European Union Reference Laboratory

for Plant Parasitic Nematodes

EURL diagnostic protocol

REFERENCE: EURL–Globo-Identification (GI)_Version 01

October/ 2021

Protocol for the identification of *Globodera rostochiensis* and *G. pallida:* Morphological and molecular methods

Foreword

These methods are recommended by:

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The consortium between both laboratories is designated the

European Union Reference Laboratory (EURL) for Plant Parasitic Nematodes

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Contents

1 Introduction

The potato cyst nematodes (PCN), *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 EURL diagnostic protocols are based on literature reviews and IPPC and EPPO standards, when applicable. This diagnostic protocol, including operational methods, was chosen as the basis, and with the scientific experience and technical expertise of the EURL team, some of these methods have been adapted, optimised and further validated by the EURL laboratory.

The purpose of this EURL recommended 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 procedures for identification 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 recommended method does not imply the exclusion of others, which may also be appropriate.

2 Terms, abbreviations and definitions

- bp: base pairs
- 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)
- Swollen stages: sedentary immobile nematode stages inside the root, e.g. third-stage juveniles (J3), fourth-stage juveniles (J4) and females of *Globodera* spp.
- Vermiform: mobile, worm-shaped, e.g. the second-stage juveniles (J2) and males of *Globodera* spp.
- ND – Not detected
- D - Detected

3 Purpose and scope

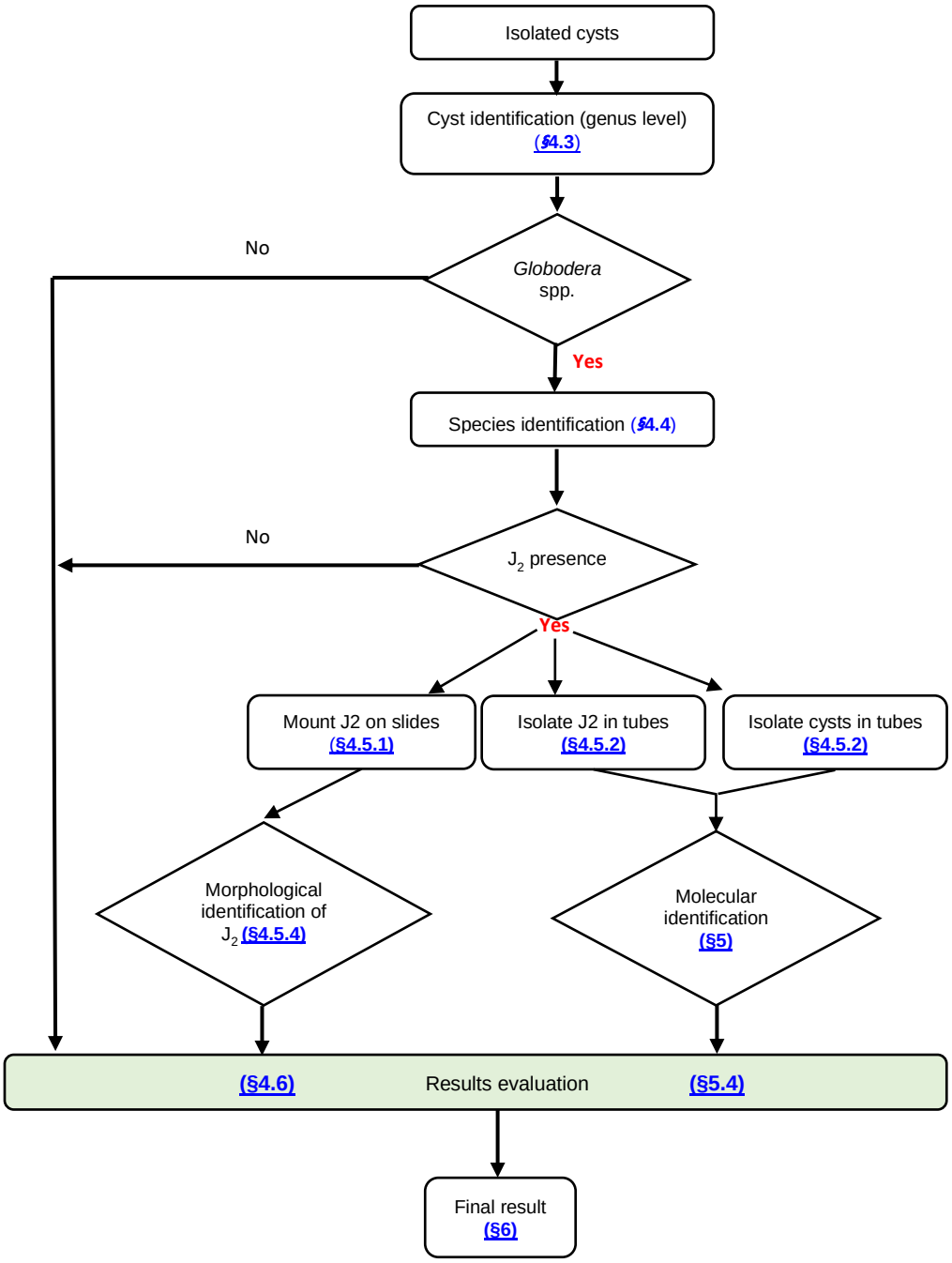
The purpose of this document is to describe a EURL diagnostic protocol for the identification of *Globodera rostochiensis* and *G. pallida* that is recommended for use in diagnostic laboratories in the EU. This protocol is based on a combination of morphological and molecular methods. This protocol can be applied to juveniles and cysts isolated from different matrices (soil, water, plant roots or tubers). Techniques to extract *Globodera* cysts are described in the protocol: EURL–Globo-Extraction (GE)_Version 01.

This protocol recommends combining a morphological method based on *Globodera* cyst morphology and species identification and the conventional PCR test from Bulman & Marshall (1997) for the identification of *G. rostochiensis* and *G. pallida*.

In Europe, other *Globodera* spp. *c*an cause confusion with *G. pallida* during morphological identification. *Globodera millefolii* and *G. artemisiae,* which does not infect potato, and the species complex of *Globodera tabacum* (e.i. *G. tabacum virginiae, G. tabacum tabacum* and *G. tabacum solanacearum*), which are found in various southern European countries and can infect solanaceous plants (CABI, 2021). Therefore, reliable differentiation between these species and *G. pallida* can only be evidenced by molecular methods. Within this context, in this protocol, a conventional multiplex PCR test based on Skantar et al. 2007 is also proposed to differentiate *G. tabacum* from *G. pallida* and *G. rostochiensis*.

The performance criteria and validation of the described morphological and molecular methods are available in Appendix **§7**.

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



4 Morphological identification

4.1 Principle

The *Globodera* cyst detection by morphological-based analysis is carried out on cysts extracted from soil, occasionally from other matrices, and isolated by visual examination at low magnification using a stereomicroscope (detailed in protocol EURL – Globo-Extraction (GE)_Version 01).

Morphological identification is based on examining the cyst shape and perineal area and the juveniles retrieved from the cysts. It is important to note that the morphological analysis permits identifying the species *G. rostochiensis* and the group of species G*. pallida/ G. tabacum* or another species misidentified with *G. pallida,* but does not make it possible to differentiate between this last group of species. Performance characteristics are available in appendix **§7.2.**

4.2 Material and consumables

4.2.1 Equipment

- Stereoscopic microscope with episcopic and diascopic illumination (magnification to a minimum of 50X)
- High definition microscope (observations at 1000X magnification)

4.2.2 Consumables and small materials

- Counting dish
- Fishing tool, small metal tip, paintbrush or other appropriate tool or instrument suitable for handling cysts and vermiform nematodes
- Small scalpel or a syringe needle
- Immersion oil
- microscope slides and coverslips
- Petri dishes
- Microtubes
- Heat source (to kill nematodes)
- A small container such as Syracuse watch glass
- Varnish (e.g. nail polish)

4.3 Identification of *Globodera* genus

The previously isolated nematode cysts (detailed in protocol EURL-GE_Version 1) are examined to identify the cysts of *Globodera* genus. This examination focuses on the shape and the perineal area (posterior part of the cysts opposite the head) (Figure 2).

1) Isolated cysts can be stored in a cold, dry place while waiting for analysis.
2) The detection of cysts belonging to the *Globodera* genus is performed using a stereoscopic microscope (magnification of 16X to 60X).
3) *Globodora* spp. are distinguished from other cysts genera by a round shape without a prominent terminal cone (Figure 1 (e.g. *G. pallida*) and 2). Sometimes confusion with *Punctodera* (Figure 1) is possible. In this case, the females' perineal pattern and/or morphology can be used to decide whether it is *Globodera* or *Punctodera.*

Figure 1 – Pictures of nematode cysts forms (source: http://nemaplex.ucdavis.edu).

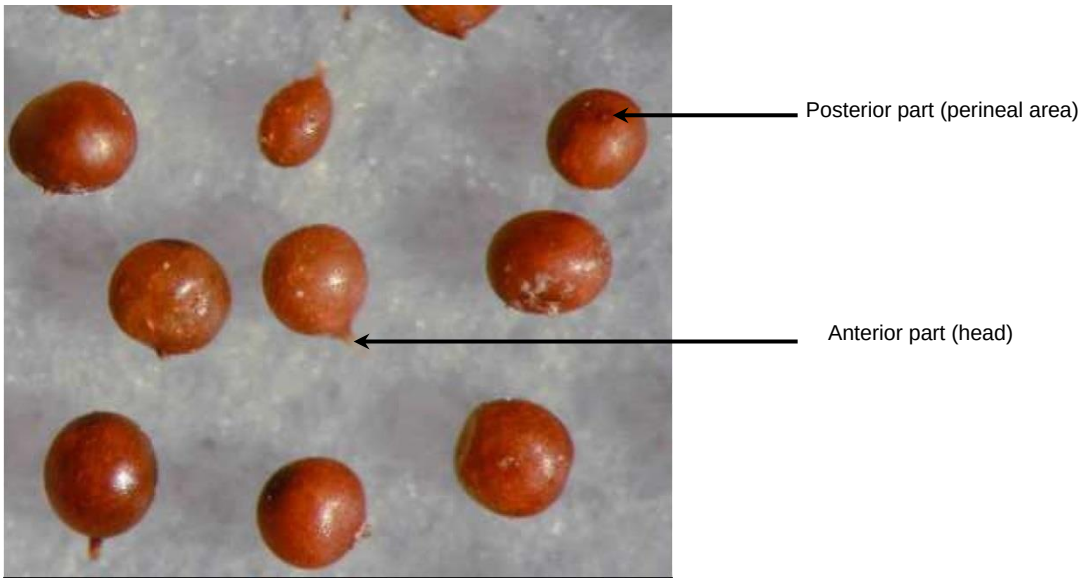


Figure 2 - *Globodera* sp. cysts with an indication of the posterior part (perineal area) and anterior part (head).

4.4 Morphological identification of cysts perineal pattern

4.4.1 Cyst hydration:

Soak the cysts in water for at least 6 hours to facilitate dissection of the perineal area.

Note: *The cysts can be soaked for more than 12 hours (up to 1 week) but should be kept cold (4 – 6 °C) to reduce fungal growth.*

4.4.2 Cyst dissection:

1) Select at least 3 cysts in good condition containing eggs, where possible.

   Note: *It is recommended to analyse* ***<u>at least 3 cysts per sample whenever possible</u>****.*

2) On each of the selected cysts, cut or pierce the anterior part of the cyst to avoid rupturing the perineal pattern. Carefully cut the posterior part of the cyst (Figure 2). Remove a maximum of eggs from the remaining cyst posterior with the syringe needle (or other appropriate tools) so that only the perineal pattern remains. Reduce the size of the cut piece by carefully cutting, approximating the perineal pattern (Figure 3E).

   Note: *Highly pigmented patterns can be put in H_2O_2 for a few minutes to bleach,*

3) Species identification by analysing the cyst perineal pattern (Figure 3). *Globodera* cyst possesses a circumfenestrate vulval region and non-fenestrate anal region (Figure 3A, E).

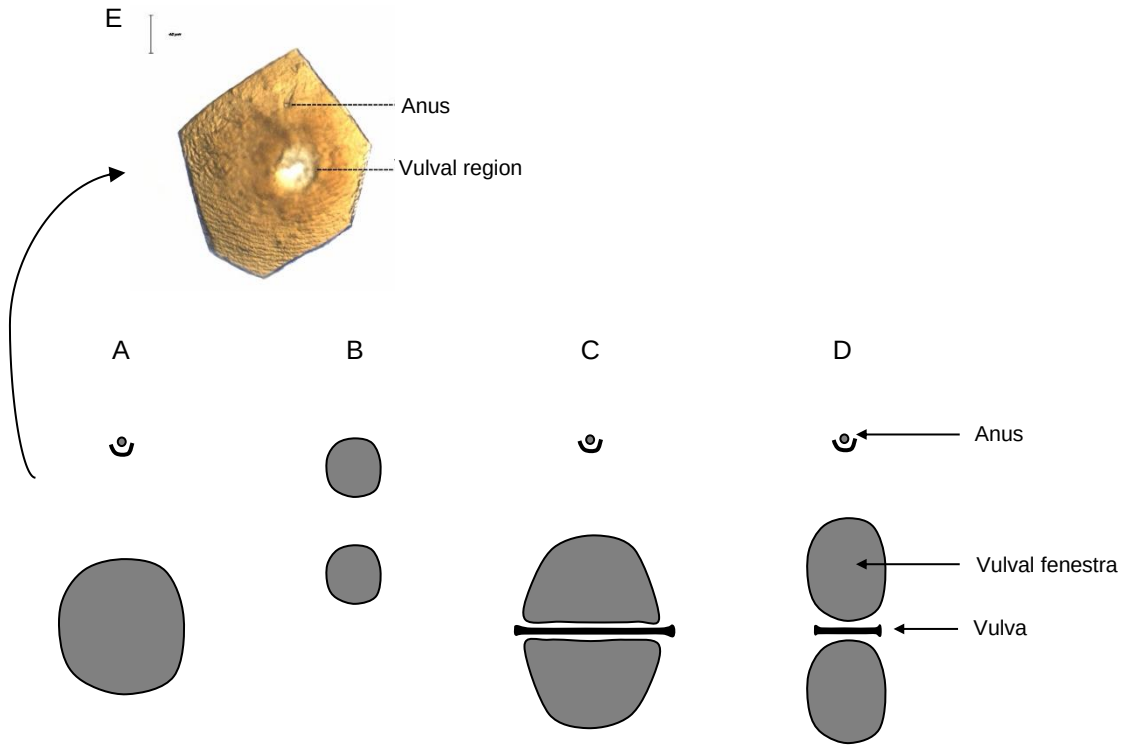


Figure 3 – Cyst perineal patterns. (A and E) Vulval region circumfenestrate and anal region non-fenestrate (*Globodera* and *Cactodera*) (Picture from ILVO-Nematology Unity). (B) Vulval region circumfenestrate and anal region fenestrate (Punctodera). (C) Vulval region semi fenestrate-ambifenestrate and anal region non fenestrate (Heterodera). (D) Vulval region semi fenestrate-bifenestrate and anal region non fenestrate (Heterodera). (Adapted from Wouts & Baldwin, 1998).

4) Most reliable diagnostic characteristics of the cysts can be seen when examining the perineal pattern using a microscope (Fig. 4 and 5):
   a. The number of cuticular ridges between the vulval basin and the anus (Fig. 4A)
   b. Granek's ratio (*i.e.* distance from the vulval fenestra to the anus / vulval fenestra diameter) (Fig. 4A and 5)

5) ***Note:*** The features measurements related to the perineal pattern for the different species, *G. rostochiensis* and *G. pallida*, are listed in Table 1 in **§4.6**

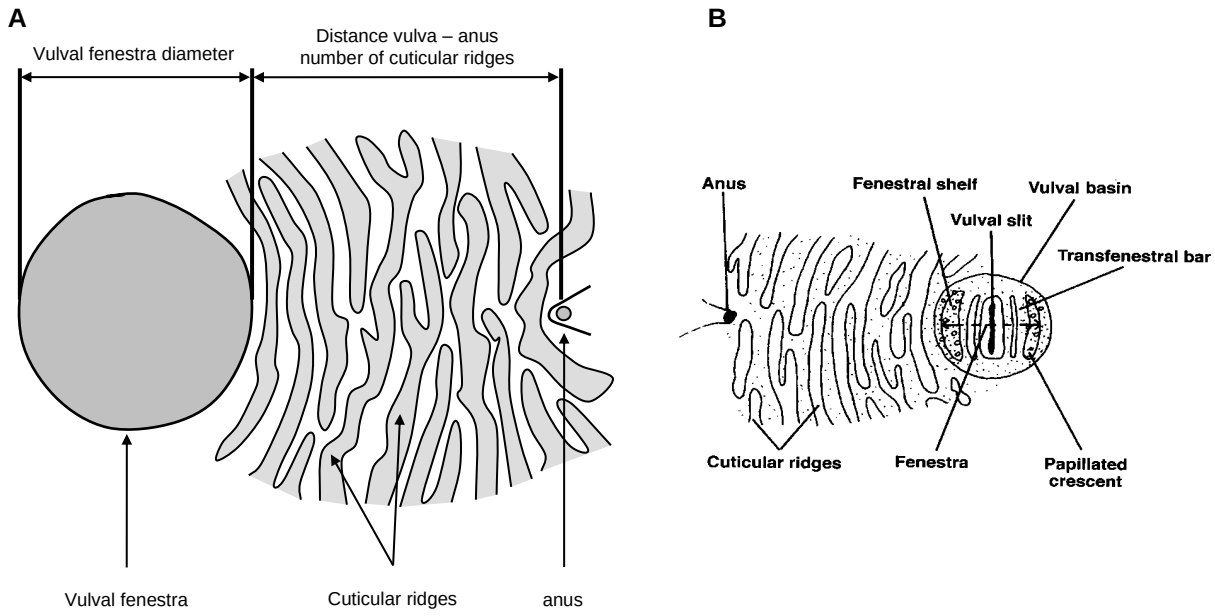


Figure 4 - (A) Perineal zone of a cyst of the *Globodera* genus and criteria used for morphological identification. (B) The perineal pattern of a *Globodera* cyst (Flemmings and Powers, 1998).

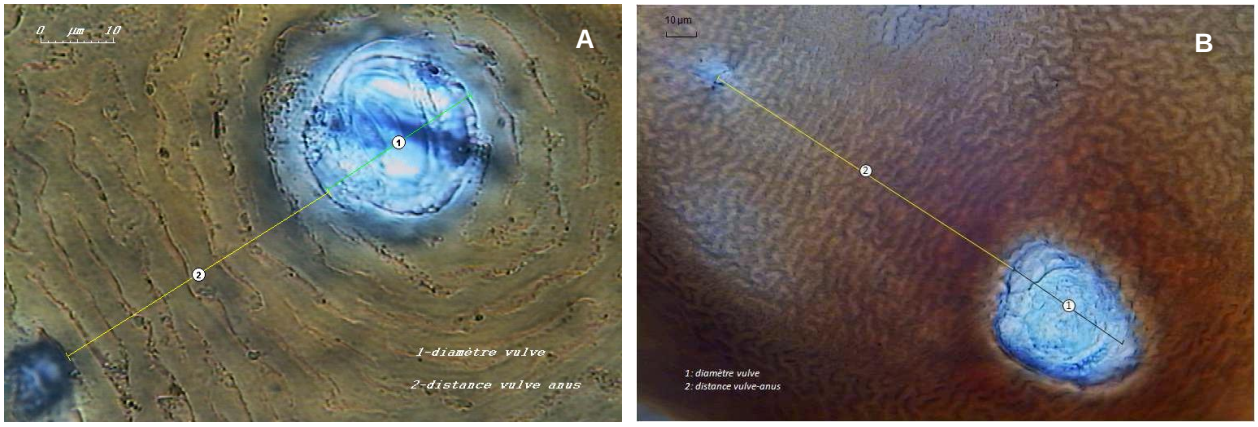


Figure 5 - Picture showing the distances for calculation of Granek's ratio of *Globodera* sp. (A) *Globodera pallida*, (B) *Globodera rostochiensis*, (1) Vulval fenestra diameter and (2) distance from the vulval fenestra to the anal fenestra. The scale varies between photos (from ANSES-Nematology Unit).

4.5 Morphological identification of juveniles

4.5.1 Sampling juveniles for mounting microscopic slides

1) J2 extracted from the cysts can be stored in water for a few days (around 1 week) at 4°C while waiting for analysis.
2) Species identification can be performed on non-viable (dead) J2 **only** if the stylet is visible and can be measured
3) For morphological identification, place some J2 (~ 3 per cyst), if present, in a drop of water on a slide for microscopic examination.
4) If none of the analysed cysts contains viable juveniles, the other cysts in the sample are crushed in water (e.g. using a crusher or between two microscopic slides). The crushed material is then rinsed into a small dish. If viable juveniles are present, proceed for morphological and/or molecular analysis depending on the number of J2 available. If no viable juveniles are present, the analysis is terminated. The result is then indicated as follows: ***Globodera pallida* not detected and *Globodera rostochiensis* not detected**.

4.5.2 Sampling for molecular analysis

Sampling for molecular analysis can be performed on juveniles and isolated *Globodera* cysts (up to 10 per reaction). Both cases are described below (**a** and **b**). Specimens should be processed directly to DNA extraction (see **§5.3.2**).

a. **Juveniles:** Take 2 to 3 juveniles per *Globodera* cyst (using a fishing tool) for molecular identification. Place them in a tube containing 100 µL of DNA extraction lysis buffer (Ibrahim *et al*., 1994) (see **§5.2.2**). Short centrifugation is carried out to place the juveniles at the bottom of the tube. The tubes can be frozen pending analysis.

Note: *If the cysts contain only 1 to 3 juveniles, they are all sampled for molecular analysis and not for morphological examination*

b. **Cysts:** Transfer the *Globodera* cysts to a tube containing 100 µL of DNA extraction lysis buffer (Ibrahim *et al*., 1994) (for 1 cyst) or 1 mL of the lysis buffer (for 2 to up to 10 cysts).

4.5.3 Cyst not containing viable juveniles

1) The identification of the cyst is interrupted.
2) **Particular case:** if none of the analysed cysts contains viable juveniles, the other cysts in the sample are crushed in water (e.g. using a crusher or between two microscopic slides). The crushed material is then rinsed into a small dish.
    a. If viable juveniles are present, proceed for molecular analysis as described in **§4.5.2.a** for juveniles OR;
    b. If viable juveniles are present, proceed for molecular analysis as described in **§4.5.2.b** for isolated cysts;
    c. If no viable juveniles are present, the analysis is terminated. The result is indicated as follows: ***Globodera pallida* not detected and *Globodera rostochiensis* not detected**.

4.5.4 Species identification of second-stage juveniles (J2)

1) Examination of 3 juveniles when possible
2) Heat the pre-mounted slide, preferably on a hot plate around 50°C, to kill the nematodes
3) Place a coverslip on the drop
4) Remove the water excess with filter paper and fix the coverslip (varnish or similar)
5) Place a drop of immersion oil on the preparation and observe the nematodes at high magnification (up to 1000x)
6) General characteristics of J2:
    a. Vermiform
    b. Body length: ± 500 µm
    c. At low magnification, the highly sclerotised head and the highly sclerotised stylet are visible
    d. Stylet length: ~ 21-25 µm
    e. Width and shape of basal knobs: ~ 3-5 µm
    f. The transition pharynx/intestine is oblique
    g. The tail is symmetric and conical, with a sharp terminus
    h. Hyaline tail part: 21-31 µm

    Note: *Most reliable diagnostic characteristics of J2 are stylet length, shape and width of the basal knobs (Fig. 7 and 8).*
7) The features measurements related to J2 for the different species, *G. rostochiensis* and *G. pallida*, are listed in Table 1 in **§4.6**

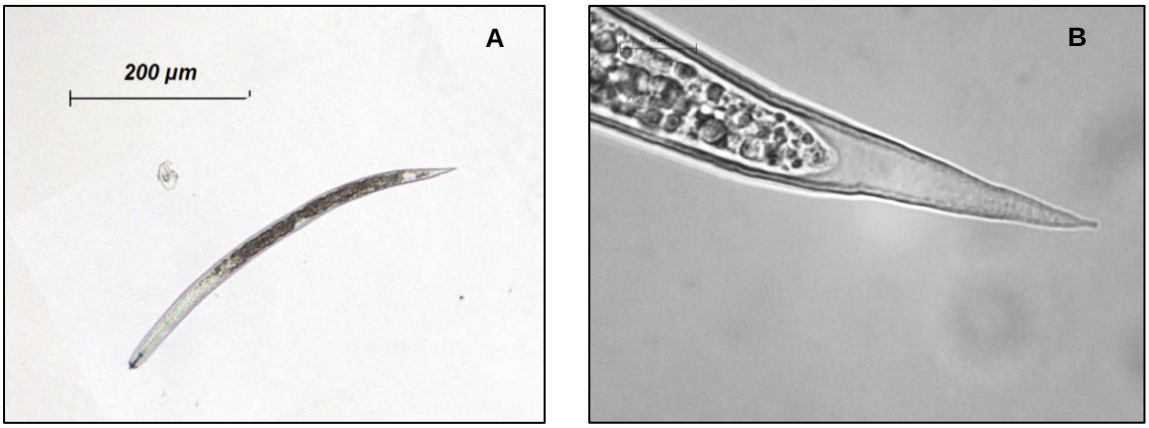


Figure 6. (A) Picture of *Globodera pallida* juvenile demonstrating the features a,b,c in point 6. (B) *Globodera* sp. tail displaying the features g and h in point 6 (from ANSES-Nematology Unit).

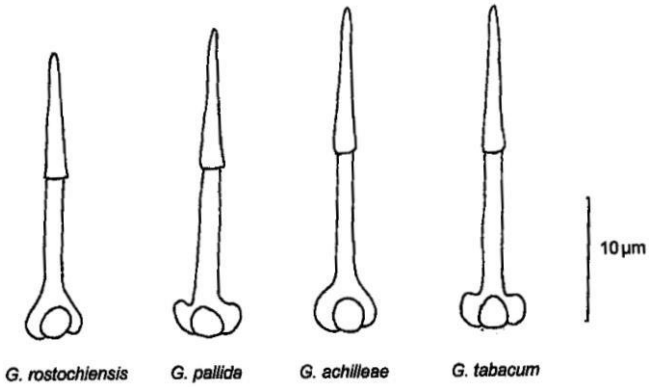


Figure 7. Stylets of 4 *Globodera* species *(*Fleming & Powers, 1998).

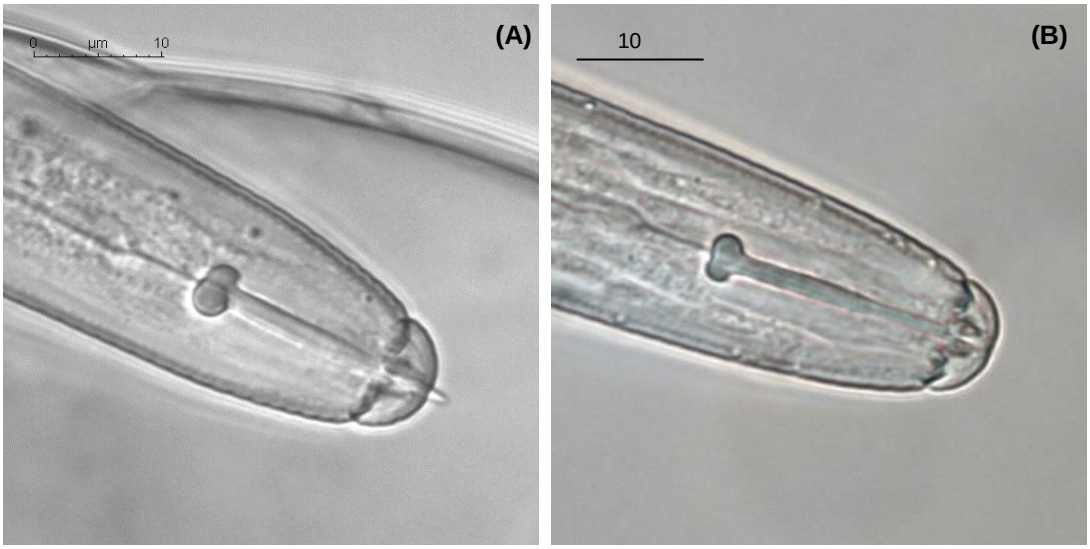


Figure 8. The stylet of second-stage juveniles (J2) of *Globodera pallida* (A) and *Globodera rostochiensis* (B) (from ANSES- Nematology Unit).

4.6 Evaluation of the morphological analysis

A combination of both the cyst and juvenile characteristics is necessary for reliable identification. Species determination of each observed cyst is performed by following the flowchart below and applying the following table.

Flowchart for morphological identification

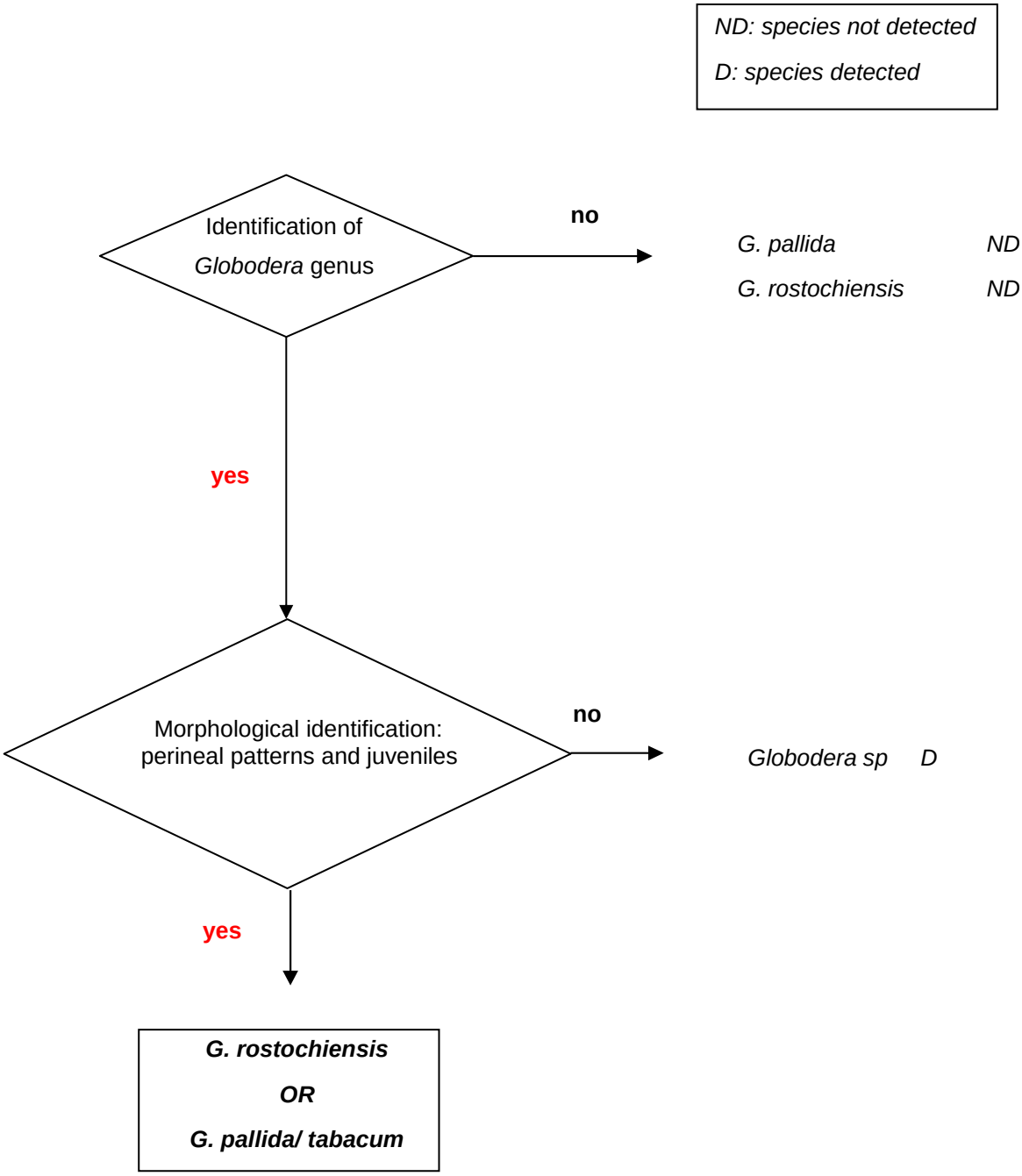


Table 1: Table's regarding the set of diagnostic features of *Globodera rostochiensis* and the group of species *G. pallida/tabacum.*

	Globodera rostochiensis	*Globodera pallida/tabacum*
Number of ridges between anus & vulval basin (N)	N > or = 20	N < or = 14
Granek's ratio (G) (1)	G > or = 3,5	G < or = 2,3
J2 stylet length (L)	L < or = 22 µm	L > or = 23 µm
Shape and width of the J2 stylet knob (B)	Small (~ 3-4 µm), round, not very prominent knobs, and not anteriorly pointed.	Large (~ 4-5 µm), prominent, anteriorly pointed knobs.
J2 body length	425 to 505 µm	440 to 527 µm

(1): G = distance from the vulval fenestra to the anal fenestra / vulval fenestra diameter

G. rostochiensis or *G. pallida* / are identified when the following 3 conditions are met for a cyst and its juveniles under evaluation:

- the 4 features N, G, L, and B could be observed,
- the L and B features for juveniles of the same cyst correspond to the same species,
- the cyst features N and G correspond to the same species.

- If one of the above 3 conditions is not fulfilled, the result is ***Globodera* sp.**

5 Molecular identification

5.1 Principle

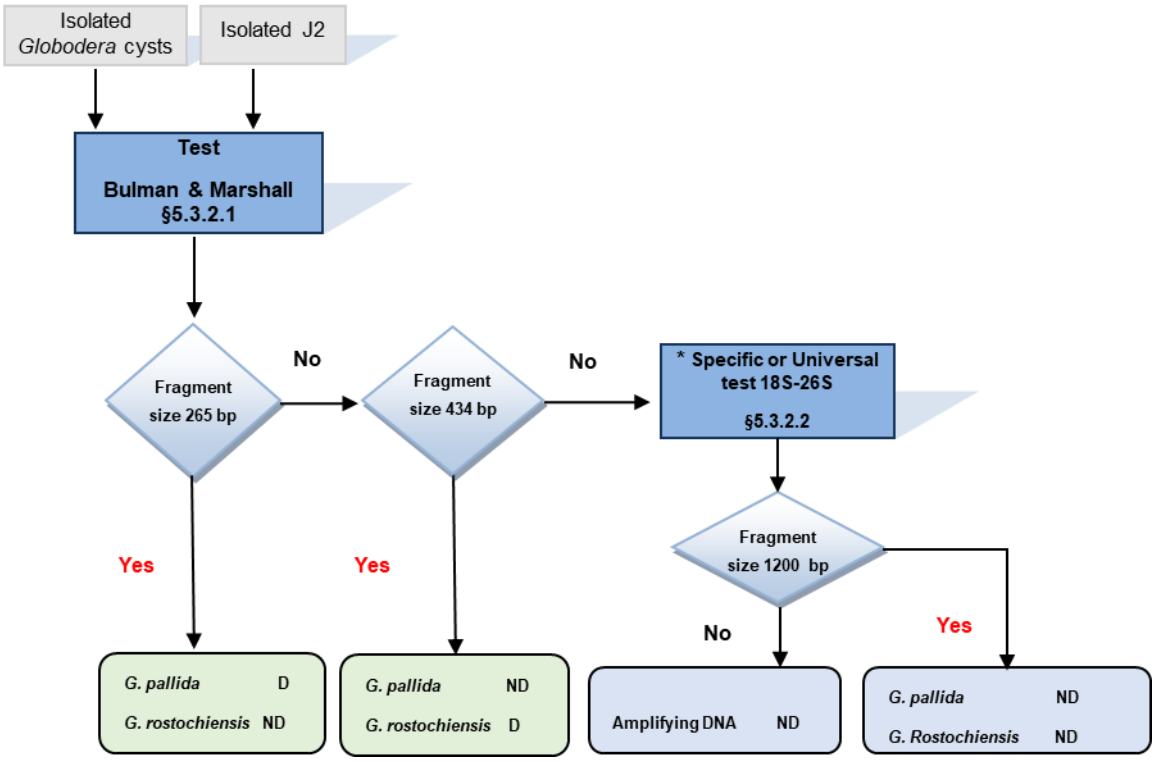
In this protocol, the conventional PCR tests from Bulman & Marshall (1997), Skantar *et al*. (2007), Thiery & Mugniery (1996) were validated by the EURL, including reagents and brand references.

The molecular identification of *G. pallida* and *G. rostochiensis* comprises the following molecular tests:

- The conventional multiplex PCR test based on Bulman & Marshall (1997). This test should be preceded by species identification based on the morphology of individual cysts and juveniles. The conventional PCR test can be performed on DNA extracted from isolated juveniles or cysts. The molecular identification test based on Bulman & Marshall (1997) amplifies a part of the 18S ribosomal DNA (rDNA) gene and the internal transcribed spacer ITS1 region with species-specific primers. Performance characteristics are available in appendix **§7.3.**
- The real-time PCR test (Gamel *et al.* 2017) can be used on the DNA extracted from second-stage juveniles replacing the conventional PCR based on Bulman & Marshall (1997).
- The universal PCR test from Thiery & Mugniery (1996) using 18S-26S primers enables the detection of the *Globodera* genus and makes it possible to show the presence of amplifiable DNA in the case of a negative result with the specific PCR test based on Bulman & Marshall (1997).
- Some *G. tabacum* populations are misidentified as *G. pallida* when using the Bulman & Marshall (1997) primers (amplification of a 265 bp fragment). Therefore, in case of suspicion of *G. tabacum*, additional testing using the conventional PCR of Skantar *et al*. (2007) can/should be applied to differentiate between these two species. This optional molecular test is available in appendix **§7.1.**

The molecular identification is carried out according to the following flowchart:

Flowchart for molecular identification of *G. rostochiensis* and *G. pallida*



* This step is performed when the Bulman & Marshall test does not produce any amplification.
In the case of *G. tabacum* suspicion, the test Skantar *et al*. (2007) can be applied (see appendix **§7.1**).
If a final result is not possible, new cysts and/or juveniles are analysed according to another specific test, either from the remaining cysts and/or juveniles or after a new extraction from the matrix.

5.2 Material and consumables

5.2.1 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 or pestle
- Shaking tissue grinder for approximately 2 mL microtubes (e.g., Tissulyser, Qiagen®) or equivalent equipment
- Conventional or real-time PCR thermal cycler
- Use pipette tips with filter plug during manipulation for PCR reactions.

5.2.2 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.

Any commercial DNA extraction kit and/or PCR mix suitable for conventional PCR application can be used as long it has been found to meet the requirements in an in-lab validation study.

Suppliers' recommendations regarding storage conditions before use should be followed as well as conservation during usage. If impossible, the laboratory should define the most optimal conditions.

The reagent or brands[2] and solution preparations are the following:

- Molecular biology grade water
- **DNA extraction lysis buffer** from Ibrahim et al., 1994 + metal/glass beads (for J2) or pestle (cysts), and as alternative freezing and defrosting the juveniles**:** 10mM Tris-HCl pH8.0, 1mM EDTA; Nonidet P40 1%; and proteinase K 100 µg mL^{-1} following Ibrahim *et al.* (1994).
- **DNA polymerase and supplied buffer:**
  - The conventional PCR assay can be performed with MP Biomedicals DNA Taq polymerase and associated buffer (ref. 11EPTQD925 or 11EPTQD025 depending on the format/size).
- **Other reagents: single reagents** (dNTP, MgCl2, etc.) or ready-to-use PCR master mixes marketed by several suppliers can be used.

- **Primers:**

Target PPNs	Test references	Primers conventional PCR	Sequence 5'- 3'
G. pallida and ***G. rostochiensis***	Bulman & Marshall (1997)	**ITS5**	GGAAGTAAAAGTCGTAACAAGG
		PITSr3*	AGCGCAGACATGCCGCAA
		PITSp4*	ACAACAGCAATCGTCGAG
All nematodes	Thiery & Mugniery (1996)	**18S**	TTGATTACGTCCCTGCCCTTT
		26S	TTTCACTCGCCGTTACTAAGG

* These primers are referred to as R3 and P4, respectively, in the remainder of the document.

[2] Brand names or suppliers may be mentioned in the description of the products necessary to implement this method. This information does not mean that the EURL recommends the exclusive use of these products. Equivalent products may be used if their performance is validated, leading to the same results.

5.3 Molecular tests

5.3.1 Controls and their purpose

Except for the PPC, which is optional, the following controls are mandatory to check the correct performance of DNA extraction and PCR steps.

Controls	Purpose	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.	Negative
Positive process control (PPC)	DNA extracted of the matrix (*Globodera* cysts) conditioned and tested similarly to the tested sample. Verify the absence of any flaw during the analytical process.	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 (viable juveniles or cysts); this control verifies that the PCR reaction has proceeded correctly, allowing the amplification of the samples containing the target.	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.	Negative
Negative specificity control (NSC)	It contains all the elements of the PCR reaction, including non-target DNA; this allows checking the absence of cross-reaction during the PCR. This type of control is not required for the universal 18S-26S molecular test.	Negative

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

5.3.2 DNA extraction

The DNA extraction procedure with the **lysis buffer** (Ibrahim *et al*., 1994) can be applied to isolated juveniles and cysts (Anthoine & Chappé, 2010).

5.3.2.1 Isolated Juveniles:

DNA extraction results from the successive action of mechanical grinding (glass or metal beads) and chemical treatment (proteinase K). The analysis is carried out on juveniles(s) that have been collected and conditioned previously according to the methodology described in **§4.5.2.a.**

1) Allow specimens conditioned in **§4.5.2.a** to defrost
2) 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 previously conditioned in the lysis buffer
3) Place the tube on a tissue grinder rack ([e.g. using a Tissulyser II (Qiagen®) shake at frequency 30 Hz for 40 sec
4) Place the tube in a water bath at approximately 55°C for at least one hour
5) Briefly centrifuge at the highest speed to precipitate the cell debris
6) Take at least 50 µl from the solution and place in a new tube
7) Incubate for 10 minutes at 95 °C to denature the proteinase K
8) The DNA can be used immediately or stored at -20 °C until use

5.3.2.2 Isolated cysts:

The *Globodera* sp. cysts isolated are conditioned in microtubes containing lysis buffer (**§4.5.2.b)**. For molecular identification, DNA extraction is performed as described below:

1) Add one or up to 10 cysts to the tube with 100 µl (for 1 cyst) or 1 ml (for 2 to 10 cysts) of DNA extraction lysis buffer
2) Crush the cysts manually using a pestle; optionally, this can be followed by a glass bead beating as described for isolated specimens on step 3 of the **§5.3.2.1**
   Note: *In the case of 2 to 10 cysts, crush the cysts first into 100 ul of lysis buffer, and then add the remaining 900 ul of lysis buffer to obtain a total volume of 1 ml*
3) Place the tube in a water bath at approximately 55°C for at least one hour
4) Briefly centrifuge at the highest speed to precipitate the cell debris
5) Take at least 100 µl from the solution and place in a new tube
6) Proceed likewise from step 7 of the **§5.3.2.1**

5.3.3 Conventional PCR tests

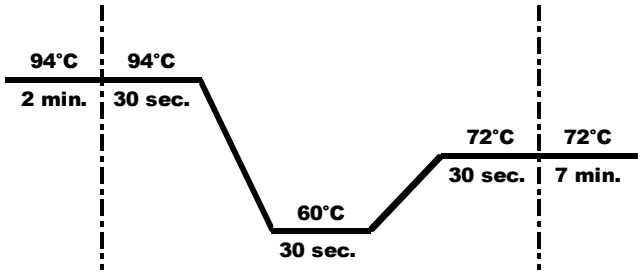
Note: *Each DNA sample is tested at least in duplicate.*

5.3.2.1 Species-specific PCR test from Bulman & Marshall (1997)

This conventional multiplex PCR test can be performed on DNA extracted with lysis buffer from isolated juveniles (J2) and isolated cysts (one or up to 10).

	Species-specific PCR test
	Test Bulman & Marshall
Reagents	**Final concentration per reaction tube**
Total volume	25 µL
Buffer Taq DNA polymerase	1 X
$MgCl_2$ (take into account the $MgCl_2$ that may be present in the Taq buffer)	2 mM
Primers	**ITS5** : 0,64 µM **R3** : 0,64 µM **P4** : 0,64 µM
dNTPs	0,25 mM
Taq DNA polymerase	0,6U/reaction
Molecular grade water water	Adjust to 20 µL
Add DNA to 20 µL of reaction mix	5 µL

PCR program:



PCR cycles 35 X

Note: *In case of DNA extraction from cysts (more than 1 cyst and up to 10), it is recommended to perform simplex PCR reactions, one containing the pairs of primers (ITS5-P4) and another reaction containing the pairs of primers (ITS5-R3). Important: Recalculate the PCR mix composition for simplex reaction.*

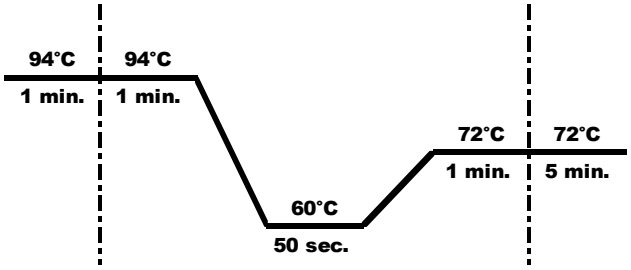
5.3.2.2 Universal PCR test

This test is carried out when the Bulman & Marshall test does not generate any amplification (see the flowchart presented in **§5.1**).

This test ensures that nucleic acid of sufficient quantity and quality has been isolated from the samples under analysis and checks the absence of PCR inhibitors.

Universal Test 18S-26S	
Reagents	**Final concentration per reaction tube**
Total volume	25 µL
Buffer Taq DNA polymerase	1 X
MgCl2 (take into account the $MgCl_2$ that may be present in the Taq buffer)	2 mM
Primer 18S	0.5 µM
Primer 26S	0.5 µM
dNTPs	0.1 mM
Taq DNA polymerase	0,5U/reaction
Molecular grade water water	Adjust to 20 µL
Add DNA to 20 µL of reaction mix	5 µL

PCR program:



PCR cycles 30 X

5.4 Evaluation and reporting the results

The result of the molecular analysis is a synthesis of the results obtained from each of the tubes analysed. The analysis for the conventional PCR is qualitative. The correct interpretation of the results is carried out by observing the amplicon generated by the PCR tests and verification of the controls. The analysis is valid if, and only if, all of the following conditions are met:

Checking the controls:

- The NPC, NTC and NSC: no amplification is observed in any of the replicates
- All replicates of PC: amplification of the amplicon in accordance with the requirements.

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

Checking the samples:

The expected fragment sizes of the amplicons are as follows:

	Multiplex test ITS5-R3-P4	**Simplex test ITS5-R3**	**Simplex test ITS5-P4**	**Universal test 18S-26S**
G. pallida	~265 bp	ø	~265 bp	~1200 bp
G. rostochiensis	~434 bp	~434 bp	ø	~1200 bp

(bp) base pairs

For all PCR tests mentioned, for each PCR reaction and after verification of the controls, the result should be as follow:

- **negative** when no amplification is observed;
- **negative** when no amplification at the expected size is observed;
- **positive** when a fragment of the expected size is observed.

The PCR test result is obtained by following the indications mentioned in the following tables:

In the case of the **species-specific Bulman & Marshall PCR test**:

Analysis		Test Results
Tube 1	**Tube 2**	
+	+	*G. rostochiensis* or *G. pallida* **detected**
+	-	The PCR is redone. After second verification, if still 1 out of 2 is positive, the result is interpreted as positive.
-	-	Universal PCR test to be performed. If universal PCR is positive, the result is: *G. rostochiensis* and *G. pallida* **not detected**

Note: + and - correspond respectively to the presence and absence of the amplification

In the case of the **Universal PCR test (18S-26S)**:

Analysis		Test Result
Tube 1	**Tube 2**	
+	+	POSITIVE
+	-	POSITIVE
-	-	NEGATIVE

Note: + and - correspond respectively to the presence and absence of the amplification

6 Final result

The final result, **per cyst,** comes from the combination of the results of the morphological and the molecular identification by applying the following decision rules:

			Morphometric identification (1)		
			Globodera sp. +	*G. pallida* +	*G. rostochiensis* +
Molecular identification	*G. pallida*	D	*G. pallida* **D**	*G. pallida* **D**	→ (2)
	G. rostochiensis	D	*G. rostochiensis* **D**	→ (2)	*G. rostochiensis* **D**
	G. pallida *G. rostochiensis*	ND ND	*G. pallida* **ND** *G. rosto*chiensis **ND**	→ (2)	→ (2)
	No molecular results		→ (2)	(2)	→ (2)

D detected; ND: not detected

(1) : If cysts of *Globodera sp*. are present without viable contents, the final result is ***G. pallida* not detected** and ***G. rostochiensis* not detected**. There is no molecular identification.

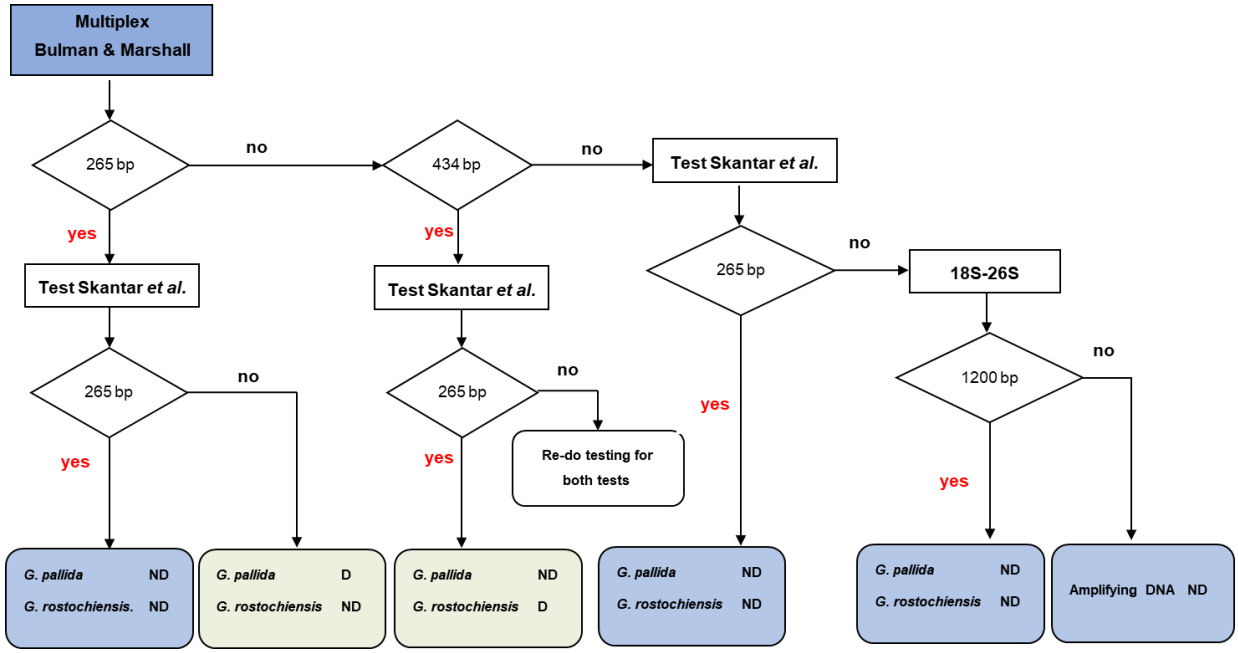
(2) : No results are obtained. New morphological and molecular analyses are undertaken. Depending on the remaining material, the following steps are taken **in order of priority**:

- In the presence of juvenile remnants of the analysed cyst, new juveniles are mounted and measured. Molecular identification is carried out;
- In the absence of juveniles remnants of the cyst in question, but the presence of cyst remaining in the sample, morphological and molecular identification are carried out on at least 3 new cysts (if possible);
- If there is no remaining material or if the discrepancy persists, the final result is **"*Globodera* sp. detected"**.

7 Appendix

7.1 Molecular test for *G. rostochiensis* and *G. pallida* in case of suspicion of *G. tabacum*

Flowchart in case of *G. tabacum* suspicion



- Positive PCR control (PC) and Negative specificity control (NSC) should be included in each PCR reaction.
- If DNA is not amplified, new juveniles and/or cysts are analysed by molecular analysis, either from the remaining extract or after a new extraction from the matrix (if available).

1) **Primers:**

Target PPNs	Test references	Primers	Sequence 5'- 3'
G. rostochiensis and ***G. tabacum***	Skantar *et al.* (2007)	**PITSt4***	ACA GCA GCA ATC GTC GGC

* This primer is referred to as T4 in the remainder of the document.

2) **DNA extraction:** The DNA extraction procedure is the same as described in **§5.3.2**.

3) **Controls:** For molecular controls, see **§5.3.1.** According to the PCR tests, the DNA extracts required to be included are from the following species:

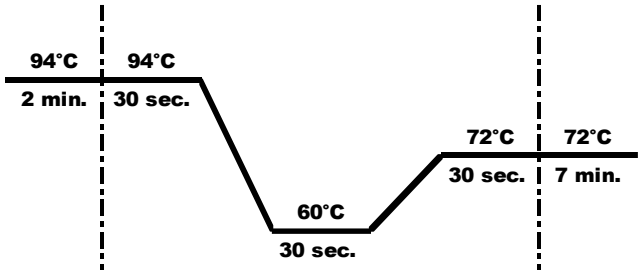
	Test ITS5-T4	Universal Test (18S-26S)
Positive PCR control (PC)	*G. rostochiensis* and *G. tabacum*	A species of *Globodera* (for example, *G. pallida* or *G.rostochiensis*)
Negative specificity control (NSC)	*G. pallida*	

-These controls make it possible to check the correct process of the DNA extraction and PCR steps.
- If available, the target species may be supplied by the EURL.

4) **Species-specific PCR test Skantar *et al.* (2007)**

	Species-specific PCR test
	Test Skantar *et al.*
Reagents	**Final concentration per reaction tube**
Total volume	25 µL
Buffer Taq DNA polymérase	1 X
$MgCl_2$ (take into account the $MgCl_2$ that may be present in the Taq buffer)	1,5 mM
Primers	**ITS5** : 0,64 µM **T4** : 0,64 µM
dNTPs	0,16 mM
Taq DNA polymerase	0,6U/reaction
Ultra-pure water	Adjust to 20 µL
Add DNA to 20 µL of reaction mix	5 µL

5) **PCR program:**



PCR cycles 35 X

2) The Universal PCR test (detailed in **§5.3.2.2**) is carried out when the species-specific **Skantar *et al.* (2007)** test does not generate any amplification (see the flowchart presented in **§7.1**). This test ensures that nucleic acid of sufficient quantity and quality is isolated from samples under analysis and checks the absence of PCR inhibitors.

3) **Evaluation and reporting the results:**

The molecular analysis result is a synthesis of the results obtained from each of the microtubes analysed. The analysis for the conventional PCR is qualitative. Regardless of the PCR test, the result is:

- **negative** when no amplification is observed;
- **negative** when no amplification at the expected size is observed;
- **positive** when a fragment of the expected size is observed.

The expected fragment sizes are as follows:

	Test ITS5-T4	**Test 18S-26S**
G. pallida	No amplification	~1200 bp
G. rostochiensis	~265 bp	~1200 bp
G. tabacum	~265 bp	~1200 bp

(bp) base pairs

4) **Final result :**

The final result, **per cyst**, comes from the combination of the results of the morphological and the molecular identification of this cyst by applying the following decision rules:

		Morphological identification (1)		
		Globodera sp. **D**	*G. pallida/tabacum* **D**	*G. rostochiensis* **D**
Molecular results: a combination of Bulman & Marshall, Skantar *et al.* and universal test	*G. pallida* **D**	*G. pallida* **D**	*G. pallida* **D**	→ **(2)**
	G. rostochiensis **D**	*G. rostochiensis* **D**	→ **(2)**	*G. rostochiensis* **D**
	G. tabacum **D**	*G. pallida* **ND** *G. rostochiensis* **ND** **(3)**	*G. pallida* **ND** *G.rostochiensis* **ND** **(3)**	→ **(2)**
	G. pallida **ND** *G. rostochiensis* **ND**	*G. pallida* **ND** *G. rostochiensis* **ND**	→ **(2)**	→ **(2)**
	No molecular results	→ **(2)**	→ **(2)**	→ **(2)**

D detected; ND: not detected

Molecular results: the combination of Bulman & Marshall (1997), Skantar *et al.* (2007) and universal tests

(1) : If cysts of *Globodera sp*. are present without viable contents, the final result is ***G. pallida* not detected** and ***G. rostochiensis* not detected**. There is no molecular identification.

(2) : No results analysis are delivered. New morphological and molecular analyses are undertaken. Depending on the remaining material, the following steps are taken **in order of priority**.

- In the presence of juveniles remnants of the cyst in question, new juveniles are mounted and measured. In addition, new morphological measurements of the cyst perineal area are taken from the preserved mounting slide. To confirm the previous measurements; a molecular identification is carried out;
- In the absence of juveniles remnants of the cyst in question, but the presence of cyst remaining in the sample, morphological and molecular identification are carried out on at least 3 new cysts (if possible);
- If there is no remaining material amount or the discrepancy persists, the final result is **"*Globodera* sp. detected"**.

(3) Result "***Globodera tabacum* detected".**

7.2 Performance criteria and validation of the morphological method

Morphological validation was applied to the following species/populations:

- 7 populations of *Globodera pallida* ;
- 10 populations of *G. rostochiensis* ;
- 3 populations of *G. tabacum.*

The morphological identification does not make it possible to differentiate between *Globodera pallida* and *G. tabacum*; thus, the method evaluation concerned the *Globodera "pallida / tabacum*" group and *G. rostochiensis*.

The method was evaluated separately for *Globodera "pallida / tabacum"* and for *G. rostochiensis.*

The tested evaluation criteria are:

- Specificity: Ability of the method to not detect the target from a range of non-target populations (absence of false positives);
- Sensitivity: Ability of the method to detect the target from a range of target populations (absence of false negatives);
- Repeatability: Ability of the method to produce identical results under identical conditions from a set consisting of one target preparation among several non-target preparations.
- Accuracy: (Intra-laboratory reproducibility): the ability of the method to produce identical results under different conditions (equipment, operators, etc.).

Note: *The detection threshold is not assessed as identification is carried out at the lowest level, a single individual (1 cyst).*

The evaluation was performed on 3 cysts for each criterium as this was considered the necessary number of cysts to carry out the analysis under optimal conditions. Cysts were obtained from pure cultures of known *Globodera* sp.

Table 1. Performance of the method according to the different criteria using 3 cysts per criterium for each group.

Performance criteria	*Globodera "pallida / tabacum"**	*Globodera rostochiensis**
Specificity	100 %	100 %
Sensitivity	76,7 %	66.7 %
Repeatability	100 %	66,7 %
Accuracy "materials"	100 %	83 %
Accuracy"operators"	92 %	67 %
Interpretation of the results	difficult	difficult

Note :

- *It is important to stress that although the evaluation of this method shows reliable specificity, the results of sensitivity, repeatability and "operator" accuracy, particularly for Globodera rostochiensis, demonstrate the importance of well-trained personnel and confirm the need to couple morphological identification with molecular tests.*

- *On the other hand, as shown in the repeatability tests, it is necessary that the observations of the mounted juveniles are made immediately after mounting since their rapid alteration can affect the quality of the measurements.*
- *Molecular tests (performed on 3 juveniles from each of the cysts observed) were systematically carried out following the morphological identifications, as foreseen by this protocol; these always conformed to the expected results.*

7.3 Performance evaluation and validation of the conventional molecular test

All molecular tests in this protocol were evaluated and validated according to the following criteria:

- Specificity: Ability of the method to not detect the target from a range of non-target populations (absence of false positives);
- Sensitivity: Ability of the method to detect the target from a range of target populations (absence of false negatives);
- Repeatability: Ability of the method to reproduce identical results under identical conditions from a set consisting of one target preparation among several non-target preparations.
- Accuracy: (Intra-laboratory reproducibility): the ability of the method to reproduce identical results under different conditions (equipment, operators, etc.).

The validation of the conventional PCR test results from a comparative evaluation of different molecular tests to identify *G. pallida*, *G. rostochiensis* and *G. tabacum.*

The molecular tests were applied to the following species/populations:

- 11 populations of *Globodera pallida* ;
- 4 populations of *G. rostochiensis* ;
- 5 populations of *G. tabacum ;*
- 1 population of *G. mexicana ;*
- 1 population of *G. artemisiae.*

The test described by Bulman and Marshall (1997) with the primers ITS5-R3-P4 makes it possible to identify *G. pallida* and *G. rostochiensis* specifically. Only a Bolivian population of *G. rostochiensis* was not detected, and the *G. tabacum virginiae* population from the USA gave an amplification corresponding to *G. pallida*. The test's sensitivity was found to be 100% for 1 *G. rostochiensis* juvenile and 96% for 1 *G. pallida* juvenile.

The combination of tests using the primers described by Bulman *et al.* (1997) and the ITS5-T4 pair described by Skantar *et al.* (2007) allowed the identification of *G. pallida*, *G. rostochiensis* and *G. tabacum*. Moreover, these tests showed a correct sensitivity with a percentage of detection reaching 100% for *G. rostochiensis* and 96% for *G. pallida* with the primers ITS5-R3-P4 and 100% for *G. rostochiensis* and 96% for *G. tabacum* with the primers ITS5-T4.

The two multiplex PCR tests with species-specific primers were selected based on their criteria performance when applied to the different *Globodera* spp. populations cited above.

The universal PCR test using 18S-26S primers from Thiéry & Mugniéry (1996) makes it possible to detect the *Globodera* genus and show the presence of amplifiable DNA in case of a negative result with the specific PCR tests.

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

Acknowledgements
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