

Video Article

Cryopreservation of Mouse Embryos by Ethylene Glycol-Based Vitrification

Keiji Mochida¹, Ayumi Hasegawa¹, Kyuichi Taguma¹, Atsushi Yoshiki¹, Atsuo Ogura¹

¹RIKEN BioResource Center

Correspondence to: Atsuo Ogura at ogura@rtc.riken.go.jp

URL: <https://www.jove.com/video/3155>

DOI: [doi:10.3791/3155](https://doi.org/10.3791/3155)

Keywords: Developmental Biology, Issue 57, mouse, embryo, cryopreservation, ethylene glycol, vitrification

Date Published: 11/18/2011

Citation: Mochida, K., Hasegawa, A., Taguma, K., Yoshiki, A., Ogura, A. Cryopreservation of Mouse Embryos by Ethylene Glycol-Based Vitrification. *J. Vis. Exp.* (57), e3155, doi:10.3791/3155 (2011).

Abstract

Cryopreservation of mouse embryos is a technological basis that supports biomedical sciences, because many strains of mice have been produced by genetic modifications and the number is consistently increasing year by year. Its technical development started with slow freezing methods in the 1970s¹, then followed by vitrification methods developed in the late 1980s². Generally, the latter technique is advantageous in its quickness, simplicity, and high survivability of recovered embryos. However, the cryoprotectants contained are highly toxic and may affect subsequent embryo development. Therefore, the technique was not applicable to certain strains of mice, even when the solutions are cooled to 4°C to mitigate the toxic effect during embryo handling. At the RIKEN BioResource Center, more than 5000 mouse strains with different genetic backgrounds and phenotypes are maintained³, and therefore we have optimized a vitrification technique with which we can cryopreserve embryos from many different strains of mice, with the benefits of high embryo survival after vitrifying and thawing (or liquefying, more precisely) at the ambient temperature⁴.

Here, we present a vitrification method for mouse embryos that has been successfully used at our center. The cryopreservation solution contains ethylene glycol instead of DMSO to minimize the toxicity to embryos⁵. It also contains Ficoll and sucrose for prevention of devitrification and osmotic adjustment, respectively. Embryos can be handled at room temperature and transferred into liquid nitrogen within 5 min. Because the original method was optimized for plastic straws as containers, we have slightly modified the protocol for cryotubes, which are more easily accessible in laboratories and more resistant to physical damages. We also describe the procedure of thawing vitrified embryos in detail because it is a critical step for efficient recovery of live mice. These methodologies would be helpful to researchers and technicians who need preservation of mouse strains for later use in a safe and cost-effective manner.

Video Link

The video component of this article can be found at <https://www.jove.com/video/3155/>

Protocol

The overall scheme of the experiment is shown in **Fig. 1**.

1. Reagent Preparation

1. Make the base medium (here in known as PB1) in a 100 ml glass bottle according to the following chart below:

| | M.W. | mM | mg/100ml |
|--|--------|--------|--------------|
| NaCl | 58.4 | 136.98 | 800.0 |
| KCl | 74.6 | 2.68 | 20.0 |
| KH ₂ PO ₄ | 136.1 | 1.47 | 20.0 |
| Na ₂ HPO ₄ ·12H ₂ O | 358.14 | 8.04 | 288.1 |
| MgCl ₂ ·6H ₂ O | 203.3 | 0.49 | 10.0 |
| Glucose | 180.2 | 5.56 | 100.0 |
| Na pyruvate | 110 | 0.33 | 3.6 |
| CaCl ₂ ·2H ₂ O | 147 | 0.9 | 13.2 |
| Penicillin G | | | 6.0 (approx) |

2. Make the Ficoll-sucrose (FS) solution :
 1. Add the following chemicals to 14 ml of PB1 solution in a 50 ml tube.

| | |
|-----------|---------|
| Ficoll 70 | 6.0 g |
| Sucrose | 3.424 g |
| BSA | 42.0 mg |

2. Mix Ficoll 70 and sucrose in PB1 solution and shake well until completely dissolved.
3. After checking complete dissolution above, add BSA on the surface of the solution and keep it at 4°C until BSA is completely dissolved (> 4 hours or overnight).
3. Make the equilibration solution (EFS20) and the vitrification solution (EFS40) in a 50 ml tube:
 - EFS20: 20%(v/v) ethylene glycol, 24% (w/v) Ficoll, and 0.4 mol/L sucrose in PB1 with BSA
 - EFS40: 40%(v/v) ethylene glycol, 18% (w/v) Ficoll, and 0.3 mol/L sucrose in PB1 with BSA
 For embryos from the BALB/c or ICR strain, increase the concentration of sucrose to 0.9 mol/L sucrose because they are more sensitive to cryodamage⁹.

| | EFS20 solution | EFS40 solution |
|-----------------|----------------|----------------|
| Ethylene glycol | 1 ml | 2 ml |
| FS solution | 4 ml | 3 ml |

4. Sterilize the solution by filtration using a 0.45 µm filter. Make aliquots in 5 ml polystyrene tubes and store at 4°C. They can be used for about 6 months.
5. Preparation of embryo thawing solution containing 0.75 M sucrose (TS1)
 1. Dissolve 7.7 g of sucrose in PB1 (prepared in step 1.1) and bring the total volume to 30 ml. Mix by gentle shaking until sucrose is completely dissolved.
 2. Add 90 mg of BSA onto the surface the solution and leave it stand until completely dissolved.
 3. Sterilize the solution by filtration.
 4. Aliquot and store at 4°C. They can be used for about 1 month.

Note: TS1 can also be prepared from commercially available M2.

 6. Dissolve 7.7 g of sucrose in M2 and bring the total volume to 30 ml.
 7. Mix by gentle shaking until sucrose is completely dissolved.
 8. Sterilize the solution by filtration.
 9. Aliquot and store at 4°C. They can be used for about 1 month.
6. TS2 (thawing solution containing 0.25 M sucrose):
 1. Dilute 10 ml TS1 with 20 ml volume of PB1 or M2, as appropriate.
 2. Aliquot and store at 4°C. They can be used for about 1 month.

2. Vitrification of 2-cell Mouse Embryos

1. Prepare 2-cell mouse embryos by natural mating or conventional *in vitro* fertilization techniques.
2. Aliquot 50 µl EFS40 into a cryotube. Hereafter, perform the rest of the vitrification procedure at the room temperature.
3. Aliquot 50 µl of EFS20 on the bottom of a 35 mm or 60 mm plastic Petri dish.
4. Transfer up to 30 embryos to EFS20 using a glass capillary with only the minimum amount of the culture medium. Start a timer for 2 min.

Note: Place embryos on the bottom of the drop. This makes efficient dehydration of embryos. Check the morphology of embryos by a stereomicroscope. Dehydrated embryos show a shrunken morphology, as shown in **Fig. 2A**. If they are not dehydrated enough, wait for more 1 or 2 min.
5. At about 1.5 min, pick up the embryos from the EFS20 drop with only the minimum amount of the solution. Transfer them to EFS40 in the cryotube prepared in step 2.1. Note: For the best results, adjust the timing of picking up embryos so that all embryos can be transferred into EFS40 at around 2 min.
6. Wait for 1 min.
7. Put the cryotube directly into liquid nitrogen (LN₂).

3. Thawing Vitrified 2-cell Mouse Embryos

1. Before thawing, prepare a dish with 10 µl drops of embryo culture medium for recovered embryos (see step 3.13). Cover the dish with oil and place in a CO₂ incubator until use. Note: Although any media for routine embryo culture may be used in this experiment, we recommend media with higher osmolarity such as M16 medium.
2. Warm TS1 to 37°C.
3. Put on a face mask and cryogloves. Open the LN₂ tank and retrieve a cryotube containing embryos.
4. Quickly open the top of the tube and discard LN₂. Wait for 30 sec to prevent TS1 from freezing at the next step.
5. Use a 1000µl pipette to add 850 µl of TS1 (37°C) into the tube and mix the solution by gentle pipettings (about ten times in 25 seconds) until the solution is evenly dissolved.
6. Transfer the entire volume of the tube to a plastic 60 mm Petri dish (or a watch glass) (**Fig. 3A**). Note: Embryos should be handled at room temperature (22-25°C) until they are placed in a CO₂ incubator at Step 3.14.
7. Start a timer for 3 min.

8. At around 2 min in TS1, gently shake the dish until the medium containing embryos is spread over the surface of the dish (**Fig. 3B**). Note: This will help the embryos go down to the bottom because they are floating near the surface when transferred to TS1.
9. Put three 50 μ l drops of TS2 onto the dish (**Fig. 3C**).
10. After 3 min in TS1, check the morphology of embryos by a stereomicroscope; they should be slightly shrunken. See the morphology of embryos in earlier (**Fig. 2B**) and later (**Fig. 2C**) in TS1. Note: If the embryos are still swollen, keep them in TS1 for more 1 to 3 min.
11. Pick up the embryos and transfer them to the first drop of TS2 (**Fig. 3D**). Start a timer for 3 min
12. After 3 min, transfer embryos to the second drop and then to the third drop (**Fig. 3E**).
13. Transfer the embryos to the culture medium prepared at Step 3.1. The embryos are in the shape shown in **Fig. 2D**.
14. Place dish in a CO₂ incubator. About 10 min later, transfer embryos to the next drop of culture medium to wash out sucrose that has been carried over from TS2.
15. Continue culture in a CO₂ incubator until embryo transfer.
Note: Transfer embryos to the oviducts of recipient females on the day of thawing. Long culture *in vitro* may decrease the viability of embryos in some strains of mice.

4. Representative Results:

In vitro- and *in vivo*-development of embryos after thawing is presented in Tables 1 and 2. The advantages of this protocol are the high survivability of embryos after thawing and its broad applicability to different strains of mice.

| Strain | Total No. of tubes | No. of embryos vitrified | Recovered (No. (%)) | Morphologically normal (No. (%)) | Development to blastocysts (No. (%)) |
|----------|--------------------|--------------------------|---------------------|----------------------------------|--------------------------------------|
| C57BL/6J | 20 | 400 | 397 (99) | 394 (99) | 342 (87) |
| BALB/cA | 15 | 300 | 296 (99) | 282 (95) | 238 (84) |
| ICR | 24 | 480 | 474 (99) | 443 (93) | 398 (90) |

Table 1. *In vitro*-development of vitrified-thawed embryos in common mouse strains

| Condition of embryos | No. of recipient females | No. of embryos transferred | Implantation sites (No. (%)) | Live offspring (No. (%)) |
|----------------------|--------------------------|----------------------------|------------------------------|--------------------------|
| Fresh | 12 | 180 | 141 (78.3) | 110 (61.1) |
| Vitrified | 16 | 242 | 202 (83.5) | 125 (51.7) |

Table 2. *In vivo*-development of vitrified-thawed embryos in C57BL/6J mice.

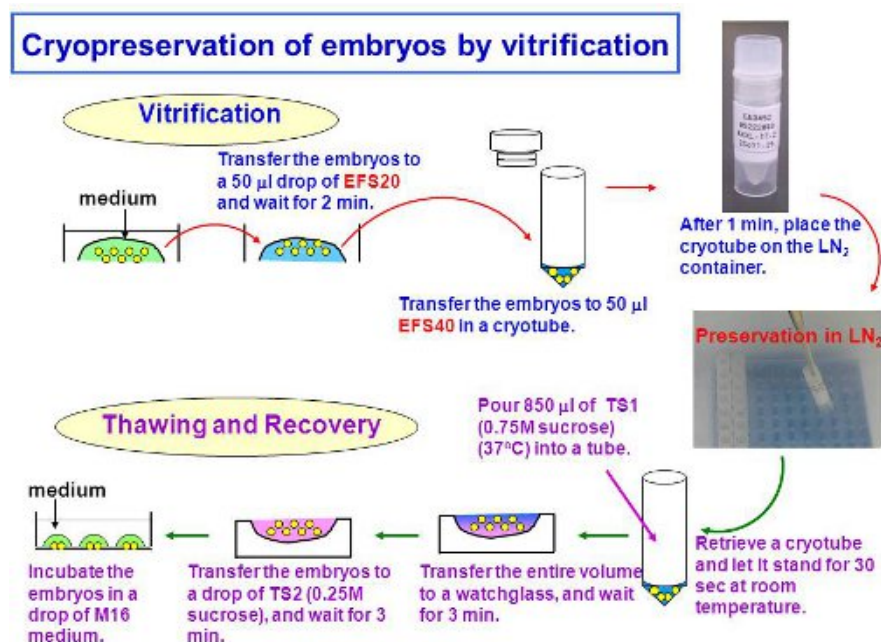


Figure 1. The overall scheme of the experiment including equilibration, vitrification, and thawing of embryos.

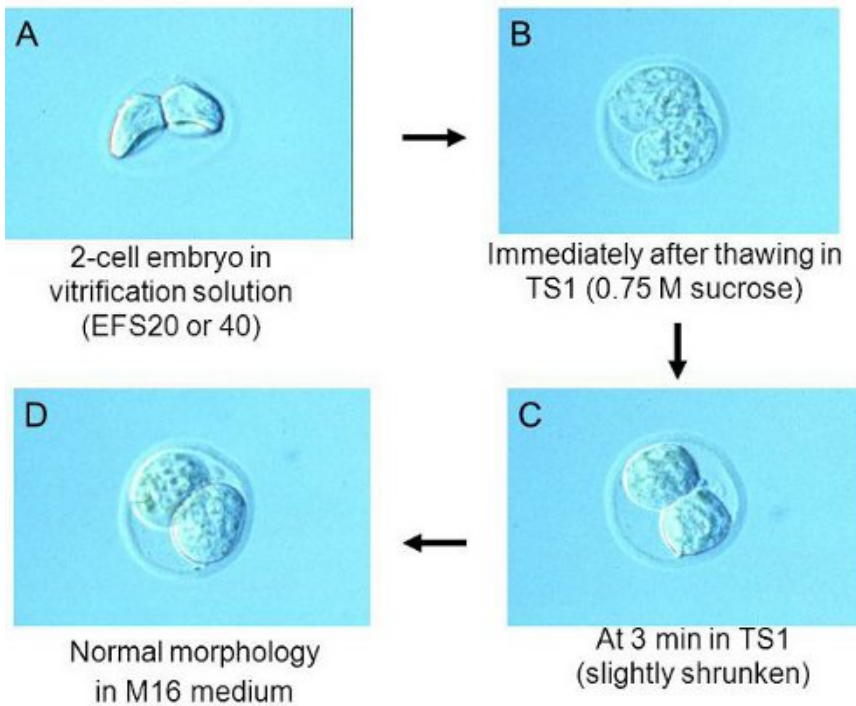


Figure 2. The morphology of embryos at each step of thawing.

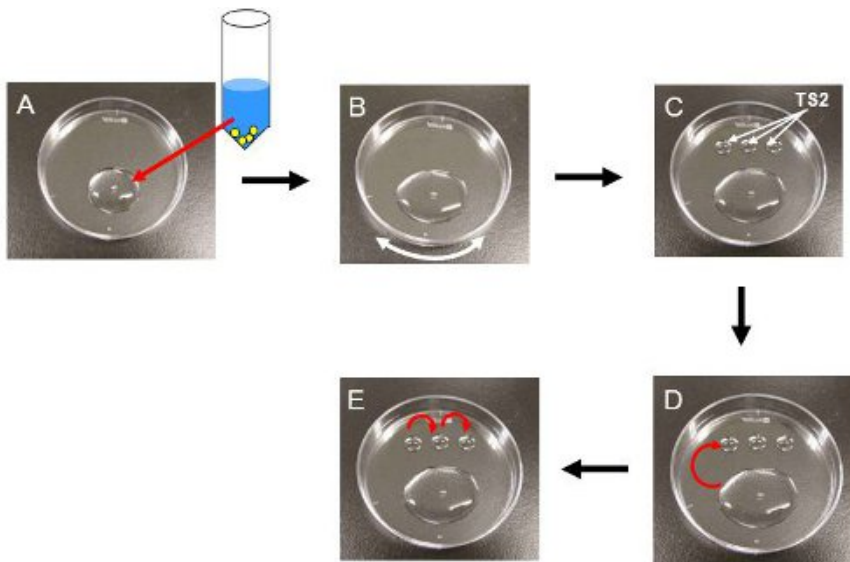


Figure 3. Thawing procedure of embryos. All procedures are performed at room temperature. (A), Add 850 μ l of TS1 (37°C) into a cryotube and transfer the entire volume of the solution in the cryotube onto a plastic dish. At this time, the embryos look swollen as shown in Fig. 2B. (B), Spread the solution over the surface of the dish by gentle shaking. (C), Place three 50 μ l drops of TS2 on the plastic dish. (D), Transfer the embryos to the first drop of TS2. After 3 min, the embryos look shrunken as shown in Fig. 2C. (E), Transfer them serially into the remaining TS2 drops and then to the culture medium.

Discussion

Since the first report of mouse embryo vitrification by Rall and Fahy in 1985², several technical improvements have been made to increase the survivability of embryos after thawing. One of the most successful modifications was achieved by use of ethylene glycol as a cryoprotectant because of its low toxicity and high membrane-permeability. Such advantages enable us to handle freezing embryos at room temperature⁴; other vitrification methods require embryo handling at cooler temperatures and are not always applicable to some strains of mice including BALB/c⁶. The first ethylene glycol-based vitrification was developed by Kasai et al. in 1990^{5,7}. Because the original method was optimized for plastic straws as containers, we have slightly modified the protocol for cryotubes, which are more easily accessible in laboratories and more resistant to physical damages. Therefore, the vitrification method described here may be applicable to many laboratories using mice as research models. The same method can also be used for mouse embryos at the morula and blastocyst stages⁸ and rat embryos⁹. However, we have recently

found that the quality of cryotubes may affect the survival rates of embryos after freezing-thawing. Thus, it is essential to examine the inside surface of the cryotubes for its smoothness before vitrifying embryos (e.g.; see at Table of specific reagents and equipment).

Vitrification methods have many advantages over conventional slow freezing methods, but they inherently have a disadvantage in respect to transportation purpose. As vitrified embryos should be kept at below -120°C to maintain their viability, dry shippers are generally used for their safe transportation. Dry shippers are heavy and bulky, and their round-trip is expensive, especially for international transportation. We are now currently developing a new vitrification method by which vitrified embryos can be stored at dry-ice temperature (about -80°C) for at least 7 days¹⁰. This method should be the vitrification of the next generation.

Disclosures

We have nothing to disclose.

Acknowledgements

This study was conducted in cooperation with the National BioResource Project, the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1. Whittingham, D.G., Leibo, S.P., & Mazur, P. Survival of mouse embryos frozen to -196° and -269°C . *Science*. **187**, 411-414 (1972).
2. Rall, W.F. & Fahy, G.M. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature*. **313**, 573-575 (1985).
3. Yoshiki, A., *et al.* The mouse resources at the RIKEN BioResource center. *Exp. Anim.* **58**, 85-96 (2009).
4. Mochida, K. & Ogura, A. Cryopreservation of embryos in laboratory species. *J. Mamm. Ova. Res.* **27**, 87-92 (2010).
5. Kasai, M., *et al.* A simple method for mouse embryos cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *J. Reprod. Fert.* **89**, 91-97 (1990).
6. Dinnyes, A., Wallace, G.A., & Rall, W.F. Effect of genotype on the efficiency of mouse embryo cryopreservation by vitrification or slow freezing methods. *Mol. Reprod. Dev.* **40**, 429-435 (1995).
7. Kasai, M. & Mukaida, T. Cryopreservation of animal and human embryos by vitrification. *Reprod. Biomed. Online*. **9**, 164-70 (2004).
8. Miyake, T., *et al.* Vitrification of mouse oocytes and embryos at various stages of development in an ethylene glycol-based solution by a simple method. *Theriogenology*. **40**, 121-134 (1993).
9. Han, M.S., Niwa, K., & Kasai, M. Vitrification of rat embryos at various developmental stages. *Theriogenology*. **59**, 1851-1863 (2003).
10. Jin, B., *et al.* Equilibrium vitrification of mouse embryos. *Biol. Reprod.* **82**, 444-450 (2010).