MBCD-GSH IVF Procedure (Standard MMRRC IVF)

IVF is a very tricky procedure. Our sense is that you are walking a knife-edged path of success, with an abyss of failure yawning on both sides. It doesn't take much to tumble from the straight and narrow. We suggest that you follow these instructions as **closely** as possible. Some of the issues in the success of IVF are:

- **Zona hardening.** The zona pellucida will, with time, become impenetrable to sperm. The longer the eggs sit in medium, the harder the zonas will become. Work fast when dissecting the eggs! This is why our IVF must be finished by 1.5 hours after female dissections start.
- **Temperature is critical**. Keep everything at 37°C as much as possible. Work quickly so that fertilization dishes have minimal exposure to room temperature and atmosphere. Always keep dishes on the microscope heating stage.
- **pH is critical.** The pH of your medium will begin to rise as soon as it is exposed to air. Try to reduce time dishes are outside of the incubator (10 min or less ideally, 20 minutes is the cut off).
- **Volatile organic compounds (VOCs)** in the air of the laboratory can greatly affect success of IVF and the viability of embryos after transfer. Do what you can to maintain good air quality. VOC's include perfume/cologne/scented lotions and shampoos. Many disinfectants are labeled "embryo toxic".
- In our hands, IVF has also failed due to bad media, bad gas, bad oil and bad luck.
- You should be able to obtain a fertilization rate (the percentage of oocytes that develop to the 2-cell stage) of >90% when using sperm and oocytes from an F1 strain such as B6D2F1 or CB6F1.
- Fertilization rates from frozen sperm will vary by strain. Thawed sperm from F1 strains will do as well as fresh strains, but inbred strains often will not do well.
- **Sperm are fragile.** Minimize shear forces by pipetting them gently using only wide-bore tips. Extended centrifugation also reduces viability.

At the end of the SOP, there is a sample IVF schedule, worksheet and timing sheet (with an explanation on timing).

Weekend Hormone Injections – TIMES ARE EXTREMELY IMPORTANT FOR IVF SUCCESS

Dosage	HyperOva (AIS)	hCG	PMS (HyperOva substitute)
½ dose	0.05cc	2.5 IU (0.05cc)	2.5 IU (0.05cc)
Full dose (dose used unless noted otherwise)	0.1cc	5.0IU (0.1cc)	5.0IU (0.1cc)
1.5 dose	1.5cc	7.5IU (1.5cc)	7.5IU (1.5cc)

(Usually Friday/Sat nights) HyperOva (HO) or PMSG (Pregnant Mare Serum Gonadotropin) Injection
Females must be injected i.p. at 7:30 PM. The dose is strain dependent and it ranges from 2.5 IU to 5.0 IU. Unless it is noted on the IVF schedule, give a dose of 5 IU (0.1cc) to each mouse. Label each cage card "H.O and date" or "PMS and date" in large print.

(Usually Sun/Mon nights) hCG (Human Chorionic Gonadotropin) injection:
Inject mice with 2.5 IU or 5.0 IU i.p. 46-48 hours after H.O./PMSG. Unless it is noted on the IVF schedule, give 5 IU (0.1cc) to each mouse. It is much better to inject earlier than to go past the 48-hour mark. Inject in the exact same order as H.O./PMS was given. Label each cage card with an "H" in different color ink than the H.O./PMS notation.

- If there is a change in mouse numbers discovered while doing injections, note the change on the IVF schedule.
- If mouse cards are not marked with "H.O. or PMS" and "H", it is assumed that they were not injected. When in doubt, do not use mice in question. Females can be used for future IVF after a 2-week wash out period.
- Never inject hormones into a male mouse as it will drastically decrease their sperm count.
- **Sunday/Monday nights before injections – place media in upper LB incubator (loosely capped)**

The rest of the SOP is for an IVF on Monday (injections done at 7:30pm Fri/Sun)

Monday (Day 1)
7am: Media and Culture Dish Preparation

Media Preparation
See pages 14-15 for media preparation SOP.
See Media Cheat Sheet for storage conditions, etc.

MBCD media

- Needs to be pre-equilibrated in the incubator at least 30 minutes prior to use. Excess media is stored in the refrigerator (up to 2 weeks after thaw date). Only enough media for the IVF day(s) should be pre-equilibrated (remainder is tossed at the end of the week).
- **Do not shake this tube.** CaCl2 can precipitate out and will settle at the bottom of the tube. If precipitate is noted in a dish, a new dish must be made with new media as the precipitate is harmful to sperm.

HTF media

- Needs to be pre-equilibrated in the incubator. Excess media is stored in the refrigerator (up to 2 weeks after thaw date). Only enough media for the IVF day(s) should be pre-equilibrated (remainder is tossed at the end of the week).

GSH needs to be added to HTF before IVF/QC dishes are made

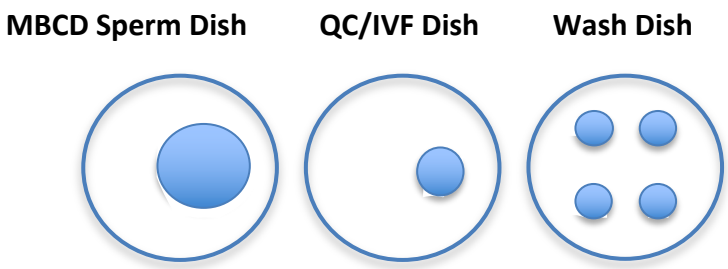
- Thaw an aliquot of 100mM GSH (Stored in the freezer).
- Add GSH to equilibrated HTF (final concentration of GSH is 1mM) in a 5mL falcon tube. Invert a few times to mix.
    - Ex: add 25uL GSH to 2.5mL pre-equilibrated HTF.
- Use immediately to make IVF/QC dishes.
- Once all IVF/QC dishes are made, place any remaining modified HTF+GSH in the incubator loosely capped (remainder is tossed at the end of the week).

Dish Preparation
Dishes must be prepared at least 30 minutes before the IVF with pre-equilibrated media or the afternoon before the IVF.
See Dish Cheat Sheet for quick guide (posted on hoods and at the end of the SOP).

The following types of dishes are used:

Dish Name	Media	# drops	#ul per drop	# to make
Fresh Sperm	MBCD	1 to side of dish	750	1 dish per strain (live male) on IVF schedule
Frozen Sperm	MBCD	1 in center	90	1 dish per cryo sample on IVF schedule
IVF Fresh Sperm	HTF + GSH	1 to side of dish	250	# dishes indicated on IVF schedule
IVF Frozen Sperm	HTF + GSH	1 to side of dish	200	# dishes indicated on IVF schedule
Wash Dish	HTF	4	150	1 dish for each IVF dish

- All of the above examples are made with 60x10mm IVF dishes (green sleeve)
- Cover all with oil once drops are made
- See Tips section (next page) for dish making tips



Mineral oil needs to be pre-gassed (left with cap loosened in the incubator for 24-48 hours) before use in dishes. Keep at least 2 bottles of mineral oil in the incubator at all times. Mineral oil (pre-gassed) is added to cover the drops using a 10mL or 25mL pipette and electric pipetter, use just enough oil to cover drops. Too much oil in dish and you risk sloshing the oil over the rim when transporting dishes. Place dishes in the top incubator at 37° C in an atmosphere of 5% CO_2. Always be very careful when transporting dishes, removing or placing dishes in incubator, putting them on or picking them up from microscope stage to avoid sloshing of oil or media which would cause drops to be of the wrong volume.

What to do with the dishes once they are made (suggested):

Top Incubator	
Top Shelf	Extra media/IVOS tubes and Mineral Oil
Middle Shelf	IVF/Wash dishes (have sperm/COC's) and 2-cells while dishes are being counted, etc.
Bottom Shelf	Empty IVF/Wash dishes (no sperm/COC's added) and MBCD dishes (with or without sperm)
Bottom Incubator	
Top Shelf	All 2-cells (Once finished counting)
Middle Shelf	Embryo QC/ET thaws
Bottom Shelf	Not used

- Always place dishes in the 100mm dishes already in the incubator. This helps keep the incubator clean and prevent possible cross-contamination.

Other Media/Tubes that need to be made at this time:

- **Male tract collection tubes**: sterile 1.5mL snap-cap tubes filled with pre-gassed mineral oil. Write the strain name on the top and mouse # on the side. Prepare 1 tube per strain using live male(s). Place in incubator (hallway in LB for GMB IVF, or 5th floor dissection room if MBRB IVF).
- **Female tract collection tubes**: sterile 50mL conical tubes with 4-6mL of pre-gassed mineral oil. On the top write the order in which the tubes will be used. For example, the 1st tube will have the #1, the 2nd tube to be used will have the #2 and so on. Write the # of females and the strain name of the female mice (this can differ from the male strain) on the side of the tube. Place in incubator (hallway in LB for GMB IVF, or 5th floor dissection room if MBRB IVF).
- **IVOS tube** (only for live males): place 200uL pre-equilibrated HTF in 1.5mL snap-cap tube. Record strain name on tube as well as "IVOS". Tube needs to remain uncapped in incubator.

Tips:

- Wipe down the surface of your hood with EtOH just before placing the dishes down. This will eliminate the static electricity, which will cause your drops to splay. May need to re-wipe with EtOH between sets of dishes.
- Different lots of dishes perform differently. If you are having trouble after the 2nd drop, get a new dish. Try to waste as little media as possible.
- Place media in 2-4 dishes, then cover those with oil. Don't work with too many at one time – when the media warms up the drops will get bigger. Change the pipet tip between sets of dishes.
- Try to space drops evenly throughout the dish and keep them from getting too close to each other and the dish sides.
- When covering with oil, point the tip of the pipet at the side of the dish so you don't distort the drops of media.
- Always be very careful when transporting dishes, removing or placing dishes in incubator, putting them on or picking them up from microscope stage to avoid sloshing of oil or media which would cause drops to be of the wrong volume.
- Tightly cap tubes immediately when removing from the incubator. They should remain tightly capped when outside of the incubator, except when actively pipetting.
- Don't lay tubes on their sides. Please use tube racks to hold tubes upright in the hood.
- Make your dishes quickly – 10 dishes shouldn't take longer than 5-7 minutes. The longer the tube of media is exposed to the air, the more the pH will change and allow precipitate to form.
- Check your dishes under the microscope before putting in incubator and before using/adding tracts/sperm

Sperm Collection – Timing is very important. Remember to take all the prep time into consideration.

Fresh Sperm/Using a live male

Capacitation time (CAP time) = 60-90 min.

Epididymide dissection should be finished at the time listed on your IVF timing sheet. This time is not when to start dissecting.

- It can be detrimental to the fertilization rate if COC's and sperm are combined before the 60-minute mark or after the 90-min mark.
- Set up all your materials (mouse room and IVF hood) before euthanizing the mouse. Everything needs to be set up to ensure the quickest dissection as possible. Keep the male tract tube in the incubator as long as possible.
- Aim to keep the time when you dissect out the epididymides to placing them onto the heating stage under 2-3 minutes.

Tools/Materials Needed

Mouse Room:

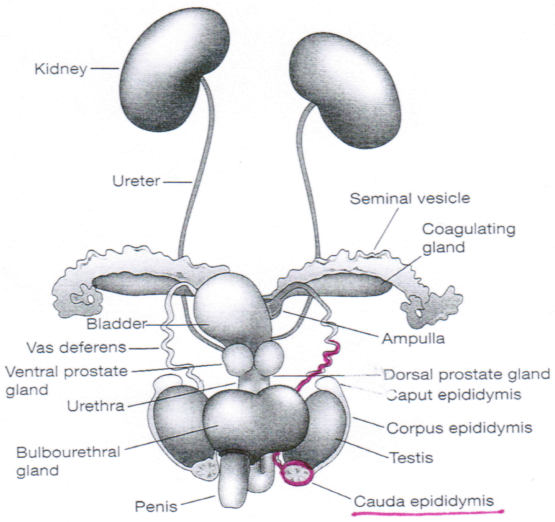
- 1.25 % TBD/Avertin and syringe (optional)
- Dissection kit
- Find the mice
- Male tract tube (in mouse room incubator)
- EtOH
- Test tube holder

IVF Hood:

- One 35mm dish (label with strain) per strain
- IVF dissection tools
- Heating stage and microscope on
- Timer
- 0-20uL pipette
- Yellow pipette tips (0-200uL)
- Kimwipes

Gross Dissection in Mouse Room:

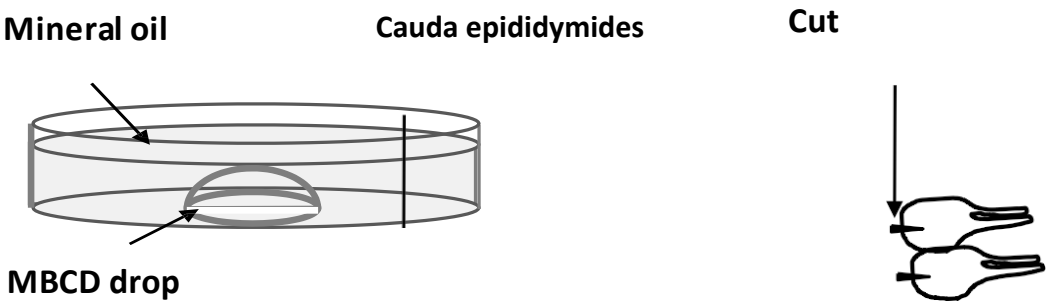
1. Double check male number on cage card against IVF schedule/timing sheet and mouse toe numbers. (This should also be done the week prior to IVF).
2. Administer 1.25% TBD/Avertin i.p. (optional), sacrifice the male by cervical dislocation, dissect out the epididymides along with the vas deferentia and place into the male tract tube. Be careful to remove as much fat and extra tissue as possible.
   a. If there are any abnormalities, please try to still dissect both epididymides. Examine both under the scope and use the tissue that appears normal.
   b. Note all abnormalities on the IVF time sheet.
3. If there are 2 strains to be done, dissect both at the same time. Be careful to keep them separate and be confident you know which strain is which.
4. Clean up the hood and room while the sperm is swimming out into the MBCD media. You need to clean up within 30 minutes of dissection. It is against IACUC regulations to leave dead animals in the rooms.
5. If someone is doing the gross dissection for you, please wait at mouse room door for the tracts ~5 minutes before your CAP time on the IVF timing sheet.



Male urogenital system

Right: Gross dissection of the epididymides from the male:

Below is the dissection under the scope:



Dissection in IVF Hood:

1. Place the tube(s) in the hallway incubator and change your gloves. Gloves that have been used in an animal room are not allowed to be used in the procedure room hoods.
2. Pour all contents of tube into the labeled 35mm dish. Do this for both males if 2 are being done at once.
3. Get a MBCD dish out of the top incubator and place on stage.
4. In the 35mm dish, dissect off any remaining fat around the epididymis.
    a. Quickly place tissue into the mineral oil on the left side of the MBCD dish.
    b. IF both epididymides look small or like they don't have any sperm, you can decrease the amount of your MBCD drop before adding sperm. This will keep the sperm suspension from being too dilute and decreasing fertilization.
        i. Can also dissect the epididymides directly into the MBCD drop and allow the sperm to swim out. After 15 min, remove tissue and discard
        ii. Note on the IVF timing sheet.
5. Repeat with 2nd epididymis.
6. Cut the epididymides, making 2-3 cuts with the IVF scissors at the apex of the epididymis. Gently pull out 1-2 droplets of sperm from each epididymis and guide them into the MBCD drop. Be careful not to drag them on the bottom of the dish. Doing so will create a trail for the media drop to splay.
7. Removed the tracts from the mineral oil and discard them in the trash.
8. Record the time on the MBCD dish lid (this is the **start of capacitation time**). This is very important.
9. Place the dish in the incubator in the area designated for sperm dishes.
10. Record the time on your IVF timing sheet as well as all other pertinent information.
11. Wipe tools with Kimwipe and EtOH to remove any sperm that may have stuck to the tools.
12. If there is another strain to be done, repeat steps 3-11.

IVOS sperm sample – For more detail, see IVOS SOP

1. At least 15 minutes after dissection into MBCD, expel air bubble into MBCD/sperm suspension and aliquot 10uL sperm from edge of drop.
    a. Be careful to keep pipette as upright as possible to prevent mineral oil from being drawn up instead of sperm.
2. Wipe oil off of pipette tip.
3. Expel sperm into IVOS tube (200uL mRVF).
4. Place uncapped IVOS tube back in incubator for at least 15 minutes to allow sperm to disperse.
5. Use IVOS to sample sperm at (1:20 dilution). See IVOS protocol for more information.
6. Calculate amount of sperm to add to each IVF drop.
    a. We want to add about 0.2M/mL sperm to each drop using the Total and Progressive concentrations for your calculations.
7. Record calculations and IVOS results on your IVF timing sheet.
8. Repeat steps to analyze next sample.
9. Your IVOS analysis can be done at a later time if needed (i.e. error in IVF timing) as long as the analysis is finished prior to sperm being added to the IVF dishes.

Frozen Sperm Preparation:

Capacitation times are shorter than fresh sperm. The capacitation time will depend on the method that the sperm was frozen. **For all frozen sperm, it is better to err on the shorter side of the capacitation time. If timing is such that you should be prepping frozen and fresh sperm at the same time, do the fresh first.**

Straws frozen with the JAX or Nakagata methods:
Ideal capacitation time: 40 minutes (acceptable range of 30-50 min.)
You can thaw up to 3 straws at the same time.

1. Move appropriate lab tank to Procedure Room B, next to the water bath.
2. Clean scissors (located in hood) with EtOH and place on clean Kimwipe.
3. Remove straw from lab tank and let stand in air for about 10-30 seconds. This prevents the straw from exploding once it is placed in the water bath.
    a. If you have another straw to thaw at the same time, place 1st on the counter and removed the next straw.
4. Slowly lower the straw(s) into water bath (37°C) until contents are thawed (about 30 seconds).
    a. Some CPA may leak out of the straw. This is normal for straws with weights, as most sperm straws with media weights fracture during the freezing process.
5. Remove MBCD dish from top incubator and place on heated stage.
6. Take straw(s) back to hood. Wipe the excess water off the straw(s). Be careful if the straw is fractured.

    If the straw has no weight, use the following expelling procedure:
    1. Cut the straw with scissors in the middle of the PVA plug while holding the straw sideways.
    2. Cut the Critoseal plug off the end of the straw at a 45-degree angle.
    3. Using a straw plunger, slowly plunge the sperm fraction into the 90uL MBCD drop.

    If the straw has a media weight (either M2 or CPA), use the following expelling procedure:
    1. Cut the straw with scissors in the middle of the large media fraction (below the plug and below the fracture if possible), hold the straw on either side of where you will cut.
    2. Cut the Critoseal plug off the end of the straw at a 45-degree angle.
    a. You must keep the straw horizontal or the sperm and media will fall out of the straw (since there is no plug holding them there).
    3. Using mouth pipette, expel only the sperm fraction into the 90uL MBCD drop, use mouth control to avoid expelling the large fraction from the straw.
7. Record the strain name, CS# and time on the dish lid. This is the **start of the capacitation time**.
8. Place dish in the incubator.
9. Record the capacitation time and all pertinent info on your IVF timing sheet.

Nunc Vials:
Ideal capacitation time: 30-50 minutes

1. Take vial out of lab tank and place in 37°C water bath for 10 min. Use Styrofoam floater to keep top of vial out of water.
2. After 10 minutes, wipe vial clean and place in IVF hood.
3. Pipette out all of the sperm and place into an empty 1.5mL snap-cap tube.
4. Add 1.2mL pre-equilibrated HTF to tube.
5. Centrifuge tube at 300g for 4 min. in the mini centrifuge on counter in 30D.
6. Once done, immediately remove as much supernatant as possible. Be careful not to draw up any of the sperm pellet (should be visible at the bottom of the tube).
7. Slowly add 100uL pre-equilibrated MBCD to tube. This is the **start of your CAP time** – be sure to record on the IVF sheet.
8. Place tube (uncapped) in incubator for 20 min.
9. After 20 min., pipette out all of the sperm suspension and create a single drop in an empty 60mm IVF dish. Cover with oil.
    a. Necessary to visualize sperm motility and draw up motile sperm (from outside of drop).
10. Label dish with strain name, CS# and time added to MBCD on dish lid.
11. Place dish in the incubator.
12. Record time and all pertinent data on your IVF timing sheet.

Collect COC's (Cumulus Oocyte Complex): Timing is very important. Remember to take all the prep time into consideration.

Another person will most likely do the gross dissection of the female reproductive tracts. This keeps the IVF within correct time parameters. **(The last group of COC's should be added to the IVF dish by 14.5-15 hours post hCG).**

- Female tract dissections start 13.5 hours post hCG injections. If hCG injections started at 7:35pm the previous night, female dissections will start at 9:05am. This timing is very important.
- Female dissections are done in the order on the IVF schedule (in the order hCG was given).
- See sample chart at the end for timing examples.

Below is what the dissection of female reproductive tracts looks like:

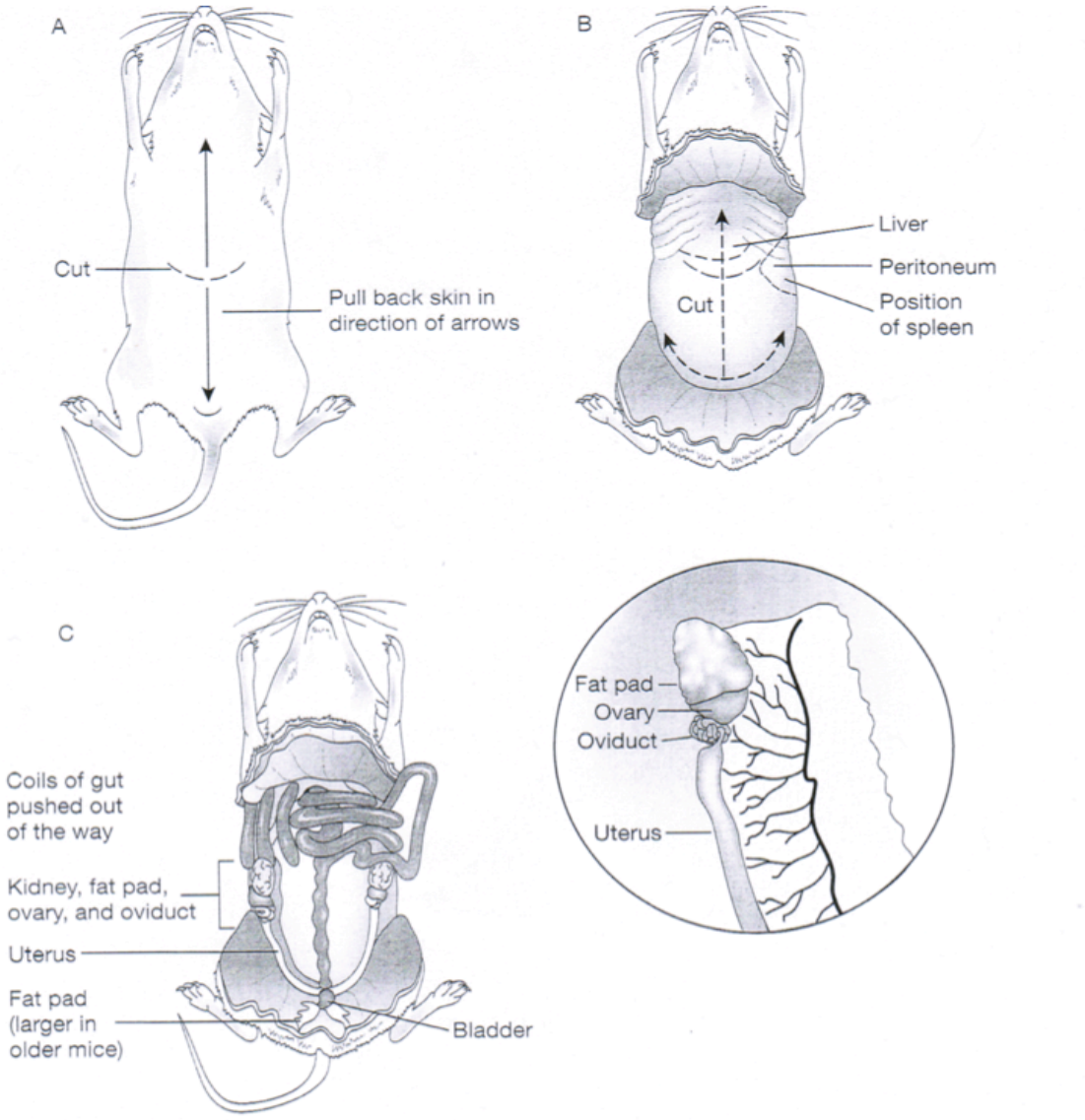
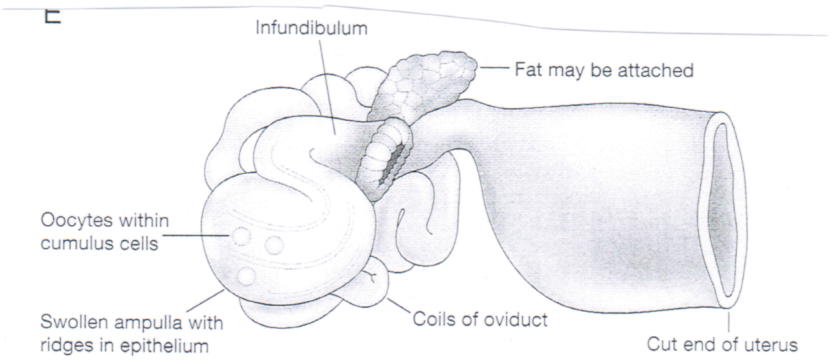


FIGURE 4.4. Dissection of reproductive organs of a female mouse. (*A*) The position of the small lateral incision in the skin is indicated by the dashed line. The skin is then pulled back in the direction of the solid arrows. (*B*) The body wall (peritoneum) is cut in the direction of the dashed arrows. (*C*) The alimentary tract displaced to reveal reproductive organs in the floor of the body cavity.

Below is what the tracts look like under the scope.



What you will need (per group of females):

- 60mm dish (blue sleeve)
- IVF dissection tools
- Heating stage and microscope on

Once you have the tube with the female tracts….

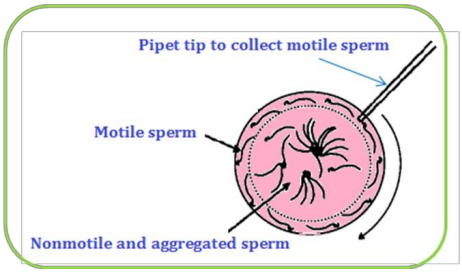
1. Pour all contents of the tube into the 60mm dish.
2. Take out the appropriate IVF dish(es) (200uL drop for frozen sperm and 250uL drop for fresh sperm).
    a. If there are multiple IVF dishes, take them all out at the same time.
3. Place the appropriate number of tracts into the left side of the IVF dish.
    a. Tracts are divided equally between IVF dishes. For example, if there are 20 females for 4 IVF dishes, you will need to place 10 tracts into each IVF dish.
4. Start dissections with your scope on the lowest magnification.
    a. With IVF forceps, grab a tract. With the other pair of forceps, rip open the ampula.
    b. Gently pull the COC into the IVF drop.
        i. Be careful to not drag the COC's on the bottom of the dish. This creates areas for the IVF media to splay.
        ii. Also look for any parts of the COC that may have stuck to the tract. They often like to hide underneath the tract.
    c. Repeat until all the COC's have been released into the IVF drop.
    d. Some ampullae are easier to see than others. Sometimes it helps to roll the tract in order to visualize the ampulla. The ampullae look like swollen footballs with horizontal striations.
    e. Count the number of COC's as you do this.
    f. Record the strain name, dish # and COC addition time on dish lid (See dish labeling sheet posted on hood).
    g. Repeat steps (a. - f.) if multiple dishes are being used in the strain's IVF.
    h. Remove the tracts from the dish(es) and discard in the trash.
    i. Record all information on your IVF timing sheet.
5. Place dish(es) in incubator.
6. The COC's will need to incubate for approximately 10-20 (15 is ideal) minutes in the IVF media before sperm is added.
    a. Your IVF timing sheet will tell you approximately what time to add sperm to dishes. It will also tell you in what order things need to be done.
    b. If there are multiple strains, you might be adding sperm to dishes you didn't do the COC dissection and vice versa.

It should take about 2-4 minutes per IVF dish from the time you receive the tract tube to placing COC's in the IVF dish(es). The less time the dishes spend outside of the incubator, the better.

Aliquot sperm to fertilization dishes (after 15 minutes of COC incubation time)

Get the appropriate IVF dishes (that correspond with the sperm you are using) and the sperm MBCD dish out of the incubator. Using a wide bore pipette tip:

1. Place pipette tip in the MBCD/sperm drop. Plunge down and blow a bubble.
    - Try to keep pipette as straight as possible – to prevent drawing up mineral oil instead of sperm.
2. Carefully and gently collect motile sperm from the peripheral part of the MBCD drop (see diagram below).
    - For fresh sperm, add amount determined by IVOS (3-5uL if no IVOS analysis available) values for each IVF drop.
    - For sperm from a crysostraw, collect 30uL sperm for each IVF drop.
    - For sperm from a cryovial, collect 30-60uL sperm for each IVF drop.
3. Wipe off the pipette tip with a Kimwipe (delicate) to remove excess oil. If you skip this step, it will be hard to expel the sperm into the IVF drop(s).
4. Add appropriate amount of sperm (refer to step #2) to the IVF drop in the IVF dish. Confirm sperm was added to the drop and not encapsulated in oil/floating on the surface. Discard pipette tip.
    - Only use a pipette tip once, then discard and get a new one (oil accumulates inside and distorts your measurement)
5. Record the time sperm was added in large red print. This is the **end of capacitation**.
6. Record time and amount of sperm used on your IVF timing sheet.
7. Repeat all steps if there are multiple IVF dishes for the strain.
    - If less than 3 min between times, leave the sperm dish on the heated stage.



Incubate fertilization dishes at 37° C under gas

For fresh sperm: 4-6 hours
For frozen sperm: 4 hours

Make sure all info is recorded on your IVF timing sheet.

- The IVF timing sheets serve as our paper record of the IVF.

Record all info in database – See MTS Experiments Module if performing this duty

You will do all embryo handling for all the dishes in which you dissected COCs. You will be responsible for all handling for the rest of the week.

Wash eggs (about 1-2pm) – Do not keep dishes out of the incubator longer than 20 minutes at a time!

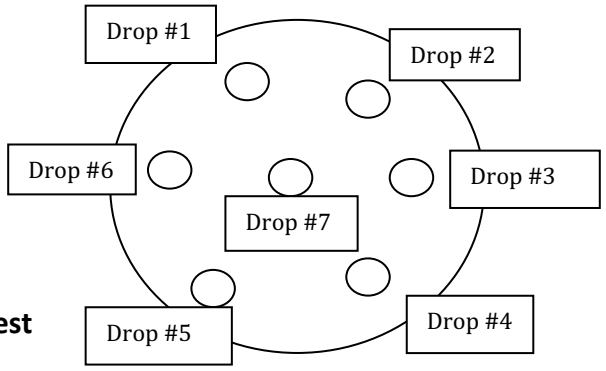
After incubation, wash the eggs to remove excess sperm/cumulus cells. Dead or dying tissue can harm the oocytes.

1. Using a capillary transfer pipette, transfer the eggs from the IVF drops to 1 drop in the "wash dish", taking care to leave behind as much debris as possible.
    a. This may take multiple transfers, depending on the number of oocytes in the dish.
2. Check the IVF drop carefully, taking care not to leave any healthy oocytes behind.
3. Wash the eggs through all 4 wash drops, each time being careful to leave as much debris behind as possible.
    a. Draw up fresh media from the drop you are moving to.
    b. Once all oocytes are moved from a drop, expel all media that remains in your pipette and blow a few bubbles.
        i. Allows you to recover any embryos close to the air bubble.
        ii. Ensures proper dilution of old media.
        iii. Bubbles serve as a marker for used drops.
4. All viable cells should be transferred to the final drop.
5. It helps to pipette the eggs gently to remove any cumulus cells and sperm that are still adhered to the zona by expelling and aspirating a few times.
6. A typical wash takes about 10 minutes.
7. After washing, write "WASH" in red on the lid with the strain info and times. Write strain info and times on the IVF dish lid.
8. Place Wash dish back in the incubator along with the IVF dish.
    a. Once you are proficient at washes (and not missing any oocytes), the IVF dishes may be discarded at this time.

Make Culture dishes for the next morning

1. Use 60mm dishes (blue sleeve). Use 1 dish per wash dish.
2. CLEAVE, KSOM or M16 can be used for culture dish media.
3. Aliquot 7 (100uL) drops per dish.
4. Cover with oil.
5. Place in the incubator.
6. Can be done immediately after the IVF or after washes.

Culture dish



Incubate Wash and Culture dishes at 37° C under gas overnight.

You will do all embryo handling for all the dishes in which you dissected COCs. You will be responsible for all handling for the rest of the week.

Tuesday (Day 2)

7-8:30am: Count embryos - Do not keep dishes out of the incubator longer than 20 minutes at a time!

We need to transfer all cells to the Culture dishes and then count the embryos.

1. Turn on scope and heating stage.
2. Take out 1 Wash dish and its corresponding IVF dish and place them on the heating stage. Take out 1 Culture dish and place on heating stage.
3. Check IVF dish for cells that were left behind. Place recovered cells in drop#1 of the Culture dish.
4. Remove all cells from the Wash dish and transfer to drop #1 in the Culture dish.
    a. Check all Wash drops just in case the dish was disrupted.
    b. Work up to transferring all cells in one pass. It may take several passes to transfer cells when learning or with a large number of oocytes.
5. Wash cells through 4 drops until you reach drop# 5.
6. Remove all 2-cell embryos and place them in drop# 6.
7. Using the counter in the hood, count all 2-cells.
    a. Record strain name, Dish #, COC addition time (female time), sperm addition time (male time) and 2-cell count on Culture dish lid.
8. Using the counter, count all the 1-cells in drop# 5.
    a. Record this number on the lid. You now have the fertilization rate for that dish (# 2-cells/ total # cells).
9. Record strain, CS# (if applicable), COC addition time, sperm addition time and any notes regarding the dish and # 2-cells/total # cells on your IVF results sheet.
10. Note any abnormalities on both the dish lid and IVF results sheet.
    a. If there are cells with missing zonas, follow the model below
        i. 53 total 2-cells, but 4 have no zonas → Recorded as 53(4) on dish lid.
11. Discard wash and IVF dishes and place Culture dish in the incubator.
12. Drop #7 is reserved for 4-cells (counted the next day).
13. Repeat until all dishes have been counted.
14. Typically, it takes 5-10 minutes to wash and count each dish. It takes practice to achieve this.
15. Once counts are finished, take the IVF results sheet(s) upstairs.
16. Record all info in database – See MTS Experiments Module if performing this duty

If an IVF was done for a rederivation or order, embryos will be transferred to a pseudopregnant female. This can be done up to the 8-cell stage. If embryos are taken out, it will be noted on the dish lid as "- # for Sx".

If an IVF was done for embryo cryo or sperm QC, embryos will be cultured further.

You will do all embryo handling for all the dishes in which you dissected COCs. You will be responsible for all handling for the rest of the week.

Wednesday (Day 3)

7-8:30am: Count 4-cells - Do not keep dishes out of the incubator longer than 20 minutes at a time!

1. Move all 4-cells to drop #7 in the Culture dish. Keep all 2/3 cells in drop #6.
2. Count 4-cells.
3. Count 2/3-cells.
4. When done counting, record # 4-cells + #3 cells + #2 cells (ex: 23 + 4 + 4) on dish lid and Embryo counts sheet.
5. Continue until all dishes have been counted.
6. If embryos are to be cryopreserved (check IVF schedule)
    a. If multiple dishes per strain, combine all 4-cells (middle drop) and all 2/3-cells (outer drop) to one dish, and then re-count all cells.
        i. We pool embryos for the person performing embryo cryo.
        ii. Record the combined total in thick black sharpie and circle on one of the dish lids.
7. Record all info in database – See MTS Experiments Module if performing this duty

Rest of the days of the week

Continue to check/count the remaining embryos. Record counts on IVF embryo count sheet and in database each day. Discard all remaining dishes Friday afternoon (unless told otherwise by JB).

IVF Schedule Timing

Using the IVF Schedule, IVF timing sheet and IVF worksheet (at the end of the SOP) as examples, here is how timing is worked out.

- It is easier to figure out timing by creating an IVF worksheet. From this sheet, you can figure out how to delegate the work and what order things should be done in.
- Assume hCG was given at 7:30pm, so the females will be dissected at 9:00am.
- When performing the IVF, these times are approximations. If a male dissection was delayed by 5 min, you need to delay your IVOS analysis by 5 min.
    - This might change the order of your tasks, but that is OK (as long as tasks are done within their specific time limits.

Figure out the female order:

1. On average, it takes about 45 sec - 1 min/mouse for female dissections. This time varies per individual, so be sure to know who is dissecting the females ahead of time.
2. In general, the female strains should be in order of least number of mice to greatest number of mice.
    a. For this example, we have put females for all cryo'd sperm first. This is to delay the male dissections to at least 8:25 (This gives everybody more time to prepare for the IVF).
    b. If putting cryo samples first does not add enough time to prep for male dissections, we can delay the female dissections for 15 min
    c. When IVFs are from MH, we send down multiple tract tubes at once if animals total less than 20.
3. Assuming a dissection time of ~60 sec/mouse, female tract tubes will be ready for COC dissection at the following times:
    a. 5 129S6/UNC → 9:05am
    b. 11 B6/UNC → 9:15am
    c. 12 B6/UNC → 9:26am
    d. 10 Btbd18-mCherry → 9:36am
    e. 11 Dlg4-tm1→ 9:46am

Figure out when the COC dissections will be finished:

1. On average, it takes 2-4 min/IVF dish for COC dissections. This time varies with each individual, so you will want to keep this in mind if there are 2 people performing the COC dissections.
2. Assuming 2-3 min/IVF dish, the COC dissections will be done at the following times:
    a. 5 129S6/UNC → 9:08
    b. 11 B6/UNC → 9:18-24
    c. 12 B6/UNC → 9:29-38
    d. 10 Btbd18-mCherry → 9:40-45
    e. 11 Dlg4-tm1 → 9:49-54

Figure out when the sperm needs to be added to each dish:

1. Sperm should be added to the IVF dishes about 15 min after the COC's are added. There is a flexible window of 10 to 20 min to add sperm, which helps with timing.
    a. If you want someone to add sperm at 10 or 20 min., please highlight that on their timing sheet.

Figure out when to dissect males/thaw sperm:

1. Here is where the ideal CAP times come into play. You can be flexible with CAP times since they are broader ranges that other times.
    a. This helps when planning timing/order of things. If things are busy, there can be 3 separate things that need to happen at one time. Being flexible will allow for all the things to be completed.
    b. If something goes awry, you will most likely still be within the ideal CAP time.
2. Using the time to add sperm, plan out when to dissect/thaw.
    a. 129 Ace-tm2 Cryo sample (JAX straws ideal 40 min. (range 30-50 min) CAP time): You want to be adding thawed sperm to the MBCD dish at about 8:50am.
    b. 3 cryo sperm samples x 11 B6/UNC → 8:55
    c. 3 cryo sperm samples x 12 B6/UNC → 9:00
    d. Btbd18-mCherry male (fresh sperm need 60-90 min. CAP time): You want to be done mincing epididymis tissue at about 8:45am. You want to plan for about 65-70 min for fresh sperm. This way, if things take longer, you have more time.
        i. Gross dissection should be done about 5-10 min prior to 8:45am.
    e. Dlg4-tm1 males (fresh sperm need 60-90 min. CAP time): You want to be done mincing epididymis tissue at about 8:50am.
        i. Close to Btbd18-mCherry in time, so moved it up to 8:45 so the person dissecting can be more efficient.

Figure out when IVOS analysis needs to be done:

1. IVOS analysis can be done 30-60 min post male dissection.
    a. Minimum amount of time is 30 min (15 min swim out in MBCD and 15 min swim out in IVOS tube).
    b. IVOS is only done for live males
2. Using the male dissection times, plan when IVOS tubes and analysis should be done.

Figure out how much media is needed for the whole IVF:

1. We do this since we need to thaw media and pre-equilibrate it in the incubator.
    a. You only want to equilibrate approximately what you are going to use.
2. We need to know how much HTF+GSH needs to be made for the IVF dishes.
3. See calculations on the IVF timing worksheet.

Notes/Tips:

- If you are preparing 2 males at once, prep both males at the time for the first group. You only want to prep two males at once if their sperm addition times are within ~5 min of each other. If the times are ~10 min apart, males should be dissected separately.
- If there are conflicting times, here is guide to help you decide the order of the tasks
    - If 2 COC groups are coming down together, do them in the order on the IVF schedule.
    - If 2 males are to be dissected at the same time, do them in the order on the IVF schedule.
    - If a COC dissection and adding sperm to another IVF dish have same time, add sperm 1st then do COC dissection.
    - If epididymides need to be dissected and a sperm sample thawed at the same time, dissect the epididymides first.

Make IVF timing sheet(s):

1. See example sheet (at end of SOP).
2. These sheets are used as our paper IVF recording, so they need to contain the same information each time.
    a. Use a template when creating, so wording/information needed stays exactly the same.
3. If there are multiple people performing IVF, try to split up all the tasks equally and in the best interest of timing.
    a. Make each person his or her own IVF timing sheet with just his or her specific tasks.
4. Assign dish making tasks
5. Write up the sheets in the order they need to happen. Having the IVF timing worksheet helps here. You already have all the times written down, you just need to put them in order for the IVF timing sheet(s).
6. Highlight any deviations to normal SOP procedures.

Color coding on IVF schedule/timing sheets

- Each strain will be a different color (8 available).
- If a group of females is used for multiple sperm QC's, all strains being QC'd will be the same color as the females.

IVF Purposes

Each IVF we do has a specific purpose and this purpose is always written on the schedule. This keeps everyone on the same page as well as helping the person creating the IVF schedule/timing sheet(s). Below are IVF purposes with their descriptions/pertinent information.

Embryo cryo: This is the main reason for doing IVF with the strains in GMB. We need to either build up our archive or replenish it. Embryos from these IVF's are almost always cryo'd on Day 3 (4-cell stage). The only reason they would not be cryopreserved is if there is less than 1 straw worth of embryos.

Rederivation: All strains that are assigned to us have to be rederived into GMB from our import facility (MBRB). These embryos will be used for embryo transfers on Day's 2-3 (2 to 4-cell stages). Any embryos not used for embryo transfers will be cryopreserved on Day 3 (4-cell stage)

Sperm QC: All sperm samples frozen need to be QC'd in order to be sold. Most sperm QC's are done in batches, grouping together samples that all have the same background strain. To do this, only 1 QC dish is used per sample (unless otherwise instructed) and a group of females will be split up equally between samples. For example, if we have 3 samples to QC, there would be a group of 12 B6/J females (4 females per sample) to divide among them. If a line is maintained by backcrossing, we will cryo the sperm QC embryos. It will be noted on the IVF schedule if you need to cryo these embryos. Normally, these culture all week long.

CC IVF (either embryo cryo or rederivation): This applies to all strains that begin with "CC" followed by a number. We do not decide whether a strain is cryo'd or used for rederivation. We are instructed by the CC group as to what needs to be done with each strain. Many of these strains perform poorly in IVF. UNC MTS is used for database entry.

UNC Work IVF: This applies to all the UNC work that we do (for internal Investigators). We will either be performing sperm QC, embryo cryo or sperm cryorecovery/rederivation. Generally, the person responsible for the UNC work will perform all tasks associated with the strain (including washes/embryo counting). UNC MTS is used for database entry.

References

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Cryopreservation of Mouse Spermatozoa and In Vitro Fertilization. Nakagata N, Methods Mol Biol. 2011;693:57-73.
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Media Preparation Guide

Immediate IVF media Prep (done 1 working day prior to IVF)

HTF

1. Thaw only amount needed for IVF and aliquot into a 15mL tube.
2. Label with "HTF and expiration date"
3. If there is any remaining media, record thaw date on bottle and place in fridge. (can be used within 2 weeks of thaw date).

KSOM

1. Thaw only amount needed for dishes and aliquot into a 15mL tube.
2. Label with "KSOM and expiration date"
3. If there is any remaining media, record thaw date on bottle and place in fridge. (can be used within 2 weeks of thaw date).

MBCD

1. Thaw only amount needed for IVF.
2. Syringe filter media (to remove precipitate) into a new, sterile 5mL faclon tube.
3. Label with "MBCD and IVF date"
4. If there is any remaining media, record thaw date on tube and place in fridge. (can be used within 2 weeks of thaw date).

Place all media tubes in the IVF media bag and place on top door shelf of upstairs lab fridge. The person doing hCG injections will make sure media is placed in the LB incubator to equilibrate.

Stock Solution Preparation

100mM GSH stock solution

1. Weigh 0.0921g GSH (L-glutathione reduced, Sigma G6013) in a 5mL falcon tube.
    i. If you do not get exactly 0.0921g, use the following formula:
    (weight value x 3) / 0.0921 = mL HTF to add
2. Add 3mL of HTF or mRVF to the 5mL falcon tube. It can take 30 min to an hour for all the GSH to fully dissolve.
3. Filter sterilize the media.
4. Aliquot 100uL media into 1.5mL snap-cap tubes.
5. Label tubes as "GSH" and expiration date of the HTF or mRVF.
6. Store in freezer.

10x Salts Solution – Use MBCD worksheet (page 20) for preparation/record keeping

1. Add 70mL embryo water (Sigma W1503) into a clean graduated cylinder.
2. Add a stir bar to the cylinder and turn on stir plate.
3. Weigh and add each component to the cylinder in the order according to Table 1 below.
4. Remove stir bar.
5. Add embryo water to bring the final volume to 100mL.
6. Cover the cylinder with parafilm and invert >5 times to mix.
7. Use fresh to make MBCD media or aliquot into 5mL falcon tubes.
8. Label aliquots as "10x Salts "with date made
9. Store aliquots in the freezer and use within 6 months.

Table 1: 10x Salts Solution

Component	g/100mL	Vendor/Catalog #
NaCl	6.97600	Sigma S-5886
KCl	0.35600	Sigma P-5405
KH_2PO_4	0.16200	Sigma P-5655
$MgSO_4 \cdot 7H_20$	0.29300	Sigma M-7774
Glucose	1.00000	Sigma G-6152
Embryo Water	To final 100mL	Sigma W1503

100x $CaCl_2 \cdot 2H_2O$ Solution - Use MBCD worksheet (page 20) for preparation/record keeping

_____ 1. Weigh 0.1255g $CaCl_2 \cdot 2H_2O$ (Sigma C-7902) in a 5mL falcon tube.
_____ 2. Add 5mL embryo water, cap tube and invert to dissolve.
_____ 3. Filter sterilize.
_____ 4. Store at 4° C with cap closed tightly.
_____ 5. Discard after 1 month.

MBCD Media - Use MBCD worksheet (page 20) for preparation/record keeping

1. Add 60mL embryo water to a sterile 100mL graduated cylinder.
2. Add 10mL 10x Salts Solution (either freshly made or thawed) to the cylinder.
3. Add stir bar and turn on stir plate.
4. Add the other components in order according to Table 2 below.
5. Remove stir bar.
6. Add embryo water to bring the final volume to 100mL.
7. Cover cylinder with parafilm and invert >5 times.
8. Test pH of media following the directions on the pH meter. pH should be 7.2-7.6.
9. Test osmolarity of media following the directions on the osmometer. The osmolarity should be 285-295.
10. If either pH is not in range or osmolarity <285, discard the media and start over.
11. If pH is in range and osmolarity is >295, adjust the osmolarity by adding embryo water. Add 0.5mL, mix solution and check osmolarity again. Repeat until media's osmolarity is in range.
12. If pH and osmolarity are in range, filter sterilize the media.
13. Aliquot the media (4mL) into 5mL sterile falcon tubes.
14. Label aliquots with "MBCD", date made and expiration date (last thaw date).
15. Store aliquots in freezer for up to 3 months.
16. Thawed aliquots are good for 2 weeks past thaw date.

Table 2: MBCD media

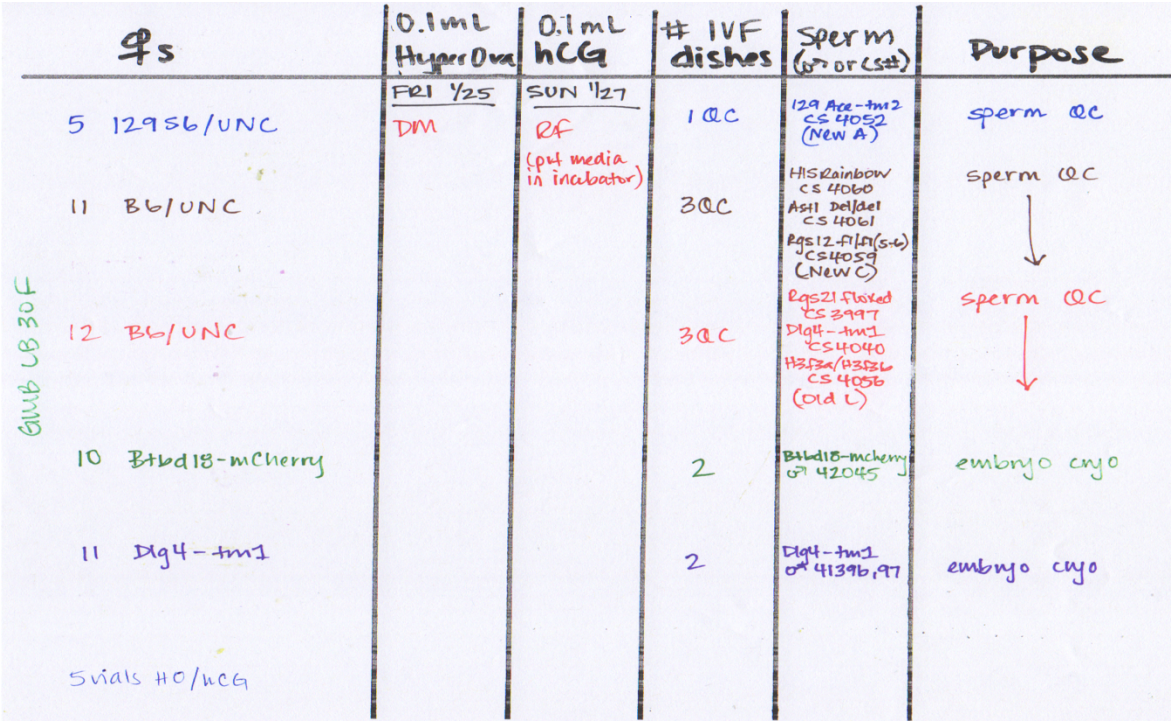
Component	g/100mL	Vendor/Catalog #
10x Salts Solution	10.0mL	Fresh or thawed
100x $CaCl_2 \cdot 2H_2O$ Solution	1.0mL	Sigma C-7902
$NaHCO_3$	0.2106g	Sigma S-5761
Sodium pyruvate	0.0055g	Sigma P-4562
Penicillin G	0.0075g	Sigma P-7794
Streptomycin sulfate	0.0050g	Sigma S-1277
MBCD	0.1000g	Sigma C-4555
PVA	0.1000g	Sigma P-8136
Embryo Water	Final volume to 100mL	Sigma W1503

Note: MBCD is 0.75mM, MW 1320

Modified RVF (mRVF)

1. Weigh 0.02275g $CaCl_2 \cdot 2H_2O$ (Sigma C-7902) in a 5mL falcon tube.
2. Add 5mL RVF (from the original 50mL bottle) to the 5mL tube. Mix to dissolve the $CaCl_2 \cdot 2H_2O$.
3. Filter sterilize the 5mL media back into the original 50mL bottle. Swirl the bottle to mix.
4. Label bottle "Modified", date of modification and your initials.

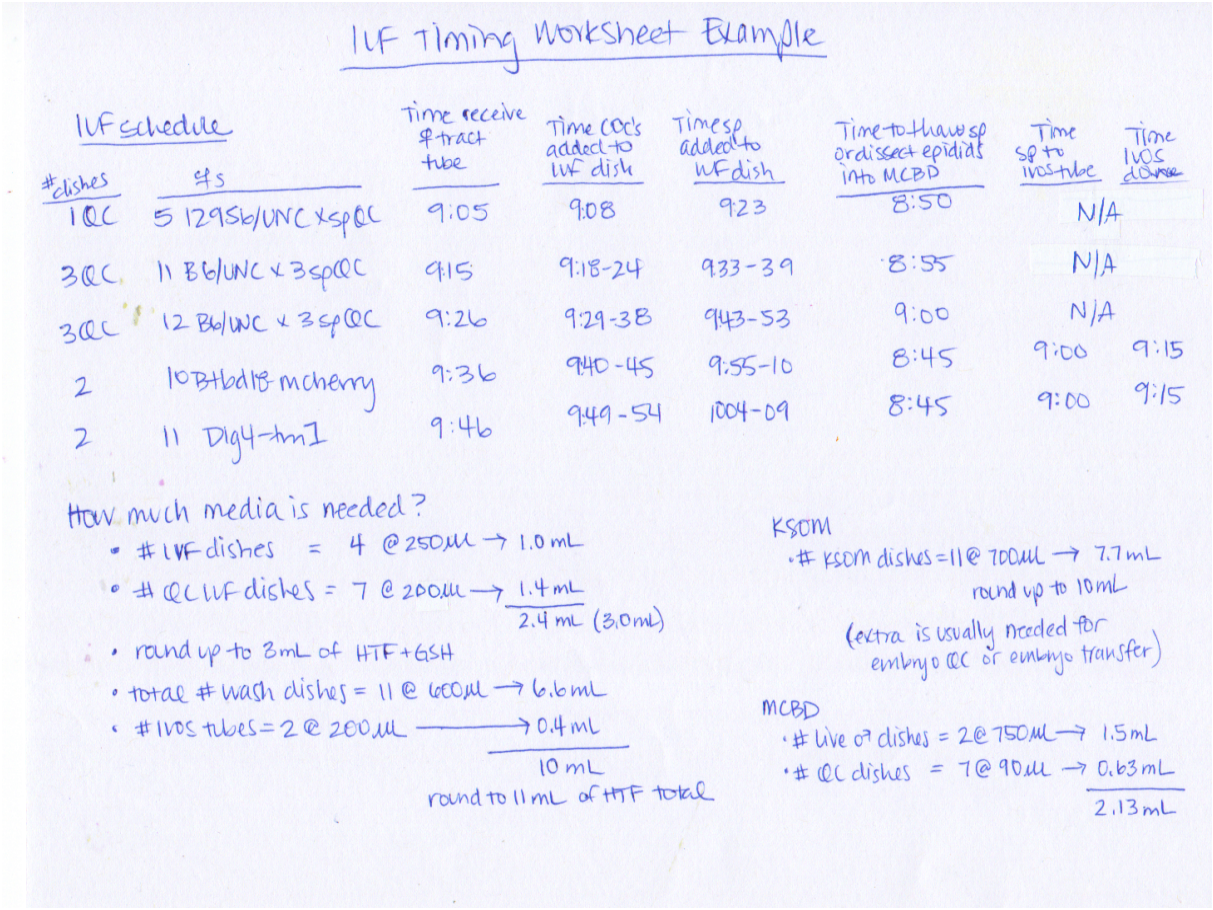
Example IVF Schedule



♀s	0.1mL Hyper Ova	0.1mL hCG	# IVF dishes	Sperm (♂ or (s#))	Purpose
	FRI 1/25	SUN 1/27			
5 12956/UNC	DM	RF (put media in incubator)	1 QC	129 Ace-tm2 CS 4052 (New A)	sperm QC
11 B6/UNC			3QC	HIS Rainbow CS 4060 Asl1 Del/del CS 4061 Rgs12-fl/fl(s-6) CS4059 (New C)	sperm QC ↓
12 B6/UNC			3QC	Rgs21 floxed CS 3997 Dlg4-tm1 CS4040 H3f3a/H3f3b CS 4056 (Old L)	sperm QC ↓
10 Btbd18-mCherry			2	Btbd18-mcherry ♂ 42045	embryo cryo
11 Dlg4-tm1			2	Dlg4-tm1 ♂ 41396,97	embryo cryo
5 vials HO/hCG					

GWB LB 30F

Example IVF Worksheet



IVF Timing Worksheet Example

IVF schedule

#dishes	♀s	Time receive ♀ tract tube	Time COCs added to IVF dish	Time sp added to IVF dish	Time to thaw sp or dissect epidids into MCBD	Time sp to IVOS tube	Time IVOS done
1QC	5 12956/UNC x spQC	9:05	9:08	9:23	8:50	N/A	
3QC	11 B6/UNC x 3spQC	9:15	9:18-24	9:33-39	8:55	N/A	
3QC	12 B6/UNC x 3spQC	9:26	9:29-38	9:43-53	9:00	N/A	
2	10 Btbd18 mcherry	9:36	9:40-45	9:55-10	8:45	9:00	9:15
2	11 Dlg4-tm1	9:46	9:49-54	10:04-09	8:45	9:00	9:15

How much media is needed?

- # IVF dishes = 4 @ 250uL → 1.0 mL
- # QC IVF dishes = 7 @ 200uL → 1.4 mL

2.4 mL (3.0mL)

- round up to 3mL of HTF+GSH
- total # wash dishes = 11 @ 600uL → 6.6 mL
- # IVOS tubes = 2 @ 200uL → 0.4 mL

10 mL

round to 11 mL of HTF total

KSOM

- # KSOM dishes = 11 @ 700uL → 7.7 mL

round up to 10 mL

(extra is usually needed for embryo QC or embryo transfer)

MCBD

- # live ♂ dishes = 2 @ 750uL → 1.5 mL
- # QC dishes = 7 @ 90uL → 0.63 mL

2.13 mL

Example IVF Timing Sheets (3 people doing IVF)

RF IVF 1/28/19

Take 1 snap cap tube of GSH from genotyping freezer before going downstairs

129 Ace-tm2 = Blue
HISRainbow = Brown
AstI Del/del = Brown
Rgs12 fl/fl(5-6) = Brown
Dlg4-tm1 = Purple

Rgs21 floxed = Orange
Dlg4-tm1 cryo sample = Orange
H3f3a/H3f3b = Orange
Btbd18-mCherry = Green

MBCD dishes:

7 frozen sperm dishes (90uL MBCD)
2 fresh sperm dishes (750uL MBCD)

IVF dishes: Place 25uL GSH and 2.5mL of HTF into tube.

4 IVF dishes = 250uL drop
7 QC IVF dishes = 200uL drop

IVOS tubes

~ 8:45 CAP time Dlg4-tm1 males (41396, 97) into 750uL MBCD. CAP time: _____#Epid_____

~ 8:50 CAP time…… thaw 1 cryo sample
129 Ace-tm2 CS4052 into 90uL MBCD. CAP time_____

~ 9:00 Dlg4-tm1 IVOS tube

~ **9:05** COC dissection for 129S6/UNC females x sp QC
total # females _____
129 Ace-tm2 CS4052 #tracts_____ #COC_____ Time COC to IVF_____

~ **9:15** IVOS Dlg4-tm1 sample **Include 2 decimal places in calculation values**
Time done _____
Total Concentration _____M/ml Calculation from Total Concentration _____uL
Static _____ M/ml and _____%
Motile _____M/ml and _____%
Progressive _____M/ml and _____% Calculation from Progressive Concentration _____uL
Slow _____M/ml and _____%

~ **9:23** (15 min post COC time on dish) Add sperm to 129S6/UNC QC dish
129 Ace-tm2 CS4052 uL sperm_____30_____ time sperm added_____

~ **9:26** COC dissection for 12 B6/UNC females x 3 sperm samples
Rgs12 floxed CS3997 #tracts_____ #COC_____ Time COC to IVF_____
Dlg4-tm1 CS4040 #tracts_____ #COC_____ Time COC to IVF_____
H3f3a/H3f3b CS4056 #tracts_____ #COC_____ Time COC to IVF_____

~ **9:46** COC dissection for Dlg4-tm1 females
total # females _____
Dish #1 #tracts_____ #COC_____ Time COC to IVF_____
Dish #2 #tracts_____ #COC_____ Time COC to IVF_____

~ **10:04** (15 min post COC time on dish) Add sperm to Dlg4-tm1 dishes
Dish #1 uL sperm_____ time sperm added_____
Dish #2 uL sperm_____ time sperm added_____

GS IVF 1/28/19

129 Ace-tm2 = Blue
HISRainbow = Brown
Astl Del/del = Brown
Rgs12 fl/fl(5-6) = Brown
Dlg4-tm1 = Purple

Rgs21 floxed = Orange
Dlg4-tm1 cryo sample = Orange
H3f3a/H3f3b = Orange
Btbd18-mCherry = Green

~ **8:45** CAP time Btbd18-mCherry (42045) male into 750uL MBCD. CAP time: _____#Epid_____

~ **8:55** CAP time…… thaw 1 cryo sample
HISRainbow CS4060 into 90uL MBCD. CAP time_____

~ **9:00 Btbd18-mCherry IVOS tube**

~ **9:15** COC dissection for 11 B6/UNC females x 3 sperm samples
HISRainbow CS4060 #tracts_____ #COC_____ Time COC to IVF_____
Astl Del/del CS4061 #tracts_____ #COC_____ Time COC to IVF_____
Rgs12 fl/fl(5-6) CS4059 #tracts_____ #COC_____ Time COC to IVF_____

~ **9:36** COC dissection for Btbd18-mCherry females
total # females _____
Dish #1 #tracts_____ #COC_____ Time COC to IVF_____
Dish #2 #tracts_____ #COC_____ Time COC to IVF_____

~ **9:44** (15 min post COC time on dish) Add sperm to 12 B6/UNC QC dishes
Rgs12 floxed CS3997 uL sperm_____30_____ time sperm added_____
Dlg4-tm1 CS4040 uL sperm_____30_____ time sperm added_____
H3f3a/H3f3b CS4056 uL sperm_____30_____ time sperm added_____

~ **9:55** (15 min post COC time on dish) Add sperm to Btbd18-mCherry dishes
Dish #1 uL sperm_____ time sperm added_____
Dish #2 uL sperm_____ time sperm added_____

JH IVF 1/28/19

129 Ace-tm2 = Blue
HISRainbow = Brown
Astl Del/del = Brown
Rgs12 fl/fl(5-6) = Brown
Dlg4-tm1 = Purple

Rgs21 floxed = Orange
Dlg4-tm1 cryo sample = Orange
H3f3a/H3f3b = Orange
Btbd18-mCherry = Green

~ **8:55** CAP time…… thaw 2 cryo samples
Astl Del/del CS4061 into 90uL MBCD. CAP time_____
Rgs12 fl/fl(5-6) CS4059 into 90uL MBCD. CAP time_____

~ **9:00** CAP time…… thaw 3 cryo samples
Rgs12 floxed CS3997 into 90uL MBCD. CAP time_____
Dlg4-tm1 CS4040 into 90uL MBCD. CAP time_____
H3f3a/H3f3b CS4056 into 90uL MBCD. CAP time_____

~ **9:15** IVOS Btbd18-mCherry sample
Time done _____
Total Concentration _____M/ml Calculation from Total Concentration _____uL
Static _____ M/ml and _____%
Motile _____M/ml and _____%
Progressive _____M/ml and _____% Calculation from Progressive Concentration _____uL
Slow _____M/ml and _____%

~ **9:33** (15 min post COC time on dish) Add sperm to 11 B6/UNC QC dishes
HISRainbow CS4060 uL sperm_____30_____ time sperm added_____
Astl Del/del CS4061 uL sperm_____30_____ time sperm added_____
Rgs12 fl/fl(5-6) CS4059 uL sperm_____30_____ time sperm added_____

Make wash and KSOM dishes

Date: _____
Initials: _____

MBCD Media Preparation Worksheet

Check to see if there are any frozen aliquots of 10x salt solution in the freezer within their expiration date.

a. Thaw 1 15mL aliquot if within expiration date at room temperature.
b. If aliquots are expired, make new batch of 10x Salts.

10x Salts Stock Solution

____ 1. Pipette 70mL embryo water (Sigma W1503) into a sterile graduated cylinder.
____ 2. Add a stir bar to the cylinder and turn on stir plate.
____ 3. Zero scale with each new weigh boat.
____ 4. Weigh and add each component to the cylinder in the order according to Table 1 below.
    i. Record the weight of each component.

Table 1: 10x Salts Solution

Vendor/Catalog #	Component	g/100mL	Weight Used
Sigma S-5886	NaCl	6.976	
Sigma P-5405	KCl	0.356	
Sigma P-5655	KH_2PO_4	0.162	
Sigma M-2773	$MgSO_4 \cdot 7H_20$	0.293	
Sigma G-6152	Glucose	1.000	

____ 5. Remove stir bar with the magnetic wand.
    i. Do not stick the wand in your media, use only on the outside of the cylinder.
____ 6. Add embryo water with sterile pipette to bring the final volume of the solution to 100mL.
____ 7. Cover the cylinder tightly with parafilm and invert >5 times to mix.
____ 8. Use fresh solution to make MBCD media or aliquot into 15mL tubes (~13-14mL per tube).
____ 9. Label aliquots as “10x Salts“ with date made and expiration date (6 months past date made).
____ 10. Store aliquots in the freezer and use within 6 months.

Make 100x $CaCl_2 \cdot 2H_2O$ Solution

_____ 1. Zero scale with new 5ml Falcon Tube.
_____ 2. Weigh 0.1255g $CaCl_2 \cdot 2H_2O$ (Sigma C-7902) in a 5mL falcon tube. Weight:_____
    i. $CaCl_2 \cdot 2H_2O$ hydrates very quickly, so lid cannot remain off while weighing out product.
    ii. If you do not get exactly 0.1255g, use:
        (weight value x 5) / 0.1255 = mL of embryo H2O to add
_____ 3. Pipette 5mL (or volume obtained from above equation) embryo water into falcon tube, cap tube and invert to dissolve.
_____ 4. Filter sterilize into a new sterile 5mL falcon tube.
    i. Use 25mm syringe filter and a 10mL syringe
_____ 5. Label tube with “100x CaCl2 and date made”.
_____ 6. Store at 4° C with cap closed tightly.
_____ 7. Discard after 1 month.

MBCD Media

____ 1. Pipette 60mL embryo water (Sigma W1503) to a sterile 100mL graduated cylinder.
____ 2. Pipette 10mL of 10x Salts Solution (either freshly made or thawed) to the cylinder.
    i. If thawed: Date made: _____ Initials of preparer: _____
____ 3. Add stir bar to cylinder and turn on stir plate.
____ 4. Zero the scale with each new weigh boat.
____ 5. Weigh and add each component to the cylinder in the order according to Table 2 below.
    i. Record the weight of each component.

Table 2: MBCD media

Vendor/Catalog #	Component	g/100mL	Weight or Amount Used
Fresh or thawed	10x Salts Solution	10.0mL	
Sigma C-7902	100x $CaCl_2 \cdot 2H_2O$ Solution	1.0mL	
Sigma S-5761	$NaHCO_3$	0.2106g	
Sigma P-4562 (fridge)	Sodium pyruvate	0.0055g	
Sigma P-7794	Penicillin G	0.0075g	
Sigma S-1277 (fridge)	Streptomycin sulfate	0.0050g	
Sigma C-4555	MBCD	0.1000g	
Sigma P-8136	PVA	0.1000g	

Note: MBCD is 0.75mM, MW 1320

____ 6. Cover cylinder tightly with parafilm while PVA dissolves.
    i. Leave stir bar spinning
____ 7. Remove stir bar with the magnetic wand.
    i. Do not stick the wand in your media, use only on the outside of the cylinder.
____ 8. Pipette embryo water into cylinder to bring the final volume to 100mL.
____ 9. Cover cylinder tightly with parafilm and invert >5 times to mix.
____ 10. Test pH of media following the directions in the manual for the pH meter.
    i. pH of your media should be 7.2-7.6. **pH: _____**
    ii. **If pH is not in range, discard media and start over.**
____ 11. If pH is in range, test osmolarity of your MBCD aliquot. (refer to Osmometer manual for procedure specifics)

Tests to see if Osmometer needs to be calibrated:

290 Clinitrol Standard Results	
Test #1	
Test #2	
Test #3	
Test #4	
Test #5	
Average	

If 290 Standard average is within 288-292, test your MBCD aliquot.
****If the average of results fall outside the range given, the osmometer needs to be calibrated****

See Osmometer manual for calibration instructions. Repeat 290 standard testing after calibration to confirm calibration.

MBCD sample test results					
Undiluted Sample		**Amount H_2O added**		**Amount H_2O added**	
Test #1		Test #1		Test #1	
Test #2		Test #2		Test #2	
Test #3		Test #3		Test #3	
Test #4		Test #4		Test #4	
Test #5		Test #5		Test #5	
Average		Average		Average	
Dilute?		Dilute?		Dilute?	

****The osmolarity should be 285-295****

If MBCD osmolarity is >295, dilute with sterile water until desired osmolarity is reached.

Approximately 500uL H_2O will lower osmolarity by approximately 5 points

If MBCD osmolarity is <285, discard CPA and start over.

____ 12. Both pH and osmolarity need to be in the proper range to keep the media, discard the media and start over if either are out of range.
____ 13. Aliquot the media (4mL) into 5mL sterile falcon tubes (aliquot will be filtered upon thaw/before use).
____ 14. Label aliquots with "MBCD" and date made.
____ 15. Store aliquots in freezer for up to 5 months.
____ 16. Save this worksheet for your records. It can be used to troubleshoot should the need arise.

Final MBCD stats

pH: _____ osmolarity: _____ Expiration date: _____