Rat Embryo Freezing and Thawing Protocol

Rat Resource and Research Center

4011 Discovery Drive, Columbia, MO, 65201, USA

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Abbreviations:

R1CC (rat 1-cell culture): A modified rat 1-cell culture medium (mR1ECM) with an osmolality of ~310 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 osmolarity 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.

NOTE: Attempt to collect and freeze zygotes first and if zygote freezing is unviable, switch to collecting and freezing morula.

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 ºC 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 6:30 am and turn off at 6:30 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, 1000 IU
	ProspecBio	HOR-272 1000IU
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 appropriately such as: PMSG RAT, 125 IU/tube, date made, date expired (3 months from aliquot date), lot number.
4) In the hood, add 200 µL of sterile phosphate buffered saline.
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^{0}C freezer up to one month.
8) **Record the PMSG in the hormone log located in binder in lab cabinet.**

Procedure for injection:

1) Diluted working solution: add 975 microliters of sterile PBS per vial.
2) Inject 0.2 cc/rat (25 IU) between 9-10 am.
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^{0}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 -20ºC 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 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 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 in 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 6 pm 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 80 g) 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 #
Water for cell culture application	Lonza	17-724Q
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
NaHCO3	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
Mineral oil	Fisher Scientific	AC41508
FBS	Gibco	16000

Supplies

- pH meter and appropriate pH stock solutions for standardization
- Sterile biosafety cabinet
- Pipet-Aid
- CO_2 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. Please make yourself familiar with protocols before proceeding to the worksheet.
2. 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)] =>186.8 g/mole
3. Please check the osmolality and pH of the medium.
4. 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, 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 Lonza's 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 an appropriate sized Griffin Beaker containing Lonza's Water for cell culture application (80% of the total volume of medium), add the following components in grams:

Company	Cat#	Reagent	FW (g)	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	100 µg/mL	0.375g		
Sigma	S1277	Streptomycin Sulfate	1457	50 µg/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 Lonza's 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 one by one as listed in Worksheet 1.2.2 to a Griffin beaker containing Lonza's 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 Lonza's water and then adding it to the volumetric flask.
3) Gently gas the solution with 5% CO_2, 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.
6) Remake the solution if the osmolality does not fall between 280-300 mOsm.
7) Sterile filter the solution using a 0.2 µm filter unit.
8) Store at 4°C for up to 4 weeks.
9) Rinse all used glassware at least ten times with **Milli-Q water only** and place on a drying rack.
    a. **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. 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% CO_2 and 37°C. Allow them to equilibrate for at least one hour prior to use.
7) Label container with make date, expiration 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	S5761	NaHCO3	84.007	25	0.5251g		
Sigma	P4562	Sodium Pyruvate	110.04	0.5	0.0138g		
Invitrogen	11140-050	MEM NEAA 100x	n/a	n/a	2.5 mL		
Invitrogen	11130-051	MEM EAA 50X	n/a	n/a	5 mL		
Invitrogen	35050-061	GlutaMAX 1	n/a	0.1	0.125 mL		

Parameter	Expected	Y or N/ Measured		
Gassed with 5% CO_2	approx... 30 min.			
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 one by one as listed in Worksheet 1.2.3 to a Griffin beaker containing Lonza's 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 Lonza's 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 290-320 mOsm.
5) Sterile filter the solution using a 0.2 µm filter unit. Store at 4°C for up to 4 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, 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.
3) Sterile filter using 0.2 µm filter.

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	S5761	NaHCO3	84.01	5	0.21g		
Sigma	P4562	Sodium Pyruvate	110.04	0.5	0.0276g		
Invitrogen	11140-050	MEM NEAA 100x	n/a	n/a	5 mL		
Invitrogen	11130-051	MEM EAA 50X	n/a	n/a	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 one by one as listed in Worksheet 1.2.4 to a Griffin beaker containing Lonza's water (80% of final volume). **Wait for PVA to dissolve before adding other components.**
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 Lonza's water and adding it to the volumetric flask.
3) Gently gas the solution with 5% CO_2, 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 4 weeks.
8) Label container with make date, expiration 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). 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% CO_2, 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 P8136		PVA	N/A	N/A	0.025		
Sigma G5761		NaHCO3	84.01	25	0.5251		
Sigma P4562		Sodium Pyruvate	110.04	0.5	0.0138		
Invitrogen 11140-050		MEM NEAA 100x	n/a	n/a	2.5mL		
Invitrogen 11130-051		MEM EAA 50X	n/a	n/a	5mL		
Invitrogen 35050-061		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 one by one according to Worksheet 1.5.5 to a Griffin beaker containing 50% of the total volume of medium with Lonza's water. **Wait for PVA to dissolve before adding other components.**
2) After all components have dissolved, check to ensure the pH of the solution is approx.7.4, adjust if needed (with 10 N NaOH).
3) Transfer the contents to the appropriate volumetric flask and bring to desired volume by rinsing out the Griffin beaker with Lonza's 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 4 weeks.
7) Label container with make date, expiration 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 µm filter.

Worksheet 1.6.5. 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	110.04	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
- CO_2 chamber for euthanasia
- CO_2 tank
- CO_2 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 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 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 CO_2 because of their fetal hemoglobin's affinity for oxygen. They may require 20-60 minutes of exposure to CO_2 with decapitation as a secondary, physical means of ensuring euthanasia.
8) Be also 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.

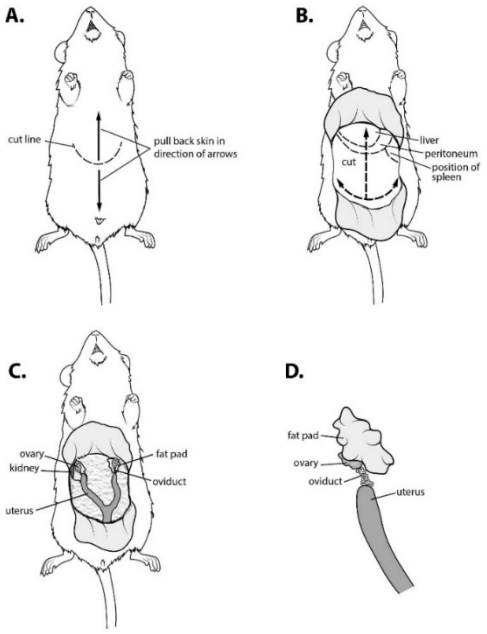

Figure 1. Reproductive organ dissection from a female rat. Cut the skin with a scissors as indicated by dashed line and then pull the skin apart in the direction of solid arrows (A). Cut the body wall (peritoneum) as indicated by dashed line in (B); the layout of internal reproductive organ of a female 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 (4rd edition), Cold Spring Harbor Laboratory Press, New York).

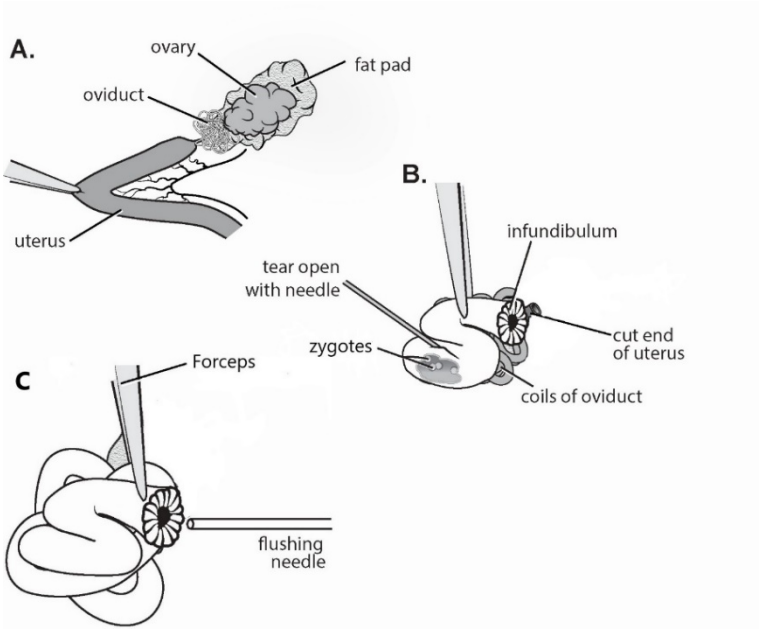

Figure 2. Collection of zygotes and cleavage stage embryos from oviduct. Excise oviduct by cutting first at oviduct and ovary junction and then a small section of uterine horn as illustrated in (A). Hold oviduct coil with a serrated forceps and release zygotes and cumulus mass by tearing the swollen 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).

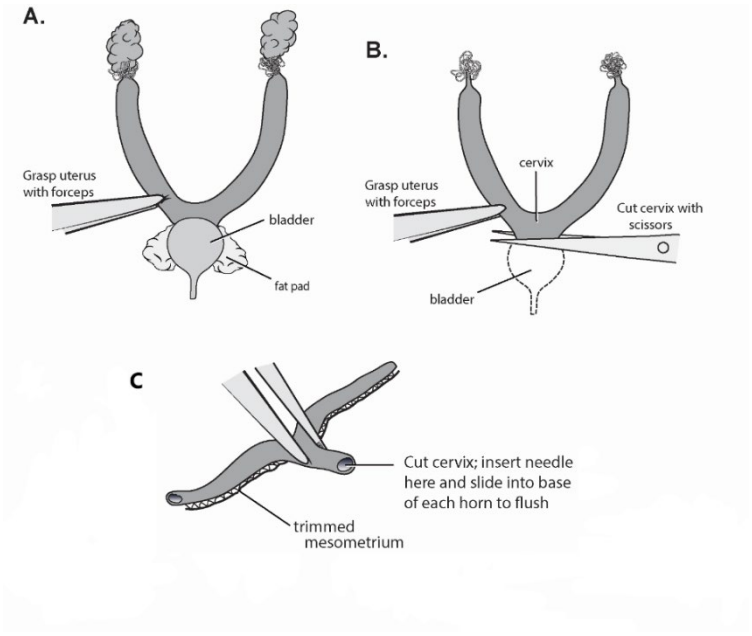

Figure 3. Collection of blastocysts from female rats. (A) Internal reproductive organs of a female rat. (B) Excise the bicornuate uterus by cutting at both the cervix and the oviducts. (C) Flush the bicornuate uterus as illustrated (adapted from Behringer et al., 2014. Manipulating the mouse embryo (4rd 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
- Hand pipette

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) Move the oviducts into a warm R1CH solution containing 1mg/mL Hyaluronidase, hold the oviducts using fine tweezers and tear the clutch using a 27 g needle to release the cumulus zygote complexes (Figure 2B).
6) Let the cumulus zygote complexes sit for about 5 min (or watch them until the cumulus cells fall apart). Using a pipette, pick up the embryos and gently wash them twice in R1CH before further usage.
7) If embryos are not going to be frozen promptly, transfer to culture plates with R1CC and culture in 37°C, 5% CO_2 incubator.

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 and wash them twice in R2CH + 10% FBS to get rid of any contaminants.
6) Under a 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 µl 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 R2CC and culture in 37°C, 5% CO_2 incubator.

Chapter 3. Embryo Cryopreservation

Section 3.1: Zygote freezing and thawing protocol

Supplies and Reagents:

- Sterile CBS Embryo/Sperm straws (0.3 mL) and weights
- 5cc syringe (with CBS straw adapter)
- Kimwipes
- 35 mm Petri dishes (Falcon 1008)
- Heat sealer
- Timer
- Microscope
- Transfer Pipette

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 = 1.8 mL FBS + 8.2 mL R1CH. Then make 1.5 M DMSO / 0.1 M sucrose = 1065 µL DMSO + 0.344 g sucrose in 10 mL 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 Crysalys at RRRC). Make sure that there is sufficient liquid nitrogen (LN_2) 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% CO_2 incubator.
7) Load 3 columns of cryoprotectant medium into the straw. 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. Embryos will be contained within the central cryoprotectant column.

8) Group the collected embryos in a final R1CH washing solution and do not freeze more than 35 embryos in 1 straw.
9) Load the embryos into the pipette being careful to keep the embryos close together, then load the straw being careful to prevent too much R1CH from entering the central cryoprotectant column.
10) Make sure that all embryos were actually loaded into the straw by checking the straw under the microscope to visually confirm that the embryos are in the central column. Repeat for each straw.
11) Seal straws and equilibrate for 10 minutes (5 minutes minimum and 15 minutes maximum if loading multiple straws at once).
12) Program (if using Cryologic or Crysalys freezer):
    a. Make sure the cryopreservation apparatus is set to the rat one-cell freezing program.
    b. Fill the Cryologic or Crysalys with LN_2.
    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 in LN_2 (Crysalys 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 LN_2.
13) Transfer straws to appropriate storage location and complete necessary paperwork/ data entry.

Zygote Thawing Procedure:

Supplies:

- R1CH (Rat 1-Cell HEPES) + 18% FBS (Fetal Bovine Serum; Invitrogen 16000)
- Equilibrated (37ºC; 5% CO_2) R1CC culture drops under oil (Five 30 µL drops per 20 embryos in each 35 mm Petri dish) – or preferred rat embryo culture medium
- Transfer pipette
- Extra dishes (depends on how many straws you are thawing)
- Long forceps (to retrieve straw from LN_2)
- 5cc syringe and syringe tip adapter for CBS straws
- Scissors
- 37°C water bath

- Kimwipe
- 35 mm Petri dishes
- Timer
- Microscope
- Frozen embryos in LN2

Procedures

1) Locate appropriate straws, verify ID and transfer to the laboratory in LN_2.
2) Prepare R1CH (without BSA) **+** 18% FBS and acquire 3 small petri dishes per straw being thawed.
3) Add 100-200µL R1CH **+** 18% FBS to a small petri dish.
4) Remove straw from LN_2 and immediately transfer to 37˚C water bath for ~10 seconds.
5) As soon as the ice dissipates, pull the straw out of the water bath and wipe off the straw with a kimwipe.
6) 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 end (below the label/weight). Immediately expel contents of straw into the drop (using a syringe fitted with straw adapter).
7) Using a timer, allow embryos to equilibrate for 10 minutes.
8) Move embryos to fresh dish containing 100-200 µL R1CH **+** 18% FBS.
9) Wash a second time by moving to fresh dish containing 100-200 µL R1CH **+** 18% FBS.
10) Identify membrane intact zygotes. Remove lysed or degenerate embryos.
11) Transfer to appropriate culture medium, rinsing through several drops of culture medium.
12) Complete documentation regarding embryo thaw.

3.2: Morula freezing and thawing protocol

Supplies:

- Sterile CBS Embryo/Sperm straws (0.3 mL) and weights
- 5cc syringe (with CBS straw adapter)
- Kimwipes
- 35 mm Petri dishes (Falcon 1008)
- Heat sealer
- Timer
- Microscope
- Transfer Pipette

Reagents:	**Company**	**Catalog #**
R2CH	N/A	N/A
FBS	Gibco/Life Technologies	16000
Sucrose	Sigma	S9378-500G
DMSO	Sigma	D8418-50ML

Freezing procedure:

1) Prepare R2CH with 10% FBS by adding 2.0 mL FBS to 18.0 mL R2CH. Then prepare the required solutions as follows: 1.5 M DMSO by adding 1065 µL DMSO to 10ml R2CH with 10% FBS and 0.5 M sucrose by adding 1.72 g sucrose to 10 mL R2CH with 18% FBS. Filter the 1.5 M DMSO and 0.5 M sucrose solutions.
2) Prepare the freezing apparatus that you will use for this procedure (we use a Cryologic or Crysalys at RRRC). Make sure that there is sufficient liquid nitrogen (LN_2) and that the machine is available and ready for use BEFORE you equilibrate morula with cryoprotectant.
3) Assemble everything you need to freeze morula, including a transfer pipette, dishes, timer, freezing solution (1.5 M DMSO), 0.5 M sucrose solution, R2CH + 10% FBS, and straw labels + weights.
4) Load the straw with freezing solution (1.5 M DMSO) and 0.5 M sucrose solution (Figure 4) using the syringe with CBS straw adaptor. 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.
5) Group the collected embryos in the final R2CH washing solution. Do not freeze more than 35 embryos in 1 straw.
6) Load the embryos into the pipette being careful to keep the embryos close together, then load the straw being careful to prevent too much R2CH from entering the 1.5 M DMSO column (Figure 4).

7) Make sure that all embryos were actually loaded into the straw by checking the straw under the microscope to visually confirm that the embryos are in the DMSO column. Repeat for each straw.
8) Seal straws and equilibrate for 10 minutes (5 minutes minimum and 15 minutes maximum if loading multiple straws at once).
9) Program (if using Cryologic or Crysalys freezer):
    a. Make sure the cryopreservation apparatus is set to the morula freezing program.
    b. Fill the Cryologic or Crysalys with LN_2.
    c. Start machine – ensure that it is at the appropriate start temperature (22°C) before adding straws to machine.
    d. Cool at -2°C/min from 22°C to -7˚C.
    e. Seed each straw at the top of the cryoprotectant column containing embryos using a large forceps precooled in LN_2 (Crysalys will prompt you to seed with loud beeping) and hold at -7°C for 10 minutes.
    f. Cool at -0.5°C/min to -40˚C.
    g. Remove from freezer and quickly plunge into LN_2.
10) Transfer straws to appropriate storage location and complete necessary paperwork/ data entry.

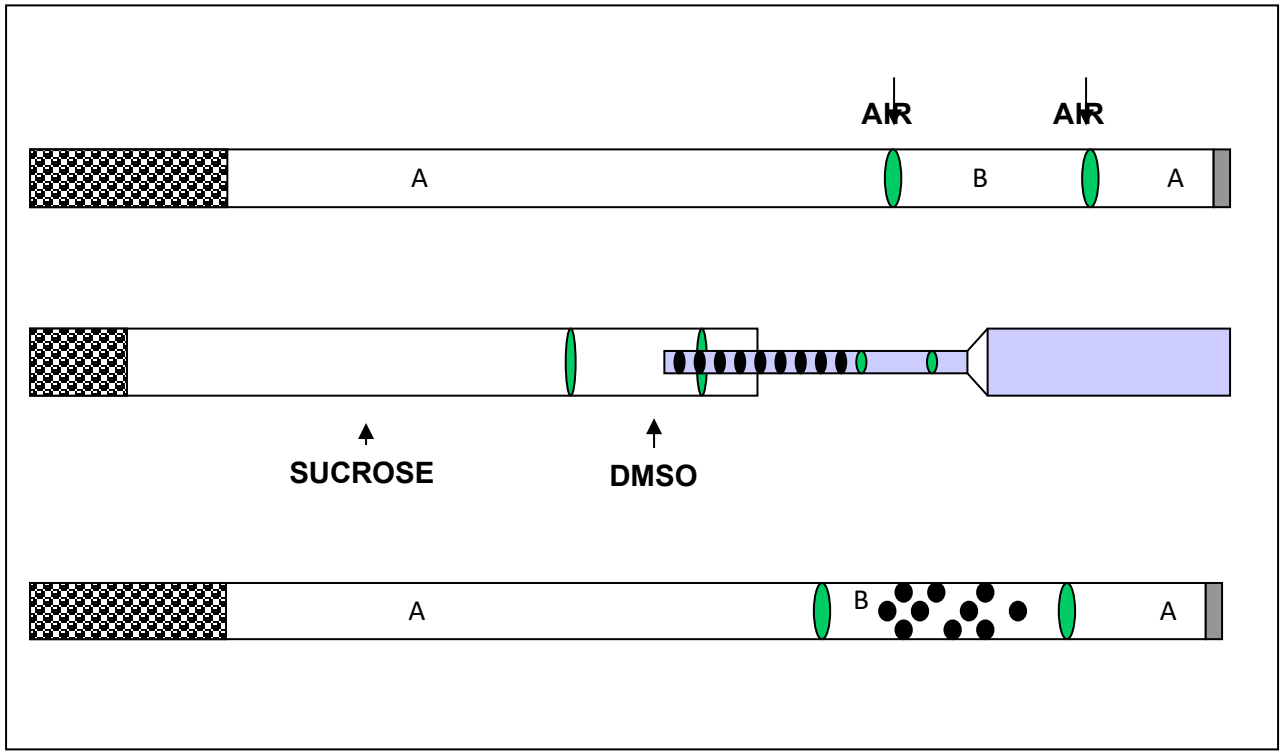

Figure 4: Loading a straw for cryopreservation of rat embryos. Add sucrose (A), air bubble, DMSO (B), another air bubble, and sucrose. Insert the pipette into the straw into the 1.5 M DMSO column and gently blow the embryos from the transfer pipette into the 1.5 M DMSO column. Also, it is very important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

Morula Thawing procedure:

Supplies:

- R2CH (Rat 2 Cell HEPES) + 10% FBS (Fetal Bovine Serum; Invitrogen 16000-036)
- Equilibrated (37ºC; 5% CO_2) 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
- Transfer pipette
- Extra dishes (depends on how many straws you are thawing)
- Long forceps (to retrieve straw from LN_2)
- 5cc syringe and syringe tip adapter for CBS straws
- Scissors
- Beaker with 22˚C (around room temperature) water
- Kimwipe
- 35 mm Petri dishes
- Timer
- Microscope
- Frozen embryos in LN2

Procedure

1) Assemble everything you need first.
2) Hold a single straw in air for 15 seconds.
3) Plunge the single straw into a beaker with 22˚C and hold for 10 seconds.
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 side with the label) with scissors. Then, hold the straw vertically (cut end down) over a small petri dish and cut the other end (below the label/weight) (Figure 1). Immediately expel contents of straw into the drop (using a syringe fitted with straw adapter).
6) Using a timer, allow embryos to equilibrate for 5 minutes.
7) Collect the embryos and transfer them into another petri dish with fresh R2CH (+10% FBS) solution. Wash the embryos 2 more times. Remove lysed or degenerate embryos.
8) Transfer to appropriate culture medium, rinsing through several drops of culture medium.
9) Complete documentation regarding embryo thaw.

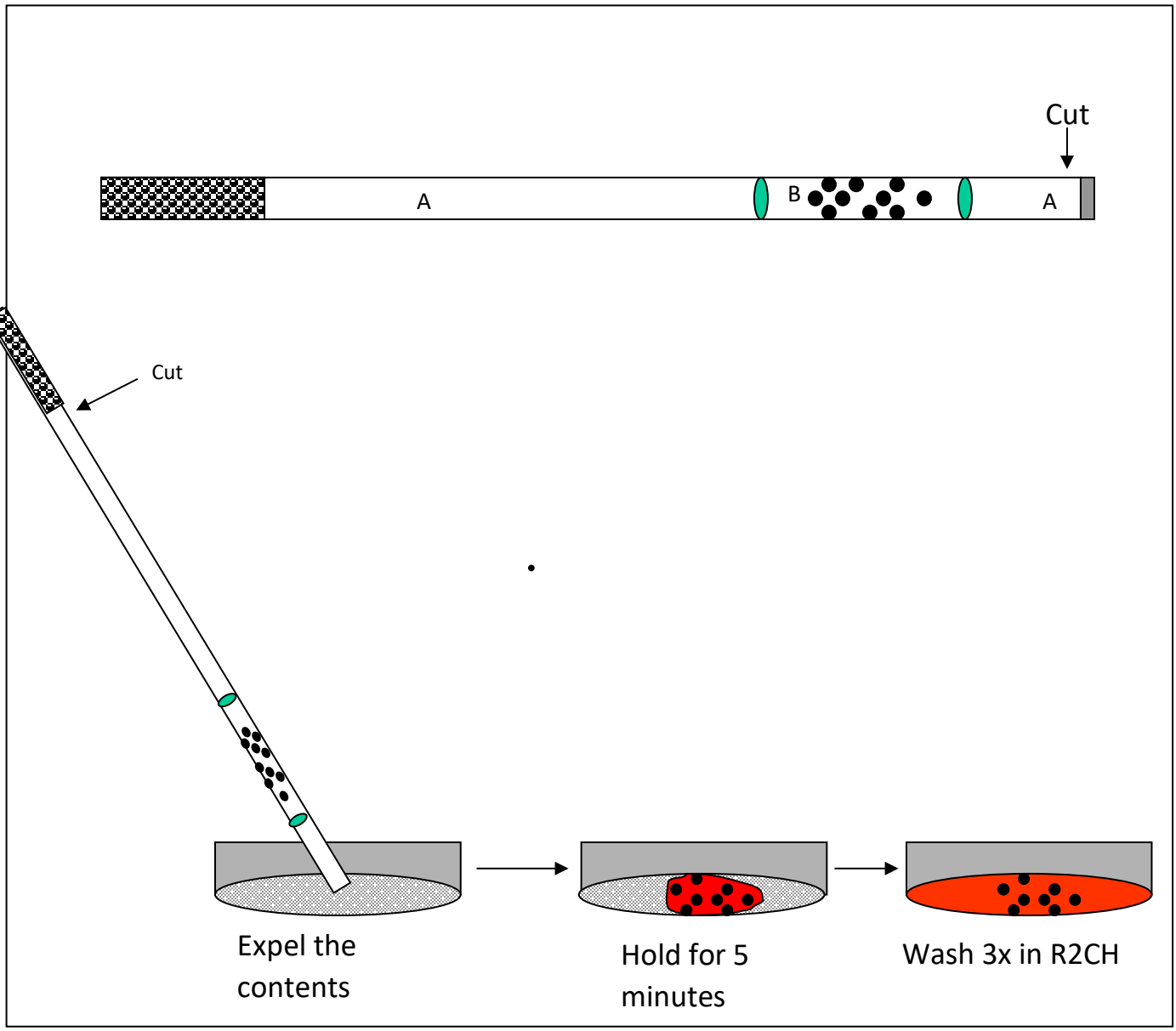

Figure 5**.** Thawing rat embryos: holding the straw horizontally, cut the sealed end opposite the end with the label. Direct the cut end into a Petri dish and then cut the straw again, just below the label/straw weight to expel the contents. Use a syringe with straw adapter/tubing to expel any remaining 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.