The procedures for in vitro production [IVP; i.e. in vitro maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC)] of embryos described here are based on procedures developed in other laboratories at the University of Wisconsin (Neal First), and University of Missouri (R. Michael Roberts) and University of Guelph (W. Allan King).

Keep in mind that the protocols described here are not fixed but rather constantly evolve as new developments take place. Therefore, practitioners of IVP will be well advised to experiment with the procedures used, especially after reading of improvements made by other laboratories.
ABBREVIATED IVP PROTOCOL

Updated 6/23/2017

This checklist can be used to ensure that each step in the IVP procedure is completed successfully.
For complete details please refer to the unabridged protocols.

DAY -2
• Preparation of Media
  ▪ Saline

• Check incubators for accuracy of temperature, O₂ and CO₂ readings

DAY -1
• Collection of ovaries from slaughterhouse

• Preparation for oocyte collection
  ▪ Place OCM and saline in the oven (2+ h prior to oocyte collection)
  ▪ Prepare OMM microdrops and cover them with mineral oil
  ▪ Place plates in the CO2 incubator (2+ h prior to oocyte collection)

• Set up for oocyte collection
  ▪ Scalpel
  ▪ Scalpel blades
  ▪ Gloves
  ▪ 400 ml beaker
  ▪ Bench paper to cover surface

• Harvest oocyte-cumulus complexes from ovaries
  ▪ Add 100 ml of warm OCM to beaker
  ▪ Slice ovaries
  ▪ Swirl ovaries in the beaker

• Collection of cumulus oocyte complexes (COCs) by filtering collected fluid

• Set up for oocyte fluid filtering and collection
  ▪ 100 um cell strainer
  ▪ Sterile transfer pipets
  ▪ 10 ml NormJect syringe
  ▪ 18 gauge needle
  ▪ Grid plate

• Set up cell strainer over 50 ml beaker
• Pour collected fluid through cell strainer to collect oocytes
• Flip cell strainer over into a grid plate on a slide warmer
• Fill needle/syringe with OCM and rinse the debris from the strainer into the grid dish

• Search for COCs
  ▪ X-plate
  ▪ Dissecting microscope
  ▪ Searching instrument (microdispensor, wiretrol, etc.)
  ▪ Slide warmer
  ▪ Transfer cumulus oocyte complexes to X-plate and rinse through two additional wells

• Oocyte maturation
After rinsing, place cleaned COCs (10/drop) into a microdrop of pre-equilibrated OMM covered in oil  
Mature cumulus oocyte complexes for 18-24 h

- Prepare media for fertilization  
  - IVF-Talp  
  - HEPES-Talp  
  - PureSperm gradient (or other gradient system)

**DAY 0**  
- Preparation of media for fertilization (2h prior to fertilization)  
  - PureSperm (place in warm oven)  
    - 1.5 ml 40% and 1.5 ml 80%  
  - HEPES-Talp (tighten cap and place in warm oven)  
    - 20 mL HEPES-Talp per fertilization plate in 15 mL conical tubes and 1 tube with 10 mL (labeled ‘Sperm Wash’)  
  - IVF-Talp (leave cap loose and place at 38.5°C in 5% CO2)  
    - 3 mL IVF-Talp in 15 mL conical tube labeled ‘Sperm Diluent’  
    - 35 mm dishes containing 1700 ul IVF-Talp  
  - PHE (place in warm oven)

- Warm-up centrifuge canisters (place in oven)  
- Plug in Citothaw - fill with distilled water

- Matured oocytes: Setup for Washing and Fertilization  
  - X-Plate with HEPES-Talp  
  - Searching instrument  
  - Dissecting microscope  
  - Heater  
  - Scissors  
  - Semen straw plunger  
  - Inverted microscope  
  - Small petri-dish  
  - Rack for tubes (place in front of the heater)  
  - Slide warmer Plastic sterile Pasteur pipets  
  - Pipette (25 ml)  
  - Pipet tips

- Matured oocytes: Washing and Fertilization  
  - Add 5 ml HEPES-Talp to each well of X-plate  
  - Transfer about 200 COCs to each well  
  - Transfer COCs to 35 mm dish with 1700 µl/plate IVF-Talp

- Sperm preparation (working in front of the heater)  
  - Place 1-3 straws of semen in citothaw  
  - Layer semen on top of Pure-Sperm gradient  
  - Place Pure-Sperm tube in a warmed centrifuge canister  
  - Centrifuge for 10 min at 1000 x g  
  - Collect semen pellet with a Pasteur pipet  
  - Place pellet into the 10 ml HEPES-Talp tube  
  - Place HEPES-Talp tube into a warmed centrifuge canister  
  - Centrifuge for 5 min at 200 x g  
  - Pipet off supernatant down to the pellet  
  - Add IVF-Talp and determine concentration
• Fertilization
  • Add 120 ul of semen to each fertilization plate containing COCs
  • Add 80 ul of PHE to each plate
  • Place plates back in the incubator and allow fertilization to proceed for 8-10 h

• Preparation of Culture medium plates
  • Prepare plates with 50 ul microdrops of SOF-BE2 and cover with mineral oil
  • Place plates in the incubator to equilibrate for at least 2 h

• Setup for removal of zygotes from fertilization plate
  • Vortexer
  • slide warmer
  • Hyaluronidase (warm in oven)
  • Sterile dolphin-nose microcentrifuge tubes (and holder)
  • Heater (in front of microscope)
  • X-Plate
  • HEPES-Talp
  • Dissecting microscope
  • Searching instrument
  • Timer

• Remove zygotes from fertilization plate
  • Add 600 uL HEPES-Talp to tube of Hyaluronidase
  • Transfer oocytes/embryos from the fertilization plate to the microcentrifuge tube
  • Allow zygotes to settle to bottom of tube for 5 minutes
  • Remove supernatant, leaving ~100-200 ul in tube with zygotes
  • Vortex the tube for 5 min

• Use Pasteur pipet to move contents of the microcentrifuge tube to a well of the X-plate
  • Search for cumulus-free zygotes
  • Wash 2X in HEPES-Talp
  • Transfer in groups of up to 30 to the SOF-BE2 microdrops

• Place culture plate in the back of the incubator

DAY 3
• Determine cleavage rate (be quick)

DAY 7-9
• Collect data on blastocyst development

this page was last updated June 23, 2017
all original material © Rocio Rivera, Peter J. Hansen et al., 2000-2017
DISHWASHING AND AUTOCLAVING

Soap Solutions

Soap Bath (to wash glassware and instruments): Dilute PCC concentrate (Pierce Chemical cat 72290) in deionized water according to manufacturer’s instructions.

7X Cleaning solution (MP Bio 0976676095): fill a squirt bottle for ease of use.

Dishwashing

Dirty dishes are rinsed in water, any tape immediately removed, and soaked in soap bath until dishes are done. Do not leave dirty dishes to air dry and do not leave them with traces of medium inside.

Glassware, caps, collection medium carboy, stir bars, and instruments

1) remove any tape/label left on the bottle/caps (this should have been done beforehand)
2) fill bottle/beaker with some dilute PCC and shake
3) clean with brush
4) rinse 5X with warm/hot tap water (make sure there is no soap left)
5) rinse 5X with deionized water
6) rinse 1X with distilled/deionized water
7) Let air dry

Plastic beakers/containers

1) squirt some 7X cleaning solution into the container
2) wash with brush or sponge
3) rinse 5X with warm/hot tap water (make sure there is no soap left)
4) rinse 5X with deionized water
5) Let air dry

Autoclaving

Perform after dishes are completely dried. Place autoclave tape on all items (turns black when successfully autoclaved). Autoclave for 30 minutes and dry cycle 15 minutes.

Bottles & oocyte collection medium carboy

place cap loosely onto the bottle. After autoclaving is done, tighten bottle caps a little bit to prevent any contaminants to enter. Do not tighten caps completely until bottles have cooled to prevent a vacuum forming in the bottle.

Stir bars/ instruments

place inside Tower DualPeel autoclave tubing (VWR) or equivalent and place autoclave tape at both ends to seal. Alternatively, heat-seal the ends using an impulse sealer (Agtech).

Beakers/volumetric flasks

Cover snugly with a double layer of aluminum foil.
STOCK SOLUTIONS

For ease of use, prepare aliquots of all solutions and keep frozen. Aliquots may be placed in a Styrofoam rack and the rack labeled with stock number, solution name, aliquot volume, and date. All solutions are to be kept for one year maximum, unless noted otherwise by manufacturer or this protocol.

The choice of water for making stocks depends upon local availability of highly-purified water. We make up stock solutions using Tissue Culture Water purchased from Sigma (W4502 aka IVF water). For all other media (OCM, saline, etc.), we use Millique water or distilled and deionized water.

Stock 1: Na lactate. Purchase as a 60% syrup. Follow manufacturer’s indications for expiration date.
   Source: Sigma L4263
   Alq Size: Keep in manufacturer’s bottle.
   Storage: Store at 4°C.

Stock 2: Na pyruvate. Dissolve 0.220 g sodium pyruvate in 100 ml IVF water. Sterile-filter.
   Source: Sigma P4562 - 5G
   Alq Size: 5 mL aliquots in 5 mL tubes and 2 mL aliquots in 3 ml tubes
   Storage: Store at -20°C.

Stock 3: Bovine Steer Serum (BSS). Thaw and aliquot prior to freezing (or refreezing).
   Source: BioreclamationIVT BOVSRM
   Alq Size: 10 ml aliquots of BSS in sterile 13 ml tubes
   Storage: Store at -20°C.

Stock 4: BSS/Hep. Prepare heparin stock (Stock 7). Dissolve 1000 USP units of sterile heparin in 3-5 mL IVF water. Filter through sterile syringe filter. Add heparin solution to 500 ml BSS (Stock 3).
   Alq Size: 10 ml aliquots in sterile 13 ml tubes
   Storage: Store at -20°C.

Stock 5: Estradiol. Dissolve 1 to 3 mg estradiol in ethanol for a final concentration of 1 mg/ml. Ex. Weigh 2.3 mg estradiol, add it to 2.3 ml ethanol. It is easier to adjust volume of ethanol than small masses of estradiol.
   Source: Sigma E2758-250MG
   Alq Size: N-A
   Storage: Store in a glass container at -20°C for up to 2 months.

Stock 6: Folltropin. Reconstitute Folltropin-V as directed by manufacturer to prepare a 20 μg/μl solution. Place aliquots into sterile 1.5 ml microcentrifuge tubes and store indefinitely at -20°C.
   Source: AgTech A30T
   Alq Size: 150 μl in sterile 1.5 ml microcentrifuge tubes
   Storage: Store at -20°C.

Stock 7: Heparin. Dissolve 20 mg heparin in 10 mL IVF water.
   Source: Sigma H3149-250KU
   Alq Size: 1010 µl aliquots in 1.5 ml microcentrifuge tubes
   Storage: Store at -20°C.

Stock 8: Gentamicin. Obtain gentamicin solution (10 mg/mL). Follow manufacturer’s indications for expiration date.
   Source: Life Technologies 15710-072
**Alq Size:** Keep in manufacturer’s bottle  
**Storage:** Store at 4°C until expiration date.

**Stock 9: PHE Mix.** Prepare fresh stock solutions.  
-0.9% Saline: 0.9g NaCl dissolved into 100mL IVF water.  
- Stock 9A (see below)  
  - 1mM Hypotaurine: 1.09mg hypotaurine dissolved in 10 mL saline.  
  - 2mM Penicillamine: 3mg Penicillamine dissolved in 10 mL saline.  
  - 250 μM epinephrine: 1.83mg epinephrine dissolved into 40 mL Stock 9A (Lactate metabisulfite solution). *Epinephrine is easily oxidized by direct light so take precautions to avoid this problem (wrap in aluminum foil or place in dark container).*  
Combine the following together to make final solution.  
- 10 ml of 1 mM hypotaurine  
- 10 ml of 2 mM Penicillamine  
- 4 ml of 250 μM epinephrine  
- 16 ml of saline  
Sterile filter.  
Upon retrieval of PHE mix for use, wrap tube in aluminum foil.  
**Source:** NaCl – Sigma S5886-500G, Hypotaurine-Sigma H1384-100MG, Penicillamine- Sigma P4875-1G, Epinephrine- Sigma E4250-1G  
**Alq Size:** 600 μl of PHE Mix into sterile 1.5 ml microcentrifuge tubes.  
**Storage:** Store in light blocking container at -20°C. Wrap tube in aluminum foil to block light upon retrieval for use.

**Stock 9A: Lactate-metabisulfite solution.** Add 77 μl of a 98% Na lactate syrup (See Stock 1 or purchase the equivalent volume if a lower percent lactate syrup is used) and 50 mg Na metabisulfite to 50 ml IVF water.  
**Source:** Sigma 13459-500G-R  
**Alq Size:** N-A  
**Storage:** Make fresh for each use.

**Stock 10A: EFAF BSA for SOF-FERT.** Dissolve essentially fatty acid free BSA in SOF base solution at a concentration of 60 mg/mL and sterile filter.  
**Source:** Sigma A6003-25G  
**Alq Size:** 10 mL aliquots in 13 mL tubes  
**Storage:** Store in at -20°C.

**Stock 10B: EFAF BSA for SOF-BE1.** Dissolve essentially fatty acid free BSA in SOF base solution at a concentration of 40 mg/mL and sterile filter.  
**Source:** Sigma A6003-25G  
**Alq Size:** 10 mL aliquots in 13 mL tubes  
**Storage:** Store in at -20°C.

**Stock 10C: BSA Fraction V for H-SOF.** Dissolve BSA fraction V in SOF base at a concentration of 60 mg/mL and sterile filter.  
**Source:** Sigma A3311-100G  
**Alq Size:** 25 mL aliquots in 50 mL tubes  
**Storage:** Store in at -20°C.

**Stock 11: Glutamax** Obtain 100X Glutamax. Follow manufacturer’s indications for expiration date.  
**Source:** Life Technologies 35050-061  
**Alq Size:** Keep in manufacturer’s bottle  
**Storage:** Store at 4°C until expiration date.
Stock 12: MgCl₂ for Percoll. Prepare 0.1 M stock by adding 0.203 g MgCl₂ to 10 ml water. Sterile filter.
   Source: Sigma M2393-100G
   Alq Size: N-A
   Storage: Store at 4°C.

Stock 13: CaCl₂ for Percoll. Prepare 1 M stock by adding 0.735 g CaCl₂+2H₂O to 5 ml water. Sterile filter.
   Source: Sigma C7902-500G
   Alq Size: N-A
   Storage: Store at 4°C.

Stock 14: Hyaluronidase. Prepare stock solution of type IV hyaluronidase at 10,000 units/ml in saline.
   Ex. Prepare 10 mL 0.9% saline (0.09g NaCl in 10mL IVF water). Dissolve 0.0533g hyaluronidase (Sigma H3884) into 10 mL saline. Filter through sterile 0.2 μm filter into sterile tube.
   Source: H3884-1G
   Alq Size: 100ul in sterile microcentrifuge tubes
   Storage: Store in at -20°C.

Stock 15: 100X Myo-inositol. Dissolve 0.998 g of myo-inositol in 20 mL of IVF water. Sterile filter.
   Source: Sigma I7508-50G
   Alq Size: 1 mL into 1.5 mL microcentrifuge tubes
   Storage: Store in at -20°C.

Stock 16: 100X Na citrate. Dissolve 0.2941g of sodium citrate in 20 mL of sterile water. Sterile filter.
   Source: Sigma C3434-205G
   Alq Size: 1 mL into 1.5 mL microcentrifuge tubes
   Storage: Store in at -20°C.

Stock 17: 100X caffeine. Dissolve 0.3884 g caffeine in 20 mL water. Sterile filter.
   Source: Sigma C0750-5G
   Alq Size: 1.2 mL into 1.5 mL microcentrifuge tubes
   Storage: Store in at -20°C.

Stock 18: Pen/Strep. Take directly from commercial bottle of 100 X pen/strep. Follow manufacturer’s indications for expiration date.
   Source: Caisson Labs PSL01
   Alq Size: Keep in manufacturer’s bottle
   Storage: Store at 4°C until expiration date.
MEDIA PREPARATION

Updated 9/27/2017

All media are to be made by two people to minimize possibilities for error.

This reflects our laboratory’s current media use. For information on media used in the past please refer to ‘Discontinued Media’ protocol.

- **Transport Saline (0.9%)**
  - Prepare 0.9% saline (90 g NaCl in 10 L double distilled water) and add 100 ml of 100X Pen/Strep. Store indefinitely at 4°C.

- **Oocyte Collection Medium - OCM**
  - Purchase from MOFA (19982/1200)

- **Oocyte Maturation Medium - OMM**
  - Purchase from IVF BioScience (BO-IVM)

- **HEPES-TL (Base Solution)**
  - To make 1 L of HEPES-TL base solution, dissolve the ingredients listed in Table 1 in 500 mL of sterile water (Sigma W4502). Adjust pH to 7.3-7.4 and bring volume up to 1 L. Sterile-filter into glass medium bottles using a Nalgene 0.2 µm FastCap filter (catalog number 298-9020) and keep for up to 6 months at 4°C. Labels should read HEPES-TL Base with initials and date made.

  **Table 1. Recipe for preparation of HEPES-TL base solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Mol Wt</th>
<th>mM</th>
<th>mg/L</th>
<th>g/L</th>
<th>Sigma Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.5</td>
<td>114</td>
<td>6669</td>
<td>6.669</td>
<td>S5886</td>
</tr>
<tr>
<td>KCl</td>
<td>74.6</td>
<td>3.2</td>
<td>238.72</td>
<td>0.2387</td>
<td>P5405</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>84</td>
<td>2</td>
<td>168</td>
<td>0.168</td>
<td>S5761</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>120</td>
<td>0.4</td>
<td>48</td>
<td>0.048</td>
<td>S9638</td>
</tr>
<tr>
<td>Na Lactate (792 g/L)(ml)</td>
<td>112.1</td>
<td>10</td>
<td>1.416</td>
<td>1416 µL</td>
<td>L4263</td>
</tr>
<tr>
<td>CaCl₂ .2H₂O</td>
<td>147.01</td>
<td>2</td>
<td>293.8</td>
<td>0.2938</td>
<td>C7902</td>
</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>203.30</td>
<td>0.5</td>
<td>101.65</td>
<td>0.1017</td>
<td>M2393</td>
</tr>
<tr>
<td>HEPES</td>
<td>238.4</td>
<td>10</td>
<td>2384</td>
<td>2.384</td>
<td>H4034</td>
</tr>
<tr>
<td>Osmolarity</td>
<td>260-270</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.3-7.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **HEPES-Talp (Working Solution)**
  - To prepare HEPES-Talp, add the ingredients listed in Table 2 to 500 mL of HEPES-TL base solution. Label the bottle HEPES-Talp with initials, date made, and expiration date and store at 4°C for up to one month. Equilibrate sample and check pH and osmolality prior to use.
Table 2. Recipe for preparation of HEPES-Talp

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Sigma Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES-TALP Working Solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES-TL Base Solution</td>
<td>500 mL</td>
<td></td>
</tr>
<tr>
<td>BSA, Fract V</td>
<td>1.5 g</td>
<td>A3311</td>
</tr>
<tr>
<td>Na pyruvate (Stock 2)</td>
<td>5 ml</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin (Stock 8)</td>
<td>375 µL</td>
<td>-</td>
</tr>
<tr>
<td>Filter in a sterile bottle</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

• IVF-TL (Base Solution)
  - To make 1 L of IVF-TL base solution, dissolve the ingredients listed in Table 3 in 500 mL of sterile water (Sigma W4502). Adjust pH to 7.3-7.4 and bring volume up to 1 L. Sterile-filter into glass medium bottles using a Nalgene 0.2 µm FastCap filter (catalog number 298-9020) and keep for up to 6 months at 4°C. Labels should read FERT-TL Base with initials and date made.

*Note: IVF-TL and IVF-TALP were previously referred to as FERT-TL and FERT-TALP

Table 3. Recipe for preparation of IVF-TL

<table>
<thead>
<tr>
<th>Component</th>
<th>Mol Wt</th>
<th>mM</th>
<th>mg/L</th>
<th>g/L</th>
<th>Sigma Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.5</td>
<td>114</td>
<td>6669</td>
<td>6.669</td>
<td>S5886</td>
</tr>
<tr>
<td>KCl</td>
<td>74.5</td>
<td>3.2</td>
<td>238.4</td>
<td>0.2384</td>
<td>P5405</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>84</td>
<td>25</td>
<td>2100</td>
<td>2.100</td>
<td>S5761</td>
</tr>
<tr>
<td>NaH₂PO₄.H₂O</td>
<td>138</td>
<td>0.4</td>
<td>55.2</td>
<td>0.0552</td>
<td>S9638</td>
</tr>
<tr>
<td>Na Lactate (792 g/L)(ml)</td>
<td>112.1</td>
<td>10</td>
<td>1.416</td>
<td>1416 µL</td>
<td>L4263</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>111.1</td>
<td>2</td>
<td>222.2</td>
<td>0.2222</td>
<td>C7902</td>
</tr>
<tr>
<td>MgCl.6H₂O</td>
<td>203.3</td>
<td>0.5</td>
<td>101.65</td>
<td>0.1017</td>
<td>M2393</td>
</tr>
<tr>
<td>Osmolarity</td>
<td></td>
<td></td>
<td>280-300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td>7.3-7.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

• IVF-Talp (Working Solution)
  - To prepare IVF-Talp, add the ingredients listed in Table 4 to 100 mL of IVF-TL base solution. Label the bottle IVF-Talp with initials, date made, and expiration date and store at 4°C for up to one month. Equilibrate sample and check pH and osmolality prior to use.

Table 4. Recipe for preparation of IVF-Talp

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Sigma Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVF-Talp Working Solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EFAF BSA</td>
<td>0.6 g</td>
<td>A6003</td>
</tr>
<tr>
<td>Na-pyruvate (Stock 2)</td>
<td>1000 µL</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin (Stock 8)</td>
<td>50 µL</td>
<td>-</td>
</tr>
<tr>
<td>Heparin (Stock 7)</td>
<td>1000 µL</td>
<td>-</td>
</tr>
<tr>
<td>Filter in a sterile bottle</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
- **SOF-BE2 (Base Solution)**
  - To make 1 L of SOF-BE2 base solution, dissolve the ingredients listed in Table 5 in 700 mL of sterile water (Sigma W4502 or Renova sterile water). Adjust pH to 7.2-7.3 and bring volume up to 1 L. Sterile-filter medium into 1 L glass medium bottles using a 0.2 µm bottletop filter. Store at 4°C for up to 6 months. Label the bottles SOF-BE2 Base Solution with initials and the date made.

  **Table 5. Recipe for SOF-BE2 Base**
<table>
<thead>
<tr>
<th>Component</th>
<th>Mol Wt</th>
<th>mM</th>
<th>mg/L</th>
<th>g/L</th>
<th>Sigma Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂·2H₂O</td>
<td>147</td>
<td>1.17</td>
<td>172.0</td>
<td>0.172</td>
<td>C7902</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>203.31</td>
<td>0.49</td>
<td>99.6</td>
<td>0.0996</td>
<td>M2393</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>136.1</td>
<td>1.19</td>
<td>161.95</td>
<td>0.1619</td>
<td>P5655</td>
</tr>
<tr>
<td>KCl</td>
<td>74.55</td>
<td>7.16</td>
<td>533.8</td>
<td>0.5338</td>
<td>P5405</td>
</tr>
<tr>
<td>NaCl</td>
<td>58.44</td>
<td>107.7</td>
<td>6294.0</td>
<td>6.2940</td>
<td>S5886</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>84.01</td>
<td>25.07</td>
<td>2106.15</td>
<td>2.1062</td>
<td>C7902</td>
</tr>
<tr>
<td>Na-lactate</td>
<td>112.06</td>
<td>5.3</td>
<td>775.5 µL</td>
<td>775.5 µL</td>
<td>L4263</td>
</tr>
<tr>
<td>Osmolarity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.3-7.4</td>
</tr>
</tbody>
</table>

- **SOF-BE2 (Working Solution)**
  - To prepare SOF-BE2 Working Solution, add the ingredients listed in Table 6 to 100 mL of SOF base solution. Sterile filter. Label the bottle SOF-BE2 with initials, date made, and expiration date and store at 4°C for up to one month. Equilibrate sample and check pH and osmolality prior to use.

  **Table 6. Recipe for preparation of SOF-BE2**
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Sigma Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>EFAF BSA</td>
<td>0.4 g</td>
<td>A6003</td>
</tr>
<tr>
<td>Glutamax (Stock 11)</td>
<td>1000 µL</td>
<td></td>
</tr>
<tr>
<td>Na-pyruvate (Stock 2)</td>
<td>50 µL</td>
<td></td>
</tr>
<tr>
<td>Myo-inositol (Stock 15)</td>
<td>1000 µL</td>
<td></td>
</tr>
<tr>
<td>Sodium Citrate (Stock 16)</td>
<td>1000 µL</td>
<td></td>
</tr>
<tr>
<td>Non-Essential AA</td>
<td>1000 µL</td>
<td>M7145</td>
</tr>
<tr>
<td>Essential AA</td>
<td>2000 µL</td>
<td>B6766</td>
</tr>
<tr>
<td>Gentamicin (Stock 8)</td>
<td>250 µL</td>
<td></td>
</tr>
<tr>
<td>Filter in sterile bottle</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### MINI-PROTOCOL BASE SOLUTIONS

Updated 9-27-2017

#### HEPES-TL Base Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Mol Wt</th>
<th>mM</th>
<th>g/L</th>
<th>Sigma Cat #</th>
<th>g/2L</th>
<th>g/4L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.5</td>
<td>114</td>
<td>6.669</td>
<td>S5886</td>
<td>13.338</td>
<td>26.676</td>
</tr>
<tr>
<td>KCl</td>
<td>74.6</td>
<td>3.2</td>
<td>0.2387</td>
<td>P5405</td>
<td>0.4774</td>
<td>0.9548</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>84</td>
<td>2</td>
<td>0.168</td>
<td>S5761</td>
<td>0.336</td>
<td>0.672</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>120</td>
<td>0.4</td>
<td>0.048</td>
<td>S9638</td>
<td>0.096</td>
<td>0.192</td>
</tr>
<tr>
<td>Na Lactate (792 g/L)(ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>147.01</td>
<td>2</td>
<td>0.2938</td>
<td>C7902</td>
<td>0.5876</td>
<td>1.175</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>203.30</td>
<td>0.5</td>
<td>0.1017</td>
<td>M2393</td>
<td>0.2034</td>
<td>0.4068</td>
</tr>
<tr>
<td>HEPES</td>
<td>238.4</td>
<td>10</td>
<td>2.384</td>
<td>H4034</td>
<td>4.768</td>
<td>9.536</td>
</tr>
<tr>
<td>Osmolarity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.3-7.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### IVF - TL Base Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Mol Wt</th>
<th>mM</th>
<th>mg/L</th>
<th>g/L</th>
<th>Sigma Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.5</td>
<td>114</td>
<td>6669</td>
<td>6.669</td>
<td>S5886</td>
</tr>
<tr>
<td>KCl</td>
<td>74.6</td>
<td>3.2</td>
<td>0.2384</td>
<td>0.2384</td>
<td>P5405</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>84</td>
<td>2</td>
<td>0.168</td>
<td>0.168</td>
<td>S5761</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>120</td>
<td>0.4</td>
<td>0.048</td>
<td>0.048</td>
<td>S9638</td>
</tr>
<tr>
<td>Na Lactate (792 g/L)(ml)</td>
<td>112.1</td>
<td>10</td>
<td>1416 uL</td>
<td>1416 uL</td>
<td>L4263</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>111.1</td>
<td>2</td>
<td>222.2</td>
<td>222.2</td>
<td>C7902</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>203.3</td>
<td>0.5</td>
<td>101.65</td>
<td>101.65</td>
<td>M2393</td>
</tr>
<tr>
<td>HEPES</td>
<td>238.4</td>
<td>10</td>
<td>2.384</td>
<td>2.384</td>
<td>H4034</td>
</tr>
<tr>
<td>Osmolarity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.3-7.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### SOF-BE2 Base Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Mol Wt</th>
<th>mM</th>
<th>mg/L</th>
<th>g/L</th>
<th>Sigma Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂·2H₂O</td>
<td>147</td>
<td>1.17</td>
<td>172.0</td>
<td>0.172</td>
<td>C7902</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>203.31</td>
<td>0.49</td>
<td>99.6</td>
<td>0.0996</td>
<td>M2393</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>136.1</td>
<td>1.19</td>
<td>161.95</td>
<td>0.1619</td>
<td>P5655</td>
</tr>
<tr>
<td>KCl</td>
<td>74.55</td>
<td>7.16</td>
<td>533.8</td>
<td>0.5338</td>
<td>P5405</td>
</tr>
<tr>
<td>NaCl</td>
<td>58.44</td>
<td>107.7</td>
<td>6294.0</td>
<td>6.2940</td>
<td>S5886</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>84.01</td>
<td>25.07</td>
<td>2106.15</td>
<td>2.1062</td>
<td>C7902</td>
</tr>
<tr>
<td>Na Lactate</td>
<td>112.06</td>
<td>5.3</td>
<td>775.5 µL</td>
<td>775.5 µL</td>
<td>L4263</td>
</tr>
<tr>
<td>Osmolarity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.3-7.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Procedures for In Vitro Production of Bovine Embryos - University of Florida

Day -1

RETRIEVAL OF OVARIES AND OOCYTE COLLECTION AND MATURATION

Revised 6/20/17

OVARY COLLECTION

Materials and Equipment Needed

- Insulated cooler with 2 1 L sealable plastic containers (0.5 L of transport saline in each)
- Optional: Temperature controlled insulated cooler set at 23°C (to ensure ovaries are not exposed to temperature extremes)
- Appropriate attire as required by the slaughterhouse
- Knives (boning knives) or other cutting tools
- Gloves

Procedure

1. Remove ovaries from the reproductive tract of cows immediately after internal organs are extracted from the carcass and place the ovaries into one of the saline containers.
2. After all ovaries have been collected, remove the excess blood from the ovaries by massaging the ovaries in the container. Then, transfer ovaries to the second container and place the containers back in the cooler.
3. Transport the ovaries to the lab immediately. Avoid exposing ovaries to temperature extremes of heat or cold. Use a temperature controlled cooler if available.

OOCYTE COLLECTION

Materials and Equipment Needed

- Preparation of Microdrops
  - Incubator (5% CO₂ and 38.5°C)
  - Laminar flow hood
  - Pipettor and pipette tips
  - Maturation medium (IVF BioScience, IVF-BO)
  - Mineral oil
  - 60 x 15 mm petri dishes
  - Plastic sterile pasteur pipet
  - 1 bottle of oocyte washing medium (MOFA) (warmed to 38.5°C)
  - 1 L saline + Pen/Strep (warmed to 38.5°C)

- Preparation for Oocyte Collection
  - Scalpel (#3)
  - Scalpel blades (#11)
  - Petri dishes with IVF-BO microdrops (pre-equilibrated in incubator)
Procedures for In Vitro Production of Bovine Embryos – University of Florida

- Bench paper to cover surface
- X-plate
- Integrid petri-dish
- 100 μm Falcon Cell Strainer
- Gloves
- 400 ml sterile beaker
- Container to discard ovaries
- Slide warmer at 38.5°C
- Dissecting microscope
- Sterile transfer pipets
- 18 gauge needle
- 10 ml NormJect syringe (all plastic, no rubber septum)
- 50 ml beaker

**Figure 1.** Some supplies plates needed for IVP. From left to right are a pair of hemostat clamps to hold the sterile cell strainer, the Falcon cell strainer, a small beaker to hold and warm OCM (also pictured), 10 mL NormJect syringe, 18 gauge needle, Falcon X plate for collecting and washing COCs, and Falcon grid plate for searching COCs.

**Procedure**

1. At least two hours before they are needed, prepare several (60 x 15 mm) plates containing up to 15, 50 μl microdrops (Figure 2) of OMM. Prepare enough OMM microdrops (10 oocytes/μl) to mature the number of oocytes expected to be collected. Cover the microdrops with 9 mL mineral oil.
Figure 2. Preparation of microdrops. Shown here are 15 50 µl drops on the bottom of a 60 mm petri dish before (left) and after (right) covering with 9 mL mineral oil. Oil is deposited using a serological pipette (or larger pipette if many plates are made).

Figure 3. Some items needed for oocyte collection. Shown on the warming plate are 2 1-L plastic beakers (one containing cleaned ovaries and one to collect ovaries after processing) and a 400 ml beaker containing 100 ml OCM. Also shown are a hemostat, scalpel handle, disposable scalpel blades and bench paper. Use of a warming plate is not required. If used, ensure warming plate does not overheat ovaries on the bottom of the bucket.

2. Upon return to the lab, classify the ovaries as Good (large number of small and medium follicles, no very large follicles, not discolored) or Marginal (others).
   a. Marginal ovaries should only be used if necessary to obtain the number of desired oocytes.
3. Wash ovaries (by massaging; Figure 4) 2-3 times with the pre-warmed saline until the majority of the blood has been washed away from the ovaries. Following the washes, place ovaries in the container containing fresh transport saline and store at room temperature until time of oocyte collection.
4. Add 100 ml oocyte collection medium to a sterile 400 ml beaker.
5. Attach a hemostat to the base of the ovary to hold the ovary firmly in place. Cut the excess tissue from the ovarian stalk. Hold ovary above beaker and make 2-3 mm deep incisions across all visible follicles (no larger than 10 mm). See Figure 5 for illustration.

As an alternative method, slash the entire surface of the ovary creating a 2-3 mm checkerboard pattern to obtain maximum yield of oocytes.

Both follicular fluid and blood in the collection medium could result in clotting of the medium, thereby rendering it impossible to retrieve oocytes. To prevent clotting of the medium, do not collect from large follicles (>10 mm). Either do not slash these follicles or rupture them before harvesting other follicles and discard the follicular fluid. Also, do not make incisions across corpora lutea. It is imperative not to cut too deep into the surface of the ovary (practice will aid with this) to avoid cutting larger blood vessels in the ovary.

If many ovaries are being slashed it may be beneficial to split them into two groups to slash. This will lessen the amount of debris present when searching for oocytes.

Figure 5. Slashing of ovaries to recover oocytes. In panel A, a hemostat is attached to the base of the ovary to hold the ovary firmly in place. In panel B the excess tissue from the ovarian stalk is removed. Panels C and D show how the ovary is held above the beaker and 2-3 mm incisions made in a downward direction with a rapid but firm movement across follicles.
6. Submerge ovary into oocyte collection medium and swirl vigorously. Repeat this process until the desired number of ovaries has been processed (See Figure 6).

*Typical yield is about 10 usable oocytes/ovary (sometimes as many as 20-30 can be obtained).*

---

**Figure 6.** Harvesting of oocytes from slashed ovaries. In the left panel, the slashed ovary is being swirled in oocyte collection medium. In the right panel, the ovary is being pressed against the side of the beaker to allow drainage.

---

**Figure 7.** Filtering the fluid collected from slashing the ovaries. Panel A depicts the supplies necessary for this step. Panel B shows pouring the aspirated pellet through the cell strainer. Rinsing of oocytes from the cell strainer into a grid petri dish is shown in Panel E.
7. Fill a 10 ml syringe with warmed oocyte collection medium (for later use in rinsing the oocytes from filter) and pour ~2 ml oocyte collection medium into an integrid petri-dish to prevent oocytes from sticking to the bottom of the plate. Pour OCM into 3 wells of an X-plate.

8. Use a forceps to hold a 100 μm cell strainer in position over a 400 ml beaker. Filter the medium with oocytes directly from the collection beaker through the cell strainer (waste fluid should be caught in the beaker below the cell strainer). Continually swirl the beaker containing the fluid with the oocytes so that the oocytes do not stick to the bottom of the glass beaker during pouring.

*Note: If you have large numbers of ovaries that are slashed, or if there will be extended time between collecting the fluid and filtering, the collected fluid containing oocytes can be poured into sterile 50-ml centrifuge tubes. Be careful not to overfill the tube which can cause oocyte loss. Place the tubes containing the oocytes and media into a water bath set to 38.5°C and allow oocytes to settle to the bottom of the centrifuge tubes for at least 5 minutes. The oocytes can then be aspirated from the bottom of the 50-mL tubes and put through the filter as described in step 8.*

If the cell strainer starts to clog during filtering rinse it with OCM using a sterile plastic Pasteur pipette. The hemostat holding the cell strainer can be tapped sideways with your finger to assist in dislodging the debris. The cell strainer can be reused if necessary to filter additional beakers of oocytes. A single strainer can normally be used to filter fluid from 4-5 beakers.

9. When all the oocyte fluid has passed through the filter, immediately turn the filter upside down (over the grid plate) and, using a 10 ml syringe fitted with an 18 g needle filled with oocyte collection medium, rinse the oocytes into an integrid petri-dish (figure 7E). Place the integrid dish on a plate warmer until ready for searching (Figure 8).

*Note: There is an alternative protocol available for processing oocytes that does not involve use of the cell strainer. See the website for the protocol under the page labeled Archives.*

10. Collect cumulus oocyte complexes (COCs) as fast as possible to prevent adverse effects of cold shock. Only COCs which have at least 3 layers of compact cumulus cells and an evenly granulated cytoplasm with no clear spaces should be used for subsequent steps. Place retrieved COCs into the first well of the X-plate containing OCM that was prepared in step 9.

11. After completing COC search, transfer oocytes from one well to the next, leaving all debris behind (repeat twice to assure that oocytes are clean of debris; Figure 8). This can be completed using a wiretrol pipet, microdispensor pipet, or the instrument of your choice for handling oocytes.
Figure 8. Washing of COCs using an X-plate.

Figure 9. Some instruments used to handle embryos and oocytes. From left to right are 1) a 1 cc syringe with an extension of rubber tubing connected to a Unopette, 2) a wiretrol (from Drummond Scientific; Fisher), 3) the same device as #1 without the rubber tubing extension and 4) a 5 ml Drummond Microdispenser (shown without pipette tip). For advice on how to use these instruments, see the section entitled ‘Guide On Use Of Instruments For Picking Up Oocytes And Embryos’.

12. After oocytes have been cleaned of debris, transfer groups of 10 to a 50 ul microdrop of OMM.

Note: It is essential that oocytes be collected, washed and incubated in OMM as quickly as possible to ensure maximum development rates.

13. Incubate for 20 to 24 h at 38.5°C and 5% CO₂ with humidity.
Figure 10. Transfer of COCs to microdrops.

Media Preparation for Day 0

1. The following media should be available on Day -1 for next day (Day 0)
   a. Hepes - TALP
   b. IVF - TALP
   c. PureSperm (or other sperm purification gradient medium)
Day 0
In Vitro Fertilization

Revised 6/22/2017

Initial Preparation for Sperm Purification and Fertilization

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

Materials and Equipment Needed

- Laminar flow hood
- PureSperm (or other 2 step density gradient)
- IVF-TALP
- HEPES-TALP
- 15 ml conical centrifuge tubes
- 3 centrifuge carriers
- Dish, 35mm x 10 mm Petri dishes, Falcon # 351008
- Thawing unit (Citothaw)
- PHE
- Pipet tips and pipettors
- Sterile serological pipets
- Plastic Pasteur pipet
- CO2 Incubator (at atmospheric oxygen with 5% CO2, 38.5 °C with humidity)
- Warming oven set at 38.5 °C (can be substituted with a water bath)

Procedures

1. For each fertilization plate, prepare 20 mL HEPES-TALP in 15 mL conical tubes. Tighten the caps and place in the warm oven.
   
   *These tubes will be used for washing before fertilization and after hyaluronidase.*

2. Add 10 mL HEPES-TALP to a 15 mL conical tube. Label it “Wash”. Tighten cap and place in the warm oven.

3. Add 3 mL IVF-TALP to a 15 mL conical tube. Label it “Sperm Diluent”. Leave cap loose and place in the incubator.

4. Prepare one 35 mm x 10 mm Petri dishes for each fertilization plate. Add 1700 µL of IVF-TALP to each plate. Place plates in incubator to allow medium to equilibrate and warm up for 2 h.

5. Place 1.5 mL of 80% PureSperm (labeled as B for Bottom layer) 1.5 mL 40% PureSperm (labeled as T for Top layer) in the warm oven.

6. Plug-in CitoThaw (i.e., thawing unit) so the water warms up.

7. Place aliquots of PHE (80 µl per plate) in the warming oven (remember to cover the tube with aluminum foil due to light sensitivity of PHE).

8. Place 2-3 centrifuge carriers in the oven to warm.
PREPARATION OF OOCYTES FOR FERTILIZATION

Materials and Equipment Needed

- 35 mm petri dishes with 1.7 mL IVF-TALP (pre equilibrated in CO2)
- X plates (source Fisher Scientific)
- HEPES-TALP (pre-warmed)
- IVF-TALP (pre equilibrated in CO2 incubator)
- PureSperm (pre-warmed)
- Dissecting microscope
- Inverted microscope
- Rack for tubes (place in front of the heater)
- Heater (small benchtop space heater)
- Scissors (wipe with ethanol)
- Semen straw plunger (wipe with ethanol)
- Plastic sterile Pasteur pipets
- Pipettors
- Pipette tips
- Instrument to pick-up oocytes
- Slide warmer (set at 38.5 °C)
- CO2 Incubator (at atmospheric oxygen with 5% CO2, 38.5 °C with humidity)
- Warming oven set at 38.5 °C (can be substituted with a water bath)
- Hemocytometer
- Eppendorf Tubes

Procedure

1. Place X plate on the slide warmer and add ~3 ml of HEPES-TALP to one well per fertilization plate.
2. Remove dishes containing matured oocytes and place on the slide warmer.
3. Transfer COCs from microdrops of OMM to one corner of a well of the X plate containing HEPES-TALP.

Repeat as necessary until all oocytes have been placed in a plate in groups of ~300. Wash the COCs by moving them from one corner to the next within the same well, completing a total of three washes. This step is most efficiently completed using a 1 mL slip tip syringe fitted with an adaptor to attach to 200 uL pipette tips to move the oocytes. See photo below.

4. Remove the dish containing pre-equilibrated IVF-TALP (1700 µl/dish) from the incubator and transfer a group of no more than 300 washed oocytes from the X-plate to the dish.
5. Return dish with the oocytes to the incubator until fertilization.
SPERM PURIFICATION USING PureSperm

Materials and Equipment Needed

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

Procedure

Note: Spermatozoa are very sensitive to changes in temperature, especially cold temperatures. It is critical to prepare the spermatozoa in a warm (but not overly hot) environment. A space heater in front of the area where sperm preparation will be performed can aid in preventing cold shock to the sperm cells (use caution to not allow the area to get too hot). 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, IVF-TALP, PureSperm).

1. Set up the gradient. Place 1.5 ml of 80% PureSperm (Bottom layer) into a 15 ml conical tube. Slowly layer 1.5 ml 40% PureSperm (Top layer) on top of the 80% PureSperm using a plastic Pasteur pipet. Cap and place in the tube rack in front of the heater.

2. Thaw 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 that it is not necessary to use 3 straws. Using this system, one straw should provide enough semen for 200-400 oocytes. Unless the choice of sire is critical, we typically pool semen from 3 bulls (1 straw per bull) to enhance the probability that sperm from at least one sire will perform well.

3. 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 PureSperm gradient (Figure 2). Care must be taken so that the gradient is not disturbed and the semen lies on top of the 40% layer.

Figure 1. Transfer of straws of semen from liquid nitrogen tank to the thawing unit (citothaw).
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.

Figure 2. Layering of sperm onto sperm purification gradient. After cutting the tip of the straw, the contents of the straw are expelled onto the top of the gradient (panel A). Removal of the semen is facilitated by using a homemade plunger. Panel B shows the layer of semen on top of the gradient before centrifugation and panel C shows the pellet of live sperm at the bottom of the gradient after centrifugation (marked by arrow).

4. Place the conical tube containing the semen and sperm purification gradient into a centrifuge carrier that has been pre-warmed to 38.5°C, and centrifuge at 1000 x g for 10 min.
5. After centrifugation, aspirate sperm pellet from the bottom of the conical tube with a plastic Pasteur pipette (Figure 3). The pellet should be collected with as little PureSperm as possible.

Figure 3. Removal of sperm from the bottom of the gradient.
6. Place the sperm pellet into a 15 ml conical tube containing 10 ml HEPES-TALP (labeled “wash”) and place in a warm centrifuge carrier before centrifuging for 5 min at 200 x g. When adding the sperm pellet to the Wash tube care should be taken to not immerse the plastic pipette into the washing medium. The exact speed at which to centrifuge is probably not critical - do a low-speed centrifugation.

7. Remove the supernatant with a Pasteur pipette, 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 HEPES-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.

8. Determine dilution required to bring sperm to a concentration of 17 x 10⁶/ml. This will produce a final concentration of sperm in the fertilization plate of approximately 1 x 10⁶/ml. Bring the semen pellet to a volume of 600 µl using pre equilibrated IVF-TALP medium. Create a 1:10 dilution of this semen by adding 10 µl of the sperm suspension to 90 µl water. Load 10 µl in the two chambers of a hemocytometer. Count the number of sperm in 5 squares of each chamber, and use the average from both chambers as the final sperm count (Figure 5).

To obtain the volume of IVF-TALP needed to bring the sperm to a concentration of 17x10⁶/ml use the following formula:

\[(Vol \times \text{cell number} \times 50,000 \times 10 / 17,000,000) - Vol\]

Where:
- Vol is the initial dilution of the sperm (600 µl)
- Cell number is the average sperm count
- 50,000 is the adjustment to determine the sperm concentration per ml
- 10 is the dilution factor of sperm before counting

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 ~17 x 10⁶/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.

FERTILIZATION

Materials and Equipment Needed

- Slide warmer (set at 38.5°C)
- Pipettors and pipette tips
- CO2 Incubator (at atmospheric oxygen with 5% CO2, 38.5°C with humidity)
- Warming oven set at 38.5°C (can be substituted with a water bath)
- Purified sperm
- PHE

Procedure

1. Remove fertilization dishes containing washed and matured oocytes from the incubator and place on the slide warmer (Fig. 6).
Figure 6. Fertilization. Shown in the left panel is removal of matured COCs from maturation drops (done during Preparation of Oocytes). Shown in the left panel is addition of 120 µl sperm to fertilization dishes.

2. Add 120 µl sperm preparation and 80 µl 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 aspirating debris that can settle to the bottom of the tube.

Prior to Step #3 the dish should be examined under the microscope to ensure that the sperm are moving and viable.

3. Return dish to incubator for 8-10 hours (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 also 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

- SOF-BE 2
- 60 x 15 mm and/or 30 x 15 mm petri dishes
- Mineral oil
- Pipettors and pipette tips
- Tri gas incubator (38.5°C, 5% CO2 5% O2 balance N, humidified)

Procedure

1. Prepare embryo culture medium (SOF-BE 2 or other culture medium) at least 2 h before removing zygotes from the fertilization plate.

2. Make 50 ul microdrops of culture medium (up to 30 zygotes per drop) in petri dishes and cover with mineral oil. For 60 X 15 mm dishes cover drops with 9 mL mineral oil. For 35 x 10 mm dishes use 4 mL mineral oil to cover drops. To culture small groups of embryos (<15 per drop) we typically prepare 25 ul microdrops of culture medium.

3. Place the dishes in the incubator to warm up and equilibrate.
TRANSFER OF FERTILIZED OOCYTES INTO EMBRYO CULTURE DROPS  
(post IVF)

Materials and Equipment Needed

- Vortexer
- Timer
- Stock 12: Hyaluronidase in a 1.5 ml Dolphin microcentrifuge tube
- X-plate (with prewarmed HEPES-TALP)
- Plastic Pasteur pipet
- Slide warmer (set at 38.5°C)
- Dissecting microscope
- Instrument to pick-up embryos

Procedure

1. Add 600 uL of HEPES-TALP to the tube of Stock 12: Hyaluronidase in the warming oven.
2. Place X-plate on the slide warmer and add ~3 ml of HEPES-TALP to 3 of the wells.
3. Remove one fertilization dish from the incubator.
4. Remove COCs (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. Allow zygotes to settle to the bottom of the tube (place in warm oven for a few minutes) and remove all but 100-200 uL (this prevents zygotes from going into the lid of the tube during vortex).

   To minimize loss of zygotes during this process, expell the removed supernatant onto the lid of the dish. It should be examined for zygotes prior to discarding it.

6. Remove cumulus cells from zygotes by vortexing (Figure 7) the tube for 5 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).
7. Transfer the putative zygotes from the tube to the X-plate and rinse the tube 2-3 times with HEPES-TALP to gather all embryos.
   
   _Additional medium can be added to the tube containing the zygotes prior to transferring them to the X plate to rinse zygotes from the walls of the tube._

8. Wash zygotes 3 times 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, 3 and 4. Add zygotes 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 well 3._

9. Finally, transfer the putative zygotes to microdrops of pre-equilibrated SOF-BE2. Return plate to incubator as quickly as possible.
DAY 1-9
EMBRYO CULTURE

Revised 6/22/2017

DAY 3 AFTER IVF - ASSESSING CLEAVAGE RATE

Assess cleavage rate of embryos by determining the number of embryos cleaved divided by the number of embryos placed initially in the microdrops. Return plates to the incubator as quickly as possible.

DAY 7-9 AFTER IVF - ASSESSING BLASTOCYST DEVELOPMENT

Assess development of embryos to the blastocyst stage by determining the number of embryos that can be classified as an early blastocyst or above, divided by the number of embryos placed initially in the microdrops. Return plates to the incubator as quickly as possible.

Information is available on the website (Big Book of Bovine Embryos) to help identify and classify embryos at various stages of development.

All original material © Rocio Rivera, Peter J. Hansen et al., 2000-2017
Edited L. Jannaman 6/22/2017
HELPFUL HINTS

Modified August 4 2017

Here are a few things that can greatly improve the chances of getting good results during IVF procedures.

**Speed is Important**
Results will improve as you become faster at performing each step.

**Water is Important**
The choice of water depends upon local availability of highly-purified water. We make up stock solutions using Tissue Culture Water purchased from Sigma. For all other media, we use deionized water that is also distilled.

**Oil is Important**
Sometimes when IVP fails, the oil is the culprit. Water-soluble contaminants in oil can kill embryos. Oil can be cleaned by incubating it with clean water or water based medium in some sort of shaker or mixer. The water soluble contaminates will migrate to the water or medium and out of the oil. We have had good luck using Sigma’s Embryo-Tested Mineral Oil but there are many other oils available.

**Keep Incubators Set Properly**
Check incubators regularly for accuracy of temperature and gas readings and to ensure the air is humidified (i.e. reservoir of water inside the incubator is about ¾ full). Water-jacketed incubators respond to changes in setpoint slowly so make adjustments well before the incubator will be used. Nitrogen can run out very frequently if not continuously monitored.

**Use Supplies for IVP Only**
Set aside glassware, plasticware, and instruments that will only be used for IVP to prevent any residue from another application to contaminate media and affect the oocytes/embryos. This could be done easily by having a set of glassware that is different than that regularly used in the laboratory.

**Keep the Work Area Warm to Prevent Cold Shock**
1. Air-conditioning vents should be covered during IVP procedures. If the room is devoted for IVP only, air-conditioning vents can be permanently sealed.
2. A space heater may be used to warm up the air near the work site as well as microscope stage and bench surfaces. Placement of a dish on a cold lab bench or microscope stage could result in a rapid cold shock of embryos. Don’t get too close to the space heater though or you can cook your embryos.
3. Before looking at embryos using a microscope, make sure stage has been pre-warmed (by turning on the space heater). Metal is an excellent conductor of cold from one material to another.
4. Whenever possible, avoid placing beakers, dishes, or tubes containing oocytes or embryos directly onto a cold surface. A slide warmer set to 38.5 °C is the best solution. If not available, place items on plastic mesh or styrofoam to insulate from the cold shock.

**Pre-Warm Media at 38.5 °C**

a) Media designed for use in air should be pre-warmed for at least 2 to 3 h in an oven, incubator, or water bath set at 38.5 °C. Make sure lid is on tight. Media to be pre-warmed in this manner includes transport saline, OCM, HEPES-TALP, PureSperm, and PHE.

b) Incubator - Media designed for use in a 5% CO₂ environment should be pre-warmed to 38.5 °C in an incubator. In order for the pH of the medium to be equilibrated, it is important to loosen the lid of any bottle/tube of medium placed in the incubator. Leaving a bottle of medium buffered for a specific CO₂ environment outside the incubator too long will result in a dramatic change of pH which could severely
affect embryonic viability and development. Media that must be pre-warmed in an incubator include OMM, IVF-TALP, and SOF-BE2.

Sterility and Cleanliness
1. All glassware, plasticware and media used should be sterile.
2. Use sterile techniques when handling media.
3. When cleaning benchtops in the oocyte collection or ovary preparation area, use a commercial cleaner (we use Windex without ammonia) to remove blood and other material from the surface and always finish cleaning by swiping all surfaces with a rag or kimwipe soaked with 70% ethanol (remember: ethanol is toxic to embryos so be careful where you splash it). When cleaning benchtops inside the IVF laboratory replace Windex with distilled water.
4. In our experience, the citothaw used to thaw semen can be a source of many nasty microorganisms. Always rinse and dry the citothaw after each use. Periodically, it is a good idea to disinfect the inside of the citothaw with bleach.

Other Tips
1. Pipet tips may contain toxins or other substances that might inhibit development of embryos. As a precaution, always fill and empty pipet tip at least once before using, especially before adding new medium or serum to a microdrop containing embryos.
2. When transferring oocytes or embryos from one medium to another, transfer the oocytes/embryos in as little medium as possible.
3. Due to repeated openings of the incubator door, temperature at the front of the incubator fluctuates. Thus, place dishes at the back of the incubator to reduce exposure to changes in temperature.
ALTERNATIVE PROTOCOL FOR PROCESSING OOCYTES

Updated 6/23/2017

This, the original protocol for washing oocytes after slashing, utilizes more medium than when the cell strainer is used to recover oocytes. It is included here as an alternative protocol for those who are already using this technique or without access to cell strainers.

1. Once a group of ovaries have been processed, place beaker in a water bath at 38.5°C and add medium until the beaker is full. Allow oocytes to settle for 5 min. Using a 25 ml sterile Pasteur pipet, remove all but the bottom 100 µl of medium (Figure 1C).

Be careful here as many oocytes can be lost during this step. An automatic pipettor makes this task quite simple. Aspirate down to the 100 ml mark (refer to Figure 1C). If the settled oocytes become disturbed, STOP IMMEDIATELY and wait a few minutes for the oocytes to settle again.

2. While oocytes are settling, add ~5 ml of OCM to an X-plate.

Figure 1. Cleaning up the preparation of oocytes collected by ovary slashing. After slashing ovaries, medium is poured into a sterile 400-ml beaker (panel A). After allowing oocytes to settle, medium is aspirated using a disposable pipette until all but 100-ml has been removed (panels B-C). The procedure is repeated several times (until the medium is clear) by filling the beaker with fresh OCM. On the last step, the medium is removed slowly until all but 50-ml has been removed (panel D).
3. Add ~350 ml of fresh OCM and repeat process until medium is clear. For the last wash, remove all but the bottom 50 ml of medium and transfer medium into an intergrid culture dish (100 x 15 mm; Figure 2). Wash the beaker with a small amount of OCM and transfer this medium to the intergrid culture dish also. Place the intergrid dish on plate warmer until ready for searching.

*Only go down to the 50 ml mark after the very last wash (it is of extreme importance that this be done very slowly so as to avoid aspirating the settled oocytes).*
ALTERNATIVE SPERM PURIFICATION PROCEDURES

Updated 6/23/2017

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
    - NaH₂PO₄·H₂O (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 MgCl₂ (Stock 12) and 1.25 mL CaCl₂ (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 heat or cold shock. A space heater in front of the area (not too close) where the sperm work will be performed can aid in preventing cold shock to the sperm cells. All media used for sperm should be warmed to 38.5°C before use. Media necessary for fertilization should be prepared at least 2 h prior to IVF.

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 15 ml conical tubes with H-SOF. Tighten the caps and place in the warm oven.

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 cito thaw 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.
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.*

9. Determine dilution required to bring sperm to a concentration of 26 x 10⁶/ml (this will produce a final concentration of sperm in the fertilization drop of 1 x 10⁶/ml). To do so, add 10 l sperm suspension to 90 l water to kill sperm. Load 10 l 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 10⁶/ml (possible with practice).*
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.
FERTILIZATION IN DROPS

Updated 7/6/17

Unless otherwise stated, procedures for sperm purification and preparation of oocytes for fertilization follow the standard protocol (Day 0).

Initial Preparation - Materials

Laminar flow hood
PureSperm gradient media
IVF-TALP
HEPES-TALP
7 x 15 ml conical centrifuge tubes
3 centrifuge carriers
35 x 10 mm or 60 x 15 mm Falcon tissue culture dishes
Thawing unit (Citothaw)
PHE
Pipet tips and pipettors
Sterile serological pipets
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 two 15 ml conical tubes (per 300 oocytes or per treatment) with HEPES-TALP. These tubes are for washing the oocytes before and after fertilization. Tighten the caps and place in the warm oven.
2. Add 10 ml HEPES-TALP to a 15 ml conical tube. Label ‘Sperm Wash’. Tighten the cap and place in the warm oven.
3. Add 3 ml IVF-TALP to a 15 ml conical tube. Label as ‘Sperm Diluent’. Leave cap loose and place in the incubator.
4. Prepare the fertilization plate. Make enough 60 uL drops of IVF-TALP for all oocytes (30 oocytes/drop). Cover with 4 mL of oil for 35 mm dishes and 9 mL oil for 60 mm dishes.

To help keep the footprint of the drops small (and improve the stability of the drop) it is recommended to make 30 uL drops, cover with oil, and then add an additional 30 µL to each drop for a final volume of 60 uL.

Preparation of Oocytes for Fertilization

1. Fill each corner of the X plate with ~200 µL HEPES-TALP. Put 3 additional ~200 µL drop of HEPES-TALP in between each corner, up against the wall of the plate. See Figure 1 below. Do not cover with oil.

Figure 1. Depiction of an X plate with media in each corner.
2. Transfer COCs from each microdrop of OMM to the X-plate containing HEPES-TALP as described in Day 0 protocol.

For ease of handling of oocytes, 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.

3. Using the warmed IVF-TALP, create a 50 µL drop for each group of 30 oocytes. Transfer groups of 30 oocytes from the X Plate to each drop of IVF-TALP to wash prior to placing in the fertilization dish. Do not cover with oil.

4. Withdraw the fertilization plate from the incubator and transfer a group of 30 oocytes from the IVF-TALP rinse drop to the fertilization drop.

5. Return plate with the oocytes to the incubator until fertilization.

Fertilization

1. Once sperm has been prepared, remove plates containing matured oocytes from the incubator and place on the slide warmer.

For conventional (unsorted) semen the final concentration of sperm in the drop should be 1 million sperm per mL. For sex-sorted semen the final concentration of sperm in the drop should be 2 million sperm per mL.

2. Add 20 µl sperm preparation and 3.5 µl PHE mix into each drop.

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 fertilization 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. We have also 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.
IN VITRO FERTILIZATION WITH SEX-SORTED SEMEN

L. G. Siqueira, N. Alves de Souza Rocha, J. Block, L. Bonilla, M.S. Ortega, A.C. Denicol, and P.J. Hansen

Ovatech, LLC, Dept. of Animal Sciences, University of Florida, and Laboratory of Physiology of Reproduction - UNESP/FMVA

Updated 7-28-2017

PREPARATION OF FERTILIZATION PLATES

Materials

- HEPES -TALP + amikacin
- IVF-TALP + amikacin
- Puresperm® gradient (Nidacon)
- Pipet tips and pipetors
- 60x15 mm Falcon culture dishes
- 15 conical tubes
- Mineral oil
- PHE
- Citothaw
- Microcentrifuge tubes
- Amikacin (see note at end of protocol)

Procedure (prepare everything at least 2 h before fertilization)

For the purpose of this protocol it is assumed that both X and Y sperm will be prepared. Other labels can be used as desired for specific purposes.

1. Prepare microcentrifuge tubes with 500 µl of IVF-TALP + amikacin and label “Sperm Wash”. Prepare one tube per straw of semen used for fertilization.
2. Prepare microcentrifuge tubes with IVF-TALP + amikacin. Calculate the volume needed based on the number of IVF drops being used (ex: if there are 10 drops to be fertilized you will need 500 uL). This will be used for washing oocytes prior to transferring into IVF drops. Label the tubes “COC Wash”.
3. Add 3 ml IVF-TALP + amikacin to a 15 ml conical tube. Label as ‘Sperm Diluent’. Leave cap loose.
4. Fill enough 15 ml conical tubes with HEPES-TALP + amikacin for the number of oocytes that were collected (2-3 tubes should be sufficient for washing 300 oocytes before and after fertilization). Place in 37°C oven.
5. Remove Puresperm® gradient (both Top and Bottom layers) from the refrigerator and 1 aliquot of PHE from the -20°C freezer (wrap in aluminum foil). Place in 37°C oven.
6. Write “Puresperm ♂” and “Puresperm ♀” on empty microcentrifuge tubes (one tube per straw of semen).
7. Write “Pellet ♂” and “Pellet ♀” on empty microcentrifuge tubes (one tube per straw of semen).
8. Prepare fertilization plates by making 60 µl drops of IVF-Talp + amikacin. 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.
9. Place the fertilization plates, “Sperm wash” tubes, “Sperm Diluent” tube, and the “COC Wash” tubes (open lids) inside the 5% CO2 incubator and allow media to equilibrate for at least 2 hours prior to fertilization.
10. Fill the citothaw with fresh deionized water and plug in so that it can warm-up.
Fertilization

Materials

X-Plate (Fisher)
HEPES-TALP + amikacin (pre-warmed)
IVF-TALP + amikacin (pre-warmed and equilibrated)
Puresperm® gradient (pre-warmed)
Plastic sterile Pasteur pipets
Pipet tips and pipettor
Microcentrifuge

Procedure

Preparation of Oocytes

1. Fill each corner of the X plate with ~200 µL HEPES-TALP + AMIKACIN. Put 3 additional ~200 µL drop of HEPES-TALP + AMIKACIN in between each corner, up against the wall of the plate. See Figure 1. Do not cover with oil.

![Figure 1. Depiction of an X plate with media in each corner.](image)

2. Transfer COCs from each microdrop of OMM to the X-plate containing HEPES-TALP + AMIKACIN as described in Day 0 protocol.

   For ease of handling of oocytes, 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.

3. Using the equilibrated “COC Wash”, create a 50 µL drop for each group of up to 30 oocytes. Transfer groups of oocytes from the X Plate to each drop of IVF-TALP + AMIKACIN to wash prior to placing in the fertilization dish. Do not cover with oil.

4. Withdraw the fertilization plate from the incubator and transfer a group of up to 30 oocytes from the IVF-TALP + AMIKACIN rinse drop to the fertilization drop.

5. Return plate with the oocytes to the incubator until fertilization.
Fertilization

1. Prepare Puresperm® gradient by slowly layering the Top layer on top of the Bottom Puresperm® layer (200 µl per layer) in a pre-warmed, pre-labeled microcentrifuge tube, return gradient to the oven.
2. Thaw the needed number of straws of semen for 30 sec in the citothaw. Dry the straws with a kimwipe.
3. 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.
4. Place the microcentrifuge tube containing the Puresperm gradient and semen into a microcentrifuge and centrifuge at 6,000 rpm for 5 min.
5. Transfer the resulting pellet to the “Sperm Wash” tube and centrifuge at 3,000 rpm for 3 min.
6. Remove the supernatant from the “Sperm Wash” tube, using care not to disturb the resulting pellet. Bring the volume of the pellet up to approximately 100 uL using IVF-TALP + amikacin from the “Sperm Diluent” tube.

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)

7. Add 20 µl of the sperm pellet suspension and 3.5 µl of PHE to each fertilization drop.
8. 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.
9. 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. The antibiotic amikacin usually resolves the problem. Amikacin can be obtained from Sigma (Cat No.). The working solution is 20 μg/ml (40 μl of a 50 mg/mL solution into 100 ml solution). All solutions used for IVF and culture should receive amikacin.
DISCONTINUED MEDIA

The following media are no longer routinely utilized in the laboratory. The recipes are provided here for those interested in the media. Note that these recipes allow many media to be made from scratch. Also, the numbering system for stock solutions for discontinued media is different than the numbering system for the current stock solutions.

Transport Saline (0.9%)

Prepare 0.9% saline (90 g NaCl in 10 L double distilled water) and add 100 ml of 100X Pen/Strep. Store indefinitely at 4°C.

Oocyte Collection Medium – OCM

1. Dissolve TCM-199 powder (w Hank’s salts and L-glutamine and without bicarbonate) (Cellgro, 50-051-PB) for 10 L and add 3.50 g NaHCO3 in 10 L ddH2O. Add 100 ml 100X Pen-Strep and Adjust pH to 7.2-7.4. Sterile-filter 400 ml medium into 500 ml glass medium bottles using a Nalgene 0.2 µm FastCap filter (catalog number 298-9020) and keep indefinitely at 4°C. Use two filters for 10 L. Labels should read “Oocyte Collection Medium” OCM - Supplements and date made”.

2. On the day of oocyte collection, add the following: 1 aliquot of stock 4: BSS+Hep and 1 aliquot of stock 11: glutamine (4 ml). Change label to “+ supplements”, and use on the same day (discard leftover medium).

Oocyte Maturation Medium - OMM

1. Prepare 44 ml aliquots of TCM-199 with Earle’s salts (Invitrogen, Cat 11150-059) in 50 ml sterile tubes and store at 4°C until used to make OMM (can be stored at least three months).

2. Prepare 5 bottles of OMM at a time. Each bottle of 44 ml of TCM-199 is supplemented with the following supplements:

   For each bottle of OMM, change label to read “Oocyte maturation medium” OMM “+ supplements” and date.

10X SP-TL (for Percoll)

1. 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
   - Na2HPO4+H2O (Sigma S9638): 0.4000 g
   - HEPES (Sigma H4034): 2.3800 g
2. 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
1. Place 16 ml of 10X SP-TL in a small beaker and add 0.3360 g sodium bicarbonate and 360 µl Na
lactate (Stock 1).
2. Stir until bicarbonate dissolves.
3. Add 144 ml Percoll, 632 µl MgCl2 (Stock 12) and 312 µl CaCl2 (Stock 13).
4. 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.
5. Filter with a 0.22 µm filter attached to a 50 ml plastic tubes (Corning catalog number 430320) so
that the final preparation is stored in four 50-ml tubes.

HEPES TALP
1. To prepare media, mix the ingredients as described in Table 2 and filter with a 0.2 µM Nalgene
bottle-top filter (catalog number 291-4520) into a glass medium bottle and store at 4°C.
2. Write expiration date on the label (use within one week) and store at 4°C.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES-TL</td>
<td>500 ml</td>
</tr>
<tr>
<td>BSA, Fract V</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Na pyruvate (Stock 2)</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Gentamicin (Stock 8)</td>
<td>750 µl</td>
</tr>
</tbody>
</table>

SOF-FERT Stock solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
<th>Sigma CAT#</th>
<th>M.W</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CaCl2.2H2O</td>
<td>1.17 mM</td>
<td>C7902</td>
<td>147</td>
<td>0.2752 g</td>
</tr>
<tr>
<td>2 MgCl2.6H2O</td>
<td>0.49 mM</td>
<td>M2393</td>
<td>203.31</td>
<td>0.1594 g</td>
</tr>
<tr>
<td>3 KH2PO4</td>
<td>1.19 mM</td>
<td>P5655</td>
<td>136.1</td>
<td>0.2591 g</td>
</tr>
<tr>
<td>4 KCl</td>
<td>7.16 mM</td>
<td>P5405</td>
<td>74.55</td>
<td>0.8540 g</td>
</tr>
<tr>
<td>5 NaCl</td>
<td>107.7 mM</td>
<td>S5886</td>
<td>58.44</td>
<td>10.0704 g</td>
</tr>
<tr>
<td>6 Caffeine</td>
<td>1.00 mM</td>
<td>C0750</td>
<td>194.19</td>
<td>0.3107 g</td>
</tr>
<tr>
<td>7 NaHCO3</td>
<td>25.07 mM</td>
<td>S5761</td>
<td>84.01</td>
<td>3.3698 g</td>
</tr>
<tr>
<td>8 Gentamicin (Stock 8)</td>
<td>5 µg/mL</td>
<td>G1397</td>
<td>50 mg/mL</td>
<td>1600 µL</td>
</tr>
<tr>
<td>9 Na-lactate</td>
<td>5.3 mM</td>
<td>L4263</td>
<td>112.06</td>
<td>1209 µL</td>
</tr>
</tbody>
</table>

1. Mix the ingredients as described in Table 3 and sterile-filter with a 0.2 µM Nalgene bottle-top filter
(Fischer catalog number 290-4520) into six 250 mL sterile plastic medium bottles (Nalgene catalog
2019-0250).
2. Write the label “SOF - FERT w/o Supplements”, date and store at 4°C.
**SOF-FERT**

**Table 4. SOF-FERT - Add the following to 250 ml SOF-FERT stock solution**

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Concentration</th>
<th>Sigma CAT#</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>EFAF BSA</td>
<td>6 mg/mL</td>
<td>A6003</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Na-pyruvate (Stock 2)</td>
<td>0.2 mM</td>
<td>P4562</td>
<td>2500 µL</td>
</tr>
<tr>
<td>Heparin</td>
<td>10 µg/mL</td>
<td>H3149</td>
<td>1250 µL</td>
</tr>
</tbody>
</table>

1. Mix the ingredients as described in Table 4 and filter into 5 aliquots with five 0.22 µM 50 mL tube top filters (Corning catalog number 430320).

2. Write the label “SOF - FERT”, date and store at 4°C.

**SOF-BE1 Stock solution**

This is based on the formulation of Ficher-Brown et al. Zygote 10:341-348 (2002) except that the concentration of Na-lactate and BSA are altered, the BSA is essentially fatty acid free BSA, and ALA-glutamine, Na-citrate, and myo-inositol were added.

**Table 5. Recipe for SOF-BE1 stock solution - Add the following to 350 ml Sigma water in sequence:**

<table>
<thead>
<tr>
<th>SOF BE1</th>
<th>Concentration</th>
<th>Sigma CAT#</th>
<th>M.W</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  CaCl₂.2H₂O</td>
<td>1.17 mM</td>
<td>C7902</td>
<td>147</td>
<td>0.0602 g</td>
</tr>
<tr>
<td>2  MgCl₂.6H₂O</td>
<td>0.49 mM</td>
<td>M2393</td>
<td>203.31</td>
<td>0.0349 g</td>
</tr>
<tr>
<td>3  KH₂PO₄</td>
<td>1.19 mM</td>
<td>P5655</td>
<td>136.1</td>
<td>0.0567 g</td>
</tr>
<tr>
<td>4  KCl</td>
<td>7.16 mM</td>
<td>P5405</td>
<td>74.55</td>
<td>0.1868 g</td>
</tr>
<tr>
<td>5  NaCl</td>
<td>107.7 mM</td>
<td>S5886</td>
<td>58.44</td>
<td>2.2029 g</td>
</tr>
<tr>
<td>6  Tri-Na-citrate</td>
<td>0.50 mM</td>
<td>C3434</td>
<td>294.1</td>
<td>0.0515 g</td>
</tr>
<tr>
<td>7  Myo-Inositol</td>
<td>2.77 mM</td>
<td>I7508</td>
<td>180.16</td>
<td>0.1747 g</td>
</tr>
<tr>
<td>8  NaHCO₃</td>
<td>25.07 mM</td>
<td>S5761</td>
<td>84.01</td>
<td>0.7371 g</td>
</tr>
<tr>
<td>9  Na-lactate</td>
<td>5.3 mM</td>
<td>L4263</td>
<td>112.06</td>
<td>264 µL</td>
</tr>
<tr>
<td>10 Gentamicin (Stock 8)</td>
<td>25 µg/mL</td>
<td>G1397</td>
<td>50 mg/mL</td>
<td>1750 µL</td>
</tr>
</tbody>
</table>

1. To prepare medium, mix the ingredients and sterile-filter with a 0.2 µM Nalgene bottle-top filter (Fischer catalog number 290-4520) into a glass culture medium bottle; pipette 50 ml of the solution into six 60 mL sterile plastic medium bottles (Nalgene 2019-0060).

2. Write label “SOF - BE1 w/o Supplements”, date and store at 4°C.
SOF-BE1

**Table 6. Recipe for SOF-BE1** - Add the following to 50 ml SOF-BE1 stock solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
<th>Sigma CAT#</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>*EFAF BSA</td>
<td>4 mg/mL</td>
<td>A6003</td>
<td>0.2000 g</td>
</tr>
<tr>
<td>ALA-glutamine</td>
<td>1 mM</td>
<td>A8185</td>
<td>500 µL</td>
</tr>
<tr>
<td>Na-pyruvate (Stock 2)</td>
<td>0.4 mM</td>
<td>P4562</td>
<td>1000 µL</td>
</tr>
<tr>
<td>Non-Essential aa</td>
<td>10 µL/mL</td>
<td>M7245</td>
<td>500 µL</td>
</tr>
<tr>
<td>Essential aa</td>
<td>20 µL/mL</td>
<td>B6766</td>
<td>1000 µL</td>
</tr>
</tbody>
</table>

*PVA (1 mg/mL) can be substituted for BSA to make modified SOF-BE1

1. Mix the ingredients and filter 10 ml aliquots into 15 mL Starstedt sterile tubes (Starstedt catalog number 62.554.205) using a single 0.2 µM syringe filter (Corning catalog number 431229).

2. Write the label “SOF - BE1”, date and store at 4°C.

**TL Solutions - For Making TALPs**

1. To prepare media, mix the ingredients as described in Table 7 (all volumes are in milliliters), adjust the pH, check osmolarity (if osmometer is available) and sterile-filter the solution.

2. Write expiration date on the label (use within one week) and store at 4°C.

**Table 7. Recipes for preparation of TL solutions**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Sp-TL</th>
<th>HEPES-TL</th>
<th>IVF-TL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (ml)</td>
<td>79.232</td>
<td>177.0</td>
<td>40.157</td>
</tr>
<tr>
<td>Stock 17: NaCl (ml)</td>
<td>4.34</td>
<td>10.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Stock 18: KCl (ml)</td>
<td>1.96</td>
<td>4.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Stock 19: bicarb (ml)</td>
<td>10.00</td>
<td>1.6</td>
<td>5.0</td>
</tr>
<tr>
<td>Stock 20: phosphate (ml)</td>
<td>1.0</td>
<td>2.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Stock 1: Na-lactate (ml)</td>
<td>0.368</td>
<td>0.372</td>
<td>0.093</td>
</tr>
<tr>
<td>Stock 21: HEPES (ml)</td>
<td>1.0</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>Stock 22: Ca chloride (ml)</td>
<td>1.0</td>
<td>2.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Stock 23: Mg chlor (ml)</td>
<td>1.10</td>
<td>1.0</td>
<td>0.25</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.3</td>
<td>7.4</td>
</tr>
<tr>
<td>Osmolarity (mOsm)</td>
<td>295-305</td>
<td>275-285</td>
<td>290-300</td>
</tr>
</tbody>
</table>

**TALP (Tyrode’s Albumin Lactate Pyruvate) Media**

Mix the ingredients as described in Table 8 and sterile-filter the solution. Write expiration date on the label (use within one week) and store at 4°C.

**Table 8. Recipes for TALP media.**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Sp-TALP</th>
<th>HEPES-TALP</th>
<th>IVF-TALP</th>
</tr>
</thead>
</table>

4 | Discontinued Media
<table>
<thead>
<tr>
<th></th>
<th>TL (ml)</th>
<th>76.0</th>
<th>500.0</th>
<th>100.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA, Fract V (g)</td>
<td>0.48</td>
<td>1.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>BSA, EFAF (g)*</td>
<td>0</td>
<td>0</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Stock 2: pyruvate (ml)</td>
<td>4.0</td>
<td>5.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Stock 8: gentamicin (μl)</td>
<td>160</td>
<td>750</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Stock 7: heparin (μl)</td>
<td>0</td>
<td>0</td>
<td>500</td>
<td></td>
</tr>
</tbody>
</table>
**KSOM-BE (Potassium Simplex Optimized Medium - Bovine Embryo Modification 2)**

1. Purchase KSOM MR-106-D from Millipore and store frozen. Once thawed, keep at 4°C for 2 weeks.

2. To 5 ml of KSOM stock add:
   - EFAF BSA - 15 mg (3.00 mg/ml)
   - Gentamicin stock 8A - 2.5 μl (0.5 μl/ml)
   - Nonessential amino acids, 100X - 25 μl

3. Sterile filter medium through a 0.22 μm syringe filter into a sterile 15 ml Starstedt tube. Use immediately.

**CR1aa (an alternative culture medium)**

*Note: the patent for this medium is held by Infigen*

1. Make CR1 stock (prepare in a 100 ml volumetric flask):
   - NaCl - 0.670 g
   - KCl - 0.023 g
   - NaHCO₃ - 0.220 g
   - Na Pyruvate - 0.004 g
   - Glutamine - 0.015 g
   - Hemi-Ca Lactate - 0.055 g

   Add first 5 ingredients to volumetric flask. Add water (~90 ml). Thoroughly dissolve constituents and then add Hemi-Ca Lactate. Add remaining water. Store for up to 2 days at 4°C.

   *Note: constituents of this medium are known to precipitate out of solution. To minimize the chances of this occurring, make sure all constituents are dissolved before adding hemi-Ca lactate and use immediately after making. If a medium appears white and cloudy, discard and start again.*

2. To prepare CR1aa, add the following to 5 ml of CR1 stock:
   - EFAF BSA - 15 mg (3.00 mg/ml)
   - Gentamicin stock 8A - 2.5 μl (0.5 μl/ml)
   - Nonessential amino acids, 100X - 50 μl
   - Essential amino acids, 50X - 100 μl

   Sterile filter medium through a 0.22 μm syringe filter into a sterile 15 ml Starstedt tube. Use immediately.

**STOCK SOLUTIONS**

*For ease of use, prepare aliquots of all solutions and keep frozen. Aliquots may be placed in a Styrofoam rack and the rack labeled with stock number, solution name, aliquot volume, and date.*

*The choice of water for making stocks depends upon local availability of highly-purified water. We make up stock solutions using Tissue Culture Water purchased from Sigma. For all other media (OCM, saline, etc.), we use Sigma water or distilled and deionized water.*

**Stock 1: Na lactate.** Purchase as a 98% syrup. Follow manufacturer’s indications for expiration date. Store at 4°C.

**Stock 2: Na pyruvate.** Dissolve 0.220 g sodium pyruvate in 100 ml water. Sterile-filter into an aluminum-foil wrapped 100 ml bottle and store at 4°C for 1 mo.
Stock 3: Bovine Steer Serum (BSS). Prepare 5 ml aliquots of BSS in sterile 13 ml tubes and store at -20°C indefinitely.

Stock 4: BSS/Hep. Add 1000 USP units of sterile heparin (dissolved in 3-5 ml of water and sterilize through a syringe filter) into 500 ml BSS (Stock 3). Store in 8 ml aliquots in sterile 13 ml tubes indefinitely at -20°C.

Stock 5: Estradiol. Dissolve 1 to 3 mg estradiol in ethanol for a final concentration of 1 mg/ml. Store in a glass container at -20°C for up to 6 months.

Stock 6: Folltropin. Reconstitute Folltropin-V as directed by manufacturer to prepare a 20 μg/μl solution. Place 150 μl aliquots into sterile 1.5 ml microcentrifuge tubes and store indefinitely at -20°C.

Stock 7: Heparin. Dissolve 20 mg in 10 ml water. Pipet 300 μl aliquots and store at -20°C in 1.5 ml microcentrifuge tubes indefinitely.

Stock 8: Gentamicin. Dilute to 5 mg/ml concentration with water and sterile filter. Pipet 600 μl aliquots into sterile microcentrifuge tubes and store at -20°C indefinitely.

Stock 8A: Gentamicin. When preparing Stock 8, prepare a few extra tubes of 10 μl aliquots in sterile microcentrifuge tubes and store at -20°C indefinitely.

Stock 9: PHE Mix. Prepare as fresh solutions primary stocks of 1 mM hypotaurine (1.09 mg in 10 ml saline), 2 mM penicillamine (3 mg in 10 ml saline) and 250 μM epinephrine [dissolve 1.83 mg in 40 ml of a lactate-metabisulfite solution (stock 9a)]. Epinephrine is easily oxidized by direct light so take precautions to avoid this problem (wrap in aluminum foil or place in dark container). Combine 10 ml of 1 mM hypotaurine, 10 ml of 2 mM penicillamine, 4 ml of 250 μM epinephrine and 16 ml of saline and sterile filter. Aliquot 400 μl of PHE Mix into sterile 1.5 ml microcentrifuge tubes and store in a light resistant container at -20°C indefinitely. Upon retrieval of PHE mix for use, wrap tube in aluminum foil.

Stock 9a: Lactate-metabisulfite solution. Add 77 μl of a 98% Na lactate syrup (or the equivalent volume if a lower percent lactate syrup is used) and 50 mg Na metabisulfite to 50 ml water. Make fresh for each use.

Stock 10: Glutamine (1ml). Prepare stock solution of 1.5 g glutamine/100 ml water, sterile filter and make 1 ml aliquots in sterile 4 ml tubes and store at -20°C indefinitely.

Stock 11: Glutamine (4 ml). Prepare stock solution of 1.5 g glutamine/100 ml water, sterile filter and store 4 ml aliquots in 13 ml tubes at -20°C indefinitely.

Stock 12: MgCl₂ for Percoll. Prepare 0.1 M stock by adding 0.203 g MgCl₂ to 10 ml water. Sterile filter and store at 4°C indefinitely.

Stock 13: CaCl₂ for Percoll. Prepare 1 M stock by adding 0.735 g CaCl₂+2H₂O to 5 ml water. Sterile filter and store at 4°C indefinitely.

Stock 14: Hyaluronidase. Prepare stock solution of type IV hyaluronidase at 10,000 units/ml in saline, sterilize through a 0.2 μm filter into a sterile tube, and prepare 100 μl aliquots in sterile microcentrifuge tubes and store at -20°C indefinitely.
Stock 15: Pen/Strep (4 ml). Thaw 100 ml bottle of pen/strep and aliquot 4 ml into sterile 5 ml tubes and store at -20°C indefinitely.

Stock 16: Pen/Strep (10 ml). Thaw 100 ml bottle of pen/strep and aliquot 10 ml into sterile 13 ml tubes and store at -20°C indefinitely.

Stocks 17-23 (prepare only if you make your own TL media)

Stock 17: NaCl. Dissolve 6.665 g in 50 mL water. Sterile filter and store at 4°C.

Stock 18: KCl. Dissolve 0.588 g in 50 mL water. Sterile filter and store at 4°C.

Stock 19: NaHCO₃. Dissolve 1.052 g in 50 mL water. Sterile filter and store at 4°C for one week only.

Stock 20: PO₄. Dissolve 0.235 g NaH₂PO₄·H₂O in 50 mL water. Sterile filter and store at 4°C.

Stock 21: 1 M HEPES. Add 119 g of HEPES to 400 mL water. Adjust pH to 7.0 and bring volume up to 500 mL. Sterile filter and cover container with aluminum foil; store at 4°C indefinitely.

Stock 22: CaCl₂ for TL. Dissolve 1.470 g CaCl₂+2H₂O in 50 mL water. Sterile filter and store at 4°C.

Stock 23: MgCl₂ for TL. Dissolve 1.017 g MgCl₂+6H₂O in 50 mL water. Sterile filter/store at 4°C.

Stock 24: Fetal calf serum. Prepare 100 µl aliquots of heat-inactivated fetal calf serum in sterile microcentrifuge tubes and store at -20°C.

Stock 25: Glutamax. Dissolve 0.434 g ALA-Gln in 20 ml Sigma water and sterile filter. Pipet 600 µL aliquots into sterile microcentrifuge tube and store at -20°C.