Cryoplunge™ 3
Plunge-freezing Instrument for Cryo TEM with GentleBlot Technology

Owner's Manual and User's Guide

Model Number 930

Part Number: 930.50501

Revision 03

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Preface

About this Guide

This *Owner's Manual and User's Guide* provides information on the Cryoplunge™3 features and functions, along with procedures for installing and operating the unit. Routine maintenance and troubleshooting procedures are also included in this manual.

Gatan, Inc., recommends following all safety precautions to prevent harm to yourself or the equipment. Please follow all warnings marked on the equipment as well.

The following typographical conventions are used for special comments:

CAUTION: Documentation must be consulted in all cases where this symbol is marked.

IMPORTANT: For Regulatory Compliance and Safety information and instructions please refer to the *Regulatory Pamphlet* provided with this product. Review this document in full before installing and operating this product.

Disclaimer

Gatan, Inc., makes no express or implied representations or warranties with respect to the contents or use of this manual, and specifically disclaims any implied warranties of merchantability or fitness for a particular purpose. Gatan, Inc., further reserves the right to revise this manual and to make changes to its contents at any time, without obligation to notify any person or entity of such revisions or changes.

Copyright and Trademarks

The text and graphics are for the purpose of illustration and reference only. The specifications on which they are based are subject to change without notice.

Returns

In the event that the instrument must be returned to the factory, please request a Returned Materials Authorization (RMA) number via email: gatan-rma@gatan.com. This RMA number must appear on all shipping documents to ensure that proper actions will be taken to repair or replace the instrument.

Support and Customer Service

Gatan, Inc. provides free technical support via phone, fax, and electronic mail. To reach Gatan technical support, contact the facility nearest you, or send an email to service@gatan.com or info@gatan.com.

Please consult the Customer Service section of the Gatan website at www.gatan.com for the latest contact information.

Important Safety Instructions - Liquid Ethane

- Cryoplunge3 makes use of liquid ethane.
- To ensure your safety, ethane gas must **only** be ordered in consultation with the Laboratory Services Manager and site Safety Officer. Please follow your laboratory and site Safety Officer's recommendations for storage and use.
- The size of ethane gas cylinder should be restricted to "lecture theater" size or the smallest size that is available for the purity rating required (at least CP grade, which is 99% pure) fitted with a 2-stage regulator that is suitable for use with ethane.
- Always check the ethane cylinder for any signs of corrosion prior to use.
- Liquefaction of ethane must only be carried out using purpose-built equipment designed to be used in conjunction with liquid nitrogen as the primary coolant.
- The ethane should be condensed in an explosion-proof (spark-proof) fume hood.
- The ethane cylinder should be supported in an approved stand within the fume hood.
- The fume hood must not contain any flammable or harmful substances and should be clear of any unnecessary equipment that could impede air flow. Other sources of ignition such as electrical equipment (including ovens) must also be removed from the fume hood before using ethane.
- The flow of ethane gas through the liquefaction apparatus must be so that only liquid ethane is produced, thus avoiding any unnecessary venting of ethane gas into the fume hood. **Use caution in high-flow fume hoods as they tend to draw the ethane out of the hood before it can become condensed into liquid form.**
- To ensure your safety, do not condense more than 10 mL of liquid ethane at one time.
- When sufficient ethane has been liquefied, the ethane cylinder valves should be closed and the 2-stage regulator should be drained of any residual ethane.
- The liquid ethane must be maintained at near liquid nitrogen temperature to prevent boil off into the atmosphere.

- The workstation of Cryoplunge3 incorporates a liquid nitrogen bath that maintains the liquid ethane at an operating temperature just above its melting point.
- The boil off of the liquid nitrogen produces an inert blanket of gas over the ethane, preventing oxygen from the atmosphere condensing on the surface of the ethane to form an explosive mixture. The liquid ethane must be kept covered by nitrogen gas at all times that are practicable to prevent dangerous oxygen/ethane mixtures from forming. Maintaining the level of liquid nitrogen within the workstation of Cryoplunge3 ensures that a layer of nitrogen gas will enshroud the ethane vessel at all times.

Equipment Ratings and Technical Specifications

Power is applied to the units via a line cord connection. Grounding is provided through this main input connection using a make-first-break-last method.

Electrical Ratings and Specifications

- Input Voltage = 100-240V~
- Input Power = 1.35A
- Frequency = 47-63Hz
- Output Voltage = 24VDC
- Output Current = 2.08A max.

NOTE: Use only with a Gatan-specified power supply.

Environmental Storage/Shipping/Operating Conditions

- Non-operating relative humidity (non-condensing) 25–85%
- Storage Temperature Range 0–33°C
- Operating Humidity Range 0–100%
- Operating maximum thermal gradient 15°C/hr.
- Operating temperature range (ambient) 4–24°C
- Ventilation: Do not block or cover ventilation holes

Assembly, Location, and Mounting Requirements

Prior to Unpacking the Instrument

The carton comprising the shipment should be inspected for any signs of damage before unpacking. If any severe damage is visible, the following procedures should be carried out:

- Photograph the extent of the damage. Digital photographs sent by email are often the best method (Gatan, Inc. info@gatan.com).

Describe the extent of the damage to Gatan, Inc. to estimate possible damage to the enclosed equipment and decide whether an insurance assessor and engineer need to be present before further unpacking.

Installation

Cryoplunge3 is intended only for use in laboratory applications and is designed specifically for workstation (table top) installations. The product is intended for operation in a controlled work environment, laboratory, or clean-room environment. It is recommended the unit not be blocked from general room ventilation.

Connection to the Power

Prior to plugging the unit in, read the *Regulatory Pamphlet* provided with this product and ensure the power switch on the unit has been turned off (**I** refers to **ON**, and **O** refers to **OFF**). Apply all applicable connections detailed above. Use only the power cord provided with this product and proper electrical power as specified on the equipment rating label. Ensure the outlet is equipped with a proper ground pin. A separate ground wire is required for connection at the main PE terminal at the back of the main column and designated by the IEC 60417-5019 symbol  . Should there be any questions as to how to connect the ground, contact a licensed electrician.

When power is applied to unit, the meters on the front panel illuminate, indicating that the unit is on.

If Cryoplunge3 is going to be moved or stored, close the pneumatic supply (nitrogen gas cylinder or house air supply connection) and vent any residual pressure in the pneumatic lines by first ensuring that the plunge piston is in its full upward (firing) position and then repeatedly pressing the **RESET** push-button. Disconnect the pneumatic supply line from Cryoplunge3. Switch the **ON/OFF** switch to **OFF** and unplug the mains connection.

Instructions

Overview

Cryoplunge™3 with GentleBlot™ technology is a semi-automated plunge freezing instrument that is used for preparing frozen hydrated specimens for cryoEM. GentleBlot technology optimizes the blotting force and pressure to provide gentle 1-side or 2-side blotting of the specimen grid to minimize breakage of even the most fragile specimen supports.

Preparing frozen hydrated specimens using Cryoplunge3 with GentleBlot technology is easy for novice or experienced cryo electron microscopists and provides excellent results. A small volume of the specimen is applied to the specimen grid, which is attached to plunging tweezers. The grid is loaded within the humidity chamber and gently blotted to produce a thin, aqueous film. The specimen grid is then plunged into a temperature-controlled ethane bath. This results in instantaneous freezing of the specimen in a layer of non-crystalline (vitreous) ice. One press of the quick disconnect allows the operator to quickly and easily disconnect the plunging tweezers from the plunge rod. The liquid nitrogen workstation provides a protective cryo environment to minimize contamination of the frozen hydrated grid during transfer to a pre-cooled cryo grid storage box within the workstation. The cryo grid storage box can then be safely transferred to a liquid nitrogen storage vessel or to the workstation of the cryo transfer holder for low dose imaging on the TEM.

Features

Easy Installation

- Everything is included for fast, easy set-up
- Light weight and portable
- Small footprint

Safe Operation

- Safety interlocks protect the user during the blotting and plunging cycle

Humidity Chamber

- Safeguards the specimen from drying during the blotting process
- Temperature/humidity metering within the specimen blotting chamber
- 98% RH within ~15 minutes
- 3 entry points for specimen loading (right, left, and front)

Specimen Blotting

- 1- or 2-side
- Single and multiple blotting
- Blot override capability
- Filter paper loading jig to minimize contact with filter paper while loading onto GentleBlot blotters
- Electronic blotting timer accurate to 0.1 sec.
- Interchangeable GentleBlot blotters
- Adjustable blotting pressure via GentleBlot blotters
- Optimal factory set GentleBlot pneumatic force
- Any filter paper type available can be used

Plunge and Tweezer Securing Mechanism

- Pneumatically assisted, multi-positional plunge piston
- Plunge speed 1.7 m/sec.
- Quick disconnect tweezer-mounting mechanism

Cryo Workstation

- Removable liquid nitrogen cryo workstation allows transport to and from explosion-proof (spark-proof) fume hood for filling and disposal of liquid ethane
- Long hold, temperature-controlled ethane pot
- Cryo grid box receptacle (round and square cryo grid boxes)
- Filter paper blot stand, kept at the temperature of the FH grid within the workstation, allows blotting excess ethane for immediate viewing on the TEM
- External funnel for filling workstation with liquid nitrogen
- Workstation covers protect frozen hydrated grid during transfer from ethane to cryo grid storage box
- Liquid nitrogen cryo grid box transfer pot (allows cryo grid box to be moved to a cryo holder workstation or a liquid nitrogen storage dewar while submerged under liquid nitrogen)

Maintenance

- Preventative maintenance plan is available

Description and Specifications

The Cryoplunge3 is a self-contained unit, as shown in the following images.

NOTE: It might be helpful to print these images and use them as a guide when reading the instruction manual.

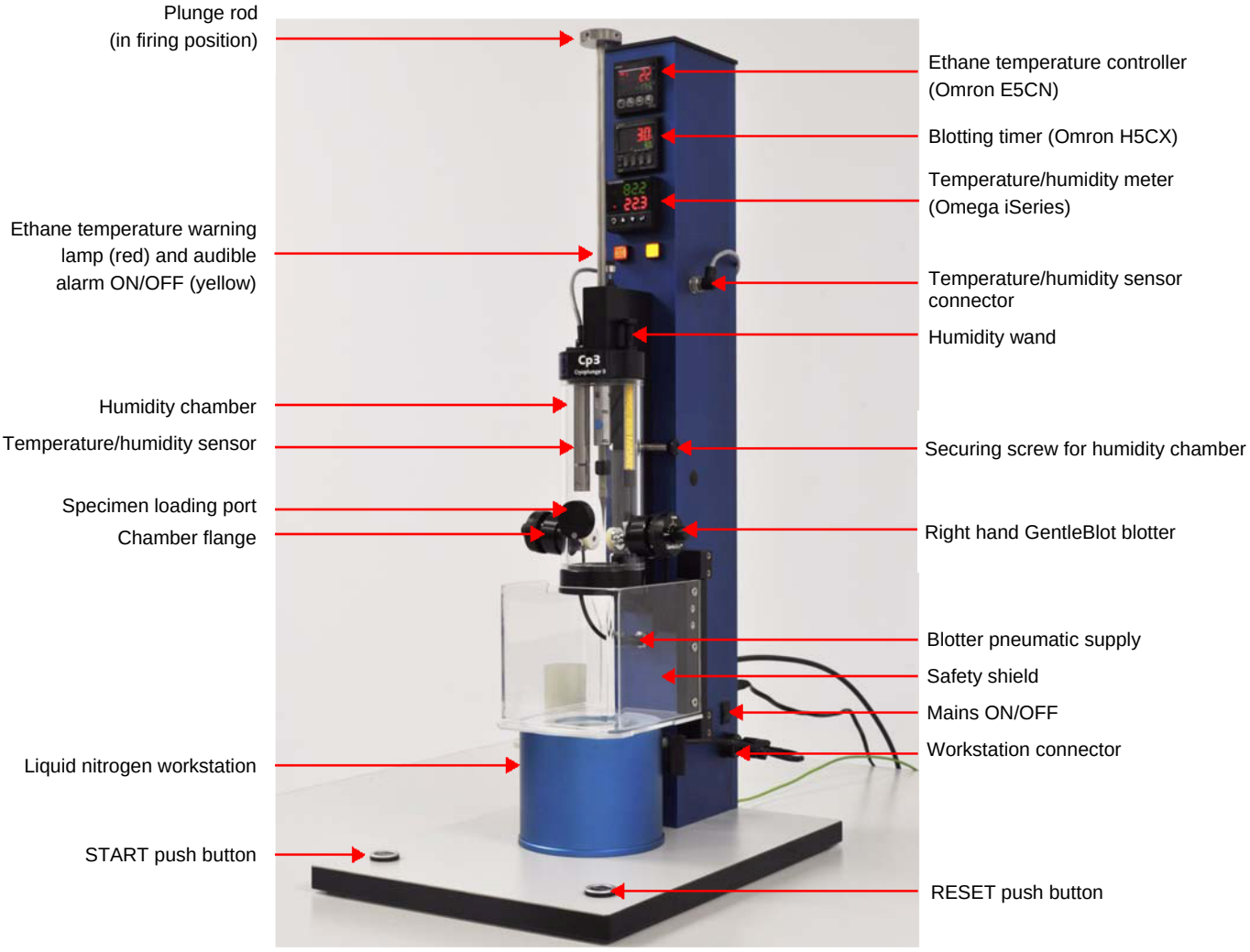


figure 1-1. Cryoplunge3 front view

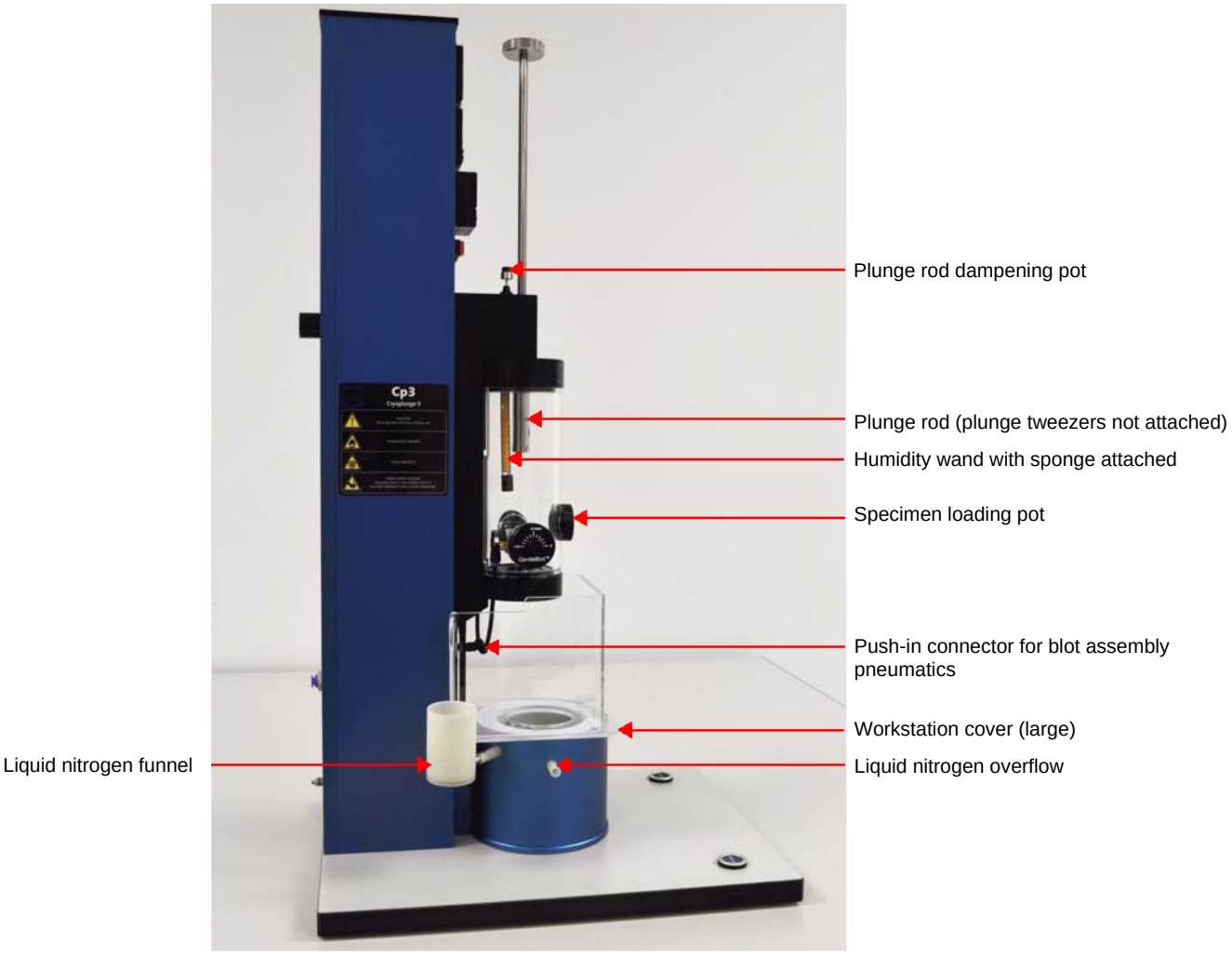


figure 1-2. Cryoplunge3 side view left

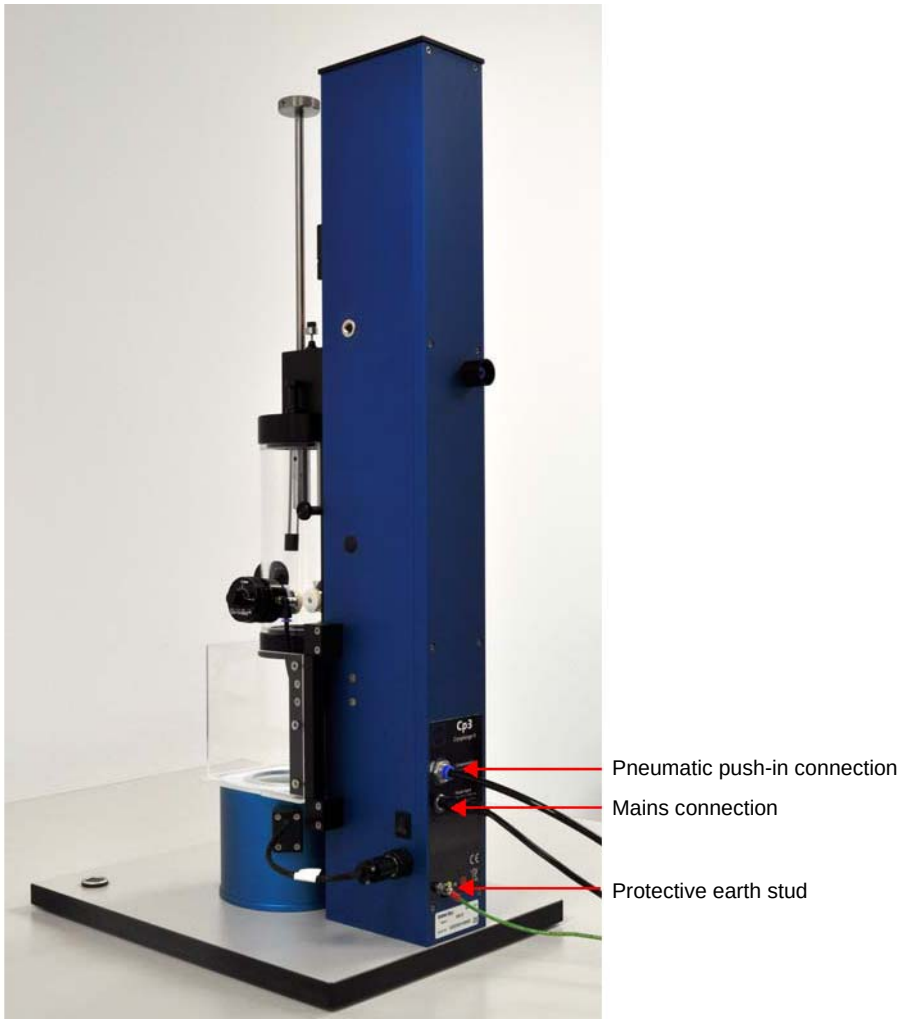


figure 1-3. Cryoplunge3 side view back and right

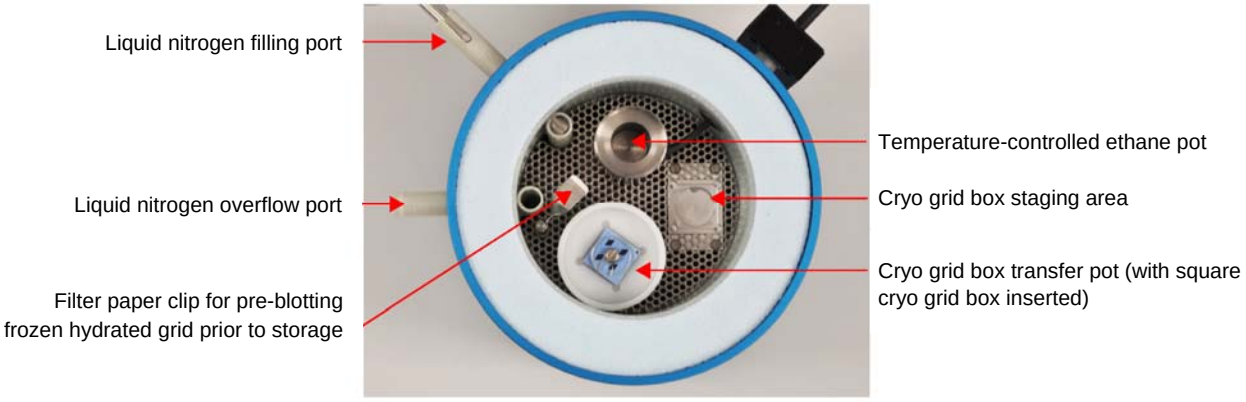


figure 1-4. Workstation

Physical Specifications

Specification	Value
Weight	12.25 kg
Dimensions (length x width x height)	41 L x 31 W x 75 H (centimeters)
Power input	24V ⎓ 2.08 max
Total weight Cryoplunge3 and shipping crate	58 lb.
Shipping crate dimensions	102 L x 64 W x 48 H (centimeters) 40 L x 25 W x 19 H (inches)

Gas Requirements and Connectors for Pneumatics

Cryoplunge3 requires a pneumatics supply. The high-pressure tubing connection to the pneumatic inlet at the back of the instrument is a 6mm (outer diameter) “push-in” fitting.

- It is recommended that a nitrogen gas cylinder is used to provide the pneumatic supply to operate Cryoplunge3. A compatible 2-stage regulator should be fitted and the second stage of this regulator must be capable of delivering 100 psi (7 bar) of pressure to achieve the operating pressures for Cryoplunge3; 70 psi (4.8 bar). Two thread to push-in fittings for the second stage of the 2-stage regulator are provided. Choose the one with the proper diameter that fits your regulator (1/4 inch or 1/2 inch are provided with Cryoplunge3).
- Alternatively, a dry, oil-free compressed air supply that can deliver 70 psi (4.8 bar) of air pressure output can also be used.

Preparing the Nitrogen Cylinder and 2-Stage Regulator

NOTE: The 2-stage regulator (not provided) must be suitable for use with nitrogen gas. The second stage of the regulator should be able to deliver 100 psi (7 bar) with an outlet fitting (1/4 inch or 1/2 inch) that accepts one of the thread to push-in fittings provided. The following instructions are intended as guidelines.

1. If you are unsure as to how to connect a 2-stage regulator to a compressed gas tank, please seek assistance from your on-site Safety Officer or laboratory advisor.
2. The tank must be secured to a wall, stand, or cart using a safety chain so that it cannot tip over during use.
3. The main tank valve should be closed (as indicated on the top of the valve).
4. Prior to attaching the 2-stage regulator to the tank, make sure the mating surfaces are clean and free of any particulate matter.
5. Wrap the threaded end of one of the thread to push-in fittings with Teflon tape and attach to the exit port of the second stage of the regulator.
6. Press one end of the pneumatic tubing (provided) fully into the thread to push-in fitting. There is an o-ring within the fitting that the tubing must go through. When fully inserted, check by pulling the tubing away from the fitting. If the tubing does not disengage from

the fitting, it is properly installed. The opposite end of this tubing fits into the push-in fitting on the back panel of Cryoplunge3.

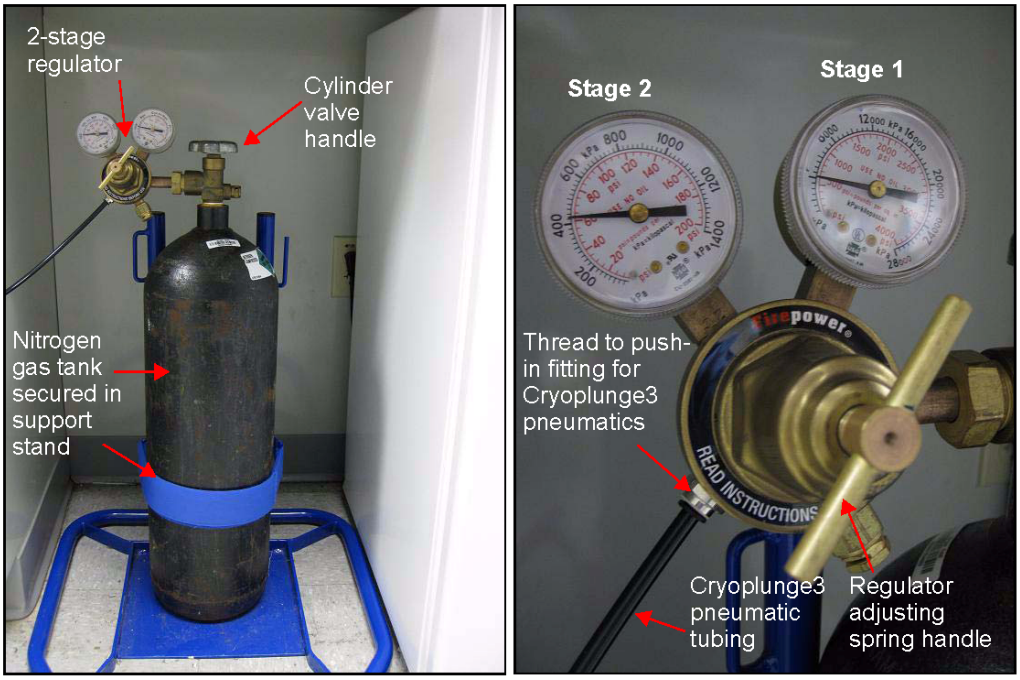


figure 1-5. Pneumatics supply for Cryoplunge3 (left), 2-stage nitrogen regulator (right)

Cryogenic Requirements and Safe Handling

- Liquid nitrogen: Please refer to the latest MSDS on the world wide web for safe handling information.
- Liquid ethane: Please refer to the latest MSDS on the world wide web for safe handling information.

Preparing the Ethane Cylinder and 2-Stage Regulator

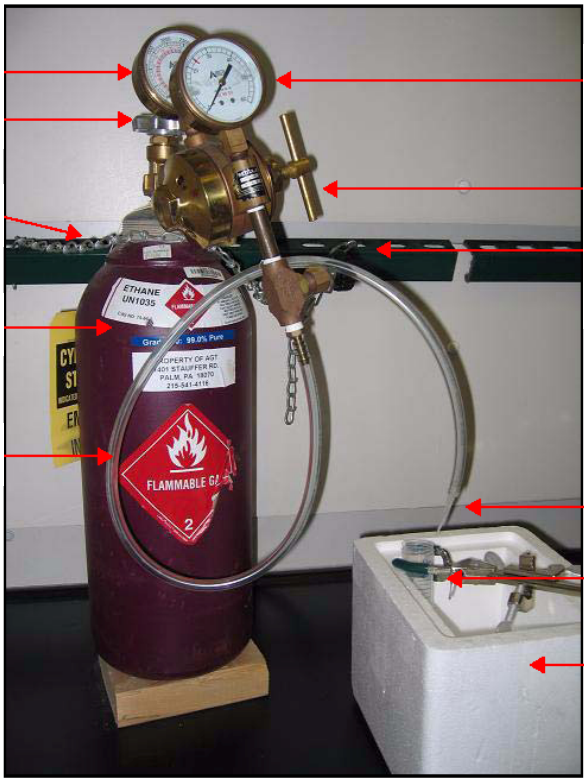


figure 1-6. Ethane tank and regulator

NOTE: The 2-stage regulator (not provided) must be suitable for use with ethane gas. The second stage of the regulator should be able to deliver 20 to 40 psi (~2 to 3 bar) with an outlet fitting that accepts narrow bore Tygon tubing (not provided).

Preparing the Ethane Cylinder and Regulator

1. Locate the ethane tank in an explosion-proof (spark-proof) fume hood
2. If you are unsure as to how to connect a 2-stage regulator to a compressed gas tank, please seek assistance from your on-site Safety Officer or laboratory advisor.
3. The tank must be secured with a safety chain so that it cannot tip over during use.
4. The main tank valve should be closed (as indicated on the top of the valve).
5. Prior to attaching the 2-stage regulator to the tank, make sure the mating surfaces are clean and free of any particulate matter.
6. Wrap the regulator's threaded mating surface with Teflon tape and screw securely into place on the ethane tank.
7. Close the outlet valve on the second stage of the regulator and open the second stage gas pressure regulating adjusting spring handle fully (i.e., no gas pressure to the second stage).
8. Affix a short length (~60 to 90 cm) of narrow bore flexible tubing (Tygon, for example) to the outlet port on the second stage of the ethane regulator so that it can reach the vessel within which the ethane will be condensed.

9. Attach a small pipette tip to the other end and cut approximately 3 mm to widen the opening at the tip of the pipette.

Leak Testing the Connections

1. Open the main tank valve. This fills the regulator with gas but no gas should escape through the flexible tubing because the outlet valve of the 2-stage regulator is closed from the previous step above. The first stage of the regulator indicates the pressure of the gas coming directly from the tank.
2. Using a dilute soap solution, leak test the fitting between the ethane tank and the 2-stage regulator to make sure that the connection is tight and that no gas is escaping from this connection (if gas is escaping at the connection, bubbles form at the connection site). If this occurs, close the main tank valve, tighten the connection, and then repeat the procedure until the connection no longer indicates a leak.

Adjusting the Gas Pressure

1. Make sure that the outlet valve of the second stage of the regulator is closed so that no gas can escape.
2. Open the main tank valve.
3. Adjust the pressure of the second stage of the ethane regulator to deliver the ethane at approximately 2 to 4 psi (0.14 to 0.28 bar) by turning the regulator adjusting spring handle clockwise.
4. At this point, the system is now ready to use for condensing ethane. However, if you are not going to immediately condense ethane for freezing your samples, the ethane tank and regulator should be left in “stand-by” mode.

Leaving the Ethane Cylinder in Stand-by Mode

Leave the ethane tank in “stand-by” mode until you are ready to condense ethane for freezing your samples. When the ethane is not being used, always close the main tank valve after use and drain the regulator of any residual ethane by following these steps:

1. Close the main tank valve
2. Open the outlet valve of the second stage of the regulator to bleed off any gas remaining in the regulator and the flexible tubing.
3. Fully open the regulator adjusting spring handle for the second stage of the regulator (turn counter clockwise until loose but not disconnected from the regulator).
4. Close the outlet valve of the second stage of the regulator.

Important Safety Precautions

- The volume of the ethane pot for Cryoplunge3 is 4.1 mL to the upper rim of the pot.
- During use, do not condense more than 10 mL of ethane at one time.
- Review the latest MSDS available on the world wide web for ethane.

Installation and Setup of Cryoplunge3

Unpacking and Inspection

NOTE: Do not discard the shipping container. Keep all packing materials.

The shipping container has been specifically designed for Cryoplunge3 and must be used in the event that the instrument must be returned to Gatan, Inc. for repair.

figure 1-7. Cryoplunge3 in the shipping crate

Removing Cryoplunge3 and Accessories from the Shipping Container

NOTE: All components of Cryoplunge3 are located in one shipping container.

To unpack Cryoplunge3:

1. Cut the two securing straps that surround the outside of the box.
2. Turn the lid locks (as indicated on the locks) to release the cover.
3. Open the cover. Cryoplunge3 and all accessories are packaged within several layers of sturdy foam.
4. Remove the layers of foam packing material to expose the contents within the packing crate.
5. Remove the accessories box and the workstation.
6. Remove the additional layers of foam packing material to fully expose Cryoplunge3.
7. Remove Cryoplunge3 by carefully and gently lifting it out of the box and placing it in the designated area where it will be used (for example, a laboratory bench top).
8. Remove all accessories.
9. Replace all of the foam layers.
10. Close the box, lock the lid, and store the shipping container in a suitable location. Do not discard **the shipping box.**

Matching the Contents against the Packing List

Make sure that these items are included in the box:

- **Cryoplunge3**
- **Instruction manuals**
  - Cryoplunge3 instruction manual
  - Digital blotting timer instruction manual (Omron H5CX)
  - Digital thermohygrometer instruction manual (Omega iSeries)
  - Digital temperature controller manual (Omron E5CN)
- **Regulatory Pamphlet**
- **Cryoplunge3 accessories kit**
  - GentleBlot blotter O-ring (2-004 Buna)
  - Tubing for pneumatic supply (5 meters)
  - 1/8 inch NPT, thread to push-in fitting, 6 mm
  - 1/4 inch NPT, thread to push-in fitting, 6 mm
  - Standard workstation (Cryoplunge3 pot assembly)
  - Liquid nitrogen filling funnel
  - Humidity wand (to retain cellulose sponges)
  - Humidity wand sponge
  - Chamber assembly
  - GentleBlot blotters (2)
  - Cryoplunge3 tweezers (2)
  - Filter paper pin pack (pack of 10)
  - Filter paper pack of 100 disks (Whatman #1)
  - Backing, plastic disk (pack of 20)
  - Blotter cushioning foam (pack of 20)
  - Cryo grid box (square)
  - Grid box handling rod
  - Grid transfer/storage tube
  - Conical tube for condensing ethane
  - Cryo grid box transfer pot
  - Filter paper loading tweezers
  - Tygon tubing for workstation overflow port (1 meter)
  - Blotter blanking plug
  - Workstation cover (small)
  - Workstation cover (large)
  - Filter paper loading jig
  - Blot pad spindle wrench

Installing and Testing Cryoplunge3

1. Place Cryoplunge3 onto a stable work surface (away from drafts and open flames) and oriented so that you can easily see the connection points at the back and right-hand side of the instrument.
2. Suggested supply for the pneumatic operation of Cryoplunge3 is a clean, dry nitrogen source.
   - **If using nitrogen gas**, ensure that the nitrogen tank is secured (follow local safety regulations for securing the tank) and that the main tank valve is closed before proceeding. Operating pressure for Cryoplunge3 is 70 psi (4.8 bar) of pressure.
   - If using house air supply, ensure that the supply valve is closed before proceeding. Operating pressure for Cryoplunge3 is 70 psi (4.8 bar).
3. Connect high-pressure tubing to the quick disconnect fitting for the pneumatic supply on the back panel of Cryoplunge3.
   - Simply press the end of the tubing fully into opening (see red arrow in image). There is a slight resistance as the tubing goes through the o-ring within the fitting.
   - Connect the opposite end of the high-pressure tubing to the thread to push-in fitting on a 2-stage nitrogen regulator or to the outlet for the house air supply. The connection to the to the thread to push-in fitting on the 2-stage nitrogen regulator was described previously in “Preparing the Nitrogen Cylinder and 2-Stage Regulator.”

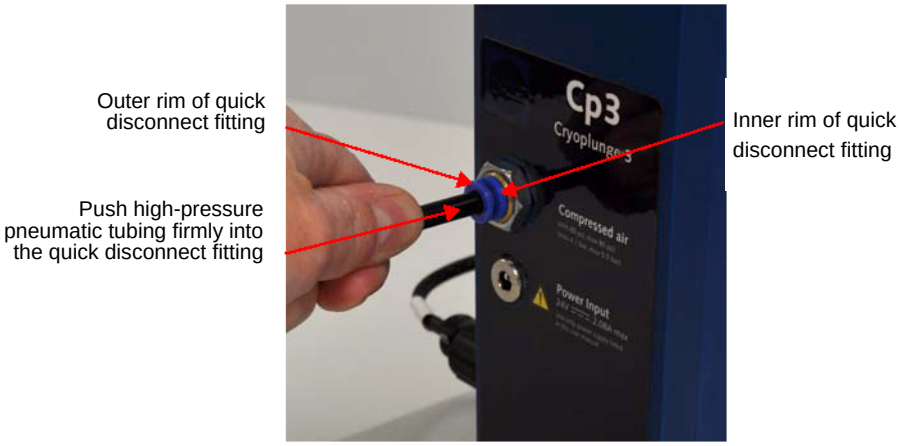


figure 1-8. Pneumatic connection

NOTE: In the event that you need to remove the tubing, always make sure the main nitrogen tank valve or house air valve is fully **off**, and while pressing the outer rim of the quick disconnect fitting in toward the inner rim of the fitting, pull the high-pressure tubing out. There will be slight resistance from the o-ring connection located within the fitting. Upon disconnecting the high-pressure tubing, the residual gas in the tubing is released.

4. Connect the protective earth cable to the protective earth post at the back panel of Cryoplunge3 and attach the opposite end to a ground source.
5. Connect the power supply to the mains cable.

6. Connect the cable to the mains receptacle on back of Cryoplunge3

figure 1-9. Pneumatic, mains, and protective earth connections

7. Connect the plug to the house electrical supply.
8. Orient Cryoplunge3 to a comfortable working position on the laboratory bench.
9. If using nitrogen gas for the pneumatics, make sure that the second stage of the regulator is not pressurized at this point.
10. Open the main tank valve on the nitrogen gas tank and note pressure on the first stage of the regulator. This is the tank pressure.
11. Adjust the second stage pressure to 70 psi (4.8 bar) by turning the regulator adjusting spring handle clockwise.

NOTE: Make sure that all connections are secure to avoid personal injury. When fully pressurized, the high-pressure pneumatic tubing carries 70 psi (4.8 bar) to Cryoplunge3. **As a safety precaution, it is recommended that** the **remainder of the tubing is secured to a stationary object.**

12. Place the liquid nitrogen workstation onto the base of Cryoplunge3, and align the groove at the base of the workstation with the workstation guide plate. Slide the workstation onto the guide plate until it stops. When the workstation is pressed fully into position, the safety interlock is engaged.

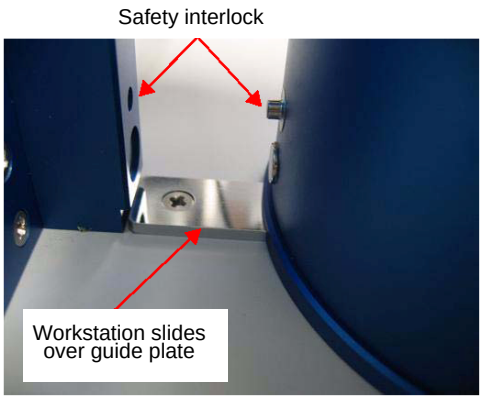


figure 1-10. Safety interlock for workstation

NOTE: There are two safety interlocks on Cryoplunge3: one is on the safety shield and the other is on the liquid nitrogen workstation.

13. Place the large cover over the top of the workstation with the detent for the small cover to the right. Place the small cover on top of the large cover so that it engages the detent in the large cover. With the small cover in place, the safety shield cannot close. This is done purposefully so that you cannot accidentally plunge the tweezers into the small cover.
14. Connect a piece of Tygon tubing (provided) to the overflow port of the workstation so that it extends beyond the edge of the base plate.

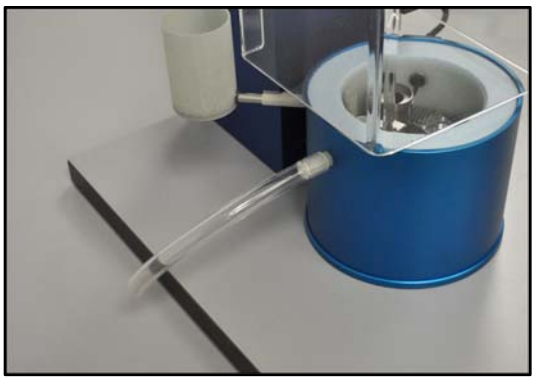


figure 1-11. Workstation overflow port with Tygon tubing attached

15. Connect the workstation connector to its receptacle on the back right-hand side of Cryoplunge3 (there is only one orientation for this connector).
16. Insert the Omega temperature/humidity sensor into the large hole on the top left-hand side of the humidity chamber. Insert the electrical connection for the sensor into its receptacle on the right-hand side of the column for Cryoplunge3. The connector for the sensor is configured so that it can only be inserted in one orientation.

figure 1-12. Insertion of Omega temperature/humidity sensor

figure 1-13. Omega temperature/humidity sensor connector

17. Insert the humidity wand into the small hole on the top right-hand side of the humidity chamber.

figure 1-14. Inserting the humidity wand

18. Remove the small workstation cover and fully close the workstation safety shield. This engages the magnetic safety interlock. In this position, the plunge rod can fire into the ethane pot.

NOTE: If the safety shield is open, pressing the **RESET** push button closes the shutter at the base of the humidity chamber. If the **START** push button is pressed when the safety shield is open, the blot pads activate, but the plunge rod does not fire into the ethane pot.

19. The instrument is delivered with the plunge rod in its full upward (firing) position (i.e., it is positioned within the humidity chamber of Cryoplunge3).

20. Place the right and left-hand blotters into their receptacles on the humidity chamber.

21. Switch mains ON:

- The meters for ethane temperature, blot time, and temperature/humidity illuminate.
- If the ethane alarm sounds, press the yellow **ethane ON/OFF** button on the main support column of Cryoplunge3 to silence the alarm.

NOTE: There is one audible alarm and two visual alarms for the ethane pot temperature.

NOTE: The cut off temperature for the audible/visual alarm is preset to -150˚C at the factory.

22. Set the blotting timer to 4 seconds using the toggle switches on the front of the timer.

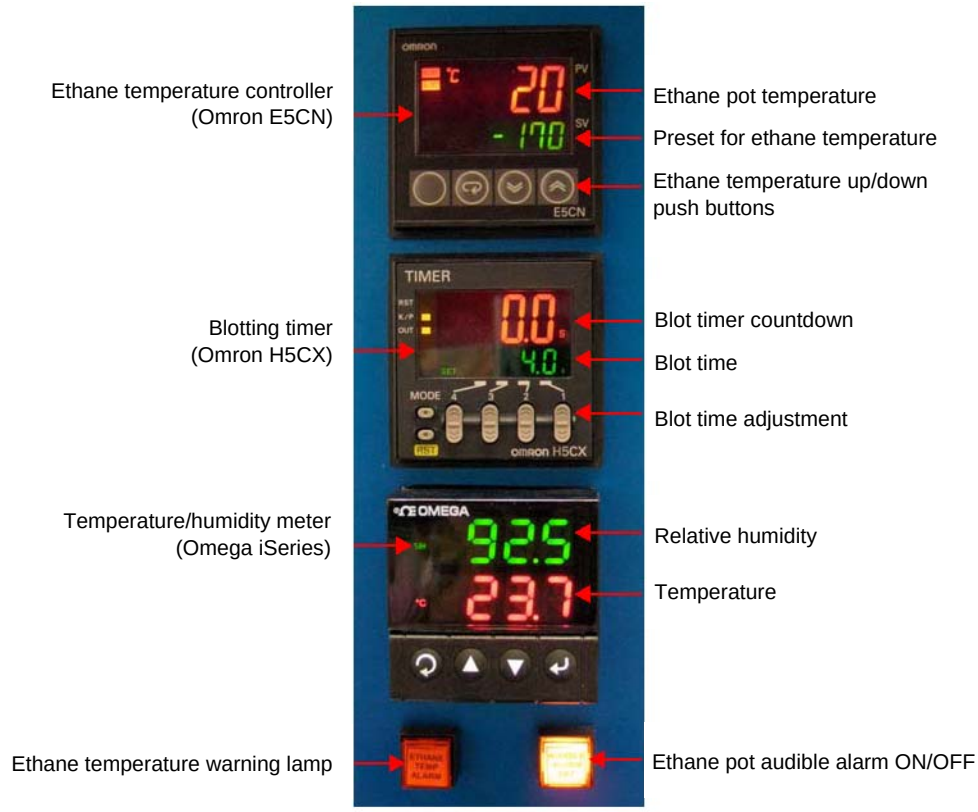


figure 1-15. Meters and alarms for Cryoplunge3

23. Check to make sure that the plunge rod is in its full upward (firing) position, then press the RESET button to close the shutter located at the base of the humidity chamber.

24. Press the START button. The blotters come together for 4 seconds and then the plunge rod fires toward the ethane pot.

25. Raise the plunge rod to the full upward (firing) position.

NOTE: Remember to return the plunge rod to its full upward (firing) position before pressing the **RESET** button or depressurizing the pneumatic supply to Cryoplunge3. Failure to do this causes the shutter at the base of the humidity chamber to close against the plunge rod. This situation can be corrected by manually moving the shutter toward the front of the instrument while raising the plunge rod to the full upward (firing) position. Refer to the "Troubleshooting" section of this manual for further instructions, if necessary.

NOTE: The plunge rod does not fire unless the following preconditions are met:

- The workstation is in the innermost position (safety interlock engaged).
- The safety shield is fully closed and the safety interlock is engaged (large cover should be in place on top of the workstation, but the small workstation cover must be removed in order for the safety shield to close).
- The pneumatic supply is on.

Preparing the Humidity Chamber

To prepare the chamber:

1. Switch mains **ON** and make sure the temperature/humidity sensor is inserted into the top of the humidity chamber.
2. Raise the plunge rod to the full upward (firing) position.
3. Press the **RESET** button to close the shutter at the base of the humidity chamber.
4. Remove the humidity wand from its receptacle on the top of the humidity chamber.
5. Insert the (compressed) cellulose sponge within the three metal supports of the wand.
6. Soak the wand sponge in hot tap water to expand the cellulose sponge.

NOTE: Soak the sponge in hot water to rapidly raise the chamber humidity.

7. Remove any excess water by blotting against absorbent paper.
8. Replace the humidity wand.
9. Wait approximately 15 minutes for the humidity chamber to reach 98% RH.
10. Resoak the humidity wand as needed to maintain 98% RH within the humidity chamber.

Filter Paper Punch 930.50000 (Optional)

NOTE: The filter paper punch is an accessory used for cutting filter paper discs for blotting the specimen.

NOTE: Always clean the surfaces of the filter paper punch using a lint-free cloth and 70% ethyl alcohol.

NOTE: During operation, keep fingers clear of the cutting surface near the die cutter to avoid injury.

1. Place the filter paper punch on a clean surface (lab bench) away from water or corrosive chemicals.
2. Clean the surfaces of the filter paper punch using a lint-free cloth and 70% ethyl alcohol.
3. Allow the surfaces to dry for several minutes.
4. From this point forward, do not touch any of the surfaces of the paper punch unless your hands are protected with gloves. Handle the filter paper to be cut with gloved hands to prevent contamination of the paper discs.
5. Place the filter paper to be cut beneath the cutting surface, making sure that the paper is pushed against the backstop.
6. Press the large black handle downward to cut the disk.
7. Rotate the filter paper until all discs have been cut.
8. Carefully transfer all of the cut discs to a clean vessel, such as a petri dish.
9. Store the filter paper punch in a dry, dust-free location when not in use.

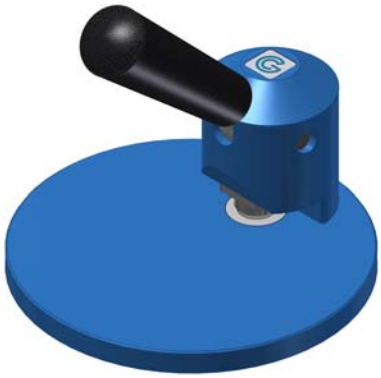


figure 1-16. Filter paper punch

Preparing the GentleBlot Blotters

To mount the blotting foam onto the blotters:

NOTE: Cryoplunge3 is shipped without the filter paper or foam attached to the GentleBlot blotters.

1. Remove the blotters from the humidity chamber by grasping the outer surface of the blotter and pulling straight out and away from the humidity chamber. There may be a slight resistance from the o-rings on the shaft of the blotter.

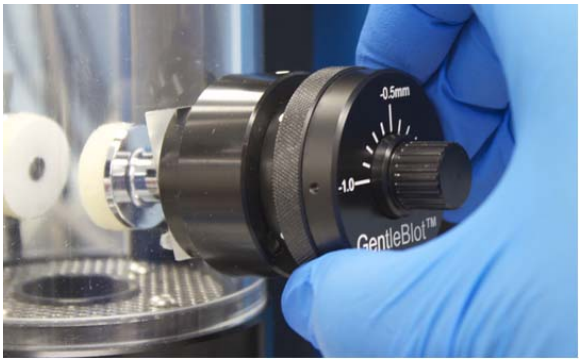


figure 1-17. Inserting/removing a blotter from the right-hand chamber flange

2. Using the blot pad spindle wrench, unscrew the top spindle. Use the filter paper loading jig as the support for the new GentleBlot blotters.

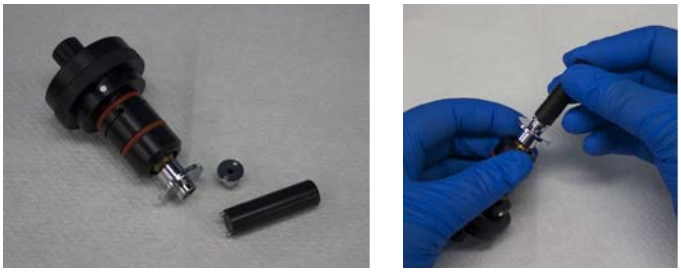


figure 1-18. Removing the spindle

3. Insert one sponge pad over the outer spindle, making sure it is properly aligned with the spindle core.

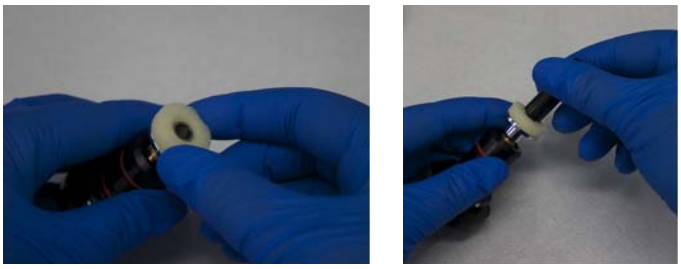


figure 1-19. Inserting the sponge pad

4. Replace the top spindle and tighten using the spindle wrench.
5. Make sure the foam pads are uniform and flat when the spindle is tightened.
6. Repeat for the other blotter.

Loading the filter paper disk onto the GentleBlot blotters

NOTE: To prevent contamination of the filter paper disks, wear protective gloves or handle the disks with pre-cleaned tweezers.

NOTE: If necessary, clean and dry the filter paper loading jig prior to loading the filter paper.

1. Remove the blotters from the humidity chamber by grasping the outer surface of the blotter and pulling straight out from the humidity chamber (see figure 1-17).
2. Place the filter paper pin flat side down into the filter paper loading jig.
3. Place a new piece of filter paper over the pin.
4. Place the plastic backer on top of the filter paper.

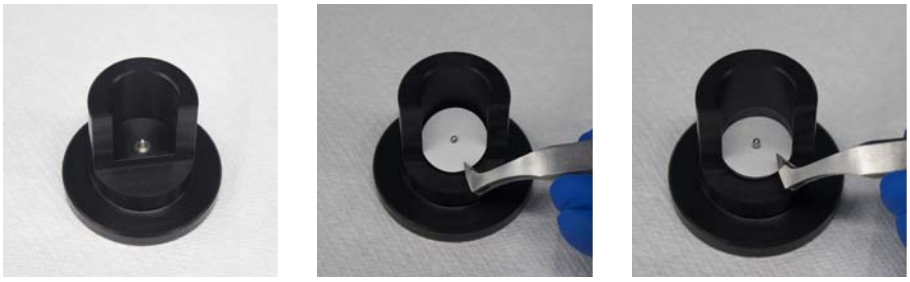


figure 1-20. Loading the filter paper disk and protective plastic backer

5. Guide the blotter down the barrel of the filter paper loading jig and press down firmly to connect the pin.
6. Remove the blotter from the jig.
7. Reinsert the blotter to the humidity chamber.
8. Repeat with the other blotter.

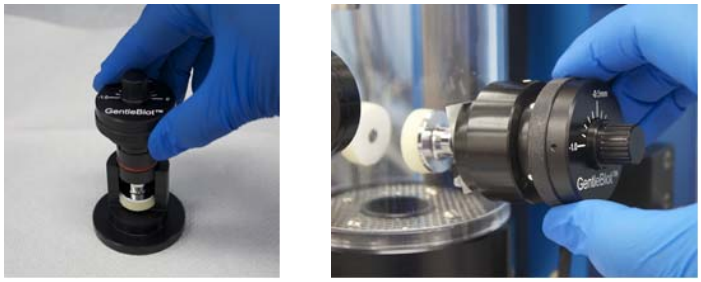


figure 1-21. Attaching the filter paper disk and inserting the blotter

Cooling the Workstation with Liquid Nitrogen

NOTE: Starting from room temperature conditions. It takes approximately 20 minutes for the workstation to stabilize at liquid nitrogen temperature. It is important for the ethane pot temperature to be at a stable temperature below -170˚C prior to condensing the ethane into the ethane pot.

To cool the workstation with liquid nitrogen:

1. Have a ready supply of liquid nitrogen available along with several small dewars or liquid nitrogen transfer vessels for the next steps.
2. Make sure that the workstation connector is plugged in.
3. Switch on the mains supply.
4. Set the ethane temperature using the up/down push buttons on the ethane temperature controller to -172˚C.

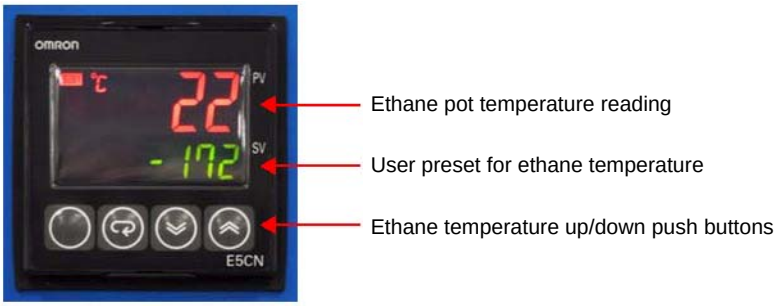


figure 1-22. Meter for ethane temperature controller

5. Remove the covers from the workstation.
6. Insert the cryo grid box transfer pot into the front of the workstation
7. Fill the workstation and the ethane pot with liquid nitrogen to start rapid cooldown of the workstation. As liquid nitrogen is added to both the workstation and the ethane pot, there will be rapid boil off of the liquid nitrogen. Continue to fill both until the workstation reaches equilibrium and the ethane pot maintains at least -170˚C. This takes at least 20 minutes.

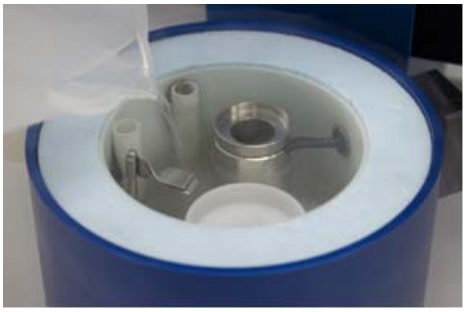


figure 1-23. Initial cool-down of the workstation and ethane pot

8. When the workstation is at the low temperature equilibrium, prepare to condense the ethane.

Condensing Ethane and Filling the Ethane Pot

NOTE: The following safety recommendations are summarized below for your convenience. You are strongly advised to review the latest MSDS available on the world wide web for safe handling procedures for liquid ethane and liquid nitrogen.

⚠ **WARNING:** REVIEW AND REMEMBER THESE IMPORATANT SAFETY PRECAUTIONS WHEN HANDLING THE LIQUID CRYOGENS USED IN THIS PROCEDURE!

- **FIRE AND EXPLOSION:** Fire and explosion are the primary hazards associated with flammable, liquefied gases such as ethane. Flammable gases can be ignited by static electricity or by a heat source (such as a flame or any hot object). Cryogens (including nonflammable nitrogen) which achieve temperatures below -183˚C can liquefy oxygen from the surrounding environment to form potentially dangerous oxygen mixtures that support violent combustion.
- **EXPLOSION DUE TO EXPANSION:** At temperatures above boiling point, all liquid cryogens expand rapidly by factors in excess of 700 times. Any cryogen contained or trapped in a sealed compartment or vessel is a potential explosion risk if allowed to warm up.
- **SPILLAGE DUE TO FAILURE OF STORAGE VESSELS:** Glass vacuum dewars can shatter when liquid cryogens are introduced, causing a spill and potential lacerations from glass shards. Take care when using glass vacuum dewars. If the vacuum integrity of the dewar is in doubt, use stainless steel vessels.
- **SPILLAGE OF ETHANE DUE TO NITROGEN ENTRAPMENT:** If the liquid nitrogen used to cool the ethane vessel overflows into the ethane, it can freeze the top layer of ethane within the ethane pot. This in turn can trap ethane gas, which can explode through the frozen layer of ethane showering the surrounding area.
- **ASPHYXIATION:** Asphyxia is a potential hazard with all cryogens if they are allowed to warm and produce large volumes of gas. Because the gases produced by ethane and nitrogen are colorless and odorless, they can escape into the atmosphere undetected and quickly reduce the concentration of oxygen below the level necessary to support life (<19.5% v/v). Victims might be unaware. At low concentrations, ethane can cause narcotic effects and symptoms can include dizziness, headache, nausea, and loss of coordination.
- **COLD BURNS:** Because of extremely low temperatures, cryogens are capable of producing severe cryogenic burns and frostbite. When spilled on a surface, they tend to cover it completely and, therefore, cool a large area. The vapors

from these liquids are also extremely cold and can produce burns. Exposure, which might be too brief to affect the skin of the face or hands, can damage delicate tissues, such as the eyes. Flesh sticks to extremely cold materials such as non-insulated pipes or metallic vessels containing cryogenic liquids. Even non-metallic materials are dangerous to touch at low temperature. In addition to the hazards of frostbite or flesh sticking to cold materials, objects that are soft and pliable at room temperature, such as rubber or plastic, become hard and brittle and are easily broken at these extremely low temperatures.

NOTE: For this next procedure, you are advised to work in an explosion-proof (spark-proof) fume hood. No flames or explosive chemicals are allowed in this hood.

NOTE: If the fume hood has an exceptionally high draw rate, you might have to minimize the draw for this procedure and then restore the draw rate at the close of the procedure.

NOTE: Have a ready supply of liquid nitrogen available to maintain the workstation at the low temperature equilibrium.

NOTE: Always wear eye protection when working with liquefied ethane or any cryogens.

IMPORTANT:

- The volume of the ethane pot for Cryoplunge3 is 4.1 mL to the upper rim of the pot.
- Do not condense more than 10 mL of ethane at one time.
- Always make sure that the containers that will receive the condensed ethane do not contain any residual liquid nitrogen. In a closed environment and upon evaporation, the liquid nitrogen pushes the liquid ethane out of the vessel at a high rate and can cause injury.
- Always position the ethane vessel away from you.
- Ethane vaporizes at -88.6˚C so it is very important to maintain the level of liquid nitrogen within the workstation of Cryoplunge3 when the ethane pot is filled.

To condense the ethane:

1. When the pre-cooled workstation has reached its low temperature equilibrium, switch mains **OFF**.
2. Disconnect the workstation connector.
3. Place the workstation in the explosion-proof fume hood.
4. Remove the workstation covers.
5. Top off the workstation with liquid nitrogen via the filling funnel.
6. Remove any residual liquid nitrogen that might be in the ethane pot. (Given time, the liquid nitrogen evaporates on its own. Alternatively, you can speed up this process by inserting a small piece of clean Tygon tubing into the ethane pot. Be sure to face the opening of the Tygon tubing away from you to prevent injury.)
7. Adjust the gas pressure for the 2-stage regulator on the ethane tank.
   a. Make sure that the outlet valve of the second stage of the regulator is closed so that no gas can escape.
   b. Open the main tank valve.

c. Adjust the pressure of the second stage of the ethane regulator to deliver the ethane at about 2 to 4 psi (0.14 to 0.28 bar) by turning the regulator adjusting spring handle clockwise.

d. At this point, the system is now ready to use for condensing ethane.

8. Two commonly used methods to condense ethane are described below:

NOTE: Make sure that the containers receiving the ethane are free of any particulate matter.

- **Method 1:** Insert the nozzle (not provided) at the end of the Tygon tubing directly into the pre-cooled ethane pot and hold it at the bottom of the pot. The "nozzle" being used in this method is made from a volumetric pipette tip that is commonly found in the laboratory. Clip the end of the pipette tip to allow an opening of approximately 2–3 mm.

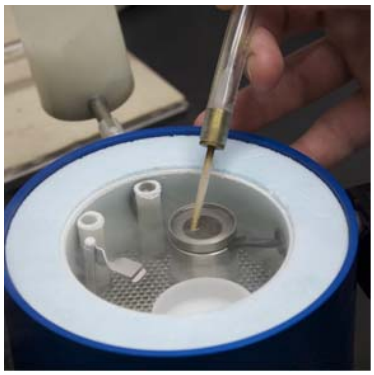


figure 1-24. Condensing ethane directly into the pre-cooled ethane pot

- **Method 2:** Pre-cool a conical tube (provided) in a small bath of liquid nitrogen. Condense 6-7 mL of ethane in the bottom of the tube. Carefully pour the condensed ethane into the pre-cooled ethane pot.

NOTE: Ethane gas within the supply tank is at ambient temperature. Condensing ethane into the pre-cooled conical tube and then pouring the ethane into the ethane pot minimizes the amount of time needed for the ethane pot to return to the preset ethane temperature on the ethane temperature controller of Cryoplunge3.

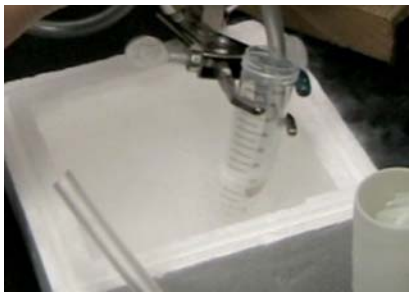


figure 1-25. Condensing ethane in the conical tube

figure 1-26. Pouring the condensed ethane into the precooled ethane pot

9. To condense the ethane, open the outlet valve on the second stage of the ethane regulator so that the ethane gas flows into the bottom of the pre-cooled ethane pot or the bottom of the conical tube depending on the chosen method for condensing the ethane.
10. As needed, adjust the outlet valve on the ethane regulator to control the rate of condensation of the ethane. It takes approximately 2 minutes to directly fill the ethane pot to the upper most rim of the pot (4 mL). If using the conical tube method to pour the ethane, condense about 6-7 mL (no more than 10 mL) to allow for some potential lost ethane on pouring into the ethane pot.
11. When the ethane pot is full or you have a suitable quantity of ethane condensed within the conical tube, close the outlet valve on the 2-stage regulator of the ethane tank and leave the ethane tank in "stand-by" mode.
12. Leave the ethane tank in stand-by mode.

Stand-by mode for the ethane tank

NOTE: Always close the main tank valve after use and drain the regulator of any residual ethane by following these steps:

a. Close the main tank valve.
b. Point the Tygon transfer tubing toward the back of the explosion-proof (spark-proof) fume hood.
c. Open the outlet valve of the second stage of the regulator to bleed off any gas remaining in the regulator and the flexible tubing.
d. Fully open the regulator adjusting spring handle for the second stage of the regulator (turn counter clockwise until loose but not disconnected from the regulator).
e. Close the outlet valve of the second stage of the regulator.

13. Add liquid nitrogen to the workstation if needed.
14. Replace the covers for the workstation.
15. If necessary, reset the flow rate for the fume hood.
16. Carefully remove the workstation and install it on Cryoplunge3.
17. Insert the workstation connector and switch the mains ON.
18. Adjust the up/down push buttons on the ethane meter until the ethane is maintained just above its melting point. For example, if the ethane freezes when the temperature reads -174˚C on the meter, press the up push button to maintain a temperature just slightly above this value until the ethane melts.

19. Wait several minutes for the ethane temperature to stabilize and add liquid nitrogen through the remote fill funnel as necessary to maintain the volume within the workstation.

20. You are now ready to freeze your sample.

Blotting Techniques for Freezing the Sample

NOTE: For best results, always pre-treat the specimen support (for example, holey carbon film or continuous carbon film) to render the surface hydrophilic or hydrophobic depending upon the needs of the sample.

NOTE: The plunge tweezers are specially designed and aligned to provide optimal contact with the GentleBlot pad assemblies.

NOTE: The black plastic securing clip for the plunge tweezers has a small and a large rectangular opening. The small opening should always be placed onto the tweezer shaft from the blunt end and the first detent position on the shaft of the tweezer should always be used to secure the specimen grid (first detent furthest from the tweezer tip). Pressing the securing clip further down on the tweezer shaft can damage the tip of the tweezer or cause the specimen grid to disengage prematurely during the plunging cycle.

NOTE: During the process of plunging the sample grids, it is necessary to monitor and/or adjust the ethane temperature, humidity of the chamber, and the volume of the liquid nitrogen in the workstation and in the cryo grid box transfer pot.

NOTE: The sample concentration, sample volumes, and blotting time generally have to be determined for each new specimen that will be plunge frozen in order to produce optimal results. The following recommendations are guidelines.

2-side Blotting

To freeze the sample using 2-side blotting:

1. Pre-cool the cryo grid box by placing it inside of the cryo grid box transfer pot within the workstation.
2. Pre-cool a second cryo grid box in the staging receptacle located on the metal mesh base of the workstation.
3. Secure the specimen support grid to the specially designed plunge tweezers and lock the grid in place by sliding the black plastic securing clip until it engages the first clickstop on the tweezer shaft.

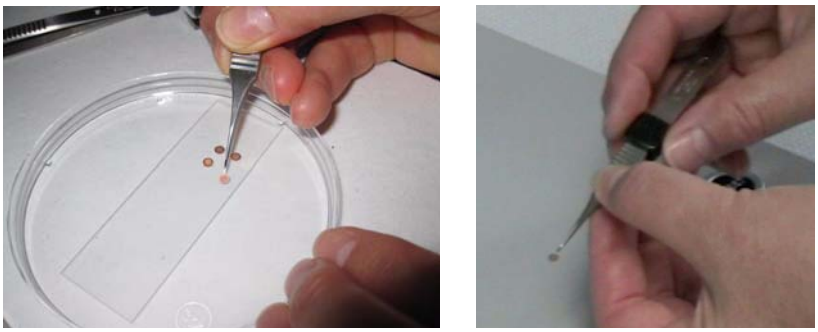


figure 1-27. Securing the specimen grid to the plunge tweezers

4. Test to make sure that the grid is held firmly in the tweezer.
5. If the plunge rod is in the full upward (firing) position, it is necessary to run a blot and plunge cycle so that the tweezer can be loaded onto the plunge rod.
    a. Remove the small workstation cover.
    f. Close the safety shield.
    b. Press **RESET** to close the shutter at the base of the humidity chamber.
    c. Press **START** to initiate the blot and plunge cycle.
    d. The plunge rod fires toward the ethane pot.
    e. Open the safety shield and replace the small cover.
6. Raise the plunge rod several centimeters and insert the base of the plunge tweezer into the slot at the base of the plunge rod and press until it clicks into place, taking care not to damage the specimen grid.

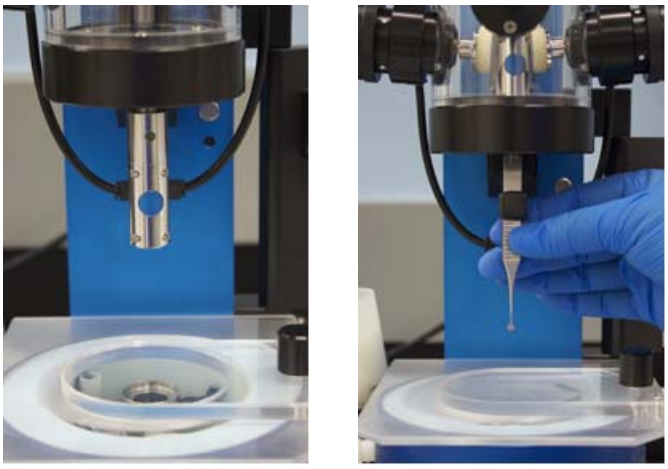


figure 1-28. Inserting the tweezers into slot at the base of the plunge rod

7. Raise the plunge rod to the full upward (firing) position until it clicks into place.

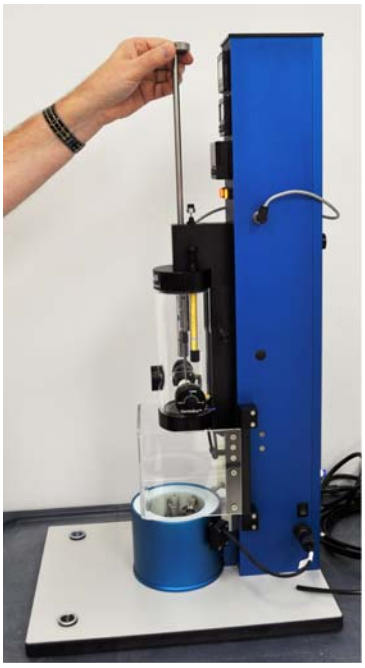


figure 1-29. Raising the plunge rod to full upward (firing) position

8. Press the **RESET** button to close the shutter at the base of the workstation
9. Position the blot pads:
   - There are four blotting positions as indicated on the GentleBlot blotters.
   - Each blotter has a white index mark.
   - Start by positioning the index mark on each blotter so that it is aligned with the 12 o'clock (uppermost) position on the chamber flange.
   - Remember to rotate the blotters after each plunge cycle so that each grid being blotted is exposed to an unused area of the filter paper (for example, after each blot/plunge cycle, rotate the blotters toward the back of the instrument).
   - Adjust the pressure for each GentleBlot blotter. Grasp the outer (large) knurled knob of each blotter and rotate the smaller knurled knob to the desired setting to apply more pressure (position 0) or less pressure (position -1), as required.
10. Open the specimen loading port on the front of the humidity chamber.
11. Apply a small aliquot of the sample suspension to the pre-treated surface of the support grid using an accurate volumetric pipetting device.
    - 3 micro liters is a good starting point.

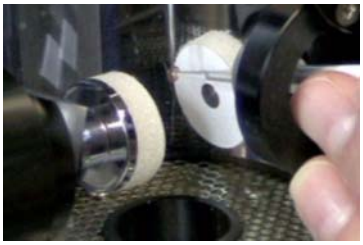


figure 1-30. Applying sample aliquot to the support film

12. Close the specimen loading port to maintain the high humidity environment within the humidity chamber.
13. Rotate the plunge rod 90 degrees to the left or right so that the grid is now aligned with the blot pads.
    - There are four detent positions for the plunge rod to allow accurate alignment of the grid with respect to the blot pads.
14. Set the timer for the desired blotting time.
    - For example, for two-side blotting where a 3 mL volume is applied to the support grid, set the blot time for 4 seconds (therefore, volume plus 1 second).
15. Remove the small workstation cover and close the safety shield.
16. Press **START**.
    a. The blot pads come together and blot the specimen for the time indicated on the timer.

figure 1-31. 2-side specimen blotting

b. The grid is plunged into the liquid ethane.

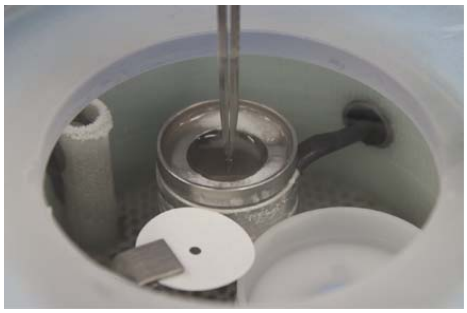


figure 1-32. Grid plunging into the liquid ethane

To remove the frozen hydrated grid from the ethane pot:

NOTE: The freshly frozen hydrated grid is protected while immersed in the liquid ethane. It is also protected during transfer within the cryogenic environment of the workstation. Prior to transferring the frozen hydrated grid from the ethane, refresh the positive outflow of cryogenic nitrogen gas from the chamber by adding a small volume of liquid nitrogen via the filling funnel. Do not breathe into the workstation when transferring the frozen hydrated grid as this will damage the grid.

NOTE: Make sure that the cryo grid box is at liquid nitrogen temperature and ready to receive the frozen hydrated grid. If necessary, top off the liquid nitrogen level within the liquid nitrogen transfer pot.

NOTE: If you wish to blot excess ethane from your frozen hydrated grid prior to storing it within the cryo grid box, insert a piece of filter paper into the paper clip within the workstation and allow it to come to liquid nitrogen temperature.

NOTE: The specimen grids are fragile and must be handled with care to avoid damage (bending of the grid or breakage of the support film).

1. Hold the plunge tweezers with one hand.
2. Press the blue quick disconnect pushbutton on the plunge rod with your other hand.

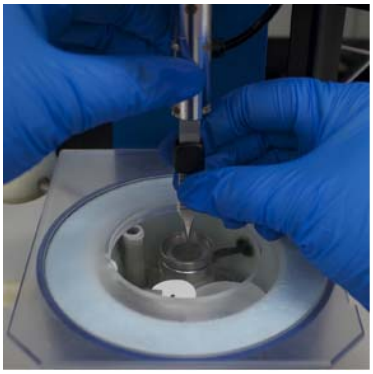


figure 1-33. Releasing the plunge tweezers from the plunge rod

3. Push the plunge rod upward approximately 3 cm to clear the top of the plunge tweezer.
4. Tilt the plunge tweezer slightly while keeping the frozen hydrated grid immersed within the center of the liquid ethane pot.

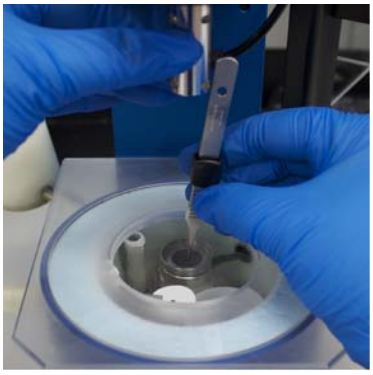


figure 1-34. Tilting the tweezers

5. Gently lower the plunge piston to its bottom-most position and leave it at this position for the next steps.
6. Remove the tweezer from the ethane pot keeping it well within the workstation to prevent devitrification of the frozen hydrated grid
7. OPTIONAL: If you want to view your grid on the same day that it is plunged, it is helpful to blot off any excess ethane that is sticking to the grid. To blot the excess ethane, place the grid just below the filter paper so that the tines of the plunge tweezers contact the filter paper without touching the grid. This action draws away any excess ethane that is trapped between the tines of the plunge tweezers and subsequently from the surface of the grid.

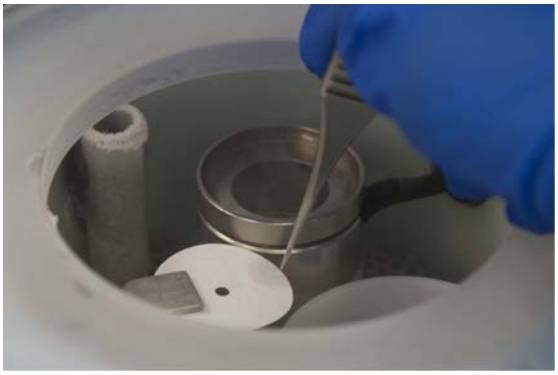


figure 1-35. Blotting excess ethane

8. Position the tweezers so that the grid is in the liquid nitrogen of the cryo grid box transfer pot and, at this point, disconnect the black plastic securing clip for the plunge tweezers taking care not to drop the grid.

figure 1-36. Store the frozen hydrated grid in the cryo grid box

9. The grid can now be placed within one of the receptacles of the cryo grid box.
10. If the cryo grid box has a rotating cover secured with a screw, position the slot of the cover and tighten the screw so that the grids cannot fall out of the box when it is transported for the next step.

To remove the frozen hydrated grids from the workstation:

NOTE: This step requires that you have a ready supply of liquid nitrogen, a liquid nitrogen transfer dewar, a liquid nitrogen storage dewar, and large tweezers to manipulate the liquid nitrogen transfer pot containing the blue cryo grid box.

NOTE: Add liquid nitrogen to the workstation through the filling funnel as necessary to maintain the temperature of the workstation.

NOTE: There are many methods for storing the frozen hydrated grids prior to viewing on the cryo TEM. A grid transfer storage tube (conical tube provided) can be used to contain the blue cryo grid boxes. This tube has holes drilled near the cap to allow liquid nitrogen to flow freely in and out of the tube. Affixing a piece of string to this tube (through the holes provided) allows transport of the storage tube, containing the cryo grid box with the frozen hydrated grids, into a large liquid nitrogen storage dewar (for example, 35 liter dewar).

1. The frozen hydrated grids should be secured within the grid box.

2. Remove the small workstation cover and ensure that the cryo grid box transfer pot is filled with liquid nitrogen.
3. Prepare a liquid nitrogen transfer dewar by filling with liquid nitrogen and waiting several minutes for the temperature of the dewar to stabilize.
4. Remove the cap on the grid transfer storage tube (conical tube provide) and immerse the tube into the dewar of liquid nitrogen.
5. Using a pair of large tweezers, remove the cryo grid box transfer pot from the workstation and place inside of the transfer dewar.
6. Transfer the grid box to the storage tube using the large tweezers.

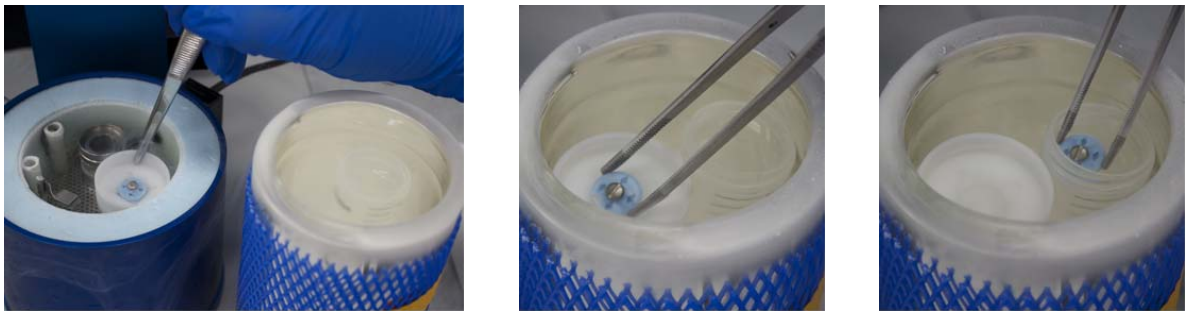


figure 1-37. Transfer the grid box to the storage tube filled with liquid nitrogen

7. Return the cryo grid box transfer pot to the workstation.
8. Refill the cryo grid box transfer pot and the workstation with liquid nitrogen to continue freezing more grids. If you no longer wish to freeze more grids, return Cryoplunge3 to stand-by for the next user.

Returning Cryoplunge3 to Stand-by Conditions

To return Cryoplunge3 to stand-by conditions for the next user:

1. Clean and dry the plunge tweezers and store them with the protective cap in place.
2. Clean and dry the filter paper loading tweezers and store them in their protective sleeve.
3. Clean and dry the filter paper loading jig and store it in its protective plastic bag.
4. Close the main tank valve on the nitrogen gas supply (pneumatics supply to Cryoplunge3).
5. Raise the plunge rod to its fully upward (firing) position within the humidity chamber.
6. Open the safety shield.
7. Press **RESET** several times to bleed the nitrogen gas from the pneumatic lines (the shutter opens and closes during this process).
8. Switch the mains **OFF**.
9. Unplug the workstation connector for the ethane pot.
10. Remove the workstation and place in the explosion-proof (spark-proof) fume hood.
11. Remove the small and large workstation covers.

12. Close the protective screen on the fume hood to allow the cryogens to vent out of the hood. When the workstation is dry (all of the cryogens have vented off), it can be returned to Cryoplunge3.

⚠ **WARNING**: Never use a heat gun to speed up the drying process. These instruments produce very high, concentrated heat that damages (melts) the workstation. If you wish to dry the workstation when all of the cryogens have vented off, it is acceptable to use a hair dryer with a low heat setting.

13. Remove the humidity wand from the humidity chamber to air dry.
14. Open the specimen loading door.
15. Remove the blotters (optional).
16. Allow the humidity chamber to equilibrate to room temperature/humidity conditions.

1-side Blotting

To freeze the sample using 1-side blotting:

NOTE: 1-side blotting is particularly useful when the sample concentration is very low or the specimen grid is very fragile.

NOTE: Test each blotting tweezer for the level of contact with each blot pad prior to conducting 1-side blotting.

NOTE: Follow the instructions for freezing the sample using 2-side blotting with the following exceptions.

1. Remove one of the blotters and insert the blotter blanking plug.
2. Apply a small aliquot of the sample suspension to the pre-treated surface of the support grid using an accurate volumetric pipetting device.
3. Orient the grid for 1-side front or backside blotting.
   a. 1-side backside blotting blots the aliquot of the sample droplet through the holes on the holey carbon film.
      - This method is helpful to use when, for example, the sample concentration is very low.
   c. 1-side frontside blotting blots the aliquot of the sample directly onto the filter paper.
4. Because only one blot pad is being used to remove excess solution from the surface of the support film, set the blotting timer for two times the blotting time that would normally be used for 2-side blotting.
   a. For example, if you add 3 microliters of your sample and normally blot for 4 seconds with 2-side blotting, use an 8 second blot time for 1-side blotting.
   b. Upon blotting, the bulk of the solution is removed within approximately the first 4 seconds, after which time the grid and filter paper disengage. The remaining 4 seconds allow the solution to slowly evaporate in the high humidity of the chamber to the desired thickness before it is finally plunged into the ethane pot.

Manual Multiple Blotting

To freeze the sample using manual multiple blotting:

NOTE: When the safety shield is left slightly ajar, pressing **RESET** closes the shutter, but pressing **START** blots the specimen without plunging the specimen grid into the ethane pot. This feature allows the user to perform manual multiple blots of the specimen grid prior to plunging it into the ethane pot.

NOTE: This technique is useful when multiple reagents or additional sample aliquots are to be added to the specimen grid prior to plunging.

NOTE: Because each blot cycle is manual for multiple blotting, different blotting times can be entered on the blotting timer to accommodate the constraints of the experiment being performed.

NOTE: Follow the instructions for freezing the sample using 2-side blotting with the following exceptions.

1. Apply a small aliquot of the sample suspension to the pre-treated surface of the support grid using an accurate volumetric pipetting device.
2. Close the sample loading port to maintain the high-humidity environment within the humidity chamber.
3. Rotate the plunge rod 90 degrees to the left or right to engage one of the four detent positions; the grid is now aligned with the blot pad(s).
4. Set the timer for the desired blotting time.
5. Remove the small workstation cover and leave the safety shield slightly ajar to override the safety interlock (the shield is still in a protective position).
6. If necessary, press **RESET** to close the shutter.
7. Allow the sample aliquot to adsorb onto the grid substrate for the desired amount of time (for example, 5 to 15 seconds).
8. Press **START**. The blot pad(s) blots the specimen for the time indicated on the timer, but the grid does not plunge into the ethane.
9. Press **RESET**. The shutter closes to maintain the high-humidity environment of the chamber.
10. Add additional sample or reagent to the grid if desired.
11. Close the safety shield completely to engage the safety interlock.
12. Press **START**. The specimen grid is blotted and plunged into the ethane pot.

No Blotting Method

Method 1: Forming the thin film by slow evaporation under high humidity conditions

NOTE: In some cases, the experiment might require that the grid not be blotted at all. There are two methods to accomplish this with Cryoplunge3.

NOTE: Cryoplunge3 is delivered with one blanking plug. Additional blanking plugs are available as an accessory.

NOTE: Follow the instructions for freezing the sample using 2-side blotting with the following exceptions.

1. Remove the blotters.
2. Insert a blanking plug into the right and left-hand chamber flanges.
3. Apply a small aliquot of the sample suspension to the pre-treated surface of the support grid using an accurate volumetric pipetting device.
4. Press **RESET** to close the shutter.
5. Set the blotting timer for the desired time (for example, 10 minutes).
6. There should be no need to orient the grid with the blot axis because the grid is not blotted.
7. Press the **START** pushbutton. The specimen slowly dries to a thin film in the high humidity of the chamber prior to plunging into the ethane. The exact time for this to occur must be determined based on the humidity in the chamber.

Method 2: Immediate plunge with no blotting

NOTE: In some cases, the specimen aliquot is applied in such small volumes that blotting or drying is not required. An example of this method would be spraying a small volume of the sample directly onto the surface of the support film within the high-humidity environment of the chamber.

NOTE: Follow the instructions for freezing the sample using 2-side blotting with the following exceptions.

1. Apply a small volume of the sample directly to the surface of the support film using a spray technique.
2. Set the blotting timer to zero (with the blotting timer set to zero, the blotters do not fire).
3. Press the **START** pushbutton.
4. The sample grid fires directly into the ethane pot.

Routine Maintenance

Removing the Humidity Chamber for Cleaning

NOTE: The humidity chamber can be removed for cleaning. Use a mild soap and water solution to clean the chamber.

To remove the humidity chamber:

1. Return Cryoplunge3 to stand-by conditions.
2. Remove the blotters.

figure 1-38. Removing the blotters

3. Remove the humidity wand.

figure 1-39. Removing the humidity wand

4. Remove the temperature/humidity sensor.

figure 1-40. Removing the temperature/humidity sensor

5. Disconnect the pneumatic supply for the chamber flanges by pressing the outer ring of the “push-in” fitting while removing the pneumatic supply tube.

figure 1-41. Disconnect the pneumatic supply for the blotter assemblies

6. Unscrew and remove the securing screw for the humidity chamber located on the right-hand side of the instrument.

figure 1-42. Removing the humidity chamber

7. Hold the humidity chamber and gently push downward approximately 2 cm.
8. Gently pull the humidity chamber forward to disconnect.

To replace the humidity chamber:

1. After cleaning and drying the chamber, reinstall on Cryoplunge3.
2. Gently press upward to engage the top of the humidity chamber with the top chamber support of Cryoplunge3.

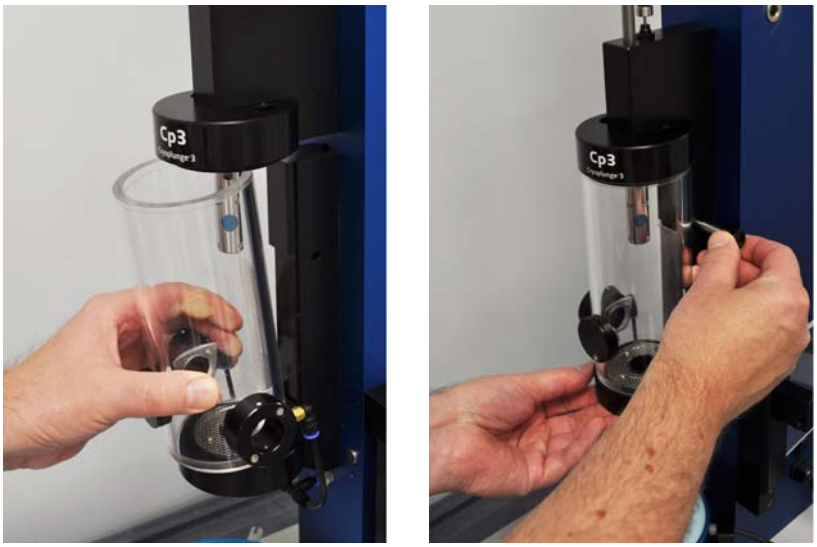


figure 1-43. Replacing the humidity chamber

3. Insert the securing screw and make finger tight.
4. Reconnect the pneumatic supply to the chamber flanges; press the pneumatic tubing inward making sure that the connection is tight within the push-in fitting.

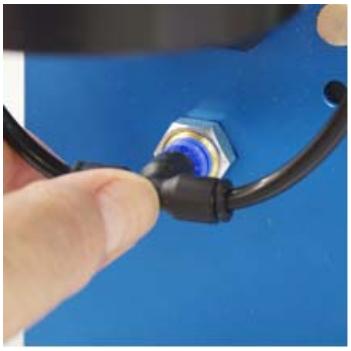


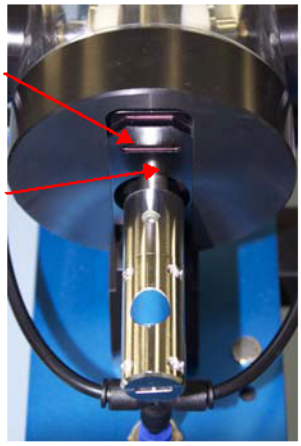
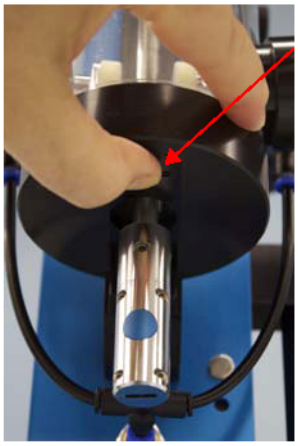
figure 1-44. Reconnecting the pneumatic supply

5. Insert the blotters.
6. Insert the humidity wand.
7. Insert the temperature/humidity sensor.
8. Maintain the instrument in stand-by conditions for the next user

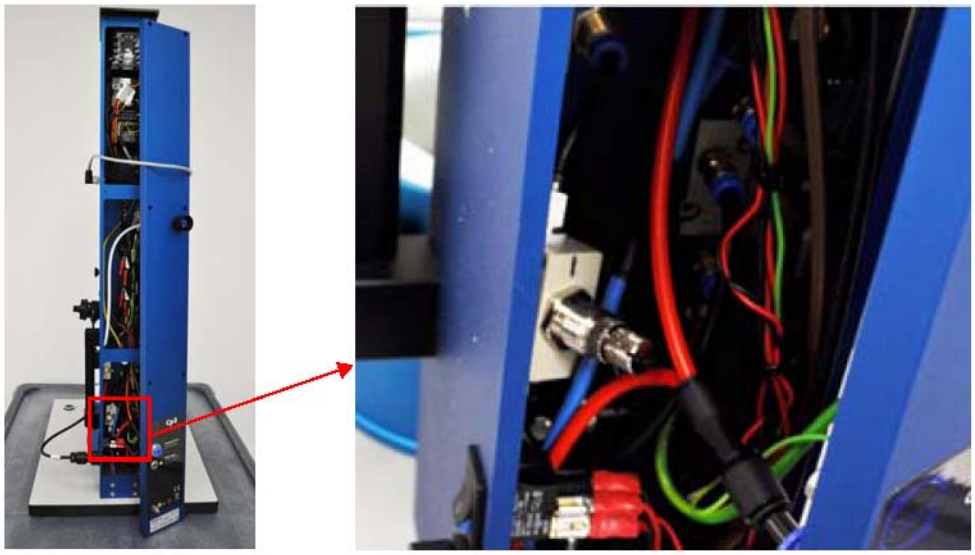
Cleaning the Outside Surfaces of Cryoplunge3

Use a mild soap solution on a soft cloth to clean the outside surfaces of Cryoplunge3. Do not use harsh chemicals or alcohol-based cleaners as this may cause damage.

Appendix A: Troubleshooting

Problem	Things to Check	Action to Take
Meters do not illuminate	• Mains power supply and **ON/OFF** switch	Check connections of mains power supply and make sure electrical connection is made. Switch **ON/OFF** switch to **ON** position.
Plunge rod does not fire	• Pneumatic supply	Make sure that the pneumatic source is on, i.e., the main tank valve for the nitrogen gas tank which supplies the pneumatic pressure is open and set the second stage of the nitrogen gas regulator on the tank to a value of 70 psi (4.8 bar). If using the house air supply, make sure that it is delivering the proper pressure.
	• Safety interlock for workstation	Make sure that the workstation is fully pressed into the innermost position on the base plate of Cryoplunge™3 and that the safety interlock for the workstation is engaged.
	• Safety shield interlock	Check that the safety shield is fully closed to ensure that the safety interlock on the door hinge of the shield is engaged.
Plunge rod cannot be raised into humidity chamber	• Power was shut off with plunge rod in downward position	Look at the bottom of the humidity chamber and, using your fingertip, gently push the shutter toward the front of Cryoplunge3 while raising the plunge rod to its full upward (firing) position. Release the shutter.
Shutter as viewed from the base of the humidity chamber The shutter is impinged upon the plunge rod. This condition results when the plunge rod is in its full downward position and the power for Cryoplunge3 is switched off. The plunge rod cannot be raised to the full upward (firing) position in this condition.		 Using your fingertip, pull the shutter forward (do not pull the plunge rod forward). This action frees the plunge rod. Hold the shutter in this position and raise the plunge rod to the full upward (firing) position. Release the shutter.
Blot pads do not come together to blot the grid	• Pneumatic supply pressure	Make sure that the pneumatic source is on, i.e., the main tank valve for the nitrogen gas tank which supplies the pneumatic pressure is open and set the second stage of the nitrogen gas regulator on the tank to a value of 70 psi (4.8 bar). If using the house air supply, make sure that it is delivering the proper pressure.
	• The blotters are not fully inserted into the chamber flange at one of the four detent positions for blotting.	Press the blotters completely into each chamber flange and align with one of the four detent positions.

Problem	Things to Check	Action to Take
Blot pads do not come together at all	• Blot pads do not come together in a uniform manner • Internal pressure regulator set too low	The force by which the blotters come together is preset at the factory at the optimal GentleBlot™ setting. If the internal pressure regulator is set too low, the blot pads will either not come together or they will come together in a "chatter like" motion. To reset the internal pressure regulator, switch the mains off, close the pneumatic supply, and bleed any residual pneumatic pressure by pressing and holding the RESET push-button. Disconnect the power cord and the pneumatic supply line from the back of Cryoplunge3. Remove the back panel and locate the internal pressure regulator. Release the locking ring of the regulator, turn the knob of the regulator fully clockwise (closed position) and then rotate the knob 1.75 turns counter clockwise to open the valve to the proper GentleBlot setting. Lock the valve in this position.



Ethane warning lamp does not illuminate	• Mains power supply and **ON/OFF** switch	Make sure the connector for the temperature/humidity sensor is inserted in the proper orientation and that it is secure and the mains power is switched **ON**.
	• The bulb is burned out.	Replace the bulb. Contact Gatan, Inc. for part number.
Ethane alarm **ON/OFF** pushbutton does not illuminate	• Mains **OFF**.	Check to make sure that the electrical connection is made and that the **ON/OFF** switch is switched **ON**.
	• The bulb is burned out.	Replace the bulb.
Temperature/humidity not indicated on meter	• Temperature/humidity connector	Make sure that the connector for the temperature/humidity sensor is inserted in the proper orientation and that it is secure
Loss of high humidity condition within humidity chamber	• The shutter and/or specimen loading port is open.	Close the shutter and/or specimen loading port by first returning the plunge rod to its full upward (firing) position and then pressing **RESET**.
	• The humidity wand sponge is too dry to maintain humidity.	Re-soak the wand with water to increase the humidity within the chamber.

Problem	Things to Check	Action to Take
Plunge tweezers do not hold grid	• The black securing clip is not properly inserted on the tweezers shaft.	Make sure that the smaller of the two openings on the securing clip is inserted onto the tweezers shaft so that it faces the tip of the tweezers and locks onto the first detent (furthest from the tweezers tip).
	• The tweezers tips are “splayed” apart due to misuse.	Remove the black securing clip and, with the tweezers opened, gently press each tip of the tweezers against a solid surface to re-align tips. (If necessary, the tips of the plunge tweezers can be gently filed against the surface of a sharpening stone as needed to improve performance and longevity.)
Grid disconnects from plunge tweezers during plunge cycle	• Black securing clip not properly inserted on tweezers shaft	Make sure that the smaller of the two openings on the securing clip is inserted onto the tweezers shaft so that it faces the tip of the tweezers and locks onto the first detent (furthest from the tweezers tip).
	• Tweezers tips are “splayed” apart due to misuse	Remove the black securing clip and, with the tweezers opened, gently press each tip of the tweezers against a solid surface to re-align tips.
	• Plunge rod dampening pot damaged	Check to make sure the plunge rod dampening pot is intact.
Ethane temperature does not display on meter	• Workstation connector	Check to be sure this connection is secure.
Ethane freezes	• Ethane temperature setting	Set the ethane temperature to a value just above its melting point of -182ûC using the up/down push buttons on the ethane meter until the desired temperature is obtained.