Cattle Embryo Transfer Procedure

An Instructional Manual for the Rancher, Dairyman, Artificial Insemination Technician, Animal Scientist, and Veterinarian

John L. Curtis
PRESIDENT, AGTECH, INC.
MANHATTAN, KANSAS

ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
San Diego **New** York Boston
London **Sydney** Tokyo Toronto

Academic Press Rapid Manuscript Reproduction

This book is printed on acid-free paper.

Academic Press, Inc
San Diego, California 93101

United Kingdom Edition published by
Academic Press Limited
24–28 Oval Road, London NW1 7DX

Library of Congress Cataloging-in-Publication Data

Curtis, John L.
Cattle embryo transfer procedure / John L. Curtis.
p. cm.
Includes bibliographical references.
ISBN 0-12-200240-7
1. Cattle--Embryos--Transplantation--Laboratory manuals
I. Title.
SF961.C87 1991
636.1'089'8178--tic30 91-4918
CIP

PRINTED IN THE UNITED STATES OF AMERICA
91 92 93 94 9 8 7 6 5 4 3 2 1

Contents

Preface

This text is designed as a basic manual for the rancher, dairyman, artificial insemination technician, animal scientist and veterinarian. In a simple, ordered fashion, all elements for a successful embryo transfer program are explained and/or illustrated.

John L. Curtis has been actively involved in the embryo transfer industry for the past 12 years. His embryo transfer training was received at Colorado State University while completing a Masters Degree in Physiology under the expert leadership of Dr. Peter Elsden and Dr. George Seidel.

Over the years, the author has logged a successful professional record of thousands of pregnancies in 12 midwestern and northeastern states. He has served as Technical Director for Twin Brook Genetics, Wilton, NH; as owner–operator of Curtis Embryo Transfer Co., Oneonta, NY; and most recently as Vice President of Embryo Operations for Dreamstreet Holsteins, Inc., Walton, NY. Dreamstreet's international market development program sent Mr. Curtis to Egypt, China, and Sudan to negotiate joint–venture semen and embryo production centers.

The author is currently a doctoral candidate in Livestock Production and Management at Kansas State University, and is also President of AGTECH Corporation. AGTECH, Inc. manufactures and distributes supplies used by the embryo transfer industry and also sponsors embryo transfer training workshops. An informational brochure on training and supplies can be obtained by writing the author at: AGTECH, Inc., P.O. Box 1222, Manhattan, Kansas 66502 (USA). FAX number (913) 537–0179.

Note: In some states, providing embryo transfer service on a fee basis for clients is restricted to veterinarians only. Check with the state attorney general's office for details.

Acknowledgements

I would like to express my appreciation to the many individuals who contributed in one way or another to the eventual production of this manual.

A special thanks goes to Dr. John McGaugh and Dr. Garry Brower for encouraging me to apply to graduate school while at Fort Hays State University. A special thanks is also extended to Dr. Peter Elsden and Dr. George Seidel for their guidance during my two years of embryo transfer training at Colorado State University; to Mr. Frank Wood and Mr. George Morgan for giving me the opportunity to work with some of the finest Holsteins in the country; and to Dr. Keith Bolsen and Dr. Duane Acker at Kansas State University for sharing their expertise and enthusiasm for livestock production and international agriculture. In addition, I would like to recognize the assistance received from Dr. Jim White, Mr. Rene Suazo, and Ms. Ginger Weir in photography and manuscript preparation.

I must also thank my wife for her continued support of the hours committed toward this project, plus her constructive criticism offered at my request throughout the manuscript. And finally, a special thank you to my parents, since without their guidance and help throughout the years, none of this would have been possible.

Overview of Embryo Transfer Procedure

For the past several decades artificial insemination has allowed genetic progress to be achieved relatively quickly through the widespread and efficient use of frozen semen. Until 15 years ago, rapid genetic progress was limited to the male side of genetic contribution because cows could realistically produce only one calf per year. Today, due to the advancement of embryo transfer techniques, cows can produce many offspring. This results in more rapid genetic gain, which complements an artificial insemination program.

The process of cattle embryo transfer begins by selecting a genetically outstanding, well–nourished, nonpregnant embryo donor (heifer or cow). At the same time, 10 to 15 nonpregnant females (embryo recipients) are identified.

The donor is superovulated by daily injections of follicle stimulating hormone and then bred artificially at estrus with high–quality semen. The estrous cycle of the recipient herd is synchronized with the donor's cycle by prostaglandin injections.

Embryos are typically recovered seven days after insemination. The embryo collection method described in this manual is an industry–proven procedure; however, other collection techniques are available. Once collected and identified, the embryos can be frozen for later use or transferred immediately as fresh embryos.

A successful embryo transfer program culminates with many embryos being transferred, and a consistently high recipient pregnancy rate. The entire process involves numerous procedures, schedules, techniques, and supplies. Attention to detail plus practice are critically important to the overall success of an embryo transfer program.

CHAPTER 1

Cattle Management

Cattle management is one of the most important components in a successful embryo transfer (ET) program. Management includes the selection and maintenance of healthy, reproductively sound donors and recipients, supported by balanced nutrition.

(1.1) HEALTH PROGRAM

All new cattle arriving at an embryo transfer center or at the producer's dairy or ranch must be accompanied by a health certificate indicating negative brucellosis and tuberculosis (TB) tests within the prior *30* days. New animals should be quarantined from existing cattle for 30 days pending verification of negative tests to TB, brucellosis, anaplasmosis and bluetongue.

To insure health and reproductive soundness, all new cattle should be processed immediately upon arrival. Donor and recipient processing includes:

(1) A thorough visual examination to exclude:
ringworm, external parasites, severe lameness, mastitis, pink–eye/blindness, etc.

(2) A thorough reproductive tract examination to verify:
- A nonpregnant, mature uterus that is free of endometritis, tumors, and adhesions
Two cycling, noncystic ovaries, both adhesion–free

(3) Evaluation of recipient age and size:
- Age and size are particularly important considerations when heifers are used as recipients. Granting that there are significant maternal influences on birth

weight, for the most part calf size at birth is genetically determined, and most embryos available for transfer tend to be from large dams and sires. Therefore, it is essential that recipients be large enough to deliver large calves. Heifers should be at least 14 months of age and 350 kg at time of transfer. Recipient cows should bc under eight years of age and at least 50 days postpartum.

- All cattle that are acceptable following the visual, internal, and age/size examination are then identified and vaccinated.

(4) Cattle identification:

- Donors and recipients are given an ear tag with accession number, beginning with 001. One scheme is to assign large white tags to donors (beginning with 001) and large yellow tags to recipients (beginning with 001). The accession number is written on the tag (front and back) with permanent ink 5 minutes prior to insertion in the ear.
- All other ear tags, neck chains, or other forms of attached identification (except metal. ear clips) should bc removed to avoid confusion.
- A lost or damaged ear tag should be replaced immediately with a tag bearing the original number.

(5) Testing and Vaccination:

- As soon as possible after arrival all cattle are tested for brucellosis, TB, anaplasmosis, and bluetongue, and are vaccinated for IBR, RVD, P13, leptospirosis (*5* antigen), vibrio fetus (Preg–Guard *9, Norden* Labs), haemophilus somnus/pasteurella haemolytica (*Somtrin P,* Affiliated *Labs*). They are also vaccinated for black–leg (*Blacklegol 7 with SPUR, Cutter Animal*

Health) three days later when the TB tests are read (Figure 1).

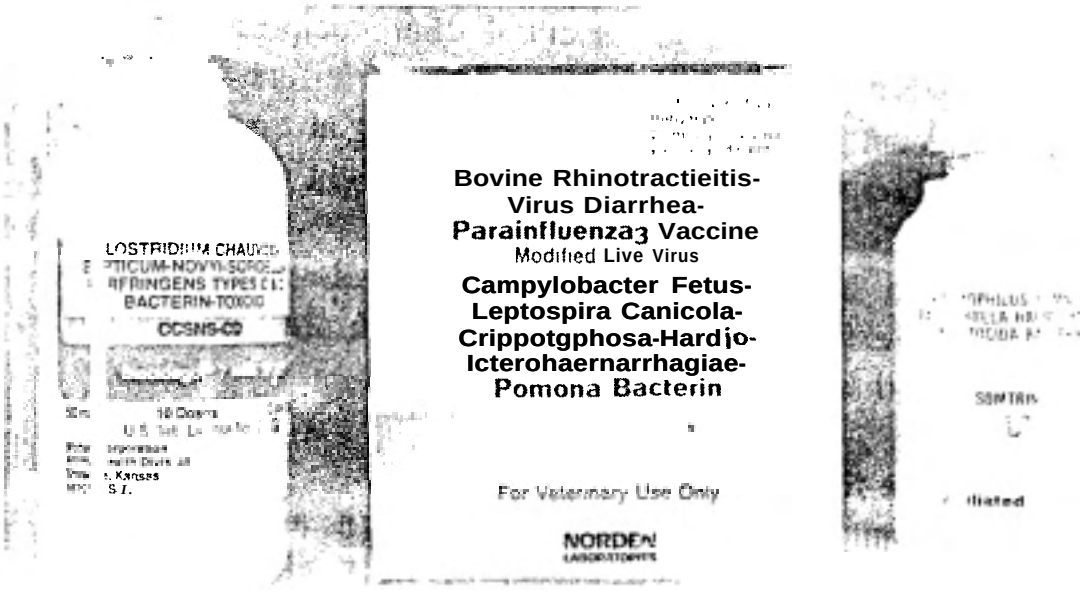


Figure 1. Donor and recipient vaccines. Blackleg01 7 (left), Preg–Guard 9 (center), and Somtrin/P. (Other equally effective brands of vaccine are available.)

- Blood samples (10 ml/animal) are drawn from the tail vein with a sterile, single–use Monoject 12 ml syringe, 16 gauge x 1 inch needle. Immediately inject the sample into a sterile, single–use 10 ml Vacutainer tube (no additives/preservatives) that has been labelled with the blood donor's eartag number. To facilitate serum/clot separation, maintain the filled vacutainer tube under refrigeration (2 to 8°C) in an inverted position (rubber stopper down) until delivery to the laboratory.
- Suspects for brucellosis and suspects and reactors for anaplasmosis arc removed from the herd (isolated). Cattle that are suspect for bluetongue will not be

culled unless they are still suspect when retested in one month. Reactors are removed. Reactors or suspects to either brucellosis or TB may result in quarantine of the herd they occur in. Therefore, care should be taken to apply sanitary TB tests to avoid caudal fold infections and unnecessary suspect responses. TB suspects are retested by comparative cervical tests by a Federal veterinarian within 10 days of the caudal fold test.

- Cattle are subjected to a final test for brucellosis and TB one month after initial processing (anaplasmosis and bluetongue also for donors) and are vaccinated a second time with Blackleg01 7.
- Anaplasmosis is readily spread between animals through reuse of needles, ear tag trocars, etc. For this reason, it is advised that sterile, single–use needles be used (18 gauge x 1 inch, Monoject), and the tagging trocar (Figure 2) be submerged in *Nolvasan* disinfectant solution between uses.
- To aid in louse control, all cattle should be treated with a pour–on systemic insecticide (*Korlan* 2), or use grubicide (*Worbex*) in September or early October.

SUMMARY OF CATTLE PROCESSING PROCEDURE

A. First Day at ET Center, Dairy or Ranch:

1. Thorough visual examination.

2. Thorough reproductive tract examination.

3. Evaluation of recipient age and size. (Cattle that are still acceptable after the age/size evaluation may continue the processing regimen.

Figure 2. Ear tag trocar (a), recipient ear tag (b), permanent ear tag marking pen (c).

4. Identify each animal with an ear tag, check for an official calf–hood vaccination shield (right ear), record tattoos and metal tag numbers.

5. Vaccinate with Preg–Guard 9 and Somtrin–P.

6. TB test (intradermal, right caudal fold).

7. Draw blood for brucellosis, anaplasmosis, bluetongue, and donor blood–typing (if necessary).

8. Pour on insecticide (Korlan 2) for lice. Use grubicide (Worbex) between September 1 and October 31.

B. Three Days Later:

1. Read TB test.

2. Vaccinate with Blacklegol 7.

C. When Blood Test Results Come Back:

1. Sell recipient reactors to bluetongue. Retest suspect bluetongue recipients, and sell if still positive. Retest donor bluetongue reactors and suspects immediately. If reactor titers persist, isolate the donor.

2. Treat anaplasmosis suspects and reactors with long–acting oxptetracycline and keep isolated until negative.

3. Place brucellosis and TB suspects in isolation. Retest TB suspects by comparative cervical test. Retest brucellosis suspects in 30 days. Reactors to brucellosis and TR are quarantined. Contact a Federal veterinarian for further instructions.

D. Thirty Days After Arrival:

1. Take blood samples for a second brucellosis test on all cattle. Test donors once again for anaplasmosis and bluetongue.

2. TB test all cattle.

3. Second vaccination of Blacklegol 7 to all cattle.

4. Palpate all cattle for pregnancy that was not detected upon examination on day 1.

(11.2) NUTRITION PROGRAM

The establishment and maintenance of an animal's health and reproductive efficiency is highly correlated to proper nutrition. Care must be taken to meet all minimum requirements for crude protein (CP), totai digestible nutrients (TDN), minerals, and vitamins.

Approximately 20 essential nutrients must be present at adequate levels in cattle rations to ensure optimum performance (Table 1). A deficiency of any one essential nutrient will adversely affect performance of an animal in some way.

Notes:

1) The quality (i.e., amino acid composition) of the crude protein (CP) in the ration is not critically important since microbes in the cow's rumen synthesize all necessary amino acids and subsequent protein molecules.

2) High quality roughage is an important component in rations for maintaining rumen function. At least 9% of the ration should be crude fiber (CF).

3) None of the B–complex vitamins need to be added to the ration since all are synthesized in the rumen.

When designing a feeding program one should group cattle by nutritional requirement and/or size, and then formulate rations to meet the requirements of each group. Ideally cattle should be weighed every two weeks, with weight gain or loss as the indicator for varying the amount of ration fed per head daily.

Occasionally, it is desirable to "slim–down" an overweight donor. This is accomplished by maintaining adequate levels and ratios of the essential nutrients while reducing the energy intake.

However, during superovulation, insemination and embryo transfer donors and recipients should be on a positive energy intake level!

Cattle can tolerate a substantial excess of most nutrients. However, the manifestations of undernutrition (levels less than National Research Council recommendations) on reproduction have been well documented, i.e.:

Table 1. Essential Nutrients in Donor and Recipient Rations

Nutrient	Approx. minimum level*
TDN	59.00% of DM
Crude protein	9.75%
Crude fiber	9.00%
Salt	0.50%
Calcium	0.30%
Phosphorus	0.20%
Potassiurn	0.75%
Magnesium	0.15%
Sulfur	0.20%
Iron	44.00 mg/kg DM
Manganese	19.80 mg/kg DM
Copper	11.00 mg/kg DM
Cobalt	0.11 mg/kg DM
Selenium	0.11 mg/kg DM
Molybdenum	0.99 mg/kg DM
Vitamin A	4,000.00 IU/kg DM
Vitamin D	880.00 IU/kg DM
Vitamin E	4.40 IU/kg DM

*See NRC for precise levels.

- Reduced conception rate postcalving
- Increased interval from calving to first estrus
- Altered estrous cycles
- Small, weak calves born to undernourished dams
- Altered reproductive hormone levels (FSH, LH, progesterone. GnRH)
- Altered insulin and glucose levels, possibly influencing ovarian steroid substrate levels
- Influences on follicular growth and subsequent ovulation

Donor and recipient rations can bc formulated by professionally qualified nutritionists from a wide range of available feedstuffs (Table 2).

Table 2. Composition of Feedstuffs Commonly Fed to Cattle

Feed name/description	TDN, %	CP, %	CF, %	Minerals, %	
				Ca	P
Alfalfa hay, S–C, early bloom	60.0	18.0	23.0	1.41	0.22
Alfalfa silage, wilted, mid–bloom	58.0	15.5	30.0	*	*
Corn silage, well–eared	70.0	8.1	23.0	0.23	0.22
Sorghum silage	60.0	7.5	28.0	0.35	0.21
Sorghum grain, flaked	92.0	10.1	3.0	0.04	0.34
Brome hay, S–C, late bloom	55.0	10.0	37.0	0.30	0.35
Wheat, fresh, early–veg.	73.0	28.6	17.0	0.42	0.40
Timothy hay, S–C, late–veg.	62.0	17.0	27.0	0.66	0.34
Cottonseed meal, solv. estd.	76.0	45.2	13.0	0.18	1.21
Soybean meal, solv. estd.	83.0	39.9	7.0	0.33	0.71

"data not available

There are direct chemical procedures by which labs can establish how potent feedstuffs are for specific nutrients. In addition, there arc fecd fractions which can be isolated chemically, but which are combinations of nutrients that have some common property permitting a chemical analysis of the group. These important fecd fractions are separated chemically by proximate analysis, and consist of:

- water
- ether extract (fats, oils)
- crude fiber (CF; i.e., cellulose, lignin)

- nitrogen free extract (simple sugars, starch)
- crude protein (nitrogenous compounds)
- ash (minerals; i.e., calcium, phosphorus)

As long as the available feedstuffs are palatable to cattle individually or in combination, they can be submitted to an analytical lab for proximate analysis. It is from the proximate analysis that the values for total digestible nutrients (TDN) are calculated.

All of the dry matter fractions of a feed that are separated by the proximate analysis, except ash, are potential sources of energy, i.e., carbohydrate, protein, and fat.

The logic behind the use of TDN is entirely straightforward. If one sums the digestible portions of crude fiber, nitrogen free extract, protein, and ether extract of a feed, each weighted in accordance with its appropriate caloric value, the resulting figure represents the total digested energy expressed in terms of calories for that feed. It is generally agreed that one gram of TDN is equal to 4,400 calories. One calorie is the amount of heat required to raise the temperature of 1.0 gram of water from 14.5 to 15.5°C.

In general, daily rations for recipients and nonlactating donors should be formulated to meet the requirements in Table 3.

Table 3. Daily Minimum Nutrient Requirements of 350 kg Recipients and 600 kg Nonlactating Donors

Body wt, kg	Daily gain, g	Feed, kg DM	TDN, %	CP, %	Minerals, g		Vitamins, 1000 IU	
					Ca	P	A	D
350	700	8.0	63.0	10.3	24	17	14.8	2.3
600	200	9.6	56.0	9.2	23	20	25.4	3.9

Note: ration proportions are expressed on a 100% dry matter basis.

Practical Demonstration of Table 3:

A typical nutrition program requires feeding hay. The following example (Table 4) illustrates feeding 9.6 kg (DM) of brome hay daily to a 600 kg donor cow. Wc assume 55% TDN and 10% crude protein content.

Calculation Note: The kilograms of hay DM divided by % DM of the hay being fed = the kilograms of hay one must feed "as-is."

Table 4. Feeding Example

Nutrient	Required for 600 kg Cow	Supplied by Feeding 9.6 **kg** of Bromc Hay
TDN	5.40 kg (56.0%)	5.30 kg (55.2%)
Crude protein	0.88 kg (9.2%)	0.96 kg (10.0%)

In this simple feeding scheme for nonlactating donors, the only supplementation needed is mineral balance. The exception would be during severe cold weather when the cow needs extra energy. Extra energy can bc provided by increased hay intake, or adding grain to the ration.

Additional practical guidelines for feeding dairy and beef cattle are available from L. R. Corah, J. R. Dunham, and E. P. Call (see references).

CHAPTER 2 _ 

Recipient Synchronization

At this point in the text, we shall assume that all cattle have been at the ET center, ranch, or dairy for at least 30 days, are receiving a balanced ration, and estrous cycles are being recorded on a twice–daily basis (6:00 A.M. and 6:00 P.M.).

Before consideration is given to superovulation and embryo recovery, the surrogate host (recipient) must be discussed. Highest conception is achieved when an embryo is transplanted to a uterine environment that most closely resembles the environment that the embryo originated from.

Embryos collected from a donor at day 7 of her cycle (estrus = day 0) should be transplanted into recipients that are day 6, 7, or 8 of their cycle. The cattle estrous cycle is typically 21 days, with estrus (visual display of heat; male receptivity) counted as day 0. In order to accommodate five fresh embryos collected from a day 7 donor, there must be at least five recipients that are at cycle day 6, 7, or 8 on embryo collection day. Since 5% of a naturally cycling recipient herd can be expected to show heat on any particular day, it is unlikely that a small recipient herd (less than 40 head) could accommodate five embryos on a particular collection day.

Fortunately, there arc five options that permit us to synchronize the cycles of recipients to donors.

Option #1: Natural Heat

One way to provide five cycle–synchronized recipients on collection day is to simply maintain a large recipient herd and observe natural heats. In a herd of 40 open (non–pregnant) cattle with randomly distributed estrous cycles, one would expect approximately two head (5%) in heat one day before the donor

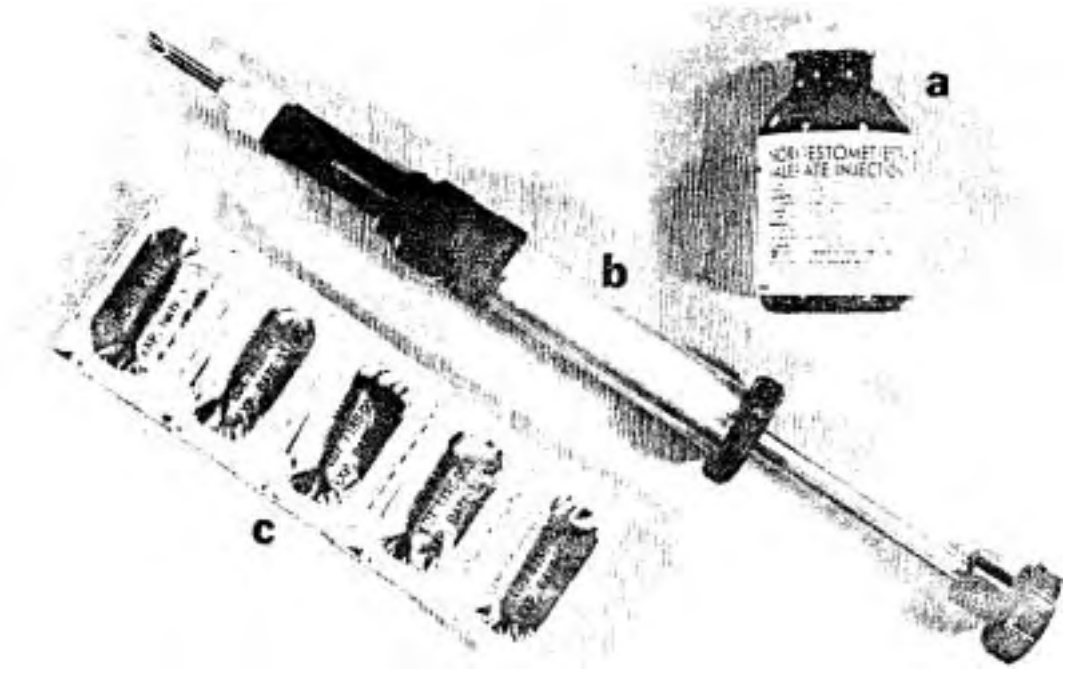


Figure 3. The Synchromate–B system. Norgestomet/ estradiol valerate injectable (a), ear implant insertion tool (b), ear implants (c).

Two synthetic prostaglandins available commercially are Lutalyse by Upjohn Co. and Estrumate by Haver (Figure 4).

Recipients that are mid–cycle (days 6 to 16) will respond to $PGF_2\alpha$ injection and show heat approximately 60 hours later. Three injection schemes are available:

a) $PGF_2\alpha$ single injection *with* palpation

Sclcct a group of recipients and palpate their ovaries. Cattle with a corpus luteum (Figure 5) are injected with $PGF_2\alpha$ (2 ml Estrumate or 5 ml Lutalyse). Approximately 90% of those injected will show heat, peaking 60 hours after the injection.

b) $PGF_2\alpha$ single injection *without* palpation

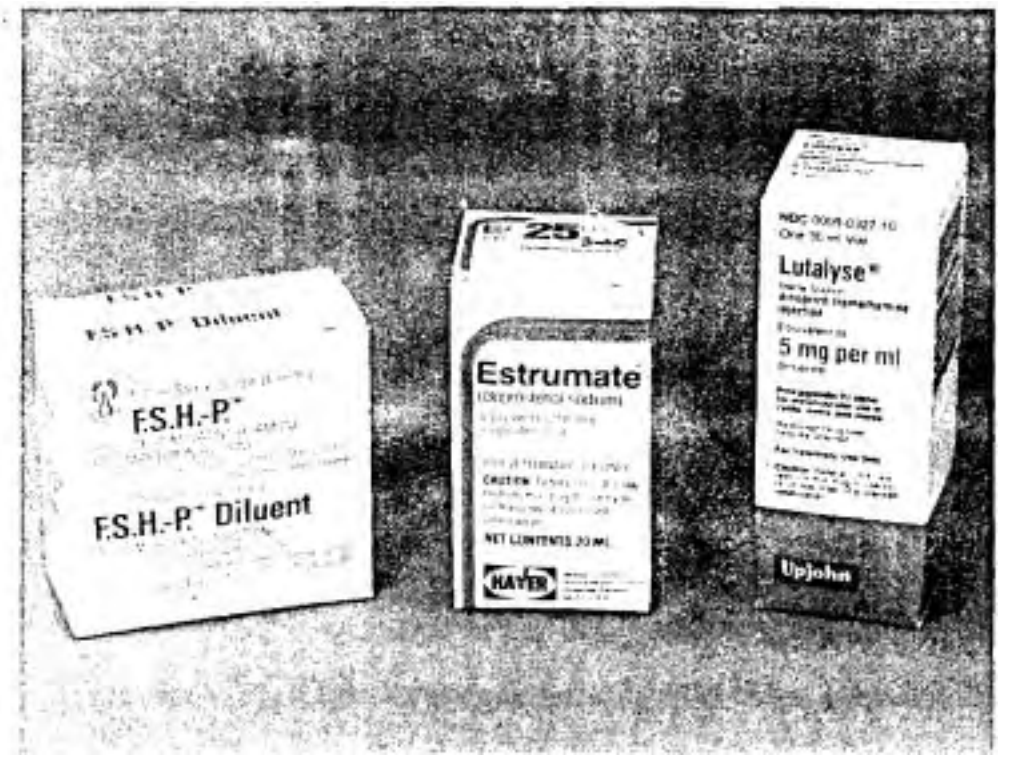


Figure 4. FSH (left), Estrumate, and Lutalyse.

Select a group of recipients and inject all with $PGF_2\alpha$. Approximately 45% of the group will come into heat 60 hours postinjection. This is the easiest method since palpation is not required, yet it requires twice as much $PGF_2\alpha$ as option 3a.

$PGF_2\alpha$ double injection without palpation

Inject all recipients with $PGF_2\alpha$ regardless of the presence or absence of a CL. Repeat the $PGF_2\alpha$ injection 11 days later. At the time of the second injection, 90% of the cattle will be mid-cycle and respond with heat 60 hours later. Obviously, this method uses twice as much $PGF_2\alpha$ as the single injection without palpation method. However, the additional drug expense may be outweighed by the fact that more recipients will be synchronized by the double injection method.

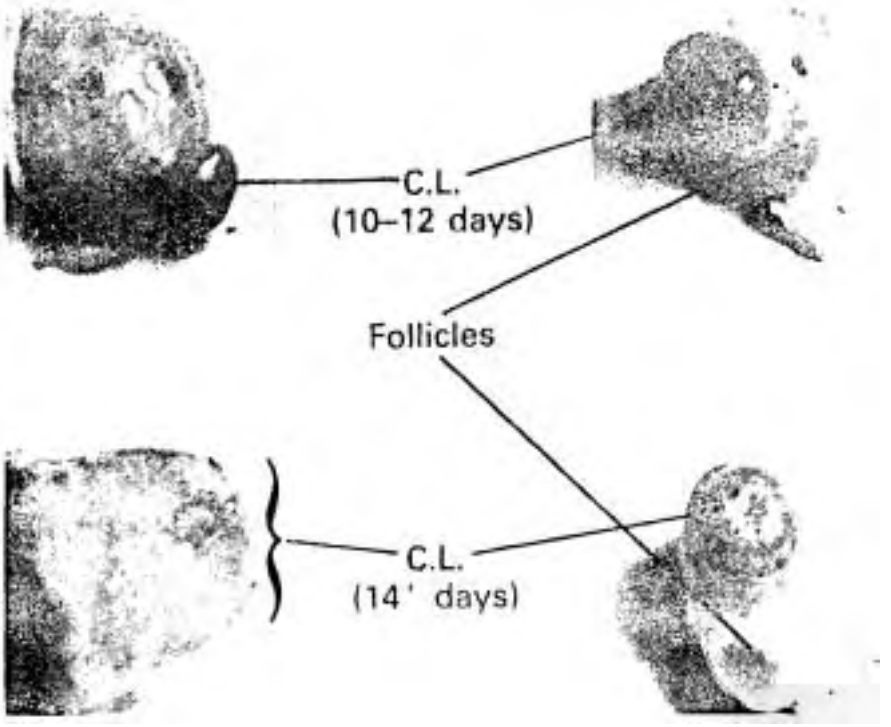


Figure 5. Normal mid–cycle ovaries.

There is no difference in pregnancy rate between recipients receiving an embryo after a natural heat versus a Synchromatc–B heat or prostaglandin heat.

Frequently, the deciding factor on which synchronization method to use is the number of available recipients currently at the ranch, dairy, or ET center. When deciding how many animals to synchronize, one must realize that approximately 25% of those showing heat will be classified after palpation on transfer day as unacceptable recipients. Typically, the rejected recipient lacks an identifiable corpus luteum.

The following examples can be used as aids in deciding which synchronization method is most appropriate, based on expected embryo production and a fixed number of recipients.

GIVEN: Two donors are superovulated, yielding 10 transferable embryos.

Example #3c: PGF$_2\alpha$ double injection without palpation

Double injecting 14 recipients with PGF$_2\alpha$ results in 13 heats, which yields 10 useable recipients on transfer day.

This method, like Synchromate–B, involves 14 recipients. Double injecting requires drugs and labor for handling the cattle twice. In addition, allowing for the interval between injections requires cattle management and feed for two to three days more than the Synchromate–B method.

SUMMARY OF RECIPIENT SYNCHRONIZATION

1. Decide if recipients are needed on a daily/weekly year–round basis, or once/twice per year.

2. Confirm how many open recipients are available at the ranch, dairy, or ET center.

3. Select a synchronization program (Examples 1 to 3c).

4. Organize the necessary drugs, supplies, and labor needs.

5. Refer to Appendix D for a comprehensive guide to scheduling, injecting, and/or Synchromating recipients.

CHAPTER 3

Donor Superovulation and Artificial Insemination

(3.1) SUPEROVULATION

Cows and heifers have a 21-day estrous cycle, with day 0 characterized by visual display of heat. Approximately 12 hours after the end of heat, a single non-fertilized egg (ova) is released (ovulated) from one of two ovaries. If the female is bull-bred during heat, or bred artificially at the end of heat, the egg will be fertilized shortly after ovulation and develop into an embryo.

The process of embryo transfer includes a donor hormone treatment referred to as superovulation, which results in approximately 10 ova ovulated at estrus, instead of the usual one egg. Typically, 50% of the ovulations result in transferable embryos.

The single most important component in ET is superovulation, yet the physiology and biochemistry involved in successful superovulation are not completely understood. In general, 85% of all "normal, fertile" donors will respond to superovulation treatment with an average of five transferable embryos, and can be repeatedly superovulated at 60-day intervals with a slight decrease in embryo production over time.

Most donors are cows with proven milk production records, or dams producing heavy weaning weight calves and/or popular showstring cattle. However, if a heifer calf carries desirable maternal and paternal genetics, she can be successfully superovulated as soon as regular estrous patterns are established.

The standard superovulatory hormone in the United States is follicle stimulating hormone (FSH, Figure 4), and is available commercially as FSH-P (Schering Corp.). A typical superovula-

tory dose in mature cattle is 35 mg, and is adjusted depending on age and breed (i.e., 24 to 28 mg in Brahman–blood cattle). Virgin heifers are usually dosed 25% lower than cows. Depending on the presence or absence of a CL on the donor's ovary, there are basically two supcrovulation regimens available.

Regimen #1: Corpus luteum present

To initiate supcrovulation, a healthy, open, well–nourished donor that is at least 50 days postpartum is observed for two estrous cycles. If the interval between heats is normal (18 to 24 days) and rectal palpation confirms a well–formed corpus luteum after the second heat, then treatment may begin.

The standard supcrovulation treatment involves four days of twice daily FSM injections. The dose declines each day and prostaglandin (Estrumate or Lutalyse) is given on treatment day 3 which causes estrus 48 hours later. For maximum fertilization, it is crucial to visually pinpoint the onset of standing heat. Only by observing the onset and duration of heat can one optimally judge when to inseminate.

Important points:

1. Donors may start the FSH regimen any time between and including cycle days 8 to 13.

2. Only donors with a distinctive, well–formed CL should start the FSH regimen.

3. The interval between FSH injections should be 12 hours.

4. Always enter the FSH bottle with a clean, sterile needle. Bacteria will quickly destroy FSH!

Regimen #2: Corpus luteum absent, cystic ovaries, or acyclic ovaries

When these conditions are present, a synchronized donor heat can be accomplished with the aid of Synchromate–B.

A cystic animal has erratic cycle lengths, with frequent and prolonged heats. Palpate the donor's ovaries. If the donor is cystic, gently attempt to manually rupture the dome–shaped, fluid–filled follicles (Figure 6) before Synchromate insertion. If the donor is acyclic, ovarian palpation will identify:

a) Small, firm ovaries with no significant structures, or

b) A dominant, persistent corpus luteum. The typical "normal" CL (Figure 7) is a firm, crown–shaped structure on the ovary surface. It is approximately 0.7 to 1.0 cm in diameter and 0.3 to 0.6 cm in height.

After identifying acyclic ovaries or manually reducing follicular ovarian cysts, insert a single Synchromate–B implant under the skin of the donor's ear (Figure 8).

Figure 6. Two fluid–filled cystic ovaries (right and left) compared to a normal ovary (center).

The Synchromate–B injection of norgestomet/estradiol valerate is NOT given to cystic donors!

The FSH injection schedule begins seven days after Synchromate–B insertion. For example, if the cystic or acyclic donor is Synchromated on Friday, FSH begins seven days later on Friday.

Figure 7. Classic CL protruding from the lower side of the ovary.

Important Points:

1. Synchromated, superovulated donors will show heat 24 to 36 hours after implant removal.

2. Synchromate–B is an excellent tool for scheduling large groups of donors for collection on a specified day.

SUMMARY OF SUPEROVULATION PROCEDURE

Refer to Appendix *D* for a comprehensive outline of scheduling and injecting donors.

With needle parallel to the ear surface, push needle between skin and cartilage (subcutaneously) until all the needle is under the skin.

Holding the needle *all* the way *in* to the ***hub,*** push the plunger all the way in; ejecting implant subcutaneously.

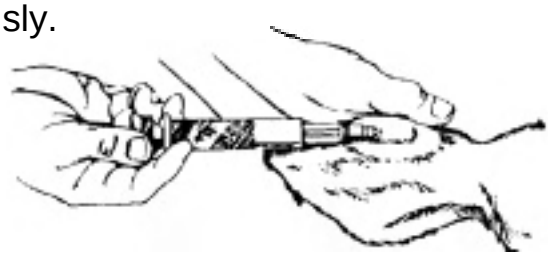


Figure 8. Diagram for inserting Synchro–mate–B ear implants.

(3.2) ARTIFICIAL INSEMINATION

As mentioned previously, successful ET requires accurate execution of many procedures, one of which is artificial insemination (AI). An AI program that yields a high embryo fertilization rate is the product of four factors:

1. Heat detection
2. Semen quality
3. Semen handling
4. Semen placement

Heat Detection

Line 5 in the schedulingiinjecting guide ***(Appendix D***) pinpoints the expected donor heat date. However, it is not unusual for the onset of "standing heat" to occur 12 hours before or after the expected heat date. A donor is in standing heat when she stands still while allowing another cow (or heifer) to climb onto (mount) her hindquarters, resembling a bull breeding a cow.

There are several guidelines to follow that will foster accurate determination of standing heat:

1. After the donor receives estrumate (or Synchromate is removed), she should not be housed alone. Keep her with approximately 10 other cows, or better yet, with a group of recipients that are due in heat.

2. The cattle lots or holding pens should not be cramped (confining) or slippery. Sexually active cattle need room to maneuver, plus sure footing for mounting.

3. Indications of approaching estrus include:

   - Donor refusing to eat (off feed)
   - Sharp drop in milk production
   - Changes in behavior; restlessness
   - Increased flow of clear mucus from the vagina

4. The best "tool" for heat detection is the naked eye. When you *see* the donor "stand" for another cow, record the date and time.

Semen Quality/Blood Typing

Prior to superovulation the semen quality should he verified.

Care should be taken to purchase semen with known high fertility. Whenever the quality of semen is in doubt, a sample should be examined *(prior* to the start of superovulation) by the producing AI organization, or a competent local veterinarian.

Positive verification of an embryo transfer calf's sire and dam requires bloodtyping of the calf, and therefore the blood–type of both sire and dam must be on record with the donor's breed association. Since some bloodtypes are quite similar and cannot be differentiated by analysis, it is advised (especially with multiple–sire matings) that the donor breed association be consulted before donor insemination to verify that a selected mating can be differ–entiated through blood–type analysis.

Semen Handling

Once semen is purchased and delivered to the ranch or dairy, it must be maintained in a semen tank submerged in liquid nitrogen. The nitrogen level should be checked every other day. If the nitrogen dissipates, then the semen is destroyed!

Care should be exercised when removing an individual straw from its goblet. Using tweezers, maintain the selected goblet within the tank, at least 5 cm below the top of the nitrogen vapor level, for no longer than 10 seconds. With this technique, the remaining goblets are not subjected to temperature fluctuations. Using a second pair of tweezers, raise the selected straw partially out of its goblet to verify the bull's name and registration number printed on the side of the straw. Once identified, the straw may be removed and placed into a water bath.

The universal water bath thaw temperature for straws is 35° C for a minimum of 40 seconds. Some semen collection centers recommend slightly different thaw procedures, so it is best to ask for specific thaw recommendations when purchasing semen.

A thermos bottle, styrofoam box, or electrified thaw unit (Figure 9) is appropriate for thawing semen. Whichever container

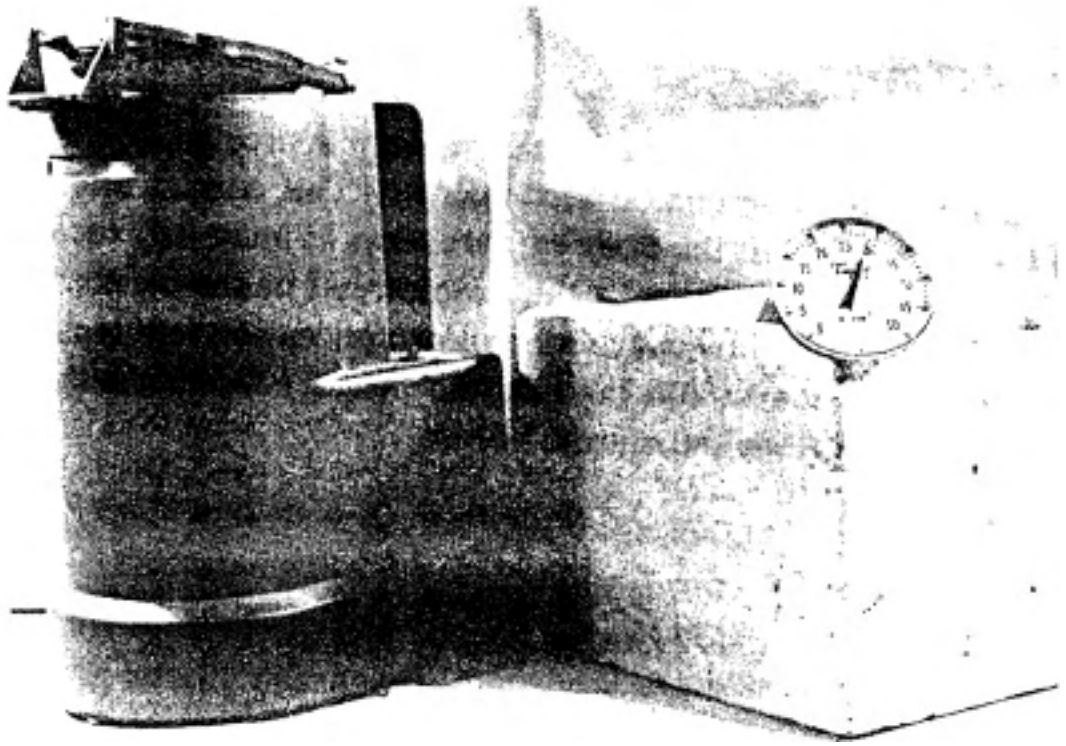


Figure 9. Cito electric thaw unit (left), styrofoam thaw box, and dial thermometer.

is used, the thaw temperature should be monitored closely, especially when several straws are being thawed simultaneously.

Once thawed, the straw should be wiped dry, the end opposite the plug squarely clipped off, and then inserted plug–first into the open end of a pre–warmed (30° C) insemination rod. When breeding outside in cold weather and/or bright sunlight, place the insemination rod into a sterile plastic sleeve, and then transport the rod under clothing to protect the semen from cold shock and exposure to direct sunlight.

The donor should be bred immediately after the semen has thawed!

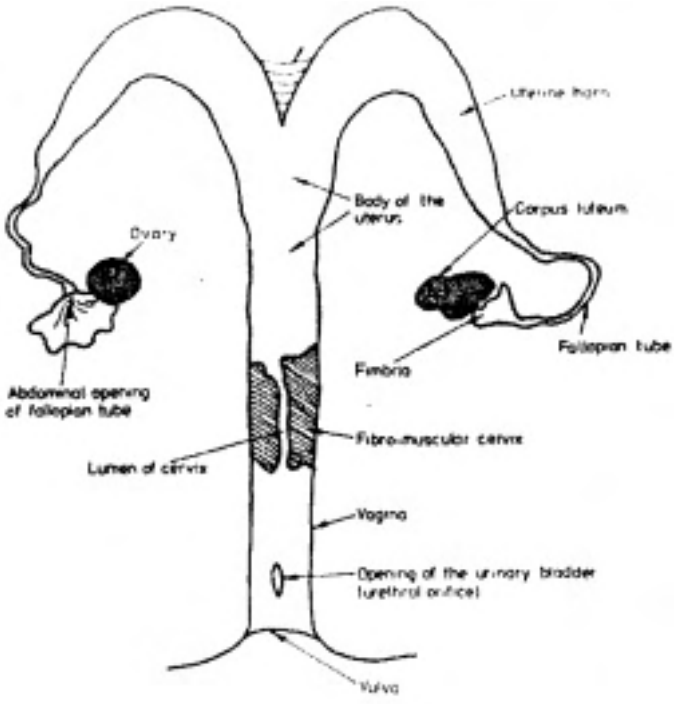
Semen Placement

Site of semen deposition is the primary source of variation among artificial insemination technicians. The correct site for semen placement is in the body of *the uterus* (Figure *10)* located approximately 2 cm in front of the anterior end of the cervix.

There is a strong tendency among low conception rate technicians to deposit semen too far into the uterus, which results in semen being placed in the uterine horns.

Sanitation

Sperm cells are living organisms and are sensitive to foreign material, including water and lubricants. Disposable plastic gloves,



Dorsal view of the reproductive tract of the cow with the uterus 'uncurled'

Figure 10. Top (dorsal) diagram of the reproductive organs within the cow.

sleeves, and breeding sheaths should never bc re–used. After thawing, dry the straw completely before clipping, and avoid contamination of the insemination rod as it passes through the vulva. A proven technique for holding the vulva open is to wedge a folded, V–shaped paper towel (V apex facing down) between the vulva lips.

Timing of Insemination

Ova are randomly released (ovulated) from superovulated ovaries over a period of six to twelve hours. For this reason, the donor must be inseminated at least twice. The first breeding should occur 12 hours after the onset of standing heat. The second breeding occurs 12 hours after the first insemination. If the donor

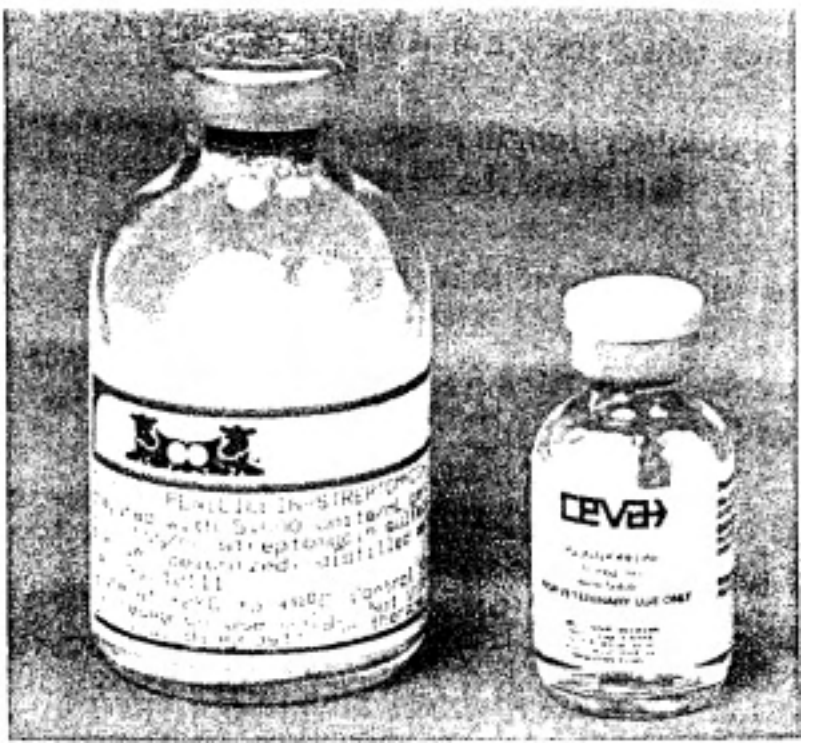


Figure 11. Lyophilized penicillin–streptomycin (left) and Cystorelin (GnRH).

is still in standing heat after the second insemination, she should then be injected with 4 ml of gonadotropin releasing hormone (Gn–RH, *Cystorelin*). Breed the donor a third time 12 hours after injecting GnRH (Figure 11).

SUMMARY OF INSEMINATION PROCEDURE

1. Verify sire and dam blood type compatibility from breed association.

2. Order semen and inventory the purchase after its arrival.

3. Pinpoint the donor's onset of standing heat.

4. Identify the sire, then thaw semen at 35° C for 40 seconds.

5. Load semen into a prewarmed insemination rod.

6. Wearing a shoulder–length plastic sleeve, clean out the rectum if necessary, then grasp the cervix.

7. Wipe the vulva clean with a paper towel.

8. Part the vulva by placing a clean, folded paper towel between the vulva lips.

9. Pass the breeding rod through the cervix, and stop when the rod has just entered the uterus.

10. Slowly (five seconds) plunge semen into the uterus. Avoid pushing the rod forward while plunging!

11. Withdraw the insemination rod. Record the breeding by noting the complete name and registration number of both sire and dam, sire code number, date, and name of inseminator.

CHAPTER 4 _

Embryo Recovery

(4.1) COLLECTION DATE AND RECIPIENT IDENTIFICATION

Bovine embryo collection is generally attempted on day 7 after estrus, which yields a development stage suitable for freezing. Embryos are frequently located in the oviducts prior to day 6 and therefore cannot be collected by the nonsurgical recovery technique. By day 8.5, the clear sphere (zona pellucida, Figures 12 and 13) surrounding the embryonic cellular mass has ruptured, leaving an embryo too mature for freezing.

Pregnancy rates from fresh (not frozen) embryos collected and transferred 6 to 8 days after heat are not different. However, in order to correctly freeze embryos produced in excess of recipients on a particular day, collection should be scheduled for day 7.

Whether collection is done on–farm or at the ET center, the first activity on collection day (or the evening before) will be recipient selection.

Assuming a day 7 collection, obtain a list of all recipients that are 6, 7, or 8 days after heat. Palpate both ovaries of each animal to identify the presence of a well–formed CL. For the most accurate and sensitive palpation, first remove the fingertips from a shoulder–length plastic sleeve. Put the sleeve on, then pull on a snug–fitting latex examination glove. This combination offers inexpensive, disposable, full–length protection and permits accurate identification of ovarian structures.

Once a well–formed CL is identified, use a cattle marking crayon to place a slash on the hip corresponding to the ovary (right or left) that the CL is located on. A record should be kept on all useable recipients (those with a well–formed CL) for convenient

recipient/embryo matching later in the collection day. The useable recipient list sum identifies how many embryos can be transferred fresh, and subsequently when to freeze excess embryos.

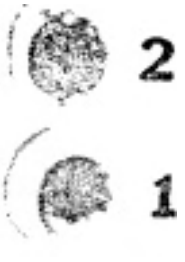


Figure 12. Unfertilized ova (1); tight morula, grade 2 (2). Low magnification (70×).

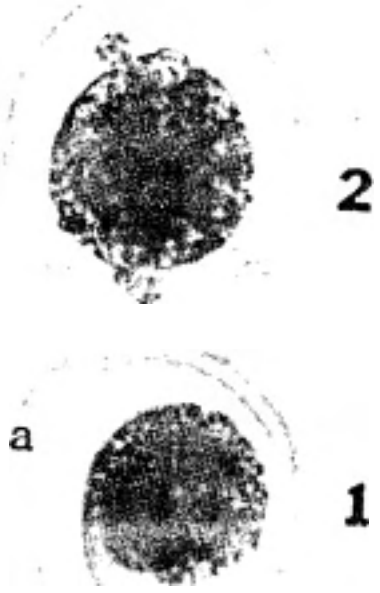


Figure 13. High magnification (400×) of Figure 12; "a" indicates zona pellucida.

SUMMARY OF COLLECTION DATE AND RECIPIENT IDENTIFICATION

1. Schedule a day 7 collection (estrus = day 0).

2. The first order of business on collection day is to palpate all recipients that showed heat 6, 7, or 8 days earlier.

3. Record ear tag number, heat date, and CL side for all useable recipients. Keep this list by the microscope!

(4.2) EQUIPMENT AND DONOR PREPARATION

After recipient identification, the next step is to organize and prepare the collection, freezing, and transfer equipment and supplies. The typical sequence is:

- Make sure that the lab work bench is clean, *free of* dust *and smoke,* and draft-free.
- Place the unopened liter bag of Dulbecco's PBS (Figure 14) upright into a small container of 37° C water. Do not submerge the serum injection port. The objective is simply to warm the collection solution.

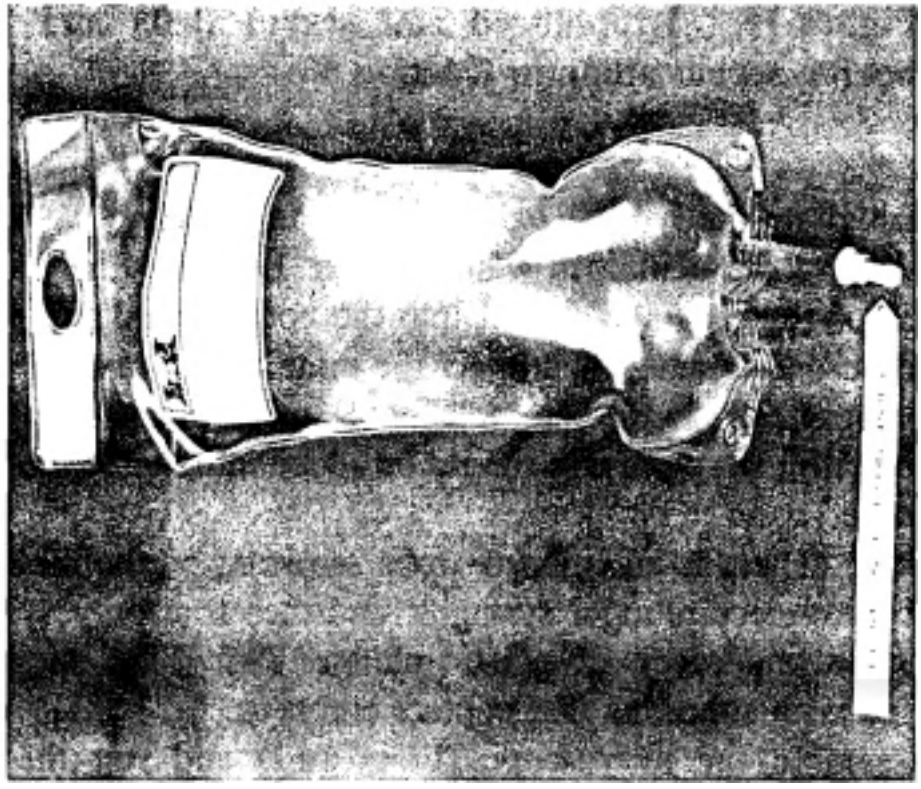


Figure 14. Dulbecco's phosphate buffered saline (PBS).

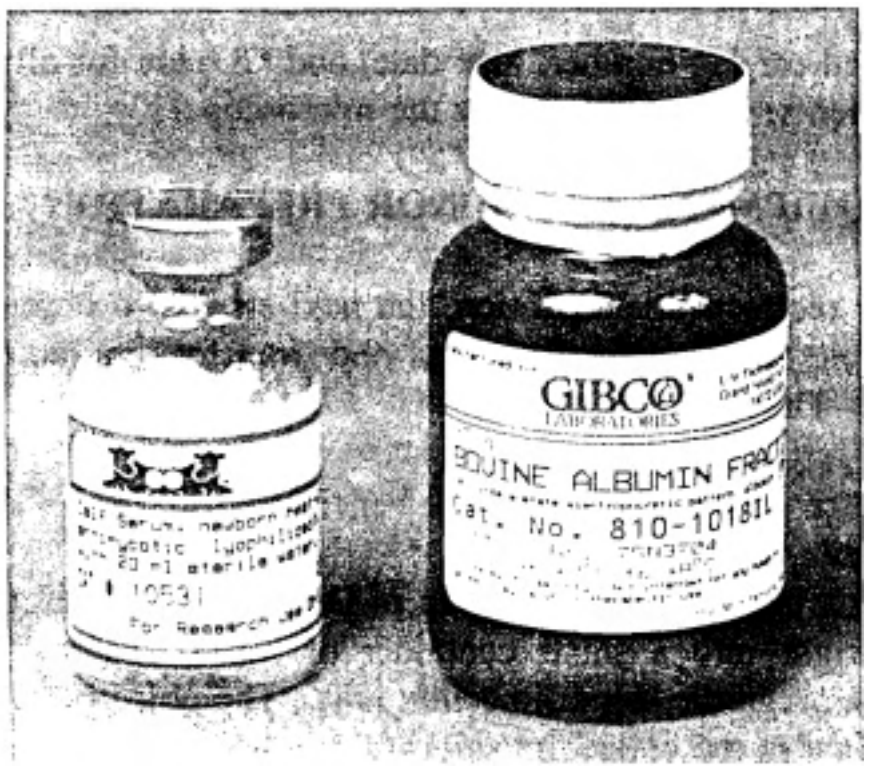


Figure 15. Lyophilized calf serum (left) and bovine serum albumin fraction V.

- Rehydrate the lyophilized serum (Figure 15) by drawing 20 ml of sterile deionized water (Figure 16) into a 30 ml Air–Tite syringe. Inject 10 ml into the bottle; gently shake the bottle one minute; then inject the remaining 10 ml of water. If not already in the serum, 2 ml of antibiotic/antimycotic or penicillin/streptomycin should be added into the 20 ml serum bottle.
- Note: There is an embryo–toxic lubricant on the rubber tip of two–piece plunger syringes; and therefore only syringes with a one–piece all–plastic plunger (Air–Tite) should be used with solutions that contact embryos.
- Assemble the microscope (Figure 17) and test the light source.

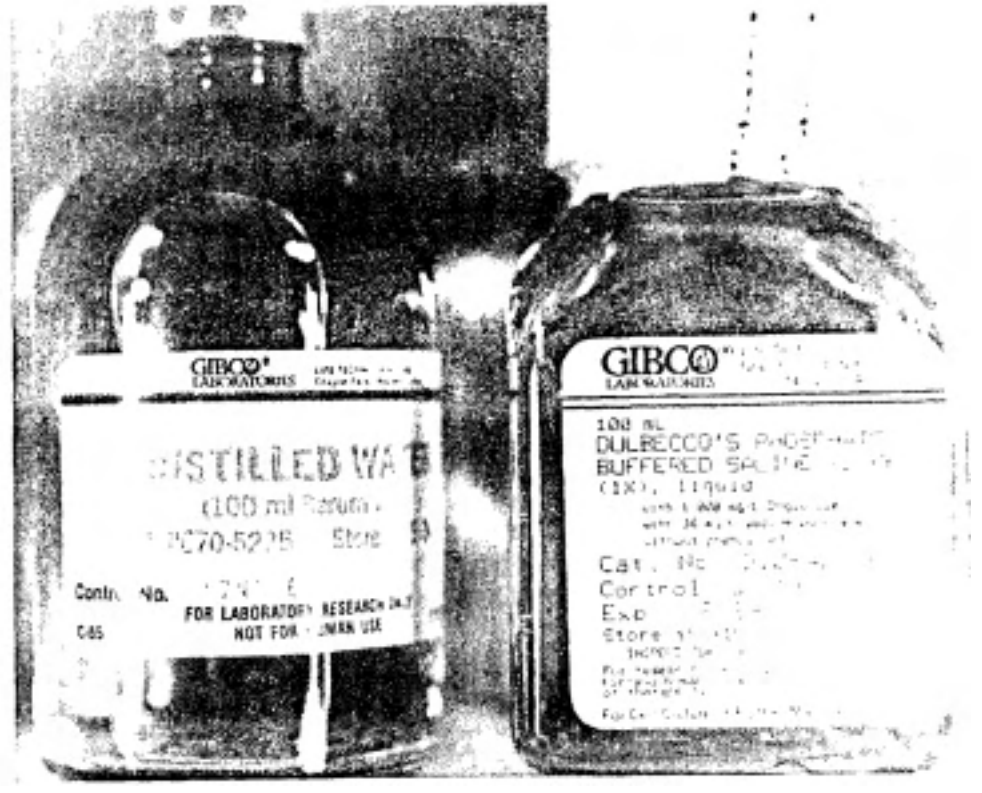


Figure 16. Deionized, distilled water (left) and Dulbecco's PBS.

- Assemble the embryo freezing instrument (Figures 18 and 19), fill its nitrogen tank with nitrogen according to directions in section 6.1, and then switch the instrument on. Verify that the preprogrammed freeze rates are correct and then test-run the program for five minutes.
- Switch on the slide-warmer (Figure 20), which should be accessible when scatcd in front of the microscope. Adjust the surfacc tempcraturc to 25° C. To control temperature fluctuation, embryos should be kept on the slide-warmer when not being examined microscopically.
- Remove the 1000 ml bag of PBS from its water bath and dry with a paper towel. Grasp the serum injection port (Figure 14) firmly with one hand, and the white rubber serum injection port plug with the other hand. Remove the plug

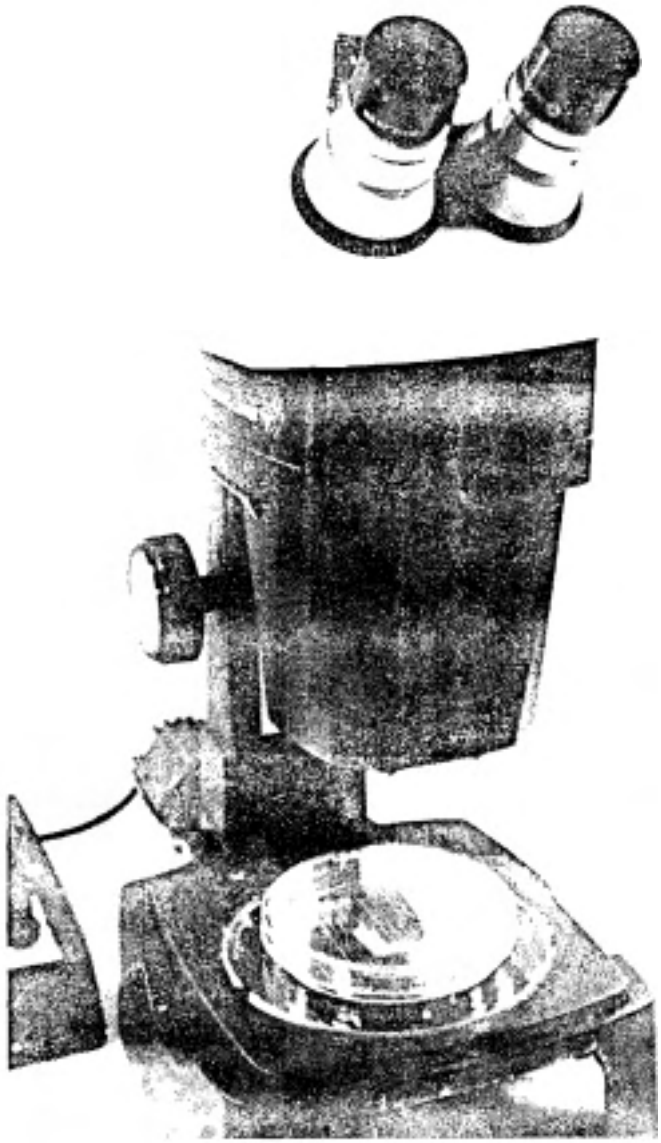


Figure 17. Bausch & Lomb stereo–zoom microscope.

with a short, quick pull. Using a sterile 10 ml Air–Tite syringe with attached 16 ga. x 1 inch needle, draw up 10 ml of newborn calf serum and inject into the serum injection port (Figure 21).

- Cut a 10 cm long piece of tubing from end (a) of the Y–junction tubing (Figures 22 and 23). Attach one end of this

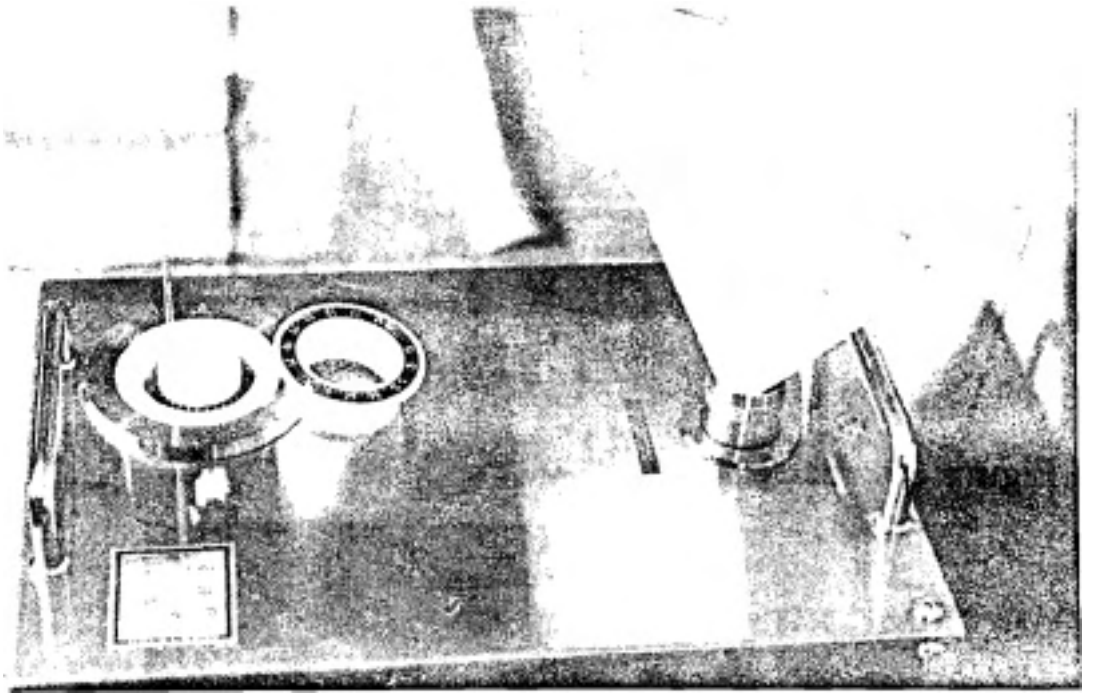


Figure 18. Liquid nitrogen reservoir of the embryo freezing unit. Nitrogen is filtered through a paper towel before entering the reservoir.

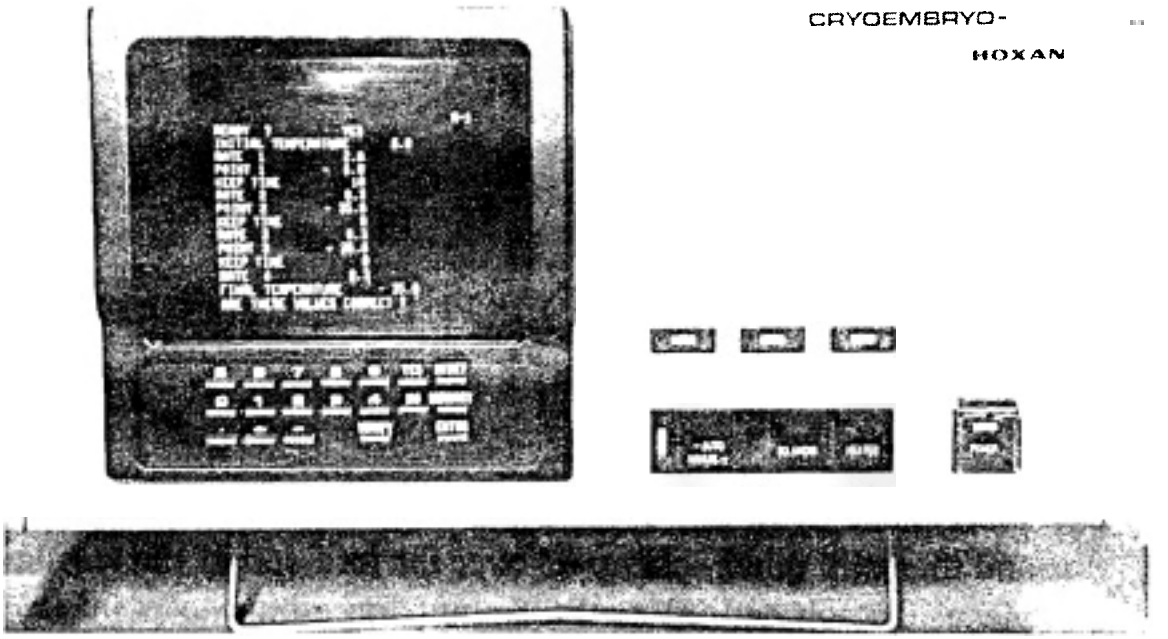


Figure 19. Cryoembryo PSP embryo freezing unit controller.

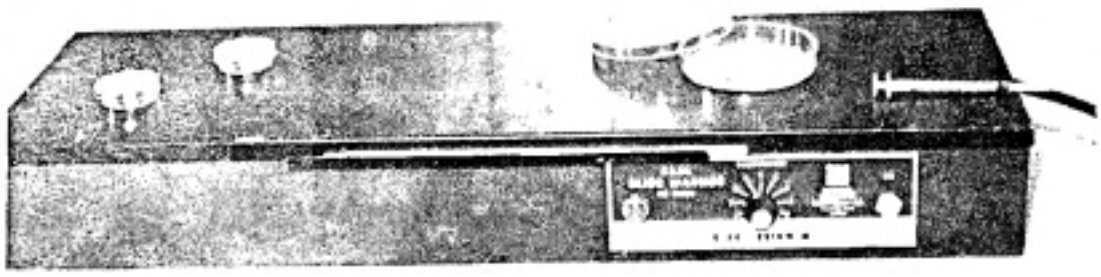


Figure 20. Slide warmer.

Figure 21. Injecting serum into the serum injection port immediately prior to use.

short piece to point (a) on the Em–Con filter (Figure 24), and then slip pinch–clamp #3 over the free end of the tubing piece. Attach end (a) of the Y–junction tubing to point (b) on the Em–Con filter. Cover tubing end (c) with a short

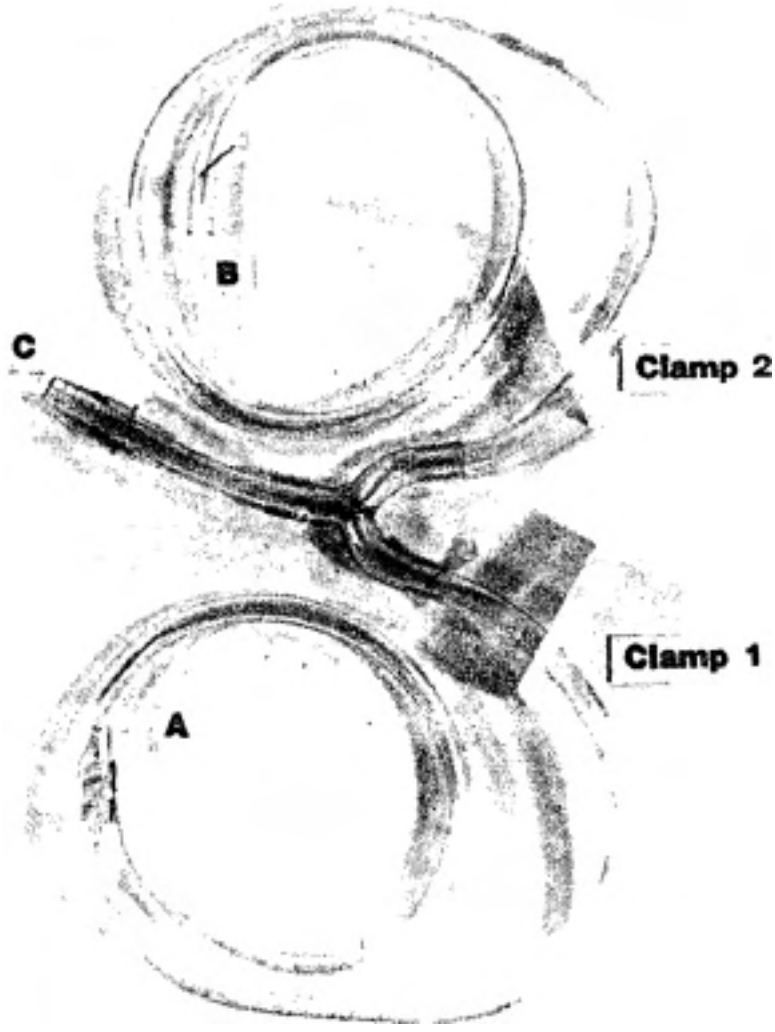


Figure 22. Y-junction tubing.

piece of autoclave tape. Firmly connect tubing end (b) to the serum injection port.

- Close off Y-junction tubing arms (a) and (b) by wedging clamps #1 and #2 into position, and close pinch-clamp #3 which regulates out-flow at Em-Con point (a). Note: If more than one donor will be collected, use a permanent marker to write the donor's name on the side of each Em-Con filter that will be used with each donor.
- Select the appropriate Folep catheter (20 ga. for cows, 16 ga. for heifers, Figure 25). Open and fold back 5 cm of the

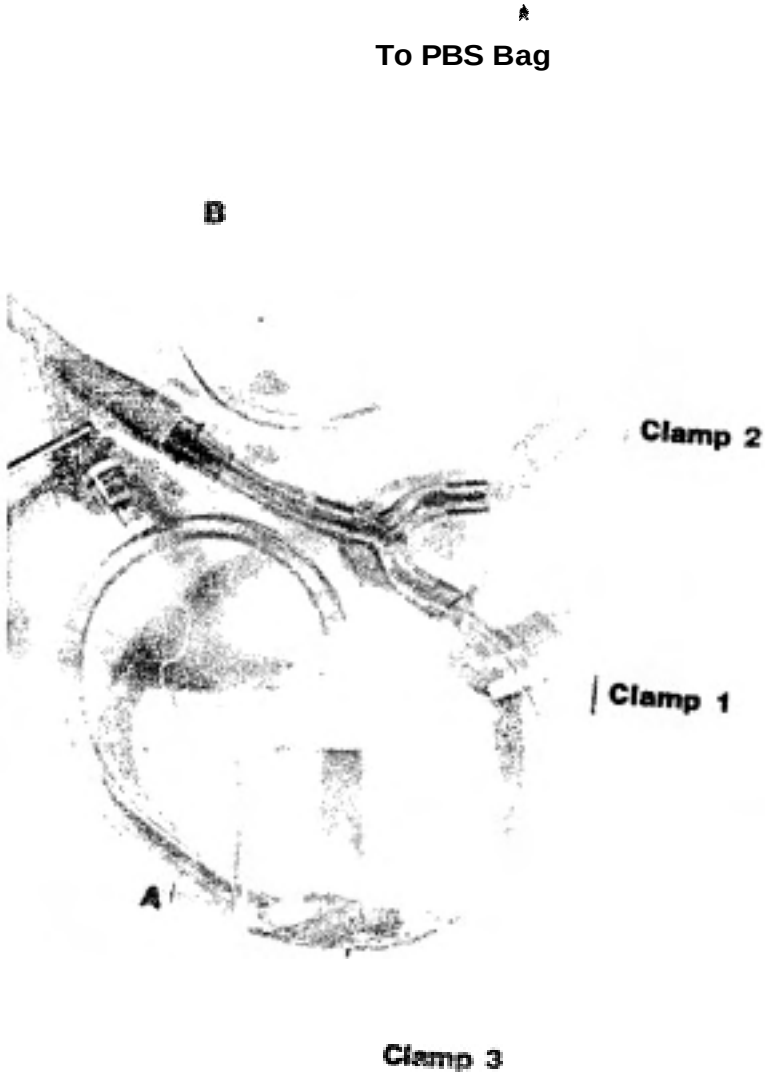


Figure 23. Y-junction tubing with attached Em-Con filter and Foley catheter.

catheter packet and then insert a sterile stylet (Figure 26) full-length into the catheter (16 ga. catheters will require a smaller diameter stylet). Holding the catheter at point "a" (Figure 27), place end "a" of the stylet against your stomach. Gently pull (stretch) the catheter 3 to 5 cm back over the stylet toward your stomach. With the catheter still stretched, use a forcep to clamp the catheter at point "b" to the stylet

(Figure 28). Draw the plunger of an empty 20 ml syringe back to the 15 ml mark. Firmly attach the syringe to the catheter air valve (point "c"). In order to verify the patency of the catheter's balloon, inject all 15 ml of air slowly through the air valve. The balloon should expand and retain its inflation (Figure 29). If the balloon holds air, pull the syringe plunger back to the 15 ml mark and detach the syringe from the catheter. All of these procedures are carried out with the catheter inside its sterile packet!

Figure 24. Em–Con filter.

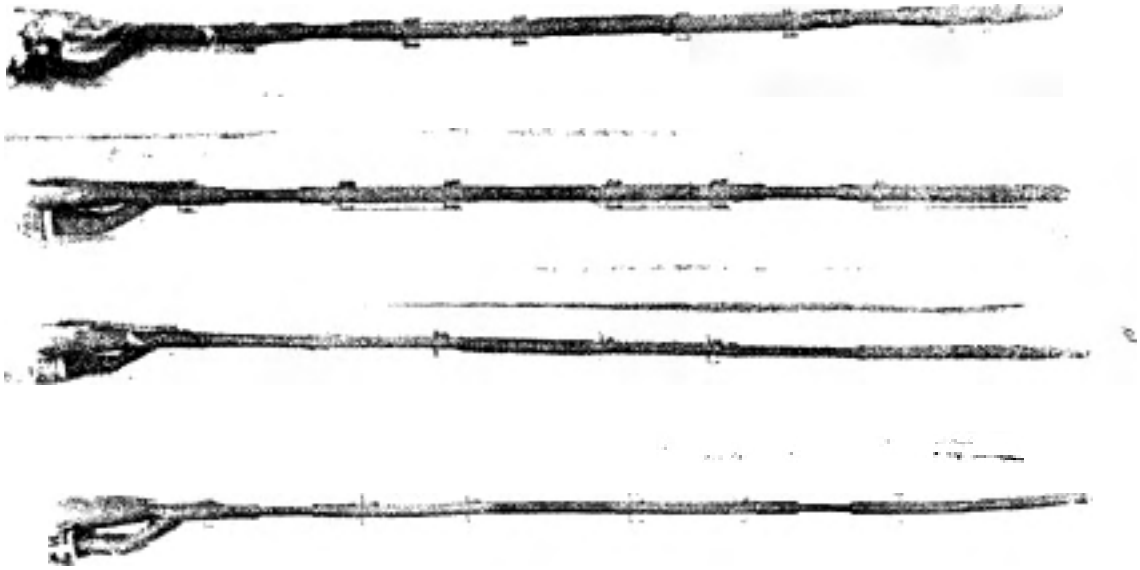


Figure 25. Assortment of Foley catheters (top to bottom); 22 fr., 18 fr., 16 fr., 14 fr.

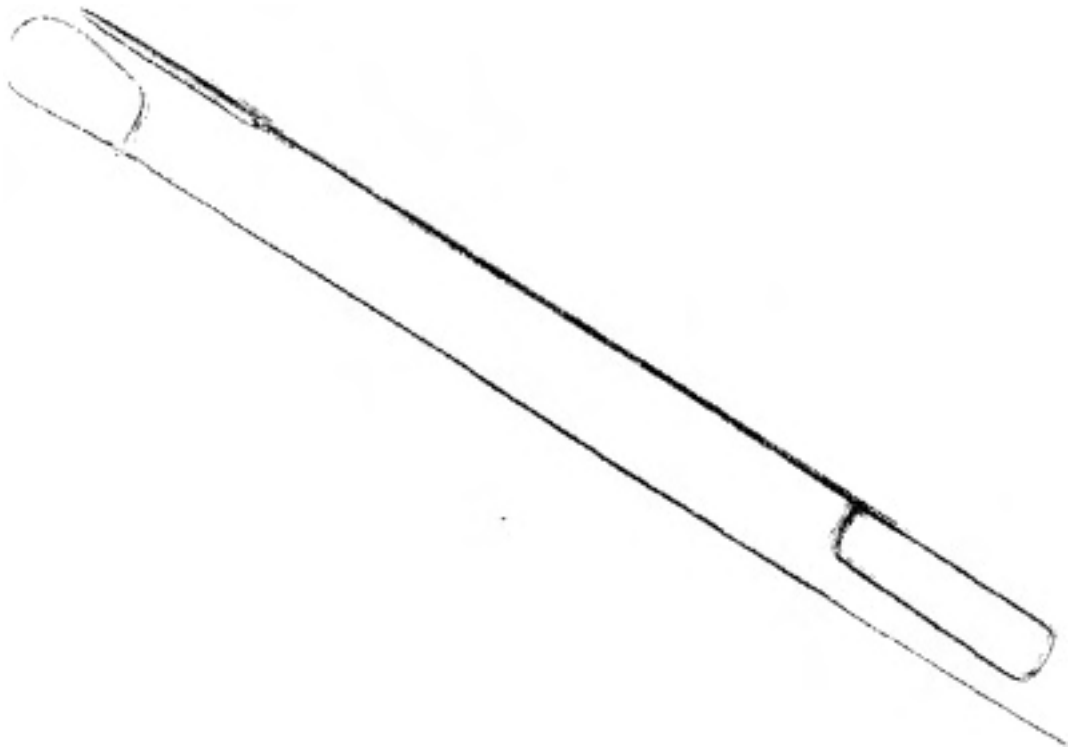


Figure 26. Stylet (bottom) that will be inserted into the Foley catheter, and cervical dilator.

- Draw 40 ml of either 0.2% nitrofurazone solution or 0.5% chlorhexidine solution into a sterile 50 ml Air–Tite syringe. Wipe any excess solution off of the syringe tip, and then attach the syringe to an infusion pipet. Keep the pipette inside its protective packet. Place this syringe/pipet combination on the instrument table next to the donor. It will be used when the collection is finished.
- Using a permanent marking pen, write the word "Estrumate" on the side of a 6 ml Monoject syringe. Draw 4 ml of Estrumate into the syringe, cap the 16 ga x 1 inch needle, and place the syringe next to the infusion pipet.
- Embryo collection can be performed more accurately and with less strain on the technician's palpation arm if the donor's rectal squeeze (peristalsis) is inhibited. This is accom–

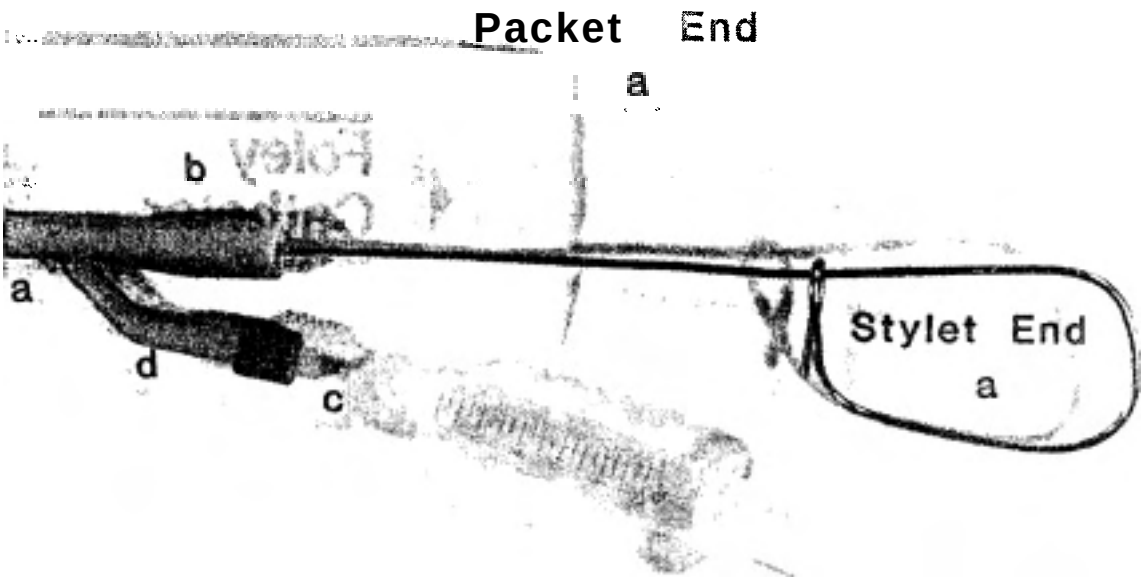


Figure 27. Foley catheter being prepared for use.

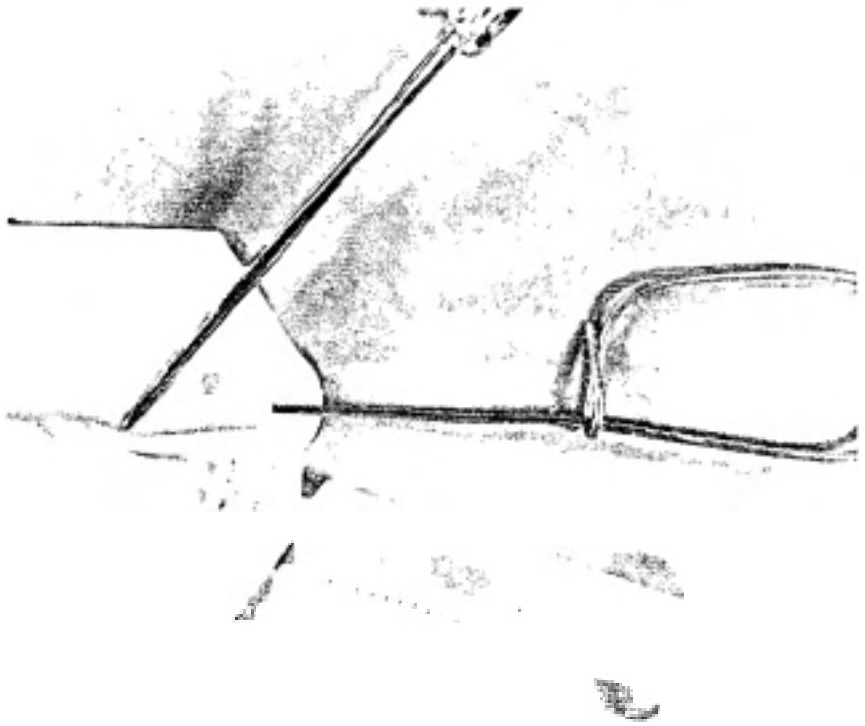


Figure 28. Foley clamped to the stylet, ready for insertion.

plished by epidural anesthesia, which should be administered at this time.

Scrub the donor's tail–head with Betadine scrub and rinse with water. Draw 6 ml of lidocaine into a 12 ml Monoject syringe (18 gauge x 1.5 inch needle). Grasp the tail firmly (just below the rectum) and elevate it 130 to 140" straight up and forward, applying constant forward pressure. Using the thumbnail of your free hand, press down firmly on the scrubbed tail–head region to locate an indentation between two vertebrae. This will be the injection site.

While still applying forward tail pressure, plunge the needle deep into the intervertebral space until the needle contacts bone. Once contact is made, withdraw the needle 1 mm and then inject the lidocaine over a 5–second interval. The lidocaine (Figure 30) will flow easily into the epidural space if the needle is positioned properly. When the injection is administered correctly, the tail will go limp in 1 minute and rectal peristalsis will essentially stop. If the tail does not become limp after 3 to 4 minutes, repeat the injection with attention given to the depth of needle placement and ease of injection (lack of backpressure) (Figure 31).

- Tie the limp tail up and out of the way.
- Pull on a shoulder–length sleeve and remove all feces from the rectum, being careful to minimize the amount of air that enters the rectum.

With a bucket of clean warm water and several drops of Betadine scrub, wash all fecal and foreign material away from the rectal sphincter and vulva. Part the vulva lips and rinse thoroughly, because scrub solution is embryo toxic! Dry with paper towels (Figure 32).

Suspend the PBS bag with attached tubing/Em–Con filter approximately 0.5 m directly above the donor's tail–head (serum injection port facing downward).

Figure 29. Testing the catheter balloon (cuff) prior to use.

Figure 30. Lidocaine HCL (left) and Rompun.

- At all times during collection, the Em–Con filter must be maintained half–full with PBS (Figure 33). This pool is initially established by opening clamps #1 and #2. Clamp #3 remains closed. Since this is a "closed" system, PBS will not enter the filter until you "open" the system by gently lifting an edge of the filter lid. Once the filter is half–full, securely seal the lid onto the filter, and close clamps #1 and #2.

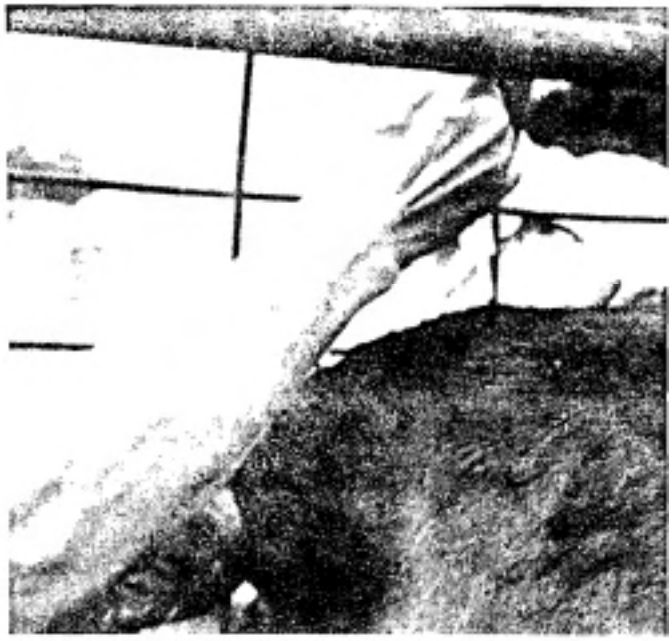


Figure 31. Administering epidural anesthesia prior to embryo collection.

SUMMARY OF EQUIPMENT AND DONOR PREPARATION

1. Select a clean lab work area that is reasonably close to the collection and transfer site.

2. Locate warm water and begin thawing and/or warming solutions.

3. Rehydrate injectables/additives.

Figure 32. Thoroughly rinsing the vulva after washing with Betadine scrub.

Figure 33. The Em–Con should be maintained half–full of PBS throughout the entire collection.

4. Assemble and test microscope and freezing unit.

5. Attach Y–junction tubing to filter; test catheter balloon.

6. Prepare post–collection infusion and Estrumate.

7. Administer epidural anesthesia; wash vulva.

8. Suspend PBS; fill Em–Con filter half–full.

(4.3) EMBRYO COLLECTION

At this point, it is convenient to have an assistant available for catheter inflation, stylet removal, and possible manipulation of the Em–Con filter.

The sequential steps for embryo collection are:

- Pull on a shoulder–length sleeve (fingertips removed) and then a latex examination glove. Using a hemostat, clamp the open end of the sleeve to your shirt at the shoulder.
- Place a small amount of lubrication jelly on the back of your examination glove and along the sleeve. Enter the rectum.
- Once inside the donor, identify the cervix and uterine horns, and then estimate the number of ovulations (CLs) and nonovulations (follicles) by gently palpating each ovary. Estimate the comparative size of each ovary (golf ball, tennis ball, baseball, etc.) Record this information for future reference.

  Grasp the catheter (point "a", Figure 27), which is still clamped to the stylet, and withdraw it from its protective packet. Place the empty packet back on the instrument table. With one hand in the rectum, grasp and extend the cervix forward. After your assistant parts the lips of the vulva, insert the catheter into the vagina and through the cervix as if the donor were being artificially bred (Figure 34).

  If you cannot gently manipulate the catheter through the cervix, you should: (a) remove the catheter and carefully manipulate the rigid cervical dilator (Figure 26) through the cervix, or (b) select a smaller–gauge catheter.
- Immediately after the catheter tip enters the uterine body, it should be placed in the right (or left) uterine horn. This is accomplished by lifting the uterus and positioning the right

horn directly in front of the catheter, rather than directing the catheter toward the horn.

- Gentle downward pressure by the middle finger will identify the Y–junction (Figure 35) where the two uterine horns separate from the uterine body. Similar downward pressure by the index finger on the right horn will identify the catheter tip. Advance the catheter tip approximately 2 to 4 cm beyond (anterior to) the palpated uterine Y–junction. The catheter is now in position, ready for inflation.
- At this point, your assistact should snugly attach a 20 ml syringe (plunger pulled back to the 15 ml mark) to the catheter inflation valve (Figure 36). Slowly inject 10 ml of air, and then maintain the plunger at the 10 ml mark while you gently palpate the balloon through the uterine horn wall. The objective is to inflate the balloon (cuff) to the point that it forms a snug seal against the uterine horn wall, and to avoid overinflation that will rupture the uterine membrane at point of contact.

Figure 34. Manipulating the Foley catheter through the cervix.

While palpating the cuff, *gently* pull backward on the catheter. If the cuff feels like it is going to slip back, you must inflate the cuff more by adding air in 1 ml increments beyond the 10 ml already in the cuff. Catheter positioning and cuff inflation are critical steps in a successful collection. The key is to avoid overinflation, which results in membrane

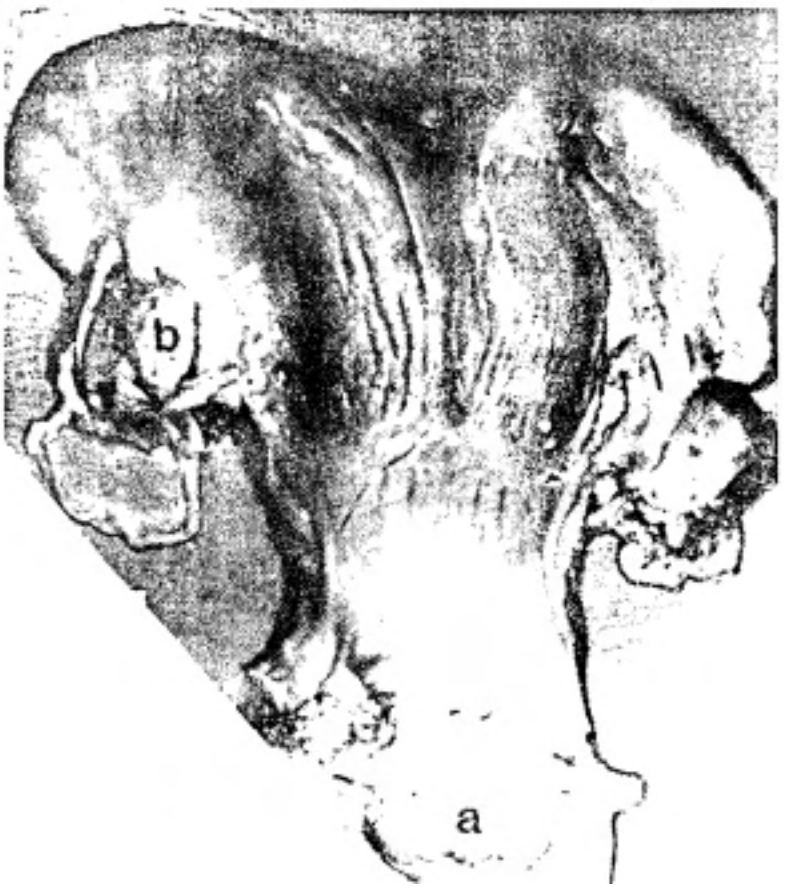


Figure 35. Photograph of the reproductive tract showing cervical entrance (a) and ovary (b).

separation and collection fluid subsequently being absorbed into the tissue.

- Once your are confident that cuff placement and inflation are correct, place a hemostat ahead of the catheter inflation valve at point "d" (Figure 27). With the hemostat in place, the assistant is free to disconnect the air syringe from the inflation plug.
- Remove the hemostat which holds the catheter to the stylet.
- While rotating the stylet, the assistant should gently pull the stylet out of the catheter, being careful not to dislodge the cuff from its position in the uterine horn. Once removed,

the stylet should be placed inside the empty catheter packet on the instrument table.

- To counteract downward pull on the catheter which could dislodge the cuff, loop a closed hemostat through one finger–ring of the hemostat clamping off the inflation valve, and then clamp it to the donor's hair coat (Figure 37).
- Remove the tape from Y–junction tubing end (c), and then plug end (c) into the catheter.
- You are now ready to introduce PBS into the uterus. The objective is to repeatedly fill and empty the right horn, remove the catheter and relocate it into the left horn, and then use the remaining 500 ml of PBS similarly in the left uterine horn.
- In order to properly monitor horn filling and emptying, it is crucial that the uterine horn is manipulated so that a majority of the horn lies in the palm of your hand. This permits sensitive finger–tip palpation of the small diameter anterior end of the horn (Figure 38), where embryos are usually located 7 days after estrus.

  Once you arc comfortable with the position of the horn, open clamp #2 which allows PBS to enter the uterus. Horn fill/expansion is constantly monitored by gently touching and tapping the entire length of the horn while it fills. Close clamp #2 as soon as the horn is full. At this point, the horn will feel hard, firm, and turgid (Figure 39).

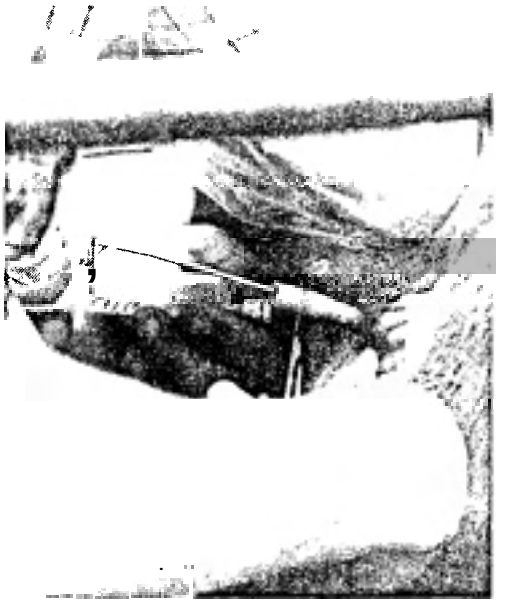


Figure 36. Clamping the air inlet valve closed prior to removing the inflation syringe.

Figure 37. The hemostat clamped to the inflation valve is attached to the donor's hair coat to minimize downward pull on the cath–eter.

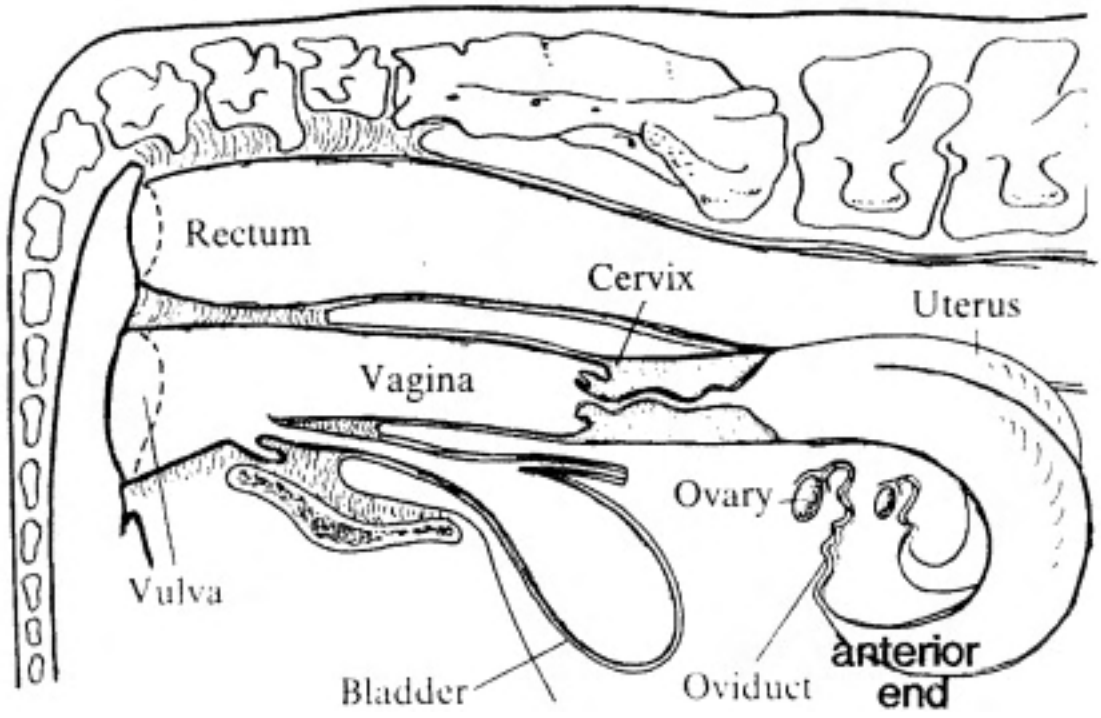


Figure 38. Right side diagram of the reproductive organs within the cow.

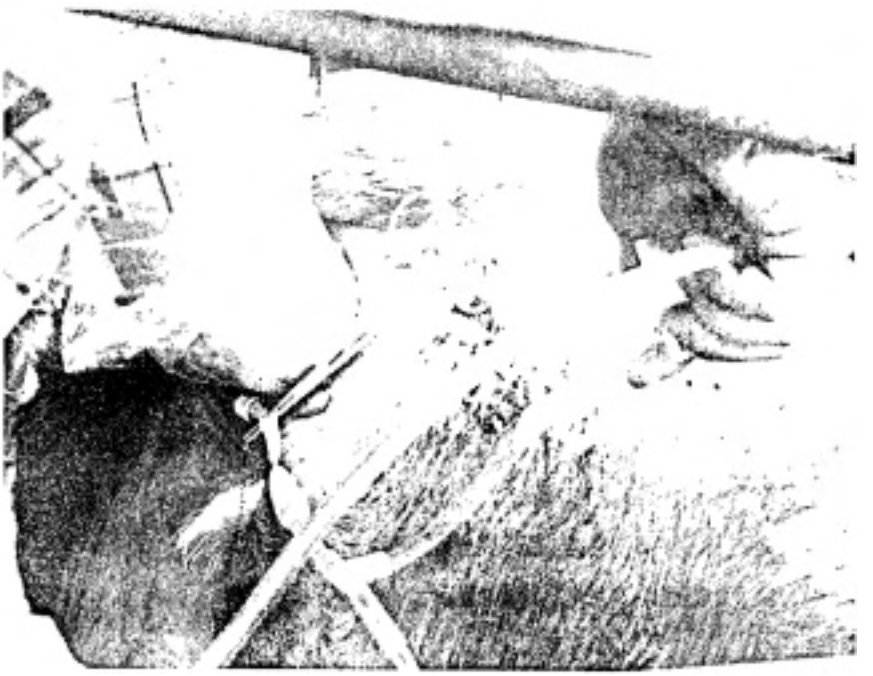


Figure 39. The right 'nand clamp will pinch the PBS inflow tube closed once the left hand palpates a full, turgid uterine horn.

Immediately open clamps #1 and *#3,* which allows *PBS* to leave the uterus and pass through the Em–Con filter (Figure 40). Once you can visualize PBS entering the filter, begin gently massaging (kneading) PBS out of the horn, starting at the anterior tip of the horn and working toward the inflated catheter cuff. The horn is considered empty once it regains a soft, flaccid feel.

NOTE: Do not massage the inflated balloon.

Close clamps #1 and *#3,* open clamp *#2,* and repeat the filling/massaging process. The horn gradually expands with each subsequent fill. Usually the horn can be filled/emptied 4 to 6 times, which leaves approximately 500 ml of PRS for the remaining left uterine horn.

- It is important to visualize fluid entering and leaving the filter during the kneading process, since this indicates *when*

and *how strong* to massage, or if a blockage has occurred in the outflow. An assistant can position the filter in your line-of-sight and also regulate the PBS level in the filter by alternately adjusting clamp #3 and/or the filter lid.

Figure 40. Uterine outflow is monitored by opening/closing the bottom clamp.

- An apparent out-flow blockage can usually be corrected by one or a combination of the following techniques/points:

  a) Check that clamps #1 and #3 are open.
  b) Straighten (uncoil) the uterine horn.
  c) Gently tap/massage the horn immediately anterior to the inflated cuff.
  d) With the horn straightened, grasp the catheter by the air inlet valve and gently pull backward (toward your body) while bouncing the uterine horn.
  e) Attach the air syringe and remove 1 to 2 ml of air from the balloon.
  f) If all else fails, deflate and reset the balloon.

- When approximately 500 ml of PBS remains in the suspended bag, the cuff should be deflated by attaching the air syringe to the catheter inflation valve and pulling back on the plunger. An alternate method is simply to press a pointed object into the catheter inflation valve. Clamps #2 and #3 should be closed and the filter lid seal slightly broken at this time.

Grasping the catheter at point "b", pull the catheter out of the donor. Allow the PBS in tubing arm (a) to drain into the filter, and then securely seal the filter lid to the filter. Separate Y-junction tubing end (c) from the catheter, insert the sterile stylet into the catheter and clamp with a hemostat.

NOTE: Be extremely careful handling the catheter outside the uterus; it must not contact any object other than the sterile stylet!

- Position the catheter into the left uterine horn and proceed as you did with the right horn.
- When no PBS remains in the suspended bag, remove the catheter and drain all remaining PBS in the tubing into the filter. After checking that the filter lid is secure and that clamp #3 is closed, detach the tubing from the filter lid (scissors map be required). Immediately transport the filter to a secure upright position adjacent to the microscope.
- Two things must still be done before the donor can be released:
  a) Pull on a clean sleeve, remove the infusion rod with attached 50 ml syringe from its clean holding packet, manipulate the rod through the cervix and infuse 20 ml of 0.2% nitrofurazone solution or 0.5% chlorhexidine solution into each uterine horn (Figure 41).
  b) Inject the donor with 4 ml of Estrumate.

- Release the donor and let her lie down until she gets her "feet back under her." The epidural anesthesia occasionally imparts a numbing influence on the hind legs, which will subside after 2 to 3 hours.
- Place all expended collection supplies into a trash receptacle (catheter, syringes and needles, infusion rod, Y-tubing and PBS bag, palpation sleeves, etc.).

Figure 41. Infusing the uterus with nitrofurazone after collection.

SUMMARY OF EMBRYO COLLECTION

1. Identify and record ovarian superovulatory response.
2. Position and inflate the catheter.
3. Flush right horn, reposition, flush left horn.
4. Place filter in a safe position next to the microscope.
5. Infuse and inject donor.
6. *Untie* ***tail*** and release the donor


المنتدى البيطري
DVD4arab
Dr.Stator®


المنتدى البيطري
DVD4arab
Distator®

- Place three petri dishes (10 x 35 mm) on the slide warmer. Using a permanent marking pen, label the dish lids A, B, and C, respectively (Figure 42). Draw 10 ml of holding medium into a sterile 20 ml Air–Tite syringe, and then plug an Acro–Disc syringe filter directly onto the syringe tip. The filter must remain in its clear plastic case when not in use to prevent contamination. Lift the syringe/filter unit from the filter case and apply pressure to the plunger. Discard the first 6 to 8 drops of PBS. Holding the syringe at a 45° angle so that only the syringe filter opening is directly over the dish, fill petri dishes A, B, and C to 60% capacity.

  NOTE: If more than one donor is being collected, write the donor's name on the lid of each set of three petri dishes and search plate assigned to that donor.
- Attach the hub of a sterile IVF catheter to the tip of a 1 ml tuberculin syringe (Figures 43 and 44). This unit will be

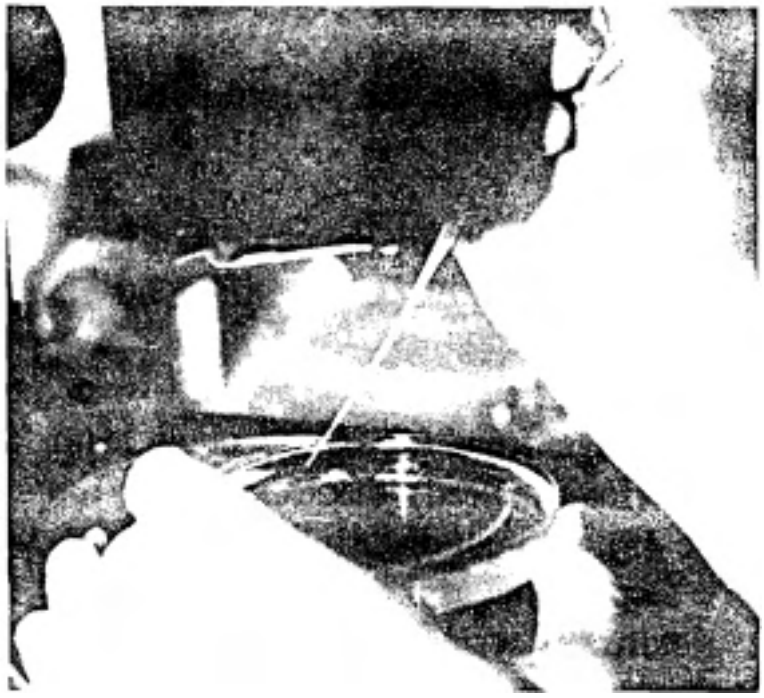


Figure 43. Aspirating an embryo from the search plate.

used for extracting and handling embryos. Place the syringe on the slide warmer so that the catheter extends over the edge of the warmer and points in a direction where it will *not contact anything.*

Figure 44. The embryo aspirated in Figure 43 is held in the IVF catheter between the two arrows,

- You are now ready to transfer the Em–Con filter PBS into the search plate.

  a) Open the plunger–end of a 10 ml Air–Tite syringe packet but do not remove the syringe.

  b) Remove the cap from the 100 ml bottle of PBS that was just prepared.

  c) Using a paper towel, dry the outside of the Em–Con filter. Gently remove and discard the filter lid.

  d) Holding the filter at eye–level with one hand and the 10 ml syringe from (a) in the other hand, aspirate the bubbles (foamy head) that remain on top of the PBS (Figure 45). Do not disturb the remaining solution in the filter! Deposit the aspirated bubbles into the search plate lid. Return the syringe to its packet.

Figure 45. Aspirating bubbles off the top of the PBS within the Em–Con filter.

Figure 46. PBS has been drained off, leaving 1.5 cm of fluid in the filter.

e) Open clamp #3 and drain off PBS until approximately 1.5 cm of fluid remains in the filter (Figure 46). Close clamp #3.

f) Gently rotate the filter, causing the PBS to swirl, and then dump the filter contents into the search plate (Figure 47). Immediately pour an equal amount of rinse PBS (Figure 48) directly from the 100 ml bottle into the filter. Holding the filter at eye level, submerge the 10 ml syringe tip into the PBS and slowly aspirate mucus/debris off of the

filter screen (Figures 49 and 50). Add the PBS in the syringe to the search plate. Pour a small amount of rinse PBS into the filter, swirl, and dump into the search platc. Repeat. The Em–Con filter and syringe may now be discarded.

Figure 47. Filter contents are dumped into a Quebec search plate.

Using both hands, carefully place the search plate onto the microscope stage. Embryos will settle to the bottom of the plate within one minute, so begin your systematic scarch by: a) adjusting the magnification knob to 1.5, and b) focusing on a piece of debris on the bottom of the platc.

From this point on the entire plate can be searched without significantly altering magnification or focus. Only when the plate contains mucus and debris suspended in solution will you need to focus up and down.

Using both hands, slide the plate on the stage so that the search begins with an upper lcft "corner". Concentrate on what you are looking for: a clear, "shiny" circle. Examine all parts of the grid in the field of view, then slide the plate lcft until the adjacent grid is exposed. Continue sliding the search plate lcft until all grids in the top row have been examined. When you reach the end of the top row, slide the plate up, exposing the row of grids directly below the top row. This row is searched by sliding the plate from lcft to right. Continue down the plate until all grids have been examined (Figure 51).

Figure 48. Pouring rinse PBS into the Em–Con filter.

Figure 49. Using a syringe to aspirate mucus/debris directly off of the filter screen.

Next, slowly rotate the plate 360° while carefully examining the junction between plate sidewall and plate bottom. This area is difficult to get in focus and may require focus adjustment as you encircle the plate.

After thoroughly searching all grids, use the IVF catheter to gently stir and mix the plate contents. Let the solution contents settle for 1 to 2 minutes, and then search the entire plate once again.

Occasionally the PBS will contain mucus and/or a large amount of uterine tissue debris. These inclusions are normal, yet they make embryo isolation and extraction more challenging. Paticncc and persistence are appropriate at this time! A sterile needle or

IVF catheter should be used as you search each grid to overturn, stretch, and rearrange the debris. Embryos can be found enveloped in debris, as well as stuck to mucus. You may have to pin down the debris or mucus and then excise the embryo, using two sterile 20 ga. x 1" needles.

Figure 50. Depositing aspirated mucus/debris into the search plate.

When an embryo is located:

1. Note which direction you are searching (right or left) to avoid duplicating or missing grids when the search resumes.

2. Grasp the 1 ml TB syringe with attached IVF catheter and pull the plunger back 0.2 ml. This air space will expel the embryo from the catheter.

3. Place the cathcter tip into petri dish C and aspirate PBS into the cathcter. PBS should extend from the catheter tip to where the wide section of the cathcter hub begins (Figure 44). *PBS should NEVER enter the TB syringe!*

4. With one hand on the plate and the TB syringe in the other hand—while looking through the microscope—carefully maneuver the catheter tip adjacent to the embryo. Slow,

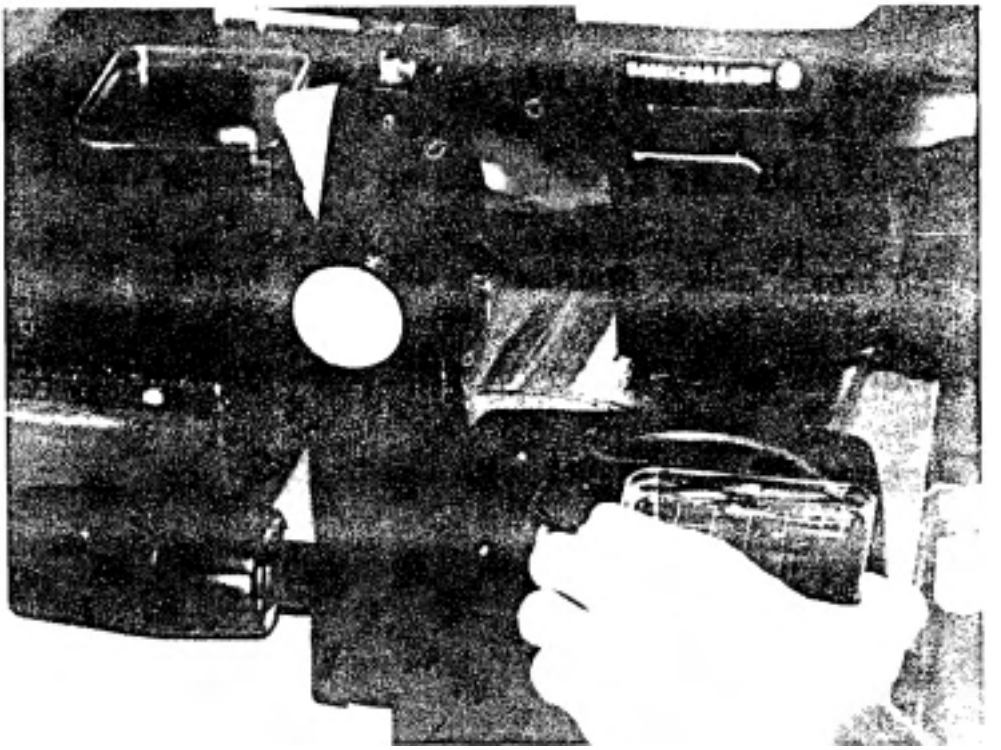


Figure 51. Searching for embryos.

controlled, one–handed aspiration will gently "walk" the embryo into the catheter tip. While maintaining slight back–pressure on the syringe, lift the syringe out of the plate. Place the catheter tip directly above (but not touching) the surface of the appropriate petri dish. Slowly expel all solution in the catheter into the center of the dish.

Petri dish A is for embryos initially identified as transferable or freezable.

Petri dish B is for nonfertilized ova and degenerate embryos.

Embryos from petri dish A should be transferred into petri dish C for final evaluation when the search is concluded.

SUMMARY OF EMBRYO HANDLING

1. Refer to Appendix E for evaluating and grading embryos.
2. Wash hands, adjust microscope and slide warmer.
3. Prepare 100 ml of PBS for filter rinse/embryo holding medium.
4. Label and fill petri dishes with sterile PBS.
5. Aspirate bubbles from top of PBS in Em–Con filter.
6. Drain excess PBS from filter, leaving 1.5 cm of PBS on top of the filter screen.
7. Swirl and transfer filter contents into Quebec search plate. Rinse filter.
8. Search the Quebec plate twice. Each time an embryo/ovum is found, transfer it into its respective petri dish (A or B).

CHAPTER 6

Embryo Freezing

The process of freezing embryos resembles the entire ET sequence in that numerous steps are involved. Attention to detail is very important. All preparations and procedures for freezing must strive to minimize the time span between collection and freezing, with highest pregnancy rates achieved when embryos are frozen within two hours of collection!

(6.1) EQUIPMENT AND SOLUTIONS

There are numerous dependable portable embryo freezing machines available today. The unit that has served the author well during the past 10 years is Cryoembryo PSP, built by the Hoxan Corp. Recently a more compact/portable freezer has been introduced from Australia (Freeze Control CL-856) which is competitively priced and is receiving excellent performance reviews.

The ET process on a particular collection day begins with recipient examination, followed by equipment set-up and embryo recovery. Assuming superovulation and collection have been successful, decisions must then be made concerning embryos in petri dish C (transferable/freezable embryos). For example, if recipient palpation earlier in the day identified eight useable recipients and petri dish C contained five transferable embryos, then all five embryos could be transferred. Three recipients would not receive an embryo.

However, if there are five useable recipients and eight embryos in petri dish C, then three embryos must be frozen or discarded.

In this chapter, we shall proceed to freeze three embryos!

Section 4.2 discussed setting up and test-operating the programmable embryo freezing machine prior to embryo collection.

At this time the Cryoembryo PSP liquid nitrogen reservoir should be filled to capacity (approximately 3 liters) by slowly unscrewing the nitrogen inlet cap. Stop unscrewing when tank pressure starts to escape (hiss) from the loose cap. Once all pressure has escaped, the cap should be removed completely.

Make a funnel filter by gently pressing a clean paper towel into the funnel mouth (Figure 18). Insert the filter–covered funnel into the nitrogen inlet and slowly fill the reservoir by pouring nitrogen from a bulk 10 liter (or similar manageable size) nitrogen tank. When full, remove the funnel and screw the inlet cap on tightly. Press the power switch to activate the freezer. Press M1 (memory #1) and verify the pre–programmed freeze steps. The freeze progression schedule should read (Figure 19):

Initial temperature	0.0 (°C)
Rate 1	2.0 (°C per minute)
Point 1	–6.0 (°C)
Keep time	10.0 (minutes)
Rate 2	0.5
Point 2	–30.0
Keep time	0.0
Rate 3	0.3
Point 3	–35.0
Keep time	0.0
Rate 4	0.3
Final temperature	–35.0

Note: This is a standard, proven cooling progression for freezing cattle embryos; however, slight variations are possible that yield equally good results.

If the display data is correct, press the power button again to turn off the freezer. It will be restarted once active freeze medium is prepared and embryos have been loaded into straws.

Immediately after verifying the freeze progression data, *active* embryo freeze medium should be prepared according to the following format:

a. Using a 16 ga. x 1" needle attached to a 2.5 ml syringe (Air–Tite), pierce the rubber stopper on a 10 ml Vacutainer tube which contains glycerol. Invert the Vacutainer tube and withdraw approximately 2.2 ml of glycerol. Remove the syringe/needle from the Vacutainer tube. Hold the syringe so the needle is pointing upward, which allows air bubbles trapped in the syringe to move toward the needle/syringe attachment point. While holding a sterile 2" x 2" gauze around the needle hub, push the syringe plunger upward, discharging air bubbles and glycerol. Stop when there is *exactly* 2 *ml* of glycerol remaining in the syringe. Use gauze to wipe the needle dry and then recap the needle.

b. Draw 20 ml of embryo freeze medium (Figure 52) into a sterile, 20 ml Air–Tite syringe (16 ga. needle). While holding the syringe upright, depress the plunger to discard 2 ml of medium. Stop when *exactly* IS *ml* of freeze medium remains in the syringe, and then create an air space in the springe by pulling the plunger back 1 cm beyond the 20 ml mark. Recap the needle, and then twist and remove the needle from the syringe.

c. Uncap the needle on the syringe containing 2 ml of glycerol. Combine glycerol and freeze medium by sliding the needle on the 2.5 ml glycerol syringe into the needle attachment channel of the freeze medium syringe (held vertically, plunger down) and inject all 2 ml of glycerol into the medium syringe.

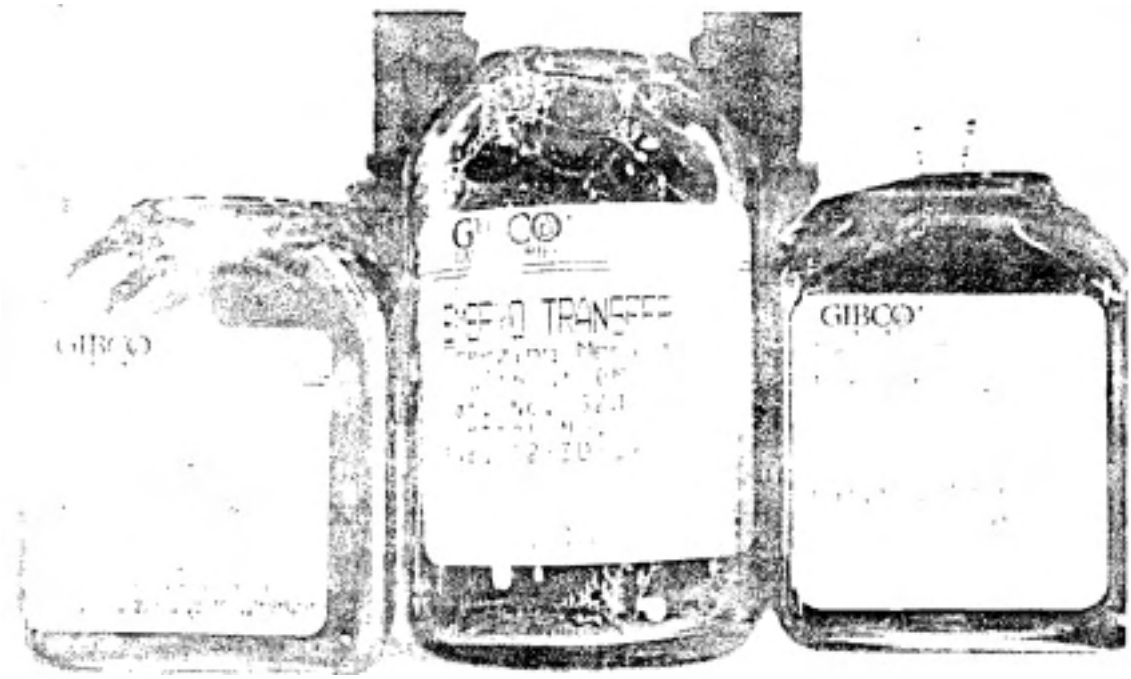


Figure 52. Dulbecco's PBS without magnesium chloride or calcium chloride (left). ET freezing medium (center), and 0.25% trypsin.

Replace the covered needle onto the freeze medium syringe, gently shake the syringe for several minutes, and then label the syringe "active" freeze medium.

Note: Freezing medium containing 10% glycerol is available commercially in vaccine–stoppered 20 ml vials.

SUMMARY OF EQUIPMENT AND SOLUTIONS

1. Use a funnel with filter to fill the liquid nitrogen reservoir.

2. Activate freezer and verify the freeze program.

3. Combine freeze medium with glycerol.

(6.2) EMBRYO SELECTION, WASHING AND FREEZING

Selection

Stage of development, embryo quality, and integrity of the zona pellucida (clear shell surrounding the embryonic mass) will determine if an embryo in petri dish C should be frozen.

a. Embryos at developmental stage No. 4 (morula), No. 5 (early blastocyst), or No. 6 (blastocyst) will yield the highest frozen/thawed pregnancy rate. There is no difference in pregnancy rate among stage of development.

b. Embryo quality should be grade No. 1 (excellent), particularly if the embryos are for export. Grade No. 2 embryos can survive freezing/thawing, yet pregnancy rate is typically reduced.

c. Since the zona pellucida is an effective pathogen barrier, it is essential that all embryos to be frozen have a zona pellucida that is intact (not cracked) and has no extraneous material attached to it.

Microbiological studies have shown the zona pellucida to be an excellent barrier to most bovine diseases. Thorough washing of embryos enclosed in intact zonae pellucidae will completely remove all traces of most pathogenic agents. Therefore it is strongly advised by the International Embryo Transfer Society (IETS), and required by importing countries, that all embryos be washed in a minimum of ten separate baths prior to exposure to active freeze medium.

Washing

The washing procedure recommended by the IETS requires transferring embryos, in groups of ten or fewer, through ten changes of PBS. A new, sterile pipette (IVF catheter) must be used to transfer embryos to each succcssivc wash.

NOTE: To maximize pathogen dilution between washes, aspirate embryos into the catheter with the least amount of solution possible.

Embryos must be gently agitated in each wash prior to moving to the next wash, and only embryos from the same donor may be washed together. The washing procedure can be carried out in ten separate petri dishes, or in a multi–well plate having at lcast ten wells.

A convenient wash method is to fill a sterile 30 ml Air–Tite syringe with 30 ml of embryo freeze medium (*NOT ACTIVE,* NO GL*YCEROL*). Using sterile technique, attach an Acro–Disc filter to the syringe tip. Apply pressure to the plunger and discard the first 6 to 7 medium drops out of the Acro–Disc, and then proceed to expel 2 ml of medium into each of 10 petri dishes/wells. Label each dish/well (1 through 10) with a permanent marker.

To ensure the integrity of the zona pellucida, each embryo must be examined over its entire surface area at not less than 50× magnification. Embryos are gently rolled in the dish so that all surfaces of the zona pellucida can be examined for cracks. This evaluation should take place *after the ten washes* and *before embryo freezing!*

Presently, only a few viral pathogens, i.e., infectious bovine rhinotracheitis virus (IBRV) and vesicular stomatitis virus (VSV) have been shown to adhere to the zona pellucida so firmly that the normal washing procedure will not remove them. Embryos exposed to these two viruses require treatment with trypsin to make them noninfectious.

If the importing country specifies IBRV and VSV free embryos, the following procedure should be followed for the combined washing and trypsin treatment of embryos:

a. Label five dishes 1 through 5, label two more dishes as T1 and T2, and finish with five dishes labelled 6 through 10 (a total of 12 separate washes). A 12-well plate can be used in place of 12 single dishes.

b. Prepare *pre-trypsin* PBS by adding 0.4 g bovine serum albumin (BSA, Figure 15) powder to a 100 ml bottle of PBS that does not contain calcium or magnesium (Figure 52). Recap the bottle and swirl gently until all BSA has dissolved.

c. Fill a sterile 20 ml Air-Tite syringe with 20 ml of pre-trypsin PBS. Label the syringe "pre-T." Fill a sterile 5 ml Air-Tite syringe with 5 ml of 0.25% trypsin solution (Figure 52). Label the syringe "T." Fill a sterile 20 ml Air-Tite syringe with 20 ml of embryo holding medium (prepared in section 5.2).

d. Using sterile technique, attach an Acro-Disc filter to each of the three syringes just prepared. Depress their plungers to discard the first several drops from each syringe.

e. Place 2 ml of "pre-T" medium into each of dishes 1 through 5. Place 2 ml of trypsin solution into dish T1 and T2. Place 2 ml of embryo holding medium into each of dishes 6 through 10.

f. Starting with dish No. 1 and using a new, sterile IVF catheter for each wash (as described earlier for washing embryos). sequentially pass the embryos through:

   Dishes 1 through 5 (1 to 3 minutes exposure time per dish)

Dish T1 (30 seconds exposure only), then T2 (30 seconds only).

Dishes 6 through 10 (1 to 2 minutes exposure per dish).

After all 10 sequential washes have been completed (or 12 if trypsin was required), embryos which still meet the developmental stage and quality criteria and have an intact, clean zona pellucida (under 50× magnification) may be frozen. For this example we shall assume that all three embryos are still acceptable, and proceed with the following sequence for freezing embryos.

Freezing

a. Peel back the protective plastic cover from the upper half of three double-length straws. With an extra-fine "Sharpie" permanent marker, label the upper half of each straw (Figure 53) according to IETS guidelines detailed in section 6.3.

Figure 53. The upper half of a freeze straw, labeled according to IETS guidelines.

With three embryos it is convenient to package them singly per 112 ml double-length (or 1/4 ml) straw. If there were 15 embryos to freeze, it would be appropriate to package them two or three per straw. The double-length straw allows ample space to print pertinent donor/sire information on the "upper half" of the straw. This half also acts as a handle for safely

identifying straws stored in nitrogen without having to raise the lower embryo–containing half of the straw out of nitrogen.

b. Place three petri dishes on the slide warmer; label them "Glycerol–1", "Glycerol–2", and the third dish "Glpcercl–3". Remove the needle from the syringe containing active freeze medium (prepared in section 6.1) and in its place attach an Acro–Disc syringe filter. Depress the syringe plunger to discard the first 4 to 6 drops of active freeze medium, and then fill all three labelled petri dishes to 60% of capacity.

c. Activate the freeze unit by pushing the power button, and then press M1. Reverify the program that appears on the screen, press "yes", and then the temperature in the freeze chamber will proceed toward its 0° initial temperature.

d. Place the petri dish containing the three washed embryos that are to be frozen onto the microscope stage. Attach a new, sterile IVF catheter to the 1 ml TB springe used for manipulating embryos. Pull the syringe plunger back 0.2 ml, insert the catheter tip into the dish and, under low magnification, locate and move all three embryos to one location in the center of the dish.

e. Aspirate all three embryos at one time into the IVF catheter, carrying a **very** *minimum amount* of *PBS* along with the embryos. Lift the catheter out of the dish and then pull the plunger back just enough to create an air space in the catheter tip.

f. While holding the IVF catheter in a horizontal position, remove the PBS dish from the stage and replace it with petri dish "glycerol–1". Remove the lid, place the catheter into solution, and focus on the catheter tip resting on the bottom of the dish.

Slowly expel and visualize all three embryos as they exit the catheter, rise to the surface, and finally settle on the bottom of the dish. Using the catheter tip, gently mix the expelled embryos/PBS with the glycerol freeze medium.

After two minutes, group all three embryos in the center of the dish and aspirate into the catheter. Remove "glycerol–1" and place "glycerol–2" on the microscope stage. Transfer the embryos into "glycerol–2" and mix gently. After 2 minutes, orient the embryos within the dish, allowing space between embryos for final individual grading and straw loading. Select the first embryo to be loaded by *moving the dish* so that this embryo is centered in the field of view. Evaluate the embryo under high magnification by recording stage of development and embryo quality on certificate C (section 6.3). Return to low magnification and refocus!

h. Snugly insert the labelled end of straw No. 1 (labelling detailed in section 6.3) into the open end of an Acro–Disc filter which has been mated to a 3 ml Monoject syringe. Pull the plunger back 0.5 ml (Figure 54).

Figure 54. Labelled end of a freeze straw inserted into an Acro-disc filter, which is mated to a 3 ml syringe.

Rinse the straw by placing its lower (unlabelled) end into "glycerol–3" and aspirating a 2 cm column of fluid. Raise the straw out of solution and pull the fluid to within 1 cm of the

white cotton plug. Depress the plunger slowly to discard the active freeze medium. Repeat the rinse procedure once more. The straw is now ready to be loaded with the following sequence of solution and air:

- Aspirate 1 cm of "glycerol–3", and then raise the straw out of solution.
- Continue gentle back (negative) pressure on the plunger with thumb and index finger until a 1 cm air space has appeared at the end of the straw.
- Lower the straw and draw up 3 cm of "glycerol–3". Raise the straw out of solution.
- Add a 1 cm air space.
- Lower the straw into "glycerol–2", adjacent to the first embryo to be loaded. Aspirate 1 cm of fluid, gently bump the embryo with the straw tip to float the embryo off the bottom of the dish, and while floating aspirate the embryo into the straw along with an additional 2 cm of fluid.
- Raise the straw and add a 1 cm air space.
- Lower the straw into "glycerol–3" and aspirate 1 cm of solution. Lift the straw out of solution and hold in a horizontal position. Draw all segments of solution and air toward the cotton plug in the center of the straw. Stop negative plunger pressure once the plug is saturated with solution.
- Lay the loaded straw on the microscope stage and focus down to verify that the embryo has been isolated in the proper segment of active freeze medium (Figure 55). After verification of embryo inclusion and air/fluid segmentation, wipe excess fluid from the straw tip with sterile gauze. Maintain the straw in a horizontal position on the slide warmer until all straws have been loaded and are ready for sealing.

Figure 55. Labelled, loaded straw ready to be placed into the freeze chamber. The arrow indicates the fluid section containing the embryo.

i. Once all three straws have been loaded, the unlabelled end of each straw (the end containing the embryo) must be sealed. Plug in the heat sealer (Figure 56) and adjust the impulse duration control knob to approximately six.

NOTE: From this point on, loaded straws must be handled very *gently* to avoid dislodging the embryo from its designated fluid segment.

Figure 56. "**Quick Seal**" **heat sealer. The sealing bar is indicated by "a".**

Grasp the middle of a loaded straw between thumb and index finger. Holding the straw in a horizontal position, rest the end to be sealed on top of the sealing bar. With your free hand, press and hold the handle down to flatten

and seal the straw end. Slowly release handle pressure two seconds after the red indicator light goes out. Roll the straw 180° between thumb and index finger and repeat the thermal impulse. With the straw still in a horizontal position, place the sealed tip on the microscope stage and verify the integrity of the seal under high magnification. The sealed straw may now be placed in a vertical position into an unoccupied freezing slot in the Cryoembryo liquid nitrogen chamber (Figure 57). Slowly lower the straw into the slot until the sealed tip contacts the bottom of the slot chamber. Seal and place the remaining two straws into the freeze chamber.

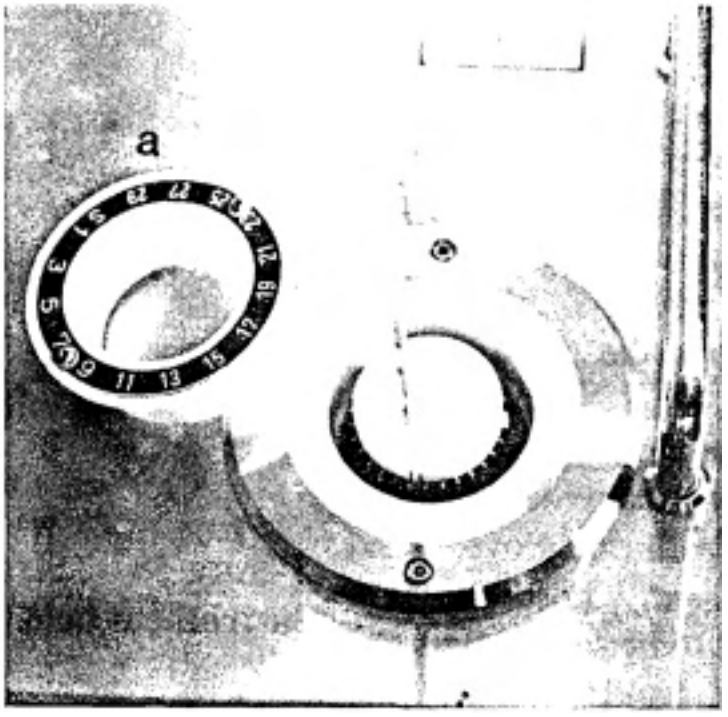


Figure 57. A sealed straw inserted into a freeze chamber slot. The straw holding ring is indicated by "a".

j. Secure the straws in place with the straw holding ring (Figure 57) and then press "yes" on the control panel to initiate temperature drop from 0 to −6° C.

k. In order to initiate ice crystal formation within the straw solution (and thus avoid harmful supercooling), each straw must be manually "seeded" during the programmed 10−minute hold which occurs at −6°.

During the temperature drop from 0 to −6°, pour a small amount of nitrogen into the insulated cup containing the seeding instrument (tweezers work well for straw seeding, Figure 58). Once the temperature reaches −6°, each straw must be seeded according to the following procedure:

Figure 58. Straw seeding instrument held in nitrogen.

- Remove the straw holding ring.
- Simultaneously remove the seeding instrument from its bath with one gloved hand and gently raise the first straw to be seeded with the other hand. Raise the straw from the freeze chamber just high enough to expose the top part of the fluid segment containing the embryo. Being careful not to crush the straw, immediately touch the seeding instrument to the straw wall surrounding the uppermost part of the embryo containing segment. Maintain contact for 3 seconds; release the seeding instrument and gently replace the straw down into its freezing slot.

Continue to seed the remaining two straws, allowing the seeding instrument to cool in the nitrogen bath for 20 to 30 seconds between seedings.

NOTE: Seeding has been accomplished when the clear freeze solution in the seeded segment turns cloudy white, indicating that crystallization has taken place.

When the programmed 10–minute hold expires, the temperature drop will automatically resume to –35°, at which point an alarm sounds indicating run completion. Straws should be removed from their freezing slots and immediately plunged into a labelled goblet (snapped to the lower end of a cane) containing liquid nitrogen. After all three straws are in the goblet, an empty goblet is inverted and placed like a hood over the labelled straw ends (Figure 59). The inverted hood snaps onto the cane, thereby holding straws securely in place on the cane.

Figure 59. Double–length ½ ml freezing straw (a), inverted "hood" goblet (b), labelled lower goblet (c), embryo transport tube (d).

SUMMARY OF EMBRYO SELECTION, WASHING, AND FREEZING

1. Select embryos to be frozen according to stage of development, quality, and integrity of zona pellucida.

2. Pass embryos through 10 sequential baths to "wash" pathogens from each embryo's zona pellucida. Some importing countries may require two additional trypsin baths.

3. When the embryo wash sequence is complete, verify the integrity of each zona pellucida at 50× magnification.

4. Decide how many embryos will be packaged per straw, and then label straws according to IETS guidelines.

5. Partition active freeze media into three labelled dishes.

6. Activate the freezer, allowing it to stabilize at 0° C.

7. Place embryos into active freeze media.

8. Load embryos into rinsed, labelled straws. Record pertinent information on certificate C.

9. Seal the unlabelled straw ends and then insert straws into freeze chamber slots.

10. Seed straws at −6°.

11. Label goblets and canes while embryos freeze.

12. When freeze run ends, plunge straws into a nitrogen–filled goblet attached to a labelled cane. Secure labelled straw ends

to the cane with an inverted goblet and then plunge the cane into its assigned canister for storage.

(6.3) CERTIFICATION, IDENTIFICATION, AND LABELLING

This section contains certification and identification guidelines approved by the International Embryo Transfer Society (IETS) and contained in the Manual of *the* International Embryo Transfer Society, 1987. The IETS was formed in 1974 in Denver, Colorado (USA) to serve as a professional forum for the exchange of information, and to further the sciences of animal embryo transfer.

Accurate identification, certification, and record keeping are absolutely essential not only to assure that buyers are receiving the embryos that they contracted to purchase, but also to assure embryo transfer offspring and parentage, to correlate embryos with correct health certificates, and to ensure a minimum of confusion when embryos are passed from one technician to another or from one country to another.

Some breed associations may require blood typing, certification, or registration of embryo transfer personnel, plus advance permission to propagate a given dam and sire by embryo transfer. The IETS recommends, as a minimum, that the donor dam and sire(s) be blood typed prior to embryo collection.

Certification/Registration

Three forms for certification of embryo recovery, freezing, and transfer (certificates A, B, and C) as well as for registration of offspring are recommended by the IETS. Whenever transferrable embryos are collected from a donor, certificate A (Embryo Recovery) should be completed and signed. When embryos are transferred (fresh or frozen), certificate B (Embryo Transfer) should

be completed and again signed by the person performing the transfers. Finally, when embryos are frozen, certificate C (Embryo Freezing) should be completed and signed by the person who froze the embryos. Certificate D (Embryo Exports) should be submitted to the appropriate breed registry in the exporting country when embryos are to be exported. Specific instructions for completing forms are included on the back of each certificate.

Certificates A and C (Embryo Collection and Embryo Freezing) should be completed by the technician for each group of embryos frozen per donor per collection. One copy of this combined form should be given to the owner of the embryo(s), one retained by the ET technician, and one sent to the appropriate breed registry. A copy of this A–C form must accompany the movement of any embryo(s) from each collection. Information on the form must correspond to the identification of each embryo in any straw or ampule.

For export purposes, it is recommended that copies of all official donor health tests be attached to the A–C form for permanent storage and reference.

A standardized code describing embryo development stages and their quality is included on the back of each certificate. The code for stage of development is based on embryo age expressed as the number of days after the donor was first observed in standing heat (heat = day 0). The quality code is also numerical, and is based on morphological integrity plus the likelihood that transferring the embryo will result in a pregnancy.

Straw Identification

The IETS recommends a standardized system for identifying containers of embryos. Essential information that must be printed in permanent ink on the upper half of each straw consists of:

1. The identification code of the person or company freezing the embryo, the breed code and donor's registration number.

   a. Person/company code. This identifies the person or company that collected and froze the embryos. For all technicians or companies throughout the world, a three-digit code preceded by "E" and starting at **500** will be assigned (for a nominal fee) by the IETS, e.g., E656.

   b. Breed code. A standardized two-letter coding system for identification of breeds is included on the back of each certificate. The breed code should follow the person/company code number, e.g., E656HO.

   c. Donor code. This consists of the donor registration number and should follow the person/company code and breed code, e.g., E656HO7947107. The donor's name is an optional addition to the straw label. The IETS recommends that the bull registration number not be recorded directly on the straw. However, if the sire's registration number is used, it should appear after the donor's registration number.

2. Freeze date. This consists of two numerals for year, two letters for month, and two numerals fur day, in that order, e.g., 89JA06 (1989 January **06).** A standardized coding system for months is included on the back of each certificate. *The freeze date* is essential for matching labelling with the **A-C** certificate, especially if embryos are collected from a donor on more than one occasion.

3. Straw number, and number of embryos per straw. The straw identification number consists of one or more numerals, and is necessary to distinguish between all straws packaged for a donor on a specific day. For example, straw #1 might contain

two excellent blnstocysts, while straw #2 has only a single grade 3 morula, all from the same collection. In addition, each straw should indicate the number of embryos contained, e.g., 1 EMB.

The IETS recommends that straw labelling information be printed on two lines. For example:

E656HO7947107	HO43421	(line 1)
89JA06	2–1 EMB	(line 2)

Explanation:

E656	Person/company code
HO	Breed code for Holstein
7947107	Registration no. of donor
HO43421	Registration no. of Holstein bull that donor was bred to (optional)
89JA06	Date of embryo collection/freezing
2–1 EMB	Straw no. 2, containing one embryo

Labelling Goblets and Canes

Proper labelling of goblets and canes is very important.

The *lower goblet* on each cane should be labelled (Figure 59) with:

1. The IETS code assigned to the person freezing the embryos.

2. The date of freezing.

3. The full registered name and number of both donor and bull she was bred to.

The head of each cane should be identified with:

1. A sequential cane number.
2. A freeze code assigned to the person freezing the embryos.
3. A donor breed code.

The *side* of each cane must show:

1. The cane number.
2. The full registered name of the donor and bull she was bred to.
3. The number of embryos in the goblet.

Furthermore, when canes are repackaged for export, they should retain the same cane number as indicated on certificate C, with the addition of a letter indicating that the cane has been repackaged, e.g., cane 1a.

SUMMARY OF CERTIFICATION, IDENTIFICATION, AND LABELLING

1. When embryos are collected, complete certificate A.

2. When embryos are transferred, complete certificate B.

3. When embryos arc frozen, complete certificate C.

4. When embryos are exported, complete certificate D.

5. Copies of all donor health test results should be attached to the embryo freezing certificate.

6. Certificates A and C must accompany frozen embryos when they are moved between locations.

7. Straws must be labelled, at a minimum, with the code assigned to the freezing party, the breed code, donor registration number, freeze date, straw number, and number of embryos per straw.

8. Goblets and canes must be labelled, at a minimum, with cane number, freezing party code, freeze date, breed code, and registered name and number of donor plus the bull that the donor was bred to.

9. The identification of any embryo in a straw must correlate with information on an A–C certificate.

CHAPTER 7

Embryo Transfer

(7.1) THAWING FROZEN EMBRYOS

Frozen embryos should be transferred immediately after they are passed through the appropriate post–thaw solutions. In order to minimize the time interval between thaw and transfer, the following preparations should be taken on transfer day prior *to* thawing.

1. After verifying on certificate C that the donor was collected 7 days after estrus, examine all recipients which presently are 6, 7, and 8 days after heat. Choose frozen embryo recipients according to criteria described previously in section 4.1.

2. Review certificate C, which correlates with the embryos that are to be thawed. This form identifies cane codes and straw numbers, how many embryos each straw contains, and should specify which nitrogen tank canister the canes are in.

3. Prepare post–thaw stock solutions:

Prior to Transfer Day

Weigh out 9.4 g of sucrose (Figure 60) and transfer into a 10 ml stoppered Vacutainer tube. Sucrose and Vacutainer tubes can be taken to a local hospital or university lab where their personnel can weigh the sucrose on a sensitive, accurate balance.

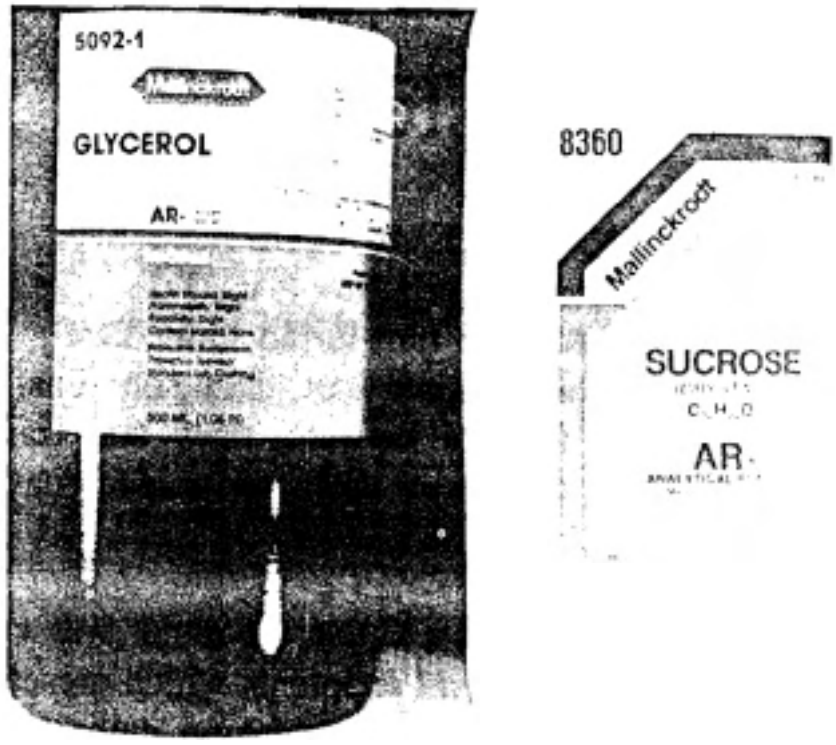


Figure 60. Glycerol (left) and Sucrose.

On Transfer Day

STOCK SOLUTION "**A**"**:**

Prepare 100 ml of embryo holding medium as described in section 5.2.

Attach a 16 ga. x 1" needle onto each of three Air–Tite 20 ml syringes. Using a permanent marker, label each syringe "Media," fill each syringe with exactly 20 ml of embryo holding medium, and then recap each needle. Discard the 40 ml of solution which remains in the 100 ml embryo holding medium bottle, recap the empty bottle and *label it "A".*

Create *stock solution A* in the empty 100 ml medium bottle by combining 9.4 g sucrose with one syringe (20 ml) of embryo holding medium. Dissolve the sucrose by swirling the bottle contents for approximately 5 minutes.

STOCK SOLUTION "B":

Remove the needle cover and press the plunger on the second "Media" syringe to discard 2 ml of solution. Stop when exactly 18 *ml* of medium remains in the syringe. Create an air space in the syringe by pulling the plunger back 1 cm beyond the 20 ml mark.

Using a sterile Air–Tite 2.5 ml syringe and attached 16 ga. x 1" needle, pierce the stopper on a Vacutainer tube containing glycerol and withdraw exactly 2 *ml* of glycerol.

With needle cover in place, twist and remove the needle from the syringe containing 18 ml of medium. Combine glycerol and medium by sliding the needle on the 2.5 ml glycerol syringe into the needle attachment channel of the medium syringe (held vertical1y, plunger down), and injecting all 2 ml of glycerol *into the medium syririge* (Figurc *61)*. Replace the covered needle onto the medium syringe, *change the syringe label to rend "B"*, and then gently shake the springe for several minutes to mix the glycerol and medium.

After thawing, embryos must be exposed to the following solutions in the sequence indicated:

First: TS (thaw solution) #1

This is stock solution "B" in the 20 ml syringe.

Second: TS #2

Attach an 18 ga. x 1.5" needle to a sterile 10 ml Air–Tite syringe. Label the syringe "TS #2".

Starting with the plunger at 0 ml, draw exactly 5 ml of stock solution A into the syringe. Next, insert a 5 ml air space

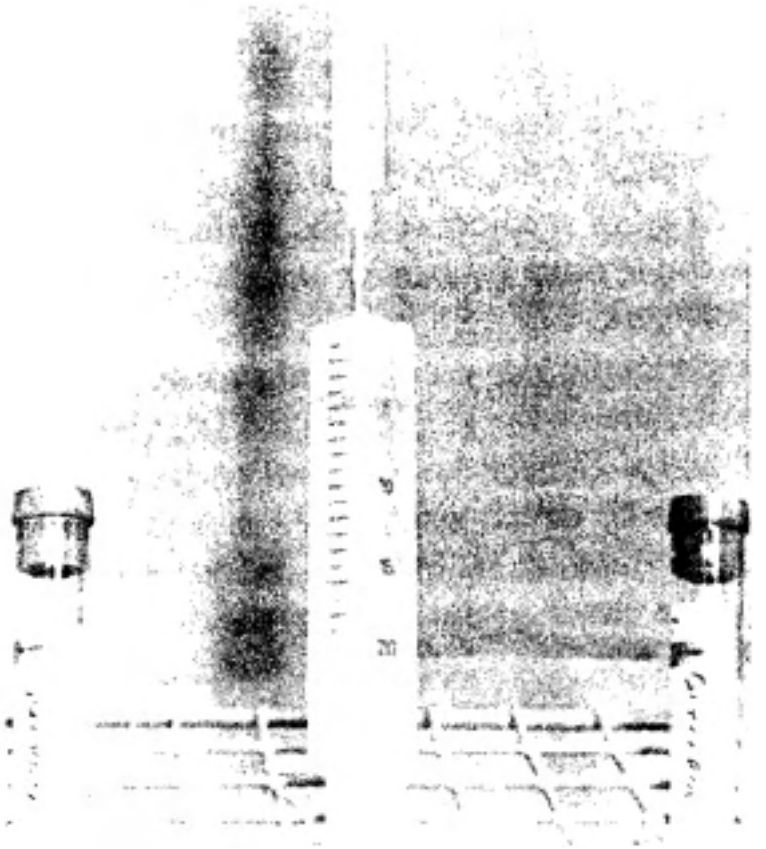


Figure 61. Injecting glycerol into a media syringe to create embryo thaw stock solution "B".

in the syringe by first removing the syringe from stock solution A, and then continuing to pull the plunger back until it is on the 10 ml mark (Figure 62). Cap and then remove the needle from the syringe.

While holding syringe TS #2 vertically (needle attachment channel up), uncap and insert the needle of *stock* solution B into the TS #2 needle channel. Inject stock solution B into TS #2 until the TS #2 syringe is full.

TS #2 is now complete.

Third: TS #3

Attach an 18 ga x 1.5" needle to a sterile 10 ml Air-Tite syringe. Label the syringe "TS #3".

Starting with the plunger at 0 ml, draw up 5 ml of stock solution A and then insert a 5 ml air space (procedure identical to TS #2 syringe preparation).

While holding syringe TS #3 vertically, inject 5 ml of embryo holding medium (from the third 20 ml medium syringe) into TS #3 until syringe TS #3 is full.

TS #3 is now complete.

Figure 62. Thaw solution syringe #2 (TS#2) with its air space that will accommodate 5 ml of stock solution "B".

Note: Embryo thaw kits are available commercially which include the three pre-mixed final solutions (TS 1,2,3) in vaccine stoppered 20 ml vials.

Fourth: TS #4

This is the approximate 15 ml of embryo holding medium which remains in the third medium syringe.

4. Place five petri dishes on tile slide warmer and label their lids 1, 2, 3, 4, and 5, respectively. Label one set of five dishes per donor being thawed.

   Attach an Acro-disc filter to the TS #1 syringe (stock solution B syringe), discard several ml, and then half-fill dish 1 with TS #1.

Attach a new Acro–disc to each of the remaining TS syringes.

After discarding the initial drops which exit each acro–disc filter, proceed to half–fill the remaining petri dishes:

- TS #2 into dish 2
- TS #3 into dish 3
- TS #4 into dishes 4 and 5

5. Straws are thawed for 30 seconds in a 30° C water bath, which should be prepared at this time. Fill the thaw box half–full with hot water and then gradually bring the temperature down to 30° C by slowly adding cold water. The water can be simultaneously mixed and temperature monitored by stirring with the probe–end of a dial thermometer (Figure 9).

6. You are now ready to thaw embryos!

   - Locate the correct canister and cane by referring to infor–mation on certificate C.

   - Center and raise the canister to a position in the lower neck of the nitrogen tank.

   - Grasp the top of the desired cane with tweezers (or between thumb and index finger) and then remove the inverted plastic goblet which covers the labelled straw ends.

   - Select the desired straw by identifying the straw number and donor registration number. This information can be verified while the embryo–containing lower portion of the straw remains immersed in liquid nitrogen within its goblet.

- Pull the straw from the nitrogen tank, hold for 4 *seconds at room temperature,* and then plunge it into the water bath. Remove the straw after 30 seconds and gently lay it on a paper towel to absorb water off the straw.

- Hold the straw firmly in a horizontal position between thumb and index finger approximately 2 cm from the heat–sealed straw end. Snip off the thermal seal with a pair of clean, sharp scissors.

- The vacuum within the straw must be released in order for embryo and solution to flow out into petri dish 1. Hold the straw at a 45° angle above petri dish 1 so that the embryo containing end of the straw is 1–2 cm above the surface of TS #1 solution. Using clean, sharp scissors cut the straw in half immediately adjacent to the end of the plug so that the entire plug remains with the upper, labelled half of the straw (Figure 63).

Figure 63. A freeze straw that has been cut, allowing the contained embryo to exit the straw.

7. Place petri dish 1 on the microscope stage and focus down to locate and evaluate the embryo(s). Embryos with a massively degenerate and/or disorganized cell mass should be discarded at this time.

Embryos which pass this initial screening should then be drawn into a sterile IVF catheter and transferred (with a minimal amount of solution) into dish 2. Use the IVF catheter tip to gently mix TS #1 (which came from the IVF catheter) with TS #2 in dish 2. After 5 minutes have passed, transfer the embryos with a new IVF catheter into petri dish 3.

Mix the catheter solution with TS #3 solution in dish 3, wait 5 minutes, and then transfer with a new catheter into petri dish 4.

8. Embryos in dish 4 which still have an intact, organized cell mass should be transferred immediately. Medium in petri dish 5 is for rinsing and partially filling the straw which the embryo will be loaded into for transfer (procedure detailed in section 7.2).

   From this point on, frozen/thawed embryos can be handled and transferred like fresh embryos.

SUMMARY FOR THAWING FROZEN EMBRYOS

1. Every effort should be made to minimize the time interval between thaw and transfer.

2. Palpate and record acceptable recipients.

3. Review certificate C information.

4. Prepare post–thaw solutions (stock and TS).

5. Label and fill one set of petri dishes per donor thawed.

6. Prepare warm water thaw bath.

7. Thaw, evaluate and then expose embryos to "TS" solutions.

(7.2) EMBRYO LOADING

If there is no need or desire to freeze embryos, then all embryos in petri dish C (from section 5.2) can be packaged in a polystyrene tube (Figure 59) for transport to a distant recipient location for transfer within 18 hours of collection; or transferred immediately on–site.

Embryos to be transferred on–site should be placed into a new petri dish containing sterile embryo holding medium (prepared in section 5.2 and filtered through an Acro–Disc). Label this dish with the donor's name or number. At the same time, prepare a second dish containing embryo holding medium and label this dish "PBS". Maintain separate dishes for each donor, and remember to label each dish with the donor's name or number!

Examine all embryos for significant differences in maturity, i.e., a "young" loose morula versus an "older" blastocyst. With the clipboard in hand containing recipient data recorded earlier in the day, the objective is to match recipient heat dates (uterine maturity) with embryological development. *Assign least mature embryos to the last recipients that showed estrus.*

REMINDER: Useable recipients are those which showed heat either one day before, the same day as, or one day after the donor's heat; and had an acceptable CL when palpated earlier in the day.

When a particular donor/sire mating is known to produce a heavy birth–weight calf, those embryos should be assigned to cow instead of heifer recipients whenever possible. If there are many embryos to transfer and recipients need to be sorted, a list should be made indicating the order that the recipients are to be placed into the transfer chute or stanchion. This allows the transfer technician to quickly match embryos with recipients, and/or load three or four straws with their assigned embryos at one microscope sitting (Figure 64).

Once embryos have been assigned and while recipients are being sorted for transfer, the following sequence should be observed:

1. Make an ET ear tag for each recipient. With a permanent tag marking pen, print the barn name (common name) of the *donor* and embryo *sire* plus the *transfer date* on the tag front (Figure 2). Print the recipient's herd number on the tag backside. Place this tag in the recipient's ear immediately *after* transfer.

2. Place the A–B certificate and recipient list next to the microscope. The recipient list identifies which embryo should be loaded first, second, . . ., while pertinent embryo and recipient information is then recorded on certificate B as each embryo is loaded. The transfer system is comprised of a ¼ ml straw, ¼ ml Cassou insemination rod, sheath with adapter to cover the loaded rod, and a polyethylene sleeve which covers the sheath during transfer (Figure 65).

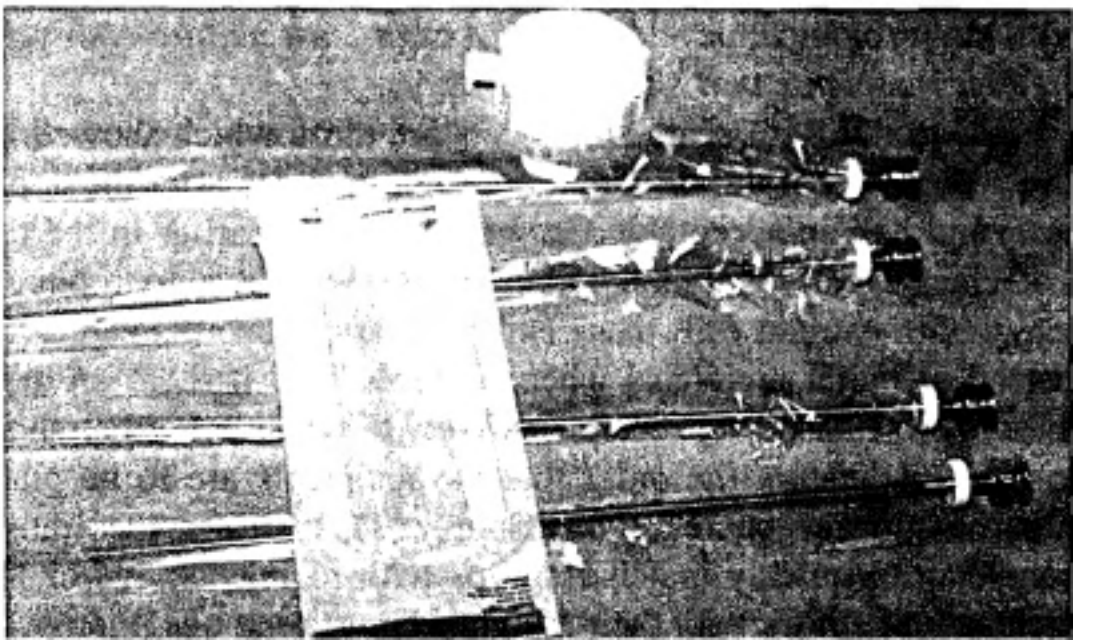


Figure 64. Several Cassou rods can be loaded at one sitting and then transported to the recipient location.

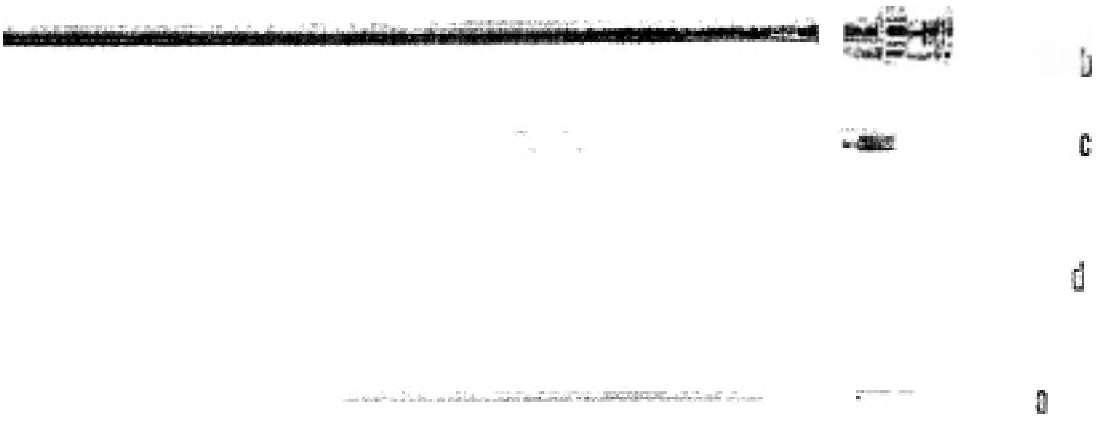


Figure 65. The transfer system: ¼ ml straw (a), ¼ ml Cassou transfer rod (b), sheath with plug insert (c), clean cover sleeve (d).

3. Place the petri dish containing embryos on the microscope stage and focus on the first embryo assigned for transfer. Record embryological and recipient data on certificate B.

4. Sheaths for the ¼ ml Cassou rod are supplied sterile, individually packaged, and must be shortened before use. With the sheath still in its protective package, clip off 0.6 cm of sheath from the end nearest the sheath insert plug. Assign a sheath to each recipient at this time by writing the recipient's tag number in permanent ink on the *protective sheath package*. The sheath (with rod) must not be removed from its protective package until immediately prior to transfer!

5. Remove a sterile ¼ ml straw from its protective packet. Hold the plugged end of the straw between thumb and index finger while inserting the open straw end into the CITO straw cutter (Figure 66). Shorten the straw by plunging the CITO blade.

Reposition the straw into the CITO and plunge the blade a *second* time.

Figure 66. Straw inserted into the Cito straw cutter.

6. Insert the plugged straw end directly into the tip of a 1 ml Monoject tuberculin syringe (Figure 67). Rinse the straw by first pulling the syringe plunger back 0.2 ml, and then placing the open end of the straw into the petri dish labelled PBS. Aspirate a 2 cm column of fluid, raise the straw out of solution, and then pull the fluid to within 1 cm of the cotton plug. Slowly depress the plunger to discard the PBS, and then repeat the straw rinse procedure once more.

7. The straw is now ready to be loaded with the following sequence of solution and air (column measurements are approximate):

Figure 67. Transfer straw ready for loading, inserted into a 1 ml syringe.

- Aspirate 1.3 cm of PBS, and then raise the straw tip out of solution.

- Apply gentle negative back–pressure on the plunger with thumb and index finger until a 0.7 cm air space has appeared at the end of the straw.

- Lower the straw and draw up 4.0 cm of PBS, and then raise the straw out of the PBS petri dish.

- Add a 0.7 cm air space.

- While viewing the embryo to be loaded under low magnification, lower the straw into the dish containing embryos. Aspirate 0.3 cm of fluid, gently bump the embryo with the straw tip to float the embryo off the bottom of the dish, and while floating, aspirate the embryo into the straw along with an additional 1.0 cm of solution.

- Add a 0.7 cm air space.

- Place the straw tip back into the PBS dish and draw up 0.7 cm of PBS. Lift the straw out of solution and hold in a horizontal position. Draw all segments of PBS and air

toward the cotton plug. Stop negative plunger pressure once the plug is saturated with solution (Figure 68).

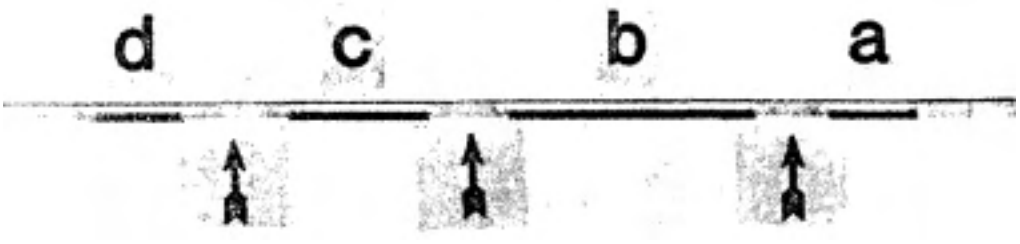


Figure 68. Loaded straw. PBS segment that wets the cotton plug (a), "push" PBS (b), PBS with the embryo (c), entrance PBS (d). Arrows indicate air spaces.

- Lay the loaded straw horizontally on the microscope stage and focus down to verify that the embryo has been isolated in the proper segment.of embryo holding medium (Figure

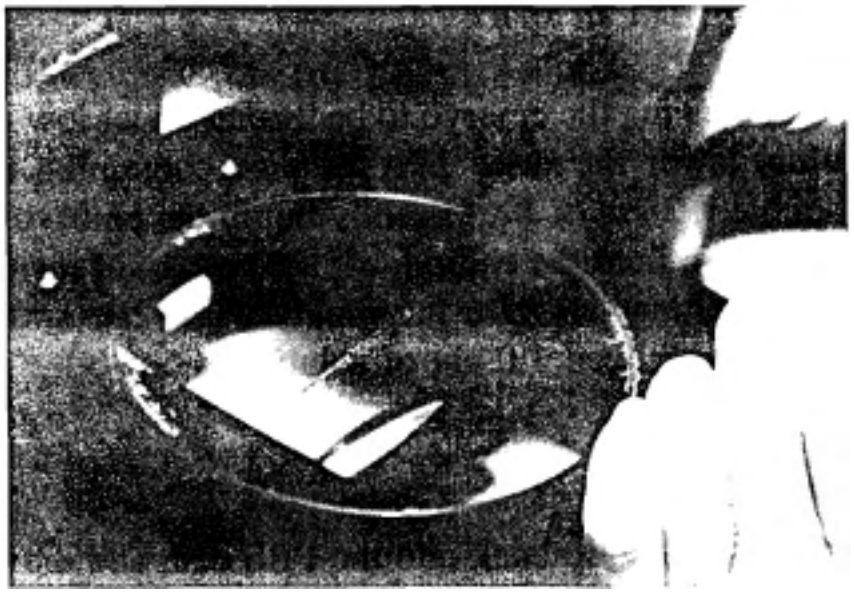


Figure 69. Examining the loaded straw to verify embryo inclusion.

69). If the embryo is not where it should be, then hold the straw in a vertical position (plug end up) over an empty petri dish. Clip the plug off with scissors, which instantly drains the straw contents into the dish. Isolate the embryo and reload into a new straw.

8. Pick up a Cassou rod and pull the plunger out approximately 15 cm. Insert the loaded straw (plugged end first) into the open end of the Cassou (Figure 70). Gently "walk" the straw down into the Cassou with the thumb and index finger, being careful to never touch the open straw end. The straw will extend approximately 0.6 to 0.7 cm beyond the Cassou when the straw is in place (Figure 71).

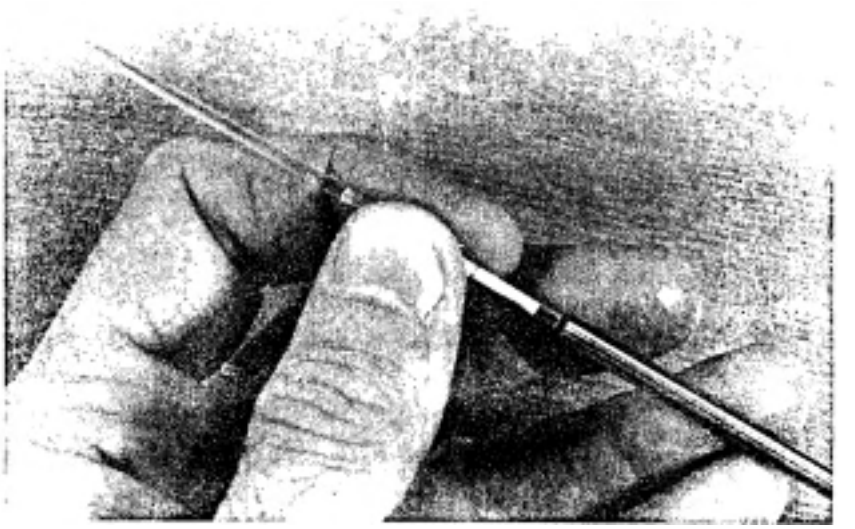


Figure 70. Inserting a loaded straw into the transfer rod.

9. Insert the loaded Cassou rod into the shortened, open (split) end of the *sheath* assigned to that particular embryo. Be very careful to gently position the exposed straw tip into the center of the sheath insert plug (Figure 72). After the straw is

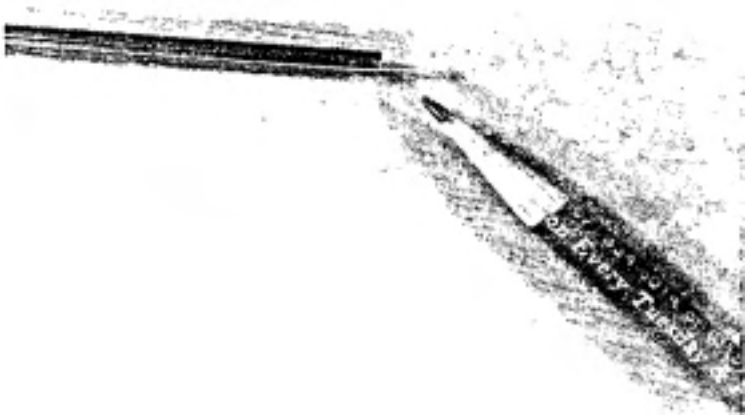


Figure 71. Approximately 0.6 cm of straw will extend beyond the Cassou when properly in place.

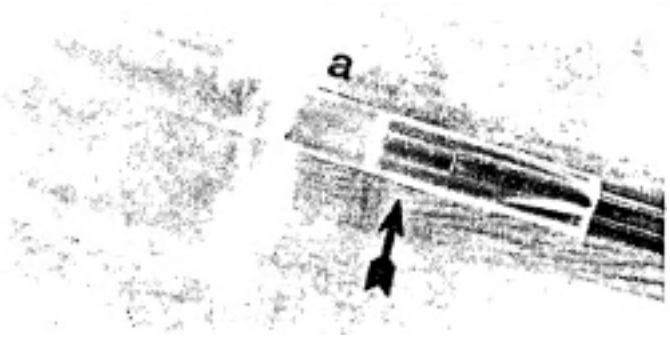


Figure 72. Placing the sheath over the loaded transfer rod. Sheath insert plug (a), and 0.6 cm of straw extending beyond the rod (arrow).

centered in the insert, the sheath (still in its package) should be pulled down to cover the entire Cassou. The sheath is correctly positioned when the insert plug is wedged flush to the sheath tip (Figure 73).

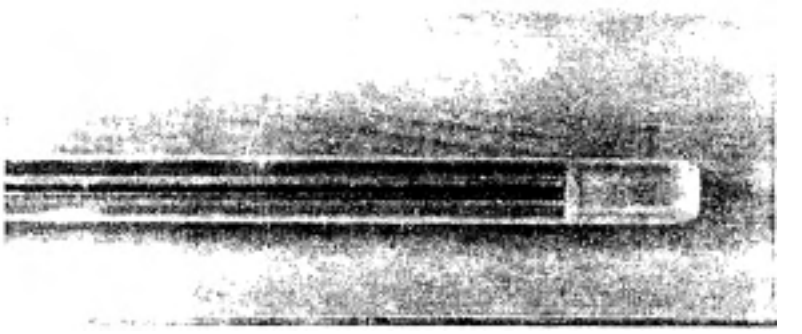


Figure 73. Sheath in place over the transfer rod. As shown here, the insert plug should fit flush against the end of the Cassou.

The sheath must now be "locked" onto the rod by the following procedure:

- With the rod pointing to your left, grasp the base flange of the Cassou (Figure 74) between thumb and index finger of the right hand.
- Lay the open left hand palm down on top of the sheath (and rod) **3** cm ahead of the base flange. Wrap this hand firmly around the rod. The white lock ring (Figure 74) should be positioned around the split part of the sheath located between both thumbs at this time. While gently pulling the sheath toward the base flange with the left hand, simultaneously twist the lock ring onto the sheath (and toward the base flange) with the left thumb and index finger.

10. The Cassou is now ready for transfer and should be placed in a horizontal position next to the microscope until transfer time. If recipients are not immediately close to where embryos are being loaded, one can load 3 or 4 Cassou rods at a microscope "sitting" and then proceed to the recipient area.

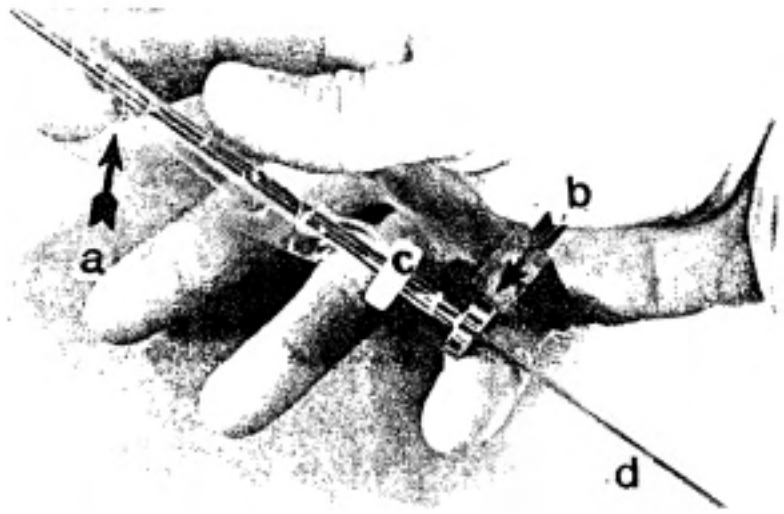


Figure 74. "Trigger" slit in the sterile sleeve (a), Cassou rod base flange (b), white lock ring (c), plunger which expels the embryo (d).

Note: The 21" IMV mini–syringe transfer rod (1/4 ml) uses a sheath featuring a two–hole side delivery tip. Conception rates with this instrument are reportedly slightly better than those achieved with the Cassou rod.

SUMMARY OF EMBRYO LOADING

1. Differentiate maturity among embryos.

2. Assign embryos to recipients.

3. Print ET tags for recipients.

4. Fill in information on certificate A–B.

5. Assign Cassou sheaths (and rods) to recipients.

6. Shorten and rinse the first ¼ ml straw.

7. Load an embryo into the first straw and record embryo morphology data on certificate A–B.

8. Load the straw into the Cassou rod.

(7.3) EMBRYO TRANSFER

Gently transport the loaded rods to the recipient area and place them in a secure location away from extreme heat, cold, and direct sunlight.

The recipient waiting in the chute (or stanchion) should be immediately prepared to receive an embryo. Sequential steps include:

1. An epidural injection *(5* ml lidocaine), administered as previously described in section 4.2.

2. Immediately after the epidural is administered (and before the tail goes limp), pull on a plastic sleeve, enter the rectum, and remove obstructive fecal material.

3. Tie the tail up and out of the way, being sure to use a quick–release knot.

4. The vulva/rectal area must be washed with warm water and several drops of Betadine scrub. NOTE: Be very careful to rinse off all Betadine scrub. Part the vulva and rinse thoroughly with water, since scrub solution is embryo toxic.

5. Pull on a clean sleeve (fingers removed) with latex glove combination.

6. Verify that the ear tag of the prepared recipient matches the recipient number written on the plastic protective sleeve surrounding the Cassou.

7. Grasp the correctly identified Cassou rod and discard the polyethylene packaging sleeve which bears the recipient's ear tag number. Thc sleeve is not sanitary enough to enter the reproductive tract, and *must be replaced* at this time with a *clean polyethylene sleeve* (Figure 65) specifically designed for entering the vagina.

   While momentarily holding the Cassou between your teeth (just ahead of the rod basc flange), squeeze a small amount of lubricating jelly onto your gloved hand, grasp the rod with your bare hand, and then enter the rectum with the gloved arm.

8. While an assistant parts the vulva (Figure 75), the Cassou is passed through the vagina and manipulated to the cervical entrance. Upon reaching the cervix, use your index finger placed through the "trigger" slit in the sterile sleeve (Figure 74) to pull the sleeve backward, causing the Cassou tip to break through the polyethylene sleeve.

   After manipulating the rod through thc cervix, lift and position the desired uterine horn (right or left horn) directly in front of the cervix. It is imperative from here on that you know exactly where the Cassou rod tip is within the uterus at all times!

   REMINDER: The Cassou is directed into the uterine horn (left or right) that is associated with the ovary (left or right) which bears the CL that was identified by palpation earlier in the day.

   The objective is to *gently* slide the rod as far into the uterine horn *until resistance* is *encountered.*

   THE EMBRYO MUST BE EXPELLED AT THIS POINT!

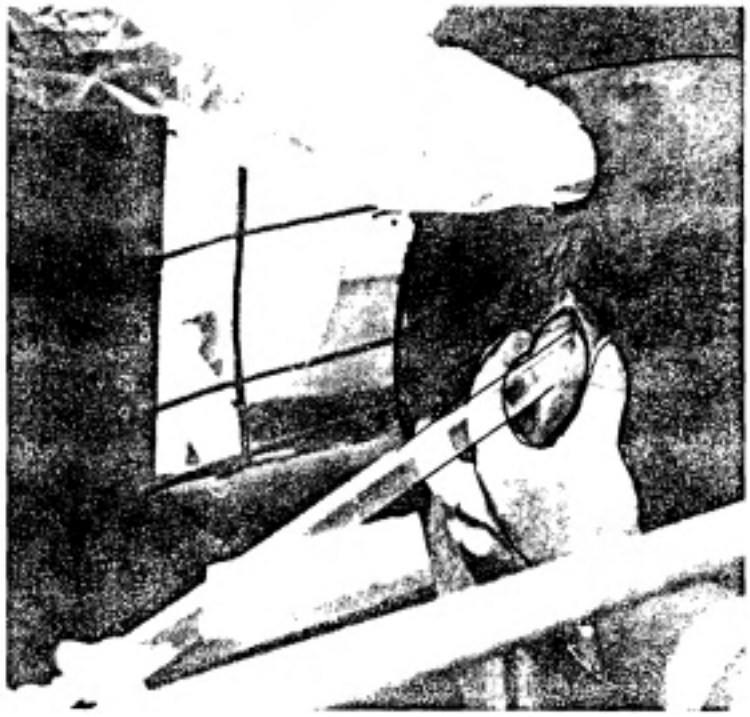


Figure 75. An assistant parts the vulva to permit a clean insertion of the transfer instrument.

When resistance (or other difficulty) to advancing the rod is encountered, the embryo should be deposited slowly (4 seconds) by pushing the plunger (Figure 74) as far as it will go into the Cassou rod. Continued manipulations to place the transfer rod deeper into the horn will result in uterine tissue damage and failure to establish pregnancy.

Withdraw the Cassou slowly after the embryo has been expelled, remove the soiled sheath from the rod, and proceed to prepare the next recipient for transfer.

NOTE: The resulting pregnancy rate—which should average 60% with fresh embryos—depends on many variables such as embryo quality, recipient preparation and management, and skill of the transfer technician.

Once a recipient is prepared for transfer, an experienced transfer technician should average about 90 seconds to implant an embryo. Problems encountered by the inexperienced technician can include:

a. Passing the Cassou through the small heifer cervix. Frequently the "tight" cervix can be opened by gently (yet firmly) manipulating a dilator rod through the cervix, removing the dilator, and then passing the Cassou.

b. Attempting to deposit the embryo high up in the uterine horn and subsequently causing damage to the uterine lining.

c. *Arm* fatigue when transferring many embryos on the same day.

SUMMARY OF EMBRYO TRANSFER

1. Administer an epidural to the first recipient.

2. Remove feces, tie tail up and away, wash vulva/rectal area.

3. Verify embryo–recipient pairing.

4. Re–sleeve the Cassou with a clean cover and then manipulate the rod through the cervix.

5. Guide the rod into the correct uterine horn.

6. Deposit the embryo when resistance is encountered!

APPENDIX A –

Embryo Collection and Transfer Supplies/Products

- Antibiotic – Antimycotic, lyophilized, 20 ml
- Antibiotic – Antimycotic, frozen, 20 ml
- Betadine, scrub, gal.
- Bovine Serum Albumin Fraction V, 25 g, 100 g, 100 nil
- Calf Serum, new born, heat inactivated, frozen, 100 ml
- Calf Serum, with Pen.–Strep., lyophilized, 20 ml
- Catheter Foley, Bard, 5 ml cuff, 12 ga – 24 ga
- Catheter, in vitro fertilization, sterile
- Cervical dilator, 22", stainless steel
- Dish, with lid, 12–well, sterile
- Ear tag, blank, size large
- Ear tag trocar
- Ear tag marking pen
- Exam glove, latex, size S, M, L, 100/box
- Filter, Em–Con, sterile
- Infusion tubes, drilled adaptor, 18"
- Lubogel, 8 oz or 1 gal
- Microscope, stereo–star, American Optical
- Microscope, stereo–zoom 7, Bausch & Lomb
- Nitrofurazone, gal.
- PBS, modified, 100 ml
- PBS, modified, bag, 1000 ml
- Penicillin–streptomycin, lyophilized, 20 ml
- Petri dish, 10 x 35 mm, 20/pkg
- Search dish, Quebec grid, 100 x 15 mm, 20/pkg
- Sheath, IMV Universal Cassou, ¼ or ½ ml, sterile
- Sheath, IMV Side–delivery, ¼ ml, sterile
- Sleeve, IMV plastic Cassou, 100/roll, 18"

Straw, ¼ ml, sterile (5/pkg)

- Stylet, catheter, stainless steel, size 16 ga and larger
- Stylct, catheter, stainless steel, size 12 ga and 14 ga
- Syringe filter, Acro–Disc, 50/box
- Tube, round bottom with cap, sterile, 12 x 75 mm
- Tubing, Y–junction, with Foley connector, and spike

APPENDIX B

Embryo Freezing, Artificial Insemination

Supplies/Equipment

- Cane, aluminum, for 10 mm straw goblet
- Freezer; Hoxan Cryoembryo PSP
- Freezer; Freeze Control CL856
- Freezing medium, not active, 100 ml
- Freezing medium, with 10% glycerol (active), 20 ml
- Glycerol, 20 ml
- Goblet, 10 mm, capacity: 5, ½ ml straws
- Insemination/transfer rod, Cassou, ¼ or ½ rnl, 18 or 21", stainless steel
- Insemination transfer rod, IMV "mini–syringe" 1/4 ml, 21"
- Nitrogen tank, 3 or 10 liter
- Paint stick, orange, 10/box
- PBS, without Ca and Mg, 100 ml
- Permanent marker, fine or broad tip
- Seeding bowl with tweezer
- Straw cutter, Cito
- Straw, ½ ml double–length sterile, (5/pkg)
- Straw sealer, impulse heat
- Thaw box, styrofoam
- Thaw box, Cito electric
- Thermometer, dial, –20 to 50° C
- Trypsin solution, 0.25%, 100 ml

APPENDIX *C*

Pharmaceuticals and Miscellaneous

- Acepromazine, 10 mg, 50 ml (+)
- Cystorelin, 2 ml, 25/box (+)
- Disinfectant, Nolvasan, gal.
- Distilled water, sterile, 100 ml
- Estrumate, 20 ml (+)
- FSH, Schering, 10 ml (+)
  Hemostat, 5.5"
- Lidocaine, 100 ml (+)
- Lutalyse, 30 ml (+)
- Needles, Air–Tite, 16×1, 18×1.5, 20×1, 100/box
- Synchromate–B, 10 dose/box
  Synchromate–B, application tool
- Syringe, Air–Tite, 2.5 ml, 5 ml, 10 ml, 20 ml, 30 ml, 50 ml, all plastic
- Syringe, Monoject, 1 ml, 3 ml, 6 ml, 12 ml
- Vacutainer, no additives or silicone coating, 10 ml

(+) Prescription required if not ordered by a licensed veterinarian. Non–veterinarians can purchase required prescription items directly from an ET product supply company by placing on–file with the company an "Authorization to Purchase ET Prescription Products" form, signed by a cooperating veterinarian.

Source of Supplies and Training Workshops

AGTECH, Inc. (USA) has a complete inventory of required pharmaceuticals, laboratory equipment, and embryo collection/freezing/transferring products, as listed in appendices A, B, and C.

In addition, AGTECH provides hands–on training workshops in embryo transfer technique.

Workshop information and a free product catalog are available by contacting AGTECH, INC., P. O. Box 1222, Manhattan, KS 66502 (USA). FAX: 913–537–0179. TELEX: 940103 WU PUBTLX BSN. Telephone: 913–776–3863.

APPENDIX D

Certificates and Forms

Scheduling Donor Superovulation and Recipient Synchronization
(Example dates have been inserted to clarify procedure sequence)

1. The donor must have a good corpus luteum (CL) prior to starting FSH, or be on Synchromate-B.
2. Carefully withdraw all diluent (water) and inject into the bottle containing powdered FSH. Refrigerate at all times.
3. Measure all doses precisely. Administer all injections in the muscle.
4. Recipients must be watched closely for estrus. Record all heat dates!
5. Donor(s) should be in heat January 23.
6. Donor(s) should be bred at least twice.
7. Inject donor with 4 cc Estrumate immediately after embryo collection.
8. The first activity on collection day will be to examine recipients.
9. Embryo collection day will be: January 30.

SPECIAL RECIPIENT INSTRUCTIONS

a. If natural heats are used, recipients do not receive Estrumate on January 20.
b. If Synchromate-B is used for recipients, it should be inserted on January 12.
c. If double injection is used for recipients, the first Estrumate injection should be given on January 09.

SPECIAL DONOR INSTRUCTIONS

a. If the donor is to be Synchromated, the implant should be inserted on January 12.

DONOR INJECTION SCHEDULE

DONOR(S) NAME: _____

DATE	TIME	DOSE	DRUG
Jan. 19	a.m.	1.3 cc	FSH (Schering FSH-P)
Jan. 19	p.m.	1.3 cc	FSH
Jan. 20	a.m.	1.0 cc	FSH
Jan. 20	p.m.	1.0 cc	FSH (Inject single and double injection recipients with 2 cc Estrumate.)
Jan. 21	a.m.	4.0 cc	ESTRUMATE (Remove Synchromate-B from each recipient.)
Jan. 21	a.m.	0.8 cc	FSH
Jan. 21	p.m.	0.8 cc	FSH (Remove Synchromate-B from each donor.)
Jan. 22	a.m.	0.6 cc	FSH
Jan. 22	p.m.	0.6 cc	FSH

OWNER'S COPY

A. CERTIFICATE OF EMBRYO RECOVERY

Breed _____
Donor Name _____ Ear Tag or Tatoo No _____
Owner _____ Address _____
_____ Onset Estrus Date AM ___ PM ___
Service Sire _____ No. _____ Breeding Date _____
I.D. Code Freeze Date or Batch No. _____ Recovery Date _____
Service Sire _____ No. _____ No. Recovered _____
No. Transferred _____
I.D. Code Freeze Date or Batch No. _____ No. Frozen _____
Signature _____ Firm _____

B. CERTIFICATE OF EMBRYO TRANSFER

Date of Embryo Transfer _____ **Recipient Identification**
Days since estrus of donor _____ Surgical __ Non-surgical __ Other __

One embryo was transferred to each of the following recipients unless It Is noted that more than one was transferred.

Ear Tag No.'s and/or Registration No.		Tattoo if appl	Breed Code	Days Since Estrus	Stage Code	Quality Code	Embryo Divided	Frozen Freeze Date on Str. or Amp.	Frozen Str. or Amp. No.	Comment
1.										
2										
3.										
4.										
5.										
6.										
7.										
8.										
9.										
10.										

Signature _____ Firm _____
ET Code _____ Phone _____

Use comment column for any special notations and/or to identify the location of the opposite half of a divided embryo. Pregnancy can be noted.

C. CERTIFICATE OF FREEZING

Type of Container
Straw A m p u l e ___ Other ___ Each container labeled to show firm code, breed, reg. no. of donor, freeze date, str. or amp. no.

Cane No.	Str. or Amp. No.	No. Embryos/ Str. or Amp.	No. X Washed	Code Stage	Code Quality	Zona Intact	Divided	Comments

Time from recovery to onset of freezing ___ (hrs.) Cryoprotectant and concentration, final molality and cooling procedure _____
How Frozen: Seed Temp. ___ Cooling Rate ___ Plunge Temp. ___ Other _____
Recommended Method of thawing _____

Signature _____ Firm _____
ET Code Phone ( ) _____

CONDITIONS FOR COMPLETING EMBRYO CERTIFICATES

A. Complete one or more Certificates of Embryo Recovery for each recovery. The responsible technician signing this certificate is attesting to the fact that the donor dam was identified with her certificate of registration, that the service sire information was taken from a written record of services, and all the information is true and correct.

B. Certificate of Embryo Transfer will be completed to the extent that is necessary and/or appropriate to identify each recipient into which an embryo is transferred. If frozen embryos are transferred, the Certificate of Embryo Recovery will be completed by the responsible technician or by transferring from the original Certificate of Embryo Recovery or by attaching a copy. The technician signing the Certificate of Embryo Transfer is attesting to the accuracy and completeness of the identification of the embryos being transferred and the identity of the recipients into which being transferred.

A complete Certificate of Embryo Transfer, with Certificate of Embryo Recovery, will be submitted to the appropriate breed office within 120 days of transfer, and before any resulting offspring will be registered.

Should any embryo identified hereon in recipient change ownership, such change will be documented by the seller completing an application for transfer with one copy submitted to the breed office and one copy provided the buyer which, in turn, will be submitted with the application to register the resulting offspring. One application for transfer is required for each change in ownership. The application for registration will be accepted from the person shown as the last owner of the recipient and/or the owner of the resulting calf at the time of its birth.

C. The Certificate of Freezing will be completed, with the Certificate of Embryo Recovery, whenever embryos are frozen. The technician signing the certificate is attesting to the identification of each embryo, with container labelling, as set forth within the Certificate, along with the accuracy of all other information.

One copy will be sent to the breed office and one copy provided the owner.

When a frozen embryo changes ownership the seller will submit one copy of an application for transfer to the breed office with a second copy provided the buyer from which an application for registration of the resulting offspring will be accepted on condition that properly completed Certificates A-C have been submitted to the breed office. Each change of ownership must be covered by a transfer.

When frozen embryos are exported a special application for embryo export will be submitted to the respective breed office, with the appropriate fee.

Use the following codes to describe the embryo, identify the breed and identify the month in all dates

STAGE OF DNELOPMENT

No.	Stage	No.	Stage
1	Unfertilized	6	Blastocys:
2	2- to 12-cell	7	Expanded Blastocyst
3	Early Morula	8	Hatched Elastocyst
4	Morula	9	Expanding Hatched Blastocyst
5	Early Elastocyst		

QUAUTY OF EMBRYOS

1	Excellent or Good	January	JA	July	JY
2	Fair	February	FE	August	AC
3	Poor	March	MR	September	SE
4	Dead or degeneratlny	April	AP	October	OC
		May	MY	Novernber	NO
		June	JN	December	DE

BOVINE

AN - Aberdeen Angus	CB - Charbray	GU - Guernsey	NR - Norwegian Red	IS - Shorthorn (dairy)
AB - Abondance	CH - Charolais	HC - Hays Converter	PA - Parthenais	SV - Simmental
AF - Afrikander	CA - Chianina	HH - Hereford (horned)	PI - Piedmont	DS - South Devon
AY - Ayrshire	DB - Danish Black & White	HP - Hereford (polled)	PR - Pie Rouge	SX - Suffolk
BA - Barzona	W - Danish Jersey	SH - Highland (Scotch Highland)	PZ - Pinzgauer	TA - Tarentaise
BE - Beefalo	HW - Danish Red & White	HO - Holstein	RA - Ranger	TG - Texas Longhorn
BF - Beef Friesian	DE - Devon	HY - Hybrid (Alberta Hybrid)	AR - Red Angus	TL - Texas Longhorn
BM - Beef Master	DX - Dexter	JE - Jersey	RB - Red Brangus	WS - Welsh Black
BB - Belgium Blue	FP - East Flemish Red Pied	KB - Kobe (Wagyu)	RD - Red Dane (Red Danish, Danish Red)	WF - West Flemish Red
BG - Belted Galloway	EA - Eringer	LO - Lulig	WW - Red Holstein	XX - Crossbreed
BD - Blonde D-Aquitaine	FA - Flamand	LM - Limousin	RP - Red Poll	
BO - Bradford	FL - Flechvieh	LR - Lincoln Red	RN - Romagnola	
BU - Brahman	FR - Fribourg	MA - Maine-Anjou	RO - Rotbunte	
BH - Brahmental	FB - Friesian (Belgium)	MR - Marchigiana	SA - Salers	
BN - Brangus	DF - Friesian (Dutch)	ME - Maremmana	XI - Santa Gertrudis	
BU - Braunvieh	GA - Galloway (beef)	MI - Meuse-Rhine Ijessel	MS - Shorthorn (milking)	
SB - Brown Swiss (beef)	GD - Galloway (dairy)	MO - Montbeliard	SS - Shorthorn (beef - Scotch)	
BS - Brown Swiss (dairy)	GS - Gascone	MG - Murray Grey	SP - Shorthorn (dairy)	
CP - Campine Red Pied	GV - Gelbvieh	NM - Normande		
CN - Canadienne	GR - Groninger			

APPENDIX E

Embryos and Ovaries

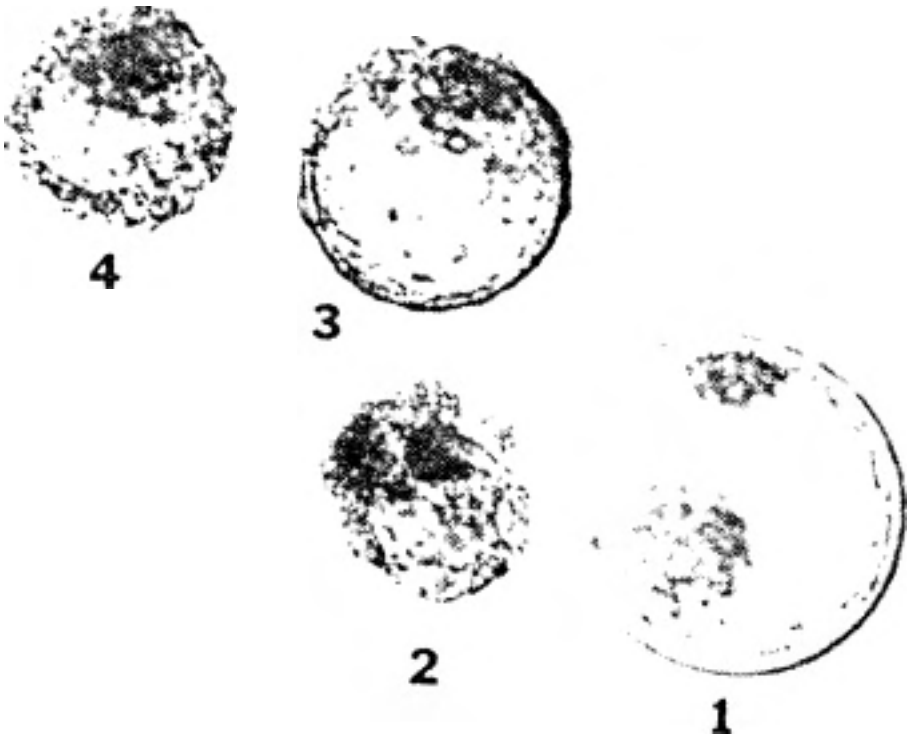


Figure E1. Expanded blastocyst, grade 1 (1); blastocyst, grade 3 (2); hatched blastocyst (3 and 4); all at 70× magnification. Embryo #1 (developmental stage #7) is generally seen in a late–7 to early–8 day collection. The expanding cell mass will "break–out" of its zona pellucida shell within one day, and then resemble embryos #3 and #4. The inner cell mass (ICM) is the unique darkened area of cells clustered near the edge of each embryo's total cell mass, and is clearly visible in all fertilized embryos. The ICM develops into the adult organism, while the other cells within the zona pellucida form placenta and embryonic membranes. Embryo #2 (blastocyst stage 6) is given a quality grade of 3 since there is a significant amount of dark, degenerate cellular debris exterior to the compact blastocyst. The embryo would receive a quality grade of 1 if the debris were absent. Embryos are classified as blastocysts once the clear, fluid–filled inner cavity (blastocoele) becomes visible.

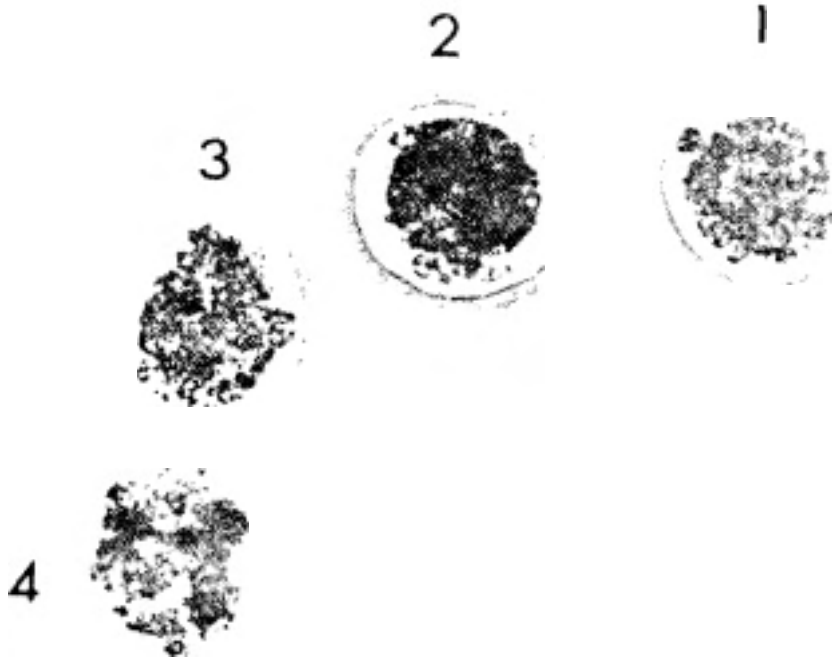


Figure E2. Tight morula, grade 2 (1 and 2); loose morula, grade 3 (3); loose morula, grade 4 (4); all at 400× magnification. A difficult task for beginning embryologists is distinguishing between a nonfertilized ovum and a tight morula. The key is to gently roll the embryo in its dish while focusing on the edge of the cell mass. or edge of the single cell if the ovum is nonfertilized. A tight morula (stage 4) will exhibit a cell mass edge that is somewhat scalloped or uneven, due to the round form of each individual cell (blastomere) packed into the cell mass. The nonfertilized ovum displays a single cell, much larger than each individual blastomere in the morula, and its edge is smooth. A stage 3 embryo (early/loose morula) has blastomeres that are larger and fewer in number than the more mature "tight" morula.

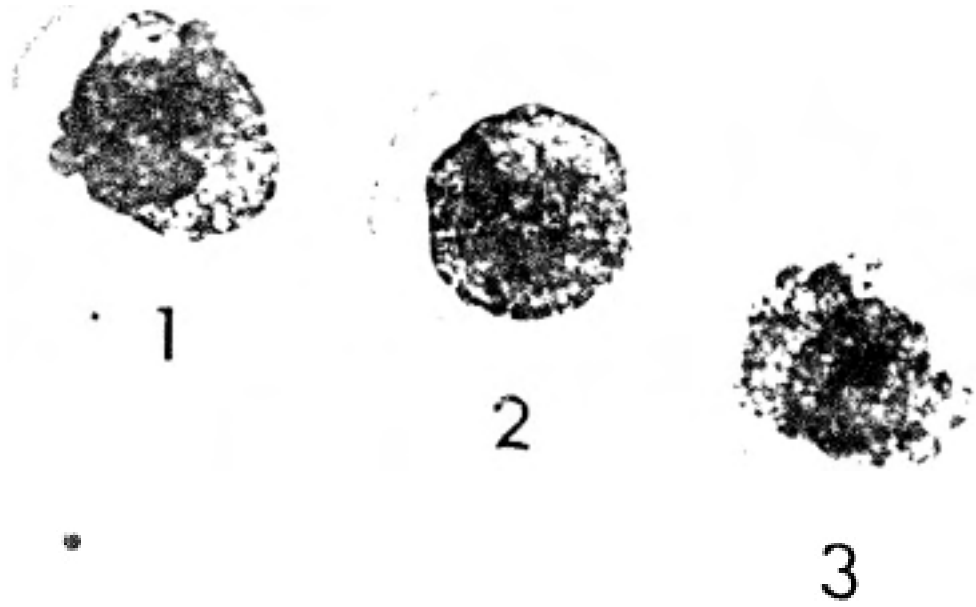


Figure E3. Early blastocyst, grade *2* (1); early blastocyst, grade 1 (2); morula, grade 3 (3); all at 400× magnification. The clear blastocoele cavity is visible in embryos #1 and *#2*. Embryo #1 is given a quality score of *2* since *2–3* blastomeres are outside of the main cell mass. Embryo *#3* shows many dark, granulated cells plus a few large clear vacuolated cells exterior to the cell mass. The embryologist must learn to "look beyond" the extraneous cellular debris and concentrate on evaluating the integrity of the intact cell mass.

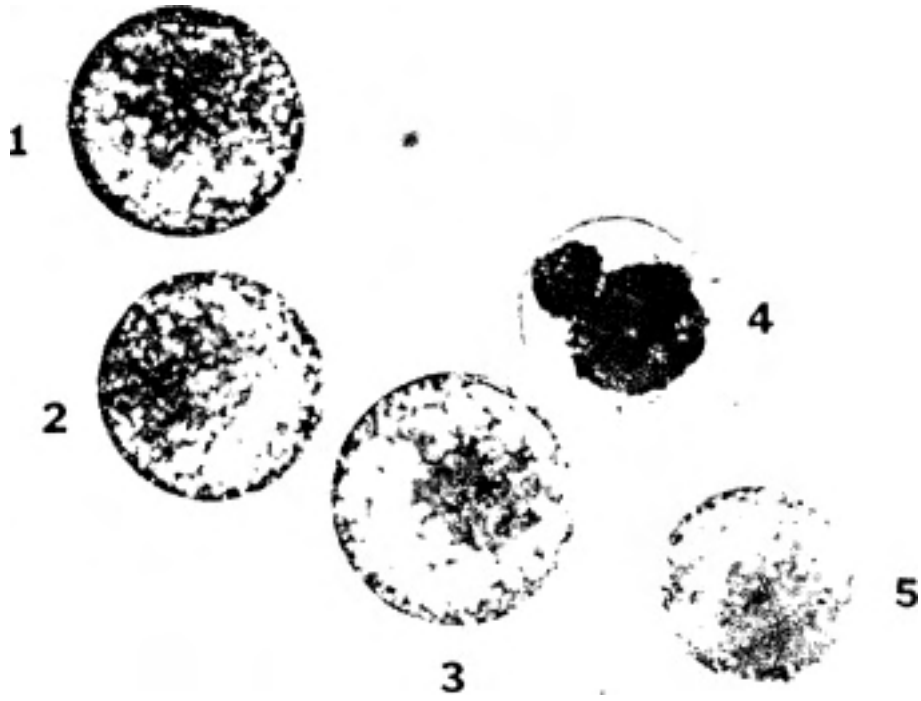


Figure E4. Expanded blastocyst, grade 1 (1, 2, and 3); tight morula, grade 2 (4); blastocyst, grade 1 (5); all at 400×. Embryo #4 has a nice tight cell mass, yet receives a quality score of 2 due to its single large extruded cell.

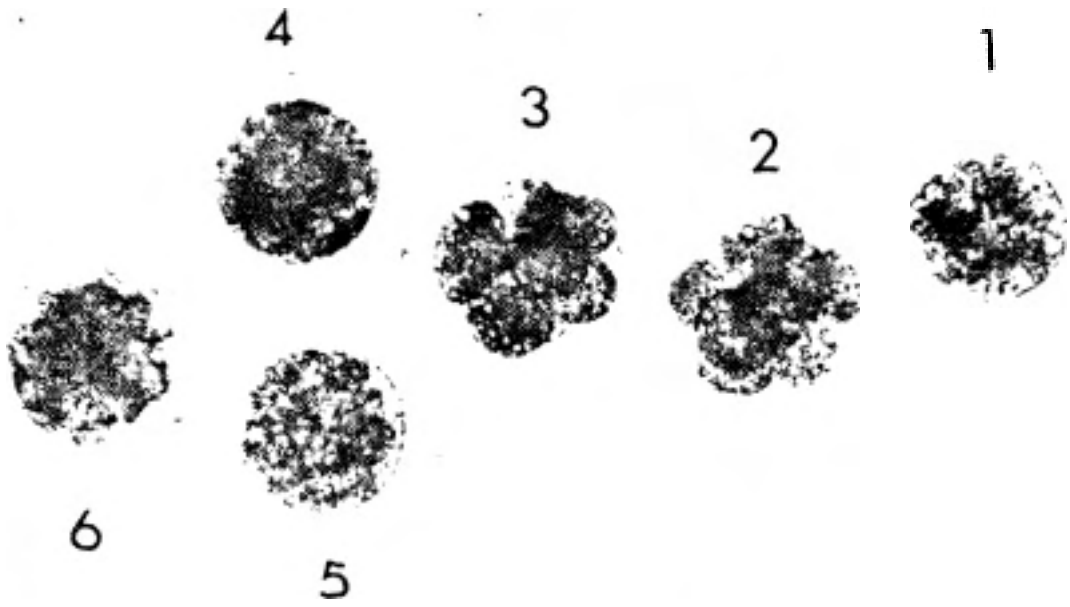


Figure E5. Unfertilized ova (1, 4, and *5);* 8–cell embryo, grade 1 (2 and 3); morula, grade 2 (6); all at 400×. Note the smooth edge of the single–cell unfertilized ovum (Nos. 1, 4, 5). Embryos #2 and #3 were collected on day 7, yet they have developed only to the morphology of a stage 2, day 3–type embryo. The viability/ survivability of a stage 2 embryo collected on day 7 is a judgment call made by the embryologist. Pregnancies have resulted from transferring stage 2 embryos into day 5.5–6 recipients.

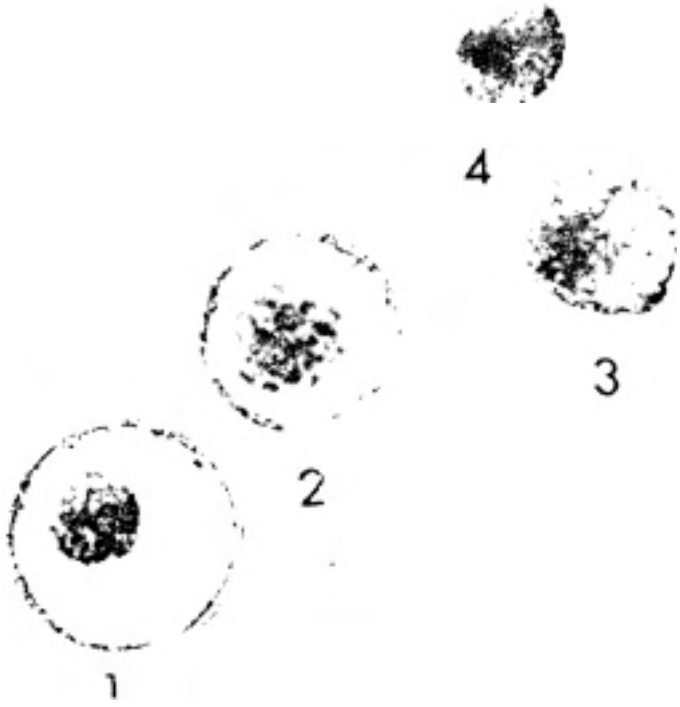


Figure E6. Expanded blastocyst, grade 1 (1 and 2); blastocyst, grade 1 (3); unfertilized ovum (4); all at high magnification.

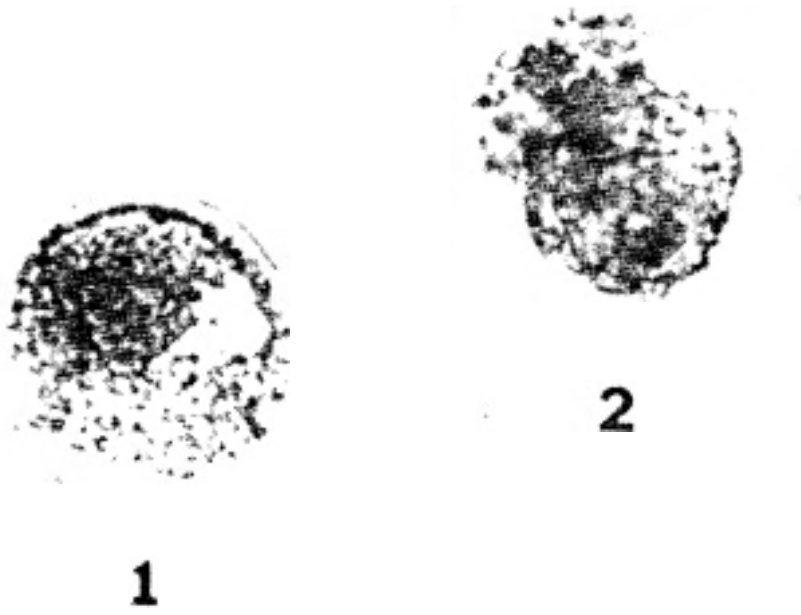


Figure E7. Hatching blastocyst (1); collapsed, previously expanded blastocyst, grade 2 (2); both at 400×.

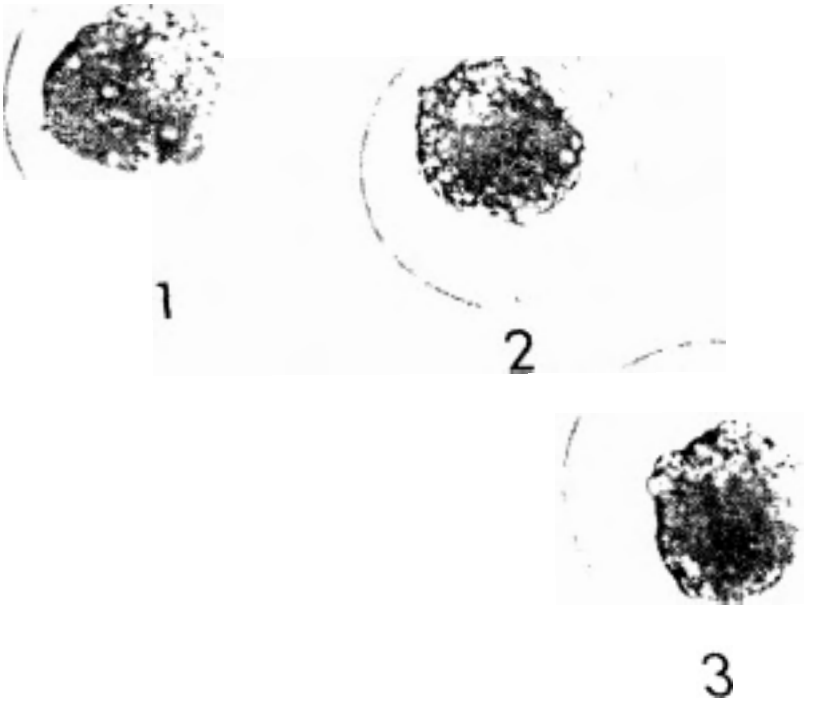


Figure E8. Collapsed, "hatching" blastocyst (1, 2, and 3), high magnification. An expanded blastocyst is like a tight balloon ready to burst. Frequently when a stage 7 embryo hatches, the "balloon" bursts (collapses) and then proceeds to re–expand its blastocoele cavity as it pushes out of the broken zona pellucida.

Figure E9. Expanded blastocyst, grade 1 (1); blastocyst, grade 3 (2); hatched blasto–cyst (3 and 4); all at 70× magnification.

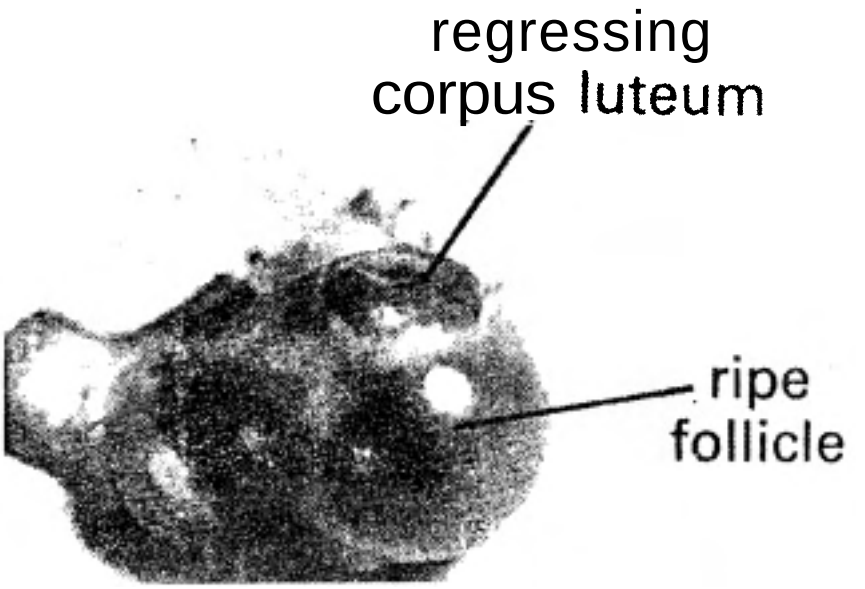


Figure E10. Ovary approximately 4 days before estrus.

Figure E11. Mid-cycle ovaries from a recipient showing a large follicle (left ovary) and mature CL on the right side of the companion ovary.

Figure E12. Superovulated ovary with numerous CLs and nonovulated follicles.

References

Bowen, J. M., R. P. Elsden and G. E. Seidel, Jr. 1978. Nonsurgical embryo transfer in the cow. Theriogenology 10:89–95.

Brand, A., M. H. Aarts, D. Zaayer and W. D. Oxender. 1978. Recovery and transfer of embryos by nonsurgical procedures in lactating dairy cattle. I : Control of Reproduction in the Cow (J. M. Sreenan, ed.). Commission of the European Communities, Galway, pp. 281–291.

Christie, W. B., R. Newcomb and L. E. A. Rowson. 1980. Nonsurgical transfer of bovine eggs: Investigation of some factors affecting embryo survival. The Veterinary Record 106:190–193.

Church, D. C. and W. G. Pond. 1988. Basic animal nutrition and feeding. Third edition, John Wiley & Sons.

Corah, L. R. 1987. Planning a nutrition program for today's productive beef cow. Cooperative Extension Service, Kansas State University, C–680.

Dunham, J. R. and E. P. Call. 1989. Feeding dairy cows. Cooperative Extension Service, Kansas State University, MF–754.

Elsden, R. P., L. D. Nelson and G. E. Seidel, Jr. 1978. Superovulating cows with follicle stimulating hormone and pregnant mare's serum gonadotropin. Theriogenology 9:17–26.

Newcomb, R. and L. E. A. Rowson. 1980. Investigation of physiological factors affecting nonsurgical transfer. Theriogenology 13:41–49.

Rowe, R. F., M. R. Del Campo, J. K. Critser and O. J. Ginther. 1980. Embryo transfer in cattle: Nonsurgical transfer. American Journal of Veterinary Research 41:1024–1028.

Schneider, H. J., R. S. Castleberry and J. L. Griffin. 1980. Commercial aspects of bovine embryo transfer. Theriogenology 13:73–85.

Tervit, H. R., M. W. Cooper, P. G. Goold and G. M. Haszard. 1980. Nonsurgical embryo transfer in cattle. Theriogenology 13:63–71.

Wright, J. M. 1981. Nonsurgical embryo transfer in cattle: Embryo–recipient interactions. Theriogenology 15:43–56.

Wright, R. W., Jr. and G. M. Lindner. 1982. Bovine embryo morphology and evaluation. Project No. 0315. College of Agriculture and Home Economics Research, Washington State University, Pullman.