IVF CARD Protocol (Preferred Method)

Reagents, supplies, and equipment:

- PMS
- hCG
- 1ml syringes for hormone injections
- FERTIUP (sperm preincubation medium: Cat# KYD-002-EX Cosmo Bio Co)
- CARD MEDIUM (fertilization medium Cat # KYD-003-EX Cosmo Bio Co)
- mHTF (incubation medium Cat# KYD-008-02-EX)
- Mineral oil (Sigma embryo tested M5310-100ml)
- Pipetman
- Pipette tips for preparation of dishes
- Wide bore pipette tips
- Plastic bulb transfer pipets
- 60mm culture dishes (Fisher 0877221)
- Dissection instruments (medium scissors, fine scissors, fine #5 forceps X2, serrated fine forceps)
- Glass capillaries for embryo handling
- Microscope
- 5% CO2 incubator
- Mice (4-6 females per fertilization dish)

**Keywords:** CARD in vitro fertilization Rederivation IVF

**IVF procedure:**

1. Day 1: Superovulate with 5IU PMS between hours of 2:00 and 6:00 pm
2. Day 2 hCG 48-52 hours following PMS injection with 5IU hCG. Place beaker of distilled water in incubator for overnight warming.
3. Day 4/Day of IVF: Prepare CARD Medium – Note that it is different prep according to type of sperm.
4. Prepare dishes and place in incubator:
   - Sperm dish – one culture dish for each straw/vial of sperm = 100 ul of FERTIUP into culture dish, cover with mineral oil and place in incubator 30 minutes before thawing sperm.
   - Fertilization dish – one culture dish for every 4-6 females. Put 200 ul of CARD MEDIUM into culture dish, cover with mineral oil and place in incubator 10 minutes before sacrificing the first female.
   - Washing Dishes – one culture dish for each fertilization dish. Put (4) 80ul drops of mHTF into a culture dish and cover with mineral oil. Place in incubator for at least 30 minutes. These culture dishes can be made when finished with insemination.
   Alternatively, RVFE (K-RVFE-50 Cook Medical) has been used successfully.
5. Collection of Oocytes:
   - Sacrifice one female (approx. 15-17 hrs after hCG).
   - Dissect the mouse to expose the ovaries/oviducts.
   - Remove the oviducts, avoiding as much fat, blood and tissue fluid as possible.
   - Place in the oil of the Fertilization (CARD) medium dish.
   - Use the #5 forceps and tear open the oviduct and release the cumulus cell-oocyte complexes (COC). Drag the COC to the drop of CARD medium and return dish to incubator. See notes on hints. Incubate for 30-60 minutes after oocytes have been added to dish.
   - Continue this process with the remaining females.
6. Thaw sperm
   - Remove a frozen straw of sperm from the liquid nitrogen and hold in the air for 5 seconds.
   - Immediately immerse straw in the pre-warmed beaker of water and leave it for 10 minutes.
   - After 10 minutes, remove the straw from the water, wipe down with alcohol, and carefully cut both ends of the straw. With a syringe or paperclip, expel the sperm portion into the FERTIUP drop and place dish back into the incubator for 30 minutes.
Insemination

1. Use a pipette to add 10 ul of the sperm suspension to the fertilization dishes. Remove the sperm from the edges of the FERTIUP drop as that is the most motile sperm and deposit on top of the cumulus cell masses.
2. Return dish to incubator.
3. In 3 hours, wash the oocytes 3 times in the mHTF drops. Leave the oocytes in the third drop.
4. In another 3 hours (6 hours post insemination) observe and discard infertile oocytes.

8. Incubate culture dishes overnight.
9. The following morning, transfer the two cell embryos to the fourth drop of mHTF. At this point they can be cryopreserved or transferred to oviducts of 0.5 dpc pseudopregnant recipients.

Variations depending on sperm source

**Fresh sperm:**

1. Sacrifice male mouse and remove cauda epididymides, avoiding as much fat, blood and tissue fluid as possible.
2. Place the removed cauda epididymides in the oil surrounding the sperm (FERTIUP) drop.
3. Cut the duct with small sharp scissors and gently press on the surface of the cauda to release the sperm.
4. Using the sharp forceps drag the clots of spermatozoa into the drop of FERTIUP.
5. Allow the sperm to capacitate by placing the suspension in the incubator for 60 minutes before insemination.
6. Inseminate COC’s with 3 ul of sperm suspension. Proceed with step 7 of the IVF protocol.

**Sperm in vials: see variation in notes below**

1. Follow steps 1 through 5 of the IVF protocol.
2. Remove vial from LN2. Open the cap and carefully discard any LN2 that is in the tube. Place vial in a 37° water bath for 10 minutes.
3. Transfer the sperm suspension using a wide bore pipette tip from the cryotube into a 1.5 ml tube. Slowly add 1.2 ml of warmed mHTF to the tube and centrifuge it at 300g at room temperature for 5 minutes.
4. After centrifugation, remove as much supernatant as possible. Add 70 ul of warmed FERTIUP to the tube. Final volume is approximately 100 ul.
5. Gently transfer all of the contents of the vial using a wide bore pipette tip into the 100 ul drop of FERTIUP (sperm dish). Place the dish in the incubator for 30 minutes.
6. Using a wide bore pipette tip, collect the pre-incubated cumulus/oocyte complexes with a minimum of CARD medium and release into drop of sperm suspension.
7. After three hours, proceed with step 7c of the IVF protocol.

**Notes:**

1. The CARD medium is prepared according to the sperm source – pay close attention to that detail and follow manufacturer’s directions.
2. Sacrifice the females one at a time and work as swiftly as possible to get them into the CARD medium and back into the incubator promptly.
3. Do not disturb sperm dishes while they are incubating prior to using them for insemination or the sperm will not attain full motility.
4. There is a third sperm method that uses sperm from thawed vials and I have found it to be more effective. Thaw sperm as directed in #2 above, using thawed sperm in pre-incubated FERTIUP drop. Proceed with the insemination step above after the 30 minute incubation. ** This is according to the CARD book provided by Cosmo Bio.