Sample Preparation

Cp3 Plunge Freezer Protocol: ***Vitrification by plunge-freezing***

Instrument Location (Room G236B, Sample Preparation room)

Following protocol are the instruction guidelines for all user, please go through regular training before using the Cp3 plunger.

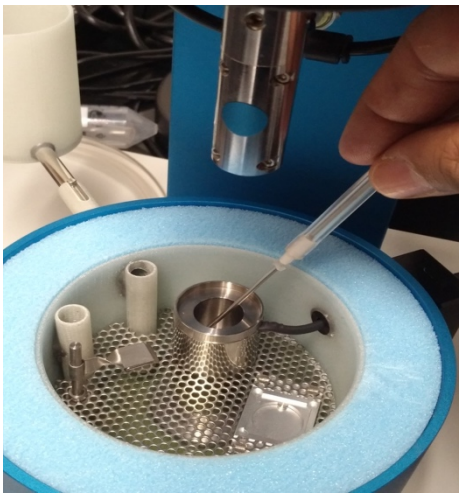
Optimization parameter: **Protein concentration and blotting time.**

Use all precautions while handling cryogenic liquids, Liquid nitrogen and ethane.

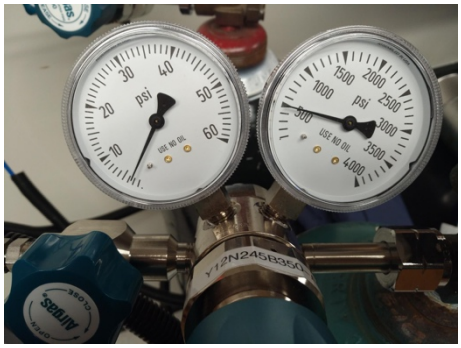
Setup:

CP3 Plunger

Nitrogen Gas Regulator (during operation)



Capillary to condense ethane in ethane chamber



Ethane Gas Regulator (during operation)

1. Turn on power switch on the right side.

2 Open the nitrogen tank (check for nitrogen pressure as marked on the valves, final pressure should be between the marked lines on the regulator: around 60 psi).

3.Test plunger without a sample first by making sure safety shield is in place and pressing 'reset', then 'start'. If there are any problems, please contact members of the core facility.

4.Replace the filter paper using the tweezers provided. (Make sure you put the plastic disc behind the filter paper and don't lose it.)

(Place inverted pin in bottom of filter paper loading station followed by new filter paper and plastic disk. Push down gently with blotter to attach. If you need to make more filter paper disks please ask a staff member, we have instrument to make/cut filter paper)

5.Insert sponge/humidity wand into chamber after wetting with hot (boiling hot)water. You may need to re-wet the sponge few times. Ideally you want the relative humidity to reach 90-92%. Keep the chamber closed all the time.

6. Add fresh, uncontaminated liquid nitrogen to the workstation. When reached temperature of -(-174ºC and is stable, get ready to condense the ethane.

7. Open the Ethane Cylinder regulator and regulate the ethane flow using the two-stage flow regulator at the cylinder, so to have minimum/optimal flow rate as described during the training (Caution: ethane could splash into your eyes and is very dangerous so don't play with it and use eye protection)

Before you start to condense Ethane, make sure you have good liquid nitrogen levels in the workstation, (during this process, Ethane chamber temperature will drop and when chamber is full remove the capillary/tube from ethane chamber holding the tube tightly so not to splash ethane. Close the Main regulator from Ethane cylinder and remove the remaining ethane gas in the tubing.
8. Set Blotting time *(optimization parameter 2-3-XX sec, optimize for the ice thickness*).
9. When set temperature (-174ºC) and relative humidity has been reached, insert cryo plunge freezer tweezers with plasma cleaned grid into plunge rod.
10.Add 2-3µl (*optimize*) of sample to grid using pipette. Rotate plunge rod 90 degrees.
11. Close safety shield, press 'reset' then 'start' to blot sample and plunge into liquid ethane.
12.Open safety shield and raise plunge rod just enough to remove tweezers by pressing blue button but not remove grid from ethane pool. Quickly transfer grid into liquid nitrogen and into cryo grid box.

Shutdown

Procedure:

1. **Lift plunge rod before turning off Cp3.**
2. Turn off nitrogen gas supply from cylinder and recheck ethane cylinder is turned off.
3. Remove sponge wand from chamber.
4. Return tools to where you found them and clean up any messes.

Optimization parameters:

1. Blotting time

In general, one will start with 2-3 sec as blotting time with 2-3 ul of your protein sample.

2. Protein concentration, please follow below chart to find a good starting point (Vinothkumar & Henderson 2016).

Concentration

M.W.	10mg/ml	2mg/ml	0.5mg/ml	0.1mg/ml	20μg/ml
10 kD	48000 (45Å)	10000 (100Å)	2500 (200Å)	500 (450 Å)	100 (1000 Å)
50 kD	10000 (100Å)	2000 (220Å)	500 (400Å)	100 (1000Å)	20 (0.2μm)
250kD	2000 (220Å)	400 (500 Å)	100 (1000 Å)	20 (0.2μm)	4 (0.5μm)
1 MD	500 (400Å)	100 (1000Å)	25 (0.2μm)	5 (0.4μm)	1 (1μm)
5 MD	100 (1000Å)	20 (0.2μm)	5 (0.4μm)	1 (1μm)	0.2 (2.2μm)
25 MD	20 (0.2μm)	4 (0.5μm)	1 (1μm)	0.2 (2.2μm)	0.04 (5μm)

Green color highlights the best concentration to start with.