Mouse In Vitro Fertilization

Mutant Mouse Resource and Research Center

University of Missouri

4011 Discovery Drive, Columbia, MO, 65201, USA

Mouse In Vitro Fertilization

Supplies:

- Fine forceps
- Scissors
- Curved Serrated Forceps
- **mHTF** Fertilization Medium
- **TYH+MBCD** Mouse Sperm Preincubation Medium
- Equilibrated mineral oil
- Embryo culture media plates with KSOM drops
- 28 G needles
- 35 mm Petri dishes
- 15 ml conical tube
- 1.5 ml Eppendorf tubes

Procedures:

Prepare Sperm (TYH+MBCD) and IVF (mHTF) Dishes

TYH+MBCD Dishes

1. Pipette a 100 µL drop of TYH+MBCD media in a 35 mm dish and cover with equilibrated mineral oil.
2. Allow to equilibrate in an incubator for at least 30 minutes prior to adding sperm.

   Note: TYH+MBCD dishes can be made the day before IVF and placed in incubator overnight.

mHTF IVF dishes

1. Pipette a single 85 µL drop of mHTF media and 5 µL GSH stock.
2. Cover with equilibrated mineral oil and allow to equilibrate in an incubator for at least 30 minutes prior to use.

   Note: mHTF dishes can be made the day before IVF and placed in incubator overnight without GSH. GSH can be added 30 minutes prior to IVF the next morning.

Sperm Preparation

Frozen

1. Remove straw from liquid nitrogen and place in a 37°C water bath for 10 minutes.
2. Place 1 mL of pre-warmed (37°C) TYH+MBCD media into 1.5 mL microcentrifuge tube.
3. Remove straw from the water bath and dry the outside with a Kimwipe.
4. Cut the end of the straw opposite the weight and label, then insert into 1.5 mL tube and cut the other end.
5. Attach the syringe with straw adapter to the straw to gently expel remaining sperm.
6. Centrifuge the tube for five minutes at 300 rcf.
7. Remove the supernatant to leave approximately 100 µL in the tube.
8. Slowly add the entire contents (~100 µL) of the Eppendorf tube to the TYH+MBCD dish.
9. Observe sperm under microscope to check for motility and place the dish in the incubator for a minimum of 30 minutes.

Fresh

1. Put 3 or 4 ml equilibrated mineral oil into a 35 mm dish.
2. Euthanize one male mouse.
3. Dissect out both cauda epididymides, carefully removing any blood and attached fat. Place directly into the oil.
4. Under a dissecting microscope, use fine tweezers to place a cauda epididymis in the oil portion of the TYH+MBCD medium dish and tear it using a 28 G needle. Holding the cauda with one set of fine tweezers, gently squeeze sperm out of the cauda and use the 28 G needle to gently drag the sperm into the media.
5. Observe sperm under microscope to check for motility and place the dish in the incubator for a minimum of 30 minutes.

Oocyte Collection and Fertilization

1. Per the *Mouse Embryo Collection and Culture SOP*, superovulate females.
2. Put 3 to 4 mL equilibrated mineral oil into a 35 mm dish.
3. Euthanize super ovulated females, dissect out oviducts, and place directly in the oil.

   Note: Cervical Dislocation is PREFERRED primary euthanasia method for oocyte donors for optimal oocyte quality and highest fertilization rate.

4. Under a dissecting microscope, use fine tweezers to place a tract in the oil portion of the mHTF medium dish and tear the ampulla using a 28 G needle. Gently drag the cumulus oocyte complex (COC) to the media.
5. Add approximately 1-4 COCs per plate.
6. Slowly add 10-20 µL of frozen sperm or 1-10 µL of fresh sperm to the appropriate mHTF medium drops containing the COCs.
7. Incubate at 37°C & 5% CO_2 for approximately 5-6 hours.
8. Using a mouth or hand micro pipette remove the embryos from the mHTF and move to a KSOM dish.
9. Wash through 2-3 drops of KSOM and place in incubator overnight.
10. The next day, evaluate the number of two cells and remove any fragmented, dead, and remaining one cells. Viable two cells can be cultured further in fresh KSOM drops for cryopreservation or transferred into pseudopregnant recipients.

Composition of high calcium HTF medium (mHTF)

To a 1000 mL glass beaker, add ~800 mL embryo water and then add the followings as detailed below.

Cat. No#	Reagent	g/1000 ml	Amount added	Lot number
Sigma S5886	NaCl	5.938		
Sigma P5405	KCl	0.3496		
Sigma M7774	MgSO4·7H2O	0.049		
Sigma P5655	KH2PO4	0.054		
Sigma C7902	$CaCl_2$-$2H_2O$*	0.756		
Sigma S5761	NaHCO3	2.100		
Sigma G6152	D-Glucose	0.500		
Sigma L7900	Sodium Lactate (60% syrup)	3.42 (ml)		
Sigma P4562	Sodium Pyruvate	0.0365		
Sigma P7794	Penicillin G Potassium Salt**	0.0075		
Sigma S1277	Streptomycin Sulfate**	0.0050		
Sigma A3310	BSA	4		

*Solubilize the $CaCl_2.2H_2O$ separately from the other reagents before combining the solutions.

** Do not add at this time for longer shelf life at 4°C. Add to working aliquots prior to use using Pen/Strep stock (1000X).

Initial pH_____Gassing with 5% CO_2_____ pH after 30 min gassing_____.

Bring to volume using a volumetric flask.

Osmolality should be between 280-295 (slightly higher is acceptable). Filter the medium using 0.22 µm filter and aliquot into 25 mL in 25 mL Eppendorf tubes. Seal the cap with paraffin film and stored the aliquots at 2-8°C for 12 months.

For working solution, add 25 ul penicillin/streptomycin stock solution (1000x) to 25 mL antibiotic free mHTF.

Composition of TYH+MBCD preincubation medium

To a 500 mL glass beaker, add ~400 mL embryo water and then add the followings as detailed below.

Component (MW)	**Vendor Cat. #**	mM	g/500 mL	Amount added	Lot #
PVA*	Sigma P8136	1 mg/ml	0.5		
NaCl (58.45)	Sigma S5886, S7653	119.37	3.488		
KCl (74.56)	Sigma P5405, P9333	4.78	0.178		
KH2PO4 (136.09)	Sigma P5655	1.19	0.081		
MgSO4·7H20 (246.5)	Sigma M5921, M7774	1.19	0.1465		
NaHCO3 (84.01)	Sigma S5761	25.07	1.053		
Glucose (180.16)	Sigma G6152	5.56	0.5		
CaCl2·2H2O (147)**	Sigma C7902	1.71	0.1255		
Na Pyruvate (110.0)	Sigma P4562	1	0.0275		
Penicillin G (Na salt) (372.5)***	Sigma P7794	0.075 g/L	0.0375		
Streptomycin sulfate (1457.4)***	Sigma S9137	0.0500 g/L	0.025		
MBCD (methyl-β-cyclodextrin, 1320)	Sigma C4555	0.75	0.5		

*Add first and wait for complete dissolving before adding other chemicals. Heating as needed to increase solubility.

**Solubilize the $CaCl_2.2H_2O$ separately from the other reagents before combining the solutions.

*** Do not add at this time for longer shelf life at 4°C. Add to working aliquots prior to use using Pen/Strep stock (1000X).

Initial_____ Gassing with 5% CO_2_____ pH after gassing_____

Bring to volume using a volumetric flask.

Osmolality (280-295 mOsm)_____

Media should then be filtered using 0.22 um filter and aliquoted into 10 mL, stored at 4°C refrigerator.

For working solution, add 10 ul penicillin/streptomycin stock solution (1000x) to 10 mL antibiotic free TYH+MBCD.

Reduced Glutathione (GSH) Stock

To a 15 mL tube add 10 mL high calcium HTF Medium and then add the following as detailed below.

Cat. No#	Reagent	g/10 ml	Amount added	Lot number
Sigma G4251	Reduced glutathione (GSH)			

Once the GSH is dissolved, aliquot 100 microliters of the solution into 0.5 mL Eppendorf tubes and store at -80°C for up to six months.

Penicillin/Streptomycin Stock Solution (1000X)

To a 50 ml conical tube, add 10 ml water for cell culture applications (Lonza 17-724Q) and then add components as detailed below.

Antibiotics	1X (1000 ml)	1X (10 ml)	**1000 X (10 ml)**
Penicillin G Potassium salt	0.0596	0.000596	**0.596**
Streptomycin Sulfate	0.0124	0.000124	**0.124**

After the components completely dissolved into solution, bring the stock to a laminar flow hood. With gloves on, sterile filter the stock using a 0.20 µm filter.

Aliquot the stock solution into appropriate volume (e.g. 0.5 mL) into sterile Eppendorf centrifuge tubes and store at -80°C for up to 12 months.

References

1. Takeo T, Nakagata N. Reduced glutathione enhances fertility of frozen/thawed C57BL/6 mouse sperm after exposure to methyl-beta-cyclodextrin. Biol Reprod. 2011 Nov; 85(5):1066-72.
2. Takeo T, Nakagata N. Combination medium of cryoprotective agents containing L-glutamine and methyl-{beta}-cyclodextrin in a preincubation medium yields a high fertilization rate for cryopreserved C57BL/6J mouse sperm. Lab Anim. 2010 Apr; 44(2):132-7.
3. Takeo T, Hoshii T, Kondo Y, Toyodome H, Arima H, Yamamura K, Irie T, Nakagata N. Methyl-beta-cyclodextrin improves fertilizing ability of C57BL/6 mouse sperm after freezing and thawing by facilitating cholesterol efflux from the cells. Biol Reprod. 2008 Mar; 78(3):546-51.
4. Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 1985; 44: 493-498.