# QualiPlate™ Kit for Pepper Mild Mottle Virus (PMMV)

*Catalog Number AP 095*

***Highlights:***

- *Ready-coated plate*
- *Two hour protocol*
- *Consistent quality*
- *Higher throughput*

***Contents of Kit:***

- *Anti-PMMV antibody-coated strip plate (12 strips of 8 wells each)*
- *PMMV Enzyme Conjugate*
- *10X Seed Extraction Buffer (makes 500 mL of 1X Seed Extraction Buffer)*
- *1X Leaf Extraction Buffer (50 mL; additional may be purchased from EnviroLogix, Cat # KR219)*
- *Packet Wash Buffer Salts (makes 1L)*
- *Substrate*
- *Stop Solution*

***Materials Needed:***

- *multi-channel pipette (100 µL)*
- *racked dilution tubes for loading samples into the plate with a multi-channel pipette (optional)*
- *marking pen (indelible)*
- *tape or Parafilm®*
- *timer*
- *distilled or deionized water for preparing Wash Buffer and for diluting 10X Seed Extraction Buffer*
- *seed grinding equipment*
- *leaf extraction equipment*
- *centrifuge (optional, for leaf extracts)*
- *wash bottle, or microtiter plate or strip washer*
- *platform orbital plate shaker with orbital diameter of ≥18mm, set to 150-200 rpm (do not use "microplate shakers" [<5 mm])*
- *microtiter plate reader or strip reader capable of reading **450 nanometers** (nm)*

## Intended Use

The QualiPlate Kit for Pepper Mild Mottle Virus screens for the presence of Pepper Mild Mottle Tobamovirus (PMMV) in seed or leaf extracts. In studies on seed lots determined to be PMMV positive by other test methods and by comparison with controls, this kit was able to consistently detect the presence of the virus (using minimum sample sizes of 3,000 seeds and minimum sub-sample sizes of 500 seeds).

## Preparation of Solutions

- **Wash Buffer:** Add the contents of the packet of Wash Buffer Salts (phosphate buffered saline, pH 7.4 – 0.05% Tween 20) to 1 liter of distilled or deionized water, and stir to dissolve. Store refrigerated when not in use; warm to room temperature* prior to assay. Additional 1L dry packets may be purchased from Sigma Chemicals, Cat#P-3563, or similar recipes may be prepared from salts on site.
- **1X Seed Extraction Buffer:** Bring 10X Seed Extraction Buffer to room temperature*, then stir or shake to dissolve precipitates completely before proceeding. To make 1X Seed Extraction Buffer, add the entire 50 mL bottle of 10X Seed Extraction Buffer to 450 mL of distilled or deionized water in a suitable container, and mix thoroughly to dissolve any remaining precipitates. Store 1X Seed Extraction Buffer refrigerated when not in use; warm to room temperature* prior to assay. Additional 10X or 35X buffer may be purchased from EnviroLogix (Cat#KR160 or Cat#KR186 respectively). See "Notes" section for preparing various volumes of Buffer.

  ***Please note: "room temperature" notation in all instructions is 18-25°C – do not expose kit components or solutions to temperatures above 25°C.**

## Sample Preparation

**Seeds:** The sample must be extracted with prepared **1X Seed Extraction Buffer** at a ratio of 1:10 (gram of seeds to mL of buffer). For example:

- 2.5 g of seed : 25 mL of 1X Seed Extraction Buffer
- 0.25 g of seed : 2.5 mL of 1X Seed Extraction Buffer

All seeds must be thoroughly ground/cracked in order for the internal tissue to come in contact with the buffer. Use equipment appropriate for the size of seed being tested (e.g. Polytron for most complete extraction, coffee grinder, rubber mallet with mesh extraction bag). Soak ground seed tissue in 1X Seed Extraction Buffer for 1 hour minimum at 4°C. Solids will settle to the bottom; use the light-colored upper layer in the assay.

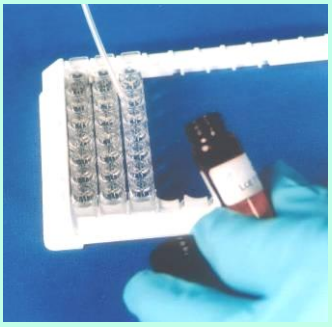
Leaf: The sample must be extracted with 1X Leaf Extraction Buffer at a ratio of 1:10 (gram of leaf tissue to mL of buffer). For example:

- 0.1 g of leaf : 1 mL of 1X Leaf Extraction Buffer

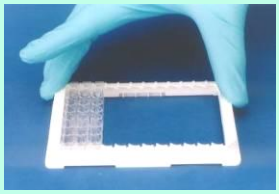
All leaf tissue must be thoroughly macerated in order for ideal sample extraction (e.g. EnviroLogix ACC 002 tube and pestle, mesh extraction bags, bead-beater apparatus). Note: extracts will be foamy.

*Prepare Wash Buffer (and Seed Extraction Buffer, if testing seed)*

*Remove unneeded strips*



*Add Extraction Buffer, controls, and sample extracts*



*Mix plate, incubate*

*All incubation steps must be performed on an orbital shaker with 18+ mm orbital diameter*

Pull off particle-free extract to run in the test. Clarification of extracts by centrifugation is recommended (10 minutes at 1800-5000 x *g*), but not required.

# How to Run the Assay

- Read all of these instructions before running the kit.
- Allow all reagents to reach room temperature before beginning (at least 30 minutes with un-boxed plates and reagents at room temperature (18-25°C) - do not remove strips from bag with desiccant until they have warmed up).
- **Organize all reagents, sample extracts, and pipettes so that step 1 can be performed in 15 minutes or less;** the use of a multi-channel pipette is strongly recommended for all reagent and extract transfers.
- If more than four strips are to be run at one time, the 15 minutes is likely to be exceeded, and the use of a multi-channel pipette is recommended (see "Note" below).
- If four or fewer strips are to be run, use a disposable-tip air-displacement pipette and a clean pipette tip to add Extraction Buffer or sample extract to the wells. Conjugate, Substrate, and Stop Solution may be added in the same manner; alternatively, use a repeating pipette with a disposable tip on the end of the Combitip for each of the three reagents.
- If fewer than all twelve strips are used, reseal the unneeded strips and the desiccant in the foil bag provided, and refrigerate.
- Use the well identification markings on the plate edge as a guide when adding the samples and reagents. It is recommended that at least two wells each of 1X Seed or Leaf Extraction Buffer and known-negative seed or leaf extract be run on each plate. Additional quality control samples may be added at the discretion of the user. Sample extracts may be run in either single or duplicate wells.

1. Add **100 µL** of **1X Extraction Buffer**, **100 µL** of any **user-prepared negative control extract,** and **100 µL** of each **sample extract** to their respective wells. Follow the same order of addition for all reagents. Treat each plate as an independently timed assay.

   **NOTE:** It is strongly recommended that a multi-channel pipette be used in steps 1, 5, 8 and 9.

2. Thoroughly mix the contents of the wells by moving the plate in a rapid circular motion on the bench top for 4-5 seconds. Be careful not to spill the contents!

3. Cover the wells with tape or Parafilm to prevent evaporation and incubate for **30 minutes** at **ambient temperature** on an **orbital shaker (with 18+ mm orbital diameter) at 150 to 200 rpm**. Note: Shaking during incubation steps is mandatory where called for. Failure to do so will result in up to 50% loss in assay sensitivity.

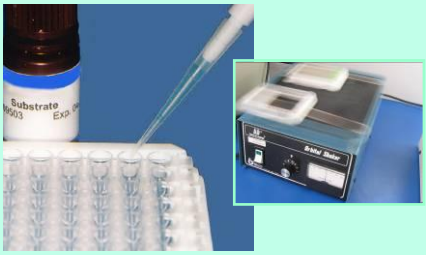
   *Protocol option: For testing convenience, at this point samples may be incubated overnight in the refrigerator (up to 16 hours at 5°C). Allow plates to come to room temperature with the rest of the kit reagents the next morning, before going on to step 4.*

4. After incubation, carefully remove the covering and empty the contents of the wells into a sink or other suitable container by inverting quickly and vigorously shaking the plate. Flood the wells completely with **Wash Buffer**, then empty as directed above. Repeat this wash step three more

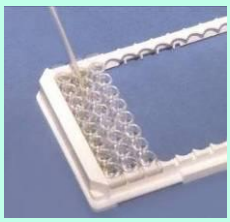
*Bottle Wash method*

*Strip Plate Wash option*

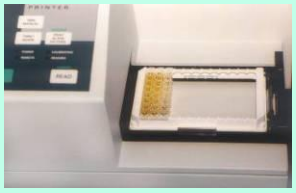
*Add conjugate, mix, incubate, wash*



*Add substrate, mix, incubate*



*Add Stop Solution*



*Read plates in a Plate Reader* ***at 450 nm*** *within 30 minutes of the addition of Stop Solution.*

times. After the final wash, keep the plate inverted and tap firmly on a dry paper towel to remove as much Wash Buffer as possible.

*If samples were incubated overnight, increase number of wash cycles to 8.*

5. Add **100 µL** of **PMMV Enzyme Conjugate** to each well.
6. Thoroughly mix the contents of the wells, as in step 2. Cover the wells with new tape or Parafilm and **incubate** for **1 hour** at **ambient temperature** on an **orbital plate shaker as described above.** Note: Shaking during incubation steps is mandatory where called for. Failure to do so will result in up to 50% loss in assay sensitivity.
7. Wash the wells again as described in step 4. Alternatively, perform four washes (300 µL/well) with a microtiter plate or strip washer.
8. Add **100 µL** of **Substrate** to each well. Thoroughly mix the contents of the wells by moving the plate in a rapid circular motion on the bench top for 20-30 seconds. Cover the wells with new tape or Parafilm and incubate for **30 minutes** (for best results) at **ambient temperature**.
9. Add **100 µL** of **Stop Solution** to each well and mix briefly. This will change the blue color in the wells to yellow. Read the plate at **450 nm**, with a reference wavelength between 600 and 650 nm. Read the stopped plate within 30 minutes; color may fade beyond that time.

**NOTE: Stop Solution is 1 N HCl. Handle carefully.**

# How to Interpret the Results

## Spectrophotometric Measurement

Set the wavelength of the microtiter plate reader to **450 nanometers** (nm). (If it has dual wavelength capability, use 600, 630 or 650 nm as the reference wavelength.)

## Interpreting Results

Compare the Optical Density (OD) of the sample extracts to those of the mean Extraction Buffer wells, or preferably, to known-negative seed or leaf extract wells, to determine presence or absence of PMMV in the sample extract. Samples with absorbances significantly greater than those of the Seed or Leaf Extraction Buffer and/or negative extract wells are presumed to be positive for PMMV.

General Guidelines:

- Mean OD of Extraction Buffer wells should not exceed 0.10.
- Mean OD of PMMV-free seed or leaf extracts should not exceed 0.15.

If test results consistently fall outside these guidelines, please contact EnviroLogix' technical service.

# Precautions and Notes

- Observe any applicable regulations, federal or state guidelines, or in-house lab safety protocols when disposing of samples and kit reagents.
- Store all QualiPlate components at 4°C to 8°C (39°F to 46°F) when not in use.
- Do not expose QualiPlate components to temperatures greater than 37°C (99°F) or less than 2°C (36°F) for optimum performance.
- Allow all reagents to reach ambient temperature (18-25°C) before use.

- Do not use kit components after the expiration date.
- Do not use reagents or test plates from one QualiPlate with reagents or test plates from a different QualiPlate type or different lot number.
- Do not use samples prepared for analysis in other test kits; do not run sample extracts prepared for this assay in other brands of test kits.
- **Do not expose Substrate to sunlight** during pipetting or while incubating in the test wells.
- **Be sure to read the results of stopped color development at 450 nm, not 405 nm.**
- Do not dilute or adulterate test reagents or use samples not called for in the test procedure.
- Quality of results is dependent upon following the assay protocol as directed.
- As with all tests, it is recommended that results be confirmed by an alternate method when necessary.

- **Preparing 1X Seed Extraction Buffer:** The following table shows the formulas for preparing alternative volumes of Seed Buffer. Always make sure the concentrated buffer is in solution before using it.

| **10X Extraction Buffer (KR160, 50 or 1000 mL)** | **Finished Volume** | | | |
|---|---|---|---|---|
| | **10L** | **5L** | **2L** | **0.5L** |
| Start with water (L) | 9 | 4.5 | 1.8 | 0.45 |
| Add 10X Extraction Buffer (mL) | 1000 (1 lg bottle) | 500 | 200 | 50 (1 sm bottle) |

Follow steps in order when diluting 35X Seed Extraction Buffer:

| **35X Extraction Buffer (KR186, 500 mL)** | **Finished Volume** | | | | | |
|---|---|---|---|---|---|---|
| | **35L** | **20L** | **17.5L** | **10L** | **5L** | **2L** |
| 1. Start with water (L) | 34 | 19.43 | 17 | 9.71 | 4.86 | 1.94 |
| 2. Add PVP (g), stir to dissolve | 700 | 400 | 350 | 200 | 100 | 40 |
| 3. Add 35X Extraction Buffer (mL) | 1000 | 571 | 500 (1 bottle) | 286 | 143 | 57 |

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