[F1 ANNEX II

TEST SCHEME FOR DIAGNOSIS, DETECTION AND IDENTIFICATION OF *RALSTONIA SOLANACEARUM* (SMITH) YABUUCHI *ET AL.*

Textual Amendments

F1 Substituted by Commission Directive 2006/63/CE of 14 July 2006 amending Annexes II to VII to Council Directive 98/57/EC on the control of Ralstonia solanacearum (Smith) Yabuuchi et al..

Appendix 6

Validated PCR protocols and reagents

3. Multiplex PCR protocol with internal PCR control (Pastrik *et al.*, 2002)

3.1. Oligonucleotide primers

Forward primer RS-1-F	5′- ACT AAC GAA GCA GAG ATG CAT TA -3′
Reverse primer RS-1-R	5′- CCC AGT CAC GGC AGA GAC T -3′
Forward primer NS-5-F	5′- AAC TTA AAG GAA TTG ACG GAA G -3′
Reverse primer NS-6-R	5′- GCA TCA CAG ACC TGT TAT TGC CTC -3′

Expected amplicon size from *R. solanacearum* template DNA = 718 bp (RS-primer set)

Expected amplicon size from the 18S rRNA internal PCR control = 310 bp (NS-primer set).

3.2. PCR reaction mix

Reagent	**Quantity per reaction**	**Final concentration**
Sterile UPW	12,625 µl	
10X PCR buffer[a] (15 mM $MgCl_2$)	2,5 µl	1X (1,5 mM $MgCl_2$)
BSA (fraction V) (10 %)	0,25 µl	0,1 %
d-nTP mix (20 mM)	0,125 µl	0,1 mM
Primer RS-1-F (10 µM)	2,0 µl	0,8 µM
Primer RS-1-R (10 µM)	2,0 µl	0,8 µM
Primer NS-5-F (10 µM)[b]	0,15 µl	0,06 µM
Primer NS-6-R (10 µM)[b]	0,15 µl	0,06 µM
Taq polymerase (5 U/µl)[a]	0,2 µl	1,0 U
Sample volume	5,0 µl	
Total volume:	25,0 µl	

a Methods were validated using *Taq* polymerase from Perkin Elmer (AmpliTaq) and Gibco BRL.

b Concentration of primers NS-5-F and NS-6-R were optimised for potato heel end core extraction using the homogenisation method and DNA purification according to Pastrik (2000) (see Section VI.A.6.1.a.). Re-optimisation of reagent concentrations will be required if extraction by shaking or other DNA isolation methods are used.

3.3. PCR reaction conditions

Run the following programme:

1 cycle of:	(i)	5 minutes at 95 °C (denaturation of template DNA)
35 cycles of:	(ii)	30 seconds at 95 °C (denaturation of template DNA)
	(iii)	30 seconds at 58 °C (annealing of primers)
	(iv)	45 seconds at 72 °C (extension of copy)
1 cycle of:	(v)	5 minutes at 72 °C (final extension)
	(vi)	hold at 4 °C.

NB: This programme is optimised for use with an MJ Research PTC 200 thermal cycler. Modification of the duration steps of cycles (ii), (iii) and (iv) may be required for use with other models.

3.4. Restriction enzyme analysis of amplicon.

PCR products amplified from *R. solanacearum* DNA produce a distinctive restriction fragment length polymorphism with enzyme *Bsm* I or an Isoschizomere (e.g. Mva 1269 I) after incubation at 65 °C for 30 minutes.**]**