Protocol for embryo vitrification using open pulled straws

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Introduction

This vitrification protocol is a slight modification of the procedure developed by Gabor Vajta (Vajta et al. Theriogenology 45: 683-689, 1996). An older version of the procedure is available in Spanish

Reagents

1. Fetal bovine serum (Atlanta Biologicals, Cat. No. S11150H)
2. Sucrose (SIGMA, S1888-500G)
3. Dimethyl sulfoxide (DMSO) (Sigma, D2650-5X5ML)
4. Ethylene glycol (Sigma, 102466-500ML)
5. DL-Dithiothreitol (DTT) (Sigma, D9163-5G)
6. Sodium bicarbonate (Sigma, S5761-500G)
7. Medium 199 Hank's (Caisson Labs, MHP04-1LT)
8. Water for embryo transfer (Sigma, W1503-500ML)

Materials

1. Styrofoam cooler
2. Liquid nitrogen (3 L)
3. Nunc IVF 4-Well Dish Nunclon Delta Surface (Thermoscientific)
4. Straws for OPS vitrification (Minitube, Ref. 19050/0050) (see figure 1)
5. 0.5 cc straws clear radiated (Agtech ITEM #F02) (see figure 1)
   (OPS straws are placed in these for storage in liquid nitrogen)
6. Drummond Wiretrol 5 µl and disposable micropipettes
7. Precision tip tweezers with curved tip (see figure 1)
8. Blunt tipped tweezer (see figure 1)
9. ¼ to ½ cc straw plug #05-088-129-1
10. 28 mm syringe filter 0.20 micrometers (CORNING 431229)
11. 50 mL Corning tube top filter (430320)
12. 15 mL Centrifuge Tube (Corning, REF 430791)
13. Screw Cap Tube Conical/Skirt 50ml (Sarstedt, No. 62.559.205)
14. 10 ml (12 ml) NORM-JECT (Henke Sass Wolf, REF 4100-000V0)

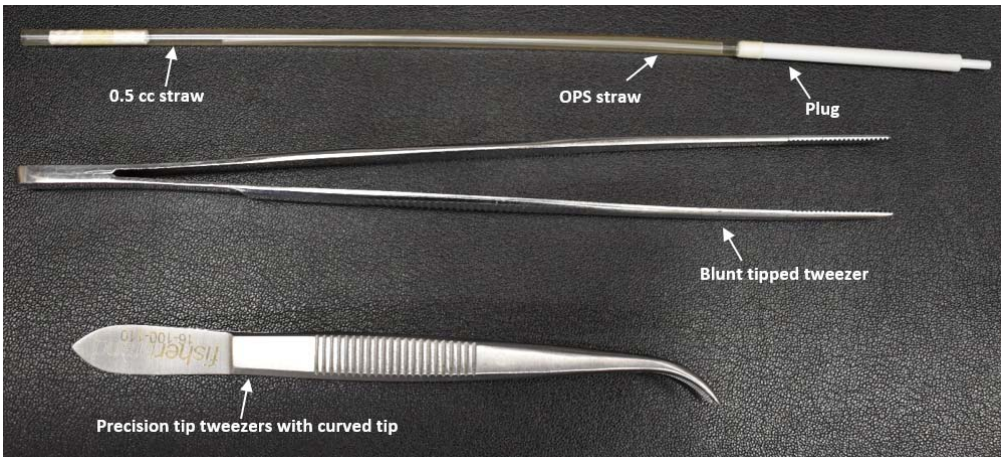

Figure 1. Devices used for vitrification and warming procedure

Media Preparation

TCM 199 medium preparation

1. Dissolve 1 Medium 199 Hank's (10.64 g/L) package with 500 ml of water in a sterile 1 L glass medium bottle.
2. Add a stir bar, place the on a stirrer, turn the stirrer on, and supplement the Medium 199-Hank's with 0.35 g sodium bicarbonate. Stir for 10-15 min.
3. Add water to the bottle containing the Medium 199-Hanks to bring to 1 L of Medium 199. Perform this step under laminar flow hood.
4. Filter the medium using a 50 mL Corning tube top filter connected to vacuum, and make 45 mL-aliquots in screw cap tubes conical/skirt 50 mL. Store at 4° C for up to 1 month.

Preparation of Holding Medium (HM)

Take a 45 mL-aliquot of TCM 199 medium, add 5 ml fetal bovine serum, mix and filter using a 50 mL Corning tube top filter connected to vacuum.

HM can be stored at 4°C for up to 2 weeks but we recommend using fresh HM each time vitrification is performed.

Preparation of Sucrose Medium (SM)

1. Weigh 3.42 g sucrose and add to the bottom of a 15 mL centrifuge tube and bring to 10 ml with HM (measured with volumetric flask). Allow sucrose to dissolve with occasional agitation if needed.
2. Filter the SM using a 10 ml NormJect syringe and a 28 mm Syringe Filter (0.20 µm).

SM can be stored at 4°C for up to 2 weeks but we recommend using freshly-prepared medium each time that the vitrification procedure is performed.

Preparation of Vitrification Solution (VS) (prepared in vitrification dish (Figure 2)

Place 670 µl SM in well 4 and add 165 µl 16.5% (v/v) ethylene glycol and 165 µl 16.5% (v/v) DMSO and gently mix by pipetting up and down.

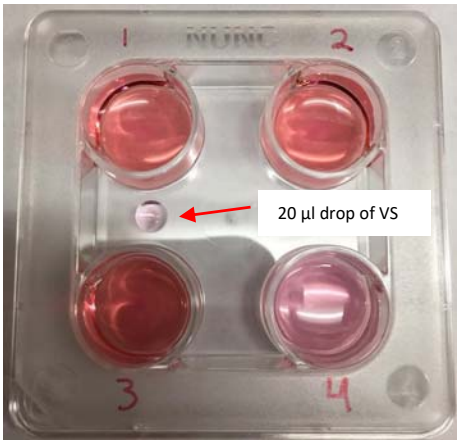

Figure 2. Vitrification Dish

Preparation of Vitrification Dishes (see Figure 2)

1. Pipette 800 µl HM into well 1
2. Pipette 800 µl HM into well 2
3. Place 850 µl HM in well 3 and add 75 µl 7.5% (v/v) ethylene glycol and 75 µl 7.5% (v/v) DMSO. Gently mix by pipetting up and down. The resultant solution is called equilibration solution (ES).
4. Place 670 µl SM in well 4 and add 165 µl 16.5% (v/v) ethylene glycol and 165 µl 16.5% (v/v) DMSO. Gently mix by pipetting up and down. The resultant solution is called vitrification solution (VS).
5. Warm vitrification dish on a slide warmer set up at 38.5°C for 10 min.
6. Cover the vitrification dish with aluminum foil.

After 10 min, gently mix the medium contained in each well. Now, it is ready to use.

Vitrification Procedure

1. Remove blastocysts from the embryo culture drop and place them in well 1 of the vitrification dish. Gently wash them three or four times and then pass them to well 2, as quickly as possible. *Pass the blastocysts to the second well with minimum amounts of medium and oil.*

2. Make a 15-20 µL drop of VS (medium from well 4) close to the center, right or left side on the vitrification dish as shown in figure 2 as convenient (*We recommend making the drop as shown in Figure 2 for right-handed practitioners).*

3. Take 4 to 5 blastocysts from the second well (with as little HM as possible), and place them in ES in well 3 for 3 min. Try to keep them together on the bottom of the well. Initially, embryos will floating but will settle to the bottom with time.

We recommend vitrifying embryos in groups of 4 until you acquire more technical skill.

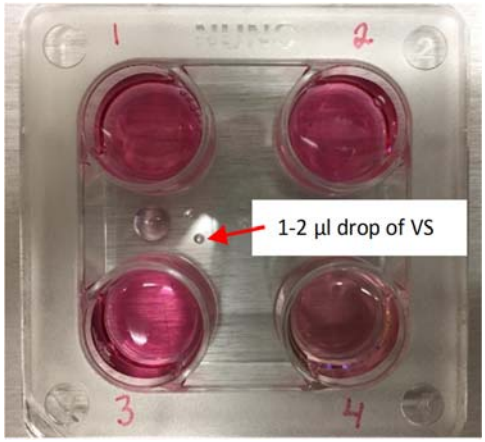

Figure 3. Vitrification dish showing the position of the 1-2 µl drop of VS placed between wells. This drop is a critical feature of the vitrification procedure.

4. After 3 min, take the embryos (with the minimum ES possible) from the ES well and transfer them to the 15-20 µL drop of VS. Expel the embryos, then aspirate them again, expel them one more time. Repeat this process twice or three times.

5. Load the embryos in 1 to 2 µL of VS, then expel the embryos anywhere (we recommend making the drop close to the 15-20 µL drop of VS, see figure 3) on the vitrification dish trying to make a 1-2 µL drop. Once the 1-2 µL drop is made, hold the OPS straw at a 60-70° angle to the horizontal bottom, and touch the 1-2 µL drop containing 4 or 5 embryos with the narrow end of the OPS straw. The OPS straw will aspirate the embryos by capillary effect.

6. Once embryos are loaded in the OPS straw, vitrify them by plunging immediately in liquid nitrogen. *We recommend taking embryos up from the ES in 1 µL volume. Try to do steps 4, 5, and 6 in a total time of ~25 sec.*

7. Keep the OPS straw immersed in the liquid nitrogen for 5-8 sec, then put the OPS straw in a 0.5 cc straw, and seal the 0.5 cc straw with a ½ cc straw plug. *This prevents the OPS straw from getting lost.*

8. Place the 0.5 cc straw containing the OPS straw in a goblet. Store the embryos in a liquid nitrogen tank until warming procedure is performed.

We make 2 or 3 holes close to the cotton seal of the 0.5 cc straw with an 18-gauge needle to allow nitrogen to go into the 0.5 cc straw. Doing so keeps the 0.5 cc straw from floating in the nitrogen container. However, be aware that the sample (embryos) will be directly in contact with liquid nitrogen and exposed to any pathogens in the liquid nitrogen. To avoid potential contamination, do not puncture the cotton seal but devised some method to secure the straws in the goblet.

Preparation of Warming Dish (see Figure 4)

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Figure 4. Warming dish

- Well 1: Place 800 µl HM + 400 µl SM
- Well 2: Place 800 µl HM + 400 µl SM
- Well 3: Place 800 µl HM + 200 µl SM
- Well 4: Place 800 µl HM

1. Warm the warming dish on a slide warmer at 38.5°C for 10 min.
2. Cover the vitrification dish with aluminum foil.

After 10 min, gently mix the medium contained in each well. It is now ready to use.

Warming Procedure

1. Place the warming dish under the microscope and focus on well 1.
2. Remove the OPS straw from the 0.5 cc straw and keep it in air for 3-5 sec holding the OPS straw between your middle finger and thumb.
3. Immerse the tip of the OPS straw in well 1. When the column where the embryos are vitrified begins melting, close the top end of the OPS straws with your index finger to expel embryos.
4. After embryos are expelled, immediately transfer the embryos to the well 2 in as little medium as possible.
5. Keep embryos in well 2 for 5 min.
6. Transfer the embryos to well 3 in as little medium as possible.
7. Keep embryos in well 3 for 5 min.
8. Transfer the embryos to well 4 in as little medium as possible.
9. Keep the embryos in well 4 for 5 min.
10. Embryos are now ready for transfer. Click the link to see details for loading embryos into straws. http://animal.ifas.ufl.edu/hansen/lab_protocol_docs/preparation_embryos_transfer.pdf

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