Real-Time PCR

Molecular biology methods for pharmacists

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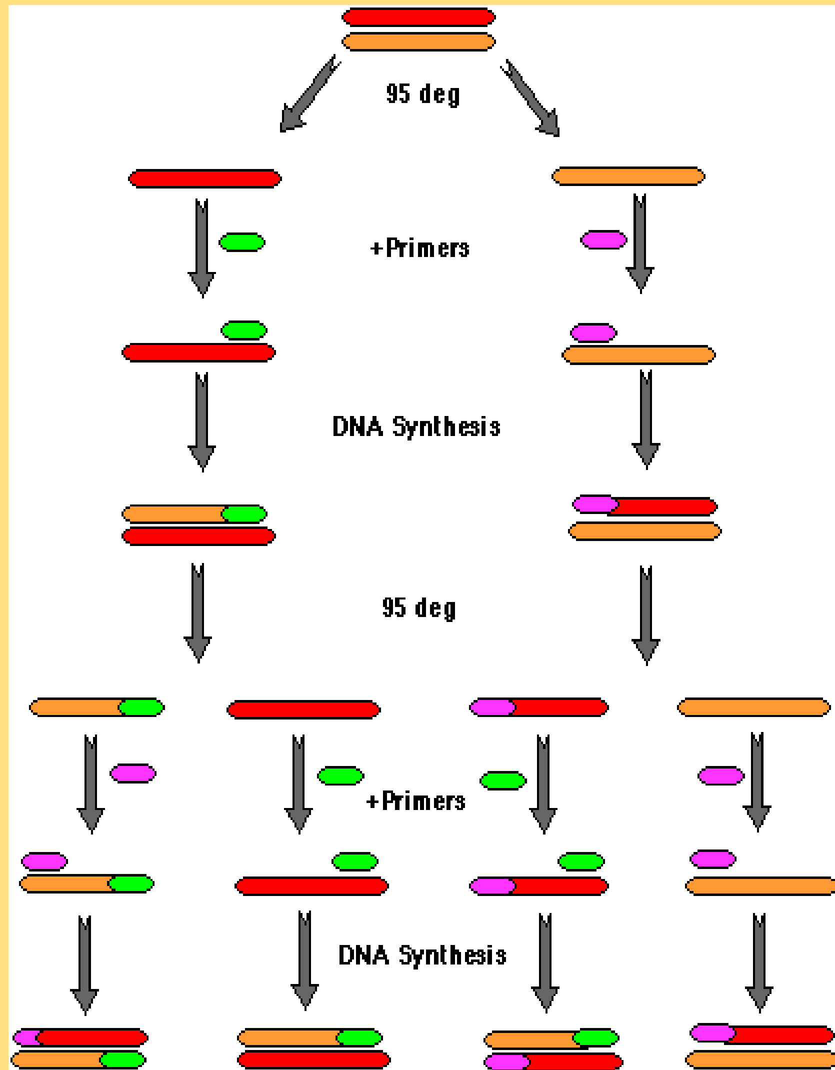
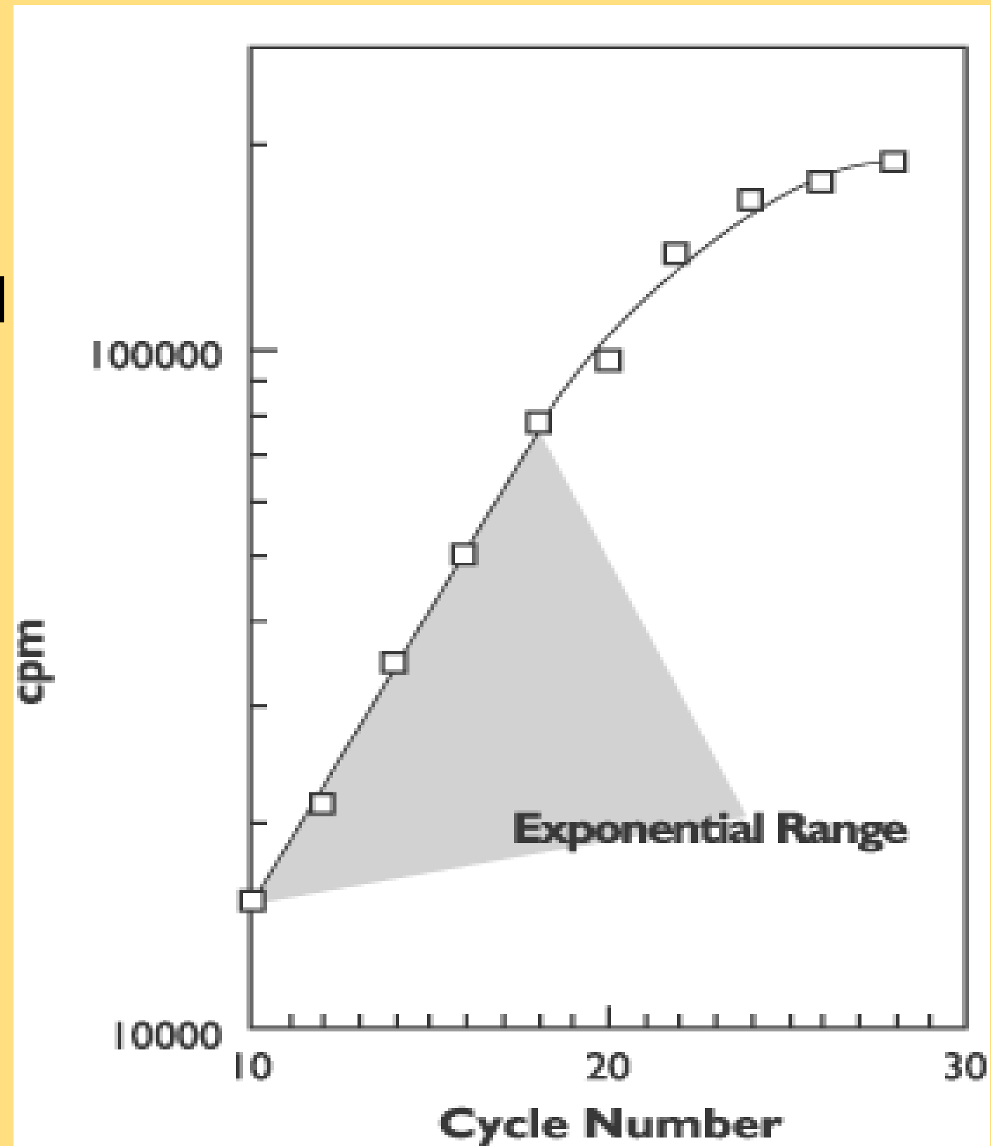
Phases of PCR amplification



Kinetics of PCR

- **Theoretically, the amount of the products is doubled during each PCR cycle**

- **Really the doubling the PCR products is only going near to 100%.**



The basic principles of the method

- **Visualisation of growing amount of the products of amplification measured by growing fluorescence during PCR**
- **Intensity of fluorescence correlates to the concentration of the template**
- **The correlation between amount of the PCR products and intensity of fluorescence is used to calculation of the templates number at the beginning of PCR**

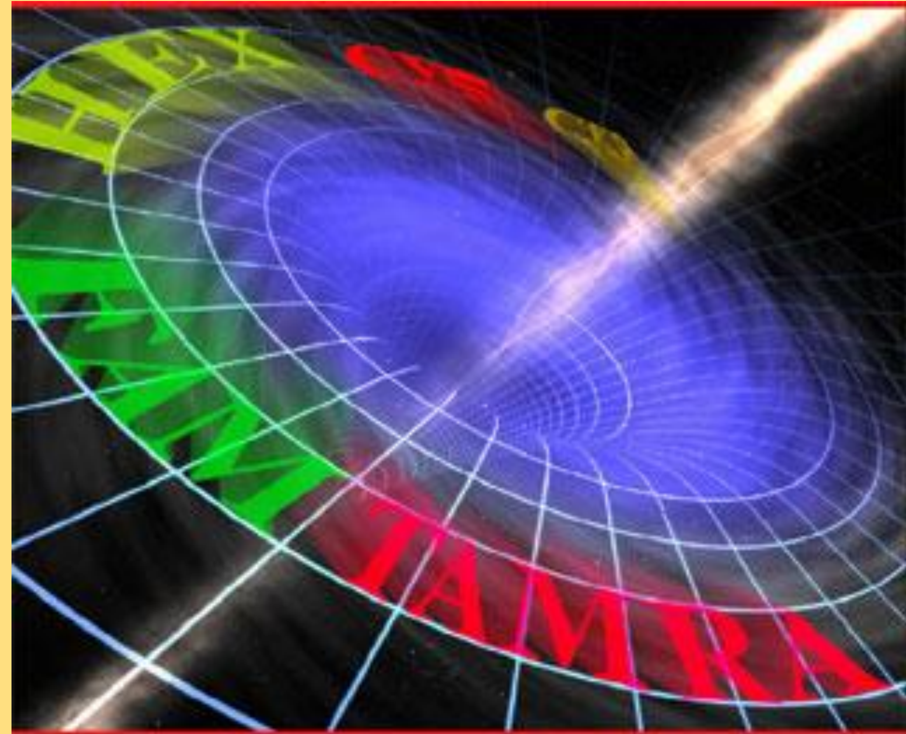
Real Time termocyclers

Real-time PCR advantages

- The same or higher sensitivity without manipulation with samples – lower contamination risk
- Analysis without electrophoresis
- Automatisation of process
- **Sample quantification** – the level of mRNA, amount of pathogen
- Possibility to run „multiplex“ reactions

Important components for performing Real Time PCR

- Fluorophores
- Quenchers
- Probes

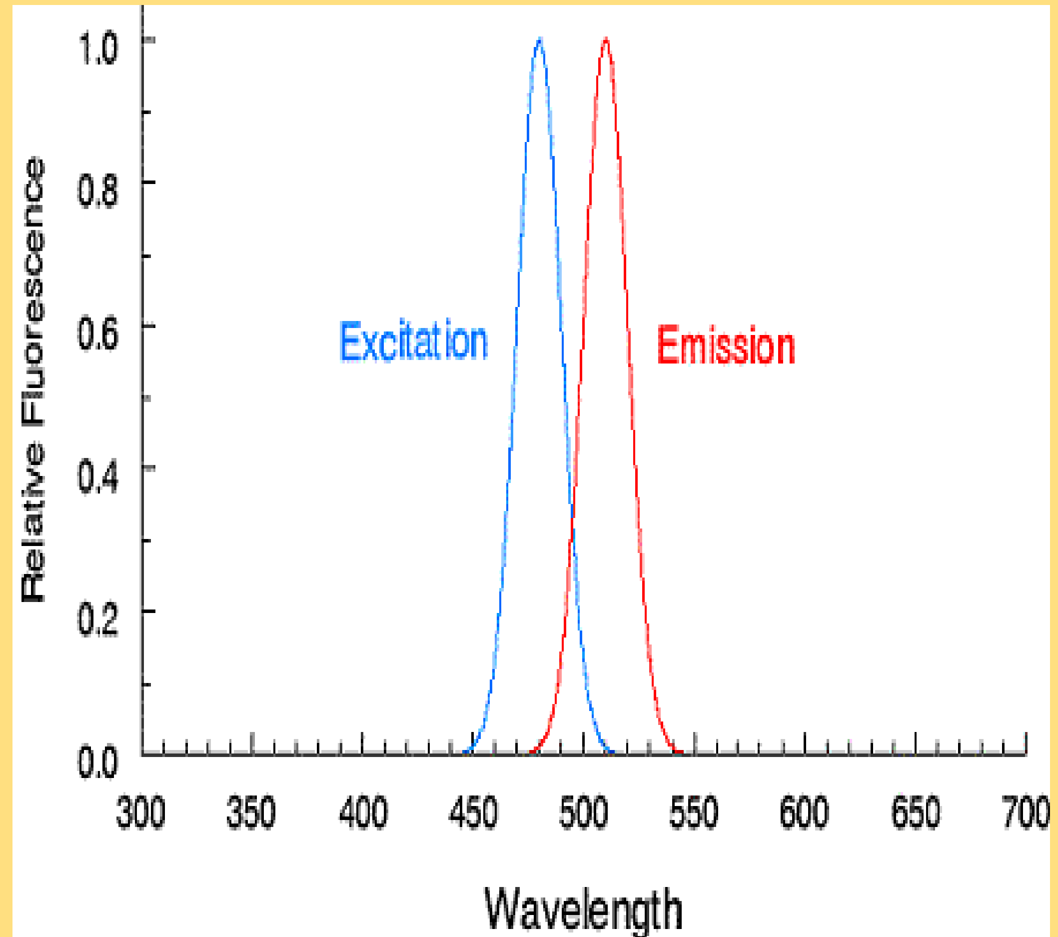


Fluorophores

- **Mostly heterocyclic polyaromatic carbohydrates**
- **Their terminal fluorescence (emission) depends on ability of fluorophore to absorb and to emit photons**
- **Emission of the fluorophore strongly depends on temperature**

The principles of fluorescence

- Absorption of light with specific wavelength by fluorophore
- Exiting the fluorophore to a state with higher energy
- Return the molecule to the basic state which is followed by emission of photon with lower wavelength



Quenchers

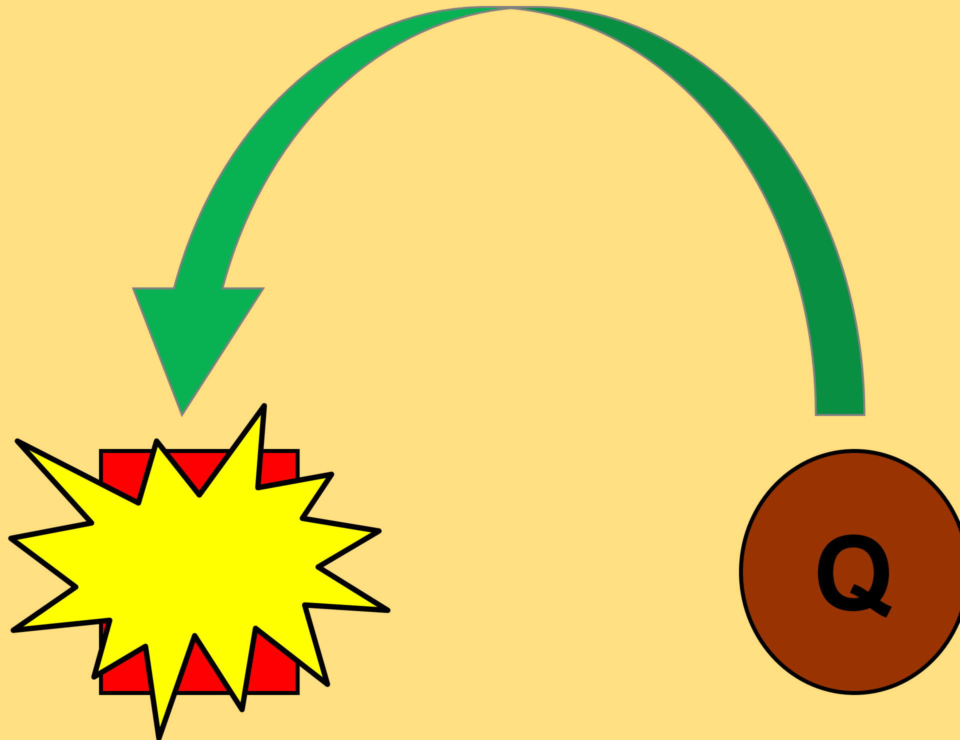
- **Molecules which are able to absorb or dissipate energy from excited fluorophore**

- **The quencher receives the energy from fluorophore and absorbs or dissipate it by the mechanism of „Proximal quenching“ or „Fluorescence resonance energy transfer (FRET)“**

Proximal quenching

- **Based on short distance between fluorophore and quencher**
- **This distance enables an effective transfer of energy which is transfer by quencher to warm and quenches the excited fluorophore by this**

Proximal quenching

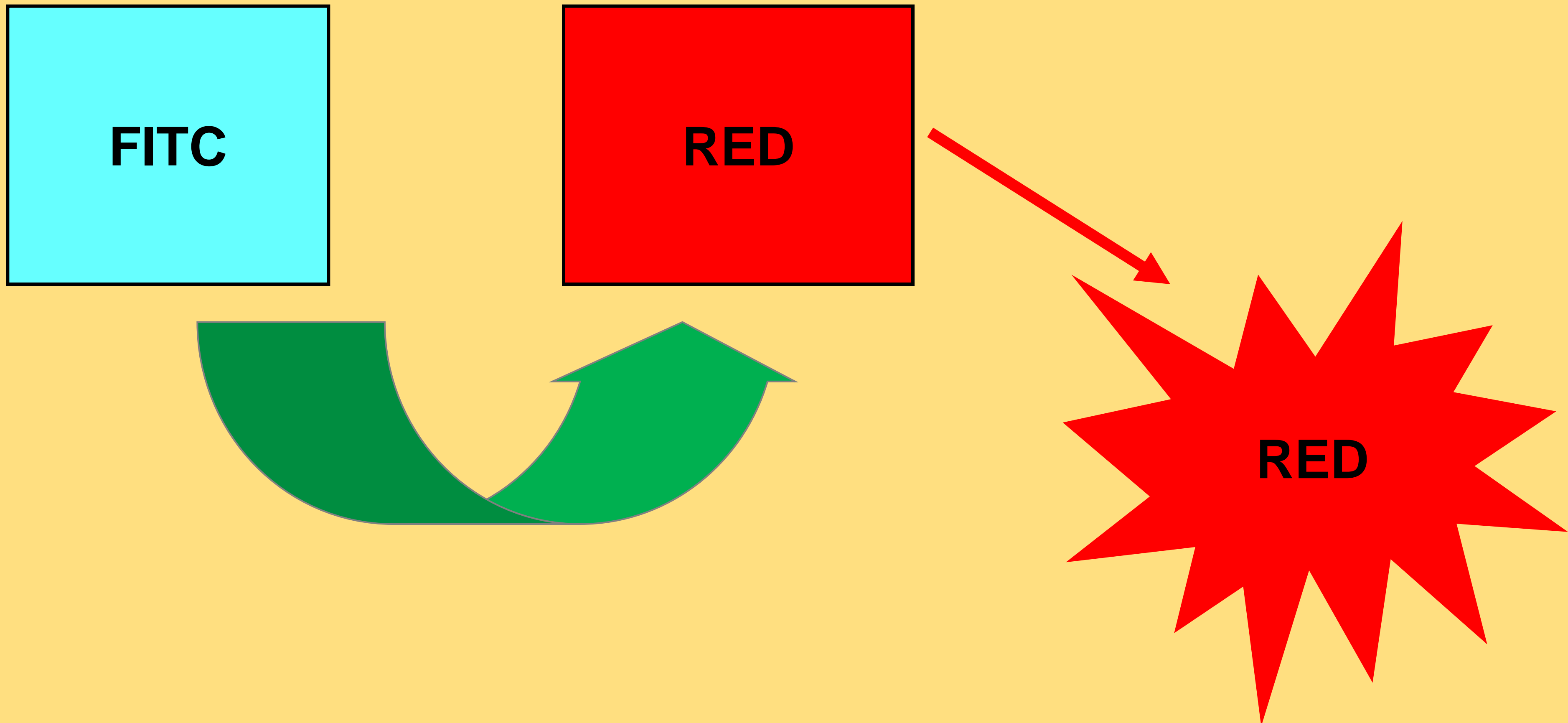


Fluorescence resonance energy transfer (FRET)

- **A donor molecule (excited by external light source) transfer a part of its energy to acceptor molecule which emits light with another wave length**

- **Efficiency of this process depends also on distance of donor and acceptor molecules (effective about 100Å, approx. 30 bp in linear probes)**

Fluorescence resonance energy transfer (FRET)

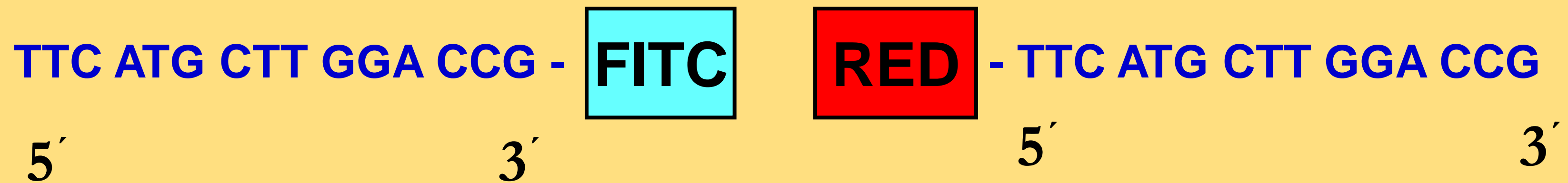


Probe

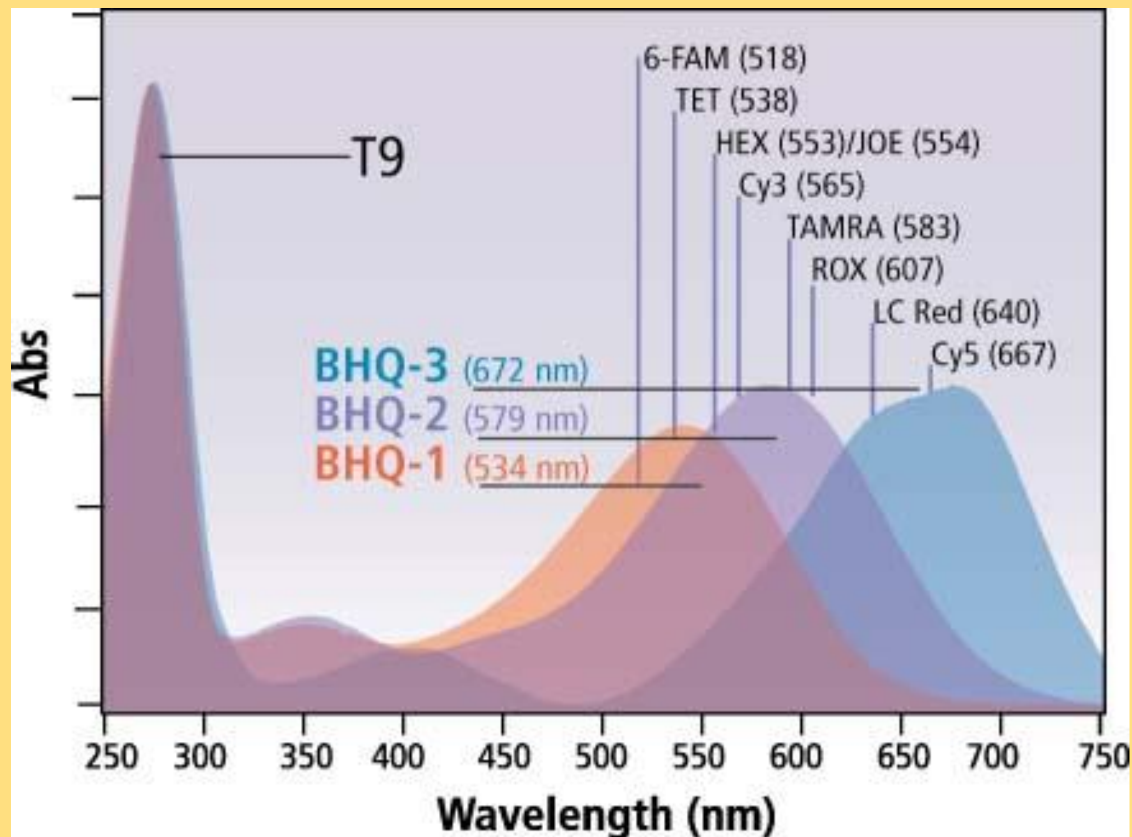
Short oligonucleotide with the similar features as PCR primer (probe binds to DNA template by the same manner)

It enables to bind fluorophore and quencher in the effective distance and ensures the process of quenching the fluorophore

Fluorophore, probe, and quencher



The most frequently used combinations of fluorophore/quencher and probe



FAM

5´

3´

BHQ1

Formats of Real Time PCR

I. **Nonspecific formats:** **based on non-specific binding of fluorophore into synthesized dsDNA molecule**

II. **Specific formats:** **based on specific binding of probe labelled by fluorophore**

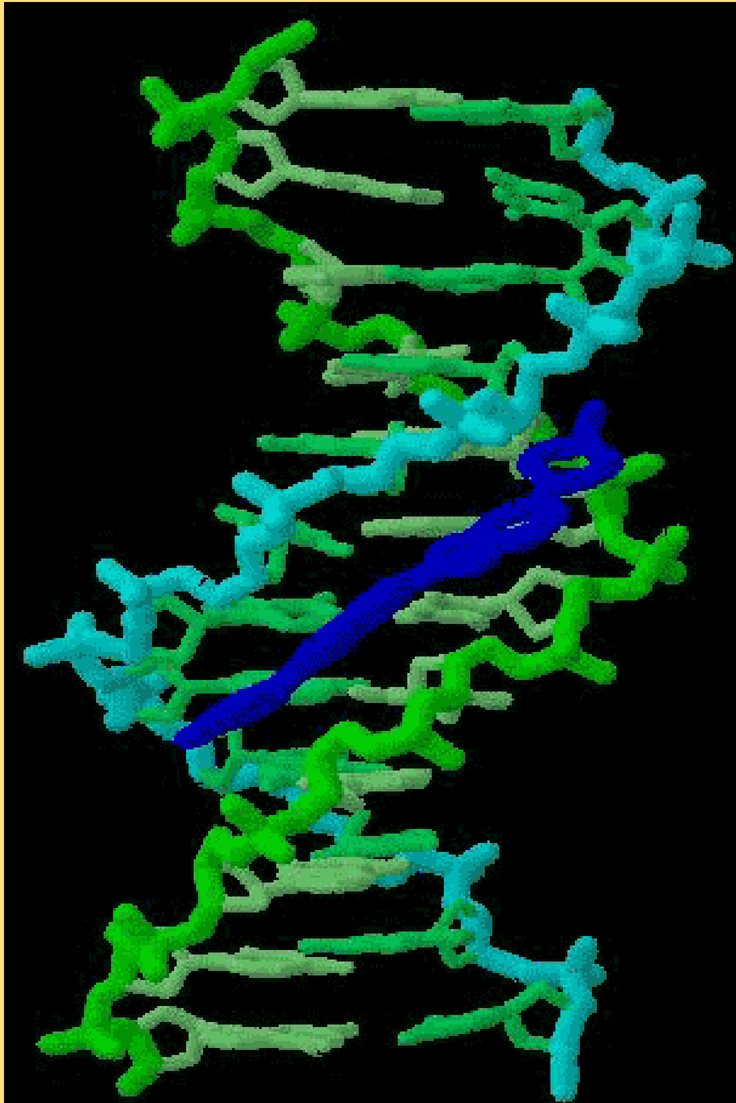
Nonspecific formats - DNA intercalators -

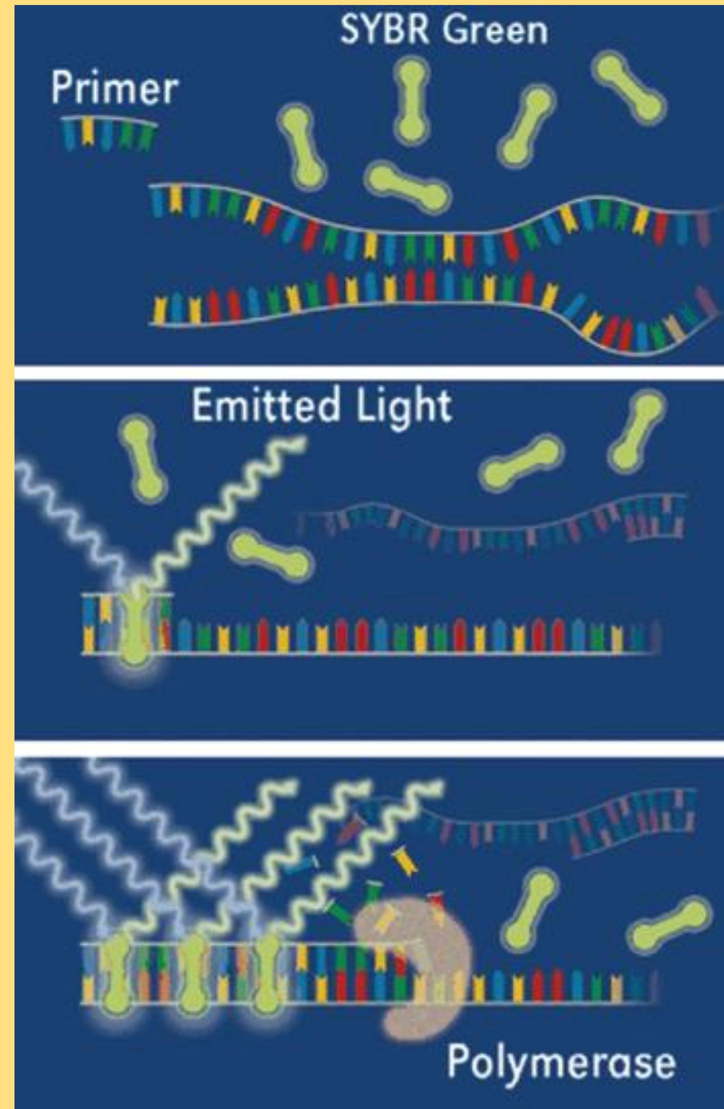
- **Fluorofore intercalates to synthesized molecule of dsDNA during PCR**
- **Binding into minor groove**

Formates

- **Quencher-Labeled Primer I**
- **Quencher-Labeled Primer II**
- **LUX™ Primers**
- **Amplifluor™**
- **SYBR Green I**

Principle of using of SYBR^{TM} Green I





Is binding of SYBR Green to dsDNA reversible or irreversible, and why?

Detection of differences in DNA sequence based on SYBR Green I
- model example -

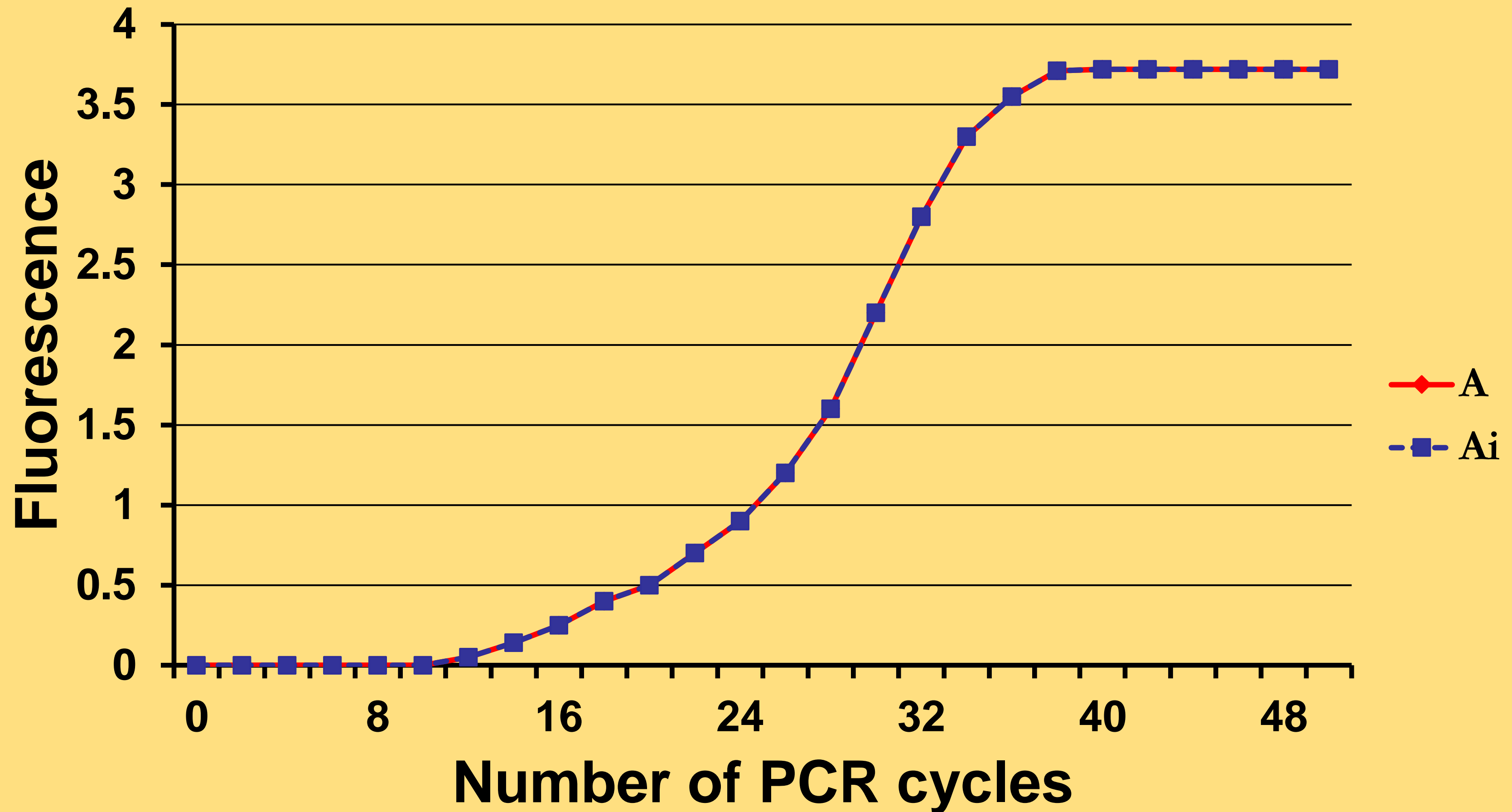
DNA sequence A that is 200 bp long

<u>CCTCCTGCCTCTACC</u>AATCGCCAGTCAGGAAGGCAGCCTACCCCGCTG
ACTCCACCTTTGAGAGACACTCATCCTCAGGCCATGCAGTGGAATTCC
ACAACCTTCCACCAAACTCTGCAAGATCCCAGAGTGAGAGGCCTGTAT
CTCCCTGCTGGTGGCTCCAGTTCAGGAACAGTAAACCCTG<u>TTCCGACT</u>
<u>ACTGCCTC</u>

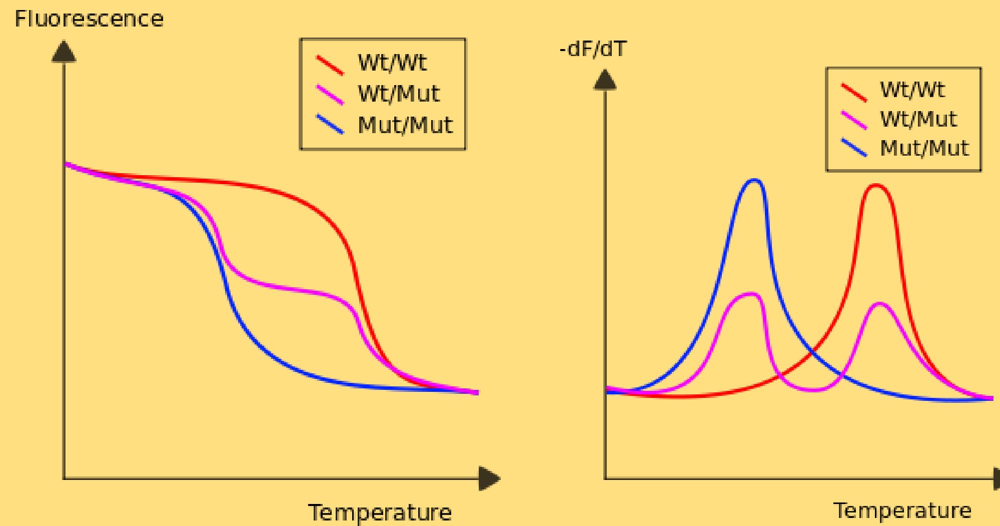
DNA sequence Ai with 5 bp insertion – total length 205 bp

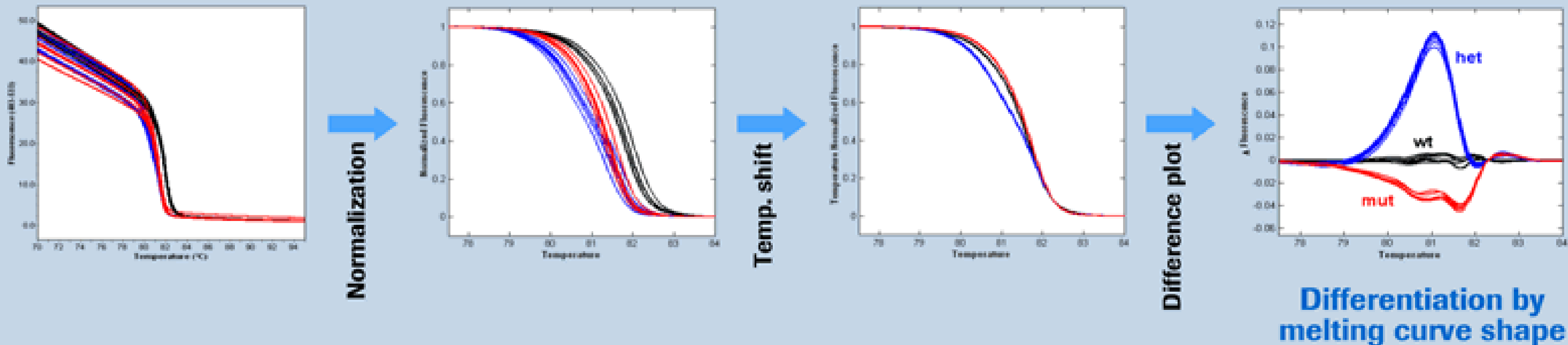
<u>CCTCCTGCCTCTACC</u>AATCGCCAGTCAGGAAGGCAGCCTACCCCGCTG
ACTCCACCTTTGAGAG<u>***ACACT***</u>ACACTCATCCTCAGGCCATGCAGTGGA
ATTCCACAACCTTCCACCAAACTCTGCAAGATCCCAGAGTGAGAGGCC
TGTATCTCCCTGCTGGTGGCTCCAGTTCAGGAACAGTAAACCCTG<u>TTCC</u>
<u>GACTACTGCCTC</u>

Result of detection by SYBR Green I - the basic data -



Result of detection by SYBR Green I - melting analysis -





http://www.gene-quantification.de/hrm.html

Specific formats
- labelled DNA probes -

Method based on primer annealing and hybridisation of probe to specific DNA locus

Two basic probes exist

- **Linear probes**
- **Structural probes**

Specific formats

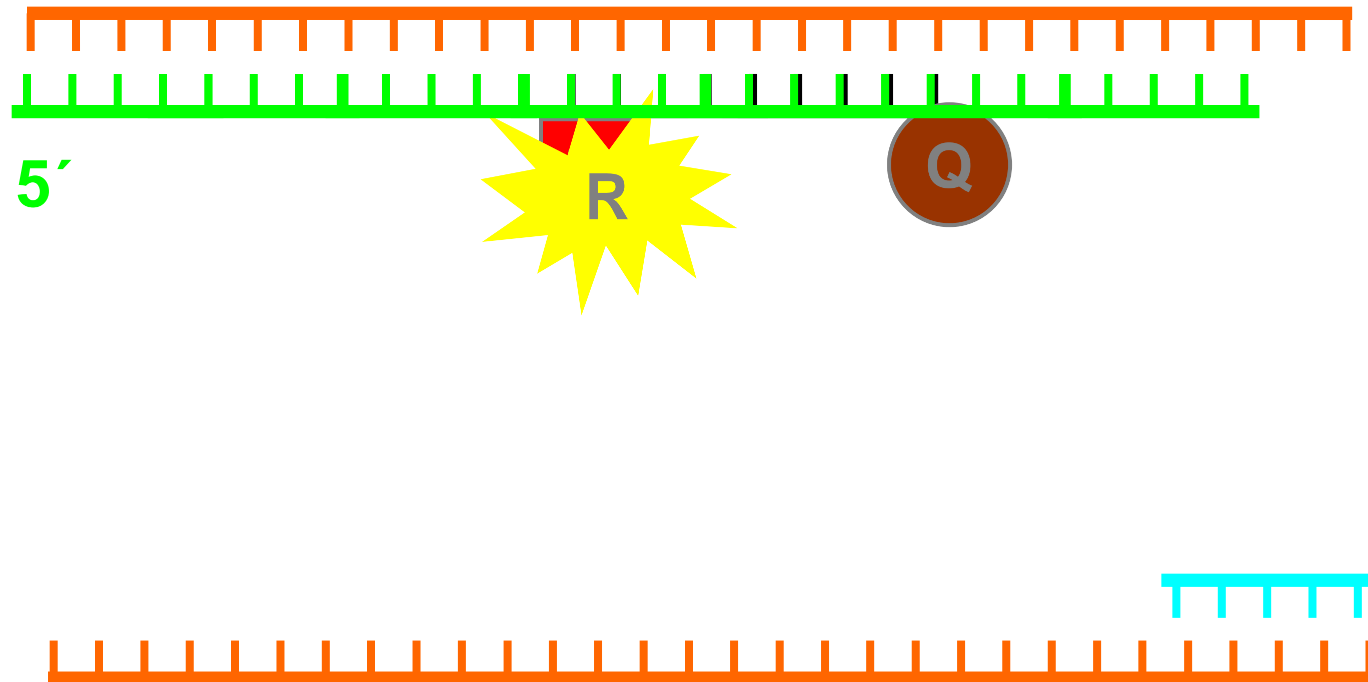
Linear probes

- ResonSense® Probes
- Angler® Probes
- HyBeacons™
- Light-up Probes
- Hydrolysis (TaqMan®) Probes
- Lanthanide Probes
- Hybridization Probes (FRET)
- Eclipse™
- Displacement Hybridization/Complex Probe

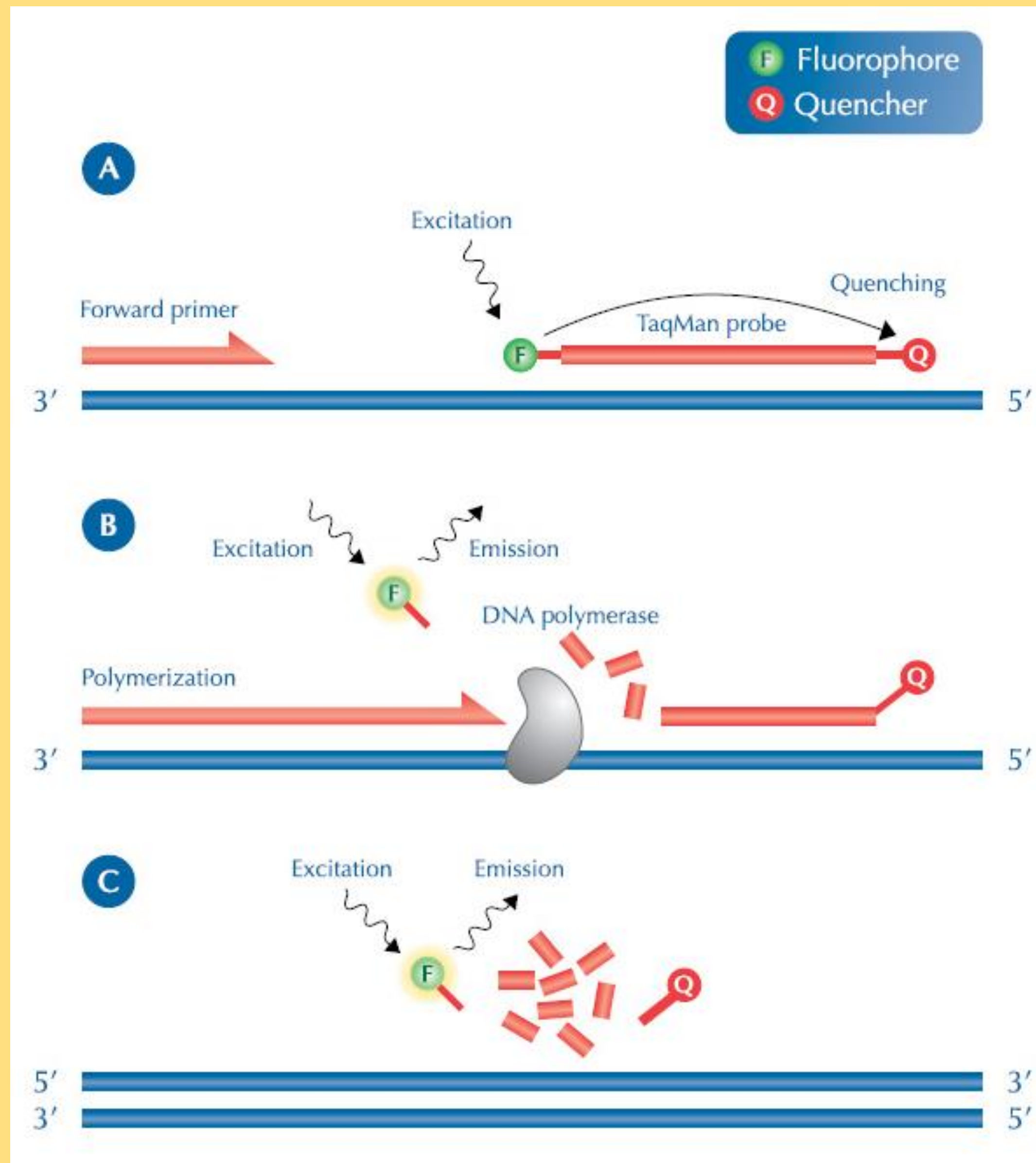
Structural probes

- Molecular Beacons
- Scorpions™
- Cyclicons™
- Nanoparticle Probes
- Conjugated Polymers/Peptide Nucleic Acid Probes

Linear probes
- Hydrolysis (TaqMan®) Probes -



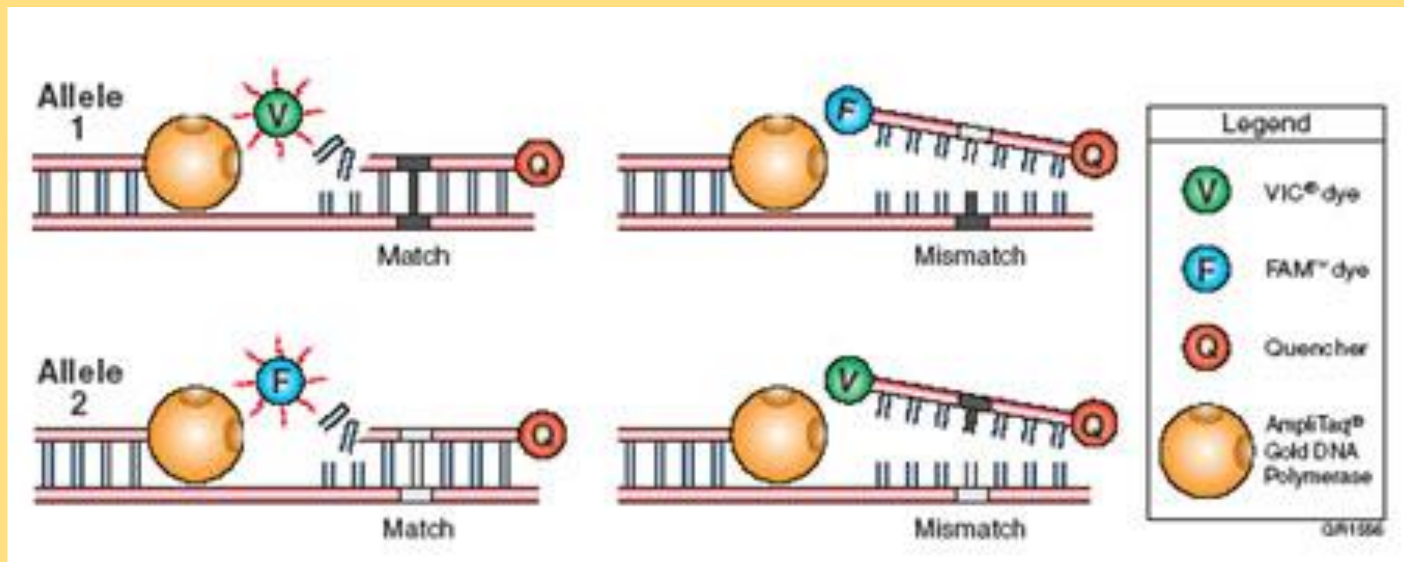
Linear probes
- Hydrolysis (TaqMan®) Probes -



www.thermoscientificbio.com

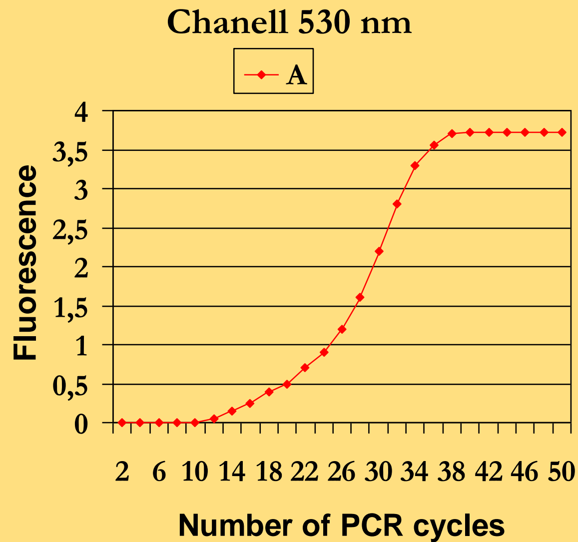
Allele specific TaqMan probes

- There are 2 probes bearing 2 different flourophores
- Each probe detects particular allele

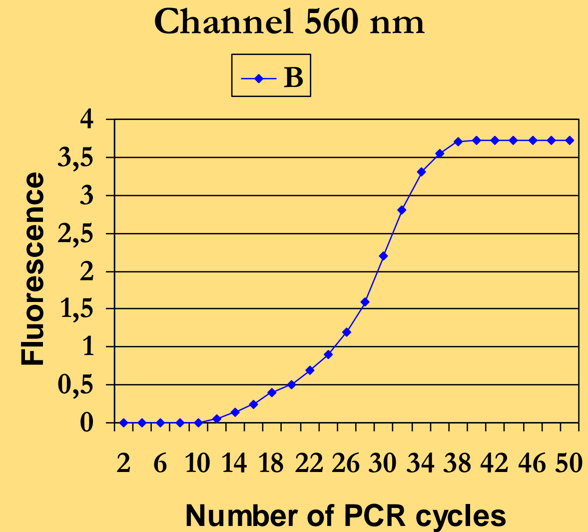


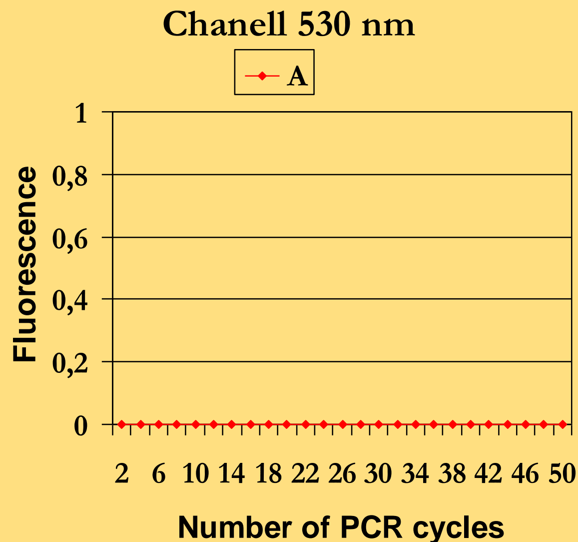
http://www.ucl.ac.uk/

Result of amplification and detection

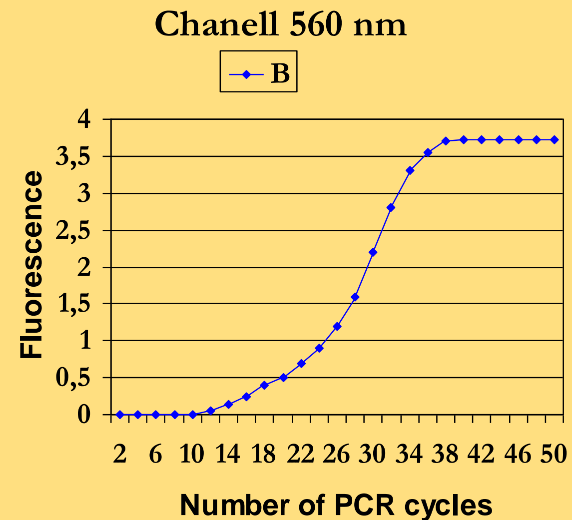


Positive reaction for A





Negative reaction for A



DNA quantification

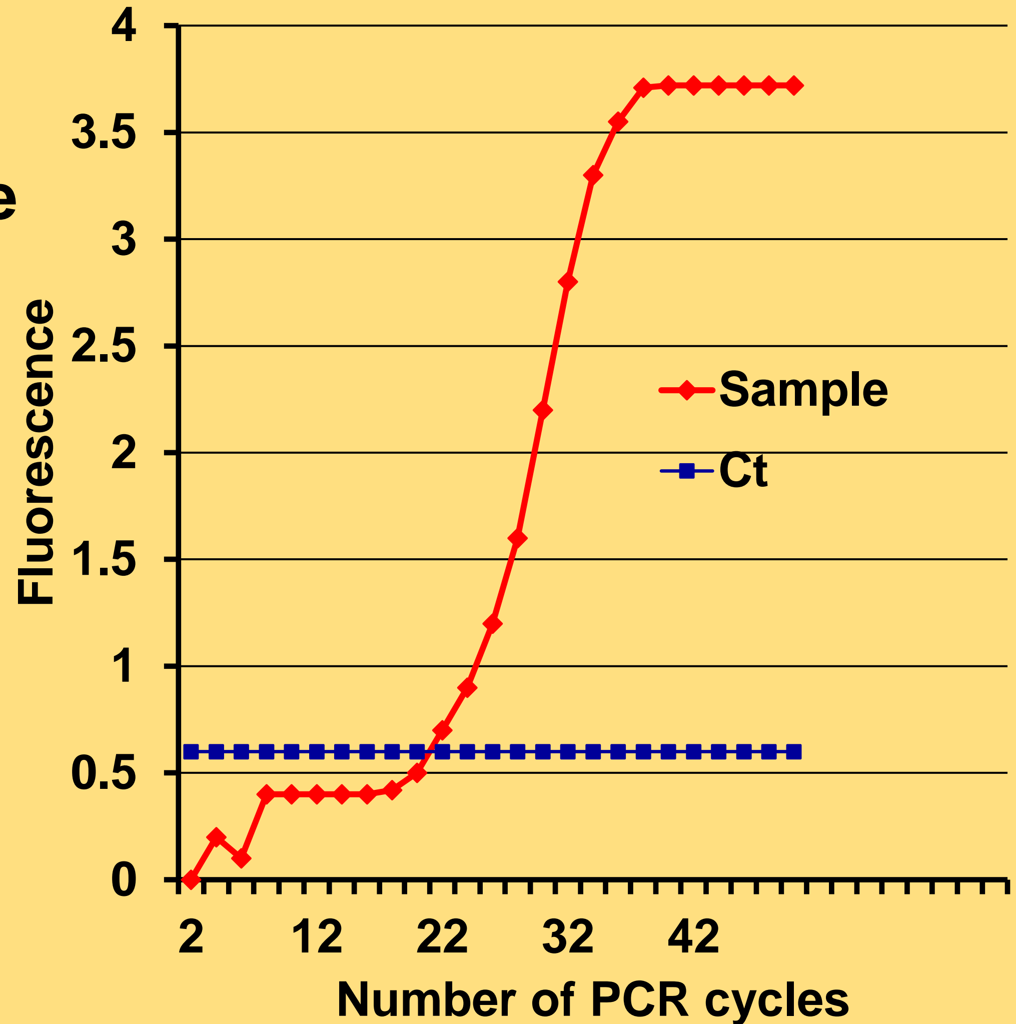
1. **Measuring Ct (threshold cycle)**

2. **Calibration curve formation**

3. **Quantification of unknown sample using the calibration curve**

Threshold cycle

- **Ct – PCR cycle in which the first change of fluorescence is detected**
- **Ct is measured by termocycler automatically**



Calibration curve

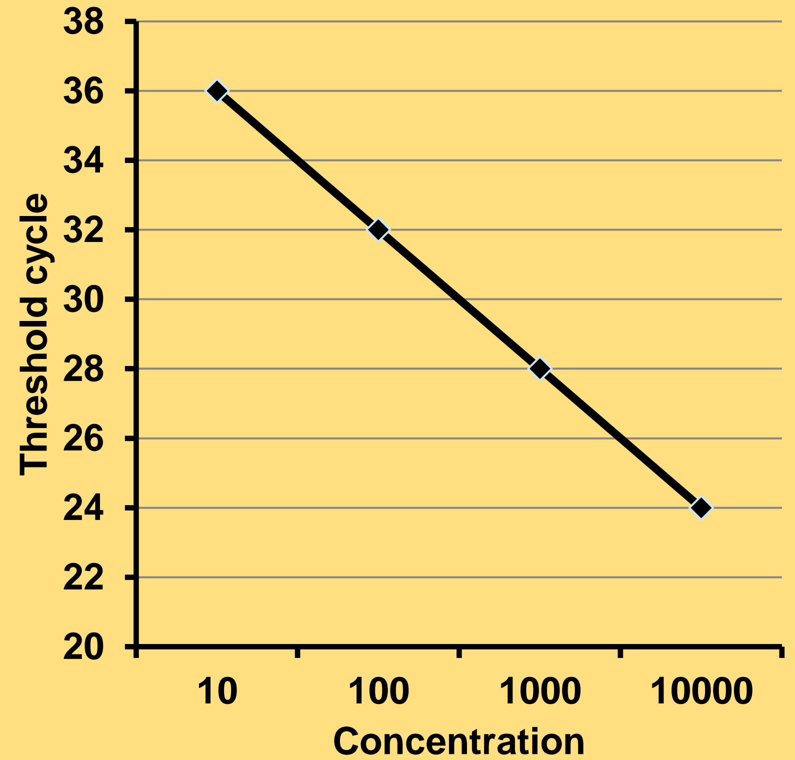
Fluorescence

4
3.5
3
2.5
2
1.5
1
0.5
0

10exp4
10exp3
10exp2
10exp1
Ct

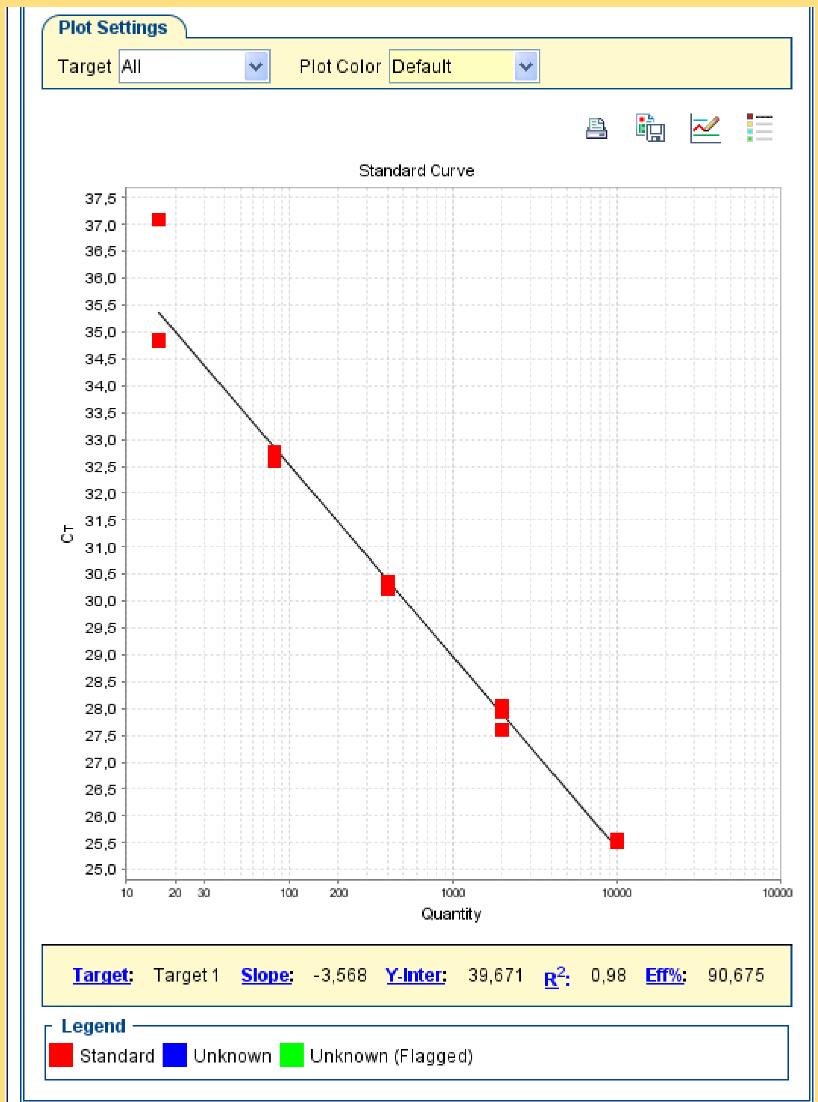
6
16
26
36
46

Number of PCR cycles



Real picture of calibration curve

Plot Settings
Plot Type: ΔRn vs Cycle
Graph Type: Log
Color: Well
Amplification Plot
0,034377
ΔRn
Cycle
Legend
A B C D E F

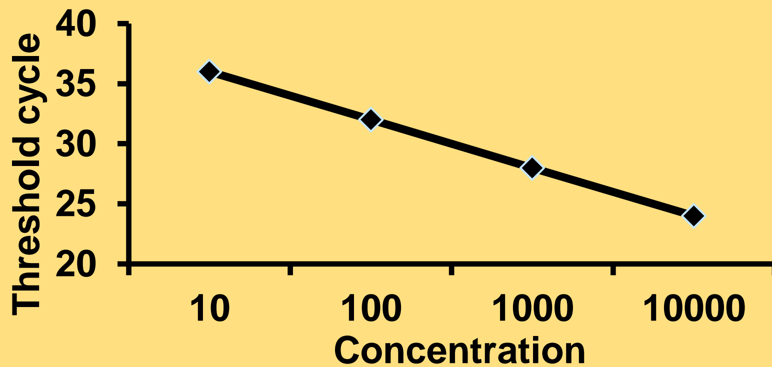


Quantitation of unknown sample

Fluorescence

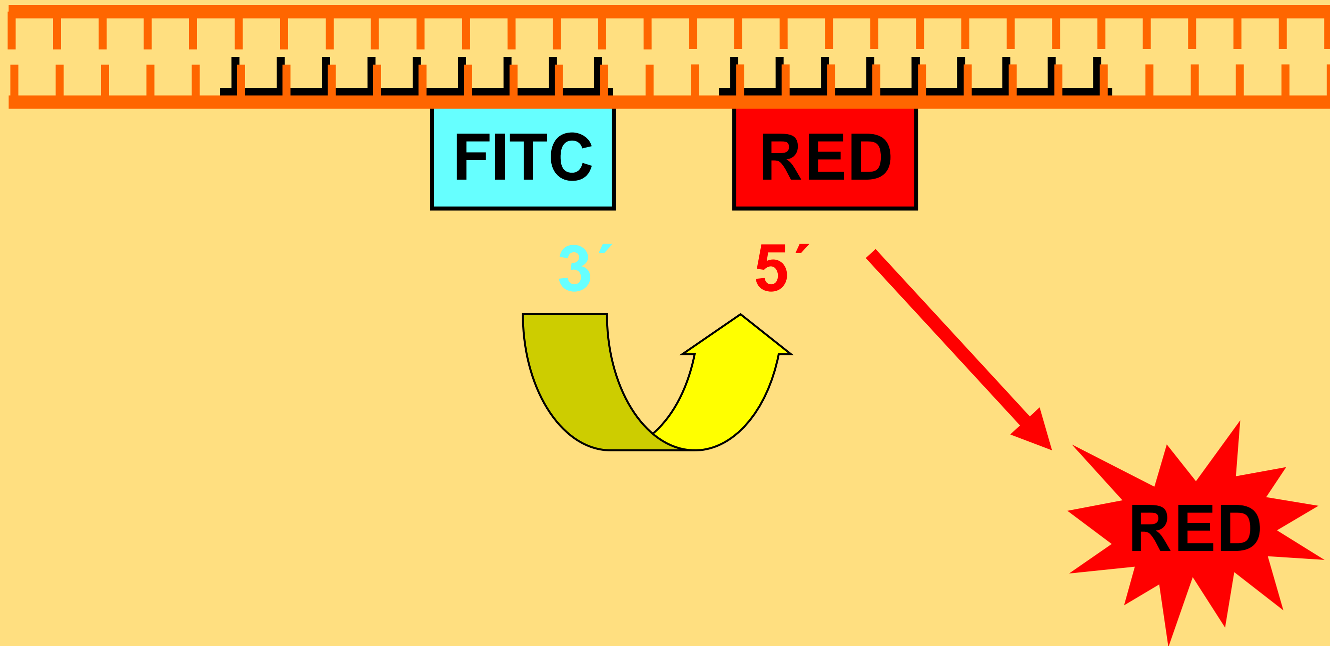
Number of PCR cycles

10exp4
10exp3
10exp2
10exp1
Ct
Sample 1
Sample 2



Sample	Type	Ct	Concentration (copies/u
1	Unknown	25,64	3,50E+03
2	Unknown	29,23	2,50E+02
K1	Standard	23,97	1,00E+04
K3	Standard	27,16	1,00E+03
K3	Standard	30,68	1,00E+02
K4	Standard	33,53	1,00E+01

Linear probes
- Hybridization Probes (FRET) -



Using FRET analysis for detection of SNP
- model example -

Standard allele 200 bp long

CCTCCTGCCTCTACCAATCGCCAGTCAGGAAGGCAGCCTACCCCGCTG
ACTCCACCTTTGAGAG**A**CACTCATCCTCAGGCCATGCAGTGGAATTCC
ACAACCTTCCACCAAACTCTGCAAGATCCCAGAGTGAGAGGCCTGTAT
CTCCCTGCTGGTGGCTCCAGTTCAGGAACAGTAAACCCTGTTCCGACT
ACTGCCTC

Mutant allele (with SNP) 200 bp long

CCTCCTGCCTCTACCAATCGCCAGTCAGGAAGGCAGCCTACCCCGCTG
ACTCCACCTTTGAGAG**C**CACTACACTCATCCTCAGGCCATGCAGTGGA
ATTCCACAACCTTCCACCAAACTCTGCAAGATCCCAGAGTGAGAGGCC
TGTATCTCCCTGCTGGTGGCTCCAGTTCAGGAACAGTAAACCCTGTTCC
GACTACTGCCTC

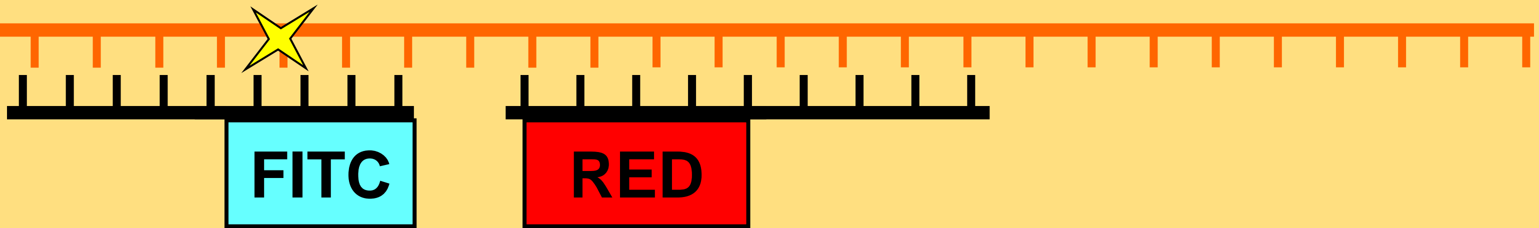
Design of probes for detection of SNP by FRET

GAGAGATCACTCA̲T-FITC RED-CCATGCAGTGGA

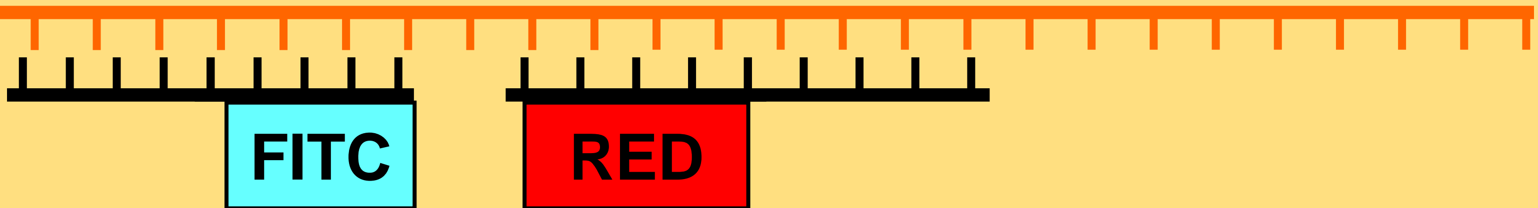
GACTCCACCTTTGAGAGATCACTCA̲(̲C̲)̲TCCTCAGGCCATGCAGTGGA

Principle of SNP analysis based of FRET probes

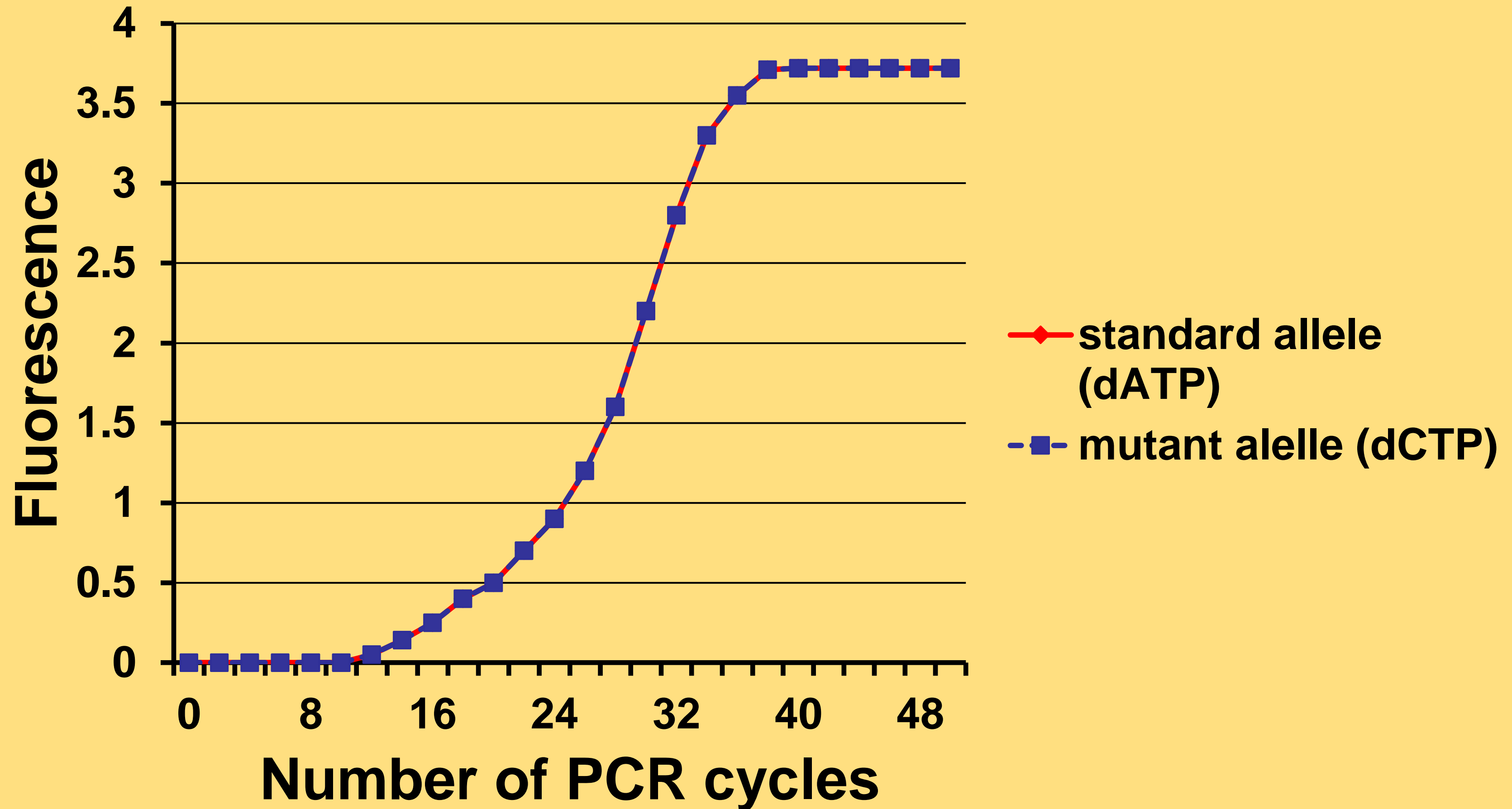
Mutant allele (dCTP) Tm= 55°C



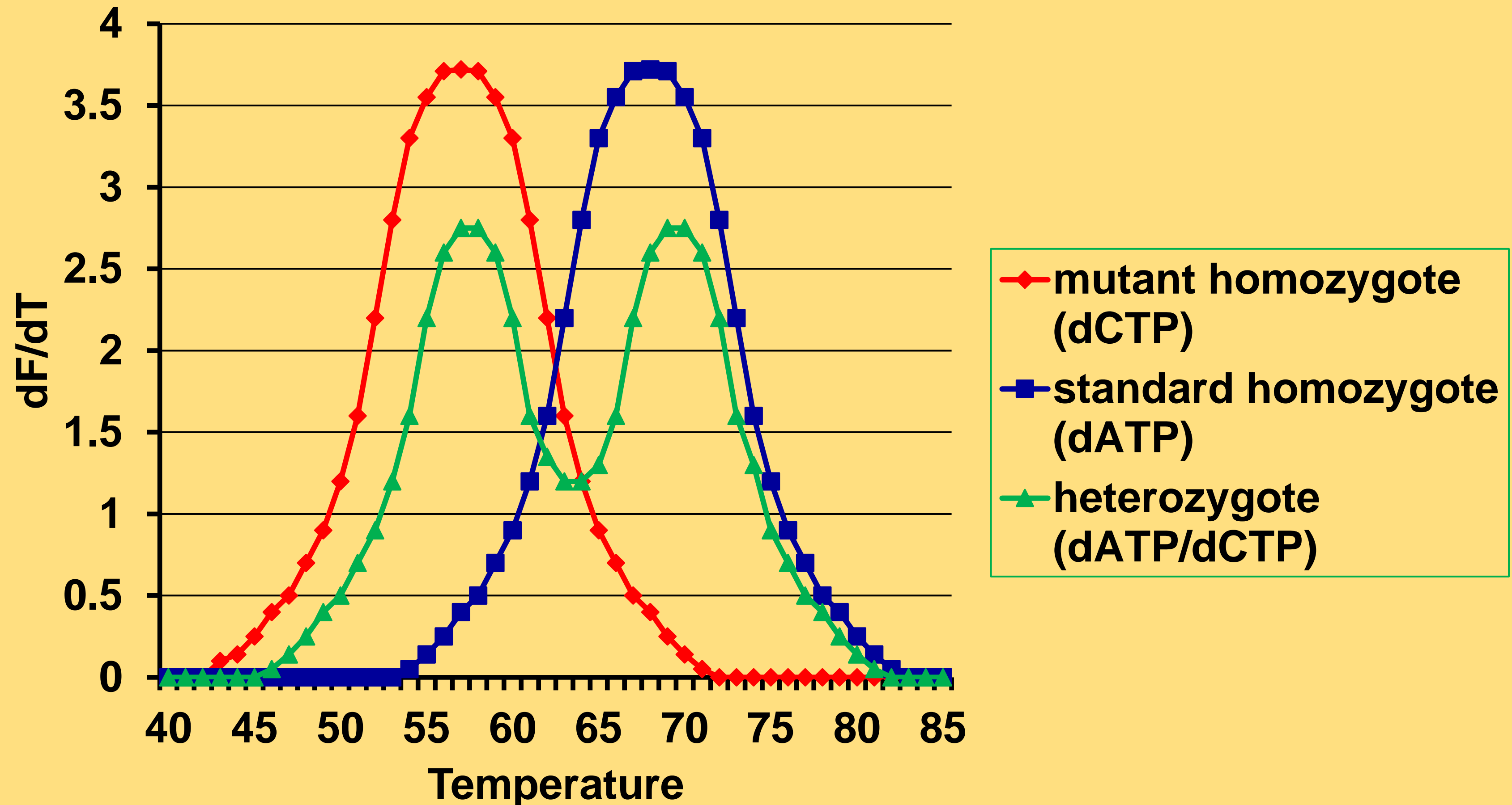
Standard allele (dATP) Tm= 62°C



Result of detection SNP by FRET - the basic data -

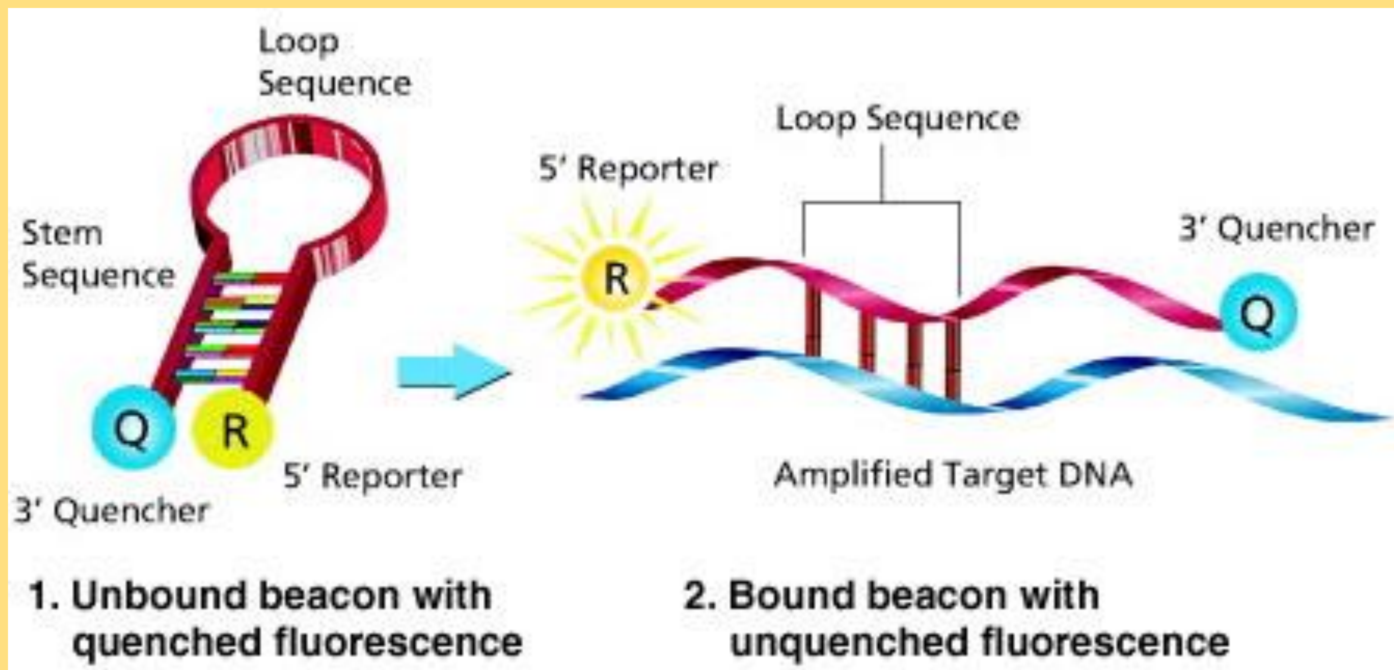


Result of detection SNP by FRET
- melting analysis -



Structure probes – molecular beacon

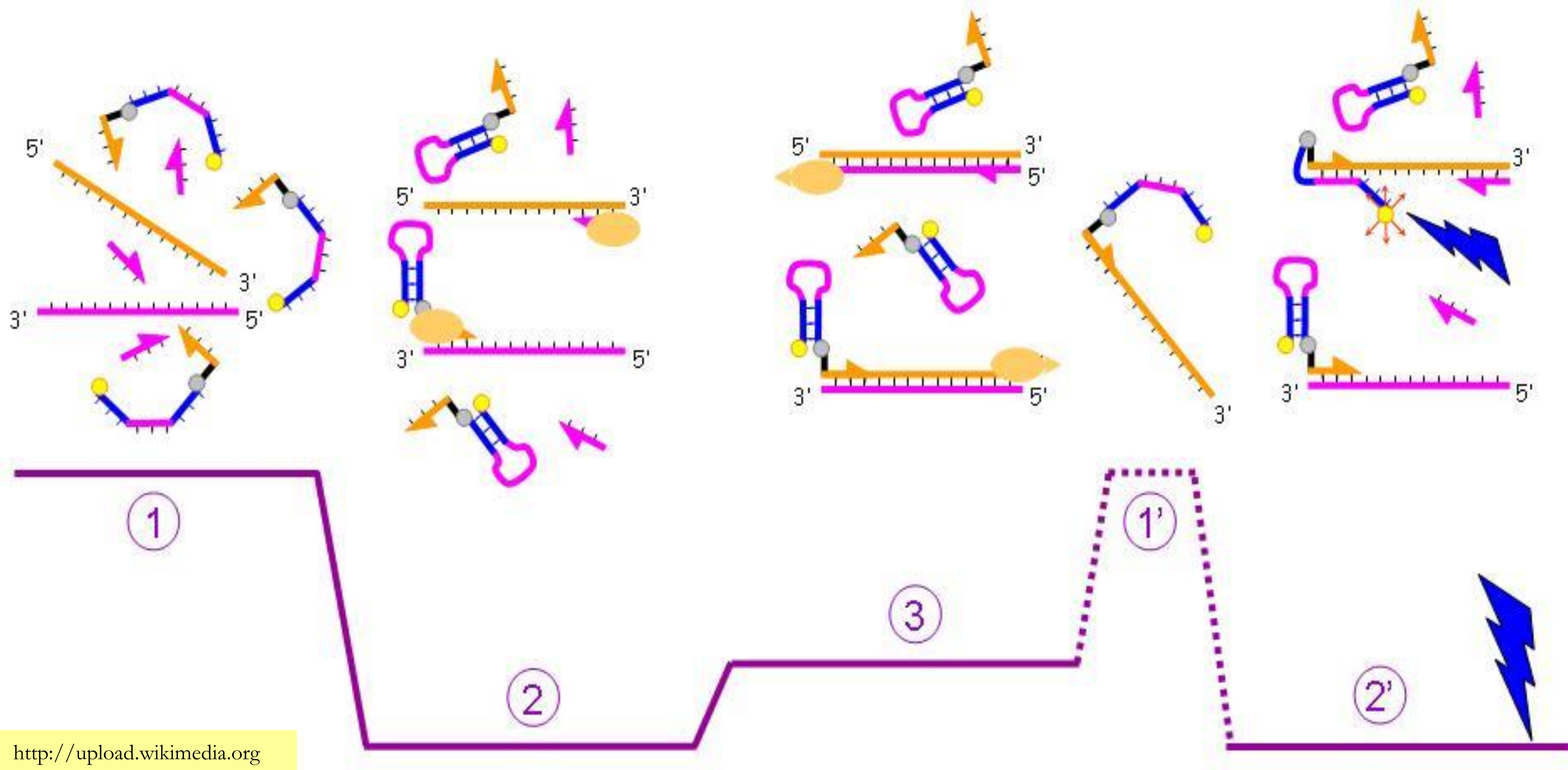
- Contains:
  - Loop with target complementary sequence
  - Stem which „closes“ the hairpin
  - Reporter and quencher
- High sensitivity – protects probe during reaction
  - SNPs detection
  - Allelic discrimination



www.sigmaaldrich.com

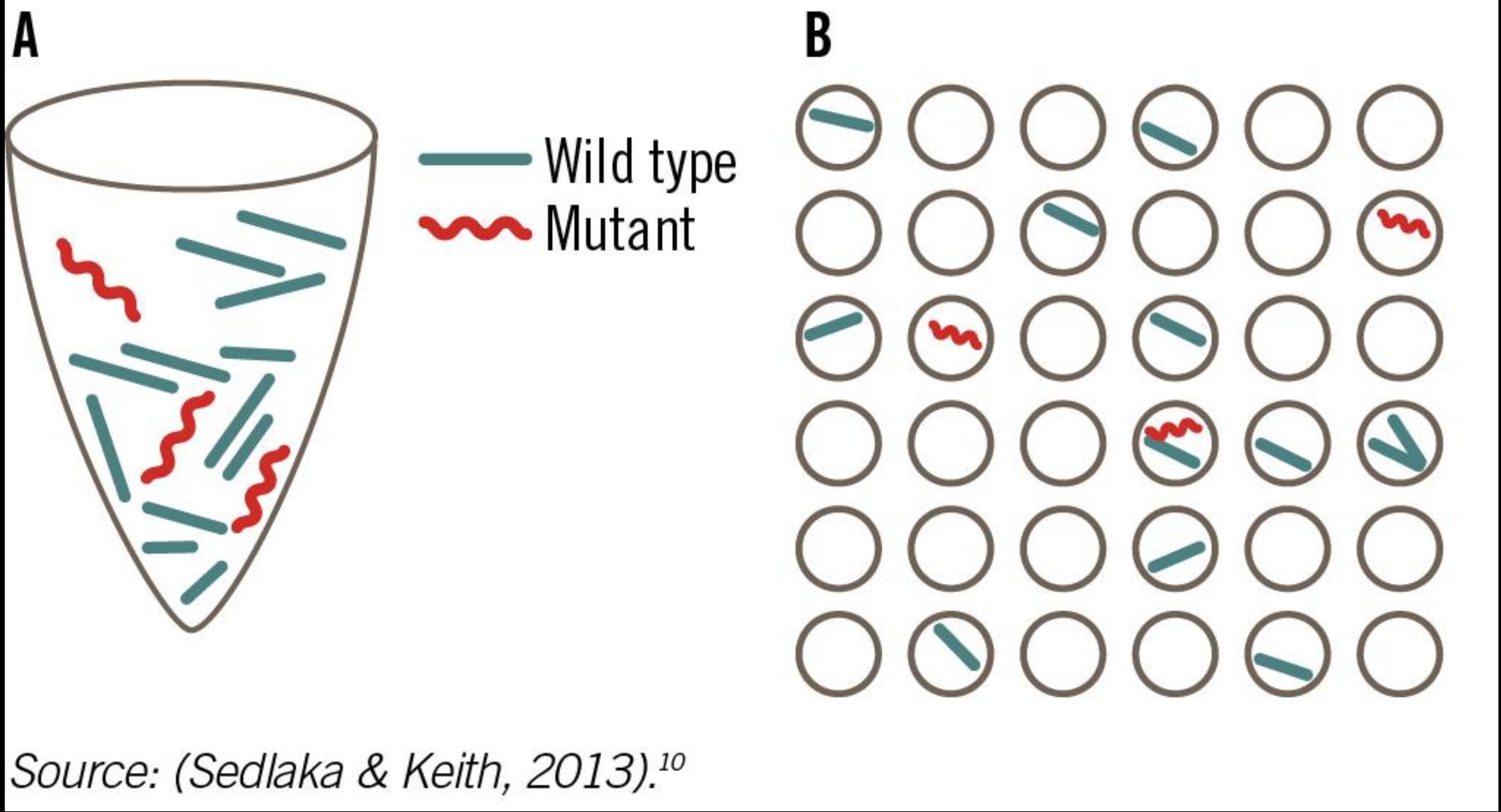
Structure probes – Scorpions

- Bi-function molecules – contain primer and probe in one molecule
- The signal is detected one cycle after probe binding



Digital PCR

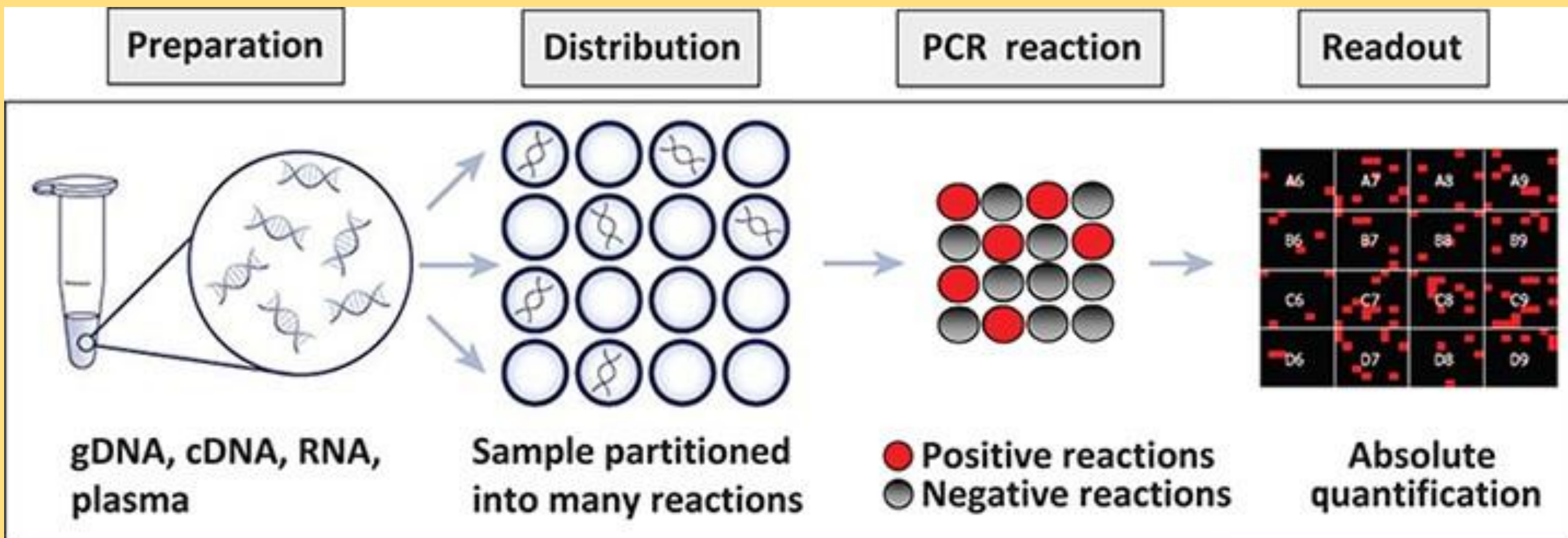
- Theorecically - **One DNA molecule** in **one reaction well**
- Molecules in wells follow Poisson distribution => „cleaning“ data by mathematical operations
- R



Source: (Sedlaka & Keith, 2013).[10]

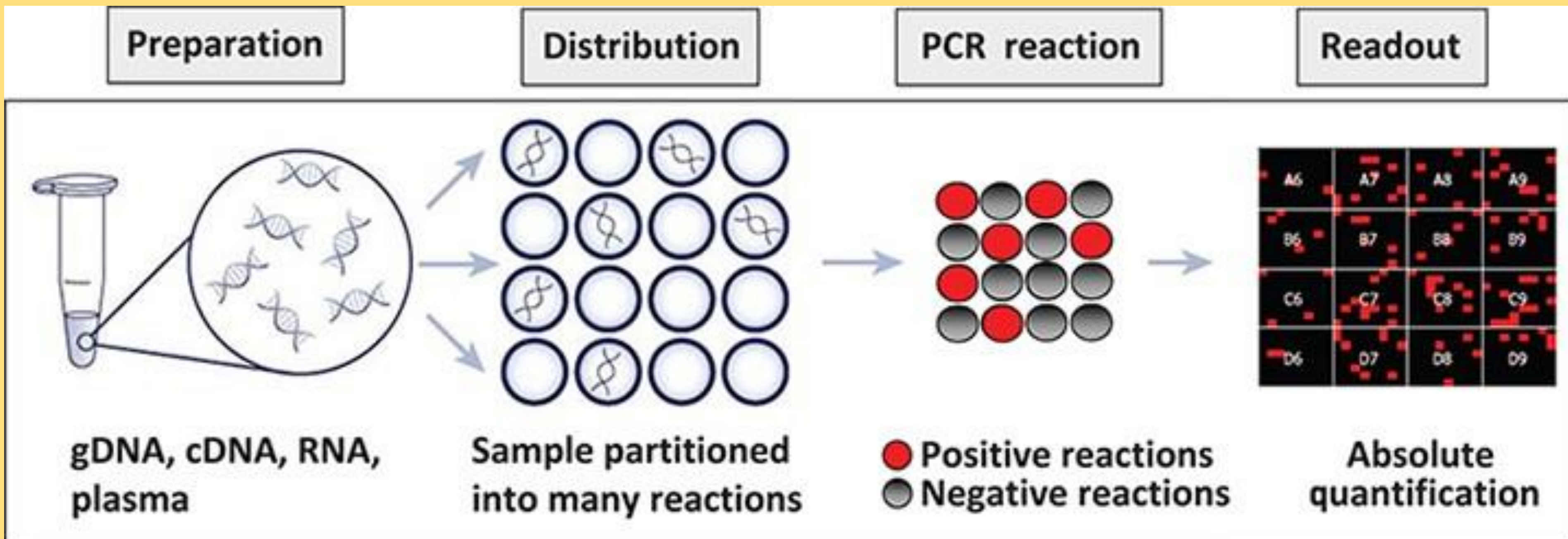
Digital PCR

- Theorecically - **One DNA molecule** in **one reaction well**
- Molecules in wells follow Poisson distribution => „cleaning“ data by mathematical operations
- Result of the reaction is **1** or **0** (positive or negative)



http://www.lifetechnologies.com/

Digital PCR



http://www.lifetechnologies.com/

Application of qPCR for ELISA

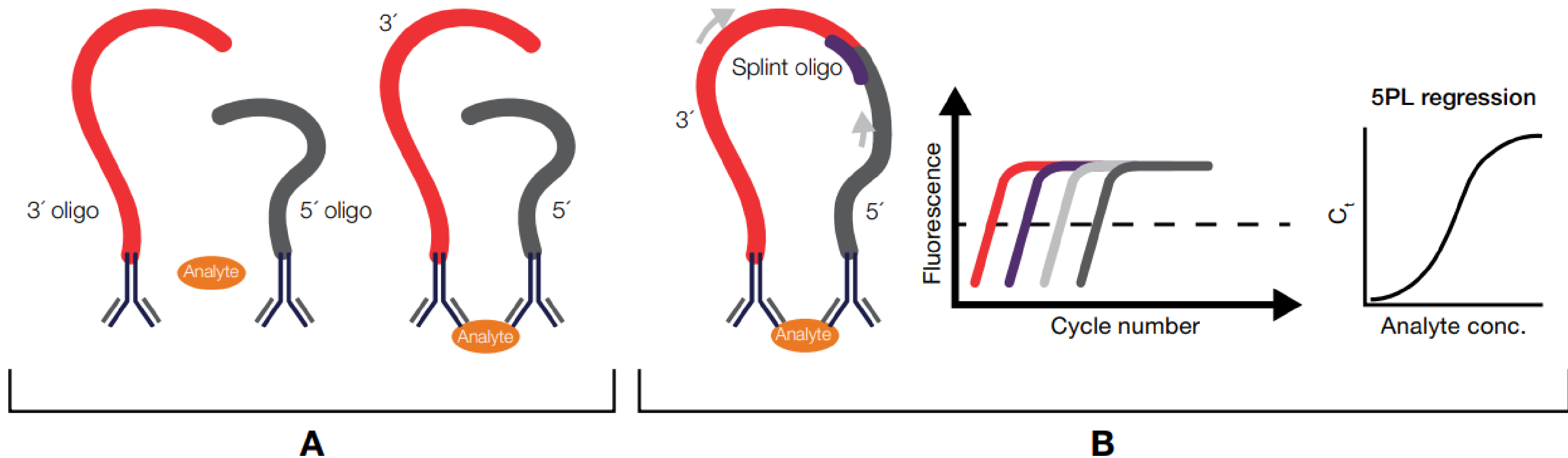


Figure 1. How ProQuantum immunoassays work.

How does it work?

ProQuantum immunoassays utilize proximity ligation assay (PLA™) technology to combine antigen–antibody binding for analyte detection with qPCR signal amplification and readout (Figure 1).

The assay is a two-step process:

A. **Analyte binding by paired antibodies conjugated to oligonucleotides**
Two antibody conjugates are provided in each kit: a 3´ end oligonucleotide and a 5´ end oligonucleotide, each conjugated to a target-specific antibody. When the antibody pair binds to two different epitopes of the protein, the 3´ and 5´ oligos come into close proximity.

B. **Ligation of the oligonucleotides by DNA ligase and amplification by Applied Biosystems™ TaqMan® qPCR Assay**
Only when the pair of antibodies binds to the analyte **(A)** can the associated oligos become bound to the complementary splint oligo and subseqently joined to each other with DNA ligase **(B)**. Following the oligo ligation, 95°C heat inactivation denatures the ligase, antibodies, and other proteins, leaving 100-base strands in concentrations proportional to the level of antibody–analyte binding in the first stage. This 100-base DNA strand serves as the amplification template for 40 cycles of qPCR using TaqMan Assays.