NATIONAL INSTITUTE OF BIOLOGY

FINAL REPORT ON THE 'NIB Proficiency Test 2015-01': PROFICIENCY TEST FOR MOLECULAR DETECTION OF *RALSTONIA SOLANACEARUM* AND *ERWINIA AMYLOVORA*

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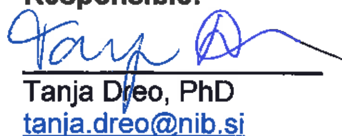

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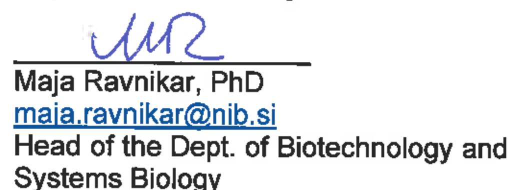

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Version 2.0

15 April 2016

How to cite:

Dreo, Tanja, 2015. Final Report on the “NIB Proficiency Test 2015-01”: Proficiency Test for Molecular Detection of *Ralstonia solanacearum* and *Erwinia amylovora* (No. 2015/001, V2.0), Proficiency Test Reports. National Institute of Biology, Ljubljana.

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 analyze them using their own methods, equipment and reagents and send results back to the organizer. Organizer analyzes 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 *Ralstonia solanacearum* and/or *Erwinia amylovora*. For each organism 5 samples (DNA) were supplied as well as one positive and one negative control. Material did not contain living bacteria and did not pose any danger to humans, animals, plants or environment. The test was organized following guidelines developed by the European and Mediterranean Plant Protection Organisation (EPPO, 2014).

In total, 20 laboratories participated in the proficiency test, 18 participants for *R. solanacearum* and 17 *for E. amylovora*. The laboratory codes assigned to each participant in the scheme are confidential.

The materials were sent out on September 22nd 2015. The deadline for the submission of the results was November 20th 2015. All laboratories performed the analysis and submitted the results within the time allocated to the proficiency testing.

3 Study Materials

Bacterial strains

The *R. solanacearum* biovar 2 strain NIB Z 1645 was isolated in 2011 from potato (*Solanum tuberosum*) cv. Marabel in Slovenia and identified and confirmed as *R. solanacearum* using immunofluorescence (IACR-PS-278, Rothamsted Research and anti-rabbit secondary antibodies Sigma F6005), its morphology on semiselective SMSA (Elphinstone *et al.*, 1996) with 2500 U per litre of bacitracin (Anonymous, Commission Directive 2006/63/EC, 2006), PCR confirmed with RFLP (Pastrik *et al.*, 2002) and real-time PCR assays targeting 16S rRNA gene (Weller *et al.*, 2000). The pathogenicity of the isolate NIB Z 1645 was confirmed on tomato (*Lycopersicon esculentum*) cv. Moneymaker and phylotype determined by PCR (Opina *et al.*, 1997, Fegan and Prior, 2005).

The bacterial strain *E. amylovora* NIB Z 903 was isolated in 2007 from a symptomatic *Malus domestica* cv. Gloster in Slovenia, The isolate was identified and confirmed as *E. amylovora* using characteristics of colony morphology on King's B (King *et al.*, 1954) and

SNA (containing 8 g/L Nutrient broth (BD 234000), 8 g/L NaCl, 5% Sucrose, 15 g/L Agar (Oxoid No.1)) media, slide agglutination test (Eam-C, Plant Research International) and real-time PCR assays targeting pEA29 plasmid (Salm and Geider, 2004), amsC gene and ITS region as described by Pirc *et al.* (2009). The pathogenicity of the isolate was confirmed by immature pear fruit inoculation.

DNA extraction

The isolates *R. solanacearum* NIB Z 1645 and *E. amylovora* NIB Z 903 were each grown in four 50 mL centrifuge tubes each containing 25 mL liquid CPG (Kelman, 1954) and King’s B media (King *et al.*, 1954), respectively, at 28 °C on an orbital shaker (app. 200 rpm). Bacteria were concentrated with centrifugation (2.500 g, 15 minutes) and washed once with 25 mL of sterile saline solution. Pellets were combined and DNA extracted from them using Power Microbial® Maxi DNA Isolation Kit (MoBio 12226-25, protocol version 04042012). The final elution of DNA was done twice giving app. 2 mL of DNA for each isolate.

The DNA and their dilutions were prepared in 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.

Real-time PCR

Real-time PCR assays were used as reference method and included real-time PCR assays developed by Weller *et al.* (2000) for the detection of *R. solanacearum* biovar 2A and by Pirc *et al.* (2009) targeting amsC of *E. amylovora*, In addition, at several stages other real-time PCR assays were used as described below. The additional assays for *E. amylovora* target pEA29 plasmid (Salm and Geider, 2004) and 16S rDNA internal transcribed spacer (ITS; Pirc *et al.*, 2009), and for *R. solanacearum* the broad-range assay targets 16S rRNA gene (Weller *et al.*, 2000).

All of the qPCR reactions were performed in triplicate on an 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, using standard temperature ramping mode. The reaction volumes of 10 µL contained, as final concentrations, 900 nM primers, 200 nM FAM reporter probes (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 manual 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).

Preparation of Samples for Proficiency Testing

For each organism five samples and two controls (positive and negative) 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.

Samples were prepared by diluting the DNA of the target organism in 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 (app. 100 rpm) until aliquoting them in 100 µL volumes in DNA LoBind Tubes (Eppendorf 022431021).

Positive control samples contained app. 10^7 target copies/µL of isolated DNA. Negative samples and negative controls were prepared in the same way as described above but omitting the target DNA. The lowest concentration used i.e. approximately $5x10^4$ target copies/µL isolated DNA was represented by two samples in each target organism series (Table 1). Aliquots of DNA were stored at < -15 °C except for the selected aliquots used for stability testing which were additionally stored at temperatures 2-8 °C and in an incubator set to 25 °C.

Homogeneity Testing

For homogeneity testing ten randomly selected aliquots of samples prepared for proficiency test were selected for each organism and each concentration level including positive controls and negative samples/negative controls.

Samples were tested with real-time PCR (in triplicates, analyzing 2 µL of DNA in each reaction) and digital PCR (in triplicates, analyzing 8 µL in each reaction) using real-time by Pirc *et al*. (2009) targeting amsC of *E. amylovora*, and by Weller *et al*. (2000) for the detection of *R. solanacearum* biovar 2A.

The results were in concordance with the true values for both organisms, all samples and controls, and for all technical replicates (30 per organisms and concentration level) with CV values below 1 % in all cases (Table 1).

Stability Testing

For stability testing three randomly selected aliquots of samples prepared for proficiency test were selected for each organism and each concentration level including positive controls and negative samples/negative controls and stored at different temperatures and times before testing: (i) for 1 week at temperatures of 2-8 °C, < -15 °C and 25 °C in the dark and (ii) for 3, 6 and 9 weeks at < -15 °C. The last stability testing of aliquots stored at < -15 °C was conducted at the deadline for reporting results.

Samples were tested with real-time PCR (in triplicates, analyzing 2 µL of DNA in each reaction) and digital PCR (in triplicates, analyzing 8 µL in each reaction) using real-time by Pirc *et al*. (2009) targeting amsC of *E. amylovora*, and by Weller *et al*. (2000) for the detection of *R. solanacearum* biovar 2A.

The results were in concordance with the true values for organisms, all samples and controls, technical replicates and at all times with CV values of the determined Cq values equal or below 1 % (Tables 2 and 3).

Table 1: Real-time PCR results of homogeneity testing of samples and controls.

Concentration level	Sample Ids	Min(Cq) - Max(Cq)	Average(Cq) ± CV
R. solanacearum			
E7	Rs-3, PC-Rs	20,6 - 20,9	20,77 ± 0,005
E5	Rs-1	27,3 - 27,8	27,59 ± 0,004
>E4	Rs-2, Rs-4	28,4 - 28,9	28,66 ± 0,004
neg	Rs-5, NC-Rs	neg (45)	NA
E. amylovora			
E7	PC Ea	21,0 - 21,3	21,12 ± 0,003
E5	Ea-1	27,9 - 28,3	28,12 ± 0,004
>E4	Ea-4, Ea-5	28,8 - 29,4	29,15 ± 0,004
neg	Ea-2, Ea-3, NC-Ea	neg (45)	NA

Table 2: Results of stability testing of aliquots stored at different temperatures for 1 week.

Concentration level	Sample Ids	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
R. solanacearum							
E7	Rs-3, PC-Rs	20,6 - 20,9	20,75 ± 0,005	20,6 - 20,9	20,73 ± 0,005	20,3 - 20,8	20,57 ± 0,008
E5	Rs-1	27,0 - 28,1	27,61 ± 0,010	27,4 - 27,7	27,51 ± 0,004	27,4 - 27,6	27,51 ± 0,003
>E4	Rs-2, Rs-4	28,5 - 28,8	28,62 ± 0,003	28,4 - 28,9	28,68 ± 0,005	28,3 - 28,7	28,57 ± 0,007
neg	Rs-5, NC-Rs	neg (45)	NA	neg (45)	NA	neg (45)	NA
E. amylovora							
E7	PC Ea	21,0 - 21,5	21,11 ± 0,007	20,8 - 21,1	20,99 ± 0,004	20,7 - 21,0	20,89 ± 0,006
E5	Ea-1	28,0 - 28,5	28,18 ± 0,006	27,9 - 28,3	28,13 ± 0,004	27,9 - 28,1	28,03 ± 0,002
>E4	Ea-4, Ea-5	29,1 - 29,7	29,34 ± 0,006	29,1 - 29,3	29,22 ± 0,003	28,9 - 29,5	29,18 ± 0,006
neg	Ea-2, Ea-3, NC-Ea	neg (45)	NA	neg (45)	NA	neg (45)	NA

Table 3: Results of stability testing of aliquots stored at temperatures < -15 °C for 1, 3, 6 and 9 weeks.

Concentration level	Sample IDs	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
R. solanacearum									
E7	Rs-3, PC-Rs	20,6 - 20,9	20,75 ± 0,005	20,7 - 21,0	20,89 ± 0,004	20,7 - 21,1	20,92 ± 0,006	20,7 - 21,0	20,83 ± 0,005
E5	Rs-1	27,0 - 28,1	27,61 ± 0,010	27,4 - 27,8	27,70 ± 0,004	27,5 - 27,9	27,75 ± 0,004	27,5 - 27,9	27,71 ± 0,005
>E4	Rs-2, Rs-4	28,5 - 28,8	28,62 ± 0,003	28,6 - 29,0	28,75 ± 0,004	28,8 - 28,9	28,85 ± 0,002	28,7 - 29,2	28,93 ± 0,006
neg	Rs-5, NC-Rs	neg (45)	NA	neg (45)	NA	neg (45)	NA	neg (45)	NA
E. amylovora									
E7	PC Ea	21,0 - 21,5	21,11 ± 0,007	21,1 - 21,2	21,15 ± 0,003	21,0 - 21,4	21,23 ± 0,006	20,9 - 21,3	21,09 ± 0,006
E5	Ea-1	28,0 - 28,5	28,18 ± 0,006	28,0 - 28,4	28,28 ± 0,005	27,9 - 28,4	28,18 ± 0,006	28,2 - 28,8	28,44 ± 0,007
>E4	Ea-4, Ea-5	29,1 - 29,7	29,34 ± 0,006	29,3 - 29,7	29,48 ± 0,003	29,2 - 30,0	29,48 ± 0,008	29,6 - 30,0	29,76 ± 0,004
neg	Ea-2, Ea-3, NC-Ea	neg (45)	NA	neg (45)	NA	neg (45)	NA	neg (45)	NA

4 Reported Results

Results, as reported are summarized in Tables 4 and 5. The methods used by the participants are listed in Appendices I and II for *R. solanacearum* and *E. amylovora*, respectively.

5 Evaluation of the Results

For *R. solanacearum* 16 out of 18 participating laboratories obtained results 100 % in concordance with the true qualitative values.

Two laboratories (Lab IDs 6 and 9) obtained correct results for negative and positive controls, and for the highest concentration of *R. solanacearum* included as a sample (log 7). Laboratories 6 and 9 reported negative results for the three samples containing log 5 or lower concentrations of the target DNA copies (samples Rs-1, Rs-2 and Rs-4; negative deviation). Laboratories reported results based on PCR modified from Pastrik *et al.* (2002) and combination of PCR (Seal *et al.*, 1993) and LAMP (Kubota *et al.*, 2008), respectively.

The PCR by Pastrik *et al.* (2002) has been used by 8 other participating laboratories with all reporting results in accordance with the true qualitative values. As well, PCR by Seal *et al.* (1993) has been used by another participant also giving results in concordance with the true qualitative values. Based on the literature data the LAMP assay may not be sensitive enough to detect lower concentrations of *R. solanacearum* (Kubota *et al.*, 2008; Lenarčič *et al.*, 2014). It is worth noting that the laboratory with Lab ID 05 also reported that LAMP (Lenarčič *et al.*, 2014) did not give positive results with samples Rs-1, Rs-2 and Rs-4.

For *E. amylovora* all results of all participants were in concordance with the true qualitative values.

Table 4: Proficiency test results as reported by the participants for *R. solanacearum* testing. Non-conforming results are shown in bold and shaded.

Sample ID	Status	Laboratory ID and Reported Results																				Percent conforming
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	
Rs-1	pos (E5)	pos	pos	pos	pos	pos	**neg**	pos	pos	**neg**	pos	pos	NA	NA	pos	pos	pos	pos	pos	pos	pos	0,89
Rs-2	pos (>E4)	pos	pos	pos	pos	pos	**neg**	pos	pos	**neg**	pos	pos	NA	NA	pos	pos	pos	pos	pos	pos	pos	0,89
Rs-3	pos (E7)	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	NA	NA	pos	pos	pos	pos	pos	pos	pos	1,00
Rs-4	pos (>E4)	pos	pos	pos	pos	pos	**neg**	pos	pos	**neg**	pos	pos	NA	NA	pos	pos	pos	pos	pos	pos	pos	0,89
Rs-5	neg (NA)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	neg	neg	neg	1,00
PC-Rs	pos (E7)	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	NA	NA	pos	pos	pos	pos	pos	pos	pos	1,00
NC-RS	neg (NA)	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	NA	NA	neg	neg	neg	neg	neg	neg	neg	1,00
Conformity of controls		yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	NA	NA	yes	yes	yes	yes	yes	yes	yes	1,00
Number of conforming results for test samples		5	5	5	5	5	2	5	5	2	5	5	NA	NA	5	5	5	5	5	5	5	

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

Sample ID	Status	Laboratory ID and Reported Results																				Percent conforming
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	
Ea-1	pos (E5)	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	NA	NA	NA	pos	pos	pos	pos	1,00
Ea-2	neg (NA)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	NA	neg	neg	neg	neg	1,00
Ea-3	neg (NA)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	NA	neg	neg	neg	neg	1,00
Ea-4	pos (>E4)	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	NA	NA	NA	pos	pos	pos	pos	1,00
Ea-5	pos (>E4)	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	NA	NA	NA	pos	pos	pos	pos	1,00
PC Ea	pos (E7)	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	NA	NA	NA	pos	pos	pos	pos	1,00
NC-Ea	neg (NA)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	NA	neg	neg	neg	neg	1,00
Conformity of controls		yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	NA	NA	NA	yes	yes	yes	yes	1,00
Number of conforming results for test samples		5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	

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Appendix I. Methods used by the proficiency test participants for *R. solanacearum*

Laboratory ID	PCR				real-time PCR			LAMP	
	Pastrik and Maiss, 2000	Pastrik *et al.*, 2002	Seal *et al.*, 1993	other	Weller *et al.*, 2000	Massart *et al.*, 2014	other	Lenarčič *et al.*, 2014	Kubota *et al.*, 2008
1		x			x				
2	x								
3	x				x				
4		x							
5	x				x (RS)			x	
6		x							
7		x	x		x				
8		x			x				
9			x						x
10	x								
11					x (RS, B2)				
12	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	NA	NA	NA	NA	NA	NA	NA	NA	NA
14		x	x						
15						x			
16		x	x	x[a]	x	x			
17							x[b]		
18		x	x						
19				x[c]	x		x[c]		
20		x							
Number of laboratories	4	9	5	2	8	2	2	1	1

[a]Boudazin *et al.*, 1999; Fegan and Prior, 2005

[b]internal method and Sherokolava, 2013

[c]PCR kit 'AgroDiagnostika' and real-time PCR kit ZAO 'Syntol'

Appendix II. Methods used by the proficiency test participants for *E. amylovora*

Laboratory ID	PCR			real-time PCR			
	Bereswill *et al*., 1992	Llop *et al*., 2000	other	Salm and Geider, 2004	Pirc *et al*., 2009	Gottsberger, 2010	other
1			x[a]		x		
2						x	
3					x	x	
4		x					
5					x (amsC, ITS)		
6	x		x[b]				
7	x	x		x			
8		x			x		
9	x	x		x			
10	x		x[c]				
11				x	x (amsC, ITS)		
12		x					
13	x		x[d]		x		
14	NA	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	NA	NA	NA
16	NA	NA	NA	NA	NA	NA	NA
17							x[e]
18		x	x[c]			x	
19			x[f]				x[g]
20			x[d]				
Number of laboratories	5	6	7	3	6	3	2

[a]Obradović *et al.*, 2007
[b]Taylor *et al.*, 2001; Stöger *et al.*, 2006
[c]Obradović *et al.*, 2007 modified by Gottsberger
[d]Taylor *et al.*, 2001
[e]internal method and Sherokolava, 2010
[f]Stöger *et al.,* 2006, PCR kit 'AgroDiagnostika'
[g]real-time PCR kit ZAO 'Syntol'