PROTOCOL: Preservation and Storage of Frozen Leaf Disks

(i) **Equipment**

- Cryo-Freezer (-80 °C)
- Freezer racks for 2" cryo-boxes

(ii) **Consumable materials**

- 2" cryo-boxes with lids
- 2 oz screw-cap Nalgene vials (cryo resistant)
- 2 ml screw-cap micro centrifuge tubes
- Cryo-labels
- Dry ice
- Nalgene trays
- Liquid Nitrogen
- Coolers

(iii) **Sample preparation and Tinfoil sorting**

- Immediately upon arrival at the Spectranomics Laboratory, remove stocking packages from the dry-shippers, and place them directly onto the sorting shelves in the -80 °C freezer.
- Label empty 2 oz Nalgene vials with cryo-labels according to the collection, sort into boxes, and place in freezer.
- Label freezer racks with letter and number (according to freezer assignment) prior to placing in freezer.
- Label two sets of 2ml centrifuge tubes in 2" boxes with dividers according to the collection and place in freezer as well (one set in blue ink for Chlorophyll /Carotenoid analysis and the other set in black in for Phenol and Tannin analysis)
- Place order for 100lbs of dry ice for every 400-600 samples the day before to arrive next day AM.
- Use two to three coolers to place dry ice blocks and temporarily store nalgene boxes during sorting. This ensures that the freezers are not opened as frequently and the temperatures are maintained between -75 °C to -80 °C
- Remove one stocking package (10 samples) from the freezer and empty the tinfoil packets contents, directly onto the dry ice.
- Sort the tinfoil packets into groups by sample, separating 'A' and 'B' replicates and then sort them in numerical order over ice.
- Be sure to note any missing, lost, duplicate samples on the logging sheet and note ANY discrepancies observed.
- Make sure that the tinfoil packets remain on the dry ice at ALL times during sorting, do not hold them up in your hand or let them fall off the ice. Thawing and refreezing will permanently disrupt the leaf tissue.
- Place the Nalgene vials that correspond to the samples on the ice and open them to receive samples.
- Put the 6 replicate 'A' and 6 replicate 'B' tinfoil packets into their respective vials.
- Seal vials, place them in their labeled boxes and put them into the freezer immediately.

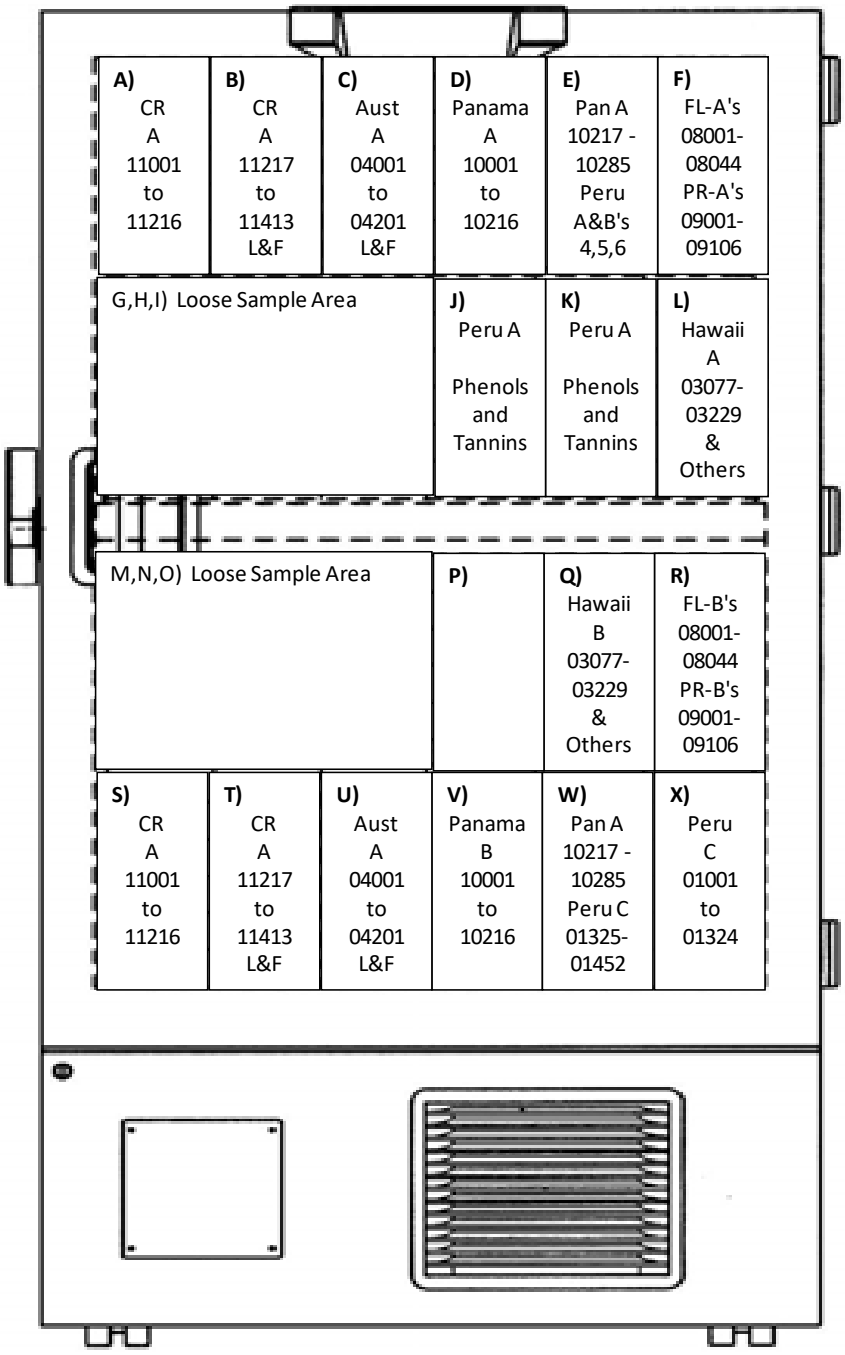
(iv) **Frozen Disc transfers**

- Once tinfoil transfer is complete, remove 4-6 boxes from the A labeled set (the working samples) to begin transferring discs for our chemical assays.
- Place the first set of labeled boxes (Chl and P&T for samples #1-81) on either side of the first box of nalgenes.
- Using tweezers, remove the XXxxx-1A tinfoil out of the nalgene, locate the best discs (green, without dead spots, rips or extra pieces..) and transfer ONE disc into the P&T box, and then another TWO discs into the CHL box.
- Cap immediately and remember to use coolers again for temporary storage while transferring.
- Every few rows of tubes capped and completed, pour some liquid nitrogen to keep samples cool. Do NOT pour into open tubes.
- Dry Ice can be stored in the -80 °C freezer overnight.

(v) **Logging procedure**

- Record the number of leaf disks in storage in the "Wet sample tracking datasheet"
- Record the location of the samples in the 'Freezer Location Tracking' worksheet and update the general freezer diagram as necessary.
- Store 'A' replicates (the working samples) in a separate location of the freezer from the 'B' replicates to ensure the integrity of the long-term samples is maintained (See diagram below).

The SPECTRANOMICS FROZEN FOREST (01-11-11)



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