Shared Vitrobot Standard Operating Procedure version 0.2

Authors:

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1. Purpose

1.1. Setting up, operating, and shutting down the ThermoFisher Scientific (TFS) Vitrobot Mark IV in a safe and effective manner for the preparation of frozen cryo-EM of samples.

2. Definitions:

2.1. ThermoFisher Scientific (TFS) Vitrobot Mark IV is a vitrification device for freezing protein samples for CryoEM analysis.
2.2. Liquid Nitrogen (LN2) is a cryogenic liquid stored under pressure.
2.3. Ethane is classified as a flammable gas.

3. Supplies & Equipment

- ❏ PPE (BSL-1)
  - ❏ Laboratory Coat
  - ❏ Nitrile Gloves
  - ❏ Goggles / Safety Glasses
  - ❏ Cryogenic Gloves
  - ❏ Face Mask
- ❏ Chemicals/Reagents
  - ❏ Liquid Nitrogen
  - ❏ Ethane
  - ❏ 70% Ethanol
  - ❏ Distilled Water
  - ❏ Sample
- ❏ Filter Paper (Pre-punched Whatman #1 or #541)
- ❏ Pipette and Tips
- ❏ Vitrobot Tweezers
- ❏ Glow Discharged / Plasma Cleaned EM Grids
- ❏ Grid Storage Boxes/Buttons
- ❏ Button Lid Tool (Screwdriver or TFS Pin Tool)
- ❏ LN2 Dewars for Transfer
- ❏ Forceps (fine tip and long tweezers)
- ❏ If BSL-2, place absorbent bench paper before starting work

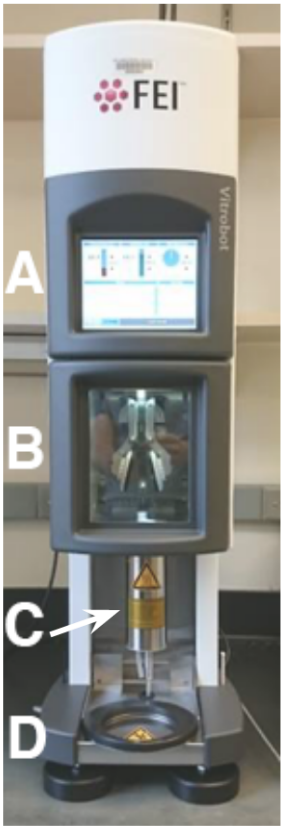

Figure 1. Vitrobot assembled and turned on. **A)** Screen. **B)** Environmental chamber with blotting pads. **C)** Humidifier. **D)** ethane lift.

4. Procedure:

4.1. Instrument setup

4.1.1. Attach humidifier to Vitrobot, sticker facing forwards (see Fig. 1C).
4.1.2. Fill humidifier with 60 mL distilled water via tube on the bottom.
4.1.3. Draw back on the syringe ~10mL to create vacuum in humidifier liquid chamber.
4.1.4. Turn on the Vitrobot via the switch on the back right of the instrument.
4.1.5. Blotting papers need to be placed before blotting. This can be done now,

or after filling the ethane cup (step 4.2 below). The amount of time the filter papers is in the environmental chamber before blotting will affect reproducibility.

4.1.6. Set desired humidity and temperature – you should let the chamber equilibrate for 15-20 min (the rest of the setup will likely take this much time).

4.1.7. Under options: set process parameters.

4.1.7.1. Enable "skip grid transfer" and "use foot pedal", if desired.

4.1.7.2. Adjust settings as needed.

4.1.7.3. Typical Settings:

Humidity	100%
Temperature	4℃ - 22℃
Blotting Time	2-5 seconds
Wait time	0-2 seconds
Blot Force	0 -15 (see troubleshooting)
Skip Grid Transfer	Yes
Use Footpetal	Yes

4.1.8. Without tweezers, test vitrification cycle by advancing through on-screen process (using foot pedal).

4.2. Cooling the Ethane

Safety note – Liquid ethane is a rapid cryogen and can burn and cause damage to your skin and eyes. You must wear proper PPE and take caution when dispensing. If the ethane surface freezes while cooling, it may build pressure and splash.

4.2.1. Assemble the ethane holder

4.2.1.1. Place brass ethane cup, gridbox holder, spider, and anti-contamination ring inside of the circular foam base (see Fig. 2).

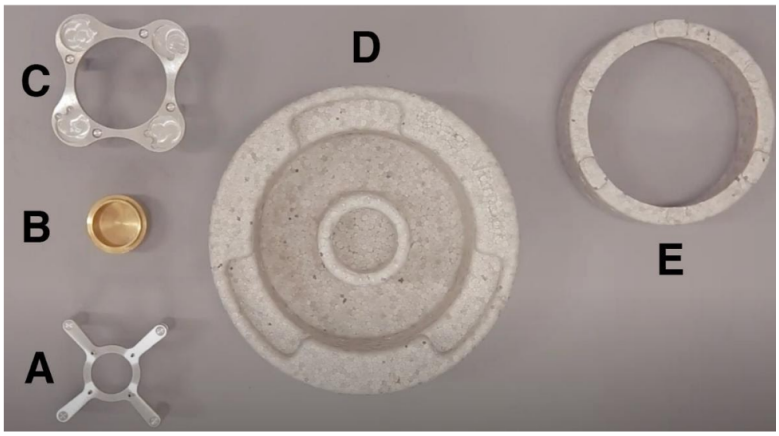

Figure 2. Ethane Holder. **A)** Spider. **B)** Brass Ethane Cup. **C)** Gridbox Holder. **D)** Base / Liquid Nitrogen Container. **E)** Anti-contamination Ring.

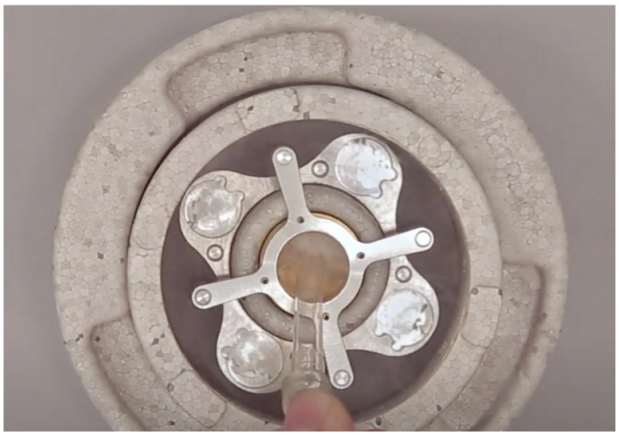

Figure 3. Ethane Holder Assembled. Proper ethane tip placement shown.

4.2.2. Fill outer ring of foam base with liquid nitrogen.

4.2.3. Place the tip of the ethane hose at the bottom of the brass ethane cup (see Fig. 3). While slowly moving tip ~1cm side to side, increase gas flow very slowly by opening the needle valve on the regulator.

4.2.3.1. Listen for bubbling gas condensing into liquid.

4.2.3.2. Adjust gas flow as necessary until liquid ethane is touching the bottom of the spider.

4.2.3.3. With tip of ethane hose submerged ~5mm, turn off gas flow and remove tip from liquid ethane. Be sure it is facing away from you and down toward the lab bench.

4.2.3.4. Allow liquid ethane trapped in the tip to evaporate before storing the hose.

4.2.4. Allow the ethane to cool. This is a good time to check vitrobot settings, glow discharge grids, etc.
4.2.5. When ethane has formed a slurry (see fig. 4), remove the spider with pliers.
4.2.5.1. If the spider is frozen to the ethane cup, place a nickel on the center of the spider to quickly melt the top. Do not remove nickel, remove the spider with the nickel instead.

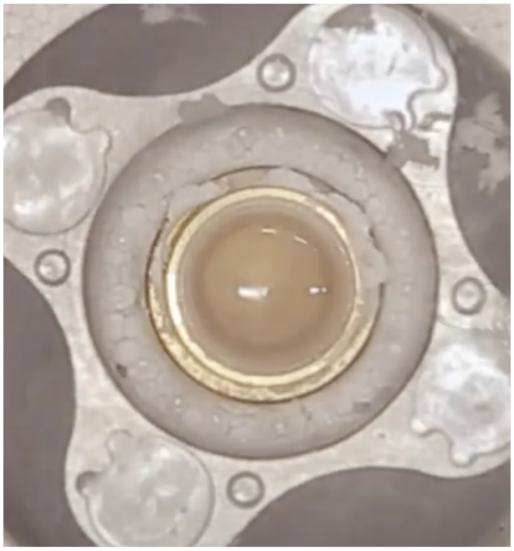

Figure 4. Slurry of Ethane, perfect for vitrification.

4.3. Freezing Cycle / Plunge Freezing

4.3.1. If you didn't attach blot papers to the blotting pads in step 4.1, do so now. Advance through the freezing cycle 1 time (without tweezers) to home blotting pads. This is essential for consistency.
4.3.1.1. Replace blot papers after each grid box or every 20 minutes, whichever occurs first.
4.3.2. Place a grid box in the gridbox holder – make sure the lid is loose enough to move with tweezers and adjust lid opening so that it's over the slot you plan to load.
4.3.3. Pick up a glow-discharged grid with Vitrobot tweezers and attach tweezers to the plunging rod.
4.3.3.1. Be sure to attach tweezers so they're oriented the same way every time (e.g. red dot to the right) to increase ice consistency.
4.3.3.2. Pick up grid and attach to plunge rod so that carbon is facing the right if you are right handed, left if left handed.
4.3.3.3. Tweezers will "click" into place when properly attached to plunge rod.
4.3.4. Press start process (or use the foot pedal). Wait for plunging arm to raise into Vitrobot and shutter to close.
4.3.5. Attach the ethane container to ethane lift by placing it in the center of the ring. Press next (or foot pedal) to advance.
4.3.6. Press next step (or use the foot pedal) to lower the tweezers into the sample loading position.
4.3.7. Open red loading port on the side of the chamber your carbon is facing.
4.3.8. Load 3 µL of sample into pipette, insert horizontally into chamber, and add your sample to your grid (see Fig. 5).
4.3.9. Remove pipette from chamber and close loading port.
4.3.10. Press next step (or foot pedal) to start the plunging process. Wait for process to complete.
4.3.11. Process is complete when ethane lift and tweezers are lowered and "place new tweezers" appears on screen.
4.3.12. Carefully detach the tweezers from plunging rod while keeping grid submerged in liquid ethane.
4.3.13. Lift tweezer slide clip to open position while keeping tweezers engaged with your fingers.
4.3.14. Quickly (1 second) move tweezers from liquid ethane to liquid nitrogen and then into gridbox slot.
4.3.15. Move gridbox lid opening to next slot.
4.3.16. Add more liquid nitrogen to ethane container (add into slot in front of guard).
4.3.17. Dry and clean tweezers with 70% ethanol + kim-wipe.
4.3.18. Freezing cycle is now complete. Repeat steps 4.3.3 to 4.3.17 for all of your grids.

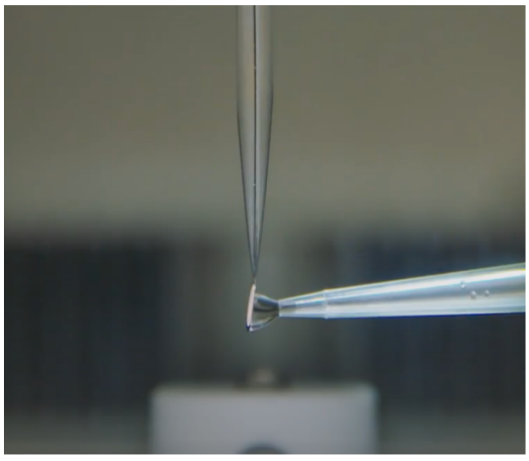

Figure 5. Pipetting sample onto CryoEM grid at sample loading position inside environmental chamber of Vitrobot

4.4. Revitalizing the Ethane

4.4.1. If too much of the ethane freezes, you run the risk of smashing your grid. Complete these steps between each grid box.
4.4.2. Place the room temperature spider upside down on top of ethane cup to thaw.
4.4.3. Once the ethane starts to melt, remove the spider.
4.4.4. Submerge spider in liquid nitrogen and allow it to cool.
4.4.5. Once enough of the frozen ethane has melted we must refreeze it.
4.4.6. Using pliers, place the cold spider on top of ethane cup, right side up (with legs in the liquid nitrogen)
4.4.7. Wait for ethane to return to slurry.
4.4.8. Remove spider.

4.5. Shutdown

4.5.1. Dry and store the Vitrobot tweezers.
4.5.2. Tap the Exit button on the Vitrobot screen.
4.5.3. Wait for the Vitribot to shut down: the screen will go completely blank and black
4.5.4. Flip off the power switch on the back of the Vitrobot.
4.5.5. Remove and dispose of blotting papers. Store rings inside chamber.
4.5.6. Remove and empty the humidifier. Invert and dump humidifier at least 3 times to clear reservoir.
4.5.7. Place the humidifier upside-down in a Styrofoam container for storage.
4.5.8. Place the foam base containing liquid nitrogen and ethane container in the hood to evaporate.
4.5.9. Ensure the ethane tank valves are closed.

5. Chemicals:

5.1. Ethane
5.2. Liquid Nitrogen
5.3. Ethanol 70%
5.4. Bleach (NaOCl)

6. Waste Disposal:

6.1. Follow facility procedure for proper disposal.
6.2. Biohazardous waste will be collected in designated bins lined with red biohazard bags.
6.3. Chemical hazardous waste will be segregated by hazard class (e.g. flammable, corrosive) and state (e.g. solid, liquid), appropriately labelled, and placed in the laboratory's hazardous waste cabinet.
6.4. Additional Waste Guidelines [BSL-2]:
6.4.1. Decontaminated solutions with 10% bleach (final conc.) will be disposed as chemical hazard liquid waste into the 10% bleach solution collection bottle.
6.4.2. Bench paper used to protect surfaces is to be folded and discarded in the designated biohazard material bin.