European Union Reference Laboratory

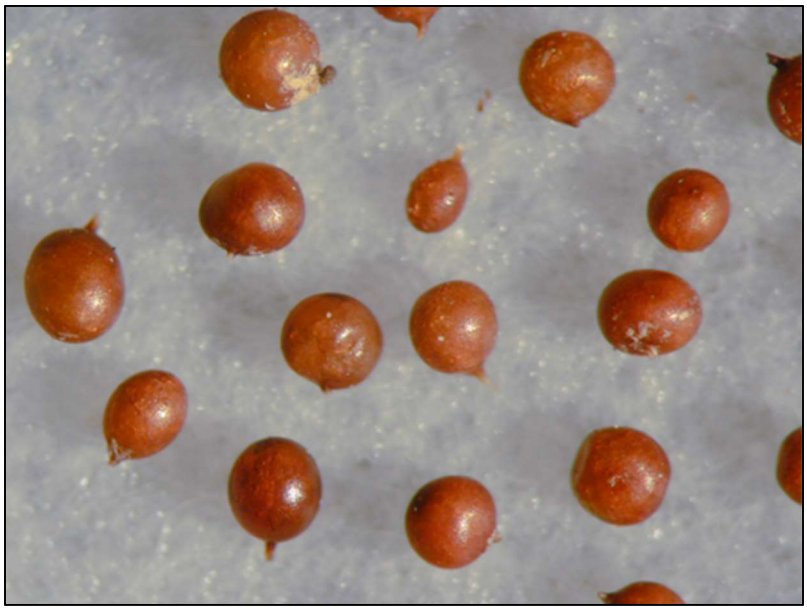
for Plant Parasitic Nematodes

EURL diagnostic protocol

REFERENCE: EURL-Globo-qPCR_Version 02

October/ 2021

Protocol for species detection of *Globodera rostochiensis* and *G. pallida* by real-time PCR from isolated nematode cysts



The table below summarises the evolution (versions) of this method, including the description of the modifications introduced.

Version	Date	Principal modifications
v1	15/10/2021	Initial Version 1 (V01)
v2	21/10/2021	Modification : • **§8**. In the table containing the concentrations of the PCR reaction, in the line molecular grade water, the adjusted value is changed from 20 µL to 15 µL.

Foreword

These methods were recommended by:

ANSES - Plant Health Laboratory - Nematology Unit

Address: Domaine de la Motte au Vicomte - BP 35327 - 35653 Le Rheu Cédex - France

ILVO - Plant Unit - Nematology

Address: Burg. Van Gansberghelaan 96 - 9820 Merelbeke - Belgium

The consortium between both laboratories is designated the

European Union Reference Laboratory (EURL) for Plant Parasitic Nematodes

Suggested citation:

European Union Reference Laboratory (EURL) (2021). EURL diagnostic protocol: Protocol for the species detection of *Globodera rostochiensis* and *G. pallida* by real-time PCR from isolated nematode cysts. EURL-Globo-qPCR_Version 01.

In-text citation: (EURL-Globo-qPCR, 2021)

Contents

1 Introduction

The potato cyst nematodes, *Globodera rostochiensis* (Wollenweber, 1923), and *Globodera pallida* (Stone, 1973) are plant-parasitic nematodes that cause significant losses in potato (*Solanum tuberosum*). Given the damage caused by these nematodes, they are regulated by a majority of countries in the world (EPPO 2017) and classified as quarantine organisms in the European Union (listed in Appendix II, Part B, of the Commission Implementing Regulation (EU) 2019/2072) in order to avoid their spread within the EU territory and the introduction of new populations.

The application of this protocol is part of the monitoring to assess the spread of these pests in Europe according to European Council Directive 2007/33/EC of 11 June 2007 (under revision).

The purpose of this EURL diagnostic protocol[1] is to assist the National Reference Laboratories (NRLs) of EU Member states in carrying out their diagnostics analyses by providing them with details on analytical operating methods for the species detection of *G. rostochiensis* and *G. pallida*.

The material linked to this diagnostic protocol, such as PowerPoint presentations and technical videos & media, can be found on the EURL Plant Parasitic Nematodes website.

Warning and safety precautions: The user of this method should be closely familiar with standard laboratory practices. It is the responsibility of the user to establish suitable health and safety practices and ensure compliance with the current regulations. All actions taken in accordance with this method must be performed by employees who have attended relevant training.

[1] The use of chemical brands or equipment on this diagnostic protocol does not imply the exclusion of others, which may also be appropriate.

2 Terms, abbreviations and definitions

- bp: base pairs
- Cycle threshold (Ct): the number of cycles required for the fluorescent signal to cross the threshold
- Cysts: the sclerotised cuticle of the deceased Heteroderinae, particularly *Globodera-, Heterodera-* and *Punctodera* females, usually filled with eggs and juveniles
- EPPO: European and Mediterranean Plant Protection Organization
- IPPC: International Plant Protection Convention
- NRLs: National Reference Laboratories
- PPN(s): Plant-Parasitic Nematode(s)
- ND: Not detected
- D: Detected

3 Purpose and scope

The purpose of this document is to describe a EURL diagnostic protocol for the species detection of *Globodera rostochiensis* and *G. pallida* that is recommended for use in diagnostic laboratories in the EU. This protocol is based on a DNA extraction from isolated nematode cysts followed by real-time PCR detection. This protocol can be applied to a mixture of nematode cysts (from one to up to 50 cysts per tube) isolated after cysts extraction from different matrices (soil, plant roots and tubers). The extraction methods and the isolation of nematode cysts from the debris are described in the protocol: EURL –Globo-Extraction (GE).

The real-time PCR test based on Gamel *et al*. 2017 was validated by the EURL, including reagents and brand references in this protocol.

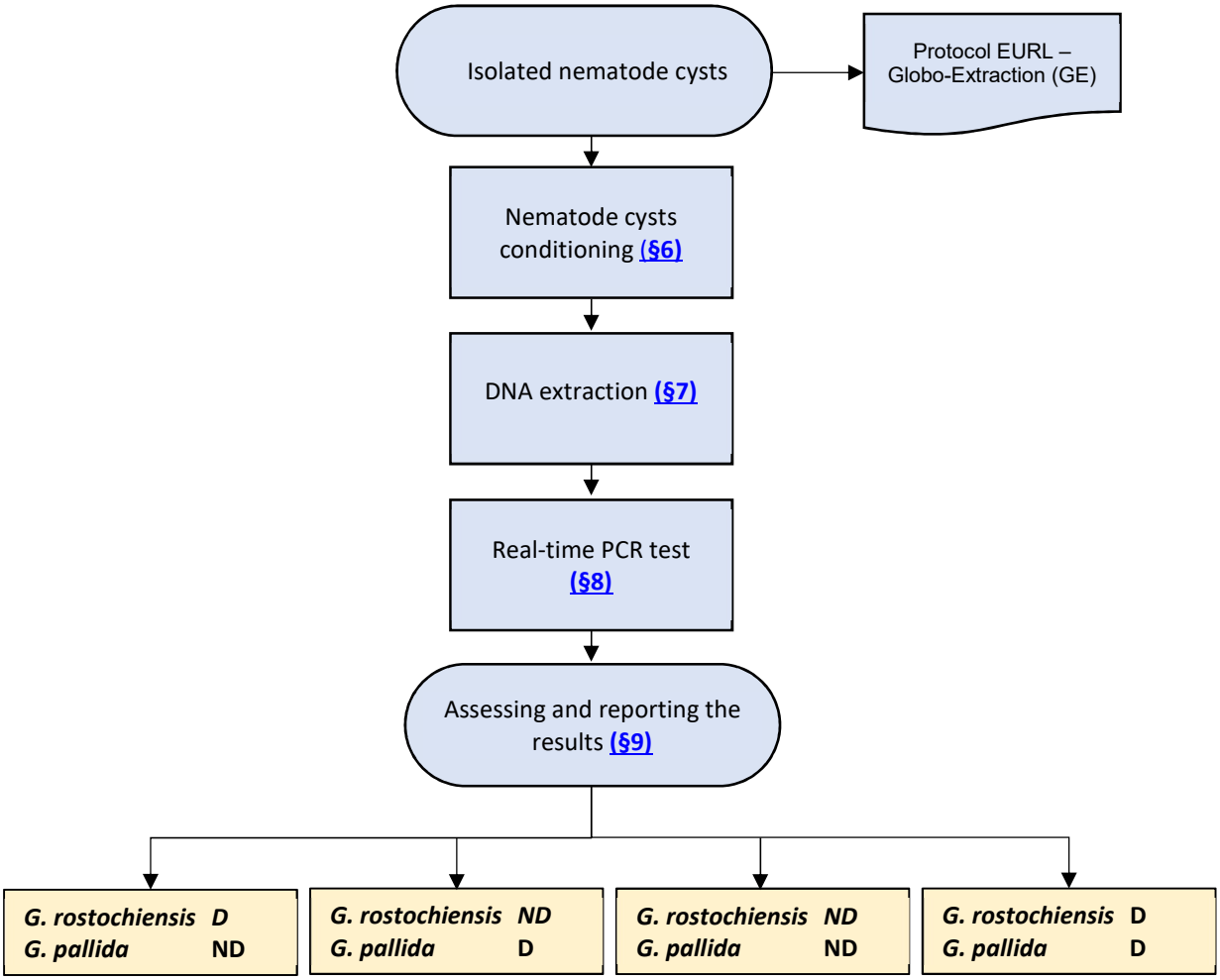
The performance characteristics of the real-time PCR method are available in appendix **§11**.

4 Principle

This protocol (based on a real-time PCR test from Gamel *et al.* 2017) can be used to detect *G. rostochiensis* and *G. pallida* from isolated nematode cysts. This molecular test can also be used on juveniles replacing the conventional PCR based on Bulman & Marshall (1997) (detailed in the protocol EURL-Globo-Identification (GI)). Performance characteristics of juveniles material are also available in appendix **§11.**

The real-time PCR detection is carried out according to the following flowchart:

Schematic procedure for the detection of *G. rostochiensis* and *G. pallida*



5 Material and consumables

5.1 Equipment and small materials

In addition to the standard equipment for molecular biology (pipettes, centrifuges, shaker, water bath, electrophoresis, etc.), the following equipment is considered necessary for certain steps of the analysis:

- Glass beads (1 and 3 mm diameter)
- Pestles adapted to the tubes
- Shacking tissue grinder for approximately 2 mL microtubes (e.g., Tissulyser, Qiagen®) or equivalent equipment
- Real-time PCR thermal cycler

5.2 Consumables

In general, the manipulator must ensure (either by the use of consumables known as molecular biology quality, or by cleaning, sterilisation or other appropriate treatment) that the water and products or consumables used are free of contamination with DNA/RNA, nuclease, inhibitor or any other element that may interfere with, the molecular analysis, and the result.

Suppliers' recommendations regarding storage conditions are followed. Failing this, the laboratory should define the most optimal conditions.

Any commercial DNA extraction kit and/or PCR master mix suitable for real-time PCR application can be used if their performances are equivalent to those obtained with products listed in this protocol.

- Molecular biology grade water.
- **DNA extraction kit:** QIAamp® DNA mini kit (Qiagen) (ref. 51306).
- **Master mix:** LightCycler® 480 Probes Master mix (Roche Diagnostics) (ref. 04707494001).
- **Primers:**

Target PPNs	Test references	Primer/probe Real-time PCR	Sequence 5'- 3'
G. pallida	Gamel *et al*. (2017)	**µsatGP-F**	AAGGAGTTGTGGTCCAGACG
		µsatGP-R	GAAGGCAATCTGTGTTCGGG
		µsatGP-P	JOE-CGCTCGTCGGCCTCCTCCTC-BHQ1
***G. rostochiensis* ***	Gamel *et al*. (2017)	**µsatGR-F**	TGACGAGGAACAGTACAAAG
		µsatGR-R	GTGTCTCTAATTTGCCATT
		µsatGR-P	FAM-AGGCATTGCTTGAGCGAACGGA-BHQ1

* The real-time probe Cy5-BHQ2 can also be used for *G. rostochiensis* as described in Gamel *et al*., 2017.

5.3 Control and their purpose

The following controls are used to check the correct performance of the DNA extraction and PCR steps.

Controls	Purpose	Control application	Expected result *
Negative process control (NPC)	DNA extraction buffer alone conditioned and tested in the same manner as the tested sample. Verify the absence of contamination during the DNA extraction process.	Mandatory	Negative
Positive process control (PPC)	Minimum of one cyst from a target species conditioned and processed as the DNA extraction step in the same manner as the tested sample. Verify the absence of any flaw during the analytical steps.	Mandatory	Positive
Positive PCR control (PC)	It contains all the elements of the PCR reaction mix, including a DNA extract from each of the target nematodes; this control verifies that the PCR reaction has proceeded correctly, allowing the amplification of the samples containing the target.	Mandatory	Positive
No Template Control (NTC)	It contains all the elements of the PCR reaction mix, but no DNA is added; this control checks the absence of contamination during the PCR preparation and reaction.	Mandatory	Negative
Inhibition control (IC)	Redo the PCR by adding in the same mastermix solution, DNA of a target species, and sample DNA. The concentration of the spiked DNA (from the target species) should be determined in each laboratory depending on the mastermix and thermocycler. This control allows checking the absence of PCR inhibitors in the sample.	Mandatory if no amplification is observed for all the microtubes corresponding to the same sample	Positive

* The results are only valid if the expected results are met.
If available, the target species may be supplied by the EURL.

6 Nematode cysts conditioning

After isolating nematode cysts from the debris, cysts can be conditioned in microtubes: one to up to 50 cysts per microtube. Depending on the number of cysts (if higher than 50 cysts in a sample), make sure to prepare a sufficient number of tubes to obtain a representative sampling of your sample. For confirmation analysis, when applicable, other cysts can be stored for further identification.

- ***Note:*** *If many cysts of different genera are present in the samples, isolate preferably cysts showing a typical Globodera morphology in order not to miss out on the target species*

Soak the cysts in a maximum of 50 µL of water for at least 6 hours.

- ***Note:*** *After this step, the cysts can be stored at <10 °C before proceeding with the analysis.*

7 DNA extraction

1) If the tube contents are frozen, allow the contents to thaw completely and then crush the cysts with a piston
2) Prepare and add 180 µL of ATL buffer and 20 µL of proteinase K according to the kit supplier's instructions.
   - ***Note:*** *Optionally prepare a solution with lysis buffer (ATL) + proteinase K and distribute 200 µL of QIAamp kit buffer to each test tube*
3) Add glass or metal beads of different diameters (e.g. 1 bead of 3 mm and a few beads of 1 mm) to the tube containing the isolated nematodes cysts.
4) Perform a second crushing: place the tube on a tissue grinder rack ([e.g. using a Tissulyser II (Qiagen®) shake at frequency 30 Hz for 40 sec
5) Place the tube in a water bath at approximately 56°C for at least one hour
6) Briefly centrifuge. Add 200 µL of AL buffer and vortex well
7) Incubate for 10 minutes at 70 °C
8) Briefly centrifuge. Add 200 µL of ethanol (96 -100%) and homogenise well
9) Transfer the contents of the tube to the column supplied with the QIAamp kit
10) Centrifuge for 1 min at 6000 g
11) Place the column on a clean collector and add 500 µl of AW1 buffer
12) Centrifuge for 1 min at 6000 g
13) Place the column on a clean collector and add 500 µl of AW2 buffer
14) Centrifuge for 3 min at 20 000 g
15) Place the column on a clean, labelled 1.5 mL microtube and add 100 µL of buffer AE or molecular grade water
16) Incubate for a few minutes at room temperature and then centrifuge for 1 min at 6000 g
17) The resulting DNA solutions are directly analysed by real-time PCR or frozen (<10 °C) until analysis.

8 Real-time PCR test (based on Gamel *et al.* 2017)

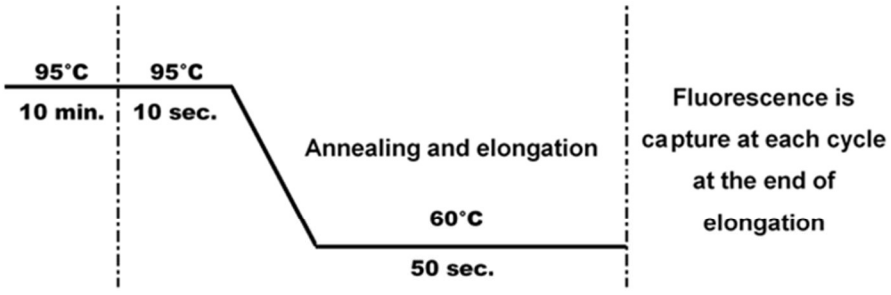
The operating procedure to perform the real-time PCR are detailed below.

Each DNA solutions have to be tested in at least duplicate.

- **Duplex reaction**

	Real-time PCR test
Reagents – Master mix	**Final concentration per reaction tube**
Total volume	20 µL
LightCycler® 480 Probe Master (Roche)	1 X
G. pallida Forward Primer (*µsat-GP-F*)	0.5 µM
G. pallida Reverse Primer (*µsat-GP-R*)	0.5 µM
G. pallida Probe (*µsat-GP-R*)	0.2 µM
G. rostochiensis Forward Primer (*µsat-GR-F*)	0.5 µM
G. rostochiensis Reverse Primer (*µsat-GR-R*)	0.5 µM
G. rostochiensis Probe (*µsat-GR-R*)	0.2 µM
Molecular grade water water	Adjust to 15 µL
Add DNA to 20 µL of reaction mix	5 µL

- **PCR program:**



PCR cycles 40 X

- **Simplex reaction**

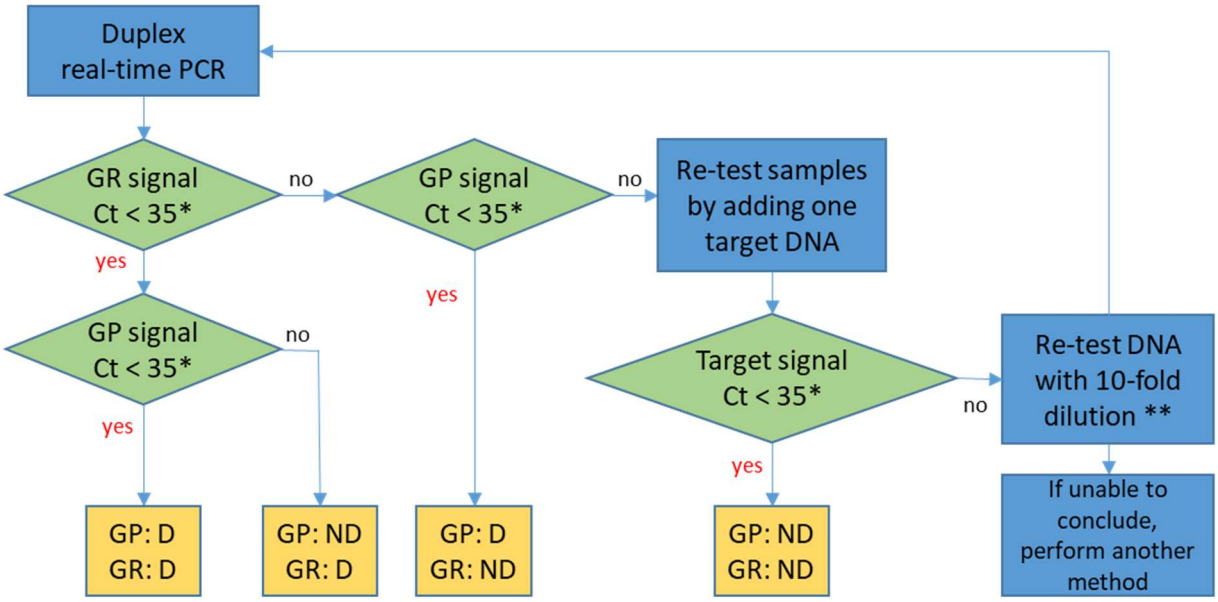
Note: *The FAM signal used to detect the target species G. rostochiensis may, in some cases, be visible on the JOE signal used to detect the G. pallida species. In this case, a low fluorescent amplification can be observed on the JOE signal. If there is any doubt on the result due to such a pattern and if an exponential curve with a Ct ≥ 35 is observed, the samples can be re-tested in the simplex reaction using only the primers and probe necessary for the detection of G. pallida.*

9 Evaluation and reporting the results

The results obtained by real-time PCR are preferably processed by an automatic software analysis or, failing that, by defining and applying the same threshold line. A Ct value must be accompanied by an exponential curve to be taken into account.

Result interpretation of the molecular analysis is carried out according to the following flowchart.

Flowchart decision for the detection of *G. rostochiensis* and *G. pallida*



GP: *G. pallida* **GR: *G. rostochiensis*** **D: Detected** **ND: Not Detected**

* Ct < 35 and exponential amplification curve observed
** If a final result is not possible, new cysts are analysed according to another protocol, either from the remaining cysts or after a new extraction from the matrix.
In the case of amplification with a Ct value > 35, it is recommended to re-test the extracted DNA in a simplex reaction to obtain higher sensitivity.

The molecular analysis result is a synthesis of the results obtained from each of the microtubes analysed. The correct interpretation of the results is carried out by observing the exponential fluorescence curves measured by the real-time PCR and generated from the various controls. The analysis is valid if, and only if, all of the following conditions are met:

Checking the controls:

- The NPC and NTC: no amplification is observed in any of the replicates
- All replicates of PC: exponential fluorescence curve observed following the requirements.

If the expected results of one or more controls do not comply (as defined above), the analysis is not valid and, depending on the non-compliance observed, all or part of the analysis must be repeated.

After validation of the controls, and for each of the fluorescence signals, observe the *Ct* value and the appearance of the amplification curve: sample results should be interpreted as follows for the two target species under investigation (*G. rostochiensis* or *G. pallida*):

Result analysis:

***G. rostochiensis* signal (FAM)**		***G. pallida* signal (JOE)**		**Sample Results**
Well 1	Well 2	Well 1	Well 2	
+	+	+	+	Positive test for both target species
+	+	-	-	Positive for *G. rostochiensis* and negative for *G.pallida*
-	-	+	+	Negative for *G.rostochiensis* and positive for *G.pallida*
+	-	+	-	Re-test DNA in simplex reactions
-	-	+	-	Re-test DNA in simplex reaction at least for *G. pallida*
+	-	-	-	Re-test DNA in simplex reaction at least for *G. rostochiensis*
-	-	-	-	Re-test samples by adding one target DNA (see **§5.3** Inhibition Control). If IC is positive (+) for target DNA, the **test result is negative for *G. pallida* and *G. rostochiensis*.** If IC is negative (-) for target DNA, a new PCR reaction is performed **with 10-fold dilution** from the DNA of the negative samples. If unable to conclude, **perform another method** (**morphology combined with conventional PCR or different specific tests).**

+: observation of an exponential fluorescence curve with a value of *Ct* < 35. Results with Ct ≥ 35 in duplex reaction requires a new PCR in simplex reaction.
- : the absence of an exponential fluorescence curve or observation of a curve that is not exponential or observation of an exponential amplification curve with a value of *Ct* ≥ 35 for simplex reaction.

***If you have any question about this protocol, please, send an e-mail to* eurl.nematodes@anses.fr.**

Acknowledgements
These recommendations were prepared by the EURL consortium composed of ANSES - Plant Health Laboratory - Nematology Unit and ILVO - Plant Unit - Nematology in the frame of EURL activities.

10 References

Bulman, S. R., & Marshall, J. W. (1997). Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Science*, *25*(2), 123–129. https://doi.org/10.1080/01140671.1997.9513998

COUNCIL DIRECTIVE 2007/33/EC on the control of potato cyst nematodes and repealing Directive 69/465/EEC. (2007). In *Official Journal of the European Union 156/12*. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32007L0033 (under revision)

Gamel, S., Letort, A., Fouville, D., Folcher, L., & Grenier, E. (2017). Development and validation of real-time PCR assays based on novel molecular markers for the simultaneous detection and identification of *Globodera pallida*, *G. rostochiensis* and *Heterodera schachtii*. *Nematology*, *19*(7), 789–804. https://doi.org/10.1163/15685411-00003086

PM 7/40 *Globodera rostochiensis* and *Globodera pallida*. (2017). *EPPO Bulletin*, *47*(2), 174–197. https://doi.org/10.1111/epp.12391

11 Appendix: Performance characteristics and validation of the real-time PCR test

This test was evaluated and validated according to the following criteria:

- Specificity (= analytical specificity-exclusivity): Ability of the method to not detect the target species from a range of populations from the non-target species (absence of false positives);
- Sensitivity (= analytical specificity-inclusivity): Ability of the method to detect the target species from a range of populations from the target species (absence of false negatives);
- Accuracy: Ability of the method to detect the target species from a range of populations from the target species and not to detect the target species from a range of populations from the non-target species.
- Limit of detection (= analytical sensitivity): the smallest amount of target species that gives a positive result in all replicates.
- Repeatability: Ability of the method to reproduce identical results under identical analytical conditions from samples at low concentrations.
- Reproducibility: Ability of the method to produce identical results under different analytical conditions (different days, operators, equipment…) from samples at low concentrations.
- Robustness: Ability of the method to produce identical results under slight but intentional variations in certain experimental conditions (annealing temperature, for example).

The specificity, sensitivity, and accuracy validation of the real-time PCR test were performed on a total of 55 populations (37 populations of *Globodera* spp. and 18 populations of *Heterodera* spp) comprising the following species/populations. If reference material available, this study could be completed by testing other populations and species.

- 14 populations of *Globodera pallida* (including 7 South American populations);
- 13 populations of *G. rostochiensis* (5 from South America and 8 from Europe);
- 7 populations of *G. tabacum ;*
- 1 population of *G. ellingtonae;*
- 1 population of *G. artemisiae;*
- 1 population of *G. mexicana ;*
- 7 populations of *H. schachtii;*
- 11 populations of *Heterodera,* including 5 species belonging to the *Schachtii* group.

All the populations (target and non-target species) were tested at a concentration between 1,5 to 5 ng/µL, depending on biological material availability. All populations of the target species tested were detected for simplex and duplex reactions. No cross-reaction was observed with the tested non-target species (only *G. artemisiae* showed amplification with a Ct value > 35).

In the tables below are the data for each criterion analysed:

Table 1. Performance of the method related to specificity, sensitivity and accuracy.

Duplex real-time PCR		
Criteria	**FAM signal**	**JOE signal**
	G. rostochiensis	***G. pallida***
Positive agreement*	26	28
Negative agreement*	84	82
Positive deviation*	0	0
Negative deviation*	0	0
Specificity*	100 %	100 %
Sensitivity*	100 %	100 %
Accuracy*	100 %	100 %

* according to the sample status

Limit of detection (LOD):

For cyst mixture:

Table 2. Performance of the method related to the limit of detection for cyst mixture.

Duplex real-time PCR		
Number of specimens per tube	**FAM signal**	**JOE signal**
	G. rostochiensis	***G. pallida***
	Number of positive wells / Total number of wells (Ct average of positive wells)	
10 juveniles of *G. rostochiensis* + 50 *G. pallida* cysts	10/12 (32,46)	12/12 (20,16)
10 juveniles of *G. pallida* + 50 *G. rostochiensis* cysts	12/12 (20,17)	12/12 (30,68)

The LOD for detecting the target *G. pallida*, is 10 juveniles of *G. pallida* within 50 cysts of *G. rostochiensis*. For *G. rostochiensis*, it was observed that in duplex reaction for 10 and 20 juveniles of *G. rostochiensis* within 50 cysts of *G. pallida*, not all replicates are positive (tables 2 and 4). However, all the replicates are positive in a simplex reaction (table 5).

For juveniles:

Table 3. Performance of the method related to the detection limit (LOD) for juveniles , DNA extraction according to the protocol EURL-Globo-Identification (GI).

Duplex real-time PCR		
Number of specimens per tube	**FAM signal**	**JOE signal**
	G. rostochiensis	***G. pallida***
	Number of positive wells / Total number of wells (Ct average of positive wells)	
1 juvenile	5/18 (33,32)	17/18 (33,12)
2 juveniles	18/18 (32,09)	18/18 (32,31)
5 juveniles	18/18 (30,95)	18/18 (30,87)

For both target species, the LOD is 2 juveniles.

Repeatability data:

Table 4. Performance of the method related to the repeatability data for cyst mixture.

Duplex real-time PCR		
Number of specimens per tube	**FAM Signal**	**JOE Signal**
	G. rostochiensis	***G. pallida***
	Number of positive wells / Total number of wells (Ct average of positive wells)	
20 juveniles of *G. rostochiensis* + 50 cysts of *G. pallida*	46% 11/24 (33,74)	100% 24/24 (20,58)
1 cyst of *G. rostochiensis* + 49 *G. pallida* cysts	100% 24/24 (27,30)	100% 24/24 (21,06)
10 juveniles of *G. pallida* + 50 cysts of *G. rostochiensis*	100% 24/24 (20,10)	100% 24/24 (30,54)
1 cyst of *G. pallida* + 49 cysts of *G. rostochiensis*	100% 24/24 (20,07)	100% 24/24 (23,97)

Table 5. Performance of the method related to the repeatability data applied to simplex real-time PCR reaction for cyst mixture.

Simplex real-time PCR	
Number of specimens per tube	**FAM Signal**
	G. rostochiensis
	Number of positive wells / Total number of wells (Ct average of positive wells)
10 juveniles of *G. rostochiensis* + 50 cysts of *G. pallida*	100% 24/24 (30,70)

Table 6. Performance of the method according to the repeatability data for juveniles (DNA extraction according to the protocol EURL-Globo-Identification (GI).

Duplex real-time PCR		
Number of specimens per tube	**FAM Signal**	**JOE Signal**
	G. rostochiensis	***G. pallida***
	Number of positive wells / Total number of wells (Ct average of positive wells)	
1 juvenile	16/18 (33,81)	18/18 (32,96)
2 juveniles	18/18 (32,02)	18/18 (32,22)
5 juveniles	18/18 (30,95)	18/18 (30,76)

Reproducibility data:

Duplicates of DNA solutions were analysed by different operators, on different days, in two different equipment and two batches of mastermix:

Table 7. Performance of the method related to the reproducibility data for cyst mixture.

Duplex real-time PCR								
Conditions	**FAM signal**				**JOE signal**			
	G. rostochiensis				***G. pallida***			
	10 juveniles GR+ 50 cysts GP	1 cyst GR + 49 cysts GP	10 juveniles GP + 50 cysts GR	1 cyst GP + 49 cysts GR	10 juveniles GR + 50 cysts GP	1 cyst GR + 49 cysts GP	10 Juveniles GP + 50 cysts GR	1 cyst GP + 49 cysts GR
Operator 1, mix 1, Day 1	0/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8
	(>35)	(32,35)	(20,18)	(20,12)	(19,94)	(20,95)	(30,66)	(23,98)
Operator 2, mix 2, Day 2	NT	8/ 8	8/8	8/ 8	NT	8/ 8	8/ 8	8/8
		(27,19)	(20,09)	(20,10)		(21,10)	(30,55)	(23,99)
Operator 2, mix 1, Day 3	0/8	NT	8/8	8/8	8/8	NT	8/8	8/8
	(>35)		(20,11)	(20,12)	(19,94)		(30,60)	(24,02)
Operator 2, mix 1, Day 4	NT	8/8	NT	NT	NT	8/8	NT	NT
		(32,60)				(21,04)		

NT: Not Tested
GR : G. rostochiensis
GP : G. pallida
Mix : correspond to the mastermix batch
The boxes marked in grey correspond to the Ct value.
The white boxes correspond to the number of positive wells / total number of wells tested.

Table 8. Performance of the method related to the reproducibility data applied to simplex real-time PCR reaction for cyst mixture.

Simplex real-time PCR	
Conditions	**Signal FAM**
	G. rostochiensis
	10 juveniles of *G. rostochiensis* + 50 cysts of *G.pallida*
Operator 1, mix 2, day 5, device 1	8/8 (30,66)
Operator 2, mix 1, day 3, device 1	8/8 (30,75)
Operator 1, mix 2, day 6, device 2	8/8 (30,69)

Mix : correspond to the mastermix batch
The numbers market between brackets corresponds to the Ct value.
The numbers above the Ct values correspond to the number of positive wells / total number of wells tested.

Table 9. Performance of the method related to the reproducibility data for juveniles (DNA extraction according to the protocol EURL-Globo-Identification (GI).

Duplex real-time PCR						
Conditions	**FAM signal**			**JOE signal**		
	G. rostochiensis			***G. pallida***		
	1 J2	**2 J2**	**5 J2**	**1 J2**	**2 J2**	**5 J2**
Operator 1/ day 1/ device 1/ mix 1	4/6 (34,13)	6/6 (32,05)	6/6 (30,96)	6/6 (32,86)	6/6 (32,17)	6/6 (30,76)
Operator 2/ day 2/ device 1/ mix 2	4/6 (34,01)	6/6 (32,08)	6/6 (30,82)	6/6 (33,26)	6/6 (32,32)	6/6 (31,05)
Operator 1/ day 2/ device 2/ mix 2	5/6 (34,05)	6/6 (31,94)	6/6 (31,05)	6/6 (33,13)	6/6 (32,24)	6/6 (30,96)

Mix: correspond to the mastermix batch
The numbers market between brackets corresponds to the Ct value.
The numbers above the Ct values correspond to the number of positive wells / total number of wells tested.

Robustness:

The method's performance has been tested with a lower and higher temperature (59°C and 61°C) instead of 60°C. The results are equivalent to those presented above.