IITA MUSA IMPROVEMENT – MOLECULAR BREEDING

STANDARD OPERATING PROCEDURE (SOP) FOR

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

1. Authors & Contributors

Teddy Oparok[1*], Noah Nyanzi[1], Catherine Nasuuna[1], Rose Simba[2], Brigitte Uwimana[1]

[1]Intrenational Institute of Tropical Agriculture – Uganda, P.O Box 7878 Kampala, Uganda
[2] International Institute of Tropical Agriculture – Tanzania, C/o NM-IST, B.O. Box 447 Arusha, Tanzania

[*]Correspondence: T.Oparok@cgiar.org, N.Nyanzi@cgiar.org, C.Nasuuna@cgiar.org, R.Simba@cgiar.org, B.Uwimana@cgiar.org.

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 the yield and quality of the DNA extracted.

Banana experiments can 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. Alternatively, the banana fields can be located away from the research station, and the collected samples cannot be processed or kept under cold conditions. This protocol covers the former situation, where samples are collected and handled in the lab for optimum DNA yield and quality.

3. Purpose

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

4. Scope

This SOP document contains procedures required in on-station banana leaf sampling. It covers preparing materials from the lab for sample collection, sample collection from the field, and sample preparation requisites for shipping to sequencing platforms.

5. Definition of terms (in alphabetical order)

- **Banana leaf sampling on-station:** This is when fresh leaf samples of banana genotypes are collected from the plants and 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:** This is a shipping document containing a detailed description of a shipment's content (consignment). It 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 breeder by coordinating molecular breeding activities including trial and experiment design and management, supervision of field, and laboratory operations, data collection, data analysis, synthesis and writing of reports. Also responsible for coordinating and supervising the field, laboratory staff and trainees.

Laboratory Technician: runs all the activities in the molecular lab such as preparation of buffers, DNA extraction and storage for genotyping, routine maintenance of machines. 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. Items needed

a. Stainless steel scissors (Figure 1)

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

Figure 1. The appropriate type and size of scissors suitable for leaf sampling.

b. Laboratory gloves

These are worn to protect (safeguard) the hands of the sampling person and prevent contamination of samples. **Always** replace your gloves immediately if they get torn or dirty.

c. 70% ethanol solution in a dispensing spray bottle (Figure 2).

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

Figure 2. Example of 70% ethanol dispensing bottles.

d. Tissue paper

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

e. Aluminium foil

These are used to keep a cigar leaf sample.

f. **Cool box and ice blocks (**

g. ***Figure 3*****)**

These are used for storing collected samples while in the field. The cool box with ice blocks help maintain sample quality by keeping them cool (lower temperature), thereby minimizing the rate of physiological deterioration. The ice blocks should be kept at -20^{o}C overnight before sampling. If not available, they can be replaced by ice.

Figure 3. Example of a cool box (A) and ice blocks (B) used for sample collection in the field.

h. Permanent marker pen

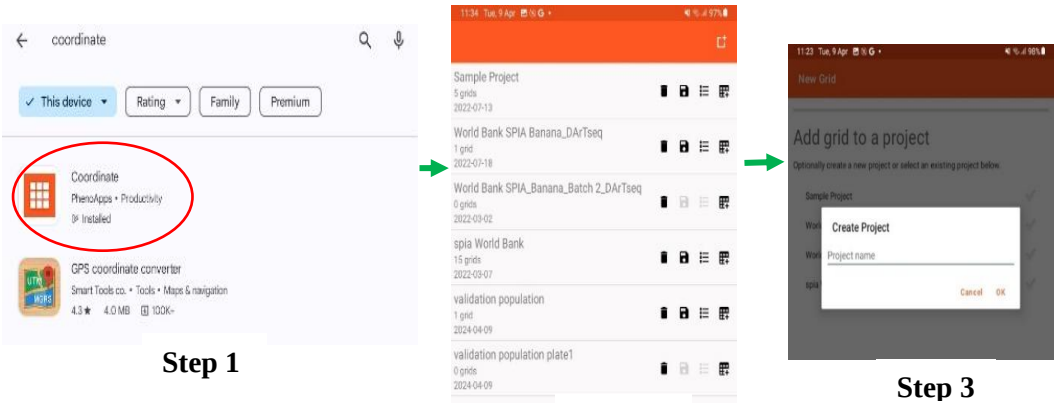
This is used for labeling the samples on aluminium foil during sample collection in the field. It should be of the type of ink that does not rub off.

i. **Field Layout**
Please use a field layout of the experimental field for guidance when collecting samples. This is crucial especially if the plants in the field do not have barcodes.

j. **Mobile device 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 leaf samples into genotyping plates. It is downloaded from the Google Play Store (for Android tablets or phones).

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 4, step 1).
ii. Open the Coordinate app icon on your mobile device and click on the "**Project**" icon (Figure 4, step2).
iii. Add "**Project name**" and click on "**Project**" created to open it (Figure 4, step3).



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

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

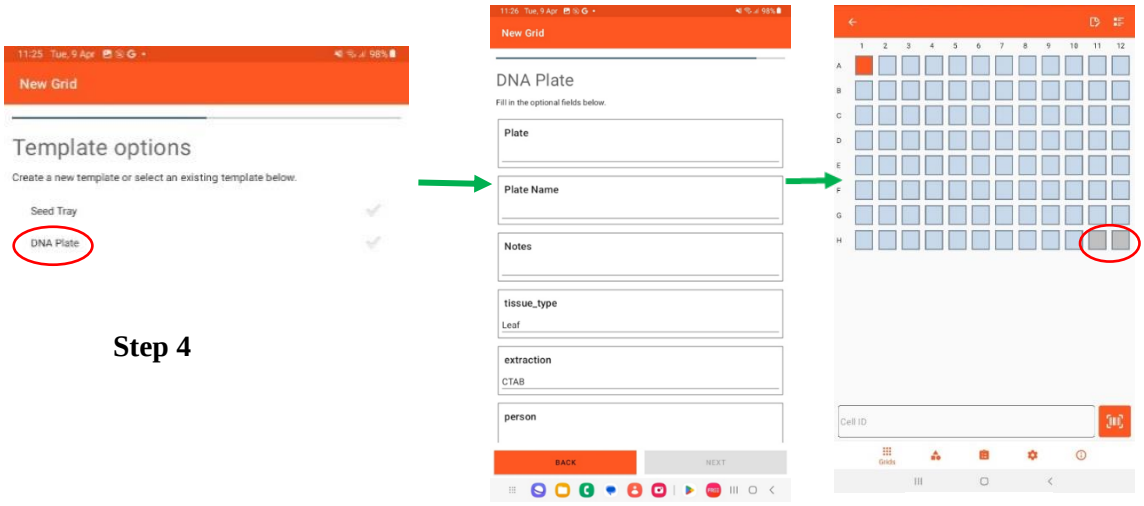


Figure 5: Process of downloading and using Coordinate app. Select "DNA plate" to add grid (Figure 5, step 4), Fill in the optional fields asked in the "DNA plate" (Figure 5, step 5), Press "Next" to go the grid layout (Figure 5, step 6).

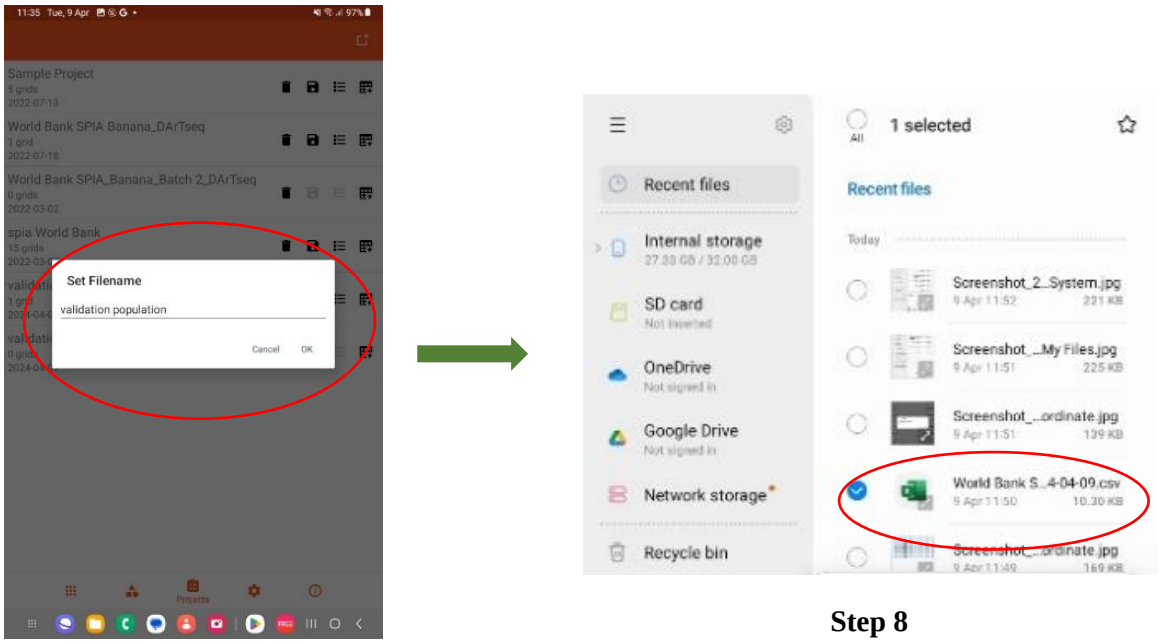
vii. During plating of the samples, prefill **H11** and **H12** wells to keep them empty (blank) if the plates are going to be sent to Intertek, or prefill **G12** and **H12** for DArTSeq plates for genotyping, by long pressing them until they turn **grey**. The two wells are left as control checks during sequencing by the respective sequencing companies (Figure 5, 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 5, step 6).

ix. To export the sample file, save the file by clicking on the "**disc**" icon, and write file name (Figure 6, step 7). The file will be saved in a "**csv**" format (Figure 6, 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 tablet screen (Figure 7, step 9).

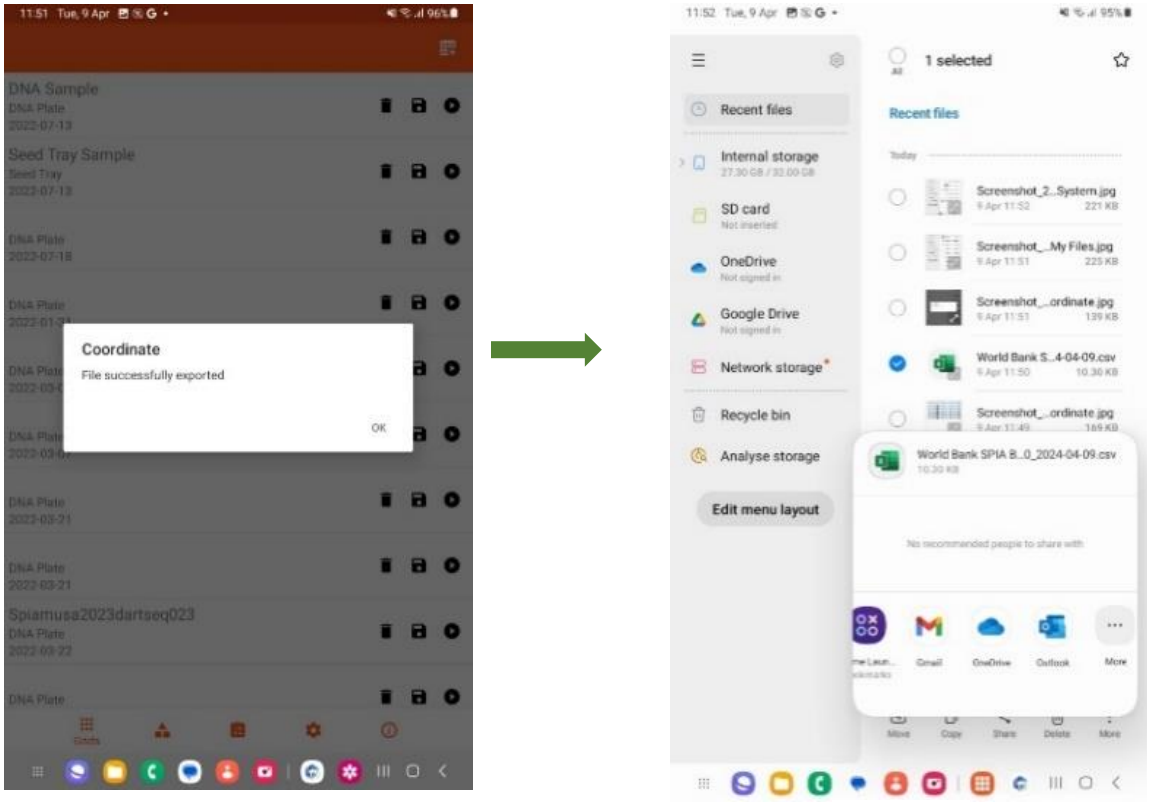
x. Do not delete your grids until you are done with your genotyping project for reference and backup.

xi. Go to the "**files**" icon on your tablet, select your saved file, and share it by email (Figure 7, step 10).



Step 7 **Step 8**

Figure 6: Process of downloading and using Coordinate app: to save file, write file name (Figure 6, step 7), The file will be saved in "csv" format (Figure 6, step 8).



Step 9 **Step 10**

Figure 7. The process of downloading and using coordinate app:" coordinate file successfully exported" message displayed on the tablet screen (Figure 7, step 9). Select saved file and share by email (Figure 7, step 10).

7.3. Preparations in the lab before going to the field

- Prior to leaf sampling, ensure that all the above items (**a-i**) 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.

7.4. Leaf sampling procedure

i. Walk to the experimental field with all the needed material for picking leaf samples from the banana plants.

ii. In the field, two scenarios may occur; some fields may have barcoded plants while others may have plants with no barcodes.

- If the plants are barcoded, scan the barcode using the coordinate android application and write the "well" number of the scanned sample on the aluminium foil in which the sample is wrapped and place it in a cool box with ice blocks.
- If the plants are not barcoded, record the name of the genotype on aluminium foil, wrap the sample and place it in a cool box. Samples without barcodes will be assigned "well" numbers accordingly when plating in genotyping plates from the laboratory.

iii. Sterilize the pair of scissors with a 70% ethanol solution sprayed on a paper towel.

iv. Identify a **healthy clean green cigar leaf** on the banana plant

v. **Figure 8**).

vi. Use sterile scissors to cut approximately 6 cm of the leaf from the leaf apex (tip of the leaf). The cigar leaf is the youngest leaf on the plant, and it yields the highest quality 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 8**.
- Samples collected from on-station can be stored in a +4°C fridge for at least 3 days after plating. Discard stored samples after 3 days because by then they are physiologically deteriorated. Since the fields are near the lab, you can go back to replace any deteriorated samples instead of plating rotten leaves.

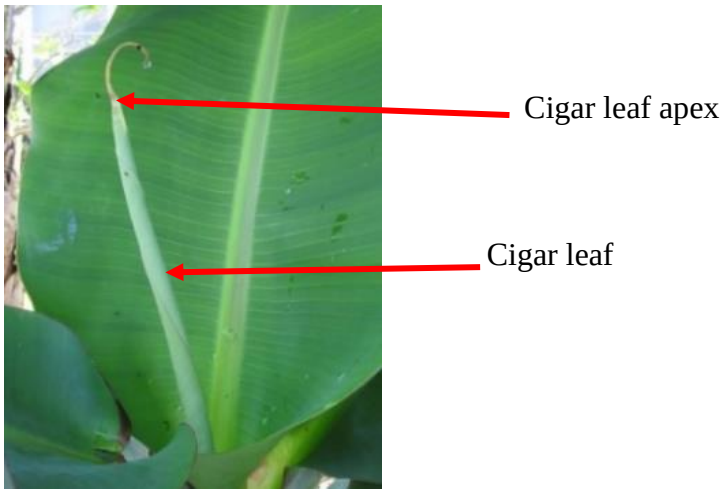


Figure 8. A cigar leaf on a banana plant.

vii. Place the cigar leaf into the aluminium foil and wrap it. Label the aluminium foil with the sample name according to **step 2 (coordinate app plate number and well or sample name)**.

viii. 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
2.
3.

4. **Figure *9***.

Figure 9. Examples of diseased cigar leaves. These are not suitable for sampling.

5. After cutting the cigar leaf, always trim off the tip of the cigar leaf (leaf apex) (**Figure *8***).
6. If the leaf is moist from rain droplets, dew or contains soil particles or dust, wipe it using tissue paper before sampling.

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

Here we consider genotyping using DArTSeq and Intertek sequencing platforms.

7.5.1. Tools and equipment required

a. Android tablets with Coordinate App installed.
b. A mat and leaf puncher (**Figure *10***)

Figure 10:A picture of a leaf puncher used for making leaf discs.

c. **Genotyping plates (**
d.
e. ***Figure 11*****)**

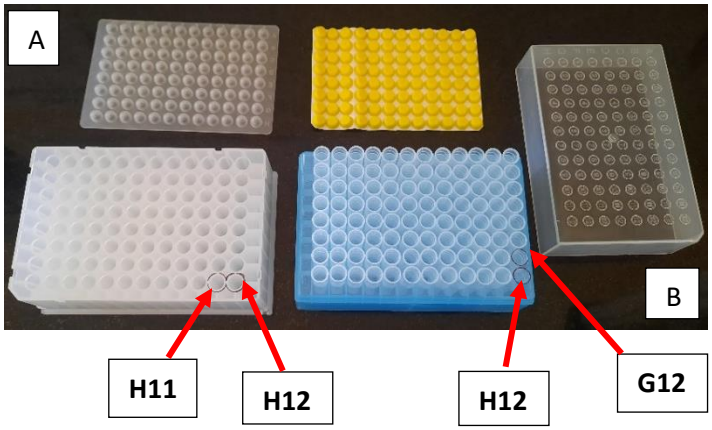


Figure 11. Types of genotyping plates used for shipping samples to sequencing companies. 12A: genotyping plate and its sealing cap used by Intertek; 12B: genotyping plate and its sealing cap and lid used by the DArTSeq sequencing.

f. A marker pen
g. 70% Ethanol in a dispenser (
h.
i.

j. **Figure *2*)**
k. **Cling film**-sealing of genotyping plate after placing in leaf discs of samples.
l. **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.
m. **Paper towel**

Spray the paper towel with 70% ethanol to disinfect the puncher and the 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 platform available at (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 are as blank for Intertek plates. For DArTSeq, these are G12 and H12. This is done by pre-filling them on Coordinate App by long pressing their positions until they turn **grey** (**Figure 5, step 6** and **Figure 12**).
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 the fresh cigar leaf 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 and position as the sample label shows.

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

b. If the samples have no barcodes, assign them well numbers in the Coordinate App grid and 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 Excel files for further use.

xi. Cover each plate with a cling film.

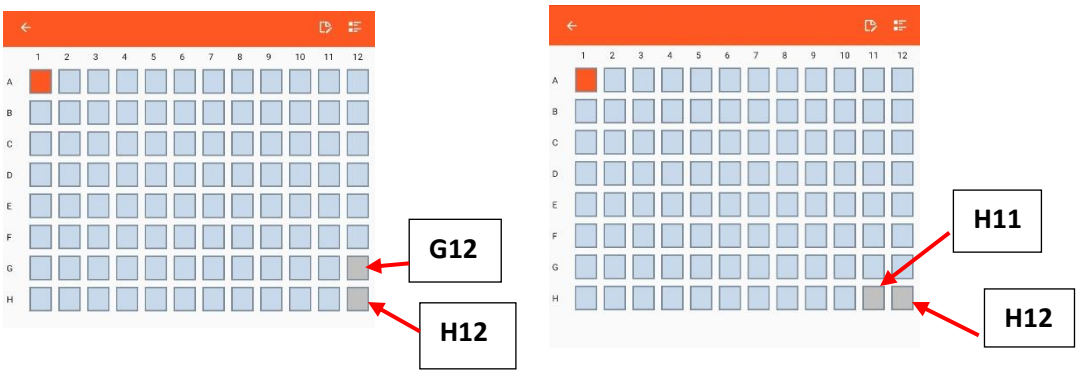


Figure 12. Interface of coordinate app grid display of prefilled well numbers G12 and H12 for DArTSeq and prefilled well numbers H11 and H12 for Intertek plate.

7.6. Freeze drying of samples

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-dryer are closed. Start the freeze-dryer. 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 cardboard pieces in the size of the plate.
- Place one cardboard at the bottom and another one at the top of each plate and secure them with strong rubber bands (**Figure *13***).
- Place the plates in a fitting cardboard box, not too large.
- Place crumbled newspapers around the plates in the box to secure them.
- Place one set of the shipping documents inside the box and the other outside the box.

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

Figure 13. A picture showing genotyping plates tied together using cardboard pieces ready for shipping.