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Standard Operating Procedure (SOP) for Maize Genotyping

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1. Introduction

Maize (*Zea mays* L.) is an important cereal that plays a crucial role in alleviating food insecurity in sub-Saharan Africa (SSA) due to its high yield potential, and low cost. It is an important staple food crop that over 900 million people depend on (https://www.cimmyt.org). Maize is highly nutritious and contains 72% starch, 10% protein, and 4% fat. It is a rich energy source, supplying about 19.5% calories, and it is the world's highest supplier of calories. However, its production is constantly hampered by a plethora of abiotic and biotic stresses. Plant breeders have used conventional breeding methods for crop improvement; however, this method can be laborious, expensive, and easily influenced by the environment. To complement conventional breeding methods, molecular markers were used. Molecular markers are not affected by the environmental factors, crop developmental stages and are also ubiquitous throughout plant genomes can be incorporated into the breeders' toolbox. Molecular markers can be used for quality control (genotype identification), marker-assisted selection, and genomic selection.

2. Purpose

The goal of this SOP is to create a step-by-step procedure and precise instructions on some laboratory operations including leaf sampling, storage, freeze-drying and DNA isolation.

3. Scope

This document covers the procedures involved in samples preparation, freeze drying and storage.

4. Definition of terms

5. Roles and Responsibilities

All staff involved in implementing genotyping activities in the Bioscience/Molecular Laboratory Unit at IITA must use the Genotyping SOP. No alteration should be made to the procedures unless approved exceptionally by the program leaders. The list of individuals responsible for each section of the SOP in the genotyping cycle is listed below.

Genotyping Lead (GL): Responsible for the overall supervision of the molecular and genotyping procedures and delegating team responsibilities. The CL is the lead Bioscience center/Molecular Geneticist and coordinator of the Bioscience Center at IITA.
Lab Technicians (LT) Data recorder performs field tasks as defined in the trial protocols such as field data collection or infield management practices. DR's responsibility is to perform tasks and use digital tools defined in the protocol for capturing, storing, transmitting, and ensuring quality of data within defined time periods.
Data Analyst (DA) Facilitates proper tissue storage and handling including freeze-drying and DNA extraction.

6. Procedure

DNA extraction

Tissue (leaf) sampling: Is a crucial step for a successful genotyping work, because any mistake made at this stage affects the final results. To minimize the danger of sample loss or mislabelling, it is crucial that leaf sampling be done conscientiously.

Materials needed for leaf sampling

- Gloves
- Ice and ice buckets
- Sample bags
- Extraction plates, punchers, ethanol and punching mats

Leaf sampling procedure

- Young leaf tissue between 2 to 3 weeks old collected from plant are sampled into the sample bag or extraction plates.
- For extraction plates, two to thirty leaf discs (depending on the genotyping platform) of each sample is punched into well labelled extraction plates. Note for each sample punched, the mat is cleaned with ethanol before the next one is punched into the plate to prevent contamination.
- The extraction plate is kept on ice throughout the sample collection process.
- Two wells are usually left empty to serve as control for the genotyping process

▲*Young tissue materials are required to get higher molecular weight DNA and also to make grinding tissue materials into a fine powder easy.*

▲*Tag numbers must be checked carefully to ensure samples are collected correctly into the sample bags or extraction plates.*

Tissue storage and freeze-drying:

- Immediately the samples are brought into the lab on ice, the extraction plates are carefully removed and covered with parafilm. The parafilm is perforated gently, while the samples in the sample bags are carefully sorted.
- The samples are then transferred into the -80^{0}C freezer before freeze-frying.
- When ready for freeze-drying the samples are removed from the freezer and transferred into a lyophilizer at a temperature of -50°C and pressure of 5.0 pas for 72 hours.
- After freeze-drying, the extraction plates are capped firmly and stored at room temperature until DHL shipment.

▲*Storage of plant tissues for genotyping is important to avoid degradation of DNA.*

▲ *Freeze-drying is done at the Bioscience Center facility at the International Institute of Tropical Agriculture*

Note: *It is important to note that some genotyping platforms prefer genomic DNA*

DNA Extraction from leaf tissue

DNA extraction is performed in the "DNA extraction laboratory/room". This laboratory is exclusively used for this purpose and is free of DNA amplicons.

▲*The laboratory must be equipped with all necessary lab equipment (set of pipettes, pipette filter tips, gloves and lab coats) that are exclusively used in this room. Lab coats and gloves must be changed when changing rooms.*

Materials and instruments

- Lab coats

- Gloves (disposable, non-sterile)

- Pipettes (0,1-10 µl, 2-20 µl, 20-100 µl, 100-1000 µl)

- DNase-free Pipette filter tips (10µl, 20 - 200µl, 1000 µl)

- DNase-free reaction tubes 1.5 ml, 2 ml

- Timer

- Tube rack

- Table centrifuge

- Vortex

- Laminar flow hood

- Water bath (65°C)

- Paper towels, soap

- Bowl of ice

Procedure

- Grind freeze dried tissue into fine powder by shaking for 2 minutes at full (1X) speed of 1500 strokes/min using GenoGrinder-2000.

Note: *prolonged grinding cause DNA degradation.*

- Add 600ul of CTAB extraction buffer to each sample and incubate at 65°C in the thermomixer shaking at moderate speed for 30 minutes.

Note: *Invert or gentle tap tubes once every 10 minutes to properly homogenize the tissue with extraction buffer (be cautious not to splash the buffer while inverting).*

- Remove tubes from the water bath and allow them to cool for 5-10mins. Centrifuge at 3500rpm for 10mins
- Transfer about 500ul aqueous phase into new tubes and add 500-600ul chloroform: isoamylalcohol (24:1) into the tubes. Mix gently with continuous rocking for 5-10min.

- Centrifuge at 3500rpm for 15 min
- Transfer the upper aqueous later to new well labeled tubes and repeat the chloroform: Isoamylalcohol wash.
- Transfer about 400ul of the upper aqueous later into new tubes.

Note:*If the aqueous phase of the sample looks dirty, repeat chloroform: Isoamylalcohol for the third time.*

- Add 600ul 100% ice-cold (stored at -20ºC) isopropanol (2-propanol), mix gently for about 5 min (or gently invert for about 50 times) to precipitate the nucleic acid.

Optional step: *Keep tubes in the freezer (-80ºC) for about 60minutes*.

- Centrifuge at 3500rpm for 30 min to form a pellet at the bottom of the tube. Discard the supernatant.

Note: *Centrifugation while the tubes are still very cold will either result to very small pellet or no pellet at all.*

- Add 400ul of 70% ethanol; flap the tubes gently to let the pellet float for ease in washing. Centrifuge for 15 min and discard ethanol by decanting.
- Air dry pellet until ethanol evaporates completely.
- Add 96ul of distilled water and 4ul of RNase into the dried pellet and store in -20°C when the pellet dissolves completely.

▲*Avoid frequent freeze thawing of the genomic DNA, required quantity should be diluted and worked with.*

DNA quality check

- Prepare 0.8% agarose gel for checking DNA quality.
- For the DNA quality check carefully weigh 0.8 grams of agarose and dissolve in 100ml of 1X TBE.

- Melt the solution in a microwave for 3 mins, then cool to about 60ºc and add 5ul ethidium bromide.
- Pour the mixture gently on to a balanced gel tray before it polymerizes.
- Add 3ul of the DNA solution to 3ul of 6x bromophenol blue loading dye. Mix briefly and load 5ul of this mix on to the agarose gel.
- Run the gel at 80volt for about 1 hour.
- Capture the gel image on the Gel documentation system
- Check the DNA concentration and purity using NanoDrop Spectrophotometer

Note:*There should not be air bubbles in the middle of the gel*

Storage and preparation of reagents

▲*All reagents are checked for their date of expiry before use.*

▲*Thaw frozen reagents completely to room temperature before use.*

▲To minimize contamination risks, ensure all reagents are aliquot in single-use-volumes and stored appropriately.

Reagent	Stock concentration	Final concentration	Per sample (ml)
Tris HCl, pH 7.5	1M	0.2M	0.14
EDTA, pH 8.0	0.5M	0.05M	0.07
NaCl	5M	2M	0.28
CTAB	-	-	0.014g
Distilled water	-	-	0.203
Mercaptoethanol			0.007

Sample shipment:

- All necessary documents must be duly filled, printed and signed (depending on the requirements of genotyping platform to be used).
- The DNA plates must be well labelled, properly arranged and padded in an appropriate and logo-free carton (because they are shipped via an air carrier).
- The carton along with the documents must be taken to the DHL office for shipment (Note: A mail must be sent to the service provider before shipment).

7. Appendix

7.1 Contacts for support

For Issues relating to Genotyping, you can contact Maize molecular breeder: Dr. Melaku Gedil M.Gedil@cgiar.org.

8. References

https://www.cimmyt.org

https://doi.org/10.12688/gatesopenres.13338.2