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SEcuRe 2.0 – A simple and economic protocol for efficient in vitro fertilization using cryopreserved mouse sperm V.5

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Abstract

The advent of genome editing tools like CRISPR/Cas has substantially increased the number of genetically engineered mouse models in recent years. In support of refinement and reduction, sperm cryopreservation is advantageous compared to embryo freezing for archiving and distribution of such mouse models. The *in vitro* fertilization using cryopreserved sperm from the most widely used C57BL/6 strain has become highly efficient in recent years due to several improvements of the procedure. However, the purchase of the necessary media for routine application of the current protocol poses a constant burden on budgetary constraints. In-house media preparation, instead, is complex and requires quality control of each batch. Here, we describe SEcuRe 2.0, a cost-effective and easily adaptable approach for *in vitro* fertilization using cryopreserved C57BL/6 sperm. This is mainly achieved by modification of an affordable commercial fertilization medium and a step-by-step description of all other necessary reagents. This protocol is compatible with frozen sperm from all major repositories, and the IVF can easily be adapted to accommodate freshly harvested sperm.

The basal fertilization medium (Cook's RVF) used in the initial SEcuRe approach has been discontinued by the manufacturer. We have validated HTF as a replacement, and SEcuRe 2.0 successfully employs HTF as the basal fertilization medium ever since.

(The last step contains a supplemental video with extra context and tips, as part of the protocols.io Spotlight series, featuring conversations with protocol authors.)

Attachments

PNG

SEcuRe 2.0 protocol ...

2.1MB

Guidelines

ANIMAL PROCEDURES

Sperm of 10- to 20-week-old C57BL/6 males and oocytes of superovulated 3-4-week-old (i.e., 12-14 g body weight) of the corresponding genetic background are collected for the IVF procedures as decribed in published protocols (Behringer, R., *et al.* Manipulating the mouse embryo: a laboratory manual, Fourth edition. ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2014.). Females are superovulated with 5 IU of PMSG (Aviva Systems Biology; OPPA01037) followed after 48 hours by 5 IU of hCG (MSD Animal Health; Ovogest 300I.E.).

We routinely pool the sperm from 2 males to compensate for variability in sperm quality between males, generate sufficient quantities of straws for archiving and distribution, and enable the optional quality control via an IVF of a single straw for each cryopreservation. Use of a single male is possible as well, but the number of straws and volume of media used needs to be reduced by 50%. The integrity of each sperm sample after cryopreservation is recommended to be evaluated by a validation IVF with oocytes from 2 superovulated females. Samples with fertilization rates of >20% can be considered as successfully archived.

The 2-cell embryo transfer is performed unilaterally according to a published protocol (Behringer, R., *et al.* Manipulating the mouse embryo: a laboratory manual, Fourth edition. ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2014.). Well tolerated inhalational anesthetics like isoflurane should be used as anesthetic and carprofen (5 mg/kg BW) Carprosol, CP-Pharma Handels-Gesellschaft mBH) as analgesic after surgery.

MEDIA PREPARATION

Use only embryo-grade reagents.
Use weighing paper and disposable spatulas only.
For precise volume measurement during c-TYH preparation use a 25 ml volumetric flask (Vitlab; 671941) washed with embryo-grade water before first use and between subsequent steps. Discard the flask after the entire procedure. Do not use detergent to clean the flask in order to re-use it as residual detergent will harm the sperm.

gCPA for sperm cryopreservation

Prepare cryoprotective agent (gCPA) containing 18% raffinose pentahydrate and 3% skim milk supplemented with 100 mM L-glutamine as follows:

- Place 0.146 g of L-glutamine in 10 ml of prewarmed (60°C) embryo-grade water and vortex for 3 min
- Place the tube back to 60°C for 5 min
- Add 1.8 g of raffinose pentahydrate and 0.3 g of skim milk, vortex the solution for 3 min and incubate for 90 min at 60°C. Vortex gCPA every 30 min for 3 min
- Centrifuge the solution at 10,000 g for 60 min (using 1 ml aliquots). The supernatant should be clear afterwards
- Carefully collect 720 µl of the supernatant of each sample from the central region of the tube and diascard the pellets
- Filter the supernatant through a 0.22 µm filter, discard first drops
- If possible, check osmolality (500-520 mOsm/kg) and store aliquots at room temperature for up to 3 months

c-TYH medium for sperm preincubation

For sperm preincubation prepare c-TYH containing 1.0 mg/ml of polyvinyl alcohol and 0.75 mM methyl-β-cyclodextrin (MBCD) from concentrated stocks as follows (alternatively commercially available FERTIUP® PM (Cosmo Bio; KYD-002-EX-X5) can be used):

1. Prepare a mixture of the following substances for concentrated stock solutions:

- Stock X:

- 10x NaCl: Weigh 1.744 g of NaCl into a 50 ml centrifuge tube and dissolve in 20 ml of embryo-grade water

- 100x Salt Mix: Weigh all the reagents into a 50 ml centrifuge tube and dissolve in 20 ml of embryo-grade water

	Reagent	Amount
	KCl	**0.890 g**
	MgSO4 × 7 H2O	0.733 g
	KH2PO4	0.405 g
	D-(+)-Glucose	2.500 g
	Penicillin G	0.188 g
	Streptomycin	0.125 g

- Rinse a new 25 ml volumetric flask with embryo-grade water before use by inverting the plugged flask

- Transfer the dissolved salt mix solution into the flask, wash the centrifuge tube with 3 ml of embryo-grade water and add it to the flask

- Fill the flask up to 25 ml with embryo-grade water

- Transfer the salt mix solution into a new centrifuge tube

- Pipette 2.5 ml of the salt mix to the centrifuge tube containing 20 ml of NaCl solution

- Wash the previously used 25 ml volumetric flask 3x with embryo-grade water

- Transfer the prepared NaCl + salt mix solution into the flask and fill it up to 25 ml with embryo-grade water

- Filter the solution through a 0.22 µm filter and store 3 ml aliquots at -20°C for a maximum of 12 months

- Stock Y: 100x $CaCl_2$ x $2H_2O$

- Weigh 0.628 g of $CaCl_2$ x $2H_2O$ into a 50 ml centrifuge tube and dissolve in 20 ml of embryo-grade water

- Wash the previously used 25 ml volumetric flask 3x with embryo-grade water

- Transfer the $CaCl_2$ solution into the flask, wash the centrifuge tube with 3 ml of embryo-grade water and add it to the flask

- Fill the flask up to 25 ml with embryo-grade water

- Filter the solution through a 0.22 µm filter, prepare 300 µl aliquots and store at -20°C for a maximum of 12 months

2. Preparation of PVA solution:

- Fill a 15 ml centrifuge tube with 2.5 ml of embryo-grade water
- Weigh 0.025 g of PVA and add it into the centrifuge tube
- To dissolve the PVA, heat the solution up to 90 °C for at least 10 min and vortex

Note: Preheating of embryo-grade water before adding PVA facilitates solving.

3. Preparation of c-TYH medium (in 50 ml centrifuge tube)

- Pipette 10 ml of embryo-grade water to a new 50 ml centrifuge tube and add to it in the following order:

	Reagent/Stock	Amount	Comment
	Stock X	**2.5 ml**	
	7.5% NaHCO3 solution	702 µl	open sterile
	100 mM Sodium pyruvate solution	125 µl	**open sterile**
	MBCD	0.0246 g	**weigh directly into the solution**
	PVA solution	2.5 ml	wash the centrifuge tube with 3 ml of embryo-grade water and add it to the prepared c-TYH
	Stock Y	250 µl	

- Transfer the medium into a 25 ml volumetric flask, wash the centrifuge tube with 3ml of embryo-grade water and add it to the flask
- Fill the flask up to 25 ml with embryo-grade water (**CAUTION:** As soon as the PVA solution is added, it starts to foam)
- Filter the solution through a 0.22 µm filter and store 1 ml aliquots at +4 °C for up to 3 months
- If possible, perform osmolality check (283-293 mOsm/kg)

HTF preparation

A ready-to-work media can be obtained commercially (e.g., Merck; MR-070-D). This product should be thawed after arrival and aliquoted. The aliquots can be stored at -80 °C for 6 months. Once the aliquots are thawed, they can be stored at 4 °C for two weeks.

HTF^{+} medium for *in vitro* fertilization

For the IVF procedure, supplement HTF medium with reduced glutathione (GSH; final concentration: 1 mM) and Ca^{2+} (final concentration: 5.14 mM) as following to obtain HTF^{+} medium:

- Prepare a 100x $CaCl_2$ x $2H_2O$ stock solution (310 mM) by dissolving 0.4558 g of $CaCl_2$ x $2H_2O$ in 10 ml of embryo-grade water
- Filter the solution through a 0.22 µm filter and store aliquots at -20°C for a maximum of 6 months
- On the day of IVF thaw an aliquot of 100x $CaCl_2$ at room temperature
- Add 60 µl of 100x $CaCl_2$ to 6 ml of HTF medium and mix gently

- HTF supplemented with $CaCl_2$ can be stored at 4°C for up to 1 week
- Place 1 ml of HTF medium supplemented with $CaCl_2$ in a tube containing 30.7 mg of GSH and vortex
- Add 50 µl of this solution to 5 ml of HTF medium supplemented with $CaCl_2$, mix gently and filter using 0.22 µm syringe end filter in order to obtain HTF^+ medium

Embryo culture media

Use **M2** medium for handling of embryos outside and embryo culture medium (**M16**, **KSOM**) for culture inside a CO_2 incubator. Many labs prefer the use of KSOM as it supports the development of embryos from many different mouse strains. All media can be prepared in-house according to the previously published method (Behringer, R., *et al.* Manipulating the mouse embryo: a laboratory manual, Fourth edition. ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2014.) or commercially purchased.

Mouse oocytes and pre-implantation embryos are incubated in embryo culture medium pre-equilibrated for at least 4 h in a CO_2 incubator (5% CO_2, 37 °C, 95% humidity).

SEcuRe 2.0 protocol applicability

The SEcuRe 2.0 protocol can be used to perform **IVF with freshly harvested sperm** after applying the following modifications:

1. 2 males of the same line should be sacrificed and sperm of better quality (based on a visual assessment after 60 min preincubation) should be utilized in an IVF procedure. If sperm of both males show the same quality, both can be used in an IVF. Sacrifice two males and dissect the cauda epididymides. Transfer the 2 cauda epididymides (after removal of fat and blood) of each male to separate dishes into the oil next to the c-TYH (or FERTIUP® PM) drop. After nicking the tissue, drag sperm with watchmaker forceps into the drop.
2. Sperm should be allowed capacitation for 60 min in c-TYH (or FERTIUP® PM).
3. 0.25 mM GSH concentration should be used in the HTF^+. For this purpose:
   - Prepare a 100x $CaCl_2$ x $2H_2O$ stock solution (310 mM) by dissolving 0.4558 g of $CaCl_2$ x $2H_2O$ in 10 ml of embryo-grade water
   - Filter the solution through a 0.22 µm filter and store aliquots at -20°C for a maximum of 6 months
   - On the day of IVF, thaw an aliquot of 100x $CaCl_2$ at room temperature
   - Add 60 µl of 100x $CaCl_2$ to 6 ml of HTF medium and mix gently
   - Place 1 ml of HTF medium supplemented with $CaCl_2$ in a tube containing 30.7 mg of GSH and vortex
   - Add 10 µl of this solution to 4 ml of HTF medium supplemented with $CaCl_2$, mix gently and filter using 0.22 µm syringe end filter
4. Oocytes from a maximum of 5 females should be placed in a 200 µl drop of HTF^+;
5. 5 µl of sperm suspension should be added to the oocytes. If the removal of cumulus cells assessed after 20 min of incubation is poor, indicating insufficient motility or concentration of sperm, an additional 5 µl of the sperm suspension should be transferred to the fertilization medium.

The SEcuRe 2.0 protocol can also be used with **samples cryopreserved according to Ostermeier *et al.*** (2008) approach routinely used by The Jackson Laboratory. In this case, the entire sperm suspension should be expelled

into the center of a 6-cm dish and 30 µl of that suspension should be added to a 90 µl drop of c-TYH (or FERTIUP PM®). Subsequently, IVF should be conducted according to the standard protocol described here (see Steps 23-28).

Update information

The basal fertilization medium (Research Vitro Fert, Cook Medical; K-RVFE-50) used in the initial SEcuRe approach has been discontinued by the manufacturer. We have validated HTF as a replacement, and SEcuRe 2.0 successfully employs HTF as the basal fertilization medium ever since.

Materials

- L-Glutamine **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G8540
- Water (for embryo transfer sterile-filtered BioXtra suitable for mouse embryo cell culture) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**W1503
- Raffinose pentahydrate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**R7630
- Skim Milk **Becton Dickinson (BD)** **Catalog #**232100
- FERTIUP Mouse Sperm Preincubation Medium: PM **Cosmo Bio** **Catalog #**KYD-002-EX-X5
- Volumetric flasks Class B transparent 25 ml **Carl Roth** **Catalog #**671941
- NaCl **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S5886
- KCl **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P5405
- MgSO4 × 7H20 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M2773
- KH2PO4 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P5655
- D-Glucose **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G6152
- Penicillin G **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P7794
- Streptomycin **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S1277
- NaHCO3 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S8761
- Gibco™ Sodium Pyruvate (100mM) **Fisher Scientific** **Catalog #**11-360-070
- Methyl-β-cyclodextrin **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**C4555
- Polyvinylalcohol (PVA) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P8136
- CaCl2 × 2H2O **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**C7902
- HTF EmbryoMax **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**MR-070-D
- L-Glutathione reduced **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G4251
- MiniStraw 0.25 ml clear **Minitube** **Catalog #**13407/0010
- Pipette tips Cell-Saver 200 µl farblos **Biozym Scientific GmbH** **Catalog #**729055
- Falcon® 35 mm TC-treated Easy-Grip Style Cell Culture Dish **Corning** **Catalog #**353001
- Falcon® 60 mm TC-treated Easy-Grip Style Cell Culture Dish **Corning** **Catalog #**353004
- Paraffin oil **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**76235
- Dulbecco's Phosphate Buffered Saline **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D8537
- Sealing ball metal for 0.25 ml straws **Minitube** **Catalog #**13400/9970
- Pipette tips Cell-Saver 200 µl farblos **Biozym Scientific GmbH** **Catalog #**729055
- Acrodisc® Syringe Filters with Supor® Membrane Sterile - 0.2 µm 32 mm **Pall** **Catalog #**4652

M2 Medium **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M7167

EmbryoMax® KSOM Mouse Embryo Media **Merck Millipore (EMD Millipore)** **Catalog #**MR-106-D

Syringe 1 ml **Terumo** **Catalog #**SS-01T1

Troubleshooting

Sperm cryopreservation

1 Prepare 20 straws for 2 sacrificed males of the same line. Use of a single male is possible as well, but the number of straws and volume of media used needs to be reduced by 50%

2 Mark the straws at 2.3 cm and 4.0 cm at the open end and label them at the other end (cotton plug)

3 Attach a 1 ml syringe to the labeled end of the straw and aspirate HTF medium until the meniscus reached the 4.0 cm mark

4 Aspirate a 2.3 cm air into the straw and store the assembly until required

5 Place a 120 µl drop of the gCPA (for preparation see Guidelines & Warnings) in a 35-mm culture dish, cover it with paraffin oil, add another 120 µl of gCPA into the drop to obtain a tall, semi-spherical 240 µl drop (for 4 cauda epididymis pooled from 2 males) and prewarm on a 37 °C hot plate

6 Sacrifice 2 males, collect the cauda epididymides and vasa deferentia in DPBS and clean them of fat and the testicular artery to avoid contaminating the semen with blood

7 Dry the cauda epididymides on a tissue (to avoid dilution of gCPA with DPBS), transfer to a 240 µl drop of prewarmed gCPA (on a 37 °C hot plate for at least 5 min) and make 6–7 cuts across the cauda epididymis with a pair of micro spring scissors

8 Place the dish on a 37 °C hot plate for 3 min and gently swirl every min for 20 sec to help the sperm disperse from the tissue

9 Divide the sperm suspension into 20 aliquots of 10 µl (using a pipette and 200 µl cell-saver tips) on a 10-cm culture dish lid, avoiding carryover of paraffin oil (clean the pipette tip from the outside with a tissue to remove the oil each time before placing a 10 µl aliquot on the dish lid) and any tissue (use microscope) into the aliquots

10 Aspirate each 10 µl drop into a separate freezing straw followed by 2.3 cm air

11 Seal the straws (e.g., with a heat sealer or metal balls) and place them in liquid nitrogen gas phase for 10 min. Note: We use a custom-made metal inlay for this purpose but self-

made or purchased freezing canisters can be used as well (e.g., http://card.medic.kumamoto-u.ac.jp/card/english/sigen/manual/spfreeze.html#canister or KYD-S018 from Cosmo Bio)

12 Transfer the straws to the liquid nitrogen tank for long-term storage

Oocyte isolation

13 Ideally, the entire IVF procedure (oocyte preincubation, sperm preincubation and fertilization) should be performed in an incubator (5% CO_2, 37 °C) at 5% O_2, but atmospheric O_2 concentration have been shown to work well, too

14 Prepare a 35-cm culture dish (for oocytes from a maximum of 3 females) with a 90 µl drop of HTF^+ (for preparation see Guidelines & Warnings) covered with oil (prewarmed to 37 °C) and equilibrate it for at least 20 min in an atmosphere of mixed gas (5% CO_2, 5% O_2, 37 °C)

15 Collect oviducts from superovulated females 15 hours after the hCG injection and clean them in DPBS

16 Transfer the oviducts into the paraffin oil next to the 90 µl drop of HTF^+

17 Release oocyte clutches into the oil by ripping the ampulla with forceps and drag them through the oil into the fertilization drop

18 Incubate oocytes for 50 min before adding the sperm suspension (at least 30 min and no longer than 60 min) in an atmosphere of mixed gas (5% CO_2, 5% O_2, 37 °C)

Sperm thawing and capacitation

19 Prepare a 35-mm culture dish (for each IVF experiment) with 90 µl c-TYH drop (for preparation see Guidelines & Warnings) covered with paraffin oil and equilibrate it overnight in an atmosphere of mixed gas (5% CO_2, 5% O_2, 37 °C)

20 Remove the required straw(s) from long-term storage in liquid nitrogen on the day of IVF, place in a dewar with liquid nitrogen. For thawing, remove the straw(s) from liquid nitrogen, keep it for 5 sec at room temperature and then quickly transfer it into a 37 °C water bath for 10 min

21 Dry the straw(s) with a tissue and cut the sealed end and the labeled end of the straw below the cotton plug

22 Expel 10µl sperm suspension into the center of a 90 µl c-TYH drop using a 1 ml syringe

23 Preincubate for 30 min in an atmosphere of mixed gas (5% CO_2, 5% O_2, 37 °C) before the IVF procedure to allow capacitation of the sperm. Note: Do not disturb the dishes containing the frozen/thawed sperm until the sperm are moving rapidly within the medium. Otherwise, the sperm will not recover full motility

In vitro fertilization

24 Add 10 µl of the sperm suspension taken from the edge of the c-TYH drop to the oocyte clutches with the help of a 200 µl cell-saver tip and incubate for 4 hours (at least 3 hours and no longer than 5 hours) in an atmosphere of mixed gas (5% CO_2, 5% O_2, 37 °C)

25 Add another 10 µl of the sperm suspension to the fertilization medium if the removal of cumulus cells assessed after 20 min of incubation is poor, indicating insufficient motility or concentration of sperm

Embryo culture and transfer

26 Wash embryos after the IVF procedure through 10 drops of preincubated embryo culture medium (e.g., M16 or KSOM) and incubate overnight in embryo culture medium in groups of 15–50 embryos per drop (a 30 µl drop of embryo culture medium covered with paraffin oil) in a CO_2 incubator (5% CO_2, 37 °C, 95% humidity)

27 The day after insemination determine fertilization rates (a percentage of the total number of inseminated oocytes that developed to the 2-cell stage)

28 Transfer 2-cell embryos into the oviducts of pseudo-pregnant 0.5 dpc females

29 (The following video contains extra context and tips, as part of the protocols.io Spotlight series, featuring conversations with protocol authors.)

https://www.youtube.com/embed/TDTZKPfThVU