INFRAFRONTIER-I3 - Cryopreservation training course

Hosted by the Frozen Embryo and Sperm Archive, MRC - Harwell

Preparation of Media and Reagents

A. Composition of high calcium HTF Medium (used for IVF) – see Kito & Ohta (2005)

Reagent Name	mg/100ml	Vendor	Cat. Number
NaCl	593.8	Sigma	S5886
KCl	35.0	Sigma	P5405
$MgSO_4 \cdot 7H_2O$	4.9	Sigma	M7774
KH_2PO_4	5.4	Sigma	P5655
$CaCl_2 \cdot 2H_2O$	**75.5**	Sigma	C7902
$NaHCO_3$	210.0	Sigma	S5761
Glucose	50.0	Sigma	G6152
Na-lactate (ml)*	0.34	Sigma	L7900
Na-Pyruvate	3.7	Sigma	P4562
Penicillin G	7.5	Sigma	P4687
Streptomycin	5.0	Sigma	S1277
BSA (Albumin Bovine Serum, Fraction V, Fatty Acid-Free)	400.0	Merck/Calbiochem	126575
0.5% Phenol Red (ml)*	0.04	Sigma	P0290

*Indicates volume of reagent

NB. There is a tendency for the calcium chloride to precipitate if dissolved in conjunction with the other components of the HTF. We therefore dissolve all the reagents except the calcium chloride together, and prepare a separate solution of calcium chloride. The two solutions are then combined before being filtered through a 0.22µm filter.

This medium is stored in 10ml aliquots at +4°C for up to three months. It is used in the preparation of fertilisation medium in the mouse IVF procedure. Pre-prepared Mouse Fertilisation medium (CARD medium) can be bought in Europe from Cosmo Bio Co., Ltd (www.cosmobio.com).

B. Composition of Sperm Preincubation Medium (TYH + 0.75mM MBCD)

Reagent Name	mg/100ml	Vendor	Cat. Number
NaCl	697.6	Sigma	S5886
KCl	35.6	Sigma	P5405
$MgSO_4 \cdot 7H_2O$	29.3	Sigma	M7774
KH_2PO_4	16.2	Sigma	P5655
$NaHCO_3$	210.6	Sigma	S5761
Na-Pyruvate	5.5	Sigma	P4562
Glucose	100.0	Sigma	G6152
$CaCl_2 \cdot 2H_2O$	25.1	Sigma	C7902
Methyl-β-cyclodextrin	98.3	Sigma	C4555
Penicillin G	7.5	Sigma	P4687
Streptomycin	5.0	Sigma	S1277
Polyvinylalcohol	100.0	Sigma	P8136

Filter the solution through a 0.22µm filter and store 1ml aliquots at +4°C for up to three months.

NB. This medium can be purchased commercially from Cosmo Bio Co., Ltd (www.cosmobio.com) as Fertiup® PM.

C. Preparation of sperm cryopreservation agent (gCPA) containing 100mM L-glutamine.

Reagent	mg/10ml	mg/20ml	mg/40ml
Raffinose pentahydrate	1,800	3,600	7,200
Skimmed Milk	300	600	1,200
L-glutamine	146	292	584

1. Add 584mg L-glutamine (Sigma: G8540) to 40ml of embryo tested water contained in a 50ml disposable tube, vortex for 3 minutes.

2. Put the tube into 60°C water bath.

3. Add 7200mg raffinose pentahydrate (Sigma: R7630) and 1200mg skimmed milk powder (Becton Dickinson: 232100) to the tube, vortex for 3 minutes;

4. Incubate the solution in a 60°C water bath for 90 minutes. Vortex for 3 minutes after every 30 minutes.

5. Divide the solution into 1.0ml aliquots and transfer to 1.5ml centrifuge tubes.

6. Centrifuge the samples at 10,000g for 60 minutes.

7. Carefully collect 0.7ml supernatant of each samples from the central region of the tube (Fig. 1).

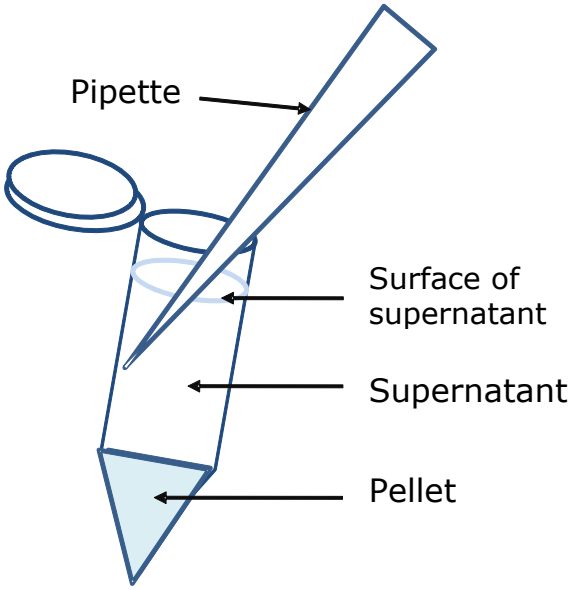

Fig. 1

8. Filter the supernatant using a disposable filter unit (pore size: 0.22 µm) discard the pellets.

9. Place 1.0ml aliquots of the filtered solution into 1.5ml microfuge tubes and seal them with parafilm. Store at room temperature (use within 3 months of preparation).

NB: this medium can be purchased separately or as part as a mouse IVF kit from Cosmo Bio Co., Ltd (www.cosmobio.com) under the trade name Fertiup® CPA.

D. Embryomax® KSOM with ½ Amino Acids, Glucose (used for embryo culture)

1. KSOM is purchased in 50ml bottles from Chemicon/Millipore (Cat No: MR-106-D) and is used as supplied. It is stored at -20°C until required.

2. Discard the medium after 7 days.

E. M2 medium (used for embryo harvest and washing)

1. M2 medium is purchased from Sigma Chemical Co. (Cat. No. M7167) in 100ml bottles which are stored at +4°C until required.

2. 100 x stock solution of Penicillin/Streptomycin (10,000 units/ml Penicillin; 10mg/ml Streptomycin) is purchased from Sigma Chemical Co. (Cat. No. P0781). On arrival, it is thawed and divided into 1.3ml aliquots which are then re-frozen at -20°C until required.

3. Before use, thaw one aliquot of Penicillin/Streptomycin. Remove 1ml of M2 from the 100ml bottle and replace with 1ml Penicillin/Streptomycin stock solution. Final concentration of Penicillin/Streptomycin is: 100units/ml Penicillin; 0.1mg/ml Streptomycin

4. Using a non-pyrogenic, rubber-free 20ml syringe (e.g. B. Braun Injekt Luer Solo) filter the medium through a 0.2µm filter into sterile 14ml falcon tubes. Push down the lids to seal tightly. Store at 4°C.

5. Discard the medium after 7 days.

F. 1.5M Propylene glycol solution (ProH) - used as a cryoprotectant for embryo freezing

1. Accurately weigh 0.57g Propylene glycol (Sigma Chemical Co. Cat No. 134368) into a sterile 14ml Falcon tube.

2. Add 4.4ml M2 (with added Penicillin/Streptomycin) to the Propylene glycol.

3. Gently mix the two solutions by rocking.

4. Using a non-pyrogenic, rubber-free 10ml syringe (e.g. B. Braun Injekt Luer Solo) filter the Propylene Glycol/M2 mixture (ProH) through a 0.2µm filter into a sterile 14ml Falcon tube. Push down the lid to seal tightly. Store at 4°C.

5. Discard after 7 days.

G. 1.0M Sucrose solution - used to dilute the Propylene glycol during embryo thawing

1. Measure 5ml M2 (with added Penicillin/Streptomycin) into a sterile 14ml Falcon Tube.

2. Add 1.71g Sucrose (Sigma Chemical Co., Cat No. S9378).

3. Mix gently until the sucrose has dissolved.

4. Using a non-pyrogenic, rubber-free 10ml syringe (e.g. B. Braun Injekt Luer Solo) filter the Sucrose solution through a 0.2µm filter into a sterile 14ml Falcon tube. Push down the lid to seal tightly. Store at 4°C, but discard after 7 days.

H. Hyaluronidase (for removing adherent cells from IVF-produced embryos)

1. 30mg Hyaluronidase Type IV-S (EC 3.2.1.35) powder is purchased from Sigma Chemical Co. (Cat no. H4272). Enzyme activity should be between 750-1500 units/mg, but each batch has a different activity which should be checked.

2. Prepare a 10 mg/ml solution of hyaluronidase in M2 (with added penicillin/streptomycin) as follows.

   2.1. Label and date 12 x 1.5ml Eppendorf microfuge tubes.

   2.2. Tap the 30 mg bottle of Hyaluronidase gently, to make the powder go to the bottom of the bottle.

   2.3. Unscrew the lid and add 3ml of M2.

   2.4. Replace the lid and swirl gently to dissolve.

   2.5. When in solution, slowly aspirate into a 10 ml syringe using a 21G needle. Invert the syringe and aspirate some air to empty the needle. Remove the needle.

   2.6. Filter sterilize through 0.2µm filter into new 14 ml tube.

   2.7. Aliquot 250µl into each of the microfuge tubes.

   2.8. Store at –20°C.

3. Prepare a working solution with a final concentration of 300µg/ml, as follows:

   3.1. Thaw a 250µl aliquot of Hyaluronidase and dilute 1:33 with M2 (30µl hyaluronidase + 970µl M2, or 200µl Hyaluronidase + 6.4ml M2).

   3.2. Mix gently.

I. Preparation of Phosphate Buffered Saline Solution (PBS)

1. Dissolve one Phosphate Buffered Saline (Dulbecco A) tablet (Oxoid, Cat No. BR0014) in 100ml distilled water.

2. Autoclave at 115°C for 10 minutes to sterilise.

J. Preparation of Pregnant Mare's Serum Gonadotrophin (PMS)

1. Pregnant Mare's Serum Gonadotrophin (PMS) is purchased from the National Hormone and Peptide Program (NHPP), Harbour-UCLA Medical Centre, 1000 W. Carson Street, Torrance, CA 90509, USA. Each ampoule contains 2000iu.

2. To prepare a working solution of PMS, at 50iu/ml, measure 40ml sterile phosphate buffered saline (PBS) into a sterile 50ml centrifuge tube.

3. Withdraw 1.0ml of the PBS using a sterile syringe and 21G needle and inject the 1.0ml PBS, by piercing the needle through the rubber cap, into the ampoule of powdered PMS.

4. Swirl the mixture gently until the powder has dissolved.

5. Invert the ampoule and using the same syringe and needle remove the hormone solution and add it to the remaining 39ml PBS.

6. Rinse the ampoule with 1.0ml of the diluted hormone, (using the original syringe and needle). Swirl again to remove any remaining hormone, and return the solution to the large tube.

7. Gently mix the PMS solution by inversion and dispense into either 1.4ml aliquots (enough for 10 females) stored in sterile microfuge tubes, or 5.2ml aliquots (enough for 50 females) stored in sterile Universal vials.

8. Freeze at -20°C until required. Once frozen, use the working solution within approximately 6 months of preparation

K. Preparation of Human Chorionic Gonadotrophin (hCG)

1. Human Chorionic Gonadotrophin (hCG) is manufactured by Intervet under the trade name Chorulon and supplied by Henry Schein Animal Health Ltd; each ampoule contains 1500iu.

2. To prepare a working solution of hCG, at 25iu/ml, measure 60ml 0.9% saline (Aqupharm No. 1) into a sterile 50ml centrifuge tube (it just fits!).

3. Withdraw 1.0ml of the saline using a sterile syringe and 21G needle and inject the 1.0ml saline into the ampoule of powdered hCG, by piercing the needle through the rubber cap.

4. Swirl the mixture gently until the powder has dissolved.

5. Invert the ampoule and using the same syringe and needle remove the hormone solution and add it to the remaining 59ml saline.

6. Rinse the ampoule with 1.0ml of the diluted hormone, (using the original syringe and needle). Swirl again to remove any remaining hormone, and return the solution to the large tube.

7. Gently mix the hCG solution by inversion.

8. Dispense the hCG solution into either 1.3ml aliquots (enough for 10 females) stored in sterile microfuge tubes, or 5.2ml aliquots (enough for 50 females) stored in sterile plastic Universal vials.

9. Freeze at -20°C until required.

Preparation of Acid Tyrode's Solution (pH3.0, 3.25 and 3.5).

1. Calibrate the pH meter as described in the S.O.P.

2. Place the probe into the Acid Tyrode's solution (supplied at pH 2.5, Sigma Chemical Co., Cat No. T1788) and wait for the reading to stabilize.

3. Prepare a 0.5M sodium hydroxide solution by adding 5ml embryo tested water to 5ml 1M sodium hydroxide in a universal tube and mix gently.

4. Add the sodium hydroxide solution drop by drop to the Acid Tyrode's solution, swirling the solution between each drop and letting the reading stabilise.

5. Once the Acid Tyrode's solution reaches the required pH (3.0, 3.25 or 3.5, as appropriate) filter the solution and dispense into 1ml aliquots.

6. Store the aliquots at -20°C until required.

M. Cook's IVF medium (used for IVF)

1. Cook's Mouse Vitro Fert IVF medium is purchased in 50ml bottles from Cook Ireland Ltd (Cat No: K-RVFE-50) and is used as supplied.

[We routinely use all 50ml of Cook's IVF medium for one IVF session but surplus can be frozen at -80°C for later use]