ANTcryo Technical Note TN-002

Grid Preparation Protocol: Thermo Fisher Vitrobot Mark IV

Field	Value
Product	ANTcryo amorphous Ni-Ti alloy support films (holey films on standard TEM grids)
Instrument	Thermo Fisher Vitrobot Mark IV plunge freezer
Version	1.1
Date	2026-01-01
Status	Released

> This document is part of the ANTcryo technical documentation series. It combines standard cryo-EM grid preparation practice (as documented for Quantifoil holey carbon/graphene oxide grids and the Vitrobot Mark IV) with ANTcryo-specific recommendations. Sources are cited in brackets; ANTcryo-specific notes are marked **[ANTcryo]**. Parameters marked "starting point" should be optimised for the individual sample.

Revision History

Version	Date	Changes
1.0	2025-01-01	Initial release: Vitrobot Mark IV protocol with ANTcryo blot parameters and after-blotting observations
1.1	2026-01-01	§4.3 parameter table streamlined to the ANTcryo standard only (Quantifoil comparison column removed); §8.1 Figure 1 re-laid out as text and image side by side

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1. Materials

- ANTcryo grids (film side is the dull, non-reflective side) **[ANTcryo]**
- Glow discharge / plasma cleaner (e.g. Gatan Solarus, PELCO easiGlow, Quorum GloQube Plus, Harrick Plasma)
- Thermo Fisher Vitrobot Mark IV plunge freezer
- Liquid nitrogen (LN2); ethane, or a 63/37 ethane/propane mixture
- Vitrobot pre-punched blotting paper (Whatman No. 1 or equivalent)
- Vitrobot tweezers and forceps clamp
- Grid storage boxes; cryo gloves and face shield

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2. Grid Storage and Handling

- Store grids in the original grid box, **dark, cool, low humidity**; use within 2 years of production to guarantee surface quality. **[ANTcryo]**
- In humid conditions, keep grids sealed in a dry container and take them out ~30 min before use to equilibrate to room temperature. **[ANTcryo]**
- Inspect grids under a light microscope before use: grids are usable as long as the film is not visibly torn and the hole pattern is intact, even if the grid was briefly blown off by a gas flow. **[ANTcryo]**
- Special note: ANTcryo For Cell grids (MS01/MS03) are shipped pre-hydrophilised and can be used directly; after long storage, give them a short plasma treatment first. **[ANTcryo]**

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3. Glow Discharge (Hydrophilisation)

Glow discharge renders the film surface hydrophilic, which is required for even ice formation and for the sample to stay in the holes. Freshly glow-discharged grids should be used **within 30 minutes** (hydrophilicity decays with time); some protocols recommend within 15 min. [Wang & Zimanyi 2024; Quantifoil HexAuFoil official]

3.1 Recommended settings

Place grids **film side up** on a glass slide (or grid holder) in the glow discharge chamber.

Instrument	Settings (industry standard)	ANTcryo recommendation [ANTcryo]
Gatan Solarus	H2/O2 mix, ~5-10 W (typical)	**H2/O2, 5 W, 30 s** (start)
PELCO easiGlow	20 mA, 30 s per side [STAR Protoc 2020]	**15 mA, 30-60 s** (start at 30 s)
Quorum GloQube Plus	45 s @ 35 mA [Nat Commun 2025]	30-60 s @ 25 mA (start)
Fischione 1070	40 W, 120-180 s, 9:1 Ar:O2, 21 mTorr [Quantifoil HexAuFoil official]	30-60 s @ 25 mA (start)

3.2 ANTcryo-specific notes

- The amorphous Ni-Ti alloy withstands glow discharge well; **time and intensity can be increased if needed**. As a reference, gold-film grids are commonly treated for ~120 s [Wang & Zimanyi 2024]. **[ANTcryo]**
- For a **new batch, first run 2-3 grids** to probe the best settings before committing the sample. **[ANTcryo]**
- Common practice for SPA (PELCO easiGlow): air glow discharge at 20-30 mA for 20 s on carbon grids, 120 s on gold grids, and 5 s on ultrathin carbon-coated grids. [Wang & Zimanyi 2024]

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4. Vitrobot Mark IV Setup

Instrument SOP follows the published Vitrobot operating procedure. [Vitrobot Mark IV User Guide; NIH Methods Mol Biol 2024; STAR Protocols 2022]

4.1 Environmental chamber

Parameter	Typical SPA setting	Notes
Chamber temperature	**4 °C** (SPA typical; up to 25 °C reported)	4 °C for very sensitive samples [Quantifoil HexAuFoil official; NIH Methods Mol Biol 2024]
Relative humidity	**100 %**	90-100 %; keep chamber ports closed when not pipetting

4.2 Before first use of the day

- Mount fresh blotting paper on the blotting pads; let it equilibrate inside the chamber for **at least 5-10 min** before blotting. Replace blot paper after **every 8-10 blots** or when it becomes visibly damp/contaminated. [STAR Protocols 2022; NIH Methods Mol Biol 2024]
- Fill the humidifier with distilled water (~60 ml); fill LN2 into the freezing chamber; condense ethane into the cryogen cup once the chamber is cold.
- Lift the tweezers/ethane pot assembly into position and check that the blotting pads align evenly with a blank grid loaded in the tweezers. Run one test blotting cycle without sample.

4.3 Blotting parameters on the Vitrobot Mark IV (difference vs Leica GP2)

The Vitrobot has a **"blot force" dial** (arbitrary units, 0-30) in addition to blot time and number of blots. The two blotting pads contact the grid from both sides during the blot step.

Parameter	ANTcryo standard **[ANTcryo]**
Blot time	**3 s**
Blot force	**2**
Blot total	**1**
Wait time	0 s
Drain time	0 s

- **Start with the ANTcryo standard: blot time 3 s, blot force 2, one blot.** This has been verified in multiple experiments and gives a controlled breakage rate while producing thin, even ice. **[ANTcryo]**
- If ice is too thick, increase blot time in 0.5 s steps or increase blot force slightly (to 3-4). If ice is too thin / holes are dry, decrease blot time or reduce force. [NIH Methods Mol Biol 2024]
- Because the Vitrobot blots from both sides, the mechanical load on the support film is higher than on the Leica GP2. Keep the blot force low (2-3) as the first optimisation axis rather than jumping to force 10. **[ANTcryo]**

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5. Sample Application

- Use **3-4 µL** of sample per grid (typical). The Vitrobot blot pads are designed for this volume range. [STAR Protocols 2022; NIH Methods Mol Biol 2024]
- Apply the drop onto the film side; do not touch the film with the pipette tip.
- Concentration starting points **[ANTcryo]**:
- **Apoferritin: 1-1.5 mg/ml** as a standard reference (screen 2-3 concentrations if needed)
- For scarce/challenging samples (e.g. membrane proteins): the low-adsorption Ni-Ti surface keeps more of the precious sample in the holes; test a small grid set before scaling up. **[ANTcryo]**
- Optional: incubate the drop on the grid for 20-30 s in the humidified chamber before blotting (adsorption/incubation improves particle density for some samples). [Nat Commun 2025; NIH Methods Mol Biol 2024]

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6. Expected Ice Behaviour (ANTcryo)

- The smooth, uniformly hydrophilic Ni-Ti film favours **even ice of 20-80 nm**, reducing thick/thin ice gradients. **[ANTcryo]**
- Under our standard Vitrobot settings (blot 3 s / force 2 / 1 blot), the success rate for ~20 nm ice is notably higher than on conventional carbon films. **[ANTcryo]**

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7. Plunge Freezing and Transfer

1. Load the glow-discharged grid on the Vitrobot tweezers (film side facing the side you will pipette onto), clamp the tweezers, and mount the assembly on the plunge rod.
2. Raise the tweezers/ethane pot into the humidity chamber and wait for temperature/humidity to stabilise.
3. Apply 3-4 µL of sample through the side port onto the film side.
4. Start the automated blot-and-plunge sequence. The grid blots, then plunges into liquid ethane.
5. After plunging, immediately transfer the grid, while submerged in LN2, from the ethane cup into an open slot of the grid box in the transfer container.
6. Store grid boxes in LN2 until screening/data collection.

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8. ANTcryo-Specific Observations After Blotting

8.1 Grey/black patches on the blot paper

After blotting an ANTcryo grid in the Vitrobot, the blot paper may show grey or black patches (Figure 1). This is a normal observation. The support film is held on the metal grid by van der Waals adhesion. **Occasional minor membrane detachment on individual grids is normal** and does not indicate a defective batch. **[ANTcryo]**

Grey/black patches on the blot paper after blotting an ANTcryo grid in the Vitrobot

Figure 1. Grey/black patches on the blot paper after blotting an ANTcryo grid in the Vitrobot.

> **[ANTcryo] Quality threshold:** If **more than ~10 % of the grids in one box** show obvious membrane detachment (capture a photo of the blot paper for reference), keep the affected grids and photos and

> contact **service@nanodim-world.com** for a one-to-one replacement.

8.2 Membrane damage visible by electron microscopy

Because the Vitrobot blots the grid from both sides simultaneously, the blotting pads exert a relatively strong mechanical force on the support film. The standard ANTcryo support film thickness is **22±3 nm**. Consequently, **some torn or damaged membrane areas are normal** after Vitrobot blotting and do not by themselves justify a product complaint. **[ANTcryo]**

> **[ANTcryo] Quality threshold:** If **50 % or more of the membrane area** on a grid is severely damaged under the microscope, save the EM images and contact **service@nanodim-world.com** for a one-to-one replacement.

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9. Troubleshooting

Symptom	Likely cause	Remedy
Wavy/corrugated square edges	Uneven glow discharge; excessive blot force or blot time	Retune glow discharge; reduce blot force to 2 or below / reduce blot time **[ANTcryo]**
Droplets / thick ice	Humidity too low, or blotting too weak	Check chamber humidity (100 %); increase blot time in 0.5 s steps **[ANTcryo]**
Particles stuck to film, few in holes	Film surface not hydrophilic enough	Re-glow discharge (longer time); check the 30-min window **[ANTcryo]**
Grid damage after blotting	Blot force too high; bent grids	Reduce blot force to 2; some edge damage is normal and does not affect data (1-2 central squares suffice); discard bent grids **[ANTcryo]**
Large membrane areas torn or missing	Normal for Vitrobot double-sided blotting; see Section 8.2	If ≥50 % of membrane is severely damaged, save EM images and contact support **[ANTcryo]**
Poor ice reproducibility between sessions	Humidity/water contamination	Replace blot paper; check humidifier fill; keep ports closed

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10. Sources

- Quantifoil Micro Tools GmbH (SPT Labtech), "Preparing HexAuFoil grids for use": handling, glow-discharge and chamber-condition guidance for holey support films. [Quantifoil official]
- Thermo Fisher Scientific, Vitrobot Mark IV User Guide: chamber operation, blot-pad replacement, and plunge-freezing workflow.
- NIH Methods in Molecular Biology (2024), RNA polymerase complexes by single-particle cryo-EM: Vitrobot Mark IV 4 °C, 100 % RH, wait 0 s, blot 5 s, drain 0 s, blot force 12; 60 mL water in humidifier; 2 × 100 s glow discharge at 15 mA for holey carbon grids.

- STAR Protocols (2022), ligand-gated ion channels: Vitrobot equilibration ~30 min; blot force 2, blot time 3.5 s, 3 µL sample; replace blot paper every ~8 blots; PELCO easiGlow 30 s @ 30 mA.
- STAR Protocols (2020), human 26S proteasome: easiGlow 20 mA 30 s per side; Quantifoil R1.2/1.3 200-mesh Cu; blot force 0, blot time 1.0 s, 2 µL sample per side.
- Kampjut, Steiner & Sazanov, iScience 24:102139 (2021): Quantifoil carbon 0.6/1 or 1.2/1.3 grids (or UltrAuFoil gold 0.6/1), glow discharge in air 2 min @ 7×10^-1 mbar, ~25 mA; Vitrobot blot force 25, blot 4-10 s.
- Wang & Zimanyi, Acta Cryst F 80:74-80 (2024): easiGlow 20-30 mA 20 s (carbon), 20-30 mA 120 s (gold), 10 mA 5 s (ultrathin carbon); use grids within 30 min of glow discharge.
- Nat Commun (2025), Chromera velia PSI structure (10.1038/s41467-025-67637-0): GloQube Plus 45 s @ 35 mA, Quantifoil R1.2/1.3 Cu 300, 3 µL sample, 30 s incubation, 4 °C / 100 % RH.
- ANTcryo support FAQ (nanodim-world.com/support, "Sample Preparation & Handling"); ANTcryo Product Data.

This technical note is provided as technical support. Parameters are starting points; final settings depend on the individual sample and instrument. For batch-specific questions, contact service@nanodim-world.com.