IETS Committee on Companion Animals, Non-Domestic & Endangered Species (CANDES)

INTERNATIONAL EMBRYO TECHNOLOGY SOCIETY

Research Subcommittee Resource Manual

http://www.iets.org/comm_candes.asp

A Compilation of Standard Operating Procedures for Embryo Transfer and Related Technologies for CANDES

Updated June 2011

To submit new protocols, contact the relevant Taxon Leader or Subcommittee Chairmen
(see p. 4 for contact information)

TABLE OF CONTENTS

IETS CANDES Research Subcommittee

E-Mail Addresses of Resource Manual Taxon Leaders

Taxon	Taxon Leader	E-Mail Address
Carnivores	Jason Herrick	jherrick@illinois.edu
Ungulates	Rebecca L. Krisher	rkrisher@fertilityresearch.org
Birds	Juan Manual Blanco	uhofleh@wanadoo.es
Amphibians/Reptiles	Andrew Kouba	akouba@memphiszoo.org
Non-Human Primates	Justine K. O'Brien	justineo@vetsci.usyd.edu.au
Invertebrates	Anita Collins	collinsa@ba.ars.usda.gov
Marsupials	Monique C.J. Paris	m.paris@uu.nl
Fish	Boris Dzyuba	bdzuba@yahoo.com
Marine Mammals	Todd Robeck	todd.robeck@seaworld.com
Rodents	Marina F. Ponzio	mponzio@mater.fcm.unc.edu.ar
Other Taxa	*Suggestions Requested*	

The IETS CANDES Research Subcommittee Resource Manual includes standard operating procedures (defined here as protocols) referring to all embryo transfer and related technologies applicable to companion animals, non-domestic and endangered species.

New information and updates will be posted in March and September annually. Authors may send copies of their protocols for posting on the IETS CANDES web page to the relevant Taxon Leader above. Each new submission must contain contact details (preferably an e-mail address) for queries concerning the protocols and they must include citations of supporting manuscripts and/or data demonstrating application of the procedures. Protocols are specifically requested for the following topics:

1. Gamete/Embryo Collection
2. Embryo Transfer
3. Artificial Insemination
4. In Vitro Fertilization/Culture
5. Cryopreservation
6. Endocrinology

Taxon Leaders are needed at this time for "Birds" and "Marine Mammals" and requests are being accepted for other taxa to be included in the Resource Manual. Queries and requests should be sent to the IETS CANDES Research Subcommittee Co-Chairmen: Rebecca L. Krisher or Monique C. J. Paris. Taxon Leaders should have some background knowledge or experience in embryo transfer and related technologies for their specific taxon, and are responsible for recruiting participation from colleagues to submit relevant protocols.

Large Felid Semen Collection by Rectal Probe Electrostimulation (Electroejaculation)

N.M. Loskutoff
Center for Conservation & Research, Omaha's Henry Doorly Zoo
September 2003

Publications using this protocol:

1) Crichton, E.G., E. Bedows, A.K. Miller-Lindhom, D.M. Baldwin, D.L. Armstrong, L.H. Graham, J. Ford, J.O. Gjorret, P. Hyttel, C.E. Pope, G. Vajta and N.M. Loskutoff (2003) The efficacy of porcine gonadotropins for repeated stimulation of ovarian activity for oocyte retrieval and in vitro embryo production and cryopreservation in Siberian tigers (*Panthera tigris altaica*). Biology of Reproduction 68: 105-113.
2) Gjorret, J.O., Crichton, E.G., Loskutoff, N.M., Armstrong, D.L. and Hyttel, P. Oocyte maturation, fertilization and early embryonic development in vitro in the Siberian tiger (*Panthera tigris altaica*). Molecular Reproduction and Development 63:79-88, 2002.
3) Nelson, K.L., E.G. Crichton, L. Doty, D. Volenec, J.M. Finnegan, R.G. Morato, C.E. Pope, B.L. Dresser, D.L. Armstrong and N.M. Loskutoff (1999) Heterologous and homologous fertilizing capacity of cryopreserved felid sperm: a model for endangered species. Theriogenology 51:290, abst.
4) Donoghue, A., A. Byers, L. Johnston, D. Armstrong, and D. Wildt. Timing of ovulation after gonadotrophin induction and its importance to successful intrauterine insemination in the tiger (*Panthera tigris*). Journal of Reproduction and Fertility, 107: pp 53-58. 1996

Materials Needed:

Rectal probe (see Fig. 1), sand paper
Electrostimulator (e.g., Platz or Beltz models – for contact details, see Technology Subcommittee Resources section)
Electrical extension cord
Lubricant (non-spermicidal)
Latex gloves
Paper towels
15 ml conical tubes (sterile)
Insulated container
Medium (e.g., TL Hepes, Bio Whittaker)
Permanent ink pen
Warming packs (if necessary)
Disinfectant

Procedure

1. Gently sand down the electrodes to ensure proper contact. ALWAYS turn the electrostimulator "ON" BEFORE inserting the rectal probe to prevent shock due to electrical surges. At that time, check the gauges to be sure the electrostimulator is functioning properly.

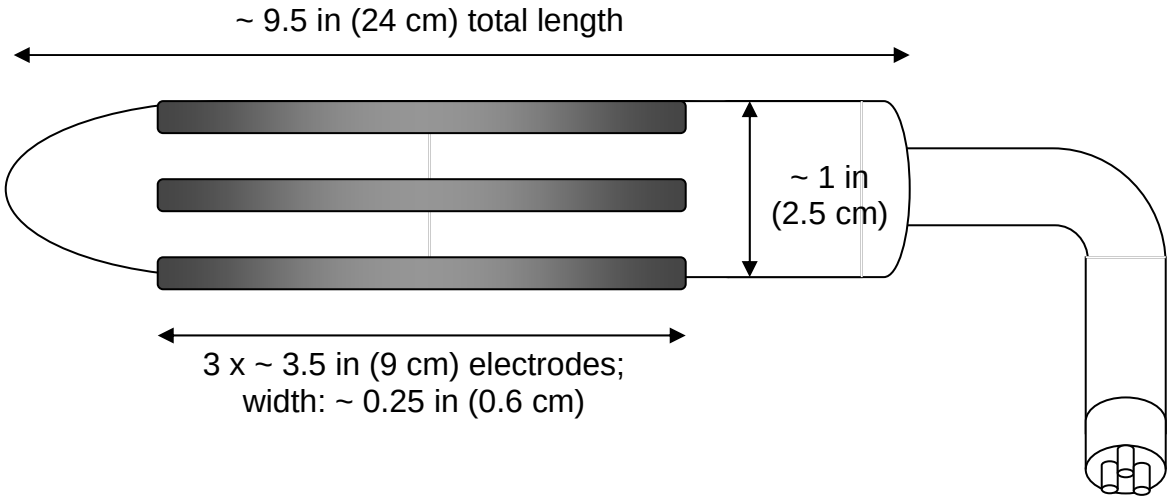


Figure 1. Diagram of rectal probe suitable for semen collection by electroejaculation from large felids (e.g., tigers and lions).

2. After the animal is chemically immobilized* and the keeper and veterinary personnel allow entrance into the animal's holding area, place lubricant on the tip of the rectal probe and gently insert it into the animal's rectum using a twisting motion. Do not force the probe if there is resistance. If the rectum need to be cleaned out, it is best to do so by inserting a water hose and flushing out the fecal material. Excess fecal material will interfere with the electrical stimulations.

   **Note that there are certain types of chemical immobilizing agents that detrimentally affect the quality of semen collected by electroejaculation, e.g., Azaperone, Medetomidine, etc.*

3. The penis should be exteriorized by carefully pushing in the skin just above (dorsally) in a forward (Cranial) manner. As soon as the penis is exteriorized, a collection tube should be placed over it. At this point the penis should constantly be kept inside a collection tube in case there are unexpected volumes of semen ejaculated. Only when the electroejaculation procedure is terminated and rectal probe removed, should the last collection tube be removed.

4. The rectal probe should be positioned so that the three electrodes are facing ventrally – the middle electrode facing the midline of the animal. The probe should not be inserted into the rectum any farther than just to the end where it can be clearly seen.
5. Before beginning electrical stimulations, be sure to ask all personnel if the procedure can begin. If it is safe, proceed with the minimum voltage limit stimulations. The stimulations are administered by increasing the voltage using the rheostat slowly to the voltage limit (e.g., count two seconds to go from 0 to 2 Volts). Hold at the voltage limit for one second, then quickly

return to 0. Once initiated, this process should continue until the operator of the electrostimulator is told to stop so that the collection tube can be exchanged for a fresh tube. Tubes should be exchanged often to avoid the possibility of urine contamination.

6. For tigers and lions, begin with a voltage limit of 2 V (5 repetitions), then increase the voltage limit by 0.5 V to a maximum of 5 V. Therefore, there should be 5 repetitions each of stimulations at 2, 2.5, 3 V – then a break of a few minutes – continued with 5 repetitions each of stimulations at 3, 3.5, 4 V – then a break of a few minutes – with the last series of stimulations of 5 repetitions each at 4, 4.5 and 5 V. Again, change tubes often to avoid urine contamination (typically, if the animal is stimulated to urinate, this will contaminate all subsequent semen samples).

7. Tiger and lion semen is clear to slightly cloudy – unlike ruminant semen which is whitish. Any yellowish tinge indicates urine contamination – pre-warmed medium (e.g., Hepes-buffered Tyrodes-lactate medium or TL-Hepes, BioWhittaker, Walkersville, MD, USA; Cat # 04-616F) should be immediately added to such tubes in an effort to recover viable sperm.

8. The electroejaculation procedure should be terminated after a maximum of 50 stimulations (to avoid creating stiffening and cramping of leg musculature) or when 5 – 7 ml of seminal fluid is obtained (average maximum volume for tigers and lions).

9. Semen fractions should be kept warm in a container or by holding the tubes against the body.

10. Each individual collection tube should be examined separately. Only those tubes that contain motile sperm should then be pooled. It is helpful to use the attached worksheet for every electroejaculation procedure performed.

11. Seminal plasma collected by electroejaculation can be harmful to large felid sperm after prolonged exposure. Therefore, prior to extension with a cryodiluent, the semen should be diluted with medium (e.g., TL-Hepes), centrifuged for 10 min at 300 x g, then the supernatant removed and discarded. The sperm rich pellet can then be reconstituted with the first cryodiluent.

12. Transport the dirty rectal probe inside a palpation sleeve or plastic bag back to the lab – wash it immediately with warm soapy water and rinse with a disinfectant.

Worksheet: Semen Collection by Rectal Probe Electrostimulation (Electroejaculation)

Date:_____ Species:_____ ID#:_____ Name:_____ Location:_____

Immobilization Protocol:
Duration Fasted:_____(feed) _____(water) Weight:_____ Pre-treatment:_____
Drug Regimen:_____
Supplementation:_____
Time Begin:_____ Time End:_____ Clinician:_____ Tech:_____

Blood Collection for:_____ Serum or Plasma (Anti-Coag?):_____ Vol:_____
Tissue/Excretus Collection:_____

Scrotal Measurements:
Length (Right):_____ Length (Left):_____ Texture:_____
Width (Right):_____ Width (Left):_____ Circumf:_____

Ambient Temp:_____ Rectal Temp:_____ Probe Size:_____
Electrostimulator:_____

Semen Collection Protocol:
Total Voltage Range:_____ Total Series:_____ Total Stimulations/Series:_____

Series:	1	2	3	4	5	6	Pool [ ]
Time Begin:							
Time End:							
Volts (range):							
mAmps (range):							
Erection (+/-):							
Volume (ml):							
Color:							
pH:							
Urine (+/-):							
Est. Motility (%):							
Linear Prog (0-5):							
Debris (type):							
Total Conc. ($x10^6$):							

Comments:_____
_____
_____
_____
_____

In Vitro Fertilization and Embryo Transfer in Felids

Charles Earle Pope
Audubon Nature Institute Center for Research of Endangered Species
September 2006

Pubications using this protocol:

1) **From:** Pope. C.E. Methods in Molecular Biology, vol. 254: Germ Cell Protocols, Volume 2: Molecular Embryo Analysis, Live Imaging, Transgenesis, and Cloning. Edited by: H. Schatten

Table 1. Pregnancies produced and kittens born after transfer of domestic cat embryos produced in vitro by our laboratory.

Embryo Derivation	Specific Treatment	Recipient Pregnant/Total (%)	Kittens Born (n)	Ref
In vivo matured/IVF	ET D4-5 ≥12 embryos/ET	11/26 (42)	21	10
In vivo matured/IVF	ET D4-5 ≥12 embryos/ET	6/23 (26)	11	10
In vivo matured/IVF	Frozen D2	2/4 (50)	3	11
In vivo matured/ICSI	ET D5	2/4 (50)	3	12
In vivo matured/SUZI	ET D5	1/4 (25)	1	14
In vitro matured/ICSI	ET D5	3/9 (33)	3	13
In vitro matured/IVF	ET D5-6	0/4 (0)	0	15
In vitro matured/IVF	ET D5-6	3/3 (100)	4	15
In vitro matured/IVF	Frozen D4-5; ET D5-6	4/10 (40)	3	20
In vitro matured/IVF	Ovaries cooled; ET D6-7	3/3 (100)	3	24

Introduction

The genome of the cat is highly conserved. In fact, of the non-primate mammalian species in which gene maps are developing, the cat genome exhibits the most similarities to that of the human (1). Biomedical studies in the domestic cat have contributed significantly to our knowledge in the areas of immunology, infectious diseases, genetics, neurophysiology, and cancer (2). Many of the heritable disorders of cats are analogous to those of humans, including hemophilia A and B, polycystic kidney disease, and several lysosomal storage diseases such as mucopolysaccharidosis, α-mannosidosis, and spingomyelinosis C. The close phylogenetic relationship, and the fact that many analogous genetic disorders have been identified and characterized, are important factors that demonstrate the advantages of domestic cats as biomedical research models for human disease when compared to other laboratory animals.

Although initial efforts to enhance reproductive rates of laboratory cats were reported more than 30 yr ago, most of the progress in development of assisted reproductive techniques (ART) has occurred during the past 10–15 yr and has been the subject of several reviews (3–6). A major focus of most recent work, including that of our laboratory, has been directed toward developing techniques that can be applied to the conservation of endangered felid species. Initially, embryos were produced from in vivo–matured oocytes after retrieval by follicular aspiration from gonadotropin treated females. Although the first in vitro–derived kittens were produced from such oocytes (7), much of the recent work on in vitro embryo production has been done using in vitro–matured (IVM) oocytes. There are several reasons for this, not the least important of which is the greater accessibility of such material. Cat ovaries are easily obtainable from local veterinary clinics, making it possible to recover several hundred oocytes per week, particularly if the laboratory is located near a large metropolitan area. Furthermore, the ability to maximize in vitro embryo production using IVM oocytes becomes of utmost importance if the technology is to be of relevance for conserving endangered felids.

Tangible evidence of progress in the development of felid ART made during the last decade is found by listing the different types of domestic kittens that have been produced in our laboratory after transfer of in vitro–derived embryos to recipient females, as shown in Table 1. Most of our embryo transfers (ET) have been done on d 5 when the embryos are mid- to late-morulae, the age and stage at which uterine entry naturally occurs. Others prefer to do oviductal ET of early cleavage stage embryos, thereby minimizing the in vitro culture interval (7,8). Oviductal ET may be advantageous in certain circumstances and possibly results in slightly higher pregnancy rates. Our decision to concentrate on uterine ET stems from it being less technically difficult and, therefore, more likely to have practical applicability, particularly if additional kittens can be produced by trans-cervical ET into the uterus, as one report has shown (9). Such an approach requires that considerable attention be devoted to continual evolution and improvement of the in vitro culture environment. For example, even though the base of our culture media for in vitro fertilization (IVF) and in vitro culture (IVC) is still Tyrode's balanced salt solution (14), a comparison of the culture media we used during the early 1990s (10,11) with that used more recently (12,13) reveals several differences. Among the most notable changes are the addition of amino acids, delayed supplementation with fetal bovine serum (FBS) and a gas atmosphere of reduced oxygen (5%). Resulting from greater interest and effort in developing ART in felids, in vitro development has shown gradual, but steady, improvement, as shown by rates of blastocyst development at d 7 increasing from 15%–30% in our earlier studies up to approx 50% in recent reports.

This chapter describes the materials and methods used for the in vitro production of cat embryos, including IVM, IVF, IVC, embryo cryopreservation, and transfer of embryos to recipient females.

Materials and Methods

Components of Culture Media

Nondisposable Plastics or Glassware

1. All reusable plastic or glassware used for media preparation or holding/processing of gametes/embryos are washed in 1% 7X detergent (ICN, Aurora, OH) as described by the manufacturer.

2. The water used for soaking/rinsing (and media preparation) is fresh sterile filtered (0.22 μm) 18 M water (Nanopure, Barnstead International, Dubuque, IA).

3. After overnight soaking in the detergent, each item is scrubbed with a brush, rinsed, and allowed to soak overnight in water before additional multiple rinses.

4. After oven drying, each item is wrapped or bagged and sterilized using a tabletop autoclave filled with reverse osmosis water and dedicated to culture labware only.

Base Media

The base salt solutions and media to which additional supplements are added to make the media used for gamete/embryo holding, culture, and storage are as follows:

1. Tyrode's balanced salt solution (Ty, Irving Scientific, cat. no. 9282, Santa Ana, CA) is the base for IVF/IVC media and a HEPES-buffered medium for diluting/maintaining spermatozoa for IVF and embryos cryopreservation .

2. TCM 199, (Irving Scientific, cat. no. 9102, with NaHCO3, without glutamine): base for IVM medium.

3. TCM 199, (Sigma, cat. no. 3769, without glutamine and NaHCO3): base for HEPES buffered holding medium for oocytes/embryos outside of the CO2 incubator.

4. TL Hepes solution, (Biowittaker, cat. no. 04-616F, Walkersville, MD): oocyte aspiration medium. 1–2 mL TL HEPES, with 10 μ/mL heparin and 50 μg/mL gentamicin added is preloaded into each 15-mL centrifuge tube to be used for collection of aspirated oocytes.

Additional Components

1. Gonadotropins. Equine chorionic gonadotropin (eCG; Calbiochem, cat. no. 367222, San Diego, CA) and human chorionic gonadotropin (hCG, Pregnyl, Organon Inc., West Orange, NJ) used in IVM medium. In a laminar flow hood, each vial of gonadotropin powder is reconstituted with sterile Ty so that each mL contains 100X final concentration used for culture (100X: eCG = 50 IU/mL; hCG =100 IU/mL). Aliquot into 1.5 mL microcentrifuge tubes, label, and store at –80°C until needed. Do not refreeze after thawing.

2. Supplement stocks. The energy sources and antibiotics in the IVF and IVC media are prepared as a 100X solution in Ty (Table 2). To prepare 10 mL 100X stock, weigh out 0.146 g glutamine, 0.040 g sodium pyruvate, 0.242 g calcium lactate, and 0.050 g gentamicin and solubilize in Ty. Sterilize by filtering into 1.5-mL tubes, label, and store at –80ºC until the day of use. Similarly, 10 mL of 100X supplement for IVM medium is prepared as described for IVF/IVC, except that glutamine is increased to 0.292 g and 0.200 g cysteine is added.

3. Bovine serum albumin (BSA; fraction V, Serological Proteins, Inc., cat. no. 82047, Kankakee, Il). BSA is added at a 6 mg/mL final concentration for IVF and 3 mg/mL for the first step of IVC (see Note 1).

4. FBS, Hyclone, Inc., cat. no. 30070, Logan, UT). BSA is replaced with 10% FBS as development approaches the early morula stage on IVC d 2 or 3. Each serum bottle is thawed upon arrival, aliquoted at 10 mL/sterile 15-mL centrifuge tube (polypropylene, Corning, Inc.,cat. no. 430052) and stored at –80°C. As needed, FBS is thawed and heat-treated at 56°C for 30 min before use.

5. Amino acids. Minimal essential medium (MEM) nonessential amino acids (NEAA) and MEM essential amino acids (EAA) are purchased as 100X and 50X solutions, respectively. Upon arrival, each 100-mL bottle is aliquoted into 1.0 mL/1.5 mL sterile microcentrifuge tubes and stored at –80°C until needed.

6. Epidermal growth factor (EGF, Sigma, cat. no. 9644). EGF powder is reconstituted with Ty to a 1000 ng/mL concentration (100X final concentration), filter sterilized into 1.5-mL microcentrifuge tubes, and stored at –80°C until needed.

Preparation of Media: Osmolality and pH

1. All culture/holding media are freshly prepared every week. Table 2 outlines the quantities of each supplement to add to bicarbonate-based media for preparing a total of 100 mL of each medium used to culture oocytes/embryos from IVM through development to the blastocyst stage.

Table 2. Quantities of base media and supplements used to prepare 100 mL of each type of culture medium used for in vitro production of cat embryos.

Item	IVM	IVF	IVC-I	IVC-II
Ty balanced salt solution	–	89.5 mL	88.5 mL	76.5 mL
Water, type I, fresh	–	8.0 mL	8.0 mL	8.0 mL
IVF/IVC 100X supplement	–	1.0 mL	1.0 mL	1.0 mL
NaHCO3, 7.5% solution	–	1.5 mL	1.5 mL	1.5 mL
100X MEM NEAAs	–	–	1.0 mL	1.0 mL
50X MEM EAAs	–	–	–	2.0 mL
FBS	–	–	–	10.0 mL
BSA Fr V	0.3 g	0.6 g	0.3 g	–
TCM 199	96.75 mL	–	–	–
IVM 100X supplement	1.00 mL	–	–	–
hCG (1 IU/mL; 100 IU/mL stock)	1.00 mL	–	–	–
eCG (0.5 IU/mL; 200 IU/mL stock)	0.25 mL	–	–	–
EGF (10 ng/mL; 100X stock)	1.00 mL	–	–	–

2. Mixing and filtering are done using a horizontal laminar flow hood. After all components have been combined and thoroughly mixed for each medium, osmolality and pH values are checked.

3. Desired osmolality is between 285 and 295 mOsm. If the medium is higher than 295 mOsm, it is adjusted by adding fresh water.

4. Before gassing with 5% CO2 in air, the pH of bicarbonate buffered media should be approx 7.7–7.8. Media not in that pH range are adjusted with 1.0 N NaOH or 1.0 N HCl, as necessary.

5. HEPES-buffered media are used for handling and processing of gametes when not maintained in a gas atmosphere of 5% CO2. After recovery of cumulus oocyte complexes, either from excised ovaries or after laparoscopic follicular aspiration, they are maintained in a HEPES buffered TCM 199 medium made in the laboratory.

6. To prepare, 15 mM NaHCO3, 15 mM HEPES, 3 mg/mL BSA, and 1% of 100X IVC supplement is added to the TCM 199 base medium described above.

7. HEPES buffered Ty (HeTy) is used for preparing embryo cryoprotectant medium and for extension and holding of sperm samples before IVF.

8. HeTy is prepared as described for He 199, except that NaHCO3 is not added because it is contained in Ty as purchased.

Filtration

1. Each type of medium, after checking/adjusting osmolality and pH, is sterilized by yringe filtration into 15-mL conical centrifuge tubes (Corning Inc., cat. no. 430052, Acton, MA) in a laminar flow hood.

2. No more than 5 mL bicarbonate-buffered culture medium is added per tube.

3. For a description of the filtration process for larger media volumes, as is done when the base medium is purchased in dry form and mixed in the laboratory (see Note 3).

Gas Equilibration

1. Immediately after filtration, while still working in a laminar flow hood, each tube of medium is gassed with a mixture of 5% CO2 in air (or 5% CO2, 5% O2, 90% N2).

2. Insert an 18 g × 3.8 cm needle, attached to the gas mixture by tubing, into the tube while it is being held at a 15° angle.

3. Allow gas to blow over the media, but not into it, which prevents bubble formation.

4. After 45–60 s, the needle is quickly withdrawn, and the tube is capped tightly.

Media Storage

After gassing, the tubes of freshly prepared media are stored in a tilt rack at 4°C until use within 7 d.

Cryopreservation Solutions

1. The cryoprotectant solution consists of 1.4 M propylene glycol (PG), 0.125 M sucrose (S), 10% Dextran 70, and 10% FBS in HeTy (CPS).

2. Previously prepared HeTy medium can be used as the ‘base’ for the cryoprotectant solution.

3. However, preparation is easier if sucrose, then dextran, are added to and mixed withTy without BSA or FBS in it.

4. After sucrose and dextran are in solution, then 1% 100X IVC supplement, 15 mM HEPES and 1.4 M PG are added.

5. Next, add BSA (3 mg/mL) powder and swirl gently, avoiding bubbles, if possible.

6. After BSA is completely in solution, add 10% FBS to complete the CPA solution.

7. Sterilize by syringe filtration into sterile 5-mL cryotubes, label, and store at –80ºC until needed.

Artificial Vagina for Semen Collection

1. The artificial vagina (AV) consists of a sleeve and collection container prepared from a 2 mL latex bulb and a 1.5-mL microcentrifuge tube, respectively.

2. After washing, the closed-end portion of the bulb is cut off, leaving an approx 3-cm length of open tube.

3. The conical base of a microcentrifuge tube is cut away from the remaining portion and fitted into the end of the latex bulb that had been cut open.

4. The AV is bagged and autoclaved along with a 50-mL glass serum bottle.

5. To use, the bottle is filled with water warmed to approx 40°C, the AV is placed into the bottle, and the open end is folded over the lip of the bottle to hold it in place.

6. A small amount of nonoil-based sterile lubricant is smeared around the AV opening.

IVC of Oocytes and Embryos

1. Oocytes and embryos are placed in 500 μL preequilibrated IVM or IVC medium in four-well culture dishes (Nunclon, cat. no., 176740 Nunc, Denmark) and cultured in a closed system.

2. The platform of the closed system is the inverted lid of a micro titer plate (Corning, Inc., cat. no. 3513) onto which are placed the four-well dishes containing the oocytes or embryos next to a 60 × 15-mm Petri dish filled with approx 7 mL sterile water.

3. Several holes are drilled in the lid of the 60-mm Petri dish to provide humidity in the sealed bag.

4. The assembled system is placed inside of a 16.5 × 20.3 cm plastic bag (Kapak SealPAK pouch, cat. no. 402, Kapak Corp, Minneapolis, MN).

5. Then, a 16-gauge × 10-cm blunt-tipped needle, connected by tubing to a premixed tank of 5% O2, 5% CO2 and 90% N2, is inserted into the open side of the bag, which, in turn, is placed in between the jaws of an impulse sealer (Model AIE-200, American International Electric, Whittier, CA).

6. The premixed gas is humidified during inflow by bubbling it through a flask containing 700 mL sterile water.

7. After the bag is inflated with the humidified gas mixture, the needle is quickly withdrawn, and the bag is heat-sealed.

8. During the sealing process, the bag and its contents sit on a heated block maintained at 38°C.

9. After checking for patency the bag containing the oocytes or embryos is placed into a water-jacketed incubator at 38°C (Forma Scientific, model 3130, Marietta, OH).

Gonadotropic Stimulation of Ovarian Follicle Development in Oocyte Donors

1. Porcine follicle stimulating hormone (FSH) (cat. no. 915, 50 IU/vial) and porcine leutinizing hormone (LH) (cat. no. 925, 25 IU/vial) are purchased from Sioux Biochemicals, Sioux Center, IA, and stored at 4°C until needed.

2. Immediately before use, lyophilized FSH is reconstituted by adding a sterile solution of 2% carboxymethylcellulose (CMC) and 1% Tween 20 (Tw20).

3. Initially, 10 mL CMC/Tw20 is added to the vial of powder.

4. To minimize bubble formation, direct flow down the inside wall, set the vial aside for a few minutes, then gently swirl for 30–60 s to ensure that all powder is completely dissolved.

5. Then, remove the 10 mL FSH solution and put into a sterile 50-mL centrifuge tube.

6. Add another 10 mL CMC/Tw20 to the original FSH vial and gently swirl before removing and adding to the 50-mL centrifuge tube.

7. Lastly, add a final 5 mL CMC/Tw20 to the FSH vial, aspirate it back into the syringe, and add it to the 50-mL tube. The final 25 mL vol of reconstituted FSH will have 2.0 IU/mL.

8. The FSH solution is then aliquoted into 3-mL glass tubes (Vacutainer, cat. no. 366381, sterile, no additive, Becton Dickinson, Franklin Lakes, NJ) and held at 4°C, if it is to be used within 2 d, or stored at –80°C for later use.

9. LH is similarly prepared, except that only 5 mL of CMC/Tw20 is added per vial to give a final 5 IU/mL concentration.

Methods

Animals

1. The cats in our facility are antibody-defined animals purchased from a US Department of Agriculture (USDA) approved, AAALAC accredited vendor (Liberty Research, Waverly, NY).

2. Potential oocyte donors are chosen from sexually mature females with most of them ranging from approx 1 to 6 yr of age.

3. They are group housed in rooms maintained at a temperature of 72–76°C, a relative humidity of 66–67%, and 14/10 h of light/dark cycle.

4. The rooms are cleaned, and fresh food and water are provided daily.

5. In addition to the animal care personnel, a veterinary staff consisting of one veterinarian and two technicians provide full-time health care.

6. Semen for laboratory use is collected from two or three sexually mature tom cats that are individually housed in stainless-steel cages.

Hormonal Stimulation of Ovarian Follicular Development

Oocyte Donors

1. Potential oocyte donors are selected from females that are interestrus as determined by vaginal cytology and lack of behavioral signs of estrus.

2. Vaginal cells are recovered from nonsedated females with a moistened vaginal swab, then rolled onto a clean microscope slide.

3. After staining, each slide is microscopically evaluated, and those with a predominance of parabasal and intermediate cells are candidates for gonadotropin treatment.

Gonadotropin Treatment of Oocyte Donors

1. The standard total amount of FSH administered to potential oocyte donors previously untreated with exogenous gonadotropins is 3.0 IU given in decreasing doses 1 time/d for 4 d.

2. At 2.0 IU/mL, the total FSH volume of 1.5 mL is typically administered subcutaneously (sc) at the rate of 0.5, 0.4, 0.3, and 0.3 mL/d.

3. FSH is given during mid to late afternoon, and injections should be done at approx. 24-h intervals. In the morning of the fifth day of hormone treatment, 3.0 IU LH is administered intramuscularly (im).

4. The LH injection is given 24 h before the scheduled oocyte retrieval.

5. For information about gonadotropin treatment for females that have previously undergone ovarian stimulation and oocyte recovery.

Oocyte Retrieval from Gonadotropin-Treated Donors

Pre-operative Procedure

1. Food and water are withdrawn on the day before laparoscopy.

2. Oocyte donors are sedated with injectable anesthetics, and the lower abdomen is closely clipped.

3. After intubation, anesthesia is maintained by inhalation of isoflurane and oxygen. The abdominal area is scrubbed with chlorohexidene and sprayed with 70% alcohol before sterile disposable drapes are placed over the animal, leaving only the surgical area exposed.

Laparoscopic Procedure

1. Oocyte retrievals are done 24 h after LH administration.

2. Three abdominal entry sites are required for insertion of the Verres' needle and two ports, one for the telescope and the other for forceps.

3. To insufflate the abdominal cavity, a small skin incision is made on the right side 2.5–3.0-cm caudal to the umbilicus and 2.0-cm lateral to the midline.

4. Skin folds on both the cranial and caudal sides of the incision are raised, and Allis tissue forceps are attached for elevating the abdominal wall.

5. A 150-mm stainless-steel Verres needle (Olympus America, Inc., cat. no. A5150.1, Melville, NY) is positioned vertically at the incision site, and with manual pressure, is carefully inserted into the abdominal cavity.

6. Usually, a 'snapping' sound is heard as the blunt stylet tip returns to its full extension beyond the needle tip after entry into the abdominal cavity.

7. When the needle tip is confirmed to be properly located, both by feel and free inflow of saline, tubing attached to an automatic insufflator (Insufflator-15 L, Olympus America, Inc.) is connected, and the abdominal cavity is insufflated to a pressure of 10 mm Hg with 5% O2, 5% CO2, 90% N2.

8. Then, a 5-mm safety trocar/cannula (Ethicon Endosurgery, cat. no. 355 SD, Cincinnati, OH) is inserted into the abdominal cavity through a skin incision approx 1-cm anterior to the umbilicus as a port for insertion of the rigid 5 mm telescope (O° angle, Olympus America, Inc., cat. no. A5290A).

9. A camera (Olympus America, Inc., cat. no. MH-972N) is connected to the telescope for visualizing the operative field on a 19"-color monitor mounted at eye-level and located at the tail-end of the operating table.

10. While visualizing the bladder and surrounding area, a second 5-mm safety trocar/cannula is inserted into the abdominal cavity through the skin incision in the midline between two most-posterior teats.

11. Even after insufflation, there is considerably less open area between the body wall and intestines at the lower puncture site.

12. Accordingly, it is important to be able to see the tip of the lower trocar as it enters the abdominal cavity to ensure that no organs or tissues are damaged during entry.

13. After the second-port entry, the surgery table is tilted (approx 15 to 20°) cranially downward so that intestines shift forward to improve access to the ovaries.

14. A 5-mm Babcock forceps (Olympus America, cat. no. A63040A) for lifting and stabilizing each ovary is inserted through the lower port.

15. If the ovaries are not immediately visible, the overlaying mesentery and/or intestinal loops are relocated using the Verres' needle.

16. Then, the forceps are placed around the base of the ovary, being careful to exclude fimbrial tissue before clamping and elevating into the correct position for visualizing and accessing follicles.

Follicle Aspiration

1. A 18-gauge, 6-cm stainless steel trocar/cannula is placed percutaneously above each ovary sequentially, not simultaneously, as a port for inserting the aspiration needle.

2. The sterile follicle aspiration units are custom -made in-house and consist of a 20 gauge (thin wall), 10 cm needle connected to a approx 0.8 m-length of 1.02 mm (id) × 1.16 mm (od) silicone tubing, the other end of which passes through a 17-mm silicone stopper with 2–3 cm of tubing extending through the exit side. The tubing is stabilized and sealed in the stopper with silicone adhesive.

3. The stopper also has a 16-gauge blunt tipped stainless-steel needle mounted in it for connecting via tubing to the vacuum pump.

4. For follicle aspiration, the stopper is seated into a 15-mL conical tube containing 1–2 mL TL HEPES solution with heparin.

5. Each visible follicle is punctured at 75 mmHg suction provided by a regulated vacuum pump specifically designed for follicle aspiration (Cook Veterinary Products, V-MAR-5115, Eight Mile Plains, Australia).

6. Most gonadotropin-stimulated follicles are from 2 to 4 mm in diameter when aspirated at 24-h post-LH injection.

7. The aspiration tubing is monitored carefully during the process to ensure that clotting has not ccurred and that the fluid is flowing into the collection tube. Periodically (after 5–15 follicle punctures), the aspiration needle is removed, placed in a tube of TL HEPES + heparin to flush the contents down into the collection tube.

8. After all visible follicles have been aspirated, each ovary is rinsed with over 50 mL of sterile saline solution infused through either the aspiration cannula or the Verres' needle. The infused saline is removed by manual aspiration back into a 30-mL syringe connected to the Verres' needle.

Post-laparoscopic Procedure

1. When both ovaries have been aspirated, the surgery table top is returned to the level position, and any remaining infused saline is removed by aspiration.

2. The insufflation tube is disconnected, and the ports on each cannula are opened so that residual gas is exhausted out of the abdominal cavity.

3. At the puncture sites for the Verres' needle and trocars, the abdominal musculature is closed with an appropriately sized absorbable suture material in a simple interrupted pattern.

4. If the animal has a thick sc fat layer, it is closed with an absorbable suture material in a simple continuous pattern.

5. The skin is closed with an absorbable suture material in a buried subcuticular pattern. In cases where there is no closure of the sc fat layer (i.e., thin animals), the subcuticular suture is intermittently tacked to the abdominal musculature to decrease the likelihood of seroma formation.

6 Poliglecaprone 25 is the preferred suture material, owing to its low reactivity and long duration of strength.

7. A small amount of tissue adhesive (cyanoacrylate) is used at the completion of the skin suture to facilitate good-knot burial.

In vitro Oocyte Maturation

Ovary Collection and Transport

Domestic cat ovaries obtained from local veterinary clinics after ovariohysterectomy are transported to the laboratory at ambient temperature in HEPES-buffered saline solution (UltraSaline A Solution, Bio-Whittaker, Walkersville, MD) supplemented with 50 µg/mL gentamicin.

Oocyte Collection and Sorting

1. Within 2–6 h, ovaries are minced and cumulus-oocyte complexes (COC) collected into HEPES-buffered TCM 199 (He-199) at 35–38°C.

2. COC recovery and handling is done within laminar flow hoods using stereomicroscopes equipped with temperature-controlled stages set at 38°C.

3. After rinsing three times in He 199, COC are sorted into three groups according to ooplasm appearance and cumulus cell presence (15):

a. Type A oocytes have uniformly dark, finely granulated ooplasm enclosed in a smooth, spherical oolemma.
b. Ooplasm of type B oocytes have slightly lighter pigmentation and the ooplasm is less uniformly and evenly granulated.
c. Oocytes of type C have pale ooplasm that is more coarsely granulated and more irregular in overall appearance.

IVC of IVM Oocytes

From 10 to 30 oocytes of each type/well are cultured using the closed system for 24 h in modified TCM 199 containing eCG/hCG and BSA and the supplements described previously.

Spermatozoa for IVF

Semen Collection

1. Semen is collected from adult domestic cats using an artificial vagina (AV; 16).

2. The collection is done in a quiet area with minimal chance of disruption. As soon as the male mounts the ‘teaser’ female, the penis is gently directed into the AV unit so that natural mating does not occur.

3. Ejaculation usually occurs rather quickly, although, on occasion, males may require more time and patience to obtain a semen sample.

4. The volume of semen samples collected using an AV usually ranges from 30 to 60 μL, although samples of 80–100 μL are obtained from some males.

Semen Extension and Cooling for Temporary Storage

1. The semen sample is held at room temperature during processing.

2. A 3–6 μL aliquot of the fresh semen sample extended in 35 μL HeTy provides more than enough spermatozoa for IVF.

3. The remainder of the semen sample is combined with 500 μL TEST yolk buffer (Refrigeration Medium, Irving Scientific, Santa Ana, CA) in a 1.5-mL microcentrifuge tube and gradually cooled to 4°C by putting the tube in a 100-mL screw-top bottle containing room temperature (22–23°C) water, then placing the sample in a 4°C refrigerator.

4. To stabilize the tube within the water bath, a hole slightly larger than the diameter of the microcentrifuge tube is drilled in the middle of the lid.

5. The bottle is completely filled with water so that the contents of the tube will be submerged during cooling.

6. Cooled semen is used for IVF for up to 3 d after collection/storage, whereas fertilizing ability is maintained for at least 7 d at 4°C (17).

7. To use spermatozoa held at 4°C, a 3–6 μLaliquot is pipetted directly from the visible sperm pellet formed by sedimentation after cooling when motility is temporarily suspended.

Estimation of Sperm Motility and Concentration

1. The percentage of motile spermatozoa in the diluted sample is estimated subjectively using bright-field microscopy, and sperm concentration is measured photometrically (SpermaCue, MiniTube of America, Veronica, WI).

2. To measure sperm concentration, an aliquot of semen sample extended in HeTy is further diluted 1:1 in a formal citrate solution consisting of 2.9% (w/v) trisodium citrate dihydrate and 0.1% (v/v) formaldehyde in deionized water (18) to inhibit motility.

3. A 25-μL aliquot of the sperm sample diluted in formal citrate is loaded into the photometer cuvette, the cuvet is placed in the chamber of the photometer, and the concentration is measured as $n \times 10^6$ sperm/mL.

4. Then, 10 μL of the extended sample is further diluted with HeTy to 10×10^6 motile sperm/mL immediately before IVF.

IVF

1. The IVF dishes are prepared by pipetting 4–40 µL drops of modified Ty containing 6 mg/mL BSA (Table 2) into a 35 × 10 Petri dish (Falcon, cat. no. 1008) and overlaying with approx 3 mL of mineral oil (Sage BioPharma, cat. no. 4008, Bedminster, NJ).

2. From 5 to 15 oocytes are pipetted into each drop in approx 5 µL medium.

3. For IVF, 5 ± 1 µLof the aliquot previously diluted to 10 × 106 motile sperm/mL is added to each droplet of IVF medium to give a final motile sperm concentration of 1×10^6/mL.

4. Oocytes and spermatozoa are coincubated in 5% CO2 + air at 38°C.

IVC

1. At 5–7 h or 15–18 h post-insemination, in vivo matured and IVM oocytes, respectively (see Note 7 for explanation of insemination durations), are rinsed four times in He 199 and cultured in 500 µL of modified Ty containing NEAA and BSA (IVC-1 medium) at 38°C in sealed bags filled with 5% CO2, 5% O2, and 90% N2.

2. Then, on d 2 or 3, uncleaved oocytes are removed and embryos are placed into fresh modified Ty containing NEAA, EAA, and 10% FBS, instead of BSA (IVC-2, Table 2).

3. Those embryos that are not transferred to recipients on d 5, 6, or 7 are evaluated on d 7 by visually determining blastocyst development using a stereomicroscope.

4. Additional information, such as number of cells per embryo, can be obtained after fixation using any of the standard staining methods, such as aceto-orceinstained wet mounts, Giemsa/air-dryed preparations, or fluorescence.

5. A simple chemically defined differential staining technique can be used to determine inner cell mass and trophectoderm cell numbers in cat blastocysts (19).

Embryo Cryopreservation

Initially, embryos were frozen on IVC d 2 at the two to four-cell stage (11), but the protocol is equally effective for embryos frozen on d 3, 4, or 5 (15,20). In fact, we are currently freezing embryos on d 5.

Equilibration and Freezing

1. The cryoprotectant solution consists of 1.4 M PG, 0.125 M S, 10% dextran 70, and 10% FBS in HeTy (CPS).

2. On d 2 (4–8 cells), d 4 (early morulae) and d 5 (morulae) of IVC, embryos are exposed to CPS at 22°C after a two-step equilibration in two parts of HeTy: 1 part CPS (1/3 CPS) and one part HeTy: two parts CPS (2/3 CPS) with 3 min/step.

3. During the 10–15 min equilibration in CPS, embryos are loaded into 0.25- mL nonirradiated straws (Ag Tech, cat. no. B4-2400, Manhattan, KS) and after heat-sealing the tip, each straw is placed into the chamber of a controlled rate-freezing unit (CryoLogic, model CL-863, Victoria, Australia) at 20°C.

4. Embryos are cooled at 2.0°C/min to –6.0°C.

5. During a 10 min hold at –6.0°C each straw is manually seeded with a cotton swab.

6. Cooling is then resumed at 0.3°C/min to –30°C, and after a 10-min hold, embryos are plunged into liquid nitrogen for storage.

Thawing and Culture

1. Straws containing the embryos are thawed for 2 min in air at 22°C, the heat-sealed tip is cut off, and the contents are expelled into a 35-mm Petri dish.

2. Cryoprotective agents are removed at room temperature using a five-step rinse consisting of 3 min each in HeTy plus: 0.95 M PG/0.25 M S; 0.95 M PG/0.125 M S; 0.45 M PG/0.125 M S; 0 PG/0.125 M S; 0 PG/0.0625 M S.

3. After a brief rinse in He 199 at 38°C, thawed embryos are cultured in IVC-2 medium until transfer to a recipient female on d 5, 6, or 7, or until evaluation/staining on d 7 or 8.

4. Such embryos are usually starting to form blastocysts by d 6 and up to half should reach the blastocyst stage by d 7, providing a convenient visual estimate of the developmental potential of the embryos being transferred.

ET

1. The recipient female is anesthetized as described earlier for laparoscopy.

2. D 5–7 in vitro-derived embryos are transferred by mid-ventral laparotomy into the uterus of gonadotropin-treated females from which follicular oocytes had been aspirated 5–7 d previously.

3. Using the aseptic technique, a section of one uterine horn is exteriorized through a 1.5-cm incision.

4. An entry site for the catheter is made by puncturing the exposed uterine horn near the anterior tip with a sterile 16-gauge stainless steel trocar with the tip ground to a round short-beveled point.

5. Embryos are loaded in approx 80 µL culture medium into a sterile 14-cm, 3.5 fr, open-ended tom-cat catheter (The Kendall Co., cat. no. 703021, Mansfield, MA) attached to a 1-mL all-plastic syringe.

6. The tip of the catheter is carefully threaded through the puncture site into the uterine lumen approx 4 cm toward the uterine body before expelling the embryos by a quick push of the syringe plunger.

7. The catheter is removed, flushed with culture medium, and the medium is checked with a stereomicroscope to ensure that no embryos are present.

8. Closure of the incision and postoperative care is the same as described for laparoscopy.

Notes

1. Each new BSA lot is tested for its ability to support in vitro cleavage of cat oocytes and development to the blastocyst stage. If in vitro embryonic development is similar to that usually obtained in our laboratory, then we request that the company set aside several bottles for us to purchase as needed.

2. Low protein binding syringe filters (Acrodisc, Pall Corp., cat. no. 1492, 0.22 μm, Ann Arbor, MI) are used for sterile filtration of all culture media prepared weekly. As a precautionary measure, for each new filter, dispose of the first 500 μL medium before collecting into sterile tubes. If using a single filter for more than one type of medium, discard the first 0.5–1.0 mL new medium.

3. If the base medium is purchased in dry form, after mixing with water, the medium (usually prepared in 1-L batches) is filtered using positive pressure. For these larger volumes (>1 L) of media, a peristaltic pump (Cole Parmer, cat. no. 77000-30, Chicago, IL) having a flow rate of 100 mL/min. with 3.9-mm id silicone tubing is used. The medium is pumped through a filter unit (AcroCap, Pall Corp. cat. no. 4480, 0.2 μm) attached to the sterile tubing and collected into 100-mL sterile glass screw-top bottles prepared as described previously. Each bottle is labeled and stored at 4°C until use.

4. The total FSH amount to be given to previously treated donors is based on their earlier follicular response to hormone treatment. As a general rule, if 20 or more oocytes were recovered during the last laparoscopic retrieval, then the total dose is not increased. However, if fewer than 20 oocytes were recovered from the potential oocyte donor at one or more previous retrievals, then the total FSH dose is increased to 3.6–4.0 IU or even 5.0 IU if only 5–15 oocytes were recovered. Another selection criterion is based on a minimum interval of 6 mo since the previous stimulation with exogenous gonadotropins. Swanson et al. (21) reported that repeated treatment (two or three times) of domestic cats with 150 IU eCG followed 84 h later with 100 IU hCG at intervals of 49–57 d resulted in development of ovarian refractoriness to follicular development and recovery of fewer oocytes at each attempt. The decrease in follicle development did not occur if the interval between eCG/hCG treatments was increased to 130–135 d. Accordingly, a minimum of 6 mo is allowed between FSH/LH treatments. Our recent data on number of oocytes recovered after repeated oocyte retrievals following follicular stimulation with FSH/LH agrees with the earlier report in which eCG/hCG was used.

5. Before each laparoscopy, the reusable equipment, such as the telescope, fiber optic cable, camera, and connecting cable, are disinfected by soaking for 60–90 min in a solution of 0.05% chlorohexidene (2% chlorohexidene diacetate, Nolvasan Solution, Fort Dodge Animal Health, Fort Dodge, IA). The instruments are then rinsed thoroughly in deionized water sterilized by autoclaving. The other instrumentation, such as Babcock forceps, 5-mm trocar/cannula, tubing/needle sets for follicle aspiration, and insufflation needles are sterilized by autoclaving or ethylene oxide. After each use, the telescope, camera, and cables are rinsed and dried. The other instruments are rinsed, cleaned by sonication in 7X detergent (1%), and rinsed thoroughly again before drying and repackaging for sterilization.

6. Ideally, training for AV semen collection should begin before sexual maturity, although sexually experienced mature males are quite trainable. An estrous female, who is compatible and cooperative with the male is used as a teaser. Also, semen can be collected from some males using a stuffed ‘dummy’ cat. Using an artificial ‘teaser’ cat eliminates the necessity of finding a cooperative female in estrus, which can occasionally be problematic.

7. IVM oocytes are coincubated with spermatozoa for 15–18 h mostly for practical reasons. Because the ovaries do not arrive in the laboratory until mid-afternoon after pick-up from the veterinary clinics, the oocytes are not placed into culture until late afternoon. Most developmentally competent cat oocytes complete IVM in approx 24 h and they are inseminated during late afternoon the following day. So, rather than returning to the laboratory later that night, spermatozoa are coincubated overnight with oocytes. Then, the next morning, approx 15 to 18 h postinsemination, oocytes are rinsed and placed in culture. On the other hand, in vivo–matured oocytes are aspirated from gonadotropin-treated donor females during the morning, and IVF is done by around mid-day. Over 90% of metaphase II cat oocytes examined between 0.5 and 3.0 h postinsemination (IVF) had undergone sperm penetration into the ooplasm (10,22). Furthermore, cleavage frequencies of oocytes following 6 vs 15 h coincubation with spermatozoa were not different (23).

Acknowledgments

I would like to express my great appreciation to Dr. Betsy L. Dresser, Senior Vice President Research/Director, Audubon Nature Institute Center for Research of Endangered Species (AICRES) for her long-term support and encouragement. I am also indebted to numerous colleagues and coworkers, both formerly at the Cincinnati Zoo Center for Research of Endangered Wildlife (CREW) and currently at AICRES, without whom this work would not have been possible. Lastly, the cooperation and assistance of local veterinary clinics in providing gonads for gamete retrieval has been invaluable.

References

1. O'Brien, S. J., Menotti-Raymond, M., Murphy, W. J., and Nash, N. G. 1999. The promise of comparative genomics in mammals. Science 286, 458–462.
2. Migaki, G. Section VIII, Compendium of inherited metabolic diseases in animals, in Animal Models of Inherited Metabolic Disease (Desnick, R., Patterson, D., and Thompson D. G., eds.), A.R. Liss, New York, 1982, pp. 473–501.
3. Goodrowe, K. L., Walker, S. L., Ryckman, D. P., Mastromonaco ,G. F., Hay, M. A., Bateman, H. L., and Waddell, W. T. (2000) Piecing together the puzzle of carnivore reproduction. Anim. Reprod. Sci. 60–61, 389–403.
4. Farstad, W. (2000) Current state in biotechnology in canine and feline reproduction. Anim. Reprod. Sci. 60–61, 375–387.
5. Luvoni G. C. (2000) Current progress on assisted reproduction in dogs and cats: in vitro embryo production. Reprod. Nutr. Dev. 40, 505–512.
6. Pope, C. E. (2000) Embryo technology in conservation efforts for endangered felids. Theriogenology 53, 163–174.
7. Goodrowe, K. L., Wall, R. J., O'Brien, S. J., Schmidt, P. M., and Wildt, D. E. (1988) Developmental competence of domestic cat follicular oocytes. Biol. Reprod. 39, 355–372.
8. Swanson, W. F., McRae, M. A., Wildt, D. E., and Rall W. F. (1999) Cryoprotectant toxicity and cryopreservation success in IVF-derived cat embryos after embryo transfer. Theriogenology (abstr.) 51, 174.
9. Swanson, W. F. and Godke, R. A. (1994) Transcervical embryo transfer in the cat. Lab. Anim. Sci. 44, 288–291.
10. Pope, C. E., Keller, G. L., and Dresser, B. L. (1993) In vitro fertilization in domestic and non-domestic cats including sequences of early nuclear events, development in vitro, cryopreservation and successful intra-and interspecies embryo transfer. J. Reprod. Fertil. (Suppl. 47), 189–201.

11. Pope C. E., McRae, M. A., Plair, B. L., Keller, G. L., and Dresser, B. L. (1994) Successful in vitro and in vivo development of in vitro fertilized two- to four-cell cat embryos following cryopreservation, culture and transfer. Theriogenology 42, 513–525.
12. Pope, C. E., Johnson, C. A., McRae, M. A., Keller, G. L., and Dresser, B. L. (1998) Development of embryos produced by intracytoplasmic sperm injection of cat oocytes. Anim. Reprod. Scs. 53, 221–236.
13. Gómez, M. C., Pope, C. E., Harris, R. F., Davis, A., Mikota, S., and Dresser, B. L. (2000) Births of kittens produced by intracytoplasmic sperm injection of domestic cat oocytes matured in vitro. Reprod. Fertil. Dev. 12, 423–433.
14. Pope C. E., Johnson, C. A., McRae, M. A., Keller, G. L., and Dresser, B. L. (1995) In vitro and in vivo development of domestic cat oocytes following intracytoplasmic sperm injection or subzonal insemination. Theriogenology (abstr.) 43, 302.
15. Pope, C. E., McRae, M. A., Plair, B. L., Keller, G. L., and Dresser B. L. (1997) In vitro and in vivo development of embryos produced by in vitro maturation and in vitro fertilization of cat oocytes. J. Reprod. Fertil. Suppl. 51, 69–82.
16. Sojka, N. J., Jennings, L. L., and Hamner C. E. (1970) Artificial insemination in the cat (Felis catus, l). Lab. Anim. Sci. 20, 198–204.
17. Harris, R. F., Gomez, M. C., Leibo, S. P., and Pope C. E. (2002) In vitro development of domestic cat embryos after in vitro fertilization of oocytes with spermatozoa stored for various intervals at 4 C. Theriogenology (abstr.) 57, 365.
18. Dott, H. M. and Foster, G. C. (1975) Preservation of differential staining of spermatozoa by formal citrate. J. Reprod. Fertil. 45, 57–60.
19. Harris, R. F., Gomez, M. C., Pope, C. E., Giraldo, A. M. King, A. L., and Dresser, B. L. (2003) Differential staining of trophectoderm and inner cell mass cells of cat in vitro derived blastocysts. Theriogenology (abstr.) 59, 448.
20. Pope, C. E., Gómez, M. C., Harris, R. F., and Dresser, B. L. (2002) Development of in vitro matured, in vitro fertilized cat embryos following cryopreservation, culture and transfer. Theriogenology (abstr.) 57, 464.
21. Swanson, W. F., Roth, T. L., Graham, K., Horohov, D. W., and Godke, R. A. (1996) Kinetics of the humoral immune response to multiple treatments with exogenous gonadotropins and relation to ovarian responsiveness in domestic cats. A m. J. Vet. Res. 57, 302–307.
22. Niwa, K., Ohara, K., Hosoi, Y., and Iritani, A. (1985) Early events of in vitro fertilization of cat eggs by epididymal spermatozoa. J. Reprod. Fertil. 74, 657–660.
23. Pope, C. E., Gelwicks, E. J., Wachs, K. B., Keller, G. L., and Dresser B. L. (1989) In vitro fertilization in the domestic cat (Felis catus): A comparison between freshly collected and cooled semen. Theriogenology (abstr.)32, 241.
24. Pope, C. E., Gómez, M. C., King, A. L., Harris, R. F., and Dresser, B. L. (2003) Embryos produced in vitro after recovery of oocytes from cat ovaries stored at 4°C for 24 to 28 h retain the competence to develop into live kittens after transfer to recipients. Theriogenology (abstr.) 59, 308.

Large Felid Semen Cryopreservation

N.M. Loskutoff
Center for Conservation & Research, Omaha's Henry Doorly Zoo
September 2003

Publications using this protocol:

1) Crichton, E.G., E. Bedows, A.K. Miller-Lindhom, D.M. Baldwin, D.L. Armstrong, L.H. Graham, J. Ford, J.O. Gjorret, P. Hyttel, C.E. Pope, G. Vajta and N.M. Loskutoff (2003) The efficacy of porcine gonadotropins for repeated stimulation of ovarian activity for oocyte retrieval and in vitro embryo production and cryopreservation in Siberian tigers (*Panthera tigris altaica*). Biology of Reproduction 68: 105-113.
2) Gjorret, J.O., Crichton, E.G., Loskutoff, N.M., Armstrong, D.L. and Hyttel, P. Oocyte maturation, fertilization and early embryonic development in vitro in the Siberian tiger (*Panthera tigris altaica*). Molecular Reproduction and Development 63:79-88, 2002.
3) Stander-Breedt, H., Schwalbach, L.M., Greylig, J. and Loskutoff, N.M. (2002). Effect of different cryodiluents and thawing methods on the motility and heterologous fertilizing capacity of African lion (*Pathera leo*) spermatozoa. Proceedings of the 9th International Symposium on Spermatology, Cape Town, South Africa (6-12 October 2002).
4) Motlomelo, K.C., Schwalbach, L.M., Greylig, J. and Loskutoff, N.M. (2002). A novel sperm cryopreservation method for the South African cheetah (*Acinonyx jubatus*). Proceedings of the 9th International Symposium on Spermatology, Cape Town, South Africa (6-12 October 2002).
5) Nelson, K.L., E.G. Crichton, L. Doty, D. Volenec, J.M. Finnegan, R.G. Morato, C.E. Pope, B.L. Dresser, D.L. Armstrong and N.M. Loskutoff. (1999) Heterologous and homologous fertilizing capacity of cryopreserved felid sperm: a model for endangered species. Theriogenology 51:290, abst.

Materials Needed:

Sterile test tubes
Medium (e.g., Hepes-TL Solution, BioWhittaker, Walkersville, MD, USA; Cat # 04-616F)
Fetal calf serum (cell culture tested; e.g., Sigma, Gibco or HyClone)
Sterile pipettes
Glass slides and cover glasses
Compound microscope
Vital stain (e.g., eosin nigrosin or eosin B – fast green)
Hemacytometer
Glutaraldehyde (2.5%)
Slide warmer
Cryodiluents: Non-glycerated and glycerated (see below)
Straws (0.25 ml)
Sealing powder
Refrigerator (walk-in, preferably)
Dry ice block (10 lb) or styrofoam "boat"
Goblets and canes
Liquid nitrogen storage tank

Cryodiluents: generally, a TES-Tris (TEST Yolk Buffer, Refrigeration Medium; Irvine Scientific, Santa Ana, CA, Tel: 800-437-5706; Cat #9972) or Tris-citrate-buffered, egg yolk-based diluent (e.g., Biladyl A, Minitube of America, Madison, WI, Tel: 608-845-1502). The Irvine cryodiluent already contains egg yolk, whereas fresh egg yolk must be added to Biladyl A just before use (one-half liter is prepared from a concentrate using distilled/deionized water that can be frozen as aliquots and stored long term).

Ideally, semen is frozen using two steps: the initial dilution in the egg yolk-based extender, then after refrigeration, the second fraction containing 8% glycerol (cell culture quality) is slowly added (for a final glycerol concentration of 4% v/v). For very poor quality samples, glycerol should be substituted with cell culture quality grade dimethyl sulfoxide (DMSO) as the cryoprotective agent.

Processing and Evaluation

1. Before leaving the lab to collect the semen by electroejaculation, organize all of the materials necessary. In addition, place a 15 ml tube of Biladyl A (Minitube) as well as one 15 ml tube each of Biladyl A containing 8% glycerol **or** 8% DMSO in the water bath (and place a tube containing 0.1 ml fetal calf serum on the counter) to thaw, and one 15 ml tube of Hepes-TL solution (BioWhittaker) to warm.

2. Collect the tiger or lion semen according to the instructions detailed on the large felid electroejaculation protocol, and be sure to change tubes often (to avoid the possibility of contaminating a semen sample with urine). A total of 10-15 separate tubes may be used for a final total volume of semen of 3-7 ml from an adult tiger. Use pre-warmed Hepes-TL solution to rinse down any droplets of semen found on the sides of the tubes. Label the individual tubes in numerical order of collection, then keep them in a pocket against your body, or in an insulated container next to warm packs until they are transported back to the lab.

3. Once back in the lab, examine each tube separately for overall motility and rate of forward progression (0= no movement to 5=fast, linear progression). Pool all of the tubes containing greater than 40% overall motility and centrifuge at 300 x g for 10 min to concentrate the sperm.

4. Discard the supernatant down to the pellet. Measure the volume of the pellet and dilute with pre-warmed Hepes-TL solution containing 10% sterile fetal calf serum (typically, the pellet is reconstituted in 500 µl (0.5 ml) of medium). A thorough examination should then be made and recorded of this pooled sample: overall motility and rate of forward progression (after 1:100 dilution in Hepes-TL and incubation on the slide warmer for 3-5 min), percentage of live and acrosome-intact sperm using eosin B-fast green vital staining, and an evaluation of structural morphology after fixation (1:100) in 2.5% glutaraldehyde). For accuracy in these assessments, be sure to gently yet thoroughly mix the sample before aliquoting (since the dead sperm will fall to the bottom of the tube).

Cryopreservation

1. At room temperature, add Biladyl A at a 2:1 dilution ratio (Biladyl A : sperm+Hepes-TL) then place this tube either in a beaker of room temperature water, or in a styrofoam holder, before placing in refrigeration (this will ensure slower cooling and avoid the possibility of cold-shock). At this time also place the cryoprotectants (Biladyl A containing 8% glycerol, or 8% DMSO if the sample is very poor quality), pre-labeled 0.25 ml straws, transfer pipettes and PVP straw

sealing powder in the refrigerator to cool. Straws should be labeled with the species, animal ID number (ISIS) and the date of collection.

2. After one hour of refrigeration (4ºC), add the pre-cooled cryoprotectants very slowly (drop by drop) using a pre-cooled transfer pipette at a 1:1 ratio to the extended semen for a final cryoprotectant concentration (glycerol or DMSO) of 4% v/v. Be sure to gently mix the sample slowly throughout the process.

3. After the cryoprotectant is added, fill the pre-labeled 0.25 ml straws and seal them with the PVP powder (5 mm column plug minimum) . Be sure to wipe off any liquid on the outer surfaces of the straws to prevent them from sticking to each other. Refrigerate the straws (in a horizontal position) an additional two hours before freezing.

4. To freeze the straws, place them directly on top of a block of dry ice contained in an insulated (e.g., styrofoam) container in the refrigerator. Be sure to hold the straws only at the cotton plug end to avoid warming the sperm by contact with your fingers. After placing all of the straws on the dry ice, wait a minimum of 10 min (maximum of 30 min) before plunging them directly and quickly into liquid nitrogen. As an alternative to dry ice, the straws can be frozen by placing them on a styrofoam "boat" (see Fig 1) that is exactly one inch above the surface of liquid nitrogen; hold for 10 min before plunging.

5. For long-term storage, goblets should be submerged and the straws inserted without removing from the liquid nitrogen. At no time should the straws be removed from the liquid nitrogen until they are thawed. Carefully and quickly place the filled goblets onto a cane and place the cane into a large liquid nitrogen storage container (flask or dewar). Record the total number of straws frozen on the semen evaluation form, along with the identification information recorded on the cane, and the number of the canister and dewar they are stored in.

Thawing

1. Have a water bath set at 50ºC and place this right next to the dewar containing the frozen straws you wish to thaw.

2. To remove straws from a cane – be sure not to pull the entire canister up beyond the neck of the dewar (the higher the canister is pulled away from the liquid nitrogen, the more chance there is to cause damage to the other sperm samples stored there – for instance, one-half an inch out of the liquid nitrogen warms the sample +100ºC and this repeated warming and re-cooling back into the liquid nitrogen damages the sperm irreparably). Select the appropriate cane, then quickly bring it up to the neck of the dewar and remove a straw using forceps (not fingers).

3. Place the straw(s) directly into the water bath and swirl for EXACTLY 8 sec. Hold the straw(s) by the PVP plug end to ensure that it does not pop out during thawing (and thereby potentially exposing the sperm directly to water).

4. Wipe the straw well to remove any water, then decant into a sterile conical tube. Place a small aliquot (e.g., 25 µl) on a glass slide and incubate on a slide warmer for 3-5 min before assessing the sample for overall motility and rate of forward progression.

5. To isolate the live and motile sperm from the cryopreserved semen sample, carefully layer the thawed sample on top of a Percoll gradient (1 ml of 90% under 1 ml of 45%) in a 15 ml conical tube and centrifuge at 700 x g for 30 min. Carefully remove the top layers down to the pellet and examine for quality before utilization.

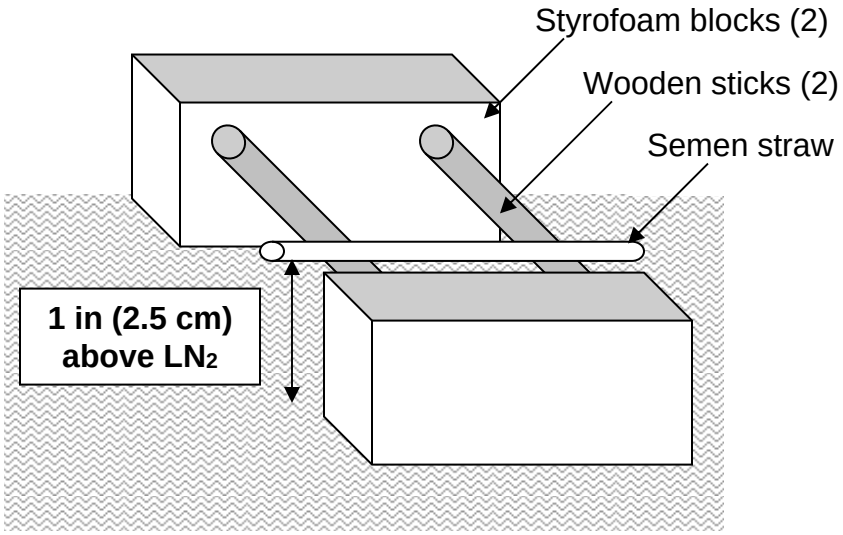


Figure 1. Styrofoam "boat" constructed from two blocks of styrofoam attached by two wooden sticks (far enough apart to securely hold the straws). Straws are laid on top of the sticks before the boat is carefully placed into an insulated container of liquid nitrogen (LN_2). The sticks are positioned so that the straws will be 1 in (2.5 cm) above the LN_2 surface. The container lid is replaced and after 10 min, the boat is lifted at an angle to plunge the straws directly into the LN_2.

Canid Semen Cryopreservation

W. Farstad
Norwegian School of Veterinary Science
September 2006

Publications using this protocol:

1) Andersen K (1975) Insemination with frozen dog semen based on a new insemination technique. *Zuchthygiene* **10,**1.
2) Farstad W (1984) Bitch fertility after natural mating and after artificial insemination with fresh or frozen semen. *J Small Anim Pract* **25,** 561-65.
3) Farstad W and Andersen Berg K (1989) Factors influencing the success rate of artificial insemination in the dog. *J Reprod Fert* **39** (Suppl), 289-92.
4) Farstad W (1996) Semen cryopreservation in dogs and foxes. Review. *Proceed, XIII Int. Congr. Anim. Reprod. and Artific. Insem. Sydney. Anim Reprod Sci* **42** 1-4, 251-60.
5) Hofmo PO (1988) *Studies on Cryopreservation of Fox Spermatozoa and Evaluation of the Fertilizing Capacity of Frozen-thawed Silver Fox Spermatozoa*. PhD thesis, Norwegian College of Veterinary Medicine, Oslo, 70p.
6) Linde-Forsberg C (1995) Artificial insemination with fresh, chilled extended and frozen-thawed semen in the dog. *Seminars in Veterinary Medicine and Surgery (Small animal),* **1,** 48-58.
7) Oettlé E (1986) Using a new acrosome stain to evaluate sperm morphology. *Vet Med **3,*** 263-66.
8) Thomassen, R, Farstad W, Krogenæs A, Fougner JA, K Andersen Berg. 2001. Artificial insemination in dogs.a retrospective study. J Reprod Fertil, **57** (suppl) 341-46.
9) Thomassen, R, Sanson G, Krogenæs A, Fougner JA, Andersen Berg,K, W.Farstad. 2006. Artificial insemination with frozen semen in dogs. A review of ten years of using the nonsurgical approach. Theriogenology (suppl) in press(available at www.sciencedirect.com)

Semen Quality Criteria

Canine semen intended for routine cryopreservation should be collected before a bitch in oestrus, and the semen should be well-fractioned, saving only the sperm dense fraction for cryopreservation. Morphology should exceed 75% progressively motile sperm prior to freezing and with less than 10% protoplasmic droplets or other major sperm defects, such as damage to the acrosome, micro-, macro- or deformed sperm heads, coiled tails and swollen mid-piece. Progressive motility should be rapid and steady and be at least 4-5 on a scale of 1-5, with 5 being the most rapid movement. Post-thaw survival should exceed 40% motility and a progressive speed of at least 3 to expect normal conception rates using the following procedures and intrauterine artificial insemination.

A variety of freezing regimes, extenders and thawing protocols have been published in the literature (for review, see Farstad, 1996). The author has worked mainly with the TRIS extender which has given good results for dog and fox semen over a number of years
(Andersen, 1975, Farstad, 1984, Farstad and Andersen Berg, 1989; Linde- Forsberg, 1995; Farstad, 1996, Thomassen et al, 2001, Thomassen et al, 2006), currently yielding conception results of 75-80% in

dogs and foxes following intrauterine deposition, 50-150 million spermatozoa per insemination on two occasions 24h apart. In the author's laboratory the following procedure for processing and freezing of dog and fox semen is used.

Preparation and Addition of Extender

The TRIS base solution with antibiotics given earlier with the addition of the cryoprotector glycerol is used as freezing solution (Table 7.1). The volume of glycerol added to the recipe in Table.7.1 is either 12ml (6v/v%) or 16ml (8v/v%) depending on the freezing regime, 6% for automatic freezing and 8% for manual freezing (see below). The distilled water content in the recipe is adjusted accordingly to, either 188ml or 184 ml, respectively, instead of the original 200 ml.

The TRIS base solution with glycerol is mixed just prior to use with 20% egg yolk. The egg yolk is obtained from specific pathogen free hens for export, and from consumer's eggs for domestic use. The yolk is separated from the egg white. Then the yolk is rolled over a clean piece of paper tissue, gently broken and allowed to run into a funnel. Then the yolk is broken down by vigorous beating with a glass rod to minimise the size of the egg yolk particles. This reduces the frequency of attachment of sperm heads to the egg particles, which makes semen motility evaluation more difficult. The egg yolk is then mixed with the TRIS buffer which is pre-warmed to 30 °C. The mixture is stirred well, and warmed to 35 °C. A premixed ready- for- use- TRIS-egg yolk extender can be frozen and kept frozen for two-three months prior to use. Gentle thawing in a water bath at 35 °C with subsequent gentle shaking is recommended.

After the microscopic evaluation of the semen according to the criteria mentioned initially, the sperm rich fraction of the ejaculate is diluted by drop-wise addition of 35 °C extender at room temperature until the desired concentration is reached. In the author's laboratory a concentration of a total of 100 million spermatozoa ml^{-1} is used routinely for freezing. However, when semen is of high quality half the concentration, i.e. 50 million ml^{-1}, may be used.

Cooling and Equilibration

After dilution, a sample is examined microscopically, and the extended semen is then poured into the previously mentioned plastic Nunc™ centrifugation tubes which are placed into a beaker holding 35°C water. The water beaker is then placed into a walk-in- refrigerator at 4 °C and left for 2h. During this time the semen is cooled to 4-5 °C, and the penetrating cryoprotectant glycerol diffuses into the sperm cell cytoplasm (equilibration).

Semen Packaging

The semen is stirred gently to remix the semen after the 2h of equilibration and cooling, and is immediately placed into 0.5ml plastic straws (IMV®, L'aigle, France or Minitüb®, Tiefenbach, Germany). This can be done by using commercially available filling devices, or by suction through a latex tube with a mouthpiece. The semen must be filled in such a way that the powder between the two filter tips at the filter end of the straw is filled with liquid and solidifies. The straws are first filled half-way, and a small air bubble is allowed into the straw before the rest of the straw is filled to approximately 1cm from the top. The air bubble prevents semen from being expelled due to change of pressure within the straw during thawing. Then the straw is sealed either by ultrasound, or by using commercially available sealing balls or sealing powders.

Freezing Regimens

There are principally two alternatives for freezing, either a manual, static protocol or an automatic, dynamic protocol, and the glycerol content added to the extender varies between the two freezing methods (see earlier for adjustment of water content in buffer base). The manual protocol involves the use of a Styrofoam box (30cm x 40cm x 30cm) with a removable metal rack placed 10cm below the edge of the box which is filled with liquid nitrogen up to a level 4cm below the rack.

The pre-filled straws, maximum 8-10 at a time, are placed horizontally on top of the rack, using a forceps, and are then left on the rack in the nitrogen vapour for 8 min. The forceps are then cooled in liquid nitrogen and applied onto the straws one by one to ensure complete crystallization (seeding). The straws are subsequently plunged into the liquid nitrogen. This freezing protocol is called static because there is a constant non-regulated flow of vaporized nitrogen during cooling and freezing.

Automatic freezing involves the use of freezing machines. It is called dynamic because vaporized nitrogen is let into the freezing chamber at variable speed by the pre-set freezing program, allowing the cooling and freezing rates to be regulated. One such freezing program using a Planer 10™ freezing machine was developed for fox semen by Hofmo (1988) and is used for dog semen in our laboratory when large quantities of semen are frozen. Straws are frozen horizontally on a rack with a removable lid, and several racks can be put into the freezing chamber. The freezing program follows the regime:

-2 °C/min from +4 °C to -7°C
-50 °C/ minute from -7 °C to -100 °C
-25 °C/minute from -100 °C to -180°C

After the program is completed the whole rack is removed and placed directly into liquid nitrogen.

Thawing

The straws are thawed in a water bath (thermos) at 70°C for 8 sec. After thawing the straw should be held vertically, filter tip down and sealed end up, and shaken to allow the air bubble in the middle of the straw to escape to the top of the straw. The straw is cut at the sealed end and a small drop of semen is placed on a slide on the warming plate for microscopic examination of post thaw quality. Often the spermatozoa need some time to start moving after thawing, but two minutes on the warming plate should ensure restoration of motility. Since other laboratories may use other freezing regimes, it is important to follow the instructions for thawing from the laboratory or company which has provided the frozen semen, since freezing and thawing regimes are closely connected.

ELISA Protocol: Measuring Anti-Gonadotropin Immunoglobulin Titers

W.F. Swanson
Center for Conservation & Research, Cincinnati Zoo
September 2002

Publications using this protocol:

1) Swanson WF, TL Roth, K Graham, DW Horohov and RA Godke. 1996. Kinetics of the humoral immune response to multiple treatments with exogenous gonadotropins and relation to ovarian responsiveness in domestic cats. American Journal of Veterinary Research 57:302-307.
2) Swanson WF, DW Horohov and RA Godke. 1995. Production of exogenous gonadotrophin-neutralizing immunoglobulins in cats following repeated eCG/hCG treatment and relevance for assisted reproduction in felids. Journal of Reproduction and Fertility 105:35-41.

ELISA Protocol

1. Exogenous gonadotropins (eCG, hCG) are diluted in 0.06M sodium bicarbonate buffer (pH 9.6) to a concentration of 10 ng protein per □l and pipetted in 50 □l aliquots (containing 500 ng protein) into each well of a 96-well flat-bottom microtiter plate (Immulon 4, Dynatech Labs).

2. Plates are sealed and maintained overnight at 4°C.

3. Plates are washed five times with an automated plate washer (Dynatech MRW) using 0.01 M PBS (containing 0.1% Tween-20; EPBS-Tween) and blotted dry.

4. Thawed serum samples are diluted in EPBS-Tween to concentrations of 1:100, 1:200 and 1:400 (or greater, depending on optical density values) and pipetted (100 □l/well) into test wells in triplicate. For appropriate control wells (see below), add 100 □l of EPBS-Tween to each well.

5. Plates are sealed and incubated at RT (22°C) for one hour.

6. Plates are washed 5X with EPBS-Tween and blotted dry.

7. Peroxidase-conjugated, rabbit anti-cat IgG (affinity purified to whole IgG; Rockland Laboratories, Gilbertsville, PA) is diluted 1:2000 in EPBS-Tween and pipetted (100 □l/well) into test wells.

8. Sealed plates are incubated for 30 minutes at 37°C.

9. Plates are washed 5X with EPBS-Tween and blotted dry.

10. OPD solution [o-phenylenediamine, Sigma; 1 mg/ml dissolved in 0.05M citrate buffer (pH 4.5) and 0.05% hydrogen peroxide) is added to each well (100 □l/well) and plates are maintained in the dark (since light sensitive) for 30 minutes at RT (22°C).

11. The color reaction is stopped with 2.5M sulfuric acid (50 □l/well) and optical density is measured at 492 nm with an automated plate reader (Dynex MRX).

Controls (for each plate - in triplicate)

1. Blank - only buffers added to wells
2. Conjugate control - no cat serum - all else the same as test samples
3. Serum control - add cat serum but no conjugate (anti-cat IgG) - all else the same
4. Substrate control - like blanks except citrate buffer contains OPD
5. Negative standard - serum from known naive cat(s) -usually domestic
6. Positive standard - serum from known positive cat(s) - usually domestic

Reagents

1. eCG or hCG (Sigma) or LH (Sioux Biochemical) - dissolve in 0.06 M sodium bicarbonate buffer (pH 9.6) to a concentration of 10 ng/□l
2. 0.06 M sodium bicarbonate - dissolve 0.5041 g sodium bicarbonate in 100 ml of milipore water - add ~200 □l 5 N NaOH to adjust pH to 9.6
3. EPBS-Tween - for 2 liters, combine 16 g NaCl, 0.4 g KCl, 2.173 g Na_2HPO_4, 0.521 g KH_2PO_4 and qs with distilled water to 1998 ml. Bring to 2 liters by adding 2 ml of Tween-20 (0.1% solution)
4. Serum samples - dilute in EPBS-Tween (diluted from 1:100 to 1:1600, depending on optical density range)
5. Conjugate (indicator or 2° antibody) - rabbit anti-cat IgG, conjugated to horseradish peroxidase (HRP) - diluted 1:2000 with EPBS-Tween
6. OPD - 10 mg OPD dissolved in 10 ml citrate buffer with 5 □l hydrogen peroxide stock (30%) added
7. Citrate buffer - 0.05 M citrate buffer (pH 4.0 - 4.5) - mix equal molar concentrations of sodium citrate and citric acid solutions (i.e., add 1.676 g sodium citrate to 100 ml water and 0.961 g citric acid to 100 ml water - mix 1:1 and pH to 4.5 with 5 N HCl)
8. Stopping solution - 2.5 M sulfuric acid - qs stock sulfuric acid with distilled water to appropriate molarity (dilute 14 ml of stock sulfuric acid to 100 ml with milipore water)

Assay Notes:

Blocking - If desired, plates may be blocked after the antigen (eCG or hCG) incubation and initial plate wash using a 0.1% BSA solution (in EPBS). Add 100 □l of BSA solution to all wells and maintain at

RT for 3 hours before washing and then proceeding with the regular assay. However, in previous studies, blocking plates did not decrease optical density values for any tested domestic cat or non-domestic cat samples and was omitted from the routine assay. For most species, the relatively high Tween concentration, multiple plate washes and use of an affinity-purified conjugate minimize non-specific binding.

Standards - A domestic cat positive and negative standard may be included on each plate to ensure that the assay is working properly and to determine the appropriate serum sample dilution (to obtain values within the linear portion of the optical density curve). For most domestic cat samples, a dilution of 1:100 to 1:400 is suitable.

Determining Positivity - For each non-domestic species, the best approach is to compare gonadotropin-binding levels (mean optical density values) from each female before gonadotropin treatment and then after each treatment and then use the mean OD value (+ 3 SD) for all naive samples as your cut-off value between negative and positive titers. In reality, true naive samples are rarely available from each no-ndomestic female so samples from the female following the first gonadotropin treatment are used instead. We assume that IgG levels don't increase much between the initial gonadotropin injection and blood sampling (usually ~5-6 days) and that IgM is not bound significantly by the conjugate. To verify this, we usually evaluate serum samples from 2-3 males per species and compare mean OD values to females treated once with gonadotropins. If significantly different, the mean OD values for males (+ 3 SD) can be used as the -/+ cut-off. If not different, the mean OD values (+ 3 SD) of 1X-stimulated females may be used instead.

Wild Cattle, Buffalo and Bison Semen Collection by Rectal Probe Electrostimulation (Electroejaculation)

N.M. Loskutoff
Center for Conservation & Research, Omaha's Henry Doorly Zoo
September 2003

Publications using this protocol:

1) Janesch, L., Rohr, J., Volenec, D., Grobler, D., Puffer, A., Prokupek, A.K., Armstrong, D.L., Dankoff, S., Curro, T., Simmons, H., Crichton, E.G., Hamilton, J., Rasmussen, L., Zimmermann, D., Lomneth, R., Wood, R., Wood, D. and Loskutoff, N.M. (2001) Post-thaw viability of wild cattle (*Bos gaurus*) and buffalo (*Syncerus caffer*) sperm cryopreserved using a novel, non-animal protein cryodiluent. Theriogenology 55:387, abst.
2) Puffer, A., de la Rey, R., de la Rey, M., Hansen, H., Grobler, D., Hofmeyr, M., Malan, J.H., Armstrong, D.L., Dankoff, S., Rasmussen, L., York, D., Zimmermann, D. and Loskutoff, N.M. (2001) Choice of chemicals used in immobilization protocols can significantly affect semen quality in ejaculates collected from free-ranging African buffalo (*Syncerus caffer*). Theriogenology 55:398, abst.
3) MacLean, R.A., Swanson, H., Hancock, J., O'Brien, E., Williams, K.R. and Loskutoff, N.M. (1998) Comparison of three techniques for separating motile sperm in cryopreserved *Bos gaurus* semen. Theriogenology 49:263, abst.

Materials Needed:

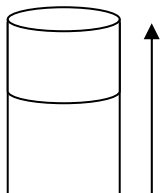
Rectal probe (see Fig. 1), sand paper
Electrostimulator (e.g., Platz or Beltz models – for contact details, see Technology Subcommittee Resources section)
Extension cord
Lubricant (non-spermicidal)
Palpation sleeves, latex gloves
Gauze, paper towels
50 ml sterile collection tubes with rack
Insulated container
Medium (e.g., TL Hepes Bio Whittaker)
Permanent ink pen
Warming packs (if necessary)
Disinfectant

Procedure

1. After the bull is chemically immobilized* and his legs are safely secured with ropes, carefully clean out the rectum as far cranially as possible.

**Note that there are certain types of chemical immobilizing agents that detrimentally affect the quality of semen collected by electroejaculation, e.g., Azaperone, Medetomidine, etc.*

~ 40 cm length



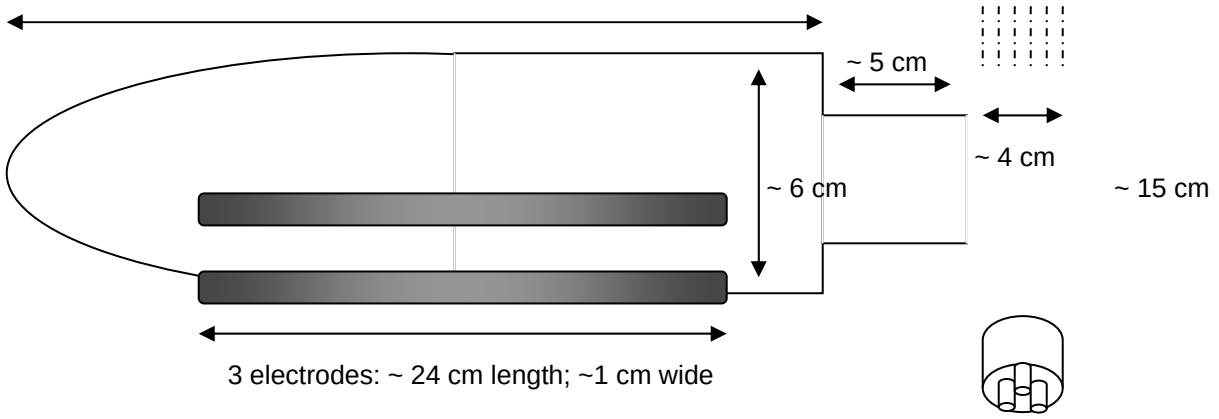


Figure 1. Diagram of rectal probe suitable for semen collection by electroejaculation from cattle (domestic and wild), buffalo and bison.

2. Gently sand down electrodes before each use to ensure proper contact.

3. After turning on the electrostimulator and checking to see if the gauges work properly, gently insert the rectal probe with the three longitudinal electrodes facing ventrally (always be sure to turn on the electrostimulator BEFORE placing the probe to avoid shock from electrical surges).

4. After checking with the veterinary personnel, the person collecting the semen can approach the sheath area. If the penis can be easily manipulated out of the sheath, it should be held gently using one piece of gauze [be sure not to apply too much pressure since this can prevent the emission of semen]. If the penis cannot be easily manipulated out of the sheath, then carefully place the 50 ml collection tube inside the prepuce [try to avoid the dirty outer areas] and clamp down on the neck of the tube using the thumb and forefinger. When the stimulations begin, be sure that the sheath is extended as far forward and straight as possible to permit unobstructed flow of semen. Commonly, the bull will extend the penis out of the sheath after the stimulations begin; if this happens, stop for a moment to secure the penis with a piece of gauze.

5. When the rectal probe is in place, the person doing the collection is ready and, most importantly, the veterinary and keeper personnel are certain that the animal is secure, begin the stimulation protocol. The stimulations are administered by taking about 2 sec to reach the voltage limit, hold for 2 sec, then quickly turning the rheostat back to 0. The voltage limit will begin at 2 V, then increase by 0.5 V increments to a maximum of 10 V. This will complete one series of stimulations. The person holding the probe should be sure that both legs are extending with equal force in response to the stimulations.

6. After about a minute of rest to change tubes, a second series can be administered. Usually only three complete series of stimulations are necessary to collect an average total volume of semen (e.g., about 20-40 ml on a first collection, about 10-20 ml on a second collection performed within 3 weeks) Semen is very commonly produced between 6 - 8 V; therefore, it is sometimes advantageous to administer 2-5 sets of stimulations at 6, 6.5, 7, 7.5, 8 & 8.5 V. If this is done, then only two complete series of stimulations are necessary.

7. Change tubes often. The color of the semen will vary from slightly opaque to milky white. If there appears to be urine contamination in the semen (yellowish appearance), then immediately dilute the sample 1:1 with medium (e.g., Hepes-buffered Tyrodes-lactate medium or TL-Hepes, BioWhittaker, Walkersville, MD, USA; Cat # 04-616F) and change to a new tube for collection.

8. It is helpful for future semen collections on specific bulls to record the responses at each voltage level and series and the time taken to achieve erection and ejaculation. A sample worksheet for recording this information is attached.

9. If the ambient temperature falls below 70^{o}F/20^{o}C, it will be important to wrap the collection tube with a warm pack to prevent cold-shock. The tubes should be maintained with warm packs in an insulated container until taken to the lab for processing.

Worksheet: Semen Collection by Rectal Probe Electrostimulation (Electroejaculation)

Date:_____ Species:_____ ID#:_____ Name:_____ Location:_____

Immobilization Protocol:
Duration Fasted:_____(feed) _____(water) Weight:_____ Pre-treatment:_____
Drug Regimen:_____
Supplementation:_____
Time Begin:_____ Time End:_____ Clinician:_____ Tech:_____

Blood Collection for:_____ Serum or Plasma (Anti-Coag?):_____ Vol:_____
Tissue/Excretus Collection:_____

Scrotal Measurements:
Length (Right):_____ Length (Left):_____ Texture:_____
Width (Right):_____ Width (Left):_____ Circumf:_____

Ambient Temp:_____ Rectal Temp:_____ Probe Size:_____
Electrostimulator:_____

Semen Collection Protocol:
Total Voltage Range:_____ Total Series:_____ Total Stimulations/Series:_____

Series:	1	2	3	4	5	6	Pool [ ]
Time Begin:							
Time End:							
Volts (range):							
mAmps (range):							
Erection (+/-):							
Volume (ml):							
Color:							
pH:							
Urine (+/-):							
Est. Motility (%):							
Linear Prog (0-5):							
Debris (type):							
Total Conc. ($x10^6$):							

Comments:_____
_____
_____
_____
_____

Superovulation and Embryo Transfer in the Dromedary Camel

J.A Skidmore
Camel Reproduction Centre, Dubai, U.A.E.
March 2003

Publications using this protocol:

1) Skidmore, J.A., Billah, M., Short, R.V. and Allen, W.R. (2002) Assisted reproductive techniques for hybridization of camelids. Reproduction, Fertility and Development 13 (8): 647-652.
2) Skidmore, J. A., Billah, M. and Allen, W.R. (2002). Factor affecting pregnancy rate after embryo transfer in dromedary camels. Reproduction, Fertility and Development 14 (2): 109-118.
3) Skidmore, J.A., Billah, M. and Allen, W.R. (1996). The ovarian follicular wave pattern and induction of ovulation in the mated and non-mated one-humped camel (*Camelus dromedarius*). Journal of Reproduction and Fertility 106, 185 – 192.
4) Skidmore, J.A., Billah, M. and Allen, W.R., (1992). Ultrasonographic and videoendoscopic monitoring of early fetal development in the dromedary camel. Proc. 1st Int. Camel Conf. pp. 193-201. Eds. W.R. Allen, A.J. Higgins, I.G. Mayhew, D.H. Snow and J.F. Wade. R & W Publications (Newmarket) Ltd. UK.

Superovulation

Superovulation treatments, to stimulate the growth of multiple follicles, include the use of exogenous gonadotrophins such as equine chorionic gonadotrophin (eCG) or FSH which may, or may not, be given after a period of progesterone priming. This progesterone priming can be given either in PRID form (progesterone releasing intravaginal device) inserted into the vagina for a period of seven days, or as daily injections of 150mg progesterone - in - oil for up to 15 days. However the best results, *i.e*. the best stimulation of the ovaries, occur if the camel is treated with exogenous gonadotrophins when there is minimum follicular activity in the ovaries. If follicles are present at the time of treatment these tend to develop into overlarge follicles before the new stimulated wave of follicles have had a chance to develop.

Follicle stimulating hormone (FSH)

FSH of **porcine or ovine** origin has been used for superovulation in camels. In the dromedary a total dose of 20 iu of ovine FSH (oFSH) or porcine FSH (pFSH) in 20ml is given over 4 days. Generally speaking two injections are given daily in gradually decreasing doses for example: Day 1: 2 x 4ml, Day 2: 2 x 3ml, Day 3: 2 x 2ml, and Day 4: 2 x 1ml.

Equine Chorionic Gonadotrophin (eCG)

Equine Chorionic Gonadotrophin is well known for its FSH activity and has been used to promote follicular development and superovulation in camels. The dosage of eCG used varies from 1500 - 6000iu. It is generally injected in a single dose one day before, or on the day of, completion of a 5 - 15 day progesterone regime.

Combined eCG and FSH

In my experience the best response is seen when a combination of both FSH and eCG are given. The eCG (2500iu) is given as a single injection on day 1 of treatment together with the first of the twice daily injections of FSH, followed by three more days of twice daily injections in decreasing doses of FSH as described above.

Problems with superovulation in camelidae

Superovulation treatments in the camelidae female are far from perfect as the ovulation response and embryo yield remain highly variable. The main problems are;

1. The high incidence of non-responsive females. Approximately 20-30% of superovulated females do not develop follicles. This is probably due to immunization against eCG in some females .

2. The high incidence of follicle luteinization before breeding. This is particularly prevalent in eCG -treated females and could be due to the LH activity of this hormone.

3. The high incidence of over stimulated ovaries. In some eCG or FSH super-ovulated females, the ovaries become very large and contain many generations of follicles with different sizes. This is probably due to an individual difference in response to the hormones.

4. Dromedary camels can become refractory to superovulation with FSH and eCG. This is probably caused by immunization against these hormones. We have observed a complete arrest of ovarian activity in some females that have been superovulated with these hormones repeatedly during several years.

Mating and induction of ovulation

In order to achieve a good ovulation rate, donors should be monitored by ultrasonography and palpation throughout the superovulation treatment and bred when the follicles reach a suitable size.

Follicles generally start to develop about 4 - 6 days after the start of treatment and reach 13 - 16mm in diameter approximately 8 - 12 days after the start of treatment.

The number of matings per donor can vary, but in our programmes we generally mate the donor twice at a 24h interval and although ovulation occurs in response to mating, we usually give donors a single intravenous injection of GnRH analogue (20□g Buserelin) at the time of the first mating in order to maximize ovulation response.

Embryo collection and evaluation

The methods of embryo collection from camelidae are similar to those described in other species.

Surgical embryo collection

After exteriorization of the uterus via laparotomy surgical embryo collection is possible, however the use of this technique is only justified when collection of embryos at the tubal stage, *ie*. at the morula stage of development, is desired.

The most widely used technique for the collection of embryos from camelidae remains the non-surgical technique.

Non-Surgical collection of embryos

The donor can either be placed in stocks or restrained sitting on the ground after being sedated (Fig 1.).

The rectum needs to be cleared of all faeces and the tail wrapped in a tail bandage before cleaning the perineal region thoroughly.

Some people like to use epidural anaesthesia which can be advantageous in llamas, alpacas and young dromedaries because of the smallness of the pelvis. However, in larger females it is not usually necessary, especially if they are already sedated.

Collection is made using a Gibbon Balloon (20 Gauge) or Foley catheter (18 - 20 Gauge for camels). Using a sterile gloved hand the catheter can be guided through the vagina; the cervix is then dilated manually and the catheter inserted. Once the catheter is through the cervix the cuff is inflated with 30-40 ml of air or PBS medium and pulled back against the internal *os* of the cervix to seal it. The uterus is then flushed repeatedly with 60 - 120ml of flushing medium, you can use commercially available bovine embryo flushing media or Dulbecco's phosphate buffered saline (DPBS) + 0.2% Bovine Serum Albumin (BSA), + kanamycin sulphate) using a total volume of approximately 500ml.

After each flush the medium is collected, by gravity flow, into sterile beakers and filtered through an embryo filter until only 20 - 30ml of medium remains. This is poured into a sterile petri dish and examined under a microscope for the presence of embryos. As many as 20 or more embryos have been recovered in a single flush but because not all the follicles will ovulate at the same time these embryos can vary in size.

Effect of timing on embryo recovery rate

It is now well established that in camelidae the embryo does not reach the uterus until day 6 or 6.5 after ovulation. Therefore any attempt to collect embryos before day 6 post-ovulation, results in low recovery rates.

In practice the best recovery rates from dromedaries are achieved when the uterus is flushed on day 7 or 8 after ovulation.

Figure 1. Transcervical embryo collection from a superovulated dromedary camel (J.A. Skidmore and M. Billah, Camel Reproduction Centre, Dubai, UAE).

Evaluation of camel embryos

Embryos recovered from the uterus in camelidae are generally at the hatched blastocyst stage. The size of the embryo is highly variable at different stages post-ovulation. However, the majority of the embryos collected at day 6.5 or 7 are already hatched blastocysts. The embryos recovered from the dromedary camel 7 days after mating are very variable in size and have a diameter ranging from 0.18 - 0.50 mm. This variability of the stage of development is probably due to the wide spread of ovulations in superovulated animals. Hatched embryos continue to grow rapidly and become easily visible to the naked eye as they expand.

The evaluation system used by most authors classifies the embryo into 5 grades according to their morphological characteristics and stage of development (Table 1; Figure 2). The clinician should look for abnormalities such as

- extruded blastomeres (i.e. individual cells which have been extruded from the cell mass).
- signs of degeneration (dark areas), and
- obvious morphological anomalies such as folding or wrinkling.

Table 1. Classification of camel embryos.

Grade	Characteristics
Grade I	Excellent quality embryo. Size corresponds to the stage of collection in relation to ovulation. Before day 8 it should be perfectly spherical with a smooth surface.
Grade II	Good embryo, same as above with some irregularities of the contour and very few protruded cells.
Grade III	Medium quality , small embryo with dark patches, irregular contour and some protruded cells.
Grade IV	Collapsed embryos showing dark areas of degeneration and many extruded cells.
Grade V	Non-transferable. Collapsed very dark embryos or embryos that are retarded, dark morulae, and all stages that are younger than morula or unfertilized ova.

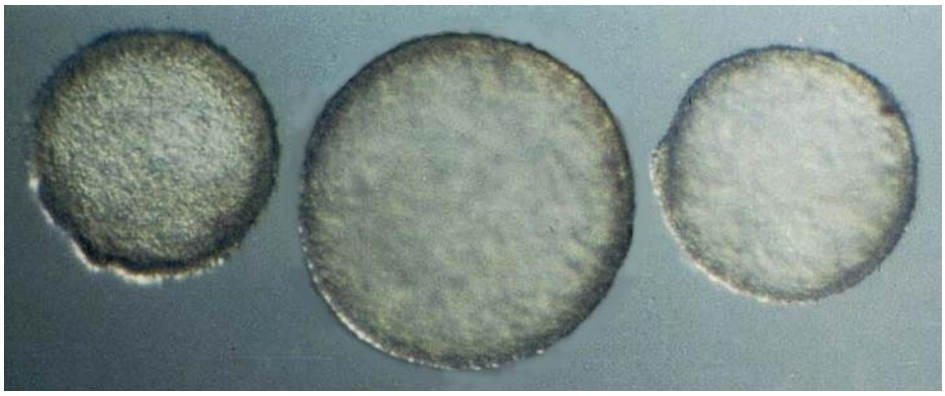


Figure 2. Hatched, Day 7 blastocysts recovered transcervically from a superovulated dromedary camel (sizes range from 175 to 500 µm in diameter).

Management of recipients

The quality of the recipient is the most important factor in the success of any embryo transfer program. The two main aspects of selection of recipients for embryo transfer are:

- the screening of reproductive and health problems and
- the preparation and synchronization with the donor.

Criteria for selection of recipients

The screening programme can be summarized as follows:

History: The potential recipient should be young (less than 12 years of age), have had at least one normal pregnancy with a normal delivery, and be either currently pregnant or recently weaned.

General examination of the recipient should focus on conformation and a good body condition and on symptoms of debilitating or contagious diseases. All potential recipients should be tested for brucellosis and trypanosomiasis.

A complete breeding-soundness examination should be done on the potential recipient including palpation and ultrasonography of the reproductive tract, uterine culture, vaginal examination and examination of the udder**.**

Synchronization with donors

Synchronization of the reproductive cycle between the donor and the recipient is very critical and embryo transfer results in the dromedary suggest that the best recipient should have ovulated 24 - 48 hours after the donor. Transfer of embryos into recipients that have ovulated one day before the donor, or three of more days afterwards, result in very low pregnancy rates.

Synchronization of follicular development and ovulation

Synchronization of ovulation between the donor and recipient can be approached using two methods:

- selection of recipients from a random group or
- preparation of recipients in such a manner that follicular development is synchronized with that of the donor.

In using the first technique a group of cycling recipients are examined 24 hours after the donor is bred and all females that have a mature follicle (1.3 - 1.7cm in diameter) are treated with GnRH or hCG. This method of selection is time consuming and can only be used if the number of donors are limited.

Synchronization of follicular development in donors and recipients has been attempted using progestagen with variable degrees of success.

Synchronization of recipients with Progesterone Releasing Intravaginal Devices (PRID's) has been attempted in the dromedary but was not successful.

Better results can be obtained when recipients are induced to ovulate with hCG or GnRH following a treatment combining progesterone and eCG. The recipients are treated daily with progesterone (100mg/day) for 10 to 15 days, to try and dampen the development of more follicles, and on the last day of progesterone treatment, 1500 - 2500 iu eCG is injected to induce follicular development. Progesterone treatment is scheduled to end on the day of injection of gonadotrophin in the donor in an attempt to synchronize the recipient and donor. The eCG treatment guarantees the presence of mature follicles in the recipient at the same time or 24 - 48 hours after the donor.

Preparation of recipients with progesterone

Synchronization between the embryo and the uterus can be obtained by progesterone therapy, without induction of ovulation. Progesterone (100mg) is given daily starting 2 days after mating of the donor. However, because there is no CL, progesterone treatment has to be continued throughout pregnancy.

Screening of recipients

All recipients should be screened on the day of transfer to ascertain that ovulation has occurred and that a mature CL is present. This can be done either by determination of progesterone concentration in the blood or by ultrasonographic visualization of the CL.

Transfer of embryos

Embryos can be transferred surgically or non-surgically.

Surgical embryo transfer

Surgical embryo transfer in the dromedary and Bactrian camels is done via the left flank laparotomy. The embryo is transferred into the uterine cavity through a puncture made in the exteriorized horn by a Pasteur pippette. However, this technique cannot be used in young and primiparous animals because the uterine horn is too short and difficult to exteriorize.

Non-surgical technique

The non-surgical technique for embryo transfer consists of placing the embryo directly into the uterine lumen through the cervix using a regular bovine insemination gun. The embryo is loaded into a 0.25ml or 0.5ml sterile plastic straw and placed in the gun for transfer. The inseminating gun is first covered by a sterile sheath with a side opening, so that the embryos can escape even if the pipette is up against the wall of the uterus, then a second plastic sanitary sheath.

The recipient is prepared in the same manner described for embryo collection. Then the embryo is transferred as follows:

1. The inseminating gun is introduced into the vagina and guided towards the cervix using a sterile gloved hand.
2. The sanitary sheath is perforated after passage of the first cervical ring, by pulling the plastic sheath backwards towards the technician, and the gun is further guided into one of the uterine horns with a hand in the rectum.
3. The plunger of the transfer pipette is pushed home and the embryo deposited into the uterus.

Bovine IVM/IVF/IVC Protocol

R.L. Krisher
Purdue University
September 2000

Publications using this protocol:

1) Krisher, R.L., Lane, M., Bavister, B.D. (1999) Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. Biology of Reproduction 60: 1345-1352.
2) Krisher R.L., Bavister, B.D. (1999) Enhanced glycolysis after maturation of bovine oocytes in vitro is associated with increased developmental competence. Molecular Reproduction and Development 53:19-26.
3) Gandhi, A.P., Lane, M., Gardner, D.K., Krisher, R.L. (2000) A single medium supports development of bovine embryos throughout maturation, fertilization and culture. Human Reproduction 15:395-401.

In Vitro Maturation

1. On average, each ovary should yield approximately 3 oocytes. On the morning ovaries are expected, prepare the working medium for the day. First, make 3 L of 0.9% saline in a large flask. The measurements do not have to be exact; this is for washing ovaries. The water should be drawn from the RO system. The flask should be placed in the dry oven to warm. Make HEPES, 100 ml for aspiration. If HEPES is pre-made, aliquot into a bottle and add PSA. Place it in the dry oven to warm. Make the appropriate maturation medium. Make maturation drops—25 ul in a 1007 dish, add 10 ml mineral oil, add another 25 ul to give 50 ul drops. Sigma embryo tested mineral oil should be used throughout this protocol. Place the dishes in the incubator to equilibrate for at least 2 hours, preferably 4. Maturation and fertilization are done at 39°C with 5% CO2 in air. Each person should have his or her own shelf or shelves in the incubator. Names can be labeled with tape on the outside of the glass door.

2. Set up for aspiration. The aspiration station should be in a room separate to the culture room. Place white absorbent paper beneath the work area. Turn on the slide warmer in the morning if it is not already on. Place the glass beakers in the warm oven, along with the styrofoam tray and several 50 ml tubes. Get out the aspiration stoppers and 18 g, 1 1/2" needles. When the ovaries arrive, remove the beakers, tray and tubes from the oven, place the stoppers on the tubes, attach the needle and filter. If it is a hand held unit, make sure the vacuum hose is attached to the upper syringe and the filter to the lower one. For the best suction, set up only as many aspiration stations as you need, so there are no suction tubes left without connections to a stopper. The number of suction tubes can be modified at the Y joint by the pump. Place several paper towels, gloves, and a plastic bag at each station.

3. When the ovaries arrive, put on a lab coat. Take the temperature of the ovaries and the saline wash. Pour the ovaries into a large beaker. Wash out the ovary container. Pour the excess blood off the ovaries. Wash them twice with the warmed saline in the large beaker, then dump them into the smaller glass beakers and fill the beaker with saline so the ovaries are covered. Place the beakers on

the slide warmer by the aspiration setup. If there are more beakers than can be aspirated at once, place them in the warm oven until use.

4. Aspirate follicles from 2-10 mm on the ovary, placing the ovaries in the plastic bag after you are through. Pierce the surface of the ovary just outside the visible boundary of the follicle. Go into the follicle under the surface of the ovary, and collect the fluid. While aspirating the fluid, use the needle to scrape the follicle walls to insure the oocyte is recovered. Collect the fluid in the 50 ml tubes. Collect about 35 ml per tube. Be careful not to aspirate any fluid into the vacuum tubing. When the tubes get full, hold them in the styrofoam tray on the slide warmer. Change the needle if it gets dull. When done aspirating, count the ovaries if necessary and place them in the plastic bag, tie/tape it closed and leave it for disposal after the oocytes are collected. Remove your lab coat, wash your hands and bring the tubes into the culture room. Also bring the HEPES medium from the dry oven. Do not wear the lab coat or gloves used for aspiration into the culture room.

5. All work with oocytes and embryos in this protocol should be done as quickly as possible. All dishes containing oocytes or embryos should be worked with on the slide warmers if possible, and time out of the incubator for all oocytes, embryos and/or dishes should be minimized. Opening of the incubator door should always be minimized in regard to number of times opened, length of time open and width of opening.

6. Set up for searching the aspiration tubes. Get out 1 1007 dish, 3 1008 dishes, several grid dishes (# of tubes of follicular fluid + 1), a drummond pipet with tips, a small bulb, and a long sterile pipette. Draw lines on the bottom of the 1007 dish. Fill the 1007 and 1008 dishes with HEPES medium—you can either pour or pipette. Sweep the bottom of one aspiration tube, using the bulb and Pasteur pipette. Pull sediment off of the bottom of the 50 ml tube into the pipette to approximately one inch past the beginning of the widened part. Place the sediment in a grid dish and add HEPES. Swirl to mix. Wipe off the drummond plunger wire with alcohol, dry with kim wipe. With a new tip, search the grid dish for oocytes. The magnification should be such that one square of the grid dish fits with corners touching the field of view. Do some selection at this time. Ultimately, only oocytes with 3 or more layers of cumulus cells which are tightly compacted, and an even cytoplasm (not pycnotic) will be selected. Do not select oocytes with expanded cumulus masses or with no or few cumulus layers. At this time only a brief look should be given each oocyte. Most oocytes will be removed from the grid dish at this time unless they are obviously poor (denuded or expanded). Use the drummond to pick the oocytes out of the grid dish and move them into the 1007 dish. Search the entire grid dish for oocytes. After the first search, swirl the dish again and re-search. When you get to the point that you are picking up 5 or less oocytes on the re-search of your dishes, you can drop the re-search and just search each grid dish one time. Repeat this procedure for each tube. Do a second sweep on all the tubes combined (all second sweeps in one grid dish) when finished with all the first sweeps. When all the oocytes have been collected in the 1007 dish, change the drummond tip. Increase the magnification and examine each oocyte for a compact cumulus mass of at least three layers, not expanded, with even cytoplasm. Pick up the selected oocytes that meet these criteria and place them into the first 1008 dish. When all oocytes are selected, wash them through the remaining two 1008 dishes. If oocytes that should not be selected become apparent during the washes, they should be discarded at this time. Remove the maturation drops from the incubator. From the last wash dish, place the oocytes with as little medium as possible into the maturation drops, 10 per drop. Be sure to open the incubator doors as little as possible, and work quickly while in the incubator. Place the maturation drops back in the incubator.

7. Clean up the microscope and aspiration areas; wipe countertop surfaces, scope bases and slide warmers with alcohol. Put all dishes into a ziplock bag, close and either place in the trash or back into the bucket if needed later in the day. Make sure all scopes and slide warmers are turned off before leaving the lab. Put the trashcan outside the culture room and lock the door if done for the day. Then go back and clean up the ovary aspiration area. Wash out the large beakers and flask with deionized water several times. Wash the aspiration stoppers with deionized water, then with alcohol, then with milliQ water. Set them in the warm air incubator to dry. Package them for autoclaving the next morning. Clean up the aspiration area by throwing away the white paper, paper towels, and used gloves into the biohazard trash can to be autoclaved. Anything not bloody should be placed into the regular trash. Wipe down the area with alcohol. Turn off the slidewarmer if it is not going to be used the next day. Take the ovaries down to the tissue cooler for incineration.

   On this day be sure to record in your notebook:
   --when the ovaries arrived
   --temperature of ovaries on arrival
   --temperature of saline
   --when aspiration began
   --when aspiration ended
   --number of ovaries you aspirated
   --when the oocytes went into maturation medium
   --how many oocytes were recovered
   --maturation treatments/medium used

In Vitro Fertilization

1. In the morning of the day of fertilization, make all necessary media and turn on the slide warmers and water bath. You will need fertilization, sperm, and HEPES medium. Make 10 ml of sperm, 5 ml of fertilization, and 50 ml of HEPES medium WITHOUT glucose. HEPES medium may be pre-made, in which case aliquot and add pen-strep. Put the sperm and HEPES into the warm air incubator to warm up. Make fertilization drops—22 ul medium in a 1007 dish, then add 10 ml paraffin oil, then add another 22 ul, for a total of 44 ul. You will later add 6 ul to this drop, for a total volume of 50 ul. Place the fertilization medium and the drops into the maturation/fertilization incubator to equilibrate for at least 2 hours, preferably 4.

2. The oocytes should be fertilized 22-24 hours after they were placed into maturation medium. Start the swim up 1 1/2 hours before you want to fertilize. Get the warmed sperm medium, place 1 ml into 5 small snap cap tubes. Thaw out the appropriate number of semen straws. To do so, remove the cap from the semen tank, draw out the appropriate canister, and use the forceps to pull up a cane while leaving the remaining canes low in the tank to avoid thawing. Quickly pull two straws out and place them immediately into a small cooler jug containing 37 degree water. Let them thaw for 1 minute. Remove them from the water bath, dry them off with a kimwipe, and cut the sealed end off. Hold the cut end over an empty small snap cap tube, and cut off the plugged end. Blow through the straw to remove all the contents. Empty all straws into one tube. Using a pipetman, SLOWLY layer 200 ul of the semen under the one ml sperm medium in each tube. Repeat until all the semen has been used. Cap the tubes tightly; place them in a 37 degree water bath for one hour. Set a timer.

3. During this time, move the oocytes from maturation to fertilization drops. Take the maturation dishes out of the incubator. Using a cleaned drummond, remove the oocytes from the maturation

drops and place them into a 1008 dish filled with warmed HEPES medium. Swirl the dish gently to wash the oocytes. Repeat the wash two times. The third wash should be in fertilization medium (500 ul under 300 ul oil in a nunc well or 3037 dish per treatment). When the oocytes are in the third wash, get the fertilization plate out of the incubator. From the third wash dish, move the oocytes into the fertilization drops with as little medium as possible (10 per drop). Place the fertilization drops back into the incubator until the sperm is ready for fertilization.

4. After one hour, remove the swim up tubes from the water bath. Get out a 15 ml centrifuge tube. Using a pipetman, carefully remove the upper 800 ul from each swim up tube, and combine into one centrifuge tube. Label the tube. Spin the tube (after balancing) at 700 g for 5 minutes. During this 5 minutes get the heparin from the -20 freezer and the PHE from the -80 freezer and place it on the slide warmer in the culture room to thaw.

5. Remove the tube from the centrifuge when done, and pull off the supernatant down to the pellet. Do not disturb the pellet. Add approximately 3 ml warm sperm medium back to the tube and spin for an additional 5 minutes at the same speed. During this time, prepare the hemocytometer for use, and place 95 ul of milliQ water into a small microcentrifuge tube. After the spin, again pull off the supernatant down to the pellet. Mix the pellet well. Do not add more medium.

6. Remove 5 ul from the pellet, and mix it with the 95 ul in the microcentrifuge tube to make a 1:20 dilution. Change tips on the pipetman. Load 10 ul of the 1:20 mix into each chamber of the hemocytometer. Count the sperm in 5 of the small center boxes on each side of the hemocytometer. Count 5 diagonally or 4 corners and the center. Get a 5 square count for each side, and average them. Divide 7500 by the average count, this is the ul of sperm needed. Subtract the ul of sperm needed from 300 to get the ul fertilization medium needed for the sperm dilution for IVF (total volume 300 ul). These calculations are based on a 1:20 dilution counted on 5 squares of a hemocytometer, and when 2 ul of the sperm/fert medium dilution is added to a 50 ul drop, the final sperm concentration is 1 M/ml. For example;

   count side 1 = 138
   count side 2 = 152
   average = 145
   7500 divided by 145 = 51.7 ul sperm pellet
   300 - 51.7 = 248.3 ul fertilization medium

   If there is not enough sperm pellet for the whole 300 ul, simply divide the amounts by one half. Usually 300 ul is much more than enough.

7. Add the appropriate amount of fertilization medium to a sterile small microcentrifuge tube. Add the appropriate amount of sperm pellet to the tube. Mix well. Place on the slide warmer with the heparin and PHE.

8. Remove the fertilization drops from the incubator. Place them on the slide warmer. Using the drummond, add 2 ul each of the sperm dilution, heparin and PHE. The order in which they are added is not critical. The tip of the drummond should be wiped with a kimwipe between each addition to remove oil. As soon as everything has been added to the drops, check to be sure you have added sperm to all drops containing oocytes and put the dishes back into the incubator. Clean up the area. Record in your notebook the time the oocytes were moved to fertilization drops, the counts, the dilution made, and the time the sperm was added to the oocytes.

In Vitro Culture

1. The presumptive zygotes should be placed into culture approximately 18 hours post insemination. Embryo culture is done at 39°C in 5% CO2 and 10% O2, balance N2. First thing in the morning the day after fertilization, you should make the appropriate culture medium. Make drops (25 ul + oil + 25 ul) in 1007 dishes and place in the incubator to equilibrate. For every 1 or 2 culture drops one drop of the same medium should be present in the dish for washing. The drops should equilibrate at least 2 hours before adding the zygotes. Also make 50 ml of HEPES and warm quickly.

2. Place 0.5 ml HEPES into a 15 ml centrifuge tube for each treatment. Add 5 ul hyaluronidase solution. Remove the fertilization plates from the incubator. Work keeping the dishes and zygotes on the slide warmer as much as possible. Add the zygotes to the tube using a clean drummond. Keep treatments separate. Vortex for 3 minutes. Add 4 ml HEPES to the tube (pouring is OK), cap the tube and swirl the tube briefly.

3. Pour out the contents of the tube into a 1008 dish. Search for the zygotes and place them in a group in the middle of the dish. Move the zygotes to a fresh 1008 dish containing 3 ml HEPES medium and swirl to wash away any cellular debris. Remove the culture drops from the incubator. Load the zygotes into the wash drops of culture medium, 10 for each culture drop so 10 or 20 per wash drop. Use the drummond to mix the medium in the drop by drawing the embryos up and down several times. Pick up 10 embryos per drop in as little medium as possible and place into the final culture drop. Place the culture dish back into the incubator.

4. Record the culture medium used, and the time the zygotes were placed into the culture drops.

5. On day 4 post insemination (72 hours of culture), make step 2 culture medium and HEPES wash. Make culture drops in the morning (25ul + oil + 25ul) and let equilibrate for at least 2 hours. Again, make wash drops as above. Move the embryos quickly from the original culture drops to 1008 dishes with HEPES. Swirl to wash. Remove culture drops from the incubator, place embryos into wash drops, mix and distribute as above. Move them in as little of the original medium as possible at each step. Place the new culture drops back into the incubator. Record the date and time when the embryos were moved.

6. On day 8 post insemination, remove the embryos from the incubator and score each embryo for cleavage and development. Scores are as follows:
   1 cell, 2 cell, 4 cell, 8 cell, 12-16 cell, compact morula, early blastocyst, blastocyst, expanded blastocyst, hatching/hatched blastocyst. Embryos from compacted morula on should be given a quality grade from 1 to 4. One is the best quality, 4 is dead.

7. Stain for cell counting if required.

Media Recipes

SOF Bovine IVM/F/C Medium

Based upon:
Liu and Foote, 1995; BOR 53:786-790
Tervit et al., 1972; JRF 30:493-497
Gardner et al., 1994; BOR 50:390-400
Gardner et al., 1997; Therio 47:278

STOCK SOLUTION COMPOSITION

STOCK A (Base) 10X 3 months expiration

Reagent	mM	g/100 ml	g/200 ml	g/500 ml	g/1000 ml
NaCl	99.7	5.827	11.654	29.135	58.270
KCl	7.16	0.534	1.068	2.670	5.340
KH_2PO_4	1.19	0.162	0.324	0.810	1.62
$MgCl_2.6H_2O$	0.49	0.0996	0.1992	0.498	0.996
Na Lactate (60% syrup)	3.30	0.616 (280.25 µl)	1.232 (560.25 µl)	3.08 (1401.25 µl)	6.16 (2802.5 µl)

STOCK B (bicarb) 10X 1 week expiration

Reagent	mM	g/10 ml	g/50 ml	g/100 ml
$NaHCO_3$	25.07	.2100	1.050	2.100

STOCK C (pyruvate) 100X 1 week expiration

Reagent	mM	g/10 ml	g/20 ml	g/50 ml
Na pyruvate	0.33	0.036	0.072	0.180

STOCK D (calcium) 100X 1 month expiration

Reagent	mM	g/10 ml	g/20 ml	g/50 ml
$CaCl_2.2H_2O$	1.71	0.252	0.504	1.260

STOCK X (glutamine) 100X 1 week expiration

Reagent	mM	g/10 ml	g/20 ml	g/50 ml
Glutamine	1.0	0.146	0.292	0.73

STOCK G (glucose) 10X 3 months expiration

Reagent	mM	g/10 ml	g/50 ml	g/100 ml
Glucose	1.50	0.0270	0.135	0.270

STOCK EDTA (edta) 100X 1 month expiration

Reagent	mM	g/10 ml	g/20 ml
EDTA**	0.1	0.029	0.058

**dissolve EDTA in 220 ul 1M NaOH, then QS to 10 ml with water.

STOCK HEPES (HEPES) 10X 3 months expiration

Reagent	mM	g/50 ml	g/100 ml	g/200 ml
HEPES, acid	21	2.9795	5.958	11.916
Phenol red (0.5% soln)		(0.69 ml)	0.0069g (1.38 ml)	(2.76 ml)

STOCK T (taurine) 100X 2 months expiration

Reagent	mM	g/10 ml	g/20 ml
Taurine	0.1	0.0125	0.025

SOF working solutions

STOCK	MAT 5 ml	FERT 5 ml	SPERM 10 ml	Step 1 culture 5 ml	Step 2 culture 5 ml
A (base)	500 µl	500 µl	1 ml	500 µl	500 µl
B (bicarb)	500 µl	500 µl	1 ml	500 µl	500 µl
C (pyruvate)	50 µl	50 µl	306 µl	50 µl	50 µl
D (calcium)	50 µl	50 µl	100 µl	50 µl	50 µl
X (glutamine)	50 µl	--		50 µl	50 µl
G (glucose)	500 µl	--		500 µl	1000 µl
T (taurine)	--	--		50 µl	--
E (EDTA)	--	--		50 µl	--
NEAA	50 µl	100 µl		50 µl	50 µl
EAA	--	--		50 µl	100 µl
Vitamins	50 ul	--		--	50 µl
Water	3250 µl	3800 µl	6960 µl	3150 µl	2650 µl
BSA	See below	0.03 g FAF-BSA	0.03 g fraction V	0.04 g Pentex BSA	0.04 g Pentex BSA

Adjust pH of all working solutions to 8 before filtering, except sperm which is adjusted to 7.4

***Note*: Vitamins, NEAA and EAA are purchased from ICN.**

Maturation medium with serum
4.4 ml above mat SOF solution
25 ul FSH
25 ul LH
25 ul EGF
50 ul PSA
500 ul BCS

Maturation medium with BSA
4.9 ml above mat SOF solution
25 ul FSH
25 ul LH
25 ul EGF
50 ul PSA
0.04 g Pentex BSA

Sperm TL
To the above listed ingredients, add:
100 µl pen-strep
34 µl lactic acid
500 µl HEPES stock solution

SOF/HEPES Working Solutions

	With glucose		Without glucose	
STOCK	50 ml	100 ml	50 ml	100 ml
A	5 ml	10 ml	5 ml	10 ml
B	800 µl	1.6 ml	800 µl	1.6 ml
C	500 µl	1 ml	500 µl	1 ml
D	500 µl	1 ml	500 µl	1 ml
X	500 µl	1 ml	500 µl	1 ml
G	5 ml	10 ml	--	--
NEAA	500 µl	1 ml	500 µl	1 ml
EAA	1 ml	2 ml	1 ml	2 ml
HEPES	4.2 ml	8.4 ml	4.2 ml	8.4 ml
PSA	500 ul	1000 ul	500 ul	1000 ul
WATER	32 ml	63 ml	37 ml	73 ml
BSA	0.150 G fraction V	0.300 G fraction V	0.150 G fraction V	0.300 G fraction V

Use HEPES with glucose except to wash eggs from maturation to fertilization medium in the bovine system. HEPES with glucose can be used for this step if a fertilization wash is included. PH to 7.4 before use

Wild & Domestic Cattle, Buffalo and Bison Semen* Cryopreservation

N.M. Loskutoff
Center for Conservation & Research, Omaha's Henry Doorly Zoo
September 2003

Publications using this protocol:

1) Shiewe, M., N. Loskutoff, B. Durrant, L. Johnston, D. Armstrong, and L. Simmons (1994). Gaur sperm cryopreservation trial: analysis of packaging type and rapid freezing method for potential field application. Theriogenology . 41: 291, abst.
2) Gross, T., T. Tharnish, M. Patton, D. Armstrong and L. Simmons (1991) Gaur semen cryopreservation: comparison of cryodiluents and freezing procedures. In Proceedings of the Wild Cattle Symposium, Omaha, Nebraska, June 13-16, 1991. pp 17-32.
3) Simmons, L., S. Junior, D. Armstrong, S. Hopkins and S. Hummel (1989) Techniques for collection and cryopreservation of gaur (*Bos gaurus*) semen. Proceedings of the American Association of Zoological Parks and Aquariums Central Regional Conference.

*The procedure for semen collection from wild cattle, buffalo and bison by rectal probe electrostimulation (electroejaculation) can be found in a separate document.

Semen Cryopreservation

Materials Needed:

Biladyl A & B fractions (Minitube of America, Madison, WI, Tel: 608-845-1502)
Microscope slides & coverslips
Micropipettor & sterile tips
Vital stain (e.g., nigrosin-eosin or eosin B-fast green)
Glutaraldehyde (2.5%)
Hemacytometer
Microcentrifuge tubes
100 ml sterile specimen cup
Sealing powder
Labeled 0.5 ml french straws, goblets & canes
Slide warmer
Refrigerator (preferably, walk-in)
Liquid nitrogen
Styrofoam "boat" or Dry ice

Procedure:

Note: detailed instructions for determining seminal characteristics (e.g., total concentration, motility, rate of forward progression, viability and acrosomal integrity) are provided in the protocol for harvesting epididymal sperm.

1. Once the overall motility and progressive forward status determinations are made, a 10 µl aliquot should be taken of the well mixed sample for the total sperm concentration. In addition, a 10 µl sample should be mixed with an equal volume of vital stain (e.g., nigrosin-eosin or eosin B-fast green) spread on a microscope slide and quickly dried (hair dryer works best) for estimations of live/dead and acrosomal status. A final 10 µl aliquot is placed into 1 ml of 2.5% glutaraldehyde for evaluation of structural morphology. When these have been determined, then the total volume after the first extension (i.e., semen plus non-glycerated Biladyl A) can be determined using the following equation:

$$\frac{\text{(Semen Volume; ml)} \times \text{(Conc.} \times 10^6\text{)} \times \text{(Motility; 0.00)} \times \text{(Morphology; 0.00)}}{120 \times 10^6}$$

Again, this equation will give you the volume of the semen plus Biladyl A; therefore, subtract the volume of raw semen to give you the amount of Biladyl A to add.

2. Add the appropriate amount of Biladyl A directly to the raw semen or epididymal sperm at room temperature. [This total volume of the semen plus Biladyl A is the amount of the glycerated Biladyl B that will be slowly added, i.e., Biladyl B contains 14% glycerol and the final glycerol concentration desired in the extended semen is 7%].

3. Carefully pipette the semen plus Biladyl A into a 100 ml specimen cup then place this into a cold room (e.g., 4-7°C) along with the Biladyl B fraction. *Be sure to place the cup on top of a styrofoam lid to prevent cold shock from a chilled surface.* After 1-2 hours, very slowly add the appropriate volume of Biladyl B, drop by drop while gently mixing the diluted, cooled semen (the high osmolarity of the glycerated diluent can cause osmotic shock in the sperm if added too quickly). Note: it is always better to add the Biladyl B after cooling to prevent the sperm from metabolizing the glycerol; however, if a cold room is not available, then the Biladyl B should be added at room temperature, straws loaded then cooled (once cooled they should NOT be warmed above 4-7°C before freezing).

4. Immediately after the final extension of the semen has been completed, load the prelabeled straws and seal the ends with the sealing powder (columns of at least 5 mm).

5. After all of the straws are filled and sealed, then wrap them in several paper towels and place them directly into a refrigerator for slow cooling down to about 4°C.

6. After at least 2 hours in an undisturbed refrigerator (maximum of about 8 hours), freeze the straws by placing them either directly onto a block of dry ice, contained in an insulated box, or in liquid nitrogen vapor by placing them on a styrofoam boat (Fig 1) so that the straws are 1 inch (2.5 cm) above the level of liquid nitrogen. The straws should remain on the dry ice or liquid nitrogen vapor for 10 min before plunging directly into the liquid nitrogen for long term storage.

7. The frozen straws should then be carefully placed into straw goblets and canes (be sure not to take them out of liquid nitrogen during this procedure) then stored in a large storage tank. The total number of straws, results of the semen evaluation and animal identification information should be recorded in the cryostorage log book.

8. To thaw, individual straws should be carefully removed from liquid nitrogen storage, and quickly placed directly into a 32°C water bath. The straw should be gently swirled in the water bath for approximately 2 min, or until completely thawed. At that time, the straw should be gently wiped and dried with a paper towel, then the two ends cut with scissors and the contents decanted into a sterile test tube. Overall motility estimations and progressive forward movement determinations should be performed at zero and two hour post-thawing to determine sperm survival after freezing.

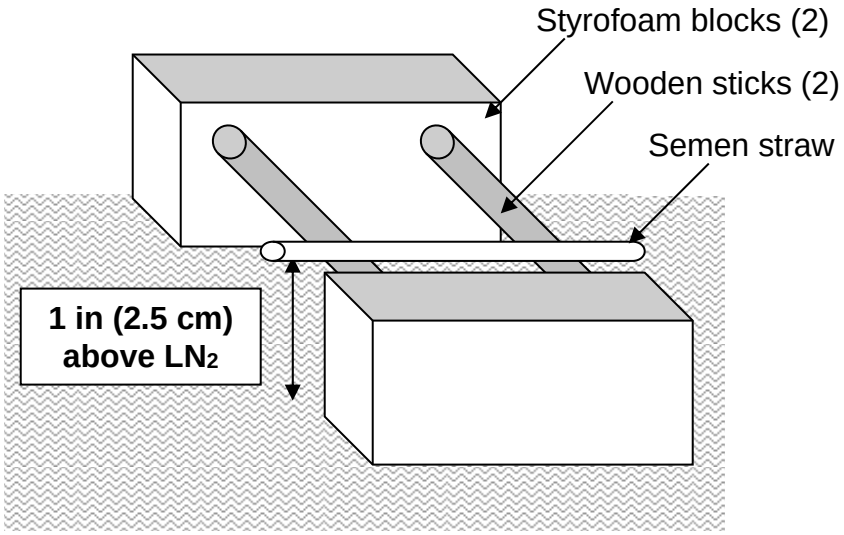


Figure 1. Styrofoam "boat" constructed from two blocks of styrofoam attached by two wooden sticks (far enough apart to securely hold the straws). Straws are laid on top of the sticks before the boat is carefully placed into an insulated container of liquid nitrogen (LN_2). The sticks are positioned so that the straws will be 1 in (2.5 cm) above the LN_2 surface. The container lid is replaced and after 10 min, the boat is lifted at an angle to plunge the straws directly into the LN_2.

Generalized Procedures for Harvesting Epididymal Sperm from Ruminants Post-Mortem for Cryobanking

N.M. Loskutoff
Center for Conservation & Research, Omaha's Henry Doorly Zoo
September 2003

Publications using this protocol:

1) Watson R., Lanterna J., Garcia A., Cooper C., Tomsett G. and Loskutoff N.M. (1997) Freezing resistances of epididymal sperm from waterbuck, greater kudu and warthog using glycerol, ethanediol or DMSO. Theriogenology 47:411 abst.
2) Rush E.M., Jewell M., Cooper C., Garcia A., Tomsett G. and Loskutoff N.M. (1997) Effect of cryoprotectant and thawing method for cryopreserving epididymal sperm from impala (*Aepyceros melampus*) for IVF. Theriogenology 47:406 abst.
3) Winger Q., Damiani P. and Loskutoff N.M. (1997) The application of standard bovine protocols for the maturation and fertilization of blesbok (*Damaliscus dorcas phillipsi*) oocytes using epididymal sperm cryopreserved in DMSO or glycerol. *Theriogenology* 47:412 abst.
4) Loskutoff, N.M., Simmons, H.A., Goulding, M., Thompson, G., De Jong, T. and Simmons, L.G. (1996) Species and individual variations in cryoprotectant toxicities and freezing resistances of epididymal sperm from African antelope. Animal Reproduction Science 42:527-535.
5) Loskutoff, N.M., Bartels, P., Meintjes, M., Godke, R.A. and Schiewe, M.C. (1995) Assisted reproductive technologies in nondomestic ungulates: a model approach to preserving and managing genetic diversity. Theriogenology 43:3-12.

Summary

Procedures are described for collecting and cryopreserving epididymal sperm from relatively healthy and fertile ruminants that die accidently or after acute trauma. Although these methods have been generally effective for a variety of species, they may not be optimal for all artiodactylids.

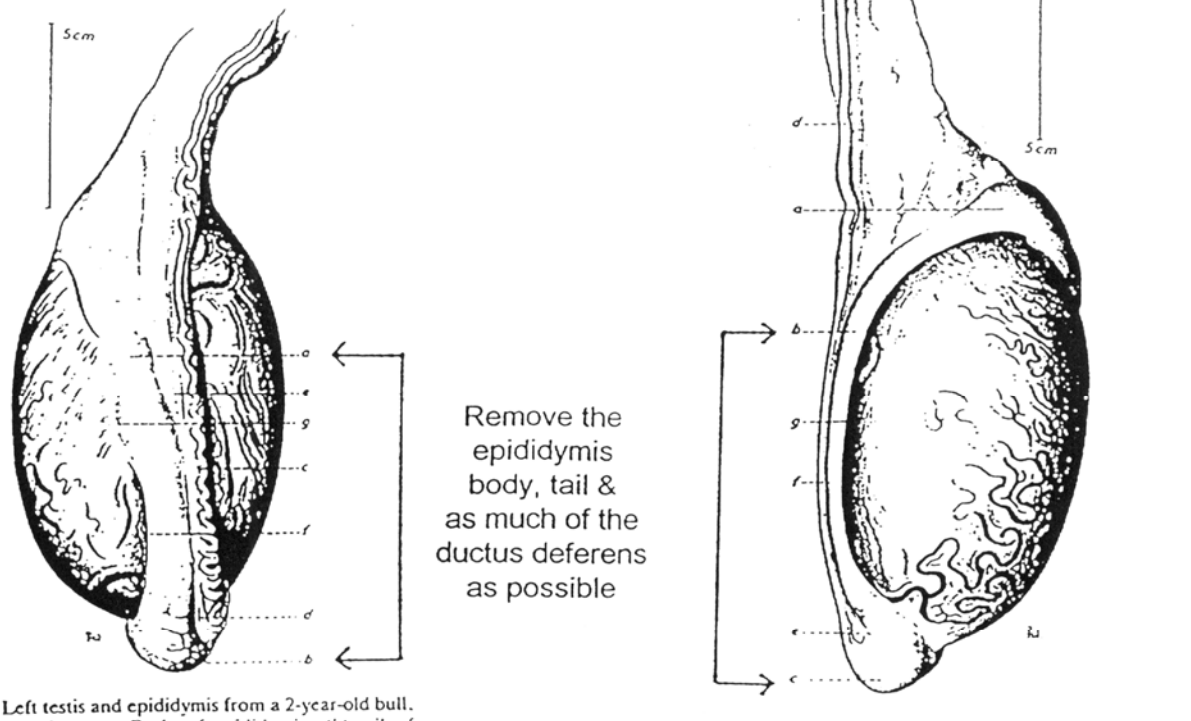
Materials Needed:

1. Sterile tissue culture dishes (e.g., 100-150 mm)
2. Sterile test tubes (e.g., 50 ml polystyrene, conical tubes)
3. Scalpel blade and holder or small scissors
4. Medium for rinsing (e.g., Hepes-TL Solution, BioWhittaker, Walkersville, MD, USA; Cat # 04-616F)
5. Sterile pipettes
6. Glass slides and cover glasses
7. Compound microscope
8. Slide warmer
9. Cell strainer (0.4-0.75 micron) or sterile gauze
10. Cryodiluents (see below)
11. Straws (0.25 ml)
12. Sealing powder
13. Refrigerator
14. Dry ice block (10 lb)
15. Liquid nitrogen storage tank
16. Goblets and canes

Cryodiluents: generally, a Tris-citrate-buffered, egg yolk-based diluent, commonly used for cryopreserving domestic bull semen, which can be purchased (e.g., Biladyl A and B, Minitube of America, Madison, WI, Tel: 608-845-1502). Fresh egg yolk must be added to each of the Biladyl fractions (A and B) at the time of preparation (QS each fraction to 400 ml with sterile, distilled/deionized water). Aliquots can then be frozen and stored for 6 mo.

Harvesting Technique

Using sterile instruments and aseptic technique, dissect the tails (caudae) of the epididymides from both testes (see Fig 1). Next, carefully remove any connective tissue layers covering the tail using forceps to first pull the tissue up and away before cutting. It is important to remove as many of the small overlying blood vessels as possible because blood can have a detrimental affect on sperm motility.



Left testis and epididymis from a 2-year-old bull, caudomedial view. *(a)* Body of epididymis, *(b)* tail of epididymis, *(c)* ductus deferens, *(d)* ligament of tail of epididymis (cut), *(e)* mesorchium (cut), *(f)* testicular bursa and *(g)* part of epididymis attached to testis; no testicular bursa is formed. (From Blom E, Christensen NO: Skandinavisk Veterinartidskrift, 1947, pp 1–45.)

Right testis and epididymis from 3-year-old bull, caudolateral view. *(a)* head of epididymis, *(b)* body of epididymis, *(c)* tail of epididymis, *(d)* ductus deferens, *(e)* ligament of tail of epididymis (cut), *(f)* mesorchium (cut) and *(g)* testicular bursa. (From Blom E. Christensen NO: Skandinavisk Veterinartidskrift, 1947, pp 1–45.)

Figure 1. Illustration of the anatomy of ruminant testes and epididymides within the scrotal sac.

Note: this will expose the tiny underlying tubules of the epididymides where the sperm are stored – be careful not to cut these tubules until most of the blood vessels are removed.

This should be done as quickly as possible – exposing the epididymides to air will result in the tubules drying out and losing the sperm. After most of the connective tissue and blood vessels have been removed, begin to cut small sections of the tubules (by lifting up with forceps and cutting with a small pair of scissors) and place these sections in a petri dish containing 5-10 ml of pre-warmed Hepes-TL

Solution. A white-milky colored fluid should be expressed. Continue cutting until the entire tail had been processed. Allow the sections to remain in the medium (preferably on a slide warmer set at 36-38°C) for at least 10 min, then use the forceps to gently squeeze then remove each section from the petri dish. Using a sterile pipette, gently pass the slurry through a cell strainer (or sterile gauze) into a 50 ml tube.

At this point, a small aliquot of the sperm suspension (i.e., 25 µl) should be placed on a pre-warmed microscope slide and evaluated for overall sperm motility and rate of forward progression. In addition, total sperm concentration, viability (percent live) and acrosomal integrity may be evaluated as described below.

Cryopreservation Procedure

Thaw aliquots of the non-glycerated (Biladyl A) and glycerated (Biladyl B) cryodiluents. At room temperature, add a volume of Biladyl A equal to that of the sperm suspension (1:1). Place this extended sample in a styrofoam box, or in a beaker containing room temperature water, and place this in a refrigerator, along with the thawed aliquot of Biladyl B (it is important for this initial cooling period to be gradual). Allow 2 hours of undisturbed refrigeration for the extended sperm sample to slowly cool to refrigeration temperature. At that time, very slowly add (e.g., drop by drop) a volume of Biladyl B equal to the total volume of the sperm suspension plus the added Biladyl A. This should be done in the refrigerator – do not allow the samples to warm during this final dilution (the cryoprotectant glycerol in Biladyl B can have toxic effects to the sperm). After adding the Biladyl B, mix the sperm sample thoroughly, but gently, and load the 0.25 ml straws. Seal the ends of the straws by dipping the end opposite to the cotton plug in PVP sealing powder. Allow the straws to remain undisturbed in the refrigerator for an additional hour before freezing. Freeze the straws by quickly placing them directly onto a block of dry ice, contained in a styrofoam box, and allow them to remain in the sealed box for a minimum of 10 min. At that time the straws should be quickly transferred directly into liquid nitrogen for long-term storage.

Note: although dry ice is preferred, if not available the straws can be frozen in liquid nitrogen vapor using the technique described in the protocol for semen cryopreservation in wild cattle, buffalo and bison.

Post-Thaw Evaluations

To determine the viability of the frozen epididymal sperm after thawing, remove a straw from liquid nitrogen storage and place in directly into a 50°C water bath for 8 sec (higher survival rates of ruminant sperm are observed with fast thawing methods; alternatively, thaw in a 36°C water bath for 2 min). Thoroughly wipe the water from the straw, then empty the contents into a sterile test tube. Examine small aliquots (i.e., 25 ul) immediately post-thawing and (if possible) at 2 and 6 hours post-thawing after warming at 36-38°C for 5-10 min.

Standardization of Procedures for Examining Sperm Samples

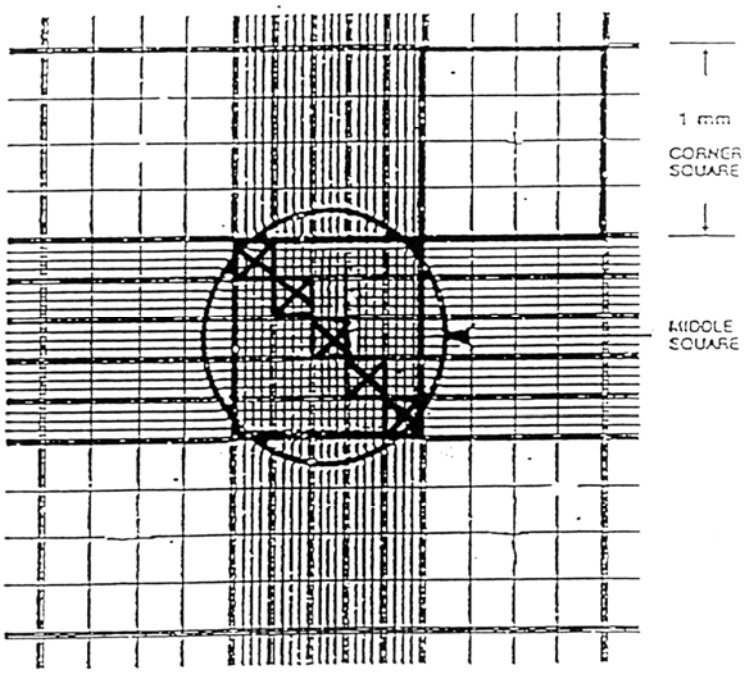
A. Total Concentration

Raw semen (either individual fractions, or pooled fractions selected by overall motility estimation) should be mixed thoroughly, <u>but gently</u>, just before sampling. A 1:100 dilution (semen:water) is made by adding 10 microliters (or 0.010 milliliter) of semen to 990 microliters (or

0.990 milliliter) of water [Note: calibrated pipettes should be used to ensure accuracy]. Dilution in water will kill the sperm making it easier to count individual cells.

Exactly 10 microliters should then be placed on a hemacytometer grid, as shown below. The sperm contained in the five diagonal squares (each comprising 25 smaller squares) in the middle large square of the grid are counted (for those sperm lying over the lines of the adjacent squares, only count those on the top and left to avoid duplication). For more accuracy, it is best to count the five inner diagonal squares on each side of the hemacytometer chamber then divide by two for an average (see next page for illustration). This average multiplied by 5 equals the total number of sperm (x one million) per milliliter. For the total concentration of sperm in the ejaculate, multiply this number by the total volume (in milliliters) of semen obtained.

STANDARD HEMACYTOMETER CHAMBER



B. Overall Motility Estimation

To ensure an accurate estimation of overall motility, the raw semen must be diluted sufficiently in a biological medium to view individual sperm. Normally, a 1:100 (semen:medium) dilution is adequate. Hepes-buffered TALP (Tyrode's medium containing albumin, lactate and pyruvate) is an excellent quality medium for maintaining the survival of sperm from a wide variety of taxa. A total of 200 sperm should be counted as moving or non-moving in several different fields on the microscope slide. For overall motility, all movement is recorded (even those sperm that may only show head movement, but no forward progression). The overall motility estimation is then recorded as the total number moving per 100 cells (expressed as % motile).

C. Rate of Forward Progression (or Kinetic Rating)

This is a very important indicator of sperm viability which should be included in the motility assessment. Since the fastest ratings are subjective, it is important that only one person performs the evaluation. As for overall motility, a total of 200 sperm are ranked as 0 to 5, using the following criteria:

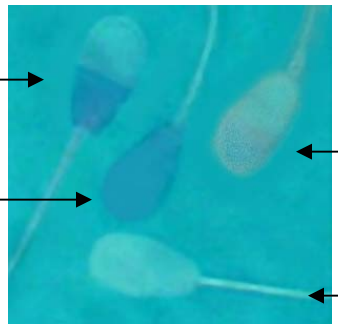
0 = no movement
1 = head movement only (no forward progression)
2 = slow forward progression (usually with labored head movements)
3 = faster forward progression
4 = faster forward progression
5 = fastest, linear forward progression

The average is determined for 100 sperm, and recorded as the average rate of forward progression (also referred to as status, or kinetic rating). This information is important because ratings of less than 3 often mean that the sperm will not be effective for use in artificial insemination.

D. Viability

The total numbers of live versus dead sperm is an important evaluation, especially for epididymal sperm recovered after death (which are live, but not motile until exposed to appropriate conditions). There are also situations where certain treatments (e.g., exposure to certain chemicals, or temperatures) can immobilize sperm temporarily. Normally, vital stains or dyes are used for this purpose – the damaged membranes of non-viable cells allow the uptake of the dye, whereas the intact membranes of viable cells do not. We have found the eosin B-fast green vital staining technique, first described for domestic bull sperm by Aalseth and Saacke (Gamete Research 15:38-81, 1986), to be very effective for a wide variety of mammalian species.

Technique: approximately 10 microliters of the sperm suspension is combined with approximately 5 microliters of eosin B-fast green dye. A smear is made and dried immediately using a hand-held hair dryer (it is important to dry the smear quickly, as the live sperm will also take up stain if they die slowly during drying). The eosin B-fast green vital stain can not only expose live versus dead sperm, but can also indicate those sperm that have intact acrosomes versus those missing acrosomes (acrosome-reacted). Acrosomal integrity must be maintained in sperm to be effective for use in artificial insemination, as acrosome-reacted sperm have very limited longevity and ability to move in a forward progression. Four categories of stained sperm can be identified using eosin B/fast green vital staining (on blue background, white = live, intact acrosome; red = dead, acrosome-reacted) e.g.,



A total of 200 sperm should be counted, assigning sperm to the four categories above, then the total number of live sperm, and the number of acrosome intact sperm, both per 100 cells total (%) should be recorded.

E. Acrosomal Integrity

In addition to the above method for determining acrosomal integrity, there are several fluorescence based stains available that are specifically targeted to the sperm acrosome region (e.g., Hoechst, PNA). Although these staining techniques are more specific for assessing acrosomal integrity, they require the use of specialized equipment for analysis (fluorescence microscopy). Nevertheless, samples of raw or treated semen can be air dried as smears on microscope slides, then shipped to appropriate facilities for analysis.

F. Structural Morphology

Ideally, normal structural morphology of sperm should be examined using the wet mount technique after fixation in glutaraldehyde (e.g., 2.5%). This is due to the fact that drying can distort sperm morphology and result in abnormalities that may be a direct result of handling. An alternative method is to air dry smears of the raw or treated semen on microscope slides, then to stain the smears using general use cytological staining kits (e.g., Diff Quick, DADE Diagnostics, Aguada, PR).

A total of 200 sperm should be characterized according to the following categories of gross abnormal morphological features suspected as primary abnormalities (those resulting during spermatogenesis in the testicle, which may be heritable), secondary abnormalities (those resulting during sperm transport, which are probably not heritable) or tertiary abnormalities (those that may have occurred as a direct result of improper handling).

Examples of gross structural abnormalities in mammalian sperm include:

<u>Primary abnormalities</u>
Head:
- Microcephalic (small head)
- Macrocephalic (large/swollen head)
- Double
- Abnormal acrosome
- Other

Midpiece:
- Swollen
- Elongated
- Abaxial
- Other

Tail:
- Double
- Short
- Other

<u>Secondary abnormalities</u>
Head:
- Detached
- Loose/damaged acrosome
- Other

Midpiece:
- Bent
- Protoplasmic droplet
- Other

Tail:
- Bent
- Shoehook
- Protoplasmic droplet
- Other

<u>Tertiary Abnormalities</u>
- Reacted acrosomes
- Coiled tails
- Other

Collection and Processing of Testicles or Ovaries Post-Mortem for Shipment

Testicles: As soon as possible after death, remove testicles enclosed within the scrotal sac and place them into a clean plastic bag and seal tightly. Place ice packs or ice sealed in a plastic bag (**not dry ice**) on the bottom of a small styrofoam box or other insulated container. Cover the ice with several layers of newspaper or paper towels, then place the testicles on top and seal the box. *Note: it is very important that the testicles cool very slowly to zero degrees (or ice bath temperature); therefore, be sure to not place them in direct contact with the ice (or the sperm will die of, or be damaged by, cold shock).* Testicles may be sent by overnight priority delivery service, if recovered from a relatively young and healthy animal. However, a significant reduction in sperm survival would be expected from aged or chronically ill animals. Ideally – and for very valuable animals – same day courier services (e.g., counter-to-counter air freight) is preferable for optimal recovery. Nevertheless, current technology has demonstrated the potential of producing live offspring from embryos derived from in vitro fertilization as well as sperm injection of either motile or non-motile sperm collected from testicles. Therefore, sperm samples should be salvaged and cryobanked as part of a conservation program for any valuable animal.

Ovaries: To avoid any loss in egg survival, ovaries must be received within 8 hours of death; therefore, they must be shipped by same day courier service (e.g., counter-to-counter air freight). Before removing ovaries, pre-warm physiological saline and several warm packs (to physiological temperature). Using sterile technique, cut the end of the oviducts at the utero-tubal junction, and remove the ovaries. Place them directly into a sterile specimen container or sealable, leak-proof plastic bag containing the physiological saline pre-warmed to body temperature. Pack the specimen cup in a thick styrofoam box containing several warm packs. Use cloth towels to ensure that the ovaries are properly insulated.

Epididymal Sperm Collection and Processing Worksheet

Date:_____ Species:_____ Animal ID:_____ Location:_____

Time of testes collection:_____ Transport temp:_____ Time of sperm recovery:_____

Sperm diluent/medium used:_____

Viability:
Motility (%):____ Progression (0-5):___ Total % Live:____ Total % acrosome intact:_____

[%Live/Acr Int:____ %Live/Acr Rx:_____ %Dead/Acr Int:_____ %Dead/Acr Rx:___]

Vital stain used:_____

Morphology:
Fixation/staining method used:_____

Total % structurally normal sperm: Total % abnormal:_____ (excl. protoplasmic droplets)

Summary of primary abnormalities (%):_____ Secondary:_____ Tertiary (handling):_____

Primary abnormalities:	*Secondary abnormalities:*	*Tertiary bnormalities:*
Head:	Head:	Type:
Microcephalic:_____	Detached:_____	_____
Macrocephalic:_____	Loose acrosome:_____	_____
Round:_____	Damaged acrosome:_____	_____
Pyriform:_____	Other:_____	_____
Elongated:_____	Midpiece:	
Double:_____	Protoplasmic droplet:_____	
Abnormal acrosome:_____	Bent:_____	
Other:_____	Other:_____	
Midpiece:	Tail:	
Elongated:_____	Protoplasmic droplet:_____	
Abaxial:_____	Bent:_____	
Thickened:_____	Shoehook:_____	
Double:_____	Other:_____	
Other:_____		
Tail:		
Coiled:_____		
Double:_____		
Short:_____		
Other:_____		

Cryopreservation Protocol:_____
Storage Location:_____
Comments:_____
_____

Semen Collection and Artificial Insemination of Cranes

Submitted by R. Lastovica & N.M. Loskutoff
Center for Conservation & Research, Omaha's Henry Doorly Zoo
September 2003

Publications using this protocol:

1) Gee, G.F. and Temple, S.A. (1978) Artificial insemination for breeding non-domestic birds. In P.F. Watson (Ed), Artificial Breeding of Non-Domestic Animals, Zool. Soc. London 43:51-72.
2) Archibald, G.S. (1974) Methods for breeding and rearing cranes in captivity. International Zoo Yearbook 14:147.
3) Gee, G.F. (1969) Reproductive Physiology of the Greater Sandhill Crane. A Progr. Rep. Admin. Rep., Patuxent Wildlife Research Center.

From: La Rue, C. (1980) Increasing Fertility of Crane Eggs. Avicultural Magazine 86: 10-15.

Introduction

Many zoos and private aviculturists keep cranes which are rendered flightless through pinioning, tenectomy, or wing clipping. The high incidence of infertile eggs in many captive cranes suggest that these procedures may have a detrimental effect on fertility. Other reasons for infertile eggs may be the absence of copulation due to incompatible pairs, interruption of precopulatory behavior by people or other animals, and improper sexing of the "pair."

In an effort to increase fertility some crane breeding centers, notably the Patuxent Wildlife Research Center and the International Crane Foundation, have implemented artificial insemination (AI) as a propagation tool. Many of the problems which produce infertile eggs can be overcome by using AI. Artificial insemination is practiced at International Crane Foundation (ICF) at Baraboo, Wisconsin, USA (http://www.savingcranes.org/) for a variety of reasons, prominent among these are when:

1. Individuals of a pair are incompatible because of behavioral problems and must be kept separated;
2. There is a shortage of unrelated sexually mature males;
3. Breeding age males are unable to copulate due to pinioning or other anatomical disabilities;
4. Birds are sexually imprinted on humans and will not pair bond with other cranes;
5. A pair is physically capable of breeding but do not copulate due to one or more of a variety of inhibiting factors; or
6. New bloodlines are desired to improve the genetic variability in the captive flock.

The technique of AI through the massage method has been described in other publications (Gee, 1969; Archibald, 1974; Gee and Temple, 1978) but will be repeated here in the hope of encouraging more aviculturists to attempt AI when applicable. AI is as much an art as a science, but it is not difficult to learn. The methods used vary somewhat between individual people and individual birds.

Handling

The capturing and handling of the bird requires a concentrated effort to prevent injuries to both the bird and the aviculturist. In several years of AI at the Foundation there has never been a fatality or serious injury during this process. Normally, the bird is herded by two or three people into a corner which has been lined with Christmas trees to act as padding. The bird is then grabbed gently by the neck just below the head and by one wing. It is important to protect both yourself and the bird's neck from being struck by their flailing feet and sharp nails. The bird is immediately placed head first between the holder's legs. The bird's head can be released and the bird held firmly pressed against the holder's thighs. Normally the bird will relax some at this point but if it continues to struggle a good may be placed over the bird's head.

Semen Collection

Three people are usually involved in semen collection although two people can do the job satisfactorily. The person holding the bird is the "stroker" whose job it is to restrain the bird while stroking the bird's thighs. The wings are allowed to remain free and should not be held between the stroker's legs. The arms are placed behind the wings with the forearms pressing the bird forward against the stroker's legs. The massage usually begins as an up and down motion on the bird's sides moving down to the inside of the thighs where the up and down strokes are continued. Normally the bird responds by raising its tail, contracting the cloaca repeatedly, and often times purring or clucking.

The second person involved is the "teaser" who squeezes the cloaca in a milking action which results in the ejaculation of a small quantity of semen. This usually occurs within a few seconds but some birds may take longer. Various methods can be employed but our preferred method is for the teaser to stand on the right side facing the opposite direction of the bird. Placing the right hand around and above the cloaca, the thumb is at the right side of the cloaca and the fingers are at the left side. The heel of the right hand helps to hold up the bird's tail pushing it over the bird's back. The left hand is placed under the cloaca, first stroking the abdomen a few times. The thumb of the left hand is then placed above the cloaca while the fingers are below the cloaca and pressure is applied in unison with the thumb and fingers of the right hand. This method results in a milking action from all four directions around the cloaca. In some techniques the left hand would be used to manipulate the cloaca while the right hand would hold the shot glass or eye cup for collecting. We use a third person to hold the small shot glass at the base of the cloaca to catch the ejaculate. Very small samples may have to be scraped off the cloaca onto the rim of the glass.

It is important to avoid contamination of the semen with urates during the collection. It helps to walk the bird and hesitate before capture to encourage the bird to defecate before handling. If the glass becomes contaminated during the collection, a second shot glass may often be substituted quickly enough to catch a clean semen sample. Uncontaminated semen is translucent with a slight frosty appearance. Normally only a few drops will be ejaculated measuring from about 0.01 to 0.3 milliliters. If the ejaculate is milky and of large volume, it is probably contaminated with urates. Because urates will kill sperm in a very short time, a contaminated sample will greatly reduce the fertilizing capability of the insemination.

Once collected in the glass, the semen is drawn into a 1 cc tuberculin syringe for measuring and subsequent insemination. If the semen is to be extended, a known amount of extender is deposited into the glass and drawn into the same syringe containing the semen. The sample is generally diluted enough

to give an insemination volume of about 0.2 milliliters. Samples larger than 1.5 milliliters are not diluted unless they are to be split.

Extender

Several semen extenders are available that can be used with cranes successfully (see Table 1 for formulations). Patuxent uses Beltsville Poultry Semen Extender while ICF uses Minnesota Turkey Semen Extender sold by the Minnesota Turkey Growers Association. The use of a semen extender is advantageous when very small samples of semen are collected and when a sample is to be split for inseminating more than one bird or to be frozen for future use. In the absence of a commercial extender, a physiological saline solution can be used for fresh inseminations.

Insemination of the Female

The actual insemination of semen can be the most difficult part of AI in cranes. While cloacal inseminations can give satisfactory results, placing the semen directly into the oviduct is preferred. At ICF, an effort is made to accomplish intra-vaginal inseminations at all times. A female in breeding condition that has laid eggs in the past or is currently laying is the easiest to inseminate. A female not in breeding condition and that has not laid before is very difficult to inseminate properly.

A reddening and enlargement of the opening into the oviduct is normally a reliable indication of breeding condition in the female crane. A relaxation of the cloaca and widening of the pubic bones often accompanies the onset of breeding condition.

The insemination of cranes at ICF follows the same pattern as semen collection with only a few differences. The bird is caught and held in the same manner as that described for the male. The female is usually stroked on the back and sides just posterior to the wings instead of the thighs. The same responses of tail raising, cloacal contractions and purring or clucking are normally elicited during the massage. In many birds, the oviduct is everted briefly and appears as a small bright pink opening, surrounded by spongy tissue, located at the lower left quadrant of the cloaca. In most birds it is necessary to gently pull the cloacal lips apart wider in an effort to expose the oviduct. This manipulation is usually done by the teaser while the third person inserts the syringe once the oviduct is located.

After locating the oviduct, the syringe is gently pushed in one to five centimeters and the semen is deposited slowly. This allows the oviduct time to accept and draw in deeper the contents of the syringe. Injecting the semen too quickly will result in some loss of semen. When the insemination is completed, the syringe is withdrawn slowly to prevent pulling semen out with the syringe. The bird is released quickly and gently to reduce the possibility of expelling semen.

***Note: More recently, one change to this protocol has been made: once the syringe is inserted into the oviduct, the stroker stops stroking the back of the female. The semen is then deposited once the female stops everting the cloaca and begins to draw inward; thereby taking up the semen.*

With the 1 cc tuberculin syringe there is generally about 0.04 ml semen left in the hub of the syringe after the insemination. This is another reason for using extender with small samples. The remaining semen is sufficient for microscopic examination for sperm cell concentration and motility. These samples can be taken to the laboratory and examined an hour or two after collection if the samples

are stored at about 5ºC and are not heavily contaminated by urates. Best results are obtained by examining samples immediately after collection with a microscope in the field at 430X magnification.

Frequency of Inseminations

Generally it is accepted that the higher frequency of inseminations, the higher the fertility rate. However, it is also true that the timing of each insemination is extremely important. Inseminations done three times a week and on days an egg is laid, a few hours after laying, should give good fertility. Currently at ICF we do AI every other day early in the morning and on days eggs are laid.

Summary

Artificial insemination can help improve fertility in many situations. The procedures are not overly complicated and can be learned in a short time through practice. A basic knowledge of AI techniques can be acquired at many University Poultry Science Departments and at commercial turkey breeding farms. These institutions provide an opportunity to develop AI skills before the cranes' breeding season begins.

Table 1. Semen extender formulations for birds (use for AI)

Diluent composition (grams/liter):	BPSE	Acetate
Fructose	5.00	8.00
Magnesium chloride x 6 H_2O	0.34	---
Tri-potassium citrate (anhydrous)	0.65	---
Sodium acetate x 3 H_2O	4.30	---
Magnesium acetate x 4 H_2O	---	0.80
Potassium acetate (anhydrous)	---	5.00
Sodium glutamate x H_2O (glutamic acid)	8.67	19.20
Potassium dihydrogen phosphate	0.65	---
Dipotassium hydrogen phosphate x 3 H_2O	12.70	---
TES	1.95	---
pH	7.50	(not specified)
Osmolality	333	333-375

Semen Collection in Snakes

Submitted by E. Viera and E.G. Crichton
Center for Conservation & Research, Omaha's Henry Doorly Zoo;
Updated September 2006

Update prepared by Kelli Mattson

Publication describing this protocol:

1) Mengden, G.A., Platz, C.G., Hubbard, R. and Quinn, H. (1980) Semen collection, freezing and artificial insemination in snakes. SSAR Contributions to Herpetology No. 1, Reproductive Biology and Diseases of Captive Reptiles; Murphy, J.B and Collins, J.T. (Eds.), Society for the Study of Amphibians and Reptiles.

Procedure

1. Expel uric acid and fecal material by holding the snake in a vertical position and gently applying pressure with the thumb along the ventral side of the animal while make a long ventral-caudal stroking motion along the lower abdomen.
2. Flush away any fecal material from the vent opening by using saline or Ringer's solution (distilled water and chlorinated water will kill sperm).
3. With the thumb, pressure is applied to the front of the anal plate and behind the hemipenes (see Figure 1 for diagram of snake anatomy). The tail is bent slightly backwards.
4. Continue stroking and rinsing if necessary.
5. Pull the cloaca open with thumb and push the tissues posteriorly. The cloacal papilla will be exposed.
6. If a dried seminal plug appears, gently remove it.
7. Repeat "stroking" procedure, semen will be expelled from the papilla.
8. Place a disposable transfer pipette between the papilla and the base of the hemipenes to extract a clean sample.
9. Add semen collected to a microcentrifuge tube containing a select volume (over 50µl and less than 500µl) TL Hepes Solution (Cambrex 04-616F). Be sure to use a new tube os Hepes with each collection to prevent contamination.
10. Males can be collected once per week during their breeding season.
11. The males should ejaculate within 5 to 10 minutes of manipulation.
12. A "trained" snake, or one that becomes accustomed to the procedure will typically ejaculate within seconds after defecating.
13. Males just starting with collections may get a little sore so be cautious and respectful, they may need some extra time between collections.

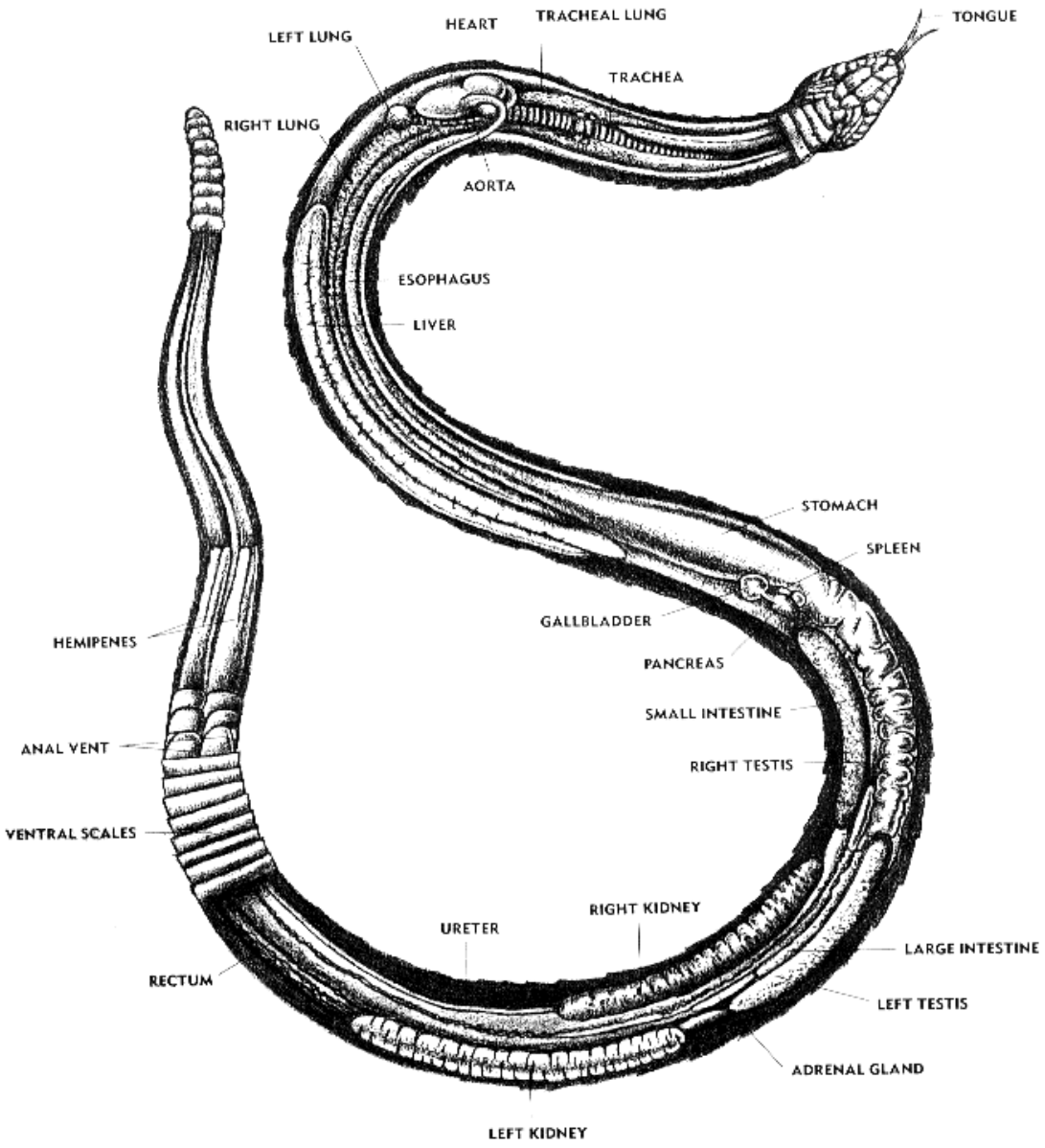


Figure 2. Diagram of anatomical features in snakes.

Artificial Insemination of Snakes

Submitted by K.J. Mattson and N.M. Loskutoff
Center for Conservation & Research, Omaha's Henry Doorly Zoo
September 2006

Publications using this protocol:

1) Quinn, H., Blasedel, T., and Platz, C.C. (1989). Successful artificial insemination in the checkered garter snake. Int Zoo Yb. 28: 177-183.
2) K.J. Mattson, A.T. DeVries, S.M. McGuire, J. Krebs, E.E. Louis, N.M. Loskutoff. (2006) Successful Artificial Insemination in the Corn Snake, Elaphe gutatta, Using Fresh and Cooled Semen. Reprod Fertil Dev (in press).

Semen collection: See "Semen Collection in Snakes" protocol

It is recommended that artificial inseminations are performed one week after the species-specific hibernation period.

Procedure

1. Immediately prior to insemination, express the feces and urates from the female following the same technique as is used with the males when collecting semen (see the "Semen Collection in Snakes" protocol).
2. The artificial insemination (AI) team should consist of three people: one person to support the head, one person to position the tail in an arc with the cloaca at the top of the arc, and the third person to perform the inseminations.
3. Draw up 100 µl of air into a 1 ml latex and silicone-free tuberculin syringe (Norm-Ject, VWR 53548-000) connected to a stainless steel feeding and dosing needle (EJAY International, FNS-20-1 ½) with a ball tip to prevent damaging any tissue during the insemination.
4. The insemination dose per oviduct (50 µl) is then drawn into the needle, and the ball tip is inserted into the cloaca.
5. The tip of the needle is slowly moved around the inside of the vent to relax the muscles and open the cloaca to allow the gentle insertion of the needle. It is best to slowly increase the speed in which the tip of the needles is moved around the cloaca as the female relaxes, however, each snake reacts slightly different so one must always be sensitive to how the female is responding to the stimulus. The opening of each oviduct is situated to either side of and dorsal to the cloaca of the snake.
6. The needle should be inserted into the oviduct and guided anteriorly until resistance is felt.
7. The insemination dose (50 µl) is then released into both oviducts and the needle slowly withdrawn. As the needle is withdrawn, release some of the air from the syringe to ensure the deposition of the entire insemination dose.

Human Sperm-Oocyte Interaction Tests

De Yi Liu and H.W. Gordon Baker
University of Melbourne Department of Obstetrics and Gynecology and Reproductive Services Royal Women's Hospital and Melbourne IVF, Melbourne, Australia
September 2002

Publications using this protocol:

1) Liu, D.Y., Lopata, A., Johnston, W.I.H. and Baker, H.W.G. A human sperm-zona pellucida binding test using oocytes that failed to fertilize in vitro. Fertil. Steril. 50: 782-788, 1988.
2) Liu, D.Y. and Baker, H.W.G. Inducing the human acrosome reaction with a calcium ionophore A23187 decreases sperm-zona pellucida binding with oocytes that failed to fertilize in vitro. J. Reprod. Fert. 89: 127-134, 1990.
3) Liu DY and Baker HWG. (1994) A new test for the assessment of sperm-zona pellucida penetration: relationship with results of other sperm tests and fertilization in vitro. Hum. Reprod., 9: 489-496.
4) Pantke, P., Hyland, J., Galloway, D.B., Liu, D.Y. and Baker H.W.G. Development of a zona pellucida sperm binding assay for the assessment of stallion fertility. Equine Reproduction VI Biol. Reprod. Mono. 1: 681-687, 1995
5) Liu DY and Baker HWG. (1996) A simple method for assessment of the human acrosome reaction of spermatozoa bound to the zona pellucida: lack of relationship with ionophore A23187-induced acrosome reaction. Hum. Reprod., 11: 551-557.

Introduction

These tests assess the capacity of populations of sperm to interact with the oocyte particularly sperm-zona pellucida (ZP) binding, the ZP induced acrosome reaction, sperm ZP penetration and, after removing the ZP, sperm-oolemma binding (Liu and Baker, 1990). The tests can be used to investigate factors which interfere with sperm function or sperm binding to the ZP and can be used clinically to provide important diagnostic information about sperm fertilising ability. Although these tests were developed with human sperm and oocytes they can be adapted for other animals such as the horse (Pantke et al., 1995).

Source of human oocytes:

Oocytes which showed no evidence of two pronuclei or cleavage at 48 to 60 h after insemination in a clinical IVF program were used for the ZP-induced AR tests. If the oocyte had sperm bound to the ZP from the IVF insemination, these were removed by aspiration using a fine glass pipette with an inner diameter (120μm) slightly smaller than the oocyte diameter. (Liu and Baker, 1994a; 1996). Most of the oocytes were obtained from patients with partial failure of fertilization in standard IVF and more than 50% of these unfertilised oocytes had one or a few sperm penetrating the ZP from the IVF insemination. We have shown previously that oocytes with <10 sperm penetrating the ZP have a similar ability for subsequent sperm-ZP binding and ZP-induced AR as those with no sperm penetration (Liu and Baker 1996). Oocytes with >10 sperm penetrating the ZP were not used. Also degenerate, activated or morphologically abnormal oocytes were not used for the sperm-ZP interaction test. Oocytes were

pooled from several patients and used for the test on the same day or kept in the incubator and used within next 2-3 days. Four oocytes are used for each sperm preparation because of variability between the oocytes. The oocytes can also be stored in 1M ammonium sulphate at 4C for up to 6 months without loss of activity of the ZP for binding sperm and stimulating the acrosome reaction.

These oocytes which have failed to fertilise are incapable of normal fertilisation during the test procedures. All patients signed consent forms permitting use of their unfertilised oocytes or sperm samples for research. The Royal Women's Hospital Research and Ethics Committees approved the project.

Sperm preparation

Motile sperm were selected by a swim-up technique as follows: 0.3 ml of semen was carefully added to the bottom of a test tube (12 x 75mm) containing 0.7 ml human tubal fluid (HTF, Irvine Scientific, Irvine, CA, USA) supplemented with 10% heat inactivated human serum (ICN Biomedicals, Irvine, CA, USA). Care was taken to avoid disturbing the interface between the semen and the medium. After incubation for 1 h, 0.5 ml of the top layer of the medium containing motile sperm was aspirated. The motile sperm suspension was then centrifuged at 1000g for 5 minutes, the supernatant removed and the sperm pellet washed again with 1 ml fresh HTF by centrifugation at 1000g for 5 minutes. The washed sperm pellet was resuspended with serum supplemented HTF to a sperm concentration of 2×10^6/ml for the ZP-interaction test.

Sperm-ZP binding

For the sperm-ZP interaction tests, motile sperm (2×10^6) in 1 ml of medium were incubated with four oocytes for 2 h at 37^{o}C in 5% CO_2 in air. After incubation, the oocytes were transferred to phosphate buffered saline (PBS), pH 7.4, containing 2 mg/ml bovine serum albumin (BSA) and washed by repeated aspiration with a glass pipette (inside diameter approximately 250 μm) to dislodge sperm loosely adhering to the surface of the ZP. Sperm binding to all 4 oocytes was assessed. Under these experimental conditions, with the high concentration of sperm in the insemination medium (20 times more than standard IVF insemination), the number of sperm bound tightly to the ZP is greater than 100/ZP with sperm from fertile or normospermic men and sperm samples with an average of $\leq$ 40 sperm/ZP were considered to have poor sperm-ZP binding and no further testing was done (Liu et al., 2001, Liu and Baker 2003). Lower concentrations of sperm can be used to get countable numbers of sperm on the ZP if the main interest is sperm-ZP binding (Liu et al., 1988).

ZP-induced AR

For sperm samples with normal ZP-binding, all sperm bound to surface of the 4 ZPs were removed by repeated vigorous aspiration with a narrow gauge pipette with an inner diameter (approximately 120 μm) slightly smaller than the oocyte (Liu and Baker 1994a, 1996). This was performed on a glass slide with about 5 μl PBS containing 0.2% BSA and the removed ZP-bound sperm were smeared in a limited area (about 16 mm^2), which was marked on the back of the slides with a glass pen to help find the sperm under the microscope. This pipetting procedure for removing sperm from the surface of ZP does not affect sperm motility, morphology and acrosome status (Liu and Baker, 1996).

The acrosome status of sperm removed from the ZP was determined with fluorescein labelled *Pisum Sativum* agglutinin (PSA, Sigma Co., St. Louis, MO) with a modification of the method of Cross et al (1986). Sperm smears were fixed in 95% ethanol for 30 minutes after drying in air and then stained in

25 μg/ml PSA in PBS for at least 2 h at 4°C. The slide was washed and mounted with distilled water and the percentage of AR was determined by scoring 200 of sperm removed from all 4 ZPs per test using a fluorescence microscope with oil immersion at a magnification of 400x. When more than half the head of a sperm was brightly and uniformly fluorescing, the acrosome was considered to be intact. Sperm with a fluorescing band at the equatorial segment or no staining of the head (a rare pattern) were considered to be acrosome reacted. As only motile sperm are capable of binding to the ZP, sperm removed from the ZP were alive and motile at the time of ZP-binding and thus no test of sperm viability was done.

Sperm-ZP Penetration Test

The procedures of this test is the same as the sperm-ZP binding test: motile sperm (2×10^6) in 1 ml of medium were incubated with four oocytes for 2 h at 37°C in 5% CO_2 in air. After incubation, the oocytes were transferred to phosphate buffered saline (PBS), pH 7.4, containing 2 mg/ml bovine serum albumin (BSA) and washed by repeated aspiration with a glass pipette (inside diameter approximately 250 μm) to dislodge sperm loosely adhering to the surface of the ZP. Sperm binding to all 4 oocytes was assessed. Then oocytes are repeatedly aspirated in and out of a pipette of inner diameter 120 μm, which is slightly smaller than the size of the oocyte. Sperm on the surface of the ZP are sheared off and only sperm with their heads embedded in the ZP, or in the PVS remain. These sperm can not be removed by further repeated pipetting. The penetrating sperm are easily counted under the microscope. Histological examination of serial cross sections of some oocytes confirmed the accuracy of this method (Liu and Baker 1994b, 2000). This test is useful for prediction of defective sperm-ZP penetration, in particular, in men with normal semen analysis and normal sperm-ZP binding.

Gorilla Sperm Cryopreservation Protocol – Non-Egg Yolk Diluent

J. K. O'Brien
University Sydney
September 2002

Publications using this protocol:

O'Brien JK, Crichton EG, Evans KM, Schenk JL, Stojanov T, Evans G, Maxwell WMC, and Loskutoff NM. (2002) Sex ratio modification using sperm sorting and assisted reproductive technology - a population management strategy. Proceedings of the Second International Symposium on Assisted Reproductive Technology for the Conservation and Genetic Management of Wildlife, Omaha's Henry Doorly Zoo, pp. 224-231.

Semen processing for samples collected by manual stimulation

1. Collect semen onto a washed (purified water) non-toxic nitrile glove (powder-free).

2. Transfer semen to either a cryovial (Nunc) or a microcentrifuge tube (ThermoTrace; Cat no. 1721-2: 2.0 ml) using a 100 ul pipette (record volume).

3. To collect semen remaining on the glove, rinse with small measured aliquots of HEPES-buffered HTF medium (Sage Biopharma, Cat. no. ART-1003) + 5%HSA (Irvine Scientific, Cat. no. 9988). This medium is termed **"mHTF" (Part A)**.

4. Record standard ejaculate characteristics:

Volume of raw semen (ml): _____
Volume of mHTF added during collection
(ml; should be no greater than 1:1; semen::mHTF): _____
Total volume (ml): _____
Sperm/ml: _____
Total sperm count: _____
Motility (%): _____
Forward progressive motility
(0-5; 0 = no forward progressive motion, 5 = rapid progressive motion): _____
Morphology: transfer 5 ul of semen to 100 ul of 1% glutaraldehyde
(40 ul 25% glut + 0.96ml PBS): _____
Acrosome integrity (FITC-PNA) and/or live dead stain: _____

Freezing procedure

1. Prior to semen collection, remove an aliquot from refrigerated Human Sperm Freezing Medium (Medicult #1067 0005; **"SFM" Part B**) and transfer to a cryovial or microcentrifuge tube. Leave tube at room temperature (so SFM will be at same temperature as semen).

2. Place in refrigerator a pipette, pipette tips, forceps, labelled straws (estimate 4 straws for every 0.25 ml of raw semen; label with Date, Male house name, studbook no., species; use a xylene-free Artline 725 permanent marker) and sealing apparatus (BD's "Seal-ease" or "ball bearings).

3. Dilute semen (which was cooled to room temperature by placing it in rack) 1:1 with Part B. **Dilute semen dropwise, very slowly with Part B – add only several drops of Part B to semen per minute so that dilution is performed over at least 5 minutes (preferably 10 min).**

Vol: _____Part A (+semen); ____Part B (1:1, diluted semen: PartB); _____Total Vol.

4. Place tube of diluted semen in a 250 ml beaker containing approximately 100ml of room temp water. Before freezing check that this apparatus will cool a tube containing 0.5-1.0ml of semen at a rate of –0.5°C/minute; ie requires **30 min** to cool from 21°C to 5°C).

5. After cooling to 5°C for 30-40 min, equilibrate for a further 30 min (for human sperm, this equilibration time can be reduced to 10 min) – at the same time fill the labelled 0.25 ml straws with semen and seal using the "Seal-ease" sealant or ball bearings. Leave 5 ul in tube for pre-freeze assessments eg motility and FPM, acrosome status

6. Record: Time start cool:_____ Time post-cool 5°C (1-1.1h): _____
   Time post-equilibration:_____

7. Place straws on a block of dry ice contained in an insulated container with a lid (eg plastic or styrofoam eski). Use forceps to handle straws at the cotton plug end. After all straws are placed on the dry ice, place lid on container and leave for **10 min**.

8. After 10 min, quickly transfer straws into liquid nitrogen using pre-cooled forceps (grasping straws at the cotton-plug end).

9. Record: Time frozen on dry ice:_____

10. Calculate no. sperm per straw: _____

11. The straws are placed in a goblet (continually submerged in liquid nitrogen) which is held on a cane that can be stored in one of several canisters in a liquid nitrogen storage dewar.

12. Record storage information:

No. straws: _____Dewar #: _____Canister: _____Cane ID: _____Straw ID: _____

Thawing procedure

1. Remove straw from liquid nitrogen storage and transfer quickly to a 37°C waterbath, shaking vigorously for **1 minute**.

2. Remove and wipe the outside of the straw with a clean tissue. Using clean scissors, cut the ends of the straw and let contents flow into a tube. Any semen remaining in the straw can be retrieved using a pipette and sterile 200ul tip (attach to straw end and de-aspirate).

Gorilla Sperm Cryopreservation Protocol – Test Yolk Buffer Diluent

J. K. O'Brien
University Sydney
September 2002

Publications using this protocol*:

1) Lambert HL, Citino S, Collazo I. Penetration of zona-free hamster oocytes by ejaculated cryopreserved gorilla spermatozoa. Fertil and Steril 1991;56:1201-1203.
2) Pope CE, Dresser BL, Chin NW, Liu JH, Loskutoff NM, Behnke EJ, Brown C, McRae Ma, Sinoway CE, Campbell MK, Cameron KN, Owens OM, Johnson CA, Evans RR, Cedars MI. Birth of a western lowland gorilla (*Gorilla gorilla gorilla*) following in vitro fertilization and embryo transfer. Am J Prim 1997;41:247-260.
3) Brown CS, Loskutoff NM. A training program for noninvasive semen collection in captive western lowland gorillas (*Gorilla gorilla gorilla*). Zoo Biol 1998;17:143-151.

*Slight differences between the protocol outlined in this document and the published protocols exist.

Semen processing for samples collected by manual stimulation

1. Collect semen onto a washed (purified water) non-toxic nitrile glove (powder-free).

2. Transfer semen to either a cryovial (Nunc) or a microcentrifuge tube (ThermoTrace; Cat no. 1721-2: 2.0 ml) using a 100 ul pipette (record volume). To collect semen remaining on the glove, rinse with small measured aliquots of HEPES-buffered HTF medium (Sage Biopharma, Cat. no. ART-1003) + 5%HSA (Irvine Scientific, Cat no. 9988). This medium is termed **"mHTF" (Part A1)**.

3. Record standard ejaculate characteristics:

Volume of raw semen (ml): _____
Volume of mHTF added during collection
(ml; should be no greater than 1:1; semen::mHTF): _____
Total volume (ml): _____
Sperm/ml: _____
Total sperm count: _____
Motility (%): _____
Forward progressive motility
(0-5; 0 = no forward progressive motion, 5 = rapid progressive motion): _____
Morphology: transfer 5 ul of semen to 100 ul of 1% glutaraldehyde
(40 ul 25% glut + 0.96ml PBS): _____
Acrosome integrity (FITC-PNA) and/or live dead stain: _____

Freezing procedure

- Prior to semen collection thaw an aliquot of the non-glycerolated (Irvine Scientific, cat #9972; **Part A2**) and glycerolated (Irvine Scientific, Cat. no. 9971; **Part B**) TEST-yolk buffered media.
- Place in refrigerator/cool room a pipette, pipette tips, forceps, labelled straws (estimate 4 straws for every 0.25 ml of raw semen; label with Date, Male house name, studbook no., species; use a xylene-free Artline 725 permanent marker) and sealing apparatus (Becton Dickinson's "Seal-ease" or "ball bearings).
- At room temperature, dilute semen (which was cooled to room temperature by placing it in rack on the benchtop) 1:1 with Part A2 (also at room temperature).
- Place the tube of diluted semen in a 250 ml beaker containing approximately 200ml of room temp water. Before freezing check that this apparatus will cool a tube containing 0.5-1.0ml of semen at a rate no greater than –0.5°C/minute.
- Cool semen to 5°C for **2 h. Then dilute semen dropwise, very slowly with Part B (using the pre-cooled pipette) - add only several drops of Part B to semen per minute so that dilution is performed over at least 5 minutes (preferably 10 min).**
- Fill the labelled 0.25 ml straws with semen and seal using the "Seal-ease" sealant or ball bearings. Leave 5 ul in tube for pre-freeze assessments eg motility and FPM, acrosome status
- Equilibrate semen for another 30 minutes ensuring that straws are held in a horizontal position.
- Record: Time start cool:___Time post-cool 5°C (2h): _____ Time post-equilibration:_____
- Place straws on a block of dry ice contained in an insulated container with a lid (eg plastic or styrofoam eski). Use forceps to handle straws at the cotton plug end. After all straws are placed on the dry ice, place lid on container and leave for **10 min**. Alternatively, straws can be placed on a rack at exactly one inch (2.54cm) above the surface of liquid nitrogen.
- After 10 min, quickly transfer straws into liquid nitrogen using pre-cooled forceps (grasping straws at the cotton-plug end).
- Record: Time frozen on dry ice:_____
- Calculate no. sperm per straw: _____
- The straws are placed in a goblet (continually submerged in luqid nitrogen) which is held on a cane that can be stored in one of several canisters in a liquid nitrogen storage dewar.
- Record storage information:

  No. straws: _____Dewar #: _____Canister: _____Cane ID: _____Straw ID: _____

Thawing procedure

3. Remove straw from liquid nitrogen storage and transfer quickly to a 50°C waterbath, shaking vigorously for **8 sec only**.
4. Remove and wipe the outside of the straw with a clean tissue. Using clean scissors, cut the ends of the straw and let contents flow into a tube. Any semen remaining in the straw can be retrieved using a pipette and sterile 200ul tip (attach to straw end and de-aspirate).

Great Ape Semen Cryopreservation: Egg Yolk-Based Cryodiluents

T.R. Bowsher[1] and N.M. Loskutoff[2]
[1]Dallas Zoo, Dallas, Texas;
[2]Center for Conservation & Research, Omaha's Henry Doorly Zoo
March 2003

Publications using this protocol:

1) Bowsher, T.R. and Loskutoff, N.M. (2001) Assisted reproductive technologies in the great apes. Proceedings of the 1st International Symposium on Assisted Reproductive Technology for the Conservation and Genetic Management of Wildlife. Henry Doorly Zoo, Omaha, NE, USA, pp. 152-156.
2) Finnegan, J.M., Loskutoff, N.M. and Brown, C.S. (1999) Cost effective method to transport manually collected gorilla semen for long term storage. Proceedings of the American Association of Zoo Veterinarians, Columbus, Ohio, pp. 362-363.
3) Kurz, S.G., Healy, M.R., Loskutoff, N.M., Brown, C.S., Crichton, E.G., Barnes, A.M., Finnegan, J.M., Volenec, D. and DeJonge, C.J. (1999) In-vitro maturation and intracytoplasmic sperm injection of western lowland gorilla oocytes. Theriogenology 51:361, abst.
4) Pope, C.E., Dresser B.L., Chin N.W., Liu J.H, Loskutoff N.M., Behnke E.J., Brown C., McRae M.A., Sinoway C.E., Campbell M.K., Cameron K.N., Owens O.M., Johnson C.A., Evans R.R. and Cedars M.I. (1997). Birth of a western lowland gorilla (*Gorilla gorilla gorilla*) following in vitro fertilization and embryo transfer. American Journal of Primatology 41:247-260.
5) Bowsher, T.R., Loskutoff, N.M., & Kraemer, D.C. (1990) Short-term storage of great ape semen. Proceedings of the Zoological Society of London, Symposium on Biotechnology and the Conservation of Genetic Diversity.

Preparation of Cryodiluents

Ingredients:
- TES buffer
- Tris buffer
- Sodium citrate
- Fructose
- Deionized/distilled water
- Fresh egg yolks
- Glycerol

Preparation*:

1. Prepare 325 mOsm solutions of each of the four ingredients in deionized/distilled water, e.g.,
   - TES: ~ 10 g in 140 ml
   - Tris: ~ 10 g in 310 ml
   - Na-citrate: ~ 10 g in 180 ml
   - Fructose: ~ 10 g in 120 ml

2. Titrate the TES and Tris solutions together to a pH of 7.0 (pour the Tris into the TES solution while stirring continuously and monitoring the pH meter).

3. Combine the following proportions into a sterile container:

60% TES-Tris solution
12.5% Na-citrate solution
10% Fructose solution
17.5% fresh egg yolk

4. Mix and freeze aliquots (e.g., 5-10 ml) of half the amount containing no glycerol (Primate Extender A).

5. To the remaining half, add 8% glycerol (cell culture quality grade), mix thoroughly, then freeze in 5-10 ml aliquots (Primate Extender B).

**Alternatively, you can purchase commercially prepared semen cryodiluents such as TES-Tris (TEST Yolk Buffer, Refrigeration Medium; Irvine Scientific, Santa Ana, CA, Tel: 800-437-5706; Cat #9972) or Tris-citrate-buffered, egg yolk-based diluent (e.g., Biladyl A, Minitube of America, Madison, WI, Tel: 608-845-1502). The Irvine cryodiluent already contains egg yolk, whereas fresh egg yolk must be added to Biladyl A just before use (one-half liter is prepared from a concentrate using distilled/deionized water that can be frozen as aliquots and stored long term). Use half of the volume of these non-glycerated cryodiluents as Primate Extender A, then to the remaining half, add 8% glycerol (cell culture quality) for Primate Extender B.*

Semen Freezing Protocol (Straw Method)

Materials Needed:

Refrigerator or cold room (4-5^{o}C)
Dry ice (5-10 lb block)
Liquid nitrogen
Sterile Pasteur or transfer pipettes
French straws (0.25 ml)
Fine permanent marker
PVP straw sealing powder
Goblets
Canes

Freezing Procedure for Straws

1. Thaw one aliquot each of the non-glycerated (Primate Extender A) and glycerated (Primate Extender B) cryodiluents. Place Primate Extender B into the refrigerator, along with a Pasteur or transfer pipette, labeled straws (estimate four straws for every 0.25 ml of raw semen) and sealing powder. Label straws with a minimum of species, animal ID number and date of collection.
2. At room temperature, add a volume of Primate Extender A equal to the semen volume (1:1 dilution ratio) directly to the raw ejaculate in a sterile test tube (large enough to contain at least four times the volume of the raw ejaculate.
3. Place the tube containing this first extension into a beaker of room temperature water, then place the beaker into a refrigerator (the water slows the cooling of the sperm to refrigeration temperature). Be sure that the refrigerator is left undisturbed during this equilibration period.

4. After a minimum of two hours in refrigeration, slowly (drop by drop) add the cold glycerated cryodiluent (Primate Extender B) using the cold pipette at a volume equal to that of the semen

plus the first extension with Primate Extender A. During the second dilution, continuously and gently mix the suspension with the pipette or by a gentle swirling motion.

5. Fill the labeled straws with the extended semen and seal using the cold polyvinylpyrrolidone (PVP) powder (at least a 5 mm column).
6. Refrigerate the filled straws for another 30 min. Be sure the straws lie in a horizontal position.
7. Freeze the samples by placing they directly and quickly on to a block of dry ice contained in an insulated (e.g., styrofoam) container with a lid. Hold the straws only at the cotton plug end to avoid warming by contact with fingers. After all the straws are placed on the dry ice, place the lid on the container and leave undisturbed for a minimum of 10 min. Alternatively, straws can be placed on a styrofoam boat (Fig 1) at exactly one inch (2.5 cm) above the surface of liquid nitrogen.
8. After 10 min, plunge the straws directly into liquid nitrogen. While submerged in liquid nitrogen, load goblets and insert them onto canes. Straws must not be lifted out of the liquid nitrogen at any time. Carefully place the canes into a liquid nitrogen storage container and record the entry into the cryobank log book including the total numbers of straws, canes, identification information and location of canister and dewar.

Thawing Procedure for Straws

1. Remove one or more straws from liquid nitrogen storage and place them immediately into a 50°C water bath. Swirl the straw(s) in the water bath for exactly 8 seconds then remove.
2. Wipe away surface moisture, then cut the ends of the straws and decant the contents into a sterile container (e.g., microcentrifuge tube).

Thawed semen should be examined immediately and two hours post-thawing for overall progressive motility and acrosomal integrity.

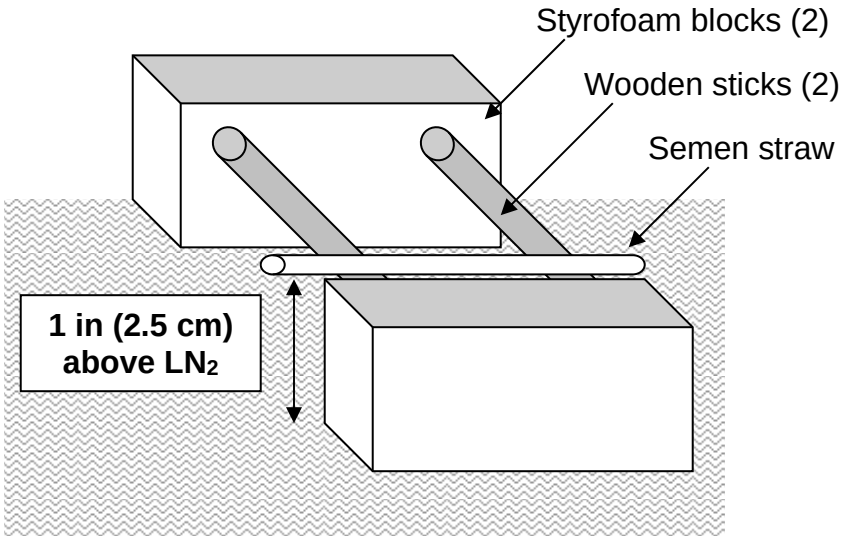


Figure 1. Styrofoam "boat" constructed from two blocks of styrofoam attached by two wooden sticks (far enough apart to securely hold the straws). Straws are laid on top of the sticks before the boat is carefully placed into an insulated container of liquid nitrogen (LN_2). The sticks are positioned so that the straws will be one inch (2.5 cm) above the LN_2 surface. The container lid is replaced and after 10 min, the boat is lifted at an angle to plunge the straws directly into the LN_2.

Semen Freezing Protocol (Pellet Method)

Materials Needed:

Refrigerator or cold room (4-5^{o}C)
Dry ice (5-10 lb block)
Cryogenic gloves
Nail board (for making ~ 5 mm depression in the dry ice block)
Sterile Pasteur or transfer pipettes
Long handled plastic spoon
Insulated container or bucket
Long forceps
Cryovials
Fine permanent marker
Canes for cryovials
Sleeves for canes
Liquid nitrogen

Freezing Procedure for Pellets

1. Thaw one aliquot each of the non-glycerated (Primate Extender A) and glycerated (Primate Extender B) cryodiluents. Place Primate Extender B into the refrigerator, along with a Pasteur or transfer pipette, labeled straws (estimate four straws for every 0.25 ml of raw semen) and sealing powder.

2. At room temperature, add a volume of Primate Extender A equal to the semen volume (1:1 dilution ratio) directly to the raw ejaculate in a sterile test tube (large enough to contain at least four times the volume of the raw ejaculate.

3. Place the tube containing this first extension into a beaker of room temperature water, then place the beaker into a refrigerator (the water slows the cooling of the sperm to refrigeration temperature). Be sure that the refrigerator is left undisturbed during this equilibration period.

4. After a minimum of two hours in refrigeration, slowly (drop by drop) add the cold glycerated cryodiluent (Primate Extender B) using the cold pipette at a volume equal to that of the semen <u>plus</u> the first extension with Primate Extender A. During the second dilution, continuously and gently mix the suspension with the pipette or by a gentle swirling motion.

5. At this time, prepare for freezing by using the nail board to make ~ 5 mm depressions on a flat surface of a dry ice block and filling the insulated container with a small volume of liquid nitrogen.

6. Use a cold Pasteur or transfer pipette to first gently mix the second extension, then drop the extended semen directly into the dry ice depressions (one drop per depression). Count the total number of pellets.

7. Allow the pellets to remain on the dry ice for 10 min.

8. Plunge the pellets directly into liquid nitrogen by using cryogenic gloves to lift the dry ice block and tapping on the opposite side to dislodge the pellets.

9. Secure labeled cryovials using long forceps and lower it into the liquid nitrogen. Using the plastic spoon, scoop up the pellets and place them into the cryovial.

10. The cryovials containing pellets should be carefully capped and placed onto canes. The canes should be covered by protective sleeves then placed into a liquid nitrogen storage container.

Thawing Procedure for Pellets

1. Transport one or more cryovials containing the frozen pellets in an insulated container (preferably not white) containing a small volume of liquid nitrogen.

2. Fill several 10 ml round bottom sterile tubes with 0.5 ml of medium (e.g., Hepes-TL Solution, BioWhittaker, Walkersville, MD, USA; Cat # 04-616F). Place the rack containing these tubes into a 37^{o}C water bath. Use one tube to thaw two to three pellets.

3. Using a flamed, 25 gauge needle, poke three holes into the body of a long handled plastic spoon.

4. Carefully uncap the cryovial and release the pellets. Using the modified plastic spoon, scoop 2-3 pellets at a time and lift them out of the liquid nitrogen. As soon as the liquid nitrogen is drained through the holes in the spoon, drop the pellets into a tube containing the pre-warmed medium and swirl the tube gently in the water bath until the pellets are thawed.

5. Thawed semen should be examined immediately and two hours post-thawing for overall progressive motility and acrosomal integrity.

Fecal Extraction Protocol for Hormone Analysis

A.C. Bellem and N. Wielebnowski
Brookfield Zoo, Chicago, IL
March 2004

Protocol prepared by: Astrid Bellem

Publications using this protocol:

1) Atsalis S, Margulis SW, Bellem A, Wielebnowski N. (submitted) Behavioral and hormonal cycles in captive geriatric lowland gorillas (*Gorilla gorilla*). American Journal of Primatology

Extraction Protocol

1. Measure 0.5g (+/- 0.05) of mixed, defrosted wet fecal sample into a 16x125 mm polypropylene test tube, being careful to remove any hair, bone, litter or debris.
2. Record exact weight on fecal extraction sheet, and record any comments (unusually wet or dry, hairball).
3. Fill a labeled 12x75mm polypropylene tube with sample and freeze for future use, if required.
4. Continue until all samples in the set are weighed out.
5. Add 5 ml of 80%EtOH in dH20 to all of the weighed fecal samples and cap tubes quickly.
6. Vortex all tubes well, making sure samples are broken up.
7. Place racked tubes in baggies (to hold tubes in rack), place in a horizontal position on a rotator and mix well for 14-18 hours (overnight).
8. Centrifuge tubes for 15 minutes at room temperature at 1500 rpm.
9. Pipette 1.0 mL assay buffer into labeled 12x75mm polypropylene test tubes, add 1 mL, of supernatant from each corresponding tube, cap tightly, mix and store frozen. Dilution tube labels should include animal ID (name), sample #, date and 1:10.

This represents a 10-fold dilution, 5-fold in EtOH, since the original volume is 5 mL, and a further 2-fold into buffer, so that the sample will freeze. Samples not diluted into buffer will evaporate, even in capped tubes stored in the freezer.

Semen/Spermatozoa Collection and Evaluation in Brushtail Possums

F. Molinia
Landcare Research, PO Box 69, Lincoln 8152, New Zealand
September 2003

Publications using this protocol:

1) Molinia FC and Rodger JC (1996). Pellet-freezing spermatozoa of two marsupials: the tammar wallaby, *Macropus eugenii* and the brushtail possum, *Trichosurus vulpecula.* In: Marsupial Gametes and Embryos. *Reprod. Fertil. Dev.* 8, 681-684.
2) Garner DL, Johnson LA, Yue ST, Roth BL and Haugland RP (1994). Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. *J. Androl.* 15, 620-629.
3) Sistina Y, Lin M, Mate KE, Robinson ES and Rodger JC (1993). The unique stability of the marsupial sperm acrosomal membranes examined by unprotected freeze-thawing and treatment with the detergent Triton X-100. *Reprod. Fertil Dev.* 5, 1-14.
4) Mate KE and Rodger JC (1991). Stability of the acrosome of the brush-tailed possum (*Trichosurus vulpecula*) and tammar wallaby (*Macropus eugenii*) *in vitro* and after exposure to conditions and agents known to cause capacitation or acrosome reaction of eutherian spermatozoa. *J. Reprod. Fertil.* 91, 41-48.
5) Rodger JC, Cousins SJ and Mate KE (1991). A simple glycerol-based freezing protocol for the semen of a marsupial, *Trichosurus vulpecula*, the common brushtail possum. *Reprod. Fertil. Dev.* 3, 119-125.
6) Rodger JC and Pollitt CC (1981). Radiographic examination of electroejaculation in marsupials. *Biol. Reprod.* 24, 1125-1134.
7) Rodger JC and White IG (1978). The collection, handling and some properties of marsupial semen. In: Artificial Breeding of Non-domestic Animals, pp 289-301. Ed. PF Watson. *Symp. Zool. Soc. Lond.* No. 43, Academic Press, London.

Semen/Spermatozoa Collection

Background

The following procedure for collecting possum semen/spermatozoa is adapted from techniques described by Rodger and White (1978), Rodger and Pollitt (1981), Rodger *et al.* (1991) and Molinia and Rodger (1996).

Safety

To avoid unnecessary exposure to biological hazards, operators should be suitably dressed in overalls and gumboots, and should wear gloves during sample collection. Avoid accidental inhalation of fluothane anaesthetic.

Procedures

Electroejaculation

a. Anaesthetise possum (using method approved by Institutional Ethics Committee).
b. Clean genital area with savlon solution. Extract penis and clean.
c. Lubricate probe with obstetrical gel and gently position probe in rectum (~ 8 cm) so that electrodes lie at the anterior end of the male reproductive tract.
d. Remove all traces of obstetrical gel from genital area and position penis in 15 ml collection vial containing 1 ml phosphate buffered saline (PBS), or EMEM media (Sistina et al., 1993), optional.
e. Activate pulse generator. This automatically applies electrical stimuli (30 pulses/sec, 6.5 V) for a period of 5 sec duration followed by 5 sec rest interval. Repeat cycle approximately 10 times until ejaculation. Resist posterior movement of probe as rectum contracts. The electrodes should lie against the posterior prostate gland that is readily palpable anterior to the pelvic bones during the breeding season.
f. Semen volumes will vary greatly (0.5 - 12 ml). Dilute semen samples, 1:1 with PBS or EMEM media, immediately after collection.
g. Between collections the probe should be thoroughly washed and soaked in savlon solution.

Epididymal flushing

a. Euthanase male possum (using method approved by Institutional Ethics Committee), and remove both testes at the base of the scrotal sac, close to the body wall.
b. Carefully dissect scrotum removing skin and outer membrane (tunica dartos). Separate epididymides from testes including a 5 cm portion of the vas deferens. Rinse tissue in PBS or EMEM media and blot with gauze sponges.
c. At the base of the tail-region of each epididymis, remove remaining outer membrane (tunica vaginalis). Tease out a single epididymal duct tubule and cut.
d. Insert a 21 G blunt needle attached to a syringe containing approximately 1-2 ml of PBS or EMEM media into the fee-end of the vas.
e. Put the tail end of the epididymis into the top-end of a 15 ml centrifugation tube, and holding the needle into the vas, flush the tissue.
f. Creamy-white epididymal spermatozoa will filter from the cut-end of the duct tubule. Stop flushing when the secretion runs clear with media. Repeat for the other side.

Notes

Electroejaculation

- Semen samples are occasionally contaminated with urine. If this occurs use a fresh sampling vial and continue collection.
- If electrical stimulation does not elicit ejaculation after 10 cycles remove, clean, re-lubricate and reposition the probe. Faecal matter may accumulate around the strip electrodes preventing good electrical contact.
- After a number of collections some possums may give only sperm-free ejaculates. Positioning the probe adjacent to the anterior prostate (by palpation and lateral movement of the prostate) and applying electrical stimulus for several 5 sec cycles may yield sperm-containing ejaculates.

Epididymal flushing

- If flush is contaminated with blood then discard the sample.
- Do not flush epididymides that appear infected.

Equipment and reagents

Probe: Perspex multipolar rectal probe (12 mm diameter) with 2 pairs of strip electrodes.
Stimulator: Portable pulse generator powered by 9 V battery producing 30 square wave DC pulses per second with an amplitude of 6-8 volts and a current of 30-60 mAmps. The pulse generator is preset to repeatedly produce pulses for 5 seconds followed by 5 seconds off.
Obstetrical gel: Veterinary lambing lubricant.
Savlon solution: Consists of 10% savlon solution.
Phosphate Buffered Saline: Containing 8 g NaCl, 0.2 g KCl, 1.44 g Na_2PO_4 and 0.24 g KH_2PO_4 in 1000 ml distilled water, pH 7.2.
EMEM media: Eagle=s minimum essential medium with Earle=s salts, L-glutamine and non-essential amino acids (M 0643 Sigma) supplemented with 20 mM HEPES buffer, 2.2 g/L sodium bicarbonate (26 mM), 0.23 mM pyruvate, 100 mg/L streptomycin, 100,000 iu/L penicillin, pH 7.4.

Semen/Spermatozoa Evaluation

Background

Integral to procedures that utilise sources of brushtail possum spermatozoa (eg: sperm cryopreservation, artificial insemination, capacitation/acrosome reaction studies) is the initial assessment of sperm quality. Sperm quality is assessed by a variety of parameters including the following: concentration, percentage and progressive motility (Rodger *et al.,* 1991; Molinia and Rodger, 1996), percentage viability (Garner *et al.,* 1994), and acrosome integrity (Mate and Rodger, 1991; Sistina *et al.,* 1993).

Procedures

Initial assessment of spermatozoa

A normal ejaculate of possum semen following electroejaculation or epididymal sperm after flushing (see SOP - No. 1) is opaque and creamy white in colour. It may appear yellow or occasionally grey/green if contaminated with urine, or pink/red if contaminated with blood, in which case the sample should be discarded.

- Aspirate sample into a clean syringe or graduated vial and record sample volume.
- Record the general appearance of the sample.

Determining sperm concentration

An improved Neubauer counting chamber is used to estimate the concentration of sperm in the semen sample. The whole grid of chamber is 1 mm square and 0.1 mm deep. Therefore the grid contains a volume of 0.1 mm^3 (= 1/10,000 ml).

a. Mix sample thoroughly and aliquot 10 µl portion into a 1 ml eppendorf tube.
b. Dilute aliquot 1:10 or up to 1:100 with counting diluent to immobilise sperm.
c. Position clean cover slip on counting chamber.
d. Thoroughly mix diluted aliquot and load the 10 µl fixed volume pipette.
e. Touch tip of pipette to edge of cover slip and slowly expel sample allowing area under cover slip to fill, but do not overfill.
f. Leave to settle for about 1 minute.
g. Using a phase contrast microscope (300 x magnification) count sperm in 5 (out of 25) large diagonal squares (equivalent to 1/50,000 ml). Include sperm lying on the lines on the bottom and right hand sides of the square.
h. Calculate concentration as:

- Sperm concentration (spermatozoa/ml) = n x df x 50,000
- n = number of sperm in 5 squares
- df = dilution factor of sample counted
- Total sperm in sample = sperm concentration x sample volume

Eg: If 5 ml sample is diluted 1:2 with PBS at collection, 1:10 with counting diluent and the total number of sperm counted in 5 large squares of the counting chamber is 56, then:

Sperm concentration = 56 x 20 x 50,000
= 5.6×10^7 sperm/ml
Total sperm in sample = 5.6×10^7 sperm/ml x 5 ml
= 2.8×10^8 sperm/sample

Assessing sperm motility

a. Dilute sperm sample (if required) with phosphate buffered saline (PBS) or EMEM media until sperm can swim freely under coverslip.
b. Place drop (15-20 µl) of diluted sample on a glass slide.
c. Gently apply a coverslip (22 mm x 22 mm) and leave to stabilise for about 1 minute on a heated stage at 36□C.
d. Observe 9-10 fields under a phase-contrast microscope, with a minimum of approximately 50-100 sperm/field and estimate percentage motility (0-100%) as the proportion of cells moving.
e. The progressive motility of the sample is graded using the following scale:

0	No movement.
1	Weak tail beat, little or no forward movement.
2	Slow forward movement usually along a circular path.
3	Moderate forward movement mainly along a curved path.
4	Moderate to rapid forward movement along a straight path.
5	Very rapid forward movement along a straight path.

Assessing sperm viability

The Fertilight live/dead sperm viability kit is used to assess the viability of a sperm sample using fluorescence microscopy. The kit has two components, stored in the freezer:
Component A = 100 µl of 1 mM SYBR-14 in 100% DMSO.

Component B = 5 ml of 2.4 mM propidium iodide in water.
To make working solutions:

Component A - a further 1:10 dilution of this is made into DMSO and can be stored in the freezer. Note: stock solutions prepared in aqueous media may result in unacceptable dye loss. On the day of use take 5 µl of this dilution and add it to 495 µl of BSA-saline media in an eppendorf tube. Note: phosphate-containing buffers may interfere with Component A staining.

Component B - on day of use take 10 µl and add to 190 µl of BSA-saline media in an eppendorf tube. Put both eppendorf tubes in the incubator at 36ºC.

To stain samples:

a. Dilute sperm sample to 25-50 million sperm/ml.
b. Aliquot 40 µl of the sperm sample in an eppendorf tube, add 5 µl of Component A working solution (final concentration of 100 nM SYBR), and 5 µl of Component B working solution (final concentration of 12 µM propidium iodide).
c. Incubate at 36ºC for 10-15 min.
d. Fix sample by adding 10 µl of 0.2% paraformaldehyde fixative.

To assess samples:

a. Turn on fluorescence lamp and once warmed up, set the (blue light) filter block commonly used for FITC.
b. Mount 10-20 µL stained fixed samples on slides under coverslip. Live sperm stain green, dead sperm stain red. Note: some sperm stain 50:50 and are classed as moribund.
c. Count several fields from 3 replicates of the sample until approximately 600 sperm are assessed, and calculate the proportion viable.

Assessing acrosome integrity

The acrosome integrity of a sample can be assessed at the light microscopy level using Bryan=s stain or at the ultrastructural level (as for any other sperm structure) using electron microscopy.

Bryan's stain

a. Dilute sperm sample in PBS or EMEM media to a concentration of around 5 million/ml.
b. Make a further 1:10 dilution of this sample with 2% paraformaldehyde fixative and incubate at room temperature for 10-15 min.
c. Load a 50 µl sample into the Cytospin chamber and centrifuge at 210 *g* for 3-4 min so that sperm attach to slides in discrete Adot@ regions. Note: If there are too many sperm attached for counting, then dilute the original sperm sample further and repeat the above steps.
d. Stain slides for 3-4 min in Bryan's stain.
e. Mount coverslips on slides and visualize acrosomes under light microscopy.
f. Assess a total of 600 sperm (3 replicates: 200 sperm per replicate) for the proportion of absent, present and altered acrosomes.

Electron microscopy

a. Dilute sperm sample 1:10 with Superfix for 10-15 min at room temperature.
b. Centrifuge sample for 10 min at 700 *g*.

c. Resuspend pellet in fresh Superfix and refrigerate.
d. Trained EM technician to continue processing.
e. Assess acrosomal status (or status of any other sperm structure) from a minimum of 100 spermatozoa per treatment by electron microscopy.

Reagents

Sperm counting diluent: Containing 50 g $NaHCO_3$, 10 ml 35% (v/v) formalin in 1L distilled water.
Phosphate Buffered Saline: Containing 8 g NaCl, 0.2 g KCl, 1.44 g Na_2PO_4 and 0.24 g KH_2PO_4 in 1000 ml distilled water, pH 7.2.
EMEM media: Eagle=s minimum essential medium with Earle=s salts, L-glutamine and non-essential amino acids (M 0643 Sigma) supplemented with 20 mM HEPES buffer, 2.2 g/L sodium bicarbonate (26 mM), 0.23 mM pyruvate, 100 mg/L streptomycin, 100,000 iu/L penicillin, pH 7.4.
Fertilight live/dead sperm viability kit: Available from Molecular Probes Inc., Eugene, Oregon. Item key: L-7011.
BSA-saline media: Contains final concentrations of 130 mM NaCl, 4 mM KCl, 1 mM CaCl, 0.5 mM MgCl, 10 mM HEPES buffer, 14 mM fructose, 1 mg/ml Bovine Serum Albumin (V), pH 7.4.
Bryan=s stain: 1% Eosin Y (E 4382 Sigma), 1% Fast Green FCF (F 7258 Sigma), 1% flavianic acid (F 6500 Sigma) in 1% acetic acid.
Paraformaldehyde fixative: Paraformaldehyde powder dissolved in PBS (ie: w/v dilution). Note: Use a magnetic stirrer to dissolve powder in PBS on hotplate (70□C), and add 1 M NaOH until solution is clear. Buffer to pH 7.4.
Superfix: Generally contains 4% paraformaldehyde and 2.5% gluteraldehyde in 0.1 M phosphate buffer.

Superovulation of Female Brushtail Possums

F. Molinia
Landcare Research, PO Box 69, Lincoln 8152, New Zealand
September 2003

Publications using this protocol:

1) Glazier AM, Mate KE and Rodger JC (2002). In vitro and in vivo maturation of oocytes from gonadotrophin-treated brushtail possums. *Molec. Reprod. Dev.* 62, 504-512.
2) Glazier AM (1999). Time of ovulation in the brushtail possum *(Trichosurus vulpecula)* following PMSG/LH induced ovulation. *J. Exp. Zool.* 283, 608-611.
3) Glazier AM and Molinia FC (1998). Improved method of superovulation in monovulatory brushtail possums *(Trichosurus vulpecula)* using pregnant mares= serum gonadotrophin-luteinizing hormone. *J. Reprod. Fertil.* 113, 191-195.
4) Molinia FC, Gibson RJ, Brown AM, Glazier AM and Rodger JC (1998a). Successful fertilization after superovulation and laparoscopic intrauterine insemination of the brushtail possum, *Trichosurus vulpecula* and tammar wallaby, *Macropus eugenii*. *J. Reprod. Fertil.* 113, 9-17.
5) Molinia FC, Gibson RJ, Smedley MA and Rodger JC (1998b). Further observations of the ovarian response of the tammar wallaby *(Macropus eugenii)* to exogenous gonadotrophins: an improved method for superovulation using FSH/LH. In: Brown JL and Wildt DE (Eds.), Reproductive Research in Wildlife Species. *Anim. Reprod. Sci.* 53, 253-263.
6) Rodger JC and Mate KE (1988). A PMSG/GnRH method for the superovulation of the monovulatory brush-tailed possum *(Trichosurus vulpecula). J. Reprod. Fertil.* 83, 885-891.

Background

Superovulation procedures are developed as a means of increasing productivity of the female and synchronising reproduction. The following procedures for superovulation of female brushtail possums are based on several published reports using exogenous gonadotrophin treatments: a) induction of growth and development of multiple ovarian follicles using pregnant mare serum gonadotrophin (PMSG) described by Rodger and Mate (1988), and Glazier *et al.* (2002); b) ovulation induction of PMSG-primed females with porcine luteinizing hormone (LH) described by Molinia *et al.* (1998a), Glazier and Molinia (1998) and Glazier (1999); and c) superovulation during seasonal anoestrus using porcine follicle stimulating hormone (FSH)/LH, based on that described for the tammar wallaby by Molinia *et al.* (1998b), and developed for the possum by Glazier and Molinia (2002).

Safety

Superovulation procedures require handling of the animals, and hormones are normally administered as intra-muscular (i.m.) injections after restraint in a hessian bag. Extreme care and experience is required to undertake these procedures. Rubber gloves are ALWAYS worn when handling animals. All hormones should be stored in a solvents cabinet, their use routinely recorded and their disposal is as for any sharps/contaminated waste.

Procedures

PMSG/LH

Possums in NZ normally breed in Autumn and to a lesser extent in Spring. The following superovulation procedure utilizes PMSG/LH and is **most** reliable from the months of April till the end of July.

i. Treat females with a single i.m. injection of 15 international units (i.u.) PMSG.
ii. 78 h later, treat females with a single i.m. injection of 4 mg LH.
iii. Onset of ovulation occurs 30-36 h post-LH treatment, and around 9-10 eggs per female can be recovered (from 48 h post-LH) using this method.

FSH/LH

During seasonal anoestrus, the PMSG/LH protocol is unreliable or fails. The following superovulation procedure (which is still in development) utilizes FSH/LH and so far is reliable from the months of November till the end of February.

i. Treat females with an i.m. injection of 3 mg FSH, twice daily (ideally 12 h apart) for four consecutive days (eight injections of FSH total).
ii. 12 h after the last FSH injection, treat females with a single i.m. injection of 4 mg LH.
iii. Onset of ovulation occurs from 27 h post-LH treatment, and around 11-12 eggs per female can be recovered (from 48 h post-LH) using this method.

Reagents

PMSG: Pregnant mare serum gonadotrophin. Intervet distribute this product under the name Folligon. Dilute sample powder using the diluent provided by the manufacturer and sterile PBS or 0.9% saline to a concentration of 50 i.u./ml and load 0.3 ml (ie: 15 i.u. dose) into 1 ml syringes attached to a 25 G x 5/8" needle. Use diluted samples immediately or they can be stored in a freezer (-20^{o}C) prior to use. If freezing, ensure there is an air-gap behind the 0.3 ml volume, and that the sample is properly thawed on the day of use.

LH: Luteinizing hormone. Vetrepharm (now called Bioniche) distribute this product as a lyophilised powder extracted from porcine pituitary glands under the name Lutropin-V. Dilute sample powder using the diluent provided by the manufacturer to a concentration of 5 mg/ml and load 0.8 ml (ie: 4 mg dose) into 1 ml syringes attached to a 25 G x 5/8" needle. Ideally, use diluted samples the same day or store briefly at room temperature.

FSH: Follicle stimulating hormone. Vetrepharm (now called Bioniche) distribute this product as a lyophilised powder extracted from porcine pituitary glands under the name Folltropin-V. Dilute sample powder using the diluent provided by the manufacturer and either sterile saline or PBS to a concentration of 10 mg/ml and load 0.3 ml (ie: 3 mg dose) into 1 ml syringes attached to a 25 G x 5/8" needle. Ideally, use diluted samples the same day or store for up to 5 days in the fridge (4^{o}C).

Superovulation in Wombat Species
(established for both common and southern hairy-nosed wombats)

M.C.J. Paris[1] & G. Druery[2]
[1]University of Glasgow, Glasgow, UK;
[2]Rockhampton, Australia
January 2006

Publications using this protocol:

1) West M, Lacham-Kaplan O, Bickell C, Cleary ML, Galloway D, Edwards G, and Paris MCJ. (2002) Successful superovulation of the Common Wombat and cleavage following ICSI of resulting oocytes. Theriogenology: 594.
2) Druery GV, Shimmin GA, Taggart DA, Temple-Smith PD, MacDonald C, Finlayson GR and Paris MCJ.(2003). Ovarian follicular superstimulation in the Southern Hairy-nosed wombat, Lasiorhinus latifrons. Theriogenology 59(1): 391.
3) West M, Paris MCJ, Cleary M, Shaw J, Galloway D, Trounson AO. (2003). In vitro maturation (IVM) of oocytes collected following hormonal ovarian stimulation of common wombat (Vombatus Ursinus). Theriogenology 59(1): 405.
4) Druery GV, Shimmin GV, Taggart D, Temple-Smith PD, MacDonals CH, Finlayson GR, Breed WG, Paris MCJ.(2003). Collection of mature oocytes from southern hairy-nosed wombats following ovarian follicular stimulation with porcine gonadotrophins. Australian Mammal Society, 49th Scientific Meeting, Sydney, New South Wales: 8.
5) McDonald CH, Taggart DA, Breed WG, Druery GV, Finlayson GR, Shimmin GA, Paris MCJ. (2003). The effect of gonadotrophins on ovarian follicular development and oocyte maturation in the southern hairy-nosed wombat. Proceedings of the 34nd Annual Conference, Australian Society for Reproductive Biology: 26, Reprod Fert Dev :15: 92 Melbourne, Victoria, Australia.
6). Druery GV, Rival MD, Taggart DA, Shimmin GA, Horsup AB, Temple-Smith PD, Paris DBBP, Paris MCJ.(2005). Timing of ovulation in the gonadotrophin-stimulated southern hairy-nosed wombat, Lasiorhinus latifrons. Reprod Fert & Dev 17: 246.
7) Druery GV, Taggart DA, Shimmin GA, Horsup AB, Temple-Smith PD, Paris DBBP, Paris MCJ. (2004). Time of ovulation and artificial insemination following superovulation in the southern hairy-nosed wombat (Lasiorhinus latifrons). Advances in Ethology 38 (supplements to Ethology) : 100. Contributions to the 5th International Symposium on Physiology, Behaviour and Conservation of Wildlife. Berlin, Germany, 26-29 September 2004.

Materials Needed for Superovulation:

<u>For Gonadotrophin Administration</u>
Needles (25G)
Syringes (1 ml)
Fridge
Hormones tested by our group:
porcine follicle stimulating hormone (pFSH; Folltropin-V; Bioniche, Canada)
Pregnant Mare Serum Gonadotrophin (PMSG; Folligon; Intervet, Australia or Bioniche, Canada)
Porcine luteinising hormone (pLH; Lutropin-V; Bioniche, Canada)

For Blood Collection:

Zoletil (Virbac Pty Ltd., Australia)
Syringes (5 ml)
Needles (21 G)
Ethanol
Gauze
Elastic band (Garrot) to visualize the vein
Heparinized blood tubes to collect plasma
Eppendorftubes
Centrifuge
Freezer for storage, minimum required temperature: –20 degrees.

Days	1	2	3	4	5	6	7	8	Ref
Short FSH/LH	2 X 6 mg FSH (im)	2 X 6 mg FSH (im)	2 X 6 mg FSH (im)	2X 6 mg FSH (im)	4 mg pLH (im)				1
Short FSH/LH	2 x 25 mg FSH (im)	2 x 25 mg FSH (im)	2 x 25 mg FSH (im)	2 x 25 mg FSH (im)	25 mg pLH (sc)				2
Long FSH/LH	2x4mg FSH (im)	2x4mg FSH (im)	2x4mg FSH (im)	2x4mg FSH (im)	2x4mg FSH (im)	2x4mg FSH (im)	2x4mg FSH (im)	4mg pLH (im)	Unpubl. Data
Long FSH/LH	2 X 13.4 mg FSH (im)	2 X 13.4 mg FSH (im)	2 X 13.4 mg FSH (im)	2 X 13.4 mg FSH (im)	2 X 13.4 mg FSH (im)	2 X 13.4 mg FSH (im)	2 X 13.4 mg FSH (im)	25 mg pLH (sc)	2
PMSG/ LH	15IU PMSG (im)			4mg pLH (im)					Unpubl. Data
PMSG	150 IU (im)			25 mg pLH (sc)					2

The efficiency of protocols above is discussed in the listed Literature. In general, it was found in the Southern Hairy nosed Wombat (so far most extensively been studied) that using the 7 day FSH/LH protocol gives oocytes the opportunity to reach metaphase 2 stage, and currently this protocol is thus recommended, also based on the fact that more precise information on the actual timing of ovulation relative to superovulation, is now available (Advances in Ethology 2004; 38 (supplements to Ethology) : 100, Reprod Fert & Dev 2005; 17: 246). Initial AI attempts have been undertaken using this information.

General comments

The injections (FSH and PMSG) are advised to be given intramuscularly, FSH injections are given 12 hourly apart, followed by either an intramuscularly or subcutaneous injection of pLH (see exact tested protocols above), 12 hours after the last FSH injection, or 72 hours after the PMSG injection.
Once prepared, the solutions can be kept for several days in the fridge, but can NOT always be frozen and can NOT be kept longer. Make sure to double check expiry date /instructions and the time that it can be stored !!

Procedure for blood collection

1. The animal will be sedated with an intramuscular injection of Zoletil. In general 3 mg/kg was generally enough to keep the animal asleep/ drousy enough for all required handling

2. Take a heparinized blood sample (ideally before start of superovulation and several times during the protocol, and also on the day of the LH administration), approximately 5 ml which will allow most hormonal assays. To do this, advisable is to visualize a vein in the peripheral circulation (forelimb or hindlimb). Alternate the leg you take blood from to avoid complications. The heparinised tube will be centrifuged at 1600 x g for 10 minutes. Plasma will then be collected from tubes, and stored at -20 degrees.

Superovulation, oocyte collection, in vitro maturation, semen collection and intracytoplasmic sperm injection in the tammar wallaby

Genevieve Magarey
Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo
September 2006

Publications using this protocol:

1) Magarey G. M. and Mate K. E. (2003). Fertilization following intracytoplamic sperm injection of *in vivo* and *in vitro* matured oocytes from an Australian marsupial, the tammar wallaby (*Macropus eugenii*). *Zygote* **11:** 339-346.
2) Magarey G. M., Rodger, J. C., Buist, J. M. and Mate K. E. (2003). Effects of repeated superovulation and surgical oocyte collection on ovarian response and natural breeding ability of the tammar wallaby (*Macropus eugenii*). *Reproduction* **125:** 701-707.
3) Magarey G. M. and Mate K. E. (2004). Comparison of glucose metabolism in *in vivo*- and *in vitro*-matured tammar wallaby oocytes and its relationship to developmental potential following intracytoplasmic sperm injection. *Reproduction, Fertility and Development* **16:** 617-623.
4) Magarey G. M. and Mate K. E. (2003). Timing and ultrastructure of events following intracytoplasmic sperm injection in a marsupial, the tammar wallaby (*Macropus eugenii*). *Reproduction, Fertility and Development* **15:** 397-406.

Procedures

Superovulation

The tammar wallaby is naturally monovular and highly seasonal, with breeding restricted to the period of decreasing day length between the summer and winter solstice (reviewed by Hinds, 1990). To collect multiple oocytes at one time wallabies are superovulated with exogenous hormones. The hormone regime varies according to whether immature (GV stage) or mature (MII stage) oocytes are to be collected.

For collection of immature oocytes

Follicular development is induced with 6mg porcine follicle stimulating hormone (pFSH) (Folltropin-V, Vetrepharm, Canada) administered intramuscularly (i.m.) twice daily at 12 hourly intervals for four days. On the fifth day, 12 hours after the last pFSH injection, immature oocytes (from follicles >1.5mm diameter) are harvested for *in vitro* maturation.

For collection of *in vivo* matured oocytes

As for immature oocytes, follicular development is induced with eight, 12 hourly pFSH injections administered i.m. over 4 days. Oocyte maturation is induced on day 5 with a single subcutaneous (s.c.) injection of 4mg porcine luteinizing hormone (pLH) (Lutropin-V, Vetrepharm) 12 hours after the last pFSH injection (Molinia et al., 1998a). This protocol induces superovulation of a similar number of eggs in both cycling and non-cycling wallabies year round (Molinia et al., 1998a). On day 6 mature oocytes are collected 24-30 hours after the pLH injection, prior to the onset of ovulation, either surgically or after euthanasia.

In vitro maturation

Oocytes collected from wallabies primed with pFSH only are left granulosa-cell-enclosed for *in vitro* culture. The *in vitro* maturation protocol is similar to that described by Mate and Buist (1999). The maturation media is Dulbecco's modification of Eagle's medium (DMEM) (Trace Biosciences NZ Ltd., New Zealand), supplemented with 10% (v/v) foetal calf serum (FCS), 10µg ml-1 pFSH, 10µg ml-1 pLH, 100IU ml-1 penicillin and 100µg ml-1 streptomycin. Granulosa-cell-enclosed oocytes are washed once in maturation media, then incubated individually in 400µl wells of maturation media at 36°C in 5% carbon dioxide (CO2) (CO2 incubator, Sanyo Electric Co. Ltd., Japan) in air for approximately 42 hours. This technique resulted in maturation of 60 – 70% of oocytes to MII stage by the end of the culture period.

Oocyte collection after euthanasia

At the time of oocyte collection, 12 hours after the last pFSH injection for immature oocytes, or 24-30 hours after the pLH injection for mature oocytes, each superovulated wallaby is euthanased. Euthanasia is performed by injection of 150mg ml-1 sodium pentobarbitone (Lethabarb: Virbac Pty. Ltd., NSW, Australia) either via the lateral tail vein, or by intracardiac injection following gaseous anaesthesia with 5% halothane (Halothane – M&B, Rhone Merieux, Victoria, Australia) or 5% isoflurane (Isoflo, Abbot Australia Pty Ltd, NSW, Australia) in oxygen. The ventral abdomen is shaved and sprayed with 70% ethanol to minimise contamination. An incision is made through the skin and linea alba from the xiphisternum to the pubis. The pouch is avoided by diverting the skin incision around the pouch opening, and moving it to one side before incising the linea alba. The median and lateral vaginae are transected at the point of attachment to the urogenital sinus, and lifted to enable excision of the entire reproductive tract cranial to the transection, including vaginae, uteri, oviducts and ovaries. The tract is then washed twice in Dulbecco's phosphate buffered saline (DPBS) (ICN Biomedicals Inc., USA) containing 12.5IU ml-1 heparin. The ovaries are dissected free of the fimbriae and ovarian ligaments and placed in a sterile petri dish. Under a dissecting microscope, each ovarian follicle over 2mm in diameter is flushed with a 26 gauge needle attached to a 3ml syringe. The flushing media is warm DMEM modified for bench-top use with reduced (0.34g l-1) sodium bicarbonate (NaHCO3) and additional 4.77g l-1 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (bench top DMEM) supplemented with heparin 12.5IU ml-1, 10% (v/v) FCS, 100IU ml-1 penicillin and 100µg ml-1 streptomycin. Oocytes are collected from the flushings under a dissecting microscope, using a finely pulled glass pasteur pipette attached to a mouthpiece and placed into fresh, warmed (35°C) DMEM supplemented with 10% (v/v) FCS, 100 IU ml-1 penicillin and 100 µg ml-1 streptomycin.

Surgical Oocyte Collection

Premedication and general anaesthesia

Prior to surgery animals receive prophylactic procaine penicillin (Table.1). Some wallabies are premedicated with the sedative midazolam. General anaesthesia is induced with 5% halothane or isoflurane in oxygen delivered via a T-piece and face-mask. When an adequate depth of anaesthesia is achieved a cuffed endotracheal tube (size 4 or 3.5mm) is placed into the trachea to more accurately maintain the depth of anaesthesia during the procedure. The anaesthetic depth is monitored closely and heart and respiratory rates are recorded every 5 minutes. The percentage oxygen saturation of the blood is monitored and maintained above 95% using a Cardio II pulse oximeter (Datex, USA) with sensor attached to the tongue. A surgical plane of anaesthesia is maintained during the procedure by adjusting

the halothane or isoflurane levels between 1.5 and 3%. Atropine sulfate is given if required during anaesthesia.

Preparation of surgical site

After induction and stabilisation of general anaesthesia wallabies are placed in dorsal recumbency and tilted, head down, to an angle of 30° to improve access to the reproductive tract. The surgical site is prepared by shaving, then scrubbing the area with 4% chlorhexidine gluconate (Chlorhexidine surgical scrub, Drion Laboratories Pty Ltd, WA, Australia) or iodine (Betadine Surgical Scrub, Faulding Pharmaceuticals, SA, Australia). The skin is sterilised by swabbing three times with 70% ethanol and three times with iodine. The surgical area is then draped. All surgical instruments and drapes are autoclaved before every use and standard aseptic techniques are used throughout the procedure.

Surgical procedure

The local anaesthetic bupivicane hydrochloride is injected at the incision site for post-operative pain management (Table.1). The incision site varied depending upon the presence and age of a pouch young. When a permanently attached pouch young is present, a 3 centimetre skin incision is made lateral to the pouch opening, at the cranial end of the epipubic bone. The pouch fold underlying the skin is pushed medially to reveal the deeper oblique abdominal muscles. These muscles are then split along the direction of the fibres (3 layers) until the peritoneum is revealed which is cut in the same direction as the last muscle layer.

When there is no pouch young (or the pouch young could be removed for the duration of the surgery and easily reattached to the teat) a 3 centimetre midline skin incision is made inside the pouch, midway between the teats and the cranial pouch border. After blunt dissection of the subcutaneous layers the linea alba is picked up with tissue forceps to allow a small stab incision into the peritoneal cavity without risk of puncturing internal organs. The linea alba incision is extended with blunt tip scissors and the peritoneum is incised. The wound is held open using small eyelid retractors. Visible immediately are the swollen vaginae, which are pushed out of the way medially to reveal the two uterine bodies. The ovaries are located dorsal to each uteri. The flat handle of a spay hook and one finger are used to manipulate one ovary, unrolling it (cranio-medially) from the fimbriae and uterine ligaments. Special care is taken not to rupture any of the mature ovarian follicles, nor tear any ovarian vessels or ligaments. The ovary is exteriorised through the laparotomy incision and gently held for follicle aspiration. Follicle aspiration is performed as described below (section 0). The ovary is then replaced to its original position in the abdominal cavity with the fimbria replaced around it. The procedure is then repeated on the opposite ovary. At the time of each surgery the ovaries are assessed visually and the number of normal mature follicles flushed and aspirated are counted. Abnormalities, such as cyst formation or adhesions, are assessed and recorded. The muscle layers or linea alba are closed using absorbable sutures (size 3.0 Dexon, Davis & Geck, USA). For lateral incisions, the pouch is replaced in its original position. A continuous absorbable suture (size 3.0 Dexon) is placed in the subcutaneous layer and the skin is closed using absorbable subcuticular sutures (size 3.0 Vicryl, Ethicon Inc., USA). The analgesic carprofen is administered during wound closure (Table.1). Vitamin E and selenium is administered as a prophylactic against myopathy.

Table.1 Drugs administered to superovulated wallabies during anaesthesia for surgical follicle aspiration

Drug	Dose	Route	Time of administration	Effect	Trade name and Supplier
Procaine penicillin	15 mg kg^{-1}	i.m.	Before surgery	Prophylactic antibiotic	Benacillin, Troy Laboratories Pty Ltd, NSW, Australia
Midazolam hydrochloride	0.25 mg kg^{-1}	i.m	Premedication (before general anaesthesia)*	Sedation	Hypnoval**,** Roche Products Pty Ltd, NSW, Australia
Isoflurane or halothane	5% for induction, 1.5-3% for maintenance in oxygen at 1.5 litre min^{-1}	Via face mask for induction; via endotracheal tube for maintenance	General anaesthesia	Induction and maintenance of general anaesthesia	Isoflo, Abbot Australia Pty Ltd, NSW, Australia; Halothane – M&B, Rhone Merieux, Victoria, Australia
Atropine sulfate	0.05 mg kg^{-1}	i.m.	If required during anaesthesia	Reduction of bradycardia or excessive salivary secretions	Atrosite, Troy Laboratories
Bupivicane hydrochloride	1mg kg^{-1}	Locally at surgical site	Before incision	Local anaesthesia (12 hours)	Bupivicane Injection B.P. 0.5%, Delta West Pty Ltd, WA, Australia
Carprofen	4 mg kg^{-1}	s.c.	After wound closure	Analgesia	Rimadyl, Pfizer Animal Health, NSW, Australia
Vitamin E and selenium	Vitamin E 3IU kg^{-1}, selenium 0.01mg kg^{-1} (0.02ml kg^{-1})	i.m.	During procedure*	Prophylactic against myopathy	Vitamin-E-Selen, Hoechst Australia Ltd, Victoria, Australia

* not given to all animals

Follicle flushing and aspiration

Based on the results of a preliminary trial in the possum (see Appendix 1), an 18 gauge double lumen needle (Cook Veterinary Products, Queensland, Australia) with flushing and aspiration systems is used for oocyte collection. One line of the double gauge needle is connected to a culture tube and a V-MAR-5100 Regulated Vacuum Pump (Cook Veterinary Products) providing follicular aspiration. The second line is connected to a 50ml syringe containing warmed bench top DMEM supplemented with heparin 12.5IU ml-1, 2.5-5% (v/v) FCS, 100IU ml-1 penicillin and 100µg ml-1 streptomycin, operated within a V-MAR-4000 Flushing System (Cook Veterinary Products) to flush the media through the

follicle. Follicles greater than 2mm in diameter are flushed and aspirated. The double lumen needle is inserted into each follicle, bevel facing down, with a suction of less than 100mm Hg applied (**Error! Reference source not found.**). The needle is twisted to ensure the entire follicular contents are aspirated and the follicle is flushed with culture media at a rate of 0.15ml per second. The flushed follicular contents and culture media are collected in a 12ml culture tube held at 36°C in a LEC 960 portable incubator (Lec Instruments, Victoria, Australia). Oocytes are susceptible to considerable damage during collection if aspiration pressures over 100mm Hg are used.

Small, unaspirated follicles (0.5 – 1.9mm) are pricked with a 26 gauge needle before the ovary is returned to the abdomen. This procedure is adopted for the second and third superovulation treatments due to the observation of large 'cystic' follicles (6-8mm) diameter after the first treatment. Small follicles are pricked in an attempt to minimise further cyst formation, since the cysts may have originated from continued growth of small unaspirated follicles stimulated by the first treatment.

Post surgical recovery and monitoring

On completion of the surgery, 100% oxygen is administered while the wallabies are placed partly in a hessian sack to recover from the anaesthetic. The endotracheal tube is only removed when consciousness is returning, and the animal showed a swallowing reflex by chewing the tube. Earlier tube removal resulted in respiratory obstruction. The wallabies are then kept in the hessian sack and closely observed for 1-2 hours. When full coordination is regained wallabies are returned to outside yards. Postoperatively, the animals are observed daily, for 5-7 days, for normal behaviour. One week after the surgery, the wallabies are caught and the wound is checked.

Sperm Collection and Preparation

Electroejaculation

Semen is collected from adult male tammar wallabies using a modified electroejaculation technique based on that previously described for marsupials (Rodger and White, 1978; Rodger and Pollitt, 1981; Mate and Rodger, 1991). Anaesthesia is induced with 5% isoflurane in oxygen delivered at a rate of 1-1.5L per minute via a T-piece and mask. The isoflurane concentration is reduced when an appropriate depth of anaesthesia is achieved. The penis is cleaned and a mild commercial microenema (Microlax, Pharmacia Australia Pty Ltd, NSW, Australia) inserted into the rectum to loosen any faeces and aid in evacuation once the procedure is complete. A lubricated rectal probe, 12 mm diameter and 200 mm long with 3 longitudinal brass electrodes at one end, is inserted into the rectum to induce electroejaculation by delivery of an electrical stimulus to the region of the prostate gland. The stimulus consisted of a 7-8 Volt, 32-45 Hertz, square wave direct current delivered in 10 cycles. The current limit is set at 100 milli-Amperes. Each cycle consisted of 5 seconds on and 5 seconds off. The stimulus is repeated once if necessary to obtain a sufficiently sperm rich sample. After the procedure the animal is allowed to recover on oxygen alone until consciousness returned. All animals are 'rested' for at least 4 weeks between electroejaculations.

Sperm preparation

The ejaculate (approximately 2-5 ml volume) is collected into a clean/sterile 50ml plastic tube and allowed to coagulate at room temperature for 30 minutes. A small sample of semen is microscopically examined immediately after collection to confirm the presence of motile spermatozoa. Spermatozoa are washed from the ejaculate by 'swim-up' (Mate and Rodger, 1991; Sistina et al., 1993b; Sistina et al., 1993a). Here, approx 5-10ml Minimum Essential Medium Eagle with Earle's Salts (Trace

Biosciences NZ Ltd.), supplemented with 4.77g l-1 HEPES, 2.2g l-1 NaHCO3, 253mg l-1 pyruvate 100IU ml-1 penicillin and 100µg ml-1 streptomycin, is gently layered over the coagulated semen and incubated at 36°C for 30 minutes to allow the motile spermatozoa to 'swim-up' into the medium, leaving behind the seminal plasma and prostatic bodies. Washed spermatozoa are then collected with a Pasteur pipette from the top layer. This 'swim up' is repeated once to provide a cleaner spermatozoon preparation. The concentration of spermatozoa is estimated using a haemocytometer and then diluted to between 2 and 5 x 106 spermatozoa per ml. This preparation is then divided into 0.5ml aliquots in 1ml centrifuge tubes and stored at 36°C in 5% CO2 until required for micromanipulation.

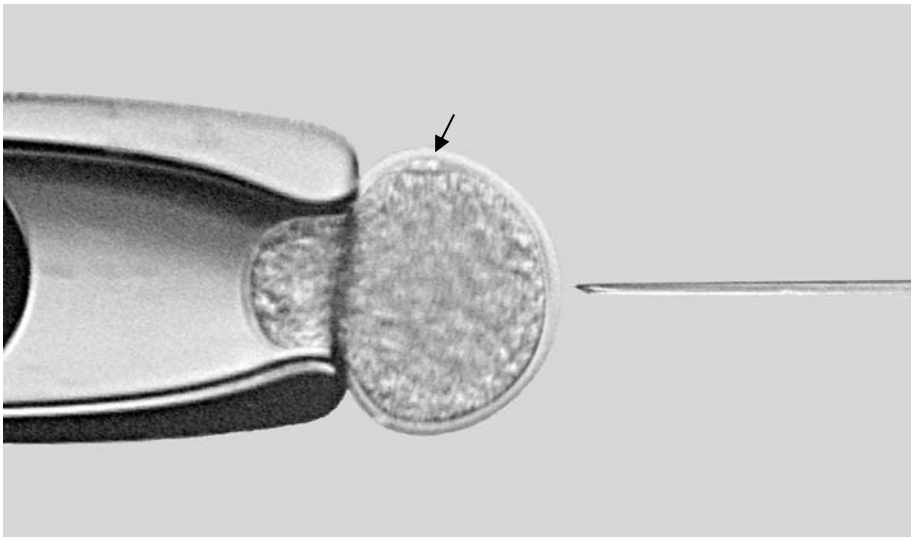
Intracytoplasmic sperm injection

Techniques for ICSI have been described previously (Van Steirteghem et al., 1993a; Catt et al., 1995; Payne, 1995; Joris et al., 1998) and a combination is employed for the tammar wallaby (Magarey and Mate 2003). Prior to micromanipulation, granulosa cells are mechanically stripped from the oocytes by gently pipetting them through a narrowly pulled glass pipette. Micromanipulations are performed in 60mm culture dishes under equilibrated tissue culture grade mineral oil (Sigma Chemical Company, Missouri, USA) pre-warmed to 36°C. Each culture dish contained 6 x 5µl individually numbered droplets of DMEM culture medium supplemented with 10% (v/v) FCS, 100IU ml-1 penicillin and 100µg ml-1 streptomycin, around a central 5µl droplet of 10% polyvinylpyrrolidone (PVP). The oocytes are washed once in DMEM before being transferred into the labelled 5µl DMEM droplets and held at 36°C in 5% CO2. Immediately prior to micromanipulation, 0.5-1µl of sperm suspension is added to the central PVP droplet and gently mixed.

Holding pipettes are either hand pulled or commercially purchased (Cook IVF, Queensland, Australia). Hand pulled pipettes are made from glass capillary tubing pulled by heating in a microflame and broken to an outside diameter of approximately 150µm. The tip is then heat polished with a microforge to an inside diameter of approximately 70µm. A bend of about 35 degrees is 'introduced' about 2mm from the tip. Commercial injection pipettes (Cook IVF) had with inside diameter 5µm and 35 degree angle at the tip.

All micromanipulations are preformed on a 36°C heated stage (Minitub HT200, Germany) of an inverted microscope (Zeiss Axiovert 35, Germany) with modulation contrast optics (Hoffman, USA). Holding and injection pipettes are each connected directly to approximately 60 centimetres of vinyl tubing (Nalge Company, USA) which are connected to 0.5ml threaded plunger syringes (Hamilton Company, USA) via bluntened 18 gauge needles. The plunger syringes and tubing are filled with Fluorinert FC40 (Sigma Chemical Company), ensuring there are no air pockets. The syringe is used to completely fill the pipettes. Dental floss is used to seal the connection between the pipettes and the tubing. The pipettes are held with H-7 Pipette Holders (Narishige Scientific Instrument Lab., Japan) which are moved using mechanical micromanipulators (Leitz, Germany). The pipettes are carefully positioned so that the tips are directly opposed and at an angle of about 10 degrees to the bottom of the culture plate. The tip of the injection pipette is lowered into a drop of 10% PVP under oil and backfilled with approximately 5mm PVP. The holding pipette is back filled with DMEM culture medium.
For ICSI, a single motile spermatozoon, in the central PVP droplet, is immobilised by striking the tail, perpendicular to the tail, with the injection pipette against the bottom of the dish. The spermatozoon is then aspirated, tail first, into the pipette and just back from the tip of the pipette. Both pipettes are then moved into a DMEM droplet containing an oocyte. The oocyte is held against the holding pipette with gentle aspiration with the first polar body positioned at 12 o'clock for mature oocytes. The holding pipette is moved up slightly so that the oocyte is no longer on the bottom of the dish then, with the sperm head immediately behind the bevel, the injection pipette is inserted through the zona pellucida

into the middle of the oocyte. Suction is applied to the injection pipette until a sudden rush of cytoplasm into the pipette past the spermatozoon, indicated the breakage of the plasmalemma. The cytoplasm and spermatozoon are then injected into the oocyte with a minimal amount of PVP. The injection pipette is withdrawn and the oocyte released from the holding pipette. The procedure is repeated for each oocyte. After micromanipulation, oocytes are transferred to individual 400µl wells of a 96 well flat bottom culture plate containing DMEM supplemented with 10% (v/v) FCS, 100IU ml-1 penicillin and 100µg ml-1 streptomycin and incubated at 36°C in humidified air with 5% CO2 for the specified time. Each oocyte is assigned with the number from it's DMEM droplet during micromanipulation and records are kept including the appearance of the oocyte and characteristics of the procedure.



Micrograph of a mature tammar wallaby oocyte with a polar body positioned at 12 o'clock (arrow) during intracytoplasmic sperm injection. The injection pipette (right hand side) contains a single wallaby spermatozoon to be injected into the cytoplasm of the oocyte.

Assessment of Oocytes After Culture

Modulation contrast and fluorescence microscopy – Hoechst stained

For fluorescence microscopy, oocytes are stained with the DNA specific dye bis-benzimide (Hoechst 33342, Sigma Chemical Company) (Pursel et al., 1985), diluted to 40µg ml-1 in DPBS. Oocytes are transferred individually into wells of a 96 well culture plate containing Hoechst staining solution and incubated in the dark for 20 minutes at room temperature. Oocytes are then rinsed 3 times in DPBS before examination.

For initial examination, oocytes are left in the 96 well plate in DPBS. Examination is conducted using an inverted microscope (Zeiss Axiovert 35) with either Hoffman modulation contrast optics or fluorescence. If details are not obvious, then the oocytes are transferred individually to a clean glass slide in a small drop of DPBS. A coverslip, supported at each corner with a drop of vaseline: wax mixture (9 parts vaseline: 1 part wax) is lowered over the oocyte and depressed until the oocyte is lightly held. When placed on the microscope stage, the oocyte could be gently rolled under the cover slip to enable observation from all angles. The slightly squashed effect improved visualisation of nuclear structures under fluorescence.

Light microscopy – acetic lacmoid stained

As an alternative method of assessment, or for confirmation of morphology after Hoechst staining and fluorescence microscopy, oocytes are stained with the DNA specific stain acetic lacmoid (Mate and Buist, 1999). Oocytes are placed on individual glass slides and lightly held under cover slips supported with a Vaseline: wax mixture, as described above (section 0). Quarter strength fixative (1 part glacial acetic acid: 15 parts 100% ethanol) is then introduced to one side of the cover slip with a glass pipette, while a tissue is held at the opposite side of the coverslip to draw the fluid through. The oocyte is observed under a dissecting microscope during this procedure, to ensure that it is not washed away with the fluid. Additional drops of fixative are added as required to prevent the oocyte from drying out. After 5 to 10 minutes, half strength fixative (1 part glacial acetic acid: 7 parts 100% ethanol) is drawn under the cover slip in a similar fashion. After a further 5 to 10 minutes, full strength fixative (1 part glacial acetic acid: 3 parts 100% ethanol) is drawn under the cover slip. The slides are then placed in a flat, shallow container of full strength fixative and stored at 4°C. After 1 to 4 days, filtered stain (1% lacmoid in 45% glacial acetic acid) is drawn under the cover slip using the pipette and tissue technique while observing the oocyte. The oocytes are assessed by light microscopy after 3 to 5 minutes. If necessary, an oocyte could be gently rolled under the cover slip to enable observation from different angles. For storage, the oocytes are washed with 45% (glacial) acetic acid and stored in 45% acetic acid.

Additional References

Catt J. W., Ryan J. P., Pike I. L., O'Neill C. and Saunders D. M. (1995). Clinical intracytoplasmic sperm injection results from Royal North Shore Hospital. *Reproduction, Fertility & Development* **7:** 255-262.

Hinds L. A. (1990). Control of pregnancy, parturition and luteolysis in marsupials. *Reproduction, Fertility & Development* **2:** 535-552.

Joris H., Nagy Z., Van de Velde H., De Vos A. and Van Steirteghem A. (1998). Intracytoplasmic sperm injection: laboratory set-up and injection procedure. *Humna Reproduction (Supplement 1)* **13:** 76-86.

Mate K. E. and Buist J. M. (1999). Timing and regulatory aspects of oocyte maturation *in vitro* in the tammar wallaby (*Macropus eugenii*). *Reproduction, Fertility & Development* **11:** 247-254.

Mate K. E. and Rodger J. C. (1991). Stability of the acrosome of the brush-tailed possum (*Trichosurus vulpecula*) and the tammar wallaby (*Macropus eugenii*) *in vitro* and after exposure to conditions and agents known to cause capacitation or acrosome reaction of eutherian spermatozoa. *Journal of Reproduction & Fertility* **91:** 41-48.

Molinia F. C., Gibson R. J., Smedley M. A. and Rodger J. C. (1998a). Further observations of the ovarian response of the tammar wallaby (*Macropus eugenii*) to exogenous gonadotrophins - an improved method for superovulation using FSH/LH. *Animal Reproduction Science* **53:** 253-263.

Payne D. (1995). Intracytoplasmic sperm injection: Instrumentation and injection technique. *Reproduction, Fertility & Development* **7:** 185-196.

Pursel V.G., Wall R. J., Rexroad J., Hammer R. E. and Brinster R. L. (1985). A rapid whole mount staining procedure for nuclei of mammalian embryos. *Theriogenology* **24:** 687-691

Rodger J. C. and Pollitt C. C. (1981). Radiographic examination of electroejaculation in marsupials. *Biology of Reproduction* **24:** 1125-1134.

Rodger J. C. and White I. G. (1978). The collection, handling and some properties of marsupial semen. *In* "The artificial breeding of non-domestic animals" (P. F. Watson, Ed.), pp. 289-301, Academic press, London.

Sistina Y., Lin M. J. and Rodger J. C. (1993a). Lysophosphatidylcholine disrupts the acrosome of tammar wallaby (*Macropus eugenii*) spermatozoa. *Molecular Reproduction & Development* **35:** 277-284.

Sistina Y., Lin M., Mate K. E. and Rodger J. C. (1993b). Induction of the marsupial sperm acrosome reaction *in vitro* by treatment with diacylglycerols. *Journal of Reproduction & Fertility* **99:** 335-341.

Van Steirteghem A. C., Nagy Z., Joris H., Liu J., Staessen C., Smitz J., Wisanato A. and Devroey P. (1993a). High fertilization and implantation rates after intracytoplasmic sperm injection. *Human Reproduction* **8:** 1061-1066.

Estrous Cycle Monitoring in Wombat Species
(established for both common and southern hairy-nosed wombats)

M.C.J. Paris[1] & G. Finlayson[2]
[1]University of Glasgow, Glasgow, UK;
[2]Institute of Wildlife Research, University of Sydney, Australia
January 2006

Publications using this protocol:

1) Finlayson GR, Shimmin GA, Taggart DA, Skinner JF, Gilmore A, and Paris MCJ (2005). The oestrous cycle of the southern hairy-nosed wombats (*Lasiorhinus latifrons*) in captivity. Anim Reprod Sci (in press).
2) Paris MCJ, White A, Reiss A, West M, Schwarzenberger F (2002). Faecal progesterone metabolites and behavioural observations for the non-invasive assessment of oestrous cycles in the common wombat (*Vombatus ursinus*) and the southern hairy-nosed wombat (*Lasiorhinus latifrons*). Anim Reprod Sci 2296:1-13.
3) West M, Galloway D, Shaw J, Trounson A, Paris MCJ (2004). The oestrous cycle of the common wombat, *Vombatus ursinus*, in Victoria. Reprod Fert Dev 16:339-346.
4) West M, Paris MCJ, Galloway D, Wright P, Shaw J, Trounson AO (2001). The oestrous cycle of the common wombat (*Vombatus ursinus*) assessed by comparing vaginal smears and plasma progesterone concentrations. Proceedings of the 32nd annual conference of the Australian Society for Reproductive Biology, Brisbane, Queensland, Australia, p. 62.

Materials needed for vaginal smear collection and assessment

Zoletil (Virbac Pty Ltd., Australia)

For Vaginal Smear Monitoring
- Glass tubes (10 cm long)
- Cotton swaps (long ones)
- Sterilization bags that will fit a 12-15 cm long object
- Sterile PBS / Saline
- Diff-Quick Stain (Laboratory Aids Pty Ltd), Staining set up available with beakers
- Microscope slides
- Microscope coverslips
- Mounting medium
- Light microscope for analysis
- Using Diff Quick: exact staining protocol is supplied with the bottles

For Blood collection
- Syringes (5 ml)
- Needles (21 G)
- Ethanol
- Gauze
- Elastic band (Garrot) to visualize the vein

Heparinized blood tubes to collect plasma
Eppendorftubes
Centrifuge
Freezer for storage, minimum required temperature: –20 degrees.

(Optional if you wish to measure weight, body temperature, pouch secretion, saliva secretion): weighing scale, rectal thermometer, and eppendorf tubes and filterpaper for saliva and pouch secretions).

Procedure

The animal will be sedated (ideally 3 times per week) with an intramuscular injection of Zoletil. In general 3 mg/kg was generally enough to keep the animal asleep/ drousy enough for all required handling

For Vaginal Assessments

A) Record status of the vaginal opening / urogenital sinus (extended, contracted/swollen/redish)

B) Make digital photos of pouch/urogenital status to get a record of cycling changes, can be correlated later on with findings in vaginal smear and Progesterone levels

C) Collect a vaginal smear, wet sterile glass tube in sterile PBS/saline, put into the vagina up to about 5 cm (mark the glass tubes before sterilisation process) and then push the cotton swab through and around and pull back before removing the glass tube again. This way you can ensure you always collect your sample from the same side, which is very important (to avoid that you get differences in results simply because you sampled from different regions !).

Blood Collection for Hormone Assessments

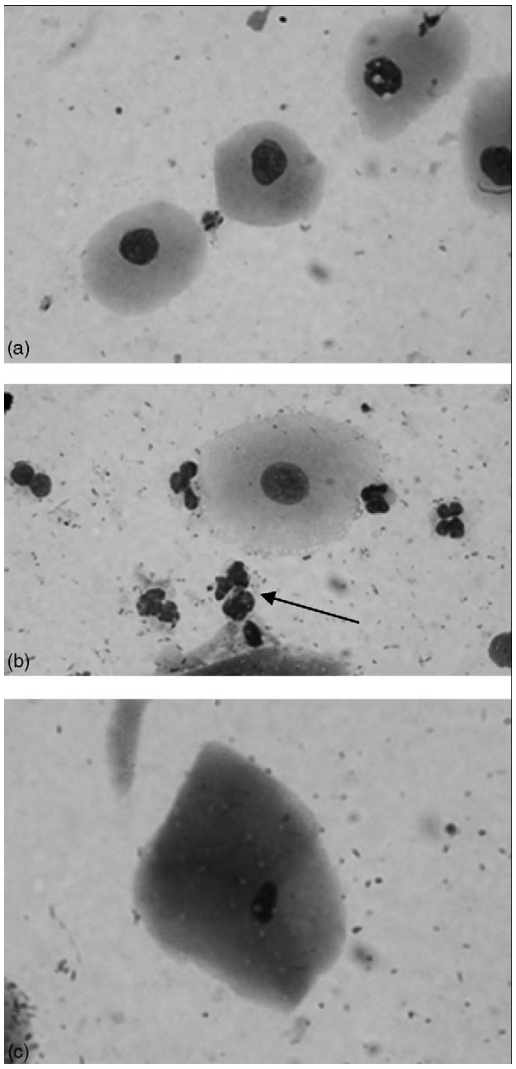
Take a heparinized blood sample, approximately 5 ml which will allow both Progesteron and Oestradiol assays. To do this, advisable is to visualize a vein in the peripheral circulation (forelimb or hindlimb). Alternate the leg you take blood from to avoid complications. The heparinised tube will be centrifuged at 1600 x g for 10 minutes. Plasma will then be collected from tubes, and stored at -20 degrees.

Optional other measurements that can be collected at that given time

A) Record Pouch Condition each time…moist/dry/dirty/clean,
B) measurement of Body Weight,
C) measurement of Body Temperature,
D) collect saliva sample (sterile on filter paper) and store at –20degrees in a microcentrifuge tube,
E) collect pouch secretion (sterile on filter paper again) and store at –20 in a microcentrifuge tube.

Vaginal smear assessment

Examine vaginal smears under a light microscope (100 X magnification) to differentiate between cell types. For analysis, cells will be grouped according to morphology as either parabasal-intermediate type cells (including basal, parabasal and intermediate) or superficial type cells (cornified cells with and without nuclei). In publications referred to above (Finlayson 2005, West 2004), vaginal smears are analysed using a 100-cell counting method, where 100 randomly selected cells were counted from each slide. The proportions of the two cell morphologies are compared to determine the oestrous cycle lengths (hormonal profiles, if collected, can be compared with relative epithelial changes found) . The number of leucocytes is also counted per 100 epithelial cells.



Vaginal cell cytology of the female southern hairy-nosed wombat:

(a) intermediate type cells;
(b) intermediate type cell and leucocytes (arrow);
(c) superficial epithelial cell with nuclei.

(a and b) typical of the luteal phase in cycling wombats and also in non-cycling or anoestrous individuals.
(c) Typical in the follicular phase of a cycling southern hairy-nosed wombat.

Wombat Faecal Progesterone Metabolite Analysis: Faecal Pregnanes: 5β-pregnane-20α-OH-pregnane assay

M.C.J. Paris[1] & F Schwarzenberger[2]
[1]Melbourne, Australia and [2]Vienna, Austria
March 2003

Protocol prepared by: Dr MCJ Paris

Publication using this protocol:

Paris MCJ, White A, Reiss A, West M, Schwarzenberger F. Faecal progesterone metabolites and behavioural observations for the non-invasive assessment of oestrous cycles in the common wombat (*Vombatus ursinus*) and the southern hairy-nosed wombat (*Lasiorhinus latifrons*). Animal Reproduction Science 2002: 2296: 1-13.

Enzyme Immunoassay (EIA) of steroid (Preganediol) on microtiter plate using a biotinylated steroid as label

Materials Needed:

- Fridge
- Centrifuge
- MTP shaker
- Pipettes
- SEP-PAK filters
- Glass beakers
- PH meter
- Washer Machine for automatic washing of microtiter plate (MTP) plates
  (If available otherwise manual washing is feasible)
- Reagents & Ingredients
- Absorbance reader

Sample preparation

Samples that were analysed in the publication "Paris et al, 2002" were collected fresh and stored at -20˚C until analysis. During the collection care was taken to collect samples that were not contaminated with urine. If samples need to be transported to another location, then please ensure the frozen status by transport on dry ice.

For the actual analysis:
Take 0.5 gram of the fecal sample (wet weight)
Put in 10 ml glass tube
Add 0.5 ml distilled water and 4 ml methanol
Shake for 30 minutes at Room temperature
Centrifuge for 15 minutes, 3700 rpm, at 5 degrees

Dilute sample (supernatant) 1:10 in assay buffer, make this dilution immediately, it is not possible to keep the concentrated sample because the methanol will evaporate.
It is this sample that is then used for the EIA.

Coating of microtiter plates

1.1 **Prepare before coating:** Coating buffer; 1.59 gram Na2CO3 (Merck 6392), 2.93 gram NaHCO3 (Merck 6329), dissolve and fill up to 1 liter with DDW adjust to pH 9.6 with (about 10 ml) HCl (1 mol/l)

1.2 HCL (1 mol/l), stable at RT (920 ml DDW + 80 ml 37% HCl (Merck 317))

Antibody coating on microtiter plate (MTP)
Nunc-Immunoplates (Nalge Nunc, International) F96 Maxisorp, 96 well plates
For one MPT prepare a solution of 50 µl coating AB **(for purchase / details contact Prof Schwarzenberger)** dissolved in 25 ml coating buffer (see 1.1). Dispense 0.25 ml/well of diluted antibody to MTP. Incubate the plate at Room temperature overnight. Discard the solution and refill each well with 0.3 ml "“second” coating buffer.

Second coating buffer :Prepare before use, store at 4 degrees if prepared before:
1.3 3.146 g Trishydroxyaminomethane (Merck 8382, 20 mmol/l)
23.3 g NaCl (Merck 6404)
13 g BSA (Sigma A-4503)
1.3 g Sodium azide (Merck 6688) dissolve and fill up to 1.3 l with DDW and adjust to pH 7.5 with (about 40 ml) HCL (1 mol/l)

Cover the filled MTP with parafilm and dust cover and keep it at room temperature until use. You can use the MTP after 3 hours, store the plates no longer than 4 weeks at room temperature.

Reagents
Keep all stock solutions frozen at –20 until use. Waterproof color label marked on small vial indicates different content as follows (when purchased from Vienna)
Blue: Antibody (AB) **for details used in the publication: contact Prof Schwarzenberger**
Red: Biotin-labelled steroid (BL) **for details used in the publication: contact Prof Schwarzenberger**
Green: Standard
for details used in the publication: contact Prof Schwarzenberger

Dilute 0.01 ml of a stock solution (1mg steroid per ml CH3OH) with 20 ml of assay buffer, mix for 2 minutes in the supersonic bath and wait 3 hours to fill it in portions of 0.05 ml. One vial has a concentration of 25,000 pg. (*this applies to the standard)*

These are the stock solutions as they can be purchased from Vienna

Preparation of dilutions for the MTP

Assay buffer

1.4 Assay buffer, store at 4 degrees:
2.42 g Trishydro-aminomethane (Merck 8382), 20 mmol/l, 17.9 g NaCL (Merck 6404), 0.3 mol/l, 1 g Bovine serum albumin (Sigma A-4503), 1 ml Tween 80 (Merck 822187), dissolve and fill up to 1 l with DDW and adjust to pH 7.5 with (about 17 ml) HCL (1mol/l), filter through SEP-PAK C18

Filtration through SEP-PAK C18. You need SEP-PAK C18 catridge column (Millipore 51910), rinse with 5 ml Methanol (Merck 6009); rinse with 10 ml DDW, connect column to tubing of peristaltic pump, discard the first 10 ml of filtrated buffer, collect buffer in a clean bottle.

DAY 1

Do the preparations as above:

Then:
Prepare standard. Add 0.15 ml of assay buffer to 0.05 ml standard vial (one portion), shake/vortex and wait 15-20 minutes.
Also both the standard, antibody and biotin label solution have to incubate 20 minutes before you can work with them so also prepare now:

AB & EL to be made up to certain concentrations depending on the batch used, check with Prof Schwarzenberger

In the mean time you can prepare other solutions/dilutions.

Such as eg for serial dilutions, dilute if already 1:10, then do 1:20, 1:40, 1:80, 1:160

Then dilute the standard, till this gives you a dilution from 500 pg till 2 pg per 0.01 ml

Plate procedure
Before use, wash coated MTP three times with washing solution.

Washing solution: 0.5 ml Tween (Merck 822184), add 2.5 l DDW (double distilled water)..mix well !!!

Stored in big plastic bottle, stored at RT during day but at night at 4 degrees because the last step of the assay (after the O/N incubation) should happen at 4 degrees.

Remove the rest of the liquid by pushing the MTP on paper towels. Do not touch the bottom side of the plate, cover directly with parafilm so the plate does not drie out !!!!! Very important !!!!

Then add samples and so on to the plate (see diagram)

NSB: 0.15 ml buffer + 0.1 ml EL
0: 0.05 ml buffer, 0.1 ml AB + 0.1 ml EL
samples: 0.05 ml samples (0.01 ml sample + 0.04 ml buffer) + 0.1 ml AB + 0.1 ml EL
the amount of sample depends, eg for cow stress hormones you take 0.05 ml sample instead of 0.01 ml + 0.04 buffer)

step 1: add buffer or samples
step 2: add 0.1 ml of antibody solution into each well but not in NSB wells
step 3: dispense 0.1 ml biotin steroid into each well.
Step 4: Cover MTP with parafilm and dust cover, shake mild) the MTP overnight at 4 degrees.
Important to do this correctly so that nothing dries out !!!!

DAY 2

After O/N incubation, decant incubated MTP, and wash MTP four times with **COLD (4 degrees)** washing solution. Between washing, shake for 10-30 seconds on a MTP-shaker

Solution needed:
Substrate buffer for peroxidase, can be stored at 4 degrees:
1.36 g Sodium acetate (Merck 6267)= 10 mmol/l, dissolve and fill up to 1 liter with DDW and adjust to pH 5.0 with 5% citric acid (Merck 244)

Stop reagent, can be prepared many weeks before usage, can be kept at RT:
2 mol/l H_2SO_4; 900 ml DDW + 100 ml H_2SO_4 (95-97 %, Merck 731)

Then:
<u>Streptavidin reaction:</u>
Prepare before usage:
0.001 ml streptavidin-POD-conjugate (=0.5 U; Boehringer 1089153, 500 U) with 30 ml assay buffer, mix on a magnetic stirrer for a few minutes IMMEDIATELY before use !

Dispense 0.25 ml of enzyme solution in each well and incubate the covered plate for 45 minutes at 4 degrees by shaking

Wash again as above

<u>Colour reaction (should turn blue)</u>

Substrate solution for peroxidase:
30 ml of substrate buffer
0.5 ml 3,3,5,5 tetramethylbenzidine (0.4%; Fluka 87748) in DMSO (Fluka 41641)
0.1 ml 0.6% H_2O_2 (0.1 ml H_2O_2, 30% Merck 8600 + 5 ml DDW), mix on a magnetic stirrer a few minutes before use

Dispense 0.25 ml of substrate solution in each well and incubate the covered plate 45 minutes at 4 degrees (shaking)

Stop reaction (should turn yellow)
Dispense 0.05 ml of stop reagent

Absorbance measuring
Reference filter: 620 nm
Measuring filter: 450 nm

The obtained results should measure pregnane values up to around 500 ng/g faeces in the elevated periods of the cycle.

LH Radioimmunoassay

Monique C.J. Paris
Murdoch Childrens Research Institute, Victoria, Australia
September 2002

Publications using this protocol:

1) Matteri RL, Roser JF, Baldwin DM, Lipovetsky V, and Papkoff H. Characterization of a monoclonal antibody which detects luteinizing hormone from diverse mammalian species. Domest Anim Endocrinol. 1987 Jul;4(3):157-65.
2) McFarlane JR, Rudd CD, Foulds LM, Fletcher TP, and Renfree MB (1997) Isolation and partial characterisation of tammar wallaby luteinizing hormone and development of a radioimmunoassay. J Reprod. Fert. 9: 475-480.

This assay has been used to measure LH in a range of marsupial species and can also be used many other species. The assay was originally published by Matteri et al (1987) and modified by us (McFarlane et al (1997) for use in marsupial species. We use ovine LH (oLH) as both standard and tracer but if authentic hormone is available this can also be used.

Preparation of the LH tracer

Reagents: Make up a stock of IODOGEN at 10ug/ml in methylene chloride or chloroform. Store in a tightly sealed bottle at –20^{o}C.

Method:

1. Evaporate 50 ul of IODOGEN in the bottom of an eppindorf tube or 12x75mm glass tube (reaction tube) with nitrogen.
2. Dissolve hormone 2-5ug in 50ul of PBS buffer.
3. Add hormone to the reaction tube.
4. Add 0.25mCi of I^{125} to reaction tube.
5. Allow the reaction to proceed for up to 10 minutes with occasional mixing.
6. Stop reaction by removing the reaction tube contents and mixing with 950ul of RIA buffer.
7. Add the 1ml reaction / RIA buffer mixture to a equilibrated PD10 column (Pharmacia), do not collect the 1ml waste.
8. Add 1ml of RIA buffer and discard waste.
9. Add a further 3ml of RIA buffer in 1ml lots collecting each eluted buffer.
10. Count 2-5ul from each fraction and record.

11. The iodinated tracer should elute in the 2 tube and may be processed further depending on hormone.

Buffers

10X PBS (O.5M Phosphate, 1.5M NaCl)	pH 7.4
Na_2HPO_4 (FW = 141.96)	57.49g
KH_2PO_4 (FW = 136.09)	12.93g
NaCl (58.44)	87.66g
Water	Make up to 1,000ml

RIA Buffer (0.05M Phosphate, 0.15M NaCl)

10x PBS stock	100ml
2mM EDTA	0.68
BSA	1g
NaN_3	1ml of 10% solution
Water	Make up to 1000ml

Normal Mouse Serum

Dilute normal mouse serum 1:10 with a 50% glycerol / PBS

Primary Antibody

The primary antibody is the Monoclonal Antibody Mab5186

Secondary Antibody

Our lab uses an in house sheep anti mouse Ig but any commercial antisera is sutiable. This is stored at 1:2 50% glycerol.

PROCEDURE: (all tubes to be done in duplicate)

Standards: Dilute the standard aliquot (100ng/ml oLH) serially in half dilutions for 9 dilutions. This will give you 10 standard tubes in duplicate (20 tubes).

Samples: Into each sample tube, in duplicate pipette 100 *u*L of plasma or serum.

QC: A quality control plasma should be prepared and used in every assay. This can be made by combining small volumes of plasma from all the samples to make a pool of plasma. This plasma should be stored frozen in small aliquots until used.

Tracer: Dilute the tracer to a concentration of 10,000 cpm/100ul.

Primary Antibody: Determine the total amount of solution to make up by the number to tubes in assay plus a couple of mLs. (100 *u*L of solution per tube). Normal Mouse serum (NMS) is stored at 1:10 and is used at 1:200. Calculate the uLs of NMS required by dividing the total mLs needed by 20, then multiplying by 1,000. This will give you the amount in ul that you need to add to the volume of RIA buffer. Before adding the primary antibody add 100ul of this to each NSB tube. The primary antibody is

stored at 1:1000 and is used at 1:250,000. Use the same calculation as you used for the NMS except divide by 250. Add this amount to the RIA/NMS buffer and vortex.

Controls: Three controls are to be run with each assay: Tc (total count), B_0, and NSB. B_0 tubes evaluate the maximum binding of tracer to antibody and NSB tubes monitor the non-specific binding.

Tc tubes: 100 *u*L tracer.
B_0 tubes: 100 *u*L RIA buffer + 100 *u*L (NMS + Ab) + 100 *u*L tracer.
NSB tubes: 100 *u*L RIA buffer + 100 *u*L (NMS) + 100 *u*L tracer.

DAY 1:

100 *u*L of Samples and Standards are each delivered into respective, labelled, duplicate tubes.

100ul of NMS/Ab (primary antibody solution) to all tubes except the TC and NSB.

100ul of Tracer to all tubes

Vortex all tubes and incubate overnight (at least 12 hrs) at room temperature covered with aluminium foil.

DAY 2:

SECONDARY ANTIBODY

To determine the total amount of secondary antibody required, multiply the volume of CTS used above by 2. This is the volume of **RIA BUFFER** needed. The secondary antibody goat-anti-mouse (GAM) is stored at 1:2 and used at 1:80. Calculate the *u*Ls of GAM needed by dividing the total volume needed by 40 and multiplying this by 1,000. Add GAM to RIA buffer and vortex.

Deliver 100 *u*L of GAM + RIA buffer to all tubes EXCEPT Tc.

VORTEX ALL TUBES.
INCUBATE OVERNIGHT (AT LEAST 12 HRS) IN COLD ROOM COVERED WITH ALUMINIUM FOIL.

DAY 3:

Add 1.6 mL of cold saline to all tubes, EXCEPT Tc, and immediately cold centrifuge all tubes, EXCEPT Tc, at 3500 RPM for 30 minutes. Only add saline to tubes for 1 centrifuge run. If more tubes, wait to add saline until just before spinning. Ensure one pair of Qc in every centrifuge run.
ASPIRATE IMMEDIATELY

Load tubes into gamma counter for counting, use Protocol 13 rack for I^{125} assays.

After counting enter and analyse data.

Ovarian Tissue Cryopreservation

J. Shaw
Monash Institute of Reproduction and Development
Monash University
September 2003

Publications using this protocol:

1) Paris MCJ., Snow, M., Cox, S-L, and Shaw JM. (2003) Xenotransplantation: can this technology provide a tool for reproductive biology and animal conservation ? Theriogenology (In press)
2) Shaw JM & Nakagata. N. (2002) Cryopreservation of transgenic mouse lines. Ch 10 in: *Methods in Molecular biology*, vol 180:207-228 "Transgenesis techniques: Principles and Protocols" 2nd Edn Editor A. Clark Humana press Totowa.
3) Shaw JM. & Trounson AO. (2002) Ovarian tissue transplantation and cryopreservation: its application to the maintenance and recovery of transgenic mouse Ch 11 in: *Methods in Molecular biology*, vol 180:229-251 "Transgenesis techniques: Principles and Protocols" 2nd Edn Editor A. Clark Humana press Totowa
4) Cleary, M. Snow, M. Wolvekamp, M. Shaw, J.M. Cox S-L and Jenkin G. (2001) Cryopreservation of mouse ovarian tissue following prolonged exposure to an ischaemic environment. Cryobiology. 42:121-133.
5) Snow, M. Cleary, M. Cox, S-L. Shaw J., Paris M. and G. Jenkin (2001) Comparison of the effects of in vitro and in situ storage on the viability of mouse ovarian tissue collected after death. Reprod. Fertil. Dev. 13:389-394.

Application

Ovarian tissue cryopreservation has been applied to a wide range of species including:

- *Eutherians*: Mouse, rat, rabbit, sheep, pig, cow, marmoset, macaque, human, cat, elephant, dog.
- *Marsupials*: Wallabies, Sminthopsis, common wombat.
- Others: e.g. Silkworm.

In all these cases the tissue has been viable after thawing (as assessed by *in vitro* or *in vivo* assays). Live young can be derived from frozen thawed grafted ovarian tissue, but to date only a small number of studies have proven that this is possible (mainly mouse and sheep). This indicates that ovarian tissue banking should be possible for a wide range of species. Most studies have used protocols that are identical or very similar to that described in this document. We recommend that it, or another proven procedure, be followed as closely as possible, because even though some variations to this protocol have relatively little impact on the outcome [e.g. using a different buffer, an equivalent cryoprotectant

(ethylene glycol instead of DMSO), or dehydrating agent (e.g. trehalose instead of sucrose) or slightly different equilibration temperatures or times], other changes can be detrimental (e.g. using glycerol rather than DMSO) or lethal (e.g. altered cooling or warming rates). The protocol provided here is a "slow cooling" procedure because this is the one that has (to date) proven most versatile and reliable.

Background

Cryopreservation of ovarian tissue is a simple procedure which can be used to store the germline of valuable female animals. In brief, it involves putting ovarian tissue into antifreeze solution and then cooling it to a low sub zero temperature (liquid nitrogen) for storage. The tissue can subsequently be thawed for use or analysis. For best results use good quality tissue. Ideally the tissue should be collected from young healthy females and processed for cryopreservation immediately after collection with sterile/ aseptic techniques. The reasons for this are:

- Age: ovarian tissue of mammals contains the maximum number of germ cells (eggs/ oocytes) at or around the time of birth, after this time there is an exponential loss with age. In some species old females may have no viable oocytes left in their ovaries.
- Health: Diseased cells present within ovarian tissue (e.g. cancer cells) can survive frozen storage. Other infectious agents can also survive frozen storage.
- Sterile/aseptic technique: Frozen material may be used to generate offspring by any of a range of techniques including in vitro maturation, in vivo maturation (following grafting to a suitable recipient), or nuclear transfer. The presence of contaminating bacteria, fungi or other infectious agents could prevent or compromise these uses.
- Rapid processing. Aim to collect ovaries by surgery or immediately after the death of an animal. Delays between the death of the animal and collection have a detrimental effect on the tissue. The viability of follicles declines as the delay between collection and cryopreservation increases. A delay of only a few hours within the body of the dead animal, is highly detrimental and kills most follicles. Ovarian tissue which is removed from an animal immediately after its death (or is removed by surgery) and placed in e.g. Phosphate buffered saline on ice or at room temperature, deteriorates less rapidly than within a dead animal, but even with this treatment few, if any, viable follicles remain 48 h after collection.

Current cryopreservation procedures focus on collecting the outermost ovarian cortex. The reason for this is that the primordial follicles (the oocyte stage which is most abundant in the ovary, and which tolerate cryopreservation very well) are mainly located within the outermost layer (2mm or less from the surface) of the ovary. Unfortunately primordial follicles are very small (<50microns) and very difficult to see except by histology or after enzymatic digestion of the ovary. A freezing protocol which preserves primordial follicles should allow later stage follicles to be generated if required because the primordial follicles grow to form mature oocytes/ follicles. In the mouse it takes approximately 3 weeks for a primordial follicles to grow and mature into a mature oocyte either in vitro or in vivo. In other species this process takes longer (months) and can (currently) only be completed in vivo (by auto, allo or xenografting). During the period of maturation many cytoplasmic and nuclear maturation (including imprinting) steps take place within the oocyte which prepare the egg for fertilization and development to term. This report details a standard slow cooling cryopreservation protocol for ovarian tissue which is known to be compatible with the survival of primordial follicles within ovarian tissue for a wide range of species. Rapid cooling procedures are available but they have only been tested on a few species.

Summary: Media and Products

a. *For collection*: PBS or other bench type handling media (100 ml per large ovary) (sterile, without protein)

    - Note: If **oocytes** are to be collected approx. 10 ml of protein containing bench solution for oocyte handling (e.g. 3-8 mg/ml Bovine Serum Albumin or 5-10% serum) should be prepared.

b. *For equilibration and freezing*: Cryoprotectant solution: one 10 ml tube per large ovary (1.1 ml DMSO; 0.32g sucrose made up to 10 ml with buffer. Sterile without protein)

c. *For dilution after thawing*:

    - First dilution solution: 0.55 ml DMSO and 0.62 g sucrose in a 10 ml tube (3ml per vial). Sterile without protein
    - Second dilution solution: 0.62 g sucrose in a 10 ml tube (3ml per vial). Sterile without protein

Solutions should be sterile. They can be sterilized by filtration through a sterile 0.22 micron filter (e.g. syringe type filter).

Summary: Large equipment

- Storage tank (liquid nitrogen or equivalent)
- A vapour shipper may be needed if specimens are to be transported frozen.
- Cooling device
- Freezing machine (Note most freezing machines require access to electricity)
- OR Passive cooling container or equivalent (this requires dry ice in an eski or a –80 freezer)
- Sterile work area (biohazard hood or equivalent is best)
- Microscope if there is a need to look for/ collect eggs.

Summary Other Materials and Equipment

Note: If the tissue has to be sterile then it is advisable to pre-sterilize items marked with *	Collection & dissection	For freezing	For thawing
ITEM			
*Gloves (mask/ cap)	Yes	Yes	Yes
Safety goggles	(Optional)	Yes	Yes
Betadine/ alcohol or equivalent to sterilize skin prior to dissection	Yes	--	--
Clippers (to remove/ shave fur /wool/hair)	(Optional)	--	--
*Dissection equipment (e.g. scalpel, scissors, forceps, retractor)	Yes	--	--
*Specimen jar/ tube (e.g. 50 ml screwcap) for transport of the ovary/ ovaries	Yes	No	No
Ice, crushed, in container (e.g. polystyrene eski)	Recommended	Yes	No
*Tubes containing the required solutions (outlined above)	Yes	Yes	Yes
*Petridishes, 10 cm (allow at least 2 per ovary)	Yes	(Optional)	No
*Petridishes, 35 mm	Yes (for oocytes)	No	Yes
*Pipettes or equivalent for measuring solutions (e.g. 1 and 10 ml)	Yes	Yes	Yes
Test tube rack (optional)	Yes	Yes	Yes
*Scalpel #20 or #22 blade (for ovary dissection)	Yes	No	(Optional)
*Scissors (small for ovary dissection)	Yes	No	No
*Forceps: Small, for holding and moving the tissue.	Yes	Yes	Yes
*Forceps: large (or equivalent, for handling and seeding)	(Optional)	Yes	Yes
*Pipettes or equivalent (e.g. pasteur pipettes with pipette bulb/ plastic transfer pipette) to move small volumes of solution	Yes	Yes	Yes
*1 ml syringe with 26 g needles or equivalent (for puncturing follicles)	Yes	No	No
Tissues	Yes	Yes	Yes
Alcohol (in squirter bottle)	Yes	Yes	Yes
Pen/ paper for taking notes	Yes	Yes	Yes
Indelible pen (for marking dishes and tubes)	Yes	Yes	Yes
Garbage bin/ bin liner	Yes	Yes	Yes
Sharps container	Yes	Yes	Yes
Hand wash soap/ water to wash hands	Yes	Yes	--
Bench cover (e.g. plastic backed paper)	(Optional)	(Optional)	(Optional)
Plastic apron/ gown/ labcoat	Advisable	Advisable	Advisable
Liquid nitrogen	No	Yes	Yes
Benchtop container for liquid nitrogen (can be eski)	No	Yes	Yes
*Cryovials 1.7 ml star-foot, external thread, conical or round base (2 per person)	No	Yes	No
Test tube rack (for Cryo tubes) (optional)	No	Yes	No
Cryo Canes	No	Yes	No
Timer	No	Yes	Yes
Insulating Cryo-gloves	No	Yes	Yes
Thermometer (for water temperature)	No	No	Yes
Waterbath or Beaker (warm water for thawing)	No	No	Yes

If eggs are to be collected then a pipettor or equivalent that can be used to move eggs between dishes is needed. If eggs are to be stored or used for ART, then additional solutions and materials (not listed here) would be required

Steps in preparing specimens for cryopreservation:

It is advisable to prepare all *equipment* and *solutions* needed for collection, dissection and cryopreservation (listed in the table above) in advance.

For collection

Prepare the media, equipment and items listed above:

Procedure

If possible remove fur/ hair/ wool and swab the skin over the intended incision site with antiseptic. Use sterilized dissection instruments. Access the ovary via one (or more) incision through the skin and abdominal wall. Excise the ovary(s) /piece of ovary and place it in a sterile pot/ tube/ dish with sterile (protein free) media e.g. saline or PBS as soon as possible after collection. If the specimen cannot be processed immediately, or has to be transported to another location then place the specimen on ice.

For dissection

Prepare the media, equipment and items listed above:

Procedures

- Very small ovaries (less than 5x5x5 mm) do not need to be dissected, as they can be frozen whole (skip to the section on freezing).
- If only a part of an ovary has been collected then identify the outer and inner surfaces. If the piece(s) is less than 1 mm thick it can be cryopreserved without further dissection. (Skip to the section on freezing).
- For larger ovaries or large ovarian pieces (> 1 mm thick) dissection is recommended as it facilitates the entry of the cryoprotectant into the tissue. The aim of the dissection is to remove the outer cortical tissue (tough, white, fibrous) for freezing.
- If a whole (large) ovary has been collected, place it ovary in a dish of PBS at room temperature (condensation makes it difficult to work with ice cold specimens). Use your instruments to separate the white firm (outermost) region from the underlying red tissue. It can be difficult to separate the two layers, but it can usually be achieved using a combination of sharp, scissors (large and small), and scalpel blades
- (e.g. #22 &/or #20). If the white tissue surrounding the ovary is normal (smooth, elastic, and tough, around 1mm thick) it can be cut into “sheets” (5 x 5 mm x 1 mm) or “strips” (1-2 mm wide x 0.5 to 1 cm long, 1 mm deep) relatively easily.
- Figure: In larger species the cortex (the outermost region of the ovary) is the region that is collected and frozen because it contains most primordial follicles.

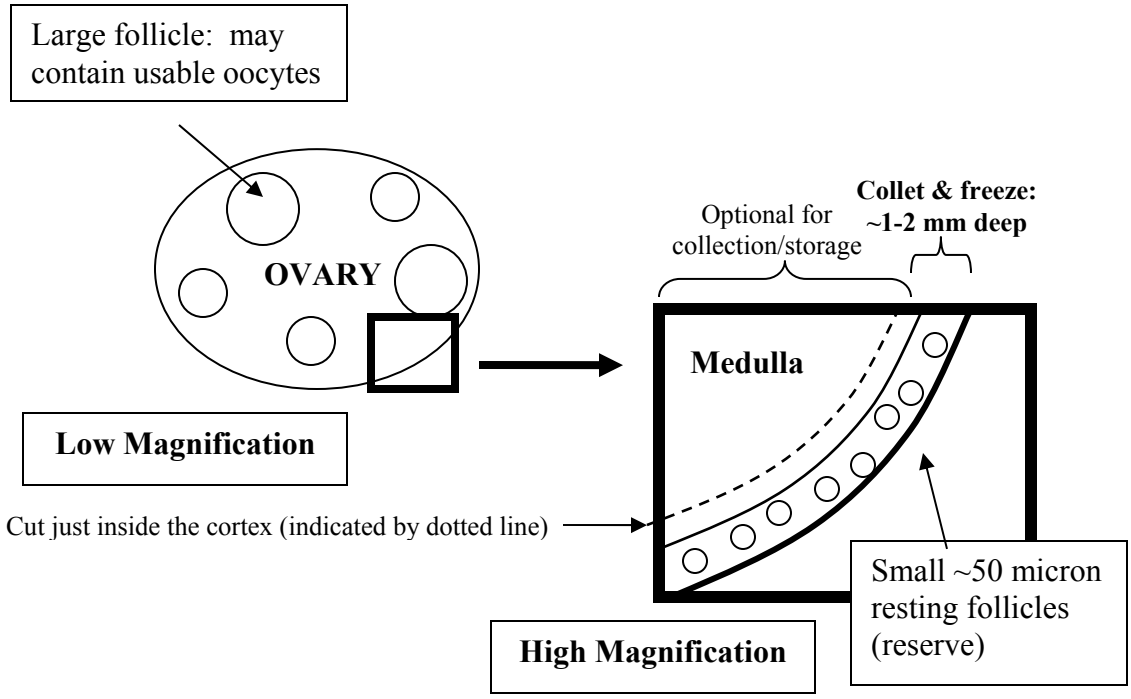


Figure: In larger species, the ovarian cortex (outermost region of ovary) is the region that is collected and frozen because it contains most of the primodial follicles and oocyte reserves.

Cryopreservation

Have the media, equipment and items listed above prepared in advance:
The protocol outlined here allows ovarian tissue to be cooled to and stored at –196C. Access to a liquid nitrogen storage tank or the equivalent (nitrogen vapour storage tank) in which this tissue can be stored long term is essential.

Preparation of the freezing machine (or equivalent)

- If a programmable freezing machine is available a suitable programme is: hold at -6C until the chamber is loaded and seeded. Once seeding is complete cool the chamber at 0.3°C/ min to -40°C, then rapidly cool to –196C.
- If a programmable freezing machine is **not** available then low cost equipment such as the "Nalgene Mr frosty" (Sigma C1562) can be used. These will, when placed in a –80C freezer, cool ampoules/vials at around 1C/ min to –80C. This is adequate for ovarian tissue freezing. Place the Mr Frosty in the refrigerator or on ice for at least 1 hr before freezing is due to commence. If there is no –80 C freezer available then the Mr Frosty can be placed in a polystyrene box with pellets (or a block) of dry ice (frozen CO2).

Preparation of the solutions and vials

Cryoprotectant should be made up, filtered and cooled to 0-1°C (in ice) before the dissection starts. Ovarian tissue is most commonly frozen in 1.5 M DMSO (i.e. 5.5 ml Dimethyl sulphoxide (e.g. Sigma

Hybrimax, D-2650)) and 0.1 M sucrose (i.e. 1.71g sucrose (e.g. Sigma S-7903)) made up to 50 ml with physiological, protein free, buffer (e.g. PBS). If filters which tolerate DMSO are available then sterilize the DMSO+ sucrose solution. If DMSO tolerant filters are not available then filter the sucrose + PBS and then add the DMSO. If DMSO is not available then 1.5 M ethylene glycol or 1.5 M propane diol can be used instead. If a different cryoprotectant is used then the dilution solutions should be made up with the same (corresponding) cryoprotectant.

- Prepare at least 10 ml cryoprotectant per ovary
- Pre-cool cryovials (e.g. 1.7 ml Nunc conical, star based, internal thread, CRYO tubes Cat # 377224) on ice. It is easiest if these are each pre-filled with 1 ml cryoprotectant.

It is best if *at least* 2 ml cryoprotectant be prepared for each tube that is frozen (1 ml for the first equilibration step, a further 1 ml for each freezing ampoule/vial). This can be made up the day before.

Equilibration procedure:

Once tissue has been dissected, placed it in a tube (e.g. 5 or 10 ml Falcon tube) containing pre-cooled (ice cold) cryoprotectant solution. *Every 5-10 minutes agitate the tube so that all pieces become exposed to the cryoprotectant.* After 15-20 min tip the contents of the tube out into a sterile dish and rapidly allocate tissue to each vial. Distribute the material as evenly as possible between all vials.

Note: label the vials before freezing commences.

Screw the cap back onto each vial and return to the ice. After the tissue has had a total of 30 min on ice they are ready to be placed in the freezing machine or in the passive cooling device ("Mr Frosty").

Passive cooling:

Place the passive cooling container in a –80 freezer or on dry ice. After 12 to 24 h, remove the vials and place them in liquid nitrogen for storage.

Cryoporeservation in a freezing machine

Ovarian tissue cryopreserved at 0.3C/ min in a biological freezer is better preserved than material cryopreserved in a passive cooling device (e.g. Mr Frosty). The difference is however not big, and must be counterbalanced against the detrimental effect of deterioration with time after collection. Thus material will be of better quality if it is frozen at 1C/ min (Mr Frosty on dry ice) as soon as possible after collection, than if the cryopreservation is delayed but then cooled at 0.3C/ min (e.g. by being transported to a site with a biological freezer).

After loading the vials containing ovarian tissue into a freezing machine they should be seeded to initiate ice formation (vials placed in a "mr frosty" are not seeded). Seeding is usually performed once the vials have reached –6ºC (this can take 5 minutes). Seed each vial by pulling it up and touching its outer surface with a large pair of forceps pre-cooled in liquid nitrogen (place the forceps just above the meniscus of the liquid, taking care to not touch the sample). After all vials have been seeded re-examine each one in turn to establish that the solution still contains ice crystals.

Cooling programme:

Once it has been confirmed that ice is present in all tubes the cooling program can start. The cooling rate should be 0.3°C/min to -40°C. On reaching -40°C the vials can be plunged directly into liquid nitrogen, and placed on labelled canes for storage. There may be a benefit from introducing a further rapid cooling ramp (e.g. 10 or 40C/ min) to a temperature of –140ºC or less before moving the vials into liquid nitrogen.

Thawing Procedure:

Solutions and a water bath should be prepared and sterilized in advance:

> The first thawing solution contains 0.75M DMSO and 0.25 M sucrose (i.e. 0.55 ml DMSO and 0.86 g sucrose/10 ml PBS), at room temperature (20-22°C). The second solution contains only 0.25 M sucrose (i.e. 0.86 g sucrose/10ml PBS) at room temperature (20- 22°C).

The water bath should be at 37°C.

After long term storage vials may have filled with liquid nitrogen. Eliminate the risk of explosions by holding the vial **deep** in the nitrogen vapour (just above the surface of the liquid nitrogen). Once there is no sign of liquid nitrogen in the vial immerse the vial into a 37°C waterbath until thawed (this may take several minutes).

Write down the details written of the vial then wipe it over with alcohol before removing the lid.

The tissue is then either grasped with sterile forceps and placed in the first thawing solution (0.75M DMSO+0.25 M sucrose), or the whole contents of the vial decanted into it. After 10 min the ovarian tissue is moved to the second solution (0.25 M sucrose), and 10 min later placed in PBS ready for grafting or culture.

Remember sterile technique should be applied throughout, all solutions, equipment, and containers should be sterile.

Oocytes

Optimized freezing protocols for immature oocytes have yet to be established. Thus it is not currently possible to recommend a strategy by which to preserve the immature oocytes which are released into the collection dish during the dissection of the ovarian tissue. If the embryologist wishes to try to preserve these oocytes for the patient, they could use an existing human oocyte slow cooling (e.g. Fabbri et al 2001; Boldt et al 2003) or rapid cooling protocol (Chen et al 2000; Liebermann and Tucker 2002) either before, or after in vitro maturation.

Other Tissues

Note that the protocol given above can be applied to tissues from other organs including skin. Note that cell suspensions may be better cryopreserved using standard cell freezing protocols.

Acknowledgements:

I thank Monash IVF and Planer Pty Ltd for support.

Additional References (for oocyte cryopreservation):

1) Boldt J, Cline D, McLaughlin D. 2003 Human oocyte cryopreservation as an adjunct to IVF-embryo transfer cycles. *Hum Reprod*. 18:1250-5.
2) Chen SU, Lien YR, Chao Kh, Lu HF, Ho HN, Yang YS. 2000 Cryopreservation of mature human oocytes by vitrification with ethylene glycol in straws. *Fertil Steril.* 74:804-8.
3) Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. 2001 Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Hum Reprod*. 16:411-6.
4) Liebermann J, Tucker MJ. 2002 Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. *Reproduction*. 124:483-9.

Fish Semen Cryopreservation.
Patterns for Common Carp (Cyprinus carpio), Barbel Sturgeon (*Acipenser nudiventris*) and Haarder (*Mugil soiuy*).

Boris Dzyuba
Institute for Problems of Cryobiology and Cryomedicine of NAS of Ukraine
March 2006

Publications using these protocols:

1) Kopeika E.F., Novikov A.N. (1983) Fish sperm cryopreservation. In Cryopreservation of cell suspensions. Kiev, Naukova Dumka (A.A. Tsutsaeva ed.), 204-239.
2) Kopeika E.F. Instructions on low temperature preservation of carp sperm (1986) Kharkov, Institute for Problems of Cryobiology & Cryomedicine, 10p.
3) Dzyuba B.B., Kopeika E.F., Cherepanov V.V., Drokin SI. (1999). Study of the quality of sturgeon semen after 6 years of cryopreservation. Journal of Applied Ichthyology, V. 15 (4-5), 312.
4) Kopieka E.F., P.Williot and B.F.Goncharov (2000). Cryopreservation of Atlantic sturgeon Acipenser sturio L., 1758 sperm: First results and associated problems. Boletin. Institututo Espanol de Oceanografia, 16(1-4), 167-173.
5) Dzyuba B.B., Kopeika E.F. (2002). Relationship between the changes in cellular volume of fish spermatosoa and their cryoresistance. CryoLetters 23, 353-360.
6) S.I. Drokin, H.Stein and T.P.Govorukha (2002). Ultrastructure of carp Cyprinus carpio Spermatozoa after cooling, dilution and freeze-thawing. CryoLetters, 24,49-55.

Introduction

Speaking about fish sperm cryopreservation we should remember some important aspects of this task:

1) Fish exhibit a wide range of reproductive strategies (from viviparity to oviparity), involving different internal or external gametes activation factors, both environmental and biological.
The ranges of external activation factors such as salinity, pH and ion composition vary significantly for different taxa.
2) Fish sperm biochemistry, morphology, ultrastructure and other biological parameters varies significantly depending on reproductive mode, fish reproduction environment and genome.
3) There is phenomenon of fish sperm quality variability within species and it is closely related to sperm cryoresistance. Parameters listed in issue 2 affect significantly fish sperm resistance and are very important for cryopreservation technology.

Basic description of sperm structure and methodology on sperm cryopreservation can be found in (Jamieson ,1991) and modern review on fish sperm cryopreservation is presented in (Tiersh and Mazik,2000, Billard,2001 and Zhang, 2004).

Described above protocols are the examples for fish species different on environmental factors of sperm motility activation (inclusive dependence on hypotonic conditions for common carp, the one on hypotonic conditions as well as certain ion composition for barbel sturgeon and no dependency on osmotic pressure for haarder) and sperm ultrastructures (no acrosome for common carp, haarder and acrosome presence in barbel sturgeon). That is why we selected these species to demonstrate some differences in sperm cryopreservation procedures.

Jamieson, B.G.M. (1991). Fish Evolution and Systematics: Evidence from Spermatozoa. Cambridge University Press, Cambridge. p.319

Tiersch, T.R. and P.M.Mazik, editors. (2000) Cryopreservation in Aquatic Species. World Aquaculture Society. Baton Rouge, Luisiana, USA. pp. 91-160

Billard R. (2001). Cryopreservation of fish spermatozoa. In: "Cryobanking the genetic resource: wildlife conservation for the future?" (P.F. Watson, W.V. Holt, Eds.) pp. 143-170. Taylor&Francis, London, New York.

Tiantian Zhang (2004). Cryopreservation of Gametes and Embryos of Aquatic Species. In "Life in frozen state"/Edited by Fuller B.J., Lane N., Benson E.E.- CRC Press LLC, pp 415-435.

Materials Needed:

1 0.5- 1.5 ml plastic conical ampoules (cryovial Nunc)
2. Sterile tubes (e.g., 50 ml polystyrene, conical tubes)
3. Sterile pipettes
4. Glass slides and cover glasses
5. Compound microscope
6. Cryodiluents (see table below)
7. Refrigerator
8. Liquid nitrogen storage tank
9.Wide necked cryocan (Dewar), height 30-50 cm
10.Thermometer (scale -80^0C $+30^0$C)
11. Support with plastic (metal) stick (length 40-50 cm, diameter 0,5-1 cm) connected to plastic (metal) net diameter of which smaller than cryocane neck (see details in fig 1.).
12. Water thermostat
13. Forceps

Sperm collection

To obtain sperm it is necessary to use mature males. Sperm may be obtained both after hormonal injections and during natural spawning period. We used the suspension of dried hypophyses of carps and sturgeons and haarder sperm was collected from males during natural spawning period. Spermiation was induced by an intramuscular injection of self-prepared carp pituitary homogenate

It is expedient to use injections in accordance with the recommendations of companies producing them. Sperm should be collected into dry vials with no contamination by urine or feces. Fish abdomen must be dry not to let water come to sperm. Sperm storage time before dilution with cryoprotective medium should be minimized.

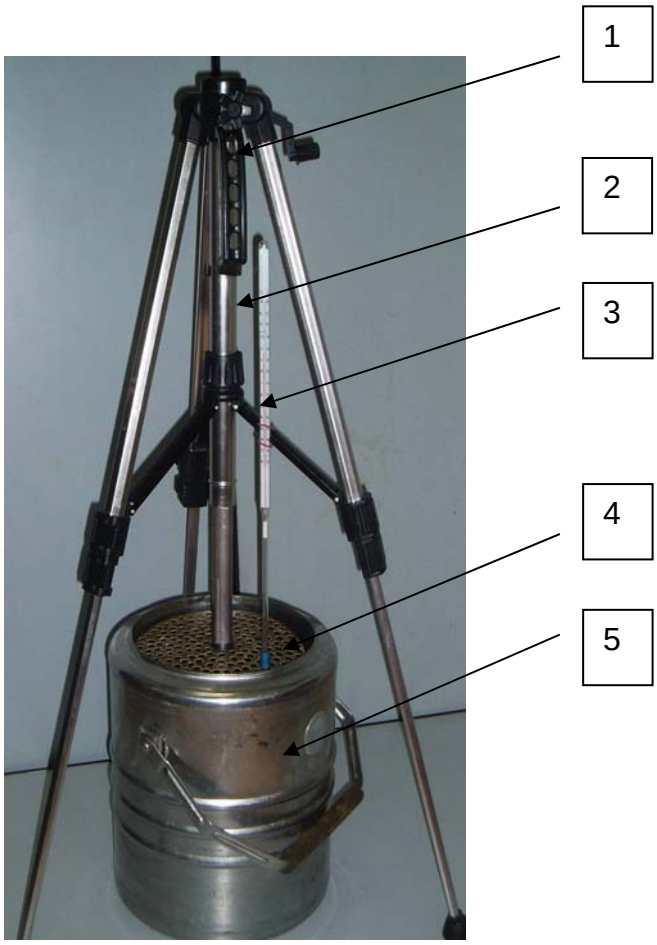


Fig.1 Example of easy assembly for sperm cryopreservation
1 – tripod, 2- guide bar, 3 – thermometer, 4 – round holder for vials
5 – Dewar vessel

Table 1. Cryodiluents' composition

Chemicals	Common Carp		Haarder		Barbel Sturgeon	
	mg	mmol/l	mg	mmol/l	mg	mmol/l
Tris–HCl, pH8.1	1700	140	242	20	600	50
$MgSo_4$	62	2.5	-	-	-	-
$CaCl_2$	18	0.8	-	-	-	-
Succrose	137	4	-	-	-	-
$NaHCO_3$	280	33	-	-	-	-
NaCl	420	72	-	-	-	-
KCl	6	0,8	-	-	-	-
Egg yolk, ml	10		10		9.6	
mannitol	1500	82	-	-	-	-
MeSO2, ml	16	2300	16	2300	14.4	2300
Glutathione reduced	56	0.002	-	-	-	-
Glucose	-	-	5400	0.3	-	-
Distilled water	Up to 100 ml		Up to 100 ml		Up to 100 ml	

Next step is express assessment of sperm motility for each male. For this aim the water solutions are used depending on species: 60 mM $NaHCO_3$ for carp, 9.3 mM tris-HCl (pH 8.0), 12 mM $NaHCO_3$ for sturgeons, 0.48 M NaCl for haarder. Haarder sperm motility is maximum in sperm plasm obtained after sperm centrifugation for 20 min at 4000g. This sperm plasm may be used as activating solution. Measurement procedure consists in as follows: 20 mcl activating solution is placed into object glass, minimal amount of sperm is put with an injection needle and percent of forwardly moving cells is assessed within not more than 20 seconds after being mixed. The best cryopreservation results can be achieved if samples with 80-100% motility are used.

Dilution

To prevent temperature and osmotic shock sperm must be diluted with cryoprotective medium of the same temperature as sperm was, cryoprotective medium should be slowly (by droplets) added to sperm and mixed carefully. Compositions of cryoprotective media are given in table 1.

For Barbel Sturgeon it is expedient after dilution to incubate sperm with cryoprotective medium for 30-40 min at 4ºC. For Haarder sperm should be diluted rapidly and frozen with no incubation.

To note: non-activating media are optimal to obtain maximum motile cell percentage after thawing.

Differences in procedures of dilution for carp, sturgeon (as one group) and in contrast to haarder are associated with the fact that for carps and sturgeons this medium is easily found and for haarder we failed to find it.

Dilution with cryoprotective medium results in activation of haarder sperm movement therefore freezing of motile cells has to be performed and so the duration of diluting-freezing procedure must be minimized.

Cryopreservation

Before sperm dilution start with cryoprotective medium round holder (4, see Fig. 1) should be placed inside Dewar vessel in the vapors of liquid nitrogen at the level of minus 80°C.
After dilution the samples are poured into 1.5ml ampoules and placed into a round holder. In 20 min's exposure in liquid nitrogen vapors thermometer is taken away and a holder with samples is placed into liquid nitrogen.

In our researches there was described more complicated three-step cooling method requiring the use of thermocouple and recording device. However we observed the differences in sperm motility after thawing when comparing these two methods only in case when the sperm initial quality was very poor.

Storage

After freezing sperm samples must be removed into labeled containers and placed into a storehouse.

Thawing

Please keep the safety instructions when working with liquid nitrogen, especially during thawing samples. Use protective gloves and eye-protection. Covers of ampoules to be thawed should be directed towards the side opposite to the researcher's face.

Samples are warmed on water bath at 40°C. For this aim with forceps the sample is taken out of a round holder for freezing and placed onto water bath with constant shaking the samples in water.

In 30-40 seconds' shaking (when about 10% of sample volume transformed into liquid phase) the sample is removed out of water bath, carefully shaked in air up to complete disappearance of liquid phase and dried and assess the motility of sperm as it was mentioned above.
Time between thawing and estimation of motility or use of sperm for insemination should be minimized. This requirement is extremely important for haarder sperm, because these species sperm will be motile just after thawing and the time of their movement will be limited as a rule by 10 min.

To obtain maximum percentage of fertilization it is expedient to increase the sperm/egg ratio 3-5 times more (depending on species) in comparison with native sperm.

Our results

This method enables to obtain following results for sperm after freeze-thawing:
20-50% of motile cells for carp, up to 10-70% fertilization
20-60% of motile cells for sturgeon, up to 5-90% fertilization
40-80% of motile cells for haarder, fertilizing ability was not examined.

Measurement Techniques for Fish Larvae Morphometric Parameters on Early Stages of Postnatal Growth

Borys Dzyuba[1], Katrien J. W. Van Look[2], Nana Satake[2] and William V. Holt[2]
[1]Institute for Problems of Cryobiology and Cryomedicine of the
National Academy of Sciences of the Ukraine,
[2] Institute of Zoology, Zoological Society of London
September 2006

Publications using these protocols:

Borys Dzyuba, Katrien J. W. Van Look, Alex Cliffe, Heather J. Koldewey, and William V. Holt Effect of parental age and associated size on fecundity, growth and survival in the yellow seahorse Hippocampus kuda. J. Exp. Biol. 2006 209: 3055-3061

Introduction

The measurements of morphometric parameters of fish on early stages of postnatal growth related with certain problems. These problems arise from small size of animals and their sensitivity to restraint during the measuring procedure. Therefore to reduce stress we elaborated techniques which allowed us to measure body length and width of fish larvae at an early stage of growth (starting from first week after hatching) without removing the animals from water and tactile contacts.

Materials Needed:

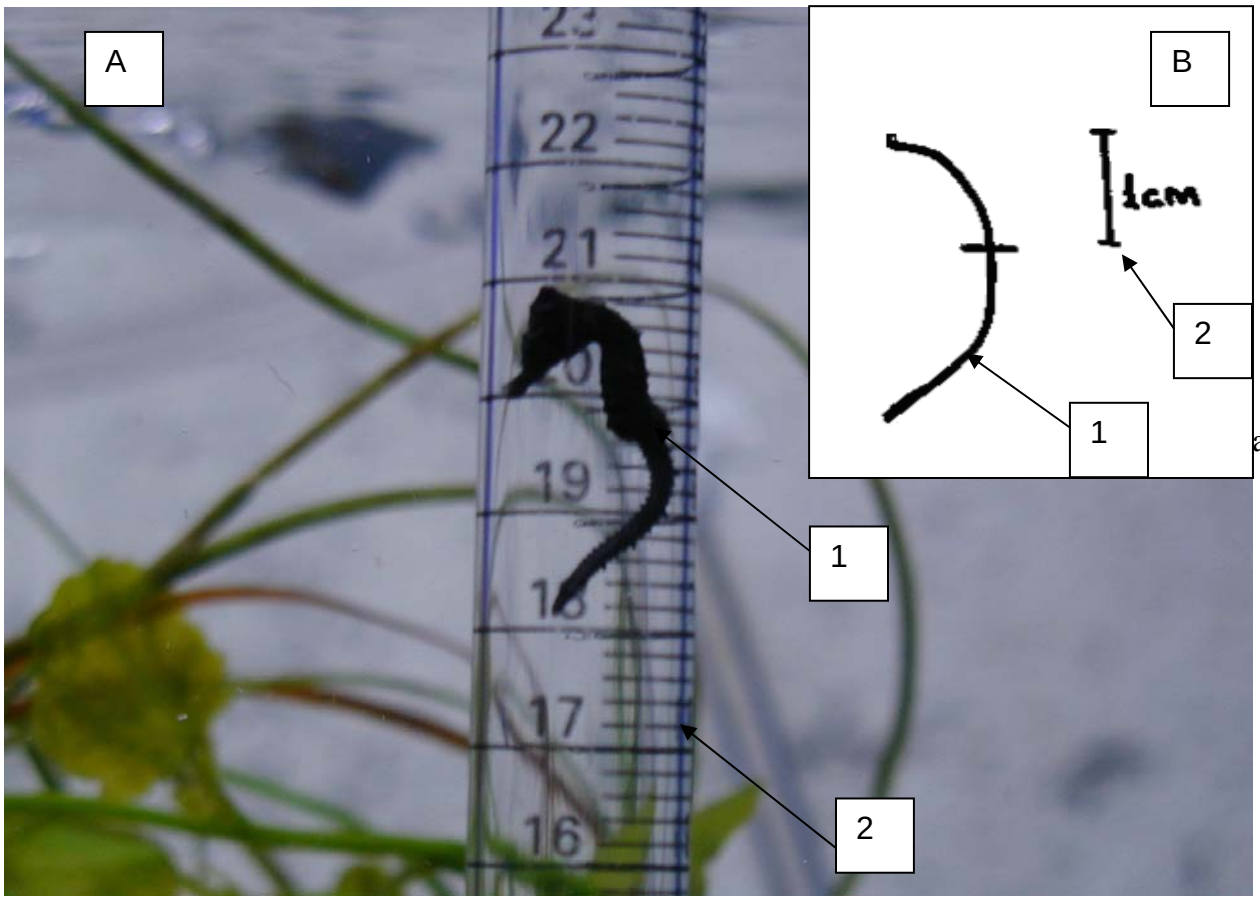
1 Wide-ended clear plastic pipette (diameter of pipette depends on size of animal)
2. Sterile tubes (e.g.: 50 ml polystyrene, conical tubes)
3. Video camcorder (Sony, Hi-8)
4. Video player with stop-frame function.
5. Image analysis software (Image Pro Plus, Media Cybernetics or Scion Image, Scion Corporation software)

Methods:

1. Individual larva (fig 1A, 1) should be captured within a wide-ended clear plastic pipette large enough to permit the juveniles free vertical movement, but sufficiently small to restrict their lateral movement (fig. 1A, 2). This pipette should be placed near the inside-front of the tank and image sequences of the captured larva should be recorded using a video camcorder. Duration of each larva recorded can differ from animal to animal but usually from 30-60 sec record is sufficient to find a suitable image.

2. The sequences are analysed and examined for identification and capture of suitable still images in which the larvae assumed an orientation where they were positioned vertically with their bodies sideways to the camera and fully extended and straight as possible. Measurements of total length, body length and width should be made manually from the still images by image analysis software. To make the procedure of measurement quick and simple, we plotted the lines that corresponds to the measured parameters by drawing them on transparent film placed against the screen of monitor with still image of larvae inside the pipette (fig.1B, 1). The image of scale, which located on the pipette should be drawn

on the same film for future calibration (fig. 1 B,2). On the same film few images can be drawn together and after that film can be scanned. The scanned image is then analysed by the image analysis software (Image Pro or Scion Image software).



an

Chinchilla lanigera

Order: Rodentia
Suborder: Hystricomorpha
Family: Chinchillidae
Genus: Chinchilla
Species: Ch. lanigera, Ch. Brevicaudata

Status: Endangered, CITES I

The following protocols were developed using the domestic chinchilla as a model.

Dra. Marina F Ponzio
Instituto de Fisiología
Facultad de Ciencias Médicas
Universidad Nacional de Córdoba
Santa Rosa 1085 – X5000ESU
Córdoba – Argentina
(54)-0351-4332019
mponzio@mater.fcm.unc.edu.ar

SEMEN COLLECTION

Semen can be obtained by electroejaculation with a probe designed in our laboratory (Ponce et al. 1998). Briefly, the chinchilla is placed in a box covering the cranial half of the body in the prone position. The caudal half rested on a metal grid, that also supported the semen collection tube. The tail is gently raised and, after cleaning the genital area, the prepuce is retracted, and the penis introduced into a 2 ml Eppendorf plastic tube. The collection tube contains 150 µl of modified Tyrodes medium in order to prevent coagulation of the sample. The bronze bipolar electrode (length 40 mm, diameter 4.2 mm) is lubricated with glycerin, inserted into the rectum to a depth of 20–30 mm and held in place by a technician. An alternating current (sinusoidal wave, 50 cycles/s) is applied for 5 of every 10 s (one pulse). The electrical potential across the poles of the electrode is controlled with a rheostat and measured with a voltimeter. The circuit is calibrated in advance using a known resister. Tree series of up to 5 pulses can be applied to each animal, yet one to 3 pulses were usually enough to produce the ejaculate. Together with the ejaculate, a white gel-like viscous plug was obtained, which was immediately discarded to prevent coagulation. All the materials coming in contact with the semen were kept at 37 C throughout the analysis. Using this technique in concious animals, 100% of the animals produce ejaculates. Anesthesia can be used, using ketamine (40 mg/kg body weight, i.m.; (Morgan et al., 1981), but ejaculates will be obtained in only 60% of the animals.

SPERM INCUBATION MEDIA (MODIFIED TYRODES MEDIUM)

Sperm obtained by electroejaculation or from epididimus (caudal section) can be incubated in a modified Tyrode's medium with the following composition (mM):

CaCl2, 1.8; MgCl2, 0.929; KCl, 2.68; NaHCO3, 11.903; NaCl, 136.86; NaH2PO4, 0.287; glucose, 5.56; penicillin 0.5 mg/mL and streptomycin 0.075 mg/mL. pH 7.0–7.5; 270–290 mOsm.

SPERM CAPACITATION

To support sperm capacitation, the incubation media has to be supplemented with 3 mg/mL BSA fraction V (Sigma Chemical Co., St. Louis, MO, USA). Capacitation is reached after 4h of in vitro incubation at 37°C.

EVALUATION OF SPERM FUNCTIONAL ACTIVITY

Motility

Can be evaluated in a Makler counting chamber (Makler 1980) (Sefi Medical Instruments, Haifa, Israel), placed over a thermostatized chamber to keep the sperm warm during the evaluation (37°C). Evaluations are made under inverted microscope at 200 X magnification (Olympus CK2, Tokyo, Japan). A drop of no more than 5-10 µl is sufficient for an even distributed cell layer. Results should be expressed as percentage of motile cells (progressive plus nonprogressive sperm).

Viability

It is assessed by supravital staining with Hoechst 33258 (1.5 µg/ml) (Yelian and Dukelow 1992) (Calbiochem, San Diego, CA, USA). Using the appropriate ultraviolet fluorescence optics, (Axiolab; Zeiss, Stuttgart, Germany) sperm having brightly blue fluorescent nuclei are scored as dead whereas those without fluorescence are scored as viable. No fewer than 200 cells should be assessed. Results are expressed as percentage of viable cells.

H33258 Stock solution

3 mg of H33258 in 1 ml of isotonic solution or DPBS, aliquot and store at -20 C.

H33258 Assay solution

10 µl stock solution + 990 µl modified Tyrodes medium, put this tube in the dark inside the incubator.
For semen viability evaluation, 950 µl of semen are mixed with 50 µl of H33258 assay solution, kept for 5 min in the incubator and then analized as described above.

Response of sperm membranes to hypo-osmotic shock

The procedure used was adapted according to Ruiz et al. (1996). An aliquot of the sperm suspensión (100 µl) are mixed with 1 ml of the hypo-osmotic solution (100 mOsm; 1 ml, pH 7.4) of fructose and sodium citrate in distilled water. After 45 min incubation (37°C), evaluations are made in a phase-contrast microscope at 400 x magnification, and the percentage of spermatozoa that showed tail swelling are determined. No fewer than 100 cells should be assessed. Results are expressed as percentage of swollen cells.

Distilled water 500 ml + Sodium citrate 3.68 g + Fructose 6.76 g

Acrosome reaction

Double staining with Pisum sativum agglutinin labelled with fluorescein isothiocyanate (FITC-PSA) as described by Cross et al. (1986) with slight modifications. Briefly, after supravital staining, samples are washed out of unbound stain by centrifuging twice at low speed (400 rpm) for 10 min with 4–6 ml of isotonic solution; the supernatant is removed, and the final sperm pellet is resuspended in 200 µl isotonic solution. Smears are produced on glass slides and dried in an incubator at 40°C. Subsequently, they are fixed with pure methanol for 30 s. Slides are then washed with a stream of distilled water for 2 min. After slides are dried, spermatozoa are stained with 30 µg/ml FITC-PSA in DPBS (stock solution: 2 mg/ml) for 30 min and washed again with a stream of distilled water for 2 min. Slides are evaluated at

1000 x magnification in an epifluorescence microscope and the viability and acrosomal status of at least 100 spermatozoa are assessed. Under blue wavelength, spermatozoa with brightly yellow fluorescents acrosomes are considered acrosome intact, whereas those with no fluorescence or only in the equatorial region are considered acrosome reacted. The filter is then changed to ultraviolet and each cell is additionally scored as viable or non-viable. The results are expressed as percentage of viable spermatozoa with intact acrosome, dead spermatozoa with intact acrosome, viable spermatozoa with reacted acrosome or dead spermatozoa with reacted acrosome.

SPERM CRYOPRESERVATION

Preparation of cryoprotective media

The procedure and preparation of the cryoprotectant media is performed as previously reported by Ponce *et al.* (1998).

A zwitterionic buffer system was prepared by titrating 325 mOsm/L TES (N-tris[Hydroxymethyl] methyl-2-aminoethanesulfonic acid) with 325 mOsm/L Tris to make the TEST solution. To prepare the cryobuffer, 48% (vol/vol) TEST, 30% sodium citrate, 20% egg yolk, and 2% fructose are mixed. The mixture then is centrifuged in an ultracentrifuge at 10.000 *g* for 10 min, and the supernatant is filtered through filter paper (Whatman I). Penicillin 0.15 mg/mL and streptomycin 0.25 mg/mL are then added and the pH (7.4-7.5) and osmolality of extenders measured. The cryobuffers are finally added with glycerol (TESTY-G) or ethylene glycol (TESTY-EG) at a 2 M concentration. Aliquots of the cryoprotectants can be stored at -20ºC for later use.

The same media can be used to store in liquid nitrogen for indefinite periods of time, or to store the sperm upo to 72 h in the refrigerator (4°C).

For 10 mL of each component
Fructose 0,6 g 323 mOsm
Tris 0,5 g 405-335 mOsm
Tes 1 g 408-334 mOsm
Sodium citrate 0,6 g 494-334 mOsm
Measure Ph in all solutions (should be near 7)
Mix Tes and Tris for the TEST solution until a Ph of 7 is reached
To obain 20 ml of cryoprotectant media:
TEST 9,6 ml
Sodium citrate 6 ml
Egg yolk 4 ml
Fructose 0,4 ml
Centrifuge, filter and add antibiotics. Mix with **ethileneglycol** or **glicerol** and store. Both cryoprotectants yield very similar sperm recovery rates.
Add 8,5266 ml of cryoprotectant media with 1,4734 ml GLICEROL
Add 8,8816 ml of cryoprotectant media with 1,1184 ETHILENE GLYCOL

Spermatozoa freezing

The sperm suspension is added drop by drop with the glycerol-based or the ethylene glycol-based cryoprotectant medium (1:1 dilution; 1 M final concentration of the cryoprotectants). The suspensions are gently shaken to achieve a complete semen-medium mixture. Plastic cryostraws (0.5 mL) are filled at room temperature with the obtained suspension, and heat sealed. Then they are slowly cooled at 4ºC at a rate of approximately 2 ºC/min. (measured with an analogical thermometer) in the refrigerator. After

10 min, the cryostraws were exposed to -20ºC for 15 min in the freezer, and afterwards cooled by exposure to liquid nitrogen gas vapor (10 cm above the liquid nitrogen level of the tank) for 10 min; the cryostraws were finally immersed into liquid nitrogen (-196ºC), and stored. In order to avoid excessive temperature fluctuations, fast transfer was performed at all steps.

The same cryopreservation media can be used to store spermatozoa at 4 C for up to 72 hs with very good sperm function recovery rates.

Thawing procedure

For thawing, straws are rapidly immersed in a water bath (37ºC) for 60 sec and wiped dry. Depending on its use, samples can be processed without removing the cryoprotectant media (the content of the straws was released directly into 500 µl of Tyrode´s buffered medium) or, the cryoprotectant can be removed by flushing the sperm suspension into an Eppendorf tube with 500 µl Dulbecco´s phosphate-buffer saline-2% (w/v) polyvinylpyrrolidone-40 (DPBS-PVP, pH 7.4, Sigma Chemical Co.) per straw. The sperm suspension is washed twice by centrifugation (100 *g*, 10 min). The precipitate is then resuspended in Tyrode´s medium and adjusted to a final sperm concentration as needed.

Cortisol Enzyme Immunoassay Protocol

Astrid Bellem[1], Coralie Munro[2] and Nadja Wielebnowski[1]
[1]Brookfield Zoo, Chicago and [2] U.C. Davis, California
March 2004

Protocol prepared by: Astrid Bellem

Publications using this protocol:

1) Shepherdson DJ, Carlstead KC, Wielebnowski N. 2004. Cross-Institutional assessment of stress responses in zoo animals using longitudinal monitoring of faecal corticoids and behaviour. Animal Welfare 13:S105-113.

Reagents

Antibody (R4866) and horseradish peroxidase (HRP) enzyme label can be purchased from Coralie Munro at the Clinical Endocrinology Laboratory at UC Davis.

Standards were prepared from ICN Cortisol (Cat. #194569) by dissolving 1.000g in 10.00mL 100% EtOH for the primary stock and diluting downward into assay buffer.

The high control was prepared by spiking assay buffer with standard stock to read about 30% on the %binding scale on the standard curve.

1. COATING BUFFER 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ per liter dH_2O, pH to 9.6

2. ASSAY BUFFER 195 mL Stock A (0.2M NaH_2PO_4), 305 mL Stock B (0.2M Na_2HPO_4), 8.7 g NaCl and 1.0 g BSA/L dH_2O, pH to 7.0

3. WASH SOLUTION CONCENTRATE (10X)
87.7 g NaCl and 5.0 mL Tween 20/L dH_2O dilute 10-fold for working wash solution

4. SUBSTRATE BUFFER 9.61 g citric acid (anhydrous)/L dH_2O, pH to 4.0

5. ABTS STOCK (40 mM) 0.55 g ABTS (azino-bis-ethylbenzthiazoline sulfonic acid)/25mL dH_2O

6. PEROXIDE (0.5 M) 500 uL H2O2 (30% solution)/8mL dH_2O

EIA PROTOCOL

1. Plate Coating
1.1 Use Nunc Maxisorb plates only
1.2 Add 40 ul antibody stock (1:100, -20°C) to 5 ml coating buffer (working dilution, 1:12500)

	1.3 Add 50 ul per well using an Eppendorf repeater pipette 1.4 Do not coat column 1 1.5 Tap plate gently to ensure that coating solution covers well bottom, being careful not to touch well bottoms at all times 1.6 Cover **tightly** with a plate sealer to avoid evaporation and incubate overnight at 4°C
2. Standards	2.1 Standard values are 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 ng/ml 2.2 Dilute high standard (20 ng/ml, -20°C) serially 2-fold using 200 ul standard and 200 ul assay buffer 2.3 Use assay buffer as 0 standard
3. Samples/Controls	3.1 Dilute samples in assay buffer to the appropriate dilution, according to linearity test 3.2 Use control as is for high control and dilute 4-fold in assay buffer for low control, ie. 150 ul buffer + 50 ul high control
4. HRP	4.1 Add 25 ul HRP stock (1:100, 4°C) to 5 ml assay buffer (working dilution, 1:20,000) 4.2 **Do not freeze** diluted HRP
5. Plate Washing	5.1 Wash the plate 5 times with wash solution using Dynex Ultrawash II 5.2 Blot on paper towel to remove excess wash solution 5.3 Do not allow coated plates to dry
6. Plate Loading	6.1 Add 50 ul standard, sample or control to each well as **quickly** and **accurately** as possible, according to plate map 6.2 Start at A2 and go down each column, pipetting samples and controls in duplicate 6.3 Using Eppendorf repeater pipette, **immediately** add 50 ul per well diluted HRP, including wells A1 and B1 (blanks), being careful not to splash 6.4 Cover plates **tightly** with plate sealer and incubate at RT (22˚C) for 1 hour
7. Plate Washing	7.1 Wash the plate 5 times with wash solution 7.2 Blot on paper towel to remove excess wash solution using plate washer 7.3 Plates are relatively stable at this point and can be left upside down for up to an hour
8. Substrate	8.1 Prepare ABTS substrate **immediately** before use 8.2 Add 40 ul 0.5M H2O2 and 125 ul 40mM ABTS to 12.5 ml substrate buffer and mix well 8.3 Add 100 ul substrate to all wells, including A1 and B1, with Eppendorf repeater pipette 8.4 Cover **tightly** and incubate at RT for 30-60 minutes with shaking (Lab-line titerplate shaker)
9. Plate Reading	9.1 Read at 405 nm (test filter 1, reference filter 4) on Dynex MRX Revelation 9.2 Maximum OD should be about 1.0