

PM 7/40 (5) *Globodera rostochiensis* and *Globodera pallida*

Specific scope: This Standard describes a Diagnostic Protocol for *Globodera rostochiensis* and *Globodera pallida*.¹ The terms used are those in the EPPO Pictorial Glossary of Morphological Terms in Nematology.²

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Authors and contributors are given in the Acknowledgements section

Specific approval and amendment: Approved as an EPPO Standard in 2003-09. Revisions approved in 2009-09, 2012-09 and 2017-02. Fourth revision approved in 2021-10.

1 | INTRODUCTION

Globodera rostochiensis and *Globodera pallida* (potato cyst nematodes, PCNs) cause major losses in *Solanum tuberosum* (potato) crops (van Riel & Mulder, 1998). The main route of spread of these nematodes is movement of infested soil (e.g. on farm machinery, adhering to tubers). Infestation occurs when the second-stage juvenile hatches from the egg and enters the root near the growing tip by puncturing the epidermal cell walls, and then the internal cell walls, with its stylet. Eventually it begins feeding on cells in the pericycle, cortex or endodermis. The nematode induces enlargement of the root cells and breakdown of their walls to form a large, syncytial transfer cell. This syncytium provides nutrients for the nematode. Infested potato plants have a reduced root system and, because of the decreased water uptake, death of the plant can eventually occur.

In this Diagnostic Protocol different tests for detection and identification are presented which can be used depending on the circumstances. In some EPPO countries, official control is in place and routine testing is required. For such routine testing in the country itself molecular techniques can be very useful. In other situations, such as the testing of imported material for potential quarantine or damaging nematodes or new infestations, identification by morphological methods performed by experienced nematologists is more suitable (PM 7/76 *Use of EPPO diagnostic protocols*).

¹Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

²http://www.eppo.int/QUARANTINE/diag_activities/EPPO_TD_1056_Glossary.pdf.

A flow diagram describing the diagnostic procedure for *G. rostochiensis* and *G. pallida* is presented in [Figure 1](#).

2 | IDENTITY

Name: *Globodera rostochiensis* (Wollenweber, 1923), Skarbilovich, 1959.

Synonyms: *Heterodera rostochiensis*, Wollenweber, 1923; *Heterodera schachtii solani* Zimmerman, 1927; *Heterodera schachtii rostochiensis* (Wollenweber) Kemner, 1929;

Taxonomic position: Nematoda, Tylenchida,³ Heteroderidae.

EPPO Code: HETDRO.

Phytosanitary categorization: EPPO A2 List no. 125, A2 Quarantine pest (Annex II B).

Name: *Globodera pallida* (Stone, 1973).

Synonyms: *Heterodera pallida* (Stone, 1973).

Taxonomic position: Nematoda, Tylenchida,³ Heteroderidae.

EPPO Code: HETDPA.

Phytosanitary categorization: EPPO A2 List no. 124, A2 Quarantine pest (Annex II B).

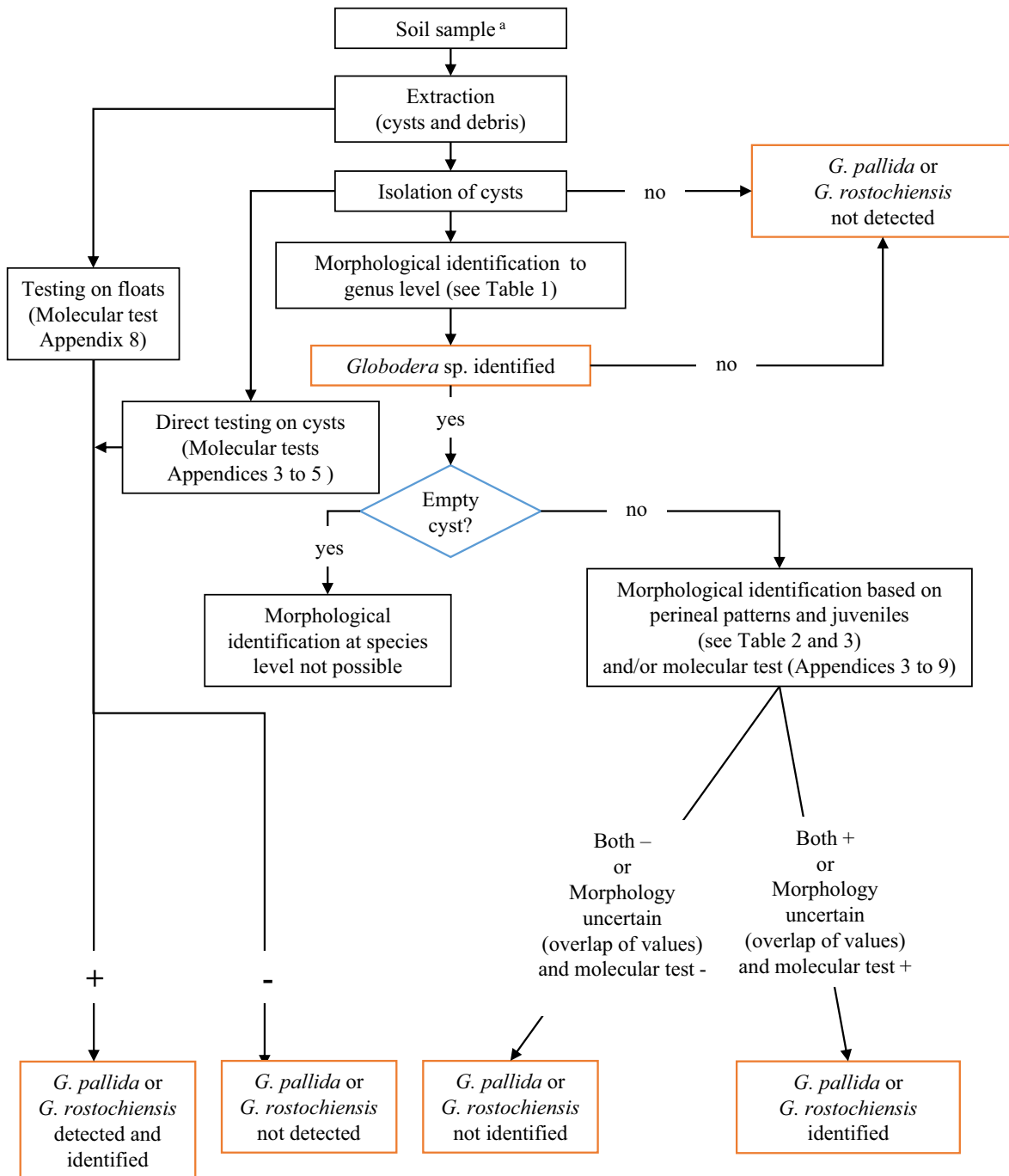
Note on the taxonomy: it should be noted that a recent study, Thevenoux et al., 2020, has shown the presence of a larger genetic diversity in *G. pallida* than previously known, suggesting the presence of a new species in the south of Peru.

3 | DETECTION

3.1 | Symptoms

Above-ground symptoms due to PCNs are not specific and often go undetected. General symptoms include patches of poor growth in the crop, with plants sometimes showing yellowing, wilting or death of foliage; tuber size is reduced and roots are extensively branched with soil stuck to them. However, there are many other causes of these symptoms. Plants should therefore be lifted for a visual check for the presence of cysts and young females on the roots, or a soil sample should be taken for testing. Young females and cysts are just visible

³Developments combining a classification based on morphological data and molecular analysis refer to 'Tylenchomorpha' (De Ley & Blaxter, 2004).



^a juveniles of cyst nematodes may be found incidentally in soil extracts (see section 4.1.1.2.) in such cases it is strongly advised to perform a cyst extraction where possible or to perform a molecular test on the juveniles (Appendices 3 to 9).

A combination of morphological and molecular methods is highly recommended for the identification of *G. rostochiensis* and *G. pallida*, especially when new introductions are suspected.

FIGURE 1 Flow-diagram for the identification of *Globodera rostochiensis* and *Globodera pallida*

to the naked eye as tiny white, yellow or brown pin-heads on the root surface (Figures 2 and 3). Detection by lifting plants is only possible for a short time as females mature into cysts and then can easily be lost at lifting, and this method is time-consuming. Soil testing is therefore the best way to determine the presence of PCNs.

3.2 | Statutory sampling procedures

Recommendations on sampling can be found in Council Directive 2007/33/EC of 11 June 2007 on the control of PCN and Repealing Directive 69/465/EEC (EU, 2007).



FIGURE 2 Potato roots infected by *G. rostochiensis*. (Courtesy: NRC-NPPO, the Netherlands.)

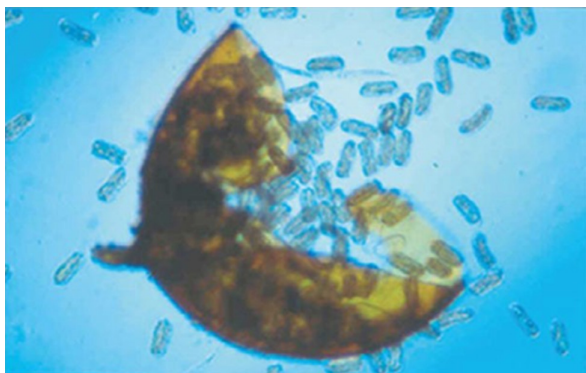


FIGURE 3 Broken cyst with eggs of *G. pallida*. (Courtesy: NRC-NPPO, the Netherlands.)

3.3 | Extraction procedures

There are various processes for extracting cysts from the soil. Simple methods based on flotation can be as good as elutriation. Extraction methods are described in PM 7/119 *Nematode extraction* (EPPO, 2013). *Globodera* cysts are generally round, which distinguishes them from most other types of nematode cysts. Prior to identification, cysts need to be removed from the floats. This process usually requires examination of the float by staff trained in separating nematode cysts from similar globular bodies in the soil. It can be time-consuming, depending upon the efficiency of extraction and whether any further clean-up has been used, e.g. acetone flotation. This process is critical to the efficiency of the diagnosis because false-negative results may result if any *Globodera* cysts are missed at this stage. The distinction between PCNs and other cysts based on morphology can only be reliably performed by trained experts.

When moist soil samples are not immediately processed and viability tests are envisaged, they should be stored above zero and below 5°C as temperature influences hatching behaviour (Muhammad, 1996; Sharma & Sharma, 1998). Soil samples should not be dried at a temperature higher than approximately 35°C as this might also influence the viability.

Educational videos on cyst extraction are available on the website of the European Union Reference Laboratory for Plant Parasitic Nematodes (<https://sites.v2.anses.fr/en/minisite/plant-parasitic-nematodes/video-s-media>).

3.4 | Bioassay

Another procedure for detecting the nematodes is bioassay (Appendix 1, test A).

3.5 | Direct testing of soil extracts or cysts

The following molecular tests can be performed on soil extracts or cysts. Appendix 2 describes nucleic acid extraction.

Test	Appendix
Multiplex real-time PCR test (Gamel et al., 2017) for the detection and identification of <i>G. rostochiensis</i> and <i>G. pallida</i>	3
High-throughput diagnosis of PCNs (<i>Globodera</i> spp.) in soil samples using real-time PCR (Reid et al., 2015)	4
Real-time PCR tests for species-specific identification as well as the detection of <i>G. rostochiensis</i> , <i>G. pallida</i> and <i>Globodera tabacum</i> (based on LSU rDNA), available as an all-inclusive real-time PCR kit (http://www.clear-detections.com)	5

4 | IDENTIFICATION

For the identification of *G. rostochiensis* and *G. pallida* cysts and other stages, it is highly recommended to combine morphological and molecular methods, especially when new introductions are suspected.

4.1 | Identification on the basis of morphological features

For morphological examination, second-stage juveniles and cysts should be obtained from the soil, plant roots or tubers. The colour of the female at the appropriate stage of development can be used as an indication of species: a female that changes during maturation from white to yellow then into a brown cyst is *G. rostochiensis*, while one that changes from white directly to brown is *G. pallida*. Differential interference contrast is highly recommended for identifying specimens mounted on microscope slides.

4.1.1 | Identification of cyst and juveniles to genus level

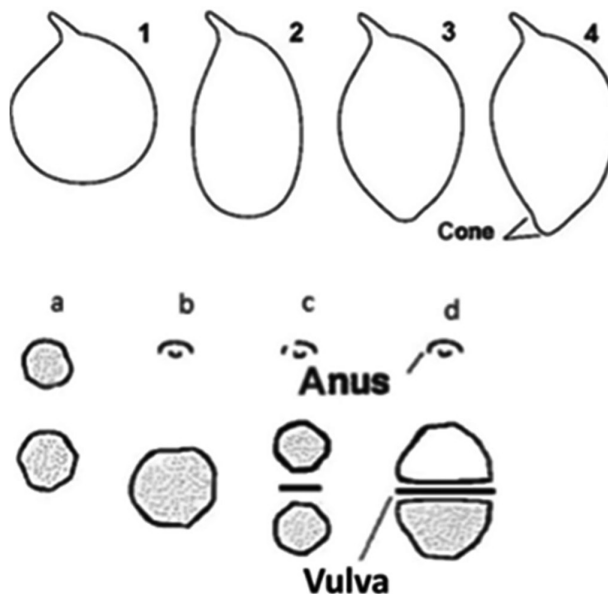
4.1.1.1 | Cysts

Identification of Heteroderidae cysts to genus level is based on the form of the cysts and the characteristics of the vulval–anal region (Table 1 and Figures 4–7). Further information is provided by the keys of Brzeski (1998), Baldwin and Mundo-Ocampo (1991), Wouts and Baldwin (1998), Siddiqi (2000) and Subbotin et al. (2010).

Globodera cysts should present the following characteristics: cysts of *Globodera* are smoothly rounded with a small projecting neck, no terminal cone, diameter $\pm 450 \mu\text{m}$, and with a tanned brown skin (Figure 6a). The cuticle surface has a zigzag pattern of ridges. The perineal area (Figures 5 and 7a) consists of a single circumfenestration around the vulval slit, with tubercles on crescents near the vulva. The anus is subterminal without fenestra, the vulva is in a vulval basin; underbridge and bullae are rarely present (Fleming & Powers, 1998), and in particular are not present in *G. rostochiensis* and *G. pallida*. Eggs are retained in the cyst, with no egg-mass present.

TABLE 1 Dichotomous key to genus of Heteroderidae cysts

1	Lemon-shaped cyst	Not <i>Globodera</i>
	Round or oval cyst	2
2	Two large, separated fenestrae of equal size	<i>Punctodera</i>
	One large vulval fenestra	<i>Globodera</i>



- 1- Spherical (*Globodera*)
- 2- Ovoid (*Punctodera*)
- 3- Lemonshape with reduced cone (some *Heterodera* or *Cactodera*)
- 4- Lemonshape including prominent cone (most *Heterodera*)

- a : Anal region fenestrate, vulva region circumfenestrate (*Punctodera*)
- b : Anal region non fenestrate, vulva region circumfenestrate, (*Globodera* & *Cactodera*)
- c : Anal region non fenestrate, vulva region semi fenestrate-bifenestrate (*Heterodera*)
- d : Anal region non fenestrate, vulva region semi fenestrate-ambifenestrate (*Heterodera*)

FIGURE 4 Form of cysts and characteristics of the vulval–anal region. (After Baldwin and Mundo-Ocampo, 1991.)

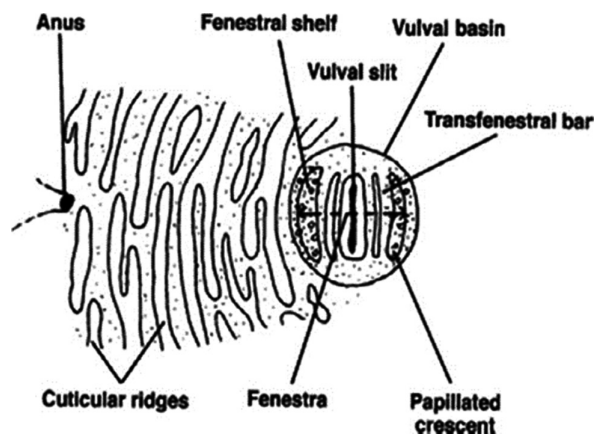
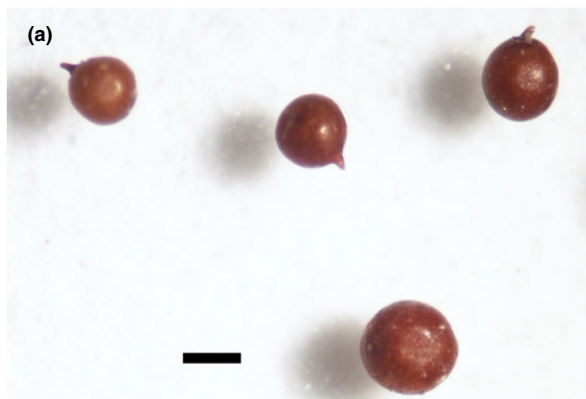


FIGURE 5 The perineal region of a *Globodera* cyst (Hesling, 1978)



Globodera spp.



Heterodera spp.

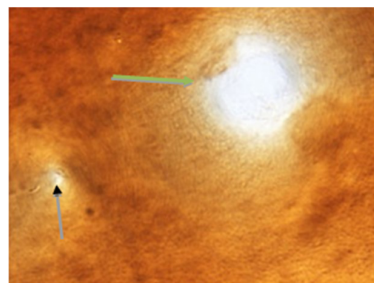


Punctodera spp.

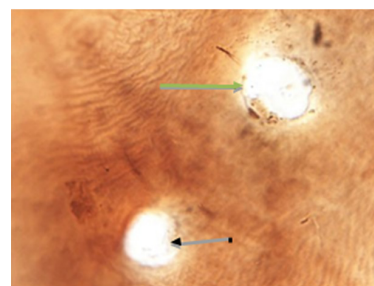
FIGURE 6 Heteroderidae cysts. Scale bar =350 μ m. (Courtesy NAK, the Netherlands.)

4.1.1.2 | Juveniles

In addition to the juveniles in cysts, juveniles of cyst nematodes may be found incidentally in soil extracts after extraction for the detection of the non-sedentary stages of nematodes. Distinction between the juveniles of *Globodera* and other Heteroderidae is difficult; in such cases it is strongly advised to perform a cyst extraction where possible or to perform a molecular test on the juveniles (see Section 4.2) and to proceed with this Diagnostic Protocol. Some information, however, is provided below.



Globodera sp. vulval fenestra/anal region non fenestrated



Punctodera sp. vulval fenestra/anal region fenestrated

FIGURE 7 Perineal region. Green arrows indicate the vulva and black arrows the anus. *Globodera* spp. vulval fenestra/anal region non-fenestrated. *Punctodera* spp. vulval fenestra/anal region fenestrated. (Courtesy NAK, the Netherlands.)

Globodera juveniles should present the following characteristics: the mobile second-stage juveniles of *Globodera* are vermiform and annulated, and taper at head and tail regions. Within the genus *Globodera*, body length ranges from 445 to 510 μ m, stylet length is 18–29 μ m, tail length is 37–55 μ m and the hyaline tail part is 21–31 μ m.

Juveniles of cyst nematodes can be distinguished from juveniles of root-knot nematodes (*Meloidogyne* spp.) by a more heavily sclerotized lip region, a relatively strong stylet, the shape of the tail and more robust appearance (Figure 8). In such cases it is advised to perform a cyst extraction or a molecular test on the juveniles.

The morphological key to *Globodera* species presented in Table 2 has used the mean average of morphometric characters to assist with differentiation, owing to the large overlap of ranges. If diagnosis of a population is carried out using morphological examination only, it is recommended to compare specimens with recent taxonomic descriptions and with the information provided in Table 3. However, as stated above, for the identification of *G. rostochiensis* and *G. pallida* it is highly recommended to combine morphological and molecular methods, especially when new introductions are suspected.

4.1.2 | Identification to species level

The identification of *Globodera* to species level based on morphology can be difficult because of the observed variability of key characteristics. Therefore, the use of a combination of cyst and second-stage juvenile

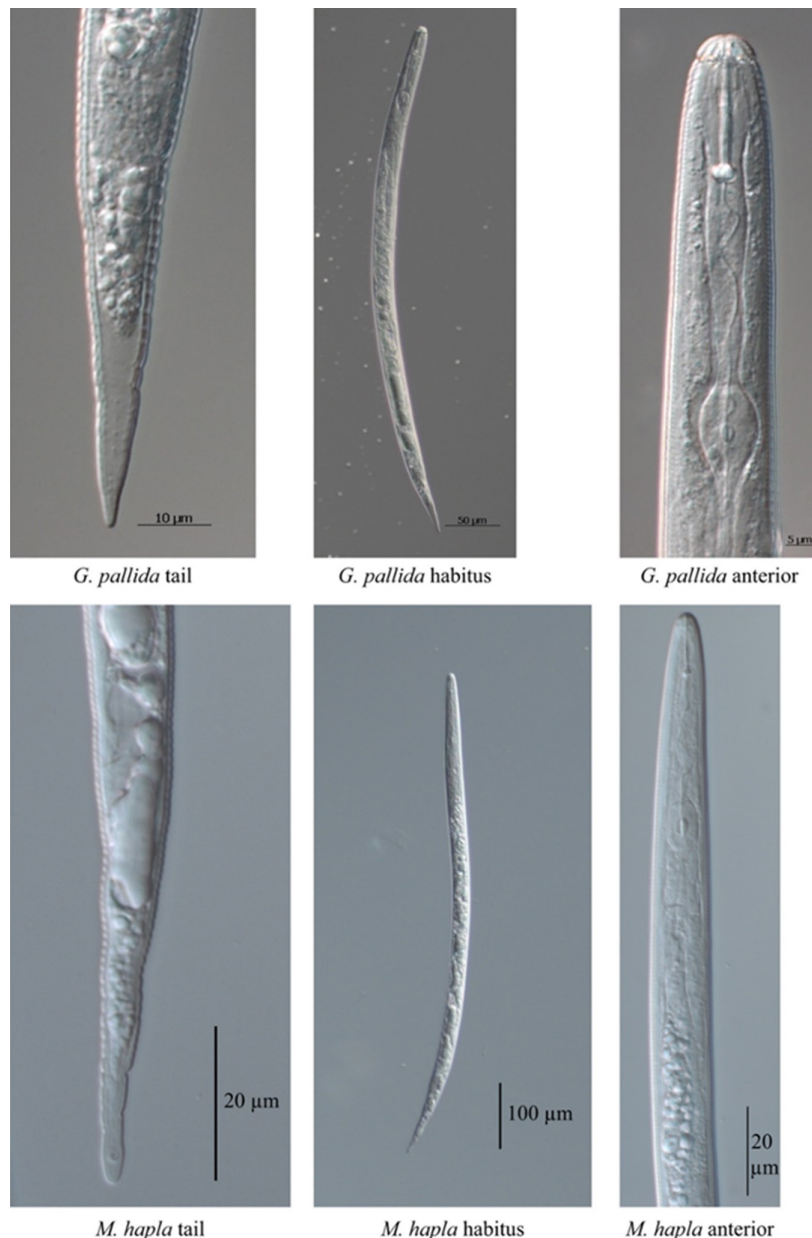


FIGURE 8 Difference between Meloidogynidae and Heteroderidae juveniles. Comparison between *Meloidogyne hapla* and *G. pallida*. (Courtesy FERA, GB)

characteristics is recommended for reliable identification. First the nematodes should be identified with the key presented in Table 2. If the nematodes are identified as PCN species, species identification should be performed using the morphological and morphometric characters presented in Table 3.

Globodera rostochiensis and *G. pallida* are morphologically and morphometrically closely related (Stone, 1973a,b). Figure 9 presents some drawings of different stages of *G. rostochiensis* (Figure 9a) and *G. pallida* (Figure 9b). For cysts, the most important diagnostic differences are in the perineal area, i.e. the number of cuticular ridges between the vulva and anus and Granek's ratio (Figure 10a,b). The second-stage juvenile characteristics are stylet length and stylet knob shape and width (Table 3,

Figure 10c). As the range of values for each of these characteristics can overlap between species, care is needed. In such cases, confirmation with molecular techniques is recommended. It should also be noted that this data is for specific populations described in the publications and that natural deviations from the range may occur.

When cysts without live content, meaning that they do not contain viable eggs or second-stage juveniles, are found, species identification is not possible.⁴

An educational video on the morphological identification of *G. pallida* and *G. rostochiensis* (perineal

⁴It should be noted that under European conditions, especially when cysts without live content have been detected in fields used for the production of potato in the past, it is highly probable that these cysts belong to either one of the PCN species *G. rostochiensis* or *G. pallida*.

TABLE 2 Dichotomous key to *Globodera* species (after Subbotin et al. (2010))

1	Cuticle of cyst thin, transparent	<i>G. mali</i>
	Cuticle of cyst thick, dark in colour	2
2	Mean length of J2 stylet $\leq 26 \mu\text{m}$	3
	Mean length of J2 stylet $\geq 27 \mu\text{m}$	<i>G. zelandica</i>
3	Mean length of J2 stylet $< 19 \mu\text{m}$	<i>G. leptonepia</i>
	Mean length of J2 stylet $\geq 19 \mu\text{m}$	4
4	Hyaline region of J2 $> 31 \mu\text{m}$	<i>G. bravoae</i>
	Hyaline region of J2 $\leq 31 \mu\text{m}$	5
5	Mean Granek's ratio usually > 2 , mostly parasites of Solanaceae	6
	Mean Granek's ratio ≤ 2 , mostly parasites of Asteraceae	11
6	Combination of: mean J2 DGO $\geq 5.5 \mu\text{m}$; mean Granek's ratio < 3 ; J2 lip region with 4–6 annules, stylet knobs rounded to slightly anteriorly projected	7
	Not with the above combination of all characters; mean J2 DGO $< 5.5 \mu\text{m}$	8
7	Cyst wall lacking a network-like pattern, ridges close; mean number of cuticular ridges = 13 (10–18); δ spicules with a pointed, thorn-like tip	<i>G. ellingtonae</i>
	Cyst wall exhibiting network-like or maze-like patterns; mean number of cuticular ridges = 7–8 (5–15); δ spicules with a finely rounded tip	<i>G. tabacum sensu lato</i>
8	Cysts with prominent bullae in the terminal region of most specimens; J2 lip region with 3 annules, mean hyaline region $> 28 \mu\text{m}$	<i>G. capensis</i>
	Cyst abullate, at most with small vulval bodies in some specimens; J2 lip region with 4–6 annules, mean hyaline region $< 28 \mu\text{m}$	9
9	J2 stylet knobs distinctly anteriorly directed to flattened anteriorly; mean J2 stylet length $> 23 \mu\text{m}$; Granek's ratio < 3	10
	J2 stylet knobs rounded to flattened anteriorly; mean J2 stylet length $< 23 \mu\text{m}$; Granek's ratio ≥ 3	<i>G. rostochiensis</i>
10	Mean Granek's ratio = 2.1–2.5	<i>G. pallida</i>
	Mean Granek's ratio = 2.8	<i>G. mexicana</i>
11	J2 lip region with 5–6 annules	12
	J2 lip region with 3 annules	<i>G. capensis</i>
12	Mean stylet $\geq 25 \mu\text{m}$ in J2, δ gubernaculum = 11.2–12.9 μm	<i>G. millefolii</i>
	Mean stylet $< 25 \mu\text{m}$ in J2, δ gubernaculum = 6.0–9.9 μm	<i>G. artemisiae</i>

TABLE 3 Morphological and morphometric characters useful for identification of *Globodera* species, range and mean values in μm (after Lownsbery & Lownsbery, 1954; Eroshenko & Kazachenko 1972; Golden & Klindic, 1973; Stone, 1973a & b, Baldwin & Mundo-Ocampo, 1991; Mota & Eisenback, 1993; Brzeski, 1998; Flemming & Powers, 1998; Manduric & Anderson, 2004)

Species	J2 stylet			Cyst measurements		
	J2 body length	Knob width	Knob shape	Stylet length	Number of cuticular ridges between anus and vulval basin	Granek's ratio
<i>G. rostochiensis</i>	468 (425–505)	3–4	Rounded to Anteriorly flattened	21.8 (19–23)	12–31 ^b (usually > 14)	1.3–9.5 (> 3)
<i>G. pallida</i>	484 (440–525)	4–5	Distinct forward projections	23.8 (22–24)	8–20 (usually < 14)	1.2–3.5 (< 3)
<i>G. tabacum</i>	476 (410–527)	4–5	Rounded to slightly anteriorly projected	24 (22–26)	5–15	1–4.2 (< 2.8)
<i>G. millefolii</i> ^a	492 (472–515)	4–5	Rounded to anteriorly projected	25 (24–26)	4–11	1.6 (1.3–1.9)
<i>G. artemisiae</i>	413 (357–490)	3–5	Rounded to anteriorly flattened	22.6 (18–29)	5–16	1.0 (0.8–1.7)

^aKrall (1978) considered *G. millefolii* (Kirjanova & Krall, 1965) Behrens, 1975 as *species inquirenda*, as the description was based on a single female. Brzeski (1998) reported on *G. achilleae*: 'it may be conspecific with *G. millefolii*'. According to Subbotin et al., 2010, 2011 *G. achilleae* is a junior synonym of *G. millefolii*. So from this point onwards the species name *G. achilleae* will not be used and *G. millefolii* instead.

^bFrom Flemming & Powers (1998); Brzeski (1998) refers to 16–31.

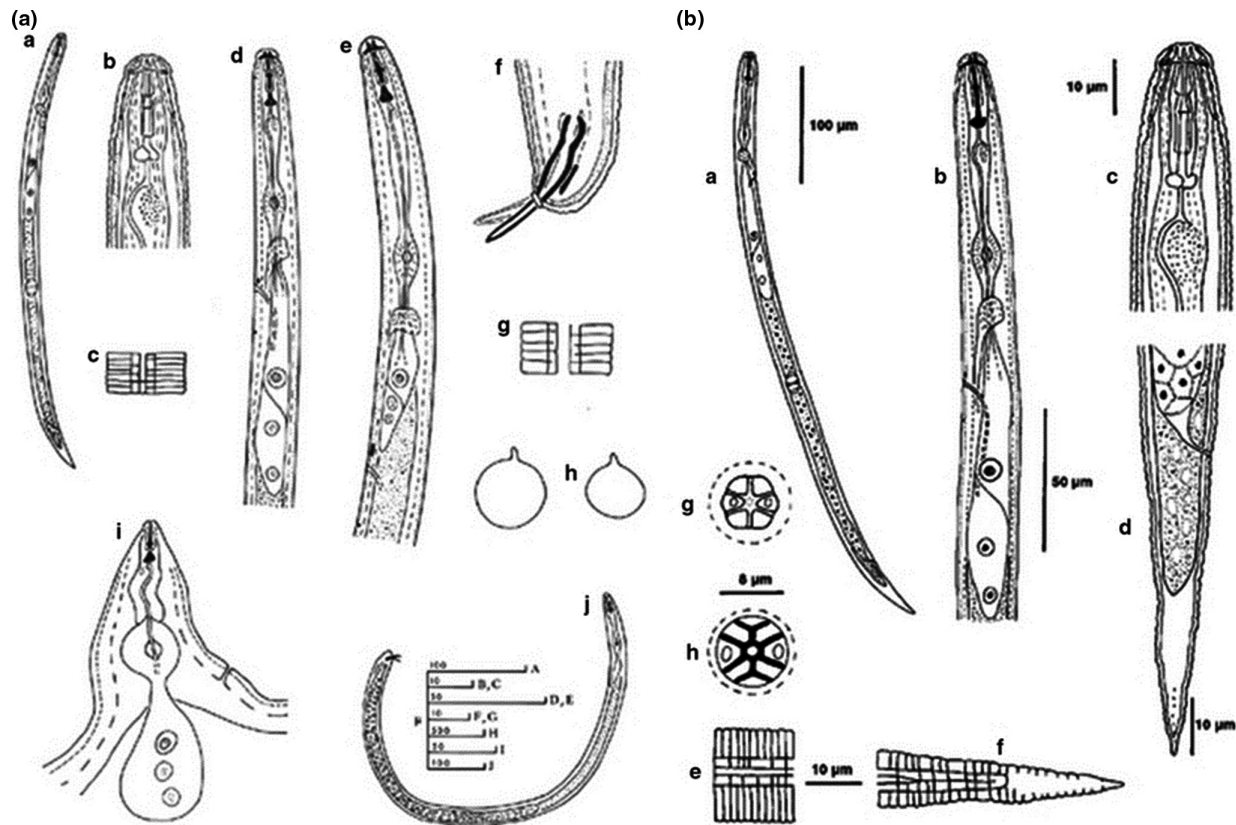


FIGURE 9 Illustrations on the left-hand side of the plate (side labelled A in bold), *G. rostockiensis*: (a) entire juvenile; (b) head region of second-stage juvenile; (c) second-stage juvenile lateral field, mid-body; (d) pharyngeal region of second-stage juvenile; (e) pharyngeal region of male; (f) tail of male; (g) lateral field of male, mid-body; (h) entire cysts; (i) head and neck of female; (j) entire male. (After C.I.H. Descriptions of Plant-Parasitic Nematodes, Set 2, No. 16.) Illustrations on the right-hand side of the plate (side labelled B in bold), *G. pallida* second-stage juvenile: (a) entire; (b) anterior; (c) head; (d) tail; (e) lateral field mid-body region; (f) lateral field tail; (g) head and face at level of lips; (h) head and face at level of base. (After Stone (1972).)

pattern and juvenile features) is available on the website of the European Union Reference Laboratory for Plant Parasitic Nematodes (<https://sitesv2.anses.fr/en/minisite/plant-parasitic-nematodes/videos-media>).

The three other *Globodera* species which could cause confusion during identification of PCNs in Europe are *Globodera millefolii* (Kirjanova & Krall, 1965) Behrens, 1975,⁵ *Globodera artemisiae* (Eroshenko & Kazachenko, 1972) Behrens, 1975, and *G. tabacum sensu lato*. The first two species are not parasitic on potato but have been recorded on *Achillea millefolium* and *Artemisia vulgaris*, respectively, in comparable agricultural areas. The *G. tabacum* species complex (*G. tabacum tabacum* (Lownsbery & Lownsbery, 1954) Skarbilovich, 1959; *G. tabacum solanacearum* (Miller & Gray, 1972) Behrens, 1975, and *G. tabacum virginiae* (Miller & Gray, 1972) Behrens, 1975) is found in North and Central America. *Globodera tabacum tabacum* is also present in Southern

Europe. It parasitizes *Nicotiana tabacum* (tobacco) and some other solanaceous plants (but not potato). Table 3 and Figure 10 provide a morphometric and morphological comparison between the PCNs *G. millefolii*, *G. artemisiae* and *G. tabacum*. See also Baldwin and Mundo-Ocampo (1991), Mota and Eisenback (1993), Brzeski (1998) Wouts and Baldwin (1998) and Subbotin et al. (2010) for more detailed information on other members of the Heteroderidae and identification keys.

Additionally, two new *Globodera* species have been described, *Globodera ellingtonae*, detected on potato in Oregon, USA (Handoo et al., 2012) and in Argentina (Lax et al., 2014), and *Globodera capensis*, detected in a potato field in South Africa (Knoetze et al., 2013). The differences between these species and PCN species are minute and molecular methods are highly recommended for a reliable distinction. The species are only locally present in the USA, Argentina and South Africa and have not been detected in Europe so far.

Two new species, *Globodera sandveldensis* and *Globodera agulhasensis*, both parasitizing

⁵Krall (1978) considered *G. millefolii* (Kirjanova & Krall, 1965) Behrens, 1975 as *species inquirenda*, as the description was based on a single female. Brzeski (1998) reported on *Globodera achilleae*: 'it may be conspecific with *G. millefolii*'. According to Subbotin et al., 2010, 2011 *G. achilleae* is a junior synonym of *G. millefolii*. So from this point onwards the species name *G. achilleae* will not be used but *G. millefolii* will be used instead.

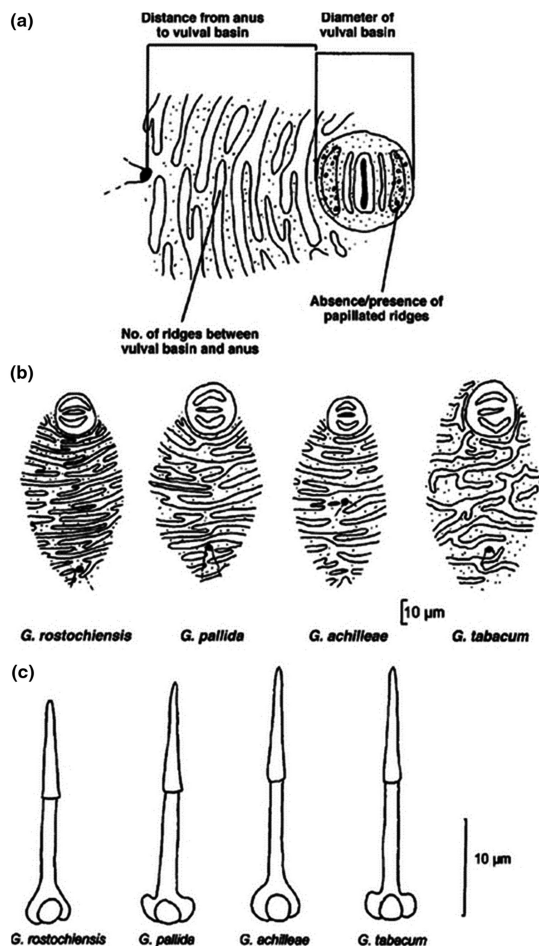


FIGURE 10 (a) Perineal measurements for *Globodera* identification. (b) Vulval–anal ridge patterns for four *Globodera* species. (c) Stylets from four species of *Globodera*. See footnote 5 (Section 4.1.2) for *G. achilleae*. (After Fleming and Powers, 1998.)

non-Solanaceae plants, have been described in South Africa (Knoetze et al., 2017a & b) and will be considered for inclusion in Table 2 in a subsequent revision.

4.2 | Molecular methods

As *G. rostochiensis* and *G. pallida* are morphologically closely related, several polymerase chain reaction

(PCR)-based tests have been developed to separate the two PCN species. The recommended molecular tests are described in Appendices 3–9. It should be noted that many tests that were developed to distinguish specifically *G. rostochiensis* from *G. pallida* have not been tested so far against species such as *G. millefolii*, *G. tabacum* and *G. mexicana*. This limitation should be noted. Tests that were developed after 2000 generally do not have these shortcomings. Specific identification of *G. millefolii* from *G. rostochiensis* and *G. pallida* is possible following the PCR restriction fragment length polymorphism (RFLP) test developed by Sirca et al. (2010). There are also differences between European and non-European populations of the two species, which might be made visible with sequencing techniques (Hockland et al., 2012). A molecular test (Skantar et al., 2007) allows a distinction to be made between *G. pallida* and *G. tabacum*.

DNA barcoding can also be used to support identification.

Identification of *G. rostochiensis* and *G. pallida* should preferably combine morphological and molecular methods, especially when new introductions are suspected.

4.2.1 | PCR tests

The PCR tests presented in Table 4 are recommended for the identification of isolated cysts or individuals from *G. rostochiensis* and *G. pallida*: as performance characteristics of the different tests presented below vary (in particular with regard to their analytical specificity) the choice of test should be made according to the circumstances of use.

Appendix 2 describes nucleic acid extraction.

4.2.2 | DNA barcoding

A protocol for DNA barcoding based on COI, 18S rDNA and 28S rDNA is described in Appendix 5 of PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests: DNA barcoding nematodes* (EPPO, 2016) and can support the identification of *G. pallida* and *G. rostochiensis*. Sequences are available

TABLE 4 PCR tests recommended for the identification of isolated cysts or individuals from *G. rostochiensis* and *G. pallida*.

Test	Appendix
Multiplex real-time PCR test (Gamel et al., 2017)	3
High-throughput diagnosis of PCNs (<i>Globodera</i> spp.) in soil samples using real-time PCR (Reid et al., 2015)	4
Real-time PCR tests for species-specific identification as well as detection of <i>G. rostochiensis</i> , <i>G. pallida</i> and <i>G. tabacum</i> (based on LSU rDNA) available as an all-inclusive real-time PCR kit (http://www.cleardetections.com)	5
A multiplex PCR test using species-specific primers based on ribosomal 18S and ITS1 sequences Bulman and Marshall (1997)	6
An internal transcribed spacer (ITS)-RFLP PCR test based on primers described by Vrain et al. (1992) (Thiéry and Mugniéry, 1996)	7
A Taqman® real-time PCR targeting the internal transcribed spacer I (ITS1) gene (Fera)	8
Identification of viable PCN (<i>Globodera</i> spp.) using RNA-specific RT-PCR (Beniers et al., 2014)	9

in databases including Q-bank (<https://qbank.eppo.int/nematodes/>).

4.3 | Pathotypes

The term ‘pathotype’ is used by the International PCN Pathotype Scheme proposed by Kort et al. (1977) but is now considered too general. Many PCN populations cannot conclusively be assigned to the pathotypes described in this scheme. There are differences in virulence between the two PCN species, in particular between populations of *G. pallida*, and they are of the utmost importance in populations from South America, but identification at this level is not adequate at the moment and it is time-consuming and expensive and requires specific analysis (Hockland et al., 2012). Any population showing signs of a new or unusual virulence (i.e. overcoming the resistance currently available in potato cultivars in Europe) should be tested as soon as possible. In practice, the virulence of populations can be tested on a set of cultivars used in each country. An EPPO Standard, PM 3/68 *Testing of potato varieties to assess resistance to Globodera rostochiensis and Globodera pallida*, is available (EPPO, 2021).

4.4 | Testing the viability of eggs and juveniles

Testing of the viability of the eggs and juveniles may be required for regulatory purposes. This can be done by different methods.

1. Visual morphological determination of viability (a table with descriptions and figures is provided in [Appendix 10](#)). These observations require trained personnel.
2. Determination of viability with a bioassay. Two tests are described in [Appendix 1](#). Such tests require more time to perform than visual morphological determination of viability and generally more time than determination of viability by hatching tests. Dormancy might play a role and should be lifted. An additional aspect of bioassays is the possibility of false-negative results owing to a very low cyst content.
3. Determination of viability by hatching tests. Three tests are described in [Appendix 11](#). Such tests require more time to perform than visual morphological determination of viability. When determining the viability with a hatching test, it should be noted that cysts which have formed recently may be dormant (e.g. when sampling is performed in the autumn after the potato harvest). To break the dormancy, cysts should be exposed to +4 °C for at least 4 months.
4. Determination of the viability of eggs using trehalose. The test is described in [Appendix 12](#), based on

the publications by van den Elsen et al. (2012) and Ebrahimi et al. (2015)

5. Determination of viability and identification on the basis of RNA. The test is described in [Appendix 9](#), based on the publication by Beniers et al. (2014).

Morphological determination of viability of eggs by staining with Meldola’s Blue is also possible, but the chemical is not easily available, so this technique is not described in this Protocol.

5 | REFERENCE MATERIAL

Reference material can be obtained from:

the National Plant Protection Organization, National Reference Centre, PO Box 9102, 6700 HC Wageningen (the Netherlands);

the Food and Environmental Research Agency (Fera), Sand Hutton, York YO41 1LZ (GB);

the Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Messeweg 11–12, 38104 Braunschweig (Germany).

the French National Institute for Agricultural Research (INRAe) Biology of Organisms and Populations for Plant Protection Domaine de la Motte, BP 35327, 35653 Le Rheu Cedex (France).

6 | REPORTING AND DOCUMENTATION

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CRITERIA

When performance criteria are available, these are provided with the description of the test. A validation data is also available in the EPPO Database on Diagnostic Expertise (<http://dc.eppo.int>), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from: E. van Heese and G. Karssen, National Plant Protection Organization, National Reference Centre, PO Box 9102, 6700 HC Wageningen (the Netherlands); e-mail: e.y.j.vanheese@nvwa.nl or g.karssen@nvwa.nl.

9 | FEEDBACK ON THIS STANDARD

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact: diagnostics@epo.int.

10 | STANDARD REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

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APPENDIX 1 - BIOASSAYS

Test A: Bioassay (method performed in Germany and Austria)

This method relies on the principle that if potato cyst nematodes (PCNs) are present in a soil sample (even in very low numbers), they will multiply when given access to the roots of growing potato plantlets in a small container. The presence of developing cysts on the roots can then be observed through the transparent walls of the special containers used.⁶

Depending on the size of the container about 100–200 mL of soil from the sample should be put into each container, ensuring that the soil remains suitably moist. Prepare as many containers as needed to process the entire sample. Eyes are cut from well-chitted certified tubers with a circular blade (diameter approximately 3 cm) and placed in the containers. Bioassay in autumn/winter requires chitting of tubers (through fumigation or treatment with gibberellic acid). To avoid growth of fungi, eye cuttings should

be left to dry for half a day at room temperature before being placed on the soil samples in the containers (eyes upwards) and covered with nematode-free soil. Control containers with known infestations are used in each test.

The square containers are placed close together on a planting table, shading each other to prevent the growth of algae on the transparent walls. To allow optimal host–parasite interaction, the air temperature in the glasshouse is ideally maintained at 22/16°C (day/night) and always kept below 25°C (possibly giving additional light in winter) and above 13°C. Containers should be watered moderately to achieve optimal root penetration of the soil. Watering may be done manually or by trickle irrigation. Surplus irrigation water can run off through a hole in the bottom of the containers. The risk of contaminating healthy samples by means of adjacent infested samples has been shown to be small. It might be necessary to take measures against foliar blight during the course of the bioassay. If an individual plant should die, the soil in the container should be tested for cysts using the Fenwick can or a related method.

Visual observation of females and cysts is done when cysts are observed in the control containers, generally after 6–10 weeks of cultivation. Before counting of

⁶They can be obtained from Ritter GmbH, Schwabenstraße 50–54, D-86836 Untermeitingen (DE)

females and cysts, potato leaves are cut with pruning shears. New cysts are visible on the roots through the transparent walls of the containers when infection levels are high. To detect low levels of infestation, it is advised to inspect roots and soil after removal from the container and to extract cysts from the soil when no infection is detected visually.

This can also be performed in closed containers kept in a dark room (Phillips et al., 1980).

Test B: Test of reproduction (method performed in Norway)

The infective success of PCNs is tested on potato plants in 500 mL pots with sand, using nylon bags with cysts (up to 20) as inoculation units. It is recommended to treat tubers with gibberellic acid in order to induce and synchronize the germination. Each pot is filled with one-third of the total soil volume and a nylon bag with cysts is placed below one potato tuber and then filled up with sand. The pots are placed in a randomized fashion in a growth cabinet with approximate day/night temperatures of 20°C/16°C and an 18 h light period. The pots should receive mineral nutrients and water as required. After 3 months the shoots are cut, and the soil and roots are air-dried. The newly formed cysts are extracted from the soil (for instance using the Fenwick can) and collected and counted. Each new cyst represents a successful infection and hence is a measure of the infection potential of the population.

APPENDIX 2 - NUCLEIC ACID EXTRACTION

Many methods or kits can be used for the extraction of nucleic acids from juveniles or cysts. The methods/kit described below have been evaluated in combination with the PCR tests described in Appendices 3, 6 and 7.

This paragraph only concerns DNA extraction. When relevant, RNA extraction is described.

DNA extracts are used immediately, stored overnight at approximately 4°C or stored at approximately -20°C until use.

1. Manual DNA extraction with lysis buffer

1.1. Tissue source.

This procedure can be applied to either isolated adults or juveniles, even one individual, but also to *Globodera* cysts (including a single cyst) (Anthoine & Chappé, 2010).

1.2. The DNA extraction procedure includes chemical treatment with a lysis buffer (Tris 10 mM pH =8, EDTA 1 mM, Nonidet P40 1%, proteinase K 100 µg mL⁻¹; Ibrahim et al., 1994) and a mechanical action.

For *Globodera* juveniles or adults, one or more nematodes are picked up and transferred to a microtube containing 100 µL of lysis buffer. Glass beads (one of 3 mm diameter and about 50 of 1 mm diameter, Sigma) are added to the microtube and the nematode(s) crushed by shaking the microtube [e.g. using a Tissulyser II (Qiagen®) at 30 beats per second for 40 s].

For *Globodera*, cysts are transferred into a microtube containing 100 µL of lysis buffer for a single cyst or 1 mL of lysis buffer for a cyst mixture (only validated for Bulman and Marshall (1997), described in Appendix 6).

The cysts are crushed with a hand pestle, which is either for unique use or disinfected between uses, followed optionally by a glass bead beating as described for juveniles and adults.

Microtubes are then incubated at approximately 55°C for about 1 h and at approximately 95°C for 10 min. The DNA supernatant obtained is transferred into a new microtube.

1.3. No further cleanup of the extracted DNA is needed.

2. Qiagen DNA extraction

Cysts are transferred into a microtube containing a maximum of 50 µL of water. The cysts are crushed with a hand pestle (single use or disinfected between samples), followed by a glass bead beating.

Glass beads (one of 3 mm diameter and about 50 of 1 mm diameter, Sigma) are added to the microtube and the nematodes crushed by shaking the microtube [e.g. using a Tissulyser II (Qiagen®) at 30 beats per second for 40 s].

DNA extraction of crushed cysts is performed using a QIAamp® DNA mini kit (Qiagen) or QIAamp® DNA micro kit (Qiagen) according to the manufacturer's instructions (QIAamp handbook, protocol for tissue).

The DNA is eluted in 100 µL of elution buffer.

APPENDIX 3 - MULTIPLEX REAL-TIME PCR TEST (GAMEL ET AL., 2017)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

An educational video on this test is available on the website of the European Union Reference Laboratory for Plant Parasitic Nematodes (<https://sitesv2.anses.fr/en/minisite/plant-parasitic-nematodes/videos-media>).

1. General information

1.1. Scope of the test: identification of *Globodera pallida* and *G. rostochiensis* using a protocol developed by Gamel et al. (2017).

1.2. The tests target the microsatellite regions and the flanking regions including the microsatellite patterns: CCT for *G. pallida*, TC for *G. rostochiensis*.

1.3. Oligonucleotides:

Primer/probe name	Sequence	Amplicon size
For <i>G. pallida</i>		
μsatGP-F	5'-AAGGAGTTG TGGTCCAGACG-3'	100 bp
μsatGP-R	5'-GAAGGCAATCT GTGTTCGGG-3'	
μsatGP-P	5'-JOE-CGCTCGTC GGCCTCCTCCTC-BHQ1-3'	
For <i>G. rostochiensis</i>		
μsatGR-F	5'-TGACGAGGA ACAGTACAAAG-3'	167 bp
μsatGR-R	5'-GTGTCTCTAATTTGCCATT-3'	
μsatGR-P	5'-Cy5-AGGCATTGCT TGAGCGAACGGA-BHQ2-3' ^a	

^a It has been noted that replacing Cy5 by FAM BHQ1 improves analytical sensitivity (Gamel S. pers comm. 2021).

1.4. LightCycler[®] 480 instrument (Roche) using probe master mix supplied by Roche Diagnostics (ref. 04707494001).

1.5. The automatic analysis option in the software that drives the thermocycler ('Abs Quant/2nd derivative Max') was used to determine the cycle threshold (C_t).

2. Methods

2.1. Nucleic acid extraction and purification.

2.1.1. Single juveniles or single cysts; see [Appendix 2](#) (Section 1).

2.1.2. One or more cysts (up to 50 cysts); see [Appendix 2](#) (Section 2).

2.1.3. Either use extracted DNA immediately or store overnight at approximately 4°C or at approximately -20°C for longer periods.

2.2. Real-time polymerase chain reaction (real-time PCR).

2.2.1. Master mix.

Each sample is tested in duplicate.

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water	NA	4.04	NA
LightCycler [®] 480 probe master (Roche)	2×	10	1×
<i>G. pallida</i> forward primer (<i>μsat-GP-F</i>)	50 μM	0.2	0.5 μM

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
<i>G. pallida</i> reverse primer (<i>μsat-GP-R</i>)	50 μM	0.2	0.5 μM
<i>G. pallida</i> probe (<i>μsat-GP-R</i>)	50 μM	0.08	0.2 μM
<i>G. rostochiensis</i> forward primer (<i>μsat-GR-F</i>)	50 μM	0.2	0.5 μM
<i>G. rostochiensis</i> reverse primer (<i>μsat-GR-R</i>)	50 μM	0.2	0.5 μM
<i>G. rostochiensis</i> probe (<i>μsat-GR-R</i>)	50 μM	0.08	0.2 μM
Subtotal		15	
DNA		5	
Total		20	

Optional: If an internal positive control (IPC) is included, add 0.5 μM of *Heterodera schachtii* forward primer (*μsat-HS-F*), *H. schachtii* reverse primer (*μsat-HS-R*) and 0.2 μM of *H. schachtii* probe (*μsat-HS-P*). *Heterodera schachtii* DNA is also added to the master mix. The volume of molecular grade water should be recalculated.

2.2.2. PCR conditions: initial denaturation 10 min at 95°C; 40 cycles of denaturation for 10 s at 95°C, annealing and extension for 50 s at 60°C; fluorescence capture at the end of the extension of each cycle.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extractions and amplifications of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a whole and full cyst of either *G. pallida* or *G. rostochiensis*.
- Negative amplification control (NAC) to rule out false positives owing to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix, in place of DNA.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism, from *G. pallida* and *G. rostochiensis* separately. This can include nucleic acid extracted from the target organisms, whole genome

amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

In addition to the external positive controls (PIC and PAC), IPCs can be used to monitor each individual sample separately. Internal positive controls can be non-target *H. schachtii* DNA added to the DNA solutions. In this case, the following oligonucleotides targeting the microsatellite regions and the flanking regions, including the pattern CAT, can be added to the master mix at the same concentration (0.5 μM for each primer and 0.2 μM for the probe):

Primer $\mu\text{satHS-F}$	5'-CCCGGACAGCCAAATTGT-3'
Primer $\mu\text{satHS-R}$	5'-GGGAAGTGAGTGGGCAGTTT-3'
Probe $\mu\text{satHS-P}$	5'-FAM-ATGGGAAGGACGGGTGGCGG-BHQ1-3'

Note that the use of the probe labelled FAM-BHQ1 for the IPC is only possible if the probe label Cy5 is used for the *G. rostochiensis* probe (see Section 1.3).

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. The same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- The NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- From the validation data, a result is considered as positive if the cycle threshold (C_t) value is below 35 for the two replicates. Results with C_t greater than 35 require further investigation (for example, repeat the DNA extraction and/or PCR and/or test by an alternative method).
- Tests should be repeated if any contradictory or unclear results are obtained.

The C_t cut-off value given above is as established in the Nematology Unit of the Plant Health Laboratory (Anses, France). As a C_t cut-off value is equipment, material and chemistry dependent, it needs to be verified in each laboratory when implementing the test.

4. Performance characteristics available

Validation data from Gamel et al., 2017. Additional validation data is available in the Eppo database on diagnostic expertise (<http://dc.eppo.int/validationlist.php>).

4.1. Analytical sensitivity data.

For simplex and multiplex reactions, the minimum number of nematodes that can be detected using the DNA extraction described in Appendix 2, Section 1 is one juvenile of *G. pallida* or *G. rostochiensis*, which represents the smallest quantity of nematode that could be encountered in a sample.

The target species *G. pallida* and *G. rostochiensis* can also be detected in mixtures, even when one of them is in a weaker concentration (10 fold diluted).

Moreover, five J2 of *G. pallida* are detected when combined with 10, 20, 40 and 50 cysts of *G. rostochiensis* (on average 130 larvae/cysts): C_t values obtained for *G. pallida* are between 31 and 32 and those obtained for *G. rostochiensis* between 22 and 25.

4.2. Analytical specificity data.

A total of 55 populations were tested: 14 *G. pallida* (including seven South American populations), 13 *G. rostochiensis* (five from South America and eight from Europe), eight *H. schachtii*, a total of seven for *G. tabacum*, *G. ellingtonae* and *G. mexicana*, and a total of 11 for other species of *Heterodera* including five species belonging to the *Schachtii* group. For simplex and multiplex reactions:

- all of the target populations tested were detected;
- no cross-reaction was observed with non-target species and with DNA extracted directly from float material;
- no competition was observed when the three target species (*G. pallida*, *G. rostochiensis* and *H. schachtii*) were present in mixture.
- Data on repeatability

Data obtained using the DNA extraction described in Appendix 2, Section 1: for *G. pallida*, 25% with one J2 and 100% with two J2 (eight replicates tested in the same run); for *G. rostochiensis*, 100% with one J2 and 100% with two J2 (duplicates tested in the same run).

In a mixture, data were obtained using the DNA extraction described in Appendix 2, Section 2: 100% with one cyst of *G. pallida* and 49 cysts of *G. rostochiensis* (six samples tested in duplicate); 100% with one cyst of *G. rostochiensis* and 49 cysts of *G. pallida* (six samples tested in duplicate); 100% with five J2 of *G. pallida* and 50 cysts of *G. rostochiensis* (two samples tested in duplicate); mean of four C_t values obtained for *G. pallida* of 32.9).

4.3. Data on reproducibility.

Only duplicates of DNA solutions were analysed with two operators, two days, two real-time PCR machines and two batches of mastermix. Data were obtained using the DNA extraction described in [Appendix 2, Section 1](#):

- for *G. pallida*, 25% with one J2 and 100% with two J2 (four replicates);
- for *G. rostochiensis*, 100% with one J2 and 100% with two J2 (four replicates).

In the mixture, data were obtained using the DNA extraction described in [Appendix 2, Section 2](#):

- 100% with one cyst of *G. pallida* (on average 250 larvae/cyst) and 49 cysts of *G. rostochiensis* (six samples tested in duplicate on two different thermocyclers); and
- 100% with one cyst of *G. rostochiensis* (on average 240 larvae/cyst) and 49 cysts of *G. pallida* (six samples tested in duplicate on two different thermocyclers);
- 100% with five J2 of *G. pallida* and 50 cysts of *G. rostochiensis* (two samples tested in duplicate on two different thermocyclers; mean of eight C_t values obtained for *G. pallida* of 32.8).

APPENDIX 4 - HIGH-THROUGHPUT DIAGNOSIS OF PCNS (*GLOBODERA* SPP.) IN SOIL SAMPLES USING REAL-TIME PCR (REID ET AL., 2015).

The test below is described as it was carried out to generate the validation data provided in [Section 4](#). Other equipment, kits or reagents may be used provided that a verification (see [PM 7198](#)) is carried out.

1. General information

- 1.1. Scope of the test: detection and identification of *G. rostochiensis* and *G. pallida* in soil samples using real-time PCR.
- 1.2. Test developed by Reid et al. (2015).
- 1.3. The test targets the internal transcribed spacer 1 region of the ribosomal DNA repeat unit (ITS1) and accession number FJ212165 for *G. pallida*.
- 1.4. Oligonucleotides:

Forward primer <i>G. pallida</i> and <i>G. rostochiensis</i>	5'-CGTTTGTTGTTG ACGGACAYA-3'
Reverse primer <i>G. pallida</i> and <i>G. rostochiensis</i>	5'-GGCGCTGTCC RTACATTGTTG-3'
Probe <i>G. pallida</i>	5'-FAM-CCGCTATG TTTGGGC-MGBNFQ-3'
Probe <i>G. rostochiensis</i>	5'-FAM-CCGCTGT GTATKGGC-MGBNFQ-3'

- 1.5. 7900HT fast real-time PCR system (Life Technologies).
- 1.6. The real-time PCR tests are run using the SDS software supplied with the 7900HT machine. Analysis settings are set to automatic.

2. Methods

2.1. Nucleic acid extraction and purification.

The following method is suitable for a float size of approximately 1 mL. For larger floats the sample should be split into several tubes.

The dry float is manually scraped off the filter paper into a Fast Funnel MINI placed over a 2 mL Safe-Lock tube and eight tungsten carbide beads are added to the tube. Batches of 48 tubes are disrupted in the TissueLyser II set at 30 Hz for 30 s and 1.5 mL of API buffer is added prior to another round of shaking at 30 Hz for 30 s. Tubes are centrifuged at 1180 g for 5 min and a minimum of 400 μ L of the supernatant is transferred using a pipette fitted with a wide-bore tip to an individual well of a 96-well S-block containing 5 μ L of RNase A. The S-block is incubated at 65°C for 10 min and centrifuged at 3000 g for 5 min.

A 340 μ L aliquot of supernatant of each sample is transferred in S-block 1 (see below) and processed using the BS96 DNA Plant program of a MagMAX Express-96 (Life Technologies). Five S-blocks (1–5) and a microplate (MP) are used in the sample processing, containing:

- S-block 1, 400 μ L isopropanol and 30 μ L MagAttract Suspension G;
- S-block 2, 400 μ L RPW buffer;
- S-block 3, 400 μ L 96% ethanol;
- S-block 4, 400 μ L 96% ethanol;
- S-block 5, 500 μ L sterile PCR-grade water (Sigma) containing 0.02% (v/v) Tween 20;
- MP containing 200 μ L sterile PCR-grade water.

The DNA sample contained in the MP can be either processed immediately or sealed with an adhesive sealing sheet and stored at approximately -20°C .

2.2. Real-time PCR.

2.2.1. Master mix.

Reagent	Working concentration	Volume per reaction (μ L)	Final concentration
Molecular-grade water	NA	4.6	NA
TaqMan [®] Environmental master mix 2.0 (Life Technologies)	2 \times	15	1 \times

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
TaqMan [®] exogenous internal positive control primer mix (Life Technologies)	10×	1.5	0.5×
TaqMan [®] exogenous internal positive control DNA (Life Technologies)	50×	0.15	0.25×
Forward primer	5 µM	1.25	0.25 µM
Reverse primer	5 µM	1.25	0.25 µM
Probe <i>G. pallida</i>	5 µM	0.625	0.125 µM
Probe <i>G. rostochiensis</i>	5 µM	0.625	0.125 µM
Subtotal		25	
DNA dilution		5	
Total		30	

2.2.2. PCR conditions:

2 min at 50°C, 10 min at 95°C, 40 cycles at 15 s 95°C, 60 s 60°C.

2.3. Real-time PCR ‘identification test’.

2.3.1. Master mix (either *pallida* or *rostochiensis*).

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	6.25	NA
TaqMan [®] environmental master mix 2.0 (Life Technologies)	2×	15	1×
Forward primer	5 µM	1.25	0.25 µM
Reverse primer	5 µM	1.25	0.25 µM
Either probe <i>G. pallida</i> or probe <i>G. rostochiensis</i>	5 µM	1.25	0.25 µM
Subtotal		25	
DNA dilution		5	
Total		30	

2.3.2. PCR conditions:

2 min at 50°C, 10 min at 95°C, 40 cycles at 15 s 95°C, 60 s 60°C.

3. Essential procedural information

3.1. Controls.

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extractions and amplifications of the target organism and target nucleic acid, respectively.

- NIC to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix (soil confirmed to be free from *Globodera*) or if not available clean extraction buffer.
- PIC to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target *Globodera* species (e.g. a float spiked with at least one of the target species, but ideally both).
- NAC to rule out false positives owing to contamination during the preparation of the reaction mix: amplification of molecular-grade water that is used to prepare the reaction mix.
- PAC to monitor the efficiency of the amplification: amplification of nucleic acid solution from *G. pallida* and *G. rostochiensis*. This can include the use of nucleic acid extracted from the target organisms, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR products). The PAC should preferably be near to the limit of detection for more appropriate control of the reaction.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation to the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2. Interpretation of results.

Verification of the controls:

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- The NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.

- Additionally, for SYBR[®] green-based real-time PCR tests the T_m value should be as expected.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

SASA (GB) performed the following validation:

4.1. Analytical sensitivity data.

A 0.1 pg aliquot was taken of PCN DNA of either *G. pallida* or *G. rostochiensis*. This level allows the detection of one viable cyst of either *G. pallida* or *G. rostochiensis*, i.e. a cyst containing a minimum of one live juvenile nematode. The test is able to detect one cyst of *G. rostochiensis* among 100 of *G. pallida* and *vice versa*.

4.2. Analytical specificity data.

Inclusivity: 641 *G. pallida* populations and 531 *G. rostochiensis* populations were tested from across Scotland.

Exclusivity: primers were designed to take account of non-target species of cyst nematodes including *Heterodera avenae* (two populations, data unpublished), *Punctodera chaltoensis* (one population), *Punctodera punctata* (two populations) and *Globodera millefolii* (six populations as *G. achilleae*), as these represent other cyst nematode genera present in the United Kingdom. No cross-reactivity with any of these species occurred. No cross-reactions were observed with *G. artemisiae* (four populations), and *G. hypolysi* (one population, now synonymized with *G. artemisiae*), which are not considered to be present in the United Kingdom. Cross-reactions occurred with *G. mexicana* (three populations), *G. tabacum* (12 populations) and *G. ellingtonae* (one population), but none of these species has been detected so far in the United Kingdom. Sequencing of positive results at the end of each testing season allows evaluation of whether any cross-reacting species were responsible for positive test results. No cross-reactions were observed.

4.3. Data on repeatability.

Not available. Some information included under Section 4.5.

4.4. Data on reproducibility.

Not available. Some information included under Section 4.5.

4.5. Other information.

Testing of soil samples from Scottish fields during 2008–2010 using morphological identification (of PCNs

from a float extracted using a Fenwick can) to species level as the diagnostic method provided a rate of 2.0% of samples testing positive for PCN. The first 3 years using the PCR diagnostic (2011–13) provided a similar rate of 2.0% of samples testing positive. (It should be noted that these were different samples taken during a different time period.)

	2008–2010	2011–2013
Mean no. of samples tested per annum	5777	16 052
Samples with only dead PCNs	5.1%	N/A
Samples with live PCNs	2.0%	2.0%
Samples with live <i>G. rostochiensis</i>	1.1%	1.0%
Samples with live <i>G. pallida</i>	1.0%	1.1%

APPENDIX 5 - REAL-TIME PCR TESTS FOR THE IDENTIFICATION OF *G. ROSTOCHIENSIS*, *G. PALLIDA* AND *G. TABACUM* BASED ON LSU RDNA (CLEARDETECTIONS KIT)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7198) is carried out.

1. General information

- 1.1. Scope of the tests: identification of *G. rostochiensis*, *G. pallida* and *G. tabacum* juveniles and cysts and detection of *G. rostochiensis*, *G. pallida* and *G. tabacum* juveniles and cysts in cyst mixtures (complex DNA background) by real-time PCR.
- 1.2. The tests target the LSU (28S) rDNA gene.
- 1.3. Amplicon sizes: *G. rostochiensis* 448 bp, *G. pallida* 86 bp and *G. tabacum* 482 bp.
- 1.4. Oligonucleotide sequences are not disclosed. These tests are available as an all-inclusive real-time PCR kit (ClearDetections, The Netherlands, www.clear-detections.com).
- 1.5. The real-time PCR kit includes target and general nematode DNA real-time PCR primer sets, positive amplification control(s) (PACs) and PCR mix with fluorescent DNA-binding dye.

2. Methods

2.1. Nucleic acid extraction.

These real-time PCR tests can be combined with any nematode DNA extraction method delivering target DNA. Validation was performed with the 'Nematode DNA extraction and purification kit' from

ClearDetections. When using these tests for nematode quantification purposes it is highly recommended to include an (internal or external) DNA standard in the extraction procedure to correct for potential DNA losses during the DNA extraction and purification process.

2.2. Real-time PCR.

2.2.1. Master mix.

See Section 1.5

2.2.2. PCR cycling conditions.

Enzyme activation: 3 min at 95°C. Amplification: 35 cycles of 10 s at 95°C, 1 min at 63°C, 30 s at 72°C. Melt curve: 0.2–0.5°C steps 72 → 95°C.

3. Essential procedural information

3.1. Controls.

For a reliable test result to be obtained the following controls should be included for each series of nucleic acid extractions and amplifications of the target organism and target nucleic acid, respectively.

- NIC to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification, preferably clean extraction buffer.
- PIC to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism. Alternatively, the all-inclusive real-time PCR kit contains a separate real-time PCR primer set for the detection of 'nematode DNA', which can be used to check for the presence and quantity of nematode DNA in the nucleic acid sample.
- NAC to rule out false positives owing to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- PACs (e.g. included in the kit) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include genomic DNA extracted from the target organism, a cloned PCR product (plasmid DNA) or synthetic DNA.

3.2. Interpretation of results.

Verification of the controls:

- The PAC and PIC amplification curves should be exponential.
- The NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.

- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- A melt curve analysis is performed, and the obtained melting temperature (T_m) value equals the T_m value of the PAC ($\pm 1^\circ\text{C}$). T_m values may vary depending on the PCR machine and PCR mix used. The T_m values obtained with the combination of a Bio-Rad CFX Connect PCR machine and ClearDetections PCR mix equal 86.0°C for *G. rostochiensis*, 85.5°C for *G. pallida* and 89.5°C for *G. tabacum*.
- The tests should be repeated if any contradictory or unclear results are obtained.
- The real-time PCR primer set included in the kit for the detection of 'nematode DNA' can be used when in doubt about the presence of nematode DNA in a DNA sample (check for possible false negatives).

4. Performance characteristics available

These real-time PCR tests are validated in line with PM 7/98.

- 4.1. Analytical sensitivity: one single juvenile or egg, against a background of 1000 juveniles or eggs of non-target cyst nematodes.
- 4.2. Diagnostic sensitivity: 100%.
- 4.3. Analytical specificity: 100% (when using the kit for the three species on one sample).

Number of populations of target organisms tested: four *G. rostochiensis* populations, three *G. pallida* populations and two *G. tabacum* populations (for details see full validation report, table 6 in the database on diagnostic expertise).

Number of non-target organisms tested: *G. artemisiae*, *G. mexicana*, *G. millefolii* (as *G. achilleae*), *Heterodera goettingiana*, *Heterodera schachtii*, *Heterodera betae* and *Punctodera stonei*.

Several target and non-target species (from different origins) were tested, and no cross-reactions were noted for the *G. tabacum* real-time PCR test. The *G. pallida* real-time PCR test is specific to the *G. pallida* populations tested, including one from South America. In addition, it picks up its close relative *G. mexicana*. The real-time PCR test for *G. rostochiensis* is specific for *G. rostochiensis* populations, including South American populations, and *G. tabacum*. These results demonstrate that in all cases where *G. rostochiensis* and *G. tabacum* cysts may be jointly found in samples and positive real-time PCR signals are found for *G. rostochiensis*, the real-time PCR test for *G. tabacum* must be used to verify possible false-positive results.

- 4.4. Diagnostic specificity: 100% (when using the kit for the three species on one sample).
- 4.5. Reproducibility: 100%.

- 4.6. Repeatability: 100%.
- 4.7. Accuracy: 100%.
- 4.8. Dynamic range: between 10–100 and 0.1 billion copies of target DNA.
- 4.9. Selectivity: 100%.
- 4.10. Robustness: no real-time PCR failure is observed when the primer combinations are exposed to a temperature gradient. With a deviation in annealing temperature (T_a) of $\pm 1.0^\circ\text{C}$ from the normal T_a (63°C), all ΔC_t values are < 1 . The real-time PCR tests for the detection of *G. pallida*, *G. rostochiensis* and *G. tabacum* are robust.

APPENDIX 6 - MULTIPLEX PCR TEST (BULMAN & MARSHALL, 1997)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

- 1.1. Identification of *Globodera* using the protocol developed by Bulman and Marshall (1997).
- 1.2. The test can only be used on nematodes morphologically identified as *Globodera* spp., as the primers are not specific for *Globodera* spp.
- 1.3. This test can be performed on single cysts or up to 10 cysts. When performed on up to 10 cysts, it is recommended to perform two simplex PCR tests (ITS5-PITSp4 and ITS5-PITSr3) Anthoine and Chappé (2010).
- 1.4. Different *G. pallida* and *G. rostochiensis* populations, from different pathotypes and geographical origins, were used. The nucleic acid source is full cysts.
- 1.5. The test is designed to the 18S rRNA gene and the internal transcribed spacer ITS1 region.
- 1.6. The PCR product of the reaction with the universal primer ITS5 and *G. pallida*-specific primer PITSp4 is 265 bp.

The PCR product of the reaction with the universal primer ITS5 and *G. rostochiensis*-specific primer PITSr3 is 434 bp.

- 1.7. Oligonucleotides.

ITS5	5'-GGA AGT AAA AGT CGT AAC AAG G-3'
PITSp4	5'-ACA ACA GCA ATC GTC GAG-3'
PITSr3	5'-AGCGCAGACATGCCGAA-3'

- 1.8. Amplification is performed in a Peltier-type thermocycler with a heated lid (e.g. Bio-Rad C1000).

2. Methods

- 2.1. Nucleic acid extraction and purification
 - 2.1.1. DNA is extracted from cysts (up to 10) or juveniles.
 - 2.1.2. For the DNA extraction procedure see Appendix 2
 - 2.1.3. Either use extracted DNA immediately or store overnight at approximately 4°C or at approximately -20°C for longer periods.
- 2.2. Polymerase chain reaction.
 - 2.2.1. Master mix.

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water	NA	to make up to 24	NA
Tris-HCl (pH 8.3)	500 mM	1	20 mM
KCl ^a	500 mM	2.5	50 mM
MgCl ₂ (Life Technologies)	25 mM	2	2 mM
dNTPs (Life Technologies)	10 mM each	0.4	0.16 mM
Forward primer ITS5	10 μM	0.625	0.25 μM^b
Reverse primer PITSp4	10 μM	0.625	0.25 μM^b
Reverse primer PITSr3	10 μM	0.625	0.25 μM^b
Taq DNA polymerase (Life Technologies)	5 U/ μL	0.12	0.6 U
Subtotal		24	
Genomic DNA extract		1	
Total		25	

^a If there are suspected low DNA amounts, for example when a low PCR amplification rate has occurred, up to 1 μL of BSA (bovine serum albumin; $c = 50 \text{ mg/mL}$ stored in 20mM phosphate buffer pH 6.8) may be added to the amplification reaction to enhance the Taq DNA polymerase activity.

^b 250 μM of each primer is mentioned in the original publication but laboratories performing the test use a final concentration ranging from 0.15 to 1 μM .

2.2.2. PCR cycling parameters.

2 min at 94°C , 35 cycles of 30 s at 94°C , 30 s at 60°C and 30 s at 72°C , 5 min 72°C

3. Essential procedural information

3.1. Controls.

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolations and amplifications of *G. pallida* and *G. rostochiensis* and nucleic acid, respectively.

- NIC to monitor contamination during nucleic acid extraction: it can be obtained by performing DNA extraction of the solution/buffer used to collect nematode specimens (e.g. DNA extraction buffer alone).

- PIC to ensure that nucleic acid of sufficient quantity and quality is isolated: solution/buffer spiked with appropriate numbers of individual cysts or juveniles confirmed as being from either *G. pallida* or *G. rostochiensis*. This control is optional as long as this test is applied on isolated nematodes (not on bulk solutions or as a screening test).
- NAC to rule out false positives owing to contamination during the preparation of the reaction mix – amplification of molecular-grade water that is used to prepare the reaction mix instead of DNA extract.
- PAC to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism – amplification of genomic DNA of individuals from both species *G. pallida* and *G. rostochiensis*; the identity of the individuals or the genomic solutions used must have been confirmed.

3.2 Interpretation of results.

Verification of the controls:

- The NIC and NAC should produce no amplicons.
- PAC, and if relevant PIC, should produce amplicons of the expected sizes (265 bp for *G. pallida*, and 434 bp for *G. rostochiensis*).

When these conditions are met:

- A test will be considered positive if amplicons of 265 bp for *G. pallida* and 434 bp *G. rostochiensis* are produced.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The following performance criteria were provided by Anses Plant Health Laboratory (France) in July 2010 with the following adaptations of the master mix: primer concentration 0.64 μM and dNTP 0.25 mM each, DNA extract 5 μL [primers, Taq DNA polymerase and MgCl_2 – MP Biomedicals (ex Qbiogene) or Sigma; dNTPs – MP Biomedicals]. The DNA extraction was performed with lysis buffer (Tris 10 mM pH 8, EDTA 1 mM, Nonidet P40 1%, proteinase K 100 $\mu\text{g mL}^{-1}$) and mechanical disruption of the cuticle [use of Tissulyser II (Qiagen) with glass beads]. See the validation report for additional information.

- 4.1. Analytical sensitivity data: one J2.
- 4.2. Analytical specificity data: the study included 11 populations of *G. pallida*, four populations of *G. rostochiensis*, five populations of *G. tabacum*

and one population each of *G. mexicana* and *G. artemisiae*. The populations cover different geographical areas.

- 4.3. Data on repeatability: 100% for *G. pallida*, 100% for *G. rostochiensis*.
- 4.4. Data on reproducibility: 96% (one J2) for *G. pallida*, 100% (one J2) for *G. rostochiensis*.
- 4.5. Diagnostic specificity data: 91% for *G. pallida* (the test cross-reacted with *G. tabacum virginiae*), 100% for *G. rostochiensis*.

APPENDIX 7 - ITS PCR-RFLP TEST (THIÉRY & MUGNIÉRY, 1996)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

- 1.1. Identification of *Globodera* spp. using a protocol developed by Thiéry and Mugniéry (1996).
- 1.2. The test can only be used on nematodes morphologically identified as *Globodera* spp., as the primers are not specific for *Globodera* spp.
- 1.3. The test is designed for the internal transcribed spacer (ITS) region.
- 1.4. The PCR product of the reaction with the ITS-specific universal forward primer 18S and reverse primer 26S is 1200 bp for *Globodera* spp. The primers are described by Vrain et al. (1992).
- 1.5. Oligonucleotides

Primer names	Sequence	Amplicon size in base pairs
Forward 18S	5'-TTG ATT ACG TCC CTG CCC TTT-3'	1200
Reverse 26S	5'-TTT CAC TCG CCG TTA CTA AGG-3'	

Note: in some publications the primer names 18S and 26S are also referred to as 5367 and 5368, respectively.

- 1.6. Amplification is performed in a Peltier-type thermocycler with heated lid, e.g. Bio-Rad C1000.

2. Methods

- 2.1. Nucleic acid extraction and purification.
 - 2.1.1. DNA is extracted from cysts or juveniles.
 - 2.1.2. Genomic DNA is isolated as described in [Appendix 2](#) (Section 1).
 - 2.1.3. Either use extracted DNA immediately or store overnight at 4°C or at approximately –20°C for longer periods.
- 2.2. Polymerase chain reaction.

2.2.1. Master mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	30.4	NA
Taq DNA polymerase buffer (MP Biomedicals)	10×	5	1×
MgCl ₂ (if not included in the Taq DNA buffer)	25 mM	4	2 mM
dNTPs	10 mM each	0.5	100 µM
Forward primer 18S	10 µM	2.5	0.5 µM
Reverse primer 26S	10 µM	2.5	0.5 µM
Taq DNA Polymerase (MP Biomedicals, ex. Appligene Oncor)	5 U µL ⁻¹	0.1	0.5 U
Subtotal		45	
Genomic DNA extract		5	
Total		50	

2.2.2. PCR cycling parameters:

2 min at 94°C, 30 cycles of 1 min at 94°C, 50 s at 60°C, 1 min at 72°C.

2.3. Restriction fragment length polymorphism (RFLP) reaction.

2.3.1. If required store products at approximately 4°C before analysis.

2.3.2. Master mix: according to the supplier's instructions.

2.3.3. Incubation temperature, time: incubation time/temperature for digestion – overnight at the recommended temperature (see supplier's instructions).

3. Essential procedural information

3.1. Controls.

For a reliable test result to be obtained the following (external) controls should be included for each series of nucleic acid isolations and amplifications of *G. pallida* and *G. rostochiensis* and nucleic acid, respectively.

- NIC to monitor contamination during nucleic acid extraction: this can be obtained by performing DNA extraction of the solution/buffer used to collect nematode specimens (e.g. DNA extraction buffer alone).
- PIC to ensure that nucleic acid of sufficient quantity and quality is isolated: solution/buffer spiked with an appropriate number of individual cysts or juveniles confirmed as being from either *G. pallida* or *G. rostochiensis*. This control is optional as long as this test is applied on isolated nematodes (not on bulk solutions or as a screening test).

TABLE 5 Sizes of RFLP fragments (Thiéry & Mugniéry, 1996).

Species	<i>Bsh1236I</i> RFLP pattern
<i>G. rostochiensis</i> (European populations)	900, 190, 110
<i>G. pallida</i> (European populations)	500, 400, 350, 190, 110
<i>G. 'mexicana'</i>	500, 400, 190, 110
<i>G. tabacum tabacum</i>	445, 400, 190, 110
<i>G. tabacum virginiae</i>	445, 400, 190, 110
<i>G. tabacum solanacearum</i>	445, 400, 190, 110

- NAC to rule out false positives owing to contamination during the preparation of the reaction mix – amplification of molecular-grade water that is used to prepare the reaction mix should be used instead of DNA extract.
- PAC to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism – amplification of genomic DNA of individuals from both species *G. pallida* and *G. rostochiensis*, the identity of the individuals or the genomic solutions used must have been confirmed.

3.2 Interpretation of results.

Verification of the controls:

- The NIC and NAC should produce no amplicons.
- PAC, and if relevant PIC, should produce amplicons of the expected sizes, namely 1200 bp for *Globodera* species.

When these conditions are met:

- A test will be considered positive if it produces the restriction fragment lengths as given in Table 5.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The following performance criteria were provided by Anses Plant Health Laboratory (France), July 2010.

- 4.1. Analytical sensitivity data: one J2.
- 4.2. Analytical specificity data: the study included 11 populations of *G. pallida*, four populations of *G. rostochiensis*, five populations of *G. tabacum* and one population of *G. mexicana* and *G. artemisiae*. The populations cover different geographical areas.
- 4.3. Data on repeatability: 100% for *G. pallida*, 100% for *G. rostochiensis*.
- 4.4. Data on reproducibility: 90% (one J2) for *G. pallida*, 93% (one J2) for *G. rostochiensis*.

- 4.5. Diagnostic specificity: 91% for *G. pallida* (one non-target species detected in 11), 100% for *G. rostochiensis*.

APPENDIX 8 - DIAGNOSIS OF *G. PALLIDA* AND *G. ROSTOCHIENSIS* PCNS USING TAQMAN® REAL-TIME PCR DEVELOPED BY FERA, GB

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

- 1.1. The test was developed by Fera, York, GB following on from the Potato Council Project R287 'Validation of quantitative DNA detection systems for PCN' and was finalized in October 2009.
- 1.2. DNA was typically extracted from either a single cyst or the anterior half of a single cyst of *Globodera* spp. Egg and/or juvenile number varies based on the given cyst being tested. A typical sample of half a single cyst would contain approximately 100 eggs (based on 315 cyst counts by Fera). DNA can also be extracted from single juveniles.
- 1.3. The real-time PCR test targets the internal transcribed spacer I (ITS1) gene, accession numbers AF016871 for *G. pallida* and EF622531 for *G. rostochiensis*.
- 1.4. The forward primer is located at base 521 of *G. pallida* AF016871 and base 356 of *G. rostochiensis* EF622531.
- 1.5. The amplicons length including primers is 71 bp.
- 1.6. Oligonucleotides:

Primers	Glob 531F	5'-TGT-AGG-CTG-CTA-YTC-CAT-GTY-GT-3'
	Glob 601R	5'-CCA-CGG-ACG-TAG-CAC-ACA-AG-3'
Probes	GP LNA	5'-FAM-TGC-CGT-ACC-(C)(A)G-CGG-CAT-BHQ1-3' ^a
	GR LNA	5'-TET-GCC-GTA-CC(T)-(T)GC-GGC-AT-BHQ1-3' ^a

^a DNA bases surrounded with brackets are locked nucleic acid (LNA) bases. Dual-labelled probes with incorporated LNA bases can be ordered from Sigma Genosys.

- 1.7. Applied Biosystems TaqMan® Universal Master Mix II, without uracil-*N* glycosylase (UNG) (4440043). The enzyme used is AmpliTaq Gold Ultra Pure DNA polymerase (Applied Biosystems), with the mix used at a final concentration of 1×.
- 1.8. No reaction additives are used.

- 1.9. Sterile water diethylpyrocarbonate (DEPC)-treated, molecular biology grade (Severn Biotech Ltd).
- 1.10. Applied Biosystems ABI Prism 7900HT Sequence Detection System.
- 1.11. Data analysis using Applied Biosystems sequence detection system, software versions 2.0, 2.2 and 2.4.

2. Methods

2.1. Nucleic acid extraction and purification.

- 2.1.1. Single cysts of *Globodera* spp., the anterior half of a single cyst containing eggs, or single juveniles are used for DNA extraction.
- 2.1.2. DNA extractions are performed using a Qiagen DNeasy Blood and Tissue Kit, following the manufacturer's protocol for animal tissues with the following modifications. The sample is placed in a 1.5 mL Eppendorf tube and homogenized with a micropestle in 180 µL of ATL buffer. A 20 µL aliquot of Proteinase K is added, and the sample is vortexed to mix and centrifuged briefly to pool. The samples are incubated at 56°C and 100 rpm for at least 3 h or overnight. The manufacturer's protocol is continued from step 3, and for elution (steps 7 and 8) each time 60 µL of AE buffer is used, giving DNA in a total volume of 120 µL.
- 2.1.3. No nucleic acid cleanup procedure is required.
- 2.1.4. Either use extracted DNA immediately or store overnight at approximately 4°C or at approximately -20°C for longer periods.

2.2. Real-time PCR.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	6.75	NA
TaqMan® Universal Master Mix II, no UNG (4440043) (Applied Biosystems)	2×	10	1×
Glob 531F	7.5 µM	0.375	112.5 nM
Glob 601R	7.5 µM	0.375	112.5 nM
GR LNA	5 µM	1.0	200 nM
GP LNA	5 µM	0.5	100 nM
Subtotal		19	
DNA		1	
Total		20	

- 2.3. PCR cycling parameters: 50°C for 2 min; initial denaturation 95°C for 10 min; cycling denaturation 95°C for 15 s; cycling annealing and extension 60°C for 1 min; heating and cooling ramp at 100%; run for 40 cycles; fluorescence capture at all steps and cycles.

3. Essential procedural information

3.1. Controls.

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolations and amplifications of the target organism and target nucleic acid, respectively.

- NIC to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of clean extraction buffer. It is recommended to perform NIC several times in a series of extracts, for example one per five samples.
- PIC to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a whole cyst of either *G. pallida* or *G. rostochiensis*.
- NAC to rule out false positives owing to contamination during the preparation of the reaction mix: amplification of PCR-grade water that was used to prepare the reaction mix, in place of DNA.
- PAC to monitor the efficiency of the amplification: amplification of nucleic acid of the target organisms, from both *G. pallida* and *G. rostochiensis* separately.

3.2. Interpretation of results.

Verification of the controls:

- The PIC and PAC amplification curves should be exponential.
- The NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- From the validation data, it was stated that a test is considered as positive if the C_t value is below 37 (owing to cross-reactions). This threshold should be re-evaluated by the laboratory at the limit of detection. For reference, one single juvenile produces an average C_t of 30 for both species; therefore, this is greater than the limit of detection.
- A C_t between 37 and 40 for either test is considered an inconclusive result and requires further investigation, such as repeating the DNA extraction and PCR and/or testing the sample by an alternative method.
- Tests should be repeated if any contradictory or unclear results are obtained.

Additional considerations:

- 1. The *G. pallida* probe is known to cross-react slightly with *G. rostochiensis* DNA. The cross-reaction will show as a slight increase in ΔR_n in the FAM channel (*G. pallida*) as the ΔR_n increases exponentially in the TET channel (*G. rostochiensis*). This cross-reaction is only observed when a sample is positive for *G. rostochiensis*.
- 2. The *G. pallida* test cross-reacts with high concentrations of DNA of *G. tabacum*; however, this generates a non-exponential amplification profile and can be differentiated from *G. pallida* and *G. rostochiensis*. *Globodera tabacum* is not known to be present in the United Kingdom; however, if *G. tabacum* is suspected then a *G. tabacum*-positive control should be run with each test to aid interpretation of the results, and any inconclusive results followed up with testing by another method.
- 3. The *G. pallida* test cross-reacts with *Punctodera* spp., generating a $C_t > 37$; however, firstly *Punctodera* cysts have a distinct morphology compared with *Globodera* and would not be selected for molecular testing and secondly this C_t value would instigate further testing.

4. Performance characteristics available

Validation data is available in the EPPO database on diagnostic expertise (<http://dc.eppo.int/validationlist.php>).

4.1. Analytical sensitivity data.

A typical sample would be half a cyst, containing approximately 100 eggs; however, the test is able to detect a single juvenile. A 100% success rate is achieved for single juveniles. Numerous half cysts with variable egg numbers have been tested. DNA from a single cyst is detectable at or at greater than a 1000-fold dilution.

4.2. Analytical specificity data.

Target organisms tested: 100+ populations of *G. pallida* from 26 counties (see validation report); 30+ populations of *G. rostochiensis* from 10 counties (see validation report).

Non-target organisms tested: populations of *G. tabacum* (see validation report); populations of *G. millefolii* (as *G. achilleae*; see validation report).

The *G. pallida* test cross-reacts with *G. tabacum*; however, this gives a non-exponential profile and can be differentiated for *G. pallida* and *G. rostochiensis*. The *G. pallida* test cross-reacts with *Punctodera* spp.;

however, firstly *Punctodera* cysts have a distinct morphology compared with *Globodera* and would not be selected for molecular testing and secondly this species generates a $C_t > 37$ which would instigate further investigation.

4.3. Data on repeatability.

Extractions were tested at both neat and 10^{-3} dilutions with eight replicates. Repeatability was 100%.

4.4. Data on reproducibility.

Extractions obtained were tested in a neat concentration by two different operators across two Applied Biosystems ABI Prism 7900HT Sequence Detection System machines available in the laboratory. Reproducibility was 100%.

4.5. Diagnostic sensitivity.

The test was compared with the UKAS-accredited conventional PCR test of Bulman and Marshall (1997) using 149 samples. This resulted in a 100% diagnostic sensitivity for both *G. pallida* and *G. rostochiensis*.

4.6. Diagnostic specificity

The test was compared with the UKAS-accredited conventional PCR test of Bulman and Marshall (1997) using 149 samples. The diagnostic specificity was 87.1% for *G. pallida* and 93.8% for *G. rostochiensis*.

APPENDIX 9 - IDENTIFICATION OF VIABLE PCNS (*GLOBODERA* SPP.) USING RNA-SPECIFIC REAL-TIME RT-PCR BENIERS ET AL. (2014).

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

- 1.1. The test was developed for the detection of viability and identification of *Globodera* spp. by real-time RT-PCR. This test is based on the article of Beniers et al. (2014).
- 1.2. Total nucleic acids are extracted from a sample containing from one to 50 *Globodera* cysts.
- 1.3. The real-time PCR test targets the elongation factor 1- α gene (EF1- α) mRNA.
- 1.4. No data on primer position.
- 1.5. No data on amplicon size.
- 1.6. Oligonucleotides:

Primers	Fw_EF_Grp_mRNA	5'-ACAAGATCGGAGGTATCG-3'
	Rv_EF_Gp_mRNA-1	5'-GTGGTTCATGATGATGACCTG-3'
Probes	EF_Gpal_probe ^a	5'-Yakimo Yellow- CGAAGA(A)(T) GACCCGGC- BHQ1-3'
	p_EF-Gros_2LNA ^a	5'-6FAM-CTCGAAGAG(C)GAC(C) CTG-BHQ1-3'

^a DNA bases surrounded with brackets are LNA bases.

1.7. Reverse transcription and real-time PCR are performed within one step using the One Step PrimeScript™ RT-PCR (TaKaRa RR064A).

1.8. No reaction additives are used.

1.9. RNase-free reagents, including molecular-grade water.

1.10. Applied Biosystems ABI Prism 7500.

1.11. Data analysis with the 7500 Fast System Software version 1.4

2. Methods

2.1. Nucleic acid extraction.

2.1.1. Extraction of RNA is performed using a MasterPure™ Complete DNA and RNA Purification Kit (Epicentre) following the manufacturer's protocol for tissue with the following modifications.

2.1.2. One to 50 cysts of *Globodera* spp. are used for nucleic acid extraction.

Collect the cysts in a 1.5 mL tube. Add 100 μ L of tap water.

Add a stainless steel ball (3 mm) or a glass bead and crush using the Retsch MM301/MM400 for 5 min at 30 Hz.

Spin off the tubes.

Then follow the manufacturer's protocol.

2.2. Real-time RT-PCR

A single-step real-time RT-PCR is performed as described below and using the One step Prime Script™ RT-PCR (TaKaRa RR064A) for a final volume of 25 μ L.

Reagent	Working concentration	Volume per reaction (μ L)	Final concentration
PCR-grade water		2.25	
One Step RT-PCR Buffer III (TaKaRa)	2 \times	12.5	1 \times
Primer Fw_EF_Grp_mRNA	10 μ M	0.75	0.3 μ M
Primer Rv_EF_Gp_mRNA-1	10 μ M	0.75	0.3 μ M
Probe EF_Gpal_probe	5 μ M	2	0.5 μ M
Probe p_EF-Gros_2LNA	5 μ M	0.5	0.1 μ M

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Prime Script™ RT Enzyme Mix II (TaKaRa)	NA	0.5	NA
TaKaRa Taq™ HS (Takara)	5 U/µL	0.5	2.5 U
ROX™ Reference Dye IIa	50×	0.25	0.5×
Subtotal		20	
DNA		5	
Total		25	

^a Linked to the cycling machine used; consult the manufacturer's instructions; NA, not applicable.

2.3. PCR cycling parameters: 42°C for 5 min; initial denaturation, 95°C for 10 s; cycling denaturation, 95°C for 10 s; cycling annealing and extension, 60°C for 1 min; run for 40 cycles; fluorescence capture at all steps and cycles.

3. Controls

3.1. Controls.

For a reliable test result to be obtained the following controls should be included for each series:

- NIC to monitor contamination during nucleic acid extraction – nucleic acid extraction and subsequent amplification preferably of clean extraction buffer/collecting solution or a *Heterodera* cyst in 100 µL of tap water.
- PIC to ensure that nucleic acid of sufficient quantity and quality is isolated – nucleic acid extraction and subsequent amplification of a matrix sample that contains viable cysts of target *Globodera* spp. (e.g. suspension of viable eggs of each species *G. pallida* and *G. rostochiensis*).
- NAC to rule out false positives owing to contamination during the preparation of the reaction mix – amplification of RNase-free molecular-grade water that is used to prepare the reaction mix.
- PAC to monitor the efficiency of the cDNA amplification – amplification of nucleic acid solution from *G. pallida* and *G. rostochiensis*.

3.2. Interpretation of results.

Verification of the controls:

- The PIC and PAC should have an exponential curve.
- The NIC and NAC control should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve.

- Tests should be repeated if any contradictory or unclear results are obtained.

4. Validation

Validation data have been generated by the Dutch General Inspection Service (NAK) according to PM 7/98.

- 4.1. Analytical sensitivity: 1 viable juvenile or egg.
- 4.2. Diagnostic sensitivity: 100%.
- 4.3. Diagnostic specificity: *G. pallida*, 100%; *G. rostochiensis*, 100%.
- 4.4. Analytical specificity:

Inclusivity 100% evaluated on six strains of *G. rostochiensis* and eight strains of *G. pallida*

Exclusivity no cross-reactions with other organisms (*H. betae*, *Heterodera glycines*, *H. schachtii*, *Heterodera trifolii*, *G. tabacum* and *Cactodera cacti*).

4.5. Reproducibility: 100%.

4.6. Repeatability: 100%.

APPENDIX 10 - VISUAL DETERMINATION

Characteristics of live and dead eggs and juveniles of the potato cyst nematodes⁷:

Live eggs (Figures 11 and 12)	Dead eggs (Figures 16 and 17)
a. Whole egg is intact	a. Egg may be damaged/broken and empty
b. Egg shell is smooth	b. Egg shell often not smooth
c. Egg is clear/transparent with distinct contents or a dark line down the middle of the egg	c. Contents have black/grey granular appearance with no structure
d. Curled juvenile filled up against the egg shell	d. Shriveled disintegrated juvenile in egg
e. Sometimes clear lip region and stylet present	e. No clear lip region or stylet present
Live juveniles (Figures 13–15)	Dead juveniles (Figures 18–20)
a. Juvenile has clear lip region, stylet visible	a. No clear lip region, partly or completely grey/black structure
b. Juvenile has strong smooth cuticle	b. Cuticle shriveled or not intact
c. Intestine is filled with grey granular structure, solid	c. Transparent, body with clear patches or completely transparent
d. Clear lopsided distinction between pharynx and intestine	d. No clear lopsided distinction between pharynx and intestine
	e. Juvenile sharply bent at an angle or lying in a half circle
Included in counts: heads	Not included in counts: tails, empty shells

⁷Based on a test performance study between laboratories in the Netherlands (L. den Nijs pers. comm.).

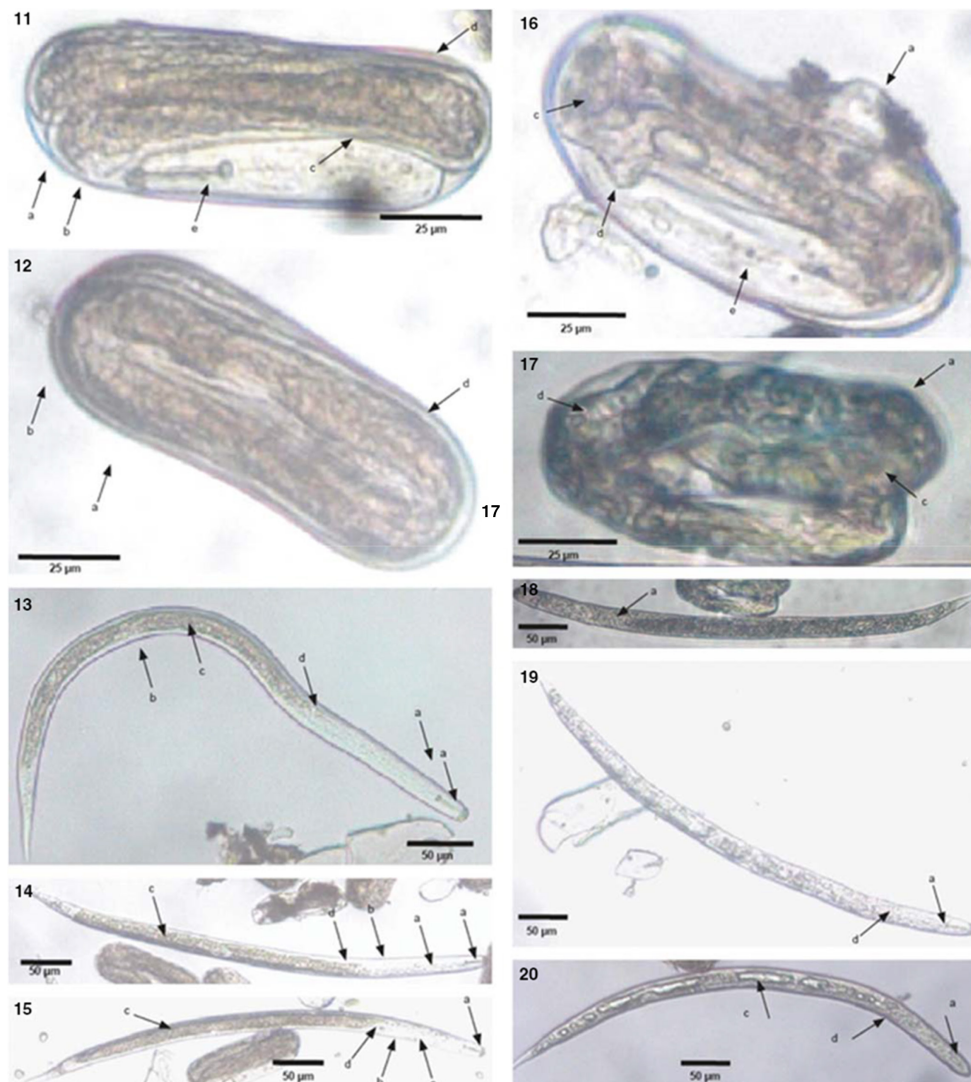


FIGURE 11-20 Characteristics of live and dead eggs and juveniles of the potato cyst nematodes

APPENDIX 11 - HATCHING TEST

(A) Hatching test (method performed in Norway)

The hatching medium, potato root diffusate (PRD), is obtained by passing 1000 mL of tap water through a 500 mL pot containing a 3 week-old potato plant growing in sand. After filtering, the PRD is stored at +3°C in the dark until needed. The PRD is used without dilution in closed glass vials (diameter = 23.5 mm, height 34 mm) functioning as hatching units. Each vial contains one cyst bag made from nylon net with 20 cysts, which is completely covered by PRD. Cysts collected in the autumn need to be exposed to +4°C for 4 months to break dormancy. After exposure to the root diffusate a high hatching frequency is reached after only 2 weeks. Each week the cyst bags are transferred to new hatching units with fresh PRD. The number of hatched juveniles is counted weekly and accumulated to form the total

hatch. At the end of the test the juveniles remaining in the cysts are counted, so the hatching can be expressed as a percentage of the total cyst content (Figure 21).

(B) Hatching test (methods performed in Sweden)

Prior to the exposure to the hatching stimulus, cysts that have been stored dry should be pre-soaked in water in Petri dishes, staining blocks or other suitable containers for 4–5 days. Whereas non-hydrated cysts tend to float, hydrated ones sink to the bottom of the Petri dish, which gives a good indication of a cyst's hydration level. During this hydration phase, the water in the containers is renewed daily to prevent bacterial and fungal growth. Repeated up and down pipetting of the cysts helps to get rid of the fungi and favours the hydration.

Potato plants for the production of the hatching medium (i.e. PRD) are grown in small (200 mL) clay pots with silver sand substrate in a greenhouse. Three-week-old plants are removed from the substrate and their roots

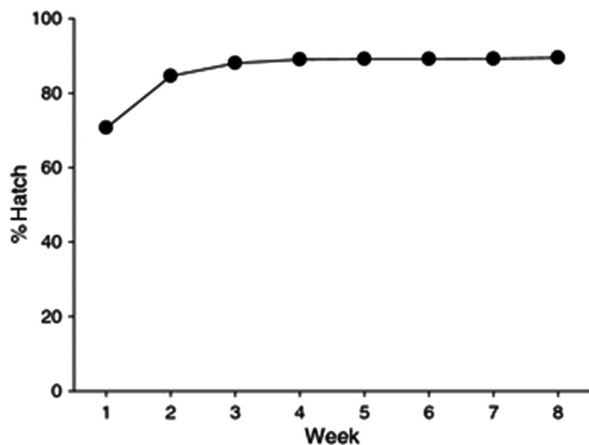


FIGURE 21 Cumulative hatch of *G. rostochiensis* in potato root diffusate from 3-week-old plants ($n = 4$)

are rinsed in water, after which the plants are transferred one by one into 200 mL beakers filled with tap water and incubated at room temperature under aeration by an aquarium air pump (only the root system is immersed in the water).

The diffusate is collected after 24 h and filtered and is then ready to use. Hydrated cysts (up to 100, depending on the number of cysts found in the sample) are soaked in the undiluted PRD. The PRD is replaced daily with a fresh sample.

The test lasts until juveniles start to hatch or for a total of 8 weeks.

(C) Hatching test (methods performed in France)

This test is shorter and follows the procedure described in test (B) with the following modifications.

PRD production: sprout tubers are placed on a funnel put on a plastic (transparent) beaker filled up with tap water. This assemblage is stored at room temperature (around 18–19°C) in the dark for 4 weeks. The water with PRD is filtered, divided into aliquot parts and frozen until use (–20°C). After freezing for 48 h, the PRD is evaluated with the previous batch of PRD (previous

production) and reference *G. pallida* and *G. rostochiensis* populations. If the test is satisfactory, the PRD can be used for the hatching test.

Hatching test: instead of being rehydrated, cysts are deposited in a fine sieve (250 µm) placed on a small dish filled with PRD. One sieve is prepared per sample to be tested. Twenty cysts of *G. pallida* and *G. rostochiensis* are put into two additional sieves as positive controls. All samples and controls are left at room temperature in the dark until they are checked for hatching. Each sample and the controls are checked for hatching every 10 days. If juveniles have hatched, the test is considered positive and the result is that the cysts are viable. New PRD is added every 10 days if necessary.

If no hatching occurs after 30 days, the cysts are crushed and the viability of juveniles is assessed by visual examination (Appendix 10). If viable juveniles are detected, the result of the test is positive, otherwise it is negative. If positive controls do not hatch the viability tests are not considered valid.

APPENDIX 12 - VIABILITY TEST BY TREHALOSE (VAN DEN ELSSEN ET AL., 2012; EBRAHIMI ET AL., 2015)

Trehalose, a disaccharide, is present in high concentrations in the perivitelline fluid of eggs in cysts and can be used as viability marker. Pre-soaked cysts (1–2 days) are boiled to free the trehalose that was present in the live eggs within the cysts. The boiling of the cysts breaks down the membrane and releases the trehalose into the solution. The presence of trehalose can be verified directly using a simple detection kit; the disaccharide is hydrolysed into two glucose molecules, and subsequently the glucose can be detected. Cutting the cysts before or after boiling facilitates the release of the trehalose into the solution. The latter is necessary when little living content is expected. When species identification is to be performed after the viability test, lysis buffer should be added after the trehalose measurement. Extraction of DNA of this solution can take place and a subsequent PCR test can be performed (see Appendices 2–8).