

DAY 0 IN VITRO FERTILIZATION

INITIAL PREPARATION FOR SPERM PURIFICATION AND FERTILIZATION

Materials and Equipment Needed

Laminar flow hood
90% Percoll
IVF-TALP
HEPES-TALP
Sp-TALP
Sp-TL
7 x 15 ml conical centrifuge tubes
3 centrifuge carriers
Dish, 35mm x 10 mm Petri dishes, Falcon # 351008
Thawing unit (Citothaw)
PHE
Pipet tips (1000 and 200 μ l) and pipettors
Sterile pipets (1 x 5 ml and 1 x 2 ml)
Plastic Pasteur pipet

Procedures

The following procedures are done on the morning of day 0 (or a minimum of 2-3 hours before fertilization) so that all supplies and media are ready when fertilization procedures are initialized.

1. Fill a total of 4-5 15 ml conical tubes with HEPES-TALP. Tighten the caps and place in the warm oven.
It may seem like 4-5 tubes is a lot but some of these tubes of HEPES-TALP will be used later in the day.
2. Add 10 ml Sp-TALP to a 15 ml conical tube. Tighten the cap and place in the warm oven.
3. Add 5 ml IVF-TALP to a 15 ml conical tube. Leave cap loose and place in the incubator.
4. Prepare enough 35 mm x 10 mm Petri dishes for the number of oocytes that are maturing (~200 oocytes/plate). Add 1700 μ l of IVF-TALP to each well and allow medium to equilibrate and warm up for 2 h.
5. Place 1.5 ml of 90% Percoll and 1.5 ml of Sp-TL to one 15 ml conical tube. Mix to make a solution of 45% Percoll. In another 15 ml conical tube, add 3 ml of 90% Percoll. Make a Percoll gradient (45% over 90%) by slowly layering the 45% Percoll over the 90% Percoll by the use a plastic Pasteur pipet. Cap and place in the warm oven.
6. Plug-in citothaw (i.e., thawing unit) so the water warms up.
7. Place 1-2 aliquots of PHE in the oven (25 μ l per well) (remember to cover the tube with aluminum foil).

8. Place 2-3 centrifuge carriers in the oven.

PREPARATION OF OOCYTES FOR FERTILIZATION

Materials and Equipment Needed

X-Plate
HEPES-TALP (pre-warmed)
IVF-TALP (pre-warmed and equilibrated in CO₂)
Sp-TALP (pre-warmed)
Percoll gradient (pre-warmed)
Dissecting microscope
Inverted microscope
Rack for tubes (place in front of the heater)
Heater
Scissors (wipe with ethanol)
Semen straw plunger (wipe with ethanol)
Light microscope
Plastic sterile Pasteur pipets
Pipettor (200 and 1000 µl)
Pipet tips
Instrument to pick-up oocytes
Slide warmer (set at 38.5°C)

Procedure

1. Place X-plate on the slide warmer and add ~5 ml of HEPES-TALP to each of the wells.
2. Remove one or two dishes containing matured oocytes and place on the slide warmer.
3. Transfer COCs from about 20 microdrops of OMM + supplement to the X-plate containing HEPES-TALP.
Repeat as necessary until all oocytes have been placed in a plate in groups of ~200.
4. Withdraw the dish (containing pre-equilibrated IVF-TALP (1700 µl/well) from the incubator and transfer a group of ~200 oocytes from a corner of the X-plate to the 35 x 10 petri dish.
5. Return dish with the oocytes to the incubator until fertilization.

SPERM PURIFICATION USING PERCOLL

Materials and Equipment Needed

The materials and equipment for Preparation of Oocytes is also used for sperm purification

Procedure

Note: It is critical that spermatozoa not be exposed to cold shock. A space heater in front of the area where the sperm work will be performed can aid in preventing cold shock to the sperm cells (make sure you don't roast the sperm by keeping it too close to the heater). Also, make sure that all media used for sperm are warmed to 38.5°C before use. Media necessary for fertilization should be prepared at least 2 h prior to IVF (HEPES-TALP, Sp-TALP, IVF-TALP, 90% Percoll).

1. Thaw 2-3 straws of semen in the citothaw for 45-60 seconds.

An alternative way to thaw semen straws is to place straws in a beaker of warm tap water (37° C). Note also that it is usually not necessary to use 2-3 straws. One straw provides enough semen for 4 wells (100-120 oocytes). Unless the choice of sire is critical, we typically pool semen from 2-3 bulls (1 straw per bull) to enhance the probability that sperm from at least one sire will perform well.



Figure 14. Transfer of straws of semen from liquid nitrogen tank to the thawing unit (citothaw).

2. Wipe the straw dry with a kimwipe, cut the tip of the straw with a scissors and expel contents of the straw onto the top of the Percoll gradient (Figure 15). Care must be taken so that the gradient is not disturbed and the semen lie on top of the 45% layer.

To facilitate removal of the semen, a homemade plunger can be devised to fit into the straw. Care should be taken not to push the cotton plug into the gradient.

3. Place the conical tube containing the semen and Percoll gradient into a centrifuge carrier that has been pre-warmed to 38.5°C, and centrifuge at 1000 x g for 10 min.

4. After centrifugation, collect sperm pellet from the bottom of the conical tube (Figure 16).

Percoll is toxic to sperm cells and the pellet should be collected with a minimum of Percoll.

5. Place the sperm pellet into a 15 ml conical tube containing 10 ml Sp-TALP and place in a warm centrifuge carrier before centrifuging for 5 min at 200 x g. *The exact speed is probably not critical - do a low-speed centrifugation.*

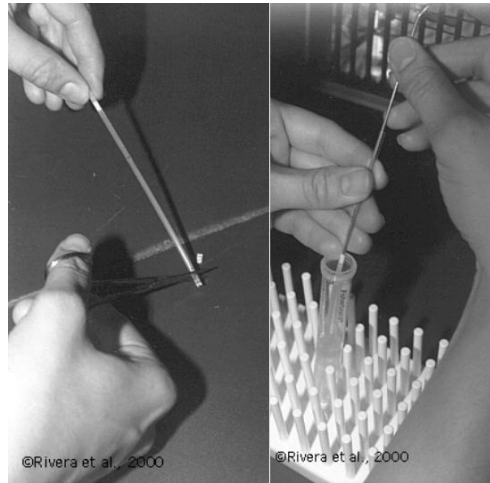


Figure 15. Layering of sperm onto Percoll. After cutting the tip of the straw (Left panel), the contents of the straw are expelled onto the top of the Percoll gradient (right panel). Here, removal of the semen is facilitated by using a homemade plunger.

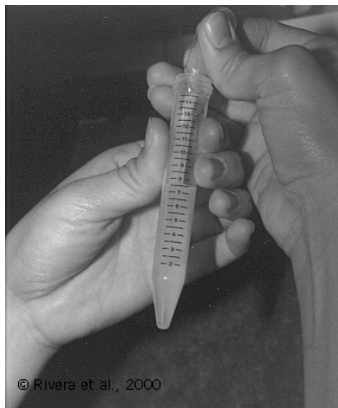


Figure 16. Removal of sperm from the bottom of the Percoll gradient.

6. Remove the supernatant with a Pasteur pipet while being careful not to disturb the pellet (Figure 17). *This step must be done quickly because motile sperm will swim out of the pellet. If the pellet is accidentally disturbed, stop the procedure and re-centrifuge.*

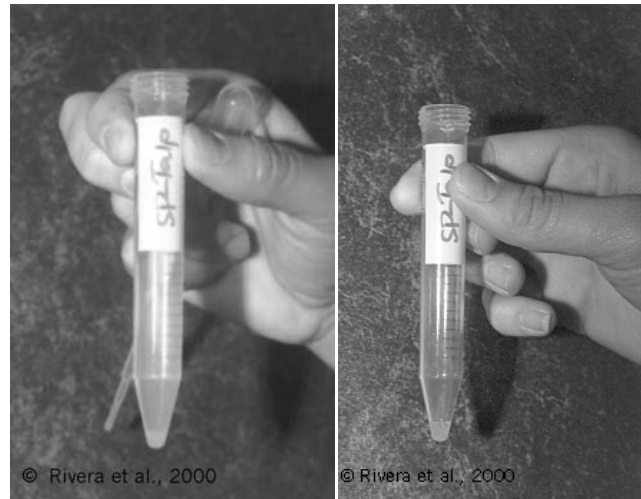


Figure 17. Washing sperm in Sp-TALP. The left panel shows the washed and centrifuged sperm. The right panel shows the pellet of sperm remaining in the tube after aspiration of the supernatant.

7. Determine dilution required to bring sperm to a concentration of $26 \times 10^6/\text{ml}$ (this will produce a final concentration of sperm in the fertilization drop of $1 \times 10^6/\text{ml}$). To do so, add $10 \mu\text{l}$ sperm suspension to $90 \mu\text{l}$ water to kill sperm. Load $10 \mu\text{l}$ of sample onto a hemacytometer. Count the number of sperm in 5 squares (Figure 18) and multiply sperm number by 500,000 to determine concentration per ml. Dilute the sperm using IVF-TALP that has been pre-equilibrated in the incubator. *Alternatively, add ~ 0.5-1.0 ml of pre-equilibrated IVF-TALP to the sperm pellet (the bigger the pellet, the larger the amount of IVF-TALP to add to the pellet) and look at the concentration of sperm cells until it appears to be ~ $26 \times 10^6/\text{ml}$ (possible with practice).*

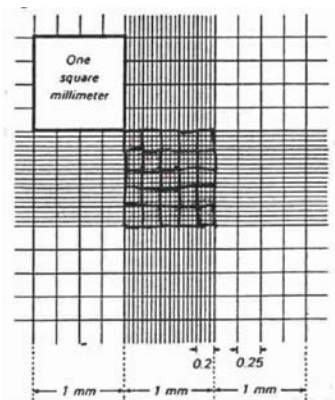


Figure 18. Hemacytometer used for counting sperm. The total number of sperm in five of the smaller boxes (outlined by freehand) are counted and multiplied by 500,000 to determine concentration per ml. For more details on how to use a hemacytometer, [click here](#).

FERTILIZATION

Materials and Equipment Needed

slide warmer (set at 38.5°C)
purified sperm
PHE

Procedure

1. Remove dishes containing matured oocytes from the incubator and place on the slide warmer.
2. Add 120 μ l sperm preparation and 80 μ l PHE mix into each dish.
When pipetting the sperm, place the pipette in the middle of the sperm suspension rather than on the bottom to avoid grabbing debris that can settle to the bottom of the tube.
3. Return dish to incubator for 8-10 h. *Many people do fertilization for 18-20 h. When we were establishing IVF in our lab, 8-10 h gave better results than longer incubation times. We have gotten good results with 18-20 h fertilization times. While longer fertilization times make it easier to remove cumulus cells after fertilization, we also see an increased rate of parthenogenesis. To determine the incidence of parthenogenesis, one well should be prepared without sperm, but with PHE. After 8 - 10 h, place these oocytes into a separate culture medium drop and culture for 3 days before looking at rate of parthenogenesis.*

PREPARATION OF EMBRYO CULTURE DROPS

Materials and Equipment

KSOM (alternatively use CR1aa or SOF)
60 x 15 mm petri dishes
mineral oil

Procedure

1. Prepare embryo culture medium (modified KSOM) at least 2 h before removing embryos/oocytes from the fertilization drops.
2. Make enough 50 μ l microdrops of culture medium (30 oocytes/embryos per drop) in 60 x 15 mm petri dishes and cover with mineral oil. *We also prepare 25 μ l microdrops when we wish to culture fewer numbers of embryo. Typically, we place 25-30 embryos in a 50 μ l microdrop and 5-10 embryos in a 25 μ l microdrop.*
3. Place the dishes in the incubator to warm up and equilibrate.

TRANSFER OF FERTILIZED OOCYTES INTO EMBRYO CULTURE DROPS (8-10 h post IVF)

Materials and Equipment Needed

Vortexer
Timer
1.5 ml Dolphin microcentrifuge tube and rack
X-plate (with prewarmed HEPES-TALP)
Plastic Pasteur pipet
Heater (placed in front of the microscope)
Slide warmer (set at 38.5°C)
Dissecting microscope
Instrument to pick-up embryos

Procedure

1. Rinse a 1.5 ml microcentrifuge tube with HEPES-TALP and leave a small (~50 µl) volume of HEPES-TALP in it.
2. Place X-plate on the slide warmer and add ~5 ml of HEPES-TALP to each of the wells.
3. After microscope and air have been warmed sufficiently, remove one fertilization dish from the incubator.
4. Remove oocyte-cumulus complexes (now called putative zygotes since many of them have been fertilized) from the fertilization dishes and place in the microcentrifuge tube. Up to 300 embryos can be loaded in one microcentrifuge tube.
5. Repeat steps 3 and 4 until all dishes have been processed.
6. Remove cumulus cells from embryos/oocytes by vortexing (Figure 18) the tube containing the embryos/oocytes for 3-4 minutes.
--A good technique is to press the tube hard so that the fluid is propelled to the top of the tube. Then rapidly, take the tube off the vortexer and repeat (i.e., kind of bounce the tube on the vortexer).
--If removal of all cumulus cells is imperative, vortex in the presence of hyalurodinase.



Figure 18. Vortexing COCs to remove cumulus cells.

7. Transfer the putative zygotes from the tube to the X-plate and rinse tube 2-3 times with HEPES-TALP to gather all embryos.

8. Wash embryos/oocytes 2-3 time by transferring them from one well to the next to clean them of cells and debris.

Notes on steps 7-8: To avoid overflow, leave well #1 empty, place HEPES-TALP in wells #2 and #4 and embryo culture medium (KSOM) in well #3. Add embryos/oocytes to well #1, and rinse the centrifuge tube 2-3 times with HEPES-TALP from well 4. Remove all bubbles with the pipette to aid in visualization of the embryos and place the bubbles in well 4 (because embryos sometimes get stuck in the bubbles). Transfer embryos sequentially from well 1 to wells 2, 4 and 3.

9. Finally, transfer the putative zygotes to microdrops of pre-equilibrated culture medium [i.e. modified KSOM (KSOM-BE) or CR1aa]. *We typically add 30 embryos/oocytes per microdrop but embryos can be cultured at other densities. We often prepare 25 μ l microdrops when we wish to 5-10 embryos per drop.*

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