SARAH MADANI | ANABELLA MARCONETTO | MARIANA DE NADAI

WEN BIN CHEN | ZOLTÁN MACHÁTY | GÁBOR VAJTA

About Oocytes and Embryos

Volume 2. Methods

2022 - 2023

PAGE DESIGNS, GRAPHICS: NARA YU, CHINA

FRONT PAGE ILLUSTRATION: ÁGNES MARCZALI, HUNGARY

CONTENT

FOREWORD TO VOLUME 2

(if boring, skip it)

Two issues motivated us to continue our joint efforts.

We received many positive feedbacks and yet had a kind of remorse: we criticised everything and everybody but gave few real answers to the "how to" type questions. Behind every nice comment, we suspected a headshake: "well, wiseguys, what about giving us something really valuable, not just small primitive tricks but methods, protocols, ways to follow... will you?"

The other reason was independent of the first volume - a problem known by many but neglected by almost everybody until recently. The differences in the overall efficiency between clinics in the same country, same region, and similar patient population. Differences that cannot be explained by anything but inappropriate interventions. No other area in human medicine could afford, no authorities would tolerate four times lower success rates in one ward compared to the other clinics - and these staggering data were officially published in countries of the great IVF pioneers, with world-renowned traditions and strict regulations. What about the rest? Random personal experiences and passing statements suggest the same - if not worse - situation in many countries throughout the globe.

We need a solution here and now.

Our small international team cannot fight certain issues, including bad features of commercialisation (competition, secrecy), lack of transparency or lack of legal consequences of obvious inefficiency. We cannot change the education and accreditation systems, either - although these factors contribute substantially to the regrettable situation.

We can use, however, the alternative strategy, the one we suggested in Volume 1 for a scientific problem: i.e. to find - and repair - the weakest link of the chain.

The "chain" analogue is more plausible than it's supposed to be. Established ways of *technology transfer* in assisted reproduction are insufficient.

- Tools, solutions, and techniques are mostly introduced in the lab by *distributors* who have no time, no basic knowledge, and only very superficial information about the product.
- At *exhibitions*, the main focus is on coffee, meal, chatting with old friends, and collecting giveaways.
- Lectures may create interest but cannot provide technical details.
- *Workshops*, even "hands-on" ones, are usually crowded and short; in lucky cases, you can make some failed attempt to go through the technique once; repeated practicing is almost always out of the question.
- *Manuals* and brochures are either too short and miss essential information; or too long, and nobody will read them thoroughly with the required focus.
- Teaching *videos* are usually superficial, with linearly arranged information; there is no way for feedback and no space for details.
- *Personal visits* to a prominent laboratory are the best way to learn, but it is the privilege of a few lucky scientists.
- Finally, *chain teaching* between individual scientists, the most frequent way of technology transfer, is also the main source of mishandling, as the technique erodes at each successive teaching demonstration.

With this Volume 2, we make a different attempt. We want to provide a streamlined, straightforward manual of complex procedures, including *bovine embryo production and vitrification* for commercial and research purposes, *laboratory issues in human assisted reproduction and somatic cell nuclear transfer in non-primate mammals*. These chapters may serve as a basic approach to achieving the given goal, with proven efficiency in different situations. Short explanations about the critical steps and choices will also be provided.

We know that our suggestions aren't the only ways to make great embryos and outcomes. But, they are simple, inexpensive, rapid, and more efficient than most methods commonly used. Some basic knowledge of laboratory embryology is indispensable

to their application. These techniques may be a good choice if you want to progress quickly by improving your inefficient and/or inconsistent systems.

However, avoid common mistakes. Firstly, follow strictly what is described here. Repeat the procedures for weeks, maybe for months. Practice is essential; all small steps should be done automatically, like changing gears in a manual car - or is it already an outdated analogue? Do not modify anything after the first two, three, or four fiascos; our instructions are correct, and our methods work well in different countries and on different continents. The rub may lie in the realisation. Better to turn to us immediately than to modify constantly. Finally, avoid elective construction of your special version with different parts collected from various protocols. Laboratory embryology is not a LEGO toy set but rather a puzzle, where all elements have their unique features and may not suit well to a different milieu.

Once you reach the appropriate efficiency, you will still have tons of opportunities to improve the system. But before that, just trust us, and follow us - or talk to us (see below).

All chapters of Volume 2 will be published online, with all the benefits and advantages of online publication. We agreed that:

- we will offer open access after free registration
- the goal is a dynamic and continuously improving guide
- all registered members can send ideas, suggestions and corrections
- all written contributions will be collected, arranged according to the subject and published
- selected ones will be incorporated into the manual (selection and incorporation will be the privilege of the authors, but opinions will always be considered)

Some additional technical and formal changes:

- in Volume 2, authors of the initial language versions of Volume 1 contribute equally to the creation of chapters and will be listed in all editions

The late Danish Institute of Agricultural Sciences, birthplace of many embryo technologies

- we welcome Wen Bin Chen, Head of VitaVitro's Experimental Laboratory, to the team, acknowledging his contribution, Chinese translation and extensive help in Volume 2
- for this volume, French translation will replace the former Arab version thanks to our most versatile author-translator, Sarah Madani
- to resolve the problem created by the extensive task versus urgent need, we will work in parallel on different parts and will publish them individually
- Linkedin, Facebook and Tweet will be used even more extensively than earlier to inform you about publications, updates and major changes

Thank you for your interest, visit, and your expected active contribution.

VitaVitro, Shenzhen, China, where new ideas are accomplished

WHAT CAN YOU EXPECT?

- from good quality ovaries, 6 to 8 developmentally competent oocytes
- around 50% blastocyst / collected oocyte (!) rates on Day 7 of embryo culture *in vitro*
- close to 100% survival and further development after cryopreservation
- good pregnancy and calving rates (mostly determined by the recipient stock and the transfer)
- no decrease in these parameters after cryopreservation and direct transfer
- no noticeable increase in developmental abnormalities or large offspring syndrome

CHAPTER I. PRODUCTION OF TRANSFERABLE STAGE BOVINE EMBRYOS *IN VITRO*

1 Collection of ovaries/oocytes, transport to the laboratory (Day -1)

The logistic arrangement depends on the local situation, the source of materials and the purpose of the work.

1.1 For abattoir-derived ovaries and oocyte

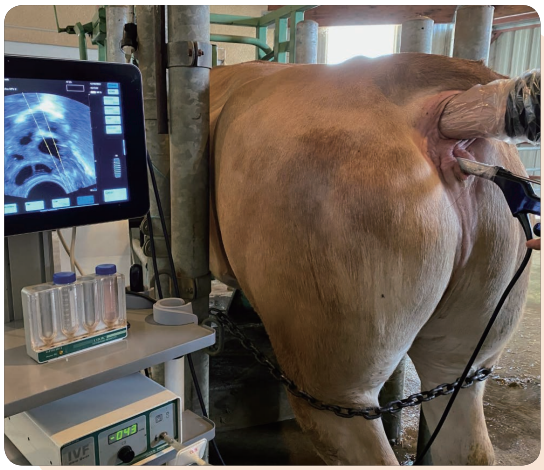
1.1.1 The lucky situation is to have a cattle abattoir willing to collaborate within 100 km, i.e. two hours driving distance that routinely slaughter 15 to 60 cows (less preferably heifers) per day routine slaughter. Please note that this collaboration is always a favour on the abattoir's side. Building a positive attitude is one of the most difficult tasks in cattle IVP requiring various techniques and creativity.

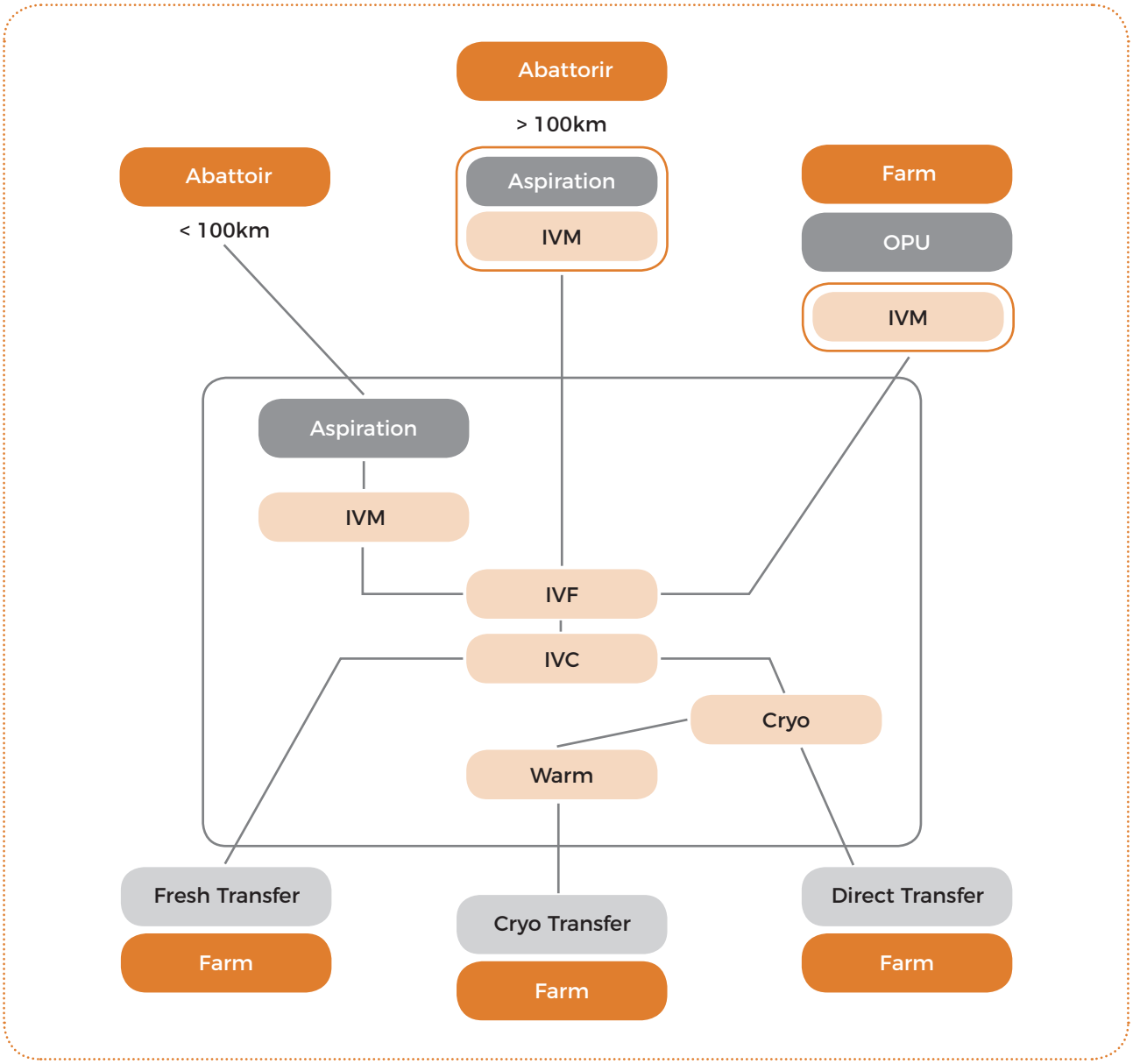
Ovaries may be collected by a skilled, reliable (and prized) local worker or by laboratory staff. In both cases, ovaries with a minimum amount of mesovarial fat tissue should be placed into a decontaminated plastic thermos flask half-filled with 25-30°C sterile physiological saline. These flasks are then safely placed in a simple cooler box (also packed with scissors, gloves, tissues, etc.) and transported by car to the lab - without any further temperature control unless extreme conditions during the transport occur. To avoid any irreversible damage to oocytes, the time from the slaughter to the start of oocyte aspiration should not exceed 5 - 6 hours, although live offspring were also produced from oocytes aspirated 24 hours after the slaughter[1].

1.1.2 The unfortunate alternative - a distant abattoir - makes things more complicated, much more expensive and, in the long term, hard to sustain. For these situations, you need to establish or hire a facility close to the abattoir: a clean room with very basic laboratory equipment required for both aspiration and oocyte recovery and used only for 1 to 2 hours a day. Also, a qualified staff, who spends the rest of the day with online work (rarely) or sightseeing with all related activities. You also need to resolve the problem of transportation. In the Golden Era of the nineties, we could go to the local train station in a remote rural area of Denmark and ask the locomotive engineer to pass the precisely heated transport box to our colleague at the platform in Copenhagen. All for free, for the sake of science. However, after 9/11, things became less relaxed, especially when air freight was involved. Although, in certain countries, professional services providing *in vitro* matured oocytes may also be available, this is not an option for most research labs with their usually tight budget. As this arrangement includes oocyte recovery in the laboratory and transportation in special incubators and tubes also used after ovum pickup (OPU), details will be discussed in the next chapter.

1.2 For ovum pickup (OPU)

The other option is the in vivo ultrasound-guided transvaginal ovum pickup. This is a mixture of the technique used in human IVF and the outcome of abattoir-related work, i.e. collection of immature oocytes without previous hormonal stimulation. The brunt of the work is done by the qualified vet; the embryologist's role is to provide pre-heated flushing media and collection tubes. Make sure the tubes are stored in heated holders, preferably kept also warm during aspiration in purpose-designed devices (as https://store.agtechinc.com/products/pocket-tube-heater-with-battery-wta). Subsequently, the oocyte recovery work is practically the same as in humans and all versions of the bovine work described above. See details in the next chapter.

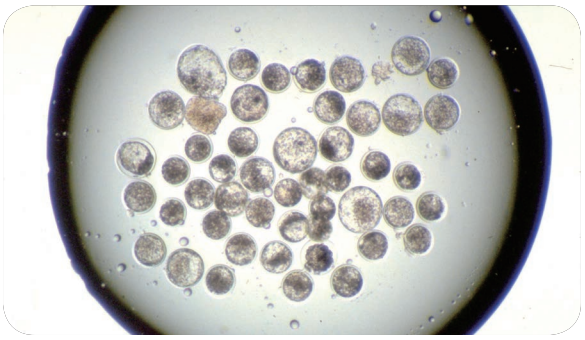




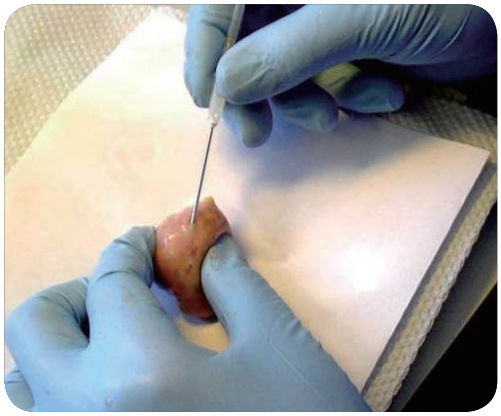
2 Recovery of oocytes from abattoir-obtained ovaries (Day -1, continued)

Two acknowledged procedures exist for recovering oocytes from abattoir-obtained ovaries: slicing with an extra sharp surgical blade and aspiration with a thick hypodermic needle using moderate negative pressure.

Although more oocytes can be collected with slicing, the work is quite laborious, requires a lot of time and solutions, creates a mess, and results in mixed-quality oocytes. Accordingly, slicing is recommended only for the ovaries of deceased valuable individual cows, where every single oocyte may mean a chance to preserve the special genetic material.



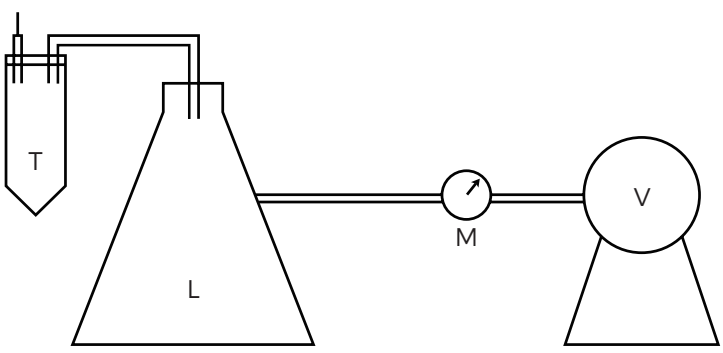
By using slicing, 47 Day 7 blastocysts obtained from the two ovaries of a slaughtered valuable cow - without hormonal stimulation.



For mass production of oocytes for experimental - and potentially commercial - purposes, the aspiration is far more practical. It is a rapid, efficient and reliable technique with a consistent outcome if properly used. From good quality, well-fed animals, 80-90% of the recovered oocytes can be used for maturation and further development.

Aspiration can be performed with a simple syringe and hypodermic needle. However, this is probably the only situation in laboratory embryo production when we suggest a more complicated setup - for obvious reasons. Holding and moving the syringe with the same hand that is supposed to create the proper negative pressure is almost impossible. Some oocytes will be denuded from the sudden aggressive vacuum, while others will remain in the follicle if insufficient sucking force is applied. Unfortunately, these facts are disregarded in most animal laboratories up till today.

To eliminate inconsistency and inefficiency, you can use a sophisticated (and disproportionally costly) *vacuum pump developed for animal OPU* and human oocyte aspiration purposes. However, a *much less expensive and at least equally efficient setup* was introduced as early as 30 years ago in some bovine embryology laboratories.



The schematic view of the suggested oocyte aspiration device. V: Vacuum pump, M: Manometer (optional), L: Large (500 or 1000 mL) glass vacuum flask to trap accidental overflow and mitigate changes of the vacuum caused by the various resistance when the needle enters the ovary, T: Tube for oocyte collection.

2.1 What is needed?

1. A separate room for the "dirty" work - a rule in several countries and a strongly advised arrangement everywhere. Bringing abattoir material into an embryo lab may seriously compromise air quality and sterility measures. On the other hand, this separate room may also be used for other non-sterile works, including those with removed reproductive organs or laboratory animals.
2. A simple oil-free, low noise, low vibration diaphragm vacuum pump with consistent vacuum

   Please note: *Precision instruments like peristaltic pumps may not have the required capacity. Purpose-designed laboratory equipment may be very expensive. A tap-water-operated vacuum pump can be used successfully; however, for improved consistency, we suggest a slightly more expensive electric pump and a few common laboratory items. A low-cost device may be perfectly suitable and can be purchased for USD 200 or less (no commercial interest).*

   To avoid vibration, the pump may be placed on the floor of the laboratory.
3. A 500 or 1000 mL glass vacuum flask with a rubber stopper
4. Ten mL glass Vacutainer blood collection tubes with rubber stopper
5. 14G (2.1x38mm) disposable hypodermic needles
6. Autoclavable silicone tubes and tube-needle connectors
7. Large heated plate with metal test-tube heating blocks
8. Large blunt laboratory forceps
9. Paper towel, tissues, gloves, face masks (if required), protective glasses (if required)
10. VitaVitro Aspiration medium

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(example)

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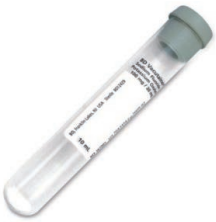

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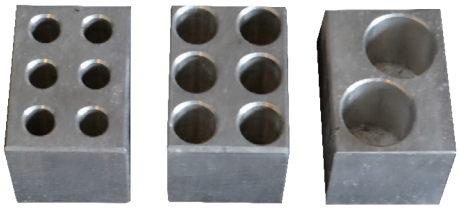

2.2 Timeframe

Preparation (including warming solutions)	1 h (effective work 30 min)
Aspiration of one ovary	1 to 2 min (for experienced operators)

2.3 Preparation

1. Warm the heating plate and heating blocks to 30°C
2. Warm the required amount (1 mL/ovary) of Aspiration medium in Vacutainer tubes in the heating blocks. Warm empty Vacutainer tubes for aspiration and for transport to the laboratory
3. Assemble the aspiration apparatus according to Fig. 1 (*see also video*).

   Please note: *for adjusting the aspiration speed, manometers and regulators of the vacuum pump may be insufficient and can only be used for rough adjustments.*

   For fine-tuning, assemble the whole setup (including the vacuum bottle) and check the aspirated volume/ time rate. The approximate suggested amount is 10 mL/30 s.

   The optimal value may differ from laboratory to laboratory. If you experience a low oocyte number (less than 6-8 per ovary, increase the flow rate. Decrease the flow rate if more than 10% of the collected oocytes have insufficient/damaged cumulus investment. For precise fine-tuning, the insertion of several needles into the rubber stopper of the vacuum bottle may be more suitable than adjusting the pump regulators. The inserted needle creates a shortcut and slightly decreases the flow rate.
4. Clean the area with 70% ethanol or appropriate laboratory disinfectant

2.4 Procedure

(see also vide: https://vimeo.com/manage/videos/772914189, first part)

1. Insert two needles into the stopper of the Vacutainer tube (as on the scheme on page 8)

   one just to pass the stopper, to be connected to the Vacuum bottle

   one pushed deeply, to be connected to the aspiration needle-tubes
2. Turn on the pump
3. Start with aspirating approximately 1mL of Aspiration medium from the pre-warmed tubes
4. Remove a few ovaries from the Thermos flask, keep them on the warm plate and cover them with a wet paper towel
5. Dry one ovary with a dry paper towel
6. By positioning the hole upward, insert the needle into the solid tissue of the ovary

   AVOID puncturing the thin membrane of a visible follicle!

   It may result in leaking and loss of the content
7. Push the needle forward and enter visible follicles from underneath

   Once in, push the needle forward and backward until the follicle completely collapses
8. Proceed with all visible follicles the same way, by using 2-4 new entrance points per ovary.

   You may also move the needle tip blindly in solid parts to hit follicles located deep in the ovarian tissue

   Do not aspirate follicles larger than 6-8 mm in diameter

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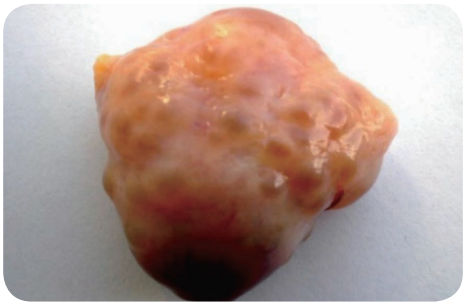

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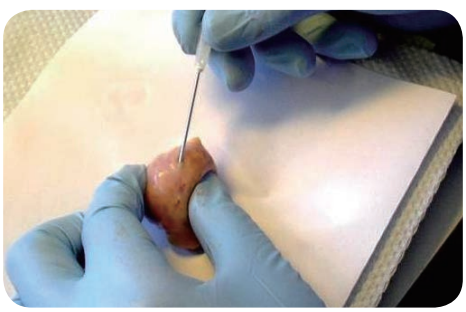


9. When this has been done for all antral follicles, start aspirating a new ovary
10. Stop the aspiration before the tube is full, and put the stopper with two needles into a new glass

    Close the full tube with the new stopper
11. Repeat steps 4 to 10
12. If a colleague in the laboratory makes the oocyte selection, make sure all full tubes are transported there in the metal tube heater as soon as possible.
13. If you work alone, stop 20 min after the start of oocyte aspiration, take the tubes to the laboratory and start oocyte selection.

    Once you finish selection and washing, put the oocytes into the maturation dish and place the dish in the incubator before you re-start another series of aspirations

    Please note: *for points 12-13: An important but often forgotten rule:*

    NOT A SINGLE OOCYTE SHOULD SPEND MORE THAN 45 MIN ON THE BENCH, i.e., outside the follicle but still not in the incubator! If it means a logistic problem, resolve it!

    Also, remember to disinfect/clean the outside of the Vacutainer tubes with oocytes before bringing them to the laboratory
14. When the last ovary is done, aspirate a little more Aspiration medium to make sure to get all the oocytes which might still be in the tube.

3 Selection, washing and *in vitro* maturation (IVM) of oocytes (Day -1, continued)

3.1 What is needed (for 100 oocytes)?

1. A basic embryo laboratory, preferably with 25°C room temperature
2. An incubator with 5% CO_2 or 5% CO_2 + 5% O_2 + 90% N_2 (5+5+90%) gas mixture, 38.5°C and maximum humidity
3. A laminar airflow, switched on during preparation steps only
4. Stereomicroscope with a 38.5°C heated stage
5. Heated plate, warmed to 38.5°C
6. A Nunc four-well dish
7. VitaVitro IVM Bicarbonate medium
8. VitaVitro Heavy Paraffin oil
9. One large (100 mm diameter) plastic Petri dish use a blade (or a thin needle) and light pressure to make fine lines 1 cm apart on the external surface of the bottom and the outer surface of the lid (marker pens produce wide dark lines, may hide oocytes)
10. Two small (33 mm diameter) plastic Petri dishes
11. 15 mL Aspiration medium
12. 20-200-1000 µL automatic pipettes with tips
13. Single-use disposable plastic Pasteur pipettes

3.2 Timeframe

The day before: dish preparation:	10 min
One hour before: warming	1 hour (5 min effective work)

The whole process of selection, washing and starting *in vitro* maturation should be finished in 20 to 25 min (in 45 min after the start of aspiration)

Please note: *better to leave some oocytes in the sediment than to collect all but compromise their developmental competence by exposing them for too long to suboptimal conditions.*

If you cannot keep this timeframe, collect oocytes from fewer ovaries next time in one round.

3.3 Preparation

The day before, prepare an IVM dish, a 4WD with 400 µL IVM Bicarbonate medium covered with 400 µL Heavy Paraffin oil in each well

Incubate at 38.5°C in 5% CO_2 in air (or 5+5+90% gas mixture) with maximum humidity overnight

One hour before the start, put a total of 15 mL Aspiration media in two 10 mL Vacutainer tubes in a metal heating block on the warm plate adjusted to 38.5°C

3.4 Procedure

(see also vide: https://vimeo.com/manage/videos/772914189, second part)

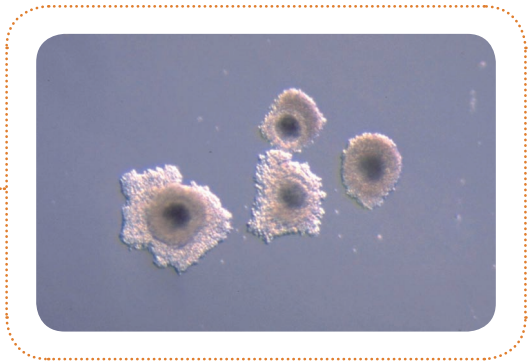
1. Fill up the two small Petri dishes with 3 mL Aspiration medium each
2. Vacutainer tubes containing the collected oocytes can be centrifuged for 2 minutes at 500 rpm (not indispensable)
3. Remove the supernatant with the plastic Pasteur pipette (avoid stirring up the loose sediment!) and add 1 mL Aspiration medium. Mix gently. Transfer the suspension to the middle of the large Petri dish, and rinse the tube twice with 2 mL Aspiration medium
4. Rotate the dish a few times to flatten the drop and distribute the sediment evenly

   Let the dish stand for about 2 minutes so that the oocytes can sink to the bottom
5. Place the Petri dish under the microscope and start collecting the cumulus-oocyte complexes (COCs) with a 20 µL pipette

   Please note: *extensive and time-consuming search and selection may be counterproductive; it may compromise the quality of all collected oocytes.*

   A focused and quick search and selection of oocytes based on the cumulus investment only (2-3 compacted layers of cumulus cells all around) is recommended.

   Cytoplasmic deviations, unless extreme, should be disregarded.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6. Place retrieved oocytes in one of the small Petri dishes

   When all the oocytes have been collected in that dish move them to the other small dish, while counting them. By doing this, the oocytes will be washed

7. After this the oocytes can be moved into the 4WD with IVM bicarbonate. Put ≤ 25 COCs into each well
8. Incubate under the conditions described above
9. Have a coffee and some dessert (not in the laboratory area)
10. *Prepare items for tomorrow (see below)*

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4 Sperm preparation and *in vitro* fertilisation (IVF) (Day 0)

4.1 What is needed? (for 100 oocytes)

1. Basic embryo laboratory, with 25°C (!) room temperature
2. Incubator with 5% CO_2 or 5+5+90% gas mixture, 38.5°C and maximum humidity
3. Laminar airflow
4. Heated plate, warmed to 38.5°C

   On the day of oocyte collection
5. One Nunc four-well dish (4WD)
6. VitaVitro IVF medium
7. VitaVitro Heavy Paraffin oil
8. 20-200-1000 µL automatic pipettes with tips
9. VitaVitro Sperm Preparation medium
10. VitaVitro Sperm Gradient medium kit
11. Fourteen mL conical Falcon centrifuge tubes with plastic caps

    23 h after the start of maturation
12. Stereomicroscope with 38.5°C heated stage
13. Laboratory centrifuge with rotor for 15 mL “blue cap” centrifuge tubes
14. Disposable plastic Pasteur pipettes
15. Thermos flask with 30-35°C water
16. Sterile scissors
17. Tweezers
18. LN_2 Dewar with frozen semen straws

4.2 Timeframe

On the day of oocyte collection	15 min
23 h after the start of maturation	90 min

4.3 Preparation

On the day of oocyte collection

1. Prepare IVF dish: add 400 µL IVF medium into each well of the four-well dish and cover it with 400 µL Paraffin oil. Incubate overnight at 38.5°C
2. Place 10 mL Sperm Preparation medium in a 14 ml Falcon tube. Also, transfer the contents of the Sperm Gradient 55% and Sperm Gradient 90% solution vials into two 14 mL Falcon tubes. Close the caps tightly, and incubate the tubes overnight at 38.5°C

23 h after the start of maturation

No preparation required

4.4 Procedure

1. Remove Sperm Gradient tubes from the incubator. Using a disposable Pasteur pipette, lift the 90% solution and enter the other Sperm Gradient tube. Holding the tube at a 30 to 45° angle, touch the lower wall with the tip of the pipette just above the level of the 55% solution, then slowly release the 90% solution. Avoid making any bubbles and turbulence. The two solutions should be separated with a very thin but visible line

   Please note: *if you find underlying the 90% Percoll difficult, you may add it to the empty tube and overlay it with the 55% solution. Use the method that results in a sharper demarcation line.*

2. Find the straw with the right semen and put it in a 30-35°C water bath (one straw for each Maturation 4WD.

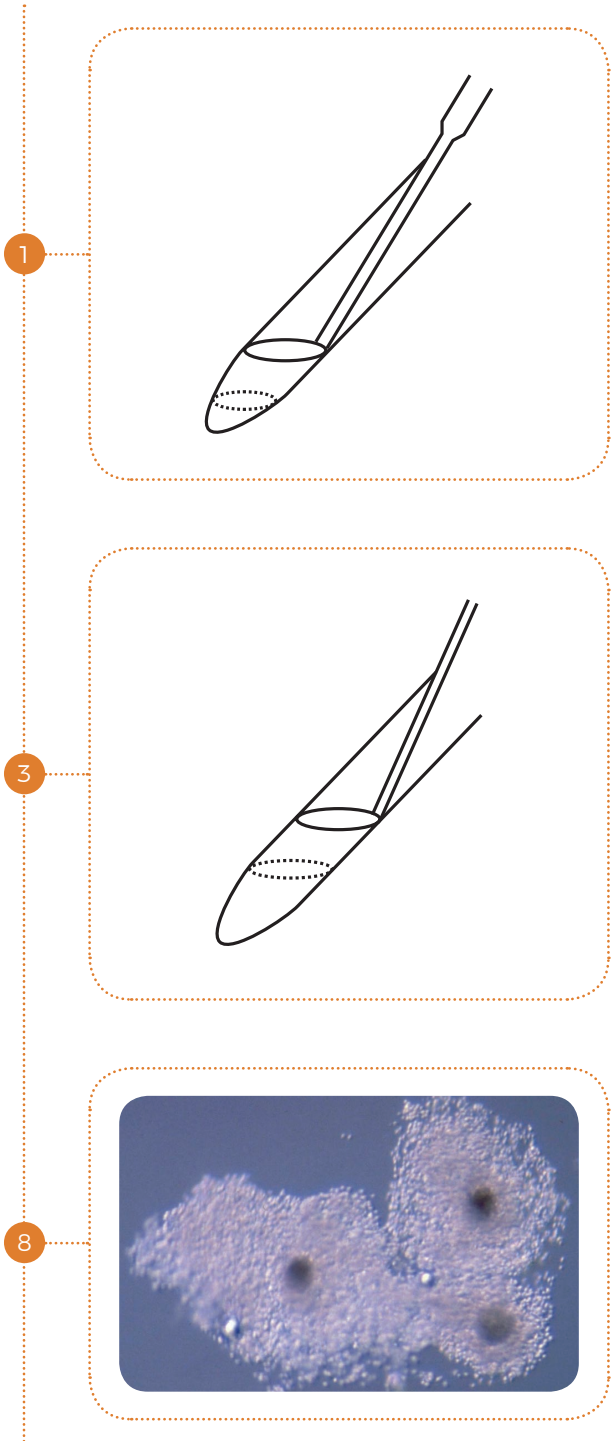
   Please note: *when taking out the straw, use forceps instead of your fingers to avoid a "heat shock " to the spermatozoa.*

   Let the straws stay in the water for a few seconds

3. Cut the straw at one end, and lower this open end into the Sperm Gradient tube, precisely as previously with the Pasteur pipette. Cut the other end. Let the content descend to the upper gradient. Get as much semen as possible out of the straw
4. Centrifuge the tube for 25 min at 1500 rpm at room temperature
5. Remove the supernatant carefully with a disposable Pasteur pipette
6. Add 4 mL Sperm Preparation medium, mix gently, then centrifuge for 10 min, 1100 rpm
7. Repeat steps 5 and 7, but this time centrifuge at 900 rpm, for 10 min
8. *During the second centrifugation*, remove IVM and IVF dishes from the incubator

   With a 200 µL automatic pipette, using the smallest possible amount of IVM medium, collect all oocytes from each well and transfer them into the corresponding IVF wells.

   Please note: *NO washing or separation of individual cumulus-oocyte complexes is required.*
   Perform the transfer as quickly as possible!



9. Remove the supernatant from the Sperm Preparation tube. Add 100 µL of Sperm Preparation medium, and resuspend the pellet.
10. Add 20 µL of the suspension to each well of the IVF dish. Incubate for 23 hours.

    Please note: *except for special situations, there is no need for counting and viability determination of a known semen lot. The optimal quantity may differ between different bulls and lots. However, experienced embryologists may make adjustments according to the stereomicroscopic view of the final sperm suspension. Slight differences in quantities do not modify the outcome and do not justify time-consuming counting and viability tests.*
11. *Prepare items for tomorrow (see below)*

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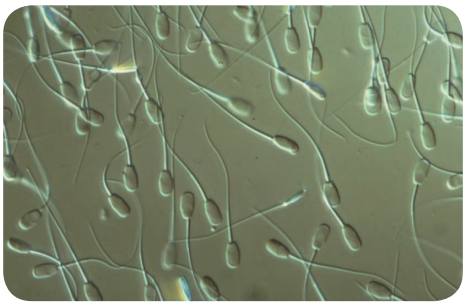


5 *In vitro* culture (IVC) of embryos (Day 1 to 7)

5.1 What is needed? (for 100 oocytes)

1. A basic embryo laboratory, preferably with 25°C room temperature
2. An incubator with 5+5+90% gas mixture, 38.5°C and maximum humidity

   Please note: *for IVC, an incubator with a low oxygen atmosphere is INDISPENSABLE!*
3. Laminar airflow, switched on during preparation steps only
4. Stereomicroscope with a 38.5°C heated stage
5. Heated plate, warmed to 38.5°C
6. Heavy duty Vortex, with rigid rubber holder, capable of aggressive shaking
7. A Nunc four-well dish
8. Two small (33 mm diameter) plastic Petri dishes
9. 14 mL conical Falcon centrifuge tube with plastic caps
10. Large (1.8 or 2 mL) conical Eppendorf tube
11. VitaVitro IVC Single medium withor without FCS (see Addendum)
12. VitaVitro Heavy Paraffin oil
13. VitaVitro TCM-HEPES
14. 20-200-1000 µL automatic pipettes with tips

5.2 Timeframe

On the day of fertilisation	15 min
23 h after the start of fertilisation	30 min

5.3 Preparation

On the day of fertilisation

1. Prepare IVC dish: add 400 µL IVC medium into well 1 and 2 of the four-well dish, then cover it with 400 µL Paraffin oil. Incubate overnight at 38.5°C
2. Place 7 mL VitaVitro TCM HEPES in a 14 mL Falcon tube, close the cap tightly, and incubate overnight at 38.5°C

23 h after the start of IVF

No preparation required

5.4 Procedure

1. Add 400 µL TCM HEPES to the Eppendorf tube
2. Add 5 mL (!) TCM HEPES to the first small Petri dish, leaving the second empty
3. With the 100 µL automatic pipette (preferably with a single pickup from all four wells), remove presumptive zygotes from the IVF dish and put them into the Eppendorf tube
4. Vortex with maximum speed for 2 min[2]

   Please note: *the required time depends on the strength of Vortex machines.*

   The outcome should be NO cumulus cells on the surface of the embryos. Otherwise, you should repeat/ prolong vortexing or add 1 to 2 µL Paraffin oil to the Eppendorf tube.

   Dispersed oil drops may help to remove mechanically the strongly attached cumulus cells.

   Please also note: *by following the subsequent steps PRECISELY, you may proceed very fast - it is essential for the proper outcome*
5. With the 1000 µL automatic pipette (adjusted to 1000 µL), remove the content of the Eppendorf tube and place it in the second (empty) small Petri dish
6. Remove 1000 µL medium from the first Petri dish, add it to the Eppendorf tube, mix with the pipette, remove, add to the second Petri dish
7. Repeat Step 6.
8. After 10 to 20 seconds (required for the embryos to sink to the bottom), swirl the dish with a careful circular movement to collect presumptive zygotes in the centre
9. Transfer them to the first Petri dish, preferably with a single pickup of the 20 µl pipette. Disperse them evenly throughout the dish.
10. After 10 to 20 seconds, with limited swirling, collect zygotes at the central third of the dish

    Quickly collect 50 zygotes and transfer them to well 1 of the IVC dish. Repeat collection and transfer the remaining zygotes into well 2
11. Incubate up to 7 days without disruption

    Please note: *EVEN THE SHORTEST VISUAL CHECK DURING DAY 1 TO 6 WILL COMPROMISE DEVELOPMENT!*

    (For practical purposes - embryo transfer arrangement - you may check embryos on Day 6 afternoon)

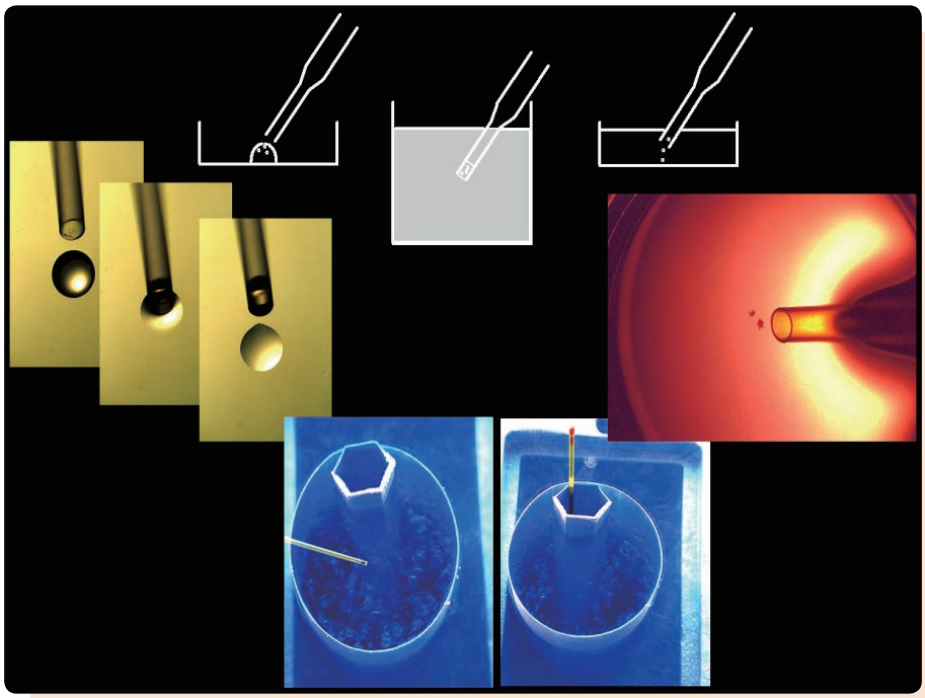
CHAPTER II. CRYOPRESERVATION OF *IN VITRO*-PRODUCED BOVINE EMBRYOS

In the title, we could replace cryopreservation with vitrification, as our chapter will not deal with traditional freezing. In contrast to human IVF, vitrification has been a strong competitor of traditional freezing since the very early days of bovine *in vitro* embryo production, say, the early '90s. Unlike their human counterparts, bovine embryos require an extended, seven-day-long culture period for successful transfer. In the early nineties, our culture media and methods were inappropriate, resulting in inferior quality blastocysts with dark cytoplasm due to lipid accumulation and compromised developmental ability. Although pregnancies and births could be achieved, the cryotolerance of these embryos was below the minimum required level of traditional freezing, so researchers were pressed to find an alternative solution.

Vitrification was a reasonable possibility, as chilling injury (damage occurring at temperatures below +14°C; Martino et al., 1996) was especially harmful to these lipid droplet-rich structures. The relatively high cooling rates (although far from those achievable today) helped to decrease chilling injury and improved the subsequent outcomes. However, the application of vitrification - as well as that of the whole bovine IVF procedure - was mostly restricted to laboratory use for decades. This regrettable delay was caused by a single practical - and questionable, or rather false - reason.

Transvaginal ultrasound-guided ovum pick-up followed by *in vitro* fertilisation (OPU-IVF) in cattle has been described as early as 1991 (Pieterse et al., 1991), but the industrial-scale application was started only ten years later, around the turn of the millennium, with streamlining the technology and adjusting it to the requirements of on-farm application. Meanwhile, due to new media and low oxygen atmosphere, the quality of *in vitro*-produced embryos has also increased significantly. Eventually, the difference between the morphology of *in vitro*-vs. in vivo-produced embryos was almost entirely eliminated, with a significant improvement in pregnancy/calving rates and cryotolerance. Accordingly, traditional slow-rate freezing has become a choice again, although the results were still variable; in general, below expectation. Consequently, approximately 80% of embryos are transferred without cryopreservation worldwide (Ferré et al., 2019) with all related logistic issues and handicaps of fresh transfers in industrial-scale applications.

Meanwhile, research in vitrification has also achieved dramatic improvements. Purpose-developed carrier tools offered a ten- to hundred-fold increase in cooling and warming rates, practically eliminating chilling injury and also enabling a radical decrease in the concentration of potentially harmful cryoprotectant solutions. After a long hesitant period, vitrification has become the obvious choice in human IVF, both for embryo and oocyte cryopreservation, restricting the use of traditional freezers to male gametes.



Curiously, in cattle IVF, where the first successes were achieved with high-speed vitrification, these improvements were completely neglected. The on-farm application requires a simple procedure for thawing-warming and transfer, without the need for laboratory equipment, microscopes, dishes, and filtered air. To meet these requirements, direct transfer after in-straw dilution of cryoprotectants was an established method in traditional freezing but was/is widely believed to be impossible after vitrification.

However, there is a simple and efficient procedure for direct transfer after vitrification. The principle was suggested by the legendary veteran scientist in animal reproduction, Dr Clifton Murphy (Missouri, USA). Based on the first purpose-made tool for high-speed vitrification, the Open Pulled Straw (OPS; Vajta et al., 1998) it was published as early as 23 years ago (Vajta, Murphy, Macháty et al., 1999). Although repeatedly mentioned in reviews, manuals and lectures, the application of this procedure was restricted to a very narrow circle of scientists and used mostly for the transfer of handmade cloned, zona-free blastocysts. On the other hand, even the limited results achieved with these highly sensitive embryos clearly prove the value of this technology.

Although alternative ways to connect vitrification to direct transfer by using different tools have been described recently, these methods miss either the essential advantage of high-speed cooling and warming, or the robustness and reliability of the OPS-related technology.

This chapter is another - maybe ultimate - attempt to eliminate this controversy, present the possibilities, and offer a simple, rapid and practical chain of methods for large-scale, possibly industrial-scale application of vitrification of *in vitro*-produced bovine embryos.

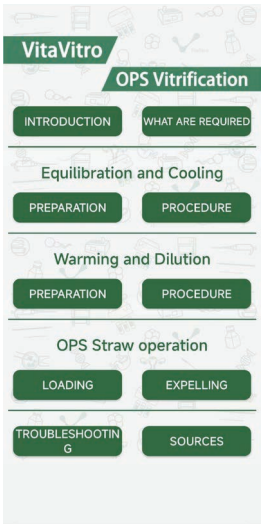
1 Vitrification: equilibration, dehydration, cooling and storage

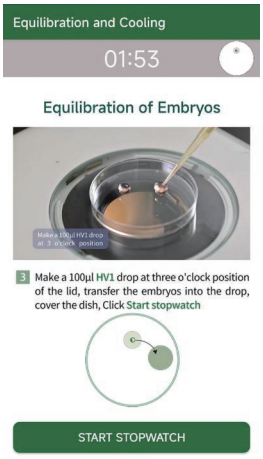
Compared to human IVF, quantities in bovine embryology may be extensive, while financial/human resources are less abundant. Accordingly, simple and short procedures using fewer disposables and solutions are preferred. An additional requirement is a flexibility regarding the number of embryos to be vitrified in a single cycle using the same carrier tool. This flexibility allows using the same technique for both on-farm single embryo (direct) transfer and large-scale storage for laboratory / experimental purposes.

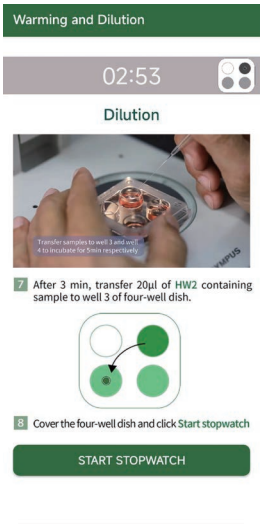
The OPS technology used for vitrification of human D5 blastocysts can be successfully applied for bovine *in vitro*-produced blastocysts, too (briefly, 9-15 min equilibration, 2 x 20 s dehydration, both at 25°C, loading, cooling; see detailed information in VitaVitro's OPS app for both iOS and android phones)

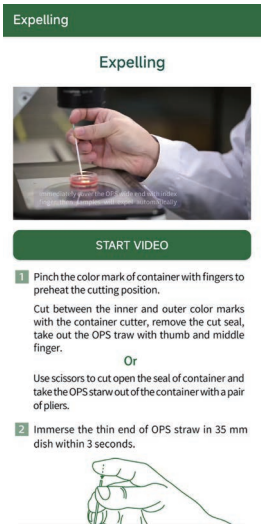
iOS

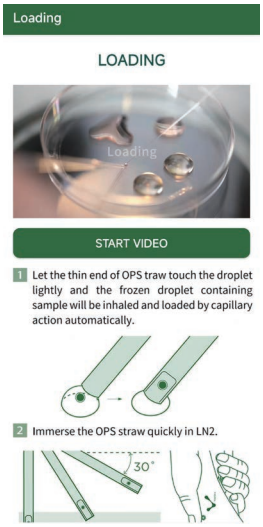
Android











However, an alternative (in fact, the original) OPS technology described below may offer more benefits for cattle embryos while preserving all advantages of OPS vitrification. Moreover, this technology was successfully applied for all developmental stages between D3 and D8 without losses in viability and developmental competence.

1.1 What is needed?

1. Embryo laboratory with clean air and basic equipment/tools for bovine *in vitro* fertilisation.

   Sterile hoods are NOT needed for vitrification - if used, the fan should be switched off

   Room temperature is not crucial, as the suggested procedure is run in a heated environment.

2. A separate bench used only for cryopreservation purposes, with ALL items required, and ONLY items required for cryopreservation

3. A stereomicroscope with heated stage and a large flat heated plate, both adjusted to 38.5°C

4. Semitransparent plastic storage boxes to cover dishes on the stage and plate

5. VitaVitro Open Pulled Straw (OPS)

6. VitaVitro Container Straw - a modified 0.5 mL plastic straw for contamination-free storage of samples in liquid nitrogen (LN2). (cat. no DA08001)

6a. or VitaVitro OPS storage tubes with perforated walls and caps for non-sterile storage of OPS (cat. no DA08007)

7. VitaVitro VitaVitro Cooling Box for safe cooling and package into container straws

   Decontaminate before use with 70% ethanol, H_2O_2 or laboratory disinfectants.

   place on a separate table or laboratory underbench storage module, 15-20 cm below the bench level (to avoid accidental cooling of the bench by LN_2 vapour)

   on the left side of your chair (for right-handed embryologists)

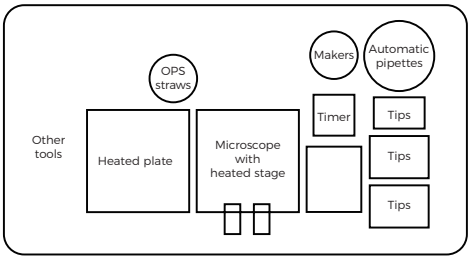
7a. or a robust styrofoam box (approx. 25 x 15 x 15 cm with at least 3 cm thick walls and lid decontaminate before use with 70% ethanol, H_2O_2 or laboratory disinfectants.

   Place it on a separate table or laboratory underbench storage module, 15-20 cm below the bench level (to avoid accidental cooling of the bench by LN_2 vapour) on the left side of your chair (for right-handed embryologists)

8. Plastic goblets for straw storage in LN_2, 65 and 15 mm diameter, respectively (your local supplier)

9. 20-200-1000 µL automatic pipette set (your preferred brand) with sterile tips

2



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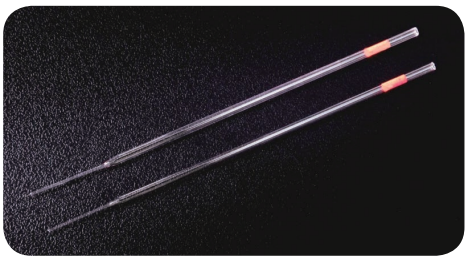

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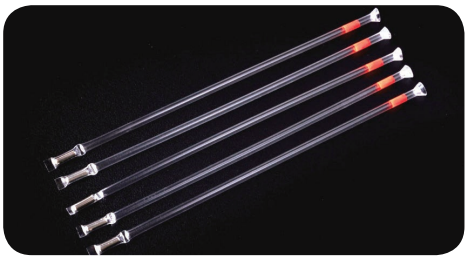
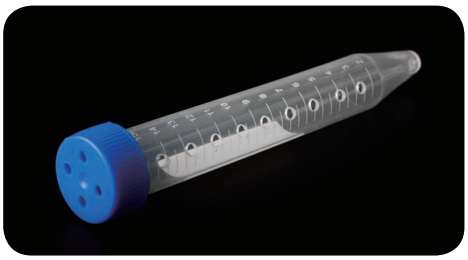


6a



7

10. Embryo handling/transfer pipette (your preferred brand)
11. Four-well dishes (4WDs) for both vitrification and warming (Nunc, Roskilde, Denmark)
12. Large blunt laboratory forceps (your local supplier)
13. VitaVitro Heat sealer for straws (cat. no VOPS01)
14. Wire denuder (your preferred brand)

14a. or stainless steel surgical straight (stich) scissors (your local supplier)

15. VitaVitro Small teeth forc
16. Timer (preferably Jadco Electronic Clock-Timer)
17. Fine tip marker pen, blue and red colour (preferably Thermo Scientific Cryo Marker Pen Set)
18. Paper and pen for notes
19. VitaVitro Vitrification and Warming solution kits

13

14

15

16

1.2 Timeframe

Preparation (including warming dishes and solutions)	30 to 40 min
One cycle in one OPS (for 1 to 5 embryos/straw)	6 min / straw see **1.4**
Parallel cycle in two OPS (for 1 to 5 embryos/straw)	4 min / straw see **1.5**

1.3 Preparation

1. Warm refrigerated bottles of CHM, CV1, CV2 on the 39°C heated plate, under a plastic box or in a 38.5°C box-type incubator for 30 min; or in a 38.5°C water bath for 20 min.

   Meanwhile, place a 4WD on the heated plate and cover it with another plastic box

1

2. Add CHM to Wells 1 and 2; CV1 to Well 3; and CV2 to Well 4

   The required quantity depends on the number of cycles

   for 1 to 2 straws (or 1 to 2 x 2 parallel straws)450 µl each well

   for 3 to 6 straws (or 2 x 3 to 6 parallel straws) 900 µl each well

   (for more than 6 straws, you have to prepare a new 4WD)

   Do not cover solutions with oil!

   Warm the dish for an additional 5 min on the heated plate, under a plastic box

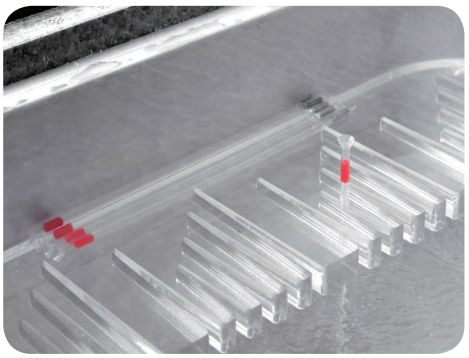
3. Meanwhile, fill the VitaVitro Cooling Box (or the foam box) with LN_2

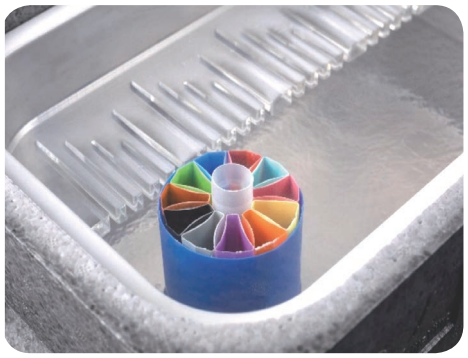
   Add the 65mm plastic goblet with coloured 15 mm small goblets in the VitaVitro Cooling Box for sterile storage *or with VitaVitro OPS storage tubes in the foam box for non-sterile storage)*

   Close the lid of the LN_2 storage box

   Keep the LN_2 storage dewar in the lab for re-filling

3





1.4 Procedures

(see also the video: https://vimeo.com/773294173 (some details - including the Cooling Box - are different)

Please note: *The maximum number of embryos to be vitrified in one OPS depends on the needs, and experience of the operator. Initially, only 1 or 2 embryos are suggested.*

Equilibration and dehydration are temperature-dependent processes. Insufficient warming of solutions and dishes may compromise the outcomes. The use of solutions at the core temperature of cattle may allow radical shortening of the time of equilibration and dehydration. At this temperature, however, evaporation may cause increased osmolality of solutions and damage to the samples.

Although only a small part of CV2 is used for the final dehydration step, the suggested large amount maintains the required temperature and osmolality. However, more than six single cycles (or more than 40 min work time in total) may result in extensive evaporation and impaired outcomes.

Please note: *for sterile storage in VitaVitro container straws: before removing embryos from the incubator, put the required number of container straws (one for each OPS straw) horizontally on the shelf of the VitaVitro Cooling Box. Switch on the heat sealer.*

Please note: *this description has been made for right-handed people. Left-handed colleagues may prefer a mirror image of the arrangement described.*

1. Pick up all embryos to be vitrified in the given 4WD and put them into Well 1

   avoid transferring an excessive amount of oil

2. Wait until all embryos sink to the bottom, then pick up all and put them in Well 2; avoid transferring any oil

3. Make a 20 µL droplet from CV2 (Well 4) at the bottom right section of the centre of the 4WD

   For sterile storage, put a container straws vertically, funnel end up, into a slot of the VitaVitro Cooling Box.

4. Transfer 1 to 5 embryos into CV1 (Well 3)with the lowest possible volume of medium. When immersed into CV1, aspirate first approx. 10 µL into the pipette, then push embryos out *slowly*. Try to keep them together and on the bottom of the well

   Keep embryos together on the bottom of the dish as close to each other as possible. Close the lid and equilibrate for a total of 3 min under a plastic box

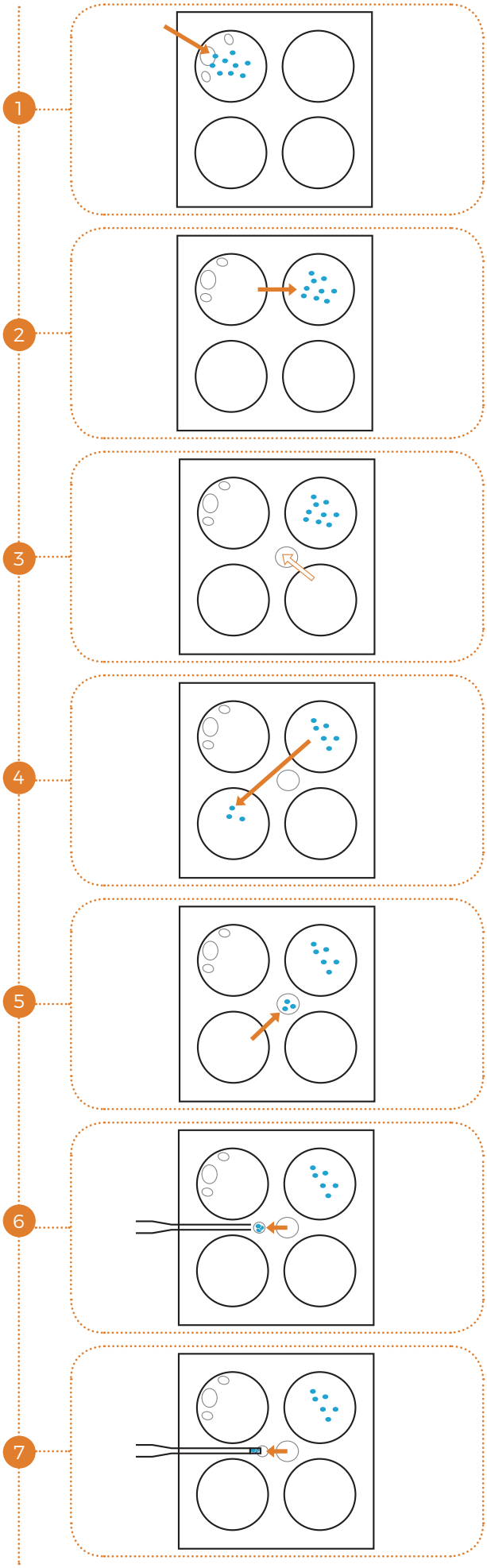
5. Hold the OPS with your left hand but avoid closing the thick end with your finger.

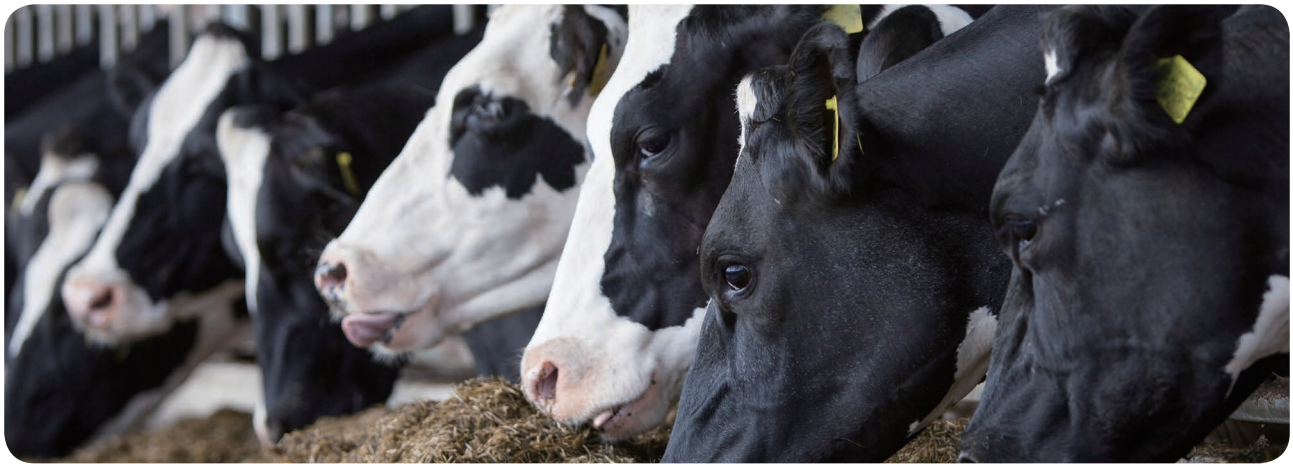
   Transfer embryos in the least possible volume into the 20 µL droplet of CV2.

   When immersed into the droplet, aspirate first approx. 10 µL of the solution into the pipette, then push embryos out *quickly*

6. Aspirate again, push out, repeat for approx. 40 s, then pick up embryos in a 2 µL volume and make a small, high droplet slightly left of the centre of the bottom of the 4WD

7. Immediately afterward, touch the left side of the droplet with the OPS, narrow end, held approx. at a 30° angle to the bottom. Don't close the wide end of the OPS. An approx. 1mm high cylinder of CV2 containing all embryos should be formed in the end of the straw



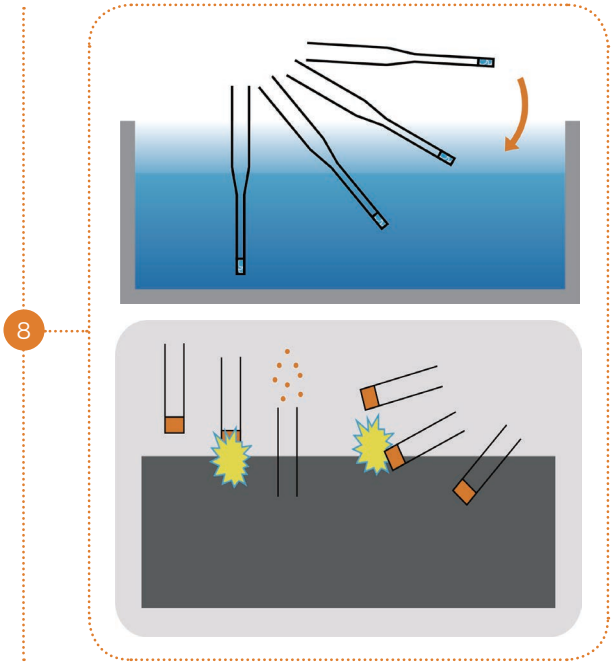


8. With a single rapid and uninterrupted movement, turning the straw from horizontal to vertical position and passing through the vapour rapidly, insert the narrow end of the OPS in the liquid nitrogen

   Please note: *If you touch the surface in a vertical position, the vapour of LN_2, generated by the warm VS2, might explode the VS2 and eject the samples.*

   Please also note: *the total time between transfer into the 20 µL drop and immersion to LN_2 should not be more than 1 min*

9. After 10 s, insert OPS either into the pre-cooled container straw, then heat seal the funnel end after insertion or into the OPS storage tube, then close the tube with the perforated blue cap



10. Start the procedure again with the transfer of new embryos from Well 2 to Well 3

    Please note: *Before each new cycle of vitrification, change the medium in the 20 µL droplet by using the same place on the bottom. On the other hand, the small drop used for loading should always be made on a new area to avoid flat drop formation.*

1.5 Accelerated vitrification procedure with parallel equilibration

Please note: *this approach is suggested only for embryologists experienced in OPS vitrification.*

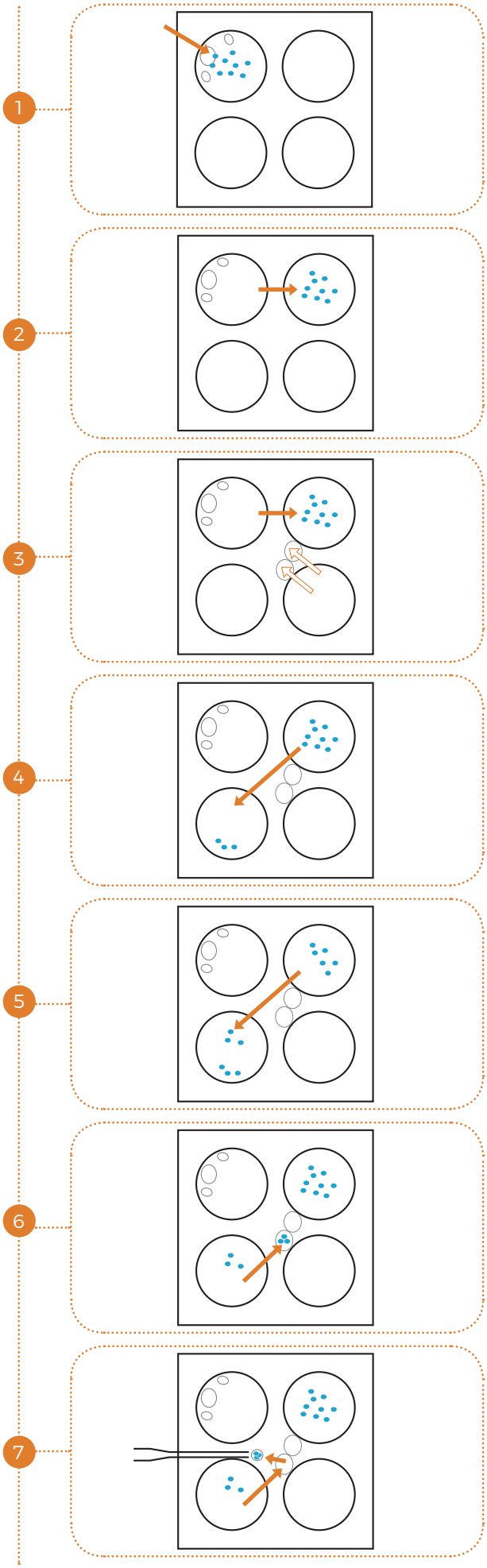
The use of the VitaVitro Cooling Box and container straws is recommended.

1. Pick up all embryos to be vitrified in the given 4WD and put them into Well 1; avoid transferring an excessive amount of oil
2. Wait until all embryos sink to the bottom, then pick up all and put them in Well 2; avoid transferring any oil
3. Make two 20 µL droplets from CV2 (Well 4) close to the centre, right side of the bottom of the 4WD

   Put two container straws vertically, funnel end up, into two slots of the shelf of VitaVitro Cooling Box.
4. Transfer 1 to 5 embryos into CV1 (Well 3)with the lowest possible volume of medium. When immersed into CV1, aspirate first approx. 10 µL into the pipette, then push embryos out slowly. Try to keep them together and on the bottom of the well, Southern quadrant
5. *Ninety seconds after* the start of equilibration of the first group, repeat the procedure with the second group, keep them together and on the bottom of the well, Northern quadrant
6. Hold the OPS with your left hand but avoid closing the thick end with your finger

   Three minutes after the start of equilibration of the first (Southern) group, transfer those embryos in the least possible volume into the *first* 20 µL droplet of CV2

   When immersed into the droplet, aspirate first approx. 10 µL of the solution into the pipette, then push embryos out quickly
7. Aspirate again, push out, repeat, then pick up embryos in a 2 µL volume and make a small, high droplet slightly left of the centre of the bottom of the 4WD
8. Immediately afterward, touch the left side of the droplet with the OPS, narrow end, held at a 30° angle to the bottom. An approx. 1 mm high cylinder of CV2 containing all embryos should be formed



9. Immerse the straw into the LN_2: first almost horizontal, then turning vertical with a single rapid movement, passing quickly through the LN_2 vapour

   Please note: *If you touch the surface in a vertical position, the vapour of LN_2, generated by the warm VS2, might explode the VS2 and eject the samples.*

   Please also note: *the total time between transfer into the 20 µL drop and immersion to LN_2 should not be more than 1 min*

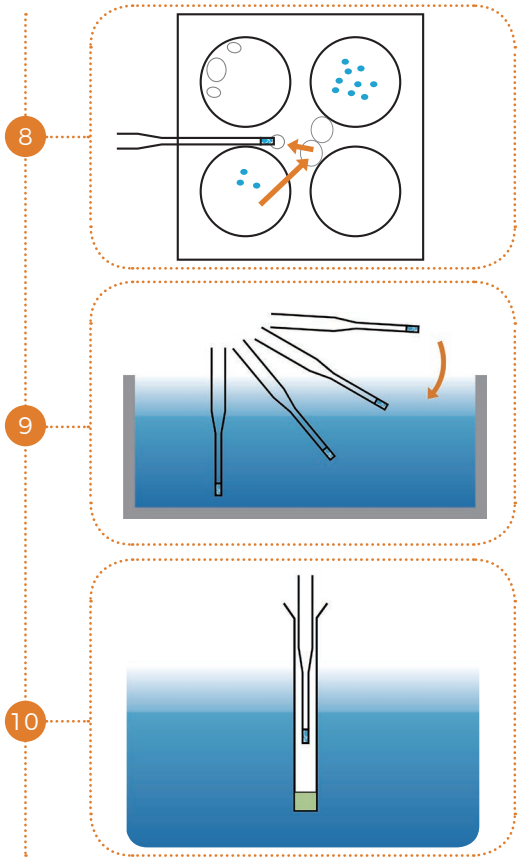
10. After 10 s, insert OPS into the vertically positioned container straw.

    Seal the end with the heat sealer

11. Immediately afterwards, repeat steps 5 to 8 with the second group (Well 3, Norther quadrant)

12. Start the procedure again with the transfer of new embryos from Well 2 to Well 3

    Please note: *Before each new parallel cycle of vitrification, change the medium in the 20 µL droplets by using the same place on the bottom. On the other hand, the small drop used for loading should always be made on a new area to avoid flat drop formation.*



Please note: *further acceleration of efficiency may be achieved by adding a third and fourth parallel cycle to the chain - however, it requires special skills and attention.*

On the other hand, if technical problems occur during the work, equilibration time in CV1 may be extended to 4 or 5 min without significant effect on the outcome.

2 Warming and rehydration of OPS vitrified bovine embryos in the laboratory

2.1 Timeframe

Preparation (including warming dishes and solutions)	30 to 40 min
One OPS straw (for 1 to 5 embryos/straw)	20 min

2.2 Preparation

1. Warm refrigerated bottles of CW1, CW2 and CHM on the 39°C heated plate, under a plastic box or in a 38.5°C box-type incubator for 30 min; or in a 38.5°C water bath for 20 min.

   Meanwhile, place a 4WD on the heated plate and cover it with another plastic box

2. Add CW1 to Wells 1 and 2; CW2 to Well 3; and CHM to Well 4; 900 µL each.

   Warm the dish for an additional 5 min on the heated plate under a plastic box.

3. Meanwhile, fill the VitaVitro Cooling Box (*or the styrofoam box*) with LN_2:

   Add the coloured plastic goblets to hold straws of vitrified embryos. Close the lid.

   Keep LN_2 storage dewar in the lab for re-filling

2.3 Procedure

Please note: *the whole process of warming is identical with that used for human oocytes/ embryos and illustrated well on the OPS app (see QR code on page 22)*

The only difference is that you need to warm the sample in Well 1, instead of a separate Petri dish.

As there is no time to follow sequential drawings during this process, we suggest you to see these links alongside with the photos below, and practice the procedure before you start using it for live material.

Please note: *During the whole procedure, avoid holding the wide end or OPS towards your eyes. Evaporating LN_2 may eject LN_2 microdrops and cause damage.*

1. Place well 1 of the 4WD in the focus of the microscope. Use low magnification, covering the well

2. After sterile storage in VitaVitro Container tubes, by using the VitaVitro Straw cutter, cut the Container straw 2 cm under the level of the end of the thick end of OPS

   Remove the sealed-cut cap and grab the wide end with the thumb and middle finger of your right hand

   THE NARROW END OF THE OPS CONTAINING THE SAMPLES SHOULD REMAIN BELOW THE LEVEL OF THE LN_2 !!!

   Alternatively, use a pair of scissors to cut the container straw over the thick end of the OPS

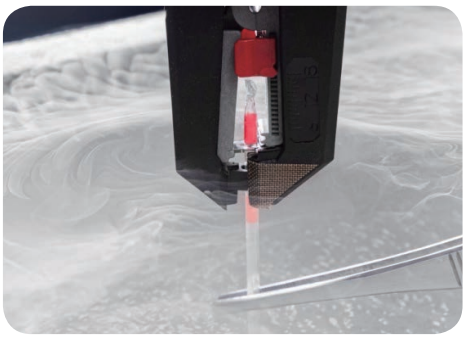
   catch OPS with the Small teeth forceps and lift it until you can grab the wide end with the thumb and middle finger of your right hand

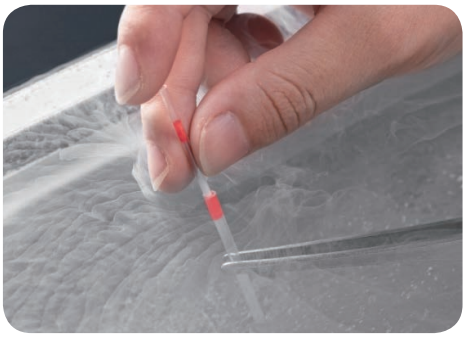
   THE NARROW END OF THE OPS CONTAINING THE SAMPLES SHOULD REMAIN BELOW THE LEVEL OF THE LN_2 !!!

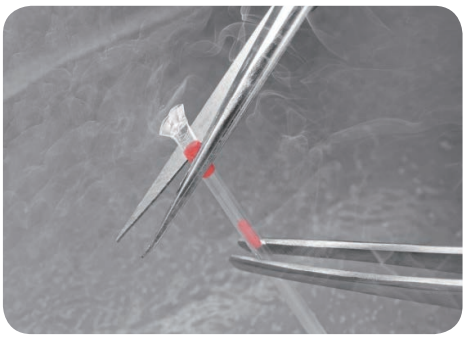
   After non-sterile storage in VitaVitro OPS storage tubes, remove the cap, catch the selected OPS with the small teeth forceps, and lift theOPS until you can grab the wide end with the thumb and middle finger of your right hand

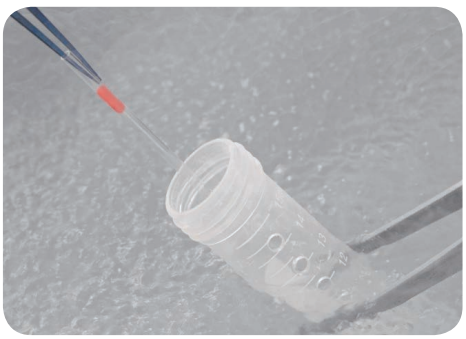
   THE NARROW END OF THE OPS CONTAINING THE SAMPLES SHOULD REMAIN BELOW THE LEVEL OF THE LN_2 !!!

2









3. Make sure the way you grab the OPS with your thumb and middle finger allows a safe closure of the wide end with your index finger; but DO NOT CLOSE the end yet

4. After a few seconds of concentration, lift the OPS and start counting while approaching Well 1 of the warming dish

   One - two - three - immerse - close. Each one for one second

   - at **"three"**, the OPS should be over the CW1 solution. Follow it under the microscope
   - at **"immerse",** lower the OPS into CW1 while holding it close to a horizontal position

     (this position allows better visual control and avoids explosion

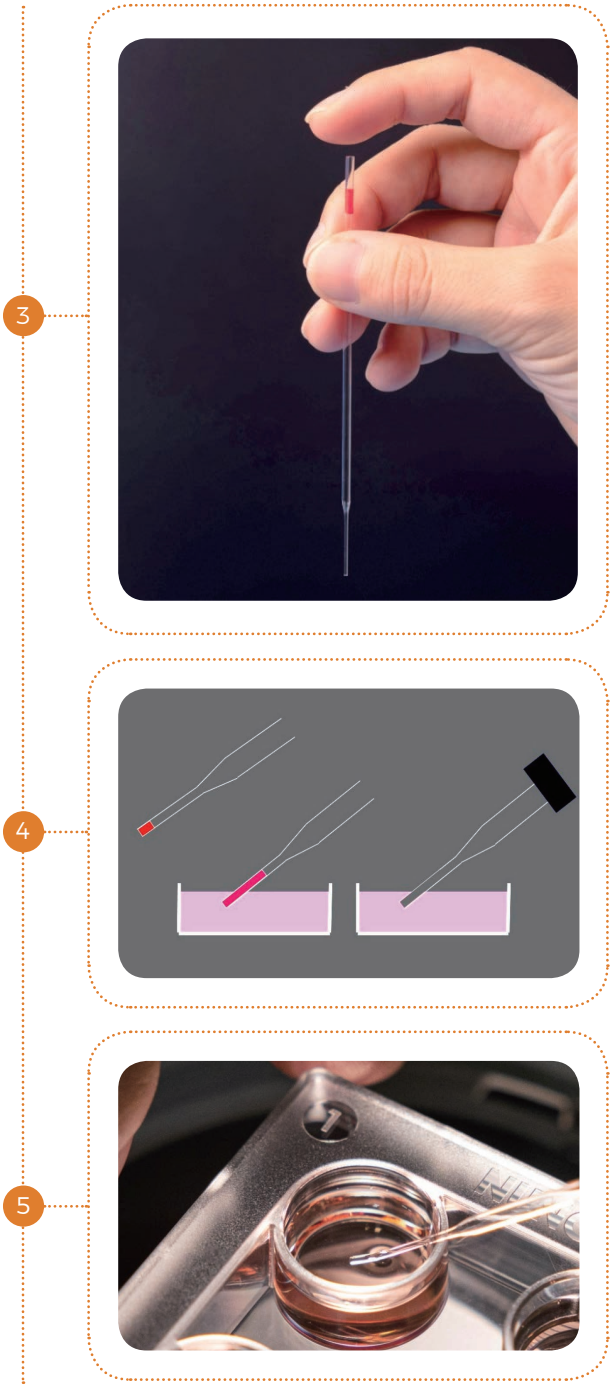
     caused by the evaporating traces of any remnant LN_2)

     You will see a short partial darkening[3] - of the solution column, then clearing up, and - as the result of the capillary effect - the column will start to move upwards
   - **"close"** immediately the wide end with your index finger, and KEEP THE CLOSURE TIGHT.

     The increasing pressure of the warming air inside the OPS will push the solution column (including the embryos) back, and it will float out calmly into CW2[4]

5. Immediately after expelling, transfer embryos into Well 2, close the lid and wait 5 min

   The blastocysts will float, then suddenly collapse and sink to the bottom



6. With a generous amount of solution (say 50 µL) , transfer embryos to Well 3, and wait 5 min

   If the cell mass forms a small ball with even outlines, the prognosis is good

7. With a generous amount of solution, transfer embryos to Well 4, and wait 5 min

   Re-formation of the blastocoel may start in Well 4

8. Transfer to the culture dish

Please note: *as mentioned before, It is strongly recommended practice first: do a few rounds of sham warming-expelling by using solutions only, then degenerated embryos.*

Don't forget: *you have to cool down the OPS properly (10-15 min in liquid nitrogen) to expel the content with the warming air inside.*

3 Warming of bovine embryos after OPS vitrification for direct transfer

3.1 What is needed?

1. VitaVitro Direct Transfer (DT) straw
2. VitaVitro Direct Transfer Warming Solution (DTWS: Holding Medium with 0.2 M sucrose)

   Keep refrigerated and warm to 35-39°C in the original bottle before use
3. A laboratory glass thermometer (preferably 300 mm long, for 0-50°C) or a reliable digital thermometer with a submersible probe
4. A 400 to 500 mL insulated jar (as Thermos Thermocafe)
5. Hot and cold water (preferably sterile water)
6. A table of a bench with a (preferably) dark surface and a chair adjusted to a convenient position.
7. Scissors (sterilised or decontaminated) for cutting the funnel end of the DT straw
8. A 10 to 100 µL automatic pipette with tips

4

3.2 Timeframe

Preparation	10 min
Procedure	1 min

3.3 Preparation

1. Decontaminate the inner surface of the jar, then fill it to 10 cm level with 39°C water.

   Control and adjust the temperature during the whole procedure.
2. Fill a DT straw with DTWS, aspirating an approx. 2.5 to 3 cm column of the medium, followed by an approx. 1.5 to 2.0 cm air column, then the medium again, leaving a 0.5 cm empty space at the open (funnel) end. Place the DT straw in the jar with the open (funnel) end up. Warm for 5 min.

3.4 Procedure

(see also the short video here: https://www.youtube.com/watch?v=L8HW30SHL6c)

3. Place the DT straw vertically in front of a dark, even surface (bench, mousepad, etc.) for better visibility

   Hold it firmly, and support your hand with the edge of the bench. Make sure you see the funnel end at a 45-degree angle

4. Remove OPS from the liquid nitrogen* by holding it with the thumb and middle finger. The index finger should be in a convenient position for closing the thick end (later)

   When the OPS leaves the liquid nitrogen, start to count:

   One - two - three - immerse - close Each for one second

   Approach the funnel end of the DT straw quickly

   **for proper removal from Storage tube or Container straw, see previous chapters*

5. At **"three"**, you should be inside the funnel. Touch the wall and proceed slowly

6. At **"immerse"**, place the end of the OPS into the warming medium, and submerge the embryo-containing column in the OPS entirely

7. In less than 1 s after immersion,

   you will see the solution ascending in the OPS because of the capillary effect.

   When you see that, close the thick end of the OPS with your index finger

   Hold firmly, do not release!

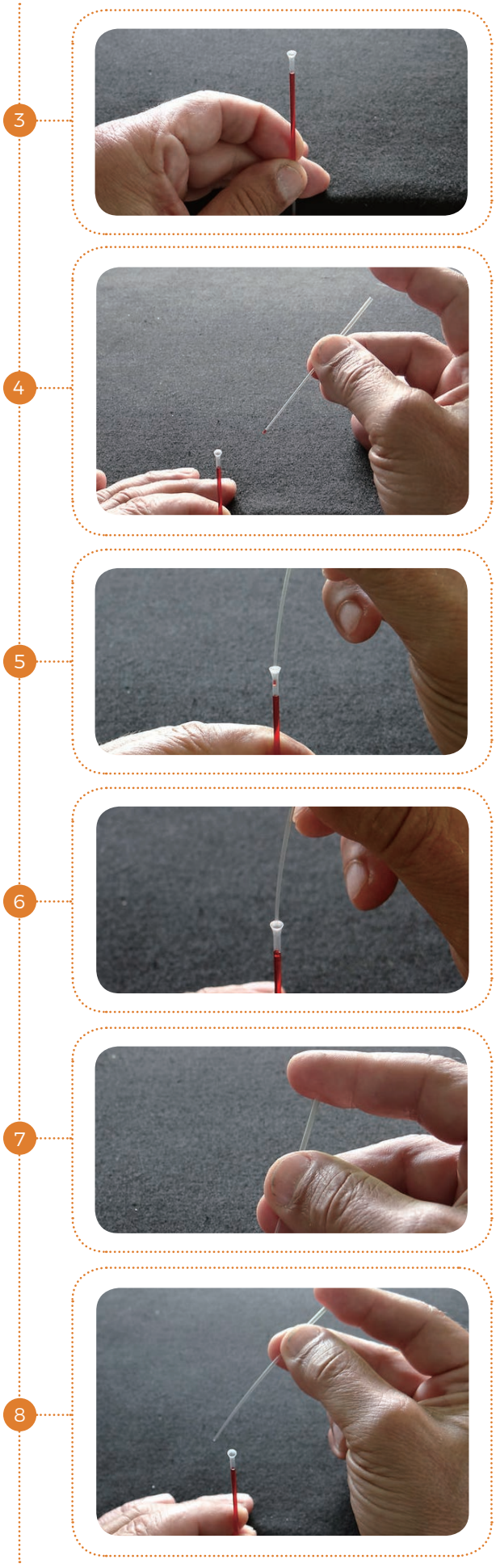
   If the closure is airtight, the warming air in the OPS will press out entirely the solution into the warm straw

8. When the air reaches the end of the OPS, but before air bubbles start to form,

   lift and remove OPS slowly from the DT straw - while holding the end firmly

   Keep the tip of the OPS in contact with the inner surface of the DT straw, it helps to get all solution - and all embryos - removed from the OPS

   No solution in OPS means all embryos are out. No microscopic control is required



Please note: *if the solution remains in the OPS, you cannot repeat the same process! Do not try to close then open it again and again. Just insert the tip of the automatic pipette into the open end of the OPS and carefully expel the medium into the warming straw. You may also flush the end of the OPS with careful pipetting in and out. This is an emergency procedure for beginners, very rarely needed in routine practice.*

It is strongly recommended to start with a little practice, a few rounds of sham warming-expelling by using degenerated embryos. Don't forget: you have to cool down the OPS properly (10-15 min in liquid nitrogen) in order to expel the content with the warming air inside.

3.5 Embryo transfer

Transfer can be performed immediately after warming or < 15 min later. Keep the DT straws warm during the waiting period by immersing it again in the jar with 39°C water.

Before the transfer, simply cut the funnel end of the DT straw with scissors, and insert the straw into the embryo transfer catheter.

ADDENDUM

1 Defined or not - the protein supplementation issue

Since the first attempt to culture mammalian embryos *in vitro*, a major goal was to create a completely defined medium, without any unknown components in order to eliminate inconsistencies, to avoid potential contamination and to make the outcome more predictable.

Defined additives, i.e., inorganic macromolecules, could provide appropriate physical support, i.e., surfactant activity to prevent adhesion or optimisation of the osmotic pressure. However, purified albumin carries vitamins, hormones, bioactive lipids and ligands; it also neutralises certain toxins that may be present in embryo culture media. These effects increase development rates both *in vitro* and after embryo transfer. Further improvement may be achieved with more complex protein supplementation, for example, the addition of globulins or using serum instead of purified proteins.

However, serum in culture medium is regarded as a capital sin nowadays - although the prosecution has a lame case.

One argument is that serum - in contrast to albumin - is not a component of the oviductal or uterine fluid. Well, is EDTA or PVA a natural constituent?

The other concern is that serum may cause developmental abnormalities summarised as "large offspring syndrome". Another urban legend, just like the "toxic DMSO" story and the need to refresh the medium during embryo culture. Other than sheep, no evidence is available that serum causes large offspring in other mammals (Jeremy Thompson, *pers. comm.*). On the other hand, subcellular morphological alterations are not related to serum but its overdose. Just as for the albumin (O'Neill, 2008) a fraction of the amount of the traditional 20% serum is more than enough to provide excellent support and does not cause any negative by-effect.

We strongly suggest you do not throw out the baby with the bath water. Use media with 5% FCS supplementation as in VitaVitro Single Culture medium .

Or, with a little extra effort, prepare and add "homemade" adult cow serum to protein-free media. *Sera collected from adult cattle* contain twice as much protein as the fetal or newborn counterpart. Adult serum may double the rate of the growth of fibroblast monolayers (Vajta et al., 2003) and improves substantially development of bovine embryos (Holm et al., 1999). The effect does not depend on the reproductive cycle or on the age of the donor animal.

Variations between animals may exist, but once you find a suitable donor, she may be used for a lifetime. According to our experience, out of four well-fed, healthy cows, at least one provides excellent serum for embryo culture. We suggest, therefore, to collect a small amount of blood from four candidates, test all four sera in three consecutive embryo culture experiments and use the best one for years. Collection of blood from cattle (up to one litre per session) and serum preparation is a routine task of vets and tissue culture experts.

(Please note - the once fashionable heat inactivation procedure is not really needed and may compromise the beneficial effect by destroying some growth-promoting factors.)

Let us provide an additional, highly subjective argument. In the 21st century, when we are not occupied with COVID issues and invading armies, we fight against anything synthetic. People prefer natural medications, clothes, and fertilisers. Back to nature. Should we deprive our embryos of dozens of supportive natural products - known and unknown components of serum - just because we do not precisely know what they are and how they work?

See additional information and arguments in: Vajta et al., 2008.

2 Liquid nitrogen and work safety

It is a sensitive issue, so we hide our comments in the Addendum. However, we must keep in mind that we violate work safety regulations when we work with liquid nitrogen in the embryo lab. Not the usual irrational bureaucratic red tape, but a rational guideline that may protect us from serious accidents. We do not break a single rule. We break almost all of them.

Our reasons are simple. Complying with all relevant regulations would seriously compromise our cryopreservation work. No single existing vitrification procedure (or a delicate pipetting) can be performed efficiently using the required thick gloves. We need naked eyes for the microscopes and would need safety goggles for rapid cooling and warming. We simply have no time to change between these work phases. And so on.

We have no idea how to resolve this conflict, and we are grateful to authorities who - either inadvertently or deliberately (for the noble purpose) tolerate our unruly behaviour. However, we should always be aware that we play with something like fire. Also, we should strictly follow rules that do not hamper our work, do not risk our oocytes and embryos and may protect us from injuries and death.

Without further arguments, let's copy-paste the relevant sentences from Volume 1, as, according to our worldwide experience, these suggestions cannot be repeated often enough.

"Rooms used for storage of liquid nitrogen dewars (more than 50 l volume in total, so practically in every human embryo laboratory) should always be equipped with safe and robust ventilation, plus a low-oxygen sensor (with displays both outside and inside the storage room) also armed with an alarm system. In case of a low (less than 18%) oxygen level, air-supplied breathing apparatus is indispensable for entering the room, even for rescuing a person in danger. One or two sets of such rescue equipment positioned close to the entry door of the storage room may save lives. Lack of these precautions has already caused a fatal accident in a European domestic animal embryo laboratory.

It should also be mentioned here that under no circumstances can liquid nitrogen be transported in an enclosed vehicle, i.e., no person should travel in the same cabin in which liquid nitrogen is being transported.

3 Efficiency and stability

Laboratory embryo production is a complicated multistep procedure with countless variables and many unpredictable factors. Accordingly, the outcome is uncertain.

We suggest two strategies to achieve an acceptable level of predictability. Firstly, disassembling the multistep procedure into small parts and evaluating the efficiency of each one individually. Secondly, standardising all contributing factors, including physical, chemical and biological constituents, all procedures, and - last but not least - the operators' skills and activities.

All these points seem obvious and evident, needless to list them. We agree. We do not claim we discovered the Philosopher's Stone. Still, according to our experience, the practical application of these principles is sparse and incidental. Therefore, we suggest a little self-analysis, then a systematic rethinking of the laboratory practice - both research and routine work - and implementing some changes if required.

3.1 Checkpoints

Fortunately, most embryo production procedures happen under a stereo- or inverted microscope, offering a possibility for morphological evaluation. We suggest using this opportunity systematically at each phase of the work. Although some points were already mentioned in the relevant paragraphs, here we list them again and complete them with other potentially useful signs.

- Day -1: Oocytes collected for maturation

  Minimum 2-3 compact layers of cumulus cells all around; no drastic morphological variations in size and the cytoplasm

- Day 0: Oocytes after maturation

  Oocytes in a single large assembled group floating in the dish; homogenously expanded cumulus investment; no differences between cumulus-oocyte complexes

- Day 1, before vortexing

  Seemingly a catastrophic look ("Landscape After the Battle"), the complexes disassembled, dispersed on the bottom of the dish; cumulus cell clusters attached to the bottom; a few sperm cells crawling around hopelessly

- Day 1, after vortexing

  Far from the expected idyllic picture: instead of the original view of MII-phase oocytes (equal size, filling approximately 90% of the space in the zona; homogenous brown cytoplasm) presumable zygotes have shrunken size, filling 70-80% of the space; dark cytoplasm with possible light areas; some dysmorphism including kidney-like shape; and in general, a kind of turbulent look. No worries, mate - these are good signs; your zygotes are happy and active.

- Day 6, afternoon

  Around 80-85% cleavage, 60-65% compacted morulae and early blastocysts, including 10-15% blastocysts

- Day 7, morning

  50% blastocyst rate, at least half of them expanded blastocysts, a few hatching or hatched

- Day 8, morning

  80 to 90% of blastocysts should be hatched

An additional, very helpful but rarely used possibility is to test an alternative route: parthenogenetic activation (PA). With the right chemicals and strictly using the correct protocol, it produces precisely the same blastocyst rates in cattle as *in vitro* fertilisation (IVF) with the most successful bull sperm.

The beauty of this approach is that you can localise the reason for compromised blastocyst rates in a single experiment.

Blastocyst rates/quality on Day 7				
PA	bad	good	bad	good
IVF	bad	bad	good	good
Problem	(oocyte or) culture	sperm or fertilisation	PA method	no problem!

If the cumulus expansion was perfect, double bad results almost always indicate embryo culture issues. Good PA and bad IVF means sperm or fertilisation problem; both are easy to identify and correct. If only the PA method is inefficient, we suggest you correct it for future tests. And if it is all good, then relax. The problem resolved itself (hopefully).

The main morphological signs of a successful procedure have been described for vitrification in the previous chapter. We need to emphasise again that - unlike in humans - the total collapse of bovine blastocysts in approx. 3-4 minutes after warming is part of the successful survival strategy; it may help rapidly remove the cryoprotectants from the blastocoel.

Following these checkpoints and controls may help to establish/improve the embryo production system and troubleshoot if the overall efficiency is compromised. Please note, just like in most biological processes (or cooking in the kitchen), good results can only be expected if all previous steps were successful. Late efforts may help to decrease the damage but won't result in impeccable outcomes.

3.2 Consistency

"If an embryo system works, DO NOT CHANGE ANYTHING, not even your underpants" (Paul J. Booth, *pers. comm.*)

Have a large supply of everything. Buy items from reliable companies, and buy large quantities from the same lot - the maximum amount that can be used within the timeframes of the expiration date.

Register every change in the supply, every event that may have the slightest effect on embryos. Not only the obligatory accident reports but unofficial, subjective notes about a strange scent or a strange temporary shade of a medium. These notes may become invaluable in retrospective troubleshooting - a nightmarish but frequent activity in embryo labs.

Try to perform everything, every small detail exactly as in the previous successful IVP-vitrification program. Try it, although it never happens so. It is impossible to repeat any embryo work exactly the same way. People who insist they can do so are superficial and disregard something which may be important. Real scientists, in contrast, do not hide their mistakes - not even unconsciously - but hunt for them, register them, and remember them.

We have heard many stories about how big discoveries arose from failed experiments. Although many of these anecdotes are colourful urban legends, some might be true. Moreover, sooner or later - if you make (and document) enough mistakes - such things will happen to you, too. Whether confessing the real story or wrapping the haphazard part in the gift paper of a bright scientific theory (a hypothesis that you have established retrospectively), your mistake may lead to the best paper of your career and a milestone in the given discipline.

But all this can only happen if the rest of your trial is performed flawlessly. Please remember: creating order and progress from chaos was the privilege of God, and even he (she?) failed in some detail.

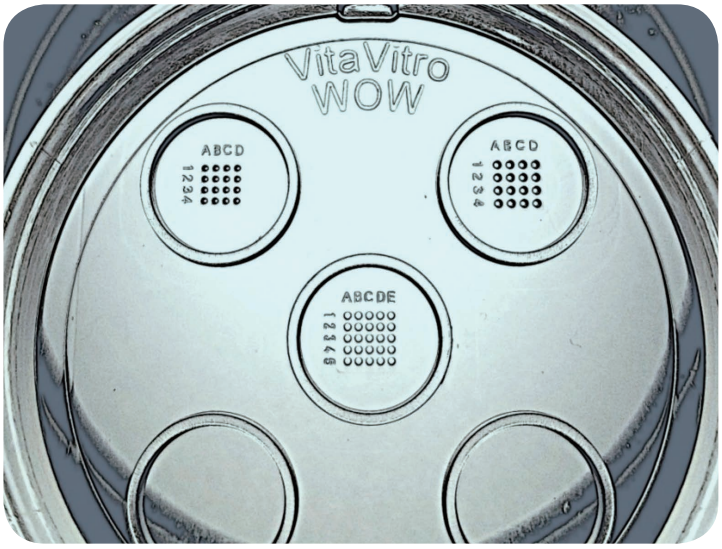
4 Special techniques for special situations

4.1 The Well of the Well (WOW) dish of VitaVitro - group vs. individual culture

(see also video: https://vimeo.com/632766530?from=outro-local)

Group culture of preimplantation stage embryos of many mammalian species, including bovine, significantly improves development *in vitro*. On the other hand, a low number of oocytes/zygotes after OPU in cattle (or stimulation in human assisted reproduction) may compromise development.

Microwell culture offers a solution to this problem. Factors secreted by the embryo are not diluted but accumulated around the surface of the embryo and support its development: paracrine factors are replaced by autocrine factors. Microwells also allow the embryo to create and maintain its optimal physical and chemical microenvironment, similar to the virtual space of the oviduct.



However, most available microwells are unsuitable for these purposes as they were produced for time-lapse investigations: their size is too large, and they have a flat bottom for optical clarity.

VitaVitro's WOW dish offers a solution for both, with microwells of various sizes and shapes, for time-lapse investigation and for optimal individual embryo culture. In the latter, a conical well with a round bottom, embryos are cultured in 0.02 µl volume. Moreover, approx. 50% of the embryo's surface is close to the wall of the well, allowing the accumulation of secreted heavy ligands, while the light products of metabolism, including ammonia, may lift and get diluted in the 50 µL volume of the drop.

By using this system, the quality and the developmental competence of individually cultured embryos are the same as those in large groups. The system also allows identification and follow-up during the whole culture period. No medium change or any other intervention during the whole culture period is required.

Dishes should be prepared the day before application. Warm both dishes, media and oil to 39°C. Add 50 µL VitaVitro Single Culture medium to each circle, including those without WOWs. Cover them with 3 mL VitaVitro Heavy Oil. Control microwells under a stereomicroscope. Bubbles may be present in a few (approx. 10%) of them but can be removed easily with a microcapillary. Incubate the dish overnight at 39°C in a 5 + 5 + 90 gas mixture and maximum humidity.

Embryos should be washed shortly in the empty drops, and then transferred close to the WOWs. With fine pipetting or pushing, each embryo should be placed into an individual well. Care should be taken to avoid mechanical shocks to the dish during transport to the incubator. Also, the door of the incubator should be opened and closed carefully during the whole culture period. Culture conditions should be the same as described above.

When removing embryos on Day 7, we suggest careful flushing instead of aspiration. Due to the conical shape, expanding blastocysts may spontaneously float out from the wells, starting on Day 7 afternoon.

See more details in: Vajta et al. 2000; Madani et al. 2022.

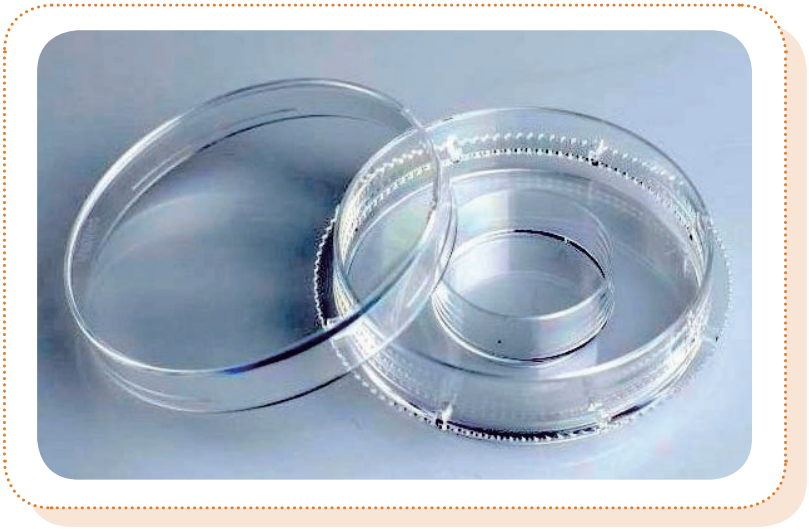
4.2 The Humdish of VitaVitro - description and instructions for use

(see also video: https://vimeo.com/manage/videos/773675929)

The VitaVitro Humdish is a purpose-designed sterile plastic dish designed for mammalian preimplantation embryo culture, especially for - but not restricted to - dry incubators or those with inconsistent, uncontrolled humidity levels.

Unique parameters of the VitaVitro Humdish ensure >95-98% humidity between Day 0 (or Day 1) to Day 7 (Day 0: day of fertilisation or ICSI) in both dry or (nominally or actually) humid incubators.

The fact that the dish itself provides humidity ensures the highest consistency, preventing any increase of osmolality during the whole culture period.



Moreover, a large amount of water stored in the outer ring also prevents temperature fluctuation during door opening or possible (but not suggested) temporary dish removal from the incubator for visual control of embryos.

Accordingly, the VitaVitro Humdish provides optimal conditions for uninterrupted culture in a single medium for single-cell embryos to develop to the blastocyst stage.

The VitaVitro® Humdish can be used in any commercially available incubator that can host standard 60 mm diameter dishes, including dry or "humid" benchtop top-loaders, box-type incubators or various new combinations of these.

Instruction for use

Suggestion: warm the Humdish to 37-38°C, and use room-temperature oil and water. Prepare the culture dish on a 37°C warm plate to avoid subsequent vapour condensation on the lid.

1. According to the actual need, make several droplets in the inner compartment of the Humdish. We suggest any volume between 5 to 20 µL. Cover the drops with 3 mL mineral oil.

   To avoid any possible increase of osmolality during drop preparation, you may consider using the drop-in method: immediately after covering the drops with oil, remove 80-90% of the drops and replace them with new medium. Use room temperature oil.

2. Add water

   Add 12 mL ultra-pure sterile water to the outer ring. Avoid splashing water into the inner ring.

3. Equilibration

   Transfer the VitaVitro Humdish into an incubator with 37°C[5], 5% CO_2, 5% O_2, and 90% N_2 atmosphere, and (preferably, to avoid any evaporation before use) maximum humidity. Incubate overnight.

4. Transfer embryos into the VitaVitro Humdish

   After washing twice in a culture drop, transfer embryos to a fresh drop and incubate them to the required stage. The Humdish provides optimal conditions for single medium-uninterrupted culture to the blastocyst stage.

Optional Supplement - Lid with a built-in humidity sensor

Warning: the supplied lid with a sensor is NOT FOR MONITORING dishes containing embryos!

The purpose of this supplementary tool is to test and prove the efficiency of the VitaVitro Humdish to establish and maintain a high level of humidity during the whole culture period in your incubator.

If you have any doubts regarding the need and efficiency of VitaVitro Humdish in your incubators,

we suggest you make two simple measurements with dishes

- with the central area filled with drops and oil, as described above, and
- with or without water in the outer ring, respectively.

Place the wire-connected control device outside the incubator and monitor the parameters during the whole period (5, 6 or 7 days) of your routine embryo cultures.

If you do not find any difference in humidity values between the two measurements, the VitaVitro Humdish is not needed in your laboratory.

See more details in: Chen et al., 2021.

REFERENCES:

Chen WB, Machaty Z, Marconetto A, Parmegiani L, Vajta G.

A Simple and Efficient Solution to Eliminate Evaporation in Mammalian Embryo Cultures.

Cell Reprogram. 2021 Oct;23(5):316-318. doi: 10.1089/cell.2021.0061.

Ferré LB, Kjelland ME, Strøbech LB, Hyttel P, Mermillod P, Ross PJ.

Review: Recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods.

Animal. 2020 May;14(5):991-1004. doi: 10.1017/S1751731119002775.

Holm P, Booth PJ, Schmidt MH, Greve T, Callesen H.

High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins.

Theriogenology. 1999 Sep;52(4):683-700. doi: 10.1016/S0093-691X(99)00162-4.

O'Neill C.

Review: The potential roles for embryotrophic ligands in preimplantation embryo development.

Hum Reprod Update. 2008 May-Jun;14(3):275-88. doi: 10.1093/humupd/dmn002.

Pieterse MC, Vos PL, Kruip TA, Wurth YA, van Beneden TH, Willemse AH, Taverne MA.

Transvaginal ultrasound guided follicular aspiration of bovine oocytes.

Theriogenology. 1991 Apr;35(4):857-62. doi: 10.1016/0093-691x(91)90426-e.

Madani S, Machaty Z, Vajta G.

An Alternative Way to Improve Mammalian Embryo Development In Vitro: Culture of Zona Pellucida-Free Embryos.

Cell Reprogram. 2022 Jun;24(3):111-117. doi: 10.1089/cell.2022.0012.

Martino A, Pollard JW, Leibo SP.

Effect of chilling bovine oocytes on their developmental competence.

Mol Reprod Dev. 1996 Dec;45(4):503-12. doi: 10.1002/(SICI)1098-2795(199612)45:4<503::AID-MRD13>3.0.CO;2-X.

Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H.

Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos.

Mol Reprod Dev. 1998 Sep;51(1):53-8. doi: 10.1002/(SICI)1098

2795(199809)51:1<53::AID-MRD6>3.0.CO;2-V.

Vajta G, Murphy CN, Macháty Z, Prather RS, Greve T, Callesen H.

In-straw dilution of bovine blastocysts after vitrification with the open-pulled straw method.

Vet Rec. 1999 Feb 13;144(7):180-1. doi: 10.1136/vr.144.7.180.

Vajta G, Peura TT, Holm P, Páldi A, Greve T, Trounson AO, Callesen H.

New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system.

Mol Reprod Dev. 2000 Mar;55(3):256-64. doi: 10.1002/(SICI)1098-2795(200003)55:3<256::AID-MRD3>3.0.CO;2-7.

Vajta G, Lewis IM, Trounson AO, Purup S, Maddox-Hyttel P, Schmidt M, Pedersen HG, Greve T, Callesen H.

Handmade somatic cell cloning in cattle: analysis of factors contributing to high efficiency in vitro.

Biol Reprod. 2003 Feb;68(2):571-8. doi: 10.1095/biolreprod.102.008771.

Vajta G, Rienzi L, Cobo A, Yovich J.

Embryo culture: can we perform better than nature?

Reprod Biomed Online. 2010 Apr;20(4):453-69. doi: 10.1016/j.rbmo.2009.12.018.

Vajta G, Parmegiani L, Machaty Z, Chen WB, Yakovenko S.

Back to the future: optimised microwell culture of individual human preimplantation stage embryos.

J Assist Reprod Genet. 2021 Oct;38(10):2563-2574. doi: 10.1007/s10815-021-02167-4.

ACKNOWLEDGEMENTS AND FINAL COMMENTS

The authors would like to thank everybody who supported this work with photographs, data, suggestions, critics, or just tolerance. We apologise to our families for the time we robbed from other - potentially more productive and helpful - activities.

Just like for Volume 1, we owe special thanks for the inspiration, continuous support and encouragement to Ms Jenny Lin, CEO of VitaVitro BioTech Co., Ltd. (Shenzhen, China), and the whole company. Some of the innovations presented and all commercial realisations were the fruits of our unique collaboration during the past eight years. We are very grateful to all members of the Embryology Laboratory of the late Danish Institute of Agricultural Sciences (Foulum, Jylland, Denmark) for the outstanding achievements in establishing many techniques described in this chapter.

We thank the creative and highly efficient designer Ms Nara Yu, for the great layout. We also owe special thanks to Dr Giselle Gamarra and Eng. Serge Lacaze, Auriva Biotechnologies, Denguin, France; Dr Morné de la Rey and Dr Robert Treadwell, Embryo Plus, Brits, South Africa, for the pictures related to animal work.

Finally, we also thank you for reaching the end of this chapter. We hope it will be helpful to your future research or commercial activities.

Volume 2 will continue with other chapters dealing with human reproductive techniques, animal somatic cell nuclear transfer and other issues. Different language versions, including Chinese, Spanish, Portuguese, French, etc., will also be available.

We hope our communication will be bi-directional. We welcome suggestions, comments and corrections. For this English version, please get in touch with Gábor Vajta via gabor.vajta@hotmail.com. Letters will be edited and added to the webpage. If necessary, we will also make relevant changes in the main text.

Items listed below can be ordered from

For more details, technical support, potential collaborations and on-site, hands-on workshops, please contact Gábor Vajta, RVT Australia, Cairns, Australia.

Email: gabor.vajta@hotmail.com

Webpage: https://gaborvajta.wixsite.com/rvt-australia

ANIMAL IVF PRODUCTS OF VITAVITRO SHENZHEN

Ordering infomation

Product Name	Specifications	Catalog No.	Shelf Life	Species
Vitrification Kit	CHM: 2 x 4.8 mL; CV1: 1 x 4.8 mL; CV2: 1 x 4.8 mL	DA01001	6 months	
Warming Kit	CW1: 2 x 4.8 mL; CW2: 1 x 4.8 mL; CHM: 1 x 4.8 mL	DA02001	6 months	
Direct Transfer Medium	4 x 4.8 mL	DA13001	6 months	
OPS and related devices	OPS + Container straw (1+1) OPS + Container straw +DT straw(1+1+1) OPS (5) OPS (10) DT straws (5) DT straws (10) Storage tube for OPS	DA08001 DA08002 DA08003 DA08004 DA08005 DA08006 DA08007	2 years	
Aspiration Medium	125 mL 250 mL 500 mL	DA04001 DA04002 DA04003	6 months	
TCM-HEPES with 2% serum	60 mL	DA05001	3 months	
Transfer Medium (with 20% serum)	4 x 4.8 mL	DA14001	3 months	
IVM-Bicarbonate Medium	4 x 4.8 mL	DA07001	3 months	
IVM-HEPES Medium	4 x 4.8 mL	DA07002	3 months	
Sperm Preparation Medium	30 mL	DA10001	6 months	
Sperm Gradient Kit	Sperm Gradient (55%) 30 mL Sperm Gradient (90%) 30 mL	DA10002	6 months	
IVF Medium	30 mL	DA06001	3 months	
IVC Single Medium	4 x 4.8 mL	DA03001	3 months	
Heavy Paraffin Oil	60 mL	DA09001	2 years	
Humdish	10 pieces/bag	DA11010	3 years	
WOW dish	10 pieces/bag	DA12010	3 years	
Cooling Box	1 Set	VOPS04	N/A	