Qualitative interpretation of quantitative qPCR results : Feedback in mycology

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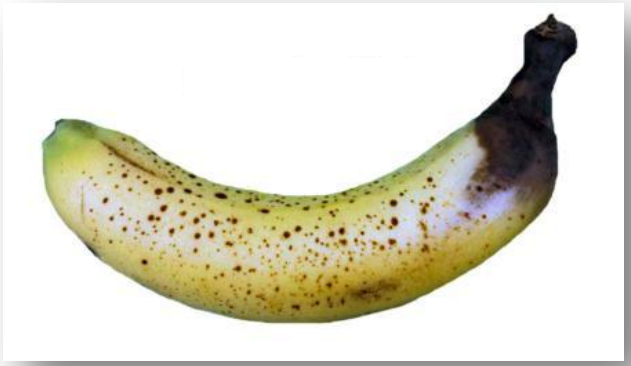
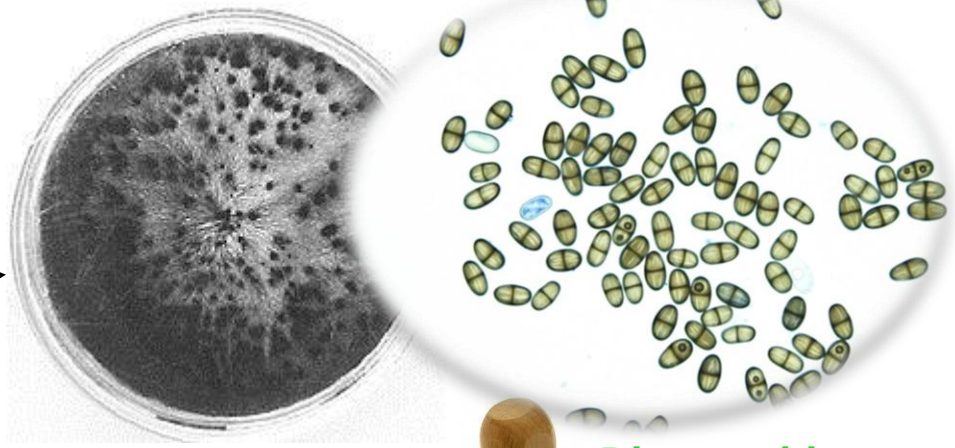
Direct vs indirect detection methods



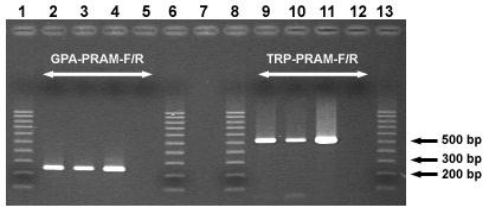


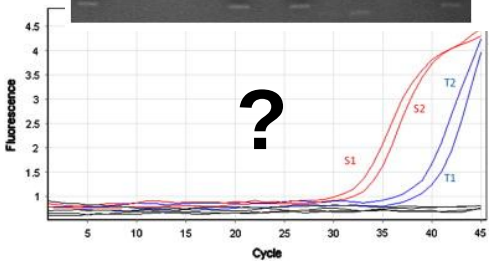
Direct evidence

Lasiodiplodia sp.

?

?





Indirect evidence

« Detected » vs « not detected »

Reliability of the results : paramount importance

Indirect analysis using PCR

Absence of quarantine fungi
=> Commodity set free

Detection of quarantine fungi
⇒Destruction
⇒Return to exporter
⇒Phytosanitary measures
⇒Ban of importation
⇒…

Reliability of a method / an analysis

1. ***a priori* validation of a qPCR method (developpement):**

- Detection of the target, when it is present, regardless of its origin and state
- No detection of the target when it is not present and no detection of a non target organism
- Use of an optimized DNA extraction protocol
- Acceptable levels of repeatability and reproducibility
- Good robustness

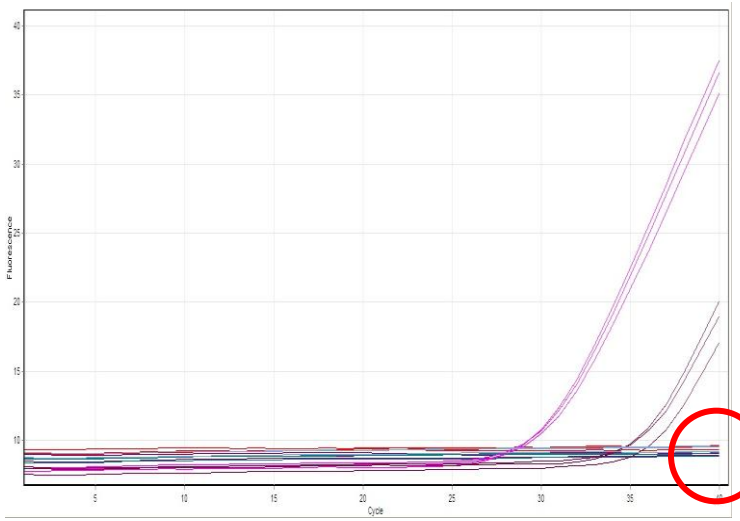
2. ***a posteriori* validation of an analysis resorting on qPCR:**

- Positive signals (Ct Cut-off value ?) are true positive
- Negative signals (Ct Cut-off value ?) are true negative
- The qPCR reaction (run) should have been optimal

Validation of qPCR results

False negative results:

- DNA shearing or loss during extraction
- Presence of inhibiting compounds
- Insufficient PCR efficiency



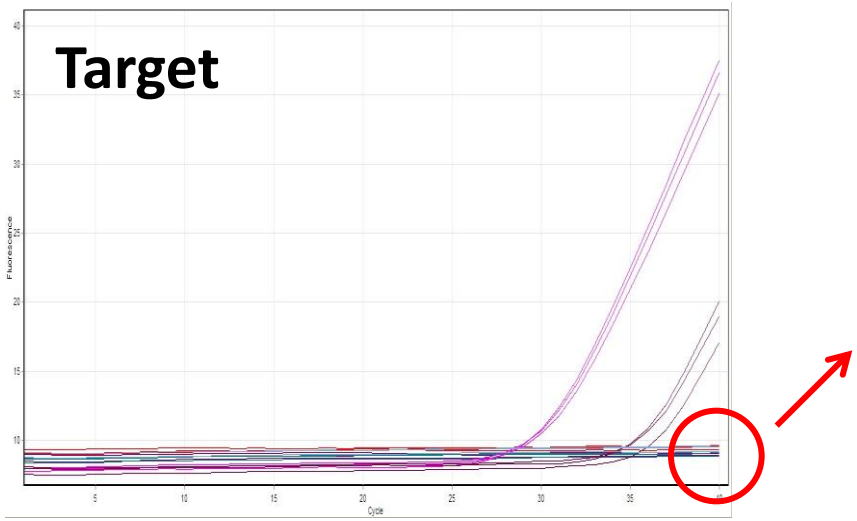
« no Ct value yielded »

- Absence of the target DNA ?
- Quantity of target DNA below the LOD ?
- Poor quality of the DNA extract ?
- Significant inhibition effect ?

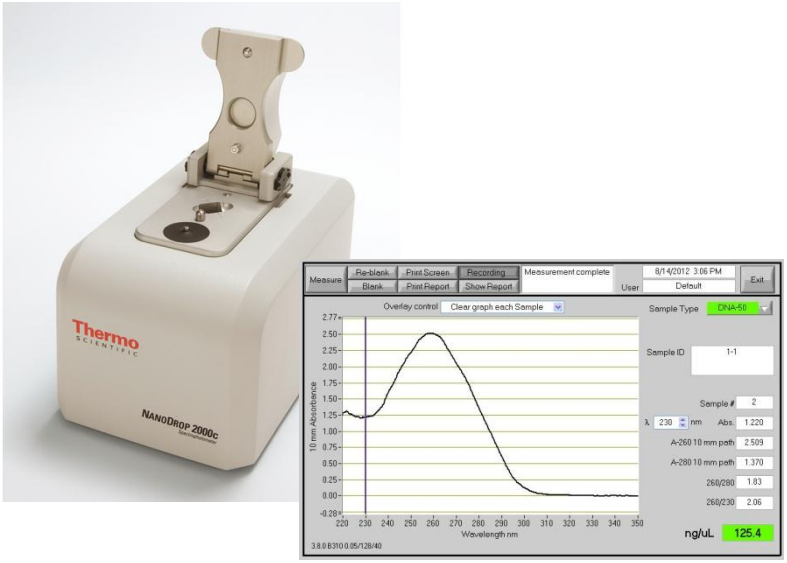
Validation of negative results

False negative results :

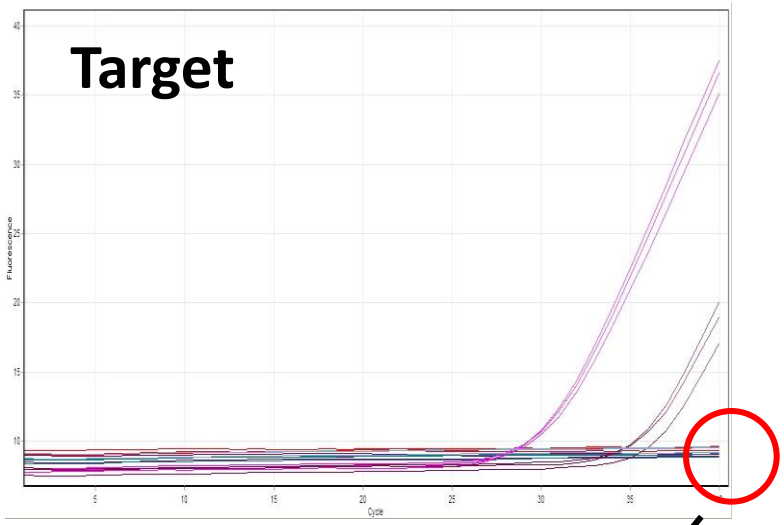
- DNA shearing or loss during extraction
- Presence of inhibiting compounds
- Insufficient PCR efficiency



Check DNA quantity with a spectrophotometer



Validation of negative results



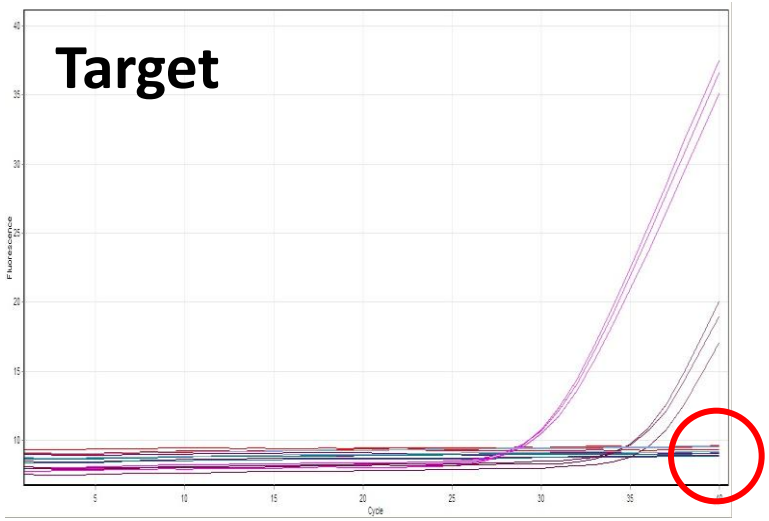
Verification of the ‘amplifiability’ of the matrix DNA (host plant DNA …) with a « universal » qPCR test
($Ct < Ct_{threhshold}$)

The additional test maybe test in duplex or separately (no loss of sensitivity)

Validation of negative results

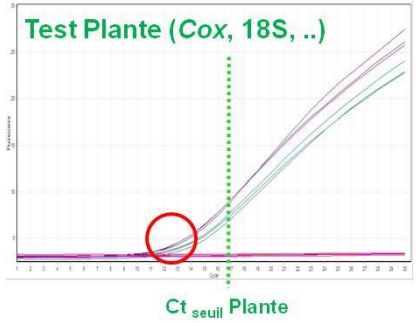
False negative results :

- DNA shearing or loss during extraction
- Presence of inhibiting compounds
- Insufficient PCR efficiency



Several options:

- Dilution of the DNA extract and re-test
- Spiking the DNA extract with target DNA and re-test
- Addition of an internal amplification control in the mastermix
- Test the DNA extract with a « universal » qPCR test (matrix DNA)



Validation of negative results

False negative results :

- DNA shearing or loss during extraction
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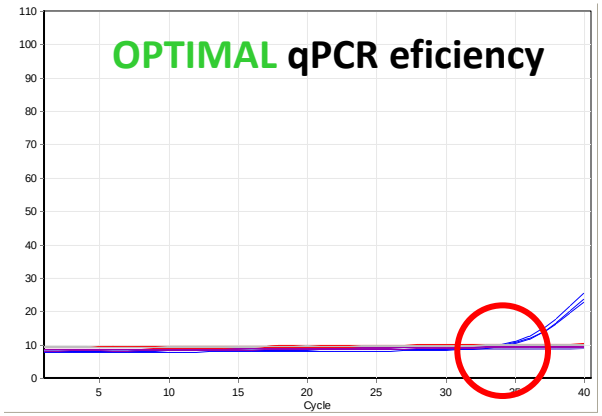
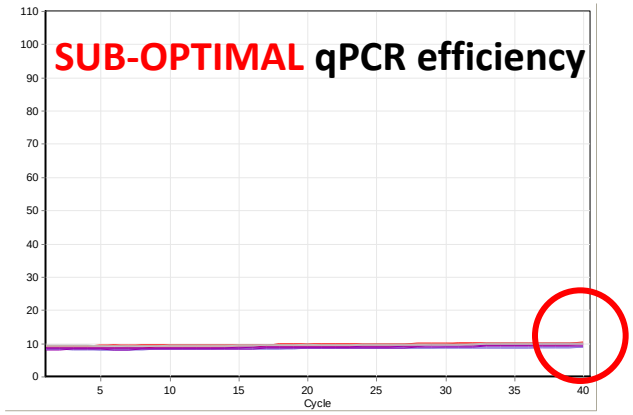
- Insufficient PCR efficiency

Regular qPCR reaction conditions



Detection
of the target DNA at low concentrations

Slight errors in pipeting volumes, temperature drift, reagents with degraded quality, etc.



Non detection
of the target DNA at low concentrations

anses

Usefulness of reference DNA samples

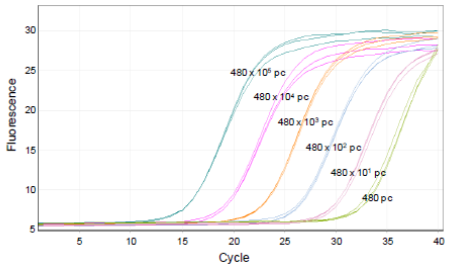
Use of reference material : Plasmidic DNA

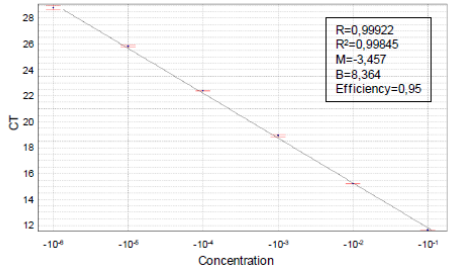
= purified bacterial plasmids containing the qPCR target DNA region)

- **Stable over time**
- **Highly homogenous**
- **Available in high amounts**
- **Easily quantifiable / transformation in copy number**
- **sequence known, verified o a regular basis/ PCR M13 –F/-R**

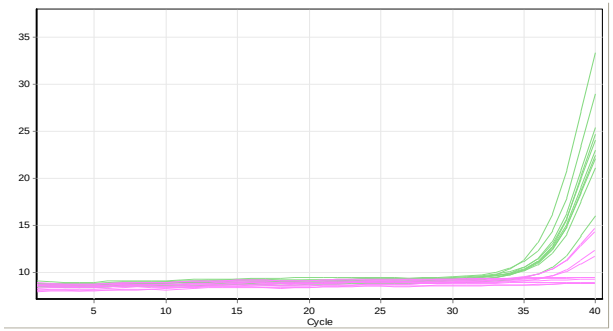
Validation of qPCR methods

- **Rough determination of the limit of detection (LOD) of the test** (wide range of 6-7 logs, 3 replicates)





- **Fine determination of the LOD** (16 replicates for the lowest amplifiable target concentration)



The LOD of the test is defined as the lowest target concentration that yields 100% of positive results (Ct values < 40 cycles)

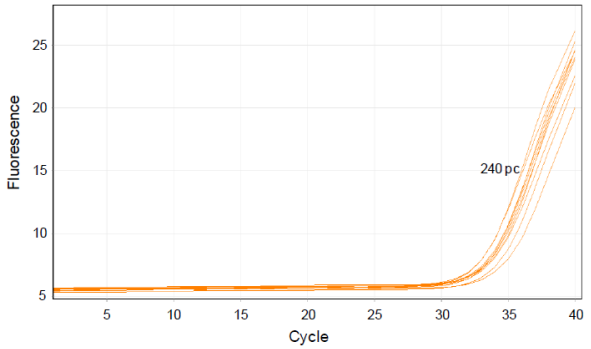
+LOD concentration remains fixed for a specific equipment (real- time machine, master mix reagents, PCR conditions)

Validation of the qPCR method

Repeatability : same samples, same reagents, same qPCR run
Reproducibility : same samples, different batch of reagents, different qPCR runs, different operator, different equipment.

Sample: target DNA with an appropriate concentration

⇒***Evaluation of the variability of the Ct value***



A high level of repeatability and reproducibility allow to use the plamidic controls as references for the test.

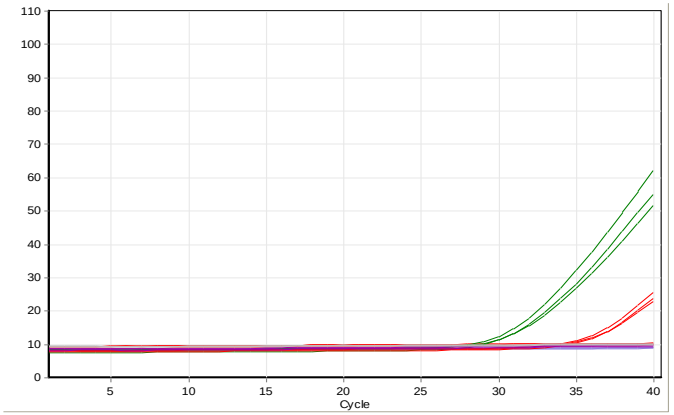
TABLE 5. Inter- and intra-assay coefficients of variation (CVs) based on mean cycle threshold values calculated for the duplex *P. halstedii* quantitative polymerase chain reaction (PCR) assay

Target	Target concentration[x]	CV (%)	
		Intra-assay	Interassay
Plasmopara halstedii qPHAL-F/-R PCR product	2.26×10^4	0.45	2.21
	2.26×10^3	0.52	1.52
	2.26×10^2[y]	1.98	1.69
P. halstedii DNA	n.d.[z]	1.74	4.04

[x] Number of plasmid copies in the PCR tube, in which was inserted the qPHAL-F/-R region, diluted in a background of *Helianthus annuus* DNA.
[y] This concentration was determined as 10 times the limit of detection of the test.
[z] Total DNA extract from a naturally infected *H. annuus* seed sample (02 FU).

Validation of a qPCR run

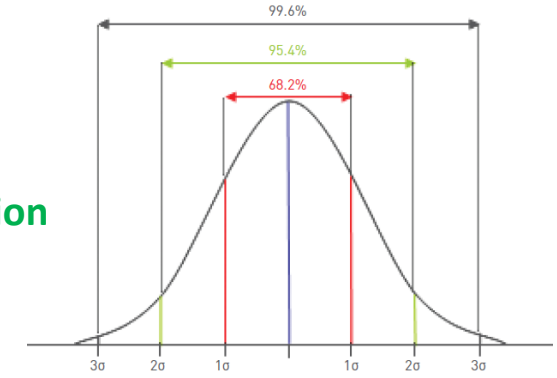
- **A positive calibration control (+CAL) is defined based on the LOD Positive control (+LOD) =>100X**



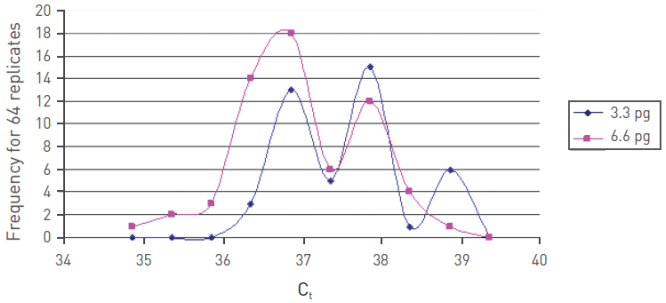
+CAL

+LOD

- **+CAL Ct values follow a normal distribution**



- **+LOD Ct values distribution is more close to a Poisson's law**



anses — Laboratoire de la Santé des Végétaux, *Unité de mycologie*

Fiche d'enregistrement

Code: PQT8-FE-030
Ind. Rév. D du 7 février 2013
Page (s): 1

Carte de contrôle en qPCR

Test qPCR :	Détection de Cibberella circinata / semences
Nature et concentration du $T+_{CALIB}$:	2 µL de solution plasmidique à la [finale] de $2,3\ 10^3$ cp / µL
Type de carte de contrôle :	**acquisition de données** ☐ **active** ☒

Opérateur	Date	Val. moy Ct $T+_{CALIB}$	M-3S→ =23,8	M-2S→ =24,6	Ct moyen (M) = 26,4 ↓	←M+2S =28,1	←M+3S =29,0	Val. moy. Ct $T+_{LOD}$	[finale] de $T+_{LOD}$
RI	7/02/13	25,7			X			35,6	23,7 cp/µL
CG/LF	19/02/13	24,37		X				32,86 (213)	23,7 cp/µL
CG/CF	20/02/13	24,31		X				30,83	23,7 cp/µL
CG	07/03/13	25,03			X			33,52	23,7 cp/µL
CG	26/03/13	24,87			X			33,51 (213)	23,7 cp/µL
CG	02/04/13	24,07		X				32,90 (213)	23,7 cp/µL

Première estimation ou réévaluation :	1ère
Nombre de valeurs de Ct acquises :	30
Moyenne M du Ct $T+_{CALIB}$:	26,4
Ecart type S :	0,9
2S :	1,8
3S :	2,7

Rappel :

- Si Ct $T+_{CALIB}$ est compris dans M±2S, la valeur Ct $T+_{CALIB}$ est conforme.
- Si Ct $T+_{CALIB}$ est compris entre M±3S et M±2S, la valeur Ct $T+_{CALIB}$ est conforme, mais attention particulière aux prochaines valeurs obtenues requise.
- Si Ct $T+_{CALIB}$ sort du tunnel de valeur M±3S, la valeur de Ct $T+_{CALIB}$ est non conforme (fiche d'écart). Le run à refaire intégralement. Si Ct $T+_{CALIB}$ est à nouveau >M+3S, l'origine de la non-conformité sera identifiée avant de reprendre les analyses.
- Si 3 valeurs consécutives de Ct $T+_{CALIB}$ sortent du tunnel M±2S, l'origine de la dérive sera être identifiée avant de reprendre les analyses (fiche d'écart)

Date, nom et visa du responsable technique :

Validation of a run :

- **Upon acceptable mean Ct value for + CAL**
- **Upon amplification of +LOD**

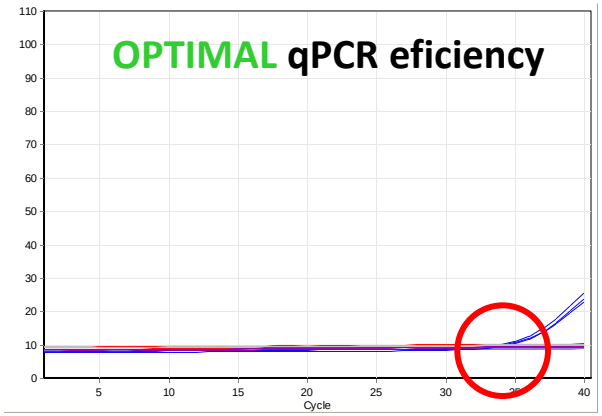
anses

Validation of qPCR results

False negative results :

- DNA shearing or loss during extraction
- Presence of inhibiting compounds
- Insufficient PCR efficiency

Regular qPCR reaction conditions



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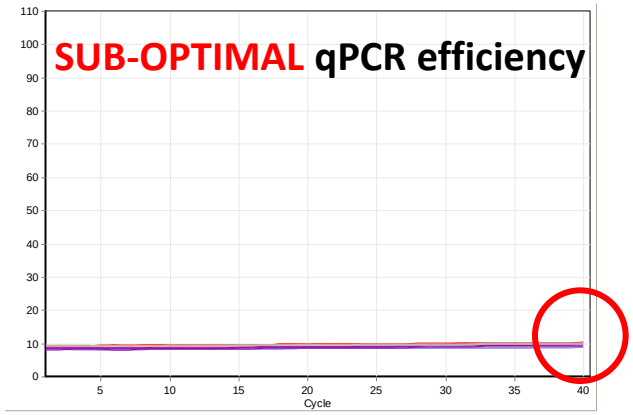


Non detection
of the target DNA at low concentrations

FA155493 ISSN 0335-3931

normalisation française

XP V 03-043
Juillet 2008

Indice de classement : **V 03-043**

ICS : 67.050

Exigences générales pour la réalisation d'analyses utilisant la biologie moléculaire pour la détection et l'identification d'organismes pathogènes, d'altération et ravageurs des végétaux et produits dérivés

E : General requirements for molecular biology analysis for detection and identification of pathogenic and destructive organisms in plants and derived products

D : Allgemeine Anforderungen für molekularbiologische Untersuchungen zum Nachweis und zur Identifizierung von pathogenen Schadorganismen für Pflanzen und Pflanzenerzeugnisse

“ … At each PCR-led detection reaction, read off the Ct of the control at limit of detection (Ct_{LOD}).
All the samples in this reaction presenting a Ct of below Ct_{LOD} +3 and showing exponentially increasing fluorescence are considered positive.”

anses

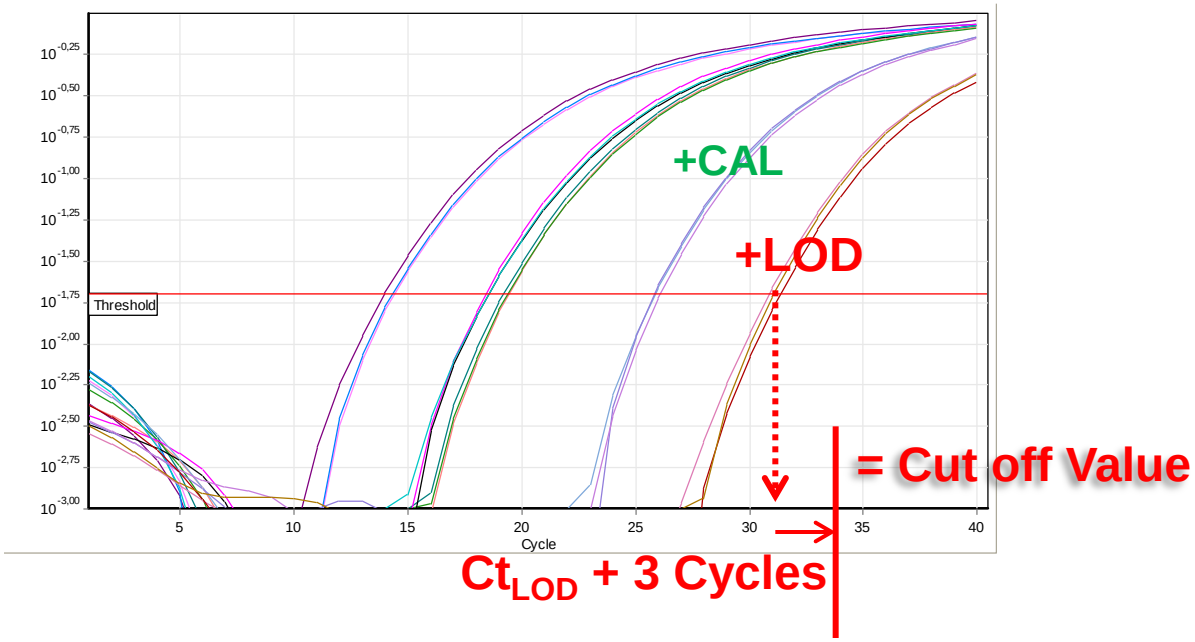
Validation of positive results

Ct values are highly dependent of equipment, software, fluorescence measurements and applied algorithms, reagents quality, brands, pipetting operations, …

=> A fixed Cut off value would mean that all the factors above are stricly conserved over time…. Nearly impossible…

Validation of positive results

“ … At each PCR-led detection reaction, read off the Ct of the control at limit of detection (Ct_{LOD}).
All the samples in this reaction presenting a Ct of below Ct_{LOD} +3 and showing exponentially increasing fluorescence are considered positive.”



=> The Cut off Value is slightly variable from run to run and is defined at the end of each reaction

System already implemented for >10 protocols in our unit, and accredited in flexible scope

Techniques

Sensitive Detection of *Fusarium circinatum* in Pine Seed by Combining an Enrichment Procedure with a Real-Time Polymerase Chain Reaction Using Dual-Labeled Probe Chemistry

Renaud Ioos, Céline Fourrier, Gabriela Iancu, and Thomas R. Gordon

Eur J Plant Pathol (2009) 125:329–335
DOI 10.1007/s10658-009-9471-x

Rapid *in planta* detection of *Chalara fraxinea* by a real-time PCR assay using a dual-labelled probe

Renaud Ioos · Tadeusz Kowalski · Claude Husson · Ottmar Holdenrieder

Techniques

Development, Comparison, and Validation of Real-Time and Conventional PCR Tools for the Detection of the Fungal Pathogens Causing Brown Spot and Red Band Needle Blights of Pine

Renaud Ioos, Bénédicte Fabre, Carole Saurat, Céline Fourrier, Pascal Frey, and Benoît Marçais

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bms British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

Fungal Biology

Optimization of a real-time PCR assay for the detection of the quarantine pathogen *Melampsora medusae* f. sp. *deltoidae*

Anne-Laure BOUTIGNY[a,], Cécile GUINET[a], Agathe VIALLE[b], Richard C. HAMELIN[b], Axelle ANDRIEUX[c,d], Pascal FREY[c,d], Claude HUSSON[c,d], Renaud IOOS[a]*

Techniques

An Optimized Duplex Real-Time PCR Tool for Sensitive Detection of the Quarantine Oomycete *Plasmopara halstedii* in Sunflower Seeds

Renaud Ioos, Céline Fourrier, Véronique Wilson, Kathryn Webb, Jean-Luc Schereffer, and Denis Tourvieille de Labrouhe

Eur J Plant Pathol
DOI 10.1007/s10658-013-0180-0

A sensitive real-time PCR assay for the detection of the two *Melampsora medusae formae speciales* on infected poplar leaves

Anne-Laure Boutigny · Cécile Guinet · Agathe Vialle · Richard Hamelin · Pascal Frey · Renaud Ioos