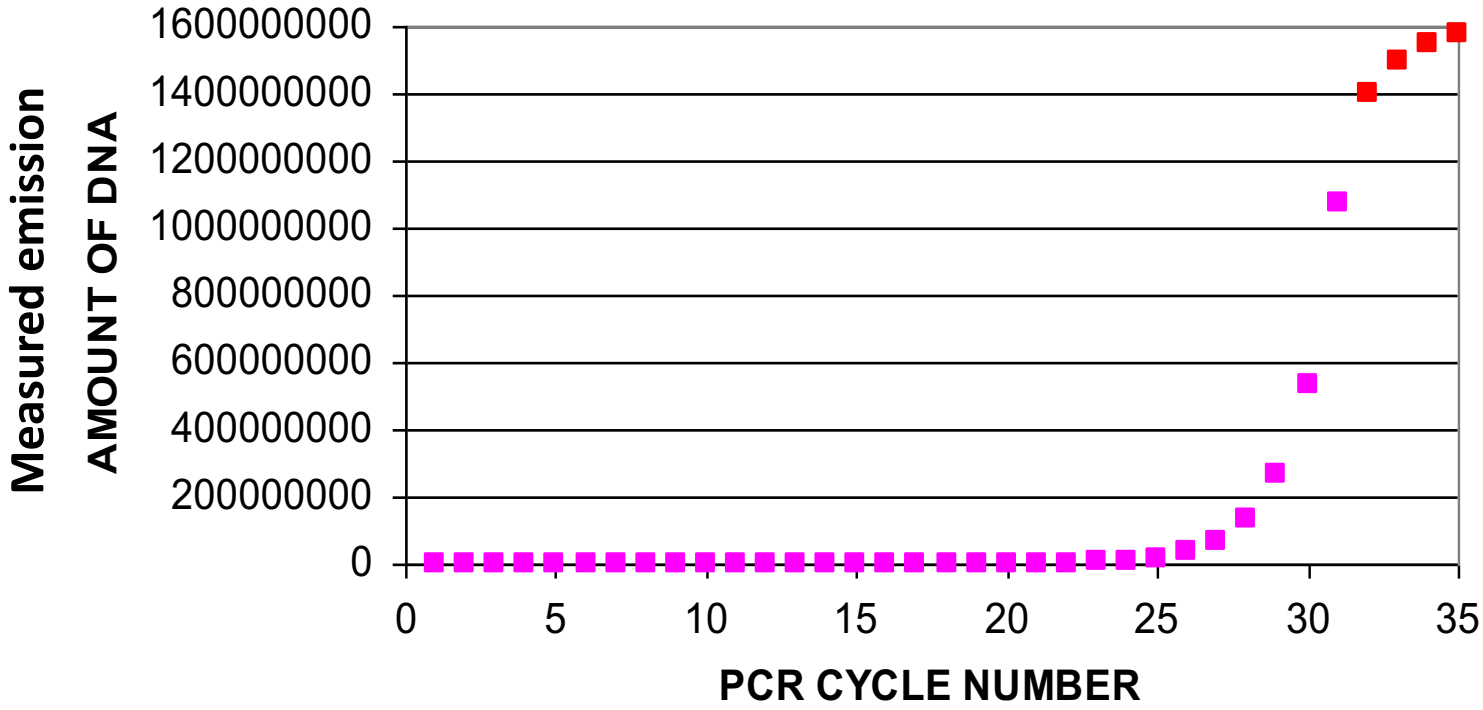
- DNA SYNTHESIS BY Taq polymerase

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!!!

Basics of real-time PCR measurements

AMPLIFICATION BLOT



Quantitative information

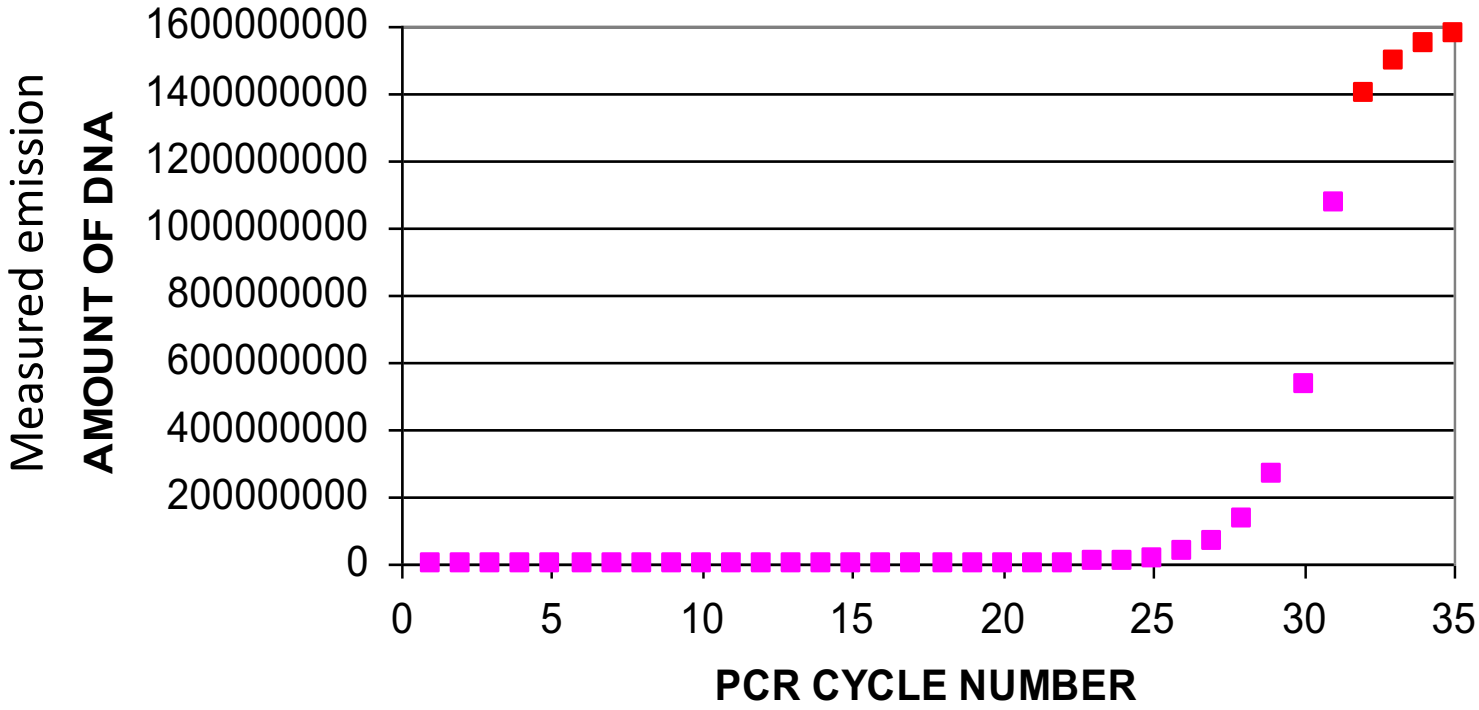
Let’s compare semiqantitative PCR and quantitative PCR

Example: gene expression analysis

- **Semi-quantitative PCR and gene expression analysis**
- **Technical details on quantitative PCR**
- **Quantitative PCR and quantitative gene expression analysis**

Analysis if quantitative RT-PCR data

AMPLIFICATION BLOT

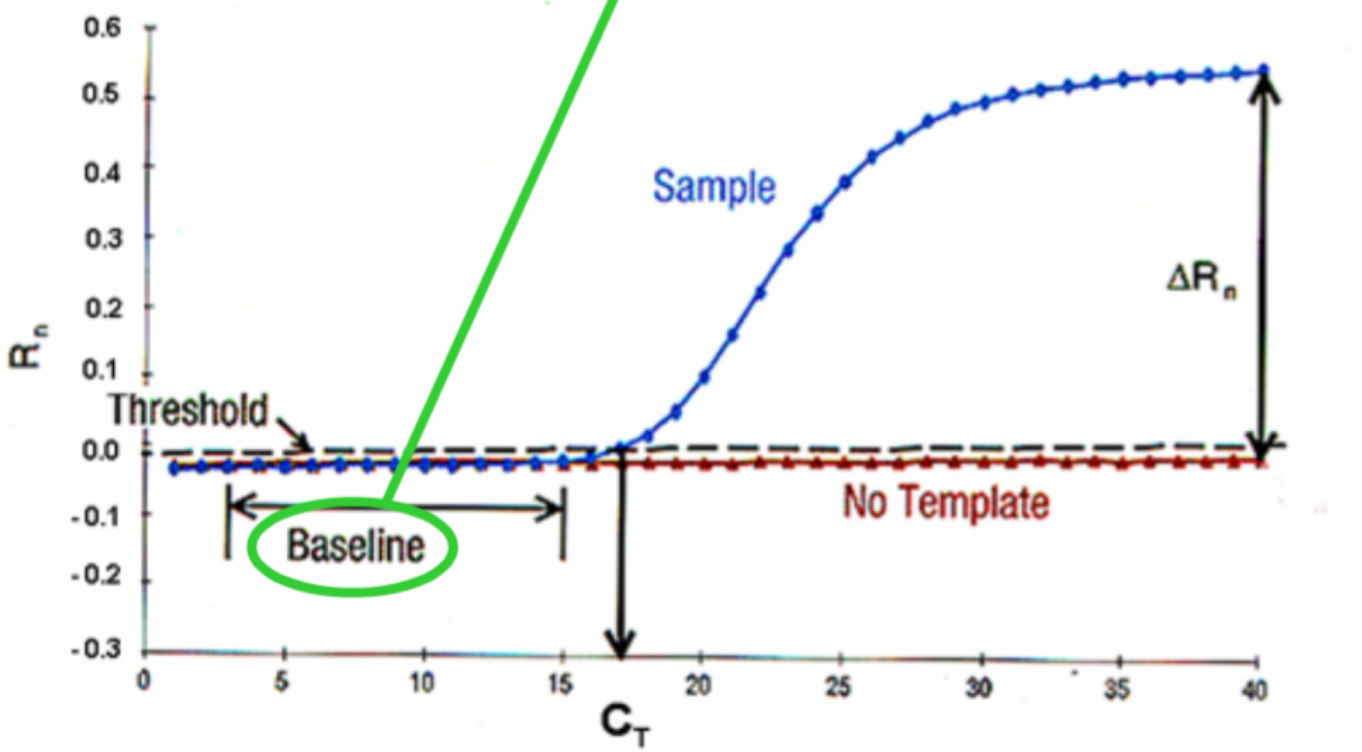


Quantitative information: number of template molecules decides at what cycle number exponential amplification is starting

Terminology of amplification blots

Terminology Baseline:

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

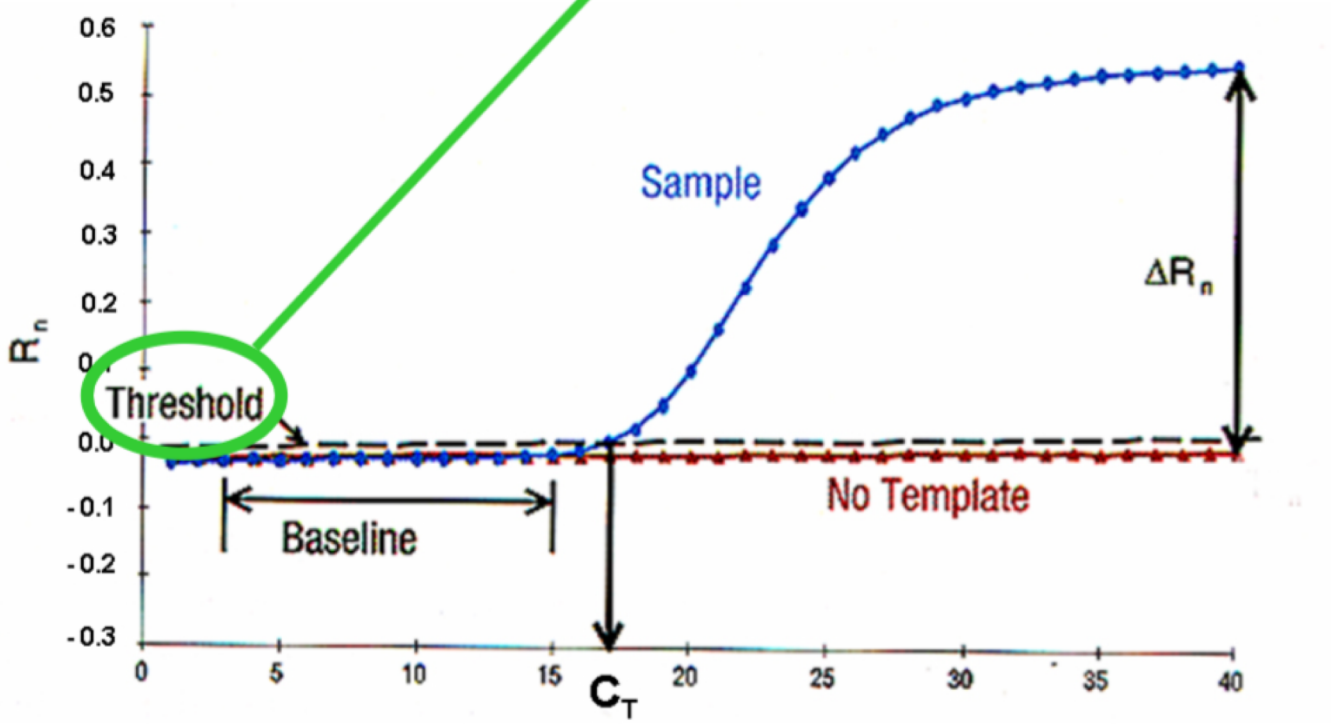


2 parallel PCR reacations shown in blot:
BLUE: PCR with template
RED: PCR without template (negative control)

R_n: reporter signal obtained from detector

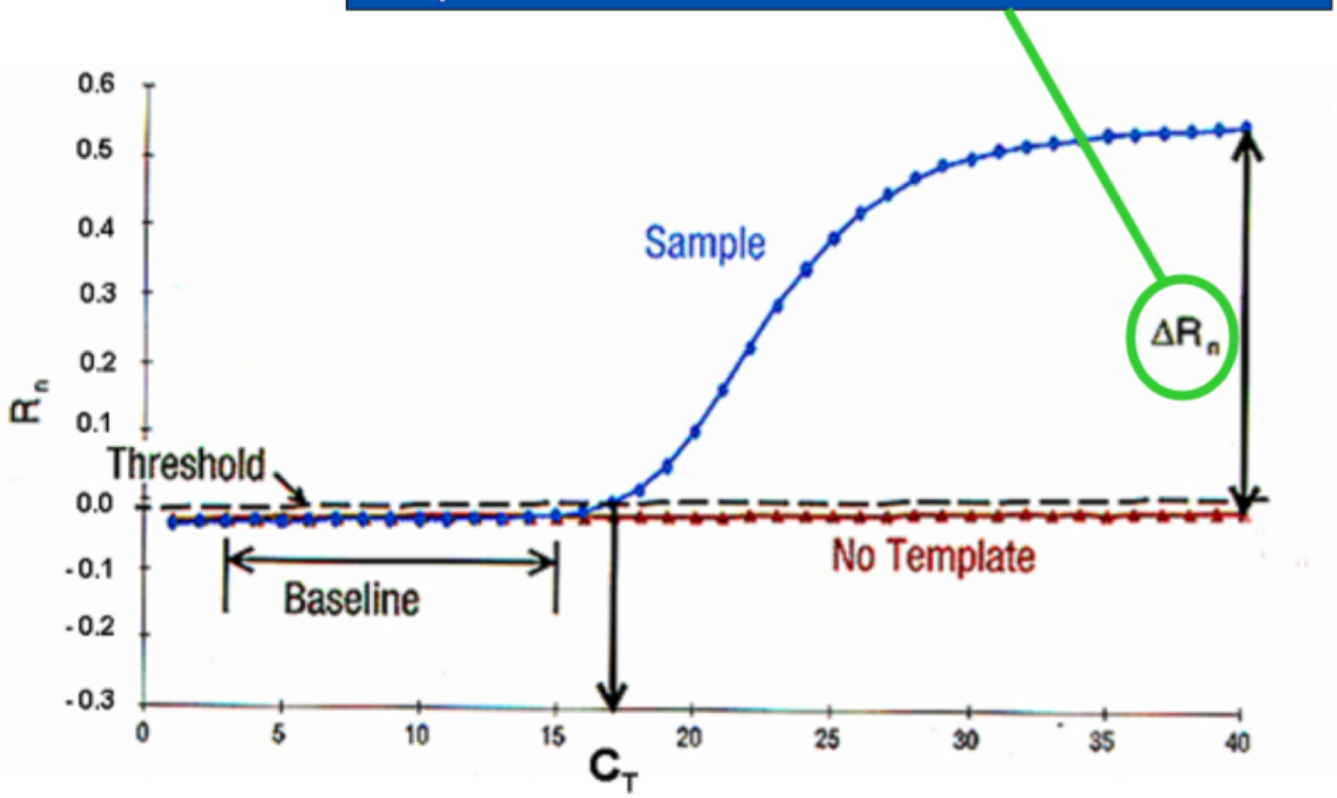
Terminology of amplification blots

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



Terminology of amplification blots

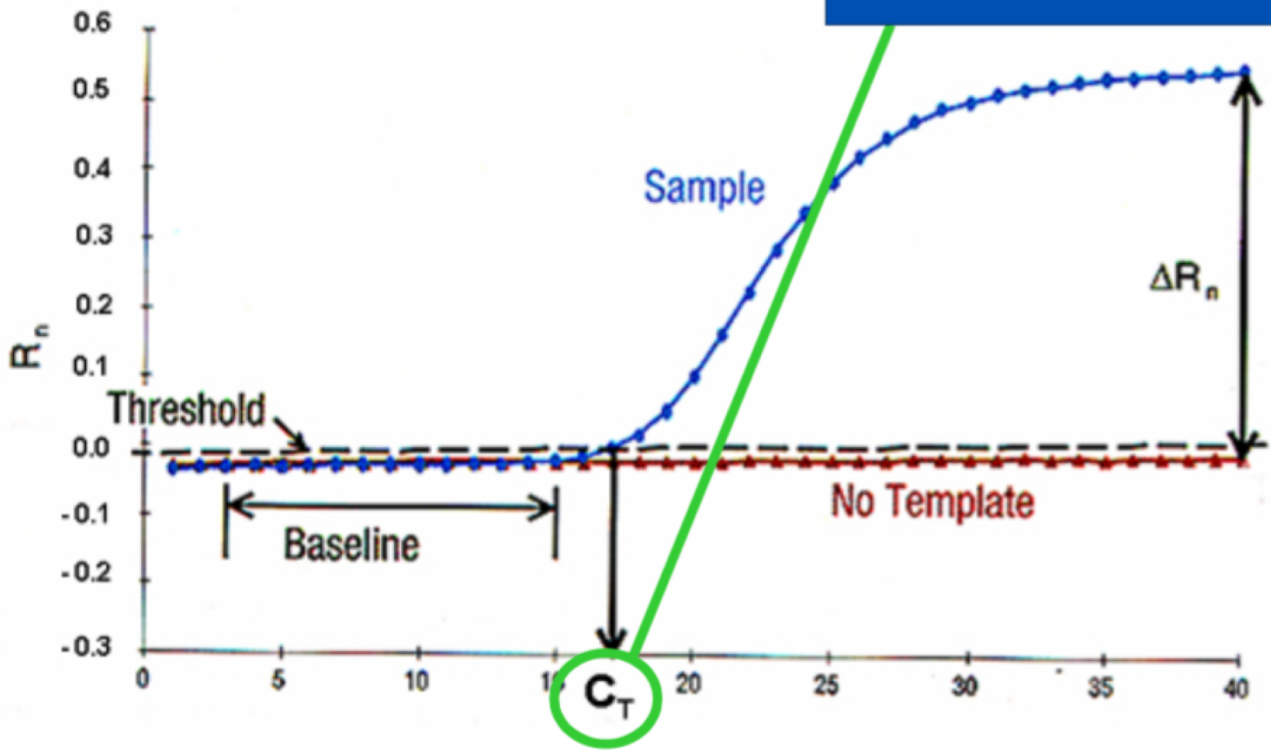
ΔR_n: **Normalized reporter signal minus background (baseline level).**



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

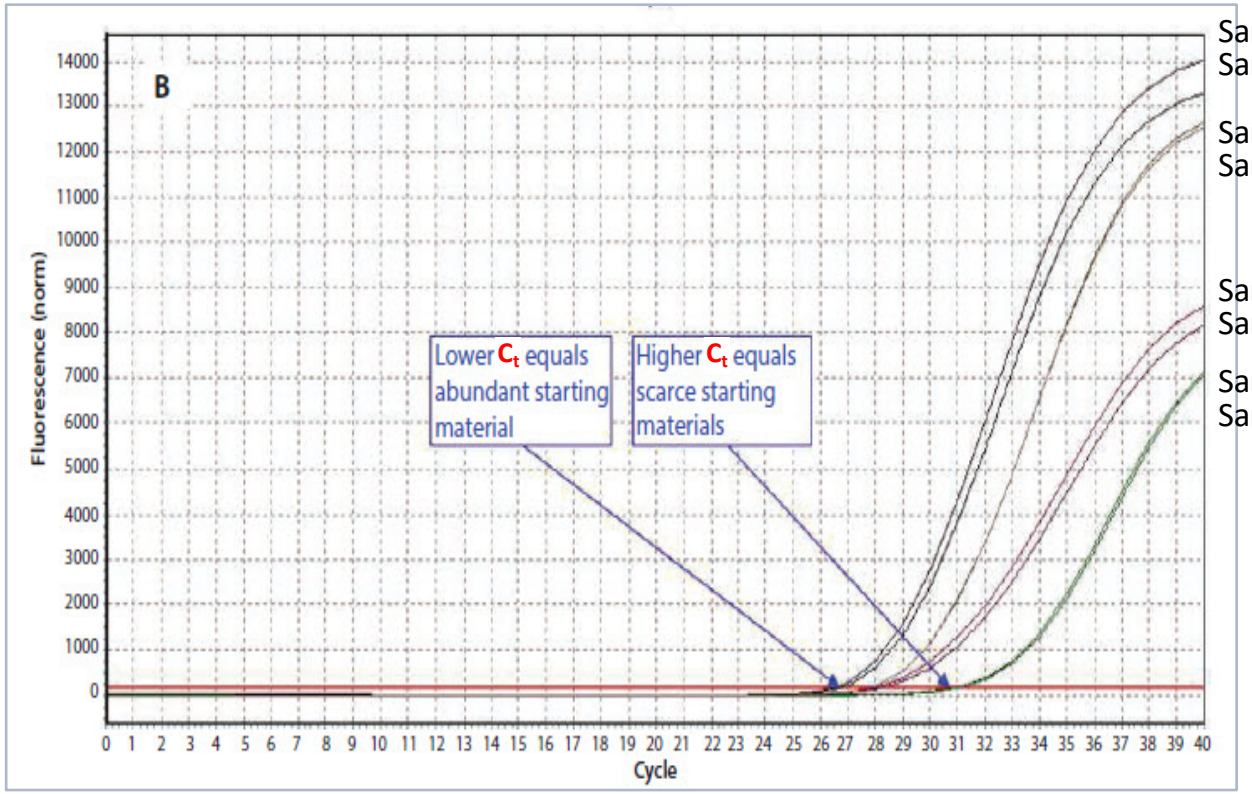
Terminology of amplification blots

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

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

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

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

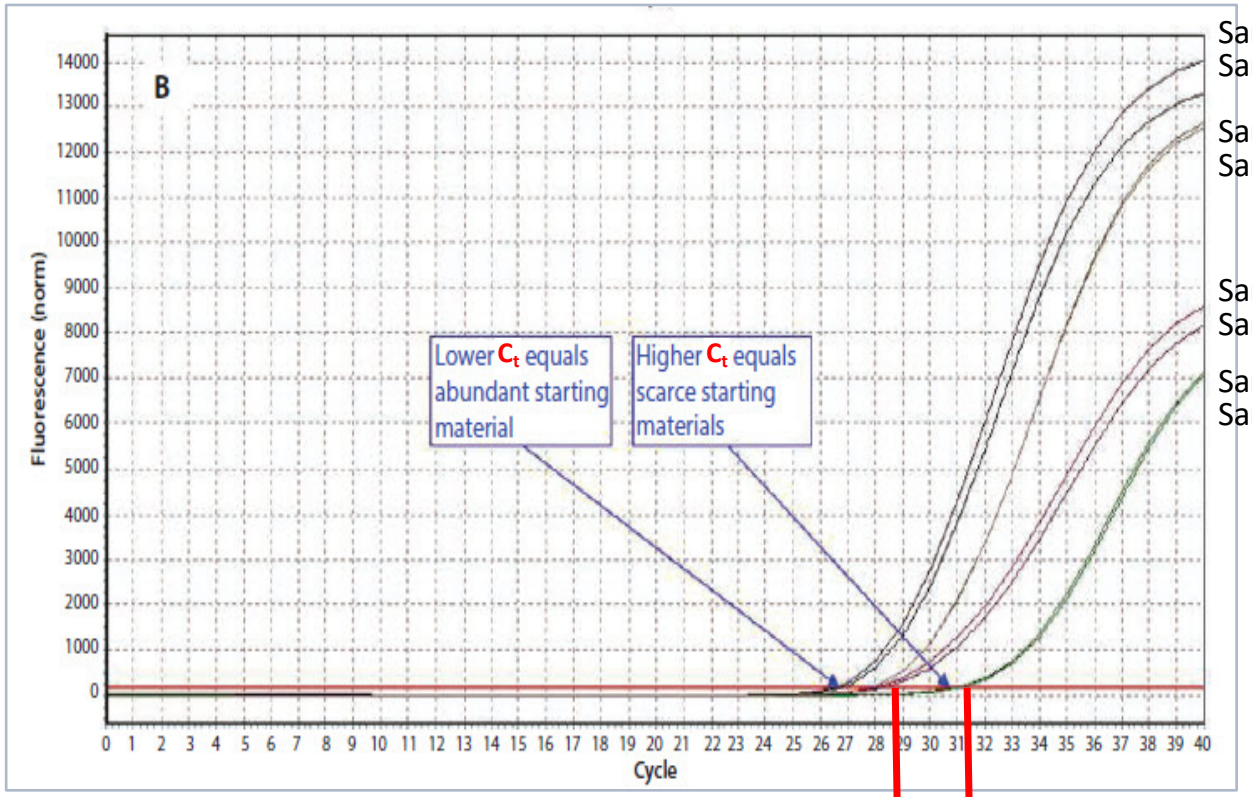
Sample 3 (duplicate 1)
Sample 3 (duplicate 2)
C_t=28,5

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

Number of target DNA molecules in different samples

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

Same primer pairs and reagents used



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 =

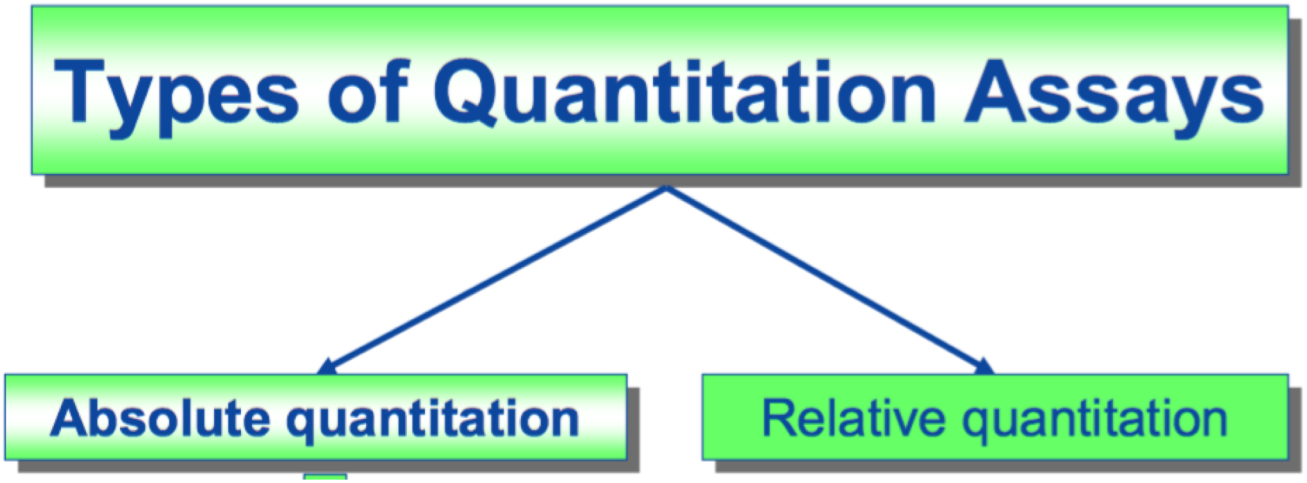
$2^{\Delta Ct}$

2^2=4

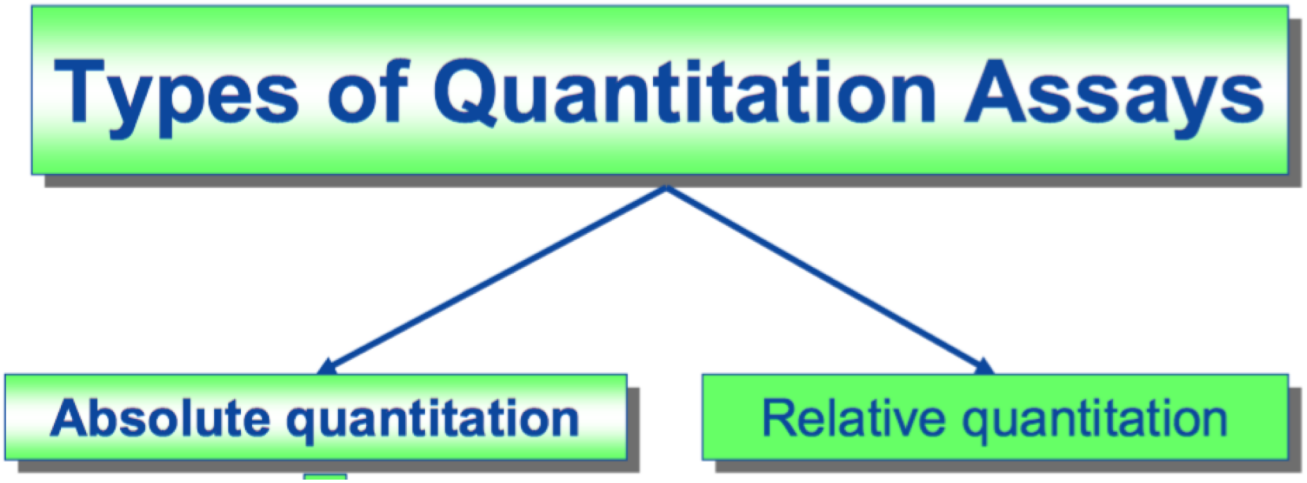
C_t (sample 4) appears 2 cycles later that C_t (sample3)
→ note: in every cycle of PCR the amout of amplified DNA doubles → 2 cycles difference =

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



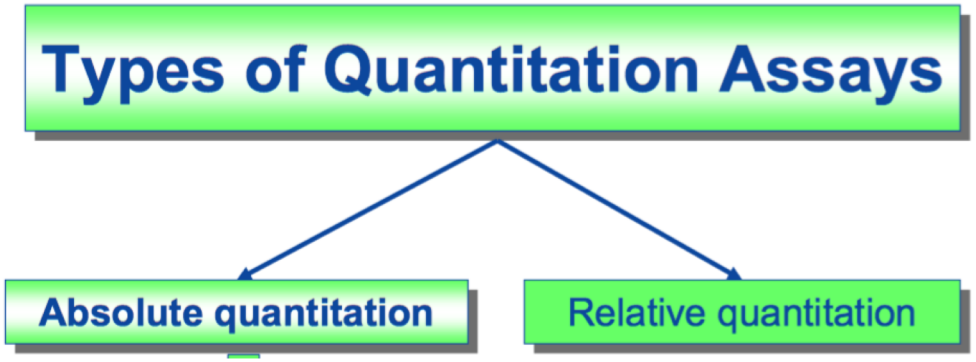
Provides absolute measurement of starting copy number

–**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

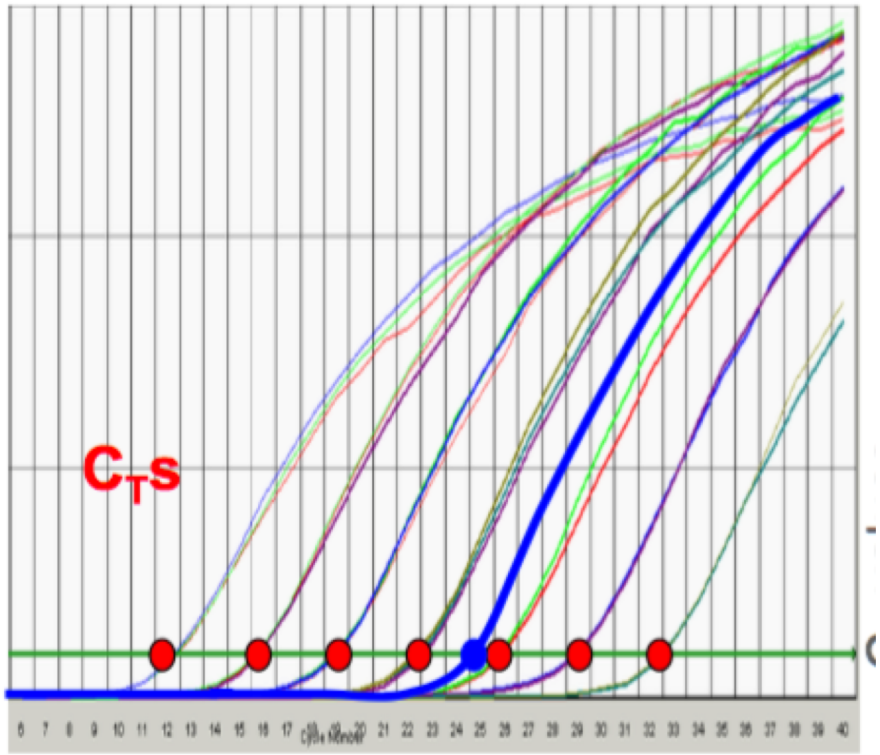
- e.g. Diagnostics: Virus titer in blood: is there virus DNA and how much is there?

Basics for the analysis of real-time PCR data - Quantitative PCR



Quantitative PCR

ABSOLUTE QUANTITATION



Example: determine the number of virus molecules in blood of patient:

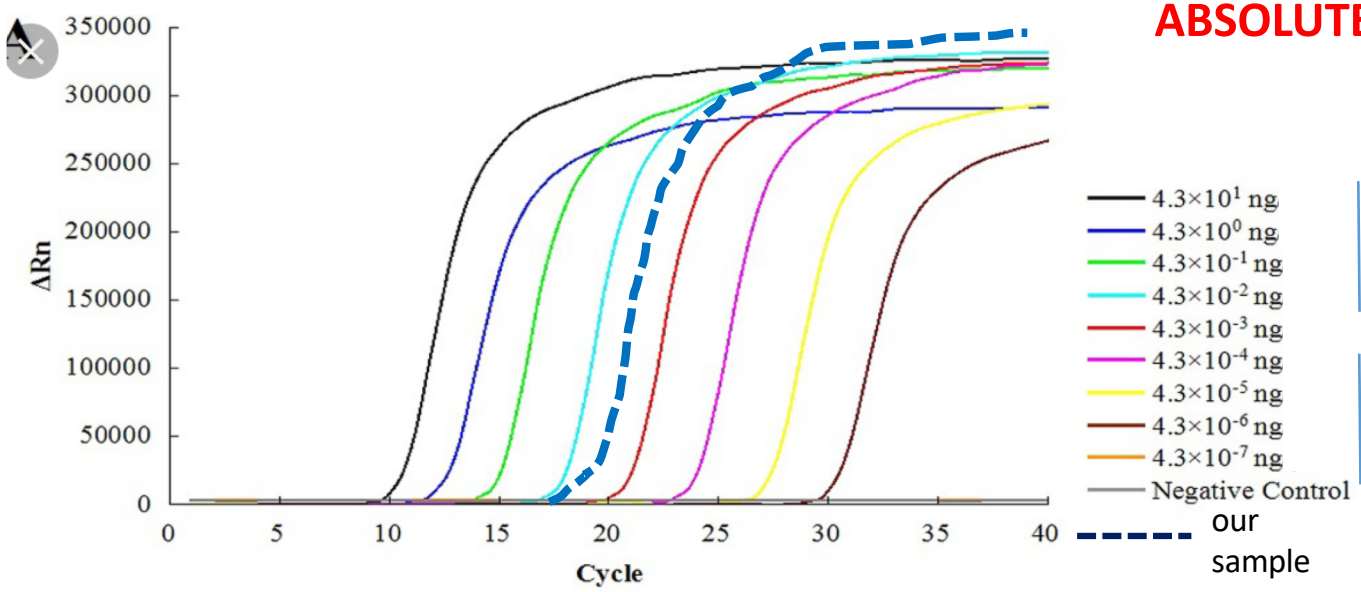
- Prepare DNA from a defined volume of blood (for example 200ul)
- Prepare Standard: serial dillution of target DNA (i.e. relevant segment of viral DNA cloned into a plasmid) concentration is known
- Run PCR with specific primer pairs (in same plate standards and patient sample)
- Analyse amplification blot

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 OF TEMPLATE 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 - Quantitative PCR

ABSOLUTE QUANTITATION



Defined amounts of target site used for PCR (standards)

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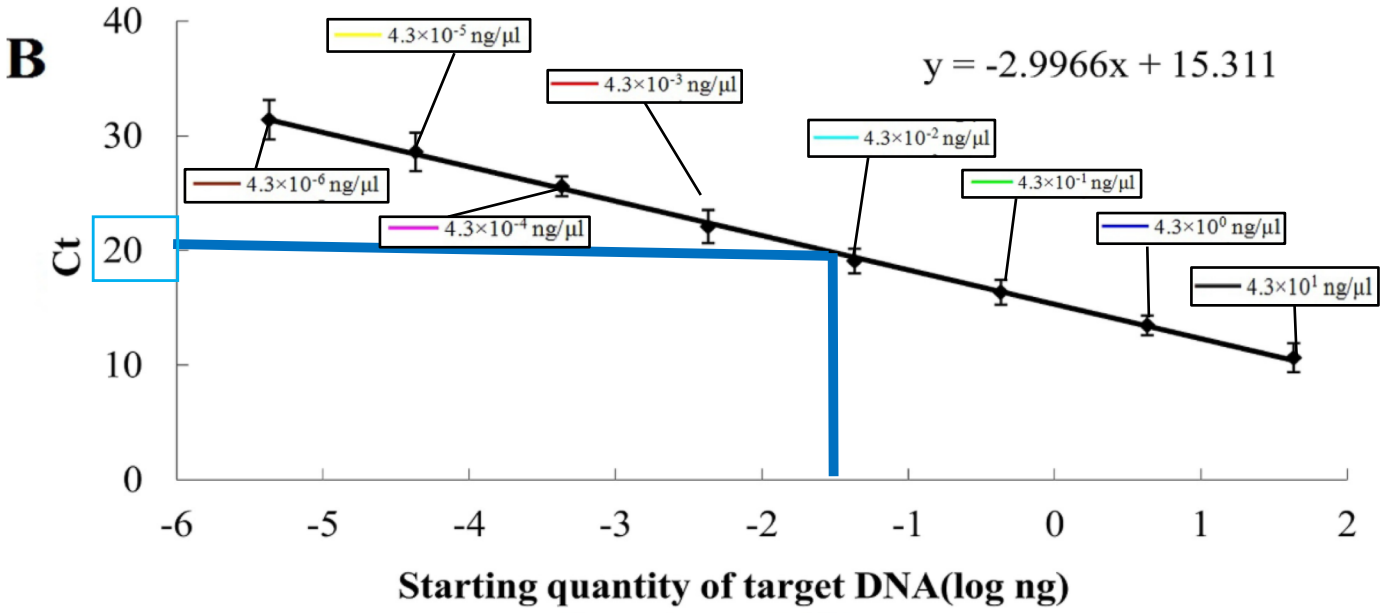
Biological sample C_t=20



C_t of all standards are used in linear regression analysis (x-axis: log ng; y-axis: C_t value)

y=-2,9966x+15.311
x =ng of experimental sample

C_t of Biological sample = 20

20=-2,9966x+15.311

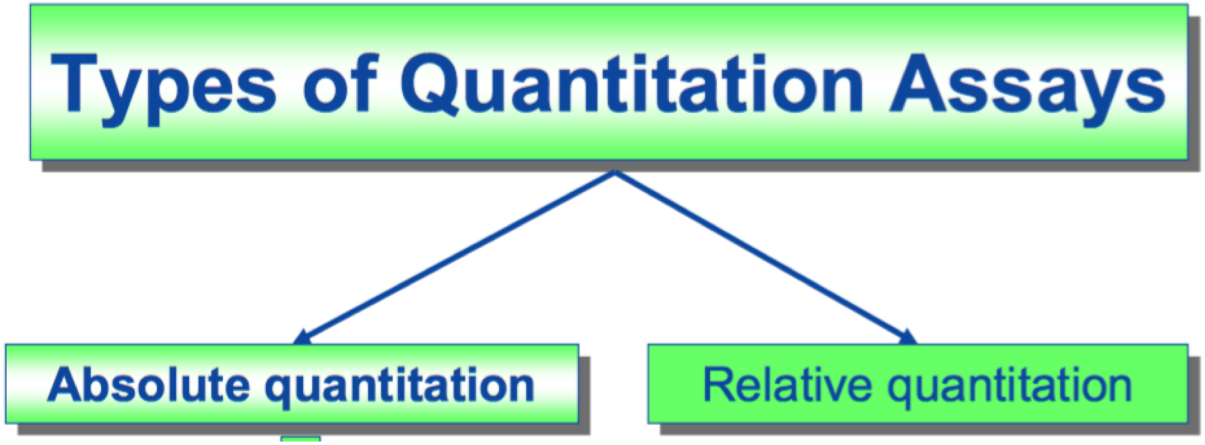
x=(20-15.311)/-2,9966

x=-1,5647 log ng

$x=10^{-1,5647}$ ng

x=0,02724 ng = $2,7x10^{-2}$ ng

Basics for the analysis of real-time PCR data - Quantitative RT-PCR (for gene expression analysis)



RELATIVE QUANTITATION

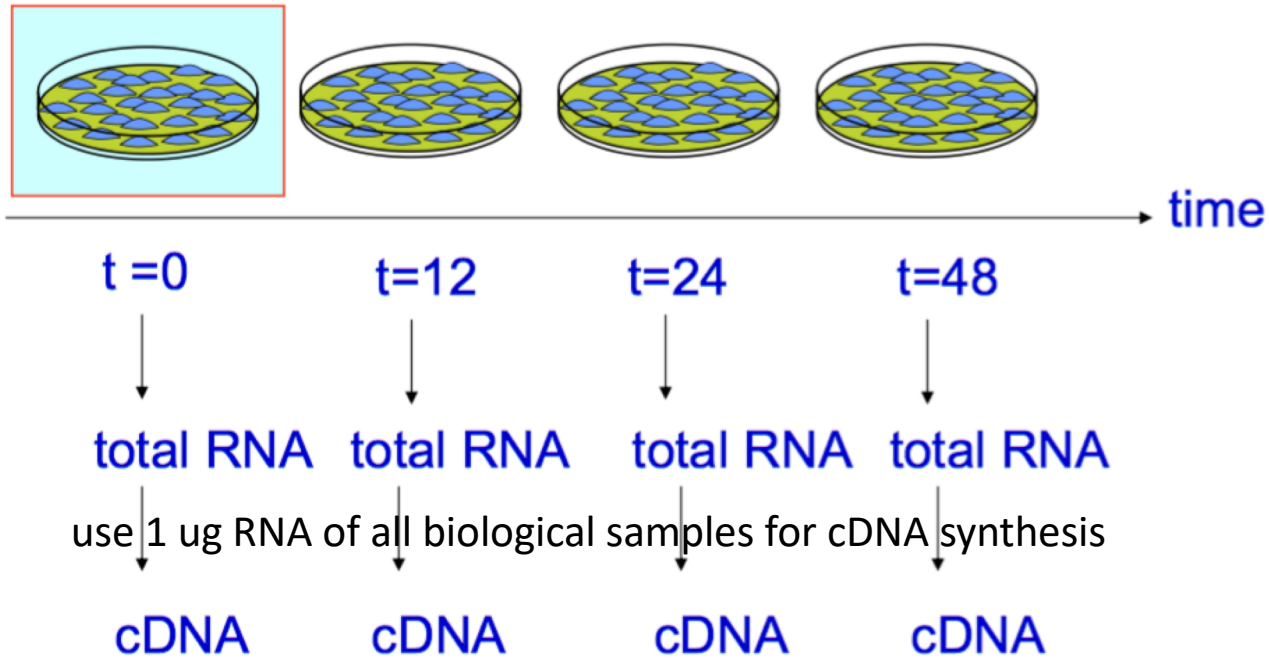
Sample 1: Control
Sample 2: Experimental alteration (drug treatment, knock-down, etc..)
Compare situation Sample 1 with that of Sample 2)

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 - Quantitative RT-PCR (for gene expression analysis)

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



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

RELATIVE QUANTITATION
Sample 1: Control
Sampe 1, 2,…: Experimental alteration (drug treatment, knock-down, etc..)

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

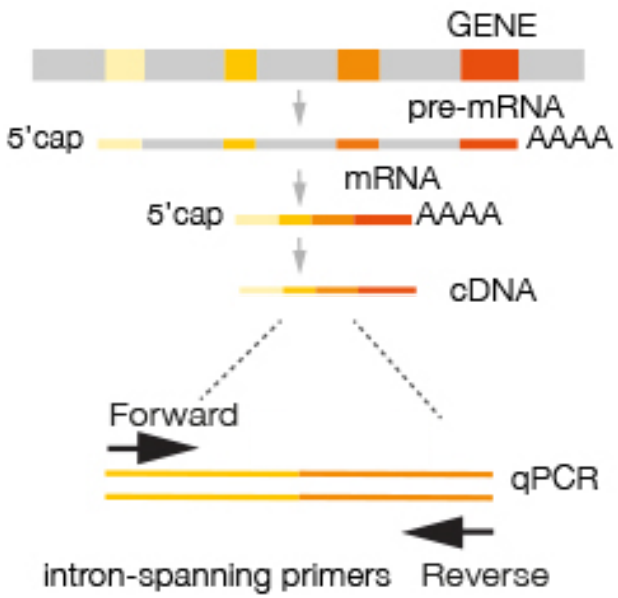
GENE OF INTEREST
(i.e. Hox gene)

REFERENCE GENE THAT IS NOT AFFECTED BY RETINOIC ACID TREATMENT

Semi-quantitative PCR and gene expression analysis

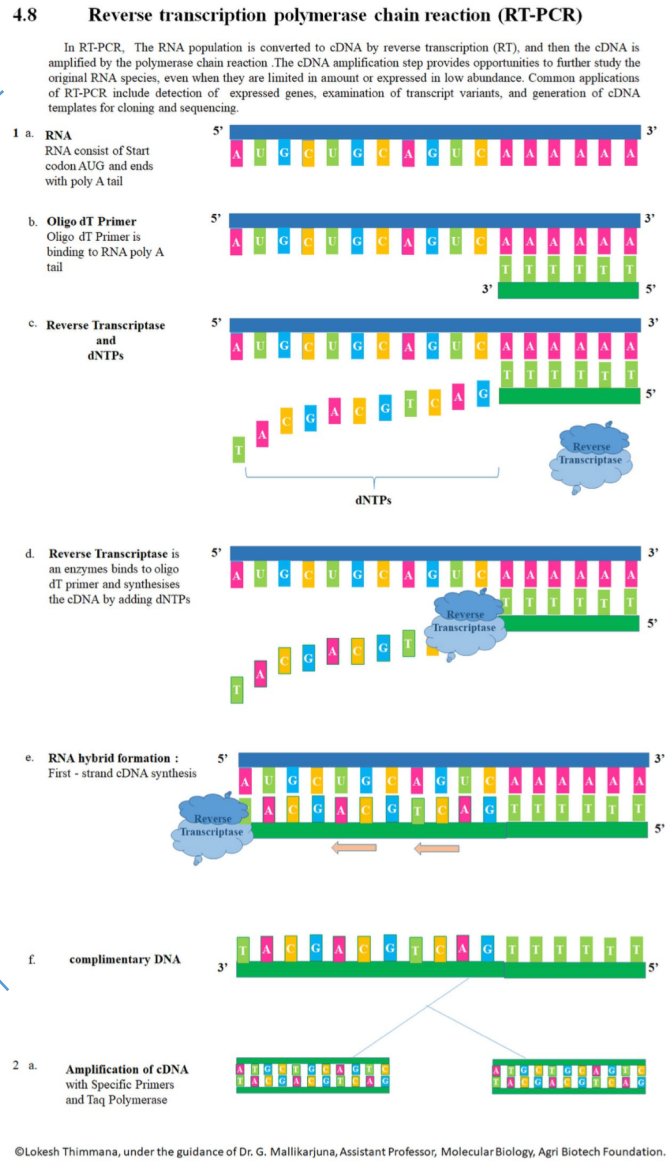
Gene expression analysis

Conversion of mRNA to complementary DNA (cDNA) using primers and reverse tran



Genes that are expressed at high levels result high number of cDNA molecules
Genes that are expressed at low leves
Result low number of cDNA molecules
→ Different template number in PCR reactions
→ Information on gene expression levels

Reverse tanscriptase (obtained from retroviruses)



Oligo-T primers or random oligonucleotide primers (9-mers) are used to prime reverse transcription of mRNA

Complementary DNA (cDNA) synthesis

RNA:cDNA hybrid

Degradation of RNA by RNaseH activity of reverse transcriptase

cDNA used as PCR template

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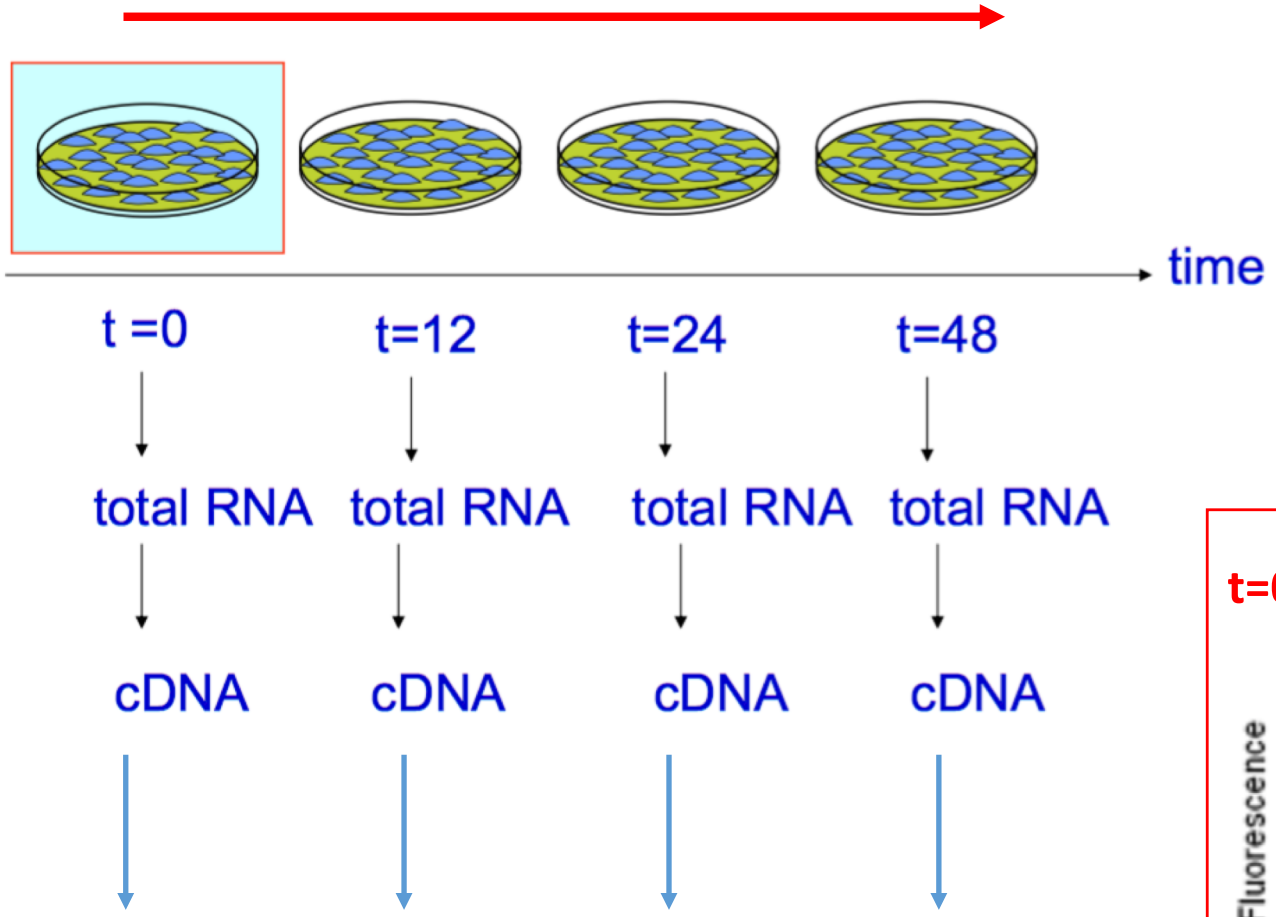
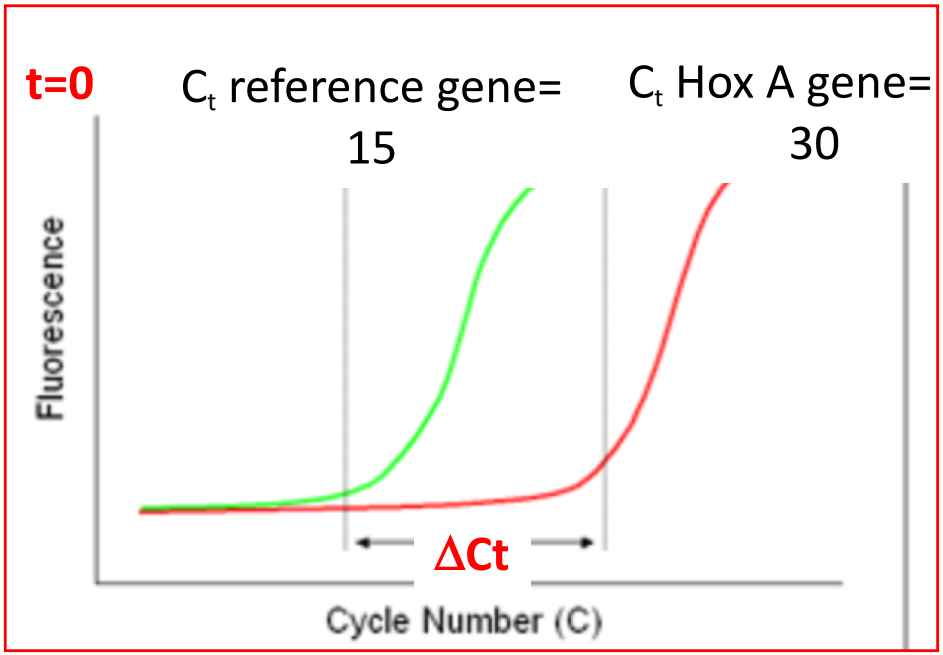


REAL TIME PCR: performed in parallel (t=0 – 48) at the same time on same plate.
Very important: precise pipetting!

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**



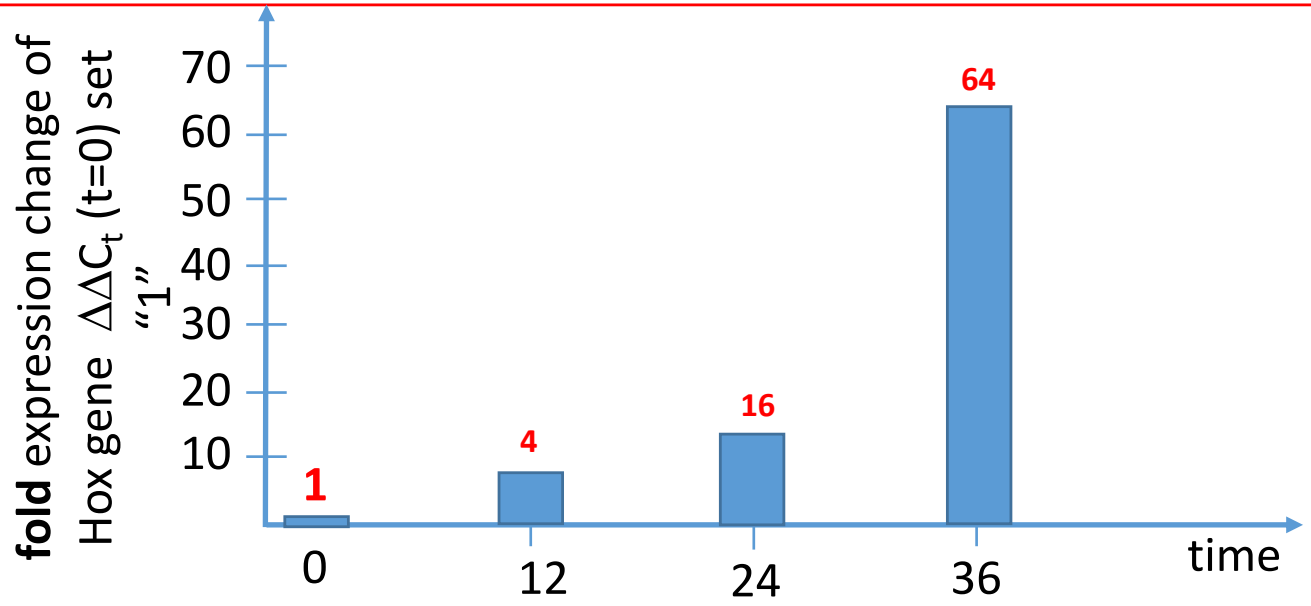
Basics for the analysis of real-time PCR data: C_t and ΔC_t and $\Delta\Delta C_t$

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

$\Delta\Delta C_t$

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

Higher expression when compared to t=0



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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

We assume a scenario where in one of the samples was not processed correctly during RNA preparation of cDNA syntehsis:

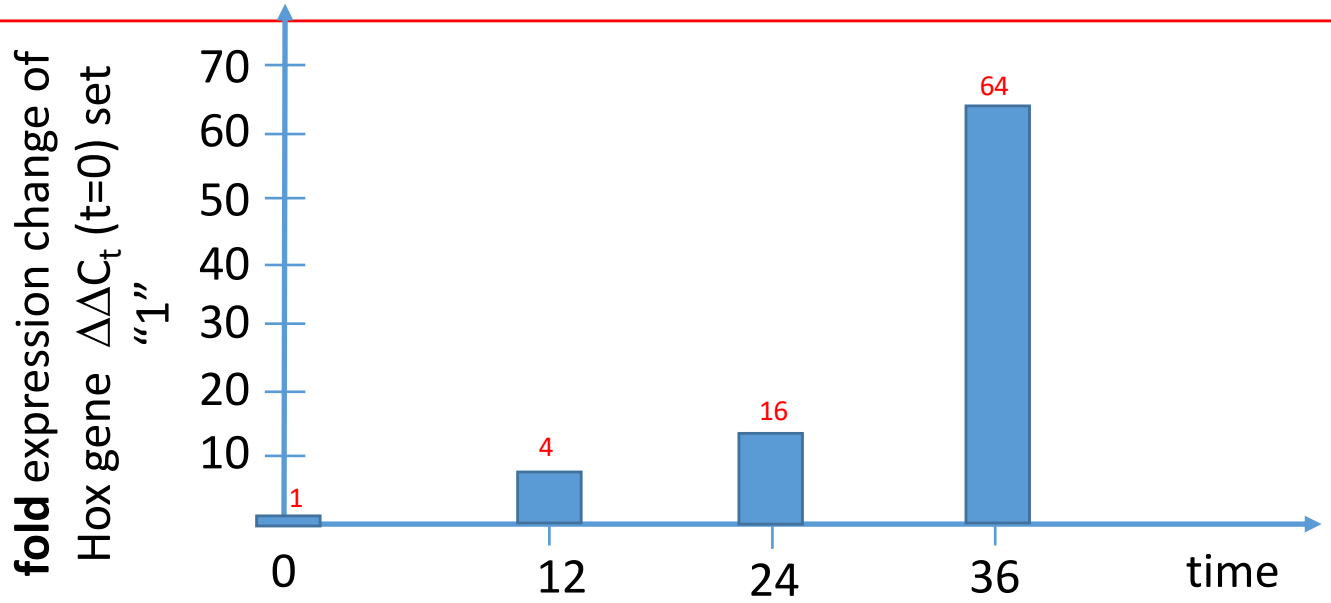
t=24:reduced cDNA levels in sample (Ct of reference =17; not 15), when compared to t=0, 12,36

→ higher Ct for reference **but also Hox gene**

→ Thus, 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, developmental biology, genetic disease….
  - Functional experiments: i.e. knock down of relevant gene → alteration of gene expression; i.e. change of condition of envirnoment → alteration of gene expression
- **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