Rat Sperm Freezing Protocol

Rat Resource and Research Center

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Abbreviations:

R1CC: A modified rat 1-cell embryo culture medium (mR1ECM) with an osmolality of ~290 mOsm (high NaCl).

R1CH: R1CC-HEPES, a modified R1CC where part of bicarbonate is replaced with HEPES buffer.

1. HEPES-buffered modified rat 1-cell embryo culture medium preparation

1.1. Equipment and supplies:

- pH meter and appropriate pH stock solutions for standardization.
- Sterile biosafety cabinet.
- Pipet-Aid.
- Weighing scale and weighing supplies (weigh paper, spatula or scoopula).
- Griffin beaker (100 mL, 250 mL, 500 mL) and stir bars.
- Volumetric flasks (100 mL, 250 mL, 500 mL).
- Serological pipettes.
- Filter bottle units (SFCA membrane, pore size: 0.22 µm).
- Steriflip-GP 50 mL filter units (pore size: 0.22 µm).
- Millex-GP 0.22 µm filter.
- Filter bottle units (SFCA membrane, pore size: 0.22 µm)
- Milli-Q water.
- 70% alcohol in wash bottle.

Reagents	Company	Cat #
Embryo Transfer water	Sigma	W1503
NaCl	Sigma	S5886
KCl	Sigma	P5405
D-Glucose	Sigma	G6152
Penicillin G K Salt	Sigma	P7794
Streptomycin Sulfate	Sigma	S1277
Sodium Lactate (60% syrup)	Sigma	L7900
$CaCl_2$-$2H_2O$	Sigma	C7902
$MgCl_2$-$6H_2O$	Sigma	M2393
$NaHCO_3$	Sigma	S5761
Sodium Pyruvate	Sigma	4562
MEM NEAA 100x	Invitrogen	11140-050
MEM EAA 50X	Invitrogen	11130-051
GlutaMAX 1	Invitrogen	35050-061
HEPES	Sigma	H6147
Fatty Acid Free BSA	Sigma	A7638

1.2. Rat stock solution:

This stock solution is intended to be used for making rat culture media and *in vitro* handling media.

1. To the appropriate sized Griffin Beaker containing Embryo Transfer water (Sigma; W1503; 80% of the total volume of media), add the components in grams according to **Worksheet 1.2**.
2. After all components have dissolved, transfer the contents to the appropriate volumetric flask and bring to desired volume by rinsing out the Griffin beaker with Embryo Transfer water and adding it to the volumetric flask. Measure the osmolality, record this result, and filter into an appropriately sized sterile bottle.
3. Label with name of solution, date made, expiration date (one month from date made) and initials. Label with osmolality and batch number. Store at 4^{o}C.

Worksheet 1.2: Rat stock solution (10X stock for making mR1ECM)

To the appropriate sized Griffin Beaker containing Embryo Transfer water (Sigma W1503, 80% of the total volume of medium), add the following components in grams:

Company	Cat #	Reagent	FW (g)	mM (Working)	500 ml	Added	Lot#
Sigma	S5886	NaCl	58.44	80	23.376g		
Sigma	P5405	KCl	74.55	3.2	1.1928g		
Sigma	G6152	D-Glucose	180.2	7.5	6.7576g		
Sigma	P7794	Penicillin G K Salt	372.2	100µg/ml	0.375g		
Sigma	S1277	Streptomycin Sulfate	1457	50µg/ml	0.25g		
Sigma	L7900	Sodium Lactate (60% syrup)	186.8	13.53	12.637g		
Sigma	C7902	$CaCl_2$-$2H_2O$	147.02	2	1.4702g		
Sigma	M2393	$MgCl_2$-$6H_2O$	203.31	0.5	0.5083g		
Osmolality							

Lot# for Embryo Transfer water	
Batch (lot)#	
Your initials:	
Today's date	
Expiry date 1 month from preparation date	

1.3. Protocol for making R1CH:

1. Add all components as listed in **Worksheet 1.3** (next page) to a Griffin beaker containing Embryo Transfer water (80% of final volume).
2. After all components have been dissolved, check pH and adjust pH to ~7.4 with 1N NaOH.
3. Transfer the contents to the appropriate volumetric flask and bring to desired volume by rinsing out the Griffin beaker with Embryo Transfer water and add it to the volumetric flask.
4. After calibrating the osmometer with a 290 mOsm standard, check the osmolality of the solution. Remake the solution if the osmolality does not fall between **280-300 mOsm**.
5. Sterile filter the solution using a 0.22 µm filter unit.
6. Label container with date made, expiration date (four weeks from date made), pH, mOsm, your initials and batch number.
7. Store at 4ºC for up to 4 weeks.

Note: If any precipitates form in the solution, discard the solution and start over.

A. Working solution:

1. Aliquot amount needed (usually 10 ml) using a sterile pipette.
2. Add 4 mg/ml (0.04 g/10 ml) Fatty Acid Free BSA (Sigma A7638).
3. Sterile filter using 0.22 µm syringe filter.

Worksheet 1.3. R1CH (high NaCl)

1. Record Embryo Transfer water used: Lot #:

2. Add the following components to Griffin Beaker containing Embryo Transfer water.

Catalog #	Reagent	Final Conc (mM)	g/500 ml	Added	Lot Number
See stock solutions	Rat Stock	n/a	50 ml		
Sigma S5886	NaCl	30	0.8766		
Sigma S5761	NaHCO3	5	0.2100		
Sigma P4562	Sodium Pyruvate	0.5	0.0276		
Invitrogen 11140-050	MEM NEAA 100x	n/a	5 ml		
Invitrogen 11130-051	MEM EAA 50X	n/a	10 ml		
Sigma H6147	HEPES	22	2.6214		
Invitrogen 35050-061	GlutaMAX 1	n/a	0.250 ml		

Parameter	Expected	Y or N/ Measured
pH of solution*	7.4	
Osmolality	290-320	
Filter Sterilized	N/A	
Expires	**4 weeks** from date made	**DATE:**

Initial pH	
Final pH	
Vol/pH Reagent Added	

**Adjust pH if needed.*

Today's date: _____

Your initials: _____

Batch # _____

Please label the bottle of "Rat 1-cell HEPES" with tape.

2. Rat sperm freezing medium preparation

Worksheet 2.1. Ingredients of rat sperm freezing medium.

Note: please wear gloves when preparing this solution.

Reagent	Company	Cat #	Amount	Added	Lot #
(D+) raffinose pentahydrate (18%)	Sigma	R-0514	18g		
Skim milk dehydrated	Fisher	232100	4.50g		
1-Thioglycerol	Sigma	M6145	4µl		
Initial volume			75mL		
Volume of supernatant to expect			50-60mL		
Volume of additional water			*20-30mL		
Final volume Embryo Transfer water (Sigma; W1503)			**100 mL**		

Protocol:

1. Dissolve the dry skim milk in 75 mL of Embryo Transfer water (Sigma; W1503; approximately ¾ of the final volume). Be sure to dissolve well. It usually dissolves in 5 minutes.
2. Aliquot the solution into two autoclaved, 50 mL round bottom centrifuge tubes.
3. Centrifuge for 1 hour at 12,000 rpm (18,500 g) at 4ºC.
4. Into a medium glassware graduated cylinder: very slowly and carefully pipette off the supernatant so that none of the pellet is disturbed or taken: leave a thin layer of supernatant above the pellet. Remove ~50 mL as identified in the table above. Do not decant as the pellet is too soft.
5. Add a small amount of additional Embryo Transfer water (see table above) so that you have a total of 80 mL volume, and then add the indicated amount of 18 g raffinose and 4 µl 1-Thioglycerol. The additional water you add will help these components dissolve.
6. Rinse the graduated cylinder with Milli-Q water 10 times and place on rack to dry.

7. Stir at room temperature in a 100 mL bottle or glass beaker until the raffinose is completely dissolved. Stir for about 3-4 hours at room temperature or 2 hours at 37ºC or stir at 60 degrees for a short time. Solution should be milky looking when you are finished. There should be no un-dissolved raffinose floating on the bottom.
8. Adjust to the final volume of 100 mL in a media graduated cylinder. Rinse the cylinder with Milli-Q water 10 times and place on drying rack.
9. Filter the sperm cryo media through a 0.22 µm filter. You may pre-filter (0.45 µm) if desired.
10. The media should have an osmolality of 420-450 mOsm. If it is higher, you may carefully add Embryo Transfer water to decrease it.
11. Label tubes with: "Sperm freezing media", osmolality, date made and your initials.
12. Aliquot 7 mL per 15 mL conical tube. Store at -20ºC for up to one month.

Today's date: _____

Your initials: _____

Supervisor's initials: _____

3. Standard cell and tissue collection preparation from euthanized rats

3.1. Euthanization

Supplies:

- VersiDry Lab Soaker for benchtop
- 35 mm Petri dishes (Falcon 1008)
- Kimwipes
- Latex Gloves
- Micro-scissors
- Fine forceps
- Curved, serrated forceps
- Stereo microscope
- Slide warmer
- Disposal bags
- CO_2 chamber for euthanasia
- CO_2 tank
- CO_2 flowmeter

Procedure:

1. Ensure that the induction box hoses are tightly connected to both the CO_2 tank, flowmeter and the induction chamber. Use only 100% CO_2 from a compressed gas cylinder.
2. Place rats into the uncharged, empty chamber. Don't overcrowd. Each animal must have enough floor space to assume its normal posture.
3. Close chamber lid, turn on CO_2 tank, and adjust flowmeter regulator to a flow rate of 30% chamber volume per minute.
4. Leave rats in chamber until breathing has ceased for several minutes.
5. Turn off flowmeter and close the tank valve to stop the flow of CO_2.
6. Remove animals from chamber and assure death by certain physical means. Bilateral pneumothorax, aortic transection, and cervical dislocation are some of the examples.
7. Also be aware that heavily loading a chamber (with animals) will result in a longer period of time for complete euthanasia to be reached.

3.2. Dissection of reproductive organs to collect tissues of interest

Supplies:

Standard cell and tissue collection set-up (see **Section 3.1**)

Procedure:

1. Euthanize the animals as described in **Section 3.1**
2. Place the animals on a VersiDry Lab Soaker on the benchtop and spray the abdominal area with 70% ethanol.
3. Grasp the abdominal skin with serrated forceps and make a lateral incision using scissors as shown in Figure 1. The skin is then pulled in opposite direction to expose the body wall. The body wall (peritoneum) is then cut to expose the testicle and accessary reproductive tracts to collect the cauda epididymis.

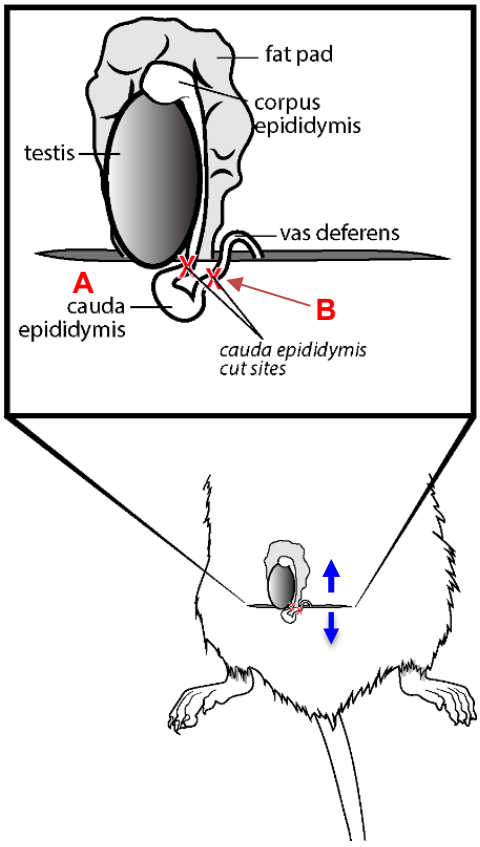


Figure 1. Dissection of reproductive organs of a male rat. The position of the small lateral incision in the skin is indicated. The skin is then pulled back in the direction of the solid blue arrows. The body wall (peritoneum) is then cut to expose the testicle and accessary reproductive tracts (adapted from Behringer et al., 2014. Manipulating the mouse embryo (4rd edition), Cold Spring Harbor Laboratory Press, New York).

4. Rat sperm freezing

Supplies:

- Raffinose cryoprotectant media (CPM) for sperm (2.0 mL needed per male)
- R1CH with 4 mg/mL BSA
- 37ºC warming plate
- 35 mm Petri dishes (3 per male)
- 0.5 mL microcentrifuge tubes (2 per male)
- 1.5 mL microcentrifuge tubes (1 per male)
- MiniSpin microcentrifuge
- Hamilton Thorne IVOS sperm analysis system
- Dissection tools
- Kimwipes
- MPB Ultra-Micro pipet tips
- Pre-labeled CBS embryo/sperm straws
- Blue weights for straws
- Pre-labeled canes
- Cane sleeves
- Heat sealer
- Liquid Nitrogen (LN_2)
- Safety glasses
- Styrofoam box labeled for sperm freezing
- 2 Plastic cane sleeves
- Metal tube rack
- Hemostats
- Timer

Procedure:

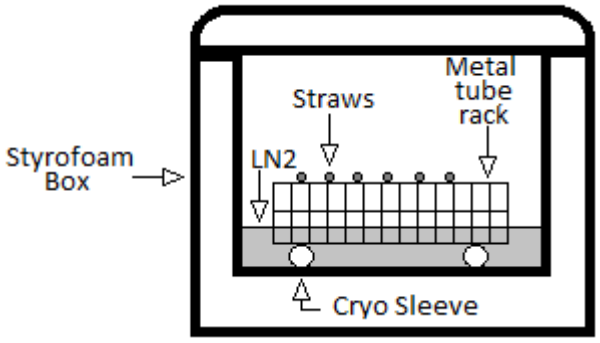
1. Make straw labels (6 per male) with the Brady label printer. The label should include barcode, sample type, freeze date, and strain number.
2. Mark labels with animal ID number and apply to straws. Pre-load blue straw weights into the straws.
3. Use the label maker to apply the cane number to the cane and put a goblet on the cane.

4. Set up your dissection area.
5. Put 4-5 mL R1CH or PBS plus BSA into a 35 mm petri dish. Prop one end of the other two dishes and add 2.0 mL raffinose CPM into one dish and 100 µL of R1CH/PBS into the other.
6. Label two 0.5 mL tubes with each animal ID number. Label one “R1CH/PBS” and the other “Raffinose”. In the R1CH/PBS tube add 100 µL of R1CH/PBS.
7. Acquire the male for sperm cryopreservation from the vivarium. Be sure that the ear tag matches the schedule and that the male is at least two months old before removing from the room.

Freezing:

1. Euthanize the animals as described in Section 3.1.
2. Place the animals on a VersiDry lab soaker on the benchtop and spray the abdominal area with 70% ethanol.
3. Open up the abdominal cavity as described in Section 3.2.
4. Dissect the cauda epididymis (CE) at point A, removing as much excess fat as possible. Cut the vas deferens (VD) at point B (Figure 1).
5. Place cauda epididymis in the R1CH/PBS plus BSA wash plate.
6. Remove the cauda epididymis from the wash and place on a Kimwipe. Remove any fat and tease out any blood. Lie the CE on a Kimwipe and straighten the VD so that the blood vessel is on one side. Use fine tip forceps to “brush” the blood out. You may eliminate blood by rinsing the tissue in R1CH quickly and drying it out using sterile 2x2 gauze.
7. Move the cauda epididymis to the 100 µL drop of R1CH/PBS plus BSA. Cut epididymis 3 to 4 times and let sit for approximately one minute.
8. Using the transfer pipette allow capillary action to acquire a small sample and place in the 0.5 mL tube labeled “R1CH/PBS”. Allow the tube to sit in a holder so it remains in a vertical orientation. **NOTE**: Handle sperm gently. Minimize agitation and pipette slowly.
9. Transfer the epididymis to the 2 mL of raffinose CPM. Move the remaining R1CH/PBS to the raffinose using the transfer pipette. Allow the sperm to swim out for 15 minutes.
10. Gently swirl the dish to evenly distribute the sperm. Then remove the epididymis from the dish.
11. Very gently pipette out 30 µL into the vial labeled “Raffinose”.
12. Load approximately 300 µL of sperm with CPM into the six pre-labeled straws.
13. Seal both ends of the straw using the heat sealer.

14. Fill the Styrofoam sperm freezing box to the marked line (approximately 1 to 1.5 inches) with LN_2. Place the metal tube rack on top of 2 plastic cane sleeves in the box. Refer to diagram below.
15. Place the straws on the metal tube rack and put the lid on the box.
16. After 5 minutes on the metal tube rack, plunge the straws directly into the LN_2 in the freezing box.
17. Put the straws into the appropriate cane and put the canes into plastic cane sleeves. Place the canes into the proper canister within the correct Dewar for long-term storage.
18. Use the R1CH/PBS plus BSA sample to measure the motility, dilute if needed. Fill a 0.5 mL tube with 90 µL of R1CH/PBS and add 10 µL from the Raffinose tube. Carefully invert the tube once and allow the sperm to diffuse. Use this 1 to 9 dilution to measure the concentration. From the non-diluted Raffinose tube make your slides to measure morphology. Count 50 sperm per male for morphology.
19. Record all the sample information in an appropriate database.



Thawing:

1. Remove straw from LN_2 and expose to room temperature for 15 seconds. Then place the straw in a 37^0C bead bath until the sample is thawed.
2. Cut both ends of straw (end opposite label first) and expel sperm slowly into a 1.5 mL microcentrifuge tube containing pre-warmed (37^0C) R1CH + 4 mg/mL BSA.

Sperm Analysis

Sperm analysis can be performed using hemocytometer. At RRRC, we use Hamilton Thorne Sperm Analysis Protocol for evaluating sperm *concentration, morphology,* and *motility*. (Protocol available upon request).