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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/41 (3)

Diagnostics
Diagnostic

PM 7/41 (3) *Meloidogyne chitwoodi* and *Meloidogyne fallax*

Specific scope

This Standard describes a diagnostic protocol for *Meloidogyne chitwoodi* and *Meloidogyne fallax*[1]. This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Terms used are those in the EPPO Pictorial Glossary of Morphological Terms in Nematology.[2]

Specific approval and amendment

Approved as an EPPO Standard in 2003-09.
Revision approved in 2008-09 and 2016-02.

This Standard was initially developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by partnership of contractor laboratories and intercomparison laboratories in European countries.

1. Introduction

At present more than 90 species of root-knot nematode have been described. All are obligate endoparasitic pests on plant roots and they are detected worldwide. About 10 species are agricultural pests, while five (*Meloidogyne arenaria*, *Meloidogyne enterolobii*, *Meloidogyne hapla*, *Meloidogyne incognita*, *Meloidogyne javanica*) are major pests and are distributed worldwide in agricultural areas. The temperate root-knot nematodes *Meloidogyne chitwoodi* and *Meloidogyne fallax* parasitize monocotyledons and dicotyledons, including several crop plants such as potatoes, carrots and tomatoes (Santo *et al.*, 1980; O'Bannon *et al.*, 1982; Brinkman *et al.*, 1996; Karssen, 2002; den Nijs *et al.*, 2004). The juveniles of the second stage are attracted to the roots and penetrate the roots closely behind the root tip, where they enter the vascular cylinder. The parasites finally start feeding on cells, which are rapidly turned into multinucleated giant cells. At the same time as the giant cells are formed, the cells of the neighbouring pericycle start to divide, giving rise to a typical gall or root knot. The root-knot nematodes are able to move only a few metres annually on their own, but they can be spread readily through the transport of infested plants and plant products, in soil, adhering to farm implements and in irrigation water.

Root-knot nematodes affect the root growth, yield and quality of their hosts. The above-ground symptoms are not readily apparent, but they may consist of various degrees of stunting, lack of vigour and wilting under moisture stress. Hosts may be heavily infested without showing external symptoms on the harvested products, for example symptomless potato tubers (Been *et al.*, 2007).

Meloidogyne chitwoodi was described from the Pacific Northwest region of the USA in 1980 (Santo *et al.*, 1980). At present this species has been recorded from Argentina, Belgium, France, Germany, the Netherlands, Portugal, the USA, Mexico and South Africa. *Meloidogyne fallax* was detected for the first time in 1992 in a field plot experiment 1 mile north of Baexem (NL) and was initially remarked as a deviating *M. chitwoodi* population (Karssen, 1994; van Meggelen *et al.*, 1994). Within Europe this species has been recorded from Belgium, France, Germany, the UK and Switzerland (Daher *et al.*, 1996; Waeyenberge & Moens, 2001; Eder *et al.*, 2010; EPPO 2013b; Sturhan, 2014). It has so far been detected outside Europe in New Zealand, Australia and South Africa (Fourie *et al.*, 2001; Marshall *et al.*, 2001; Nobbs *et al.*, 2001). Updated information on distribution is available in the EPPO Global Database (https://gd.eppo.int/). Another closely related species was described from potato in 2004 as *Meloidogyne minor*. This species has so far been detected in the UK, Ireland, Belgium and the Netherlands. It is particularly damaging on golf courses where it induces the yellow patch disease, but is also found on other mono- and dicotyledonous hosts (Karssen *et al.*, 2004).

[1]the 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.

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

A flow diagram describing the diagnostic procedure for *M. chitwoodi* and *M. fallax* when found in soil, roots or tubers is presented in Fig. 1.

2. Identity

Name: *Meloidogyne chitwoodi* Golden *et al.* 1980
Taxonomic position: Nematoda: Tylenchida[3] Meloidogynidae
EPPO Code: MELGCH
Phytosanitary categorization: EPPO A2 List no. 227, EU Annex designation I/A2.

Name: *Meloidogyne fallax* Karssen 1996
Taxonomic position: Nematoda: Tylenchida[3], Meloidogynidae
EPPO Code: MELGFA
Phytosanitary categorization: EPPO A2 List no. 295, EU Annex designation I/A2.

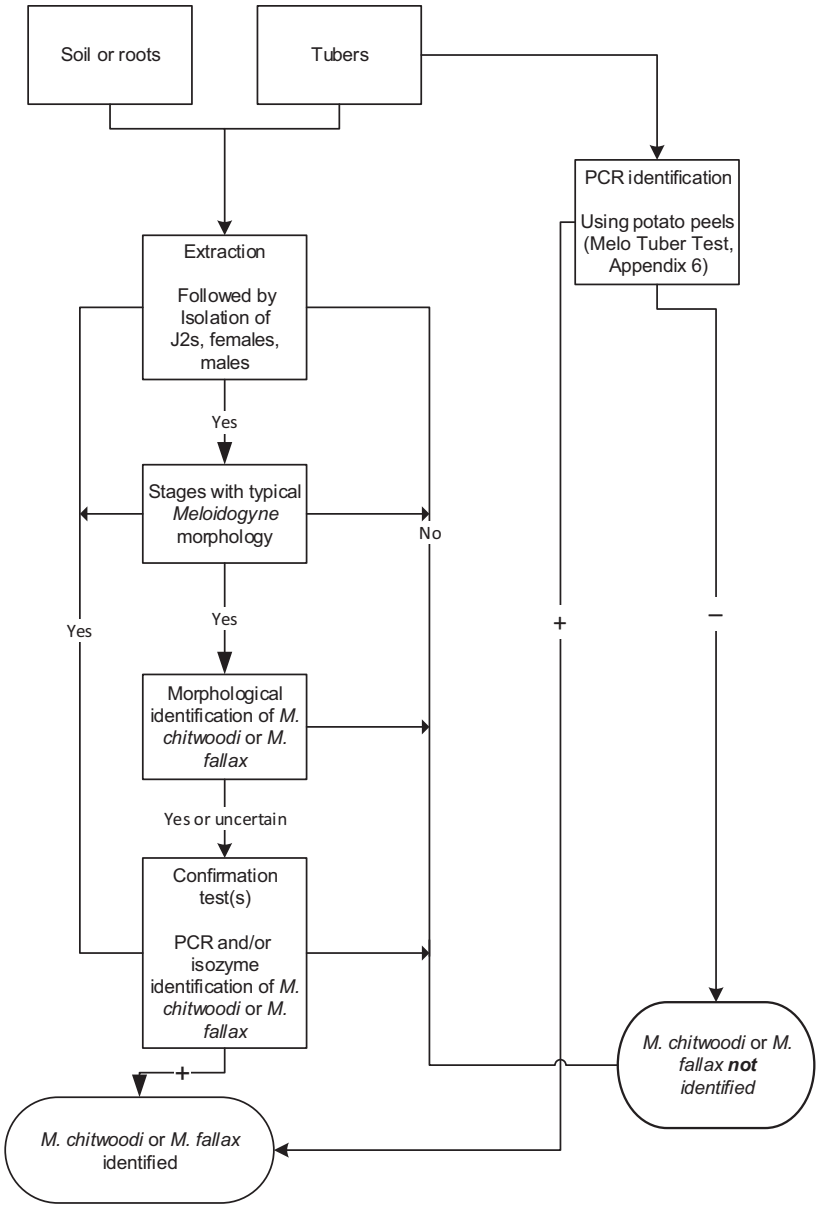

Fig. 1 Flow diagram for the detection and identification of *M. chitwoodi* and *M. fallax* from soil, roots or tubers.

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

3. Detection

3.1 Symptoms

Above-ground symptoms of heavily infested plants include stunting and yellowing, while below ground galling is typical (Fig. 2 shows symptoms on carrot and potato). Pictures of other affected crops are available in the EPPO Global Database (https://gd.eppo.int/). The root galls produced by *M. chitwoodi* and *M. fallax* are comparable to those produced by several other root-knot species, i.e. relatively small galls in general without secondary roots emerging from them (as in *M. hapla*). On potato tubers, *M. chitwoodi* and *M. fallax* cause numerous small pimple-like raised areas on the surface (in *M. hapla* these swellings are not evident). Some potato cultivars, although heavily infested, may be free from visible external symptoms, while the internal potato tissue is necrotic and brownish, just below the peel.

Fig. 2 Top left: carrot infested by *Meloidogyne fallax* (courtesy: National Plant Protection Organization, NL). Top right: the top half of the picture shows potatoes infested by *Meloidogyne chitwoodi* compared with healthy ones (bottom part of the picture) (courtesy: National Plant Protection Organization, NL) and brownish necrotic spots in the flesh just below the peel of potato infested with *M. chitwoodi* (courtesy: Main Inspectorate of the State Plant Health and Seed Inspection Service (PL)).

3.2 Sampling

Specific guidance on sampling of potato tubers for the detection of *M. chitwoodi* and *M. fallax*. is given in the Phytosanitary Procedure PM 3/69 (EPPO, 2006), and for soil sampling in the National Regulatory Control System PM 9/17 (EPPO, 2013a) .

3.3 Extraction

Guidance on extraction is given in PM 7/119 *Nematode extraction* (EPPO, 2013b).

3.4 Molecular tests

A triplex real-time TaqMan® polymerase chain reaction (PCR) test called the MeloTuber Test, has been developed to simultaneously detect *M. chitwoodi* and *M. fallax* directly in secondary potato tuber peelings (see Appendix 6). This real-time PCR has only been tested on peelings of potato, not on those of other crops.

4. Identification

In order to identify nematodes that may be present on a commodity, it is necessary to extract specimens from the roots, potato tubers or from soil or growing medium and

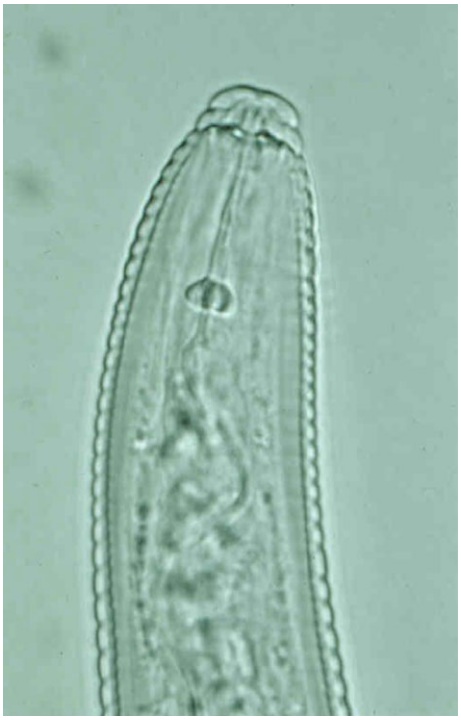

Fig. 3 *Meloidogyne chitwoodi* male (Karssen, 1996a).

Table 1. Morphological and morphometric differences between *M. chitwoodi*, *M. fallax*, *M. minor* and *M. hapla* (in µm) (Karssen, 2002; Karssen *et al.*, 2004)

	M. fallax	*M. chitwoodi*	*M. minor*	*M. hapla*
♀ stylet length	14.0–14.5	10.5–13.5	12.5–15	14.0–17.0
♂ stylet length	18–21	16–18	17–19	17–23
♀♂ stylet knob	Prominent	Small	Prominent	Small
Shape of stylet knob	Rounded	Irregular	Ovoid	Rounded
J2 body length	368–410	336–417	310–416	357–517
J2 tail length	46–55.5	39–47	49–63	46–69
J2 hyaline tail length	12–16	8–12.5	12–22	12–19

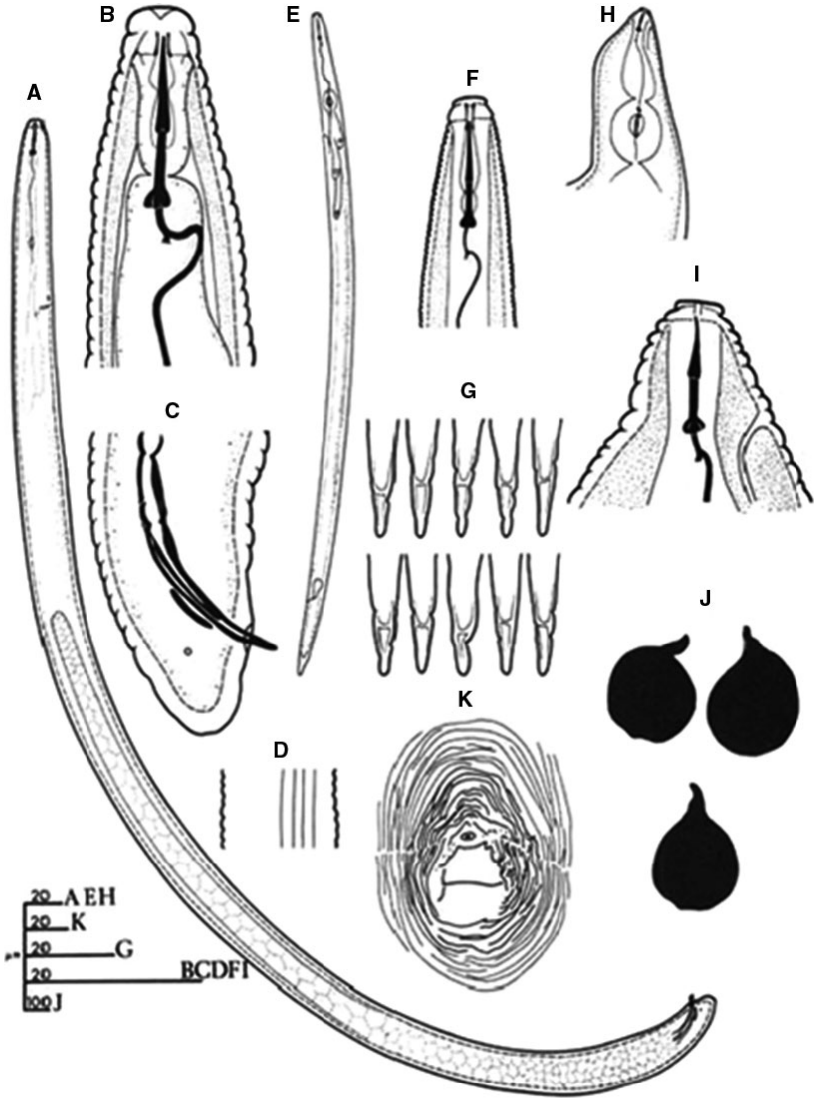

Fig. 4 *Meloidogyne chitwoodi*. Male: (A) entire, lateral view; (B) anterior end, lateral view; (C) posterior end, lateral view; (D) lateral field. Second-stage juvenile (J2): (E) entire, lateral view; (F) anterior end, lateral view; (G) tails. Female: (H), (I) anterior end, lateral view; (J) entire; (K) perineal pattern (Santo *et al.*, 1980).

follow the procedures described in the flow diagram (Fig. 1). Extraction is not necessary in cases where detection and identification of the nematodes will take place directly in tissue of the commodity (Fig. 1).

If knots are found on roots, all stages of the nematode should be obtained, particularly mature swollen females, males and second-stage juveniles. These should have the characters described for *M. chitwoodi* or *M. fallax*. If root

knots are not found but motile nematode stages are obtained from soil (particularly second-stage juveniles), these should be distinguished from all other soil-inhabiting nematodes. Mature females can be observed within the roots by means of a dissecting microscope using transmitted light. Identification to species level is based on a combination of morphological/morphometric characters and biochemical or molecular methods (isozymes or PCR). Differential interference contrast is recommended to identify specimens mounted in fixative on microscope slides.

4.1 Morphology

4.1.1 Meloidogyne morphology

Sedentary females are thin, annulated, pearly white and globular to pear-shaped, 400–1300 µm long and 300–700 µm wide. The stylet is dorsally curved, 10–25 µm long, with rounded to ovoid stylet knobs, set off to sloping posteriorly. The non-sedentary males (see Fig. 3) are vermiform, annulated, slightly tapering anteriorly, bluntly rounded posteriorly, 700–2000 µm long and 25–45 µm wide. The stylet is 13–30 µm long, with stylet knobs, variable in shape. The non-sedentary second-stage juveniles are vermiform, annulated, tapering at both ends, 250–700 µm long, 12–18 µm wide, tail length 15–100 µm and hyaline tail part 5–30 µm in long.

4.1.2 M. chitwoodi and *M. fallax*

Morphological and morphometric differences between *M. chitwoodi* and *M. fallax* were noticed when the two species were cultivated on the same host under comparable conditions (Karssen, 1995, 1996a). The most striking differences for males, females and juveniles of different species are shown in Table 1.

4.1.3 Additional characters that may be observed

With the scanning electron microscope, it was observed that the head of *M. fallax* males has an elevated labial disk. Secondary differences were recorded in the female perineal pattern (*M. fallax*: relatively higher dorsal arch and thicker striae) and second-stage juvenile hemizonid position to the excretory pore (same level for *M. fallax*, anterior adjacent for *M. chitwoodi*). Based on morphology, the two species are closely related, and this misleading morphological resemblance to *M. chitwoodi* was the reason for the name given to *M. fallax* (Peterson & Vrain, 1996). Figures 4–7 present drawings of different stages of *M. chitwoodi* and *M. fallax*. However, for identification, biochemical techniques and/or molecular methods should be used in addition to morphology and morphometrics (Appendices 1–8).

4.2 Possible confusion with similar species

Meloidogyne hapla differs from *M. chitwoodi* and *M. fallax* in (female) perineal pattern (rounded with low dorsal arch

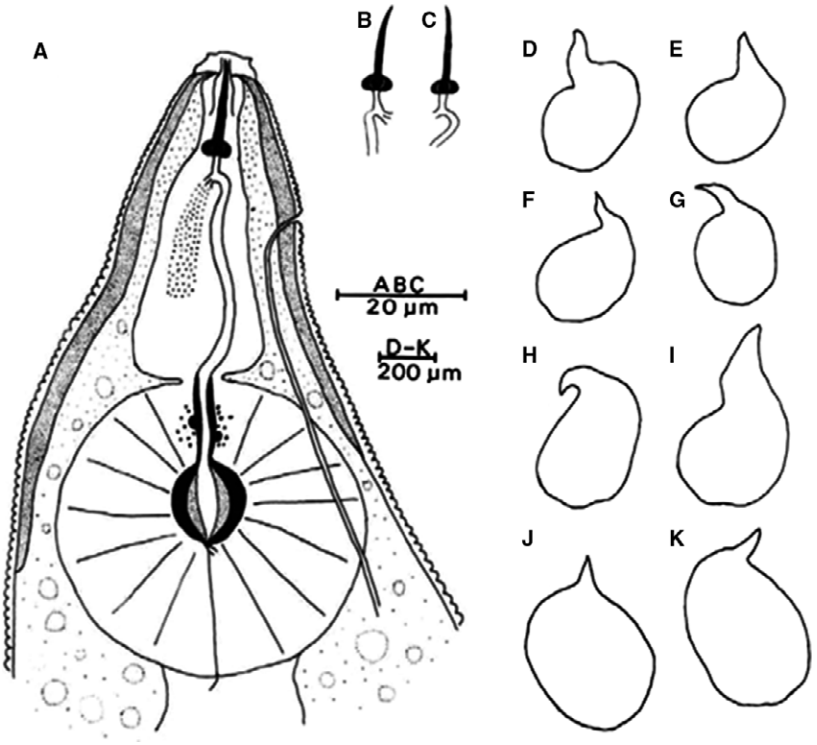

Fig. 5 *Meloidogyne fallax*. Female (lateral view): (A) anterior end; (B), (C) stylets; (D)–(K) entire (Karssen, 1996a).

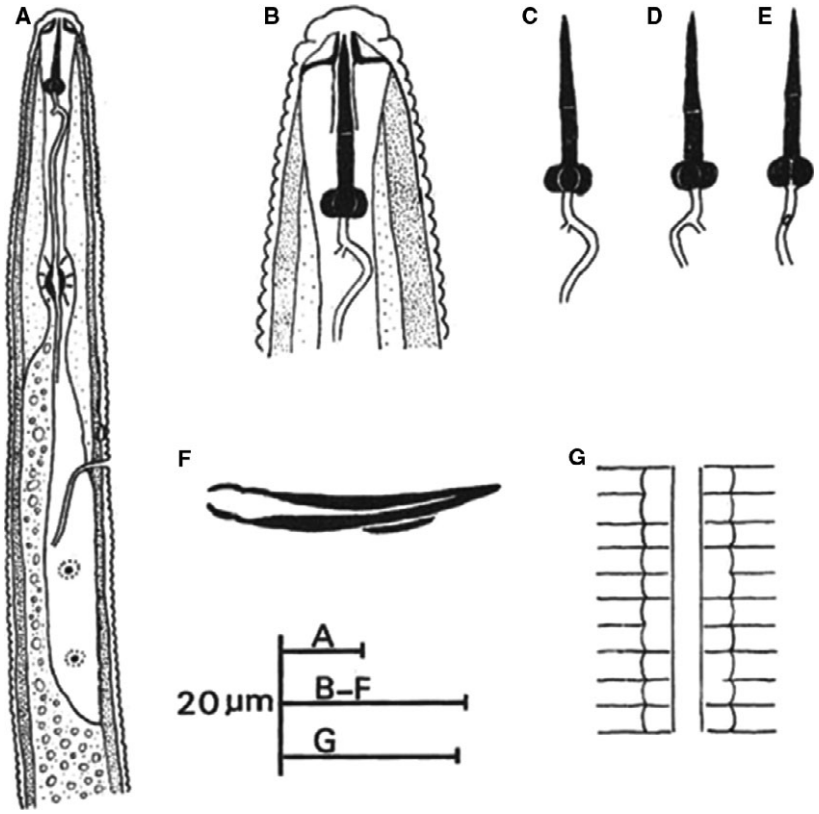

Fig. 6 *Meloidogyne fallax*. Male: (A), (B) anterior end; (C)–(E) stylets (E, ventral view); (F) spicule and gubernaculum; (G) lateral field (Karssen, 1996a).

and lateral lines; punctations often present in the tail terminal area); male and female stylet knobs (small rounded and set off from the shaft); male head shape (head region not in contour with the first body annule, but slightly wider); and second-stage juvenile tail shape (long, slender tail with a narrow tapering terminus, tip finely rounded to pointed, hyaline terminus not clearly demarcated). *Meloidogyne minor* differs from *M. chitwoodi* and *M. fallax* in (female) perineal pattern (small, rounded with fine striae); stylet knob shape (prominent and rounded); and second stage juvenile (J2) hemizonid position to the excretory pore (posterior).

Jepson (1987), Karssen (2002) and Karssen *et al.* (2013) provide a comparison with other *Meloidogyne* species and Siddiqi (2000) a comparison with other genera of Tylenchida.

4.3 Biochemical techniques

Esbenshade & Triantaphyllou (1985) have described a useful method for identification of the females of several *Meloidogyne* species (including *M. chitwoodi*) by isozyme electrophoresis. Esterase (EST; EC 3.1.1.1) and malate dehydrogenase (MDH; EC 1.1.1.37) isozyme patterns discriminated *M. fallax*, *M. chitwoodi*, *M. hapla* and *M. minor* females (Karssen *et al.*, 1995, 2004; Karssen, 1996b). Additionally, isozymes of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) are useful to differentiate the two species (Van der Beek & Karssen, 1997). The test is described in Appendix 1.

4.4 Molecular methods

Recommended molecular tests are any of the following PCR tests:

- A PCR method using species-specific primers based on sequence-characterized amplified regions (SCARs) designed from random amplified polymorphic DNA fragments (Zijlstra, 2000) (Appendix 2)
- A PCR method based on species-specific primers designed from ribosomal intergenic spacer (IGS) regions (Wishart *et al.*, 2002) (Appendix 3)
- An internal transcribed spacer (ITS) PCR-restriction fragment length polymorphism (RFLP) method based on the Vrain primers (Vrain *et al.*, 1992), Zijlstra *et al.* (1997) (Appendix 4)

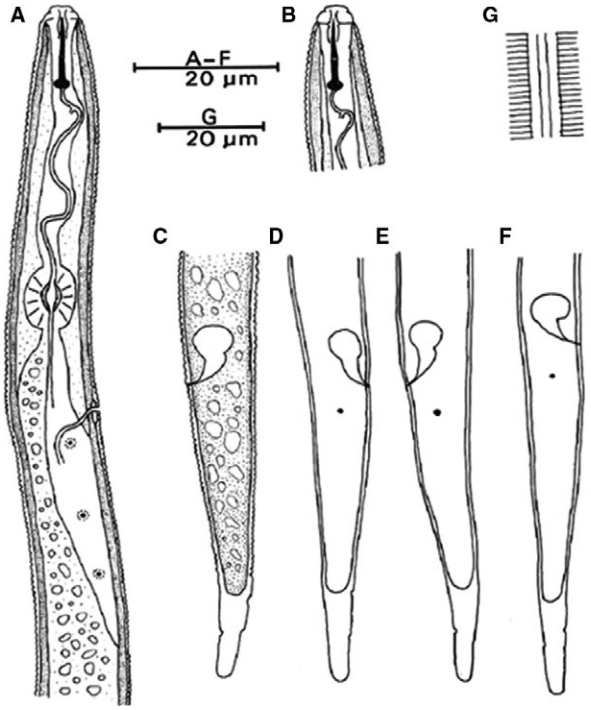

Fig. 7 *Meloidogyne fallax*. Second-stage juveniles (J2) (lateral view): (A), (B) anterior end; (C)–(F) tails; (G) lateral field (Karssen, 1996a).

- A real-time TaqMan® test based on ITS sequences (Zijlstra & van Hoof, 2006) (Appendix 5)
- A triplex real-time TaqMan® PCR directly in secondary potato tuber peelings (de Haan *et al.*, 2014) (Appendix 6)
- A real-time PCR based on rDNA (large subunit, LSU) sequences (Appendix 7)
- A high-resolution melting curve analysis as a tool for root-knot nematode diagnostics, based on Holterman *et al*. (2012) (Appendix 8).

PCR tests can be performed on all developmental stages of nematodes.

A molecular diagnostic key for the identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.) has been published by Adam *et al.* (2007). Another publication for *Meloidogyne* species identification with species-specific primers is by Petersen *et al.* (1997).

5. Reference material

Reference material can be obtained from:

National Plant Protection Organization, PO Box 9102, 6700 HC Wageningen (NL).

Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Toppheideweg 88, 48161 Münster (DE) (for *M. chitwoodi*).

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) *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:

G. Karssen & L.J.M.F. den Nijs, National Plant Protection Organization, National Reference Centre, PO Box 9102, 6700 HC Wageningen (NL). G.karssen@nvwa.nl or l.j.m.f.dennijs@nvwa.nl

9. Feedback on this Diagnostic Protocol

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. Protocol 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

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Appendix 1 – Isozyme electrophoresis for identification of *M. chitwoodi* and *M. fallax*

1. General information

1.1. Several reliable isozyme electrophoresis methods are available for the identification of single young egg-laying *Meloidogyne* females. The following method was originally developed by Esbenshade & Triantaphyllou (1985) and modified and adapted for PhastSystem, i.e. an automated electrophorectic apparatus, by Karssen *et al.* (1995). It is preferable to run two gels at a time with a total of 24 females per electrophoresis run. The applied method uses native gradient polyacrylamide gel electrophoresis in a discontinuous buffer system.

1.2. The PhastSystem apparatus, prefabricated gels and the sample well stamps are manufactured by Amersham Electrophoresis and sold by GE Healthcare.

2. Samples

2.1. Roots infested with root-knot nematode are placed in 0.9% NaCl solution and young egg-laying females are isolated under a dissecting microscope and placed in 0.9% NaCl solution on ice or stored in a freezer at a maximum of −20°C.

2.2. Before electrophoresis the females are transferred from the NaCl solution to reagent-grade water on ice for a few minutes for desalting.

3. Sample preparation

3.1. After desalting, a sample well stamp (on ice) with twelve wells is filled with one female per well.

3.2. The two middle wells are each filled with reference material (preferably female *Meloidogyne javanica*).

3.3. To each well 0.6 µL of extraction buffer is added (20% sucrose, 2% Triton X-100 and 0.01% bromophenol blue).

3.4. The females are carefully macerated with a small glass rod and loaded on two 12/03 sample applicators (0.3 µL per well).

3.5. Both applicators are inserted at the cathode slot into the left and right applicator arm.

4. Electrophoresis

4.1. Before electrophoresis two PhastGel gradient gels (8–25) are placed on the gel bed and pre-cooled at 10°C.

4.2. The following adapted programme is used for electrophoresis:

Sample applicator down at step 3.2, 0 Vh
Sample applicator up at step 3.3, 0 Vh
Step 3.1: 400 V, 10 mA, 2.5 W, 10°C, 10 Vh
Step 3.2: 400 V, 1 mA, 2.5 W, 10°C, 2 Vh
Step 3.3: 400 V, 10 mA, 2.5 W, 10°C, 125 Vh.

4.3. After adding the sample applicators the programme can be started.

4.4. After electrophoresis the gels are placed in a Petri dish for staining.

5. Staining

5.1. One gel is stained for esterase (EST, EC 3.1.1.1) activity, the other for malate dehydrogenase (MDH, EC 1.1.1.37)

5.2. Staining solutions are prepared according to Table 2.

Table 2. Esterase and malate dehydrogenase staining solutions

Esterase:	
0.1 M phosphate buffer, pH 7.3	100 mL
Fast Blue RR salt	0.06 g
EDTA	0.03 g
Alpha-naphthyl acetate (dissolved in 2 mL of acetone)	0.04 g
Malate dehydrogenase:	
Beta-NAD	0.05 g
Nitro blue tetrazolium	0.03 g
Phenazine methosulphate	0.002 g
0.5 M Tris, pH 7.1	5 mL
Stock*	7.5 mL
Reagent-grade water	70 mL

*10.6 g Na_2CO_3 + 1.34 g L-malic acid in 100 mL water.

5.3. Staining solution is added to each Petri dish with gel and placed in an incubator at 37°C.

5.4. The total staining times for EST and MDH are 60 min and 5 min, respectively.

6. Results

6.1. The band patterns are compared with Fig. 8 or the relative band movement compared with the buffer front (relative mobility, Rm%) is measured and compared with Table 3.

6.2. The esterase isozyme pattern for *M. fallax* is usually absent, but after prolonged staining (more than 1 h) a weak three-banded pattern will appear.

6.3. Deviating patterns of *M. chitwoodi* and *M. fallax* have not been observed so far. If these appear they can be

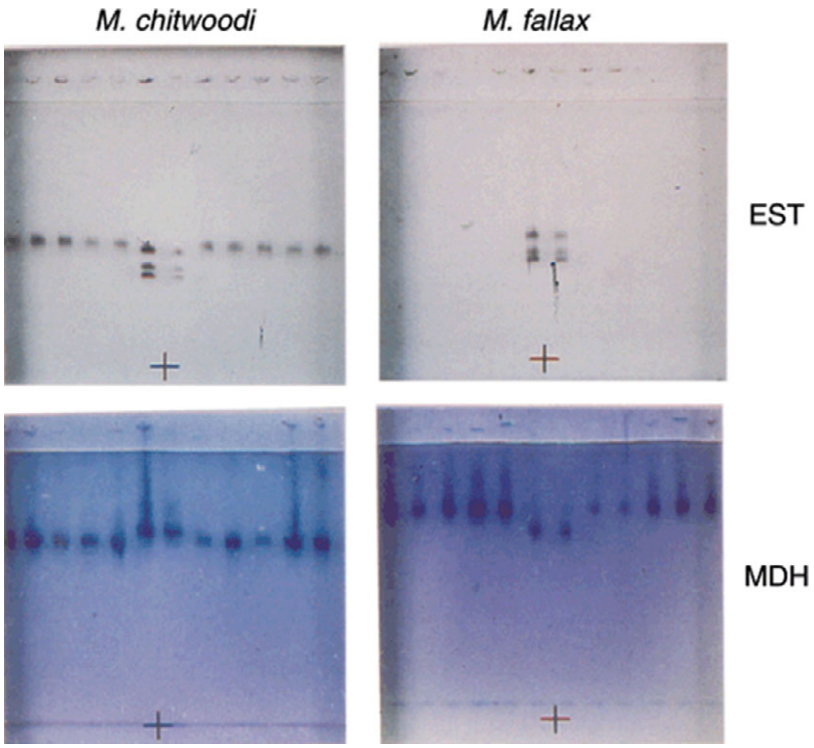

Fig. 8 Isozyme patterns for esterase (EST) and malate dehydrogenase (MDH) of *M. chitwoodi* and *M. fallax*.

Table 3. The relative mobility (Rm%) and coding for esterase (EST) and malate dehydrogenase (MDH) isozymes

Species	MDH	EST
M. chitwoodi	N1a 31.5	S1 51
M. fallax	N1b 20	F3 31, 40, 58*
M. javanica	N1 28	J3 54.5, 62, 65.5
M. hapla	H1 47.5	H1 57.5
M. minor	N1a 31.5	VS1 38

*The weak F3 EST type will only appear after prolonged staining. Usually no EST activity will be detected for *M. fallax*.

best compared with the isozyme data of Esbenshade & Triantaphyllou (1985).

Appendix 2 – SCAR marker-generated sequence based PCR tests for *M. chitwoodi* and *M. fallax*

1. General information

1.1. Identification of *M. chitwoodi* and *M. fallax* using the protocol developed by Zijlstra (2000).

1.2. The targeted regions are derived from SCARs specific for *M. chitwoodi* and *M. fallax*, respectively.

1.3. The PCR product of the reaction with the *M. chitwoodi*-specific forward Fc and reverse primer Rc is 800 bp. The PCR product of the reaction with the *M. fallax*-specific forward primer Ff and reverse primer Rf is 515 bp.

1.4. Forward and reverse primers for *M. chitwoodi* and *M. fallax*: Fc 5′-TGG AGA GCA GCA GGA GAA AGA-3′
Rc 5′- GGT CTG AGT GAG GAC AAG AGT A-3′
Ff 5′-CCA AAC TAT CGT AAT GCA TTA TT-3′
Rf 5′-GGA CAC AGT AAT TCA TGA GCT AG-3′

1.5. 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. Nematodes (J2 juveniles, females, eggs, males) are extracted from the roots, potato tubers or from soil as described in PM 7/119. When selected nematode specimens are not used for isozyme analysis (Appendix 2), specimens can be collected in molecular-grade water (MGW) instead of 0.9% NaCl solution.

2.1.2. DNA from nematodes (reliably for 5 or more) can be extracted using the High Pure PCR Template Preparation Kit (Roche) according to the mammalian tissue protocol. DNA is eluted in 40 µL of elution buffer [10 mM Tris-HCl, pH 8.5 (at 25°C)]. Crushing of the nematodes prior to DNA isolation is not necessary.

2.1.3. No DNA clean-up is required.

2.1.4. Use extracted DNA immediately or store overnight at 4°C or at −20°C for longer periods.

2.2. Conventional PCR

2.2.1. Master mix for PCR

Reagent	*M. chitwoodi*		*M. fallax*		
	Working concentration	Volume per reaction (µL)	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	15.40	N.A.	15.40	N.A.
PCR buffer (reaction buffer, containing 15 mM $MgCl_2$ (Roche)	10×	2.50	10×	2.50	1×
dNTPs (Roche)	10 mM each	0.50	10 mM each	0.50	0.2 mM
Forward primer (Fc)	10 µM	0.75	–	–	0.3 µM
Forward primer (Rc)	10 µM	0.75	–	–	0.3 µM
Reverse primer (Ff)	–	–	10 µM	0.75	0.3 µM
Reverse primer (Rf)	–	–	10 µM	0.75	0.3 µM
Taq DNA Polymerase (Roche)	5 U μL^{-1}	0.1	5 U μL^{-1}	0.1	0.5 U
Subtotal		20		20	
Genomic DNA extract or cDNA (dilution of the amplicons derived from a first PCR if appropriate)		5		5	
Total		25		25	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2. PCR cycling parameters.

M. chitwoodi: 2 min 94°C, 45 cycles of 30 s 94°C, 30 s 60°C, 1 min 72°C, and a final extension of 10 min 72°C.

M. fallax: 2 min 94°C, 45 cycles of 30 s 94°C, 30 s 58°C, 1 min 72°C, and a final extension of 10 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 extraction and amplification of *M. chitwoodi* and *M. fallax* and nucleic acid, respectively:

- Negative isolation control (NIC): DNA extraction of solution used to collect nematode specimens in either MGW or 0.9% NaCl solution
- Positive isolation control (PIC): if available, DNA from either *M. chitwoodi* or *M. fallax* specimens (reliably for 5 or more nematodes included) in either MGW or 0.9% NaCl solution
- Negative amplification control (NAC): MGW that was used to prepare the reaction mix
- Positive amplification control (PAC): genomic DNA of 5 (or more), or whole genome amplified DNA of, *M.chitwoodi* and *M. fallax* nematodes.

3.2. Interpretation of results

In order to assign results from the PCR-based test the following criteria should be applied:

- NIC and NAC should produce no amplicons
- PAC (and if relevant PIC) should produce amplicons of 800 bp for *M. chitwoodi* and/or 515 bp for *M. fallax*. When these conditions are met:
- A test will be considered positive if amplicons of 800 bp for *M. chitwoodi* and 515 bp for *M. fallax* 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 criteria available

4.1. Analytical sensitivity data.

At the French Agency for Food, Environmental and Occupational Health & Safety (ANSES, FR), one J2 stage for *M. fallax* and two for *M. chitwoodi* could be detected reliably.

4.2. Analytical specificity data.

At ANSES (FR) the specificity data was 100% for *M. fallax* and 100% for *M. chitwoodi*. Two populations of target organisms were tested for *M. fallax* and 4 for *M. chitwoodi*. Thirty-one non-target nematode populations were tested and no cross-reactions occurred. The full validation report is available from ANSES (FR).

4.3. Data on repeatability: 75% (1 J2), 100% (2 J2) for *M. fallax*
62% (2 J2); 100% (5 J2) for *M. chitwoodi.*

4.4. Data on reproducibility:

91% (1 J2), 100% (2 J2) for *M. fallax*;
83% (2 J2), 100% (5 J2) for *M. chitwoodi*.

Appendix 3 – IGS-based PCR test for *M. chitwoodi* and *M. fallax*

1. General information

1.1. Identification of *M. chitwoodi* and *M. fallax* using a protocol developed by Wishart *et al*. (2002).

1.2. The targeted regions are derived from the IGS regions specific for *M. chitwoodi* and *M. fallax*, respectively.

1.3. Amplicon location: the forward primer JMV1 anneals in the *5S* gene and reverse primer JMV2 anneals in the IGS region.

1.4. The PCR product of the reaction with the forward primer JMV1 and reverse primer JMV2 is 540 bp for *M. chitwoodi* and 670 bp for *M. fallax*.

1.5. Primers: JMV1 5′-GGA TGG CGT GCT TTC AAC-3′ JMV2 5′-TTTCCCCTT ATG ATG TTT ACC C-3′.

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. Nematodes (J2 stage, females, eggs, males) are extracted from the roots, potato tubers or from soil as described in PM 7/119. When selected nematode specimens are not used for isozyme analysis (Appendix 2), specimens can be collected in MGW instead of 0.9 % NaCl solution.

2.1.2. DNA from nematodes (reliably for 5 or more) can be extracted using the High Pure PCR Template Preparation Kit (Roche) according to the mammalian tissue protocol. DNA is eluted in 40 µL of elution buffer [10 mM Tris-HCl, pH 8.5 (at 25°C)]. Crushing of the nematodes prior to DNA isolation is not necessary.

2.1.3. No DNA clean-up is required.

2.1.4. Use extracted DNA immediately or store overnight at 4°C or at −20°C for longer periods.

2.2. Conventional PCR

2.2.1. Master mix for PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	11.30	N.A.
PCR buffer (containing 15 mM $MgCl_2$) (Promega)	10x	2.50	1x
dNTPs (Promega)	10 mM each	5	2 mM
Forward primer (JMV1)	10 µM	0.5	0.2 µM
Reverse primer (JMV2)	10 µM	0.5	0.2 µM
Taq DNA polymerase (Promega)	5 U μL^{-1}	0.2	1 U
Subtotal		20	
Genomic DNA extract or cDNA (dilution of the amplicons derived from a first PCR if appropriate)		5	
Total		25	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2. PCR cycling parameters: 35 cycles of 30 s 94°C, 30 s 55°C, 90 s 72°C, and a final extension of 10 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 extraction and amplification of *M. chitwoodi* and *M. fallax* and nucleic acid, respectively:

- Negative isolation control (NIC): DNA extraction of solution used to collect nematode specimens in either MGW or 0.9% NaCl solution
- Positive isolation control (PIC): if available, DNA from either *M. chitwoodi* or *M. fallax* specimens (reliably for 5 or more nematodes included) in either MGW or 0.9% NaCl solution
- Negative amplification control (NAC): MGW that was used to prepare the reaction mix
- Positive amplification control (PAC): genomic DNA of 5 (or more nematodes included), or whole-genome amplified DNA, *M. chitwoodi* and *M. fallax*.

3.2. Interpretation of results

In order to assign results from the PCR-based test the following criteria should be applied:

- NIC and NAC should produce no amplicons
- PAC (and if relevant PIC) should produce amplicons of 540 bp for *M. chitwoodi* and 670 bp for *M. fallax*.

When these conditions are met:

- A test will be considered positive if amplicons of 540 bp *M. chitwoodi* and 670 bp for *M. fallax* 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 criteria available

The tests carried out at ANSES (FR), for which the validation data was produced, used a modified test: the dNTP concentration was reduced. The full validation report is available from ANSES.

4.1. Analytical sensitivity data: at ANSES one J2 stage for *M. fallax* and one for *M. chitwoodi* could be detected reliably.

4.2. Analytical specificity data: at ANSES the specificity data was 100% for *M. fallax* and 97% for *M. chitwoodi*. Two populations of target organisms were tested for *M. fallax* and 5 for *M. chitwoodi*. Twenty-eight non-target nematodes populations were tested. Cross-reactions were observed with some populations of *M. javanica* (1 population), *M. enterolobii* (1 population), *Heterodera schachtii* and *Xiphinem*a sp. These were detected as *M. chitwoodi*.

4.3. Data on repeatability: 100% for *M. fallax*, 100% for *M. chitwoodi*.

4.4. Data on reproducibility: 100% for *M. fallax*, 100% for *M. chitwoodi*.

Appendix 4 – ITS-based PCR-RFLP test for *M. chitwoodi* and *M. fallax*

1. General information

1.1. Detection and identification of *M. chitwoodi* and *M. fallax* using the protocol developed by Zijlstra *et al*. (1997).

1.2. The assay is designed for the ITS region of rDNA sequences of *Meliodogyne* spp.

1.3. The PCR product of the reaction with the ITS-specific universal forward primer 18S and reverse primer 26S is 760 bp for both *M. chitwoodi* and *M. fallax*. The primers are described by Vrain *et al*. (1992).

1.4. Primers: 18S 5′-TTG ATT ACG TCC CTG CCC TTT-3′ 26S 5′-TTT CAC TCG CCG TTA CTA AGG-3′. (Note: 18S and 26S are also referred to in publications as 5367 and 5368, respectively.)

1.5. 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. Nematodes (J2 stage, females, eggs, males) are extracted from the roots, potato tubers or from soil as described in PM 7/119. When selected nematode specimens are not used for isozyme analysis (Appendix 2), specimens can be collected in MGW instead of 0.9% NaCl solution.

2.1.2. DNA from nematodes (reliably for 5 or more) can be extracted using the High Pure PCR Template Preparation Kit (Roche) according to the mammalian tissue protocol. DNA is eluted in 40 µL of elution buffer (10 mM Tris-HCl, pH 8.5 (at 25°C)]. Crushing of the nematodes prior to DNA isolation is not necessary.

2.1.3. No DNA clean-up is required.

2.1.4. Use the extracted DNA immediately or store overnight at 4°C or at −20°C for longer periods.

2.2. Conventional PCR

2.2.1. Master mix for PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	13.80	N.A.
PCR buffer (buffer, containing 15 mM $MgCl_2$) (Qiagen)	10×	2.50	1×
dNTPs (Qiagen)	10 mM each	0.5	0.2 mM
Forward primer (18S)	10 µM	1.50	0.6 µM
Reverse primer (26S)	10 µM	1.50	0.6 µM
Hot Start Taq DNA polymerase (Qiagen)	5 U μL^{-1}	0.2	1 U
Subtotal		20	
Genomic DNA extract or cDNA (dilution of the amplicons derived from a first PCR if appropriate)		5	
Total		25	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2. PCR cycling parameters: 15 min 94°C, 45 cycles of 15 s 94°C, 30 s 58°C, 1 min 72°C, and a final extension of 10 min 72°C.

2.2.3. RFLP reaction (according to Zijlstra et al., 1995):

- If required store products at 4°C before analysis
- Incubation: 1 h at 37°C for both restriction enzymes.

Reagent	Working concentration	Volume per reaction *Rsa*I (µL)	Volume per reaction *Dra*I (µL)	Final concentration
Molecular-grade water*	N.A.	3.5	3.5	N.A.
Restriction enzyme buffer (Promega)	10×	1.0	1.0	10×
Restriction enzyme (*Rsa*I)	10 U μL^{-1}	0.5	-	5 U
Restriction enzyme (*Dra*I)	10 U μL^{-1}	–	0.5	5 U
Subtotal		5	5	
(Purified) PCR product		5	5	
Total		10	10	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

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 *M. chitwoodi* and *M. fallax* and nucleic acid, respectively:

- Negative isolation control (NIC): DNA extraction of solution used to collect nematode specimens in (either MGW or 0.9% NaCl solution)
- Positive isolation control (PIC): if available, DNA from either *M. chitwoodi* or *M. fallax* specimens (reliably for 5 or more nematodes included) in either MGW or 0.9% NaCl solution
- Negative amplification control (NAC): MGW that was used to prepare the reaction mix
- Positive amplification control (PAC): genomic DNA of 5 (or more nematodes included), or whole-genome amplified DNA, *M. chitwoodi* or *M. fallax* nematodes.

3.2. Interpretation of results

In order to assign results from PCR-based test the following criteria should be applied:
- NIC and NAC should produce no amplicons
- PAC (and if relevant PIC) should produce amplicons of 760 bp for *M. chitwoodi* as well as for *M. fallax*
- PAC should give a restriction pattern with restriction enzymes *Dra*I and *Rsa*I according to Table 4. When these conditions are met:
- A test will be considered positive if amplicons of 760 bp and a *Meliodogyne*-specific restriction pattern 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.

Table 4. Restriction patterns of *M. chitwoodi* and *M. fallax* with *Dra*I and *Rsa*I

	M. chitwoodi	*M. fallax*
*Dra*I	660, 100	650, 110
*Rsa*I	760	630, 130

3.3. The method can only be used on nematodes morphologically identified as *Meloidogyne* spp., as the primers are not specific for *Meloidogyne* spp.

Appendix 5 – ITS-based real-time (TaqMan) assay for *M. chitwoodi* and *M. fallax*

1. General information

1.1. Detection and identification of *M. chitwoodi* and *M. fallax* using the real-time PCR protocol developed by Zijlstra & van Hoof (2006).

1.2. The assay is designed to the ITS2 region of rDNA sequences of *Meloidogyne* spp.

1.3. The PCR product of the reaction with the forward primer FC612ITS and reverse primer RcFTAQ and *M. chitwoodi*-specific MGB TaqMan probe pMcFAM and *M. fallax*-specific MGB TaqMan probe pMfVIC is 75 bp.

1.4. Primers and probes: FC612ITS 5′-TGT ATA CTT TAT AAT TTTTCT GTT TTG-3′
RcFTAQ 5′-AAA AAA TAA AGC ATA TTTGATACA A-3′;
pMcFAM (5′-6FAM-TGC AAT TTT ATT GAA TAA-MGB-3′);
pMfVIC (5′-VIC-TAC AAT TTG TTG AAT AAT-MGB-3′).

1.5. Amplification is performed in a real-time PCR thermocycler, e.g. CFX96 Real-Time PCR system (Bio-Rad Laboratories).

1.6. Real-time PCR results are analysed using automatic thresholds and baselines for VIC and FAM, e.g. CFX manager 2.0 for the CFX96 Real-time PCR system (Bio-Rad).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Nematodes (J2 stage, females, eggs, males) are extracted from the roots, potato tubers or from soil as described in PM 7/119. When selected nematode specimens are not used for isozyme analysis (Appendix 2), specimens can be collected in MGW instead of 0.9% NaCl solution.

2.1.2. DNA from nematodes (reliably for 5 or more) can be extracted using the High Pure PCR Template Preparation Kit (Roche) according to the mammalian tissue protocol. DNA is eluted in 40 µL of elution buffer [10 mM Tris-HCl, pH 8.5 (at 25°C)]. Crushing of the nematodes prior to DNA isolation is not necessary. DNA from potato peel can be extracted using the Plant DNA isolation kit from BioNobile or the Wizard Magnetic DNA purification System for food (Promega). DNA is eluted in 50 µL of elution buffer. Disruption of the potato tissue can be performed by freeze drying or mechanically (e.g. Grindomix MM-200, Retsch).

2.1.3. No DNA clean-up is required.

2.1.4. Use extracted DNA immediately or store overnight at 4°C or at −20°C for longer periods.

2.2. Real-time PCR

2.2.1. Master mix for PCR

Reagent	Working concentration	*M. chitwoodi* Volume per reaction (µL)	*M. fallax* Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	18.35	18.35	N.A.
Real-time PCR buffer (Eurogentec)	10×	3.0	3.0	1×
$MgCl_2$ (Eurogentec)	50 mM	3.0	3.0	5.0 mM

(continued)

Table *(continued)*

Reagent	Working concentration	*M. chitwoodi* Volume per reaction (µL)	*M. fallax* Volume per reaction (µL)	Final concentration
dNTPs (Eurogentec)	5 mM each	1.2	1.2	0.2 µM
Forward primer (FC612ITS)	10 µM	0.9	0.9	0.3 µM
Reverse primer (RcFTAQ)	10 µM	0.9	0.9	0.3 µM
Probe pMcFAM	10 µM	0.45	–	0.15 µM
Probe pMfVIC	10 µM	–	0.45	0.15 µM
HotStarGold DNA polymerase (Eurogentec)	5 U μL^{-1}	0.2	0.2	1 U
Subtotal		28	28	
DNA dilution		2	2	
Total		30	30	

*Molecular-grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2. PCR cycling parameters: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 56°C for 1 min.

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 *M. chitwoodi* and *M. fallax* and nucleic acid, respectively:

- Negative isolation control (NIC): DNA extraction of solution used to collect nematode specimens in (either MGW or 0.9% NaCl solution)
- Positive isolation control (PIC): if available, DNA from either *M. chitwoodi* or *M. fallax* specimens (reliably for 5 or more nematodes included) in either MGW or 0.9% NaCl solution
- Negative amplification control (NAC): MGW that was used to prepare the reaction mix
- Positive amplification control (PAC1): genomic DNA of 5 (or more nematodes included), or whole-genome amplified DNA, *M. chitwoodi* and *M. fallax* nematodes
- Positive amplification control (PAC2): Dilution of PAC1.The PAC should preferably be near to the limit of detection. To check for false negative reactions caused by inhibition of the amplification reaction an internal amplification control (IAC; 10 fg), 150 nM of IAC forward primer FPgfp (5′-TGG CCC TGT CCT TTT ACC AG-3′, 150 nM of IAC reverse primer RPgfp (5′-TTT TCG TTG GGA TCT TTC GAA-3′), 150 nM of IAC MGB TaqMan probe pIAC (5′-ACA CAA TCT GCC, NED label and quencher dye Eclipse Dark Quencher (Klerks et al., 2004) can be added to the reaction mixes. The IAC is a green fluorescent protein (GFP) plasmid construct and can be obtained from Dr C. Zijlstra, Plant Research International, Wageningen (NL).

3.2. Interpretation of results

In order to assign results from a PCR-based test the following criteria should be adopted:

Verification of the controls

- PAC (as well as PIC, 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
- Tests should be repeated if any contradictory or unclear results are obtained. As a Ct cut-off value is dependent on equipment, material and chemistry it needs to be verified in each laboratory when implementing the test.

3.3. Note: this method has been used with nematodes, and has also been used directly on potato tubers, crocus bulbs and iris bulbs.

3.4. It should be noted that ordering fluorescent probes with MGB quenchers is essential for the correct use of this test. When using other quenchers such as TAMRA or BHQ, the probe melting temperature (T_m) will be too low and no, or unreliable, results can be expected.

Appendix 6 – The MeloTuber Test

1. General information

1.1. This protocol is based on the article by Zijlstra & van Hoof (2006) as described in Appendix 6 and was further developed by the Dutch General Inspection Service for Agricultural Seed and Seed Potatoes (NAK) according to de Haan *et al*. (2014).

1.2. The so-called MeloTuber Test was developed to simultaneously detect and identify *M. chitwoodi* and *M. fallax* directly by triplex real-time TaqMan® PCR in secondary potato tuber peelings.

1.3. The test is designed for the ITS2 region of rDNA sequences of *Meloidogyne* spp.

1.4. Oligonucleotides:

FC612ITS 5′-TGT ATA CTT TAT AAT TTTTCT GTT TTG-3′
RcFTAQ 5′-AAA AAA TAA AGC ATA TTTGATACA A-3′
Probe McFAM 5′-6FAM-TGC AAT TTT ATT GAA TAA-MGB-3′ (Life Technologies)
Probe MfVIC 5′-VIC-TAC AAT TTG TTG AAT AAT-MGB-3′ (Life Technologies)
COX-F 5′-CGT CGC ATT CCA GAT TAT CCA-3′
COX-R 5′-CAA CTA CGG ATA TAT AAG RRC CRR AAC

TG-3′
Probe COX-SOL1511T 5′-Cy5-AGG GCA TTC CAT CCA GCG TAA GCA - BHQ3-3′.

1.5. The PCR product of the reaction with the forward primer FC612ITS and reverse primer RcFTAQ and *M. chitwoodi*-specific MGB TaqMan probe McFAM and *M. fallax*-specific MGB TaqMan probe MfVIC is 75 bp.

1.6. Amplification is performed in a real-time PCR thermocycler, e.g. the ABI 7500 sequence detection system (Applied Biosystems), using a QuantiTect multiplex PCR Kit (Qiagen)

2. Methods

2.1. Nucleic acid extraction and purification

For the MeloTuber Test, secondary (second layer) peelings are sampled from unwashed tubers by first peeling the periderm (±2 mm thickness) and subsequently cutting a secondary peeling of the clean underlying cortex layer (±1 mm thickness). A maximum of 100 secondary peelings are pooled (weighing 93 to 130 g) and homogenized in 75-mL reverse osmosis (RO) water using a Grindomix® GM 200 Knife Mill (Retsch) equipped with a 1-L autoclavable grinding container and a gravity lid with overflow channels, for 10 s at 224 *g* and then 30 s at 3578 *g*. Two hundred microlitres of the tuber homogenate is used for DNA extraction using the sbeadex maxi plant kit (LGC Genomics) in the KingFisher™ mL or the KingFisher™ 96 system (Thermo Scientific). DNA is eluted in 100 µL of elution buffer.

2.2. Polymerase chain reaction

2.2.1. Meloidogyne chitwoodi primer/probe mix

Reagent	Stock concentration	Volume (µL)	Final concentration
Fc612ITS	100 µM	10	4 µM
RcfTaq	100 µM	10	4 µM
Probe McFAM	100 µM	10	4 µM
Molecular-grade water	N.A.	220	

2.2.2. *Meloidogyne fallax* primer/probe mix

Reagent	Stock concentration	Volume (µL)	Final concentration
Probe MfVIC	100 µM	10	4 µM
Molecular-grade water	N.A.	240	

2.2.3. COX primer/probe mix

Reagent	Stock concentration	Volume (µL)	Final concentration
COX-F	100 µM	10	4 µM
COX-R	100 µM	10	4 µM
Probe COX-SOL1511T	100 µM	10	4 µM
Molecular-grade water	N.A.	220	

2.2.4. Real-time PCR master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*		6.75	
PCR master mix (Qiagen)	2×	12.50	1×
M. chitwoodi primer/probe mix	4 µM	1.25	0.2 µM
M. fallax primer/probe mix	4 µM	1.25	0.2 µM
COX primer/probe mix	4 µM	1.25	0.2 µM
Subtotal		23.00	
DNA		2.00	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.5. PCR cycling conditions: an initial step at 95°C for 15 min, followed by 40 cycles (95°C for 15 s and 58°C for 60 s). Step for fluorescence capture: 58°C for 60 s.

3. Controls

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 uninfected host tissue or 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 or a sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism)
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of MGW that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product)
- Internal control (IC) to monitor inhibitory effects introduced by the nucleic acid extract: COX PCR.

3.2. Interpretation of results

In order to assign results from PCR-based test the following criteria should be followed.

Verification of the controls:
- The PAC amplification control and the PIC isolation control should have an exponential curve
- The NIC and NAC control should give no amplification
- The internal PCR control COX should have a Ct-value < 32

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

4. Performance criteria available

The real-time PCR is validated in line with PM 7/98 (EPPO, 2014).

4.1. Analytical sensitivity: 1 female in a sample of 100 peelings.

4.2. Diagnostic sensitivity 100%.

4.3. Diagnostic specificity 100%.

4.4. Analytical specificity 100%.

4.4.1 As described in the article of Zijlstra & van Hoof (2006): isolates of *M. chitwoodi* (8) and *M. fallax* (4) tested positive. Isolates of *M. incognita* (4), *M. javanica* (3), *M. arenaria* (1), *M. hapla* (5), *M. minor* (1), *M. naasi* (3), *Globodera pallida* (1), *Globodera rostochiensis* (1) and *Caenorhabditis elegans* (1) tested negative.

4.4.2 From validation experiments at NAK: isolates of *M. chitwoodi* (24) and *M. fallax* (6) tested positive. Isolates of *M. incognita* (1), *M. javanica* (1), *M. hapla* (6), *M. minor* (10), *M. naasi* (7), *G. pallida* (2), *G. rostochiensis* (6), *Globodera tabacum* (1) and *Globodera artemisiae* (1) tested negative.

4.5. Reproducibility 100%.

4.6. Repeatability 100%.

4.7. Robustness: stability testing. Secondary peelings and/or homogenate can be stored at −20°C without affecting the analytical sensitivity.

4.8. Selectivity: the MeloTuber Test is insensitive to variation in sample material (potato varieties). The different performance criteria such as analytical sensitivity, repeatability, reproducibility, diagnostic sensitivity and diagnostic specificity do not seem to be influenced by matrix effects caused by the different varieties and this confirms the selectivity and the robustness of the molecular test.

Appendix 7 – Diagnostic real-time PCR tests for identification and detection of *M. chitwoodi* and *M. fallax*, based on LSU rDNA

1. General Information

1.1. Scope of the tests: identification of *M. chitwoodi* and *M. fallax* nematodes by real-time PCR and detection of *M. chitwoodi* and *M. fallax* nematodes in both simple (plant material) and complex (soil or substrate) DNA backgrounds by real-time PCR.

1.2. These tests target the LSU (28S) rDNA gene.

1.3. Amplicon sizes: *M. chitwoodi* 189 bp, *M. fallax* 263 bp.

1.4. Oligonucleotide sequences are not disclosed. These tests are available as an all-inclusive real-time PCR kit (ClearDetections, NL, http://www.cleardetections.com/)

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 this test for the purpose of nematode quantification 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

The real-time PCR kit includes target and general nematode DNA real-time PCR primer sets, PACs and PCR mix with fluorescent DNA-binding dye.

2.3. PCR cycling conditions

Enzyme activation: 3 min 95°C. Amplification: 35 cycles of 10 s 95°C, 1 min 63°C, 30 s 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 MGW that was used to prepare the reaction mix

- PACs (included in the kit) to monitor the efficiency of the 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

In order to obtain results from PCR-based tests the following criteria should be met.

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 produces no exponential amplification curve or if it produces a curve which is not exponential
- A melt curve analyses is performed and the obtained 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 a ClearDetections PCR mix are 82.5°C for *M. chitwoodi* and 83.0°C for *M. fallax*
- 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 criteria available

These real-time PCR tests are validated in line with PM 7/98. (EPPO, 2014)

4.1. Analytical sensitivity: less than one individual nematode (about 3 cells of target nematode).

4.2. Diagnostic sensitivity 100%.

4.3. Analytical specificity: specificity value 100%.

4.3.1 *In silico* primer design: an alignment of 105 LSU rDNA sequences from 25 different known *Meloidogyne* species (*M. chitwoodi*, *M. fallax*, *M. minor*, *M. naasi*, *M. hapla*, *M. dunensis*, *M. duytsi*, *M. incognita*, *M. ardenensis*, *M. paranaensis*, *M. trifoliophila*, *M. exigua*, *M. konaensis*, *M. arenaria*, *M. thailandica*, *M. javanica*, *M. graminicola*, *M. maritima*, *M. artiellia*, *M. baetica*, *M. mali*, *M. ulmi*, *M. ichinohei*, *M. hispanica*, *M. enterolobii*) and 20 LSU rDNA sequences of unknown *Meloidogyne* species ('*Meloidogyne* sp.') was used to identify species-specific sequence motives for *M. chitwoodi* and *M. fallax*

4.3.2. Specificity of the primer sets was tested with plasmid DNA (LSU rDNA) of relevant non-target nematode species: *M. chitwoodi*, *M. fallax*, *M. minor*, *M. naasi*, *M. hapla*, *M. ichinohei*, *M. arenaria*, *Pratylenchus penetrans*

4.3.3. Target nematode species were identified microscopically and originated from the Netherlands.

4.4. Diagnostic specificity 100%.

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 remain < 1. The real-time PCR tests for the detection of *M. chitwoodi* and *M. fallax* are robust.

Appendix 8 – High-resolution melting curve analysis as a tool for root-knot nematode diagnostics according to Holterman *et al.* (2012)

1. General Information

1.1. Protocol according to Holterman *et al*. (2012).

1.2. Scope of the test: identification of *M. chitwoodi* and *M. fallax* in a single-tube assay by high-resolution melting curve (HRMC) analysis.

1.3. Test is for the second intergenic spacer region (IGS2).

1.4. Amplicon size in base pairs: *M. chitwoodi* 499; *M. fallax* 631; *M. hapla* 400 (excluding primer sequences).

1.5. Oligonucleotides:

JMV1: 5′-GGATGGCGTGCTTTCAAC-3′

JMV2: 5′-TTTC CCCTTATGATGTTTACCC-3′

JMVhapla: 5′-AAAAATCCCCTCGAAAAATCCACC-3′.

1.6. LightCycler 480 (Roche, Basel, CH).

1.7. LightCycler 480 software version 1.3.0.0705 (automatic).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. J2 juveniles, females, males.

2.1.2. DNA is extracted by transferring an individual specimen to 0.2-mL PCR tubes containing 25 µL of MGW. An equal volume of lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% (vol/vol) β-mercaptoethanol and 800 µg mL^{-1} of proteinase K is added. Lysis takes place in a Thermomixer (Eppendorf, Hamburg, DE) at 65°C at 750 r.p.m. for 2 h followed by a 5-min incubation at 100°C (Holterman *et al.*, 2006).

2.1.3. DNA to be stored at −20°C.

2.2. Real-time PCR and HRMC analysis

2.2.1. Master mix for PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	7.1	N.A.
HotStar Taq Mastermix (Qiagen)	2×	10	1×
Reso-Light dye (Roche)	20x	1.0	1x
Primer 1 (JMV1)	20 µM	0.3	0.3 µM
Primer 2 (JMV2)	20 µM	0.3	0.3 µM
Primer 3 (JMVhapla)	20 µM	0.3	0.3 µM
Subtotal		19	
Genomic DNA		1.0	
Total		20	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.3. PCR conditions: 95°C, 20 min; 95°C, 30 s, 55°C 30 s, 70°C 90 s, 45 cycles; 72°C, 10 min; temperature ramp for HRMC analysis: 65° to 95°C for 47 min with 100 data acquisitions per °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 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
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of MGW that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole-genome amplified DNA or cloned PCR product.

3.2. Interpretation of results

In order to assign results from PCR-based test the following criteria should be met:

Real-time PCR tests:

- HRMC is a combination of real-time amplification followed by the high-resolution melting curve analysisVerification 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
- Additionally for SYBR® Green-based real-time PCR and HRMC tests the T_m values should be as expected: *M. chitwoodi*, 82.77 ± 0.11°C*; *M. fallax*, 83.43 ± 0.09°C; *M. hapla*: 79.66 ± 0.06°C
- Tests should be repeated if any contradictory or unclear results are obtained. *Note: based on 15 replicates per run. As the T_m value is dependent on equipment, material and chemistry it needs to be verified in each laboratory when implementing the test.

4. Performance criteria available

Minimum requirements should be made in line with PM 7/98:

4.1. Sensitivity is 1 individual specimen (female, juvenile, male).

4.2. Specificity is high. T_m values for *M. chitwoodi* and *M. fallax* were significantly different from each other and the closely related species *M. hapla*.

4.3. Repeatability is high (100%).

4.4. Reproducibility is high (100%).