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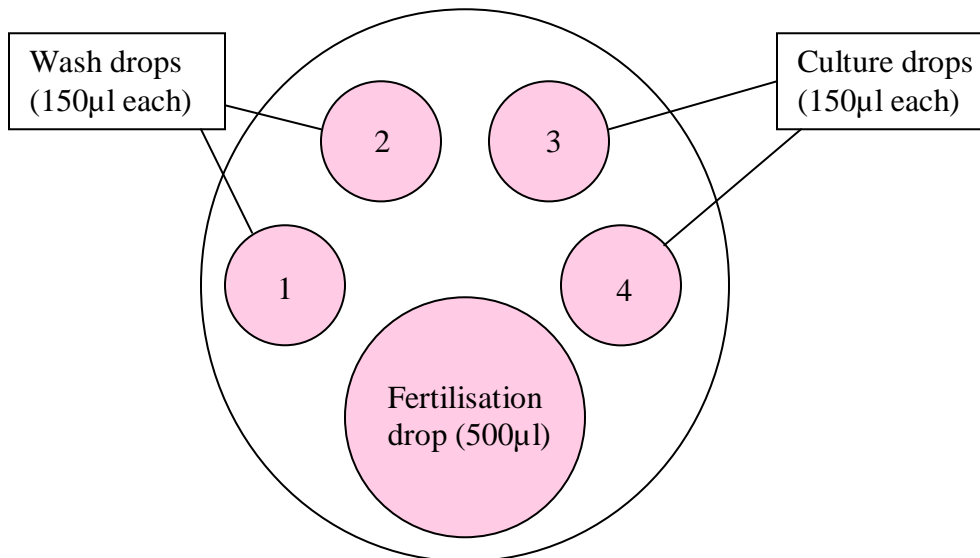
In vitro fertilisation of mouse oocytes using Cook's IVF medium

A. Preparation of oocyte harvest dishes

1. Add approximately 2-3ml of Cook's IVF medium (K-RVFE-50) to a numbered 35mm Petri Dish (Falcon 351008). One dish is required for every three superovulated females.
2. Place the dishes in the incubator overnight at 37°C, in 5% CO₂ in air to equilibrate.

B. Preparation of Fertilisation/Wash/Culture dishes

1. Prepare one 60mm Petri Dish (Falcon 351016) for every three superovulated females.
2. Into each dish, carefully pipette 5 drops of Cook's IVF medium as follows:
 - 1 x 500µl for fertilisation
 - 4 x 150µl for washing and overnight culture



3. Carefully overlay the drops with Mineral Oil (Sigma Chemical Co.; Cat. No. M8410, embryo culture tested) ensuring that they do not run together.
4. Equilibrate the dishes overnight at 37°C, in 5% CO₂ in air.



C. Preparation of sperm dispersal dish (for freshly harvested sperm)

1. Pipette 500µl Cook's IVF medium into the centre of a 60mm Petri Dish (Falcon 351016).
2. Overlay with Mineral oil and equilibrate overnight at 37°C, in 5% CO₂ in air.

D. Preparation of sperm samples:

Freshly harvested sperm

1. The selected male should be at least 8 weeks old, and not have been used for mating for at least 3 days before sperm collection.
2. Sacrifice the male and dissect the cauda epididymides.
3. Place on a clean paper tissue, and remove as much adipose and vascular tissue as possible, using size 5 watchmakers' forceps. Work under a dissecting microscope lit from above, dissecting quickly to prevent the material from desiccating.
4. Place the cauda epididymides into the oil next to the dispersal drop. Nick the tip of the cauda epididymides with a pair of fine scissors. Gently palpate the tissue to expel the sperm.
5. Drag the blebs of sperm into the dispersal drop.
6. Allow the sperm to disperse into the medium for between 10 and 90 minutes, at 37°C in the CO₂ incubator.
7. Place IVF dishes on a heated stage or hot pad, then pipette 10µl of the sperm suspension into each fertilisation drop using a wide-bore tip and return the dishes to the incubator until the oocytes are harvested.

Cryopreserved sperm

1. Using forceps, hold the cryotube in air for 30 seconds. If liquid nitrogen is present in the cryotube, wait for it to evaporate and escape by rolling the cryotube around on the bench.
2. Take **special care** that the cryotube is not filled with liquid nitrogen before plunging into the water bath (such tubes may explode).
3. Thaw the sperm sample rapidly by placing in a 37°C water bath.
4. Once thawed, pipette 10µl of the sperm suspension into each fertilisation drop using a wide bore pipette tip and return the dishes to the incubator.





E. Harvesting oocytes

1. Dissect the oviducts from three superovulated female mice and place into a preincubated dish of Cook's IVF medium (for superovulation methods, see the protocol for harvesting and cryopreservation of *in vivo* derived embryos and the table below).
2. Under a dissecting microscope, hold each oviduct down with forceps and gently tear the swollen ampulla with a second pair of forceps to release the cumulus masses into the Cook's IVF medium, then remove the oviduct from the dish.
3. When all the cumulus masses have been extracted, gently draw them up into a wide bore pipette tip in a maximum of 30µl Cook's IVF medium. To stop them from sticking to the plastic, first wet the inside of the pipette tip by drawing up and expelling some Cook's IVF medium. Hold the pipette close to vertical to ensure the cumulus masses are aspirated with a minimal amount of medium.
4. Transfer the cumulus masses to a fertilisation drop (containing sperm) being careful to transfer as little Cook's IVF medium as possible.
5. Incubate the dishes at 37°C, in 5% CO₂ in air for approximately 5-7 hours to allow fertilisation to occur.
6. Repeat steps 1-5 for each fertilisation dish in succession (i.e. complete all of the steps from collecting the oviducts to returning the fertilisation dishes to the incubator for one batch of females before starting the next batch). Aim to take no more than 5 minutes from collecting the oviducts to returning the fertilisation drop (including oocytes) to the incubator.

F. Washing and culturing the fertilised oocytes

1. Between 5-7 hours after the cumulus masses were placed in the fertilisation drop, remove all of the oocytes and place them in wash drop 1 (see diagram).
2. Move the good quality oocytes from wash drop 1 to wash drop 2, cleaning the oocytes as much as possible in the process. Leave poor quality oocytes in wash drop 1.
3. Divide the washed oocytes approximately equally between the two culture drops (3 and 4).
4. Incubate overnight at 37°C, in 5% CO₂ in air.





G. Preparing fertilised oocytes for embryo transfer or freezing

1. Next morning, separate the 2-cell embryos from those which have not fertilised or cleaved, or have degenerated. Place all the 2-cell embryos in drop 4 and the 1-cell or degenerated oocytes/embryos in drop 3.
2. Prepare a drop of hyaluronidase solution (300µg/ml made up in M2) in a Falcon 351008 petri dish. **NB: This hyaluronidase washing step is only required when it is necessary to remove the adherent cumulus cells from the zona pellucida.**
3. Collect the 2-cell embryos into a drop of M2 in a Falcon 351008 petri dish.
4. Transfer these embryos into the drop of hyaluronidase.
5. Incubate at 37°C for a few minutes with gentle agitation at intervals using a glass pipette until any adherent cells or sperm have fallen off.
6. Wash the embryos through two drops of M2.
7. Either transfer the 2-cell embryos to the oviducts of 0.5d pseudopregnant foster mothers, or:
8. Prepare the 2-cell embryos for cryopreservation according to the standard protocol for *in vivo* derived embryos, or:
9. Culture the embryos in KSOM.

Timetable of events for IVF

Day -3 (e.g. Saturday)	Day -1 (e.g. Monday)	Day 0 (e.g. Tuesday)	Day 1 (e.g. Wednesday)
Superovulate between ten and thirty 3-4 week old females by injecting 0.1ml (5iu) PMS at 17.30-18.00.	Prepare dishes for oocyte harvest, fertilisation/wash/culture and sperm dispersal (if using a freshly harvested sample).	07:45 Thaw cryopreserved sperm sample, or collect and disperse freshly harvested sperm.	Morning: score the IVF success (2-cell vs others).
	Induce ovulation in the females by injecting 0.1ml (2.5iu) hCG at 18:00	08:00-09:00 Harvest oocytes and place into diluted sperm preparation.	Prepare the 2-cell embryos for cryopreservation, embryo transfer or culture.
		15:00 Wash the presumptive zygotes and place into culture drops.	

This timetable assumes that the mice are exposed to 12 hours of darkness between 19:00 and 07:00.

