

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 ·7H ₂ O	4.9	Sigma	M7774
KH ₂ PO ₄	5.4	Sigma	P5655
CaCl ₂ ·2H ₂ O	75.5	Sigma	C7902
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
NaHCO ₃	210.0	Sigma	S5761
0.5% Phenol Red (ml)*	0.04	Sigma	P0290
BSA (Albumin Bovine Serum, Fraction V, Fatty Acid-Free)	400.0	Merck/Calbiochem	126575

*Indicates volume of reagent

Osmolality: 300-310mOsm/kg

This medium is stored in 8ml 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 CARD MEDIUM® Mouse Fertilization 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 ·7H ₂ O	29.3	Sigma	M7774
KH ₂ PO ₄	16.2	Sigma	P5655
CaCl ₂ ·2H ₂ O	25.1	Sigma	C7902
Na-Pyruvate	5.5	Sigma	P4562
Glucose	100.0	Sigma	G6152
Methyl-β-cyclodextrin	98.3	Sigma	C4555
Penicillin G	7.5	Sigma	P4687
Streptomycin	5.0	Sigma	S1277
NaHCO ₃	210.6	Sigma	S5761
Polyvinylalcohol	100.0	Sigma	P8136

Osmolality: Approx. 283-293mOsm/kg

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® Preincubation Medium.

C. Preparation of sperm cryopreservation agent (qCPA) 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. Warm 40ml embryo tested water contained in a 50ml disposable tube in a 60°C water bath.
2. Add 584mg L-glutamine (Sigma: G8540) to the warmed embryo tested water, vortex for 3 minutes.

3. Place the tube back into 60°C water bath.
4. Add 7200mg raffinose pentahydrate (Sigma: R7630) and 1200mg skimmed milk powder (Becton Dickinson: 232100) to the tube, vortex for 3 minutes;
5. Incubate the solution in a 60°C water bath for 90 minutes. Vortex for 3 minutes after every 30 minutes.
6. Divide the solution into 1.0ml aliquots and transfer to 1.5ml centrifuge tubes.
7. Centrifuge the samples at 10,000g for 60 minutes.
8. Carefully collect 0.7ml supernatant of each sample from the central region of the tube (Fig. 1).

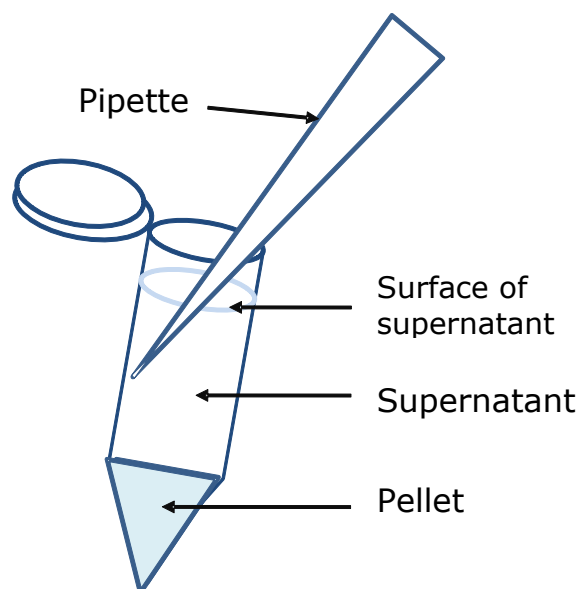


Fig. 1

9. Filter the supernatant using a disposable filter unit (pore size: 0.22 μm), discard the pellets.
10. 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).
11. Osmolality should be tested and be within the range 500-520mOsm/kg

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® Cryoprotectant.

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. 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.
3. Discard the medium after 7 days.

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

1. Accurately weigh 1.14g Propylene glycol (Sigma Chemical Co. Cat No. 134368) into 10ml volumetric flask.
2. Make up the solution to 10ml with M2 (with added Penicillin/Streptomycin) .
3. Gently mix by inversion.
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.
5. Store at 4°C, discard after 7 days.

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

1. Add 3.42g Sucrose (Sigma Chemical Co., Cat No. S9378) to a 10ml volumetric flask containing 5ml of M2 (with added Penicillin/Streptomycin).
2. Mix gently, by inversion, until the sucrose has dissolved.
3. Make up solution to 10ml with M2.
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.
5. Store at 4°C, 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 stock solution of hyaluronidase in M2 (with added penicillin/streptomycin) as follows.
 - 2.1. Label and date 96 x 1.5ml Eppendorf tubes.
 - 2.2. Tap the 30 mg bottle of hyaluronidase gently, to make the powder go to the bottom of the bottle.
 - 2.3. Remove seal from the bottle and remove bung.
 - 2.4. Dispense 3ml of filtered M2 media into the ampoule with a 10ml Gilson pipette
 - 2.5. Replace bung and invert several times to ensure the powder has dissolved.
 - 2.6. Once dissolved, dispense 30µl aliquots of the hyaluronidase solution into 1.5ml Eppendorfs with a P100 Gilson pipette.
 - 2.7. Store at -20°C for 6 months.

3. Prepare a working solution with a final concentration of 30µg/ml, as follows:
 - 3.1. Take one Eppendorf containing 30µl of 10mg/ml hyaluronidase stock solution out of the freezer for every stock to be treated and place in a hot block set at 37°C.
 - 3.2. Dilute with 970µl of M2 media to make a 30µg/ml working solution and leave it in the hot block to warm up for a minimum of 5mins before use.

NOTE: We have found 56µg/ml (made by diluting 30ul of 10mg/ml hyaluronidase in 500µl M2 is also satisfactory.

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)

Calculation:

(strength of 1 vial of PMS divided by strength required), divided by 10 equals vol. of **STERILE WATER** required per vial.
E.g. $(2,000/5)/10 = 40\text{ml}$

Method:

1. Pregnant Mare's Serum Gonadotrophin (PMS) at a concentration of 2000iu.
2. To prepare a working solution of PMS, at 5iu/ml, measure 40ml sterile water for injection into a sterile 50ml centrifuge tube.
3. Withdraw 1.0ml of the sterile water using a sterile syringe and 21G needle and inject the 1.0ml sterile water, 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 sterile water.
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 Eppendorf tubes, or 4ml aliquots (enough for 30 females) stored in sterile Universal tubes.
8. Freeze at -20°C until required. Once frozen, use the working solution within approximately 3 months of preparation.

Preparations for other suppliers:

NHPP (2,000iu)

5iu = 1 vial of PMSG + 40ml sterile water for injection
200ml sterile water for injection + 5 vials of PMSG

Prospec (5000iu)

5iu = 1 vial of PMSG + 100ml sterile water for injection
200ml sterile water for injection + 2 vials of PMSG

K. Preparation of Human Chorionic Gonadotrophin (hCG)

Calculation:

(strength of 1 vial of hCG divided by strength required), divided by 10
equals vol of **STERILE WATER** required per vial.

E.g. $(10,000/5)/10 = 200\text{ml}$

Method:

1. Human Chorionic Gonadotrophin (hCG at a concentration of 10000iu.
2. To prepare a working solution of hCG, at 5iu/ml, measure 200ml sterile water for injection into a sterile 500ml media bottle.
3. Withdraw 1.0ml of the sterile water using a sterile syringe and 21G needle and inject the 1.0ml sterile water 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 sterile water.
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 1.4ml aliquots (enough for 10 females) stored in sterile Eppendorf tubes, or 4ml aliquots (enough for 30 females) stored in sterile plastic Universal tubes.
9. Freeze at -20°C until required.

Preparations for other suppliers:

NHPP (10,000iu)

5iu = 1 vial of hCG + 200ml sterile water for injection
200ml sterile water for injection + 1 vial of hCG

Chorulon (Intervet) (1,500iu)

5iu = 1 vial of hCG + 30ml sterile water for injection
210ml sterile water for injection + 7 vials of hCG

Sigma (2,500iu)

5iu = 1 vial of hCG + 50ml sterile water for injection
200ml sterile water for injection + 4 vials of hCG

L. 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. Preparation of PB1 Solution for Vitrification

Composition of PB1 medium. Osmolality should be 285-295mOsm/Kg.

Reagent Name	mg/100ml	Vendor	Catalogue Number
NaCl	800	Sigma	S-5886
KCl	20	Sigma	P-5405
CaCl ₂	12	Sigma	C-5670
KH ₂ PO ₄	20	Sigma	P-5655
MgCl ₂ 6H ₂ O	10	Sigma	M-2393
Na ₂ HPO ₄	115	Sigma	S 5136
Na-Pyruvate	3.6	Sigma	P-4562
Glucose	100	Sigma	G-6152
Penicillin	7.5	Sigma	P-4687
Streptomycin	5.0	Sigma	S-1277
BSA	300	Sigma	A-4378

1. To prepare PBS (-), dissolve 4.8 g Dulbecco's PBS (-) in embryo transfer water with a final volume of 490 ml. Sterilise the solution by autoclaving (121°C, 15 min.) or filtration (22 µm filter). Store in the refrigerator at 4 °C.
2. To prepare CaCl₂·2H₂O (100x concentration), dissolve 66 mg CaCl₂·2H₂O in 5 ml of embryo transfer water. Sterilise the solution by filtration.
3. To prepare MgCl₂·6H₂O (100x concentration), dissolve 50 mg MgCl₂·6H₂O in 5 ml of embryo transfer water. Sterilise the solution by filtration.

4. To prepare PBS (+), add 5 ml of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution to 490 ml of PBS (-) solution and mix gently.
5. Add 5 ml of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ solution and mix gently. Store in the refrigerator
6. To prepare PB1 (BSA free), add the following chemicals to **100ml PBS (+)** solution described above:

Na Pyruvate	3.6 mg/100ml	Sigma (#P-8574)
Penicillin G	6.4 mg/100ml	Sigma (#P-4687)
Glucose	100 mg/100ml	Wako (#041-00595)

7. To prepare PB1 medium, add 300 mg of BSA (Bovine Serum Albumin) to 100ml of PB1 (BSA free), let the solution sit until the BSA has dissolved, do not mix.
8. Sterilize the solution by filtration and store in the refrigerator. This solution has a shelf life up to 1 month at 4°C.

N. 1M DMSO Solution for Vitrification

1. Prepare the 1M DMSO solution by adding 1.56ml DMSO into 18.44ml of PB1 medium.
2. Filter the solution through a 0.22µm syringe end filter. This solution has a shelf life up to 6 months at 4°C.

O. 0.25M Sucrose Solution for Vitrification

1. Prepare the 0.25M sucrose solution by adding 1.711g of sucrose to 10ml of PB1 medium.
2. Make up the solution to 20ml and mix gently.
3. Filter the solution through a 0.22µm syringe end filter. This solution has a shelf life up to 6 months at 4°C.

P. DAP213 Solution for Vitrification

Composition of DAP213 vitrification solution

Solution A

Reagent Name	ml/10ml	Vendor	Catalogue Number
PB1	2.31	-	-
DMSO	3.13	Sigma	D-2650
Propylene glycol	4.56	Sigma	P-1009

Solution B

Reagent Name	mg/10ml	Vendor	Catalogue Number
Acetamide	1181.4	Sigma	A-0500

1. To prepare 25ml of solution A, measure out 5ml of PB1 solution and add 7.1ml 4M DMSO and mix. Then add 10.975ml of 6N Propylene Glycol and mix. Make up the solution to 25ml with PB1.
2. To prepare 25ml of solution B, measure out 15ml of PB1 solution and add 2.95g Acetamide. Mix gently by stirring and make up the solution to 25ml with PB1.
3. Add equal amounts of each solution to a media bottle and mix by inversion.
4. Aliquot out 1ml in 1.5ml Eppendorf tubes and centrifuge at 10,000rpm for 10 mins.
5. Take off 900µl of the supernatant and discard the tube. Filter the solution through a 0.22µm syringe end filter. This solution has a shelf life up to 6 months at 4°C.

This media can be purchased commercially from Cosmo Bio Co., Ltd (www.cosmobio.com).

Q. Supplementation of Lifor with Sphingosine Monophosphate (S1P)

1. The Lifor solution is to be made in a Laminar Air Flow (LAF) cabinet.
2. Measure out 2.638ml of methanol and add to a 14ml falcon tube.
3. Take one 1ml of methanol from the falcon tube and add to a 1mg vial of S1P, mix and add contents to the rest of the methanol in the falcon tube giving the solution a concentration of 0.379mg/ml.
4. Rinse the vial 2-3 times with the solution from the falcon tube.
5. Measure out 200ml of Lifor Preservation Solution, and using a 1ml Gilson pipette discard 2ml and place the rest into a 250ml media bottle.
6. Add 2ml of S1P + methanol solution and mix by inversion. Ensure the solution is mixed well.
7. Aliquot 1.2ml into autoclaved Eppendorfs, label with 'Lifor + S1P', date, and store at -80°C . The Lifor solution + S1P solution, is not sterilised by filtration therefore it is essential that the solution is aliquoted into autoclaved 1.5ml Eppendorfs.
8. The solution has a shelf life of 12 months in the -80°C freezer.