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Determination of LOD and cycle cut-off in real-time PCR detecting culturable and non-culturable target organisms

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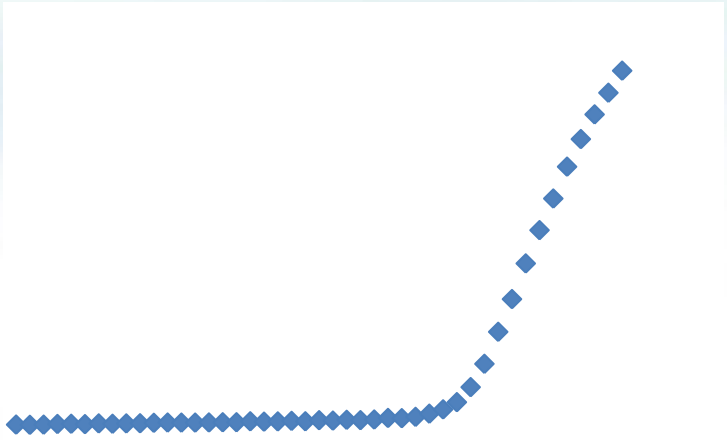
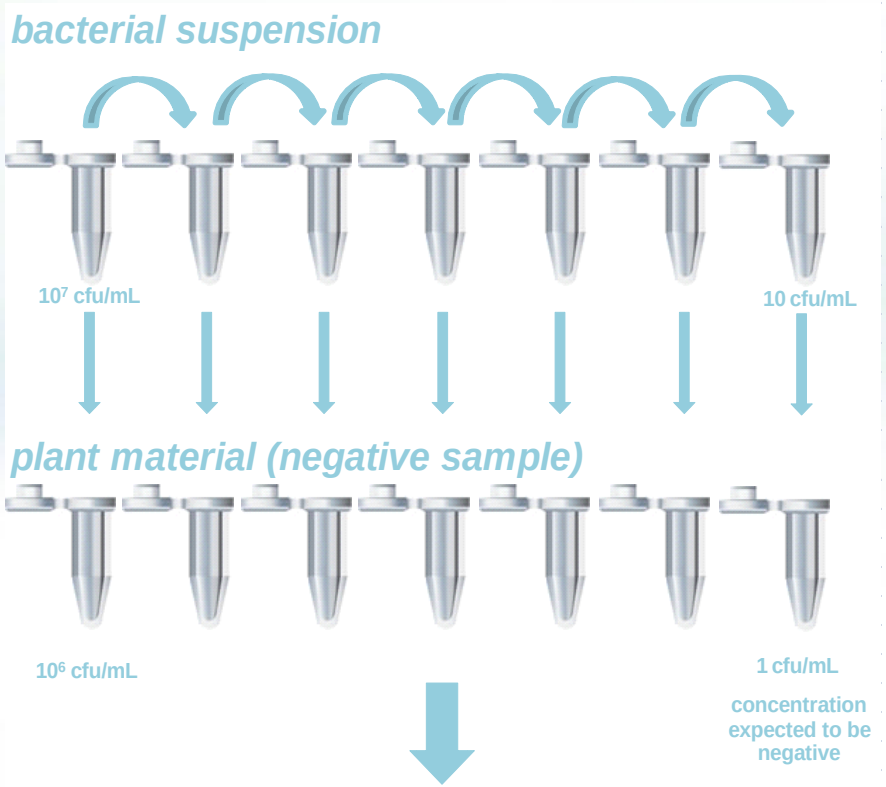


EPPO Workhop on Setting Ct cut-off values for real-time PCR
11th – 12th November 2013

Analytical sensitivity in bacteriology

EPPO PM 7/98, spiked samples: negative samples + target HO

Minimum 3 different samples

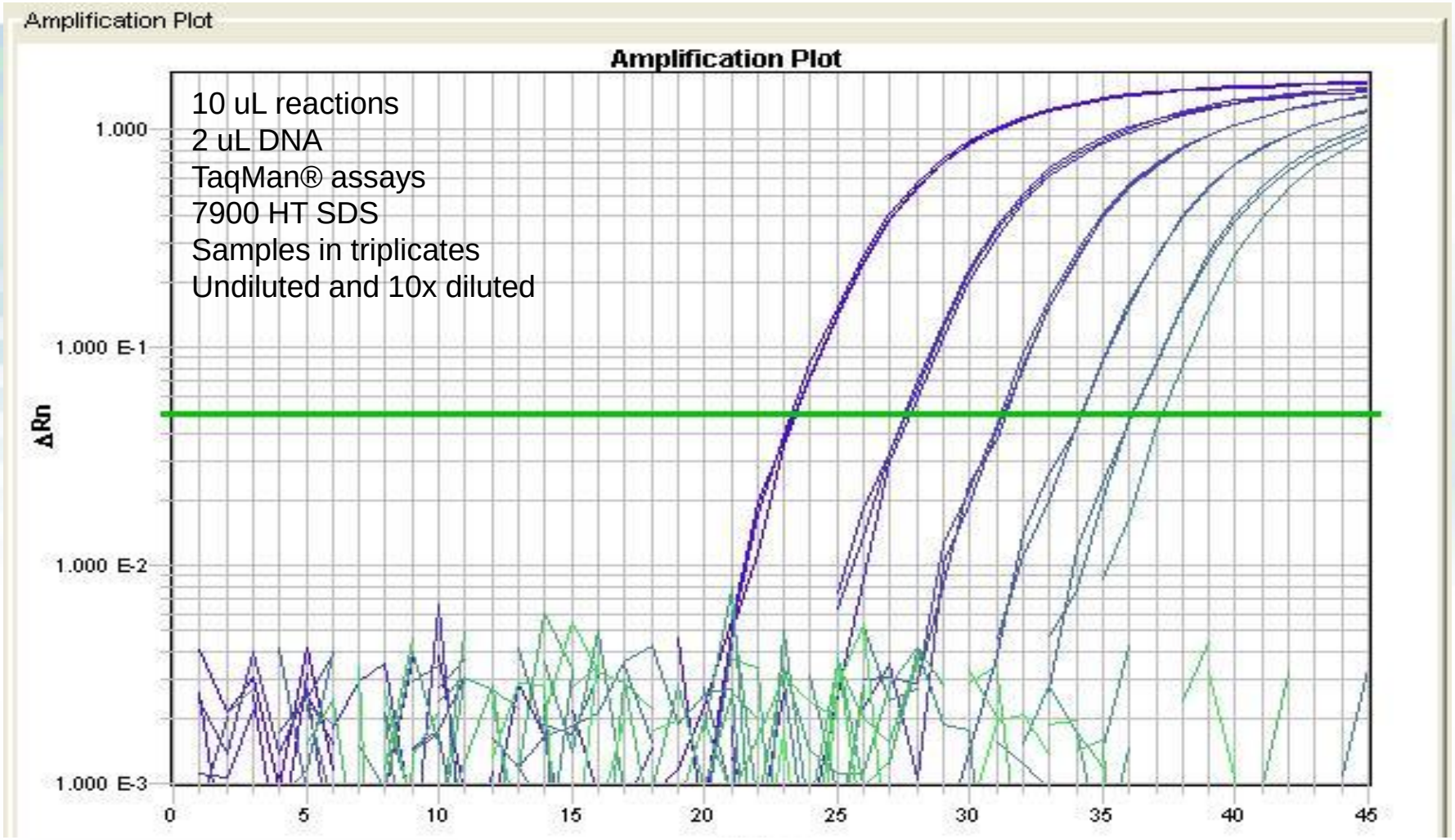


DNA extraction

real-time PCR

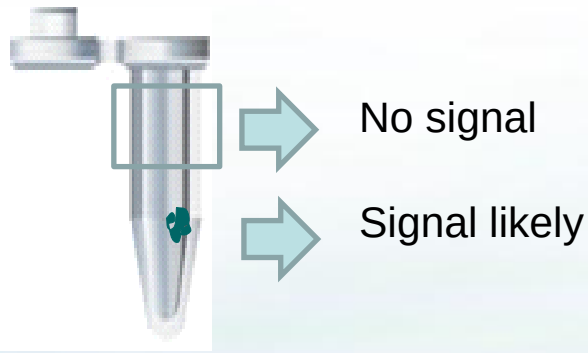
PM 7 /76 (2)
Smallest amount of target that can be detected reliably (this is sometimes referred to as 'limit of detection')

From curves to a numerical LOD value



Low level detection

- **Stochastic effect**



- **Poisson probability distribution**
- In practice, **10 copies** of target DNA per PCR vessel is **the lowest concentration** which is **amplified each time** a PCR assay is performed (Vaermann et al., 2004).

Cq values at low level detection

- **Monte Carlo effect - larger variations** in quantified amount / **Cq values**: *"an inherent limitation of PCR amplification from small amounts of any complex template due to differences in amplification efficiency between individual templates"*

- **What is reliable?**

  In-house definition: “LOD = target copy concentration with probability of detection **P(det) 0.9*, ****”

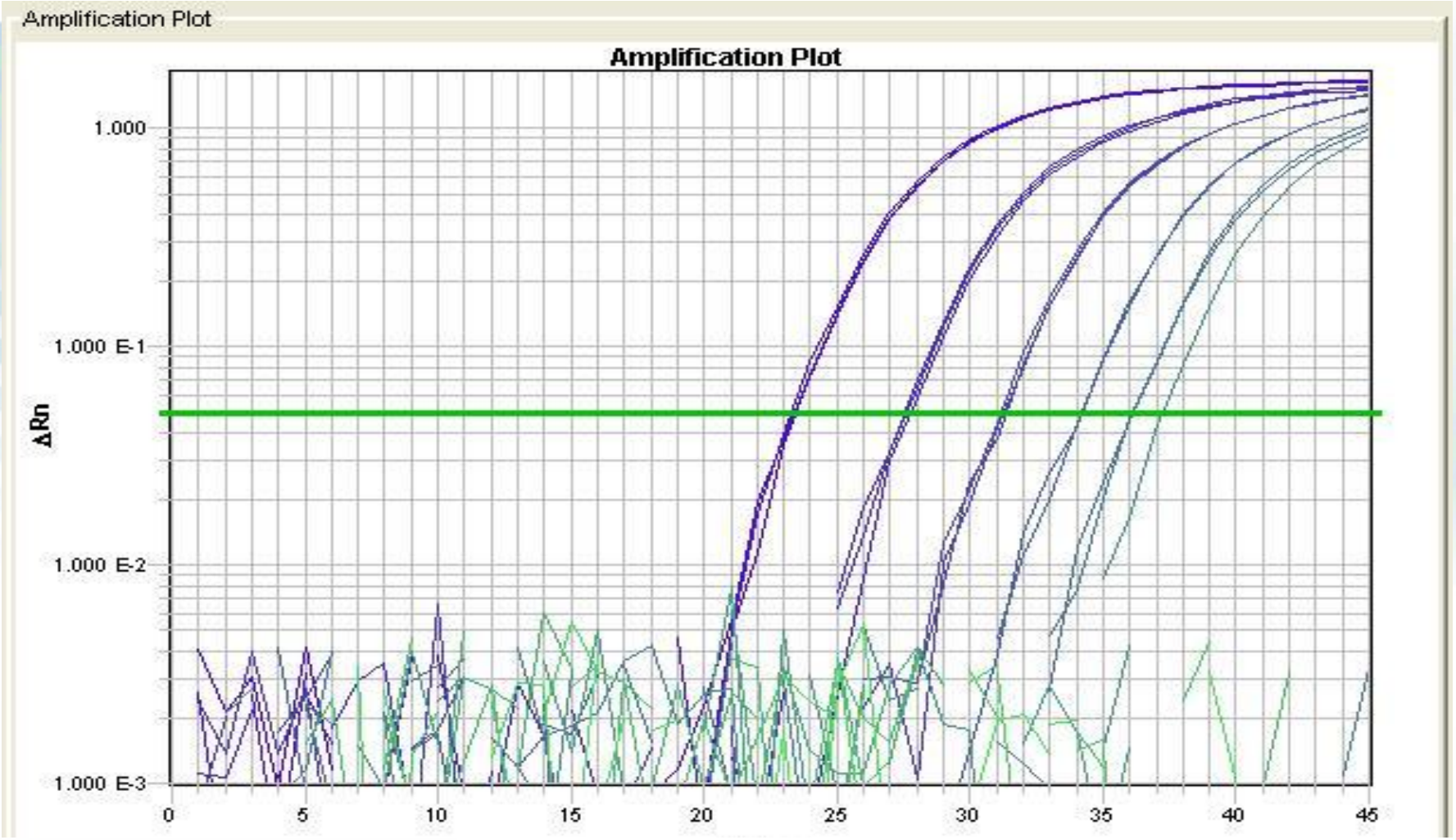
$$= \frac{\text{number of positive reactions}}{\text{number of all reactions}}$$

*0.95 required by Codex Allimentarius

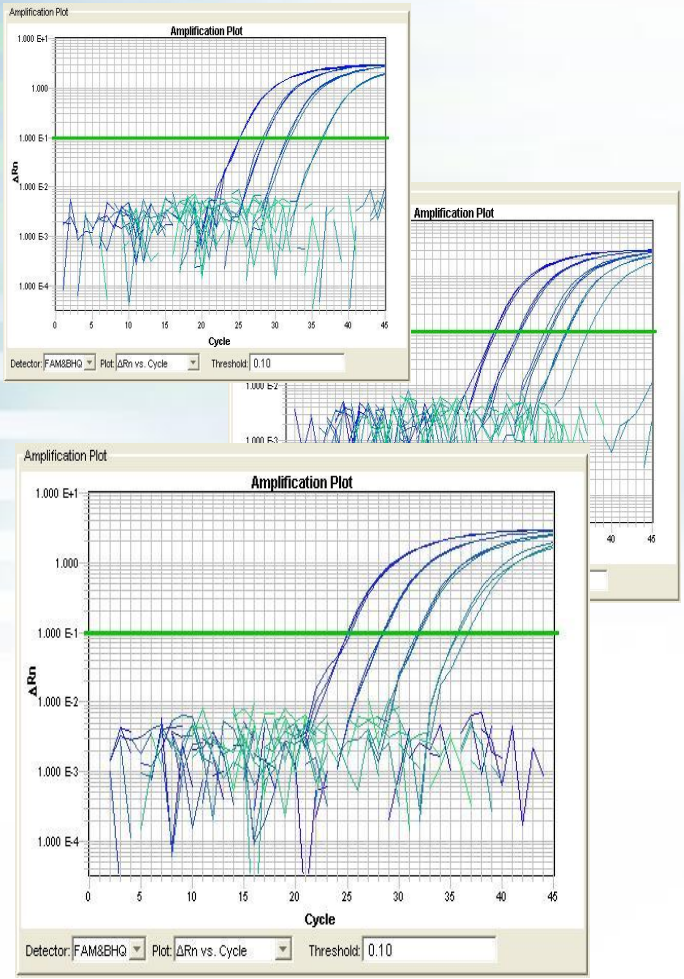
** in a sample population behaving in the same way we expect to miss 10 %

- (Also influenced by target copy number, volume of sample processed, E (DNA extraction),...)

Limited dilutions assay

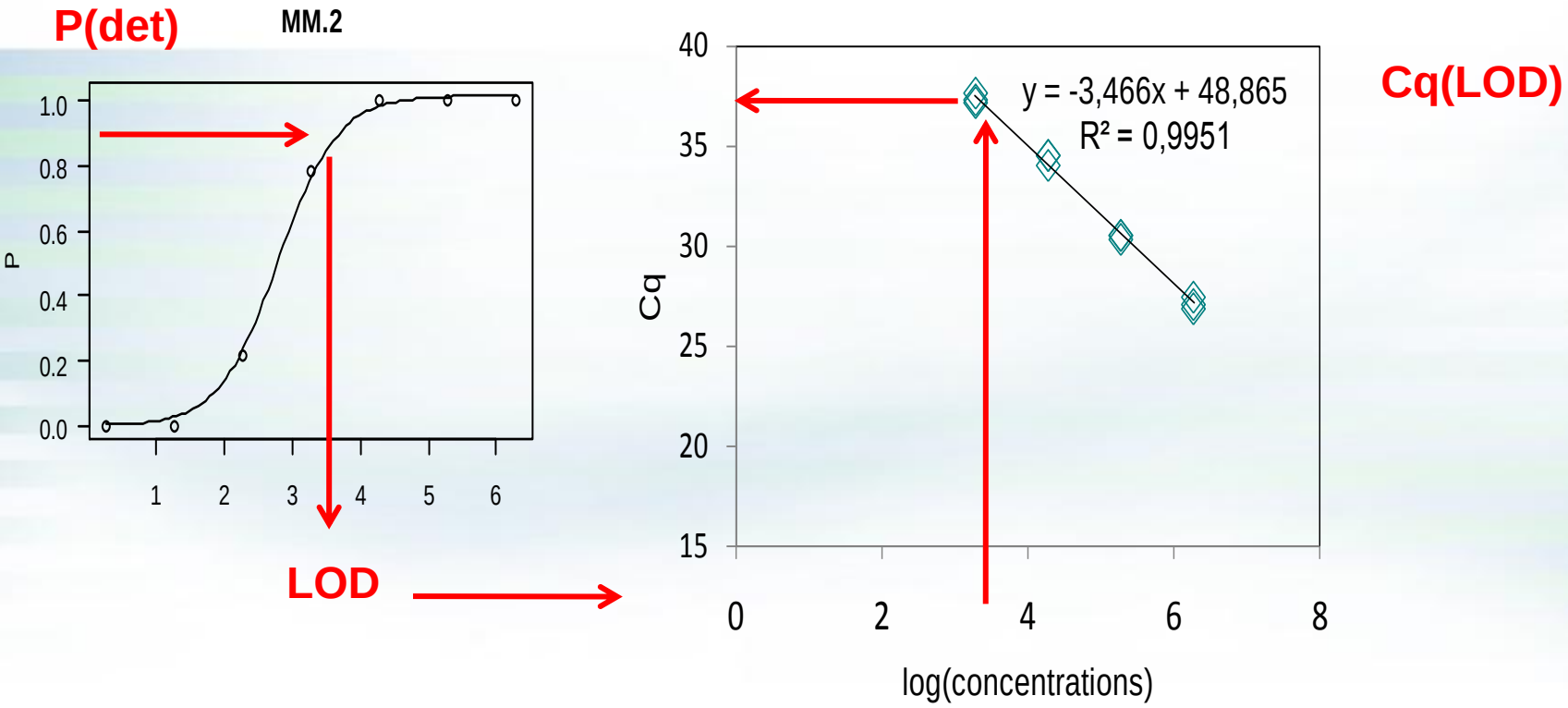


Nonlinear modelling: data transformation



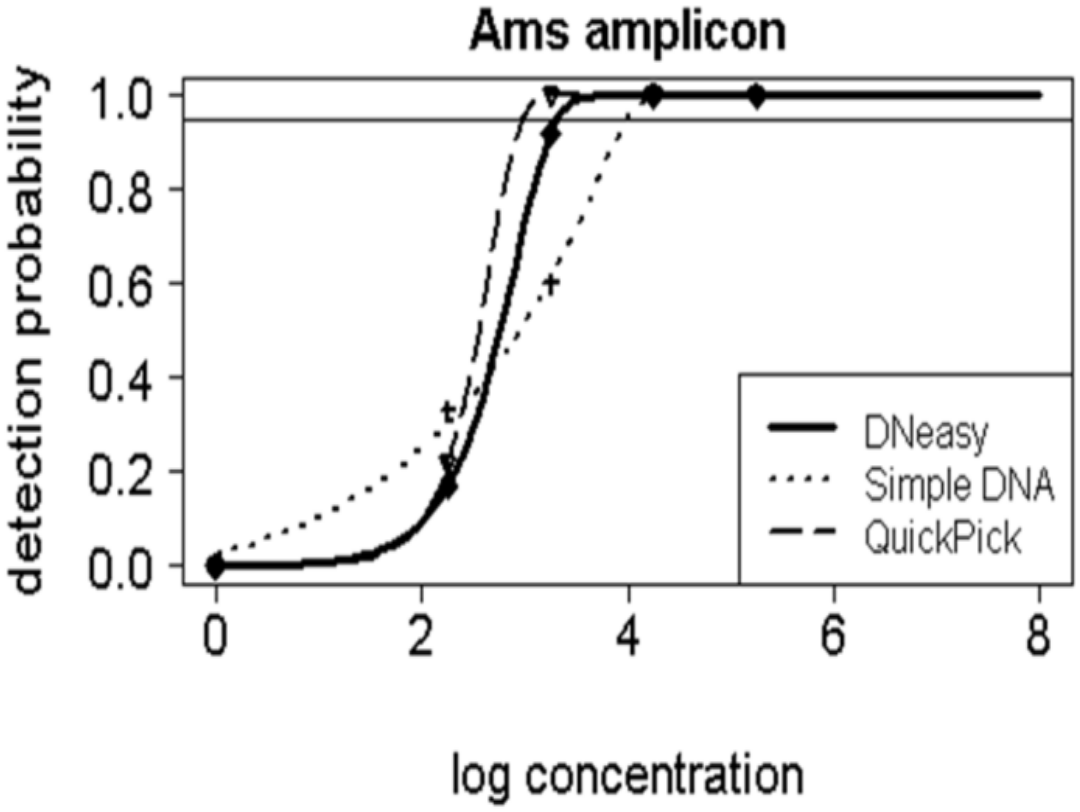
Level of Ea contamin	Ea in reaction	log 7900HT		positive/all parallel reactions			
		CFU/rxn	CFU/mL	SC1	SC2	SC3	all
E6	3184,8	3,5	6,3	3/3	3/3	3/3	1,00
E5	318,5	2,5	5,3	3/3	3/3	3/3	1,00
E4	31,8	1,5	4,3	3/3	3/3	3/3	1,00
E3	3,2	0,5	3,3	1/3	3/3	3/3	0,78
E2	0,3	-0,5	2,3	1/3	1/3	0/3	0,22
E1	0,0	-1,5	1,3	0/3	0/3	0/3	0,00
E0	0,0	-2,5	0,3	0/3	0/3	0/3	0,00

R statistical (http://www.r-project.org/), drc package (Ritz & Streibig, 2005)



- **LOD of the method** (and not practical / sample LOD)
- **Cq(LOD) is not cycle cut-off; practically it is determined from a series of $PCI_{e,\ LOD}$**

AmsC assay for *Erwinia amylovora*: influence of DNA extraction



*P(det) = 0.95

Calculated LOD* concentrations	
DNeasy	2,E+03
Simple DNA	1,E+04
QuickPick	9,E+02

Having numerical values at the same P(det) helps us choose the best assay for the purpose.

Pirc et al., 2012. In: DNA Binding and DNA Extraction: Methods, Applications and Limitations.

560/10 10x	29,386944
	29,369022
	29,343899
	29,347807
	29,274107

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Approach in phytoplasma diagnostics

	36,33966
	38,13234
560/10 10^3	36,74732
	36,320198
	37,39178
	38,378628
	37,255714
560/10 3x10^3	36,967052
	Undetermined
	37,274067
	37,40168
	37,368053
560/10 9x10^3	37,23105
	Undetermined
	37,27828
	Undetermined
	Undetermined
560/10 2.7x10^4	Undetermined
	38,28518
	Undetermined
	Undetermined
	Undetermined
560/10 8.1x10^4	Undetermined
	Undetermined
	Undetermined
	Undetermined
	Undetermined
	Undetermined
560/10 24.3x10^4	Undetermined
	Undetermined
	Undetermined

- analyze serial dilutions, 5 replicates
- search for the last group of samples in which there is no amplification in some of the replicates
- take the range of the highest Cq values observed
- round this Cq value up to the next half value
- add 0.5 to this Cq value (to take into account the difference in threshold chosen between runs)

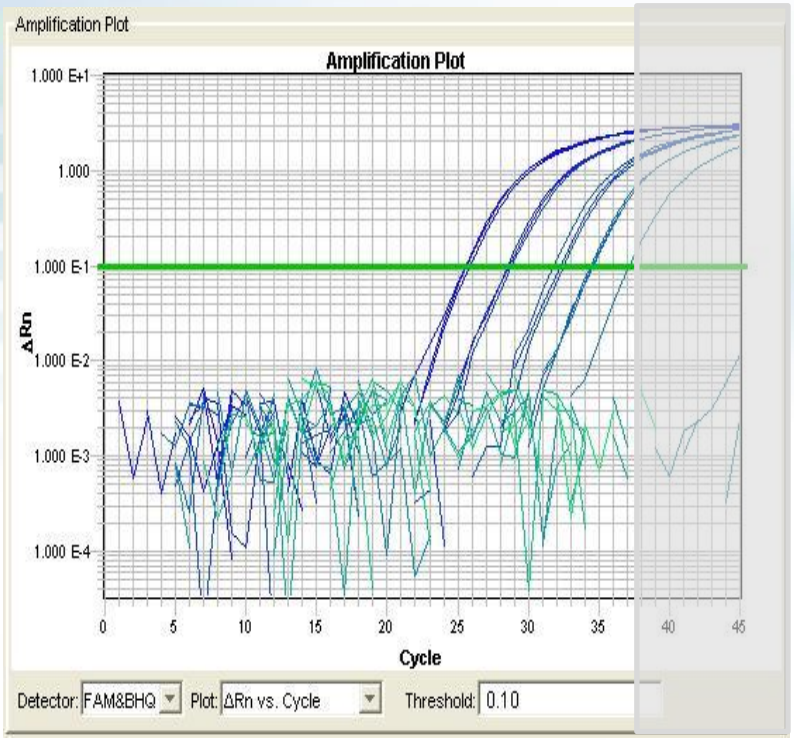
38.5 → +0.5 → Cut-off value:

BNgen: 39.0
UniRNA: 37.5
FDgen: 38.5

Always used and interpreted in combination

Cycle cut-off value

- Cq above which signals are considered negative



Area in which we cannot be certain that a signal is a consequence of a target DNA amplification (considered false positive)

» **Everything above arbitrary Cq**

» **Cq(LOD/LOQ) + 1 Cq**

GMO and food (allergen) analysis

LOD (method) -> LOD (sample)

- **Differences in amplification efficiencies, efficiency of DNA extraction etc.** between the sample and material used in standard curves
- **High standardization of sample preparation** (necrotic tissues, different varieties, different physiological ages)
- Bustin & Nolan:
  - Cq (10 copy) = 43.3-43.9 (Xamp ~ 10E3 = 37)
  - Cq (1 copy) = 43.3-48.8 (Xamp < 10E3 = 37-43)
- **No background signals**
- **Unspecific amplification?**

Zero tolerance pathogens

- proof of absence (testing all, not practical/possible)
- Certain reliability of detection
- Low prevalence
- Aim: to detect as low concentrations of the target pathogen as possible / acceptable risk

Technical justification for Cq cut-off in pathogen detection?

Our experience:

- Signals with high Cq values very rare, usually issues with DNA extraction/sample preparation
- **Confirmation by qPCR with other targets**
- **Reported as suspicious in the absence of pure culture**

Confirmation of LOD level results: other targets

sample		Sample 1				Sample 2			
enrichment	dilution	AmsC (1)	AmsC (2)	pEA29	ITS	AmsC (1)	AmsC (2)	pEA29	ITS
King's B medium	undiluted	neg	neg	neg	neg	neg	neg	neg	neg
		neg	neg	neg	neg	neg	neg	neg	neg
		neg	neg	neg	neg	neg	neg	neg	neg
	1:10 dilution	neg	neg	neg	neg	neg	neg	neg	neg
		neg	neg	neg	neg	neg	neg	neg	neg
		neg	neg	neg	neg	neg	neg	neg	neg
CCT medium	undiluted	neg	neg	36.24	38.48	35.50	36.11	35.65	34.75
		37.18	neg	36.15	36.84	34.08	36.18	36.36	34.63
		neg	neg	36.81	36.97	34.81	35.41	34.34	34.17
	1:10 dilution	neg	neg	neg	neg	37.38	35.62	35.40	35.57
		neg	neg	neg	neg	36.19	neg	37.39	35.85
		neg	neg	37.54	38.39	35.83	neg	35.50	36.78

Dreo et al. Trees (Berl. West) 2012, 26(1): 165-178

Cq cut-off valuable

How to determine it?

What are the criteria?

Biological and epidemiological significance of low concentrations is known

- Qualitative pathogens
- Zero tolerance pathogens with certain treshold needed to cause disease (e.g. 5 x $10E^3$ for potato pathogens)

Other possible criteria: can be confirmed by other methods, risk,..

- **Cq cut-off is an in-house characteristic:** value linked to cycler, analysis, sample preparation and laboratory – **defining a common target concentration (organisms/copies) is viable**
  - Can we agree on the approach how to determine it?
- Cq cut-off is adopted - the **number of samples above** this Cq **should be followed** to detect an inscrease in their incidence
- Good assay design and absence of contamination is a pre-requirement

Current status

- Reporting on the **method LOD with asociated P(det) + relevant experimental data** (MIQE guidelines)
- Calibrating laboratories: use of reference material quantified by dPCR

Thanks to all colleagues

Thank you for your attention