Report on the experiences gathered from establishing real-time PCR assays to detect *Dickeya* spp. (detection of DNA) and potato viruses (detection of RNA)

Dr. Karl-Heinz Pastrik
Chamber of Agriculture Lower Saxony
Plant Protection Office Hanover

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1. Real-time PCR assay for the detection of *Dickeya* spp.

Description of the assay:

- Real-time (qPCR) assay described by Pritchard et. al., (2013), Plant Pathology

- qPCR-Assay: ECH (specific for *Dickeya* spp.)

- Multiplex qPCR: - ECH primers and probe (pathogen-specific)
  - Cox primers and probe (internal PCR control)

- Matrix: homogenates of potato tubers (heel ends)

- Template for qPCR: same DNA extracts prepared for Cms/Rsol surveys

- DNA extraction method: Easy DNA kit (Invitrogen)

Description of the test parameter of the qPCR:

- Source of primer/probes: Eurogentec
  - ECH probe labeled with FAM
  - Cox probe labeled with YY
- Real-time PCR system: AB step-one
- qPCR-Master-Mix: DyNAmo Flash Probe qPCR kit (Finnzyme)
- PCR cycle conditions: 40 cycles

Determination of the limit of detection (LOD)

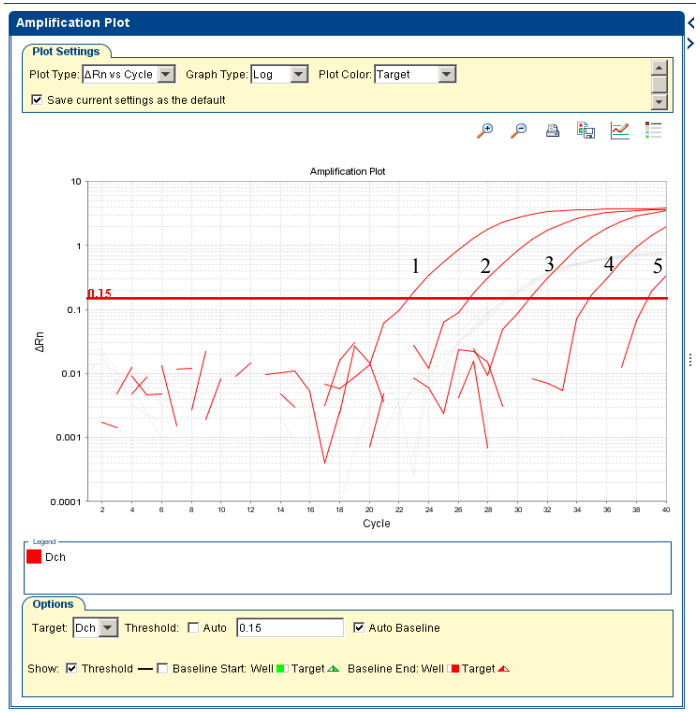
- For the determination of the LOD 3 series of sample extracts were analysed, which were spiked in a range of 10^7 – 10^1 *Dickeya* cells/ml potato homogenate (according to PM 7/98).

- Each series was tested in 5 replicates (in different qPCR-runs).

- The LOD was determined by the lowest cell density giving consistent positive results in all replicates.

- Additionally, 50 negative samples were tested in 3 replicates. These samples were tested negative in previous studies with conventional PCR (Nasar et. al., 1996).

- Based on the results of the spiked samples, the fluorescence threshold was set manually in the log linear phase of the amplification at a constant value of 0.15 ΔRn units.



Detected *Dickeya* cells / ml potato homogenate:

1 = 10^7

2 = 10^6

3 = 10^5

4 = 10^4

5 = 10^3

- **Results of the 3 series of sample extracts spiked with *Dickeya* cells:**

Series 1

No. of *Dickeya* spp. cells / ml potato homogenate	Ct-values in different qPCR runs					Mean Ct-value	Standard deviation from mean Ct-value
	run 1	run 2	run 3	run 4	run 5		
10^7	22.1	22.3	22.2	21.8	22	22.08	0.19
10^6	26.2	26.1	26.4	25.9	25.6	26.04	0.3
10^5	30.3	30.6	30	30.1	29.8	30.16	0.31
10^4	34.1	33.6	34.2	34.3	34.7	34.18	0.4
10^3	37.5	37.6	38.2	**37**	37.9	**37.64**	**0.45**

-> The lowest cell density giving consistent positive results in all replicates was **10^3** *Dickeya* cells/ml potato homogenate.

Series 2

No. of *Dickeya* spp. cells / ml potato homogenate	Ct-values in different qPCR runs					Mean Ct-value	Standard deviation from mean Ct-value
	run 1	run 2	run 3	run 4	run 5		
10^7	22.4	21.5	21.9	21.8	22.3	21.96	0.37
10^6	25.4	25.8	26.2	26.4	26.5	26.06	0.45
10^5	29.6	29.9	30.4	30.7	30.6	30.24	0.47
10^4	33.9	33.6	34.5	34.2	33.9	34.02	0.34
10^3	37.1	37.7	38.2	38.3	37.2	**37.7**	**0.55**

-> Ct values generated with this cell density were between **37** and **38.9**.

Series 3

No. of *Dickeya* spp. cells / ml potato homogenate	Ct-values in different qPCR runs					Mean Ct-value	Standard deviation from mean Ct-value
	run 1	run 2	run 3	run 4	run 5		
10^7	22.6	21.8	22.4	22.3	21.9	22.2	0.34
10^6	25.8	26.5	26.2	26.7	25.8	26.2	0.4
10^5	30.8	30.7	30.1	29.6	30.6	30.36	0.5
10^4	34.3	34.9	33.9	33.7	34.3	34.22	0.46
10^3	37.5	37.9	37.4	38.1	**38,9**	**37.96**	**0.59**

Results of the 50 negative samples:

-> The majority of the negative samples tested had **no** amplification,

-> However, a limited number of the negative samples generated Ct-values between **38.2** and **39.9**.

-> These Ct-values were **not** consistent in all 3 replicates and were possibly generated by:

- the degradation of the probe-based fluorophore by cross contamination,

or

- by non specific amplification of background nucleic acids.

(Burns et. al., 2008)

Determination of the Ct cycle cut-off value

- For the determination of the Ct cycle cut-off value the results achieved with the spiked and healthy samples were analysed.

- The analysis of the Ct values generated revealed an overlapping distribution of the positive and negative samples.

- The same observation was described at Chandalier et. al., 2010:

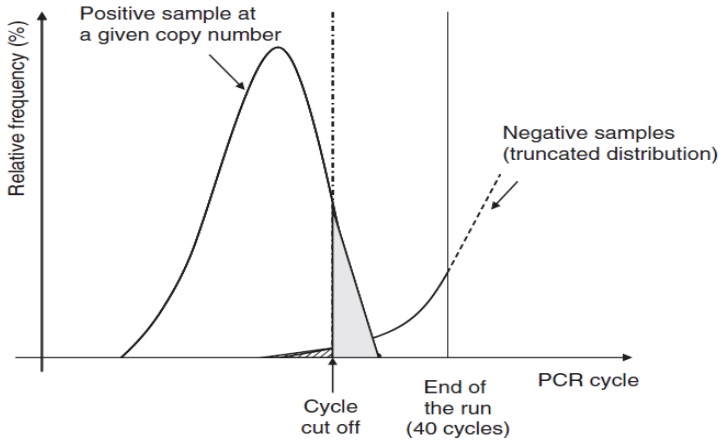


Fig. 1 Representative plot of the PCR cycles against the relative frequency (%) for a set of positive samples with a given copy number and a set of negative samples. The grey area represents the risk of false negatives, and the hatched area represents the risk of false positives.

- A literature research has been made and demonstrated that no standardised way exists to estimate the Ct cycle cut-off value for DNA analysis using real-time PCR.

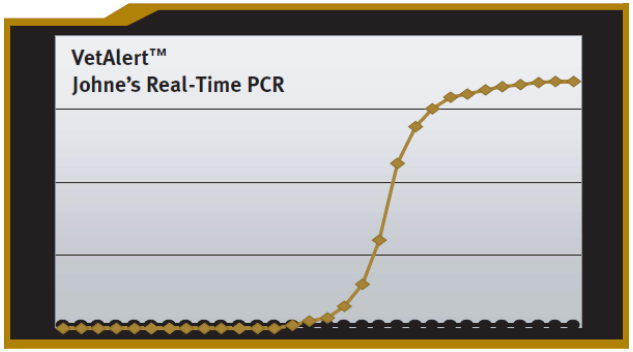
- In most of the publications monitored no statement was made about the criteria the authors used for setting the Ct cycle cut-off value.

- Publications were found with formal calculations of the Ct cycle cut-off value:
  - Caraguel et. al. (2011), Journal of Veterinary Diagnostic Investigation,
  - Chandelier et. al. (2010), Bulletin OEPP/EPPO Bulletin,
  - Burns & Valdiva (2008), European Food Research and Technologiy.

- For qualitative detections also publications were found in the field of human medicine with a determination of the Ct cycle cut-off value, based on a less formal approach:

Tetracore®

Mycobacterium Paratuberculosis DNA Test Kit, Polymerase Chain Reaction



For qualitative detection of *Mycobacterium paratuberculosis* DNA extracted from bovine fecal samples or culture

Ct cutoff value for positive samples:

A sample is considered positive if:

- Ct ≤ 38 for ABI and BioRad series of real-time PCR thermocyclers
- Ct ≤ 42 for Cepheid, Roche and Stratagene real-time PCR thermocyclers

The Ct for a positive result has been set at the upper range of the Ct values at which one gene copy of DNA can be detected (in an uninhibited sample).

A sample that appears to cross the threshold after the cutoff Ct value can not definitely be considered positive ("suspect" result). Such a sample may represent either a true negative or a low positive which was inefficiently amplified. To confirm such a suspect sample, the test should be repeated in duplicate, taking care to minimize any potential contamination. If at least one out of the two re-tested replicates is positive at an equal or earlier Ct value, the suspect sample can be classified as a "low positive." Low positive samples should be confirmed by culture.

NOTE: Caution should be used in interpreting positive samples at or below the cutoff value, since such results might be the result of transient passage of consumed organisms rather than a low-level infection.

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- In our approach, **ranges** of Ct cut-off values were empirically determined, similar to the procedure used in the example of human medicine, based on the results of the spiked and negative samples.

- Following **Ct ranges** were defined:

  - Ct value **<** 38 -> **positive**
    (**<** any Ct value scored in negative samples)

  - Ct value **≥** 38 and **<** 39 -> **doubtful**
    (Ct values are located in the overlapping distribution of positive and negative samples)

  - Ct value **≥** 39 -> **negative**
    (**>** any Ct value scored at the positive samples)

Robustness of the assay

- During the method validation process critical performance characteristics of the qPCR assay were checked, 2 example are illustrated below:

-> Impact of the measurement instrument:

-> The same samples were tested with 2 different real-time PCR machines of the same manufacturer (StepOne, Applied Biosystems).

Results:

-> A constant deviation of **0.3** Ct values was recorded with the samples tested in different real-time PCR machines.

-> For this reason, verification of the test is required, if different measurement instruments are used.

-> Impact of PCR reagents (qPCR Master Mix)

-> Different brands of qPCR Master-Mixes were tested with a set of samples:

- DyNAmo Flash Probe qPCR kit (Finnzyme)
- TaqMan universal PCR Master Mix (AB)
- SensiFast Probe kit (Bioline)

Results:

-> Significant differences were observed between the qPCR Master Mixes tested in respect to intensity of the signals and background amplification.

-> For this reason, verification of the test is required, if PCR reagents are replaced, because this variation can have an impact on the criteria defined before (e.g. fluorescence threshold and Ct cycle cut-off value).

2. Assays for the real-time PCR detection of potato viruses on tubers

Description of the assay:

- RNA potato viruses investigated: PVY, PVS, PLRV.

- For the detection one-step RT qPCR assays were used published in literature.

- Multiplex PCRs were carried out with an internal PCR control (Cox system).

- The objective of the work was to detect one virus-infected potato tuber out of 10 tubers.

Determination of the limit of detection (LOD)

- In these experiments healthy and naturally infected potato tubers were used, that are also reference material for virus indexing tests of seed potatoes.

- For the determination of the LODs for the different assays spiked samples were tested.

- Different numbers of infected potato tubers were added to healthy tubers in a sample size of 10 tubers and analysed in the one-step RT qPCR.

- A total of 60 samples were tested for the different viruses respectively:
    - 30 samples from healthy potatoes
    - 30 spiked samples
      (15 samples spiked with one infected tuber)

Results:

-> The lowest possible infection level was detected in all samples tested (corresponding to one infected tuber out of 10).

-> Based on the data obtained in the experiments of healthy and infected samples Ct cut-off values were determined empirically for the different viruses.

Robustness of the assay

-> The robustness of the assays was checked by the variation of the matrix (potato leaves instead of tubers).

-> Using the assays it was also possible to detect the viruses in samples of potato leaves.

-> However, the change of the matrix resulted in a significant modification of the Ct cut-off values determined: e.g. Cut-off value for PVY: - matrix tuber: **28**

- matrix leaves: **19**

3. Summary and conclusion

- Qualitative real-time PCR assays were established for the detection of pathogens in potato tubers.

- Test criteria of the assays were determined empirically, based one data obtained in experiments.

- The robustness of the assays were checked by the variation of test parameters of the real-time PCR assay.

- The experiments showed, that verification of the assay is required, if essential real-time PCR parameter are replaced (e.g. PCR reagents or matrix), because this variation can have an impact on the test criteria defined before.

Thank you for your attention !