Quantitative Real-Time PCR

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Real-Time PCR: principle

1. The real time PCR is based on the detection and the quantification of a fluorescent transmitter during the process of amplification.

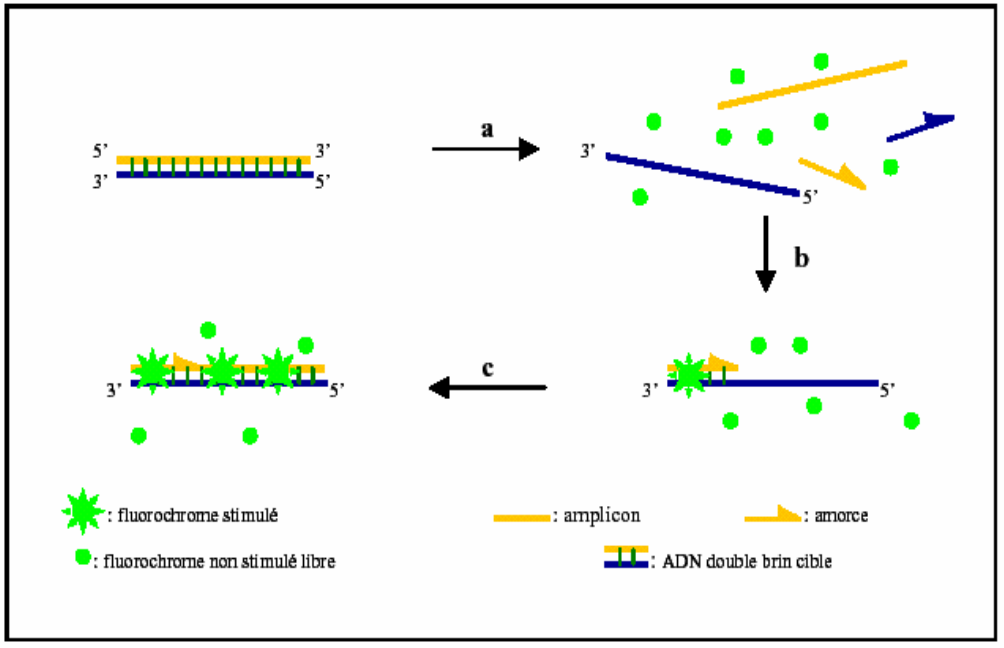
2. The increase in the fluorescent signal is directly proportional to the quantity of amplicons produced during the reaction.

3. Two general principles for the quantitative detection of amplicons:
   - agents binding to the double-stranded-DNA (SybrGreen I)
   - fluorescent probes (FAM, TAMRA, JOE, ROX,…)

4. For the fluorescent probes, there are 4 main technologies:
   - probe hydrolysis
   - hybridisation of 2 probes
   - molecular beacons
   - scorpion primer

   } Equivalent sensitivity
   Different Specificity

Agents binding to the double-stranded-DNA (SYBR Green I)

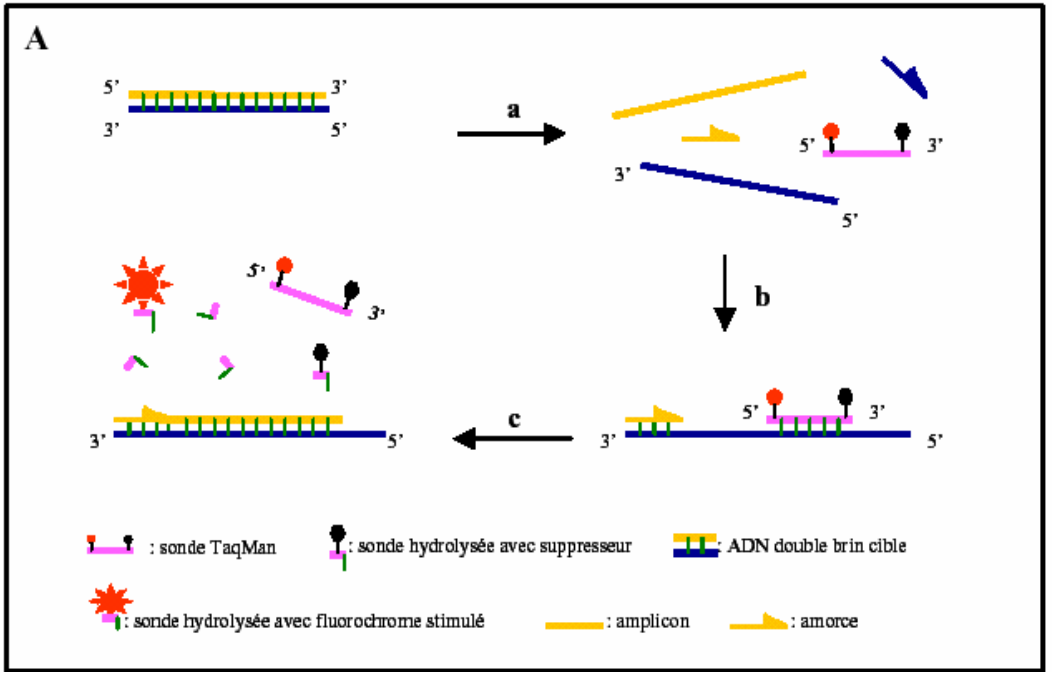


a. The free SYBR Green exhibits little fluorescence at the time of the denaturation.

b. With the temperature of pairing, some molecules bind to the nascent double-stranded-DNA.

c. During the polymerisation step, more and more of molecules bind to the nascent strand and the increase in fluorescence can be followed in real time.

Hydrolysis probes (Taqman)

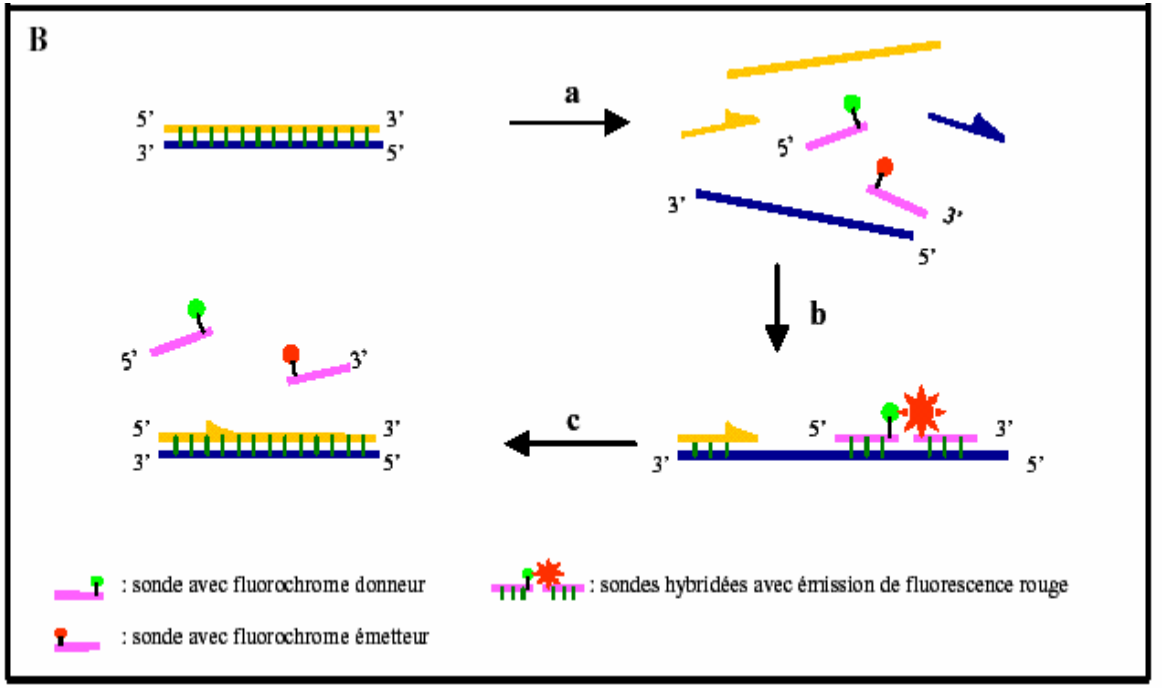


a. During the denaturing step, the probe is free on solution.

b. During the annealing step, both probes hybridise to their target sequence. The proximity of the fluorochrome allows the inhibition of fluorescence.

c. The polymerase moves and hydrolyses the probe. The transmitting fluorochrome is released from the environment of the suppressor thus allowing the emission of fluorescence.

Hybridisation probes (HybProbes)

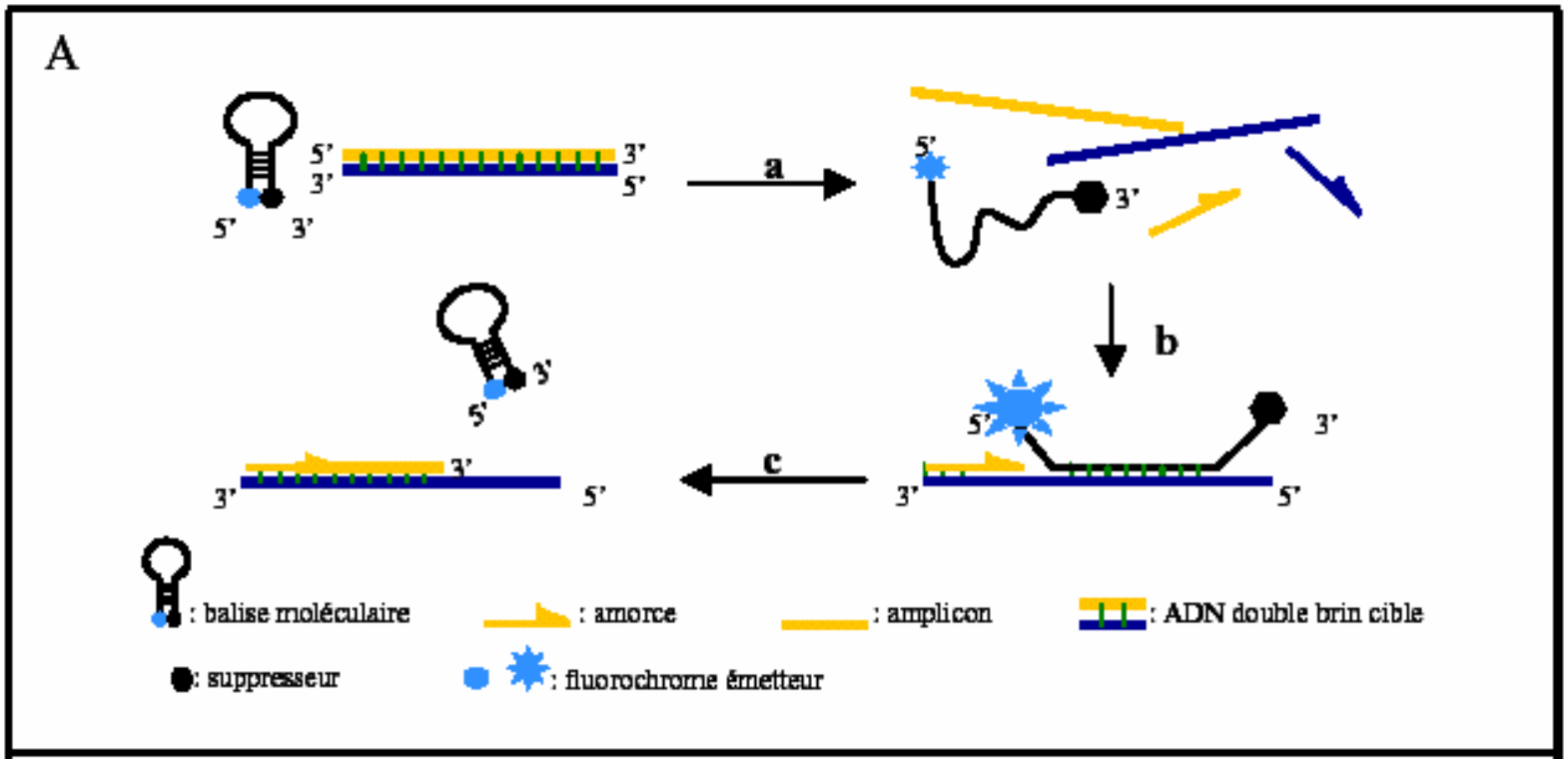


a. During the denaturing step, in solution the 2 probes are apart.

b. During the annealing step, both probes hybridise to their target sequence. The proximity of the fluorochrome allows the red emission of fluorescence.

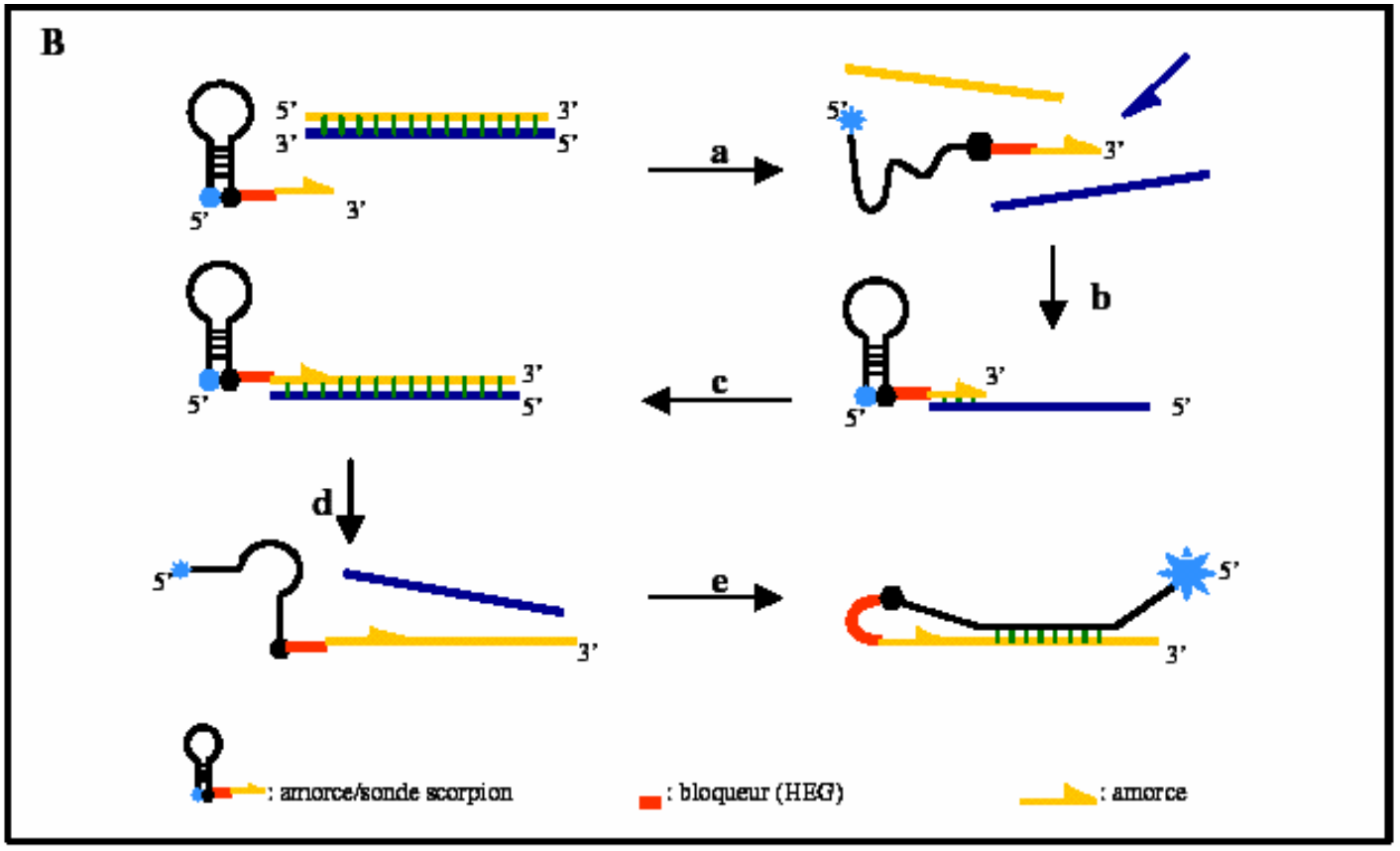
c. The probes turn over free in solution.

Molecular beacons



- High specificity + high precision
- When T°C<Tm, hairpin structure → no hybridisation and no fluorescence

Scorpion primer



- Suppressor HEG prevents the replication of the molecular beacon
- One of the best method

The SYBR Green: advantages and disadvantages

1.Advantages:

- Economic
- Easy to use
- Has more sensibility than the ethidium bromide (another intercalating agent)
- Does not inhibit the reaction of amplification
- Does not require any fluorescent probe, thus does not require any particular expertise for the design of the probes
- Is not affected by mutations in the target DNA

2.Disadvantages:

- Impossible to make sure of specificity of amplicons
- Bad pairing can lead to positive forgeries or an over-estimate of the quantification
- The emission of fluorescence can be skewed by the molecular mass of the DNA amplified by a longer amplicon which will fix more fluorescent molecules compared to a shorter amplicon in the same reaction
- Still unspecified mutagen capacity

Hydrolysis probes: advantages and disadvantages

1. Advantages:

- Increased specificity: the specificity of hybridisation between the fluorescent probe and the sequence of DNA significantly reduces the emission of non-specific fluorescence due to bad pairings or primers dimers.

- Better capacity of multiplexing: reactions multiplex can be elaborate by using distinct transmitting fluorochromes related to different probes in a PCR reaction.

2. Disadvantages:

- Taqman technology is less effective and less flexible device that other technologies in real time for the detection of specific mutations.

- To respect the principles of design of the probes

Probe design

1. 30 bases in length
2. G-C content of around 50 %
3. The probes should not overlap with, or have sequence complementarity with either of the primers
4. Probes should not contain a G at their 5’ ends, because such an arrangement quenches reporter fluorescence, even after cleavage
5. A Tm of 5 to 10°C greater than that of the primers

Hybridisation probes: advantages and disadvantages

1. **Advantages**:

    - High specificity
    - High flexibility for probe design
    - As the probes are not hydrolysed, they are used at each cycle

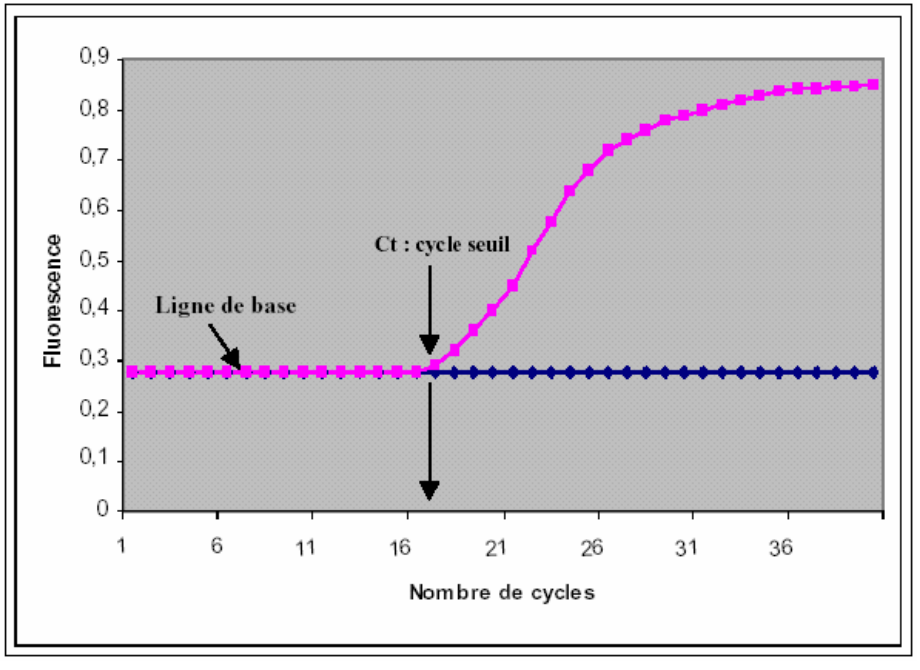
2. **Disadvantages**:

    - Taqman probe design

Threshold cycle = Ct (1)

-The concept of the threshold cycle is at the heart of accurate and reproducible quantification using fluorescence-based PCR.

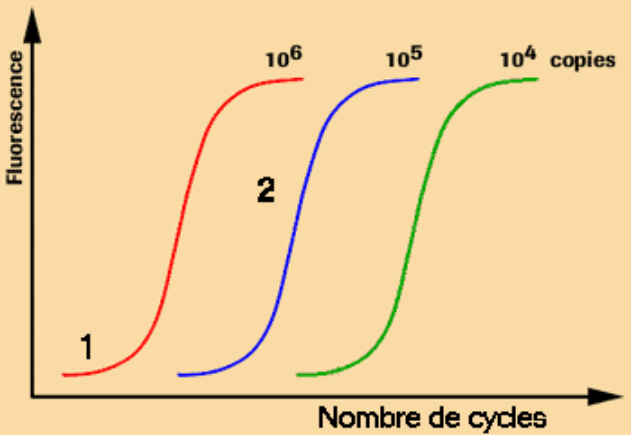
-It corresponds to the cycle from which one observes a statistically significant increase in standardized fluorescence



Threshold cycle = Ct (2)

- The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point for which the fluorescence signal is first recorded as statistically significant above background. This point is defined as the Ct.

- The threshold cycle will always occur during the exponential phase of amplification.



Threshold cycle = Ct (3)

-Quantification is not affected by any reaction components becoming limited in the plateau phase
-The Ct value can be translated into a quantitative result by constructing a standard curve

$$R = 2^{-(\Delta Ct1 - \Delta Ct2)}$$

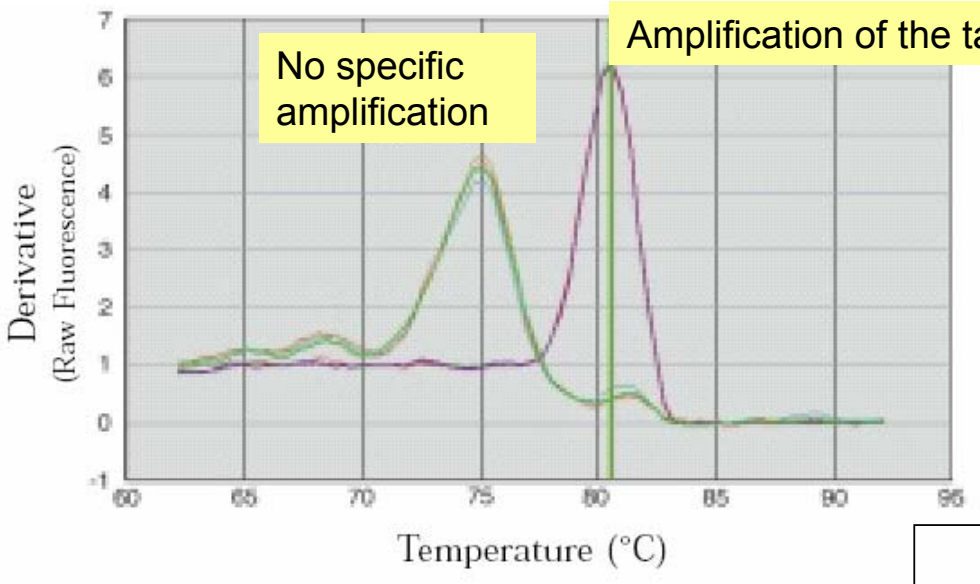
ΔCt1 = ΔCt target gene = Ct target gene with treated sample – Ct same gene with sample calibrator

ΔCt2 = ΔCt standardizing gene = Ct standardizing gene with treated samples – Ct same gene with sample calibrator

⇒ The difference between two samples is considered as significant from one Ct.

Development

Dissociation curve



Standard curve