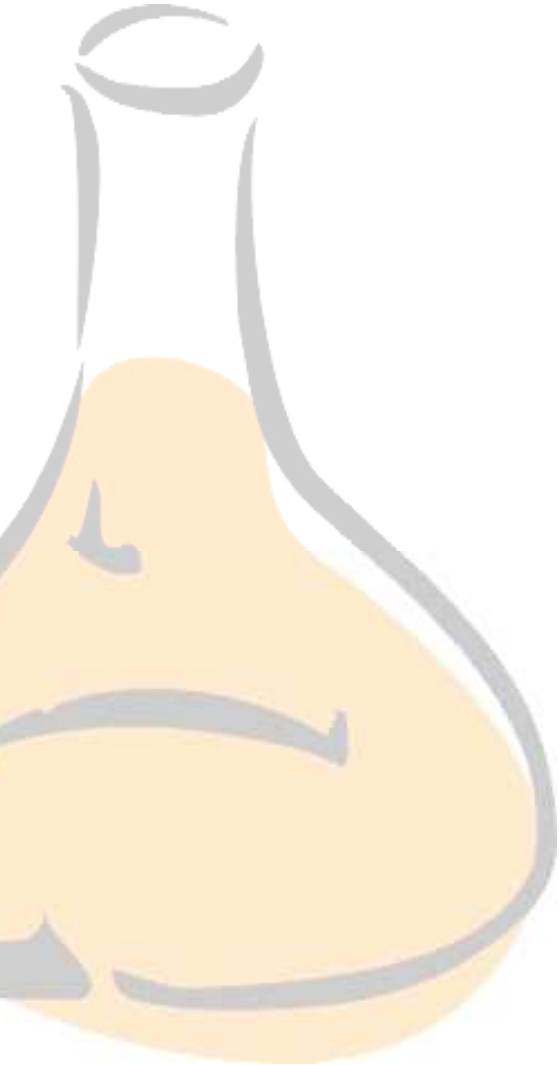
Validation report of a real-time PCR assay for the identification of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) isolates



Groen Agro Control
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Summary

In this report, we present complete validation data related to recently developed primer-probe combinations for the identification of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) isolates with the use of a real-time PCR assay. The primers and probes were developed on two loci: on the intergenic spacer between the 16S and 23S ribosomal RNA genes (IGS) and on the pathogenity-1 gene (Pat-1), which is located on the plasmid pCM2. The validated method is qualitative and consists of a DNA extraction from pure colony material followed by a duplex real-time PCR assay based on the primer-probe combinations for IGS and Pat-1.
The method is very specific, because only the 20 tested pathogenic *Cmm* isolates were positive for both primer-probe combinations. Negative results were obtained from 12 *Cmm* look-alikes, 4 *Cm* isolates and 9 other tomato pathogens. Tests on repeatability, reproducibility and robustness of this assay performed well.

However, when the validation of the assay described here was completed, three newly isolated *Cmm* isolates scored negatively for the Pat-1 primer-probe combination, but still positive for the IGS assay. Nevertheless, these three isolates were positive for earlier described Pat-1 primer-probe combinations. Therefore, the assay based on the Pat-1 primer-probe combination assay described here cannot be used as a conclusive assay yet and it has to be improved. The assay based on the IGS primer-probe combination however is performing well in identifying all pathogenic *Cmm* isolates. Confirmation of the *Cmm* identity with the IGS primer-probe combination is achieved at a Ct-value lower than 35.5.

1 Introduction

Clavibacter michiganensis subsp. *michiganensis*. (*Cmm*) is the causal agent of bacterial canker of tomato (1). As one of the most important bacterial diseases of tomato, *Cmm* infection can lead to substantial economic losses. The pathogen can survive on plant debris in soil for up to 2 or 3 years and can be transmitted through seed (2). In order to prevent outbreaks of tomato bacterial canker, the quarantine status of *Cmm* is internationally regulated in the European Union by Commission Directive 2000/29/EC (3). Early identification of *Cmm* is therefore crucial and can be realized by application of specific and highly-sensitive primers for *Cmm* in a real-time PCR approach. The primer sets developed so far for the detection of *Cmm* have showed false positive or false negative results in several cases (4, 5).

This validation report has been directed in underlining the important performance characteristics, through a method validation, of the real-time PCR assay for the identification of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in bacterial colony material.

Real-time PCR is a well-known technique and is considered as a reference method. In the real-time PCR assay described here, two new primer sets with accompanying probes have been validated. The primer-probe combinations recognize different DNA locations in described sequences of *Cmm*: at the genome (IGS; intergenic spacer between 16S and 23S ribosomal RNA genes) and at the plasmid pCM2. The primer-probe combination developed for the pCM2 plasmid is localised on the pathogenicity gene Pat-1.

The experiments have been performed during the period 29th of April till 14th of May 2009.

For the validation, the following performance characteristics have been considered:

- Analytic sensitivity
- Analytic specificity
- Selectivity
- Repeatability
- Reproducibility
- Robustness

The results of the real-time PCR assay were interpreted qualitatively, because the test is developed for the identification of *Cmm* in pure colony material.

2 Analytic sensitivity

2.1 Introduction

Analytic sensitivity is defined as the range within the analysis, which can be applied with certain reliability. The limit of detection represents the smallest amount of substance in a sample that can be measured accurately by an assay.

2.2 Material and methods

The limit of detection is determined for a *Cmm* isolate of Groen Agro Control. The reason for using this isolate, instead of the reference isolate (NCPPB 382), was the large differences in Ct-values between the assays based on the IGS and Pat-1 loci for the reference isolate (derived from Plant Protection Service, Wageningen). This can be due to frequent subculturing of the isolate, which can cause the loss of plasmids (containing Pat-1). The analytic sensitivity was determined by using three dilution series of *Cmm*. The dilutions were prepared separately by homogenizing colony material from plate (D2ANX; 6) in saline and diluting these samples further (10x) with a minimum of 8 dilutions for each series. Around the limit of detection the dilution was performed with smaller steps (2x) to determine the exact limit of detection. The DNA of the different dilutions was extracted according the standard method of Groen Agro Control (appendix 1).

The total number of colony forming units (CFU's) in the dilution used for the DNA extraction was determined by counting the colonies on plate (D2ANX (6), 7 days by 28℃) after plating 100 µl of each stock dilution. The same amount was also used for the DNA-extraction. The real-time PCR assay was performed according to the standard protocol of Groen Agro Control (appendix 2) with the two primer sets and probes. The limit of detection is determined by the average of the detection limit of the three series plus 3 times the standard deviation (guideline of NRL).

2.3 Results

Table 1 shows the limit of detection for the *Cmm* isolate of Groen Agro Control. The limit of detection when using both primer-probe combinations is $7 * 10^4$ CFU (total amount of CFU in the extraction). The limit of detection, when using only the IGS primer set, is also $7 * 10^4$ CFU. When using only the Pat-1 primer set the detection limit is $9 * 10^3$ CFU.
Only 10% of the extracted DNA is used in the real-time PCR assay (appendix 1 and 2). This results in the detection limit of $7 * 10^3$ CFU when using either both primer-probe sets or only the IGS primer-probe set, and in $9 * 10^2$ CFU when only the Pat-1 primer set is used.

Table 1: Limit of detection of Cmm isolate

Dilution	Serie 1		Serie 2		Serie 3		Number of CFU		
	IGS	Pat-1	IGS	Pat-1	IGS	Pat-1	Series 1	Series 2	Series 3
1	27.8 (+)	27.7 (+)	27.6 (+)	27.2 (+)	28.0 (+)	28.3 (+)	$4,2 * 10^6$	$4,2 * 10^6$	$5,1 * 10^6$
2	30.3 (+)	30.7 (+)	31.2 (+)	31.0 (+)	31.4 (+)	31.9 (+)	$4,2 * 10^5$	$4,2 * 10^5$	$5,1 * 10^5$
3	33.8 (+)	35.4 (+)	33.0 (+)	31.9 (+)	33.5 (+)	36.4 (+)	$4,2 * 10^4$	$4,2 * 10^4$	$5,1 * 10^4$
4	36.8 (-)	35.9 (+)	35.3 (+)	34.1 (+)	34.7 (+)	36.3 (+)	$2,1 * 10^4$	$2,1 * 10^4$	$2,6 * 10^4$
5	35.6 (-)	38.1 (-)	36.6 (-)	34.7 (+)	34.1 (+)	35.0 (+)	10^4	10^4	$1,3 * 10^4$
6	36.1 (-)	0 (-)	36.9 (-)	37.2 (+)	36.3 (-)	35.6 (+)	$5,2 * 10^3$	$5,2 * 10^3$	$6,4 * 10^3$
7	37.7 (-)	36.3 (+)	37.5 (-)	37.4 (+)	37.9 (-)	35.0 (+)	$2,6 * 10^3$	$2,6 * 10^3$	$3,2 * 10^3$
8	37.6 (-)	0 (-)	39.4 (-)	38.3 (-)	37.8 (-)	0 (-)	$1,3 * 10^3$	$1,3 * 10^3$	$1,6 * 10^3$
Reference GAC	+	+							

2.4 Discussion

The limit of detection is relatively high. This is probably due to the DNA-extraction method (DNAzol) used in this validation. Groen Agro Control uses this extraction method as a quick method to isolate DNA from pure cultures. This extraction method was chosen, because this validation is intended for the identification of *Cmm* from pure colony material. Another reason for a relative high limit of detection is that the results of the PCR are defined to be positive when the related Ct-values are below a certain level (see conclusion). Low concentrations of *Cmm* are still measurable with both primer sets, but are scored negative because of the high Ct-values.

3 Analytic specificity

3.1 Introduction

Analytical specificity is defined as the ability of a detection method to distinguish the pathogen from other organisms (whether or not related) and the extent to which the analysis can detect variants of the organism. To determine the specificity of a detection method, representative isolates must be tested. Furthermore, cross-reactions with related organism (within the same host plant) must be excluded.

3.2 Material and methods

Several different isolates were tested for the specificity of the 2 primer-probe sets;

- 20 different *Cmm* isolates (derived from Naktuinbouw), which are all positive in a pathogenicity assay on tomato.
- 12 different *Cmm* look-alikes (derived from Syngenta), which are all negative in a pathogenicity assay on tomato.
- 4 different *Cm* isolates from different host plants: *CMI* (*Clavibacter michiganensis* subsp. *insidiosus*), *CMT* (*Clavibacter michiganensis* subsp. *tessellarius*), *CMN* (*Clavibacter michiganensis* subsp. *nebraskense*) and *CMS* (*Clavibacter michiganensis* subsp. *sepedonicus*).
- 9 other pathogens from tomato different from *Cmm*

The primers and probes were also compared for possible homologous with related or unrelated sequences using BLAST.

3.3 Results

Table 2 shows the results for both primer-probe combinations for all the different isolates included in the present validation study.

Table 2: Results acquired from the specificity test for the identification of Cmm

Strain number	Strain designation	Origin	Source	Pathoge-nicity assay	IGS primers	Pat-1 primers
NAK Cmm 10	ALV4588	Washington	A. Alvarez	+	18.7	15.8
NAK Cmm 11	ALV2057	Idaho	A. Alvarez	+	22.6	23.6
NAK Cmm 14	ALV4000	Ohio	A. Alvarez	+	22.5	21.2
NAK Cmm 15	ALV3994	Iowa	A. Alvarez	+	21.7	22.5
NAK Cmm 19	ALV4759	Chili	A. Alvarez	+	18.6	23.0
NAK Cmm 20	ALV4690	Portugal	A. Alvarez	+	16.8	21.6
NAK Cmm 21	ALV4681	Portugal	A. Alvarez	+	17.1	21.7
NAK Cmm 23	ALV4763	Marocco	A. Alvarez	+	23.7	17.0
NAK Cmm 24	ALV4768	Marocco	A. Alvarez	+	18.5	16.0
NAK Cmm 25	ALV4750	South Africa	A. Alvarez	+	21.4	23.1

NAK Cmm 56	ALV2645	California	A. Alvarez	+	17.8	15.7
NAK Cmm 59	ALV2700	North Carolina	A. Alvarez	+	20.5	21.6
NAK Cmm 60	ALV2701	North Carolina	A. Alvarez	+	20.4	21.8
NAK Cmm 62	ALV4004	Ohio	A. Alvarez	+	18.0	15.4
NAK Cmm 86	ALV4874	California	A. Alvarez	+	17.9	23.1
NAK Cmm 87	ALV4877	Caliornia	A. Alvarez	+	17.7	21.5
NAK Cmm 96	ZUM3059	ALV4000 rif resistant	Syngenta	+	21.4	16.0
NAK Cmm 277	NCPPB 382	UK	A. Alvarez	+	21.3	17.3
NAK Cmm 280	ZTO525		A. Alvarez	+	19.6	19.1
NAK Cmm 281	ZTO 520		A. Alvarez	+	29.9	21.6
CMI 1	LMG3663			–	0	0
CMT 1	LMG7294			–	0	0
CMN 1	LMG3700			–	0	0
CMS 1	LMG2889			–	0	0
Cm 4	IPO 543			–	0	0
Cm 82	ALV4681		A. Alvarez	–	0	0
Cm 101	?			–	0	0
Cm 102	?			–	0	0
Cm 218	?			–	0	0
Cm 219	?			–	0	0
Cm 220	?			–	0	0
Cm 61	ALV2974		A. Alvarez	–	0	0
Cm 106	?			–	0	0
Syn Cm 1	Nvt		Syngenta	–	0	39.7
Syn Cm 2	Nvt		Syngenta	–	35.5	0
Syn Cm 3	Nvt		Syngenta	–	0	0
Syn Cm 4	Nvt		Syngenta	–	0	0
Syn Cm 5	Nvt		Syngenta	–	0	0
Syn Cm 6	Nvt		Syngenta	–	0	0
Syn Cm 7	Nvt		Syngenta	–	0	0
Syn Cm 8	Nvt		Syngenta	–	0	0
Syn Cm 9	Nvt		Syngenta	–	38.5	0
Syn Cm 10	Nvt		Syngenta	–	37.4	0
Syn Cm 11	Nvt		Syngenta	–	0	0
Syn Cm 12	Nvt		Syngenta	–	0	0
Phytophthora infestans	CBS43090	Netherlands			0	0
Pythium ultimum	PPO strain (2004)	Netherlands			0	0
Pythium aphanidermatum	CBS116664	unknown			0	0
Fusarium oxysporum subsp. *lycopersici*	MUCL39788	Belgium			0	0
Erwinia carotovora spp. *carotovora*	MDI050125113	Netherlands			0	0
Verticillium albo-atrum	Isolate GAC/ MDI040621043	Netherlands			0	0
Pseudomonas syringae tomato	LMG5508	Belgium			0	0
Erwinia cypripedii	LMG1268	unknown			0	0
Agrobacterium tumefaciens	Isolate GAC/ MDI050527576	Netherlands			0	0

The real-time PCR assay described here showed very good results on the specificity test. No homologous sequences were found for both primer-probe combinations with related or unrelated species.

3.4 Discussion

Both primer-probe combinations were positive for all the *Cmm* isolates and negative for all the *Cm* look-a-likes and other pathogens than C*mm* when using the criteria that IGS is positive with a Ct-value below 35.5 and Pat-1 with a Ct-value below 37.5. These values were obtained (by the lowest Ct-values) from the isolates showing a false positive result in this validation work.

4 Selectivity

4.1 Introduction

The selectivity of a method refers to the extent to which it can determine the pathogen in a complex mixture without interference from other components in the mixture (matrix effects).

4.2 Discussion

Selectivity is not applicable in this validation, because it deals with identification of *Cmm* from pure colony material. For this reason it is not relevant to investigate the influence of plant material on the results.

5 Repeatability

5.1 Introduction

Repeatability is defined as the variation in measurements taken by a single person or instrument on the same item and under the same conditions. A measurement is due to be repeatable when this variation is smaller than a certain agreed limit.

5.2 Material and methods

The same person extracted DNA from colony material of the reference strain NCPPB 382 8-times in duplicate in one day and performed the real-time PCR analysis.

5.3 Results

Table 3 shows the results for repeatability.

Table 3: Results for repeatability acquired with reference strain NCPPB 382.

Number of repetition	Duplicate			
	1		2	
	IGS	Pat-1	IGS	Pat-1
1	21.3	27.3	20.3	26.8
2	21.7	28.6	20.2	27.3
3	20.6	27.3	20.1	26.9
4	20.8	27.5	20.3	27.5
5	21.5	29.1	20.5	27.9
6	20.2	27.1	21.1	28.3
7	20.8	28.8	20.8	27.8
8	20.4	27.6	20.5	27.6
Reference GAC	26.7	25.5		

5.4 Discussion

Repeatability was found to be optimal. All results were positive for *Cmm.* The test can be repeated and shows always the same results.

6 Reproducibility

6.1 Introduction

Reproducibility is defined as the ability of a method to be accurately reproduced or replicated by different people working independently at different days.

6.2 Material and methods

Different persons extracted DNA from colony material of the reference strain NCPPB 382 on different days and performed a real-time PCR. The samples were tested in duplicate.

6.3 Results

Table 4 shows the results for reproducibility.

Table 4: Results for reproducibility acquired with reference strain NCPPB 382..

Date	Number of repetition	Person	Duplicate					
			1		2		Reference GAC	
			IGS	Pat-1	IGS	Pat-1	IGS	Pat-1
010509	1(repeatability)	A	20.5	26.4	21	27.2	25.0	24.0
040509	2	B	20.4	27.8	20.6	27.4	26.0	25.4
050509	3	C	20.3	25.7	21	27	25.1	24.5
060509	4	A	21.3	29.1	20.9	27.1	24.6	23.6
070509	5	B	21.4	27.8	21.4	27.8	25.7	25.0
080509	6	C	22.9	28.9	22.5	28.9	24.9	24.7
110509	7	A	20.9	27.2	21.0	27.2	24.6	24.0
130509	8	B	20.5	26.4	21	27.2	25.0	24.0

6.4 Discussion

Repeatability was found to be optimal. All results were positive for *Cmm.* Different people are able to reproduce the test at different days.

7 Robustness

7.1 Introduction

Robustness measures how insensitive a method is for deviations in performance, conditions and the quality of material. Robustness can be determined by varying the most important parameters that may affect the results of the test.

7.2 Material and methods

The tests were performed with DNA acquired from repetition 1 of repeatability and reproducibility (reference strain NCPPB 382) and with DNA acquired from a *Cmm* reference isolate of Groen Agro Control.

The following tests were performed:

- Variation in template amount (50%. 75%. 100%. 125% en 150%)
- Variation in primer- and probe concentration (50%. 75%. 100%. 125% en 150%)
- Variation in annealing temperature (57˚C. 59˚C. 60˚C. 61˚C en 63˚C)
- Variation in PCR apparatus (thermocyclers ABI 7000 versus ABI 7300)

7.3 Results

The results for robustness are represented in table 5.

Table 5: Results for robustness acquired with strain NCPPB 382 and the reference of Groen Agro Control.

Robustness	Sample	IGS primers	Pat-1 primers
50% template	1		27.7
75% template	1	20.6	27.7
100% template	1	20.2	27.8
125% template	1	20.0	28.3
150% template	1	19.8	28.6
50% primer/probe	1	21.2	30
75% primer/probe	1	20.8	28.6
100% primer/probe	1	20.2	27.8
125% primer/probe	1	20.4	28.2
150% primer/probe	1	20.1	27.7
57˚C annealing	1	19.1	22.9
59˚C annealing	1	19.3	24.6
60˚C annealing	1	19.9	30.6
61˚C annealing	1	20.5	Not detectable
63˚C annealing	1	25.1	24.5
ABI 7000	GAC reference	25.0	24.0
ABI 7300	GAC reference	21.0	27.7

7.4 Discussion

Reduction of 50% in the primer and probe concentration and annealing temperatures from 61˚C influenced the Ct-values negatively (higher Ct-value). The results are mostly positive, but the Ct-values are higher compared to the normal situation. An annealing temperature of 63˚C showed a negative result for *Cmm* when using the Pat-1 primers and probe. Excessive variations in primer/probe concentrations, template and annealling temperature should therefore be avoided.

8 Discussion

At the time this validation was performed, all *Cmm* isolates gave a positive result for both primer-probe combinations. However, when the validation work of the assay described here was completed, three newly isolated Cmm isolates from Dutch greenhouses, scored negatively for the Pat-1 primer-probe combination, but still positive for the IGS assay.
An explanation for this result can be a mutation in the plasmid of the three isolates, whereby the Pat-1 primers are not able to anneal anymore to the plasmid. Other Pat-1 primers (4) confirmed this hypothesis, because they showed a positive result in a conventional PCR. At this moment new primer-probe combinations for Pat-1 are in development to pick up all *Cmm* isolates.
To avoid false negatives with the Pat-1 assay, it is recommended at this moment to use only the IGS assay for the identification of *Cmm*. The current Pat-1 assay can be used to confirm the identification of *Cmm,* when for example the IGS gives Ct-values between 30 and 40, but is not yet conclusive.

The limit of detection ($7*10^4$ cfu) is relatively high, but is probably lower when using a more sensitive DNA extraction method. It's important to keep the annealing temperature between 57˚C and 61˚C. Low detection signals were sometimes present for *Cm* look-alikes. The Ct-values for these isolates were always above a certain Ct-value (35.5) and one of the two primer and probe sets (either IGS or Pat-1) was always negative. Therefore the criterion to confirm the presence of DNA from *Cmm*, the real-time PCR assay should show Ct-values below 35.5 for the IGS primer-probe combination and below 37.5 for the Pat-1 primer-probe combination.

9 Conclusions

- The results of this validation indicate that the real-time PCR method with the IGS primer-probe combination can be used as an identification tool of *Cmm* in pure colony material.
- It is recommended to use the Pat-1 primer-probe combination only to confirm the results and not as a conclusive identification.
- The recommended criterion to confirm the presence of DNA from *Cmm* using the real-time PCR assay should be Ct-values below 35.5 for the IGS primer-probe combination and below 37.5 for the Pat-1 primer-probe combination.

10 Literature references

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Appendix 1: Protocol DNAzol extraction

The DNA extractions in this validation are all performed with “Plant DNAzol Reagent” (Invitrogen. 10978-021). DNAzol is a complete. ready to use reagent for the isolation of genomic DNA. The DNAzol procedure is based on the use of guanidine-detergent lysing solution which hydrolyzes RNA and allows the selective precipitation of DNA from the lysate. The extractions are performed according the protocol of Invotrogen for DNAzol:

1. Add 400 µl sterile demi water to a 2 ml tube
2. Homogonize some colony material in the water
3. Add 0.5 ml DNAzol
4. Shake genlty for 10 minutes
5. Add 0.5 ml chloroform. invert gently to mix and incubate for 10 minutes
6. Spin for 10 minutes at 12.000 rpm
7. Transfer the aqueous phase to a fresh 2 ml tube
8. Add 0.8 ml 100% EtOH (freeze)
9. Incubate for 6 minutes
10. Spin for 6 minutes at 11.000 rpm
11. Remove the supernatant
12. Add 0.5 ml 70% EtOH (refrigerator)
13. Shake gently and spin for 10 minutes at 10.000 rpm
14. Remove the supernatant
15. Dry pellet for 10 minutes
16. Add 20 µl PCR water and dissolve DNA

Appendix 2: Protocol real-time PCR

The standard real-time PCR mix used by Groen Agro Control is Sigma Jumpstart Taq Readymix for quantitative PCR (D7440-400RXN). The standard real-time PCR devices used by Groen Agro Control are thermocyclers ABI 7000 and ABI 7300

The following chemicals are applied In one reaction of the real-time PCR:

- 12.5 µl Sigma Jumpstart Taq Readymix for Quantitative PCR
- 4.25 µl PCR-water
- 1 µl 25 mM $MgCl_2$
- 0.25 µl intern standard (reverence dye R4526. Sigma)
- 0.75 µl 10 pmol/µl F-primer IGS
- 0.75 µl 10 pmol/µl R-primer IGS
- 0.75 µl 10 pmol/µl F-primer Pat-1
- 0.75 µl 10 pmol/µl R-primer Pat-1
- 1 µl 5 pmol/µl probe IGS (VIC)
- 1 µl 5 pmol/µl probe Pat-1 (FAM)
- 2 µl DNA-template

The standard PCR program of Groen Agro Control consists:
1 cycle of 2 minutes at 95ºC (initialisation) followed by 40 cycli of 15 seconds at 95ºC (denaturation) and 1 minute at 60ºC (annealling and elongation).

The validated primer-probe-combinations are:

IGS
Forward: 5'-TGTCGAGGGCATGTTGCACG-3'
Reverse: 5'-GTTTCGCCTCCCCGAAG-3'
Probe: 5'-FAM-TCGTCCTGTTGTGGATG-MGB-3'

Pat-1
Forward: 5'-CTCTCGATTGTCCGGATC-3'
Reverse: 5'-CTCGTAGTCATTGACGAGAG -3'
Probe: 5'-VIC-AGTTGTTATCCGACTTCG-MGB-3'

The real-time PCR analyses are performed in duplex.

The result of an analyses is considerd positive when the Ct-value for IGS < 35.5 and the Ct-value for Pat-1 < 37.5