

PLEASE READ: Since high concentration of cryoprotective agents impair both sperm capacitation and fertilization and fertilization relies on the presence of appropriate amount of sperm with good motility, cryopreserved sperm with different volumes should be recovered by using different IVF methods. It is recommended to use our SOP for “**Mouse Rescue IVF Using 70-100µL of Cryopreserved Sperm**” (pages 1-6) when using a sperm sample with volume > 50 µl (for example 70-100 µL), and use our SOP for “**Mouse IVF Using 40-50µL of Cryopreserved Sperm**” (pages 7-11) when using a sperm sample with volume 30-50 µl. If using a small volume (10 µl) sperm sample in a cryostraw, it is recommended to use our SOP for “**Mouse IVF Using Small Volume of Cryopreserved Sperm (10µL)**” (pages 12-17).

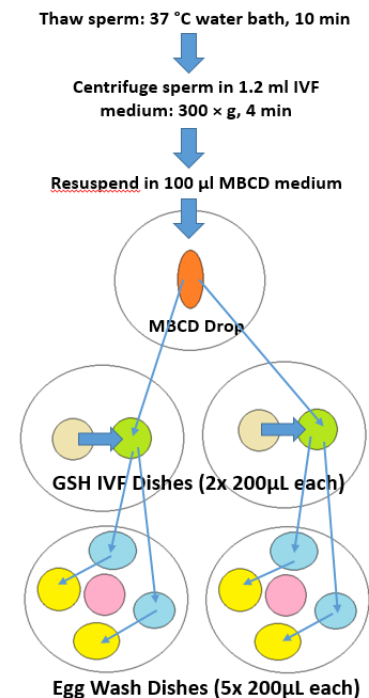
Mouse Rescue IVF Using 70-100µL of Cryopreserved Sperm

This protocol is for IVF using a cryovial containing 70-100 µL of cryopreserved sperm. Oocytes are collected and pre-incubated in IVF medium containing GSH for 30-70 min before insemination. Thawed sperm are washed and concentrated by centrifugation and resuspension, and then pre-incubated in MBCD medium for 30-40 min before insemination.

A. Materials

1. **CO₂ incubator:** 5.5% CO₂ in humidified air, 37 °C, and >90% relative humidity. Check temperature and CO₂ level daily using a thermometer and a Fyrite CO₂ analyzer, and calibrate when temperature is not 37 °C or CO₂ not within 5.0-6.0%. Change water biweekly and clean incubator when needed.
2. **High calcium medium:** Research Vitro Fert medium (RVF, Cook #K-RVFE) with 5.14 mM Ca **or** Mouse HTF medium (Fisher Scientific # MR070D). See **Appendix 1** for preparing RVF medium with high calcium (5.14 mM) and see **Appendix 3** for Mouse HTF formula (5.14 mM calcium).
3. **MBCD medium:** See **Appendix 4** for MBCD medium formula.
4. **Mineral oil** (for example, Fisher Scientific O1211)
5. **GSH** (L-glutathione reduced, Sigma G6013). See **Appendix 2** for preparing 100 mM GSH.
6. **Petri dishes:** Falcon 35x10mm (Fisher Scientific 08757100A) and 60x15mm (Fisher Scientific 08757100B)
7. **Superovulated mice:** ~15 mice, 3-4 weeks old. Light cycle 7am on and 9pm off. Inject each female with 5 IU of PMSG around 8pm, and 47-48 hours later inject 5 IU of HCG by IP injection. Oocytes are collected 14-16 hours post HCG injection. If using 6 weeks or older mice, inject 7.5 IU PMSG and 7.5 IU HCG each mouse.
8. **Digital microcentrifuge**

Note: IVF Kit (including RVF with 5.14 mM calcium or Mouse HTF medium, MBCD medium and GSH) is available for an additional charge. One kit is enough for two IVF procedures.



B. Prepare media and dishes for pre-equilibration in CO₂ Incubator (overnight or at least 1 hour)

1. **MBCD medium:** 0.5 or 1 ml in a sterile tube with cap loosely closed for resuspending sperm after centrifugation. Note: Mix MBCD medium after thaw by gentle inversions.
2. **Sperm washing medium:** 1.5 ml high calcium RVF or Mouse HTF in a sterile 5ml Falcon tube with cap loosely closed.
3. **Egg collection dishes:** For every group of 5 to 8 females, prepare a 35mm Falcon dish with ~ 3 ml RVF medium (high or regular Ca concentration) or Mouse HTF medium.
4. **High calcium RVF or Mouse HTF medium for GSH IVF dishes:** Add 2.5 ml medium to a 5ml sterile Falcon tube, cap the tube loosely, and stand on a rack inside CO₂ incubator.
5. **Mineral oil:** Add 40-50 ml mineral oil to a sterile 50ml Falcon tube, cap the tube loosely, and stand on a rack inside CO₂ incubator.

C. Prepare dishes first thing on the IVF morning

1. **GSH IVF dishes:**
 - 1) Thaw an aliquot of 100 mM GSH solution and mix.
 - 2) Add 20 µl GSH to the 2.5 ml pre-equilibrated high calcium (5.14 mM) IVF medium, and invert the tube gently a few times to mix.
 - 3) Prepare 2 or 3 Falcon 60x15mm dishes with 2x 200µl drops of GSH IVF medium each dish covered with pre-equilibrated mineral oil, and place in CO₂ incubator.
2. **Egg wash dishes:** For each GSH IVF dish, prepare a 60mm Falcon dish with 5x 200µl drops of high calcium RVF or HTF medium (**without GSH**) covered with mineral oil. *Note: Egg wash dishes can be prepared after insemination.*

D. Harvest eggs and incubate in GSH IVF medium

1. After preparing the GSH IVF dishes, retrieve a sperm sample from liquid nitrogen storage and bring to the water bath at 37 °C using a small dewar containing liquid nitrogen. **Do not thaw sperm now.**
2. Between 9am and 10am, start to sacrifice the first group of superovulated females (5-8 mice) by CO₂ inhalation and/or cervical dislocation. *Note: Sterilize surgical tools in advance.*
3. Disinfect the abdominal skin of each animal with 70% alcohol, and wipe away extra alcohol.
4. Pull or cut the abdominal skin open for each mouse, and pull apart to expose the body wall.
5. Lift up the body wall of a mouse using forceps and cut open using scissors, and then carefully move the digestive tract from inside the abdomen to expose the uterus, oviducts, and ovaries. *Note: Cut skin using one set of surgical tools and manipulate/cut body wall and tissues using another set of surgical tools; do not mix them.*
6. Dissect out both oviducts from the mouse and place in a pre-equilibrated egg collection dish. *Note: Do not take out the dish from CO₂ incubator until the group of females are skin-disinfected and cut/pulled open to minimize pH and temperature changes. Keep the egg collection dish on warm stage (37 °C) when outside of CO₂ incubator.*

7. Repeat the above steps 5 and 6 **quickly** to dissect out oviducts from the remaining mice of the group.
8. Under a dissecting microscope with warm stage, **quickly** collect egg clutch (cumulus-oocyte-complexes, COCs) from each oviduct by using forceps to hold down the oviduct against the dish, and a needle to tear open the ampulla to release the egg clutch. Remove the tissue from dish after its egg clutch is released.
9. After collecting egg clutches from all oviducts in the dish, **immediately** transfer them into the first GSH IVF drop in an IVF dish with minimum medium using a wide-bore pipette tip, and mix. Then transfer the egg clutches into the second GSH IVF drop.
10. Return the GSH IVF dish into CO₂ incubator.
11. Repeat the above steps 2-10 to collect eggs from the remaining group(s) of superovulated females.
12. Incubate the COCs for 30-70 min before insemination.

E. Thaw, centrifuge and incubate sperm

➤ **If using a cryovial sperm sample:**

- 1) After egg collection, **immediately** remove the cryovial of sperm from liquid nitrogen, loosen its cap, pour out any liquid, then put the cap back on the vial and stand on a floating rack in water bath at 37 °C to thaw sperm for 10 min. *Note: Sperm can start to be thawed before sacrificing the second or third group of females (egg donors).*
- 2) While thawing sperm, pipette 1.2 ml pre-equilibrated high Ca RVF or Mouse HTF medium into a sterile 1.5ml microcentrifuge tube (the same type as the balance microcentrifuge tubes), and close the tube.
- 3) After thawing sperm, remove the cryovial from water bath, and wipe dry. Mix the thawed sperm by gently swirling once or twice.
- 4) Transfer all of the sperm from the cryovial into the microcentrifuge tube containing 1.2 ml high Ca RVF or Mouse HTF slowly and carefully using a **wide-bore** pipette tip. Cap the tube and gently invert 1 or 2 times to mix the sperm.
- 5) Very carefully select or prepare a balance microcentrifuge tube of the same type containing equal volume of water, and load the microcentrifuge rotor symmetrically with the sperm tube and the balance tube.
- 6) Centrifuge the sperm at 300x g for 4 min.
- 7) Identify the sperm pellet at the tube bottom, and very carefully remove as much of the supernatant as possible using a 1000µl pipette tip and then a 200µl tip. Do not let pipette tip touch the sperm pellet!
- 8) Slowly add 100 µl of pre-equilibrated warm MBCD medium to the tube, and very gently resuspend the sperm pellet by pipetting a few times using a wide-bore pipette tip. Do not let pipette tip touch the sperm pellet!
- 9) Using the wide-bore pipette tip prepare a long-flat drop of sperm suspension in a 60mm Falcon dish and cover with pre-equilibrated mineral oil. *Note: Use all of the resuspended sperm for the MBCD drop.*

10) Incubate sperm for 30-40 min before insemination.

➤ **If using a cryostraw sperm sample:**

- 1) After egg collection, **immediately** thaw the sperm straw by removing it from liquid nitrogen, holding in the air for 5 seconds, and placing in a 50ml Falcon tube filled with 37 °C DH₂O in a water bath for 10 min. *Note: Sperm can start to be thawed before sacrificing the second or third group of females (egg donors).*
- 2) While thawing sperm, pipette 1.2 ml pre-equilibrated high Ca RVF or Mouse HTF medium into a sterile 1.5ml microcentrifuge tube (the same type as the balance microcentrifuge tubes), and close the tube.
- 3) After thawing the sperm, remove the straw from water bath, and wipe it dry.
- 4) Disinfect the straw with a Kimwipe sprayed with 70% ethanol, and wipe it dry completely.
- 5) Hold the straw horizontally and firmly, and carefully and gently cut off the heat seals at both ends using a pair of 70% ethanol-disinfected scissors (disinfect the scissors in advance and wipe dry completely).
- 6) Carefully and slowly push the straw plug using a metal rod or similar device to expel all sperm suspension directly into the microcentrifuge tube containing 1.2 ml IVF medium. Cap the tube and gently invert 1 or 2 times to mix the sperm.
- 7) Very carefully select or prepare a balance microcentrifuge tube of the same type containing equal volume of water, and load the microcentrifuge rotor symmetrically with the sperm tube and the balance tube.
- 8) Centrifuge the sperm at 300x g for 4 min.
- 11) Identify the sperm pellet at the tube bottom, and very carefully remove as much of the supernatant as possible using a 1000µl pipette tip and then a 200µl tip. Do not let pipette tip touch the sperm pellet!
- 12) Slowly add 100 µl of pre-equilibrated warm MBCD medium to the tube, and very gently resuspend the sperm pellet by pipetting a few times using a wide-bore pipette tip. Do not let pipette tip touch the sperm pellet!
- 13) Using the wide-bore pipette tip prepare a long-flat drop of sperm suspension in a 60mm Falcon dish and cover with pre-equilibrated mineral oil. *Note: Use all of the resuspended sperm for the MBCD drop.*
- 14) Incubate sperm for 30-40 min before insemination.

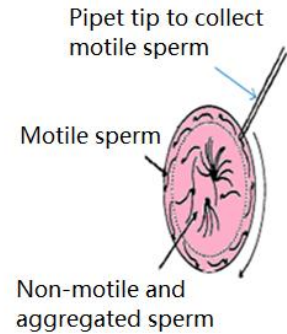
F. Inseminate and wash eggs

1. After the sperm have been incubated in MBCD medium for 30-40 min, carefully take the MBCD dish and the first GSH IVF dish out from CO₂ incubator and place on warm stage under a dissecting microscope.

- Using a P20 pipettor and a 200 μ l regular pipette tip carefully and slowly collect 20 μ L sperm while moving and aspirating along the MBCD drop edge, and then expel the sperm **slowly and directly** onto **each** of the egg clutches in the IVF drop under the microscope.

Note: Rotate the dish so that the pipette tip is facing the direction in which it is moving while aspirating along the drop edge slowly.

- Return the inseminated IVF dish into CO₂ incubator.
- Repeat the above steps 1-3 to inseminate the eggs in the remaining IVF dish(es) using sperm collected from different locations of the MBCD drop.
- Repeat the above steps 1-4 to collect and inseminate more sperm into each IVF dish if needed (each IVF drop can receive up to 50 μ L sperm).
- Co-culture the eggs with sperm in the IVF dishes for 3-4 hours without disturbance.
- After 3-4 h of co-culture, pick up the eggs from an IVF drop on warm stage (37 °C) and distribute into two drops in an egg wash dish. Mix the medium in each drop using the pipette, and then distribute into two other drops in the same dish. *Note: Wash eggs quickly to reduce changes of pH and temperature.*
- Repeat step 7 to wash eggs in the remaining IVF dishes.
- Score dishes the next day morning for IVF rate (2-cell rate). Take care to ignore degraded or fragmented oocytes. Transfer all 2-cell embryos in drop 5 or 6 and continue incubation in CO₂ incubator before embryo transfer microsurgery or embryo cryopreservation on the same day.



Appendix 1. Prepare high calcium (5.14 mM) RVF or ZHTF medium

- To increase calcium concentration of a 50ml bottle of RVF medium from 2.04 mM (regular concentration) to 5.14 mM (high calcium), weigh 0.02275 g (~23 mg) CaCl₂·2H₂O (Sigma C-7902) and add to a sterile Falcon tube.
- Pipette 5 ml medium from an original 50ml bottle to the tube, and mix to dissolve the CaCl₂·2H₂O.
- Filter-sterilize the 5 ml medium back into the original 50ml bottle slowly using a 0.2 μ m syringe filter while swirling the bottle to mix.
- Close the bottle with cap tightly, store the medium at 4 °C, and use within 2-3 weeks.

Appendix 2. Prepare 100 mM GSH Stock Solution

- Dissolve 0.18450 g GSH (L-glutathione reduced, Sigma G6013) in 6 ml RVF or Mouse HTF medium.
- Filter-sterilize using a 0.2 μ m syringe filter.
- Aliquot the GSH stock solution (200 μ l each), and store at -80 °C.

Appendix 3. Formula of Mouse HTF Medium (5.14 mM Ca):

Component (MW)	Vendor Cat. #	mM	g/L
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NaCl (58.45)	Sigma S5886, S7653	101.6	5.9375
KCl (74.56)	Sigma P5405, P9333	4.69	0.3496
KH ₂ PO ₄ (136.09)	Sigma P5655	0.37	0.0504
MgSO ₄ ·7H ₂ O (246.5)	Sigma M5921, M7774	0.20	0.0492
NaHCO ₃ (84.01)	Sigma S5761	25.00	2.1000
Na-Lactate (60% syrup, 112.1)	Sigma L1375, L7900	21.4	3.42 ml
Glucose (180.16)	Sigma G6152	2.78	0.5000
CaCl ₂ ·2H ₂ O (147)	Sigma C7902	5.14	0.7556
Na Pyruvate (110.0)	Sigma P4562	0.33	0.0365
Penicillin G (Na salt) (372.5)	Sigma P7794	0.0750 g/L	0.0750
Streptomycin sulfate (1457.4)	Sigma S9137	0.0500 g/L	0.0500
Phenol red (1%)	Sigma P0290	0.20 ml/L	0.20 ml
BSA	Sigma A3311	4 mg/ml	4 mg/ml

Appendix 4. Formula of MBCD Medium:

Component (MW)	Vendor Cat. #	mM	g/L
NaCl (58.45)	Sigma S5886, S7653	119.37	6.97600
KCl (74.56)	Sigma P5405, P9333	4.78	0.35600
KH ₂ PO ₄ (136.09)	Sigma P5655	1.19	0.16200
MgSO ₄ ·7H ₂ O (246.5)	Sigma M5921, M7774	1.19	0.29300
NaHCO ₃ (84.01)	Sigma S5761	25.07	2.1060
Glucose (180.16)	Sigma G6152	5.56	1.00000
CaCl ₂ ·2H ₂ O (147)	Sigma C7902	1.71	0.25100
Na Pyruvate (110.0)	Sigma P4562	1	0.0550
Penicillin G	Sigma P7794	7.5 mg/100 ml	0.0750
Streptomycin sulfate	Sigma S1277	5.0 mg/100 ml	0.0500
MBCD (methyl-β-cyclodextrin, 1320)	Sigma C4555	0.75	1.0000
PVA (polyninylalcohol)	Sigma P8136	100 mg/100 ml	1.0000

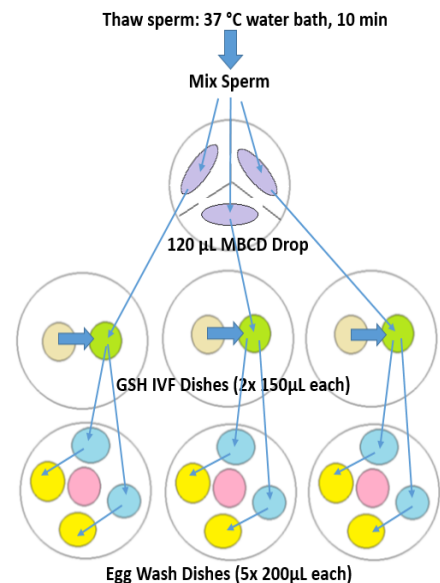
Mouse IVF Using 40-50 μ L of Cryopreserved Sperm

This protocol is for IVF using a cryostraw or cryovial containing 40-50 μ L of cryopreserved sperm. Oocytes are collected and pre-incubated in IVF medium containing GSH for 30-70 min and sperm are thawed and pre-incubated in MBCD medium for 30-40 min before insemination.

A. Materials

9. **CO₂ incubator:** 5.5% CO₂ in humidified air, 37 °C, and >90% relative humidity. Check temperature and CO₂ level daily using a thermometer and a Fyrite CO₂ analyzer, and calibrate when temperature is not 37 °C or CO₂ not within 5.0-6.0%. Change water biweekly and clean incubator when needed.
10. **High calcium medium:** Research Vitro Fert medium (RVF, Cook #K-RVFE) with 5.14 mM Ca or Mouse HTF medium (Fisher Scientific # MR070D). See **Appendix 1** for preparing RVF medium with high calcium (5.14 mM), and see **Appendix 3** for Mouse HTF formula (5.14 mM calcium).
11. **MBCD medium:** See **Appendix 4** for MBCD medium formula.
12. **Mineral oil** (for example, Fisher Scientific O1211)
13. **GSH** (L-glutathione reduced, Sigma G6013). See **Appendix 2** for preparing 100 mM GSH.
14. **Petri dishes:** Falcon 35x10mm (Fisher Scientific 08757100A) and 60x15mm (Fisher Scientific 08757100B)
15. **Superovulated mice:** ~15 mice, 3-4 weeks old. Light cycle 7am on and 9pm off. Inject each female with 5 IU of PMSG around 8pm, and then 47-48 hours later inject 5 IU of HCG by IP injection. Oocytes are collected 14-16 hours post HCG injection. If using 6 weeks or older mice, inject 7.5 IU PMSG and 7.5 IU HCG each mouse.

Note: IVF Kit (including RVF with high calcium or Mouse HTF medium, MBCD medium and GSH) is available for an additional charge. One kit is enough for two IVF procedures.



B. Prepare media and dishes for pre-equilibration in CO₂ Incubator (overnight or at least 1 hour)

6. **Egg collection dishes:** For every group of 5-8 females, prepare a 35mm Falcon dish with ~ 3 ml RVF medium (high or regular Ca concentration) or Mouse HTF medium.
7. **High calcium RVF or Mouse HTF medium for GSH IVF dishes:** Add 2.5 ml medium to a 5ml sterile Falcon tube, cap the tube loosely, and stand on a rack inside CO₂ incubator.
8. **Mineral oil:** Add 40-50 ml mineral oil to a sterile 50ml Falcon tube, cap the tube loosely, and stand on a rack inside CO₂ incubator.

9. **MBCD dish:** In a 60mm Falcon dish, prepare 3x 120 μ l long-flat drops of MBCD medium covered with mineral oil.

C. Prepare dishes first thing on the IVF morning

3. **GSH IVF dishes:**
 - 4) Thaw an aliquot of 100 mM GSH solution and mix.
 - 5) Add 20 μ l GSH to the 2.5 ml pre-equilibrated high calcium IVF medium, and invert tube gently a few times to mix.
 - 6) Prepare three Falcon 60x15mm dishes with 2x 150 μ l drops of GSH IVF medium each dish covered with pre-equilibrated mineral oil, and place in CO₂ incubator.
4. **Egg wash dishes:** For each GSH IVF dish, prepare a 60x15mm Falcon dish with 5x or 6x150 μ l drops of high calcium RVF or Mouse HTF medium (**without GSH**) covered with mineral oil. *Note: Egg washing dishes can be prepared after insemination.*

D. Harvest eggs and incubate in GSH IVF medium

1. After preparing the GSH IVF dishes, retrieve a sperm sample from liquid nitrogen storage and bring to the water bath at 37 °C using a small dewar containing liquid nitrogen. **Do not thaw sperm now.**
2. Between 9am and 10am, start to sacrifice the first group of superovulated females (5-8 mice) by CO₂ inhalation and/or cervical dislocation. Note: Sterilize surgical tools in advance.
3. Disinfect abdominal skin of each animal with 70% alcohol, and wipe away extra disinfectant.
4. Pull or cut the abdominal skin open for each mouse, and pull apart to expose body wall.
5. Lift up the body wall of a mouse using forceps and cut open using scissors, and then carefully move the digestive tract from inside the abdomen to expose the uterus, oviducts, and ovaries. *Note: Cut skin using one set of surgical tools and manipulate/cut body wall and tissues using another set of surgical tools; do not mix them.*
6. Dissect out both oviducts from the mouse and place in a pre-equilibrated egg collection dish. *Note: Do not take out the dish from CO₂ incubator until the group of females are skin-disinfected and cut open to minimize pH and temperature changes. Keep the egg collection dish on warm stage (37 °C) when outside of CO₂ incubator.*
7. Repeat the above steps 5 and 6 **quickly** to dissect out oviducts from the remaining mice of the group.
8. Under a dissecting microscope with warm stage, **quickly** collect egg clutch (cumulus-oocyte-complexes, COCs) from each oviduct by using forceps to hold down the oviduct against the dish, and a needle to tear open the ampulla to release the egg clutch. Remove the tissue from dish after its egg clutch is released.
9. After collecting COCs from all oviducts in the dish, immediately transfer them into the first GSH IVF drop in an IVF dish with minimum medium using a wide-bore pipette tip, and mix. Then transfer the COCs into the second GSH IVF drop.
10. Return the GSH dish into CO₂ incubator.

11. Repeat the above steps 2-10 to collect eggs from the remaining group(s) of superovulated females.
12. Incubate the COCs for 30-70 min before insemination.

E. Thaw sperm and incubate in MBCD medium

➤ **If using a cryovial sperm sample:**

- 1) After egg collection, **immediately** remove the cryovial of sperm from liquid nitrogen, loosen its cap, pour out any liquid, then put the cap back on the vial and stand on a floating rack in water bath at 37 °C to thaw sperm for 10 min.
- 2) Remove the cryovial or straw from water bath, and wipe dry.
- 3) Swirl the cryovial gently once or twice to mix sperm.
- 4) Distribute all of the sperm slowly and evenly into the 3x pre-equilibrated MBCD drops using a **wide-bore** pipette tip under a dissecting microscope. Make sure the pipette tip is inserted into the MBCD drops before sperm suspension is expelled.
- 5) Return the MBCD dish into CO₂ incubator, and incubate for 30-40 min before insemination.

➤ **If using a cryostraw sperm sample:**

- 9) After egg collection, **immediately** thaw the sperm straw by removing it from liquid nitrogen, holding in the air for 5 seconds, and placing in a 50ml Falcon tube filled with 37 °C DH₂O in a water bath for 10 min.
- 10) Remove the straw from water bath, and wipe it dry.
- 11) Disinfect the straw with a Kimwipe sprayed with 70% ethanol, and wipe dry completely.
- 12) Hold the straw horizontally and firmly, and carefully and gently cut off the heat seals at both ends (sterilize scissors in advance), and then use a metal rod or similar device to expel all sperm suspension slowly into an empty culture dish.
- 13) Mix the sperm suspension and distribute the sperm **slowly** and **evenly** into the 3x pre-equilibrated MBCD drops using a **wide-bore** pipette tip under a dissecting microscope. Make sure the pipette tip is inserted into the MBCD drops before sperm suspension is expelled.
- 14) Return the MBCD dish into CO₂ incubator, and incubate for 30-40 min before insemination.

F. Inseminate and wash eggs

10. After the sperm have been incubated in MBCD medium for 30-40 min, carefully take the MBCD dish and the first GSH IVF dish out from CO₂ incubator and place on warm stage under a dissecting microscope.

11. Using a P20 pipettor and a 200µl regular tip carefully and slowly collect 20 µL sperm while moving and aspirating along the first MBCD drop edge, and then expel the sperm **slowly and directly** onto **each** of the egg clutches in the IVF drop under the microscope.

Note: Rotate the dish so that the pipette tip is facing the direction in which it is moving while aspirating along the drop edge slowly.

12. Return the inseminated IVF dish into CO₂ incubator.

13. Repeat the above steps 1-3 to inseminate the eggs in the remaining two IVF dishes using sperm from the remaining two MBCD drops.

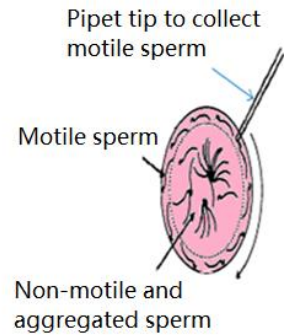
14. Repeat the above steps 1-4 to collect and inseminate more sperm into each IVF dish if needed (each IVF drop can receive up to 40 µL sperm).

15. Co-culture the eggs with sperm in the IVF dishes for 3-4 hours without disturbance.

16. After 3-4 h of co-culture, pick up the eggs from an IVF drop on warm stage (37 °C) and distribute into two drops in an egg wash dish. Mix the medium in each drop using the pipette, and then distribute into two other drops in the same dish. *Note: Wash eggs quickly to reduce changes of pH and temperature.*

17. Repeat step 7 to wash eggs in the remaining IVF dishes.

18. Score dishes the next day morning for IVF rate (2-cell rate). Take care to ignore degraded or fragmented oocytes. Transfer all 2-cell embryos in drop 5 or 6 and continue incubation in CO₂ incubator before embryo transfer microsurgery or embryo cryopreservation on the same day.



Appendix 1. Prepare RVF + 5.14 mM Ca

- 5) To increase calcium concentration of a 50ml bottle of RVF medium from 2.04 mM (regular concentration) to 5.14 mM (high calcium), weigh 0.02275 g (~23 mg) CaCl₂·2H₂O (Sigma C-7902) and add to a sterile Falcon tube.
- 6) Pipette 5 ml medium from an original 50ml RVF bottle to the Falcon tube, and mix to dissolve the CaCl₂·2H₂O.
- 7) Filter-sterilize the 5 ml medium back into the original 50ml bottle slowly using a 0.2 µm syringe filter while swirling the bottle to mix.
- 8) Close the bottle with cap tightly, store at 4 °C, and use within 2-3 weeks.

Appendix 2. Prepare 100 mM GSH Stock Solution

- 1) Dissolve 0.18450 g GSH (L-glutathione reduced, Sigma G6013) in 6 ml RVF or Mouse HTF medium.
- 2) Filter-sterilize using a 0.2 µm syringe filter.
- 3) Aliquot the GSH stock solution (200 µl each), and store at -80 °C.

Appendix 3. Formula of Mouse HTF Medium (5.14 mM Ca):

Component (MW)	Vendor Cat. #	mM	g/L
NaCl (58.45)	Sigma S5886, S7653	101.6	5.9375

KCl (74.56)	Sigma P5405, P9333	4.69	0.3496
KH ₂ PO ₄ (136.09)	Sigma P5655	0.37	0.0504
MgSO ₄ ·7H ₂ O (246.5)	Sigma M5921, M7774	0.20	0.0492
NaHCO ₃ (84.01)	Sigma S5761	25.00	2.1000
Na-Lactate (60% syrup, 112.1)	Sigma L1375, L7900	21.4	3.42 ml
Glucose (180.16)	Sigma G6152	2.78	0.5000
CaCl ₂ ·2H ₂ O (147)	Sigma C7902	5.14	0.7556
Na Pyruvate (110.0)	Sigma P4562	0.33	0.0365
Penicillin G (Na salt) (372.5)	Sigma P7794	0.0750 g/L	0.0750
Streptomycin sulfate (1457.4)	Sigma S9137	0.0500 g/L	0.0500
Phenol red (1%)	Sigma P0290	0.20 ml/L	0.20 ml
BSA	Sigma A3311	4 mg/ml	4 mg/ml

Appendix 4. Formula of MBCD Medium:

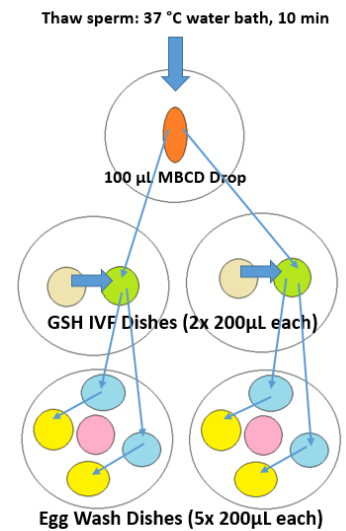
Component (MW)	Vendor Cat. #	mM	g/L
NaCl (58.45)	Sigma S5886, S7653	119.37	6.97600
KCl (74.56)	Sigma P5405, P9333	4.78	0.35600
KH ₂ PO ₄ (136.09)	Sigma P5655	1.19	0.16200
MgSO ₄ ·7H ₂ O (246.5)	Sigma M5921, M7774	1.19	0.29300
NaHCO ₃ (84.01)	Sigma S5761	25.07	2.1060
Glucose (180.16)	Sigma G6152	5.56	1.00000
CaCl ₂ ·2H ₂ O (147)	Sigma C7902	1.71	0.25100
Na Pyruvate (110.0)	Sigma P4562	1	0.0550
Penicillin G	Sigma P7794	7.5 mg/100 ml	0.0750
Streptomycin sulfate	Sigma S1277	5.0 mg/100 ml	0.0500
MBCD (methyl-β-cyclodextrin, 1320)	Sigma C4555	0.75	1.0000
PVA (polyninylalcohol)	Sigma P8136	100 mg/100 ml	1.0000

Mouse IVF Using Small Volume of Cryopreserved Sperm (10 μ L)

This protocol is for IVF using a cryostraw containing 10 μ l of cryopreserved sperm. Oocytes are collected and pre-incubated in IVF medium containing GSH for 30-70 min and sperm are thawed and pre-incubated in MBCD medium for 30-40 min before insemination.

A. Materials

16. **CO₂ incubator:** 5.5% CO₂ in humidified air, 37 °C, and >90% relative humidity. Check temperature and CO₂ level daily using a thermometer and a Fyrite CO₂ analyzer, and calibrate when temperature is not 37 °C or CO₂ not within 5.0-6.0%. Change water biweekly and clean incubator when needed.
17. **High calcium medium:** Research Vitro Fert medium (RVF, Cook #K-RVFE) with 5.14 mM Ca or Mouse HTF medium (Fisher Scientific # MR070D). See **Appendix 1** for preparing RVF medium with high calcium (5.14 mM), and see **Appendix 3** for Mouse HTF formula (5.14 mM calcium).
18. **MBCD medium:** See **Appendix 4** for MBCD medium formula.
19. **Mineral oil** (for example, Fisher Scientific O1211)
20. **GSH** (L-glutathione reduced, Sigma G6013). See **Appendix 2** for preparing 100 mM GSH.
21. **Petri dishes:** Falcon 35x10mm (Fisher Scientific 08757100A) and 60x15mm (Fisher Scientific 08757100B)
22. **Superovulated mice:** ~15 mice, 3-4 weeks old. Light cycle 7am on and 9pm off. Inject each female with 5 IU of PMSG around 8pm, and then 47-48 hours later inject 5 IU of HCG by IP injection. Oocytes are collected 14-16 hours post HCG injection. If using 6 weeks or older mice, inject 7.5 IU PMSG and 7.5 IU HCG each mouse.



Note: IVF Kit (including RVF with high calcium or Mouse HTF medium, MBCD medium and GSH) is available for an additional charge. One kit is enough for two IVF procedures.

B. Prepare Media and Dishes for Pre-equilibration in CO₂ incubator (overnight or at least 1 hour)

1. **Egg collection dishes:** For every group of 5-8 females, prepare a 35mm Falcon dish with ~ 3 ml RVF or HTF medium (high or regular Ca concentration).
2. **High calcium RVF or Mouse HTF medium for GSH IVF dishes:** Add 2.5 ml medium to a 5ml sterile Falcon tube, cap the tube loosely, and stand on a rack inside CO₂ incubator.
3. **Mineral oil:** Add 40-50 ml mineral oil to a sterile 50ml Falcon tube, cap the tube loosely, and stand on a rack inside CO₂ incubator.

4. **MBCD dish:** Prepare 1x 100µl long-flat MBCD medium drop and cover with mineral oil. Note: Mix MBCD medium after thaw by inversions prior to use!

C. Prepare Dishes first thing on the IVF Morning:

1. **GSH IVF dishes:**

- 1) Thaw an aliquot of 100 mM GSH solution and mix.
- 2) Add 20 µl GSH solution to the 2.5 ml pre-equilibrated high calcium RVF or Mouse HTF medium, and invert tube gently a few times to mix.
- 3) Prepare two Falcon 60x15mm dishes with 2x 200µl drops each and cover with oil.

2. **Egg wash dishes:** For each GSH IVF dish, prepare a 60x15mm Falcon dish with 5x 200µl drops of high calcium RVF or Mouse HTF medium (without GSH) covered with mineral oil. *Note: Egg washing dishes can be prepared after insemination.*

D. Harvest eggs and incubate in GSH IVF medium

1. After preparing the GSH IVF dishes, retrieve a sperm sample from liquid nitrogen storage and bring to the water bath at 37 °C using a small dewar containing liquid nitrogen. **Do not thaw sperm now.**
2. Between 9am and 10am, start to sacrifice the first group of superovulated females (5-8 mice) by CO₂ inhalation and/or cervical dislocation. *Note: Sterilize surgical tools in advance.*
3. Disinfect abdominal skin of each animal with 70% alcohol, and wipe away extra disinfectant.
4. Pull or cut the abdominal skin open for each mouse and pull apart to expose body wall.
5. Lift up the body wall of a mouse and cut open using scissors, and then carefully move the digestive tract from inside the abdomen to expose the uterus, oviducts, and ovaries. *Note: Cut skin using one set of surgical tools and manipulate/cut body wall and tissues using another set of surgical tools; do not mix them.*
6. Dissect out both oviducts from the mouse and place in a pre-equilibrated egg collection dish. *Note: Do not take out the dish from CO₂ incubator until the group of females are skin-disinfected and cut open to minimize pH and temperature changes. Keep the egg collection dish on warm stage (37 °C) when outside of CO₂ incubator.*
7. Repeat the above steps 5 and 6 quickly to dissect out oviducts from the remaining mice of the group.
8. Under a dissecting microscope with warm stage, quickly collect egg clutch (cumulus-oocyte-complexes, COCs) from each oviduct by using forceps to hold down the oviduct against the dish, and a needle to tear open the ampulla to release the egg clutch. Remove the tissue from dish after its egg clutch is released.

9. After collecting COCs from all oviducts in the dish, immediately transfer them into the first GSH IVF drop in an IVF dish with minimum medium using a wide-bore pipette tip, and mix. Then transfer the COCs into the 2nd GSH IVF drop.
10. Return the GSH dish into CO₂ incubator.
11. Repeat the above steps 2-10 to collect eggs from the remaining group(s) of superovulated females.
12. Incubate the COCs for 30-70 min before insemination.

E. Thaw sperm and incubate in MBCD medium

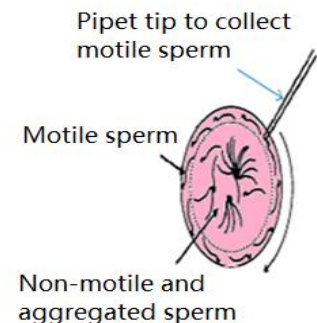
1. After egg collection, **immediately** thaw the sperm straw by removing it from liquid nitrogen, holding in the air for 5 seconds, and placing in a 50ml Falcon tube filled with 37 °C DH₂O in a water bath for 10 min. *Note: Sperm can start to be thawed before sacrificing the second or third group of females (egg donors)*
2. Remove the straw from water bath, and wipe it dry.
3. Disinfect the straw with a Kimwipe sprayed with 70% ethanol, and wipe dry completely.
4. Hold the straw horizontally and firmly, and carefully and gently cut off the heat seals at both ends (sterilize scissors in advance), and then use a metal rod or similar device to expel all sperm suspension slowly and directly into the MBCD drop in the MBCD dish under a dissecting microscope. Make sure the sperm suspension is expelled into the MBCD drop.
5. Incubate sperm for 30-40 min in CO₂ incubator before insemination.

F. Inseminate and wash eggs

1. After the sperm have been incubated in the MBCD medium for 30-40 min, carefully take out the MBCD dish and the two GSH IVF dishes from incubator and place on a warm stage under a dissecting microscope.
2. Using a P20 pipettor and a 200µl regular tip carefully and slowly collect 20 µL sperm while moving and aspirating along the MBCD drop edge, and then expel the sperm slowly and directly onto each of the egg clutches in the IVF drop under the microscope.

Note: Rotate the dish so that the pipette tip is facing the direction in which it is moving while aspirating along the drop edge slowly.

3. Return the inseminated IVF dish into CO₂ incubator.
4. Repeat the above steps 1-3 to inseminate the eggs in the remaining IVF dish(es) using sperm collected from different locations of the MBCD drop.
5. Repeat the above steps 1-4 to collect and inseminate more sperm into each IVF dish if needed (each IVF drop can receive up to 50 µL sperm).



6. Co-culture the eggs with sperm in the IVF dishes for 3-4 hours without disturbance.
7. After 3-4 h of co-culture, pick up the eggs from an IVF drop on warm stage (37 °C) and distribute into two drops in an egg wash dish. Mix the medium in each drop using the pipette, and then distribute into two other drops in the same dish. *Note: Wash eggs quickly to reduce changes of pH and temperature.*
8. Repeat step 7 to wash eggs in the remaining IVF dishes.
9. Score dishes the next day morning for IVF rate (2-cell rate). Take care to ignore degraded or fragmented oocytes. Transfer all 2-cell embryos in drop 5 or 6 and continue incubation in CO₂ incubator before embryo transfer microsurgery or embryo cryopreservation on the same day.

Appendix 1. Prepare RVF + 5.14 mM Ca

- 9) To increase calcium concentration of a 50ml bottle of RVF medium from 2.04 mM (regular concentration) to 5.14 mM (high calcium), weigh 0.02275 g (~23 mg) CaCl₂·2H₂O (Sigma C-7902) and add to a sterile Falcon tube.
- 10) Pipette 5 ml medium from an original 50ml RVF bottle to the Falcon tube, and mix to dissolve the CaCl₂·2H₂O.
- 11) Filter-sterilize the 5 ml medium back into the original 50ml bottle slowly using a 0.2 µm syringe filter while swirling the bottle to mix.
- 12) Close the bottle with cap tightly, store at 4 °C, and use within 2-3 weeks.

Appendix 2. Prepare 100 mM GSH Stock Solution

- 1) Dissolve 0.18450 g GSH (L-glutathione reduced, Sigma G6013) in 6 ml RVF or Mouse HTF medium.
- 2) Filter-sterilize using a 0.2 µm syringe filter.
- 3) Aliquot the GSH stock solution (200 µl each), and store at -80 °C.

Appendix 3. Formula of Mouse HTF Medium (5.14 mM Ca):

Component (MW)	Vendor Cat. #	mM	g/L
NaCl (58.45)	Sigma S5886, S7653	101.6	5.9375
KCl (74.56)	Sigma P5405, P9333	4.69	0.3496
KH ₂ PO ₄ (136.09)	Sigma P5655	0.37	0.0504

MgSO ₄ ·7H ₂ O (246.5)	Sigma M5921, M7774	0.20	0.0492
NaHCO ₃ (84.01)	Sigma S5761	25.00	2.1000
Na-Lactate (60% syrup, 112.1)	Sigma L1375, L7900	21.4	3.42 ml
Glucose (180.16)	Sigma G6152	2.78	0.5000
CaCl ₂ ·2H ₂ O (147)	Sigma C7902	5.14	0.7556
Na Pyruvate (110.0)	Sigma P4562	0.33	0.0365
Penicillin G (Na salt) (372.5)	Sigma P7794	0.0750 g/	0.0750
Streptomycin sulfate (1457.4)	Sigma S9137	0.0500 g/	0.0500
Phenol red (1%)	Sigma P0290	0.20 ml/L	0.20 ml
BSA	Sigma A3311	4 mg/ml	4 mg/ml

Appendix 4. Formula of MBCD Medium:

Component (MW)	Vendor Cat. #	mM	g/L
NaCl (58.45)	Sigma S5886, S7653	119.37	6.97600
KCl (74.56)	Sigma P5405, P9333	4.78	0.35600
KH ₂ PO ₄ (136.09)	Sigma P5655	1.19	0.16200
MgSO ₄ ·7H ₂ O (246.5)	Sigma M5921, M7774	1.19	0.29300
NaHCO ₃ (84.01)	Sigma S5761	25.07	2.1060
Glucose (180.16)	Sigma G6152	5.56	1.00000
CaCl ₂ ·2H ₂ O (147)	Sigma C7902	1.71	0.25100
Na Pyruvate (110.0)	Sigma P4562	1	0.0550
Penicillin G	Sigma P7794	7.5 mg/10 ml	0.0750
Streptomycin sulfate	Sigma S1277	5.0 mg/10 ml	0.0500

MBCD (methyl- β -cyclodextrin, 1320)	Sigma C4555	0.75	1.0000
PVA (polyninylalcohol)	Sigma P8136	100 mg/1 ml	1.0000