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VitroEase™ Cryo-EM Training Kit

USER GUIDE

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B.0	26 July 2022	Correcting catalog number to be A51363.
A.0	11 January 2022	New manual for VitroEase™ Cryo-EM Training Kit new product.

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1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product description

Sample preparation can be difficult in cryo-electron microscopy (cryo-EM) single-particle analysis (SPA). Currently, sample optimization is often performed in a non-systematic way, extending optimization time over days or weeks. The VitroEase™ Cryo-EM Training Kit is designed to provide the components to begin cryo-EM analysis of protein samples. The kit includes Apoferritin as a positive control protein for grid optimization and cryo-EM analysis and accessories used for grid preparation, including tweezers, grids, and grid boxes. The control and components may be used as a comparison for success for new users, or QC with expert users.

Contents and storage

Contents	Amount	Storage
VitroEase™ Apoferritin Protein (3.5–4 mg/mL)[1]	5 × 20 µL	–80°C
EM grids, Quantifoil™ R 1.2 / 1.3, 300-mesh copper	25 grids	Room temperature
Tweezers, Dumont HP Number 5 Carbon steel, 0.08-mm × 0.04-mm tip (or equivalent)	1	
Tweezers, ESD Soft Grip Tweezers, 115 mm, Extra Fine Tip	1	
Round Cryo TEM Grid Box with pin-type lid	2	

[1] Apoferritin protein is shipped separately on dry ice. For optimal results, avoid multiple freeze-thaws to minimize aggregation and loss of protein quality.

Required materials not supplied

- Adjustable pipettes, 10 µL
- Pipette tips, 10 µL tip for sample loading and 100 µL tip for ethane preparation
- Laboratory tissues
- Microcentrifuge tubes, 1 mL
- Liquid nitrogen storage carriers

- Liquid nitrogen benchtop flasks, 2-3 L
- Plasma cleaner with grid slide
- Vitrobot™ Mark IV System
- Vitrobot™ tweezers, filter pad, and foam holder
- Ethane cup, ring, and metal spindle
- Ethane source and tube connection
- *(Optional)* Heating cabinet and hair dryer

Workflow

Workflow

Prepare the Vitrobot™ instrument

1. Connect and fill the Vitrobot™ humidifier.
2. Adjust vitrification settings.
3. Cool the Vitrobot™ to 4°C and adjust the chamber humidity to 100%.

Glow discharge the EM grids

Prepare for vitrification

1. Thaw one Apoferritin aliquot.
2. Place the Vitrobot™ filter pads.
3. Perform a trial run to confirm Vitrobot™ tweezer centering.

Prepare liquid ethane

1. Prepare the LN2 dewar and flask.
2. Cool the Ethane Coolant Container and ethane cup with LN2.
3. Prepare the liquid ethane.

Plunge the grid

1. Plunge freeze the Apoferritin sample.
2. Transfer and store the vitrified grid.

Screen vitrified grid for ice assessment

2 Methods

Prepare the Vitrobot™ humidifier

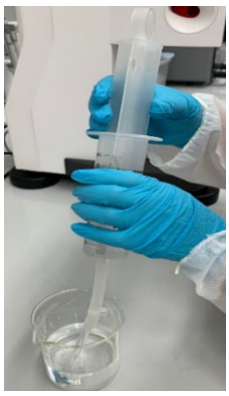
IMPORTANT! Wear appropriate Personal Protective Equipment (PPE) when dispensing liquid cryogens. Minimal PPE are cryo gloves and goggles or face shield. Beware of heavy tanks and thermos containers and ensure proper venting. Consult also your institutional safety procedures for working with cryogenic and flammable gasses (please see the safety information section in this manual)

IMPORTANT! Ensure the Vitrobot™ Mark IV System is installed in a well-ventilated area with no live electrical switches near the fume hood or spaces surrounding the instrument. Ethane gas present in the instrument can form an explosive mixture in air. See the Vitrobot™ Mark IV System user guide for more information.

Note: Only use distilled water in the humidifier.

1. Connect the humidifier (silver cylinder) under the humidity chamber and fill with distilled water.
   a. Draw 60 mL of distilled water into a plastic syringe and inject the water into the tube connected at the bottom of the cylinder.

   b. Keep the syringe attached and pull back ~10 mL of air until no air is drawn back from the cylinder.



Note: The draw of air creates a slight vacuum to ensure that the humidifier properly fills and works during the vitrification procedure.

2. Ensure the O-ring (orange ring in image below) is aligned on top of the humidifier before connecting to the Vitrobot™ system.

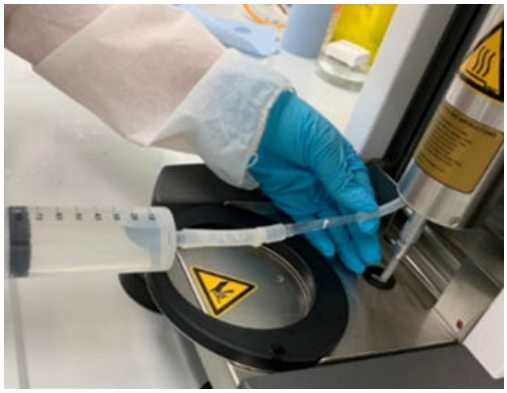
3. Connect the Vitrobot™ cable to the humidifier while pressing on the port.

4. Place the humidifier with the label outward.

5. Turn the humidifier to the right.

6. Fill the humidifier with distilled water using a syringe to ensure no air is trapped in the humidifier.

Note: Overfilling the water container can result in a water leak in the Vitrobot™ chamber, resulting in overheating and burning the Vitrobot™ instrument connection.



Glow discharge

1. Using Dumont HP tweezers, gently transfer an EM grid to a glow discharge glass slide or metal net with the carbon side (dark grey) facing up (Fig. 1D).

Note: We recommend practicing the procedure several times with one grid to confirm accuracy without damaging the EM grids.

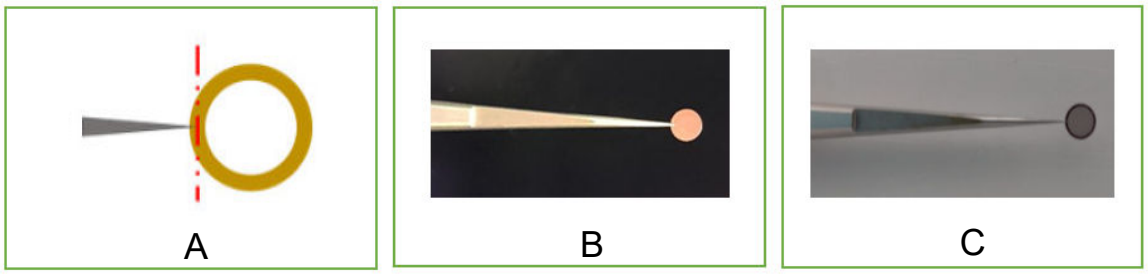
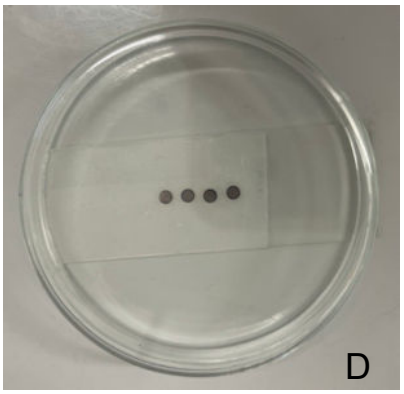


Figure 1 Handling EM grids.

(A) Diagram showing positioning of the tweezers tip while grasping the EM grid. The tweezers tips should be on the center of the grid and engaged ~0.5 mm from the grid edge. (B) Image of proper grid grasp with tweezers (copper face up). (C) Preparing the EM grid for glow discharge with carbon face up. (D) Grids placed on a glass slide for glow discharging.



2. Open the vacuum chamber and carefully place the metal mesh or glass slide with grids inside the chamber with the carbon side up (Fig. 1D).

3. Gently close the vacuum chamber to prevent the grids from flipping or moving.

4. Adjust the default glow discharge setting on the device touchscreen.

Table 1 Glow discharge default settings.

Parameter	Setting
Glow discharge time	30 seconds
Plasma current	20 mA
Glow discharge head polarity	Negative
Atmosphere	Residual Air

Note: Glow discharge settings are dependent on the glow discharge model. See manufacturer recommendations for the appropriate settings. After achieving success with a given set of glow discharge conditions, we recommend maintaining the same settings for future samples.

Note: Default settings in Table 1 are useful for glow discharge models of Emitech K100X/Quorumtech Q150R and GloQube systems (Quorum).

5. Run the default glow discharge program until complete.

6. Run the vent program, wait for the program to complete, then carefully open the door to retrieve the EM grids.

Note:

- Do not retrieve grids from the glow discharge instrument until the samples and the ethane cryo cup in the Coolant Container are ready.
- Use EM grids within 30 minutes after glow discharging. Grids may go through the glow discharge procedure one more time as needed (support film facing up).
- Only use flat, intact EM grids. Use caution to not bend the grid during sample application. Consult Figure 1 for proper EM grid handling with tweezers.
- Prior to vitrification, protect freshly glow-discharged grids from dust.

Prepare the Vitrobot™ instrument

Attach filter papers

1. Thaw Apoferritin Protein on ice while preparing the Vitrobot™ instrument.

2. Attach Vitrobot™ filter papers to the blot pads. Use the white circular clipping rings and one filter paper per pad (Fig. 2).

Note: Wear clean, dry gloves when touching filter papers. Gently apply blot papers on blot pads.

Note: Attach the Vitrobot™ filter paper immediately before starting the vitrification process.

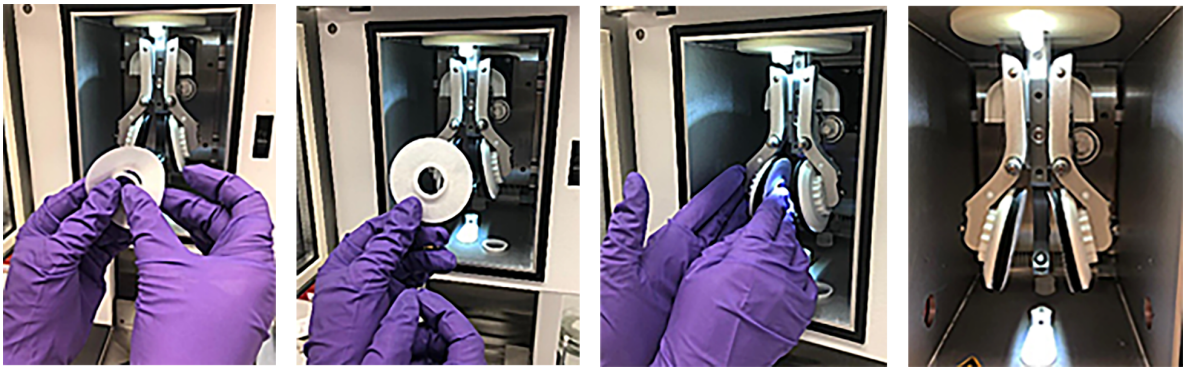
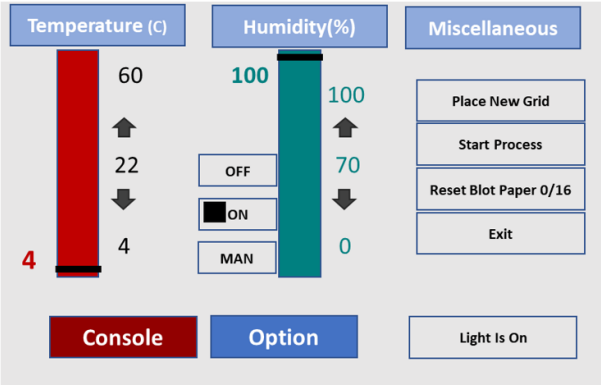


Figure 2 Attaching filter papers to Vitrobot™ blot pads.

Set Vitrobot™ user interface settings

The Vitrobot™ user interface has Console and Option screens for entering vitrification parameters.



Note: Temperature and humidity can be set using the **+** and **–** buttons or by dragging the black band across the bar up or down.

1. Set the temperature at 4°C and humidity at 100%. Touch **ON** next to the humidity bar. To avoid over-saturation of the chamber, allow 5–10 seconds of manual (**MAN**) humidity before increasing to 100%.

   Note: The humidifier water reservoir contains a sensor that sends a warning message to the console interface if the water level is too low. If the humidity level does not rise, check for this error condition. If the error condition shows, add distilled water to the reservoir per the instructions in “Prepare the Vitrobot™ humidifier” on page 7.

2. In the **Options** screen, set the sample blotting parameters: **Blot time** and **Blot force**.

Parameter	Setting
Temperature	4°C
Humidity	100%
Blot force	0
Blot time	3–5 seconds
Sample volume (on grid)	3 µL
Drain time	—
Wait time	—
Blot total	1

3. Set the **Miscellaneous** parameters. For optimal use, select **Use Foot Pedal**, **Humidifier OFF**, and **Skip Grid Transfer**.

Prepare liquid ethane

For an optimal freezing temperature, both liquid nitrogen and ethane are used. If the ethane is too warm, the freezing process will slow and affect the sample quality and final reconstruction. If the ethane is too cold, the ethane will become too viscous. Liquid nitrogen is first used (via the “four-legged spindle") until it is frozen solid at the rim of the ethane cup, followed by ethane that forms a thin turbid frozen film on the liquid surface. Remove the spindle and wait for the solid film on the surface to thaw until the ethane is clear.

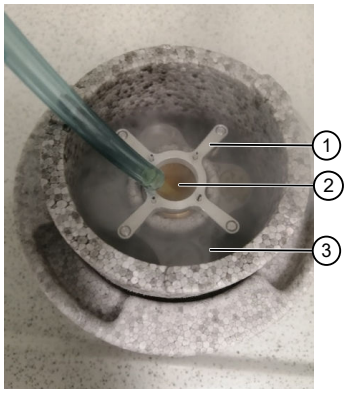


Figure 3 Ethane Coolant Container assembly.

① Metal spindle
② Liquid ethane
③ Liquid nitrogen

1. Assemble the Coolant Container and fill the outer ring and ethane vessel with liquid nitrogen to cool the container. Allow the liquid nitrogen to settle as it fills to full. Complete equilibration is obtained when the liquid nitrogen slowly boils away in a foam container and the bubbling sound stops. Avoid pouring liquid nitrogen on the copper ethane cup.

2. Slowly open the ethane gas source and wait until a thick white fog appears indicating ethane gas flow. Place the tip of the hose on the bottom of the copper ethane cup and adjust the ethane flow. When bubbling starts, carefully increase the ethane flow.

Note: Make sure the tip connected to ethane tube is cut at a 45° angle. The 45° tip makes it easier for the ethane to flow, reduces the pressure inside the tube, and prevents freezing to the bottom.

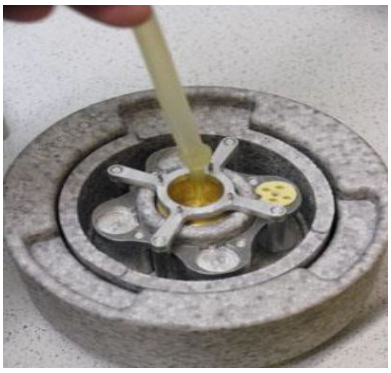


Figure 4 Liquid ethane preparation.

3. Raise the ethane level and fill the space to 2 mm below the edge of the copper ethane cup.

4. Cover the whole assembly (reduce contamination by water/ice) for 2–3 minutes. Once solidified, the ethane is cold enough for plunging.

5. Thaw the metal spindle using an extra metal piece (e.g., another clean ethane cup at room temperature) touching the spindle between the top pins for 5 seconds. Observe the top ethane layer thawing.

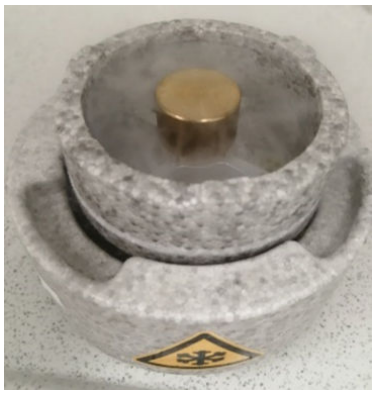


Figure 5 Metal spindle thawing using a spare ethane cup.

6. Remove the metal spindle using dry room-temperature long tweezers (>200 mm) by gripping the spindle between pins to avoid tipping into the ethane when lifting.

Figure 6 Removing metal spindle using tweezers.

Vitrification steps using the Vitrobot™ Mark IV System

Grab grid using Vitrobot™ tweezers

To grab the grid without damage, the tweezers must be correctly clamped using the tweezers slider (black slider). As shown here, the slider must be placed in the middle of the tweezers for appropriate pressure. Any position to the left or right of the middle can cause clamping issues.

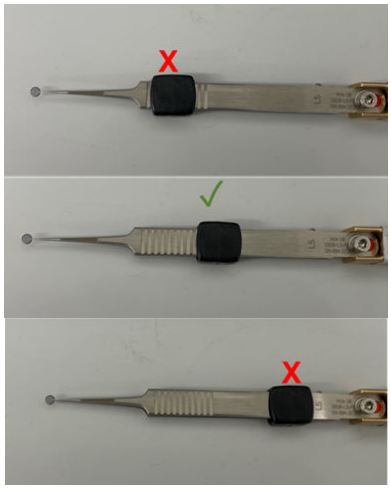


Figure 7 Correct tweezer slider position for EM grid handling (green check).

Center the grid using Vitrobot™ tweezers

Tweezers must be properly centered to ensure proper blotting functionality and avoid Vitrobot™ damage.

1. With the temperature and humidity equilibrated, click **Place New Grid** (moves the central axis into the position for attaching the tweezers).
2. Ensure slider is properly aligned on tweezers (see Figure 7).
3. Clean tweezers with ethanol and mount to the Vitrobot™ shown in Figure 8 by docking onto the connection groove in the central axis rod.

4. Center tweezers in between the blot pads.

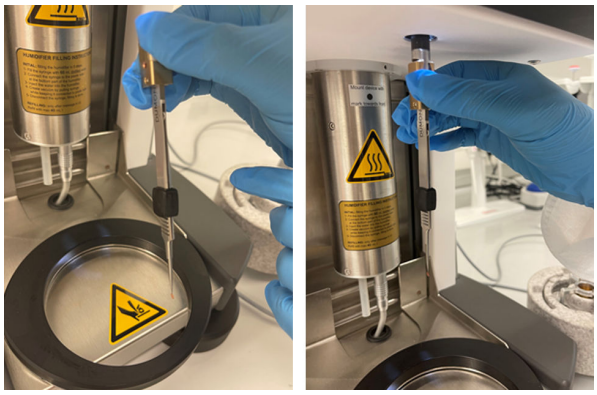


Figure 8 Tweezer docking procedure.

5. Click **Continue** or use the foot pedal to move the tweezers into the climate chamber.

6. Place the coolant container on the platform ring. Click **Place Ethane Container** or use the foot pedal.

Figure 9 Ethane compartment placement in vitrification steps.

7. Click **Continue** or use the foot pedal to activate the blotting and plunge-freezing procedure with the previously specified wait and drain times. The Vitrobot™ will then lower the coolant container and the tweezers for access.

Note: Check centering by observing if the tweezers moves when repeatedly blotting (typically 5 blots) with the same vitrification parameters.

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8. Undock the tweezers carefully from the central axis rod and remove the coolant container. Dry the tweezers using a clean lint-free dry tissue.

Note: Test at least one run of the vitrification procedure by using tweezers with no grid attached.

Load the grid and apply the sample

1. Grasp a freshly glow-discharged EM grid with the tweezers and move the black clamping ring down to the first groove on the tweezers.

2. Click **Place New Grid** and mount the tweezers with the EM grid to the Vitrobot™. Orient the grid so the support carbon layer faces the side (left/right) the sample is applied (Fig. 10).

3. Refill the Coolant Container with liquid nitrogen. Avoid splashing liquid nitrogen on to the ethane cup.

4. Click **Continue** or use the foot pedal to move the tweezers into the climate chamber.

5. Place the Coolant Container on the platform ring. Click **Place Ethane Container** or use the foot pedal.

6. Click **(Start) Process** or use the foot pedal to lower the tweezers slightly to allow easier application of a sample to the grid through the side entry port using a pipette (Fig. 10). The sample must be applied to the carbon film side of the grid and can be applied from either side of the climate chamber.

7. Carefully pipet 3 µL of thawed Apoferritin and dispense on the carbon film side of the grid.

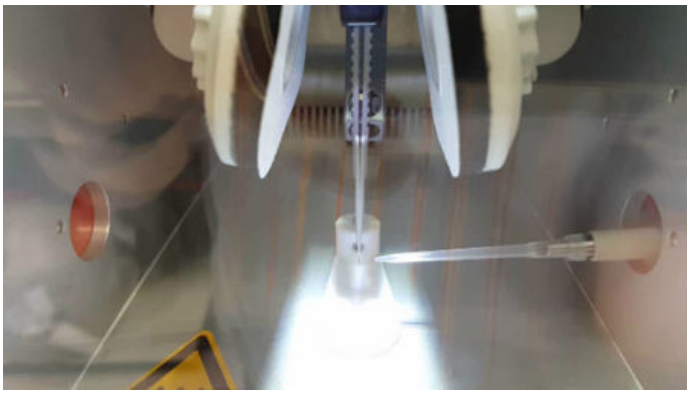

Figure 10 Sample application on the carbon film side of the grid.

Note: Pipet accuracy is critical for optimal results. Hold the pipet vertically and immerse the tip ~1 cm into the liquid. Deeper immersion or pipetting at an angle lead to increased inaccuracies.

8. Click **Continue** or use the foot pedal to activate the blotting and plunge-freezing procedure with the previously specified blotting time, force, and grid plunging. The Vitrobot™ will lower the Coolant Container and the tweezers for access.

9. Carefully undock the tweezers from the central axis. Keep the vitrified grid under the surface of the liquid ethane (Fig. 11A). Bring the Coolant Container down and move the black clamping slider up without loosening the tweezers to transfer the grid to a free position in the grid box (Fig. 11B).

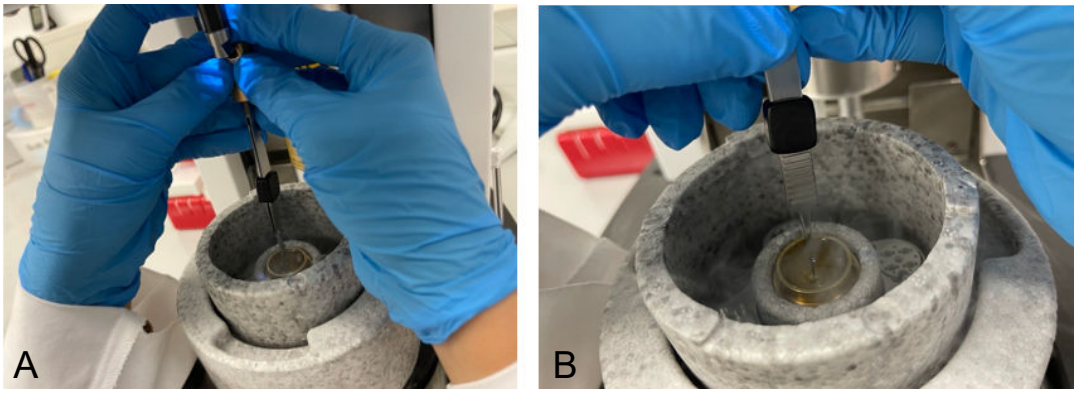

Figure 11 Grid transfer from ethane to grid box.

Note: Complete this step with the Coolant Container on the support ring of the Vitrobot™. Do not transfer the Coolant Container to the bench top while keeping the grid in liquid ethane as handling the container with one hand can cause accidents.

If the liquid ethane solidifies during vitrification, immerse clean, dry 200-mm metal tweezers into the ethane cup for 5 seconds to melt the frozen ethane ring. Avoid using icy ethane.

---

10. Repeat the steps as needed for additional grids.

11. When all grids (maximum of 4) are placed in the grid box, tighten the pre-cooled screw to seal it (cool the screw in the same Coolant Container before sealing grid box).

12. Transfer the grid box to storage or alternatively mount into Autogrids before loading in the microscope.

13. Place the Coolant Container under a fume extractor for evaporation of liquid ethane and nitrogen.

14. To turn off the Vitrobot™, touch **Exit** on the console screen. Ensure the tweezers have been removed from the central axis.

15. When the instrument light turns off, the central axis has moved into the parked position and the interface shuts down. Turn off the switch on the back of the Vitrobot™.

16. Remove the filter papers from the pads and leave the chamber door slightly open to prevent mold growth.

17. Empty the humidifier unless the Vitrobot™ is to be used again within 24 hours. Disconnect the electrical cable from the humidifier by pressing on the ring near the cable and pulling down the cable connector (Fig. 12).
    a. Apply pressure to the connection ring on the bottom of the humidifier to disconnect the cable.
    b. Disconnect the connector while applying pressure to the lock.

c. Remove the humidifier assembly while lifting and carefully turning.

Figure 12 (A) Apply pressure to connection ring. (B) Disconnect the connector. (C) Remove assembly.

A

Troubleshooting

Observation	Possible cause	Recommended action
Thick ice on vitrified grid	Blotting time was too short.	Increase the blotting time in the Vitrobot™ while keeping the blot force constant.
Losing carbon support	Tweezers were not centered on the Vitrobot™.	Adjust tweezers to center on the Vitrobot™. See Figure 7.
Hexagonal ice on grid	Ethane cup was touching the chamber.	Re-position the ethane cup to avoid touching chamber.
	Liquid ethane was not sufficiently cooled.	Confirm liquid ethane maintains appropriate temperature for the procedure.
Too many dry holes on vitrified grid	Carbon was hydrophobic.	Use a longer glow discharge time.
	Insufficient sample applied to grid.	Perform correct sample pipetting and/or increase the sample volume.
	Blot force setting was too strong and Blot time was too long.	Adjust the Blot force and Blot time.
Tweezers tip indented on vitrified grid	Carbon was hydrophobic.	Use a longer glow discharge time.
	Insufficient sample applied to grid.	Perform correct sample pipetting and/or increase the sample volume.
	Blot force setting was too strong and Blot time was too long.	Adjust the Blot force and Blot time.
Grid falls off the tweezers inside the Vitrobot™ chamber	Grid was handled improperly.	Use the correct slider position on the tweezers. See Figure 7.
	Tweezers slider was not correctly positioned.	Use the correct slider position on the tweezers. See Figure 7.
	Tweezers were damaged.	Use a new tweezers to avoid grid damage.
Vitrified grid is damaged	Grid was improperly handled.	Use correct grid handling. See Figure 1.
	Tip was incorrectly positioned during sample pipetting onto the grid.	See Figure 1.
Irregular behavior of Vitrobot™ when moving tweezer to the chamber	The Vitrobot™ tweezers were not centered.	Re-center the tweezers and confirm the correct position is used. See Figure 8.
Humidifier not working correctly	Insufficient water level in the humidifier.	Fill the humidifier to the correct level.
	Excess water in the humidifier.	Empty excess water to achieve the correct level.

Observation	Possible cause	Recommended action
Humidifier not working correctly *(continued)*	Humidifier was not connected to the chamber.	Check all connections between the humidifier and chamber.
	Air bubbles were found in the tubing.	Purge air bubbles from tubing. See the correct procedure for humidifier connection in "Prepare the Vitrobot™ humidifier" on page 7.
	O-ring on Vitrobot™ was incorrectly positioned before connecting to the humidifier.	Reposition O-ring in connection. Confirm no damage to the O-ring.

B

Glow discharge effect

Glow discharge is used to charge the carbon EM grid and make the surface more compatible with hydrophilic samples before vitrification. Upon sample application, a successful glow-discharged grid will result in a drop of sample that spreads evenly over the entire surface.

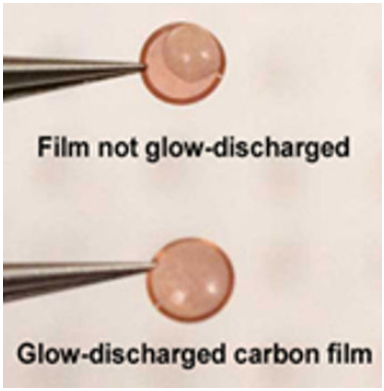

Figure 13 Even sample dispersion on film after glow discharging.

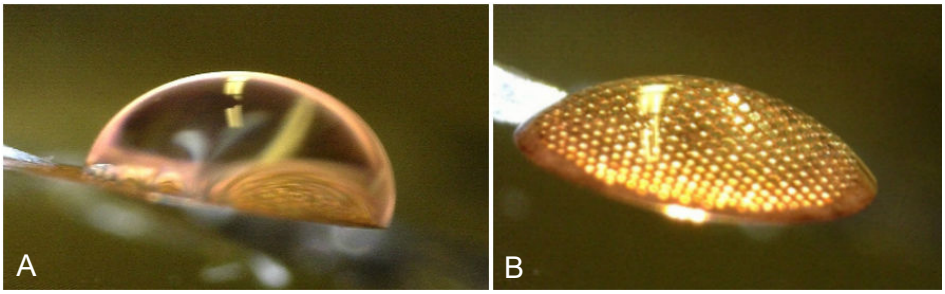

Figure 14 Comparison of grids (A) before (hydrophobic) and (B) after (hydrophilic) glow discharging.

C Ice quality assessment

For successful protein reconstruction, it is critical to embed the protein sample in amorphous glass-like ice. Non-vitreous ice may result if the sample was not vitrified rapidly; if the ethane was not at the optimal temperature; or if the grid warmed during handling. Such ice is not suitable for high resolution data collection because the protein native structure may not be preserved. The optimum ice thickness is 2–3 times greater than the protein size. Thinner ice is required to achieve high-resolution data collection, and ice thickness is typically assessed by looking at gray values in the image. For optimal selection of ice, review the grid square selection shown in Figure 16 and Figure 17.

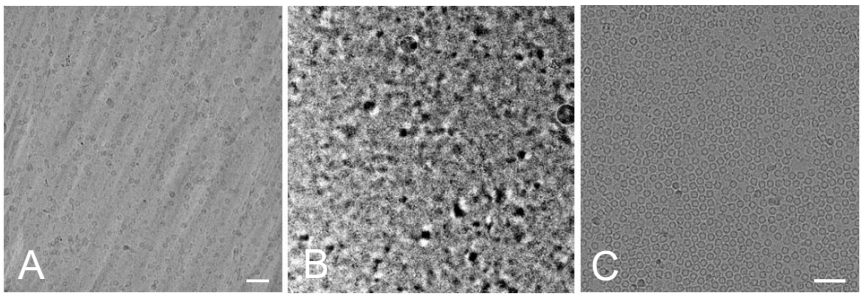

Figure 15 Examples of vitreous and non-vitreous ice in a vitrified grid.

(A) Hexagonal ice not suitable for imaging. (B) Cubic ice not suitable for imaging. (C) Amorphous ice suitable for imaging. Scale bar equals 20 nm for images A and B and 10 nm for image C.

D

Ice quality assessment on the grid and grid square levels

Ice quality assessment on the grid level

The grid overview image (Atlas) shown below indicates various types of grid square quality as a reference for optimal grid square selection. In this Atlas image, the visible differences in the ice thickness gradient are because of the blotting position of the Vitrobot™ MK4 instrument. The thick areas (not optimal) appear as darker areas due to reduced electron transmission.

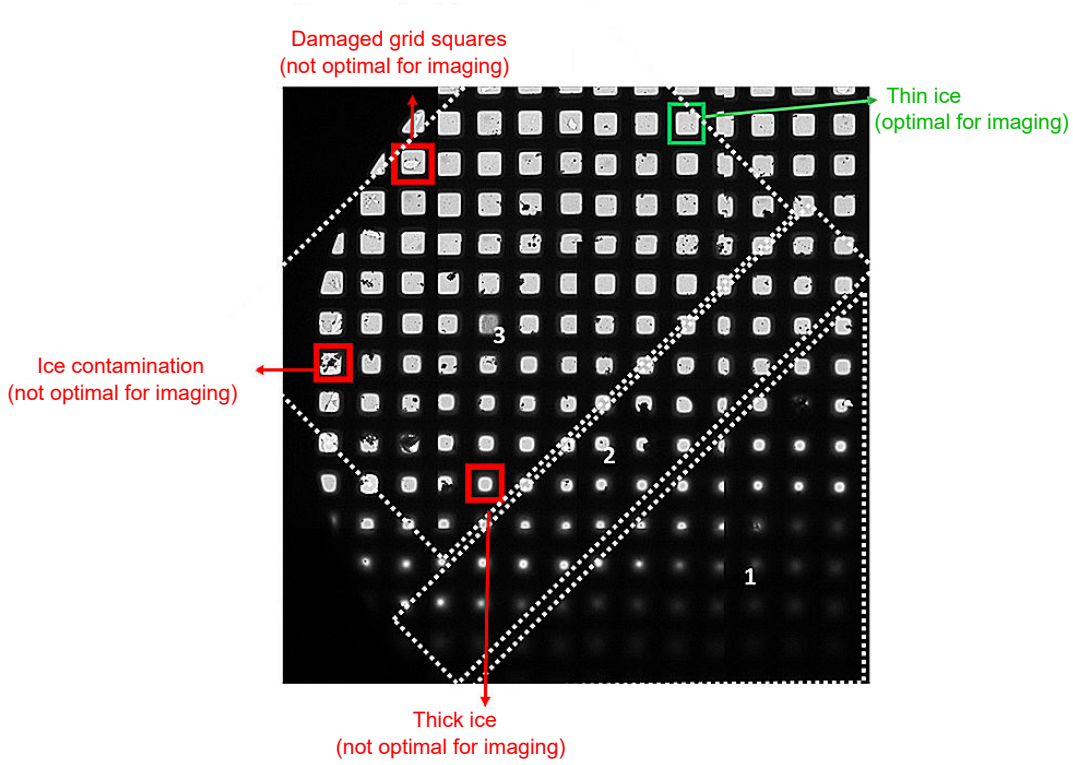

Figure 16 Optimal and degraded grid squares.

Atlas image from a vitrified grid using a Quantifoil™ R1.2/1.3 grid at 500,000x magnification.

Ice quality assessment on the grid square level

The grid squares shown below illustrate variable ice thickness (thin and thick ice, square with the damaged carbon foil, and grid square with surface ice contamination). The optimum grid square for high-resolution data collection is the grid square with thin ice.

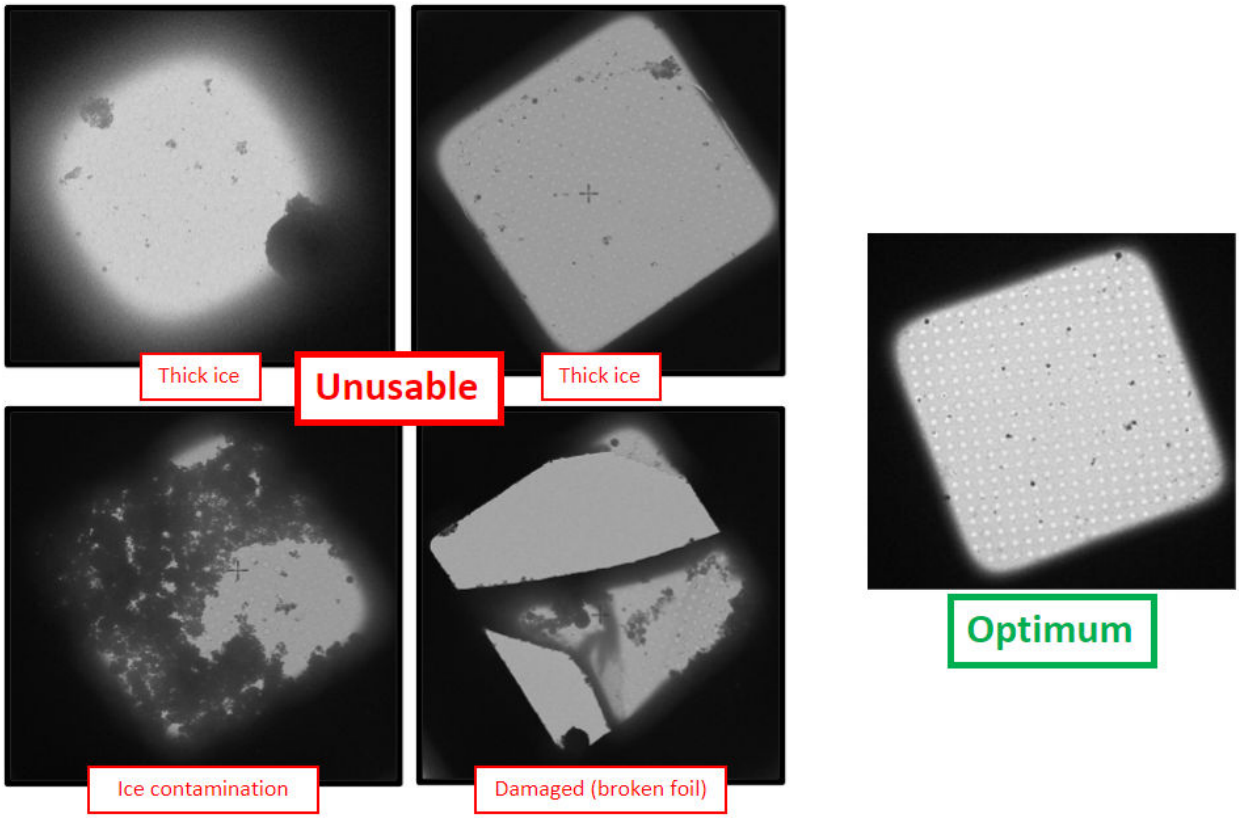

Figure 17 Detailed images of grid squares.

Grid square images from a vitrified Quantifoil™ grid R1.2/1.3.

E

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

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  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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