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DEPARTMENT	Bacteriology Laboratory		
EFFECTIVE DATE	3 rd May 2023		Version Number: 03

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1. Introduction

This is an immuno enzymatic assay which uses a nitrocellulose membrane as a support for the sample and reagents. NCM-ELISA is a sensitive, easy and quick method. It is important as the nitrocellulose membrane with the samples can be stored for several weeks before they are analysed. The membrane can also be sent to other laboratory that can perform the assay.

2. Purpose

This Standard Operation Procedure (SOP) guides the laboratory in testing of *Ralstonia solanacearum* in potato tubers and stems by Nitrocellulose Membrane ELISA.

3. Scope

This sop covers all the steps involved in the analysis of *Ralstonia solanacearum* from potato samples preparation to results analysis and reporting.

4. Definitions

NCM- Nitrocellulose Membrane

ELISA-Enzyme Linked Immuno-sorbent Assay

5. Equipments

- Biosafety Cabinet
- Autoclave
- Vortex
- Micropipettor (1000µl, 100µl, 200 µl)
- Conical flasks (2000ml, 1000ml, 500ml, 250ml, 100ml)
- Media Botttles (1000mls,500mls, 250mls 100mls)
- Graduated cylinder
- Beakers
- Buckets to wash and disinfect the tubers
- Plastic paper bags
- Sharp knives or blades (scalpels)
- Serviettes
- Surgical gloves
- Burnsen Burners

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- Lighter or match box
- Distilled water
- Self-adhesive labels
- Eppendorf tubes
- Micropipette tips
- Plastic Racks
- Aluminum foil
- Wooden roller or hammer
- Crushed ice
- pH metre
- Incubator shaker or incubator
- Refrigerator

6. Serological Tests (ELISA Test)

6.1.1.1 This test method utilizes specific antibodies in the detection of *Ralstonia solanacearum* and is mainly used in the detection of latent infection. This method is used for testing *Ralstonia. solanacearum* in potato tubers and stems.

Reagents: NCM-ELISA Kits
Ethanol
Sodium Hypochlorite

7. PROCEDURE

7.1 Sample preparation

7.1.1 Buffer preparation

The buffers are prepared, autoclaved at 121°C for 15 minutes and then kept in the refrigerator at 4°C.

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7.1.2 Preparation of extraction Buffer

7.1.2.1 Dissolve the content of the extraction buffer(citric acid) packet into a 1000mls distilled water, and the slowly add the content of sodium citrate while agitating.

7.1.2.2 Autoclave to sterilize for 15 minutes at 121⁰C.

7.1.2.3 Check and ensure the pH is 5.6 for the first time it is prepared.

7.1.3 Sample extraction

7.1.3.1 From tubers:

7.1.3.2 The sample size should be 400 tubers per field (0.5 to 2 hectares)

7.1.3.3 Wash the potato tubers samples with tap water to remove soil

7.1.3.4 Dip the potato tubers in 1% Sodium hypochlorite for 3-5 minutes

7.1.3.5 Divide the sample into 16 sub-samples of 25 tubers each.

7.1.3.6 Dry the samples on a clean serviette and sterilize them further by wiping the stolon end with 70% ethanol.

7.1.3.7 Flame sterilize the knives or blades after each sample.

7.1.3.8 Cut a thin slice at the stolon end with sterile blade or knife and scoop a thin layer from the vascular ring.

7.1.3.9 Place the thin tuber pieces in a polythene bag standing in ice.

7.1.3.10 Weigh the pieces and add the sterile extraction buffer in the ratio of 1:2 (1g to 2ml buffer).

7.1.3.11 Crush the material with a wooden roller or hammer.

7.1.3.12 Allow the plastic bags with samples to stand vertically on crushed ice for not more than 1 hour to avoid oxidation of the phenols before starting enrichment.

7.1.4 From Stems

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7.1.4.1 From a plant exhibiting wilt, cut 3cm of the lower part (if wilting is just beginning) or from the apical region (if the symptoms are severe), and put in a tube containing 5mls of sterile water.

7.1.4.2 Incubate for 30 minutes at room temperature to allow the bacterial exudates to come out of the vessels.

7.1.5 Apply 20ul the turbid water suspension on the nitrocellulose membrane (without enrichment).

7.1.6 For evaluation of latent infection in a stem apparently healthy from an infected field, cut 3cm-stem fragment at the lower part of the plant, put it in a plastic bag, and crush it with citrate buffer as described for tubers. Before performing NCM-ELISA, the stem extract should be enriched for 48 hours at 30°C.

7.2 Enrichment buffer preparation

7.2.1 Dilute the enrichment broth SMSA 10X by adding 180ml of distilled sterile water to the 20ml broth provided.

7.2.2 Enrichment procedure

7.2.2.1 Dispense 500µl (0.5 ml) of SMSA 1X in sterile 1.5ml Eppendorf tubes

7.2.2.2 Mix plant extracts with enrichment broth (SMSA) at the ratio of 1:1 and incubate for 48 hours at 30°C under constant agitation in an incubator shaker or agitated manually at least 3 times a day if there is no incubator shaker.

7.2.3 TBS Buffer preparation

7.2.3.1 Dissolve the content of designated for TBS buffer with 997.5 ml of distilled water. Mix thoroughly. Verify pH and adjust to pH 7.5 by dropwise addition of HCl solution provided in flask (approximately 2.5 ml).

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7.3 Dot blotting

7.3.1.1 This is the process of putting the samples on the membrane and involves the following steps:-

7.3.1.2 Label the membrane appropriately before wetting it.

7.3.1.3 Prepare the membrane by dipping in TBS buffer for 5 minutes.

7.3.2 With dot-blot apparatus

7.3.2.1 Soak a piece of filter paper in the prepared TBS Buffer and place it on top of the perforated plate of the apparatus.

7.3.2.2 Remove the wet membrane from the buffer and place it on the wet filter paper

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7.3.3 Without dot-blot apparatus

7.3.3.1 Place 2 sheets of dry filter paper on a laboratory bench and put two wet filter papers humidified with TBS on top of them.

7.3.3.2 Carefully avoid the formation of air bubbles while placing the wet membrane on top of the wet filter papers. Wait for a few seconds until the liquid on the surface of the membrane has been absorbed.

7.3.3.3 Roll a clean and sterile pipette or glass tube on the membrane to ensure the good contact between the membrane and the filter papers

7.3.4 Application of the samples on the membrane

7.3.4.1 With a micro-pipetor take 20µl of the enriched tuber extract and put it slowly on the membrane by carrying the pipette vertically and allowing a slight contact of the pipette end on a membrane at the drop makes contact.(do not let it fall because it will expand a lot and the dot will not be uniform.

7.3.4.2 After dot blotting, transfer the membrane to a dry filter paper and let it dry for 60 minutes.

7.3.5 Membrane Storage:

7.3.5.1 Put the membrane between two dry filter papers protected between two pieces of cardboard for storage or mailing. This membrane can be stored for several weeks before doing the serology.

7.3.6 Serological Test

7.3.6.1 300 ml of TBS data will be required per membrane

7.3.6.2 All incubations and washing steps are done at room temperature, with a rotary shaker at 50 rpm (gentle agitation) and washings at 100 rpm. If no shaker is available, incubations can be done with frequent manual rotary agitation but constant rotary agitations must be done for washings.

7.3.6.3 **NB:** Do not forget to perform ELISA of NCM strips dotted with positive and negative controls .

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7.3.7 Blocking

7.3.7.1 Prepare the blocking solution (non fat powdered milk) by mixing content in packet with 30 ml of TBS buffer.

7.3.7.2 Pour 30 ml of the solution into a container and then slowly slide the membrane into the solution ensuring no formation of air bubbles.

7.3.7.3 Slide the positive control strip and incubate at room temperature with gentle agitation at 50 rpm on an orbital shaker for one hour.

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7.3.8 Rs-antibody binding

7.3.8.1 Dissolve the contents of packet designated for antibody buffer with 30ml of TBS buffer

7.3.8.2 Add the antibody 100µl of (Rs)-specific rabbit antibodies with antibody buffer solution.

7.3.8.3 Discard the blocking solution and add the antibody solution

7.3.8.4 Incubate for at least 2 hours under gentle agitation or overnight without impairing the results.

7.3.9 Washing step 1

7.3.9.1 Preparation of T-TBS: mix 250µl of tween 20 with 500 ml of TBS buffer. NB: the tween 20 is very viscous and should be taken up slowly with a micropipettor.

7.3.9.2 Discard the antibody solution and remove the unbound Rs antibodies by washing the membrane in 30 ml of T-TBS.

7.3.9.3 Repeat this three times for 3 minutes each.

7.3.9.4 Rs-antibody complex with the conjugated goat anti-rabbit antibodies.

7.3.9.5 During the last washing prepare the conjugate buffer by mixing packet 5 (skimmed milk) with 30ml of TBS buffer then add 100µl of Rs-antibody complex to the conjugate buffer.

7.3.9.6 Add the conjugated antibody solution

7.3.9.7 Incubate the membrane for one hour under gentle agitation.

7.3.9.8 Preparation of substrate buffer

7.3.9.9 Dissolve the content of the packet designated for substrate in 100 ml of distilled water

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7.3.9.10 Mix thoroughly and adjust the pH by drop wise addition of concentrated HCl solution until reaching pH 9.6

7.3.10 Preparation of the NBT

7.3.10.1 (Nitroblue Tetrazolium)/BCIP (Toluidine salt of 5-bromo, 4-chloro, 3-indolyl) solutions

7.3.10.2 Caution: DMF is highly toxic and it is absorbed by the skin. Wear gloves when preparing the NBT/BCIP substrate solution.

7.3.10.3 Dissolve the content of Eppendorf tube for NBT by adding 800µl of solvent solution DMF/NBT as per the user manual. Agitate until completely dissolved

7.3.10.4 Dissolve the content of Eppendorf tube for BCIP by adding 800µl of solvent solution DMF/BCIP. Agitate until completely dissolved

7.3.10.5 Store the NBT and BCIP solutions at 4o C (not more than a month)

7.3.10.6 DMF/NBT: 70% Dimethylformamide in H₂O

7.3.10.7 DMF/BCIP: 100% Dimethylformamide

7.3.11 Washing step 2

7.3.11.1 After incubation discard the conjugate solution.

7.3.11.2 Wash the membrane three times for 3 minutes each

7.3.11.3 During the last washing, prepare the substrate buffer solution.

7.3.11.4 Preparation of the NBT/BCIP substrate solution

7.3.11.5 Draw 25ml of the previously prepared substrate buffer and put in a dark flask.

7.3.11.6 Take 100µl of the NBT solution and put in the dark flask containing the substrate buffer, dropwise while agitating gently.

7.3.11.7 Add 100µl of the BCIP solution dropwise while agitating gently.

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7.3.12 Coloration development

7.3.12.1 Add the substrate solution to the container with the membrane and allow the reaction to take place for 5 to 20 minutes while agitating.

7.3.12.2 Check the development of the purple color of the positive controls. If no reaction is visible within this time, it is unlikely that a reaction will take place

7.3.12.3 After 30 minutes, a background reaction may start to appear.

7.3.12.4 Stop the reaction once the positive strip at 10^6 starts appearing by discarding the substrate and solution and by rinsing thoroughly the membrane with tap water.

7.3.12.5 Dry the membrane on filter paper and store between two dry filter papers.

7.3.12.6 Results Interpretation

7.3.12.7 The enzymic reaction gives rise to a purple coloration in positive samples, ranging in intensity between light to dark depending on the concentration of Rs in the samples. Therefore the positive

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7.3.13 Colour development (enzymatic reaction)

7.3.13.1 Prepare the substrate buffer.

7.3.13.2 Prepare BCIP and NBT if not prepared before.

7.3.13.3 Prepare substrate solution by mixing 25ml of substrate buffer with 100µl of NBT agitating and 100µl of BCIP in a dark flask (Cover the whole flask with aluminum foil).

7.3.13.4 Discard the conjugate solution, wash the membrane 3 times with T-TBS and add the substrate solution.

7.3.13.5 Incubate for 5-20 minutes and stop the reaction once the positive strip at 10^6 starts appearing.

8. Interpretation of results

8.1.1.1 Stop the reaction after colour development is observed on the positive strip at 10^6 . The enzymatic reaction gives rise to a purple colouration in positive samples, ranging in intensity between light to dark depending on the concentration of *R. solanacearum* in the sample.

8.1.1.2 Only samples with a purple colour of the same intensity as the positive control (10^7 & 10^8 bacteria per ml) are considered infected with *R. solanacearum*.

8.1.1.3 Sample with a light purple colour (equal or lighter than concentration on 10^6 bacteria per ml), nor green or brown colour (colour of tuber or stem extract) should be considered as positive.

8.1.1.4 If positive, confirmation of any positive from ELISA should be done by:-

- Isolating *Ralstonia* from enrichment extracts in Kelman' and or SMSA media and,
- SOP for Molecular Detection of *Ralstonia solanacearum* –TE/03
- Pathogenicity test using susceptible tomato plants.

9. References

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1. CIP NCM-ELISA Kit for the detection of Ralstonia solanacearum in potato.

10. Records

2. Sample reception book
3. Raw data book
4. Raw data Maps and membranes
5. Laboratory reports

11. Annex (Sampling plan)

12. Revision list

Current Version number	Superseded version number	Revision summary	Contributors
01	None	New	L. Thungu, E.Ringera, J.Wanjiku
02	01	Inclusion of sampling plan as an Annex. Typographical errors	J.Wanjiku
03	02	Editorial changes to comply with the new kit	J. Wanjiku

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Annex 1.

SAMPLING PLAN FOR *RALSTONIA* SPP.

1.0 *Sampling procedures for the detection of Ralstonia spp.*

1.1 Sampling Procedure for Seed Potatoes as a requirement for Seed certification

1.1.1 Sampling for seed potatoes is usually done on seed fields that have fulfilled all seed certification requirements (according to the Seeds and Plant Varieties ACT and any other prevailing legal or administrative requirement governing seed production).

1.1.2 Asymptomatic potato plants are to be sampled randomly from the field. Zigzag sampling pattern is preferred.

1.1.3 Symptomatic potato plants/tubers are to be sampled individually in a suitable container, avoiding leakage and cross-contamination of any potentially infected material

1.1.4 The amount to be sampled depend on the class or type of seed potato to be analysed (e.g. basic, pre-basic or clone i.e. breeder's material).

a) **Basic seed** potato including clone C and D. (Each sample is a composite of 25 potatoes).

A total of 400 tubers are required for 0.5 to 2 ha of seed potato.

Note: 400 potatoes have been proven to give the best result when testing for bacterial wilt using ELISA based techniques.

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b) **Pre-basic** (each sample is a composite of 2 tubers fragment). 0.5% of the plants from the same variety, same green house or same multiplication plot will be analysed.

c) From each plant selected randomly take 2 tubers at random, process them together and analyse them as one composite sample. For example, in a lot of 5,000 plants, 25 samples of 2 tubers each will be analysed (a total of 50 tubers).

Note: The tubers to be submitted as 25 samples of 2 potatoes each harvested from one plant. Each of the 2 potatoes is from an individual plant.

d) Sampling of **Potato Germplasm** and small-scale trials: -

- Each sample is a single tuber sample
- For clones, 30 tubers are analysed individually
- 30 tubers are selected (any size above 30mm) at random in the experimental plot or 2 tubers are taken randomly per plant in 15 plants.

1.1.5 All the seed potato samples well labelled to be submitted for analysis.

1.1.6 **Soil samples** should be collected randomly from the field, preferably from a depth of 10cm and below (avoid collecting soil that is too close to the surface to reduce chances of false negatives). A total of 50 primary samples per ha collected randomly is recommended. The samples are comprised of a composite prepared by mixing randomly collected soil samples. The soil should not be too wet or too dry and should be submitted to the laboratory for analysis immediately.

1.1.7 **Water samples** should be collected close to the irrigation source. At least one liter of water should be submitted to the laboratory for analysis. The sample should be collected at least 10cm below the

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surface in a clean, sealable bottle and submitted to the lab within 24hrs for best results.

1.2 Procedure for *Ralstonia* spp. extraction from stem cuttings of suspected wilting potato plants

1.2.1 Sampling procedure

The ideal sampling time is start of flowering to full bloom of the potato crop.

- 1.2.2 Select potato fields/households in pre-selected villages in a zigzag pattern while crossing the village
- 1.2.3 Wear a hand glove/ disinfect hands with 70 % ethanol before entering the potato field
- 1.2.4 Give a given sample number to one potato field/household and fill in the information in the attached data sheet
- 1.2.5 Label a paper towel to wrap stem samples with and a paper bag with corresponding sample number on the data sheet (in at least three places)
- 1.2.6 If the potato field has a lot of wilting plants, then select 5 wilting potato hills randomly and then cut a fragment of approximately 10 cm with a horticultural scissors at the base of the plant of just one principal stem per hill of the 5 hills selected.
- 1.2.7 wipe-out mud from the 5 composite stem cuttings and wrap them in the labelled paper towel, and put the wrap in the corresponding labelled paper bag.

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1.2.8 clip/stitch the opening of the paper bag with stapler before placing it on the vehicle.

1.2.9 Disinfect the scissors with 1% hypochlorite solution

1.2.10 change gloves/disinfect hands before entering the next potato field.

1.2.11 Analyze the sample the same day or keep it no more than one day in a cool place (around 15°C); do not keep it in the refrigerator.

1.3 Procedure for tuber sampling during routine surveys.

The ideal sampling time is at full bloom of the potato crop.

1.3.1 Select potato fields/ in a zigzag pattern while crossing the village

1.3.2 Wear a hand glove/ disinfect hands with 70 % ethanol before entering the potato field

1.3.3 Give a given sample number to one potato field/household and fill in the information in the attached data sheet

1.3.4 Label a paper bag with corresponding sample number on the data sheet (in at least three places)

1.3.5 If the potato field a lot of wilting plants, then select 5 hills randomly and sample 2 tubers from each hill (reject rotten tubers)

1.3.6 Wipe out mud from the selected tubers and put them in the labelled paper bag

1.3.7 clip/stitch the opening of the paper bag before placing it on the vehicle (keep ventilating the vehicle if it is closed; if it is a pickup truck, be careful of rains wetting the paper bags)

1.3.8 Do not forget changing gloves/disinfecting hands before entering the next potato field.