

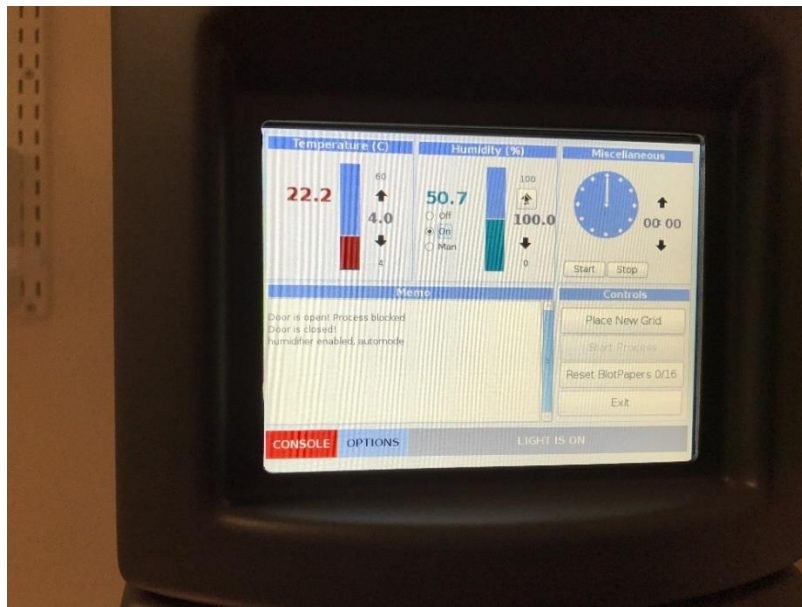
SBL Vitrobot Protocol (based on the Hibb's lab protocol, modified by Xiaochen and James)

Materials Required (your own supply or from SBL):

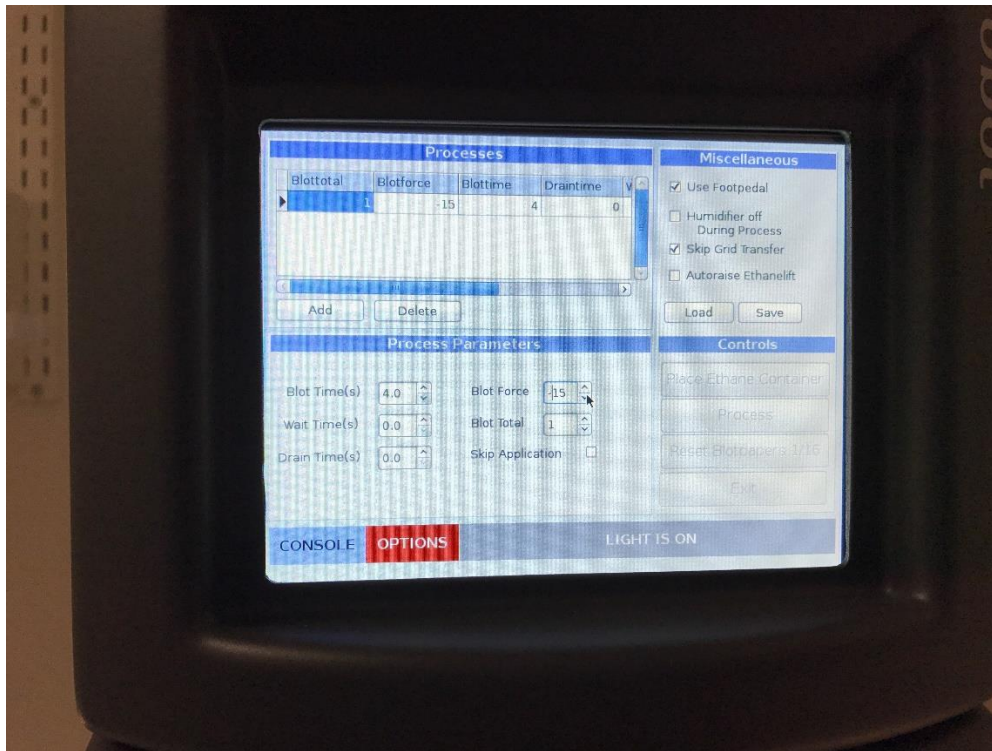
- Grids.
- Vitrobot Tweezer Assembly.
- Grid containers (buttons) (we prefer the white ones, as they allow more space for the tweezers and are more forgiving), and a screw driver for opening/closing the lid.
- Dewar with 50ml falcon tube on a string to store and transport grid containers.
 - o make sure that the cap of the falcon tube is pre-drilled with holes.

Vitrobot prep

1. Turn on vitrobot. Set temperature to 4 degrees and wait for it to cool. The humidity would be set to 70% by default, wait until the temperature reaches 4 degrees, then set the humidity to **95%** and select **"ON"**.
 - a. Fill the chamber with **Milli-Q water**. Don't overfill. Attach it to the Vitrobot.



2. Gently place blotting papers on pads, without pushing blotting pads in either direction.
3. Under “OPTIONS”: check “Use Foot pedal” and “Skip grid transfer”. Set parameters: 4 sec blot, 5-15 blot force (0 wait, 1 Blot Total, 0 drain), .



4. Do a “dry run” with the tweezers. Pay special attention that the blotting is symmetrical and does not push the tweezers in either direction. If it does, inform SBL (to adjust with little gray wrench and repeat).

Glow Discharge

1. Turn on the glow discharger. Check the protocol. Our favorite is 30 mA, 60-90” glow discharge. Make sure the vent goes to 1 ATM (1,042 mBar) at the end.
2. Carefully lift the chamber and place it upside down.
3. Transfer your grids, top side up, on a glass cover slip, or a glass cover slip with wrapped parafilm for ease of lifting grids.
4. Press “autorun” on your protocol.
5. Make sure the chamber has vented completely before you lift it! If it isn’t unsealed, run the protocol again but press the “stop and vent” button immediately.

Ethane Setup

1. Make sure the LN2 hose is dry- use a hair dryer and paper towels if you must. Make sure the dewars are dry. Any water you introduce is going to introduce ice on your samples. Ice = bad!
2. Place the brass ethane container into the large gray cup. Place the aluminum grid box holder over it. Place a grid box in there. Add the aluminum heat-transfer “spider” on top.



3. Add nitrogen to the outside and inside of the gray cup. At the start, splash some nitrogen into the ethane cup to cool it rapidly. Use a metal rod to dispense the liquid N₂ inside the ethane cup completely.
4. After the nitrogen has stopped bubbling, top it up and start piping in the ethane.
5. With the ethane, be careful to not go too fast- it'll blow out into the nitrogen- or too slow- it'll freeze the tip.
6. When the ethane cup is full, take the tip out and close the ethane gas simultaneously.
Ideally there should be a layer of white solid ethane along the inside walls as well as the bottom of the copper holder. Use the metal rod to gently scrape the bottom if needed (to warm it up and dissolve the solid ethane).
7. add the grid box (white one) into the assembly.



Use the Vitrobot

1. Start the vitrobot at least 20 minutes before freezing. Make sure the temperature is 4C and humidity is 95%. Change the filter paper.
2. Prepare the ethane tank (see "Ethane setup")
3. Do the glow discharge for grids (See "Glow discharge")
4. Place a grid in the vitrobot tweezer assembly. Don't slide the black slider down past the first notch, or your grid will fall out. Pay attention to which side you orient the top of the grid- it depends on which hand you pipette with. Do that consistently.
5. Place the ethane container carefully. Top it off with nitrogen to just the level of the Styrofoam surrounding the brass. **Do NOT overfill the liquid nitrogen over the Styrofoam around the brass unless instructed!!! YOU WANT A LAYER OF WHITE ETHANE ICE ON THE inside OF THE BRASS CONTAINER.** If it's solid, you will be out \$1000 after you've destroyed the tweezers by plunging them into it. If it's all liquid, you are wasting money on freezing grids b/c it's not cold enough to vitrify the ice and you will have a cloudy grid on the scope.

6. **Make sure that the column of the vitrobot is not lowered to the lowest position (press the foot pedal to slightly raise it if needed)**. Place the tweezers on the vitrobot so the little ball joint is “clicked” into the position. Step on the footpedal to raise the tweezers.
7. If you haven’t selected “autoraise ethane lift”, keep pressing the foot pedal until the tweezers are in the “load” position.
8. Open the red slider on either side and load your sample with your foot on the pedal. We like 3 ul of 3-6 mg/ml for a membrane protein of 250kD (3 mg/ml) to 400 kD (6mg/ml). You will need to determine your optimal concentration empirically. Soluble proteins tend to require much less material than this.
9. Immediately, press the footpedal and allow the plunger to run its course.
10. Carefully transfer the grid to your grid box. Slide the black slider up with one hand, and hold the tweezers with your other hand. Maybe try this on a dry run many times before you try it for real. Don’t push the grids into the small hole in the grid box. Let the grid drop into the hole to prevent it from bending. The truth is that the remaining ethane on the grid would allow the grid to “stick” to the tweezers even if the grid is not physically “clamped” down.
11. Wipe dry the tweezers before moving to the next grid.

You’re done! Repeat grid freezing until you finish.

Clean-up

1. Transfer your grids to a long-term storage dewar.
2. Remove the tweezers, and blot dry. Carefully store tweezers in their box with their tips protected.
3. Click “exit” on Vitrobot interface.
4. Turn off Vitrobot.
5. Remove blot papers, and wipe out chamber if any moisture has accumulated. The chamber can be left open to release the humidity if needed.
6. Empty the Milli-Q water in the humidifier/metal container.
7. If you haven’t already, turn off glow discharger.
8. **!!! Completely turn off (close) the gas valve on the ethane tank !!!**
9. **Log usage for both the Vitrobot as well as the glow discharger.**
10. Leave the ethane and nitrogen holders on the bench top table to dry.
11. Empty the liquid Nitrogen dewar and place it upside down in the storage area. If the liquid Nitrogen is fresh, you may choose to top off the storage dewar.

