In Vitro Production of Bovine Embryos

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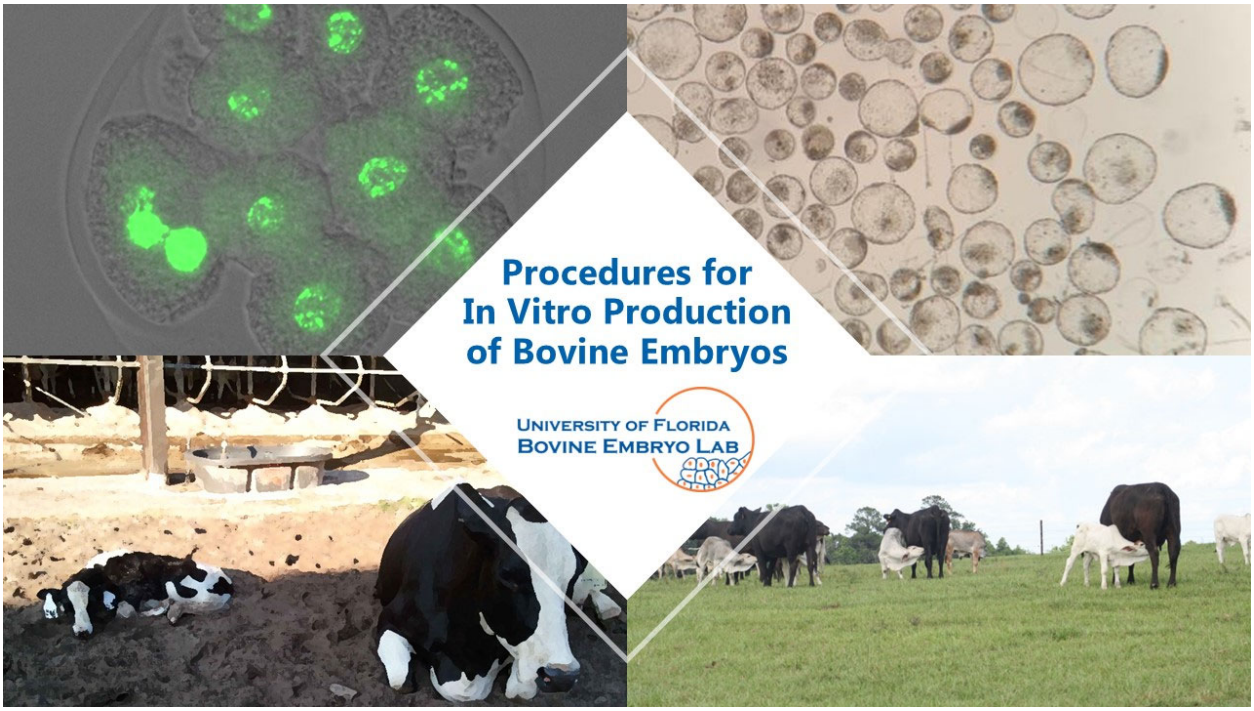

The procedures for in vitro production [IVP; i.e. in vitro maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC)] of embryos described here are based on procedures developed in other laboratories at the University of Wisconsin (Neal First), and University of Missouri (R. Michael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

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ABBREVIATED IVF PROTOCOL

Revised 3/15/2023

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

- Prepare buckets and saline for the ovary collection person:
  - Filled up 8 plastic buckets ¼ with saline (place in the walk-in fridge) and leave it in the cooler
  - Filled up 1L bottle with saline and place in the walk-in fridge with Oocyte Washing Medium (Oocyte Washing Medium with BSA – 19982/1200 Minitube)
  - Place 2 to 3 bottles of Oocyte Washing Medium in the walk-in fridge

DAY -1 (Slashing and in vitro maturation - IVM)

- Collection of ovaries from slaughterhouse
  - Preparation for oocyte collection (at least 2 hours prior to oocyte collection)
  - Place Oocyte Washing Medium and saline in the oven (located in the slashing area)

  Note: this step is already done by the person going to the slaughterhouse
  - Prepare OMM microdrops (50 µL/drop – total 18 to 20 drops) in a 60 mm petri dish and cover with mineral oil (7.5 mL)
  - Place plates in the CO_2 incubator (Sanyo conventional incubator -38.5°C in 5% CO_2)

- Set up for oocyte collection
  - Hemostat
  - Scalpel
  - Scalpel blades
  - Gloves
  - 400 mL beaker
  - Bench paper to cover surface

- Harvest oocyte-cumulus complexes from ovaries
  - Add 150 mL of warm MOFA to beaker
  - Slice ovaries
  - Swirl ovaries in the beaker

- Set up for oocyte fluid filtering and collection
  - 100 µm cell strainer
  - 10 mL NormJect syringe
  - 18 gauge needle
  - Integrid plate

- Oocyte collection
  - Set up cell strainer over 400 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 Oocyte Washing Medium and rinse the debris from the strainer into the integrid dish
  - Collection of cumulus oocyte complexes (COCs) by filtering collected fluid

- Search for COCs
  - X-plate
  - Dissecting microscope
  - Searching instrument (microdispensor, wiretrol, etc.)
  - Slide warmer
  - Transfer COCs to X-plate and rinse through three additional wells

Note: Do not use overmature COCs

- Oocyte maturation
  - After rinsing, place cleaned COCs (10/drop) into a microdrop of pre-equilibrated OMM covered in oil
  - Mature COCs for 22-24 h

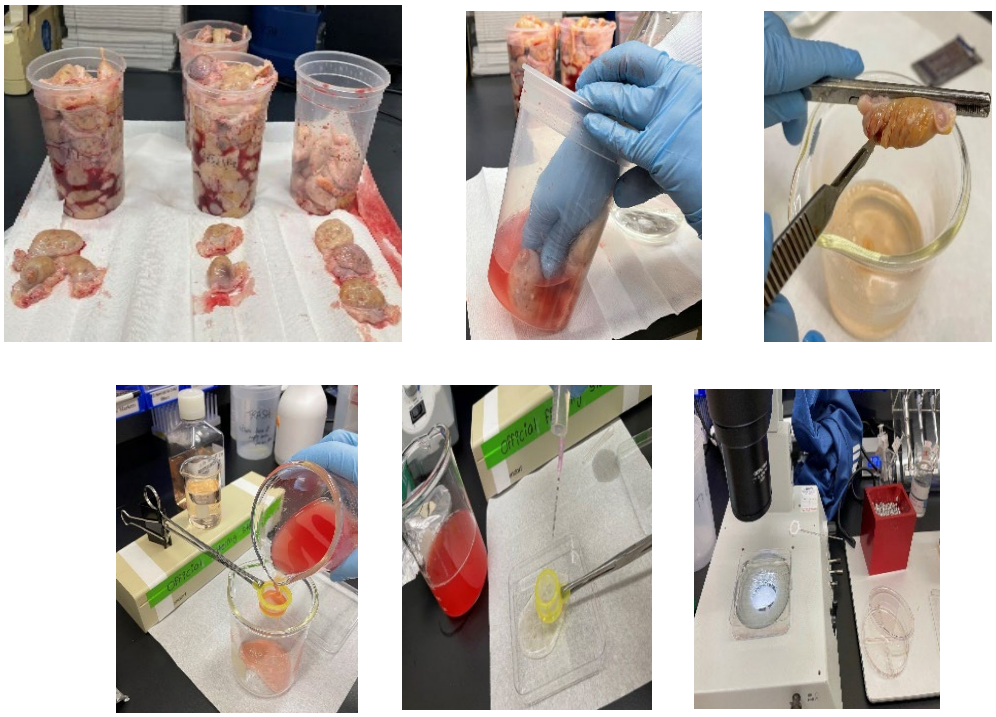


Figure 1. *Overall slashing, filtering and searching of oocytes. From left to right on top are images of 1) ovary selection, 2) washing and massaging selected ovaries with saline and 3) incision of follicles across surface of ovary. From left to right on bottom are 1) filtration of medium with oocytes, 2) rinsing the filter into an integrid petri-dish and 3) integrid dish containing COCs to be searched and X-plate to place the COCs and wash it 3x prior to transfer to maturation dish.*

DAY 0 (in vitro fertilization - IVF)

- Preparation of media for fertilization (at least 2 hours prior to fertilization)

All reagents are placed in the conventional incubator - 38.5°C in 5% CO_2

- HEPES-TALP (tighten cap once placed in incubator)
  - 15 mL HEPES-TALP (labeled "Oocyte Wash") in 15 mL conical tubes and 1 tube with 10 mL (labeled "Sperm Wash")
  - 15 mL of HEPES-TALP (labeled "Zygote wash" - 1 tube/person), wrap it with parafilm
- IVF-TALP *(leave cap loose* once placed in incubator)
  - 3 to 4 mL IVF-TALP in 15 mL conical tube labeled "Sperm Diluent"
  - 35 mm dishes containing 1700 µL IVF-TALP

  - PHE (aliquots stored in -20 freezer in the slashing area) - cover with aluminum foil
  - PureSperm
    - 1.5 mL 40% and 1.5 mL 80% (aliquots stored in walk-in fridge)

- Make sure there are warm centrifuge canisters (placed in conventional incubator)

- Plug in Citothaw – fill with distilled water

- Setup for COCs washing and Fertilization
  - X-Plate with HEPES-TALP ("Oocyte Wash")
  - 1 mL syringe with adaptor
  - Dissecting microscope
  - Semen straw plunger
  - Scissors
  - Inverted microscope (located in main lab)
  - Small petri-dish (35 mm)
  - Rack for tubes
  - 15 mL conical tube
  - Plastic sterile Pasteur pipets
  - Pipet tips

- Matured oocytes: Washing and Fertilization
  - Add ~ 3.5 mL HEPES-TALP to each well of X-plate (1 well/person)
  - Transfer COCs to the well and wash 3 times (1x/corner) in the same well
  - Transfer COCs to 35 mm dish with 1700 µL/plate IVF-TALP

- Sperm preparation
  - Prepare PureSperm gradient: add 1.5 mL of bottom and slowly add 1.5 mL of top to a 15 mL conical tube
  - Place 1-3 straws of semen in citothaw (take it from IVF tank – tank #8) and wait 30-45 sec
  - Layer semen on top of PureSperm gradient using the semen plunger
  - Place PureSperm tube with semen in a warmed centrifuge canister
  - Centrifuge for 10 min at 1000 x g (centrifuge mark: 2.5)
  - Collect all semen pellet with a Pasteur pipet
  - Place pellet into the 10 mL HEPES-TALP tube ("Sperm Wash")
  - Place HEPES-TALP tube into a warmed centrifuge canister
  - Centrifuge for 5 min at 200 x g (centrifuge mark: 1.5)
  - Pipet off supernatant down to the pellet
  - Measure volume of pellet and add IVF-TALP (IVF-TALP to be added = 600 µL - volume of pellet)
  - Add 10 µL of the mix (IVF-TALP + semen pellet) to 90 µL of water. Pipet up and down to mix and immediately add 10 µL of this volume to each side of the hemocytometer to determine sperm concentration (average of counts from five squares among the middle 25 - four squares on corner and one in middle)
  - Sperm Dilution:
    Total µL of IVF-TALP to be added to 600 µL sperm = (17.647 x sperm count in five squares) - 600

- Fertilization
  - Add 120 µL of diluted sperm to each fertilization plate containing COCs
  - Add 80 µL of PHE to each plate
  - Place plates back in the incubator and allow fertilization to proceed for ~ 10 to 18 h

- Preparation of culture medium plates
  Note: This step can be done after/during IVF procedure when time allows it or at least 2 hours prior to culture (Day 1)
  - Add 50 µL microdrops of SOF-BE2/culture medium in 35 mm dish (total: 4 to 6 drops/dish) and cover with 3 to 3.5 mL mineral oil. Place dish in benchtop incubator (5% CO_2 and 5% O_2 at 38.5C)

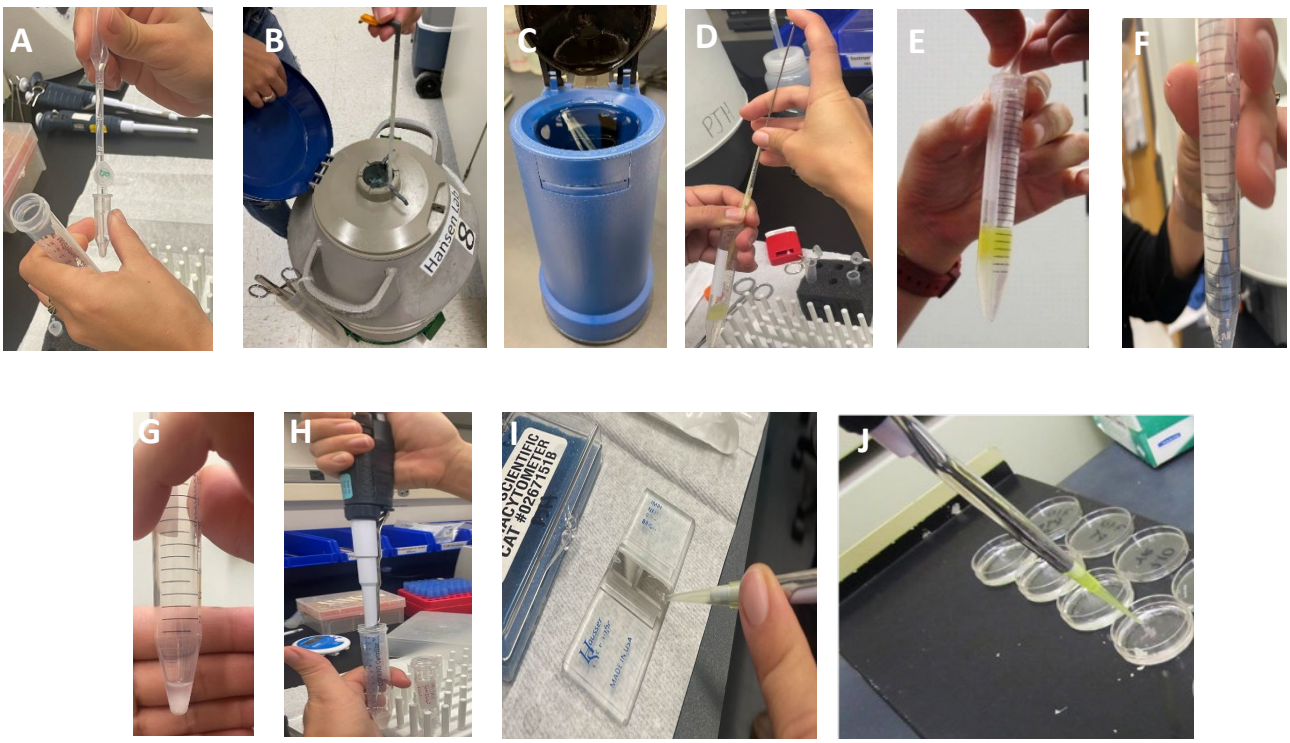


Figure 2. *Overall semen preparation for IVF. A) Semen gradient preparation (note addition of bottom and top of Eppendorf tube to a 15 mL tube). B) Removal of semen straws from nitrogen tank. C) Thawing of semen straws in cytothaw. D) Adding semen from straw to tube prepared on figure A. E) Aspiration of sperm pellet after first centrifugation. F) Addition of sperm pellet to HEPES-TALP ("Sperm Wash"). G) Pellet after second centrifugation and removal of supernatant. H) Addition of IVF-TALP to sperm pellet to bring up volume of 600 µL. I) Addition of dead sperm (sperm + water) to hemocytometer. J) Addition of diluted sperm to petri dish containing the mature oocytes.*

DAY 1 (in vitro culture - IVC)

- Setup for removal of putative zygotes from fertilization plate
  - Vortex mixer
  - Slide warmer
  - Hyaluronidase (stored in -20 freezer – slashing area)
  - X-Plate
  - HEPES-TALP (tube labeled "Zygote Wash")
  - Dissecting microscope
  - Searching instrument (wiretrol)
  - 1 mL syringe with adaptor
  - Timer

- Remove zygotes from fertilization plate
  - Add 300 µL HEPES-TALP to the microcentrifuge tube of Hyaluronidase (needs to be thawed and warm) and pipette up and down to mix
  - Transfer zygotes from the fertilization plate to the microcentrifuge tube using the syringe
  - Allow putative zygotes to settle to bottom of tube for 2 minutes (place it in the tube warmer or conventional incubator)
  - Remove supernatant and discard it on the lid of the X-plate, leaving ~100-200 µL in tube with zygotes
  - Check on lid of X-plate if there is any zygote left behind. If there is, take it and put it back to tube
  - Vortex the tube for 5 min (maximum speed)

- Washing
  - Take "Zygote Wash" tube and add HEPES-TALP to the 4 wells of X-plate (add less to first well). Leave about 3 mL to the next step.
  - Use Pasteur pipet or syringe with adaptor to move contents of the microcentrifuge tube to the first well of the X-plate.
  - Wash tube 1 to 2x to make sure there is no putative zygote left behind
  - Search for cumulus-free zygotes
  - Wash zygotes (3X) – move from well 1 to well 2, then 3 and lastly 4.
  - During the washing steps, best zygotes need to be selected based on morphology (homogeneous and dark cytoplasm and without COCs)
  - Transfer in groups of up to 30 to the SOF-BE2 or another culture media microdrops
  - Place culture plate in benchtop incubator

DAY 3.5

- Determine cleavage rate (be quick)

DAY 7.5

- Determine blastocyst rate
  Note: If not using blastocyst for your project, place blastocyst to tube with paraformaldehyde labeled as "blastocyst for teaching"

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.

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.

The glassware used for embryo work and in the IVF lab must be free of soap residue as it will harm the embryos. It is essential to be certain there is no soap residue left in or on any of this glassware.

- Scrub bottles and beakers with brush and PCC soap solution.
- Rinse vigorously 5 times with tap water and 5 times with D.I. water.
- Be certain there is no soap residue left in or on any of the dishes!!!!!
- Change the water and soap in the tubs weekly.
    - Fill tub with D.I. water and add 25 pumps or 700 ml of PCCP-free soap.
    - Label the tubs with the date the water and soap were changed.
- Set dishes to dry in racks above IVF sink.
- Cover beakers with 2 layers of aluminum foil.
- Place lids **loosely** on bottles.
- Package stir bars and utensils in autoclave bags and seal shut.
- Place autoclave tape on everything to be autoclaved (see page 3 for autoclave instructions).
- Put autoclaved dishes and supplies away in the cabinets and drawers in the IVF laboratory.

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.

this page was last updated 3-8-2023 by Peter J. Hansen

STOCK SOLUTIONS

*For ease of use, prepare aliquots of all solutions and keep frozen. **Aliquots** may be placed in a Styrofoam rack (15 mL) or small box (bullet tubes). The **rack or box should be labeled with stock number, solution name, aliquot volume, date, and initials of the person making the solution**. 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 Water for embryo transfer purchased from Sigma (W1503 - IVF water). For all other media (i.e., saline), we use Millique water or double distilled and deionized water.

Stock 1: Na lactate. Purchase as a 60% syrup. Follow manufacturer's indications for expiration date.
Source: Sigma L7900 – 100 mL
Aliquot Size: Keep in manufacturer's bottle
Storage: Store at 4°C (walk-in fridge)

Stock 2: Na pyruvate. Dissolve 0.220 g sodium pyruvate (stored in the walk-in fridge) in 100 mL IVF water. Sterile filter.
Source: Sigma P4562 – 5G
Aliquot Size: 5.2 mL aliquots (~ 15 total) and 1100 µL aliquots in 2 mL microcentrifuge tubes (~ 20 total)
Storage: Store at -20°C (main lab)

Stock 3: Fetal bovine Serum (FBS)*. Thaw and aliquot prior to freezing (or refreezing).
Source: Gibco – 10437010 (supplier: Thermo Fisher)
Aliquot Size: 10.1 mL aliquots of FBS in sterile 15 mL tubes
Storage: Store at -20°C (slashing area)

**The old name for this stock was BSS (bovine steer serum). We now use FBS as a replacement because steer serum is hard to find.*

Stock 5: Estradiol. Dissolve 1 to 3 mg estradiol (main lab: reagent's cabinet) 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 it is to weigh small masses of estradiol.
Source: Sigma E2758 - 250MG
Aliquot 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 2 mL microcentrifuge tubes and store indefinitely at -20°C.
Source: A30T – Vetoquinol (supplier : AgTech)
Aliquot Size: 30 µL in sterile 2 mL microcentrifuge tubes
Storage: Store in -20°C freezer – IVF box (slashing area)

Stock 7: Heparin. Dissolve 20 mg heparin (heparin sodium salt from porcine intestinal mucosa) stored in the walk-in fridge) in 10 mL IVF water. Don't need to filter it.
Source: Sigma H3149 - 100KU
Aliquot Size: 1010 µl aliquots in 2 mL microcentrifuge tubes
Storage: Store at -20°C

Stock 8: Gentamicin. Obtain gentamicin solution (10 mg/mL – stored in the walk-in fridge). Follow manufacturer's indications for expiration date.
Source: Sigma G1272 – 10 mL
Aliquot Size: Keep in manufacturer's bottle
Storage: Store at 4˚C until expiration date (walk-in fridge)

Stock 9: PHE Mix.

- Step 1: Prepare saline:
  - 0.9% Saline: 0.45 g NaCl dissolved into 50 mL IVF water.
- Use saline (prepared in step 1) to make hypotaurine and penicillamine:
  - 1mM hypotaurine: 1.09 mg (0.00109 g) hypotaurine dissolved in 10 mL saline.
  - 2mM penicillamine: 3 mg (0.003 g) penicillamine dissolved in 10 mL saline.
- Step 2: Prepare Stock 9A (Lactate-metabisulfite solution):
  - Add 125.77 µl of 60 % Na DL-lactate syrup and 50 mg of Na metabisulfite (both stored in the walk-in fridge) to 50 mL of IVF water.
- Use Stock 9A (prepared in step 2) to make epinephrine:
  - 250 uM 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 (prepared in step 1)
- Sterile filter
- Upon retrieval of PHE mix for use, wrap tube in aluminum foil

Source: *NaCl* - Sigma S5886-500G, *Hypotaurine* - Sigma H1384-100MG, D-*Penicillamine* - Sigma P4875-1G, *Epinephrine* - Sigma E4250-1G (All are stored in the walk-in fridge, except NaCl which is stored in the main lab: reagent's cabinet)
Aliquot Size: 350 µl of PHE Mix into sterile 2 mL microcentrifuge tubes
Storage: Store in light blocking container at -20˚C freezer (slashing area). Wrap tube in aluminum foil to block light upon retrieval for use

Stock 9A: Lactate-metabisulfite solution. Add 125.77 µl of 60% Na lactate syrup and 50 mg Na metabisulfite (both stored in the walk-in fridge) to 50 mL of IVF water.
Source: Sigma L7900 – 100 mL (60% syrup) and 13459 – 500G (Na metabisulfite)
Aliquot Size: N-A
Storage: Make fresh for each use

*****If 98% Na DL-lactate (Thermo Fisher - L14500.06): Add 77 µl of a 98% Na lactate syrup and 50 mg Na metabisulfite (both stored in the walk-in fridge) to 50 mL of IVF water.

Stock 11: Glutamax. Obtain 100X Glutamax (stored in the walk-in fridge). Follow manufacturer's indications for expiration date.
Source: Gibco - 35050-061 (supplier: Thermo Fisher)
Aliquot Size: Keep in manufacturer's bottle and seal it with parafilm after each use
Storage: Store at 4˚C until expiration date (walk-in fridge)

Stock 12: $MgCl_2$ for Percoll. Prepare 0.1 M stock by adding 0.203 g $MgCl_2$ +$6H_2O$ (stored in the main lab: IVF reagent's cabinet) to 10 mL water. Sterile filter.
Source: Sigma M2393 - 100G
Aliquot Size: N-A
Storage: Store at 4°C

Stock 13: $CaCl_2$ for Percoll. Prepare 1 M stock by adding 0.735 g $CaCl_2$+$2H_2O$ (stored in the main lab: IVF reagent's cabinet) to 5 mL water. Sterile filter.
Source: Sigma C7902 - 500G
Aliquot 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 from bovine testes (stored in -20°C freezer: slashing area inside IVF box) into 10 mL saline. Sterile filter.
Source: Sigma H3884 - 1G
Aliquot Size: 100µL into 2 mL microcentrifuge tubes
Storage: Store in at -20°C

Stock 15: 100X Myo-inositol. Dissolve 0.998 g of myo-inositol (stored in the main lab: IVF reagent's cabinet) in 20 mL of IVF water. Sterile filter.
Source: Sigma I7508 - 50G
Aliquot Size: 1 mL into 2 mL microcentrifuge tubes
Storage: Store in at -20°C

Stock 16: 100X Na citrate. Dissolve 0.2941g of sodium citrate tribasic dihydrate (stored in the main lab: IVF reagent's cabinet) in 20 mL of sterile water. Sterile filter.
Source: Sigma C3434 - 205G
Aliquot Size: 550 µL into 2 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: Gibco – 15140-122 (supplier: Fisher)
Aliquot Size: Keep in manufacturer's bottle (1 mL)
Storage: aliquots in -20°C freezer (slashing area)

Stock 19: Human EGF. Dissolve in DPBS-BSA to 100 ug/mL in IVF water. Store indefinitely.
Source: Invitrogen - BMS320 (supplier: Fisher)
Aliquot Size: 100 µL into 2 mL microcentrifuge tubes
Storage: Store at -20°C freezer (slashing area) indefinitely

Currently, Stocks: 4 (BSS/Hep), 10A (EFAF BSA for SOF-FERT), 10B (EFAF BSA for SOF-BE1, 10C (BSA Fraction V for H-SOF) are not in use and have been added to the Discontinued Media file.

MEDIA PREPARATION

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**
  - Boviplus Oocyte Washing Medium, Minitube (19982/1200)

- **Oocyte Maturation Medium – OMM**
  - To prepare OMM, combine the ingredients listed in Table 1. Label the bottle OMM with initials, date made, and expiration date and store at 4˚C for up to one month. Equilibrate sample prior to use.

Table 1. Recipe for preparation of OMM working solution

OMM Working Solution				
Ingredient	**Stock Concentration**	**Final Concentration**	**Preparation of 100 mL**	**Source and Catalog #**
Gibco M199	-	-	86.925 mL	Thermo Fisher, 11150
Fetal Bovine Serum	-	10% (v/v)	10 mL	Atlanta Biologicals, S11150H
Pen Strep	100 x	1% (v/v)	1 mL	Stock 18
Na pyruvate	200 mM	0.2 mM	1 mL	Stock 2
Glutamax	375 µl	-	1 mL	Stock 11
Human Recombinant EGF	100 µg/mL	50 ng/mL	50 µL	Stock 19
FSH (Folltropin)	20 mg/mL	5 µg/mL	25 µL	Stock 6
Filter in a sterile bottle				

- **HEPES-TL (Base Solution)**
  - To make 1 L of HEPES-TL base solution, dissolve the ingredients listed in Table 2 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 RapidFlow filter (Thermo Fisher; catalog number 291-4520) and keep for up to 6 months at 4˚C. Labels should read HEPES-TL Base with initials and date made.

- **HEPES-TALP (Working Solution)**
  - To prepare HEPES-TALP, add the ingredients listed in Table 3 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-TL base solution

HEPES-TL Base Solution					
Component	**Mol Wt**	**mM**	**mg/L**	**g/L**	**Sigma Cat #**
NaCl	58.5	114	6669	6.669	S5886
KCl	74.6	3.2	238.72	0.2387	P5405
$NaHCO_3$	84	2	168	0.168	S5761
$NaH_2PO_4.H_2O$	120	0.4	48	0.048	S9638
Na Lactate (792 g/L) (ml)	112.1	10	1.416	1416 µL	L4263
$CaCl_2$.$2H_2O$	147.01	2	293.8	0.2938	C7902
$MgCl.6H_2O$	203.30	0.5	101.65	0.1017	M2393
HEPES	238.4	10	2384	2.384	H4034
Osmolarity	260-270				
pH	7.3-7.4				

Table 3. Recipe for preparation of HEPES-TALP

Ingredient	Amount	**Sigma Cat #**
HEPES-TL Base Solution	500 ml	
BSA, Fract. V	1.5 g	A3311
$NaHCO_3$	84	
Na pyruvate (Stock 2)	5 ml	
Gentamicin (Stock 8)	375 µl	
Filter in a sterile bottle		

- **IVF-TL (Base Solution)**
  - To make 1 L of IVF-TL base solution, dissolve the ingredients listed in Table 4 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 Rapid Flow filter (Thermo Fisher; catalog number 290-4520) and keep for up to 6 months at 4°C. Labels should read IVF-TL Base with initials and date made.

Table 4. Recipe for preparation of IVF-TL

IVF -TL Base Solution					
Component	**Mol Wt**	**mM**	**mg/L**	**g/L**	**Sigma Cat #**
NaCl	58.5	114	6669	6.669	S5886
KCl	74.5	3.2	238.4	0.2384	P5405
$NaHCO_3$	84	25	2100	2.100	S5761
$NaH_2PO_4.H_2O$	138	0.4	55.2	0.0552	S9638
Na Lactate (792 g/L)(ml)	112.1	10	1.416	1416 µL	L4263
$CaCl_2$.$2H_2O$	111.1	2	222.2	0.2222	C7902
$MgCl.6H_2O$	203.3	0.5	101.65	0.1017	M2393
Osmolarity	280-300				
pH	7.3-7.4				

- **IVF-TALP (Working Solution)**
  - To prepare IVF-TALP, add the ingredients listed in Table 5 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 5. Recipe for preparation of IVF-TALP

IVF-TALP Working Solution		
Ingredient	**Amount**	**Sigma Cat #**
EFAF BSA	0.6 g	A6003
Na-pyruvate (Stock 2)	1000 µL	-
Gentamicin (Stock 8)	50 µL	-
Heparin (Stock 7)	1000 µL	-
Filter in a sterile bottle		

- **SOF-BE2 (Base Solution)**
  - To make 1 L of SOF-BE2 base solution, dissolve the ingredients listed in Table 6 in 700 mL of sterile water (Sigma water for embryo collection; cat no. W4502). 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 6. Recipe for SOF-BE2 Base

SOF-BE2 Base Solution					
Component	**Mol Wt**	**mM**	**mg/L**	**g/L**	**Sigma Cat #**
$CaCL_2.2H_20$	147	1.17	172.0	0.172	C7902
$MgCl_2.6H_2O$	203.31	0.49	99.6	0.0996	M2393
KH_2PO_4	136.1	1.19	161.95	0.1619	P5655
KCl	74.55	7.16	533.8	0.5338	P5405
NaCl	58.44	107.7	6294.0	6.2940	S5886
$NaHCO_3$	84.01	25.07	2106.15	2.1062	C7902
Na-lactate	112.06	5.3	775.5 µL	775.5 µL	L4263
Osmolarity	260-270				
pH	7.3-7.4				

- **SOF-BE2 (Working Solution)**
  - To prepare SOF-BE2 Working Solution, add the ingredients listed in Table 7 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.

See next page for recipe

Table 7. Recipe for preparation of SOF-BE2

SOF-BE2 Working Solution		
Ingredient	**Amount**	**Sigma Cat #**
EFAF BSA	0.4 g	A6003
Glutamax (Stock 11)	1000 µL	-
Na-pyruvate (Stock 2)	50 µL	-
Myo-inositol (Stock 15)	1000 µL	-
Sodium Citrate (Stock 16)	1000 µL	-
Non-Essential AA	1000 µL	M7145
Essential AA	2000 µL	B6766
Gentamicin (Stock 8)	250 µL	-
Filter in sterile bottle		

PROTOCOL FOR PREPARATION OF BASE SOLUTIONS

HEPES-TL Base Solution in IVF water							
Component	**Sigma Cat #**	**Mol Wt**	**mM**	**g/L**	**g/2L**	**g/4L**	**g/6L**
NaCl	S5886	58.5	114	6.669	13.338	26.676	40.014
KCl	P5405	74.6	3.2	0.2384	0.4774	0.9548	1.4322
$NaHCO_3$	S5761	84.01	2	0.168	0.336	0.672	1.008
NaH_2PO_4. H_2O	S9638	137.99	0.4	0.048	0.096	0.192	0.288
Na DL-Lactate	L7900	112.06	10	1416 uL	2832 uL	5664 uL	8496 uL
$CaCl_2.2H_2O$	C7902	147.01	2	0.2938	0.5876	1.175	1.763
$MgCl_2.6H_2O$	M2393	203.30	0.5	0.1017	0.2034	0.4068	0.6102
HEPES	H4034	238.4	10	2.384	4.768	9.536	14.304
Osmolarity	**255 – 270**						
pH	**7.3 – 7.4**						

IVF-TL Base Solution in IVF water					
Component	**Sigma Cat #**	**Mol Wt**	**mM**	**mg/L**	**g/L**
NaCl	S5886	58.5	114	6669	6.669
KCl	P5405	74.6	3.2	238.4	0.2384
$NaHCO_3$	S5761	84.01	25	2100	2.100
NaH_2PO_4. H_2O	S9638	137.99	0.4	55.2	0.0552
Na DL-Lactate	L7900	112.06	10	1.416	1416 uL
$CaCl_2.2H_2O$	C7902	147.01	2	222.2	0.2222
$MgCl_2.6H_2O$	M2393	203.30	0.5	101.65	0.1017
Osmolarity	**280 – 300**				
pH	**7.3 – 7.4**				

SOF-BE2 Base Solution in IVF water					
Component	**Sigma Cat #**	**Mol Wt**	**mM**	**mg/L**	**g/L**
$CaCl_2.2H_2O$	C7902	147.01	1.17	172.0	0.172
$MgCl_2.6H_2O$	M2393	203.30	0.49	99.6	0.0996
KH_2PO_4	P5655	136.09	1.19	161.95	0.1619
KCl	P5405	74.6	7.16	533.8	0.5338
NaCl	S5886	58.5	107.7	6294.0	6.2940
$NaHCO_3$	S5761	84.01	25.07	2106.15	2.1062
Na DL-Lactate	L7900	112.06	5.3	775.5 uL	775.5 uL
Osmolarity	**260 – 270**				
pH	**7.3 – 7.4**				

NOTES

- All reagents are stored in the IVF reagent cabinet in the main lab
- ALL bases are made with IVF water (W1503 – Sigma) + the components listed above
- All ingredients are added to IVF-water outside the hood (Main Lab), once pH and osmolarity is good, filter (inside hood) with a 0.2 µM Nalgene bottle-top filter (catalog number 09-741-09) into a glass bottle and store at 4°C (maximum 6 months)

this page was last updated by Tatiane Maia August 30, 2022

Day -1

RETRIEVAL OF OVARIES AND OOCYTE COLLECTION AND MATURATION

OVARY COLLECTION

Materials and Equipment Needed

- Slaughterhouse materials:
    - Insulated cooler with car charger (temperature controlled insulated cooler set at 23°C (to ensure ovaries are not exposed to temperature extremes)
    - Cooler for transport of plastic buckets
    - Appropriate attire as required by the slaughterhouse (lab coat, apron, ear protection, boots)
    - Knives (boning knives) or other cutting tools
    - Gloves
    - Wypall or other bench paper
- Preparation for slaughterhouse (day before):
    - Cover both coolers with wypall bench paper or paper towels
    - Filled 8 plastic buckets ¼ full with saline (place in the walk-in fridge) and leave it in a cooler or large refrigerator
    - Filled up 1 L bottle with saline

***Figure 1**. Materials to prepare for slaughterhouse*

    - Place the following in the walk-in fridge: 1 L bottle containing saline and 2-3 bottles of oocyte washing medium (BoviPlus Oocyte Washing Medium with BSA – 19982/1200 Minitube).

***Figure 2**. Saline and Oocyte Washing Medium placed in the walk-in fridge*

Slaughterhouse procedure

- When arriving in the laboratory (AM):
  - Place saline and Oocyte Washing Medium bottle (located in walk-in fridge) and place both in the oven (slashing area). Take both coolers to the slaughterhouse.
- In the slaughterhouse facility:
  - 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 plastic buckets
  - 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.
  - If needed, reproductive tracs will be collected.
- Transport the ovaries to the lab immediately. Avoid exposing ovaries to temperature extremes of heat or cold. Use the temperature-controlled cooler if available.

Figure 3. *Ovaries upon arrival in the laboratory*

OOCYTE COLLECTION

Materials and Equipment Needed

- **Preparation of Microdrops**
  - Incubator (5% CO_2 and 38.5°C)
  - Laminar flow hood
  - Pipettor and pipette tips
  - Maturation medium (OMM – prepared in the lab)
  - Mineral oil (Cat # 9305, Irvine)
  - 60 x 15 mm petri dishes (Cat # 351007, Falcon)
  - Oocyte washing medium (warmed to 38.5°C) – Cat # 19982/1200, Minitube
  - 1 L saline + Pen/Strep (warmed to 38.5°C)

- **Preparation for Oocyte Collection**
  - Scalpel (#3)
  - Scalpel blades (#11)
  - Petri dishes with 50 µL OMM microdrops (pre-equilibrated in incubator)
  - Bench paper to cover surface
  - X-plate (Cat # FB087582, Fisher)
  - Integrid petri-dish
  - 100 µm Falcon Cell Strainer (Cat # 08-771-19)
  - Gloves
  - 400 mL sterile beaker
  - Container to discard ovaries
  - Slide warmer at 38.5°C
  - Dissecting microscope
  - 18 gauge needle
  - 10 mL NormJect syringe (all plastic, no rubber septum – cat # 14-817-30, Fisher)
  - 50 mL beaker

Preparation of maturation dishes

1) At least two hours before they are needed, prepare several (60 x 15 mm) plates containing up to 20 drops in 50 µL microdrops (Figure 4) of OMM. Prepare enough OMM microdrops (10 oocytes/microdrop) to mature the number of oocytes expected to be collected. Cover the microdrops with 7 to 7.5 mL of mineral oil. Place dishes in incubator (5% CO_2 and 38.5°C).

Figure 4. *Preparation of microdrops. Left panel show the materials to be used for preparation of maturation dishes (OMM medium and mineral oil). Right panel shows 15 50 μL drops on the bottom of a 60x15 mm petri dish before (left) and after (right) covering with 7 mL mineral oil. Oil is deposited using a glass serological pipette (or larger pipette if many plates are made)*

Ovary Selection and Slashing

1) Upon return to the lab, classify the ovaries as Good (> 20 small and medium follicles, no very large follicles, not discolored), Marginal (> 10 follicles) or bad (< 10 follicles). Avoid ovaries with cystic follicles.

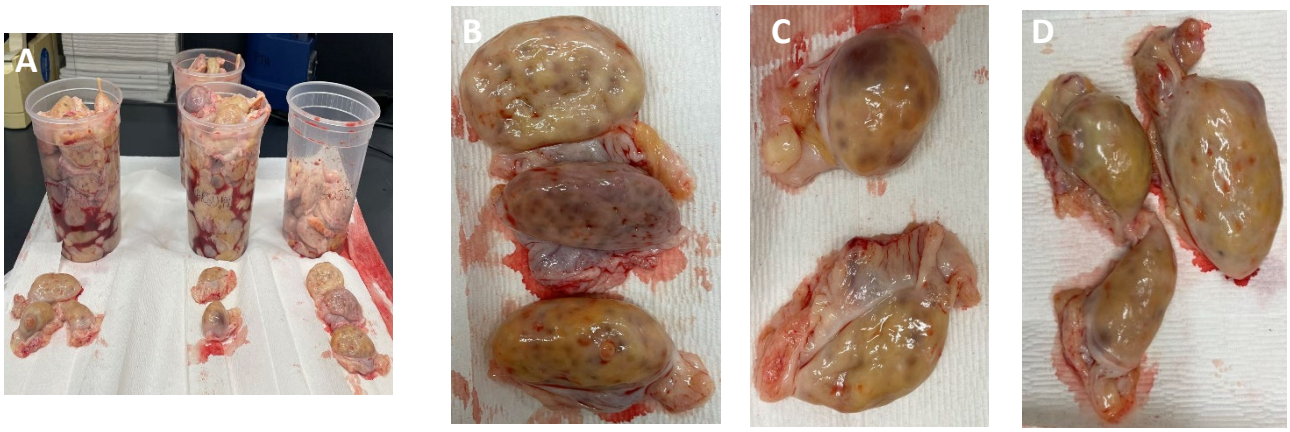


Figure 5. *Left panel (A) show separation of ovaries based on follicle number and ovary morphology. Right panel show 3 classification of ovaries "good" (B), "marginal" (C) and "bad" (D)*

Note: Marginal ovaries should only be used if necessary to obtain the number of desired oocytes. Bad ovaries should not be used. If a CL is present, either remove manually or avoid hitting with the scalpel.

2) Wash ovaries (by massaging; Figure 6) 2-3 times with the pre-warmed saline until most of the blood has been washed away from the ovaries. Following the washes, place ovaries in the container containing fresh saline and store at warmer until time of oocyte collection (Figure 7A). *Use of a warming plate is not required. If used, ensure warming plate does not overheat ovaries on the bottom of the bucket.*

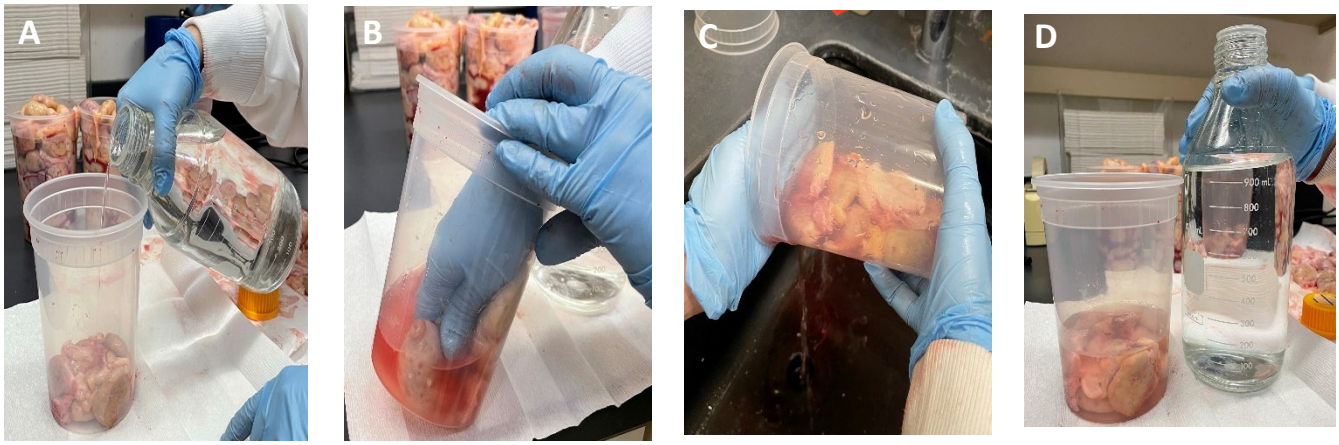


***Figure 6**. Washing and massaging the ovaries to remove blood. A: addition of saline to selected ovaries. B: massaging the ovaries. C: removal of saline. D: ovaries ready to be used after been washed for 2-3x with saline*

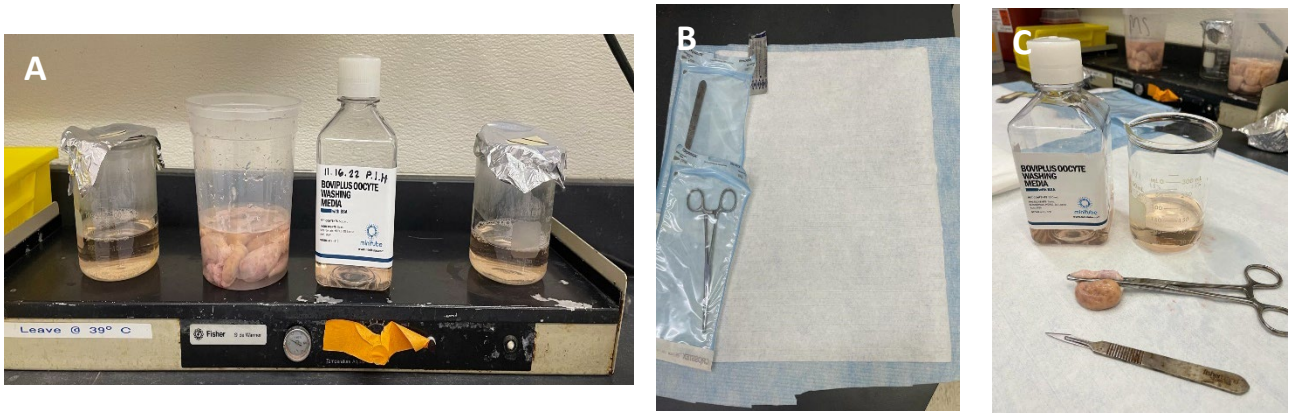


***Figure 7**. A: Plastic bucket containing ovaries with saline and oocyte washing medium with beaker placed in warmer until ready to slash. B: Instruments and materials used to slash ovaries: hemostat, scalpel handle, disposable scalpel blades and bench paper. C. Beaker with 100 mL oocyte washing medium and instruments prior to slash.*

3) Add 100 to 150 mL oocyte washing medium to a sterile 400 mL beaker (Figure 7A).
4) Attach a hemostat to the base of the ovary to hold the ovary firmly in place (Figure 7C). 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) and then on the entire surface of the ovary on both surfaces and sides of ovary. See Figure 8 for illustration.

Note: Typical yield is ~20 usable oocytes/ovary (sometimes as many as 30-35 can be obtained)

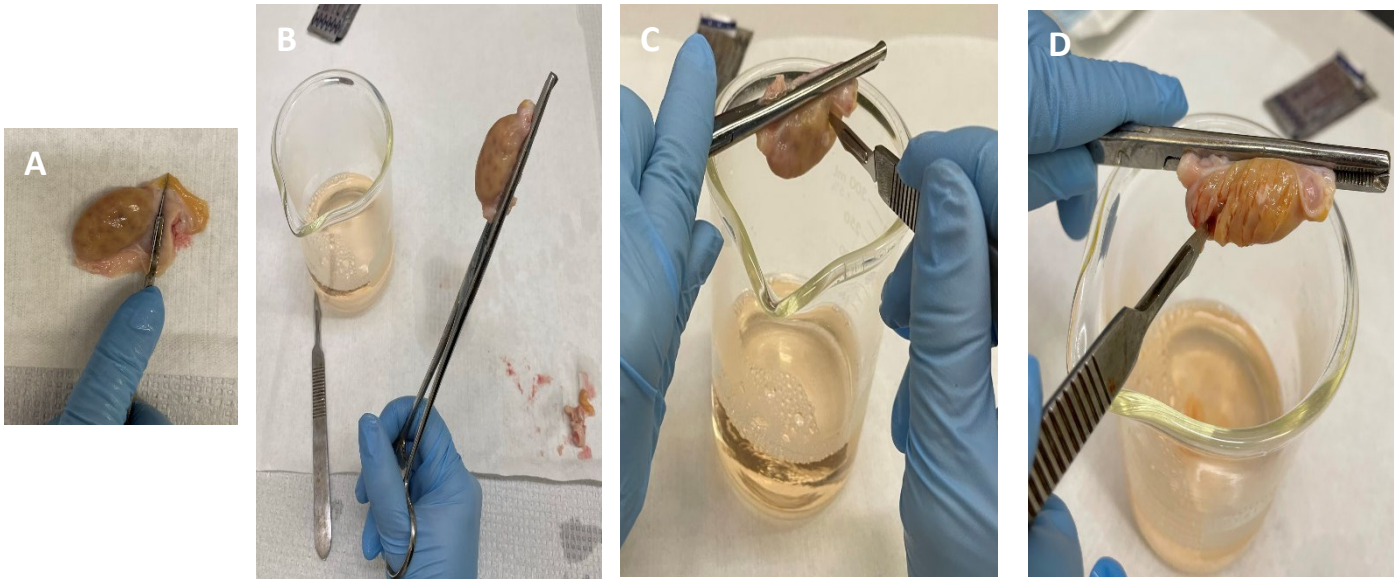


Figure 8. *Steps in slashing of ovary to obtain oocytes. A: Removal of excess tissue in ovary using a scalpel blade. B: A hemostat is attached to the base of the ovary to hold the ovary firmly in place. Panels C and D show how the ovary is held above the beaker and 2-3 mm incisions made in a downward direction with a rapid but firm movement across follicles. C: Incisions across all visible follicles. D: Incision on the entire surface of ovary.*

5) Once done with incisions (slashing), submerge ovary into oocyte collection medium and swirl vigorously and squeeze the ovary against the wall of the beaker (Figure 9). Repeat this process 2-3 times for each ovary until the desired number of ovaries has been processed. Be careful to not do it very aggressively and break the beaker.

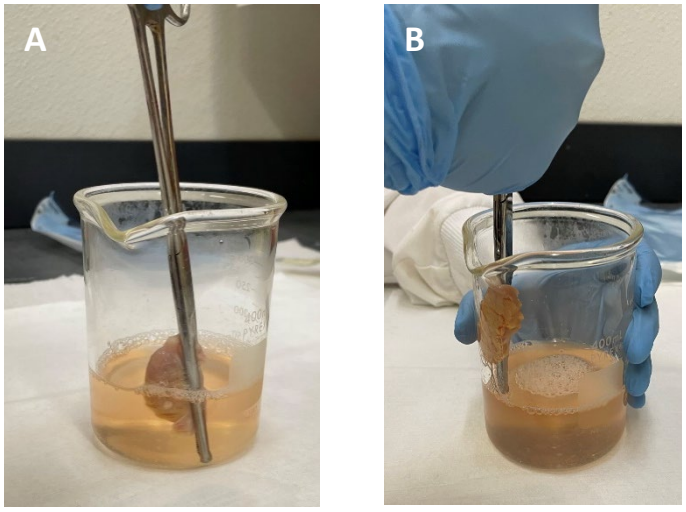


Figure 9. *Harvesting of oocytes from slashed ovaries. In the left panel (A), the slashed ovary is being swirled in oocyte collection medium. In the right panel (B), the ovary is being pressed against the side of the beaker to allow drainage.*

Note: 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 (CL). When CL is present, remove it if possible or avoid slashing near the area. 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 (> 10) are being slashed it may be beneficial to split them into two groups to slash. This will lessen the number of debris present when searching for oocytes.

6) Place beaker with follicular fluid from slashed ovaries in a warmer until ready to filter (Figure 10).

***Figure 10**. Beaker placed in warmer (with beads) containing follicular fluid from slashed ovaries*

Filtering and Searching

1) Prepare materials for filtering and searching (Figure 11). You will need an empty 400 mL beaker, 2 mL beaker, forceps, cell strainer, binder clip (used to hold the forceps), a stand, integrid petri-dish, 10 mL syringe, 18 gauge needle, warmed oocyte collection medium (Figure 10) and an X-plate dish.

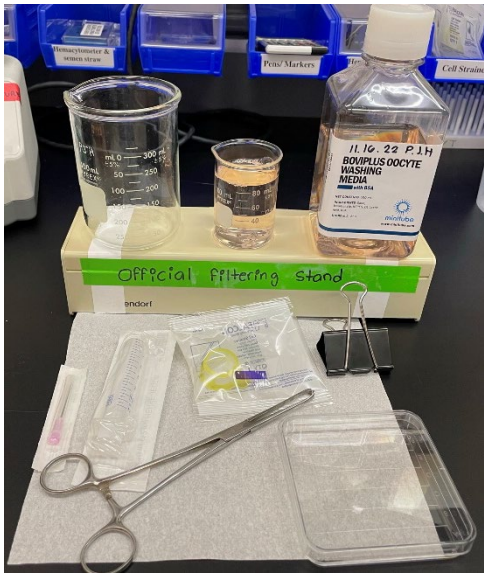


***Figure 11**. Materials to be used for filtering and search of oocytes*

2) 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 warmed oocyte collection medium into 3 wells of an X-plate (Figure 11).

3) Use a forceps to hold a 100 µm cell strainer in position over a 400 mL beaker (Figure 11).

Figure 11. *Illustration of setup for filtering medium containing oocytes. Shown are a 10 mL syringe fitted with an 18 g needle and smaller beaker containing oocyte washing medium after collection of oocytes (ready for filtering). The binder clip is attached to the hemostat to hold the filter at the same level as the empty beaker.*

4) 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 (Figure 12A).

5) Once all the liquid is filtered, rinse the beaker 1-2 times with a syringe filled with oocyte washing medium (Figure 12B) and filter it. This step is done to make sure there is no oocyte left that got stick to the beaker.

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.

If the cell strainer starts to clog during filtering rinse it with oocyte washing medium using a sterile plastic Pasteur pipette. The forceps 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 3 beakers.

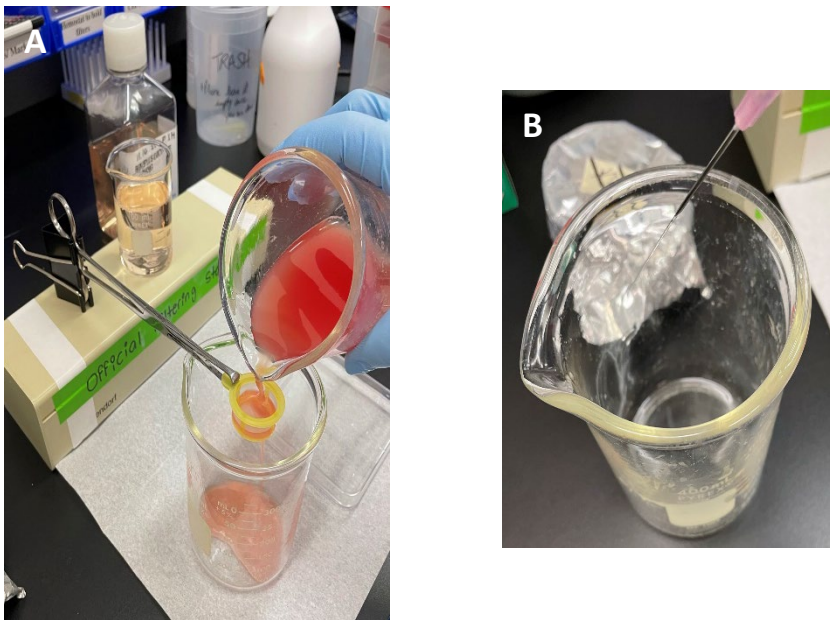


***Figure 12**. Filtering of oocytes. A. Filter of ovary fluid using a cell strainer. B. Rinsing the beaker with oocyte washing medium after filter the ovary fluid.*

6) When all the ovary fluid has passed through the filter, immediately turn the filter upside down (over the grid plate – figure 13A) and rinse the oocytes into an integrid petri-dish (figure 13B). Place the integrid dish on a plate warmer until ready for searching (Figure 14A).

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 "Alternative protocol for processing oocytes".

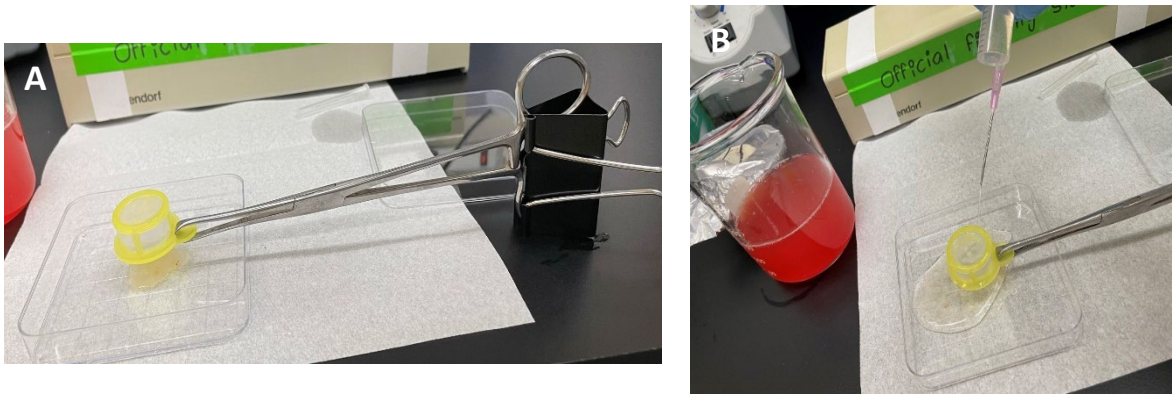


***Figure 13**. Removal of oocytes from cell strainer. Forceps holding the cell strainer upside down. B. Rinsing the filter on top of the integrid petri-dish with a syringe filled with oocyte washing medium.*

7) Collect cumulus oocyte complexes (COCs) as fast as possible to prevent adverse effects of cold shock. Place retrieved COCs into the first well of the X-plate containing oocyte washing medium (Figure 14C). 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.

8) After completing COC search starting the washing step. Transfer oocytes from the first well to the second well, then to third and then fourth (last well), leaving all debris behind. Moving oocytes from one well to another assure that the oocytes are clean of debris (Figure 15). This can be completed using a wiretrol pipet, microdispensor pipet, or the instrument of your choice for handling oocytes.

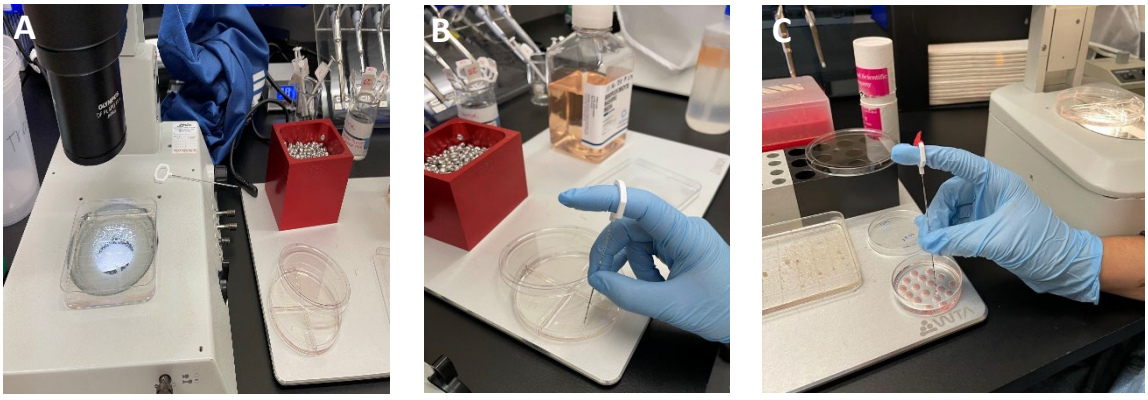


***Figure 14**. Searching for COCs. A. Both integrid plate (searching dish) and X-plate place in the warmer. B. Adding COCs from the integrid plate to the first well of X-plate. C. Placing groups of 10 COCs into maturation drops.*

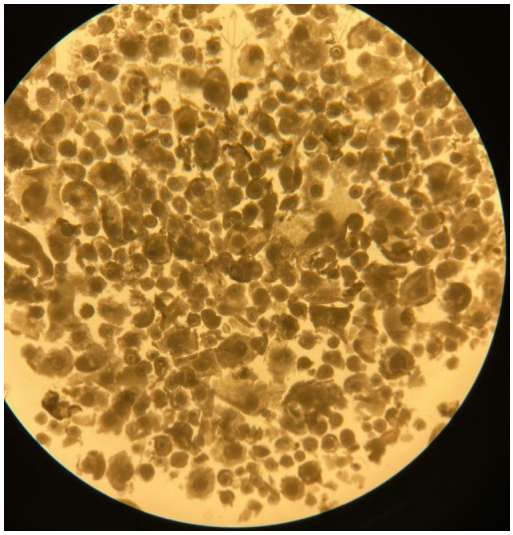


***Figure 15**. COCs after being washed and prior to move to maturation dish.*

10) After oocytes have been cleaned of debris, transfer groups of 10 to a 50 µL 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.

11) Incubate for 20 to 24 h at 38.5°C and 5% CO_2 with humidity.

DAY 0
IN VITRO FERTILIZATION

INITIAL PREPARATION FOR SPERM PURIFICATION AND FERTILIZATION

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 (aliquots of 400 µL placed in -20 IVF freezer)
- Pipet tips and pipettors
- Sterile serological pipets
- Plastic Pasteur pipet
- CO_2 Incubator (at atmospheric oxygen with 5% CO_2, 38.5°C with humidity)
- Warming oven set at 38.5°C (can be substituted with a water bath or incubator)

Preparation of in vitro fertilization dishes and tubes

Note: 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.

1) For each fertilization plate, prepare 15 mL HEPES-TALP in 15 mL conical tubes. Tighten the caps, rap it with parafilm and place in the incubator. Label it "Zygote Wash". Additionally, for every 4 people, prepare 15 mL HEPES-TALP in 15 mL conical tubes and place in the incubator with cap close. Label it "Oocyte Wash" (Figure 1).

Note: 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 "Sperm Wash" (Figure 1). Tighten cap and place in the incubator.

3) Add 4 mL IVF-TALP to a 15 mL conical tube. Label it "Sperm Diluent" (Figure 1). Leave cap open and place in the incubator.

4) Prepare one 35 mm x 10 mm Petri dishes for each fertilization plate. Add 1700 µL (2x850 µL) of IVF-TALP to each plate. Label it "IVF and date" (Figure 1). Place dishes in incubator to allow medium to equilibrate and warm up for at least 2 h.

Note: Add maximum of 300-350 COCs in each fertilization dish. If greater, make an additional dish.

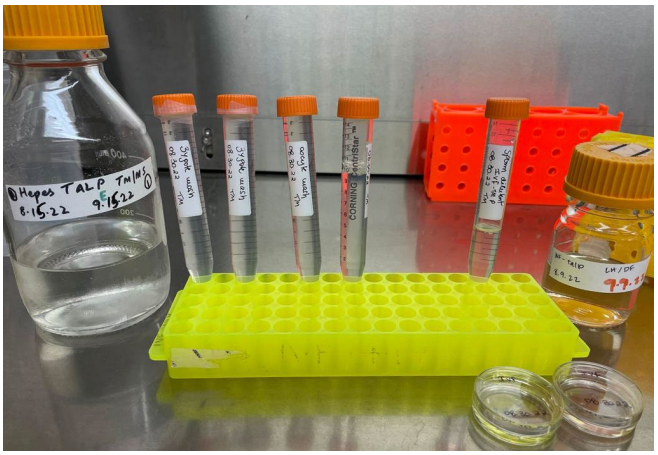


Figure 1. *IVF media preparation sufficient for two people. There are tubes of HEPES-TALP including 2 zygote wash tubes (15 mL/tube), 1 oocyte wash tube (15 mL) and 1 sperm wash tube (10 mL). There is one sperm diluent tube of IVF-TALP (4 mL) and dishes (1700 µL/dish).*

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

6) Fill up with distilled or tap water the CitoThaw (i.e., thawing unit) and plug-in so the water warms up. Temperature should reach ~ 35.5 ºC.

7) Place aliquots of PHE (80 *µL* per dish) in the incubator (remember to cover the tube with aluminum foil due to light sensitivity of PHE).

8) Place 2-3 centrifuge carriers (in our lab, carries are already in the incubator) to warm.

PREPARATION OF OOCYTES FOR FERTILIZATION

Materials and Equipment Needed

- 35 mm petri dishes with 1.7 mL IVF-TALP (pre equilibrated in CO_2)
- X plates (source Fisher Scientific)
- HEPES-TALP (pre-warmed)
- IVF-TALP (pre equilibrated in CO2 incubator)
- PureSperm (pre-warmed) – catalog #: PS-40; PS-100 Nidacon
- Dissecting microscope
- Inverted microscope
- Rack for tubes
- Scissors (wipe with ethanol)
- Semen straw plunger (wipe with ethanol)
- Plastic sterile Pasteur pipets
- Pipet tips and pipettors
- Instrument to pick-up oocytes (1 mL syringe: Z683531 - Sigma and adaptor: DI-0040/5 – Partnar Animal Health)
- Slide warmer (set at 38.5˚C)
- CO_2 Incubator (at atmospheric oxygen with 5% CO_2, 38.5˚C with humidity)
- Warming oven or incubator set at 38.5˚C (can be substituted with a water bath)
- Hemocytometer 0.1 mm deep (catalog #: 0267151B - Fisher)
- Eppendorf Tubes

Procedure

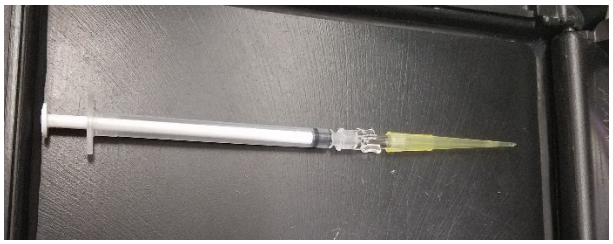
1) Place X plate on the slide warmer and add ~3 mL of HEPES-TALP to one well per fertilization plate.

2) Remove dishes containing matured oocytes and place on the slide warmer.

Note: If one person has 2 IVF dishes you can combine all the oocyte in one well of the X-plate and wash it all together.

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 ~300-350 maximum. 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 μL 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~300-350 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. If the room where the sperm preparation is carried out is cold (i.e, well air conditioned), it can be advantageous to use a small space heater in front of the area where sperm preparation will be performed to prevent 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 (Toplayer) on top of the 80% PureSperm using a plastic Pasteur pipet. Cap and place in the tube rack in front of the heater (Figure 2).

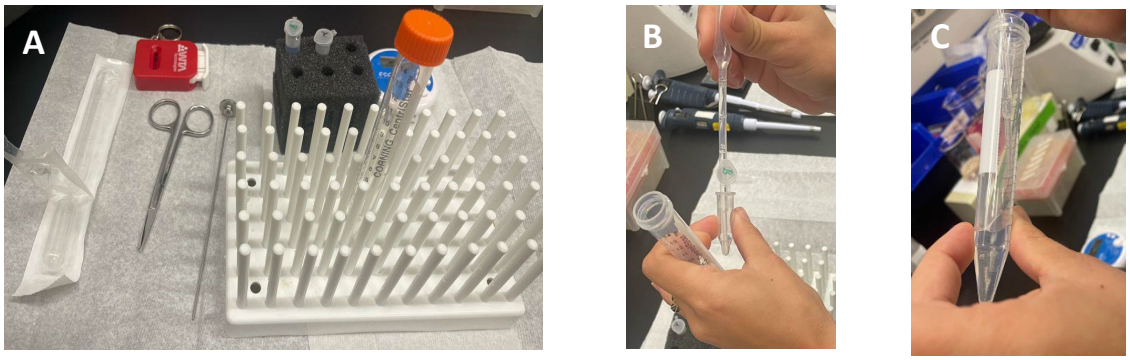


Figure 2. *Preparing pure sperm gradient. Setup for first centrifugation (panel A). Adding 1.5 mL of PureSperm (bottom layer; panel B). Adding 1.5 mL of PureSperm (top layer; panel C).*

2) Thaw 3 straws of semen in the citothaw for 30-45 seconds (Figure 3).

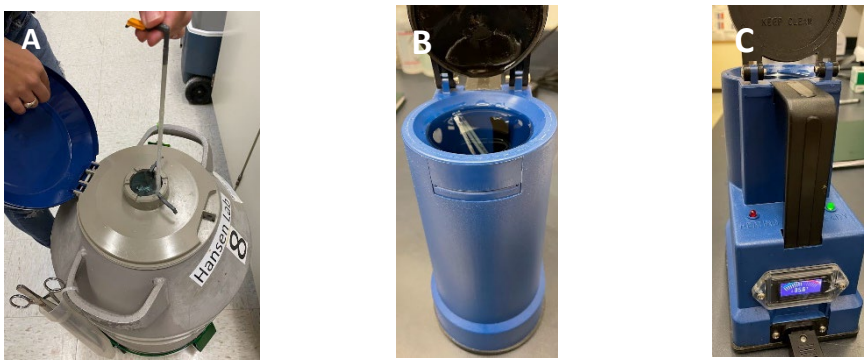


Figure 3. *Collection of straws of semen from liquid nitrogen tank to the thawing unit (citothaw). Removing semen straws from nitrogen tank (panel A). Thawing a pull (3) of semen straws (panel B). Citothaw unit (panel C).*

Note: 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. 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 or semen straw cutter and expel contents of the straw onto the top of the PureSperm gradient (Figure 4). Care must be taken so that the gradient is not disturbed and the semen lies on top of the 40% layer.

Note: 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.

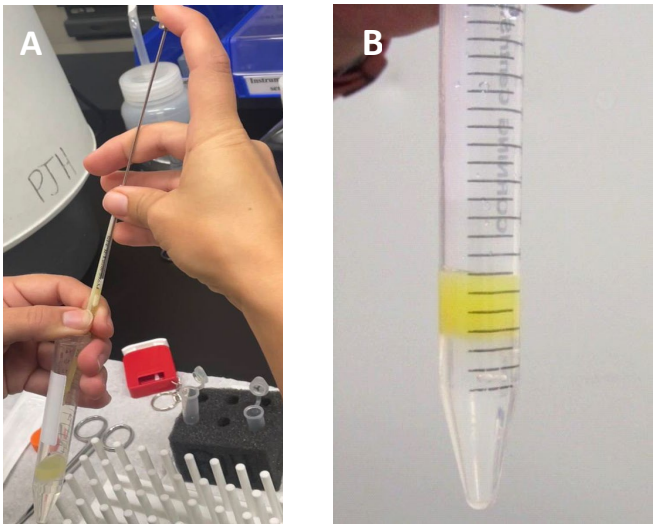


Figure 4. *Layering of sperm onto sperm purification gradient. After cutting the tip of the straw, the contents of the straw are expelled onto the top of the gradient (panel A). Removal of the semen is facilitated by using a homemade plunger. Panel B shows the layer of semen on top of the gradient before centrifugation.*

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 5). The pellet should be collected with as little PureSperm as possible.

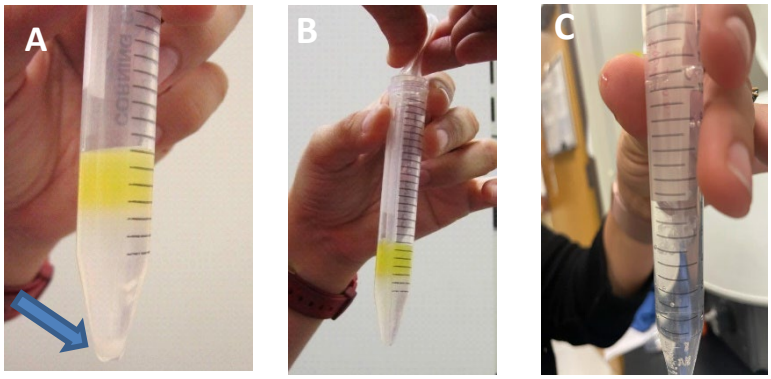


Figure 5. *Pellet after first centrifugation followed by addition to HEPES-TALP. Panel A: sperm pellet after centrifugation on step 4. Panel B: removal of sperm from the bottom of the gradient. Panel C: adding sperm pellet to sperm wash tube containing 10 mL of HEPES-TALP.*

6) Place the sperm pellet into a 15 mL conical tube with 10 mL HEPES-TALP (labeled "Sperm Wash"; Fig.5C) and place in a warm centrifuge carrier before centrifuging for 5 min at 200 x g.

Note: When adding the sperm pellet to the Sperm 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 (as much as possible) with a Pasteur pipette, being careful not to disturb the pellet (Figure 6). Then, set the 1000 µL pipet to 400 µL and start to twist the plunger to

measure the volume of the pellet (Figure 6D). Example: if your pipet starts with 400 µL and after twisting the plunger you have a total 520 µL, then the volume of your pellet is 120 µL.

Note: 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.

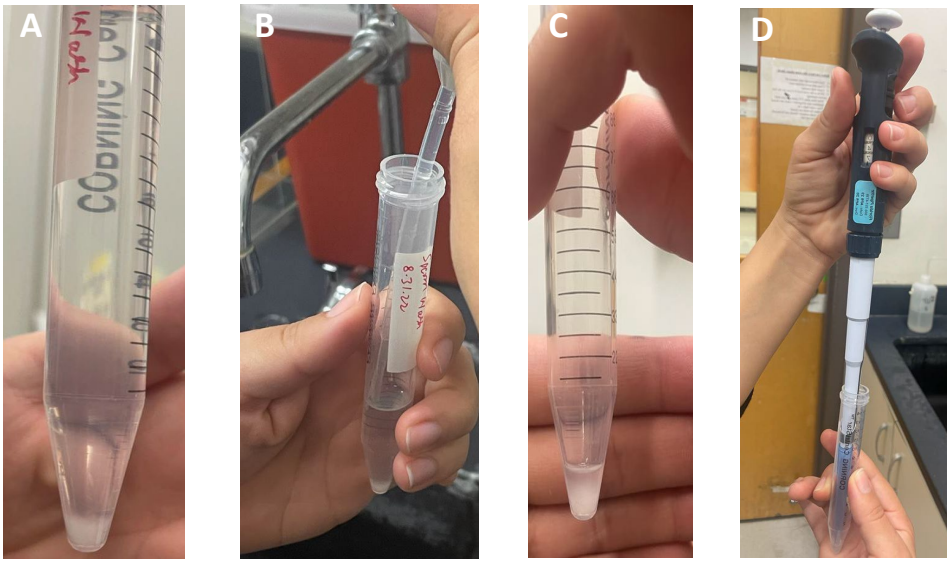


***Figure 6**. Panel A: sperm pellet after centrifugation. Panel B: supernatant removal. Panel C: final sperm pellet. Panel D: setting up pipet to start to measure the volume of the pellet.*

8) Determine dilution required to bring sperm to a concentration of 17 x 10^6/mL. This will produce a final concentration of sperm in the fertilization plate of approximately 1.1 x 10^6/mL.

9) Bring the semen pellet to a volume of 600 µL using pre-equilibrated IVF-TALP medium (figure 7). So, if the volume of the pellet was 120 µL, then add 480 µL (600 µL – 120 µL) of IVF-TALP to the tube with your pellet (already previous label as "Sperm Wash").

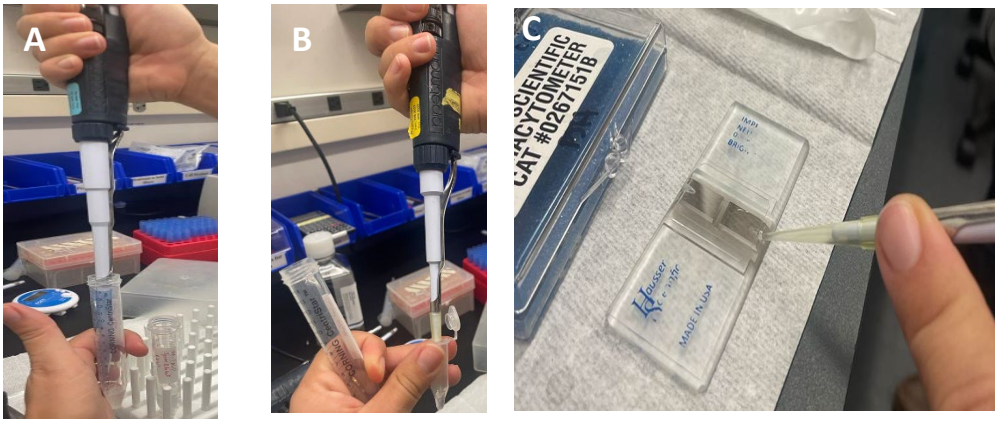


***Figure 7**. Preparation of sperm pellet for counting sperm. Panel A: addition of IVF-TALP to sperm pellet to bring up to a final volume of 600 uL. Panel B: mixture of 10 uL of the sperm pellet with 90 µL water. Panel C: Load 10 µL of mix (sperm + water) to hemocytometer.*

10) Create a 1:10 dilution of this semen by adding 10 µL of the sperm suspension (IVF-TALP + pellet = 600 µL) to 90 µL of tap water (figure 7B). Load 10 µL in the two chambers of a

hemocytometer (figure 7C). Count the number of sperm in 5 squares of each chamber and use the average from both chambers as the final sperm count (Figure 8).

Note: Make sure you mix (pipet up and down) very well the sperm diluted in water to avoid sperm clump during sperm count.

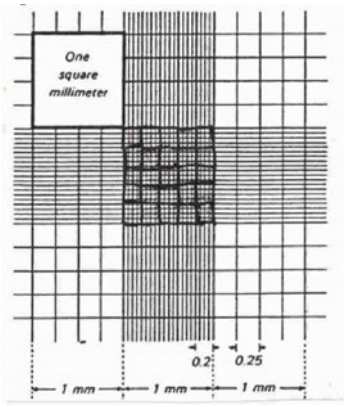


***Figure 8**. Hemacytometer used for counting sperm. The total number of sperm in five of the smaller boxes (outlined by freehand) are counted and multiplied by 500,000 to determine concentration/mL.*

Note: 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 10^6/mL (possible with practice).

To obtain the volume of IVF-TALP needed to bring the sperm to a concentration of 17x10^6/mL use the following formula:

(Vol * sperm number * 50,000 * 10 / 17,000,000) - Vol

= (17.647 x sperm number) - 600

Where:
- Vol is the initial dilution of the sperm (600 µL)
- sperm 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

Example: If the average sperm count was 80, then (17.647 x 80) - 600 = 1411.76 – 600 = 811.76 µL. So you will need to add 811.76 µL of IVF-TALP to your sperm pellet (already bring up to a volume of 600 µL). The final concentration of sperm will be 17x10^6/mL.

FERTILIZATION

Materials and Equipment Needed

- Slide warmer (set at 38.5°C)
- Pipettors and pipette tips
- CO2 Incubator (at atmospheric oxygen with 5% CO_2, 38.5°C with humidity)
- Incubator or warming oven set at 38.5°C (can be substituted with a water bath)
- Purified sperm
- PHE (aliquots of 400 µL are already prepared and is stored in -20°C freezer - IVF lab)

Procedure

1) Remove fertilization dishes containing washed and matured oocytes from the incubator and place on the slide warmer (Figure 9).

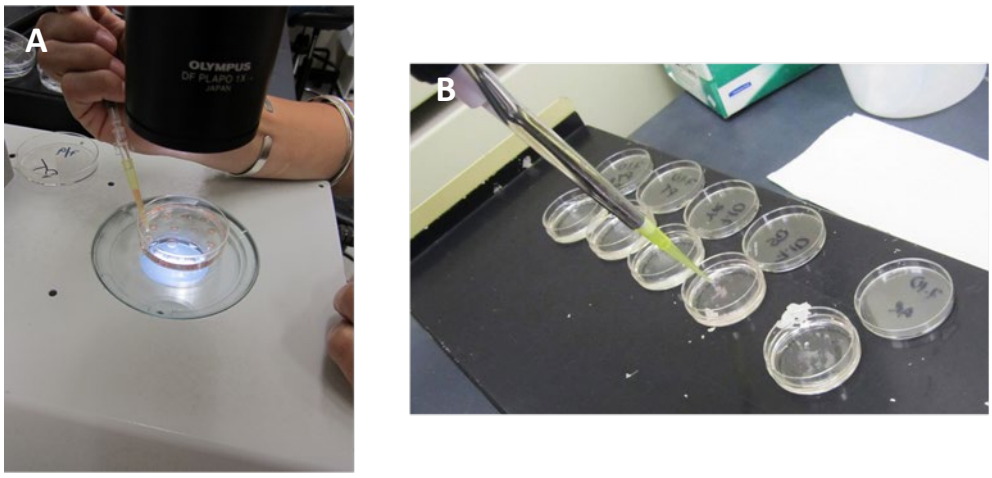


Figure 9. *Fertilization. Shown in the left panel (A) is removal of matured COCs from maturation drops (done during preparation of oocytes). Shown in the right panel (B) is the addition of 120 µL sperm to fertilization dishes.*

2) Add 120 µL sperm preparation and 80 µL PHE mix into each dish to produce a final sperm concentration of 1.1 x 10^6 sperm/mL (Figure 9B).

Note: 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 10-18 hours (h).

Note: Many people do fertilization for 15-16 h or 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-BE2
- 60 x 15 mm and/or 30 x 15 mm petri dishes
- Mineral oil
- Pipettors and pipette tips
- Bench top or Trigas incubator (38.5°C, 5% CO_2 5% O_2 balance N, humidified)

Note: the standard embryo culture medium we use is SOF-BE2. We make it but a very similar medium can be purchased from Caisson. Other commercial media also work as well or better than SOF-BE2 in terms of blastocyst yield. One drawback of many commercial media is that the formulation is a trade secret which, for experimental purposes, can sometimes limits uses of the media.

Procedure

Note: Prepare embryo culture medium (SOF-BE2 or other culture medium) at least 2 h before removing zygotes from the fertilization plate. We typically prepare culture medium during or after IVF (i.e., during first sperm centrifugation during the 10 min break or once IVF is over)

1) Make 50 µL 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 7 mL mineral oil. For 35 x 10 mm dishes, use 3.5 mL mineral oil to cover drops. To culture small groups of embryos (<15 per drop), we typically prepare 25 µL microdrops of culture medium.

2) Place the dishes in the incubator (38.5°C, 5% CO_2 5% O_2 balance N_2, humidified) to warm up and equilibrate.

DAY 1
TRANSFER OF FERTILIZED OOCYTES INTO EMBRYO CULTURE DROPS

Materials and Equipment Needed

- Vortexer
- Timer
- Stock 12: Hyaluronidase in a 1.5 mL microcentrifuge tube (aliquots of 100 µL are already prepared and is stored in -20 freezer - IVF lab)
- X-plate (with pre warmed HEPES-TALP)
- Syringe (1 mL) with pipet tip (1 mL syringe: Z683531 - Sigma and adaptor: DI-0040/5 – Partnar Animal Health)
- Slide warmer (set at 38.5˚C)
- Dissecting microscope
- Instrument to pick-up embryos (catalog #: 5-000-1005 – Drummond)
- Bench top or Trigas incubator (38.5°C, 5% CO_2 5% O_2 balance N, humidified)

Procedure

1) Warm hyaluronidase stock 12 in the water bath (see Figure 1 for photo of beads block – catalog number: 52-100 Lab Armor containing metal beads-catalog number: 12L038 Lab Armor).

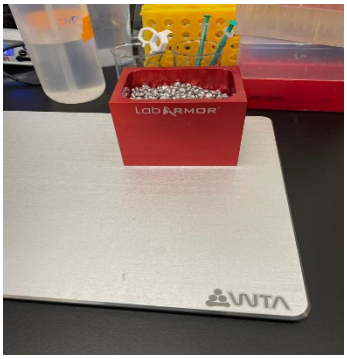


Figure 1*. Small bead blocks with metal beads. Use of beads eliminates the algal contamination often seen with water baths.*

2) Add 300 µL of HEPES –TALP to the tube of Stock 12 (Hyaluronidase already thawed) and pipet up and down to mix it.

3) Place X-plate on the slide warmer and add ~3 mL of HEPES-TALP to 3 of the wells.

4) Remove one fertilization dish from the incubator.

5) Remove COCs (now called putative zygotes since many of them have been fertilized) from the fertilization dishes using a syringe with a pipet tip adaptor and place in the microcentrifuge tube. Up to 350-400 embryos can be loaded in one microcentrifuge tube.

6) Allow zygotes to settle to the bottom of the tube (place in incubator for 1-2 minutes or warmer) and remove all but 50-100 μL or enough to cover the pellet (this facilitates cumulus removal and prevents zygotes from going into the lid of the tube during vortex). Discard the remaining fluid and pipette onto the lid of the X-plate to allow recovery of any zygotes accidentally discarded (Figure 2).

Note: To minimize loss of zygotes during this process, expel the removed supernatant onto the lid of the X-plate dish. Check if there are any zygotes with the supernatant and if there are a few, just take them and put it back in the microcentrifuge tube.

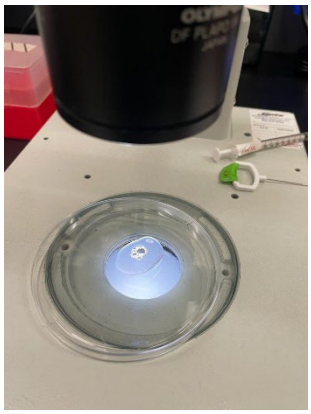


Figure 2. Discarded fluid from the tube of putative zygotes. Placing the fluid on the X-plate allows an extra search to recover any putative zygotes that were accidentally removed.

7) Remove cumulus cells from zygotes by vortexing (Figure 3) the tube for 5 minutes in maximum speed.

Note: A technique used by some is to press the tube hard so that the fluid is propelled to the top of the tube. Then the tube is rapidly removed from the vortexer and the process repeated (i.e., kind of bounce the tube on the vortexer).

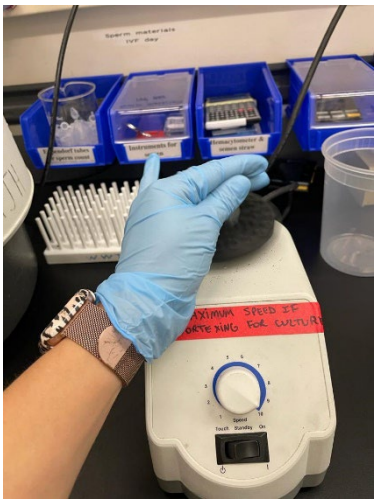


***Figure 3**. Vortexing COCs to remove cumulus cells.*

8) 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 and transfer the zygotes to X plate (Figure 4). You can look at the tube under the microscope to make sure there is no zygotes left. One can exclude transfer putative zygotes that are obviously degenerate (clear or mottled cytoplasm or that still retain cumulus cells.

9) Wash zygotes 3 times by transferring them from one well to the next to clean them of cells and debris (Figure 4).

Notes on steps 8-9: 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.

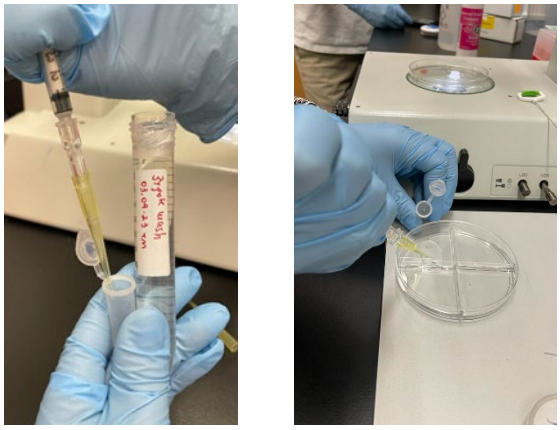


***Figure 4**. Placement of zygotes into the X-plate. The left panel shows rinsing of the tube with HEPES-TALP. The right panel shows the rinsed liquid with putative zygotes placed in the first well of X-plate for washing.*

10) Finally, transfer the putative zygotes to microdrops of pre-equilibrated SOF-BE2. Return plate to incubator as quickly as possible.

Note: One can place up to 30 embryos in a 50 μL drop. For smaller numbers of embryos (10-15), embryos are placed in a 25 μL drop. Culture of less than 10 embryos can be done in a 10 μL drop.

DAY 1-9
EMBRYO CULTURE

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 AFTER IVF - ASSESSING BLASTOCYST DEVELOPMENT

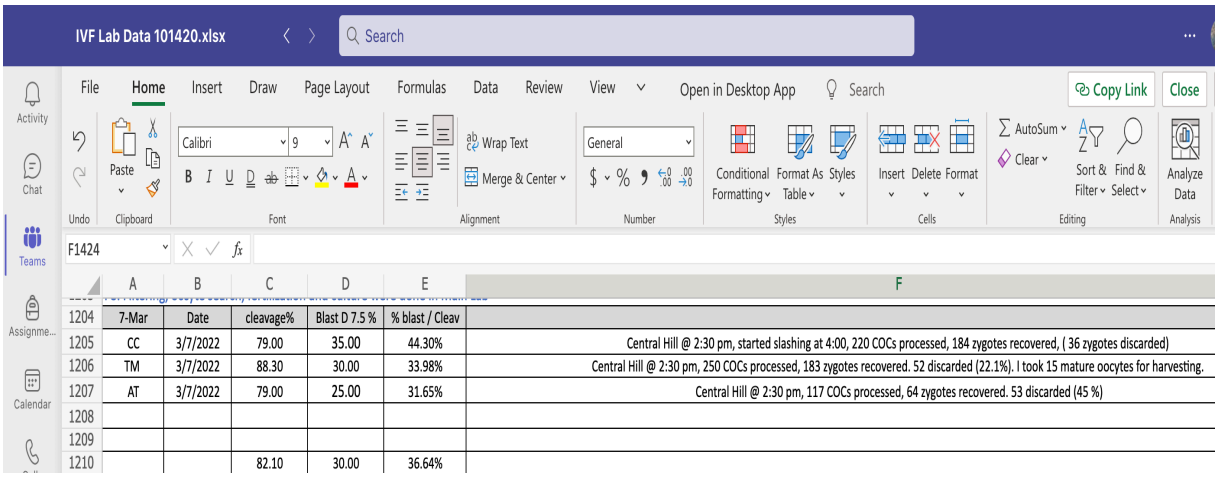
Assess development of embryos to the blastocyst stage by determining the number of embryos that can be classified as an early blastocyst or above, divided by the number of embryos placed initially in the microdrops. Return plates to the incubator as quickly as possible.

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

AT THE END OF IVF - ENTER DATA IN TEAMS PAGE

Adding data to TEAMS page:

Data should be added every week.



	A	B	C	D	E	F
1204	7-Mar	Date	cleavage%	Blast D 7.5 %	% blast / Cleav	
1205	CC	3/7/2022	79.00	35.00	44.30%	Central Hill @ 2:30 pm, started slashing at 4:00, 220 COCs processed, 184 zygotes recovered, (36 zygotes discarded)
1206	TM	3/7/2022	88.30	30.00	33.98%	Central Hill @ 2:30 pm, 250 COCs processed, 183 zygotes recovered. 52 discarded (22.1%). I took 15 mature oocytes for harvesting.
1207	AT	3/7/2022	79.00	25.00	31.65%	Central Hill @ 2:30 pm, 117 COCs processed, 64 zygotes recovered. 53 discarded (45 %)
1208						
1209						
1210			82.10	30.00	36.64%	

Figure 1. Example of record data on TEAMS page – IVF lab data.

- Column: A
  - Add your initials
- Column: B
  - Add the date you were slashing
- Column: C
  Cleavage Rate is usually checked on D3.5 (D0: IVF Day): total number of cleaved embryos/total number of “embryos” in each drop

- When checking cleavage rate:
  - Write the number of each drop on you IVF dish (i.e, 1, 2, 3 …)
  - Count the total number of "embryos" in each drop and the total that cleaved (minimum 2 cell stage). Example: if you found 27 that cleaved out of 30 embryos, then your ratio for that drop is 27/30 = 90%. If you have more than one drop, then use total cleaved/total in each drop as your result (add as a percent).

- Column: D
  Blastocyst Rate is checked on D7.5 (D0: IVF Day): total number of blastocyst/total number of "embryos" in each drop

  - When checking blastocyst rate:
    - Count the total number of "embryos" in each drop and the total that became a blastocyst. Your blastocyst rate will include early blastocyst, blastocyst, expanded blastocyst, hatching blastocyst and hatched blastocyst. Use total blastocyst/total in each drop as your result (add as a percent).

Notes for column C and D: Use the contrast on the microscope and the wiretrol (to roll the potential zygotes) if in doubt.

The word "embryos" was used as a reference to the total number of putative zygotes that was added during culture to each drop.

- Column: E
  - Ratio of cleaved/blastocyst (add as percent)

- Column: F
  - Add location and time that you got the ovaries (i.e., Central Hill @2:30 pm)
  - Add how many oocytes you added to your maturation dish on Day-1 (i.e., 300 COCs processed)
  - Add how many potential zygotes you have recovered and discarded during IVC. For example, if you have processed 300 COCs, but you only cultured (Day 1) 250 COCs, then you recovered 250 and discarded 50 zygotes = 50/300 = 16.6% discarded

ALTERNATIVE PROTOCOL FOR PROCESSING OOCYTES

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.

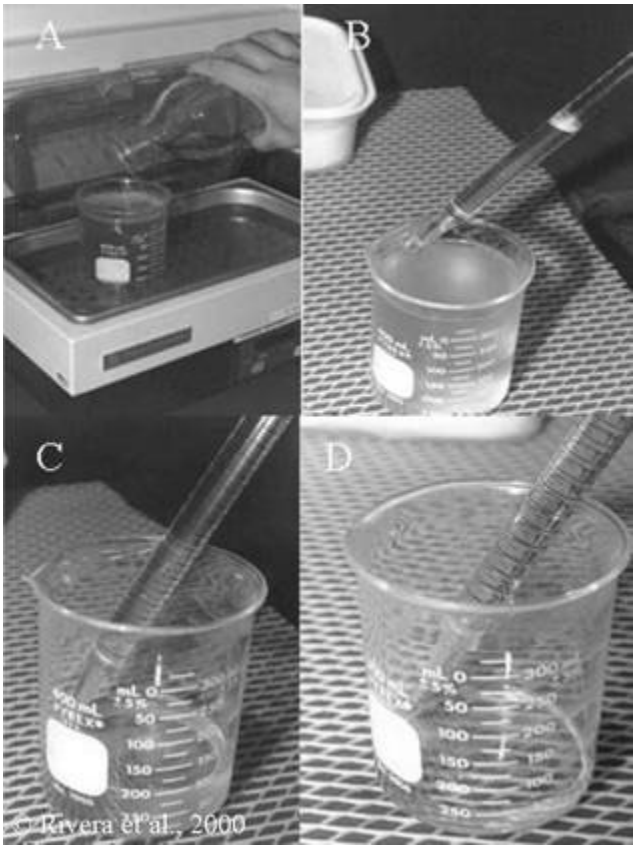


Figure 1. *Cleaning up the preparation of oocytes collected by ovary slashing. After slashing ovaries, medium is poured into a sterile 400-ml beaker (panel A). After allowing oocytes to settle, medium is aspirated using a disposable pipette until all but 100-ml has been removed (panels B-C). The procedure 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).*

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

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

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 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 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.

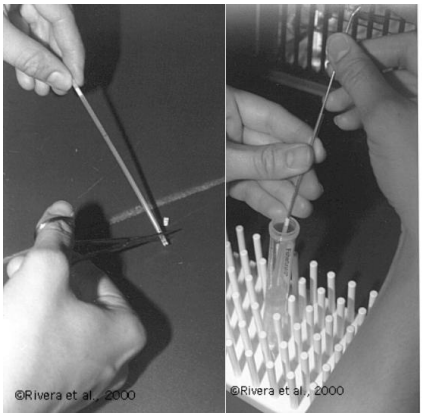


Figure 2. *Layering of sperm onto Percoll. After cutting the tip of the straw (Left panel), the contents of the straw are expelled onto the top of the Percoll gradient (right panel). Here, removal of the semen is facilitated by using a homemade plunger.*

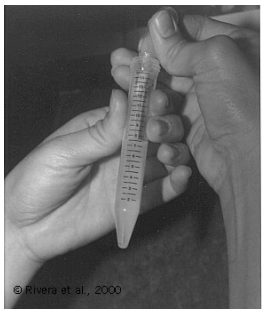


Figure 3. *Removal of sperm from the bottom of the Percoll gradient.*

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.

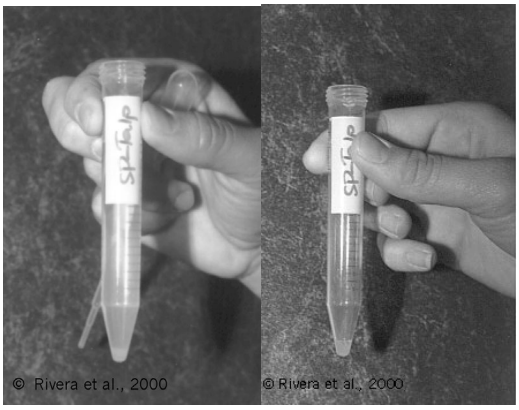


Figure 4. *Washing sperm in H-SOF. The left panel shows the washed and centrifuged sperm. The right panel shows the pellet of sperm remaining in the tube after aspiration of the supernatant.*

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 ¤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 10^6/ml (possible with practice).

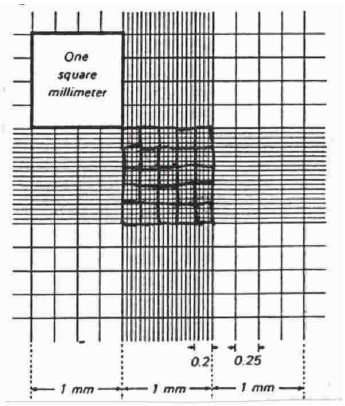


Figure 5. *Hemacytometer used for counting sperm. The total number of sperm in five of the smaller boxes (outlined by freehand) are counted and multiplied by 500,000 to determine concentration per ml.*

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 ¤l 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 ¤l from each tube and combine samples. Centrifuge (1000 rpm) the combined sample for 5 minutes. Discard all but the bottom 500 ¤l 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

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.

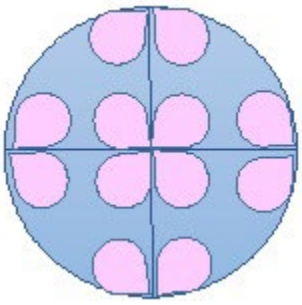


Figure 1. Depiction of an X plate with media in each corner.

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.

This page was last updated July 7, 2017 by E. Jannaman

IN VITRO FERTILIZATION WITH SEX-SORTED SEMEN

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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 of 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 37^0C 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 37^0C 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 Ocoytes

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.

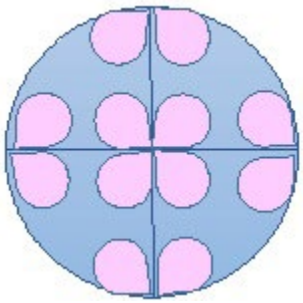

Figure 1. Depiction of an X plate with media in each corner.

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. Brint 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.

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 $NaHCO_3$ in 10 L ddH_2O. Add 100 ml 100X Pen-Strep anb 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 (old version)

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:

Table 1. Recipes for preparation of OMM

Ingredient for each bottle	Amount		To make 5 bottles, take the following amount
BSS (stock 3)	5	ml	5 aliquots of 5 mL each
Gentamicin (stock 8)	500	µl	3 aliquots of 1 mL each
Na Pyruvate (stock 2)	500	µl	1 aliquot of 3 mL
Glutamax (stock 25)	500	µl	1 aliquot of 2.5 mL
Folltropin (stock 6)	63	µl	1 aliquot of 400 µL
Estradiol (stock 5)	100	µl	pipette what is needed (save unused portion)

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
NaH_2PO_4+H_2O (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 $MgCl_2$ (Stock 12) and 312 µl $CaCl_2$ (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.

SOF-FERT Stock solution

Table 3. Recipe for SOF-FERT stock solution - Add the following to 1600 ml Sigma water in sequence:

	Ingredient	Concentration		Sigma CAT#	M.W	Amount	
1	**$CaCl_2.2H_20$**	1.17	mM	C7902	147	**0.2752**	**g**
2	**$MgCl_2.6H_2O$**	0.49	mM	M2393	203.31	**0.1594**	**g**
3	**KH_2PO_4**	1.19	mM	P5655	136.1	**0.2591**	**g**
4	**KCl**	7.16	mM	P5405	74.55	**0.8540**	**g**
5	**NaCl**	107.7	mM	S5886	58.44	**10.0704**	**g**
6	**Caffeine**	1.00	mM	C0750	194.19	**0.3107**	**g**
7	**$NaHCO_3$**	25.07	mM	S5761	84.01	**3.3698**	**g**
8	**Gentamicin (Stock 8)**	5	µg/mL	G1397	50 mg/mL	**1600**	**µL**
9	**Na-lactate**	5.3	mM	L4263	112.06	**1209**	**µL**

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

Supplement	Concentration		Sigma CAT#	Amount	
EFAF BSA	6	mg/mL	A6003	**1.5**	**g**
Na-pyruvate (Stock 2)	0.2	mM	P4562	**2500**	**µL**
Heparin	10	µg/mL	H3149	**1250**	**µL**

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:

	SOF BE1	Concentration		Sigma CAT#	M.W	Amount	
1	$CaCL_2.2H_20$	1.17	mM	C7902	147	**0.0602**	**g**
2	$MgCl_2.6H_2O$	0.49	mM	M2393	203.31	**0.0349**	**g**
3	KH_2PO_4	1.19	mM	P5655	136.1	**0.0567**	**g**
4	KCl	7.16	mM	P5405	74.55	**0.1868**	**g**
5	NaCl	107.7	mM	S5886	58.44	**2.2029**	**g**
6	Tri-Na-citrate	0.50	mM	C3434	294.1	**0.0515**	**g**
7	Myo-Inositol	2.77	mM	I7508	180.16	**0.1747**	**g**
8	$NaHCO_3$	25.07	mM	S5761	84.01	**0.7371**	**g**
9	Na-lactate	5.3	mM	L4263	112.06	**264**	**µL**
10	Gentamicin (Stock 8)	25	µg/mL	G1397	50 mg/mL	**1750**	**µL**

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

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

*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

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

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.

Ingredient	Sp-TALP	HEPES-TALP	IVF-TALP
TL (ml)	76.0	500.0	100.0
BSA, Fract V (g)	0.48	1.5	0
BSA, EFAF (g)*	0	0	0.6
Stock 2: pyruvate (ml)	4.0	5.0	1.0
Stock 8: gentamicin (µl)	160	750	100
Stock 7: heparin (µl)	0	0	500

- **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
$NaHCO_3$ -	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 8A: 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.

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

Stock 5: Estradiol. Dissolve 1 to 3 mg estradiol (main lab: reagent's cabinet) 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 12: $MgCl_2$ for Percoll. Prepare 0.1 M stock by adding 0.203 g $MgCl_2$ (stored in the main lab: reagent's cabinet) to 10 ml water. Sterile filter.
Source: Sigma M2393-100G
Alq Size: N-A
Storage: Store at 4°C

Stock 13: $CaCl_2$ for Percoll. Prepare 1 M stock by adding 0.735 g $CaCl_2$+$2H_2O$ (stored in the main lab: reagent's cabinet) to 5 ml water. Sterile filter.
Source: Sigma C7902-500G
Alq Size: N-A
Storage: Store at 4°C

this page was last updated by Peter J Hansen March 15 2023

HELPFUL HINTS

Here are a few things that can 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 and Fujifilm Irvine Science Oil for Embryo Culture (Catalog 9305) 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.

Record all Details of Materials and Supplies Used Each Week
We use a log sheet to record exactly what supplies and media were used during the week. This information is crucial when a crash occurs. We also record the cause of each crash when identified (helpful for diagnosing future crashes)

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.
We currently do not use a space heater. Whether to use one depends on the air conditioning in the room.
3. Before looking at embryos using a microscope, make sure stage is not cool (turn on the space heater if needed). 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% CO_2 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 CO_2 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.

Figure 1. Examples of use of metal beads in a water bath (top) or plastic carrier on a heating plate (bottom).

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.
5. Historically, our water baths were a great place to grow fungus. Accordingly, the water in the baths has been replaced with metal beads that are a great (although expensive) substitute for water (Figure 1). They can be easily cleaned by autoclaving or washing in ethanol. We have also placed the metal beads into a plastic carrier placed on a heating plate to hold tubes at 38.5°C. There are several suppliers including Sheldon Manufacturing which produces Lab Armor ® beads.

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.

last edited by Tatiane Maia and Peter J Hansen, March 15 2023.

IVF USING OOCYTES COLLECTED BY OOCYTE PICKUP (OPU)

Searching for Oocytes Collected by OPU

1. Clean searching area with windex and alcohol.
2. Plug in portable incubator (set to 38.5 C) and place maturation tubes inside to thaw.
3. Tape down sterile blue paper and white wipeall towels where water bath will be placed.
4. Plug in power strip, microscopes and slide warmers.
5. Fill water bath half way, plug in and turn on, let it warm up before placing media in.
6. Sanitize all wiretrols and microscopes
7. Mix media (aspiration and searching) and place one of each in the warm water bath.
8. Uncap aspiration media, place horse insemination catheter into media and cover with plastic.
9. Place parafilm around the open bottle top.
10. Aspirate media into 30 mL syringes and put 10 mL into 50 mL tubes.
11. Fill 35 mm dishes with searching media and place on warm plates with the cap partially on.
12. Take ovary bucket and cut a hole in the lid where you will place the filter.
13. Cut the tube of the filter to appropriate length and place on ovary bucket
14. Filter aspiration fluid from each cow immediately by gently mixing tube and draining the contents into the filter (if you are running behind make sure to place the tube into the warming bath until ready).
15. Slowly release aspiration fluid into bucket- **DO NOT LET THE FILTER GET DRY**
16. With a thin layer of fluid covering the filter, take a 30 mL syringe full of aspiration media and rinse the filter, making sure to rinse the sides as well. Slowly release fluid until there is only a thin layer covering the bottom. Repeat until the liquid is free of blood.
17. Label a grid plate and small 35 mm dish with donor number.
18. At an angle, dump the contents of the filter into the grid plate while simultaneously rinsing the bottom of the filter with a needle topped 30 mL syringe. Repeat until filter is clean and no leftover tissue remains.
19. Add a thin layer of aspiration fluid to the filter and cap.
20. Take grid plate full of aspiration media and search for cumulus oocyte complexes (COC). Place COCs into small 35 mm dish. Search the entire plate at least twice before giving it to a second person to search once more.
21. After searching is completed, clean all collected COCs into another 30 mm dish with warm searching media.
22. Grade all COCs based on a scale of 1-4: **1-** the best quality cytoplasm, numerous layers of granulosa cells, regular in shape, **2-** cytoplasm is dark and uniform, not as many granulosa cells, **3-** cytoplasm regular in shape, not as dark, very few granulosa cells, **4-** cytoplasm in not uniform in shape and possesses a light color, no granulosa cells surrounding the oocyte.
23. Once all COCs are washed and graded, place them into maturation tubes (1 ml of maturation media, 200 μl of mineral oil). Place up to 30 COCs per tube.
24. Cap maturation tube tightly, label the cap clearly with the donor ID, donor order and number of COCs present.
25. Place into the incubator and shut.
26. Upon arrival back to the main lab, place all tubes into the permanent incubator with the cap off (if using boviteq media keep the cap on).
27. Allow the COCs to mature for 22 hours.

IVF Procedure

Prepare all media at least two hours before IVF procedure takes place

1. Fill one 15 mL conical tube with IVF-TALP and place in the incubator uncapped.
2. Prepare four 2 mL microcentrifuge tubes for the IVF procedure per bull. All tubes should be left uncapped.
    a. One labeled 'PureSperm'
    b. One labeled 'Wash'
    c. Once labeled 'Sperm'
    d. One labeled 'IVF'
3. Fill 'Wash' tube with 1000 μL of IVF-TALP.
4. Fill 'IVF' tube with 2 mL of IVF-TALP.
5. Place one top and one bottom of PureSperm per bull in the incubator to warm.
6. Into a 35 mm dish, place 3 60 μL drops of IVF-TALP. Cover all drops with mineral oil. Each donor will have its own dish.
7. Prepare two 15 mL conical tubes of HEPES TALP and place in the incubator, cap on.
8. Prepare extra mineral oil in a 15 mL conical tube in the incubator.
9. Fill citothaw with water and plug in to warm.
10. At the time of fertilization, retrieve one x plate and one Fisher sterile transfer pipette.
11. With your transfer pipette, fill the three corners of one quarter of an x plate with HEPES.
12. Once 22 hours of maturation has taken place, get one maturation tube and place it on the slide warmer.
13. With a sterile tip and 1 mL syringe, collect all matured COCs from the bottom of the maturation tube and place in the first corner of the x plate.
14. Double check under the microscope that all COCs have been collected.
15. With a clean wiretrol, count and wash each matured COC individually, passing them from one drop to the next letting them all sit in the third drop before being placed in a 60 μl drop of IVF-TALP. Maximum of 30 COCs per fertilization drop
16. After all COCs are washed, place 200 μl of bottom Puresperm® and 200 μL of bottom Puresperm® into the warmed 2 mL tube labeled 'PureSperm' creating a gradient.
17. Collect semen straw from the liquid nitrogen tank and place directly into citothaw for 30-45 seconds.
18. Dry the semen straw with a paper towel, cut tip (opposite of the cotton plug) and release semen into 'PureSperm' tube.
19. Carefully close the tube and place in the microcentrifuge for 5 minutes at 6000 RPM.
20. After the 5 minutes is complete, transfer 100 μL of the sperm pellet and place in the warmed 'Wash' tube.
21. Centrifuge the 'Wash' tube for 3 minutes at 3000 RPM.
22. After the 3 minutes is complete, transfer 100 μl of the pellet to the warmed tube labeled 'Sperm'.
23. In a new 2 mL tube place 30 μl of water and 2 μl of the washed sperm from the 'Sperm' tube.
24. From this 40 μl dilution, fill each side of the hemocytometer with 10 μl.
25. With a tally counter, count 5 squares on each side of the hemocytometer and average the two.
26. With your average sperm number calculate how much to dilute your 'Sperm' tube with this formula: **[(Volume x 5 x cells x 20)/ 850]- Volume**. Volume is 100 μL. The result from this formula will be the amount of IVF-TALP to add. Your final concentration will be 1 x 10^6 sperm/mL
27. After gently mixing the IVF-TALP and sperm add 20 μL of sperm and 3.5 μL of PHE to each drop containing matured COCs.
28. Allow COCs and sperm to coincubate for at least 9 hours.
29. If fertilization drops become too large and become partially uncovered with oil, gently add 1 mL of oil into the fertilization dish until all drops are completely covered.

30. After IVF procedure, prepare culture dishes and place at least two full 15 mL tubes of HEPES TALP in the incubator in preparation for culture.

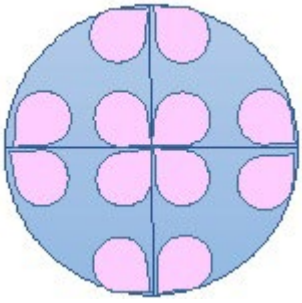

***Figure 1**. Depiction of an X plate with media in each corner.*

Washing procedure

1. After at least 9 hours of coincubation of COCs and sperm, thaw one tube of hyaluronidase (amount will depend on the number of donors)
2. Once thawed, add 300 μL of HEPES TALP into the hyaluronidase tube and gently mix to avoid bubbles.
3. Take 3-4 empty sterile 2 mL tubes and fill each with 35 μL of the hyaluronidase+HEPES.
4. Retrieve an x plate, labeling each quarter with the donor ID. Fill each corner with HEPES, leaving the middle empty.
5. With a sterile pipette tip and 1 mL syringe (or wiretrol), collect all zygotes from fertilization drops and place them into the 35 μL hyaluronidase+HEPES tubes.
6. Vortex zygotes for 3 minutes.
7. After vorexing, rinse the walls of the 2 mL tube with HEPES and let the zygotes settle to the bottom for 1 minute.
8. With a transfer pipette, collect all the liquid and zygotes and place into the empty space in the middle of the x plate.
9. Using a wiretrol, collect all zygotes with no remaining granulosa cells into the bottom left corner of the x plate.
10. If not all zygotes are present, rewash the 2 mL tube, repeating the same process as above (repeat only 2-3 times, keep in mind some zygotes will explode or disintegrate during vortexing).
11. Once all zygotes are accounted for and washed place them into their respective culture dishes (maximum of 30/dish).

this page was prepared by Lané Haimon, Eliab Estrada-Cortés and Thiago Amaral
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PREPARATION OF IN-VITRO DERIVED BOVINE EMBRYOS FOR TRANSFER INTO RECIPIENTS

Selection of Recipients

Recipient cows should be on day 7 of the estrous cycle (d 0 = estrus). Cows can be selected based on estrus detection (with or without synchronization) or after synchronization of ovulation without estrus detection. There are many ways to achieve estrous synchronization. See Estrous Synchronization - A Reproductive Management Tool from Select Sires, Estrous Synchronization of Cattle from *Brangus World* and Synchronization Programs from ABS among other sources.

One way to synchronize recipients is by giving one injection of GnRH (100 μg, i.m.) followed seven days later by an injection of $PGF_{2\alpha}$ (25 mg/ml) followed by estrus detection. Cows seen in estrus (most within 48-96 h after PGF) are scheduled to receive an embryo on day 7 of the estrous cycle. Transfer without estrous detection can also be performed by using the OvSynch program typically used for timed artificial insemination. This timed embryo transfer (TET) procedure, which is still under development, may be useful when estrus detection is difficult such as during heat stress (see Ambrose et al., 1999 and Al-Katanani et al., 2002). Cows used for TET receive an intramuscular injections of 100 μg GnRH (Cystorelin) on day 0 followed by 25 mg $PGF_{2\alpha}$ on day 7 and 100 μg GnRH on day 9. Embryos are transferred 8 days after last GnRH injection (note: the optimal time for transfer has not been determined experimentally).

Harvesting and Transport of Embryos

1. Embryos produced by in vitro techniques (see Procedures for In Vitro Production of Bovine Embryos by Rivera et al) are harvested on day 7 or 8 after fertilization (day 0=day of insemination). For this purpose, excellent and good quality blastocysts (as described in the Manual of the International Embryo Transfer Society) are identified using a dissecting microscope and transferred into a sterile tube containing embryo transfer medium (we use HEPES-TALP using HEPES-TL from Biowhittaker but other media probably work well also). *Note: Many types of tubes will work for transport – we frequently use a microcentrifuge tube with 1 ml of medium and as many embryos as available.*
2. Tubes are sealed by placing a strip of Parafilm around the cap and loaded in a device to keep embryos warm during transport. *Note: Use a portable incubator from Minitub (catalog # 19180/0000) (inexpensive but less reliable) or Cryologics (INC-RB1) (more expensive but excellent) that can maintain temperature at 39.0°C. Both operate on batteries. The Minitub incubator an operate from a car cigarette lighter.*

Preparation of Materials for Transfer

The following is prepared on the day of transfer and transported to the embryo transfer site:

a. Slide warmer set at 39°C (if not available use a Styrofoam rack or other piece of Styrofoam as a platform for Petri dishes containing embryos to keep embryos from cold surfaces)
b. Bench paper (not necessary but helpful at creating a clean area to work on)
c. 70% Ethanol (wipe all areas with ethanol before starting any procedure)
d. Petri-dish to place embryos prior to pick up into transfer straw (Intergrid plate works very well because of the demarcations on the plastic)

e. Round Petri dish to transfer embryos from the tube were the embryos were transported (60 x 15 Petri dish works very well due to its small size)
f. 1 ml tuberculin syringes
g. Instrument to handle embryos – many are available as described in the Guide on Use of Instruments for Picking Up Oocytes and Embryos
h. Water bath
i. 200 μl pipet tips (yellow)
j. 0.25 ml French straw
k. Pipettor set at 70 μl
l. Embryo transfer rods (we use IMV 21" deep chamber rods from Agtech cat# F17)
m. Sheaths for embryo transfer rod (these have sideways openings to reduce contamination of the uterus – from Agtech; catalog number F18A)
n. Oversleeve Sanitary Chemise – a plastic sleeve used to cover the sheath and transfer rod manufactured by IMV (available from Agtech; catalog #F27A)
o. Additional sterile wrap (Dualpeel tubing from VWR works well for this step) for embryo transfer rods
p. Dissecting microscope
q. Plastic Pasteur pipets
r. Transfer medium (HEPES-TALP - ~ 15-30 ml)

Setup of Work Area at Transfer Site

a) Prepare a clean area at the farm were the preparation of straws will take place. A temperature controlled room works best but any draft-free area can be sufficient.
b) Wipe all surfaces with 70% ethanol.
c) Place bench paper (if available) on area where the embryo manipulations will take place.
d) Set slide warmer at 39°C (or set up Styrofoam rplatform)
e) Place round and Intergrid Petri-dishes on slide warmer
f) Set water-bath set at 38.5°C - *Alternatively, one could use the portable incubator to the tube containing transfer medium warm. If a portable incubator or a water-bath is not available, another alternative is to place the drops of medium into an Intergrid plate that is on top of the slide warmer. Place the lid on the plate to prevent evaporation. In about 5-10 minutes, the 70 μl drops of medium should be warmed up and ready for use.*
g) Transfer embryos from the tube in which they were transported into a round Petri dish with a plastic Pasteur pipet.

Loading Embryos into Straws (see Figure 1)

a) Locate, count and evaluate embryos for quality.
b) Insert 200 μl yellow tip onto the 1 ml syringe as shown (see Figure 1).
c) On a Petri-dish (i.e. Intergrid plate) place 3 microdrops of 70 μl holding medium side by side (**A**).
d) Place one blastocyst into the middle drop (**B**).
e) Insert the cotton plug side of a 0.25 ml French straw into the wide end of the pipet tip (**C-D**).
f) Aspirate one empty drop (**E**).
g) Aspirate air to create a ~0.5 cm column (**E**)
h) Aspirate the microdrop containing embryo (make sure that the embryo is aspirated by observing under the microscope; **F**)
i) Aspirate air to create a ~0.5 cm column (**F**)
j) Aspirate the remaining empty drop until the cotton plug is wet (**G**).
k) Remove transfer straw from syringe and place on slide warmer until use (**H**).

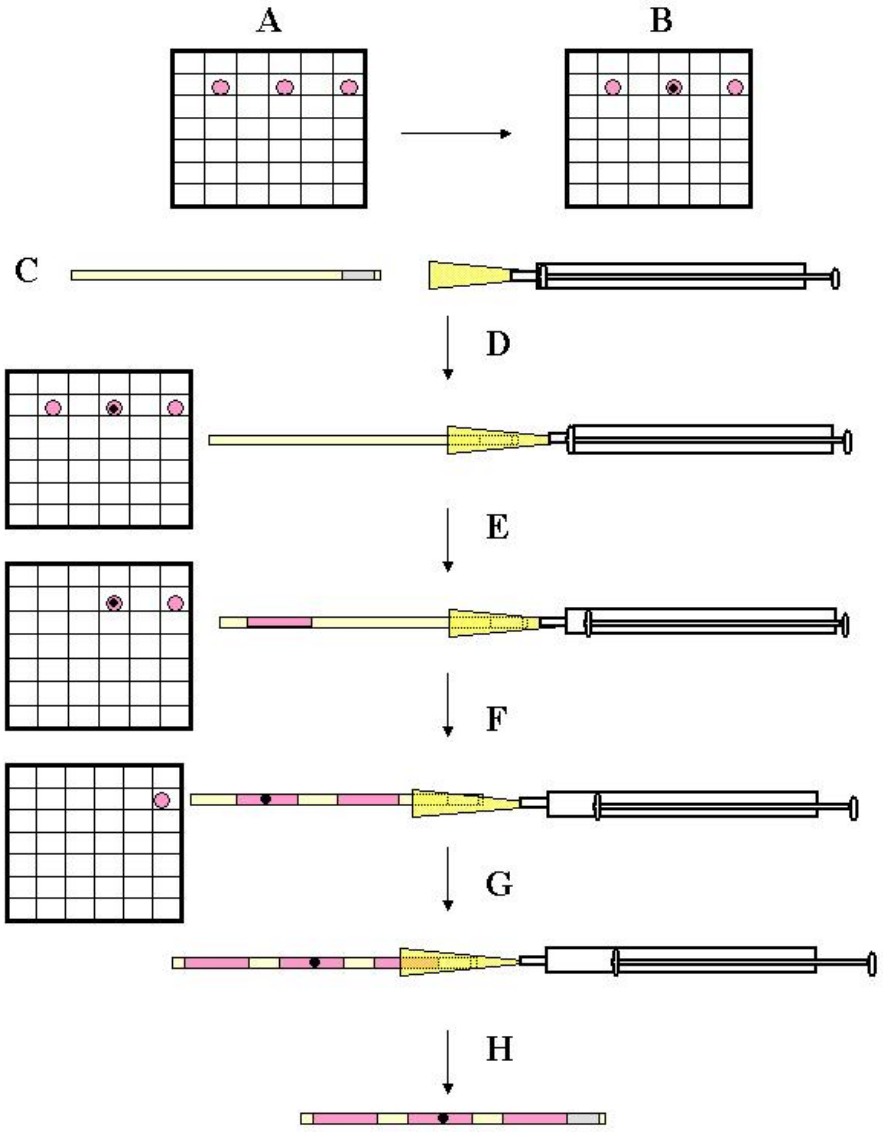

Figure 1. Loading embryos into straws.

Loading Straws into Transfer Pipettes (see Figure 2)

a) Open bag containing sterile transfer rod (**A**) *(use rod designed for 0.25 cc straws)*
b) Pull the plunger from the back of the transfer rod (**B**)
c) Insert the embryo containing French straw into the transfer rod cotton side first (do this by holding the straw *from the side of the cotton plug to minimize contamination of the straw). If resistance is encountered stop immediately and check if the plunger if pulled back* (**C**).
d) Move blue ring towards the center of the gun. Place a disposable blue sheath over the transfer gun without touching the front end. Push the sheath all the way to

the back of the rod (you may need to apply some pressure). Move blue ring to the back of the rod to fasten the sheath in place (**D**).

e) Place a chemise over the gun without touching the front end (**E**).
f) Place loaded rod into a protective bag (**F**).
g) Transport to the site of transfer in a horizontal position.
h) Remove rod from protective bag and keep plastic bag clean to reuse for next transfer
i) After completion of transfer, bring transfer rod back to the preparation area and wipe with ethanol. Do this by performing a single movement from the tip of the rod (cleanest area) to the back of the rod (dirtiest area).
j) Wait until ethanol has evaporated completely.

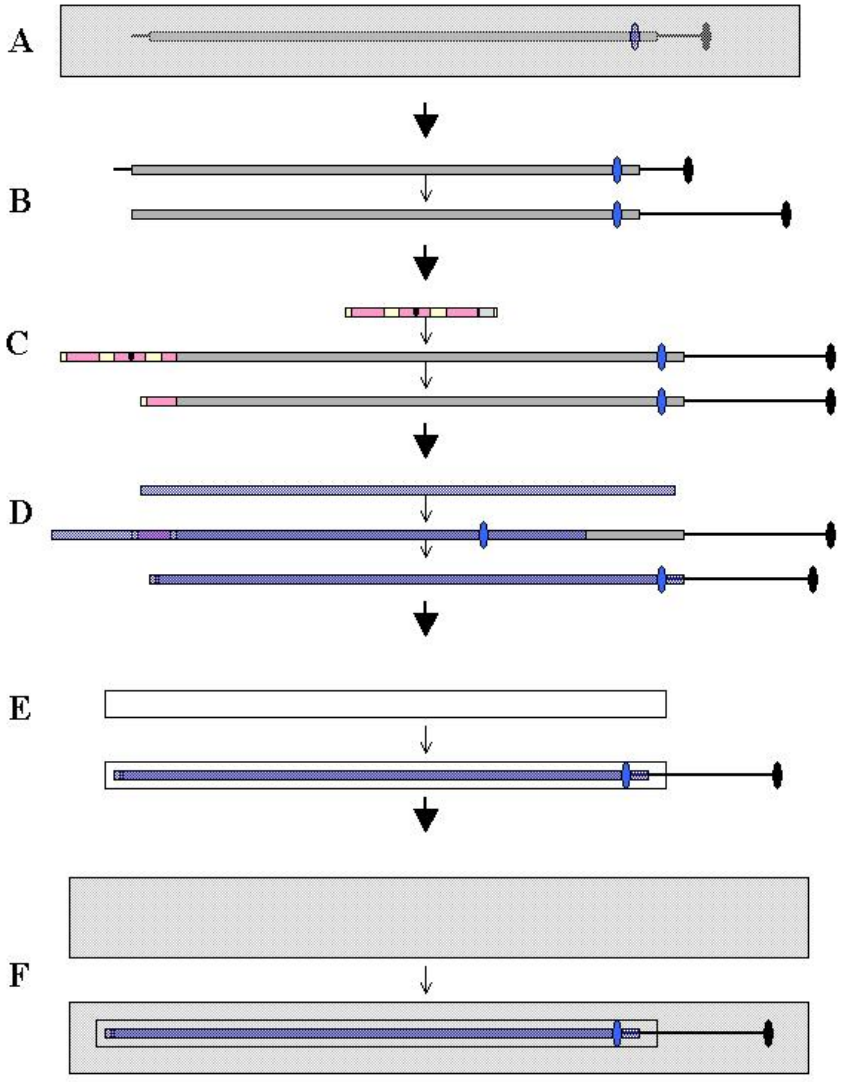

Figure 2. Loading straws into embryo transfer pipettes

Preparation of Cows for Transfer

a) Restrain the cow
b) Palpate recipient cows for the presence of the corpus luteum (CL) – if no CL present, do not use the recipient.
c) With chalk mark the side were the CL is present
d) Shave hair on the tailhead
e) Prepare tailhead for epidural by cleansing with betadine scrub followed by alcohol (70% ethanol)
f) Give an epidural by injecting 5 ml of lidocaine into the epidural space. *When the tail is relaxed, the recipient is ready for the embryo to be transferred.*

Embryo Transfer Procedure

In general, transfer is performed as for AI (see Artificial Insemination Technique *by Michael O'Connor. Special modifications and concerns are listed below*

a) Care must be taken to avoid contamination with feces. Clean the vulva thoroughly before inserting the transfer pipette. In addition, the vulvar lips should be opened before insertion of the pipette. This can be accomplished by the technician (by pushing the arm in the rectum downwards and back slightly) or by an assistant (by grabbing the vulvar lips and pulling backward).

b) The tip of the transfer pipette is placed approximately one-third of the way up the uterine horn ipsilateral to the corpus luteum. The embryo is then gently expelled from the pipette.

Bovine embryo vitrification and thawing

based on protocol provided by Acceligen

Materials Needed:

- 15 mL conical tubes (Cat #: 05-538-59B, Corning)
- Syringe filter (Cat #: CLS431229, Corning)
- Bottle filter (Cat #: 595-4520, Thermo Scientific)
- Sterile Pipet tip
- Wiretrol (Cat #: 50002005, Drummond)
- 1X DPBS + 0.2% PVP
- Dehydration solution
- Vitrification solution
- NUNC 4-well dish (Cat #: 144444, Thermo Scientific)
- 35 mm petri dish (Cat #: 351008, Corning)
- Culture Media: BO-IVC (Cat #: 71004, IVF Biosciences) or SOF-BE2 from our lab
- Oil for embryo culture (Cat #: 9305, Irvine Scientific)
- Cryolock (Cat #: CL-R-TC-B, Biotech)
- Goblets
- Racks for embryos (Cat #: 2019-13-106, IVF Store)
- Foam box
- Liquid Nitrogen
- Forceps
- Tags for goblets
- Incubator (38.5C, 5% CO2, 5% O2)
- Bench top warmer

Additional information:

- Embryos from Day 6 to 8 can be cryopreserved
- Wash hands well and before beginning to work prepare all the working solutions and the plates
- Clean work area with 70% ethanol
- Turn on microscope and its warming plate (38.5ºC)
- Select only blastocysts grade 1, according with the IETS manual
- Do one embryo at a time. Follow steps 1 – 8 (Vitrification steps session) for each embryo

Vitrification Buffers:

- Add the following items in 15 mL conical tubes to prepare the solutions. Once all reagents are diluted, then filter the solution using a syringe filter
- It is recommended to always use these solutions fresh. Expiration date: 1 week since date made

Dehydration Solution - total volume 10 mL:

Product	Cat # - Manufacturer	Concentration	volume
Ethylene Glycol	102466 - Sigma	10%	1 mL
DMSO	D2650 - Sigma	10%	1 mL
DPBS (1X)* 0.2% PVP	2230811 - Gibco		8 mL

Vitrification Solution - total volume 10 mL:

Product	Cat # - Manufacturer	Concentration	volume
Ethylene Glycol	102466 -Sigma	20 %	2 mL
DMSO	D2650 - Sigma	20 %	2 mL
DPBS (1X)* 0.2% PVP	2230811 - Gibco		6 mL
Sucrose	S1888 - Sigma	0.25M = 0.8558 g	

*DPBS can be made by adding 1 packet of DPBS to 1 liter of ddH20. Then filter using a bottle filter.

Culture Plate (BO-IVC or SOF-BE2 from our lab):

- IVC Plates should already be in incubator with the embryos.
- IVC Plates preparation: Add 50 µL drops with culture media (BO-IVC/SOF-BE2) for every 30 embryos or by donor into 35 mm petri dish. Label it as "IVC", and the date on the bottom. Cover with 3.5 mL mineral oil in the incubator
- The embryos can be held in this plate before vitrification.

Vitrification Plates:

- Using a 4-well dish, add the following solution to the wells as described below (**figure 1**):
    - Well 1: 500 µL of DPBS with 0.2% PVP
    - Well 2: 500 µL of DPBS with 0.2% PVP
    - Well 3: 500 µL of Dehydration solution (ES)
    - Well 4: 500 µL of Vitrification solution
    - Small drops: Using Vitrification solution make a 10µL drop in the middle of the plate and second one of 1 µL (show in yellow)

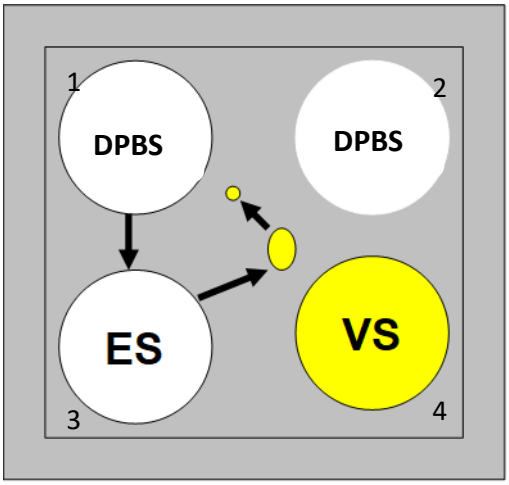

Figure 1. 4 well dish with the respective solutions to be used during vitrification

Vitrification Steps

1) Label cryolock for each embryo with number, name of donor, etc.
2) Add liquid nitrogen (-196°C) to a foam box (size will vary, we use 12 x 8 x 8 in)
3) Wash the embryo (3x) in well 2 using the wiretrol by pipetting up and down

3) Move embryo to well 1 until ready for step 4
4) Move embryo to well 3 (show as ES in figure 1) and leave it for 1 minute

Steps 5, 6 and 7 should be done as quickly as possible.

5) Place embryo into the 10 µL drop, pipet up and down with wiretrol (~10x)
6) Move embryo to the 1 µL drop and place the embryo with as little volume as possible on the tip of the cryolock
7) Using a forceps, immediately submerge cryolock into liquid nitrogen and cap the cryolock while submersed in nitrogen (**Figure 2**)
8) Place 3 to 4 cryolocks per goblet. Then, put another goblet on top to avoid losing any device in the nitrogen tank during storage
9) Identified the cane with the cryotag (including the date and name of the experiment)
10) Move cryolock to a tank

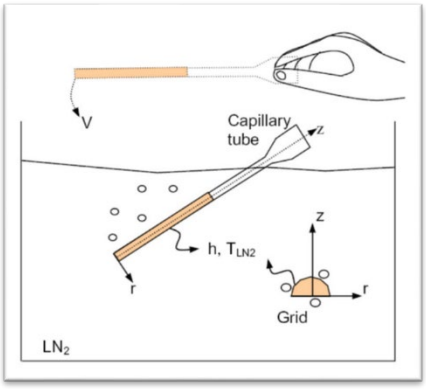

Figure 2. Submersion of cryolock into liquid nitrogen

Thawing Steps:

1. Prepare TCM 199 and sucrose solutions:

TCM 199 - total volume 500 mL:

Product	Cat # - Manufacturer	Amount (g)	Volume
IVF Water	W3500 - Sigma		500 mL
Medium 199	M2520 - Sigma	7.35 g	
Sodium Bicarbonate	S5761 - Sigma	1.1 g	

Once TCM is prepared, adjust pH for 7.2 – 7.4

0.5 M sucrose solution - total volume 10 mL:

Product	Cat # - Manufacturer	Amount (g)	Volume
TCM 199	Already prepared		10 mL
Sucrose	S1888 - Sigma	1.712 g	

0.25 M sucrose solution - total volume 10 mL:

Product	Cat # - Manufacturer	Amount (g)	Volume
TCM 199	Already prepared		10 mL
Sucrose	S1888 - Sigma	0.8558 g	

Both sucrose solutions should be prepared fresh. If not, keep in the fridge for maximum 1 wk.

2. Prepare ET medium

ET medium - total volume 100 mL:

Product	Cat # - Manufacturer	Volume
HEPES-TALP	Already prepared	90 mL
Fetal Bovine Serum	10437-028 - Gibco	10 mL
DTT	D9163 - Sigma	5 μL*

*DTT: 5 μL of 0.154g of DTT in 1 mL of IVF water

Once medium is prepared, aliquot 15 mL ET medium and place into the incubator

3. Place 0.5 M and 0.25 M sucrose solutions in water bath to warm up to 38.5 C.
4. In a 4 well plate, place 500 μL of 0.5 M sucrose solution in well 1 and 500 μL of 0.25 M sucrose solution in well 3.
5. Fill a small Styrofoam box containing semen straw canisters with liquid nitrogen.
6. Place all cryolock straws into canisters while they await thawing.
7. Place the warmed 4 well plate with sucrose solutions under the microscope.
8. Take the desired cryolock straw and fully submerge the side containing the embryo in the liquid nitrogen.
9. With a hemostat, unlock the cryolock leaving the hemistaw still fully submerged.
10. Quickly transfer hemistraw from the liquid nitrogen to the well containing the 0.5 M sucrose solution, watching as the embryo rolls of the hemistraw.
11. Leave the thawing embryo in the 0.5 M sucrose solution for 5 minutes.
12. About 20 seconds before the 5-minute time frame is finished, locate the embryo and be ready with a clean wiretrol for transfer.
13. After 5 minutes has passed, transfer the thawing embryo to well 3 containing the 0.25 M sucrose solution. Leave for 5 minutes.
14. As the embryo thaws, fill two quarters of an x plate with warm ET medium.
15. Transfer thawed embryo into warmed ET and load into an ET straw. Place in a warmed incubator set for 38.5 C.

Modified by PJ Hansen and Tatiane Maia 3.20.2023
Written by Tatiane Maia and Lané Haimon 10.17.2022

ONLINE TRAINING RESOURCES FROM THE LAB AND ELSEWHERE

Several online materials have been made by lab members to assist in learning parts of the protocol.
YouTube subscription: https://www.youtube.com/user/ufpete100

Big Book of Bovine Embryos

The purpose of this collection of images of bovine oocytes and embryos is to assist new bovine embryologists with identification of structures encountered under the microscope.

Video: oocyte slashing

How to slash ovaries with a scalpel to obtain oocytes. The video was prepared by Luis Armando Davila.

Video: oocyte selection for bovine IVF (searching)

This video was produced for a class at UF by Heather Rosson. Very well done.

Video: making drops

It can be a little tricky to make microdrops. Video by Elizabeth Jannaman and Paula Tribulo.

Video: picking up embryos

Another useful video from Liz and Paula.

Evaluation and classification of bovine embryos

A paper by Bo and Mapletoft published in Animal Reproduction 10:344 (2013) that is an excellent guide.

https://www.animal-reproduction.org/article/5b5a604cf7783717068b46a2/pdf/animreprod-10-3-344.pdf

IVF Media Record and Recipe

Weekly IVF Log

Week of ….. to ….., 2023

MEDIA USAGE DURING THE WEEK

	Batch at start week		Second batch		
Medium	Lot # or date made	Tech or Brand	Lot # or date made	Date opened	Tech or Brand
MOFA					
How many bottles of MOFA ?:		Does it need to be ordered?			
OMM					
IVF -TALP					
Pure - Sperm					
HEPES-TALP					
SOF-BE2					
Mineral Oil					
How many bottles of Oil ?:		Does it need to be ordered?			
IVF H_2O					
PHE					
Hyaluronidase					

PLASTICWARE

	Batch at start week		Second batch		
Supply	Lot #	Brand	Lot #	Date opened	Brand
50 ml tubes					
15 ml tubes					
Transfer pipettes					
Grid Plates					
X-Plates					
Cell Strainer					
Maturation Plates (60 mm-1008) (Big)					
Fertilization/ Culture plates (35 mm-1007) (Small)					

Dishwasher Checklist

Task	Completed	Notes from Dishwasher
Mop Floor 10% Bleach (Water ONLY inside IVF Lab!)		
Check 70% Ethanol Jug Fill squeeze bottles. Make new if needed.		

Notes: Please make sure there are enough aliquots (> 15) of PHE, hyluronidase and pure sperm

Please make sure ovaries have been discarded properly in the Meat Lab

MEDIA PREPARATION									
Week of …. to …., 2023									
Complete Medium						**Ingredients - enter lot # or date made for aliquots**			
OMM	Date made	Initials	Volume	PH 38.5°C	Osm.	TCM 199		Na Pyruvate	
						FBS		Glutamax	
						Folltropin		Pen/Strep	
						EGF			
IVF-TALP	Date made	Initials	Volume	PH 38.5°C	Osm.	IVF-TALP base		Na Pyruvate	
						BSA EFAF		Gentamicin	
						Heparin			
HEPES-TALP	Date made	Initials	Volume	PH 38.5°C	Osm.	HEPES-TALP base		Na Pyruvate	
						BSA FractV		Gentamicin	
SOF-BE2	Date made	Initials	Volume	PH 38.5°C	Osm.	SOF Base		Na Citrate	
						EFAF-BSA		Non-Esst. AA	
						Glutamax		Esst. AA	
						Na Pyruvate		Gentamicin	
						Myo-Inositol			
Base Medium						**Ingredients - enter lot # or date made**			
HEPES-TL Base	Date made	Initials	Volume	PH RT	Osm.	Hepes		KCl	
						CaCl2.2H20		NaCl	
						MgCl2.6H20		NaHCO3	
						NaH2PO4.H2O		Na-Lactate	
						Sigma H_2O			
SOF-Base	Date made	Initials	Volume	PH RT	Osm.	CaCl2.2H20		KCL	
						MgCl2.6H20		NaCl	
						Na-Lactate		NaHCO3	
						KH2PO4		Sigma H_2O	
IVF-TL Base	Date made	Initials	Volume	PH RT	Osm.	NaCl		Na-Lactate	
						KCl		CaCl2.2H20	
						NaHCO3		MgCl2.6H20	
						NaH2PO4.H20		Sigma H_2O	
Aliquots						**Ingredients - enter lot # or date made**			
PHE	Date made	Initials	Aliquot size			Penicilla.		NaCl	
						Hypotaurine		Na Lactate	
						Epinephrine		Na Metabisu.	
BSS+Heparin	Date made	Initials	Aliquot size			BSS		Sigma H_2O	
						Heparine			
Pen/Strep	Date made	Initials	Aliquot size			Pen/Strep			
Hyaluronidase	Date made	Initials	Aliquot size			NaCl		Sigma H_2O	
						Hyaluronidase			
BSS	Date made	Initials	Aliquot size			BSS Lot			
Folltropin	Date made	Initials	Aliquot size			Folltropin			
Heparin	Date made	Initials	Aliquot size			Heparin		Sigma H_2O	
100X Myo-Inositol	Date made	Initials	Aliquot size			Myo-Inositol		Sigma H_2O	
100X Na Citrate	Date made	Initials	Aliquot size			Na- Citrate		Sigma H_2O	
Na Pyruvate	Date made	Initials	Aliquot size			Na Pyruvate		Sigma H_2O	

Note: If any medium needs to be prepared, once media is done, an aliquot of 3 mL is placed in incubator for at least two hours and then pH and osmolarity is checked.

OMM 50 mL stock solution = starting volume

FILTER ALL MEDIA INTO STERILE BOTTLES

TEST OSMOLARITY & PH FOR ALL MEDIA - RECORD IN LOG BOOK

Ingredient	Amount	Date	Date	Date	Date	Date	Date	Date	Date	Date
TCM 199	43.463 mL									
FBS	5 mL									
Pen/Strep (Stock 18)	500 µL									
Na-pyruvate (Stock 2)	500 µL									
Glutamax	500 µL									
EGF	25 µL									
FSH (Folltropin)	12.5 µL									
Filter in a sterile bottle										
Technician										

Ingredient	Amount	Date	Date	Date	Date	Date	Date	Date	Date	Date
TCM 199	43.463 mL									
FBS	5 mL									
Pen/Strep (Stock 18)	500 µL									
Na-pyruvate (Stock 2)	500 µL									
Glutamax	500 µL									
EGF	25 µL									
FSH (Folltropin)	12.5 µL									
Filter in a sterile bottle										
Technician										

Note: OMM is the only medium that is prepared fresh every time. Expiration date: 1 month

IVF-TALP 100 mL stock solution = starting volume FILTER ALL MEDIA INTO STERILE BOTTLES TEST OSMOLARITY & PH FOR ALL MEDIA - RECORD IN LOG BOOK										
Ingredient	**Amount**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**
EFAF BSA	0.6 g									
Na-pyruvate (Stock 2)	1000 µL									
Gentamicin (Stock 8)	50 µL									
Heparin (Stock 7)	1000 µL									
Filter in a sterile bottle										
Technician										
Ingredient	**Amount**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**
EFAF BSA	0.6 g									
Na-pyruvate (Stock 2)	1000 µL									
Gentamicin (Stock 8)	50 µL									
Heparin (Stock 7)	1000 µL									
Filter in a sterile bottle										
Technician										
Ingredient	**Amount**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**
EFAF BSA	0.6 g									
Na-pyruvate (Stock 2)	1000 µL									
Gentamicin (Stock 8)	50 µL									
Heparin (Stock 7)	1000 µL									
Filter in a sterile bottle										
Technician										

Note: Start volume: 100 ml of IVF-TALP Base + reagents listed above. Expiration date: 1 month.

HEPES-TALP 500 mL stock solution = starting volume

FILTER ALL MEDIA INTO STERILE BOTTLES

TEST OSMOLARITY & PH FOR ALL MEDIA - RECORD IN LOG BOOK

Ingredient	Amount	Date	Date	Date	Date	Date	Date	Date	Date	Date
BSA, Fract V	1.5 g									
Na-pyruvate (Stock 2)	5 mL									
Gentamicin (Stock 8)	375 µL									
Filter in a sterile bottle										
Technician										
Ingredient	**Amount**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**
BSA, Fract V	1.5 g									
Na-pyruvate (Stock 2)	5 mL									
Gentamicin (Stock 8)	375 µL									
Filter in a sterile bottle										
Technician										
Ingredient	**Amount**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**	**Date**
BSA, Fract V	1.5 g									
Na-pyruvate (Stock 2)	5 mL									
Gentamicin (Stock 8)	375 µL									
Filter in a sterile bottle										
Technician										

Note: Start volume: 100 ml of HEPES-TALP Base + reagents listed above. Expiration date: 1 month.

SOF-BE2 50 mL stock solution = starting volume

FILTER ALL MEDIA INTO STERILE BOTTLES

TEST OSMOLARITY & PH FOR ALL MEDIA - RECORD IN LOG BOOK

Ingredient	Amount	Date	Date	Date	Date	Date	Date	Date	Date	Date
EFAF BSA	0.2 g									
Glutamax (Stock 11)	250 µL									
Na-pyruvate (Stock 2)	1000 µL									
Myo-inositol (Stock 15)	500 µL									
Sodium Citrate (Stock 16)	500 µL									
Non-Essential AA	500 µL									
Essential AA	1000 µL									
Gentamicin (Stock 8)	125 µL									
Filter in a sterile bottle										
Technician										

Ingredient	Amount	Date	Date	Date	Date	Date	Date	Date	Date	Date
EFAF BSA	0.2 g									
Glutamax (Stock 11)	250 µL									
Na-pyruvate (Stock 2)	1000 µL									
Myo-inositol (Stock 15)	500 µL									
Sodium Citrate (Stock 16)	500 µL									
Non-Essential AA	500 µL									
Essential AA	1000 µL									
Gentamicin (Stock 8)	125 µL									
Filter in a sterile bottle										
Technician										

Note: Start volume: 100 ml of SOF-BE2 Base + reagents listed above. Expiration date: 1.5 months.