

Mouse In Vitro Fertilization (IVF)

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In Vitro Fertilization

1.1 Introduction

The following protocol has been adopted from the Institute of Experimental Genetics, in the GSF – Germany¹. The protocol provides a full set of instructions for both ‘simple’ *in vitro* fertilization and for cryopreservation and the usage of thawed spermatozoa for *in vitro* fertilization.

In vitro fertilization can be used to produce large colonies of mice in a relatively short period of time, saving also on mouse caging space. Moreover, fewer males are needed to fertilize the same number of females and the procedure naturally overcomes problems such as periods of ‘no breeding’ or some fertility difficulties encountered especially in mutated strains.

The usage of cryopreserved spermatozoa enables long-term preservation of strains but has its limitations (mice on a C57Bl/6 pure background can not be used in this procedure, also maintenance of certain strains such as congenics and consomics requires matching oocytes).

Concept: sperm from **one** ♂  is taken to fertilize oocytes from **15-20** **superovulated** ♀ . On average, C3H females produce 15 oocytes per female. If cleavage rate is 60%, ~200 two-stage cells are attained, and 20 are retrieved per mouse – using **8-10 pseudopregnant** ICR ♀ . As a mouse usually conceives up to 8 mice, usually at least 50 mice are generated in one reaction (80 being the upper limit).



At first it seems as if it is much too much work for generating backcross mice for mapping. But considering mouse room limitations, and the F1 males limitation, the thought that in one week using 8 F1 mutants 400 progeny can be attained is quite encouraging. (ref 9 & 10 original protocol).

Snapshot protocol for IVF

Day 1

<input type="checkbox"/>	1	PMSG: 100µl (C3H)/ 75µl (BL6) per mouse, 16:00.
<input type="checkbox"/>	2	Prepare CPA if there isn't any.

Day 3

<input type="checkbox"/>	1	Prepare HTF and KSOM.
<input type="checkbox"/>	2	Prepare equilibrated mineral oil.
<input type="checkbox"/>	3	HCG: 100µl (C3H)/ 75µl (BL6) per mouse, 18:00.

Day 4

<input type="checkbox"/>	1	Prepare fertilization plates.
<input type="checkbox"/>	2	Prepare 4 well plates + sperm evaluation plate.
<input type="checkbox"/>	3	Take 4 well plates on Ice + cryotube for spleen.
<input type="checkbox"/>	4	07:10 Dissect cauda epididymis + spleen/ tail tip.
<input type="checkbox"/>	5	Prepare sperm sample (clean in NaCl+ cut in CPA).
<input type="checkbox"/>	6	Add 1µl of fresh sperm to each fertilization plate + 2µl of sperm to sperm evaluation plate, notice the time. [if using thawed sperm – 2-4µl]
<input type="checkbox"/>	7	Prepare sample for freezing (straws + liquid N ₂ O).
<input type="checkbox"/>	8	Sacrifice females and dissect out oviducts – 14 hours after hCG injection (7:40-8:30am). Put oviducts in 60 mm dish with regular mineral oil.
<input type="checkbox"/>	9	Transfer the oviducts to a 60mm dish with 400µl HTF – covered with mineral oil.
<input type="checkbox"/>	10	Drag the oocytes with cumulus complex from the ampullae into the HTF drop.
<input type="checkbox"/>	11	Dispense the oocytes between the 4 fertilization dishes.
<input type="checkbox"/>	12	Incubate for 4-6 hours. ☀️ 😊 → 🕒
<input type="checkbox"/>	13	Evaluate sperm quality.
<input type="checkbox"/>	14	Wash in 4 drops of KSOM.
<input type="checkbox"/>	15	Transfer all oocytes to the o/n KSOM culture dish.

Day 5

<input type="checkbox"/>	1	Wash fertilized oocytes in three drops of M2 medium (ok from 08:00am).
<input type="checkbox"/>	2	10:00-15:00 Transfer the 2 cell oocytes to the 100µl M2 drop covered with mineral oil. Leave on 37°C plate or in incubator until transfer.
<input type="checkbox"/>	3	Embryo transfer

Prepare

<input type="checkbox"/>	hCG
<input type="checkbox"/>	PMSG
<input type="checkbox"/>	CPA

<input type="checkbox"/>	HFT + mineral oil equilibrated
<input type="checkbox"/>	KSOM + mineral oil equilibrated
<input type="checkbox"/>	Glass capillary pipettes

<input type="checkbox"/>	Fertilization plate: 4 X35mm plates, 200µl HTF + 3ml HTF equilibrated oil.
<input type="checkbox"/>	4 well plate – 3 drops of NaCl + 170µl CPA for each mouse.
<input type="checkbox"/>	Prepare sperm evaluation plate: 100µl HTF + equilibrated oil.
<input type="checkbox"/>	60 mm dish with regular mineral oil.
<input type="checkbox"/>	60 mm dish with a drop of 400µl HTF covered with regular mineral oil.

<input type="checkbox"/>	Wash plates(1) – 4 drops of KSOM
<input type="checkbox"/>	Overnight culture dish – 100µl KSOM + KSOM equilibrated oil.

<input type="checkbox"/>	Wash plates(2) – three drops of M2 medium (50µl each) + 10µl hyaluronidase in the first drop.
<input type="checkbox"/>	100µl M2 drop covered with mineral oil.
<input type="checkbox"/>	Anesthesia for ICR females.

1.3 General considerations – Notes

- Reproductive differences between mouse strains seen in vivo are also evident in vitro.
- Hybrids normally have a better fertility than inbred strains.
- For cryopreservation – male mice age between 3-5 months are usually taken, because spermatozoa of younger males sometimes are usually less vigorous.
- Before freezing – should be **kept single for two weeks** and should not be mated.
- Ideally the males have already proven fertility before.

2.0 Preparation of solutions for IVF –

As a general rule, the solutions for IVF should be made fresh before each reaction.

KSOM – must be made fresh and used within 1-2 days.

HTF – need not be made 1 day in advance but never use HTF that is more than 4 days old for IVF.

CPA – can be kept for 2-3 months. 3 months is considered old and should not be used. The KSOM and HTF equilibrated mineral oils must be fresh and made the day before the IVF.

2.1 HTF

HTF medium is used for sperm quality evaluation and in vitro fertilization.

Prepare one day before IVF.

Preparation:

Component	mg/100ml	Source	cat.no.
<input type="checkbox"/> Sodium-Pyruvate	3.65	Sigma	P-4562
<input type="checkbox"/> MgSO ₄ X 7H ₂ O	4.93	Sigma	M-9397
<input type="checkbox"/> Streptomycin	5.0	Sigma	S-1277
<input type="checkbox"/> KH ₂ PO ₄	5.04	Sigma	P-5655
<input type="checkbox"/> Penicillin G	7.5	Sigma	P-4687
<input type="checkbox"/> KCl	34.96	Sigma	P-5405
<input type="checkbox"/> Glucose	50.0	Sigma	G-6152
<input type="checkbox"/> NaHCO ₃	210.0	Sigma	S-5761
<input type="checkbox"/> NaCl	593.75	Sigma	S-9888
<input type="checkbox"/> Sodium lactate 60%	342µl	Sigma	L-7900
<input type="checkbox"/> CaCl ₂ X 2H ₂ O	60.0	Sigma	C-7902
<input type="checkbox"/> BSA	400.0	Sigma	A-4378

1. Dissolve everything in 75ml of ultrapure water (except CaCl₂ X 2H₂O and BSA). (use a 150-300 ml glass beaker).
2. Dissolve 60.0mg of **CaCl₂ X 2H₂O** in 25ml water and add it to the rest of the medium.
3. Gas the medium using a pipette immersed in the solution with mixture of 5% CO₂, 5%O₂ and 90% N₂ for 10 min.
 - Turn osmometer on and weigh the BSA while the medium is being gassed.
4. Add **BSA** – measure 5mg more than required (as it is measured on a separate plate).
5. **Sterilize** the solution before storage (non pyrogenic filter 0.22µm; Millipore cat.no.SLGVR25KS) using a 20ml syringe into 2 50ml tubes.

6. Measure the **osmolarity** of the medium – should be **260-280mOsm**.
[First calibrate the osmometer to 0 using ultrapure water and then measure 150µl of the solution. When the needle starts shifting to the right, press on the ‘vibrator’ button].
7. Store at **37°C** for no more than one week; leave lid open (just put lid on it to close but do not secure close it).
8. **Equilibrate** an aliquot of the final medium with mineral oil (Sigma cat.no.M-8410), e.g. put 25ml HTF medium and 25ml oil in a tube and shake it carefully, put in the incubator over night (open lid); use this equilibrated oil to cover drops of medium.

2.2 KSOM

KSOM medium is used for over night culture of the embryos.

Preparation:

Component	mg/100ml	Source	cat.no.	
<input type="checkbox"/> EDTA	0.38	Sigma	E-5134	
<input type="checkbox"/> Sodium-Pyruvate	2.2	Sigma	P-4562	
<input type="checkbox"/> Glucose	3.6	Sigma	G-6152	
<input type="checkbox"/> KH ₂ PO ₄	4.75	Sigma	P-5655	
<input type="checkbox"/> MgSO ₄ X 7H ₂ O	4.95	Sigma	M-9397	
<input type="checkbox"/> Streptomycin	5.0	Sigma	S-1277	
<input type="checkbox"/> Penicillin G	6.3	Sigma	P-4687	
<input type="checkbox"/> Glutamine	14.5	Sigma	G-5763	
<input type="checkbox"/> KCl	18.5	Sigma	P-5405	
<input type="checkbox"/> NaHCO ₃	210.0	Sigma	S-5761	
<input type="checkbox"/> NaCl	559.5	Sigma	S-9888	
<input type="checkbox"/> Sodium lactate 60%	174µl	Sigma	L-7900	Is very viscous, pipette slowly.
<input type="checkbox"/> Ess.-AminoacidX50	1000µl	Gibco/BRL	11130-036	
<input type="checkbox"/> Non-ess. AminoacidX100	500µl	Giibco/BRL	11140-035	
<input type="checkbox"/> Phenol red	0.1	Sigma	P-3532	Is used as an indicator - add using a tip.
<input type="checkbox"/> CaCl ₂ X 2H ₂ O	25.0	Sigma	C-7902	
<input type="checkbox"/> BSA	100.0	Sigma	A-4378	

1. Dissolve everything in 70ml of ultrapure water (except CaCl₂ X 2H₂O and BSA). A good size beaker is 150-300 ml.
2. Add essential amino acids and non essential amino acids and bring up to a total volume of 75ml.
3. Dissolve 25mg of CaCl₂ X 2H₂O in 25ml of water and add it to the rest of the medium (→ solution’s total volume is 100ml).
 - Steps 1-3 can be done at once.

4. **Gas** the medium using a pipette immersed in the solution with mixture of 5% CO₂, 5%O₂ and 90% N₂ for 10 min (should have salmon color). Cover beaker with aluminium foil, should bubble lightly.
 - Turn osmometer on and weigh the BSA while the medium is being gassed.
5. Add **BSA** – measure 5mg more than required (as it is measured on a separate plate).
6. **Sterilize** the solution before storage (non pyrogenic filter 0.22µm; Millipore cat.no. SLGVR25KS) using a 20ml syringe into 2 50ml tubes.
7. Measure the **osmolarity** of the medium (**250-270mOsm**).
8. Store the medium at **37°C** for not more than two weeks; leave the lid open.
9. **Equilibrate** an aliquot of the final medium with mineral oil (25ml KSOM + 25ml mineral oil); leave with an open lid overnight.
 - KSOM can substitute for M16 (transgenics).
 - For preparing the solutions (measuring weights) use a metal spatula and clean with alcohol in between components. Start with the lightest component and end with the heaviest.

Osmometer

The osmometer can be used only after the green light is turned on.

First set ref to 0 using 150µl of ultrapure water.

Next, put 150µl of sample in the vial.

As soon as the dial starts shifting to the right, press on the vibrator button.

The first reading value is the correct one.

Write the osmolarity values on the solution tubes + the date they were made.

2.3 CPA

CPA – Cryoprotecting agent for sperm cryopreservation

Component	Concentration	g/40ml	Source	cat.no.
<input type="checkbox"/> Raffinose	18%	7.2	Sigma	R-7630
<input type="checkbox"/> Skim-milk	3%	1.2	DIFCO	0032-17-3

1. Warm approx. 50ml of ultrapure water up to 60°C.
2. Weigh the Raffinose and Skim milk. Transfer them to a 50ml tube (will fill about 15ml of it).
3. Dissolve the components (shake) in 30ml warm water; then fill it up to 40ml.
4. Allot the solution to 2ml tubes. Mount the tubes on a tabletop centrifuge. (use gloves!)
5. Centrifuge the solution at 13,000 rpm for 60min. (20°C?)
6. Collect the supernatant from the 2ml tubes into a 50ml tube (do not let pellet go in).
7. Take supernatant (has to be clear) and sterilize it (non pyrogenic filter 0.45µm) using a 20ml syringe into a 50ml tube.
8. Measure the osmolarity of the medium (480-500).

9. Make aliquots convenient for your usage and write date (1.5ml = 10mice); store them at -20°C for no more than 3 months.
10. De-freeze fast (put in 37°C incubator). Once thawed – can not be frozen again.

2.4 M2 medium

M2 medium is purchased from Sigma (cat.no.M5910).

It should be stored in the dark at 2°C - 8°C .

Essentially, it is a variation of M16 medium containing HEPES in place of the sodium bicarbonate, is used to collect mouse embryos and to handle them for extended periods of time outside the incubator.

The powdered media are extremely hygroscopic and should be protected from atmospheric moisture. The entire contents of each package should be used immediately after opening. It is not recommended to prepare a concentrated solution of medium as precipitates may form.

1. Measure 90% of final required volume of water. Water temperature should be $15\text{--}20^{\circ}\text{C}$.
2. While gently stirring the water, add the powdered medium. Stir until dissolved. **Do NOT heat.**
3. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 1.
4. Add the following components to the solution in step 3:

	Component	g/1000ml	
1	Lactic acid (60% syrup) ¹	4.349	Stir until dissolved
2	Sodium bicarbonate	0.35	Stir until dissolved
3	Penicillin	0.06	
4	Streptomycin	0.05	

5. While stirring, adjust the pH of the medium to 0.1-0.3 pH units **below the desired pH** (it may rise during filtration – desired pH is 7.2-7.4; according to the book, the pH has to be adjusted with 0.2N NaOH. There are NaOH ‘grains’ in the cupboard – even one grain has a DRASTIC effect on pH, therefore be cautious).
6. Add additional water to bring the solution to final volume (1L).
7. Sterilize immediately by filtration using a membrane with a porosity of $0.22\mu\text{m}$.
8. Aseptically dispense medium into sterile container.

The medium can be stored for 6 up to months at 4°C .
Complete volume to 1L and make aliquots of 50ml.

2.5 Hyaluronidase

Hyaluronidase is purchased from Sigma (cat.no. H3884).

Prepare a stock solution (10mg/ml M2 medium or water).

¹ The lactic acid is at a 1.32g/ml concentration. Therefore – $3.295\text{ml} = 4.349\text{g}$.

Sterilize with a 0.22 μ m filter.
Make aliquots and store them at -20°C .
For usage dilute them to approximately 300 $\mu\text{g}/\text{ml}$ in M2 medium.

The Hyaluronidase we have prepared in the lab (by Nadav) is at a 6mg/ml concentration and aliquoted to 0.1ml aliquots. Therefore, in order to get to a 300 $\mu\text{g}/\text{ml}$ concentration it should be diluted X 20. → **for every 50 μl add 0.95ml of M2.**

2.6 Fertilization dishes

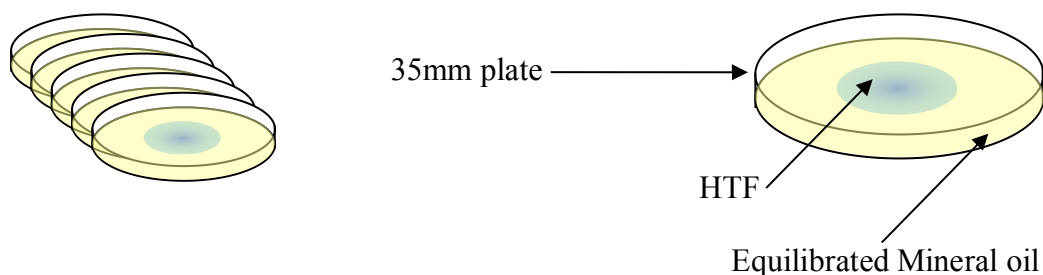
Prepare **4 fertilization plates 35mm** and number them 1-4 (on the side).
Put 200 μl of HTF medium in each plate = fertilization medium.
HTF can be prepared up to 1 week in advance but for IVF should be prepared one day in advance.

Essentially, the more fertilization dishes you prepare – the better it is for the IVF as the proteins that are released during the cumulus disintegration are toxic to the sperm. Nonetheless, the more fertilization dishes used, the more washes are needed later.

Preparation:

Prepare fertilization dishes the morning of the IVF. The oil should be already equilibrated over night. After preparation of the dish, leave in incubator for 10 minutes for media to warm back before adding sperm to it.

1. Put 1 drop (**200 μl**) in the middle of the plate.
2. Cover drops completely with **equilibrated** mineral oil – sigma.
3. Keep in the 37 $^{\circ}\text{c}$ incubator with the lid a bit open – to allow contact with the CO_2 .



2.1 Superovulation, Pseudopregnancy & selection of mice for IVF

♀ **for superovulation C3H/C57BL/6**: can be used at age 6-12 weeks. **8 weeks** is the best age, 6 week old mice are still a bit premature and 12 are a bit old.

♀ **for fostering the embryos ICR**: best at age **6-8 weeks**, after 10 weeks are considered 'old'.

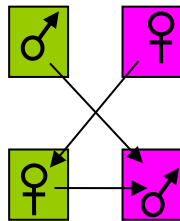
♂ **for sperm collection**: can be as young as 8 weeks (not younger) and as old as 1-1.5 years. It is possible to perform IVF with older mice but the yield is lower.

Pseudopregnancy

Pseudopregnant mice are generated by mating the ♀ ICR mice with vasectomized males. In order to augment the ratio of plug positive mice the following measures can be taken:

9. If two females are put together with each male – the highest plug rate will be on the third day.

10.



- i. Put the ♀ mice in separate cages two-three days before the mating.
- ii. The day before the mating put the males in their counterpart female cages and the females in the males cages.
- iii. The next day, for the mating – put the females in their original cages, now the males have learned their odor and have been waiting for them.

Ovulation induction

The following section refers to a mouse room with a light cycle of: **07:00-19:00**.

Materials:

Intergonan 1000 I.U. (Intervent, Toenisvorst, Germany).

Oovogest 1500 I.U. (Intervent).

Physiological salt solution – **NaCl 0.9%**.

For injection: **1ml syringe** and 26G needle.

Hormone preparation:

- Add **20ml 0.9% NaCl to Intergonan (PMSG)** and mix well.
- Add **30ml 0.9% NaCl to Oovogest (hCG)** and mix well.
→ Your final concentration is 50IU/ml.
- Make aliquots convenient for the number of females you want to inject; these aliquots **can be stored up to 6 months at -20⁰c and 1 week after thawing – in refrigerator**. Do not refreeze!
- 3 days before IVF inject the females intraperitoneally with **PMSG, 4PM** is recommended as injection time.
- 1 day before IVF inject the females intraperitoneally with **hCG, 6PM** is recommended as injection time.

PMSG – 16:00
50 hours between
PMSG and hCG
hCG – 18:00
oocyte collection 14-
16h afterwards –
8:00-10:00am.

Inject 15-20 female
mice.

Dosage:

For C3H mice 100µl of each; For C57Bl/6 75µl of each.

Essentially, for both hormones 5-7.5 IU per mouse are most commonly used, but for C57BL/6 3.75 IU are used.

hCG should be injected between 48 and 50 hours after PMSG injection. A longer or shorter interval may interfere with the response.

The oocytes are collected between 14-15 hours after hCG injection. If they are collected earlier than 13.5 hours after injection, insemination may fail. Collection after 16 hours or later is also unsuitable – at this time oocytes already begin to age and can no longer be fertilized. Due to this injection schedule the collection of spermatozoa (in vitro capacitation) should be timed as well.

'our PMSG and hCG' –

PMSG – the solution is already ready in the –80°C freezer, every Eppendorf has enough for 4 mice (although it says 5).

hCG – every Eppendorf says 3 – add 300µl saline (0.9% NaCl).

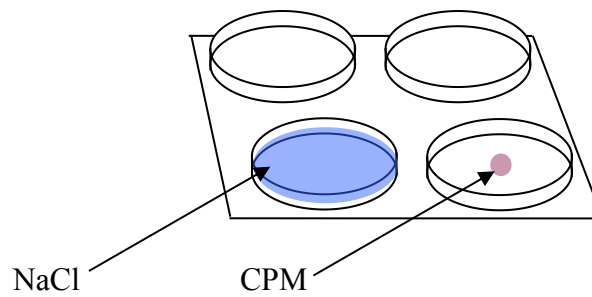
2.2 Collection of spermatozoa

Sacrifice male by cervical dislocation / CO₂ chamber and dissect the two caudae epididymis, removing as much fat as possible. Place the tissue into 0.9% NaCl in the appropriate well of the 4-well dish (on ice).

- *Males need not be isolated/taken out of breeding cages before being sacrificed for collection of spermatozoa.*

Dissection of cauda epididymis

1. Prepare a four well plate – put some **NaCl** (3mm deep) in one well. [2 wells are used for each male].
 2. Put **170µl** of **CPM** in a second well.
- It is better to write numbers on the side of the wells.
 - From the time sperm is taken, all procedures are performed **on ice**.



Bring with you to the dissection:

For dissection of caudae epididymis:

- **Scissors.**
- **Fine scissors.**
- **Fine forceps.**
- **Watchmaker forceps.**

For sample preparation:

- **Spring scissors.**
- **Watchmaker forceps.**
- **Ice.**
- **HTF medium.** → fertilization plates.

If also cryopreserving:

- 0.25ml french type mini straws.²
- 1ml syringe.
- Self-adhesive labels for straw identification.
- Liquid nitrogen.

² Minitub

Tel: 49 (0) 87 09/ 92 29 0
Fax: 49 (0) 87 09/ 92 29 39

French type ministraw 0.25cc; 2000 pcs/bg Art.No. 13407/0010
minitube@minitube.de

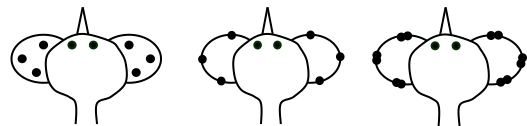
- Freezing canister (insert polystyrene block into the bottom of a 50ml syringe, heat seal the syringe tip, attach the syringe to an acrylic handle – ‘figure 3’).
- For sample storage:
 - i. Liquid nitrogen tank.
 - ii. Cryopreservation cups.
 - iii. Grid drawer.
- For thawing:
 - i. Waterbath 37^oc.
 - ii. 35mm culture dishes (Nunc).
 - iii. HTF medium.
 - iv. HTF-equilibrated mineral oil for IVF.

Time estimation – it should take about 1h to dissect and freeze sperm from 4 mice.

Protocol:

Dissection of Cauda epididymis

1. **Sacrifice** the mice with cervical dislocation or using CO₂ chamber. For CO₂ chamber, pressure should be ~60bar.
2. **Arrange** mice in a row, each mouse on a separate tissue, according to their numbers.



*Mouse identification using ear punching method:
Right ear in the picture (mouse left ear)
represents numbers 1-9 and left ear numbers 10-
90. left mouse right ear 1 (front), 2 (middle) & 3
(back), middle mouse right ear 4 (front) etc.*

3. **Spray** their abdomen with a disinfectant solution or alcohol 70%, mainly to wet the mouse hair.
4. If necessary – take tails and spleen for DNA (spleen should be put in a cryotube and thrown into a liquid nitrogen beaker).
5. Make a transverse incision in the mouse abdomen and pull skin in both directions.
6. Open abdominal wall.
 - a. Reveal testis by pulling the fat pad.
 - b. Pull testis toward the mouse legs. The Vas will show as a white string running toward the abdomen.
 - c. **Cut vas deference** (not too close as the vas contains sperm that can be used for the IVF).
 - d. Separate the fat from the vas deference.
 - e. Pull the vas towards the mouse legs and cut the muscle.
 - f. **Cut between the cauda epididymis and the fat pad** (the testicle is the next structure after the fat pad).

- g. Clean cauda and vas on tissue from auxiliary blood vessels.
- h. **Put the vas+cauda in the NaCl well** (gather both sides in the same well³). The NaCl well should contain about 3 drops of NaCl and 170µl CPA.

Sample preparation

This part of the dissection is done using a **light microscope**. There are two things to keep in mind:

- Work as quick as possible.
 - Remove as much fat as you can.
1. Take the cauda in the NaCl well to a light microscope.
 2. Use a 1.4 magnification.
 3. **Cut** (do not pull) **the fat** from around the cauda and vas using spring scissors.
 4. **Cut the vas at the point where there is no more sperm** (the sperm is seen as an opaque density within the clear vas).
 5. **Dry** cauda and vas on the border (NaCl should not be transferred to the next plate).
 6. **Transfer** the cauda and vas after drying it, to **CPA** well.
 - a. Release the sperm out of the vas using the forceps to anchor the cauda and the spring scissors to **'milk' the vas**.
 - b. **Cut the cauda** a few cuts in order for the sperm to disperse from the tissue.
 - c. Cover the wells with the lid and **tap** the wells on the side to homogenize the solution.
 - d. **Leave for 3-5 minutes on ice** for the solution to homogenize (according to original protocol – on ice/37°C; according to GSF – on ice). To get a homogenous suspension shake the dish carefully (**do not pipette the suspension** because this can destroy the spermatozoa!). It is a bad sign if the spermatozoa do not start to disperse by themselves (they may be already dead or immotile; → the 'grey clouds' should become more homogenous).
 7. **Add 1µl of sperm to each fertilization plate** (as it is fresh sperm). **2µl** can be added routinely instead of 1 or in cases the sperm count looks low.

If it were **frozen sperm**, the concentration of viable spermatocytes would have been lower and therefore **2-4µl of thawed sperm** should have been added.

 - a. Take the fertilization plates out of the incubator.
 - b. Put the correct amount of sperm in the HTF medium which is covered with equilibrated mineral oil (If the sperm is very concentrated you'll see the drop).

³ 160µl of CPA if only cryopreserving; 170 if also fresh sperm IVF is preformed.

Put back in incubator for 30min (optimal time) – 2 hours (if you leave it for a longer period, sperm will die).

Evaluation of sperm quality –

Put **2µl** of the homogenous **sperm** suspension in a drop of **100µl HTF** covered with equilibrated mineral oil. Put in incubator for **60 minutes** to capacitate spermatozoa.

Then take out the sample and pipette 15µl into the chamber of the sperm analyzer or look at the sperm using a light microscope. 60% motility and 30% Progressiveness represent good sperm. Anyway, never count only on the sperm analyzer. The important measures are: concentration, motility and **Progressiveness**.

When evaluating the sperm by eye, one should evaluate:

1. The ratio of living:dead spermatozoa.
2. The straightness of the swimming spermatozoa (can be seen better at the border of the drop).
3. The velocity of the spermatozoa.

Of all parameters, Progressiveness is the most important one.

Sperm freezing

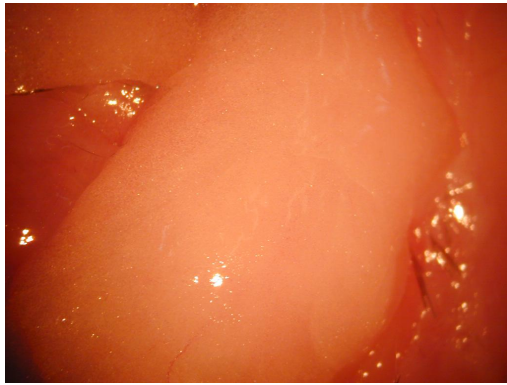
1. Pipette 10 sperm drops of 15µl each (for making 10 freezing straws) on top of the lid of the 4 well plate.
2. Connect the 1ml syringe with a straw and aspirate –
 - a. ~100µl HTF medium – up to ½ of the straws length.
 - b. An air bubble.
 - c. The sperm aliquot.
 - d. Another air bubble.
3. Weld both ends of the straw.
4. Mark the straws by attaching the label on one end of the straw.
5. When done – put on ice.
6. Once all straws are ready - put on the yellow cover with the ‘name’.
7. Put the straws in a curette; don’t drop into the liquid nitrogen! Lean it on the side for 10min (that’s what the polystyrene block is for) and then drop into the liquid nitrogen. Use a liquid nitrogen pot that is (minimum) 50cm high + a cover.

The sperm is put into two cups saved in two different locations. Each cup has 5 straws. To be on the safe side, if you intend to freeze a strain – make 3 cryopreservations for each strain, with 10 straws per mouse.

The GSF uses the following table to follow the cryopreserved spermatozoa:

ID	Strain	Location in tank	Motility	Progressiveness	Concentration

It must be take into consideration that after thawing, 80% of the sperm might be dead.



fat pad



epididymis



cauda in NaCl



Sperm coming out of cauda

Oocyte collection

Phase I oocytes can be fertilized.
Phase II oocytes divide on their own.
Therefore the oviducts should be taken earlier than 8am.

P15

Put mineral oil in a 60mm dish.

To get cumulus – we dissect the oviducts (take ampulla).

Usually inject 15-20 female mice.

Theoretically:

20♀ mice → 200 two stage cells → retrieve 20 per mouse (10 mice) → 50 pups.

This also depends on the strain. When considering the amount of mice to superovulate, take into consideration the amount of embryo transfers you are capable of performing in one day!

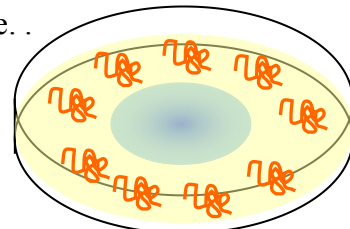
Make batches of 5 females.

1. Open body wall.
2. Cut peritoneum.
3. Cut part of the uterus + between the oviduct and ovary.
(ovary is red, then you'll see the oviduct and the uterus is next).
4. Collect cumulus in HTF medium –
tools required:
 - forceps
 - two needles
 - a. Put 300-400µl HTF medium in a 6cm plate.
 - b. Cover plate with mineral oil (keeps the medium pH).
 - c. Put the oviducts on the border. If you aren't going to put all immediately, the ones waiting should be kept at the 37°C incubator, closed.

Hold the uterus with the preparation needle. .

Break the ampulla.

Simply drag the oocytes with the cumulus into the drop.



from looking at the cumulus you can not know how many fertilized oocytes you'll be getting.

we just put 50% in each dish.

collect the oocytes into the fertilization dishes.

- d. Put fertilization dishes in the incubator for **4 hours** (for fertilization).


Procedure of In Vitro Fertilization

- Transfer the washed oocyte-cumulus-complexes in the fertilization dishes (already containing capacitated spermatozoa).
 - Incubate oocytes and spermatozoa for 4 hours (37°C, 5% CO₂).
 - After this time wash the oocytes of each fertilization dish in 3-4 drops (50µl each) of KSOM medium. Always make sure to first wet the pipette and only then pick up the oocytes. The oocytes should be pulled up by capillary pressure, do not suck them in. also, do not make bubbles..
 - After washing transfer the oocytes in 100µl KSOM medium covered with oil for overnight culture (37°C, 5% CO₂).

 - On the next day evaluate the amount of 2-cell embryos. Only embryos which have two symmetrical blastomeres are taken for the embryo transfer.
 - * you can look at the oocytes and start washing them from 08:00am, but transfer should be at 10:00am-15:00.
 - ** if you are unable to transfer the embryos, you can freeze them in 2cell stage. The problem – you loose 20-30% of cells. 2-4 cell embryos are transferred to the infundibulum.
 - *** for transferring in the next day – leave o/n in the incubator.

 - When looking at the oocytes observe:
 - i. How much dirt is there in the medium.
 - ii. Oocyte development.

 - For the embryo transfer the 2-cells **are washed three** times in drops of **M2 medium** (50µl each). Add approximately **10µl Hyaluronidase in the first drop** to remove remaining cumulus cells and spermatozoa on the surface.
- Some oocytes still have some cumulus remnants around them. It is important to clean them otherwise their development will be inhibited.*

A diagram showing a circular oocyte with several small dots representing cumulus remnants clustered around its base. An arrow points from the text above towards the oocyte.
- After washing transfer the oocytes in 100µl M2 medium covered with oil and put them on a warming plate (37°C) until you transfer them.

Embryo transfer

To complete their development embryos have to be transferred into the reproductive tract of a pseudopregnant female. Pseudopregnancy is important to guarantee that implantation can occur. It is as well indispensable to choose the right time of pseudopregnancy.

- Embryos from one cell to morula stage are transferred in the oviduct of 0.5-day post coitum females.
- Embryos at the blastocyst stage have to be transferred in the uteri of 2.5 day post coitum females.

Especially outbred and F1 females are suitable as recipients because they reliably raise even small litters.

Pseudopregnancy is generated by mating the females (8-10 weeks old, weight below 30g) to vasectomized or genetically sterile males. It is recommended to mate at least 10 females per planned embryo transfer. Two females can be mated to one vasectomized male. Without the possibility of fertilization a vaginal plug is produced which allows a precise timing of the mating (has to be checked until noon on the morning after the mating). Females which have no plug or are not need can be used again (females with plug 10-14 days after plugging).

Before surgery you can load the embryos in a mouth pipette (embryos should be in M2 medium). Try to minimize the time the embryos spend in the mouth pipette.

1. *Anesthetize the female.*

i. In the GSF the following solution is used:

1. 0.25ml **Rompum**⁴.
2. 0.5ml **Ketamine**⁵.
3. 4.25ml **NaCl 0.9%**.

250µl are injected to each mouse.

Anesthesia lasts for about 20 minutes.

ii. In Tel Aviv University we use Avertin solution:

1. 10 gr of **Avertin** (2,2,2 tribromoethanol, Fluka).
2. 10ml **Tert-amyl alcohol** (Aldrich) 1g/ml – dissolving solution.

mix for 30 minutes and filter, store in refrigerator wrapped in aluminium foil (stock). The stock solution is 100% and should be diluted to 2.5% with PBS or ddH₂O. As it tends to form crystals, shake well before diluting.

The stock solution is kept in a dark bottle at room temperature. The working solution, 25mg/ml (dissolved in ddH₂O or PBS), is kept at 4 °C, with a shelf life of up to one month).

⁴ Rompum 2% - Bayer, 25ml, (xylazine-hydrochloride).

⁵ Ketamine 10% - (ketamine-hydrochloride).

700 μ l are injected to each mouse.

Avertin solution 15 μ l/gr mouse at a working concentration of 25mg/ml.

After anesthesia, put a couple of Saline drops on the mouse eyes, so they do not dry during the operation.

2. Ovary, oviduct and a part of the uterus are exposed.
3. Open the bursa covering the oviduct and ovary with a fine spring scissor.
4. Expose the infundibulum and fix it with a small pad.
5. Introduce the capillary already containing the embryos carefully.
6. Expel the embryos in the infundibulum.
7. Return the ovary and oviduct carefully in the abdomen.
8. Close body wall with one stitch and close skin with a wound clip.
9. After surgery keep the mouse warm and undisturbed until it wakes up.

- Prepare operation.
- Put the culture dish with the fertilized oocytes on a 37°C heated plate.
- Draw some M2 medium into the pipette.
- Anesthetize female.
- **Put NaCl 0.9% drops in mouse eyes** to avoid their dehydration.
- Have the mouse lying on its abdomen; **legs and hands down away the sides of its body.**
- Make a short diagonal skin incision in medio-caudal to lateral-rostral direction.
- Cut the muscles and fascia under the skin incision using a clean pair of sharp scissors.
- Identify ovarian fat pad and draw it out of the mouse in a midline direction, the ovary, oviduct and uterus will follow.

For transfer to the fallopian tube:

- Place the mouse under the microscope in a position that will enable a clear view of the oviduct and ovary.
- For the majority of the manipulations, **use the fine forceps closed.**
- Identify the uterus connection to the fallopian tube.
- **Load 9-11 embryos** into the mouth pipette, and from now on keep the mouth pipette in your mouth, for the time **leave the pipette in a diagonal plane on the table.**
- The infundibulum lies on the exact opposite side, with its entrance facing caudo-anteriorly and towards the ovary. There is a small fat pad between the ovary and oviduct that lies rostrally to it.
- Make a tear in the membrane between the ovary and oviduct.
- Identify the infundibulum.
- Without using forceps of other hand (or by using them closed), reach the infundibulum with the pipette, remember the entrance might be facing towards the table, enter the infundibulum and inject the

fertilized oocytes. (before doing ‘the real thing’ it is advised to practice for a while on ICR females using blue loading buffer).

- Slowly and gently, without manipulating the fallopian tube, return ovary, oviduct and fallopian tube into the mouse.
- Close skin with a wound clip.

For transfer to the uterus:

- Prepare the mouse in a similar manner to the preparation for transfer to the fallopian tube.
- Place the mouse with the uterus facing away from you.
- Make a hole at the entrance to the uterus, using a 26G needle (hold the needle with your left hand).
- Hold needle inside the whole and draw the uterus upwards.
- Place the injecting end of the mouth pipette under the needle and enter the uterus while taking out the needle.
- Inject the fertilized oocytes into the uterus.
- Return organs into the mouse and close skin with a wound clip.



Embryo transfer –

E0.5 – two cell stage → oviduct (wall/infundibulum).

E1.5 – compact morula → uterus.

E3.5 – blastocyst → uterus.

Sperm quality and concentration

Sperm quality and concentration evaluation should always be performed.
There are small dishes that consist of a chamber and a cover disk.
Take aliquots of 15 μ l from the fertilization plate.

A concentration of 100 M/ml is good.
Motility should be higher than 60.
Progressiveness should be higher than 30.
These parameters are required to make sure sperm can be frozen.

If concentration is 100M/ml – put 1 μ l in IVF reaction.
If concentration is 50M/ml – put 2 μ l.

The same can be done with the frozen sperm.

PZD – Partial Zona Dissection

IVF using C57BL/6 mice does not work as efficiently as C3H mice IVF. The main reason is the relative ‘weak’ sperm of this mouse strain. In order to get around this limitations there are two possible approaches:

1. **ICSI – Intra Cytoplasmic Sperm Injection.**
Is by far the most effective way of doing IVF, every oocytes that had the sperm injected will be fertilized. Yet, this technique requires using a specific micromanipulator.
2. **PZD – Partial Zona Dissection.**
This technique involves **sticking the oocytes** to the bottom of a culture dish using a **hypo osmotic sucrose solution** that also causes an inflation in the perizonal space. Next, a **tear** is made in the zona, and the oocytes are **washed** and IVF continues as regular. Using this technique raises the fertilization rate from C57BL/6 sperm to 60-70%.
 - A. Take the oocyte-cumulus complex. Instead of putting it into a fertilization dish, treat it with hyaluronidase.
 - B. **Oocytes free from cumulus cells are introduced into a 100µl drop of 0.3M sucrose solution without BSA** on a dish. The oocytes are initially located in the top of the solution and then descend and attach to the bottom of the dish. This attachment is caused by difference of charge between the oocytes and dish surface.
 - C. **A single downward motion with 30G needle dissect** partially the zona pellucida of attached oocytes.
 - D. Following PZD, the electrostatic attraction of zona surface are neutralized by addition of **20µl of 0.3M sucrose solution with 4% BSA.**
 - E. To remove PZD oocytes from the dish surface, **the sucrose solution is sprayed to the oocytes by a micropipette.** Note, the flow of spray should be from opposite side of the split, to prevent the escape of ooplasm from the zona pellucida.
 - F. PZD oocytes are **washed gently 3 times in HTF** medium to remove sucrose.

Miscellaneous

- Mice should not be stressed during/before/after the hormone injections.
- The weather has an effect on the success of the IVF, usually does not work so well in the winter and the summer...

¹ The Transgenic Mollusc: Methods and Protocols; Chapter 3: In vitro fertilization/ Cryopreservation; Susan Marschall and Martin Hrabe de Angelis; Institute of Experimental Genetics, GSF Research Center for Environment and Health, 85764 Neuherberg, Germany.