

## FERTILIZATION IN DROPS

The original protocol for fertilizing in microdrops is included here. Unless otherwise stated, procedures for sperm purification and preparation of oocytes for fertilization follows the standard protocol.

### Initial Preparation - Materials

Laminar flow hood  
90% Percoll  
IVF-TALP  
HEPES-TALP  
Sp-TALP  
Sp-TL  
7 x 15 ml conical centrifuge tubes  
3 centrifuge carriers  
4 well plates from Nunc or 5 well plates from Minitube (*both work well*)  
Thawing unit (Citothaw)  
PHE  
Pipet tips (1000 and 200  $\mu$ l) and pipettors  
Sterile pipets (1 x 5 ml and 1 x 2 ml)  
Plastic Pasteur pipet

### Initial Preparation - 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 4-well plates (Figure 2) for the number of oocytes that are maturing (30 oocytes/well). Add 600  $\mu$ 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 ml per well) (remember to cover the tube with aluminum foil).
8. Place 2-3 centrifuge carriers in the oven.

### Preparation of Oocytes for Fertilization

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 each microdrop of OMM + supplement to the X-plate containing HEPES-TALP. *For ease of handling of oocytes and to speed up this step, transfer the contents of 3 microdrops (30 matured oocytes) into each corner of the X plate. Repeat as necessary until all oocytes have been placed in the corners of the X-plate in groups of 30.*
4. Withdraw 4-well plate (containing pre-equilibrated IVF-TALP (600 ml/well) from the incubator and transfer a group of 30 oocytes from a corner of the X-plate to a well of a 4-well plate.
5. Return plate with the oocytes to the incubator until fertilization.

### Fertilization

1. Remove plates containing matured oocytes from the incubator and place on the slide warmer.
2. Add 25 ml sperm preparation and 25 ml PHE mix into each well. *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 4-well plate 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. Recently, however, we have gotten good results with 18-20 h fertilization times. In addition, longer fertilization times make it easier to remove cumulus cells after fertilization. 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 2 days before looking at rate of parthenogenesis.*

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