

Vitrobot Mk IV Procedure

Grid preparation:

1. Use a piece of Parafilm to cover the top of a glass slide.
2. Use tweezers to transfer the desired number of grids from the grid box onto the glass slide, placing them carbon side up (dark side).
3. Place the glass slide into the glow discharge chamber, but don't start it yet.
4. Use the 'Enter' button on the glow discharge machine to cycle through the parameters and set the values to the desired settings. Be sure to only change the polarity, current, and time. Typical ranges/settings are:
 - a. Polarity: negative for proteins; positive for nucleic acids
 - b. Current: 10 – 30 mA
 - c. Time: 30 – 90 sec
 - d. Note: Beyond these values you can thin out the carbon too much!
5. If you are using a grid box that has the removable lid, it is recommended that you do not use gloves when handling grids after this point at the risk of statically charging the grids.
6. After you have prepared the liquid ethane, start the glow discharger.

Vitrobot setup:

1. Change the blotting paper.
 - a. While wearing gloves, open the door of the Vitrobot and remove the white circular clipping rings.
 - b. Discard the used blotting paper.
 - c. Attach new blotting paper to the blot pads using the clipping ring, making sure that the concave side is facing the blotting pad.
 - d. Close the door of the Vitrobot.
2. Fill the humidifier.
 - a. Attach the humidifier to the Vitrobot if it is not already attached. With the black dot facing towards you, align the groove on the top of the humidifier with the screw on the Teflon ring (you may need to guide some of the cable back through the hole on the bottom). Press up and then turn slightly counterclockwise till you feel the humidifier click into place. (You may or may not hear the click, but you should feel it).
 - i. The cable should NEVER be disconnected from the humidifier!
 - b. Fill the syringe with exactly 60 mL of distilled or MilliQ water.
 - c. Connect the syringe to the tubing at the bottom of the humidifier and inject the 60 mL of water.
 - d. Pull back on the plunger until some water is removed. This creates a vacuum in the tubing and ensures the humidifier is properly filled.
3. Turn on the Vitrobot using the switch at the back, located on the bottom left side.
4. Once the operating system has booted up, use the stylus to adjust the temperature and humidity to the desired settings.

- a. Most people use 4 – 6° for temperature and 100% humidity.
5. Initially, turn on the humidifier using the 'Manual' setting. You should immediately see a large puff of mist.
 - a. If you do not see the mist, turn the humidifier off. Then connect the syringe to the bottom of the humidifier and draw out some more air. Then try turning it on again.
 - b. If you do see the mist, switch the humidifier setting from 'Manual' to 'ON'.
 - c. Note that the temperature does not need to be turned on or off; the system will start adjusting the temperature as soon as you change it.
6. You want the filter paper to sit in the humid environment for at least 20 – 25 minutes to ensure the best consistency.

Liquid Ethane Preparation

1. While waiting for the temperature and humidity to stabilize, prepare your liquid ethane in the foam cooling holder.
2. Fill a dewar with liquid nitrogen if you haven't done so already.
3. Bring the liquid nitrogen and the required number of grid boxes with you to the large bay hood (446C).
4. Place the grid boxes into the grid holder, then place it into the cooling holder.
5. Next add the brass ethane cup to the center, add the spider, and lastly the foam ring (with the ridges facing up).
 - a. The ridges ensure that the N₂ vapor can escape and not contaminate the ethane when the cooling cup is raised on the Vitrobot.
 - b. The foam ring also helps create a small, cold area of vapor and minimizes contamination.
6. Cool all the components with liquid nitrogen, pouring directly into the middle of the cooling container.
7. Wait for all the liquid nitrogen to evaporate from the brass cup.
 - a. If it's taking too long, you can use a metal rod to evaporate any residual drops.
8. Fill up the cooling holder with liquid nitrogen from behind the foam ring to prevent any liquid nitrogen from getting into the brass cup.
9. Fill the brass cup with liquid ethane:
 - a. Place the tip of the ethane nozzle into the cup so that it touches the bottom at an angle (not perpendicular to the bottom).
 - b. Open the main valve on top of the ethane bottle.
 - c. If necessary, use the adjustment screw to increase the pressure to around 10 psi on the second gauge.
 - d. Open the black knob to start the flow of ethane.
 - e. Adjust the flow rate till you hear the ethane bubbling.
 - f. Fill the liquid ethane up to the edge of the cup without overflowing.
 - g. Close the small valve, then remove the nozzle from the cup.

- h. Close the main valve on the tank, then open the small valve until the charged gas drained out of the regulator, then close the small valve.
10. Wait for the ethane to solidify before removing the spider.
 - a. Use a nickel or flip an empty brass cup on top of the spider to thaw the solid ethane, then remove the spider.
11. Cover the cooling holder with foil and carefully carry to the Vitrobot.

Plunging

1. Glow discharging your grids.
2. Set up your plunging conditions using the 'Options' tab. The most common ranges are listed below and some recommended values from Veer are given in parentheses.
 - a. Blot time: 2 – 7 s (4 s)
 - b. Blot force: -25 to 25 (recommends close to zero, ~4)
 - i. This is what used to be the 'offset' on the old Vitrobot, but the control range seems much finer here.
 - ii. Negative value: more force, thinner ice
 - iii. Positive value: less force, thicker ice
 - c. Wait time: 5 – 10 s
 - i. This is the time between applying the sample and blotting.
 - d. Blot total: if you are using viscous organic solvents, more blots are recommended
 - i. Set to 0 if using fibrous samples and manually blot on the backside of the grid before plunging
 - e. Drain time: 0 s
 - i. This is the time between blotting and plunging.
 - f. Skip Application: if checked, the tweezers will not lower and sample cannot be applied to the grid.
 - g. Use Foot Pedal: Personal preference here. If Off, you will need to use the stylus.
 - h. Humidify OFF during process: This should be checked to prevent no pockets of mist interact with the sample during the process.
 - i. Skip Grid Transfer: if unchecked, the grid is lifted and transferred to the vapor above the grid box (checking this is recommended to minimize time in the vapor)
 - j. Auto Raise Ethane Lift: Recommended to be left off for better control.
3. Grab a glow-discharge grid with the tweezers. Lower the black clamp to just before the notches. Gently tap the tweezers to make sure the grid is secure.
4. Click the 'Place New Grid' button on the control panel. Once the rod lowers, attach the tweezers to the clamp making sure they are centered.

- a. You should be consistent about how which side the carbon side of the grid faces inside the Vitrobot. It may be best to face it towards your dominant hand side for ease of pipetting your sample on.
5. Click the 'Continue' button, which will raise the tweezers into the chamber.
6. Place the ethane container into the black ring, then click 'Place Ethane Container' button.
7. After the ethane has been raised, click the 'Process', which will lower the tweezers (unless Skip Application has been checked).
8. Apply your sample to the carbon side of the grid using one of the pipettes next to the Vitrobot.
 - a. 3 μ L is the recommended amount.
 - b. Be sure your sample is adequately prepared (e.g. thawed if frozen, vortex mixed, centrifuged, etc.) depending on the nature of your sample.
9. Click the 'Continue' button. The tweezers will raise, then wait time (if any), then blotting, then drain time (if any), then plunging into the ethane and the cup will lower.
10. Immediately after plunging and lowering, fill the ethane container with liquid nitrogen to cover the grid boxes.

NOTE: During the grid transfer process, it's important to not push down on the foam ring to keep the vapor inside the ring and prevent external humidity contamination.

11. Carefully unclamp the tweezers from the rod without touching the sides of the brass cup.
12. Keeping the tweezers steady and the grid submerged in the ethane, use your other hand to grab the ethane container and carefully transfer both to the benchtop.
 - a. It is helpful to rest your pinky and/or ring finger on the side of the ethane container to keep the tweezers steady.
13. Unclamp the tweezers while still submerged in the ethane.
14. Quickly transfer to any area of liquid nitrogen and keep the grid submerged there for a few seconds to equilibrate.
15. If necessary, use a second pair of cooled tweezers to rotate the grid box opening to the desired slot.
16. Move the grid to above the desired slot.
17. Open the tweezers (the grid should stick to one side only).
18. Lower the grid into the slot.
19. Repeat Steps 4 – 18 as needed for additional grids.

NOTE: You should swap the tweezers in between plunging and ensure that they are thoroughly dry with a hair dryer before use.

20. When finished, quickly transfer the grid box to a Falcon tube for storage. Samples can then be stored in the storage tank or brought to the scope for imaging.

Shutting Down

1. After the tweezers have been removed, click the 'Exit' button on the control panel. Click 'Yes' to proceed. This will shut off the operating system.
2. Turn off the instrument using the switch at the back.
3. Open the chamber door to prevent growth from occurring.
4. Remove the humidifier by rotating clockwise and then pulling down slightly.
5. Invert and pour out the water. Turn right side up and shake a bit, then invert again. Repeat this a few times. Then leave the humidifier on the bench next to the machine to dry out fully.
 - a. NEVER unplug the cable connecting the humidifier to the Vitrobot!

Maintenance

1. Bleach can be used to clean the chamber. This will depend on the nature of the samples used and whether any growth is observed.
2. The oil on the glow discharge pump should be changed ~1x per year.