

IN VITRO FERTILIZATION WITH SEX-SORTED SEMEN

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PREPARATION OF FERTILIZATION MEDIA

Materials

Hepes-Talp

Hepes Wash medium - Hepes-Talp containing 10 mM caffeine

IVF-Talp containing 2 mM caffeine

90% Percoll

Pipet tips and pipetors

60 x 15 mm petri dishes

7 x 15 mL conical tubes

Mineral Oil

PHE

Citohaw

Procedure

- 1) Prepare 2-3 tubes of Hepes-Talp using 15 mL conical tubes. The number of tubes will depend on the total number of oocytes that were collected.
- 2) Prepare 300 μ L of 90% Percoll in a 2 mL microcentrifuge tube.
- 3) Prepare 300 μ L of 45% Percoll by mixing 150 μ L of 90% Percoll and 150 μ L of Hepes-Talp in a 2 mL microcentrifuge tube.
- 4) Prepare 1 mL of Hepes Wash medium in a 2 mL microcentrifuge.
- 5) Make 1 tube of 90% Percoll, 45% Percoll and Hepes Wash medium for each straw of semen that will be used for fertilization.
- 6) Place the Percoll, Hepes-Wash, and Hepes-Talp in the oven to warm.
- 7) Prepare fertilization plates by making 60 μ L drops of IVF-Talp + caffeine in a 60 x 15 mm petri dish. Cover drops with mineral oil. Prior to fertilization, 30 oocytes will be added to each microdrop of IVF-Talp so the number of drops will depend on the number of oocytes that need to be fertilized.
- 8) Place 500 to 1000 μ L of IVF-Talp + caffeine into a 15 mL conical tube. This will be used for diluting the sperm to the desired final concentration so the total amount will depend on the amount of sperm that needs to be diluted. This volume will usually be enough for 3-4 straws of sex sorted semen.
- 9) Place the fertilization plates and IVF-Talp + caffeine inside the 5% CO₂ incubator and allow the media to equilibrate for at least 2 hours prior to fertilization.
- 10) Remove 1 aliquot of PHE from the -20°C freezer, wrap in aluminum foil and place inside the oven to warm.
- 11) Fill the citohaw with fresh deionized water and plug in so that it can warm-up.

Fertilization

Materials

X-Plate

HEPES-TALP (pre-warmed)

Hepes Wash Medium (pre-warmed)

IVF-TALP + caffeine (pre-warmed and equilibrated in CO₂)

90% and 45% Percoll gradients (pre-warmed)

Plastic sterile Pasteur pipets
 Pipet tips and pipetor
 hemacytometer

Procedure

- 1) Place an X-plate on the slide warmer and add ~2-3 ml of HEPES-TALP 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 about 3 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 ~30.
- 4) Withdraw the dish (containing pre-equilibrated IVF-TALP+caffeine) from the incubator and transfer a group of ~30 oocytes from a corner of the X-plate to each fertilization drop.
- 5) Use only a wire trol or Drummond microdispenser to transfer the oocytes so that the minimum volume of HEPES-TALP is transferred to the fertilization drops.
- 6) Return dish with the oocytes to the incubator until fertilization.
- 7) Prepare 45/90% Percoll gradient by slowly layering the 45% Percoll gradient on top of the 90% Percoll gradient. Return 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 top of the Percoll gradient. Use one Percoll gradient per straw of semen,
- 10) Place the microcentrifuge tube containing the Percoll gradient and semen into a microcentrifuge and centrifuge at 600 x g for 10 min.
- 11) Following centrifugation, remove the sperm pellet from the microcentrifuge using a Pasteur pipet and slowly place sperm pellet into the microcentrifuge tube containing the HEPES Wash Medium.
- 12) Place the tube containing the HEPES Wash Medium and sperm into the microcentrifuge and centrifuge at 300 x g for 5 min.
- 13) Following centrifugation, remove the supernatant leaving the sperm pellet undisturbed at the bottom of the microcentrifuge tube.
- 14) If more than one straw of semen was used combine the sperm from each HEPES Wash tube into one microcentrifuge tube.
- 15) Add 50 to 100 µL of IVF-TALP + caffeine to the sperm suspension (this volume will depend on the total number of straws of semen used) and then measure the total volume in the microcentrifuge tube.
- 16) Remove 2 µL of the sperm suspension and place into a microcentrifuge tube containing 38 µL of water. This will be used to determine the sperm concentration using a hemacytometer. Place the sperm suspension back into the incubator until the sperm concentration is calculated.
- 17) Add 10 µL of the sperm/water suspension to a hemacytometer to determine the sperm count. Calculate the sperm concentration using the equation below and dilute the sperm suspension with the pre-equilibrated IVF-TALP + caffeine to achieve the desired concentration of sperm of 4×10^6 /mL.

$$\left[\frac{\text{Sperm count from hemacytometer} \times 20 \text{ (dilution factor)} \times 50,000 \times \text{volume of sperm suspension}}{4 \times 10^6} \right]$$

- volume of sperm suspension
 = volume in mL of IVF-TALP + caffeine that need to be added to the sperm suspension to achieve a concentration of 4×10^6 /mL.

This will yield a final concentration of 1×10^6 /mL sperm in the fertilization drop.

- 18) Once the sperm suspension has been diluted to the desired concentration, add 20 μL of the sperm suspension to each fertilization drop (see [Alternative Protocols for Fertilization](#) for fertilizing in drops).
- 19) Add 3 μL of PHE to each fertilization drop.
- 20) View the fertilization drops under a microscope to confirm that sperm has been added to each drop and also that the sperm are motile.
- 21) Place fertilization plates back into the incubator and allow the sperm and oocytes to co-incubate for 8 hrs.
- 22) Follow procedures for washing oocytes after fertilization.

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 $\mu\text{g}/\text{ml}$ (40 μl into 100 ml solution). All solutions used for IVF and culture should receive Amikacin.

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