# Maize Lethal Necrosis (MLN):

## A Technical Manual for Disease Management

**Editor**
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CIMMYT MR
International Maize and Wheat Improvement Center

CGIAR RESEARCH PROGRAM ON Maize

In collaboration with international and national research and development partners

Chapter 5

# Diagnostic Protocols for MCMV and SCMV

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## 1. Introduction

Early and accurate detection of plant viruses is important in surveillance, seed multiplication, and seed exchange. Maize chlorotic mottle virus (MCMV) infection may be difficult to diagnose based on symptoms alone as some of them (stunting, chlorosis) resemble those caused by nutrient deficiencies, moisture stress or other maize-infecting viruses like maize mosaic virus, maize streak virus and maize stripe virus. There are several diagnostic tools that are available for detection of MCMV and sugarcane mosaic virus (SCMV). The most reliable methods for detecting MCMV in host tissues include ELISA (enzyme-linked immunosorbent assay), immunostrips, and polymerase chain reaction (PCR). In this chapter, we present several validated MCMV and SCMV detection methods and suggest their point of use depending on the objective.

## 2. MCMV and SCMV Detection in Leaf Samples using Immunostrips in the Field

### 2.1. Requirements

1. MCMV and SCMV detection immunostrip kits
2. Gloves
3. Scissors
4. Tweezers
5. 1 lt of 10% bleach water solution
6. Cotton wads or gauze
7. Containers (big plastic boxes for carrying out the test under clean conditions (to protect the material and the reagents from dust, dirt etc.)

8. Tissue homogenizer/sap extractor

### 2.2. Procedure

- Collect the leaf tissue using the same procedure and precautions as described under “Leaf sampling” and according to the company providing the immunostrips.
- Try to find a site in the field with shade, with little or no wind, and with no movement around you.
- Open the plastic box in no. 7 of 2.1 and use it as a bench.
- Wear clean gloves.
- Open the Immunostrips kit and dispense all the reagents inside the plastic box.
- Holding the collected tissue with the tweezers, cut with the scissors a piece of leaf tissue of the size of a coin (approximately 0.1–0.15g) or what is recommended by the provider and place it in the plastic extraction bag provided with the kit.
- Disinfect carefully the scissors and the tweezers with a cotton wad soaked in the bleach solution.
- Carry out the test following the procedure described with the kit.
- Record the results on the survey form.

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In case of a positive result for MCMV (Figure 1), follow-up by sending the sample to the recommended laboratory for ELISA or PCR testing for further confirmation.

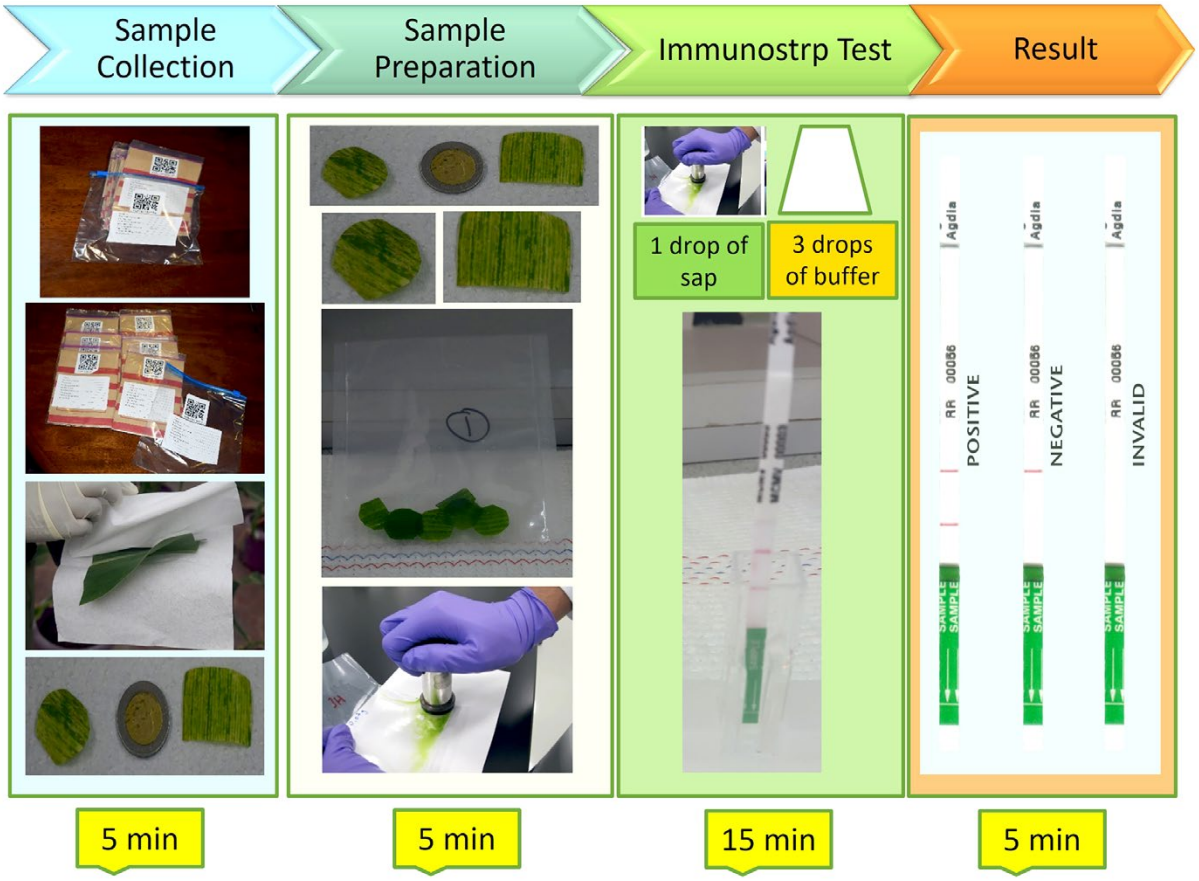



**Figure 1.** MCMV immunostrip assay using maize leaf samples.

## 3. MCMV and SCMV Detection using ELISA

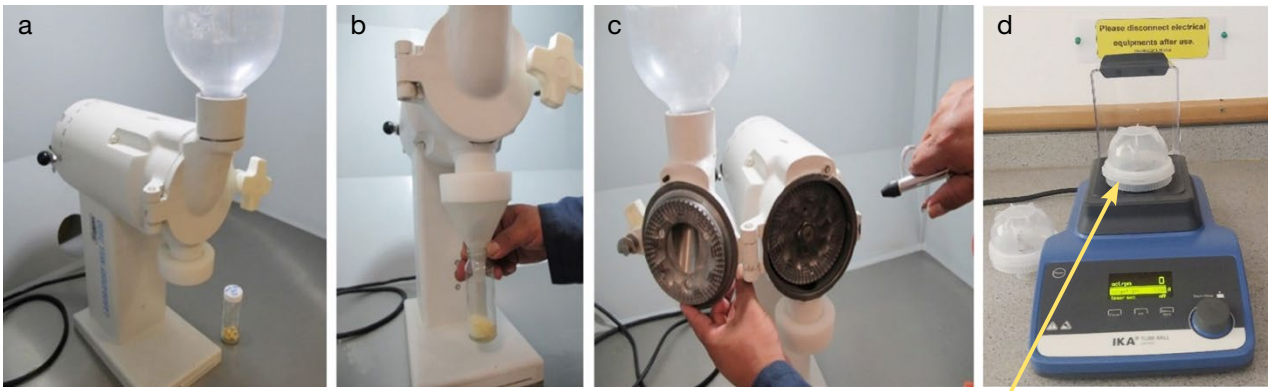
### 3.1. Antisera, Protocols and Recipes

There are several companies that provide kits (antisera) for ELISA testing for the MLN-causing viruses. Usually, the protocol for the testing is provided along with the kits. It is important to follow the company instructions to perform the test always. The antisera must be (a) stored as per instructions, (b) used at the indicated dilution, (c) not mixed with antisera sourced from different companies, and (d) not to use expired antisera.

### 3.2 Sample Preparation

The leaf samples obtained must be extracted with a tissue homogenizer or sap extractor. The amount of leaf material needed for the test must be calculated based on the instructions provided by the kit company. The extraction of the virus from the sample usually requires a dilution of the leaf sample at a ratio of 1:10 weight/volume (w/v) in extraction buffer, unless the protocol given with the kit gives different instructions.

The seed samples must be ground with a seed grinder (two examples of seed grinder are given in Figure 2; any other type is suitable so long as it allows a scrupulous cleaning between samples). Proper caution must be taken in cleaning thoroughly between samples, using a brush or by blowing compressed air, followed by cleaning of all surfaces that were in contact with the test material (seed) with 70% ethanol. The amount of ground material needed for the test must be calculated based on the instructions provided by the kit company. The extraction of the virus from the sample usually requires a dilution of the ground sample at a ratio of 1:10 w/v in extraction buffer, unless the protocol given with the kit gives different instructions.




Position of the disposable grinding chamber in the tube mill

**Figure 2. (a)** Seed grinder; **(b)** collection of flour after grinding through the funnel; **(c)** cleaning of the grinder between samples with a shot of high-pressure air; **(d)** seed grinder with disposable grinding chamber.

## 3.3. Materials and Equipment

- ELISA reagents
  - ◊ Buffers: Carbonate coating buffer 1X; General extraction buffer 1X; PBST wash buffer 1X; Conjugate buffer 1X; Substrate buffer 1X
  - ◊ Antisera [Capture antibody and alkaline phosphatase enzyme conjugate (enzyme- labelled antibody)]
  - ◊ PNP tablets
- Both positive and negative controls purchased from the company.
- Local healthy and infected controls (leaf tissue or seed) should also be added in the test.
- 96-well microtiter plates (usually these are provided with the kit)
- Plate covers/parafilm
- Distilled or purified water
- Paper towels
- Micropipettes 0-10 µl and 20-200 µl and Micropipette tips
- Tissue homogenizer and seed grinding devices
- Plastic bags, tube mill or disposable grinding chambers
- Plate washer bottle
- Airtight container for incubations
- ELISA reader
- Refrigerator (2-8°C)

## 3.4. DAS–ELISA Procedure

A general DAS-ELISA procedure is described here (Figure 3), but the protocol provided with the kit must be strictly followed to make sure that the antisera will perform at their best sensitivity and specificity levels.

**Buffer preparation:** Prepare in advance (at least one day before carrying out the test) carbonate coating buffer, PBST buffer, general extraction buffer, conjugate buffer and PNP buffer, as described in **Annex 1.**

**The day of the experiment:**
Prepare the list of the samples to be tested with proper identification and then prepare the layout of the experiment on a log sheet (Fig. 2) that will help to fill the microtiter plate and record the results. Remember that in the 96-wells plate you must use:

- 2 wells per each sample
- 2 wells for the positive control
- 2 wells for the negative control
- 2 wells for extraction buffer only (this will determine whether there are background reactions)
- 2 wells may be left empty (“blank) to standardize the reading of the ELISA reader; this is optional; it depends on the instructions of the equipment.

Therefore, in each plate you will have room for testing 44 or 45 different samples.

- It is not necessary to fill a complete plate in every experiment; if you do not have enough samples, you can use only the wells that you need, preparing the quantity of each reagents according to the number of wells needed. Unused wells can be left empty.
- Remember that in each well you will dispense 100µl of each reagent; therefore, for 96 ells you will need 9.6ml of coating, conjugate and PNP buffers. Usually, 10ml of each reagent is prepared for convenience in the calculation of the dilutions of the antisera and to cope with any pipetting error.

**STEP 1: Coating: binding specific antibodies**

- Dilute the coating antibody 1:1000 or as recommended by the kit provider in coating buffer (e.g., 10ul of antibody in 10m of coating buffer)
- Pipette 100µl of the diluted antibody to each well of the microtiter plate.
- Cover the plate with a rubber plate cover or a layer of parafilm.
- Incubate the plate at room temperature (between 20-30°C) for 4h or as recommended by the kit provider in an incubator shaker or overnight in the refrigerator at 4°C.

**Note:**

- All antibodies and enzyme conjugates should be prepared in a container made of either polyethylene or glass that does not readily bind antibodies. Do not use polystyrene.
- Do not store coated plates longer than 24 hours.

**Leaf and seed samples preparation**

During the incubation time of STEP 1 prepare the samples to test.

- Weigh the amount of leaf tissue or the ground seed necessary to satisfy the ratio with the general extraction buffer as indicated on the kit protocol: for example, if it is 1:10 w/v it will be 1g of leaf tissue or 1g of ground seed in 10ml of extraction buffer and so on.
- Transfer the amount of leaf in a small plastic bag and the amount of ground seed into a 50ml centrifuge tube.
- Add the required amount of the general extraction buffer (GEB) (1g of the sample: 10ml of GEB).
- Extract the sap from the leaf tissue with a sap extractor; mix the ground seed and the GEB 1X thoroughly.
- While you wait for the incubation of STEP 1 to be over, store the extracted samples (leaf and seed) in the refrigerator at 4°C.

**WASH THE PLATE:** Use a quick flipping motion to empty the wells into a sink without mixing the contents. Fill the wells completely with 1X PBST using the wash bottle and quickly empty them again. Repeat the washing 3 times. Hold the plate upside down and tap firmly on a folded paper towel to remove excess wash buffer.

**STEP 2: Adding samples**

- Dispense 100µl of the mixed ground seed sample or leaf extract into each well with a micropipette, changing the tips between samples.
- Dispense 100µl each of positive control and negative control into the positive and negative wells, respectively.
- Cover the plate and incubate at 4°C overnight.
- Wash the plate 6-8 times and blot dry on paper towel. Inspect the wells for presence of sample/plant residue; if present, repeat the wash step and dry the plate.

**STEP 3: Prepare enzyme conjugate**

- Dilute the conjugate 1:1000 in conjugate buffer or at a ratio indicated by the provider.
- Mix the enzyme conjugate solution thoroughly.

**Note:** Always prepare the enzyme conjugate within 10min before use.

- Pipette 100µl of the diluted conjugate to each well.
- Cover the plate and incubate at room temperature for 5h.
- Wash the plate thoroughly and blot dry on a paper towel.

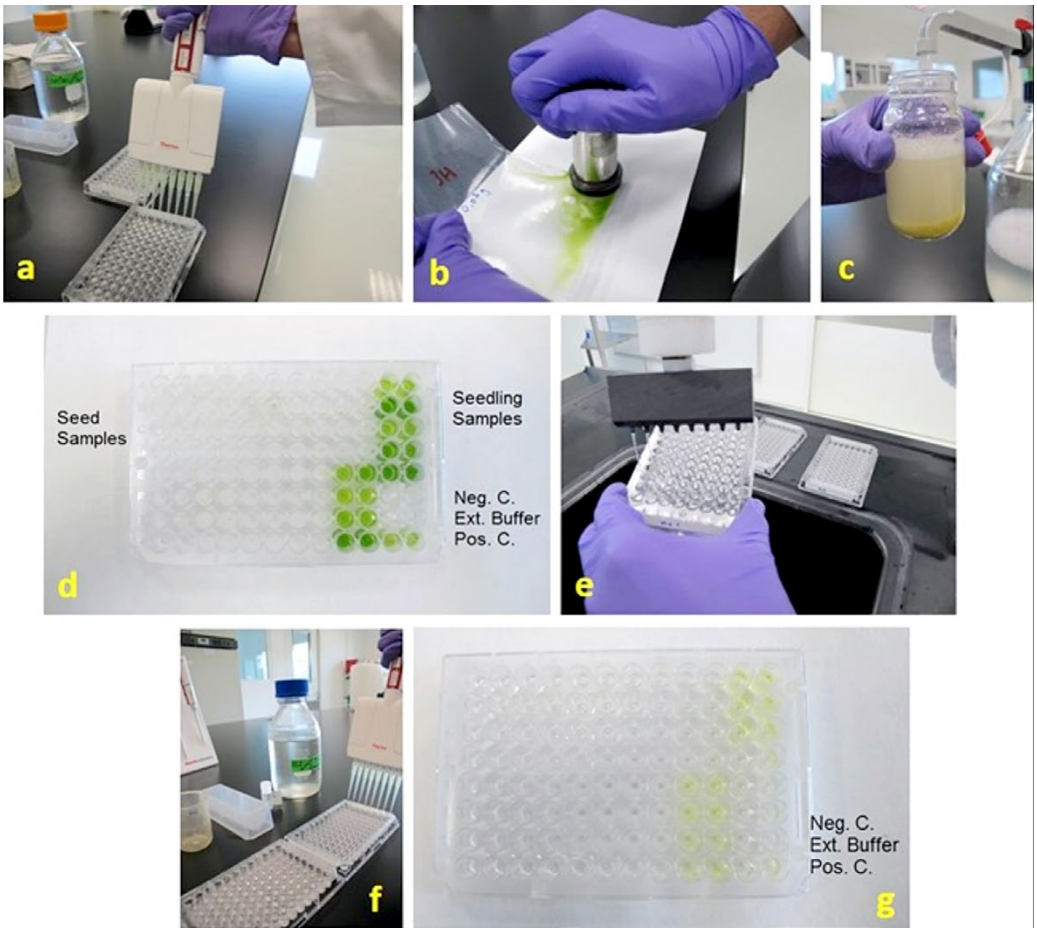



**Figure 3.** ELISA protocol: (a) plate coating; extraction from (b) leaves or (c) seed; (d) sample distribution in the plate; (e) washing of the plate; (f) adding conjugate; and (g) results. Yellow colored reactions in the plate indicate here MCMV +ve samples.

**STEP 4: Substrate preparation**

Prepare the PNP solution about 15min before the end of the above incubation step.

- Dissolve the pNPP tablet in substrate buffer to give a final concentration of 1mg/1ml.
- Add 100µl of the substrate solution to each well.
- Cover the plate and incubate at room temperature in the dark.
- Monitor for color change from transparent to yellow both visually and/or photometrically at 405nm (for ELISA readers with individual filters) or at 405/492 nm (for ELISA readers with dual filters).
- Measure optical density values after 60min or when indicated by the kit protocol.

  **Note:** Eliminate all air bubbles present at the time of reading the plate because they can alter results, if in the light path. Add 60ul of 3M NaOH to stop the reaction.

**Quality Control**

How to assess if ELISA has worked:

1. Wells in which color changes to yellow indicate positive results while wells in which there is no significant color development indicates negative result. Test results are valid only if the positive control wells give a positive result while the negative control and buffer wells remain colorless.
2. Get the average value of the negative controls and multiply by two. Get the average of each sample and compare with the healthy (negative) control.
   - If the average value of the sample is equal to or above **2x** healthy control = **positive result.**
   - If **1.5-2x** healthy control = **tentatively positive.** In this case the recommendation is to: REPEAT THE TEST or retest using an RT-PCR assay.
   - If equal or less than healthy control = **negative result.**

   **Note:** Buffer control must be less than or equal to the healthy control.

### Interpretation of ELISA Results

The ELISA results can be interpreted visually based on the color development in the wells of the ELISA plates or with the help of a spectrophotometer, which is more accurate. The wavelength at which the reading should be taken is recommended on the ELISA kit protocols, but not the threshold for establishing whether a sample is positive or negative. There are no easy ways to establish positive-negative thresholds in ELISA, although the threshold to establish positive or negative sample is often 2x the value of the healthy control used in the experiment. Sutula et al. (1986) and Fenlop and Sopp (1991) provided useful guidelines for interpreting ELISA data and determining positive-negative thresholds. In case of ELISA test for MLN viruses, one needs to specifically watch out for "false negatives" (i.e., a test result improperly indicates that there is no pathogen) as these could be more problematic than "false positives" (i.e., a test result improperly indicates presence of the pathogen) because samples can generally be retested to eliminate false positives.

## 4. Sensitivity and Utility of Diagnostic Methods for MCMV Detection in Commercial Maize Seed Lots

SCMV and other potyviruses are prevalent in sub-Saharan Africa. Their aphid vectors are also prevalent in the region. While control of overall aphid populations might have minor effects on potyvirus transmission, spraying insecticides on the fields infested with aphids does not always reduce potyvirus spread, and is not recommended. Because the viruses are not transmitted through seed, there is no need to test seed for their presence. Therefore, we focus on testing for MCMV.

MCMV is known to contaminate seed of infected plants and could be transmitted through seed (although at very low rates), thus making identification of MCMV-infested seed lots important for preventing long-distance virus spread. We have adapted two commonly used diagnostic approaches for the detection of diverse MCMV isolates in maize seed: ELISA and RT-PCR. Although quantitative RT-PCR (RT-qPCR) has been used for diagnosis of MCMV in seed, experiments in the US indicated that this diagnostic assay does not have increased sensitivity over RT-PCR and the cost is about almost twice higher than RT-PCR (Bernardo et al., 2021; Table 1). The selection of positive and negative controls for all diagnostic assays is quite important. For ELISA especially, the seed extract can produce relatively high background.

Bernardo et al. (2021), based on an analysis of three different diagnostic assays for MCMV detection in seed, indicated that:

1. RT-PCR and RT-qPCR were several orders of magnitude more sensitive than ELISA on a "per virion" basis; however, all the three assays detect viral components, and do not distinguish between infectious and non-infectious virus.
2. ELISA is sufficiently sensitive for most seed diagnostic applications, and has the advantage of the lowest cost per sample (Table 1), with less specialized equipment or training required. Although ELISA is much less sensitive than RT-PCR, the assay is sufficiently sensitive to detect a single infested seed in a sample of 500 seeds.
3. Other diagnostic platforms for MCMV detection are available, including reverse transcription loop-mediated isothermal application (Chen et al., 2017) and Immunostrips, but these diagnostics have relatively lower throughput and require adaptation for use with seed.

**Table 1.** Assay detection thresholds and costs for MCMV detection. (Source: Bernard et al., 2021).

| Assay | Material Detected | Cost/sample[a] | Detection Limit | |
|---|---|---|---|---|
| | | | ng[b] | Virions[c] |
| ELISA | Coat Protein | US$ 0.94 | 1.00E-04 | 13,300,000 |
| RT-PCR | RNA | US$ 4.97 | 1.00E-08 | 4,100 |
| RT-qPCR | RNA | US$ 10.72 | 1.00E-08 | 4,100 |

[a] Approximate cost of reagents per sample.
Costs were calculated based on US market costs, as of February 2020 (details provided by Bernard et al., 2020).
[b] Lowest amount of coat protein or RNA always detected across isolates in each assay.
[c] Theoretical number of virions detected based on virion RNA and coat protein contents of 2.4E-12 and 7.5E-12 µg/virion, respectively.

In case of Kenya, the calculated cost per sample for ELISA tests ranges between US$ 5.98 and US$ 6.31 per sample depending on the source of the MCMV antibody kits. This is relatively higher compared to the cost in the USA (US$ 0.94) as indicated by Bernardo et al. (2021). In Kenya, the cost of the reagents is the most expensive category (50%) followed by overheads (a third of the total costs). Overheads comprises importation costs (taxes and duties, clearing and forwarding and agents' costs). Molecular-based tests are more expensive (3-4-fold) than ELISA, in general, in Kenya.

## 4.1. Sample Preparation

There are three options for seed preparation. The seed soak solution (SSS) and seed extract (SExt) produce essentially identical results (Table 2). However, SSS has very low background and does not require grinding. Because many labs test for multiple pathogens or traits in dry ground seed, extracts of ground seed were also tested for MCMV detection. For all the three methods, care must be taken to prevent contamination with MCMV. Air-borne contamination with dust or aerosols from infected seed must be avoided; this is more difficult for dry ground samples.

**Table 2.** MCMV detection in soaked and dry ground maize seed.

| Grind Type | SSS | Sext |
|---|---|---|
| Soaked seed | 54/54 | 54/54 |
| Dry seed | - | 23/24 |

## 4.2. SSS and SExt Preparation

Materials

- 1 quart mason jars (standard mouth) with metal disk and open-center screw lid (or screw-top plastic lids) (link)
- 44 jars = 1 ELISA plate
- 1,000g unknown seed, divided into 100g samples
- 100-200g of healthy background seed, divided into 100g samples
- 5 Liters (lt) 1X GEB (link)
- Agdia ELISA reagent set for MCMV (link)
- 1.5ml tubes
- 200µl and 1000µl pipette tips
- Lab tape
- Weigh boats
- Scale
- Vedco D-256 (viricide for clean-up; link)

Preparation

- Prepare 5 lt 1X GEB (**Annex 1**).
- Label jars with tape.
- Weigh healthy control seed (100g) and place in corresponding jar.
- Weight unknown seed (100g) and place in jar.
- Shake each jar by hand for 10 seconds vertically and for 10 seconds horizontally (this is an attempt to mimic mixing that may occur in a seed lot bag).
- Starting with the healthy control jars, pour 1X GEB into each jar. The GEB should cover the seed and be about 2cm above the level of seed. For 100g, this is about 150ml, depending on the size of seed. Use the same amount of GEB for all samples.
- Cover the jars. To minimize laboratory contamination, lids may be sealed with strips of parafilm.
- Jars can be left on bench overnight at room temperature (~23°C).
- Prepare for ELISA next day (Section 3.4).

**ELISA using SSS**

- Start sampling with healthy control samples, then move to the test samples.
- Cover the jar lid with paper towel; to prevent leaking, shake the jar by hand, first vertically (up and down) for 10sec, then side to side (with the jar horizontal) for 10sec.
- Collect 600µl of the soaking solution from each jar and put into sample tubes.
- Store samples at 4°C until you are ready to load ELISA plate.
- Sample jars should be stored at 4°C until after ELISAs are run, in case resampling is required.

## 4.3. MCMV Detection using RT-PCR

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) is a sensitive technique that amplifies the virus' genomic nucleic acid. For MCMV and SCMV, the viruses RNA genome is copied into DNA using reverse transcriptase. Then, short nucleic acid sequences (referred as "primers"), in the presence of Taq DNA polymerase, hybridize to opposite strands of the target sequence, and amplify segments during repeated cycles of thermal cycling (warming and cooling). Each amplified segment serves as a new template for amplification in the subsequent thermal cycle. As a result, very small quantities of nucleic acids may be amplified relatively quickly. The results of the amplification may be visualized on an agarose gel, following electrophoresis and staining.

Seed samples (100g) containing a single MCMV-infested seed were either soaked overnight in buffer or dry ground. For soaked seed, the seed soak solution (SSS) and seed extract (SExt) were tested for the presence of MCMV by ELISA. Extracts of dry ground seed were similarly tested. Three independent experiments with a total of 54 soaked seed and 24 dry seed samples were tested. Data presented are the number of positive samples/total number of samples.

To eliminate the need for isolating RNA, samples may consist of seed soak solutions (SSS) prepared as outlined above. If seed extracts (SExt) or ground seed extracts are used, then total RNA should be isolated using a suitable commercial kit (e.g., Direct-zol RNA MiniPrep Plus kit, Zymo Research Corporation). New primers for amplification of a portion of the viral replicase gene that are highly conserved across MCMV isolates were developed that eliminate spurious amplification in RNA from healthy maize. Commercial kits are used for RT-PCR reactions; the instructions below are based on a protocol developed by Peg Redinbaugh (Ohio State University, USA).

**Primers**

- Specific primers are required for each specific pathogen. Primer specificity and validation are the most difficult steps for obtaining reliable PCR results. The following primers for MCMV and SCMV, as reported by Wangai et al. (2012), can be used to identify MCMV and SCMV:
  MCMV Forward Primer: 5'-ATGAGAGCAGTTGGGGAATGCG-3'
  MCMV Reverse Primer: 5'-CGAATCTACACACACACACTCCAGC-3'
  Size of amplicon: 550bp

  Primer pair MCMV-2452F (5'-AGTGGAGGTAGGCAGAGTCA-3') and MCMV-3111R (5'-TCCAACAGCAATGTTTTCCA-3') produces 660 bp amplicons using one-step RT-PCR was shown to be highly sensitive and specific for MCMV detection in SSS (Bernardo et al., 2021).

  SCMV Forward Primer: 5'-GCAATGTCGAAGAAAATGCG-3'
  SCMV Reverse Primer: 5'-GTCTCTCACCAAGAGACTCGCAGC-3'
  Size of amplicon: 900bp

- Make a master stock solution of each lyophilized primer by adding x µl of Molecular Biology Grade (MBG) to separate primer tubes, according to the concentration declared on the primer manufacturer sheet, to reach a concentration of 100 pmol/µl (µM).
- Mix thoroughly, and centrifuge briefly (2-3sec) in a microfuge.
- Make from the stock solution 100µl of working solutions 1:10 (10µl of stock solution in 90µl of MBG water).
- Store both the residual primer stock solution and the working primer solutions at -20°C for further use.

### Sample and Control Preparation

- **Samples**: The seed soak solution (SSS) may be used directly in RT-PCR. If soaked seed extracts (SExt) are used, then RNA should be extracted from 500µl extract using a commercial kit. Similarly, RNA can be extracted from a thoroughly mixed 10mg sample of dry ground seed and used as samples. Laboratory contamination from grinding should be avoided.
- **Negative controls:** No matter what type of sample is selected, a similarly treated sample from known MCMV-free maize seed should be used as a negative control. A 'water' control should also be included to monitor environmental contamination of reagents with MCMV.
- **Positive control:** RNA isolated from MCMV infected leaf tissue.

### RT-PCR amplification

The master-mix for the detection of MCMV by RT-PCR is prepared with reagents as indicated in Table 3. The steps below are followed to make the master-mix.

- Reaction mix: RT-PCR reactions (25µl) are carried out according to the manufacturer's instructions. Add 4 to the number of samples that will be tested to determine how much master mix to make.

**Table 3.** Master-mix preparation for RT-PCR detection of MCMV.

| Component | Initial Conc. | Volume (µl) | Final Conc. | n Rx (µl) |
|---|---|---|---|---|
| GoTaqBuffer[a] | 5X | 10 | 1X | |
| DTT | 100 mM | 1.25 | 5 mM | |
| Forward Primer | 20 µM | 1 | 800 nM | |
| Reverse Primer | 20 µM | 1 | 800 nM | |
| dNTPs | 10 mM | 0.5 | 200 µM | |
| RNAseOUT[a] | 40 U/µl | 0.1 | 4 Units/Rx | |
| GoTaq DNA Polymerase[b] | 5 U/µl | 0.25 | 1.25 Units/Rx | |
| Superscript III[c] | 200 U/µl | 0.035 | 7 Units/Rx | |

[a] Promega Madison, WI, USA
[b] Invitrogen Carlsbad, CA, USA
[c] Invitrogen

- Label PCR tubes for each reaction: one for each sample, plus one each for the negative control, water control and positive control.
- Pipet out 23µl of reaction mix into each tube and close the lids.
- Open the tube lids one at a time, add 2µl sample or control into tube. Mix by gently pipetting up and down. Change tips between tubes and take care not to generate aerosols.
- Place tubes into a thermal cycler programmed for 55°C for 40 min, 94°C for 2 min, followed by 33 cycles of 94°C for 15 secs, 55°C for 20 sec and 72°C for 40sec, and then a final elongation step of 72°C for 7 min.
- Amplicons are analyzed in a 1% agarose gel containing ethidium bromide. Use a 100 bp ladder to verify amplicon size of 660 bp.

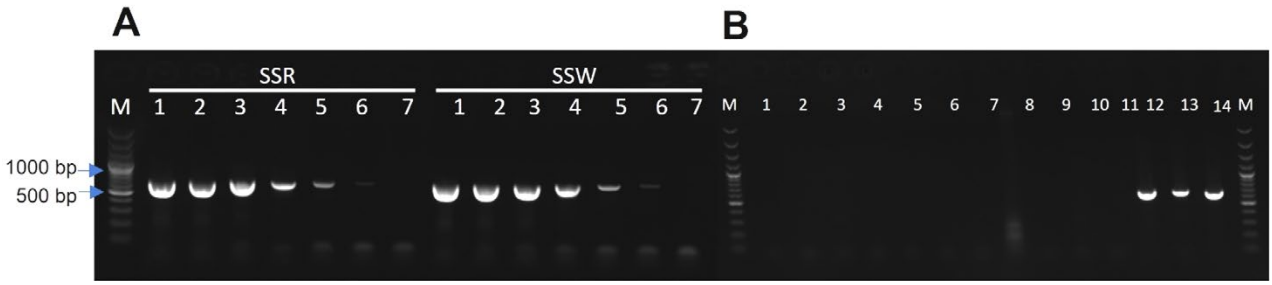



**Figure 4.** Sensitivity and specificity of RT-PCR for detection of MCMV. **A:** RT-PCR was carried out using serial dilution of RNA purified from MCMV-Kansas virions into Seed Soak RNA (SSR) and Seed Soak Water (SSW) using the MCMV-2452F and MCMV-3111R primers. M, 100bp DNA ladder; lane 1, 100pg MCMV RNA; lane 2, 10pg; lane 3, 1pg; lane 4, 100fg; lane 5, 10fg; lane 6, 1fg; lane 7, water control. **B:** RT-PCR was carried out using total RNA isolated from leaves of maize plants infected with: lane 1, wheat mosaic virus; lane 2, Johnsongrass mosaic virus; lane 3, maize chlorotic dwarf virus; lane 4, maize dwarf mosaic virus; lane 5, maize fine streak virus; lane 6, maize necrotic streak virus; lane 7, maize rough dwarf virus; lane 8, maize rayado fino virus; lane 9, sugarcane mosaic virus; lane 10, wheat streak mosaic virus; lane 11, water control; lane 12, MCMV-Kenya; lane 13, MCMV-Kansas; lane 14, MCMV-Mexico; M, 100bp DNA ladder (ThermoScientific, O'GeneRuler 100bp Plus DNA ladder used above).

## 5. References

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# Annex 1. Preparation of Buffers for MLN Diagnostic Protocols

| 1. Carbonate Coating Buffer (1X): | |
|---|---|
| Dissolve in distilled water | 1000ml |
| Sodium carbonate (anhydrous) | 1.59 g |
| Sodium bicarbonate | 2.93g |
| Sodium azide | 0.2g |
| Adjust pH to 9.6 and store at 4°C | |

| 2. PBST* (Wash Buffer) (1X): | |
|---|---|
| Dissolve in distilled water | 1000ml |
| Sodium chloride | 8 g |
| Sodium phosphate, dibasic (anhydrous) | 1.15g |
| Potassium phosphate, monobasic (anhydrous) | 0.2g |
| Potassium chloride | 0.2g |
| Tween™-20 | 0.5g |
| Adjust pH to 7.4. | |

* PBST is phosphate-buffered saline solution with a low-concentration detergent solution, such as Tween™ 20.

| 3. ECI (Enzyme Conjugated Immunoglobin) Buffer (1X): | |
|---|---|
| Add to PBST (1X) | 1000ml |
| Bovine Serum Albumin (BSA) | 2.0g |
| Poly vinyl pyrolidone (PVP) MW 24-40,000 | 20.0g |
| Sodium azide | 0.2g |
| Adjust pH to 7.4 and store at 4°C. | |

| 4. General Extraction Buffer (GEB; 1X) | |
|---|---|
| Add to PBST (1X) | 1000ml |
| Sodium sulfite (anhydrous) | 1.3g |
| Poly vinyl pyrolidone (PVP) MW 24-40,000 | 20.0g |
| Sodium azide | 0.2g |
| Powdered egg (chicken) albumin, Grade II | 2.0g |
| Tween-20 | 20g |
| Adjust pH to 7.4 and store at 4°C. | |

| 5. PNP (Substrate) Buffer (1X) | |
|---|---|
| Dissolve in distilled water | 800ml |
| Magnesium chloride hexahydrate | 0.1g |
| Sodium azide | 0.2g |
| Diethanolamine | 97.0ml |
| Adjust PH to 9.8 with hydrochloric acid. | |
| Adjust the final volume to 1000ml with distilled water and store at 4°C. | |