

Vitrobot Standard Operation Protocol

The Vitrobot is a PC-controlled semi-automated plunging device with humidity and temperature control for making vitreous ice (an amorphous, noncrystalline state) more reproducible by rapid cooling of a thin layer of aqueous sample e.g. protein/ virus in a liquid cryogen, commonly ethane, such that it is cooled about 200K in $<10^{-4}$ s. The process of blotting, plunging and vitrification is automated to ensure reproducibility.

1. Preparation of Vitrobot
2. Preparing grid
3. Condensing ethane
4. Sample vitrification
5. Shutting down Vitrobot and end of the session.

PPE (BSL-1)

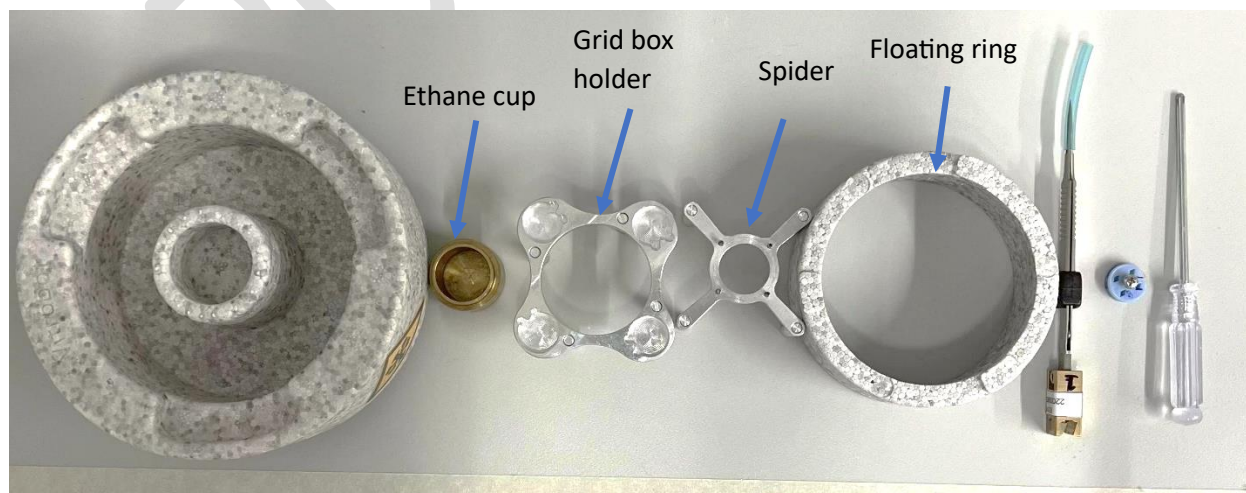
1. Laboratory Coat
2. Nitrile Gloves
3. Goggles / Safety Glasses
4. Cryogenic Gloves
5. Face Mask

1. Preparation of Vitrobot

- Get everything ready. Label the grid box.

Note: To keep your experiment condition consistent, please only use tweezer #1 for Vitrobot #1 and tweezer #2 for Vitrobot #2.

Note: Check the Vitrobot tweezers tips, make sure they are not bent and are symmetrically aligned. Please report to EM staff immediately if any problems are found.



- Assemble the central (ethane) cup, the grid box holder, spider, and floating ring inside the coolant container. Put the grid box in the right position.

Note: Make sure the lid of the grid box is loose enough to move with tweezers and adjust lid opening so that it's over the slot you plan to load.

Note: The orientation of the floating ring.



X

✓



- Attach the humidifier cable to the humidifier by aligning the red dot on the cable connector and the red dot on the locking ring on the underside of the humidifier (NOT on the main body of the humidifier) and pushing the connector gently upwards to lock in place.

Note: The dried humidifier is placed on the bench beside the Vitrobot. Please report to EM staff if it is still attached to the Vitrobot or you cannot find it.

Please only use humidifier #1 for Vitrobot 1 and humidifier #2 for Vitrobot 2.



- Attach the humidifier to the bottom of the climate chamber by pushing up at the indicated position and turning it to the right.

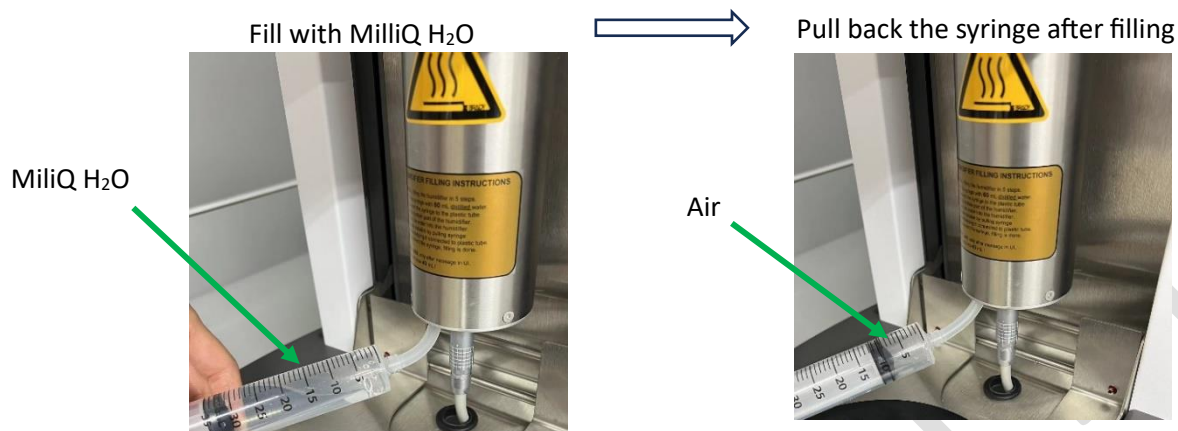
Note: The labels are facing the front.

This side faces the front!



Align the grave (J shape) on the humidifier with the screw (small circle) position on the main body.

- Fill the humidifier with 30 ml of fresh Milli-Q water using the syringe. After filling with water, pull back on the syringe to about 10ml (while it is still attached to the humidifier) to create a “vacuum”, which is required for it to be properly filled. 30ml of water is enough to be used for at least 3 hrs at 37C and 100% humidity.



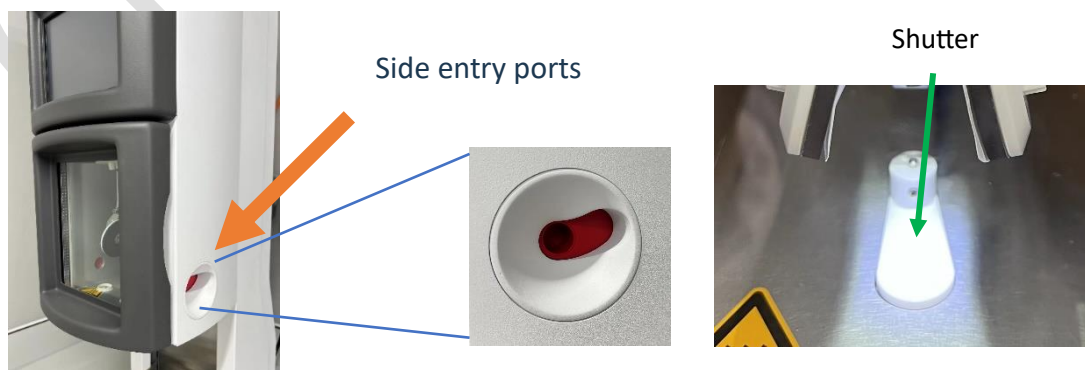
Note: Refill only after the “Empty” message in the UI. Refill by directly connecting the syringe with a maximum of 40 ml! No need to remove the humidifier from the Vitrobot.



- Turn the Vitrobot on by using the switch on the back right-hand side of the instrument (indicated by the blue arrow on the Vitrobot body).

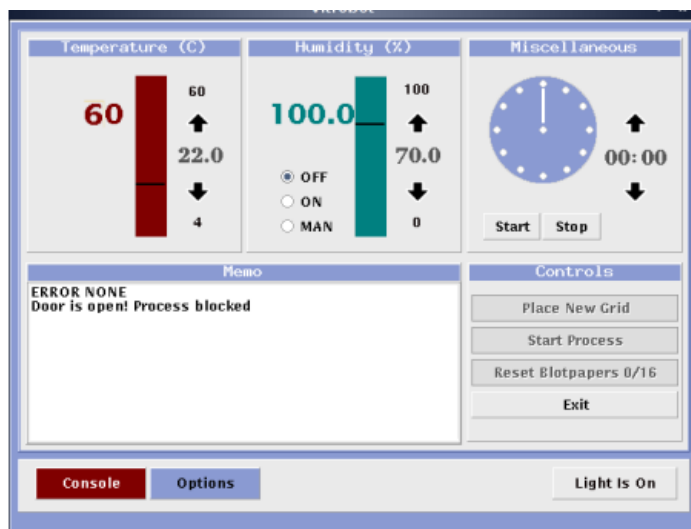


Note: Make sure the front door, shutter and side entry ports at both the left and right of the chamber are closed.



- Set Vitrobot to the required temperature and relative humidity. It is not necessary to switch on the humidifier before the desired temperature has been reached.

Note: 4C will need ~20min to reach. The humidity will reach the set value within 1-4 min after turning it “on” if the temperature has been reached.



- Click [Options] on the screen and select the appropriate settings and vitrification parameters for your sample plunge cycle.

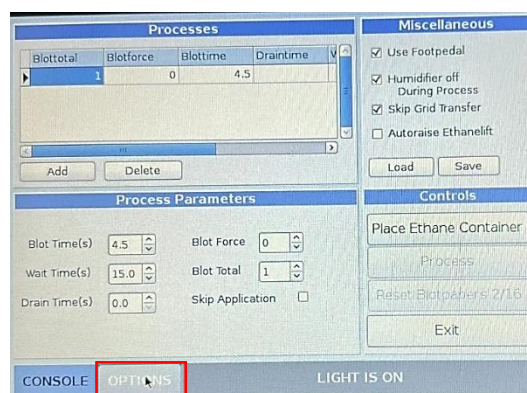
You can select “Use foot pedal”, which will always follow the plunging sequence.

Tick “Humidifier off during process” to switch off the humidifier during manual application and plunge freezing.

Tick “Skip grid transfer” to avoid the movement of the ethane container after plunging.

Do NOT tick “Auto raise ethane lift”: If ticked, the coolant container will be lifted towards the bottom of the climate chamber simultaneously with the uplift of the tweezer. To operate in a more safe way, these two steps should be separated.

Blot time	The time of each individual blot
Blot force	The force at which the excess of fluid is removed from the grid.
Wait time	the relaxation time before the blotting
Blot Total	the number of blottings on one sample
Drain time	Intermediate time between blotting and plunge freezing

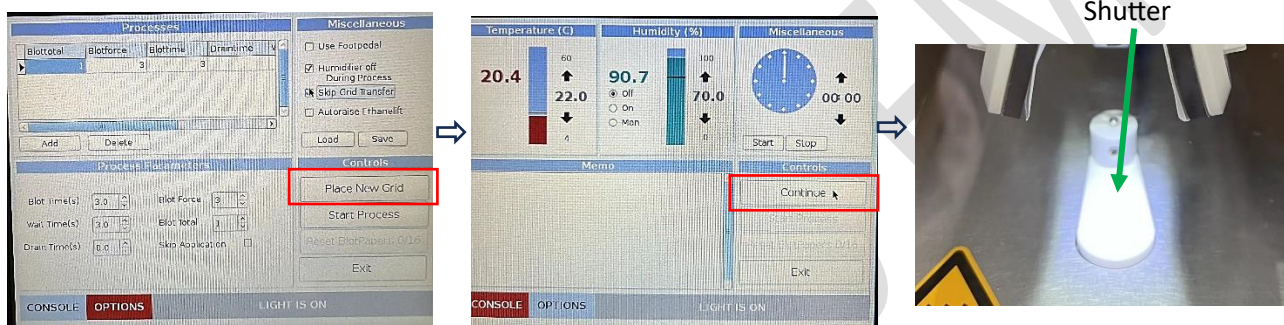


As a good starting point for vitrification...

- For 1.2/1.3 grids: 3.5–4.5 seconds blot time and -5/0 blot force
- For 2/2 grids: 3.0–3.5 seconds blot time and -5/0 blot force

For optimization, adjust the blotting time to get good ice thickness, or adjust the blot force (- means less force and + means more force; at 0 the pad should be just touching the grid). Blot time has more influence on ice thickness than blot force!

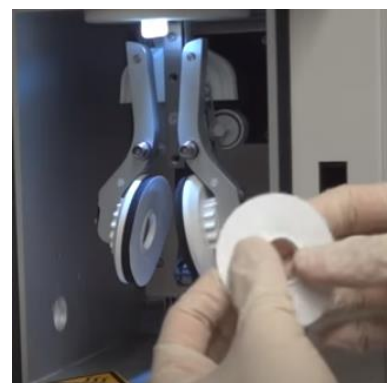
- Perform a test-run of the plunging cycle to check everything is working correctly.
- Seal the chamber by clicking on [Place New Grid] and then clicking on [Continue] to move the plunging rod all the way up to the chamber. This closes the shutter at the bottom of the chamber to keep the chamber at a consistent temperature and humidity.



- Open the chamber and attach the filter paper with the white circular clipping rings to the blotting pads. The inner edge of the paper has a ridge and should be placed towards the pads, NOT the sample.

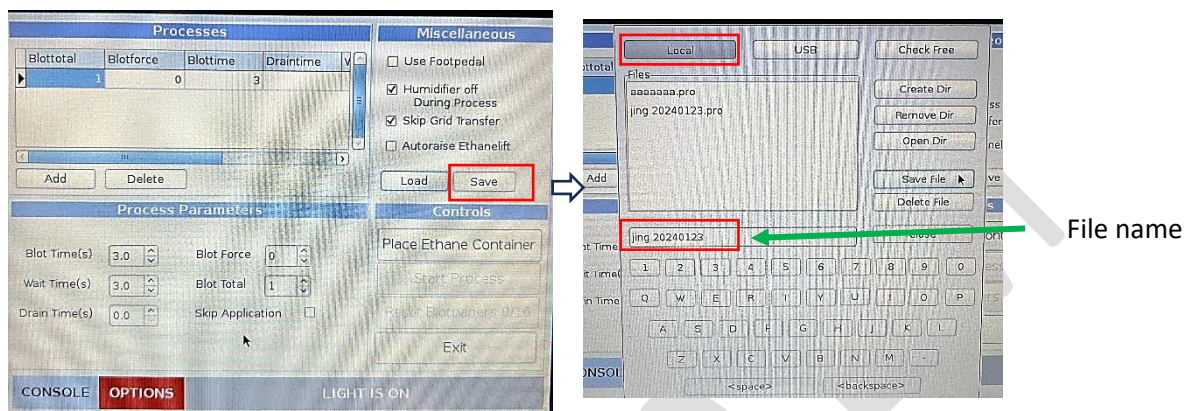
Note: You must wear gloves to avoid contamination of the filter paper.

Option: Blotting papers need to be placed before blotting. This can be done now, or after filling the ethane cup. The amount of time the filter papers is in the environmental chamber before blotting will affect reproducibility.

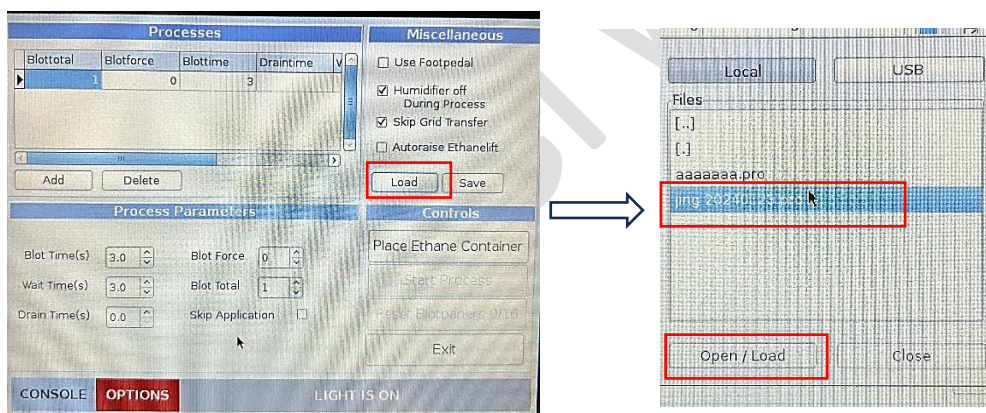


- You can save your settings by clicking [save], inputting the name, and saving the file in "local."
- For loading the setting, click [load], then choose the file name you would like to load, then [open/load].

Save settings



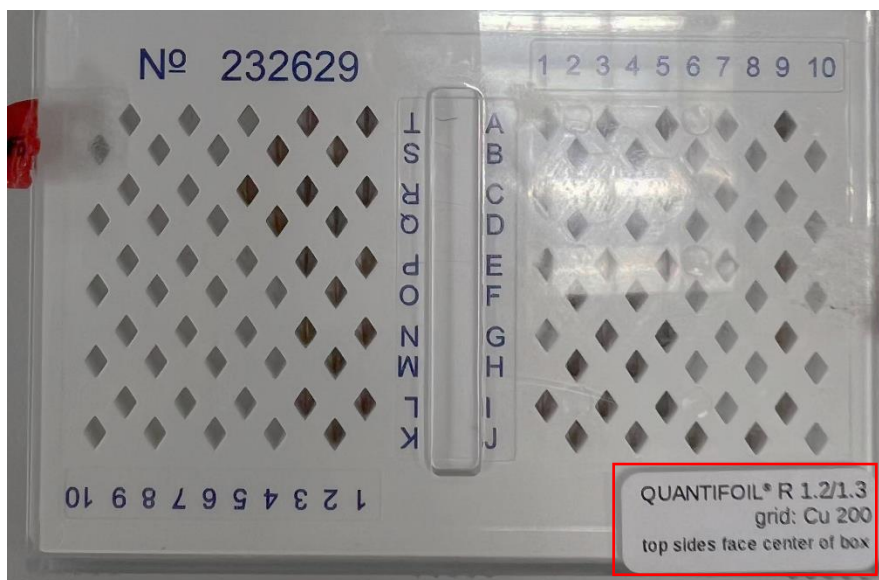
Load settings



2. *Preparing grid – Glow discharge with Easiglow*

- Please refer to the SOP of Easiglow

Note: The coating (e.g. carbon) side of the grid should be facing up. Please refer to the instructions on the grid box to find out which side is coating side.

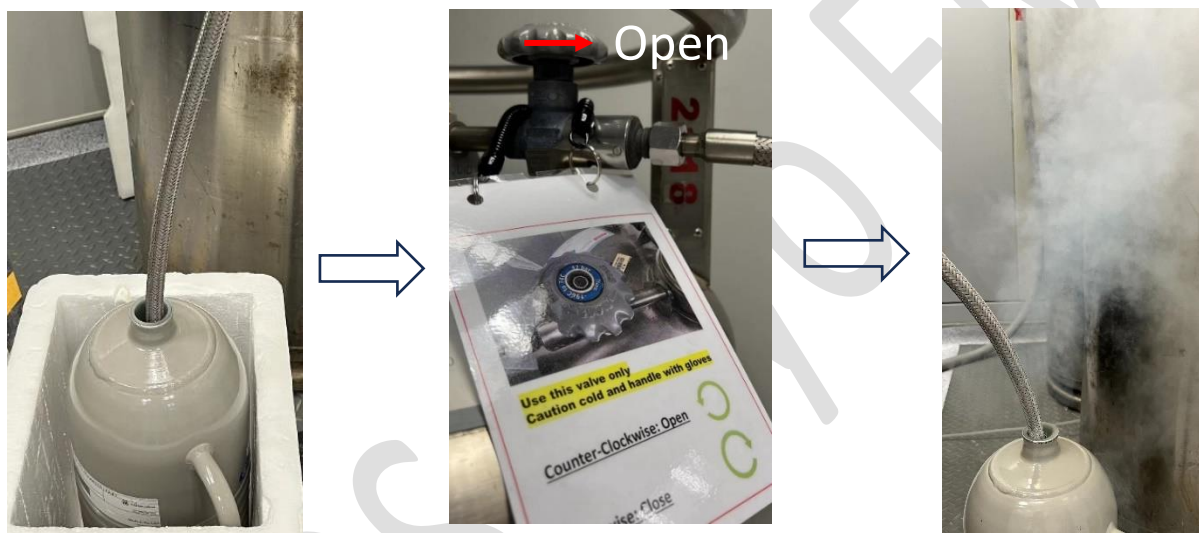


3. Condensing ethane

Warning: Liquid ethane is highly flammable and can cause severe burns and blindness if splashed in the eye. Always wear safety glasses and lab coat when handling.

- Prepare the coolant container and put it on the bench near the ethane tank.
- Take an empty dry 4L LN2 dewar flask. Put it into the foam box. Wearing cryogenic protection gloves and face shield.
- Put the LN2 tank hose into the 4L LN2 dewar flask. Turn on the switch a bit anticlockwise. After 1-2min, turn it bigger to fill up faster.

Warning: Note the nitrogen gas. Keep proper distances with nitrogen gas to avoid inhaling nitrogen gas!



- When it is almost full, turn off the main switch on the LN2 tank. Cover the 4L LN2 dewar flask with the blue cap. Put the cryogenic protection gloves back in the right place and sign the logbook.
- Pour the LN2 into the small LN2 flask (~500ml) with a loose lid. They are much easier to handle than the 4l dewar flasks.
- Cool down the assembled container with liquid nitrogen.

Note: The label faces the front.

Pour LN into the inner space of the container to speed up the cooling of everything. **The center cup needs to be filled with LN2 to let it cool down as well.** Once the floating ring floats up, add LN2 to the outer space.

It needs about 300ml LN2 to fill up the coolant container.





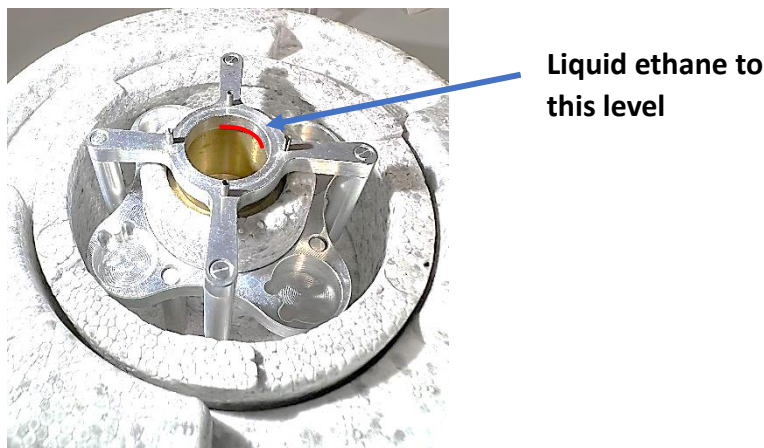
- Wait until all parts of the coolant container, especially the central ethane cup, have reached liquid nitrogen temperature. This is the case after the four legs of the spider have each experienced a boiling effect, after which the ethane cup has reached at least 185K (-88°C), suitable for liquefying ethane. Make sure the level of LN2 is kept up during the filling.
Note: The boiling of spider legs happens at ~ 40-45 sec after pouring the LN2 into the coolant container.
The ethane cup will take 2.5-3min to dry if you fill it up with LN2.
- When boiling stop, fill LN2 to the outer space to full. Now, it is ready for ethane condensing.
Note: Make sure not to spill any LN2 inside the floating ring since LN2 might spill into the brass ethane container.



- Check the ethane gas. Before you turn on any valves, the needle on the gauge should be at "0". Open the gas main switch main main valve 1 and the needle will immediately turn to a certain level.
Note: The right-hand side gauge should always be set at 5 psi. Please contact EM staff if it is not set at 5 psi.
Note: open valve: anticlockwise; close valve: clockwise.



- Put the tip of the gas outlet into the ethane brass cup at an angle and stay as close to the bottom as possible. Turn on flow valve 2 slowly so that there is a steady even flow of ethane. It should take a few minutes for the cup to fill depending on the flow rate. The liquid ethane level should be touching the bottom of the spider.



Note: Ethane gas vapor will appear immediately after turning on flow valve 2 and it takes about 60 sec to disappear. The flow is bigger, taking less time to disappear.

When ethane vapor disappears, turn flow valve 2 a bit bigger. It will take about 2.5-3.5min to fill up the cup. The flow is bigger, less time is needed.

Warning: Never point the tip of the ethane outlet at yourself or another person!

Important Note: To prevent the ethane liquid from blowing out of the cup and to avoid backflow into the tip caused by pressure changes, it is crucial to follow these steps when finishing filling:

1. Before removing the tip, reduce the flow of flow valve 2 to a lower setting.
2. After removing the tip from the cup, ensure that the tip is facing downwards towards the floor.
3. Turn off main valve 1.
3. To drain the ethane inside the regulator and tubing, gradually increase the flow of flow valve 2.
4. Keep an eye on the pressure gauges. When the needle on both gauges reaches "0," it indicates that the draining process is complete.

Warning: Ethane valves must be off after use.

- Wait for ethane to start showing cloudy and white ice. The optimum freezing temperature of the ethane is the mixture of solidified ethane ice with liquid ethane in the central cup. After the appearance of ethane ice, the metal spider must be removed.

Note: The spider is easily frozen to the ethane cup. Get the 50 cent coin and the big forcecep ready. When the ice of the ethane shows up, place a 50-cent coin on the center of the spider and to quickly melt the top. Do not remove coin, remove the spider with the coin quickly from the coolant chamber to prevent the ethane from freezing.



@~ 3 min



@~ 5-10 min

Note: The timing of removing the spider is critical. Keep observing the ethane cup after stopping the ethane gas. At ~2 minutes, you may see a small patch of ethane starting to grow at the surface of the ethane cup. When the ice spreads to the bottom of the cup, put the 50 cent coin on the spider and quickly remove the spider from the cup.



Ice starts growing.

Note: There will be ice accumulation on the floating ring. Please change to a dry one when there is ice.

Warning: the metal spider must be removed from the container prior to vitrification!



- Carefully transfer the coolant container with the liquid ethane to your workstation.

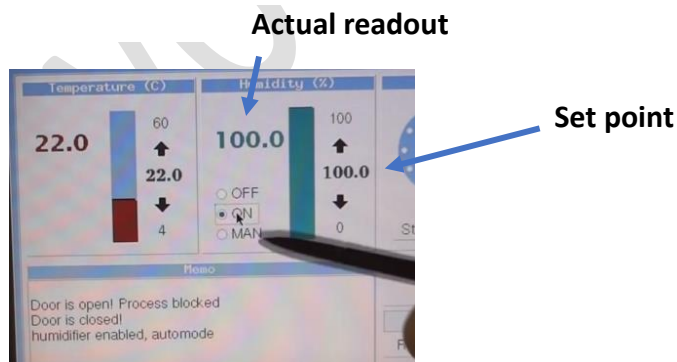
4. Sample vitrification

Warning: Make sure the ethane is liquid and not fully frozen before starting the vitrification cycle or else it may bend your grids and damage the Vitrobot tweezers!!!

Note:

1. Vitrobot tweezers are very delicate and expensive (~HKD10,000 each). Please always handle them with care. It is for grid pickup ONLY. Using it for other tasks may damage the tip and result in inconsistent or failed sample freezing.
2. Always use dry tools to reduce the ice contamination of liquid nitrogen.
3. Anything which comes into contact with the frozen grid (e.g. the cap of the grid box, tweezers etc.) needs to be pre-cooled to prevent it from warming up.
4. Click [Place New Grid] to abort the vitrification process, using a foot pedal will continue in sequence!
5. Check the humidity and temperature to make sure they reach the set point before running.

- Set humidity “on” if it is at “off” mode.



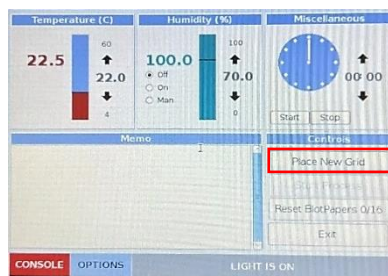
The humidity will reach the set value within 1-4 min after turning it “on” if the temperature has been reached.

Note: If you are using glow discharged grids or require special grid treatment, the best time for treatment is after filling the ethane cup to ensure good quality grids. However, you need to keep an eye on the level of LN2. To prevent evaporation, cover the plunging cup with a sheet of lint-free paper. This is only recommended for experienced users since it requires smooth operation.

If you are a new user, it is recommended that you have the glow discharge before preparing the ethane container.

There is no need to wear gloves for any stage of the procedure, other than installing the Vitrobot filter paper on the blotting pads. Gloves may transfer static to the grids, and cause charging later, even when using antistatic tweezers.

- Click [Place New Grid] / use the foot pedal to put the central axis of the tweezers in the correct position for mounting.



Note: Before picking up the grid, please ensure that the lid of the grid box has either been removed or loosened, depending on the type of grid box.

- Pick up a grid with the Vitrobot tweezers.

Note: 1. Make sure that the black clamping ring is fixed down to the first grooves on the tweezers. 2. Make sure to orientate the desired side of the grid on the side where you are going to load your sample (e.g. for application through the right-hand port, the carbon side should be facing to the right) and also take into account the correct mounting orientation of the tweezers. Number labeling faces to the front!

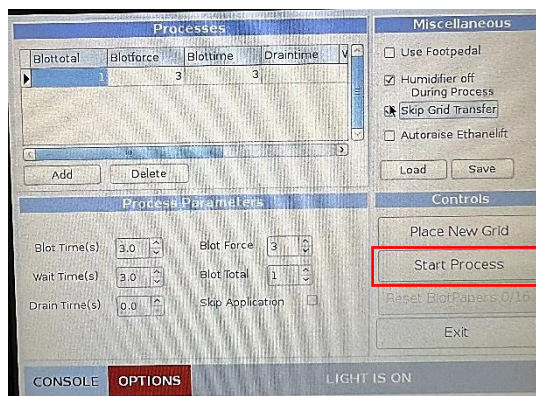
Front



- Mount the tweezer onto the connection groove of the plugging rod.



- Click [Start Process] / use the foot pedal and wait for the tweezers to be lifted up into the climate chamber.

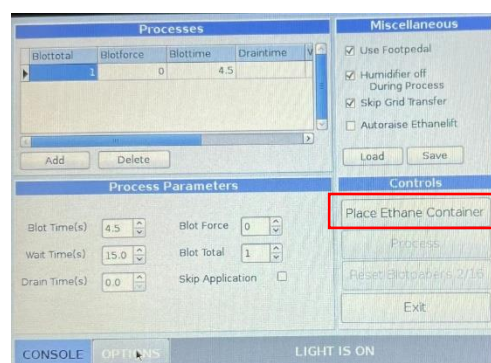


- Place coolant container onto the lift and check the liquid nitrogen level.

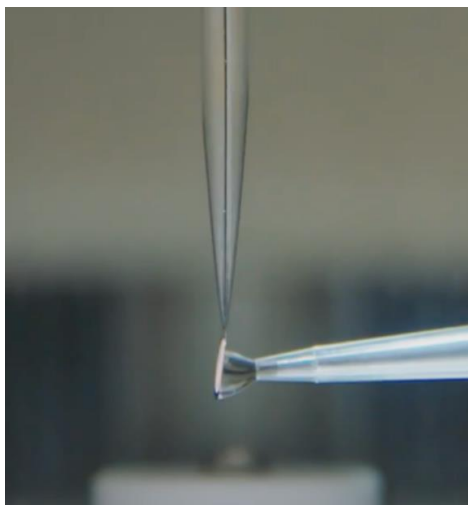
Note: fill the level of LN2 to cover your gridboxes. If it is too full, there is a danger of overflow and nitrogen burns, or if nitrogen comes into contact with the central ethane cup, the ethane will solidify.



- Click [Place Ethane Container] / use the foot pedal to raise the coolant container towards the climate chamber.



- Click [Continue] / use the foot pedal to proceed, the tweezers will be slightly lowered to allow the application of sample through either of the side-entry ports using a pipette.
- Apply 3-3.5 μ l sample to the grid and close the side-entry port.



- Click [Continue] / use the foot pedal to start blotting and plunging the grid in the liquid ethane. After each blot, the blot pads undergo a slight rotation to ensure a clean, new area of filter paper for the next blot session.
- After plunge freezing both the liquid coolant container and the tweezers with grid are automatically and simultaneously lowered while keeping the grid inside the liquid ethane. This prevents any contamination of the freshly frozen sample.
- Once the coolant container has stopped after the plunging motion, fill the level of LN2 to cover your gridboxes making sure not to spill any LN2 inside the floating ring and/or the ethane cup.
- Carefully detach the tweezers from plunging rod while keeping grid submerged in liquid ethane.
- Lift the tweezers slide clip to open position while keeping the tweezers engaged with your fingers.
- Quickly move the tweezers from liquid ethane to LN2 and then into gridbox.

Note: taking care to neither bump the grid inside the ethane cup, nor to lift it out of the ethane, and either transfer the grid directly to the LN2 or to the grid box (don't leave the protective atmosphere of N2 gas!)

- Move grid box lid opening to next slot.
- Dry and clean tweezers by using 70% ethanol and a kim-wipe. You may use a hair dryer to dry it if necessary.

Note: Do NOT use hot air when blowing the tweezers! Only cold air is applied.

- The freezing cycle is now complete. Repeat the steps for all of your grids.
- After finishing freezing all your samples, seal your grid box(es) and transfer to a labelled cryo EM puck etc., pre-cooled in liquid nitrogen, for storage.

Note: Please refer to the SOP for storage of grid to the LN2 tank.

Note during multiple grids freezing:

1. To prevent ice crystals forming outside the floating ring from dropping into the LN2 and contaminating it, change the floating ring frequently, taking care not to drip LN2 into the ethane cup. (extra floating rings are available for each Vitrobot)



2. **Warning:** If too much of the ethane freezes, you run the risk of smashing your grid and damaging the tweezers.

If too much of the ethane freezes in the plunging cycle, the spider can be placed in reverse on top of the ethane cup to warm up the cup enough to avoid ethane freezing solid. (DO NOT LEAVE IN PLACE DURING PROCESSING!)

Note: Only keep the reversed spider for 1-2 seconds, then remove it immediately. Otherwise, it may thaw all the ethane ice into liquid.

Note: Due to the volume expansion of ethane ice, the liquid ethane may spill over the cup. The ethane liquid level might be lower after you warm up the cup with a reversed spider. Make sure the ethane liquid level is high enough to cover the grid when freezing.



3. Excess ethane in the central cup (a meniscus appears outside the horizontal plane of the cup) may be removed with some filter paper.



4. Replace the filter papers on the blotting pad whenever necessary. e.g. after blocking a maximum of 16 grids, or the filter paper is too humid after being inside 100% humidity chamber for a long time.

CPOS Cryo EM

5. Shutting down Vitrobot and end of the session

- When you have finished, carefully transport the LN2 container back to the fume cupboard. Leave it assembled and allow the ethane to evaporate naturally in a well-ventilated area.

Note: Never use force to disassemble the parts if ice forms between each part. Force will damage the container!

- Clean the tweezers with 70% ethanol and store them in their case.
- Tap [exit] on the screen. A reminder message of removing the tweezers will pop up. Please double check if you remove the tweezers. Then click “Yes”.



- Wait for the system to shut down and the screen to go dark. After the software has shut down, make sure you turn off the Vitrobot with the switch at the back!



Note: If the Vitrobot is not turned off with the switch at the back, the system WILL come back online after a while and stay on! Please make sure you double check!

- Remove and dispose of the blotting paper in the Bio-Waste bin and leave the pair of plastic rings inside the chamber.



- Wipe the condensation water inside the chamber gently with Kim-wipes and leave the door slightly ajar to allow the chamber to air-dry completely.



- Disconnect the humidifier cable from the humidifier by pushing the locking ring on the underside of the humidifier upwards and gently disconnect the cable by pulling from the metal connector part, not the cable itself.



Locking ring

- Remove the humidifier by turning left to unleash the bajonet-connection and pulling it downwards. Empty all the water in the central reservoir into the beaker.

Note: Emptying the humidifier is a two-step process: 1. Empty all the water in the central reservoir into the beaker. 2. Put the humidifier upside-down on the bench and re-connect the syringe to the plastic tube at the bottom part to remove the water from the outside reservoir.

- Empty 500ml LN2 flask if any LN2 is left by pouring the LN2 into the Styrofoam box. Then put it into oven to dry.
- Perform a general cleanup of the workspace with 70% ethanol and leave the Vitrobot and the room in the state which you would wish to encounter it.
- Sign on the logbook.

References

1. FEI Vitrobot Mk IV User manual.
2. Passmore LA, Russo CJ (2016) Specimen Preparation for High-Resolution Cryo-EM *Methods in Enzymology*

Checklist before leaving the lab:

- Turn off the ethane tank to prevent any potential leaks or accidents.
- Shut down the Vitrobot following the proper procedures.
- Remove the filter paper and dispose of it in the designated waste bin.
- Empty the humidifier of any remaining water.
- Place the Vitrobot tweezers back in the case.
- Place the coolant container in the fume hood to ensure proper ventilation and dry.
- Place the thermos bottle and its lid in the oven to dry.
- Tidy and clean the bench area.
- Sign the logbook.
- Remember to take all of your personal belongings with you when you leave.