Journal of Visualized Experiments

Minimum volume vitrification of immature feline oocytes

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61523R1
Full Title:	Minimum volume vitrification of immature feline oocytes
Section/Category:	JoVE Biology
Keywords:	cat; Banking; conservation; cryopreservation; Cumulus-oocyte complex; Felid; Female; freezing; Gamete; Germinal Vesicle; Germplasm; Rapid
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Additional Information:	
Question	**Response**
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TITLE:

Minimum Volume Vitrification of Immature Feline Oocytes

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KEYWORDS:

Cat, Banking, Conservation, Cryopreservation, Cumulus-oocyte complex, Felid, Female, Freezing, Gamete, Germinal Vesicle, Germplasm, Rapid

SUMMARY:

This manuscript describes a protocol for the minimum volume vitrification of immature cat oocytes with laboratory-made media on commercial supports. It covers every step from oocyte isolation from ex vivo gonads to vitrification and warming.

ABSTRACT:

In wild animals' conservation programs, gamete banking is crucial to safeguard genetic resources of valuable individuals and rare species and to promote biodiversity preservation. In felids, most species are threatened with extinction, and domestic breeds are used as a model to increase the efficiency of protocols for germplasm banking. Among oocyte cryopreservation techniques, vitrification is more and more popular in human and veterinary assisted reproduction. Cryotop vitrification, which was at first developed for human oocytes and embryos, has demonstrated to be well-suited for cat oocytes. This method offers several advantages, such as the feasibility in field conditions and the speed of the procedure, which can be helpful when several samples need to be processed. However, the efficiency is strongly dependent on the operator's skills, and intra- and inter-laboratory standardization are needed, as well as personnel training. This protocol describes minimum volume vitrification of immature feline oocytes on a commercial support in a step by step field-friendly protocol, from oocyte collection to warming. Following the protocol, preservation of oocyte integrity and viability at warming (as high as 90%) can be expected, although there is still room for improvement in post-warming maturation and embryonic development outcomes.

INTRODUCTION:

Cryopreservation has become a key step of assisted reproduction techniques (ARTs). In humans, it allows preservation of fertility or postponement of parenthood for medical or

personal reasons. In animals, it is necessary to overcome distance and time in planned matings, especially in farm animals and pets, or to preserve genetic material of valuable subjects in conservation programs, particularly in wild endangered species. Gamete cryopreservation is the best choice when the individuals to be bred have not been chosen yet or in order to avoid ethical issues associated with embryo freezing, especially in human medicine[1]. Spermatozoa are relatively easy to preserve and give satisfactory outcomes after thawing, but oocytes, due to their structural features, might be more complex to store. Indeed, the low surface/volume ratio, as well as the presence of the zona pellucida surrounding the ooplasma, limits the movement of cryoprotectants and water across the cell[2]. Moreover, in domestic animals including felids, they are characterized by a lipid-rich cytoplasm, which is thought to make them more sensitive to cryopreservation[3].

Most felids are threatened, and the domestic cat is used as a model to develop protocols for germplasm preservation thanks to the availability of gonads from routine ovariectomy. In wild animals, gonads can be obtained after elective surgeries or (more frequently) *post-mortem*, and immature (germinal vesicle) gametes can be retrieved. Hormonal stimulation aimed to obtain mature (metaphase II) oocytes is not as common as in human ARTs because of the ethical issues and the species- and individual-specific response to treatments[4].

Therefore, the development of cryopreservation strategies has focused on immature gametes, which can usually be retrieved after the unexpected or sudden death of rare individuals. From a biological point of view, there are some differences in the cryopreservation of immature or mature gametes, each having its advantages. Firstly, DNA is more protected in immature oocytes, whose germinal vesicle contains chromosomes surrounded by a nuclear membrane, while the meiotic spindle of metaphase II oocytes could be more vulnerable to cryoinjuries[5]. Secondly, cold-induced cytoskeleton damages might affect spindle rotation, polar body extrusion, pronuclear migration and cytokinesis, which could have different impacts according to oocyte developmental phase, influencing meiosis progression or post-fertilization events. Finally, and perhaps most importantly, whereas mature oocytes are ready to be fertilized, immature gametes rely on the support of the surrounding cumulus cells to go through nuclear and cytoplasmic maturation[6], and this is the reason why whole cumulus-oocyte complexes (COCs) are cryopreserved. However, the loss of cumulus cells and/or the loss of functional connection between the gamete and the surrounding somatic cells are probably the most detrimental effect of cryopreservation of immature COCs.

Among cryopreservation techniques, vitrification is one that can be applied more easily in field conditions. Compared to slow (or controlled rate) freezing, vitrification is faster and does not require specific equipment, such as a programmable freezer. In order to satisfy the three fundamental principles of vitrification (i.e., high viscosity, connected to high cryoprotectant concentration, small volumes and ultra-rapid temperature decrease), several media and supports especially have been developed and used in cats for both immature and mature oocytes. Beginning with simple straws[7], devices were then developed to reach the “Minimum volume” goal. Cryoloop[8], open pulled straws (OPS)[9], plastic gutters (modified straw)[10] and cryotubes[11] have been used, until a more efficient device (i.e., Cryotop) was employed[11],

improving survival and meiosis resumption. Cryotop (**Supplemental Figure 1**) is a commercially available support which has become the elective open system for vitrification. Developed for the vitrification of human oocytes and embryos, it consists of a small film strip attached to a hard plastic holder, protected by a plastic tube cap during storage[12]. Thanks to its usability and to the extreme reduction in vitrification volume (as little as 0.1 µL), which also leads to extremely rapid cooling and warming rates, this vitrification support has been increasingly applied in several species, including the domestic cat, in which it has been used with a variety of media[13–17].

The purpose of this manuscript is to describe a collection-vitrification-warming protocol, with minor modifications from the one originally developed for human oocytes, which employs laboratory-made media and commercial supports for minimum volume vitrification and can be easily applied in field conditions for the cryopreservation of immature feline COCs.

PROTOCOL:

The procedures hereby depicted did not undergo ethical approval since cat ovaries were collected at veterinary clinics as byproducts from owner-requested routine ovariectomy or ovariohysterectomy.

1. Oocyte collection

1.1. Before starting the experiments, prepare solutions for ovary and oocyte collection. Prepare ovary collection solution with phosphate buffered saline (PBS) with a mixture of antibiotics and antimycotics (100 IU/mL of penicillin G sodium, 0.1 mg/mL of streptomycin sulphate, 0.25 µg/mL of amphotericin B). Prepare oocyte collection solution (i.e., PBS/PVA) with PBS with 100 IU/mL of penicillin G sodium, 0.1 mg/mL of streptomycin sulphate and 0.1% (w/v) polyvinyl alcohol (PVA).

1.2. Store solutions for ovary and oocyte collection at 4 °C until use.

1.3. On the day of queens' spaying, a few hours before processing the samples, take part of ovary and oocyte collection solutions and let them warm up at room temperature (RT; 25 ± 2 °C).

1.4. When samples arrive to the lab, isolate the ovaries from the surrounding connective tissue and from the oviduct and wash them in fresh ovary collection medium in a Petri dish.

1.5. Fill one 35 mm Petri dish for each queen with about 3 mL of RT PBS/PVA, and one more dish to collect the oocytes.

1.6. Place a pair of ovaries in a Petri dish and mince the cortex with a surgical scalpel. Ensure all the follicles have been broken with the help of a stereomicroscope (magnification 8x).

1.7. Collect COCs with a pipette and move them in fresh PBS/PVA in the allocated Petri dish. Select good qualities COCs, with a homogenous, dark cytoplasm and surrounded by several compact layers of cumulus cells (grade I[18]).

2. Vitrification

2.1. Prepare equilibration and vitrification media.

2.1.1. Prepare equilibration solution (ES) with 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethylsulphoxide (DMSO) in Medium 199, with 20% fetal bovine serum (FBS).

2.1.2. Prepare vitrification solution (VS) with 15% (v/v) EG, 15% (v/v) DMSO and 0.5 M sucrose in Medium 199, with 20% FBS (modified from [19]).

2.1.3. Let ES and VS warm up at RT before use.

NOTE: Cryoprotectants (e.g., EG, DMSO) are known to be cytotoxic. A strategy to counter their toxicity is to reduce the temperature at which the cells are exposed to them[20], and oocytes are usually exposed to cryoprotectants at RT. Thus, the whole procedure, from oocyte collection to vitrification, is carried out at RT in this protocol to avoid temperature fluctuations.

2.2. Prepare the vitrification dish (i.e., a special dish consisting of six conical wells divided in two rows, known as “Repro plate”). Prepare one row (three wells) for each vitrification support.

2.2.1. Add 20 µL of ES on the bottom of the first well

2.2.2. Add 300 µL of VS on the bottom of the second (i.e., 1VS) and third (i.e., 2VS) wells.

2.3. Equilibrate COCs in ES.

2.3.1. With a small-bore pipette (e.g., a Pasteur pipette pulled on a Bunsen beak to make it thinner – diameter should be at least 200 µm), transfer one (or more) COC(s) on the bottom of the first well.

NOTE: Choose the size of the pipette according to the number of layers of cumulus cells surrounding the oocytes, so to COCs dimensions, aiming to reduce as much as possible the volume of media which is transferred with COCs in each step. With the present protocol, trained operators can vitrify successfully up to eight feline COCs per each vitrification support.

2.3.2. Slowly add 20 µL of ES on the border of the drop. Wait for 3 minutes.

2.3.3. Slowly add other 20 µL of ES on the border of the drop. Wait for 3 minutes.

2.3.4. Slowly add 240 µL of ES on the border of the drop. Wait for 9 minutes.

2.3.5. While waiting, prepare a box with liquid nitrogen (LN_2), label a vitrification support with the experiment/cat identification code and leave it open. Put both of them near the stereomicroscope, so that they can be easily reachable while working.

CAUTION! Wear personal protective equipment when handling LN_2.

2.3.6. Alternatively, if several COCs have to be vitrified, begin the first equilibration step (see step 2.3.1 - 2.3.2) for another group of COCs (which will be loaded onto a new vitrification support).

2.4. Vitrify COCs in VS in less than 90 seconds.

2.4.1. Using the same small-bore pipette, fill it with 1VS, take the COCs from the bottom of the first well (ES) and move them to the surface of the second well (1VS).

2.4.2. Wash the pipette with 1VS.

2.4.3. Take the COCs and move them in another area (on the bottom) of the well; mix the medium surrounding them with the pipette.

2.4.4. Fill the pipette with 1VS in another area of the well, take the COCs, move them and mix the medium surrounding them with the pipette.

2.4.5. Repeat step 2.4.4.

2.4.6. Wash the pipette with 2VS.

2.4.7. Take the COCs and move them on the bottom of the third well (2VS); mix the medium surrounding them with the pipette.

2.4.8. Fill the pipette with 2VS in another area of the well, take the COCs, move them and mix the medium surrounding them with the pipette.

2.4.9. Repeat step 2.4.8.

2.4.10. Fill the pipette with 2VS in another area of the well, take the COCs and load them on the strip of the vitrification support, as close as possible to the tip. Aspirate excess medium to reduce the volume of VS as much as possible.

2.4.11. Immediately plunge the vitrification support in LN_2, moving it. Close it with the help of some clamps, making sure it always remains immersed in LN_2.

NOTE: Keeping the right timing is crucial due to cryoprotectant cell-toxicity. Because of their

high concentration in vitrification media, cell exposure needs to be controlled, also by flawless execution of the technique[21]. If samples are abundant, consider dividing them to process them more quickly and minimize the amount of time from oocyte collection to vitrification.

2.5. Store the loaded vitrification supports in a goblet and keep them in a storage LN_2 tank until warming.

NOTE: All the previous steps of the protocol can also be applied in field conditions if LN_2 is available. A dry shipper will be necessary to transport the samples to the lab safely and then proceed there with the following phases.

3. Warming

3.1. Prepare warming media.

3.1.1. Prepare thawing solution (TS) with 1 M sucrose in Medium 199, with 20% FBS.

3.1.2. Prepare dilution solution (DS) with 0.5 M sucrose in Medium 199, with 20% FBS.

3.1.3. Prepare washing solution (WS) with Medium 199 with 20% FBS.

3.1.4. Before use, warm up TS to 38 °C and DS and WS at RT.

3.2. If necessary, prepare the culture medium for the subsequent use of warmed COCs (e.g., in vitro maturation, IVM).

3.3. Place the heating stage close to the stereomicroscope and turn it on (38 °C). Put the lid of a Petri dish to warm up.

3.4. When everything is ready, transfer the vitrification supports which must be warmed from the storage tank to a box with LN_2, and put the box near the stereomicroscope.

3.5. Prepare the “Repro plate”. Prepare a row for each vitrification support that must be warmed.

3.5.1. Add 300 µL of DS on the bottom of the first well

3.5.2. Add 300 µL of WS on the bottom of the second (i.e., 1WS) and third (i.e., 2WS) wells.

3.6. Make a drop of TS (100 µL) on the lid of the Petri dish.

3.7. With clamps, open one vitrification support in the LN_2.

3.8. Put the TS drop under the stereomicroscope and with one fast movement take the

vitrification support from the LN_2 and immerse its strip in the drop, moving it until all COCs detach. Remove the support from the drop as soon as it is empty, but leave the COCs in TS for 1 minute in total (from immersion until the following step).

3.9. Take a pipette similar to that used for vitrification and fill it with DS. Take the COCs from the drop (TS) and move them to the bottom of the first well (DS). Wait for 3 minutes.

3.10. Wash the pipette with 1WS. Take the COCs and move them on the bottom of the second well (1WS). Wait for 5 minutes.

3.11. Wash the pipette with 2WS. Take the COCs and move them on the surface of the third well (2WS). Wait for them to touch the bottom of the well.

3.12. Repeat step 3.11 using another area of the well.

3.13. Wash the pipette with culture medium and move the oocytes to the culture dish for the following use (e.g., IVM).

REPRESENTATIVE RESULTS:

Following cat oocyte vitrification and warming according to the present protocol (**Figure 1** and **Supplemental Figure S1**), the vast majority of gametes survive. After vitrification, among other techniques, viability can be evaluated at the optical microscope as morphological integrity[22] or with the use of vital stains. One of the latter is fluorescein diacetate/propidium iodide (FDA/PI), which allows the identification of viable (bright green fluorescence) and dead cells (red fluorescence). **Figure 2** shows a representative picture of vitrified-warmed cat COCs stained with FDA/PI right after warming. Data from the experiments showing the percentage of survival after vitrification are reported in **Table 1**, which depicts post-warming data of oocytes intended for in vitro maturation[17] or in vitro embryo production[16]. On the whole, in the two experiments used as examples here[16,17], 395 out of 435 oocytes survived, scoring an overall 90.8% post-warming viability.

However, some morphological changes can be noticed after warming (**Figure 3**). In gametes vitrified-warmed following this protocol, the most frequent morphological abnormalities are changes in ooplasm shape and granulation, partial (or, rarely, total) loss of cumulus cells and (rarely) zona pellucida fractures. On the other hand, as reported in our previous works on minimum volume vitrification with the same support, these vitrified COCs can mature[17] and develop into embryos in vitro[16], even if at lower rates than fresh COCs. In addition, they do not usually present zona pellucida hardening (which is another well-known consequence of cryopreservation), since in vitro fertilization (IVF) is successful[16].

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic depiction of oocyte vitrification-warming protocol. Please refer to the manuscript text for complete indications. COCs= cumulus-oocyte complexes; ES=equilibration solution; VS=vitrification solution; TS=thawing solution; DS=dilution solution; WS=washing

solution; LN_2=liquid nitrogen.

Figure 2. Viability of vitrified cat oocytes after warming. Vitrified cumulus-oocyte complexes stained with fluorescein diacetate/propidium iodide (FDA/PI) show green fluorescence when viable (**A**) or red or weak fluorescence when dead (**B**). Excitation/emission wavelengths: 495 nm/517 nm for FDA; 538 nm/617 nm for PI. Scale bar: 50 µm.

Figure 3. Light micrographs of fresh and vitrified cat oocytes. (**A**) Fresh cumulus-oocyte complexes after collection, before vitrification. (**B**) Vitrified cumulus-oocyte complexes after warming, showing some vitrification-induced injuries (changes in shape and loss of cumulus cells, black arrow). Scale bar: 50 µm.

Table 1. Representative results of viability in cat vitrified-warmed oocytes. Data from †Colombo et al. 2019[16] and ‡ Colombo et al. 2020[17].

Supplemental Figure 1. Representative picture of the supports used for oocyte vitrification. The commercial support measures 13 cm and is easy to handle and store. Oocytes have to be loaded on the thin plastic strip at the top of the device, as close as possible to the black mark near the tip. Copyright: Kitazato Corporation.

DISCUSSION:

Oocyte cryopreservation is a crucial germplasm conservation technique, especially in taxa where many species are endangered, such as Felidae family. In this manuscript, a simple and field-friendly protocol for the vitrification of immature cat oocytes was presented. Laboratory-made media, minimum volume vitrification supports and trained personnel are the key factors for the success of this method, which allows obtaining viable oocytes consistently and repeatedly, as shown by the representative results hereby reported.

Beginning with media preparation, the protocol should be followed accurately to ensure the best results, but the operator's skills are the major issue. Media should be prepared the same day of the vitrification procedure, or, if that is not possible, they should be prepared the day before and stored at 4 °C. In any case, before sample processing, enough time should be left to allow the solutions to warm up to RT. Oocyte collection and COCs selection are quick and simple procedures that are routinely performed by every ARTs laboratory. However, cryopreservation is the most delicate procedure. While the warming procedure is not so critical, if the protocol and the timings are followed, vitrification might be more complex. After equilibration, in which care must be taken just in respecting timings, the most challenging phase is likely to be the vitrification step (see 2.4. of the present protocol). Indeed, oocyte transfers and pipette washings need to be well-timed and, all together, performed in less than 90 seconds. For beginners, it is advisable to time the procedure with a stopwatch while training, while also for trained individuals it might be useful to have a timer beeping after 60 seconds as an alert that time is running up. In addition, this would allow a higher standardization, since operators vitrifying groups of 6-8 COCs should be close to the transfer of the oocytes from the second to the third well of the Repro plate (see step 2.4.7 of the present protocol) when the

alarm goes off. Hopefully, with the aid of this article, a higher standardization between individuals and between laboratories could be achieved.

The major limitations of the protocol remain the intrinsic features of cryopreservation procedures themselves. As previously noted, vitrification exposes oocytes to non-physiological and potentially damaging conditions, even if it is performed flawlessly. The incubation with cryoprotectants and the decrease of temperature are the most critical events[20] and they need to be kept under strict control. Among the most common cryoinjuries, some are easily observable at the optical microscope (e.g., cumulus cell loss, alteration of cellular shape and size), while others are not visible and often affect subcellular structures (e.g., zona pellucida hardening, oxidative stress, cytoskeleton damages, meiotic spindle and DNA alterations)[23,24]. Although mostly viable, COCs vitrified following this protocol often present some evident morphological anomalies after warming. However, there is no correlation between morphology and viability, which might also be hampered because of subcellular damages[3,25], but morphological alterations are likely a consequence of cryoinjuries and partially a reason for the in vitro developmental outcomes of vitrified oocytes, which are quite poor if compared with that of fresh oocytes. The careful preparation of vitrification media and the precise observation of protocol timing contribute to obtain good post-warming viability and morphological integrity, but improvements in further in vitro maturation of oocytes and embryo development are still needed, since viability often decreases during the following culture[16].

Cold-induced injuries unfortunately occur with every cryopreservation method[24]. The minimum volume vitrification support used in this protocol has been developed to reduce the vitrification volume as much as possible, resulting in better cooling and warming rates compared to other cryopreservation devices (-23,000 °C/minute and 42,000 °C/minute respectively, according to the manufacturer specifications). As a consequence, in human medicine, where clinical studies assessing both in vitro (i.e., fertilization and embryo development) and in vivo parameters (i.e., pregnancy rates and live births) are available, minimum volume vitrification is the most common choice for oocyte cryopreservation due to its incomparable results in terms of post-warming survival and, finally, live births[12] from vitrified mature human oocytes. In addition, compared to other vitrification devices, the one used in this protocol is easier to use and safer to store[12], since it is comfortable to handle and is protected by a cap during storage. For instance, the Cryoloop is also an efficient support in order to reach the minimal volume goal, but its structure (a loop supporting a film of solution on which the oocytes are loaded) is fragile and prone to accidental warming[12]. Straws (0.25 mL volume) or open pulled straws (OPS) can also be used, but they do not allow a significant reduction in the vitrification volume and are also challenging to fill and empty, as some oocytes might be lost[26]. Many other supports have been developed[2], but each of them has its drawbacks. Instead, compared to slow freezing, which previously was the most used cryopreservation technique, the main advantages of minimum volume vitrification on commercial supports are its feasibility and speed. As previously mentioned, vitrification does not require a programmable freezer to be performed, and while slow freezing of oocytes takes about one hour and half to be concluded[25], vitrification can be accomplished in about 17 minutes following the present protocol.

In conclusion, staff skills and inter-operator standardization might be the most challenging requirements when starting a vitrified oocyte bank, but they will be achieved with personnel training. This minimum volume vitrification protocol for cat immature oocytes gives consistent and repeatable results when performed by experienced operators. However, there are still chances for improvement in oocyte developmental rates, even if post-warming viability and integrity are satisfactory. With further optimization, the present protocol could also be applied in field conditions for wild felids, for which published data concerning oocyte cryopreservation is still scarce[27]. Widening the application of this method not only would increase the possibility to improve cryopreservation protocols, but would also allow establishing vitrified oocyte biobanks for fertility and biodiversity preservation in felids.

ACKNOWLEDGMENTS:

This work was partly supported by EVSSAR (European Veterinary Society for Small Animal Reproduction) Grant 2016 and by Università degli Studi di Milano, Piano di Sostegno alla Ricerca 2019 (Linea 2 Azione A). We also wish to thank Dr. MariaGiorgia Morselli for her contribution to the experiments hereby depicted and to image acquisition.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Bankowski, B., Lyerly, A., Faden, R., Wallach, E. The social implications of embryo cryopreservation. *Fertility and Sterility*. **84** (4), 823–832 (2005).
2. Saragusty, J., Arav, A. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction*. **141** (1), 1–19 (2011).
3. Luvoni, G.C. Gamete cryopreservation in the domestic cat. *Theriogenology*. **66** (1), 101–111 (2006).
4. Thongphakdee, A., Tipkantha, W., Punkong, C., Chatdarong, K. Monitoring and controlling ovarian activity in wild felids. *Theriogenology*. **109**, 14–21 (2018).
5. Parks, J.E. Hypothermia and mammalian gametes. *Reproductive Tissue Banking*. 229–261 (1997).
6. Carabatsos, M.J., Sellitto, C., Goodenough, D.A., Albertini, D.F. Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Developmental Biology*. **226** (2), 167–179 (2000).
7. Murakami, M. et al. Blastocysts derived from in vitro-fertilized cat oocytes after vitrification and dilution with sucrose. *Cryobiology*. **48** (3), 341–348 (2004).
8. Merlo, B., Iacono, E., Regazzini, M., Zambelli, D. Cat blastocysts produced in vitro from oocytes vitrified using the cryoloop technique and cryopreserved electroejaculated semen. *Theriogenology*. **70** (1), 126–130 (2008).
9. Luciano, A.M. et al. Effect of different cryopreservation protocols on cytoskeleton and gap junction mediated communication integrity in feline germinal vesicle stage oocytes. *Cryobiology*. **59** (1), 90–95 (2009).
10. Comizzoli, P., Wildt, D.E., Pukazhenthi, B.S. In vitro compaction of germinal vesicle chromatin is beneficial to survival of vitrified cat oocytes. *Reproduction in Domestic*

Animals. **44** (SUPPL. 2), 269–274 (2009).
11. Alves, A.E., Kozel, A.C., Luvoni, G.C. Vitrification with DAP 213 and Cryotop of ex situ and in situ feline cumulus-oocyte complexes. *Reproduction in Domestic Animals*. **47** (6), 1003–1008 (2012).
12. Kuwayama, M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method. *Theriogenology*. **67** (1), 73–802007).
13. Pope, C.E. et al. In vivo survival of domestic cat oocytes after vitrification, intracytoplasmic sperm injection and embryo transfer. *Theriogenology*. **77** (3), 531–538 (2012).
14. Galiguis, J., Gómez, M.C., Leibo, S.P., Pope, C.E. Birth of a domestic cat kitten produced by vitrification of lipid polarized in vitro matured oocytes. *Cryobiology*. **68** (3), 459–466 (2014).
15. Fernandez-Gonzalez, L., Jewgenow, K. Cryopreservation of feline oocytes by vitrification using commercial kits and slush nitrogen technique. *Reproduction in Domestic Animals*. **52**, 230–234 (2017).
16. Colombo, M., Morselli, M.G., Tavares, M.R., Apparicio, M., Luvoni, G.C. Developmental competence of domestic cat vitrified oocytes in 3D enriched culture conditions. *Animals*. **9** (6), 329 (2019).
17. Colombo, M., Morselli, M.G., Apparicio, M., Luvoni, G.C. Granulosa cells in three-dimensional culture: A follicle-like structure for domestic cat vitrified oocytes. *Reproduction in Domestic Animals*. (2020).
18. Wood, T.C., Wildt, D.E. Effect of the quality of the cumulus-oocyte complex in the domestic cat on the ability of oocytes to mature, fertilize and develop into blastocysts in vitro. *Journal of Reproduction and Fertility*. **110** (2), 355–360 (1997).
19. Cobo, A., Kuwayama, M., Pérez, S., Ruiz, A., Pellicer, A., Remohí, J. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. *Fertility and Sterility*. **89** (6), 1657–1664 (2008).
20. Mullen, S.F., Critser, J.K. The science of cryobiology. *Oncofertility - Fertility preservation for Cancer Survivors*. 83–109 (2007).
21. Fahy, G.M., Wowk, B. Principles of cryopreservation by vitrification. *Methods in Molecular Biology*. **1257**, 21–82 (2015).
22. Apparicio, M., Ruggeri, E., Luvoni, G.C. Vitrification of immature feline oocytes with a commercial kit for bovine embryo vitrification. *Reproduction in Domestic Animals*. **48** (2), 240–244 (2013).
23. Brambillasca, F., Guglielmo, M.C., Coticchio, G., Mignini Renzini, M., Dal Canto, M., Fadini, R. The current challenges to efficient immature oocyte cryopreservation. *Journal of Assisted Reproduction and Genetics*. **30** (12), 1531–1539 (2013).
24. Moussa, M., Shu, J., Zhang, X., Zeng, F. Cryopreservation of mammalian oocytes and embryos: current problems and future perspectives. *Science China Life Sciences*. **57** (9), 903–914 (2014).
25. Luvoni, G., Pellizzari, P., Battocchio, M. Effects of slow and ultrarapid freezing on morphology and resumption of meiosis in immature cat oocytes. *Journal of Reproduction and Fertility. Supplement*. **51**, 93–98 (1997).
26. Calvo, J.J., Robert, D., Viqueira, M., Lombide, P., Calvo, J.J. *Vitrified and* in vitro *matured*

485 *oocytes viability from adult housecat (Felis catus) in reproductive season*. *International*
486 *Journal of Morphology*. **33** (4) (2015).
487 27. Nowak, A. et al. The viability of Serval (Leptailurus serval) and Pallas Cat (Felis manul)
488 oocytes after cryopreservation using the Rapid-I Method. *Cryo Letters*. **40** (4), 226–230
489 (2019).

Figure 1

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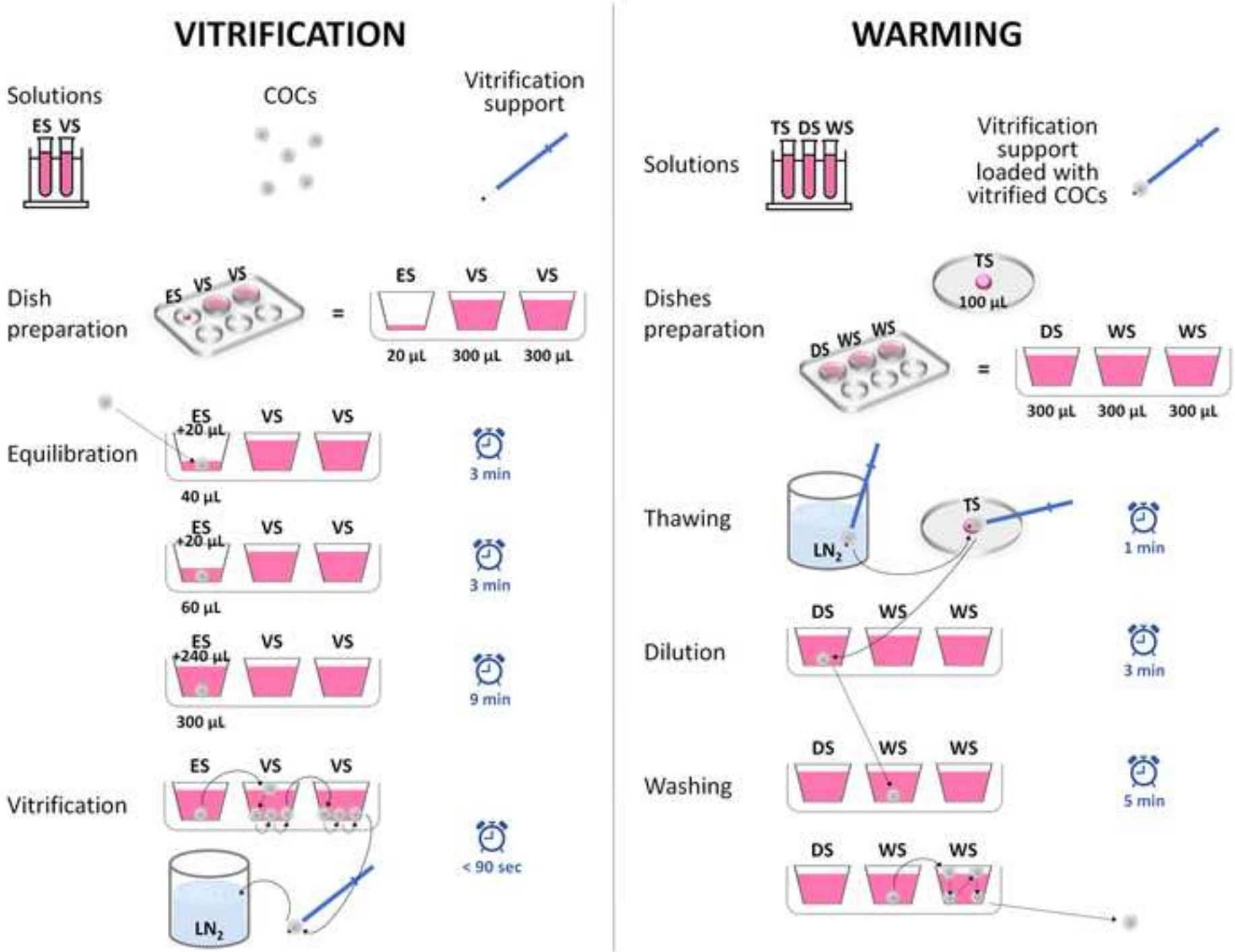

Figure 2

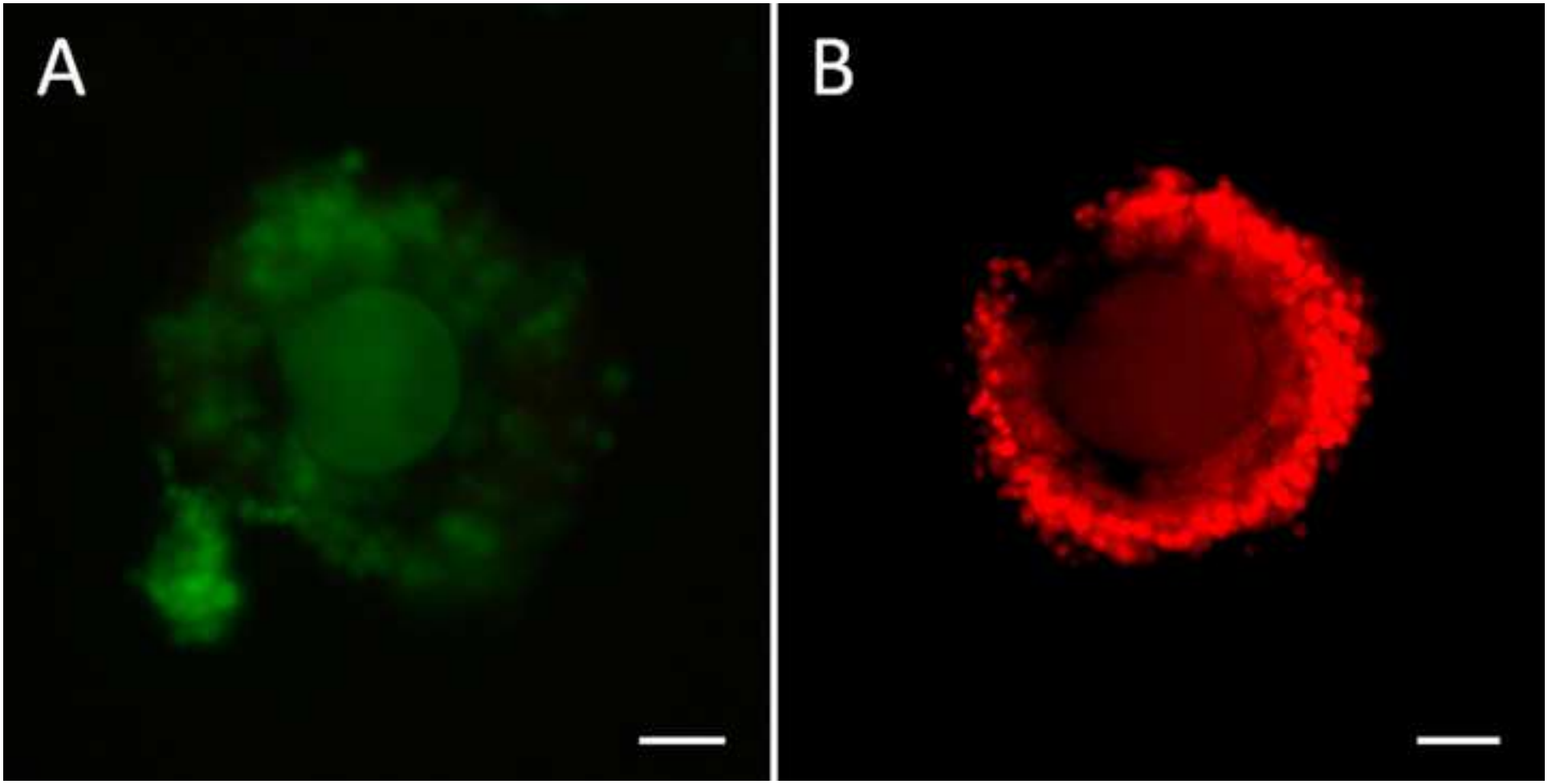

Figure 3

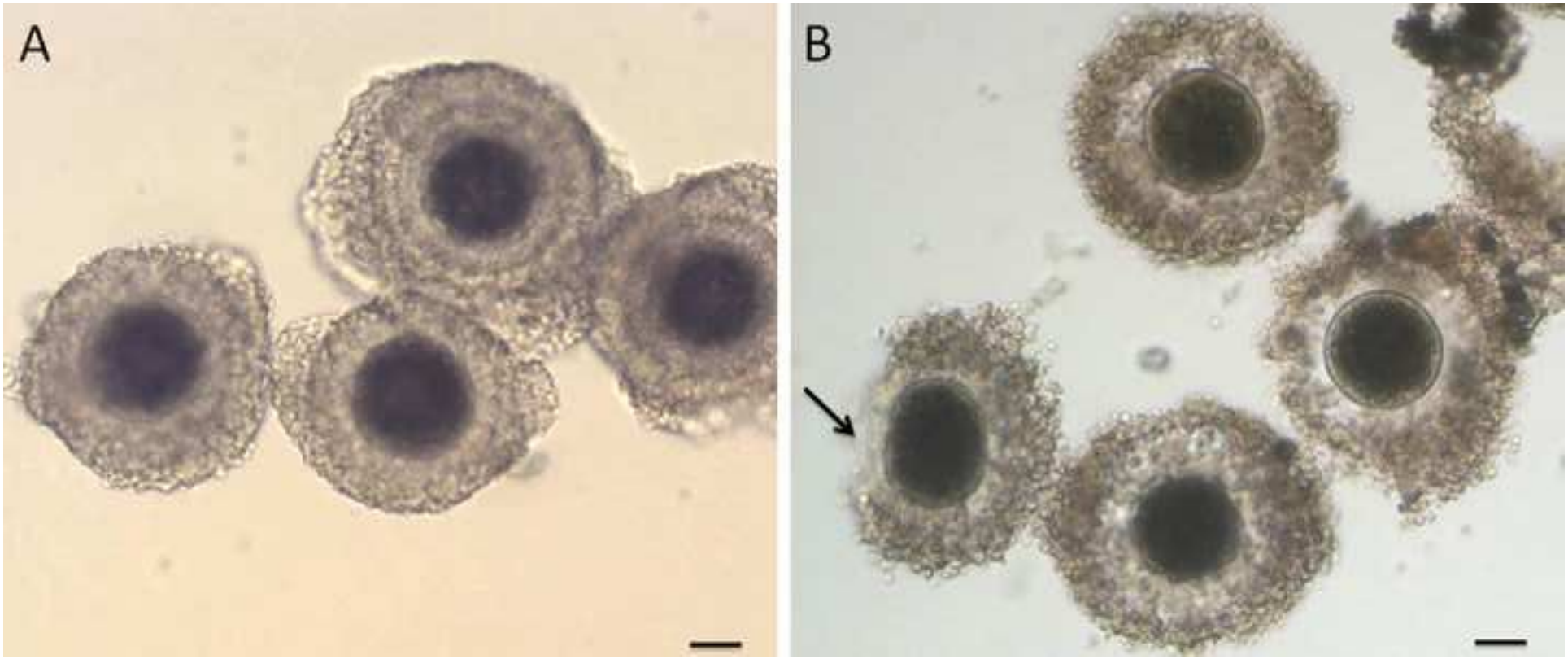

Table 1

Experiment	Group	Warmed oocytes (n)	Viable oocytes (n)	Viability (%)	Viability (mean % ± SD)
1†	1	13	12	92.31	91.54 ± 3.66
	2	47	44	93.62	
	3	52	45	86.54	
	4	26	24	92.31	
	5	41	40	97.56	
	6	21	19	90.48	
	7	50	44	88.00	
2‡	1	17	17	100.00	91.51 ± 9.16
	2	17	17	100.00	
	3	9	8	88.89	
	4	21	21	100.00	
	5	30	23	76.67	
	6	26	23	88.46	
	7	17	13	76.47	
	8	10	10	100.00	
	9	15	14	93.33	
	10	23	21	91.30	

Name of Material/Equipment	Company
Amphotericin B	Sigma-Aldrich
Automatic pipettes & tips	/
Surgical scalpels	/
Bunsen beak	/
Clamps	/
Cryotop	Kitazato (distributor: MBT - Medical Biological Technologies)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Ethylene glycol (EG)	Sigma-Aldrich
Fetal bovine serum (FBS)	Sigma-Aldrich
Glass Pasteur pipettes	/
Gobelets	/
Heating stage	/
Liquid nitrogen	/
Medium 199	Sigma-Aldrich
Phosphate-buffered saline (PBS)	Sigma-Aldrich
Penicillin G sodium	Sigma-Aldrich
Polyvinyl alcohol (PVA)	Sigma-Aldrich
Repro plate	Kitazato (distributor: MBT - Medical Biological Technologies)
Stereomicroscope	/
Storage tank	/
Streptomycin sulphate	Sigma-Aldrich
Styrofoam/nitrogen resistant box	/
Sucrose	Sigma-Aldrich
Timers	/

Catalog Number	Comments/Description
A2942	/
/	Needed to pipette 20, 100, 240 and 300 µL
/	Size 10 is usually ok
/	/
/	Some small (Mosquito clamp) for ovary isolation, some bigger (Klemmer clamp) to work in liquid nitrogen
01.CR	Distributors and catalog number may change in different countries
D2650	/
E9129	/
F9665	/
/	Advised lenght 230 mm (100+130)
/	According to the canisters of the storage tank
/	All heating stages are ok, as long as they can keep 38ºC
/	/
M4530	/
D8662	/
P3032	/
P8136	/
01.K-2	Distributors and catalog number may change in different countries
/	As long as the operator can select the oocytes, other stereomicroscopes are ok
/	Any regularly filled tank is ok
S9137	/
/	All boxes which can contain liquid nitrogen are ok, as long as the operator is comfortable. Kitazato box is called "Cooling Rack"
S1888	/
/	All timers which can be set on times until 9 minutes are ok

Manuscript JoVE61523 – Revision 1

The manuscript has been revised according to the Editor's and Reviewers' suggestions.
We thank for the constructive criticisms which contribute to improve the quality of the work.

Lines indicated in the Authors' replies are referred to the HIGHLIGHTED version of the manuscript.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Authors: The manuscript has been proofread.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
Authors: Formatting has been checked.

3. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please use generic term for "Cryotop" both in the title and throughout the text. The term can be introduced once in the introduction.
Authors: The term "Cryotop" has been deleted or replaced with "Minimum volume (vitrification)" / "Commercial support" / "Vitrification device" or similar expressions in the title and throughout the text (Lines 2, 21-22, 36-37, 101, 107, 169, 170, 187, 209, 216, 244, 247, 269, 294, 297, 306, 326, 327, 411, 455-456, 461, 464, 473, 475, 485) and changes to the text have been applied accordingly. "Cryotop" was left once as explanation in the introduction and once in the abstract to facilitate paper retrieval through bibliographic databases (e.g. PubMed, CABI, WoS…).

4. Please remove the commercial term from the title and from the list of keywords.
Authors: The term has been removed.

5. Please expand all abbreviations during the first time use.
Authors: One abbreviation explanation (PVA) was added (Line 122).

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Cryotop, Cryotop Repro plate, etc.
Authors: Changes have been applied in the manuscript text as described in the third Editorial comment. In the Table of Materials and Reagents, lines of Cryotop and Repro Plate have been slightly modified to further clarify materials availability.

7. Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol.
Authors: Done.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone

how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
Authors: Necessary changes have been applied (Lines 117, 120, 156, 159, 170, 215, 280, 282, 284, 297, 327).

9. The Protocol should contain only action items that direct the reader to do something.
Authors: Checked.

10. Please ensure you answer the “how” question, i.e., how is the step performed?
Authors: Done.

11. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
Authors: Necessary changes have been applied (Lines 156-162, 280-286).

12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Authors: Following some modifications of the protocol (Editorial comment 11), the highlighting of some of the text has been deleted (media preparation, Lines 156-162, 280-289).

13. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
Authors: Done.

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Authors: Figures 1, 2 and 3 in the present manuscript have never been published elsewhere, thus copyright permission is not necessary. For Supplemental Figure 1 (S1), which is copyrighted by Kitazato Corporation, a permission has been obtained by the owner. The permission has been uploaded in the Editorial Manager.

15. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Authors: Done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The paper gives a very detailed description on oocyte vitrification in cats. It is well written and clearly structured

Major Concerns:

none

Minor Concerns:

none

Authors: We thank the Reviewer for her/his contribution.

Reviewer #2:

The manuscript is extremely interesting for the conservation area of animal germplasm. In addition, the Felidea family needs studies in this area, especially to be a model for endangered wild species. However, before publication, some questions need to be revised for a better quality of the manuscript.

1. Abstract: What do the authors want to tell us about vitrification being the most popular method of cryopreservation? It is known that vitrification is a promising methodology, but there are still many works with slow freezing. Therefore, specify the term "the most popular" better.

Authors: We thank the Reviewer for her/his contribution. To give the idea of an increasing use of vitrification, even if still flanked by slow freezing, “the most popular” has been changed with “more and more popular” (Line 30).

2. Protocol: Why does manipulation occur at 25°C and not at 37°C?

Authors: Higher temperatures increase the cytotoxicity of cryoprotectants and reducing the temperature of exposure to high concentrations of cryoprotectants is a strategy to decrease/control the toxicity of the vitrification solutions (Mullen & Critser, 2007), therefore vitrification procedures are usually carried out at room temperature, and so is oocyte selection, to avoid temperature fluctuations. A note has been added at Lines 164-167, as well as the appropriate reference (which was already in the reference list). The note at Lines 250-264 has been modified accordingly.

3. Protocol: It would be interesting figures of the devices and a figurative scheme of the whole process.

Authors: A picture of the vitrification support has been added (Supplemental Figure 1 – S1) and referenced in the text at Lines 96 and 346. A scheme of the vitrification-warming process has been added (Figure 1) and referenced in the text at Line 345. The legends of the new figures (1 and S1) were added at Lines 367-387 and 402-405. Numbering of the other figures has been changed accordingly.

4. What other techniques of oocyte quality after cryopreservation could be employed?

Authors: Among other techniques to evaluate oocyte quality, the first assessment is usually viability. In addition to morphological evaluation and the fluorescent viability staining (e.g. FDA/PI) we chose in the manuscript, other stainings (fluorescent or not) might be employed (e.g. calcein or methylthiazolyldiphenyl-tetrazolium bromide – MTT), or viability might be indirectly evaluated assessing mitochondrial activity or activation of apoptotic pathways. Moreover, in vitro maturation, fertilization and culture might be another way to assess oocyte quality, yet other variables play a role in this. Morphology and FDA/PI are the only techniques mentioned in the manuscript because data or figures presented were based on these. We feel that the addition of information about other techniques might be redundant, since it is not included in the

aim of the manuscript, which focuses on the vitrification protocol itself. To better specify that morphology and FDA/PI are not the only techniques available, the sentence at Line 346 has been modified with the addition of “among other techniques”.

5. Data were compared with which experimental group? Control (not cryopreserved)? Control with other cryopreservation techniques? I believe that comparisons of this nature would be extremely valid in the present study.
Authors: Cryotop oocyte vitrification gives >90% post-warming viability rates in other species, and the data presented in the manuscript were collected in our lab as internal control of our vitrification efficiency. Our aim was not to compare with other techniques but to present specifically Cryotop vitrification.

Reviewer #3:
Major Concerns:
Protocol
- indicate which temperature should be used for vitrification as temperature markedly influences on CPA permeability/toxicity. Would it be needed to work on warm-stage (37 C) or can be done with room temp as indicated in the MS. This has been indicated only for warming process.
Authors: We thank the Reviewer for her/his contribution. The information has been added in a note at Lines 164-167.

- 2.4. Vitrify COCs in VS in 60-90 seconds: too large time variation for vitrification. This is very important step, and such high concentration of CPA in vitrification solution can be very toxic. Exact time (or with narrowing time window) is necessary for successful vitrification.
Authors: The timing we wrote in the manuscript was inspired by the Cryotop-Kitazato original protocol, where they advise a time of max 30 seconds for each of the two vitrification wells. Oocyte loading on the Cryotop strip is not included in these 60 seconds, thus our protocol says 60-90 seconds for the whole vitrification phase (until immersion in liquid nitrogen), but we agree with the Reviewer this time variation might be too large. In our lab practice, the time slightly changes according to the number of oocytes vitrified at the same time, but as mentioned in the discussion (Lines 424-434), we usually are at around 1 minute when the oocytes are moved to the third well of the Repro Plate, yet completing the vitrification step in less than 90 seconds. Therefore, Line 218 has been modified with “in less than 90 seconds”.

Result
- The data (result/outcome/efficiency) of vitrified/warmed immature oocytes showing in this manuscript is very limited. Although authors show high post-warming viability (90.8%), these viability was assessed immediately after warming. It would be better to demonstrate the ability of vitrified/warmed oocytes to be matured/fertilized. In addition, it is better to compare success rates of vitrification of feline immature oocytes using different vitrification techniques (compare with other publications) in terms of maturation, fertilization and blastocyst formation rates. This should be addressed in results/discussion
Authors: We agree with the Reviewer, but we chose to show only post-warming viability for several reasons. For instance, this is the data usually showed in other species, such as humans, for which the vitrification support is marketed. In addition, maturation and embryo development rates of “our” vitrified oocytes are showed elsewhere. Now these data are more clearly referenced in the manuscript (Lines 352-353). Maturation, fertilization and embryo development rates also depend on the media employed for these steps, and this is another reason why we would prefer not to add these data to the present manuscript, which focuses on the vitrification procedure itself. For the same reason, the comparison with other vitrification techniques in other publications is tricky, even if some is made in the discussion just concerning the use of different vitrification devices (Lines 463-471).

- What are experiments 1 and 2 in table 1? Please indicate in manuscript
Authors: Experiments 1 and 2 are referenced in the table legend, but now this issue was addressed also in the manuscript text at Lines 352-353.

- Fig 1 : A vs B Is oocyte A is the same oocyte as oocyte B but with different wavelength? Please indicate excitation/emission for FDA/PI
Authors: This figure (now Figure 2), represents two different oocytes. “Oocytes” (plural) is reported in the figure legend, and excitation/emission for FDA/PI have been also added in the legend at Lines 391-392.

Minor Concerns:
Line 58 consider "routine ovariectomy"
Authors: The suggested change has been applied (Line 62).

Line 104-105 consider "routine ovariectomy or ovariohysterectomy"
Authors: The suggested change has been applied (Line 112).

Line 109 consider "prepare solutions for ovary and oocyte collection"
Authors: The suggested change has been applied (Line 117).

Line 139 15% (v/v) DMSO
Authors: The suggested change has been applied and v/v has been added at Lines 156 and 159.

Reviewer #4:
This is a really interesting manuscript that describes the vitrification of feline oocytes by using the cryotop method, which is largely recommended for publication after only minor revision. At general, it is well written. Introduction clearly justifies the use of the protocol, which is clearly detailed, step by step. This will certainly help the reader to conduct it, but some images or even a video will certainly be welcome to ilustrate it. Discussion is well written and highlights the advantages of using such protocol.

Manuscript Summary:
Both summary and abstract are adequate and clearly reports the main contents of the manuscript.

Minor Concerns:
I only have few statements. For the protocol description, authors should provide ilustrations or organograms showing the steps, and also a figure of the cryotop device used for vitrification in this protocol. Moreover, at the representative results section, authors report that vast majority of oocytes will survive to the protocol. At my point of view, they should provide some numeric results in percentage to ilustrate the protocol efficiency at vitrifying feline oocytes, mainly because such average values are presented at the Table 1. Moreover, authors should provide a better image to ilustrate the damaged oocyte, since the labeling at figure 1B is really clear and difficult to be visualized after IP impregnation.

Authors: We thank the Reviewer for her/his contribution.
A figure to illustrate the protocol has been added (Figure 1), and a video will be produced by JoVE upon manuscript acceptance. A picture of the cryotop device has also been added (Supplemental Figure 1 – S1). New figure legends have been written (Lines 367-387 and 402-405), and the numbering of the old figures has been changed accordingly.

In the results section of the text, a general sentence is used to begin the first paragraph, but then numerical data are reported at the end of the same paragraph (Lines 353-355). All numeric results of oocyte viability are provided both as percentage and as mean % ± SD in Table 1, together with the absolute numbers, from which readers will be able to calculate all the percentages they wish.
We agree with the Reviewer that Figure 1B (now 2B) was not bright enough for publication, so it has been changed with a brighter one.

Reviewer #5:
Manuscript Summary:
The methods article by M. Colombo and G.S. Luvoni describes the protocol of immature domestic cat oocyte collection, vitrification, and thawing. This scientific group is among the recognized leaders in the field of feline oocytes cryopreservation. Therefore, demonstration and dissemination of their practices, especially in methods that require special skills such as vitrification, is a relevant task. As it is noted in the manuscript, the Felidae family consists of mainly endangered species and the domestic cat is an important intermediate step for cryopreservation of more rare species. Moreover, cat oocytes are used as a model for the investigation of lipid-related cryopreservation effects.
The article is written in a detail and concise manner. All the important steps and materials are presented. I find the current version of the manuscript acceptable, however, few minor improvements can be recommended:
Authors: We thank the Reviewer for her/his contribution.

Line 116: it is obvious that at 4°C organs can not be stored eternally, thus some recommendations for storage time should be given.
Authors: The sentence was not clear, we meant the storage of the solution for ovaries. So, it has been rephrased as follows: “Store solutions for ovary and oocyte collection at 4ºC until use.” (Line 124).

Line 128: a recommended microscope magnification might be useful
Authors: Advised magnification of the stereomicroscope is 8X and it was added at Line 136.

I suppose that manipulations with Repro plate will be in the video part, however, it will not be superfluous to add a static scheme of Repro plate with oocyte movement and transfer between wells. This will help the readers unfamiliar with Kitazato Cryotop Kit.
Authors: A figure depicting the protocol (Figure 1) and its legend (Lines 367-387) have been added. A video will be produced by JoVE upon manuscript acceptance.

Lines 145: Step 2.2.1 seems to contain a misprint: BS should be instead of ES.
Authors: There are three commercial media marketed by Kitazato for oocyte vitrification, and one of them is a solution called BS. However, in our protocol we just prepare 2 solutions (ES and VS), and we use ES also in the step where they use BS in the commercial kit. To avoid confusion in the readers, we would like to avoid mentioning BS.

Lines 269-277: Authors noted vital staining and bright-field microscopy observation for evaluation of survival rate after cryopreservation. Since many oocytes do not survive further culture, it is important to specify the time passed between thawing and vital staining.
Authors: We agree, the oocytes were stained right after warming. We have added the information at Lines 350, 351, 352.

If some additional indicators of the effectiveness of the described protocol were available, it would be useful to those readers who want to use this protocol and want to check themselves. For example, I suggest adding some numbers describing the in-vitro maturation capability of thawed oocytes.
Authors: We agree with the Reviewer, but we chose to show only post-warming viability in the present manuscript because maturation (and embryo development) rates also depend on maturation medium, while this manuscript focuses on vitrification itself, and because these data are already published. We hope we have clarified this and made the data easier to find in the previous papers with the changes we have made at Lines 352-353.

Supplemental Figure 1 (S1)

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S1 R1.psd

Martina Colombo graduated in Veterinary Biotechnology Sciences (MSc) at the University of Milan. Currently, she is a PhD student in Veterinary and Animal Science at the same University, and her research concerns reproductive biotechnologies in animal models. Specific scientific skills include in vitro cell culture in two and three-dimensional systems, in vitro embryo production and gamete cryopreservation.

Gaia Cecilia Luvoni is a Full Professor in Veterinary Obstetrics and Gynaecology and Head of the Department of Health, Animal Science and Food Safety at the University of Milan.
ECAR diplomat (European College of Animal Reproduction), her scientific work concerns Theriogenology and Biotechnology of Reproduction in Carnivores: assisted reproduction, cryopreservation of gametes and gonadal tissue, in vitro embryo production and ultrasonographic foetal biometry. Author of several papers in international journals and invited speaker at international meetings.

To Journal of Visualized Experiment (JoVE) Editors

We, the undersigned Kitazato Corporation, address: 1-1-8 Shibadaimon Minato-ku, Tokyo, 105-0012 JAPAN, e-mail: trading@kitazato.co.jp

Hereby give explicit consent

To Ms Martina Colombo and Prof. Gaia Cecilia Luvoni, Università degli Studi di Milano, address: via Celoria, 10, 20133 Milan, Italy, e-mail: martina.colombo@unimi.it

For

The use of copyrighted images depicting Cryotop® Vitrification Device (Open System) on the webpage https://www.kitazato.co.jp/en/products/vitrification/cryotop

And for

the publication of the aforementioned images in their manuscript on feline oocyte vitrification, submitted to the Journal of Visualized Experiments with the ID JoVE61523.

Thank you.
Yours sincerely,

Place and Date

1-1-8 Shibadaimon Minato-ku, Tokyo, 105-0012 JAPAN
2020/05/18

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Author(s):	Martina Colombo, Gaia Cecilia Luvoni

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1st April 2020

On behalf of all the authors