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# NEXUS

Indian Fertility Society
& ORIGIO India Initiative

## Vitrification: An Enigma

## Frozen life

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JOINT SECRETARY - IFS
EDITOR - NEXUS

## PRESIDENT'S MESSAGE

It gives me immense pleasure to present to you this edition of NEXUS which covers the vast topic of vitrification in its entirety. Today, the technology of vitrification forms the backbone of a successful ART programme, whether it is the cryopreservation of supernumerary embryos or freeze-all embryos as a part of segmentation in cases of imminent ovarian hyperstimulation syndrome.

Freezing of embryos as a part of biopsy and preimplantation genetic diagnosis or screening, or gamete cryopreservation either electively, or those diagnosed with malignancy and about to start a potentially sterilizing treatment are other common indications .

Optimising of the vitrification technique is required to achieve excellent pregnancy and live birth rates comparable, and sometimes superior to transfer in a fresh cycle. This involves a deep understanding of the media used, the importance of the adherence to the protocols formulated by different manufacturers, and an in-depth knowledge of the various carrier devices used for oocyte and embryo loading.

It is a great pleasure and privilege to write this message for the 6th E-bulletin of IFS Nexus. I also sincerely thank "Origio India Private Ltd" for supporting us in this academic endeavor.

**Dr. Sohani Verma**
**President-IFS**

## SECRETARY'S MESSAGE

This is an honor for me to write best wishes message for this edition of Nexus bulletin on "Vitrification".

With the advent of vitrification in the field of assisted reproduction, it has become possible to achieve a near 100% survival of oocytes and embryos following cryopreservation. Oocytes and all embryonic stages from zygote to blastocysts are amenable to vitrification with excellent post warming recovery.

This is a potentially revolutionary technology as it helps the fertility specialists to overcome the obstacles of ovarian hyperstimulation, elevated progesterone in late follicular phase, poor endometrium following stimulation, bleeding and difficult embryo transfer. However, it is necessary to adhere to the recommended protocols and manufacturer's guidelines when using any vitrification media or carrier device to achieve optimal recovery.

This bulletin will offer the reader the finer nuances involved in achieving high post warming survival rates, and the tips and tricks to optimize your current vitrification program.

Indian Fertility Society feels proud and congratulates the editors of the 6th edition of Nexus E-bulletin. It would not only help to disseminate scientific & ethical content but also constantly update the readers with new developments and research across the world.

I wish the editorial team best of luck in this endeavor.

**Dr. K.D. Nayar**
**Secretary General-IFS**

# EDITOR'S MESSAGE

To start with, the editorial team would like to express heartfelt thanks to all the readers for appreciating and acknowledging previous bulletins of nexus. **The bulletin has been named NEXUS which means bridging gaps.**

Such bulletins , being call of the day not only bridges the gap between existing knowledge and recent advances but also covers all essential details related to infertility with nice algorithms.

Cryobiology has been very dear to my heart since years. Things were not very simple in our era of learning and we did not have good teachers too. You Tube and Google were not very helpful either and learning was with one to one interaction with a kind teacher if we could find one. It took me a long time to understand nuances of vitrification which I started 2005 onwards with multiple learning failures in the beginning.

**With nexus on my side I am trying to percolate down what ever I know to you all in simple and lucid way without hiding any details.**

Our present edition is focussed on vitrification process which is a rapidly emerging technique of cryopreservation. Embryos were routinely cryopreserved by slow freezing methods a decade back. However, there are some constraints with these protocols which includes -longer time taking, intracellular ice crystal formation leading to cellular damage and need for expensive programmable freezing equipments.

Vitrification leads to ultra rapid cooling of a solution containing high concentration of cryoprotectant, inducing a glass like state, as a result avoiding ice crystal formation and cellular damage. It also favours short equilibration time, fast cooling rates and no expensive equipments. Vitrification has come a long way and till date the results obtained with this process are equal or better than conventional slow freezing techniques.
In this issue, the intricacies of the vitrification technique are discussed along with an in-depth comparison of various vitrification-warming media and the carrier devices available in the market. We sincerely hope that this bulletin would benefit senior, junior & trainee embryologists and clinicians with a keen interest in the nuances of gamete cryopreservation.

Our aim remains **"Empowering Knowledge"** and we sincerely hope that you would enjoy reading this write up. Feel free to communicate with us any point of time and your critical contribution would attribute to the fineness of bulletin. Your valuable comments would be published in the next bulletin which is titled **"deciphering Embryos".**

I am thankful to **Dr Pranay Ghosh** and **Miss Sapna** for helping me in designing this piece of art. We would also like to express our truthful thanks to Origio India Limited who are helping us in the publication of this bulletin.

Wishing you all a very happy reading and yes don't forget to file this issue.

Jaihind

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# Guest Editors For The Edition

Having reached a bottleneck in ART in terms of success rates, the endeavor to improve the pregnancy and livebirth rates has intensified. Whether it is the transfer of a single genetically normal embryo deemed disease free and euploid by preimplantation genetic diagnosis and screening, the transfer of embryo/s in a more physiological uterine environment as a part of "freeze-all" and "segmentation" strategy or simply achieving a higher cumulative success rate by freeze-thaw-transfer of supernumerary embryos produced by a single stimulation, the cornerstone of all these modalities is vitrification.This bulletin provides an in-depth review of the various vitrification-warming media and carrier devices available today, and the tips to optimize your current practice.

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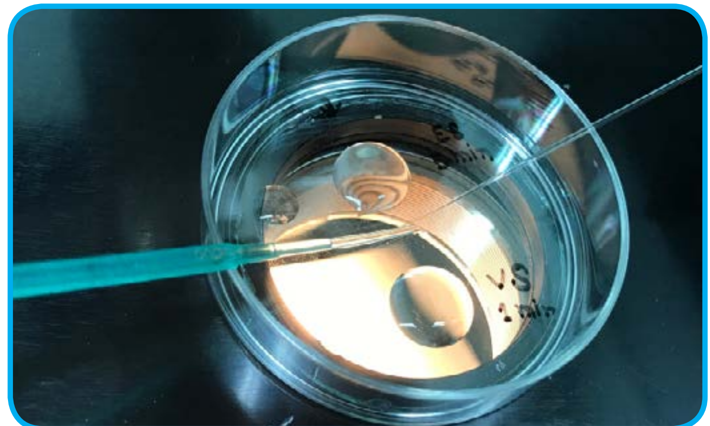
I would take this opportunity to thank Dr. Pankaj Talwar for the immense faith he has shown in me and endowed me with the responsibility of being a part of this edition of the Nexus E-bulletin. I tried my best efforts to complete the assigned role under his able guidance.

**Sapna**
B.Tech (Biotechnology)



# Part-1

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## 01 What is vitrification and how does it work?

Vitrification is defined as "the instant solidification of a solution brought about by an extreme elevation in viscosity during cooling, without ice crystal formation". It is a kinetic process in which a liquid solidifies into a glassy state (derived from "vitri", meaning glass in Greek).Upon cooling to extremely low temperatures, the liquid reaches its glass transition temperature at which it changes to solid state. Vitrification is faster and lacks some of the typical disadvantages seen in traditional slow freezing.
The recent improvements in vitrification techniques include reduction of concentration of cryoprotectants, increase of cooling and warming rates, and use of novel cryodevices.

The measure of internal energy in a physical system is temperature. It is this internal energy that allows the molecules in fluids to tumble, twist, dissociate from one another, move from place to place in fluid, and chemically react with other molecules. On reducing the temperature, the energy to drive this molecular motion decreases. In systems such as pure water, temperature reduction below a certain point results in abrupt reorganization of the fluid into an organized solid lattice called crystal. This is referred to as freezing. However, in other systems, temperature reduction just causes more slowing of molecular motion, less molecular mobility and lowering of chemical reaction rates until a critical temperature is reached below which the system almost completely loses its fluidity. This temperature is known as the "glass transition temperature", and the fluid becomes a "liquid solid" or more formally, a "glass" that is said to have "vitrified".

The cryopreservation of gametes and embryos involves an initial exposure to cryoprotectants, cooling to subzero temperatures, storage, thawing, and finally, dilution and removal of the cryoprotectants, with a return to a physiological environment which allows their further development.

## 02 Why vitrification is more efficient than slow-freezing?

Vitrification is now regarded as a potential alternative to the conventional slow freezing method as it has the advantage of preventing ice crystal formation and this rapid freezing technique also minimizes chilling injury and osmotic shock to the embryo. Two conditions usually required to allow vitrification are an increase in the viscosity and a depression of the freezing temperature. These conditions are achieved by addition of CPA which acts like antifreeze. Vitrification usually requires the addition of CPA prior to cooling. Other components required for vitrification are basal medium buffered with phosphate or HEPES, macromolecules, sugars and proteins. The technique of vitrification has undergone various improvements by using less toxic and more permeable chemicals, by using combination of CPA to reduce toxicity, using a stepwise approach to equilibration and increasing cooling and warming rates[1].

**Table:1. Comparison of embryo vitrification with conventional slow freezing[1]**

| FACTORS | VITRIFICATION | CONVENTIONAL SLOW-FREEZING |
|---|---|---|
| Time consumed | Less (10 minutes) | More (3 hours) |
| Instruments | Inexpensive | Expensive |
| Sample volumes | < 4 nL | 100 to 250 μL |
| Cooling rates | 15,000 to 30,000$^{O}$ c/min | 0.3 to 0.6$^{O}$ c/min |
| CPA concentration | High | Low |
| Ice crystallization | No | Yes |
| Direct contact with $LN_2$ | Depends on the carrier being used | No |
| Mechanical damage | Less or none | More |
| Chemical damage | More | Less |

## 03 What are the laboratory pre-requisites for vitrification?

As opposed to slow-freezing, vitrification does not require any special equipment. The following disposables and media are required to carry out successful vitrification:

**1. Cryoprotective agents** A cryoprotectant is a substance used to protect biological tissue from freezing damage (i.e. that due to ice formation). Though they may have varying chemical compositions, all CPAs are highly water soluble, but possess a concentration-dependent toxicity. They cause the lowering of the freezing point of the solution, displace the water from intracellular to extracellular environment and hence alter the solute concentration in the liquid phase[2]. There are two classes of CPAs:

- **Permeating CPA** These agents penetrate the cell membrane, and have low molecular weights (<400 g/mol). They cross the membrane easily thereby creating an osmotic gradient, leading to shift of water from intracellular to extracellular compartment, and hence further lowering the freezing point. This class includes glycerol, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and 1,2 propanediol (PROH).
- **Non-permeating CPA** These agents do not cross the cell membrane, and have large molecular weights (>1000 g/mol). They increase the concentration of extracellular solutes, thereby creating an osmotic gradient and hence causing cellular dehydration. This class includes sugars like sucrose, fructose, dextrose, trehalose, raffinose etc.

We commonly use equilibration and vitrification solutions for the 2-step vitrification procedure. The equilibration solution contains 20-50% lower concentration of permeating cryoprotectants as compared to second step vitrification solution. The lower concentration of permeating cryoprotectants in the equilibration solution makes it much less toxic than the vitrification solution. Oocytes/embryos in the equilibration solution shrink initially and gradually re-expand to their original volume after brief exposure to the cryoprotectants. Re-expansion of the cells depicts healthy oocyte/embryo with intact and functional membranes, and ensures successful vitrification-warming outcome. Quick re-expansion reduces the time needed for exposure of the gametes and embryos to the vitrification solution. Vitrification provides better chances of survival for oocytes and other cells with high water content and those that have a low surface / volume ratio[3]. Single step vitrification without equilibration step leads to the possibility of insufficient permeation of the cryoprotectants that may result in intracellular ice formation during cooling or warming.

**2. Vitrification carrier devices** After short exposure of the embryos to the equilibration and the vitrification solution they may be loaded on the cryo-devices of various capacities. These can be open or closed and work with variable volumes of cryofreezing solutions. Some of them are enumerated below:

- **Conventional straw** For the conventional straw method for vitrification, the embryos are loaded in 0.25 ml plastic straws. The embryos are loaded the same way as done for the slow conventional freezing technique. The straw is then directly plunged into liquid nitrogen. During warming procedure, the straw is taken out of the $LN_2$ and held in the air for 5 seconds, then plunged into water bath at 37 °C for 10 seconds.The vitrification solution in the straw after cooling should remain transparent in the liquid nitrogen and air depicting minimal or no water molecules in the cryosolution. A whitish discoloration of vitrification medium in the straw indicates ice formation and unsuccessful outcome.
- **Electron microscope copper grids** Use of Electron microscope copper grids is not a popular method for vitrification any more. Biological EM work is done on small (several millimeters) copper discs called grids cast with a fine mesh. This mesh can vary a lot depending on the intended application, but is usually about 15 squares per millimeter (400 squares per inch). On top of this grid, a thin layer of carbon is deposited by evaporating carbon graphite onto it. Thin carbon film on the grid holds the sample during the procedure. Embryos are placed on the electron microscope copper EMgrids (IGC 400; Pelco International, CA, USA). The grids act as a physical support and maximize cooling rates when the embryos are directly plunged into liquid nitrogen ($LN_2$) .It is recommended that 2-3 embryos are transferred onto the electron microscope copper grid at a time . To further reduce the volume of vitrification solution and thus the cooling rates, the underside of the grid is blotted on a filter membrane . The grid supporting the oocytes is now plunged into liquid nitrogen using fine forceps, and stored in a cryovial filled with liquid nitrogen. During warming, the grid is extracted and directly submerged in the warming solution[4].
- **Open pulled straw** 0.25 ml plastic straw is heated, softened and pulled manually. The pulled straw is cut at the tapered end. The inner diameter of the tip is approximately 0.8 mm with a wall thickness of 0.77 mm. Commercial sterile pulled straws have recently become available. Embryos are loaded into the tip of the pulled straw through the capillary effect by simply touching a microdrop (1-2 µL) of vitrification solution containing the embryos. Plunging the OPS directly into liquid nitrogen leads to the cooling of the embryos. For warming procedure, the tip of OPS is submerged into the dilution solution, and the positive pressure of the expanding gas column in the straw expels the embryos spontaneously in the media droplet.
- **Cryoloop** The cryoloop consists of a nylon loop (0.5-0.7 mm diameter) mounted on a stainless steel rod that is inserted into the lid of a cryovial loop is dipped into vitrification solution to make a filmy layer of solution on the nylon cryoloop by surface tension and embryos are place over this film in minimal volume of the vitrification solution. Now the cryoloop is directly plunged into liquid nitrogen. At the time of warming the vial is opened, and the cryoloop is placed directly into the warming solution[5].
- **Cryotop** The cryotop has a fine polypropylene strip (0.4 mm wide x 20 mm long x 0.1 mm thick), attached to a plastic holder and equipped with a protective plastic tube. Embryos are loaded on the strip with minimal vitrification solution.The Cryotop is immersed directly into liquid nitrogen. For warming, the protective cover is removed from the cryotop and the strip is placed directly into the warming solution to release the embryos[6].

**3. Liquid nitrogen**
**4. Dewars for liquid nitrogen storage**
**5. Patient identification system**
**6. Security measures against accidental warming (including an appropriate sensor alarm for each cryocan)**
**7. Specially designed reservoir boxes with foam insulation to hold $LN_2$. Alternatively, clean Styrofoam boxes can be used.**

The other equipment that are required for carrying out vitrification are standard part of an embryology laboratory e.g. Laminar flow hood with a heated microscope stage, warming plate, stereozoom microscope, micropipettes and micropipette tips, flexipets, stripper handle, benchtop stop watch, forceps of various sizes, and fine-tip permanent markers.

## 04 What is the composition of media used for vitrification?

Vitrification cooling solutions contain a mixture of permeating and non-permeating cryoprotectants in increasing concentrations and are basically composed of HEPES or MOPS buffered media, whereas warming solutions are composed of decreasing concentrations of sucrose.

## 05 What is the survival rate of vitrified warmed embryos?

The post warming survival rates depend on various factors e.g. the stage of embryo cryopreservation, embryo morphology, the type of vitrification media used, the type of carrier device used and additional factors like blastocoel collapse for blastocyst vitrification, temperature at which vitrification is carried out etc.

Vitrification warming of early cleavage-stage embryos and blastocysts has produced high post warming survival rates, and with the current media and protocols we can expect at least 90% cryosurvival rates.

**Table:2. Vitrification of early-stage embryos**

| YEAR | AUTHORS | STAGE | CPA USED | CARRIER | NUMBER OF VITRIFIED EMBRYOS | Survival % |
|---|---|---|---|---|---|---|
| 2015 | Debrock et al[7] | 6-8 cell | DMSO/EG | CBS-Vit High security | 217 | 84.3 |
| 2014 | Fasano et al[8] | 2-12 cells | DMSO/EG<br>EG/PE | High security straw<br>High security straw | 330<br>330 | 89.4<br>87.6 |
| 2012 | Wang et al[9] | 6-8 cell | EG/DMSO/S | Cryoleaf | 825 | 86.6 |
| 2009 | Rama Raju et al[10] | 6-8 cell | EG based | Cryoloop | 907 | 90.37 |
| 2008 | Balaban et al[11] | 6-8 cell | EG/PE | Cryoloop | 234 | 94.8 |
| 2007 | Al Hasani et al[12] | Zygote | EG/DMSO/S | Cryotop | 339 | 98 |

## 06 When should we vitrify an oocyte or an embryo? At which cell stage is it recommended to freeze an embryo?

### OOCYTE

The optimal stage of vitrifying oocytes is 2-4 hours post oocyte retrieval. Optimal timing for carrying out ICSI on human oocytes has been found to range from 37 hours to 41 hours after administration of human chorionic gonadotropin (hCG)[13]. In most vitrification programmes, oocyte retrieval is carried out between 34-36 hours post hCG trigger, and oocyte vitrification is carried out 2 hours after oocyte retrieval. This is carried out immediately after removal of cumulus cells (denudation). After warming, oocytes need to be incubated for 2-4 hours before carrying out ICSI, as there is severe spindle disorganization immediately post warming which may affect the fertilization and cause increased rates of digynic fertilization if ICSI is carried out immediately post warming. Amongst the oocytes, MII oocytes are more resistant to cryodamage as compared to GV stage oocytes due to the differences in the properties of their cytoskeletal elements. While the microtubules and microfilaments in MII stage oocytes are undulating and flexible, these cytoskeletal elements appear straight and rigid in GV stage oocytes.

### EARLY STAGE EMBRYOS

Vitrification can be successfully carried out with excellent post warming recovery for embryos cryopreserved from pronuclear stage to 8-10 cell stages. There are several protocols available for cleavage stage embryo vitrification. However, they have the same underlying principle, and the differences in the protocol are related to the type and concentration of CPAs and the duration of exposure to CPAs.
Few studies have reported superior results with day 3 vitrification as compared to day 2 vitrification[14]. It may be argued that day 3 embryo vitrification inherently offers a better selection as compared to day 2 embryo vitrification, and hence the better results. Moreover, blastomere loss post warming may be more critical for a day 2 4-cell embryo as compared to a 6-8 cell day-3 embryo, since the loss of a single blastomere translates to the reduction of cellular machinery by 25% in a 4-cell embryo as compared to about 12.5% in an 8-cell embryo.

### MORULA AND BLASTOCYST STAGE EMBRYOS

Embryo vitrification on day 4 at the morula or the compaction stage has been described in the literature and is associated with comparable outcomes as compared to the cleavage-stage embryo and blastocyst vitrification[15]. The presumed advantages of day 4 vitrification over day 3 vitrification are better embryo selection and the fact that embryonic genome activation is completed. Another presumed advantage over day 5 vitrification is that blastocoel collapse is not required as compared to early or expanded blastocysts.

Blastocyst vitrification is optimally carried out when the blastocyst size is between 170 to 220 μm. Some of the media and protocols advocate the artificial collapse of blastocoel cavity either by applying a LASER shot or mechanically by needle (micromanipulation injection pipette or a PZD pipette) e.g. Origio Medicult, Vitrolife etc. whereas others do not e.g. Kitazato, Cryotech etc.

There is no single recommendation regarding the optimal stage to freeze the embryo, and every clinic has to adapt the vitrification protocol to their post warming cryo survival for different embryonic stages. However, with the advent of newer media & protocols and an increasing trend towards trophoectoderm biopsy for preimplantation genetic diagnosis/screening, day 5 blastocyst vitrification is emerging as the embryonic stage of choice for vitrification, and all vitrification programs must attempt to optimize it. Arguably, if there are supernumerary embryos following day 2/3 embryo transfer, then a day 5 culture would have offered a better selection to enable a single embryo transfer, and it would be recommended to vitrify any supernumerary embryos then.

## 07 Which surface among cold or warm is better for vitrification?

Apart from exposure time and toxicity of cryoprotectant used, the other factor determining the outcome of vitrification is the temperature at which vitrification is carried out. There are two schools of thought regarding the best temperature to carry out vitrification. The first one advocates the use of lower temperature (room temperature) to reduce the cryoprotectant toxicity while carrying out vitrification. On the other hand, some argue that based on oocyte meiotic spindle studies, vitrification carried out at 37°C results in a faster spindle recovery as compared to when conducted at room temperature[16] (Table 3).

Table:3

| VITRIFICATION / WARMING TEMPERATURE | RT/RT | RT/37 °C | 37 °C/37 °C |
|---|---|---|---|
| SPINDLE RECOVERY TIME (MINS) | 180 | 30 | 15 |

However, due regard should be paid to the manufacturer guidelines and the vitrification and warming processes should be carried out at the temperatures mentioned in the product insert.

## 08 For how long can oocyte/embryos be vitrified?

Ample studies have been carried out that conclude that there is no significant impact of storage on clinical pregnancy, implantation, miscarriage or live birth rates, and that the cryostorage duration does not affect post thaw survival or pregnancy outcome [17,18].

Storage at -196°C virtually eliminates all movement of atoms or molecules, and it is speculated that at these temperatures only the background cosmic radiation induced modifications to the stored tissue is possible.

Based on complex mathematical calculations elucidating the Tα (glass transition temperature) for various cryoprotectant solutions, the projected safe storage times in the amorphous state are as follows:

Table:4. Projected safe storage times in the amorphous state[19]

| TEMPERATURE | CRYOPROTECTANT SOLUTION | | | TIME UNITS |
|---|---|---|---|---|
| | M22 | $DAP_{10}$ | DMSO | |
| -22 °C | 1 | 1 | 1 | Min |
| -80 °C | 13.2 | 23.2 | 2.7 | Hours |
| -90 °C | 5.5 | 12.0 | 0.62 | Days |
| -100 °C | 18.0 | 53.3 | 0.85 | Weeks |
| -115 °C | 652 | 4379 | 3.18 | Years |
| -121 °C | 82,374 | 990,388 | 81.7 | Years |

## 09 What happens if the oocytes/embryos as exposed to longer duration in the media than the defined time period?

On prolonged exposure to cyoprotectants, there may be excessive movement of water across cell membrane, and hence excessive dehydration resulting in cell contraction or expansion beyond safe osmotic limits. Though osmotic buffers are added to cryoprotectant solutions, it is still prudent to limit the exposure to cryoprotectants within the specified time mentioned in the individual protocols. Prolonged exposure of oocytes to cryoprotectant solutions may lead to oocyte degeneration and parthenogenetic activation[20].

## 10 What is the maximum time period to transfer the device from work-station to the $LN_2$ storage tank?

After loading of the oocyte(s) or embryo(s) on the cryodevice and removal of the excess cryoprotectant, the cryodevice is immediately plunged into a box containing $LN_2$. As long as the cryodevice remains immersed into $LN_2$ at all times, the device can be transferred to the main $LN_2$ storage tank without any rush. However, care must be taken not to prolong this in order to avoid the excessive evaporation of $LN_2$ from the styrofoam box, and the inadvertent warming of oocytes/embryos.

## 11 How many oocytes/embryos can be vitrified on one device?

Different carrier devices specify different maximal capacity of oocytes/embryos that can be loaded on the device. Roughly, up to 3-4 oocytes can be loaded on a single cryodevice, while up to 2-4 embryos can be loaded on different cryodevices according to the manufacturer's recommendations. However, there is a move towards loading 1 or maximally 2 embryos on a single device so as to facilitate single embryo transfer and avoid the unnecessary re-vitrification associated with warming and survival of 3 or 4 embryos warmed from a single device, and the supernumerary embryos left after single embryo transfer.

## 12 What are the risks involved with oocyte/embryo vitrification?

The process of cryopreservation is associated with inadvertent cryodamage to various cellular organelles (Table 5).

**Table:5. Cellular freezing and cryodamage [21]**

| Cellular Structure | CRYODAMAGE |
|---|---|
| Nucleus | Cryopreservation can affect the structural integrity of the nuclear envelope & affect DNA replication and transcription. |
| Microfilaments | Microfilaments are uniformly organized in a layer enveloping the cortex in human oocytes and play an important role during oocyte maturation esp. polar body extrusion, pronuclear migration, intracellular movement of organelles and cell division. These processes may be perturbed by cryopreservation. |
| Microtubules | In oocytes and blastomeres, the major microtubular structure is the meiotic spindle which is responsible for spatial organization and migration of chromosomes during meiotic and mitotic divisions. Damage to this microtubular network may lead to altered chromosome complement within the cell, and ultimately aneuploidy. |
| Mitochondria | Mitochondrial swelling & abnormal distribution has been noted following cryopreservation. |
| Zona pellucida | Cryopreservation may lead to premature cortical granule exocytosis and subsequent zona hardening. |

There are various kinds of cryoinjuries which the oocytes/embryos incur while undergoing cryopreservation.

**Table:6. Various types of cryo-cellular injuries [21]**

| Type of injury | OUTCOME |
|---|---|
| Chilling injury (-5 to -50°C) | Irreversible damage to cellular structures – lipid droplets, proteins, membranes and microtubules. Different mammalian species and different developmental stages of oocytes contain varying amounts of lipid & thus varying sensitivities to chilling injury. |
| Osmotic shock during equilibration (-5 to -50°C) | Osmotically driven water efflux that occurs in the cells during freezing is responsible for causing rupture of plasma membrane, and leading to extracellular ice entering the cytoplasm. |
| Zona pellucida hardening | Premature cortical granule exocytosis due to cryopreservation may cause zona hardening, and this situation in case of oocytes may be circumvented by the use of ICSI. |
| Fracture injuries (-50°C to -150°C) | Zona fracture is a known consequence of cryopreservation and is observed both in oocytes and embryos. Fracture injuries are more common with the use of closed systems than open systems, as open systems use extremely small volumes of CPA and there is avoidance of mechanical forces associated with extreme pressure changes as observed with closed systems. |
| Safe zone (-196°C) | All cellular activity is suspended at this temperature as there is complete cessation of atomic and molecular movement. The only potential risk of DNA degradation is from background terrestrial radiation levels (0.1 centigray/year) which are too low to harm the stored tissue. |

## 13 What are the indications and advantages of oocyte/embryo freezing?

### INDICATIONS FOR OOCYTE CRYOPRESERVATION

- In cases following oocyte retrieval where the male partner is unavailable/unable to produce semen sample
- To circumvent the ethical and legal concerns associated with embryo freezing
- Women who wish to delay childbearing
- Oncofertility patients who are due to receive chemotherapy and/or radiation therapy for malignancy prior to commencement of treatment
- Oocyte cryobanking for oocyte donation programs
- Patients at risk of premature ovarian failure
- Better synchronization of donor-recipient cycles
- Cryoaccumulation (Accu-vit) strategy for poor responders

### INDICATIONS FOR EMBRYO CRYOPRESERVATION

- Supernumerary embryos left after transfer of selected embryos in a fresh cycle
- Avoiding fresh embryo transfer in stimulated cycles in patients at risk of OHSS, elevated progesterone, thin endometrium, fluid in the endometrial cavity on day of transfer, polyps detected incidentally following stimulation, bleeding and difficult embryo transfers.
- Blastocyst cryopreservation following trophoectoderm biopsy for PGS/PGD.

### ADVANTAGES OF VITRIFICATION

- The timing of the whole cryopreservation procedure is greatly reduced with vitrification as compared to conventional slow freezing
- Vitrification involves the use of high concentrations of CPA which allows shorter exposure times to the CPA
- Minimal volume used for vitrification increases the probability and rate of freezing
- Vitrification eliminates intracellular ice crystal formation and avoids lethal cellular injury
- It is associated with minimal osmotic injuries
- The protocols for vitrification are simple, easy to use, and do not require any expensive equipment like programmable freezer used for slow freezing

## 14 What is Leidenfrost effect?

Leidenfrost effect is a physical phenomenon in which a liquid that is in near contact with a mass significantly hotter than the liquid's boiling point, produces an insulating vapour layer that keeps the liquid from boiling rapidly. Due to this repulsive force, the droplet hovers over the surface rather than making physical contact with it ***(Fig.1)***.

The vitrification events are accompanied by a Leidenfrost phenomenon, which impedes the heat transfer to cool the liquid, when the liquid droplet comes into direct contact with liquid nitrogen [22]. The vapour layer formed around the specimen in effect insulates it from the cold liquid nitrogen.

The Leidenfrost effect can be reduced by decreasing the temperature of $LN_2$ to -210ºC, hence eliminating vapour formation and achieving even higher cooling rates.

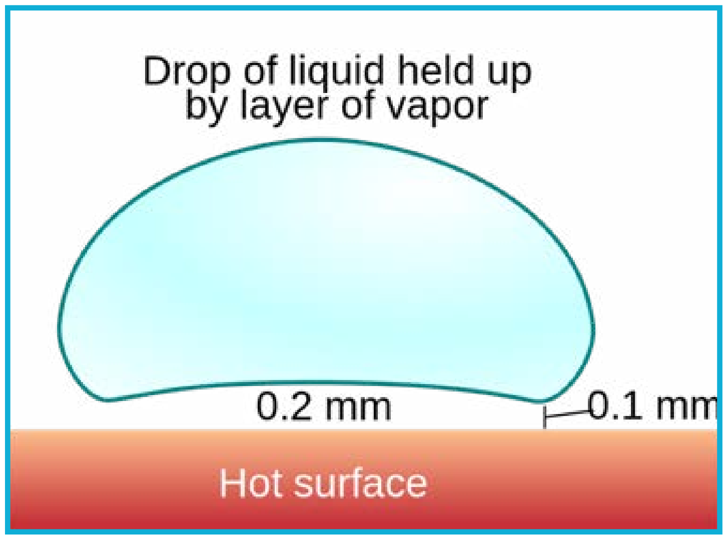



**Figure-1 Leidenfrost effect**

## 15 What is the most important step in vitrification? What should be the time permitted to move the carrier device from $LN_2$ to the thawing solution?

The seminal work of Peter Mazur and his colleagues has shown that the cryosurvival of oocytes and embryos depends almost entirely on the warming rate [23]. The faster the warming rate, higher the survival. Rapid warming is essential to minimize both the formation of intracellular ice crystals by devitrification and their growth to lethal size by recrystallization [24]. Rapid warming involves abrupt transfer of the carrier device from under the $LN_2$ to the thawing solution within under a second (without any brief intermediate hold in air).

## 16 How long can we cryopreserve gametes and embryos?

It is currently unknown what the maximum storage period might be for oocytes and embryos. Cryopreservation of human gametes and embryos has been around since 1980's, and this implies that the longest time that a human embryo has been stored is 25-30 years. Patients that have cryopreserved their gametes and embryos this long back are unlikely to return and request these embryos to be used. Live births have been reported from frozen human sperm stored for 40 years [25]. Similarly, successful live births have been reported from oocytes stored for more than 14 years in cryopreservation [26], and from embryos frozen over 20 years ago.

## 17 How has the technique of cryopreservation progressed on from slow-freezing of oocyte and embryos to vitrification?

The earliest pregnancy from frozen thawed human embryos was reported by Trounson in 1983 [27], followed closely by the first live birth in 1984 by Zeilmaker et al [28]. Similarly, the first pregnancies resulting from frozen oocytes were reported in 1980s, but this was followed by a quiet period with only sporadic case reports [29]. While most of the ART laboratories were using slow-freezing for gametes and embryos with mixed results and a subpar post thaw survival, a lot of research was carried out by various groups in the field of vitrification as an alternative technique to slow-freezing for cryopreservation. Mukaida et al. reported the first successful vitrification of human 4-8 cell embryos based on the methods developed for murine embryos [30] and this was confirmed by other groups as well. With the standardization of protocols, vitrification has been adopted by almost all the clinics worldwide as the standard method for oocyte and embryo vitrification. Successful vitrification of human oocytes was first described by Kuleshova et al in 1999, and though the results of oocyte vitrification are far superior to slow-freezing, the number of live births following embryo vitrification far exceeds those following oocyte vitrification.[31]

## 18 What are the differences between vitrification of oocytes, embryos and blastocyst?

The vitrification protocols for cryopreservation of oocytes and blastocysts differ from those for preserving cleavage-stage embryos, and this is reflected in the differences in the equilibration time for the oocytes, embryos or blastocysts.

### Peculiarities of OOCYTE vitrification

1. The mature human oocyte is the largest cell in the body (120 µm, ooplasm measuring around 100 µm and zona thickness around 16-18 µm). Its surface area to volume ratio is around 0.05 (surface area: 4.5 x $10^4$ $\mu m^2$; volume: 9 x $10^5$ $\mu m^3$). This, along with the fact that the oocyte is spherical in shape, accounts for poor permeation of CPAs due to lower surface area, as compared to a cell like spermatozoa.
2. The metaphase II oocyte possesses a temperature sensitive meiotic spindle which is crucial for events following fertilization in the completion of meiosis, second polar body extrusion, pronuclei migration, and the formation of first mitotic spindle. This spindle is susceptible to depolymerization following vitrification-warming procedure, and this may compromise the ability of the oocyte to undergo fertilization and normal preimplantation development.
3. The mature oocytes possess an actin scaffolding and regulatory secretory granules called cortical granules, that are synthesized in the centre of the oocyte but translocate to the plasma membrane during meiosis in preparation for fertilization. Following fertilization, a signaling pathway induces these granules to fuse with the oolemma and release their content in to the oocyte's extracellular matrix. However, during vitrification, these cortical granules may undergo premature exocytosis and consequently, zona hardening. This issue can be circumvented by the routine use of ICSI for vitrified warmed oocytes.
4. Finally, the oocyte is a single cell in contrast to the cleavage-stage embryos (composed of blastomeres) or blastocysts (containing numerous inner cell mass and trophoectodermal cells). Hence, the result of vitrification-warming in terms of cryosurvival is all or none.

### Peculiarities of BLASTOCYST vitrification

1. Unlike oocytes and cleavage-stage embryos, the blastocyst possesses a blastocoel cavity which is a fluid-filled cavity formed after cavitation. Since the basic premise of vitrification is the gradual dehydration of cellular contents, the presence of a blastocoel cavity is a deterrent to the process of vitrification. Some groups advocate the artificial collapse of the blastocoel cavity prior to vitrification by either the application of LASER pulse, manual pipetting through narrow bore pipettes or by mechanical collapse using an ICSI or a PZD pipette. The safety of these artificial shrinkage methods has been well demonstrated. A study by Wang et al. showed that the differences in the blastocyst survival rate were comparable between the LASER assisted shrinkage (LAS) and the micro-needle assisted shrinkage (MNAS) groups. However, LAS was found to be associated with increased implantation and clinical pregnancy rate, live birth rate, but also a significantly higher rate of monozygotic twinning [32].

However, now, there are various protocols that avoid the issue of blastocoel collapse by increasing the equilibration time e.g. Kitazato, Cryotech etc.

2. In contrast to the cleavage-stage embryos where the post warming morphology gives a rapid and clear impression of the cryosurvival, in case of blastocysts the immediate post warming morphology may not be unambiguous. Hence, it is recommended to culture the blastocysts for 1-2 hours post warming to allow for re-expansion and a better morphological assessment.

3. The results with day 5 vitrification may depend on the degree of blastocyst expansion, and a recommended size of blastocysts amenable to vitrification with consistent survival rates is between 170 to 220 µm.
4. It is also argued that day 3 embryo vitrification is associated with a higher cumulative pregnancy rate as there are fewer embryos that reach the blastocyst stage, and can be vitrified.

## 19 What magnification do we work at when vitrifying oocytes or embryos?

The working magnification while carrying out oocyte or embryo vitrification is subjective. However, working at lower magnifications [(0.8 – 1.2 X) x 10X eyepiece] is preferable since if more than one oocyte or embryo is put in the equilibration or vitrification solution, it is easier to track them in lower magnification, and the risk of losing any oocytes or embryos in the vitrification solution is minimal. This should be noted as the oocytes/embryos have a tendency to float when moved from equilibration to vitrification solution, and may temporarily disappear on visual inspection. However, a lower magnification helps to avoid this problem. It is also easier to load the oocytes/embryos on the carrier device in lower magnification. However, the degree of collapse and re-expansion is best assessed by changing to a higher magnification temporarily.

## 20 What is the role of vitrification in ovarian tissue cryopreservation?

Though vitrification is now the method of choice for cryopreservation for gametes and embryos, less is known about its performance in ovarian tissue cryopreservation, for which slow-freezing is the current convention. A recent meta-analysis of 14 studies suggested that vitrification may be more effective than slow-freezing, with less primordial follicular DNA strand breaks and better preservation of stromal cells[33]. Hence, vitrification may result in improved ovarian function after transplantation.To avoid the cryoprotectant associated toxicity which is the chief concern while using vitrification, relatively low concentrations of different cryoprotectants can be combined. However, more research is required to optimize the protocols for ovarian cortical vitrification.

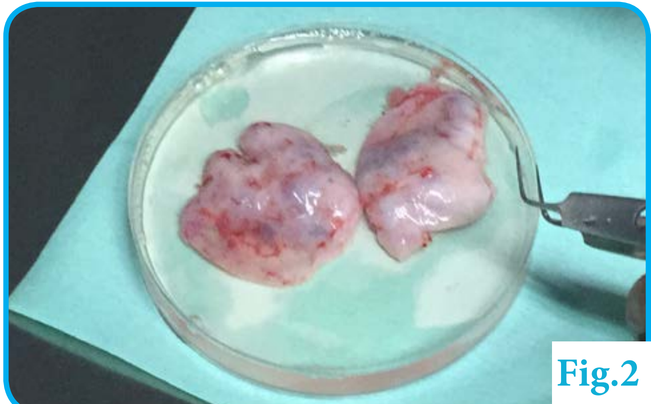


Fig.2

Fig.3

Fig.4

**Figure-2.** The ovary specimen being rinsed in the buffer solution. Using a fine tissue forceps, the specimen is held and we can see the outer cortical and inner medullary tissue of the ovary.

**Figure-3.** Approx 5 mm long x 5 mm wide x 1 mm deep slices of cortical tissue are sliced and rinsed in the washing solution

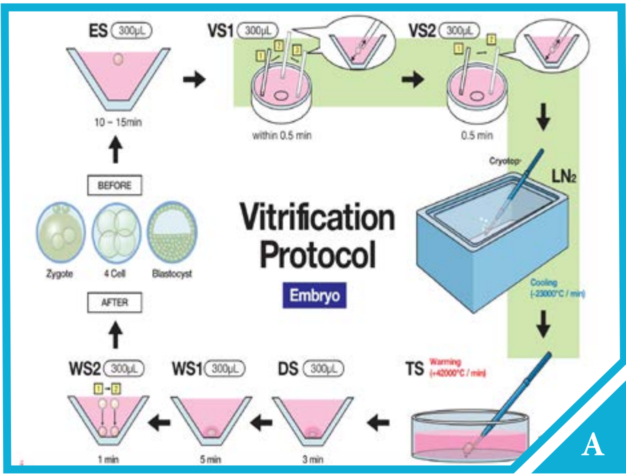
**Figure-4.** Strips of cortical tissue are loaded on specifically designed devices e.g. Ova Cryo Device™.

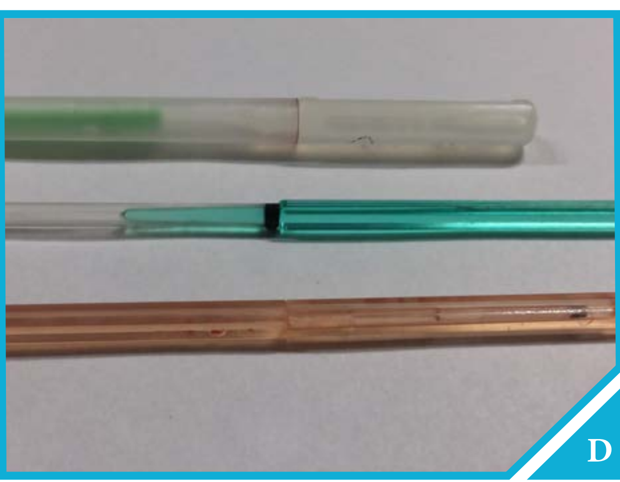
## 21 Can the technique of vitrification be used for sperm cryopreservation?

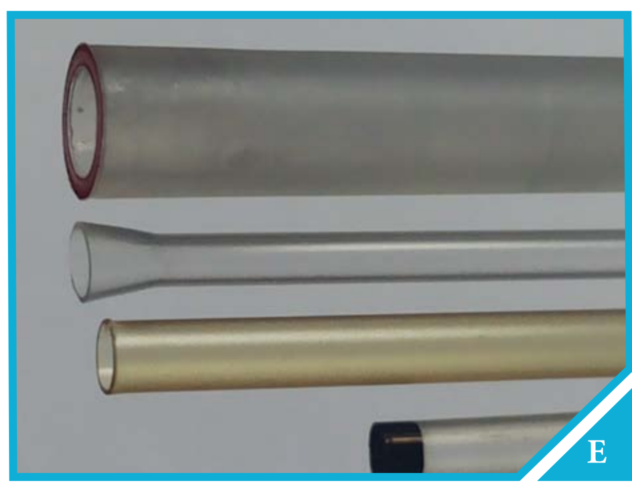
During cryopreservation, the cooling and warming processes during slow and rapid freezing associated with the intermediate zone of temperature (-10 to -60°C), which the cells must traverse twice (once during cooling and then during warming), can be lethal to the sperm. Vitrification does not require either the use of specially devised cooling programs or CPAs, and is much faster, simpler and cheaper. The method is based on cooling of sperms by direct immersion into $LN_2$, thereby avoiding intracellular ice crystal formation. Optimal cooling rates are obtained with the following specifically designed packaging systems: Open pulled straws, the Flexipet denuding pipette, micro- drops, electron microscope copper grids, the Hemi-straw system, Cryotop, Cryoleaf, Cryotip and other carrier devices. Another modification of vitrification is direct dropping of spermatozoa suspension in $LN_2$.

## 22 What are some tips to optimize vitrification results

**Foremost, strict adherence to the manufacturer's guidelines, protocols and time duration is extremely important.**





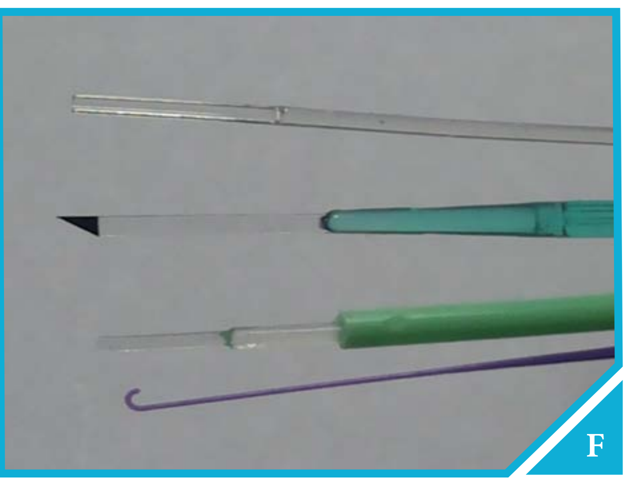



**Fig.1a:** Example of vitrification protocol from one of the manufacturers (Kitazato Dibimed) with step by step description of each process and recommended times for equilibration and vitrification solutions.

**Fig.1b:** Vitrification and warming kits from various manufacturers.

**Fig.1c:** Vitrification carrier devices from various manufacturers

**Fig.1d:** Locking mechanisms of different carrier devices.

**Fig.1e:** Differences in the outer protective sheath with different identification labels to facilitate easier manipulation under liquid nitrogen.

**Fig.1f:** Loading surfaces of different carrier devices.

**Fig.1g:** A dedicated Medical grade refrigerator to store the vitrification and warming media.

**Pre-freezing embryo morphology is a strong predictor of post warming cryosurvival. Hence, only grade A embryos should be vitrified. For oocytes, the timing of vitrification is important, and oocytes must be vitrified after 2-4 hours of incubation after retrieval, and immediately after denudation.**

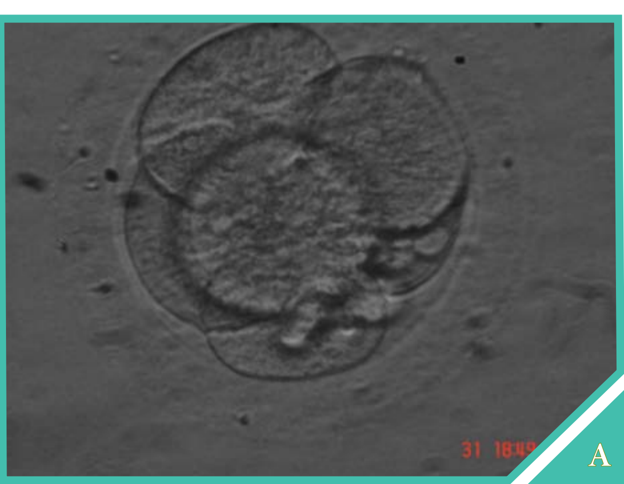



**Fig 2a:** A day-3 6-cell grade A embryo with less than 10% fragmentation.

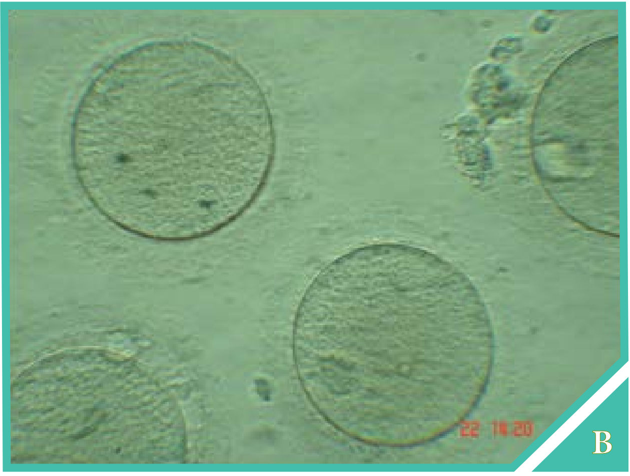
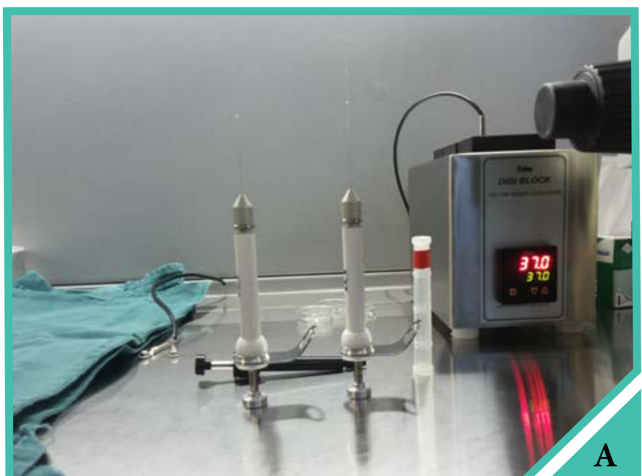
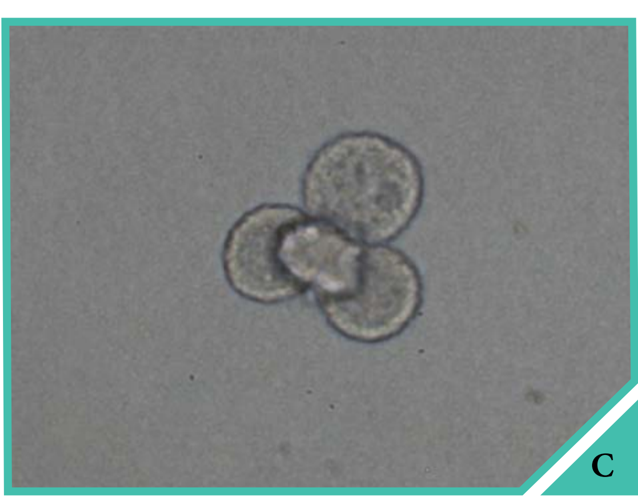



**Fig 2b:** Mature metaphase 2 oocytes should be selected for vitrification for optimal outcome.

**Regarding blastocyst vitrification, some protocols advocate the collapse of blastocoel cavity prior to vitrification, while others do not. The protocol should be strictly followed and tweaking or deviation from the protocol is not recommended.**

**It is recommended that a pipette with an inner diameter of 140-150 μm be used for the oocyte. Likewise, pipettes of optimum diameter should be used for embryos and blastocysts.**





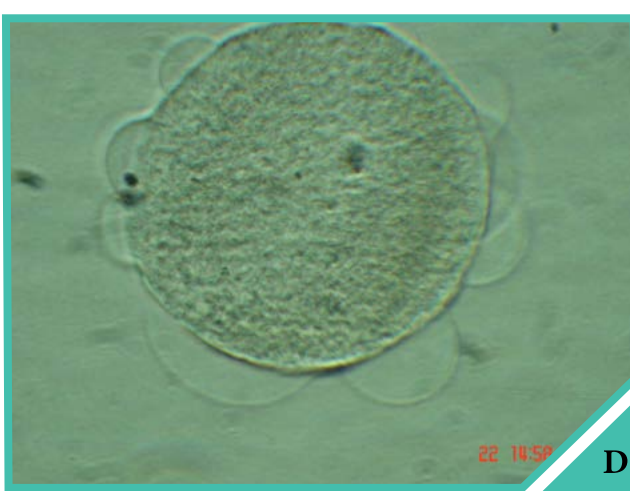



**Fig.4a:** Denudation pipettes with stripper handles of various sizes.

**Fig.4b:** Stripper handles from different manufacturers.

**Fig.4 c&d:** Cryoinjuries in the form of zona rupture and blebbing of oolemma.

**Fig.4e:** Manipulation of oocyte/embryo in equilibration and vitrification solutions

**5** **The vitrification process should be carried out at room temperature (unless specified otherwise). Similarly, the warming process (first step) should be carried out at 37°C (unless specified otherwise).**

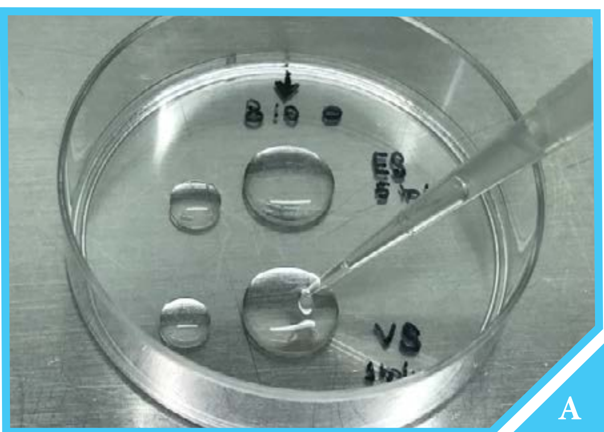


**Fig 5a:** Preparation of drops of equilibration and vitrification solution.

**Fig 5b:** Use of specified media volumes (300 μl) for equilibration and vitrification solutions in specially designed repro/vitriplates

**6** **Before commencing vitrification, all the solutions should be brought to room temperature at least 1 hour before starting.**

**7** **Similarly, before commencing warming, the thawing solution should be incubated to 37°C at least 2-3 hours before use (varies according to protocol followed). Some protocols recommend overnight incubation.**

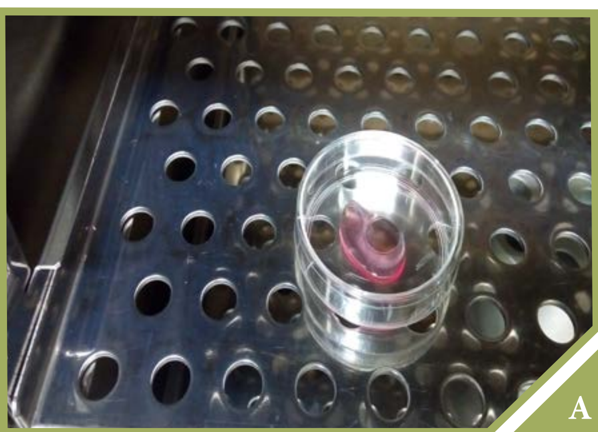


**Fig.7a&b:** Thawing solution (TS) being warmed to 37°C at least 1 hour prior to use.

**Fig.7c:** Do not keep TS at room temperature or for periods longer than intended use as it may cause media evaporation and sudden shifts in osmolality.

**8** **Patient identification is of utmost importance. Hence, the cryo devices should be clearly labelled with 2 unique identifiers, and once vitrification is completed and the device is stored in the cryotank, the location of the device including the number of oocytes/embryos vitrified and the media and carrier device used should be clearly documented in records.**

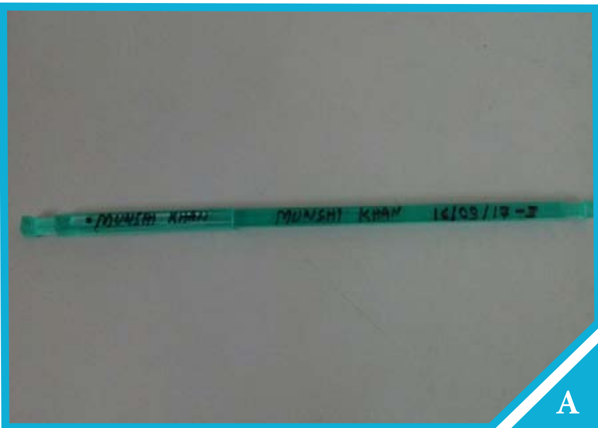



**Fig.8a:** Proper labelling of the cryodevice with 2 unique patient identifiers.

**Fig.8b:** Proper labelling of the aluminium cryocanes for easier identification of the location of carrier devices.

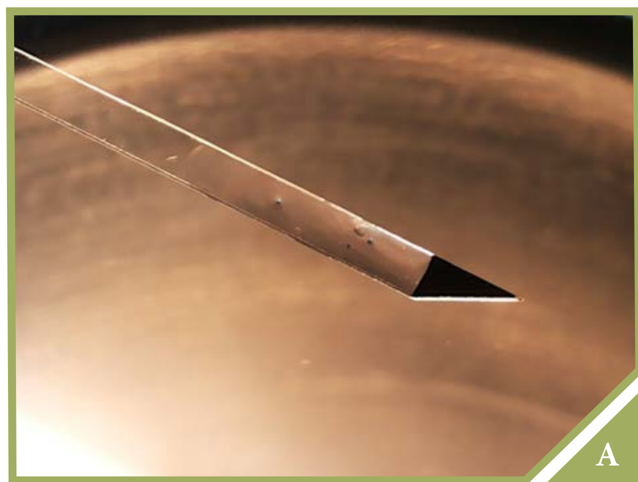
**Fig.8c:** If the devices are not marked properly or marked with sub quality pens the writing comes off and it is difficult to identify the device later at the time of warming.

**Fig.8d:** If the aluminium Cryocanes are not marked properly or marked with sub quality pens the writing comes off and it is difficult to identify the device later at the time of warming.

**9** **There should be at least 1 assistant to keep note of the time and to assist during the vitrification and warming steps.**

**Fig.9 a&b :** There should be at least 1 assistant to keep note of time and to assist during the vitrification and warming steps.

**During vitrification, after loading the embryos from the vitrification solution on to the carrier device and removal of excess CPA, the device should be immediately immersed into $LN_2$. Similarly, while warming, the cryodevice should be moved from $LN_2$ to the thawing solution under one second with one swift motion.**



A

B

C

D

E

F

**Fig.10a :** Figure showing carrier device with embryos and excess media.

**Fig.10b :** Embryos in minimal media.

**Fig.10c :** Loading of oocytes/embryos on the cryodevice with an appropriately sized pipette.

**Fig.10d :** Rapid immersion of the carrier device into liquid nitrogen.

**Fig.10.e&f:** The fig. shows adequately filled styrofoam box . Second is our innovation to save loss of liquid nitrogen during the procedure as lid can be place over the box safely.

**Fig.10g:** Placing the carrier device into a visotube attached to a cryocane.

**11** **During the first step of warming, care should be taken that the entire tip of the loading surface of the carrier device should be immersed in the thawing solution, and no bubbles should be created.**

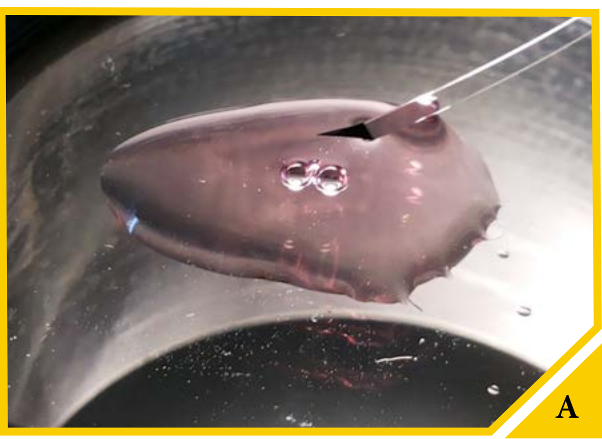


**Fig 11a&b:** Care should be taken while warming that the entire loading surface of the carrier device should be immersed into the thawing solution and attempts should be made to minimize making bubbles.

**12** **During warming, usually the embryos float when the device is immersed in the thawing solution. However, if the embryos remain on the surface of the carrier device, they should not be manipulated by sucking through the manipulation pipette. Rather, some media should be taken into the pipette and gently sprayed over the embryos to detach them from the device.**

**Fig 12 a&b :** It should be made sure that the entire loading surface is submerged under the thawing solution, and not just the tip of the carrier device.

Though classically 2-4 embryos have been loaded per carrier device, now it is recommended that no more than 2 embryos be stored per carrier device.

The recommended volumes of vitrification and warming solutions should be used as any deviation from the volume will alter the osmolality of the solution and is detrimental.

## The journey of an Embryo from Cryocan to Womb

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# Notes

# Part-2

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Table-7 :.Comparative analysis of different Cryo- devices from different OEM

| DEVICE | TYPE OF DEVICE | COMPONENTS OF DEVICES | HANDLE | LOADING SURFACE | LOCK | COLORS | LOGO | LABELING / PATIENT ID | REMARKS |
|---|---|---|---|---|---|---|---|---|---|
| **Mc Gill Cryoleaf™** <br><br> **(REF : 40771401)** | **Open device** <br><br> (oocytes / embryo are in direct contact with $LN_2$) | **3 parts** <br><br> **Carrier straw** <br><br> **Protective sleeve** (green) to protect oocytes over the tip & lock it by turning <br><br> **Outer cover** | **Circular handle** <br><br> (made up of polypropylene) | Flat loading surface | Rotating lock | Green | McGill Cryoleaf ™ on carrier straw for orientation of loading surface <br><br> Red marking for $LN_2$ level on the sleeve. | Patient ID is labelled on outer cover | **Maximum load is 2-3 oocytes** with minimum media volume <1 µl <br><br> Open system vitrification carrier for maximum cooling <br><br> Easy handling & efficient storage <br><br> Small surface available for labelling. |
| **VitriFit™** <br><br> **(REF : 42802 001A)** | **Open device** | **2 parts** <br><br> **Body** <br><br> **Cap** <br><br> Both body and cap made up of same material and are designed for a perfect fit at all temperature. | **Hexagonal Handle** <br><br> This shape allows VitriFit-™ carrier to be packed tighter during storage than popular vitrification carriers. <br><br> And improved storage efficiency by up to 50 % | **Curved** <br><br> **concave** loading surface | Plug in lock | Clear <br><br> Blue <br><br> Green <br><br> Yellow | ORIGIO is engraved on handle for the orientation of loading surface <br><br> Flat surface having orientation mark at the top indicates concave side up. | Long flat surface provided on VitriFit™ for recording patient ID | Maximum **specimen loading is 3** with minimal volume of media (<1µl) (use black mark as reference for loading) <br><br> Concave tip have a loading guidance <br><br> Cap has a dark ring to ease assembly |

| DEVICE | TYPE OF DEVICE | COMPONENTS OF DEVICES | HANDLE | LOADING SURFACE | LOCK | COLORS | LOGO | LABELING / PATIENT ID | REMARKS |
|---|---|---|---|---|---|---|---|---|---|
| **Rapid-i™ Kit**<br><br>**(REF : 94419)** | **Closed device**<br><br>Oocytes / embryos are not in direct contact with $LN_2$, therefore lowering the chances of cross contamination. | **80 mm stick** made from polymethyl methacrylate **(Rapid-i™)**<br><br>**130 mm thermoplastic elastomer storage straw** equipped with a stainless steel weight **(Rapid straw)**<br><br>**115mm stainless steel** rod inserted in Rapid straw | No handle | **30 nano - litre loading hole** at the tip of the straw, designed to produce surface tension to make loading of device easy. | **Ultrasonic sealing using ultrasonic sealer.**<br><br>Make sure that the seal covers the entire diameter of the rapid straw and that no holes / defects are present | Blue<br><br>Green<br><br>Yellow<br><br>Red<br><br>White | Logo is not engraved on device.<br><br>Black Indicators at bottom to help keep the embryo in safe zone<br><br>Red Markings at top to visualize where to cut **(approximately 30mm above the red mark)**<br><br>The lowest black mark of rapid straw must always be immersed in $LN_2$<br><br>The other edge of the Rapid-i™ is marked black to increase the visibility under $LN_2$ | Label the rapid straw with the **Patient ID between the 2 black marks** on the rapid straw. | Consistent sub-micro litre volume of media and minimised risks of unintentional warming<br><br>Minimum thermo insulation around the droplet & embryo.<br><br>Simple design and easy handling.<br><br>Avoid over filling the hole or else the embryos may float out.<br><br>Metal weight at the bottom keeps the Rapid-i™ in place by the magnets in the Smart Box™ and storage tank. |
| **Cryotop®**<br><br>**(REF : 81113)** | **Open device** | **2 parts**<br><br>**Body**<br><br>**Cap**<br><br>**To protect the Cryotop® once vitrified. Tip with weight to prevent floating in $LN_2$** | **Plastic handle**<br><br>Manufactured in malleable plastic resistant to $LN_2$ | **0.4mm** wide, **20mm** long and **0.1mm** thick fine strip of transparent film<br><br>**Made up of polypropylene** attached to a plastic handle | Plug-in lock | Yellow<br><br>White<br><br>Green<br><br>Blue<br><br>Pink | KITAZATO/Cryotop® is engraved on the flattened area of handle for the orientation of loading surface.<br><br>Black marking on external covering signifies the $LN_2$ level<br><br>Black marking on fine strip is loading guidance | Patient ID is labelled on the handle/ straw cap of Cryotop® | Best cooling and warming rates (-23000 ˚C /min) & (42000˚C/min) respectively which in turn lead to 90% survival rates.<br><br>Valid for all stage of development: oocytes, embryos, blastocysts.<br><br>**Allows upto 4 specimen to be loaded per device** with a minimum volume of media (0.1 µL) |

| DEVICE | TYPE OF DEVICE | COMPONENTS OF DEVICES | HANDLE | LOADING SURFACE | LOCK | COLORS | LOGO | LABELING / PATIENT ID | REMARKS |
|---|---|---|---|---|---|---|---|---|---|
| HSV Kit<br><br>(REF: 025463) | **Closed device**<br><br>(high security closed system) | **3 parts**<br><br>High security ultra thin **ionomeric resin straw** having pre-sealed weighted end and flared opening (40 mm length)<br><br>A **capillary tube** with a pre-formed fully **clear gutter** co-injected with its coloured identification rod.<br><br>A **blue plastic insertion** and **removal device** | No handle | **Curved**<br><br>(**concave** loading surface) | Heat sealing using **SYMS sealer** | Yellow<br><br>White<br><br>Red<br><br>Blue<br><br>Purple<br><br>Green | CryoBio Systems is engraved on blue plastic insertion device for orientation of loading surface | Patient ID is labelled approximately 20mm from the flared end of the straw.<br><br>When labelled this way, it does not cover the entirely of the coloured rod or the biological sample zone. | Maximum **2 specimen** can be loaded per device<br><br>Traceability by colour coding and $LN_2$ resistant labels for easy storage and identification<br><br>To properly seal HSV straws, the **right combination of heat, pressure, time and position of the straw** must be guaranteed. CryoBio system has validated this for **SYMS, SYMS II, SYMS III.** |
| Cryo lock™<br><br>(REF: CL-R-CT) | **Open device** | 2 parts<br><br>**Body**<br>A square shape stick **( 4.56 ” length, 0.118 ” width and 0.118 ” height)**<br><br>Made up of polystyrene medical grade with curved loading surface<br><br>**Cap**<br>**1.78 ” length, 0.118 ” width and 0.118 ” height**<br><br>Made of same material Provides an air tight seal by coupling of two tapered surfaces in a **0.250” sealing surface** and even temperature conduction at low temperatures. | Handle made of polystyrene medical grade. | **Curved**<br><br>(**concave** loading surface)<br><br>**Concave** tip allows secure loading ofembryos and isolation against displacement of the samples during closing | Plug-in lock | Green<br><br>Yellow<br><br>Orange<br><br>White<br><br>Blue | Cryolock™ is engraved on handle for orientation of loading surface.<br><br>Also patient ID is to written on same surface.<br><br>Black mark on tip is used as reference. | Patient ID is labelled on same surface where Cryolock™ logo is engraved. | Wide labelling surface for clear identification<br><br>Maximum loading of **2 specimens** for vitrification with a minimum volume of media (≤1 µl)<br><br>Body & cap have gaps on their extremes that allow easy grip with forceps during manipulation<br><br>Secure, hermetic seal keeps tip isolated from $LN_2$<br><br>Cooling and warming rates (≈-1494˚C/min) and (≈+21000˚C/min) respectively.<br>Cryolock™ vials are sterilized by gamma radiation |

| DEVICE | TYPE OF DEVICE | COMPONENTS OF DEVICES | HANDLE | LOADING SURFACE | LOCK | COLORS | LOGO | LABELING / PATIENT ID | REMARKS |
|---|---|---|---|---|---|---|---|---|---|
| Cryotech | **Open and closed device** | **1)** Plastic body with an attached filmstrip (20mm long) and<br>**2)** 30mm long plastic cap with a metal barrel at the opposite tip to prevent floating, and a plastic seal to avoid liquid nitrogen flow. | Plastic handle resistant to liquid nitrogen | Transparent PET filmstrip 1.0 mm wide, 20 mm long and 0.075 mm thick.Triangle black mark at the tip for recognition of loading side and easy view inside the liquid nitrogen. | **Open System:** plug-in lock<br>**Closed System:** heat seal | Red, Blue, Green, Yellow, White | CRYOTECH* logo engraved on one of the faces of the handle on the recommended loading side.<br>Black triangle mark on the loading filmstrip for loading and covering ease.<br>Black mark on the upper of the cap to ease recognition under liquid nitrogen. | Three flat sides of the handle are available for clear labelling of patient ID | Improved version which leads to 100% survival rate.<br>Endotoxin free, simple and reliable use. Can be used for all stages of development.<br>Cooling rates of 24,000 and 1,500 °C/min in open and closed systems respectively; and 43,000 °C/min warming speed. |

## Table-8 : Differences between Reproplate and Vitriplate

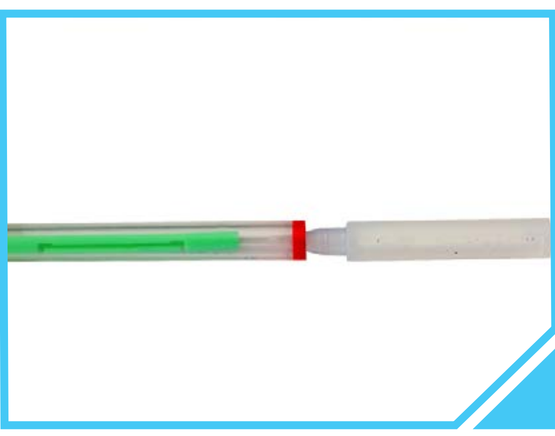
| REPROPLATE | VITRIPLATE & WARMPLATE |
|---|---|
|  | |
| **Manufactured by Kitazato Dibimed** | **Manufactured by Cryotech Reprosius** |
| Reproplate has 6 wells, and can be used for either vitrification or warming. | Vitriplate (3 wells) & warming plate (3 wells and a TS well) by Cryotech are separate, and are used for the respective steps. |
| The wells of reproplate are comparatively more conical. | The wells of vitriplate are comparatively more rounded. |
| - | The TS well of warming plate has a slope to facilitate the immersion of the device. |
| **Reproplate:** Does not have a groove to hold the carrier device. | **Vitriplate:** Has a groove to hold the carrier device. |

## Table-9: Commercially Available Cryo - Devices

### 1. MC GILL CRYOLEAF™

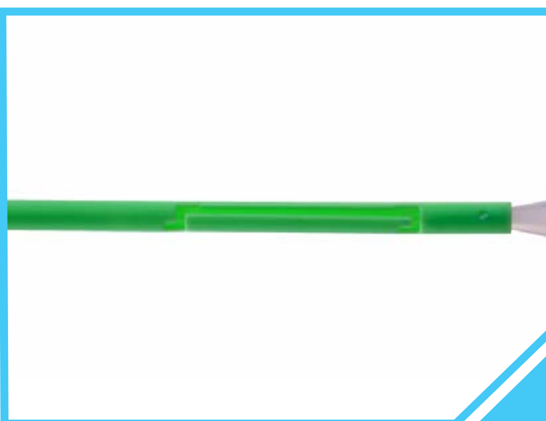
**Mc Gill Cryoleaf™** is an **open** device which means oocytes/embryo are in direct contact with $LN_2$.

It has 3 parts:
**Carrier straw**
**Protective sleeve** (green) to protect oocytes over the tip & lock it by turning
**Outer cover**
McGill Cryoleaf™ should stay dry during the process.



#### HANDLE

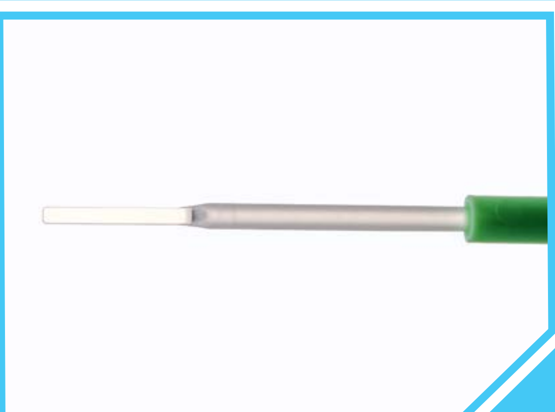
**Circular handle** made up **of Polypropylene**



#### LOCK

**Rotating lock**

The tip of McGill Cryoleaf™ is protected by sliding the green sleeve over the tip and locking it into place by turning.



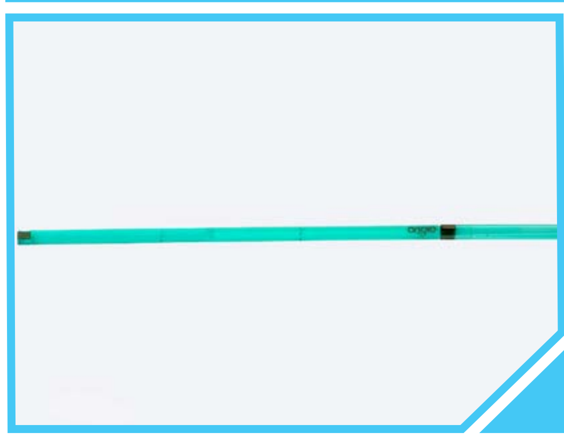
#### LOADING SURFACE

Flat loading surface

## LABELLING SURFACE

Patient ID is labelled on outer cover and handle.

# 2. VITRIFIT™



**VitriFit™** is an **open** device

It has 2 parts:
**Body**
**Cap**

Both body and cap made up of same material and are designed for a perfect fit at all temperature.

Cap has a dark ring to ease assembly

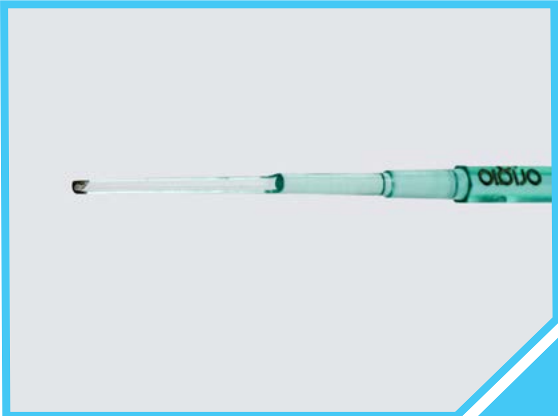
## HANDLE

**Hexagonal Handle**

Hexagonal shape allows VitriFit™ carrier to be packed tighter during storage than popular vitrification carriers and improved storage efficiency by up to 50 %

## LOCK

Plug in lock



## LOADING SURFACE

**Curved** i.e. concave loading surface for specimen protection and to ease loading and removal of excess medium.

Concave tip have a black loading guidance.

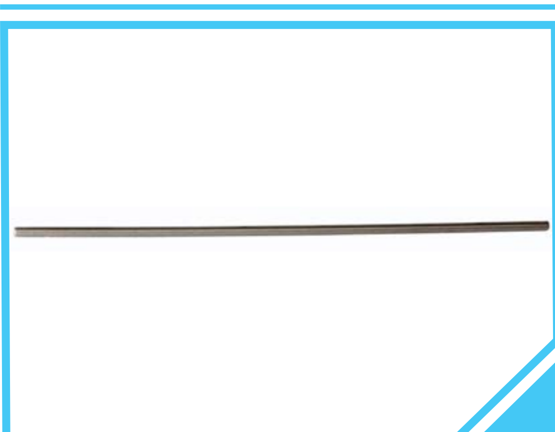
## LABELLING SURFACE

Long flat surface provided on VitriFit™ for recording Patient ID

# 3. RAPID-I™ KIT

**Rapid-i™** Kit is a **closed** device which means oocytes/ embryos are not in direct contact with $LN_2$, therefore lowering the chances of cross contamination.

It has 3 parts:
**80 mm** stick made from Polymethyl methacrylate (**Rapid-i™**)
**130 mm** thermoplastic elastomer storage straw equipped with a stainless steel weight, (**Rapid straw**)
**115 mm stainless steel rod** inserted in Rapid straw



## INSERTER

No handle but it has an inserter to insert the Rapid-i™

## LOCK

**Ultrasonic sealing using ultrasonic sealer,** meaning no heat is emitted to the straw and the specimens remains safe.

## LOADING SURFACE

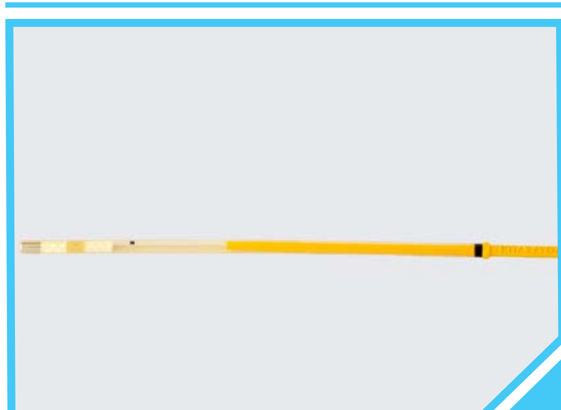
**30 Nanolitre loading hole** at the tip of the straw, designed to produce surface tension to make loading of device easy.

## LABELLING SURFACE

Label the rapid straw with the patient **ID between the 2 black marks** on the rapid straw.

Red marking for $LN_2$ level on the sleeve.

# 4. CRYOTOP$^{R}$



**Cryotop**$^{R}$ is an **open** device

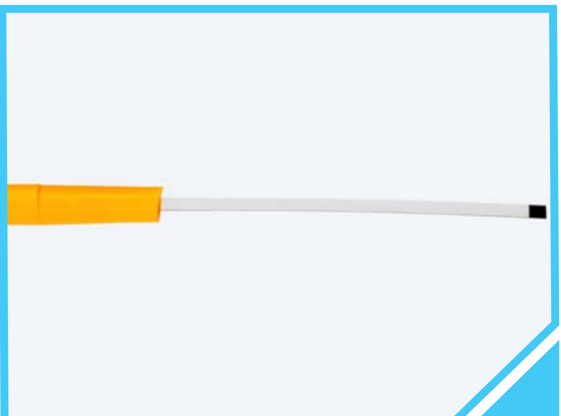
It has 2 parts:
**Body**
**Cap** (to protect the Cryotop$^{R}$ once vitrified. Tip with weight to prevent floating in $LN_2$)

## HANDLE

**Plastic handle** manufactured in **malleable plastic** resistant to $LN_2$

## LOCK

**Plug-in lock with a black marking for $LN_2$ level.**



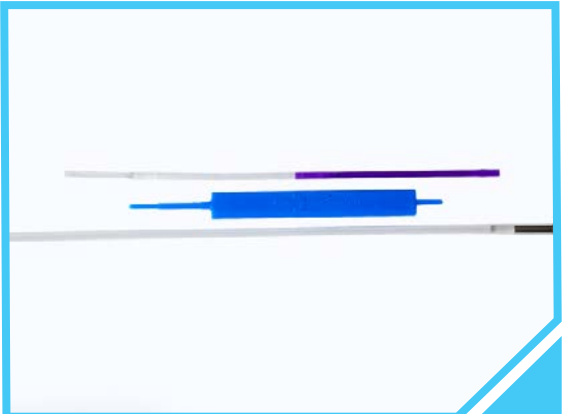
## LOADING SURFACE

Loading surface is a **0.4 mm wide, 20 mm long and 0.1 mm thick** fine strip of transparent film made up of Polypropylene attached to a plastic handle.

## LABELLING SURFACE

Patient ID is labelled on the handle/straw cap of Cryotop$^{R}$.

# 5. HSV KIT



**HSV (High Security Vitrification)** is a **Closed** device

It has 3 parts:

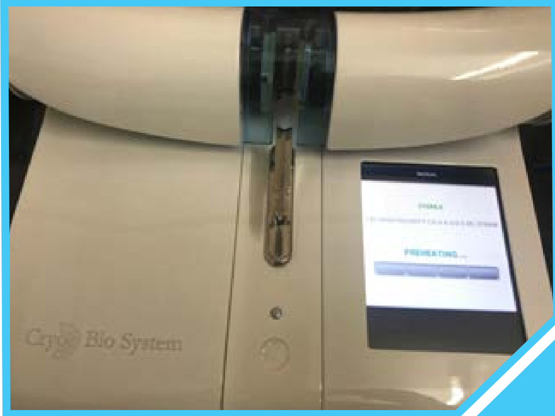
High security ultra thin **ionomeric resin straw** having pre-sealed weighted end and flared opening **(40 mm length)**

A **capillary tube** with a pre-formed gutter co-injected with its coloured identification rod.

**A blue plastic insertion and removal device**

## INSERTION REMOVAL DEVICE

No handle but it has an insertion and removal device.




## LOCK

**Thermal sealing using SYMS sealer**
It is a **benchtop unit** that can be used under a laminar flow cabinet.
It **uses a thermal process** to seal CBS™ High Security 0.3 ml, 0.5 ml and 2 ml straws, CBS™ High Security tubes as well as HSV High Security Vitrification straws.
**It consists of:**
Protective cover
Touch screen
Presence sensor
Product support
ON/OFF switch and USB port

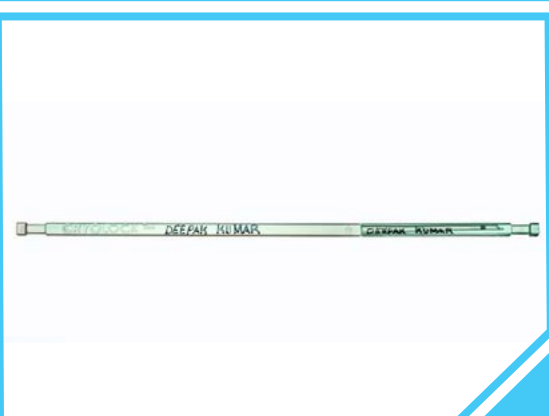
## LOADING SURFACE

Curved i.e. **concave** loading surface

## LABELLING SURFACE

Patient ID is labelled approximately 20 mm from the flared end of the straw

# 6. CRYOLOCK™

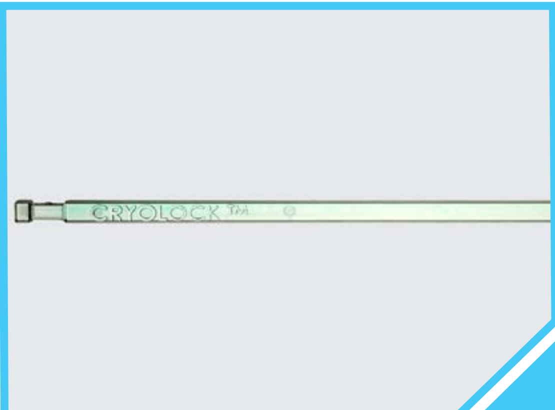



**Cryolock™** is an **open** device

It has 2 parts:

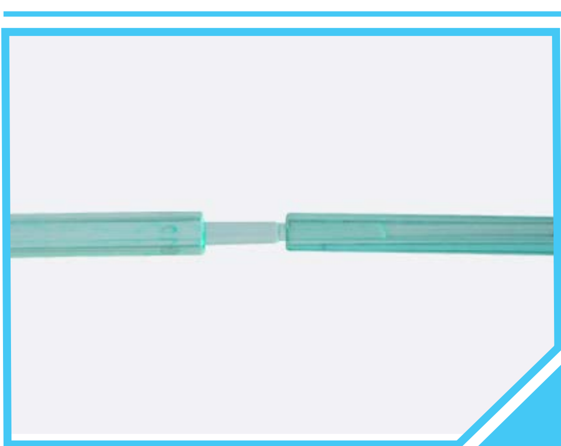
**Body:** A square shape stick (**115.8 mm length, 2.794 mm width and 2.794 mm height)** made up of Polystyrene medical grade with curved loading surface.

**Cap:** A square shape cap **(45.212 mm length, 2.794 mm width and 2.794 mm height)** made of same material.



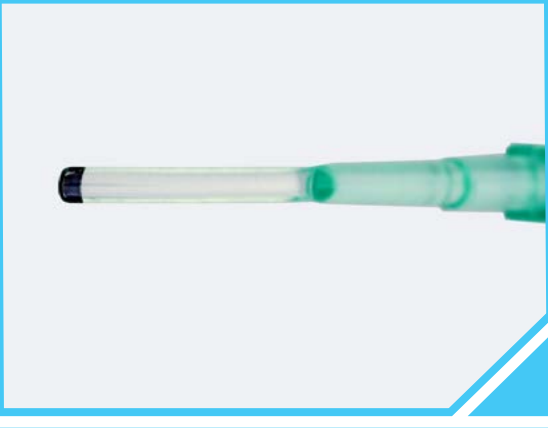

## HANDLE

**Square handle** made of **Polystyrene** medical grade and CRYOLOCK™ is engraved on it for orientation of loading surface.



## LOCK

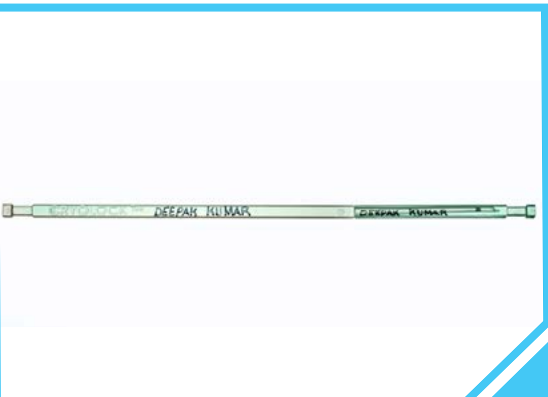
**Plug-in lock**



## LOADING SURFACE

**Curved** i.e. concave loading surface with a black loading guidance.

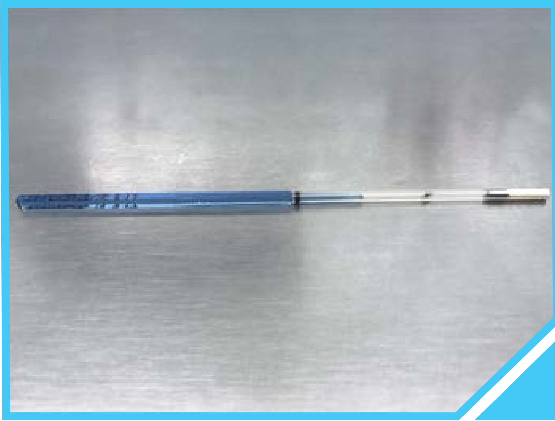
Concave tip allows secure loading of embryos and isolation against displacement of the samples during closing.




## LABELLING SURFACE

Patient ID is labelled on same surface where Cryolock™ logo is engraved.

# 7. CRYOTEC



**Cryotec** is an **Open** device.

It has 2 parts:

**Body**

**Cap**
(inside cap there is a balancer ball)

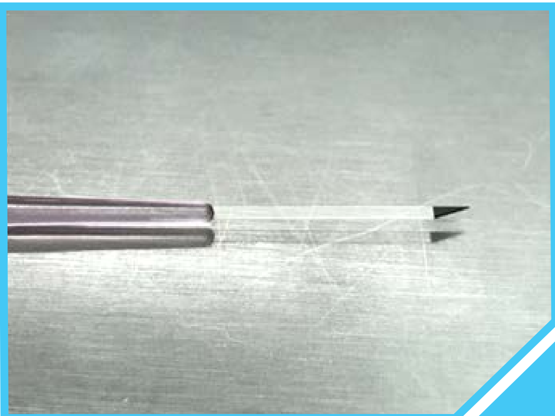
## HANDLE

**Long and wide handle.**

CRYOTEC* is engraved on handle for orientation of loading surface.

## LOCK

Plug-in lock



## LOADING SURFACE

**Fine, thin film loading surface** slightly cut from edge with a black loading guidance.

## LABELLING SURFACE

**Patient ID is labelled on the cap and handle** (on same surface where Cryotec* is engraved.)

## Table-10 : Composition of Vitrification & Warming Media from Different OEM

| S.NO. | ORIGINAL EQUIPMENT MANUFACTURER (OEM) | MEDIA | COMPOSITION | BASIC COMPOSITION |
|---|---|---|---|---|
| 1. | **KITAZATO** | **Vitrification Media** | | HEPES with Basic Culture Media |
| | | Basic Solution **(BS)**<br>Equilibration Solution **(ES)**<br>Vitrification Solution **(VS)** | Ethylene Glycol<br>Dimethyl Sulfoxide (DMSO)<br>Trehalose | |
| | | **Warming media** | | |
| | | Thawing Solution **(TS)**<br>Diluent Solution **(DS)**<br>Washing Solution **(WS)** | Ethylene Glycol<br>Dimethyl Sulfoxide<br>Trehalose | |
| 2. | **SAGE (ORIGIO)** | **Vitrification media** | | It is a MOPS buffered solution of modified HTF containing :<br><br>Essential amino acids<br>Non-essential amino acids<br>Gentamicin Sulfate (0.01g/L) |
| | | Equilibration Solution **(ES)** | 7.5% (v/v) Dimethyl sulphoxide<br>7.5% (v/v) Ethylene Glycol<br>Human Albumin (12mg/ml) | |
| | | Vitrification Solution **(VS)** | 15% (v/v) Dimethyl sulphoxide<br>15% (v/v) Ethylene Glycol<br>Human Albumin (12mg/ml)<br>0.6 M Sucrose | |
| | | **Warming media** | | |
| | | 1.0 M Sucrose Warming Solution | Human Albumin (12mg/ml)<br>1.0 M Sucrose | |
| | | 0.5 M Sucrose Warming Solution | Human Albumin (12mg/ml)<br>0.5 M Sucrose | |
| | | MOPS Solution | Human Albumin (12mg/ml) | |
| 3. | **CRYOTECH** | **Vitrification media** | | Modified HEPES Buffered MEM |
| | | Equilibration Solution **(ES)** | Ethylene Glycol<br>DMSO<br>Hydroxy Propyl Cellulose | |
| | | Vitrification Solution **(VS)** | Trehalose<br>Ethylene Glycol<br>DMSO<br>Hydroxy Propyl Cellulose | |
| | | **Warming media** | | |
| | | Thawing Solution **(TS)** | Trehalose<br>Hydroxy Propyl Cellulose | |
| | | Diluent Solution **(DS)** | Trehalose<br>Hydroxy Propyl Cellulose | |
| | | Washing Solution **(WS)** | Hydroxy Propyl Cellulose | |

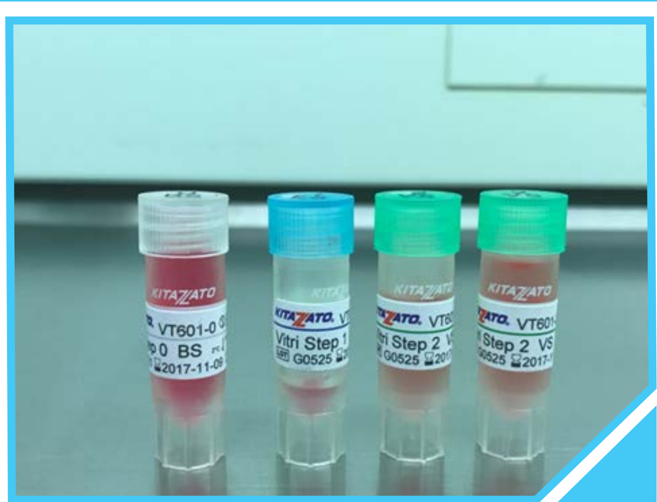
| S.NO. | ORIGINAL EQUIPMENT MANUFACTURER (OEM) | MEDIA | COMPOSITION | BASIC COMPOSITION |
|---|---|---|---|---|
| 4. | IRVINE | **Vitrification media** | | M – 199 HEPES Buffered Medium<br><br>Gentamicin |
| | | Equilibration Solution **(ES)** | 7.5% (v/v) Dimethyl sulphoxide<br>7.5% (v/v) Ethylene Glycol<br>Human Albumin (12mg/ml) | |
| | | Vitrification Solution **(VS)** | 15 % DMSO<br>15 % Ethylene Glycol<br>20% Dextran substitute supplement<br>0.5 M Sucrose | |
| | | **Warming media** | | |
| | | Thawing Solution **(TS)** | 1.0 M Sucrose<br>20 % Dextran Substitute Supplement | |
| | | Diluent Solution **(DS)** | 0.5 M Sucrose<br>20 % Dextran Substitute Supplement | |
| | | Washing Solution **(WS)** | 20 % Dextran Substitute Supplement | |
| 5. | COOK Blast | **Vitrification media** | | Cryobase buffer<br><br>10 mm HEPES Buffered Solution<br><br>20.0 mg/ml Human Serum Albumin<br><br>0.01 mg/ml Gentamicin |
| | | Blastocyst vitrification solution 1 | Cryobase buffer | |
| | | Blastocyst vitrification solution 2 | Cryobase buffer<br>8 % Dimethyl Sulphoxide<br>8% Ethylene Glycol | |
| | | Blastocyst vitrification solution 3 | Cryobase buffer<br>16 % Dimethyl Sulphoxide<br>16 % Ethylene Glycol<br>0.68 M Trehalose | |
| | | Blastocyst vitrification solution 4 | Dimethyl Sulphoxide (DMSO) | |
| | | **Warming media** | | |
| | | Blastocyst warming solution 1 | Cryobase buffer with 0.33 M Trehalose | |
| | | Blastocyst warming solution 2 | Cryobase buffer with 0.2 M Trehalose | |
| | | Blastocyst warming solution 3 | Cryobase buffer | |
| 6. | GLOBAL Blast | **Vitrification media** | | Sodium chloride , Potassium chloride , Calcium chloride , Potassium phosphate , Magnesium sulfate , Sodium Bicarbonate , Glucose , Lactate Na Salt , Sodium Pyruvate , Glycine , L-Alanine , L-Arginine HCL , L-Asparagine , L-Aspartic Acid L-Cystine, L-Glutamic Acid, Glycyl- |
| | | Solution 1 | Dimethyl sulphoxide (7.5 % v/v)<br>Ethylene Glycol (7.5 % v/v)<br>Human Serum Albumin (10 mg/ml) | |
| | | Solution 2 | Dimethyl sulphoxide (15 % v/v)<br>Ethylene Glycol (15 % v/v)<br>Sucrose (0.5 M)<br>Human Serum Albumin (10 mg/ml) | |

| S.NO. | ORIGINAL EQUIPMENT MANUFACTURER (OEM) | MEDIA | COMPOSITION | BASIC COMPOSITION |
|---|---|---|---|---|
| | GLOBAL Blast | **Warming media** | | Glutamine , L-Histidine, L-Isoleucine , L-Leucine , L-Lysine HCL, L-Methionine , L-Phenylalanine , L-Proline , L-Serine , L-Threonine , L-Tryptophan , L-Tyrosine , L-Valine , EDTA , Phenol Red , HEPES, Gentamicin Sulfate (10μg/ml) |
| | | Warm 1 solution | Sucrose (1.0 M)<br>Human Serum Albumin (10 mg/ml) | |
| | | Warm 2 solution | Sucrose (0.5 M)<br>Human Serum Albumin (10 mg/ml) | |
| | | Warm 3 solution | Human Serum Albumin | |
| 7. | **VITROLIFE Oocyte** | **Vitrification media** | | MOPS Buffered Solution |
| | | Vitri 1™ **oocyte** | No cryoprotectant | |
| | | Vitri 2™ **oocyte** | Ethylene Glycol<br>Propanediol | |
| | | Vitri 3™ **oocyte** | Ethylene Glycol<br>Propanediol<br>Sucrose | |
| | | **Warming media** | | |
| | | Warm 1™ **oocyte** | Sucrose | |
| | | Warm 2™ **oocyte** | Sucrose | |
| | | Warm 3™ **oocyte** | Sucrose | |
| | | Warm 4™ **oocyte** | No Sucrose | |
| | **VITROLIFE Cleavage** | **Vitrification media** | | MOPS Buffered Solution<br>Hyaluronan<br>Amino Acids |
| | | Vitri 1™ **Cleave** | Contains no cryoprotectant | |
| | | Vitri 2™ **Cleave** | Ethylene Glycol as cryoprotectant | |
| | | Vitri 3™ **Cleave** | Ethylene Glycol<br>Propanediol<br>Ficoll<br>Sucrose as cryoprotectant | |
| | | **Warming media** | | |
| | | Warm 1™ **Cleave** | Sucrose | |
| | | Warm 2™ **Cleave** | Sucrose | |
| | | Warm 3™ **Cleave** | Sucrose | |
| | | Warm 4™ **Cleave** | No cryoprotectant | |

| S.NO. | ORIGINAL EQUIPMENT MANUFACTURER (OEM) | MEDIA | COMPOSITION | BASIC COMPOSITION |
|---|---|---|---|---|
| | **VITROLIFE Blastocyst** | **Vitrification media** | | MOPS Buffered Solution<br>Amino Acids<br>Hyaluronan |
| | | Vitri 1™ **Blast** | No cryoprotectant | |
| | | Vitri 2™ **Blast** | Ethylene Glycol<br>Propanediol | |
| | | Vitri 3™ **Blast** | Ethylene Glycol<br>Propanediol<br>Ficoll | |
| | | **Warming media** | | |
| | | Warm 1™ **Blast** | Sucrose | |
| | | Warm 2™ **Blast** | Sucrose | |
| | | Warm 3™ **Blast** | No cryoprotectant | |
| | **VITROLIFE Omni** | **Vitrification media** | | MOPS Buffered Solution<br>Human Albumin Serum<br>Amino Acids<br>Hyaluronan<br>Gentamicin<br>Energy substrates |
| | | Vitri 1™ **Omni** | No cryoprotectant | |
| | | Vitri 2™ **Omni** | Ethylene Glycol<br>Propanediol | |
| | | Vitri 3™ **Omni** | Ethylene Glycol<br>Propanediol | |
| | | **Warming media** | | |
| | | Warm 1™ **Omni** | Sucrose | |
| | | Warm 2™ **Omni** | Sucrose | |
| | | Warm 3™ **Omni** | Sucrose | |
| | | Warm 4™ **Omni** | No sucrose | |
| | | | | |

## Table-11: Commercially available Media Kits

### KITAZATO




### VITRIFICATION MEDIA

The range of sequential vitrification media bottles are packed in sterile condition with cap of different colour codes for differentiation between each media for different stages of the vitrification process.

**The media range includes:**
One vial of Vitri Step 0 BS **(White cap) (1.5 ml)**
One vial of Vitri Step 1 ES **(Blue cap) (1.5 ml)**
Two vials of Vitri Step 2 VS **(Green cap) (2 x 1.5 ml)**

### WARMING MEDIA

The range of sequential vitrification media bottles are packed in sterile condition with cap of different colour codes for differentiation between each media for different stages of the vitrification process.

**The media range includes:**
One vial of Vitri Step 0 BS **(White cap) (2 x 4 ml)**
One vial of Vitri Step 1 ES **(Blue cap) (4 ml)**
Two vials of Vitri Step 2 VS **(Green cap) (4 ml)**

**Material of the vial/ bottle: Plastic**
**Expiry: 3 months** after the date of manufacture.
**Stability:** These solutions are stable until the expiration date shown on the vial label.

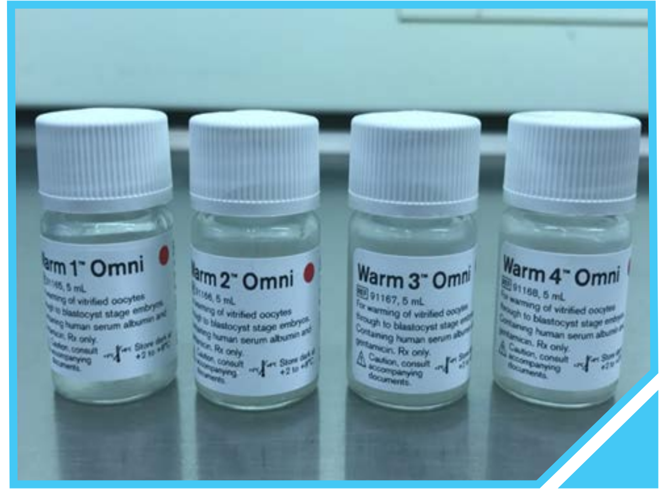
**Quality assurance:**
**pH**
**Osmolality**
**Endotoxins: <0.25EU/ml by LAL Methodology>**
**Sterility (Bacteria, Fungi): Current USP Sterility Test**
**MEA (Mouse Embryo Assay): 1- cell assay ≥80 % after 96 hours.**

### VITROLIFE Omni

### VITRIFICATION MEDIA

The sequential vitrification media includes:
One vial of Vitri 1™ Omni **(5 ml)**
One vial of Vitri 2™ Omni **(5 ml)**
One vial of Vitri 3™ Omni **(5 ml)**

All media bottles are packed in glass bottles in sterile condition with cap.




### WARMING MEDIA

The sequential Warming media contains:

One vial of Warm 1™ Omni **(5 ml)**
One vial of Warm 2™ Omni **(5 ml)**
One vial of Warm 3™ Omni **(5 ml)**
One vial of Warm 4™ Omni **(5 ml)**

**Material of the vial/ bottle: Glass**
**Expiry: 2 weeks after first opening**
**Stability:** Solutions are stable until the expiration date shown on the container labels and the LOT specific Certificate of analysis.

**Quality Assurance:**

**Sterile filtered (SAL)** $10^{-3}$
**Mouse Embryo Assay** (1-cell)
[% expanded blastocyst within 96 hours] ≥80
**Bacterial Endotoxins** (LAL Assay) [EU/ml] <0.5

## IRVINE SCIENTIFIC

### VITRIFICATION MEDIA

The sequential Vitrification media includes:
One vial of Equilibration Solution **(White cap) (2 x 1 ml)**
One vial of Vitrification Solution **(Blue cap) (2 x 1 ml)**

### WARMING MEDIA

**The media range includes:**
Four vials of Thawing Solution **(Yellow cap) (4 x 2 ml)**
One vial of Dilution Solution **(Orange cap) (2 ml)**
One vial of Washing Solution **(Red cap) (2 ml)**

**Material of the vial/ bottle: Plastic**

**Expiry: 8** weeks after opening
**Stability:** Solutions are stable until the expiration date shown on the vial tubes.
**Storage: 2 - 8˚C** in dark place

**Quality Assurance:**

**Endotoxin** by LAL Methodology
**Biocompatibility** by Mouse Embryo Assay (1- cell)
**Sterility** by current USP Sterility Test
**Albumin Test**

## SAGE

### VITRIFICATION MEDIA

**The media range includes:**
One vial of Equilibration Solution **(Blue cap) (2 ml)**
One vial of Vitrification Solution **(Purple cap) (2 ml)**

### WARMING MEDIA

**The warming media contains:**
One vial of 1.0 M Sucrose Warming Solution **(Blue cap) (4 ml)**
One vial of 0.5 M Sucrose Warming Solution **(Purple cap) (2 ml)**
One vial of MOPS Solution **(Pink cap) (6 ml)**

Do not use if the product becomes discoloured, cloudy, turbid, or shows any evidence of microbial contamination.

**Material of the vial/ bottle: Glass**

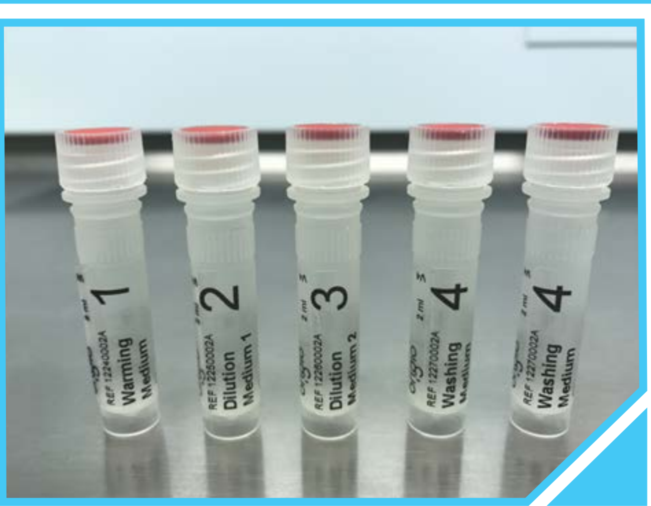
**Expiry: 1 year** from the date of manufacturing.

**Stability:** The solutions are stable until the expiration date shown on the labels
**Storage:** store unopened containers refrigerated at **2˚C to 8˚C** (Do not freeze or expose to temperatures greater than 39˚C.)

**Quality Assurance:**

**Endotoxin**
**Biocompatibility** by the 1- cell Mouse Embryo Assay (MEA)
**Sterility:** The solutions are **membrane filtered** and aseptically processed according to cGMP procedures which have been validated to meet a **sterility assurance level (SAL) of $10^{-3}$**.

## MEDICULT




### VITRIFICATION MEDIA

**The sequential vitrification media contains:**
Two vials of Equilibration Medium **(1 ml)**
Two vials of Vitrification Medium **(1 ml)**

### WARMING MEDIA

**The Warming media includes:**
One vial of Warming Medium **(Vial 1) (2 ml)**
Two vials of Dilution Medium as
Dilution Medium 1 **(Vial 2) (2 ml)**
Dilution Medium 2 **(Vial 3) (2 ml)**
Two vials of Washing Medium **(Vial 4) (2 x 2)**

**Material of the vial/ bottle: Plastic**

**Expiry: Minimum 8 weeks** from the date of shipment

**Stability:** Solutions are stable until the expiration date shown on the vial tubes.

**Quality Assurance:**

**Sterility** tested
**Endotoxin** tested ≤0.5 EU/ml
**Mouse Embryo Assay (MEA)** tested
**pH** tested
**Osmolality**

## VITROLIFE Oocyte

### VITRIFICATION MEDIA

**The sequential vitrification media contains:**
One vial of Vitri 1™ Oocyte **(10 ml)**
One vial of Vitri 1™ Oocyte **(10 ml)**
One vial of Vitri 1™ Oocyte **(10 ml)**

### WARMING MEDIA

**The sequential Warming media contains:**
One vial of Warm 1™ Oocyte **(10 ml)**
One vial of Warm 1™ Oocyte **(10 ml)**
One vial of Warm 1™ Oocyte **(10 ml)**

**Material of the vial/ bottle: Glass**

**Expiry: 2 weeks** after first opening
**Stability:** Solutions are stable until the expiration date shown on the container labels and the LOT- specific Certificate of analysis.
**Storage: 2 - 8˚C** in dark place

**Quality Assurance:**

**Sterile filtered (SAL) $10^{-3}$**
**Mouse Embryo Assay** (1-cell)
[% expanded blastocyst within 96 hours] ≥80
**Bacterial Endotoxins** (LAL Assay) [EU/ml] <0.5

## VITROLIFE Blastocyst

### VITRIFICATION MEDIA

**RapidVit™ Blast** contains
Vitri 1™ Blast **(10 ml)**
Vitri 2™ Blast **(10 ml)**
Vitri 3™ Blast **(10 ml)**
For the vitrification of human blastocyst stage embryos.

### WARMING MEDIA

**RapidWarm™ Blast** contains
Warm 1™ Blast **(10 ml)**
Warm 2™ Blast **(10 ml)**
Warm 3™ Blast **(10 ml)**
For the warming of the vitrified human blastocyst stage embryos.

**Material of the vial/ bottle: Glass**

**Expiry: 2 weeks** after first opening

**Stability:** Solutions are stable until the expiration date shown on the container labels and the LOT- specific Certificate of analysis.

**Storage: store dark at 2 - 8˚C**

**Quality Assurance:**

**Sterile filtered (SAL) $10^{-3}$**
**Mouse Embryo Assay** (1-cell)
[% re-expanded blastocyst within 24 hours post-test] ≥70
**Bacterial Endotoxin** (LAL Assay) [EU/ml] <0.5

## GLOBAL Blastocyst

### VITRIFICATION MEDIA

**The sequential Vitrification media contains :**
One vial of DMSO Equilibration Solution **(Blue cap) (5 ml)**
One vial of Vitrification Solution **(White cap) (5 ml)**

### WARMING MEDIA

**The Warming media contains:**
One vial of Warm 1 Solution **(Green cap) (1 x 5 ml)**
One vial of Warm 2 Solution **(Yellow cap) (2 ml)**
Two vials of Warm 3 Solution **(Red cap) (2 x 5 ml)**
For the recovery and rehydration of the human blastocyst stage embryos.

**Material of the vial/ bottle: Plastic**

**Expiry: 1 year** from the date of manufacture.
**Stability:** Solutions are stable until the expiration date shown on the container labels
**Sterility**: 2- 8°C and protected from light

**Quality Assurance:**
**Sterility: Membrane filtered (SAL) 10-3**

**Physiochemical test:**
**pH** – 7.1- 7.2
**Osmolality** (mOsM)
**Biological test:**
**Endotoxin** (LAL Assay - <0.5 EU/ml
**Mouse Blastocyst Re- expansion Assay** (% re-expanded blastocysts at 24th of culture after step- in, step- out of all of cryopreservation and thawing solutions)- ≥80 %
**MEA (1-cell)** has very low permeability to glycerol and therefore 1-cell Mouse Embryo assay is inappropriate for testing of this kit.

## CRYOTECH

### VITRIFICATION MEDIA

**The vitrification media includes:**
One vial of Equilibration Solution as **Vitri Step 1 with blue cap (1 ml)**
Two vials of Vitrification Solution as **Vitri Step 2 with green cap (1 ml each)**

### WARMING MEDIA

**The Warming Solution contains:**
Thawing Solution as **Warm Step 1 with red cap (1.8 ml)**
Diluent Solution as **Warm Step 2 with yellow cap (.5 ml)**
Washing Solution as **Warm Step 3 with white cap (1 ml)**

**Material of the vial/ bottle: Plastic**

**Expiry: 12 months (1 Year)**
**Stability:** Solutions are stable **at room temperature for 90 days (3 months)** since it contains Endotoxin free Trehalose.
**Storage: 2- 8°C** protected from light.

**Quality assurance:**

**Sterility:** Sterility Test
**Endotoxin by ES methodology** (Each component)
**Efficiency:** Survival of 30/30 Porcine oocytes.

## COOK Blastocyst

### VITRIFICATION MEDIA

**The media range includes**
Blastocyst Vitrification Solution 1 with **Orange cap (20 ml)**
Blastocyst Vitrification Solution 2 with **Orange cap (20 ml)**
Blastocyst Vitrification Solution 3 with **Orange cap (20 ml)**
Blastocyst Vitrification Solution 4 with **Blue cap (10 ml)**

### WARMING MEDIA

**The media range includes**
Blastocyst Warming Solution 1 with **Orange cap (20 ml)**
Blastocyst Warming Solution 2 with **Orange cap (20 ml)**
Blastocyst Warming Solution 3 with **Orange cap (20 ml)**

**Material of the vial/ bottle: Glass**

**Stability: Solutions are stable until the expiration date shown on the vial label.**
**Expiry: 8 weeks** from date of manufacture.

**Quality Assurance:**
**Sterility:** These products are supplied sterile and are sterilized by Aseptic Filtration.
**Each LOT of product is tested for:**
**Endotoxin** by LAL Methodology
Biocompatibility by Mouse Embryo Assay (MEA)
**Osmolality and pH**

## Table-12 : Description of Vials available in Vitrification & Warming Kits

| | TYPE OF FREEZING | BRAND NAME | VITRIFICATION MEDIA | | | WARMING MEDIA | | |
|---|---|---|---|---|---|---|---|---|
| | | | VIALS | VOLUME | COLOR OF VIAL CAP | VIALS | VOLUME | COLOR OF VIAL CAP |
| 1. | OOCYTE FREEZING | CRYOTECH | Equilibration Solution **(ES)** | 1 x 1 ml | Blue | Thawing Solution (TS) | 1 x 1.8 ml | Red |
| | | | Vitrification Solution **(VS)** | 2 x 1 ml | Green | Diluent Solution (DS) | 1 x 0.5 ml | Yellow |
| | | | | | | Washing Solution (WS) | 1 x 1 ml | White |
| | | VITROLIFE | Vitri 1™ oocyte | 10 ml | White | Warm 1™ oocyte | 10 ml | White |
| | | | Vitri 2™ oocyte | 10 ml | White | Warm 2™ oocyte | 10 ml | White |
| | | | Vitri 3™ oocyte | 10 ml | White | Warm 3™ oocyte | 10 ml | White |
| | | | | | | Warm 4™ oocyte | 10 ml | White |
| 2. | EMBRYO FREEZING | SAGE (ORIGIO) | Equilibration Solution **(ES)** | 1 x 2 ml | Blue | 1.0 M Sucrose warming solution | 1 x 4 ml | Blue |
| | | | Vitrification Solution **(VS)** | 1 x 2 ml | Purple | 0.5 M Sucrose warming solution | 1 x 2 ml | Purple |
| | | | | | | MOPS solution | 1 x 6ml | Pink |
| | | VITROLIFE | Vitri 1™ Cleave | 1 x 10 ml | White | Warm 1™ Cleave | 1 x 10 ml | White |
| | | | Vitri 2™ Cleave | 1 x 10 ml | White | Warm 2™ Cleave | 1 x 10 ml | White |
| | | | Vitri 3™ Cleave | 1 x 10 ml | White | Warm 3™ Cleave | 1 x 10 ml | White |
| | | CRYOTECH | Equilibration Solution **(ES)** | 1 x 1 ml | Blue | Thawing Solution (TS) | 1 x 1.8 ml | Red |
| | | | Vitrification Solution **(VS)** | 2 x 1 ml | Green | Diluent Solution (DS) | 1 x 0.5 ml | Yellow |
| | | | | | | Washing Solution (WS) | 1 x 1 ml | White |
| | BLASTOCYST FREEZING | COOK Blast | Blastocyst vitrification solution 1 | 20 ml | Orange | Blastocyst warming solution 1 | 20 ml | Orange |
| | | | Blastocyst vitrification solution 2 | 20ml | Orange | Blastocyst warming solution 2 | 20 ml | Orange |
| | | | Blastocyst vitrification solution 3 | 20ml | Orange | Blastocyst warming solution 3 | 20 ml | Orange |

| S.NO | TYPE OF FREEZING | BRAND NAME | VITRIFICATION MEDIA | | | WARMING MEDIA | | |
|---|---|---|---|---|---|---|---|---|
| | | | VIALS | VOLUME | COLOR OF VIAL CAP | VIALS | VOLUME | COLOR OF VIAL CAP |
| 3. | BLASTOCYST FREEZING | COOK Blastx` | Blastocyst vitrification solution 4 | 10ml | Blue | Blastocyst warming solution 3 | 20 ml | Orange |
| | | SAGE (ORIGIO) | Equilibration Solution **(ES)** | 1 x 2 ml | Blue | 1.0 M Sucrose warming solution | 1 x 4 ml | Blue |
| | | | Vitrification Solution **(VS)** | 1 x 2 ml | Purple | 0.5 M Sucrose warming solution | 1 x 2 ml | Purple |
| | | | | | | MOPS solution | 1 x 6ml | Pink |
| | | VITROLIFE | Vitri 1™ Blast | 1 x 10 ml | White | Warm 1™ Blast | 1 x 10 ml | White |
| | | | Vitri 2™ Blast | 1 x 10 ml | White | Warm 2™ Blast | 1 x 10 ml | White |
| | | | Vitri 3™ Blast | 1 x 10 ml | White | Warm 3™ Blast | 1 x 10 ml | White |
| | | GLOBAL | Dimethyl Sulphoxide Equilibration Solution ( DMSO **ES**) | 1 x 5 ml | Blue | Warm 1 solution | 1 x 5 ml | Green |
| | | | Vitrification Solution **(VS)** | 1 x 5 ml | White | Warm 2 solution | 1 x 5 ml | Yellow |
| | | | | | | Warm 3 solution | 2 x 5 ml | Red |
| 4. | OOCYTES, EMBROYS, BLASTOCYST | VITROLIFE omni | Vitri 1™ omni | 1 x 5 ml | White | Warm 1™ omni | 1 x 5 ml | White |
| | | | Vitri 2™ omni | 1 x 5 ml | White | Warm 2™ omni | 1 x 5 ml | White |
| | | | Vitri 3™ omni | 1 x 5 ml | White | Warm 3™ omni | 1 x 5 ml | White |
| | | | | | | Warm 4™ omni | 1 x 5 ml | White |
| | | KITAZATO | Basic Solution **(BS)** | 1 x 1.5 ml | | Thawing Solution (TS) | 2 x 4 ml | Red |
| | | | Equilibration Solution **(ES)** | 1 x 1.5 ml | Blue | Diluent Solution (DS) | 1 x 4 ml | Yellow |
| | | | Vitrification Solution **(VS)** | 2 x 1.5 ml | Green | Washing Solution (WS) | 1 x 4 ml | White |
| | | IRVINE | Equilibration Solution **(ES)** | 2 x 1ml | White cap | Thawing Solution (TS) | 4 x 2 ml | Yellow |
| | | | Vitrification Solution **(VS)** | 2 x 1ml | Blue cap | Diluent Solution (DS) | 1 x 2 ml | Orange |
| | | | | | | Washing Solution (WS) | 1 x 2 ml | Red |

## Table-13 : VITRIFICATION PROTOCOL FOR DIFFERENT OEM

| S.NO. | OEM | COOLING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | TIME | |
| 1. | CRYO-TECH | **Equilibration Solution (ES)**<br>Dispense 300 µl of **ES** on the vitri plate and transfer oocyte on the surface of **ES** | < 15 mins | **Equilibration Solution (ES)**<br>Fill the vitri plate well with 300 µl of **ES** and the embryo on the surface of **ES** | < 12 mins | **Equilibration Solution (ES)**<br>Dispense 300 µl of **ES** on the vitri plate and transfer blastocyst on the surface of **ES** | < 15 mins | Bring **ES** and **VS** vials to room temperature (25-27˚C) at least 1 hour before vitrification<br>The entire procedure must be carried out at room temperature (25-27⁰C).<br>Do NOT use heated stage. |
| | | **Vitrification Solution (VS)**<br>Transfer oocyte to the first drop of 300 µl of $\mathbf{VS_1}$ | 30-40 sec | **Vitrification Solution (VS)**<br>Transfer embryo to the first drop of 300 µl of $\mathbf{VS_1}$ | 30-40 sec | **Vitrification Solution (VS)**<br>Transfer blastocyst to the first drop of 300 µl of $\mathbf{VS_1}$ | 30-40 sec | |
| | | Finally transfer oocyte to the second drop of 300 µl of $\mathbf{VS_2}$ | 10- 20 sec | Finally transfer embryo to the second drop of 300 µl of $\mathbf{VS_2}$ | 10- 20 sec | Finally transfer blastocyst to the second drop of 300 µl of $\mathbf{VS_2}$ | 10- 20 sec | |
| 2. | KITAZ-ATO | **Basic Solution (BS)**<br>Dispense 20 µl of **BS** on the repro plate and transfer oocyte to the bottom of the **BS** | 3 mins | **Equilibration Solution (ES)**<br>Dispense 300 µl of **ES** on the repro plate and transfer the embryo to the top center of **ES** | 10- 15 mins | **Equilibration Solution (ES)**<br>Dispense 300 µl of **ES** on the repro plate well and transfer the blastocyst to the top center of **ES** | 12- 15 mins | Bring BS, **ES** and **VS** to room temperature (25-27˚C) |
| | | **Equilibration Solution (ES)**<br>Add 20 µl of **ES** gently on top of **BS** with the oocyte | 3 mins | **Vitrification Solution (VS)**<br>Dispense 300 µl of **VS** on the repro plate and transfer embryo from **ES** to $\mathbf{VS_1}$ | 0.5 min | **Vitrification Solution (VS)**<br>Dispense 300 µl of **VS** on the Repro plate and transfer blastocyst from **ES** to $\mathbf{VS_1}$ | | |

| S.NO. | OEM | COOLING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | TIME | |
| 3. | KITAZ-ATO | Add another 20 µl of **ES** gently on the top of **BS** | 3 mins | Drop another 300 µl of **VS** and transfer embryo from **$VS_1$** to **$VS_2$** | 0.5 min | Drop another 300 µl of **VS** and transfer blastocyst from **$VS_1$** to **$VS_2$** | 0.5 min | |
| | | Add another 240 µl of **ES** gently on top of the **BS** | 6-9 mins | | | | | |
| | | **Vitrification Solution (VS)**<br><br>Dispense 300 µl of **VS** on the Repro plate and transfer oocyte from **ES** to **$VS_1$** | 0.5 min | | | | | |
| | | Dispense another 300 µl of **VS** and transfer oocyte from **$VS_1$** to **$VS_2$** | 0.5 min | | | | | |
| | IRVINE | **HEPES buffered medium with protein (H)**<br><br>Dispense 20 µl of **H** and place oocytes into **H** | 1 min | **Equilibration solution (ES)**<br><br>Dispense 50 µl of **ES** and transfer embryos to **ES** | 6- 10 mins | **Equilibration solution (ES)**<br><br>Dispense 50 µl of ES and transfer blastocysts to **ES** | 6- 10 mins | All procedure must be performed at room temperature (22 - 27°C)<br><br>Do not use **HEAT-ED STAGE.**<br><br>The timing for exposure to VS is **CRIT-ICAL** for embryo vitrification. |
| | | **Equilibration Solution (ES)**<br><br>Dispense three 20 µl drops of **ES** (**$ES_1$**, **$ES_2$** and **$ES_3$**)<br>Merge **$ES_1$** with **H** and allow spontaneous mixing | 2 mins | **Vitrification Solution (VS)**<br><br>Dispense 50 µl of **VS** and transfer embryos from **ES** to **VS** | 30 sec | **Vitrification Solution (VS)**<br><br>Dispense 50 µl of **VS** and transfer blastocyst from **ES** to **VS** | 30 sec | |
| | | Then merge **$ES_2$** with **H+$ES_1$** and allow spontaneous mixing | 2 mins | | | | | |
| | | Transfer oocytes from merged drop to **$ES_3$** | 6- 10 mins | | | | | |
| | | **Vitrification Solution (VS)**<br><br>Dispense 50 µl drop of **VS** and transfer oocytes from **$ES_3$** to **VS** | 30 sec | | | | | |

| S.NO. | OEM | COOLING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | TIME | |
| 4. | VITRO-LIFE | **Vitri 1™ Oocyte**<br><br>Dispense 1 ml of Vitri 1™ oocyte into a 5-well plate and transfer oocytes from culture medium into Vitri 1™ Oocyte. | 5- 20 mins | **Vitri 1™ Cleave**<br><br>Dispense 0.5- 1ml of Vitri 1™ Cleave into separate wells of 5-well plate and transfer embryos from culture medium into the Vitri 1™ Cleave | 5- 10 mins | **Vitri 1™ Blast**<br><br>Dispense 0.5- 1 ml of Vitri 1™ Blast into separate wells of a 5-well plate and transfer collapsed blastocysts from culture medium into Vitri 1™ Blast | 5- 20 min | All procedures should be performed at +37˚C and ambient atmosphere.<br><br>Deviations from +37˚C will alter the permeability of cryprotectants, which may compromise specimen survival.<br><br>Warm all solutions to +37˚C |
| | | **Vitri 2™ oocyte**<br><br>Dispense 1 ml of Vitri 2™ oocyte into same plate and transfer oocytes from Vitri 1™ oocyte to Vitri 2™ oocyte | 2- 5 mins | **Vitri 2™ Cleave**<br><br>Dispense 0.5- 1 ml of Vitri 2™ Cleave in a separate well of same plate and transfer embryos into Vitri 2™ Cleave | 2 mins | **Vitri 2™ Blast**<br><br>Dispense 0.5- 1ml of Vitri 2™ Blast in a separate well of same plate and transfer blastocyst from Vitri 1™ Blast to Vitri 2™ Blast | 2 min | |
| | | **Vitri 3™ oocyte**<br><br>Dispense 20 µl droplet of Vitri 3™ oocyte on a 40 mm culture dish and transfer oocytes from Vitri 2™ oocyte to Vitri 3™ oocyte | 25- 30 sec | **Vitri 3™ Cleave**<br><br>Dispense 20 µl droplet of Vitri 3™ Cleave on a 40 mm culture plate and transfer embryos into Vitri 3™ Cleave | 30 sec | **Vitri 3™ Blast**<br><br>Dispense 20 µl droplet of Vitri 3™ Blast on a 40 mm culture plate and transfer blastocyst into the Vitri 3™ Blast | 45 sec | |
| 5. | SAGE ORIGIO | **Details Not Available** | **NA** | **Equilibration solution (ES)**<br><br>Prepare an inverted lid of a Petri dish by aseptically dispensing one 20 µl drop of **ES** on the lid and transfer embryos from culture medium to the top of the drop of **ES** | 5-15 mins | **Equilibration solution (ES)**<br><br>Prepare an inverted lid of a Petri dish by aseptically dispensing one 20 µl drop of **ES** on the lid and transfer embryos from culture medium to the top of the drop of **ES** | 5-15 mins | Vitrification procedure is to be performed at room temperature (20-25˚C)<br><br>Do not use a **HEATED** microscope stage |
| | | | | **Vitrification Solution (VS)**<br><br>Dispense four 20 µl drops of **VS ($VS_1$, $VS_2$, $VS_3$ and $VS_4$)** and transfer embryos from **ES** into the centre of **$VS_1$** | 5 sec | **Vitrification Solution (VS)**<br>Dispense four 20 µl drops of **VS ($VS_1$, $VS_2$, $VS_3$ and $VS_4$)** and transfer embryos from **ES** into the centre of **$VS_1$** | 5 sec | |

| S.NO. | OEM | COOLING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | TIME | |
| | SAGE ORIGIO | Details Not Available | NA | Quickly transfer the embryos from the $VS_1$ to the centre of the $\mathbf{VS_2}$ | 5 sec | Quickly transfer the embryos from the VS1 to the centre of the $\mathbf{VS_2}$ | 5 sec | Minimize exposure of specimens to light during incubation in ES and VS.<br><br>Bring the solutions to room temperature before use. |
| | | | | Next, transfer the embryos from $VS_2$ to the centre of the $\mathbf{VS_3}$ | 10 sec | Next, transfer the embryos from $\mathbf{VS_2}$ to the centre of the $\mathbf{VS_3}$ | 10 sec | |
| | | | | Finally, transfer the embryos from $VS_3$ to the bottom of the $\mathbf{VS_4}$ | 90 sec | Finally, transfer the embryos from $\mathbf{VS_3}$ to the bottom of the $\mathbf{VS_4}$ | 90 sec | |
| 6. | GLOBAL Blast | Details Not Available | NA | Details Not Available | NA | **Equilibration Solution (ES)**<br><br>Dispense 20 µl of **ES** into the embryo GPS[R] dish and transfer the blastocyst to the top of the drop of **ES** | 5- 15 mins | The vitrification procedure is to be performed at room temperature (20-27˚C)<br><br>Bring the ES and **VS** to room temperature before use.<br><br>Do not use **HEATED** microscope stage<br><br>Minimize exposure of specimens to light during exposure to the ES and **VS.** |
| | | | | | | **Vitrification Solution (VS)**<br><br>Dispense four 20 µl of **VS** into the same plate ($\mathbf{VS_1}$, $\mathbf{VS_2}$, $\mathbf{VS_3}$ and $\mathbf{VS_4}$) and transfer blasto-cysts from ES into the center of the $\mathbf{VS_1}$ | 5 sec | |
| | | | | | | Quickly, transfer the blastocysts from $\mathbf{VS_1}$ to the center of the $\mathbf{VS_2}$ | 5 sec | |
| | | | | | | Next, transfer the blastocysts from $\mathbf{VS_2}$ to the center of the $\mathbf{VS_3}$ | 10 sec | |
| | | | | | | Finally, transfer the blastocysts from the $\mathbf{VS_3}$ to the bottom of the $\mathbf{VS_4}$ | 90 sec | |

| S.NO. | OEM | COOLING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | TIME | |
| 7. | COOK Blast | **Details Not Available** | **NA** | **Details Not Available** | **NA** | **Blastocyst vitrification solution 1**<br>Prepare the vitrification solutions in a 4-well dish by adding 800 µl of Vitrifiation Solution 1 into well 1 and 2 and place blastocysts to be vitrified into well 1. | | Aseptic technique should be used.<br><br>DMSO is a frozen solid at 2-8˚C. Before use equilibrate Blastocyst Vitrification Solution 4 (DMSO) to room temperature prior to use.<br><br>Equilibrate the three Vitrification Solutions to 37˚C prior to use. |
| | | | | | | Move the specific blastocysts to be vitrified into well 2 | 1 min | |
| | | | | | | **Blastocyst Vitrification Solution 2**<br>Prepare the vitrification solution by adding 400 µl of Blastocyst Vitrification Solution 4 to 4.6 ml of Blastocyst Vitrification Solution 2 and mix well.<br>Dispense 800 µl of this prepared solution into well 3 and place the blastocysts to well 3. | 2 mins | |
| | | | | | | **Blastocyst Vitrification Solution 3**<br>Prepare the vitrification solution by adding 1 ml of Blastocyst Vitrification Solution 4 to 5.25 ml of Blastocyst Vitrification Solution 3 and mix well<br>Dispense 800 µl of this prepared solution into well 4 and place the blastocysts to well 4 | 20-30 sec | |
| 8. | **ORIGIO Medicult** | **As Discussed In Part 3** | | **As Discussed In Part 3** | | **As Discussed In Part 3** | | |

## Table-14 : WARMING PROTOCOL FOR DIFFERENT OEM

| S.NO. | OEM | WARMING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | TIME | |
| 1. | CRYOTECH | **Thawing Solution (TS)**<br>Dispense 1.8 ml of **TS** into the first square well of the warm plate and quickly (within 1 sec) put the Cryotech into the **TS** | 1 min | **Thawing Solution (TS)**<br>Dispense 1.8 ml of **TS** into the first square well of the warm plate and quickly (within 1 sec) put the Cryotech into the **TS** | 1 min | **Thawing Solution (TS)**<br>Dispense 1.8 ml of **TS** into the first square well of the warm plate and quickly (within 1 sec)put the cryotech device into the **TS** | 1 min | |
| | | **Diluent Solution (DS)**<br>At the end of 1 min. aspirate the oocyte and 3 mm long column of **TS** into the pipette.<br>Transfer **TS** to the bottom center of **DS** and expel the oocyte slowly at the bottom of **TS** layer in **DS** well. This is for most gradual displacement from **TS** to **DS.** Wait for 3 min. While waiting, fill the **$WS_1$** and **$WS_2$** well with **300 µl** of Washing Solution. | 3 mins | **Diluent Solution (DS)**<br>At the end of 1 min. aspirate the embryo and 3 mm column long of **TS** into the pipette.<br>Transfer **TS** to the bottom center of **DS** and expel the embryo slowly at the bottom of **TS** layer in **DS** well. This is for most gradual displacement from **TS** to **DS.** Wait for 3 min. While waiting, fill the **$WS_1$** and **$WS_2$** well with **300 µl** of Washing Solution. | 3 mins | **Diluent Solution (DS)**<br>At the end of 1 min. aspirate the blastocyst and 3 mm long column of **TS** into the pipette.<br>Transfer **TS** to the bottom center of **DS** and expel the blastocyst slowly at the bottom of **TS** layer in **DS** well. This is for most gradual displacement from **TS** to **DS.** Wait for 3 min. While waiting, fill the **$WS_1$** and **$WS_2$** well with **300 µl** of Washing Solution. | 3 mins | The whole procedure should be made under room temperature (20- 27˚) |
| | | **Washing Solution (WS)**<br>Aspirate the oocyte and 3 mm long column of **DS** into the pipette.<br>Transfer **DS** to the bottom center of **$WS_1$**, and expel the oocyte slowly at the bottom of **DS** layer in **$WS_1$** well. This is for most gradual displacement from **DS** to **$WS_1$**. Wait for 5 min.<br>Give a survival judgment at the end of this step depending on the recovery of the shrunken oocyte. | 5 mins | **Washing Solution (WS)**<br>Aspirate the embryo and 3 mm long column of **DS** into the pipette.<br>Transfer **DS** to the bottom center of **$WS_1$**, and expel the embryo slowly at the bottom of **DS** layer in **$WS_1$** well. This is for most gradual displacement from **DS** to **$WS_1$**. Wait for 5 min.<br>Give a survival judgment at the end of this step depending on the recovery of the shrunken embryo. | 5 mins | **Washing Solution (WS)**<br>Aspirate the blastocyst and 3mm long column of **DS**into the pipette.<br>Transfer **DS** to the bottom center of **$WS_1$**, and expel the blastocyst slowly at the bottom of **DS** layer in **$WS_1$** well. This is for most gradual displacement from **DS** to **$WS_1$**. Wait for 5 min.<br>Give a survival judgment at the end of this step depending on the recovery of the shrunken blastocyst. | 5 mins | Use a Pasteur pipette with the right diameter for oocytes, embryos **(140- 150 µm)**<br>Limit exposure to light while moving the specimens through the solutions. |
| | | Aspirate the oocyte with minimal volume of **$WS_1$**<br>Put the oocyte on the surface of the **$WS_2$** well. When oocyte/embryo sinks to bottom, aspirate and put on the surface. It will again sink to the bottom, thus washing is done twice.<br>Put the oocyte in the droplet of the culture media for the recovery for ICSI and ET. | 1 min | Aspirate the embryo with minimal volume of **$WS_1$**<br>Put the embryo on the surface of the **$WS_2$** well. When oocyte/embryo sinks to bottom, aspirate and put on the surface. It will again sink to the bottom, thus washing is done twice.<br>Put the embryo in the droplet of the culture media for the recovery for ICSI and ET. | 1 min | Aspirate the blastocyst with minimal volume of **$WS_1$**<br>Put the blastocyst on the surface of the **$WS_2$** well. When oocyte/embryo sinks to bottom, aspirate and put on the surface. It will again sink to the bottom, thus washing is done twice.<br>Put the blastocyst in the droplet of the culture media for the recovery for ICSI and ET. | 1 min | |

| S.NO. | OEM | WARMING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | TIME | |
| 2. | KITAZATO | **Thawing Solution (TS)** Dispense the full contents into the Petri Dish and quickly immerse (within 1 sec) Cryotop sheet into **TS** and dispense oocyte from the sheet. | 1 min | **Thawing Solution (TS)** Dispense the full contents into the Petri Dish and quickly immerse (within 1 sec) Cryotop sheet into **TS** and dispense embryo from the sheet. | 1 min | **Thawing Solution (TS)** Dispense the full contents into the Petri Dish and quickly immerse (within 1 sec) Cryotop sheet into **TS** and dispense blastocysts from the sheet. | 1 min | |
| | | **Dilution Solution (DS)** Dispense 300 µl of **DS** into the Repro Plate well and blow out only **TS** in the Pasteur pipette into the bottom center of **DS** slowly, then gently place oocyte on the bottom of the **TS** layer. | 3 mins | **Dilution Solution (DS)** Dispense 300 µl of **DS** into the Repro Plate well and blow out only **TS** in the Pasteur pipette into the bottom center of **DS** slowly, then gently place embryo on the bottom of the **TS** layer. | 3 mins | **Dilution Solution (DS)** Dispense 300 µl of **DS** into the Repro Plate well and blow out only **TS** in the Pasteur pipette into the bottom center of **DS** slowly, then gently place blastocysts on the bottom of the **TS** layer. | 3 mins | Warm **TS** vial (sealed) with a Petri Dish in an incubator to 37°C (>1.5 hours) |
| | | **Washing Solution (WS)** Dispense two 300 µl drops of **WS** (**$WS_1$** and **$WS_2$**) into the Repro Plate well and blow out only DS in the Pasteur pipette into the bottom center of **$WS_1$** slowly, then gently place the oocyte on the bottom there. | 5 mins | **Washing Solution (WS)** Dispense two 300 µl drops of **WS** (**$WS_1$** and **$WS_2$**) into the Repro Plate well and blow out only **DS** in the Pasteur pipette into the bottom center of **$WS_1$** slowly, then gently place the embryo on the bottom there. | 5 mins | **Washing Solution (WS)** Dispense two 300 µl drops of **WS** (**$WS_1$** and **$WS_2$**) into the Repro Plate well and blow out only **DS** in the Pasteur pipette into the bottom center of **$WS_1$** slowly, then gently place the blastocysts on the bottom there. | 5 mins | Bring DS and **WS** to room temperature (25- 27°C) |
| | | Aspirate oocyte with minimal volume of **$WS_1$** with Pasteur pipette and transfer it to the top center of **$WS_2$**. After the oocyte free falls to the bottom of **$WS_2$**, do same work again in **$WS_2$** | 1 min | Aspirate embryo with minimal volume of **$WS_1$** with Pasteur pipette and transfer it to the top center of **$WS_2$**. After the embryo free falls to the bottom of **$WS_2$**, do same work again in **$WS_2$** | 1 min | Aspirate blastocysts with minimal volume of **$WS_1$** with Pasteur pipette and transfer it to the top center of **$WS_2$**. After the blastocysts free falls to the bottom of **$WS_2$**, do same work again in **$WS_2$** | 1 min | |

| S.NO. | OEM | WARMING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | | |
| 3. | IRVINE | **Thawing Solution (TS)**<br>Dispense 1 ml of **TS** and warm to 37°C and quickly plunge the curved spatula (gutter) of the holding rod into the 37°C **TS** and gently swirl to detach oocyte and leave oocyte in the **TS** | 1 min | **Thawing Solution (TS)**<br>Dispense 250 µl of **TS** and warm to 37°C and quickly plunge the curved spatula (gutter) of the holding rod into the 37°C **TS** and gently swirl to detach embryo and leave embryo in the **TS** | 1 min | **Thawing Solution (TS)**<br>Dispense 250 µl of **TS** and warm to 37°C and quickly plunge the curved spatula (gutter) of the holding rod into the 37°C **TS** and gently swirl to detach embryo and leave embryo in the **TS** | 1 min | |
| | | **Dilution solution (DS)**<br>Dispense one 50 µl drops of **DS** on a sterile Perti Dish and transfer oocyte to **DS** | 4 mins | **Dilution solution (DS)**<br>Dispense one 50 µl drops of **DS** on a sterile Perti Dish and transfer embryo to **DS** | 4 mins | **Dilution solution (DS)**<br>Dispense one 50 µl drops of DS on a sterile Perti Dish and transfer embryo to **DS** | 4 mins | Warm **TS** at 37°C (incubator without $CO_2$) at least 30 minutes prior to starting warming procedure. |
| | | **Washing Solution (WS)**<br>Dispense two 50 µl drops of **WS** (**$WS_1$** and **$WS_2$**) and transfer oocyte to **$WS_1$** | 4 mins | **Washing Solution (WS)**<br>Dispense two 50 µl drops of **WS** (**$WS_1$** and **$WS_2$**) and transfer embryo to **$WS_1$** | 4 mins | **Washing Solution (WS)**<br>Dispense two 50 µl drops of **WS** (**$WS_1$** and **$WS_2$**) and transfer embryo to **$WS_1$** | 4 mins | Limit exposure to light while moving specimens through solutions. |
| | | And finally transfer oocyte to **$WS_2$** from **$WS_1$** and after 4 mins transfer warmed oocyte to pre-equilibrated culture medium. | 4 mins | And finally transfer embryo to **$WS_2$** from **$WS_1$** and after 4 mins transfer warmed embryo to pre-equilibrated culture medium. | 4 mins | And finally transfer embryo to **$WS_2$** from **$WS_1$** and after 4 mins transfer warmed embryo to pre-equilibrated culture medium. | 4 mins | |

| S.NO. | OEM | WARMING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | TIME | |
| 4. | VITROLIFE | **Warm 1™ Oocyte**<br>Dispense 1 ml of the media into the separate wells of a 5-well plate and quickly remove Rapid-i from the Rapid Straw and plunge the tip and the hole of the Rapid-i into the Warm 1™ Oocyte solution and allow oocytes to fall from device and sink at the bottom. | 1 min | **Warm 2™ Cleave**<br>Dispense 0.5- 1 ml media into the separate wells of a 5-well plate and place the vitrified embryos quickly into the Warm 1™ Cleave and allow the embryos to fall from device and sink at the bottom | 10- 30 sec | **Warm 1™ Blast**<br>Dispense 0.5- 1 ml media into the separate wells of a 5-well plate and place the vitrified blastocyst quickly into the Warm 1™ Blast and allow the embryos to fall from device and sink at the bottom | 2 min | All manipulations of the specimens are carried out at 37°C<br>The recommended volumes should not be changed. Volume changes will affect temperature control in the first warming solution as well as the osmolality, which may result in suboptimal oocyte survival |
| | | **Warm 2™ Oocyte**<br>Dispense 1 ml of the media and transfer oocytes into the Warm 2™ Oocyte and let the oocytes remain in the solution | 3 min | **Warm 2™ Cleave**<br>Dispense 0.5- 1 ml of the media and transfer the embryos into the Warm 2™ Cleave<br>And let the embryos remain in the solution | 1 min | **Warm 2™ Blast**<br>Dispense 0.5- 1 ml of the media and transfer the blastocyst into the Warm 2™ Blast<br>And let the blastocyst remain in the solution | 3 min | |
| | | **Warm 3™ Oocyte**<br>Dispense 1 ml of the media and transfer the oocytes into Warm 3™ Oocyte and let the oocytes remain in the solution | 5 mins | **Warm 3™ Cleave**<br>Dispense 0.5- 1 ml of the media and transfer the embryos into the Warm 3™ Cleave<br>And let the embryos remain in the solution | 2 mins | **Warm 3™ Blast**<br>Dispense 0.5- 1 ml of the media and transfer the blastocyst into the Warm 3™<br>And let the blastocyst remain in the solution | 5- 10 mins | |
| | | **Warm 4™ Oocyte**<br>Dispense 1 ml of the media and transfer the oocytes to Warm 4™ Oocyte and let the oocytes remain in the solution | 5- 10 mins | **Warm 4™ Cleave**<br>Dispense 0.5- 1 ml of the media and transfer the embryos into the Warm 4™ Cleave and let the embryos remain in the solution | 5 mins | | | |

| S.NO. | OEM | WARMING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | TIME | |
| 5. | SAGE (ORIGIO) | Details Not Available | NA | **1.0M Sucrose Warming Solution (1 M WS)**<br>Dispense one 20 µl drop of media on the sterile Petri Dish and place the vitrified embryos quickly into the **1.0 M WS** and allow the embryos to fall from device and float to the top of the drop. | 1 min | **1.0 M Sucrose Warming Solution (1 M WS)**<br>Dispense one 20 µl drop of media on the sterile Petri Dish and place the vitrified blastocysts quickly into the **1.0 M WS** and allow the embryos to fall from device and float to the top of the drop. | 1 min | The warming and dilution procedure is to be performed at 35-37°C. |
| | | | | **0.5 M Sucrose Warming Solution (0.5 M WS)**<br>Dispense two 20 µl of the 0.5 M **WS** (0.5 M **$WS_1$** and **0.5 M $WS_2$**) and transfer embryos from the drop of **1.0 M WS** to the bottom of **0.5 M $WS_1$**. | 2 mins | **0.5 M Sucrose Warming Solution (0.5 M WS)**<br>Dispense two 20 µl of the **0.5 M WS (0.5 M $WS_1$** and **0.5 M $WS_2$**) and transfer blastocysts from the drop of **1.0 M WS** to the bottom of **0.5 M $WS_1$**. | 2 mins | Use a **HEATED** microscope stage for the following procedure. |
| | | | | Then transfer to the bottom of the 0.5 M **$WS_2$** | 2 mins | Then transfer to the bottom of the **0.5 M $WS_2$** | 2 mins | |
| | | | | **MOPS Solution**<br>Dispense three 20 µl drops of MOPS Solution (**$MS_1$**, **$MS_2$** and **$MS_3$**). Transfer the embryos to the bottom of the $MS_1$ | 3 mins | **MOPS Solution**<br>Dispense three 20 µl drops of MOPS Solution (**$MS_1$**, **$MS_2$** and **$MS_3$**). Transfer the embryos to the bottom of the **$MS_1$** | 3 mins | Minimize exposure of specimens to light during incubation in warming solutions.. |
| | | | | Then transfer the embryos to the top of the **$MS_2$** | 3 mins | Then transfer the embryos to the top of the **$MS_2$** | 3 mins | Bring the solutions to 35- 37°C before use. |
| | | | | Now, transfer the embryos to the top of the **$MS_3$** | 3 mins | Now, transfer the embryos to the top of the **$MS_3$** | 3 mins | |
| | | | | After 3 mins finally transfer embryos to the pre-equilibrated culture medium. | | After 3 mins finally transfer embryos to the pre-equilibrated culture medium. | | |

| S.NO. | OEM | WARMING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | TIME | |
| **6.** | **GLOBAL Blast** | **Details Not Available** | **NA** | **Details Not Available** | **NA** | **Warm 1 Solution (WS 1)**<br>Dispense two 20 µl drops of **WS 1** (**WS 1a** and **WS 1b**) and place the vitrified embryos quickly into WS 1b and draw 1 µl solution from **WS 1a** and dispense it adjacent to **WS 1b** and merge the drops with gradual mixing | 1 mins | Whole procedure to be performed at 20-27°C |
| | | | | | | Transfer blastocyst from WS1b to the bottom of WS1a and allow it to float to the top of the drop | 1 mins | |
| | | | | | | **Warm 2 Solution (WS 2)**<br>Dispense two 20 µl drops of **WS 2** (**WS 2a** and **WS 2b**) and transfer blastocyst from WS1a to the bottom of the **WS 2a** and hold. | 2 mins | Minimize exposure of specimen to light during incubation in the warming solutions |
| | | | | | | Transfer blastocyst from WS2a to the bottom of WS2b and hold. | 2 mins | Warm all solutions (Warm 1, Warm 2 and Warm 3) to 20-27°C before use. |
| | | | | | | **Warm 3 Solution (WS 3)**<br>Dispense three 20 µl drops of **WS 3** (**WS 3a**, **WS 3b** and **WS 3c**) and transfer blastocyst from **WS 2b** to the bottom of **WS 3a** and hold. | 3 mins | |
| | | | | | | Then, transfer the blastocyst from WS 3a to the top of WS 3b and hold | 3 mins | Make sure the contents of each vial are well mixed by gentle inversion several times before use. |
| | | | | | | Next, transfer the blastocyst from **WS 3b** to the top of the **WS 3c** and hold. | 3 mins | |
| | | | | | | Finally, transfer the blastocyst to an Embryo GPS[R] dish of pre-equilibrated culture medium. | | |

| S.NO. | OEM | WARMING PROTOCOL | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|
| | | OOCYTE | TIME | EMBRYO | TIME | BLASTOCYST | TIME | |
| 7. | COOK Blast | Details Not Available | NA | Details Not Available | NA | **Blastocyst Warming Solution 1**<br>Prepare the warming solution in a 4- well plate by adding 800 µl of Blastocyst Warming Solution 1 into the well 1 and 2 and immediately extract the blastocysts from the vitrification device and place them into the well 1 and stir IMMEDIATELY until the bead dissolves. | | Aseptic technique should be used. |
| | | | | | | Transfer the blastocysts to the well 2. | 5 mins | Equilibrate the three Warming Solutions to 37˚C before use. |
| | | | | | | **Blastocyst Warming Solution 2**<br>Dispense 800 µl of Blastocyst Warming Solution 2 into well 3 and move the blastocysts into well 3 and wash. | 5 mins | Prepare a suitable volume of Blastocyst Medium in an incubator with 6 % $CO_2$ environment at 37˚C prior to use, for the culture of the blastocysts post warming. |
| | | | | | | **Blastocyst Warming solution 3**<br>Dispense 800 µl of Blastocyst Warming Solution 3 into well 4 and move the blastocysts into well 4 and wash. | 5 mins | |
| | | | | | | Finally, place the blastocysts in the dish containing equilibrated Blastocyst Medium for hatching. | | |
| 8. | ORIGIO Medicult | As Discussed In Part 3 | | As Discussed In Part 3 | | As Discussed In Part 3 | | |

# Notes

# Part-3

# VITRIFICATION - WARMING PROTOCOLS

## Vitrification protocol followed in our centre

Vitrification is an exciting new technique which is used to cryopreserve (freeze) supernumerary oocytes and embryos. Using ultra rapid freezing techniques, it is now possible to efficiently store spare oocytes and embryos, so they can be used later on to achieve a pregnancy after warming. Survival rates after vitrification are better than they were with conventional slow freezing.

**Principle** The word "vitrification" comes from Latin term of glass, **vitrum**. It is the process of freezing so rapidly that the water molecules do not have time to form ice crystals, and instead instantaneously solidify into a glass-like structure.

Vitrification uses an extremely quick freezing rate (approximately 15,000°C/min) for near instantaneous freezing. Furthermore, vitrification suspends cryopreserved samples in a crystalline lattice structure that does not have ice crystal formation as a side effect.

### Materials Required

**Media:** Medicult Vitrification Media

**Cryo-devices used:** McGill Cryoleaf™ and VitriFit™

The vitrification kit is a DMSO (Dimethyl Sulphoxide) free, instead containing ethylene glycol and propanediol along with Human Albumin Solution (HAS) and Gentamicin Sulphate (10 μg/ml).

### The kit has 2 vials

**Vial 1:** Equilibration Solution (2 x 1 ml)
**Vial 2:** Vitrification Solution (2 x 1 ml)

### Disposables

1. Sterile Tissue Culture Dishes (60 x 15 mm)
2. Transfer Pipettes (170 μl)
3. Tweezers
4. 1.8 ml cryovial base
5. Scissors or scalpel
6. Timer or stopwatch
7. Liquid Nitrogen reservoir (Styrofoam container with lid)
8. Liquid Nitrogen (sufficient volume to cover the plugged in 1.8 ml cryovial base)

## Vitrification Protocol (Using Origio Medicult Media and McGill Cryoleaf as carrier device)

1. Prepare a 60 x 15 mm tissue culture dish and label it with Equilibration Solution (ES) and Vitrification Solution (VS). ***(Fig. 1)***
2. Mix the contents of the Equilibration Solution and Vitrification Solution vials by a few gentle inversions. Dispense 100 µl droplets of each solution in a tissue culture dish and allow them to rest for 30 minutes at room temperature for equilibration of media.***(Fig. 2)***
3. Prepare a reservoir with enough liquid nitrogen to allow complete submersion of 1.8 ml cryovial base on the cryocane. Label the cryocane with specific patient identification number and attach 1.8 ml cryovial base to the bottom of it and submerge in the liquid nitrogen.***(Fig. 3)***
4. Prepare the number of cryo-devices according to the specimens to be vitrified and label them with the Patient ID and place near the microscope. ***(Fig. 4)***
5. Select embryos to be vitrified from the culture dish and using a suitable pipette, transfer 2-3 embryos into the Equilibration Solution and start the timer for 5 minutes just after the transfer. ***(Fig. 5)***
6. With 30 seconds remaining on the timer transfer the embryos in minimum volume into the Vitrification Solution and start the timer for 1 minute. ***(Fig. 6)***
7. Meanwhile, plunge the outer cover of the McGill Cryoleaf™ into the $LN_2$ reservoir and let the air come out.
8. With 30 seconds remaining, quickly load the vitrified embryos in minimum volume (<1 µl) onto the McGill Cryoleaf™ using a suitable pipette. Make sure to remove excess medium carefully and quickly using the pipette.***(Fig. 7)***
9. Quickly immerse the McGill Cryoleaf™ with the embryos directly into the liquid nitrogen. ***(Fig. 8)***
10. Carefully slide the protective sleeve (green) over the tip with the embryos and lock it into the place by turning. Take care that the McGill Cryoleaf™ remains immersed in $LN_2$ at all times.
11. Insert the McGill Cryoleaf™ into the outer cover and press tightly. Take care that the McGill Cryoleaf™ remains immersed in $LN_2$ at all times. ***(Fig. 9)***
12. Repeat the steps for all set of embryos.
13. Transfer to the storage tank while keeping the McGill Cryoleaf™ immersed in $LN_2$. ***(Fig. 10)***
14. Keep records in a record book mentioning date of vitrification, cryocane identification number, medium used, cell-stage of the embryos and the number of embryos vitrified in a cryo-device.

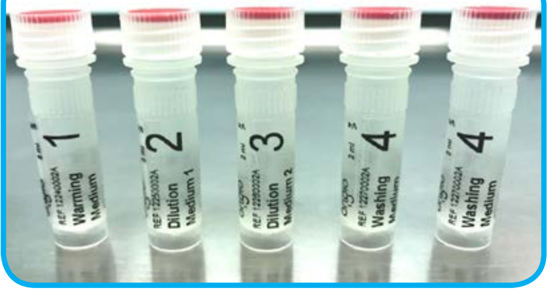
## Warming of Vitrified Embryos

**Media used:** Medicult Warming Media

The warming kit contains decreasing concentration of sucrose along with Human Albumin Solution (HAS) and Gentamicin Sulfate (10 µg/ml).

## The kit has 4 vials

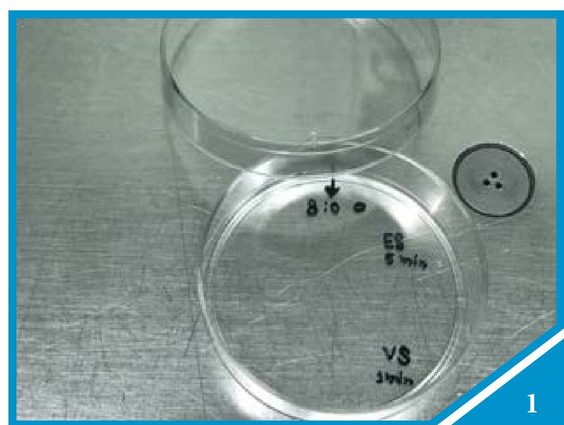
**Vial 1:** Warming Solution (1 x 2ml)
**Vial 2:** Dilution Solution 1 (1 x 2 ml)
**Vial 3:** Dilution Solution 2 (1 x 2 ml)
**Vial 4:** Washing Solution (2 x 2 ml)
**Cryo-device used:** VitriFit™ & McGill Cryoleaf™




## Warming Protocol

1. Prepare a 60 x 15 mm tissue culture dish label it with Dilution Solution 1, Dilution Solution 2 and Washing Solution and 35 x 10 mm tissue culture dish for Warming Solution. ***(Fig. 11)***
2. Mix the content of the individual vials by a few gentle inversions prior to use and dispense 100 µl droplets each of Dilution Solution 1, Dilution Solution 2 and Washing Solution and allow them to rest for 30 minutes to room temperature for equilibration. Meanwhile, 300 µl of warming solution should be warmed to 37°C at least 1 hour prior to commencing warming procedure ***(Fig. 12).*** However, the expertise involved in using smaller volumes comes with vast experience, and beginners are requested to stick to the use of larger volumes as per manufacturer's guidelines.
3. Meanwhile, prepare a reservoir with enough liquid nitrogen to allow complete submersion of **1.8 ml cryovial base** on a cryocane and collect the cryocane and **1.8 ml cryovial base** containing the cryo-device with the vitrified embryos from the storage tank and quickly transfer them to the liquid nitrogen reservoir. Make sure the cryo-device is submerged under liquid nitrogen ($LN_2$). ***(Fig. 13)***
4. Using forceps twist and pull the VitriFit™ handle and cap apart. Take care all parts of the VitriFit™ except the handle remain immersed in liquid nitrogen at all times. ***(Fig. 14)***
5. Quickly, within few seconds take the VitriFit™ out of the $LN_2$ and quickly plunge the VitriFit™ tip with vitrified embryos facing up into Warming Solution and start the timer for 1 minute. ***(Fig. 15)***
6. With 30 seconds remaining, using a suitable pipette and minimum volume, get ready to transfer the embryos into the Dilution Solution 1 at room temperature and start the timer for 3 minutes. ***(Fig. 16)***
7. Next, with 30 seconds remaining and minimum volume, get ready to transfer the embryos into the Dilution Solution 2 and again start the timer for 3 minutes. ***(Fig. 17)***
8. Next, in minimum volume transfer the embryos into the Washing Solution for 3 minutes. ***(Fig. 18)***
9. Repeat the washing step by transferring the embryos to another Petri dish with washing solution.
10. Finally, transfer the embryos into the preferred equilibrated culture medium and allow them to rest in the incubator for minimum two hours before visual inspection.
11. Repeat the steps for all set of embryos.

## Vitrification Protocol




1

### FIGURE - 1

Figure shows a 60 x 15 mm Tissue Culture Dish made up of Polystyrene (REF: 353002), manufactured by BD Falcon, Corning, USA is appropriately labeled with following details:

**Time** at which we start preparing the plate

Equilibration Solution **(ES)**

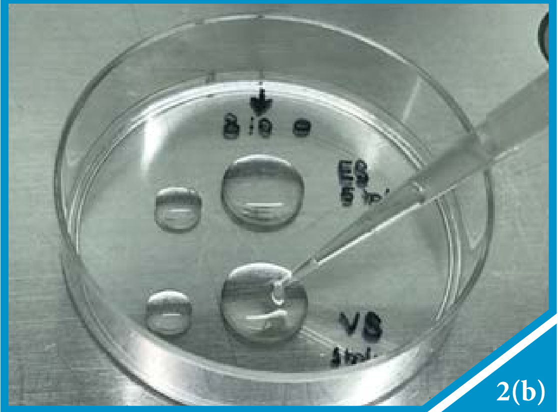
Vitrification Solution **(VS)**

2(a)

### FIGURE - 2 (a)

A variable pipette in a **volume range of 10 µl to 100 µl** is set at 100 µl.

It is used to dispense media in desired volume.

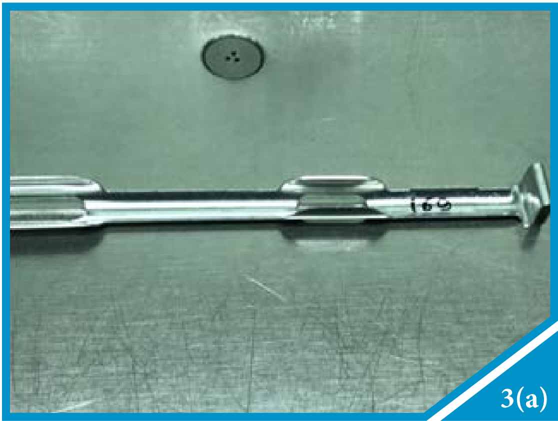



2(b)

### FIGURE - 2 (b)

Dispense **100 µl droplets** each of Equilibration Solution (ES) and Vitrification Solution (VS) separately onto the labeled tissue culture dish and two **small droplets for the washing of** the embryos.

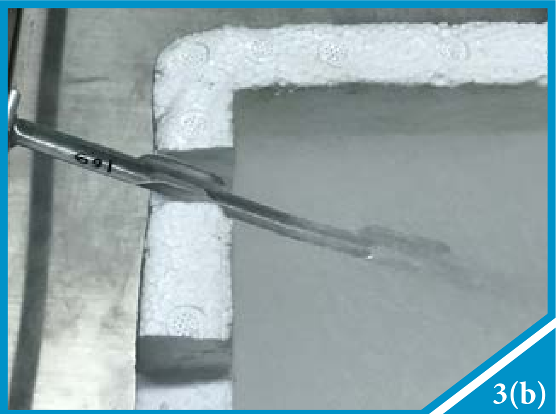
Allow the media to **rest for 30 minutes at room temperature for equilibration.**



3(a)

### FIGURE - 3 (a)

Label the cryocane **with specific Patient identification number** and attach a **1.8 ml cryovial base** to the bottom of it for the safe keeping of the cryo-devices in liquid nitrogen storage tanks.


3(b)

## FIGURE - 3 (b)

Prepare a reservoir with enough liquid nitrogen in a **Styrofoam container with certain incurvates** so that it provides more depth to the cryocane and the attached **1.8 ml cryovial base** in liquid nitrogen.

It should be noted that the Patient identification number should be visible in liquid nitrogen.

4

## FIGURE - 4

Prepare the Cryo-devices, as we have prepared one McGill Cryoleaf™ and one VitriFit™ for the same patient and label its **handle and cap with Patient ID.**

Number of Cryo-devices is prepared according to the embryos to be vitrified.

5(a)

## FIGURE - 5 (a)

Select embryos to be vitrified from the culture dish using a stripper with suitable microtips (usually 170 µm) and **transfer 2-3 embryos into the Equilibration Solution (ES).**

The embryos **initially shrink before re-expanding** to their original size.

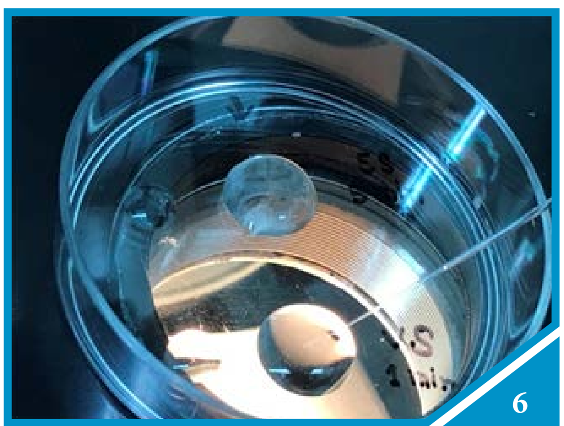
Equilibration is completed once the embryos have re-expanded

5(b)

## FIGURE - 5 (b)

The timer is set on 5 minutes for the Equilibration step.

Just after the transfer of the embryos from culture dish into the Equilibration Solution, start the timer and allow the embryos to re-expand to their original size.




## FIGURE - 6

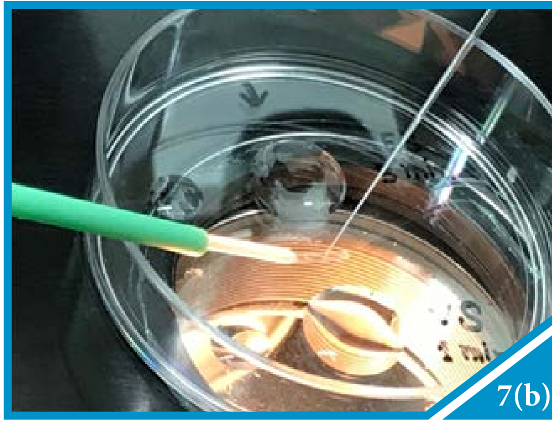
After equilibration, transfer the embryos from Equilibration Solution (ES) **in minimum volume into the Vitrification Solution (VS).**

The **embryos shrink again** in Vitrification Solution.

The time from the transfer of the embryos into the Vitrification solution until vitrified must not **exceed 1 minute**

## FIGURE - 7 (a)

Figure shows a stripper handle from COOK Medical which is set at **3 µl volume** of media so that embryos are vitrified in minimum volume of the media.

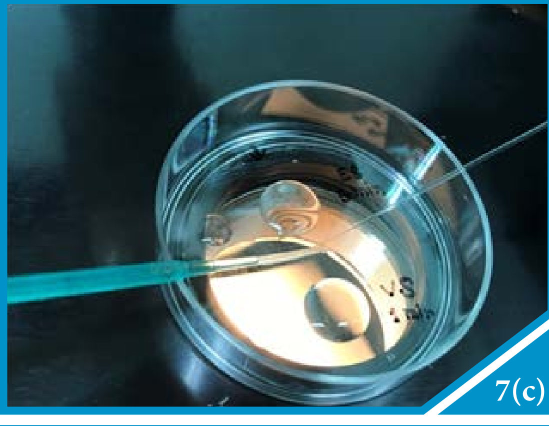



## FIGURE - 7 (b)

With 30 seconds remaining into the Vitrification Solution, quickly **load 2-3 vitrified embryos** in minimum volume (<1 µl) unto the McGill Cryoleaf™ using a 170 µm stripper.

Make sure to remove excess medium carefully and quickly using the stripper and **quickly immerse the McGill Cryoleaf™ into the liquid nitrogen**

McGill Cryoleaf™ should stay dry during the process.




## FIGURE - 7 (c)

Quickly **load the remaining 2-3 vitrified embryos** in minimum volume (<1 µl) into the **concave** loading surface of VitriFit™.

Use **black mark at the tip of loading surface as reference for loading.**

Make sure to remove excess medium carefully and quickly using the stripper and immediately insert the VitriFit™ into liquid nitrogen.

8(a)

## FIGURE - 8 (a)

Plunge the outer cover of McGill Cryoleaf™ into the liquid nitrogen and let the air come out.

Carefully **slide the protective sleeve (green) over the embryo loading edge** with vitrified embryos by turning and **lock it** in place.

Take care that the McGill Cryoleaf™ remains immersed in liquid nitrogen at all times.

8(b)

## FIGURE - 8 (b)

Plunge the cap of the VitriFit™ into the liquid nitrogen and allow the air to come out.

Take care that the VitriFit™ remains immersed in liquid nitrogen at all times.

9(a)

## FIGURE - 9 (a)

Insert the McGill Cryoleaf™ into its outer cover and press tightly to ensure locking.

Patient ID should be matched for both the handle and the outer cover to avoid chances of cross contamination.

Part of McGill Cryoleaf™ till the red marking should remain immersed in liquid nitrogen.

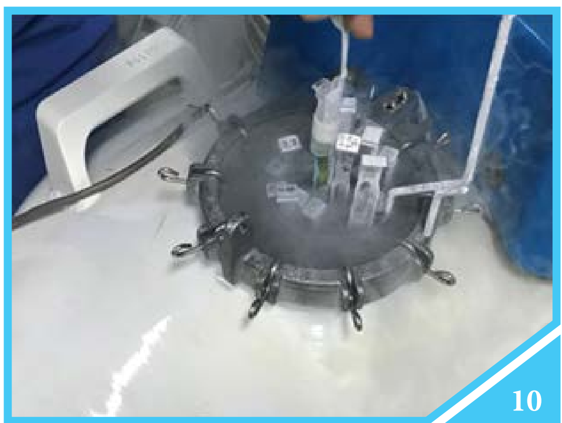
9(b)

## FIGURE - 9 (b)

Insert the VitiFit™ into its cap and press tightly to ensure locking.

Patient ID should be matched for both body and the cap to avoid chances of cross contamination.

Take care that VitriFit™ remains immersed in liquid nitrogen.



10

## FIGURE - 10

Fit the Cryo-devices into the 1.8 ml cryovial base on a labeled aluminium cryocane.

Next, quickly transfer the cryocane to the storage tank.

Make sure that the vitrified embryos are submerged under liquid nitrogen at all times.

## Warming Protocol

11(a)

## FIGURE - 11 (a)

A variable pipette in a **volume range of 100 µl to 1000 µl** is used to dispense Warming Media in desired volume.

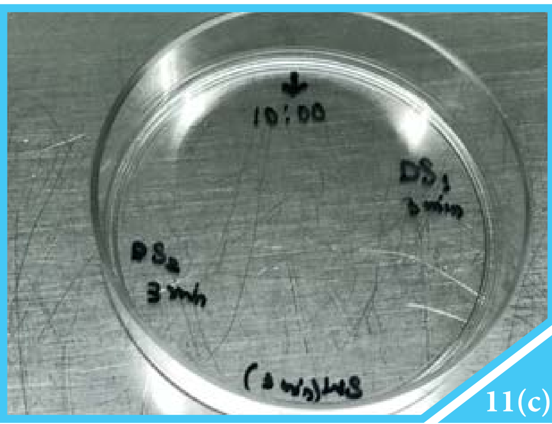
11(b)

## FIGURE - 11 (b)

Figure shows a 35 x 10 mm Tissue Culture Dish made up of Polystyrene (REF: 353001), manufactured by BD Falcon, Corning, USA is appropriately labeled with following details:

**Time** at which we start preparing the plate

Warming Media **(WM)**

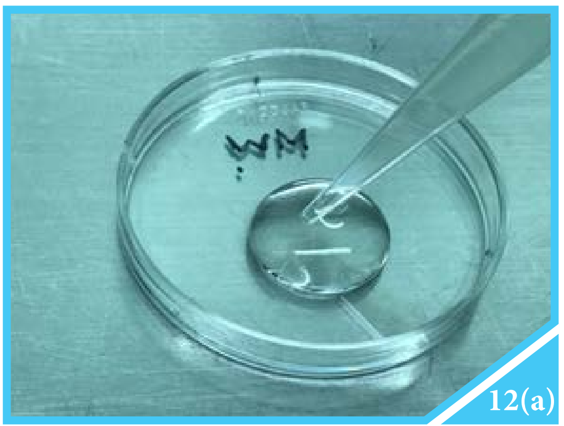



11(c)

## FIGURE - 11 (c)

Figure shows a 60 x 15 mm Tissue Culture Dish made up of Polystyrene (REF: 353002), manufactured by BD Falcon, Corning, USA is appropriately labeled with following details:

**Time** at which we start preparing the plate
Dilution Solution1 **(DS 1)**
Dilution Solution 2 **(DS 2)**
Washing Solution **(WS)**



12(a)

## FIGURE - 12 (a)

Dispense **300 µl droplet of Warming Media** onto the same labeled tissue culture dish

This should have been warmed to 37ºC for at least 1 hour prior to starting warming.

12(b)

## FIGURE - 12 (b)

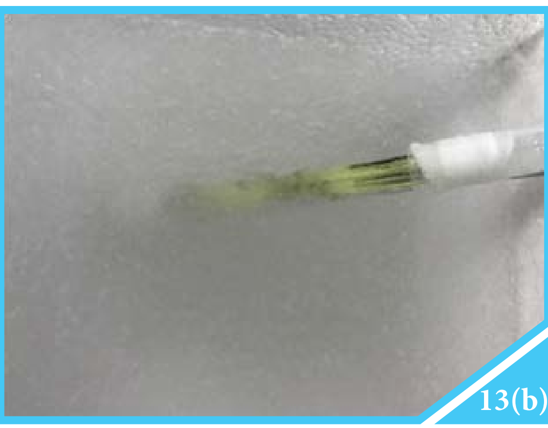
Dispense **100 µl droplets each** of Dilution Solution 1 **(DS 1)**, Dilution Solution 2 **(DS 2)** and Washing Solution **(WS)** separately onto the same labeled tissue culture dish and two small droplets for the washing of the embryos.

Allow the media to **rest for 30 minutes at room temperature** for equilibration.

13(a)

## FIGURE - 13 (a)

Prepare a reservoir with enough liquid nitrogen in a Styrofoam container with incurvates on one side to allow complete submersion of the aluminium cryocane with plugged in 1.8 ml cryovial base.


13(b)

## FIGURE - 13 (b)

Collect the cryocane and the cryo-device with the vitrified embryos from the storage tank and quickly transfer them to the liquid nitrogen reservoir.

Make sure the cryo-device is submerged under liquid nitrogen.

## FIGURE - 14

Using forceps **twist and pull the VitriFit™ handle and cap apart** while keeping the tip region fully immersed in liquid nitrogen.

## FIGURE - 15

Quickly, within 1 second, **plunge** the VitriFit™ tip with the vitrified embryos facing up **into the Warming Media** and under microscopic observations, gently move the VitriFit™ until the embryos are released from the tip.

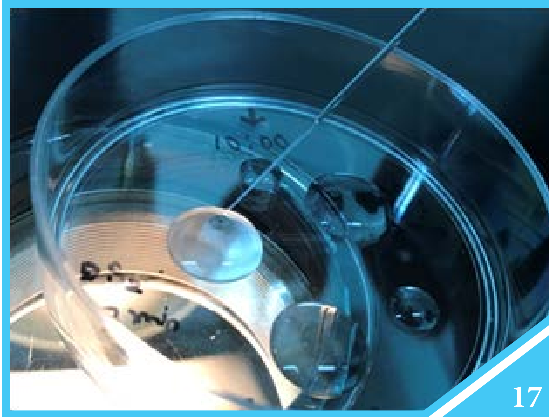
Leave the embryos for **maximum of 60 seconds (at this point, embryos are still shrunken)**

## FIGURE - 16

Using **170 µm** stripper tightly mounted unto the handle and minimum volume of Warming media, **transfer the embryos into the Dilution solution** 1 at room temperature.

Leave the embryos into the Dilution Solution 1 for **3 minutes (at this point, embryos will start to re-expand).**

Before transferring the embryos into the Dilution Solution 2, wash the embryos in small droplet of Dilution Solution 1



## FIGURE - 17

In minimum volume of Dilution Solution 1, **transfer the embryos into the Dilution Solution 2.**

Leave the embryos into the Dilution Solution 2 for **3 minutes (at this point, embryos will continue the re-expansion).**

Before transferring the embryos into the Washing Solution, wash the embryos in small droplet of Dilution Solution 2.

18

## FIGURE - 18

Finally, in minimum volume of Dilution Solution 2, **transfer the embryos into the Washing Solution** and leave embryos into it **for 3 minutes.**

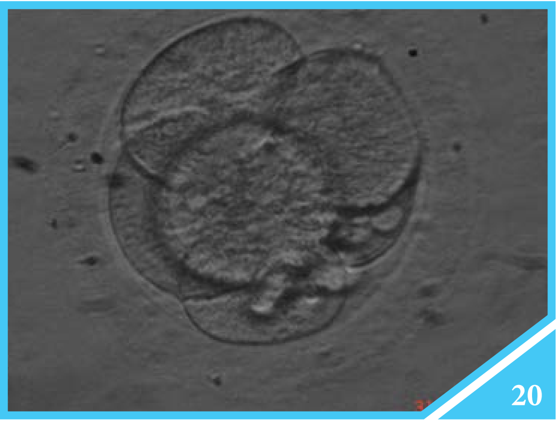
Embryos at this stage **are fully re-expanded** and transfer them into the preferred pre-equilibrated culture medium and allow them to rest in incubator for minimum of two hours before visual inspection.

19

## FIGURE - 19

The working magnification of the stereozoom microscope is set at 0.8 to 1.2 X (X 10x for eyepiece) as embryo manipulation and loading is easiest at low magnifications. However, the magnification may be increased to assess for collapse and re-expansion as necessary

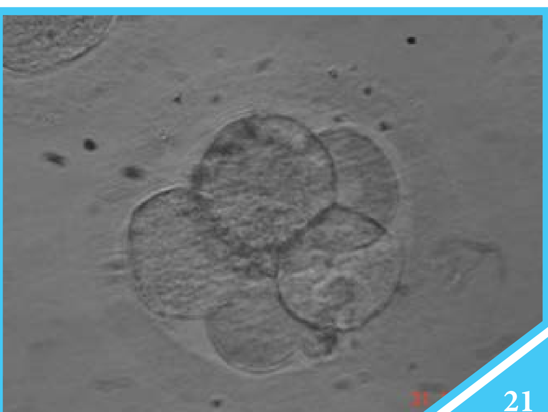
## The effect of Vtrification and Warming on Embryos: Under the Microscope



20

## FIGURE - 20

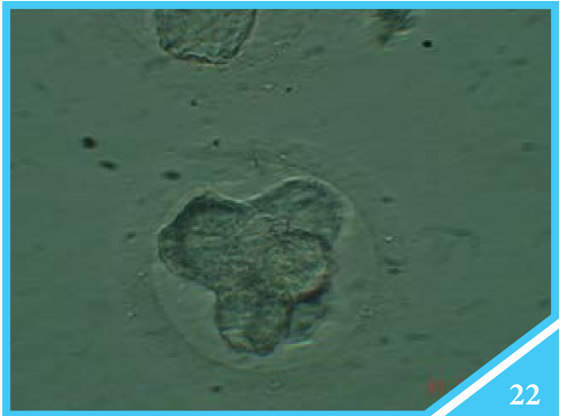
Grade 1, 6-cell embryo with minimal fragments.



21

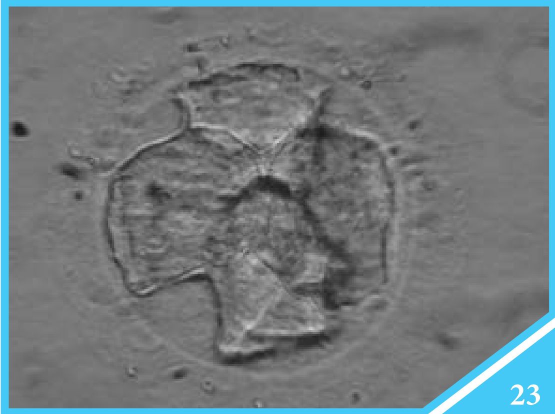
## FIGURE - 21

A 6 cell, grade 1, embryo in Equilibration Solution. Contact period of the embryos with media is 5 minutes.


22

## FIGURE - 22

A 6-cell grade I embryo now in Equilibration Solution at the end of 2 minutes. We can appreciate sudden contraction of the blastomeres. Perivitelline space is contracted. Zona pellucida looks regular. Embryos are very fragile at this stage and require gentle handling. Use appropriate size pipettes to rinse the embryos.


23

## FIGURE - 23

A 6-cell, embryo in ES after 1 minute of contact. Embryos gradually expand as equilibration occurs over period of 5 minutes.

24

## FIGURE - 24

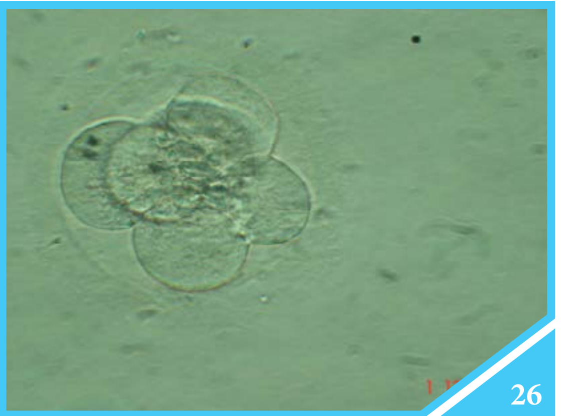
Quickly load 2-3 vitrified embryos in minimum volume (<1 µl) into the **concave** loading surface of VitriFit™. Use black mark at the tip of loading surface as reference for loading.Make sure to remove excess medium carefully and quickly using the stripper and immediately immerse the VitriFit™ into liquid nitrogen

# Embryo Warming

25

## FIGURE - 25

Quickly, within 1 second, plunge the VitriFit™ tip with the vitrified embryos facing up into the Warming Media and under microscopic observations, gently move the VitriFit™ until the embryos are released from the tip. Leave the embryos for maximum of 60 seconds (at this point, embryos are still shrunken).



26

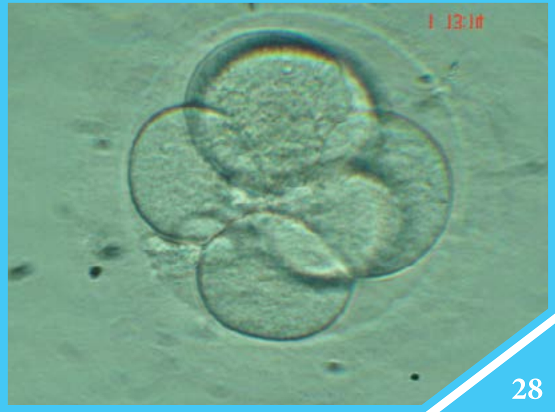
## FIGURE - 26

Transfer the embryos into DM1 and let the embryos remain in this solution for 3 minutes. Embryos are still shrunken and difficult to visualize and they move towards the periphery of the media well. These are very fragile and should be handled very gently with appropriate sized pipette. We should work at low light and less magnification. This makes embryo aspiration and handling easier. Embryos are still dehydrated and difficult to visualize.

27

## FIGURE - 27

A six - cell embryo in wash solution. Embryo is recovering its original shape. Gradually the blastomeres become becoming more translucent and clear. The blastomeres are regular and compact. Zona pellucida is healthy.

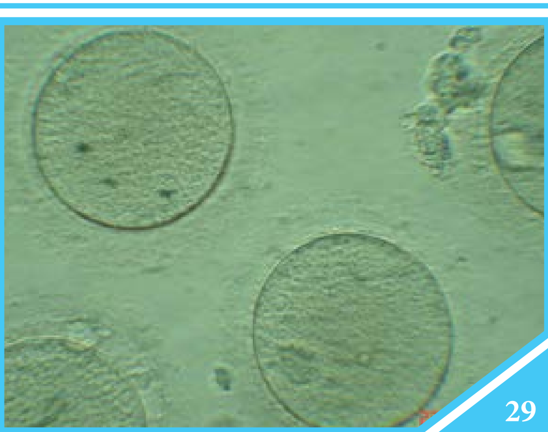


28

## FIGURE - 28

A six cell, grade 1, fully recovered embryo after culture in cleavage stage Media. Blastomeres are regular and normal shape. Zona pellucida is intact and regular.

# Oocyte Vitrification



29

## FIGURE - 29

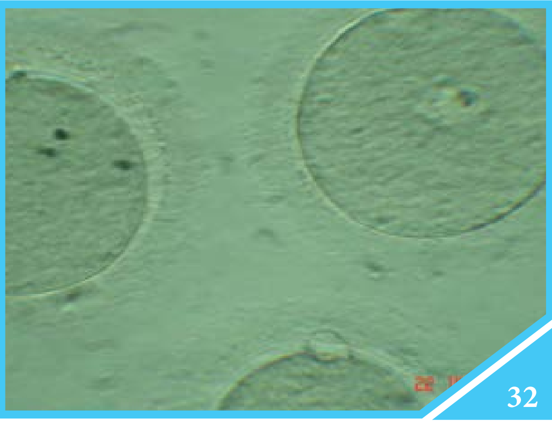
Oocytes denuded using hyaluronic acid. Any commercial brand may be used in concentration of 80 IU/ml.

## FIGURE - 30

Oocyte in equilibration media. We can visualize shrinking and irregularity of oolemma occurring immediately after contact of oocyte with the media .Infolding of the oolemma is seen.Intact polar body is seen at 11’Oclock position.

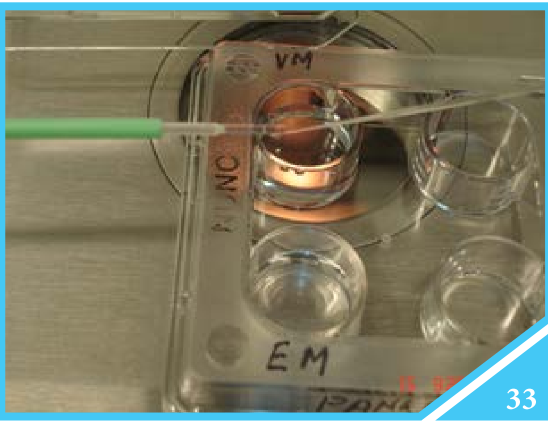
## FIGURE - 31

After 3 minutes of contact period of ES and the oocyte , we can see the oolemma returning to the original state.The ooplasm looks healthy though some amount of crenations along the edges are still seen.Polar body is at 12’Oclock position and is non fragmented.




## FIGURE - 32

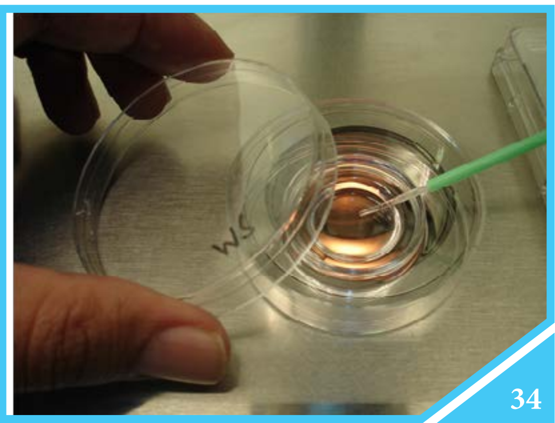
Fully expanded oocytes that have returned to the original state after a contact period of 4 minutes in ES.




## FIGURE - 33

Oocytes are now shifted to the vitrification media. Spray minimal volume of VS on oocytes in ES. These are now aspirated in minimal volume of ES and transferred to well 2 containing VS. Oocytes are to be handled very gently in Vitrification media. From transfer in of oocytes to the VS and to the loading on the cryoleaf oocytes should not spend more than 60 seconds in VS.

## Oocyte Warming

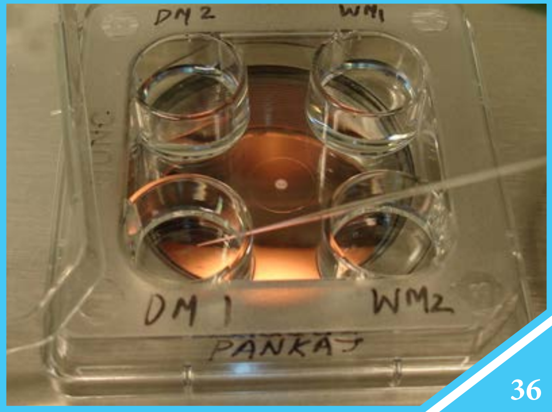



### FIGURE - 34

Outer sheath of the cryoleaf is removed under liquid nitrogen. Green sleave is unlocked and the tip of cryoleaf containing the oocytes is dipped in the 400 microlitre warming medium in a single well dish. Alternatively, 100 microlitre droplet could be made and warming carried out.The vitrification media are not recommended to be covered with oil as it will create contamination and coat the oocytes leading to delayed osmotic changes.

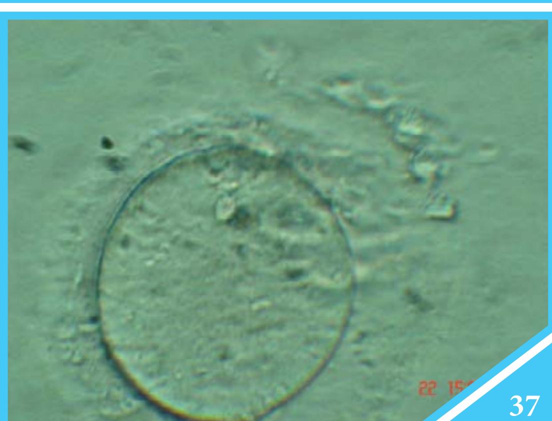
### FIGURE - 35

Oocyte as seen in the warming media. Maximum contact period of the oocyte with warming media permitted is 1 min. Ooplasm here is contracted and oolemma is wrinkled. Wide perivitelline space is observed and zona pellucida is intact.



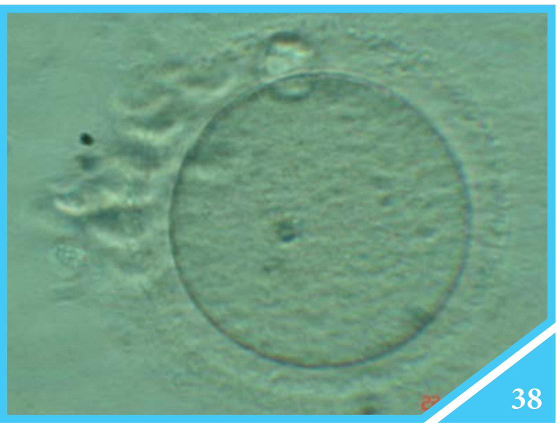

### FIGURE - 36

Oocytes being transferred from diluent media 1 and 2 to washing media 1and 2 as shown in the figure. Oocytes are kept in each media for 3 min each and then are finally transferred to the IVF media.




### FIGURE - 37

Oocytes gradually recover in the diluting media 1. Maximum contact period permitted here is 3 minutes. Oocyte now is gradually expanding, looks normal and oolemma appears to be healthy. Polar body is seen at the 1 O'clock position.



### FIGURE - 38

Oocyte is further recovering in the washing media 1. Perivitelline space is decreasing and oolema is expanding circumferentially. Zona pellucida is healthy and polar body is seen at 11 O'clock position.

## Notes

# Part-4

## TABLE- 15: OEM & Vendor Related Contact Information

| S.NO. | ORIGINAL EQUIPMENT MANUFACTURER (OEM) | INDIA DISTRIBUTOR | DEVICE BRAND | PRICE PER DEVICE | CONTACT PERSON | PHONE | EMAIL |
|---|---|---|---|---|---|---|---|
| 1. | **ORIGIO** a/s Knardrupvej2 2760 Malov Denmark | Origio India Pvt.Ltd. | **Mc Gill Cryoleaf™** (REF: 40770000A) | Not disclosed | Mr. Piyush Jain | +91 7042396972 | pjain@origio.com |
| 2. | **ORIGIO** a/s Knardrupvej 2 2760 Malov Denmark | Origio India Pvt. Ltd. | **VitriFit™** (REF: 42802001A) | Not disclosed | Mr. Piyush Jain | +91 7042396972 | pjain@origio.com |
| 3. | **Vitrolife** Sweden AB Gustaf Werners gata 2 SE-421 32 Vasta Frolunda Sweden | Vision Diagnostics Pvt. Ltd. | **Rapid-i™ Kit** (REF: 94419) | INR 1100 | Mr. Punit Khatnani | +91 9910188771 | punit@vision-groups.com |
| 4. | **KITAZATO** BioPharma Co., Ltd 278-7 Mtonima, Fuji city, Shizuoka JAPAN | SAR Health Line Pvt.Ltd | **Cryotop®** (REF: 81113) | INR 1900 | Mr. Atul Walia | +91 9958029696 | info@sarhealth.com |
| 5. | **CryoBio System** Groupe I.M.V Technologies France | CryoBio System India | **HSV Kit** (REF: 025463) | INR 850 | Mr. Jitender Kumar | +91 9650602424 | jitender@cryobiosystemindia.com |
| 6. | **Biotech Inc.** 11539 Parf Woods Cir. Suite 202 Alpharentta, GA, USA | SAR Health Line Pvt.Ltd. | **Cryolock™** (REF: CL-R-CT) | INR 1248 | Mr. Atul Walia | +91 9958029696 | info@sarhealth.com |
| 7. | **Cryotech Japan** Reprolife, 2-5-3 9F, Shinjuku, Shinjuku-ku, Tokyo, 160-0022 | Cryo Tech India Mumbai | **Cryotech™** (REF: TEC: 010R, 010W, 010B, 010B, 010Y) | INR 1,680 | Dr. Chetan Sowani<br>Mrs. Goral Gandhi | +91 9819855095<br>+91 9821618106 | info@cryotechindia.com |

## TABLE- 16: OEM & Vendor Related Contact Information For Media

| S.NO. | ORIGINAL EQUIPMENT MANUFACTURER (OEM) | INDIA DISTRIBUTOR | PACK-BRAND | PRICE PER PACK | CONTACT PERSON | PHONE | EMAIL |
|---|---|---|---|---|---|---|---|
| 1. | **ORIGIO** a/s Knardrupvej2 2760 Malov Denmark | Origio India Pvt. Ltd. | **Origio Medicult** | Not disclosed | Mr. Piyush Jain | +91 7042396972 | pjain@origio.com |
| 2. | **Vitrolife Sweden** AB Gustaf Werners gata 2 SE-421 32 Vasta Frolunda Sweden | Vision Diagnostics Pvt. Ltd. | **Vitri Omni** | INR 18000/- | Mr. Punit Khatnani | +91 9910188771 | punit@vi-sion-groups.com |
| 3. | **KITAZATO** Bio-Pharma Co., Ltd 278-7 Mtonima, Fuji city, Shizuoka JAPAN | SAR Health Line Pvt.Ltd | **Kitazato** | Vitrification: INR 9500/- (plus tax)<br><br>Warming: INR 8500/- (plus tax) | Mr. Atul Walia | +91 9958029696 | info@sarhealth.com |
| 4. | **CryoBio System** Groupe I.M.V Tech-nologies France | CryoBio System India | **Irwine** | INR 16,000/- (plus tax) | Mr. Jitender Kumar | +91 9650602424 | jitender@cryobio-systemindia.com |
| 5. | **Cryotech Japan, Reprolife, 2-5-3 9F Shinjuku, Shin-juku-ku, Tokyo, 160-0022** | **Cryotech India** (Indo Nippon Medical Trading Co.) | **Cryotech India** Mumbai | Vitrification: INR 13,440/- (plus tax)<br><br>Warming: INR 6,720/- (plus tax) | Dr. Chetan Sowani<br><br>Mrs. Goral Gandhi | +91 9819855905<br><br>+91 9821618106 | info@cryotechindia.com |
| 6. | **Life Global**, LLC 393, Soundview Road, Guilford, CT, 06437, USA | Shivani Scientific India Pvt. Ltd. | **Global Blastocyst** | INR 32,000/- | Mr. Amol Sharan | +91 9990199933 | amols@shivanisci-entific.com,<br><br>amolsharan@gmail.com |
| 7. | **Cook Medical Inc.** P.O Box 4195, Bloomington, In 474024195, USA | Intermedics | **Sydney IVF Blastocyst vitrifica-tion** | INR 19,850/- | Mr. Gopal | +91 9212798185 | projects@intermed-ics.in |

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# ORIGIO Vitrification

## A Full Range of Cool Products

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## Our Range of Media Products

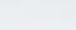
### DMSO - NEW

**SAGE Vitrification Kit (ART-8026*)**

- 2x 2ml Equilibration Solution
- 2x 2ml Vitrification Solution

**SAGE Warming Kit (ART-8031*)**

- 2x 2ml 1M Sucrose WS
- 2x 2ml 0.5M Sucrose WS
- 2x 2ml MOPS Solution



*CE pending: available from first half 2017 in registered countries.

### DMSO FREE

**Medicult Vitrification Cooling (1228)**

- 2x 1ml Equilibration Medium
- 2x 1ml Vitrification Medium

**Medicult Vitrification Warming (1229)**

- 1x 2ml Warming Medium (1M Sucrose)
- 1x 2ml Dilution 1 Medium (0.5M Sucrose)
- 1x 2ml Dilution 2 Medium (0.25M Sucrose)
- 2x 2ml Washing Medium

Until registered, we recommend ART-8025 and ART-8030.

### Open System Vitrification Carrier

New VitriFit (pack of 20)

Clear (4278) • Green (4280) • Blue (4279) • Yellow (4281)

McGill Cryoleaf (Pack of 14)

**Documented Efficiency**

SAGE
- 94% oocyte survival (Selman et al., 2010)
- 97% embryo survival (Selman et al., 2009)

origio
- 92% oocyte survival (Cao et al., 2009)
- 94% embryo survival (Bern et al., 2011)

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