European Union Reference Laboratory

for Plant Parasitic Nematodes

Diagnostic protocols and analytical methods

REFERENCE: EURL – MeloIdentification Version 02

October/ 2020

Identification methods for *Meloidogyne chitwoodi* and *M. fallax*

Foreword

These methods were recommended by:

ANSES - Plant Health Laboratory - Nematology Unit

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

ILVO - Plant Unit - Nematology

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

The consortium between both laboratories is designated the

European Union Reference Laboratory (EURL) for Plant Parasitic Nematodes

Contents

1 Introduction

The root-knot nematodes (RKN), *Meloidogyne chitwoodi* and *Meloidogyne fallax* are plant-parasitic nematodes which cause significant damage to susceptible agronomic important crops. Both root-knot nematode species are classified as quarantine organisms in the European Union (listed in Appendix II, Part B, of the Commission Implementing Regulation (EU) 2019/2072) and are subject to mandatory control measures to avoid their introduction into and spread within the EU territory in geographical areas where these pests are not present.

The purpose of these EURL recommended protocols[1] is to assist the National Reference Laboratories (NRLs) of EU Member states in carrying out their diagnostics analyses, by providing them with details on analytical operating procedures for the detection and identification of *M. chitwoodi* and *M. fallax*.

These protocols are suitable for official testing, in particular in the framework of plant health controls for these pests under the EU territory, including import and export surveillance.

2 Method validation

These EURL recommended protocols are based on EPPO standards. Some of these diagnostic protocols, including operational procedures, were chosen as the basis, and with the scientific experience and technical expertise of the EURL team, some of these protocols have been modified, optimised and further validated by the EURL laboratory.

Warning and safety precautions: The user of these methods should be closely familiar with standard laboratory practices. It is the responsibility of the user to establish suitable health and safety practices and ensure compliance with the current regulations.

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

3 Terms, abbreviations and definitions

- Bp: Base pairs
- DGO: Dorsal Gland Opening
- EPPO: European and Mediterranean Plant Protection Organization
- IPPC: International Plant Protection Convention
- NRLs: National Reference Laboratories
- PPN(s): Plant-Parasitic Nematodes
- RKN: Root-Knot Nematodes
- Swollen stages: sedentary immobile stages inside the plant, third-stage juveniles (J3) and fourth-stage juveniles (J4) and females.
- Vermiform: second-stage juveniles (J2) and males

4 Identification of *Melodoigyne chitwoodi* and *M. fallax*: morphometric and molecular analysis.

4.1 Purpose and scope

The purpose of this protocol is to describe the methods for detection and identification of *Meloidogyne chitwoodi*, and *M. fallax* based on a combination of morphological/morphometric and molecular analyses. This method applies to the *Meloidogyne* sp. stages (juveniles, male and female stages) isolated from different matrices (soil, roots, bulbs, tubers or rhizomes). In the case of egg isolation, these should be placed to hatch, and the analyses performed on the J2 hatched from the eggs.

In this protocol, the conventional PCR test was validated by ILVO; and the Real-Time PCR test was validated by ANSES, both tests including reference to brands.

The laboratory procedures presented in the protocol may be adjusted to laboratory standards provided they are properly validated.

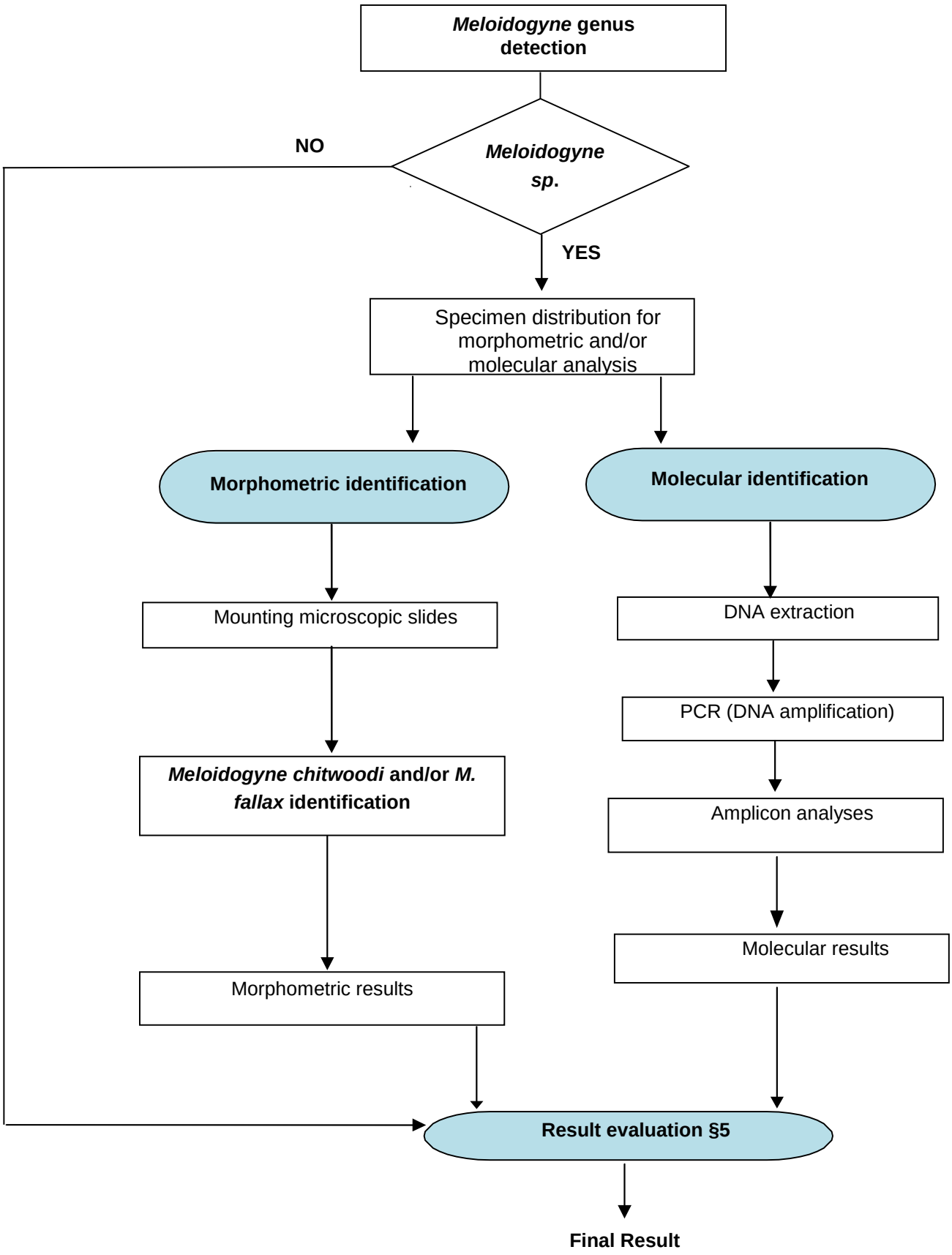
Several morphological and morphometric features of *Meloidogyne chitwoodi* and *M. fallax* can also be found in EPPO PM 7/41 (3).

4.2 Principle

The identification by morphological and morphometric-based analysis is carried out after nematode extraction. It is performed on the different stages of the nematode (J2, female and/or male) present in the obtained nematode extract. The RKN morphological and morphometric characteristics that can be observed through a stereomicroscope for general morphological characteristics and by an interference microscope for morphological and morphometric features.

The molecular identification of *Meloidogyne chitwoodi* and *M. fallax* with the conventional PCR is performed by amplifying a part of the IGS (Intergenic Spacer) region of the ribosomal DNA (rDNA) using species-specific primers, followed by electrophoresis with staining immediately in the gel, and visualisation. The real-time PCR test is based on the amplification of the LSU rDNA followed by data analysis.

Schematic procedure for *Meloidogyne* sp. detection and identification



4.3 Protocol for morphometric identification

4.3.1 General requirements

1) Nematode extracts can be stored for a few days (maximum 1 week) at 4°C while waiting for analysis.
2) Homogenise the nematode suspension.
3) Let the nematodes settle for a few minutes before observation, and ensure that nematodes no longer float in the suspension or on the surface.
4) Observe the entire plate.

4.3.2 Materials and consumables

4.3.2.1 Equipment and small materials

- Stereoscopic, dissection and compound microscopes with episcopic and diascopic illumination (magnification of 16X to a minimum 60X)
- High definition microscope (observations at 1000X magnification)

4.3.2.2 Consumables

- Counting dish
- Fishing needle or any other instrument suitable for handling filiform nematodes
- Brush or other appropriate tools for handling swollen nematodes
- Small scalpel or a syringe needle
- Immersion oil
- Slides and coverslips
- Microtubes
- Heat source (to kill nematodes)
- Lysis buffer (**§4.4.2.2**)
- Varnish

4.3.3 Detection of *Meloidogyne* genus

The analysis should cover all nematodes present in the extract (suspension) or isolated specimens.

1) Transfer the suspension into a counting dish. If the counting dish is too small, distribute the suspension over several dishes.

2) Detect if specimen of the genus *Meloidogyne* are present using a stereoscopic microscope (magnification of 16X to 60X). The main morphological and morphometric criteria for the *Meloidogyne* genus are :

- **J2:** vermiform, small with a length between 250 - 600 µm, stylet hardly visible, lips fused to dumbbell shape (visible with scanning microscopy), oblique overlap pharynx/intestine, presence of a clear area at the level of the anus, sharp and slim tail with a hyaline part, and often assumes a moon-shaped or stretched position (Fig 6).
- **Female:** globular or pear-shaped white body, diameter between 295 - 4250 µm, sometimes elongated, with a neck of varying length (Fig. 6); eggs outside the female in a gelatinous matrix
- **Male:** vermiform, large: 700 - 1900 µm, head often prominent, strongly sclerotised head skeleton; robust stylet 13-30 µm, ventral overlap of the intestine; terminal spicules and rounded tail without bursa (Fig. 6).

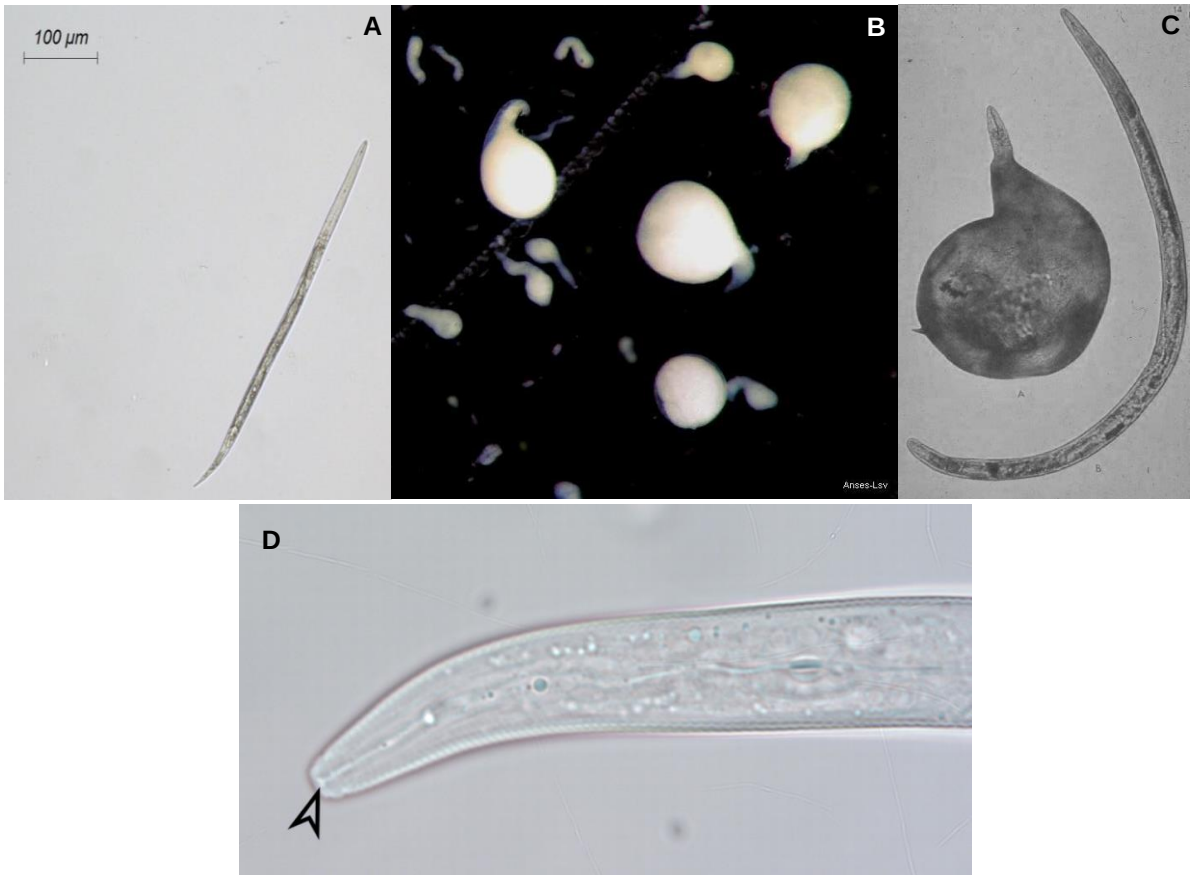


Figure 6. *Meloidogyne* sp. different stages. **(A)** Second-stage juveniles (J2); **(B)** pear-shaped white females; **(C)** Male and female of *Meloidogyne* sp; and **(D)** lips fused to dumbbell shape, which is visible with scanning microscopy (Pictures from North Carolina State University, ANSES-LSV, France and ILVO, Belgium).

3) When the genus *Meloidogyne* is detected, nematodes are fished out with the help of a fishing needle (J2 and male), or a brush (females) and (i) placed in a drop of water on a slide for further morphological analyses or (ii) conditioned per stage in microtubes containing the lysis buffer (see **§4.4.3.2.1/4.4.3.3.1**) for molecular identification (see **§4.4**).

- ***Note:*** *A maximum of 10 J2 or 1 female or 1 male is conditioned per tube. The test sample is then either immediately subjected to DNA extraction or kept at a temperature of -18°C until DNA extraction.*

4) **How to divide the number of specimens for molecular and morphological analysis** (table 1):

Table 1.Distribution of the number of specimens to undergo molecular and morphological analysis.

Stage	Number of available specimens	Molecular analysis	Morphometric analysis
J2	1 to 10	Total (= all specimens)	0
	11 to 19	10	Total** – 10
	20 or more	***Total (=minimum 50 specimens, if available) - 10	10
White females and males	only ♀ translucent*	0	Total (min. 5 if available)
	1 to 4 per sex	Total	0
	5 to 8	4	Total - 4
	9 or more	Between 5 and 20	min. 4

* Translucent females contain little or no DNA; no results can be obtained in a PCR test.

If different stages are present in the sample, analyses (morphological and molecular) are performed on all of them.

** (- 10) means less 10 specimens that have been removed.

*** Examples: If 25 specimens, 10 are morphologically analysed, and 15 specimens are used for molecular analysis. If 100 specimens are found, 10 are morphologically analysed and a minimum of 50 specimens used for molecular analysis.

- ***Note:*** *If only 1 specimen is found in the sample, an attempt is made to obtain additional specimens by extracting the remaining original sample so that the DNA extraction can be done with at least 2 specimens.*

4.3.4 Morphological identification of *Meloidogyne chitwoodi and M. fallax*

4.3.4.1 Mounting slides

4.3.4.1.1 J2 and males

1) After fishing out the individual nematodes, place them in a drop of water on a slide (at least 10 specimens, **see table 1**)
2) Heat the slide, preferably a hot plate at 60°C, in order to kill the nematodes
3) Place a coverslip on the drop
4) Remove excess of water with filter paper and fix the coverslip (varnish, etc.)

4.3.4.1.2 Females

5) After fishing out the individual female, place in a plate (e.g. petri dish lid) or on a slide
6) Cut off the head with a micro-scalpel or syringe needle and empty the contents of the female
7) Cut off the vulval end, as close to the vulva as possible
8) Place the vulval end as flat as possible in a drop of water on a slide, place it external side up in order to observe the perineal pattern
9) Place a coverslip, remove the excess of water and fix the coverslip

4.3.4.2 Species identification

4.3.4.2.1 Second-stage juveniles (J2)

The morphological and morphometric key features are observed at high magnification (up to 1000 x). The key features presented below allows to differentiate *Meloidogyne chitwoodi* and *M. fallax* species from other *Meloidogyne* sp, and also between them.

- ***Note:*** *ANSES has validated this morphological key for J2 identification, and the conclusion of the implemented tests highlights a fairly low sensitivity, hence the need to combine morphometrical identification with molecular analysis.*

Table 2. Performance criteria of the ANSES validation data represented on the identification key below for J2 morphometric identification.

Performance criteria	*M. chitwoodi*	*M. fallax*
Specificity	96,67	100%
Sensitivity	50%	36,67%
Reproducibility	100%	100%

(a) The validation data was obtained by analysing 60 specimens (N=60) for each of the target species.

(b) Specificity (%) = number of negative results / 60

(c) Sensitivity (%) = number of positive results / 60

1. Tail length (Figure 7) < 40 µm and hyaline part (Figure 8) < 8 µm.....*M. artiellia*

No.....**2**

2. Tail length > 65 µm, hyaline part > 19 µm (Figure 9)
And the presence of granules in the median bulb (Figure 9 and 10).....*M. naasi*

No.....**3**

3. Deformed or irregular tail with not clearly delimitated hyaline part (Figure 11,12,13)..*M. hapla*

No deformed tail and a clearly delimitated hyaline part (Figure 14).....**4**

4. Large, parallel-edged, rounded tail tip (Figure 15)
And hyaline part length between 13,0 et 13,91 µm .....*M. fallax*

No.....**5**

5. Conical tail with a rounded end (Figure 16), hyaline tail part between 11,5 and 12,5 µm and
Hemizonid anterior and adjacent to S-E pore .....*M. chitwoodi*

No.....**6**

6. Conical tail with finely rounded tail terminus, hyaline tail part clearly demarcated (Figure 17)
Hemizonid posterior and adjacent to S-E pore (Figure18) .....*M .minor*

No.....*Meloidogyne sp.*

To implement the identification key, the following criteria can be observed on the pictures below (ANSES-Plant Health Laboratory Nematology Unit pictures and pictures 17 and 18 from ILVO):

Tail length: distance from the anus to the tail tip

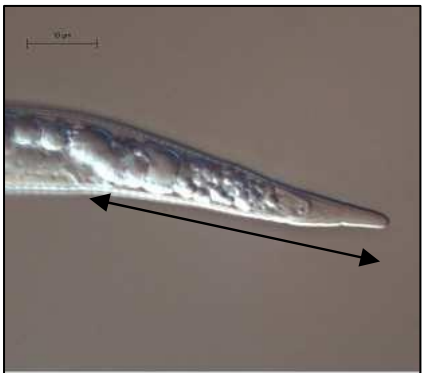


Figure 7: Tail length

Length of hyaline part of the tail: distance from the end of the tail opaque area to the tail tip

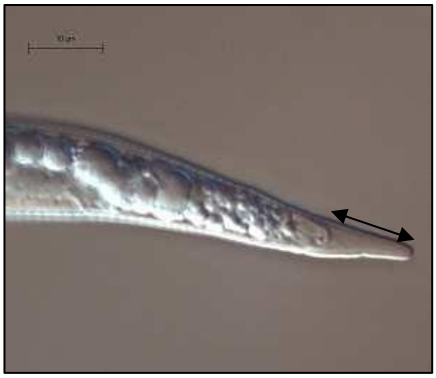


Figure 8: Length of hyaline part of the tail

Granules present at the median bulb anterior part and **long finger-shaped tail**

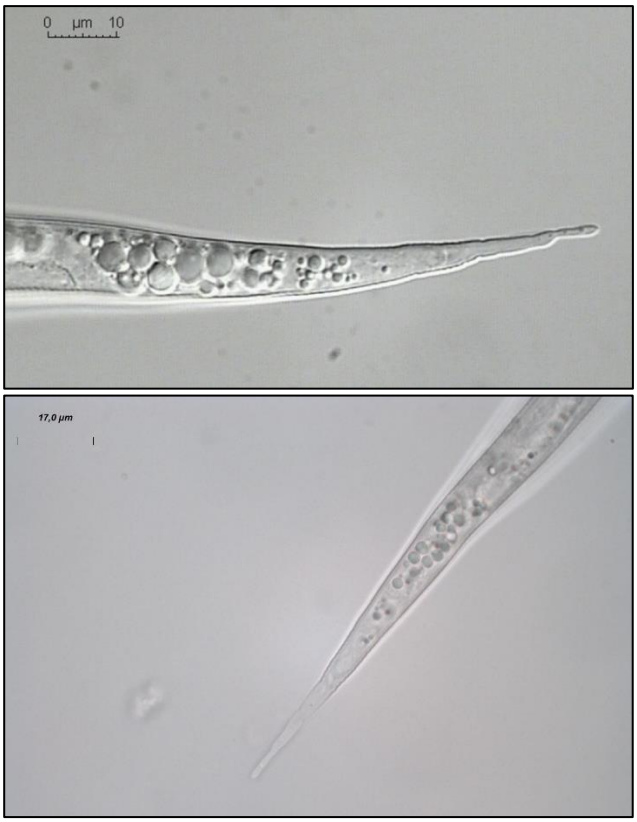


Figure 9: granules anterior the median bulb and long shaped tail

Granules at the medial bulb anterior part

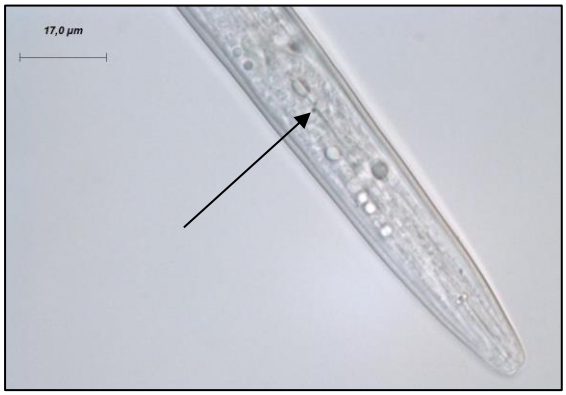


Figure 10: median bulb granules

Shape of the tails tip

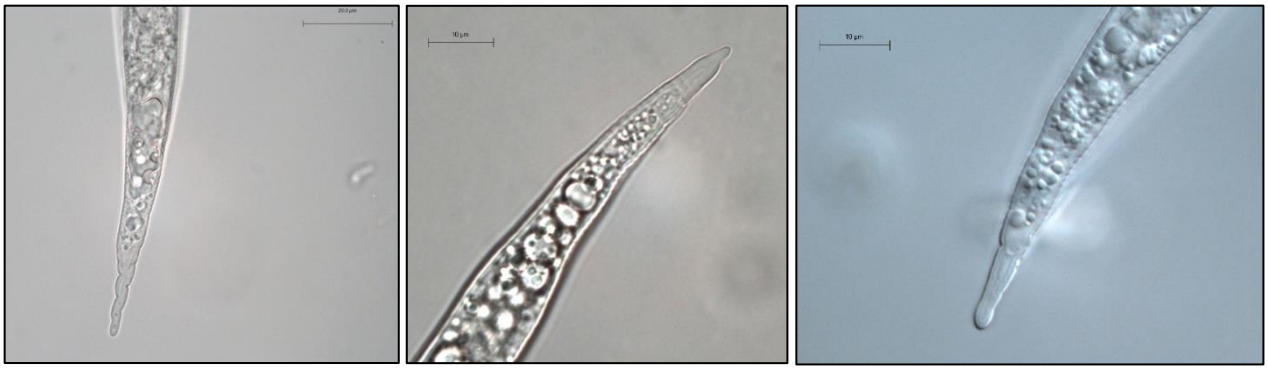


Figure 11: Irregular tails end

Shape of the tail tip

Figure 12: Irregular club-shaped tail ends

Characteristic of the hyaline part

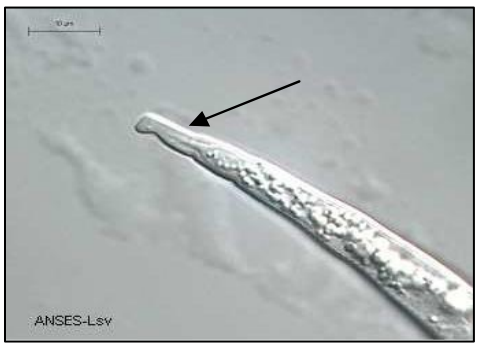


Figure 13: Not clearly delimitated hyaline part

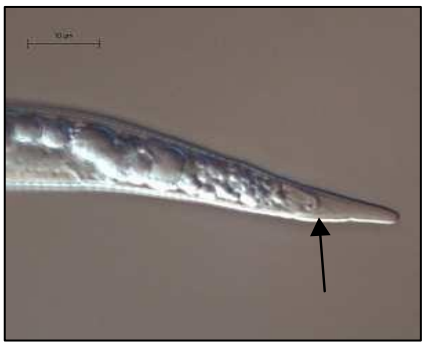


Figure 14: Clearly delimitated hyaline part

Characteristic of the tail tip

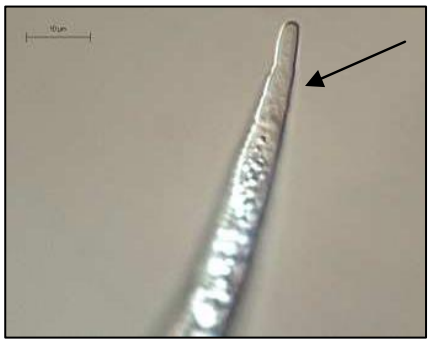


Figure 15: Large tail with parallel edges and rounded tip

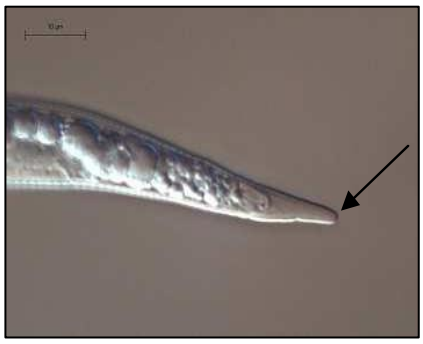


Figure 16: Conical tail with rounded end

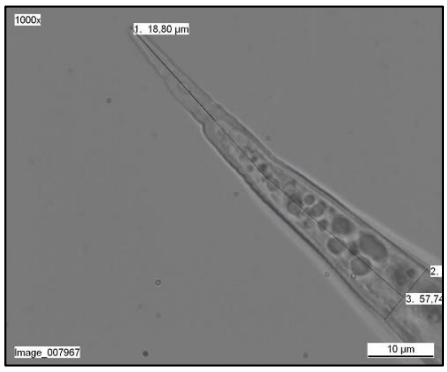


Figure 17: Rounded tail terminus with hyaline tail part clearly demarcated

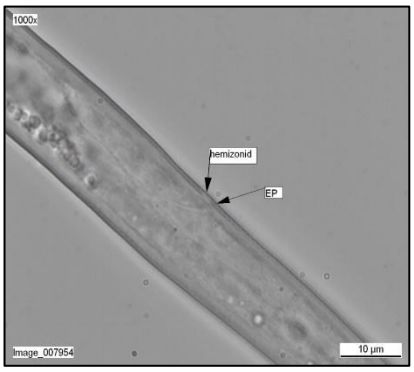


Figure 18: Hemizonid posterior to S-E pore

4.4 Protocol for molecular identification

4.4.1 General requirements

1) Use pipette tips with filter plug during manipulation for PCR reactions.
2) Each DNA sample is tested at least in duplicate.
3) Let all the PCR reagents melt entirely, mix them well and keep them on ice.
4) Any commercial PCR mix (e.g. polymerase buffer, DNA polymerase, dNTP, MgCl2, etc.) that is suitable for conventional and real-time PCR applications can be used as long they have been used in an in-lab validation study.

4.4.2 Material and consumables

4.4.2.1 Equipment and small materials

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

- Shacking tissue grinder for approximately 2 mL microtubes (e.g., Tissulyser, Qiagen®) or equivalent equipment
- Conventional PCR equipment and real-time PCR equipment

4.4.2.2 Consumables

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

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

The reagent and primer brands[2] and solution preparations indicated in these protocols are not mandatory.

- Molecular biology quality Ultrapure (Milli-Q) water
- Two DNA lysis buffers are recommended for DNA extraction:
  - **For conventional PCR:**
    - WLB +**:** Mix 860 µl of WLB with 100 µl of DTT (DL-dithiothreitol 1 M) and 40 µl of ProtK (20mg/ml) in a 1.5 ml Eppendorf. Store for a maximum of 1 month at -20 ° C. This buffer is used at the ILVO laboratory.
      Note: *DTT is toxic, it should be handled in a fumehood*
    - Worm Lysis Buffer (WLB)**:** Mix 2 ml 1 M NaCl with 2 ml 1 M Tris-HCl pH8.0 and 4.6 ml ultra pure water in a 15 ml sterile Falcon tube. Store at 4 °C for an unlimited time.
    - 20 mg/mL Proteinase K (ProtK) storage solution: Dissolve the ProtK in a certain amount of ultrapure water to obtain a concentration of 20 mg/mL: e.g. batch of 100 mg of ProtK in 5 ml of ultrapure water. Divide into 1.5 ml Eppendorf. Store the dissolved ProtK at -20 ° C for a maximum of 1 year. Also, note that ProtK as a lyophilised powder can be stored for a maximum of 2 years.
  - **For real-time PCR**:
    - Lysis buffer from Ibrahim et al., 1994 + metal/glass beads (for J2) or piston (female), and as alternative freezing and defrosting the specimens.

[2] The use of certain brands of chemicals and primers in this recommended protocol does not imply the exclusion of other brands, which may also be appropriate.

- **Commercial PCR mix**: The conventional PCR test was validated using the FastStart™ Taq DNA polymerase (Ref. 04738403001) from Sigma – Aldrich.
- **Commercial Diagnostic Kit:** The real-time PCR test was validated using the Cleardetection's Real-Time PCR diagnostic kits for *Meloidogyne chitwoodi* (RT-N-D-1305) and *M. fallax* (RT-N-D-1309). https://www.cleardetections.com/index.php/service/nematode-real-time-pcr-diagnostic-kits/
- **Primers:**

Target-nematodes	Primers Conventional PCR	Sequence 5' - 3'
M. chitwoodi and M. fallax	**JMV1** **JMV2**	GGA TGG CGT GCT TTC AAC TTT CCC CTT ATG ATG TTT ACC C

Target-nematodes	Primers Real-time PCR Clear®Detections	Sequence 5' - 3'
M. chitwoodi, **and** ***M. fallax***	Primers **RT-ND-1305** ***M. chitwoodi*** Primers **RT-ND-1309** ***M. fallax***	Primer sequences are not published; commercially available ready-to-use solutions can be ordered from the company Clear®Detections*

Target-nematodes	Primers PCR tests	Sequence 5' - 3'
All nematodes	**18S** **26S**	TTG ATT ACG TCC CTG CCC TTT TTT CAC TCG CCG TTA CTA AGG

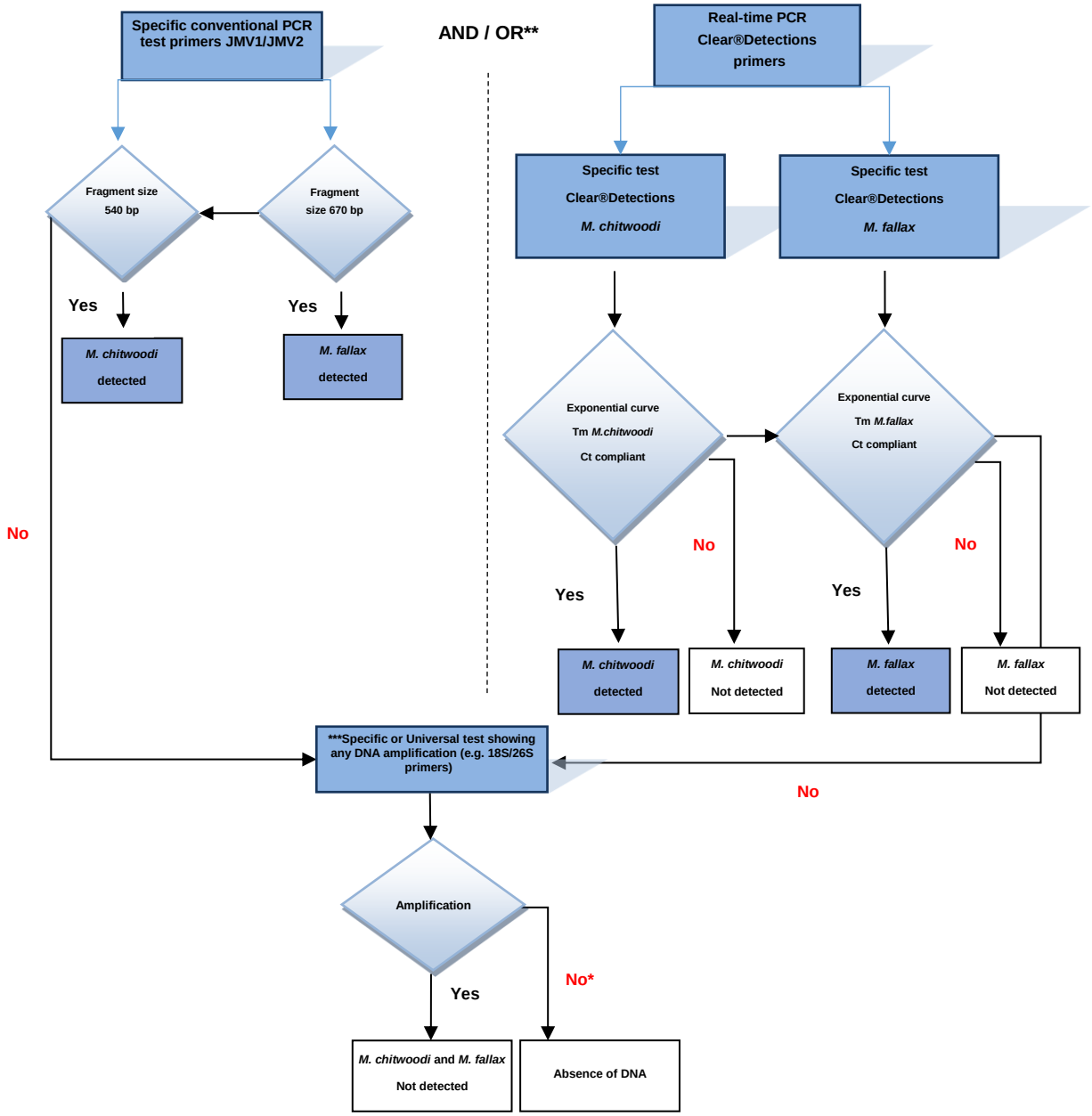
- ***Note:*** *It is possible to convert the conventional PCR into a multiplex PCR, by adding the JMV hapla primer (sequence: 5' AAA AAT CCC CTC GAA AAA TCC ACC 3') which results in an amplicon of 440bp.*

4.4.3 Molecular tests

Two molecular tests are recommended: the conventional PCR specific JMV1/JMV2 test (Wishart *et al*. 2002), and the real-time test using Clear®Detections commercial Kit. The laboratory can apply one of the tests to routine analysis, and if required confirm the result using the other proposed test or any other different test of its choice, as long as these are performed under different conditions (i.e. primers amplifying different genome regions).

The molecular analysis results evaluation is carried out according to the following schematic procedure

Schematic procedure for molecular tests analyses



* If DNA is not amplified, new specimens are analysed by molecular biology, either from the remaining extract or after a new extraction from a matrix (if available).

** If confirmation is required the EURL advises a confirmation with two different specific tests or the same test, as long as these are performed under different conditions (i.e. primers amplifying different genome regions).

*** This test is performed when the specific PCR tests do not produce any fragment amplification or do not generate an amplification corresponding to *M. chitwoodi* and *M. fallax* targets.

4.4.3.1 Control and their purpose

Reference samples must be included during the molecular process to validate the different steps of the tests. The following controls are used to check the correct performance of the DNA extraction and PCR steps (amplification of DNA from *M. chitwoodi* and/or *M. fallax* for positive control and absence of contamination on negative controls).

Controls	Purpose	Control application	Expected result *
Negative process control (NPC)**	DNA extraction buffer alone conditioned and tested in the same manner as the tested sample. Verify the contamination of the DNA extraction process.	Mandatory	Negative
Positive PCR control (PC)**	Contains all the elements of the PCR reaction including a DNA extract from each of the nematode targets; this control verifies that the PCR reaction has proceeded correctly and has allowed amplification of the samples containing the target.	Mandatory	Positive
No Template Control (NTC)	Contains all the elements of the PCR reaction, but no DNA is added; this control allows checking the absence of contamination during the PCR reaction.	Mandatory	Negative
Negative specificity control (NSC)	Contains all the elements of the PCR reaction, including a non-target nematode DNA; this allows to check the absence of reaction Inhibitors during the PCR. This type of control is not required for the universal 18S-26S test.	Mandatory when no amplification is observed	Positive for a universal test or other non-target specific tests

* The results are only valid if the expected results are met.

** Possibility to use the negative and positive extraction control described on the Meloextraction protocol (see **§4.3)** as NPC and PC, respectively.

According to the PCR tests, the DNA extracts required to be included are from the following species:

Controls	Specific PCR	Universal PCR
PC	*M. chitwoodi* and *M. fallax*	*M. chitwoodi* or *M. fallax*
NSC	Non-target *Meloidogyne* species	

4.4.3.2 Specific conventional PCR test

4.4.3.2.1 DNA extraction

The analysis is carried out on specimen(s) that have been collected and conditioned previously according to the methodology described in **§4.3.3 (table 1)**. **The following step is applied to both stages, vermiform and swollen (females).**

1) Add the nematodes to a PCR tube with 25 µl of ultra-pure water.
2) Add 25 µl Worm Lysis buffer + (WLB +) (composition see **§4.4.2.2**).
3) Incubate in a thermocycler for 90 minutes at 65 °C, followed by 5 minutes at 99 °C.
4) The DNA can be used immediately or stored at -20 °C until use.

4.4.3.2.2 PCR reaction

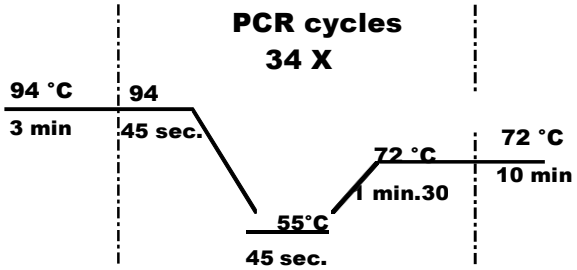
- JMV1/JMV2 test (Wishart *et al*. 2002)

	JMV1/JMV2/ test*
Reagents	**Final concentration for 1 reaction**
Total volume	50 µL
Taq DNA polymerase buffer	1 X
$MgCl_2$ (take into account the MgCl2 possibly present in the Taq buffer)	3 mM
Primers	JMV1: 0,3 µM JMV2: 0,3 µM
dNTPs	0,2 mM
FastStart Taq DNA polymerase (Roche)	1U/25 µl
Ultra-pure water	35 µL (or adjust accordingly)
DNA	2 µL

*This test can be turned into a multiplex PCR test which simultaneously detects *Meloidogyne hapla* by adding the JMVhapla primer (same concentration as the JMV1 and JMV2 primers). Adjust the volume of water to reach a final volume of 50 µl PCR reaction mix (Wishart *et al.*, 2002).

JMVhapla sequence 5' - AAA AAT CCC CTC GAA AAA TCC ACC - 3'. This primer gives an amplicon of 440 bp.

- PCR program:



4.4.3.2.3 Evaluation and reporting the results

- The molecular analysis result is a synthesis of the results obtained from each of the microtubes analysed.
- The analysis for the conventional PCR is qualitative. Regardless of the PCR test, the result is:
  - negative when no amplification is observed,
  - negative when no amplification at the expected size is observed,
  - positive when a fragment of the expected size is observed.
- The expected fragment sizes are as follows:
  - The JMV1/JMV2 and Universal primers described in **§4.4.2.2** allow a specific amplification fragment of approximately **540 bp** for ***M. chitwoodi*****, 670 bp** for ***M. fallax*** and **760 bp** for other *Meloidogyne* genus.

4.4.3.3 Real-time PCR - Clear®Detections test

4.4.3.3.1 DNA extraction

DNA extraction results from the successive action of mechanical grinding (glass or metal beads or piston) and chemical treatment (proteinase K).

The analysis is carried out on specimen(s) that have been collected and conditioned previously according to the methodology described in **§4.3.3 (table 1)**.

In the case of vermiform stages:

1) Add glass or metal beads of different diameters (e.g. 1 bead of 3 mm and about 50 µL of beads of 1 mm diameter) to the microtube containing the nematodes and 100 µL of lysis buffer (Ibrahim *et al*., 1994).
2) Using a Tissue Lyser (for example QIAGEN) or equivalent: agitate for about 40 seconds at a frequency of 30 pulses per second (thaw microtubes prior to agitation).

In the case of females:

3) Grind, using a disposable piston, the previously conditioned individuals in microtubes containing 100 µL of lysis buffer (Ibrahim et al., 1994).

The following step applies to both stages, vermiform and swollen (females):

4) Incubate the microtubes in a water bath (between 50 and 60°C) for at least one hour to optimise the proteinase K activity.
5) Inactivate the ProtK by 10 minutes at 95 ° C
6) The DNA extract can be used immediately or stored at -20 ° C until use.

4.4.3.3.2 PCR reaction

PCR reaction is performed following the supplier's recommendations from the preparation of the PCR mix

- ***Note:*** *As the detection tests are simplex, two separate PCRs are performed with each primer mix* ***(Clear®Detections M. chitwoodi or Clear®Detections M. fallax)*** *for each nematode target.*

4.4.3.3.3 Evaluation and reporting the results

The correct interpretation of the results is carried out by observing the fluorescence curves measured by the real-time PCR and generated from the various controls (NTC and PC).

- The analysis is valid if, and only if, all of the following conditions are met:

1) All replicates of PC, generate an amplicon in accordance with the requirements.
2) None of the NTC replicates generate an amplicon corresponding to the desired target.
3) Analysis of amplification curves and melting curves are combined.

4) If the results of one or more controls do not comply with those expected (as defined above), the analysis is not valid and, depending on the non-compliance observed, all or part of the analysis must be repeated.
5) After validation of the controls and the series, and for each of the PCR reactions, observe the *Ct* value, the appearance of the amplification curve and the *Tm* value: sample results should be interpreted as follows for the target organism under investigation (*M. chitwoodi* or *M. fallax*):

PCR analysis		Sample Results
+	+	Positive Test
+	-	PCR is re-done. If at least 1 out of 2 is positive, the result is interpreted as positive.
-	-	Negative Test

+: observation of an exponential amplification curve with a value of *Ct* < 35 and with a *Tm* value corresponding to the desired target.

- : the absence of an exponential amplification curve with a value of *Ct* < 35 or observation of an exponential amplification curve with a value of *Ct* value < 35 but, with a *Tm* value different from that of the desired target.

- The molecular analysis result is a synthesis of the results obtained from each of the microtubes analysed.
- Result Interpretation of the molecular analysis is carried out according to the following flowchart **§4.4.3.**

5 Sample final result: Interpretation and evaluation

The final identification result for *M. chitwoodi* and/or *M. fallax* results from the combination of the morphological/morphometric and molecular results.

Table 3. Synthesis analyses and final result.

Morphometric analysis result			
Genus verification	**Species identification**	**Molecular analysis results**	**Final result**
Meloidogyne sp. (-)	*None*	*None*	*M. chitwoodi and M. fallax not detected*
Meloidogyne sp. (+)	*- Meloidogyne sp.* *- None (absence of specimens to conclude the analysis)* *-Other than M. chitwoodi and M. fallax*	*M. chitwoodi and M. fallax (-)*	*M. chitwoodi and M. fallax not detected*
		M. chitwoodi and M. fallax (+)	*M. chitwoodi and M. fallax detected*
	M. chitwoodi	*M. chitwoodi (+)* *M. fallax (-) or (+)*	*M. chitwoodi detected* *M. fallax (not) detected* **(a)**
		M. chitwoodi (-) *M. fallax (-) or (+)*	**(1)**
	M. fallax	*M. chitwoodi (-) or (+)* *M. fallax (-)*	**(1)**
		M. chitwoodi (-) or (+) *M. fallax (+)*	*M. chitwoodi (not) detected* **(a)** *M. fallax detected*

(-): negative test

(+): positive test

(a): Respectively according to the result of the molecular analysis

(1) In the situation where the morphological/morphometric results are inconsistent with the molecular results, or if no amplification is obtained (absence of DNA), the following actions shall be carried out:

- *New specimens are analysed (morphology and molecular), if still available from the same sample. If specimens are not available, only the positive molecular test is taken into account and the species can be declared present in the sample. If PCR is negative, check the presence/absence of DNA using the Universal primers; if an amplification is obtained – results correspond to "species other than M. chitwoodi and M. fallax. If no amplification is obtained, take another specimen, if available.*
- *Two negative molecular results for the same species indicate the absence of the species, hence a negative result. If no molecular results are obtained during the second analysis or in the absence of another specimen, the client is informed, and an analysis report is issued with the result "Meloidogyne sp. detected".*

- The analysis result on the report will be, when applicable:
  - ***Meloidogyne chitwoodi "detected" or "not detected" and/or Meloidogyne fallax "detected" or "not detected"***
  - ***Meloidogyne sp. "detected".***

If you have any question about the identification protocols for Meloidogyne chitwoodi and M. fallax, please, send e-mail to eurl.nematodes@anses.fr.

Acknowledgements

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

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