

Nuclear Transfer in Sheep

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Summary

Somatic cell nuclear transfer is a complex and intricate procedure with a low success rate. Despite advances in embryo culture and the production of specialized tools and equipment success rate has remained poor. The procedure remains basically the same despite technical innovations but is now easier to learn and requires less technical expertise. Oocytes now come from an in vitro system, which may make the procedure more economical and flexible than before.

Oocytes are produced from ovaries collected at an abattoir. The cumulus oocyte complexes (COCs) are recovered from the ovary with hypodermic needle and syringe. Selected COCs are matured for 18 to 20 h in medium that is supplemented with hormones. Cumulus cells are stripped from the oocytes using hyaluronidase, and mature oocytes with polar bodies, selected for enucleation. These oocytes are treated with a cytoskeletal inhibitor to prevent lysis of the oocyte during enucleation and with a DNA-specific dye to visualize the chromosomes with the aid of ultraviolet light. These permit removal of the metaphase II chromosomes and polar body prior to reconstruction of the embryo. A diploid cell is injected under the zona pellucida of the enucleated oocyte, which can then be fused to the cytoplasm using an electrical pulse. The fused embryos are activated and allowed to develop in culture to the morula or blastocyst stage, when they are surgically implanted into previously prepared synchronized, recipient animals.

Key Words: Micromanipulation; in vitro maturation; recipient animals; enucleation; electrofusion; embryo culture; embryo transfer.

1. Introduction

Nuclear transfer (NT) is an inefficient method of replicating animals. Originally, cleavage-stage embryos were used, usually before compaction of the blastomeres, and this meant that it was only possible to produce small numbers of identical animals (1). This changed in 1995, when animals were produced from cells that had been grown in culture (2), greatly simplifying the production of identical animals. These experiments were conducted on sheep oocytes

From: *Methods in Molecular Biology*, vol. 325: *Nuclear Reprogramming: Methods and Protocols*
Edited by: S. Pells © Humana Press Inc., Totowa, NJ

because they were relatively cheap to use and because efficient methods of handling the animals existed at the Roslin Institute. The cells used as nuclear donors were cultured from the inner cell mass (ICM) of sheep embryos. The following year, similar experiments were performed using three different cell types: ICM cells, fetal cells, and cells from the udder of a 6-yr-old adult animal. The three cell lines produced four lambs, two lambs, and one lamb, respectively; the animal produced from the udder cells was “Dolly” (3).

Current methods of NT have not changed to any great extent, but the process has become easier as a result of the development of specific sheep culture medium (4) and the production of specialized tools and equipment. NT is still inefficient with somatic cell nuclear transfer (SCNT) efficiency running at 1–5%, but it is much easier because of the development of new technology (5,6).

NT consists of the removal of the maternal chromosomes and the replacement of these chromosomes with those from a diploid cell. The oocytes are usually used at the metaphase II stage; they have their maternal deoxyribonucleic acid (DNA) removed using cytoskeletal inhibitors and a DNA-specific dye. A cell is placed in the perivitelline space and the cell is then fused to the cytoplasm using an electric current. The reconstructed embryo is then activated and allowed to develop before the transfer into a surrogate mother (7).

2. Materials and Equipment

2.1. Media

1. Synthetic oviduct fluid + amino acids + bovine serum albumin (SOFaaBSA; *see Table 1 [4]*).
2. Calcium-free synthetic oviduct fluid + amino acids + bovine serum albumin (SOFaaBSA-Ca), which is the same as SOFaaBSA except for the omission of CaCl_2 .
3. Hepes-buffered synthetic oviduct fluid (HSOF; *see Table 2*).
4. Hepes-buffered calcium-free synthetic oviduct fluid. (HSOF-Ca), which is the same as HSOF except for the omission of CaCl_2 .
5. Hepes-buffered synthetic oviduct fluid + 1% fetal calf serum (HSOF/FCS).
5. Phosphate-buffered saline + 1% fetal calf serum (PBS + 1% FCS).
6. Fusion medium: 0.3 M manitol, 0.1 mM MgSO_4 , and 0.05 mM CaCl_2 in double-distilled water (dd water). Osmolarity adjusted to 280 mosmoles with dd water.
7. Cell culture medium: Dulbecco's modified essential medium (DMEM) + 10% FCS.
8. Serum deprivation medium: DMEM + 0.5% FCS.
9. Oocyte wash medium (*see Table 3*).
10. Maturation medium (*see Table 4*).

2.2. Additional Reagents

1. Hyaluronidase stock. Aliquots of 300 U in 20 μL of dd water.

Table 1
SOFAaBSA Composition

Component	Sigma cat. no.	g (or mL)/1000 mL
NaCl	S-5886	6.29
KCl	P-5405	0.534
KH ₂ PO ₄	P-5655	0.162
MgSO ₄ · 7H ₂ O	M-1880	0.182
Sodium lactate	L-7900	0.6 mL
Penicillin	P-4687	0.06
NaHCO ₃	S-5761	2.1
Phenol red	P-5530	0.01
Na pyruvate	P-4562	0.0357
CaCl ₂ · 2H ₂ O	C-7902	0.262
L-Glutamine	G-5763	0.3
β-Mercaptoethanol	B-6766	20 mL
MEM Nonessential amino acid solution	M-7145	10 mL
Bovine serum albumin	A-6003	4

Table 2
HSOF Composition

Component	Sigma cat. no.	g (or mL)/1000 mL
NaCl	S-5886	6.29
KCl	P-5405	0.534
KH ₂ PO ₄	P-5655	0.162
MgSO ₄ · 7H ₂ O	M-1880	0.182
Sodium lactate	L-7900	0.6 mL
Penicillin	P-4687	0.06
NaHCO ₃	S-5761	0.42
Phenol Red	P-5530	0.01
Na pyruvate	P-4562	0.0357
CaCl ₂ · 2H ₂ O	C-7902	0.262
HEPES	H-3784	5.208
BSA	A-6003	4

2. Cytochalasin B stock. Aliquots of 7.5 µg in 10 µL of dimethyl sulfoxide.
3. Bisbenzimidazole (Hoechst 33342) stock; 5 µg in 1 mL of dd water.
4. Petroleum jelly.
5. Sigmacote.
6. Mannitol.

Table 3
Oocyte Wash Medium

Component	Sigma cat. no.	g (mL or U)/1000 mL
TCM199 ^a	M-0650	100 mL
NaHCO ₃	S-5761	0.400
HEPES	H-6147	2.98
Heparin	H-3149	2500 U
Sheep serum	Prepared from our animals	20 mL

^aTissue Culture Medium 199.

Table 4
Maturation Medium

Component	Sigma cat. no.	g (or mL)/1000 mL
TCM199 ^a	M-0650	100 mL
NaH CO ₃	S-5761	2.100
FSH (follicle-stimulating hormone)	Ovagen ^b	0.005
LH (ovine leutinizing hormone)	L-5269	0.005
Estradiol 17-β	E-1024	0.001
Sheep serum	Collected from our animals	200 mL

^aTissue Culture Medium 199.

^bOvagen; Ovine Pituitary Extract, UK Distributor: David Maharg, Wiltshire, England.

- 7. Dimethyl sulfoxide.
- 8. Veramix sponges from Upjohn Ltd., for the synchronization of ewes.

2.3. Equipment

2.3.1. Microscope and Manipulators (see Fig. 1)

A Nikon TE300 inverted microscope fitted with differential interference contrast (DIC) optics and epiflourescence capability is used. This is used with 4× phase contrast, 10× phase contrast, 20× DIC, and 40× DIC lenses (*see Note 1*). The microscope should be fitted with two Nikon Narishige MO-188 “Joy-stick Hydraulic Micromanipulators” and two IM-188 Microinjectors. The volume per turn of the injector can be altered by replacing the 3-mL syringe fitted with a 250-μL pipet for the enucleation pipet and a 500-μL pipet for the holding pipet.

A three-way tap (Vigon VG1) in the hydraulic line between the microinjector and the tubing allows the system to be filled using a suitable syringe thus over-



Fig. 1. Equipment used for NT: inverted microscope, two micromanipulators, and two microinjectors.

coming any need to take the equipment apart for filling (*see* **Notes 2** and **3**). Embryos are handled using Leica M7.5 and M12.5 microscopes fitted with transmitted light stands and Linkam CO102 temperature controllers and warm stages.

2.3.2. Miscellaneous Equipment

1. Siliconized glass slides.
2. Glass strips approx $20 \times 3 \times 2$ mm.
3. Glass holding pipet.
4. Glass enucleation pipet (18 μ m, Humagen).
5. Gilson or similar micropipettors with 10- μ L, 20- μ L, 100- μ L, 200- μ L, and 1000- μ L capacities.
6. A BLS CF 150/B cell fusion machine is used for electrofusion of the cell couplets. The fusion machine is connected to a BLS fusion chamber that can be placed in a sterile 100-mm disposable Petri dish for fusion.

2.4. Isolation and In Vitro Maturation of Oocytes

1. Collect ovaries from the slaughterhouse in warm PBS in a vacuum flask to maintain their temperature.
2. Wash the ovaries in clean PBS and return to the flask to maintain their temperature.
3. Take a small quantity of ovaries from the flask and replace the lid to maintain the temperature.

4. Using an 18-gage hypodermic needle and a 10-mL syringe, aspirate the follicles on the ovaries to recover the cumulus oocyte complexes (COCs).
5. Insert the needle into the ovary and push the point into the follicles, at the same time maintaining gentle suction on the plunger of the syringe (*see Note 4*).
6. Once an appropriate volume of follicular fluid has been extracted and collected in the syringe remove the needle and gently expel this into tubes containing warm oocyte wash medium (*see Table 3* and *Note 5*).
7. Pour the fluid into a 90-mm Petri dish that has been previously marked with a grid pattern to assist in searching for the COCs.
8. If necessary, add more oocyte wash, and select the COCs that have at least three layers of cumulus cells (*see Note 6*).
9. Wash the COCs three times in oocyte wash medium and once in maturation medium (*see Table 4*) before placing 40 COCs in 800 μ L of prewarmed and gassed maturation medium in Nunc 4-well plates.
10. Cover the medium with oil and mature for 18 to 20 h at 38.5°C in a humidified atmosphere of 5% CO₂ in air (8).

3. Method

3.1. Prepare the Recipient Animals

Recipient animals: prepare in advance because the procedure takes several days to complete.

1. Select ewes with reasonable body condition (condition score >3) and are sound in mouth and udder.
2. At day 0, insert a 60-mg medroxyprogesterone acetate sponge (Veramix /Upjohn) into the vagina of each ewe.
3. Remove the sponge on day 13.
4. Check for estrus between days 14 and 16 using a vasectomized ram.
5. NT (*see Subheading 3.2.* onwards) will take place on day 15, which is heat day of the recipient.
6. Starve the animal on the afternoon of day 21.
7. Conduct embryo transfer on the morning of day 22.

3.2. Media Preparation

On the day of the procedure, make up the following culture media volumes/dishes, i.e., SOFaaBSA culture medium dishes for enucleation, fusion, and culture, and place in the incubator (37°C, 5% CO₂ in air) to gas.

1. Enucleation medium dish: prepare 25- μ L drops of SOFaaBSA-Ca under oil in a 60-mm culture dish.
2. Fusion medium dish: to 1 mL of SOFaaBSA, add 4.5 μ L of Cytochalasin B stock. Prepare 25- μ L drops of SOFaaBSA under oil in a 60-mm culture dish.
3. Culture medium dish: place 800 μ L of SOFaaBSA in a 4-well Nunc culture plate.

4. HSOF culture medium for the removal of residual cumulus cells (Hyaluronidase treatment: *see Subheading 3.4., steps 1–3*), washing, micromanipulation, and Hoechst staining (These medium dishes should be warmed in a temperature-controlled “Hot box” set at 37°C).
5. Add 5 μ L of hyaluronidase stock to 500 μ L of HSOF–Ca for hyaluronidase treatment.
6. Washes: add 500 μ L of fetal calf serum (FCS) to 4.5 mL of HSOF–Ca.
7. Micromanipulation medium: to 1 mL of HSOF–Ca/FCS (*see Subheading 2.1. and Table 2*), add 4.5 μ L of Cytochalasin B stock.
8. Hoechst/CB medium dish: take 500 μ L of HSOF–Ca/FCS + CB from **step 8** and add 5 μ L of Hoechst stock.

3.3. Manipulation Chamber Preparation (*see Fig. 2*)

1. Clean a siliconized glass slide with a paper towel and 70% alcohol.
2. Apply a small quantity of petroleum jelly along both sides of the slide for a distance corresponding to the length of the glass strips.
3. Using sterile technique place a glass strip on each of the areas with the petroleum jelly.
4. Apply another small quantity of petroleum jelly along the top of the glass strips.
5. Using a pipettor, place 300 μ L of the micromanipulation medium in the middle of the glass on the slide.
6. Place a clean sterile cover slip on the top of the chamber and push down to make sure that a liquid-tight seal has been made.
7. Fill each end of the chamber with mineral oil or Dow Corning Silicone Fluid.
8. Mount the slide on the microscope.
9. Mount a holding pipet on the left manipulator arm of the micromanipulator workstation. Fluorinert is used as a hydraulic fluid in this system (*see Note 7*).
10. Flush all of the air from the system and push the end of the pipet into the chamber (*see Note 8*).
11. Suck back slightly to put a little medium into the pipet.
12. Mount an enucleation pipet in the right manipulator arm of the micromanipulator workstation. Fluorinert is used as a hydraulic fluid in this system.
13. Remove the pipet from its packaging using sterile technique and mount in the tool holder.
14. Expel all of the air from the microinjection system.
15. Insert the pipet into the manipulation chamber.
16. Rotate the pipet until the bevel of the pipet is seen, and tighten the tool holder in this position (*see Notes 9 and 10*).

3.4. Preparation of the Oocytes

1. Remove any residual cumulus cells from the oocytes by culturing in the hyaluronidase treatment medium (*see Subheading 3.2., step 6*) for up to 10 min in the hot box until the cumulus cells begin to loosen.
2. Pipet repeatedly with an automatic pipettor to remove the cumulus cells (*see Note 11*).

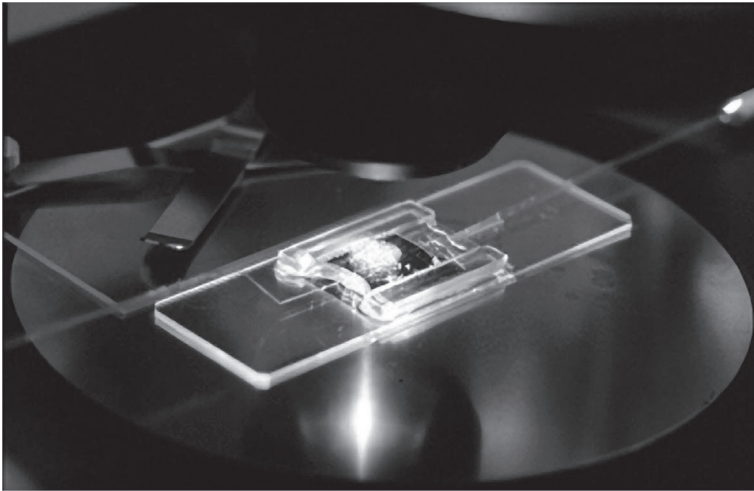


Fig. 2. Micromanipulation chamber with the holding pipet entering the chamber from the left side and the enucleation pipet entering from the right side.

3. Wash several times in wash medium to leave the cumulus cells behind.
4. Examine the oocytes under a dissecting microscope while turning the oocytes over to select mature oocytes with polar bodies.
5. Lift these oocytes out of the dish and store in the enucleation medium dish until required.
6. Take a manageable group of oocytes and place in the Hoechst/CB dish and culture for 15 min in the hot box.

3.5. Enucleation (see Fig. 3)

1. Start the mercury vapor lamp on the microscope.
2. Remove the group of oocytes from the Hoechst + CB dish and place in the manipulation chamber.
3. Lift an oocyte from the group and find an area in the chamber in which there are no other oocytes visible.
4. Using the enucleation pipet, turn the oocyte until the polar body is at 1-o'clock or 5-o'clock position, depending on whether the bevel of the pipet is facing up or down.
5. Insert the pipet through the zona pellucida (ZP), taking care to avoid damaging the cytoplasm of the oocyte.
6. Aspirate the polar body and the adjoining cytoplasm into the pipet.
7. Remove the holding pipet with the oocyte out of the field of view.
8. Expose the enucleation pipet to the ultraviolet light. If low light levels are used on the microscope, it is not necessary to turn the light off. The polar body will glow brightly, and the metaphase chromosomes less brightly.
9. Turn off the ultraviolet light.

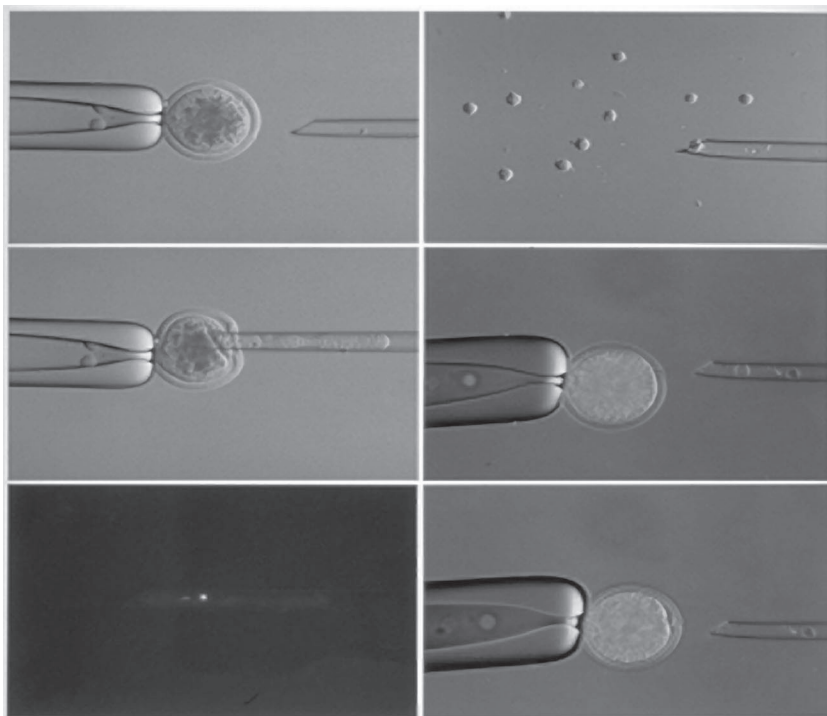


Fig. 3. Left column: Enucleation of oocyte; removal of the polar body and surrounding cytoplasm; checking to see that the polar body and metaphase chromosomes are fluorescing in the pipet. Right column: cell transfer; picking up cells; transfer of a cell to the PV space of an enucleated oocyte.

10. If the metaphase chromosomes and polar body cannot be seen, the process of checking enucleation can be repeated with the light turned off.
11. Repeat the enucleation process if the metaphase chromosomes are not present in the pipet.
12. Deposit the successfully enucleated oocytes in a group at the right side of the manipulation chamber.
13. After enucleation of each group, return them to culture in the “Enucleation” dish.
14. Repeat with suitably sized groups until complete.

3.6. Preparation of Nuclear Donor Cells

1. Use suitable cells for reconstruction of the couplet. These cells may be of many different lines, and these may require different strategies to make them suitable for supplying the donor nucleus.
2. Thaw the cells and culture in DMEM with serum.
3. Serum-deprive the cells (*see Note 12 [9]*).

4. Create a single-cell suspension by Trypsin treatment and resuspension in DMEM.
5. Store the trypsinized cells in the appropriate conditions for the cell type being used.

3.7. Cell Transfer

1. Prepare the manipulation chamber as before (*see Subheading 3.3.*) but with HSOF only.
2. Deposit a small number of cells in the top right corner of the chamber.
3. Deposit a small number of enucleated oocytes in the centre of the chamber.
4. Use an appropriately sized pipet on the right tool holder to pick up cells without damage. An 18- μm pipet is suitable for most cell types (*see Note 13*).
5. Pick up an enucleated oocyte with the holding pipet.
6. Push the pipet through the ZP at a position opposite the holding pipet.
7. Deposit a single cell in the perivitelline (PV) space (*see Note 14*).
8. Make sure that the cell is in good contact with the cytoplasm.
9. Repeat the process for all of the enucleated oocytes.

3.8. Electrofusion

1. Immediately after cell transfer, conduct the electrofusion (*see Note 15*).
2. Wash the couplets in fusion buffer to remove excess ions, making sure that the minimum amount of medium is transferred with the couplets (*see Note 16*).
3. Move the couplets individually between the electrodes and align the couplets either manually or using an AC pulse of electricity.
4. Give a fusion pulse of 0.25 kV/cm AC for a few seconds followed by three pulses of 1.25 kV/cm DC for 80 μs .
5. Immediately lift the pulsed couplet out of the fusion buffer and place in HSOF to prevent lysis.
6. Repeat the procedure, taking care not to introduce ions into the fusion chamber, until all of the couplets have been pulsed.
7. Transfer the fusing couplets into SOFaaBSA and culture for 1 h in a 5% CO_2 incubator.
8. Check for fusion of the couplets after 1 h.
9. Repeat the fusion procedure on unfused couplets if appropriate.

3.9. Culture

1. Culture the fused oocyte-cell couplets in SOFaaBSA in Nunc 4-well plates overlaid with mineral oil in a humidified 5% CO_2 : 5% O_2 : 90% N_2 gas atmosphere.
2. On day 7 of culture, select those embryos that have developed normally for transfer to surrogate recipient animals.

3.10. Embryo Transfer

1. Anesthetize the animal using a short-acting barbiturate.
2. Intubate the animal and maintain anesthesia using a mixture of Halothane, nitrous oxide, and oxygen.
3. Conduct a mid-ventral laparotomy to expose the uterus.

4. Using a blunt needle (16 gage \times 1 in.) puncture the uterus near the utero-tubal junction.
5. Using a positive displacement pipet (Drummond 20- μ L pipet) transfer the NT blastocysts through the hole made by the blunt needle (*see Note 17*).

4. Notes

1. It is easier to arrange the lenses in the nosepiece so that those which are used most often are adjacent to each other. This prevents having to move the nosepiece past lenses which are not in use. I keep the 4 \times and 20 \times lenses adjacent to each other as these are the most frequently used lenses for this technique.
2. I control the holding pipet mounted on the left of the chamber with my right hand and the enucleation pipet mounted on the right side of the chamber with my left hand. This allows both the joystick and injector on one side to be used simultaneously.
3. Make sure that there are no air bubbles in the tubing because this can prevent the pipet from working smoothly. Any trapped air can be compressed like a spring, making it difficult to control the system. The metal pipet holder can also obscure air bubbles so flush a little of the hydraulic fluid through the system to prevent this from happening.
4. Take care when collecting oocytes from the ovaries, because the follicles are small. It is advisable to insert the needle a few millimeters from the follicle so that the bevel of the needle is completely covered, thus preventing air from entering the syringe.
5. Always remove the needle from the syringe before emptying the syringe of follicular fluid as squeezing the COCs through the needle at high pressures can remove some of the cumulus cells.
6. When selecting COCs, discard those that have too few cumulus cells, which appear to have very patchy cytoplasm, or which show other abnormalities.
7. Prepare the holding pipet by pulling a GC10 glass capillary over a small gas flame to make a long parallel length of glass of approx 150 mm. Bend the glass in a micro forge four times to make the pipet fit under the cover slip of the manipulation chamber. Fire polish the end of the pipet until it is approx 20- μ m wide.
8. When setting up the microscope for manipulation use the 4 \times lens to set up the glass microtools, then use the 20 \times lens for micromanipulation.
9. Successful micromanipulation relies on control of the glass micropipet. Keep the hydraulic fluid near the end of the pipet, so that the viscosity of the fluid and the narrow diameter of the pipet give friction which helps to control the suction.
10. If there is not sufficient control over suction in the enucleation pipet, pick up some drops of oil. The oil increases the friction in the pipet and so increases control of suction.
11. When stripping cumulus cells from the oocytes using a pipettor, keep the pipettor at an angle so that the oocytes are not squeezed against the bottom of the dish.
12. Deprive the cells of serum until they enter G0. The time for this to occur is variable, depending on the cell line used. It must therefore be determined empirically for each new cell line. The status of the cells can be determined by using proliferating cell nuclear antigen (i.e., PCNA) staining.

13. Select bright cells without vacuoles and even cytoplasm. Several cells can be aspirated into the pipet, leaving a small gap between each of the cells.
14. When transferring cells into the PV space, make sure that there is not excess pressure that can force the cell out of the hole. This can be prevented by withdrawing the pipet slowly, allowing any pressure to dissipate.
15. Change the fusion buffer frequently or flood the fusion chamber with fusion buffer so that the fusion buffer does not change osmolarity.
16. Wash reconstructed couplets prior to electrofusion to prevent ions being carried into the fusion buffer and causing lysis. Make sure that no medium is transferred into the fusion chamber as this can cause lysis of the couplets.
17. When transferring NT embryos using a positive displacement pipet, suck up a small quantity of air followed by the embryos in a small amount of medium and then more air in the end of the pipet. The first air bubble prevents the embryos from sticking to the piston, and the air bubble at the end prevents the embryos from coming out when the end of the pipet touches the uterus of the animal.

Acknowledgments

With thanks to all of my colleagues past and present at the Roslin Institute, also for all of the financial support from The Roslin Institute, MAFF, DEFRA, EC, and others without whom none of this work could have taken place. My thanks especially go to Professor Ian Wilmut for the facilities to carry out the work, to Dr. Jane Taylor for expert help, and Marjorie Ritchie for critical reading and checking of the manuscript.

References

1. Willadsen, S. M. (1986) Nuclear transplantation in sheep embryos. *Nature* **320**, 63–65.
2. Campbell, K. H. S., McWhir, J., Ritchie, W. A., and Wilmut, I. (1996) Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380**, 64–66.
3. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., and Campbell, K. H. S. (1997) Viable offspring derived from foetal and adult mammalian cells. *Nature* **385**, 810–813.
4. Walker, S. K., Hill, J. L., Kleemann, D. O., and Nancarrow, C. D. (1996) Development of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations. *Biol. Reprod.* **55**, 703–708.
5. Denning, C., Burl, S., Ainslie, A., Bracken, J., Dinnyes, A., Fletcher, J., et al. (2001) Deletion of the alpha (1,3) galactosyl transferase (G. G.T. A.1) gene and the prion protein (PrP) gene in sheep. *Nat. Biotechnol.* **19**, 559–562.
6. Schnieke, A., Kind, A. J., Ritchie, W. A., Mycock, K., Scott, A. R., Ritchie, M., et al. (1997) Human factor I. X. transgenic sheep produced by transfer of nuclei from transfected foetal fibroblasts. *Science* **278**, 2130–2133.
7. Young, L. E., Sinclair, K. D., and Wilmut, I. (1998) Large offspring syndrome in cattle and sheep. *Rev. Reprod.* **3**, 155–163.

8. Wells D. N., Misica P. M., Day A. M., and Tervit, H. M. (1997) Production of cloned lambs from an established embryonic cell line: a comparison between in vitro and in vivo matured cytoplasts. *Biol. Reprod.* **57**, 385–393.
9. Campbell K. H. S., Loi P., Otaegui P. J., and Wilmut, I. (1996) Cell cycle co-ordination in embryo cloning by nuclear transfer. *Rev. Reprod.* **1**, 40–46.



<http://www.springer.com/978-1-58829-379-4>

Nuclear Reprogramming

Methods and Protocols

Pells, S. (Ed.)

2006, XIV, 334 p., Hardcover

ISBN: 978-1-58829-379-4

A product of Humana Press