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DATASHEET

Instrumentation and Resources for the Preparation of Cryo-EM Samples

A vital step in the cryo-EM workflow

This datasheet covers the main steps of the cryo-EM single particle analysis (SPA) sample-preparation workflow and provides a comprehensive list of all tools and instrumentation that are recommended for bringing cryo-EM sample preparation to your lab.

A cryo-EM SPA sample is typically a vitrified suspension of biological material consisting of proteins, protein complexes, viruses or other macromolecules.

As sample preparation is a vital step in the single particle analysis workflow, it is important to have a good understanding of the steps involved and to have the right instrumentation. To help you prepare the best possible cryo-EM grids, Thermo Fisher Scientific offers:

- A dedicated sample preparation website
- The Scientific Workflows App (for iOS and Android), which provides step by step guidance through the main steps of cryo-EM sample preparation
- A Customer Success Manager who will provide practical recommendations for sample preparation laboratories
- A full list of instruments required for cryo-EM sample preparation (provided below)
- A comprehensive overview of the sample preparation protocols that have been developed and published (see reference list at the end of this document). This allows you to choose the best protocol based on the type of sample you are working with.

Sample preparation steps

To prepare a cryo-EM sample suitable for high-resolution data collection, the following steps are typically followed:

Biochemistry
Protein expression

Biochemistry
Protein purification and optimization

Biochemistry Screening
Sample quality assessment

Vitrification
Sample preservation

Cryo-EM
Grid screening

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Protein purification

Although the single particle analysis workflow can resolve partial heterogeneity in the sample via 3D classification procedures, it is necessary to biochemically purify the sample in order to obtain a solution containing the isolated target proteins.

Cryo-EM samples are typically prepared using 2-5 µL of 50 nM – 5 µM protein solution, depending on the sample, EM grids, and conditions used for vitrification. For structural studies, the sample should remain active and stable[1] in optimized, *in vitro* conditions (i.e. buffer composition). A suitable biochemical or other functional assay might also be exploited to test the stability and activity[2] of the protein.

The quality of the sample prior to vitrification is critical for the success of later cryo-EM imaging. The ideal protein sample for cryo-EM fulfills the following requirements:

- Very high (>99%) sample purity (a single band in an SDS-PAGE gel)
- Minimal compositional heterogeneity (a single peak in an SEC chromatogram, or a single mass distribution in native mass spectrometry)
- Minimal conformational heterogeneity (i.e. locked in one state or only a few different states)
- Contains very low levels (<2%) of cryo-preservants (e.g. glycerol) and has low ionic strength (<500 mM)

1. Stability is defined by the propensity for the sample to aggregate or disassemble and is relevant for all samples.

2. Activity is applicable only for samples such as enzymes.

Common biochemical/biophysical methods used to assess protein sample composition and homogeneity are:

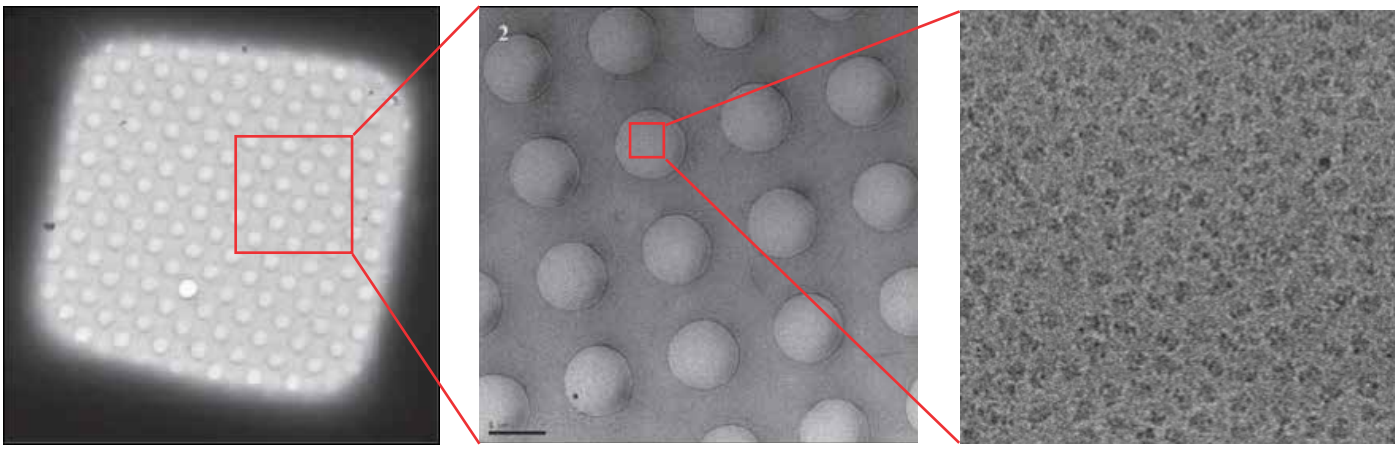
- Polyacrylamide gel electrophoresis (SDS-PAGE)
- Size-exclusion chromatography (SEC)
- Native mass spectrometry (nMS)
- Dynamic light scattering (DLS)

Typically, most of these techniques would be available in regular biochemistry labs. Thus, little or no additional investment would be required for this portion of cryo-EM sample preparation.

Sample quality assessment

An easy and straightforward method to assess the quality of purified biological samples at the microscopic scale is negative stain electron microscopy, which qualitatively assess a specimen's compositional and conformational homogeneity. Often, this assessment is done on a side-entry microscope (e.g. the Thermo Scientific™ Talos™ L120C or F200C TEM) or a semi-automated loading system (e.g. the Thermo Scientific Tundra™ Cryo-TEM) since screening is usually done one grid at a time, and the actual time spent on the microscope is short. Additionally, native mass spectrometry can speed up cryo-EM sample screening and compliments successful sample preparation during vitrification. An application note and webinar are available for more information.

Alternatively, the screening for biochemical quality can be done at cryogenic conditions, effectively combining it with screening for frozen hydrated samples, as described in the next section. Cryo-screening can take advantage of the Thermo



Perfect grid square: homogeneous, clean, thin vitreous ice.

Scientific VitroEase™ Buffer Screening Kit to find the optimal conditions that produce a stable sample in vitreous ice. Note that these conditions can differ from the optimal buffer found using solution methods or negative staining, as they account for additional challenges commonly encountered in cryo-EM, such as preferred particle orientation or protein denaturation at the airwater interface.

Sample preservation

Freezing is essential to cryo-EM as it allows the sample to be compatible with the vacuum of the microscope, locks the individual particles in place, and reduces the radiation damage from the beam. In order to preserve macromolecular structures, freezing must happen quickly enough to avoid crystalline ice formation. This is accomplished by rapidly plunging the grid into liquid ethane; the sample is then kept in liquid nitrogen to preserve the amorphous nature of the embedding ice layer and to avoid damage to the biological particles. The resulting frozen hydrated sample has individual sample molecules embedded and evenly distributed within a thin layer of amorphous (vitreous) ice.

This entire procedure can be simplified using semi-automated plungers such as the Thermo Scientific Vitrobot™ System. Using a set of key parameters (i.e. sample blotting time, blotting force, relative humidity, and temperature) allows for reproducible preparation of high-quality vitrified samples. Thermo Scientific cryo-electron microscopes also use patented AutoGrid sample carriers, which are the industry standard for robust and reliable loading and unloading of cryogenic samples using a robotic sample loader. AutoGrids also enable the seamless interchange of samples between different microscopes in the workflow, so that sample preparation can be performed in a standard biochemistry laboratory. The equipment listed below is recommended for an optimal cryo-EM sample preparation setup.

Vitrobot Mark IV System: state-of-the- art sample preparation unit for cryo-EM.

A typical glow discharge unit.

Reference list:

1. K. Sader, R. Matadeen, P. Castro Hartmann, T. Halsana and C. Schlichten, **Industrial cryo-EM facility setup and management**. *Acta Crystallographica Section D* (2020) D76, 313–325. doi: org/10.1107/S2059798320002223
2. L.A. Passmore, C.J. Russo, **Specimen preparation for high-resolution cryo-EM**. *Methods in Enzymology* (2016), 579: 51-86. doi: 10.1016/bs.mie.2016.04.011
3. Y. Cheng, N. Grigorieff, P. A. Penczek, and T.s Walz, **A Primer to Single-Particle Cryo-Electron Microscopy**. *Cell* (2015), 161(3):438-449. doi:10.1016/j.cell.2015.03.050.
4. R.F. Thompson, M. Walker, C.A. Siebert, S.P. Muench, N.A. Ranson. **An introduction to sample preparation and imaging by cryo-electron microscopy for structural biology**. *Methods* (2016), 100:3-15. doi: 10.1016/j.ymeth.2016.02.017
5. B. Carragher, et al. **Current outcomes when optimizing "standard" Cryo-EM specimen preparation for single-particle cryo-EM**. *Microsci* (2019), 276(1):39-45. doi: 10.1111/jmi.12834
6. I. Drulyte, et al. **Approaches to altering particle distributions in cryo-electron microscopy Cryo-EM specimen preparation**. *Acta Crystallogr. Sect. D, Struct. Biol.* (2018), 74(6):560–571. doi: 10.1107/S2059798318006496
7. L.Y. Kim, et al. **Benchmarking cryo-EM single particle analysis workflow**. *Front. Mol. Biosci.* (2018) 5:1-8. doi: 10.3389/fmolb.2018.00050
8. D. Kampjut, et al. **Cryo-EM grid optimization for membrane proteins**. *iScience* (2021). 24(3). doi: 10.1016/j.sci.2021.102139.
9. P.D.B. Olinares, et al. **Native Mass Spectrometry-Based Screening for Optimal Sample Preparation in Single-Particle Cryo-EM**. *Structure* (2021). 29(2):186-195. doi: 10.1016/j.str.2020.11.001

Recommended equipment for cryo-EM sample preparation

Device	Purpose	Suggested supplier
Vitrobot Mark IV System	Plunge freezing of grids	Thermo Fisher Scientific
Quorum Glocube glow discharge unit/plasma cleaner/carbon evaporator	Preparation of grids • Hydrophilicity of the EM grid support film is achieved and controlled by glow discharge or plasma treatment to optimize the distribution of particles in ice • Provides an additional, optional carbon layer	Mitigen via Thermo Fisher Scientific[3]
ESD soft grip tweezers (electrostatic discharge) 15mm, Extra Fine Tips (or equivalent)	General manipulation of grids prior to freezing	Agar Scientific
Worthington Industries LD4 4 Liter LN2 Dewar	Easy nitrogen pouring	Mitigen via Thermo Fisher Scientific[3]
Taylor Wharton LN2 120l tank with connector and cryohose	Liquid nitrogen storage	Linde Group
Explosive cabinet for ethane, including installation and connection to exhaust	Safety cabinet for ethane cylinder	Asecos via Fisher Scientific
Ethane gas cylinder 20l	Source of ethane for grid plunging	Airliquide
Worthington Industries HC34 High-Capacity Liquid Nitrogen Refrigerator with roller base	Long-term storage for vitrified grids	Mitigen via Thermo Fisher Scientific[3]
Cryo-EM Grid Storage Pucks and Puck System	Organized storage of vitrified grids; fits into HC35	Mitigen via Thermo Fisher Scientific[3]
Foam dewar: 800 mL	Transfer vitrified grids into pucks	Mitigen via Thermo Fisher Scientific[3]

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Recommended equipment for cryo-EM sample preparation		
Device	**Purpose**	**Suggested supplier**
Worthington Industries CX100 Dryshipper and Shipping Case	Plunge freezing of grids	Mitigen via Thermo Fisher Scientific[3]
Digital dry bath with block heaters	Dry cryo-tools during grid clipping and loading	Thermo Fisher Scientific
Pipettes 0.5-10 µL	Apply sample on a grid	Thermo Fisher Scientific
Drying cabinet	Accelerated and thorough drying of small tooling	MilliporeSigma
Liquid nitrogen tipper	Easy nitrogen pouring into dewars	BOC
Accessories provided by Thermo Fisher Scientific, as part of the cryo-electron microscope delivery • C-clip insertion tool (4x) • AutoGrid alignment tool • AutoGrid containers • Grid container box • Auto grid assembly workstation	Small tools for assembling and storing AutoGrids	Thermo Fisher Scientific
Consumables – a starter set will be provided by Thermo Fisher Scientific as part of the cryo-electron microscope delivery • C-clip rings (AutoGrids) • C-clips • EM grids	Source of ethane for grid plunging	Thermo Fisher Scientific
Vitroease Buffer Screening Kit	Sample optimization for optimal cryo-EM grid preparation	Thermo Fisher Scientific

Optional equipment for cryo-EM sample preparation		
Device	**Purpose**	**Suggested supplier**
Thermo Scientific EPU Multigrid Software	Increase screening throughput by automated image acquisition for up to 12 grids on Autoloader TEM systems	Thermo Fisher Scientific
Cryo Tweezer Assembly (Vitrobot Compatible)	Alternative tweezers for the Vitrobot Mk 4 System	NanoSoft via Thermo Fisher Scientific
Igloo (Vitrobot Compatible	Provides a more controlled environment for sample transfer and reduce contamination	NanoSoft via Thermo Fisher Scientific
AutoGrid Inspection Tool	Facilitates inspection of AutoGrids prior to loading into the autoloader cassette	NanoSoft via Thermo Fisher Scientific
Ethane Condenser (Vitrobot compatible)	Increases the efficiency of ethane condensing	NanoSoft via Thermo Fisher Scientific

3. Available as part of a Cryo-EM sample preparation bundle.

All equipment, accessories and consumables are commercially available.

Find out more at **thermofisher.com/em-sample-prep**

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