



# Development and evaluation of two TaqMan assays for generic detection of *Dickeya* species

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**Abstract** Two TaqMan assays were developed for generic detection of *Dickeya* species in plant material. The assays enabled detection of all strains of *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. solani* and *D. zea* tested, but only weak reactions were found with strains of *D. paradisiaca*. The assays enabled detection of as low as 100 fg of target DNA in a background of potato DNA. The assays were evaluated with DNA extracted from potato tuber extracts, in which different *Dickeya* species can be present. Results of both assays were highly similar indicating that they can be used reliably in potato seed testing programs.

**Keywords** Diagnostics · Blackleg · Seed potato · Specificity · Accuracy · Sensitivity · Refused sample material

*Dickeya* species belong to the family of soft rot *Pectobacteriaceae* (Adeolu et al. 2016) from which

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many if not all can cause plant diseases. In 2005, *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. paradisiaca*, *D. solani* and *D. zea* were described as species (Samson et al. 2005). More recently *D. aquatica* *D. fongzhangdai* and *D. lacustris* were described (Parkinson et al. 2014; Tian et al. 2016; Hugouvieux-Cotte-Pattat et al. 2019). From the various *Dickeya* species, *D. dianthicola*, *D. dadantii* and *D. solani* have been described as causative agents of potato blackleg (Czajkowski et al. 2011, Toth et al. 2011). For detection of the pathogen, various *Dickeya* species-specific TaqMan assays have been developed, such as for *D. solani* and *D. dianthicola* (Pritchard et al. 2013, van der Wolf et al. 2014), *D. dadantii*, *D. paradisiaca*, *D. chrysanthemi*, *D. zea* and *D. dieffenbachiae* (van der Wolf et al. 2014). However, detection of multiple species with individual assays in large-scale testing programs is laborious and expensive. There is obviously a need for assays that enable faster and more cost-effective testing of seed lots. In 1996, a conventional PCR assay for the generic detection of all *Dickeya* species was developed (Nassar et al. 1996). Combined with an enrichment step to increase densities of *Dickeya* species in plant extracts, this PCR test seemed to be suitable as a tool in a disease management program (Tsrör et al. 2012). However, conventional PCR assays require the use of gel electrophoresis as a post amplification step to analyse the amplicons, which make the assays less suitable for high throughput screening. Because TaqMan assays involve fluorescence measurements that can be performed without opening the PCR tube, the risk of carry-over product is greatly reduced

(Kalinia et al. 1997). Moreover, the use of a fluorescent probe in TaqMan assays allows real time measurements which reduces labour costs and increases specificity. The aim of this study was to develop a TaqMan assay that detects all *Dickeya* species that can affect potato, i.e. *D. dadantii*, *D. dianthicola* and *D. solani*, but not (related) non-target bacteria, and that can be successfully applied for testing of potato tissues for blackleg-causing *Dickeya* species.

In order to design primers and probes that detect all known species of *Dickeya* present on potato in Europe, they need to be conserved in *Dickeya*, but they should not be found in non-target organisms. To find these conserved regions, the genome of a *D. solani* strain (IPO2222) was selected as the reference as it consisted of a single 4.9 Mb contig (Khayati et al., 2016). This reference sequence was split up in 500 bp fragments, resulting in 9898 sequences (set A). After mapping the sequences from set A with BWA MEM (Li, 2013) to *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. dieffenbachiae*, *D. solani* and *D. zaeae*, sequences aligning to all genomes were selected (conserved set B). Sequences from set B mapping to *Pectobacterium atrosepticum*, *P. brasiliensis*, *P. carotovorum*, *P. odoriferum*, *P. betavascularum* and *P. wasabiae* were discarded. The remaining conserved 500 bp regions (set C) were sorted based on the number of mismatches within the group of blackleg-causing *Dickeya* species.

Two sets of primers and probes for the TaqMan PCRs for generic detection of all *Dickeya* species were designed on the basis of the selected conserved regions (set C) in *Dickeya* using Primer Express 3.0 (Applied Biosystems). The MGB TaqMan probes were labelled with a 5'-end reporter dye (FAM-6-carboxyfluorescein (FAM)) and a 3'-end quencher dye (BHQ-1). The first set was derived from a gene encoding a ferredoxin subunit involved in aerobic respiration. It consisted of forward primer Fw109 (5'-GCGCGCAGCACTHGAT-3'), H=A or C or T; reverse primer Rv109 (5'-CGACGGCACGCTCAGAAT-3') and probe P109 (5'-AAGCCGCGGAAAT-3'). The other set was derived from a potassium transporter Kup involved in potassium-ion uptake under hyper osmotic stress at a low pH. It consisted of forward primer Fw284 (5'-TGTGCGTTTTCGGGCTASTC-3'), S=C or G; reverse primer Rv284 (CCYTGTCTTCTGTTATCAATTCATTAAC-3'), Y=C or T and probe P284 (5'-

AACCAGAATAAGGCCC-3'). DNA was isolated from bacterial strains listed in Table S1. DNA was extracted from pure cultures of bacteria grown for 48 h on TSA at 27 °C. The bacteria were scraped from the agar surface and stored at -20 °C until DNA extraction. The DNA was extracted using the Wizard Magnetic DNA purification System for Food (Promega, Leiden, The Netherlands). DNA yield was determined by fluorescence using the Pico® Green I dye (Invitrogen) and a TECAN Infinite® M200 pro microplate reader (Tecan Group Ltd., Switzerland). The selected primer sets and corresponding probes were tested in TaqMan assays performed in a 96-well format in an ABI Prism 7500 apparatus. For each TaqMan assay, 2 µl sample was mixed with 23 µl reaction mix containing 12.5 µl 2X Premix Ex Taq (Takara Bio Inc. Otsu, Shiga, Japan), 0.5 µl 50X ROX reference Dye II (Takara Bio Inc.), 100 nM probe and 300 nM of each forward and reverse primer. The reaction conditions were: an initial incubation step at 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s followed by 60 °C for 60 s. Analysis of the data was done by automatic threshold calculation within the 7500 system software version 1.2.3. Ct-values were interpreted qualitatively. Reactions with Ct-values below 35 were considered positive.

The specificity of the generic *Dickeya* TaqMan assays was evaluated using 0.4 ng DNA of pure cultures of target and non-target strains. Results are summarized in Table 1 and full results are shown in Table S1. All 36 strains of the 6 target species, i.e. *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. dieffenbachia*, *D. solani* and *D. zaeae*, were detected by both TaqMan assays. TaqMan reactions of the 77 strains of more than 15 non-target species tested were all negative. In general, Ct-values of both assays were similar. Ct-values of the tested strains of *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. dieffenbachia*, *D. solani* and *D. zaeae* varied between 15.4 and 23.2 when TaqMan set 109 was used and between 15.8 and 24.2 for TaqMan set 284. However, Ct-values of the *D. paradisiaca* strains were higher and varied between 29.3 and 36.0. Three out of four strains of *D. paradisiaca* were positive when TaqMan assay set 109 was used and two out of four were positive when TaqMan set 284 was used. None of the 77 non-target strains were positive, including 61 strains of various taxonomically related *Pectobacterium* species.

**Table 1** Results of TaqMan assays (with set 109 and set 284), developed for generic detection of *Dickeya* species, with target and non-target bacteria

		Numbers of strains tested	Numbers of strains positive (Ct < 35) <sup>1</sup>	
			Set 109	Set 284
Target bacteria	<i>D. chrysanthemi</i>	4	4	4
	<i>D. dadantii</i>	6	6	6
	<i>D. dianthicola</i>	11	11	11
	<i>D. solani</i>	6	6	6
	<i>D. zeae</i>	7	7	7
	<i>D. paradisiaca</i>	4	3 <sup>2</sup>	2 <sup>2</sup>
Non-target bacteria	<i>Pectobacterium</i> sp.	61	0	0
	Other bacteria	14	0	0
	Water control	2	0	0

<sup>1</sup> 0.4 ng of DNA per reaction was tested

<sup>2</sup> The Ct-values for *D. paradisiaca* strains were relatively high (between 29.3 and 36), whereas all other positive Ct values were < 25

TaqMan assays 109 and 284 were highly specific and detected all plant pathogenic *Dickeya* species involved in the studies, with the exception of *D. paradisiaca* for which relatively high Ct-values were found. Also in other studies, *D. paradisiaca* was found divergent from the other *Dickeya* species (Naushad et al. 2014). This pathogen has a narrow host range, being isolated only from *Musa* species and *Zea mays* that were grown in tropical regions. It has never been described in association with potato.

The TaqMan assays were further evaluated using ten lots of seed potatoes from different places in the Netherlands and belonging to different cultivars, i.e. Amorosa (Groningen), Binella (Zeeland), Diamant E (Groningen), Elmundo (Noord Holland), Fabula E (Noordoostpolder), Kondor (Zeeland), Monalisa A (Friesland), Monalisa (Friesland), Picasso A (Noord Holland) and Zafira A (Noordoostpolder). From the same ten seed lots regular (commodity) samples were collected as done by seed inspectors, as well as samples of refused tubers that do not meet the quality standards. The refused tubers showed bruises, were misshaped and/or showed decay. We hypothesized that *Dickeya* would be more readily detected in refused samples. Two times ten subsamples of 20 tubers per seed lot were analysed, ten subsamples for the commodity samples and ten subsamples for the regular samples per seed lot. These 20 tubers from each subsample were put in 100 µm thick bags (Topa, Lelystad) and 5 ml water was added. The bags were vacuumed and incubated for

7 days at room temperature. Subsequently 1 ml of the rotten extract was used for DNA extraction using the Wizard Magnetic DNA purification System for Food (Promega, Leiden, The Netherlands). One hundred regular potato subsamples and 97 refused potato subsamples were analysed with the TaqMan assays 109 and 284 for generic detection of *Dickeya* species with TaqMan assays 109 and 284.

Full results are shown in Table S2 and results are summarized in Table 2. Of 100 regular subsamples 90 subsamples were negative and four subsamples were positive for the presence of *Dickeya* species by both generic assays (Set 109 and Set 284). Four subsamples were positive with Set 109 and negative for set 284. Conversely, two subsamples were positive with set 284 and negative with set 109. Ct-values of the positive regular subsamples were relatively high (between 30 and 35) except for a highly positive sample of Elmundo with Ct-values of 17.0 and 17.7 for set 284 and set 109 respectively. Of the 97 refused subsamples, 82 (84.5%) were tested negative for the presence of *Dickeya* species by both generic TaqMan assays. Thirteen subsamples (13.4%) were tested positive by both generic assays. Two subsamples (2.1%) were positive with set 109 and negative with set 284.

The sensitivity, specificity and accuracy of both assays are summarized in Table 3. Results of both assays are similar and the calculated values of the selected characteristics are high. With set 284 there was a lower number of subsamples positive, which

**Table 2** Evaluation of TaqMan assays for detection of plant pathogenic *Dickeya* species with potato tubers collected from seed lots

Seed lot	Type	Nr. of subsamples	Numbers of positive subsamples (Ct < 35) in TaqMan assay:		Type	Nr. of subsamples	Numbers of positive subsamples (Ct < 35) in TaqMan assay:	
			Set 109 <sup>1</sup>	Set 284 <sup>1</sup>			Set 109	Set 284
Zafira A	Regular <sup>2</sup>	10	1 <sup>3</sup>	0	Refused <sup>4</sup>	10	0	0
Fabula E	Regular	10	1	0	Refused	10	7	6
Monalisa A	Regular	10	1	0	Refused	10	0	0
Diamant E	Regular	10	0	0	Refused	10	0	0
Amorosa	Regular	10	1	0	Refused	9	3	2
Elmundo	Regular	10	1	1	Refused	10	3	3
Picasso A	Regular	10	0	0	Refused	8	2	2
Monalisa	Regular	10	3	3	Refused	10	0	0
Binella	Regular	10	0	1	Refused	10	0	0
Kondor	Regular	10	0	1	Refused	10	0	0
	Total	100	8	6	Total	97	15	13

<sup>1</sup> Set 109 and Set 284 were developed in this study for detection of plant pathogenic *Dickeya* species

<sup>2</sup> Subsamples of a regular seed lot consisted of 20 tubers as collected by NAK inspector

<sup>3</sup> Ct-values of positive regular samples were relatively high (between 30 and 35), except for the single positive sample of Elmundo (17.0 and 17.7)

<sup>4</sup> Refused samples, tubers that did not meet quality standards (misshaped, damaged, or diseased tubers)

may be a disadvantage if the generic assay is used for initial screening. In the refused samples more *Dickeya* was found than in the regular samples (Table 2) which confirmed our hypothesis that *Dickeya* is more frequently present in rejected tubers that are misshaped, wounded or rotten.

In order to determine the detection ranges of the *Dickeya* TaqMan assays in a background of potato extract, 10-fold dilution series (10 fg – 10 ng) of

genomic DNA of *D. solani* strain IPO 2187 were prepared in a background of ca. 34 ng of DNA extracted from potato extract from regular tubers tested negative for the presence of *Dickeya*. When set 109 was used, the response was linear with a slope of  $-3.29$ , an  $R^2$  of 0.996 and a Y-intercept of 39.0 (Fig. 1A). The response was also linear when set 284 was used (Fig. 1B) with a slope of  $-3.58$ , an  $R^2$  of 0.975 and a Y-intercept of 41.5.

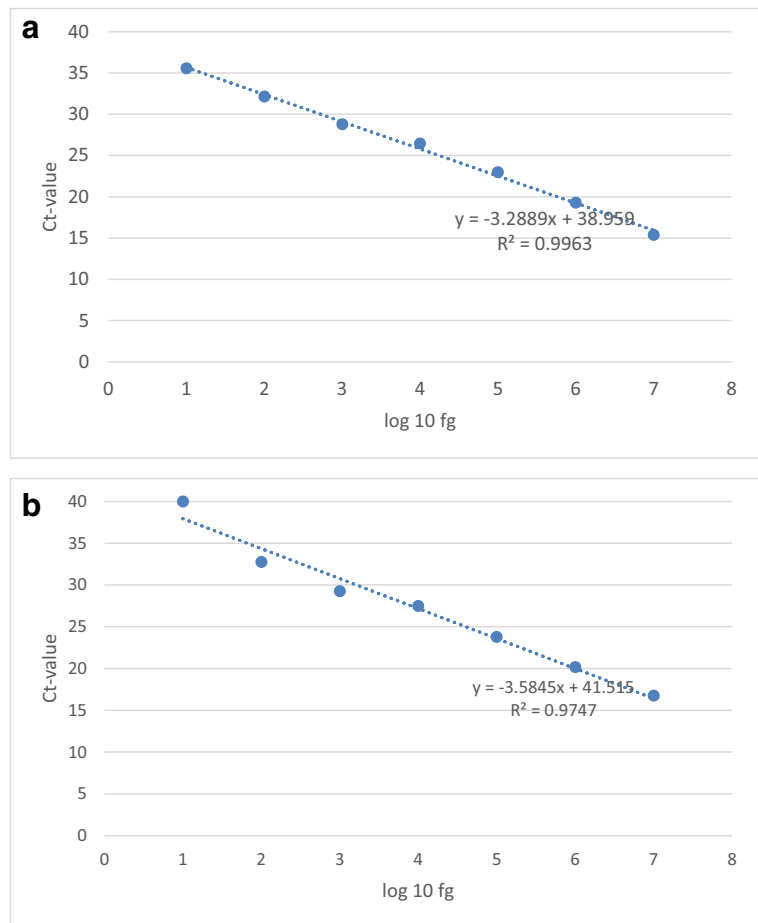
**Table 3** Characteristics of the TaqMan assays for generic detection of *Dickeya* species in potato seed lots

Definition	Formula <sup>1</sup>	Value (%) <sup>2</sup>
Set 284 as reference for set 109		
Sensitivity of set 109	$TP/(TP + FN)$	89
Specificity of set 109	$TN/(TN + FP)$	97
Accuracy of set 109	$(TN + TP)/(TN + TP + FN + FP)$	96
Set 109 as reference for set 284		
Sensitivity of set 284	$TP/(TP + FN)$	74
Specificity of set 284	$TN/(TN + FP)$	99
Accuracy of set 284	$(TN + TP)/(TN + TP + FN + FP)$	96

<sup>1</sup> TP = True positive, FP = False positive, TN = True Negative, FN = False Negative

<sup>2</sup> 197 subsamples of seed potatoes were analysed with two TaqMan assays for the presence of *Dickeya* species. Ct-values <35 were considered positive

**Fig. 1** Standard curves showing the relationship between Ct (cycle threshold) and quantity of genomic DNA of *Dickeya solani* strain IPO 2187 in a background of DNA from potato extract using the generic *Dickeya* TaqMan assays. (A) Ct-values for set 109 plotted against the quantity (log base 10) of *Dickeya* DNA (fg). (B) Ct-values for set 284 plotted against the quantity (log base 10) of *Dickeya* DNA (fg)



For both TaqMan assays the detection level was 100 fg bacterial DNA per reaction in a background of potato DNA.

This study has shown that two TaqMan assays have been developed that can detect all *Dickeya* species found on potato. If necessary, samples positive in the generic assays can be further analysed with *Dickeya* species-specific assays.

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#### Compliance with ethical standards

**Conflict of interest** The authors herewith declare that they have no conflict of interest. This study does not contain studies with human participants or animals performed by any of the authors.

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