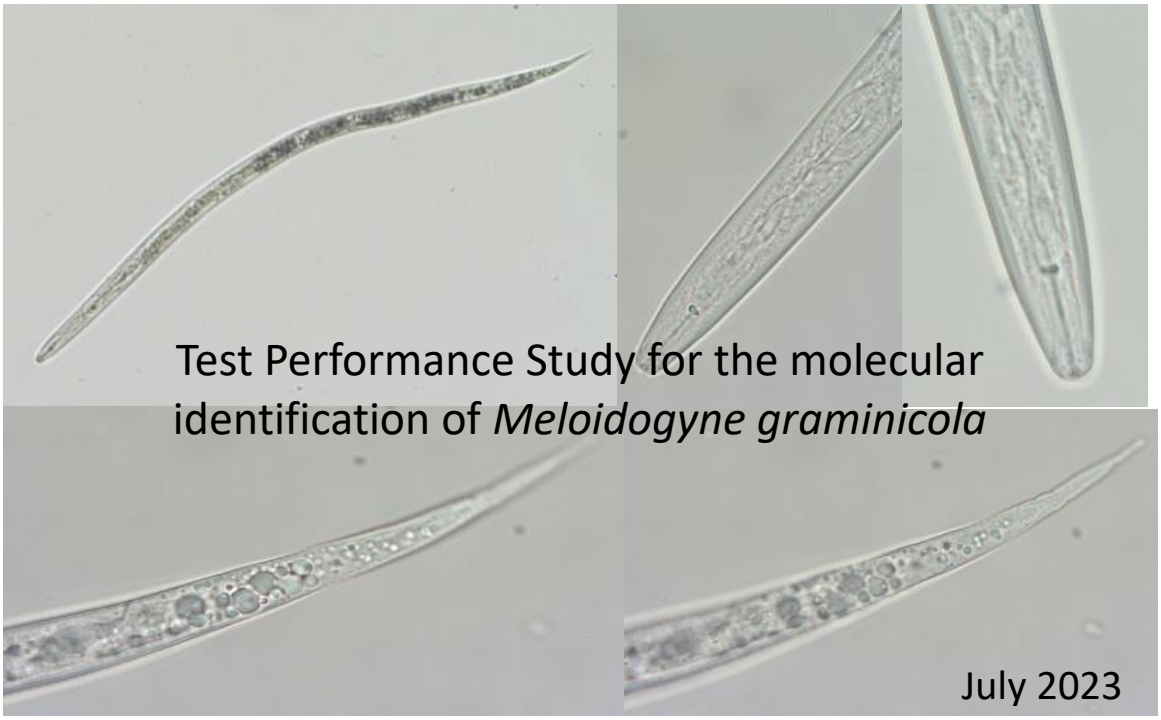
Plant Health Laboratory

European Union Reference Laboratory
for plant-parasitic nematodes

TEST PERFORMANCE STUDY REPORT

22MG



Final report
Test Code: 22MG

	First name and Last name	Function	Date and Signature
Approval	Nicole Damme	Test Performance study Coordinator	Nicole Damme (Signature) Digitaal ondertekend door Nicole Damme (Signature) Datum: 2023.07.07 10:32:07 +02'00'
Authorization for distribution	Nicole Viaene	Test Performance study Coordinator 2	Nicole Viaene (Signature) Digitaal ondertekend door Nicole Viaene (Signature) Datum: 2023.07.07 14:08:45 +02'00'

This report is distributed by the organizer to the TPS participants and all National Reference Laboratories (NRLs) in the framework of the Work Programme of the EURL for Pest of Plants on Nematodes in 2022.

Content

1. Introduction

Meloidogyne graminicola is a major plant-parasitic nematode on rice in Asia, where it is present in most countries in South, South-East and East Asia. The nematode is also present in other parts of the world, including Southern Africa (South Africa, Madagascar), and South America (Brazil, Colombia and Ecuador). It has been found in Europe in 2016 and 2018 in rice fields in Italy.

Besides rice, *M. graminicola* can also multiply on several other Poaceae such as oat, pearl millet, wheat, barley, sugar cane, corn, and barnyard grass. Also, vegetables like onion, pea and tomato are reported as hosts.

The European Union (EU) emergent nematode *Meloidogyne graminicola* (rice root-knot nematode) fulfils the pest criteria set out in Subsection 2 of Section 3 of Annex I to Regulation (EU) 2016/2031. Therefore the Implementing Regulation (EU) 2022/1372 established temporary measures to prevent the entry into, the movement and spread within, the multiplication and release of this phytonematode in the Union. *Meloidogyne graminicola* is also in the EPPO Alert List 2023.

Within the framework of its Work Programme 2020 , the European Union Reference Laboratory (EURL) for plant-parasitic nematodes (ILVO), evaluated published molecular identification tests for this pest. Due to a validation study that was performed by the EURL in the framework of the EU-funded project EURLs-EURCs 2021-2022 (grant SI2.870859), 2 tests, in combination with a third test, were selected on the basis of their performance in the EURL laboratory for validation in this TPS. NRLs from the Mediterranean countries of the EU, where rice-growing is important, and NRLs from other countries that had explicitly indicated being willing to participate in this test performance study (TPS), organised by ILVO in December 2022, were invited to participate.

2. General organisation of the test

2.1. Purpose of the test

The test performance study (TPS) aimed to generate additional validation data and assess the performance criteria of two conventional PCR tests, Htay et al., 2016, and Bellafiore et al., 2015, each in combination with a third conventional PCR test (Mattos et al., 2019) (since no primer set was found to be specific) to identify the presence of *Meloidogyne graminicola* in samples of known status by evaluating the accuracy of the results. Further on in this report, these tests will be referred to as the "Htay primers", the "Bellafiore primers" and the "Mattos primers", respectively. The accuracy of the results was assessed through the ability to give positive results on status-positive samples (sensitivity) and negative results on samples with negative status (specificity).

In addition to this, participants were also asked to determine the analytical sensitivity of the two primer pairs, i.e., the Htay primers and the Bellafiore primers.

Primer sequences were communicated to the participating laboratories prior to the shipment of the samples to allow laboratories to order them and be ready for the TPS when it was sent out.

The samples consisted of nematodes in suspensions. Each sample contained only one species and this was communicated to the participants.

The protocols for the tests were provided to the participating laboratories.

Each laboratory was allowed to apply its routine method for DNA extraction, its routine chemicals and equipment. Hence, the robustness of the proposed tests was thoroughly tested.

Testing the robustness of the PCR protocols under different laboratory conditions and by different laboratory personnel is critical for successful out-house validation. In addition, the knowledge and experience gained in this TPS will support the EURL for Plant parasitic nematodes to better advise NRLs on the PCR-based identification test(s). Furthermore, the organisation of this TPS potentially identified challenges for the identification of *M. graminicola*, and the organization of a Proficiency Test (PT).

2.2. Identification of the test performance study coordinator and the staff involved in the study

The test performance study was organised and coordinated by the ILVO Diagnostic Center for Plants, Nematology Laboratory, which is accredited according to EN ISO/IEC 17025:2017 for the detection (morphology) of *Meloidogyne spp.*, which enabled it to establish the assigned value of the samples and to carry out the homogeneity and stability studies.
Table 1 indicates the staff involved in the conception and management of the TPS and the function held by each person.

Table 1: Contributors to the organisation of the test performance study

Functions	First and last name
Test Performance Study Coordinator	Nicole Damme Nicole Viaene
Quality manager	Annemie Hoedekie
Technical operator	Anne-Marie Deeren Lirette Taning Niels Vermassen

Contact details of the proficiency test coordinator
Nicole Damme e-mail: nicole.damme@ilvo.vlaanderen.be Tel: +32 (0)9 272 24 44 **Nicole Viaene** e-mail: nicole.viaene@ilvo.vlaanderen.be Tel: +32 (0)9 272 24 25

2.3. Participating laboratories

The test performance study was opened to the National Reference Laboratories (NRLs) from the EU member states in the Mediterranean region where rice growing is important (participant type 1) and to the National Reference Laboratories (NRLs) from the EU member states that had prior to the invitation, indicated being willing to participate in this TPS (participant type 2), including the Belgian NRL. In total, 10 laboratories were invited, and only 9 registered for participation.

All laboratories are coded as LXX (XX being a two-digit number), to ensure anonymized participation and results confidentiality. Each participant's laboratory code was individually communicated when their panel was sent and in the TPS's individual summary sheet transmitted at the moment of the final report's release.

The profile of the invited and participating laboratories is presented in Table 2:

Table 2: Profile of invited and participating laboratories

Participant Type*	Laboratory invited	Country	Participated
1	Central Laboratory for Plant Quarantine	Bulgaria	yes
1	Croatian Agency for Agriculture and Food Centre for Plant Protection Laboratory for Nematology	Croatia	yes
1	ANSES	France	yes
1	INIAV	Portugal	yes
1	KIS	Slovenia	yes
1	CREA	Italy	yes
1	National Phytosanitary Laboratory	Romania	no
2	ILVO	Belgium	yes
2	DAFM	Ireland	yes
2	NVWA	The Netherlands	yes
TOTAL	**10**		**9**

*Type 1= Mediterrranean country , type 2= volunteering

2.4. Instructions to participants

The organiser paid particular attention to the information provided to the participants. The aim was to ensure that the participants would register for the test performance study in full awareness of the participation conditions and that they would be clearly informed of the operations they had to carry out at each stage of their participation.
The participating laboratories undertook the analyses according to the organiser's specifications under normal and usual working conditions.
The shipment of the panel with samples included various documents, which were also provided by e-mail:

-a technical instruction sheet, specifying the procedures for receiving and storing the samples, carrying out the analyses, recording and transmitting the results;
-an acknowledgement receipt form, including lines to report to the organiser any problem concerning the sample integrity;
-a result form , standardising the presentation of results and enabling to report of any associated technical information needed by the organiser for interpreting the results (critical steps of the analysis, critical consumables used, critical equipment used, etc.)

2.5. The timeline of the test performance study

The TPS was conducted according to the key steps summarized in the table below:

Table 3: Schedule of the key stages of the test.

Steps	Deadlines
Call for registration	6 December 2022
Closing of registration	12 December 2022
Homogeneity study	12-16 December 2022
Shipping of samples	19 December 2022, 9 and 16 January 2023*
Deadline for reporting results	15 March 2023
Stability Study	20-24 March 2023
Sending of the TPS report	July 2023

*: Some participants asked to ship their panel at the beginning of January 2023.

3. Test Performance Study items

3.1. Identification of *Meloidogyne graminicola*

3.1.1. Test selection

Several tests were evaluated for the EURL at ILVO:
- 4 conventional PCR tests: Htay *et al*. (2016, rDNA), He *et al*. (2021, SCAR), Bellafiore *et al*. (2015, SCAR) and Mattos *et al*. (2019, SCAR)
- 2 RT-PCR methods: He *et al*. (2021, SCAR) and Htay *et al*. (2016, rDNA)
- 1 LAMP method: He *et al*., (2021, SCAR)

Based on their performance in the EURL laboratory, two conventional PCR tests, i.e. Htay *et al*. and Bellafiore *et al*. were chosen for further validation in this TPS.

3.1.2. Composition of the panel of samples and criteria being evaluated

A panel consisting of 16 coded falcon tubes (15ml) with nematodes suspended in water, was sent to each participant. The number of nematodes (second-stage juveniles) per sample ranged from 65 to 425. The detailed composition of the panel of samples is shown in the table below.

Table 4: Description of the samples in the panel

List of Samples	Nature of samples	Packaging	Characteristics	Type of Sample Identification	Assigned Value Identification
Sample 1	*Meloidogyne graminicola*, population 1 (Italy), suspension in water	Around 10 ml of suspension in a 15 ml Falcon tube	*M. graminicola*	Target	Detected
Sample 2				Target	Detected
Sample 3				Target	Detected
Sample 4	*Meloidogyne graminicola*, population 2 (the Philippines), suspension in water	Around 10 ml of suspension in a 15 ml Falcon tube	*M. graminicola*	Target	Detected
Sample 5				Target	Detected
Sample 6				Target	Detected
Sample 7	*Meloidogyne naasi*, suspension in water	Around 10 ml of suspension in a 15 ml Falcon tube	*M. naasi*	Non-Target	Not Detected
Sample 8				Non-Target	Not Detected
Sample 9				Non-Target	Not Detected
Sample 10	*Meloidogyne oryzae*, suspension in water	Around 10 ml of suspension in a 15 ml Falcon tube	*M. oryzae*	Non-Target	Not Detected
Sample 11				Non-Target	Not Detected
Sample 12				Non-Target	Not Detected
Sample 13	*Meloidogyne incognita*, suspension in water	Around 10 ml of suspension in a 15 ml Falcon tube	*M. incognita*	Non-Target	Not Detected
Sample 14				Non-Target	Not Detected
Sample 15				Non-Target	Not Detected
Sample 16	Lure	Around 10 ml of suspension in a 15 ml Falcon tube	Differs from panel to panel, result not evaluated		

The panel was chosen to enable the organiser to evaluate the following performance criteria for each target species:

Diagnostic Sensitivity: 6 samples infested with *Meloidogyne graminicola* (3 samples from population 1 and 3 samples from population 2).

Specificity: the presence of 9 non-target samples, among which:

- 3 samples infested with *M. naasi,* which can be found in European fields
- 3 samples infested with *M.oryzae,* present in South-America (Brazil), and which cross-reacts with the *M. graminicola* primers
- 3 samples infested with *M. incognita,* present in warm climates in many countries worldwide.

Accuracy: which summarises the two above-mentioned criteria.

The criteria of sensitivity, specificity and accuracy are defined in paragraph 5.1.1.

A lure sample was introduced into each panel in order to modify the proportion of positive and negative samples from one panel to another. The results of these lures were not considered for evaluation.

The following table shows which samples were used to evaluate each performance criterion.

Table 5: Identification of the samples used for the evaluation of the different performance criteria

Panel Samples	Nature of samples	Identification of *Meloidogyne graminicola*		
		Evaluated criteria *M. graminicola*		
		Sensitivity	Specificity	Accuracy
Sample 1	Suspension containing *M. graminicola* (population 1)	x		x
Sample 2		x		x
Sample 3		x		x
Sample 4	Suspension containing *M. graminicola* (population 2)	x		x
Sample 5		x		x
Sample 6		x		x
Sample 7	Suspension containing *M. naasi*		x	x
Sample 8			x	x
Sample 9			x	x
Sample 10	Suspension containing *M. oryzae*		x	x
Sample 11			x	x
Sample 12			x	x
Sample 13	Suspension containing *M. incognita*		x	x
Sample 14			x	x
Sample 15			x	x
Sample 16	Lure	Not evaluated		

3.1.3. Sample codification

The samples were randomly coded and labelled with a three-figure number from 001 to 278 samples, preceded by the TPS code, i.e. 22MG. For instance, 22MG-213 being sample number 213. Each sample was identified with two adhesive labels affixed, one on the falcon tube lid and the other on the falcon tube side, mentioning its code. After labeling, all the samples were verified for the presence of double labeling and the agreement between the sample codes and their status.

For each laboratory, the panel was composed of 16 falcon tubes of nematode suspension samples (6 containing *M. graminicola* from 2 populations, 3 samples per population, 3 containing *M.naasi*, 3 containing *M. oryzae , 3* containing *M. incognita* and 1 lure sample) randomly selected and then placed in a cardboard box with partitioning for falcon tubes, which was then placed in a box for dispatching. The laboratories were anonymously identified by a laboratory code.

Prior to the shipment, each panel was verified by an operator who ensured (I) the presence of all the samples constituting the panel, (II) the exactness of the codes in the sample codification table, (iii) the accordance between the laboratory code and the name of the participating laboratory.

Appendix 1 shows the sampling plan for the distribution of the test material to the participating laboratories. It enables the laboratories to decode their results.

3.1.4. Sample validation

Samples were validated in terms of status (assigned value), homogeneity and stability, to ensure that the test performance assessment was reliable.

3.1.4.1. Assigned value

The assigned value is the value assigned to a particular property of an entity under test performance testing.
The assigned value of samples results from the experimental work of the ILVO Nematology Group. It was defined independently of the participants' results. It is established as originating from a certified culture.
This value was confirmed during the homogeneity test (see 3.1.4.2) by repeated analyses of the samples conditioned in their final form. The analytical methods used to determine the assigned value were based on morphological and molecular identification of *Meloidogyne graminicola.*

3.1.4.2. Homogeneity

The homogeneity assessment was performed after the samples had been packed in their final format. The homogeneity study was performed on 10 falcons per production batch of samples with *Meloidogyne* sp., resulting in 50 samples. The analytical methods used to analyse the homogeneity study samples were based on morphology and on molecular identification tests (Htay test in combination with Mattos test, on 10 second-stage juveniles per sample). The homogeneity study demonstrated that all samples were sufficiently homogenous to validate the assigned values defined *a priori*, to all samples in the panels.

3.1.4.3. Stability

The stability study covered the period from the date of the homogeneity study to the deadline for the participating laboratories to perform the analysis. It concerned 4 samples per production batch, kept under the same storage conditions as the participating parcels and simulated transport conditions (storage at ambient temperature until receipt of the TPS parcels). Upon “receipt”, the samples were placed in a refrigerated room (12±2°C) until the stability analyses. The stability study was performed on 4 falcons per production batch of samples with *Meloidogyne* sp., resulting in 20 samples. The analytical methods used to analyse the stability study samples were based on morphology and described in ILVO/W03N08. The samples were analysed end of March 2023. The stability study was evaluated based on qualitative and quantitative results.
The stability study demonstrated that all the sample types concerned in this study were sufficiently stable and served as intended to evaluate the performance of the tests. Nevertheless, most nematodes in the suspensions had died during storage for 3 months at 12± 2°C.

3.2. Determining the Analytical Sensitivity

The analytical sensitivity of a test is the smallest amount of target that can be detected reliably (this is sometimes referred to as the ‘limit of detection’).
This analytical sensitivity evaluation was not done "blindly". Participants were asked, for each test, i.e. Htay and Bellafiore, to choose a sample identified as *M. graminicola* in the previous part of the TPS (see 3.1) and to determine the lowest amount of target needed for a positive result, by preparing DNA from 1, 2, 5 and 10 nematodes, in 3 replicates. As each laboratory used its routine method for DNA extraction, the robustness of the molecular test is being evaluated as well.

4. Practical implementation of the test performance study

4.1 Registration

The call for registration for participation in the test performance study 22MG was launched via e-mail on 6 December 2022, with a closing date for registration on 12 December 2022. Nine laboratories registered for the test performance study.

4.2 Shipment and receipt of the parcels

The proficiency test parcels were dispatched on 19 December 2022 (for 5 laboratories), on 9 January 2023 (for 3 laboratories) and on 16 January 2023 (for 1 laboratory). The shipments were postponed on demand of the participating labs. Shipment from ILVO (Merelbeke, Belgium) to the participant happened by courier.

4.3 Condition of samples

The samples were sent in a cardboard storage box for 15 ml falcon tubes, with partitions. This box was packed in a second cardboard box. The box contained 16 falcon tubes with nematodes suspended in water.
The boxes were shipped at ambient temperature. Any package or sample damaged due to transport had to be reported to the organiser within 24 hours after receipt. All participants stated that they had received the samples in good condition.

4.4 Delay for analysing the samples and submitting the results

The deadline for submitting the TPS results was 15 March 2023 for all participants. All participants respected this deadline except one laboratory **L03** which delivered the results on 31 March 2023. The laboratory notified the organiser before the deadline indicating difficulties to meet the deadline.

4.5 Implementation of the analyses

4.5.1. Identification of *M. graminicola*

All laboratories carried out the analyses in accordance with the organiser's instructions. Hereafter are the instructions that were communicated to the participating laboratories:
- The analyses must be performed on all samples received.
- The samples must be analysed under normal laboratory operating conditions (except for the molecular identification tests, which were specified by the organizer).
- Each sample contains only one species.

It is asked to indicate whether a sample contains *M. graminicola* or not, using the molecular identification tests mentioned in the "TPS Technical Instruction document".
Two conventional PCR methods are to be executed on the samples for the identification of the nematodes. However, as we have not found a species-specific primer set for *M. graminicola* among the published primers, these are to be combined with a third primer set to differentiate between *M. graminicola* and *M. oryzae*.

The following primer sets had to be ordered by the participating laboratory.

Table 6: Primer sequences

Publication	Primer name	Forward/Reverse	Sequence (5'→3')
Htay *et al.*, 2016	Mg-F3	Forward	TTATCGCATCATTTTATTTG
	Mg-R2	Reverse	CGCTTTGTTAGAAAATGACCCT
Bellafiore *et al.*,2015	SCAR-MgFW	Forward	GGGGAAGACATTTAATTGATGATCAAC
	SCAR-MgRev	Reverse	GGTACCGAAACTTAGGGAAAG
Mattos *et al.*, 2019	ORYA12F	Forward	CCAGCATCCGCTGTTGTAT
	ORYA12R	Reverse	AACAGGCTCCAGGTGAAAAG

As laboratories could apply their routine protocol for DNA extraction, details about their protocol were asked to be communicated to the organiser. Each participant was entitled to use the Taq-polymerase of its own choice (See Appendix 3).

The details on mastermix composition used during EURL validation and cycler conditions were communicated to the participants in the TPS technical instruction (Table 7).

Table 7: Mastermix composition, cycler conditions and amplicon size for Htay *et al.*,2016.

Ingredient	Stock concentration ingredient	**Concentration ingredient in the mastermix**	Volume(µl) 1 reaction
ddH2O	-	**-**	17,7
PCR buffer with $MgCl_2$	10x	**1x**	2,5
dNTPs	10mM	**200 µM**	0,5
Mg-F3	50µM	**2 µM**	1
Mg-R2	50µM	**2 µM**	1
Fast Start Taq DNA polymerase	5U/µl	**1.5U/25µl**	0,3
Template DNA			2
Total volume			25

- Cycler conditions for Htay *et al.*,2016 :
  95°C for 4 min
  35 cycles: 95°C for 30s, 51°C for 30s, 72°C for 30s
  final extension: 72°C for 5 min

- Amplicon size: 369 bp

Based on a previous validation performed by the EURL for Plant Parasitic Nematodes, the Htay primers can also be used in qPCR with Sybr-green fluorescence technology; melt curve with a single peak at 83,7°C

Table 8: Mastermix composition (adapted), cycler conditions and amplicon size for Bellafiore *et al.*, 2015.

Ingredient	Stock Concentration Ingredient	**Concentration ingredient in mastermix**	Volume (µl) for 1 reaction
ddH2O			19.05
10x PCR buffer with MgCl2	10x	**1x**	2.5
dNTPs	10mM	**200 µM each**	0.5
SCAR-MgFW	100µM	**0.5 µM**	0.125
SCAR-MgRev	100µM	**0.5µM**	0.125
FastSTartTaq DNA polymerase	5U/µl	**1U/25µl**	0.2
DNA			2.5
Total volume			25

- Cycler conditions for Bellafiore *et al.*,2015 :
  4 min at 95°C
  35 cycles : 30 s at 95°C, 30s at 60°C, 1 min at 72°C
  Final extension: 10 min at 72°C

- Amplicon size: 640 bp

Table 9: Mastermix composition (adapted), cycler conditions and amplicon size for Mattos *et al*., 2019:

Ingredient	Stock concentration Ingredient	**Concentration ingredient in mastermix**	Volume (µl) for 1 reaction
ddH2O			20.3
PCR reaction buffer with 20mM MgCl2	10x	**1x**	2.5
dNTPs	10mM	**200µM**	0.5
Primer FW : ORYA-12F	100µM	**1µM**	0.25
Primer Rev: ORYA-12R	100µM	**1µM**	0.25
FastStartTaqpolymerase	5U/µl	**1U/25µl**	0.2
DNA			1
Total Volume			25

- Cycler conditions for Mattos *et al*.,2019 :
  5 min at 95°C
  35 cycles : 30 s at 95°C, 45s at 56°C, 1 min at 72°C
  Final extension: 8 min at 72°C

- Amplicon size: 120bp

Participants were asked to test the nematodes in all samples using both PCRs: Htay and Bellafiore. In cases where an amplicon of the expected size was generated, a second PCR (Mattos) had to be executed to discriminate between *M. graminicola* and *M. oryzae*, knowing that *M. graminicola* does not amplify with these Mattos primers (based on EURL validation study).

4.5.2. Analytical sensitivity

In addition, for one sample (of the laboratories own choice) that had been identified as *M. graminicola*, the laboratory was asked to determine the sensitivity of both primer sets, i.e. Htay *et al*. and Bellafiore *et al*. by using DNA from 1, 2, 5 and 10 nematodes in 3 replicates, and to evaluate the amplification.

4.6 Transmission of the results

The participants were requested to record their results on the TPS Results entry form to standardise the presentation of the results.
Participants were invited to report any problems encountered during the TPS to the organiser via the results form.

5. Analysis of the results

5.1. Identification of *M. graminicola*

5.1.1. Statistical criteria used to interpret the results

The test performance was evaluated based on the qualitative results submitted by the participants. These results are available in **Appendices 2A and 2B**.
The results were interpreted for each test by calculating the number of positive agreements (PA), negative agreements (NA), positive deviations (PD) and negative deviations (ND), according to the following table.

Table 10: Definition of the parameters of positive agreement (PA), negative agreement (NA), positive deviation (PD) and negative deviation (ND).

Reference / Test	Positive assigned value	Negative assigned value
Positive test result	PA=positive agreement	PD= positive deviation
Negative test result	ND = negative deviation	NA = negative agreement

These parameters were used to calculate the following performance criteria:

Table 11: The definition and calculation of performance criteria were based on PM7/122 (2).

Performance criteria	Definition	Calculation*
Accuracy (AC)	Closeness of agreement between the test result and the assigned value (definition adapted from EN ISO 16140-1)	**AC= (sum PA + sum NA)/N x 100%**
Diagnostic Sensitivity (SE)	Closeness of agreement between the test result and the assigned value for samples for which the assigned value is positive (definition adapted from EN ISO 16140-1)	**SE = sum PA/N^+ x 100%** Note: the result of the calculation (1-SE) gives the number of false negatives obtained by the laboratory
Diagnostic Specificity (SP)	Closeness of agreement between the test result and the assigned value for samples for which the assigned value is negative (definition adapted from EN ISO 16140-1)	**SP = sum NA/N^- x 100%** Note: the result of the calculation (1-SE) gives the number of false positives obtained by the laboratory

* N = total number of samples; N^+ = number of positive samples; N^- = number of negative samples

5.1.2. Criteria for exclusion of data for analyses

In the homogeneity study, it was shown that the a priori assigned values to the samples were true. Results that deviated from the assigned value and where it was obvious that the deviation was caused by human error were excluded from the result analyses.

5.1.3. Assessment of test results

The criteria presented in paragraph 5.1.1, were applied to each laboratory and test.
Detailed results of PA, PD, NA and ND values are available in **Appendices 2A and 2B** for the Htay and Bellafiore tests, respectively. A summary, together with the percentages SE, SP and AC, are given in Tables 12 and 13, for Htay and Bellafiore tests, respectively.

As each laboratory was allowed to use its routine DNA extraction protocol, there was no limit to the number of nematodes that were being used for the DNA extraction in the identification test. Each sample contained plenty of nematodes, so each laboratory could use the number of nematodes normally being used for identification, in a certain volume, hence DNA concentration and quality (purity) differed from laboratory to laboratory. For details on the DNA extraction performed in each laboratory, see Appendix 3.

5.1.3.1. Identification of *Meloidogyne graminicola* with the combination of Htay and Mattos PCR results

Table 12: Evaluation of performance criteria for each laboratory concerning the detection and identification of *M. graminicola* with the **Htay and Mattos primers**.

Lab Code / Criteria	L01	L03	L06	L08	L09	L10	L11	L12	L13
PA Number	6	6	6	6	5	5	6	6	6
PD Number	0	0	0	0	0	1	0	0	0
NA Number	9	9	9	9	9	8	9	9	9
ND Number	0	0	0	0	1	1	0	0	0
Sensitivity (SE)	100%	100%	100%	100%	83%	83%	100%	100%	100%
Specificity (SP)	100%	100%	100%	100%	100%	89%	100%	100%	100%
Accuracy (AC)	100%	100%	100%	100%	93%	87%	100%	100%	100%

Laboratory L10 clearly switched two samples, i.e. sample 22MG-072 with assigned value "*M. graminicola*" and sample 22MG-052 with assigned value "*M. oryzae*". The laboratory scored sample 22MG-072 as being negative for *M. graminicola*, and sample 22MG-052 as being positive for *M. graminicola*. Therefore the results from laboratory L10 are not taken into account for the test performance evaluation. L10 is considered an outlier.

Laboratory L09 failed to identify one sample i.e. 22MG-137, as *M. graminicola*. As both tests (see Appendix 2A and 2B) failed to identify this sample as *M. graminicola*, in this laboratory, it is assumed that the DNA extraction of the sample was not optimal. L09 was not considered an outlier and was included in the evaluation of the performance of the tests.

The diagnostic sensitivity of the combination of the **Htay** test with the **Mattos** test is 100% in 7 out of 8 participating laboratories. The diagnostic specificity is 100% in all participating laboratories. The accuracy is 100% in 7 out of 8 participating laboratories.

By combining the results from the participating laboratories, it is the robustness of the combination of the tests that is being evaluated (different personnel, different chemicals and different equipment).

Diagnostic Sensitivity of the combination of the **Htay-Mattos tests**:

$\sum PA/\sum N^{+}$: (6+6+6+6+5+6+6+6)/(6+6+6+6+6+6+6+6) x 100 = 47/48 x 100 = **98%**

Diagnostic specificty of the combination of the **Htay-Mattos tests**:

$\sum NA/\sum N^{-}$: (9+9+9+9+9+9+9+9)/(9+9+9+9+9+9+9+9) x 100 = 72/72 x 100 = **100%**

Accuracy of the combination of the **Htay-Mattos tests**:

$(\sum NA + \sum PA) / (\sum N^{-} + \sum N^{+})$: (6+6+6+6+5+6+6+6) + (9+9+9+9+9+9+9+9)/(6+6+6+6+6+6+6+6) + (9+9+9+9+9+9+9+9) = 47+72/48+72= 119/120 = **99%**

5.1.3.2. Identification of *Meloidogyne graminicola* with the combination of Bellafiore and Mattos PCR results

Table 13: Evaluation of performance criteria for each laboratory concerning the detection and identification of *M. graminicola.* with the **Bellafiore and Mattos primers.**

Criteria \ Lab Code	L01	L03	L06	L08	L09	L10	L11	L12	L13
PA Number	6	/	6	6	5	5	6	6	6
PD Number	0	/	0	0	0	1	0	0	0
NA Number	9	/	9	9	9	8	9	9	9
ND Number	0	/	0	0	1	1	0	0	0
Sensitivity (SE)	100%	/	100%	100%	83%	83%	100%	100%	100%
Specificity (SP)	100%	/	100%	100%	100%	89%	100%	100%	100%
Accuracy (AC)	100%	/	100%	100%	93%	87%	100%	100%	100%

Similar to the above test, laboratory L10 clearly switched two samples, i.e. sample 22MG-072 with assigned value "*M. graminicola*" and sample 22MG-052 with assigned value "*M. oryzae*". The laboratory scored sample 22MG-072 as being negative for *M. graminicola*, and sample 22MG-052 as being positive for *M. graminicola*. Therefore the results from laboratory L10 are not taken into account for the test performance evaluation. L10 is considered an outlier.

Laboratory L09 failed to identify one sample, i.e. 22MG-137, as *M. graminicola*. As in this laboratory, both tests (see Appendix 2A and 2B) failed to identify this sample as *M. graminicola*, it is assumed that the DNA extraction of this sample was not optimal.

Laboratory L03 was not able to generate amplicons with the Bellafiore primers: a positive isolation control (PIC, from their own population) which was positive in the Htay test, did not generate any amplicon with the Bellafiore test, so results are inconclusive. The results of **L03** were not taken into account for the evaluation of the Bellafiore primers.

In summary, the diagnostic sensitivity of the combination of Bellafiore and Mattos was 100% in 6 out of 7 participating laboratories. The diagnostic specificity is 100% in all participating laboratories. The accuracy is 100% in 6 out of 7 participating laboratories.

By combining the results from the participants, the robustness of the combination of Bellafiore and Mattos was evaluated.

Diagnostic Sensitivity of the combination Bellafiore-Mattos:

$\sum PA/\sum N^{+}$: (6+6+6+5+6+6+6)/(6+6+6+6+6+6+6) x 100 = 41/42 x 100 = **98%**

Diagnostic specificty of the combination Bellafiore-Mattos:

$\sum NA/\sum N^{-}$: (9+9+9+9+9+9+9)/(9+9+9+9+9+9+9) x 100 = 63/63 x 100 = **100%**

Accuracy of the combination Bellafiore-Mattos:

$(\sum NA + \sum PA) / (\sum N^{-} + \sum N^{+})$: (6+6+6+5+6+6+6) + (9+9+9+9+9+9+9)/(6+6+6+6+6+6+6) + (9+9+9+9+9+9+9) = 41+63/42+63= 104/105 = **99%**

5.1.3.3. Conclusion concerning identificaton

Table 14: The result of both primers sets for identification of *M. graminicola*, in combination with the Mattos *M. oryzae* primers

Primer set*	Diagnostic Sensitivity	Specificity	Accuracy
Htay et al.	98%	100%	99%
Bellafiore et al.	98%	100%	99%

*Each primer set had to be combined with the Mattos *M. oryzae* primers to discriminate *M. graminicola* from *M. oryzae*

Both primer sets are able to identify *M.graminicola,* in combination with the second primer pair for *M. oryzae* from Mattos. Results of diagnostic sensitivity, specificity and accuracy are comparable for both primer pairs, i.e. Htay and Bellafiore.

The specificity towards other closely related nematodes from the "graminis group", other than *M. naasi* and *M. oryzae* , has not been evaluated in this TPS as it is not obvious to acquire material from these nematodes, but when identifying nematodes obtained from the field, it should always be kept in mind what other nematodes from the "graminis group" could be present in that region.

5.2. Determination of the analytical sensitivity

Participants were asked to determine the analytical sensitivity of both primer pairs, i.e. Htay and Bellafiore.
Therefore, the participants could chose a sample, identified in the identification part (see above), as being *M. graminicola*.
Laboratories were asked to extract DNA from 1, 2, 5 and 10 juveniles (J2), in 3 replicates and to perform PCR on these extracts for both primer pairs and to evaluate the amplification.

5.2.1. Analytical sensitivity of the Htay *M. graminicola* primers pair

Table 15: Evaluation of the amplification with the Htay primers for 1, 2, 5 and 10 nematodes, in 3 replicates.

Lab	Sample	1 nematode			2 nematodes			5 nematodes			10 nematodes		
		Rep.1	Rep. 2	Rep. 3	Rep.1	Rep.2	Rep. 3	Rep.1	Rep.2	Rep. 3	Rep.1	Rep.2	Rep. 3
~~L01~~	~~22MG-204 (pop.1)~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~
L03	22MG-089 (pop.2)	yes	no	no	yes	yes	yes	yes	yes	yes	yes	yes	yes
L06	22MG-259 (pop.2)	yes	yes	yes*	yes	yes	yes*	yes	yes	yes	yes	yes	yes
L08	22MG-085 (pop.2)	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
L09	22MG-023 (pop.2)	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
L10	22MG-034 (pop.1)	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
L11	22MG-196 (pop.2)	yes	no	no	yes	yes	no	yes	yes	yes	yes	yes	yes
L13	22MG-014 (pop.2)	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes

*: weak amplicon

L12 did not participate in this part of the test.
L01 could not generate any amplicon in this test, although in the diagnostic sensitivity part (identification analysis), DNA extracted from 10 nematodes resulted in amplification: for this reason, the results of L01 were not taken into account and considered outliers.

Analytical sensitivity for 1 nematode: amplicon in 17 out of 21 replicates: 81%
Analytical sensitivity for 2 nematodes: amplicon in 20 out of 21 replicates: 95%
Analytical sensitivity for 5 nematodes: amplification in all replicates (21 on 21): 100%
Analytical sensitivity for 10 nematodes: amplification in all replicates (21 on 21) 100%

5.2.2. Analytical sensitivity of the Bellafiore *M. graminicola* primer pair

Table 16: Evaluation of the amplification with the Bellafiore primers for 1, 2, 5 and 10 nematodes, in 3 replicates.

Lab	Sample	1 nematode			2 nematodes			5 nematodes			10 nematodes		
		Rep.1	Rep. 2	Rep. 3	Rep.1	Rep.2	Rep. 3	Rep.1	Rep.2	Rep. 3	Rep.1	Rep.2	Rep. 3
~~L01~~	~~22MG-204 (pop.1)~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~
~~L03~~	~~22MG-089 (pop.2)~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~	~~no~~
L06	22MG-259 (pop.2)	no	no	no	no	no	no	no	no	no	Yes*	Yes*	Yes*
L08	22MG-085 (pop.2)	no	no	no	yes	yes	no	yes	yes	yes	yes	yes	yes
L09	22MG-023 (pop.2)	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
L10	22MG-034 (pop.1)	no	no	no	no	no	no	no	no	no	yes	no	no
L11	22MG-196 (pop.2)	no	no	no	no	no	no	no	yes	no	yes	yes	yes
L13	22MG-014 (pop.2)	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes

*: weak amplicon

L12 did not participate in this part of the test.
L01 could not generate any amplicon in this test, although in the diagnostic sensitivity part (identification analysis) , DNA extracted from 10 nematodes resulted in amplification: for this reason, the results of L01 will not be taken into account and will be considered as outliers.
L03 was not able to generate amplicons with the Bellafiore primers. The results of **L03** were not taken into account for the evaluation of the Bellafiore primers.

Analytical sensitivity for 1 nematode: amplicon in 6 out of 18 replicates: 33%
Analytical sensitivity for 2 nematodes: amplicon in 8 out of 18 replicates: 44%
Analytical sensitivity for 5 nematodes: amplication in 10 out of 18 replicates: 56%
Analytical sensitivity for 10 nematodes: amplication in 16 out of 18 all replicates: 89%

5.2.3. Conclusion concerning analytical sensitivity

Table 17: Results for both primers sets on analytical sensitivity for *M. graminicola*.

Nr. of nematodes (J2)	Analytical Sensitivity (%)	
	Htay *M. graminicola* primers	Bellafiore *M. graminicola* primers
1	81	33
2	95	44
5	100	56
10	100	89

The primers from Htay *et al*. are more analytical sensitive than those from Bellafiore *et al*.: 1 nematode could generate an amplicon in 81% of cases for the Htay primers, compared with only 33% for Bellafiore primers. With 5 nematodes, there is always amplification for the Htay primers, while for the Bellafiore primers, even with 10 nematodes, there was not always amplification.

In general, the identification of *M. graminicola* using the Htay *et al*., 2016 test can be applied using 5 juveniles. However for Bellafiore *et al.,* 2015 test, the number of juveniles used for DNA extraction should be more than 10. The identification using the Htay primers is more robust, more laboratories were able to identify *M.graminicola* when the number of nematodes were limited.

5.3. Htay in qPCR

Although the Htay primers are published for use in conventional PCR, preliminary validation trials by the EURL showed that they could be used in qPCR as well, as mentioned in 4.5.1. Two laboratories, L12 and L13 tried the Htay primers in qPCR (SYBR green technology) in this TPS, although specifications on how to execute the qPCR were not given.
Lab L13 was very successful and showed a perfect linearity between the Ct and the log Concentration and showed that it was possible to discriminate between *M. graminicola* and the other nematode species in this test, based on the different melting temperatures (for *M. graminicola* 80.8°C) and also based on the Ct (for *M. graminicola* Ct <27).
Lab L12 could not generate any amplification. The reason for this is not clear, maybe it is due to the low concentrations of the primers that were being used: 0.4 µM compared to 2 µM during the try-out at theEURL ; the DNA amount was also different: 10,5µl on 25µl mastermix, compared to 2 µl on 20µl mastermix during the try-out at theEURL.

No other lab tried the Htay primers in qPCR.

5.4 Summary of the test results

5.4.1. Identification

Diagnostic sensitivity, specificity and accuracy

Both primer pairs i.e. Htay and Bellafiore, can identify *M. graminicola*, in combination with the Mattos primers. The diagnostic sensitivity, specificity and accuracy are equal for both primer pairs: 98, 100 and 99% respectively.
The diagnostic sensitivity was evaluated with two populations of *M. graminicola*, one from Italy and one from the Philippines.

5.4.2. Analytical sensitivity

When nematode numbers are limited, the Htay primers scored better than the Bellafiore primers.
No conclusions were made concerning the different populations, most participants used a sample belonging to the Philippine population (Batangas).

5.4.3. Robustness

Identification using the Htay primers is more robust: with small numbers of nematodes available (low DNA concentration), more laboratories, regardless of the DNA extraction method used, are able to identify *M. graminicola.*

5.4.4 qPCR with Htay primers

The results with the Htay primers in qPCR with SYBR green technology look very promising.
There was perfect linearity between the concentration and the Ct value, and it might be possible to discriminate species based on different melting temperatures. Further research is warranted.

6. Comments from the participating laboratories and remarks for improvement

L06 remarked that they observed a second weaker amplicon around 450-500 bp for the Htay primers.

L08 had to increase the amount of DNA (from 1 to 2.5µl) in the Mattos *M. oryzae* PCR mastermix to be able to generate amplicons.

L08 suggested including a positive control sample (DNA or nematodes).

L09 remarked that they observed a second amplicon (around) 700bp for the Htay primers in the case of *M. oryzae.*

L10 suggested that it would be better to have two separate sets of samples, one for each primer set, so that there is no bias possible between results for the tested primer pairs.

L10 also suggested to number the pages in the results form.

L11 remarked that the Mattos amplicons are not very strong. Sensitivity was not evaluated for the Mattos primers.

It would have been better to (own comments for improvements):

- have a more detailed results form allowing for reporting all the results from each primer pair and not only for the combination of the two primer pairs with the third primer pair.
- To ask to include the gel pictures.
- To specify whether replicates are biological or technical replicates.
- To have the analytical sensitivity of the Mattos *M. oryzae* primers determined.

7. Future validation and research

- Specificity: These primer pairs should be tested against more *Meloidogyne* species from the "*Meloidogyne graminis*" group, but these are not easy to obtain. It would also be interesting to test more than 2 populations of *M. graminicola.*

- Htay in qPCR:
  The primer pairs of Htay looked promising to apply in qPCR. One lab was successful and showed that this qPCR is specific when melting temperatures are considered. It would be interesting to look into this further. Future validation should focus on establishing the analytical sensitivity and the diagnostic specificity of these primers in qPCR.

- To develop a probe for *M. graminicola*, based on the sequence of the amplicon, obtained with the Htay primers, to differentiate *M. graminicola* from *M. oryzae* in qPCR, and possibly other *Meloidogyne* spp. from the "graminis" group.

- Some preliminary (EURL internal validation) tests were done with the He et al., 2021, Lamp primers and the results looked promising but have to be repeated and further validated.

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Appendices

Appendix 1: Sampling plan for the distribution of the samples to the participating laboratories

Laboratory Code		L01	L03	L06	L08	L09	L10	L11	L12	L13
Samples		Code	Code	Code	Code	Code	Code	Code	Code	Code
Sample 1	*M. graminicola* Italian population	22MG-180	22MG-100	22MG-066	22MG-127	22MG-008	22MG-034	22MG-068	22MG-144	22MG-162
Sample 2	*M. graminicola* Italian population	22MG-204	22MG-163	22MG-099	22MG-183	22MG-029	22MG-072	22MG-095	22MG-165	22MG-184
Sample 3	*M. graminicola* Italian population	22MG-217	22MG-223	22MG-186	22MG-241	22MG-137	22MG-154	22MG-168	22MG-201	22MG-209
Sample 4	*M. graminicola* Philippine population	22MG-121	22MG-089	22MG-135	22MG-010	22MG-023	22MG-074	22MG-053	22MG-050	22MG-014
Sample 5	*M. graminicola* Philippine population	22MG-221	22MG-097	22MG-151	22MG-085	22MG-225	22MG-175	22MG-196	22MG-102	22MG-125
Sample 6	*M. graminicola* Philippine population	22MG-267	22MG-129	22MG-214	22MG-118	22MG-239	22MG-219	22MG-249	22MG-103	22MG-132
Sample 7	*M. naasi*	22MG-200	22MG-022	22MG-251	22MG-192	22MG-039	22MG-017	22MG-160	22MG-041	22MG-205
Sample 8	*M. naasi*	22MG-262	22MG-098	22MG-260	22MG-210	22MG-164	22MG-084	22MG-236	22MG-138	22MG-218
Sample 9	*M. naasi*	22MG-270	22MG-153	22MG-271	22MG-227	22MG-174	22MG-131	22MG-253	22MG-275	22MG-237
Sample 10	*M. oryzae*	22MG-064	22MG-051	22MG-071	22MG-042	22MG-021	22MG-030	22MG-075	22MG-111	22MG-147
Sample 11	*M. oryzae*	22MG-142	22MG-130	22MG-155	22MG-136	22MG-148	22MG-052	22MG-232	22MG-126	22MG-172
Sample 12	*M. oryzae*	22MG-220	22MG-149	22MG-240	22MG-177	22MG-265	22MG-212	22MG-258	22MG-187	22MG-211
Sample 13	*M. incognita*	22MG-057	22MG-077	22MG-027	22MG-105	22MG-108	22MG-035	22MG-115	22MG-002	22MG-024
Sample 14	*M. incognita*	22MG-222	22MG-110	22MG-093	22MG-215	22MG-116	22MG-158	22MG-140	22MG-013	22MG-169
Sample 15	*M. incognita*	22MG-276	22MG-173	22MG-161	22MG-277	22MG-234	22MG-199	22MG-278	22MG-194	22MG-273
Sample 16	Lure	22MG-244	22MG-026	22MG-259	22MG-096	22MG-235	22MG-001	22MG-020	22MG-058	22MG-092

Appendix 2A: Descriptive analysis of the results submitted by the laboratories for the identification of *Meloidogyne graminicola,* using the combination Htay-Mattos (1/3)

Laboratory Code			L01			L03			L06			L08		
Samples		Assigned value	Code	Result	A/D	Code	Result	A/D	Code	Result	A/D	Code	Result	A/D
Sample 1	*M. graminicola* Italian population	**Positive**	22MG-180	Positive	PA	22MG-100	Positive	PA	22MG-066	Positive	PA	22MG-127	Positive	PA
Sample 2	*M. graminicola* Italian population	**Positive**	22MG-204	Positive	PA	22MG-163	Positive	PA	22MG-099	Positive	PA	22MG-183	Positive	PA
Sample 3	*M. graminicola* Italian population	**Positive**	22MG-217	Positive	PA	22MG-223	Positive	PA	22MG-186	Positive	PA	22MG-241	Positive	PA
Sample 4	*M. graminicola* Philippine population	**Positive**	22MG-121	Positive	PA	22MG-089	Positive	PA	22MG-135	Positive	PA	22MG-010	Positive	PA
Sample 5	*M. graminicola* Philippine population	**Positive**	22MG-221	Positive	PA	22MG-097	Positive	PA	22MG-151	Positive	PA	22MG-085	Positive	PA
Sample 6	*M. graminicola* Philippine population	**Positive**	22MG-267	Positive	PA	22MG-129	Positive	PA	22MG-214	Positive	PA	22MG-118	Positive	PA
Sample 7	*M. naasi*	**Negative**	22MG-200	Negative	NA	22MG-022	Negative	NA	22MG-251	Negative	NA	22MG-192	Negative	NA
Sample 8	*M. naasi*	**Negative**	22MG-262	Negative	NA	22MG-098	Negative	NA	22MG-260	Negative	NA	22MG-210	Negative	NA
Sample 9	*M. naasi*	**Negative**	22MG-270	Negative	NA	22MG-153	Negative	NA	22MG-271	Negative	NA	22MG-227	Negative	NA
Sample 10	*M. oryzae*	**Negative**	22MG-064	Negative	NA	22MG-051	Negative	NA	22MG-071	Negative	NA	22MG-042	Negative	NA
Sample 11	*M. oryzae*	**Negative**	22MG-142	Negative	NA	22MG-130	Negative	NA	22MG-155	Negative	NA	22MG-136	Negative	NA
Sample 12	*M. oryzae*	**Negative**	22MG-220	Negative	NA	22MG-149	Negative	NA	22MG-240	Negative	NA	22MG-177	Negative	NA
Sample 13	*M. incognita*	**Negative**	22MG-057	Negative	NA	22MG-077	Negative	NA	22MG-027	Negative	NA	22MG-105	Negative	NA
Sample 14	*M. incognita*	**Negative**	22MG-222	Negative	NA	22MG-110	Negative	NA	22MG-093	Negative	NA	22MG-215	Negative	NA
Sample 15	*M. incognita*	**Negative**	22MG-276	Negative	NA	22MG-173	Negative	NA	22MG-161	Negative	NA	22MG-277	Negative	NA
Sample 16	Lure	**Lure**	22MG-244	Negative	not evaluated	22MG-026	Positive	not evaluated	22MG-259	Positive	not evaluated	22MG-096	Negative	not evaluated

Key: PA = positive agreement, NA = negative agreement, PD = positive deviation, ND = negative deviation. The red font is used to indicate the discordant results (deviations).

Appendix 2A: Descriptive analysis of the results submitted by the laboratories for the identification of *Meloidogyne graminicola,* using the combination Htay-Mattos (2/3)

Laboratory Code			L09			L10			L11			L12		
Samples		Assigned value	Code	Result	A/D	Code	Result	A/D	Code	Result	A/D	Code	Result	A/D
Sample 1	*M. graminicola* Italian population	**Positive**	22MG-008	Positive	PA	22MG-034	Positive	PA	22MG-068	Positve	PA	22MG-144	Positive	PA
Sample 2	*M. graminicola* Italian population	**Positive**	22MG-029	Positive	PA	22MG-072	Negative	ND	22MG-095	Positve	PA	22MG-165	Positive	PA
Sample 3	*M. graminicola* Italian population	**Positive**	22MG-137	Negative	ND	22MG-154	Positive	PA	22MG-168	Positve	PA	22MG-201	Positive	PA
Sample 4	*M. graminicola* Philippine population	**Positive**	22MG-023	Positive	PA	22MG-074	Positive	PA	22MG-053	Positve	PA	22MG-050	Positive	PA
Sample 5	*M. graminicola* Philippine population	**Positive**	22MG-225	Positive	PA	22MG-175	Positive	PA	22MG-196	Positve	PA	22MG-102	Positive	PA
Sample 6	*M. graminicola* Philippine population	**Positive**	22MG-239	Positive	PA	22MG-219	Positive	PA	22MG-249	Positve	PA	22MG-103	Positive	PA
Sample 7	*M. naasi*	**Negative**	22MG-039	Negative	NA	22MG-017	Negative	NA	22MG-160	Negative	NA	22MG-041	Negative	NA
Sample 8	*M. naasi*	**Negative**	22MG-164	Negative	NA	22MG-084	Negative	NA	22MG-236	Negative	NA	22MG-138	Negative	NA
Sample 9	*M. naasi*	**Negative**	22MG-174	Negative	NA	22MG-131	Negative	NA	22MG-253	Negative	NA	22MG-275	Negative	NA
Sample 10	*M. oryzae*	**Negative**	22MG-021	Negative	NA	22MG-030	Negative	NA	22MG-075	Negative	NA	22MG-111	Negative	NA
Sample 11	*M. oryzae*	**Negative**	22MG-148	Negative	NA	22MG-052	Positive	PD	22MG-232	Negative	NA	22MG-126	Negative	NA
Sample 12	*M. oryzae*	**Negative**	22MG-265	Negative	NA	22MG-212	Negative	NA	22MG-258	Negative	NA	22MG-187	Negative	NA
Sample 13	*M. incognita*	**Negative**	22MG-108	Negative	NA	22MG-035	Negative	NA	22MG-115	Negative	NA	22MG-002	Negative	NA
Sample 14	*M. incognita*	**Negative**	22MG-116	Negative	NA	22MG-158	Negative	NA	22MG-140	Negative	NA	22MG-013	Negative	NA
Sample 15	*M. incognita*	**Negative**	22MG-234	Negative	NA	22MG-199	Negative	NA	22MG-278	Negative	NA	22MG-194	Negative	NA
Sample 16	Lure	**Lure**	22MG-235	Positive	not evaluated	22MG-001	Negative	not evaluated	22MG-020	Negative	not evaluated	22MG-058	Negative	not evaluated

Key: PA = positive agreement, NA = negative agreement, PD = positive deviation, ND = negative deviation. The red font is used to indicate the discordant results (deviations).

Appendix 2A: Descriptive analysis of the results submitted by the laboratories for the identification of *Meloidogyne graminicola,* using the combination Htay-Mattos (3/3)

Laboratory Code			L13		
Samples		Assigned value	Code	Result	A/D
Sample 1	*M. graminicola* Italian population	**Positive**	22MG-162	Positive	PA
Sample 2	M. graminicola Italian population	**Positive**	22MG-184	Positive	PA
Sample 3	*M. graminicola* Italian population	**Positive**	22MG-209	Positive	PA
Sample 4	*M. graminicola* Philippine population	**Positive**	22MG-014	Positive	PA
Sample 5	*M. graminicola* Philippine population	**Positive**	22MG-125	Positive	PA
Sample 6	*M. graminicola* Philippine population	**Positive**	22MG-132	Positive	PA
Sample 7	*M. naasi*	**Negative**	22MG-205	Negative	NA
Sample 8	*M. naasi*	**Negative**	22MG-218	Negative	NA
Sample 9	*M. naasi*	**Negative**	22MG-237	Negative	NA
Sample 10	*M. oryzae*	**Negative**	22MG-147	Negative	NA
Sample 11	*M. oryzae*	**Negative**	22MG-172	Negative	NA
Sample 12	*M. oryzae*	**Negative**	22MG-211	Negative	NA
Sample 13	*M. incognita*	**Negative**	22MG-024	Negative	NA
Sample 14	*M. incognita*	**Negative**	22MG-169	Negative	NA
Sample 15	*M. incognita*	**Negative**	22MG-273	Negative	NA
Sample 16	Lure	**Lure**	22MG-092	Negative	not evaluated

Key: PA = positive agreement, NA = negative agreement, PD = positive deviation, ND = negative deviation. The red font is used to indicate the discordant results (deviations).

Appendix 2B: Descriptive analysis of the results submitted by the laboratories for the identification of *Meloidogyne graminicola, using the combination Bellafiore-Mattos* (1/3)

Laboratory Code			L01			L03			L06			L08		
Samples		Assigned value	Code	Result	A/D	Code	Result	A/D	Code	Result	A/D	Code	Result	A/D
Sample 1	*M. graminicola* Italian population	**Positive**	22MG-180	Positive	PA	22MG-100	Negative	ND	22MG-066	Positive	PA	22MG-127	Positive	PA
Sample 2	*M. graminicola* Italian population	**Positive**	22MG-204	Positive	PA	22MG-163	Negative	ND	22MG-099	Positive	PA	22MG-183	Positive	PA
Sample 3	*M. graminicola* Italian population	**Positive**	22MG-217	Positive	PA	22MG-223	Negative	ND	22MG-186	Positive	PA	22MG-241	Positive	PA
Sample 4	*M. graminicola* Philippine population	**Positive**	22MG-121	Positive	PA	22MG-089	Negative	ND	22MG-135	Positive	PA	22MG-010	Positive	PA
Sample 5	*M. graminicola* Philippine population	**Positive**	22MG-221	Positive	PA	22MG-097	Negative	ND	22MG-151	Positive	PA	22MG-085	Positive	PA
Sample 6	*M. graminicola* Philippine population	**Positive**	22MG-267	Positive	PA	22MG-129	Negative	ND	22MG-214	Positive	PA	22MG-118	Positive	PA
Sample 7	*M. naasi*	**Negative**	22MG-200	Negative	NA	22MG-022	Negative	NA	22MG-251	Negative	NA	22MG-192	Negative	NA
Sample 8	*M. naasi*	**Negative**	22MG-262	Negative	NA	22MG-098	Negative	NA	22MG-260	Negative	NA	22MG-210	Negative	NA
Sample 9	*M. naasi*	**Negative**	22MG-270	Negative	NA	22MG-153	Negative	NA	22MG-271	Negative	NA	22MG-227	Negative	NA
Sample 10	*M. oryzae*	**Negative**	22MG-064	Negative	NA	22MG-051	Negative	NA	22MG-071	Negative	NA	22MG-042	Negative	NA
Sample 11	*M. oryzae*	**Negative**	22MG-142	Negative	NA	22MG-130	Negative	NA	22MG-155	Negative	NA	22MG-136	Negative	NA
Sample 12	*M. oryzae*	**Negative**	22MG-220	Negative	NA	22MG-149	Negative	NA	22MG-240	Negative	NA	22MG-177	Negative	NA
Sample 13	*M. incognita*	**Negative**	22MG-057	Negative	NA	22MG-077	Negative	NA	22MG-027	Negative	NA	22MG-105	Negative	NA
Sample 14	*M. incognita*	**Negative**	22MG-222	Negative	NA	22MG-110	Negative	NA	22MG-093	Negative	NA	22MG-215	Negative	NA
Sample 15	*M. incognita*	**Negative**	22MG-276	Negative	NA	22MG-173	Negative	NA	22MG-161	Negative	NA	22MG-277	Negative	NA
Sample 16	Lure	**Lure**	22MG-244	Negative	not evaluated	22MG-026	Negative	not evaluated	22MG-259	Positive	not evaluated	22MG-096	Negative	not evaluated

Key: PA = positive agreement, NA = negative agreement, PD = positive deviation, ND = negative deviation. The red font is used to indicate the discordant results (deviations).

Appendix 2B: Descriptive analysis of the results submitted by the laboratories for the identification of *Meloidogyne graminicola, using the combination Bellafiore-Mattos* (2/3)

Laboratory Code			L09			L10			L11			L12		
Samples		Assigned value	Code	Result	A/D	Code	Result	A/D	Code	Result	A/D	Code	Result	A/D
Sample 1	*M. graminicola* Italian population	**Positive**	22MG-008	Positive	PA	22MG-034	Positive	PA	22MG-068	Positive	PA	22MG-144	Positive	PA
Sample 2	*M. graminicola* Italian population	**Positive**	22MG-029	Positive	PA	22MG-072	Negative	ND	22MG-095	Positive	PA	22MG-165	Positive	PA
Sample 3	*M. graminicola* Italian population	**Positive**	22MG-137	Negative	ND	22MG-154	Positive	PA	22MG-168	Positive	PA	22MG-201	Positive	PA
Sample 4	*M. graminicola* Philippine population	**Positive**	22MG-023	Positive	PA	22MG-074	Positive	PA	22MG-053	Positive	PA	22MG-050	Positive	PA
Sample 5	*M. graminicola* Philippine population	**Positive**	22MG-225	Positive	PA	22MG-175	Positive	PA	22MG-196	Positive	PA	22MG-102	Positive	PA
Sample 6	*M. graminicola* Philippine population	**Positive**	22MG-239	Positive	PA	22MG-219	Positive	PA	22MG-249	Positive	PA	22MG-103	Positive	PA
Sample 7	*M. naasi*	**Negative**	22MG-039	Negative	NA	22MG-017	Negative	NA	22MG-160	Negative	NA	22MG-041	Negative	NA
Sample 8	*M. naasi*	**Negative**	22MG-164	Negative	NA	22MG-084	Negative	NA	22MG-236	Negative	NA	22MG-138	Negative	NA
Sample 9	*M. naasi*	**Negative**	22MG-174	Negative	NA	22MG-131	Negative	NA	22MG-253	Negative	NA	22MG-275	Negative	NA
Sample 10	*M. oryzae*	**Negative**	22MG-021	Negative	NA	22MG-030	Negative	NA	22MG-075	Negative	NA	22MG-111	Negative	NA
Sample 11	*M. oryzae*	**Negative**	22MG-148	Negative	NA	22MG-052	Positive	PD	22MG-232	Negative	NA	22MG-126	Negative	NA
Sample 12	*M. oryzae*	**Negative**	22MG-265	Negative	NA	22MG-212	Negative	NA	22MG-258	Negative	NA	22MG-187	Negative	NA
Sample 13	*M. incognita*	**Negative**	22MG-108	Negative	NA	22MG-035	Negative	NA	22MG-115	Negative	NA	22MG-002	Negative	NA
Sample 14	*M. incognita*	**Negative**	22MG-116	Negative	NA	22MG-158	Negative	NA	22MG-140	Negative	NA	22MG-013	Negative	NA
Sample 15	*M. incognita*	**Negative**	22MG-234	Negative	NA	22MG-199	Negative	NA	22MG-278	Negative	NA	22MG-194	Negative	NA
Sample 16	Lure	**Lure**	22MG-235	Positive	not evaluated	22MG-001	Negative	not evaluated	22MG-020	Negative	not evaluated	22MG-058	Negative	not evaluated

Key: PA = positive agreement, NA = negative agreement, PD = positive deviation, ND = negative deviation. The red font is used to indicate the discordant results (deviations).

Appendix 2B: Descriptive analysis of the results submitted by the laboratories for the identification of *Meloidogyne graminicola, using the combination Bellafiore-Mattos* (3/3)

Laboratory Code			L13		
Samples		Assigned value	Code	Result	A/D
Sample 1	*M. graminicola* Italian population	**Positive**	22MG-162	Positive	PA
Sample 2	*M. graminicola* Italian population	**Positive**	22MG-184	Positive	PA
Sample 3	*M. graminicola* Italian population	**Positive**	22MG-209	Positive	PA
Sample 4	*M. graminicola* Philippine population	**Positive**	22MG-014	Positive	PA
Sample 5	*M. graminicola* Philippine population	**Positive**	22MG-125	Positive	PA
Sample 6	*M. graminicola* Philippine population	**Positive**	22MG-132	Positive	PA
Sample 7	*M. naasi*	**Negative**	22MG-205	Negative	NA
Sample 8	*M. naasi*	**Negative**	22MG-218	Negative	NA
Sample 9	*M. naasi*	**Negative**	22MG-237	Negative	NA
Sample 10	*M. oryzae*	**Negative**	22MG-147	Negative	NA
Sample 11	*M. oryzae*	**Negative**	22MG-172	Negative	NA
Sample 12	*M. oryzae*	**Negative**	22MG-211	Negative	NA
Sample 13	*M. incognita*	**Negative**	22MG-024	Negative	NA
Sample 14	*M. incognita*	**Negative**	22MG-169	Negative	NA
Sample 15	*M. incognita*	**Negative**	22MG-273	Negative	NA
Sample 16	Lure	**Lure**	22MG-092	Negative	not evaluated

Key: PA = positive agreement, NA = negative agreement, PD = positive deviation, ND = negative deviation. The red font is used to indicate the discordant results (deviations).

Appendix 3: Details of the methodologies used per participating laboratory

Laboratory	DNA extraction		Polymerase	Thermal cycler and other equipment	Amplicon Detection
	# nematodes in identification test	extraction method			
L01	10 (Fixated with TAF)	kit	Qiagen Taq DNA polymerase 5U/µl	Applied Biosystems 2720	gel stained with Olerup SSPGelRedTM and UV illumination
L03	10, in duplicate	Crude DNA extract	FastStart Taq DNA polymerase (Roche, 5U/µl)	C1000 Touch Thermal Cycler Bio-Rad	not specified
L06	15 nematodes	Kit: Eluted in 20 µl for 1 and 2 nematodes , in 30µl for 5 nematodes and in 40µl for 10 and 15 nematodes	Promega GoTaq G2 Flexi DNA Polymerase	Appled Biosystems GeneAmp PCR system 9700	not specified
L08	15 nematodes	Kit, eluted in 100µl elution buffer provided with the kit, independent of nr. of nematodes	Promega GoTaq Hot Start Green Master mix	MJ Research PTC-200 Applied Biosystems Veriti	not specified
L09	20 nematodes	Kit: elution volume 50µl	HS DreamTaq PCR Master Mix (2x) as manufacturer instruction (ThermoFisher Catalog number K1071)	Applied BioSystem 2720	capillary electrophoresis gel using TapeStation 4200 Instrument
L10	10 nematodes, in duplicate	Lysis buffer, in 50µl	FastStart Taq DNA polymerase	Sensoquest labcycler	gel
L11	10 nematodes in duplicate	Lysis buffer, in 100µl	MP Biomedicals Taq DNA polymerase	Biometra TAdvanced Applied Biosystems 2720 Thermal Cycler	gel
L12	total of the sample	Kit, in 100 µl	GoTaq G2 Flexi DNA Polymerase (Promega)	Veriti 96-well Fast Thermal Cycler (Applied Biosystems)	not specified
L13	1,2,5 and 10 from each sample	kit	Supreme NZYTaq II DNA polymerase (Nzytech)only add primers , DNA and water	Biometra Tgradient thermocycler	gel (VersaDoc Gel imaging System, Bio-Rad)

End of the report