PATHSENSORS

Detecting Pathogens at the Speed of Light

Phytophthora CANARY® Zephyr Assay

User Manual

(catalog 903-153)

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1 Zephyr Assay Components

1.1 Assay Kit Contents

Phytophthora Assay Kit (Catalog 903-153): Sufficient materials are supplied to perform 20 assays based on the protocol outlined in Section 5.

Table 1. Phytophthora CANARY® Zephyr assay kit components (for 903-153).

Material	Amount
Phytophthora Biosensor Reagent	1 X 1 mL
Reconstitution Buffer 2	3 mL
Negative Control Tube (NC)	2 tubes
Positive Control Tube (PC)	2 tubes
Assay Buffer (ND)	60 mL
Extraction Buffer (PL)	60 mL
Phytophthora Capture Beads	2 mL
Foil Sleeve with Assay Barcode	1 sleeve
Assay Tubes	50 tubes

1.2 Reagents, Consumables, and Equipment to be Supplied by User

Reagents:

- 70% Ethanol or 70% Isopropanol Solution

Consumables:

- Filter (Barrier) Pipette Tips (P20, P200, P1000)
- Gloves, Non-Powder
- Light Duty Wipes
- 1.5-2.0 mL Microcentrifuge Tubes
- Disposable, Absorbent Bench Underpads

Equipment:

- Zephyr System (PathSensors Cat. No. 904-124)
- Manual Pipettors (P20, P200, P1000)
- Microcentrifuge(s); Range 200 – 10,000 x *g*
- Liquid Nitrogen Storage Unit
- 37°C Water Bath
- 4°C Refrigerator
- Microcentrifuge Rotisserie*
- Magnetic Stand*
- Personal Protection Equipment

**Note: If the user does not already have these pieces of equipment, they may be ordered from PathSensors using the ordering information provided in Appendix B.*

Note: Additional materials and equipment are required for plant sample preparation, depending on desired sample preparation method. Please refer to Appendix A for recommended sample preparation methods. Sample preparation as outlined in Appendix A will require several additional standard lab materials that will need to be supplied by the user.

1.3 User Manual

It is strongly recommended that the end user read the detailed protocol sections of this user manual before using the kit for the first time. Experienced users may refer to the Quick Reference Guide provided with each kit for an abridged version of the assay protocol.

Look for the Canary Logo throughout the user manual for helpful tips and cautions!

2 Zephyr Assay Description

2.1 Principle of Detection

CANARY® is an immuno- and cell-based biosensor detection platform. Unlike a standard immuno-based assay (i.e., ELISA or lateral flow), the antibodies used for target detection are bound to the surface of a living cell. Target binding by the antibodies stimulates a rapid signal transduction process within the cell, resulting in the release of intracellular calcium. The calcium release excites an intracellular calcium-sensitive luminescent protein, which generates a measurable luminescent light output. By utilizing the cell-based signal transduction process, CANARY® technology allows target detection within seconds after contact between the target and the biosensor, without the use of any additional detection and signal amplification reagents.

2.2 Assay Specifications

Analytical Specificity: The Phytophthora CANARY® Zephyr assay detects all *Phytophthora* species tested below (Table 2) and does not cross-react with *Pythium* or other common soil fungi tested at a concentration of 100 μg/mL or greater of pure culture.

Analytical Sensitivity: The Phytophthora CANARY® Zephyr assay detects down to 1% *Phytophthora ramorum*-infected rhododendron leaves spiked into healthy control leaves with 99% sensitivity and 99% specificity (both with a 95% confidence interval lower bound at 95%).

Sample Matrices: The Phytophthora CANARY® Zephyr assay can detect *Phytophthora* in a variety of plant matrices. PathSensors has validated this assay for use in the following matrices: the leaves of rhododendron, camellia, bay laurel, oak tree, potato and tomato plants; crown and root tissue of strawberry and lavender plants; and fruits of bell peppers and eggplants. Please contact PathSensors about using this assay with plant matrices not listed here.

Sample to Results: 20 minutes (batch sample processing possible to minimize assay time for 20 samples)

Table 2. Analytical specificity of Phytophthora Biosensor to various *Phytophthora* species and co-occurring oomycetes and fungi.

Phytophthora spp.	*Pythium spp.*	Common Co-occurring Fungi
Tested positive at 100 μg/mL **of pure culture**	Tested negative at 100 μg/mL **of pure culture**	Tested negative at 1 mg/mL **of pure culture**
Phytophthora cactorum	*Pythium amasculinum*	*Fusarium oxysporum*
Phytophthora cambivora	*Pythium aphanidermatum*	*Mortierella elongata*
Phytophthora capsici	*Pythium graminicola*	*Sclerotinia sclerotiorum*
Phytophthora cinnamomi	*Pythium helicandrum**	*Trichoderma harzianum*
Phytophthora citrophthora	*Pythium myriotylum*	
Phytophthora kernoviae	*Pythium inflatum*	
Phytophthora infestans	*Pythium irregulare**	
Phytophthora nicotianae	*Pythium spinosum*	
Phytophthora niederhauserii	*Pythium sylvaticum*	
Phytophthora ramorum	*Pythium ultimum*	
Phytophthora sojae		

** low signal observed at 1 mg/mL of pure culture*

2.3 Assay Intended Use

PathSensors' Phytophthora CANARY® Zephyr assay kit is designed to provide rapid and sensitive detection of *Phytophthora spp.* in diseased and asymptomatic samples from ornamental and agricultural plants, utilizing CANARY® technology. The assay kit is designed for use as a primary rapid detection and identification method, in order to allow the initiation of prompt action in the case of an actionable event. Secondary confirmation testing in accordance with industry regulations is strongly recommended.

2.4 Storage and Handling

Kit Component Storage: Upon receipt, transfer the Biosensor Reagents from the dry shipper unit to a permanent on-site liquid nitrogen storage unit. Store the remaining kit components at 2-8°C. Do not mix the reagents from different assay kit lots together.

Biosensor Reagent Handling: The Biosensor Reagent contains biologically active ingredients that must be handled with care. **Under no circumstances should the Biosensor Reagent be vortexed or vigorously agitated.** When thawing, do not leave the reagent in the 37˚C water bath for longer than 2 minutes. Biosensor Reagent should be mixed via gentle trituration or tube inversion. When pipetting the reagent, use gentle pipetting motions. After the reagent is resuspended, it must be equilibrated at room temperature for 30 minutes before use, and it should be used within 8 hours after reconstitution. The Biosensor Reagent should be stored in a stable horizontal position when not in use, and stored in the provided foil sleeve if the reagent is not going to be used for more than 1 hour.

Before running an assay, PSI recommends gently mixing the Biosensor Reagent by gentle inversion 1 -2 times immediately before its first use, and to pull reagent from the middle of the reagent tube. Turning the reagent tube from a horizontal to vertical position just prior to its use for each subsequent sample is adequate for mixing the reagent. If the Biosensor Reagent will not be used for more than 1 hour between samples, it should be mixed by gentle inversion 1 -2 times before use.

3 Safety Information

Safety Data Sheets: Safety data sheets for the kit components are available upon request from PathSensors.

Biosafety Consideration: Follow facility's biosafety/biohazard guidelines when working with plant pathogens and potentially infected materials.

Dry Shipper Safety: The dry shipper used to transport the Biosensor Reagent does not contain liquid nitrogen in a form that can splash or spill. The liquid nitrogen has been absorbed into a porous material lining the dry shipper, and is held in a gas/vapor state within the dry shipper. However, the temperature maintained in the dry shipper is extremely cold (approximately -196˚C) so always **wear insulated gloves when removing contents from the dry shipper.**

4 Quality Assurance

The reagents in the Phytophthora CANARY® Zephyr assay kit have been manufactured following good manufacturing practices and tested with pathogen

to assure proper function. Please see the certification sheet in the kit for further details.

5 Zephyr Assay Protocols

5.1 Frozen Biosensor Reconstitution

Before beginning, review Section 2.4 on how to properly handle the Biosensor Reagent. Follow institution's biosafety/biohazard guidelines for proper disposal of biosensor-contaminated liquid and solid wastes.

1. Remove assay kit from 4°C and allow reagents to come to room temperature (approximately 20 minutes).
   If you have multiple Phytophthora Zephyr kits on hand, be sure to not mix the reagents from different assay kit lots together.

2. Remove the vial of frozen **Phytophthora Biosensor Reagent** from the liquid nitrogen storage unit.
   Wear insulated gloves and face protection when removing reagents from a liquid nitrogen storage container.

3. Place the frozen vial of **Phytophthora Biosensor Reagent** into a 37˚C water bath and thaw until just melted for 2 minutes.
   It is okay if a sliver of ice remains in the tube at the end of 2 minutes.

4. Open the vial and transfer the contents to a separate 1.5-2.0 mL microcentrifuge tube using a P1000 pipettor. If ice crystals are still present, gently mix the solution until the ice has fully melted before transferring.

5. Centrifuge the microcentrifuge tube in a microcentrifuge at **200 x *g* for 5 minutes**.

6. Remove the liquid using a P1000 pipettor, leaving the cell pellet in the bottom of the tube.
   Dispose of liquid waste in accordance to institution's biohazard guidelines.

7. Pipet 1 mL of the room temperature **Recon Buffer (PC)** into the microcentrifuge tube and resuspend the cells by gentle pipetting using a P1000 pipettor.

8. Using a P1000 pipettor, transfer the contents of the microcentrifuge tube back into the **Recon Buffer (PC)** tube, cap the tube, and gently invert a few times to mix the **Phytophthora Biosensor Reagent** with the remaining **Recon Buffer (PC)**.

9. Place resuspended **Phytophthora Biosensor Reagent** inside of the provided **Foil Sleeve with Assay Barcode** and place in a stable horizontal position at room temperature in the dark for **at least 30 minutes** before use.

   *The resuspended reagent should be used **within 8 hours** after reconstitution. If the biosensor reagent is to be left at room temperature for an extended period of time before use, it should be protected from light. Leave the resuspended **Phytophthora Biosensor Reagent** in a horizontal resting position while running assays and mix by **GENTLE** inversion before each use.*

10. Discard remaining resuspended **Phytophthora Biosensor Reagent** at the end of 8 hours.

5.2 Running Reagent Positive and Negative Control Samples

*One positive and one negative control must be run for each reconstituted **Phytophthora Biosensor Reagent** tube to ensure that the reagent from Section 5.1 was reconstituted properly.*

1. Remove a **Negative Control Tube (NC)** and a **Positive Control Tube (PC)** from the kit.

2. Add **200 µL** of **Assay Buffer (ND)** to each tube using a P200 pipettor.

3. Start the **Zephyr System Software** and follow the software prompts to complete system self-diagnostics.

4. Select the **Run 1-Sample** button on the **Home** screen.

5. Scan or enter your **Operator ID** using the barcode scanner or computer keyboard.

6. Press the **Right Arrow** button to progress to the **Assay Selection** screen. Scan the **Phytophthora Cartridge ID Barcode** located on the foil sleeve of the resuspended **Phytophthora Biosensor Reagent** tube using the barcode scanner.

   

   The Zephyr software remembers the last entered barcode in that session, making it unnecessary to rescan the barcode for each sample run from one assay kit.

7. Press the **Right Arrow** button to progress to the **Assay Confirmation** screen. Confirm that the assay type listed on the screen is correct (**Phytophthora**).

8. Select the **Negative Control** sample type button.

9. Advance to the next screen and enter a unique **Sample ID** name for your sample (example: NC).

   

   Any sample ID up to 32 characters can be entered. For ease of use, the software will remember the last entered Sample ID name in that session, making it easy to quickly enter and modify sample names in a given series.

10. Mix the resuspended **Phytophthora Biosensor Reagent GENTLY** by inversion.

11. Transfer **20 µL** of the resuspended **Phytophthora Biosensor Reagent** into the cap of the **Negative Control Tube (NC)** using a P20 pipettor.

12. Gently close the cap and place the tube into the **microcentrifuge** of the Zephyr System with a balance tube in the opposite position. Press the **Right Arrow** button on the computer screen to start the centrifuge. The centrifuge will spin for **5 seconds**.

13. After centrifugation is complete, *quickly* transfer the **Negative Control Tube (NC)** to the **Luminometer** and immediately close the **Luminometer** door to initiate data collection.

    

    As outlined in Section 2.1, light emission from the biosensor reagent begins within moments after interaction with the target antigen. Measuring this early light emission is necessary for accurate software analysis. Therefore the sample transfer from the Zephyr

*system microcentrifuge to the luminometer must be completed within **8 seconds**. Failure to do so will result in an aborted run, and the user will be required to prepare and run a fresh assay tube.*

14. After the data collection is complete, follow the onscreen instructions to reach the **Results** screen. This screen will display the results of the **Negative Control** test.

15. Use the **Home** button located in the upper right hand of the **Results** screen to return to the **Home** screen.

16. If the **Negative Control** has passed, proceed to process the **Positive Control Tube (PC)** in the same manner as the Negative Control (NC) was processed (Section 5.2.1 through 5.2.14).

17. When both the Negative (NC) and Positive (PC) controls have passed, proceed to process unknown plant samples as outlined in Section 5.3.

*If the reconstituted **Phytophthora Biosensor Reagent** fails any of the controls, DO NOT proceed with sample testing and contact technical support at (443) 557-6150 or support@pathsensors.com.*

5.3 Plant Sample Processing

*Before beginning the Phytophthora CANARY® Zephyr assay, crude plant extract samples must be prepared. The method of plant sample preparation is up to the user. However, the supplied **Extraction Buffer (PL)** must be used to prepare the extract to ensure optimal assay performance, and a sample volume of approximately 100 µL or 1 mL is required for each Phytophthora CANARY® assay sample (depending on preparation method used). Plant sample preparation protocols validated by PathSensors are provided in Appendix A: Plant Sample Preparation Methods. Results obtained from other plant sample preparation protocols or plants and tissues not listed in Appendix A are not guaranteed by PathSensors.*

Twenty unique plant samples can be processed and assayed with one Phytophthora CANARY® Zephyr Assay kit. As the reconstituted Phytophthora Biosensor Reagent expires 8 hours after reconstitution, it is recommended that the user collects and groups samples before utilizing the assay kit.

When possible, use aseptic technique for plant sample processing to minimize sample cross-contamination.

Remember to follow user institution's biosafety/biohazard guidelines for proper handling and disposal of plant pathogens, infected plant material, and pathogen-contaminated liquid and solid wastes.

5.3.1 Phytophthora Capture Bead Preparation

1. Label a 1.5-2.0 mL microcentrifuge tube for each plant sample to be tested.

2. Flick the bottom of the **Phytophthora Capture Beads** tube for 10 seconds to thoroughly resuspend the capture beads.

3. Transfer **100 µL** of the **Phytophthora Capture Beads** to each of the labeled microcentrifuge tubes using a P200 pipettor. Cap and set aside while preparing the crude plant extracts.

   *For simplicity, the microcentrifuge tube containing the capture beads and plant sample extract is referred to as the **Capture Tube** for the rest of 5.3.*

5.3.2 Phytophthora Antigen Capture

1. After plant sample extract has been added, flick the bottom of each **Capture Tube** to thoroughly mix the contents.

2. Place the **Capture Tubes** on a microcentrifuge rotisserie and gently rotate the tubes **for 10 minutes** at room temperature.

3. Remove the tubes from the rotisserie and tap them on the bench to ensure no residual liquid remains in the cap of the tube.

4. Place the **Capture Tubes** on a microcentrifuge magnetic stand for **2 minutes.**

   *Brown-colored magnetic beads should be visible on the side of the **Capture Tubes** after this step. If the magnetic beads are not clearly visible due to the presence of plant material, disperse the plant material by gently pipetting the material/bead pellet inside **the Capture Tube** a few times using a P1000 pipettor. Then place the **Capture Tube** back onto the magnetic stand for an additional 2 minutes to recapture the magnetic beads.*

5. Slowly remove the supernatant from the **Capture Tubes** *while they are on the magnetic stand* with a P1000 pipettor.

   Liquid waste contaminated with Phytophthora should be properly disposed according to institute's biosafety guidelines.

   The Capture Bead pellet may slide down the magnet a little and this is okay as long as the pellet does not fall off of the magnet. If the pellet falls off of the magnet, resuspend the beads in the supernatant, repeat step 5.3.2.4 to recollect the Capture Beads, and proceed to step 5.3.2.5.

6. While the **Capture Tubes** are on the magnetic stand, gently rinse the **Phytophthora Capture Beads** by slowly adding **1 mL of Assay Buffer (ND)** along the tube wall opposite of the bead pellet using a P1000 pipettor.

   DO NOT *disturb the Capture Bead pellet while rinsing. Doing so will result in loss of sample material. If the beads are accidentally disturbed or resuspended, repeat step 5.3.2.4 to recollect the Capture Beads, and then proceed to step 5.3.2.7.*

7. Using a P1000 pipettor, slowly remove the supernatant from the **Capture Tubes** *while they are on the magnetic stand.*

   The Capture Bead pellet may slide down the magnet a little and this is okay as long as the pellet does not fall off of the magnet. If the pellet falls off of the magnet, resuspend the beads in the Assay Buffer (ND) wash, repeat step 5.3.2.4 to recollect the Capture Beads, and proceed to step 5.3.2.7.

8. Using a P1000 pipettor, add **1 mL of Assay Buffer (ND)** to each **Capture Tube**, close the lid, and remove from the magnetic stand.

9. With a pen or marker, label the lid of an **Assay Tube** for each plant sample to be tested.

   DO NOT *place labels on the side of the assay tubes, as this will interfere with the luminometer detection of the luminescence signal from the biosensors.*

10. Flick the bottom of each **Capture Tube** for **10 seconds** and then immediately transfer **200 µL of the solution** to the corresponding **Assay Tube** using a P200 pipettor.

11. Proceed to Section 5.4.

5.4 Running Samples on Zephyr System

After plant samples have been processed, proceed to run the samples on the Zephyr System.

1. Select the **Run 1-Sample** button on the **Home** screen.

2. Scan or enter your **Operator ID** using the barcode scanner or computer keyboard.

3. Press the **Right Arrow** button to progress to the **Assay Selection** screen. Scan the **Phytophthora Cartridge ID Barcode** located on the foil sleeve of the resuspended **Phytophthora Biosensor Reagent** tube using the barcode scanner.

   

   The Zephyr software remembers the last entered barcode in that session, making it unnecessary to rescan the barcode for each sample run from one assay kit.

4. Press the **Right Arrow** button to progress to the **Assay Confirmation** screen. Confirm that the assay type listed on the screen is correct (**Phytophthora**).

5. Proceed to the **Sample Type** screen and select **Sample** as your sample type.

6. Continue to the **Sample ID** screen and enter a unique identification name for your sample.

   

   Any sample ID up to 32 characters can be entered. For ease of use, the software will remember the last entered Sample ID name in that session, making it easy to quickly enter and modify sample names in a given series.

7. Mix the resuspended **Phytophthora Biosensor Reagent** by gentle inversion.

8. Transfer **20 µL** of the resuspended **Phytophthora Biosensor Reagent** into the cap of the sample **Assay Tube** using a P20 pipettor.

9. Gently close the cap and place the tube into the **Microcentrifuge** of the Zephyr System with a balance tube in the opposite position. Press the **Right Arrow** button on the computer screen to start the centrifuge. The centrifuge will spin for **5 seconds**.

10. After centrifugation is complete, *quickly* transfer the **Assay Tube** to the **Luminometer** and immediately close the **Luminometer** door to start data collection.

    

    *As outlined in Section 2.1, light emission from the biosensor reagent begins within moments after interaction with the target antigen. Measuring this early light emission is necessary for accurate software analysis. Therefore the sample transfer from the Zephyr system microcentrifuge to the luminometer must be completed within **8 seconds**. Failure to do so will result in an aborted run, and the user will be required to prepare and run a fresh assay tube.*

11. At the end of the 1-minute data collection, follow the onscreen instructions to reach the **Results Screen** where a positive or negative result for the sample will be indicated on screen.

12. Use the **Home** button located in the upper right hand corner of the **Results** screen to return to the **Assay Start** screen.

13. Repeat steps 1-12 from Section 5.4 until all unknown plant samples have been tested on the Zephyr system.

Appendix A Plant Sample Preparation Methods

The following plant sample processing protocols have been validated for use with the Phytophthora CANARY® assay with the described plant matrices (see Section 2.2). Use of other plant sample processing protocols and testing of other plants or other tissues of listed plant types will require user testing and optimization, and the results are not guaranteed by PathSensors. Plant samples can be processed using BioMasher-I (Section Appendix A.1), a high-throughput tissue homogenizer (e.g. FastPrep®-24) (Section Appendix A.2), or by mesh bags (Section Appendix A.3). After samples are processed, proceed to Section 5.3 Plant Sample Processing.

When possible, use aseptic technique for plant sampling and processing to minimize sample cross-contamination. We recommend processing samples inside a decontaminated biosafety cabinet, on top of a clean paper towel or duty wipes placed on a disposable absorbent bench underpad. Use clean gloves, razor blades, weigh boats, and paper towels between the processing of each sample to minimize sample cross-contamination.

Remember to follow user institution's biosafety/biohazard guidelines for proper handling and disposal of plant pathogens, infected plant material, and pathogen-contaminated liquid and solid wastes.

While it is recommended that plant samples be processed right away, whole tissues can be stored at 4°C for 2-4 days, while harvested lesions can be stored at -80°C for up to two weeks.

Appendix A.1 BioMasher-I Sample Processing

The following protocol has been validated for testing of leaf tissue from oak trees, rhododendron, camellia, bay laurel, and potato. Testing of other plants or other tissues of listed plant types will require user testing and optimization, and are not guaranteed by PathSensors.

Additional Materials and Equipment:

- Balance, Capable of Weighing 100-200 mg
- Weigh Papers or Weigh Boats
- Disposable Single-edge Razor blades or Scalpels
- Microcentrifuge(s); Range 200 – 15,000 x *g*

- BioMasher-I, 1.5 mL Disposable Homogenizers Without O-Ring, With Polypropylene Filter (catalog # NIP-30-1.5 or NIP-120-1.5, Nippi)
- PowerMasher II (catalog # 891300, Nippi)

1. Label a **BioMasher-I tube assembly** for each sample to be tested. One **BioMasher-I tube assembly** consists of a collection microfuge tube, a filter column, and a pestle.
   
   Label tubes on the side to avoid smearing or loss of the labels during processing.

2. Remove approximately **100 mg of tissue** from the plant sample, ideally from the lesions (from lesion margins if possible) of symptomatic plant tissue. Chop the tissue finely with a clean razor blade, and transfer into the **filter column** of a labeled **BioMasher-I tube assembly** from previous step.

3. Add **200 µL of Extraction Buffer (PL)** into the filter column of each of the **BioMasher-I tube assembly** containing a plant sample using a P200 pipettor.
   
   Compatibility of the PathSensors' Phytophthora CANARY® assay with other extraction buffers should be determined by the end users.

4. Insert a clean pestle and homogenize the plant tissue in **BioMasher-I tube assembly** by simultaneously pressing the pestle down towards the filter and rotating the pestle against the tissue. This can be done by hand or with the aid of a **PowerMasher for approximately 30 seconds to 1 minute.** The sample is adequately homogenized when the tissue has been broken up and the extraction buffer takes on a uniform green or brown color.
   
   Do not pull the pestle or filter column out once homogenization begins, as the liquid tends to wick up between the pestle, column, and the collection tube.

5. Centrifuge the entire **BioMasher-I tube assembly** in a microcentrifuge at **15,000 x *g* for 30 seconds**.

6. Remove **BioMasher-I tube assembly** from the centrifuge and discard the pestle and filter column, keeping the collection tube.

7. Without touching the pellet, transfer **100 µL of supernatant** from the collection tube to the appropriate **Assay Tube** from Step 5.3.1.3 using a using a P200 pipettor.

8. Add **1 mL of Extraction Buffer (PL)** to the **Assay Tube** using a P1000 pipettor.

9. Proceed to Section 5.3.2.1.

Appendix A.2 FastPrep®-24 Sample Processing

The following protocol has been validated for testing of leaf tissue from oak trees, rhododendron, camellia, bay laurel, and potato. Testing of other plants or other tissues of listed plant types will require user testing and optimization, and are not guaranteed by PathSensors.

Additional Materials and Equipment:

- Balance, Capable of Weighing 100-200 mg
- Weigh Papers or Weigh Boats
- Disposable Single-edge Razor blades or Scalpels
- Microcentrifuge(s); Range 200 – 15,000 x *g*
- High-Throughput Tissue Homogenizer, e.g. FastPrep®-24 Instrument (catalog # 116004500, MP Biomedicals)
- Lysing Matrix A Tubes (catalog # 116910, MP Biomedicals)

1. Label a **Lysing Matrix A tube** for each sample to be tested.
   Label tubes on the side to avoid smearing or loss of the labels during FastPrep®-24 processing.

2. Remove approximately **100 mg of tissue** from the plant sample, ideally from the lesions (from lesion margins if possible) of symptomatic plant tissue. Chop the tissue coarsely with a clean razor blade, and transfer into a labeled **Lysing Matrix A tube** from previous step.

3. Add **1 mL of Extraction Buffer (PL)** to each of the **Lysing Matrix A tube** containing a plant sample using a P1000 pipettor.
   Compatibility of the PathSensors' Phytophthora CANARY® assay with other extraction buffers should be determined by the end users.

4. Homogenize the plant tissue in **Lysing Matrix A tubes** using a **FastPrep®-24 instrument set at 6.0 m/s for 40 to 60 seconds.**

Optimum processing time and speed should be determined for each sample type. PathSensors recommends 40 seconds for potato leaves and 60 seconds for rhododendron, camellia, and bay laurel leaves at 6.0 m/s.

5. Centrifuge the **Lysing Matrix A tubes** in a microcentrifuge at **200 x *g* for 1 minute**.

6. Without touching the pellet, transfer **100 µL of supernatant** from the matrix tube to the appropriate **Assay Tube** from Step 5.3.1.3 using a using a P200 pipettor.

7. Add **1 mL of Extraction Buffer (PL)** to the **Assay Tube** using a P1000 pipettor.

8. Proceed to Section 5.3.2.1.

Appendix A.3 Mesh Bag Sample Processing

The mesh bag protocol has been validated for testing of rhododendron, camellia, tomato, and lavender leaf tissue, crown and root tissue of strawberry and lavender plants, and eggplant and bell pepper fruit. Testing of other plants or other tissues of listed plant types will require user testing and optimization, and are not guaranteed by PathSensors.

Additional Materials and Equipment:

- Balance, Capable of Weighing 100-200 mg
- Weigh Papers or Weigh Boats
- Disposable Single-edge Razor blades or Scalpels
- Microcentrifuge(s); Range 200 – 15,000 x *g*
- Sample Mesh Bags, e.g. Agdia Sample Mesh Bags (catalog # ACC 00930)
- Handheld Tissue Homogenizer or Dead Blow Hammer

1. Remove approximately **150 mg of tissue from the lesions** (from lesion margins if possible) of symptomatic plant tissue and place into a mesh bag.

2. Add **3 mL of Extraction Buffer (PL)** to the mesh bag using a pipette or P1000 pipettor, and homogenize the sample with a hand-held tissue homogenizer

or dead blow hammer. The sample is adequately homogenized when the tissue has been broken up and the extraction buffer takes on a uniform green or brown color.

Compatibility of the PathSensors' Phytophthora CANARY® assay with other extraction buffers should be determined by the end users.

3. Using a P1000 pipettor, transfer the plant extract from the mesh bag to a 1.5-2.0 mL microcentrifuge tube and centrifuge the tubes in a microcentrifuge at **200 x *g* for 1 minute**.

4. Without touching the pellet, transfer **1 mL of supernatant** from the microcentrifuge tube to the appropriate **Assay Tube** from Step 5.3.1.3 using a using a P1000 pipettor.

5. Proceed to Section 5.3.2.1.

Appendix B Ordering Information

To place an order for PathSensors' Phytophthora CANARY® Zephyr Assay Kits, Sample Preparation Accessory Kits, or lab equipment required for the assay, please call or email us and reference the following product numbers.

Item Name	Catalog Number
Phytophthora Zephyr Assay Kit (20 assays)	903-153
BioMasher-I Sample Preparation Kit (20 units)	903-166
Capture Bead Accessory Equipment Kit	904-140

For further information regarding the Phytophthora CANARY® Zephyr Assay Kit, the Zephyr system, troubleshooting assistance, or information on other PathSensors' CANARY® assay kits please visit our website or contact our office:

PathSensors, Inc.
701 East Pratt Street
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Appendix C Terms and Conditions

1. **AGREEMENT.** Only the product ("Product(s)"), price, quantity, and delivery terms contained in Buyer's purchase order, if accepted by Seller, together with the terms and conditions herein constitute an agreement between the parties ("Agreement"). Buyer represents and warrants that it intends to use the Products for its internal use and is not purchasing the products with the intent to resell or distribute the Products, unless authorized by Seller.

2. **CONTROLLING TERMS AND CONDITIONS.** This Agreement shall govern the sale of the Products to Buyer. If Buyer submits any other document that contains terms and conditions which are inconsistent with or in addition to this Agreement then any such term or condition shall not alter these Terms and Conditions or be part of this contract unless expressly accepted or agreed to by Seller in writing. The sale is expressly conditioned upon Buyer's acceptance of this Agreement.

3. **PAYMENT TERMS.** Prices are stated in U.S. dollars. All sales are F.O.B. Origin and payments are due and payable net thirty (30) days after invoice date. Pro rata payment shall be due on partial shipments as made and invoiced. Payments not received by the date due shall bear interest at the rate of twelve percent (12%) per annum or the maximum rate allowed by law, whichever is less, until paid in full. The purchase prices of the Products are exclusive of all applicable sales and use taxes, value added taxes, export fees and duties or other similar fees and taxes. All shipping, handling, taxes, custom duties, tariffs and similar charges shall be at Buyer's expense and, at Seller's option, added to the price of the goods.

4. **SHIPPING TERMS.** Title to and all risk of loss of or damage to the goods shall pass to and be assumed by Buyer F.O.B. Origin. Buyer is responsible for all transportation charges and for filing timely and proper claims against carriers if goods are lost or damaged in transit. Seller's estimated delivery date(s) stated on the front of this contract are approximate and subject to reasonable scheduling changes made after the date hereof. Seller shall not be liable for any loss or expense, whether by contract or tort, incurred by Buyer resulting from failure to meet the estimated delivery date.

5. **MODIFICATION AND CANCELLATION OF ORDERS.** Orders may not be modified or cancelled in whole or in part except by mutual written agreement of the parties. Cancellation of orders shall be effective only at seller's option, and if Seller agrees to any cancellation, Buyer shall be responsible for return freight costs and all costs incurred by Seller as a result of such cancellation, including, but not limited to, a fifteen percent (15%) restocking fee and all labor and material costs incurred prior to termination.

6. **RETURNS.** All returns must be authorized by Seller. A Returned Material Authorization (RMA) number issued by Seller must accompany all returned goods or parts unless otherwise directed by Seller. All goods or parts returned to Seller must be shipped transportation charges prepaid. Seller does not accept collect or C.O.D. shipments.

7. **PACKING.** Unless a preferred packing method is provided for in the purchase order, all goods shall be packaged and packed for shipment and storage in accordance with good commercial practices. Preferred packing charges shall be paid by Buyer.

8. **COMPLIANCE WITH LAWS.** Buyer acknowledges that the goods supplied herewith may be subject to laws, regulations and executive orders ("Laws") of the U.S. relating to export controls. Buyer represents and warrants that the Products will not be used for any purpose prohibited by the Laws and Buyer shall

comply with the Laws as well as all laws and regulations in Buyer's jurisdiction and any other location related to the import, export, re-export, transfer, shipping, and/or use of the Products.

9. **SELLER'S REMEDIES.** If Buyer fails to make timely payments for goods or services accepted, or fails to perform any other of Buyer's obligations set forth herein, Seller may, at its option, defer further shipment(s), revise its terms of payment, cancel the unshipped balance, or pursue any other remedy set forth in this contract or provided by law.

10. **LIMITED WARRANTY.** Unless superseded by individual Product warranty terms formally set forth by Seller to Buyer in writing and/or included in Product Packaging, Seller's standard warranty terms shall be limited to those set forth in this paragraph. For a period of one (1) year after shipment of the Product, Seller will at its option repair or replace on an exchange basis any Product or component part thereof returned to Seller to be defective in material or workmanship. All costs associated with the transportation, troubleshooting, installing or removing the Product or component part thereof shall be paid by Buyer. Repair or replacement of any Product or component thereof will not extend the original warranty period. This limited warranty extends only to Buyer and is not transferable to any other party and any transfer made in violation of this provision shall be void. This limited warranty does not apply to any Product or component which (i) has been subject to misuse, neglect, accident or improper storage; (ii) has been installed, repaired, maintained or altered in any way that in the judgment of Seller has adversely affected the condition of the Product; (iii) has been used, operated or maintained inconsistent with Seller recommendations or with normal practice and conditions, or (iv) has been changed or modified from its original condition. For consumable Product components such as BioDiscs or assay kits, this limited warranty shall be modified as follows: (a) the unopened shelf life shall be the stated Expiration Date marked on the packaging; (b) the opened shelf life shall be 1 day from the date the product packaging is opened; THIS LIMITED WARRANTY IS ONLY A LIMITED WARRANTY TO REPAIR OR REPLACE AND NOT A WARRANTY OF THE CONDITION OR FUTURE PERFORMANCE OF THE PRODUCT. ALL OTHER WARRANTIES, EXPRESS OR IMPLIED, SPECIFICALLY INCLUDING, BUT WITHOUT LIMITATION, WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE AND NON-INFRINGEMENT ARE EXPRESSLY DISCLAIMED. BUYER WAIVES AND IN NO EVENT WILL SELLER BE RESPONSIBLE FOR ANY INCIDENTAL, CONSEQUENTIAL, INDIRECT, SPECIAL, PUNITIVE OR EXEMPLARY DAMAGES OF ANY KIND. No person is authorized to give any other warranty or to assume any additional obligation or liability on behalf of Seller. In no event will Seller's liability hereunder exceed the purchase price of the defective Product. This limited warranty shall not apply if Buyer has unpaid invoices. Buyer agrees that the warranty disclaimer and limited liability set forth herein are fundamental elements of this Agreement and Seller would not be able to provide the Product on an economic basis without such limitations.

11. **GRANTING OF RIGHTS.** Nothing contained herein shall be construed as granting Buyer any right to any intellectual property rights, including but not limited to patents, trademarks, trade secrets, or copyrights, owned or licensed by Seller. Further, Buyer agrees that it shall not, directly or indirectly, reverse engineer, decompile, modify, alter, disassemble or emulate the functionality of the product.

12. **INDEMNIFICATION.** Buyer shall indemnify Seller in the case of any Product misuse by Buyer.

13. **PATENTS.** If Buyer modifies the Product or provides Seller with designs, specifications, or instructions to modify the Product, then Buyer shall hold Seller harmless from and against any expense or loss resulting from infringement of patents or trademarks arising therefrom. Except as otherwise provided in the preceding sentence, Seller shall defend any suit or proceeding brought against Buyer so far as based on a claim that the Products furnished under this Agreement constitute an infringement of any patent of

the United States, issued on or before the date of shipment, if notified promptly in writing and given authority, information and assistance (at Seller's expense) for the defense of same, and Seller shall pay all damages and costs awarded therein against Buyer. In case such goods, or any part thereof, are held to infringe and the use of said goods or part is enjoined, or Seller shall reasonably conclude that the goods are infringing. Seller shall, at its own expense, either procure for Buyer the right to continue using said goods or parts, or replace same with non-infringing goods; or modify such goods so they becomes non-infringing; or remove said goods from the market and refund the purchase price and the transportation and installation costs thereof. The foregoing states the entire liability of seller for patent infringement by the said goods or any part thereof.

14. **FORCE MAJEURE.** Delay in performance or non-performance of any obligation contained herein, other than Buyer's obligation to pay, shall be excused to the extent such failure or non-performance is caused by a force majeure. For purposes of this Agreement, force majeure shall mean any cause or event preventing performance of an obligation under the Agreement which is beyond the reasonable control of Seller or Buyer, as the case may be, including without limitation, fire, flood, power shortage, mechanical breakdown, sabotage, shipwreck, embargo, explosion, strike or other labor trouble, accident, riot, acts of government authority (including, without limitations, acts based on laws or regulations now in existence as well as those enacted in the future), acts of God, war or acts of terrorism, and other events or conditions beyond the reasonable control of the affected party. In the event a force majeure continues for more than ninety (90) days, this Agreement may be terminated without any liability by either party upon written notice thereof to the other.

15. **NO ASSIGNMENT.** Buyer may not assign this Agreement, or its right to receive Products, without the prior written consent of Seller. Any such attempted assignment shall be void.

16. **GOVERNING LAW.** This contract shall be governed by and construed according to the laws of the United States of America and the State of Maryland, including the Uniform Commercial Code as enacted therein, without regard to its laws regarding conflicts of law.

17. **JURISDICTION AND VENUE.** All proceedings relating to this Agreement shall be maintained exclusively in the federal or state courts of Maryland and the parties hereby irrevocably and unconditionally submit and consent to exclusive jurisdiction and venue herein and expressly waive any right to object to personal jurisdiction or venue. Each of the parties hereby consents to the service of process by registered mail or by an express delivery service providing a return receipt at its address set forth above and agrees that its submissions to jurisdiction and its consent to service of process by mail are made for the express benefit of the other party.

18. **SEVERABILITY.** If any provision of this Agreement is held invalid or otherwise unenforceable, the enforceability of the remaining provisions shall not be impaired thereby.

19. **NO WAIVER.** The failure by any party to exercise any right provided for herein shall not be deemed a waiver of any rights hereunder.

20. **ENTIRE AGREEMENT.** This Agreement constitutes the entire agreement between the parties relating to the matter contained herein. This Agreement may not be amended, extended or modified in any matter, orally or otherwise, except by an instrument in writing signed by a duly authorized representative of each party.