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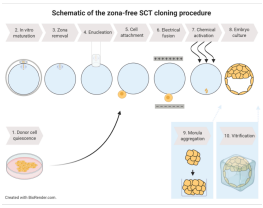
Generating cloned sheep embryos by zona-free somatic cell transfer



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Abstract

This protocol summarises zona-free sheep cloning by somatic cell transfer (SCT), adapted from a similar SCT method in cattle (https://doi.org/10.1089/153623003772032763). It describes the workflow that is required for reprogramming somatic cells into cloned blastocysts of high morphological quality, including two optional steps of embryo aggregation and vitrification. The resulting embryos can then be transferred into surrogate ewes for producing live cloned animals. Compared to conventional zona-intact procedures, this zona-free embryo reconstruction system increases throughput of cloned embryo production and ease of operation.

Guidelines

A routine SCT cloning experiment involves 3 people: 1 for enucleation and/or electrical fusion and 2 for all remaining steps. Each SCT run will typically be on the following scale:

- 200 slaughterhouse-derived ovaries
- 400 oocytes
- 300 usable metaphase II (MII) oocytes with a polar body and even cytoplasm
- 220 oocytes enucleated (the rest used as parthenogenetic activation controls)
- 220 fused SCT reconstructs (fusion rate 95–100%)
- 180 cleaved embryos (cleavage rate ~80%)
- 20–40 compacted morulae/blastocysts (morulae/blastocysts development rate 10–20%)

Materials

MATERIALS

- Ionomycin calcium salt from Streptomyces conglobatus **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**I0634
- Dulbecco's Phosphate Buffered Saline **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D5652
- DMEM/F-12, GlutaMAX™ supplement **Thermo Fisher Catalog #**10565018
- Trypsin-EDTA (0.25%), phenol red **Thermo Fisher Catalog #**25200056
- Medium 199, Earle's Salts, powder **Thermo Fisher Catalog #**31100035
- Oxoid™ Saline Tablets **Thermo Fisher Catalog #**BR0053G
- 6-(Dimethylamino) purine (6-DMAP) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D2629
- Ficoll 70 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F2878
- Hoechst 33342 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**B2261
- Hyaluronidase **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3506
- Lectin phytohemagglutinin (PHA-P) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L9017
- Mineral oil **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M5310
- Pronase (Protease from Streptomyces griseus) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P8811
- Bovine Serum Albumin (BSA) fatty acid free Gamma-irradiated **MP Biomedicals Catalog #**ABIVP, lot 5226
- Fetal bovine serum (FBS) **Moregate Biotech Catalog #**FBSF, Lot 48827103
- PVA (Polyvinyl Alcohol) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P8136
- Kanamycin monosulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**K1377
- Folltropin-V (Follicle-stimulating hormone) **Vetoquinol Catalog #**Batch 76965
- DBL Heparin Sodium (porcine mucous) 5000 I.U / 1 mL Injection BP **Pfizer (Hospira) Catalog #**Batch 157357A
- 17β-Estradiol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E2758

If the chemical was not listed above, it was supplied by Sigma-Aldrich.

MEDIA AND SOLUTIONS:

- Aspiration medium: H199, 925 IU/ml heparin, 20 µl/ml of 2% (w/v) FBS.
- B199: Medium 199 with 25 mM $NaHCO_3$, 0.2 mM pyruvate, 0.086 mM kanamycin monosulfate.
- B199+FBS: B199 with 10% (v/v) FBS.
- Disaggregation medium: HSOF (without calcium and BSA), 0.1 mg/ml PVA, 0.2 g/ml EDTA, 5 µg/mL cytochalasin-B.
- Embryo-hold: LSOF with 20 mM MOPS, 5 mM $NaHCO_3$.
- Early Synthetic Oviduct Fluid (ESOF): (Wells et al. (2003), https://doi.org/10.1016/S0093-691X(02)01273-6).

- Fusion buffer: 260 mM D-mannitol, 0.1 mM $MgSO_4$, 0.5 mM HEPES, 0.1 mg/mL polyvinyl alcohol (PVA).
- H199: Medium 199 with 15 mM Hepes, 5 mM $NaHCO_3$, 0.086 mM kanamycin monosulfate.
- H199+FBS: H199 with 10% (v/v) FBS.
- H199+BSA: H199 with 3 mg/mL BSA.
- Hepes-buffered Synthetic Oviduct Fluid (HSOF): 107.7 mM NaCl, 7.15 mM KCl, 0.3 mM KH_2PO_4, 5 mM $NaHCO_3$, 3.32 mM sodium lactate, 0.069 mM kanamycin monosulfate, 20 mM Hepes, 0.33 mM pyruvate, 1.71 mM $CaCl_2.2H_2O$, 3 mg/ml fatty-acid free BSA.
- In vitro maturation (IVM) medium: B199+FBS with 10 µg/ml ovine follicle-stimulating hormone, 1 µg/ml 17-ß-estradiol, 0.1 mM cysteamine.
- Late Synthetic Oviduct Fluid (LSOF): (Wells et al. (2003), https://doi.org/10.1016/S0093-691X(02)01273-6).
- Pronase solution: Protease (from Strep. griseus), HSOF (without calcium and BSA), 1 mg/ml PVA, 0.1 mg/ml $CaCl_2.2H_2O$, 0.1 mg/ml $MgCl_2.6H_2O$.

PLATE PREPARATION:

The following plates can be prepared the **day before** SCT and stored at 4°C. If different treatments or groups are used, additional plates can be prepared to physically separate the embryos.

	Plate name	Medium	Dish size (mm)	Drop configuration under mineral oil	Maximum embryos in plate	Final location
	Oocyte	H199+BSA +Caffeine	60	12 × 30 µl drops	360	Warm stage
	Stain	H199+BSA +Caffeine +Hoechst33342	60	12 × 30 µl drops	~400	Warm stage
	Cytoplast	H199+BSA +Caffeine	60	12 × 30 µl drops	360	Warm stage
	Couplet	H199+BSA +Caffeine	60	12 × 30 µl drops	120	Warm stage
	Post-fusion	H199+BSA +Caffeine	60	12 × 30 µl drops	120	Warm stage

The following plates should be prepared on the **day of** SCT. If different treatments or groups are used, additional plates should be prepared to physically separate the embryos. These solutions are made on the day of SCT to prevent ESOF components from precipitating. Prepare appropriate numbers of each plate to ensure that there are enough single culture drops/dimples to hold the number of reconstructs produced.

	Plate name	Medium	Dish size (mm)	Drop configuration under mineral oil	Maximum embryos in plate	Final location
	Lectin	H199+BSA+Caffeine+phytohemagglutinin	60	12 × 30 µl drops		Warm stage
	ESOF -Ca	ESOF-Ca+10% FBS +Caffeine	60	3 × 40 µl wash drops & 30 × 5 µl single culture drops	30	Gassed incubator

DMAP	ESOF+6-DMAP	60	3 × 40 µl wash drops & 30 × 5 µl single culture drops	30	Gassed incubator
ESOF	ESOF	35	2 × 40 µl wash drops & 7 × 20 µl culture drops	70	Modular incubation chamber

EQUIPMENT:

- Aspiration module: 21-gauge needle (Vacutainer® blood collection needle, BD, USA) pushed through a rubber bung for collection in 15 ml Falcon® tubes (Thermo Fisher Scientific, USA).
- Electrofusion chamber: custom-made chamber with two parallel surgical-grade titanium electrodes 2 mm deep, 3 mm separation, and 35 mm long, mounted on a glass microscope slide.
- Enucleation microscope: standard inverted research microscope (e.g. Nikon Diaphot, Olympus IX 70) with epifluorescence illumination and the appropriate fluorescence filters (e.g. for Hoechst 33342).
- Enucleation needle: 25–30 µm outer diameter, perpendicular break, no bevel or spike, with a ˜30° bend at 80 µm thickness, made from thin wall borosilicate capillaries (GC100T-15, Harvard Apparatus Ltd., UK), using a horizontal puller (P-87, Sutter Instruments, CA) and a microforge (MP-9, Narishige, Japan).
- Fusion machine: Standard electrofusion/electroporation system (e.g. ECM 200 (BTX, USA)).
- Micromanipulators: Standard three-axis oil hydraulic hanging joystick micromanipulators (MO-188, Nikon Narishige, Japan) and a 0.2-mL Gilmont® micrometer syringe (Cole-Parmer Instruments, IL).
- Modular incubation chambers (MIC-101, QNA International Pty Ltd., Australia).
- Petri Dishes (35 mm, 60 mm, 90 mm, Falcon®).
- Pulled glass Pasteur pipettes: Glass Pasteur pipette (unplugged D810, Volac, UK) hand-pulled over a flame to slighter wider than the diameter of a zona-intact oocyte, scored to make a perpendicular break and edges smoothed by flame polishing.
- Separation needle: 100–150 µm outer diameter, perpendicular break, closed firepolished tip, with a ˜30° bend, made from borosilicate capillaries, using a horizontal puller and a microforge.
- Standard stereo microscope with warm stage at 38.5°C.
- Standard thermos for ovary collection, and a wide-mouth thermos for maintaining temperature during aspiration.
- Tissue culture plates (4-well, 48-well, 96-well, Falcon®).
- Vacuum pump: IVF Ultra Quiet VMAR-5100 (Cook veterinary products, Switzerland).
- Vortex: MS1 Minishaker Vortexer (IKA, Germany).

Before start

Information on media, equipment and plates can be found in the materials section.

To save time on the day of SCT, prepare some of the solutions and plates in advance. Carry out media preparations in the same week as SCT, but if several experiments are planned within a few weeks, bulk media can be prepared a few weeks in advance.

Induce donor cell quiescence by serum starvation

1 Induce donor cells into quiescence (or G_0) by serum starvation

1.1 Seed cells at approximately 2.5 × 10^4 cells/cm^2 in duplicate wells (equivalent to 5×10^4 cells per well in a 4-well plate or 5 ×10^3 cells in a 96-well plate). Proceed to serum starvation within 6-20 hours after seeding.

1.2 Wash cells once with phosphate-buffered saline (PBS) and add starvation medium DMEM/F12+0.5% FBS for 4-6 days. Do not change medium during the starvation period.

In vitro maturation of oocytes

2 Retrieve oocytes from slaughterhouse ovaries

Note

Sheep (ovine) is a seasonally reproductive species and SCT experiments are best carried out during the extended breeding season (February-July) when ewes are in estrous in our geographic region (latitude ˜37°S, Waikato, New Zealand). During that time, we collected ovaries from local slaughterhouses. Experimental outcomes are more consistent with ovaries from mature ewes rather than lambs. In slaughterhouses, designated “lambs” during the above specified collection period range from prepubescent to 13-months old. By contrast, “two-tooth” sheep (identified by the two adult central incisors which appear at approximately 13 months of age) are considered adult. On average, expect to recover 2 good quality oocytes per mature ewe ovary.

2.1 Dissect ovaries from the reproductive tract and place in a thermos in saline at 30 °C for aspiration within 2–4 hours of collection.

2.2 Rinse the ovaries very briefly in 70% ethanol to sterilise, followed by a thorough rinsing in 30 °C saline 2-3 times.

2.3 Aspirate follicles that range in size between 1 mm - 5 mm with the aspiration module (see materials section) using negative pressure from a vacuum pump (~43

mmHg) into 2 mL - 3 mL of oocyte aspiration medium in a 15 mL tube.

Note

In order to keep oocytes warm, change tubes after filling to approximately 7 mL and keep at 30 °C .

2.4 Transfer the aspirated follicular content (the sediment containing oocytes) from the 15 mL tube to a 90 mm dish containing fresh, warmed aspiration medium. Search for oocytes using a stereomicroscope with warm stage at 38.5 °C , selecting only cumulus-oocyte complexes (COCs) with intact layers of cumulus and relatively homogenous ooplasm.

Note

All dishes used throughout protocol are Petri dishes to prevent adherence of cells.
Wash dishes throughout protocol are 35 mm in diameter.
All microscopes had a warm stage of 38.5 °C unless otherwise stated.

2.5 Wash COCs serially through two dishes of H199+FBS, then once in B199+FBS. Transfer 10 COCs in 10 µL B199+FBS into a 40 µL drop of IVM medium in a 60 mm diameter dish overlaid with mineral oil (maximum of 12 drops per plate).

Note

It is important that B199+FBS and IVM dishes containing COCs do not stay out of the gassed incubators for so long that the pH changes (as indicated by colour change from red to pink).

2.6 Mature oocytes overnight at 38.5 °C in humidified 5% CO_2 with atmospheric O_2 concentration.

Collection of mature oocytes and zona removal

3 Collection of mature oocytes and zona removal

3.1 Move MII-arrested oocytes at 19 hours post-onset of maturation (hpm) out of IVM drops into a wash dish of H199+FBS using a pipette. Transfer maximally 200 oocytes in as little medium as possible into 500 µL [M] 1 mg/mL hyaluronidase (in H199) in a 1.5 ml tube.

3.2 Vortex COCs for 00:01:30 (2000 rpm - 2200 rpm) to disperse cumulus cells. Briefly centrifuge to collect oocytes and transfer back into H199+FBS. Rinse the inside of the hyaluronidase tube with H199+FBS to recover any remaining oocytes.

3.3 Quickly wash oocytes through a fresh dish of H199+FBS to remove hyaluronidase. Then transfer to a dish of H199+BSA.

3.4 Screen oocytes for the presence of a polar body (which in sheep is often flattened against the zona pellucida).
Using a finely pulled glass pipette, rotate oocytes until the polar body is observed. After all oocytes have been screened, re-check those that were initially without a polar body. If there is still no polar body present, discard.

Note

All following manipulations are performed using a finely pulled glass pipette attached to a mouth piece unless otherwise stated.

3.5 Remove the zona pellucida by placing the polar body containing oocytes into a 50 µL drop of pronase solution for 30 s – 2 min. Approximately 50 oocytes can be processed at a time, collecting misshaped oocytes with dissolving zonae first and exposing more pronase-resistant oocytes for longer.

Note

Sheep zonae tend to flatten to the bottom of the dish, making the oocytes harder to collect. Inexperienced users should start with smaller numbers until confident at lifting flattened oocytes off the bottom of the dish. Alternatively, flattened oocytes can be pushed to the edge of the pronase drop where surface tension lifts them from the base of the plate.

3.6 Wash oocytes in H199+BSA and allow 5–15 min for them to recover to a normal spherical shape before enucleation.

Note

Some zonae are more resistant to pronase and will not be fully removed with pronasing alone. In these situations:

- If there is a small break in the zona, the oocyte can be released with a pipette whose tip is slightly smaller than the diameter of an oocyte (~120 µm). Alternatively, suck the oocyte up and down the shank of a standard pulled glass pipette until it is released from the zona.
- If the zona has loosened but is still intact, pin it to the bottom of the dish with a glass pipette (works best if the tip is not fire polished) while gently applying some suction. This pulls the oocyte and tears the zona, making a hole and releasing the oocyte.

3.7 Transfer oocytes into H199+BSA with caffeine. Maintain the oocytes in media containing [M] 5 millimolar (mM) caffeine before activation. Activate around 26.5 hpm, so include caffeine from 20.5 to 26.5 hpm.

Note

The only steps that are carried out in media without caffeine are enucleation and fusion. In our experience, 20.5 hpm coincides with post-pronasing and recovery time.

3.8 Transfer zona-free oocytes in groups of 30 into drops in the 'oocyte plate' and hold them until enucleation.

Enucleation of zona-free oocytes

4 Enucleate zona-free oocytes

Note

Enucleation occurs between 20-22 hpm.

4.1 DNA stain oocytes in groups of ~40 for 00:05:00 in [M] 5 µg/mL Hoechst 33342 in H199+BSA+Caffeine ('stain plate').

4.2 Wash DNA-stained oocytes in H199+FBS and transfer into a H199+FBS droplet in the enucleation dish, covered by mineral oil, on the enucleation microscope stage (30 °C).

4.3 Observe the DNA-stained oocytes under the 20x objective, with the fluorescence lamp diaphragm reduced as much as possible. Locate the 'bump' on the oocyte surface overlying the metaphase plate under brightfield-transmitted light illumination, then confirm presence of the metaphase chromosomes by briefly exposing them to UV light.

4.4 Using micromanipulation, aspirate the metaphase chromosome-spindle complex into an enucleation needle (see materials section) with a minimal volume of cytoplasm. Aspirated material may include the first polar body, if still present. Separate the aspirated material (karyoplast) from the enucleated oocyte (cytoplast) by lightly tapping on the stage to vibrate the needle. Alternatively, the enucleation needle can be pushed past a separation needle (see materials section) to pinch off the karyoplast. Confirm aspiration of the chromosomes by brief UV exposure. As long as you can maintain suction control, proceed to enucleate the next oocyte without expelling the previous karyoplast.

4.5 Transfer batches of 40 cytoplasts into the 'cytoplast plate' (30 per drop) until all oocytes are enucleated.

Cell preparation and attachment

5 Cell preparation and attachment

> Note
>
> Cell preparation can start around 20 minutes before finishing enucleation.

5.1 Passage cells by washing with PBS, followed by trypsinisation. Inactivate the trypsin with warmed H199+0.5% FBS. Lift cells and centrifuge. After centrifugation, remove supernatant and resuspend at approximately 1×10^5 cells/mL in H199+0.5% FBS. Aliquot the cell suspension into 40 µL drops under oil.

5.2 Distribute a few hundred cells across four drops of the 'lectin plate' with [M] 20 µg/mL phytohemagglutinin (PHA-P). While cells settle to the bottom of the dish, transfer

approximately 80 cytoplasts into the remaining drops (10 cytoplasts per drop) and incubate in the lectin solution for a few minutes before cell attachment.

Note

Inexperienced users may start with 5 cytoplasts per drop, as you gain experience this can increase to 10-12 per drop. Note that moving the dish around will slowly bring the cytoplasts closer to together, resulting in unattended cytoplasts sticking to each other.

5.3 Attach a cytoplast by dropping it on top of a cell, rolling it a few times to increase adherence, and then re-depositing the couplet back into a drop devoid of donor cells. Incubate couplets in lectin for at least 5 minutes.

Note

All couplets should be kept separate to avoid them sticking together. If this happens, cytoplasts have to be manually separated, which often creates membrane protrusions that look like donor cells, complicating scoring of successful cell fusion later on.

If more than one cell sticks to a cytoplast, or two cytoplasts stick together, immediately pipette them up and down within the lectin drop until they separate. If they are attached more firmly (due to longer incubation in lectin), transfer the couplet to a wash dish or drop under oil of H199+BSA+Caffeine and pipette up and down until they separate. Strongly attached extra cells can be blown against the drop/oil interface to help knock them off.

5.4 After incubation in lectin, wash couplets through H199+BSA+Caffeine and then store in the 'couplet plate' with a maximum of 10 per drop to prevent cytoplast-cytoplast adhesion (couplets are still adherent after removal from lectin).

Cell electrofusion (SCT)

6 Cell electrofusion (SCT)

Note

The actual SCT step occurs by electrofusion at approximately 22-24 hpm

6.1 Prepare for electrofusion by attaching the chamber (see materials section) to a 90 mm dish with Blu-Tack and flooding it with Room temperature fusion buffer. To reduce evaporation, fusion is performed at room temperature, not on a microscope warm stage. Use a pipette to remove any bubbles within the chamber and electrodes. Connect the electrodes to the fusion machine (see materials section).

6.2 Remove batches of 20-40 couplets from drops under oil and wash through a dish of H199+BSA+Caffeine, then wash through two dishes of fusion buffer (see materials).

Note

It is critical to use fusion buffer without calcium ions to prevent premature cytoplast activation.

6.3 Transfer 5-20 couplets to the electrofusion chamber. Couplets are automatically aligned by applying an alternating current (AC) field (83 V/cm) for 5–10 s. If any couplets do not automatically align, remove them from the chamber. Apply 2 × 10 µs, 2 kV/cm direct current (DC) pulses to the automatically aligned couplets. Manually align remaining couplets and apply the same DC pulses.

6.4 Return couplets to fusion buffer dish until all have been exposed to the DC fusion pulse, then wash through H199+BSA+Caffeine. Place the couplets in the 'post-fusion plate' (10 couplets per drop) for scoring cell fusion 10–30 minutes post-DC pulses.

6.5 Wash fused reconstructs through HSOF-Ca^{2+}+10% FBS, and wash drops in 'ESOF-Ca plate', then transfer individual reconstructs to single 5 µL drops (to prevent adhesion between reconstructs). Incubate at 38.5 °C in a humidified 5% CO_2 incubator with atmospheric O_2 concentration.

Chemical activation

7 Chemical activation

Note

Chemical activation is carried out 3–4 hours post-fusion to prolong the donor nucleus exposure to the MII cytoplast environment.

7.1 Equilibrate the reconstructs by transferring them from their single drops in the ESOF-Ca plate to a dish of HSOF +1 mg/ml BSA at 5–30 minutes before activation.

7.2 To activate, collect all of the reconstructs with a pipette and deposit into a dish containing freshly prepared solution of [M] 5 micromolar (µM) ionomycin in HSOF+1 mg/mL BSA for 00:04:30, followed by a wash through HSOF+30 mg/ml BSA for at least 3 minutes.

> Note
>
> We recommended using the ionomycin solution within 10-30 minutes of thawing.

7.3 Transfer the reconstructs through series of three wash drops of 6-DMAP before placing individual reconstructs into single 5 µL drops of [M] 2 millimolar (mM) 6-DMAP ('DMAP plate'). Incubate for 3–4 hours at 38.5 °C in a humidified 5% CO_2 incubator with atmospheric O_2 concentration.

7.4 After 6-DMAP incubation, wash reconstructs thoroughly through two dishes of HSOF and culture as described below.

In vitro embryo culture

8 Embryo culture

> Note
>
> The following embryo culture incubation steps are at 7% O_2 in modular incubation chambers with 5% CO_2 in N_2. (Previous incubation was at atmospheric O_2 concentrations.)

8.1 Following HSOF washes, transfer the zona-free embryos to the 'ESOF plate'. Wash embryos serially through two 40 µL wash-drops. Single embryos are then placed in manually prepared micro-wells (dimples) to prevent the embryos from aggregating. Embryos are cultured in groups of 10 in 20 µL and stored in 7% O_2 in modular incubation chambers.

Note

Micro-wells are made by using a blunt needle to push dimples in the plastic in the base of the Petri dish after drops have been made. Sterilise the blunt needle first with 70% ethanol and rinse in HSOF.

Embryos can be successfully cultured in groups of 8-12, if necessary.

8.2 On day two of culture, refresh half of the medium (10 µL) with warm, gassed ESOF to reduce the accumulation of ammonium and return to 7% O_2 in modular incubation chamber.

8.3 On day four of culture, replace three quarters of the medium (15 µL) with warm, gassed LSOF and return to 7% O_2 in modular incubation chamber.

8.4 On day five of culture, remove the embryos from the micro-wells and place them into single 5 µL drops of LSOF and return to 7% O_2 in modular incubation chamber.

If aggregating embryos on day five, select all morulae (pre-compacting, compacting and compact) and go to step 9.

Note

Micro-well culture ends at this time point. As embryos develop into blastocysts, they expand to fill the micro-well and tightly adhere to its sides, so they can be easily damaged upon removal.

8.5 Embryo culture ends on day six or seven before the blastocysts collapse and start degenerating.

Embryo quality is morphologically graded using modified International Embryo Transfer Society (IETS) guidelines. Briefly, grade 1 embryos are excellent/good and have greater than 85% of their cellular material intact, and a large, regular inner cell mass (ICM); grade 2 are fair with some moderate irregularities but still have a robust ICM; grade 3 are poor, with major irregularities and/or little ICM.

CITATION

Robertson I, Nelson RE (1998). Certification and identification of the embryo. In: Manual of the International Embryo Transfer Society. 3rd edition.

Morula aggregation

9 Morula aggregation

9.1 Select competent morulae with little fragmentation on day five of embryo culture. Compact morulae are the preferred stage for aggregation, but pre-compacting and compacting morulae can also develop into good quality aggregate blastocysts, albeit at lower efficiency.

Note

Compact morulae often adhere to the micro-well surface, which helps in their identification during transfer into LSOF droplets. In some instances, morulae may also be found and aggregated on days four and six of culture.

9.2 Pair morulae of similar developmental stage and morphological grade for aggregation.

9.3 Incubate paired morulae in disaggregation media (see materials section) for 00:02:00 , wash in HSOF, and transfer to 5 µL drops of LSOF containing one micro-well.

9.4 Gently disaggregate the embryos by blowing up and down with a pipette smaller than the embryo (inner diameter: 50-70 µm) until they break into 2-3 fragments.

9.5 Re-aggregate the embryo fragments with a pipette approximately the diameter of the reconstituted embryo and place into a micro-well. Aggregated embryos are then incubated in the modular incubation chamber for 5–6 hours.

9.6 Remove each aggregated embryo from the micro-well after 5–6 hours, before blastulation occurs. Within this time, the pieces usually form a single compacted morula.

Each individual aggregated embryo is then placed into a single LSOF drop for culture until day six or seven at 7% O_2 in a modular incubation chamber. Grade blastocysts as in 8.5.

Note

Blastocysts are removed at this stage since the trophoblast adheres tightly to the edges of the micro-well and often becomes damaged upon removal.

Cryopreservation by vitrification

10 Cryopreserve by vitrification

10.1 Cryopreserve blastocysts using the CryoLogic Vitrification Method (CryoLogic, Australia), following an adapted protocol from our lab.

CITATION

Wei J, Wagner S, Maclean P, Brophy B, Cole S, Smolenski G, Carlson D, Fahrenkrug S, Wells D, Laible G (2018). Cattle with a precise, zygote-mediated deletion safely eliminate the major milk allergen beta-lactoglobulin. Sci Rep.
LINK
https://doi.org/10.1038/s41598-018-25654-8

10.2 Wash blastocysts twice through base medium (Embryo-hold (no BSA)+20% FBS), then incubate in a solution comprised of 7.5% (v/v) each of ethylene glycol and DMSO in base medium for 00:02:00 .

10.3 Transfer the embryo to a solution of base medium with the addition of [M] 0.1 millimolar (mM) Ficoll 70, [M] 1 Molar (M) sucrose and 15% (v/v) each of ethylene glycol and DMSO for 00:00:30 for equilibration. Collect embryos in 2.5 µl and carefully deposit as a single droplet on the nylon hook of a CryoLogic fibreplug.

Note

Embryos can be vitrified in groups of 1-4 per hook.

10.4 Place the hook containing the droplet of embryos onto the surface of a stainless-steel cooling block (which has been equilibrated in a bath of liquid N_2 for 15–20 seconds. Vitrification should be carried out within 60 seconds from the first exposure to the final vitrification solution, usually occurring after around 45 seconds.

10.5 Sheath the fibreplug in a cooled plastic cover and store in liquid N_2 until day of embryo transfer.

10.6 On the morning of embryo transfer, warm the blastocysts by submerging the vitrified droplet into a [M] 0.27 Molar (M) sucrose solution in base medium for 00:02:00, transfer and incubate in [M] 0.16 Molar (M) sucrose solution in base medium for 00:03:00, followed by two incubations in the base medium for 00:02:30.

10.7 Leave the blastocysts to recover in standard Embryo-hold medium (with BSA), re-grade morphology according to IETS guidelines above and transfer within 2 hours.

Citations

Step 10.1

Wei J, Wagner S, Maclean P, Brophy B, Cole S, Smolenski G, Carlson D, Fahrenkrug S, Wells D, Laible G. Cattle with a precise, zygote-mediated deletion safely eliminate the major milk allergen beta-lactoglobulin **https://doi.org/10.1038/s41598-018-25654-8**

Step 8.5

Robertson I, Nelson RE. Certification and identification of the embryo