

Maize Lethal Necrosis (MLN): A Technical Manual for Disease Management



Editor
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In collaboration with international and national research
and development partners

Chapter 4

MLN Surveillance, Leaf and Seed Sampling Protocols

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1. Introduction

Continuous surveillance for MLN causing viruses is required to monitor the disease incidence in farmers' maize fields and seed production fields. Surveillance informs decisions on deployment of management practices to limit the effect of the disease at the farm-, country- and regional levels. Effective diagnostics and surveillance of the possible incidence of MLN in the seed production fields is essential for producing and exchanging MLN-free seed (see Chapter 8).

2. Field Survey Protocol

For a successful MLN field survey, the following elaborate strategy should be adopted.

2.1. Checklist for Field Surveys

Prior to starting field survey work, field survey teams should have the following:

- Pre-printed field survey forms (sufficient number plus spares)
- Sample collection envelopes and labels (sufficient number plus spares)
- Pens/Pencils (at least 3 per team member; pencils should be used for writing if there is a lot of moisture in the environment)
- GPS Unit (1 per team) – with standard settings for units
- Spare AA batteries (at least 4 per team)
- Tablet (or smartphone) and charger (if using electronic survey)
- Pocket knife/scissors
- Tape
- Sampling plastic bags
- Diagnostic tools (e.g., MCMV immunostrips kit)
- Sample storage equipment (e.g., cool box for temporary storage of samples, if needed)
- Hand counter
- MLN diagnostic illustrations

2.2. Field Inspection/Sampling Pattern

Make a preliminary survey of the field to identify and collect the survey information using survey forms in **Annex 1A and/or 1B**. Give particular attention to:

- a. Possible micro-climates in the field that appear different enough to warrant special attention when inspected. This may include locations in which high moisture levels may be retained due to proximity to rivers and streams, drainage areas, low spots, etc.
- b. Weedy areas
- c. Areas of the field affected by borders, such as field edges, tree lines in the field, adjacent fields of a similar crop, presence of buildings etc.
- d. Drought-stressed areas in the field

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The field inspection pattern must ensure that all parts of the field are adequately and proportionately represented in the plants inspected within the various possible microclimates of the field. For maize field inspections and surveys, the staggered “X” pattern is recommended (CDFA, 1985). It requires:

- i. Examination of plants along one side of the field.
- ii. Then diagonally in a staggered pattern across rows to the far corner, and across the far side of the field.
- iii. Diagonally back to starting corner (Fig. 1).

Additional examinations may be necessary for field environments not covered by the inspection pattern. Counting the number of plants between a sample and another with the hand counter maybe useful in case positives are detected. Counting the number of plants between a sample and another with the hand counter maybe useful in case positives are detected.

2.3. Sampling the Plants for Virus Testing

1. Operators should wear laboratory gloves while sampling. Sample should be collected before pesticide application, if any, is done.
2. Samples should be collected from the youngest rapidly growing leaves of plants.
 - 2.1. Invert the plastic sample bag over one hand, grasp the leaf to be sampled through the bag and revert (Fig. 2a-c)
 - 2.2. Using your other hand, grasp the leaf below the bag and cut it into the plastic bag (Fig. 2d). Always use separate bags for different plots.
 - 2.3. Important caution: Do not touch the interior of the plastic bag with fingers, implements or any other leaves; remove any leaf exudate (sap) from hand and cutting implements immediately after sampling to prevent cross-contamination of samples.
 - 2.4. Place a completed sample label on the side of the sample bag and record the unique sample code with details, as shown in Fig. 3A and B. Place the labelled sample bag inside another plastic bag to protect the label from any possible damage.
 - 2.5. Store the labelled samples in a styrofoam or thermos-cool box with ice packs. Individual, labeled samples from the same plot maybe placed inside one large plastic bag to keep them altogether (Fig. 2e-f).
 - 2.6. Send the styrofoam with samples secured in a cardboard box to the recommended laboratory for ELISA testing for confirmation., Keep these in paper layers and put it in a labelled polythene bag with a few holes, and place the polythene bag in a carton.
 - 2.7. Please mention on the box “Plant sample“ “RUSH IMMEDIATELY”.

If samples cannot be mailed immediately, keep them refrigerated (preferred) or in a cool dark place.
3. Label each individual sample bag with a computer-generated adhesive label with all the relevant information (date, site, plot number, crop stage etc.) (Fig. 3A). A computer generated unique QR code label must also be attached to each individual sample bag (Fig. 3B) and the unique code recorded on the corresponding field survey form.
4. The labeled leaf samples must be put immediately in a cooler containing freezer blocks.
5. If the samples are not processed immediately, they must be refrigerated at 4°C and no longer than 48h. After that time, leaf samples can deteriorate, and the results will not be reliable.

Note: Although symptomatic plants must be tested, it is critical to analyze asymptomatic plants as well. In general, it is preferable to undertake systematic sampling across the field (including both symptomatic and symptom-free/asymptomatic plants) for analysis.
6. For every symptomatic plant sampled, it is required to sample at least three neighboring symptom-free plants into separate sampling bags and with the same procedure as described above.
7. Complete the Sample Collection Form (Annex 2) and include it with the sample.

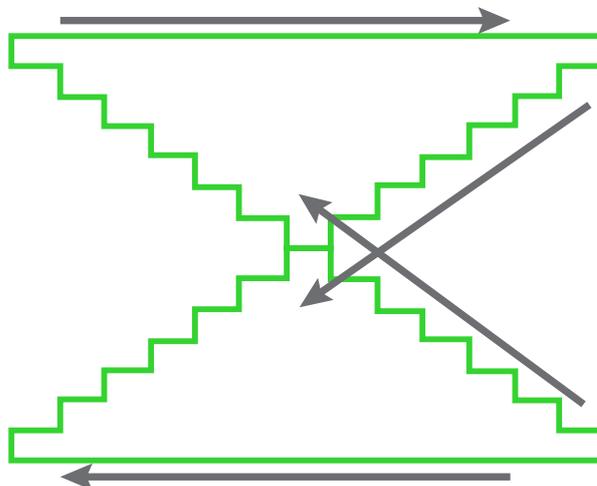


Figure 1. Staggered sampling in the field.



Figure 2. Leaf sampling in the field (a-d) and storage (e-i) for testing of the MLN viruses.

Field location:
Farmer's or Seed Producer's Name:
Trial / Plot Number (if any)
Sample type (leaf / Seed):
Sample number:
Virus symptoms present: Yes / No
Date of sampling (d/m/y):
Name of the person who sampled:
Remarks, if any:

A



1SQsaFOR

B

Figure 3. Example of the sample label (A) and the unique QR code (B).

2.4. Seed Sampling

This procedure describes how to obtain a suitable sample size in which probability of a constituent (MLN-causing virus) being present is determined only by its level of occurrence in the seed lot (**Annex 1C**). The seed sample to be tested must represent as homogeneously as possible the composition of the whole seed lot. The International Seed Testing Association (ISTA, 2004) has provided procedures and tables with number of seed to be sampled according to the size of the seed lot under evaluation. Composite samples can be used to help overcome issues with possible irregular distribution of virus across a seed lot.

2.4.1. Sampling threshold for MLN testing

Generally, if the threshold for tolerance of a pathogen transmitted through seed is 1%, then the sample size taken for laboratory analysis after the procedure for reduction of the sample withdrawn from the whole seed lot should be 400 seeds per seed lot according to ISTA (ISTA, 2004). If the threshold is much lower than 1%, the sample size is scaled up accordingly. The sensitivity of test used (ELISA or PCR) and the incidence of infected seed in a lot are the factors that play a role in the equation for the calculation of the sample size to withdraw from a seed lot (Morrison, 1999).

In case of MLN, different strategies may be required depending on where the seed is being distributed to: (a) MLN-free countries, the tolerance (column in Table 1 named “proportion of MCMV infection”) level should be zero; and (b) for MLN prevalent areas, a higher level of tolerance can be used depending on the guidelines from specific phytosanitary agencies.

2.4.2. Sampling procedure for primary samples

- Generally, if the threshold for tolerance of a pathogen transmitted through seed is 1%, then the sample size should be 300-400 seeds per seed lot according to ISTA (ISTA, 2004). If the threshold is much lower than 1%, the sample size is scaled up accordingly.

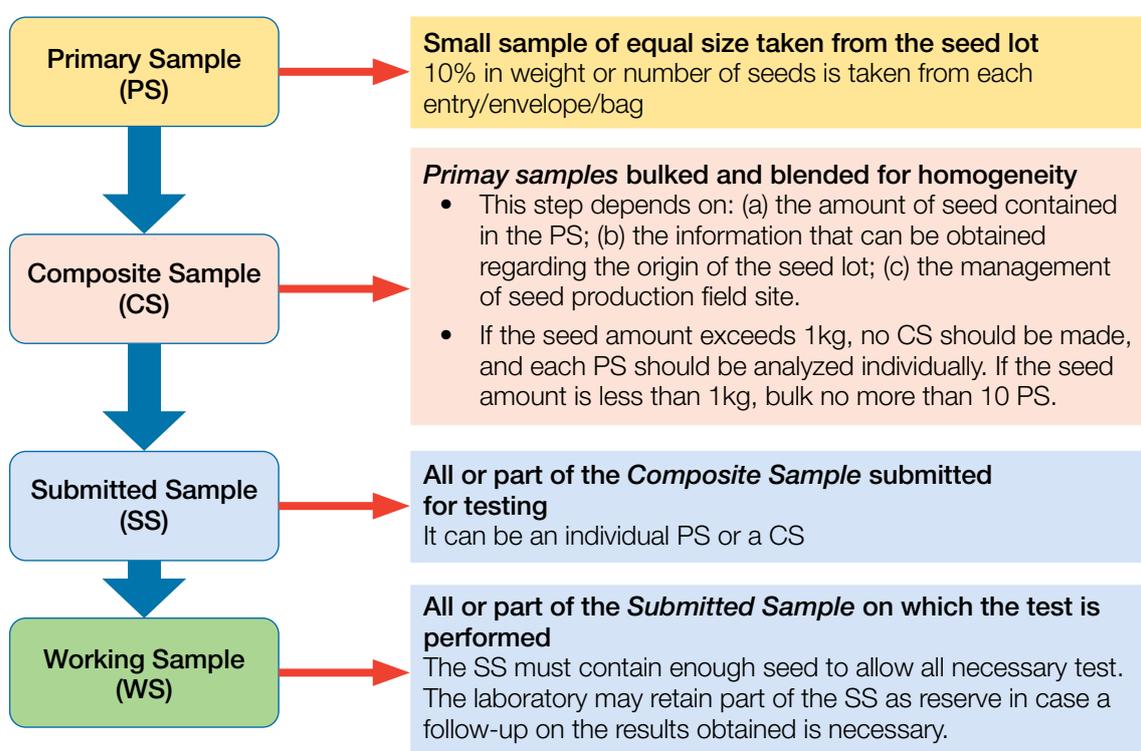


Figure 4. Definition of a primary sample, composite sample, and submitted sample.
Source: Mezzalama et al. (2015).

- For experimental seed lots for hybrids, inbred lines and OPVs:
 - i. Sample between 100 g to 1 kg, 10% of the total seed quantity in weight or number of kernels. However, for seeds quantities less than this, 5 % is recommended under the condition that the seed has been produced in an area where the plants were inspected and found free of MLN causing viruses.
 - ii. Collect between 1-14 kg, 10% from each entry
 - iii. Quantities between 15-100 kg.

Table 1. Minimum sampling intensity for seed lots in containers of up to 15-100 kg capacity (inclusively).

Number of containers in the lot	Minimum number of primary samples to be taken
1-4	3 primary samples from each container
5-8	2 primary samples from each container
9-15	1 primary samples from each container
16-30	15 primary samples from each seed lot
31-59	20 primary samples from each seed lot
60 or more	30 primary samples from each seed lot

- iv. Draw small amounts of seed from 100 kg and above (usually commercial seed lots) from as many points in the seed lot as possible.

Table 2. Number of primary samples to be taken from seed lots of more than 100 kg or from the seed stream.

Lot size	Number of primary samples to be taken
Up to 500 kg	At least five primary samples
501 to 3,000 kg	One primary sample for each 300 kg but not less than five
3,001 to 20,000 kg	One primary sample for each 500 kg but not less than 10
20,001 kg and above	One primary sample for each 700 kg, but not less than 40

2.4.3. Seed sampling in the laboratory

In the laboratory there are two categories of samples:

(a) Single samples: For seed quantity greater than or equal to 500 g, a single sample of 10% of the seed quantity either by weight or number of kernels is collected after thoroughly mixing the seed in the bag to homogenize it.

(b) Composite samples: Depending on the number of entries in the list submitted and the quantity of seed (less than 500g in weight or number) per entry, take 10% from each entry and homogenize to constitute a composite sample.

In both cases, take 50% of the total amount of seed and use it for analysis and the remaining 50% should be kept as a reserve.

Sample preparation in the laboratory

Once a representative seed sample is obtained, identify and store it in plastic or paper bags until you reach the laboratory.

3. MLN Electronic Survey Forms

Electronic versions of the MLN Field Survey Forms (**Annex 1A & 1B**) and the MLN Seed Survey Form (**Annex 1C**) have been developed using ODK software. The survey tools may be downloaded without cost and will work on any Android device that has GPS (smartphone or tablet). No internet connection is required to collect data in the field, as data and data forms will be stored on the device and sent when a connection becomes available.

The survey data collected using these tools will be stored by CIMMYT on a secure server in the MLN Toolbox Data Management System developed in partnership with Aarhus University, Denmark. Survey data will not be released to the public domain prior to approval of a country's authorized official (i.e., the country's designated national plant protection officer).

3.1. Download and Installation

Download ODK Collect using the link below; <https://play.google.com/store/apps/details?id=org.odk.collect.android&hl=en>

Download ZXing barcode scanner using the link below; <https://play.google.com/store/apps/details?id=com.google.zxing.client.android&hl=en>

Note: Device GPS MUST be switched on in order to complete the survey forms.

3.2. Device Settings (First time Use Only)

Click on menu icon on main ODK page (see Fig. 5). Select "General Settings"; Click on "Configure platform settings" and enter the following

- i. URL: <https://kc.kobotoolbox.org>
- ii. Username: mlnsurvey_yourcountryname (e.g., mlnsurvey_malawi)
- iii. Password: mlnsurvey_yourcountryname (e.g., mlnsurvey_malawi)

The password can be changed by going to kc.kobotoolbox.org, then login using old password. Click on the three lines at the top left corner of the page and choose settings among the list in the left column. Click on change password and submit the new password and click ok.

In "AUTO SEND". It is recommended to select "Auto send with Wi-Fi" and "Auto send with network" [This will ensure automatic sending of data forms when connected to internet].

Load the MLN FIELD and SEED survey forms (**Note:** For first time use only, or if an updated version of the forms is available).

- On main ODK page. Click on "Get Blank Form" (Fig. 5).
- Enter username and password if needed. Click OK for server authentication.
- Select MLN FIELD survey form v1.0 and MLN SEED survey form v1.0.
- Click on "Get Selected".

Open the MLN FIELD Survey Form v1.0

- On main ODK page. Click on "Fill Blank Form" (Fig. 5).
- Select MLN FIELD Survey Form v1.0.
- Swipe screen and fill in form. Enter text or select options from lists.
- To collect GPS coordinates. Click on "Record Location" button. Once the GPS signal has been received the latitude, longitude and elevation will be automatically recorded.
- If MLN symptoms are observed in a plot, you have the option to take a photo of a symptomatic leaf/plant. It is recommended to take a photo of a leaf that has clear symptoms.
- For ALL leaf samples collected the unique QR code for the sample MUST be recorded. Stick a unique QR code label on each sample bag and also on the bulk sample bag. Click on the "Get Barcode" button in the survey form. The camera will open. Align the camera directly over the QR code. Once the QR code is scanned the code will automatically appear in the survey form. Check that this matches the code on the sample bag (If it does not match – click on "Replace barcode" and repeat).

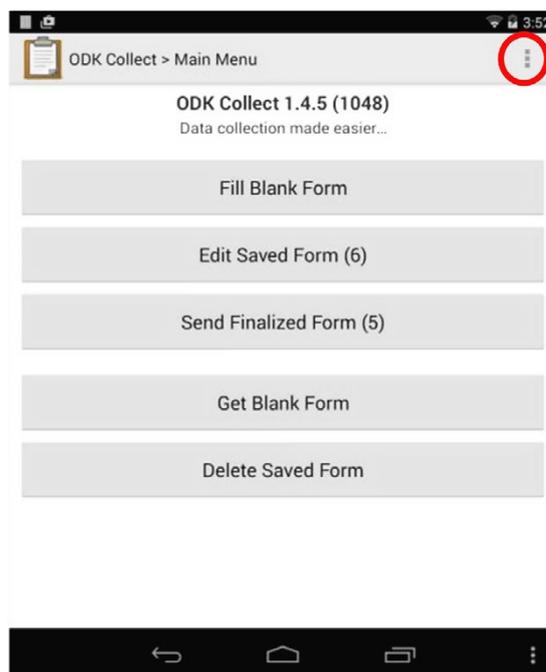


Figure 5. ODK Collect Main Menu screen. Settings menu icon highlighted in red circle.

- Please note some of the questions on the survey form are conditional – selection of a response will lead to additional relevant questions.
Note: If a number (e.g., number of other diseases etc.) needs to be changed. It is possible to scroll back, edit the number and add the additional data without losing any previously entered data.

Once the survey is completed click on “Save Form and Exit”. The completed forms will also be sent when a WI-FI connection is available. Open ODK. Saved forms will either be sent automatically or click on “Send Finalized Form” button on the main ODK menu screen (Fig. 5).

3.3. Using the MLN Seed Survey Form

Open the MLN SEED Survey Form v1.0 or the newest version on main ODK page. Click on “Fill Blank Form” (Fig. 5). Select MLN SEED Survey Form v1.0. Swipe screen and fill in form. Enter text or select options from lists. To collect GPS coordinates. Click on “Record Location” button. Once the GPS signal has been received the latitude, longitude and elevation will be automatically recorded.

It is essential that you enter the number of seed samples collected. For ALL seed samples collected the unique QR code for the sample MUST be recorded. Stick a unique QR code label on each sample bag. Click on the “Get Barcode” button in the survey form. The camera will open. Align the camera directly over the QR code. Once the QR code is scanned the code will automatically appear in the survey form. Check that this matches the code on the sample bag (If it does not match – click on “Replace barcode” and repeat).

Please note some of the questions on the survey form are conditional – selection of a response will lead to additional relevant questions.

3.4. MLN Field Survey using a GPS

If surveys are conducted using paper forms, a handheld GPS should be used to record field location (latitude and longitude). Display features vary depending on the GPS model being used. General operating procedure for handheld GPS units is as follows:

- Turn on the GPS and get a satellite signal. Once in the field, switch on GPS unit by pressing the power button.
- View the main satellite display page. Wait for 2-3 minutes for the GPS to get a location fix using the satellites overhead. Once a fix has been obtained, satellite symbols and signal strength bars will turn black. Once signals from at least 4 satellites have been received, Latitude and longitude data (and GPS accuracy) will be displayed.
- Once latitude and longitude are displayed and accuracy is 10m or less, you can now record the location on the survey form.
- Turn off the GPS after recording the location, to switch off GPS – press and hold Power key. Complete the MLN Survey Form and sampling and move to next survey location.

Note:

The first time you use a GPS in a completely new region it can take up to 5+ minutes to receive satellite signals. After initial use, signal reception will be much faster – about a minute or less. The more satellites you receive signals from the more accurate will be the location. However, the maximum accuracy possible with handheld units is +/- 4 or 5 meters. Anything less than 10 meters is good enough.

4. References

- CDFA (1985) California Department of Field and Agriculture (CDFA) Phytosanitary Field Inspection Manual.
- ISTA (2004) International Seed Testing Association (ISTA) Handbook on Seed Sampling, 2nd Edition. ISTA, Zurich, Switzerland.
- Mezzalama M, Das B, Prasanna BM (2015) MLN Pathogen Diagnosis, MLN-free Seed Production and Safe Exchange to Non-endemic Countries. CIMMYT, Mexico DF. <https://repository.cimmyt.org/xmlui/bitstream/handle/10883/4284/56880.pdf?sequence=3&isAllowed=y>
- Morrison RH (1999) Sampling in seed health testing. *Phytopathology* 89: 1084-1087.
- Redinbaugh MG, Stewart LR (2018) Maize lethal necrosis: an emerging, synergistic viral disease. *Annual Review of Virology* 5: 301-322. <https://doi.org/10.1146/annurev-virology-092917-043413>

Annex 1A. Field Survey Form for MLN Detection

Country/Institution: _____

Date of Survey (d/m/y): _____ / _____ / _____

Location: _____

Latitude (decimal degrees): N S

		.						
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Longitude (decimal degrees): E W

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Elevation: _____ (meters above sea level)

Survey Site: Farmer's field Seed Production Field Maize trial

Growth Stage: Vegetative (VE-VT) _____ Reproductive: R1. Silk R2. Blister R3. Milk
R4. Dough R5. Dent R6. Maturity

Plot Seed Source: 1. Farmer saved seed 2. Neighbour/Friend/Relative 3. Purchased from Agro Dealer
4. Donation (Gov/Project/NGO) 5. Other _____

Date of Planting (d/m/y): _____ / _____ / _____

Field area size: _____ ha Variety: _____

Disease	Present (Y/N)	Plot Incidence (% of plot infected)	Plot Severity (Avg % severity on plants)
1. MLN (visible symptoms)		L M H	L M H
2.		L M H	L M H
3.		L M H	L M H
4.		L M H	L M H
5.		L M H	L M H

L (Low) = 1-20%

M (Moderate) = 20-40%

H (High) = more than 40%

[Note: If other diseases / symptoms observed – record in disease column. If no diseases observed leave table blank]

Insects present: Thrips Whitefly Aphids Leaf beetles Others _____

Visible Insect Damage: Leaf: L M H Stem: L M H

MLN Control Measures: None Insecticide Removal of Infected Plants

Insecticide used: _____ Dose (l/ha): _____ Date of Last Application: _____ / _____ / _____

MLN-infected Leaf samples collected: Y N Number of Leaf samples collected: _____

Leaf Sample ID	Source* (Variety)	MLN Visible Symptoms (Y / N)	Bulk Sample ID (6 leaves)	Sent for ELISA Assay (Y / N)	Immunostrip used (Y / N)	Bulk Immunostrip Result (+ / -)
1						Test 1.
2						
3						
4						Test 2. (if test1 +ve)
5						
6						
7						Test 1.
8						
9						
10						Test 2. (if test1 +ve)
11						
12						
13						Test 1.
14						
15						
16						Test 2. (if test1 +ve)
17						
18						
19						Test 1.
20						
21						
22						Test 2. (if test1 +ve)
23						
24						

*Indicate specific source from where the leaf sample was collected (e.g., Variety X if several varieties are grown in the same field)
 [Record exactly same sample ID's as QR label used on sample bags/bulk bag. NB: Codes are case sensitive]

Annex 1B. Survey Form for MLN Detection in Farmer's Field

Farmer's Name:

Farmer's Tel Number (if available):

Is maize cultivated continuously? Y N Previous Crop:

Is maize planting synchronized in the locality? Y N

Has the farmer seen MLN symptoms before? Y N

Does the farmer have access to extension agent? Y N

No. of extension visits / season?

Additional Comments / Observations:

Notes on filling MLN Field Survey Form:

- **Disease Table:** Primary focus of survey is MLN, but if other diseases are observed and can be identified, record them in the disease column and score the plot incidence and severity. If unknown viral symptoms are observed, use the following 6 symptom categories for recording – unknown virus symptoms – Mosaic; Chlorotic stripes; Yellowing; Necrotic leaf margin; Dead heart; Dead plant.
- **Sample Table:** Take 6 leaf samples, create a bulk and test bulk sample with Immunostrip. Record sample IDs and bulk ID and the Immunostrip result. If the Immunostrip result is positive, take another aliquot from the same bulk and re-test using another Immunostrip. If both tests are positive, re-sample and re-test infected plants. If positive tests are obtained, sample 3-4 surrounding / neighboring plots.
- Only send the samples that tested +ve with Immunostrips for follow-up ELISA analysis.

Annex 1C: Survey Form for MLN Detection in Commercial Seed

Name of the Surveyor:

Country/Institution:

Date of Survey (dd/mm/year):

Latitude (decimal degrees): N S

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Longitude (decimal degrees): E W

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Elevation: _____ (meters above sea level)

Name of the Agro Dealer:

Location:

Tel:

If commercial seed samples are collected, provide the following details:

Seed Sample ID (QR code)	Name of the Variety	Company	Seed Lot Number	Seed Source (Country /Location as per label)	Weight of seed lot / bag sampled (kg)	Approx. weight of sample (kg)	Sent for ELISA Assay (Y/N)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

Additional Comments / Observations:

Annex 2. Maize Sample Collection and Submission Form for MCMV/MLN Diagnosis in the Laboratory

Maize Sample Collection for MCMV/MLN Diagnosis in the Laboratory	
To be completed by field staff collecting samples	
1. Collection Number:	2. Date of Collection:
3. Submitting Organization:	
4. Name and address of the sample collector:	
5. Place of collection (Name / Station / GPS coordinates etc.):	
6. Type of sample (leaf/seed):	
7. Packaging:	
8. Number of samples submitted:	
9. Name and signature of the sample collector/sender:	

To be completed by laboratory staff analyzing samples	
10. Name and address of the Laboratory:	
11. Remarks by the Laboratory Analyst:	
12. Lab testing method:	
13. Pathogen identified (Common name, abbreviation):	
14. Description notes, if any:	
15. Name and Signature of Laboratory Analyst	
Place: Date:	