CRYOPRESERVATION MANUAL

Recommended use of Vitrolife Cryopreservation products

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CRYOPRESERVATION

Improving success rates

Vitrolife is dedicated to improving the success rates of Human Assisted Reproduction. Long term research in reproductive physiology and studies of embryo development has resulted in the most advanced IVF media products available. This manual describes the use of Vitrolife's Rapid-i Vitrification System™, RapidVit™ and RapidWarm™ solutions as well as G-Freeze™/Thaw Kit Cleave™ and SpermFreeze Solution™.

We are well aware that there are many ways of practicing cryopreservation. The methods we describe below have resulted in high success rates with our products.

Our cryopreservation products

Vitrolife offers a full range of cryopreservation products. Every LOT undergoes rigorous Quality Control testing before release to customers. With every released LOT, a Certificate of Analysis is issued.

Quality Control together with quality assured operations (ISO 13485:2012 and 21 CFR Part 820) guarantees LOT-to-LOT consistency. All raw materials used for the manufacturing of our products are selected as the best available on the world market. All raw materials are tested and evaluated individually by stringent quality control and MEA procedures before use in the manufacturing of our cryopreservation products.

We are certain you will agree that the "creation" of new human life deserves the best possible environment. We are committed to bringing you the highest quality products.

GENERAL

Opening your cryopreservation products

When you receive your delivery of Vitrolife products you may notice that they are packaged in a special way. There are specific reasons for this:

- All Vitrolife products are tamper-proof sealed. The packaging ensures that it is impossible to enter the bottle without visible evidence.
- All materials used in the packaging are non-toxic so that nothing may interfere with the final product. PETG bottles, screw caps, specially designed labels and pharmaceutical sealing are all part of this tamper-evident protocol.

Hands on

1. Open all products in a clean laminar air flow (LAF) cabinet.
2. Before bringing bottles into the LAF cabinet, assure the bottles are clean on the outside. It is recommended that the bottles are wiped with a lint free cloth and an appropriate disinfectant.
3. Identify the product and check the expiry date. Break the tamper-proof seal and discard.
4. Remove the cap and make sure to maintain sterility, for example place the cap face down in a sterile petri dish.
5. Remove the desired volume with a sterile non-toxic pipette. Replace bottle cap and screw on firmly. Do not touch the inner sides of the cap.

6 Keep the media cold as much as possible. Prepare vitrification or warming dishes immediately after the bottles have been removed from the refrigerator. Media bottles should not be left at ambient temperature for a longer period of time than it takes to prepare dishes.

Aseptic working techniques means to work in the absence of microorganisms capable of causing infection or contamination.

CAUTION
Never pour the contents out of the bottle, as the lip of the bottle may not be sterile once opened.

INTRODUCTION TO CRYOPRESERVATION

This manual describes procedures for cryopreservation of human gametes and embryos at different developmental stages. Since its introduction in the 80's, cryopreservation became an integral part of ART treatment and contributes significantly to the overall success.

Cryopreservation temporary stops all biological activity and allows for usage the preserved material at a later time point assisting in fulfilling the dream of many infertile couples. Cryopreservation involves the cooling, storage and warming of cells with minimal effect on viability. For this, cryoprotectants that dehydrate cells and avoid lethal ice formation are mandatory. It is important to know that cell damage does not occur in the storage phase if continuously stored below the glass transition temperature. It is the cooling and warming processes that are crucial to cell survival.

Cryopreservation of gametes and embryos can be trough slow freezing or vitrification. In both methods, crystallisation of water during cooling is avoided. While extracellular ice is present extracellular in slow freezing, it is avoided during vitrification. In both methods cells have to be dehydrated allowing them to survive the procedures. Introduction of cryoprotectant(s) is done in a fashion that is designed to reduce the risk of osmotic rupture and toxic effects on the cells by high osmolality and high concentrations of cryoprotectant.

From its introduction in the 80's until the middle of the last decade,slow freezing was the method of choice. For this, the choice of cryoprotectants used depends on the developmental stage of the embryos. Propanediol (PrOH) is commonly used for cleavage stage embryos and glycerol is commonly used for the blastocyst stage of development. Together with these penetrating cryoprotectants, sucrose is commonly used for additional dehydration by osmosis.

Despite the increasing success of vitrification, slow freezing was also improved. The work of Edgar et al. inspired Vitrolife to improve existing products resulting in FreezeKit™ Cleave and ThawKit™ Cleave. These products are built on Vitrolife´s experience with culture media and combined with the current knowledge on slow freezing, providing embryos with the best possible environment to survive the freezing and thawing process.

Today, vitrification is more commonly used. It requires higher levels of cryoprotectants and different rates of cooling and warming. The use of 2 different penetrating cryoprotectants, reducing potential toxic effects from individual cryoprotectants, is frequently used. For this, Vitrolife uses ethylene glycol (EG) and PrOH. The availability of kits designed for different developmental stages meets the specific requirements of the cells to be cryopreserved. An example of this is omitting calcium in the vitrification media for oocytes. All vitrification media contain hyaluronan for additional membrane protection.

Except for the solutions, also the carrier device is important. To avoid potential safety issues and comply with regulations in different countries, Vitrolife developed Rapid-i™, a carrier allowing vitrification without direct contact with liquid nitrogen. Today, Rapid-i™ is increasingly used and shows to be a reliable carrier device for vitrification of oocytes and embryos at different developmental stages.

Finally, also spermatozoa are frequently stored for later use. So far, these cells are still preserved using slow freezing. For this, Vitrolife developed a product that is egg-yolk free and thus meeting regulatory requirements in many countries.

VITRIFICATION

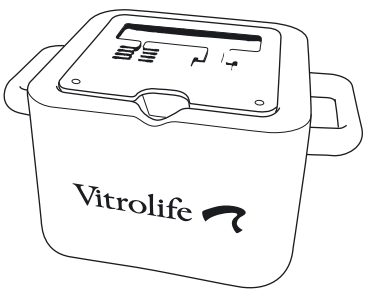
Vitrification using Rapid-i Vitrification System

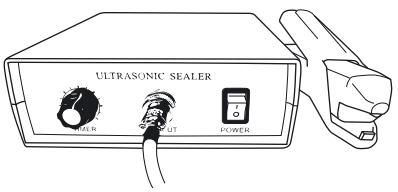
To obtain vitrification of oocytes and embryos it is essential to obtain sufficiently high cooling and warming rates. This is obtained by using a device carrying the samples in a very small volume of vitrification solution. It is important to minimise potential risks of contamination by choosing a closed device.

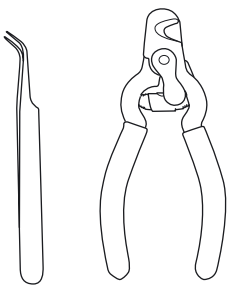
Timing with vitrification procedures is critical to ensure you follow the protocol precisely. Rapid-i™ Kit is a cryopreservation device intended to be used to contain, vitrify and maintain embryos and/or oocytes. It consists of a stick with a hole into which the sample to be vitrified is placed, a straw and a metal rod. The Rapid-i holds the sample in a volume of 30 nanolitres.

Rapid-i Vitrification System consists of the following items in addition to the Rapid-i™ Kit device:

- SmartBox™ for liquid nitrogen
- Ultrasonic sealer
- Needle nose tweezers
- Rapid-i™ Cutter







Essentials

- Plan for transfer of the Rapid-i™ to and from the long term storage without leaving liquid nitrogen, prior to starting the procedure.
- Place the Ultrasonic sealer on the lab bench and turn it on.
- Place the SmartBox™ on the lab bench close to the microscope.

Hands on

1 Fill the SmartBox™ with liquid nitrogen up to 1 cm from the box's rim and place the lid on top of the box. Always keep the top of the RapidStraw at a sufficient distance from the liquid nitrogen.

NOTE: Always maintain a sufficient level of liquid nitrogen in the SmartBox™.

2 Prepare the dishes with vitrification media according to the instructions below. Place the dishes close to the microscope.

3 Check that the Rapid-i™ Kit packaging is intact and that the use by date has not expired.

4 Open the package using aseptic technique.

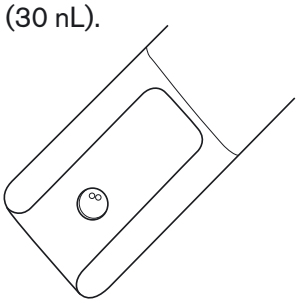
5 Label the exact number of RapidStraws needed with the patient's identification or according to local regulations. Place the label below the top, black mark of the RapidStraw.

6 Prepare oocytes/embryos for vitrification by following the instructions provided with the vitrification media.

7 After moving the oocytes/embryos to Vitrification solution 2, place the RapidStraw with metal rod in the SmartBox™to cool down. Remove the metal rod 20-30 seconds before insertion of the Rapid-i™ into the RapidStraw.

8 Place the Rapid-i™ on the microscope stage with the flat side down. Locate the correct plane of focus so that the hole of the Rapid-i™ is in view, for easy loading.

9 Collect the oocytes/embryos with a Vitrolife micropipette. Slide the hole of the Rapid-i™ into view, in the microscope. Move the tip of the pipette close to the wall of the hole in the Rapid-i™ and apply slight pressure to expel the oocytes/embryos into the hole. The maximum volume of the vitrification solution should not exceed the volume of the hole (30 nL).



NOTE: Avoid overfilling the hole or else the embryos may float out.

10 Slide the Rapid-i™ off the microscope stage and quickly, but carefully, place it vertically into the precooled RapidStraw sitting in the SmartBox™. Cover the straw opening immediately after insertion for a few seconds to prevent that the Rapid-i™ accidentally pops out. Make sure that no more than 8-12 mm of the Rapid-i™ is visible above the lid when fully inserted. The oocytes/embryos are now vitrified and should not be warmed again until the warming procedure is planned.

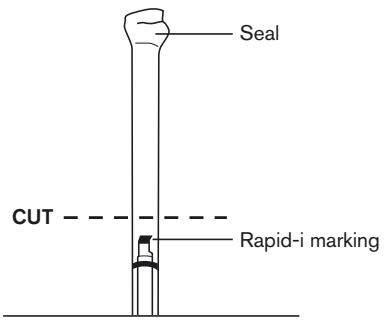
WARNING: From this point, inappropriate handling of the sealed RapidStraw may cause warming which can lead to de-vitrification of the oocytes/embryos. The sealed RapidStraw may not be removed from liquid nitrogen.

11 Seal the top of the RapidStraw using the Ultrasonic sealer. Inspect the seal to ensure that sealing was correctly performed. Make sure that the seal covers the entire diameter of the RapidStraw and that no holes or defects are present. If appropriate sealing has not occurred, attempt to reseal the RapidStraw.

12 Place the storage vessel into the SmartBox™. Move the sealed RapidStraw from the lid and into the storage vessel, so that the RapidStraw with the oocytes/embryos does not leave the liquid nitrogen. The lowest black mark of the RapidStraw must always be immersed in liquid nitrogen.

Warming

1. Prepare the dishes with warming media according to the instructions provided with the media. Place the dishes close to the microscope.
2. Move the storage vessel containing the RapidStraws into the liquid nitrogen in the SmartBox™.
3. Remove one RapidStraw from the storage vessel and place it in the lid, without leaving the liquid nitrogen. Make sure that the RapidStraw is secure in the lid and that the lower end touches the bottom of the SmartBox™.
4. Warm the RapidStraw with your fingers around the black mark to get a better view of the black tab on the Rapid-i™.
5. Hold the RapidStraw well above the black mark and cut it 3 mm above the back end of the Rapid-i™. Do not lift the RapidStraw from the lid and make sure it stays up-right in liquid nitrogen.



6. Lift the Rapid-i™ (using needle nose tweezers) out of the RapidStraw just enough to enable you to grasp the end with your finger tips. Then quickly (as fast as possible), but carefully, remove the Rapid-i™ from the RapidStraw and plunge the tip and hole of the Rapid-i™ into the first warming solution immediately. While watching under the microscope, gently move the Rapid-i™ back and forth. Verify that the oocytes/embryos are free in the warming medium and then remove the Rapid-i™.
7. Proceed with the warming according to the instructions provided with the warming media. Discard the Rapid-i™ Kit and RapidStraw according to lab routines.

Vitrification general

Essentials

- Vitrification and warming should only be performed by staff trained in such procedures.
- Note: Timing during the first warming step is crucial.
- **Oocyte vitrification and warming using RapidVit™/RapidWarm™ must be carried out at +37°C and ambient atmosphere.** Deviations from 37°C will alter the permeability of cryoprotectants, which may compromise oocyte survival.
- **The time frames set in the protocol must be very carefully followed**
- **The recommended volumes of solutions should not be changed.** Failure to use the correct volume of media may result in osmolality changes, which could cause suboptimal survival.

Vitrification and warming of oocytes and all embryo developmental stages

Vitrolife´s products for vitrification and warming of oocytes through to blastocyst stage embryos are called RapidVit™ Omni and RapidWarm™ Omni.

Vitrification

RapidVit™ Omni contains three solutions. The solutions consist of a MOPS-buffered medium containing gentamicin and human serum albumin.
- Vitri 1™ Omni contains no cryoprotectants.
- Vitri 2™ Omni contains ethylene glycol and propanediol as cryoprotectants.
- Vitri 3™ Omni contains ethylene glycol and propanediol as cryoprotectants and sucrose.

Hands on

1 Place 0.5–1 ml of each of the following media into separate wells of a multi-well dish and warm to 37 °C in ambient atmosphere:
Vitri 1™ Omni
Vitri 2™ Omni
Vitri 3™ Omni

2 All manipulations are carried out at 37 °C (on the heated stage) in ambient atmosphere. It is very important to keep the temperature of all vitrification solutions at 37°C at all times.

3 **Blastocysts:** Collapse the blastocysts by using a laser beam or by inserting an ICSI pipette.

4 **Oocytes and all embryos:** Transfer the oocytes or embryos from culture to Vitri 1™ Omni and let them remain in the solution for 5-20 minutes.

NOTE: You can use one dish for loading of maximum 4 Rapid-i before an increase in osmolality above specification will occur.

5 Move an appropriate number of oocytes/embryos into Vitri 2™ Omni.

6 **Oocytes:** Let the oocytes remain in Vitri 2™ Omni for 2-5 minutes. The oocytes will tend to float to the surface, if so, collect and replace them onto the bottom of the dish. The oocytes are expected to re-expand to their original volume within 5 minutes.

NOTE: If re-expansion is not observed within 5 minutes this could indicate that the temperature of the medium is not at 37°C. Temperature will affect the rate at which the cryoprotectants enter the oocyte and therefore the observed re-expansion.

Embryos: Embryos should remain in Vitri 2 for 2 minutes.

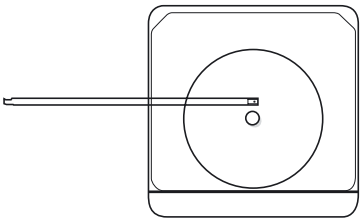
7 Prepare the cryodevice to be used.

8 When 30 seconds of exposure to Vitri 2™ Omni remain, make a 20µL droplet of Vitri 3™ Omni on a non-toxic surface, preferably a culture dish. Making a small droplet of Vitri 3™Omni enables easy loading onto the cryodevice.

NOTE: The 20 µl droplet can only be used once.

9 When 10 seconds of exposure to Vitri 2™ Omni remain, begin collecting the oocytes/embryos and transfer them to the 20µL droplet of Vitri 3™ Omni and let them remain in this solution for 30-45 seconds including the time it takes to load the cryodevice and vitrify.

NOTE: The total time from transferring the oocytes or embryos into the droplet of Vitri 3™ Omni until they are vitrified should be between 30-45 seconds.



10 Perform the actual vitrification according to the instructions above if Rapid-i Kit is used. (If another device is used follow the instructions for use provided with the device.)

11 Continue storage according to laboratory practice.

Warming of vitrified oocytes and embryos after the use of RapidVit™ Omni

RapidWarm™ Omni contains four solutions for the warming of vitrified oocytes and embryos. The solutions consist of a MOPS- buffered medium containing gentamicin and human serum albumin.

- Warm 1™ Omni contains sucrose
- Warm 2™ Omni contains sucrose
- Warm 3™ Omni contains sucrose
- Warm 4™ Omni contains no sucrose

Hands on

1 Place 0.5–1 ml of each of the following media into separate wells of a multi-well dish and warm to 37 °C in ambient atmosphere:
Warm 1™ Omni
Warm 2™ Omni
Warm 3™ Omni
Warm 4™ Omni

NOTE: The recommended volumes should not be changed. Volume changes will affect temperature control in the first warming solution as well as osmolality, which may result in suboptimal survival.

NOTE: It is very important to keep the temperature of all vitrification solutions at 37°C at all times. If more than one Rapid-i™ is to be warmed in the same dish, make sure that the temperature reaches 37°C after each warming procedure.

2 Remove the cryodevice containing the vitrified oocytes/embryos from the cryostorage container.Follow the warming instructions for the specific cryodevice used.

3 Immediately, transfer oocytes/embryos into Warm 1™Omni and allow them to fall from the device and sink to the bottom. Leave for 1 minute.

4 Transfer the oocytes/embryos into Warm 2™ Omni and let them remain in the solution for 3 minutes.

5 Transfer the oocytes/embryos into Warm 3™ Omni and let them remain in the solution for 5 minutes.

6 Transfer the oocytes/embryos to Warm 4™ Omni and let them remain in the solution for 5-10 min.

7 Rinse the oocytes/embryos in culture media several times and continue culture according to laboratory practice.

Oocytes

Perform ICSI 1-2 hours after warming. The total time between HCG injection and ICSI should not exceed 42 hours. Continue culture according to standard laboratory practice.

Vitrification and warming of oocytes

Vitrolife´s products specifically for vitrification and warming of oocytes are called RapidVit™ Oocyte and RapidWarm™ Oocyte.

Vitrification

RapidVit™ Oocyte contains three solutions for the vitrification of oocytes. The solutions consist of a MOPS-buffered medium containing gentamicin and human serum albumin.

- Vitri 1™ Oocyte contains no cryoprotectants.
- Vitri 2™ Oocyte contains ethylene glycol and propanediol as cryoprotectants.
- Vitri 3™ Oocyte contains ethylene glycol and propanediol as cryoprotectants and sucrose.

Hands on

1. Place 1 ml of each of the following media into separate wells of a multi-well dish and warm to 37 °C in ambient atmosphere:
   - Vitri 1™ Oocyte
   - Vitri 2™ Oocyte
   - Vitri 3™ Oocyte
2. All manipulations of the oocytes are carried out at 37 °C (on the heated stage) in ambient atmosphere. It is very important to keep the temperature of all vitrification solutions at 37°C at all times.
3. Transfer the oocytes into Vitri 1™ Oocyte. Oocytes should remain in the solution for at least 5 minutes, but a maximum of 20 minutes.
4. Move an appropriate number of oocytes into Vitri 2™ Oocyte. The oocytes will be exposed to this solution for 2-5 minutes. The oocytes will tend to float to the surface, if so, collect and replace them to the bottom of the dish. The oocytes are expected to re-expand to their original volume within 5 minutes.

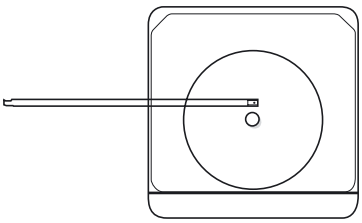
NOTE: If re-expansion is not observed within 5 minutes this could indicate that the temperature of the medium is not at 37°C. Temperature will affect the rate at which the cryoprotectants enter the oocyte and therefore the observed re-expansion.

5. Prepare the cryodevice for use.
6. When the oocytes have fully re-expanded make a 20 µl droplet of Vitri 3™ Oocyte on a non-toxic surface, preferably a culture dish. Making a small droplet of Vitri 3™ Oocyte enables easy loading onto the cryodevice.
7. Perform the actual vitrification according to the instructions above if Rapid-i Kit is used. (If another device is used follow the instructions for use provided with the device.)

NOTE: The 20 µl droplet can only be used once.

8. Transfer the oocytes in a minimal volume of Vitri 2™Oocyte to avoid dilution of the droplet.

9 Transfer the oocytes into the 20 µl droplet of Vitri 3™Oocyte. Mix the oocytes within Vitri 3™ Oocyte with the pipette. After 15 seconds, start to collect the oocytes and place them onto the cryodevice.



NOTE: The total time from transferring the oocytes into the droplet of Vitri 3™ Oocyte until the oocytes are vitrified should be between 25-35 seconds.

10 Immediately vitrify the oocytes according to the cryodevice instruction.

11 Continue storage according to laboratory practice.

Warming of vitrified oocytes

RapidWarm™ Oocyte contains four solutions for the warming of vitrified oocytes. The solutions consist of a MOPS- buffered medium containing gentamicin and human serum albumin.

- Warm 1™ Oocyte contains sucrose
- Warm 2™ Oocyte contains sucrose
- Warm 3™ Oocyte contains sucrose
- Warm 4™ Oocyte contains no sucrose

Hands on

1 Place 1 ml of each of the following media into separate wells of a multi-well dish and warm to 37 °C in ambient atmosphere:
   - Warm 1™ Oocyte
   - Warm 2™ Oocyte
   - Warm 3™ Oocyte
   - Warm 4™ Oocyte

NOTE: It is very important to keep the temperature of all vitrification solutions at 37°C at all times. If more than one Rapid-i™ is to be warmed in the same dish, make sure that the temperature reaches 37°C after each warming procedure.

2. Remove the cryodevice containing the vitrified oocytes from the cryostorage container. Follow the warming instructions for the specific cryodevice used.
3. Immediately, transfer oocytes into Warm 1™ Oocyte. It is important to use 1ml of Warm 1™ Oocyte to ensure the temperature of the medium is not perturbed by successive cryodevice submersions.
4. Allow the oocytes to fall from the device and sink to the bottom. Leave for 1 minute.
5. Transfer the oocytes into Warm 2™ Oocyte and let the oocytes remain in the solution for 3 minutes.
6. Transfer the oocytes into Warm 3™ Oocyte and let the oocytes remain in the solution for 5 minutes.
7. Transfer the oocytes into Warm 4™ Oocyte and let the oocytes remain in the solution for 5 to 10 minutes.
8. Following warming, oocytes should be held in an incubator in equilibrated G1™ PLUS at 37°C at 6% CO_2. The oocytes should be washed several times through this medium.
9. Perform ICSI 1-2 hours after warming. The total time between HCG injection and ICSI should not exceed 42 hours. Continue culture according to standard laboratory practice.

Vitrification and warming of cleavage stage embryos

Vitrolife´s products specifically for vitrification and warming of cleavage stage embryos are RapidVit Cleave and RapidWarm Cleave.

Vitrification

RapidVit™ Cleave contains three solutions for the vitrification of day 3 cleavage stage embryos. The solutions consist of a MOPS buffered medium containing gentamicin and human serum albumin.

- Vitri 1™ Cleave contains no cryoprotectants.
- Vitri 2™ Cleave contains ethylene glycol as a cryoprotectant.
- Vitri 3™ Cleave contains ethylene glycol, propanediol, ficoll and sucrose as cryoprotectants.

Hands on

1 Place 0.5-1 ml of each of the following solutions into separate wells of a multi-well dish and warm to 37 °C:
- Vitri 1™ Cleave
- Vitri 2™ Cleave
- Vitri 3™ Cleave

3 Transfer the embryos from culture into the Vitri 1™Cleave and let the embryos remain in the solution for at least 5 minutes but a maximum of 10 min.

4 Move an appropriate number of embryos into Vitri 2™Cleave. The embryos remain in this solution for 2 minutes. The embryos will tend to float to the surface; if so, collect them and replace them to the bottom of the dish.

4 Prepare the cryodevice for use.

5 When 30 sec remain, make a 20 µl droplet of Vitri 3™Cleave on a non-toxic surface, preferably a culture dish. This enables easy loading onto the cryodevice.

NOTE: Due to evaporation and dilution, the 20 µl droplet can only be used once.

6 When 10 sec remain, begin collecting the embryos. Transfer the embryos in a minimal volume of Vitri 2™Cleave to avoid dilution of the Vitri 3™ Cleave droplet.

7 Transfer the embryos into the 20 µl droplet of Vitri 3™ Cleave and let them remain in this solution for 30 sec. To allow proper exposure of the embryos to the Vitri 3™ Cleave solution, move the embryos around 2–3 times within the droplet.

8 When 5–10 sec remain, collect the embryos in a minimal volume and place them onto the cryodevice.

9 Immediately vitrify the embryos.

NOTE: The total time from transferring the embryos into the droplet until the embryos are vitrified must not exceed 30 sec.

10 Continue cryostorage according to laboratory practise.

Warming of vitrified cleavage stage embryos

RapidWarm™ Cleave contains four solutions for the warming of vitrified day 3 cleavage stage embryos. The solutions consist of a MOPS buffered medium containing gentamicin and human serum albumin.

- Warm 1™ Cleave contains sucrose.
- Warm 2™ Cleave contains sucrose.
- Warm 3™ Cleave contains sucrose.
- Warm 4™ Cleave contains no cryoprotectants.

Hands on

1. Place 0.5-1 ml of each of the following media into separate wells of a multi-well dish and warm to 37 °C:
   - Warm 1™ Cleave
   - Warm 2™ Cleave
   - Warm 3™ Cleave
   - Warm 4™ Cleave
2. In a cryodewer, bring the cryodevice containing the vitrified embryos close to the prepared wells.
3. Place the vitrified embryos quickly into Warm 1™Cleave. Allow the embryos to fall from the device and sink to the bottom. Leave for 10–30 sec.
4. Transfer the embryos into Warm 2™ Cleave and let the embryos remain in the solution for 1 minute.
5. Transfer the embryos into Warm 3™ Cleave and let the embryos remain in the solution for 2 minutes.
6. Transfer the embryos into Warm 4™ Cleave and let the embryos remain in the solution for 5 minutes.
7. Rinse the embryos in culture media several times and continue culture according to laboratory practice.

Vitrification and warming of blastocysts

Vitrolife´s products specifically for vitrification and warming of blastocysts are RapidVit™Blast and RapidWarm™Blast.

Vitrification

RapidVit™ Blast contains three media for the vitrification of human blastocyst stage embryos. The media consists of a MOPS buffered medium containing gentamicin and human serum albumin.

- Vitri 1™ Blast contains no cryoprotectants.
- Vitri 2™ Blast contains ethylene glycol and propanediol as cryoprotectants.
- Vitri 3™ Blast contains ethylene glycol, propanediol, ficoll and sucrose as cryoprotectants.

Hands on

1. Collapse the blastocysts by using a laser beam or by inserting an ICSI pipette.

2. Place 0.5-1 mL of each of the following media into separate wells of a multiwell dish and warm to 37 °C:
   - Vitri 1™ Blast
   - Vitri 2™ Blast
   - Vitri 3™ Blast

3. Transfer the blastocysts from culture into Vitri 1™Blast and let the blastocysts remain in the solution for at least 5 minutes but a maximum of 20 min.

4. Move an appropriate number of blastocysts into Vitri 2™ Blast. The blastocysts remain in this solution for 2 minutes. The blastocysts will tend to float to the surface, if so, collect them and replace them to the bottom of the dish.

5. Prepare the cryodevice for use.

6 Towards the end of the 2 minutes, make a 20 µL droplet of Vitri 3™ Blast on a non-toxic surface, preferably a culture dish. Making a small droplet of Vitri 3™ Blast enables easy loading onto the cryodevice.

NOTE: The 20 µL droplet can only be used once.

7 When 10 sec remain, begin collecting the blastocysts.

8 Transfer the blastocyst in a minimal volume of Vitri 2™ Blast to avoid dilution of the droplet.

9 Transfer the blastocysts into the 20 µl droplet of Vitri 3™ Blast and let them remain in this solution for 45 sec. Mix the blastocysts within the Vitri 3™ Blast with the pipette. When 5–10 sec remains, collect the blastocysts and place them onto the cryodevice.

NOTE: The total time from transferring the blastocysts into the droplet until the blastocysts are vitrified must not exceed 45 sec.

10 Immediately vitrify the blastocysts according to the cryodevice instruction.

11 Continue storage according to laboratory practice.

Warming of vitrified blastocysts

RapidWarm™ Blast contains three media for the warming of vitrified human blastocyst stage embryos.

The media consists of a MOPS buffered solution containing gentamicin and human serum albumin.

- Warm 1™ Blast contains sucrose.
- Warm 2™ Blast contains sucrose.
- Warm 3™ Blast contains no cryoprotectants.

Hands on

1. Place 0.5-1 mL of each of the following media into separate wells of a multi-well dish and warm to 37 °C:
   - Warm 1™ Blast
   - Warm 2™ Blast
   - Warm 3™ Blast
2. Remove the cryodevice containing the vitrified blastocysts from the cryostorage container. Follow the warming instructions for the specific cryodevice used.
3. Immediately after warming, transfer blastocysts into Warm 1™ Blast.
4. Allow the blastocysts to fall from the device and sink to the bottom. Leave for 2 minutes.
5. Transfer the blastocysts into Warm 2™ Blast and let the blastocysts remain in the solution for 3 minutes.
6. Transfer the blastocysts into Warm 3™ Blast and let the blastocysts remain in the solution for 5-10 minutes.
7. Rinse the blastocysts in culture media several times and continue culture according to laboratory practice.

FREEZING AND THAWING

Slow freezing of cleavage stages embryos

Equipment list

This is a suggested list and alternatives may exist.

1. Cryopreservation machine: Base your purchase decision on cost, available space and ability for maintenance and service.
2. Storage vessel: This should be a vessel used only for human embryos. Liquid nitrogen submersion is the traditional method but storage in nitrogen vapour phase is also an option. Separate vessels should be used if you cryopreserve embryos from patients with infectious diseases.
3. Storage devices for freezing: These need to be sterile, appropriately tested and approved for human IVF and of high quality.
4. The amount of liquid nitrogen (LN_2) used is dependent upon the choice of equipment and storage system.

More information about the Cryo protocols can be found in:

- Edgar DH, Karani J, Gook DA. Increasing dehydration of human cleavage-stage embryos prior to slow cooling significantly increases cryosurvival. Reprod Biomed Online. 2009 Oct;19(4):521-5
- Gardner DK, Maybach J, Lane M (2001) Hyaluronan and RHSA increase blastocyst cryosurvival. Proc 17th World Congress on Fertility and Sterility, Melbourne. pp 226
- Lane et al (2003) Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan. Mol Reprod Dev 64:70–78
- Gardner DK et al (2003) Changing the start temperature and cooling rate in a slow-freezing protocol increases human blastocyst viability. Fertil and Steril 79 (2): 407–410

Cryopreservation of cleavage stage embryos using Freezekit™ Cleave and Thawkit™ Cleave

This method of cryopreservation can be used for pronuclear stage oocytes up to the 8-cell stage.

Freezing procedure

FreezeKit™ Cleave contains two solutions for freezing of pronuclear-stage oocytes and cleavage-stage embryos. The solutions are MOPS buffered and contain human serum albumin.

Equilibration solution, ES, contains no cryoprotectants

Freezing solution, FS, contains propanediol and sucrose as cryoprotectants for dehydration of cells.

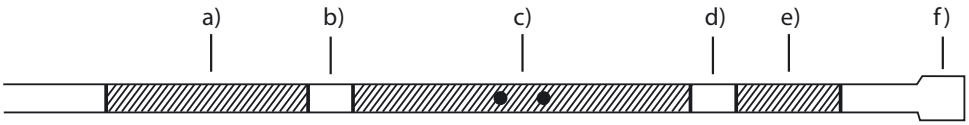
Essentials

- Check patient´s identity and prepare all paperwork before you start the procedure.

Hands on

1. Label dishes and straws, pipette appropriate volumes (0.5-1.0 ml) of ES and FS into respective dishes.
2. Equilibrate to room temperature. To avoid dilution, always transfer the embryos in a minimum amount of solution.
3. Place the embryos selected for freezing in ES and rinse properly. The embryos can stay in ES for 10 minutes.

4 Transfer the embryos into the FS and expose for 10 minutes. Loading of the prepared straws can start immediately after the embryos have been transferred to FS. The total exposure time for the embryos to the FS should be at least 10 minutes. Load the straws in accordance with manufacturer´s recommendation or as shown below.



a) FS, 2cm
b) Air bubble 1/4 cm
c) FS + embryos, 2–3 cm
d) Air bubble 1/4 cm
e) FS, 1 cm
f) Seal: plastic plug

5 Place the straws in the freezing machine at room temperature and initiate the freezing program.

Use the following freezing program

Starting temperature	+18.0 to +25°C
Step 1	–2.0°C/min to –6.0°C
Step 2	Hold at –6.0°C and manually seed after 2 minutes. Keep the straw at –6.0°C for a total time of 10 minutes. Do not seed close to the embryo
Step 3	–0.3°C/min to –30°C
Step 4	–50°C/min to –150°C

Plunge the straw into liquid nitrogen and arrange for storage at -196°C.
Store in liquid nitrogen.

Thawing procedure

ThawKit™ Cleave contains three solutions for thawing of pronuclear-stage oocytes and cleavage-stage embryos. The solutions are MOPS buffered and contain human serum albumin.

Thawing solution 1, TS1, and Thawing solution 2, TS2, are buffered solutions containing decreasing concentrations of sucrose allowing removal of propanediol and gradual rehydration of the cells.

Equilibration solution, ES, is a buffered solution that does not contain any cryoprotectants and is used for final rehydration before culture.

Essentials

- Thaw straws one at a time.
- Check patient identity carefully and prepare all paperwork accordingly
- Perform all steps at ambient temperature.

Hands on

1. Label dishes, pipette appropriate volumes (0.5-1.0 ml) of TS1, TS2 and ES into respective dishes. Equilibrate to room temperature. To avoid dilution, always transfer embryos in a minimum amount of solution.
2. Remove the straw from liquid nitrogen and expose to air for 30 seconds.
3. Place the straw in a 30°C water bath for 45 seconds.
4. Remove the straw and wipe it carefully. Open the straw using aseptic technique and gently expel the embryos. into TS1. Leave for 5 minutes.
5. Move the embryos into TS2 and expose for 5 minutes.
6. Move the embryos into ES and expose for 5 minutes.
7. Transfer the embryos to accurately equilibrated culture medium, rinse embryos properly and culture according to standard laboratory procedure.

Cryopreservation of semen

Semen cryopreservation is a procedure to preserve spermatozoa. The cryopreserved spermatozoa can be used for sperm donation where the recipient wants the treatment in a different time or place, or as a means of preserving fertility for men undergoing vasectomy or treatments that may compromise their fertility, such as chemotherapy, radiation therapy or surgery.

The most common cryoprotectant used for semen is glycerol. Often sucrose or other di-, trisaccharides are added to glycerol solution. Older cryoprotectant media for semen are often supplemented with egg yolk while more modern products are egg yolk free. Egg yolk contain cholesterol which has a membrane stabilising function that will improve sperm survival rates.

Vitrolife´s product for cryopreservation of semen is SpermFreeze™ Solution. To avoid potential risks of viral contamination, the product is free from egg yolk. Instead, the product contains free fatty acids which also contain cholestererol.
Semen samples are frozen either using a freezing machine or in liquid nitrogen vapour phase. The sample can be frozen either in straws or in vials.

Freezing

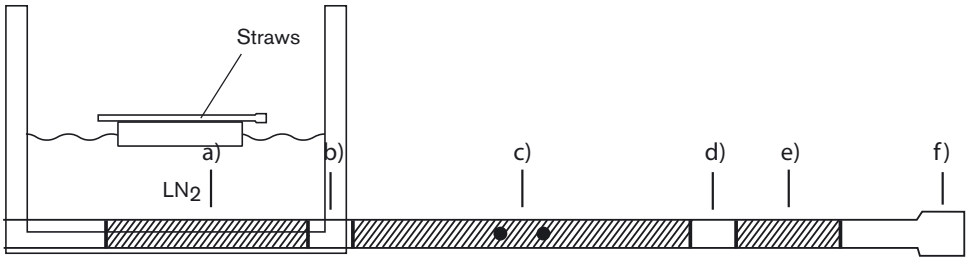
SpermFreeze Solution™ is a bicarbonate and MOPS buffered medium containing gentamicin and human serum albumin.

Essentials

- Allow the semen to liquefy for at least 10 minutes prior to preparation
- Both semen and SpermFreeze Solution™ should be at room temperature before initiation of the freezing procedure

Hands on when using straws

1. Dilute with equal volume of semen and SpermFreeze Solution™. SpermFreeze Solution™ should be added slowly and dropwise to the semen and the mixture carefully tilted after each drop added.
2. Close the lid tightly and turn the tube upside down 20 times, being careful not to create bubbles. The semen mixture is left at room temperature for 10 minutes.
3. Mark 0.5mL straws with patient ID and load the semen mixture into straws. Ensure that some air space is left in the lower part of the straw for sealing as well as to allow expansion of the semen mixture during freezing.
4. Seal the straw according to the straw manufacturer's instructions.
5. Place the straws horizontally on a 1-3 cm styrofoam board in a liquid nitrogen bath to allow for freezing in vapour phase. Leave for 30 minutes*.



6. Transfer the straws quickly into liquid nitrogen and store at -196 °C.

* Optional, this step can be performed using a slow-freeze machine programmed for sperm freezing.

Use the following freezing program for machine

Start temperature: +20°C

1. -5 °C/min to -8 °C
2. Hold 1 min
3. -10 °C/min to -25 °C
4. -25°C/min to -150°C

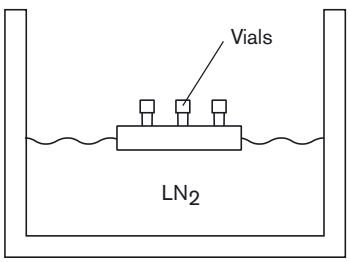
Thawing of straws

1. Remove straws from -196 °C and place them in a water bath at 35 ± 2 °C for 30 seconds.
2. Wipe the straws dry with a clean paper towel and open according to instructions from the manufacturer of the straws.
3. Expel semen mixture into clean test tubes and dilute with equal amount of equilibrated G-IVF™ PLUS. G-IVF™ PLUS should be added dropwise to the semen mixture and the solution carefully mixed after each addition.
4. Continue with gradient separation according to the G-series manual.

When using cryovials

Freezing

1. Measure the total volume of the semen and carry out semen analysis as required.
2. Dilute with equal volume of semen and SpermFreeze Solution™. SpermFreeze Solution™ should be added slowly dropwise to the semen and the mixture carefully tilted after each drop added.
3. Close the lid tightly and turn the tube upside down 20 times, being careful not to create bubbles. The semen mixture is left at room temperature for 10 minutes.
4. Mark 1.5mL cryovials with patient ID and load the semen mixture into cryovials. Do not fill cryovials completely to allow for expansion.
5. Place the cryovials upright on a 1-3 cm styrofoam board in a liquid nitrogen bath to allow for freezing in vapour phase. Leave for 30 minutes*.



6. Transfer the cryovials quickly into the liquid nitrogen and store at -196 °C.

* Optional, this step can be performed using a slow-freeze machine programmed for sperm freezing.

Use the following freezing program for machine:

Start temperature: +20°C

1. -5 °C/min to -8 °C
2. Hold 1 min
3. -10 °C/min to -25 °C
4. -25°C/min to -150°C

Thawing

1. Remove cryovials from -196 °C and place them in a water bath at 35 ± 2 °C for 10 min.
2. Wipe the cryovials dry with a clean paper towel.
3. Transfer semen mixture to clean test tubes.
4. Dilute the semen mixture with equal amount of equilibrated G-IVF™ PLUS. G-IVF™ PLUS should be added dropwise to the semen mixture and the solution carefully mixed after each addition.
5. Continue with gradient separation according to the G-series manual.

QUALITY CONTROL PROGRAM

All raw materials used for manufacturing Vitrolife products are dedicated for human medical use and whenever applicable are of US Pharmacopeia (USP) and European Pharmacopoeia (Ph Eur) grade. Each lot of raw materials and final products are tested and evaluated by stringent quality control procedures. All production takes place in a manufacturing environment classified for aseptic production that is under strict control and monitoring. Quality Control, together with quality assured operations (ISO 13485:2003 and 21 CFR Part 820:QSR) result in excellent LOT-to-LOT consistency.

Physicochemical, biological and functional tests are performed according to the characteristics of each product. In addition to recognised standards for test performance, internal Standard Operating Procedures according to the Quality Handbook of Vitrolife are followed. The Quality Control Program of Vitrolife includes all necessary tests to guarantee both the safety and efficacy of the media.

All instruments and standard solutions used in the Quality Control system are acquired from qualified manufacturers.

Validation and calibration

All quality control procedures performed by Vitrolife are performed in accordance with quality assured operations (ISO 13485:2003 and 21 CFR Part 820:QSR) by highly qualified personnel. All methods and equipment used are subject to a continuous and extensive validation and calibration program.

Physicochemical tests

pH

The pH measurement is performed using a validated method according to USP and Ph Eur. The range of acceptance for each product is kept as small as possible, considering the nature of the product and its intended use. Samples are pre-incubated at the appropriate temperature and atmosphere before measurement. Media equilibration at 6 % CO_2 is achieved by the use of a gas mixture with a certified CO_2 level.

Osmolality

The osmolality measurement is performed according to USP and Ph Eur using a validated method based on freezing-point depression. The range of acceptance for each product is kept as small as possible, considering the nature of the product and its intended use.

Biological tests

Bacterial endotoxin

The absence of toxic levels of endotoxins is verified for all raw materials and each LOT manufactured. The validated bacterial endotoxin test (LAL assay) that is used by Vitrolife is the most sensitive of the currently used methods for endotoxin testing with a minimum sensitivity of 0.005 EU or IU/mL. The validated procedure is performed in accordance with USP, Ph Eur, and FDA guideline “Guideline on validation of the limulus amebocyte lysate test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices”.

Sterility test - membrane filtration

The sterility of all media is confirmed by a membrane filtration in accordance with USP and Ph Eur. The test period for media is 2 weeks and for oil it is 3 weeks. No detectable bacterial or fungal growth is accepted, neither in fluid thioglycollate medium nor in soya-bean casein digest medium. A strict requirement of the sterility assurance level (SAL) provides a safe application for users.

Functional tests

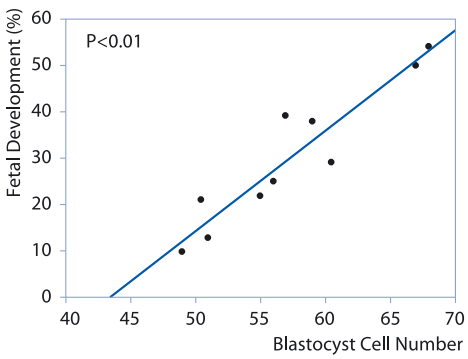
Mouse embryo assay

Mouse embryo assay (MEA) is a functional test method. All media, medium components and critical devices used for media manufacturing are tested by culturing one-cell stage mouse embryos to the expanded blastocyst stage. The developmental stages of the embryos are recorded according to the specifics of each test. The safety and efficacy of the media is determined by observing the number of embryos that develop to defined developmental stages with appropriate cell numbers in a predetermined time period. In addition, the determination of cell numbers and the use of specific cut off values set for all products increase the sensitivity of the MEA as cell numbers of the blastocysts are linked to their subsequent viability upon transfer to the uterus.

Validation of Mouse Embryo Assay for Screening Medical Devices in Clinical ART

A bioassay, such as the one cell Mouse Embryo Assay (MEA), is required for laboratory certification by the College of American Pathologists which governs Reproductive Clinics in the United States. All commercial media and those media made in house, must be evaluated prior to clinical use by such an assay.[1] The MEA is the most widely used assay for media components, culture media, and equipment used in clinical ART.[2] The MEA has been proven effective in screening for several potential embryo toxins present in the equipment used in clinical work, new LOTs of oil used as a media overlay, consumables and each new LOT of medium. Many steps have been taken to maximise the sensitivity of the MEA. For example, when testing contact materials we use media without albumin as it can chelate potential embryo-toxins. Furthermore, we use 1-cell mouse embryos as opposed to 2-cell stage.[3]

Determining cell numbers of blastocysts in the MEA is indicative of embryo viability. As shown in the figure below, fetal development is proportional to blastocyst cell number.[4] Using the MEA it is possible to quantitate the product properties of culture media used in clinical ART.



REFERENCES

1. College of American Pathologists (1998), Reproductive Laboratory-Section 90 Proposed Checklist, p26
2. Gardner DK, Reed L, Linck D, Sheehan C and Lane M (2005) Quality control in human in vitro fertilization. Sem. Reprod. Med. 23, 319-324.
3. Davidson, A., Vermesh, M., Lobo, R.A., and Paulson, RJ (1998), Mouse embryo culture as quality control for human in vitro fertilisation: the one-cell versus the two-cell model. Fertil. Steril., 49: 516-521.
4. Lane M. and Gardner DK (1997), Differential regulation of mouse embryo development and viability by amino acids. Reprod Fertil., 109: 153-164.

Cryopreservation - and related products from Vitrolife

Product	Intended purpose
G-MM™	G-MM™ contains Recombinant Human Albumin solution (50 mg/mL) and is intended for use in assisted reproductive procedures which include gamete and embryo manipulation. These procedures include the use of G-MM™ as a supplement for culture medium. Not for use as an injectable product.
HSA-solution™	HSA-solution™ contains Human Serum Albumin solution (100 mg/mL) and is intended for use in assisted reproductive procedures which include gamete and embryo manipulation. These procedures include the use of HSA-solution™ as a supplement for culture medium. Not for use as an injectable product. CAUTION: All blood products should be treated as potentially infectious. Source material from which this product was derived was found negative when tested in accordance with current FDA required tests, HIV, types 1 and 2; HBV; HCV, and HTLV types I and II. No known test methods can offer assurance that products derived from human blood will not transmit infectious agents.
FreezeKit™ Cleave * †	Solutions for freezing of pronuclear oocytes and cleavage-stage embryos.
ThawKit™ Cleave * †	Solutions for thawing of frozen pronuclear oocytes and cleavage-stage embryos.
Rapid-i™ Kit	Cryopreservation device intended to be used to contain, vitrify and maintain embryos and/or oocytes. In the US - for 4-8 cell stage embryos only.
RapidVit™ Oocyte * †	Media for vitrification of oocytes.
RapidWarm™ Oocyte * †	Media for warming of vitrified oocytes.
RapidVit™Cleave	Media for vitrification of cleavage stage embryos (US: day 3 only).
RapidWarm™Cleave	Media for warming of vitrified cleavage stage embryos (US: day 3 only).

continued on next page

Product	Intended purpose
RapidVit™Blast	Media for vitrification of blastocyst stage embryos.
RapidWarm™Blast	Media for warming of vitrified blastocyst stage embryos.
RapidVit™ Omni	Media for vitrification of oocytes through to blastocyst stage embryos
RapidWarm™ Omni	Media for warming of vitrified oocytes through to blastocyst stage embryos.
Rapid-i™ Cutter	To cut Rapid-i™ .
Rapid-i™ Forceps	To remove the Rapid-i™ from the straw during warming
Rapid-i™Sealer PS-202, 230V	For sealing of Rapid-i™ straws during vitrification (230V)
Rapid-i™Sealer PS-202, 120V	For sealing of Rapid-i™ straws during vitrification (120V)
Rapid-i™ Goblet	To store Rapid-i™ in liquid nitrogen
Rapid-i™ Cryocane	To store Rapid-i™ in liquid nitrogen with goblet
SmartBox	To support the vitrification process with Rapid-i™

* Not available in Australia

† Not available in the US

Correspondence

Vitrolife welcomes any comments on the text in the Manual.
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