EURL European Union Reference Laboratory for Plant Pathogenic Bacteria

ILVO

Health Food Chain Safety Environment

Validation report

TaqMan real-time PCR assay for the detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in seed extracts of common bean

EURL-BAC-2024-CORBFL-TR-01

Version 1.0

23/12/2024

Colophon

Validation report - TaqMan real-time PCR assay for the detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in seed extracts of common bean

EURL-Bacteriology code: EURL-BAC-2024-CORBFL-TR-01
Version : 1.0

Authors: Jolien Venneman, Johan Van Vaerenbergh

Contact details
Flanders Research Institute for Agriculture, Fisheries and Food (ILVO)
Plant Sciences Unit
Diagnostic Centre for Plant Health – NRL
Burgemeester Van Gansberghelaan 96
9820 Merelbeke, Belgium

Notices
The validation data were generated in the framework of the CurtoALERT project funded by the Belgian Federal Public Service Health, Food Chain Safety and Environment (RF 23/08), and in support of the activities of the European Union Reference Laboratory (EURL) for Plant Pathogenic Bacteria.

Suggested citation
Venneman J, Van Vaerenbergh J (2024). Validation report - TaqMan real-time PCR assay for the detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in seed extracts of common bean. EURL-BAC-2024-CORBFL-TR-01.

Contents

1. Specific scope ... 4
2. Introduction ... 4
3. Analytical specificity ... 5
4. Sensitivity ... 8
4.1. Determination of analytical sensitivity using spiked seed extracts ... 8
4.2. Determination of diagnostic sensitivity using field samples ... 11
4.3. Determination of LOD using artificially inoculated seed ... 11
5. Repeatability ... 13
6. Reproducibility ... 13
7. Cited literature ... 15
Appendix 1: DNA extraction with QuickPick™ SML Plant DNA Kit and KingFisher Flex ... 16
Appendix 2: PCR protocols ... 18
Appendix 3: Differences in sensitivity between ISHI-Veg TaqMan PCR and Tegli conventional and real-time PCRs for leaf and seed extracts from a Belgian survey (CurtoALERT project) ... 21

1. Specific scope

This report presents validation data for the method EURL-BAC-2024-CORBFL-TM-01, which was drafted by the European Union Reference Laboratory (EURL) for Plant Pathogenic Bacteria (1). The EURL method aims to assist EU National Reference Laboratories with the detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff) in seeds of common bean (*Phaseolus vulgaris*).

2. Introduction

Curtobacterium flaccumfaciens pv. *flaccumfaciens* (Cff) infects several plant species within the Fabaceae or legume family, with *Phaseolus vulgaris* (common bean) being the main host, where the disease is known as bacterial wilt. Infected seeds are considered the most important way for introduction and dissemination of the pathogen over long and short distances (2). When present in the field, Cff is able to survive on bean debris, soil and alternative hosts (3,4,5). Since 1921, the disease has occurred intermittently as an endemic and economically important constraint in the bean-producing regions of the USA (2). Widespread findings have also been reported on *Vigna radiata* (mung bean) and *Vigna unguiculata* (cowpea) in the eastern states of Australia (6), on common bean and soybean in Brazil (7,8), and on common bean and cowpea in Iran (9). In order to prevent introduction and establishment of Cff in the EU, the pathogen is currently regulated as a quarantine organism under Regulation (EU) 2016/2031 (10).

Early detection of Cff in (imported) seeds is therefore crucial and can be realized by a seed extract TaqMan PCR and/or by isolation on semi-selective medium. This validation report underpins the TaqMan real-time PCR method. Two tests based on serine protease genes have been designed by Naktuinbouw (Roelofarendsveen, The Netherlands) in the framework of an ISHI-Veg standard on detection of Cff in bean seeds (not published at the time of writing). The PCR can be run as simplex with one of the two Cff primer/probe combinations, or as multiplex. The P967F/P1194R/Gpos primer/probe combination (11) is recommended as an internal amplification control for Gram-positive bacteria.

The validation experiments have been performed during the period November 2023 – December 2024. The following performance characteristics have been considered:

- Analytical specificity
- Analytical and diagnostic sensitivity
- Repeatability
- Reproducibility

3. Analytical specificity

3.1. Introduction

Analytical specificity is defined as the ability of a detection method to distinguish the pathogen from other organisms and the extent to which the analysis can detect variants of the organism. To determine inclusivity, representative target isolates covering genetic diversity and different geographical origin must be tested. Furthermore, cross-reactions with non-targets (e.g. closely related organisms, contaminants) must be excluded (exclusivity).

3.2. Experimental approach

Specificity of the ISHI-Veg TaqMan tests has been assessed by Naktuinbouw. During primer and probe design, *in-silico* inclusivity and exclusivity checks were performed, and several targets and non-targets were included in wet lab PCR testing (validation data not yet published at time of writing).

ILVO obtained additional validation data on inclusivity and exclusivity, in particular the test specificity for *C. flaccumfaciens* strains isolated from common bean, soybean and broad bean but not pathogenic on common bean. DNA was obtained from thermal lysis (15 min at 95°C and cooling down on ice) of cell suspensions at about 10^8 cells/mL, or using the protocol in Appendix 1.

- Inclusivity testing on:
    - 26 Cff strains from official collections (CFBP & LMG)
    - 17 Cff strains from the ILVO GBBC collection
    - 10 strains received as Cff from other lab collections
- Exclusivity testing on:
    - 9 strains from other pathovars within *C. flaccumfaciens* (Cf): *Cf* pv. *betae* (1), *Cf* pv. *beticola* (1), *Cf* pv. *basellae* (1), *Cf* pv. *oortii* (1), *Cf* pv. *poinsettiae* (3) and *Cf* pv. *euphorbiae* pv. nov. (2)
    - 31 other Cf strains not pathogenic to bean
    - 4 *Frigoribacterium* isolates from *Phaseolus* bean and soybean

Specificity of the two ISHI-Veg TaqMan real-time PCR tests was set against the specificity of the conventional and real-time PCR tests of Tegli (12,13) (Appendix 2). The instruments used were a QuantStudio 5 PCR System for real-time PCR and a MiniAmp Plus Thermal Cycler for conventional PCR (both from Applied Biosystems). Amplicons of conventional PCR were resolved by capillary electrophoresis (QIAxcel system).

3.3. Results

Table 1 and 2 show the results of the inclusivity and exclusivity tests, respectively, for the target and non-target isolates included in the present validation study.

Two out of the 53 Cff targets were not detected in the different PCR tests (AGQB#44 and LPPA 393). Although these strains were originally reported as pathogenic on bean (14,15), they did not develop symptoms in the pathogenicity assays at ILVO. It was concluded that they had lost their virulence, underpinning the negative results in the PCR tests based on pathogenicity genes. The other strains all tested positive, with Ct values typically below 16 in both the TaqMan and SYBR assays.

In contrast, the 44 non-target strains were either not detected (no Ct value) or showed high Ct values in real-time PCR (11 non-targets with Ct > 30 in ISHI-Veg TaqMan and 17 non-targets with Ct >28 in Tegli SYBR but no amplicon in Tegli conventional PCR). The late signals are probably due to the high concentration of bacterial DNA used in the PCR reactions (>10 ng).

3.4. Conclusion

The specificity of the ISHI-Veg TaqMan tests and the Tegli PCRs are considered equivalent for the tested isolates and strains. All virulent Cff strains tested positive with Ct < 16, and for the non-targets either no Ct or a high Ct value not attributable to Cff was obtained (>10 Ct difference with the Cff strains in real-time PCR).

Table 1. *Results of inclusivity testing. cPCR = conventional PCR*

Strain	Taxon	Biological origin	Geogr. origin - country of isolation	Year of isolation	Isolate reference	ISHI-Veg TaqMan PCR 1*	ISHI-Veg TaqMan PCR 2*	Tegli cPCR*	Tegli real-time PCR (SYBR)*
CFBP 3455	*Cff*	*Phaseolus vulgaris*	Canada	1954	Hagborg	1	1	1	1
CFBP 3461	*Cff*	*Phaseolus vulgaris*	USA		Vidaver	1	1	1	1
CFBP 3462	*Cff*	*Phaseolus vulgaris*	USA		Vidaver	1	1	1	1
CFBP 6913	*Cff*	*Phaseolus vulgaris*	Brazil	1996	A.C. Maringoni	1	1	1	1
CFBP 8371	*Cff*	*Triticum aestivum*	Brazil	2012	A.C. Maringoni	1	1	1	1
CFBP 8372	*Cff*	*Avena strigosa*	Brazil	2013	A.C. Maringoni	1	1	1	1
CFBP 8373	*Cff*	*Avena sativa*	Brazil	2013	A.C. Maringoni	1	1	1	1
CFBP 8374	*Cff*	*Hordeum vulgare*	Brazil	2013	A.C. Maringoni	1	1	1	1
CFBP 8391	*Cff*	*Brassica napus*	Brazil	2013	A.C. Maringoni	1	1	1	1
CFBP 8393	*Cff*	*Phaseolus vulgaris*	Brazil	2006	A.C. Maringoni	1	1	1	1
CFBP 8394	*Cff*	*Phaseolus vulgaris*	Brazil	2002	A.C. Maringoni	1	1	1	1
LMG 3640	*Cff*	*Phaseolus vulgaris*	Germany	1958	R.A. Lelliott	1	1	1	1
LMG 3645[PT]	*Cff*	*Phaseolus vulgaris*	Hungary	1957	Z. Klement	1	1	1	1
LMG 3647	*Cff*	*Phaseolus vulgaris*	Romania	1965	D.C. Graham	1	1	1	1
LMG 3648	*Cff*	*Phaseolus* sp.	USA		F. Hedges	1	1	1	1
LMG 3649	*Cff*	*Phaseolus* sp.	USA	1923	W. Burkholder	1	1	1	1
LMG 3650	*Cff*	*Phaseolus vulgaris*	USA	1956	M. Schuster	1	1	1	1
LMG 3651	*Cff*	*Phaseolus vulgaris*	USA	1957	M. Schuster	1	1	1	1
LMG 7242	*Cff*	*Phaseolus vulgaris*	UK		W.J. Dowson	1	1	1	1
LMG 7243	*Cff*	*Phaseolus vulgaris*	USA		M. Schuster	1	1	1	1
LMG 7244	*Cff*	*Phaseolus vulgaris*	USA	1957	M. Schuster	1	1	1	1
LMG 7245	*Cff*	*Phaseolus vulgaris*	USA		M. Schuster	1	1	1	1
LMG 7247	*Cff*	*Phaseolus vulgaris*	USA	1923	W. Burkholder	1	1	1	1
LMG 7248	*Cff*	*Vigna angularis*	USA	1919	W. Burkholder	1	1	1	1
LMG 7249	*Cff*	*Phaseolus vulgaris*	USA	1922	W. Burkholder	1	1	1	1
LMG 7250	*Cff*	*Vigna radiata*	USA	1934	W. Burkholder	1	1	1	1
GBBC 3406	*Cff*	*Vicia faba*	Belgium	2021	ILVO	1	1	1	1
GBBC 3581	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3582	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3621	*Cff*	*Phaseolus vulgaris*	Belgium	2024	ILVO	1	1	1	1
GBBC 3622	*Cff*	*Phaseolus vulgaris*	Belgium	2024	ILVO	1	1	1	1
GBBC 3627	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3628	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3629	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3630	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3632	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3633	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3634	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3635	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3636	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3637	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3642	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
GBBC 3643	*Cff*	*Phaseolus vulgaris*	USA	2024	ILVO	1	1	1	1
AGES 1212/18	*Cff*	*Vigna radiata*	Austria	2018	R. Gottsberger	1	1	1	1
AGQB#44	*Cff*	*Glycine max*	Germany	2011	JKI	0	0	0	0
CARLOS 1	*Cff*	*Zea mays*	USA	2006	R. Harveson	1	1	1	1
CARLOS 2	*Cff*	*Phaseolus vulgaris*	USA	2007	R. Harveson	1	1	1	1
CARLOS 3	*Cff*	*Triticum aestivum*	USA	2006	R. Harveson	1	1	1	1
CARLOS 4	*Cff*	*Phaseolus vulgaris*	USA	2006	R. Harveson	1	1	1	1
CARLOS 5	*Cff*	*Phaseolus vulgaris*	USA	2005	R. Harveson	1	1	1	1
CARLOS 6	*Cff*	*Glycine max*	USA	2005	R. Harveson	1	1	1	1
CARLOS 7	*Cff*	*Phaseolus vulgaris*	USA	2004	R. Harveson	1	1	1	1
LPPA 393	*Cff*	*Phaseolus vulgaris*	Spain	2015	A. Gonzales	0	0	0	0

*1 = Ct-value typically < 16 or amplicon generated; 0 = no Ct-value or no amplicon

Table 2. *Results of exclusivity testing. cPCR = conventional PCR*

Strain	Taxon	Biological origin	Geogr.origin - country of isolation	Year of isolation	Isolate reference	ISHI-Veg TaqMan PCR 1*	ISHI-Veg TaqMan PCR 2*	Tegli cPCR*	Tegli real-time PCR (SYBR)*
LMG 3596[PT]	*Cf* pv. *betae*	*Beta vulgaris* subsp. *vulgaris* var. *rubra*	UK	1955	Keyworth	0*	0*	0	0*
ATCC BAA-144	*Cf* pv. *beticola*	*Beta vulgaris* subsp. *vulgaris*	China	1995		0*	0*	0	0*
ATCC BAA-143	*Cf* pv. *basellae*	*Basella rubra*	China	1994		0*	0*	0	0*
LMG 3702[PT]	*Cf* pv. *oortii*	*Tulipa gesneriana*	Netherlands	1967		0	0	0	0
LMG 3712	*Cf* pv. *poinsettiae*	*Euphorbia pulcherrima*	USA	1959	McFadden	0	0	0	0
LMG 3715[PT]	*Cf* pv. *poinsettiae*	*Euphorbia pulcherrima*	USA		Starr	0	0	0	0
LMG 3716	*Cf* pv. *poinsettiae*	*Euphorbia pulcherrima*	USA		Burkholder	0	0	0	0
AGQB#46	*Cf* pv. *euphorbiae*	*Euphorbia pulcherrima*	Germany	2021	JKI	0	0	0	0
GBBC 3458[PT]	*Cf* pv. *euphorbiae*	*Euphorbia pulcherrima*	Belgium	2021	ILVO	0	0	0	0
CAL-093	*Cf*	*Phaseolus vulgaris*	Belgium	2024	ILVO	0	0	0	0*
CAL-106	*Cf*	*Phaseolus vulgaris*	Belgium	2024	ILVO	0	0	0	0*
CAL-127	*Cf*	*Vicia faba*	Belgium	2024	ILVO	0*	0*	0	0*
CAL-130	*Cf*	*Vicia faba*	Belgium	2024	ILVO	0*	0*	0	0*
CAL-133	*Cf*	*Vicia faba*	Belgium	2024	ILVO	0*	0*	0	0*
CAL-136	*Cf*	*Vicia faba*	Belgium	2024	ILVO	0*	0*	0	0*
CAL-138	*Cf*	*Vicia faba*	Belgium	2024	ILVO	0*	0*	0	0*
CAL-139	*Cf*	*Vicia faba*	Belgium	2024	ILVO	0	0	0	0
CAL-157	*Cf*	*Phaseolus vulgaris*	Belgium	2024	ILVO	0	0	0	0
CAL-159	*Cf*	*Phaseolus vulgaris*	Belgium	2024	ILVO	0	0	0	0
CAL-160	*Cf*	*Phaseolus vulgaris*	Belgium	2024	ILVO	0	0	0	0
CAL-173	*Cf*	*Phaseolus vulgaris*	Belgium	2024	ILVO	0	0	0	0
CAL-179	*Cf*	*Glycine max*	Belgium	2024	ILVO	0	0	0	0
CAL-181	*Cf*	*Glycine max*	Belgium	2024	ILVO	0	0	0	0
CAL-184	*Cf*	*Glycine max*	Belgium	2024	ILVO	0	0	0	0
CAL-197	*Cf*	*Phaseolus vulgaris*	Belgium	2024	ILVO	0	0	0	0*
GBBC 3558	*Cf*	*Phaseolus vulgaris*	Belgium	2023	ILVO	0	0	0	0*
GBBC 3559	*Cf*	*Vicia faba*	Belgium	2023	ILVO	0*	0*	0	0*
GBBC 3580	*Cf*	*Phaseolus vulgaris*	USA	2024	ILVO	0	0	0	not tested
GBBC 3618	*Cf*	*Phaseolus vulgaris*	Belgium	2023	ILVO	0	0	0	0
GBBC 3619	*Cf*	*Vicia faba*	Belgium	2024	ILVO	0	0	0	0
GBBC 3623	*Cf*	*Glycine max*	Belgium	2024	ILVO	0	0	0	0*
GBBC 3624	*Cf*	*Glycine max*	Belgium	2024	ILVO	0*	0*	0	0*
GBBC 3625	*Cf*	*Glycine max*	Belgium	2024	ILVO	0	0	0	0*
GBBC 3631	*Cf*	*Phaseolus vulgaris*	Belgium	2024	ILVO	0*	0*	0	0*
GBBC 3639	*Cf*	*Vicia faba*	Belgium	2024	ILVO	0	0	0	0
GBBC 3640	*Cf*	*Glycine max*	Belgium	2024	ILVO	0	0	0	0
GBBC 3641	*Cf*	*Glycine max*	Belgium	2024	ILVO	0	0	0	0
NIB Z 3363	*Cf*	*Phaseolus vulgaris*	Slovenia	2021	NIB	0	0	0	0
NIB Z 3364	*Cf*	*Phaseolus vulgaris*	Slovenia	2021	NIB	0	0	0	0
NIB Z 3404	*Cf*	*Glycine max*	Slovenia	2021	NIB	0	0	0	0
CAL-107	*Frigoribacterium* sp.	*Phaseolus vulgaris*	Belgium	2024	ILVO	0	0	0	0
CAL-158	*Frigoribacterium* sp.	*Phaseolus vulgaris*	Belgium	2024	ILVO	0	0	0	0
CAL-174	*Frigoribacterium* sp.	*Glycine max*	Belgium	2024	ILVO	0	0	0	0
NIB Z 3358	*Frigoribacterium* sp.	*Phaseolus vulgaris*	Slovenia	2021	NIB	0	0	0	0

0 = no Ct-value or no amplicon; 0 = high Ct value, typically > 30 in ISHI-Veg TaqMan and >28 in Tegli SYBR

4. Sensitivity

4.1. Determination of analytical sensitivity using spiked seed extracts

4.1.1. Introduction

Analytical sensitivity is defined as the range within which the analysis can be applied with a certain level of reliability. The smallest amount of target that can be detected reliably is also referred to as the 'limit of detection' (LOD).

4.1.2. Experimental approach

In accordance with the seed testing workflow described in EURL-BAC-2024-CORBFL-TM-01 (1), a large volume of 50-times concentrated extract was prepared from a Cff-free seed lot of common bean (thousand seed weight (TSW) = 160 g) from which 1 mL aliquots were spiked with Cff reference strain LMG 3651 to the final concentrations of **9 x 10^5, 9 x 10^4, 9 x 10^3, 4.5 x 10^3, 9 x 10^2 and 4.5 x 10^2 CFU/mL**. The same concentrations were also prepared without seed background (Cff cells in 10 mM phosphate buffer, PB) to evaluate the effect of the matrix. Cell density was assessed by plating the appropriate dilutions of the stock suspension on Difco Pseudomonas Agar F supplemented with 10 g/L sucrose and counting the colonies after 3 days at 28°C.

The samples of spiked extract and pure Cff cells were pelleted (10 min at 16,000 x g) and frozen without supernatant at ± -20°C for at least 12 h. DNA was extracted from the pellets following the protocol in Appendix 1. DNA extraction was done by two analysts on different days, each processing three biological replicates per Cff concentration. Subsequently, analyst 1 performed a triplex ISHI-Veg TaqMan real-time PCR and compared its performance with that of the ISHI-Veg PCR 1 run as simplex and the conventional PCR of Tegli (Appendix 2). Analyst 2 only tested his DNA extracts in the ISHI-Veg 1 simplex assay. Two technical replicates were included in all PCR reactions. TaqMan PCRs were done on the QuantStudio 5 Real-Time PCR System and conventional PCR on the MiniAmp Plus Thermal Cycler (both from Applied Biosystems). Amplicons of conventional PCR were resolved by gel electrophoresis on a 2% agarose gel and by capillary electrophoresis with the QIAxcel system.

In addition to the analytical sensitivity and LOD, the efficiency of the TaqMan PCR reactions was determined based on the curve of the dilution series [$E = -1+10^{(-1/slope)}$].

4.1.3. Results

Table 3 shows the analytical sensitivity of the ISHI-Veg 1 and ISHI-Veg 2 tests performed simultaneously by analyst 1 in a triplex assay. The values of the internal amplification control for Gram-positive bacteria are not included in this overview. The triplex results are compared with those of the ISHI-Veg 1 simplex assay and the Tegli conventional PCR. The TaqMan real-time PCR results are also visualized in Figure 1, with reaction efficiencies calculated for the spiked seed extract samples. The slopes of the curves are all between -3.1 and -3.6 (E between 90 and 110%), which means that the efficiency of the simplex and multiplex tests can be considered acceptable.

In terms of sensitivity, taking into account the in-house applied cut-off at Ct 35 for a positive result, TaqMan detection is still possible at the lowest Cff concentration tested: 4.5 x 10^2 CFU per mL of concentrated seed extract, equivalent to 9 CFU per mL of the unconcentrated (crude) extract. With a Ct divergence of approximately 1 between comparable samples with and without seed background (Figure 1a-c), there appears to be a slight negative effect of the seed matrix. This is not considered an issue given the generally high sensitivity of the TaqMan tests. Furthermore, it can be observed that the performance of the three ISHI-Veg TaqMan assays is quite similar, with a maximum Ct divergence of ±1.5 (Figure 1d). The ISHI-Veg 1 simplex assay is generally the most sensitive, while the ISHI-Veg 2 assay is the least sensitive in the triplex setup.

Importantly, for seed extracts, the TaqMan real-time PCRs outperform the Tegli conventional PCR which has long been the gold standard for Cff testing in seeds. While pure Cff cells without seed background can still be confidently detected by conventional PCR at concentrations of 9.0 x 10^2 CFU per mL of sample, the detection limit in concentrated seed extracts is somewhere between 4.5 x 10^3 and 9.0 x 10^4 CFU/mL. The results of a Belgian survey with seed and leaf samples collected in the field also show this difference in sensitivity between real-time and conventional PCR (Appendix 3).

Table 3. *Analytical sensitivity of ISHI-Veg simplex and triplex assays as compared to Tegli conventional PCR (ANALYST 1). Ct values of samples A, B and C are the average of two technical replicates. ΔRn threshold = 0.07.*

Spike (CFU/mL)[*1]	Copies in reaction[*2]	Sample type[*3]	PCR test	Biological replicate (DNA extraction & PCR)			Mean Ct	STDEV Ct
				A	B	C		
9.0E+05	1.8E+04	Pv + Cff	ISHI-1 simplex	22.31	22.43	22.46	**22.40**	0.08
			ISHI-1 triplex	23.24	21.91	23.10	**22.75**	0.73
			ISHI-2 triplex	23.73	22.54	23.74	**23.34**	0.69
			Tegli conventional	POS	POS	POS	**POS**	
		Cff	ISHI-1 simplex	21.11	21.19	21.38	**21.23**	0.14
			ISHI-1 triplex	22.14	22.32	22.18	**22.21**	0.09
			ISHI-2 triplex	22.46	22.76	22.56	**22.59**	0.15
			Tegli conventional	POS	POS	POS	**POS**	
9.0E+04	1.8E+03	Pv + Cff	ISHI-1 simplex	26.00	25.60	25.90	**25.83**	0.21
			ISHI-1 triplex	26.62	25.81	26.78	**26.41**	0.52
			ISHI-2 triplex	27.02	25.97	27.14	**26.71**	0.64
			Tegli conventional	POS	POS	POS	**POS**	
		Cff	ISHI-1 simplex	24.57	24.62	24.61	**24.60**	0.03
			ISHI-1 triplex	25.67	25.61	25.49	**25.59**	0.09
			ISHI-2 triplex	26.01	25.95	25.84	**25.93**	0.08
			Tegli conventional	POS	POS	POS	**POS**	
9.0E+03	1.8E+02	Pv + Cff	ISHI-1 simplex	29.47	29.08	28.60	**29.05**	0.43
			ISHI-1 triplex	29.84	29.85	29.96	**29.88**	0.06
			ISHI-2 triplex	30.29	30.38	30.70	**30.45**	0.22
			Tegli conventional	NEG	WEAK	WEAK	**WEAK**	
		Cff	ISHI-1 simplex	27.93	28.61	28.09	**28.21**	0.36
			ISHI-1 triplex	29.12	28.98	29.10	**29.07**	0.08
			ISHI-2 triplex	29.32	29.34	29.45	**29.37**	0.07
			Tegli conventional	POS	POS	POS	**POS**	
4.5E+03	9.0E+01	Pv + Cff	ISHI-1 simplex	29.74	29.91	30.23	**29.96**	0.25
			ISHI-1 triplex	30.16	30.91	32.04	**31.04**	0.94
			ISHI-2 triplex	30.69	31.32	32.61	**31.54**	0.98
			Tegli conventional	NEG	WEAK	WEAK	**WEAK**	
		Cff	ISHI-1 simplex	29.08	28.76	28.95	**28.93**	0.16
			ISHI-1 triplex	29.79	29.51	29.83	**29.71**	0.18
			ISHI-2 triplex	30.24	30.10	30.21	**30.18**	0.08
			Tegli conventional	POS	POS	POS	**POS**	
9.0E+02	1.8E+01	Pv + Cff	ISHI-1 simplex	32.57	31.59	32.73	**32.30**	0.62
			ISHI-1 triplex	32.69	32.82	34.00	**33.17**	0.72
			ISHI-2 triplex	33.44	33.64	33.90	**33.66**	0.23
			Tegli conventional	NEG	NEG	NEG	**NEG**	
		Cff	ISHI-1 simplex	32.06	31.12	31.49	**31.55**	0.47
			ISHI-1 triplex	32.83	32.30	32.31	**32.48**	0.30
			ISHI-2 triplex	33.36	32.63	32.62	**32.87**	0.42
			Tegli conventional	WEAK	WEAK	WEAK	**WEAK**	
4.5E+02	9.0E+00	Pv + Cff	ISHI-1 simplex	34.82	33.56	33.21	**33.86**	0.85
			ISHI-1 triplex	35.17	34.00	33.99	**34.39**	0.67
			ISHI-2 triplex	34.68	34.60	34.62	**34.63**	0.04
			Tegli conventional	NEG	NEG	NEG	**NEG**	
		Cff	ISHI-1 simplex	31.62	32.65	32.81	**32.36**	0.65
			ISHI-1 triplex	33.89	33.09	34.18	**33.72**	0.57
			ISHI-2 triplex	34.28	33.80	34.38	**34.15**	0.31
			Tegli conventional	VERY WEAK	VERY WEAK	VERY WEAK	**VERY WEAK**	

[*1] LMG 3651 cells added to 1 mL sample
[*2] From 1 mL sample, 100 µL DNA extract was prepared of which 2 µL was used in PCR reaction (factor 50)
[*3] Pv + Cff = 50x concentrated *Phaseolus vulgaris* seed extract spiked with LMG 3651; Cff = LMG 3651 cells only

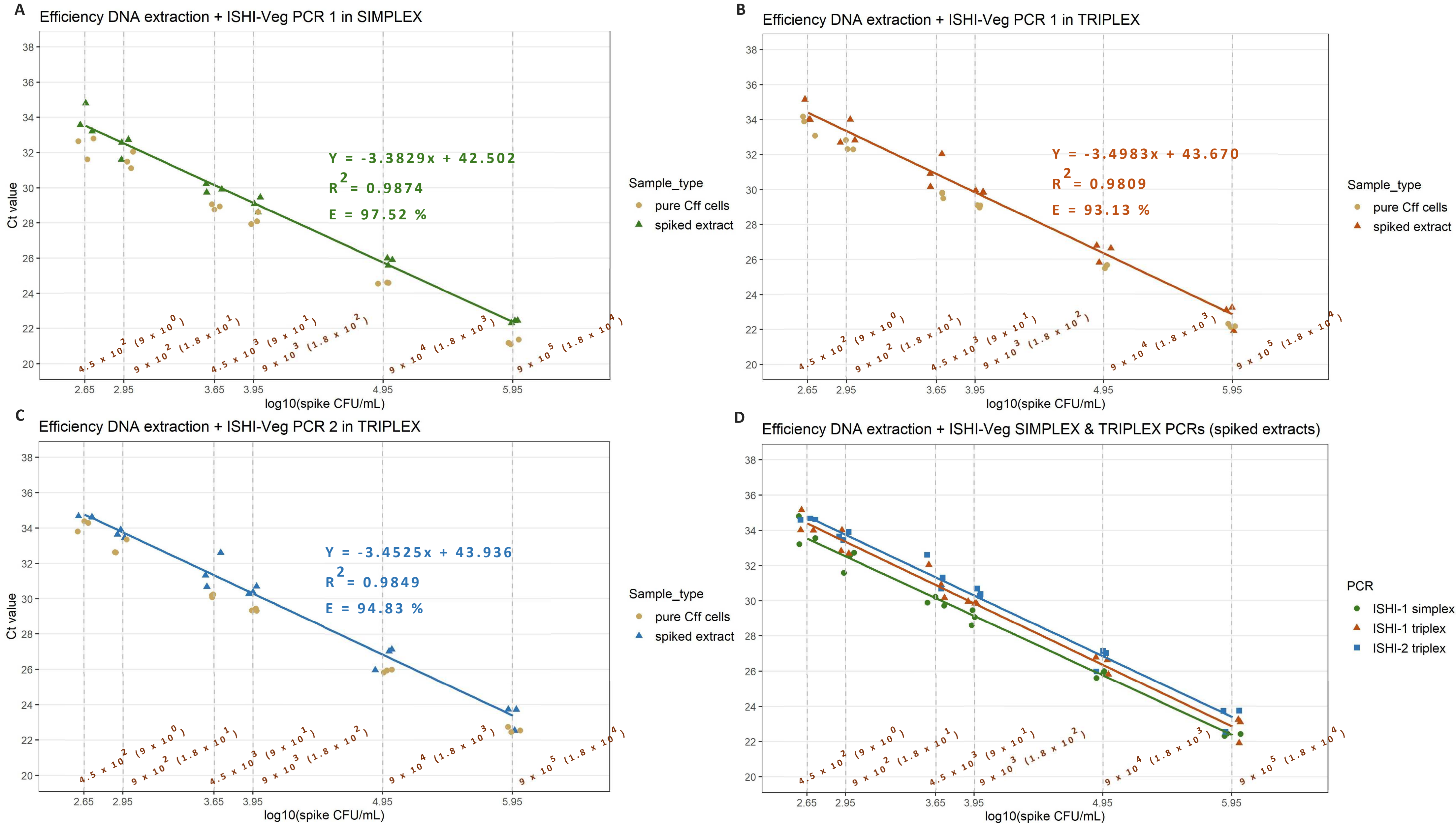

Figure 1. *Efficiency and analytical sensitivity of ISHI-Veg 1 simplex assay (A) and ISHI-Veg 1 and 2 run as multiplex assay (B and C, respectively). In panels A-C, the small impact of the seed matrix is visualized as the Ct difference between spiked extracts and samples without seed background (Cff only). In panel D, results of the seed extracts are set out for all TaqMan tests to assess differences in sensitivity. The Cff concentration in the spiked samples is indicated in red (CFU per mL). The values in brackets specify what the Cff level would be in the unconcentrated seed extract.*

4.1.4. Conclusion

The ISHI-Veg TaqMan tests, both as simplex and triplex assay, are considered fit for purpose with satisfactory reaction efficiency and high analytical sensitivity. The ISHI-Veg TaqMans outperform the Tegli conventional PCR for detection of Cff in extracts of bean seeds. The LOD is set at 4.5 x 10^2 to 9.0 x 10^2 CFU per mL of concentrated seed extract, equivalent to 9-18 CFU per mL of crude extract (see also 6. Reproducibility).

4.2. Determination of diagnostic sensitivity using field samples

4.2.1. Introduction

Diagnostic sensitivity measures the proportion of samples that test positive out of all truly positive samples as determined by the standard test.

4.2.2. Experimental approach

Diagnostic sensitivity of the DNA extraction method and ISHI-Veg TaqMan PCRs was assessed using seed and leaf samples collected during a field survey in Belgium in 2024 (CurtoALERT project). Seed extracts were prepared from subsamples of 1000 seeds according to EURL-BAC-2024-CORBFL-TM-01 (1). For the extraction of bacteria from leaf material, pooled samples consisting of 25 to 30 surface-sterilized 2-cm petiole fragments were homogenized with a hammer in an extraction bag containing 10 mM PB. For plants showing typical symptoms, leaves were treated individually by macerating small pieces of leaf tissue or petiole in PB. After 30 minutes of incubation, an aliquot of the extract was pelleted and stored at ± -20°C. DNA was extracted from the seed and leaf pellets according to Appendix 1, after which ISHI-Veg simplex and triplex assays were performed and compared with Tegli conventional and real-time PCR (Appendix 2).

4.2.3. Results

Results for a subset of the survey samples are presented in Appendix 3. Of the 29 samples included, 15 tested positive in Tegli conventional PCR, which is one of the two validated standard detection tests (both conventional PCRs) in the current version of EPPO PM 7/102 (16). These 15 Tegli positives were also detected by the ISHI-Veg TaqMan real-time PCRs, giving 100% diagnostic sensitivity. Additionally, several Tegli-negative samples tested positive in the TaqMan PCRs as well as in the Tegli SYBR-based assay. With the conventional PCR and also the plating method being less sensitive than real-time PCR, it is difficult to determine whether these are true or false positives.

4.2.4. Conclusion

Diagnostic sensitivity is considered 100% when TaqMan results are compared with those from Tegli conventional PCR as the standard test. However, because conventional PCR and also plating are less sensitive and more prone to inhibiting contaminants than real-time PCR, it is difficult to determine the true accuracy of the ISHI-Veg TaqMan method using natural samples with unknown Cff status.

4.3. Determination of LOD using artificially inoculated seed

4.3.1. Introduction

In this section, the LOD is defined as the minimum number of pathogen propagules per seed which can be detected in a subsample.

4.3.2. Experimental approach

The ability of the seed testing method EURL-BAC-2024-CORBFL-TM-01 (1) to detect one infected seed in a subsample was determined by adding 1 artificially inoculated seed at different levels of contamination to 1000 healthy seeds based on the TSW of 160 g.

First, *Phaseolus* seeds were loaded with Cff by soaking them in three different concentrations of Cff inoculum (10^6, 10^7 and 10^8 CFU/mL; strain LMG 3651) and immediately subjecting them to vacuum for 5 min. Vacuum infiltration in 10 mM PB served as a negative control. The seeds were air-dried overnight in a laminar flow, after which ten seeds from each concentration were individually tested

via real-time PCR and plating to determine the actual infestation level per seed (number of CFU). Next, artificially contaminated subsamples were prepared by adding 1 infected seed to 160 g of healthy *Phaseolus* seeds. For each inoculum level and the PB control, ten 1000-seed subsamples were tested by both plating (CFFKB medium) and TaqMan real-time PCR according to the standard workflow described in EURL-BAC-2024-CORBFL-TM-01 (1).

4.3.3. Results

The Ct values of the tested subsamples contaminated with one artificially infected seed are shown in Table 4. The lowest Cff inoculum, i.e. 1 seed carrying approximately 10^4 CFU in a 160 g seed subsample is readily detected in ISHI-Veg real-time PCR 1 (Ct range 27.37 to 30.43). In contrast, the plating method yields a CFU count near the limit of detection for this inoculum dose, with only 5 to 28 colonies growing on CFFKB when plating 100 µL of the undiluted crude extract.

4.3.4. Conclusion

One seed carrying approximately 10^4 Cff cells is readily detectable in a subsample of approximately 1000 seeds using ISHI-Veg TaqMan real-time PCR. For the plating method, on the other hand, this level of contamination is considered to be near the LOD.

Table 4. *Detection of Cff in seed subsamples of 160 g (TSW) contaminated with 1 artificially infected seed by both ISHI-Veg TaqMan PCR and plating on CFFKB. nd= not determined.*

Median infestation level of added infected seed (CFU/seed)*1	ID of contaminated 1000-seed subsample	Ct of 50x concentrated seed extract	Plating unconcentrated seed extract		
			# colonies	Dilution used*2	CFU/mL in unconc. extract
4.28E+05	1-A	22.87	169	10x	16 900
	1-B	23.74	136	10x	13 600
	1-C	23.73	92	10x	9 200
	1-D	22.06	330	10x	33 000
	1-E	23.64	105	10x	10 500
	1-F	24.60	nd		
	1-G	23.11	nd		
	1-H	24.93	nd		
	1-I	23.18	nd		
	1-J	22.17	nd		
9.33E+04	2-A	26.48	66	0x	660
	2-B	25.05	260	0x	2 600
	2-C	26.96	58	0x	580
	2-D	26.86	61	0x	610
	2-E	27.05	53	0x	530
	2-F	27.48	nd		
	2-G	27.08	nd		
	2-H	26.93	nd		
	2-I	26.36	nd		
	2-J	26.97	nd		
1.46E+04	3-A	**29.66**	**14**	0x	140
	3-B	**30.43**	**7**	0x	70
	3-C	**30.22**	**5**	0x	50
	3-D	**28.40**	**22**	0x	220
	3-E	**28.11**	**28**	0x	280
	3-F	28.40	nd		
	3-G	29.26	nd		
	3-H	28.49	nd		
	3-I	27.37	nd		
	3-J	29.64	nd		

*1 Infestation level was determined for 10 individual seeds per inoculum dose; median values are shown

*2 100 µL of each used dilution was plated

5. Repeatability

5.1. Introduction

Repeatability is defined as the level of agreement between replicates of a sample tested simultaneously by a single person using the same instruments under the same conditions.

5.2. Experimental approach

The same person extracted DNA from three biological replicates of seed extract spiked with different concentrations of Cff cells (LMG 3651) and from comparable samples without seed background. ISHI-Veg simplex and triplex PCR tests were then performed. All procedures are described under section 4. Sensitivity.

5.3. Results

The results of repeatability are presented as biological replicates A, B and C in Table 3 and Table 5. Except for the negative controls, all test results are positive for Cff. Based on the standard deviation, the variation between the replicates is minimal.

5.4. Conclusion

Repeatability of the DNA extraction procedure and the ISHI-Veg TaqMan tests on spiked concentrated bean seed extracts and on cell suspensions of Cff is considered 100%.

6. Reproducibility

6.1. Introduction

Reproducibility is the ability of a method to provide consistent results when applied to aliquots of the same sample tested under different conditions (e.g. time, persons, equipment, location).

6.2. Experimental approach

At a different moment, a second operator independently extracted DNA from three biological replicates of the same material as above and performed the ISHI-Veg 1 TaqMan test as a simplex reaction.

6.3. Results

The results of reproducibility are presented in Table 5 and Figure 2. Analyst 2 obtained measurable Ct values for the entire Cff concentration range, but with lower analytical sensitivity compared to the data of analyst 1. This is because the second analyst was not as familiar with the DNA extraction procedure as the first analyst who optimized the protocol. Taking into account the in-house applied cut-off at Ct 35 for a positive result, the lowest Cff concentration that could still be reliably detected in concentrated seed extract by analyst 2 is 9.0×10^2 CFU/mL, as compared to 4.5×10^2 CFU/mL for analyst 1.

6.4. Conclusion

Despite the difference in analytical sensitivity between the tests of operator 1 and 2, reproducibility is considered 100%. Except for the lowest spike, different people are able to reliably reproduce the results at different days.

Table 5. *Reproducibility of DNA extraction and ISHI-Veg 1 simplex PCR. Ct values of samples A, B and C are the average of two technical replicates. ΔRn threshold = 0.07.*

Spike (CFU/mL)[*1]	Copies in reaction[*2]	Sample type[*3]	Analyst	Biological replicate (DNA extraction & PCR)			Mean Ct	STDEV Ct
				A	B	C		
9.0E+05	1.8E+04	Pv + Cff	Analyst 1	22.31	22.43	22.46	**22.40**	0.08
			Analyst 2	24.88	25.12	25.28	**25.09**	0.20
		Cff	Analyst 1	21.11	21.19	21.38	**21.23**	0.14
			Analyst 2	23.30	23.27	23.29	**23.29**	0.01
9.0E+04	1.8E+03	Pv + Cff	Analyst 1	26.00	25.60	25.90	**25.83**	0.21
			Analyst 2	27.99	28.35	27.90	**28.08**	0.24
		Cff	Analyst 1	24.57	24.62	24.61	**24.60**	0.03
			Analyst 2	26.55	26.66	26.80	**26.67**	0.13
9.0E+03	1.8E+02	Pv + Cff	Analyst 1	29.47	29.08	28.60	**29.05**	0.43
			Analyst 2	30.38	31.16	31.44	**30.99**	0.55
		Cff	Analyst 1	27.93	28.61	28.09	**28.21**	0.36
			Analyst 2	30.65	30.33	30.44	**30.47**	0.16
4.5E+03	9.0E+01	Pv + Cff	Analyst 1	29.74	29.91	30.23	**29.96**	0.25
			Analyst 2	32.38	32.87	31.91	**32.39**	0.48
		Cff	Analyst 1	29.08	28.76	28.95	**28.93**	0.16
			Analyst 2	31.34	31.10	31.08	**31.17**	0.14
9.0E+02	1.8E+01	Pv + Cff	Analyst 1	32.57	31.59	32.73	**32.30**	0.62
			Analyst 2	34.25	34.98	34.44	**34.56**	0.37
		Cff	Analyst 1	32.06	31.12	31.49	**31.55**	0.47
			Analyst 2	34.90	33.40	33.65	**33.98**	0.81
4.5E+02	9.0E+00	Pv + Cff	Analyst 1	34.82	33.56	33.21	**33.86**	0.85
			Analyst 2	36.03	35.49	36.19	**35.91**	0.37
		Cff	Analyst 1	31.62	32.65	32.81	**32.36**	0.65
			Analyst 2	34.92	34.57	34.39	**34.63**	0.27

[*1] LMG 3651 cells added to 1 mL sample
[*2] From 1 mL sample, 100 µL DNA extract was prepared of which 2 µL was used in PCR reaction (factor 50)
[*3] Pv + Cff = 50x concentrated *Phaseolus vulgaris* seed extract spiked with LMG 3651; Cff = LMG 3651 only

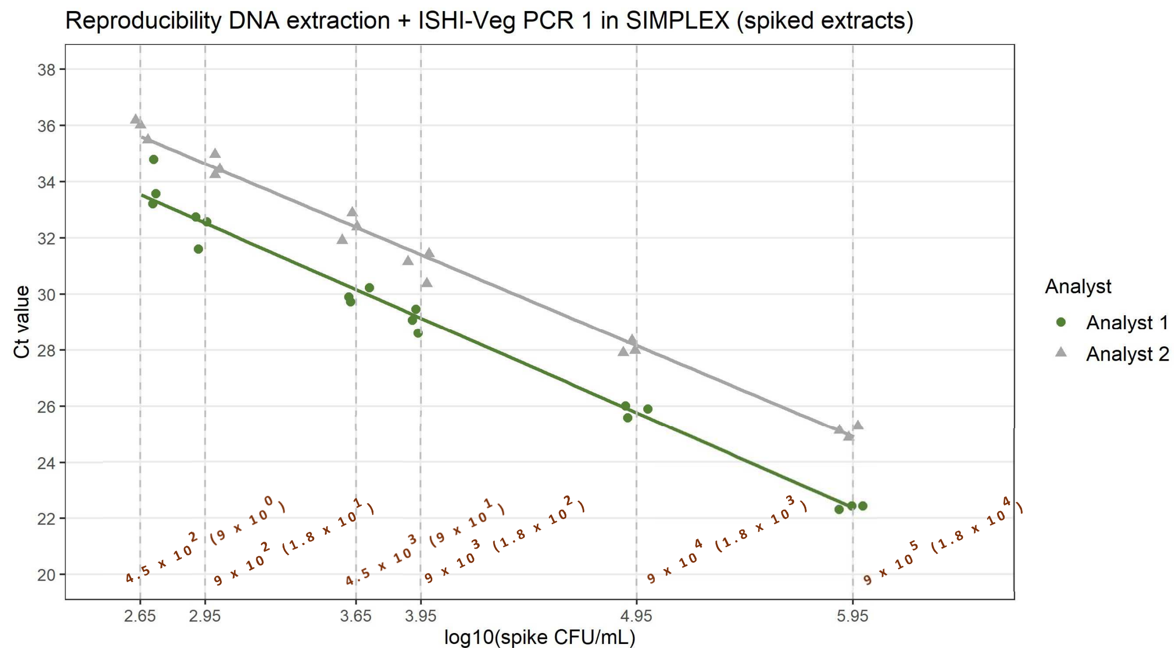

Figure 2. *Analytical sensitivity and reproducibility of DNA extraction and ISHI-Veg 1 simplex PCR for seed extracts spiked with different Cff concentrations. The number of Cff cells in the spiked samples is indicated in red (CFU per mL). The values in brackets specify what the Cff level would be in the unconcentrated seed extract.*

7. Cited literature

1. Venneman, J., Vreeburg, R.A.M., Bergsma-Vlami, M., Van Malderghem, C., De Paepe, B. and Van Vaerenbergh, J. (2024). Detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in common bean seeds. EURL-BAC-2024-CORBFL-TM-01.
2. Harveson, R.M. , Schwartz, H.F. , Urrea, C.A. and Yonts, C.D. (2015). Bacterial wilt of dry-edible beans in the central high plains of the US: Past, present, and future. Plant Disease 99, 1665–1677.
3. Silva Júnior, T.A.F., Negrão, D.R., Italo, A.T., Soman, J.M. and Maringoni, A.C. (2012). Survival of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in soil and bean crop debris. Journal of Plant Pathology 94, 331–337.
4. Gonçalves, R.M., Schipanski, C.A., Koguishi, L., Soman, J.M., Sakate, R.K., Silva Júnior, T.A.F. et al (2017). Alternative hosts of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, causal agent of bean bacterial wilt. European Journal of Plant Pathology 148, 357–365.
5. Gonçalves, R.M., Soman, J.M., Krause-Sakate, R., Passos, J.R., Silva Júnior, T.A.F. and Maringoni, A.C. (2018). Survival of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in the soil under Brazilian conditions. European Journal of Plant Pathology 152, 213–223.
6. Wood, B.A. and Easdown, W.J. (1990). A new bacterial disease of mungbean and cowpea for Australia. Australasian Plant Pathology 19, 16–21.
7. Soares, R.M., Fantinato, G.G.P., Darben, L.M., Marcelino-Guimarães, F.C., Seixas, C.D.S. and Carneiro, G.E.S. (2013). First report of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* on soybean in Brazil. Tropical Plant Pathology 38, 452–454.
8. Puia, J.D., Ferreira, M.G.D.B., Hoshino, A.T., Borsato, L.C., Canteri, M.G. and Vigo, S.C. (2021). Occurrence of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in the state of Paraná and its pathogenicity in beans. European Journal of Plant Pathology 159, 627–636.
9. Osdaghi, E., Taghavi, S.M., Fazliarab, A., Elahifard, E. and Lamichhane, J.R. (2015). Characterization, geographic distribution and host range of *Curtobacterium flaccumfaciens*: An emerging bacterial pathogen in Iran. Crop Protection 78, 185–192.
10. Document 02016R2031-20191214 - Regulation (EU) 2016/2031 of the European Parliament of the Council of 26 October 2016 on protective measures against pests of plants, amending Regulations (EU) No 228/2013, (EU) No 652/2014 and (EU) No 1143/2014 of the European Parliament and of the Council and repealing Council Directives 69/464/EEC, 74/647/EEC, 93/85/EEC, 98/57/EC, 2000/29/EC, 2006/91/EC and 2007/33/EC. http://data.europa.eu/eli/reg/2016/2031/2019-12-14
11. Wu, Y.D., Chen, L.H., Wu, X.J., Shang, S.Q., Lou, J.T., Du, L.Z. and Zhao, Z.Y. (2008). Gram stain-specific probe-based real-time PCR for diagnosis and discrimination of bacterial neonatal sepsis. Journal of Clinical Microbiology 46, 2613–2619.
12. Tegli, S., Sereni, A. and Surico, G. (2002). PCR-based assay for the detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in bean seeds. Letters in Applied Microbiology 35, 331–337.
13. Tegli, S., Gaudiosa, D. and Stefanucci, D. (2022). Innovative detection of the quarantine plant pathogen *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, causal agent of bacterial wilt of leguminous plants. In: Luchi, N. (ed) Plant Pathology. Methods in Molecular Biology, vol 2536. Humana, New York, NY.
14. González, A.J., Tello, J.C. and Rodicio, M.R. (2005). Bacterial wilt of beans (*Phaseolus vulgaris*) caused by *Curtobacterium flaccumfaciens* in Southeastern Spain. Plant Disease 89, 1361.
15. Sammer, U.F. and Reither, K. (2012). *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* on soybean in Germany - a threat for farming. Journal of Phytopathology 160, 314–316.
16. EPPO (2011). PM 7/102 (1) - *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*. Bull. OEPP 41, 320–328.

Appendix 1: DNA extraction with QuickPick™ SML Plant DNA Kit and KingFisher Flex

This DNA-extraction procedure for *Phaseolus* seed extracts to be tested for the presence of *Cff* is based on the validated ILVO protocol for *Clavibacter sepedonicus* and *Ralstonia solanacearum* in potato tubers. It relies on the QuickPick SML Plant DNA Kit in combination with the KingFisher Flex extraction robot.

- Resuspend thawed pellets in 250 µL 1x TE buffer.
- Add 40 µL of lysozyme solution (50 mg/mL). Mix by vortexing.

  Alternatively, add 200 µL of 0.1-mm glass beads to the resuspended pellet and process by bead beating at 30 Hz/s for 6 min.
- Centrifuge for 10 s @ 10,000 x g to avoid contamination from the lid.

  Add 300 µL of Bio-Nobile Lysis Buffer and mix well.

  Incubate for 30 min at 37°C.
- Centrifuge for 10 s @ 10,000 x g to avoid contamination from the lid.

  Add 10 µL Proteinase K solution and pulse vortex.

  Incubate for 30 min at 65°C.
- During the final lysis step, QuickPick™ SML Plant DNA reagents to be used in the KingFisher apparatus are pipetted into sample plates A to D:

Plate	Plate type	Process	Well content	volume per well
A	Deep well	Binding	500 µL Binding Buffer + 20 µL Magnetic Particles, mix 10s	520 µL
B	Deep well	Wash 1	Wash Buffer	500 µL
C	Deep well	Wash 2	Wash Buffer	500 µL
D	Standard	Elution	Elution Buffer	100 µL

- Centrifuge the lysates for 5 min at 16,000 x g.
- Carefully transfer 500 µL of the supernatant to the wells of Plate A
- Load the plate onto the KingFisher Flex instrument and run the protocol described hereafter:

1. MIXING (Plate A)
   Beginning of step
   - Release beads = Yes, time = 10 s, speed = Slow

   Mixing/Heating parameters
   - Mix time = 8 min, speed = Medium

   End of step
   - Collect beads = No
2. BINDING (Plate A)
   Beginning of step
   - Release beads = No

   Mixing/Heating parameters
   - Mix time = 2 min 30 s, speed = Slow

   End of step
   - Collect beads = Yes, count = 4, collect time = 30 s
3. WASH 1 (Plate B)
   Beginning of step
   - Release beads = Yes, time = 5 s, speed = Slow

   Mixing/Heating parameters
   - Mix time = 2 min, speed = Medium

   End of step
   - Collect beads = Yes, count = 4, collect time = 30 s

4. WASH 2 (Plate C)
   Beginning of step
   - Release beads = Yes, time = 5 s, speed = Slow

   Mixing/Heating parameters
   - Mix time = 2 min, speed = Medium

   End of step
   - Collect beads = Yes, count = 4, collect time = 30 s

5. ELUTION (Plate D)
   Beginning of step
   - Release beads = Yes, time = 5 s, speed = Slow

   Mixing/Heating parameters
   - Mix time = 10 min, speed = Slow, heating during mixing = Yes, preheat = Yes, block temperature = 75°C

   End of step
   - Postmix = Yes, time = 5 s, speed = Slow
   - Collect beads = Yes, count = 4, collect time = 5 s

- Transfer the DNA from the Elution plate to microtubes for PCR analysis.
- Store the DNA at refrigeration temperature for up to 2 weeks, or at ± -20°C for later use.

Appendix 2: PCR protocols

The following PCR protocols were used for the validation experiments presented in this report.

1. ISHI-Veg real-time TaqMan PCR by Naktuinbouw

Two tests based on serine protease genes have been designed by Naktuinbouw that can be used for detection and identification of bean-pathogenic *Curtobacterium*. The PCR can be run as simplex with one of the two Cff primer/probe combinations. The P967F/P1194R/Gpos probe primer/probe combination is an internal amplification control for Gram-positive bacteria (11).

Oligonucleotides

Primers and probes	Sequence	Amplicon size
Forward primer Cff1-F	5′-ACG TAG CCG CAA TCC GTT AT-3′	
Reverse primer Cff1-R	5′-CGA CAC TTT CTC CCG CTA CT-3′	87 bp
Probe Cff1-P	5′-FAM-GCC CAC TGC GCA TCA CGA GTC GGG CA- BHQ1-3′	
Forward primer Cff2-F	5′-GTT CCA GAG GTC GCA AGG AA-3′	
Reverse primer Cff2-R	5′-ATG CCG TAG AAC TTG CCT GA-3′	92 bp
Probe Cff2-P	5′-VIC-AGG AGA TTC CGG CGG ACC AGT GGT GTC GC-BHQ1-3′	
Forward primer P967F	5′-CAA CGC GAA GAA CCT TAC C-3′	
Reverse primer P1194R	5′-ACG TCA TCC CCA CCT TCC-3′	227 bp
Probe Gpos probe	5′- Cy5-ACG ACA ACC ATG CAC CAC CTG-BHQ2-3′	

Master mix composition for simplex

Reagent	Working concentration	Volume per reaction µL	Final concentration
PCR grade water	N.A.	6.4	N.A.
Quantabio PerfeCTa qPCR ToughMix UNG Low ROX	2 x	10.0	1x
Forward primer Cff1-F or Cff2-F	10 µM	0.6	0.3 µM
Reverse primer Cff1-R or Cff2-R	10 µM	0.6	0.3 µM
Probe Cff1-P or Cff2-P	10 µM	0.4	0.2 µM
Subtotal		18.0	
DNA from sample		2.0	
Total		20	

Master mix composition for multiplex

Reagents	Working concentration	Volume per reaction µL	Final concentration
PCR grade water	N.A.	5.6	N.A.
PerfeCTa qPCR ToughMix	2 x	10.0	1x
Forward primer Cff1-F	10 µM	0.3	0.15 µM
Reverse primer Cff1-R	10 µM	0.3	0.15 µM
Probe Cff1-P	10 µM	0.2	0.10 µM
Forward primer Cff2-F	10 µM	0.3	0.15 µM
Reverse primer Cff2-R	10 µM	0.3	0.15 µM
Probe Cff2-P	10 µM	0.2	0.10 µM
P967F	10 µM	0.3	0.15 µM
P1194R	10 µM	0.3	0.15 µM
Gpos probe	10 µM	0.2	0.10 µM
Subtotal		18.0	
DNA from sample		2.0	
Total		20	

PCR conditions

Cycles	Time	Temperature
1x	5:00	45°C
1x	3:00	95°C
40x	0:15	95°C
	1:00	60°C

2. Tegli conventional PCR (Tegli *et al.*, 2002; 12)

The amplicon of this test is derived from a 550 bp ERIC-PCR fragment obtained from *Cff* strains (GenBank records AJ307048 to AJ307051). In the annotated NCBI RefSeq assembly GCF_013359815.1, it is identified as 234 bp at the 3' end of a trypsin-like serine protease and 72 bp in the upstream intergenic region (contig 27 of NCBI WGS accession JABMCF01 from LMG 3645^{PT}).

Oligonucleotides

Primer	Sequence	Amplicon size
CffFOR2	5'-GTT ATG ACT GAA CTT CAC TCC-3'	306 bp
CffREV4	5'-GAT GTT CCC GGT GTT CAG-3'	

Master mix for conventional PCR[1]

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
PCR grade water	N.A.	10.8	N.A.
GoTaq Flexi Buffer	5 x	5.0	1x
$MgCl_2$	25 mM	2.5	2.5 mM
dNTPs	10 mM	0.5	200 µM
Forward primer CffFOR2	10 µM	0.5	0.2 µM
Reverse primer CffREV4	10 µM	0.5	0.2 µM
GoTaq G2 Flexi DNA Polymerase	5 U/µL	0.2	1 U
Subtotal		20.0	
DNA from sample		5.0	
Total		25.0	

[1] The master mix composition is different from the original publication by Tegli *et al.* (2002).

PCR conditions[1]

Cycles	Time	Temperature
1x	5:00	94°C
35x	0:30	94°C
	0:30	62°C
	0:30	72°C
1x	5:00	72°C

[1] The PCR conditions are different from the original publication by Tegli *et al.* (2002).

3. Tegli real-time PCR (Tegli *et al.*, 2022; 13)

The amplicon of this test is derived from a 550 bp ERIC-PCR fragment obtained from several Cff strains (GenBank records AJ307048 to AJ307051). In the annotated NCBI RefSeq assembly GCF_013359815.1, it is identified as 164 bp at the 3' end of a trypsin-like serine protease (gene HP445_00770 with CDS NUU08805.1) and 68 bp in the upstream intergenic region (contig 27 of NCBI WGS accession JABMCF01 from LMG 3645^{PT}).

Oligonucleotides

Primer	Sequence	Amplicon size
CffF3	5'-CGT TAG TGA AGG CTG ACG AA-3'	232 bp
CffB3	5'-TTC CCG GTG TTC AGT TGA C-3'	

Master mix for real-time PCR[1]

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	3.0	N.A.
PerfeCTa SYBR Green Low ROX FastMix or SsoFast EvaGreen Supermix	2 x	5.0	1x
Forward primer CffF3	10 µM	0.5	0.5 µM
Reverse primer CffB3	10 µM	0.5	0.5 µM
Subtotal		9.0	
DNA from sample		1.0	
Total		10.0	

[1] The master mix composition is different from the original publication by Tegli *et al.* (2022).

PCR conditions[1]

Cycles	Time	Temperature
1x	3:00	95°C
40x	0:05	95°C
	0:30	60°C

Melt curve analysis from 65°C to 95°C with 0.2°C steps each 10 seconds.

[1] The PCR conditions are different from the original publication by Tegli *et al.* (2022).

Appendix 3: Differences in sensitivity between ISHI-Veg TaqMan PCR and Tegli conventional and real-time PCRs for leaf and seed extracts from a Belgian survey (CurtoALERT project) [Green lines= positive in all tests]

Sample	ISHI-Veg triplex TaqMan 2024-11-25			ISHI-Veg 1 simplex TaqMan 2024-09-20 (ΔRn thresh. 0.07)	Tegli PCRs			Plant species	Geogr. origin	Year of analysis	Cff isolates obtained?
	Gram+ (ΔRn thresh. 0.04)	ISHI-Veg PCR 1 (ΔRn thresh. 0.07)	ISHI-Veg PCR 2 (ΔRn thresh. 0.07)		Conventional PCR 2024-12-04	SYBR Perfecta 2024-12-02 Ct (ΔRn thresh. 0.16)	SYBR Perfecta 2024-12-02 Tm (thresh. 80 000)				
Leaf extract 1	14.33	15.99	16.48	15.75	POS	12.30	85.2	*Phaseolus vulgaris*	Belgium	2024	Y
Leaf extract 2	20.73	27.32	27.99	26.87	POS	22.81	85.2	*Phaseolus vulgaris*	Belgium	2024	Y
Leaf extract 3	20.30	UND	UND	35.94	NEG	33.20	85.3	*Phaseolus vulgaris*	Belgium	2024	N
Leaf extract 4	18.35	33.67	35.18	33.75	NEG	35.27	80.9	*Phaseolus vulgaris*	Belgium	2024	N
Leaf extract 5	18.15	34.46	33.50	34.26	NEG	30.55	85.1	*Phaseolus vulgaris*	Belgium	2024	N
Leaf extract 6	17.40	UND	UND	UND	NEG	37.67		*Phaseolus vulgaris*	Belgium	2024	N
Leaf extract 7	17.28	37.13	37.66	UND	NEG	31.42	86.7	*Glycine max*	Belgium	2024	N
Leaf extract 8	19.76	24.68	25.08	24.27	POS	20.62	84.9	*Phaseolus vulgaris*	Belgium	2024	Y
Seed extract 1[a]	22.84	33.04	34.06	32.88	NEG	30.55	85.1	*Phaseolus vulgaris*	USA	2024	N
Seed extract 2[a]	23.35	31.14	30.92	30.44	NEG	27.11	85.6	*Phaseolus vulgaris*	USA	2024	N
Seed extract 3[a]	16.64	18.11	18.37	17.39	POS	13.77	85.4	*Phaseolus vulgaris*	USA	2024	Y
Seed extract 4[a]	23.22	UND	38.72	UND	NEG	38.10		*Phaseolus vulgaris*	USA	2024	N
Seed extract 5[b]	22.00	24.29	24.90	24.05	POS	20.31	85.4	*Phaseolus vulgaris*	USA	2024	N
Seed extract 6[a]	21.30	32.02	32.67	32.20	NEG	28.55	85.2	*Phaseolus vulgaris*	USA	2024	N
Seed extract 7[a]	22.88	36.70	UND	UND	NEG	31.58	85.4	*Phaseolus vulgaris*	USA	2024	N
Seed extract 8[b]	22.02	32.01	32.45	31.60	WEAK	28.21	85.2	*Phaseolus vulgaris*	USA	2024	N
Seed extract 9[a]	20.11	35.24	37.65	34.77	NEG	32.76	85.3	*Phaseolus vulgaris*	USA	2024	N
Seed extract 10[a]	21.30	33.38	33.65	32.61	NEG	29.55	85.3	*Phaseolus vulgaris*	USA	2024	N
Seed extract 11[b]	18.71	20.17	20.81	19.87	POS	15.57	85.2	*Phaseolus vulgaris*	USA	2024	Y
Seed extract 12[c]	22.99	33.21	34.81	32.51	NEG	30.37	85.2	*Phaseolus vulgaris*	n/a	2024	N
Seed extract 13[a]	19.70	21.00	21.46	20.50	POS	16.56	85.1	*Phaseolus vulgaris*	n/a	2024	Y
Seed extract 14[a]	19.99	30.22	30.75	29.80	WEAK	26.77	85.5	*Phaseolus vulgaris*	n/a	2024	Y
Seed extract 15[a]	21.13	28.71	30.48	29.37	WEAK	28.84	85.7	*Phaseolus vulgaris*	n/a	2024	N
Seed extract 16[a]	20.49	28.55	29.30	28.54	NEG	26.17	85.4	*Phaseolus vulgaris*	n/a	2024	N
Seed extract 17[d]	16.05	18.11	18.21	17.60	POS	13.31	84.8	*Phaseolus vulgaris*	USA	2024	Y
Seed extract 18[d]	17.68	27.22	27.96	26.60	WEAK	23.10	85.1	*Phaseolus vulgaris*	USA	2024	N
Seed extract 19[d]	17.31	19.85	20.02	18.69	POS	14.81	85.0	*Phaseolus vulgaris*	USA	2024	Y
Seed extract 20[d]	14.33	15.97	16.44	15.04	POS	10.91	84.9	*Phaseolus vulgaris*	USA	2024	Y
Seed extract 21[d]	18.77	27.91	28.48	27.68	WEAK	23.69	85.0	*Phaseolus vulgaris*	USA	2024	Y

[a] Seeds coated with Fludioxonil
[b] Seeds coated with Apron XL
[c] Seeds coated with Chlorpyrifos + Thiram
[d] Five subsamples from the same seed lot; not coated