

Benchmarks

Mouse embryo cryopreservation utilizing a novel high-capacity vitrification spatula

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Embryo cryopreservation is an indispensable technique in reproductive programs and in animal facilities where genetically modified mice are used extensively. Here we report the use of a vitrification spatula (VS) that can be readily homemade and has a large holding capacity to vitrify preimplantation mammalian embryos in a micro-drop employing ultra-rapid cooling in liquid nitrogen (LN₂). Vitrified one-cell embryos and morulae have high survival rates after thawing, and the fertility of the derived progeny is comparable to that of the control unvitrified group. The large holding capacity (up to 50 embryos per VS) does not only allow rapid expansion of storage capacity for additional mouse strains but also opens up the possibility to streamline transgenic mice generation procedures in transgenic facilities.

Cryopreservation of mammalian preimplantation-stage embryos serves a wide range of purposes, including routine assisted reproduction programs for humans and livestock, and animal management in institutional animal facilities. The first case of successful mouse embryo cryopreservation by slow controlled-rate freezing was reported in 1972 (1), but a more effective cryopreservation method—vitrification, or ultra-rapid freezing—was developed in 1985 (2). The success of vitrification is based on procedures that minimize the formation of intra-cellular ice crystals when an embryo and the surrounding vitrification solution are “glassified.” The reduced exposure time of the embryos to osmotic stress and toxic cryoprotectant leads to a high revival rate of the stored embryos.

Different devices were used as containers for preimplantation embryo vitrification. Plastic straws (3) are widely used for vitrification. Open pulled straws (4) and the double straw system (5) were

produced to improve sample cooling rate and to isolate samples from potential infectious agents accumulated in the storage vessels. Electron microscope grids (6), Cryoloop (7), Cryotop (8), nylon mesh (9) and metal mesh (10) were recently adopted as an open device to maximize the cooling rate. However, these devices are only available commercially. They do not have a large holding capacity nor can they achieve a high embryo revival rate after vitrification is performed as a routine procedure in mouse facility. We hereby report an inexpensive and easy-to-assemble device—a vitrification spatula (VS)—as an alternative. This device, which allows easy handling, does not only allow an ultra-rapid cooling of samples, but also stores vitrified samples in a closed system. Most important, the VS has the highest effective embryo holding capacity ever reported.

To assemble a VS, the tip of an autoclaved gel-loading tip (Cat. no. 1022-000; USA Scientific, Ocala, FL, USA) was crushed with a pair of fine forceps (N4, Regine,

Switzerland) that had been gently heated with a Bunsen burner (Model no. F4003; R & L Enterprises, Bramley, Leeds, UK) to generate a petal-like plate of 1 mm² (see Supplementary Materials for the monitoring of the heating status of the forceps and the softening of the spatula). The distal edge was heat-sealed to avoid liquid infiltration. The other end of the gel-loading tip was removed to shorten the spatula stalk. The cut end was mounted onto the underside of a cryogenic vial cap (Cat. no. 430488; Corning, Corning, NY, USA) by heat (Figure 1; see Supplementary Materials for a detailed protocol of VS assembly, vitrification and thawing of embryos). On average, a VS can be assembled in 1–2 min. We did not observe any detachment of the stalk from the cap, even when an assembled spatula, with or without liquid N₂ (LN₂) cooling, was allowed to free-fall to the ground from 1 m.

To evaluate the efficacy of vitrifying preimplantation embryos on a VS, one-cell embryos, morulae, and blastocysts were first harvested from super-ovulated and mated C57BL/J6/CBAF1 females and were vitrified as follows. Since it is the most commonly used in transgenic mouse generation by DNA microinjection (11), this mouse strain was tested to enable a good comparison with other studies. To eliminate the fluctuating contribution of low-quality one-cell embryos to the survival assay, only one-cell embryos with two prominent pronuclei and polar bodies confirmed under inverted DIC microscopes were selected for experimentation. After ~20 embryos were incubated in previtrification and vitrification solutions [Ethylene glycol (Cat. no. 102466; Sigma-Aldrich, St. Louis, MO, USA), DMSO (Cat. no. D2650; Sigma-Aldrich), Ficoll PM70 (Cat. no. F2878; Sigma-Aldrich), and M2 medium (Cat. no. MR-010P-5F; Specialty Media, Phillipsburg, NJ, USA)] (each for 30 s), the embryos were loaded onto the surface of a VS with approximately 0.5 μL vitrification solution and cooled down by dipping the droplet into LN₂ by holding the attached cryovial caps. The VS was then inserted into a precooled cryogenic vial and stored in a LN₂ cell storage vessel for 1–3 months before their viability was assessed. The embryos were then thawed and released from the VS by dipping the tip containing the droplet in 2 mL of 0.5 M sucrose solution. After the embryos had fallen from the spatula, they were transferred to a droplet of 20 μL 0.5 M sucrose solution and then 20 μL 0.25 M sucrose solution (each for 2 min) to remove the vitrification medium. The embryos were then washed with one

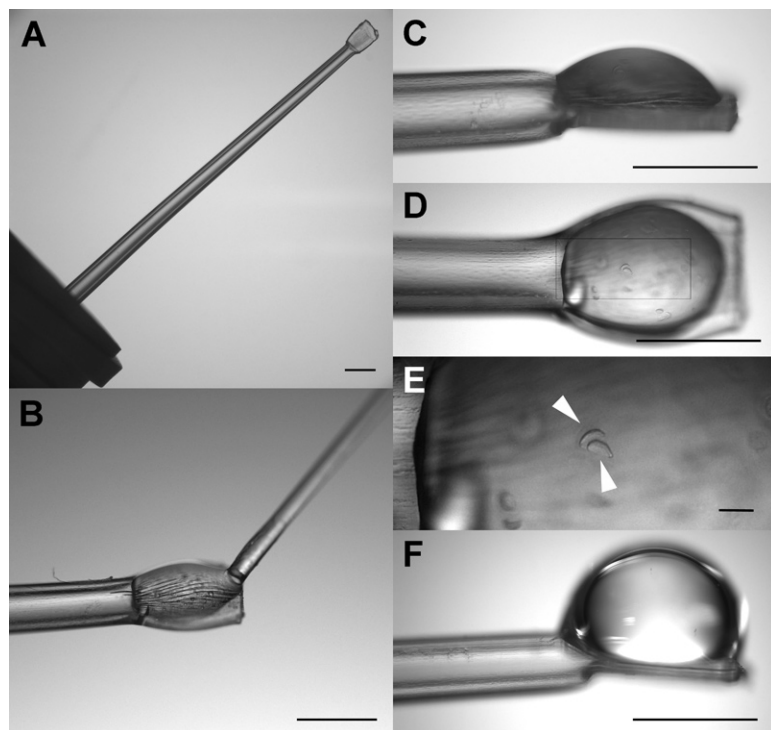


Figure 1. Vitrification of preimplantation embryos with a VS. (A) A VS with the thin plate at the upper right and the attached cap at the bottom left. (B) Loading of embryos onto a VS with a micropipet. Lateral view (C) and top view (D) of a VS with embryos in ~0.5 µL medium. (E) Higher magnification at the selected area in (D) with dehydrated embryos in focus (arrowheads). (F) A VS containing a droplet of ~2 µL, the volume required to hold 50 preimplantation embryos. All scale bars represent 1 mm, except that of 0.1 mm in (E).

drop of M2 medium before they were transferred to M16 medium (Cat. no. MR-015P-D; Specialty Media) for in vitro survival tests and subsequently implanted into the foster mother for in vivo survival tests.

In culture, >98% of both vitrified-thawed one-cell embryos and morulae survived and developed into expanded blastocysts, a rate matching that of respective unvitrified controls (Table 1). However, a dramatic decrease in hatching

rate was observed on vitrified-thawed embryos (41.2% for one-cell embryos; 34% for morulae) when compared with those in the corresponding unvitrified control (65.9% for one-cell embryos; 60.7% for morulae). There was, however, no significant drop in survival rate of implanted vitrified one-cell embryos and morulae in vivo (Table 2). Approximately 48% of the implanted vitrified-thawed one-cell embryos and 65% of the vitrified-thawed morulae developed to

term with no significant difference with respect to the unvitrified control groups (45.4% for one-cell embryos; 67.2% for morulae) (Table 2). The drop in blastocyst hatching rate may be accounted for by the hardening of the zona pellucida by vitrification as previously reported (12–14). Both blastocyst-derived (15,16) and uterine-derived (17,18) proteolytic factors are involved in breaking the zona pellucida during hatching. The hardening of zona pellucida by vitrification may be compensated by the uterine-derived proteolytic factors in the in vivo survival test. On the contrary, the hatching of the blastocysts in the in vitro survival test rely only on the blastocyst-derived proteolytic factors. Therefore, the in vitro hatching rates may not fully represent the viability of the thawed vitrified embryos. Indeed, there are currently no reports indicating the correlation between the hatching rates and in vivo survival rate of vitrified embryos. On the contrary, only 66.7% ($n = 120$) of the vitrified blastocysts re-expanded and survived in culture after thawing. The high lethality may be due to the trapping of toxic cryoprotectant in the blastocoels, which could have been circumvented by artificially collapsing the blastocoels before cryopreservation (19). Out of the re-expanded blastocysts, 67.8% of them developed to term after implantation, a rate similar to that of the unvitrified control group (70.8%) (Table 2). Nevertheless, a reduction of hatching rate (71.3% for vitrified blastocysts versus 81.7% for the unvitrified control group) was observed. In addition, fifteen pairs of born pups from all of the vitrified test groups and the unvitrified control group of morulae were tested for their fertility upon reaching adulthood. Their first litter sizes were similar with an average of 6–8 pups (Table 2).

Table 1. In Vitro Recovery of Vitrified Embryos

	1-cell		Morula		Blastocyst	
	Vitrified	Unvitrified	Vitrified	Unvitrified	Vitrified	Unvitrified
1-cell	119 (100%)	123 (100%)	NA	NA	NA	NA
2-cell	117 (98.3%)	121 (98.4%)	NA	NA	NA	NA
Morula	117 (98.3)	119 (96.7%)	106 (100%)	112 (100%)	NA	NA
Expanded blastocyst	117 (98.3%)	119 (96.7%)	104 (98.1%)	112 (100%)	80 (66.7% ^a)	126 (100%)
Hatched blastocyst	49 (41.2%)	81 (65.9%)	36 (34.0%)	68 (60.7%)	57 (71.3% ^b)	103 (81.7%)

^aPercentage of re-expanded blastocysts after warming of vitrified blastocysts ($n = 120$); ^bpercentage base on the re-expanded blastocyst after warming ($n = 80$). NA, not applicable.

Table 2. In Vivo Recovery of Vitrified Embryos

	1-cell		Morula		Blastocyst	
	Vitrified	Unvitrified	Vitrified	Unvitrified	Vitrified	Unvitrified
Transferred embryos	128	119	120	134	121	106
Born pups	61 (47.7%)	54 (45.4%)	78 (65%)	90 (67.2%)	82 (67.8%)	75 (70.8%)
Average litter sizes*	7.3	ND	7.0	6.7	7.5	ND

*Average litter sizes of 15 pairs of animals recovered from each vitrified embryo group. ND, not determined.

Finally, we challenged the holding capacity of the VS with 50 one-cell embryos per device. Although the time that embryos spent in each vitrification solution was extended from 30 s to 2 min due to prolonged handling, results indicated that 91% ($n = 150$) of the vitrified one-celled embryos developed into blastocysts in vitro, which is only slightly below that of the unvitrified control group (97%, $n = 100$).

By in vitro and in vivo survival tests and fertility tests, we demonstrated that VS is a reliable alternative for storing mammalian preimplantation embryos. It is inexpensive and easy to assemble. Similar methods of vitrifying embryos in a droplet have been reported previously (20,21), wherein a droplet of vitrification solution containing embryos is cooled by direct dipping into LN₂. With this method, we frequently encountered problems of retrograde filling of the droplet into the pipet tip, due to the contraction of air volume within the pipet tip when the sample is lowered but before reaching the LN₂ surface. Irreversible adhesion of the vitrified droplet on the pipet tip was also noted. Furthermore, after storing the vitrified droplets in cryovials in a LN₂ storage vessel, the vitrified droplets often stuck to the inner wall of the cryovial which made the thawing and retrieving procedures extremely difficult. However, all these technical problems were circumvented using the VS, which provides an attachment for the droplet handling all the way from the cooling to thawing procedure.

In addition, the VS has the largest effective holding capacity ever reported (4,7,9,22). The previous record holding capacity reported was 65 unfertilized bovine oocytes on a nylon mesh (9). However, the survival rate of these vitrified oocytes after fertilization was only 9%, one fourth of the unvitrified control. With a large effective holding capacity of VS reported here, bulk cryopreservation of mouse strains could be performed easily for the rapid expansion of mouse-strain storage. In conventional practice, expansion of the recovered mouse colony relies on breeding with the recovered founders. With bulk-thawing, one generation's worth of time (i.e., more than 2 months) could be saved. It also opens up the possibility that embryos at different stages can be stored in bulk and thawed anytime for genetically modification purposes, such as for pronuclear microinjection of transgenes, embryonic stem (ES) cell aggregation in morulae, or ES cells introduction into blastocysts. Without being limited by the time of

hormone administration and embryo harvesting, increased flexibility can be achieved to streamline routine embryo manipulation experiments in transgenic facilities. Similarly, excess oocytes collected from super-ovulated females could also be stored for future experiments.

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All experimental procedures involving live animals complied with Animal (Control of Experiment) Regulations of HKSAR.

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The authors declare no competing interests.

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