NATIONAL INSTITUTE OF BIOLOGY

FINAL REPORT ON THE 'NIB Proficiency Test Round 2016-02: PROFICIENCY TEST FOR MOLECULAR DETECTION OF *Xylella fastidiosa*

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1 Background

Proficiency test is a way in which competence of laboratories is assessed and demonstrated. In proficiency testing standardized samples are prepared with known status regarding the presence of harmful organisms. These are sent out to participating laboratories that analyse them using their own methods, equipment and reagents and send results back to the organizer. Organizer analyses the results and provides a report detailing all participants' results in confidential manner together with actual sample status.

2 Organisation of the proficiency test

This proficiency test covered molecular detection of *Xylella fastidiosa*, providing each participant with five (5) samples. The test was organised following guidelines developed by the European and Mediterranean Plant Protection Organisation (PM 7/122 (1)).

In total, 29 laboratories participated in the proficiency test. The laboratory codes assigned to each participant are confidential.

The materials were sent out on October 11th and October 17th 2016. The deadline for the submission of the results was December 2nd 2016.

3 Study Materials

Bacterial strains

The *Xylella fastidiosa* CoDiRO isolate was maintained on BCYE agar plates at 28 °C and exhibited typical growth for *X. fastidiosa*. The isolate was confirmed as *X. fastidiosa* using real-time PCR analysis (Francis *et al*., 2006; Schaad *et al*., 2002).

DNA extraction

The *Xylella fastidiosa* CoDiRO isolate was grown on several plates of BCYE until good growth and the DNA isolated from colonies scraped from the agar and suspended in sterile phosphate buffer saline (pH = 7,2) to form milky-white suspension. Bacterial cells were concentrated with centrifugation (10.000 g, 130 seconds) from 5 tubes of 1800 µL of bacterial suspension, and pellets washed once with 1 mL of PBS each. DNA was extracted from pellets using UltraClean® Microbial DNA Isolation Kit (MoBio 12224-50, protocol version 08102016). The final elution of DNA was done in 50 µL buffer for each tube and the eluted DNA from all five tubes combined giving app. 250 µL of extracted DNA in total.

The DNA dilutions were prepared in Tris-EDTA (TE) buffer with salmon sperm DNA (25 ng/µL; UltraPure™ Salmon Sperm DNA Solution, Invitrogen 15632-011) in DNA LoBind Tubes (Eppendorf 022431021) and stored at < -15 °C unless otherwise specified. Real-time PCR and

digital PCR were used to test the quality (amplifyability) of the DNA, and digital PCR was used to determine the concentration of the target copy numbers.

Preparation of samples

Five (5) samples were prepared for each participant of the proficiency test. The target concentrations in samples were selected in such a way to represent middle and high target concentrations, all above the theoretical and reported analytical sensitivity of various molecular methods of detection. The target concentrations were 10^6 and 10^5 target copies per mL, corresponding to equal number of cells when the target is present in single copies per genome. Samples were prepared by diluting the DNA of the target organism in Tris-EDTA (TE) buffer with salmon sperm DNA (25 ng/µL; UltraPure™ Salmon Sperm DNA Solution, Invitrogen 15632-011). All mixtures were vortexed for 30 seconds and kept at room temperature on orbital shaker (100 rpm) until aliquoting them in 100 µL volumes in DNA LoBind Tubes (Eppendorf 022431021). Samples were stored < -15 °C before further analyses and distribution. The samples did not contain plant material or plant DNA.

Real-time PCR

Real-time PCR assays were used to assess the homogeneity and stability of the samples, and included real-time PCR assays developed by Francis *et al*. (2006) and Schaad *et al*. (2002) with modifications as described below.

All of the qPCR reactions were performed in triplicate on a ViiA™ 7 Real-Time PCR System (Life Technologies) using the following universal cycling conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, and 1 min at 60 °C, and using standard temperature ramping mode. The reaction volumes of 10 µL contained, as final concentrations, 900 nM primers, 200 nM FAM/BHQ1 probe (Integrated DNA Technologies), 1×TaqMan Universal PCR Master Mix (Life Technologies), and 2 µL sample DNA. The qPCR data were analysed using the Viia7™ Software v. 1.2.4 (Life Technologies) with automatic baseline and a manually selected threshold of 0,1 for all assays. Positive and no template controls were used in each run. The qPCR data are given as minimum and maximum Cq values (i.e. qPCR quantification cycle) and as the average Cq values together with the associated coefficient of variation (CV) value. Validation data on the assays with the described modifications is available at the EPPO Database on Diagnostic Expertise (http://dc.eppo.int/; Dreo *et al*., 2016).

Assigning reference values to samples with digital PCR (dPCR)

The *X. fastidiosa* assay developed by Francis *et al*. (2006) was directly transferred to digital PCR probe with HEX as a reporter dye and Internal ZEN™ Quencher (Integrated DNA Technologies). The digital PCR was used to determine the absolute concentration of the target

copies in the isolated bacterial DNA using the QX100TM Droplet Digital™ PCR system (Bio-Rad) as described previously (Dreo *et al*., 2014). The sample volume analysed in each reaction was 8 µL. The software package provided with the dPCR system (QuantaSoft™ Software, verison 17.4.0917, Bio-Rad) was used for data acquisition. A minimum of 10.000 accepted droplets per reaction was required for the reaction to be considered valid. A fixed manual global threshold discriminating between negative and positive droplets was set at 2.200 relative fluorescence units. A reaction was interpreted as positive if the number of positive droplets was ≥2. Positive and no template controls were used in each run. The data from the dPCR are given in target copies/µL reaction and as log (copies/mL of DNA). For real-time PCR by Francis *et al*. (2006) these are expected to correspond to the concentration of cells.

The assigned values (target concentrations) for samples were determined as an average concentration of ten sample aliquots per each concentration level, each in triplicates. Samples contained an average target concentration of 761,1 and 40,5 target copies/µL at concentration levels E6 and E5, respectively (Table 1).

Table 1: Concentration levels of samples as determined with digital PCR using primers and probes designed by Francis *et al*. (2006). Cps = target DNA copies, CV = coefficient of variation, NA = not applicable.

Conc. level	Sample ID	cps/µL DNA		log(cps/mL DNA)	
		Min - Max	Average ± CV	Min - Max	Average ± CV
E6	Xyf 3	715 - 815	761,1 ± 0,032	5,85 - 5,91	5,881 ± 0,00245
E5	Xyf 1, Xyf 4	35 - 44,8	40,5 ± 0,06	4,54 - 4,65	4,607 ± 0,00582
neg	Xyf 2, Xyf 5	neg	NA	neg	NA

Homogeneity and stability testing

Homogeneity and stability of the samples were tested with real-time PCR assays (Francis *et al*., 2006; Schaad *et al*., 2002) analysing 2 µL of DNA in each reaction.

For homogeneity testing ten randomly selected aliquots of samples prepared for proficiency test were selected for each concentration level, and tested in three technical repeats (wells) each. The results were in concordance with the true values (target copies determined with dPCR) in both assays (Francis *et al*., 2006; Schaad *et al*., 2002), for all samples and concentration levels with coefficients of variations below 1 % in all cases (Table 2). As expected because of the differences in the number of the target copies per cells in these two assays, the Cq values obtained with Francis *et al*. (2006) were considerably higher than the ones obtained with Schaad *et al*. (2002).

Table 2: Real-time PCR results of homogeneity testing of samples. Homogeneity testing was done with real-time PCR tests developed by Francis *et al*. (2006) and Schaad *et al*. (2002). Ten aliquots per concentration level were tested in three technical repeats (wells) each. Cq = cycle of threshold, CV = coefficient of variation, NA = not applicable.

Concentration level	Sample ID	Min(Cq) - Max(Cq)	Average(Cq) ± CV
Francis et al., 2006			
E6	Xyf 3	29,5 - 30,1	29,75 ± 0,005
E5	Xyf 1, Xyf 4	33,9 - 34,9	34,43 ± 0,007
neg	Xyf 2, Xyf 5	neg (45)	NA
Schaad et al., 2002			
E6	Xyf 3	24,8 - 25,3	25,03 ± 0,005
E5	Xyf 1, Xyf 4	29,1 - 29,9	29,51 ± 0,006
neg	Xyf 2, Xyf 5	neg (45)	NA

Short term and long term stability of the samples were tested. Short-term stability was tested after mimicking conditions during transport by incubating three randomly selected aliquots of samples of each concentration level for one week at different temperatures (< -15°C, 2-8 °C, and 25 °C) in the dark. Long-term stability was tested on aliquots stored < -15 °C for 3, 6 and 9 weeks, the latter corresponding to the deadline for reporting results. Samples were stable in all cases (Tables 3 and 4).

Table 3: Results of short-term stability testing of aliquots stored at different temperatures for 1 week. Three aliquots per concentration level were tested in three technical repeats (wells) in real-time PCR after one week of incubation at temperature below -15 °C, 2-8 °C and 25 °C. Cq = cycle of threshold, CV = coefficient of variation, NA = not applicable.

Concentration level	Sample ID	T < -15 °C		2 - 8 °C		25 °C	
		Min(Cq) - Max(Cq)	Average(Cq) ± CV	Min(Cq) - Max(Cq)	Average(Cq) ± CV	Min(Cq) - Max(Cq)	Average(Cq) ± CV
Francis et al. (2006)							
E6	Xyf 3	29,5 - 29,9	29,62 ± 0,006	29,4 - 30,0	29,37 ± 0,006	29,9 - 29,5	29,65 ± 0,004
E5	Xyf 1, Xyf 4	34,3 - 34,7	34,46 ± 0,005	33,9 - 34,9	34,51 ± 0,009	34,7 - 34,0	34,33 ± 0,006
neg	Xyf 2, Xyf 5	neg (45)	NA	neg (45)	NA	neg (45)	NA
Schaad et al. (2002)							
E6	Xyf 3	24,9 - 25,1	24,99 ± 0,004	24,8 - 25,2	24,97 ± 0,004	25,3 - 24,9	25,08 ± 0,004
E5	Xyf 1, Xyf 4	29,3 - 29,6	29,51 ± 0,004	29,3 - 29,9	29,78 ± 0,006	29,7 - 29,2	29,46 ± 0,005
neg	Xyf 2, Xyf 5	neg (45)	NA	neg (45)	NA	neg (45)	NA

Table 4: Results of long-term stability testing of sample aliquots stored at temperature of < -15 °C. Three aliquots per concentration level were tested in three technical repeats (wells) in real-time PCR after 1, 3, 6 and 9 weeks. Cq = cycle of threshold, CV = coefficient of variation, NA = not applicable.

Concentration level	Sample ID	Week 1		Week 3		Week 6		Week 9	
		Min(Cq) - Max(Cq)	Average(Cq) ± CV	Min(Cq) - Max(Cq)	Average(Cq) ± CV	Min(Cq) - Max(Cq)	Average(Cq) ± CV	Min(Cq) - Max(Cq)	Average(Cq) ± CV
Francis et al. (2006)									
E6	Xyf 3	29,5 - 29,9	29,62 ± 0,006	30,2 - 30,5	30,41 ± 0,003	29,7 - 29,9	29,81 ± 0,002	29,6 - 30,0	29,80 ± 0,004
E5	Xyf 1, Xyf 4	34,3 - 34,7	34,46 ± 0,005	35,0 - 35,5	35,30 ± 0,005	34,4 - 35,0	34,66 ± 0,007	34,3 - 34,9	34,63 ± 0,006
neg	Xyf 2, Xyf 5	neg (45)	NA	neg (45)	NA	neg (45)	NA	neg (45)	NA
Schaad et al. (2002)									
E6	Xyf 3	24,9 - 25,1	24,99 ± 0,004	25,0 - 25,3	25,13 ± 0,003	25,0 - 25,3	25,12 ± 0,004	24,9 - 25,2	25,10 ± 0,004
E5	Xyf 1, Xyf 4	29,3 - 29,6	29,51 ± 0,004	29,2 - 30,8	29,67 ± 0,015	29,3 - 29,8	29,52 ± 0,005	29,4 - 29,7	29,53 ± 0,004
neg	Xyf 2, Xyf 5	neg (45)	NA	neg (45)	NA	neg (45)	NA	neg (45)	NA

4 Reported results

Results, as reported, are summarized in Table 5.

Table 5: Proficiency test results as reported by the participants for *X. fastidiosa* testing. No non-conforming results were reported.

Sample ID / Status		Xyf 1 pos (E5)	Xyf 2 neg	Xyf 3 pos (E6)	Xyf 4 pos (E5)	Xyf 5 neg	Number of conforming results
Laboratory ID and Reported Results	3	pos	neg	pos	pos	neg	5/5
	4	pos	neg	pos	pos	neg	5/5
	6	pos	neg	pos	pos	neg	5/5
	7	pos	neg	pos	pos	neg	5/5
	8	pos	neg	pos	pos	neg	5/5
	9	pos	neg	pos	pos	neg	5/5
	10	pos	neg	pos	pos	neg	5/5
	11[a]	pos	neg	pos	pos	neg	5/5
	12	pos	neg	pos	pos	neg	5/5
	13[b]	pos	neg	pos	pos	neg	5/5
	14	pos	neg	pos	pos	neg	5/5
	15	pos	neg	pos	pos	neg	5/5
	17	pos	neg	pos	pos	neg	5/5
	18	pos	neg	pos	pos	neg	5/5
	20	pos	neg	pos	pos	neg	5/5
	22	pos	neg	pos	pos	neg	5/5
	23	pos	neg	pos	pos	neg	5/5
	24	pos	neg	pos	pos	neg	5/5
	25	pos	neg	pos	pos	neg	5/5
	26	pos	neg	pos	pos	neg	5/5
	27	pos	neg	pos	pos	neg	5/5
	28	pos	neg	pos	pos	neg	5/5
	29	pos	neg	pos	pos	neg	5/5
	30	pos	neg	pos	pos	neg	5/5
	32	pos	neg	pos	pos	neg	5/5
	34	pos	neg	pos	pos	neg	5/5
	35	pos	neg	pos	pos	neg	5/5
	36	pos	neg	pos	pos	neg	5/5
	37	pos	neg	pos	pos	neg	5/5
Percent of conforming results[c]		**100,0%**	**100,0%**	**100,0%**	**100,0%**	**100,0%**	**100,0%**

[a]Results were reported on January 10th 2017, after the deadline for the submission of the results.

[b]Results were reported on December 7th 2016, after the deadline for the submission of the results.

[c]Results reported after the deadline were not taken into account.

5 Evaluation of the results

In total, 29 laboratories participated in the proficiency test for *Xylella fastidiosa*. Of these, 27 laboratories (93 %) reported results within deadline for the submission of results. Two laboratories (7 %, laboratories 11 and 13) reported results after the deadline.

All results of all participants were in concordance with the true qualitative values.

6 Methods used

Among the methods used for reporting the overall results most commonly used were real-time PCR developed by Harper *et al*. (2010a, 2010b) and PCR developed by Minsavage *et al*. (1994) (Table 6).

Table 6: Methods used for reporting results on *X. fastidiosa* in this proficiency test

Method	Reference	Times used
Real-time PCR[a]	Harper *et al*., 2010	13
	Harper *et al*., 2010 err. 2013	7
PCR	Minsavage *et al*., 1994	15
Real-time PCR[b]	Francis *et al*., 2006	9
Real-time PCR[c]	Schaad *et al*., 2002	2
LAMP[d]	Harper *et al*., 2010	2
PCR product sequencing[e]	NA	2
PCR	Firrao and Bazzi, 1994	1
PCR	Rodrigues *et al*., 2013	1
MLST	Yuan *et al*., 2010	1

[a]It is not clear whether laboratories indeed use probe as reported in Harper *et al*., 2010, or the one reported in the erratum (Harper *et al*., 2010b) which corrected the probe sequence to TCGCATCCCGTGGCTCAGTCC, and have simply omitted the reference to the erratum.

[b]Both intercaleting dyes and probes were used, some laboratories did not specify which. The number of times used includes one laboratory reporting on using Francis *et al*. (1994) which could not be identified.

[c]Also includes one laboratory reporting on using Schaad *et al*. (2006) which could not be identified.

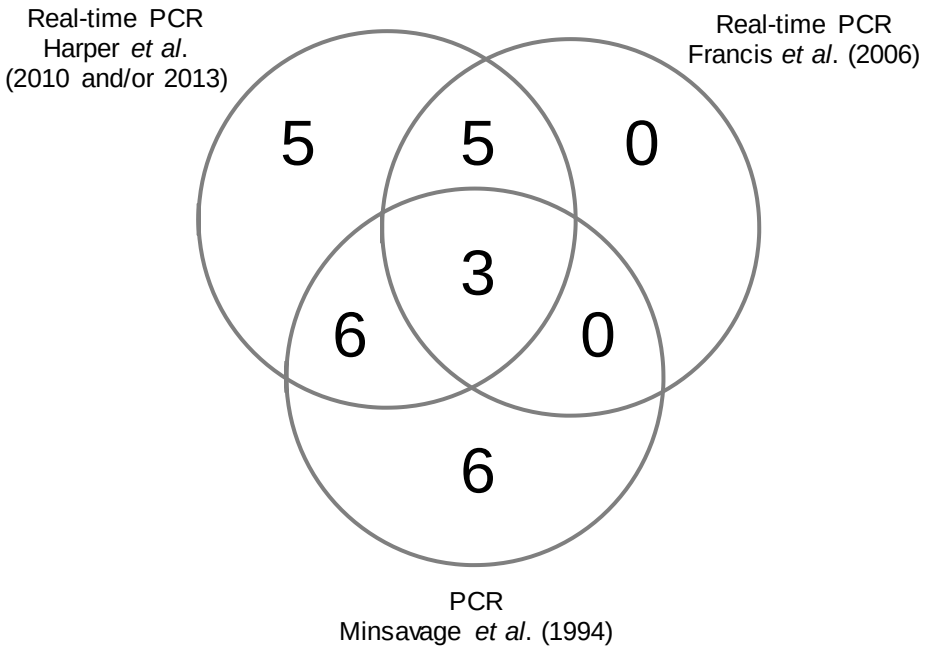
[d]LAMP was reported to be used by two laboratories however, the results of LAMP were not reported.

[e]Sequencing of PCR products was reported by two laboratories. Sequencing was used to confirm PCR products of Firrao and Bazzi (1994) and Minsavage *et al*. (1994).

Many laboratories reported using a combination of methods. Of the 29 participating laboratories, 2 (7 %), 3 (10 %) and 12 (41 %) laboratories reported using 4, 3 or 2 methods, respectively. Most frequently used combinations of methods are shown in Figure 1. Common combinations include real-time PCR by Harper *et al*. (2010a/2010b) with classical PCR

(Minsavage *et al*., 1994), and combination of real-time PCR assays by Harper *et al*. (2010a/2010b) with Francis *et al*. (2006).

Figure 1: Most frequent combinations of methods as reported by the participants. Note, that not all data is included e.g. number of times these tests were used in combination with methods not shown here.



Twelve laboratories (55 %) relied on single method. Of these, real-time PCR tests developed by Harper *et al*. (2010a, 2010b) was used by five (5) laboratories, the PCR by Minsavage et al. (1994) by six (6) laboratories and the one by Schaad *et al*. (presumably 2002, reported as 2006) by one (1) laboratory).

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