European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

21-27082 (21-26497)
PM 7/40 (5)

Diagnostics

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

Specific scope

This Standard describes a diagnostic protocol for *Globodera rostochiensis* and *Globodera pallida*. Terms used are those in the EPPO Pictorial Glossary of Morphological Terms in Nematology[1].
This Standard should be used in conjunction with PM 7/ 76 *Use of EPPO diagnostic protocols* [2].
Authors and contributors are given in the Acknowledgements section

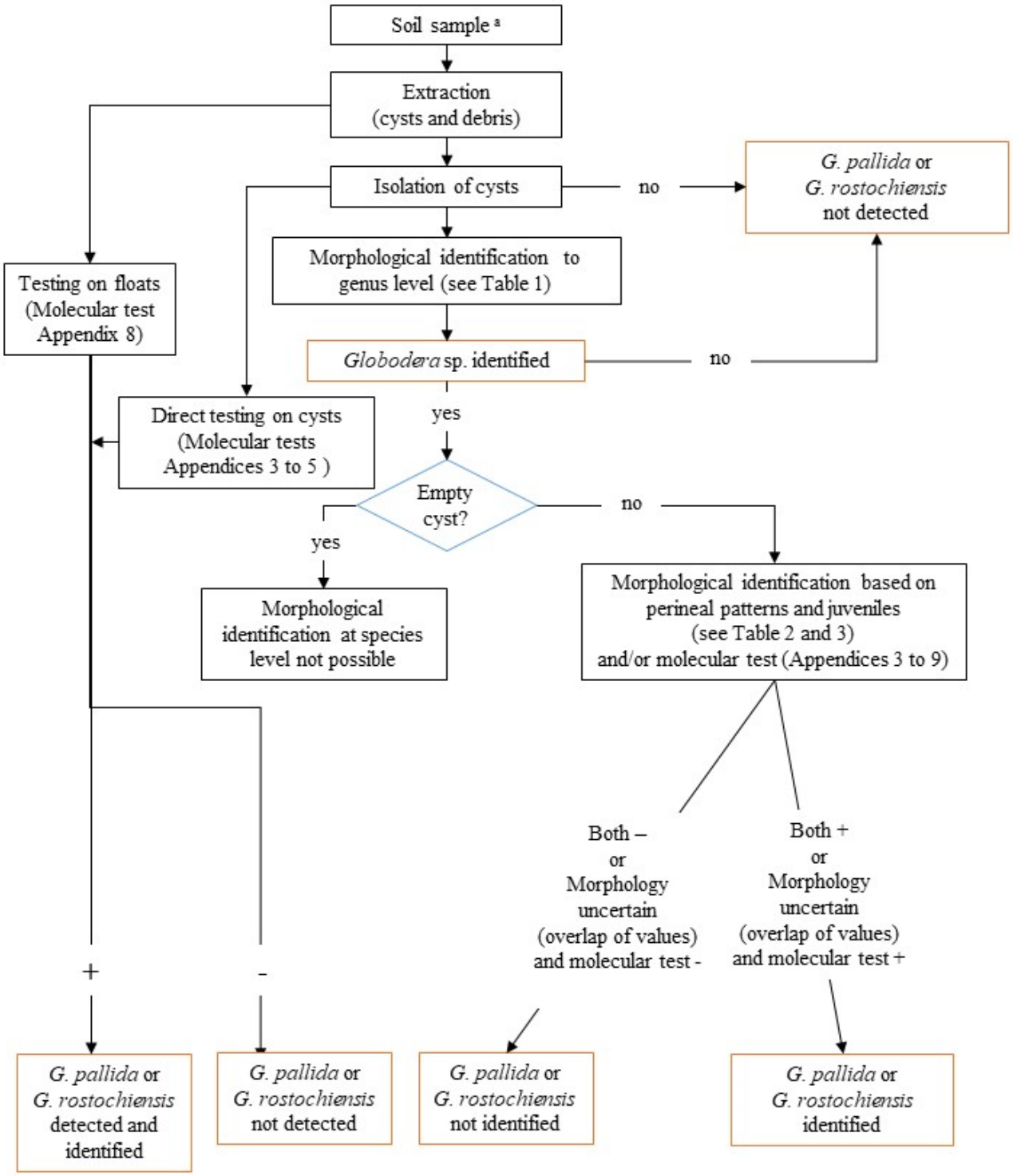
Specific approval and amendment

Approved as an EPPO Standard in 2003-09. Revisions approved in 2009-09, 2012-09 and 2017-02. 4th revision approved in 2021-XX.

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 routes 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 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*).
A flow diagram describing the diagnostic procedure for *G. rostochiensis* and *G. pallida* is presented in Fig. 1.

[1] http://www.eppo.int/QUARANTINE/diag_activities/EPPO_TD_1056_Glossary.pdf
[2] Use 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.



[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.

Fig. 1 Flow-diagram for identification of *G. rostochiensis* and *G. pallida*.

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.* 2019, 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 to the naked eye as tiny white, yellow or brown pin-heads on the root surface (Figs 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 it is time-consuming. Soil testing is therefore the best way to determine the presence of PCNs.

Fig. 2 Potato roots infected by *G. rostochiensis*. (Courtesy: NRC-NPPO, NL.)

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

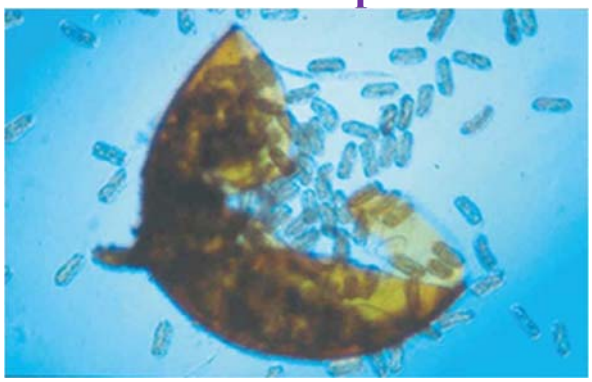


Fig. 3 Broken cyst with eggs of *G. pallida*. (Courtesy: NRC-NPPO, NL.)

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).

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://sitesv2.anses.fr/en/minisite/plant-parasitic-nematodes/videos-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 *G. rostochiensis*, *G. pallida.*	3
Reid *et al*., (2015) High-throughput diagnosis of PCNs (*Globodera* spp.) in soil samples using real-time PCR	4
Real-time PCR tests for species specific identification as well as detection of *G. rostochiensis*, *G. pallida* and *G. tabacum* (based on LSU rDNA) available as an all-inclusive real-time PCR kit (http://www.cleardetections.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 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 which changes during maturation from white to yellow then into a brown cyst is *G. rostochiensis*, while one which 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 Figs 4 – 7). Further information is provided by keys of Brzeski (1998), Baldwin & Mundo-Ocampo (1991), Wouts & Baldwin (1998), Siddiqi (2000) and Subbotin *et al.* (2010).

Table 1 Dichotomous key to genus of Heteroderidae cysts is presented below:

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

Globodera cysts should present the following characteristics:

Cysts of *Globodera* are smoothly rounded with a small projecting neck, no terminal cone, diameter ± 450 µm, and with a tanned brown skin (Fig. 6A). The cuticle surface has a zigzag pattern of ridges. The perineal area (Figs 5 and 7A) consists of a single circumfenestration around the vulval slit, with tubercules 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 not present in *G. rostochiensis* and *G. pallida.* Eggs retained in cyst, no egg-mass present.

4.1.1.2 Juveniles

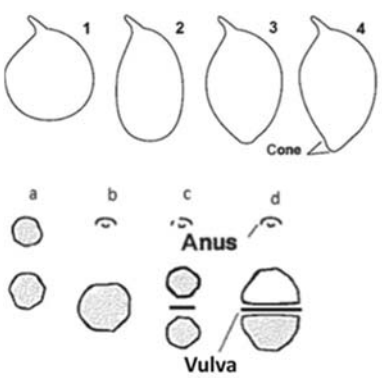
In addition to the juveniles in cysts, juveniles of cyst nematodes may be found incidentally in soil extracts after extraction for the detection of non-sedentary stages of nematodes.

Distinction between 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 juveniles should present the following characteristics:

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

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



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)*

Fig 4. Form of cysts and characteristics of the vulval-anal region. (After Baldwin & Mundo-Ocampo (1991).)

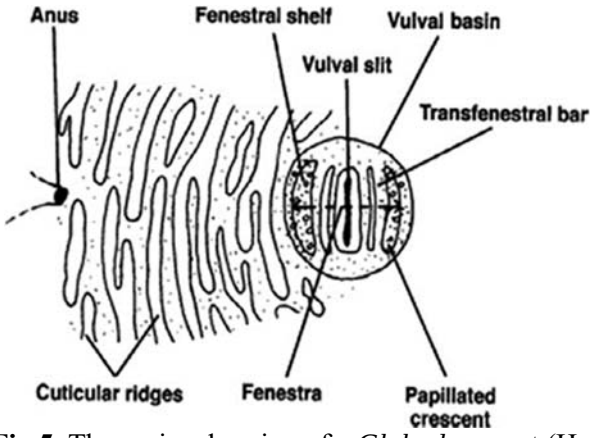
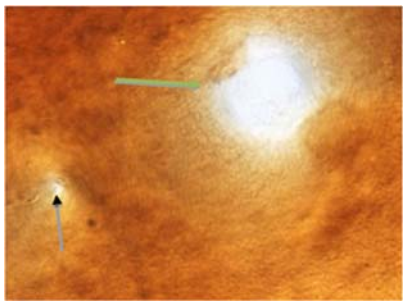


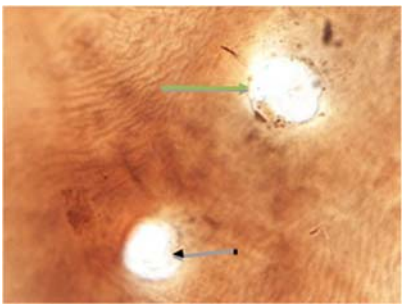
Fig 5. The perineal region of a *Globodera* cyst (Hesling, 1978).

Globodera spp. *Heterodera* spp. *Punctodera* spp.

Fig 6. Heteroderidae cysts. Scale bar = 350 µm. (Courtesy NAK, NL.)



Globodera sp. vulval fenestra/anal region non fenestrate



Punctodera sp. vulval fenestra/anal region fenestrate

Fig 7. Perineal region. Green arrows indicate the vulva, black arrows the anus. *Globodera* spp. vulval fenestra/anal region non-fenestrate. *Punctodera* spp. vulval fenestra/anal region fenestrate. (Courtesy NAK, NL.)

Table 2. Dichotomous key to *Globodera* species. (After Subbotin *et al*. (2010).)

1	Cuticle of cyst thin, transparent	*G. mali*
	Cuticle of cyst thick, dark in colour	2
2	Mean length of J2 stylet ≤26 µm	3
	Mean length of J2 stylet ≥27 µm	*G. zelandica*
3	Mean length of J2 stylet <19 µm	*G. leptonepia*
	Mean length of J2 stylet ≥19 µm	4
4	Hyaline region of J2 > 31 µm	*G. bravoae*
	Hyaline region of J2 ≤ 31 µm	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 ≥5.5 µm; 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 µm	8
7	Cyst wall lacking a network-like pattern, ridges close; mean number of cuticular ridges = 13 (10–18); ♂ spicules with a pointed, thorn-like tip	*G. ellingtonae*
	Cyst wall exhibiting network-like or maze-like patterns; mean number of cuticular ridges = 7–8 (5–15); ♂ spicules with a finely rounded tip	*G. tabacum sensu lato*
8	Cysts with prominent bullae in the terminal region of most specimens; J2 lip region with 3 annules, mean hyaline region >28 µm	*G. capensis*
	Cyst abullate, at most with small vulval bodies in some specimens; J2 lip region with 4–6 annules, mean hyaline region <28 µm	9
9	J2 stylet knobs distinctly anteriorly directed to flattened anteriorly; mean J2 stylet length >23 µm; Granek's ratio <3	10
	J2 stylet knobs rounded to flattened anteriorly; mean J2 stylet length <23 µm; Granek's ratio ≥3	*G. rostochiensis*
10	Mean Granek's ratio = 2.1–2.5	*G. pallida*
	Mean Granek's ratio = 2.8	*G. mexicana*
11	J2 lip region with 5–6 annules	12
	J2 lip region with 3 annules	*G. capensis*
12	Mean stylet ≥25 µm in J2, ♂ gubernaculum = 11.2–12.9 µm	*G. millefolii*
	Mean stylet <25 µm in J2, ♂ gubernaculum = 6.0–9.9 µm	*G. artemisiae*

The morphological key to *Globodera* species presented in Table 2 above has used the mean average of morphometric characters to assist with differentiation, due 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.

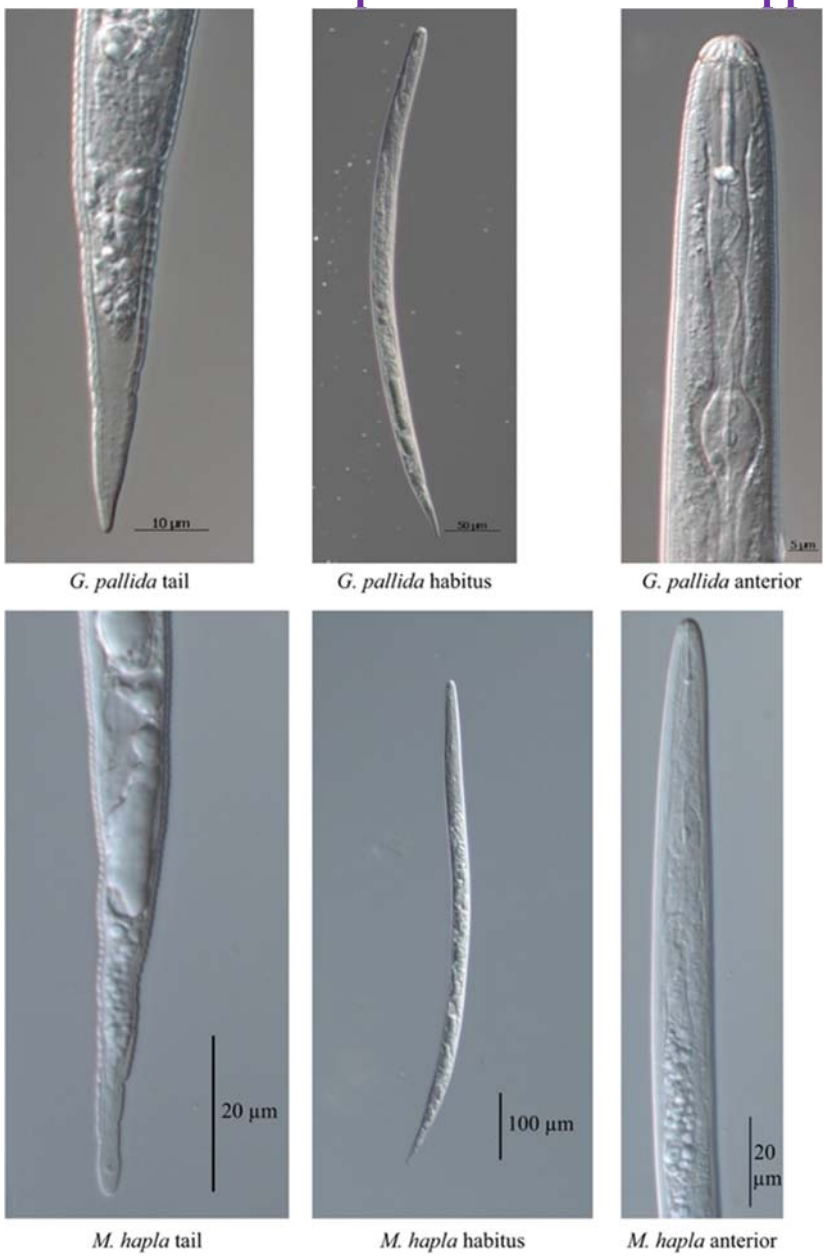


Fig 8. Difference between Meloidogynidae and Heteroderidae juveniles. Comparison between *Meloidogyne hapla* and *G. pallida*. (Courtesy FERA, UK.)

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 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.

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 1973 a and b, Baldwin & Mundo-Ocampo, 1991; Mota & Eisenback, 1993; Brzeski, 1998; Flemming & Powers, 1998; Manduric & Anderson, 2004).

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

* From Flemming and Powers 1998; Brzeski 1998 refers to 16-31

[a] Krall (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.

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* (Fig. 9A) and *G. pallida* (Fig 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 (Fig. 10A, B). The second-stage juvenile characteristics are stylet length and stylet knob shape and width (Table 3, Fig. 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.

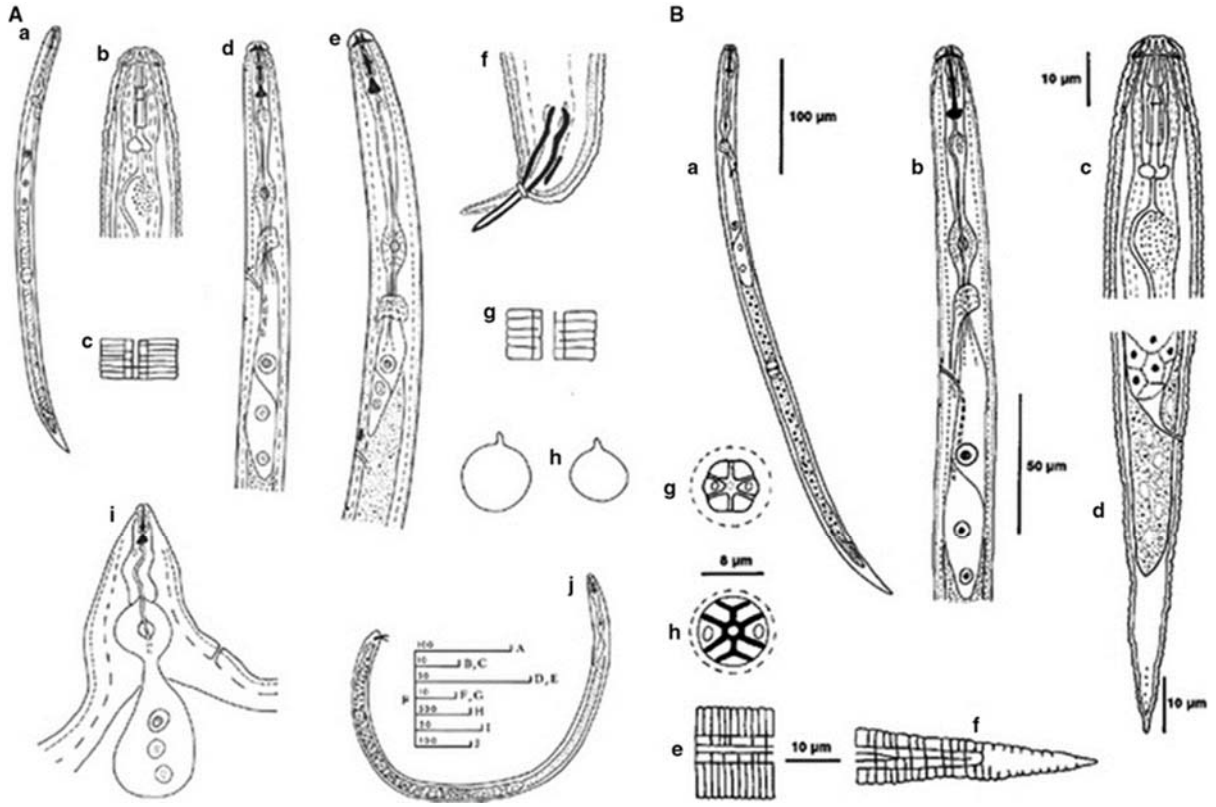


Fig 9. Illustrations on the left-hand side of plate (side labelled A in bold), *G. rostochiensis*: (a) entire juvenile; (b) head region of second-stage juvenile; (c) second-stage juvenile lateral field, mid-body; (d) pharyngeal region of second-stage juvenile; (e) pharyngeal region of male; (f) tail 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 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).)

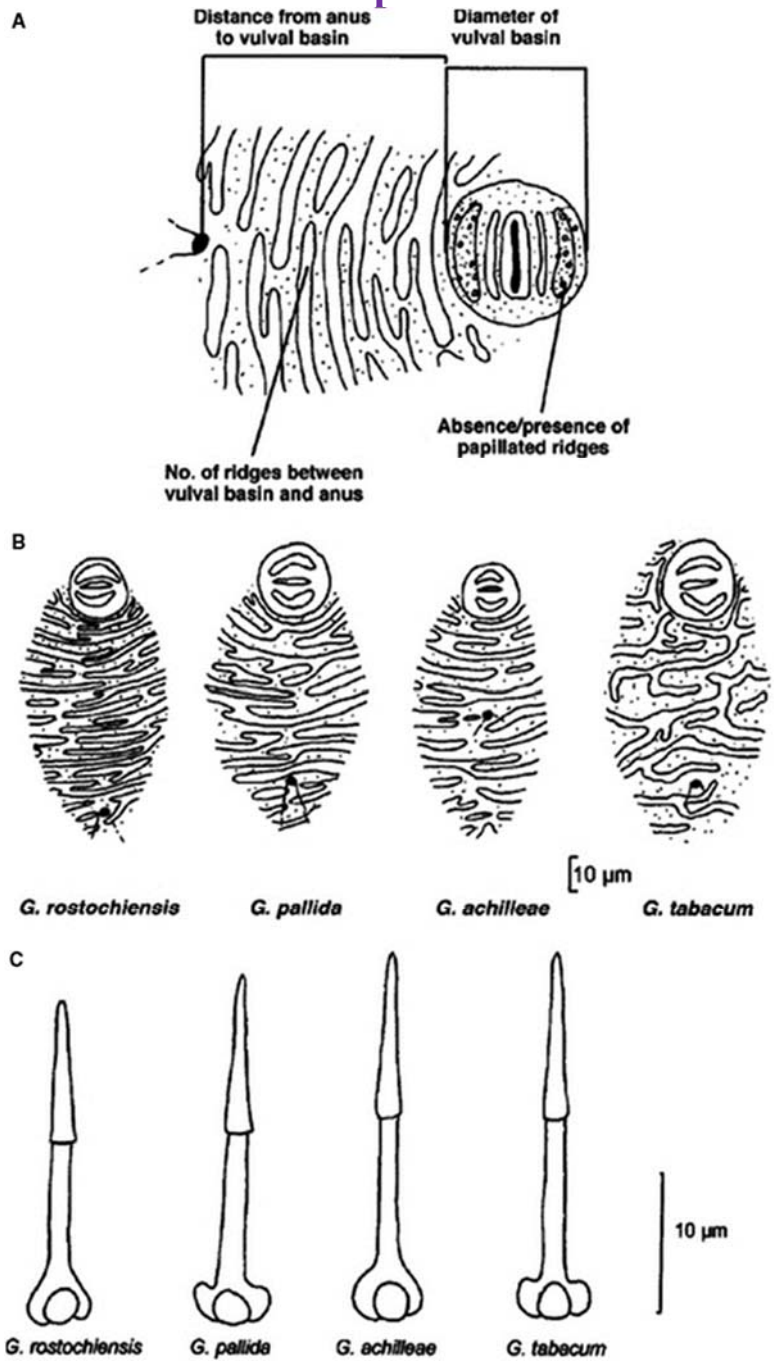


Fig 10. (A) Perineal measurements for *Globodera* identification. (B) Vulval-anal ridge patterns for four *Globodera* species. (C) Stylets from four species of *Globodera*. See footnote 6 (section 4.1.2) for *G. achilleae*[5]. (After Fleming & Powers (1998).)

When cysts without live content, meaning 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 *Globodera pallida* and *G. rostochiensis* (perineal 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.

[4] It 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.*

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 *Globodera tabacum sensu lato*. These 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 Fig. 10 provide a morphometric and morphological comparison between the PCNs *G. millefolii*[5], *G. artemisiae* and *G. tabacum*. See also Baldwin & Mundo-Ocampo (1991), Mota & Eisenback (1993), Brzeski (1998) Wouts & 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 *G. sandveldensis* and *G. agulhasensis*, both parasitizing non *Solanaceae* plants, have been described in South Africa (Knoetze *et al*., 2017 a & 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. 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* or *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 the distinction 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.

[5] Krall (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.

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

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 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 utmost importance in populations from South America, but identification at this level is not adequate at the moment and it is time-consuming, 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 due 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 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:
National Plant Protection Organization, National Reference Centre, PO Box 9102, 6700 HC Wageningen (NL).
Food and Environmental Research Agency (Fera), Sand Hutton, York YO41 1LZ (GB).
Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Messeweg 11–12, 38104 Braunschweig (DE).
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 (FR).

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. 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 (NL). 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@eppo.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.

11. Acknowledgements

This Standard was originally developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by a partnership of contractor laboratories and intercomparison laboratories in European countries. The first draft was prepared by: L den Nijs and G Karssen, National Plant Protection Organization, National Reference Laboratory, PO Box 9102, 6700 HC Wageningen (NL). It was revised by G Anthoine (Anses, Plant Health Laboratory, Angers, FR), L Kox, G Karssen, B van den Vossenberg and L den Nijs (National Plant Protection Organization, National Reference Center, Wageningen, NL).
Methods performed in Germany and Austria provided by D Kaemmerer, Bayerische Landesanstalt für Landwirtschaft. Methods performed in Norway provided by C Magnusson. Methods performed in Sweden provided by S Manduric. This revision was prepared to include a new molecular test (Appendix 3) and to include references in Table 3. The test description was prepared by S Gamel (Anses, Plant Health Laboratory, Rennes, FR), the revision of Table 3 was prepared by I Gabl (Austrian Agency for Health and Food Safety, Vienna, AT) and E van Heese (National Plant Protection Organization, National Reference Center, Wageningen, NL).
The Standard was reviewed by the Panel on Diagnostics in Nematology.

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Appendix 1: - Bioassays

Test A Bioassay (method performed in Germany and Austria)

This method relies on the principle that if 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 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 unlikely. 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 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 1/3 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 soil (for instance by 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.

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

Appendix 2 - Nucleic acid extraction

Many methods or kits can be used for 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 either 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 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 cyst mixture (only validated for Bulman & 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 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 *G. pallida*		
µsatGP-F	5'-AAGGAGTTGTGGTCCAGACG-3'	100 bp
µsatGP-R	5'-GAAGGCAATCTGTGTTCGGG-3'	
µsatGP-P	5'-JOE-CGCTCGTCGGCCTCCTCCTC-BHQ1-3'	
For *G. rostochiensis*		
µsatGR-F	5'-TGACGAGGAACAGTACAAAG-3'	167 bp
µsatGR-R	5'-GTGTCTCTAATTTGCCATT-3'	
µsatGR-P	5'-Cy5-AGGCATTGCTTGAGCGAACGGA-BHQ2-3' *	

* 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 (Ct).

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	N.A.	4.04	N.A.
LightCycler® 480 Probe Master (Roche)	2 x	10	1x
G. pallida Forward Primer (*µsat-GP-F*)	50 µM	0.2	0.5 µM
G. pallida Reverse Primer (*µsat-GP-R*)	50 µM	0.2	0.5 µM
G. pallida Probe (*µsat-GP-R*)	50 µM	0.08	0.2 µM
G. rostochiensis Forward Primer (*µsat-GR-F*)	50 µM	0.2	0.5 µM
G. rostochiensis Reverse Primer (*µsat-GR-R*)	50 µM	0.2	0.5 µM
G. rostochiensis Probe (*µsat-GR-R*)	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 *H. schachtii* Forward Primer (*µsat-HS-F*) and *H. schachtii* Reverse Primer (*µsat-HS-R)*, 0.2 µM of *H. schachtii* Probe (*µsat-HS-P*). *H. schachtii* DNA is also added to

the mastermix. The volume of molecular grade water should be recalculated.

2.2.2. PCR conditions: initial denaturation 10 min at 95°C; 40 cycles: denaturation 10 sec at 95°C; annealing and extension 50 sec at 60°C; fluorescence capture at the end of 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 extraction and amplification 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 due 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), internal positive controls (IPC) can be used to monitor each individual sample separately. Internal positive controls can be non-target *Heterodera 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 µsatHS-F	5'-CCCGGACAGCCAAATTGT-3'
Primer µsatHS-R	5'-GGGAAGTGAGTGGGCAGTTT-3'
Probe µsatHS-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 1.3).

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. 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.
- 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 Ct value is below 35 for the two replicates. Results with Ct greater than 35 requires 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 cycle threshold (C_t) cut off value given above is as established in the Nematology Unit of the Plant Health Laboratory (Anses, France). As a Ct 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, 5 J2 of *G. pallida* are detected when combined with 10, 20, 40 and 50 cysts of *G. rostochiensis* (on average 130 larvae/cyst): Ct 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 7 South American populations), 13 *G. rostochiensis* (5 from South America and 8 from Europe), 8 *H. schachtii*, a total of 7 for *G. tabacum*, *G. ellingtonae* and *G. mexicana*, a total of 11 for other species of *Heterodera* including 5 species belonging to the *Schachtii* group. For simplex and multiplex reactions,

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

4.3. Data on Repeatability

Data obtained using the DNA extraction described in Appendix 2, section 1: for *G. pallida*: 25% with 1 J2 and 100% with 2 J2 (8 replicates tested in a same run)
for *G. rostochiensis*: 100% with 1 J2 and 100% with 2 J2 (duplicates tested in a same run)
In mixture, data obtained using the DNA extraction described in Appendix 2, section 2:
100% with 1 cyst of *G. pallida* + 49 cysts of *G. rostochiensis* (6 samples tested in duplicates),
100% with 1 cyst of *G. rostochiensis* + 49 cysts of *G. pallida* (6 samples tested in duplicates),
100% with 5 J2 of *G. pallida* + 50 cysts of *G. rostochiensis* (2 samples tested in duplicates; mean of 4 Ct values obtained for *G. pallida* 32.9),

4.4. 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 obtained using the DNA extraction described in Appendix 2, section 1:

- for *G. pallida*: 25% with 1 J2 and 100% with 2 J2 (4 replicates),
- for *G. rostochiensis*: 100% with 1 J2 and 100% with 2 J2 (4 replicates).

In mixture, data obtained using the DNA extraction described in Appendix 2, section 2:

- 100% with 1 cyst of *G. pallida* (on average 250 larvae/cyst) + 49 cysts of *G. rostochiensis* (6 samples tested in duplicates on two different thermocyclers) and
- 100% with 1 cyst of *G. rostochiensis* (on average 240 larvae/cyst) + 49 cysts of *G. pallida* (6 samples tested in duplicates on two different thermocyclers).100% with 5 J2 of *G. pallida* + 50 cysts of *G. rostochiensis* (2 samples tested in duplicates on two different thermocyclers; mean of 8 Ct values obtained for *G. pallida* 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 7/98) 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), accession number FJ212165 for *G. pallida*.

1.4 Oligonucleotides:

Forward primer *G. pallida* and *G. rostochiensis*	5′ CGTTTGTTGTTGACGGACAYA 3′
Reverse primer *G. pallida* and *G. rostochiensis*	5′ GGCGCTGTCCRTACATTGTTG 3′
Probe *G. pallida*	5′-FAM-CCGCTATGTTTGGGC-MGBNFQ-3′
Probe *G. rostochiensis*	5′-FAM-CCGCTGTGTATKGGC-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 8 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 AP1 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 µL 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 µL of RNase A. The S-block is incubated at 65°C for 10 min and centrifuged at 3000 g for 5 min.

340 µL 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). 5 S-blocks (1–5) and a microplate (MP) are used in the sample processing, containing:

S-block 1, 400 µL isopropanol and 30 µL MagAttract Suspension G;
S-block 2, 400 µL RPW buffer;
S-block 3, 400 µL 96% ethanol;
S-block 4, 400 µL 96% ethanol;
S-block 5, 500 µL sterile PCR-grade water (Sigma) containing 0.02% (v/v) Tween 20;
MP containing 200 µL 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 (µL)**	**Final concentration**
Molecular grade water	N.A.	4.6	N.A.
TaqMan® Environmental Master Mix 2.0 (Life Technologies)	2×	15	1×
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 *G. pallida*	5 µM	0.625	0.125 µM
Probe *G. rostochiensis*	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	N.A.	6.25	N.A.
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 *G. pallida* or Probe *G. rostochiensis*	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 extraction and amplification 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 preferably of a sample of uninfected matrix (soil confirmed to be free from *Globodera*) or if not available 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 matrix sample that contains the target *Globodera* species (e.g. a float spiked with at least one of the target species, but ideally both).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that is used to prepare the reaction mix.
- Positive amplification control (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.
- 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. 4.1 Analytical sensitivity data

0.1 pg of PCN DNA of either *G. pallida* or *G. rostochiensis*. This level allows detection of 1 viable cyst of either *G. pallida* or *G. rostochiensis*, i.e. a cyst containing a minimum of 1 live juvenile nematode. The test is able to detect 1 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 have been tested from across Scotland.
Exclusivity: primers were designed to take account of non-target species of cyst nematodes including *Heterodera avenae* (2 populations, data unpublished), *Punctodera chalcoensis* (one population), *Punctodera punctata* (2 populations) and *Globodera millefolii* (6 populations as *G. achilleae*), as these represent other cyst nematode genera present in the UK. No cross reactivity with any of these species occurred. No cross reactions were observed with *G. artemisiae* (4 populations), and *G. hypolysi* (1 population, now synonymized *G. artemisiae*) which are not considered to be present in the UK. Cross-reactions occur with *G. mexicana* (3 populations), *G. tabacum* (12 populations) and *G. ellingtonae* (1 population), but none of these species have been detected so far in the UK. 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 4.5.

4.4. Data on reproducibility

Not available. Some information included under 4.5.

4.5. Other information

Testing of soil samples from Scottish fields during 2008–10 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 at a different time period.)

	2008–10	**2011–13**
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 *G. rostochiensis*	1.1%	1.0%
Samples with live *G. pallida*	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 7/98) 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, *G. tabacum* 482 bp.

1.4 Oligonucleotide sequences are not disclosed. These tests are available as all-inclusive real-time PCR kit (ClearDetections, The Netherlands, www.cleardetections.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 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°C → 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 extraction and amplification 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, preferably 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 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.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification controls (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.
- 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 analyses is performed, and the obtained melting temperature (T_m) value equals the T_m value of the PAC (±1°C). T_m values may vary depending on the PCR machine and PCR mix used. 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.*

- 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: 4 *G. rostochiensis* populations, 3 *G. pallida* populations and 2 *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*, *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 for 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 ±1.0°C from the normal T_a (63°C), all ΔCt 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 & 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 (ITS5-PITSp4 and ITS5-PITSr3) Anthoine & 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′-AGCGCAGACATGCCGCAA-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	N.A.	to make up to 24	N.A.
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 mg/mL stored in 20mM phosphate buffer pH 6.8) may be added to the amplification reaction to enhance 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 isolation and amplification of *G. pallida* and *G. rostochiensis* and nucleic acid, respectively.

- Negative isolation control (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).
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: solution/buffer spiked with 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).
- Negative amplification control (NAC): to rule out false positives due 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.
- Positive amplification control (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.

Interpretation of results:

Verification of the controls:

- 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 (FR) in July 2010 with the following adaptations of the master mix: primer concentration 0.64 µM and dNTP 0.25 mM each, DNA extract 5µL (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 µg mL^{-1}) and mechanical disruption of 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,* 4 populations of *G. rostochiensis,* 5 populations of *G. tabacum*, 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% (1 J2) for *G. pallida,* 100% (1 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 & 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	N.A.	30.4	N.A.
Taq DNA polymerase buffer (MP Biomedicals)	10×	5	1×
MgCl2 (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 isolation and amplification of *G. pallida* and *G. rostochiensis* and nucleic acid, respectively.

- Negative isolation control (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).
- Positive isolation control (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).
- Negative amplification control (NAC): to rule out false positives due 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.
- Positive amplification control (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:

- 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.

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

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

4. Performance characteristics available

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

4.1 Analytical sensitivity data: one J2.

4.2 Analytical specificity data: The study included 11 populations of *G. pallida,* 4 populations of *G. rostochiensis,* 5 populations of *G. tabacum*, 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% (1 J2) for *G. pallida*, 93% (1 J2) for *G. rostochiensis*.

4.5 Diagnostic specificity: 91% for *G. pallida* (1 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 (ITSI) 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* glycoslyase (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. 20 µL of Proteinase K is added, the sample is vortexed to mix and centrifuged briefly to pool. The samples are incubated at 56°C and 100 r.p.m. 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	N.A.	6.75	N.A.
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 isolation and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (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.
- 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 cyst of either *G. pallida* or *G. rostochiensis*.
- Negative amplification control (NAC) to rule out false positives due 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.
- Positive amplification control (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.
- 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 Ct value is below 37 (due 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 Ct of 30 for both species; therefore, this is greater than the limit of detection.
- A Ct 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 ΔRn in the FAM channel (*G. pallida*) as the ΔRn 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 UK; 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 Ct >37; however, firstly *Punctodera* cysts have a distinct morphology compared with *Globodera* and would not be selected for molecular testing and secondly this Ct 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 Ct >37 which would instigate further investigation.

4.3 Data on repeatability

Extractions were tested at both neat and 10^{-3} dilutions with 8 replicates. Repeatability was 100%.

4.4 Data on reproducibility

Extractions obtained were tested at 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 & 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 & Marshall (1997) using 149 samples. The diagnostic specificity was *G. pallida* 87.1% 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 1 to 50 *Globodera* cysts.

1.3 The real-time PCR test targets the elongation factor 1-α gene (EF1-α).

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 *	5′-Yakimo Yellow- CGAAGA(A)(T)GACCCGGC- BHQ1-3′
	p_EF-Gros_2LNA*	5′-6FAM-CTCGAAGAG(C)GAC(C)CTG-BHQ1-3′

* 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. 1–50 cysts of *Globodera* spp., are used for nucleic acids extraction.

Collect the cysts in a 1.5 mL tube. Add 100 µL 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 µL.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water		2.25	
One Step RT-PCR Buffer III (TaKaRa)	2×	12.5	1×
Primer Fw_EF_Grp_mRNA	10 µM	0.75	0.3 µM
Primer Rv_EF_Gp_mRNA-1	10 µM	0.75	0.3 µM
Probe EF_Gpal_probe	5 µM	2	0.5 µM
Probe p_EF-Gros_2LNA	5 µM	0.5	0.1 µM
Prime Script TM RT Enzyme Mix II (TaKaRa)	N.A.	0.5	N.A.
TaKaRa Taq™ HS (Takara)	5 U/µL	0.5	2.5 U
ROX™ Reference Dye II[a]	50×	0.25	0.5×
Subtotal		20	
DNA		5	
Total		25	

[a] Linked to the cycling machine used; consult the manufacturer's instructions; N.A.: 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:

- Negative isolation control (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.
- Positive isolation control (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*).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of RNase-free molecular-grade water that is used to prepare the reaction mix.
- Positive amplification control (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

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:* 72.2%, *G. rostochiensis* 86.7%, *Globodera* spp. 68.2%

4.4. Analytical specificity:

Inclusivity 100% evaluated on 6 strains of *G. rostochiensis* and 8 strains of *G. pallida*

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

4.5. Reproducibility: 100%

4.6. Repeatability: 100%

Appendix 10 - Visual determination[7]

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

Live eggs (Figs. 11 & 12)	**Dead eggs (Figs. 16 & 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 fill up against the egg shell	d. Shrivelled disintegrated juvenile in egg
e. Sometimes clear lip region and stylet present	e. No clear lip region or stylet present
Live juveniles (Figs. 13, 14 & 15)	**Dead juveniles (Figs. 18, 19 & 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 shrivelled 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 half circle
Included in counts: heads	Not included in counts: tails, empty shells

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

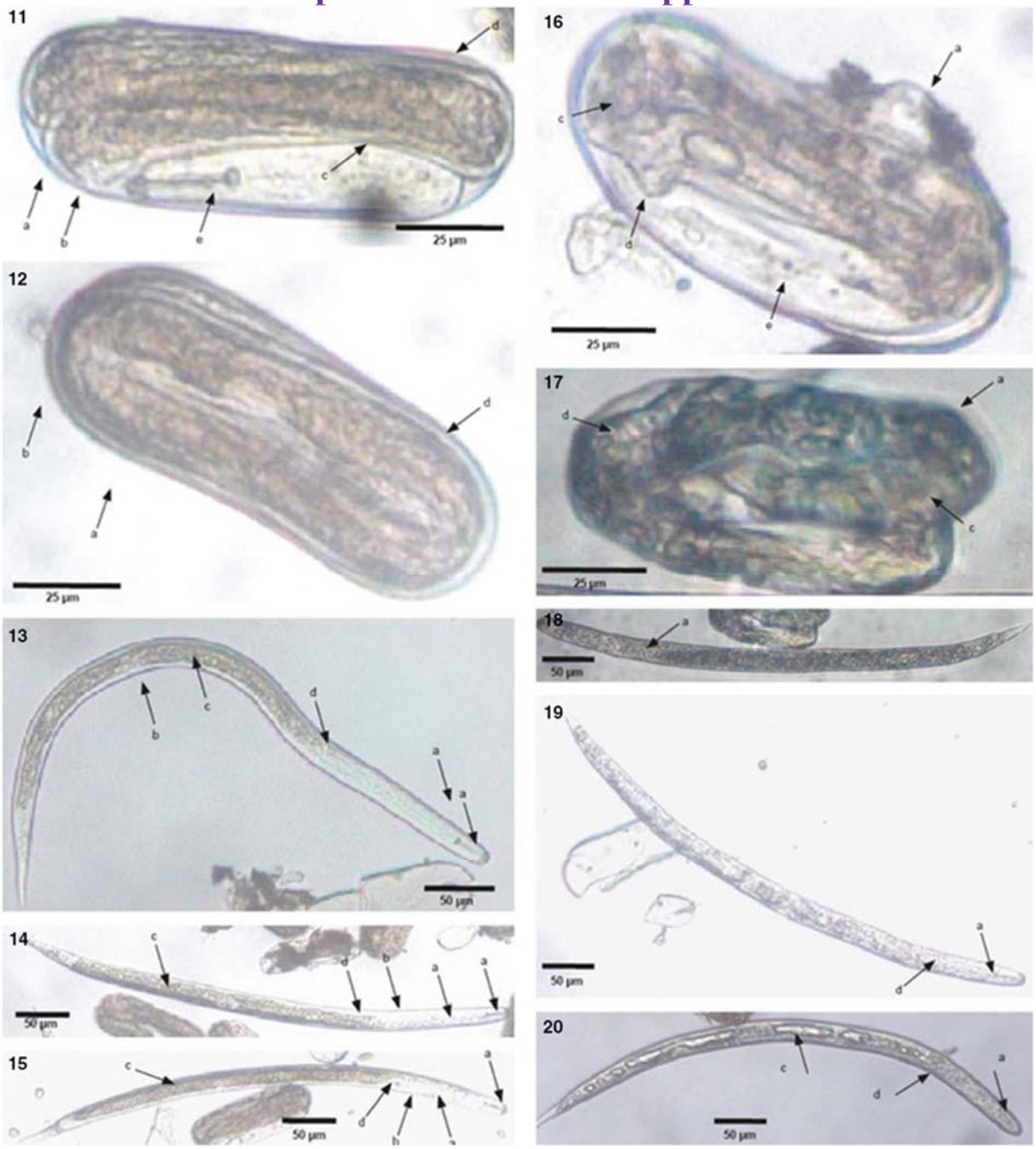

Figure 11-20. Characteristics of live and dead eggs and juveniles of the potato cyst nematodes.

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 (Ø = 23.5 mm, height 34 mm) functioning as hatching units. Each vial contains one cyst bag made of nylon net with 20 cysts, which is completely covered by PRD. Cysts collected in the autumn need to be exposed +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 (Fig. 21).

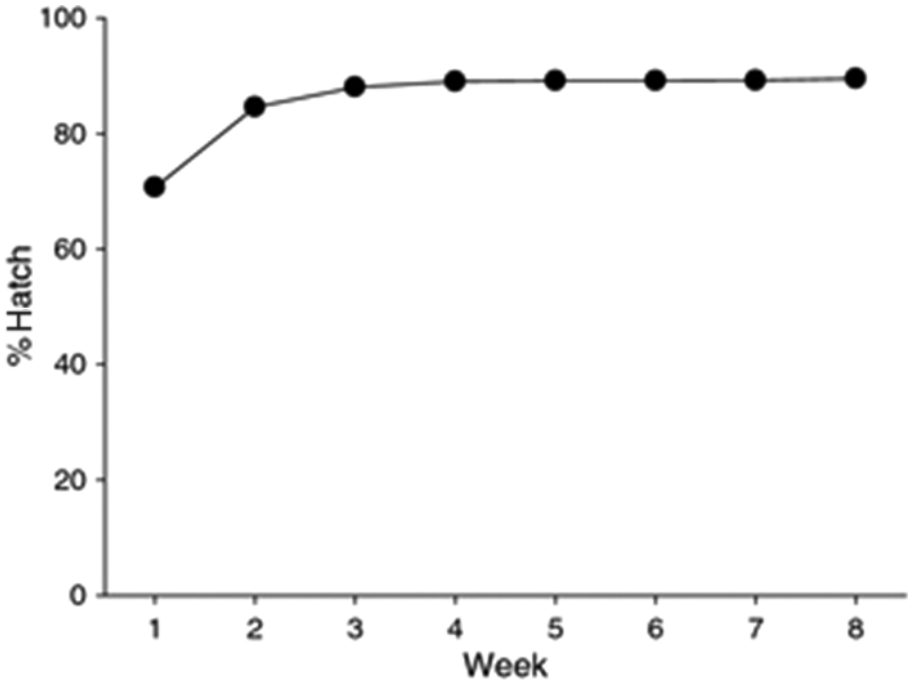

Figure 21. Cumulative hatch of *G. rostochiensis* in potato root diffusate from 3-week-old plants (n = 4).

(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 trend to float, hydrated ones sink to the bottom of the Petri dish, which gives a good indication of a cyst's hydrate on 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 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 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).

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. 20 cysts of *G. pallida* and *G. rostochiensis* are put respectively in 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 Elsen *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).