UNC-CH CryoEM Core Vitrobot Protocol

This protocol is for individuals that have been certified to operate the UNC-CH Vitrobot. If you like to get certified please contact the core director.

Before preparing cryo-grids:

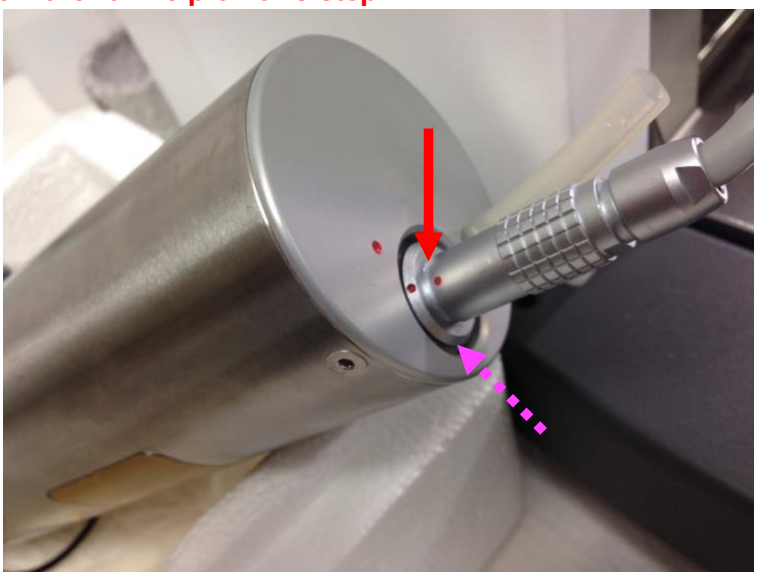
1. Make sure you have everything you need before starting this experiment.
2. Check the LN2 tank and propane-ethane tank in the Vitrobot room.
3. Check LN2 dewar make sure it does not contain ice or water.
4. Check the Vitrobot make sure it is working.
5. Check the Pelco EasiGlow or Tergeo-EM make sure both are working.
6. Make sure you have enough sample for your experiment.

Steps to follow when using the Vitrobot:

1. Setup the Vitrobot and allow it to equilibrate at high humidity (30-60 mins)
2. Assemble Vitrobot Pot and fill it with liquid nitrogen and ethane propane (15 mins)
3. Glow-discharge or plasma clean the TEM grids (5 mins)
4. Prepare cryo-grids (30-60 mins)
5. Transfer cryo-grids to storage (5 mins)
6. Shutdown Vitrobot and clean-up after yourself (5 mins)
7. Enter your information into the Vitrobot Log Notebook (2 mins)

1. Setup the Vitrobot and allow it to equilibrate at high humidity (30-60 mins)

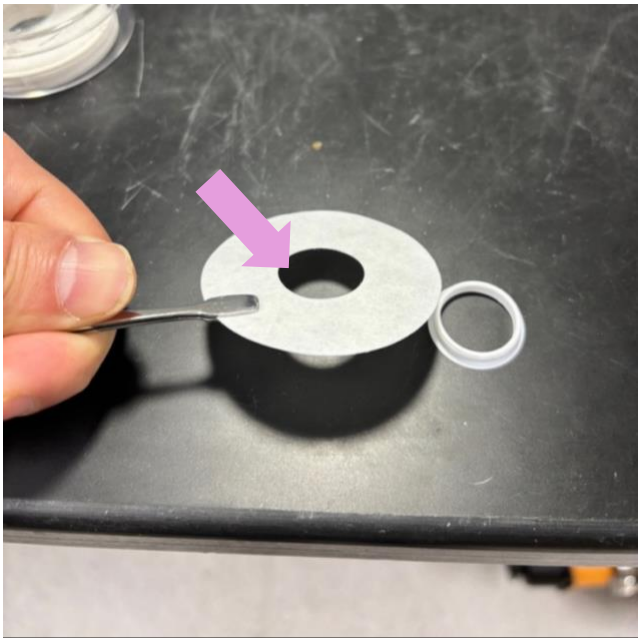
i. Normally the Humidifier is plugged into the Vitrobot. If it is not if not plug in the cable into the bottom of the Vitrobot Humidifier. Align the red dot on the cable with the red dot on the bottom of the Vitrobot Humidifier. To remove the plug push down on the button (indicated in purple), as you pull the cable away from the humidifier. Failure to do so will damage the Humidifier. **This part is critical! Ask the Core for Help on this step!!!**

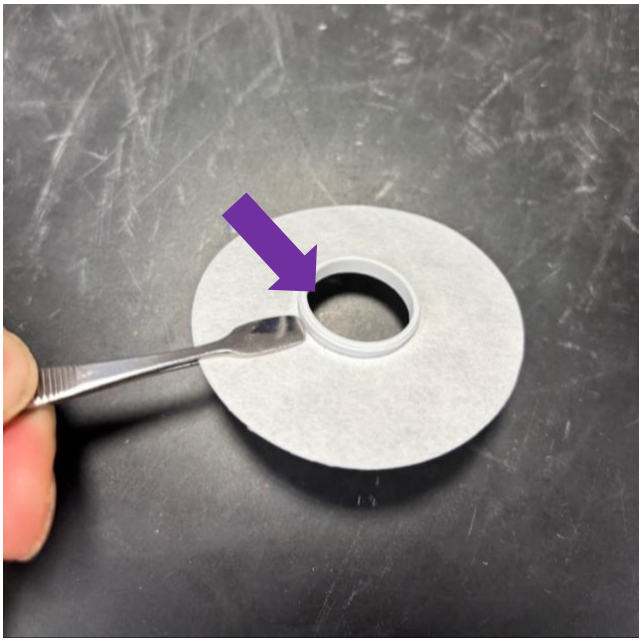


ii. Make sure that the orange rubber O-ring is mounted on the top of the Vitrobot Humidifier before sliding it into the Vitrobot. Align the notches on the Vitrobot humidifier with the pins on the Vitrobot. It should be snug and not wiggle. Place the Vitrobot Humidifier into the Vitrobot so that the sticker faces you (as shown in the picture).

iii. **Fill the humidifier with 20-30 ml of dH_20 with the 60 ml Syringe.** After injecting the water into the humidifier, pull back on the syringe to suck the water into the humidifier.

iv. **Put filter paper on Vitrobot Blotting pads.** Put the Vitrobot Filter Paper onto the Plastic Ring. Don't touch the filter paper with your bare hands. Wear gloves and use forceps. Orientate the filter paper with the lip of the filter paper facing up (pink arrow). Place the Plastic Ring inside the filter paper as shown (purple arrow)





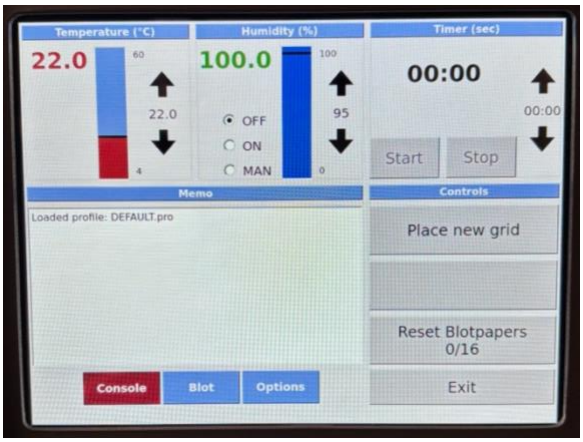
v. **Mount the Plastic Rings with Filter Paper on to the Vitrobot Blotting Pads.** Make sure that the rod is in the upright position inside the chamber. Its easer to do this when the Vitrobot is off. Make sure the filter paper and plastic rings are flush with the blotting pads. Wear gloves when handling the Filter Paper and/or use forceps, don't touch the filter paper with dirty hands.

vi. **Turn on the Vitrobot main power switch**, located in the back of the unit. Wait for the computer to boot up and the main control screen to appear before starting the experiment.

vii. **Set parameters in the Vitrobot Control Screen (Console and Options Tab).** *The Vitrobot Mark IV+ has a touch screen. Use your fingers to operate the touch screen.*

Console Tab:

Set Temperature to the desired temperature of the sample. Once it reaches that temperature set the **Humidity to 95**%.

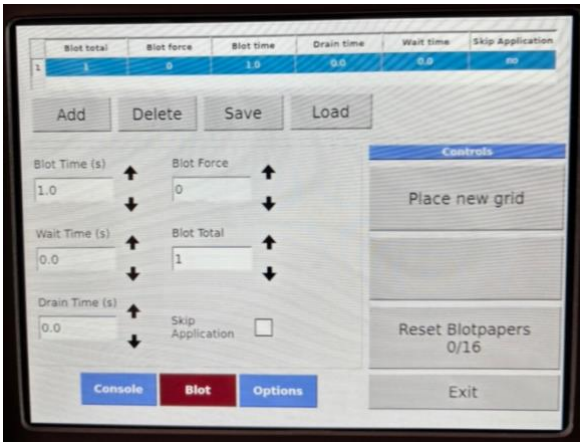


Blot Tab: Process Parameters

Blot time(s): time that grid is blotted.
Wait Time(s): time that sample sits on grid before blotting
Drain Time (s): time in-between blotting and plunging
Blot Force: separation of blot pads when blotting.
Blot Total: number of blots.
Check "use foot pedal" and "humidifier off during process"

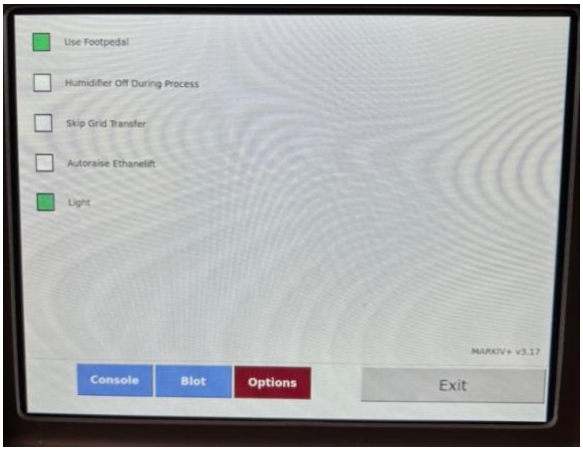
Typical Parameters we use are:

- *Blot time 3-5 seconds.*
- *Single blot*
- *Drain time 0*
- *Apply 3-4 µL of sample*
- *We use either 4, 22 or 38 °C depending on the sample.*
- *Humidity to 95%*
- *Check "Use Foot pedal"*
- *Check "Humidifier off during process"*
- *Blot force -10 or 0*



Options Tab:

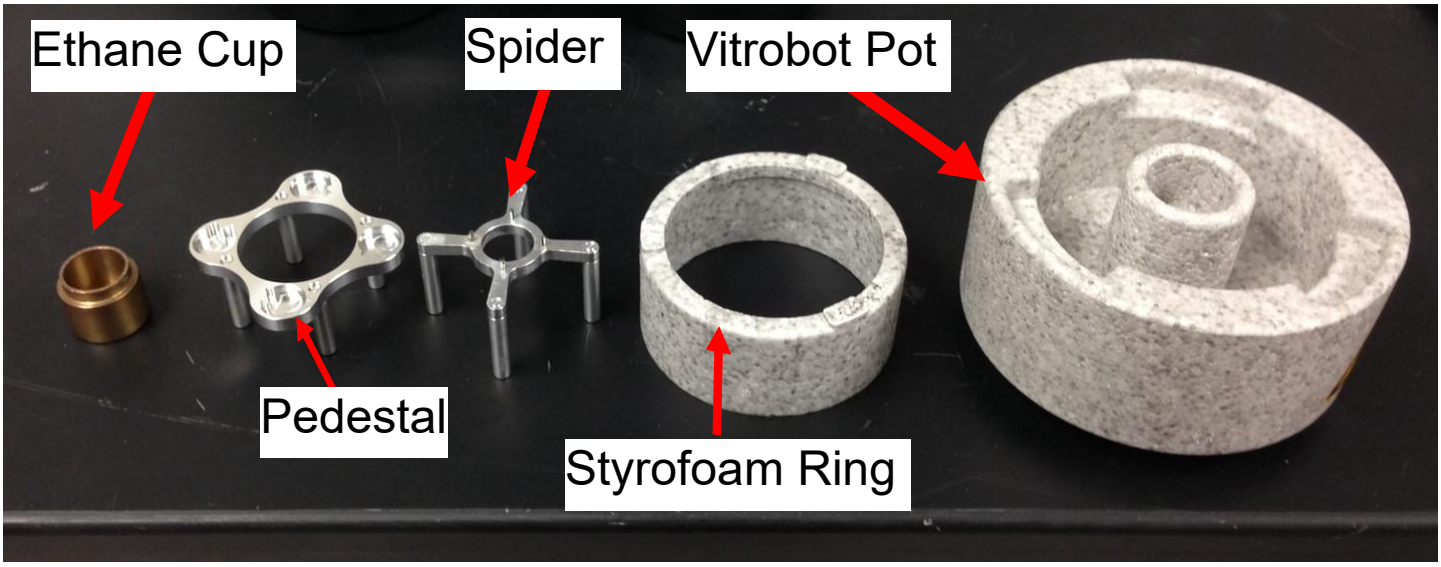
- Use Footpedal (select)
- Humidifier Off During Process (optional)
- Skip Grid Transfer (optional)
- Autoraise Ethanelift (optional)
- Light (select)



We recommend turning the humidifier off and set the temperature. It normally takes ~ 30 mins to cool the Vitrobot to 4 °C. Once the Vitrobot is cool, set the humidity to 95%. It normally take ~ 5 mins to reach high-humidity. ***We recommend letting the Vitrobot filter paper sit in the chamber at high-humidity for ~ 1 hour before preparing cryo-grids.***

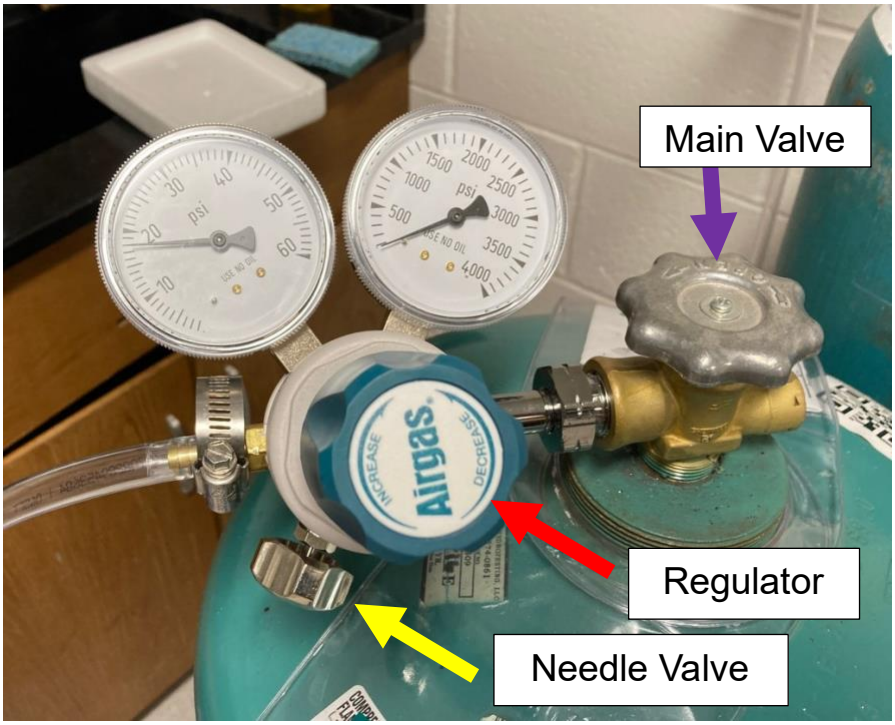
2. Assemble Vitrobot Pot and fill it with liquid nitrogen and ethane propane (15 mins)

i. Assemble the Vitrobot Pot. Make sure all the components are clean and not damaged. Check for dirt, old grids, water and old grid boxes.

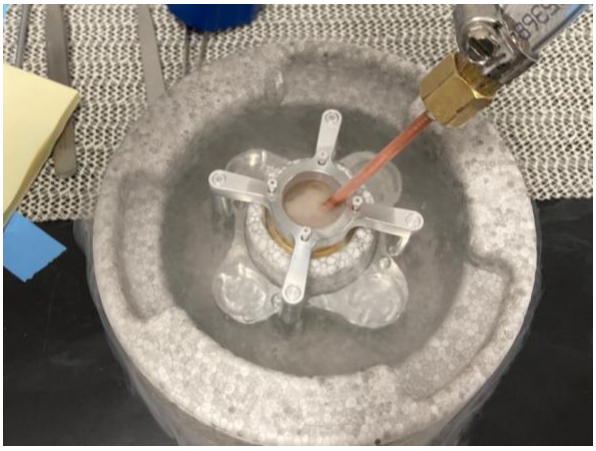


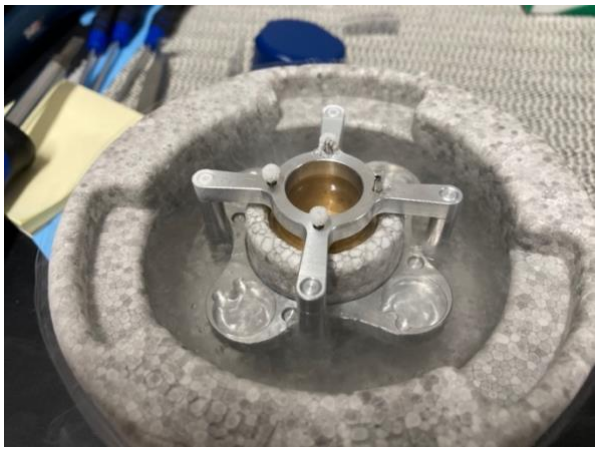
ii. **Fill the pot with liquid nitrogen (LN2).** It should take ~10 mins to cool the ethane cup, during this time add more LN2, and cover the top of the Vitrobot Pot with a plastic beaker or ice box to prevent frost from building up in it. Alternatively, pre-cool the ethane pot and Spider with LN2 in a separate container then assemble using pliers to handle the metal components. Once the liquid nitrogen stops boiling it is ready to dispense the ethane or ethane propane into the ethane cup.

iii. **Fill ethane cup with ethane propane.** Open the main valve on the ethane propane tank. The PSI should be ~15. To dispense the ethane propane, slowly open the needle valve; the PSI on the regular will drop and gas will come out of the needle tip. If you notice the tank is at low pressure it might be running low or empty. If that is the case tell the core personal.



Before dispensing the ethane propane mixture, place the needle tube inside the ethane cup, orientate the tube at an angle touching the bottom of the ethane pot. Then open the needle valve to dispense the ethane propane gas. The gas should flow out slowly and will condense inside the ethane cup. Fill the pot the top, the level of ethane propane should be just below the spider. **When you are finished using the ethane propane, make sure to close the needle valve.**

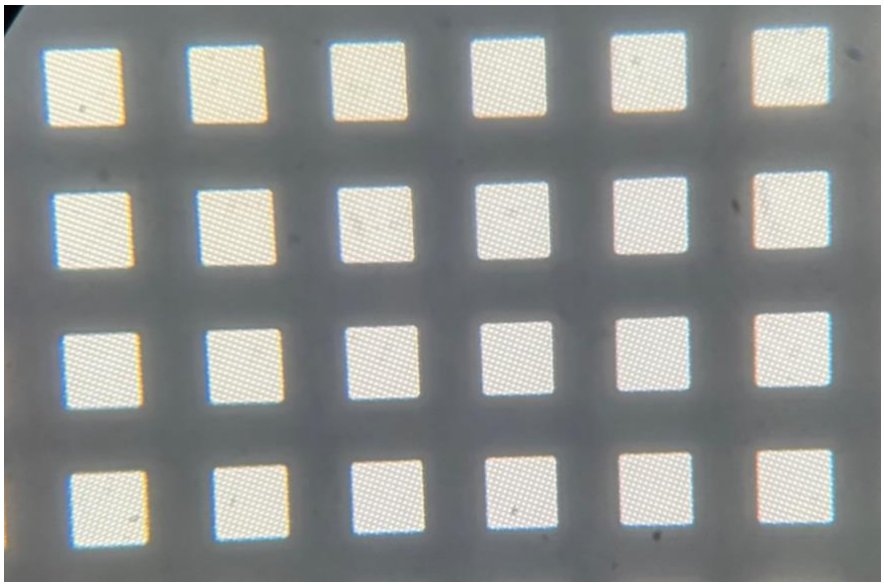




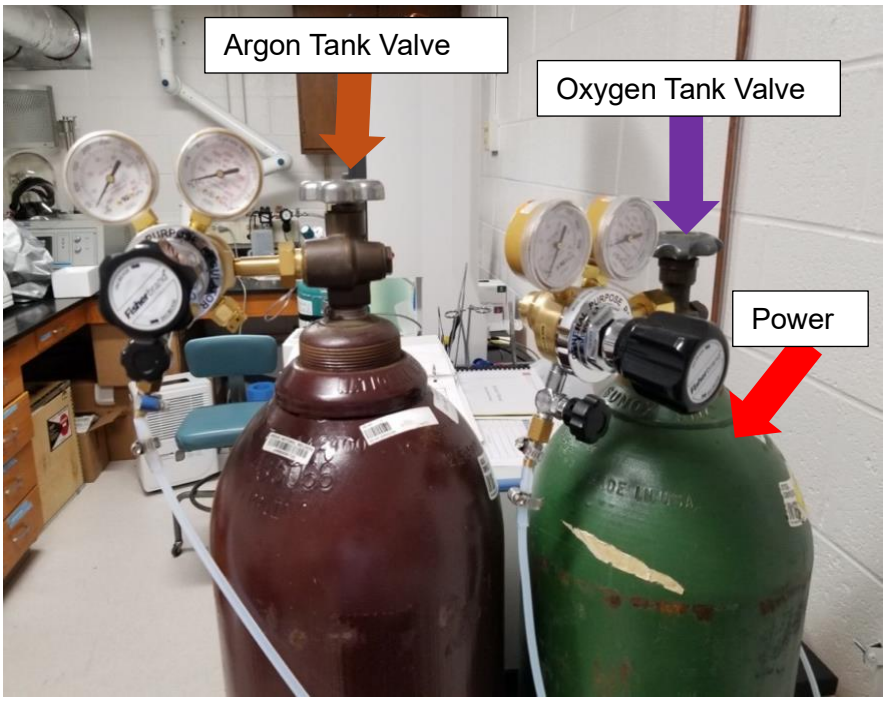
iv. Wait a few mins for the ethane propane mixture to cool down to -190 C°. Check the temperature of the ethane-propane using a thermometer. Once the ethane propane mixture is cooled you can remove the Spider and place it on the hot plate.

3a. Plasma clean the TEM grids (5 mins)

i. **Examine the TEM grids with the light microscope before and after plasma cleaning.** If the grids have defects, cracks, or were damaged during the plasma cleaning stop the experiment. This what the a Quantifoil grid should look like, if you see cracks in the support film or broken grid squares *don't use the grid.*



ii. **To Plasma Clean turn on the TergeoEM plasma cleaner** using the power switch on the back side of the machine. The main valves on the Oxygen and Argon tanks (next to the plasma cleaner) should be closed to open turn counterclockwise. You should not normally need to adjust the regulator valves; the regulators should read ~50. If the pressure to the tanks is low tell the core staff, the tank might be empty, and the TergeoEM will not make good quality plasma.

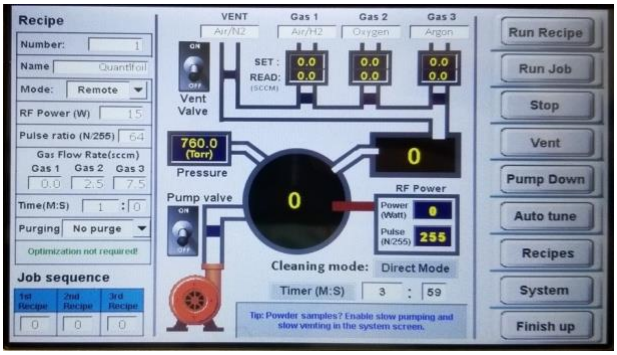


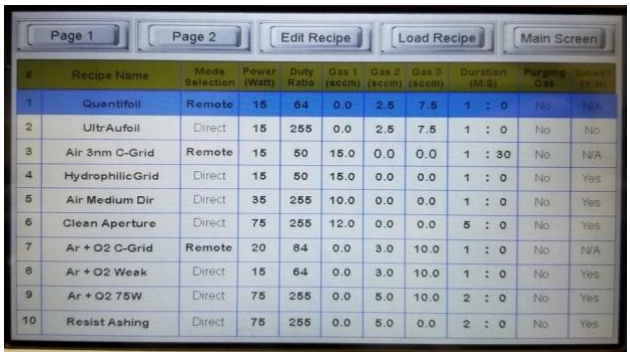
iii. **Open the Tergeo-EM chamber door** by turning it counterclockwise. Place the grids to be plasma cleaned on a glass slide or metal pedestal (both located in the small drawer next to the PELCO easiGLow) and **set the glass slide or metal pedestal inside the chamber** on top of the clear glass holder, pushing the slide or pedestal a couple of inches back into the chamber.

iv. Place the chamber door back on the plasma cleaner and turn clockwise until it stops. The door will feel loose until the vacuum is applied.

v. On the touchscreen, **select "Recipes"** and find the recipe that is suitable for the type of grids you are using.

vi.





vii. **Select the recipe (highlight blue) by clicking on it and selecting "Load Recipe" at the top of the screen.** At the home screen, once the grids are inside the chamber and the door is on, select **"Run Recipe"** and monitor the screen until complete. The vacuum pump will turn red to indicate that it is on, and the pressure will start to drop as indicated on the screen. Once the vacuum is at the appropriate pressure, the gas will begin to flow, and the plasma will ignite. At this point, the timer will start to count down. After the timer reaches zero, the system will be in a standby state with the vacuum pump on. Tap the pump icon on the screen (shown in red) to turn off the pump; the icon should turn gray.

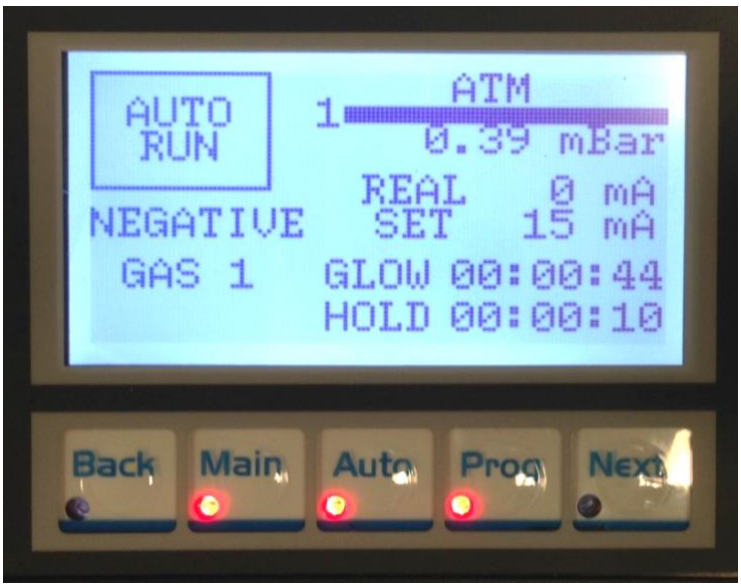
viii. Press the "**Vent**" icon on the screen and wait until the chamber reaches atmospheric pressure, then press "**Stop**". You can now **remove the chamber door and retrieve your grids**.

ix. After using the TergoEM put the chamber door back on and **click "Finish up".** This will pump down the chamber.

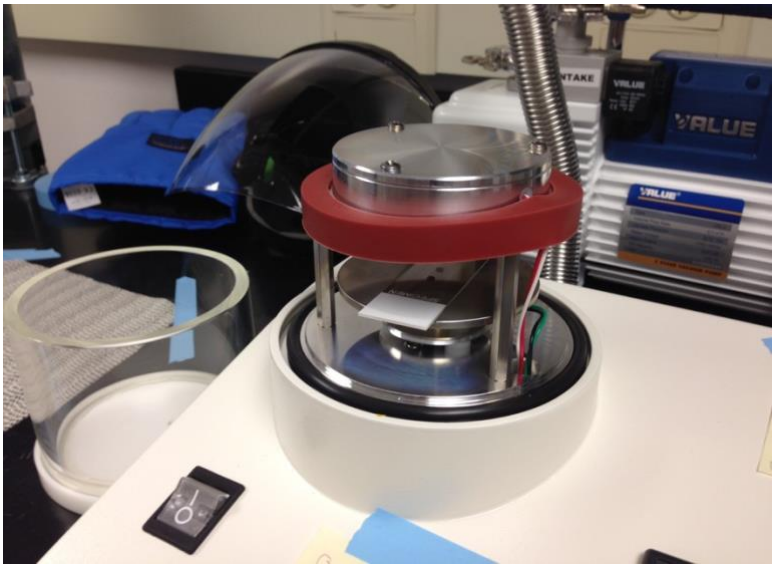
x. **Turn off the plasma cleaner**, the power switch on the back of the machine. Leave the Oxygen and close the main vales to the argon and oxygen.

3b. To glow discharge TEM grids use the Pelco EasieGlow

i. **Check Settings on the Viewing Screen.** You can change the settings by pressing the "**Prog**" button. Refer to the manual (located in the draw below the unit) for details on operating it. To glow-discharge, the TEM grids click "**AUTORUN**" on the touch screen. If you change the settings please change it back to default parameters of 25 seconds at 15 mA. For UltrAufoil grids set the time to 45 seconds.



ii. Place the TEM grids carbon side facing up on a glass slide or metal pedestal. Carefully remove the bell-jar, place it on the bench with the plastic top-down, to avoid damaging the glass bell-jar.



iii. Check the O-ring for dust before placing the bell-jar back on the unit. **To glow discharge the grid press "Auto Run" on the touch screen.** The unit will automatically vent the bell-jar after glow discharging the grids. You can remove the slide or pedestal from the unit only after it vents.

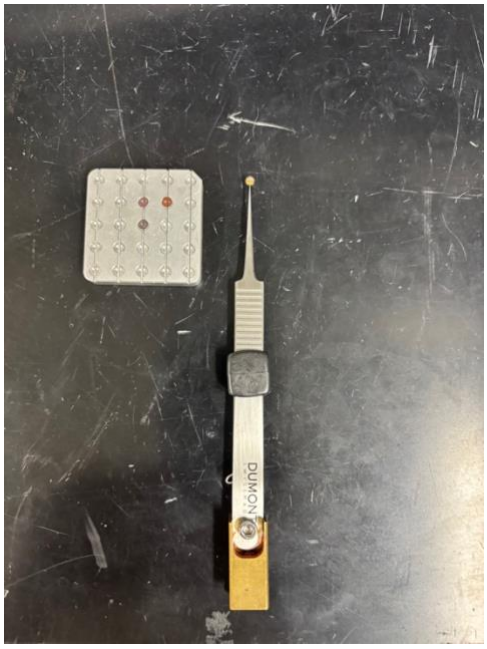
4. Prepare cryo-grids (30-60 mins)

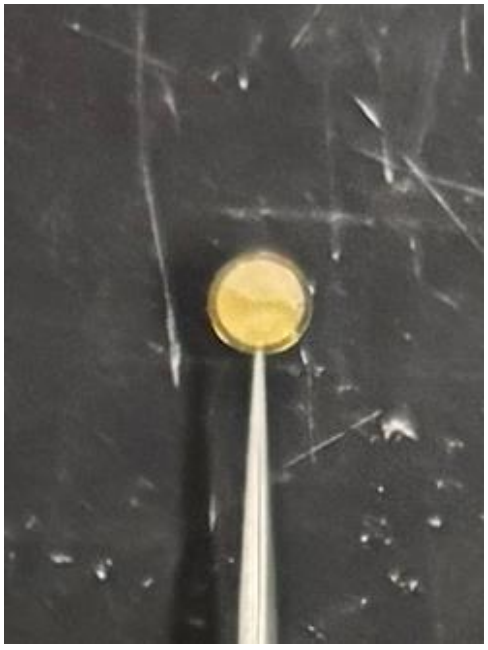
i. **Label the side of a cryo-box with a unique identifier**, we recommend your initials + date + alpha-numeric character. (ie JDS 5/1/2025 A).

ii. **Place the grid boxes on the pedestal**. Make sure that the screw-top is loose.

iii. **Put a Styrofoam ring in the Vitrobot Pot.** It will help keep a layer of cold nitrogen gas over the grids. Keep the LN2 level slightly above the grid boxes. We took off the Styrofoam ring in this image to make it easier to see the grid boxes. You should keep the Styrofoam in the Vitrobot pot during the experiment. If it gets frosty remove it and put a new ring on.


iv. **Pickup a TEM grid with the Vitrobot Forceps.** Grab the grid at it edge at the 6'clock position. Slide the clip down to the first notch to secure the TEM grid, then gently tap the forceps to verify that the TEM grid is secure. Keep track of the orientation of the TEM grid relative to the forceps. As

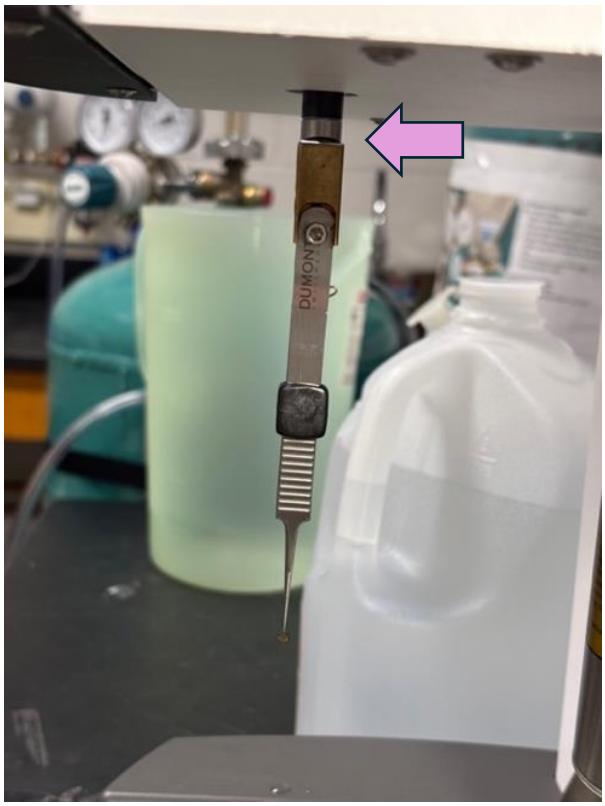


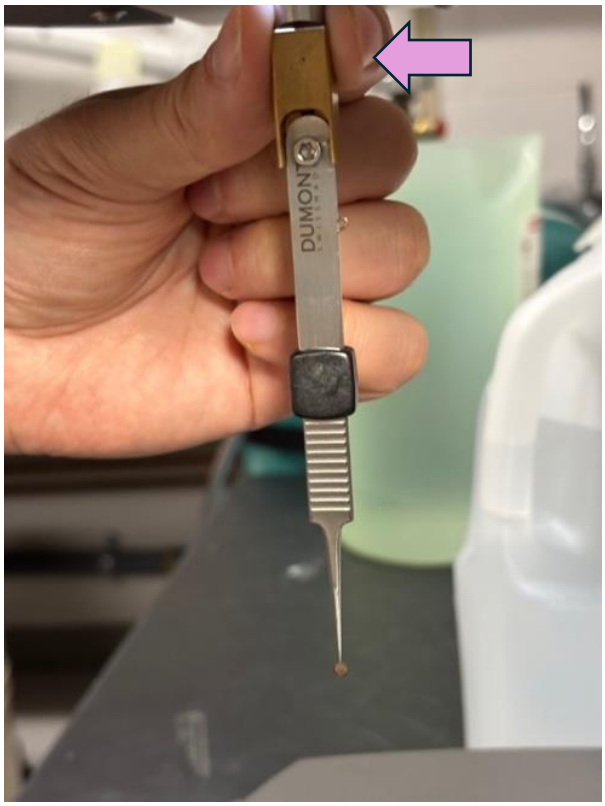


I am right-handed, I prefer to pick-up the TEM carbon side facing the Right Sample Port of the Vitrobot. Make sure the grid is not bent or damaged.

v. On the Vitrobot Control Screen select "**place new grid**" or hit the foot-pedal, this should raise the rod.

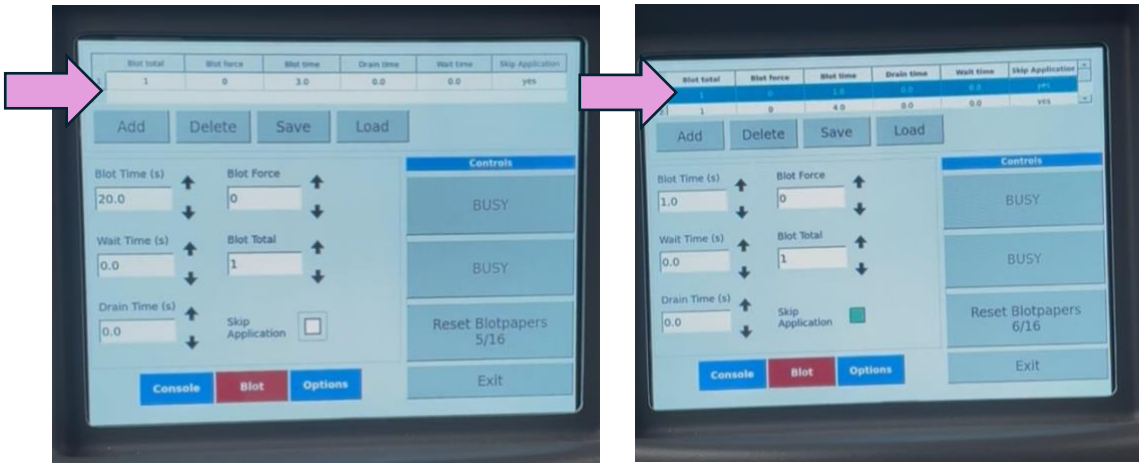
vi. **Place the Vitrobot Forceps on the rod, keep track of the orientation of the forceps and TEM grid. Make sure that the dovetail is flush with the rod…This is critical!!! Or you might damage the Vitrobot!!!**





vii. Place the Vitrobot Pot on the Vitrobot, then select **"place ethane" or use the foot pedal to raise it up.**

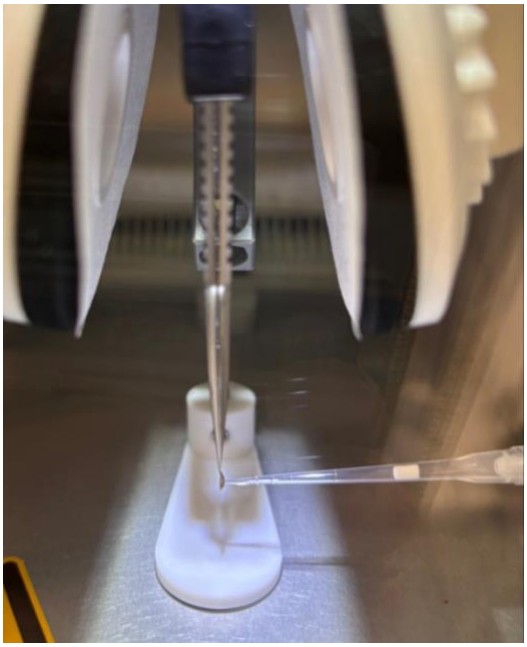
Make sure the top of the Blot Tab is highlighted blue (image on the right). If the top is white (Image on the left) the Vitrobot will not use the correct blot time or settings. This will happen if you delete the settings and forget to press it.



viii. Select **"Continue" or use the foot pedal** to raise the rod into the chamber.

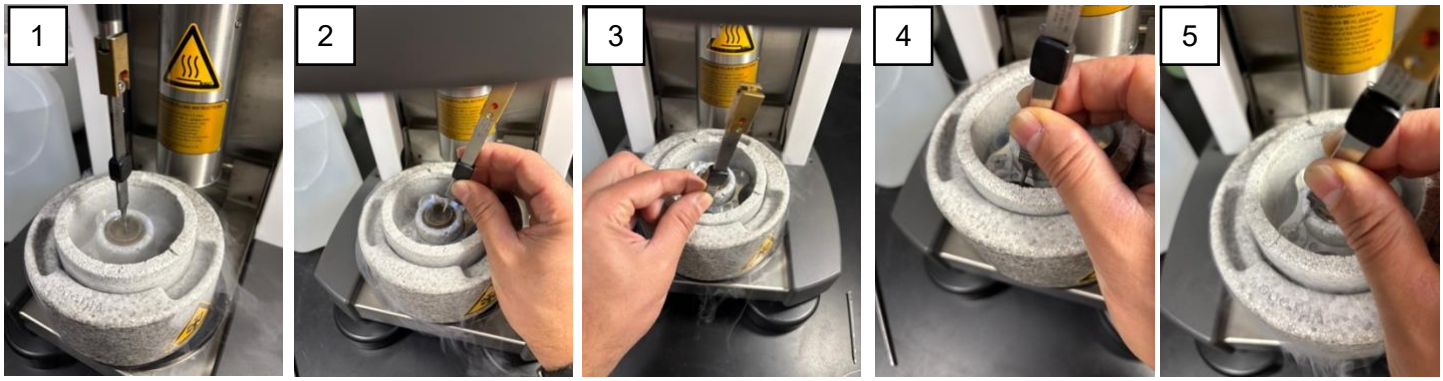
xi. Select **"Continue" or use the foot pedal** to lower the rod and forceps to the level of the blotting pads and filter paper.

xi. **Apply the sample onto the surface of the TEM grid**. Avoid having the pipette make direct contact with the TEM grid. The liquid should coat the grid and not ball up. If the grid is hydrophobic the liquid will ball up and not evenly coat the grid.



xii. Select "Continue" or foot pedal to blot the grid.

xiii. After the Vitrobot plunges the TEM grid into liquid ethane propane, **transfer the grid into the cryo-box**. When transferring the cryo-grid from the Ethane or ethane propane into the cryo-grid box, make sure to work quickly and keep the grid under a cryogen for every second possible.



xiv. **Continue this process (steps 4-12) until you have multiple cryo-grids.** We normally prepare 3-4 for each sample or condition. We don't recommend making more then 12 cryo-grids for one session

5. Shutdown procedure

i. **Transfer cryo-grids to storage or ask a core staff for help with this.** Tighten the screw on the cryo-boxes. Transfer the cryo-boxes to storage in LN2. Put the grid boxes into your puck or falcon tube or ask the core staff for help.

ii. **Drain Vitrobot Humidifier.** Unmount the Humidifier from the Vitrobot, pour the water from it into the foam box and place the humidifier inside the foam box to dry. Use the syringe to remove any remaining water. Do not unplug the humidifier……. this is critical!!!!

iii. Vitrobot shutdown…. On the Vitrobot Control Screen select "shutdown Vitrobot", this is critical do not skip this step!!! After the computer shuts off, turn the main power switch off located in the back of the unit.

iv. **Clean up…**Put all the tools and equipment back where you found them. Put the Vitrobot Pot under the snorkel to evaporate. Remove filter paper form the blotting pads. Clean the rings with 70% EtOH, leave rings in the chamber. Open the Vitrobot chamber so the moisture will evaporate. Clean Vitrobot tweezers with 70% EtOH then place tweezers back into its box.

v. **Enter your information into the Vitrobot Log Notebook.** Include your name, who to charge to (ie Lab), Institute, number of hours you used the Vitrobot, settings you used, and notes. If you notice an error with the Vitrobot make sure to write it down in this notebook and tell the core director or a staff personal. If you notice a critical error with the Vitrobot or other equipment, tell the core director.