European Union Reference Laboratory

for Plant Parasitic Nematodes

Diagnostic protocols and analytical methods

REFERENCE: EURL – MeloRT-PCR-Soil Version 01

August/ 2020

Detection of *Meloidogyne chitwoodi* and *M. fallax* on soil extracts by Real-Time PCR

Foreword

This method is recommended by:

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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 this EURL recommended protocol[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 direct detection and identification of *M. chitwoodi* and *M. fallax* via real-time PCR.

This protocol is 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.

1.1 Purpose and scope

This method describes the different steps required for the direct detection and identification of *M. chitwoodi* and *M. fallax* from the soil extracts using the real-time PCR technique.

The method is applicable to soil extracts.

1.2 Principle

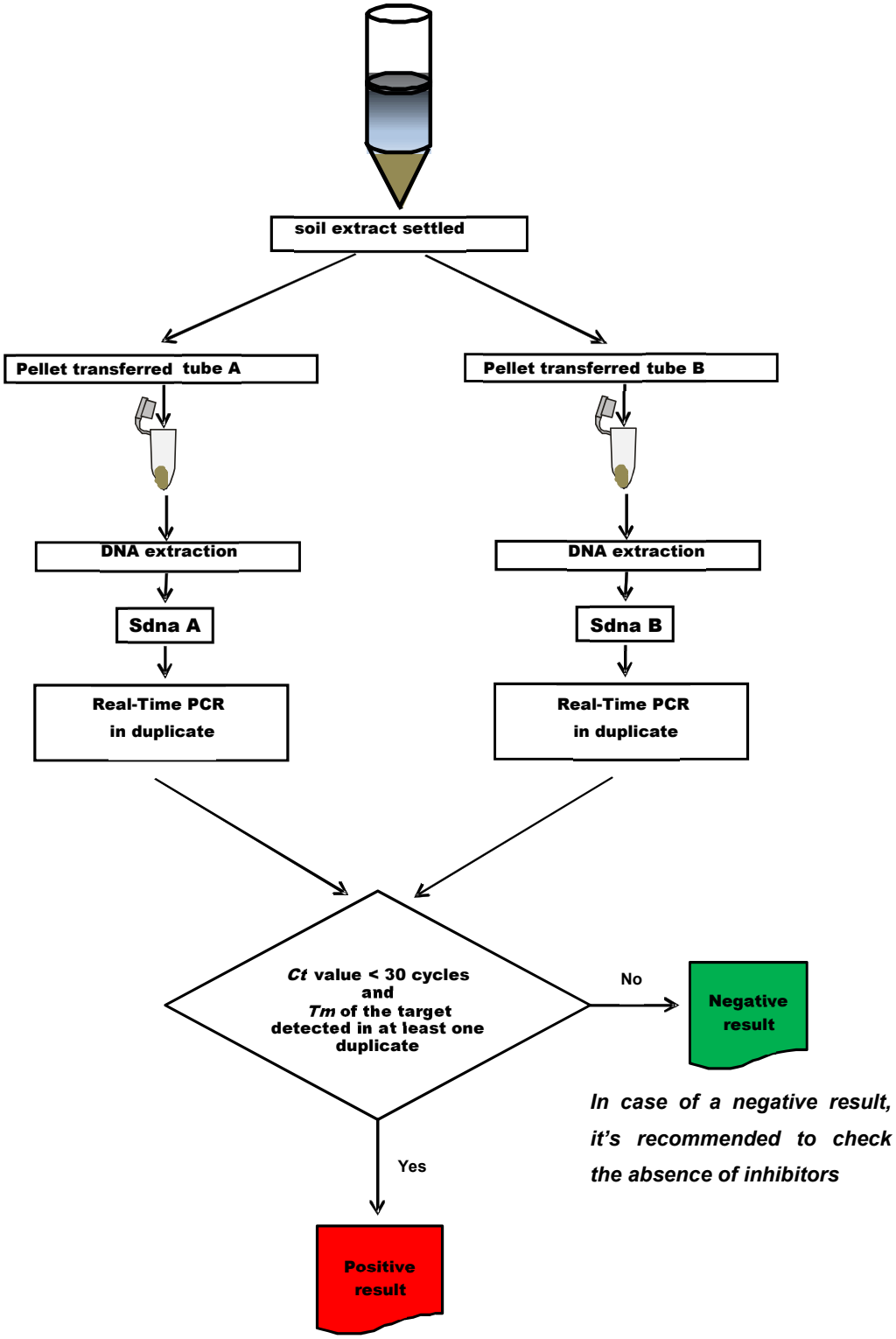
This method relies on the direct detection and identification of *Meloidogyne chitwoodi* and *M. fallax* from soil extracts by direct DNA extraction and specific amplicon amplification using a real-time PCR test.

The present method was developed and validated by ANSES - Plant Health Laboratory-Nematology Unit and is based on real-time PCR tools developed by BLGG AgroXpertus - Binnenhaven 5 - 6709 PD Wageningen, (The Netherlands). The validation was performed with the reagent brands and solution preparations indicated in this protocol. However, laboratory procedures and commercial brands presented in this protocol may be adjusted to laboratory standards provided they are properly validated.

The detection threshold determined during validation is on 5 juveniles (*M.chitwoodi* or *M.fallax*) per soil extract, in 100% of cases.

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

Schematic procedure for direct detection of *M.chitwoodi* and *M. fallax* on soil extract



In case of a negative result, it's recommended to check the absence of inhibitors

2 Material and consumables

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 specific steps of the analysis:

- Oscillating tissue grinder for approximately 2 mL microtubes (e.g., Tissulyser, Qiagen®) or equivalent equipment
- Real-time PCR thermal cycler
- A processor of magnetic particles (= automate Thermo Electron KingFisher® mL). This equipment allows the simultaneous processing of 15 samples
    - ***Note:*** *Others Thermo Scientific KingFisher can be used.*

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
- **DNA extraction kit**: Kit Wizard® Magnetic DNA Purification System for Food (Promega, ref. FF3750 et FF3751) - DNA extraction is performed using the KingFisher® mL automated system
- **Commercial Diagnostic kits**: ClearDetection's Real-Time PCR diagnostic kits :
    - RT-ND-1305 for *Meloidogyne chitwoodi*
    - RT-ND-1309 for *Meloidogyne fallax*

https://www.cleardetections.com/index.php/service/nematode-real-time-pcr-diagnostic-kits/

[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. Any commercial Kit can be used as long as suitability and performance have been demonstrated, or have been used in an in-lab validation study.

Commercial kits have been validated on Roche Real-time PCR thermal cycler (LightCycler 480 Roche, 96 plates). When using another Real-time PCR thermal cycler, the laboratory will have to validate the obtaining of an equivalent sensitivity (5 juveniles detected, in 100% of cases) and determine the *Tm* (Temperature melting) of each target.

- **Primers:**

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*

*Clear®Detections, Binnenhaven 5, 6709PD Wageningen, The Netherlands or website contact: http://www.cleardetections.com/en

- **Disposable consumables:**
  - Adapted microtubes and glass or metal beads (e.g. 1 mm and 3 mm in diameter)
  - Use pipette tips with filter plug during manipulation of Real-time PCR reactions.
  - Plastic consumables for automated KingFisher: tube bars and magnet bar protectors
  - PCR Plates

3 Controls and their purpose

Soil extracts free from *Meloidogyne chitwoodi,* and *M. fallax* must be included during the analytical process to validate the different steps of the method and the entire process. Controls must be included during the molecular process to validate the different steps of the test. The following controls are mandatory to check the correct performance of the DNA extraction and real-time PCR steps:

Controls	Use	Type and Purpose
Negative process control (NPC)	Mandatory for each sample series	• Soil extract free from the targets • This control verifies that no cross-contamination between samples occurs during this analytical step.
Positive process control (PPC)	Mandatory for each sample series	• Soil extract (or plant extract) contaminated with *M. chitwoodi* and *M. fallax* • DNA extraction is performed under the same conditions as samples; this control allows verifying the absence of any flaw during the analytical step
Positive PCR control (PC)	Mandatory for each sample series	• Contains all the elements of the PCR reaction, including a DNA extract from the target; this control verifies that the PCR reaction has proceeded correctly and has allowed amplification of the samples containing the target.
Negative PCR control NTC (No template control)	Mandatory for each sample series	• Contains all the elements of the PCR reaction but no DNA is added; this control verify the absence of cross-contamination during the PCR reaction

Additionally, to these controls, it is highly recommended to check the negative results and the absence of inhibitors. This verification can be done, for example :

- Using an Internal Amplification Control (IAC) (could be punctually provided by the LRUE);
    - ***Note:*** *The IAC construction was based on Langrell, 2002 publication. If more information or the specific protocol for this control, please send an email to* *eurl.nematodes@anses.fr*
- Including in each negative result a DNA target, and perform another PCR;
- Performing another PCR with universal primers

4 DNA extraction on soil extracts

After nematode extraction from soil samples **(see EURL – MeloExtraction** §Extraction from soil samples**),** the soil extracts are settled and conditioned for DNA extraction, as detailed below.

4.1 Preparation of soil extracts

1) Transfer the soil extract (approx. 10 to 20 ml) into a conical bottom tube
2) Allow settling for at least 3 hours at room temperature
    - **Note:** *The extract may be placed at 4°C for a more extended period, but it should not exceed 7 days.*
3) Pipette the bottom of the decanted extract (≈3 ml), and divide the 3 ml into 2 microtubes 1.5 ml
4) Centrifuge of about 10 min at 12000 g
5) Gently remove the supernatant retaining the pellet
    - **Note:** *Following this step, tubes containing the pellet may be frozen (< -18°C) before proceeding with further analysis.*

4.2 DNA extraction

DNA extraction is performed with the Kit Wizard® Magnetic DNA Purification System for Food (Promega, réf. FF3750 et FF3751). Therefore all reagents cited below belong to the Kit.

DNA extraction is performed using the KingFisher® mL automated system.

Add to the frozen pellet:

1) 300 µl of Lysis Buffer A
2) 150µl of Lysis Buffer B
3) Glass or metal beads (e.g. 2 beads of 3 mm and a few beads of 1mm)
4) Place the microtubes on a tissue grinder rack; shake at frequency 30 Hz for 40 sec
5) Incubate for at least 10 min at room temperature
6) Add 450 µl of precipitation solution (blue solution) and vortex
7) Centrifuge about 10 min at 12000 g
    - ***Note:*** *The Magnesil™ PMPs solution must be shaken to be well homogenised before use.*
8) Depending on the number of samples to be processed, place the required number of tube strips on the tray of the machine; fit the protective covers for the magnetic strips of the machine
9) For each tube strip, distribute:
    - Position A, 30 µl of Magnesil™ PMPs and 350 µl of isopropanol
    - Position B, 250µl of Lysis Buffer B
    - Positions C et D, 1 ml of 70% ethanol

10) Retrieve the centrifuged microtubes; for each sample, transfer 400 µl of the supernatant (DNA solution) into the first tube (position A) placed on the tray of the machine (already containing 30µl of MagnesilTM PMPs and 350µl of isopropanol)

11) Position the tube tray in the machine (make sure that the magnet bar protectors are securely in place) and start the "Promega food 65 C" program

- ***Note:*** *Program script in appendix* ***§8***

12) At the end of the drying step, remove the tray and dispense 100 µl of water at 65°C (Biomol grade) into the E position of each tube strip

13) Re-position the tube tray in the machine and restart the program

14) At the end of the extraction process, remove the tray and collect the eluate

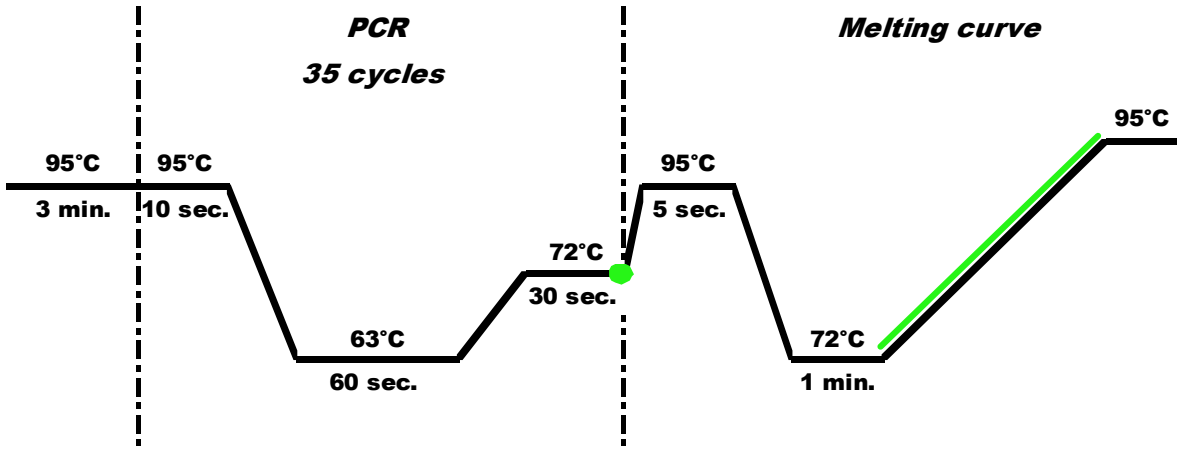
- ***Note:*** *the eluate is the DNA solution (S_{DNA}) that will be directly analysed by the real-time PCR. The eluted DNA can be stored at 4°C if used within 24 hours or in the freezer (<-10°C) for later use*

5 DNA amplification via Real-Time PCR

As the detection tests are simplex, two separate PCRs are performed with each primers mix for each nematode target **(see §2.2)**.

Users have to follow the provider's recommendations of the commercial diagnostic kits **(see §2.2)** and in particular instructions for the preparation of the PCR mix, and real-time PCR amplification program.

All S_{DNA} have to be tested in duplicate.



- ***Note:*** *The SYBR Green fluorescence is measured punctually at the end of each cycle and continuously during the melting curve (symbolised in green on the program above).*

In case of using the combination of Roche Real-time PCR thermal cycler (LightCycler 480 Roche, 96 plates) and Cleardetection's Real-Time PCR diagnostic kits, the expected *Tm* values for the targets are indicated in table 1. For another combination, the mean value of *Tm* should be defined through an assessment by the lab.

Table 1. Mean values of *Tm*

	mean values *Tm*
M. chitwoodi	84.2 (± 1°C)
M. fallax	84.5 (± 1°C)

6 Results of the validation

The results are obtained by an automatic analysis of LightCycler® 480 Software without manual intervention to set the threshold or background line. A quantitative analysis is used to determine the cycle threshold (*Ct*), and the melting curve analysis is used to determine the *Tm* (the temperature at which 50% of the DNA is presented at single-strand) of the amplified products.

Analysis validation 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:

- All replicates of PC, generate an amplicon: *Ct* value < 30 and a *Tm* value in accordance with the requirements.
- None of the NTC replicates generate an amplicon with a *Ct* value < 30 and a *Tm* value corresponding to the desired target.

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.

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: samples results should be interpreted as follows for the target organism under investigation:

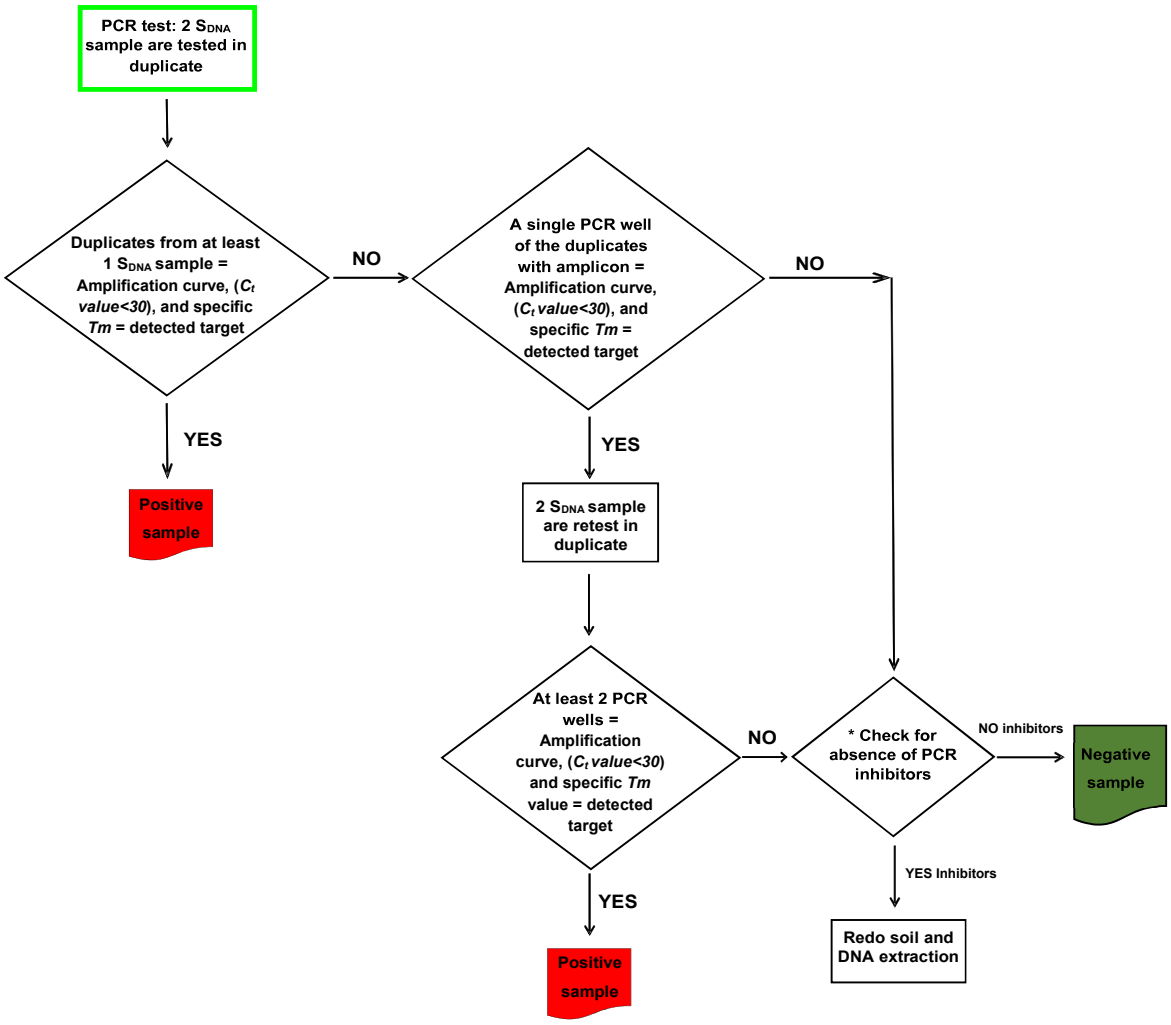
S_{DNA} n° 1		S_{DNA} n° 2		**Sample Results**
Well 1	Well 2	Well 1	Well 2	
+	+	+	+	Positive Test
+	-	+	+	Positive Test
-	-	+	+	Positive Test
+	-	+	-	PCR is retest. If at least 2 out of 4 are positive, the result is interpreted as positive.
+	-	-	-	PCR is retest. If at least 2 out of 4 are positive, the result is interpreted as positive
-	-	-	-	Negative Test

+: observation of an exponential amplification curve with a value of *Ct* value < 30 with a *Tm* value corresponding to the desired target.

- : the absence of an exponential amplification curve with a value of *Ct* value < 30 or observation of an exponential amplification curve with a value of *Ct* value < 30 but, with a *Tm* value different from that of the desired target.

The overview of the result analysis is illustrated by the decision flowchart below:

Schematic procedure for results analysis



*Checking the absence of PCR inhibitors is a control that is highly recommended by the EURL.

7 Reporting results

The final result of the analysis is expressed in qualitative form as follows and according to the test carried out:

"**Detection of *Meloidogyne chitwoodi* by real-time PCR**", or "**Detection of *Meloidogyne fallax* by real-time PCR**":

- "**Negative test**" when the analysis result of the test sample is negative for the target nematode.
- "**Positive test**" when the analysis result of the test sample for analysis is positive for the target nematode.

If you have any question about this method 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.

8 Appendix

Automate programme script

Name = promega food 65C
Protocol template version = 2.6.0
Instrument type = KingFisher mL
Creator = LNPV LNN
Created = 27/8/2009 14:58:19
Description = surnageant 400µl + 350 isopro + 30 billes
Kit = PROMEGA FOOD for soil sols
Plate layouts = Default

PLATE LAYOUTS

DEFAULT

Plate type = KingFisher tubestrip 1000 ul Plate change message = Change Default

A:
- volume = 400, name = surnageant lysat centrifugé
- volume = 350, name = ISOPROPANOL 0.8 V
- volume = 30, name = Magnesil

B:
- volume = 250, name = lavage Lysis buffer B

C:
- volume = 1000, name = Lavage EtOH 70

D:
- volume = 1000, name = Lavage EtOh 70

E:
- volume = 100, name = Eau BM à 65 C°

STEPS

BIND

Step parameters
- Name = Binding
- Well = A, Default

Beginning of step:
- Premix = Yes

Bind parameters:
- Bind time = 5min 0s, speed = Fast dual mix
- **End of step :**Collect beads = Yes, count=3

WASH
Step parameters
- Name = Wash 1 lysis buffer
- Well = B, Default

Beginning of step:
- Release = Yes, time = 10s, speed = Fast

Wash parameters:
- Wash time = 2min 0s, speed = Fast dual mix

End of step:
- Collect beads = Yes, count = 3

WASH

Step parameters

- Name = Wash 2 ethanol
- Well = C, Default

Beginning of step:

- Release = Yes, time = 10s, speed = Fast

Wash parameters:

- Wash time = 2min 0s, speed = Fast dual mix

End of step:

- Collect beads = Yes, count = 3

WASH

Step parameters

- Name = Wash 3 ethanol
- Well = D, Default

Beginning of step:

- Release = Yes, time = 10s, speed = Fast

Wash parameters:

- Wash time = 2min 0s, speed = Fast dual mix

End of step:

- Collect beads = Yes, count = 3

DRY

Step parameters

- Name = Dry
- Well = D, Default
- Dry time = 10min 0s
- Tip position = Outside well

PAUSE

Step parameters

- Name = Pause
- Well = D, Default
- Message = Ajout 100 eau BM 65C
- Dispense:Elution buffer, volume=ul

ELUTION

Step parameters

- Name = Elution
- Well = E, Default

Beginning of step:

- Release = Yes, time = 10s, speed = Fast

Elution parameters:

- Elution time = 2min 0s, speed = Fast

Pause parameters:

- Pause for manual handling = No

Remove beads:

- Remove beads = Yes, collect count = 3, disposal well = B

9 References

EPPO (2016). PM 7/41 (3) *Meloidogyne chitwoodi and Meloidogyne fallax*. *EPPO Bulletin*/*Bulletin OEPP 46*(2), 171–189. https://doi.org/10.1111/epp.12292

Ward, L. I., Beales, P. A., Barnes, A. V. & Lane, C. R. (2004). A real-time PCR assay based method for routine diagnosis of *Spongospora subterranea* on potato tubers. J. *Phytopathology* 152, 633–638.

Zijlstra C., & Van Hoof R. A. (2006). A multiplex Real-Time Polymerase Chain Reaction (Taqman) assay for the simultaneous detection of *Meloidogyne chitwoodi* and *M. fallax*. *Phytopathology* Vol.96, 1255-1262.

Langrell, S. R. H. (2002). Molecular detection of Neonectria galligena (syn. Nectria galligena). *Mycological Research*, *106*(3), 280–292. https://doi.org/10.1017/S095375620200552X