ThermoFisher SCIENTIFIC

Vitrobot Mk IV

User Manual

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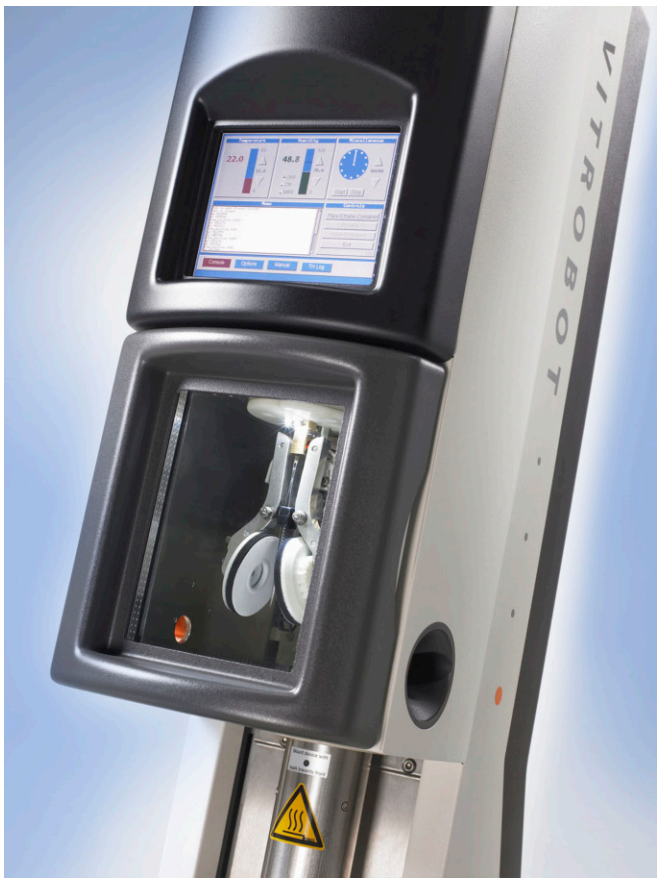
thermo scientific

Contents

1 Introduction

The Vitrobot™ (Vitrification Robot) is a fully PC-controlled device for vitrification (= rapid cooling) of aqueous samples. Minimal, yet essential, training is required when operating the instrument for research purposes in a laboratory environment. The process of plunging, blotting and vitrification is fully automated upon placing a vial in the chamber and setting up the ethane holder. Operation is strictly controlled by dedicated hardware and software, implying that parameters and the results are reproducible. This enables a high throughput of vitrified samples, with an easy and straightforward control of the vitrification process. The parameters to be influenced are: temperature, humidity, the number of blots, and a number of critical time settings. The instrument will consistently produce excellent specimens for cryo-electron microscopy. Research can now rely on specimen preparation allowing the investigator to concentrate on structural studies of the specimen.



1.1 Design Change

In 2017 the design of the Vitrobot Mk IV was updated to the Thermo Scientific branding. There are no mechanical or functional changes.

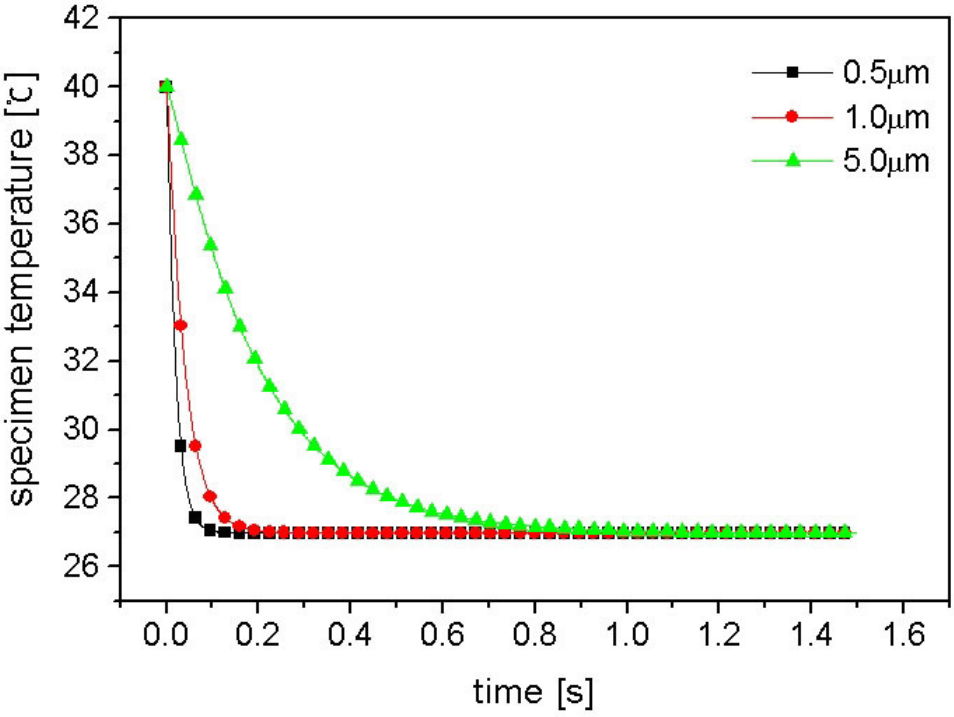
1.2 Design Philosophy

Sample preparation for cryo-electron microscopy involves a few steps; application of the sample to a specimen grid, blotting away excess liquid and shooting the thin (about 100 nm) sample into liquid ethane.

In the study of thermal behavior of liposomes and intact cells by cryo-EM we found unpredictable thermal and osmotic effects. These effects were ascribed to the dual effect of evaporation; cooling the specimen to the dew point and concentration of solutes. Theoretical analysis as well as experimental evidence indicated that the heat and mass exchange are rapid processes in an aqueous thin film with a high surface to volume ratio (see figures below). Time constants involved are a few tenth of a second or shorter. The environmental humidity is a key parameter for sample evaporation. After preliminary experiments with environmental controlled chambers it was decided to design and construct a fully self-contained system that included sample application, blotting and rapid cooling into ethane. A first prototype was built and automatic blotting was one of the key features of this PC controlled prototype. After 4 years of experience with this prototype it became clear that the cryo-EM world was in demand of reliable specimen preparation unit. The Vitrobot™ as presented should fulfill this demand. The Vitrobot (patent pending) is the result of a complete redesign, involving hardware, electronics and software. The instrument is tested in research groups with an established reputation in cryo-EM.

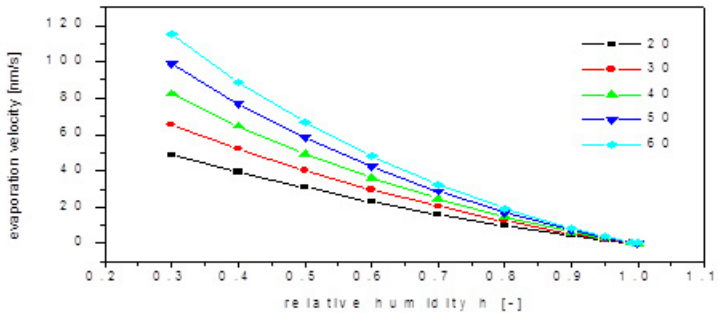
1.2.1 Theoretical analysis of specimen thermodynamics

The visualized experiment describes the behavior of a thin film of an aquous specimen at various temperatures in an environment of 40 °C and a relative humidity of 40 %. Thickness of water layer is as indicated.

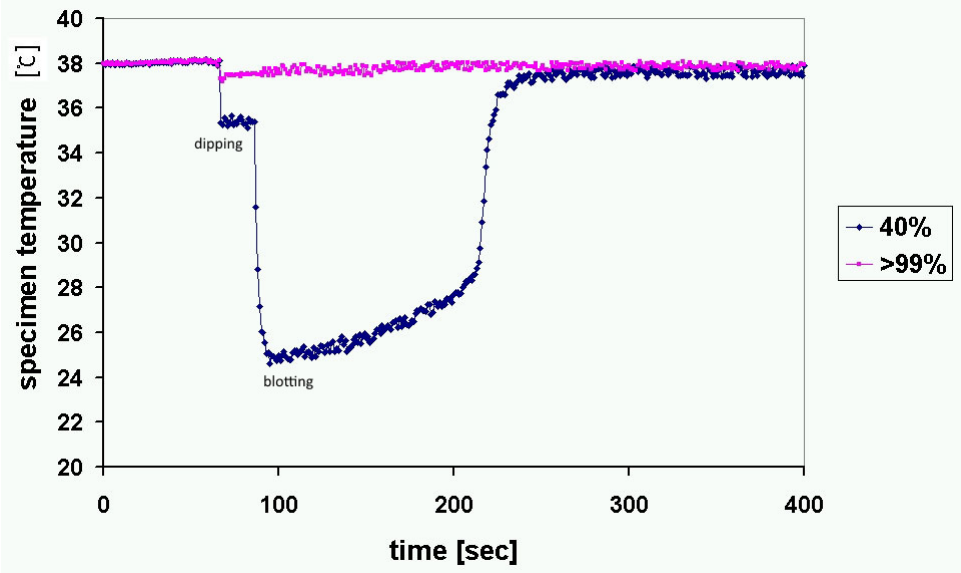


1.2.2 Thermodynamic analysis of specimen evaporation

The underlying graph illustrates that the evaporation velocity is independent of film thickness but that the evaporation depends on the relative humidity in the reaction/climate chamber. A constant ice thickness is therefore easier to achieve at higher humidity. Furthermore it is clear that thin film preparation under room conditions (e.g. 20 °C and a relative humidity of 40 %) may result in an appreciable loss of water; in the two seconds of a typical preparation 80 nm of the thickness of the thin film is evaporated. This evaporation has not only an effect on the temperature (preceding paragraph) but also on the solute concentration (N.B. osmotic effects!) and the concentration of the suspended particles.



Inside the climate chamber of the Vitrobot, a small micro-thermocouple is present which measures the temperature in the chamber. In the figure below the temperature of the specimen was measured during the vitrification process i.e. during dipping in a vial, withdrawal, blotting and the last moments before the freezing. The thermal history was estimated with an environmental humidity of 40 % (blue) and > 99 % (magenta) respectively while the chamber temperature was at 38°C. Also note the slow/ insufficient equilibration of the temperature of the sample in the vial.



1.3 Technical Specification

1.3.1 Key Features

- Temperature: software controlled, direct read out, and range: 4 - 60°C. Peltier type of heating/ cooling.
- Humidity: software controlled, direct read out, and range: room conditions up to 100 % rH. Ultrasonic humidifier.
- Sample application: software controlled dipping/withdrawing from a vial. Small sample volumes can be applied manually through a side port allowing access to a pipette. The time between sample application and blotting (“wait time”) is controlled by software.
- Blotting: removal of excess liquid by a controlled blotting action using filter paper on (rotating) foam pads. The number of blotting actions (1 - 16) as well as the duration of the blotting action is software controlled. Time between blotting and vitrification (“drain time”) can be set.
- Vitrification: smooth acceleration of the thin film to the liquid ethane container. A shutter opens the chamber automatically to give the specimen access to the liquid/solid ethane (-178°C). Tailored damping action prevents bouncing of the specimen during vitrification. A lift for the ethane container brings the coolant as close as possible to the shutter to ensure optimal vitrification. The carriage of the vitrified specimen is coupled to the ethane lift and by lowering this assembly the vitrified specimen can be retrieved and stored for further investigations.
- Process control: the entire vitrification process is controlled by a built in PC. Dedicated software is used for process control and this program allows tailoring of relevant parameters to fit the specimen and the design of experiments.

1.3.2 Instrument Specifications

The Vitrobot can be run in the fume hood or on a lab bench in a well ventilated and spark free environment, with Oxygen sensor present. Filling of the ethane cup can exclusively be performed in a hume food.

Main Item	Details	Specification
Power supply	Voltage	● 100 - 240 Vac ● 50 - 60 Hz
	Fuse	● 6.3 mains fuse (90 - 150 V) ● 3.15 mains fuse (150 - 260 V)
	Full load	0.25 kVA
Conditions of use	Ambient temperature	18 - 25 °C
	Storage temperature	- 40 - 70 °C
	Relative ambient humidity	lower than 85 % rH, not condensing
Dimensions and connections	Power cable	90 - 250 V, euro female entry
	Wiring configuration	2 wires + ground, single phase
	Weight	31 kg
	Dimensions (h x b x d) in mm	890 x 260 x 413
	Footprint in mm	310 x 450

2 Safety

2.1 General Safety

- One should abide by the rules of the laboratory and local authorities to secure safe working conditions.
- The Vitrobot has been designed for use in a fume hood and is to be operated by trained personnel only.
- Working with explosive materials like ethane and propane requires working in a spark free fume hood or in a well-ventilated environment where an Oxygen level detector is installed and no open flames or high-risk sources of ignition are used. Working with these materials is at own risk!
- Only use the Humidifier with demineralized, distilled water. Any other liquids may cause damage to the Vitrobot.
- To prevent any formation of bacteria in the pre-heated water of the humidifier, it is advisable to dispose the water in the humidifier at the end of each working day!
- Working with Biohazard materials is at the user's own risk and responsibility!
- Do not perform internal trouble shooting unless you are a trained service person and unless another person, capable of rendering first aid or resuscitation, is in the immediate vicinity.
- Be aware of the location of the nearest phone.
- Be aware of the emergency services number.
- Always ensure a safe environment when performing service or maintenance.
- Make sure your hands are dry and that you are standing on a dry, insulated surface, capable of withstanding the accessed voltages.
- Make sure the Vitrobot has been leveled to avoid the chance of toppling over.
- Thermo Fisher Scientific cannot be held responsible for the consequences of improper handling!
- Filling of the brass cup with Ethane should be done in a fume hood. When the cup is filled and liquid ethane is equilibrated near its freezing point (-183°C) by cooling liquid nitrogen, ethane evaporation is minimal and the Styrofoam container with liquid ethane and liquid nitrogen can be moved from the fume hood to the Vitrobot Mk IV
- At the end of sample preparation, the brass cup with liquid ethane should be placed back to the fume hood and let evaporate at room temperature inside the fume hood with turned exhaust on

2.2 Messages and Symbols

Messages

The following messages are used throughout the manuals to highlight information.

Note Text

A Note message indicates the information in it requires special attention.

CAUTION!

Text

A Caution message indicates a potentially hazardous situation that, if not avoided, may result in moderate or minor injury. It may also be used to alert against unsafe practices.

WARNING!

Text

A Warning message indicates a potentially hazardous situation that, if not avoided, may result in death or severe injury.

DANGER!

Text

A Danger message indicates an imminently hazardous situation that, if not avoided, will result in death or severe injury.

Explanation of warning symbols

CAUTION!

Cold substances could be present. Extremely low temperatures are reached using Liquid Nitrogen. Precautions must be taken where this sticker is visible.

Location: On the LN_2 container.

CAUTION!

Heated surfaces. Extreme temperatures may be present. Take necessary precautions.

Location: In climate chamber.

CAUTION!

Pinch hazard! Hand surface injuries can occur when the Ethane container lift is raised or lowered. Keep hands away below or above the Ethane container lift when it is operating.

Location: Below Ethane container lift.

DANGER! **Indications that there are lethal voltages present in the electronics compartment. Take necessary precautions when removing the covers.**

Location: In electronics compartment.

CAUTION! **Protect ESD-sensitive devices from electrostatic discharge.**

Always work with:

- **An ESD table mat.**
- **An ESD wristband.**
- **ESD-safe gloves.**

Make sure that the ESD wristband and ESD table mat are connected to the same electrical ground as the ESD sensitive device.

Location: On ESD bags of spare parts.

2.3 Mechanical Moving Parts

CAUTION! **Pinch hazard! Hand surface injuries can occur when the Ethane container lift is raised or lowered. Keep hands away below or above the Ethane container lift when it is operating.**

- Always keep hands away from the hole below the climate chamber. A pinch hazard can occur if the ethane container is missing on the ethane lift, tweezer is mounted and a plunging cycle is performed.
- During the regular use of the Vitrobot, mechanical parts will move. The chamber door needs to be closed for the vertical metal rod to operate. The rod will be plunged at high speed in the vertical downward direction. Until the metal rod is decelerated and completely stopped in its most downward position, keep your hands away from the space right underneath the Vitrobot chamber.
- Vitrobot tweezers have a pointy, sharp tip; care must be taken to handle the tweezers with care, as direct contact with the sharp tip will result in a skin puncture and cut. Handle the Vitrobot tweezers as any other sharp object.
- If there is any suspected malfunction of any of the mechanical moving parts, do not attempt to fix it yourself. Always turn off the instrument and contact the Thermo Fisher Scientific service to schedule an Thermo Fisher Scientific service engineer on-site visit.

2.4 Electronics

DANGER! **Indications that there are lethal voltages present in the electronics compartment. Take necessary precautions when removing the covers.**

Note	Users are not allowed to take of any cover. Lethal voltages are present inside the Vitrobot.

- Users should notify Thermo Fisher Scientific service immediately when any electrical error occurs.

Fuses

Only trained service personnel should replace fuses. Replace only with fuses of the same type, voltage rating, and current.

2.5 Thermal

CAUTION! **Cold substances could be present. Extremely low temperatures are reached using Liquid Nitrogen. Precautions must be taken where this sticker is visible.**

Metallic parts of Vitrobot liquid nitrogen container

The Vitrobot will be provided with metallic parts, which are cooled with liquid nitrogen during use. During normal use, these get extremely cold (-196 °C). These metallic accessories, once cold, should not be handled by hand directly, but with appropriate tweezers covered with an insulating material, to avoid skin burn.

Liquid Ethane/Propane

When used in the appropriate small container provided with the Vitrobot accessories, a small volume (~4 ml) of liquid ethane/propane is formed by cooling the gases at liquid nitrogen temperature; there is a risk of skin burns upon direct contact with liquid ethane or liquid propane or a mixture of both. Use protective eyewear during the entire session.

- Ethane and Propane gases are highly flammable and, along with Nitrogen, pose an asphyxiation hazard. They should only be used in a well-ventilated, spark-free environment with an Oxygen level detector. The Vitrobot can be used on the lab bench, but Ethane filling/condensation is to be performed in a fume hood
- It is recommended that a small Ethane/Propane gas bottle is used in the lab where the Vitrobot is installed; usually 2.5 l – 5.0 l; the reduced dimensions should facilitate storage of the gas bottle in a fire-proof cabinet when not in use.
- A pressure regulator with two dedicated gauges (100 psi to 5 psi) is needed to extract the gas in a regulated, constant and safe manner.

Liquid nitrogen (LN2)

Liquid Nitrogen is a cryogen (-196 °C) and should be handled with care and with appropriate personal protective equipment (eye protection, cryo gloves,lab coat). Direct contact with liquid nitrogen will cause severe frostbite of skin and eyes. A 4.0 l container for the Liquid Nitrogen is recommended to work near the Vitrobot, larger volumes are usually not required for a Vitrobot session aiming at freezing 4-12 samples (= 4 to 12 grids).

The Vitrobot has a LN_2 container with a cup filled with liquid ethane to vitrify samples. Only authorized personnel should carry out replenishment and the SDS for liquid Nitrogen (refer to SDS overview) should be read and understood. The use of liquid Nitrogen can give a risk of asphyxiation. Be careful when handling pressurized gas of any kind. Thermo Fisher Scientific advises to install a

separate oxygen detector in the room. Most cryogenic liquids are odorless, colorless, and tasteless when vaporized. When cryogenic liquids are exposed to the atmosphere the cold boil-off gases condense the moisture in the air, creating a highly visible fog.

2.5.1 Liquid Nitrogen specific safety precautions

Wear safety goggles or a full faced mask and cryogenic gloves when handling Liquid Nitrogen. Special clothing may be advisable. It is preferable to wear trousers outside of boots or work shoes. Cryogenic gloves are for indirect or splash protection only, they are not designed to protect against immersion into cryogenic liquids!

- Always handle these liquids carefully to avoid skin burns and frostbite. Dewars must be moved carefully. Sloshing liquid into warmer regions of the container can cause sharp pressure rises. Carry the vessel with both hands and as far away from your face as comfortably possible.
- Use a cryogenic glove and/or tongs to handle any object going into or out of the Liquid Nitrogen.
- Dewar flasks are under vacuum to provide insulation and can collapse from thermal shock or slight mechanical shock.
- Before usage, the inside of the Dewar flask should be inspected for etched surfaces, cracks or pinholes which could cause an explosion. **When damage is observed the flask should be replaced.** If there is a small leak in the flask e.g. developed when it is frozen and some liquid “enters” the flask vacuum compartment it will expand when the flask is warmed up, (expansion ratio is 696:1) and cause an explosion.
- If cryogenic liquid or cold boil off contacts a worker's skin or eyes, frozen tissues should be flushed or soaked with tepid water (60-100 F, 16-38 °C). DO NOT USE HOT WATER. Cryogenic burns which result in blistering or deeper tissue freezing should be seen promptly by a physician.

Note For more information about possible dangers, causes and recommendations when using a Dewar flask, see manufacturer’s safety instructions.

2.6 Vitrobot Climate Chamber & Humidifier

CAUTION! **Heated surfaces. Extreme temperatures may be present. Take necessary precautions.**

The Vitrobot chamber and humidifier can be operated at a temperature range between 4 °C and 60 °C; when used at hot temperatures, care should be taken not to touch hot surfaces to avoid skin burn.

2.7 Safety Data Sheets (SDS)

For Safety Data Sheets, please refer to your chemicals supplier.

2.8 Biohazard

- When used in a ML-II (BSL-2) or higher category lab, especially when working with infectious biological material, the Vitrobot pads can be protected to avoid contamination by covering them with a piece of Parafilm M® plastic sheet, carefully placed between the sponge material of the pads and the filter paper disc used for blotting the samples prior to the plunging session.
- After use, both the filter paper disc and the Parafilm M® sheet should be disposed according to the EH&S rules and regulations in vigor depending on the BioSafety level and local officer guidelines.

2.9 Decontamination and Decomissioning

Some of the system construction materials are recyclable. The system is comprised mainly of steel, aluminum, copper, and lead. Standard PVC insulation is used on most cables. Additionally, the system contains components included in standard electronic equipment.

Consult local environmental agencies for decontamination and cleaning procedures and for recycling and disposal of electrical equipment. Process chemicals must be disposed of according to local environmental requirements.

According to the Waste Electrical and Electronic Equipment (WEEE) directive, these products of Thermo Fisher Scientific are classified as “monitoring and control instruments” (WEEE-category 9). In accordance with the requirements of the WEEE-category classification, the following applies:

For its products sold in the European member States, Thermo Fisher Scientific guarantees the users of Thermo Fisher Scientific equipment that collection, treatment, removal, and environmentally sound disposal of this product at end of life is part of the transaction.

Go to https://www.thermofisher.com/us/en/home/electron-microscopy/recycling.html for details.

Unless Member State legislation requires a different approach, Thermo Fisher Scientific policy for older equipment that was on the market before 2005-08-13 (referred to as “historical waste”) is that all costs shall be borne by the users.

Customer is responsible for materials, compound and biological materials which are analyzed with the system. The proper decontamination is in responsibility of device user.

3 Operating Instructions

Vitrobot should only be operated by trained personnel. Vitrobot users must have a basic knowledge of the vitrification process.

In this chapter, the complete operation of the Vitrobot, both hardware and software is stepwise explained and where possible visualized.

3.1 Starting Up – Filling the Humidifier

Prior to activation of the Vitrobot, the humidifier must be filled with distilled water. For this, the syringe needs to be filled with 60 ml of water before filling the humidifier through the plastic tube at the bottom part. After injection of the water into the humidifier, it is important that an “under-vacuum” is created inside the syringe (by “de-filling or pulling” the syringe while keeping attached to the plastic tube). By pulling out “air” from the humidifier, the humidifier is properly filled with water (see also the instruction movie for further details).

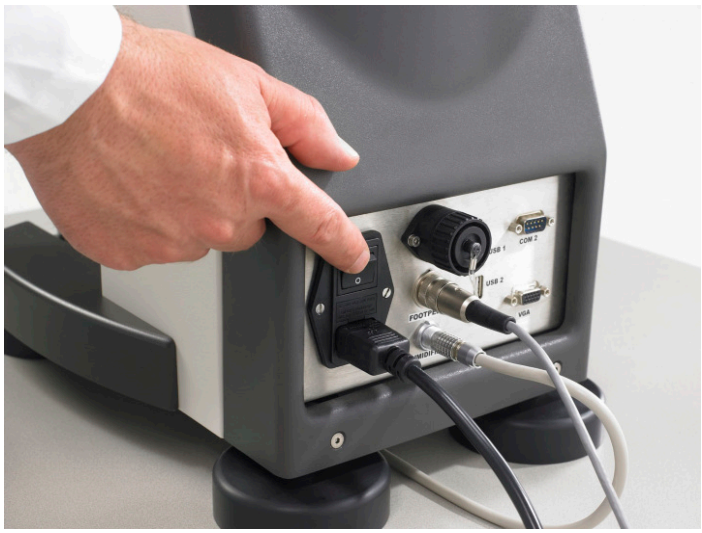
The humidifier is bajonet-attached to the bottom of the climate chamber. By turning and pulling, the humidifier can be removed. For further details on the exchange or refreshment of the water see:

3.2 Starting Up – Switching on the Vitrobot

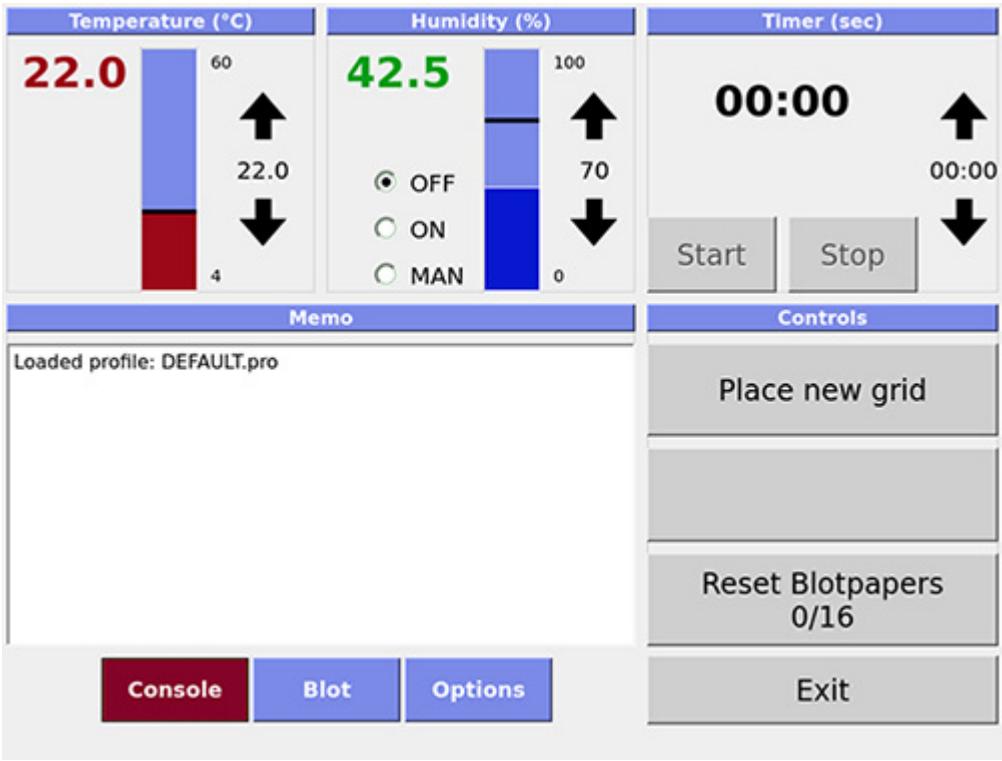
Prior to switching on the Vitrobot by using the hard lock switch on the backside of the Vitrobot, make sure all cables and wires are properly connected.

Note Be aware to fill the humidifier beaker with sufficient distilled water prior to enabling the ultrasonic humidification!

Note The humidifier has to operate on distilled water. Any other liquids used may cause damage to the Vitrobot™.

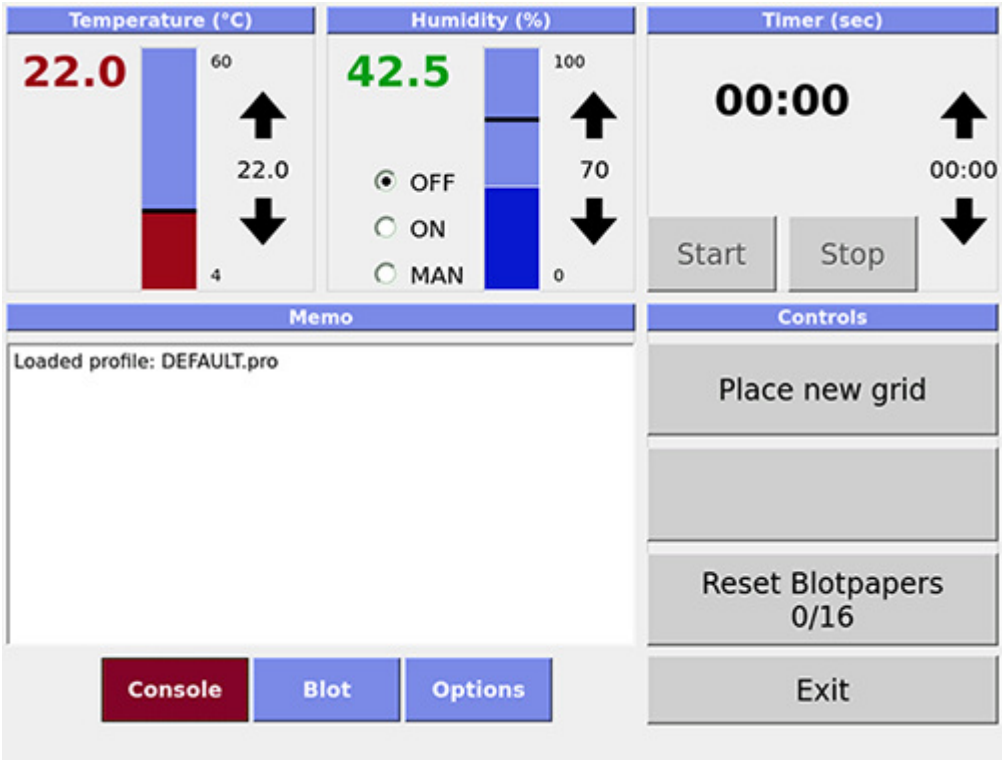


After activation of the hard lock switch the embedded PC with Linux operating system is automatically starting up. The Vitrobot User Interface page will appear after a few seconds.



3.3 Vitrobot User Interface

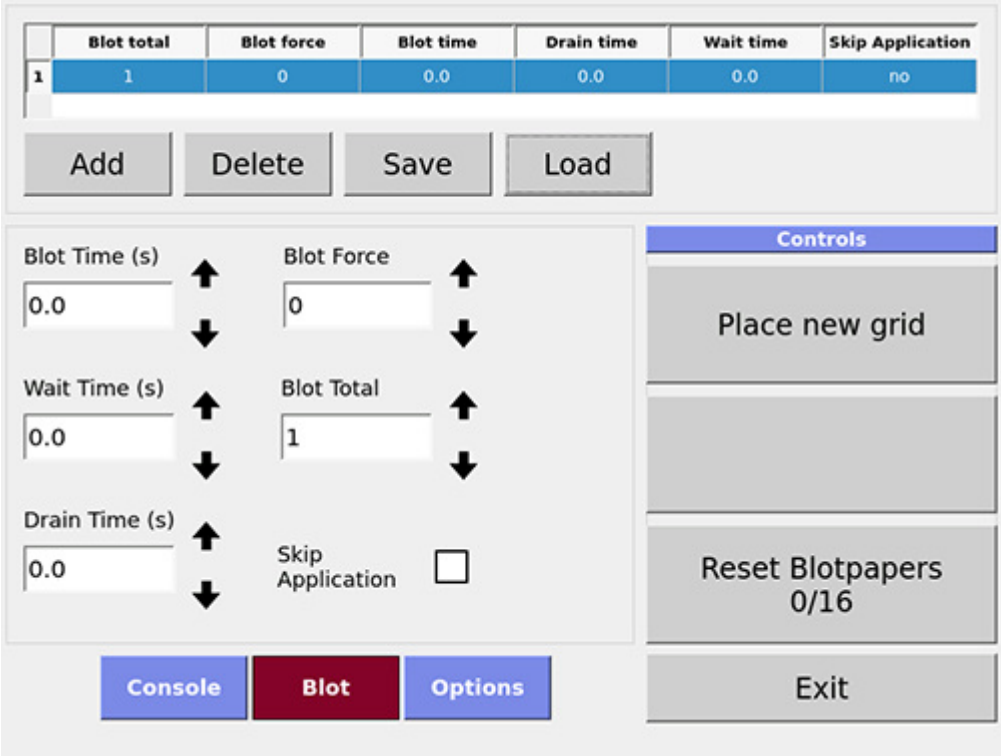
The Vitrobot User Interface consists of following pages: the Console, Blot and Options screens. In all pages, a variety of vitrification parameters can be set.



In the **Console** screen the temperature can be set towards any value between 4 °C and 60 °C with the + and – buttons or drag the black marker up or down. The actual temperature read-out is displayed in red.

Similarly, the humidity – displayed in green - in the climate chamber can be set. Select the desired humidity value of choice with the + and - buttons or drag the black marker up or down and enable the humidity switchbox to start the evaporation (On/Off switch).

A chronometer is added to record experimental times. Once a specific time is set using the up and down arrows, the chronometer starts to count down.



In the **Blot** screen, additional vitrification parameters can be set. For example the relaxation time before the blotting (Wait Time) and the intermediate time between blotting and plunge freezing (Drain Time).

Parameters that affect the blotting process are the number of blottings (Blot Total), the time of each individual blot (Blot Time) and the force of the blotpads onto the grid (Blot Force). The latter determines the force at which the excess of fluid is removed from the grid.

The last possibility is to Skip Application of sample.

One of the features is the option to do repetitive sample application onto the grid and subsequent blotting prior to plunge freezing. To activate this function press Add and define the application parameters of the first substance that is to be applied onto the grid. In the Processes section of the screen, the parameters are displayed. By again pressing ADD, the application parameters for the second substance can be defined – as displayed in Processes. Up to 20 application cycles can be added in this way (1,2,3,4….20).

The Console Screen also gives the opportunity to control the mouse sequence of the complete vitrification process (Place new grid, Start Process, etc) or to Exit the interface. After 16 sequential blottings, the Reset Blotpaper button becomes red pointing out that the blot papers need to be replaced.

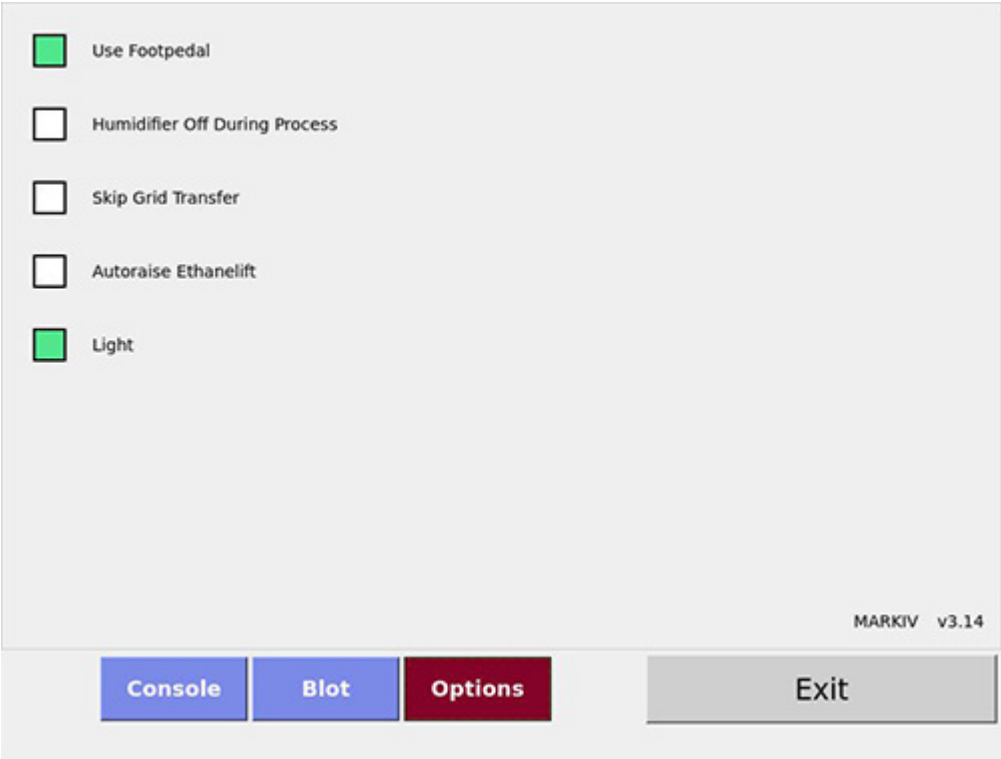
The memobox on the left side of the interface functions as an event logger. All major actions and warnings are displayed there.

By pressing Delete selected application parameters can be removed from the sequence list.

In addition, all essential freezing parameters can be saved (Save) and loaded (Load) according to the type of sample or experiment that is performed.

In the **Options** screen, the use of a foot pedal switch (as alternative for the stylus on the touchscreen), and the possibility to switch off the humidifier during manual application and plunge freezing can be chosen. The semi-automatic grid transfer (i.e. the automatic movement of the grid

from the liquid ethane/propane towards the grid box in the liquid nitrogen atmosphere) is default activated but can be de-activated by checking the skip box. In case the auto raise ethane lift option is checked, the coolant container will be lifted towards the bottom of the climate chamber simultaneously with the uplift of the tweezer (you can also do this in separate actions).



The light in the climate chamber can be switched off as well (“Light is On” changes into “Light is Off” upon activation).

Summary of Parameters that can be set

Item	Parameter	Range	Increment
Blot-time (s)	0 - 99999	± 0.1	± 0.5
Blot process	0 - 20	1	1
Blot force	-25 - +25	± 1	± 1
Drain time (s)	0 - 99999	± 0.1	± 0.5
Wait time (s)	0 - 99999	± 0.1	± 0.5
Skip application process	on / off		
Use foot pedal	on / off		
Hum off during process	on / off transfer		
Skip Grid Transfer	on / off		
Auto raise Ethane lift	on / off		
Light	on / off		

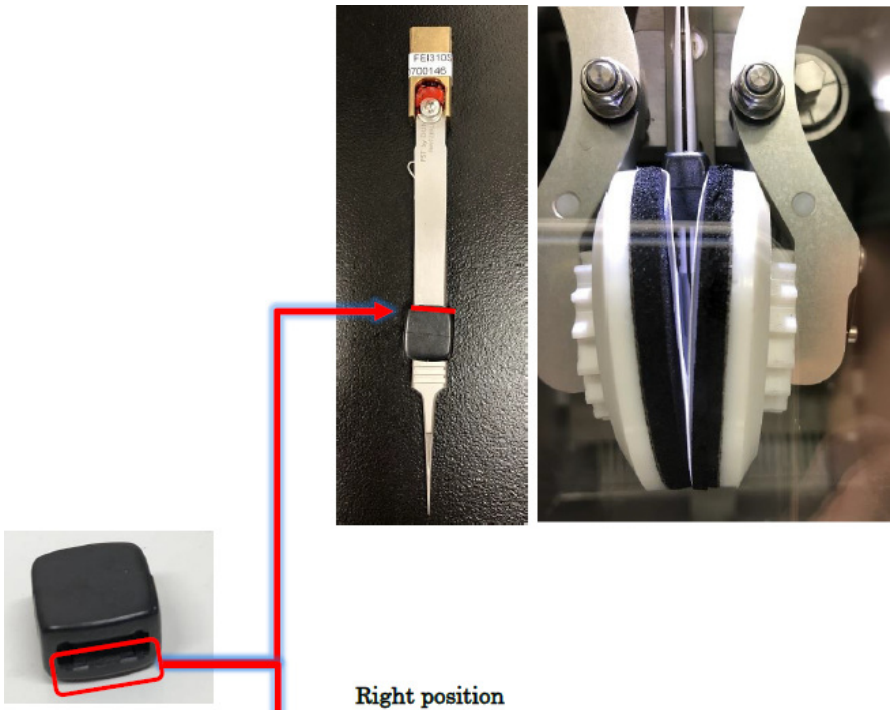
3.4 Preparation of the Climate Chamber

The climate chamber needs to be prepared for the vitrification process. Besides setting the proper parameter conditions, the LCD lights are switched on, pneumatic pressure control must be active and the blotting papers are put on either side of the blot pads. The papers can be attached to the blot pads by using the white circular clipping rings.

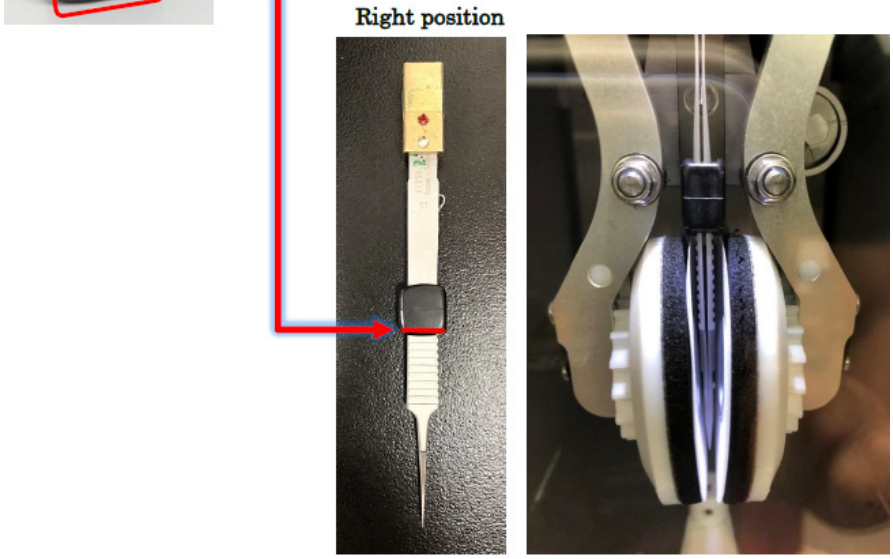
For details about setting the proper parameter conditions.see: Starting Up – Switching on the Vitrobot on page 14

3.5 Preparation of the Grid

A glow-discharged grid (preferably a quantifoil or lacey carbon film grid) is attached to the tweezer. Make sure that the black clamping ring is fixed in such a way that the grid does not fall off in its vertical position (down to the first groves in the tweezers).



Right position



Mount the tweezer onto the connection grove in the central axis. To do this, first select the ‘Place New Grid’ button in the Vitrobot User Interface to put the central axis in the right position for mounting the tweezer to it.

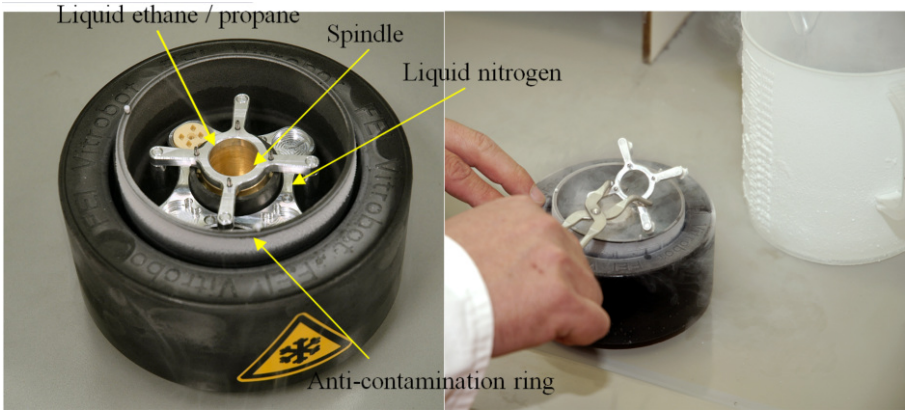
The tweezers with grid are subsequently lifted into the climate chamber by selecting the ‘Start Process’ button in the User Interface, or alternatively, use the foot pedal switch. The environmental and process parameters (i.e. temperature, humidity, number of blots etc) can now be set as described in: Starting Up – Switching on the Vitrobot on page 14

3.6 Preparation and Lifting of the Coolant Container

Prior to setting the coolant container in the proper position for vitrification it needs to be pre-cooled in advance.

To do so, the outer ring of the container must be filled with liquid nitrogen. The central cup can be pre-cooled with liquid nitrogen before filling with ethane or propane. Cooling is a clear two-step process, to be carried out in a fume-hood; first the peripheral reservoir will attain liquid nitrogen temperature; in a second stage the central part having a higher heat capacity will cool down. Vigorous boiling (“Leidenfrost effect”) followed by a “calm” equilibrium indicates that the metal parts have attained liquid nitrogen temperature.

When the central cup is at liquid nitrogen temperature, ethane or propane can be condensed in this cup (check for remaining liquid nitrogen in the center part, wait for complete evaporation). While condensing the ethane or propane keep monitoring the peripheral ring; refilling this reservoir with liquid nitrogen might be necessary. If the ethane or propane does not condense, the metal parts are not at liquid nitrogen temperature yet. To speed up the condensation and temperature decrease of liquid ethane/propane, the metal spindle can be positioned on top of the central cup (with its “feet” into the pre-cooled peripheral ring). The central cup should be filled up to the brim with liquid ethane or propane for optimal vitrification. Indicative for the optimum freezing temperature of the ethane may be a white solidified halo of ice on the inner side of the central cup. Immediately after the appearance of this halo of ice, the metal spindle must be removed. See also the instruction video for more visualized details.



When the ethane/propane container is ready for vitrification the holder can be placed on the platform-ring under the Vitrobot.

Note Make sure that liquid nitrogen remains present in the peripheral ring throughout the entire vitrification procedure.

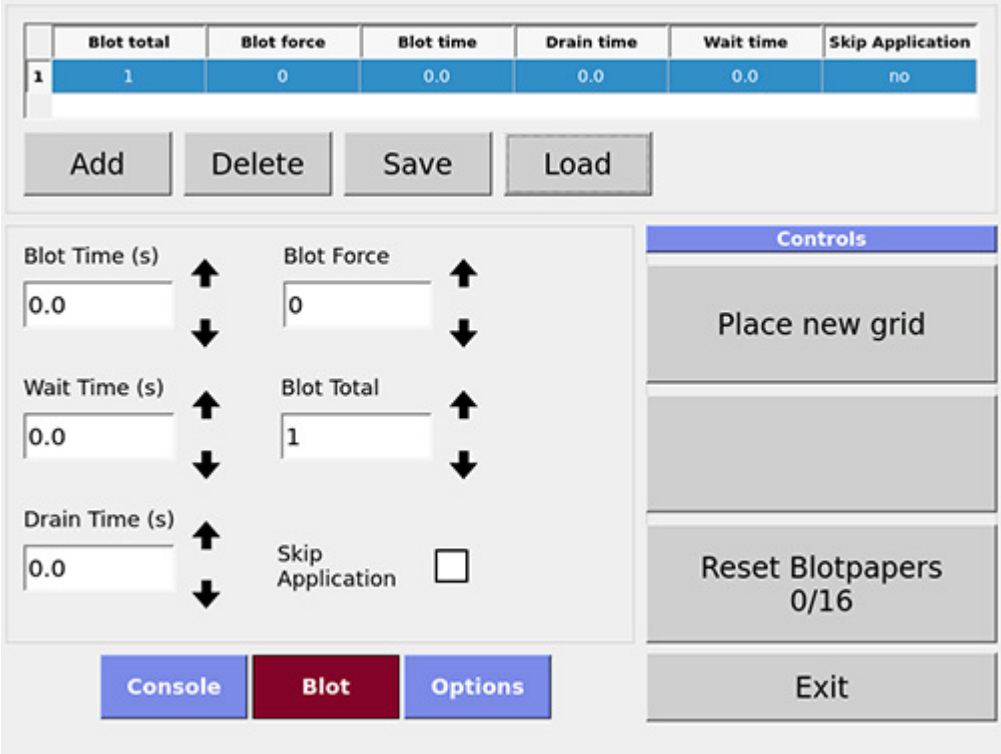
Note Make sure that the metal spindle is removed from the coolant container prior to the vitrification procedure.

Note Working with explosive materials like ethane and propane requires working in a spark free-fume hood!

After the coolant container has been placed on the platform-ring, the foot pedal switch or the ‘Continue’ button in the User Interface must be pressed in order to raise the container towards the bottom of the climate chamber.

3.7 Sample Application

It is possible to skip application if the sample is already applied onto the grid.



For manual application of the sample onto a grid, select with the foot pedal switch or the stylus ‘Continue’ to proceed. The tweezers is slightly lowered as to allow the application of suspension through the side-entry port using a pipette. As a consequence of this method only one side of the grid is inoculated with suspension. The advantage of this method is that only small volumes of the sample (typically 3 µl) are used.

3.8 Blotting and Vitrification

The excess of suspension must be removed from the grid prior to plunge freezing. To do so, select ‘Continue’ in the UI or use the foot pedal. This activates a slight uptake of the grid towards the correct position between the blot pads and a subsequent blotting of the grid. The blotting conditions can be set in the ‘Options page’ of the UI (. After each blot, the blot pads undergo a slight rotation to ensure a clean, new area of filter paper for the next blot session.

For details about the UI see: Starting Up – Switching on the Vitrobot on page 14

Note After a maximum of 16 blottings, the filter papers must be replaced. The UI displays the message: ‘Replace Blotpapers’.

The blotting procedure is immediately followed by injection of the tweezers with grid into the liquid ethane or propane. The only delay between blotting and plunge freezing is determined by the drain time that can be set in the ‘Options’ page of the UI and the time required for removing the shutter from the hole in the climate chamber. The actual plunging mechanism is mediated by a pneumatics (air pressure) at the central axis combined with the gravitational force.

After plunge freezing both the liquid coolant container and the tweezers with grid are automatically and simultaneously lowered while keeping the grid inside the liquid ethane. This prevents any contamination of the freshly frozen sample.

3.9 Transfer of Vitrified Grid

After vitrification the frozen grid must be transferred into a storage box or cryo holder.

With the semi-automated grid transfer default active, the grid is automatically transferred from the liquid ethane/propane towards the liquid nitrogen atmosphere. Prior to positioning the grid into the grid box, the tweezers must be carefully disconnected from the central axis. To make the grid transfer more convenient, the coolant container should be lifted from the support ring and positioned next to the Vitrobot.

It is recommended to remove the access of liquid ethane – that may be capillary present in the groove of the tweezers that hold the grid – with some filter paper. The anti-contamination ring, which floats on the liquid nitrogen, creates a cold gaseous atmosphere which facilitates the transfer and minimizes possible ice contamination on the grid. The outer ring contains a circular storage grid box for four grids underneath a layer of liquid coolant gas.

After the grids have been transferred into the grid box, they are sealed with a special screw and usually stored into a somewhat larger dewar for liquid nitrogen prior to the transfer into a cryo holder and subsequently a TEM or SEM.

The transfer of the grid box towards nitrogen container should be done fast and swiftly as to minimize the risk of sudden and unwanted temperature shifts.

3.10 Summary - Vitrification Control through UI

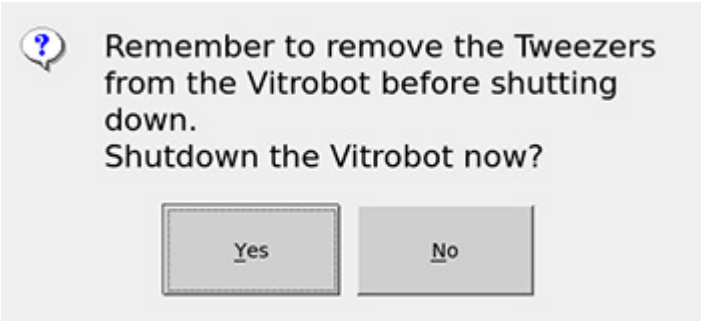
This table summarizes the instrument action in relation with the activated knobs and buttons in the Vitrobot User Interface.

Console screen	**Instrument action**
Humidity disabled/enabled	Humidity will be brought to the set value when enabled
Place new grid	Shutter opens and forceps are presented or can be placed. Make sure the application side of the grid is at the right or left side (equal to the application orientation), when the forceps number on the brass dovetail is visible.
Continue	Forceps enter the chamber, shutter closes (time for thermal equilibration) and the ethane container is raised (optional). “Place new grid” will still be active in case grid replacement is considered essential.
(Continue)	When “auto raise ethane lift” is switched off from the options page; “continue” (or foot pedal) should be pressed again before the next step can be activated.
Start process	Specimen preparation and vitrification will proceed according to the parameters set from the software (see guidelines above). Processing ends by sample vitrification and lowering the vitrified sample in the ethane for sample retrieval. The humidifier will be shut off automatically at the end of the process to prevent excessive use of water.

Console screen	Instrument action
Place new grid	Once vitrification is completed processing of a new specimen can be started. By activating “place new grid” the forceps is raised above the ethane level to place a new grid (a closed loop in instrument operation). At this stage varying of instrument parameters should be considered to meet the requirements of the specimen.
Reset blot paper	Resets the counter for the remaining blots left on a piece of blot paper after replacement.
Exit	Shuts down the Vitrobot system after use.

3.11 Shutting down - Switching off the Vitrobot

At the end of the working day, the Vitrobot can be switched off. To do this, press the “Exit” button in the interface. The following question appears:



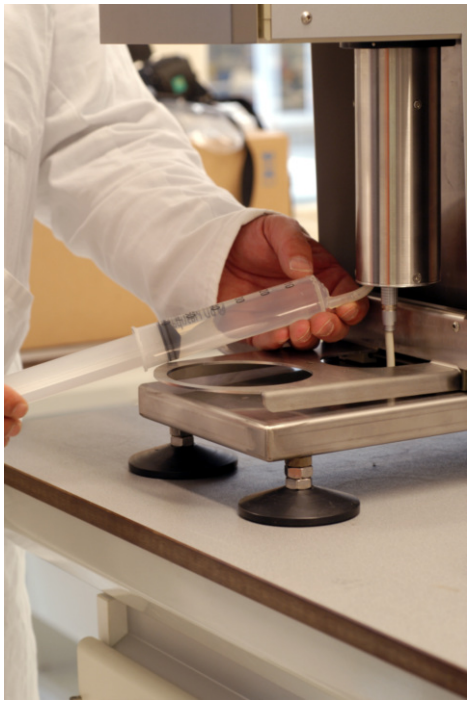
After removal of the tweezers, the central axis is moving into its parking position (inside the climate chamber), the LED is switched off and the user interface shuts down. The LCD will light up brightly. The Vitrobot may be shutdown subsequently.

3.12 Shutting down – Emptying the humidifier

After shutting down the Vitrobot PC, the remaining water in the humidifier may be removed. To do this, pull the metal ring connector downwards to disconnect the electronic cable from the humidifier. Subsequently, twist and pull the humidifier in order to unleash the bajonet-connection. Emptying the humidifier is a two step process:

1. Pouring the humidifier will empty the central reservoir.

2. Re-connecting the syringe to the plastic tube at the bottom part and removing the water from the outside reservoir is the second essential step



Note To prevent any formation of bacteria in the water of the humidifier, it is advisable to dispose the water supply in the humidifier at the end of each working day!

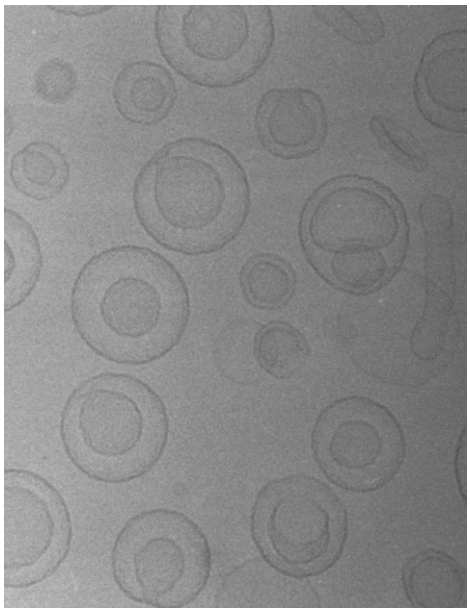
4 Troubleshooting

4.1 Guidelines for Optimal Specimen Preparation

The instrument performance has been tested with phospholipid vesicles (dipalmitoyl-phosphatidylcholine; DPPC 10mg/ml) prepared by extrusion through a 100 nm filter.

The shape of these vesicles varies with temperature; spherical above 41 °C (lipid in the Lα phase), facetted and rippled between 37 and 41 °C (lipid in the P□' phase), and facetted below 37 °C (lipid in the Lα phase). To obtain a large area for cryo-electron microscopy (thickness below 100 nm, good vitrification) two short blotting actions were employed. With low viscosity samples, such as a DPPC suspension, we recommend to increase the number of blotting actions (two blotting actions instead of one) when vitrified films are too thick. With high viscosity samples we recommend to increase the blotting time (e.g. 2-3 sec) before deciding for an increase in the number of blotting actions.

Also note the critical effects of the environmental humidity prior to a vitrification action. With an unsaturated environment the actual temperature of the sample will be lower than the set temperature of the chamber (dew-point effect, demonstrated with the shape of DPPC vesicles!). Evaporation will also accelerate the thinning of aqueous thin films. Thinning may result in concentration of particulate material by a sweeping action of two concave air-water interfaces approaching each other and eventually flattening out in an equilibrium configuration. The effects of thinning are recognized in the image after some experience with cryo-observation. Temperature effects and osmotic effects are often not readily observed in vitrified specimens. An evaporation of 50 % of the water volume during preparation of a thin film (10 sec, 40 % rH) was experimentally determined from volume changes in liposomes (pictured the collapse of spherical liposomes into two interconnected concentric spheres).



The dew point (= temperature) and osmotic effects of water evaporation can be prevented by using the humidifier to obtain a relative humidity > 99 %. Concentration of particulate material, inherent to conventional cryo-preparation (and not necessarily a disadvantage), is virtually absent (or slowed down) when preparation is carried out at water saturating conditions. Increasing the concentration/ density of the material may be necessary to obtain a useful vitrified specimen with chamber conditions at an rH > 99 %. Under these conditions it is the blotting that determines the initial thickness of the thin film; further thinning will occur during the drain process. Gentle and reproducible

blotting as performed by the Vitrobot™ has to be tailored (e.g. blotting time, number of blotting actions) to the requirements of the specimen, when an even film is obtained, the thickness can be optimized by choosing an appropriate drain time (e.g. 10 sec).

4.2 General Remarks on the Operational Procedures

One should abide by the rules of the laboratory and local authorities to secure safe working conditions. The Vitrobot™ has been designed for use in a fume hood exclusively and is to be operated by trained personnel only. Operation of the Vitrobot™ requires basic knowledge of the vitrification process.

The instrument operates only by (build in) PC control. Check PC screen (messages) and connections before starting an experiment. For temperature and humidity equilibration some 20-30 minutes are required (also see test read-outs provided with the instrument). Experiments performed at high humidity require sufficient water in the water container. If the humidity fails to reach the set value (normally within 1-4 min.) check the water level first. Increasing the relative humidity in the chamber will affect the temperature and for critical experiments an additional period for thermal equilibration is thus required. Do not switch on the humidifier before the desired temperature has been reached.

Remember that a sample in a vial takes more than 20-30 minutes for thermal equilibration within the chamber. Pre-heating (incubator, water bath) is recommended for critical samples.

Blotting papers (∅ 55 mm with hole) should be mounted at an early stage in the procedure. Replacing the blot papers when putting a new sample in the chamber is a good routine to prevent unnecessary opening of the chamber. Make sure that the concave side of the paper is put against the blot pads. The filter papers are fixed against the blot pads with the white clip ring.

Excess of ethane in the central cup (a meniscus appears outside the horizontal plane of the cup) may be removed with some filter paper.

Special applications

On the web-site www.thermofisher.com/fei procedures are given for some special applications. Please consult the website.

4.3 Troubleshooting

In the following paragraph a number of possible errors are described and some points for action are considered.

Problem description	**Solution**
The Vitrobot™ is not switching on	Check if the power entry is turned on.
The blot paper does not hold	Check the clipring mechanism. The clipring should fix the paper onto the blotpads.
The humidity will not rise	The water level in the humidifier cup. The humidifier contains a sensor, which notifies (by a message in the interface) in case the water level is too low. Re-filling of water may be needed. Call service if the water level is ok, but the humidity is still not increasing upon activation.

Problem description	Solution
The Vitrobot™ does not reach the desired temperature	A certain ΔT (downwards of approximately 20 °C, upwards of approximately 55 °C), depending on the rH of the environment and climate chamber, can be reached. Check the temperature of the environment. Extra cooling can be attained by gently providing cool air into the backside of the Vitrobot™. Extra heating can be attained by gently providing warm air. The side door for application may be still open.
A broken grid	Make sure the correct volume of the specimen is accounted for on the option screen. Defining the level too low will cause the forceps to plunge too deep. This will catch liquid between the tip of the forceps resulting in improper blotting and poor release of the vitrified grid from the forceps. The necessary force to remove the grid later in the ethane holder can cause damage to the grid
Film is not located in the correct orientation on a quantifoil	Make sure the application side of the quantifoil is in sync with the carbon-site of the grid. Application can be done both through the left and right hand side opening of the climate chamber. Check the blot off-set value in the options page. It may be that the blot pressure is not properly defined.
Quantifoil or lacey carbon film is damaged during processing	Centering of forceps between the blot pads is incorrect. This is likely the result of either one of the two causes: 1 the tip of the forceps is misaligned with respect to the rod catching brass part (accidental dropping or undue force?) or alternatively 2 the blotting mechanism is misaligned with respect to the vertical movements of the forceps. Check mechanical centering of the tip of the forceps and the brass part with the alignment tool (alignment tool is not standard delivered with each system). If this is alignment is correct and problems remain the mechanical alignment of the blotting mechanism is the next probably cause of specimen damage. Consult the service instructions.

Revision History

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To provide feedback on this document, reach out to MSD.SerDocumentation@thermofisher.com.

Revision	Date	ECO number	Description of Changes
A	-		Initial Release
B	10-Feb-2016		Document migrated to CMS and updated
C	28-Feb-2018	SDR14165	Change in temperature value of tepid water
D	07-Jan-2019	SDR12047	Updated Decontamination and decommissioning chapter
E	19-Jul-2019		Corrected a typo on the front cover page
F	02-May-2024	SDR42834 SDR47443	Updated link to website for WEEE (Recycling) Updates after review by Product Manager
G	30-Jun-2024		Updated the use of Vitrobot on the lab bench Update of working parameters
H	31-Jul-2024		New UI update

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