Rat Embryo 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 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.

1. 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
$NaHCO_3$	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
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 beakers and stir bars
- Volumetric flasks
- 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
- Sterile Pasteur pipettes
- Osmometer

1.1 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 (**except BSA**) as listed in Worksheet 1.1 to a Griffin beaker containing Lonza's water (80% of final volume) while stirring with a stirring bar.
2) The solution should be clear after all components have been dissolved. If any precipitation occurs, discard the solution and start over.
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 then adding it to the volumetric flask.
4) Gently gas the solution with 5% CO_2, using a sterile Pasteur pipette, for 15-30 minutes.
5) Check to ensure the pH of the solution is approx.7.4 - if pH is <7.0 remake solution.
6) 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.
7) Add BSA and let BSA slowly dissolve into medium without stirring.
8) Sterile filter the solution using a 0.2 µm filter unit.
9) Label container with make date, expiration date, pH, mOsm, initials and batch number and batch number (Your Initial, mm/dd/yyyy).
10) Store at 4°C for up to 4 weeks.
11) Take an aliquot of the medium (usually 40-45 ml in 50 ml conical tube) and make 30 µL drops in 35 mm culture plates and cover with mineral oil. 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.
12) Place dishes in incubator preset to 37°C, 5% CO_2 with maximal humidity. Allow them to equilibrate for at least one hour prior to use.

Worksheet 1.1: R1CC (high NaCl) for 1-cell rat embryo culture

Reagent	Company	Cat#	FW (g)	mM	500 mL	Added	Lot#
NaCl	Sigma	S5886	58.44	110	3.2142 g		
KCl	Sigma	P5405	74.55	3.2	0.11928 g		
NaHCO3	Sigma	S5761	84.007	25	1.05 g		
$MgCl_2$-$6H_2O$	Sigma	M2393	203.31	0.5	0.0508 g		
D-Glucose	Sigma	G6152	180.2	7.5	0.67575 g		
Na^+ Pyruvate	Sigma	P4562	110.04	0.5	0.0275 g		
Penicillin G K Salt	Sigma	P7794	372.2	100µg/mL	0.05 g		
Streptomycin Sulfate	Sigma	S1277	1457	50µg/mL	0.025 g		
Sodium Lactate (60% syrup)	Sigma	L7900	186.8	13.53	1.2637 g		
MEM NEAA 100x	Invitrogen	11140-050	n/a	n/a	5 mL		
MEM EAA 50X	Invitrogen	11130-051	n/a	n/a	10 mL		
GlutaMAX 1	Invitrogen	35050-061	n/a	0.1	0.25 mL		
$CaCl_2$-$2H_2O$*	Sigma	C7902	147.02	2	0.14702 g		
BSA	Sigma	A3311		4 mg/mL	2 g		

***dissolve in a separate small beaker and add last.**

Gassed with 5% CO_2 (Approx 30 min)_____ Expected pH___7.3~7.4____ Actual pH_____

Expected osmolality_280~300 mOsm__ Actual osmolality_____

Today's date: _____

Your initials: _____

Batch # _____

1.2: 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 **(except BSA)** as listed in Worksheet 1.2 to a Griffin beaker containing Lonza's wtaer (80% of final volume) while stirring with a stirring bar.
2) The solution should be clear after all components have been dissolved. If any precipitation occurs, discard the solution and start over.
3) Check to ensure the pH of the solution is approximately 7.4. Adjust if needed (with 10N NaOH).
4) 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.
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 290-320 mOsm.
6) Add BSA and let BSA slowly dissolve into medium without stirring.
7) Sterile filter the solution using a 0.2 µm filter unit. Store at 4°C for up to 4 weeks.
8) Rinse all used glassware at least ten times with **Milli-Q water only** and place on a drying rack.
9) Label container with make date, expiration date, pH, mOsm, initials and batch number (Your Initial, mm/dd/yyyy).

Worksheet 1.2: R1CH for 1-cell rat embryo flushing and handling

Reagent	Company	Cat#	FW (g)	mM	500 mL	Added	Lot#
NaCl	Sigma	S5886	58.44	110	3.2142 g		
KCl	Sigma	P5405	74.55	3.2	0.11928 g		
NaHCO3	Sigma	S5761	84.007	5	0.21 g		
$MgCl_2$-$6H_2O$	Sigma	M2393	203.31	0.5	0.0508 g		
D-Glucose	Sigma	G6152	180.2	7.5	0.67575 g		
Na^+ Pyruvate	Sigma	P4562	110.04	0.5	0.0275 g		
Penicillin G K Salt	Sigma	P7794	372.2	100µg/mL	0.05 g		
Streptomycin Sulfate	Sigma	S1277	1457	50µg/mL	0.025 g		
Sodium Lactate (60% syrup)	Sigma	L7900	186.8	13.53	1.2637 g		
MEM NEAA 100x	Invitrogen	11140-050	n/a	n/a	5 mL		
MEM EAA 50X	Invitrogen	11130-051	n/a	n/a	10 mL		
GlutaMAX 1	Invitrogen	35050-061	n/a	0.1	0.25 mL		
HEPES	Sigma	H6147	238.31	22	2.6214g		
$CaCl_2$-$2H_2O$*	Sigma	C7902	147.02	2	0.14702 g		
BSA	Sigma	A3311		4 mg/mL	2 g		

***dissolve in a separate small beaker and add with stirring.**

Expected pH 7.3~7.4 Actual pH_____

Expected osmolality 280~300 mOsm Actual osmolality_____

Today's date: _____

Your initials: _____

Batch # _____

1.3: 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 1.3 to a Griffin beaker containing Lonza's water (80% of final volume) while stirring with a stirring bar. **Wait for PVA to dissolve before adding other components**.
2) The solution should be clear after all components have been dissolved. If any precipitation occurs, discard the solution and start over.
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) Gently gas the solution with 5% CO_2, using a sterile Pasteur pipette, for 15-30 minutes.
5) Check to ensure the pH of the solution is approx.7.4, if pH is <7.0 remake solution.
6) 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.
7) Sterile filter the solution using a 0.2 µm filter unit.
8) Store at 4°C for up to 4 weeks.
9) Label container with make date, expiration date, pH, mOsm, and initials.
10) Rinse all used glassware at least ten times with **Milli-Q water only** and place on a drying rack.
11) 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. For 2-cell to 8-cell, do not add FBS. Sterile filter using 0.2 µm 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) Place the culture plates in a calibrated incubator preset to 5% CO_2, 37°C with maximal humidity 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 1.3: R2CC for 2-cell or later stage rat embryo culture

Reagent	Company	Cat#	FW (g)	mM	500 mL	Added	Lot#
PVA	Sigma	P8136	N/A	N/A	0.05		
NaCl	Sigma	S5886	58.44	80	2.3376 g		
KCl	Sigma	P5405	74.55	3.2	0.11928 g		
NaHCO3	Sigma	S5761	84.007	25	1.05 g		
$MgCl_2$-$6H_2O$	Sigma	M2393	203.31	0.5	0.0508 g		
D-Glucose	Sigma	G6152	180.2	7.5	0.67575 g		
Na^+ Pyruvate	Sigma	P4562	110.04	0.5	0.0275 g		
Penicillin G K Salt	Sigma	P7794	372.2	100µg/mL	0.05 g		
Streptomycin Sulfate	Sigma	S1277	1457	50µg/mL	0.025 g		
Sodium Lactate (60% syrup)	Sigma	L7900	186.8	13.53	1.2637 g		
MEM NEAA 100x	Invitrogen	11140-050	n/a	n/a	5 mL		
MEM EAA 50X	Invitrogen	11130-051	n/a	n/a	10 mL		
GlutaMAX 1	Invitrogen	35050-061	n/a	0.1	0.25 mL		
$CaCl_2$-$2H_2O$*	Sigma	C7902	147.02	2	0.14702 g		

***dissolve in a separate small beaker and add with stirring.**

Gassed with 5% CO_2 (Approx 30 min)_____ Expected pH___7.3~7.4____ Actual pH_____

Expected osmolality_235~255 mOsm__ Actual osmolality_____

Today's date: _____

Your initials: _____

Batch # _____

1.4: 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.

Protocol

1) Add all the components one by one according to Worksheet 1.4 to a Griffin beaker containing 80% of the total volume of medium with Lonza's water while stirring with a stirring bar. **Wait for PVA to dissolve before adding other components**.
2) The solution should be clear after all components have been dissolved. If any precipitation occurs, discard the solution and start over.
3) Check to ensure the pH of the solution is approx.7.4, adjust if needed (with 10 N NaOH).
4) 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.
5) 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.

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. Label also with the batch number which is the Julian date.
9) Rinse all used glassware at least ten times with Milli-Q water only and place on a drying rack.

Worksheet 1.4. R2CH for 2-cell or later stage rat embryo flushing and handling

Reagent	Company	Cat#	FW (g)	mM	500 mL	Added	Lot#
PVA	Sigma	P8136	N/A	N/A	0.05		
NaCl	Sigma	S5886	58.44	110	3.2142 g		
KCl	Sigma	P5405	74.55	3.2	0.11928 g		
NaHCO3	Sigma	S5761	84.007	5	0.21 g		
$MgCl_2$-$6H_2O$	Sigma	M2393	203.31	0.5	0.0508 g		
D-Glucose	Sigma	G6152	180.2	7.5	0.67575 g		
Na^+ Pyruvate	Sigma	P4562	110.04	0.5	0.0275 g		
Penicillin G K Salt	Sigma	P7794	372.2	100µg/mL	0.05 g		
Streptomycin Sulfate	Sigma	S1277	1457	50µg/mL	0.025 g		
Sodium Lactate (60% syrup)	Sigma	L7900	186.8	13.53	1.2637 g		
MEM NEAA 100x	Invitrogen	11140-050	n/a	n/a	5 mL		
MEM EAA 50X	Invitrogen	11130-051	n/a	n/a	10 mL		
GlutaMAX 1	Invitrogen	35050-061	n/a	0.1	0.25 mL		
HEPES	Sigma	H6147	238.31	22	2.6214g		
$CaCl_2$-$2H_2O$*	Sigma	C7902	147.02	2	0.14702 g		

***dissolve in a separate small beaker and add with stirring.**

Expected pH 7.3~7.4 Actual pH_____

Expected osmolality 240~255 mOsm Actual osmolality_____

Today's date: _____

Your initials: _____

Batch # _____

Zygote Thawing Procedure:

Supplies:

- R1CH (Rat 1 Cell HEPES) + 18% FBS
- Equilibrated (37°C; 5% CO_2) R1CC culture drops under oil
- 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 LN_2

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 100-200 µL 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.

Morula Thawing procedure:

Supplies:

- R2CH (Rat 2 Cell HEPES) + 10% FBS
- Equilibrated (37°C; 5% CO_2) R2CC + 10% FBS culture drops under oil
- 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 LN_2

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.

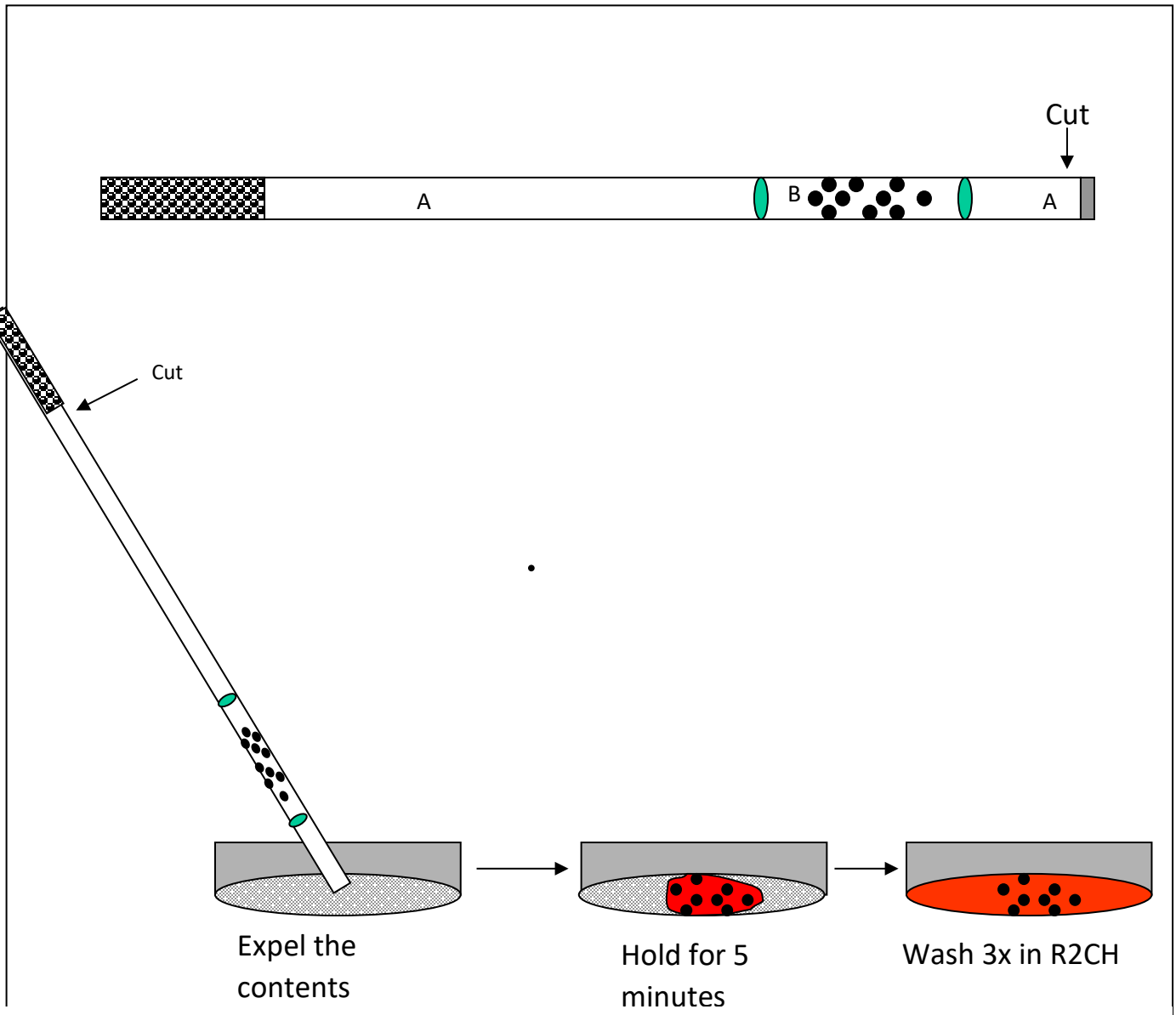

Figure 5**.** Thawing rat embryos: holding the straw horizontally, cut the sealed end opposite the end with the label. Direct the cut end into a Petri dish and then cut the straw again, just below the label/straw weight to expel the contents. Use a syringe with straw adapter/tubing to expel any remaining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