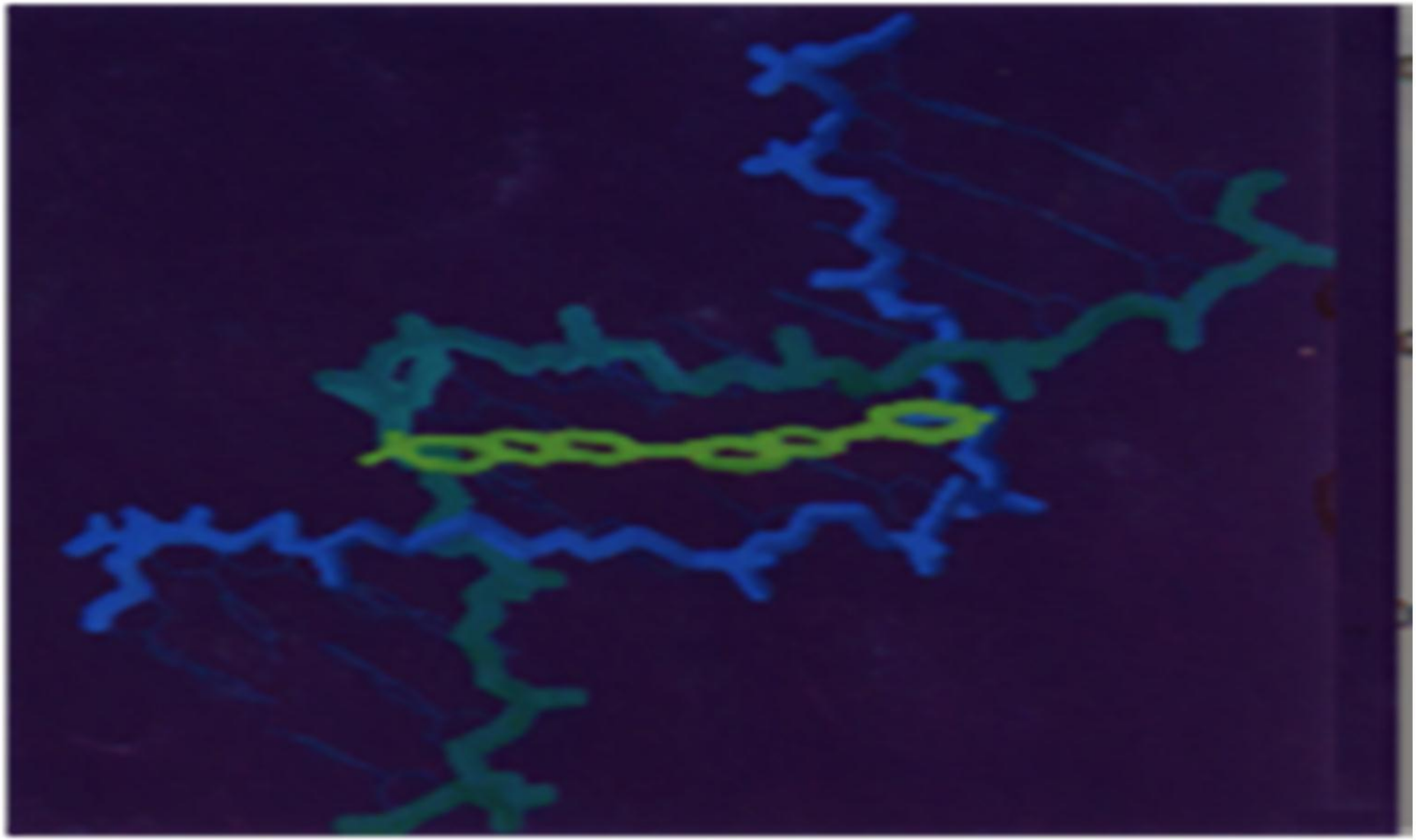
REAL TIME PCR

SALWA HASSAN TEAMA



Contents

- Gene Quantitation
- Principles of Real-Time Quantitative PCR Techniques
- Quantitation Assay
- Application in Molecular Diagnostics

Gene Quantitation

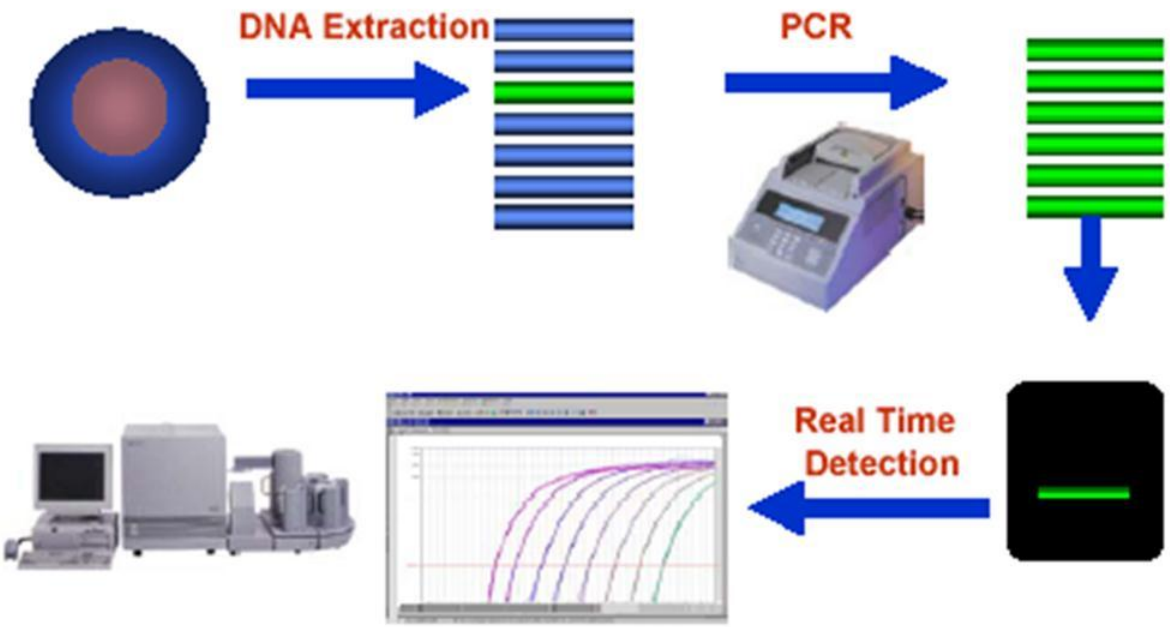
- Gene quantitation achieves an accurate estimation of DNA and RNA targets.

- Monitoring of DNA/RNA amplification reaction:
- Endpoint analysis
- Real-Time analysis

Detection Strategies

End Point	Real Time
▪ DNA/RNA amplification reaction is monitored after completion of thermocycling ▪ Useful for +/- studies ▪ Does not provide a good measure of the starting number of DNA targets. ▪ Allelic discrimination	▪ Accurate quantitation ▪ Reproducible data ▪ Ct calculated

Real Time PCR



Traditional PCR has advanced from detection at the end-point of the reaction to detection while the reaction is occurring (Real-Time).

Disadvantages of Traditional PCR

- Poor Precision
- Low sensitivity
- Short dynamic range < 2 logs
- Low resolution
- Non-Automated
- Size-based discrimination only
- Results are not expressed as numbers
- Ethidium bromide for staining is not very quantitative
- Post-PCR processing

Advantages of Real-Time PCR

- Increased dynamic range of detection
- High technical sensitivity
- High precision
- No post-PCR processing
- Detection is capable down to a 2-fold change
- Collects data in the exponential growth phase of PCR
- An increase in reporter fluorescent signal is directly proportional to the number of amplicons generated
- Minimize risk of cross contamination.

REAL-TIME PCR

Advantage	Wide dynamic range of quantification(7-8 log decades)
	High technical sensitivity (<5 copies)
	High precision (<2% SD)
	No post PCR steps, thus minimized risk of cross contamination
	High throughput
	Multiplex approach possible.
Limitation	PCR product increases exponentially
	Variation increases with cycle number
	Increased variation after transformation to linear values
	Overlap of emission spectra
	Maximal four simultaneous reaction
	Increased risk of false negative results

Real-Time PCR

DNA amplification analysis is monitored simultaneously over the course of thermocycling, the amplification product is detected as it accumulate.

Real-time PCR quantitates the initial amount of the template most specifically, sensitively.

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time). The higher the starting copy number of the nucleic acid target, the sooner significant increase in fluorescence is observed.

Real-Time PCR Protocol

Sample ← Blood, Serum, Plasma, CSF, Pleural fluid, Ascites, Urine, Stool, Pus, Sputum, Swabs, Bronchial lavage, Tissue, FNAC *etc.*

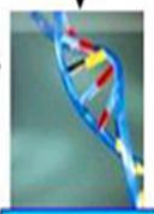
DNA extraction

Addition of premix reagents

Automated amplification +

- SNP
- Allelic discrimination
- Detection
- Quantification
- Genotyping
- Mutation detection

Premix (Primers, probes, Enzymes, dNTPS, PCR Buffer buffers enhancers, etc) + Mg solution + IC (internal control gene)



Template

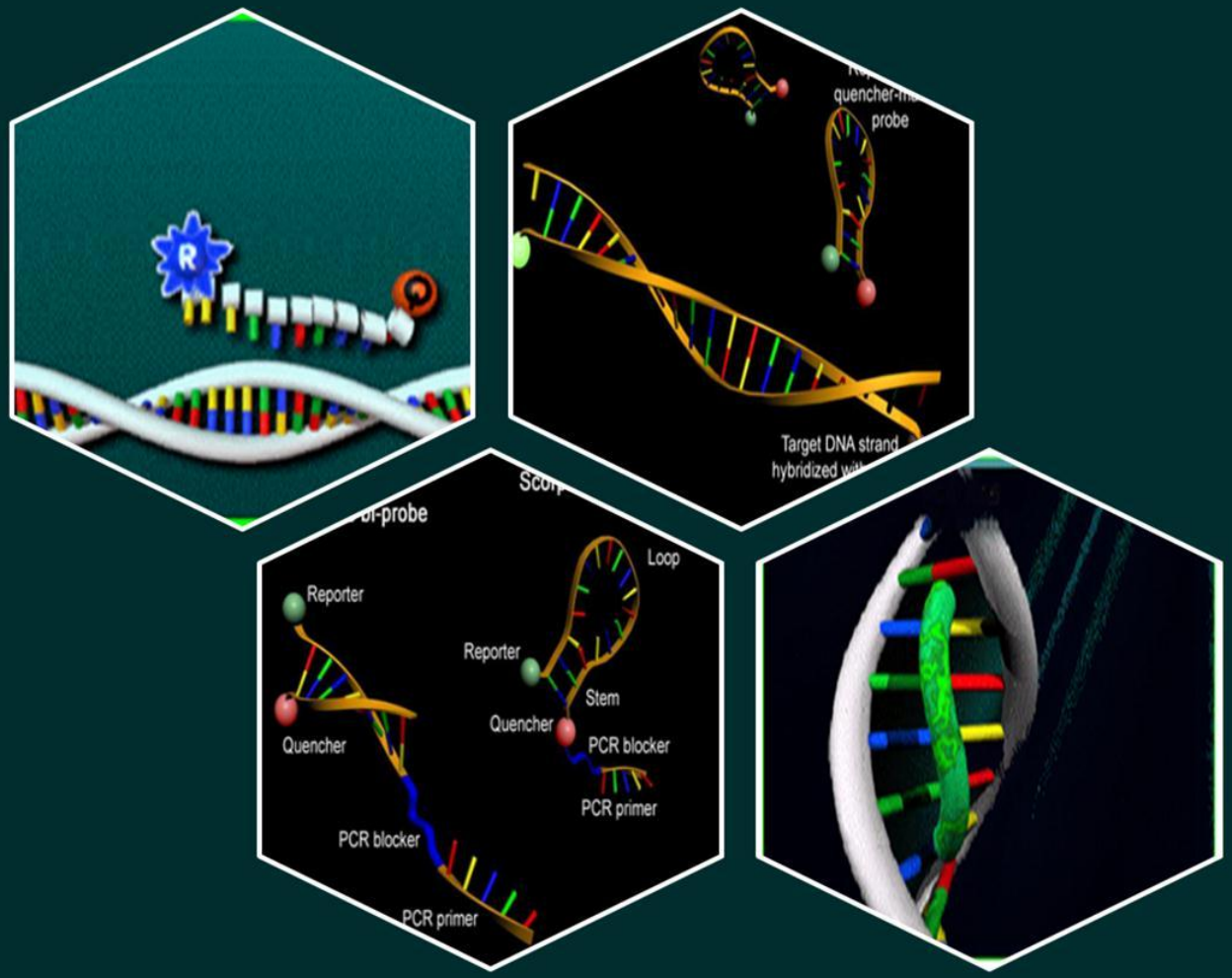
Real Time PCR Machine For Amplification

PCR Chemistries

- Intercalator based PCR, The simplest and cheapest principle is based on interaction of double stranded DNA binding dyes.

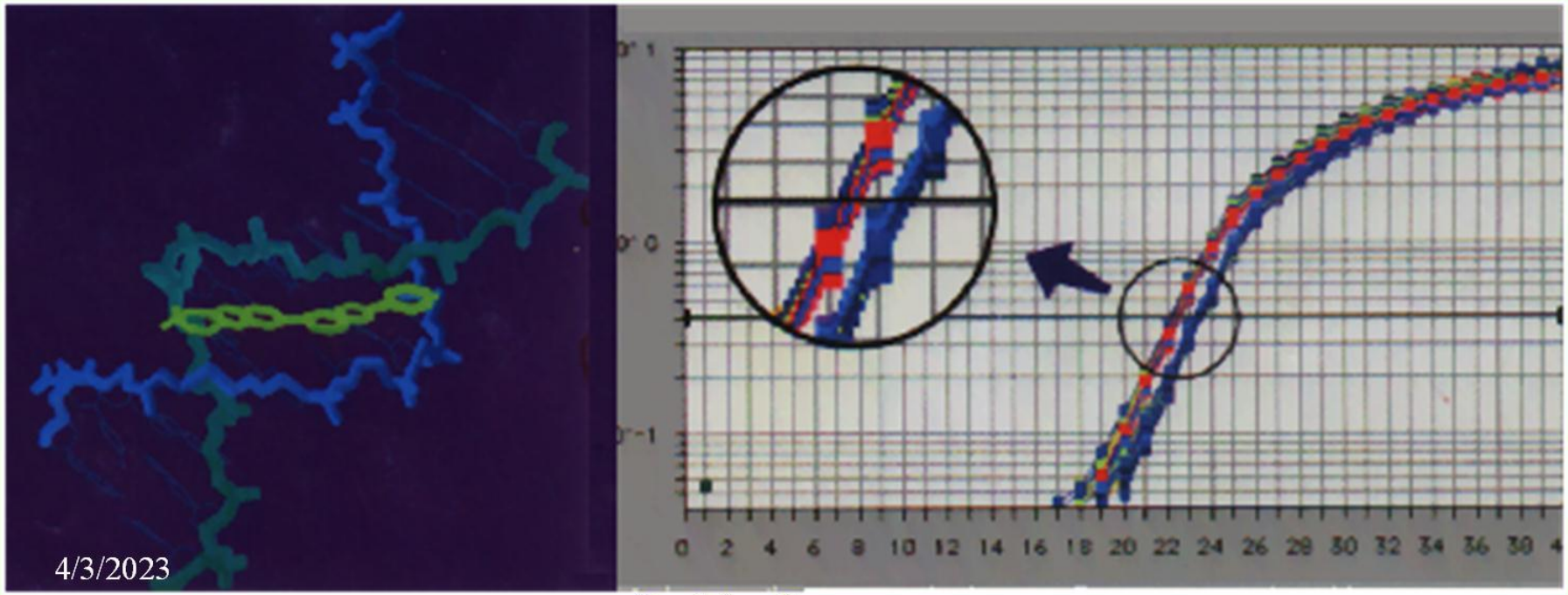
- Probe based PCR, The principles are based on the introduction of an additional fluorescence labeled oligonucleotide. Sufficient amount of fluorescence are only released either after cleavage of the probe (hydrolysis probes) or during hybridization of one (molecular beacon) or two (hybridization probes). (Ref. Trends in Molecular Medicine vol. 8 No.6 June 2002).

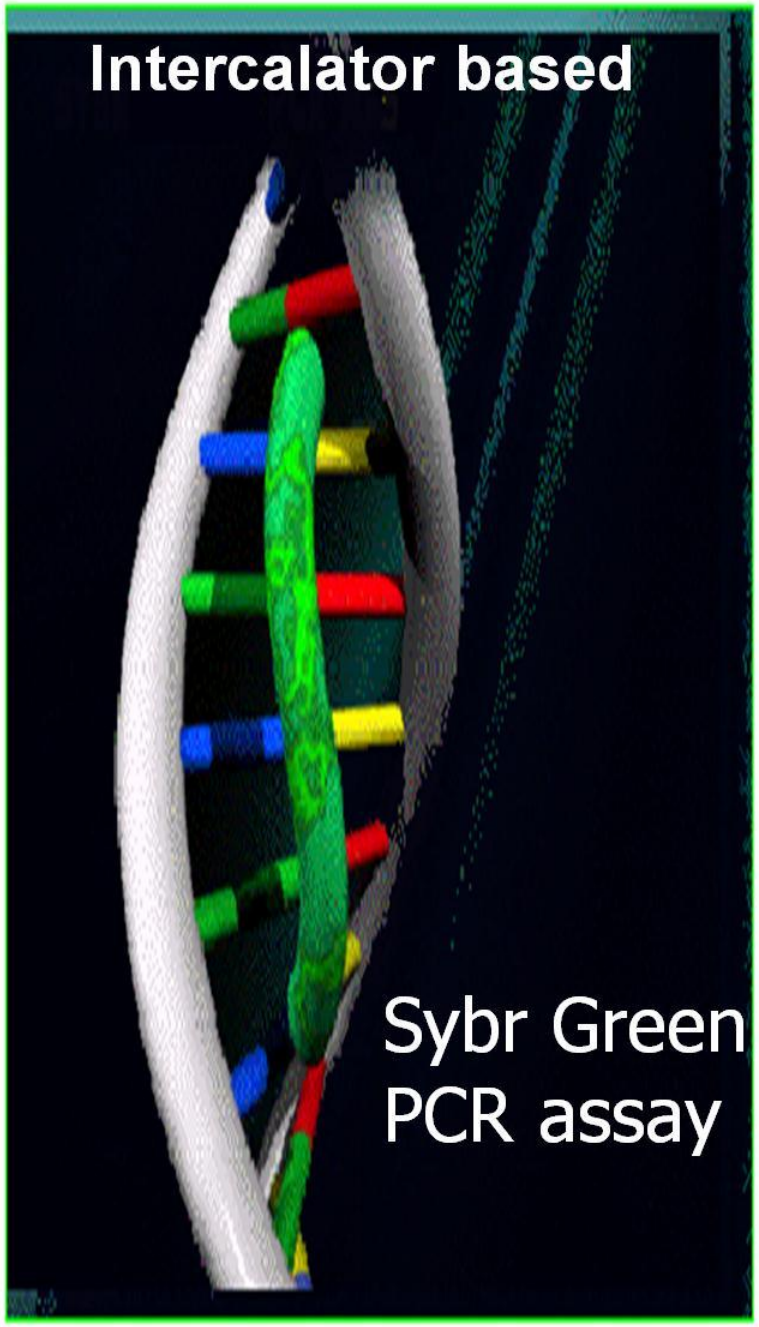
Principles of Real-Time Quantitative PCR Techniques



Source:Wikipedia
Source: Applied Biosystem

All technologies are based on the measurement of florescence during the PCR. The amount of emitted fluorescence is proportional to the amount of PCR product and enables the monitoring of the PCR reaction. The resulting PCR curve is used to define the exponential phase of the reaction, which is a prerequisite for accurate calculation of the initial copy number at the beginning of the reaction.





- SG is a DNA binding dye that insert itself into dsDNA. When SG added to the sample it immediately binds with all dsDNA present in the reaction including any unwanted amplicons.

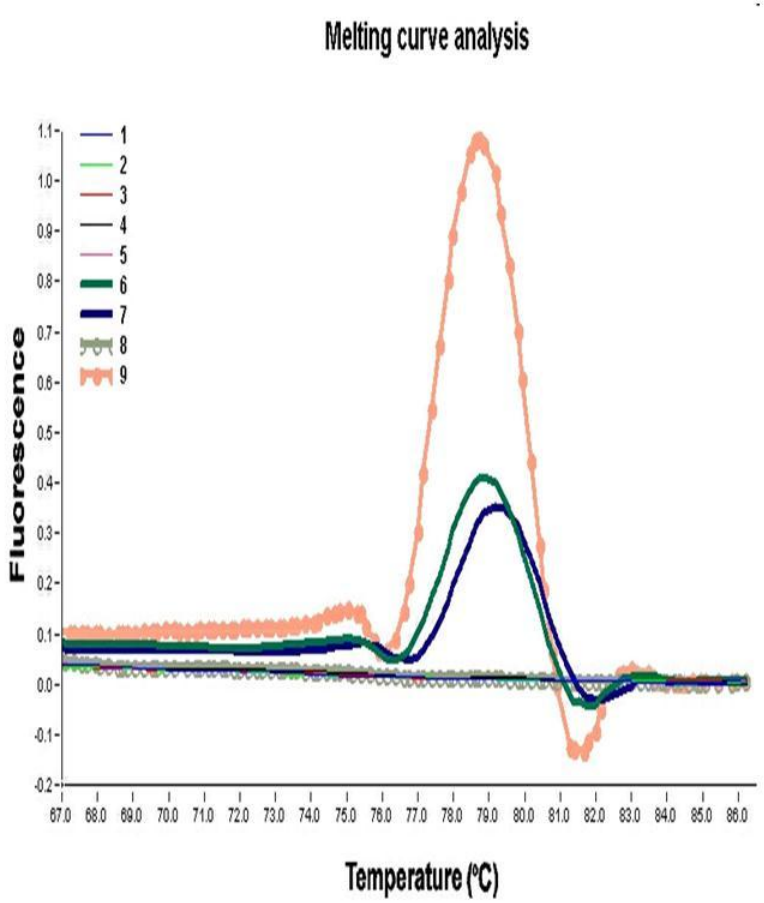
- SG binds to each new copy of dsDNA increase in copies yield increase in fluorescence which read by sequence detector on the system.

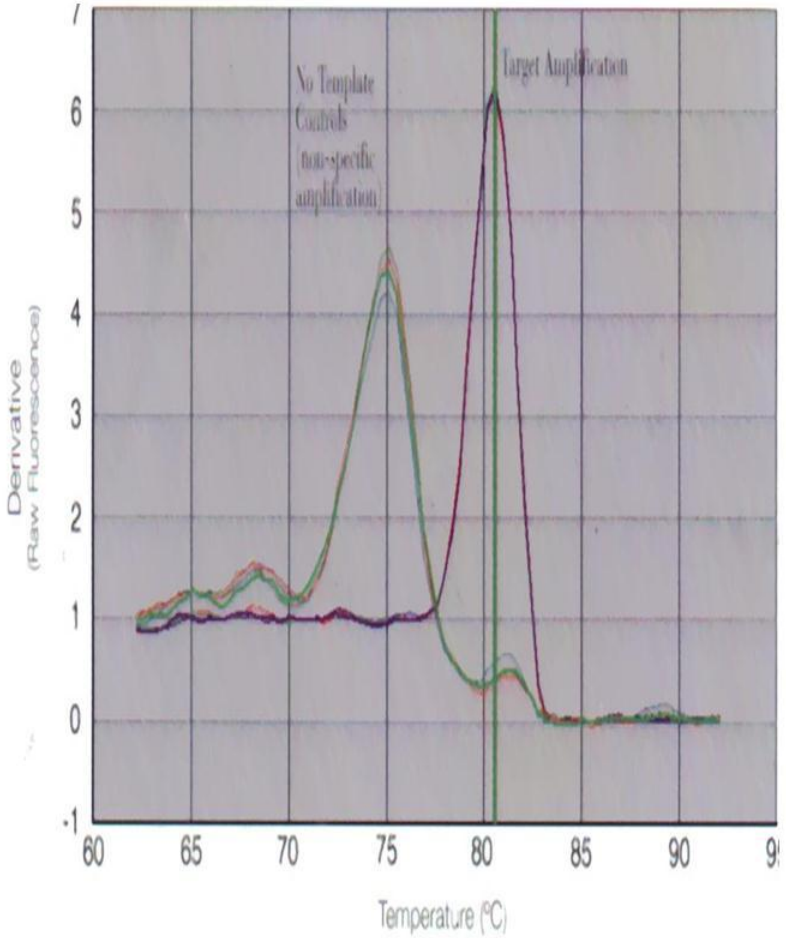
Dissociation Graph

- **However, Sybr Green PCR assay both specific and non specific PCR products are both detected, therefore the assay require careful optimization of the PCR conditions and clear differentiation between specific and nonspecific PCR products using melting curve analysis (Dissociation Graph).**

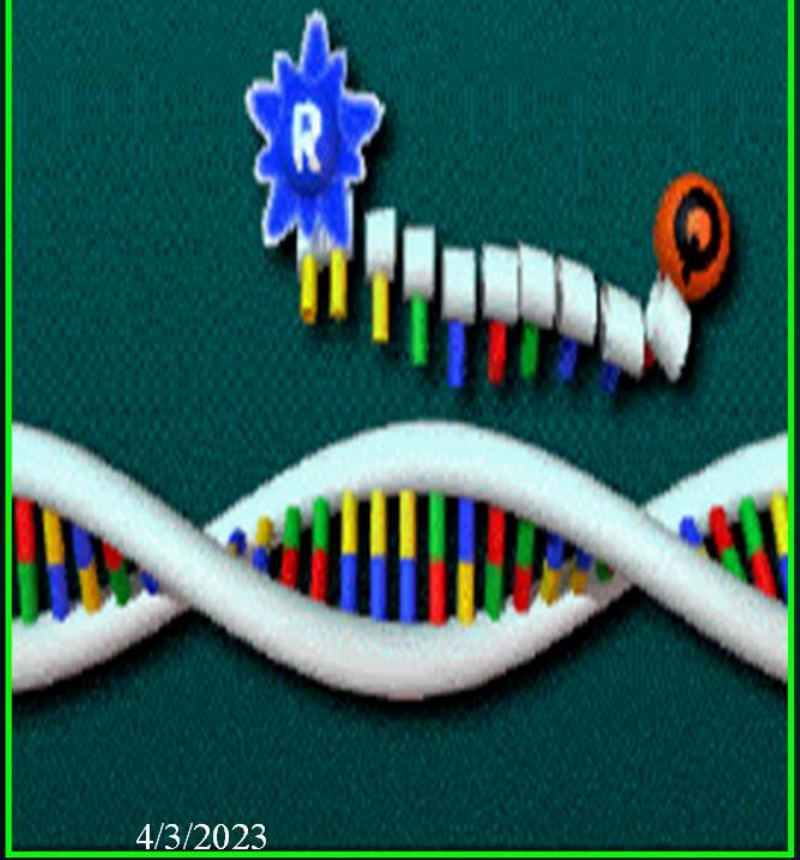
- Dissociation Protocol can be added to the thermal cycling parameters, allows detection of non-specific products

Dissociation Curve





TaqMan assay



TaqMan assay use flourogenic probe to detect RCR reactions. These probe emit fluorescence when they cleaved from copied DNA strand.

During denaturation phase of PCR, the two DNA strand are split apart. Next, the probe and primer anneal themselves to the single strands and DNA polymerase enzyme complete the polymerzation.

TaqMan Primers and Probes

TaqMan Primer

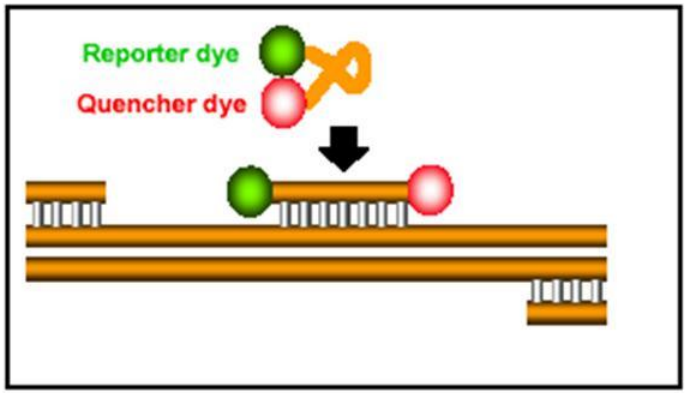
- * Equal Tm (58-60 °C)
- * 15-30 bases in length
- * G+C content 30-80%
- * No runs of four or more Gs (any nucleotide)
- * No more than two G+C at the 3’ end
- * No G at the 5' end
- * Amplicon size 50-150 bp (max 400)
- * span exon-exon junctions in cDNA

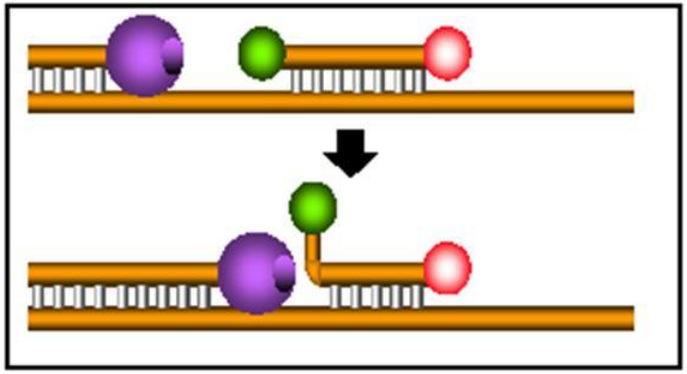
TaqMan Probes

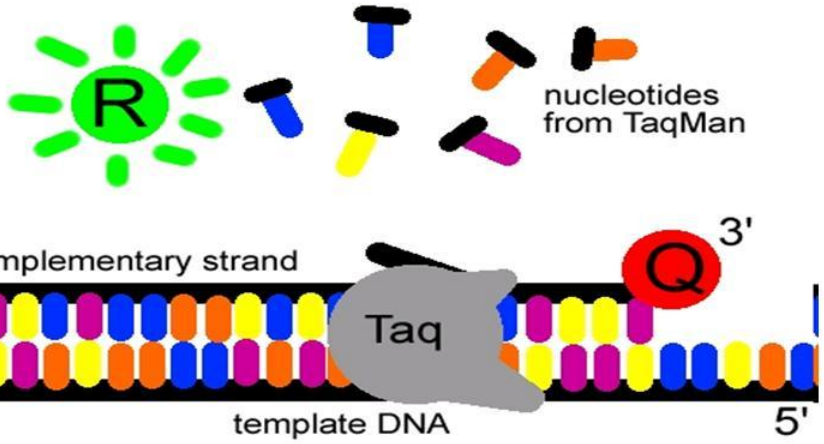
- *Tm value 10 C higher than primers
- * Runs of identical nucleotides (no consecutive Gs)
- * G+C content 30-80%
- * More Cs than Gs
- * No G at the 5' end

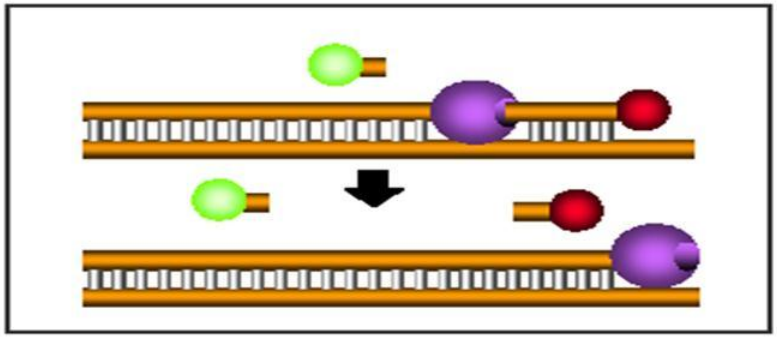
Target specific probe

- 5' reporter and 3' quencher
  - Reporters: FAM,TET,VIC,JOE
  - Quenchers: TAMRA, MGB.
- The probe conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact.

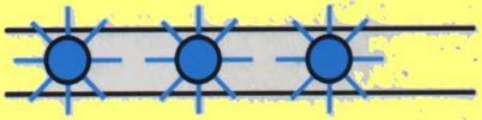




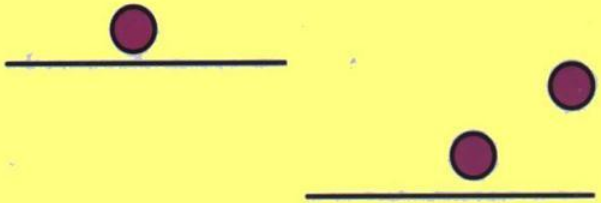




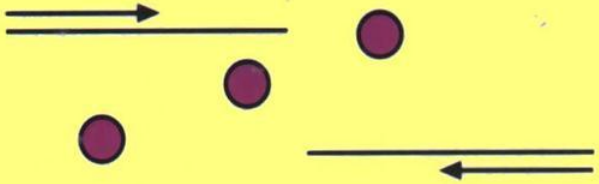
The probe sits in the path of the enzyme as it starts to copy DNA or cDNA. When the enzyme reaches the annealed probe the 5` exonuclease activity of the enzyme cleaves the probe, this separates the reporter dye from the quencher dye, generating an increase in the reporter dye's fluorescence intensity.



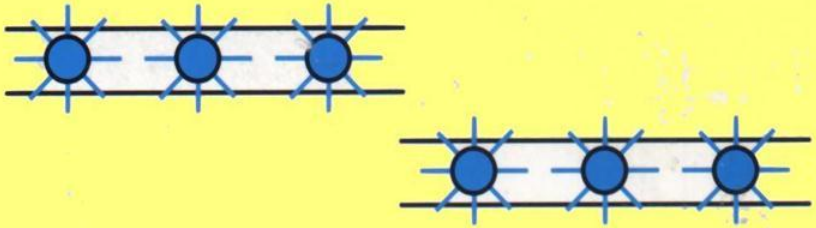
SYBR Green 1 dye fluoresces when bound to double-stranded DNA.



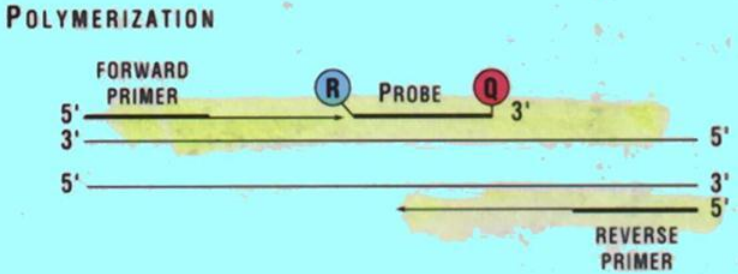
When DNA is denatured, the SYBR Green 1 dye is released and fluorescence is drastically reduced.



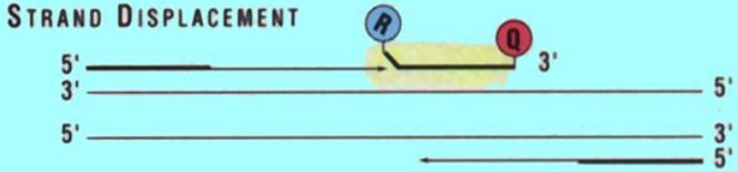
During extension phase, primers anneal and PCR product is generated.



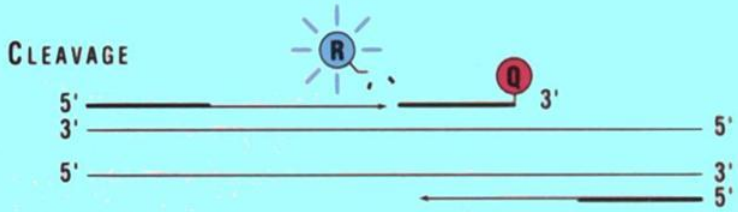
Polymerization is complete and SYBR Green 1 dye binds, resulting in a net increase in fluorescence.



The reporter (R) and the quencher (Q) dyes are attached to the probe.



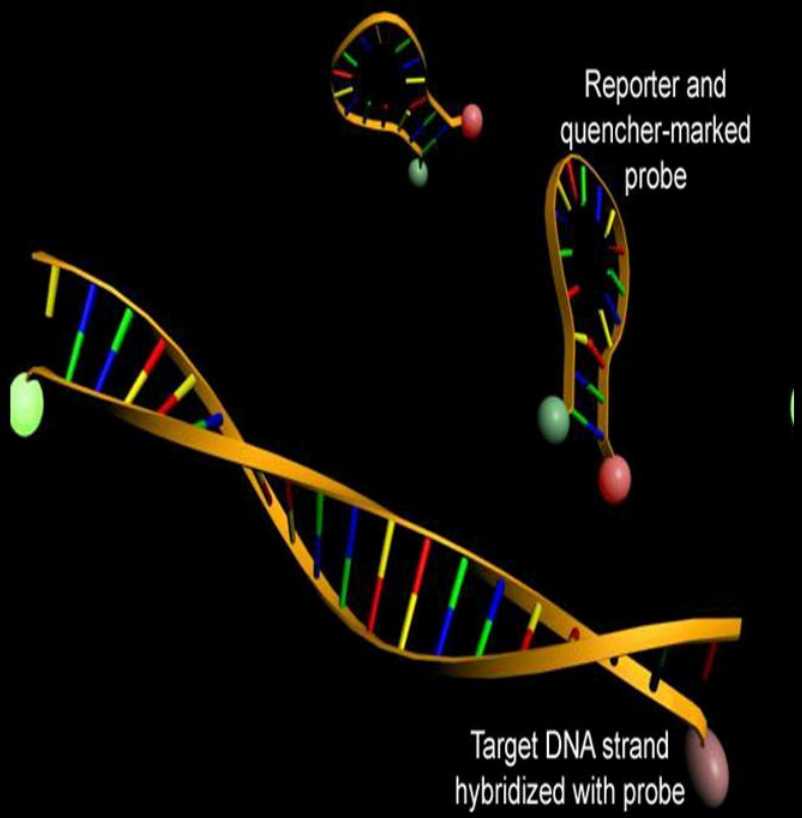
When both dyes are attached to the probe, reporter dye emission is quenched.



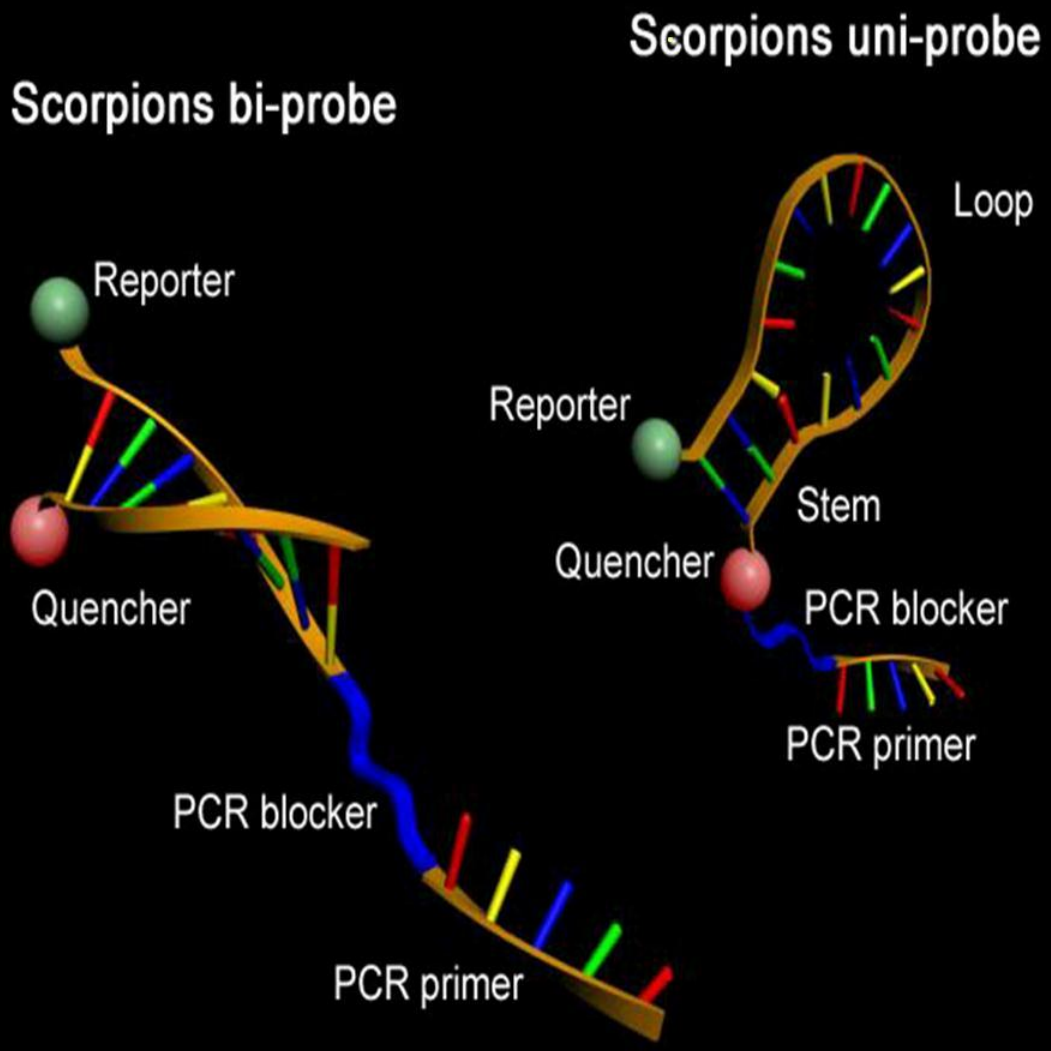
During extension, DNA polymerase cleaves the reporter dye from the probe.

Sybr Green PCR	
Advantage	Inexpensive, easy to use, and sensitive. No probe is required, which reduces assay setup and running costs.
Disadvantage	Sybr Green PCR assay both specific and non specific PCR products are both detected, therefore the assay require careful optimization of the PCR conditions and clear differentiation between specific and nonspecific PCR products using melting curve analysis (Dissociation Graph).
TaqMan Assay	
Advantage	TaqMan probes add specificity to a PCR reaction. Non specific amplification due to mis-priming or primer dimer artifact does not generate signal. Allow the development of multiplex reaction.

Molecular beacons



Molecular beacons do not fluoresce when they are free in solution. However, when they hybridize to a nucleic acid strand containing a target sequence they undergo a conformational change that enables them to fluoresce brightly.


Scorpions bi-probe
Reporter
Quencher
PCR blocker
PCR primer
Scorpions uni-probe
Loop
Reporter
Stem
Quencher
PCR blocker
PCR primer

FRET (Fluorescent Resonance Energy Transfer)

- This technology is utilized in the 5`nuclease assay. The principle is that when a high-energy dye is in close proximity to a low-energy dye, there will be a transfer of energy from high to low.

- The TaqMan Probe is designed with a high-energy dye termed a Reporter at the 5` end, and a low-energy molecule termed a Quencher at the 3` end. When this probe is intact , the Reporter dye's emission is suppressed by the Quencher dye as a result of the close proximity of the dyes, When the probe is cleaved by the 5` nuclease activity of the enzyme, the distance between the Reporter and the Quencher increases causing the transfer of energy to stop. The fluorescent emissions of the reporter increase and the quencher decrease.

Quantitative Assay

- It measures (quantitates) the amount of a nucleic acid target during each amplification cycle of the PCR.

- The target may be:
- DNA,
- cDNA, or
- RNA.

How Real-Time PCR Quantitation Assays Work

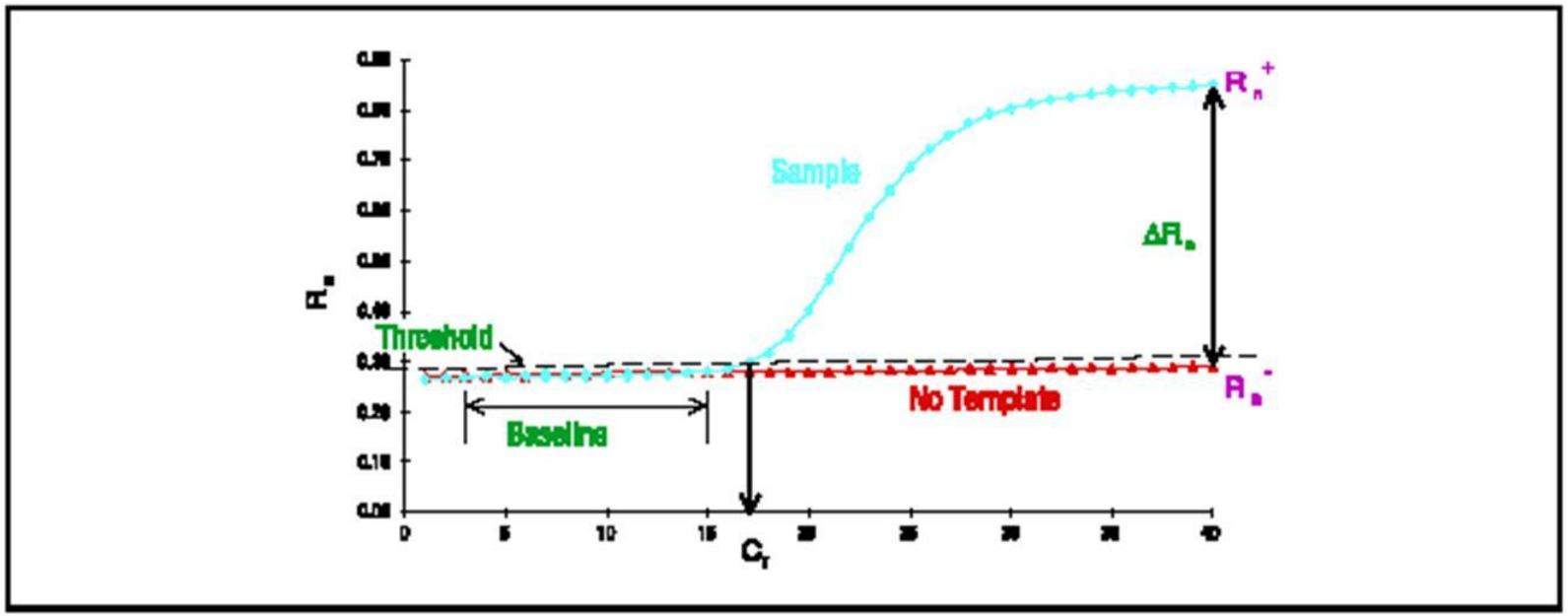


Figure 2. Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR.

In **the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline. For the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated target. A fixed fluorescence threshold can be set above the baseline. The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.**

A Quantitation Assay

•It measures (quantitates) the amount of a nucleic acid target during each amplification cycle of the PCR. The target may be DNA, cDNA, or RNA.

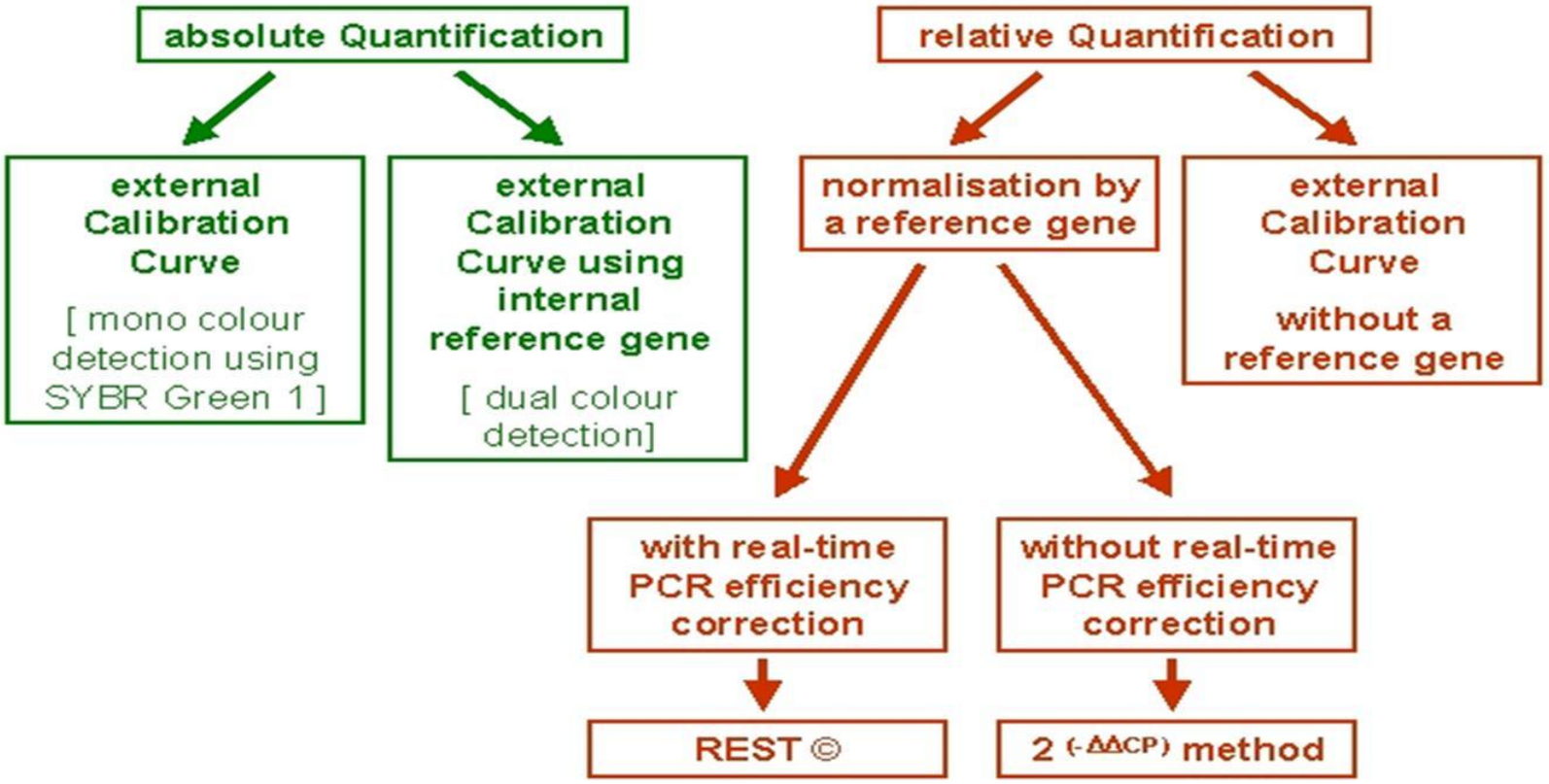
There are three types of Quantitation Assays

•• DNA/cDNA quantitation

•• RNA quantitation using one-step reverse transcription polymerase chain reaction (RT-PCR)

•• RNA quantitation using two-step RT-PCR

Quantification Strategies in Real Time RT-PCR



Quantification strategies in real-time RT-PCR

Absolute quantification using a calibration curve:

- recombinant DNA (recDNA) calibration curve *(Pfaffl & Hageleit, Biotechnol.Lett. 2001)*
- recombinant RNA (recRNA) calibration curve *(Pfaffl & Hageleit, Biotechnol.Lett. 2001)*
- calibration curve using a synthetic oligo-nucleotide *(Bustin, JME 2000)*
- calibration curve using a purified RT-PCR product

Relative quantification versus a reference gene

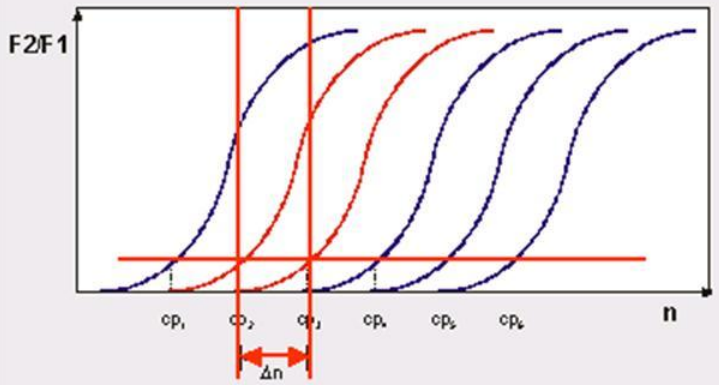
(housekeeping gene):

$$\text{relative expression} = \frac{E_{target}^{\Delta CP_{target}\,(control\,-\,sample)}}{E_{reference}^{\Delta CP_{ref}\,(control\,-\,sample)}}$$

Pfaffl, Nucleic Acids Research 2001

Calculation of real-time PCR efficiency

$E = 10^{-1/slope}$ => $E = 10^{-1/-3.337}$ => $E = 10^{0.299}$ => $E = 1.99$



$N_2 = N_{0,2} \times E^{n_2}$ $N_3 = N_{0,3} \times E^{n_3}$

For $N_2 = N_3$:

$N_{0,2} / N_{0,3} = E^{(n_3 - n_2)} = E^{\Delta n}$

For $N_{02} = 10 \times N_{0,3}$ (10-fold dilutions):

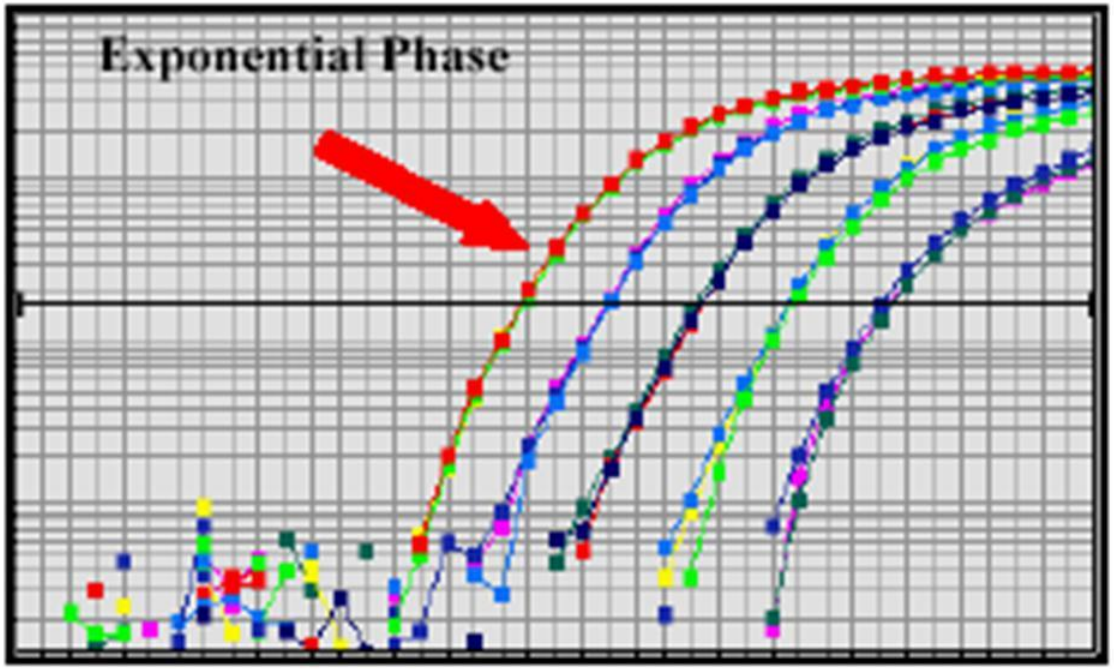
$\Delta n = 1 / \log E$ $E = 10^{-1/slope}$

e.g. E = 2.0 Δn = 3.32
E = 1.9 Δn = 3.58
E = 1.8 Δn = 3.91

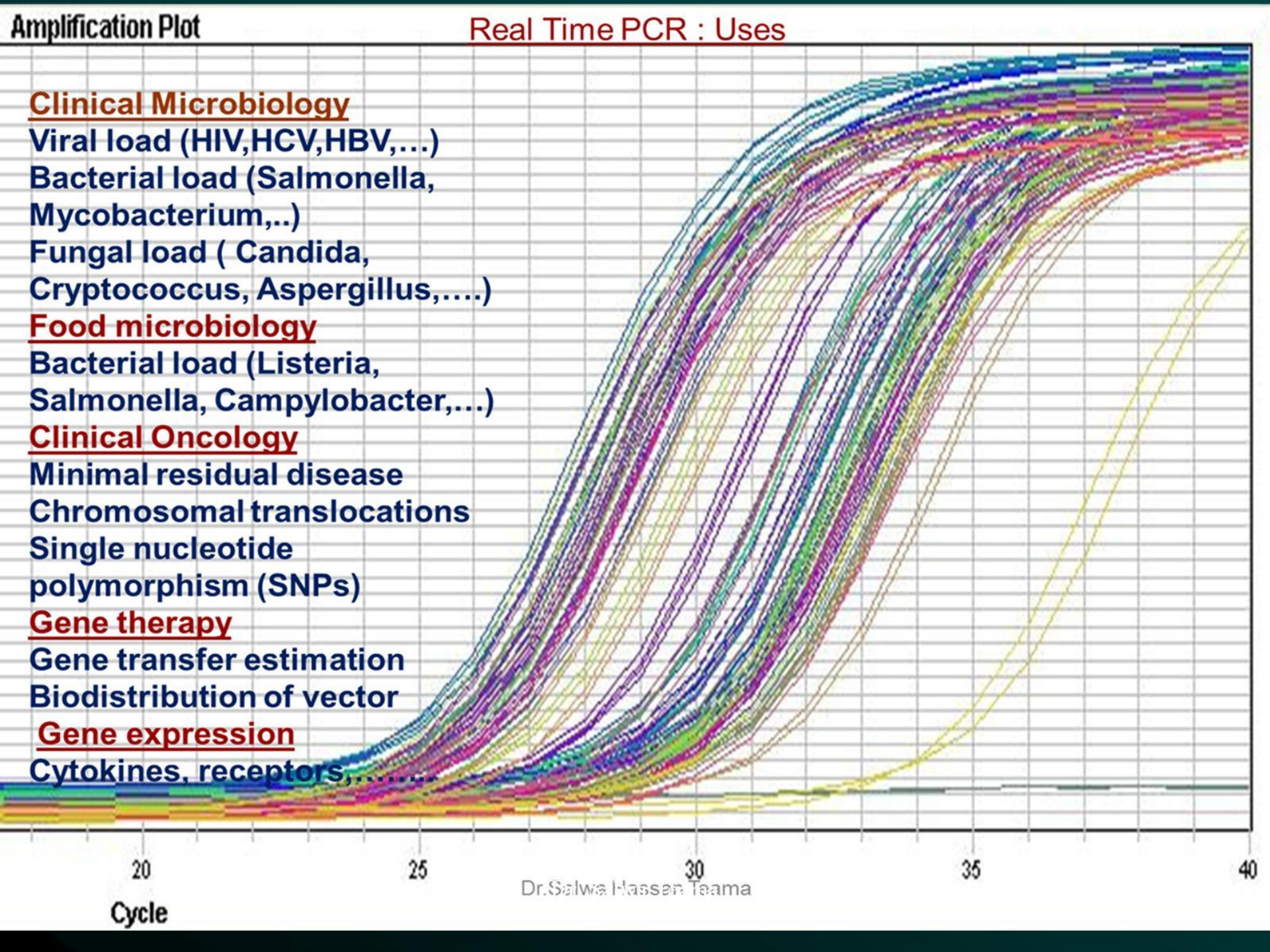
Roche Diagnostics, LC rel. Quantification software, March 2001

Rasmussen, R (2001) *Quantification on the LightCycler. In: Meuer, S, Wittwer, C, Nakagawara, K, eds. Rapid Cycle Real-time PCR, Methods and Applications Springer Press, Heidelberg; page 21-34*

Amplification Curve



Real Time PCR : Uses



Clinical Microbiology

Viral load (HIV,HCV,HBV,…)

Bacterial load (Salmonella, Mycobacterium,..)

Fungal load (Candida, Cryptococcus, Aspergillus,….)

Food microbiology

Bacterial load (Listeria, Salmonella, Campylobacter,…)

Clinical Oncology

Minimal residual disease

Chromosomal translocations

Single nucleotide polymorphism (SNPs)

Gene therapy

Gene transfer estimation

Biodistribution of vector

Gene expression

Cytokines, receptors,……..

REAL TIME PCR :USES

- **Detection of nucleotide mutation and polymorphism.**

e.g. Allelic Discrimination detects different forms of the same gene that differ by nucleotide substitution, insertion or deletion.

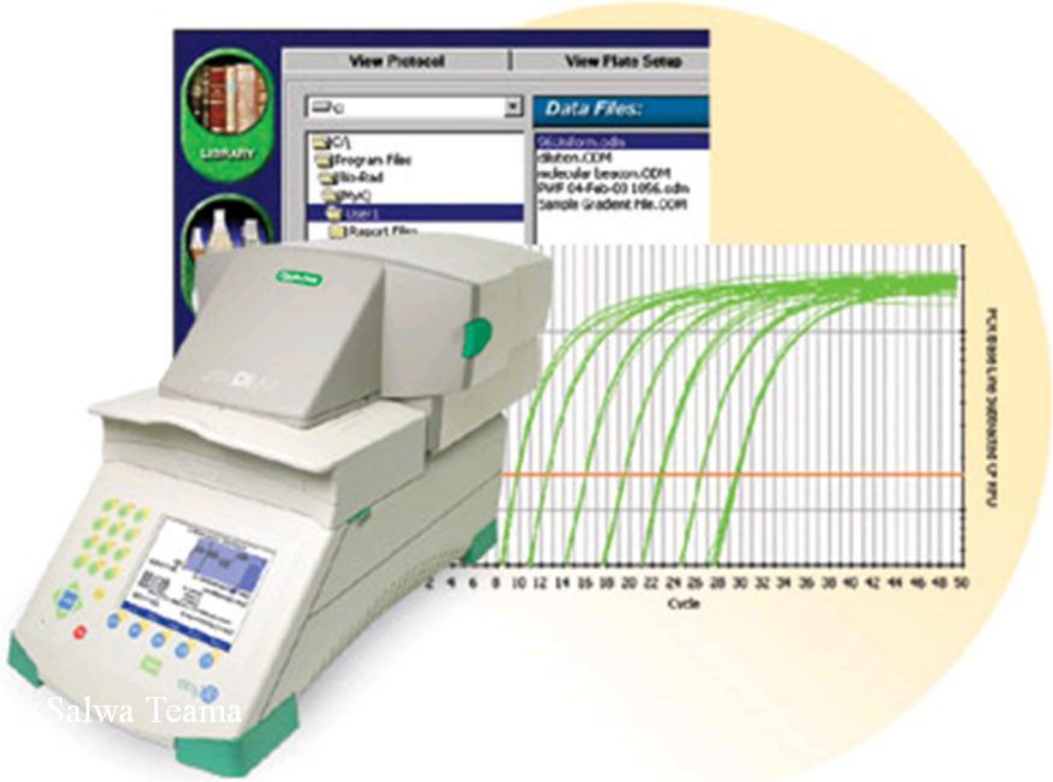
REAL TIME PCR :USES

- Molecular beacons are ideal probes for use in diagnostic assays designed for genetic screening, SNP detection, and pharmacogenetic applications.
- Drug therapy efficacy / drug monitoring
- DNA damage (microsatellite instability) measurement
- *In vivo* imaging of cellular processes
- Mitochondrial DNA studies
- Methylation detection
- Detection of inactivation at X-chromosome
- Microdeletion genotypes
- Quantitative microsatellite analysis
- Prenatal diagnosis / sex determination using single cell isolated from maternal blood maternal circulation
- Prenatal diagnosis of hemoglobinopathies

The development of real time PCR has eliminated the variability traditionally associated with quantitative PCR, thus allowing the routine and reliable quantification of PCR product.

Real-time PCR quantitation (qPCR) eliminates post-PCR processing of PCR products (which is necessary in competitive RT-PCR). This helps to increase throughput and reduce the chances of carryover contamination. In comparison to conventional RT-PCR, real-time PCR also offers a much wider dynamic range of up to 10^7-fold (compared to 1000-fold in conventional RT-PCR).

THANK YOU



References and Further Reading

- Holland, PM., Abramson,RD.,. et al.,1992. Clinical Chemistry.38, 462-463.
- www.appliedbiosystem.org
- http://www.gene-quantification.de/strategy
- Trends in Molecular Medicine vol. 8 No.6 June 2002


THANK YOU