Phosphate buffered saline (modified Dulbecco's)

For a final volume of 100 ml of solution weigh out the following into approximately 75 ml of culture grade water:

NaCl	800.0 mg
KCl	20.0
KH_2PO4	20.0
$MgCl_2.6H_2O$	10.0
Dextrose	100.0
Na_2HPO_4	115.0
Sodium pyruvate	3.6
Penicillin G	7.5
Streptomycin sulfate	5.0

1. Dissolve 10.0 mg $CaCl_2$ (anhydrous) in about 10 to 20 ml of culture grade water.
2. Add the dissolved $CaCl_2$ to the above ingredients and bring the total volume up to 100 ml.
3. Use 20 ml of the above solution for making up a 2 M solution of DMSO (Aldrich).
4. Add 240.0 mg of crystallized Bovine Serum Albumin (BSA) and 0.1 ml of a 1% solution of Phenol red to the remaining 80 ml.
5. Sterilize the solutions by filtration.

Return to Page One

HTF

Add each chemical in order and mix carefully.

Gas for 5 minutes with 5% CO2, 5% O2 balanced with N2, using a 1 ml pipette submerged in the media before adding BSA. Add BSA after gassing and then incubate to dissolve. Filter with .22um filter and gas before refrigerating.

		1000 ml	**200 ml**
NaCl	(FW 58.44, Sigma S-5886)	5.9375	1.1875
KCl	(FW 74.55,Sigma P-5405)	0.3496	0.06992
$MgSO_4$ $7H_20$	(FW 246.5, Sigma M-1880)	0.0492	0.00986
KH_2PO4	(FW136.09, Sigma P5655)	0.0504	0.01008
$CaCl_2$ $2H_2O$	(FW 147, Sigma C-7902	0.3	0.06
(add directly)			
NaHCO3	(FW 84.01, Sigma S-5761)	2.1	0.42
Glucose	(FW180.16, Sigma G-6152)	0.5	0.1
Na-pyruvate	(FW110.0, Sigma P-4562)	0.0365	0.0073
Na-lactate	(FW 112.1, Sigma L-1375)	3.42 ml	0.683 ml
Penicillin	(FW 372.5, Sigma P-7794)	0.075	0.015
Streptomycin	(FW1457.4, Sigma S-9137)	0.05	0.01
Phenolred (1%)	(Sigma P-0290)	0.20ml	0.04
*BSA	(Equitech-Bio BAC62-0050	4.0	0.8

Always gas the medium after every use.

Reference

Quinn P, Kerin JF and Warnes GM (1985) Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertility and Sterility 44: 493-498.

Return to Page One

KSOM medium

For a final volume of 100 ml of medium weigh out the following into approximately 75 ml of double distilled water:

EDTA, disodium salt **(ADD FIRST)**	(FW 372.2, Sigma)	0.38 mg
NaCl	(FW 58.44, Fisher)	559.5 mg
KCl	(Sigma)	18.5 mg
KH_2PO_4	(FW 136.09, Sigma)	4.75 mg
$MgSO4.7H_20$	(Sigma)	4.95 mg
DL-Lactic Acid, sodium salt	(MW 112.1, Sigma L-1375)	0.174 ml
D-Glucose	(FW 180.16, Sigma G-6152)	3.60 mg
$NaHCO_3$	(FW84.01, Sigma)	210.0 mg
L-Glutamine	(MW 146.1, Sigma G-5763)	14.5 mg
Pyruvic Acid, sodium salt	(FW 110.0, Sigma P-4562)	2.2 mg
Penicillin G	(FW 372.5, Sigma P-7794)	6.3 mg
Streptomycin Sulfate	(FW1457.4, Sigma S-9137)	5.0 mg
MEM Essential Amino Acids	(GibcoBRL 11130-051	1.0 ml
MEM Non-essential AA	(Sigma M-7145)	0.5 ml

1. Dissolve 25 mg $CaCl_2$ (anhydrous) in about 25 ml of double distilled water.
2. Add the dissolved $CaCl_2$ to the above ingredients and bring the total volume up to 100 ml.
3. Add 0.1 ml of a 1% solution of Phenol red to the solution.
4. Gas the solution using a pipette immersed in the solution with 5% CO_2, 5% O2, Balance N_2, for 10 minutes (should be a salmon color).
5. Add 100 mg of crystallized Bovine Serum Albumin (BSA).
6. Sterilize the solution by filtration, and gas before storage (do not immerse the pipette).

References

Lawitts, J.A. Biggers, J.D. (1991). Optimization of mouse embryo culture media using simplex methods. J Reprod Fert 91:543-556.

Lawitts, J.A. Biggers, J.D. (1992). Joint effects of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media. Mol Reprod Dev 31:189-194.

Lawitts, J.A. Biggers, J.D. Culture of preimplantation embryos. In Methods in Enzymology: Guide to Techniques in Mouse Development. P.M. Wasserman and M.L. DePomphilis (eds.) 225:153-164. Academic Press. 1993

Erbach, G.T. Lawitts, J.A. Papaioannou, V.E. Biggers, J.D. (1994).Differential growth of the mouse preimplantation embryo in chemically defined media. Biol. Reprod. 50:1027-1033.

M2 media preparation

M2 medium is conveniently made from stock solutions.

Return to Page One

M2 stock solutions

Stock A 10X concentration (expires in 3 months)

Stock B 10X concentration (expires in 2 weeks)

Stock C 100X concentration (expires in 2 weeks)

Stock D100X concentration (expires in 3 months)

Stock E 100X concentration (expires in 3 months)

Preparing M2 stock solutions:

Prepare stocks A, B, C, and D

1. Zero scale with the beaker on it.
2. Add approximately one half of the distilled water necessary for the stock solution.
3. Add the proper amounts of each component stirring occasionally.
4. Add distilled water until scale reads desired grams. (example: Stock A = 200 g, Stock B = 20 g)
5. Filter through a 150 or 250 ml filter system depending on the amount of stock solution made.

Prepare stock E

1. Repeat steps 1-3 from above.
2. Adjust the pH to 7.4 with 0.2 N NaOH.
3. Add distilled water until scale reads desired grams.
4. Filter through a 250ml Filter System.

Preparing M2 from stock solutions

1. Zero scale with the beaker on it.
2. Add the proper amount of each stock solution starting with Stock A and ending at the distilled water. Scale will read 200 g.
3. Add 0.8 grams of Bovine Albumin and let sit until dissolved.
4. Adjust pH to 7.2 – 7.4 if necessary, using 0.2 N NaOH.
5. Filter sterilize with a 22 um filter
6. Measure the osmolality of the medium. It will range between 265 and 280.

Return to Page One

M2

per 200 ml

Stock A(x10)	20 ml
Stock B(x10)	3.2 ml
Stock C(x100)	2 ml
Stock D(x100)	2 ml
Stock E(x10)	16.8
Distilled H2O	156 ml
BSA	0.8 g

*Store stock solutions and M2 in the refrigerator at 4 degrees C.

1 Molar sucrose in M2

Used as an osmotic buffer for embryo cryopreservation.

1. Measure 10ml of M2.
2. Add 3.42 g of sucrose.
3. Mix by gently rocking media until sucrose is completely dissolved.

Sucrose Sigma Cat. # S-1888

Miscellaneous supplies & equipment

Component	Sigma cat. #	Size ordered	g/200 mL
NaCl	S-5886	500g	11.068
KCl	P-5405	250g	0.712
KH_2PO_4	P-5655	100g	0.324
$MgSO_4*7H_2O$	M-1880	500g	0.586
Sodium Lactate	L-7900	100g	8.698
Glucose	G-6152	100g	2
Penicillin	P-4687	10,000,000 u	0.12
Streptomycin	S-1277	5g	0.1

Component	g/20 mL		
$NaHCO_3$	S-5761	500g	0.4202
Phenol red	P-5530	5g	0.002

Component	g/10 mL		
Sodium Pyruvate	P-4562	25g	0.036

Component	g/50 mL		
CaCl2*2H2O	C-7902	500g	1.26

Return to Page One

Component		g/250 mL	
HEPES	H-6147	100g	14.895
Phenol red			0.025
Albumin, bovine	A-2153	50g	0.8

Fisher Cat.#			
150 ml filter system	SCGP U01 RE	12 pack	.22 micrometers
250 ml filter system	SCGP U02 RE	12 pack	.22 micrometers

Return to Page One

Propylene glycol

1. Measure 8.8 ml of M2.
2. Add 1.2 ml of 1,2-Propanediol.
    a. 1,2-Propanediol has a high viscosity.
    b. Make sure that all excess Propanediol is removed from the outside of the pipette.
    c. After adding the initial amount of Propanediol, allow it to collect at the end of pipette before expelling the remaining Propanediol into the solution.
3. Mix by gently rocking media.

Component	Sigma cat. #
1,2-Propanediol	P-1009

Store at 4°C, discard after 1 week.

Return to Page One

MEM medium

For a final volume of 200 ml of medium, measure out the following into 174 ml double distilled water:

10X EBSS (see below)	20 ml
E. Amino Acid 50X	4 ml
100X Vitamins	2 ml
Na Pyruvate	5 mg
K Penn	15 mg
Strep	10 mg
L-Glutamine	58.4 mg
$NaHCO_3$	0.44 g
EDTA	0.76 mg

1. Add 0.2 ml of 1% Phenol red solution.
2. Gas for ten minutes with 5% CO_2, 5% O_2, balance N_2, using a pipette immersed in the solution.
3. Add 600 mg crystallized Bovine Serum Albumin (BSA), and allow to dissolve.
4. Sterilize by filtration, and re-gas before storage.

Return to Page One

10X EBSS

200 ml

$CaCl_2$ 2 H_2O	(Sigma C-7902)	0.53 g
KCl	(Sigma)	0.80 g
$MgSO_4$ 7 H_2O	(Sigma)	0.40 g
NaCl	(Fisher)	13.60 g
NaH_2PO_4	(Sigma S-5011)	0.25 g
D-Glucose	(Sigma G-6152)	2.0 g

Calcium dihydrate should be dissolved first in a separate container with Millipore H^2O .

**Add the $CaCl_2$ last after all the other chemicals have dissolved.

Return to Page One

Avertin

Components:

- 2, 2, 2 Tribromoethanol
- Tertiary amyl alcohol

Stock solution:

25 g Tribromoethanol in 15.5 ml amyl alcohol.

Store in dark (wrap bottle in foil).

Solution for injection:

1 ml of stock solution to 50 ml of isotonic saline.

Warm to 40°C, shake well, then store in refrigerator.

Dose:

0.015 ml per gram body weight or 0.4 ml per average adult mouse.

Sperm cryoprotectant

- (D+) raffinose pentahydrate (Sigma R-0250) 18%
- Skim Milk Dehydrated (Difco* 0032-17-3) 3%
- Culture grade water
- *(Difco was acquired by Becton-Dickinson)

Here are two recipes for making the cryoprotectant (CPA). They differ in how clear the solution will be before filtering; we can detect no difference in functionality.

Version 1

1. Warm to dissolve the raffinose completely and then dissolve the dehydrated skim milk.
2. Centrifuge at 13,000 x g for 15 minutes
3. Filter the supernatant through a 0.22 micron pore size filter.
4. Aliquot and store frozen.

Version 2

1. Dissolve 18 g dry milk in ultrapure water with a final volume of 300 ml. Dissolve well. The final concentration will be 6%.
2. Centrifuge 1 hour, 12,000 rpm (18,500 x g), 4 deg C.
3. Carefully decant supernatant so that none of the pellet is taken. Using a pipette is a good way to do this. Pouring off the supernatant is not a good method (unless you have very steady hands) because the pellet is not packed very tightly.

Return to Page One

4. Take exactly 200 ml of the supernatant. Add 125 ml water and 72 g raffinose. Add water to 400 ml. Stir at room temperature until the raffinose dissolves.
5. Filter through a 0.2 micron filter. (If you've been sloppy about your decanting step, you might need to pre-filter through a 0.45 micron filter to prevent clogging at this step.)
6. Aliquot using sterile technique and freeze.

Notes:

- The second recipe makes a clearer CPA because centrifuging in the absence of raffinose allows the milk solids to pellet through a much less dense solution. In other words, the first recipe is essentially a density centrifugation.
- You can make the CPA even clearer by centrifuging longer and/or at higher speeds.
- Filtering the CPA is easier with a luer-type cartridge that fits on the end of a syringe than with a vacuum unit, because you can generate higher pressures. However, the second recipe results in a CPA that is clear enough to use with a vacuum unit.
- Remember that CPA is essentially milk and sugar and as such will be a good medium for any bacterial contaminants that you might introduce. Discard any unused solution.

Reference:

Nakagata, N. (1996). Use of cryopreservation techniques of embryos and spermatozoa for production of transgenic (Tg) mice and for maintenance of Tg mouse lines. Lab-Anim-Sci 46(2): 236-8.

Return to Page One

Gonadotropins

PMSG (pregnant mare serum gonadotropin) is available from Sigma Chemical Company

It is available in 10,000 I.U. vials Cat. #G-4877. Working solutions are made up by adding 400 ml of isotonic saline to a 10,000 I.U. vial of powdered hormone, which is then stirred with a magnetic stirrer for 5-6 minutes. This solution can then be packaged into 12 x 75 mm disposable plastic tubes with caps (we use BIORAD tubes as these are available in colors for coding purposes - PMS and HCG can be kept in different color tubes to avoid mix up) and frozen until needed. Frozen preparations are not kept for longer than three months.

HCG (human chorionic gonadotropin) is also available from Sigma Chemical Company

It is also available in 10,000 I.U. vials Cat. #CG-10. It is diluted in the same way as PMS described above.

For a working solution inject the 10 ml of diluent supplied into the vial of powdered hormone (10,000 I.U.) with a syringe and needle, withdraw the mixed solution and add to 390 ml of isotonic saline. This solution can then be packaged into 12 x 75 mm disposable plastic tubes with caps as for the PMS, but in different color tubes if possible.

These dilutions will yield 2.5 I.U. of hormone per 0.1 ml.

Return to Page One