European Union Reference Laboratory

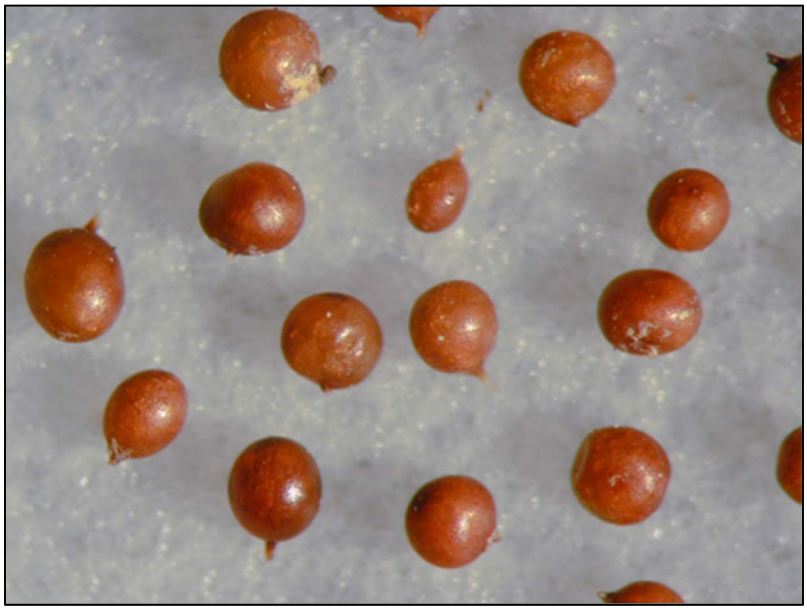
for Plant Parasitic Nematodes

EURL diagnostic protocol

REFERENCE: EURL–Globo-Extraction (GE)_Version 01

October/ 2021

Protocol for extraction and isolation of *Globodera cysts*



Foreword

These methods are recommended by:

ANSES - Plant Health Laboratory - Nematology Unit

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

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The consortium between both laboratories is designated the

European Union Reference Laboratory (EURL) for Plant Parasitic Nematodes

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Contents

1 Introduction

The potato cyst nematodes, *Globodera rostochiensis* and *Globodera pallida* are plant-parasitic nematodes (PPNS) that cause significant losses in potato (*Solanum tuberosum*) crops. Given the damage caused by these nematodes, they are regulated by a majority of countries in the world (EPPO 2017) and classified as quarantine organisms in the European Union (listed in Appendix II, Part B, of the Commission Implementing Regulation (EU) 2019/2072) to avoid their introduction into and spread within the EU territory.

The application of this protocol is part of the monitoring to follow and assess the spread of these pests in Europe according to European Council Directive 2007/33/EC of 11 June 2007 under revision.

The EURL recommended protocols are based on literature reviews and IPPC and EPPO standards, when applicable. These extraction methods, including operational procedures, were chosen as the basis, and with the scientific experience and technical expertise of the EURL team, some of these methods have been adapted, optimised and further validated by the EURL laboratory.

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 extraction of *Globodera* cysts.

The material linked to this diagnostic protocol, such as PowerPoint presentations and technical videos & media, can be found on the EURL Plant Parasitic Nematodes website.

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. All actions taken in accordance with this method must be performed by employees who have attended relevant training.

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

2 Terms, abbreviations and definitions

- Cysts: the sclerotised cuticle of the deceased *Globodera-, Heterodera-* and *Punctodera* females, usually filled with eggs and juveniles
- Container: bowl, beaker, vial, tube, pot, jar, device, etc
- Extraction: separating nematodes, and more specifically cysts from soil and underground plant organs
- Extract: cysts + organic material collected with the extraction process
- EPPO: European and Mediterranean Plant Protection Organization
- IPPC: International Plant Protection Convention
- L/h: litres per hour
- ML/min: millilitres per minute
- NRLs: National Reference Laboratories
- PPN(s): Plant-Parasitic Nematodes

3 Purpose and scope

The purpose of this document is to describe a EURL diagnostic protocol for the extraction of potato cysts nematodes from soil and underground plant organs and their isolation from the debris. These extraction methods also extract the cyst forms of other Heteroderinae genera, particularly *Globodera, Heterodera* and *Punctodera*. A protocol to determine cyst viability is described in the protocol EURL-GV_version 01).

This protocol is suitable for the extraction of *Globodera* species in their cyst form. However, the choice of the method depends, among other variables, on the purpose of the extraction, equipment available, and preferences of the person performing the extraction.

These extraction methods are applied to the following matrices:

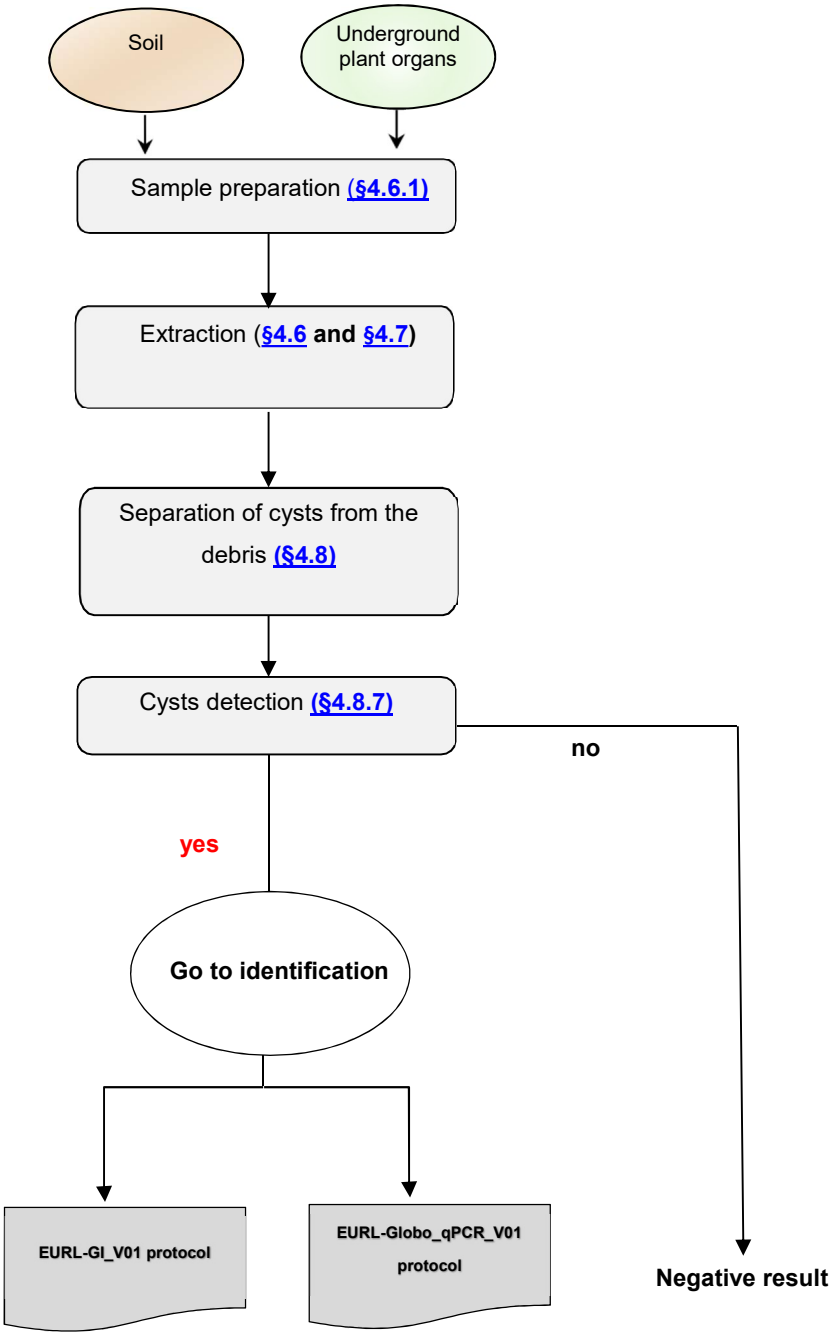
- any soil or soil product (excluding organic substrates such as peat, coconut fibres, potting soil)
- underground plant organs such as bulbs, rhizomes, tubers and roots.

4 Cyst extraction methods

4.1 Principle

The cysts are separated from the other sample fractions (soil or organic particles) by differences in the rate of settling in water due to their density (less than 1 gram/cm^3 for dry cysts, slightly more than 1 gram/cm^3 for wet cysts), their shape and size (between 250 and 600 µm).

The following flowchart represents the different steps of this protocol:



4.2 Homogenising samples

1) The items reused when preparing the samples, such as extraction equipment: spoon, sieves, etc., are thoroughly rinsed before starting sample extraction and between samples.
2) Homogenising: the sample must be thoroughly mixed before a sub-sample is taken. Sample homogenisation can be done either in a plastic bag or in a mixing container.
3) Plastic bag or mixing container: Make sure that the volume is at least 4 times larger than the sample.

4.3 Controls and their purpose

These controls are added to evaluate if the cyst extraction process was correctly performed and serves as a control for further morphometric and molecular identification analysis.

<table>
<tr><th>Extraction Control</th><th colspan="2">Purpose</th><th>Control application</th><th>Expected Results</th></tr>
<tr><td>Negative process control*</td><td colspan="2">When analysing a series of samples or between samples, water is considered a negative control. The water undergoes the same operations as a sample. However, the material (e.i. sieves, etc.) is also changed between a series of samples.</td><td>Mandatory</td><td>No cysts</td></tr>
<tr><td rowspan="3">Positive process control**</td><td rowspan="3">When possible, with each series of samples to be analysed, one sample from the same matrix infested with Globodera cysts is also analysed.</td><td>When analysing potato tubers for Globodera cysts, one infected tuber is treated like the rest of the samples.</td><td>Optional</td><td>Presence of Globodera cysts</td></tr>
<tr><td>Use positive infected soil samples.
✓ In the absence of a positive soil sample, Globodera cysts from culture may be added to a negative soil sample to simulate a positive soil sample.</td><td>Optional</td><td>Presence of Globodera cysts</td></tr>
<tr><td>Use positive infected root samples.
✓ In the absence of a positive root sample, Globodera cysts from culture may be added to a negative root sample to simulate a positive root sample.</td><td>Optional</td><td>Presence of Globodera cysts</td></tr>
</table>

*During the cysts extraction process, the samples are in the following order: samples, positive control, followed by the negative control.
**The *Globodera* cysts are detected on the positive control before starting the identification analysis of the remaining samples. In this way, one is sure that the cysts were extracted from the samples. The presence of other cysts and/or nematodes in the samples is an additional guarantee for the extraction.

4.4 General requirements

1) Between sample reception and the start of the analysis, soil samples are preferably stored at cool temperatures, at most room temperature. High temperatures can influence the content of the cysts, including juveniles which are necessary for species identification.
2) Extracted cysts can be stored in a cold, dry place before and after analysis.
3) Ensure that sieves are in good condition.
4) **Important**: Sieves and extraction equipment must be cleaned with the utmost care after each use and between samples.
5) With extraction equipment, the initiation of an extraction cycle ("blank extraction") between samples is mandatory to reduce the risk of contamination.
6) The sieves used for cyst recovery and sample preparation are cleaned in the following way:
    a. Rinse sieves thoroughly so that soil and/or organic material is removed. A clean sieve is used for each sample. You can clean the sieves using a dishwasher or an ultrasonic bath for 10 minutes to remove sand grains and nematodes, avoiding cross-contamination between samples.
    b. 250-µm sieves should be checked regularly using a stereomicroscope for cysts that are stuck between the wires
    c. **Important:** If using the ultrasonic bath, it is advised to change the bathwater between samples.

4.5 Material and consumables

4.5.1 Equipment

- Equipment for drying soil samples (e.g. heated and/or ventilated chamber etc.)
- Proper safety equipment should be in place when working with toxic products see **§4.8**
- Extraction equipment[2] for soil samples:
    - ✓ Fenwick can
    - ✓ Schuiling centrifuge
    - ✓ Kort elutriator
    - ✓ Seinhorst elutriator
- Stereomicroscope with episcopic illumination

[2] The use of other extraction devices is possible provided that they are recognised or verified as having at least equivalent performance to the equipment recommended in this protocol.

4.5.2 Consumables and small materials

For extraction:

- Brush or other appropriate instruments suitable for handling cysts
- Petri dishes
- Beakers, bowls and containers
- Sieves
  - ✓ 250 µm sieves for cyst recovery
  - ✓ 4 mm sieves (sample preparation)
  - ✓ specific sieves integrated into the extractors (mesh 1 to 2 mm),
  - ✓ 800 µm sieve (optional for sample preparation).
- Filter paper (19 cm diameter is being used in the EURL)

For cleaning cysts from debris (see §4.8):

- Glass beaker (option 2)
- Detergent (option 2)
- Strips of white blotting paper (option 2)
- Acetone (recovery and preparation of the extract according to option 3)
- 500 ml Erlenmeyer conical flask (option 3 and 4)
- Round filter MN 615 Ø 185mm (option 4)
- Ethanol 99% denatured (recovery and preparation of the extract according to option 4)

4.6 Cyst extraction from soil

In the interval between sample reception and the start of the analysis, soils are kept at cold positive temperatures (fridges, cold rooms such as basements or similar). Higher temperatures are prohibited to avoid alteration of the juvenile's content of the cysts, which is necessary for species identification and/or if viability tests are envisaged, as this might also influence the viability (i.e. hatching behaviour).

4.6.1 Soil sample preparation

1) Manually remove large plant debris and stones, disintegrate clods and homogenise by manual stirring. If necessary, pass over a 4-mm sieve (for dry samples only) and remove large elements.
2) The maximum quantity of soil that can be analysed by each extraction equipment described below may vary according to the type of soil and the characteristics of the equipment used.
   - ***Note:*** *If the volume of the sample is greater than the maximum analysable quantity of the extraction equipment, the sample is divided into volumes analysable for each equipment and passed through the extractor several times until the sample is fully analysed.*
3) When the sample consists of clay soil, it is soaked in tap water during a period of a minimum of 8 and a maximum of 24 hours. One can add two tablespoons of sodium oxalate (approximately 12 g) to improve the dispersion of the clay particles.
4) Samples should be dried prior to extraction when using the Fenwick can or the Schuiling centrifuge. The sample can be left to dry at room temperature or higher but not exceeding 35 °C. The soil can be crumbled to speed up the drying process and facilitate subsequent sieving. Drying soil is not required for the Seinhorst and Kort elutriator.

4.6.2 Fenwick can

This method is based on the floating characteristic of dried cysts and the size difference compared to other fractions of the soil sample.

The Fenwick can is a can tapering at the top with a sloped base. The can has a sloping collar below the rim. The dried soil sample is washed through a sieve into the can in which water is constantly flowing. Heavy soil particles fall to the bottom of the can, whereas dried cysts and light soil debris float to the surface, pass over the rim into the collar and end up on a collecting sieve (Fenwick, 1940).

1) **Required:** dried soil sample.
2) **Volume of sample to analyse**: Up to 250 mL
3) The can is filled to the rim with water.
4) The dried soil is carried by a water jet through the upper sieve with a 1 to 2 mm mesh into the can.
5) The cysts and light soil debris float and are dragged by overflowing water into the collection collar, under which a 250 µm sieve is placed.
6) The water supply is maintained until the sample is exhausted and clear water overflows.
7) Collect the extract on the 250 µm sieve and rinse with a water jet to remove the finest soil particles.

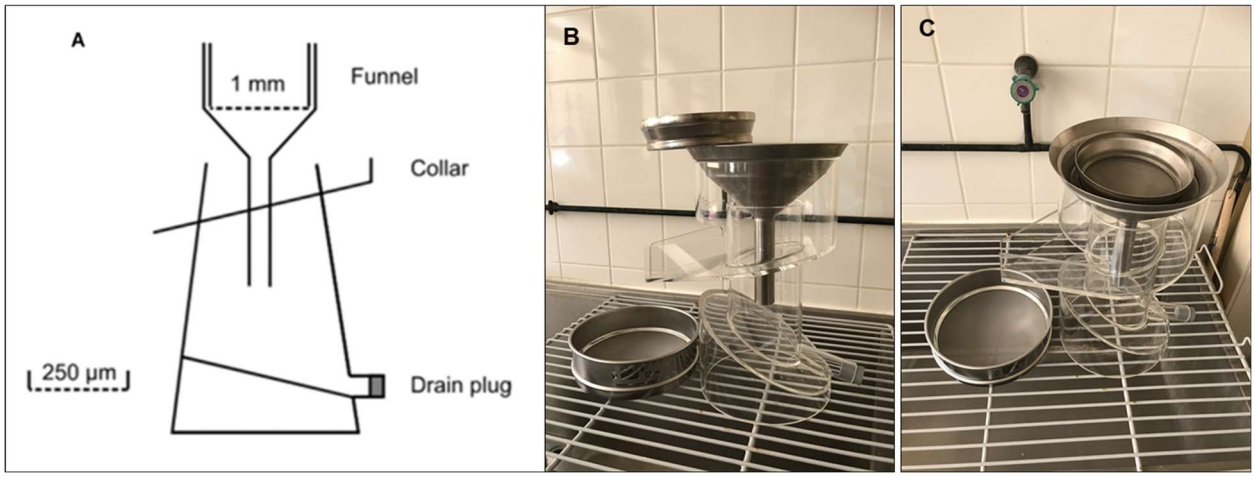


Figure 1. (A) Vertical-section diagram of Fenwick can. **(B)** Acrylic Fenwick can with a 1 mm top sieve and a 250 µm bottom sieve, **(C)** top view of the Fenwick (Diagram: adapted from EPPO pm7/119; Picture: ANSES-LSV, France).

4.6.3 Schuiling centrifuge

This cysts extraction device uses a semi-automatic flotation method described by Turner (1998) and is also based on flotation proprieties of dried cysts and size differences between soil sample fractions.

1) **Required:** dried soil sample.
2) **Volume of sample to analyse**: Approximately 400 mL of dry soil (or approximately 500 g) maximum.
3) The dried soil is poured into the transparent cylinder (between the wall and the central sieve), partly filled with water. The contents are swirled with a rotating two-pronged fork at high speed, creating a vortex.
4) The lighter elements (cysts, debris and organic particles) float and are forced to the center and filtered through a wire-mesh (1 to 2 mm) cylinder.
5) Collect the extract on a 250 µm sieve and rinse with a water jet to remove the finest particles.

Figure 2. Schuiling centrifuge to extract cysts from soil samples at ANSES-LSV (Picture ANSES-LSV, France).

4.6.4 Kort and Seinhorst elutriators

These methods are based on the differences in density between cysts and soil particles.

The fixed upward water current in the Seinhorst elutriator ensures the cysts float while the soil particles sink. The cysts flush through a collar onto a sieve with a mesh size smaller than the diameter of the cysts. According to Seinhorst (1964), the light (semi) empty cysts and the heavy full cysts remain in the upper half of the elutriator. The device, therefore, has a side outlet so that the contents of the upper half of the elutriator can also flow onto the sieve, catching cysts that do not float as well as empty or dry cysts.

1) The technique is suitable for moist or dry soils.
2) **Volume of sample to analyse**:
    - The maximum amount of soil that can be analysed by the semi-automated Seinhorst (Manufacturer: Meku) (Figure 3) is approximately 200 mL.
    - The maximum amount of soil that can be analysed by manual Seinhorst elutriator (Figure 4A and B) is up to 500 mL for the standard model (Seinhorst, 1964).
    - The maximum amount of soil that can be analysed by the Kort (Figure 5) depends on the device's size.
3) The elutriators are equipped with a flow meter so that the upward water current water can be adjusted:
    - Semi-automated Seinhorst: pressure of **~ 2 bars**
    - Manual Seinhorst: water flow of **~ 3.5L/min**
    - Kort: water flow of **~ 4L/min**
4) The soil sample is added to the device over an upper sieve (1 to 2 mm) using a water jet (Seinhorst or Kort). Take care that the sample is only added when a certain water level in the device is reached.
5) Cysts with a density less than 1 (dry cysts) or slightly more than 1 (wet cysts) float or are carried along by the rising water flow. Cysts are then overflowed into the collection ring, under which a 250 µm sieve is placed.
6) The water flow is maintained for at least 2 min (manual devices) until clear water overflow is reached.
7) Open the second outlet (manual Seinhorst elutriator).
8) Close the upward water current for manual devices.
9) Rinse and collect the collected product in the recovery 250 µm sieve.

Figure 3. Semi-automated Seinhorst Meku Elutriator to extract cysts from soil samples at ANSES-LSV (Picture ANSES-LSV, France).

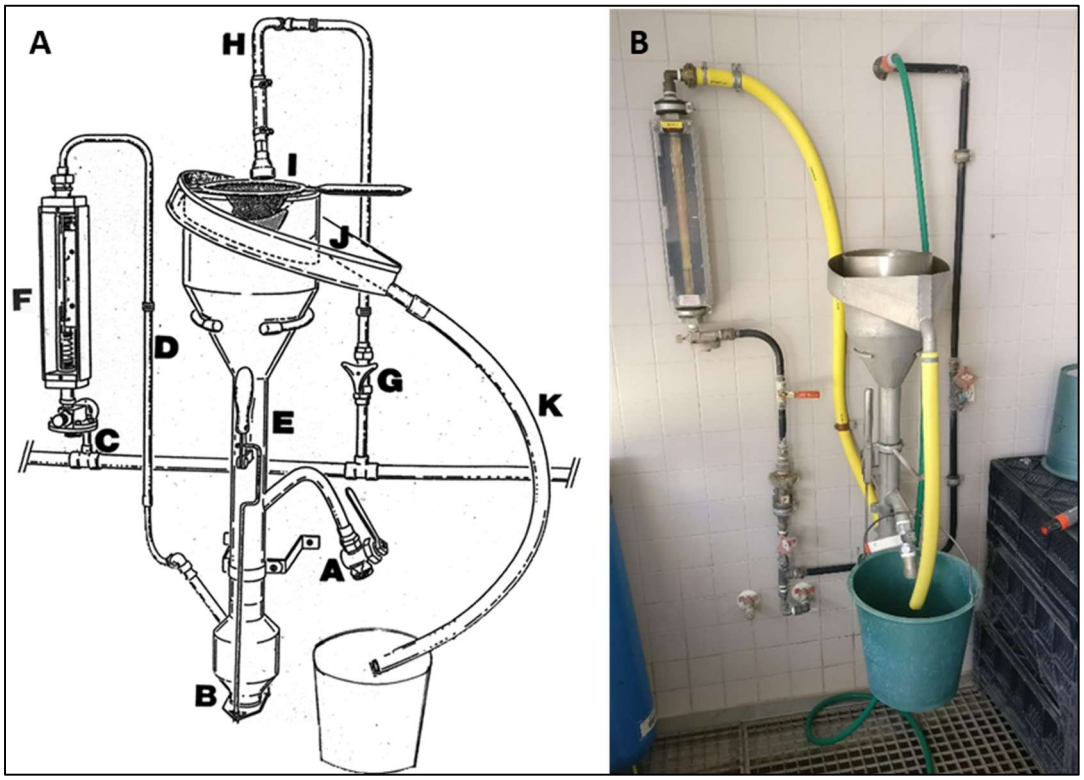


Figure 4. **(A)** Seinhorst elutriator diagram: Aside outlet, B bottom valve, C flowmeter supply valve, D upward current supply hose, E open/close bottom valve, F flowmeter, G flushing hose supply valve, H flushing hose, I sieve (+/- 2mm), J collar, K collar hose (Seinhorst, 1964). **(B)** Manual Seinhorst elutriator at ILVO (Picture ILVO, Belgium).

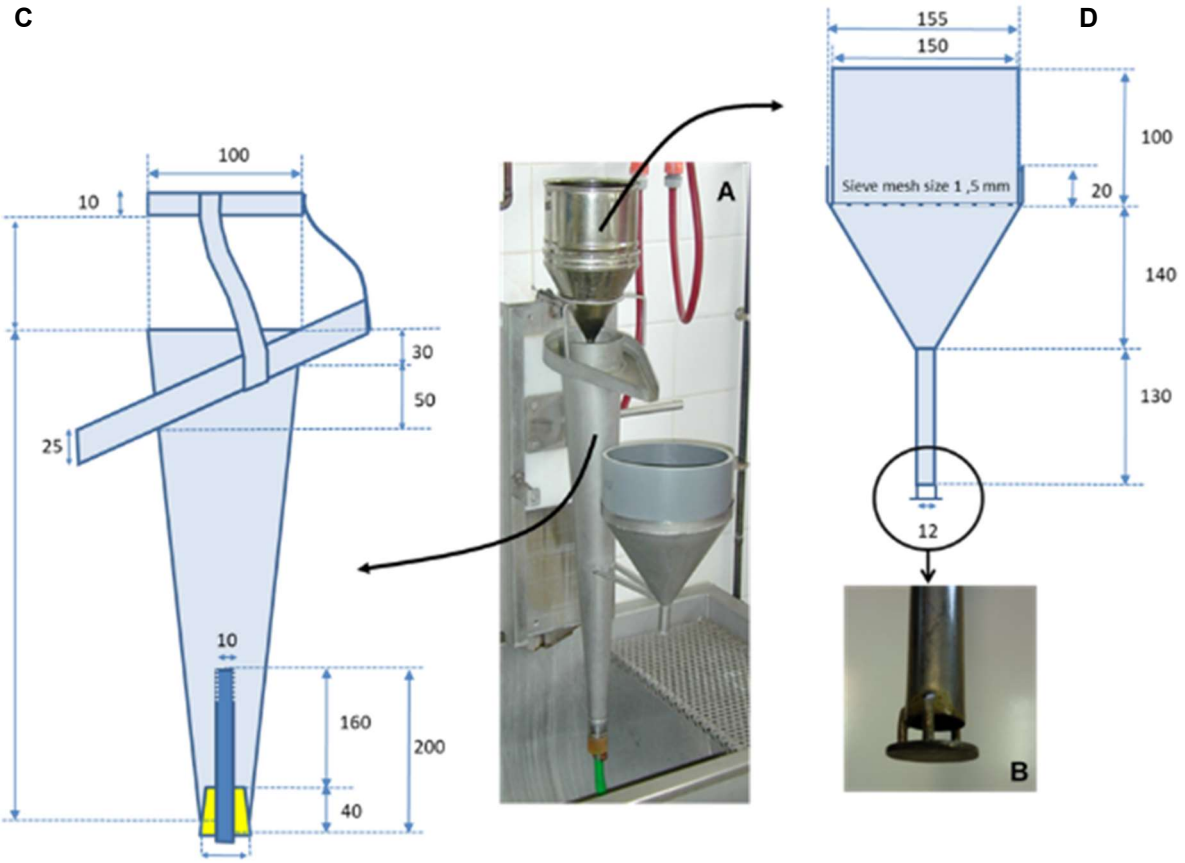


Figure 5. Left **(C):** a drawing showing the elutriator Kort column, and the right drawing **(D)** demonstrates the funnel to support the sieve to place the soil samples. The dimensions are in millimetres. **(A)** Photo of the elutriator kort at ANSES-LSV. **(B)** Photo of the bottom of the funnel, which allows a better dispersion of the soil sample in the water column (Picture ANSES-LSV, France).

4.7 Cyst extraction from underground plant organs

This method is applied to bulbs, tubers, rhizomes, tuberous roots and non-tuberous roots.

In the interval between the reception of the sample and the start of the analysis, the perishable underground plant organs are kept at a temperature (~ 4°C) that guarantees their preservation and to avoid alteration of the juvenile's content of the cysts, which is necessary for species identification.

1) In water, wash the sample by soaking and brushing and/or spraying with a strong jet of water. This can be done in any container/bowl of sufficient volume.
2) Collect the rinsed soil + organic materials, and water in a bowl.
3) Transfer the bowl contents onto two sieves, one above the other. The top one has a mesh size of 850 µm (or 1 mm mesh size) and the bottom with 250 µm.
4) Rinse the bowl thoroughly and pour the contents on top of the two sieves.
5) Carefully spray on the 850 µm sieve with the water jet to push any residual cysts between the organic materials through the sieve.
6) The content of the 850 µm sieve may be discarded.
7) Spray carefully on the 250 µm sieve to distribute the organic material and the cysts evenly onto the surface of the sieve.

4.8 Separation of cysts from the debris

4.8.1 Drying of cysts

1) Either leave the extract on the collecting sieve to dry, or transfer the extract (cysts and organic debris) collected in the last sieving on a filter paper using water (water bottle)
2) Allow the extract to dry in the open air or an incubator at a maximum of 25°C. Be careful that (dry) cysts are not blown away by air currents such as ventilation systems.
   - ***Note**: The cysts can be picked out immediately from the wet extract, without drying, for example, when the extract has little organic matter.*

The following operational procedures describe the cleaning of cysts from other particles, mostly organic debris extracted and captured on the 250 µm-sieve. This treatment is carried out when there is too much debris in the extract and facilitates cysts' isolation.

4.8.2 Option 0: dry separation of cysts and debris

The dried extract is rubbed on the collecting sieve using a clean flat brush. Small debris will pass through the sieve, but cysts and debris > 250 µm remain. The remaining mixture is then transferred and distributed on a tray made of wood and in which equidistant shallow ridges are made. Cysts are then counted and/or picked out ridge by ridge under a stereomicroscope.

4.8.3 Option 1: Cleaning with Arvo can

The extract is passed through the Arvo can (Figure 6) in the following situations:

1) If the extract has been previously dried as indicated in **§4.8.1**
2) Immediately after the extract is obtained from the devices that used dry soil at the beginning of the extraction

For using the arvo can:

1) Open the water supply tap, check the pressure of the water inlet (about 0.5 bar)
2) Rinse the inside of the column with a jet in the upper position
3) Place a 250 µm sieve or the strips blotting paper at the outlet of the Arvo can and recuperate the extract

Figure 6. Arvo can[3] equipment to clean the extract obtained with the Schuiling centrifuge.

4.8.4 Option 2: Cyst separation via the paper strip method

1) A strip of blotting paper is placed on the inside of the vertical walls of a wide, short glass beaker (type crystalliser beaker, Figure 7), with the upper part slightly higher than the rim.
2) Fill the beaker with water to about three-quarters of its volume.
3) Gently crush the extract, rub off the elements adhering to the filter paper, then pour into the beaker.
   - ***Note:*** *When the extract is rich in large particles (seeds, root fragments, for example), it can be passed over an 800 µm sieve placed on the beaker to improve the separation of the cysts.*
4) The suspension is gently stirred (e.g. with the help of a spatula or spoon) to disperse the elements of the extract and then left to stand until the floating particles are immobilised (Figure 7A).
5) Apply a drop of detergent in the centre of the beaker to disperse the floating particles towards the blotting paper strip.
6) The paper strip is removed from the beaker by turning in one direction and placed on a suitable support (e.g. a board) to pick out the cysts (Figure 7B and C).

[3] Arvo can : The device was bought from the company Fa A Volkers & Sons in The Netherlands; http://volzonpotatoes.nl/

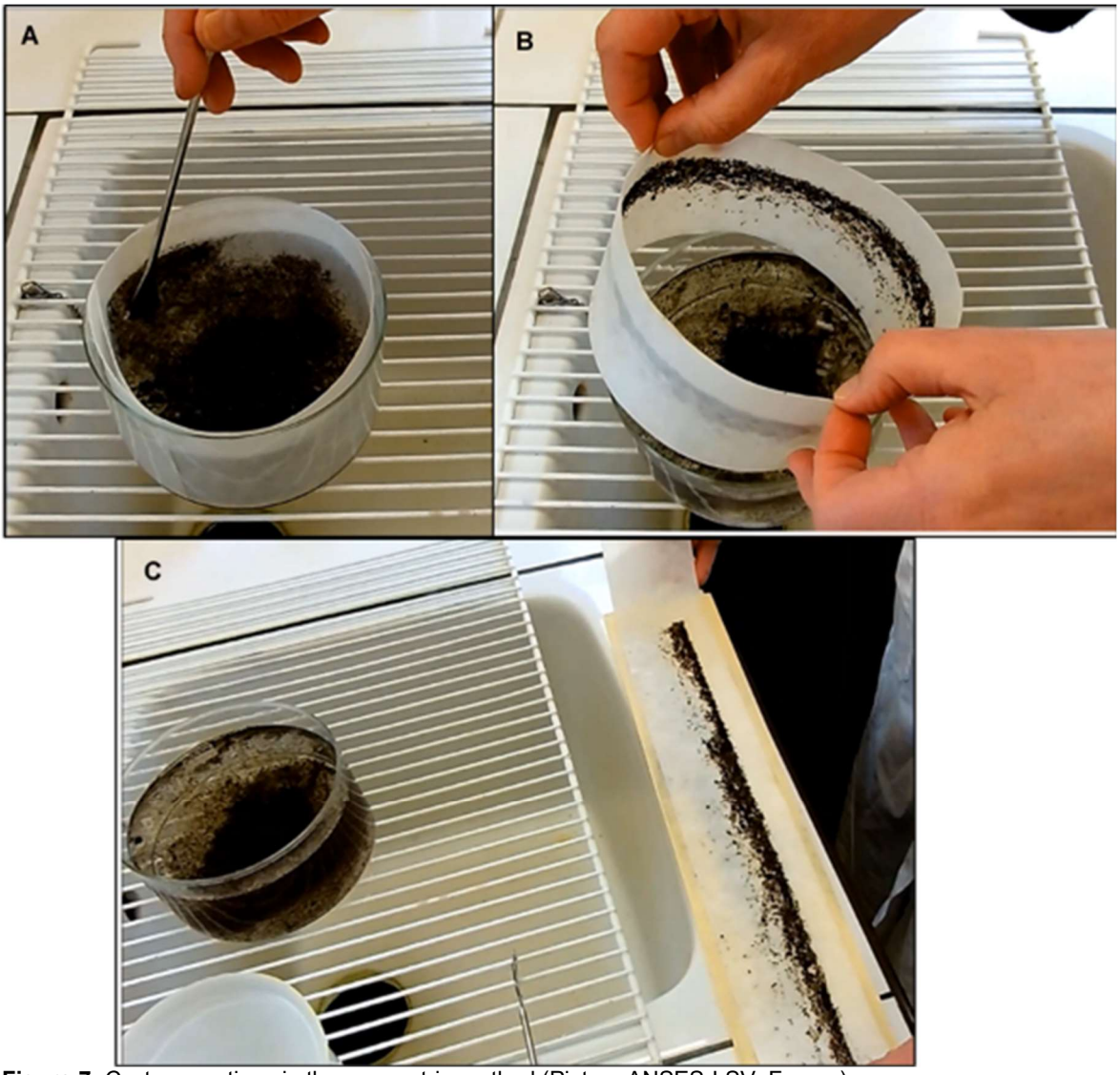


Figure 7: Cyst separation via the paper strip method (Picture ANSES-LSV, France).

4.8.5 Option 3: Separation of cysts with acetone

- ***Note:***
  - *This preparation makes it possible to obtain an extract with a low quantity of residual organic elements (sedimentation of most organic debris at the bottom of the flask).*
  - *Working under the fume hood. The use of acetone is recommended for soils rich in organic material.*

1) Start the acetone extraction when the filter paper has completely dried. Manually break aggregates by rubbing the filter paper with the fingers.
   - **Optional:** Do this over a second unfolded filter paper laid down on the table to avoid losing parts of the filter content.
2) The dry and previously gently crushed extract is placed into a 500 ml Erlenmeyer flask partially filled with acetone with the help of a clean and dry glass funnel.
3) After shaking, the Erlenmeyer is filled with acetone up to about 1 cm under the rim.
4) The supernatant in the upper part of the Erlenmeyer is poured over a paper filter in a funnel, and the deposit at the bottom of the Erlenmeyer is removed.

4.8.6 Option 4: Separation of cysts with ethanol (EtOH) (Method provided by the NRL from Germany)

- ***Note:***
  - *The Ethanol 99% denatured can be used several times if it is ensured that it is not exposed to the ambient air for too long and is stored in a closed container for longer storage periods – risk of evaporation/ reduction of the percentage of alcohol.*
  - *Separation of cysts with ethanol should be carried out under a fume hood.*

1) Start the EtOH extraction when the filter paper is completely dried. Filter paper is folded two times to have an overall triangle shape.
2) Manually break aggregates by rubbing the filter paper with the fingers.
   - **Optional:** Do this over a second unfolded filter paper laid down on the table to avoid losing parts of the filter content.
3) Place the dried and previously gently crushed extract into a 500 ml Erlenmeyer conical flask with the help of a clean and dry glass funnel.
4) Add approximately 250 ml of EtOH to the extract in the Erlenmeyer flask. Close the flask with a plug and shake the content vigorously for solving the particles.
5) Afterwards, fill the Erlenmeyer flask **nearly** completely with EtOH 99% up to 1 cm under the margin.
6) Wait a couple of minutes to let the particles settle/separate in different sections/" height" of the solution in the Erlenmeyer flask. (Intact) cysts are automatically separated within the upper section of the Erlenmeyer flask by its specific density.

7) Carefully pull up the plug avoiding strong vibrations, and separate in this way, the cysts ascended into the bottleneck.
8) Prepare another funnel equipped with a new filter paper. Place a beaker under this funnel for collecting the EtOH. Pass the supernatant of the cyst containing the upper section/phase of the solution carefully through this funnel. Remove any remaining cysts from the inside of the Erlenmeyer flask with EtOH using a wash bottle (**i.e. not by shaking the Erlenmeyer flask**).
9) Shake the Erlenmeyer flask again, carefully remove the plug to fill up again with EtOH, and repeat steps 6 to 8 for the second round of decantation.
10) EtOH can be filtered and re-used.

4.8.7 Cyst detection

1) The band of filter paper or other support of the extracted cysts and debris is placed under the stereomicroscope to search for nematode cysts (Figure 8A-B) that may belong to the genus *Globodera* (Figure 7D and Figure 8D).
   - ***Note:*** *Thoroughly moisten the filter paper band to keep the cysts on the filter preventing the cysts from "jumping".*
2) Nematode cysts are separated from the debris of the extract using, for example, a paintbrush or small metal tip. In this way, the operator can easily observe the typical shape of the cyst.
3) Nematode cysts are recognised based on various morphological features:

   - Diameter between 250 and 600 µm, on average ± 450 µm, can sometimes be smaller
   - Colour depending on the age of the cyst: straw yellow to tanned brown (Figure 8)
   - Appearance: smooth, no ornaments. Ornamentation is common on seeds.
   - Presence of a head (Figure 7)
   - Compared with similar soil debris, cysts have a certain elasticity (appreciated by exerting a slight pressure with a mounted needle) (characteristic of Heteroderinae cysts)
   - Select round shape cysts belonging to *Globodera* genus
   - If in doubt between a cyst and a seed, cut the cyst/seed open and observe its content (cysts general contain eggs, seeds are full inside)

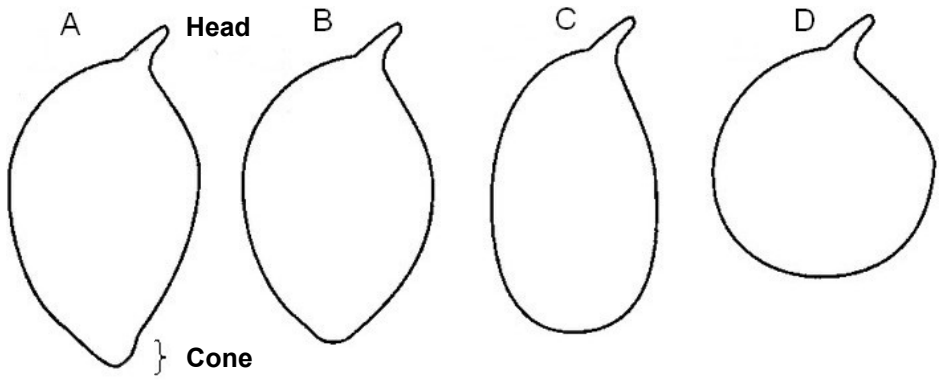


Figure 7 – Drawing of nematode cyst forms (Wouts & Baldwin, 1998). **(A)** Lemon-shaped with prominent terminal cone (Heterodera); **(B)** - Lemon-shaped with not very prominent terminal cone (Heterodera or Cactodera); **(C)** - Ovoid, without terminal cone (Punctodera); **(D)** - Spherical, without terminal cone (*Globodera*).

Figure 8 – Picture of nematode cyst forms. **(A, B)** - band of filter paper containing a *Globodera* sp.(red arrow) among the debris; (C) band of filter paper containing a *Heterodera* cyst (orange arrow) among the debris; (D) - Spherical, without terminal cone *Globodera* sp. cysts. Empty *Globodera* cysts can also be observed in the picture (blue arrow); (E, F) - Lemon-shaped with not very prominent terminal cone *Heterodera* cysts; (E) – Cysts of the group *Heterodera schachtii*; and (F) Cysts of the group *Heterodera goettingiana* (Picture ANSES-LSV, France).

If you have any question about this protocol, please, send an e-mail to eurl.nematode@enses.fr

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

5 References

Bellvert, J., Crombie, K., & Horgan, F. G. (2008). Effect of sample size on cyst recovery by flotation methods: Recommendations for sample processing during EU monitoring of potato cyst nematodes (Globodera spp.). *EPPO Bulletin*, *38*(2), 205–210. https://doi.org/10.1111/j.1365-2338.2008.01204.x

Fenwick, D. W. (1940). Methods for the Recovery and Counting of Cysts of Heterodera schachtii from Soil. *Journal of Helminthology*, *18*(4), 155–172. https://doi.org/10.1017/S0022149X00031485

PM 7/119 (1) Nematode extraction. *EPPO Bulletin*, *43*(3), 471–495. https://doi.org/10.1111/epp.12077

Kort J (1960) A technique for the extraction of Heterodera cysts from wet soil and for the estimation of their egg and larval content.Verslagen en Mededelingen, Plantenziektenkundige Dienst, Wageningen Netherlands 233, 6.

Seinhorst, JW (1964): Methods for the extraction of *Heterodera* cysts for not previously dried soil samples. Nematologica 10, 87 – 94.

Turner, S. J. 1998. Sample preparation, soil extraction and laboratory facilities for the detection of potato cyst nematodes. Pp. 75- 90 in R. J. Marks and B. B. Brodie, eds. Potato cyst nematodes: Biology, distribution and control. UK: CABI Publishing