ALTERNATIVE SPERM PURIFICATION PROCEDURES

Percoll Purification

10X SP-TL (for Percoll)

- Prepare 10x SP-TL stock solution by dissolving the following in 100 ml water:

NaCl (Sigma S5886):	4.6750 g
KCl (Sigma P5405):	0.2300 g
$NaH_2PO_4+H_2O$ (Sigma S9638):	0.4000 g
HEPES (Sigma H4034):	2.3800 g

- Adjust pH to ~7.3, filter with a 0.2 µM Nalgene bottle-top filter (catalog number 290-4520) into a glass medium bottle and store for at least 6 months at 4°C.

90 % Percoll

- Place 64 ml of 10X SP-TL in a small beaker and add 1.344 g sodium bicarbonate and 1.44 mL Na lactate (Stock 1). Stir until bicarbonate dissolves.

- Add 576 ml Percoll, 2.53 mL $MgCl_2$ (Stock 12) and 1.25 mL $CaCl_2$ (Stock 13).
  While stirring, adjust pH to 7.3-7.45. If a precipitate forms in the Percoll solution, continue to stir. If compounds do not re-dissolve, then start over.

- *It is very easy to get precipitation if acid or base is added too rapidly during the adjustment of pH. Therefore, it is recommended that this step be done slowly.*

- Filter with a 0.22 µm filter attached into a 500 ml glass media bottle (Corning catalog number 430320) and aliquot into 50 mL plastic tubes. Store for up to 6 months at 4°C.

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 (H-SOF, SOF-FERT, 90% Percoll).

Steps 1 and 2 are done on the morning of day 0 (or a minimum of 2-3 hours before fertilization).

1. Fill a total of 4-5 15 ml conical tubes with H-SOF. 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 H-SOF will be used later in the day.

2. Place 1.5 ml of 90% Percoll and 1.5 ml of H-SOF 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.

3. Thaw 2-3 straws of semen in the citothaw for 45-60 seconds (Figure 1).
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 1. Transfer of straws of semen from liquid nitrogen tank to the thawing unit (citothaw).

4. 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 2). 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.

5. 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.

6. After centrifugation, collect sperm pellet from the bottom of the conical tube (Figure 3).
Percoll is toxic to sperm cells and the pellet should be collected with a minimum of Percoll.

7. Place the sperm pellet into a 15 ml conical tube containing 10 ml H-SOF 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.*

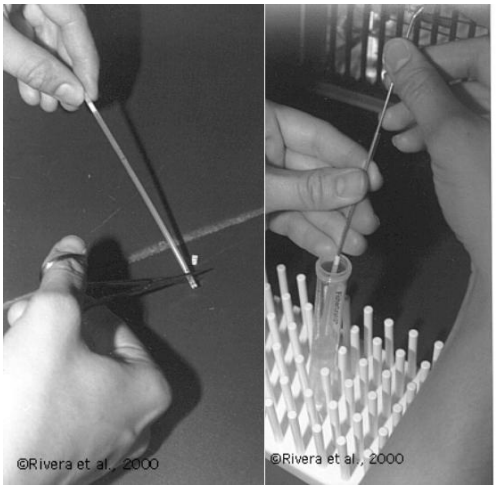

Figure 2. Layering of sperm onto Percoll. After cutting the tip of the straw (Left panel), the contents of the straw are expelled onto the top of the Percoll gradient (right panel). Here, removal of the semen is facilitated by using a homemade plunger.

Figure 3. Removal of sperm from the bottom of the Percoll gradient.

8. Remove the supernatant with a Pasteur pipet while being careful not to disturb the pellet (Figure 4). *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.*

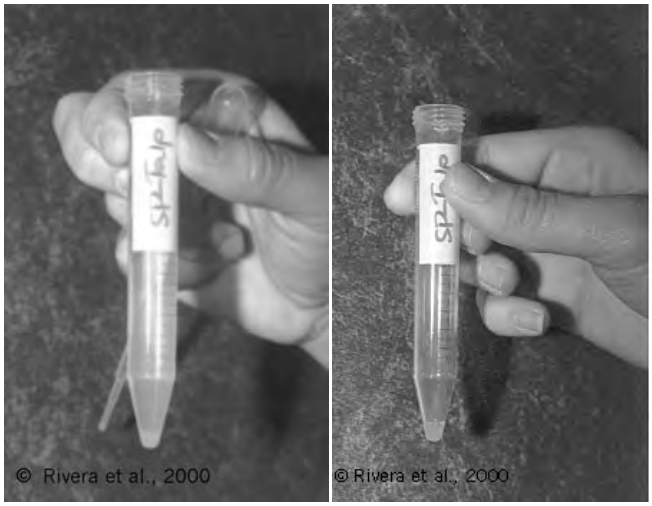

Figure 4. Washing sperm in H-SOF. The left panel shows the washed and centrifuged sperm. The right panel shows the pellet of sperm remaining in the tube after aspiration of the supernatant.

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

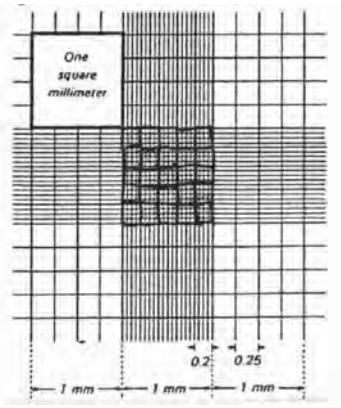

Figure 5. Hemacytometer used for counting sperm. The total number of sperm in five of the smaller boxes (outlined by freehand) are counted and multiplied by 500,000 to determine concentration per ml.

Sperm Swim-up

It is slower than the Percoll procedure and, in our hands, does not give better results. We have, however, used it as a method for evaluating treatment effects on sperm motility. We have not used the procedure since SP-TALP was replaced with H-SOF for routine sperm work but the procedure should work with H-SOF.

1. Thaw 6 to 8 straws of frozen semen in the cyto-thaw for 60 seconds. If possible, use semen from different bulls.

2. Combine contents of straws in 5 ml SP-TALP. Place sample into the incubator (38.5°C) for 5 minutes.

3. Centrifuge semen (200 x g; 5 min) and discard all but the bottom 1 ml of supernatant.

4. Prepare 4 to 5 test tubes containing 1 ml SP-TALP. Add approximately 250 μl of sperm suspension very slowly to the bottom of each tube using a 20 gauge needle and 1 ml syringe. Place tubes in incubator (38.5°C) for 1 h.

5 At the end of sperm swim-up, aspirate the top 800 μl from each tube and combine samples. Centrifuge (1000 rpm) the combined sample for 5 minutes. Discard all but the bottom 500 μl of supernate.

Glass- wool Filtration

This filtration procedure usually requires 10-15 minutes and generally yields nearly 100% viable sperm. Probably, HEPES-TALP and Sp_TALP can be replaced with H-SOF.

1. Prepare in advance 0.2 ml glass wool columns in 1 ml syringes that are rinsed 10X with Milli-Q water and autoclaved.

2. Immediately before starting purification, rinse column several times with HEPES-TALP and finally with Sperm-TALP to equilibrate column.

3. Frozen-thawed semen (3-5 straws) is washed twice with 10-15 ml Sperm-TALP by centrifugation at 200 x g (10 min) and then resuspended in 0.6-0.8 ml IVF-TALP.

4. Sperm suspension is then layered over the wet column and allowed to filter by gravity.

5. The number and viability of filtered sperm is determined.

this page was last updated April 15, 2013