United States Department of Agriculture

Pale Potato Cyst Nematode National Survey and Diagnostic Cyst Sample Forwarding Protocols

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United States Department of Agriculture (USDA)
Animal Plant Health Inspection Service (APHIS)
Plant Protection and Quarantine (PPQ)

Emergency and Domestic Programs (EDP)
National Identification Service (NIS)
Center for Plant Health Science and Technology (CPHST)

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Pale Potato Cyst Nematode National Survey Plan

Introduction

On April 19, 2006 officials of USDA‟s Plant Protection and Quarantine (PPQ) and the Idaho State Department of Agriculture (ISDA) announced the detection of pale potato cyst nematode (PCN), *Globodera pallida*, a major pest of potato crops. The original soil sample came from an ISDA-sampled tare soil sample. This is the first detection of the pest in the United States. PPQ and ISDA implemented statewide surveys to determine the level of distribution of PCN in fields in Idaho and launched regulatory and eradication programs in the infested area. The potato industry has requested that PPQ devise a national survey for PCN on certified seed potato. The implementation of this survey is critical to safeguarding the U.S. potato industry.

PPQ recommends implementing a national survey plan for the detection of PCN in all potato-producing states. The national survey plan is designed to survey a portion of the annual seed potato acreage. In addition, all other land used for seed potato research, including land owned by universities, government or other research organizations, will be surveyed in the same manner as land used for commercial seed potato production. This will help ensure that the entire seed potato supply, including seed from research trials, variety evaluations, post-harvest evaluations and farm-saved seed, is free from PCN. Fields will be sampled in their entirety in a fixed grid pattern, at a minimum of 5,000 cc soil/ha (5 lb or 2,000 cc soil /acre).

The rationale for focusing on seed potato fields is that seed potatoes pose the greatest risk (pathway) for PCN introduction and contamination from one field to another (EPPO, 1998; EPPO, 2000). Early detection may help prevent the spread of PCN to other areas. Surveying one percent of the commercial potato acreage as well as all other land used for seed production (universities, government or other research organizations) would provide the necessary data to demonstrate area freedom of PCN to trading partners. This will restore lost markets resulting from the PCN find in Idaho and perhaps provide the opportunity for opening new markets. Any seed potatoes traded between the US and Canada must be sampled on a full field basis using at least one 2000 cc (approx 5 lbs) sample per acre.

The SPHD and SPRO in each state will determine which entity or entities are to implement the survey. The state department of agriculture may decide to implement the survey in part or in its entirety. The state may also decide to enter into agreement with the seed potato certification agency, agriculture extension service, or others to implement the survey. However, all sampling must be conducted under the supervision of a federal or State plant regulatory agency so that the samples are “official samples.” This may vary from one state to another and hence the decision should be made locally. Although the proposed plan is designed to survey for PCN, soil samples can be examined for other cysts, such as golden nematode. Officials in each State are to decide how best to leverage the resources in the most effective manner.

General Definitions (for reference only)

1. **Headlands** - this portion of the field is often used as the "staging area" of the field during planting (*i.e.*, seed potatoes are loaded into planters in this area, harvested potatoes are often transloaded into trucks here). This portion of the field is usually planted into potatoes and may have a higher likelihood of containing PCN cysts as this area comes into contact with large quantities of seed potatoes and soil from various parts of the field.
2. **Perimeter** - this is the outside edge of the field, including the headlands and turnarounds. The degree to which the perimeter will extend into the field will depend on the size of the field and the subsequent number of samples that need to be taken.
3. **Turnarounds** – these are areas at the ends of rows where farm equipment (tractors, harvesters, etc.) turn around to enter the next row. These areas may have a higher likelihood of containing PCN cysts as soil is deposited in these areas from other locations in the field and possibly from other fields as well. The size and shape of these areas will depend on the size and shape of the field.

Sampling Methodology

The survey will take place on certified seed potato acreage, commercial potato acreage and all other land used for seed potato research (universities, government and other research organizations) and it is designed to encompass a general field detection survey. The PCN technical working group recommends implementing a full field survey at the highest amount of soil per acre that is operationally practical. We have determined that 2000 ccs of soil per acre is the appropriate sample size to balance detection sensitivity against survey resource costs. Appendix 2 provides an acceptable soil sampling protocol.

Certified Seed Potato Acreage

The plan is to survey a portion of the annual seed potato acreage in each country. Land used for seed potato production, including land owned by universities, government or other research organizations, and any seed potatoes traded between the US and Canada must be sampled on a full field basis using at least one 2000 cc sample per acre.

Full field (grid) survey
Fields chosen for survey will be surveyed in their entirety using a grid survey. In past years perimeter sampling was acceptable on some fields. Recent reviews of sampling efficacies dictate that **full field** sampling is necessary for reliable detection survey.

One composite sample, comprised of at least 100 sub-samples, will be taken per acre of each surveyed field. This sampling method will produce at least 2000cc (a little more than 5 pounds) of soil per acre if 100 subsamples of approximately 20 grams are collected. Efficacy of sampling is increased by increasing the number of subsamples and decreasing the size of each subsample.

Each 2000cc sample will be processed in its entirety using an approved processing system. Procedures for the processing of soil samples are available from the USDA.

Sampling Options

The following two options can be utilized for sampling on both seed potato and commercial potato fields. The sampling method to be used will be decided on by the state based on staff and resource availability.

1. Full field (grid) survey:

A) Mechanical Sampler Survey:

This survey method is primarily used on fields which are fallow or recently harvested. Given entry permission, this method can also be utilized on recently planted grain fields with minimal impact by some mechanical sampling devices. Mechanical samplers include small units towed by all terrain vehicles as well as large, tractor mounted units. Wheel diameter, number of chisels per wheel, and number of wheels per sampling unit determine the rate of sampling. It is important to calibrate the samplers so that at least 2000 cc of soil is collected in at least 100 subsamples per acre with subsamples being collected uniformly across the length and width of the field.

Some commercially available samplers use mechanisms other than chisels on wheels, and they are acceptable as long as they collect soil down to a depth of approximately 4 inches and collect at least 2000 cc of soil per acre with at least 100 subsamples.

Mechanical sampling table (based on the Golden Nematode Program Manual):

Cysts per acre[1]	Number of chisels/wheels	Swath width (in square feet)	Area per sample point (in square feet)	Volume per Acre (lbs)[2]
50,000	8	1.15	1.25	74.9
100,000	8	3	2.5	37.4
200,000	4	3	5	18.7
300,000	4	4.5	7.5	12.5
400,000	4	6	10	9.3
500,000	4	7.5	12.5	7.4
750,000	3	7.5	18.75	5.0
1,000,000	2	7.5	25	3.75
2,000,000	1	7.5	54	1.875
4,000,000	1	15	108	0.938

[1] Detection level based on sampling from top 4 inches of soil, with vertical homogeneity of cysts assumed within the plow layer. Soil density assumed to be 86.09 lb. per square foot. Detection probability is 95 percent as determined by the Poisson approximation.
[2] Based on approximately one gram of soil per sample point.

The Golden Nematode Manual can be found at the following website:
http://www.aphis.usda.gov/import_export/plants/manuals/domestic/index.shtml

Sources for Mechanical sampling wheels:

Haines Manufacturing Co.
P.O. Box I
20 Carrington Street
Avoca NY 14809
Phone: 607-566-2234
Website: http://www.hainesequipment.com/

AMS
105 Harrison St. American Falls, ID 83211
1-800-635-7330
www.ams-samplers.com

Prices on mechanical soil sampling wheels available upon request.

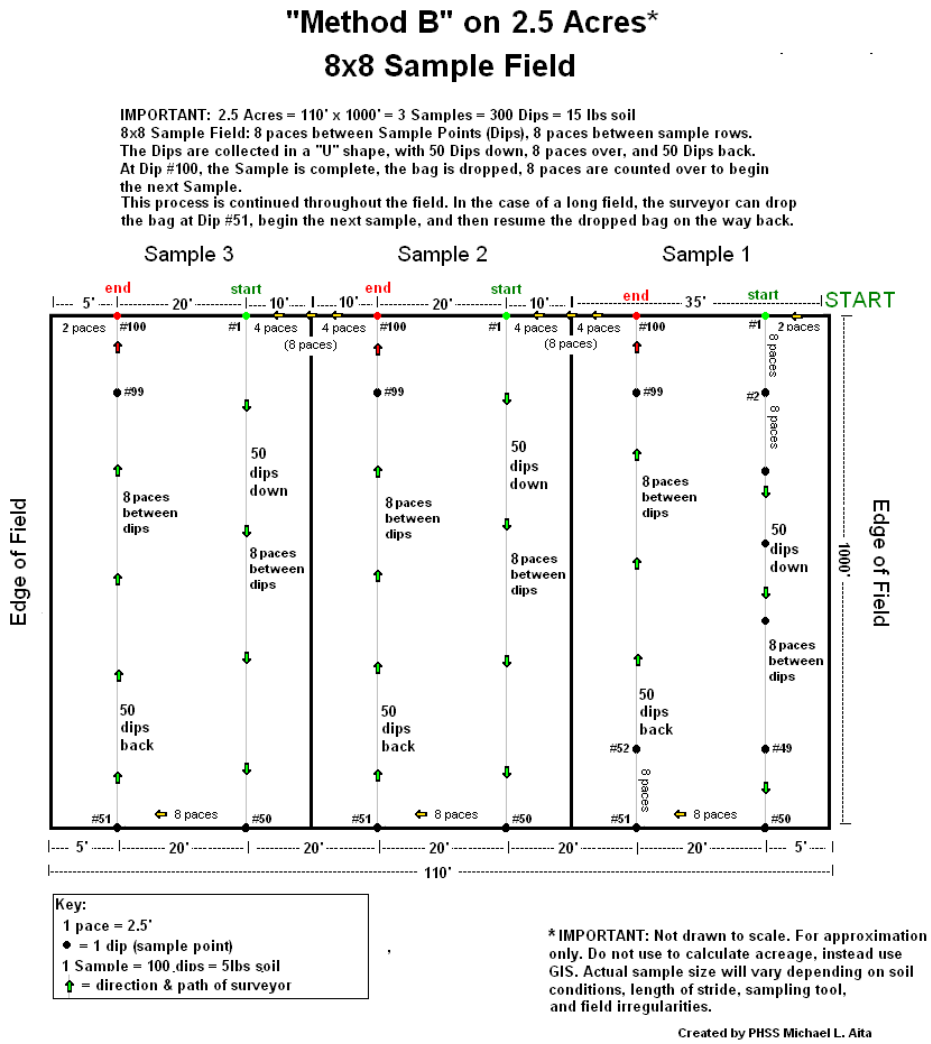
B) Hand Sampling Survey:

To be completed on all fields which are recently harvested or on any field in which entry will be granted. Sampling in this manner will result in minimal or no crop damage. Whenever possible, surveys will be conducted after harvest to take advantage of the soil mixing that takes place during harvesting operations.

One 2000 cc composite sample, comprised of 100 sub-samples, will be taken per acre of field. Subsamples may be taken with an auger or with a long handled trowel. The following 8 x 8 sampling diagram reflects the collection of 2000cc or approximately 5lbs of soil.

Regardless of whether the mechanical or hand sampling method is used, each 2000cc sample must be processed in its entirety using an approved processing system. Procedures for the processing of soil samples are available from the USDA.

Sampling diagram 8x8 (Diagram by Michael Aita of APHIS)



Further guidance is provided on how to perform the 8 x 8 soil survey are found in Section 2 "Procedures" in the Golden Nematode Program Manual, pages 2-3-4 through 2-3-6. The Golden Nematode Manual can be found at: http://www.aphis.usda.gov/import_export/plants/manuals/domestic/downloads/gnpm.pdf

Data Management

It is important to properly identify soil samples as they are taken, and it is imperative that sample identity must be preserved during collection, transportation, drying and processing.

Integrated Plant Health Information System (IPHIS) is being used to facilitate the data reporting element of the National Survey and is presented in a separate document for the purpose of clarity and readability.

References Cited

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EPPO Standards. Guidelines on Good Plant Protection Practice. Potato. September 2000.

European Union. 1969. Council Directive 69/465/EEC of December 1969 on control of potato cyst eelworm. Official Journal of the European Communities L323/3, 563-564. (*currently in the process of a new proposal which has not been ratified*)

European Union. 2007. Council Directive 2007/33/EC of 11 June 2007 on the control of potato cyst nematodes and repealing Directive 69/465/EEC.

Evans, K., Barker, A.D.P. 2004. Economies in nematode management from precision agriculture – limitations and possibilities. Nematology Monographs & Perspectives 2, 23-32.

Trudgill, D., Elliott, M., Phillips, M. 2001. Make sure you are sampling with a purpose. Potato Review 11, 10-12.

USDA APHIS PPQ. 2008. Golden Nematode Program Manual: Final Edition http://www.aphis.usda.gov/import_export/plants/manuals/domestic//index.shtml

Appendix 1. Processing of samples

Introduction

Laboratory methods that are acceptable for cyst extraction include sugar centrifugation, USDA cyst extractor, Fenwick can sieving, wet sieving, and elutriation. The following protocol, as part of Appendix 2 and currently used in Idaho, is recommended for use in extracting potato cyst nematodes, *Globodera* spp., from soil samples. (Note: the Golden Nematode Program in New York uses a slightly different protocol and can be found in the Golden Nematode Manual at: http://www.aphis.usda.gov/import_export/plants/manuals/domestic/index.shtml)

The protocol is to be used as a guidance document with the understanding that it may be necessary to modify the procedure depending upon soil type, equipment, personnel, and other mitigating factors specific to your laboratory. Laboratory directors should use extant knowledge of their unique system and environment when making modifications, noting and recording the changes. Any soil should be thoroughly mixed prior to extraction procedures to ensure sample homogeneity. Furthermore, these procedures are specific only to mature nematode cysts and not to vermiform nematodes. Additional extraction procedures will need to be utilized to extract and collect vermiform nematodes.

The SPHD and SPRO in each state will determine which laboratories will provide the extraction and the initial screening support. All suspect samples will be forwarded to the appropriately identified authority for confirmation. Final confirmation of any PCN cysts from outside of the regulated area requires ARS Nematology laboratory confirmation, which will be accomplished after secondary screening. Laboratory capacity may vary from one state to another. The SPHD and SPRO may elect to leverage local resources, including in some cases, lab capacity within the state department of agriculture, Nation al Plant Diagnostic Network, university systems, and others. Again, the decision regarding which entity will provide the diagnostic support should be made locally. States without diagnostic infrastructure may be able to leverage laboratory capacity of neighboring states. APHIS PPQ will provide the necessary protocols and other technical and logistical support.

The initial cost estimate per sample will vary by state depending on the circumstances, resources required and the needed logistics. APHIS PPQ will work with states that may require additional support, especially in the area of diagnostic infrastructure. Contact information is provided later in this document.

Disposal of Soil and Processing Water

Soil and water should be disposed of in accordance with permit requirements for regulated samples. To reduce the risk of spreading any potential pests through soil movement, plans for processing of soil should contain methods for adequately dealing with processing water and processed soil. Solid processing effluent should be captured in soil traps and disposed in approved landfills. Liquid processing effluent should be released into a sewer system which connects to a water treatment system or an approved drain field. Soil should be sterilized by any approved method or collected and deposited according to all pertinent regulations in an approved landfill for burial.

Appendix 2. Acceptable soil sampling protocol

1. Chain of Custody (COC)

Soil samples are usually shipped via commercial shippers (e.g. FedEx, UPS, etc.) or delivered from survey crews via enclosed trailer. In either case, the approved laboratory responsibilities begin when the soil samples and a completed PCN SAMPLE CHAIN-OF-CUSTODY FORM (including a signature, organization, and data/time under "Relinquished By" in SECTION 3 of the form) are turned over to laboratory/COC personnel.

Signing for receipt of soil samples

All soil samples indicated on the PCN SAMPLE CHAIN-OF-CUSTODY FORM must be individually accounted for by COC/laboratory personnel.

After all samples are accounted for, COC/laboratory personnel sign the PCN SAMPLE CHAIN-OF-CUSTODY FORM under "Received by" in SECTION 3 of the form to indicate receipt of the samples.

Two photocopies are made of the signed PCN SAMPLE CHAIN-OF-CUSTODY FORM. One copy is filed and one is given to a representative of the submitting party (this may require the transmission of a facsimile if the sample were shipped via a commercial shipper).

Soil sample storage

Samples are to be placed on secured soil sample storage racks.

Sample handling

Soil sample bags are opened just prior to processing. Sample bags are cut open in the sample washing area and are transferred directly to Fenwick cans (or equivalent) for washing and initial processing.

2. Cyst Extraction

Equipment and apparatus

Fenwick can soil washer (10” diameter X 16” deep).

Sieves:

No. 20 (850µm mesh), 8” diameter, 2” deep (top screen)

No. 60 (250µm mesh), 8” diameter, 2” deep (bottom screen)

Plastic beaker with handle (2 liter capacity)

Rubber apron

Soil sample flotsam (floating material) extraction

Soil samples must be completely dry prior to extraction. Do not wash more samples than can be sorted within three hours of washing on the same day. Flotsam (including cysts, if present) from soil samples are extracted using Fenwick can soil washers and a series of sieves. Soil samples are washed and prepared in batches for reading. A batch consists of soil samples from the same field that can be extracted/sorted in one day.

Place two eight-inch sieves (No. 20 on top, No. 60 on bottom) into the wash station sieve receptacle.

Select an empty polypropylene beaker (600ml) and a soil sample.

Squeeze the soil sample bag to check for any large clumps of soil. If there are clumps, break them apart by placing the soil sample in a plastic bag and pounding with a rubber mallet.

Take the soil sample and beaker to a wash station. Partially open the water valve to begin filling the bottom funnel of the Fenwick can. Ensure that the fan in the hood (if present) above the Fenwick can is turned on.

Open the soil sample bag carefully and pour the entire soil sample into the Fenwick can. Try to minimize dust. Discard the paper sample bag into the garbage.

Fully open the water valve. Allow high-pressure water to flow until it is just below the lip of the spout of the washer can, mixing the soil sample and causing floating material to rise to the top. DO NOT leave the soil sample unattended while the high-pressure water is flowing, as the can and/or sieves could overflow resulting in sample loss.

Turn the valve to a lower setting as the can volume reaches the lip of the spout. This will allow water and flotsam to gently flow over the spout and through the sieves. Keep the water flowing slowly and evenly throughout the washing process. Any PCN cysts present in the sample will float to the water surface, flow over the spout, through the top (No. 20) sieve and be caught on the bottom (No. 60) sieve. Using the hose and sprayer, carefully spray the remaining flotsam over the Fenwick can spout onto the sieves. Take care to not spray outside of wash station and/or into the ventilation hood filters. (Wet filters will not draw air efficiently.)

After at least two minutes have passed, again spray the flotsam over the Fenwick can spout.

Turn the water valve off and thoroughly rinse the top sieve, washing smaller flotsam down into the bottom sieve. Remove the top sieve and empty the debris into a garbage can. Thoroughly backwash this sieve and replace it on the top of the 60-mesh sieve. Rinse through the top sieve to the bottom sieve again.

Gently wash flotsam out of the No. 60 sieve into an appropriately labeled 600ml beaker.

Using the hose, gently fill the beaker with water until approximately two (2) inches from the top and set aside for recording and further processing.

Return to Fenwick can wash station and turn can upside down to clean out residual soil. Turn the water valve on fully and use the sprayer to thoroughly rinse all remaining soil out of the can.

Thoroughly wash and backwash sieves with clean water before placing back into the washer. Carefully wash all residual soil and debris from sink area.

Thoroughly rinse hands before starting another soil sample. It is possible to process two soil samples simultaneously if soil types and equipment availability permit. While the first sample is being processed, a second soil sample bag can be initiated in a second washing unit. Do not mix up the two soil samples and beakers and never mix soil samples together unless specifically directed to do so!

Extraction room and equipment cleaning

In between soil samples belonging to the same field, a thorough rinse of all equipment is sufficient.

In between soil samples from different fields or at least daily, thoroughly clean and disinfect all equipment.

Sweep the extraction/wash room floor (this works well when floors are dry, first thing in the morning).

Sweep aisles after moving soil sample bags from storage.

Remove rubber apron, hose it off in the sink, and hang to dry.

Hose down the entire wash area. Use a clean sponge to wipe down the inside and outside surfaces of washers, sinks, etc.

Ensure there is NO residual soil left clinging to the sides and/or in the bottom of the washer sinks.

Remove all soil from counters.

Wash countertops and backsplashes with laboratory disinfectant solution.

Mop the extraction/wash room floor with laboratory disinfectant solution. Squeegee to remove excess water if necessary.

Wipe down soil carts with sponge and laboratory disinfectant solution.

Wash and rinse hands thoroughly.

3. Sorting

Initial screening is based on gross morphological characters (mainly size and shape). This step will be referred to as "sorting" and the individual as a "sorter". An employee cannot prepare/sort extracted samples until they have passed the proficiency test.

When cysts or cyst-like structures are found, the sorter forwards the specimen to another more experienced individual for further verification. This step will be referred to as "verifying" and the individual as a "verifier".

Equipment

Stereomicroscope with light source

Hand-held laboratory counter

No. 60 (250µm mesh), 3-inch sieve

Stainless steel spatula

Teasing needle

400 and 600ml polypropylene beakers

Wash bottles containing tap water, 500ml

Plastic sorting tray

Petri dish lid

Cyst removal loop

Cyst collection vials with lid

Vial holder

Adhesive labels

Lab coat

Preparation of extracted samples for sorting

Take a No. 60 (250µm mesh), 3-inch sieve to a sink with an appropriately labeled 600ml beaker containing the soil sample extract.

While rotating the beaker, slowly pour the soil sample extract through the 3-inch sieve.

Place the sieve atop a 400ml beaker to allow the flotsam to drain.

Using a clean spatula, carefully scrape flotsam from the sieve into a plastic sorting tray. Rinse spatula into the sieve using a water bottle.

Gently wash all remaining flotsam from the sieve into the reading tray.

Sorting extracted samples

Place the reading tray on the stereomicroscope stage at the sorting station.

Fill the trays with water from a squirt bottle until the flotsam is at the level of the top of the tray but not overflowing. All cysts will float, whether viable (alive) or nonviable (old, dead, or flattened).

PCN cysts are round or spherical objects that appear to have a short spout or protrusion. They are barely visible to the unaided eye. Color can vary from brown (typically viable cysts) to black (typically non-viable cysts).

Scan the entire tray by methodically moving the tray across the field of view. Move the tray in small increments to maintain a landmark. Use the teasing needle probe to gently move material aside so the entire flotsam

volume will be examined. Continue until all of the material has been examined.

When cysts or debris of any type (including *Heterodera sp.* cysts) that resembles PCN cysts are found, sorters must forward the sample for verification that these cysts are, or are not *Globodera sp*. cysts before proceeding.

The forwarded specimen is reviewed by a more experienced individual referred to as a "verifier" who has been trained and specifically given discard authority.

The verifiers have been trained to recognize additional morphological characters that would exclude *Globodera sp*. (such as look-alike propagules, debris, etc.) or to determine that forwarding is necessary for final identification. Cysts will only be collected when the verifier cannot rule out the possibility of cysts being *Globodera sp*.

If *Globodera sp*. cysts are identified, continue to the instructions for removing cysts described below in "Collecting Cysts".

When you have finished examining the tray and have removed any presumptive PCN cysts, dump the flotsam and water through an eight-inch No. 60 sieve into a sink.

Be sure to document the sample results.

Clean all lab equipment (e.g. teasing needles, cysts extraction loops, 3-inch sieve, three-cornered beaker, sorting tray, etc.) used during the sorting process with a thorough rinse and by scrubbing with a toothbrush when necessary. Ensure that no residue remains on/in any equipment.

Repeat above steps until all extracted samples have been read/examined.

In between extracted samples of different fields, all equipment (spatula, sieve, probe, loop, beaker, and stereomicroscope base) should be washed with a laboratory disinfectant solution.

Collecting Cysts

If one or more *Globodera sp*. cysts are verified when sorting a particular soil sample from non-eradication fields, follow the instructions below.

Put on a lab coat.

Get a cyst collection vial and lid.

Use only one vial per extracted soil sample.

Do not mix cysts from multiple extracted samples into one vial unless specifically instructed to do so.

All possible *Globodera sp.* cysts/sample will be collected, and the total number of cysts in each extracted sample is data required to be documented. Hand counters are useful when counting cysts.

Use a loop to remove all cysts from the flotsam, transfer into the collection vial, and securely fasten the lid.

Prepare and attach a label to the collection vial. The label should include the soil sample number, the number of cysts isolated, and the date and initials of the sorter who collected the cysts.

Note the final cyst count for that particular sample.

Place the vials of cysts (if any) in an appropriate vial storage box and do the following:

Examine the cysts using a high-power stereomicroscope (e.g. Nikon 1500 MZC) outfitted with a digital camera (e.g. Nikon Digital Sight DS-Fi1) and take digital photos of the cysts.

When cysts are forwarded, please follow the directions below.

When fewer than 20 *Globodera sp.* cysts are present, all will be forwarded; however, when greater than 20 cysts are found, only 20 representative cysts in good condition will be forwarded for final identification. The remaining cysts will be archived and stored.

Complete PPQ Form 391, Specimens for Determination for each vial sent for confirmation. This form must be completed with all available information before the extracted cysts are sent.

Pack the vials according to the directions below:

Each sample should be submitted as dry cyst(s) placed in a screw-top plastic vial.

The cap should be wrapped with parafilm.

The vial(s) should be wrapped in bubble wrap and double-bagged in sealed 4ml plastic bags.

The double-bagged sample should be placed in a sturdy, cardboard tube.

The tube(s) should be packed with bubble wrap or newspaper in a sturdy cardboard box along with a copy of the lab permit (PPQ Form 526) for the party to which you are shipping, and PPQ Form 391.

Avoid Thursday or Friday shipping to ensure package arrives on a normally scheduled business day.

The laboratory officer or supervisor should inform the recipient that a shipment is in the mail; including a brief description of what is being sent, a mail service tracking number, and the expected delivery date/time.

4. Disposal Practices

Sample packaging

Cardboard boxes used to transport samples are collected and transported directly to a Landfill/Transfer Station.

Paper sample bags are collected in plastic sacks and transported directly to a Landfill/Transfer Station.

Washing station effluent

Effluent from the Fenwick can cyst extraction process is flushed from the washing area. Soil and other particulates that are collected are disposed of in a landfill.

Clarified effluent may be discharged to a subsurface drain field or sewage system connected to a water treatment facility.

Sample flotsam

Sample flotsam from the reading/cyst extraction process is sieved (No. 60, 250µm mesh) prior to dumping down sinks. The sieved flotsam is routinely autoclaved (see "Preventing sample-to field-contamination") and disposed of in a landfill.

5. PCN Contamination Prevention

There are several steps that laboratory/COC personnel must take to decrease the likelihood of sample-to-sample and sample-to-field contamination.

Preventing sample-to-sample contamination/confusion

In the wash area:

Always check that all sample labels match the soil sample bag/container.

Water flow from the Fenwick cans must be such that splashing and overflow is minimized.

All equipment must be thoroughly cleaned free of soil between samples.

Floors, aprons, wash stations, mats, etc., must all be cleaned in between samples from different fields, and at least on a daily basis when completion of a field requires more than one day of processing.

While sorting:

During cyst extraction, lab coats must be worn, and lab coats should be laundered at least weekly.

When recording cyst counts, double check that data is entered for the correct sample number.

Frequently wash hands, especially between soil samples from different fields.

Vials containing cysts should have lids firmly secured at all times except when in use.

Vials containing cysts should never be set down on a countertop/lab bench, unless placed in a vial holder.

Vials should be clearly and neatly labeled with the correct sample number, sorter initials, date cysts were extracted, and cyst count.

Preventing sample-to-field contamination

All people entering a PCN Laboratory must wear lab dedicated footwear (i.e. footwear that is not used in any other setting for any other purpose) or shoe coverings that are removed upon exiting the lab and remain at the lab.

Shoe baths filled with laboratory disinfectant or 'sticky mats' (placed at every lab exit) should be stepped on with a gentle scrubbing motion upon exiting the lab. Lab carts should also roll over these mats. Mats should be monitored regularly and kept filled with disinfectant at all times.

Cysts should be stored in a locked cabinet at all times except when in use.

No visitors are allowed to enter the PCN laboratory without prior approval and without an escort.

Flotsam from samples previously sorted should not be dumped directly down a sink drain, but should be first dumped through a No. 60 sieve placed in a sink to drain. The sink sieves should be dumped into a specially labeled receptacle and autoclaved using the following process:

Transfer flotsam to an autoclavable bin or bag, ensuring that flotsam is no more than about one inch deep.

Place a Diack sterilization monitor into the middle of flotsam and autoclave at 121ºC for 60 minutes.

Remove autoclaved material and check the Diack sterilization monitor. If the pellet inside the glass tube has melted, the flotsam reached an appropriate sterilization temperature and may be discarded into the trash. If not, autoclaving must be repeated until an appropriate sterilization temperature is reached.

In case of spills

Soil spills in the laboratory must be contained immediately. All spilled soil and materials used to clean up spilled soil must be autoclaved and then disposed of in the trash. To avoid inadvertent cross-contamination, do not use anything to clean up spilled soil (such as brooms, mops) that cannot be sterilized or thrown away. Following clean up; sterilize the affected area with laboratory disinfectant.

Cyst spills must be contained immediately. Clean packaging tape is the most useful tool in retrieving spilled cysts from counter tops, floors, clothing, and shoes. If the quantity of spilled cysts is known, retrieval should continue until as many cysts as possible are accounted for. Following clean up; sterilize the affected area with laboratory disinfectant.

Quality Control

Globodera tabacum cysts will be used to spike a previously sorted negative extracted sample. This testing is especially important during prolonged stretches of time when sorters are not regularly seeing and collecting *Globodera* cysts. Sorters will be given

three chances to identify all *Globodera* cysts present in spiked samples. If sorters fail in all three chances, they will be required to be retrained.

Note: Dry down *G. tabacum* cysts for reuse after training takes place.

References:

1. Wang, X., and Thurston, D. May 2006. Method for PCN Cyst Extraction Using the Fenwick Can.
2. Wang, X. and Thurston, D. May 2006. PCN/GN Cysts and Acetone Purification.
3. Barker, K.R., Carter, C.C., and Sasser, J.N. 1985. An Advanced Treatise on Meloidogyne. Raleigh, NC.

Appendix 3: Specimen Forwarding Protocol

Introduction

State programs will forward all samples needing identification through the PPQ Survey Identifier assigned to their region. A suspect sample is a sample that was processed by an approved laboratory method for cyst extraction and verified as being a sample that contains what appear to be *Globodera* cysts. The overriding principle is that if there is any doubt about the identity of a sample, it should be submitted for further testing. The following protocol describes the procedures, roles, and reporting requirements for the screening and confirmation diagnostic laboratories in support of the PCN National Survey:

1. **Designated State Laboratories** -
    a. Cooperators will enter pest submission information into the ISIS Database.

    b. Package all nematode samples requiring additional identification to PPQ as detailed below:
        i. Each nematode sample should be submitted as dry cyst(s) placed in a screw-top plastic vial.
        ii. Label each vial with the soil sample ID number. We are suggesting the states use the following nomenclature for identifying the samples:

            Example Sample Bag Label: **07SDFAR-00001-001**

            07 = Year
            SD = Submitting State
            FAR = County Code where sample was taken
            00001 = Number of the field; this would sequentially increase each time a **new** field is surveyed
            001 = Sample Number; this would sequentially increase each time a sample bag is filled from **within the same field**

        iii. The cap should be wrapped with parafilm.
        iv. The vial(s) should be double-bagged in 4-ml plastic bags and sealed.
        v. The double-bagged sample should be placed in a sturdy, cardboard tube packed with bubble wrap or newspaper.
        vi. The tube should be placed in sturdy, cardboard box and a copy of the lab permit (PPQ Form 526) to which you are shipping, PPQ Form 391 (attached below), and Chain of Custody documents (attached below) needs to accompany the shipment.

    c. Submitted samples will be forwarded priority overnight via a traceable shipping service (FedEx, UPS, etc.) to the designated PPQ Survey Identifier for additional diagnostic identification (Contact the identifiers for a copy of the permit & any shipping questions):

Dr. Craig Webb
USDA, APHIS, PPQ – Plant Pathologist Identifier
Department of Plant Pathology, Kansas State University
4024 Throckmorton Plant Sciences, Manhattan, KS 66506-5502
Office (785) 532-1349, Cell (785) 633-9117, Fax (785) 532-5692

d. Identification results will be reported to the submitting laboratory by PPQ. If necessary, specimens may be forwarded to the ARS Nematology Laboratory, Beltsville and PPQ Molecular Diagnostics Laboratory, Beltsville for final confirmation. Federal confirmatory testing includes identification on the basis of cyst morphology and testing of DNA isolated from J2 juveniles from eggs from one or more cysts.

This report is authorized by law (7 U.S.C. 147a). While you are not required to respond your cooperation is needed to make an accurate record of plant pest conditions. *See reverse for additional OMB information.*

FORM APPROVED
OMB NO. 0579-0010

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE

SPECIMENS FOR DETERMINATION

Instructions: Type or print information requested. Press hard and print legibly when handwritten. Item 1 - assign number for each collection beginning with year, followed by collector's initials and collector's number. Example (collector, John J. Dingle): 83-JJD-001.
Pest Data Section – Complete Items 14, 15 and 16 or 19 or 20 and 21 as applicable. Complete Items 17 and 18 if a trap was used.

FOR IIBIII USE
LOT NO.
PRIORITY

1. COLLECTION NUMBER

2. DATE — MO | DA | YR

3. SUBMITTING AGENCY
☐ State Cooperator ☐ PPQ ☐ Other _____

SENDER AND ORIGIN

4. NAME OF SENDER

6. ADDRESS OF SENDER

ZIP

INTERCEPTION SITE

5. TYPE OF PROPERTY *(Farm, Feedmill, Nursery, etc.)*

7. NAME AND ADDRESS OF PROPERTY OR OWNER

COUNTRY/ COUNTY

PURPOSE

8. REASON FOR IDENTIFICATION *("x" ALL Applicable Items)*

A. ☐ Biological Control (Target Pest Name)	E. ☐ Livestock, Domestic Animal Pest
B. Damaging Crops/Plants	F. Possible Immigrant *(Explain in* REMARKS*)*
C. Suspected Pest of Regulatory Concern *(Explain in* REMARKS*)*	G. Survey *(Explain in* REMARKS*)*
D. ☐ Stored Product Pest	H. ☐ Other *(Explain in* REMARKS*)*

9. IF PROMPT OR URGENT IDENTIFICATION IS REQUESTED, PLEASE PROVIDE A BRIEF EXPLANATION UNDER "REMARKS".

HOST DATA

10. HOST INFORMATION
NAME OF HOST *(Scientific name when possible)*

11. QUANTITY OF HOST
NUMBER OF ACRES/PLANTS
PLANTS AFFECTED *(Insert figure and indicate* ☐ Number ☐ Percent*):*

12. PLANT DISTRIBUTION
☐ LIMITED
☐ SCATTERED
☐ WIDESPREAD

13. PLANT PARTS AFFECTED

☐ Leaves, Upper Surface	☐ Trunk/Bark	☐ Bulbs, Tubers, Corms	☐ Seeds
☐ Leaves, Lower Surface	☐ Branches	☐ Buds	
☐ Petiole	☐ Growing Tips	☐ Flowers	
☐ Stem	☐ Roots	☐ Fruits or Nuts	

PEST DATA

14. PEST DISTRIBUTION
☐ FEW
☐ COMMON
☐ ABUNDANT
☐ EXTREME

15. ☐ INSECTS ☐ NEMATODES ☐ MOLLUSKS

NUMBER SUBMITTED	LARVAE	PUPAE	ADULTS	CAST SKINS	EGGS	NYMPHS	JUVS.	CYSTS
ALIVE								
DEAD								

16. SAMPLING METHOD

17. TYPE OF TRAP AND LURE

18. TRAP NUMBER

19. PLANT PATHOLOGY – PLANT SYMPTOMS *("X" one and describe symptoms)*
☐ ISOLATED ☐ GENERAL

20. WEED DENSITY
☐ FEW ☐ SPOTTY ☐ GENERAL

21. WEED GROWTH STAGE
☐ SEEDLING ☐ VEGETATIVE ☐ FLOWERING/FRUITING ☐ MATURE

22. REMARKS

23. TENTATIVE DETERMINATION

24. DETERMINATION AND NOTES *(Not for Field Use)*

SIGNATURE

DATE

FOR IIBIII USE
DATE RECEIVED
NO.
LABEL
SORTED
PREPARED
DATE ACCEPTED
RR

PPQ FORM 391 *Previous editions are obsolete.*
(AUG 02)

OMB Information

According to the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0579-0010. The time required to complete this information collection is estimated to average .25 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.

Instructions

Use PPQ Form 391, Specimens for Determination, for domestic collections (warehouse inspections, local and individual collecting, special survey programs, export certification).

BLOCK	INSTRUCTIONS
1	1. Assign a number for each collection beginning the year, followed by the collector's initials and collector's number **EXAMPLE** In 2001, Brian K. Long collected his first specimen for determination of the year. His first collection number is 01-BLK-001 2. Enter the collection number
2	Enter date
3	Check block to indicate Agency submitting specimens for identification
4	Enter name of sender
5	Enter type of property specimen obtained from (farm, nursery, feedmill, etc.)
6	Enter address
7	Enter name and address of property owner
8A-8L	Check all appropriate blocks
9	Leave Blank
10	Enter scientific name of host, if possible
11	Enter quantity of host and plants affected
12	Check block to indicate distribution of plant
13	Check appropriate blocks to indicate plant parts affected
14	Check block to indicate pest distribution
15	• Check appropriate block to indicate type of specimen • Enter number specimens submitted under appropriate column
16	Enter sampling method
17	Enter type of trap and lure
18	Enter trap number
19	Enter X in block to indicate isolated or general plant symptoms
20	Enter X in appropriate block for weed density
21	Enter X in appropriate block for weed growth stage
22	Provide a brief explanation if Prompt or URGENT identification is requested
23	Enter a tentative determination if you made one
24	Leave blank

Distribution of PPQ Form 391

Distribute PPQ Form 391 as follows:

1. Send Original along with the sample to your Area Identifier.
2. Retain and file a copy for your records.

SUBMITTER:

(Please complete Sections 1 & 2 and relinquish in Section 3.)

Document all evidence transfers in Section 3 (page 2).

SECTION 1

Program Name:			**Date Submitted:**
Agency:			**Agency Case No.:**
Address:			
City/County:		**State:**	**ZIP Code:**
Phone No.:	**Fax No.:**	**E-mail:**	
Emergency Contact:		**Phone No.:**	

Submitter: **(Print Name):**	**Agency:** **Telephone:**	**Date:**

SECTION 2

Shipping Site:		**Site Address:**
Collected By:		**Agency:**
Submitter Description: Include the number of containers, identification number(s) and a physical description of each sample submitted for testing. **{Relinquish sample(s) on page 2.}**		
Submitter Comments:		
Lockbox Evidence Seal Number:		

NOTE: PLEASE DOCUMENT TRANSFER OF SAMPLES ON PAGE 2 (SECTION 3)

SECTION 3

Chain of Custody: Persons relinquishing and receiving samples: Provide signature, organization and date/time to document sample transfers. (Start with Box Number 1 below)

Relinquished By (Submitter)	Organization	Date/Time	Received by	Organization	Date/Time
1.			**2.**		

Relinquished By	Organization	Date/Time	Received by	Organization	Date/Time
3.			**4.**		

Relinquished By	Organization	Date/Time	Received by	Organization	Date/Time
5.			**6.**		

Relinquished By	Organization	Date/Time	Received by	Organization	Date/Time
7.			**8.**		

Relinquished By	Organization	Date/Time	Received by	Organization	Date/Time
9.			**10.**		

SECTION 4 **Laboratory Use Only**

Laboratory Description of Submitted Samples: Include the number of containers, identification number(s) and a physical description of each item submitted for testing.	
Signature:	**Date:**

SECTION 5 –Disposal-(if appropriate) {To be completed by Laboratory Evidence Custodian}

Disposition Site:	**Disposition No.:**	**Method of Disposition/Date:**
Performed by:		**Date:**
Witnessed by:		**Date:**

SECTION 6

Supplemental Information (i.e. sample description, comments, other)

Questions??

Please contact the following National Program Managers for additional information and/or assistance:

Phillip Mason, Ph.D.
National Operations Manager
USDA, APHIS, PPQ - 2150 Centre Avenue
Building B, 3E10, Ft. Collins, CO 80526
Phone (970) 494-7565
phillip.a.mason@aphis.usda.gov

Jonathan M. Jones
National Policy Manager
4700 River Road, Unit 137, Suite 4C-01.62
Riverdale, MD 20737
Phone (301) 851-2128
mailto:mjmjones@aphis.usda.gov