Protocol

Mouse Embryo Cryopreservation by Slow Freezing

Robert Taft

This embryo cryopreservation protocol is used by The Jackson Laboratory and several other repositories around the world. This protocol has clearly withstood the test of time; >2 million embryos from thousands of strains have been cryopreserved using this method. It requires a controlled-rate freezer, but is simple, effective, and can be applied to cryopreserve embryos from the two-cell to the blastocyst stage. Thawing requires neither special media nor equipment because the insemination straw contains all of the needed elements, making this very useful for distributing cryopreserved embryos. Embryo survival rates of greater than 90% can be expected when this method is used, making it reliable and enabling staff with prior embryo-handling experience to become proficient quickly.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Cryoprotectant solution, 1.5 M 1,2-propanediol <R>
Dilution solution, 1 M sucrose <R>
Embryo culture medium (e.g., KSOM^{AA}; Zenith Biotech ZEKS-050 or similar)
HEPES-buffered medium (e.g., M2 Millipore MR-015-D)
Pseudopregnant females at 0.5 or 2.5 d postcoitum (dpc) for oviduct or uterine transfer of cryorecovered embryos
Superovulated and mated embryo donors or two-cell-stage embryos obtained by in vitro fertilization (IVF)

Equipment

Controlled-rate freezer (e.g., Thermo CryoMed, FTS Biocool IV, Planar, etc.)

There are many models available that are effective and reliable. The minimum requirements are (1) programmable, that is, able to accept multistep freezing protocols, (2) ability to cool samples at 0.5°C/min, and (3) ability to cool straws to at least −30°C.

*If a controlled-rate freezer is beyond your budget, consider using a nonequilibrium method (e.g., Protocol: **Mouse Embryo Cryopreservation by Rapid Cooling** [Shaw 2018] or Protocol: **Mouse Embryo Cryopreservation by High-Osmolality Vitrification** [Mochida 2018]) or a low-technology approach such as the Nalgene Nunc (5100-0001) cryo container available from Fisher (15-350-50), VWR (55710-200), or Sigma-Aldrich (Mr. Frosty C1562).*

From the Manipulating the Mouse Embryo collection, edited by Richard Behringer, Marina Gertsenstein, Kristina Vintersten Nagy, and Andras Nagy.

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When placed into a −80°C freezer overnight, it provides a cooling rate of 1°C/min. If using this device, avoid spilling isopropanol onto the specimens.

Embryo-handling pipette
Forceps (large) to manipulate samples in LN_2
Gloves and goggles for handling LN_2
Incubator, humidified at 37°C, 5% CO_2, 95% air, or 6% CO_2, 5% O_2, and 89% N_2
Labels (e.g., Brady LAT-17-361-2.5)

Any self-laminating labels rated to withstand exposure to extreme cold (−196°C) and LN_2 are acceptable. Self-laminating labels adhere better in extreme conditions, and the lamination protects printing to maintain legibility. If using an alcohol-based freezer, be careful that neither the ink used nor the labels used are affected by alcohol. A cryo pen (e.g., Thermo Scientific TF4000221) can be used to write on the straws.

Liquid nitrogen (LN_2)
LN_2 containers (Dewar flasks or Styrofoam box) for benchtop work
LN_2 storage containers, goblets and canes or cassettes (e.g., Cryo Bio System, IMV, Minitube, MVE, Taylor-Wharton Cryogenics)
Metal rod (wire that can fit inside the straw to push the plug)
Petri dishes (e.g., 35 mm; BD Falcon 351008)
Sealing material for straws: Critoseal (e.g., Fisher 02-676-20, VWR 15407-103), PVA powder (e.g., IMV 018818), balls (e.g., Minitube 13400/9970), heat sealer (e.g., MTG 13135/0100), or ultrasonic sealer
Stereomicroscopes with transmitted and reflected light or fiber-optic illumination (with 20× and 40× magnification)
Straws, clear plastic, insemination (0.25 cc, 133 mm) (e.g., IMV 005565 or Minitube 13407/0010)
Surgical instruments
Syringe

Monoject (Atlantic Healthcare, Portland, SHA501400, Covidien 1188100555) fits the straw without any additional modifications. Alternatively, use any type of 1-cc syringe with an inserted cutoff yellow tip or connected with flexible tubing to fit the straw with an airtight joint and serve as aspirator to fill the straw.

Water bath at room temperature

METHOD

Cryopreserving Embryos by Slow Freezing

1. Precool the controlled-rate freezer to −7°C.
2. Collect the embryos as described in Protocol: **Collecting Two-Cell- to Compacted Morula-Stage Embryos** (Nagy et al. 2006a). Count and remove abnormal embryos. Keep the embryos at room temperature in HEPES-buffered medium. Alternatively, prepare two-cell-stage embryos obtained by IVF (Protocol: **In Vitro Fertilization in Mice** [Taft 2017] or Protocol: **In Vitro Fertilization in Mice** [Takeo and Nakagata 2018]).

   It is not necessary to move immediately from collection to cryopreservation, but the time embryos are held in HEPES-buffered medium at room temperature should be minimized. If required, embryos can be kept in the incubator in the appropriate medium until one is ready to start the cryopreservation process.

3. Prepare the straws for loading the embryos. Straws can be prepared before or after embryo collection.
   i. Using a metal rod, push the plug until it is ∼45 mm from the plugged end of the straw and ∼75 mm from the open end where embryos will be loaded (Fig. 1A).
   ii. Label the straw.
   iii. Using a fine felt tip permanent marker and a ruler, mark the straw at 20, 27, and 32 mm from the plug as marks 1, 2, and 3 (Fig. 1A).
4. Fill a straw with solutions (Fig. 1B).

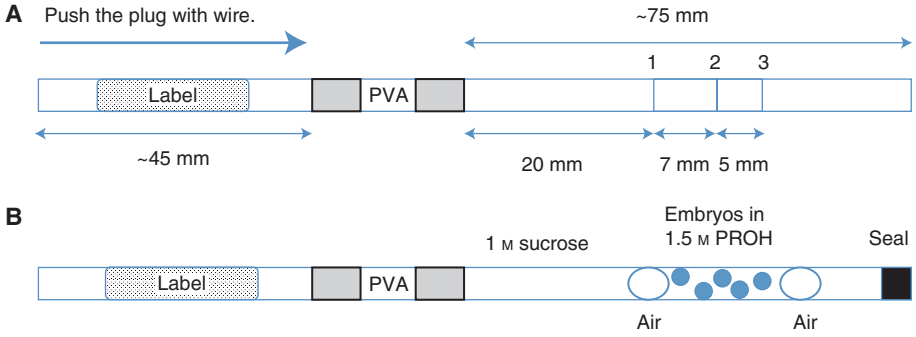

FIGURE 1. Preparation and loading of the straw for embryo cryopreservation. (*A*) The plug is pushed into the straw with the metal rod, so it is ~45 mm from the plugged end. The straw is labeled and marked 1, 2, 3 relative to the cotton plug. (*B*) A straw is filled with sucrose, an air bubble, and cryoprotectant and drawn until sucrose wets the plug. Embryos are loaded into the cryoprotectant column and the straw is sealed.

i. Attach the labeled end of the straw to a Monoject syringe by inserting it into the tip of the syringe where a needle would normally be located.

ii. Aspirate 1.0 M sucrose to mark 3, closest to the end of the straw.

iii. Aspirate air until the sucrose meniscus reaches mark 2.

iv. Aspirate the 1.5 M 1,2-propanediol solution until the sucrose meniscus reaches mark 1, closest to the plug.

v. Aspirate air until the column of sucrose enters the cotton plug and wets the PVA in the middle of the plug. This seals the straw.

vi. Remove the syringe carefully and repeat with the rest of the straws.

vii. Set the straws aside carefully; handle them gently to make sure not to mix sucrose and cryoprotectant.

5. Add ~2 mL of 1.5 M 1,2-propanediol to a 35-mm Petri dish.

6. Using a finely drawn pipette, transfer the embryos in a minimal volume of medium (to avoid diluting the cryoprotectant) to the dish of propanediol.

7. Equilibrate the embryos in this solution for 15 min at room temperature (20°C).

8. Move the embryos into groups of about 20–40, or whatever number to be loaded into each straw. The number of embryos placed into one straw depends on their expected viability; it is often convenient to have a sufficient number for transfer into two recipients.

9. Load the embryos into the straws.

i. Aspirate one group of embryos into a finely drawn pipette.

ii. Under a stereomicroscope and while holding the pipette in one hand and a straw in the other, insert the pipette into the column of 1.5 M 1,2-propanediol in the straw and gently expel the embryos.

A very small air bubble can be introduced into the pipette before the embryos are aspirated; this makes it easier to visualize when the embryos have been expelled. Do not introduce a large volume of medium or large bubbles.

iii. Repeat until all of the straws have been loaded.

Embryos should not be left in propanediol at room temperature for >30 min.

10. Seal the straws using a sealer, Critoseal, PVA powder, or a similar material.

Straws can be sealed as they are loaded rather than sealing all straws at one time.

11. Place the loaded straws into the controlled-rate freezer set to −7°C, and equilibrate them for 5 min.

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12. Using a cotton swab or the tips of forceps cooled in liquid nitrogen, touch the column of sucrose away from the embryos (near the plug) to initiate the seeding process. Ice formation will start spreading along the straw.
13. Repeat until all of the straws have been seeded.
14. Wait 5 min. Check the straws to ensure that ice has migrated and visibly formed in the columns containing sucrose and the embryos in propanediol. Reseed if necessary.

    The propanediol section will look opaque.

    See Troubleshooting.
15. Cool the straws at 0.5°C/min until the temperature reaches −30°C.
16. Once the temperature reaches −30°C, remove the straws from the controlled-rate freezer and plunge them directly into liquid nitrogen.

    Straws cannot be stored at −30°C but can be held at this temperature until it is convenient to plunge them into liquid nitrogen.
17. Place the straws into a small container filled with liquid nitrogen, making sure to keep the embryo fraction submerged. Transport the embryos in this manner to the long-term LN_2 container. Record all relevant information, such as strain information, number of embryos, location, and so on.

Thawing Embryos

18. Transfer a straw from the LN_2 refrigerator to a smaller container of liquid nitrogen.
19. Prepare a water bath at room temperature. Use fresh water for each thaw to prevent exposure to bacteria.
20. Using forceps, hold the straw near the label and hold it in the air for 40 sec, then submerge it in the room-temperature water bath and hold it there until the ice disappears.

    See Troubleshooting.
21. Wipe the straw dry.

    If there are concerns about cross-contamination, the exterior of the straw can be disinfected (e.g., with 70% ethanol), but make sure to rinse the straw carefully to remove traces of disinfectant. Note that alcohol will dissolve many types of ink, so make sure that the details on the straw are recorded before wiping with alcohol.
22. Holding the straw firmly, cutoff the seal and cut through the PVA plug, leaving about half the cotton plug in place to act as a plunger.
23. Using a metal rod, expel the entire liquid contents of the straw into a 35-mm dish. Do not let the plug drop into the dish. Both sections of medium need to be expelled into the same drop to mix.
24. Wait 5 min. The embryos will shrink considerably
25. Transfer the embryos to a new 35-mm Petri dish filled with ~2 mL of HEPES-buffered medium (e.g., M2), and wait another 5 min. They will rapidly take up water and assume a normal appearance.
26. Wash the embryos through a fresh drop of medium. They are now ready for transfer into pseudopregnant females. If they are being used for rederivation, they should be washed more following the recommendation of the International Embryo Transfer Society, and only embryos with intact zonae should be used.

    Culturing for an additional 24 h may provide another test of embryo viability as judged by their development. It has the additional benefit of minimizing the transfer of potential pathogens (Hill and Stalley 1991). Embryos with damaged blastomeres often continue to develop in culture and can develop to term.

    See Troubleshooting.
27. Transfer the embryos into the oviducts or uteri of pseudopregnant females, depending on the embryo stage, as described in Protocol: **Oviduct Transfer** (Nagy et al. 2006b) or Protocol: **Uterine Transfer** (Nagy et al. 2006c).

TROUBLESHOOTING

Problem (Step 14): Straws fail to seed.
Solution: Check all solutions and validate temperatures in the controlled-rate freezer.

Problem (Step 14): Ice formation is initiated, but ice fails to propagate into the column of fluid containing the embryos.
Solution: Reduce the time between when straws are prepared and when embryos are loaded.

Problem (Step 20): Straws explode during thawing.
Solutions: Exploding straws indicate problems with sealing. Consider the following:

- If using a heat sealer, the intensity or duration of the pulse can lead to inadequate sealing or to holes from excessive melting of the straw. Adjusting the settings until a consistent seal is achieved may help. The model of heat sealer may also impact success, with a wider heating element often yielding more consistent results than a sealer with a thin wire element.
- If using balls/beads, minor variations in the size of the balls or straws may lead to inadequate sealing. Trying different batches or straws may yield better results.
- If problems arise using Critoseal, it is often best to buy a new batch and keep it in a cool, dark location because the sealing effectiveness seems to decrease with exposure to heat or high humidity.

Problem (Step 26): All embryos are dead upon thawing.
Solutions: The most frequent causes of embryo death during cryopreservation are physical damage from ice formation and osmotic stress. The causes and solutions include the following:

- The medium is improperly made. Verify that all solutions are made correctly.
- There is embryotoxicity of the medium components. Verify that no components of the medium used are toxic to the embryos.
- The embryos were transferred to incorrect medium. Verify that embryos have been put into 1.5 M 1,2-propanediol and not 1 M sucrose before initiating freezing.
- Straws failed to seed. Verify that seeding is complete.
- The controlled-rate freezer is malfunctioning. Verify that the controlled-rate freezer is functioning properly. Verify both the accuracy of the temperature measurements and that the programs execute correctly.

RELATED INFORMATION

This embryo cryopreservation protocol is based on the method originally developed by Renard and Babinet (1984).

RECIPES

Cryoprotectant Solution, 1.5 M 1,2-Propanediol

1,2-Propanediol, Analar grade (e.g., Sigma-Aldrich, MW 76.1, P-1009)	0.6 mL
HEPES-buffered embryo medium (e.g., M2 Millipore MR-015-D)	4.4 mL

Make before use, filter-sterilize, keep at room temperature, and store for up to 1 wk at 4°C.

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Dilution Solution, 1 M Sucrose

Sucrose (cell culture tested; e.g., Sigma-Aldrich S1888)	1.7 g
HEPES-buffered embryo medium (e.g., M2 Millipore MR-015-D)	5 mL

Make before use, filter-sterilize, keep at room temperature, and store for up to 1 wk at 4°C.

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