Nederlandse Voedsel- en Warenautoriteit
Ministerie van Economische Zaken

EPPO workshop on Ct cut-off values

Setting Ct cut-off values using international test performance studies (TPS)

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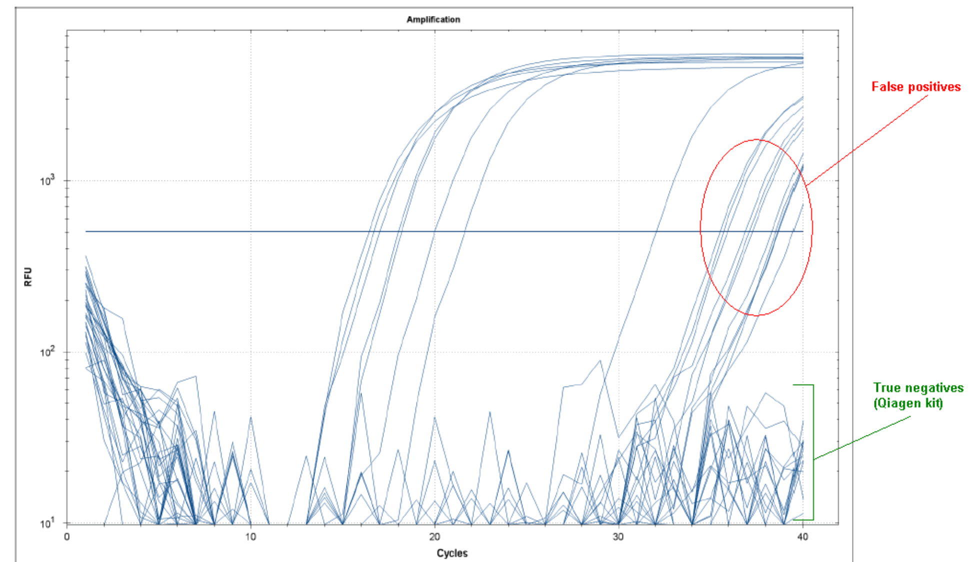
Why do we need Ct cut-off values

Lack of analytical specificity or selectivity of a real-time PCR test

Amplification curves obtained from non-target organisms (related organisms, look-alikes or matrix) can lead to false postive results

Usually mentioned in literature, or uncovered during method validation



Applies to a-specific signals that cannot be distinguished based on the amplification plot

Why do we need Ct cut-off values

Detection tests versus identification tests

Detection in complex matrix increases the risk of a-specific amplification and false positive results

Setting a too stringent cut-off value will lead to false negative results

When an additional identification test is used, no cut-off might be needed

Setting cut-off values by individual labs

A-specific amplification might be influenced by:

- Reaction mix
- Thermocycler
- Consumables
- Analysis method (passive reference dyes, background correction)

Validation (verification) has to be used to determine the need and position of a cut-off value

- Analytical specificity, Selectivity, Analytical sensitivity

No guidelines available for cut-off determination in qualitative tests

Setting cut-off values by individual labs

Standard text EPPO instructions to authors (appendix 3)

The cycle cut off value for target X is set at xx, and was obtained using the equipment/materials and chemistry used as described in this appendix. When necessary the Ct cut off value should be determined for the required control. ***The cycle cut off value needs to be verified in each laboratory when implementing the test for the first time****.*

Verification of cut-off values can be difficult

- Reference material of cross-reacting species or matrixes is hard to obtain
- Test verification is (usually) time consuming
- Has to be repeated when new equipment or reagents are used

International Test Performance Studies

International Test Performance Studies (TPS) can be used to determine a generic cut-off value for specific tests.

Strengths of TPS

- Large number of (international) participants (x > 8)
- Relevant (known) cross reacting non-target organisms and matrices can be included
- Different real-time PCR machines and analysis settings are used
- Additional robustness experiments: in-house available nucleic acid extraction kits and reagents

International Test Performance Studies

TPS set-up has to be reliable to benefit from TPS strengths

Relevant samples (diagnostic or spiked)

Homogeneity (√n samples) and stability of samples

Proficiency of participants (training session)

Provide as much consumables and reagents as possible to allow comparison

Clear instructions (what is allowed and what not)

Provide standardised controls to detect outliers

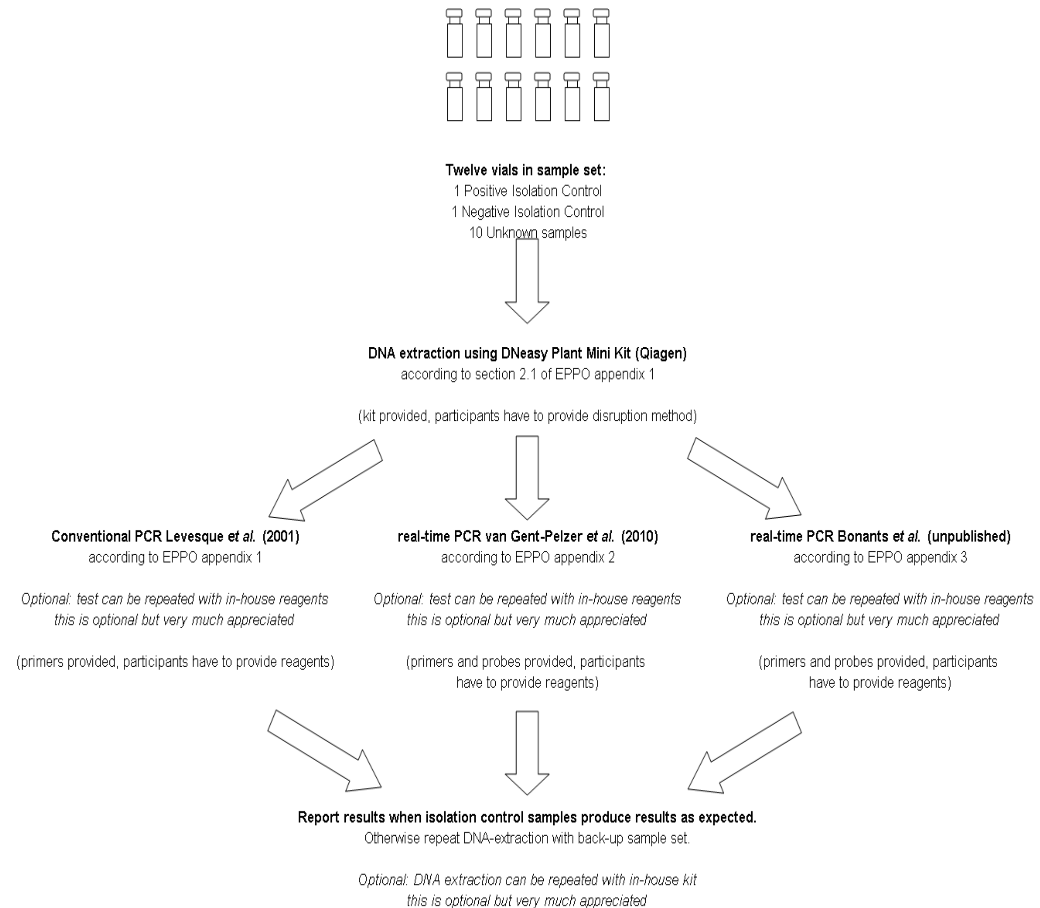
An example: EUPHRESCO SENDO 3a - TPS

15 partners from 10 different countries

Determining performance criteria for molecular detection and identification tests for *S. endobioticum*

12 samples per partner in duplicate

3 tests (1 conventional, 2 real-time)



An example: EUPHRESCO SENDO 3a - TPS

Draft PM7/28 - 3 appendixes according to latest version of instructions to authors

Detailed description of test interpretation

Instruction booklet, TPS sample set, Return sample, LoA, Primers and probes, Positive amplification controls, DNA extraction kit

Appendix 1. Detection of *S. endobioticum* in potato wart material using conventional PCR

1. General Information

1.1. Detection of *S. endobioticum* in potato warts using conventional PCR developed by Levesque *et al.* 2001

1.2. The conventional PCR was first published by Levesque *et al.* in 2001, but described in detail by van den Boogert *et al.* in 2005. The PCR reaction mix was updated by NPPO-NL in 2013 and validated in an international test performance study as such.

1.3. Primers F49 (5'-CAACACCATGTGAACTG-3') and R502 (5'-ACATACACAATTCGAGTTT-3') amplify 472 bp of the internal transcribed spacer (ITS) region.

1.4. Amplification is performed in a thermal cycler with heated lid, e.g. T100 Thermal cycler (Bio-Rad).

2. Methods

2.1. Nucleic Acid Extraction and Purification

Potato wart material (max. 100 mg) is extracted using a modified Plant Tissue mini protocol from the DNeasy® Plant Mini Kit (Qiagen).

2.1.1. Transfer wart material (max. 100 mg) to a 2 mL microcentrifuge tube. Add two steel beads (e.g. 4 mm diameter) to the vial when mechanical disruption is used

2.1.2. Add 400 µL Buffer AP1 and 4 µL RNase A stock solution (100 mg/mL). Buffer AP1 may form precipitates upon storage. If necessary, warm to 65°C to redissolve

2.1.3. Disrupt the sample either manually (e.g. with a micro pestle) or mechanically (e.g. with a BeadBeater or TissueLyser)

2.1.4. Incubate the mixture for 10 min at 65°C. Mix 2 or 3 times during incubation by inverting tube

2.1.5. Centrifuge 1 min at 20,000 x g and transfer the supernatant (= lysate) to a new 1.5 mL microcentrifuge tube

2.1.6. Add 130 µL Buffer P3 to the lysate and mix.

2.1.7. Incubate for 5 min on ice

2.1.8. Centrifuge the lysate for 5 min at 20,000 x g

2.1.9. Pipet the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 mL collection tube

2.1.10. Centrifuge for 2 min at 20,000 x g

2.1.11. Transfer 450 µL of the flow-through fraction from step 10 into a new 1.5 mL microcentrifuge tube

2.1.12. Add 675 µL (1.5 volumes) of Buffer AW1 to the cleared lysate, and mix by pipetting.

2.1.13. Pipet 650 µL of the mixture from step 12, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 mL collection tube.

2.1.14. Centrifuge for 1 min at 6000 x g

2.1.15. Place the DNeasy Mini spin column into a new 2 mL collection tube and repeat step 11 with remaining sample.

2.1.16. Place the DNeasy Mini spin column into a new 2 mL collection tube, add 500 µL Buffer AW2,

2.1.17. Centrifuge for 1 min at 6000 x g.

2.1.18. Place the DNeasy Mini spin column into a new 2 mL collection tube, add 500 µL Buffer AW2 to the DNeasy Mini spin column.

2.1.19. Centrifuge for 2 min at 20,000 x g to dry the membrane.

2.1.20. Transfer the DNeasy Mini spin column to a 1.5 mL microcentrifuge tube, and pipet 50 µL Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature (15 - 25°C).

2.1.21. Centrifuge for 1 min at 6000 x g. The flow through contains the extracted DNA.

An example: EUPHRESCO SENDO 3a - TPS

TPS set-up had to be followed

Alternative DNA extraction and reaction mix set was allowed using the identical back-up sample set (robustness)

Data reported in a standardised form

Data collection, clean-up and determination of performance criteria (and cut-off values)

Detection test: van Gent Pelzer *et al.*, 2010 – known issues with false positive signals in healthy potato and NACs

Real-time PCR van Gent Pelzer *et al*., 2010 (5)

Real-time PCR machines used following draft EPPO appendices

#	Real-Time PCR Machines
1	Mx3005P (Stratagene) (Sendo)
	7900HT Fast Real-Time PCR system (Applied Biosystems) (Sendo and P1/non-P1)
2	7900HT Fast Real-Time PCR system (Applied Biosystems)
3	7900HT Fast real-time PCR system (Applied Biosystems)
4	Mastercycler® ep realplex (Eppendorf)
8	7500 Real-Time PCR system (Applied Biosystems)
9	LightCycler®480 (Roche)
11	CFX96™ (Bio-Rad)
12	CFX96™ (Bio-Rad)
13	7500 Real-Time PCR system (Applied Biosystems)
14	N.A.
15	7500 Real-Time PCR system (Applied Biosystems)

Real-time PCR van Gent Pelzer *et al.*, 2010

SENDO

RAW DATA PROVIDED BY PARTNER

test results using the TPS set-up. When the back-up sample set was used because of contamination or failed DNA extraction, these results are used in this table.

			Partner 1	2	3	4	5	6	7	8	9b	10	11	12	13	14	15	HT average	SD
NAC			N/A	N/A	N/A	N/A	N/A		N.A.	N/A	N/A		N/A	N/A	37,0	N/A	N/A	N/A	N/A
PAC1 P1			18,8	17,0	16,8	18,8	16,12		17,99	19,71	16,1		16,2	19,25	16,1	19,25	17,13	16,7	1,4
PAC2 P1			26,3	24,7	24,0	20,93	23,81		24,49	26,57	23,83		24,1	17,84	23,3	17,84	23,96	24,0	1,4
NIC	healthy potato "eersteling"	N.A.	**N/A**	**39,9**	**N/A**	**38**	**N/A**		**34,06**	**N/A**	-		**N/A**	**38,08**	**31,2**	**38,08**	**N/A**	N/A	N/A
PIC	S. endo 1 (D1)	MB42	**20,1**	**15,8**	**17,3**	**16,4**	**14,1**		**21,1**	**19,6**	**18,5**		**19,5**	**16,0**	**15,4**	**16,0**	**15,3**	16,3	1,0
sample 01	S.endo 2 (G1)	MB08	**21,6**	**19,1**	**24,5**	**19,4**			**18,1**	**20,4**			**22,4**	**17,2**	**16,2**	**17,2**	**N/A**	19,6	2,2
sample 02	S.endo 1 (D1)	MB42	**21,0**			**20,2**	**15,5**		**17,7**	**19,0**	**16,6**		**19,0**	**15,0**	**14,2**	**15,0**	**19,2**	16,3	1,0
sample 03	S.endo 18 (T1)	MB86		**20,8**	**20,8**	**16,7**	**17,4**		**18,9**		**17,1**		**19,8**		**18,8**		**20,0**	18,1	1,7
sample 04	S.endo 38 (Nevsehir)	MB56		**17,5**	**20,9**		**20,2**		**18,4**	**20,6**	**18,6**		**15,7**	**13,9**		**13,9**	**16,8**	18,7	1,3
sample 05	healthy potato "eersteling"	N.A.	**N/A**	**33,5**	**N/A**	**N/A**	**N/A**		**32,7**	**37,3**	**35,0**		**38,6**	**32,6**	**32,7**	**32,6**	**N/A**	N/A	N/A
sample 06	S.endo 6 (O1)	MB10	**18,5**	**14,9**	**19,7**	**19,1**	**15,1**				**16,0**		**15,1**	**15,3**	**13,8**	**15,3**	**17,2**	15,5	1,2
sample 07	S.endo 18 (T1)	MB86	**23,9**		**19,0**	**18,2**	**16,3**		**16,3**	**19,5**	**17,9**		**16,9**	**15,7**	**15,8**	**15,7**	**17,1**	18,1	1,7
sample 08	S.endo 6 (O1)	MB10	**19,5**	**16,6**	**16,8**	**17,4**	**16,0**		**16,7**	**17,9**	**15,1**		**15,1**				**16,0**	15,5	1,2
sample 09	S.endo 2 (G1)	MB08	**21,5**	**17,6**	**17,7**		**18,1**		**17,7**	**20,0**			**17,4**	**15,9**	**17,5**	**15,9**		19,6	2,2
sample 10	S.endo 38 (Nevsehir)	MB56	**23,3**	**17,7**		**17,4**	**17,0**			**19,4**	**17,9**			**14,9**	**17,9**	**14,9**	**N/A**	18,7	1,3
sample 11	S.endo 1 (D1)	MB42	**22,5**	**15,7**	**18,9**	**20,5**			**18,6**	**20,2**	**17,0**			**15,1**	**15,9**	**15,1**	**N/A**	16,3	1,0
sample 12	healthy potato "eersteling"	N.A.	**N/A**	**34,9**	**N/A**	**35,6**	**N/A**		**34,1**	**37,4**	**N/A**		**36,7**	**N/A**	**34,0**	**N/A**		N/A	N/A
			10	10	10	10	10	0	10	10	10	0	10	10	10	10	10		

		sum correct	n analysed	% correct
NAC	MGW	12	13	92%
PAC1 P1	(MB42)	13	13	100%
PAC2 P1	(MB42)	13	13	100%
MB42	S.endo 1 (D1)	34	35	97%
MB08	S.endo 2 (G1)	20	21	95%
MB86	S.endo 18 (T1)	21	21	100%
MB56	S.endo 38 (Nevsehir)	19	20	95%
MB10	S.endo 6 (O1)	21	21	100%
Healthy	Eersteling	18	38	47%

Color codes dataset:
Green + Bold = within -3SD and +3SD limits
Blue + Bold = below -3SD limit
Red + Bold = above +3SD limit

Data clean-up: significant outliers in PACs and “non-proficient” lab(s)

Real-time PCR van Gent Pelzer *et al*., 2010 (2)

40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0

S.endo P1 MB42

S.endo P2 MB08

S.endo P6 MB10

S.endo P18 MB86

S.endo P38 MB56

Healthy potato “eersteling”

No outliers in healthy potato detected with Grubbs test (Extreme Studentised Deviate) with significance values 0.01 and 0.05

Real-time PCR van Gent Pelzer *et al.*, 2010 (3)

Diagnostic sensitivity and diagn. Specificity – influence of Ct cut-off

Lowest false positive Ct-value = 31.2

Arbitrarily

Cut-off = 40

Sendo			sum correct	n analysed	% correct
NAC	MGW		12	13	92%
PAC1 P1	(MB42)		13	13	100%
PAC2 P1	(MB42)		13	13	100%
MB42	S.endo 1 (D1)		34	34	100%
MB08	S.endo 2 (G1)		20	21	95%
MB86	S.endo 18 (T1)		21	21	100%
MB56	S.endo 38 (Nevsehir)		19	19	100%
MB10	S.endo 6 (O1)		21	21	100%
Healthy	Eersteling		18	38	47%

Cut-off = 40

			Standard (HT)	
			+	-
TPS		+	115	20
		-	1	18

Diagnostic sensisitvity	99%		(false negatives)
Diagnostic specificity	47%		(false positives)

Cut-off = 31

Sendo			sum correct	n analysed	% correct
NAC	MGW		13	13	100%
PAC1 P1	(MB42)		13	13	100%
PAC2 P1	(MB42)		13	13	100%
MB42	S.endo 1 (D1)		34	34	100%
MB08	S.endo 2 (G1)		20	21	95%
MB86	S.endo 18 (T1)		21	21	100%
MB56	S.endo 38 (Nevsehir)		19	19	100%
MB10	S.endo 6 (O1)		21	21	100%
Healthy	Eersteling		38	38	100%

Cut-off = 31

			Standard (HT)	
			+	-
TPS		+	115	0
		-	1	38

Diagnostic sensisitvity	99%		(false negatives)
Diagnostic specificity	100%		(false positives)

Real-time PCR van Gent Pelzer *et al*., 2010 (4)

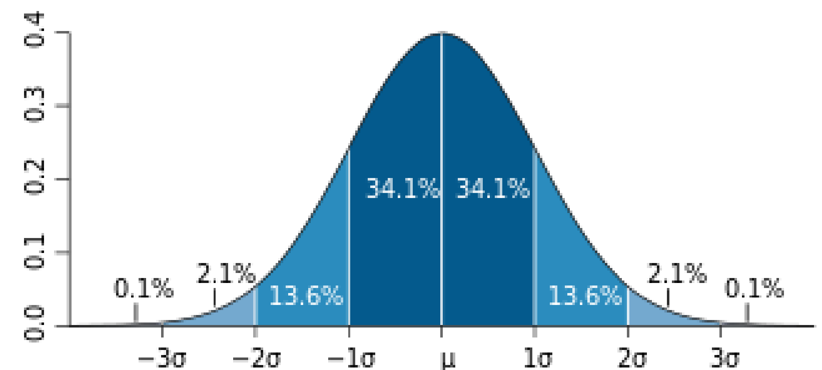
Introducing confidence levels for cut-off values (semi-quantitative)

clean-up of dataset (Grubbs outlier test)

Average and StDev of a-specific signals

Zijlstra test: average = 35.5, StDev = 2.5

Average Ct value	confidence level	Cut-off Zijlstra test
- 1 SD	84.0 %	33.0
- 2 SD	97.6 %	30.5
- 3 SD	99.7 %	28.0



Real-time PCR van Gent Pelzer *et al*., 2010 (2)

40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0

S.endo P1 MB42

S.endo P2 MB08

S.endo P6 MB10

S.endo P18 MB86

S.endo P38 MB56

Healthy potato “eersteling”

Cut-off value : 28.0 (99,7% confidence)

Real-time PCR van Gent Pelzer *et al*., 2010 (6)

Robustness – changes to EPPO appendix

40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0

1 2 3 4 5

S.endo P2 MB08

S.endo P1 MB42

S.endo P18 MB86

S.endo P38 MB56

S.endo P6 MB10

Healthy potato "eersteling"

1 3 4 5

COX

1:TaqMan Universal master mix (Applied Biosystems)
2:1 µl i.o. 3 µl template
3:PCR mastermix 2x (Qiagen), duplex, primers and probes 200 nm each
4:PCR mastermix 2x (Qiagen); 2x simplex, primers and probes as TPS
5:Sbeadex maxi plant kit (LGC Genomics) on KingFisher

Discussion Points

Is it possible to establish a truly generic cut-off value for a specific test?

Does this remove the need for (elaborate) test verification?

Does a TPS provide added value in terms of cut-off value determination?

Detection tests that are followed by specific identification (verification) tests do not require cut-off values.

A confidence level will aid the determination of a cut-off value and will give information for test implementation.

Thank you for your attention

Questions, discussion, any other business