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Cryo-EM Grid Preparation Manual

JEC-3000FC and Vitrobot Mark IV

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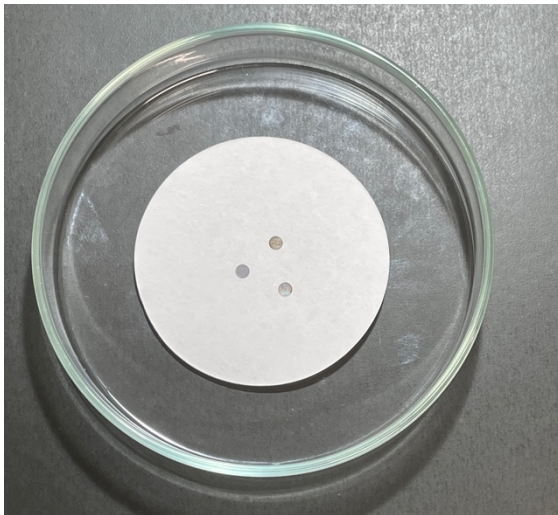
I. Acetone treatment of the grid

*The purchased grid may contain residual plastic components from the time of manufacture.

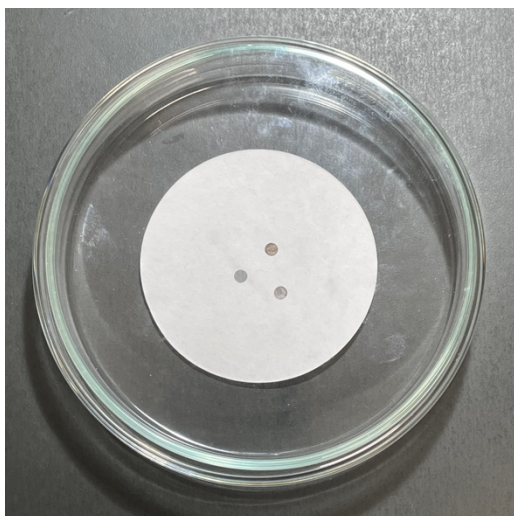
In that case, the effect of the hydrophilic treatment in the next step is reduced, so the plastic component is removed.

If you don't need it, you can omit it.

- ☐ Place the filter paper soaked in acetone in a glass petri dish and place the grid removed from the case. Turn the surface (carbon face = slightly blackish side) upwards.



- ☐ Cover with a lid, and let it stand for 10–20 minutes, then slightly open the lid to let the acetone evaporate. Or, cover and let it sit overnight. Since the acetone evaporates completely, it proceeds to the next hydrophilic treatment.



II. Starting Vitrobot and Hydrophilic treatment of the grid

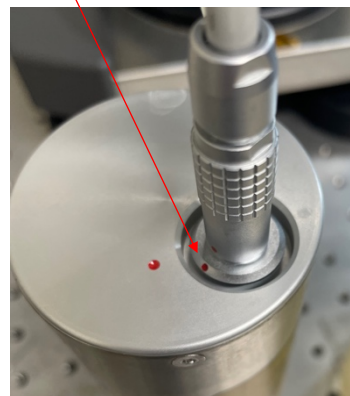
1. Starting Vitrobot

- Removed the humidifier and checked the milliQ water inside. Turn the cylinder slightly to the left and lower it down to remove it.

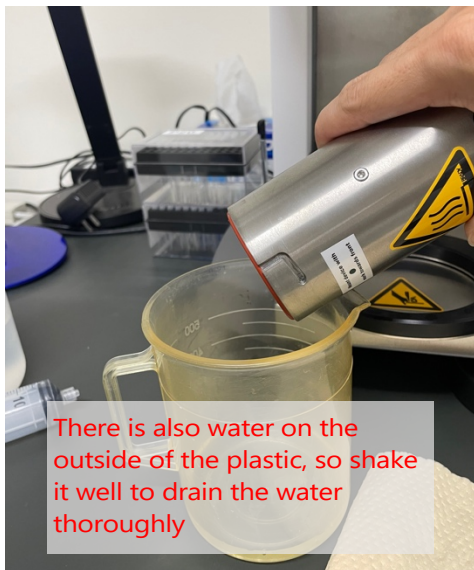


The cable is long and can be pulled out.

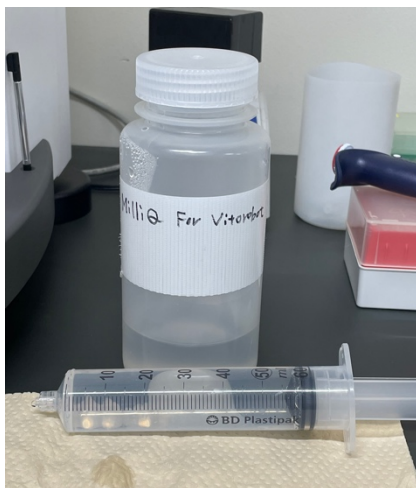
When unplugging the cable, pull it out while pushing in the ring-shaped part.



- ☐ If the milliQ water is low or looks old, throw it away.



- ☐ Reinstall it in the same way as when removing the humidifier.
If it is not properly installed, it is not possible to control the humidity, so check carefully to see if there is any rattling.
- ☐ Fill a 50 ml syringe with milliQ water (a little more than 50 ml) and attach it to the Humidifier water injection tube.



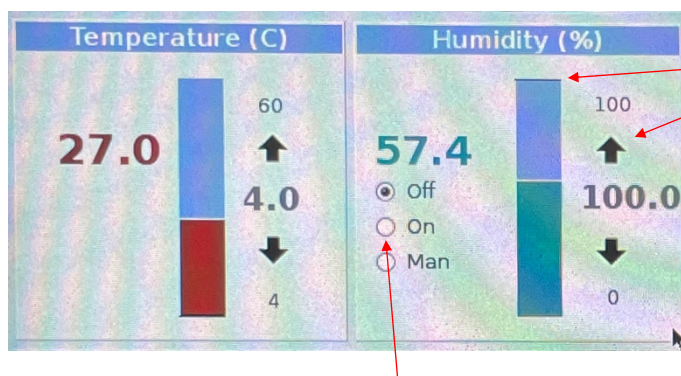
- ☐ After injecting all the milliQ water in the syringe, flow back about 5 ml (the valve closes).
- ☐ Remove the syringe.

- Turn on the power of the Vitrobot main unit (lower back).

After the OS boot screen is displayed, the main screen is displayed.



- Enter the desired temperature and humidity

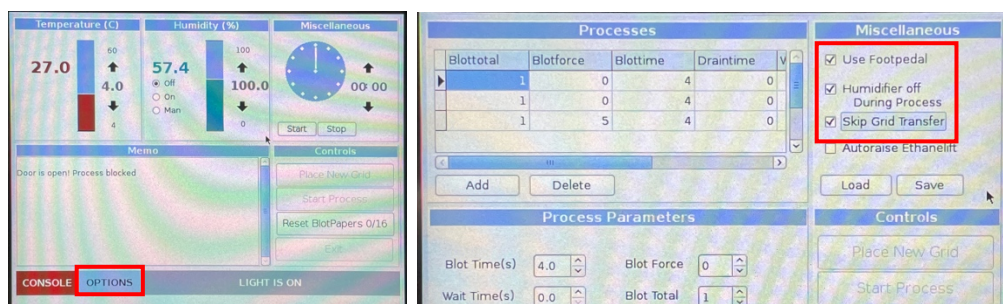


Use the arrow keys to move the numbers or slide the black bars

* When setting the humidity to a low temperature, turn on the humidity control after the temperature drops.

* It takes several tens of minutes for the temperature to stabilize.

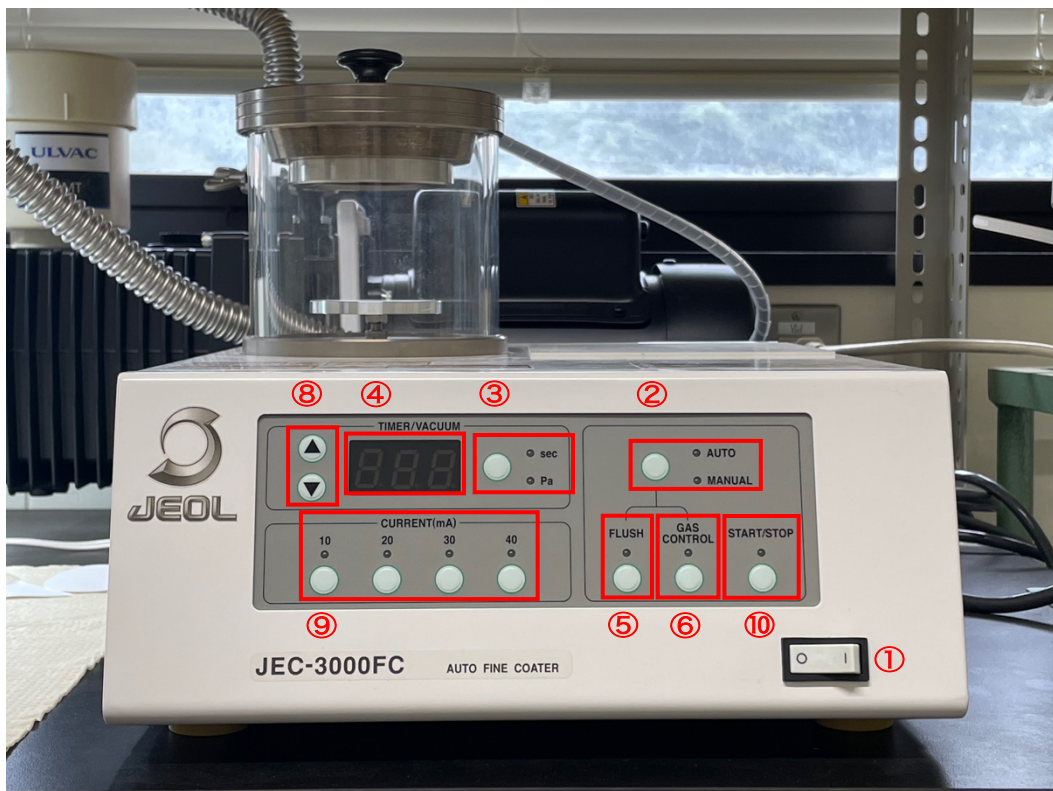
- Press **OPTIONS** button and check the three boxes on the right side of the screen, from the top.



*Hydrophilic treatment is performed immediately before grid freezing

2. **Blank of the equipment**

- ☐ JEC-3000FC is used.
- ☐ When using it for the first time that day, it is blank and empty without putting anything in the chamber.
- ☐ Close the chamber cover and lid and turn on the power switch (1).



- ☐ Confirm that the indicators are lit for (2) MANUAL and (3) Pa.
* If not, press the ○ button to switch
- ☐ (4) The current pressure in the chamber is displayed on the digital display.
Wait for it to reach 3 or less, and press (5) FLUSH.
- ☐ (4) Wait for the digital display to reach 3 or lower again, and (6) press GAS CONTROL.

- ☐ (4) The digital display indicates the desired pressure (7 Pa, 20 Pa, etc.). Wait until it is.
If necessary, adjust the (7) needle valve on the back of the main unit.

- ☐ (3) Press the ○ button and confirm that the indicator is lit on the SEC.

- ☐ (8) Press the up and down keys for the desired time (10 sec, 60 sec, etc.). Align with

- ☐ (9) Set CURRENT to the desired value (10 mA, 40 mA, etc.) button.

- ☐ (10) Press START/STOP to start hydrophilic treatment.

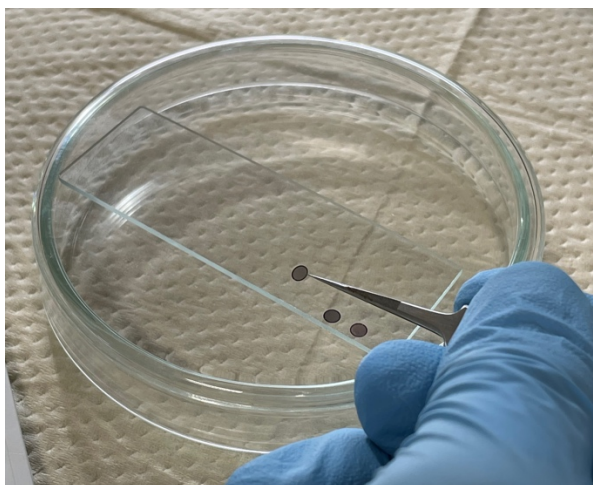
- ☐ After finishing, (1) turn off the power switch. Wait a moment until the pressure in the chamber is released to atmospheric pressure.

- ☐ (After the blank strike is completed, put the grid in the chamber and perform the above operation in the same way.)



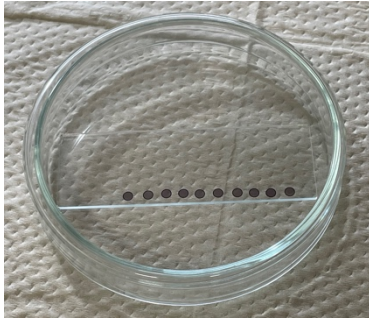
3. Hydrophilization of the grid

- ☐ The grid removed from the case (or after acetone treatment) is lined up on a glass slide. Surface (carbon face) facing up.

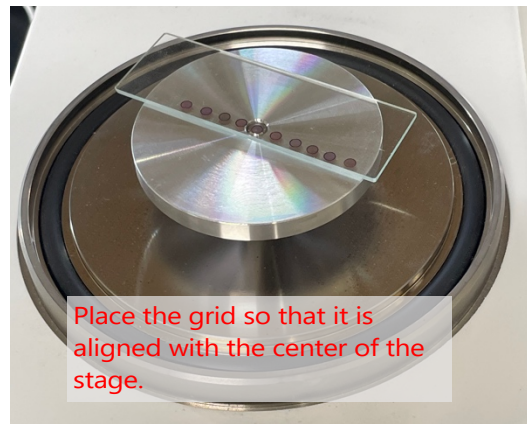
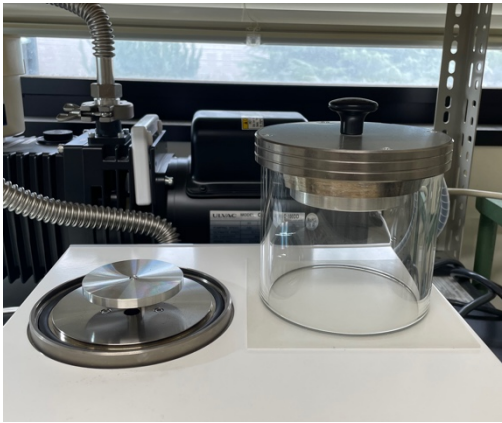


When pinching the grid with tweezers, pinch the outer periphery to avoid scratches.
Pay close attention to the grid, as it is easy to bend (**bent grids cannot be used for measurements**).

- It is a good idea to arrange them on the edge of the glass slide for easy access later.

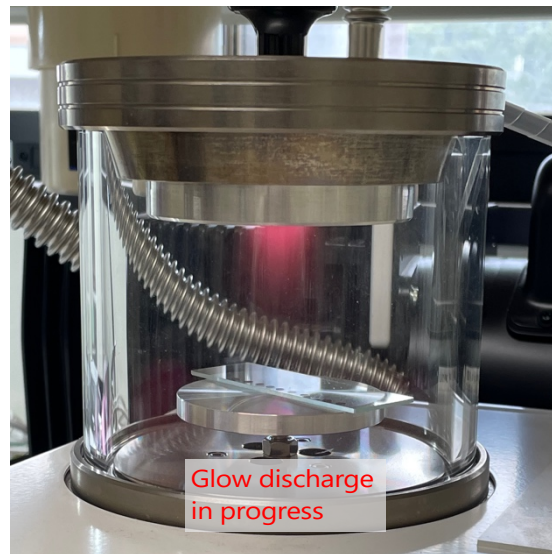


- Remove the chamber of the JEC-3000FC and place it on the stage of the equipment while it is lined up on a glass slide.



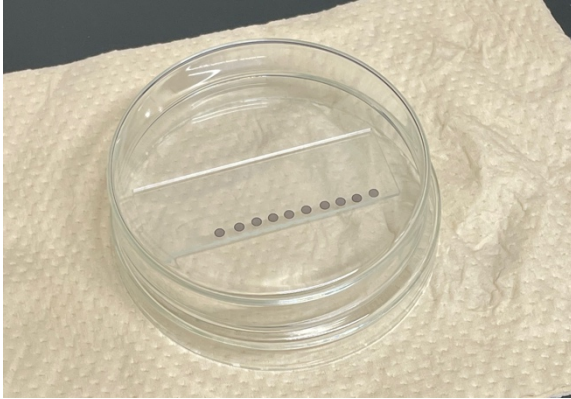
Place the grid so that it is aligned with the center of the stage.

- Set the lid of the chamber firmly and **2. Hydrophilization is performed in the same operation as when the device is idle.**



Glow discharge in progress

- ☐ After hydrophilic treatment, it is recommended to cover it with a glass petri dish or the like so that it does not cover dust.



4. Typical Hydrophilic Parameters

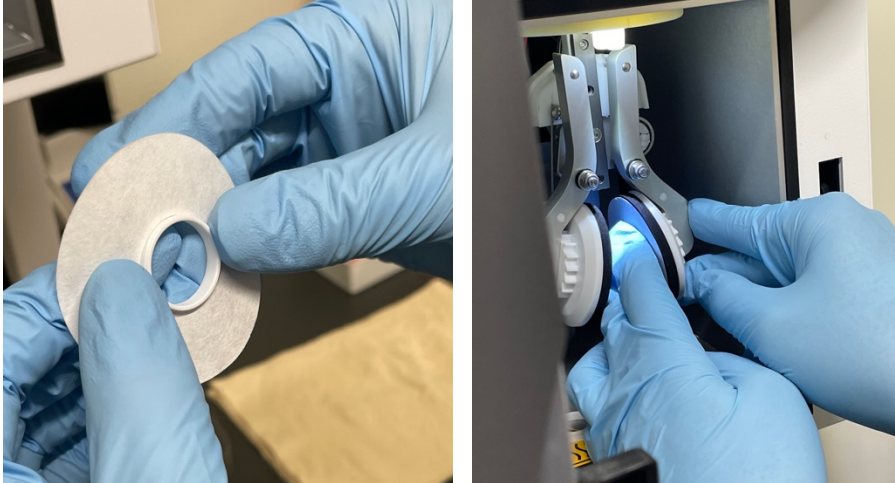
- ☐ Standard: 7 Pa, 10 mA, 10 sec
- ☐ Stable particles in thin ice: 20 Pa, 20 mA, 45 sec
- ☐ +2 nm C: 20Pa, 10mA, 30s (one side)
- ☐ UltraAuFoil: 20 Pa, 40 mA, 60 sec

**In the case of UltraAuFoil, both sides of the grid are hydrophilically treated.*

III. Cryogrid Fabrication with Vitrobot

1. Attach the filter paper

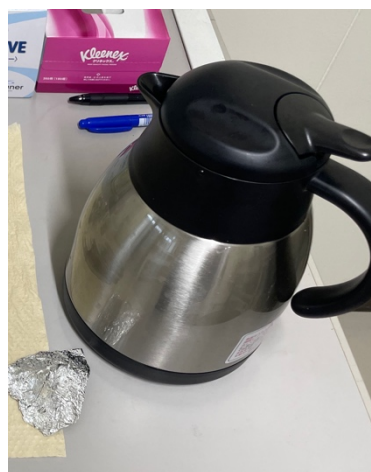
- ☐ Wear gloves and attach the filter paper to the pads on each side, one at a time.



- * It is safer to align the front and back of the filter paper in a certain direction each time.
- * There are different ways to use it, such as whether to use it in an environment with 100% humidity or to wear it just before blotting and use it in a relatively dry state, but it is important to use the same method every time.
- * When the time is long, such as when the number of grids is large, it is good to change the filter paper in the middle. After replacing, the pad may be moving a little, so I will empty it once.

2. Preparation of ethane container and liquefaction of ethane

- ☐ Checked the components of the ethane container, tweezers, grid case, pot for liquid nitrogen, etc.



- ☐ Assemble the ethane container, fill it with liquid nitrogen and cool it.



* The inside of the ethane cup is also filled with liquid nitrogen (evaporates in a short time).

* Replace liquid nitrogen as the liquid nitrogen boils violently until it is sufficiently cooled. Allow the boiling to subside, and the container to be sufficiently filled with liquid nitrogen.

- ☐ Confirm that the liquid nitrogen in the ethane cup has completely evaporated.
- ☐ wear gloves, safety glasses (if liquid ethane gets into the eyes, there is a risk of blindness);
- ☐ Make sure that the two taps ((2) and (3)) on the downstream side of the regulator of the ethane cylinder are closed, and open the main tap ((1)) slightly.



- ☐ Open the plug (2) on the downstream side of the regulator. The downstream needle only needs to move slightly (up to 0.01 MPa).
- ☐ Open the stopper (3). If necessary, adjust the stopper (2). It is good if there is a slight gas emitted.
- ☐ Insert the tip of the tube to the bottom of the ethane cup to liquefy the ethane.

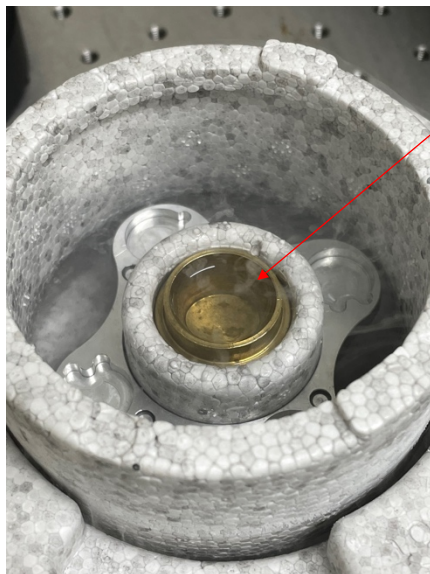


- * When liquefaction begins, the sound changes
- * Adjust the tip of the tube to keep slightly below the liquid level as the liquid level rises.
- * Liquefy to the very edge of the boundary between the ethane cup and the spider.



spider

- ☐ When liquefaction is completed, the main valve of the cylinder is closed, and then the downstream spigot is also closed.
- ☐ Immediately remove the spider.



Make sure the ethane cup is filled with liquid ethane

- * If you leave the spider as it is, it will freeze and you will not be able to remove it.

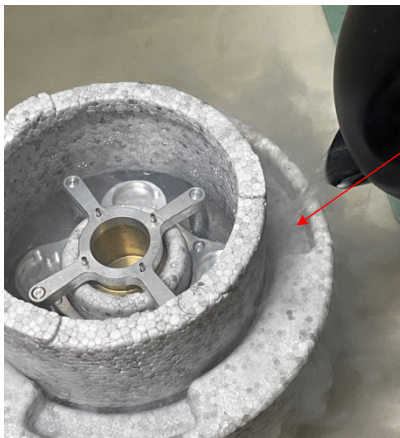
* If the ethane solidifies and the white stuff appears to be sinking, place the room-temperature spider upside down to transfer heat and melt.

As soon as it melts, remove the spider.



- Cover with a transparent cover (a large beaker is acceptable) to prevent frost from entering.

If necessary, add liquid nitrogen from the edge of the container.



Pouring through the
recess at the end

3. Quick freezing operations with Vitrobot

* Make sure the humidity control is turned on.

- Vitrobot main unit screen > **OPTIONS** > Process Parameters
Set the desired conditions

The screenshot shows the 'Process Parameters' screen with the following settings:

Parameter	Value
Blot Time(s)	4.0
Wait Time(s)	0.0
Drain Time(s)	0.0
Blot Force	0
Blot Total	1
Skip Application	<input type="checkbox"/>

*Blot time: 2 – 10 s, Blot Force: 0 – 10 is standard?

*Blot Force uses empirical values for each device because there are large individual differences in the device.

*Processes can also be set to blot once and then add the sample again.

The screenshot shows the 'Processes' screen with a table of process parameters:

	Blottotal	Blotforce	Blottime	Draintime	V
▶	1	0	4	0	
	1	0	4	0	
	1	5	4	0	

Buttons: Add, Delete

- Confirm that **Place New Grid** is displayed on the Vitrobot main unit screen.

* If the status is different, step
on the foot pedal several times to
make **Place New Grid** visible.

The screenshot shows the 'Controls' screen with the following buttons:

- Place New Grid** (highlighted with a red box)
- Process
- Reset Blotpapers 6/16
- Exit

- Pinch the edges of the grid with Vitrobot tweezers and secure them with black fasteners.



* Try shaking it a little to see if the grid doesn't fall.

- Secure the tweezers to the Vitrobot arm.



If it fits properly,
there is a clicky
feeling.

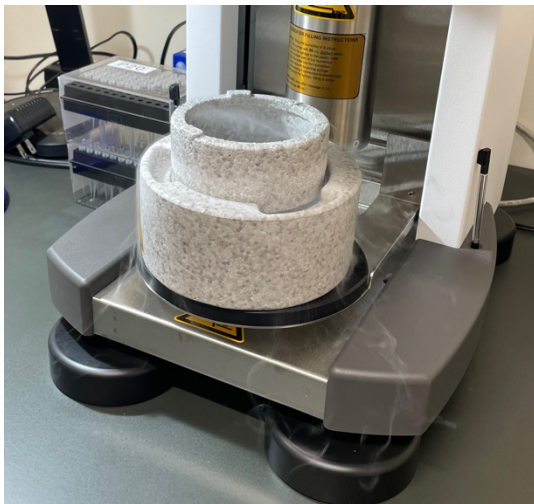
Install so that the carbon
side is facing the
dominant side.

- Step on the foot pedal twice. Tweezers rise into the box.

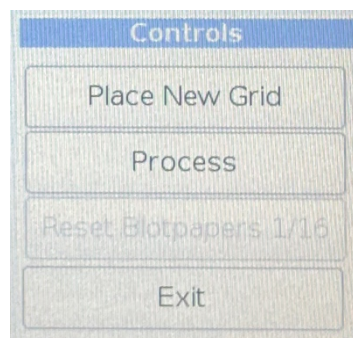


* The display will change to Place Ethane Container

- Add liquid nitrogen as needed and place the ethane container on the stage.



- Step on the foot pedal. The ethane container lifts up and the screen display changes.



* If you step on the Foot pedal in this state, it will proceed to the next step as if you pressed Process.

*When you press Place New Grid, the stage of the ethane container goes down and the tweezers also come down = return to the previous step

- ☐ If there is no problem, step on the foot pedal.

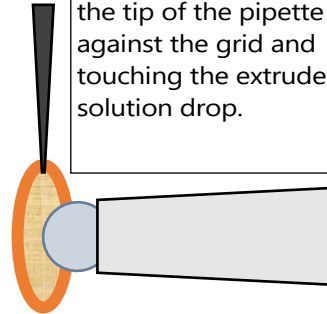
As the tweezers go down, apply around 3 μ l of sample.



The small window next to the main unit
Open and insert the pipette.



* Push out the entire volume by not touching the tip of the pipette tip against the grid and touching the extruded solution drop.



- ☐ Step on the Foot pedal and perform blotting -> plunge freezing at the set value.
- ☐ When the tweezers and ethane container come down, remove the tweezers and lower the ethane container from the stage.



* The tweezers come off even if you pull them toward you or push them in the back.

* Be very careful not to bend the grid against the wall of the ethane cup when removing it.

* When unloading the ethane container, be careful not to let the grid come out of the ethane.
Hold your elbows on the desk and your wrists against the edge of the ethane container.

- ☐ Quickly move the grid from ethane to liquid nitrogen.



* Be careful to minimize the height at which the grid is lifted from the liquid level.

- ☐ The grid is raised to about 5-10 mm above the liquid nitrogen surface (it does not rise to room temperature), and the residual liquid ethane is vaporized to remove it.

* When the temperature rises, the carbon surface of the grid turns white as if it had been frosted in a few seconds.

* If you wait as it is, the whitish stuff will disappear, so wait a few more seconds after it disappears completely before returning it to liquid nitrogen.

* Repeat the above several times to remove as much of the ethane remaining on the grid as possible.

- ☐ While the grid is immersed in liquid nitrogen, the black fasteners of the tweezers are shifted upwards to release the fastening. Hold the tweezers firmly so as not to drop the grid.

- ☐ Store the grid in a grid box.



* Be careful to minimize the height at which the grid is lifted from the liquid level.

* Be careful not to bend the grid when storing it in the grid box.

* When you put about half of the bottom of the grid, remove the tweezers and drop it.

- ☐ Repeat the operation from p.14 for the number of samples.

* Once used, the tweezers should dry well and then use again.

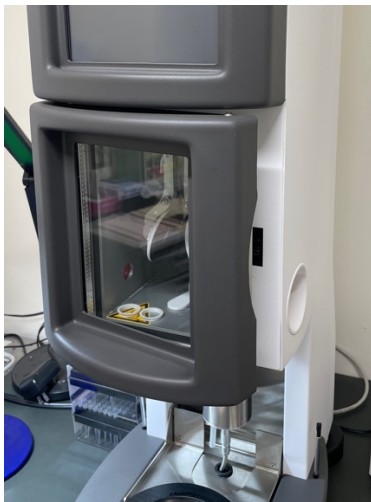
- ☐ Store the grid case in liquid nitrogen.

4. Vitrobot Shutdown

- ☐ Vitrobot screen > **Exit** > **Yes**



- ☐ Turn off the power switch on the back of the main unit.
 - ☐ Remove the filter paper and leave the chamber door slightly open (to dry the inside).
- * If you have only used 2-3 filter papers, you may leave them.



* Be careful not to lose the filter paper fasteners.

- ☐ If you do not use it for a while, discard the milliQ water in the humidifier.
The cylinder may be removed.

