MANUAL

Jena Bioscience

Cryo-EM V-Kit

Vitrification Starter Kit

MiTeGen
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Cat. No.	Amount
X-CEM-301	1 Kit

For general laboratory use

Storage Conditions: store at -20 °C / ambient temperature

Shelf Life: 12 months

Kit Contents

Surfactants and Cryoprotectants	Amount	Concentration	CMC	Class
Fluorinated Octyl Maltoside (FOM)	100 µl	0.41% (w/v)	0.07% (w/v)	Non-ionic detergent
Hexadecyltrimethylammonium Bromide (CTAB)	100 µl	0.34% (w/v)	0.03% (w/v)	Cationic detergent
n-Decyl-β-D-Maltoside (DM)	100 µl	0.87% (w/v)	0.09% (w/v)	Non-ionic detergent
n-Decyl-α-D-Maltoside (DαM)	100 µl	0.46% (w/v)	0.08% (w/v)	Non-ionic detergent
n-Dodecyl-β-D-Maltoside (DDM)	100 µl	0.09% (w/v)	0.01% (w/v)	Non-ionic detergent
Sodium Deoxycholate	100 µl	1.66% (w/v)	0.17% (w/v)	Anionic detergent
Triton X-100	100 µl	0.15% (w/v)	0.01% (w/v)	Non-ionic detergent
Tween 20	100 µl	1% (w/v)	0.01% (w/v)	Non-ionic detergent
CHAPSO	100 µl	2.5% (w/v)	0.5% (w/v)	Zwitterionic detergent
Amphipol A8-35	100 µl	5% (w/v)		Anionic surfactant
Glycerol	1 ml	30% (w/v)		Cryoprotectant

Quantifoil™ Holey Carbon Films

20x Quantifoil™ R 1.2/1.3 on 200 copper mesh
20x Quantifoil™ R 1.2/1.3 with ultrathin (2 nm) continuous layer of carbon on top on 200 copper mesh
20x Quantifoil™ R 2/1 on 300 copper mesh
20x Quantifoil™ R 2/1 with ultrathin (2 nm) continuous layer of carbon on top on 300 copper mesh

Store the Quantifoil™ grids at an ambient temperature.

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Introduction

Single particle electron cryo-microscopy (cryo-EM) is a powerful technique to determine the structures of protein complexes down to atomic resolution. The sample in solution is flash-frozen in liquid ethane, forming a thin layer of vitreous ice, in which the sample particles are ideally evenly distributed in random orientation far enough from the air-water interface (Fig. 1).

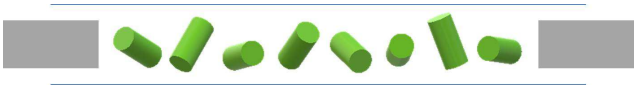

Fig. 1 Schematic cross section of a grid hole with ideally distributed single particles in random orientation

Protein sample vitrification for cryo-EM is still a major bottleneck. Only the combination of choosing the right grid type, vitrification conditions, protein concentration and additives enables high quality data acquisition in cryo-EM.

Description

The Cryo-EM V-Kit offers both a selection of surfactants and cryoprotectants which have been successfully applied in cryo-EM sample preparation and a selection of Quantifoil Holey Carbon Films to facilitate the search for the optimal vitrification condition for both soluble and membrane proteins.

Preconsiderations

A successful specimen preparation in cryo-EM is highly dependent on the protein sample quality, kind of protein, buffer composition, working environment, instruments, and last but not least, the user. The protocol provided here is well-established but might require further optimization and/or amendments by the user. Preliminary considerations should be taken into account for the following points:

Protein Sample

A stable, homogeneous and monodisperse protein sample is the key to success. It has been shown that only high-quality samples lead to good results and ease the process for high resolution structure determination. During sample preparation, a high protein concentration might be useful, since it is better to dilute the sample down to the right concentration and particle density. The final protein concentration can vary from 0.1 up to 15 mg/ml.

It is highly recommended to observe the protein sample quality for homogeneity and concentration using negative stain EM before vitrification. As a rule of thumb, the starting concentration for the optimization of cryo-EM grid preparation is about 2 to 5 times the concentration of a saturated negative stain grid.

Surfactants

In summary, the usage of surfactants can be reduced to two benefits:

1. Ice quality: The protein solution will better wet the grid because it reduces the surface tension. This ultimately leads to an even and thin vitrified ice layer (if the blotting parameters fit).
2. Protein interactions: Proteins tend to adhere to the air-water-interface. This can induce denaturation of parts of the protein and can limit the resolution of the final reconstruction. Surfactants will cover the air-water-interface and therefore prevent proteins from being

exposed to the "deadly" air phase[1-4]. Some proteins also adhere to the carbon foil. Addition of detergents can help to "wash" the protein of interest into the hole of the grid, increasing the number of particles per image, which is beneficial for later data processing.

Surfactants can influence the structure and function of your protein. Therefore, make sure that the used surfactant does not impair your protein by biochemical assays and/or negative stain EM. For membrane proteins, lowering the concentration of the additional surfactant might be necessary.

Fluorinated Octyl Maltoside (FOM) was successfully used to determine high resolution cryo-EM maps[5,6] and is therefore the first choice in optimizing frozen membrane protein specimens.

Grids

Choose a grid type from the kit. In general, start with Quantifoil™ R 1.2/1.3 on 200 mesh and continue to additional carbon grids. After glow discharging, work quickly to have a freshly glow discharged grid for your experiment.

Protocol

Materials

- Stable and homogeneous protein sample
- Cryo-EM V-Kit
- Filterpaper for ThermoFisher Scientific Vitrobot MarkIV (Store at 4°C)
- Reaction tubes

Instruments

- Plunge freezer: ThermoFisher Scientific Vitrobot MarkIV

Note: The applied sample volumes and instrument settings can vary and need to be optimized carefully

- Pipettes and Tips
- Plasmacleaner e.g. GloQube
- Cryo-TEM for cryogenic specimen screening

Sample Vitrification

1. Prepare plunge freezer (follow the manufacturer's protocol), briefly:
   a. Set Vitrobot to 4°C, 100% humidity and activate manual humidity control
   b. Replace blotting paper
   c. Let temperature and humidity settle for 30 minutes
   d. Cool down nitrogen/ethane pot and condensate ethane or ethane/propane mixture
   e. Regularly refill nitrogen to avoid ice contaminations
2. Prepare grid
   a. Plasma clean the desired amount of grids in a plasma cleaner of choice
   b. Fix the grid into the cryoplunger forceps
3. Prepare sample
   a. Dilute the respective screening surfactant (and/or glycerol) into the sample buffer to achieve 2x the final concentration
   b. Mix 5 µL of the 2x surfactant (/glycerol) solution and 5 µL of your sample in a fresh tube
   c. Avoid long incubation
   d. Apply 2 to 4 µL of the final protein/surfactant solution to the freshly glow discharged grid and start the plunge freezing process
   e. As a starting procedure, use 4 s blotting time and a blotforce of 0 to -2
   f. Transfer the vitrified specimen into a storage box and store at nitrogen temperature until the sample can be screened in a cryo-TEM

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Sample Assessment

The most important step to successful cryo-EM grid preparation is the evaluation of the prepared grids. Vitrified specimens should have an even ice distribution, but also a gradient throughout the grid can be useful in finding the right ice thickness. For successful data acquisition, the vitrified specimen should be created under the following guidelines (in macroscopic to nanoscopic order):

1. Carefully handle the grid at all times in order to ensure that it remains flat and unbent. Sharp forceps are a prerequisite and grids should be held at the thick rim of the grid.
2. Acquire an Atlas (EPU), Montage (SerialEM) or a comparable grid overview to assess the overall ice thickness and quality.

Note: The ice thickness of squares can vary from being too thick (black or only a small area is visible) to too dry (visible empty holes or the edges of the square are sharply visible).

3. Thin ice should be evenly distributed within the square (Figure 2).
4. Acquire images at high magnification to check the particle distribution and contrast at different ice thicknesses.

Note: Figure 3 shows a micrograph of a mixture of Apoferritin (Sigma) and TcdA toxin. The overall particle density is too low. In addition, the roundish Apoferritin particles tend to clump together.

5. Perform tomography to assess if particles adhere to the air-water-interface.
6. The optimal ice thickness is a balance between contrast and particle distribution. If the ice is too thin, particles could be omitted from the ice and cluster in thicker ice at the edge of the hole. On the contrary, thick ice can lead to overlapping particles and poor signal-to-noise ratio.

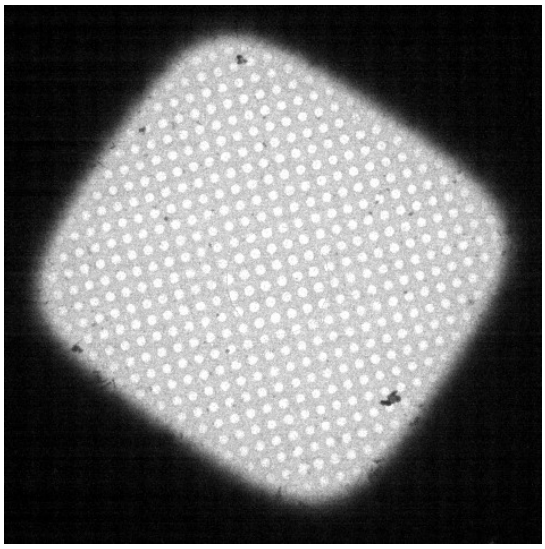

Fig. 2: Typical square overview. The edges are roundish indicating the presence of an ice layer. By comparing the grey values of the holes with an empty area (broken carbon foil for example), the presence or absence of the ice and its thickness can be estimated.

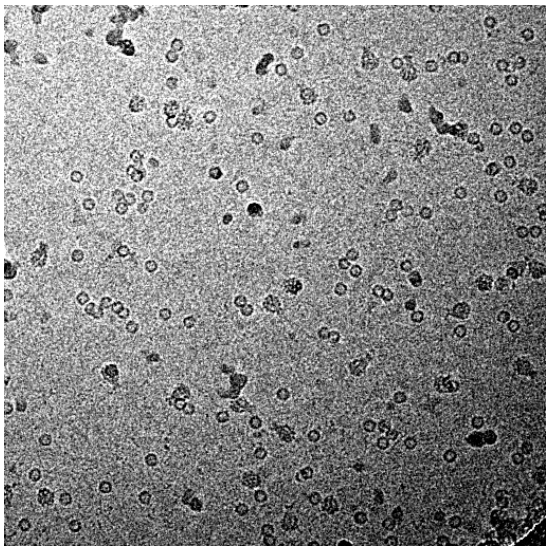

Fig. 3 The electron micrograph shows a vitrified specimen of Apoferritin (Sigma) and TcdA1 toxin from *P. luminescens* (Dept. Structural Biochemistry, MPI Dortmund). The particle density is sufficient to distinguish between the two proteins. However, for data acquisition, the density of single particles should be twice to three times higher. Modern tools to select single particles (Automated particle picker, i.e. crYOLO of the SPHIRE package) can be used to select hundreds of thousands of particles within minutes. High particle densities and even non-homogeneous samples can be picked, because deep learning algorithms are trained to recognize proteins in a vitrified ice layer.

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Optimization

The buffer of a protein sample may be the first thing that can be adjusted. Usually, the buffer needs to be optimized in order to preserve the protein's structural and biochemical integrity. But some components such as high salt, high glycerol or reducing agents, can reduce the contrast and it might be necessary to lower the concentration of the buffer components in order to obtain the best image quality possible.

The major task during the process of optimization is the establishment of a robust and reproducible vitrification protocol. While there is no general approach, a well-established procedure is to stick to a fixed volume of protein sample and blot force, only varying the blotting time. Changing the grid type can help to facilitate thinner ice and ultimately define how many images per hole and holes per square can be acquired. It can occur that only a few squares are usable for data acquisition and therefore a 200 mesh is favorable over a 400 mesh. A small hole size (R 1.2/1.3) can be useful in reducing charging effects, while larger hole sizes (R 2/1) might be useful for proteins that are pushed to the rim of the hole or span the hole like filamentous protein complexes.

Ice quality depends on protein concentration. A thin ice film spanning the hole will only be created if the protein solution has the right concentration. Therefore, it should be optimized in a way that the occupancy in the hole is high, but the particles should not overlap.

If the protein concentration is too low and/or particles have a preferred orientation, the preparation of graphene oxide coated grids is recommended.
Proteins tend to bind to graphene oxide and therefore a lower concentration - compared to negative stain - can be used. In addition, it can help to prevent preferred orientations and facilitate higher quality data.

Selected References

[1] Noble *et al.* (2018) Routine Single Particle CryoEM Sample and Grid Characterization by Tomography. DOI: 10.7554/eLife.34257.
[2] Thonghin *et al.* (2018) Cryo-electron microscopy of membrane proteins. *Methods* **147**:176.
[3] Drulyte *et al.* (2018) Approaches to altering particle distributions in cryo-electron microscopy sample preparation. *Acta Cryst. D* **74**:560.
[4] Glaeser *et al.* (2017) Opinion: hazards faced by macromolecules when confined to thin aqueous films. *Biophys Rep* **3**:1.
[5] Gatsogiannis *et al.* (2016). Membrane insertion of a Tc toxin in near-atomic detail. *Nat. Struct. Mol. Biol.* **23**:884.
[6] Efremov *et al.* (2015) Architecture and conformational switch mechanism of the ryanodine receptor. *Nature* **517**:39.