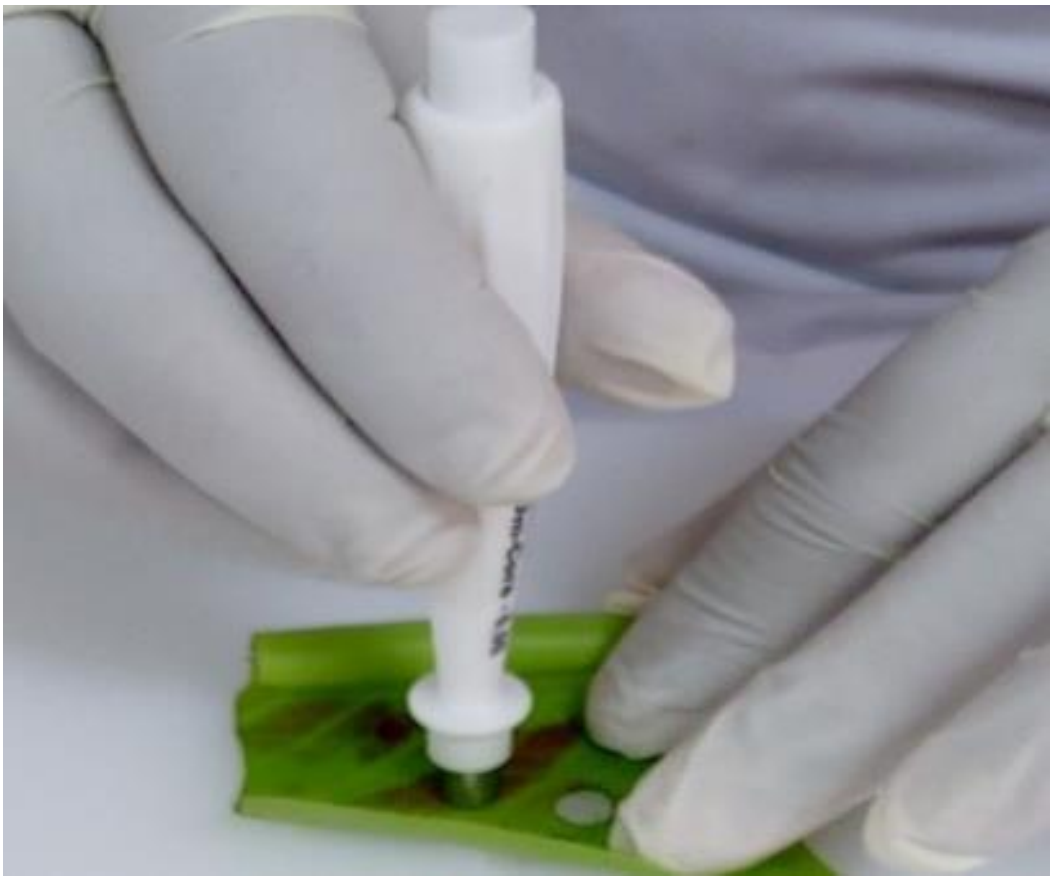
March 1, 2025

SOP14:

Standard Operating Procedure (SOP) for Off-Station banana leaf sampling, handling, and processing for DNA extraction



IITA MUSA IMPROVEMENT – MOLECULAR BREEDING

STANDARD OPERATING PROCEDURE (SOP) FOR

OFF-STATION BANANA LEAF SAMPLING, HANDLING, AND PROCESSING FOR DNA EXTRACTION

1. Authors & Contributors

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2. Introduction

Genotyping involves studying an organism's genetic makeup based on its DNA. Banana leaves are commonly used for DNA extraction due to their abundance, accessibility, and the relatively high yield of DNA they provide. Proper leaf sampling and handling are important for optimal yield and good quality of the DNA extracted.

Banana experiments may be located near the research station, where the collected samples are taken to the molecular laboratory (hereafter referred to as "the lab") on the same day for processing. However, banana fields needed for sample collection for DNA extraction may be located away from the research stations (hereafter referred to as "off-station"), where the collected samples cannot be processed right away or kept under cold conditions. This protocol covers the latter situation, where samples collected cannot be processed or stored under cold conditions, and or delivered to the lab on the same day.

3. Purpose

The purpose of this SOP is to describe the procedure to be followed during fresh banana leaf sampling off-station, and the necessary documentation for shipping to genotyping platforms.

4. Scope

This SOP document contains processes and procedures required for off-station banana leaf sampling. It should be used when sampling is done far from the station and the samples cannot be delivered to the lab on the same day of sampling. This includes preparation of the sampling materials from the laboratory for sample collection from off-station fields, sample preparation requisites for shipping and the shipping process.

5. Definition of terms (in alphabetical order)

- **Banana leaf sampling off-station:** Is when fresh leaf samples of banana genotypes cannot be delivered to the laboratory on the same day of sampling.
- **Commercial invoice:** this is a shipping document that specifies the commercial value of the samples. It is used for the export and import clearance and forwarding process of consignments at customs.
- **Customer declaration**: This is a shipping document, required by some countries. It gives additional specifications on the source of the samples such as the species, the protocol used for DNA extraction (in the case of DNA), whether the samples come from genetically modified organisms, and the purpose and use of the samples.
- **Order form**: This is a document filled by a client (breeder/researcher/technician) sending samples for genotyping. The order form is generated online from the website of the vendor (genotyping platform or company) or shared by the vendor after placing the order. It includes the contact details of the person sending the samples, information about the samples, type of sequencing services, cost etc.
- **Packaging list:** Is a document containing a detailed description of a shipment's content (consignment) but does not include any information about pricing or value. It contains information such as a list of sample ID or names, quantity, size, weight, and packaging information.

- **Phytosanitary certificate:** This is a shipping document that indicates that the leaf samples were collected from plants that meet specific import phytosanitary requirements of the destination country for a particular species (bananas in this case). The document can be replaced by an exemption certificate issued by the destination country.

6. Roles and responsibilities

Molecular banana breeder: Responsible for overseeing all molecular breeding activities, data production, analysis and reporting at the trait discovery stage, and across the other stages wherever molecular markers are applied in the Banana Breeding Programme.

Research Associate: Support the molecular banana breeder by coordinating molecular breeding activities including trial and experiment design and management, field and laboratory operation supervision, data collection, data analysis, synthesis, and writing of reports. Also responsible for coordinating and supervising the field, laboratory staff and trainees.

Laboratory Technician: Performs tasks such as preparation of buffers, DNA extraction and storage for genotyping, routine maintenance of laboratory machines and equipment. Conducts sample preparation processes, including field collection of leaf samples, plating of leaf samples in genotyping plates, freeze drying of samples for shipping to genotyping platforms, reporting the results.

7. Procedure

7.1. Required tools and equipment

a. **Mobile devices with Coordinate App**
   Mobile phones or tablets installed with the Coordinate app help with correctly labeling the samples. Coordinate app has two functions in leaf sampling. It is used to keep track of sample collection from the field and for plating of leaf samples into genotyping plates. It is downloaded from the Google Play Store (for Android tablets or phones).

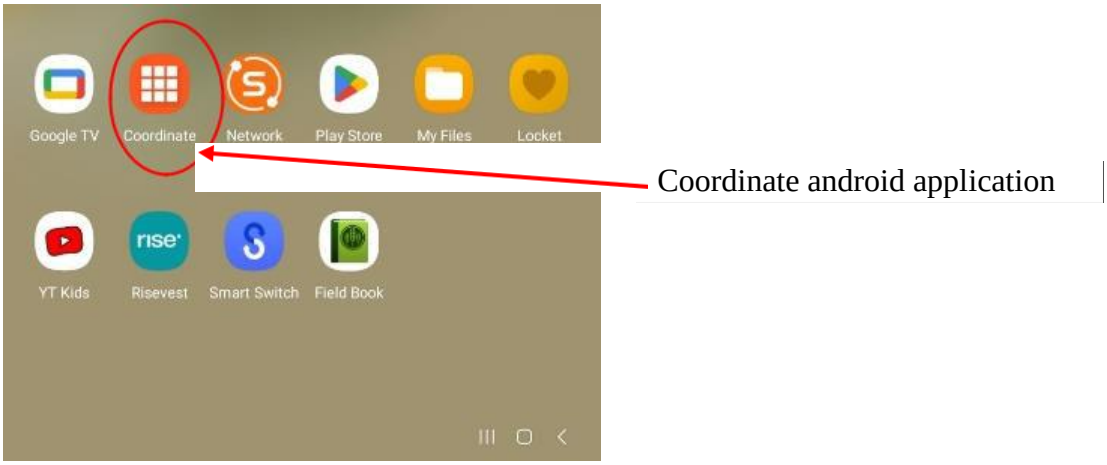


Figure 1: Display of the Coordinate android application icon.

b. A pair of stainless-steel scissors

This is used for cutting the banana cigar leaf into a sizeable portion.

Figure 2: The appropriate type and size of scissors suitable for leaf sampling.

c. Laboratory gloves

These are worn to protect your hands and to also avoid contaminating leaf samples. When working, **always** replace your gloves immediately if they get torn or dirty during work.

d. 70% ethanol solution in dispensing spray bottles

This is used for sterilizing scissors. It is **highly important to sterilize** scissors before cutting any plant to avoid cross contamination of samples and **spreading of banana diseases, such as banana bacterial wilt.**

Figure 3: Examples of the dispensing bottles that can be used.

e. Tissue paper

This is used for cleaning scissors along with 70% ethanol. Tissue paper is also necessary to clean leaf samples that have water droplets or soil particles in case it rained prior to sampling.

f. Falcon tubes

These will be used to keep/store cigar leaf samples. Do a quality control check on tubes provided first before use.

Figure 4: Examples of the dispensing bottles that can be used.

g. Silica gel

This is a drying agent. Silica gel is blue in colour when completely moisture free. Use blue silica gel when sampling. If the silica gel is pink in colour, this means it has absorbed moisture and is not suitable for use in sampling. Pink silica gel can be reheated in an oven to lose moisture, left to cool and re-used for sample storage (Figure 11).

h. Cotton wool

This is used to form a barrier between the silica gel and the cigar leaf sample in a falcon tube to avoid contamination.

i. Bar codes

This is a readable representation of data/information for sample identification in form of numerals and characters.

Figure 5*:* A QR (quick response) code used for sample identification.

j. Ziplock bags

These are used for storage of collected samples while in the field.

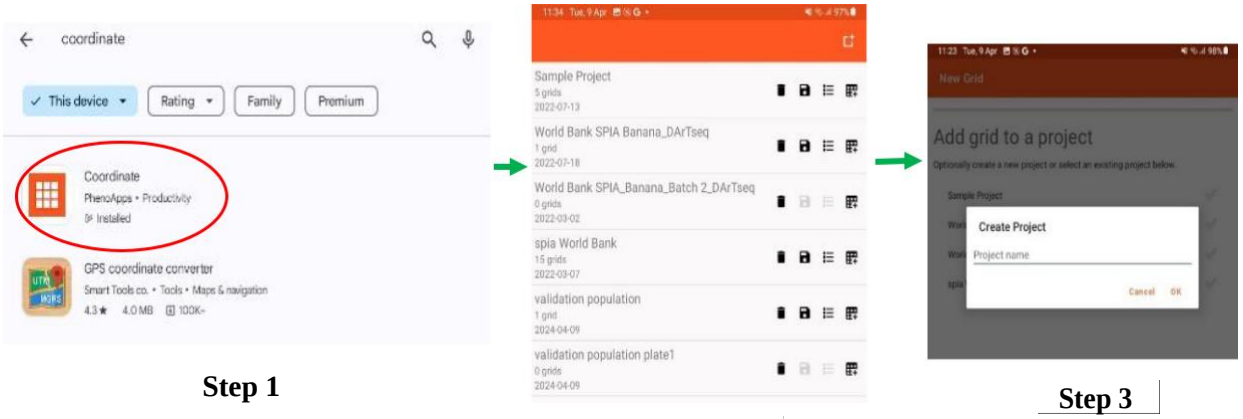
Figure 6: Example of Ziplock bags

k. A permanent marker pen

This is used for labelling samples during sample collection in the field. It should be of the type with ink that does not rub off.

7.2. Step-by-step guide on how to download and use Coordinate app

i. Go to "Google play store" and search for "**Coordinate**" and download it (Figure 7, step 1).
ii. Open the coordinate android application icon on your tablet and click on "**Project**" icon (Figure 7, step 2).
iii. Add "**Project name**" and click on "**Project**" created to open it (Figure 7, step 3).



Step 1 **Step 2** **Step 3**

Figure 7: Process of downloading and using Coordinate app. Downloading coordinate app from Google play store (step 1), open coordinate app (step 2), create project by adding project name (step 3).

iv. Add grid to created project by selecting "**DNA plate**" (Figure 8, step 4).
v. Fill in the "**optional fields**" details displayed in the "DNA plate". Key fields to fill are "**Plate**" and "**Plate Name**" (Figure 8, step 5).
vi. Press "**Next**" to go to the "**grid layout**" (Figure 8, step 6).

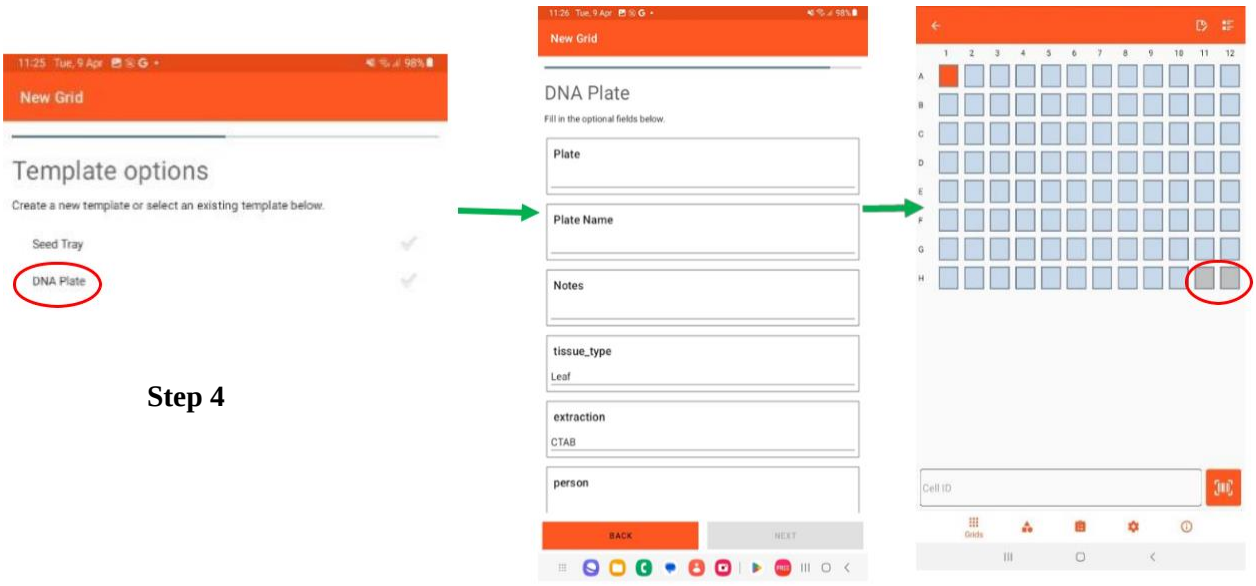


Figure 8: Process of downloading and using Coordinate app. Select "DNA plate" to add grid (step 4), Fill in the optional fields asked in the "DNA plate" (step 5), Press "Next" to go the grid layout (step 6).

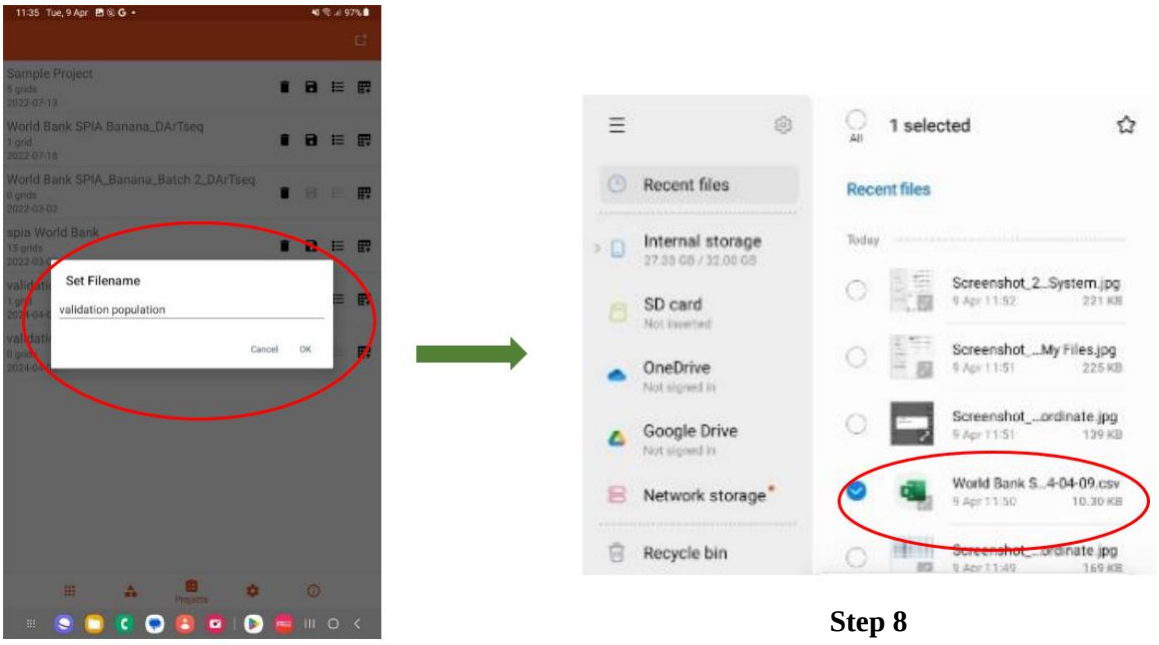
vii. During plating of the samples, prefill **H11** and **H12** wells to keep them empty (blank) if the plates are going to be sent to Intertek, or prefill **G12** and **H12** for DArTSeq plates for genotyping, by long pressing them until they turn **grey**. The two wells are left as control checks during sequencing by the respective sequencing companies (Figure 8, step 6).

viii. Fill the plates **vertically** (A1-H1) and not horizontally (A1-A12). After filling the plate with samples, use the back arrow on the top left of the grid layout to go back to the "projects" (Figure 8, step 6).

ix. To export file, save file by clicking on "**disc**" icon, write file name (Figure 9, step 7). The file will be saved in "**csv**" format (Figure 9, step 8). Export the coordinate grid layout for the scanned samples into excel files for further use. At this point, a message "Coordinate file successfully exported" will be displayed on the mobile device screen (Figure 10, step 9).

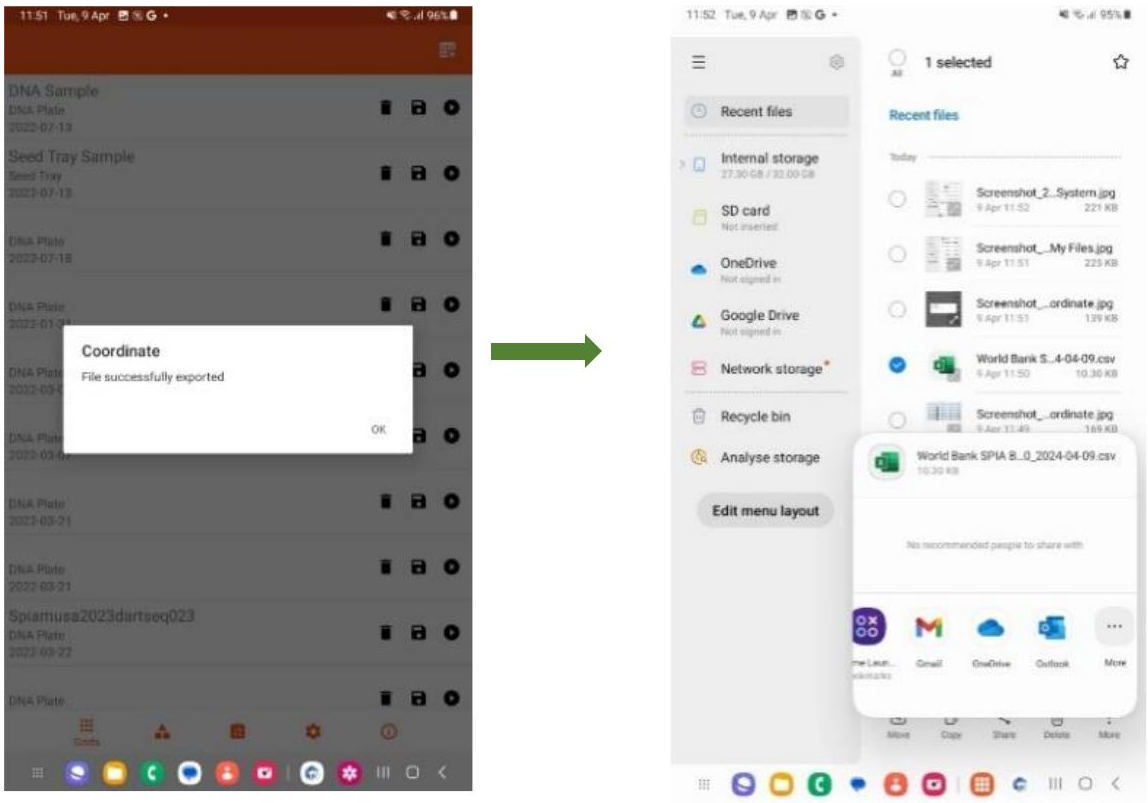
x. Do not delete your grids until you are done with your genotyping project for reference and use as back up.

xi. Go to "**files**" icon in your tablet, select your saved file and share file via email (Figure 10, step 10).



Step 7 **Step 8**

Figure 9: Process of downloading and using Coordinate app: to save file, write file name (step 7), The file will be saved in "csv" format (step 8).



Step 9 **Step 10**

Figure 10: The process of downloading and using coordinate app:" coordinate file successfully exported" message displayed on the tablet screen (step 9). Select saved file and share by email (step 10).

7.3. Preparations in the lab before going to the field

- Prior to leaf sampling, ensure that all the above items (**a-k**) have been assembled as this makes the sampling process easier in the field.
- Before working, wear laboratory gloves.
- It is also advised to wear a green coat to protect your clothes from sap staining.

7.3.1. Filling the falcon tubes with silica gel

a. Before working, wear laboratory gloves to avoid cross contamination.
b. Add about 200 g of silica gel to the falcon tubes.

c. Place a small piece of cotton wool on top of the silica gel.
d. Close the falcon tube **tightly** with its cap. Avoid closing the cap loosely as this allows air into the falcon tube and the silica gel will absorb moisture, reducing its efficiency to properly dry the leaf sample.
e. An example of a falcon tube filled with silica gel that hasn't absorbed moisture and cotton wool is shown in the figure below (Figure 11A).

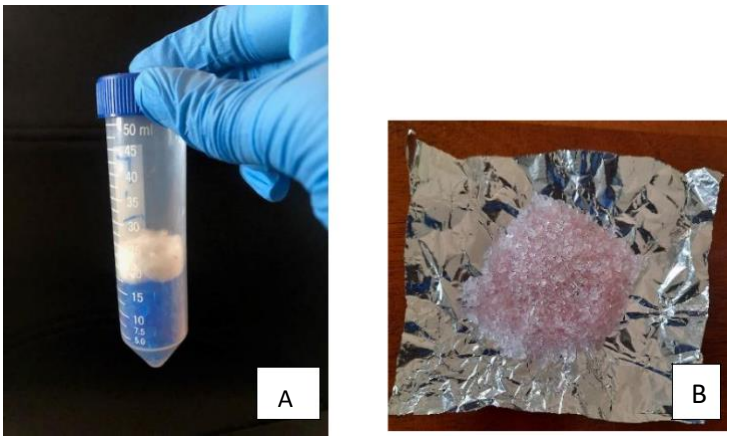


Figure 11:A falcon tube filled with silica gel and cotton wool. Picture B shows pink colour of silica gel (when it has absorbed moisture).

7.4. Leaf sampling procedure

i. Walk with the farmer with all the needed material to their "main plot" (farmer decides) for banana.
ii. Ask the farmer to show you examples of the different banana varieties they cultivate in that plot.
iii. For each different variety that the farmer says s/he has in the plot, follow the steps below.
iv. Ask questions in the CAPI DNA fingerprinting module for that banana variety.
v. Sterilize the pair of scissors with 70% ethanol solution.
vi. Identify a **healthy clean green cigar leaf** on the banana plant and cut about 6 cm of the leaf, from the leaf apex (tip of the leaf) using sterilized scissors. The cigar leaf is the youngest leaf on the plant, and it gives the best yield and quality of DNA.

N.B:

- Do not sample mature leaves. Cigar leaves should be sampled, but if a cigar leaf is not present, sample the youngest leaf available. See the example in **Figure 12**. **Sample each identified variety in duplicate**.
- When collecting samples from off-station, endeavour to collect enough leaf tissue per sample and store the remaining leaf tissue per sample well until the genotyping results and genotypic data analysis is complete. This will be used as back up where need arises.

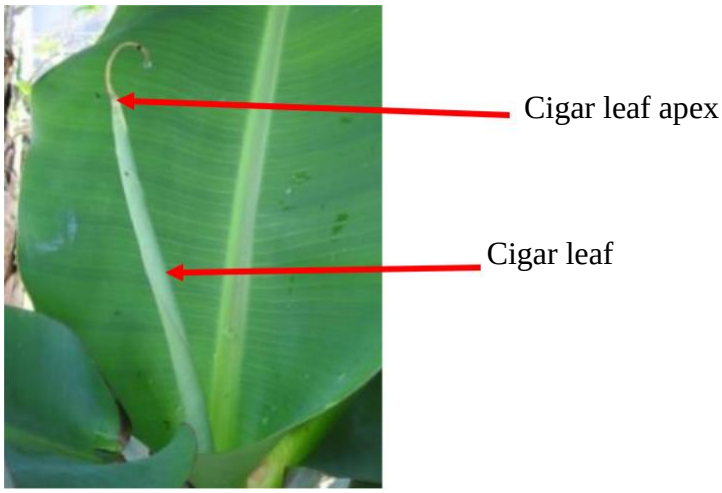


Figure 12: A cigar leaf on a banana plant.

vii. Place the cigar leaf into the falcon tube and close the cap tightly. Ensure that the falcon tube is labelled with the right barcode and that it contains blue silica gel.

viii. Place the falcon tube into a Ziplock bag and close it. Ensure the Ziplock bag is labelled with the right barcode too (same barcode on the falcon tube).

ix. Sterilize the scissors by spraying them with 70% ethanol and move to the next plant to be sampled.

Pre- caution

1. Do not sample diseased or physically damaged cigar leaves. Examples of diseased cigar leaves are shown in **Figure 13**.

Figure 13: Examples of diseased cigar leaves. These are not suitable for sampling.

2. After cutting the cigar leaf, always trim off the tip of the cigar leaf(leaf apex) (**Figure 12**).
3. If the leaf is moist from rain droplets, dew or contains soil particles or dust, wipe it using tissue paper before sampling.

7.4. Quality control of samples received in the lab

a. Perform a quantity and quality control check of samples received by counting and noting the number of all samples received.
b. The quality control check involves; checking that all samples contain two barcodes that match while confirming that the sample in the containers are banana leaf samples.
c. If the samples have no barcodes, assign them well numbers in an excel sheet in the coordinate app grid layout (format). Use this coordinate app grid layout to place the leaf discs in genotyping plates accordingly.
d. Check the sample integrity; discard browning and blackish/moulded or rotten samples (**Figure 14A** and **14B**)

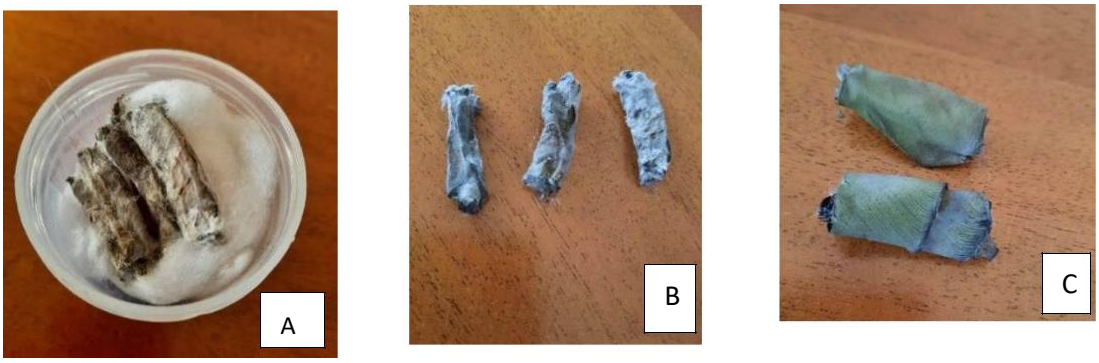


Figure 14: Figure 14 A and 14 B shows examples of leaf tissue with mold and browning that are discarded. Figure 14 C shows an example of good leaf tissue of banana sample that has dried well under silica gel and is acceptable.

e. Moisture status of the sample; check if the samples are completely dry or still in the drying process. **N.B:** Samples that are totally dry should be brittle (**Figure 14 C**). For samples that still drying, reduce the cotton wool to a reasonable amount as this can hinder effective drying of a sample.

f. Change of silica gel. The good/ideal colour of silica gel is blue in colour. If the silica gel turns pink, this means that it has absorbed moisture and must be replaced. In samples which are not yet dry, change the silica gel, and reduce cotton wool to fasten the drying of samples.

NB: Make a report of the total number of samples. i.e., good samples, bad samples, samples with labelling errors (missing barcodes or barcodes that do not match), and samples that do not contain banana leaf samples.

7.5. Processing of samples collected off-station for shipment to sequencing platforms

7.5.1. Items needed

Here we consider genotyping using DArTSeq and Intertek sequencing platforms.

a. Android tablets with Coordinate App installed.
b. A mat and leaf puncher (**Figure 15**)

Figure 15:A picture of a leaf puncher and mat used for making leaf discs.

c. Genotyping plates (**Figure 16**)

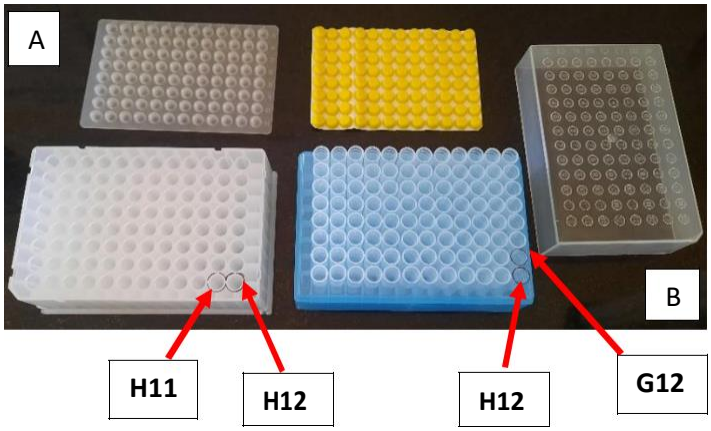


Figure 16: Shows types of genotyping plates used for shipping samples to sequencing companies. Figure 17A shows the genotyping plate and its lid used by the Intertek sequencing company and Figure 17B is a genotyping plate and its lid used by the DArTSeq sequencing.

d. A marker pen
e. 70% ethanol in a dispenser (Figure 2)
f. Cling film-sealing of genotyping plate after placing in leaf discs of samples.
g. A syringe or micropipette tip: Used to perforate (punch holes) in the cling film covering a filled genotyping plate with samples. This is purposely to aid moisture evaporation during the freeze-drying process of samples.
h. Paper towel

Spray the paper towel with 70% ethanol for disinfecting the puncher and punching mat after working on each sample.

7.5.2. Transferring samples to genotyping plates

i. Label the genotyping plates using the plate names from step 3 of the online genotyping platformavailableat(https://cimmyt-genotyping-prd.azurewebsites.net/ldsgrequest/create) for Intertek and from the assigned names for DArTSeq[1].
ii. Label the plate covers for DArTSeq with the same labels as the plates.
iii. Label the individual tubes (wells) for the DArTSeq plates as A01, A02, etc. This is because the tubes are removable and can be easily mixed up.
iv. Set up a coordinate app grid with a specific project title.
v. Mark wells H11 and H12 blank for Intertek plates. For DArTSeq, these are wells G12 and H12. This is done by pre-filling them on Coordinate App by long pressing their positions until they turn **grey** (Figure 8, step 6, Figure 16).
vi. For the remaining wells, each well is filled with 1 sample: 2 leaf discs for Intertek and 4 leaf discs for DArTSeq by pinching out the discs out of fresh cigar leaves using a puncher. Pick out the discs with sterilised forceps and place them in the plate well.
vii. Place the genotyping plate on ice while working.
viii. Label the sample in the well on the Coordinate App grid as follows:
    a. If samples have barcodes, they are labelled in Coordinate App in the field at the time of sampling and the Coordinate App position is written on the aluminium foil (see step 3.ii above). Place the discs of each sample in their designated plate well number position as the sample label shows.
    b. If the samples have no barcodes, assign them well numbers in the coordinate app grid and place the discs in the plates accordingly.
ix. Fill the plates vertically (**A1-H1**) and not horizontally (**A1-A12**).
x. Export the coordinate grid layout for the scanned samples into an excel file for further use.
xi. Cover each plate with a cling film.

[1] DArTSeq is not covered under online genotyping platform for now.

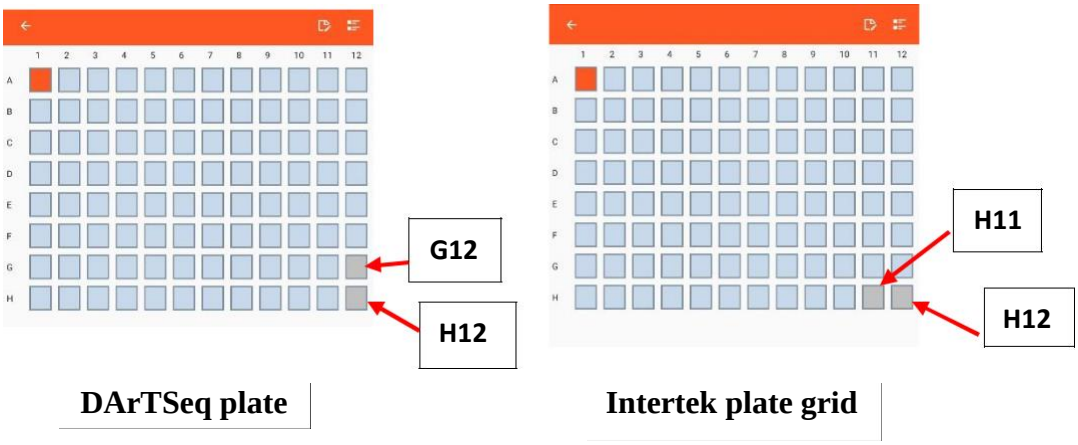


Figure 17: Interface of coordinate app grid display of prefilled well numbers G12 and H12 for DArTSeq plate and prefilled well numbers H11 and H12 for Intertek plate.

7.6. Freeze drying of samples

Seal each genotyping plate with a cling film.

i. After sealing each genotyping plate with a cling film, perforate a hole through the cling film on top of each well using a syringe.
ii. Place the plates in a -80°C freezer overnight or longer.
iii. Ensure that the valves on the drying chamber of the freeze-drier are closed. Start the freeze drier. Program it to 0.214 Pa.
iv. Once the freeze-dryer has reached the right pressure and -50°C, place at most 3 plates in the drying chamber and dry them for at least 24 hours (this applies to a drying chamber of 18 litres).
v. Once the samples are fully dry (they should be brittle when touched with forceps), remove them from the drying chamber promptly, remove the cling film, and seal the plates with sealing caps immediately to limit air exchange.
vi. If the plates are not shipped right away, store them in airtight containers at room temperature.

7.7. Sample preparation for shipping

i. Documents for shipping: Print each one of them in duplicate.

- A Phytosanitary Certificate or an exemption
- Commercial invoice
- Packaging list
- Shipping address
- Customer declaration (when shipping to Australia)
- Genotyping order forms

ii. Packaging

- Make sure the caps are pressed firmly to close each plate.
- Cut rectangular card box pieces in the size of the plate.
- Place one card box at the bottom and another one at the top of each plate and secure them with strong rubber bands (**Figure 18**).
- Place the plates in a fitting card box, not too large.
- Place crumbled newspapers around the plates in the box to secure them.
- Place one set of the documents inside the box and the other outside the box.

  N.B: Attach the air waybill number on the documents for easy tracking of the consignment. (The air waybill number is provided by the shipping company).

Figure 18:A picture showing genotyping plates tied together using cardboard pieces ready for shipping.

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