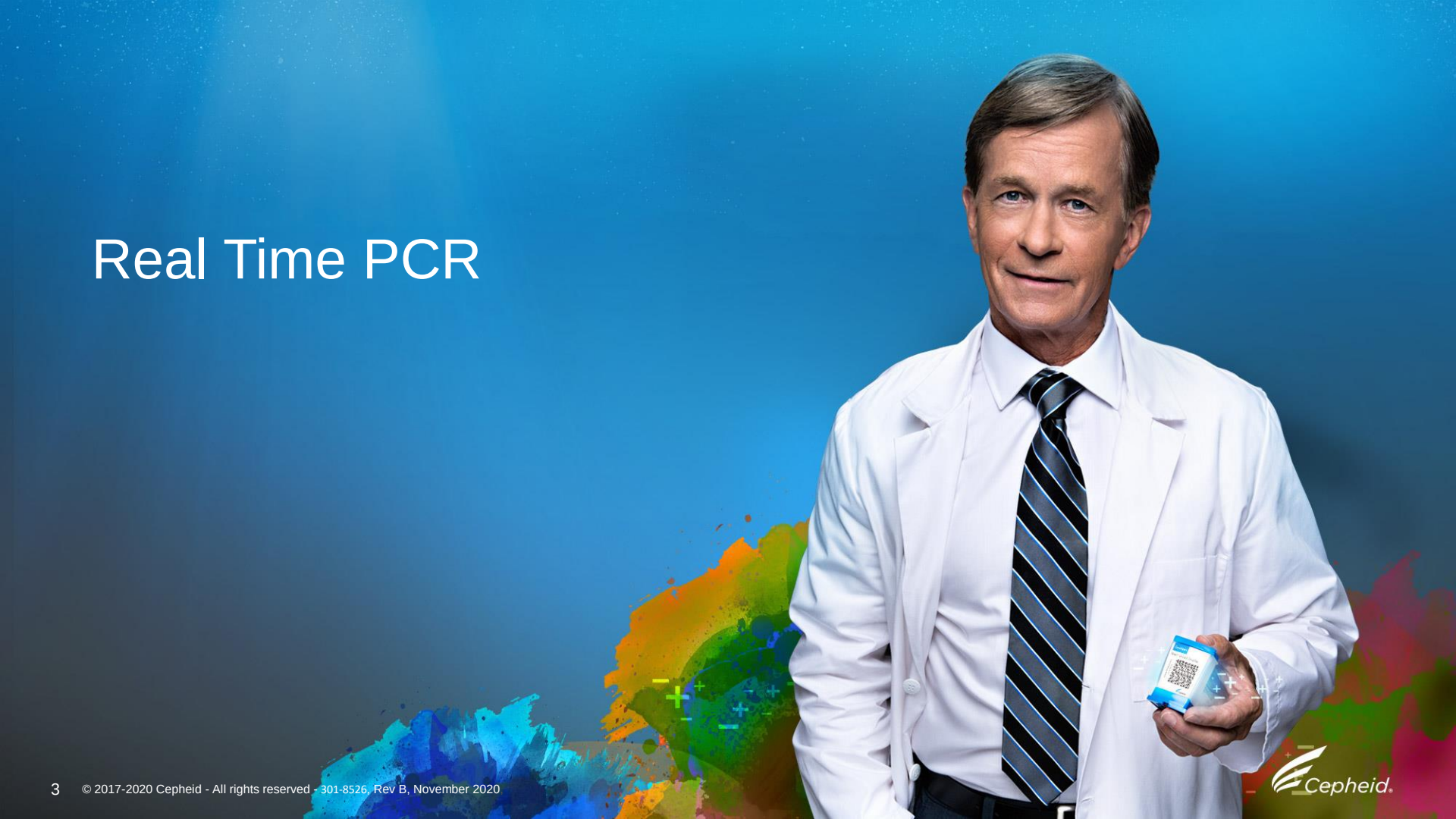
Basics of Polymerase Chain reaction (PCR) - II

Agenda

PCR Basics Part I	Basics of Molecular Biology
	Definition of PCR
	The phases of PCR
PCR Basics Part II	**Definition of Real Time PCR**
	Qualitative Real time PCR
	Quantitative Real Time PCR
PCR Basics Part III	Definition of Melting Temperature
	Melt Curve Analysis

Real Time PCR



Real-Time PCR (RT-PCR)

- Real-time PCR is a regular PCR reaction using an additional oligonucleotide marked with a fluorescent molecule : It is called a probe.
- The probe is a single –stranded DNA, matching a target sequence
- The fluorescent probe emits a fluorescent signal when activated by hybridization

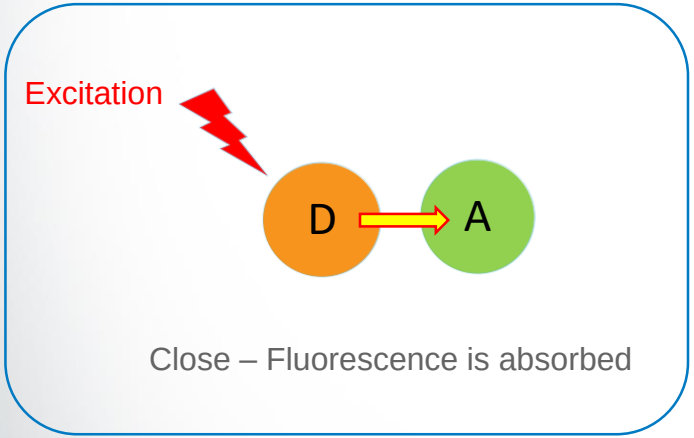
Probe

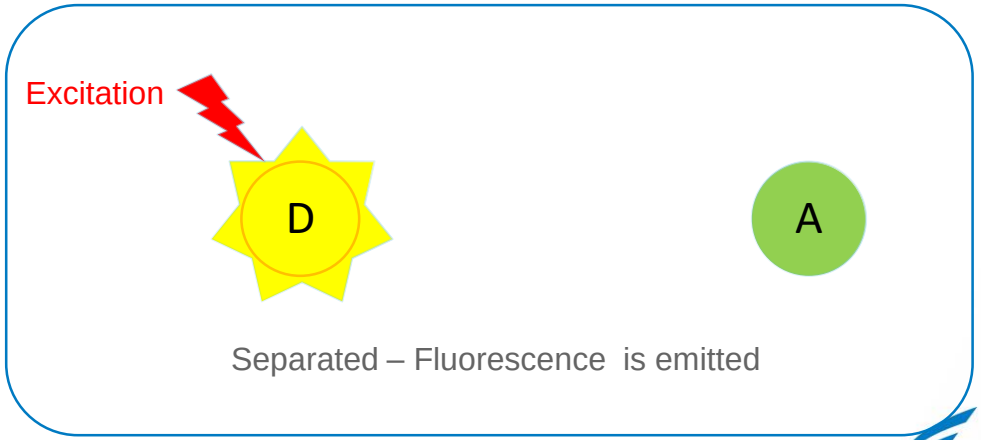
- One copy of target DNA activates one probe molecule, hence the fluorescence signal will be directly proportional to the number of copies of target DNA generated
- Examples of probes used for real-time PCR: Taqman, Molecular beacon, Scorpion probes…

FRET Technology:

- Fluorescence Resonance Energy Transfer (FRET) is a distance-dependent interaction between two dye molecules.
- Excitation energy is transferred from a donor molecule to an acceptor molecule without emission of a photon.
- FRET has many applications, including PCR.

D=Donor/Reporter
A=Acceptor/Quencher





Taqman probe

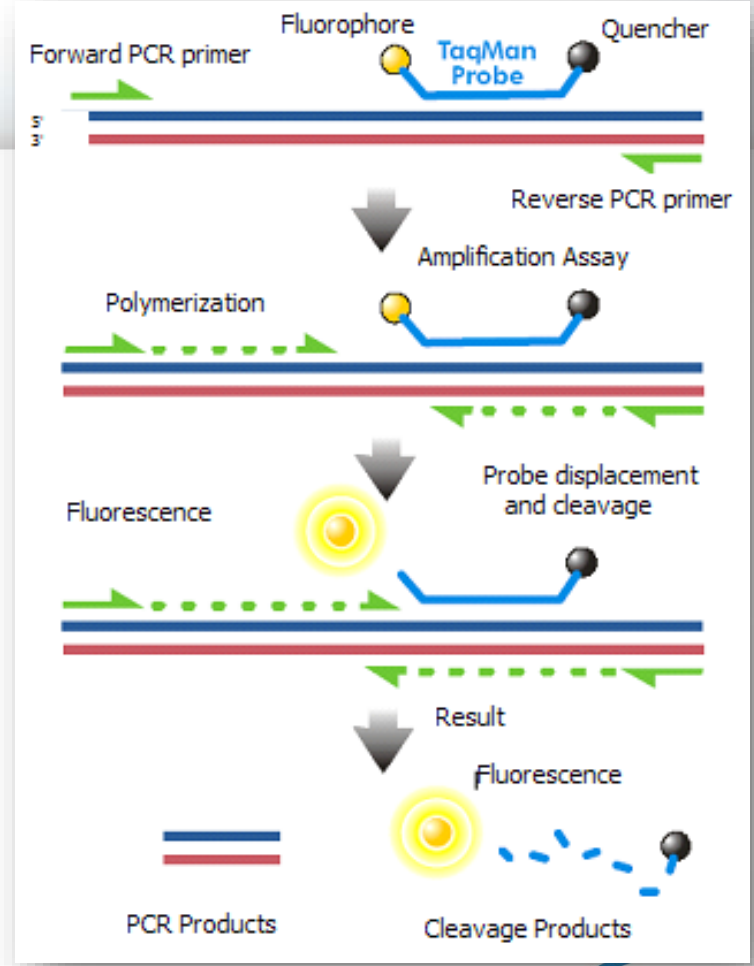
- A Taqman probe is a short oligonucleotide (15-30 bases long) probe labeled with a fluorescent dye at the 5' end and a quencher at the 3' end.
- As long as the reporter and the quencher are in close proximity, the quencher will absorb the fluorescence from the reporter
- The probe is designed by DNA sequence to anneal to the target
- During the extension phase of PCR, the Taq polymerase will degrade the probe
- This will physically separate the reporter and quencher, allowing fluorescence to be emitted and measured

Probe

1 free fluorophore/ DNA amplicon

Cepheid

Taqman Probe

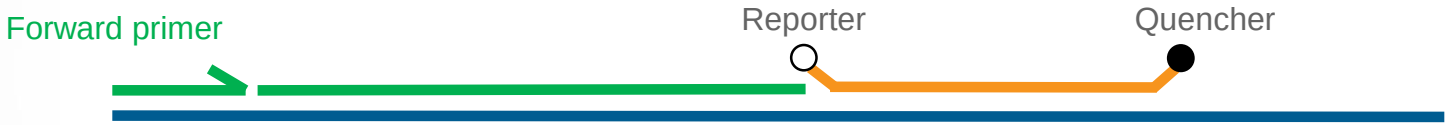


Taqman probe

Taqman probe

Forward primer

Taqman probe

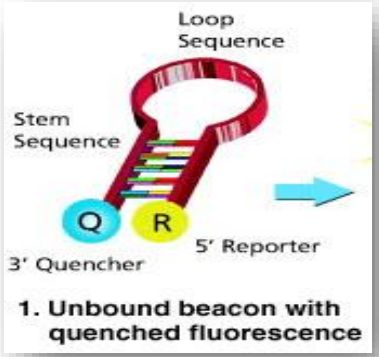


Taqman probe

Forward primer

Molecular beacon probe

- A molecular beacon probe is an hairpin-shaped molecule that consists of a fluorophore (reporter) and a quencher
- The probe sequence is about 17–21 bases long. The Stem sequence to form a stable duplex that is 5-8 bases long
- When free in solution, the two extremities stands close, leading to quenching of fluorescence.
- In presence of target DNA, the probe anneals to the target and separates the fluorophore and quencher, leading to emission of fluorescence



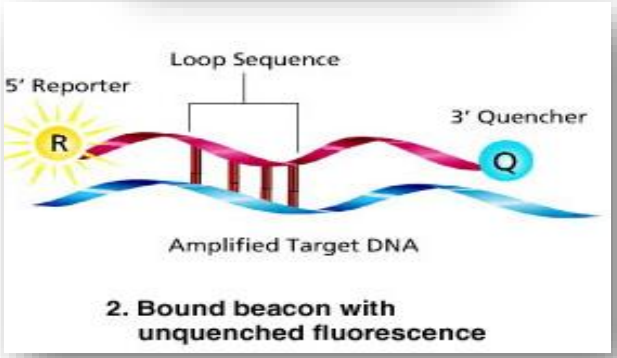
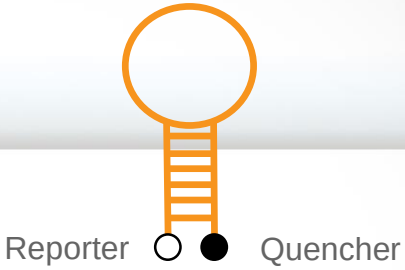


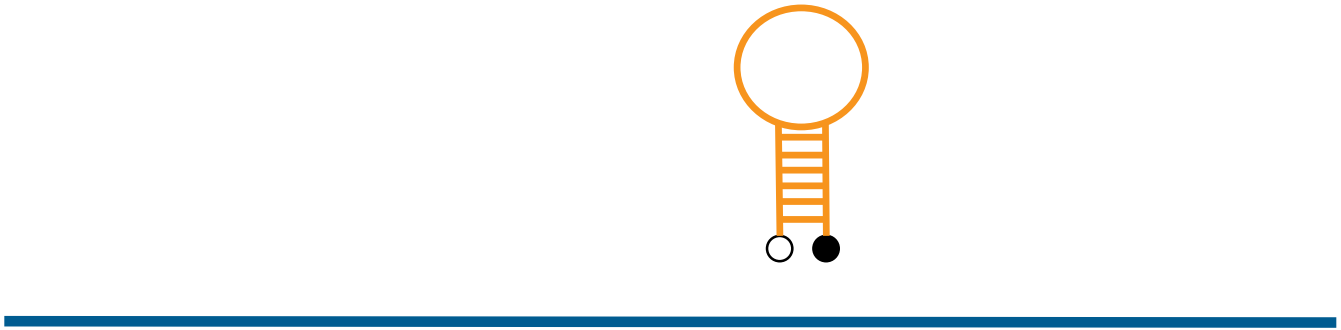
Image: Sigma-Aldrich

Molecular Beacon Probe

Molecular Beacon Probe



Molecular Beacon Probe



Molecular Beacon Probe

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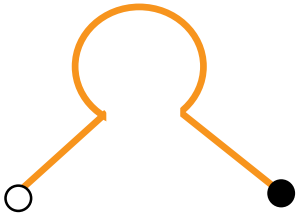
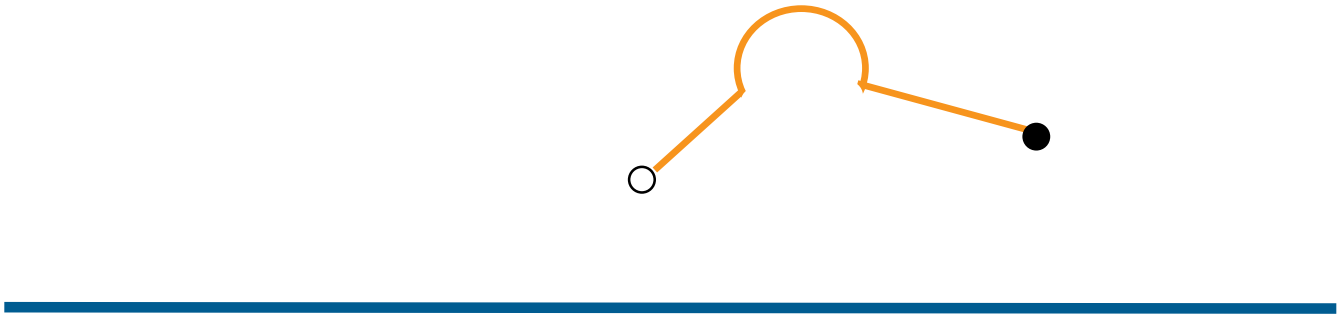


Molecular Beacon Probe



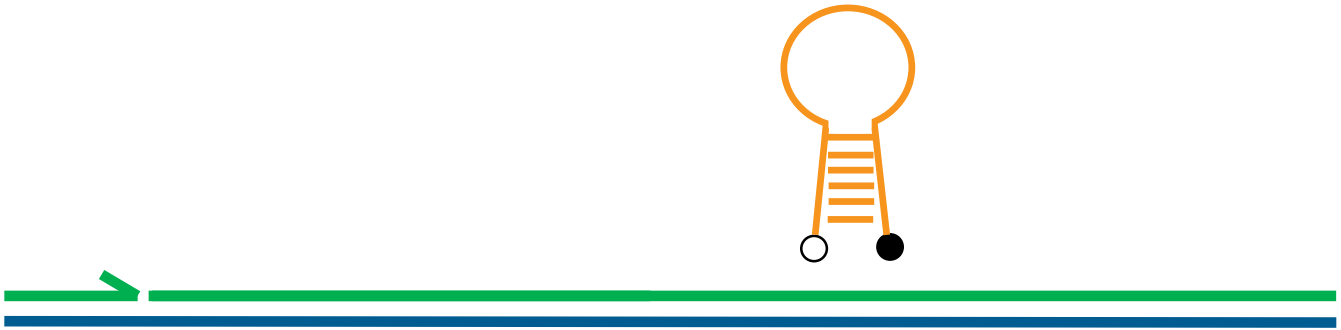
Molecular Beacon Probe

Forward primer

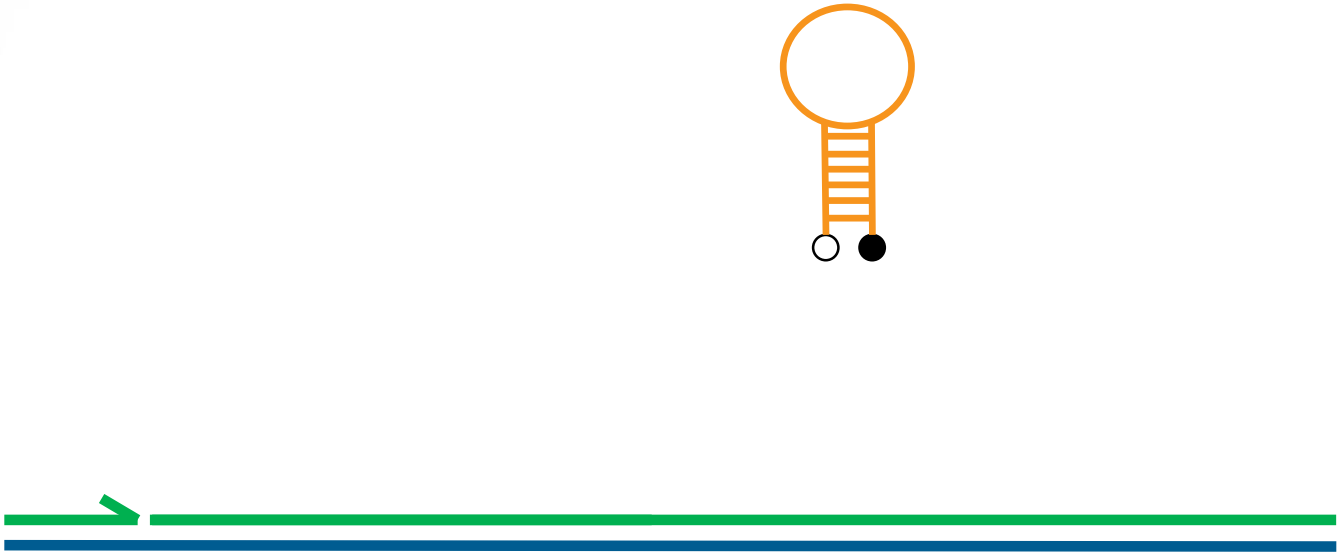
Molecular Beacon Probe

Molecular Beacon Probe

Molecular Beacon Probe

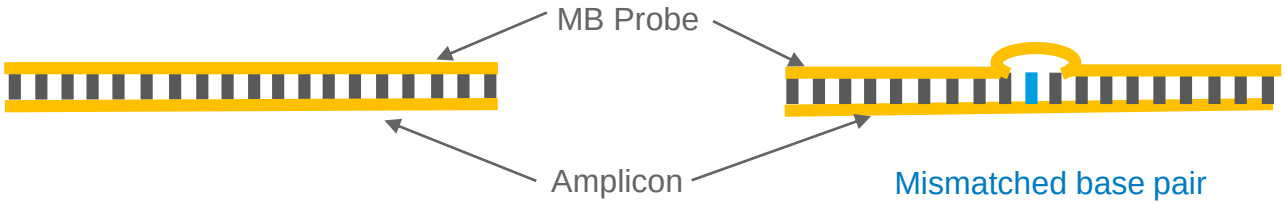


Molecular Beacon Probe

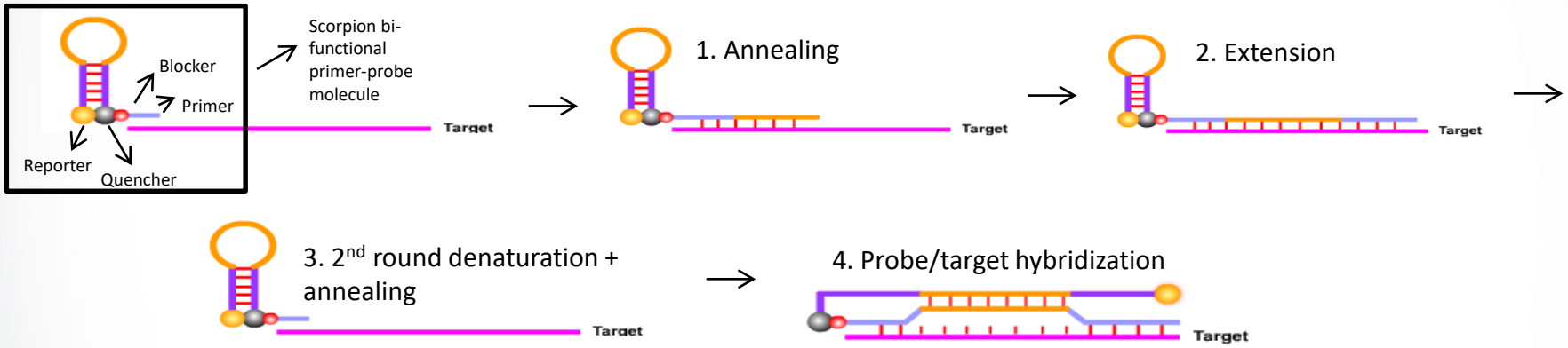


Sloppy Molecular Beacon

- Sloppy molecular beacons possess relatively long probe sequences (about 30-40 bases long), enabling them to form hybrids with amplicons from many different species despite the presence of mismatched base pairs.



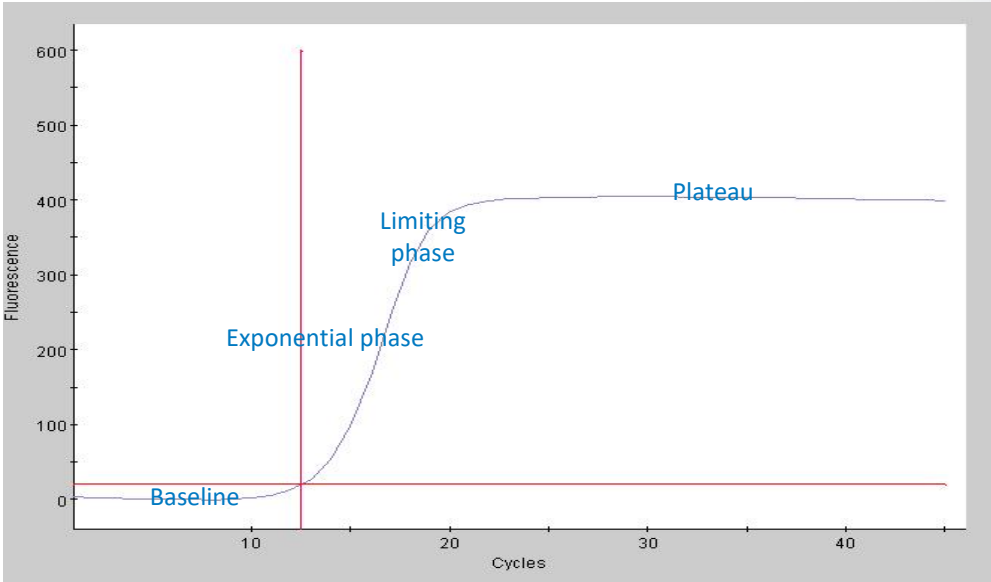
Scorpion probe



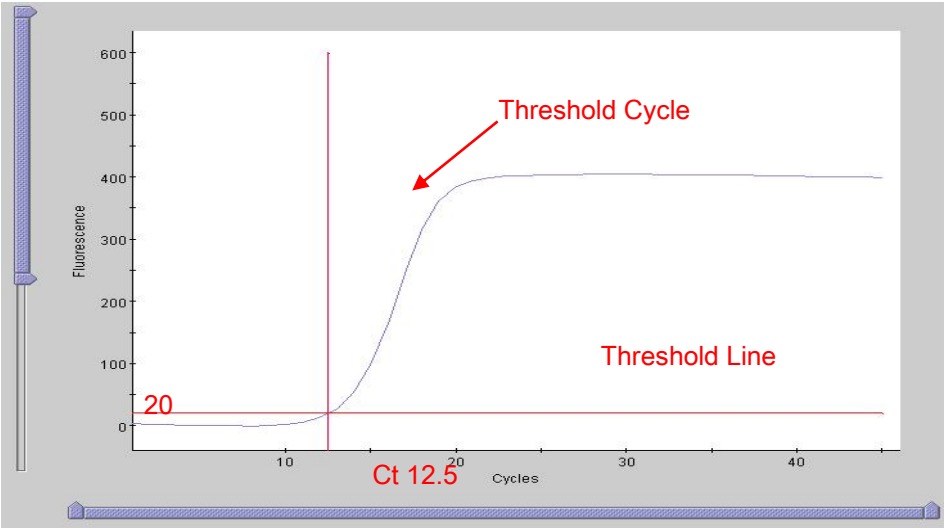
The probe sequence should be 17–27 bases long.

Changes in fluorescence during PCR

- The amplification curve consists of 4 phases :



Definition of Ct – based on threshold



- Threshold cycle (Ct): the first cycle which crosses a defined fluorescence threshold
- This cycle value can be fractional

Validation criteria of a PCR: Ct range and end-point fluorescence

- Ct range
  - It is the acceptable range for a Ct value
  - It is limited by Ct_{min} and Ct_{max}
- End point fluorescence
  - The fluorescence value at the end of the PCR (Plateau)

For Xpert tests, outside the range, the amplification curve is not validated : the result cannot be provided

Multiplex PCR

- Multiplex PCR is the amplification of **multiple DNA targets** simultaneously
  - Each target has its own set of primers
  - Each target is detected or quantified by its own probe, labeled with a different dye, detected at a specific fluorescence wavelength
- When designing a multiplex PCR, competition between targets must be avoided

Detection of multiple dyes – 6 dyes until 2020

- Different dyes (reporters) are selected
- They are excited and they emit at distinct wavelengths

Analyte	Reporter	Excitation (nm)	Emission (nm)
Target 1	Dye 1	375-405	420-480
Target 2	Dye 2	450-495	510-535
Target 3	Dye 3	500-550	565-590
Target 4	Dye 4	630-650	665-685
SPC	Dye 6		>700
Target 5	Dye5	555-590	606-650

Detection of multiple dyes – 10 dyes now

- Different dyes (reporters) are selected
- They are excited and they emit at distinct wavelenghs

		iCore Detection			
	iCore Optical Channels	Blue + IR (420-477 nm + > 700 nm)	Green + Deep Red (510-535 nm + 660-680 nm)	Yellow (565-585 nm)	Red (620-645 nm)
iCore Excitation	UV (400 nm)	CF1			
	Blue (470 nm)		FAM	CF7 (FAM-CF3)	CF9
	Green (520 nm)	CF10 (CF3-CF6)		A532 (CF3)	CF8 (CF3-CF4)
	Yellow (574-584 nm)				TxR (CF4)
	Red (635 nm)	CF6	A647 (CF5)		

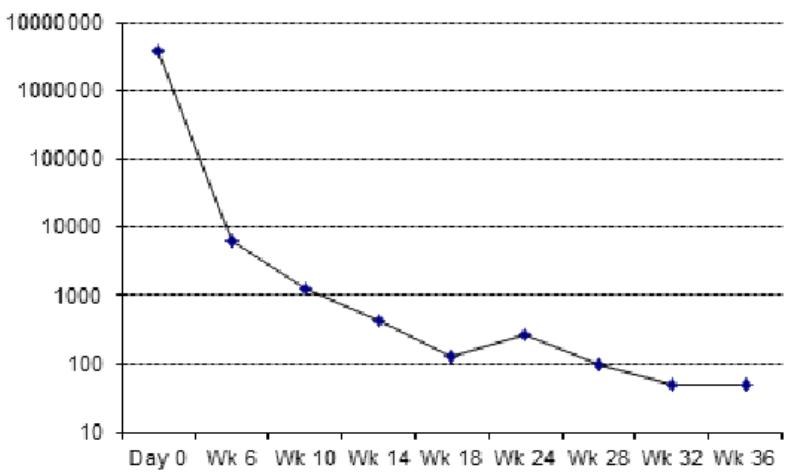
Quantitation by Real Time PCR

Quantitation

- Absolute quantitation : result reported as a concentration (copies/mL, IU/mL, etc…):

Xpert HIV-1 VL and Xpert HCV

- Relative quantitation : result reported as a ratio: Xpert BCR-ABL

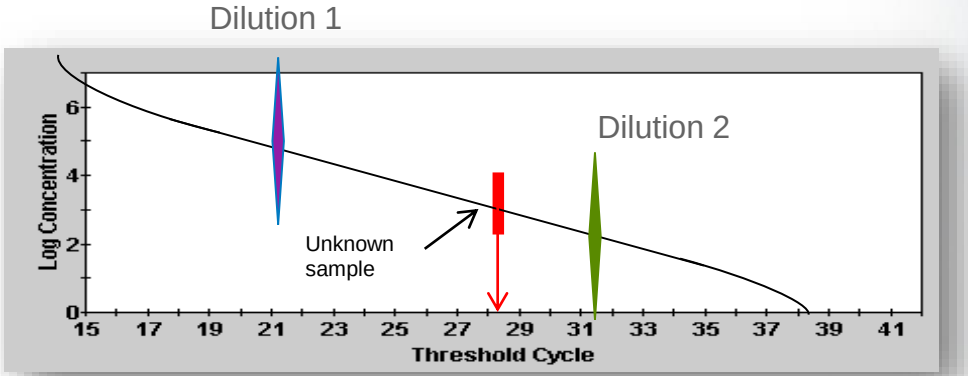


HIV-1 VL viral load decrease on ART
(other method than GeneXpert).
Graphic : hivbook.com

Photo: South Africa 2012 © Treatment Action Campaign - MSF

Absolute quantitation using external standards

1. Prepare dilutions of a sample containing the target DNA at a known concentration.
2. These dilutions will be run along with your unknown sample, each in a separate tube
3. For each dilution, the Ct is reported
4. The standard curve is drawn: Ct versus concentration
5. The Ct of the unknown sample is used to extrapolate its concentration from the standard curve



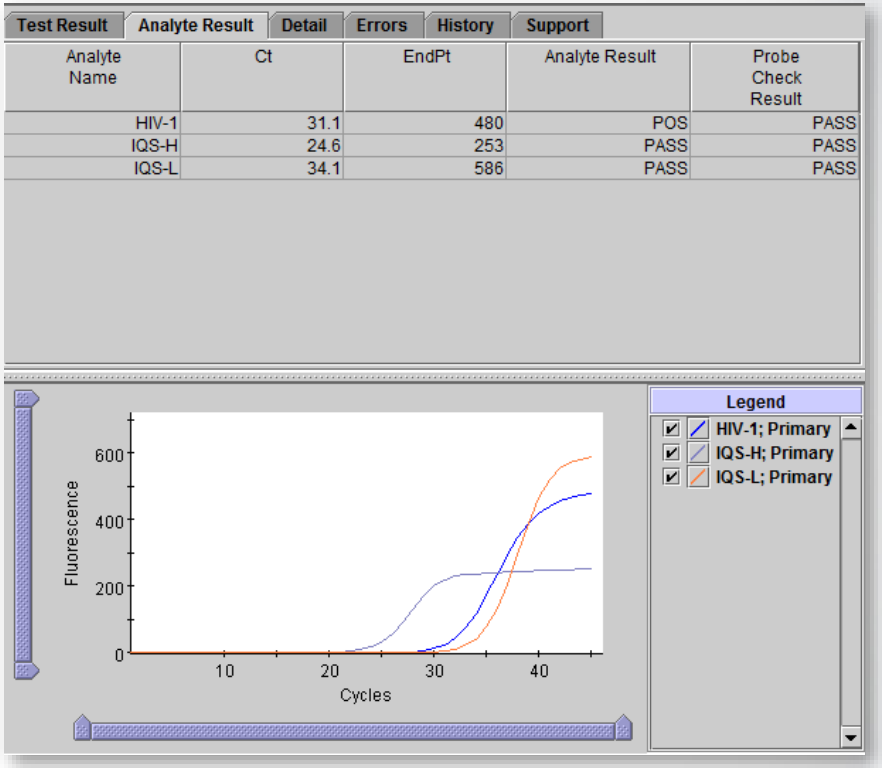
Within the linear range of concentration, 2 standards are sufficient

Absolute Quantitation using internal standards.

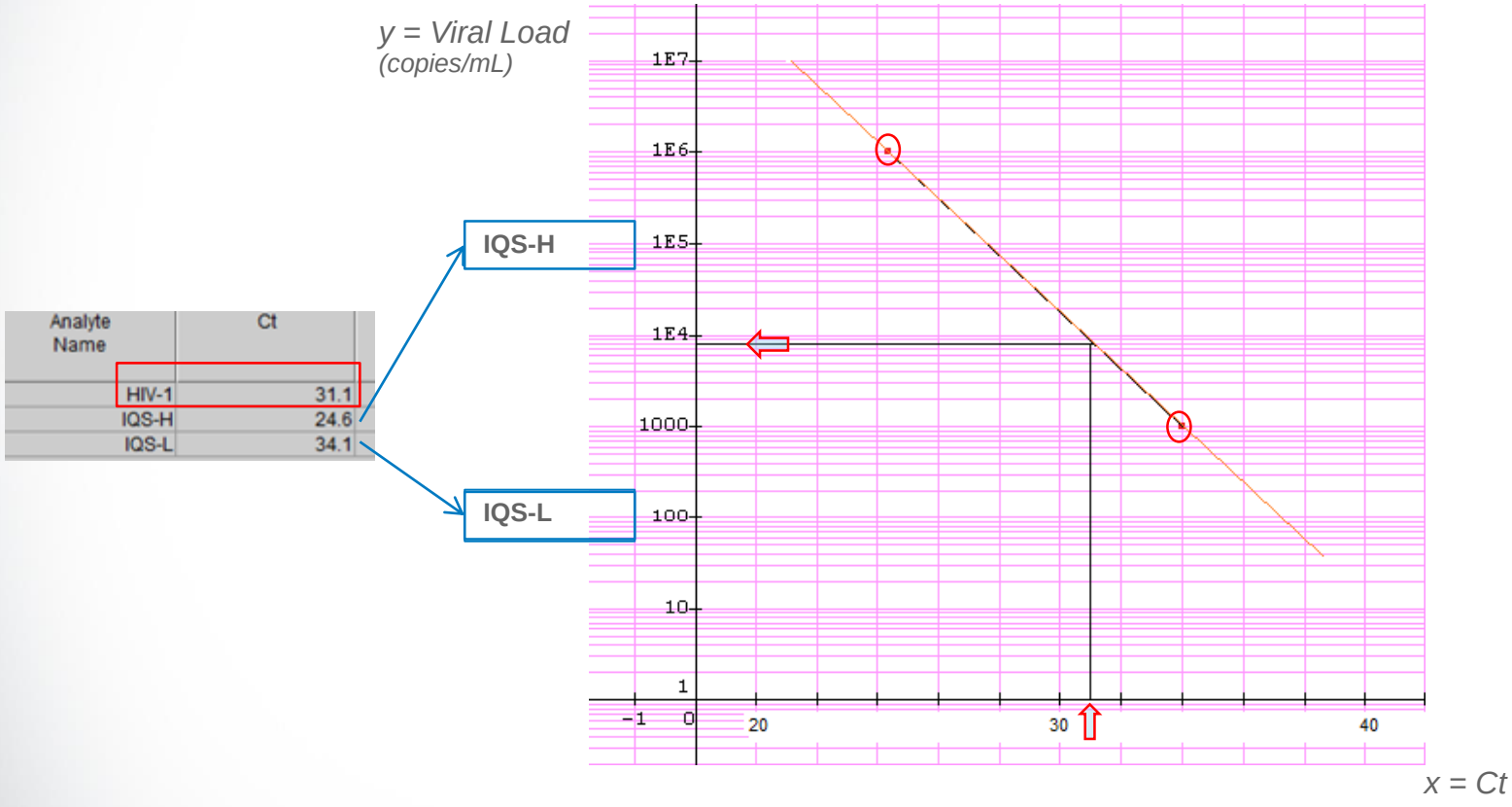
For Xpert HIV-1 VL :
2 standards are used to calculate the concentration of the sample:

- 1 high standard (IQS-H) = 10^6 copies/mL
- 1 low standard (IQS-L) = 10^3 copies/mL

- Based on Cts and known concentration of each standard and the Ct of the unknown sample, the concentration of the unknown sample will be calculated by the GeneXpert software.

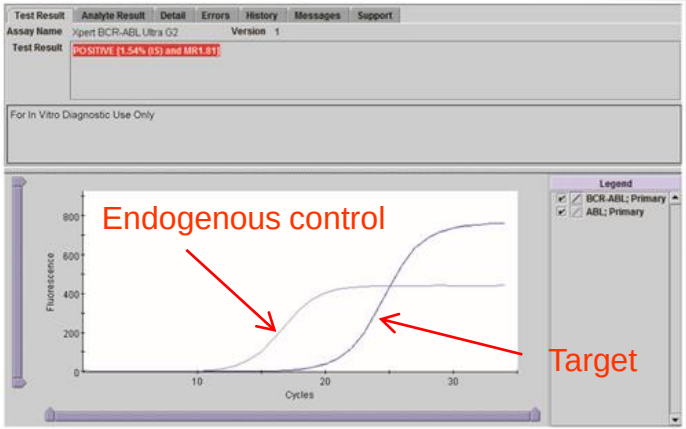


Calculation of the sample concentration



Relative quantitation with Real Time PCR
(Ex: Xpert BCR-ABL)

- Relative quantitation measures the level of a target and expresses it relative to the level of an internal control (reference gene)
- The reference gene can be endogenous. As such, it can also ensure that sufficient sample is used in the test.
- Because of its low variability, the endogenous control can also be used to indicate PCR inhibition.



POSITIVE [1.54% (IS) and MR1.81]

Example of an Xpert BCR-ABL Ultra test result

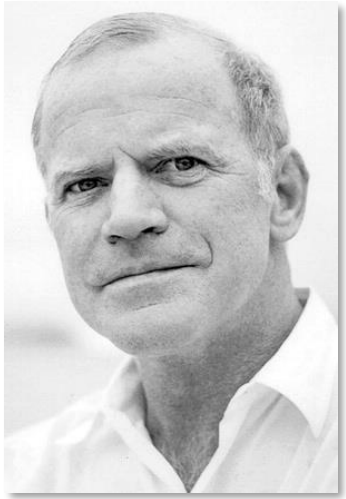
Conclusion

RT-PCR is:

- Fast
- Sensitive
- Precise
- Easy to perform
- Can be quantitative

Science consistently produces a new crop of miraculous truths and dazzling devices every year.

Kary Mullis

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