

Rat Embryo Freezing and Thawing Protocol

Rat Resource and Research Center

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Abbreviations:

R1CC (rat 1-cell culture): A modified rat 1-cell 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.

R2CC (rat 2-cell culture): A modified rat 1-cell culture medium (mR1ECM) with an osmolality of ~246 mOsm for rat 2 cell culture (R2CC) or later stage embryo culture.

R2CH: R2CC-HEPES, a modified R2CC where part of bicarbonate is replaced with HEPES buffer for handling and flushing 2 to pre-compacted rat embryos.

R2CH/FBS: R2CH with 10% FBS for handling and flushing morula and blastocyst stage rat embryos.

Chapter 1: Hormone and Medium Preparation

Notes: Immature rats can be superovulated by either PG600 or PMSG. We primarily use PG600 and use PMSG as a substitute when PG600 is unavailable.

1.1: PG600 stock preparation

Purpose: PG600 is used to superovulate immature female rats.

Supplies and reagents:

- Sterile 1.5 mL microcentrifuge tubes
- Pipettes and sterile tips

Reagents	Company	Cat #
PG600 2000 IU	Valley Vet	16820
DPBS w/o CaCl ₂ , MgCl ₂	Gibco	14190-144

Reconstitution and aliquoting:

- 1) Dilute the 2000 IU bottle with 2.0 mL (2000 µL) of diluent (DPBS) for a concentration of 100 IU /100 µL.
- 2) Aliquot 80 µL/ 2.0 mL microcentrifuge tube. This should generate 25 tubes of concentrated PG600.
- 3) Appropriately label the tubes and place in box and label the box with PG600, make date, expiration date, lot number, and dilution instructions. Place the box in the -80 °C freezer.

Procedure for superovulation:

Thaw one tube. Add 720 µL of saline to a final volume of 800 µL. This will give a working concentration of 80 IU/0.8 mL or 20 IU/0.2 mL. Each immature female rat should be injected intraperitoneally (IP) with 0.2 mL diluted PG 600 between 9-10 am (on a light cycle where the lights come on at 6 am and turn off at 8 pm).

- 1) Each tube should then be enough for 4 rats (although practically speaking, will only be enough for 3 animals due to losses in the syringe/ needle).
- 2) Once you have thawed and diluted a PG600 concentrated vial, discard any unused portion.
- 3) *PG600 is appropriate for sexually immature female rats (~50-80 grams; little to no vaginal opening).

1.2: Rat Pregnant Mare Serum Gonadotropin (PMSG) stock preparation

Purpose: To provide a step-by-step procedure for reconstituting and aliquoting PMSG for superovulation of immature rats.

Supplies and Reagents:

Ice bucket and ice

1.5 mL clear and sterile Eppendorf tubes

Small freezer storage box with lid

Pipettor, and pipette tips

Reagents:	Company	Catalog #
PMSG hormone	Calbiochem	367222, 1000IU
	ProspecBio, 1000IU	HOR-272
DPBS w/o CaCl ₂ , MgCl ₂	Gibco	14190-144

Procedure for reconstitution and aliquoting:

1. Obtain ice bucket and PMSG hormone from -20°C freezer. Please note that if there are less than three vials of PMSG left, then we need to order more.
2. Obtain clear 1.5 mL sterile Eppendorf tubes and small box with lid.
3. Label the box with PMSG RAT, 125 IU/tube, date made, date expired (3 months from aliquot date), lot number, *instructions for diluting.
4. In the hood, add 200 µL of sterile phosphate buffered saline (14190-144 Dulbecco's Phosphate Buffered Saline without calcium chloride and without magnesium chloride) at neutral pH.
5. Pipette up and down to mix.
6. Keeping the hormone on ice, aliquot 25 microliters per Eppendorf tube, adding the tubes to ice as you go.
7. Place the tubes in the small box and store in the -80°C freezer up to one month.
8. **Record the PMSG in the hormone log located in binder in lab cabinet.**

1.2.2: Procedure for injection:

1. Diluted working solution: add 975 microliters of sterile PBS (14190-144 Dulbecco's Phosphate Buffered Saline without calcium chloride and without magnesium chloride) per vial.
2. Inject .2cc/rat (25 IU) between 9-10am.
3. Do not leave the diluted hormone at room temperature for longer than 30 minutes. Put hormone tube/s in a container of ice if there are several animals to inject.

1.3: GnRH (LHRH) stock preparation

Purpose: GnRH is used to synchronize mature female rats.

GnRH is used for mature rat synchronization without superovulation to maximize embryo donors since mature rats do not respond to superovulation well. GnRH is also used to synchronize rats used for embryo transfer recipients to maximize pseudopregnant females.

Supplies and Reagents:

- 1cc syringes with caps or with detachable needles
- Freezer storage box

Reagents:	Company	Catalog #
des-Gly ¹⁰ , [D-Ala ⁶]-Luteinizing Hormone Releasing Hormone Ethylamide	Sigma	L-4513 (5 mg).
DPBS w/o CaCl ₂ and MgCl ₂	Gibco	14190-144

Procedure for reconstitution/aliquoting:

- 1) Make 200 µg/mL stock by diluting 5 mg in 25 mL of DPBS.
 - a) Unscrew the top and inject 1 mL of the DPBS to the vial. Mix well by rolling vial between the palm of your hands and tapping vial so that powder at top of vial goes into liquid and should be completely dissolved.
 - b) This should be performed in a laminar flow hood. Transfer all of the liquid containing the hormone from the vial to the sterile glass cylinder. Add an additional 1 mL DPBS into the vial and mix well, then add to the glass cylinder. Repeat once more. Add PBS until the volume is 25 mL.
- 2) Aliquot 1 mL per 1 cc syringe and store in -80°C for up to three months.
- 3) Label the box with GnRH, made date, expiration date, and lot number of the hormone.

Procedure for injection: (Synchronization of mature female rats)

- 1) 2-3 hours after light induction, give 40 µg LHRH (0.2 mL) intraperitoneally (IP) per mature female rat (8 weeks or older). Transport the hormones on ice if there are several animals to inject.
- 2) Four days after injection, mate with a mature intact male rat (at least 10 weeks of age) for embryos OR with a sterile, vasectomized male rat (at least 10 weeks of age) for embryo transfer recipient.
- 3) Collect morula from 3.5 day rats (4 days after mating). Or use the plug positive females from vasectomized male mating for embryo transfer. Any plug negative females which have been mated to vasectomized males can be recycled (use again after 14 days or completion of pseudopregnancy as shown by vaginal cytology).

1.4: Human chorionic gonadotropin (hCG) stock preparation

Purpose: Human Chorionic Gonadotropin (hCG) is used to induce ovulation.

Supplies:

- Microcentrifuge tube, 1.5 mL
- Pipette-Aid
- Micropipettes
- 15 mL tubes

Reagents:	Company	Catalog #
hCG	Calbiochem	230734
DPBS w/o CaCl ₂ , MgCl ₂	Gibco	14190-144

Procedure for reconstitution and aliquoting:

- 1) Obtain ice bucket and hCG (1 mg, 3,100 IU) hormone from -20°C freezer.
- 2) Obtain clear 1.5 mL microcentrifuge tubes and obtain small box with lid (for tubes).
- 3) Label the box appropriately, concentration: 200 IU/1 mL, date expires (3 months from aliquot date),
- 4) In the laminar flow hood, peel the foil off from around the top of the vial. Remove the rubber stopper, taking care that some of the hormone may be sticking to the rubber. Add 2.0 mL of DPBS at neutral pH to dilute the Hcg and pipette up and down to mix.
- 5) Add the rubber stopper and invert the solution a couple of times.
- 6) Remove the 2 mL into an empty 50 mL graduated cylinder.
- 7) Add additional 2 mL of saline to the vial and rinse the vial.
- 8) Remove the 2 mL into an empty 50 mL graduated cylinder.
- 9) Add 11.5 mL DPBS to the graduated cylinder, for a total of 15.5 mL saline and pipette several times to mix.
- 10) Keeping the hormone on ice, aliquot 1000 µl per microcentrifuge tube (adding the tubes to ice as you go) resulting in 200 IU/vial (3100 divided by 15.5 = 200).
- 11) Place the tubes in the labeled container and store in the -80°C freezer up to three months.
- 12) Each rat gets 0.2 mL hCG IP, at ~11 am – 2 pm. This is 40 IU.

Procedure for superovulation:

Procedure for injection:

1. Remove the frozen tube of hormone from the freezer and allow it to thaw.
2. Inject 0.2 cc/animal (40 IU) between 12-2 pm for embryo collection.
3. Do not leave the diluted hormone at room temperature for longer than 30 minutes. Put the hormones on a container of ice if injections will take longer than 30 minutes.

1.5: Hyaluronidase stock (1 mg/mL) preparation for denuding of rat zygotes

Purpose: Hyaluronidase is used to remove cumulus cells from rat zygotes also called as denuding.

Supplies and Reagents:

- 1.5 or 2.0 mL microcentrifuge tubes

Reagents:	Company	Catalog #
Hyaluronidase	Sigma	H4272-30 mg
BSA, Fatty acid free	Sigma	A7638
R1CH with 4 mg/mL BSA (Fatty Acid Free)	N/A	N/A

Procedure:

- 1) Wear nitrile gloves during preparation of enzyme (sterile technique).
- 2) Clean area in hood for preparation of aliquoting hyaluronidase.
- 3) Prepare working solution of R1CH (4 mg/mL of Fraction-V BSA, Fatty Acid Free)*.
 - * Add 0.20 g BSA to 50 mL of R1CH in 50 mL conical tube. Let the tube stand in the hood until all BSA go into solution and then filter.
- 4) Aliquot into 1.5 or 2.0 mL microcentrifuge tubes placed in the microcentrifuge tube stand and place in the hood.
- 5) Remove Hyaluronidase from -20°C freezer.
- 6) First take the metal off around the vial of hyaluronidase to expose the cap. Open the cap carefully since some of the powder might be stuck on the cap.
- 7) Set cap aside in laminar flow hood.
- 8) Add 15 mL of working solution of R1CH (4 mg/mL BSA).
- 9) Replace cap and invert to dissolve.
- 10) Taking care not to insert your pipet too far in the vial so as to cause overflow of liquid, add another 15 mL of the working solution.
- 11) Replace cap and invert to mix.
- 12) Pipette 1mL hyaluronidase into 1.5 or 2.0 mL microcentrifuge tubes.
- 13) Label lid of each tube with the letter "R" (for "Rat").

Label box with DATE, NAME OF TECHNICIAN,

HYALURONIDASE FOR RAT ZYGOTES

CATALOG NUMBER and LOT NUMBER, HYALURONIDASE 1 mg/mL

Table 1: Timing of hormone injection and embryo collection

Day	Immature rat	Mature rat
1	PG600 or PMSG 9 am	GnRH 9 am
2		
3	hCG and mate 11am	
4	zygote	
5	2-cell	Mate
6	2-cell – 4-cell	zygote
7		2-cell
8		4-cell
9		morula
10		blastocyst

Rules:

- 1) Rule of thumb #1: Mature rat embryo development is about 4 hours later on average than the immature rat embryo development since these are synchronized, not super-ovulated. Therefore, we do not collect zygotes from mature rats (since we don't want to collect at 5 or 6pm at night)
- 2) Rule of thumb #2: Immature rat embryo development is abnormal past the initial fertilization stage, because immature rats cannot maintain a normal pregnancy. Therefore, embryo quality and numbers may reduce dramatically past the 2-cell stage. We collect embryos at the zygote stage in immature rats.
- 3) Rule of thumb #3: Immature rats must have a vaginal opening (average weight when this occurs is around 80g) before superovulation to ensure mating- unless the endpoint is just oocytes.

1.6: Rat embryo culture and handling media

Reagents and supplies:

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 ₂ O	Sigma	C7902
MgCl ₂ -6H ₂ O	Sigma	M2393
NaHCO ₃	Sigma	S5761
Sodium Pyruvate	Sigma	P4562
MEM NEAA 100x	Invitrogen	11140-050
MEM EAA 50X	Invitrogen	11130-051
GlutaMAX 1	Invitrogen	35050-061
HEPES	Sigma	H6147
PVA	Sigma	P8136
Fatty Acid Free BSA	Sigma	A7638
Embryo tested mineral oil	Sigma	M8410
FBS	Gibco	16000

Supplies

- pH meter and appropriate pH stock solutions for standardization
- Sterile biosafety cabinet
- Pipet-Aid
- 5% CO₂ Cylinder
- 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)
- Kimwipe

- Serological pipettes
- Filter bottle units (SFCA membrane, pore size: 0.2 μm)
- Steriflip-GP 50 mL filter units (pore size: 0.2 μm)
- Millex-GP 0.22 μm filter
- Filter bottle units (SFCA membrane, pore size: 0.2 μm)
- Sterile Pasteur pipettes
- Osmometer
- pH meter

General notes regarding preparation of media

1. Prepare all reagents on the day or at least one day prior to usage. RRRC uses embryo transfer water for all stock solutions requiring sterile water. **Please wear gloves when preparing these solutions.**
2. Please follow the medium recipe and preparation instructions **exactly** as it is written in this SOP. Please make yourself familiar with protocols before proceeding to the worksheet.
3. For the reagent Sodium Lactate (60% syrup) the formula weight (FW) is calculated as follows:
FW of 112.1 g/M. Given a 60% w: w solution = $[112.1 / (0.6)] \Rightarrow 186.8 \text{ g/mole}$
4. Please check the osmolality and pH of the medium.
5. After calibrating the osmometer with 290 mOsm standard, check the osmolality of the solution. If the osmolality does not fall between desired levels, please remake the solution.
7. Sterile filter the solution using a 0.2 μm filter unit.
8. Rinse all used glassware at least ten times with Milli-Q water only and place on proper drying rack.
9. Label container with date made, expiration date (two weeks from make date), pH, mOsm, initials and batch number (YourInitialmm/dd/yyyy).

1.6.1. Rat stock solution (10X)

Purpose: this will be used in preparation for rat 1-cell or 2- cell media.

Protocol

- 1) After all components have been dissolved, transfer the contents to the appropriate volumetric flask and bring to desired volume by rinsing out the Griffin beaker with embryo water and adding it to the volumetric flask. Measure the osmolality, record this result, and filter into an appropriately sized sterile bottle.
- 2) Label with name of solution, make date, expiration date (one month from make date) and initials. Label with osmolality and batch number (Your Initial, mm/dd/yyyy). Store at 4°C.
- 3) Rinse all used glassware at least ten times with **Milli-Q water only** and place on a drying rack.

Worksheet 1.6.1: Rat stock solution (10X)

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)	Working concentration mM	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 ₂ O	147.02	2	1.4702g		
Sigma	M2393	MgCl ₂ -6H ₂ O	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	

1.6.2: R1CC (high NaCl) for 1-cell rat embryo culture

Purpose: R1CC is used for rat 1-cell embryo culture.

Protocol:

1. Add all components as listed in Worksheet 1.2.2 to a Griffin beaker containing Embryo water (80% of final volume).
2. After all components have been dissolved, transfer the contents to the appropriate volumetric flask and bring to desired volume by rinsing out the Griffin beaker with embryo water and then adding it to the volumetric flask.
3. Gently gas the solution with 5% CO₂, using a sterile Pasteur pipette, for 15-30 minutes.
4. Check to ensure the pH of the solution is approx.7.4 - if pH is <7.0 remake solution.
5. 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.
6. Sterile filter the solution using a 0.2 µm filter unit.
7. Store at 4°C for up to 2 weeks.
8. Rinse all used glassware at least ten times with **Milli-Q water only** and place on a drying rack.

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

Working Solution:

1. If medium has been stored in the refrigerator, it must be gassed for approximately 1 minute prior to aliquoting.
2. Aliquot amount needed (usually 10 mL) using a sterile pipette and gas the top of the bottle before closing and storing at 4°C.
3. Add 4 mg/1 mL (0.04 g/10 mL) Fatty Acid Free BSA (Sigma A7638).
4. Sterile filter using 0.2 µm syringe filter.
5. Make 30 µL drops in 35 mm culture plates and cover with embryo tested mineral oil (Sigma M8410) which has been filtered, washed and pre-equilibrated in the incubator. Do not make more than five plates at a time before adding mineral oil, since drops can evaporate in the flow hood in a short amount of time, and there can be a sharp rise in pH which is deleterious to embryo development.
6. Immediately place dishes in incubator preset to 5% CO₂ and 5% O₂ (low O₂) and 37°C. Allow them to equilibrate for at least one hour prior to use.
7. Label container with make date, expiration date (two weeks from make date), pH, mOsm, initials and batch number and batch number (Your Initial, mm/dd/yyyy).

Worksheet 1.6.2: R1CC (high NaCl) for 1-cell rat embryo culture

Company	Cat #	Reagent	FW (g)	[mM]	250 mL	Added	Lot #
	Rat Stock Solution		N/A	N/A	25 mL		
Sigma	S5886	NaCl	58.44	30	0.4383g		
Sigma	G6152	NaHCO3	84.007	25	0.5251g		
Sigma	P7794	Sodium Pyruvate	372.2	100µg/mL	0.0138g		
Invitrogen	11140-050	MEM NEAA 100x	1457	50µg/mL	2.5 mL		
Invitrogen	11130-051	MEM EAA 50X	186.8	13.53	5 mL		
Invitrogen	35050-061	GlutaMAX 1	147.02	2	0.125 mL		

Parameter	Expected	Y or N/ Measured
Gassed with 5% CO ₂	approx... 30min.	
pH of Solution	7.4	
Osmolality	280-300	
Filter sterilized	Y/N	

Today's date: _____

Your initials: _____

Batch # _____

1.6.3: R1CH for flushing and handling 1-cell rat embryos

Purpose: R1CH is used as a holding medium to manipulate rat 1-cell embryos.

Protocol:

- 1) Add all components as listed in Worksheet 1.2.3 to a Griffin beaker containing Embryo water (80% of final volume).
- 2) After all components have dissolved, check to ensure the pH of the solution is approximately 7.4. Adjust if needed (with 10N NaOH).
- 3) Transfer the contents to the appropriate volumetric flask and bring to desired volume by rinsing out the Griffin beaker with embryo water and adding it to the volumetric flask.
- 4) After calibrating the osmometer with a 290 standard, check the osmolality of the solution.
 - a. Remake the solution if the osmolality does not fall between 290-320 mOsm.
- 5) Sterile filter the solution using a 0.2 μm filter unit. Store at 4°C for up to 2 weeks.
- 6) Rinse all used glassware at least ten times with **Milli-Q water only** and place on a drying rack.
- 7) Label container with make date, expiration date (two weeks from make date), pH, mOsm, initials and batch number (Your Initial, mm/dd/yyyy).

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

Working Solution:

- 1) Aliquot amount needed using a sterile pipette (usually 50 mL).
- 2) Add 4 mg/mL (0.04 g/10mL or 0.2 g/50 mL) Fatty Acid Free BSA (Sigma A7638). Allow the BSA to dissolve into solution in the 37 degree Celsius water bath.
- 3) Sterile filter using 0.2 μm filter (preferably a Steriflip, Millipore SCGP00525).

Worksheet 1.6.3: R1CH for 1-cell rat embryo flushing and handling

Company	Catalog #	Reagent	FW (g)	[mM]	500 mL	Added	Lot#
	Rat Stock Solution		N/A	N/A	50 mL		
Sigma	S5886	NaCl	58.44	30	0.8766g		
Sigma	G6152	NaHCO ₃	84.01	5	0.21g		
Sigma	P7794	Sodium Pyruvate	110	100µg/mL	0.0276g		
Invitrogen	11140-050	MEM NEAA 100x	N/A	50µg/mL	5 mL		
Invitrogen	11130-051	MEM EAA 50X	N/A	13.53	10 mL		
Sigma	H6147	HEPES	238.31	22	2.6214g		
Invitrogen	35050-061	GlutaMAX 1	N/A	0.1	0.25 mL		

Parameter	Expected
Gassed with 5% CO ₂	
pH of Solution	7.4
Osmolality	290-320
Filter sterilized	N/A

Today's date: _____

Your initials: _____

Batch # _____

1.6.4: R2CC for 2-cell or later stage rat embryo culture

Purpose: R2CC is used in culturing rat embryos from 2-cells to blastocysts.

Protocol:

- 1) Add all components as listed in Worksheet 1.2.4 to a Griffin beaker containing Embryo water (80% of final volume).
- 2) After all components have been dissolved, transfer the contents to the appropriate volumetric flask and bring to desired volume by rinsing out the Griffin beaker with embryo water and adding it to the volumetric flask.
- 3) Gently gas the solution with 5% CO₂, using a sterile Pasteur pipette, for 15-30 minutes.
- 4) Check to ensure the pH of the solution is approx.7.4, if pH is <7.0 remake solution.
- 5) 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 235-255 mOsm.
- 6) Sterile filter the solution using a 0.2 µm filter unit.
- 7) Store at 4°C for up to 2 weeks.
- 8) Label container with make date, expiration date (two weeks from make date), pH, mOsm, and initials with green tape.
- 9) Rinse all used glassware at least ten times with **Milli-Q water only** and place on a drying rack.
- 10) Note: If any precipitates form in the solution, discard the solution and start over.

Working Solution:

- 1) Using a sterile Pasteur pipette, gas bottle for approximately 1 minute prior to aliquoting medium.
- 2) Aliquot amount needed using a sterile pipette and gas the top of the bottle before closing and storing at 4°C.
- 3) For 16 cell or morulae, make up a working solution of 10% FBS (Gibco 16000). For 2-cell to 8-cell, do not add FBS.
Sterile filter using 0.2 µL syringe filter.
- 4) Make the 35 mm culture plates the day before use (30 µl drops for culture) and use only embryo tested mineral oil (Sigma M8410) which has been filtered/washed/ and pre-equilibrated in the incubator. Do not make more than 5 plates at a time before adding mineral oil, since drops can evaporate in the flow hood in a short amount of time, and secondly, there can be a sharp rise in pH which is deleterious to embryo development.
- 5) Immediately place the culture plates in a calibrated incubator preset to 5% CO₂ and 5% O₂ (low O₂) 37°C and allow them to equilibrate overnight. Place the tube of unused medium in the incubator so that it can be used for making additional culture dishes if needed.

Worksheet 1.6.4: R2CC for 2-cell or later stage rat embryo culture

Company	Cat #	Reagent	FW (g)	[mM]	250 mL	Added	Lot#
	Rat Stock Solution		N/A	N/A	25 mL		
Sigma S5886		PVA	N/A	N/A	0.025		
Sigma G6152		NaHCO ₃	84.01	25	0.5251		
Sigma P7794		Sodium Pyruvate	110	0.5	0.0138		
Invitrogen 11140-050		MEM NEAA 100x	N/A	0.5x	2.5mL		
Invitrogen 11130-051		MEM EAA 50X	N/A	0.5x	5mL		
Sigma H6147		GlutaMAX 1	N/A	0.1	0.125mL		

Parameter	Expected
Gassed with 5% CO ₂	approx... 30min.
pH of Solution	7.4
Osmolality	235-255 mOsm
Filter sterilized	

Today's date: _____

Your initials: _____

Batch # _____

1.6.5: R2CH for 2-cell or later stage rat embryo flushing and handling

Purpose: R2CH is used in handling and manipulating rat embryos from 2-cells to blastocyst.

Supplies:

- Griffin beaker
- Volumetric flask
- Stir bar
- Weigh paper
- Spatula
- Scoopula

Protocol

- 1) Add all the components according to Worksheet 1.5.5 to a Griffin beaker containing 50% of the total volume of medium with Embryo water (Sigma W1503).
- 2) After all components have dissolved, check to ensure the pH of the solution is approx.7.4, adjust if needed (with 10N NaOH).
- 3) Transfer the contents to the appropriate volumetric flask and bring to desired volume by rinsing out the Griffin beaker with embryo water and adding it to the volumetric flask.
- 4) After calibrating the osmometer with a 290 mOsm standard, check the osmolality of the solution.
 - a. Remake the solution if the osmolality does not fall between 240-255 mOsm.
- 5) Sterile filter the solution using a 0.2 μm filter unit.
- 6) Store at 4°C for up to 2 weeks.
- 7) Label container with make date, expiration date (two weeks from make date), pH, mOsm, and initials with green tape. Label also with the batch number which is the Julian date.
- 8) Rinse all used glassware at least ten times with Milli-Q water only and place on a drying rack.

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

Working Solution:

- 1) Aliquot amount needed using a sterile pipette. This is usually 50 mL.
- 2) Sterile filter using 0.2 μL filter.

Worksheet 1.2.5. R2CH for 2-cell or later stage rat embryo flushing and handling

Catalog #	Reagent	FW (g)	[mM]	500mL	1000mL	Added	Lot #
Sigma P8136	PVA	N/A	N/A	0.050	0.1		
Sigma S5761	NaHCO ₃	84.01	5	0.2100	0.4201		
Sigma H6147	HEPES	238.31	22	2.6214	5.2428		
Sigma P4562	Sodium Pyruvate	110	0.5	0.0276	0.0550		
Invitrogen 11140-050	MEM NEAA 100x	N/A	N/A	5 mL	10mL		
Invitrogen 11130-051	MEM EAA 50X	N/A	N/A	10 mL	20mL		
Invitrogen 35050-061	GlutaMAX 1	N/A	0.1	0.250 mL	0.5mL		
See stock Solutions	Rat Stock Solution	N/A	N/A	50 mL	100 mL		

Parameter	Expected	Adjusted
pH:	7.3~7.4	
Osmolality	240~255 mOsm	

Today's date: _____

Your initials: _____

Batch # _____

Chapter 2. Embryo Collection

2.1: Standard Cell and Tissue Collection preparation from euthanized rats

(To euthanize rats with a minimum amount of pain or distress.)

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
- CO₂ chamber for euthanasia
- CO₂ tank
- CO₂ flowmeter
- 70% Ethanol.

Reagents:

- mR1ECM for culture of Rat embryos of 1-cell (R1CC), 2-cell and later stages (R2CC) or appropriate HEPES-buffered medium.

Procedure:

- 1) Ensure that the induction box hoses are tightly connected to both the CO₂ tank, flowmeter and the induction chamber. Use only 100% CO₂ 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 CO₂ 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 CO₂.
- 6) Remove animals from chamber and assure death by some certain physical means. Bilateral pneumothorax,

aortic transection, and cervical dislocation are examples.

- 7) Young rats (“pinkies” or “fuzzies”) may be extremely difficult to euthanize via CO₂ because of their fetal hemoglobin’s affinity for oxygen. They may require 20-60 minutes of exposure to CO₂ with decapitation as a secondary, physical means of ensuring euthanasia.
- 8) Also be aware that heavily loading a chamber (with animals) will result in a longer period of time for complete euthanasia to be reached.

2.2: Dissection of reproductive organs to collect tissues of interest

Supplies:

- Standard Cell and Tissue Collection Set-Up (see Section 2.1)

Procedure:

- 1) Euthanize the animals as described in Section 2.1.
- 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 at the midline using scissors (see Figure 1). Push the intestines away to reach the uterine horns (they are positioned laterally) to collect the tissues of interest (Figure 2, Figure 3). The complete reproductive tract may be removed in its entirety – the ovaries, oviducts, uterine horns and cervix.

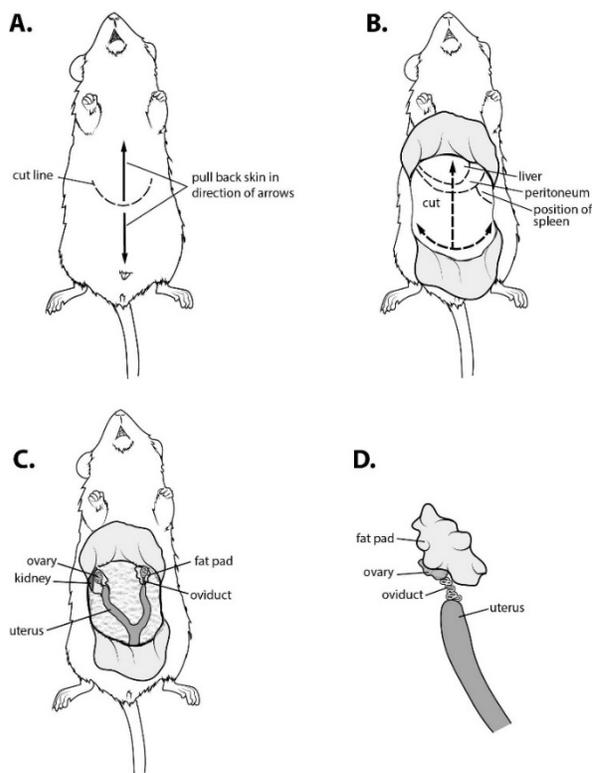


Figure 1. Reproductive organ dissection from a female rat. Cut the skin with a scissors as indicated by dashed line and then pull the skin apart in the direction of solid arrows (A). Cut the body wall (peritoneum) as indicated by dashed line in (B); the layout of internal reproductive organ of a female rat (C). An enlarged view of one side of internal reproductive tract of a female rat (adapted from Behringer et al., 2014. *Manipulating the mouse embryo* (4th edition), Cold Spring Harbor Laboratory Press, New York).

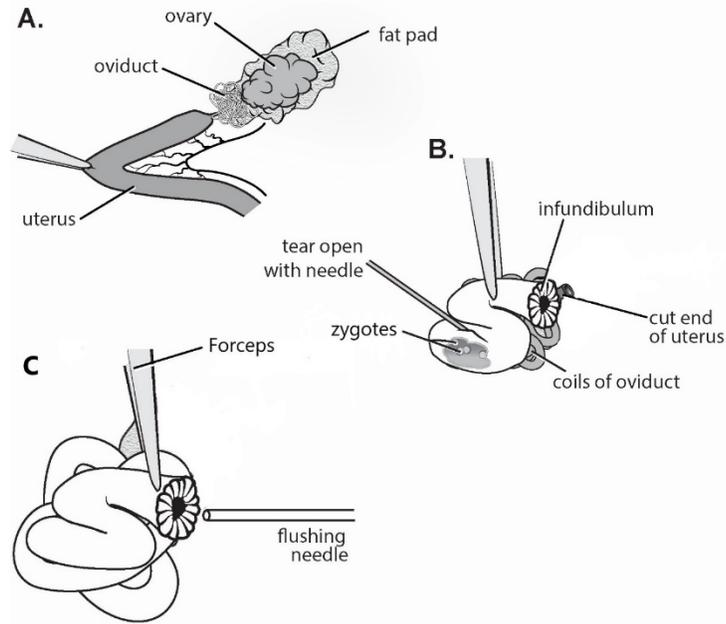


Figure 2. Collection of zygotes and cleavage stage embryos from oviduct. Excise oviduct by cutting first at oviduct and ovary junction and then a small section of uterine horn as illustrated in (A). Hold oviduct coil with a serrated forceps and release zygotes and cumulus mass by tearing the swollen ampulla with an insulin needle as indicated in (B). Cleavage stage embryos (2-cell to morula) are collected by flushing oviduct through the infundibulum (C) (adapted from Behringer et al., 2014. *Manipulating the mouse embryo* (4rd edition), Cold Spring Harbor Laboratory Press, New York).

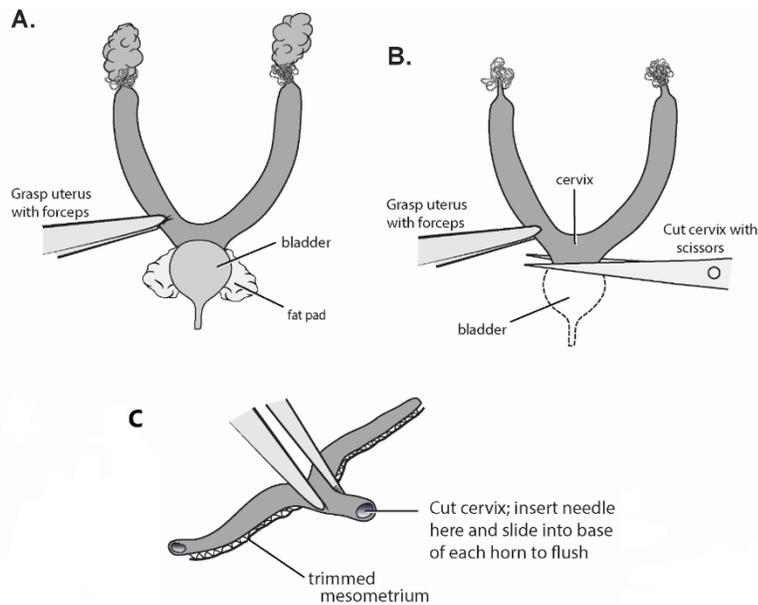


Figure 3. Collection of blastocysts from female rats. (A) Internal reproductive organs of a female rat. (B) Excise the bicornuate uterus by cutting at both the cervix and the oviducts. (C) Flush the bicornuate uterus as illustrated (adapted from Behringer et al., 2014. *Manipulating the mouse embryo* (4rd edition), Cold Spring Harbor Laboratory Press, New York).

2.3. Zygote-cumulus complexes collection from the oviduct

Supplies and reagents:

- Standard Cell and Tissue Collection Set-Up (see Section 2.1)
- R1CH/BSA (4 mg/mL)
- Insulin syringes with needles
- Fine forceps
- Stripper pipette tips (Origio MXL3-125)
- Hyaluronidase (1 mg/mL in R1CH) optional, for cumulus cell removal only

Procedure:

- 1) Euthanize the animals as described in Section 2.1.
- 2) Place the animals on a VersiDry lab soaker on the benchtop and spray the abdomen with 70% Ethanol.
- 3) Open the abdominal cavity as described in Section 2.2, Figure 1.
- 4) Remove the oviducts and place them into R1CH, washing them twice with R1CH.
- 5) In the last washing step, grab the oviducts using fine tweezers and tear the clutch using a 27 g needle to release the cumulus zygote complexes (Figure 2B). Wash them twice in R1CH before further usage.
- 6) If you want to remove the cumulus cells, put the cumulus zygote complexes into a warm R1CH solution containing 1mg/mL Hyaluronidase for about 5 min (or watch them until the cumulus cells fall apart and then gently pipette them to remove the remaining cumulus cells).

2.4: Embryo Isolation: Collection of embryos from the uterus by flushing the oviduct through the infundibulum.

Materials:

- Standard Cell and Tissue Collection Set-Up (see Section 2.1)
- R2CH-PVA (0.1 mg/mL)
- Hamilton needles -30 g, blunt (for flushing)
- Fine forceps
- Hand pipette
- Pulled glass pipette tips or plastic pipette tips if you prefer

Procedures:

1. Please refer to **Table 1** for timing of embryonic development.
2. Euthanize the animals as described in Section 2.1.
3. Place the animals on a VersiDry lab soaker on the benchtop and spray the abdominal area with 70% Ethanol.
4. Open up the abdominal cavity as described in Section 2.2.
5. Remove the oviducts and uteri (as described in Section 2.2) and place in R2CH + 10% FBS medium, and wash them twice in R2CH + 10% FBS to get rid of any contaminants.
6. Under the stereo microscope, using fine tweezers, gently grab the infundibulum, insert a 30 g Hamilton needle attached to a 1cc syringe (pre-loaded with R2CH + 10% FBS) and push the plunger to release 500 μ l of the solution per horn to expel cleavage stage embryos prior to blastocysts (Figure 2C). Blastocysts can be collected by puncturing the uterus close to the utero-tubal junction and flushing with 1 mL of the medium (Figure 3).
7. Collect the embryos into a clean Petri dish containing R2CH + 10% FBS. Wash them 3 times in R2CH + 10% FBS before further usage. (Refer to the following table to find out which embryonic stage you want to collect following mating.)
8. If embryos are not going to be frozen promptly, transfer to culture plates with R1CC and culture in 37°C, 5% CO₂, 5% O₂ incubator.

Chapter 3. Embryo Cryopreservation

Section 3.1: Zygote freezing and thawing protocol

Supplies and Reagents:

- Collection Dishes
- 0.3mL Sterile CBS Embryo/Sperm straws and straw weights
- Device to pipette embryos

Reagents:	Company	Catalog #
R1CH without BSA	N/A	N/A
FBS	Gibco/Life Technologies	16000
Sucrose	Sigma	S9378-500G
DMSO	Sigma	D8418-50ML

Freezing Procedure:

1. Prepare cryoprotectant medium as follows: 18% FBS in R1CH = 1.8mL FBS + 8.2 mL R1CH. Then make 1.5M DMSO / 0.1 M sucrose = 1065 μ L DMSO + 0.344g sucrose in 10mL R1CH with 18% FBS. Filter and load straw with cryo medium.
2. Prepare the freezing apparatus that you will use for this procedure (we use a Cryologic or Crysalyt at RRRRC). Make sure that there is sufficient liquid nitrogen (LN₂) and that the machine is available and ready for use BEFORE you equilibrate zygotes with cryoprotectant.
3. Collect zygotes using standard procedure. For QC and some projects it is necessary to collect each female separately.
4. Treat zygotes with hyaluronidase, if needed, to remove adherent cumulus cells.
5. Examine zygotes for signs of fertilization (pronuclei, sperm tail emerging from ooplasm, polar body, etc.).
6. Remove unfertilized oocytes from the pool of zygotes. If embryos are not going to be frozen promptly, transfer to culture plates with R1CC drops covered with mineral oil and culture in 37°C, 5% CO₂, 5% O₂ incubator.
7. Load 3 columns of cryoprotectant medium into the straw. The columns should be separated by an air bubble. Embryos should be contained within the central cryoprotectant column.
8. Load embryos into straws, seal straws, and equilibrate for 10 minutes in cryoprotectant medium.
9. Program (if using Cryologic or Crysalyt freezer):
 - a. Make sure the cryopreservation apparatus is set to the rat one-cell freezing program.

- b. Fill the Cryologic or Crysalyz with LN₂.
 - c. Start machine – ensure that it is at the appropriate start temperature (4°C) before adding straws to machine.
 - d. Cool at -2°C/min from 4°C to -7°C.
 - e. Seed each straw at the top of the cryoprotectant column containing embryos using a large forceps precooled at LN₂ (Crysalyz will prompt you to seed with loud beeping) and hold at -7°C for 10 minutes.
 - f. Cool at -0.5°C/min to -35°C.
 - g. Remove from freezer and plunge into LN₂.
10. Transfer straws to appropriate storage location and complete necessary paperwork/ data entry.

Thawing Procedure:

- 1) Locate appropriate straws, verify ID and transfer to the laboratory in LN₂.
- 2) Set up necessary thaw reagents and dishes.
- 3) Small petri dishes.
- 4) R1CH (without BSA) + 18% FBS.
- 5) Add 100-200µL R1CH + 18% FBS to a small petri dish.
- 6) Remove straw from LN₂ and immediately transfer to 37°C water bath for ~10 seconds.
- 7) Wipe the exterior of the straw with a Kimwipe to remove water. Holding the straw horizontally, cut the sealed end (opposite the side with the label) with scissors. Then, hold the straw vertically (cut end down) over a small petri dish containing the R1CH + 18% FBS and cut the other sealed end. Immediately expel contents of straw into the drop (using a syringe fitted with straw adapter).
- 8) Hold for 10 minutes.
- 9) Move embryos to fresh dish containing 100-200 µL R1CH + 18% FBS.
- 10) Wash a second time by moving to fresh dish containing 100-200 µL R1CH + 18% FBS.
- 11) Identify membrane intact zygotes for culture or embryo transfer. Remove lysed or degenerate embryos.
- 12) For embryo transfer: transport thawed zygotes to procedure area and perform embryo transfer.
- 13) For culture: transfer to appropriate culture medium, rinsing through several drops of culture medium.
- 14) Complete documentation regarding embryo thaw/ recovery culture and or transfer.

3.2: Rat morula freezing and thawing protocol

Supplies:

- Sterile CBS Embryo/Sperm straws (0.3 mL) and weights
- 1cc syringe (with CBS straw adapter) to load the straws with DMSO (cryoprotectant) and sucrose
- Kimwipes
- 35mm Petri dishes (Falcon 1008)
- Heat sealer
- Timer
- Microscope

Reagents:

- R2CH + 10% FBS.
- 1.5 M DMSO (1,065 μ l DMSO in 10 mL R2CH, filtered).
- 0.5 M sucrose (1.72g sucrose in 10 mL R2CH, filtered).

Freezing procedure:

IMPORTANT: RECEIVE PROPER TRAINING ON HOW TO USE THE CRYOLOGIC OR PROGRAMABLE FREEZER YOU ARE USING:

- 1) Start out: Check that the Cryologic is working properly. To do this, monitor the temperature digital readout as you add LN₂. The temperature should go down. Press reset to activate the thermo-regulating device. The controller should show that the straw holder is returning to start temperature. Please see Cryologic manuals for this aspect of the freezing process.
- 2) Assemble everything you need to freeze embryos, including a transfer pipette, dishes, timer, freezing solution (1.5M DMSO), 0.5M sucrose solution, R2CH + 10% FBS, and straw labels + weights if using CBS straws.
- 3) Load the straw with freezing solution (1.5M DMSO) and 0.5M sucrose solution (Figure 4). Leave plenty of room between the two ends of the straw to avoid liquid touching either end. Also, leave plenty of air between columns so that you keep the embryos isolated within a single column.
- 4) Group the collected embryos (average of 30 embryos per straw) in the final R2CH washing solution. Do not freeze more than 35 embryos in 1 straw.
- 5) Load a small volume of R2CH into a fine glass pipette, followed by an air column. Load the embryos into the pipette being careful to keep the embryos close together, preventing too much R2CH from entering the 1.5M DMSO column 2 (Figure 4). Leave liquid behind the air column to prevent air bubbles from entering the straw.

- 6) Insert the transfer pipette into the straw and gently expel the embryos and air bubble from the transfer pipette into the 1.5M DMSO column. Be sure to see the air bubble to verify that the embryos are in. After loading embryos into each straw, go back to your R2CH washing dish and expel any cryoprotectant that is left in your pipette. Make sure that all embryos were actually loaded into the straw by checking under the microscope that there is nothing in your pipette. Repeat for each straw. Also, if you are collecting more than one strain of animal, expel all liquid from your pipette in between strains to avoid cross-contamination between types of embryos.
- 7) Seal the straws with a heat sealer.
- 8) From the time you start loading, the embryos should be in the straw no longer than 15 minutes before placing in the planar or Cryologic. Also, it is best that the straws be exposed to DMSO at room temp for about 10 minutes to equilibrate, before the program begins.
- 9) Place the straws into a programmable freezer using the morulae-freezing program which will cool the straws to -7°C ($-2^{\circ}\text{C}/\text{min}$), hold at -7°C for 1 minute (soaking), alarm for manual seeding*, then hold for an additional 10 minutes before proceeding to -40°C at a rate of $-0.5^{\circ}\text{C}/\text{min}$.
 - a. ***Note: Manual seeding is done by touching pre-cooled forceps to the very top of the DMSO column. Forceps are pre-cooled by plunging the tips of the forceps in LN_2 for approximately ten seconds.**
- 10) When the program has reached -40°C , straws should be quickly plunged into LN_2 . When using the Cryologic, straws may be plunged directly into the LN_2 bath. When using the Planer, you will need to fill a LN_2 flask with LN_2 and plunge the straws inside the straw holders into the LN_2 . Take the straws out of the holders under LN_2 .
- 11) Transfer the straws to their appropriate cane and storage location.

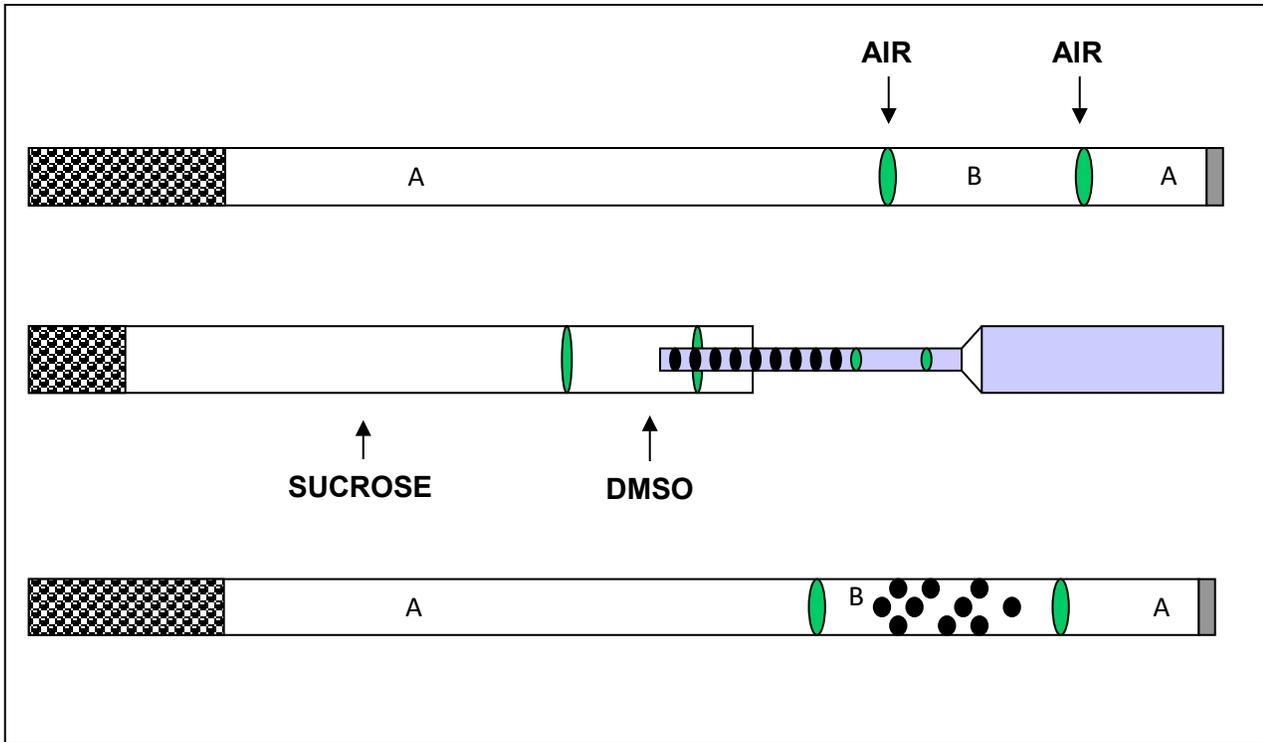


Figure 4: Loading a straw for cryopreservation of rat embryos. Add sucrose (A), air bubble, DMSO (B), another air bubble, and sucrose. Insert the pipette into the straw into the 1.5 M DMSO column and gently blow the embryos and air bubble from the transfer pipette into the 1.5 M DMSO column. Be sure to see the air bubble to verify that the embryos are in. Also, it is very important to have 3x more sucrose than DMSO. Otherwise you will need to empty the straws into 0.5 M sucrose when thawing.

Thawing procedure:

Reagents:

- R2CH (Rat 2 Cell HEPES) + 10% FBS (Fetal Bovine Serum; Invitrogen 16000-036)
- Equilibrated (37°C; 5% CO₂) R2CC + 10% FBS culture drops under oil (Five 30 µL drops per 20 embryos in each 35 mm Petri dish) – or preferred rat embryo culture medium
- Paper and pen
- Transfer pipette
- Extra dishes (depends on how many straws you are thawing)
- Long forceps (to retrieve straw from LN₂)
- 1cc syringe and syringe tip adapter for CBS straws (or appropriate sized rubber tubing) to empty the straws
- Scissors (small scissors work best)
- Beaker with 22°C (around room temperature) water
- Kimwipe
- 35 mm Petri dishes (Falcon 1008 is what we use)
- Timer
- Microscope
- RRRC embryos

Procedure

- 1) Assemble everything you need first.
- 2) Hold a single straw in air for 15 seconds.
- 3) Plunge the single straw into a water bath at 22°C and hold for 10 seconds. A beaker of water will work for this step.
- 4) As soon as the ice dissipates, pull the straw out of the water bath and wipe off the straw with a kimwipe.
- 5) Holding the straw horizontally, cut the sealed end, opposite the plug/straw weight.
- 6) Quickly direct the cut end into a Petri dish and then cut the opposite end (below the label/weight) to expel the contents (Figure 1). It is important to expel all the liquid from the straw. This can be done using a 1 or 3 cc syringe with a syringe tip or cut piece of rubber tubing which will fit around the end of the straw. Do not touch the expelled liquid with the cut end of the straw. Otherwise, capillary action will pull some liquid and embryos back into the straw.
- 7) Using a timer, allow embryos to equilibrate for 5 minutes.

- 8) Collect the embryos and transfer them into another petri dish with fresh R2CH (+10% FBS) solution. Wash the embryos 2 more times before culture/transfer/etc. Record how many embryos have been recovered and how many have lysed.
- 9) Transfer to equilibrated culture medium drops.

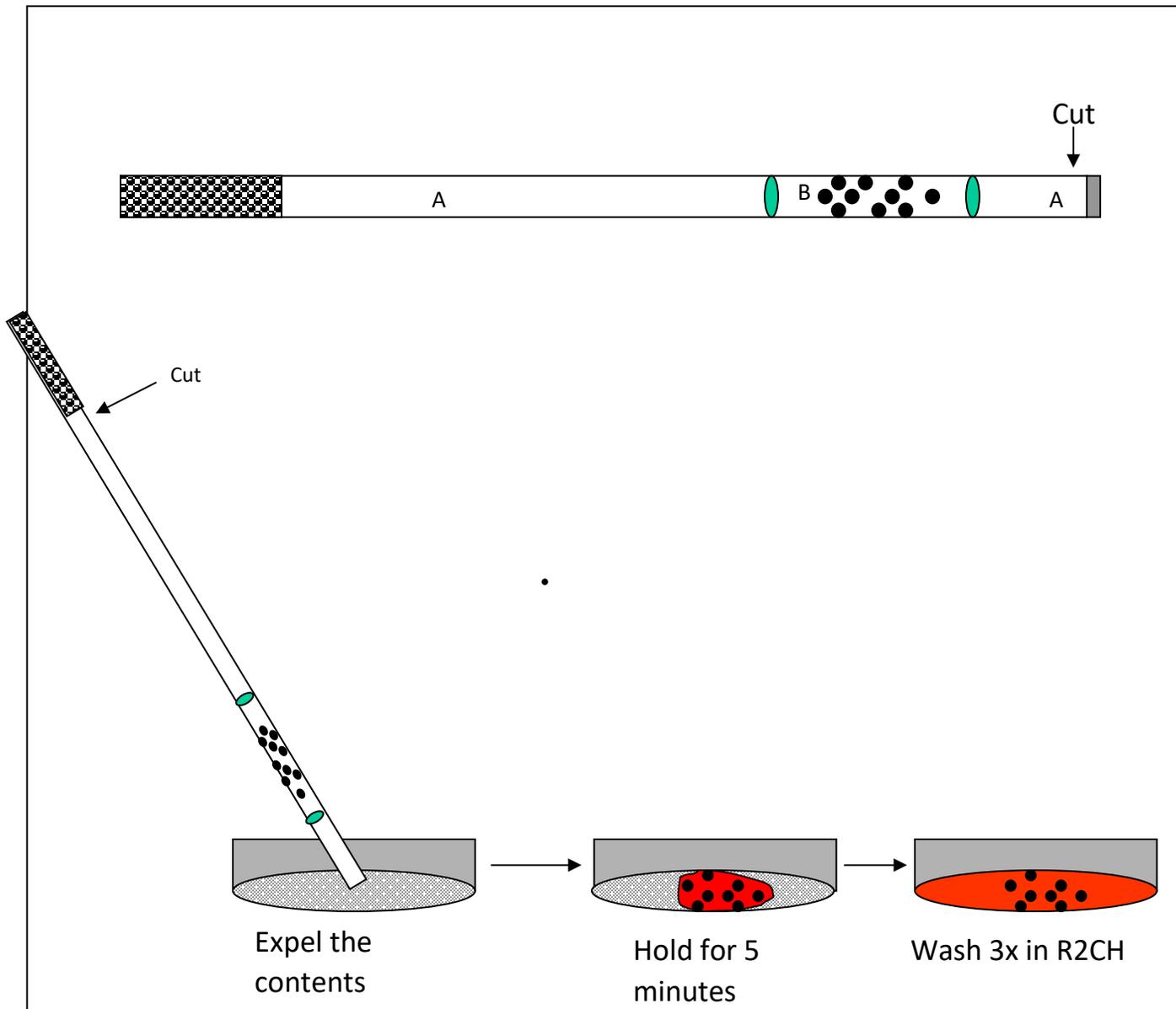


Figure 5. Thawing rat embryos: holding the straw horizontally, cut the sealed end opposite the end with the label. Direct the cut end into a Petri dish and then cut the straw again, just below the label/straw weight to expel the contents. Use a syringe with straw adapter/tubing to expel any remaining contents. Allow embryos to equilibrate for 5 minutes. Collect the embryos and transfer into another petri dish with fresh R2CH +10% FBS solution. Wash the embryos 2 more times before further culture/transfer/etc.

RRRC provides many resources for scientists. The following website contains useful information for SOP for protocols standardized for RRRC cryobiology lab including printable *Worksheet* and information about ordering animals, embryonic stem cells and services.

<http://www.rrrc.us/>

Disclaimer

This protocol is intended for use as an internal SOP at the RRRC. Each laboratory should amend this protocol to be consistent with the specific aspects and procedures of their individual laboratory.