saône-et-loire
LE DÉPARTEMENT

Real-time PCR in animal health diagnosis according to the french standard XP U47-600




Introduction

- Presentation of the results of a collective work on PCR as a diagnostic tool of animal pathogens

- Interesting to compare experiences and point of view in two areas that are not so distinct

- XP U47- 600 = request of the French Ministry of Agriculture for the accreditation of laboratories performing the detection of regulated animals pathogens

- It was developed in accordance with the recommendations of the OIE standards, the World Organization for Animal Health

Introduction

- The Working Group met from 2006 to 2010 and was composed of:

reference laboratories

developers of PCR tests

users of PCR kits

suppliers of reagents

equipment manufacturers

- XP U47- 600 = a consensus of knowledge and experience of all members

Introduction

- It is intentionally generalist and educational

- It is divided into two interrelated parts:

Part 1 for users of PCR tests: it describes the minimum requirements necessary to set up and use a PCR method

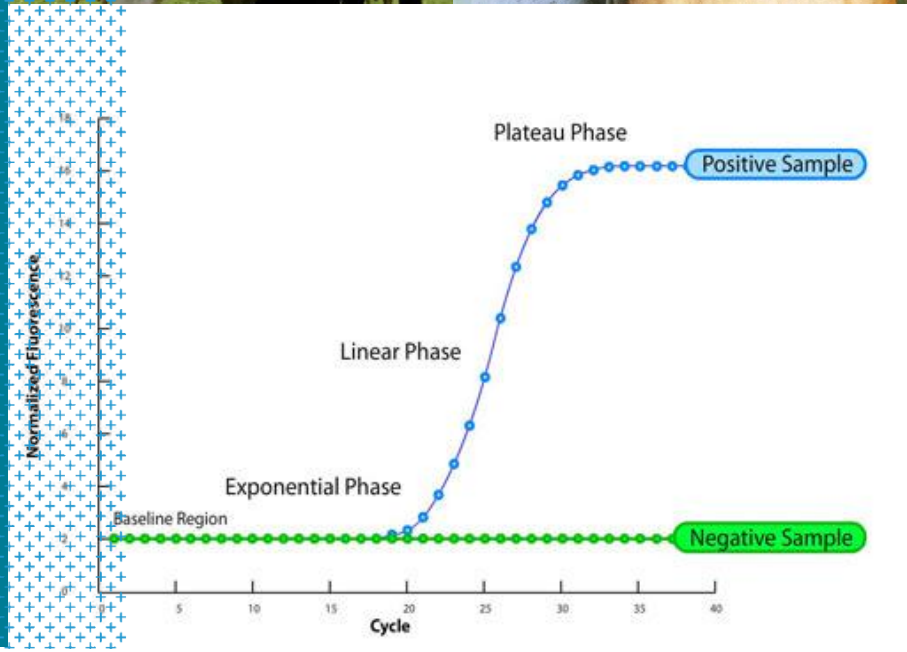
Part 2 for developers of PCR tests: it describes the characterization process of the PCR and the complete method

- Field of application not restricted to animal health

Real-time PCR in animal health diagnosis according to the french standard XP U47-600:

no Ct cut-off value but LOD in number of copies of target sequences

1 Why this approach?



Why this approach?

PCR: direct diagnostic method of the presence or absence of a pathogen by amplifying a specific target sequence

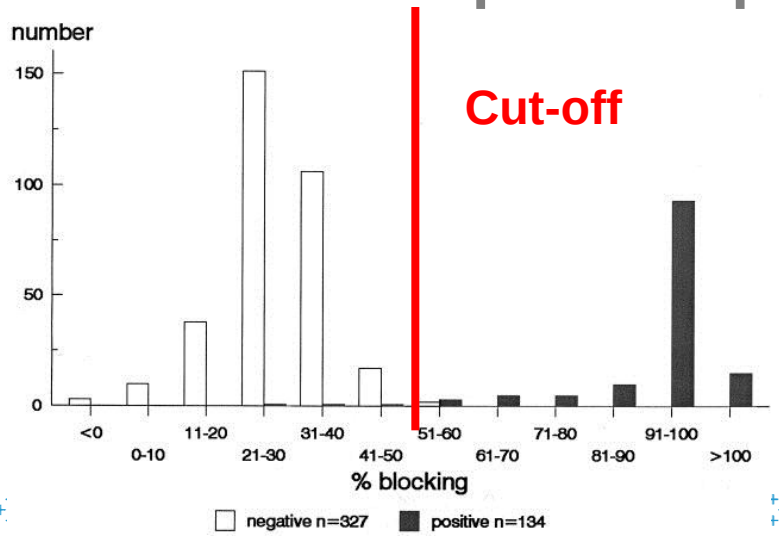
+The presence of a **signal having the expected characteristics** (size of bands for the endpoint PCR, delta of fluorescence and appearance of real-time PCR amplification curves, Tm of dissociation curve) is **directly correlated with the presence of the amplified target sequence**, and therefore **the presence of pathogen in the field of the analytical specificity covered by the primers-probe used**

Why this approach?

PCR: direct diagnostic method of the presence or absence of a pathogen by amplifying a specific target sequence

+Unlike methods based on antibody-antigen recognition as the ELISA, **there is no need to establish a cut-off** that distinguishes between the background of nonspecific antibody-binding on the negative population and the signal level observed with the positive population

ELISA



Why this approach?

PCR: direct diagnostic method of the presence or absence of a pathogen by amplifying a specific target sequence

+In real-time PCR, where we follow an **increase in fluorescence** at each cycle, the **signal analysis software already eliminates the background of fluorescence** observed in each well at the beginning of the PCR run

Why this approach?

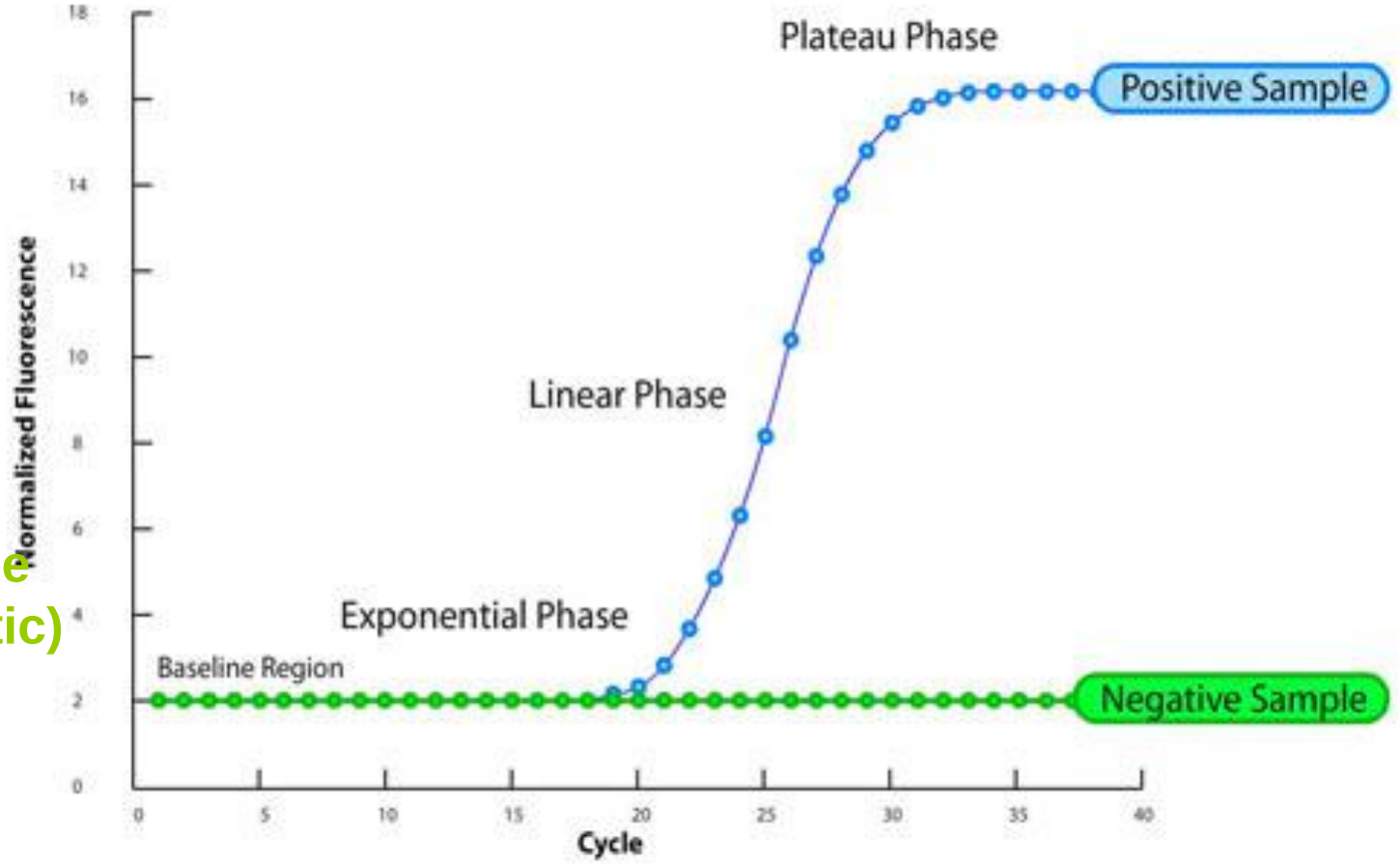
How is made the analysis of signal observed in real-time PCR?

+The real-time thermocycler measures at each cycle the **level of fluorescence** and the analysis software allows to view graphically the evolution of levels of fluorescence

+In some thermocycler, the observed **fluorescence is normalized (Rn)** with respect to a passive ROX fluorescence contained in the master mix

Why this approach?

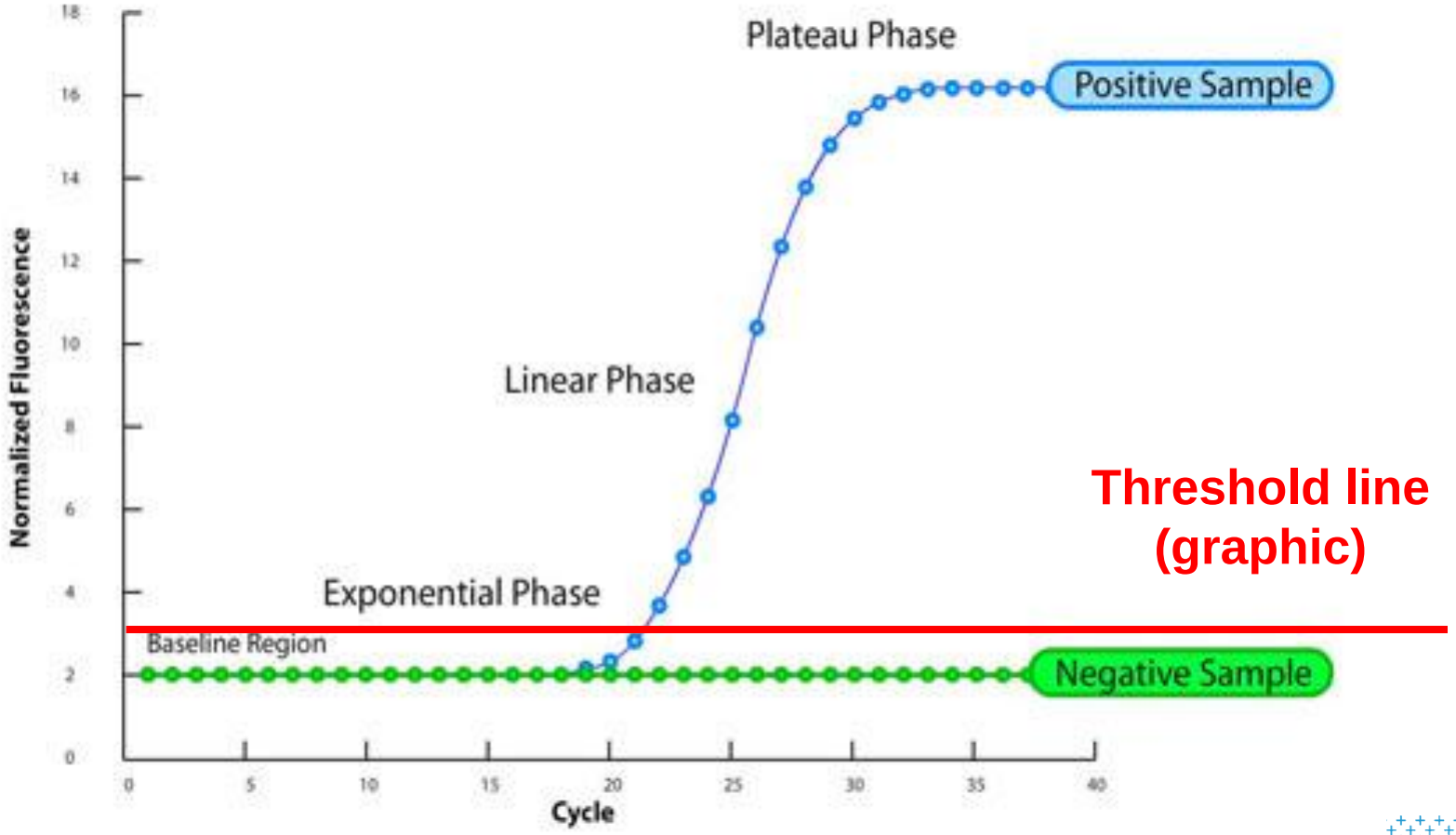
How is made the analysis of signal observed in real-time PCR?



Baseline (automatic)

Why this approach?

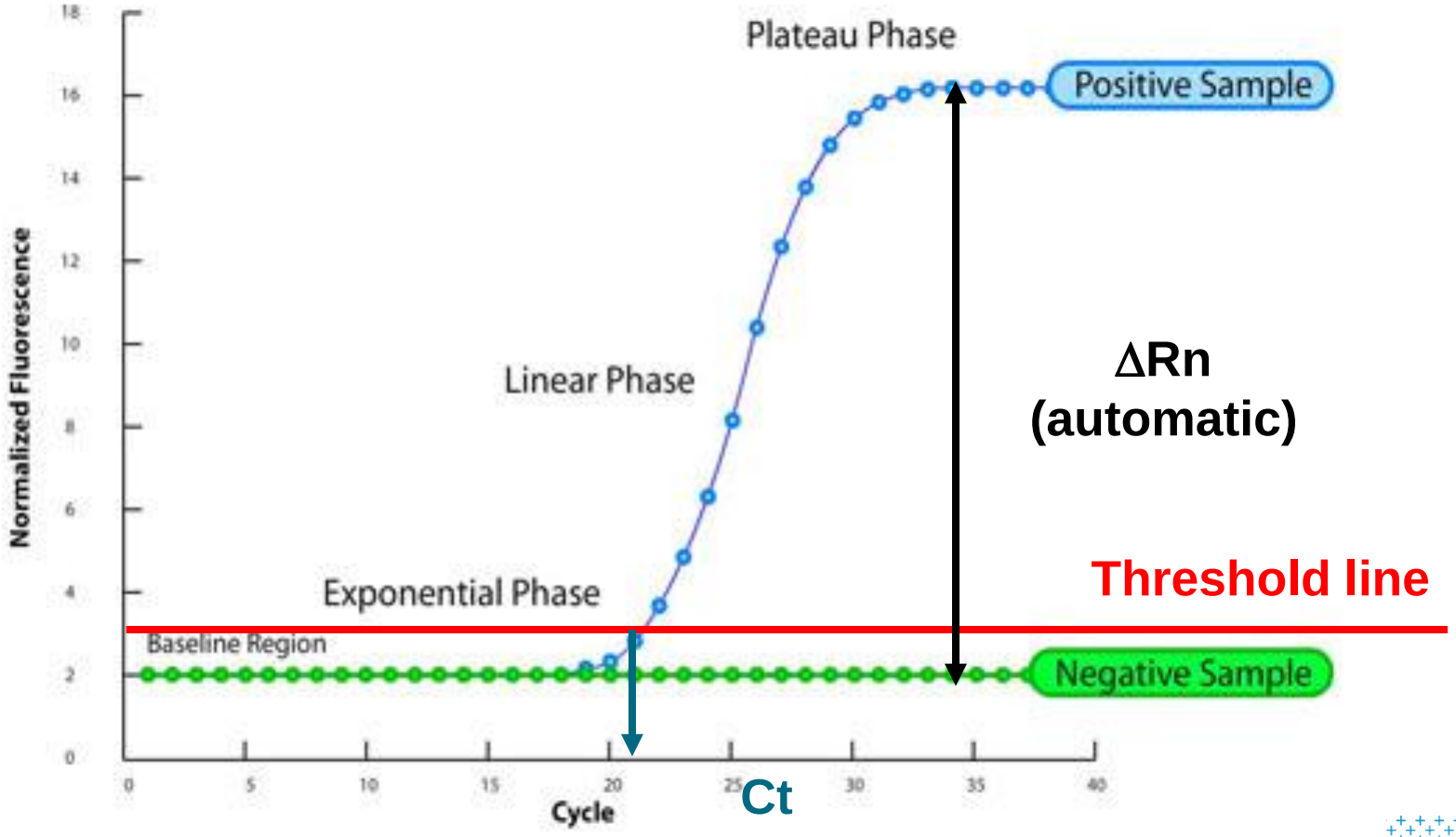
How is made the analysis of signal observed in real-time PCR?



Threshold line (graphic)

Why this approach?

How is made the analysis of signal observed in real-time PCR?



Why this approach?

What do we know about Ct value?

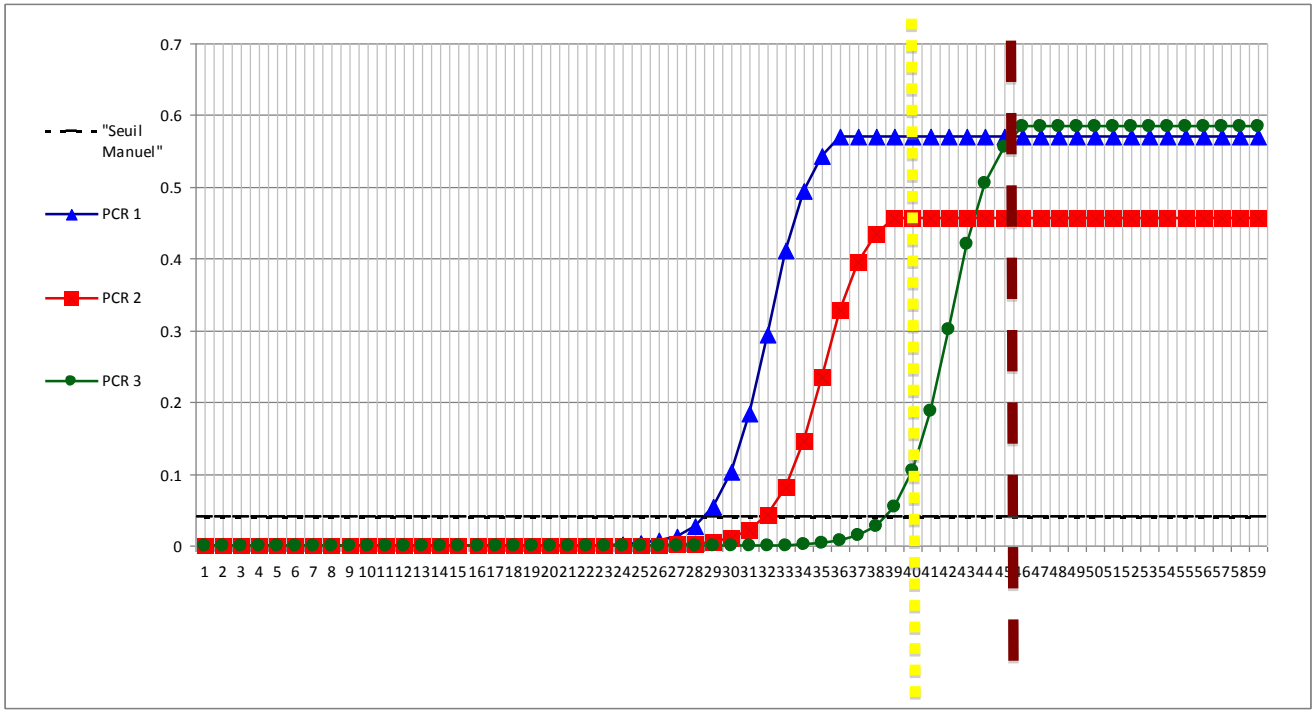
+Ct value is **a relative graphic measure** which **depends on the concentration of target sequence** in PCR reaction

+**But** many others factors impact the Ct value such as:

- components of master mix
- amount of ROX passive dye
- **efficiency of PCR reaction**

Why this approach?

What do we know about Ct value?



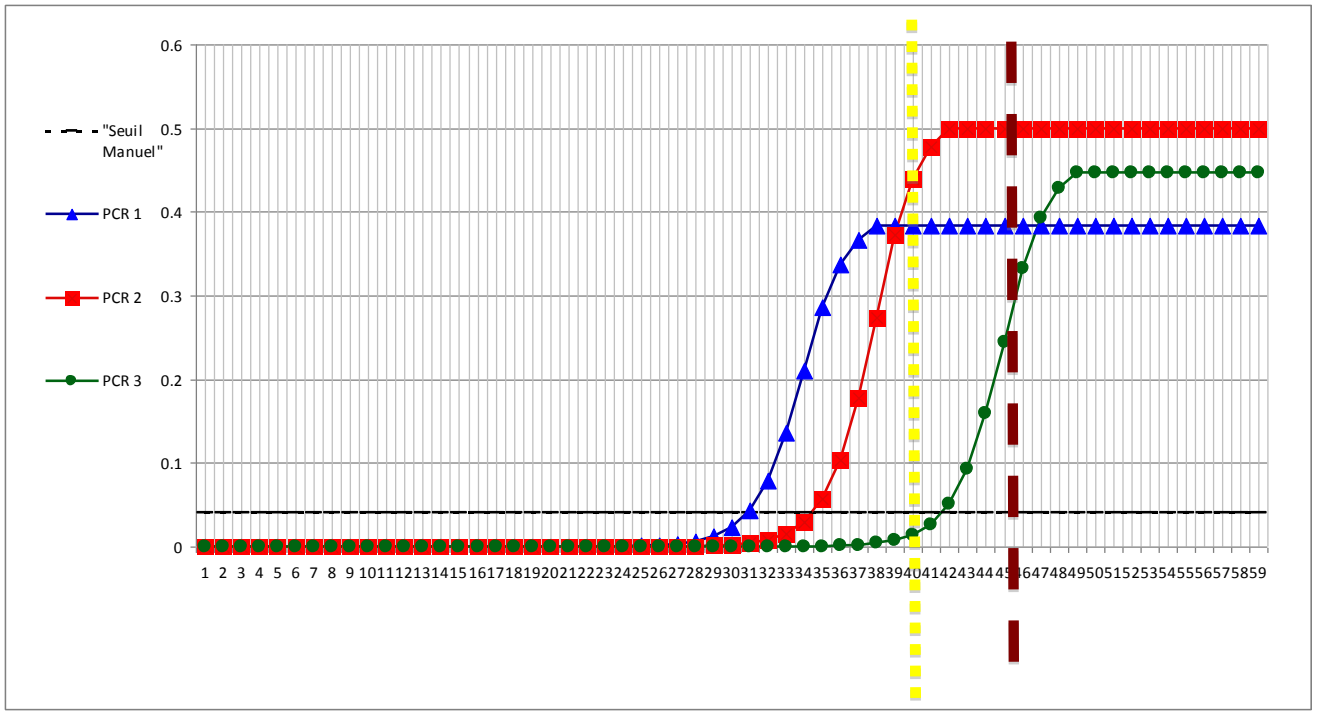
E = 100%

1000 copies: Ct 29
100 copies: Ct 32.2
1 copy: Ct 38.6

45 cycles = End of PCR run

Why this approach?

What do we know about Ct value?



E = 90%

1000 copies: Ct 31
100 copies: Ct 34.5
1 copy: Ct 41.7

45 cycles = End of PCR run

Why this approach?

What do we know about Ct value?

+ If we want to use the absolute value of Ct to evaluate the performance of PCR, it is necessary that the **reaction efficiency and all other components are stable** (but they always vary a little)

+ And the performance determined by one laboratory is not comparable with another one, **which is not compatible with a network of approved laboratories for the detection of regulated pathogens**

Why this approach?

What do we know about Ct value?

+The validation of a **quantitative PCR** and **a calibration range added to each PCR run**, are absolutely essential conditions to allow the **correlation between the Ct value and the quantity of target sequence**

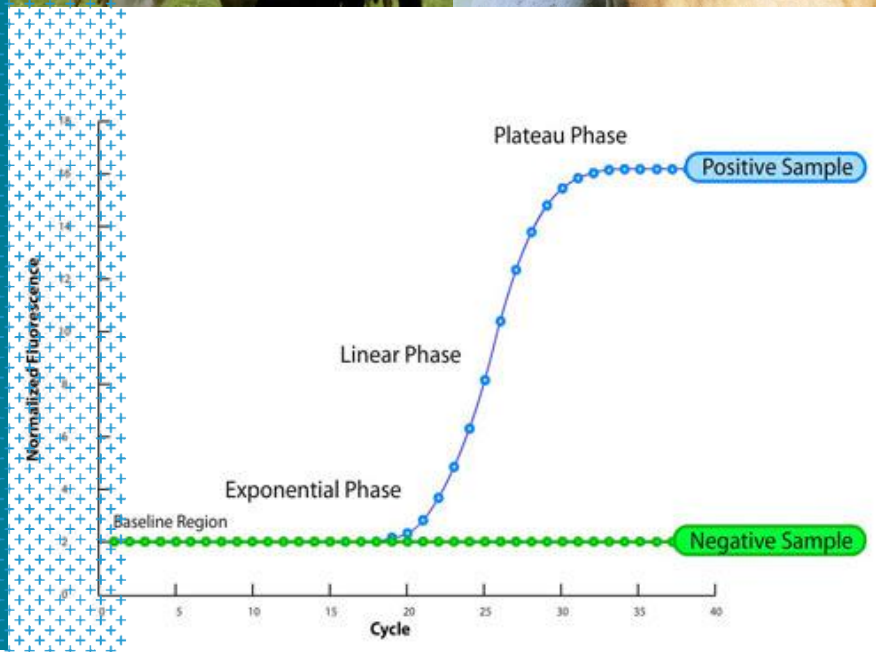
+**Ct value alone can not** be attributed to a PCR to **rule on the positivity or negativity of a sample** as it is the analysis of the measured signal **(ΔRn and appearance of the amplification curve)** that allows deciding on positivity or negativity

Why this approach?

Conclusion

The only way to determine the performance of a PCR based on criteria that could be extrapolated within a network of analytical laboratories is to determine these criteria in units of nucleic acids (number of copies of target sequence or number of units of the genome) since the PCR is an amplification of a target sequence.

2 How to evaluate the performance of a qualitative real-time PCR?



How to evaluate the performance of a qualitative real-time PCR?

The protocol is described in Part 2 of the standard.

Determination of the **PCR characteristics** on one side, then **characterization of the whole method** (which comprises the preparation of the sample, the nucleic acid extraction and PCR).

These characterizations are performed **on a fixed and optimized analytical process**.

How to evaluate the performance of a qualitative real-time PCR?

Characterization of the PCR:

+ **Analytical specificity of the PCR** determined *in silico* by comparison with nucleic sequences must be verified experimentally
  - on strains, nucleic acids extracts **representative of the genetic variability** of the agent to detect **(inclusivity)**
  - and on strains, nucleic acids extracts **representative from other pathogens close genetically or within the same ecological niche (exclusivity)**

to determine the field of the analytical specificity of the PCR = the level of taxonomic precision that matches an amplification signal (positive result): group, family, genus, species, genotypes, serotypes ...

How to evaluate the performance of a qualitative real-time PCR?

Characterization of the PCR:

+ **Limit of detection (LOD_{PCR}):**

  - different dilutions of nucleic acids flanking the value of LOD_{PCR} assumed are tested in terms of repeatability intra-test (replicates) and inter-test (independent sessions)

  - a minimum of six dilutions around the estimated value of LOD_{PCR} give sufficient accuracy on the estimation of the LOD_{PCR}

How to evaluate the performance of a qualitative real-time PCR?

Characterization of the PCR:

+ **Limit of detection (LOD_{PCR}):**

Minimum number of independent sessions	Minimum number of operators	Minimum number of replicates per dilution tested
3	1	8

the approximate value of the LOD_{PCR} to 95%, is the concentration of target sequence giving at least 23 positive results out of 24

How to evaluate the performance of a qualitative real-time PCR?

	A	B	C	D	E	F	G	H	I
1	Nombre de copies	Log_{10} (Nb copies)	Positifs séance 1	Positifs séance 2	Positifs séance 3	Cumul nombre détection	Nombre total d'essais	Fréquence de détection	Fréquence de détection corrigée
2	8	=LOG10(A2)	8	8	8	=C2+D2+E2	24	=F2/G2	=(F2-0,5)/G2
3	4	=LOG10(A3)	8	8	8	=C3+D3+E3	24	=F3/G3	=(F3-0,5)/G3
4	2	=LOG10(A4)	7	8	7	=C4+D4+E4	24	=F4/G4	=(F4-0,5)/G4
5	1	=LOG10(A5)	8	7	5	=C5+D5+E5	24	=F5/G5	=(F5-0,5)/G5
6	0,5	=LOG10(A6)	6	3	4	=C6+D6+E6	24	=F6/G6	=(F6-0,5)/G6
7	0,25	=LOG10(A7)	1	3	0	=C7+D7+E7	24	=F7/G7	=(F7-0,5)/G7
8									
9	Données de constructic								

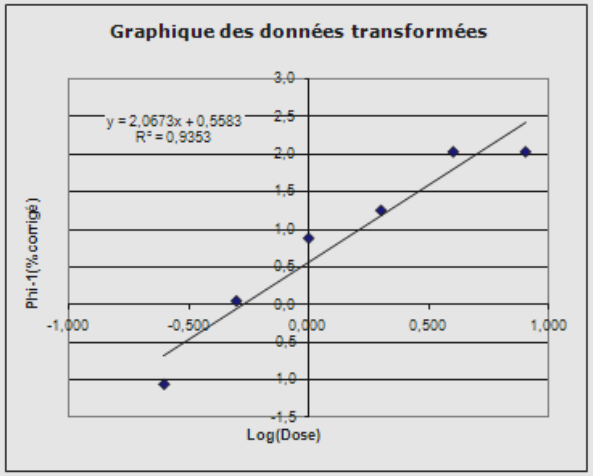
10	y calculé	Log_{10}(dose)	% calculé
11	=$F$19*B11+$F$20	-4	=LOI.NORMALE.STANDARD(A11)
12	=$F$19*B12+$F$20	-3,8	=LOI.NORMALE.STANDARD(A12)
13	=$F$19*B13+$F$20	-3,6	=LOI.NORMALE.STANDARD(A13)
14	=$F$19*B14+$F$20	-3,4	=LOI.NORMALE.STANDARD(A14)
15	=$F$19*B15+$F$20	-3,2	=LOI.NORMALE.STANDARD(A15)
16	=$F$19*B16+$F$20	-3	=LOI.NORMALE.STANDARD(A16)
17	=$F$19*B17+$F$20	-2,8	=LOI.NORMALE.STANDARD(A17)
18	=$F$19*B18+$F$20	-2,6	=LOI.NORMALE.STANDARD(A18)
19	=$F$19*B19+$F$20	-2,4	=LOI.NORMALE.STANDARD(A19)
20	=$F$19*B20+$F$20	-2,2	=LOI.NORMALE.STANDARD(A20)
21	=$F$19*B21+$F$20	-2	=LOI.NORMALE.STANDARD(A21)
22	=$F$19*B22+$F$20	-1,8	=LOI.NORMALE.STANDARD(A22)
23	=$F$19*B23+$F$20	-1,6	=LOI.NORMALE.STANDARD(A23)
24	=$F$19*B24+$F$20	-1,4	=LOI.NORMALE.STANDARD(A24)
25	=$F$19*B25+$F$20	-1,2	=LOI.NORMALE.STANDARD(A25)
26	=$F$19*B26+$F$20	-1	=LOI.NORMALE.STANDARD(A26)
27	=$F$19*B27+$F$20	-0,8	=LOI.NORMALE.STANDARD(A27)
28	=$F$19*B28+$F$20	-0,6	=LOI.NORMALE.STANDARD(A28)
29	=$F$19*B29+$F$20	-0,4	=LOI.NORMALE.STANDARD(A29)
30	=$F$19*B30+$F$20	-0,2	=LOI.NORMALE.STANDARD(A30)
31	=$F$19*B31+$F$20	0	=LOI.NORMALE.STANDARD(A31)
32	=$F$19*B32+$F$20	0,2	=LOI.NORMALE.STANDARD(A32)
33	=$F$19*B33+$F$20	0,4	=LOI.NORMALE.STANDARD(A33)
34	=$F$19*B34+$F$20	0,6	=LOI.NORMALE.STANDARD(A34)
35	=$F$19*B35+$F$20	0,8	=LOI.NORMALE.STANDARD(A35)
36	=$F$19*B36+$F$20	1	=LOI.NORMALE.STANDARD(A36)
37	=$F$19*B37+$F$20	1,2	=LOI.NORMALE.STANDARD(A37)
38	=$F$19*B38+$F$20	1,4	=LOI.NORMALE.STANDARD(A38)
39	=$F$19*B39+$F$20	1,6	=LOI.NORMALE.STANDARD(A39)
40	=$F$19*B40+$F$20	1,8	=LOI.NORMALE.STANDARD(A40)
41	=$F$19*B41+$F$20	2	=LOI.NORMALE.STANDARD(A41)
42	=$F$19*B42+$F$20	2,2	=LOI.NORMALE.STANDARD(A42)
43	=$F$19*B43+$F$20	2,4	=LOI.NORMALE.STANDARD(A43)
44	=$F$19*B44+$F$20	2,6	=LOI.NORMALE.STANDARD(A44)
45	=$F$19*B45+$F$20	2,8	=LOI.NORMALE.STANDARD(A45)
46	=$F$19*B46+$F$20	3	=LOI.NORMALE.STANDARD(A46)
47	=$F$19*B47+$F$20	3,2	=LOI.NORMALE.STANDARD(A47)
48	=$F$19*B48+$F$20	3,4	=LOI.NORMALE.STANDARD(A48)
49	=$F$19*B49+$F$20	3,6	=LOI.NORMALE.STANDARD(A49)
50	=$F$19*B50+$F$20	3,8	=LOI.NORMALE.STANDARD(A50)
51	=$F$19*B51+$F$20	4	=LOI.NORMALE.STANDARD(A51)
52			

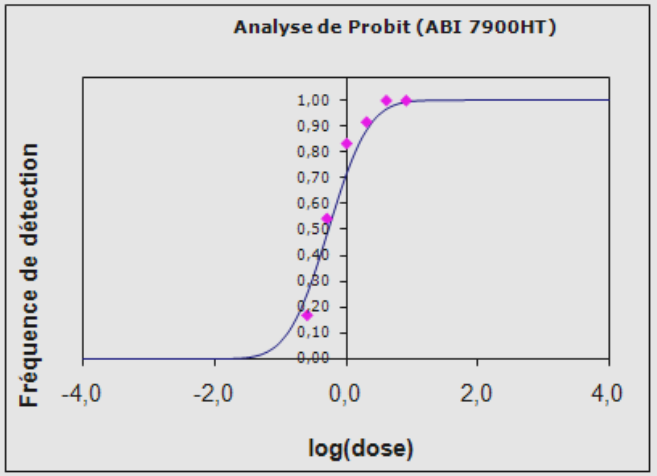
Log_{10}(dose)	Φ^{-1}(% corrigé)
=B2	=LOI.NORMALE.STANDARD.INVERSE(I2)
=B3	=LOI.NORMALE.STANDARD.INVERSE(I3)
=B4	=LOI.NORMALE.STANDARD.INVERSE(I4)
=B5	=LOI.NORMALE.STANDARD.INVERSE(I5)
=B6	=LOI.NORMALE.STANDARD.INVERSE(I6)
=B7	=LOI.NORMALE.STANDARD.INVERSE(I7)

Pente	=PENTE(F11:F16;E11:E16)
Ordonnée Origine	=ORDONNEE.ORIGINE(F11:F16;E11:E16)
R^2	=COEFFICIENT.DETERMINATION(F11:F16;E11:E16)

Precision (%)	0,95
Φ^{-1}(Précision)=	=LOI.NORMALE.STANDARD.INVERSE(I10)

log(dose seuil)	=(I11-$F$20)/$F$19
dose seuil	=PUISSANCE(10;I13)

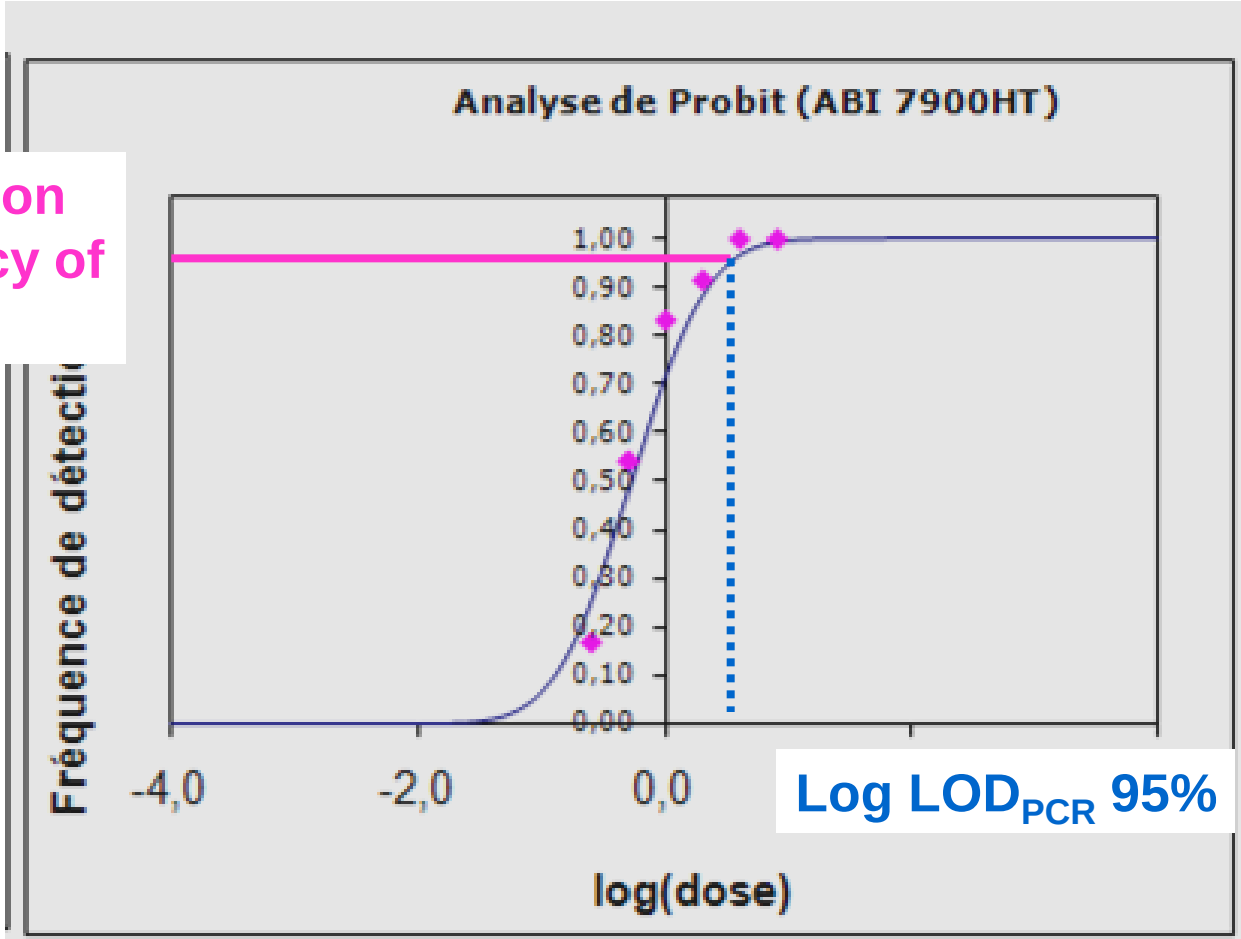




Cases à renseigner

How to evaluate the performance of a qualitative real-time PCR?

Detection frequency of 95%



Log LOD_{PCR} 95%

How to evaluate the performance of a qualitative real-time PCR?

Characterization of the PCR:

+ **Limit of detection (LOD_{PCR}):**

  - With the probit model, the log of the **concentration of target sequence detected with a frequency of 95%** is statistically determined = log of **LOD_{PCR} 95%**

  - But this **value must be experimentally verified** by the analysis of 10 replicates of **LOD_{PCR} 95%** to be found positive 9 times out of 10.

How to evaluate the performance of a qualitative real-time PCR?

Characterization of the whole method:

+ The characterization of the complete method is carried out **on samples** and is determined **for each type of analytical matrix** (same distribution of the agent and same nucleic acid extraction protocol)

How to evaluate the performance of a qualitative real-time PCR?

Characterization of the whole method:

+ **Limit of detection (LOD_{METHOD}):**

  - The LOD_{METHOD} is evaluated using dilutions of **a positive sample calibrated in number of target sequence per unit of mass or volume**. The dilutions are tested in reproducibility conditions

Minimum number of independent sessions	Minimum number of operators	Minimum number of replicates per dilution tested
2	1	4

How to evaluate the performance of a qualitative real-time PCR?

Characterization of the whole method:

+ **Limit of detection (LOD_{METHOD}):**

  - The LOD_{METHOD} **is estimated** as the last dilution level where the 8 repetitions are found positive.

  - **This is an estimated value** : the absolute detection limit of the method is at a lower concentration of target sequences per unit of sample **so samples can be found positive beyond this value**

How to evaluate the performance of a qualitative real-time PCR?

Characterization of the whole method:

+ **Diagnostic sensitivity and diagnostic specificity of the whole method:**

  - On **positive and negative samples**

  - The positive and negative status of these samples has previously been validated **by a reference method** if it exists, **or by a method or set of methods different than the PCR evaluated**.

  - The **use of standard additions** on negative samples is not appropriate, because these samples would **not be representative of a natural infection** ("OIE Terrestrial Manual").

How to evaluate the performance of a qualitative real-time PCR?

Characterization of the whole method:

+ **Diagnostic sensitivity and diagnostic specificity of the whole method:**

  - For positive samples, it is recommended to have **three levels of positivity**: low, medium, high.

  - DSe_{METHOD} = % of positive results obtained from the positive samples

  - DSp_{METHOD} = % of negative results obtained from the negative samples.

How to evaluate the performance of a qualitative real-time PCR?

Characterization of the whole method:

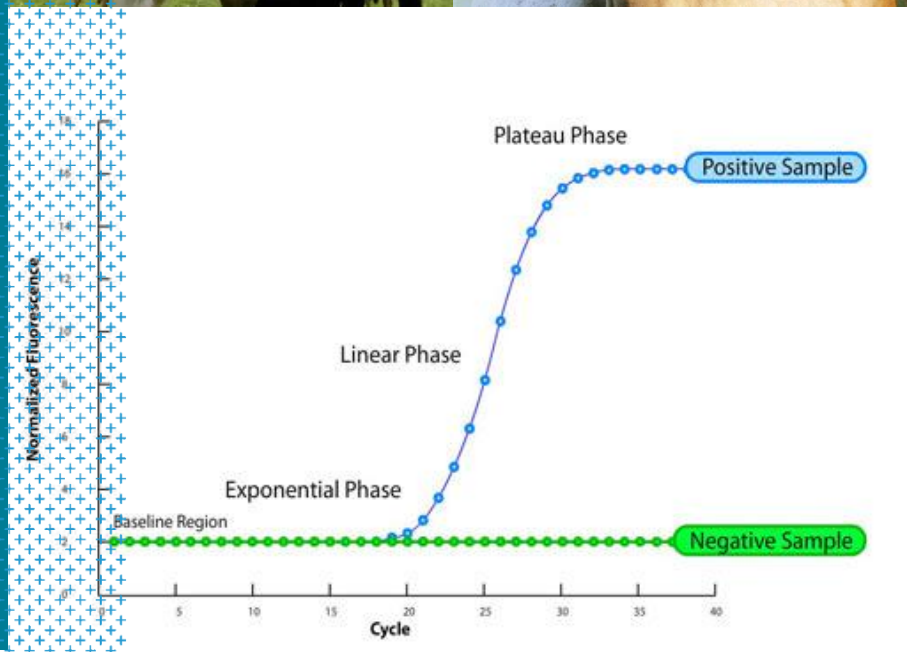
+ **Diagnostic sensitivity and diagnostic specificity of the whole method:**

Real-time PCR = **better detectability as reference techniques used to determine the status of the samples**

- the diagnostic sensitivity of the method is generally 100%

- whereas **the diagnostic specificity is less than 100%, the samples weakly positive being more easily detected with real-time PCR**

3 How the analysis laboratory confirms its ability to implement a validated PCR?



How the analysis laboratory confirms its ability to implement a validated PCR?

Protocol of a method adoption :

+ described in the part 1 of the document

+ to confirm that the **laboratory is able to reproduce the performances** of the **PCR** and of **the complete method**

+The laboratory should have **two types of reference materials:**

- a **nucleic acid solution at the LOD_{PCR}**
- and **a sample at the LOD_{METHOD}**

How the analysis laboratory confirms its ability to implement a validated PCR?

Protocol of a method adoption :

+**The laboratory confirms its ability to detect the LOD_{PCR}**

by depositing on each of its thermocyclers,

3 replicates of a level of NA solution corresponding to 3 times the LOD_{PCR}95% determined by the developer

Cyclers giving 100% of positive results are qualified to perform routine analysis

How the analysis laboratory confirms its ability to implement a validated PCR?

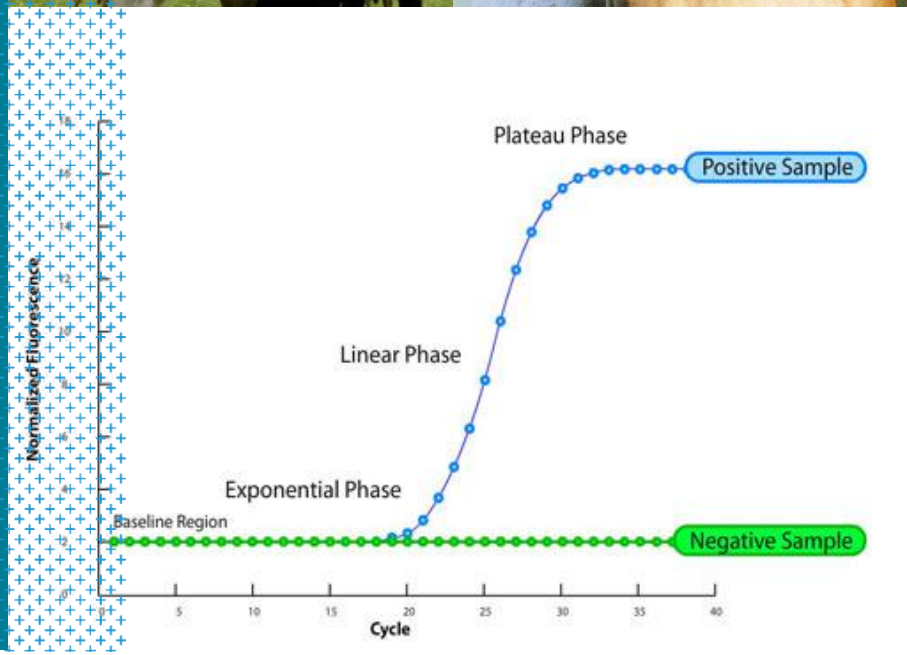
Protocol of a method adoption :

+**The laboratory confirms its ability to detect the LOD_{METHOD}**

by performing twice the complete analytical protocol on two replicates of a sample containing 1 to 10 times the LOD_{METHOD} determined by the developer

The 4 results must be positive.

4 How the analysis laboratory routinely uses a qualitative PCR validated?



How the analysis laboratory routinely uses a qualitative PCR validated?

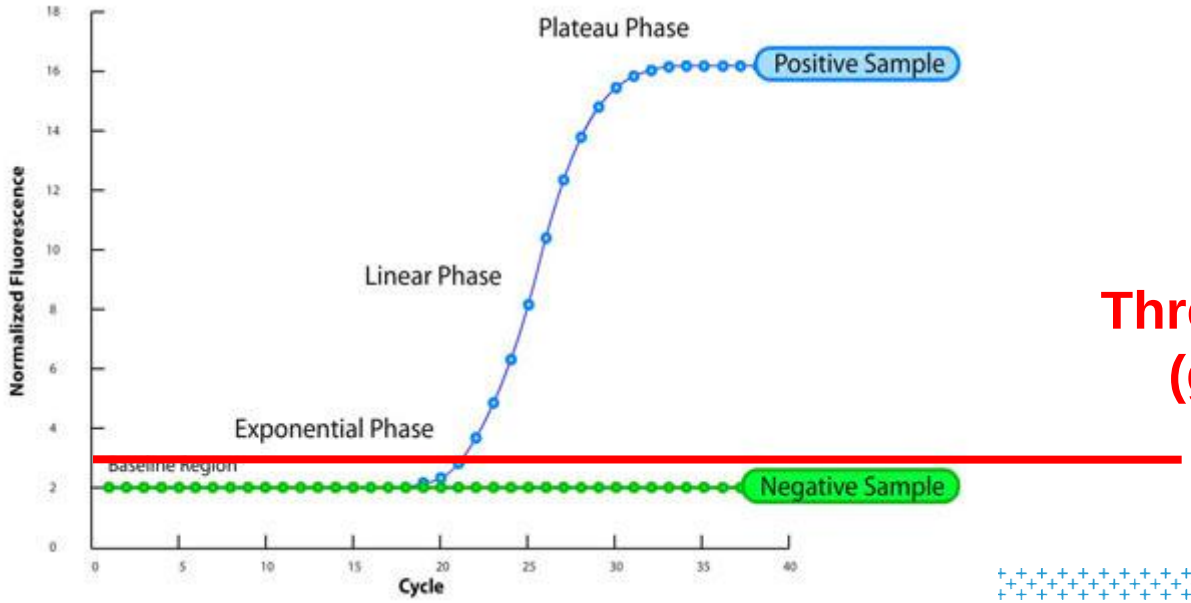
Controls used to confirm the smooth running of the analytical process in time:

- **Positive sample** with a stable level of the concentration in copies of target sequence, **close to the LOD_{METHOD}** (10 to100 times), is **analyzed at each series**
- **Negative sample (or water)** is used at each series in **number appropriate to the risk of cross contamination during the analysis**. This negative control is subject to all of the analytical process
- **Internal Positive Control**: endogenous or exogenous **nucleic acid sequence** to the sample analyzed and usually **co-amplified with the target sequence in each sample**

How the analysis laboratory routinely uses a qualitative PCR validated?

Interpretation of the results obtained for the controls:

Reminder: a **positive or negative result** is **interpreted** according to the presence or absence of a signal characteristic in real-time PCR, therefore **the presence or absence of a characteristic amplification curve intersecting the threshold line**



Threshold line (graphic)

How the analysis laboratory routinely uses a qualitative PCR validated?

Interpretation of the results obtained for the controls:

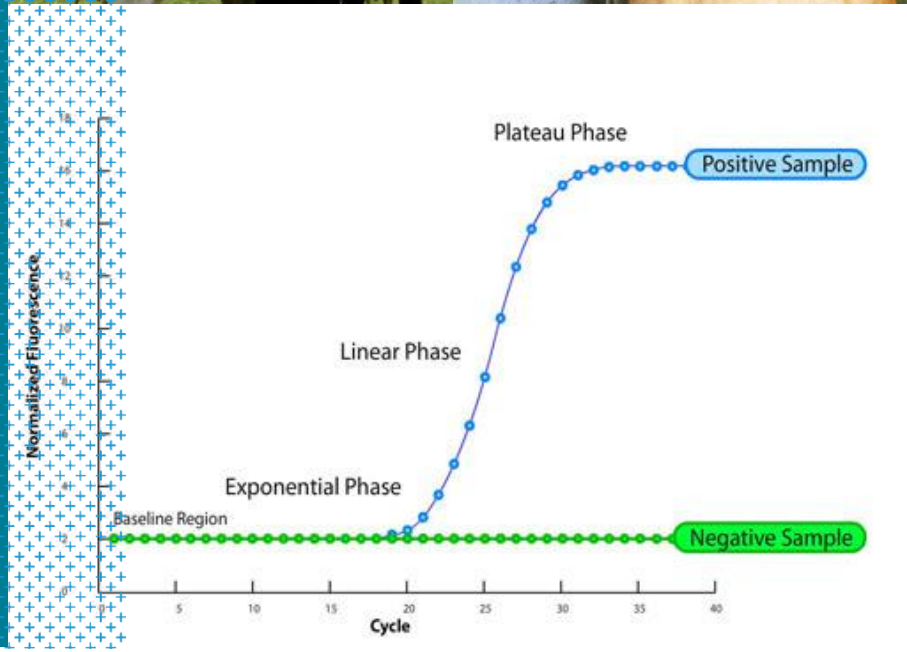
- **Positive sample** must be found Positive = **presence of amplification curve.** It is recognized that for a well controlled method conducted under standardized conditions, the **Ct values** obtained in the laboratory can be followed on **a control chart (Shewhart)**
- **Negative sample** must be found Negative = **absence of amplification curve**
- **Internal Positive Control** must be found positive in each sample = **presence of amplification curve with a signal** (ΔRn and Ct) **consistent with the signal expected**

How the analysis laboratory routinely uses a qualitative PCR validated?

Interpretation of the results obtained for each sample:

- **Positive =** presence of a characteristic amplification curve intersecting the threshold line. If the signal appears later than the signal of the positive control, it is specified that **the sample is found positive within the detection limit of the method**
- **Negative =** absence of a characteristic amplification curve intersecting the threshold line and signal of IPC consistent with the signal expected
- **Not conclusif =** IPC signal not consistent or absent; or amplification curve not characteristic

5 PCR relative on a diagnostic or regulatory threshold of interpretation



PCR relative on a diagnostic or regulatory threshold of interpretation

The requirements to implement a relative PCR are:

+PCR is a **quantitive PCR method**, properly **validated** (PCR: LOD_{PCR}, LOQ_{PCR}, **linearity**, **efficiency**; Method: LOD_{METHOD}, LOQ_{METHOD}, **accuracy profile**) and **adopted**

+The laboratory has a **certified reference material** (CRM) on the threshold of diagnostic interpretation **(to distinguish between healthy carriage of the pathogen and the clinical level)** or of regulatory interpretation **to inform the risk manager on field**

PCR relative on a diagnostic or regulatory threshold of interpretation

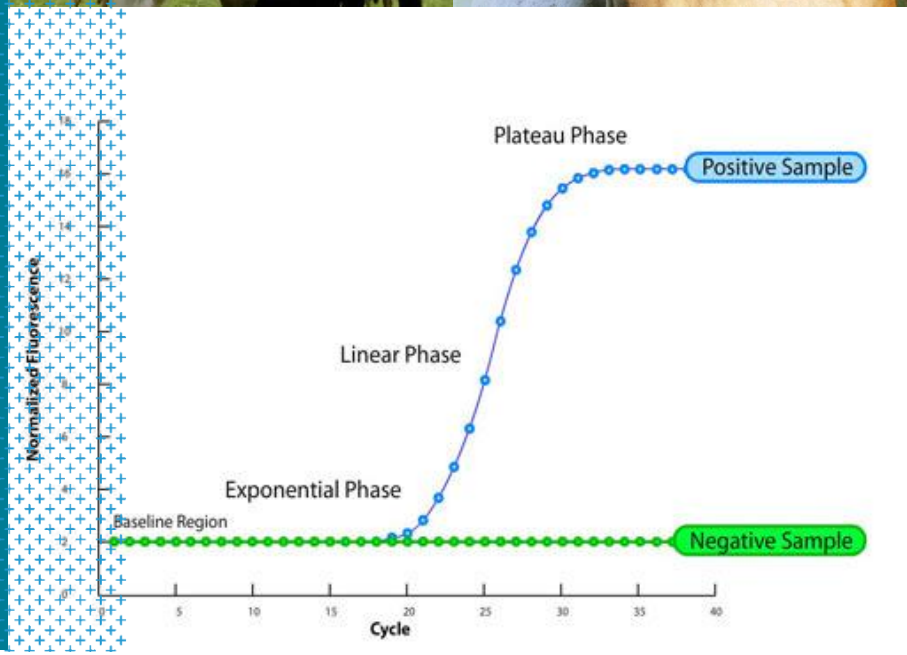
Sample results are interpreted in terms of the position of their signal (Ct) to the CRM :

+If **Ct is earlier**, we can say that the quantity of target sequence in the sample is **greater** than that of **CRM**

+If **Ct is later**, we can say that the quantity of target sequence in the sample is **less** than that of **CRM**

+If **Ct is earlier or later but in the interval of delta Ct determined in accuracy profile for CRM**, we can say that the quantity of target sequence in the sample **is close to** that of **CRM**

6 Examples of the risk of giving a false negative result on the basis of a value of Ct



Examples of the risk of giving a false negative result on the basis of a value of Ct

Blue tongue virus detection:

- The official recommandations, established before the drafting of the standard, provided that **any positive sample found with a Ct value greater than 35 was of no consequence for the risk management**
- We analyzed a sample of an **animal showing clinical signs of the disease**, but we found a **positive result** with a Ct value **greater than 35**
- Alerted by this surprising result, we asked our kit supplier to determine the target sequence of the virus present in our sample.

Examples of the risk of giving a false negative result on the basis of a value of Ct

Blue tongue virus detection:

+The sequencing revealed a **mutation in the site of attachment of the probe**, which explains the loss of efficiency of PCR.

+The incorporation of this mutation in the sequence of the probe allowed to obtain for the sample a positive result with an earlier Ct (24).

Thus, even if the analytical process is under control, a late signal must be interpreted in the epidemiological context because it can be due to a mutation in the « wrong place », especially for RNA genomes

Examples of the risk of giving a false negative result on the basis of a value of Ct

Grapevine Flavescence Dorée phytoplasma :

- « Triplex real-time PCR assay for sensitive and simultaneous detection of grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous analytical control » , Pelletier C. et al, *Vitis*, 48(2),87-95, 2009
- In the French official method derived from this publication, when the laboratory found **a positive result with a Ct greater than 35**, it has to make **two additional PCR analysis of the nucleic acid extract**:

  - if at least **2 out of 3 PCR analysis are positive**, the results is **Positive**

  - if **only the first PCR analysis is found positive**, the result is **Negative**

Examples of the risk of giving a false negative result on the basis of a value of Ct

Grapevine Flavescence Dorée phytoplasma :

+When we renew the PCR analysis on these samples, we confirm, that depending on the initial Ct observed, results follows

- either the normal Gauss distribution **(100% of confirmation for Ct around 35-37)**
- or the law of very low copy number, the Poisson distribution **(around 50% of confirmation for Ct exceeding 38)**

Examples of the risk of giving a false negative result on the basis of a value of Ct

Grapevine Flavescence Dorée phytoplasma :

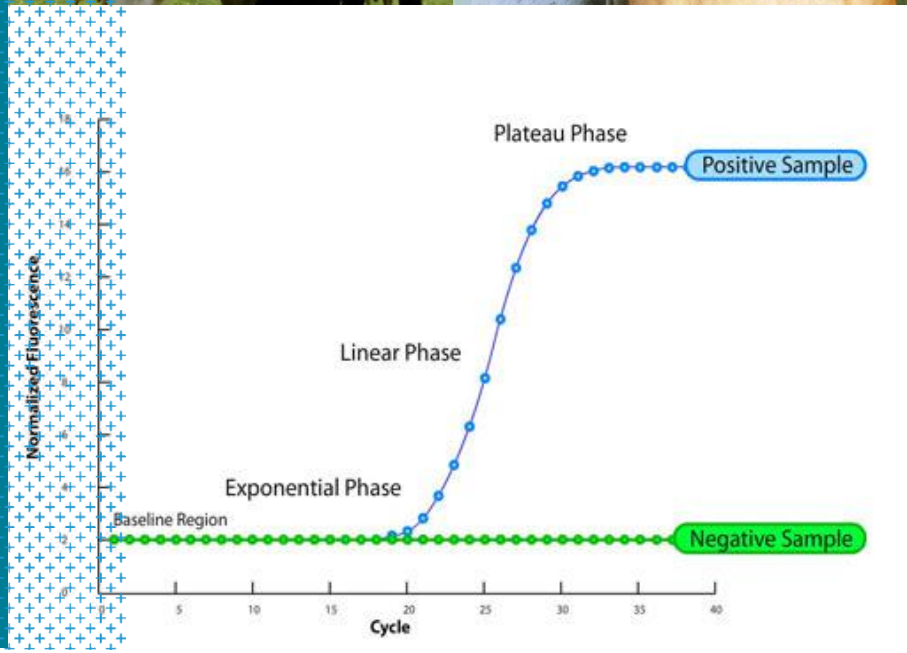
+we made wrongly negatives conclusions:

- For analysis of **pool of five vine stocks (where only one of the stocks subsequently analyzed individually was found positive with Ct under 35)**

- For analysis **of vine stocks from new infected areas** or **from varieties which multiply less the FD phytoplasma**. The **vine stocks not uprooted the year n are positive the year n +1**

- For analysis at the end of sampling campaign, **when the autumn leaf senescence began more precociously**, **the sap which multiplies the phytoplasmas is less present in the phloem**

Conclusion


Plateau Phase
Positive Sample
Linear Phase
Exponential Phase
Baseline Region
Negative Sample
Normalized Fluorescence
0
5
10
15
20
25
30
35
40
Cycle

Conclusion

The presence of a characteristic amplification curve, whatever the level of precocity of the signal, corresponds to a positive result in the field of analytical specificity determined during the validation of PCR

With a qualitative PCR, using a positive control near to the LOD_{METHOD} allows to inform the risk manager if a sample is found positive, close to the LOD_{METHOD}

With a relative PCR (derived from a quantitative PCR), a CRM used at each series allows to inform the risk manager on the relative quantity of target sequence present in a sample

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