Vitrification and warming of rat morulae

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Time schedule

Collection and vitrification of rat morulae

Day 1 (after 16:00) A proestrous female rat is mated with a mature male rat of the same strain.

Day 2 (9:00-11:00) Mating is confirmed with the presence of a vaginal plug and smear test.

Day 5 (13:00-15:00) Collection and vitrification of morulae

Restoration

Day 1 (after 13:00) A sexually mature female rat is housed together with a vasectomized male.

Day 2 (9:00-11:00) Vaginal plug confirmation and smear test are performed.

Day 4 (13:00-15:00) Preserved morulae are warmed and transferred into the uterus.

Day 25 (afternoon) When spontaneous delivery does not occur, caesarian section is performed.

*Animal room: lighting cycle (light-on: 7:00-21:00), 55 ± 5% humidity, 24 ± 2°C temperature

1. Preparation

1.1) Preparation of freezing medium

Materials

Distilled water, ethylene glycol, Ficoll 70, sucrose, BSA, 18G syringe needle, 1-ml syringe, 10-ml syringe, filter for sterilization (0.45 μm), glass ampoule

Preparation of modified PB1 (Table 1)

1. All reagents shown in Table 1 are dissolved with 70 ml of distilled water, and the volume is adjusted to 100 ml.

Table 1. Composition of PB1

Reagent	100mL
NaCl	800.0 mg
KCl	20.0 mg
$CaCl_2$-$2H_2O$	13.2 mg
KH_2PO_4	20.0 mg
$MgCl_2$-$6H_2O$	10.0 mg
Na_2HPO_4-$12H_2O$	290.0 mg
Na-pyruvate	3.6 mg
Glucose	100.0 mg
Streptomycin	5.0 mg
Penicillin G	7.5 mg
BSA	300 mg

2. The solution is sterilized by filtration through a 0.20-μm filter.
3. The solution is aliquoted (10 ml each) into tubes and stored at -20°C.
4. BSA is added at 3 mg/ml at the time of use, and the solution is sterilized by filtration through a 0.20-μm filter.

Preparation of EFS40

1. To 35.1 ml of PB1 (before BSA addition), 15.0 g of FicollTM PM70 is added, and the solution is stored (several hours-overnight).
2. The solution is combined with 8.56 g of sucrose and stirred to completely dissolve sucrose (final volume 50 ml).
3. BSA (105 mg) is added to the solution and dissolved (FS, Table 2).
4. Using 18G needle-attached 1-ml syringes, ethylene glycol and FS are added into a tube with a cap at a volume ratio of 2:3 (4 ml + 6 ml) (EFS40, Table 3).
5. To measure accurately, identical syringes with identical position of graduations should be used.
6. The two solutions are completely mixed by slowly inverting the tube, and sterilized by filtration through a 0.45-μm filter. Note: Clogging occurs when a 0.20-μm filter is used.
7. The solution is aliquoted into sterile glass ampoules, sealed, and stored at room temperature.

Table 2. Composition of FS

Ficoll 70	15.0 g
sucrose	8.56 g
BSA	105 mg
PB1 (- BSA)	35.1 mL

Table 3. Composition of EFS40

40 % (v/v) ethylene glycol
18 % (w/v) Ficoll 70
0.3 M sucrose
in PB1

Preparation of S-PB1 (0.5M sucrose-supplemented PB1)

1. Sucrose (19.19 g) is dissolved with 100 ml of PB1 (supplemented with BSA), and the solution is sterilized by filtration through a 0.20-μm filter.

2. Vitrification

2.1) Preparation of the donor rat

1. The estrus cycle of a sexually mature female rat is confirmed by smear test.
2. A proestrous female rat is housed together with a sexually matured male rat from the evening.
3. The rats are separated in the following morning, and vaginal plug and sperms are confirmed.

2.2) Collection of morulae

Materials

PB1, mineral oil, perfusion needle (26-30 G), 1-ml syringe, plastic dish, capillary for embryo manipulation, mouthpiece, sterile filter paper, forceps, surgical scissors, ophthalmologic scissors, stereoscopic microscope

Method

1. Four 100-μl drops of PB1 are formed and covered with mineral oil in a plastic dish (35 mm), and equilibrated to room temperature.
2. In the afternoon on day 3 after plug confirmation, the oviducts and uterus are excised from the mated female rat.
3. The oviducts and uterus are placed on a sterile filter paper, and adhered fat and blood are removed as much as possible. The half of the uterus is cut, remaining the half on the oviduct side.
4. PB1 is aspirated into a syringe attached with a perfusion needle. The perfusion needle is inserted into the infundibulum of the oviducts, and morulaes are flushed out.
5. Morulae are collected and washed 3 times or more by transferring into the PB1 drops to remove cells and blood.

2.3) Vitrification of morulae

Materials

EFS40, S-PB1, mineral oil, liquid nitrogen, plastic dish (35 mm), capillary for embryo manipulation, mouthpiece, 1-ml syringe, 18G syringe needle, silicon tube for straw connection, plastic tube for artificial insemination (0.25 ml), styrene foam plate (6-10 mm thickness), timer, thermometer, forceps, heat sealer, stereoscopic microscope, liquid nitrogen container (wide-mouthed Dewar vessel or polystyrene foam container in which straws can be horizontally placed)

Method

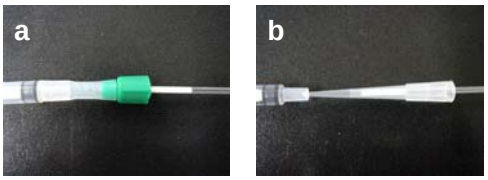
1. The room temperature in which embryo manipulation for vitrification is performed is set to 25°C. Since the permeation velocity of cryo-protective substances is altered by temperature, attention should be paid to toxicity on embryos.
2. EFS40 is aspirated into a 1-ml syringe attached with an 18G syringe needle, and equilibrated to room temperature.

3. EFS40 (about 0.1 ml) and S-PB1 (about 1-3 ml) are added into a plastic dish (35 mm).
   Note: Since EFS40 is easily evaporated and concentrated, a necessary volume is poured from the syringe each time for individual samples.
4. The cotton plug side of the straw (0.25-ml straw for insemination) is connected to a 1-ml syringe, and S-PB1 (~60 mm), air (~20 mm), EFS40 (~5 mm), air (~5 mm), and EFS40 (~12 mm) are sequentially aspirated (Fig. 1).

Fig. 1. Preparation of a freezing straw

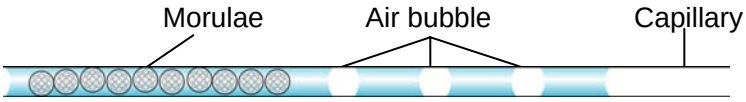
   Note: When a silicon tube for straw connection (Fig. 2a) is not available, it can be substituted by a 200-µl pipette tip (Fig. 2b). The straw tip is wiped with tissue paper each time before aspirating solutions to avoid contamination of the solutions.

Fig. 2. Connection of a syringe for aspiration of morulas and a straw



5. Liquid nitrogen is prepared, and a polystyrene foam plate (6-10 mm thickness) is floated.
6. Embryos to be frozen are pooled. When the procedure is performed under a microscope, microscope lighting should be reduced as much as possible to prevent temperature elevation.
7. Embryos are packed in a pipette and introduced with a small volume of PB1 into the center of an EFS40 column (~12 mm) of a horizontally placed straw, at which the timer is started: 0 m 0 s. Since movement of air bubbles in the pipette serves as an indicator, several air layers are aspirated into the pipette, followed by densely packed embryos in the pipette tip (Fig. 3). To maintain a constant temperature (room temperature), the EFS40 region (~12 mm) of the straw must not be touched.

Fig. 3. Packing of morulae into a capillary



8. The straw content is aspirated by a syringe until S-PB1 reaches the powder section in the cotton plug. The straw opening is closed using a heat sealer (Fig. 1) and kept at room temperature.

9. At 30 seconds after introducing morulae into EFS40, the straw is placed on a

polystyrene foam plate on liquid nitrogen (0 m 30 s). Attention should be paid to the duration of this process because ambient temperature alters the permeation velocity of freeze damage-protective substances into embryos and affects the developmental ability of the embryos (1 minute when temperature is 20°C).

10. After keeping the straw inside the liquid nitrogen gas for 3 minutes or longer, the straw is immersed in liquid nitrogen and transferred into the storage tank (3 m 30 s).

3. Warming

Materials

PB1, S-PB1, mineral oil, tap water at room temperature, plastic dish (35 mm), capillary for embryo manipulation, mouthpiece, 1-ml syringe, 18G syringe needle, thermometer, forceps, straw cutter, stereoscopic microscope, wide-mouthed container (tap water container)

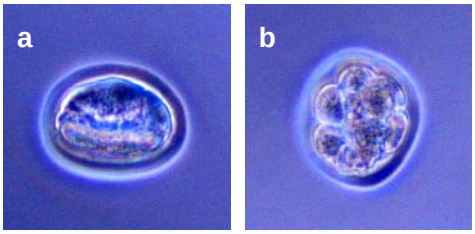
Method

1. S-PB1 and PB1 are equilibrated to room temperature.
2. Four drops each of S-PB1 and PB1 are formed and covered with mineral oil in a dish.
3. Tap water at room temperature is prepared in a wide-mouthed container.
4. S-PB1 (0.8-1 ml) is aspirated into a 1-ml syringe attached with an 18G syringe needle.
5. The straw is taken out from the liquid nitrogen into the air, at which the timer is started (0 m 00 s).
6. After 10 seconds, the straw is immersed in water at room temperature and lightly shaken (0 m 10 s).
7. When the S-PB1 region in the straw starts to thaw (~ 0 m 18 s), the straw is immediately taken out, and water on the straw surface is wiped off with tissue paper. Be cautious not to touch the EFS40 column region of the straw.
8. Holding the S-PB1 region, the straw is kept horizontal and the end on the EFS40 side and the cotton plug is cut off.
9. The straw is slightly tilted, keeping the EFS40-side end above the dish. An 18G syringe needle attached to a syringe containing S-PB1 is inserted from the opposite end, and the straw content is washed out into the dish (~ 1 m 00 s). The process from thawing to washing out should be performed as quickly as possible.
10. Embryos are collected using a pipette, and transferred into S-PB1 drops for washing (~2 m 30 s). In case only few embryos are recovered, S-PB1 that is left in the straw is spilled out and searched for remaining embryos.

11. At 5 minutes after washing out with S-PB1, the embryos are transferred into the PB1

drops and washed (6 m 00 s). Normal embryos shrink in S-PB1 (Fig. 4a) and return to the normal shape in PB1 (Fig. 4b). The embryos are transferred into R1ECM medium and kept in a CO_2 incubator at 37°C until uterine transfer.

Fig. 4. Morulas in S-PB1 (a) and PB-1 (b)



4. Uterine Transfer

Materials

Nembutal, physiological saline, Giemsa stain solution, capillary for embryo manipulation, mouthpiece, 1-ml syringe, 26G syringe needle, cotton swab, slide glass, forceps, surgical scissors, ophthalmologic scissors, clamp, autoclip, suture needle, stereoscopic microscope

Method

1. After confirming the estrus cycle of sexually mature female rats, a proestrous female rat is mated with a vasectomized male rat.
2. The rats are separated in the following morning, and a vaginal plug is confirmed.
3. Morulae are implanted into the uterine horns of the pseudopregnant (recipient) rat on day 2 after vaginal plug confirmation.
4. Nembutal solution is intraperitoneally administered to the recipient rat.
5. Hair of the dorsolateral region is clipped.
6. An about 1-cm incision is made along the midline in the dorsolateral skin. The tips of ophthalmologic scissors are compressed to the incised region, and the epidermis and muscle layer are separated by opening the scissors.
7. An about 1-cm incision is made in the muscle layer, and the ovary and uterus are pulled out by carefully pulling the attached fat tissue out of the abdominal cavity, which is then placed on sterile gauze, and retained with a clamp.
8. A 26G syringe needle is inserted into a site about 1 cm from the uterine horn-oviduct junction. The pipette is inserted into the needle hole, and morulae are injected.
   Note: Several air layers are aspirated into a pipette, followed by densely packed morulae in the pipette tip.
9. The ovary and uterus are returned into the abdominal cavity, the muscle layer is sutured, and the outer skin is closed with autoclips.
10. Morulae are similarly transferred into the other uterine horn.
11. After the operation, the rat is warmed on a warm plate until arousal from anesthesia, and transferred into a cage when the animal starts to move. The animal is maintained until the expected date of delivery.

5. List of instruments and reagents

Vitrification of morulae

Tools	Maker	Code No.
Straight scissors, one-side sharp and the other blunt (NAPOX)	Natsume Seisakusho	B-3
Small straight scissors, both sides sharp (NAPOX)	Natsume Seisakusho	B-12
Iris forceps without hooks, straight (NAPOX)	Natsume Seisakusho	A-7
Forceps, fine points (INOX-1) (DUMONT)	Natsume Seisakusho	MA-41
Perfusion needle, 28G	Taguchi Chusha-shin Seisakusho	28G (custom-made)
1-ml syringe	Terumo	SS01T
Dish, 35 mm	Corning	430588
Dish, 60 mm	Falcon	35-1007
Capillary	Drummond Science	2-000-050
Tube, 15 ml	Corning	430053
Tube, 50 ml	Falcon	35-2070
Freeze straw, 0.25 ml	My Science Corp.	AAA201
Drugs	Maker	Code No.
NaCl	Nacalai Tesque	313-20
KCl	Nacalai Tesque	285-14
$CaCl_2$-$2H_2O$	Nacalai Tesque	06731-05
KH_2PO_4	Nacalai Tesque	28721-55
$MgCl_2$-$6H_2O$	Nacalai Tesque	20909-42
Na_2HPO_4-$12H_2O$	Nacalai Tesque	31723-35
Na-pyruvate	Nacalai Tesque	298-06
Glucose	Nacalai Tesque	168-06
Streptomycin	Meiji Seika	-
Penicillin G	Meiji Seika	-
Sucrose	Nacalai Tesque	30403-55
Ficoll 70	Amersham Biosciences	17-0310-10
Ethlene Glycol	Nacalai Tesque	15209-85
Mineral oil	SIGMA	M8410-1L
BSA	Nacalai Tesque	01863-77

Intrauterine morulae implantation

Tools	Maker	Code No.
Straight scissors, one-side sharp and the other blunt (NAPOX)	Natsume Seisakusho	B-3
Small straight scissors, both sides sharp (NAPOX)	Natsume Seisakusho	B-12
Forceps, very fine points, straight	Natsume Seisakusho	A-3-1
Forceps, fine points (INOX-1)	Natsume Seisakusho	MA-41
Iris forceps without hooks, straight (NAPOX)	Natsume Seisakusho	A-7
Arterial clamp, small, straight, grooved (NAPOX)	Natsume Seisakusho	C-17
Autoclip (**Still**)	Natsume Seisakusho	C-29
Hair-clipping scissors (small) (NAPOX)	Natsume Seisakusho	B-39
Carbide Still needle holder (NAPOX)	Natsume Seisakusho	C-35-T
1-ml syringe	Terumo	SS01T
Dish, 35 mm	Corning	430588
Dish, 60 mm	Falcon	35-1007
Capillary	Drummond Science	2-000-050
Tube, 15 ml	Corning	430053
Tube, 50 ml	Falcon	35-2070
Suture thread	Akiyaka Seisakusho	CA1370N2
Drug	Maker	Code. No.
BSA	Nacalai Tesque	01863-77
Mineral oil	SIGMA	M8410-1L
Nembutal injection (50 mg/ml pentobarbital)	Dainippon Pharmaceutical	-
Bosmin	Daiichi Pharmaceutical	872451
Physiological saline 5–ml ampoule x 100	Fuso Pharmaceutical Industry	-
500-ml bottle	Otsuka Pharmaceutical	024-3L1