Peste des Petits Ruminants: Diagnosis training

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LA RECHERCHE AGRONOMIQUE POUR LE DÉVELOPPEMENT

Peste des Petits Ruminants diagnosis

Part 1

ELISA

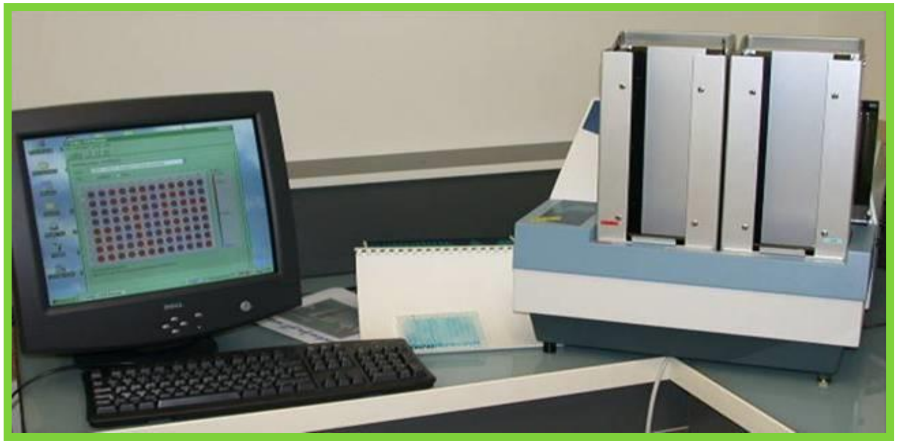
ELISA

→ **ELISA = Enzyme Linked ImmunoSorbent Assay**

- **The Enzyme Linked ImmunoSorbent Assay is an immuno-enzymatic test of detection**

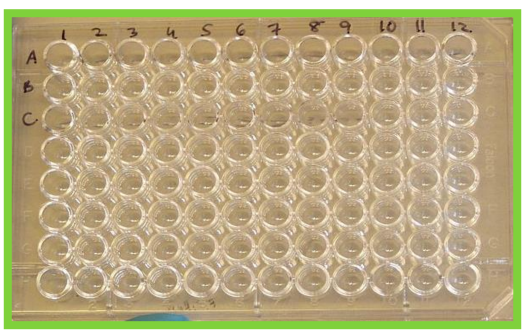
- **The reaction antigen/antibody is visualized by a colored reaction of a substrate with an enzyme linked to the antibody**

Materials and equipment required



Spectrophotometer

Multichannel pipette



96- well polystyrene plate

micropipettes

Important points for ELISA test

- **Pipetting → the pipette must be regularly verified**

- **Washing → use high quality purified water for preparation of reagents**

  → be careful for the cleaning of the material

- **Temperature → incubate at the correct temperature (37± 5)°C or (21± 5)°C**

- **Timing → use a timer**

- **Spectrophotometer → use the correct wavelength**

PPR c-ELISA

IDVET innovative diagnostics

ID Screen®
PPR Competition

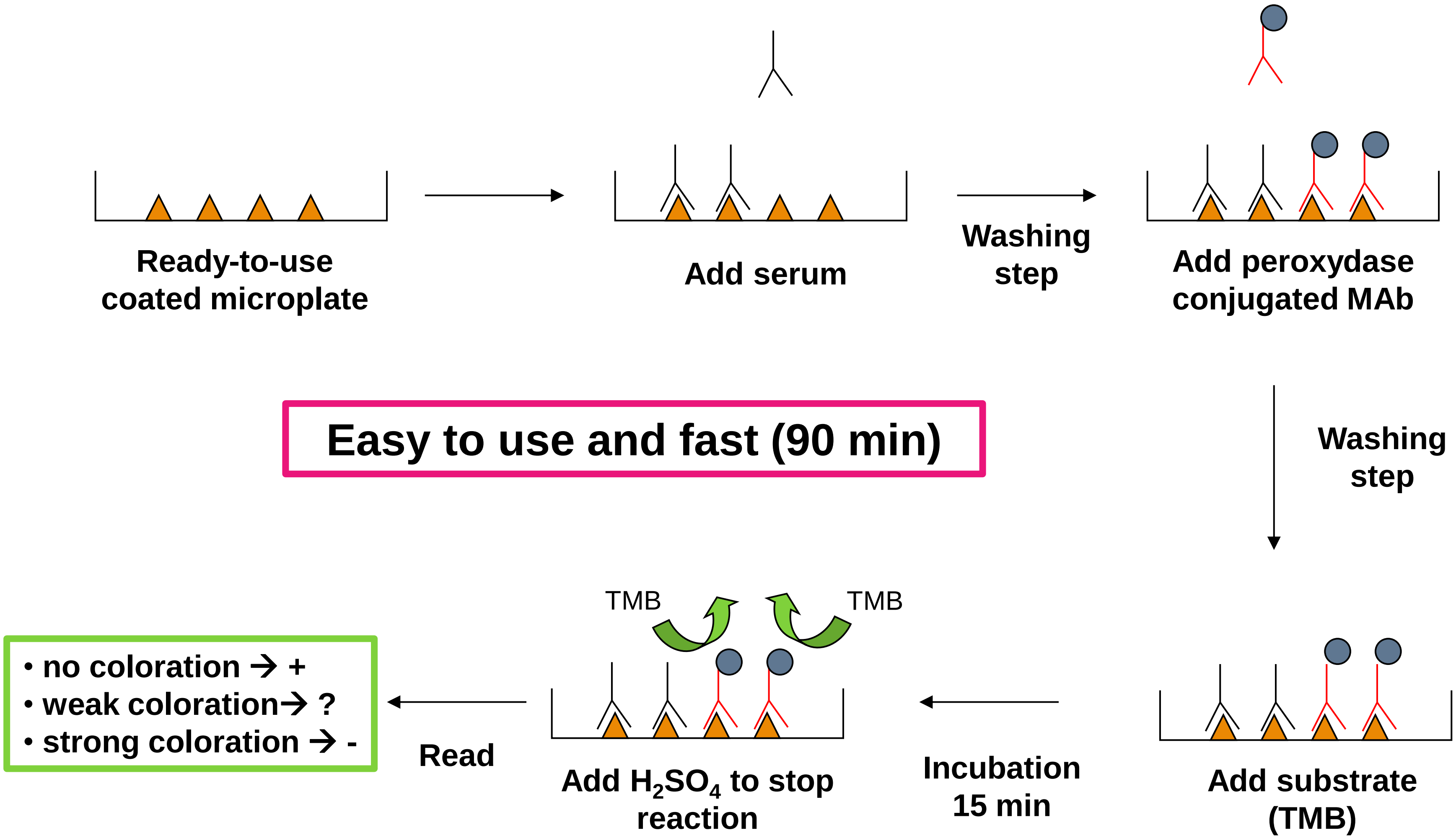
Kit for the detection of antibodies against the Peste des Petits Ruminants (PPR) virus in sheep and goat serum and plasma by competitive screening ELISA

For *in vitro* use

AFAQ ISO 9001 VERSION 2000

PPRC ver 1209 GB

PPR c-ELISA


Ready-to-use
coated microplate
Add serum
Washing
step
Add peroxydase
conjugated MAb
Easy to use and fast (90 min)
Washing
step
TMB
TMB
• no coloration → +
• weak coloration→ ?
• strong coloration → -
Read
Add H_2SO_4 to stop
reaction
Incubation
15 min
Add substrate
(TMB)

Testing procedure

- Allow all the reagents to come to room temperature (21±5)°C before use
- Homogenize all reagents by inversion or vortex

1. add 25 µL of dilution buffer 13 to each well
2. add 25 µL of the positive control to well A1 and B1
3. add 25µL of negative control to well C1 and D1
4. add 25 µL of each sample to be tested to the remaining wells
5. Incubate 45 min at 37°C
6. Wash each well 3 times with approximately 300 µL of Wash solution. Avoid drying of the wells between washings
7. Prepare the conjugate 1X by diluting the conjugate 10X to 1/10 in dilution buffer 4
8. Add 100 µL of the conjugate 1X to each well
9. Incubate 30 min at 21°C
10. Wash each well 3 times with approximately 300 µL of Wash solution. Avoid drying of the wells between washings
11. Add 100 µL of the substrate solution to each well
12. Incubate 15 min at 21°C in the dark
13. Add 100 µL of stop solution to each well in order to stop the reaction
14. Read and record the O.D. at 450 nm

Validation of the test

The test is validated if:

- **The mean value of the negative control O.D. is greater than O.7**

→ $OD_{NC} > 0.700$

- **The mean value of the positive control O.D. is less than 30% of the OD_{NC}**

→ $OD_{PC} / OD_{NC} < 0.3$

Interpretation

- **For each sample, calculate the competition percentage:**

$$S/N = (OD_{sample}/OD_{NC}) \times 100$$

Result	Status
S/N ≤ 50%	POSITIVE
50% < S/N ≤ 60%	DOUBTFUL
S/N > 60 %	NEGATIVE

Peste des Petits Ruminants diagnosis

Part 2

PCR

PPR diagnosis by RT – PCR (1)

→ RT = Reverse Transcription

- **to synthesize cDNA from RNA samples**

→ PCR = Polymerase Chain Reaction

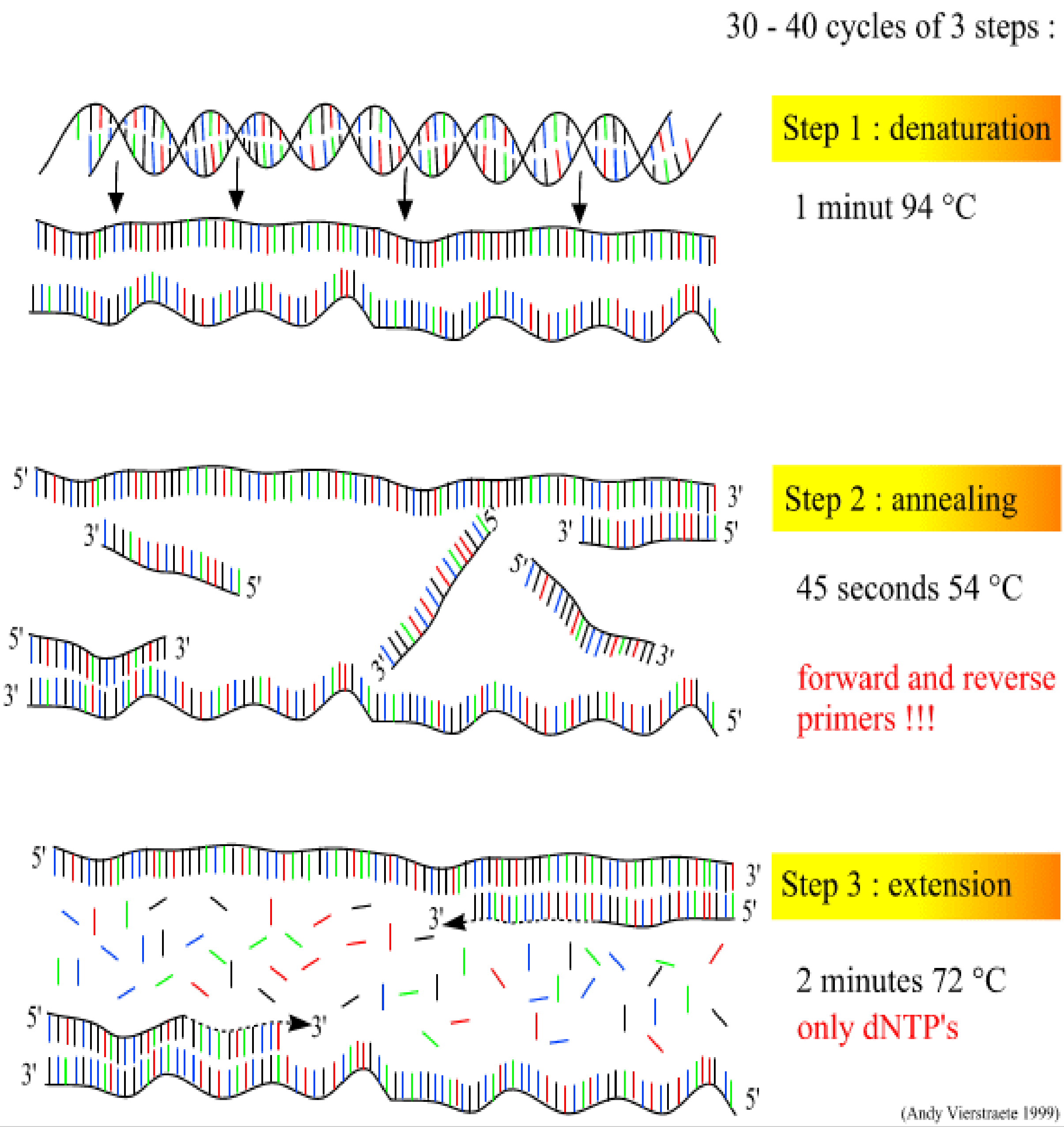
- **to amplify DNA**

Pcr.exe

→ RT - PCR = Reverse Transcription + Polymerase Chain Reaction

- **to synthesize cDNA from RNA samples and then amplify DNA**

PCR : Polymerase Chain Reaction



Reaction tube content:

-DNA to amplify : 50ng to 500ng

-2 sépcifics primers of the targed DNA

-dNTP

-Taq Polymerase

Final volume : 20 µl to 100 µl

Equipment : thermocycler

PPR diagnosis by RT – PCR (2)

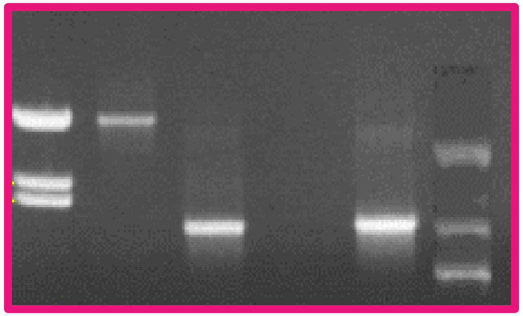
→ 3 main steps

1. extraction | **2. amplification** | **3. analysis**

RNA extraction from samples

cDNA synthesis (RT)

One-step RT - PCR



Electrophoresis on 1.5% agarose gel

Limits of this technique

- need results visualization on agarose gel
- need use of Ethium Bromide (= risk)
- could be a source of contamination for the laboratory
- difficulties to record data
- qualitative results only (- or +)
- need 4 to 8 hours to obtain final results for tested samples

→ **Development of Real-Time PCR protocols**

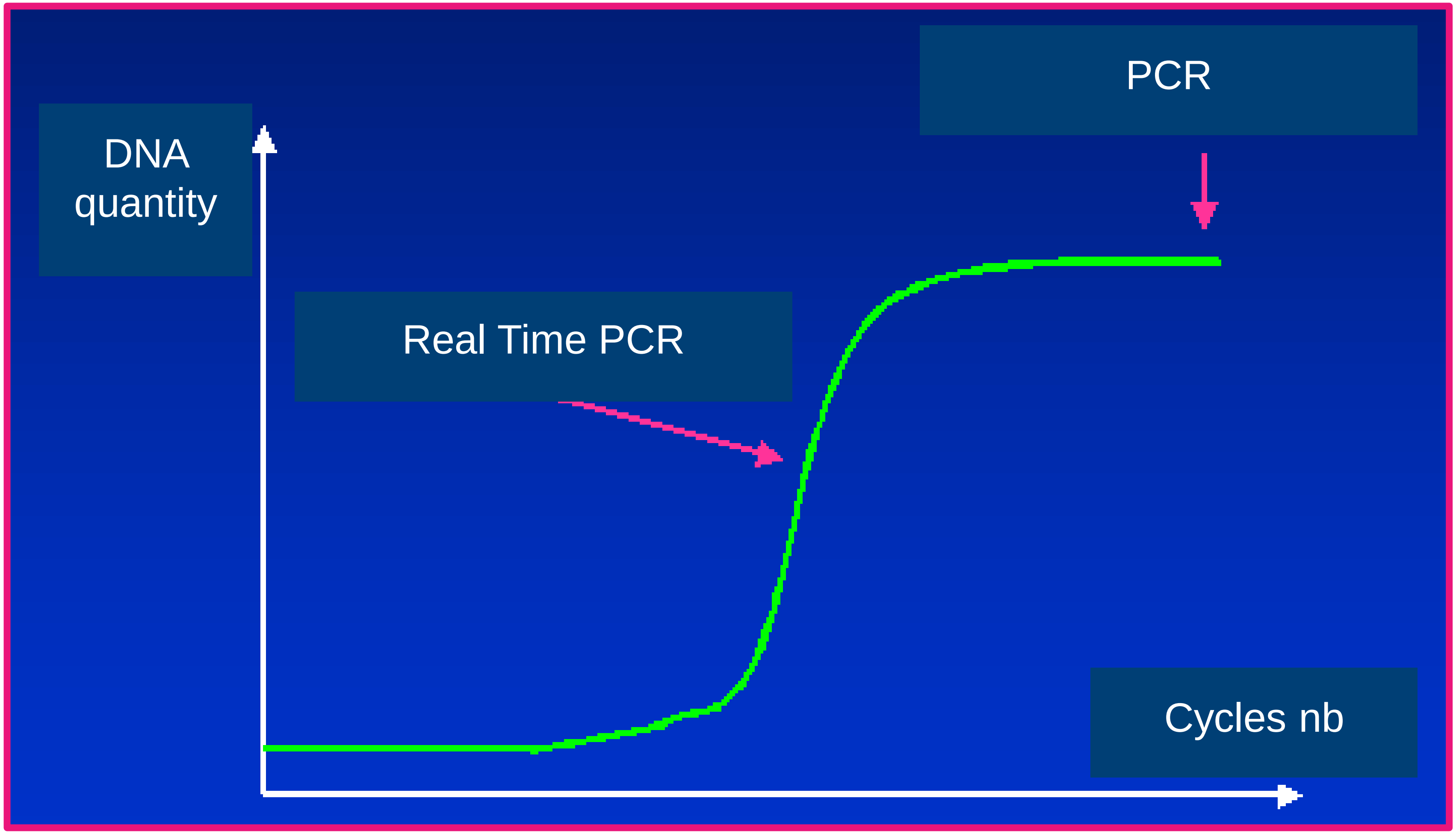
PPR one-step RT-PCR protocol

- Extract RNA from samples using 150 µl of the viral suspension
- One-step RT-PCR

	One step Qiagen Mix 1 Reac (50µl)	Mix10 reactions	Mix 20 reactions
RNA	5	5	5
5X Tp qiagen	10	100	200
dNTPmix qiagen	2	20	40
Q solution	10	100	200
NP3 10uM	3	30	60
NP4 10uM	3	30	60
Qiagen enz mix	2	20	40
H_2O	15	150	300

Programme	
RT	30 min 50°C
Denaturation	15 min 95°C
40 cycles	30 sec 94°C 30 sec 60°C 1 min 72°C

PCR versus Real Time PCR



The Realtime PCR

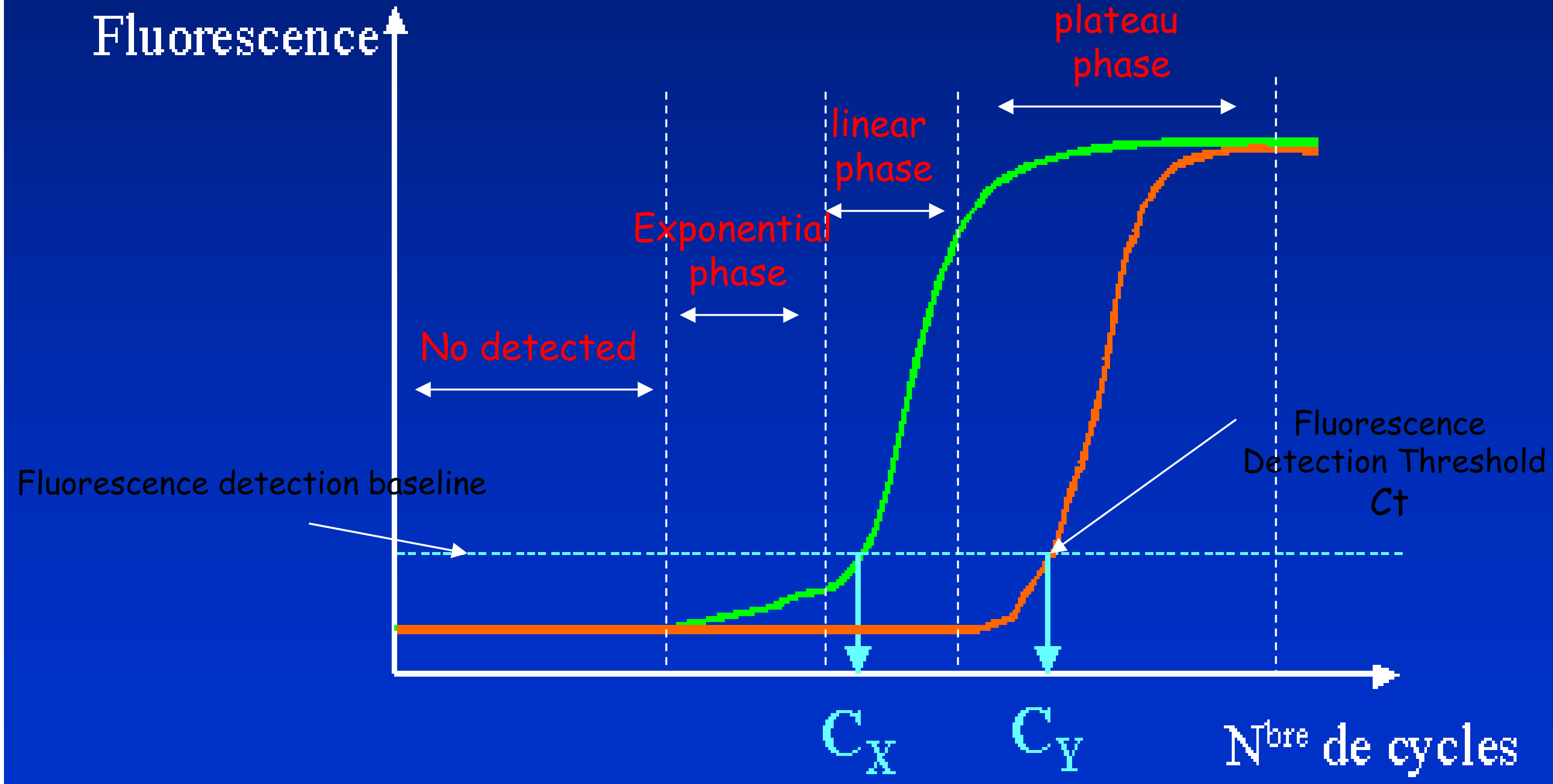
« Quantitative PCR », « Real time PCR », « Kinetic PCR », « Real time RT PCR » (sur ADNc), « FRET PCR » *(Förster resonance energy transfer)*

•PCR product formation followed over the time by in situ fluorescence emission (many possible approaches).

•Determination of the number of cycles from which the PCR product is detectable : threshold cycle number = "Cycle threshold" (or "crossing point" = Ct.

•Time of appearance of the threshold signal (= Ct) depending on the quantity of matrix initially present in the amplified sample.
Linearity over 5 to 7 orders of magnitude.

Monitoring de l'amplification



Used for quantify:

- **Genomic DNA : for example, detection/quantification of microorganisms in tissue**
- **Or cDNA produce from mRNA** after reverse transcription

Quantitative PCR relies on continuous measurement of fluorescence release during PCR cycles reflecting PCR product accumulation.

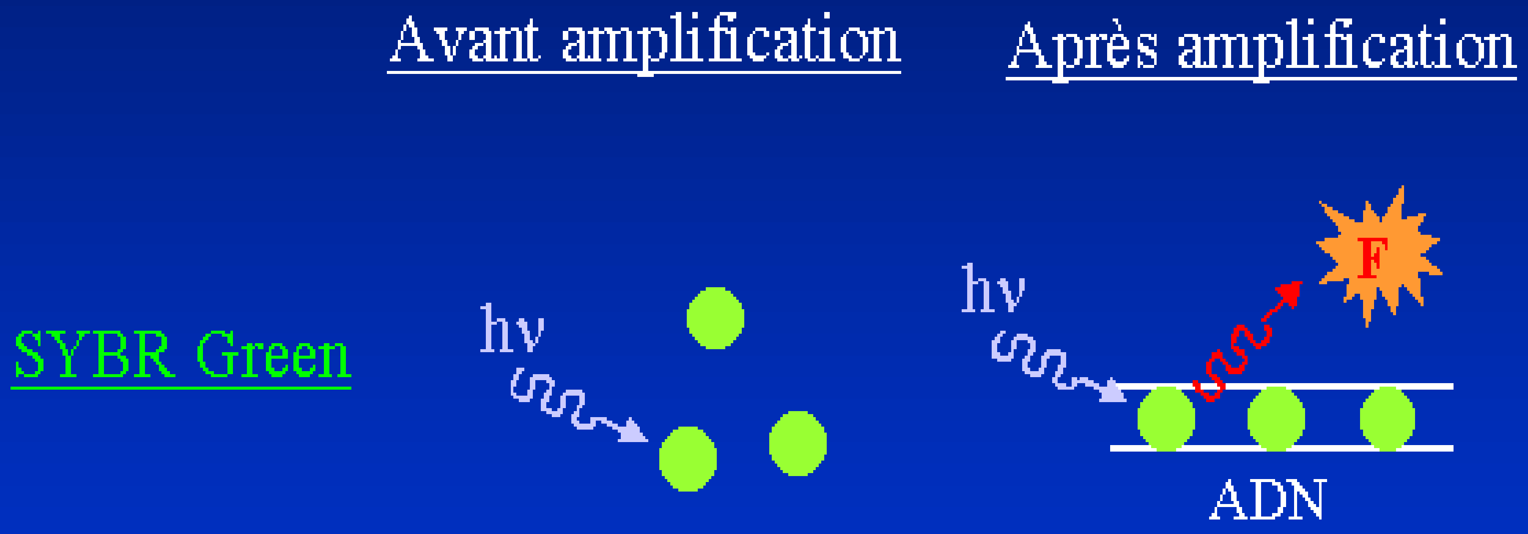
Main techniques of fluorescence emission during PCR

We distinguish several approaches of QPCR according to the mechanisms leading to the increase of the fluorescence during the amplification:

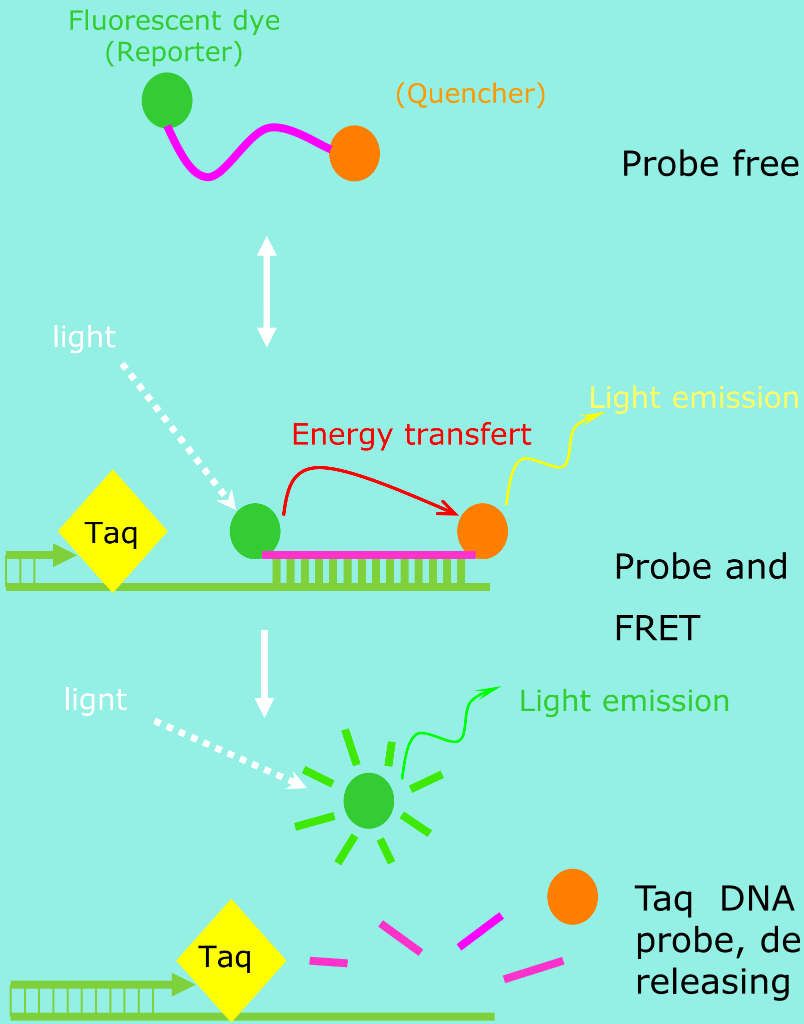
- Fluorescence emission by an intercalating agent (e.g. SybrGreen® I) inserted into double-stranded DNA

- Fluorescence release based on :
    - The 5'-3 'exonuclease activity of the Taq polymerase exerted on a probe (eg TaqMan®), the degradation of which during the elongation of the PCR fragment makes it possible to remove the inhibitory action of a quencher on a fluorophore

Génération de la fluorescence (1)



TAQMAN PROBES ®



Probe free in solution

Probe and primers annealing

FRET

Taq DNA polymerase encounters the probe, deplaces and degrades it, releasing free reporter in solution

Fluorescence emission

Fluorochromes *reporters* nature:

- FAM : blue
- VIC : green
- NED : black
- PET : red

(FAM and VIC:) Best *quenching*

Advantages of Q-PCR with probes:

- Very specific (this PCR requires the use of two primers and one or more specific probes)

- Possibility of multiplexing i.e. several PCRs in the same tube: detection of different fluorochromes

Limit : Cost of probes (especially if many genes to quantify)

Two technics of quantification :

-The absolute quantification: it consists in the determination of the exact number of copies of target matrix. This approach requires a calibrated range of which the exact number of copies (e.g., a range realized using a perfectly defined quantity of a plasmid carrying the gene of interest) is known.

-The relative quantification: it consists in the direct comparison of two given conditions.
Example: in the determination of the ratio of the target quantity between the two conditions (in this case the ratio of the amount of amiF transcripts after culture at pH 7 and at pH 5 , 5).
This approach requires the quantification in parallel for each condition of the amount of a reference gene. This reference is sometimes tricky to choose. In fact, this must be a gene whose expression does not vary between the two conditions compared.
The genes most often used as references are so-called "housekeeping" genes such as bacterial RNA16S, gapdh (encoding glyceraldehyde-3-phosphate dehydrogenase) or the gene encoding eukaryotic actin

Standard curve technique

- Draw the standard curve : y = Ct
  $x = \log(\text{concentration})$
- Calculate a and b such as : $y = ax + b$
- Report on the curve the ct values of your samples

C_T-value

- CT “cycle treshold”
- C_T is inversely proporttional to the initial copy number
- The Ct value can be directly correlated to the starting target concentration.