

Vitrobot Mark IV

Standard Operating Procedure

Automated Vitrification System for Cryo-EM Sample Preparation

1. Overview

The ThermoFisher Scientific Vitrobot Mark IV is a PC-controlled semi-automated plunging device designed for vitrification of aqueous samples for cryo-electron microscopy. The instrument provides precise control of temperature, humidity, and blotting parameters to ensure reproducible vitrification of biological samples in liquid ethane.

This procedure covers setup, operation, and shutdown protocols for safe and effective sample preparation.

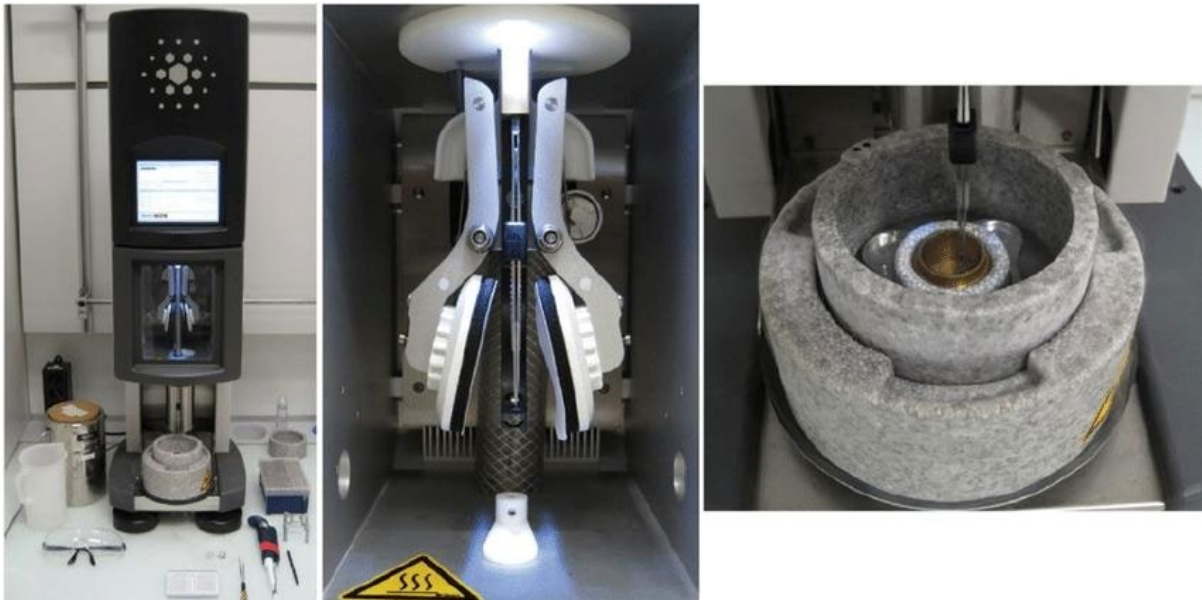


Figure 1. Vitrobot Mark IV System: (A) Full system showing touchscreen interface and cryo workstation, (B) Interior view with blotting pads and tweezers, (C) Cryo workstation close-up showing ethane cup and LN2 container

2. Equipment Components

Main Vitrobot Unit

- **Climate Chamber:** Environmentally controlled enclosure with humidity and temperature control (4-60°C)
- **Humidifier:** Attaches to bottom of climate chamber; ultrasonic humidification maintains up to 100% RH
- **Sample Ports:** Left and right side entry ports allow manual sample application via pipette
- **Blotting Pads:** Located inside climate chamber; hold filter paper for excess fluid removal
- **Tweezers/Plunge Rod:** Holds EM grid during blotting and plunging; operates via automated vertical motion
- **Touchscreen Interface:** Controls all vitrification parameters and process steps
- **Power Switch:** Located on back right of instrument

Cryogenic Workstation

The coolant container sits on a motorized lift and contains the following components:

- **Ethane Cup:** Central container holding liquid ethane (coolant for vitrification)
- **Spider:** Three-legged metal device for cooling/heating the ethane cup; controls ethane temperature
- **Liquid Nitrogen Tank:** Surrounds ethane cup; maintains cryogenic temperature (-196°C)
- **Floating Ring:** Anti-contamination barrier that floats on liquid nitrogen surface
- **Grid Box Holder:** Holds cryo-grid storage box within liquid nitrogen environment

3. Safety Requirements

Personal Protective Equipment (PPE)

- Laboratory coat
- Nitrile gloves (for general handling)
- Cryogenic gloves (for handling liquid nitrogen)
- Safety glasses or goggles
- Face mask (as needed)

Hazards

- **Liquid Nitrogen:** Cryogenic liquid (-196°C); risk of cold burns and asphyxiation
- **Ethane:** Flammable gas; ensure tank valves are properly controlled
- **Moving Parts:** Keep hands clear during automated plunging and lift operations

4. Setup Procedure

1. **Humidifier Installation:** Attach humidifier to bottom of climate chamber by aligning red dots on cable connector and locking ring. Push upward and twist clockwise. Sticker should face forward.
2. **Fill Humidifier:** Using syringe, inject 30-45 mL distilled water through bottom tube. After filling, pull back syringe plunger to ~10 mL to create vacuum. This prevents water leakage.
3. **Power On:** Turn on power switch located on back right side of instrument.
4. **Install Blotting Paper:** Open chamber door. Attach pre-punched filter paper (Whatman #1 or #541) to blotting pads using white circular clipping rings. Ensure inner ridge of paper faces pads, NOT the sample.
5. **Set Environmental Parameters:** Using touchscreen interface, set desired temperature (typically 20-25°C) and humidity (typically 100%). Press up arrow to adjust humidity from default 70% to 100%.
6. **Configure Options:** Press 'Options' and enable 'use foot pedal' and 'skip grid transfer.' Set blot time (typically 3-5 seconds), wait time, and blot force as needed for your sample.
7. **Prepare Cryogenic Workstation:** Assemble ethane cup, grid box holder, spider, and floating ring in coolant container. Fill container with liquid nitrogen until it covers grid box positions.
8. **Condense Ethane:** Submerge spider in liquid nitrogen to cool. Place cooled spider (legs down) on top of ethane cup. Slowly introduce ethane gas into cup. You will hear gurgling as gas condenses. When ethane forms a liquid slurry, remove spider with pliers.

IMPORTANT: Verify ethane is liquid (not frozen solid) before beginning vitrification to prevent grid damage.

5. Operation Procedure

1. **Check Parameters:** Verify temperature and humidity have reached set points before processing grids.
2. **Load Grid:** Mount glow-discharged EM grid in Vitrobot tweezers. Ensure grid orientation matches intended sample application side. Attach tweezers to plunge rod.
3. **Position Workstation:** Place coolant container on motorized lift below climate chamber.
4. **Start Cycle:** Press foot pedal or 'Start Process' to raise plunge rod into climate chamber.
5. **Apply Sample:** Pipette 3-3.5 μL sample through side entry port onto grid surface. Close port immediately.
6. **Automated Vitrification:** Press foot pedal to continue. The Vitrobot will automatically execute: wait time $\rightarrow$ blotting $\rightarrow$ drain time $\rightarrow$ plunging into liquid ethane $\rightarrow$ lowering into liquid nitrogen.
7. **Grid Transfer:** Carefully disconnect tweezers from plunge rod. Transfer grid from ethane cup through liquid nitrogen into grid storage box. Use pre-cooled forceps.
8. **Monitor Ethane:** If ethane begins to freeze excessively, briefly place spider upside-down (legs up) on ethane cup for 1-2 seconds to warm, then remove immediately.

Note: For multiple grid processing, change floating ring frequently to prevent ice crystal contamination.

6. Shutdown Procedure

1. **Close Ethane Tank:** Ensure ethane gas cylinder valves are fully closed.
2. **Clean Tweezers:** Dry and properly store Vitrobot tweezers. Handle with care as they are delicate and expensive (~\$10,000 each).
3. **Exit Software:** Press 'Exit' button on touchscreen interface. Confirm shutdown when prompted.
4. **Power Off:** Wait for screen to go completely black, then flip power switch off on back of instrument.
5. **Remove Blotting Papers:** Open chamber and dispose of filter papers. Store clipping rings inside chamber.
6. **Empty Humidifier:** Remove humidifier from climate chamber. Invert and dump water at least 3 times to clear reservoir completely. Store humidifier upside-down in Styrofoam container.
7. **Evaporate Cryogens:** Place coolant container with remaining liquid nitrogen and ethane in fume hood to evaporate safely.

7. Troubleshooting & Tips

Optimizing Blot Parameters

Starting parameters for standard grids: 3-4.5 seconds blot time, -5 to 0 blot force. Blot time has more influence on ice thickness than blot force. Adjust based on ice quality observed in cryo-EM screening.

Ethane Temperature Control

Proper ethane consistency is critical. Liquid ethane should appear as a slushy mixture. If too frozen: use reversed spider briefly. If too liquid: cool with spider legs-down.

Grid Handling

Always use dry, pre-cooled tools when handling frozen grids. Vitrobot tweezers are for grid pickup ONLY - using them for other tasks will damage the tips and compromise sample quality.

Document Version: 1.0

Last Updated: October 2025