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# Quality Control Factors Influencing the Successful and Reliable Implementation of Oocyte and Embryo Vitrification

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Mitchel C. Schiewe

Additional information is available at the end of the chapter

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## Abstract

Clinical vitrification evolved slowly, with interests and acceptance being commercially driven by the development of unique devices, safer solutions, and the misconception that ultra-rapid cooling in an “open” system was a necessity to optimizing vitrification success. Furthermore, the dogma surrounding the importance of cooling rates has led to unsafe practices subject to excessive technical variation and risky modifications to create closed-storage devices. The aim of this chapter is to highlight important quality control factors (e.g., ease of use, repeatability, reliability, labeling security, and cryostorage safety) into the selection process of which device/solution to use, independent of commercial manipulations. In addition, we provide clinical and experimental evidence in support of warming rates being the most important factor determining vitrification survival. Lastly, we exhibit indisputable support that aseptic, closed vitrification systems, specifically microSecure vitrification ( $\mu$ S-VTF), can achieve success with attention to quality control details often lacking in open vitrification devices.

**Keywords:** blastocyst, cryopreservation, device type, oocyte, quality control, vitrification

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## 1. Introduction

The early successes of William F. Rall, PhD, and coworkers with mammalian embryo vitrification (VTF) were based on extensive experimentation, meticulous solution and straw-handling preparations, and precise straw sealing [1, 2]. Although there was less overt cellular

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damage, these early investigations simply proved that vitrification was a potentially effective alternative cryopreservation procedure, but not necessarily more effective than conventional slow-freezing methodologies. The degeneration experienced with visually intact vitrified embryos could have been due to the potential cryotoxicity of high-molarity vitrification solutions (e.g., VS3a = 6.5 M glycerol) [3]. An alternative consideration involved the importance of warming rates to prevent recrystallization events that could adversely effect cellular survival of vitrified blastomeres [4]. In the early to mid-1990s, most investigations focused on developing safer, less toxic solutions [5–7] to improve vitrification success. It was widely accepted that the combined use of less concentrated permeating cryoprotective agents (CPAs) made for safer vitrification solutions [6]. Indeed, by combining permeating CPAs (e.g., dimethyl sulfoxide (DMSO), ethylene glycol (EG), and glycerol (GLYC)), and adding other nonpermeating CPAs (e.g., sucrose and ficoll) to create moderately concentrated vitrification solutions, brief intervals of exposure proved to be safe to embryonic blastomeres and oocytes. Combined with the commercial development of novel vitrification devices [8], and the proposed relative importance of cooling rate to vitrification success [9–12], vitrification technology has essentially replaced slow-freezing procedures for human oocytes and embryos in the twenty-first century.

The history and general discussion of vitrification's application to human oocytes, zygotes, and embryos have been previously reviewed [13]. Cryopreservation in the absence of damaging ice-crystal formation (i.e., vitrification) efficiently preserves cell membrane integrity, typically yielding high complete survival rates (>90%) for oocytes and embryos utilizing various vitrification methods. Live birth rates associated with the vitrified embryo transfer cycles are considered equal to or higher than those of fresh blastocysts [14–16], and others claim that the use of vitrified donor oocytes is comparable to fresh donor oocytes [17, 18]. Yet, vitrification success is susceptible to procedural variation between programs referred to as “technical signature” [19]. Variation associated with technical repeatability and reliability between individuals, and a multitude of vitrification devices and methods have resulted in inconsistencies between programs applying vitrification. To optimize the application of vitrification industry-wide, several quality control factors should be taken into account. This chapter describes those quality control factors and problematic events/examples associated with the development of different vitrification devices. In addition, we detail the successful implementation of a noncommercial, simple, and secure aseptic-closed procedure (i.e., microSecure-VTF) which has aimed to minimize quality control-related variation. Furthermore, there is a growing need to educate reproductive biologists about the complexity of the vitrification process and understand the relative importance of warming rate to cooling rate and their relationship to the vitrification solution used.

## 2. Quality control considerations

In the last decade, vitrification technology has rapidly supplanted conventional freezing practices. To a great extent, this was due to the commercial industry's development of specialized vitrification devices. However, the overall safety, efficiency, and effectiveness of clinical

vitrification have been handicapped by these same commercial influences introducing inherent design flaws in devices used in the in vitro fertilization (IVF) industry. Indeed, specific differences in devices and their utilization have introduced significant technical variation between individuals and programs. Although the efficacy of any single vitrification method/device can be optimized within a program (i.e., intra-program variation), through extensive training and experience, its adoption throughout the assisted reproductive technology (ART) industry may be less effective (i.e., inter-program variability). It is this inability to easily and successfully apply a vitrification method between laboratories (i.e., technical signature) that warrants attention, if an optimized universal method(s) is to be executed throughout the IVF industry. When attempting to integrate an effective vitrification system into your clinical laboratory, several quality control factors should be taken into account to fully assess the completeness of a vitrification device and its potential pitfalls. These quality control considerations include the following:

**1. Labeling potential**

- a. Can labels be securely adhered and easily identified?
- b. Do they offer dual color identification potential?
- c. Are they tamperproof and fail safe?
- d. Does it require a secondary label and can the label be easily removed for record-keeping purposes (i.e., patient verification) postwarming?

**2. Technical ease and reliability**

- a. Can embryos be easily loaded into or onto the device in a timely and repeatable manner?
- b. Can the device be easily and safely extracted to facilitate rapid warming (i.e., achieve a warming rate >its cooling rate)?
- c. Can embryos be simply identified and tracked postwarming?

**3. Procedural simplicity and repeatability**

- a. Does the vitrification method offer simplicity and reliability?
- b. Does it easily allow for repeatable applications within and between patients which minimizes variation between technicians (internal) and programs (external)?

**4. LN<sub>2</sub> storage capacity**

- a. Can the devices be easily and safely handled and identified?
- b. Is the device's storage potential space efficient?
- c. Does the device offer security and safety from physical damage?
- d. Does the container provide safety and reliability from possible pathogenic contaminants as an aseptic closed system?

## 5. Recovery potential/survivability

- a. Is the device design prone to potential problems in the guaranteed recovery of embryos?
- b. Will the system reliably vitrify and maintain complete cellular integrity postwarming?

### 2.1. Vitrification device development

New concepts in vitrification device/container design began emerging in the assisted reproductive technology industry the late 1990s through mid-2000s, as previously reviewed and discussed by Vajta and Nagy [8]. Dr. Vajta and his coworkers created an “open-pulled straw” (OPS) which tapered from the conventional 0.25-ml straw diameter to a >50% reduction in diameter over an approximate 4-cm length [20]. This novel design effectively increased the surface-to-volume ratio which increased its cooling rate capacity in a lower volume, which reduced cryoinjury to vitrified bovine oocytes. The OPS was simple to use (i.e., load) for animal scientists and veterinarians familiar with handling and sealing 0.25-ml straws, yet it was difficult to label and store in a secure, organized, and effective manner. The labeling of the plastic straw with a fine sharpie or cryomarker was subject to being partially rubbed off in cryostorage and becoming un-identifiable. Then, there was also concerns on how to safely and securely store these opened OPS units. One good alternative was to enclose and seal them inside a larger 0.5-ml semen straw [21], preferably an ionomeric resin CBS straw capable of achieving reliable weld seals. Thus, the former quality control issues were resolved at the expense of the insulated OPS having slower cooling rates without direct contact to liquid nitrogen (LN<sub>2</sub>). A couple of years later, another adapted straw procedure emerged, called the hemi-straw, which involved supercooling a microdroplet on the inner edge of a transverse cut 0.25-ml straw plunged into LN<sub>2</sub> [22, 23]. Like the OPS, the hemi-straw could be inserted into a 0.5-ml semen straw before LN<sub>2</sub> exposure (as a closed system) or following ultra-rapid cooling and then plug-sealed [24]. The hemi-straw concept led to the commercial development of the aseptic-closed high-security vitrification device (HSV; CryoBioSystem, France) which used a plastic wand device with an elongated trough tip (i.e., gutter) to support a vitrified microdroplet, which was then inserted into a 0.25-ml CBS™ ionomeric resin straw [25].

In the years between the development of the OPS and hemi-straw, a unique carrier system called the “Cryoloop” was adapted from X-ray crystallography applications (Hamilton Research Instruments, USA). A nylon loop, barely detectable to the eye, supported the suspension of a thin film of vitrification solution to facilitate the supercooling (>10,000°C/min) of an embryo or oocyte in the confines of a liquid nitrogen-filled cryovial [26]. Although cryovial labeling and storage were standard practices, the precise loading of the fluid-embryo combination onto and handling of the delicate loop affixed to a metal post was subject to technical variation. Despite the cryoloop's clinical success over the next decade [27, 28], the device required assembly (i.e., glue adhesion of the loop/post to the cap) and the use of specialized instruments (e.g., curved grasping forceps, an extended rod with magnet) to facilitate handling. Although a published comparison between the hemi-straw method and the cryoloop ultimately revealed no differences in survival rates or pregnancy outcomes [24],

the proposed importance of ultra-rapid cooling rates to insuring high vitrification success rates dominated the commercial push to integrate vitrification into the human IVF industry. While other novel thin-film, supercooling procedures were proven effective (e.g., electron microscopy (EM) grid, nylon mesh; see review [8]), it was the development of an open-system, plastic wand-flat-blade device called the “Cryotop” [29, 30] that would have the greatest impact on the adoption of clinical vitrification (Kitizato, Japan), as a routine cryopreservation method used for human embryos and oocytes [31]. Promoting the importance of ultra-rapid cooling rates in a micro-volume (0.1  $\mu$ l), the popularity of open-blade methods grew (e.g., Cryoleaf, Cryolock, and Cryotech) and, like the Cryotop, each device was subject to technical variation and other unique quality control issues discussed below.

While open-system advocates minimized concerns over the potential risks of pathogen cross-contamination among LN<sub>2</sub>-stored samples [32], there are others who express strong apprehension over the long-term cryostorage of embryos/oocytes in containers or devices which are unsealed (i.e., leaky, open container, and protected device systems) or poorly/improperly sealed due to disease transmission risk assessments, based on animal model research [33]. Although LN<sub>2</sub> vapor-phase storage tanks offset these concerns, they were and still are not common to, nor practical, in most clinical IVF laboratory settings. Thus, there were additional commercial efforts to produce effective closed vitrification systems in the mid-2000s. During the development of the CBS™ HSV device (mentioned above), an ultra-fine OPS system, called the Cryotip™, was marketed by Irvine Scientific as the first Food and Drug Administration (FDA)-approved vitrification device. This modified closed micropipette system produced comparable postwarming embryo outcomes compared to the Cryotop [30]. Unfortunately, the Good Manufacturing Practice (GMP) focus by the FDA was strictly on the effectiveness of the device to achieve a reliable seal, and not on other important quality control issues influencing gamete and embryo safety. Indeed, the Cryotip™ was a mass-marketed flawed vitrification device that proved to be technically challenging to use (i.e., “technical signature” concept applied) due to aspiration, bubbling, and sealing issues, as well as biosecurity and cryostorage identification issues. Shortly thereafter, another closed micropipette device called the Cryopette™ (Mid-Atlantic Instruments-Originio, USA) was developed to overcome loading and dual-sealing problems associated with the Cryotip™. In addition, this device added color coding, a positive feature originally found in CBS™ 0.3-ml embryo straws. By mounting a colored, cryo-resistant bulb on one end of a shortened flexipette, it strived to control technical aspiration and expulsion of embryos, and simultaneous close one end. Again, FDA's approval of this device insured that the open end of the flexipette could be effectively sealed without harm to its cellular contents, but did not address concerns regarding labeling, cryostorage safety, or bulb reliability. These quality control flaws were left to the consumer to discover, as discussed below.

The Cryotip and Cryopette devices are both considered “closed systems” as the gametes and embryos they contain are sealed in an environment away from any potential contact with liquid nitrogen. However, the outer surface of their micropipettes still reside in direct contact with LN<sub>2</sub>, and thus are still at potential risk of being a carrier of pathogens found free floating in stored LN<sub>2</sub> (e.g., adherent bacteria [34, 35]). Although the risk of transfection is unproven, risk

assessment potential is virtually eliminated in an “aseptic, closed vitrification system” such as the HSV [25] and enclosed OPS or cut standard straw [36, 37] approaches (described previously). Unlike many original suboptimal designs, CryoBioSystems made improvements in their HSV system enhancing the ease and reliability of device extraction to reduce warming variation, as well as improving device identification by offering color coding. Subsequently, a similar device, referred to as Vitrisafe (Astro-med Tec, Austria), has also produced a high level of clinical success [38, 39], similar to open systems. In the last decade, three additional novel approaches were developed, with two in particular, the rapid-i [40] and microSecure vitrification [41] being clinically validated. The rapid-i (Vitrolife, USA/Sweden) is a hybrid-designed device mimicking both the flat-blade wand of a cryotop possessing a micro-hole drilled in the center of the surface to suspend the vitrification solutions, like the Cryoloop. The advantage of the hole in the plastic blade was the ability to directly view the embryo in a 0.05- $\mu$ l volume, with residual vitrification solution easily aspirated from the blade surface. However, technical precision is still required in terms of embryo/oocyte handling, but with less concern aspirating residual fluid off the blade. The rapid-i system has a special LN<sub>2</sub> bath that allows closed bottom-weighted straws to be supported upright in LN<sub>2</sub> with the open end being accessible above a covered surface. Each rapid-i wand could then be supercooled inside each straw, theoretically in a rapid manner, followed by heat sealing and LN<sub>2</sub> storage. Unfortunately, one unexpected problem was the latent conversion of LN<sub>2</sub> vapor to liquid inside the straw during the equilibration period, resulting in the transfer of kinetic energy to a warm wand dropped into the straw. To prevent the initial expulsion of the device, the company adopted a procedural step to cover the straw opening upon device insertion, followed by sealing. Other than that, the straw does not have any colorized component, or system for secure or duplicate labeling.

The growing high level of success and undeniable security advantages of some aseptic closed systems [38–42] has prompted another new and potentially problematic development of hybridizing vitrification systems. Attempting to gain the benefits of direct LN<sub>2</sub>-mediated ultra-rapid cooling, some innovative embryologists and at least one commercial company have begun sealing LN<sub>2</sub>-exposed open devices into plastic straws. Unlike the safety and security of weld-sealing an ionomeric plastic straw under ambient (20–22°C) conditions (i.e., HSV and mS-VTF), the compliance of supercooled straws to effective heat sealing may be compromised leading to suboptimal, unsecure closure. Without the specialized LN<sub>2</sub> bath lid of the rapid-i device, the sealing of straws while primarily submerged in LN<sub>2</sub> could lead to the incomplete heat sealing of straws and/or the partial trapping of N<sub>2</sub> gas inside a straw. Upon rapid warming, the consequences of such a scenario could be disastrous, as the rapid expansion of N<sub>2</sub> gas from a liquid phase can be explosive in a closed container [43]. In an at-risk situation, as described above, the straw should be cut and the vitrification device removed while still partially submerged in LN<sub>2</sub>. Furthermore, if the warming rate of a hybrid device does not exceed its initial cooling rate, the viability of its vitrified gametes or embryos will be compromised.

## 2.2. Relative importance of warming rates

Over 60 years ago, Dr. Peter Mazur first discussed the relative importance of warming rates to cellular solutions which had been cooled very rapidly [44]. He proposed that unstable ice

crystals can grow to a size damaging cells if the warming velocity was not sufficiently high to melt the unstable ice formation. Then, with the development of vitrification in the 1980s, both Drs. Greg Fahy [45] and William Rall [4] warned us about the importance of warming for cellular survival. Yet, it was Dr. Mazur again, with his postdoctoral fellows and scientific colleagues, who defined our path to successful vitrification over the past decade. The answer today is definitive, and the efficacy of vitrification success is more highly dependent on warming rates than cooling rates [46–50]. Independent of the vitrification device or open/closed system used, the warming rate must exceed the cooling rate to insure high survival rates. Using a slow warming model, Dr. Brian Wowk has demonstrated the relationship of ice nucleation during cooling and recrystallization of ice growth upon warming relative to cryoprotective agent concentration [50], as well as the thermodynamics behind vitrification [51]. Under low-warming conditions, today's typical commercial vitrification solutions (e.g., 30–32% (total permeable cryoprotective agents), EG/DMSO or EG/PPG) are classified as “unstable” and are highly dependent on rapid cooling and higher warming rates for cell survival. Whereas metastable solutions (e.g., 50–70% (total permeable cryoprotective agents)) have a lower temperature of heterogeneous ice nucleation ( $T_h$ ) where the warming rate does not need to outrun the temperature of devitrification ( $T_d$ ) to inhibit (i.e., melt) the potentially damaging recrystallization of ice, as originally eluded to by Mazur [44].

Although commercial vitrification solutions work well in both open and closed systems, the use of metastable solutions (e.g., VS3a, 6.5 M glycerol or ICE-BL, >7.9 M glycerol/EG) may offer aseptic closed vitrification systems a higher level of biosafety. As we learned in the 1990s, the mixture of cryoprotective agents reduces the cytotoxicity for a potential vitrification solution [52]. Additional research from Mazur's laboratory [53] has shown that infrared laser technology can be used to exponentially increase warming rates and achieve high oocyte survival using a threefold diluted vitrification solution. But is there really a need to make solutions even less concentrated at the expense of becoming warming rate dependent? Concerns over the potential toxicity of vitrification solutions are likely as misunderstood, as the importance of cooling rates to successful vitrification. Recently, we have shown that human blastocysts (BL) diluted into a more concentrated ICE-BL non-DMSO vitrification solution (Innovative Cryo Enterprises, USA) are as viable as those in 30% EG/DMSO (LifeGlobal, USA/Canada) and 32% EG/PPG (Vitrolife, USA/Sweden) solutions for up to 10-min exposure [54], revealing that human blastocysts are more resilient to vitrification toxicity than previously believed. Furthermore, we conducted a series of revitrification (rVTF) studies aimed at understanding the cryotoxicity and osmotic stress associated with different vitrification solutions [54]. Using our control metastable vitrification solution (ICE-BL), no difference in 0-h survival or 24-h development was exhibited after up to 5X rVTF, with or without sucrose elution between treatments, proving how cryotolerant human blastocysts are to metastable vitrification in an aseptic closed system. Interestingly, ongoing unpublished data using a common EG/DMSO solution revealed no difference in survival, but a significant decline in sustained viability at 24 hr after the second rVTF treatment. Like Wowk's slow-warming model, our data may be revealing the vulnerability of unstable solutions to cryoinjury when exposed to a cryostress model. These studies demonstrate interesting findings in support of theoretical vitrification principles regarding the relationships of cooling and warming rates relative to

molar concentration of cryoprotective agents. In addition, it reveals that the commercial industry should seriously reevaluate vitrification formulations to optimize their product for the IVF industry [55].

### 2.3. Identifying and troubleshooting device-related quality control problems

A huge problem among early non-straw or cryovial products was in how a particular device was labeled. Most experienced embryologists with good laboratory practices found effective ways to properly label a device (as described below in Section 3). When using Brady labels to wrap around an open-system handle or a 0.25-ml straws, care is needed to insure the font size is readable. A horizontal wrap may be more secure than a vertical placement, but will likely not provide sufficient space for readable text. Therefore, validation testing should be performed to confirm that the vertical surface is reliably adherent. Alternatively, the label could be adhered horizontally to create an external flag on the device, which optimizes the labeling surface. If a flag label is used, be cautious to not overcrowd samples causing possible breakage of the flag. In most cases, a secondary ID on the device is warranted to prevent possible identity loss in storage. For example, the Cryotip was a poorly planned device in terms of labeling and storage, but there was a simple solution suggested to the company after their FDA approval and marketing commenced. The user had the option of placing or handwriting (with sharpie) the label onto the metal protector or handwriting of the upper straw by retracting the metal protector in a sterile manner during setup. Difficulty in the ease and safety of extracting these miniature devices from a shortened goblet for identification/selection was a commonly experienced storage problem (discussed further below). The simple solution was the use of 0.5-ml straws crimped at the open end and slid over the sealed end of the device, as used for conventional one-step straws. Each straw handle could be used to correspondingly label each device to facilitate safe and easy identification under ambient conditions while the device remained safely submerged in LN<sub>2</sub>. Such a solution was not possible to the alternative Cryopette system, which may have improved the system for aspirating embryos into the device but did so at the expense of the important factor of labeling. Perhaps, the most outlandish experience I witnessed was with a shipment of OPS units ( $n = 8-10$  OPS) simply stored in a flat cartridge meant to hold straws. Each OPS only had a hand-printed last name and a date, without any further identification distinguishing them. Then to make matters worse, when I attempted to slide the wand upward to systematically extract them, they did not move up like a straw but instead the tapered tips slid down and jammed up the glide track creating a real problem. These possible conditions must be carefully thought out before implementation.

Technical ease and reliability of the methods used are an important consideration. This factor is important within your group, but perhaps even more important outside your program. Often times patients move or simply change physicians (i.e., clinics/laboratories), resulting in the transport of your embryos and oocytes to another laboratory. To avoid possible liability issues associated with lost embryos, faulty devices, or nonsurvival issues of the patients' only/last embryo, it is critical to insure the end user will also be successful. Thus, simple and reliable products are essential. Early open systems such as EM grids and nylon mesh units were difficult to use for an individual unfamiliar with the device. Even a more established product

such as Cryoloops presented challenges to the unknowing user. For example, upon warming if the supercooled metal post holding the loop, containing the embryo(s), contacted the warming medium it would cause excessive vaporization and bubbling which would hinder an efficient recovery process. Worst yet, the Cryoloop device required assembly (i.e., glue adhesion of the loop/post to the cap) and was susceptible to device error associated with loose/fallen posts, broken loops, as well as variable microdroplet sizes. The Cryotip has also been known to be susceptible to recovery problems associated to excessive internal bubbling and damaged tips. Then, there are the popular open-blade methods that predominate the world-wide ART industry. Tremendous technical variation exists with respect to the amount of vitrification solution to retain on the surface with the embryo or oocyte(s). If too large, the droplet could disengage from the surface during storage, or in a closed system such as the HSV the droplet could displace itself to the inner straw surface. At least in the closed system, there is still the opportunity to recover the lost embryo or oocytes from the sterile inner straw surface. If the microdroplet surrounding the embryo/oocyte(s) is aspirated too much (i.e., nearly dry), it places the embryo/oocyte(s) at risk of dehydration and osmotic injury prior to vitrification. One final example worth acknowledging here is a more recently developed device called the Cryotech, made of a lighter weight, more transparent film with a 90° angle to the embryo/oocyte-loading surface. On at least three occasions, involving the international shipment of oocytes, the oocytes were lost upon warming. The last shipment was actually tracked by the same experienced, senior embryologist performing both vitrification and warming events in two different countries. In the latter situation, the device failed, suggesting that the excessive handling dislodged the droplet from the surface. These are the types of very unpleasant circumstances that typically leave each party, or worse the patient, blaming negligence of one laboratory or the other, but could have simply been the fault of the device design. In these scenarios, a closed pipetting device, such as microSecure-vitrification ( $\mu$ S-VTF), is ideal in its simplicity and reliability to retain the cellular products they contain.

Emphasis on procedural simplicity and repeatability cannot be underestimated. Ideally, you want to minimize the time and effort required to place the embryo/oocyte(s) onto or into a device. We have already seen problematic examples above that can create significant hardship for all parties involved. Thus, it is critical that we strive to use fail-safe systems, and that the procedures involved are also safe for the embryologist to perform. An important technical example here is the sealing or securing of a device once the embryo/oocyte(s) are loaded onto the device. Most closed systems are loaded at room temperature and their straws heat sealed, preferably in an ionomeric-resin straw (e.g., CBS™) and using an automatic sealer. However, if using a manual impulse sealer or other miscellaneous approaches (e.g., heated forceps, curling iron, etc.) then a meticulous quality control practice must be implemented to insure the completeness of each straw seal by each technician. One approach I learned from Dr. Rall over 30 years ago on the sealing of conventional 0.25-ml plastic straws with a standard impulse sealer was to flip them 180° several times until it adheres to the Teflon surface (over the electrode) and then do it one more time (requiring a slight delay to gently pry the straw from the cooling surface). That technique was repeatable and teachable, and more importantly created reliable and secure seals that never resulted in an exploding straw post rapid warming. If the seal is incomplete due to a poor sealing technique or a noncompliant plastic due to LN<sub>2</sub>

vapor conditions (described above), these straws will allow LN<sub>2</sub> seepage into the container to occur. If these containers are warmed too rapidly, the vaporization pressure could be excessive and damaging. One other noteworthy example here is a device requiring two different heat settings to optimally seal two different size openings (e.g., Cryotips). The latter was simply a formula for repeated errors, frequently resulting in bent and burnt tips. It is also possible that the overheating of the fine tip ends may have been partially responsible for the excessive and problematic bubbling experienced in these devices.

Another problem experienced by many inexperienced user of the Cryotip device was the overcrowding of devices within a given storage goblet. Unlike a compact arrangement of straws, the tight opposition of Cryotips could cause their protective shields to rise, leaving their delicate tips exposed to damage (e.g., bending, breakage). Similarly, although Cryopettes were not as delicate, they were completely unexposed in storage without support. The potential for breakage or fracturing its bulb connection, if accidentally compressed in frozen storage, was a real risk. Another important practical factor to consider is the LN<sub>2</sub> storage capacity of a device. If we consider that 0.25-ml straws (e.g., HSV, Vitrisafe) or perhaps Cryotop devices in large goblets is an optimal standard of 10 units/goblet, then the storage of 8 units of 0.5-ml straw-size devices (e.g., rapid-i,  $\mu$ S-VTF) or square-capped Cryolock devices is very good. However, the safe storage of five Cryotips per goblet begins to become inefficient, while one or two Cryoleaf devices are completely impractical. Lastly, we have already discussed the ability to safely access and visualize samples in storage/LN<sub>2</sub>-filled dewar flasks or specialized bathes, but what about the safety of the handler. Most open-system methods require the placement of a protective straw cover (e.g., Cryotop, Cryotech) or a plastic cap (e.g., Cryolock) over the supercooled device end for storage protection. Likewise, these protective covers must be removed in LN<sub>2</sub> prior to warming to facilitate high warming rates. However, these covers can be difficult to unlock and remove under freezing conditions. Any miscues in the insertion or removal of the protective covers could adversely influence the stability of the embryo/oocyte(s) on the surface of the open blade. Both vitrification and warming events entail the coordinated handling of device components, with fingers and forceps, in close proximity to LN<sub>2</sub>, thus creating reoccurring safety issues. Although the use of protective liners provides delicate finger agility in handling and reduces potential contact burns, it does eliminate a mishandling event (e.g., connecting or sealing hybrid devices) that could result in the accidental LN<sub>2</sub> spillage of a full dewar flask. In short, the unnecessary handling and manipulation of devices in LN<sub>2</sub> creates biosafety issues for the user. By contrast, the assembly and sealing of aseptic closed devices under ambient conditions eliminates similar safety concerns.

Our final end point consideration is the recovery and survival rate potential of a given device. As we have already discussed above, there are several quality control factors that can ultimately influence the final outcome. One issue we have not touched on is the advantage of being able to visualize the embryo or oocyte(s) upon warming. This is particularly important with oocytes as they do become highly translucent during their initial exposure to the T1 sucrose solution. Therefore, methods that allow you to distinctly image and account for the expected number of embryo(s)/oocyte(s) present (e.g., Cryoloops, rapid-i and pipetting

methods) offer distinct advantage to efficiently locate the desired cell products. Blade and hemi-straw microdroplet methods can leave the technician wondering if the unfound embryo(s) and oocyte(s) are still on the device or free floating on the surface of the sucrose solution or attached to an air bubble. The fact is problems can arise and some methods simply make it easier to troubleshoot the issue at hand. Unfortunately, most technical and clinical publications failed to discuss recovery rate potential and associated problems, but instead choose to disguise that outcome among the nonsurvival group. It is unclear why that has been a scientifically acceptable practice, considering rare embryo losses using conventional slow-freezing technology typically warranted an incident report. If we are to fully evaluate the efficacy of a vitrification device or our ability to efficiently apply the technology, we must be willing to honestly share our mistakes and device experiences, as touched on by Vajta and others [56].

### **3. The microSecure-VTF ( $\mu$ S-VTF): a quality control solution**

Having a firm grasp of the cryobiological principles of vitrification, we developed an aseptic closed vitrification device aimed at insuring the simplicity, efficacy, and reliability of vitrification success [57]. It was developed in 2008 as an inexpensive, noncommercial, FDA-compliant method which optimized quality control aspects of vitrification to reduce or eliminate technical variation. Using the CBS<sup>TM</sup> 0.3-ml embryo straw (with hydrophobic plug) as our model, our system uniquely offers tamperproof internalized, dual-colored labeling. The use of different label and rod colors allows for quick identification of patient samples based on day of cryopreservation, whether blastocyst biopsy was performed, or blastocyst quality, for example. In contrast to the HSV system, we maintained secure labeling by not reducing the straw diameter. Having internalized labels allows us to use nonpermanent adhesion labels (GA International, USA) that can be easily removed postwarming and placed onto the patients' Cryo-data sheet record to confirm identification with the patient at the time of ET. Furthermore, in the case of a preimplantation genetic screening (PGS) cycle with discard aneuploidy embryos, the placement of removed labels onto the Cryo record is an excellent quality assurance practice. Finally, in terms of labeling it is essential that an accurate description of the patient sample is conveyed, including the last and first name, secondary ID, embryo description (#, stage, quality grade; Ex: 1x4AA or 1x8cB), and the cryopreservation date. Upon receiving other devices in our laboratory, it is so surprising to witness how little information some programs actually provide on a device. Out of respect to all IVF laboratories, proper labeling is essential to avoid possible liability issues.

Since  $\mu$ S-VTF uses shorten sterile flexipettes to pipette, load, and directly store embryo(s) or oocytes, there is no secondary device surface to introduce technical variation. Thus,  $\mu$ S-VTF embryos and oocytes are simply loaded and easily visualized upon removal to insure >99.9% recovery rates. To achieve rapid warming after safe patient sample identification in a dewar flask, the straw is cut below the plug/seal (below the ID rod) and quickly tipped (60° angle) and tapped to promote the free fall of the flexipette into a warm sucrose bath (see You Tube video "microSecure vitrification warming"). On rare occasion, if an embryo is missing upon

pipetting into T1 solution it has invariably been found in the sucrose bath, due to it having been loaded to close to the tip. From this rare experience, we have learned that although there is capillary drift into flexipettes while resting on the sidewall of a 60-mm dish in the sucrose bath (for 5–10 s, as the pipette fluid volume will attempt to equilibrate to the sucrose level), the initial plunge into the bath may create an initial force that pulls a fraction of fluid from the tip. It is important that biologists remain mindful to load the embryo(s)/oocytes approximately mid-way in the fluid column. Again, we control this by aspirating a full, fresh 3- $\mu$ l column of vitrification solution into the pipette (i.e., plunger released, no technical variation) and then expel one-third to a half of the fluid upon picking up the embryo(s)/oocytes, followed by controlled plunger release (to preset fill volume). Upon pipette removal and tip drying (i.e., sterile gauze wiping), the capillary volume in the flexipette is stable during handling procedures. Our rare loss of an embryo has been exclusively related to hatched blastocysts post-biopsy. These embryos can be extremely adherent on contact with any plastic (i.e., charged surface) and potentially difficult to ID in their completely collapsed state. Thus, as with our standard blastocyst biopsy of trophectoderm cells, we suggest pre-coating the surface of all pipettes with human serum albumin (HSA) before handling to minimize cellular stickiness and possible loss of hatched blastocysts.

Next, the  $\mu$ S-VTF system uses CBS<sup>TM</sup> ionomeric-resin straws that completely weld seal using an automated sealer, which again effectively eliminates technical variation. By not worrying about the quality of the seal, our system offers repeatability and reliability only found in CBS<sup>TM</sup> straw products (e.g., HSV). Prior to sealing, we make sure the tip of the flexipette has dropped down to the plug end, insuring at least 1 cm of air space to safely seal the straw. Next, we suggest supporting the straw at the point of sealer contact (as opposed to the natural instinct to hold the end of the straw) to minimize any abrupt vibration stimulated by the automatic sealer. Upon inverting the straw label-end up, we check the quality of the seals and whether any fluid remnant/discharge appears in the upper straw air space (as the flexipette base should now be resting against the bottom seal). The upper air space near the plug/labeling rod insures safety to cut the straw postwarming. If any fluid was visualized, we check to make the flexipette did not accidentally get sealed into the straw. If on a rare occasion this happened: (1) if the seal is incomplete then you must extract the flexipette and attempt to find the embryo in the residual fluid droplet before reloading; or (2) if the seal is complete, simply make a note on the record (for that straw #) of the situation, so that proper care is taken postwarming to rinse the inner straw for possible extruded oocytes/embryo(s). Upon storing the straws in LN<sub>2</sub> on canes with large open goblets, up to eight straws can be stored/cane (i.e., good storage capacity). Furthermore, there is no need for an upper cover on the cane, as each straw is weighted, unless they are transported and susceptible to not maintaining their upright position. Coincidentally, if a straw is ever to accidentally drop into an LN<sub>2</sub> tank, they are easily recovered as the weighted rod drops the tank bottom and sticks straight upward (due to air buoyancy in the straw), as opposed to lying on the bottom somewhere in the residual N<sub>2</sub> debris.

As an aseptic closed system whose vitrification device (i.e., a sterile flexipette) is stored in an outer straw container, the  $\mu$ S-VTF device achieves a cooling rate of 1391°C/min and

corresponding warming rate of over 6000°C/min. As an insulated device with lower cooling rates than an open device system, it has proven to be more resilient to accidental room temperature exposures (Ovation Fertility, unpublished data). Overall, the  $\mu$ S-VTF device has been systematically validated to be a simple and reliable approach that minimizes intra- and inter-laboratory technical variation, while providing maximum cryosecurity using sterile products [41]. In addition, it has been developed without commercial influence and marketing pressure, thus providing the added benefit of substantial cost-savings. In today's IVF industry, which is increasingly reliant of biopsying and vitrifying every fair to excellent quality blastocyst to optimize pregnancy success [58], costs are an increasingly important factor to consider. This is especially true when one realizes that 50–75% of the genetically tested blastocysts will be aneuploidy and destined to be discarded after short-term storage. In conclusion, the  $\mu$ S-VTF system has proven to be a highly effective procedure that may offer “universal” acceptance to alleviate current quality control concerns with the handling, storage, and shipment of vitrified oocytes and embryos.

#### 4. Conclusion

Vitrification is the single most impactful assisted reproductive technology in the IVF industry since the development of intracytoplasmic sperm injection (ICSI). Today, we faithfully cryopreserve blastocysts and oocytes without regard to possible loss. We have had to reeducate ourselves, and our infertility patients, that fresh ET is no longer better than vitrified ET cycles, especially in combination with blastocyst biopsying and preimplantation genetic screening. By adhering to strict quality control standards and quality assurance practices, we can continue to improve the reliability of our laboratory outcomes, and help avoid future liability issues together.

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## Author details

Mitchel C. Schiewe

Address all correspondence to: mschiewe@ovationfertility.com

Ovation Fertility, Newport Beach, CA, USA

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