
United States Department of Agriculture
Animal and Plant Health Inspection Service
Plant Protection and Quarantine


# 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)

# Table of Contents

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# 2007 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 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 have implemented statewide surveys to determine the level of distribution of PCN in fields in Idaho. The potato industry has requested that PPQ devise a national survey for PCN on certified seed potato and commercial potato acreage. The implementation of this survey is critical to safeguarding the U.S. potato industry.

PPQ recommends implementing a national survey for the detection of PCN in all potato-producing states. The plan is designed to survey the 2006 potato acreage. Although part of the survey will be implemented in 2007 and perhaps in 2008, field selection should be made based on the acreage available from 2006 crop year statistics. The timeframe for implementing the proposed survey plan is two years. The goal is to begin implementing the survey in the fall of 2006, continue surveying in the spring of 2007, and complete the survey in the fall of 2007 or, if necessary, the spring of 2008. The current survey plan is designed to sample targeted potato fields (sampling 100% of seed potato and 10% of commercial production) only one time over the two year timeframe.

The rationale for surveying all 2006 seed potato fields is that seed potatoes pose the greatest risk as a 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 ten percent of the 2006 commercial potato acreage over the span of the next two years would provide the necessary data to demonstrate domestic 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.

The SPHD and SPRO in each State are to decide which entity or entities are to implement the survey. Any State's department of agriculture may decide to implement the survey in part or in its entirety. A State may also decide to enter into agreement with the seed potato certification agency, agriculture extension service, or others to implement the survey. 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.

## Definitions

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. For the purpose of this survey, 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.) turns 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 and commercial potato acreage and it is designed to encompass a general field detection survey. The amount of acreage to be surveyed will differ between seed potato and commercial potato fields. The PCN technical working group recommends implementing a field survey.

### Certified Seed Potato Acreage

The plan is to target all seed fields in each state for sampling (EU, 1969; USDA APHIS PPQ, 2006).

### Commercial Potato Acreage

The plan is to randomly select approximately 10% of the production fields within each county of production within the state. This will limit bias in the distribution of fields across counties to be sampled. Though the selection is meant to be random, special attention may be paid to fields with a higher likelihood of infestation and detection. This includes fields that have been planted to potatoes consecutively for the longest amount of time (USDA APHIS PPQ, 2006), fields that have been in potato production for the longest period of time (including rotation) (USDA APHIS PPQ, 2006), and fields that have had exposure to equipment that has been purchased from Europe.

For both certified seed and commercial potato acreage, there are two options for sampling:

***1. Perimeter***

At each field, **at least** 10% of the acreage should be sampled at a rate of 3 five-pound composite samples per surveyed acre. The entire five-pound samples will be collected and transported from the field. Each five-pound sample will be processed in its entirety using an approved processing system. Procedures for these processes are available from the USDA (Appendix 4).

The rationale for taking 3 five-pound samples per acre (at 112 sampling points per 1 five-pound sample) is consistent with the experience of European PCN experts who recommend taking as many small cores as possible (EPPO, 2000; Trudgill et al., 2001, Evans 2004). The collection of many small cores is necessary due to the patchy distribution of PCN cysts in the field, which can vary from one part of the field to another (Trudgill et al., 2001; Evans, 2004).

Sampling should focus on the **headlands, turnarounds, and perimeter** of the field. For the purpose of this survey, 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. However, it is imperative that the full perimeter be surveyed. The 10% of acreage to be sampled is a minimum. If sampling of the whole perimeter requires an increase in the percent of field to be surveyed, then additional samples should be taken. GIS, when available, can be utilized in the determination of the sampling areas and sampling points.

***2. Full field (grid) survey***

Fields chosen for full field survey will be surveyed in their entirety using a grid survey (10% of fields for commercial production and all seed fields). One composite sample, comprised of at least 100 five gram sub-samples (cores), will be taken per acre of field. This sampling method will produce 1.07-1.10 pounds of soil (486-500 grams) per acre. Cores should be collected from a rectangular grid of 18 x 24 feet (or approximately 6 by 8 paces). Each approximately 1 pound sample will be processed in its entirety using an approved processing system. Procedures for these processes are available from the USDA (Appendix 4).

The total amount of soil collected per acre is in accordance with the European Union (EU) Council Directive 2007/33/EC (European Union, 2007). Under this directive, a rate of 1500 ml/ ha (486 g/ acre) is used. In the PCN National Survey, the number of cores per acre has been increased from the EU Council Directive rate of 100 cores/ha (40 cores/acre) to 100 cores per acre. This could increase the likelihood of detection without increasing the amount of soil removed from the field and processed in the lab.

## 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. Perimeter Survey:

### A) Mechanical Sampler Survey:

This survey method is to be completed on all fields which are fallow or recently harvested. Given entry permission, this method can also be utilized on recently planted grain fields with minimal impact. The 10% of the field identified for sampling will be sampled with a mechanical (wheel) sampler with 4 chisels and a swath width of 3 square feet (USDA APHIS PPQ, 2006) (Appendix 2). This calibration will result in the collection of 3 samples per acre (approximately 18.7 pounds of soil total) which can detect a level of infestation of 200,000 cysts per acre with 95% confidence (USDA APHIS PPQ, 2006) (Appendix 2). (For mechanical wheel sampler ordering information see Appendix 2).

### B) Hand Sampling Survey:

To be completed on all fields which are recently planted or any field in which entry will be granted. Sampling in this manner will result in minimal or no crop damage. The number of samples per field will be determined based on the 3 samples per acre rate (*i.e.*, a 100 acre field x 10% = 10 acres x 3 samples per acre = 30 samples per 10 acre of headlands). A 4 pace x 4 pace (1 pace = 2.5 feet) sampling method should be utilized to sample the identified headlands. An ideal 4 x 4 sampling method would result in the collection of approximately 4 five-pound samples per acre (USDA APHIS PPQ, 2006). Each soil sample should be collected from a rectangular block of the field in the headlands (Appendix 1). A dip of soil (1 dip = approximately 20 grams) will be taken by trowel or soil probe every 4 paces (1 pace = 2.5 feet), from a total of 112 sampling points in a rectangular block (Appendix 1). The surveyor will walk into the headlands area of the field 4 paces and take 1 sample every 4 paces at 56 sampling points. The surveyor will then walk 4 paces deeper into the field and take 56 more samples, moving in the direction of the starting point. The surveyor will return to the original starting point, 4 paces deeper into the field (Appendix 1). To achieve the an average of three samples per 10% of field acreage target, additional sampling blocks can be added by moving 4 paces to the side or into the field from the first sampling block (Appendix 1). This sampling regime can detect a level of infestation of approximately 200,000 - 300,000 cysts per acre when the entire sample is processed (Appendix 2). Lastly, it is imperative that the number of samples taken per field equal at least an average of three five pound samples per acre for 10% of the field (*i.e.*, 30 samples per 100 acres).

Regardless of whether the mechanical or hand sampling method is used, each five-pound sample will be processed in its entirety using an approved processing system. Procedures for these processes are available from the USDA (Appendix 4).

## 2. Full field (grid) survey:

**A) Mechanical Sampler Survey:** This survey method is to be completed on all fields which are fallow or recently harvested. Given entry permission, this method can also be utilized on recently planted grain fields with minimal impact. The entire field will be

sampled with a mechanical (wheel) sampler with 1 chisel per wheel and a swath width of 7.5 square feet. The sampler should begin the first pass 7.5 feet into the field. To achieve the sample size of approximately 1 pound of soil per acre, the mechanical sampler will sample from every other pass in the field. This will increase the area per sampling point from 54 square feet to 108 square feet and will reduce the weight of soil collected per acre from 1.875 pounds to 0.938. The mechanical sampler will take 2 cores at a time (1 from each wheel); therefore, each pass of the wheels (2 cores) will be from a sample area of 216 square feet. Approximately 200-201 sub-samples (2 cores each) should be taken per acre. This calibration will result in the collection of 1 sample per acre of approximately 0.938 pounds of soil total. This sampling rate can detect a level of infestation of approximately 4,000,000 cysts per acre with 95% confidence (USDA APHIS PPQ, 2006) (Appendix 2). This is an extrapolation from the Golden Nematode Program Manual's Mechanical Sampling Table and communications with the Golden Nematode Program Director and staff (USDA APHIS PPQ, 2006) (Appendix 2). This calibration has not been used in the Golden Nematode Program but will be field tested by the program's staff in the fall of 2007. (For mechanical wheel sampler ordering information see Appendix 2).

**B) Hand Sampling Survey:**

To be completed on all fields which are recently planted or any field in which entry will be granted. Sampling in this manner will result in minimal or no crop damage. One composite sample, comprised of 100 sub-samples (cores), will be taken per acre of field. Each core will be approximately 5 grams (4.86 g is the ideal core size). Cores should be taken with an auger that has been calibrated to the 5 g rate. This sampling method will produce 1.07-1.10 pounds of soil (486-500 grams) per acre. Cores should be collected from a rectangular grid of 18 x 24 feet. The surveyor should begin taking samples at the edge of field, taking a sample every 24 feet until reaching the end of the field. The surveyor should then walk 18 feet into the field and begin taking cores every 24 feet.

It is important to properly identify the potato shipments being delivered as either seed or non-seed lot. Pertinent information, including field name, field number, grower name, and date of survey, etc., should be recorded.

## Processing of Samples and Data Management

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 ARS Nematology Laboratory for confirmation. 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, NPDN, 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 (Appendix 3).

The initial cost estimate of $69 per sample was calculated based on actual costs in the Idaho and the New York programs. Reducing the amount of soil processed in the diagnostic process may help in maintaining the $69 per sample for the national survey. While this may work for states currently equipped with the necessary diagnostic infrastructure, it may not for other states. APHIS PPQ will work with states that may require additional support, especially in the area of diagnostic infrastructure. Suspect samples should be submitted using the forms and instructions provided in Appendix 4.

It is important to note that the long term goal of the plan will be based on field survey and that entire samples (approx. 5 lbs.) will be processed. We recognize that in order to accomplish this, it will be necessary to leverage existing infrastructure. The proposed methodology is to be used as a minimum. The 10% acreage of 100% of seed fields will not detect cysts that are potentially present in the additional 90% of the seed acreage within that field. The collection and processing of 3 five-pound soil samples per acre (approximately 15-18.7 lbs. of soil, depending on sampling method and soil type) can detect a level of infestation of approximately 200,000 - 300,000 cysts per acre with 95% confidence (USDA APHIS PPQ, 2006) (Appendix 2). If the entire soil sample is not processed and only two packed 500 cc sub-samples are processed per five pound sample, (500 cc = approximately 1 lb, 3 samples per surveyed acre x 2 lbs. of soil processed = 6 pounds of soil processed per surveyed acre), the effective sampling detection rate is lowered to between 500,000 and 1,000,000 cysts per acre. It is important to keep in perspective that this rate is still only in relation to the approximately 10% of the field that is sampled and does not apply to the other 90% of the field.

## References Cited


EPPO Standards. Phytosanitary Procedures. *Globodera pallida* and *G. rostochiensis*. Soil Sampling Methods. 1998.

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

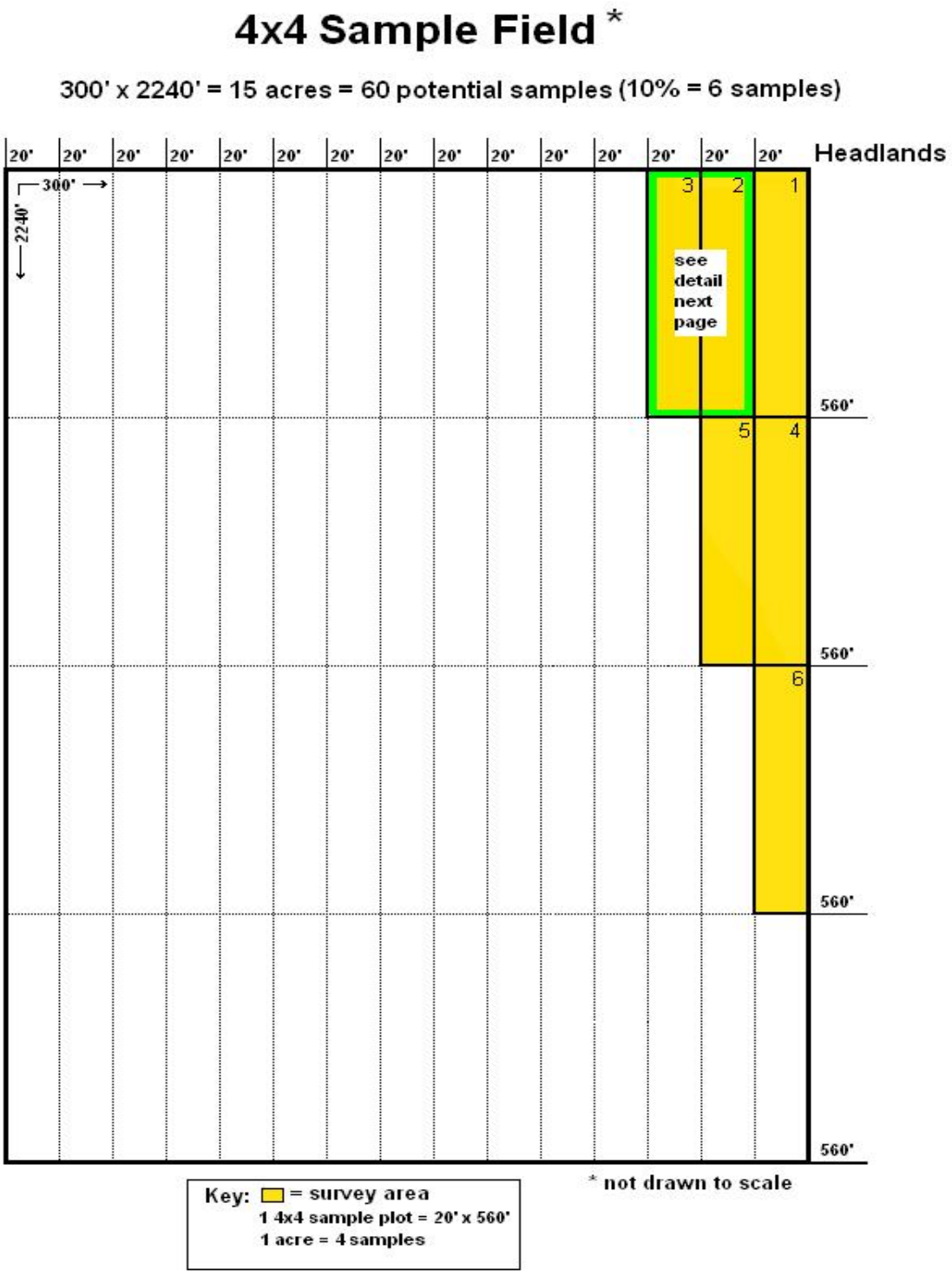
Evans, K., Barker, A.D.P. 2004. Economies in nematode management from precision agriculture – limitations and possibilities. Nematology Monographs & Perspectives 2, 23-32.

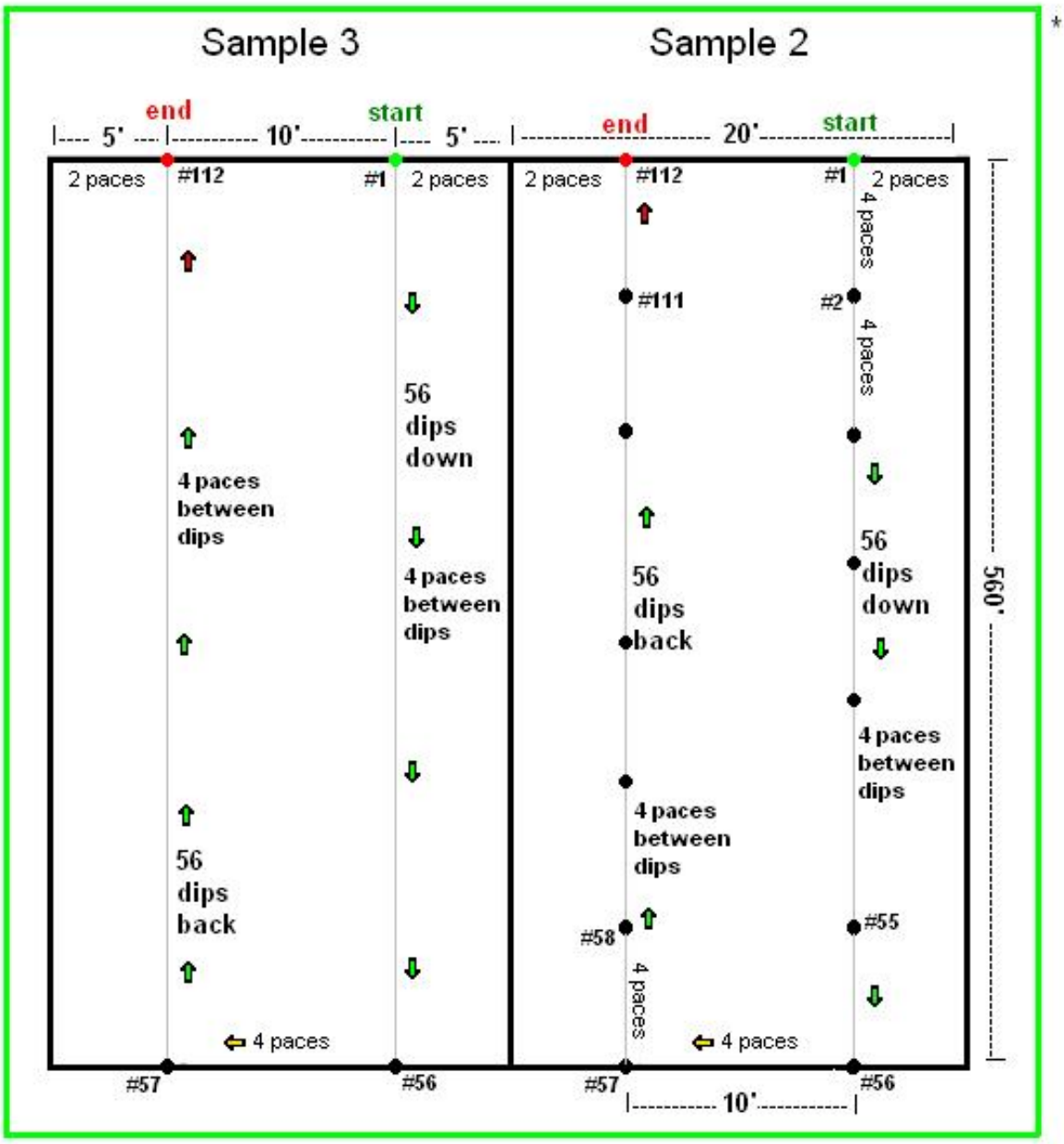
EU (1969) Council Directive 69/465/EEC of December 1969 on control of potato cyst eelworm. Official Journal of the European Communities L323/3, 563-564. (*currently in the process of a new proposal which has not been ratified*)

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

USDA APHIS PPQ. 2006. Golden Nematode Program Manual: Interim Edition. http://www.aphis.usda.gov/ppq/manuals/online_manuals.html#Golden

**Appendix 1. Hand sampling diagram (4 X 4).** **Diagram by Michael Aita (APHIS).**






* not drawn to scale

Key:
1 pace = 2.5'
● = 1 dip (sample point)
1 Sample = 112 dips
⇧ = direction & path of surveyor

**Appendix 2. Golden Nematode Program Manual: Mechanical Sampling Table**

Figure 2-3-4 contains a selection guide for collecting samples by machine.

| Cysts per Acre[1] | Number of Chisels/Wheels | Swath Width (in square feet) | Area per Sample Point | Pounds of Soil Per Acre[2] |
|---|---|---|---|---|
| 50,000 | 8 | 1.15 | 1.25 | 74.9 |
| 100,000 | 8 | 3 | 2.50 | 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 |
| 1,000,000 | 2 | 7.5 | 5 | 3.75 |

**FIGURE 2-3-4 Selection Guide for Collecting Soil Samples by Machine**

1 Detection level based on sampling from top 4 in, 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 one gram of soil per sample point.

## Appendix 3. Laboratory Methods

### Introduction

Laboratory methods that are acceptable for cyst extraction include sugar centrifugation, USDA cyst extractor, and Fenwick can sieving. The following protocols are recommended by APHIS-PPQ-CPHST for use in extracting potato cyst nematodes, *Globodera* spp., from soil samples. The protocols should 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.

### 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. Processing effluent should be treated in soil traps or released into sewer system which connects to a water treatment system. Soil should be sterilized by any approved method or collected and deposited in landfill for burial.

**I. Recommended USDA-Cyst-Extractor w/ or w/o Acetone Purification Protocol for Extraction of *Globodera* spp. Cysts.**

**A. <u>Materials and Equipment</u>**

a. Materials needed
- Acetone (optional)
- Milk filter sock or filter paper

b. Equipment needed
- USDA-Cyst Extractor apparatus
- No. 4 (4.75 mm), No. 20 (850 µm) and No. 60 (250 µm) US Standard mesh sieves
- Jars

**B. <u>Extraction for each sample</u>**

a. Screen thoroughly air-dried soil sample through a No. 4 (4.75 mm) sieve.
b. Record the weight of the sample and add the entire (approx. 5-12 lb) sample of screened soil into the container.
c. Place the nested No. 20 sieve over the No. 60 (250 µm) sieve setup under the overflow spout.
d. Turn on the fast and slow water control valves, allowing water to flow into the container from the bottom. Use sticks or other appropriate utensils, which can be cleaned between samples, to help mix the soil and water.
e. When the soil/water mixture is within five inches of the container top, turn off the fast water control valve and let the mixture slowly rise until it reaches the overflow spout.
f. Floats (cysts and organic material) will be collected on a No. 20 sieve nested over a No. 60 sieve.
g. Rinse the sides of the container to wash any cysts or material off of the walls as water continues to overflow into the nested sieves.
h. Continue to wash until the water runs clear (usually less than 10 minutes depending on soil type and amount).
i. While the water is running, rinse the No. 20 sieve thoroughly to completely wash the cysts through the No. 20 sieve.
j. Remove the No. 20 sieve and clean thoroughly to prepare for the next sample.
k. After the water runs clear, remove the No. 60 sieve, tilt at an angle, and concentrate the collected material and cysts on the No. 60 sieve by carefully washing toward the edge of the sieve.
l. Carefully rinse the material from the No. 60 sieve into a funnel that is attached to a milk filter sock suspended inside a jar.
m. Rinse the cysts and organics into the bottom of the filter sock, squeeze off excess water, and place a label inside the sock.
n. Thoroughly clean the Extractor apparatus, funnel, and No. 60 sieve in preparation for the next sample.
o. Let the filter sock air dry overnight.
p. Break apart the clumps of extract using hands or a spatula.

q. Pay strict attention to maintaining sample identification and record keeping throughout the process.

**Optional, to further separate the cysts from the collected material:**

r. Under a fume hood, slowly place the milk sock into a jar of acetone. Cysts and some debris will float to the perimeter of the sock.
s. Mix the sediments with a spatula and slowly lift the sock up and down several times (time in acetone should not exceed 4 minutes).
t. Squeeze off excess acetone and carefully cut off the bottom of the sock containing the junk material.
u. Dry the extract in the remaining section of the sock under a fume hood.
v. Once dry, cysts may be counted directly on the milk sock using a dissecting microscope or stored until reading occurs.

**References:**


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

**II. Recommended Centrifugation and Sugar Flotation Protocol for Extraction of *Globodera* spp. Cysts.**

**A. Materials and Equipment**

a. Materials needed
- Parafilm
- Sucrose

b. Equipment needed
- Large metal bowls or other suitable mixing container
- No. 20 (850 µm), No. 60 (250 µm) and No. 70 (212 µm) US Standard mesh sieves
- 50 and 150 mL beaker
- 50 mL round bottom centrifuge tubes
- Centrifuge
- Collection bucket or bowl

**B. Extraction for each sample**

a. Prepare sucrose solution: 680 g of sucrose is dissolved in 1 liter of water.
b. 500 cc of thoroughly mixed (with any clumps broken apart) and homogeneous soil is added to a metal bowl or other mixing container of sufficient capacity to cover the soil by several inches of water.
c. Water is added to the bowl to a sufficient volume to completely cover the soil by several inches of water, and the mixture allowed to soak for 20 minutes.
d. After the soaking period, soil and water should be thoroughly mixed by hand (or other stirring device) and clumps broken apart,
e. Pour the water/soil mixture through a No. 20 (850 µm) mesh sieve screen suspended over a clean catch bowl or bucket in 4 aliquots if necessary.
f. Briefly rinse from above the debris caught on the No. 20 mesh sieve into the catch bowl.
g. Allow to settle for 1 minute. Pour the water portion carefully through a No. 60 mesh sieve.
h. Carefully rinse to concentrate collected material and cysts and wash the cysts from the No. 60 mesh sieve into a 1 clean labeled 50 ml beaker using water.
i. Pour beaker contents into 50 ml round bottom centrifuge tubes.
j. Centrifuge tubes at 400 *g* for 5 minutes.
k. Carefully decant the supernatant from tubes and discard.
l. Add the sucrose solution to centrifuge tubes and mix thoroughly.
m. Centrifuge tubes at 400 *g* for 1 minute.
n. Decant tubes over a No. 70 mesh sieve.
o. Carefully and thoroughly rinse the sucrose solution off of the sieve with water.
p. Rinse and clean all sieves and bowls completely in order to prepare for the next sample to avoid any cross contamination from the previous sample.
q. Repeat b-o if processing the entire soil sample
r. Using tap water in a water bottle, carefully rinse the cysts from the sieve into a 50 ml beaker and prepare for storage or rinse directly into a reading chamber if sample is to be examined immediately.

s. Pay strict attention to maintaining sample identification and record keeping of entire volume of soil processed for each sample throughout the protocol.

**References:**


1. Barker, K.R., Carter, C.C., and Sasser, J.N. 1985. An Advanced Treatise on Meloidogyne. Raleigh, NC.
2. Hafez, S.L. May 2006. SOP: Soil Processing for Nematode Analysis. University of Idaho – Parma.
3. Hafez, S.L. May 2006. SOP: Sugar Floatation Technique for Nematode Extraction. University of Idaho – Parma.

## III. Recommended Fenwick Can Protocol for Extraction of *Globodera* spp. Cysts.

### A. Materials and Equipment

a. Materials needed
   - Parafilm
   - Acetone (optional)
   - Milk filter sock

b. Equipment needed
   - Fenwick can apparatus
   - No. 8 (236 mm) and No. 60 (250 µm) US Standard sieves
   - Jars

### C. Extraction for each sample

a. Screen thoroughly air-dried soil through a No. 8 sieve (236 mm).
b. Add 500cc of screened soil that has been thoroughly mixed and is a representative homogeneous sample into the top container/screen (1 mm) of the Fenwick can.
c. Place the No. 60 (250 µm) sieve under the outflow spout.
d. Wash the soil sample through the top screen via the funnel apparatus into the Fenwick.
e. The majority of the cysts and organic material will float up and over the lip of the apparatus while soil and other heavy particles settle to the bottom of the apparatus. The floating particles will collect on the No. 60 (250 µm) sieve located below the outflow. Continue to wash until the water exiting the can runs clear.
f. Conduct a final thorough wash of the top container through the screen into the middle funnel apparatus. (Make sure to completely clean out the screen mesh between samples)
g. Remove the top screen and wash the middle funnel thoroughly.
h. Remove the No. 60 sieve with the collected material from the area of the Fenwick can and thoroughly clean the Fenwick can apparatus in preparation for the next sample. Remove the stopper near the base of the Fenwick at this point if it has one. **Replace stopper after cleaning**.
i. Gently tilt the No. 60 sieve and using a water stream, preferably from a plastic squeeze bottle, rinse the cysts and collected material down to a concentration point on the lower section of the sieve in order to prepare the sample for step j.
j. Carefully wash the cysts and organic layer from the No. 60 sieve into a funnel that is attached to a milk filter sock suspended inside a jar.
k. Rinse the cysts and organics into the bottom of the filter sock, squeeze off excess water and place a label inside the sock.
l. Thoroughly clean the funnel and No. 60 sieve in preparation for the next sample.
m. Repeat steps a-l as necessary to process the entire soil sample.
n. Being careful to record the total volume of soil processed for the entire sample.
o. Let the filter sock air dry overnight.
p. Break apart the clumps of extract using hands or a spatula.
q. Pay strict attention to maintaining sample identification and record keeping throughout the process.

**Optional, to further separate the cysts from the collected material:**

r. Under a fume hood, slowly place the milk sock into a jar of acetone. Cysts and some organic debris will float to the perimeter of the sock.
s. Mix the sediments with a spatula and slowly lift the sock up and down several times (time in acetone should not exceed 4 minutes).
t. Squeeze off excess acetone and carefully cut off the bottom of the sock containing the waste material.
u. Dry the extract in the remaining section of the sock under a fume hood.
v. Once dry, cysts may be counted directly on the milk sock using a dissecting microscope or stored until reading occurs.

**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.

## IV. Recommended USDA Semi-Automatic Elutriator Protocol w/ Centrifugation and Sugar Flotation for Extraction of *Globodera* spp. Cysts.

**Introduction:**

The following protocol is recommended by APHIS-PPQ-CPHST for use in extracting potato cyst nematodes, *Globodera* spp., from soil samples. The protocol should 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 if entire sample is not being processed. Furthermore, this procedure is specific to only to mature nematode cysts and not to vermiform nematodes. Additional extraction procedures will need to be utilized to extract and collect vermiform nematodes.

### A. Materials and Equipment

a. Materials needed
   - Sucrose
   - Parafilm

b. Equipment needed
   - Semi-Automatic Elutriator
   - No. 10 (2.00 mm), No. 20 (850 µm) and No. 60 (250 µm), No. 70 (212 µm) US Standard mesh sieves
   - 50 and 150 mL beaker
   - 50 mL round bottom centrifuge tubes
   - Centrifuge

### B. Extraction for each sample

a. Place clean No. 60 mesh sieves in the appropriate locations on the elutriator for each sample to be collected.
b. Add 500 cc of thoroughly mixed and homogenous soil per sample to the elutriator with air and water flowing (minimum 0.6 l/min) at the appropriate rates.
c. Allow elutriator to run for 3-4 minutes, catching cysts on the No. 60 sieve.
d. Prepare sucrose solution
   - 680 g of sucrose is dissolved in 1 liter of water.
e. Wash the collected material and cysts from the No. 60 mesh sieve into a clean labeled 150 ml beaker using water.
f. Pour beaker contents into 50 ml round bottom centrifuge tubes.
g. Centrifuge tubes at 400 *g* for 5 minutes.
h. Carefully decant the supernatant from tubes and discard.
i. Add the sucrose solution to the centrifuge tubes and mix thoroughly.
j. Centrifuge tubes at 400 *g* for 1 minute.
k. Decant tubes over a No. 70 mesh sieve.
l. Carefully and thoroughly rinse the sucrose solution off of the sieve using tap water.
m. Repeat steps a-l until the entire sample is processed.

n. Using tap water in a water bottle, carefully rinse the cysts from the screen into a 50 ml beaker and prepare for storage or rinse directly into a reading chamber if sample is to be examined immediately.
o. Thoroughly wash and clean the elutriator and sieves in preparation for the next set of samples to be processed.
p. Pay strict attention to maintaining sample identification and record keeping throughout the process.

**References:**


1. Barker, K.R., Carter, C.C., and Sasser, J.N. An Advanced Treatise on Meloidogyne. Raleigh, NC. 1985.
2. Hafez, S. Sugar-Flotation Technique for Nematode Extraction. May 2006.

**V. Recommended USDA Semi-Automatic Elutriator Protocol w/ Acetone Purification for Extraction of *Globodera* spp. Cysts.**

**Introduction:**

The following protocol is recommended by APHIS-PPQ-CPHST for use in extracting potato cyst nematodes, *Globodera* spp., from soil samples. The protocol should 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 if entire sample is not being processed. Furthermore, this procedure is 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.

**A. Materials and Equipment**

a. Materials needed
   - Acetone
   - Milk filter socks

b. Equipment needed
   - Semi-Automatic Elutriator
   - No. 10 (2.00 mm), No. 20 (850 µm) and No. 60 (250 µm) US Standard mesh sieves
   - Jars

**B. Extraction for each sample**

a. Place clean No. 60 mesh sieves in the appropriate locations on the elutriator for each sample to be collected.
b. Add 500 cc of thoroughly mixed and homogenous soil per sample to the elutriator with air and water flowing (minimum 0.6 l/min) at the appropriate rates.
c. Allow elutriator to run for 3-4 minutes, catching cysts on the 60 mesh sieve.
d. Wash the collected material and cysts from the No. 60 sieve into a clean funnel that is attached to a milk filter sock suspended inside a jar.
e. Thoroughly wash and clean the elutriator and sieves in preparation for the next set of samples to be processed.
f. Rinse the cysts and organics into the bottom of the filter sock, squeeze off excess water and place a label inside the sock.
g. Repeat steps a-f until entire sample is processed if necessary.
h. Pay strict attention to maintaining sample identification and record keeping throughout the process.
i. Let the filter sock air dry overnight.
j. Break apart the clumps of extract using hands or a spatula.
k. Under a fume hood, slowly place the milk sock into a jar of acetone. Cysts and some organic debris will float to the perimeter of the sock.
l. Mix the sediments with a spatula and slowly lift the sock up and down several times (time in acetone should not exceed 4 minutes).

m. Squeeze off excess acetone and carefully cut off the bottom of the sock containing the waste material.
n. Dry the extract under a fume hood.
o. Once dry, cysts may be counted directly on the milk sock using a dissecting microscope or stored until reading occurs.

**References:**


1. Barker, K.R., Carter, C.C., and Sasser, J.N. An Advanced Treatise on Meloidogyne. Raleigh, NC. 1985.
2. Wang, X. and Thurston, D. PCN/GN Cysts and Acetone Purification. May 2006.

**Appendix 4. PCN National Survey Diagnostics Reporting Protocol**

# Potato Cyst Nematode (PCN) National Survey

*Globodera pallida*

## Diagnostics Reporting Protocol

## May 2007

Samples shall be collected from the field in a manner consistent with the national survey sample collection protocol. State programs will forward all samples needing identification through the PPQ Survey Identifier assigned to their region. 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. Conducts the initial cyst extraction and identification diagnostics of all soil samples according to recommended methods unless other arrangements have been made. Recommended extraction methods are listed at: http://www.aphis.usda.gov/plant_health/plant_pest_info/potato/pcn.shtml

   b. Cooperators will enter pest submission information into the ISIS Database.

   c. 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.

d. 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):

**Samples from Eastern Region States:**

Grace Okeefe
USDA, APHIS, PPQ – Plant Pathologist Identifier
105 Buckhout Lab, Penn State University, University Park, PA 16802
Office (814) 865-9896, Cell (814) 450-7186, Fax (814) 863-8265

**Samples from Western Region States:**

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

e. Identification results will be reported to the submitting laboratory by PPQ. If necessary, specimens may be forwarded to the ARS Nematology Laboratory, Beltsville for final confirmation.

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 | |
| ☐ Leaves, Lower Surface | ☐ Branches | ☐ Buds | ☐ Seeds |
| ☐ 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)*

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

SIGNATURE

DATE

**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<br><br>**EXAMPLE** In 2001, Brian K. Long collected his first specimen for determination of the year. His first collection number is 01-BLK-001<br><br>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<br>• 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:**<br><br>**(Print Name):** | **Agency:**<br><br>**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.}** | | |
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| | | |
| **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. | |
|---|---|
| | |
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| | |
| **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) |
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## *Chain of Custody (COC)Forms*

The best and most efficient method of documenting sample movement and keeping track of sample locations is proper use of COC forms. Proper documentation of sample movement assures maximum possible security and accountability for sample material. Chain of custody forms should be filled out/signed each time samples change hands. COC's should be treated as sensitive and confidential documents.

Filling out a COC should proceed as follows:

1. Originating facility shall fill out Section 1 completely. In some cases, there will be a standard COC with this section already filled out. The box titled "Date Submitted" should always be filled out on the day samples are initially released for movement.
2. Emergency Contact refers to emergencies regarding samples (loss of, damage to, etc).
3. The originating collection facility should clearly fill out Section 2 with the following information: Field Name(s), Sample Numbers, any important comments. Use a new line for each field name. If necessary, attach another sheet of paper if you have more field names than lines in Section 2. For example:

   **07SDFAR-00001, #1-100 (some samples may be wet**)
   **07SDFAR-00005, #1-50, 52, 67-68 (gaps in sample numbers due to …..**)
4. When information about the shipment contents has been completed, the relinquishing facility shall sign and date line 1 in section 3.
5. In the case that the relinquishing party is not handing the COC directly over to the receiving party, a note should be made below the signature indicating this. Example: "COC form left in locked trailer for pickup." In the event that COC forms must be left unattended for later pick-up, they should always be placed in a secure, locked area. The receiving party should be given notice of where to find the COC when they arrive.
6. A separate COC must accompany each box being shipped.
7. Either fax the COC to the receiving PPQ Domestic Identifier, or call to notify that samples are being forwarded. Place a copy of the COC inside of the box to be shipped prior to taping or sealing the box.
8. Maintain a record of the shipping or tracking number in case the shipment needs to be located.
9. The PPQ Domestic Identifier will sign and date the COC when the samples are received. A signed copy of the COC will be faxed to the submitting office. This will be your confirmation that the samples have been received. If there is any discrepancy on the COC with what samples have been received, the PPQ Domestic Identifier will contact you immediately for resolution.
10. In the event that samples from one shipment are separated and moved after being delivered, a new chain of custody form shall be made for each resulting shipment. This should be noted in Section 6 of the new forms. Example:

"Samples in this shipment were part of a previous larger shipment from Idaho Falls to Boise. This section of that previous shipment is now being forwarded on to the Parma facility."

11. Both relinquishing and receiving parties should keep in contact with the appropriate authorities.