Cattle Embryo Transfer Procedure

An instructional manual for the veterinarian, beef cattle breeder, dairyman, artificial insemination technician and animal scientist

By

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Preface

This text is designed as an instructional manual for the veterinarian, beef cattle breeder, dairyman, AI technician, and animal scientist previously skilled in bovine artificial insemination technique. In a stepwise sequence, elements required to implement embryo transfer technology into your herd (or to offer embryo transfer service to your clients) are explained or illustrated. The accompanying DVD attached to the inside back cover provides visual components of instruction which complement this manual. **If your desire is to only transfer frozen / thawed embryos, this information can be found in sections 2.3, 3.1, 3.3, all of chapter 6, plus the included DVD.**

Dr. Curtis received his formal embryo transfer training at Colorado State University while completing a Master's Degree in Physiology and Biophysics under the guidance of Dr. Peter Elsden and Dr. George Seidel. His Ph.D. in Animal Science is from Kansas State University. Prior to creating Agtech, Inc., the author logged a successful twelve-year professional record of thousands of pregnancies in the United States throughout fourteen Midwestern and northeastern states. He has served as technical director for Twin Brook Genetics, Wilton, New Hampshire, as owner/operator of Curtis Embryo Transfer Co., Oneonta, New York, as vice president of Embryo Operations for Dreamstreet Holsteins, Inc., Walton, New York and is the founder and president of Agtech, Inc. in Manhattan, Kansas, USA.

Dr. Curtis continues to conduct hands-on bovine embryo collection, freezing and transfer courses domestically and internationally. He has formally trained more than 500 students (veterinarians and cattle breeders) in ET technology. He serves as a consultant to emerging embryo transfer clinics and cooperatives and directs the daily business activities of Agtech including negotiating with overseas manufacturers onsite, taking an active role in marketing, and product research and development.

Agtech, Inc. provides ET and AI instruction at its Training Center in Manhattan, Kansas - USA and also distributes ET and AI instruments and supplies throughout the United States and seventy-nine other countries. Additional information can be found at www.agtechinc.com.

Definition of Terms

A.I.	Artificial insemination. The act of placing frozen/thawed semen into the uterus.
CC	Cubic centimeter, a measurement of volume. The abbreviation represents the same volume as milliliter (ml) and is used interchangeably with ml.
CIDR™	A controlled internal drug releasing (progesterone) silastic vaginal implant, for use in regulating the cow's cycle.
CL	Corpus luteum, or yellow body. A progesterone producing structure which develops on the ovary where ovulation occurred.
Cyst	A fluid-filled structure on the ovary usually greater than 20mm in diameter, and persistent (usually for more than 10 days). Generally believed to be the result of a follicle which matured yet failed to ovulate, a cyst is often classified based on its production of progesterone (luteal) or estrogen (follicular).
Donor	The cow or heifer which is superovulated for embryo production.
DT	Direct transfer. A technique for freezing embryos in ethylene glycol, which allows the frozen/thawed embryo to be placed directly into the uterus without requiring microscopic examination.
EG	Ethylene glycol; the chemical cryoprotectant that direct transfer embryos are frozen in.
ET	Embryo transfer. Can refer to the specific act of placing an embryo into the uterus, or the term can refer to the combined processes of donor superovulation, embryo recovery and transfer, and recipient estrous cycle synchronization.
Flush	The act of infusing the uterus with medium with the intent of extracting embryos. Also referred to as collecting or flushing.
Follicle	A fluid-filled blister (cavity) in and protruding above the surface of the ovary, containing an oocyte.
FSH	Follicle stimulating hormone such as Response™, Folltropin™, Pluset® or local equivalents.
GnRH	Gonadotropin releasing hormones, such as Cystorelin, Ovacyst, Factrel, Fertagyl or local equivalents.
IETS	The International Embryo Transfer Society
Infundibulum	The "ball-glove" like structure which gathers and funnels ovulated oocytes into the oviduct.
Oocyte	A non-fertilized ova (egg, or ovum).
PG	Prostaglandin, a hormone produced by the uterine endometrial tissue which targets the corpus luteum. Also referred to as PGf2 alpha, Lutalyse, Prostamate or local equivalents. The prostaglandin analog Estrumate (Cloprostenol Sodium) targets the CL in a similar fashion.
Recipient	The surrogate host (cow or heifer) which receives the donor's embryo into its uterus.
Superovulation	Exogenous administration of FSH resulting in the maturation and ovulation of multiple follicles.
Zona pellucida	The gelatin-like sphere encircling and enclosing the embryo until approximately day 8.5.

Overview of the Embryo Transfer Procedure

For the past seventy years artificial insemination has allowed genetic progress to be achieved in cattle relatively quickly through the widespread and efficient use of frozen semen. Until thirty years ago, rapid genetic progress was limited to the male side of genetic contribution via A.I. because cows can realistically produce only one calf per year. Today due to the refinement of embryo transfer techniques, cows can produce twenty-five or more offspring annually. The addition of ET technology to a successful AI program results in more rapid genetic gain than could be realized from AI alone.

The process of cattle embryo transfer begins by selecting a genetically outstanding, well-nourished, non-pregnant embryo donor (heifer or cow). At the same time, eight to twelve non-pregnant females are identified as embryo recipients.

The donor is superovulated by daily injections of follicle stimulating hormone and then bred artificially using high-genetic-value frozen semen. Simultaneously the estrous cycles of the recipient females are synchronized with the donor's cycles by prostaglandin injections.

Embryos are typically recovered seven days after donor insemination. The embryo collection method described in this manual is an industry-proven procedure. However, modifications to this technique 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** is critically important to the success of an embryo transfer program.

Chapter 1:
Cattle Management

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

1.1 HEALTH PROGRAM

If you are boarding resident donors and/or recipients at your clinic, farm, or ET business, the arriving cattle should be accompanied by a health certificate indicating negative brucellosis and tuberculosis (TB) tests within the prior thirty days. New animals should be quarantined from existing cattle for thirty days pending verification of negative tests for TB, brucellosis, anaplasmosis, and bluetongue. Of course the same standards would be expected for cattle within your own herd for which you are collecting and transferring embryos into.

To insure health and reproductive soundness, all new cattle should be processed immediately upon arrival to the clinic or at the dairy or ranch. Donor and recipient processing would include:

a. A thorough visual examination to detect and treat ringworm, external parasites, severe lameness, mastitis, pink-eye/blindness.
b. A thorough reproductive tract examination to confirm:
   - A non-pregnant, mature uterus free of endometritis, tumors, and adhesions. Palpate all cattle thirty days later to identify pregnancies not detected on arrival day.
   - Two normal sized, non-cystic ovaries, each adhesion-free.
c. Evaluation of recipient age and size.
   - Age and size are important considerations when heifers are used as recipients. Granted 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. Ideally heifers would be at least fourteen months of age and approaching 800 lbs. (365kgs). Recipient cows should be under eight years of age and at least fifty days post-partum.
   - Cattle that are acceptable following the visual, internal, and age/size examination are then identified and vaccinated.
d. Cattle Identification.
   - Donors and recipients should be easily identified by a simple visual marking scheme. A common method is to insert a large numbered tag into the ear. One system might assign large white tags to donors (pre-numbered in permanent ink) and large yellow tags to recipients. The number should be large and bold and appear on both sides of the tag.
   - All other ear tags, neck chains, or other forms of attached identification (except metal vaccination ear clips) should be removed to avoid confusion. A lost or damaged tag should be replaced immediately with a tag bearing the same number.
e. Testing and Vaccination.
   The following tests and vaccinations are indicated and normal procedure in the United States. Outside the United States, the protocol which you implement would be tailored to your country and local conditions.
   - As soon as possible after arriving as resident donors and recipients at the clinic (or into your personal herd), all cattle are tested (blood draw) for brucellosis, anaplasmosis, and bluetongue, and also TB tested (intradermal, right caudal fold). Recommended vaccinations include IBR, BVD, PI3, leptospirosis (five antigens) and vibrio fetus. Three days later when the TB tests are read, cattle would be vaccinated against blackleg (seven-way).
   - Based on the results of the blood tests, consult with your local or Federal veterinarian for further instructions regarding quarantine, retesting, selling reactors, treatments, and additional vaccinations.

1.2 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), total digestible nutrients (TDN), minerals and vitamins.

Approximately twenty 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.

TABLE 1. Essential Nutrients in Donor and Recipient Rations

Nutrient	Min. Level	Nutrient	Min. Level
TDN	59% of DM	CP	9.75%
CF	9.00%	Salt	0.50%
Calcium	0.30%	Phosphorus	0.20%
Magnesium	0.15%	Sulfur	0.20%
Iron	44.0 mg/kg DM	Manganese	19.8 mg/kg DM
Copper	11.0 mg/kg DM	Cobalt	0.11 mg/kg DM
Selenium	0.11 mg/kg DM	Molybdenum	0.99 mg/kg DM
Vitamin A	4000 iu/kg DM	Vitamin D	880 iu/kg DM
Vitamin E	4.40 iu/kg DM		

*see most current NRC for precise levels

Notes:

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

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

c) 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 requirements and /or size, and then formulate rations to meet the requirements of each group. Ideally, cattle would be weighed monthly, 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 can be accomplished by maintaining adequate levels and ratios of the essential nutrients while reducing the energy intake. However, for at least three weeks prior to superovulation and/or thawing-transferring, both donors and recipients should be on a positive energy intake level (i.e. gaining).

Cattle can tolerate a substantial excess of most nutrients. However, the manifestations of under-nutrition (levels less than National Research Council recommends) on reproduction have been well documented, contributing to:

- Reduced conception rate post-calving
- 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 be 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	TDN %	CP %	CF %	Ca %	P %
Alflafa hay, 2nd cut 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	24.0	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, 2nd cut 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, 2nd cut, late veg	62.0	17.0	27.0	0.66	0.34
Cotton seed meal, solv. Extd.	76.0	45.2	13.0	0.18	1.21
Soybean meal, solv. Extd.	84.0	49.9	7.0	0.33	0.71

There are direct chemical procedures which labs can use to establish how potent feedstuffs are for specific nutrients. In addition, there are feed fractions which can be isolated chemically, but which are combinations of nutrients that have some common properties permitting a chemical analysis of the group. These important feed fractions are separated chemically by a procedure named proximate analysis, which determines the following components:

- Water
- Ether extract (fats, oils)
- Crude fiber (CF; i.e. lignin, cellulose)
- Nitrogen free extract (simple sugars, starch)
- Crude protein (nitrogenous compounds)
- Ash (minerals; i.e. calcium and 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 are separated by the proximate analysis, except ash, and are potential sources of energy (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 extracts 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 1.0g 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. Note that ration proportions are expressed on a 100 percent dry matter basis.

TABLE 3. Daily Minimum Nutrient Requirements of 350kg Recipients and 600kg Nonlactating donors

	Daily gain, g	Feed, kg DM	TDN, %	CP, %	Ca g	P g	Vitamin A 1000iu	Vitamin D 1000iu
350 kg recipient	700	8.0	63.0	10.3	24	17	14.8	2.3
600 kg dry donor	200	9.6	56.0	9.2	23	20	25.4	3.9

A typical nutrition program requires feeding hay. Table 4 is a practical demonstration of Table 3, as Table 4 illustrates feeding 9.6kg (DM) brome hay daily to a 1,320 lb (600kg) donor cow. We assume 55 percent TDN and 10 percent crude protein content.

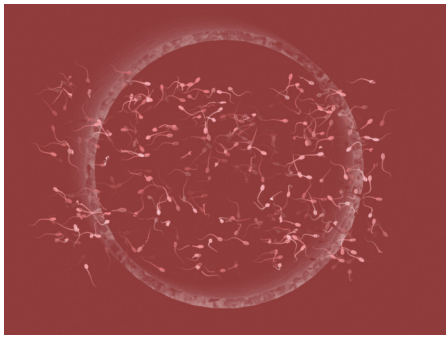
Calculation note: The kilograms of hay DM divided by percent DM of the hay being fed equals the kilograms of hay one must feed "as-is."

TABLE 4. Feeding Example

Nutrient	Required for 600 kg cow	Supplied by feeding 9.6 kg Brome
TDN	5.40 kg (56%)	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 severely cold weather when the cow needs more energy. Extra energy can be provided by increased hay intake or adding grain to the ration. (Corah,1987; Dunham and Call, 1989)

Chapter 2:
Superovulation, A.I., Synchronization



2.1 DONOR SUPEROVULATION

Cattle have a twenty-one day estrous cycle with day 0 characterized by visual display of heat. Approximately twelve hours after the end of heat, a single non-fertilized ova (commonly referred to as an egg), is released (ovulated) from one of the 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 on average results in six oocytes (non-fertilized “eggs”) and/or degenerate embryos, and six transferrable embryos. Generally speaking, a transferrable embryo will be a stage 4 morula through a stage 7 expanded blastocyst. More discussion on this can be found in section 4.3. Normally cattle release one ova per cycle. The single most important component in the ET protocol is superovulation, yet the physiology and biochemistry involved in successful superovulation are not completely understood. In general, 85 percent of all “normal” donors will respond to superovulation treatment averaging six transferable embryos, and these donors can be repeatedly superovulated at forty-five to sixty day intervals with only a slight decrease in embryo production over time.

Most embryo donors are dairy cows with above average milk production records, or beef cattle dams proven to produce heavy weaning weight calves and/or popular show string cattle. However, if a heifer carries desirable maternal and paternal genetics, she can be successfully superovulated as soon as regular estrous patterns are established.

Normal superovulated ovaries on flush day will be approximately the size of golf balls (40-45 mm diameter) with five to seven CLs, and one to two follicles greater than 7mm diameter per ovary. An over-response to FSH is characterized by large ovaries (tennis ball size or larger), many ovulations, and a high percent of non-fertilized oocytes and/or degenerate embryos. By knowing the width of your fingers, you should measure and record the volume (length x width x thickness) of each ovary on flush day, and also record the number of CLs and retained follicles (non-ovulations). This information should be considered when deciding how to adjust the FSH dose on subsequent stimulations.

If the donor over-responded, reduce the total FSH by 30 percent for the next stimulation. If the donor under-stimulated (ovaries on flush day are normal size and display essentially no CLs), increase the total FSH by thirty to forty percent for the next stimulation.

Described below are two protocols for superovulating cattle. Each method has been used successfully for many years on thousands of donors. Variations of each method regarding FSH dosage and the use of GnRH (or estradiol benzoate or estradiol 17 beta where allowed by law instead of GnRH) are expected as you record and study the yield of transferrable embryos (the response) from each subsequent superovulation treatment on a specific donor. (Chesta, Marana, Peres, & Bo, January 2006)

Two Cattle Superovulation Methods

#1) Superovulation based on no known previous heat date using a CIDR plus GnRH or estradiol (Table 5, *preferred method*).

#2) Superovulation based on donor's observed heat date (natural or prostaglandin-induced) and confirmed CL.
(estrus = Day 0, & no CIDR for donors) Table 5-1

As specified in Table 5, the combination of the CIDR vaginal progesterone implant and a 2cc injection of GnRH (Cystorelin, Factrel, or Fertagyl) have been shown to positively influence bovine superovulation, i.e. more transferable embryos in the search dish. A donor can be superovulated anytime during her cycle with this method as long as ovaries are normal size.

Superovulation dose; 8 injections of FSH over 4 days, decreasing dose

Bos taurus (Holstein)
400mg: 4cc morning & evening day 1, then 3cc am & pm, 2cc am & pm, 1cc am & pm (20cc total)

Bos taurus (i.e. Angus)
300mg: 3cc morning & evening day 1, then 2cc am & pm, 1.5cc am & pm, 1cc am & pm (15cc total)

Bos indicus (i.e Zebu, Nelore)
200mg: 2cc morning & evening day 1, then 1.5cc am & pm, 1cc am & pm, 0.5cc am & pm (10cc total)

Heifers
200mg more/less: 2cc morning & evening day 1, then 1.5cc am & pm, 1cc am & pm, 0.5cc am & pm (10cc total)

Example dates are shown on the following page in calendar format, to clarify when each step occurs.

FSH = Folltropin, Pluset GnRH = Cystorelin, Factrel, Fertagyl or Ovacyst
EB = estradiol benzoate E17b = estradiol 17beta
*EB and E17b are not approved for use in the US

The donor should be bred twice, 12 hours apart. Example: If the onset of standing heat is Sunday morning, you'd breed her Sunday evening and Monday morning. Alternatively, timed AI (no visual heat detection) as designated in Table 5 below yields conception rates very similar to "observed heat" breeding.

RED text below refers to DONOR instructions. Yellow highlighted text refers to recipient treatment.

Table 5 is the ***preferred method***

TABLE 5. Superovulation based on no known previous heat date: CIDR w/ GnRH (or EB or E17b instead of GnRH)

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
	1	**2**	**3**	**4**	**5**	**6**
7 AM = morning PM = evening	**8**	**9**	**10**	**11** Insert CIDR in recips. GnRH recips (2cc). Donor: Insert CIDR & inject 2.5mg EB or E17b	**12**	**13** GnRH donor (2cc) *DO NOT give GnRH if EB or E17b was given 2 days prior.
14	**15** FSH AM & PM	**16** FSH AM & PM	**17** FSH AM & PM	**18** FSH AM & PM Estrumate recips (2cc AM) & remove CIDR. Estrumate 2cc PM Remove CIDR PM	**19** Heat check recips beginning PM. Record ear tag number, date & time standing heat begins. Estrumate 2cc PM GnRH 2 cc PM	**20** (Donor due in heat AM) A.I. donor AM A.I. donor PM GnRH recips 2cc AM Heat check recips AM & PM.
21 Heat check recips AM & PM.	**22**	**23**	**24**	**25**	**26**	**27** FLUSH DAY Palpate each recipient on flush day (or day before) to confirm a CL. Transfer embryos into recipients with a defined CL, and which showed heat +/- 1 day of when the donor started standing heat.

- Research shows that giving a 2nd injection of Lutalyse or Estrumate 24 hours after the 1st injection contributes significantly to reducing circulatory progesterone (P4) levels to around 0.3-0.4ng/ml at time of AI. It's critical that P4 is low at/around AI time to yield high fertilization and a high percentage of quality score 1 & 2 embryos.
- Research shows that follicle growth and associated oocyte maturation occurs best during superovulation when circulatory progesterone is sustained throughout the entire 4-day FSH regimen. Therefore CIDR removal and prostaglandin injection should be the last events on day 4.

TABLE 5-1. Superovulation based on donor's observed heat (natural or prostaglandin-induced) & confirmed CL.
(estrus = Day 0, & no CIDR for donors)

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
	1	**2**	**3**	**4**	**5** Donor observed in standing heat.	**6**
7 AM = morning PM = evening	**8**	**9**	**10**	**11** Insert CIDR in recips. GnRH recips (2cc).	**12**	**13** Palpate donor. If CL is present, inject GnRH (2cc). If no CL is present, do not superovulate.
14	**15** FSH AM & PM	**16** FSH AM & PM	**17** FSH AM & PM	**18** FSH AM & PM Estrumate recips (2cc AM) & remove CIDR. Estrumate 2cc PM Remove CIDR PM	**19** Heat check recips beginning PM. Record ear tag number, date & time standing heat begins. Estrumate 2cc PM GnRH 2cc PM	**20** (Donor due in heat AM) A.I. donor AM A.I. donor PM GnRH recips 2cc AM Heat check recips AM & PM.
21 Heat check recips AM & PM.	**22**	**23**	**24**	**25**	**26**	**27** FLUSH DAY Palpate each recipient on flush day (or day before) to confirm a CL. Transfer embryos into recipients with a defined CL, and which showed heat +/- 1 day of when the donor started standing heat.

The CIDR vaginal implant is added into the superovulation schedule according to the dates shown in Table 5.

NOTE:

1. Given that the typical FSH dose is 20cc, you will encounter donors through sequential stimulations which demonstrate that they respond nicely to a significantly smaller total dose (i.e. 10-15cc). This historical donor response information allows you to rehydrate the lyophilized FSH pellet with all 20cc diluent, then immediately draw the extra 5-10cc of FSH that won't be used into a sterile syringe. Record the hydration date on the syringe barrel and then store the syringe in a kitchen freezer for future superovulations.

Figure 1. CIDR vaginal implant & insertion tool

2. For superovulated donors that are known to not completely ovulate (presence of multiple retained follicles on flush day) or those demonstrating extended heats (more than fourteen hours), you are encouraged to inject those donors with 2-4cc GnRH at the onset of the superovulated heat.

2.2 DONOR INSEMINATION

Embryo transfer technology is the combination of many small steps, each performed correctly. Attention to detail is essential. One crucial step is artificial insemination (AI) of the donor. An AI program that yields a high embryo fertilization rate is the result of three factors:

1. Dedicated heat detection
2. High quality (high fertility) semen
3. Correct AI technique (semen handling and placement)

HEAT DETECTION

Normally the donor would be in standing heat 36 to 48 hours after removing the CIDR and injecting Estrumate. However, it is not unusual for the onset of the donor's standing heat to occur twelve hours before or after the expected time. 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. The following guidelines will foster accurate determination of standing heat.

a) After the donor receives Estrumate (and the CIDR is removed), she should not be housed alone. Keep her with several other cows, or with a group of recipients that are due in heat.
b) The cattle lots or holding pens should not be cramped or slippery. Sexually active cattle need room to maneuver, plus sure footing for mounting.
c) Indications of approaching estrus include:
   - Donor refusing to eat (off feed)
   - Sudden drop in milk production

- Changes in behavior; restlessness
- Increased flow of clear mucus from the vagina

d) The best "tool" for heat detection is your eye. When you see the donor "stand" for another cow, record the date and time.

SEMEN QUALITY AND BLOOD TYPING

Prior to superovulation the semen quality should be 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 by a veterinarian experienced in evaluating semen motility.

Positive verification of an embryo transfer calf's sire and dam requires blood typing 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 blood types 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 differentiated by blood type analysis.

SEMEN HANDLING

Once semen is purchased and delivered to the ranch, dairy, or clinic, it must be maintained in a semen tank and submerged in liquid nitrogen. The nitrogen tank should be checked every other day to confirm that the tank is at least half-full of nitrogen.

Care should be exercised when removing an individual straw from its goblet. Using tweezers, grab on to the desired goblet within the neck of the tank, keeping the goblet at least 5cm (2") below the top of the nitrogen vapor level for no longer than ten seconds. With this technique, the remaining goblets in the canister are not subjected to temperature fluctuations. Using a second pair of tweezers, grasp the desired straw and raise it partially out of its goblet to verify the bull's name and registration number. Once identified, the straw may then be removed from the goblet and placed into a water bath.

The universal water bath thaw temperature for semen straws is 35°C (95°F) for a minimum of forty 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 is appropriate for thawing semen. Whichever container is used, the thaw temperature should be monitored closely, especially when several straws are thawed simultaneously. Once thawed, the straw should be wiped dry, the end opposite the plug squarely clipped off, and the inserted cotton-plug end 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. Breed the donor immediately after the semen has been thawed.

SEMEN PLACEMENT

The correct site for semen placement is in the body of the uterus. There is a tendency among low conception rate technicians to deposit semen too far into the uterus (perhaps an attempt to "horn" breed) resulting in bruising or small tears to the endometrial tissue. Sperm cells are living organisms and are sensitive to foreign material, including water and lubricants. Disposable plastic sleeves and AI sheaths should never be reused. After thawing, dry the straw completely before clipping and avoid contamination of the insemination rod as it passes through the vulva. A common technique for holding the vulva open is to wedge a folded V-shaped paper towel (apex of V facing downward) between the vulva lips.

TIMING OF INSEMINATION

Ova are randomly released (ovulated) from superovulated ovaries over a period of approximately twelve hours, beginning more or less twenty-four hours after the onset of standing heat. For this reason, the donor must be inseminated at least twice. The first breeding should occur twelve hours after the onset of standing heat, with the second breeding twelve hours after the first breeding. For example if the donor begins standing heat on Wednesday morning, you would breed her Wednesday evening and again Thursday morning. She would be flushed the next week on Wednesday.

If the donor is still in standing heat at the second breeding, she should be injected with 4cc GnRH and inseminated a third time twelve hours after the GnRH injection.

2.3 RECIPIENT SYNCHRONIZATION

Poor physical condition of the surrogate host (the recipient) will negatively influence the ET pregnancy rate. Ideally the recipients will have been at the clinic, ranch, or dairy for at least thirty days receiving a balanced ration resulting in a slight (a half-kilo per day) weight gain. Beyond providing for nutritionally sound, healthy recipients, the highest conception is achieved when an embryo is transplanted to a uterine environment which most closely resembles the environment that the embryo originated from.

Embryos collected from a donor on day 7 of her cycle (estrus = day 0) should be transplanted (transferred) into recipients that are day 6, 7, or 8 of their cycles. For example, to provide recipients for six embryos on flush day (or on thaw/transfer day), there must be at least six recipients that are at cycle day 6, 7, or 8 on that day with a CL. Since 5 percent of a naturally cycling (no drug synchronization involved) recipient herd can be expected to show heat on any particular day, it is unlikely that a small recipient herd

(less than forty head) can accommodate six embryos on a particular collection day. Certainly natural heats yield embryo recipients, however large herds and associated cattle feed expenses would be required to provide enough recipients for when multiple donors are collected on the same day, or when large numbers of frozen embryos are transferred on a specific day.

Fortunately, tables 6 and 6-1 outline methods which allow us to synchronize the cycle of recipients to donors.

TABLE 6. Transfers with heat detection *(or without = FTET)* **using CIDR and GnRH.** Expect about 85% to show heat.

- FTET (fixed time embryo transfer / no heat dates recorded) following the protocol below can be successful yielding average rates.
- For highest overall conception (10% above average), recording heat dates allows you to transfer less mature stage 4 embryos into day-6 recipients, & mature stage 7 embryos into day-8 recipients.

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
	1	**2**	**3**	**4**	**5**	**6**
7 AM = morning PM = evening	**8**	**9**	**10**	**11** Insert CIDR in recips. GnRH recips (2cc).	**12**	**13**
14	**15**	**16**	**17**	**18** Estrumate recips (2cc AM) & remove CIDR. ▲	**19** Heat check recips beginning PM. Record ear tag #, date & time standing heat begins. No heat checking required if FTET is used.	**20** GnRH recips 2cc AM Heat check recips AM & PM. No heat checking required if FTET is used.
21 Heat check recips AM & PM. No heat checking required if FTET is used.	**22**	**23**	**24**	**25**	**26**	**27** Palpate recips to confirm CL. FTET: Transfer into all recipients with a CL. Or, If recip has heat date & CL, match embryo stage to age of uterus.

▲ New: For low body condition (BCS<3) or acyclic cattle, inject 400 IU eCG (PMSG) at CIDR removal, *& again 7 days after ET* (= 14 days after AI). Note: eCG (equine chorionic gonadotropin) is not available in the US as of Jan 2012. cCG has FSH & LH activity and stimulates continued growth of the dominant follicle when given at CIDR removal. It stimulates increased progesterone production from the CL when given 7 days post transfer.

TABLE 6-1. Transfers with heat detection *(or without = FTET)* **using 1 or 2 injections of prostaglandin (PG), i.e. Estrumate or Lutalyse. No CIDR.**

- FTET (fixed time embryo transfer / no heat dates recorded) following the protocol below can be successful yielding average rates.
- For highest overall conception (10% above average), recording heat dates allows you to transfer less mature stage 4 embryos into day-6 recipients; & mature stage 7 embryos into day-8 recipients.

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
AM = morning PM = evening	**1**	**2**	**3**	**4**	**5**	**6**
7 Estrumate recips (2cc) Do not heat check.	**8**	**9**	**10**	**11**	**12**	**13**
14	**15**	**16**	**17**	**18** Estrumate recips (2cc AM) ▲	**19** Heat check recips beginning PM. Record ear tag #, date & time standing heat begins. No heat checking required if FTET is used.	**20** GnRH recips 2cc AM Heat check recips AM & PM.
21 Heat check recips AM & PM.	**22**	**23**	**24**	**25**	**26**	**27** Palpate recips to confirm CL. FTET: Transfer into all recipients with a CL. Or, If recip has heat date & CL, match embryo stage to age of uterus.

1. Expect approximately 70% to show heat if you inject PG twice. In the example above, you'd inject all animals on the 7th and 18th.
2. Expect 55% to show heat if you only inject PG on the 18th.

The minimum prerequisites for cattle on either schedule prior to injecting are: not pregnant, clean uterus, & free of reproductive diseases.

▲ New: For low body condition (BCS<3) or acyclic cattle, inject 400 IU eCG (PMSG) with the 2nd PG dose, and again *7 days after ET* (= 14 days after AI). Note: eCG (equine chorionic gonadotropin) is not available in the US as of Jan 2012. cCG has FSH & LH activity and stimulates continued growth of the dominant follicle when given, and stimulates increased progesterone production from the CL when given 7 days post transfer.

Chapter 3:
Embryo Recovery

3.1 COLLECTION DATE AND RECIPIENT IDENTIFICATION

Bovine embryo collection (flushing) is usually attempted on day 7 after estrus, which yields a development stage suitable for freezing. Embryos are normally located in the oviducts prior to day 6 and therefore cannot be collected nonsurgically. By day 8.5, the clear sphere (zona pellucida) surrounding the embryonic cell mass has fractured, leaving an embryo too mature for freezing. Pregnancy rates from fresh (not frozen) embryos transferred six to eight days after heat are not different. However, to collect embryos that are most likely to survive freezing and thawing, collection should be scheduled for day 7.

Leading up to estrus, the dominant follicle (a fluid-filled, fluctuant, blister-like structure on the ovarian surface; contains the ova) continues to grow. Approximately twenty-four hours after the onset of standing heat, the follicle ruptures releasing its oocyte.

The CL develops where the follicle ruptured, and by day 5 is normally large enough to be detected by palpation. The released ova is captured by the infundibulum and funneled into the oviduct where fertilization occurs. Around day 5.5 the embryo migrates into the uterine horn.

Whether collection is done on-farm or at the clinic, the first activity on collection day will be recipient examination. Assuming a day 7 collection, you would organize a list of all recipients that are 6, 7, or 8 days after heat. For the most accurate and sensitive palpation, pull on a plastic sleeve, remove the fingertips, then pull on a snug-fitting wrist-length latex examination glove. This combination offers inexpensive, disposable full-length protection and permits accurate identification of ovarian structures.

Palpate both ovaries of each animal to identify the presence of a CL and then use a marking crayon to place a mark on the hip corresponding to the uterine horn associated with the CL (CL right = slash on right hip). Nearly 65 percent of the time, cattle ovulate from the right ovary, and normally the active ovary for that cycle is significantly larger in size than the opposite ovary and the larger ovary would be expected to carry the CL for that cycle. Several structures can be encountered as you palpate ovaries, i.e. a CL, a retained follicle, a large cyst, or a combination of these structures.

Palpation on day 7 will identify CLs that vary in size. The classic “text-book” CL will resemble the eraser on a pencil, approximately 8mm in diameter, generally round, and 3-5mm in height. Larger CLs are commonly found that are 10-13mm diameter and 8mm in height. As your fingers gently glide over the surface of each ovary, you are trying to identify a circular, firm structure that stands up/away from the ovarian surface and displays a rather flat top. The CL generally resembles the shape of a plateau.

Fortunately, there is no difference in pregnancy rates between recipients displaying a large compared to a small CL on transfer day. Therefore, recipient selection requires that you identify all animals that have a CL on transfer day. If you cannot identify a CL, the animal should not receive an embryo.

After all recipients have been examined, place a paper copy of the results next to the microscope for convenient recipient/embryo matching later in the collection day. The list of useable recipients identifies how many embryos can be transferred fresh, and how many must be frozen.

Note: The average pregnancy rate will be slightly higher using virgin heifers vs. cows, if all other variables are equal.

3.2 INSTRUMENTS, SUPPLIES AND SETUP

After identifying useable recipients, the next step is to organize the instruments and supplies listed below for collection.

- D-PBS
- Catheter (Fig. 3) and stylet
- 20cc and 6cc syringes
- Lidocaine
- Shoulder-length disposable sleeves
- Y-tubing (Fig. 2)
- Filter (Fig 4)
- 18ga x 1.5" needles
- Hemostats and scissor (Fig. 5)
- Latex exam gloves
- Stereozoom binocular microscope (Fig 6)
- Electronic controlled rate freezer (Fig 7)

Figure 2. Y tubing

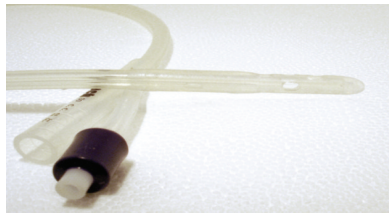


Figure 3. Vortech™ Catheter

Figure 4. ZONA collection filter

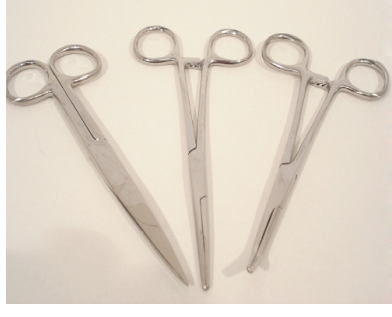


Figure 5. Hemostats and scissor

Figure 6. Binocular microscope

Figure 7. Controlled rate freezer

Be sure that the lab work bench is clean, free of dust and smoke, draft-free and (ideally) temperature controlled. Most on-farm collections require that you set up the lab equipment in the client's barn, milk tank room, under a shaded area next to the squeeze chute, at the client's kitchen table, or occasionally the client has built a room dedicated for your searching requirements.

Whenever possible it's best to work in cooler (50-75°F) lab room temperatures vs. warmer. As the microscopic embryo leaves the uterus and enters the filter device, warm temperatures hasten the death of embryos outside the uterus by increasing the metabolism of the embryo. Unfortunately, the collection medium is a non-perfect substitution for the uterine fluid which the embryo has been living in.

This instructional manual teaches the technique referred to as horn flushing where the collection medium enters the uterus via gravity. Embryos on day 7 in superovulated donors are normally located in the upper one-third of each uterine horn. Therefore horn flushing directs 100% of the recovery medium into the anterior half of the horns where embryos are most likely to be found. The following sequence describes preparing the items used for collection.

Select a 1-liter bag of D-PBS (Dulbecco's phosphate buffered saline) containing the surfactant poly vinyl alcohol (PV). Earlier versions of collection medium required that you add a serum product immediately prior to collection, however today 95 percent of all collections use D-PBS supplemented with PV and low levels of the antibiotics gentamycin and kanamycin. An example of this is BioLife Advantage™ Collection Medium.

Attach a Y-tubing set to the flush bag. Close both pinch clamps, and then insert the spike-end of the tubing into the spike port of the bag. Attach a filter device to the other long arm of the tubing set. If more than one donor is being collected, write the donor's name on the filter sidewall with a permanent marker. Keep the catheter adapter end of the tubing set sealed or clamped shut.

Purge air from the Y-tubing set and at the same time allow PBS to flow into the filter device until there is a 20-30mm depth of PBS in the filter. This is accomplished by opening both tubing pinch clamps, closing off the short tubing section leading to the Foley connector with a hemostat, and opening the pinch clamp on the filter outflow port. Next, lay the liter bag on its side on a table and then lower the tubing and filter below the table surface. Hold the cup filter so that the open end of the outflow port points upward. Gravity will cause medium to flow through the tubing and into the filter cup. When the cup contains a 20-30mm depth of medium, close all pinch clamps and return the tubing and filter to the table surface.

Select and test a two-way Foley catheter. Long (23") silicone catheters are popular because they can be autoclaved and re-used, and were designed specifically for cattle embryo collection, having at least six large off-set eyelets to maximize collection efficiency. An example of this is the Vortech™ catheter. Alternatively, 18" long disposable latex catheters are available with two eyelets.

You must also select a catheter diameter, referred to as its French (fr) size or its gauge. Most bovine ET catheters are available in 12fr to 24fr (even-numbered increments). I suggest that you select the largest diameter catheter that will pass the specific donor's cervix using normal manipulation efforts. The 16fr catheter will pass most virgin heifers, while 18fr and larger are popular choices for cows.

Catheters are then selected based on the balloon (cuff) capacity, either 5cc or 30cc balloon capacity. Injecting 15cc of air into the 5cc model causes the cuff to inflate high/tall with a horizontal width of approximately 20mm, compared to the 30cc cuff which inflates lower in height and carries a wider horizontal profile. Balloon selection is personal preference, based on how easily you can palpate the inflation and location of the cuff inside the uterine horn.

Allow several drops of flush medium to drip from the Foley end of the Y-tubing down into the catheter, and then insert the sterile stainless steel stylet into the catheter. Keep the catheter in its sterile bag at all times when not in the donor, to keep it clean.
Using a 20ml syringe and with the catheter in its packet, inflate the balloon with 20cc of air and then pull all 20cc of air back out of the balloon. Catheter preparation is complete after you stretch the catheter 0.5" back toward the hub of the stylet and then gently clamp the 0.25" posterior end of the catheter hub onto the stylet using a hemostat. Prepare the epidural syringe by attaching an 18ga x 1.5" needle to a 6cc syringe. Aspirate 5-6cc of 2% lidocaine and then cover the needle.

Prepare a 6cc syringe (18ga x 1.0" needle) with 4cc Estrumate, which will be given to the donor after collection. This serves to initiate regression of the multiple CLs and should cause estrus within 4-6 days. Causing the donor to cycle after collection prevents her from becoming pregnant due to an embryo that wasn't recovered during the flush process.

Other items that should be placed on your collection tray include several shoulder-length sleeves and latex exam gloves, three hemostats, the lidocaine bottle and several more needles, and paper and pencil to record ovarian structures and volume.

Assemble the microscope and test the illuminator.

Assemble the electronic embryo freezer but do not add nitrogen to the bath.

3.3 DONOR PREPARATION FOR FLUSHING

The collection procedure normally takes twenty to thirty minutes, so if the donor is restless, confine her in a squeeze chute or stanchion. If the donor is a large, deep-bodied animal (uterus extends low into the body cavity and is heavy), the collection process will proceed easier if the donor's front half can be elevated 10-12". For example, the floor of the chute can be designed so that it inclines as she walks into the chute. Or if collecting in a tie stall dairy barn, tie the donor's neck to the horizontal bar in front of her (with slack included) and then ease her backward until her back legs step into the gutter that runs behind her stall. To prevent her from moving back up onto the stall, place a horizontal bar under her neck and secure each end to the dividers on each side of her stall.

After securing the donor, remove any significant debris from her tail head where the epidural will be administered. Wash this area with an antiseptic liquid such as iodine scrub. Rinse with clean water. **For descriptive purposes in this text, we will assume that you palpate with your left hand.**

Pull on a full-length sleeve and remove all of the manure from her rectum. Then with the same gloved hand, grasp the tail firmly 3" below the rectum and elevate the tail to a position slightly above horizontal. This tail control and position allows you to control her movements and reduces her desire to kick when administering the epidural. Using the thumbnail of your ungloved right hand, press down firmly on the scrubbed tail-head region to locate an indentation between two vertebrae. This will be the injection site. Plunge the needle (attached to the lidocaine syringe) deep into the intervertebral space until the needle contacts bone. Once contact is made, begin to inject lidocaine. Initially there will be back pressure on the plunger, so withdraw the needle slowly in 1mm increments as you continue to push the plunger. As the needle opening enters the intervertebral space, the plunger will push easily and all 5-6ml lidocaine will flow quickly into the space. When this injection is administered correctly, the tail will become limp after one minute and rectal peristalsis essentially stops. If the tail does not become limp, repeat the injection (with a new needle) giving attention to the depth of needle placement and ease of injection (lack of back pressure). Tie the limp tail up and out of the way to her left side.

Strip off the dirty sleeve and pull on a clean, fingerless sleeve and latex exam glove. Using clean water, wash all fecal material away from the rectal sphincter and the vulva. Part the vulva lips and rinse thoroughly. Blot dry with paper towels.

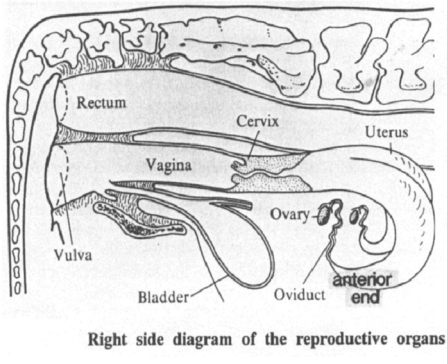
This same preparation sequence would be used on **recipients** immediately prior to transfer.

Suspend the 1L bag of D-PBS with attached Y-tubing and filter so that the lower edge of the bag is approximately one meter above her tail head.

3.4 RECOVERY PROCEDURE

Lubricate your left (palpation) hand and arm with water and enter the rectum. If you require a lubricant other than water, be sure that if lubricant drips down over the vulva, the lubricant is not allowed to get inside the catheter (or the transfer rod) as you enter the animal. All lubricant is toxic to the embryo!

Once inside the donor, identify the cervix and uterine horns (Fig. 8).



Right side diagram of the reproductive organs

Figure 8. Diagram of cattle reproductive organs

Estimate the number of ovulations (CLs) and retained follicles, and measure the volume (length x width x height) of each ovary. By measuring the width of your palpation fingers, you can quickly lay them over each ovary and estimate volume. Record this information for future FSH dosing adjustments.

Remove the catheter from its protective packet, part the vulva, insert the catheter into the vagina, and manipulate it through the cervix as if you were performing AI. As you work with the body and horns from this point onward, you want to scoop and retract as much of the uterus as possible up/onto the pelvic floor. Otherwise, the tract will continue to stretch away from you and if the donor is rather large and deep, catheter placement and fluid recovery can be challenging. Immediately after the catheter tip enters the uterine body, manipulate the tract so that the right (or left) horn is directly in front of the catheter, rather than directing the catheter toward the horn.

Downward pressure by the middle finger will identify the y-split area where the right and left horns separate (the palpable bifurcation). As you continue to lift and position the right horn, gentle downward pressure by your index finger on the right horn will identify the catheter tip location as it slides into the right horn. Advance the catheter tip 6-7cm beyond the bifurcation. The catheter is now in the correct location and is ready for inflation. Successful horn flushing technique focuses on location and inflation, i.e. (1) your ability to locate the catheter far enough anterior to (ahead of) the bifurcation so that the cuff will accurately seal against the inside wall of the horn and not dis-

lodge during the flush; and (2) insuring the correct inflation volume of the cuff. Too much air and the endometrial tissue will split. Not enough air and the cuff will slip out of position, resulting in a body flush and less accurate fluid recovery.

At this point, ask your assistant to snugly attach a 20cc syringe to the catheter inflation valve (plunger pulled back to the 20 mark) and then push 12cc of air into the balloon. The assistant holds the plunger at this mark while you palpate the horn to locate the partially inflated balloon. If the balloon feels prominent/easy to identify/easy to define its location, then there is enough air in the balloon. If the balloon is not easy to identify, add air in 1cc increments until you like the "feel" of the balloon. *If the location is correct,* you will not require more than 16cc of inflation in the largest donor to seal off the interior of the horn and maintain the location.

Most cows require 14-16cc of air in the cuff while virgin heifers will only need 12-13cc of inflation. Do not pull backward on the catheter at this stage to determine if the cuff is properly seated, since this only serves to dislodge the cuff from its location. Learn to trust what you feel with your fingers as you gently palpate the inflated balloon. If the balloon feels prominent/easy to identify/easy to define its location, then there is enough air in the balloon. It's important to avoid over-inflation, which results in membrane separation and the flush fluid being absorbed into the tissue. Catheter **location** within the horn, and cuff **inflation** are critical factors for a successful collection.

Once you are confident that cuff location and inflation are correct, remove the hemostat which is holding the catheter to the stylet and clamp the hemostat ahead of the catheter inflation valve (i.e. on the short section of tubing connecting the inflation valve to the shaft of the catheter). With the hemostat in place, the assistant then disconnects the air syringe from the inflation valve.

To remove the stylet, place your thumb and index finger around the opening of the catheter to serve as a "back-stop" so the catheter does not pull backward as your assistant gently rotates and pulls the stylet out of the catheter. Place the stylet inside the empty catheter packet on the instrument tray.

To counteract downward pull on the catheter which could dislodge the cuff, slip the tips of a second closed hemostat through one finger-ring of the first hemostat, which is clamping off the inflation valve, and then clamp the second hemostat to the donor's hair coat near her tailhead. Using two hands, your assistant then firmly inserts the Foley adapter end of the Y-tubing into the open end of the catheter. You are now ready to introduce D-PBS into the uterine horn.

The objective is to repeatedly fill and empty the right horn, remove the catheter, and relocate into the left horn. Then repeat the process with the remaining 500ml of flush medium. In order to properly monitor horn filling and emptying, it is crucial that the uterine horn is manipulated so you can place your fingers around the anterior half of the horn (the section of the horn farthest from the cervix), so that the upper (smaller diameter) half 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, where embryos are located seven days after estrus.

Once you are comfortable with the position of the horn, close the clamp on the Y-tubing leading to the filter, and open the clamp which allows DPBS to enter the uterus. Remember, the uterine horn is a muscle and must be allowed to gradually stretch. Therefore the first fill must be conservative, basically to establish flow, which allows you to visualize fluid flowing down the tube, into the uterus, and then exiting and entering the filter device. Be sure the outflow port on the filter is open. Horn fill/expansion is constantly monitored by gently tapping (thumping) the upper third of the horn as it fills.

When your fingers tell you the horn is full (it is tight, firm, turgid), close the inflow clamp and open the outflow clamp which allows fluid to rush toward the filter. Immediately begin to gently massage (knead) the horn using a motion similar to hand-milking a cow's teat, beginning at the smallest diameter area of the horn and finishing the stroke at the middle of the horn. Repeat this massage procedure until fluid stops flowing into the filter. Note: It normally only takes sixty to ninety seconds to fill the horn. However, it will empty in twenty to thirty seconds.

When 500cc of medium remains in the suspended bag, the catheter should be relocated into the other horn according to the following steps. If there is fecal material around the vulva, wash if off with water. Confirm that the filter's outflow port is open, the clamp leading to the bag is closed, and the clamp leading to the filter is open.

Remove the hemostats attached to the catheter and deflate the cuff by attaching the air syringe to the catheter inflation valve and pulling back on the plunger. Alternatively, press one of the hemostat tips into the valve for two to three seconds, which will release the air in the cuff. Grasp the catheter between thumb and index finger where the catheter joins the Y-tubing. Pull the catheter (still connected to the Y-tubing) out of the cow and elevate it so that the liquid remaining in the down flow arm of the Y-tubing enters the filter.

Be careful handling the catheter outside the uterus so that it remains clean. On flush day cattle are "mid-cycle" and prone to uterine infection, more than cattle at estrus which are being artificially inseminated.

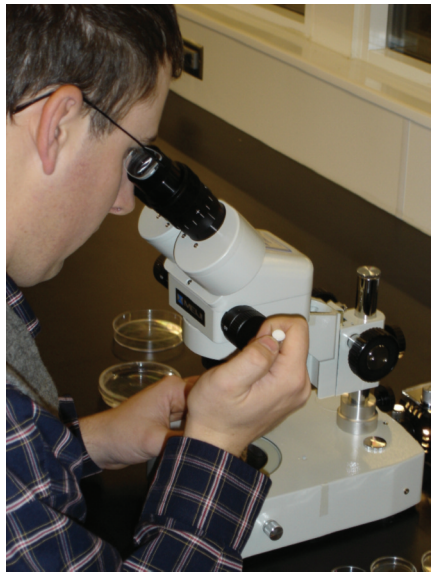
Your assistant then separates the Y-tubing from the catheter, inserts the stylet into the catheter, clamps the open end of the catheter onto the stylet, and hands the catheter to you. Insert the catheter into the horn which has not been flushed and follow the same flush procedure you used on the first horn. When the suspended bag is empty, remove the catheter, detach the filter from the y-tubing, and transport the filter to a secure location next to the microscope. Before releasing the donor, some technicians will infuse the uterus with an antimicrobial product (i.e. dilute chlorhexidine). However, this is not necessary if your flush technique is clean and correct.

I would suggest you search for embryos *before* infusing (if this is your choice) or releasing the donor. If the search yields significantly less embryos or oocytes than what was indicated by the CL count, put the donor in the stall and flush her again. You will always learn or gain something from this second flush, like the following:

A) If you recover embryos from the second flush, your first flush efforts were less than adequate.

B) If the second flush goes well (as you thought the first flush went) and no more embryos are recovered, you can conclude there were no more embryos/oocytes to recover from the uterine horns. The non-recovered eggs associated with the ovulations most likely were retained in the oviducts, or they were never captured by the infundibulum. Before releasing the donor, inject her with 4cc Estrumate. This injection serves to lyse the multiple CLs and return the ovaries to normal size. It also keeps the donor from becoming pregnant if an embryo remained in the uterus.

Chapter 4:
Embryo Handling



Accurate interpretation of embryo morphology is imperative for:

a. Separating fertilized embryos from nonfertilized ova.
b. Matching recipient estrous cycle maturity with embryo maturity.
c. Selecting the embryo stage of development that most likely will survive freezing and thawing.

When examining embryos, a helpful technique is to roll the embryo by bumping it with a sterile in-vitro fertilization (IVF) catheter tip, or by gently moving the liquid in the dish causing the embryo to roll. The embryo should be viewed from all sides before making a final determination on stage and quality. To help interpret variance in embryo morphology, please refer to the embryology section of the accompanying DVD.

4.1 RINSING THE FILTER

By following the sequential steps in this section you will discover whether the super-ovulation and collection work has been fruitful! Begin by washing and drying your hands, then turn on the microscope illuminator to high power. Adjust the sub-stage reflector so the light bounces off the frosted (not mirrored) side of the reflector. If you collected into a cup-style filter like the ZONA™ filter, you must transfer the filter contents into a 90mm diameter round gridded search dish.

Using a permanent ink marker, write the donor's name on the lid and sidewall of the search dish, then remove the lid and place it upside down next to the dish. Remove a 20cc all plastic (two-part) syringe (CellSafe™ or HSW) from its package, attach a 16ga needle and fill the syringe with 20cc of flush medium, rinse medium, or holding medium. The filter rinse process requires 40-60cc to properly rinse the filter and screen.

Please note that you must only use two-part syringes (CellSafe™ brand or HSW brand) if the liquid in the syringe will contact embryos. Syringes that include a black rubber or butyl stopper around the end of the plunger are toxic to embryos. The stopper is coated with a silicone-type surfactant that will mix with the liquid in the barrel of the syringe, leading to significantly reduced pregnancy rates.

Holding the filter at eye level, open the outflow port and drain off liquid until 6-7mm of fluid depth remains. Close the outflow port. Gently rotate the filter, which will cause the medium to swirl, and then invert the cup and dump the contents into the search dish (swirl and dump).

Use the 20cc syringe to wash down the filter sidewalls, leaving a 6-7mm fluid depth in the filter. Remove the needle from the syringe, hold the filter at eye level, and then place the syringe nipple *directly onto the screen.* Move the nipple over the screen as you slowly aspirate, drawing mucus and uterine debris (and embryos) off the screen and into the syringe. Gently expel the syringe contents into the search dish. If there are bubbles in the syringe, expel them into the inverted lid for later searching (after the bubbles have resolved to liquid).

If there is still significant mucus and debris on the screen, draw another 20cc of new rinse medium into the syringe and perform the direct-aspirate procedure again on the screen. Finish the filter rinse process with one more swirl and dump process. The filter and syringe can now be discarded.

4.2 SEARCHING AND EXTRACTION

Using both hands, carefully place the search dish onto the microscope stage. Embryos will settle to the bottom of the plate within one to two minutes. Begin a systematic

search of the dish by adjusting the zoom magnification knob to low power yielding 10-15x total magnification. Turn the back focus knob up/down until the bottom of the search dish is in sharp focus. 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 or down.

Move the plate on the stage so the search begins with the grid (cell) in the upper left corner and proceeds to the right in a Z-pattern, i.e. over, down, over, down, etc. The cells are 10mm x 10mm, and low magnification allows you to examine an entire cell in a single field of view. Concentrate on what you are looking for: a clear, shiny circle (the zona pellucida). Move through the entire dish examining all cells and extracting embryos that are in the open spaces, not entangled in debris. Next, slowly rotate the plate 360° while carefully examining the dark area where the vertical sidewall meets the horizontal bottom. This area is difficult to get in focus and may require focus adjustment as you encircle the plate.

After searching all grids, use the IVF catheter tip to gently stir and mix the plate contents. Let the solution settle for one to two minutes, and then search the entire dish one more time. Mucus and/or uterine tissue debris are part of a normal flush, yet they make embryo isolation and extraction more challenging. Patience and persistence are appropriate at this time.

For this second time through the dish, use a 20ga x 1.5" needle to move, stretch, and overturn each piece of debris and mucus, as you search more closely for embryos embedded in the debris. To remove an embryo embedded in mucus, use two 20ga x 1.5" needles. Viewing the embryo on low power, use one needle to pin down mucus on one side of the embryo, and then place the other needle into the mucus on the opposite side of the embryo, and gently pull/stretch the mucus away from the embryo. This action repeated several times will release the embryo from the mucus and/or debris.
TIP: If an embryo with/without mucus attaches itself to the needle tip and you can't get the embryo to release, raise the needle up and out of the liquid. As the needle tip breaks the surface tension, the embryo will remain behind in the dish of medium.

When an embryo is located, firmly attach an IVF catheter to a 1cc three-part syringe (Fig. 9). I prefer the three-part syringe because the black rubber stopper with silicone allows the plunger to move easily and smoothly through the barrel. Note that **you will not need to and must not draw medium into the syringe barrel.** The IVF catheter provides adequate internal volume for aspirating and expelling (manipulating) embryos, therefore medium never has to enter the syringe.

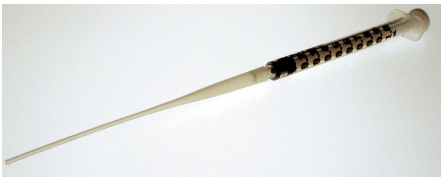


Figure 9. IVF catheter attached to a 1cc three-part syringe

TIP: Sometimes the open hub of the IVF catheter is slightly longer than the luer slip nipple of the 1cc syringe, thus a snug, airtight attachment is not possible. This is easily corrected by removing 2-3mm of the catheter hub length using a sharp scissor.

Prepare a petri dish (Solution™ 35x10mm) or a Solution™ 6-well dish to accept the embryo by writing the donor's name on the dish lid and sidewall. **The use of embryo toxicity certified plastic-ware is essential for optimum embryo viability** (Lane, Mitchell, Kashman, Feil, Wakefield, & Zander-Fox, January 2008). At a minimum, plasticware should be bioassay certified for the one-cell mouse embryo assay (Tucker & Jansen, 2002). Additional levels of confidence are achieved by HSSA (human sperm survival assay) certification and also low-level endotoxin certification. The Solution™ plasticware line displays all three certifications.

Draw 8-10cc of holding/transfer medium into a new 10 or 20ml CellSafe™ syringe, remove the needle, and attach a syringe filter to the syringe. Packaged, manufactured holding/transfer media are sterile, however I prefer the added insurance of using a syringe filter (0.2u ETMedia™) to sterile-filter any liquid that I place an embryo into that will eventually be deposited into the uterus. Apply pressure to the plunger to discard the first four to five drops of medium from the syringe filter, and then allow six to eight drops to fall into the dry petri dish. Rinse the dish by swirling the droplets in the dish and then discard the medium. Continue to fill the dish from the syringe filter until the dish is 65 percent full.

Grasp the 1cc syringe with attached IVF catheter and pull the plunger back 0.2ml. This air space will serve to expel the embryo from the catheter. Lower the catheter tip into the top layer of medium in the search dish and aspirate a 15mm column of liquid into the small open end of the catheter.

As you look through the microscope on low power with the embryo positioned under the center of the microscope head, lower the catheter tip adjacent to the embryo. Applying a slow, controlled one-hand aspiration technique, gently "walk" the embryo into the catheter tip. Once the embryo is 2-3mm inside the catheter, stop aspirating.

Lift the catheter out of the search dish, and then position the tip directly over the center of the prepared petri dish or multi-well dish. Lower the tip until it contacts the surface of the liquid, and then slowly push the plunger and expel all of the liquid contained

in the catheter tip (a 15-20mm length of medium). Do not expel air into the petri dish liquid, which would result in bubbles that embryos can attach to, and also obscures your ability to view into/through the liquid. Continue the second search of the dish, removing and placing all embryos found into the single petri dish (or multi-well dish).

4.3 EMBRYO GRADING

Refer to Appendix A. to familiarize yourself with numerical codes describing stage of development. Even though some visual subjectivity will influence the stage code numbers that we assign, it is the best system available to our industry. Therefore it is crucial that you take time to accurately examine embryos from various angles. One dimensional pictures and drawings are good learning tools. However, it is best to examine the live, three-dimensional embryo from several angles to determine the most correct stage of development and quality score.

Stage numbers 1-9 refer to the maturity of the main cell mass, i.e. the aggregation of individual cells (blastomeres). Roll the embryo over as you examine it at low power, and then at higher (50-90x) magnification. As magnification increases, you will want to increase the brightness of illumination and perhaps adjust the sub-stage reflector. Assign a stage code number to the embryo which most closely resembles the stages illustrated in Appendix A.

Usually the most difficult stages for the beginning student to correctly identify are the stage 1 (1-cell) nonfertile vs. the stage 4 morula. To help make this distinction, remember that the nonfertile is a single, large cell enclosed in a membrane. Imagine the single cell as an inflated beach ball having a uniformly smooth, tight, shiny "skin." And surrounding the beach ball is the zona pellucida! Contrast this to the stage 4 morula, which looks like a uniformly tight cluster of grapes, or a soccer ball with its tightly sewn patchwork covering. As you compare these two stages, concentrate on the edge of the mass. The edge line of the nonfertile will be smooth and continuous, whereas the edge of the morula will have a scalloped appearance.

A good rule of thumb is that if you can count thirty blastomeres more or less, it's a stage 3 early morula. Commercial ET protocol describes stage 2 (2-16 cell) embryos as degenerate (dead) and discards them, and of course stage 1 ova also.

Quality numbers 1-4 generally refer to the composition and quantity of "debris" which is not part of the main cell mass. Debris is primarily comprised of extruded blastomeres that failed to be included in the primary cell mass. These extruded blastomeres are located in the perivitelline (PV) space, which is the area between the main cell mass and the zona pellucida. Stages 3, 4, and 5 frequently receive a quality score other than 1. As the blastomeres in these younger stages continue to divide, cells which fail to join

the main mass are easily visible in the PV space. If there are no extruded blastomeres in the PV space, the quality score is 1, otherwise assign a score of 2 or 3.

Note that the guidelines suggested by the Manual of the International Embryo Transfer Society (Stringfellow & Seidel, 1998) relative to quality scoring are based on subjective visual interpretation. 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. The organization's Web site is www.IETS.org.

There is no difference in pregnancy rate between stages 4, 5, 6, or 7 quality 1 embryos compared to the same stages scored quality 2. The "strength" of the embryo comes from the integrity and maturity of the main cell mass, and is not significantly influenced by the extruded blastomeres, vacuoles, or dead cells in the PV space.

Good embryos for commercial ET purposes would normally be developmental stage 4 (morula), 5 (early blastocyst), 6 (blastocyst) and 7 (expanded blastocyst). A stage 3 early morula should not be frozen. However, good results can be achieved with an early morula when transferred fresh to a day 6 recipient. The accompanying DVD includes an embryology section showing developmental stages of numerous embryos. Stage of development, embryo quality, and integrity of the zona pellucida determines if the embryo is suitable for freezing. Another excellent reference which includes photographs of graded embryos is the *Manual of the International Embryo Transfer Society.*

4.4 EMBRYO ASSIGNMENT

Prepare two new petri dishes, each labeled on the lid and sidewall with the donor's name. A multi-well dish can also be used. Rinse and then fill each dish 65 percent full with filtered holding medium. Write "transfer" on one dish lid, and write "freeze" on the other lid. At the completion of this evaluation/sorting process, what remains in the original dish will be non-fertile ova and degenerate embryos, otherwise referred to collectively as "bad eggs or bad embryos." Good embryos are those that yield average pregnancy rates when frozen or transferred fresh. Average pregnancy rates are 65 percent for fresh transfers and 55 percent for frozen/thawed embryos.

With all of the embryos in our example now isolated into a single petri dish, the next step is to assign a stage and quality score to each. A typical collection would be comprised of various stages of development acceptable for transfer or freezing, plus several degenerate embryos and (unfortunately) one to two nonfertile ova.

For this example we'll assume that we have five useable recipients, and the collection yielded twelve eggs total. Grading the twelve eggs resulted in the following: seven

transferrable embryos (two stage 7 expanded blastocysts, two stage 6 blastocysts, three stage 4 morulae); three stage 2 degenerate embryos, and 2 nonfertile ova. Therefore we must decide which embryos will be transferred fresh and which will be frozen. Because the frozen/thawed survival rate for stage 7 expanded blastocysts can be 5-10 percent less than blastocysts and morulae, I would suggest the following assignment.

a) Freeze the two stage 6 blastocysts.
b) Transfer the two stage 7 expanded blastocysts and also the three stage 4 morulae.

Note that the stage 7 and 4 embryos would yield the same good results transferred fresh, assuming you had recipient heat dates similar to the "age" of the embryos. This would mean transferring the stage 7 embryos into recipients that were in heat eight days earlier, and transferring the stage 4 embryos into recipients that cycled six days earlier.

4.5 LOADING STRAWS

Embryos must be aspirated into 0.25cc straws prior to freezing or before transferring. Your ability to load straws correctly, efficiently, and repetitively is partially an art that gets better and easier with practice. Whether loading straws with EG for freezing, or with holding medium for transferring fresh embryos or thawed glycerol embryos, the loading sequence is essentially the same. Refer to Fig. 10 for images of loaded straws.

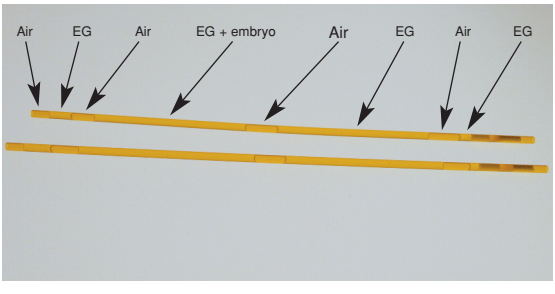


Figure 10. Loaded straws showing medium and air

a. If you are transferring fresh embryos or thawed glycerol embryos, label one petri dish on the side and lid with the word HOLDING and then syringe-filter holding medium into the dish. The dish will be used to isolate the embryo that you are loading, and for rinsing the straw and drawing the sections of holding medium into the straw.

   The sequence that follows describes loading embryos for fresh transfer, or for reloading thawed glycerol embryos for transfer. However, the steps are the same if you are loading embryos for freezing. The difference is that EG would be used instead of holding medium. Note that essentially all segments of medium and air are loaded visually (not looking through the microscope) so that the segment lengths are correct. Only as you guide the embryo into the straw will the microscope be used.

b. Place the dish containing all of the embryos to be transferred onto the microscope stage. Aspirate one of the embryos from the group into the IVF catheter. Remove this dish from the stage and replace it with the newly labeled dish of holding medium. Release the embryo into the dish and then maneuver it to the center of the dish. Grade the embryo for stage and quality and record this information under section B-Certificate of Transfer.

c. Insert the cotton-plugged end of the new, dry 0.25cc straw directly into the tip of a 1cc 3-part luer-slip syringe. You may have to hold the straw at a slight angle as you push and turn the straw against the tip opening. Most of the length of the cotton plug will insert into the syringe tip, resulting in a snug, air-sight seal that allows you to draw a vacuum through the straw.

d. **Rinse the straw** by first pulling the syringe plunger back 0.2cc, and then lower the open end of the straw into the dish of holding medium containing the single embryo. Place the straw tip near the edge of the dish, away from the embryo which is located in the center of the dish. Aspirate a 20mm column of medium, raise the straw out of the medium, and then pull the medium to within 10mm of the cotton plug. **Do not wet any part of the plug!** Slowly depress the plunger to discard the medium.

e. The straw is now ready to load with segments of medium, air, and the embryo (column length measurements that follow are approximate). Aspirate 10mm of medium and then raise the straw tip out of the dish. **This segment will eventually wet the cotton plug.**

f. Gently push up/back on the plunger using your thumb and index finger until a 6mm air space has appeared at the end of the straw.

g. Lower the straw into the dish and aspirate 40mm of medium, and then raise the straw tip out of the dish. **This segment is referred to as the "push" liquid.**

h. Gently push up/back on the plunger using your thumb and index finger until a 6mm air space has appeared at the end of the straw.

i. Lower the straw into the dish and aspirate 3mm of medium. This creates the beginning of the segment that will hold the embryo. While viewing the embryo on low magnification, place the edge of the straw adjacent to the embryo. Gently bump (push) the embryo and lift the straw tip slightly upward, similar to a scooping action. This motion will cause the embryo to lift off the bottom of the dish and "float" directly in front of the straw, allowing you to aspirate the embryo into the straw.

As soon as you see the embryo enter the straw, move your eyes away from the eyepieces and visually monitor the column length, as you continue to pull holding medium into the straw until the section is **45mm long more/less.** Maintain the straw at an approximate 45° angle as you continue to lengthen this segment, to help the embryo remain in the center of the segment. Raise the straw tip out of the dish. **This segment contains the embryo.**

j. Gently push up/back on the plunger using your thumb and index finger until a 6mm air space has appeared at the end of the straw.

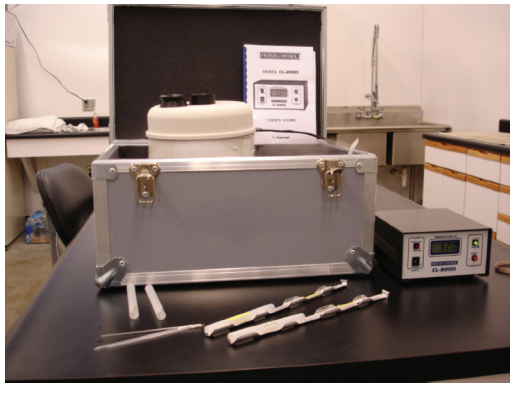
k. Lower the straw into the dish and aspirate 5mm of medium, and then raise the straw tip out of the dish. This is referred to as the entry segment of medium. The remaining 4-5mm of straw should contain no medium and will accept the sealing plug. Do not wet the plug yet.

l. Before wetting the plug, you must confirm that the embryo is captured in the proper segment of medium. Remove the dish of holding medium from the stage. Lay the loaded straw horizontally on the microscope stage and hold it there with an index finger. The embryo will sink to the bottom edge of the straw. However, it is not possible to focus on the bottom of the straw. Therefore, focus (low magnification) on the top edge of the straw, at one end of the segment which contains the embryo. As you look through the microscope, slowly pull the straw across the stage and simultaneously roll the straw. If the embryo is in the segment, you will be able to see it as the embryo momentarily floats at the top edge of the straw. If you do not see the embryo in the middle portion of the segment, be sure to closely examine each end of the segment in the darkened bend of the meniscus.

m. If you identify the embryo and it is in the correct segment of medium, you should then pull the syringe plunger back until the center of the cotton plug is saturated with medium. The center of the cotton plug contains poly vinyl chloride (PVC) powder which swells and expands when wet, serving as a semi-permanent seal at the end of the straw. Detach the syringe from the straw, which is ready for transfer.

If the examination determines the embryo is in the wrong position or is not in the straw, you can gently push the plunger and expel the contents back into the petri dish and reload. If you cannot expel the contents because the plug is already wet, simply cut off the cotton plug. This releases the vacuum and allows the contents to drain into the petri dish.

Chapter 5:
Embryo Freezing



Embryos frozen commercially twenty to thirty years ago were frozen in combinations of glycerol, DMSO, and/or sucrose. The thaw process required that the embryo pass through numerous dilutions of medium before being reloaded into a straw for transfer. This required time, media formulation, and setting up a microscope. Yet it was the best technique available at the time.

Fortunately, private as well as academic research and numerous field trials over the last ten to fifteen years utilizing ethylene glycol instead of glycerol resulted in acceptable direct transfer methodology for freezing embryos. This allows frozen embryos to be thawed and transferred in a fashion very similar to frozen semen. Today, more than 95 percent of embryos frozen worldwide are frozen by direct transfer (DT) methodology. However, there are still hundreds of glycerol embryos in semen tanks around the world.

To avoid confusing glycerol straws from DT straws and thawing one or the other incorrectly, the IETS recommends that all DT embryos be frozen and stored in yellow containers, i.e. yellow straws, plugs, labels, and goblets.

To achieve the best frozen/thawed results, many steps must be performed accurately and in a timely manner prior to freezing and also immediately after thawing. Embryos are dying from the moment they flow out of the donor. Therefore they must either be seeded

in the cryochamber *within two hours of collection,* or transferred fresh shortly thereafter. As soon as embryos have been designated for freezing, place them in sterile holding medium and set them aside as you prepare the following instruments and consumables.

5.1 EQUIPMENT AND SUPPLIES

The following instruments and supplies should be available on flush day if you anticipate freezing embryos.

- Cryologic model 2200 controlled rate freezer. Requires 110v or 220v electricity, or battery power module. There are several dependable portable electronic-controlled rate embryo freezers on the market today. Some models utilize alcohol, while others use liquid nitrogen as the cooling agent.
- Liquid nitrogen (3-4 liters); 0.25 liter insulated cup; semen tank w/ canisters for holding frozen embryos
- Microscope (the same that was used for searching)
- 0.25cc yellow straws, 47mm yellow straw plugs, 13mm yellow goblets, aluminum canes
- Brady label printer w/ yellow label stock; permanent ink marker
- ABC Embryo collection-transfer-freezing form; information on donor and flush sire(s)
- 35mm petri dishes, or 6-well dishes
- 20ml CellSafe™ syringes, 16gax1" needles
- IVF catheters, 1ml three-part syringes, straight tip 5.5" long hemostats
- Timing device
- 20ml ethylene glycol (EG) freeze medium
- ETMedia™ syringe filters
- Trypsin kit and holding medium (if embryos are for export)

5.2 CERTIFICATION OF MATING, TRANSFERS AND FREEZING

Accurate identification, certification, and record keeping are absolutely essential, not only to assure buyers that they 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.

A form developed by the IETS and used universally for recording collection, transfer, and freezing information is referred to as the "ABC" form, included herein as Appendix B.

When transferable embryos are collected, 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 (Freezing) should be completed and signed by the person or company freezing the embryos. Certificate D (Embryo Exports) is available and should be submitted to the appropriate breed registry in the exporting country where 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 embryos, 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 embryos from each collection. Information on the form must correspond to the identification of the embryo in the straw.

Also 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.

5.3 LABELING

The IETS recognizes a standardized system for identifying containers of embryos. Essential information that must be printed in permanent ink either on the upper half of the straw or on the straw plug should be on two lines and include the following information.

1 E656HO 7947108 Bessie (this information on line #1)
09AU22 28495 Elevation (this information on line #2)

Explanation of the above labeling information:

1	straw #1 of this flush
E656	The unique code number assigned by the IETS to the person or business freezing the embryo
HO	Holstein (or whatever breed is being frozen)
7947108	Registration number of the donor
Bessie	Barn or common name of the donor
09AU22	Year, month, day of the collection
28495	Registration number of the flush sire
Elevation	Barn or common name of the flush sire

In addition to labeling the embryo container, proper labeling of goblets and canes is required. The same information that you record on the straw should be recorded on the lower goblet (minus the straw number) and on the side of the aluminum cane. Normally only the lower goblet contains embryo straws and the upper goblet is inverted and then slid down over the straws to insure they do not float out of 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.

5.4 CRITERIA FOR FREEZING

Stage of development and integrity of the zona pellucida will determine if an embryo should be frozen. Stages 4,5,6 and 7 are acceptable for freezing, noting that stage 7 expanded blastocysts are 5-10 percent less likely to result in frozen/thawed pregnancies than the other stages.

Freezing damages a certain percent of blastomeres in all stages, which is primarily caused by ice crystal formation within the cells. Embryos younger than stage 4 have poor frozen/thawed survival rates since they only have a few blastomeres to begin with and losing cells to ice crystal damage results in a large percentage loss. By inducing ice crystal formation via seeding at -6°C, the ice crystals are smaller and less damaging than if we allowed the EG medium to spontaneously crystallize at temperatures below -15ºC.

Since the zona pellucida is an effective pathogen barrier, it is essential that all frozen embryos have an intact zona pellucida (no fractures/no cracks). Microbiological studies have shown the zona pellucida to be an excellent barrier to most bovine diseases. Thorough washing of embryos having an intact zona will completely remove all traces of most pathogenic agents. Therefore it is strongly advised by the IETS, and required by importing countries, that all embryos be washed in twelve separate baths prior to exposure to EG freeze medium.

To ensure the integrity of the zona, each embryo post-wash must be examined over its entire surface area at no less than 50x magnification. Embryos are gently rolled in the dish so that all surfaces of the zona can be examined for cracks. *This evaluation should take place after the twelve washes and before freezing.* If the embryos are not destined for export, washing is not required prior to freezing.

The washing procedure recommended by the IETS requires transferring embryos in groups of ten or fewer, all from the same donor, through twelve changes of medium utilizing a 1:100 dilution. For example, if the dish well contains 1cc of wash medium, aspirate the embryo(s) in 10ul or less medium and transfer the embryo(s) to the next well, changing sterile tips between washes.

Presently, only infectious bovine rhinotracheitis virus (IBRV) and vesicular stomatitis virus (VSV) have been shown to adhere to the zona 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. Therefore, if the importing country specifies IBRV- and VSV-free embryos, a twelve-step trypsin wash must be used. Refer to Appendix C for step-by-step trypsin wash instructions.

5.5 Sequence for freezing DT (ethylene glycol) embryos

The cryoprotectant ethylene glycol might be considered a "necessary evil." Without it, bovine embryos cannot survive freezing. However, **exposure to EG for longer than ten minutes significantly reduces the survivability of frozen embryos. Therefore, the very last thing you do is place the embryo into ethylene glycol.**

1) Wash embryos through the twelve-step Trypsin sequence if required for export. The embryos to be frozen are then placed into a separate dish of sterile holding medium. Label the dish lid and sidewall with the donor's name, and write "holding" on the lid also.

2) Prepare everything ahead of time, i.e. straws are labeled (or straw plugs are labeled), freezer is holding at -6°C, 1 petri dish filled with syringe-filtered EG medium (dish sidewall and lid are labeled EG). The EG dish will be used for rinsing the straw and drawing the initial sections of EG into the straw, and to expose the embryo to EG. The timing device has been set at seven minutes.

3) Place the dish of holding medium containing the embryos onto the microscope stage. Using the IVF catheter, maneuver the first embryo to be frozen to the center of the dish. Evaluate it for stage and quality, and then record this information under section C-Certificate of Freezing.

4) Aspirate the embryo into the IVF catheter. As soon as the embryo enters the catheter, stop aspirating. Lift the catheter out of the holding medium, push the holding medium dish ahead, and place the EG dish on the center of stage. Looking through the microscope *on low magnification,* gently lower the tip of the IVF catheter to the bottom of the EG dish, and then gently push the plunger until you see the embryo exit the catheter. Do not expel anymore holding medium into the EG than is required to release the embryo. Gently "stir" the catheter tip past/around the embryo to mix the expelled holding medium with the EG. The embryo will settle to the bottom of the dish.

5) Activate the timing device so that it starts counting down from seven minutes. With four minutes remaining on the timer, load the embryo into the yellow

0.25cc straw by the method described in section 4.5. **VERY IMPORTANT:** (a) the segment of medium containing the embryo must be 45mm long more or less, and (b) you must confirm that the location of the embryo is at or very near the center of this EG segment. If it is not at or very near the center of its assigned segment, you must reload this segment. Otherwise, there is a significant chance that seeding will instantly freeze and kill the embryo.

6) If the embryo is safely in the center of its EG segment, you can then wet the plug. Note that because this straw will be frozen, *it is crucial that **100 percent** of the PVC powder (middle of the cotton plug) is saturated with EG.* If you pull the plunger back too slowly, the initial contact of EG with PVC will result in only the face of the PVC section becoming wet, yielding a very weak seal. If the PVC seal is weak, it most likely will blow out during the thaw process and the embryo will be lost.

7) Therefore, pull the plunger aggressively so the EG rapidly enters and saturates 100 percent of the PVC powder. The instant you see that all of the powder is wet, stop pulling! Otherwise, you can dislodge and fragment the entire cotton plug. Holding the straw at the cotton plug, gently rotate and pull the straw out of the syringe.

8) Insert the nipple end of the labeled yellow plastic sealing plug (Fig. 11) into the end of the straw opposite the cotton plug. Be sure to **seat the plug all the way to its hub,** being careful not to bend or crush the straw. Correct insertion of the plastic plug will be difficult to do if your fingers are oily therefore a latex glove wrapped over this part of the straw will help you fully insert the plug into the straw. Be aware that if the plastic plug is not fully inserted into the straw, the plug might blow out of the straw the moment it is placed into the thaw bath.

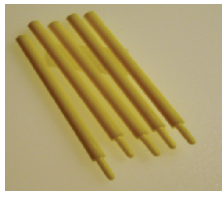


Fig. 11. Sealing plug

9) Move the straw to the cryochamber and gently insert it vertically, cotton plug down, all the way down into one of the twenty-three slots. If necessary, two straws can fit into each vertical slot thereby doubling the cryochamber capacity to 46 straws. The freezer should be holding at -6ºC.

10) When the timer expires and rings at seven minutes, seed the straw. **Do not seed before seven minutes.** Seeding is the act of inducing ice crystal formation at the uppermost edge (the meniscus) of the column of EG containing the

embryo. Assuming the straw was loaded correctly, the embryo will be located in the lower half of its EG section. Place the tips (excluding the jaw) of a straight hemostat into the liquid nitrogen bath surrounding the cryochamber for twenty seconds.

11) With the hemostat tips still in the nitrogen, grasp the straw by its plastic labeled plug, slowly raise the straw out of the cryochamber *until the meniscus of the section containing the embryo is visible.* As you hold the straw in this position with one hand, raise the hemostat tips out of the nitrogen with your other hand and gently close the hemostat tips around the sidewalls of the straw 1mm below the meniscus, being careful not to crush the straw. Maintain contact for ten seconds then remove the hemostat. You should see a small, frosty-white patch of EG medium just below the meniscus confirming that seeding was successful. Gently lower the straw back down into the cryochamber slot.

12) If there is only one straw in the chamber, slide an empty "dummy" straw into the slot alongside the embryo straw to insure the embryo straw remains vertical and contacts the steel chamber wall (not the foam lifter core). If two embryo straws are in the slot, no empty "dummy" straw is required. Continue loading and seeding until all straws are in the cryochamber.

13) *CRITICAL!* (a) The time between placing the embryo into the EG dish and seeding must not exceed ten minutes. (b) Therefore, place one embryo into EG at a time so that you can easily load *and seed the straw* at seven minutes, allowing three minutes of "safety margin" for corrections/reloads before the ten minute deadline. **Do not** load and place multiple straws into the cryochamber and then seed all of the straws at one time. The correct technique is to load one embryo, place it into the cryochamber, and immediately seed that single straw.

14) After the final straw has been seeded, release the hold button to the run position. Approximately one hour later the freeze run will be finished, holding at -32°C.

15) Attach the labeled goblet to the lower position on a cane and then place the cane into the nitrogen bath surrounding the cryochamber. Add nitrogen into the bath until the goblet is submerged. One by one, pull each straw up/out of its slot and transfer the straw into the submerged goblet (straw plug upward). Slide the open end of a goblet over the top of the straw plugs and then snap the goblet into the upper clips of the cane. The cane can now be transferred to a nitrogen tank for long-term holding.

Chapter 6:
Embryo Transfer

The overall process of embryo transfer *when referred to as the entire technology* requires the execution of many skills in sequence, attention to detail, comprehension of cattle reproductive physiology, and an appreciation for different cattle reproduction management objectives. While one cattle owner's objective might be to thaw and transfer 400 embryos over two days every spring and fall, another client may ask you to transfer all embryos fresh from a collection and conclude the day transferring frozen embryos purchased and imported from another country. The management plan of a dairy breeder may be to thaw and transfer single embryos weekly throughout the year as cattle cycle naturally. All of these management plans require inserting an embryo into the uterine horn. This chapter describes how to transfer embryos.

6.1 IDENTIFYING USEABLE RECIPIENTS

Criteria for selecting a useable recipient on transfer day are the same for fresh and frozen thawed embryos. The animal must not be pregnant, and she must have a CL on at least one of her ovaries. Lacking an identifiable CL, the transferred embryo will not result in a pregnancy. Refer back to Section 3.1 to review CL identification, as well as the accompanying DVD sections showing CLs on excised tracts.

If the day's plan is to flush several donors and transfer embryos, you should examine recipients before flushing. This knowledge about useable recipients allows you to quickly move from searching to freezing, minimizing the time between collection and freezing (or transferring).

If the day's work is at a remote pasture where 200 synchronized recipient cows have been rounded up and you must thaw and transfer 150 embryos, the thaw/transfer pace will be accelerated. Assuming adequate help, as the cow enters the squeeze chute you step in behind and quickly palpate. If a CL is identified, you would immediately prep the recipient as an assistant notes her heat date, selects and thaws an embryo, loads a transfer rod and hands it to you.

6.2 MATCHING EMBRYOS TO RECIPIENTS

Taking time to match stage 4 and stage 7 embryos with recipients having known heat dates will pay huge dividends through more pregnancies via an overall higher pregnancy rate. This is a frequently overlooked detail. If you purchase frozen embryos, you **must** obtain a copy of the ABC form if you want the opportunity to match embryos with recipients to the best of your ability. The Code Stage column in section C-Certificate of Freezing will tell you what developmental stage the embryo was when it was frozen.

Stages 5 and 6 embryos yield the same pregnancy rate when transferred into recipients that are day 7. To maximize success considering all embryo stages, you should strive to place stage 4 embryos (fresh or frozen/thawed) into day 6 recipients, and transfer stage 7 embryos into cattle that are day 8.

For example, if the paperwork indicates a frozen stage 4 morula and the recipient was observed in standing heat on Sunday, you should thaw the embryo on Saturday (six days after the recipient cycled). Similarly, if the frozen embryo was a stage 7 expanded blastocyst, you should get the recipient in on Monday (eight days after the recipient cycled), palpate and confirm a CL, then thaw and transfer the embryo. Use this same strategy for assigning fresh embryos to recipients.

Frequently you will not have the luxury of knowing recipient heat dates. The client may advise that he attempted to synchronize the recipients yet was not available to check and record heat dates. The best you can do in this situation is palpate CLs, confirm that the animal is not pregnant, and transfer embryos with no regard to developmental stage. Some technicians routinely practice "timed transfers" like this with no regard to heat dates. Results will normally be acceptable, yet not as good as they could have been if visual heat dates had been recorded.

The emphasis is that **if you want your results to be better than average, you should match the embryo's stage of development with uterine age whenever possible.**

6.3 THAWING GLYCEROL EMBRYOS

Embryos frozen in glycerol are traditionally thawed by either a three-step or a one-step method. Both methods require you to set up a microscope and pass the embryo through medium and the pregnancy rate is the same between methods. Refer to Appendix D, "Methods for thawing glycerol embryos" for technical details.

6.4 THAWING DT EMBRYOS

The advent of DT technology made transfer day for frozen embryos quite easy and quick, which is why the majority of embryos frozen worldwide today utilize EG as the cryoprotectant. The pregnancy rate between glycerol and ethylene glycol is not different. The DT thaw process requires only a thaw bath and no microscope is required.
At thaw time remove the straw from the nitrogen tank and place it into a warm (30°C/86°F) water bath for thirty seconds. Remove the straw from the bath, dry it with a paper towel, and re-confirm the information printed on the straw plug. Remove the plastic plug, insert the straw cotton-plug-end first into the transfer rod, and proceed with the transfer.

Some technicians prefer to air-thaw the straw for five to six seconds before placing it into the warm water bath. However, the pregnancy rate (all thawed embryos considered) is not different among techniques. Also note that embryos are thawed at a temperature that is approximately 10 °F cooler than semen thaw temperature. If you are thawing many embryos sequentially, it is convenient to use an electric water thaw unit like the CITO brand thaw box. The unit is preset to thaw semen, so be sure to adjust the temperature control knob on the bottom of the unit until the resulting temperature is stable at 86°F.

Remember, once thawed, the DT embryo must be transferred immediately. Ideally, the embryo will be deposited into the uterus within three to four minutes after thawing. At room temperature, EG is quite toxic to the embryo, therefore the last thing you should do is thaw the embryo. All prep work should already be accomplished, i.e. a useable CL has been palpated and the recipient has been prepared to receive the embryo. It is also wise to thaw and transfer only one embryo at a time. You don't want thawed embryos lying inside transfer rods, *essentially dying,* waiting to be transferred.

6.5 TRANSFER INSTRUMENTS

When the practice of commercial embryo transfer began thirty-five years ago, all embryos were transferred surgically. Then university researchers began trials transferring embryos nonsurgically using 18" long 0.5cc insemination rods, and then using 0.25cc insemination rods. Initially pregnancy rates were poor but quickly technicians learned the art and science of nonsurgical transfer.

During this same period the IMV Company in France developed an improved transfer system comprised of a 21" long rod (essentially identical to a semen rod) that utilized a steel-tipped side-delivery "blue-colored" sheath (Fig. 12) combined with a sanitary over-chemise. This blue sheath system was well received by the industry and today the majority of embryos worldwide are transferred through a side-delivery sheath method. Today some transfer technicians continue to use only the 0.25cc (18") insemination rod for embryo transfer with excellent results. However, most persons transferring embryos report an approximate 10 percent higher pregnancy rate using the side-delivery system vs. the standard insemination rod.

Regardless of which transfer rod or system is used, the greatest single influence on pregnancy rate is the skill of the person inserting the embryo.

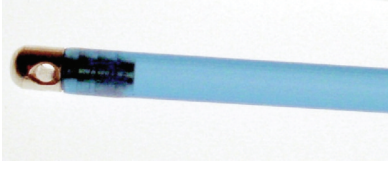


FIGURE 12.
Side-delivery tip of blue sheath

6.6 TRANSFER PROCEDURE

The act of transferring is more art than science therefore your results will improve as you transfer more embryos. Successful transfer technique requires mental focus, as if you are "seeing" through the fingertips of your palpation hand as you guide and slide the rod down toward the small anterior tip of the horn. On day seven the embryo is normally located in the upper one-third of the uterine horn which is the smallest diameter section of the horn, most-distant from the cervix. Therefore the objective is to deposit the fresh or frozen/thawed embryo as deep as you can into the horn associated with the CL while causing the least amount of damage to the uterine endometrial lining.

The key to transfer success depends on (A) where you place the embryo, and (B) your ability to minimize trauma to the endometrial lining of the horn during the transfer.

(A) To achieve average success (65 percent fresh and 50 percent frozen) you must deposit the embryo at least half-way down the horn associated with the

CL. Halfway refers to the mid-point between the palpable external horn bifurcation and the end of the horn. In many cattle this mid-point is where the horn bends downward. To achieve conception rates that are 10 percent or more above average, you must consistently deposit the embryo into the upper one-third of the uterine horn, which is the smallest diameter section of the horn most-distant from the cervix.

(B) Unfortunately, it is impossible to guide the tip of the transfer rod deep into the horn and not contact the endometrial lining of the horn. What you are trying to avoid is blunt, direct contact of the side-delivery tip with the uterine wall. The following sequence and advice will help you reach the desired upper one-third of the uterine horn with minimal trauma to the uterine lining.

Prepare the recipient the same way as described in section 3.3 for the donor.

While an assistant parts the vulva, the transfer instrument with over-chemise is passed through the vagina and manipulated to the cervical entrance. Before entering the cervix, grasp the chemise with the thumb and index finger of the right hand and pull the chemise backward, causing the tip of the sheath to pass through the poly bag. Manipulate the rod through the cervix and stop just before entering the uterine body. Allow the cervix to shield the sheath tip from entering and damaging the uterine wall. Release the cervix and focus on retracting the horns.

Extend the hand forward and retract both horns back toward you into the floor of the pelvis. Release both horns, and then rapidly pass your fingers forward, over, down and around the horn that you wish to transfer into, being careful not to compress the horn. From this point forward, it is crucial that you always know where the tip of the sheath is. This is accomplished by shielding the sheath tip in the palm of your hand as you manipulate the horn and gently slide the rod down into the horn.

No matter where you are inside the uterine horn, you must not allow the sheath tip to extend outside the area of your palm. By realizing where the tip is at all times, you will be able to control (minimize) blunt contact to the uterine wall.

With your fingers encircling (but not compressing) the horn, you can maneuver the horn up/down, left/right as you straighten/extend an approximate 50mm length of horn in the palm of your hand, so that the lumen of the horn is directly in front of the sheath tip. When you are confident that the opening (the lumen) of the horn is directly in front of the tip, gently advance the rod forward. Slide your fingers forward to the next section of horn and repeat this 50mm advancement process.

When you reach the location for deposition, allow four to five seconds to slowly depress the plunger and expel the embryo into the uterus. Remove your fingers from around the horn and withdraw the transfer rod. Be careful not to alter the withdrawal path in a manner that would cause the sheath tip to push or drag against the wall of the horn.

Each horn will present a different challenge for manipulation. If the horn is thick, rather short, and difficult to lift and straighten in front of the sheath, you may not be able to place the embryo as deep into the horn as desired without causing significant trauma. In this situation you must deposit the embryo mid-horn. If the horn presents long and straight, it will be easier to slide the sheath in a very controlled manner deep into the horn.

When palpating with the left hand, it will be more difficult to manipulate the left horn. Occasionally when you try to pass the transfer rod (or the flush catheter) into the left horn, it repeatedly diverts into the right horn. Most likely the sheath tip is being diverted into the right horn by the vertical septum of tissue located inside the uterus where the horns split left and right. To avoid this problem, place the palm of your left hand against the side of the left horn and push the uterus to the right. Stop when the left horn is aligned under the spine of the animal. Pull the sheath tip back into the uterine body so that it almost reenters the cervix. Then gently guide the tip forward against the left wall of the uterine body. This will bypass the internal septum and allow entry into the left horn.

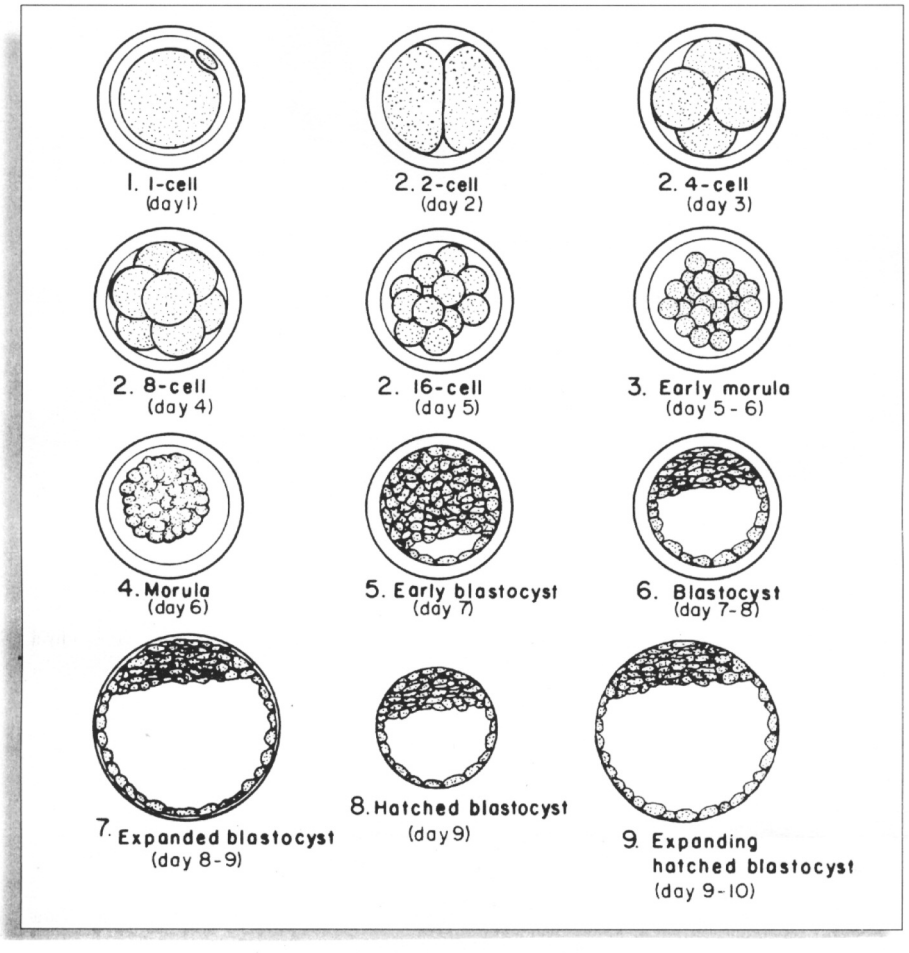
TIP: Some recipients will have a CL on each ovary upon examination. In this situation, transfer into the horn that you are most comfortable manipulating. If the recipient has a CL and also a significant (8mm+) retained follicle, it is acceptable to transfer into the horn ipsilateral to (same side as) the CL, and then inject the recipient with GnRH. The injection will induce the follicle to rupture and also stimulate increased progesterone production from the existing CL. The corpus luteum produces progesterone until the uterus determines it is not pregnant, whereupon prostaglandin is released causing CL lysis (regression) and the cycle repeats.

TIP: Timed transfers with no heat detection have been used by many technicians, resulting in an average of 55 percent pregnancy rate with frozen/thawed embryos, and 65 percent from fresh transfers. The recipient treatment sequence for this would be:

- GnRH and insert CIDR into all recips
- PG and remove CIDR seven days later
- GnRH (2cc) all recips two days after CIDR removal (expected day of estrus)
- Seven days after GnRH, transfer embryos into all animals with a CL

Another day of high-country ET work begins in Montana.

APPENDIX A. Graphical illustration depicting stages of development
Reproduced by permission of the IETS.



APPENDIX B. Form ABC

A. CERTIFICATE OF EMBRYO RECOVERY

Page ___ of ___

Breed _____

Donor Name _____ No. _____ Ear Tag or Tattoo _____

Owner _____ Address _____

_____ Onset Estrus Date _____ AM PM (Yr. Mo. Day)

Service Sire _____ No. _____ Breeding Date _____ (Yr. Mo. Day)

ID Code _____ Freeze Date or Batch No. _____ Sexed Semen X or Y ___ Recovery Date _____ (Yr. Mo. Day)

Service Sire _____ No. _____ Total Recovered _____

No. Cleaved/Degen. _____

ID Code _____ Freeze Date or Batch No. _____ Sexed Semen X or Y ___ No. Unfertilized _____

No. Transferred _____

No. Frozen _____

Signature _____ Firm _____

Practitioner or Leader of the Embryo Production Team recovering Embryos

ET Code _____

B. CERTIFICATE OF EMBRYO TRANSFER (see reverse side for coding instructions)

ACCOMODATES DIRECT TRANSFER

Date of Embryo Transfer if all trans. at one time _____ (Yr. Mo. Day) Surgical ____ Non-surgical ____ Other ____

Days since Estrus of Donor _____

IF FROZEN

Freeze Date on straw _____

Practitioner I.D. _____

Straw No.'s _____

One Embryo was transferred to each of the following recipients unless it is noted that more than one was transferred.

	RECIPIENT IDENTIFICATION Ear Tag, Registration or Tattoo No.	Breed Code	Days Since Estrus	Stage Code	Qual. Code	Embryo Manipulated N, D, F, M or U	Straw No.	Emb. Trans. Date	Comments*
1.									
2.									
3.									
4.									
5.									
6.									
7.									
8.									

Signature _____ Firm _____

Technician/Practitioner or Team Leader that transferred the Embryos

ET Code _____ Phone ( ) _____

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

C. CERTIFICATE OF FREEZING (see reverse side for coding instructions)

Type of Container: Straw ____ Other ____ Each container labeled to show firm code, breed, reg. no. of donors, freeze date and straw no.

Cane No.	Straw No.	No. Embryos/ Straw	No. X Washed	Trypsin Treated	Code Stage	Code Quality	Zona Intact	Embryo Manipulated N, D, F, M or U	Comments

Time from recovery to onset of freezing _____ (hrs.) Cryprotectant and concentration, equilibration, final molality and cooling procedure

_____

How Frozen: Seed Temp. _____ Cooling Rate _____ Plunge Temp. _____ Other _____

Recommended method of thawing and dilution _____

_____

Signature _____ Firm _____

Practitioner or Leader of the Embryo Production Team Freezing the Embryos

ET Code _____ Phone ( ) _____

APPENDIX C. TRYPSIN TREATMENT PROCEDURE (simplified) **for bovine embryos**
Using a 5ul (0.05ml) pipettor like the EHP11, move embryos (zona intact / no cracks) through each of 12 washes, changing tips (EHP12) between each wash.

Move embryos in groups of ten or fewer, all from the same donor

Insure a 1:100 dilution between wells, changing tips between wells

- i.e. use a 10ul or smaller pipet tip to move embryos through a series of 1ml (1000ul) baths. Refer to pages 41-43 of the 1990 IETS Procedural Guide if needed.

Wash 1-5:
Use BioLife C15 or C15C transfer medium (or similar) which contains BSA as the serum component. Make up 5 wells or dishes, each containing at least 1ml of transfer medium. Sequentially move embryos through each well (30 seconds per exposure) changing tips between each well or dish. NOTE: Using two of Agtech's 6-well dishes (item D18) is convenient for this entire wash process which features a 1ml capacity per well.

Wash 6 and 7:
Thaw and bring to room temperature Agtech's Trypsin C22 (0.25% Trypsin, ready-to-use, 10ml vial - store frozen). You will note that this liquid has a pink color to it which is a visual indicator that the pH is correct. If the color turns light blue, the pH is not correct and the contents should not be used. The pink color is phenol red, an inert, embryo-friendly pH indicator used routinely in cell culture work.

Make up 2 wells or dishes, each containing at least 1ml of C22. Sequentially move embryos through each droplet (30 seconds per exposure maximum), changing tips between each droplet. Discard any remaining unused trypsin solution.

Wash 8-12:
Use BioLife C15 Transfer Medium (or C15C) which contains BSA as the serum component. Make up 5 wells or dishes, each containing at least 1ml of transfer medium. Sequentially move embryos through each well (30 seconds per exposure), changing tips between each well. All embryos still with an intact zona can now be frozen for export.

APPENDIX D. Methods for thawing glycerol embryos

One-Step Method

1) Remove frozen straw from nitrogen tank and place directly into a 30c (86f) water bath. Hold in the bath for thirty seconds.
2) Remove the plug from the straw end and hold the straw at a 45-degree angle above a dry, empty petri dish. Using scissors, clip off the cotton plug end of the straw.
3) Liquid in the straw will drain out of the straw and form a droplet in the dish.
4) Immediately place the dish on the scope stage and examine the droplet to confirm that the embryo is in the droplet.
5) Place Vigro 1-step thaw EVM247 solution (3-5ml) into a petri dish.
6) Transfer the embryo from the droplet into the bath of 1-step thaw. Gently move the embryo throughout the dish for four to five minutes at room temperature.
7) Transfer the embryo to a new petri dish containing holding/transfer medium. Evaluate/grade the embryo and then load it into a straw for transfer.

Three-Step Method

1) Remove frozen straw from nitrogen tank and place directly into a 30c (86f) water bath. Hold in the bath for thirty seconds.
2) Remove the plug from the straw end, hold the straw at a 45-degree angle above a dry, empty petri dish. Using scissors, clip off the cotton plug end of the straw.
3) Liquid in the straw will drain out of the straw and form a droplet in the dish.
4) Immediately place the dish on the scope stage and examine the droplet to confirm that the embryo is in the droplet.
5) Place four new petri dishes on the counter top. Label each accordingly (Vigro EVM248 thaw #1, #2, #3), and transfer medium (like C15).
6) Working at room temperature and using a sterile, new 10cc all plastic syringe w/ 16gax1” needle, draw 5-7cc out of thaw #1 and fill thaw #1 petri dish with solution.

7) Using other new syringes, fill petri dishes #2 and #3 with their respective thaw solutions. Fill the transfer petri dish with transfer solution (i.e. C15, or EVM024).

8) Using the D17 IVF catheter attached to the 1ml syringe, aspirate the embryo from the droplet into the catheter. Solution should NEVER enter the syringe. Always keep the embryo inside the polyethylene catheter.

9) Deposit the embryo into thaw #1. Allow it to stay in this dish for three to four minutes. Then, aspirate the embryo back into the catheter and transfer it into thaw #2 (three to four minutes), then thaw #3 (three to four minutes), then into transfer medium.

10) Evaluate and grade the embryo, then load into a straw for transfer.

APPENDIX E. Professional organizations

International Embryo Transfer Society, Inc., 1111 N. Dunlap Ave., Savoy, IL 61874 USA
www.iets.org

American Embryo Transfer Association, 2441 Village Green Place, Champaign, IL 61822 USA
www.aeta.org

Canadian Embryo Transfer Association, Box 2000, 595 County Road #44, Kemptville, ON, K0G 1J0, Canada
www.ceta.ca

European Embryo Transfer Association
www.aete.eu

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