IN VITRO FERTILIZATION WITH SEX-SORTED SEMEN

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PREPARATION OF FERTILIZATION PLATES

Materials

Talp-Hepes
IVF-Talp
Puresperm® gradient (Nidacon)
Pipet tips and pipetors
60x15 mm petri dishes
15 conical centrifuge tubes
Mineral oil
PHE
Citothaw
Microcentrifuge tubes

Procedure (prepare everything at least 2 h before fertilization)

1. Prepare one microcentrifuge tube with 500 µl of IVF-Talp and label the tube "wash" (prepare one tube per straw to use).
2. Prepare one microcentrifuge tube with 500-1000 µl of IVF-Talp (the range depends on the number of groups of COCs to be washed. Pprepare one tube per person doing IVF. This tube will be used for washing oocytes prior to transferring into IVF drops.
3. Remove Puresperm® gradient (both 40 and 80 layers) from the refrigerator.
4. Write "Puresperm ♂" and "Puresperm ♀" on empty microcentrifuge tubes (one tube per gradient to be prepared) *Note this step assumes that both X and Y sperm will be prepared. Other labels can be used as desired for specific purposes.*
5. Write "Pellet ♂" and "Pellet ♀" on empty microcentrifuge tubes (one tube for each sex).
6. Fill enough 15 ml conical tubes with Talp-Hepes for the number of oocytes that were collected (2-3 tubes should be sufficient for washing 300 oocytes before and after fertilization).
7. Remove 1 aliquot of PHE from the -20°C freezer, wrap in aluminum foil .
8. Place the tubes containing Talp-Hepes, Puresperm® gradient, empty "Puresperm ♂ and ♀" microcentrifuge tubes, pellet ♂ and ♀ tubes, and PHE in the oven and allow to warm for at least two hours prior to fertilization.
9. Prepare fertilization plates by making 60 µl drops of IVF-Talp. First make 30 µl drops, cover with mineral oil, then add an additional 30 µl. A total of 30 oocytes will be added to each drop, so the number of drops will depend on the number of oocytes that need to be fertilized.
10. Place the fertilization plates, "wash" tubes, and the tube with 500-1000 µl of IVF-Talp (open lids) inside the 5% CO_2 incubator and allow media to equilibrate for at least 2 hours prior to fertilization.
11. Fill the citothaw with fresh deionized water and plug in so that it can warm-up.

Fertilization

Materials

X-Plate Fisher (# FB087582)
Talp-Hepes (pre-warmed)
IVF-Talp (pre-warmed and equilibrated in 5% CO_2)
Puresperm® gradient (pre-warmed)
Plastic (LDPE) sterile (transfer) pipets
Blue and yellow pipet tips and pipetors
Microcentrifuge

Procedure

1. Place an X-plate on the slide warmer and add ~2-3 ml of H-SOF to each of the wells as necessary.
2. Remove one or two dishes containing matured oocytes and place on the slide warmer.
3. Transfer COCs from 3 microdrops (~30 COCs) to the X-plate containing Talp-Hepes, repeat as necessary until all oocytes have been placed in a plate in groups of ~30 COCs and wash the groups of COCs two times by transfer from one corner to the next of the X-plate well.
4. Withdraw the fertilization plate and the tube with 500-1000 µl of IVF-Talp from the incubator. Using Pasteur pipets, make drops of IVF-Talp on the lid of the X-plate (the number of drops is the same as the number of COCs groups being washed).
5. Transfer a group of ~30 oocytes from a corner of the X-plate to each IVF-Talp drop for a final wash. Then, transfer COCs to fertilization drops on fertilization plates. *Use only a wiretrol or Drummond microdispenser to transfer the oocytes from IVF-Talp to fertilization plates so the minimum volume of medium is transferred to the fertilization drops.*
6. Return fertilization plate with the oocytes to the incubator until the moment of fertilization.
7. Prepare Puresperm® gradient by slowly layering the 40 layer on top of the 80 Puresperm® layer (200 µl per layer) in a pre-warmed, pre-labeled microcentrifuge tube, return gradient to the oven.
8. Thaw the needed number of straws of semen for 30 sec in the citothaw. Make sure to dry the straws with a kimwipe to remove all of the water.
9. Slowly expel the semen from the straw on the top of the Puresperm® gradient. Use one Puresperm® gradient per straw of semen. *Ensure that the tip of the straw is in contact with the wall of the tube to avoid spraying the sperm on to the gradient.*
10. Place the microcentrifuge tube containing the isolate gradient and semen into a microcentrifuge and centrifuge at 6,000 rpm for 5 min.
11. Following centrifugation, remove most of the isolate gradient with a 1000 µl pipettor (blue tips) leaving ~ 100 µl at the bottom; then with a new tip mix the sperm pellet from the bottom of the tube with IVF-Talp (half of the volume from the wash tube) and slowly place the pellet into the unused microcentrifuge "wash" tube.
12. Place the tube containing the IVF-Talp and sperm into the microcentrifuge and centrifuge at 3,000 rpm for 3 min.
13. During this second centrifugation, calculate the volume of semen necessary to fertilize all the drops you have. Define the volume of semen to be collected (20 µl per drop) and adjust a 200 µl pipetor for this defined volume (e.g. if you have 4 drops to fertilize, 80 µl of semen are needed. Adjust the pipetor to ~90 µl).
14. Following centrifugation, slowly take the sperm pellet at the bottom of the microcentrifuge tube with your adjusted pipetor (for the example above, take ~90 µl of the pellet). Transfer the pellet to the pre-warmed microcentifuge tubes (labeled "pellet").
15. If more than one straw of semen was used, combine the sperm pellet from each wash tube into one microcentrifuge tube (so, if two straws are to be combined, and needed 80 µl, we would add 45 µl to each pellet then combine them to have 90 µl) .

16. In the "pellet" tube, the sexed semen is ready to fertilize.

Since sperm concentration is low in sexed-sorted straws, there is no need to calculate sperm concentration in the drops. Generally, one straw of sexed semen should be enough to produce a 100 µl sperm pellet after both centrifugation, which is enough for five IVF drops (this will depend very much on the bull used)

17. Add 20 µl of the sperm "pellet" suspension and 3.5 µl of PHE to each fertilization drop.
18. View the fertilization drops under a microscope to confirm that the sperm has been added to each drop and also that the sperm are motile.
19. Place the fertilization plates back into the incubator and allow the sperm and oocytes to co-incubate for 12-18 h.

Note on Contamination of Semen

Some straws of sexed semen contain a bacterium that is resistant to the antibiotics commonly used in IVF media. Often, a brown cloud of microorganisms is seen surrounding COCs after fertilization. Such contamination has severe deleterious effects on the outcome of IVF. Personal communication from Fuliang Du (Evergen) indicates that the antibiotic Amikacin can sometimes resolve the problem. Amikacin can be obtained from Med-Shop Total Care Pharmacy (Longview TX 75605; 1-888-769-4710) at a concentration of 50 mg/ml. The working solution is 20 µg/ml (40 µl into 100 ml solution). All solutions used for IVF and culture should receive Amikacin.

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