## Mouse sperm extraction:

This method of extraction is used for acrosome reaction assays, immunocytochemistry and biochemical assays.

Collect two cauda epidydimus from one male, cut them 5 times and place them in a 2.0 ml eppendorf tube with 500 ul of warm Whittens-HEPES media (pH 7.2-7.4), incubate them at 37 C for 10-15 min (to allow sperm to swim out).

**For Capacitation**:

-Prepare Whittens-HEPES media supplemented with $NaHCO_3$ 30 mM and BSA 10 mg/ml (Sigma A0281, fatty acid free). This is a capacitation media 2X.
-Adjust pH to 7.2 – 7.4 with NaOH (one pellet per 1 ml $H_2O$ mili-Q).
-After swim out, add 1 volume of sperm to 1 volume of capacitation media and incubate at 37 C for 60-90 minutes.

Whittens HEPES Medium

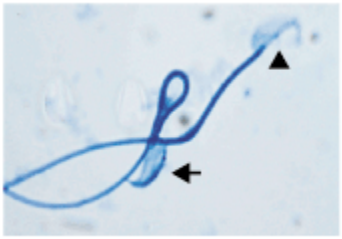
| Compound | Mol Weight | Final [mM] | Quantity (mg) for 250 ml |
|---|---|---|---|
| NaCl | 58.44 | 100 | 1461 |
| KCl | 74.55 | 4.4 | 87.6 |
| $KH_2PO_4$ | 136.1 | 1.2 | 40.8 |
| $MgSO_4$ | 120.4 | 1.2 | 36.1 |
| Glucose (dextrose) | 180.2 | 5.4 | 247.8 |
| Pyruvic Acid | 110 | 0.8 | 22.01 |
| Lactic Acid (hemi-Ca) | 109.1 | 4.8 | 130.92 |
| HEPES (acid) | 238.3 | 20 | 1191.5 |

Use Mili-Q water
Filter to sterilize and make 50 ml aliquots (use a fresh aliquot per experiment), store at 4 C.

## Mouse acrosome reaction

-**Ionophore induction**: After 1 h of capacitation, add 5-10uM of A23187, 8-Bromo (Calbiochem 100107, 2mM Stock) and incubate for additional 30 min.
-Take 20ul of sperm and fix with 20ul of formalin 10% (5% final concentration) for 10min at RT. Smear 15ul of sperm on slides and let dry.
- Put the slide in the Coomasie blue staining solution for 1 min. Wash, add 10ul of PBS-Glycerol 50% and cover. Assess for AR under phase contrast microscope.
**Alternatively:**
-For PNA staining permeabilize cells with 0.5% TritonX100 for 5 min at RT. Wash with PBS-T20.
-Block with PBS+0.1%T20+1%BSA for 2 h.
-Add PNA-Alexa 1/200 in blocking solution and incubate for 1 hour.
-Wash with PBST20, mount and assess for AR under fluorescence microscope.

**Coomasie solution:**
0.22% (m/v) Coomasie in 50%Methanol+10% Acetic acid.



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## Mouse IVF protocol

**Day 1:** Inject females with 5IU(100µl) of PMSG at 21:00 hrs.

**Day 3:** Inject females with 5IU(100µl) of hCG at 21:00 hrs. (44-48 hrs after PMSG)

a) Prepare Pyruvic acid 1000X concentrate (25 mg/ml) and add to media Wittens/$HCO_3$- and Wittens/HEPES (0.23 mM final concentration, 1ul of 1000X/ml of media)

b) Add $NaHCO_3$ :

| | | | |
|---|---|---|---|
| * for + bicarb (22 mM) | 1.9 mg/ml | (9.2 mg/5 ml) | (18.5 mg/10 ml) |
| * for + HEPES (7 mM) | 0.59 mg/ml | (2.9 mg/5 ml) | (5.9 mg/10 ml) |

c) Add BSA to the Media 15 mg/ml BSA (we use BSA fatty acid free, SIGMA).

d) Bring Wittens/HEPES media to pH 7.4

e) Add 40 µl 1M NaOH to 10ml of $HCO_3$/BSA Media

Sterile filter using Nalgene 0.2 µm syringe filter into new 15 ml tube and place the Wittens/HCO3-/BSA media in incubator 5% CO2 overnight to equilibrate.

- Prepare wells NUNC with 500 ul of media covered with mineral oil. As many as sperm treatment you have ( take in account that each well will have two epididymis).
- Prepare the wells for the dilutions with 450ul of media.
- Egg equilibration drop of 200ul.
- Prepare fertilization drops of 200ul each.
- Prepare post fertilization drops of 50ul each.

**Day 4:**

**8:00 AM**

a) To warm Whittens-HEPES medium

b) To prepare Whittens/HEPES/PVA medium containing 0.1% hyaluronidase (stock 100X stored at -20 ˚C) and place at 37˚C .

10ul hyal + 1000ul Wittens/HEPES/PVA/BSA

**9:00 – 9:30 AM** Sperm collection

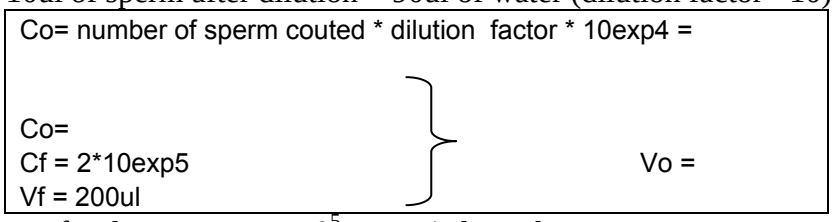
Sacrifice male (CD9 male, 3-8 months old). Clip cauda epididymes and upper portions of the vas deferens free from surrounding tissue. Using scissors and forceps, make 3-4 cuts across the epididymes and several cuts along the length of the vas deferens. *NOTE - sperm should not be stripped out of these tissues, but allowed to swim out instead (alternatively, squizze sperm out in 500 µl Whitten's-HCO3 under oil using forceps and needle). Place the dish in 5% $CO_2$ incubator for 10-15 minutes. Dilute sperm 1:10 (50 µl sperm + 450 µl medium) in Whittens/HCO3/BSA. Be sure to remove sperm

from very top or sides of sperm drop to obtain the sperm with highest motility. Return the dish to the 5% $CO_2$ incubator and allow sperm to capacitate for 1-3 hrs prior to use for insemination.

**9:30 AM** Capacitation begins.
**9:30 – 10:00 AM** Counting of sperm

Add 10 µl of the 1:10 sperm dilution to 90 µl water, mix gently, and place 10 µl of this under cover slip of hemocytometer. Count the number of sperm in the central 5 x 5 triple line-bordered box, or average the count in 4 boxes of that same size. Sperm count x dilution x $10^4$ = number sperm/ml.

10ul of sperm after dilution + 90ul of water (dilution factor =10)




For fertilization use $2x10^5$ sperm/ml per drop

**10:30 – 11:00AM** Egg collection

Sacrifice females (CD9, 6-8 weeks old, superovulated with PMSG then hCG 44-48 hr later) between 12.5-14 hr after hCG administration. Remove oviducts and place in large drop of Whittens/HEPES/PVA to rinse off blood and loose tissue. Transfer oviducts individually to a drop of Whittens/HEPES/PVA + hyaluronidase and puncture oviduct to release cumulus/egg mass. Place oviduct aside, and proceed to the next oviduct. When all cumulus masses are in the hyaluronidase drop, use a large bore pipette to assist mechanically in dispersion of the cumulus cells. As soon as the eggs are free of cumulus cells, wash through 2-3 drops of Whittens/HEPES/PVA and then place into Whittens/HCO3/PVA incubation drops.

**11:00 – 12:00** Fertilization starts

Place eggs in fertilization drops (Up to 200 eggs may be placed in a 500 µl fertilization drop). Inseminate using sperm capacitated for 1-3 hrs from the 1:10 dilution in Whittens/HCO3/BSA to obtain a final concentration in the fertilization drop of 2 x $10^5$ sperm/ml. Co-incubate eggs and sperm in a 5-5-90 chamber for 3-4 hr, and then wash sperm off eggs using a fine-bore pipet. After washing, place fertilized eggs in the post fertilization drops and incubate up to 6 to 7 hours total. In the same media the eggs can be incubated ON and then observe 2cell-stage.

**Criteria to assign fertilized eggs:**

- two polar bodies (at two hours) and two pronucleous (at four hours).
- Perivitelin sperm.
- Fused heads.
- Two cell-stage.

Whitten's IVF medium

**Stock:**

| | 100 ml | 250 ml | 500 ml | 1 liter |
|---|---|---|---|---|
| 109.5 mM NaCl | 640 mg | 1600 mg | 3.2 g | 6.4 g |
| 4.7 mM KCl | 35.6 mg | 89 mg | 178 mg | 356 mg |
| 1.2 mM $KH_2PO_4$ | 16.2 mg | 40.5 mg | 81 mg | 162 mg |
| 1.2 mM $MgSO_4$-$7H_2O$ | 29.4 mg | 73.5 mg | 147 mg | 294 mg |
| 5.5 mM glucose | 100 mg | 250 mg | 500 mg | 1 g |
| 4.8 mM L(+) lactic acid (hemicalcium salt; $C_3H_5O_3$-1/2 Ca) | 52.7 mg | 131.75 mg | 263.5 mg | 527 mg |
| Antibiotic cocktel Gibco BRL (10 mg/ml streptomycin) | 0.1 ml | 0.25 ml | 0.5 ml | 1 ml |
| Phenol Red (1% stock) | 0.1 ml | 0.25 ml | 0.5 ml | 1 ml |

**Wittens/HEPES**

| | | | | |
|---|---|---|---|---|
| 15 mM HEPES (acid) | 357 mg | 894 mg | 1.787 g | 3.575 g |

0.01% PVA
(polyvinyl alcohol, MW 30-70,000) 10mg/100ml

Filter both media in Nalgene Filterware (0.2 μm cellulose nitrate filter)

## Oocytes staining

1- Oocytes washing with PBS/0.1%BSA.
2- Fixation:. Mix 500ul of PBS/BSA with 500ul of PFA 7.4%. Place the eggs in drops of 3.7% Paraformaldehyde for 30 minutes at 4°C.

**Fixation solution**: 7.4% (74mg/ml) PFA stock at -80°C.

740mg PFA + 10ml PBS + 2drops of NaOH 5N ----→ warm up until solved.
Bring to pH=7.4, make aliquots of 500ul and store at -80°C.

3- Wash 3 times in PBS/BSA.
4- Incubate the eggs with Hoechst 33342 (final concentration 10ug/ml) for 10 minutes at RT. (Stock 1000X, dilute in PBS/BSA).
5- Wash 3times in PBS/BSA and mount in slides with mounting solution. Add drops in the corners of the cover-slip before mounting. Keep in dark and refrigerador.

## Culture of embryos *in vitro*

Females should be weeks old or more.
Male should be older than 10 weeks.

-Induce super-ovulation and put the male and female together at the same moment of injection of hCG. It is possible to mate 1 male with 2 females.

-Next morning check for the formation of the “plug” in the female vagina. If mating was successful, separate male from female.

-24 h after fertilization, morning after plug was observed, extract 2-cell embryos from female oviduct.

**Flushing of the embryos:**

-Prepare a tube with 9.5 ml of Whittens-Hepes and add 500 ul of FCS (aliquots of 500ul aliquots kept at -20C). Separate 500 ul of the WH-FCS for oviducts. And keep the rest at 37C
-Use KSOM-BSA to prepare incubation drops (30ul) and keep at 37C with 5% CO2 for at least 2 hs.
-Dissect oviducts from females (try to keep some of the uterus to facilitate the recognition of the ampulla path). Place oviducts in the eppendorf with 500ul of WH-FCS.
-Cut the tip of a 30G1/2 needles up to half its size. Fill up a syringe with WH-FCS and flush the oviducts. You should be able to see the fimbria in the ampulla path and flush the embryos out through that side.
-Incubate the embryos in KSOM drops for 24, 48, 72 and 96 h to obtain 4-cell, 8-cell, morula (12-32 cells) and blastocyst stage respectively.

KSOM

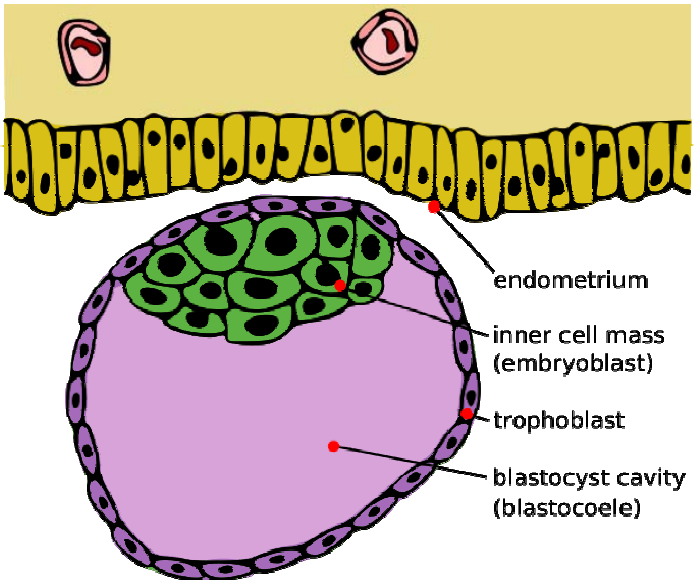
Stock A (10X)

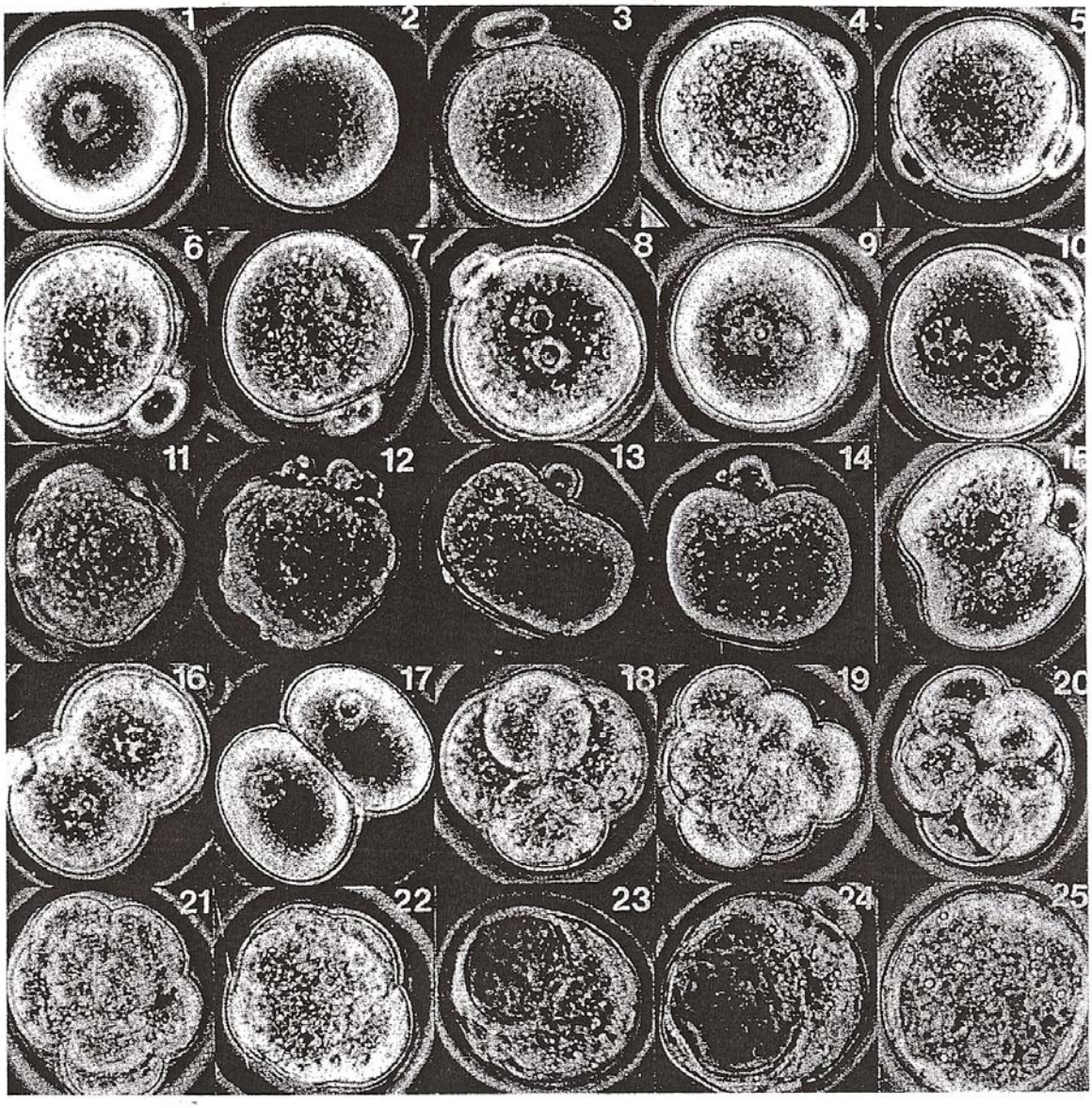
| | mM (final) | g/100ml |
|---|---|---|
| NaCl | 95 | 5.55 |
| KCl | 2.5 | 0.1864 |
| KH2PO4 | 0.35 | 0.0476 |
| MgSO4 (7H20) | 0.2 | 0.0492 |
| CaCl2(2H2O) | 1.71 | 0.2514 |

| | mM (final) | /100ml |
|---|---|---|
| Stock A | | 10 ml |
| NaHCO3 | 25 | 0.21 g |
| EDTA stock 500mM | 0.01 | 2ul |
| Glucose | 0.2 | 0.0036g |
| Glutamine | 1 | 0.0146g |
| Lactate | 10 | 143.6ul |
| Pyruvate | 0.2 | 0.0022g |
| Penicillin/Streptomycin stock | | 500ul |

Finally add BSA at 1mg/ml and then filter. Keep up to 2 weeks at 4C

You can keep Stock A in the freezer as 11 ml aliquots





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