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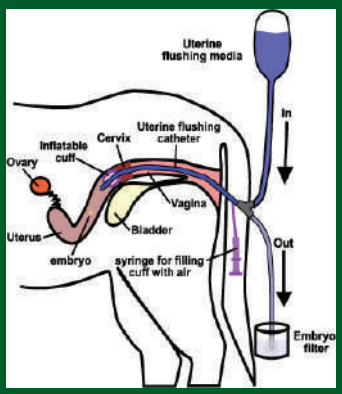
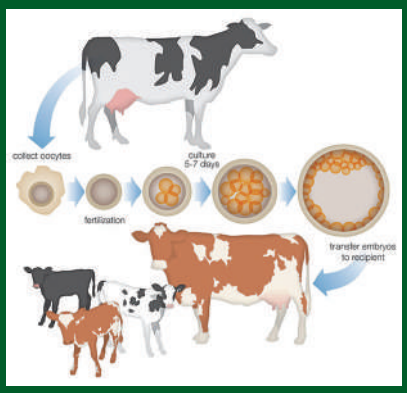
NATIONAL DAIRY RESEARCH AND DEVELOPMENT CENTRE
DEPARTMENT OF LIVESTOCK
MINISTRY OF AGRICULTURE & FORESTS
YUSIPANG, THIMPHU

GUIDELINES FOR EMBRYO TRANSFER (ET) PROCEDURES IN BHUTAN





Dr. Dorji, Mr. YR Giri, Mr. Chungsila, Mr. Abi NK, NDRDC, Yusipang
Dr. NB Tamang, Program Director, NDRDC, Yusipang
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Procedures In Bhutan

Contact Address:
Specialist Head/Program Director
National Dairy Research & Development Centre,
Yusipang, Department of Livestock, MoAF

Layout and Design:
Lokey Thapa, Dy. Chief LPO

PO Box: 1058
Office Phone: +97517116985, 17116985 (PABX) Lab: 17116986
Email:ndrc17@gmail.com

Forward

I am pleased to note that National Dairy Research & Development Centre is coming up with the Guidelines for Embryo Transfer (ET) procedures. This is necessary to conduct ET procedures as per set standard in the country.

Embryo transfer (ET) is a reproductive biotechnology that has great potential for speeding up genetic improvement and an important research tool for animal reproduction and embryology in cattle. The main use of ET in cattle is to amplify reproductive rates of valuable females. Because of low reproductive rates and long generation intervals, ET is especially useful in Bovine. Cattle may be valuable for many reasons, including scarcity, proven genetic value, or having unique characteristics such as disease resistance. It has been said that to dramatically improve the genetic base of a given cattle population it will take 10 - 20 years to accomplish using only natural service. By incorporating Artificial Insemination (AI), these improvements can be seen in 7 - 8 years. Through the use of an intensive ET program this change is accelerated to 4 - 5 years. Furthermore, ET is the method of choice to introduce quality genetic material into cattle populations as it circumvents the high costs, lengthy quarantine periods and risk of infectious diseases linked to the importation of live animals. Moreover, offspring from ET are 100 % of desired genotype and will adapt more readily to the new environment because of passive immunity acquired from the recipient mothers.

Through AI technology, it is possible to exploit the vast numbers of sperm produced by a genetically superior bull. Like AI has done for the bull, ET is a technique that can greatly increase the number of offspring that a genetically superior cow can produce. ET techniques have improved over the years so that frozen thawed embryos can be transferred to suitable recipients as easily and simply as AI.

The development of the Guidelines on ET procedure is timely as the Centre embarks on bovine ET technology in the country. ET procedures are very structured programs that require considerable time and significant planning, as well as commitment from all parties in order to obtain successful results. A successful program will require close attention to all the minute components because just a small deviation can cause an entire program to fail. The Guideline outlines the basic steps and standard operating procedures (SOP) for all the activities to be followed for a sound ET program in the country. The document represents current recommended good practices that will guide staff and ensure standard ET procedures as per World Organization for Animal Health (OIE).

Tashi Delek!

Dr. Tashi Yangzome Dorji
DIRECTOR

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1. SCOPE

2. The guideline shall provide framework for basic steps and standard operating procedures for all the activities to be followed for a sound bovine ET program in the country. Guideline shall provide good practices that will guide staffs and ensure standard ET procedures as per World Organization for Animal Health (OIE).

3. INTRODUCTION

What is Embryo Transfer

The process of assisted reproduction in which embryo(s) collected from the Donors are transferred / placed into the uterus of recipient(s) with the intent to establish pregnancy is generally referred to as embryo transfer (ET), without restriction to the transfer procedure only. It is the technique by which multiple embryos are collected from super-ovulated donor females and are transferred to recipient females, which serve as surrogate mothers. Embryos may be transferred shortly after collection (Fresh Transfer), or frozen after collection for thawing and transfer at a later date (Frozen Transfer). ET can be used to rapidly produce multiple progenies from desirable genetic combinations of sire and dam. The superovulation of donor animals by exogenous gonadotrophin treatment and the preparation of synchronized recipients are the essential prerequisites of successful bovine ET program.

History of Embryo Transfer

Embryo transfer was first performed and recorded by Walter Heape, a German scientist in 1890 (Betteridge 1981, 2003; Hasler 2003), when he successfully transferred two Angora rabbit embryos into a recipient Belgian doe. Since the first successful transfer of fertilized rabbit ovum it was known that a surrogate mother could raise another's embryo. Considerable research on the collection and transfer of bovine embryos was conducted during the 1930s and 1940s. However, most of the modern applicable ET technology was developed in the 1970s and 1980s. Umbaugh reported the first transfer of a bovine embryo in 1949 (Umbaugh 1949). Although Umbaugh reported four pregnancies resulting from the transfer of bovine embryos, all the recipients aborted prior to term. The first calf born from ET (surgical transfer of slaughter house derived day five embryo) was reported in 1951 by Willett (Willett et al 1951).

The commercial bovine ET industry started in North America in the early 1970s (Betteridge 1981, 2003). This was due to European dual-purpose breeds and exotic

cattle (which had become popular in North America, Australia and New Zealand) commanding very high prices when imported in small numbers from Europe. Breeders and producers sought means to circumvent the high costs and lengthy quarantine periods linked to the importation of European breeding stock and to capitalize on premium prices that progeny from these valuable dams and sires could command. Thus, ET offered a means by which their numbers could be multiplied rapidly. For several years, the most common use of ET in animal production program was the proliferation of desirable phenotypes. However, in 1987, Smith at the University of Guelph introduced the concept of Multiple Ovulation and Embryo Transfer (MOET). The term MOET was coined by Nicholas and Smith (Smith 1988a, 1988b) to consider ET and related technology in the context of optimizing genetic improvement of cattle. They showed how well designed MOET programs could lead to increased selection intensity and reduced generation intervals, resulting in improved genetic gains in livestock. The resulting genetic improvement can be disseminated to the general population by ET, AI or more practically by young ET bulls to be used in natural breeding. MOET procedures rely on advanced technology, which at first seems inappropriate for less developed countries. However with improvement in procedures over the years, all advanced technical procedures could be carried out at one site.

The first commercial ET programs relied on mid-ventral surgical exposure of the uterus and ovaries with the donor under general anesthesia. This necessitated surgical facilities and limited the use of the technology particularly in the dairy industry as the udder of dairy cows hindered mid-ventral access to the reproductive tract. However, the introduction of nonsurgical embryo recovery (flushing) in the mid 1970s and nonsurgical transfer techniques in the late 1970s (Betteridge 1977) allowed ET to be practiced on the farm and made it especially attractive to dairy farmers. The improved development of effective methods of freezing embryos has made ET a much more promising and applicable technology. For many years, embryos were frozen very successfully in glycerol, but as glycerol penetrates cell membranes rather slowly, it also had to be removed slowly during the thawing procedures. This necessitated lengthy microscopic examination in a laboratory setting. The development and use of highly permeating cryoprotectants such as ethylene glycol has allowed the direct transfer of bovine embryos. With this approach, the embryo straw is thawed in a water bath and its contents are deposited directly into the uterus of the recipient, much like AI. ET has been applied to nearly every species of domestic animal and many species of wildlife and exotic animals, including humans and non-human primates. Thus the degree of sophistication of ET procedures has evolved to permit complete utilization of non-surgical embryo retrieval / transfer in the cow and cryopreservation of frozen embryos.

Why Embryo Transfer in cattle

The reproductive potential of each normal newborn calf is enormous. There are an estimated 150000 potential eggs or ova in the female and countless billions of sperm produced by each male. By natural breeding, only a fraction of the reproductive potential of an outstanding individual can be realized. The average bull will sire around 15 - 50 calves per year and the average cow will have one calf per year (usually 8 – 10 calves in her lifetime) under well-managed conditions. With AI, it is possible to exploit the vast numbers of sperm produced by a genetically superior bull. Like AI has done for the bull, ET is a technique that can greatly increase the number of offspring (average 5 viable embryos / cow / flush) that a genetically superior cow can produce. Furthermore, ET is the method of choice to introduce quality genetic material into cattle populations as it circumvents the high costs, lengthy quarantine periods and risk of infectious diseases linked to the importation of live animals. Moreover, offspring from ET are 100 % of desired genotype and will adapt more readily to the new environment because of passive immunity acquired from the recipient mothers.
The main purpose of ET in cattle is to amplify reproductive rates of valuable females. Cattle in Bhutan are valuable for many reasons including scarcity, agricultural value (dairy / draught) and possess unique characteristics to combat local challenges, such as good resistance to common diseases that are found in Bhutan. With current ET techniques, it is possible to increase reproductive rates of valuable cows by an average of tenfold or more in a given year and this amplification will increase substantially as new technologies are perfected.

ET is an ever changing and expanding field and today this technology has gained considerable popularity and is becoming commonplace. Over the years, commercial ET in cattle has become a well-established industry with more than 500,000 embryos being transferred on an annual basis throughout the world. Although this results in a very small number of offspring on an annual basis, its impact is large due to the quality of animals being produced. ET is now being used for real genetic improvement, especially in the dairy sector and most semen used today comes from bulls produced by ET. Bovine embryos with intact zona pellucidae can be specified as pathogen free through washing procedures, making this an ideal procedure for disease control programs and in the international movement of animal genetics. Also, techniques have improved over the years so that frozen thawed embryos can be transferred to suitable recipients as easily and simply as AI. It has been said that to dramatically improve the genetic base of a given cattle population it will take 10 - 20 years to accomplish using only natural service. By incorporating AI, these improvements can be seen in 7 - 8 years. Through the use of an intensive ET program this change is accelerated to 4 - 5 years.

ET procedures have minimal impact on both donor and recipient cattle. However, donor animals are required to be restrained for some time during collection of embryos, and those with poor temperament may need particular attention. Generally non-surgical procedures are not perceived to cause pain and thus relief measures are mostly non applicable. The use of an epidural anesthetic is of primary benefit for ease of manipulation of the reproductive tract. The main issue with repeated treatments and embryo collection is the response in recoverable embryos, since there do not appear to be any adverse effects on the animals provided the procedures are done by competent personnel. Published work has shown satisfactory results with up to 10 repeat super ovulation treatments per annum. This frequency is unlikely to be reached in programs currently used in Bhutan and the more common practice is 3 - 4 collections per donor per year. Embryo production / recovery after flushing vary greatly from donor to donor and flush to flush. Generally, five freezable embryos (excellent and good) and eight transferable (excellent, good, fair and poor) embryos per super ovulation can be recovered. Pregnancy rates for fresh embryos are reported to be 60 - 70% on average, while for frozen embryos the average is 50 - 60% (Hasler et al 1983, 1987). However, many factors affect pregnancy rates such as embryo quality, condition of recipients, competence of transfer personnel etc. Further, some donors consistently produce embryos with higher pregnancy rates than others with embryos of a similar grade. This last factor seems to be uncontrollable and unpredictable.

There is also a widespread perception that both fresh and frozen embryos from Bos indicus result in lower pregnancy rates compared to embryos from Bos taurus cattle. There is also variation within Bos taurus cattle. Significantly lower pregnancy rates were reported from Jersey embryos compared to Holstein embryos frozen either in ethylene glycol or glycerol (Steel & Hasler 2004). An important point is that calves from ET are physiologically normal. Over 1900 embryo transfer pregnancies were studied, and found no differences from non embryo transfer calves in abortion rates, congenital abnormalities, birth weight, sex ratio (51% male), neonatal death, gestation length, calf hood disease or any other characteristics (King et al 1985). ET procedures are very structured programs that require considerable time and significant planning, as well as commitment from all parties in order to obtain successful results. A successful program will require close attention to all the minute components because just a small deviation can cause an entire program to fail. This SOP outlines the basic steps to be followed for a sound ET program in Bhutan.

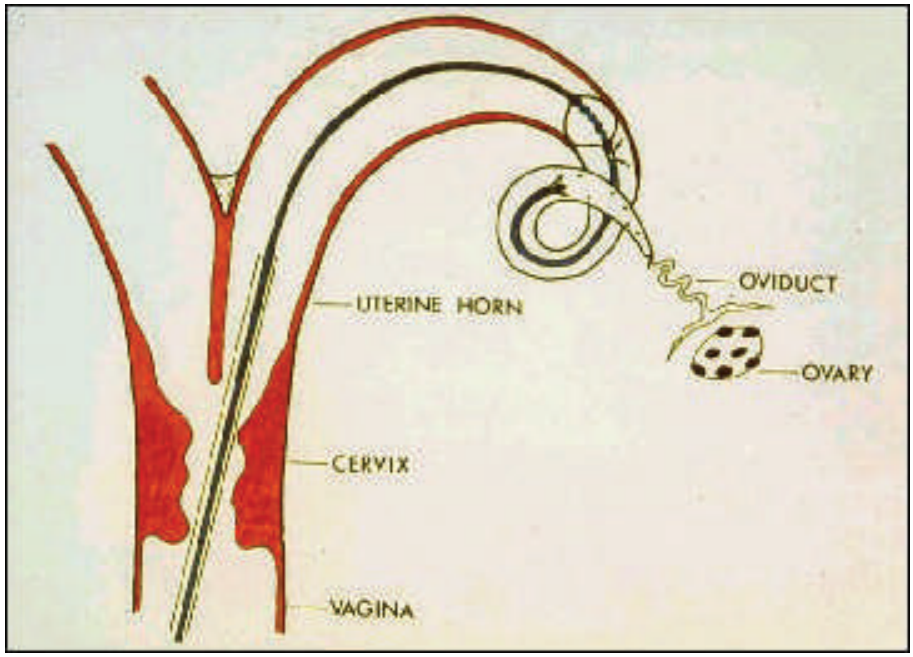
4. BENEFITS OF ET TECHNOLOGY

- ET techniques allow top quality female cattle to have a greater influence on the genetic advancement of a herd in the same way AI has allowed greater use of superior sires.

- A high yielding cow normally produces 8 - 10 high yielding calves during her lifetime while ET technology can produce 80 - 200 calves during her lifetime.

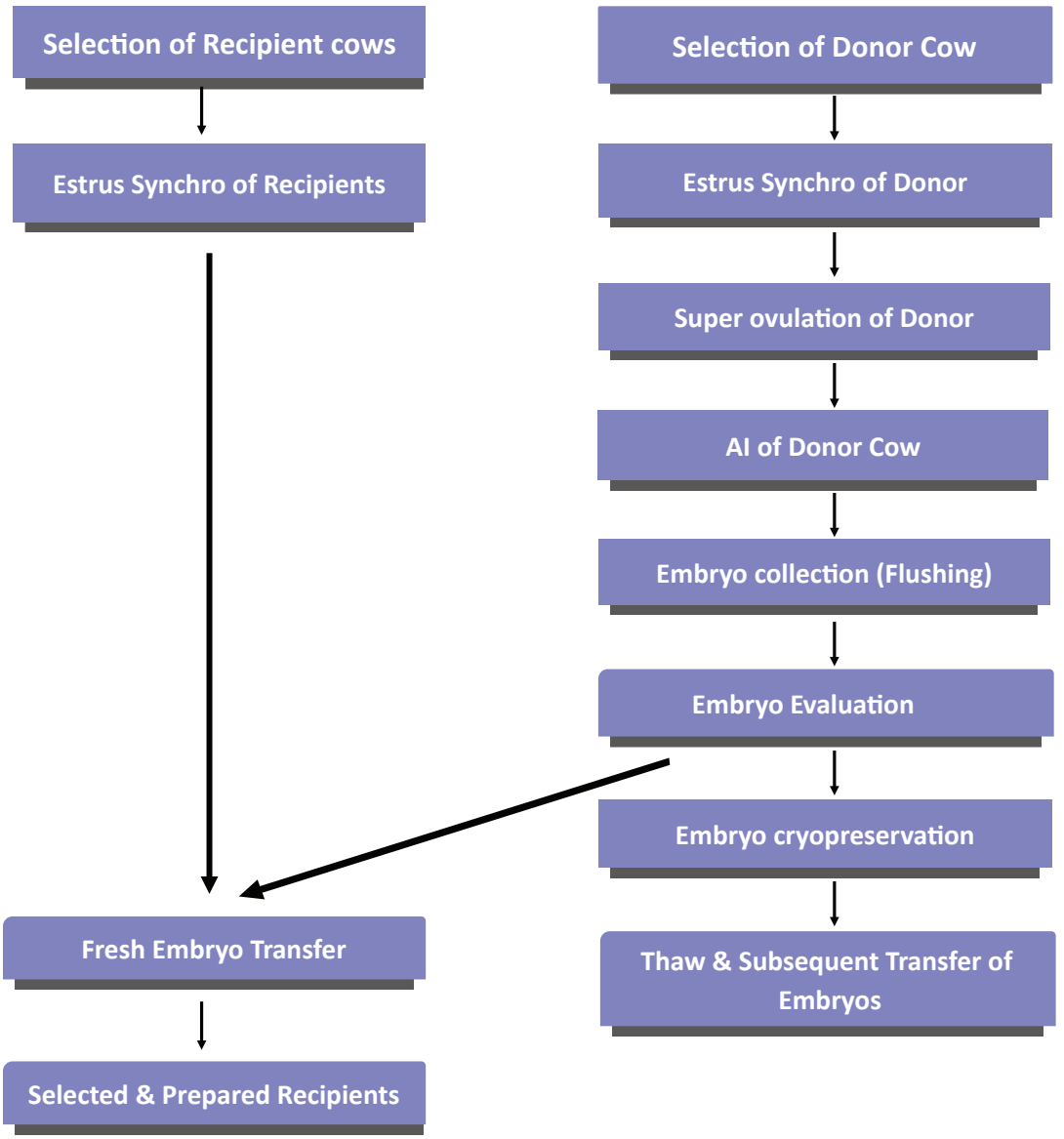
- ET is an important technology for the conservation of unique cattle breeds which are endangered.

- The general epidemiological aspects of ET indicate that the transfer of embryos provides the opportunity to introduce genetic material into populations of livestock while greatly reducing the risk for transmission of infectious diseases.



ET Flushing Procedure:
Correct placement of Foleys Catheter into the uterine horn

5. SUMMARY OF ET PROCEDURE IN CATTLE



6. EQUIPMENTS / MATERIALS REQUIRED IN ET PROCEDURES

i. For preparation of Donor and Recipient cows

- Follicle Stimulating Hormone (FSH)
- Gonadotropin Releasing Hormone (GnRH)
- Prostaglandin ($PGF_2\alpha$)
- Controlled Internal Drug Release Bovine (CIDR - B) 1.38g progesterone implants
- TRIU - B 0.772 g progesterone implants
- Disposable Syringe (1 ml, 2 ml, 5 ml, 10 ml, 20 ml, 50 ml)
- Disposable needle (16G, 18G)
- Cattle Marker paint (for marking the site of CL)

ii. For Embryo flushing

- Paper towel
- Embryo Flushing medium (1000 ml)
- Water boiler / heater
- Marker pen
- Permanent pen
- Embryo Filter cup
- 2 Way Foley catheter (# 16 for Jersey, # 18 for Siri / Nublang)
- Y flushing tube (connecting to Foley catheter)
- Stylette (for 2 Way Foley catheter)
- Disposable Syringe (20 ml for AIR infusion)
- Disposable Syringe (50 ml for medium flushing)
- Plastic measuring cylinder (1000 ml)
- Cervix dilator
- AI Gloves
- Surgical gloves (for hormonal drugs / preparation)
- Epidural anesthesia (2.5 ml) – Lignocaine
- Sanitary cotton wool
- Surgical table
- Polythene table cloth / Surgical table cloth
- KY gel for lubrication of 2 Way Foley catheter / Cervical dilator
- IV stands
- Cello tape for fixing the embryo filter cup to the measuring cylinder
- Scissors
- Artery forceps / Clamps
- Distilled water
- Alcohol (70 %)
- Protective kits (Gum boots, apron, mouth mask etc)

- Stainless steel Bucket (20 L), Jugs
- Basket (2 nos.) for holding & carrying the equipments / chemicals

iii. For Embryo Searching

-
- Slide warmer
- Embryo Holding solution (for embryo culture) 20 ml
- Stereo microscopes
- Petri dish 90 x 20 mm
- Culture dish 35 x 10 mm
- Micro pipettors (10, 20, 50, 100, 200, 1000 micro L)
- Pipette tips (20, 100, 200, 1000 micro L)
- Pulled micro haematocrite tubes (BRIS Ref. no. 160231)
- Silicon tube (connecting to the mouthpiece for embryo pickup)
- Pasteur pipette
- Lab Alcohol burner / Alcohol lamp
- 70% alcohol
- Paper towel
- Plastic bucket (20 L) with washing soap for hand sanitization
- Rubbish bin
- Extension cord (electric line)

iv. For Embryo loading for Fresh Transfer

- Tuberculin syringe
- Convertor (for fixing French mini straw to tuberculin syringe)
- French mini straw (0.25 ml) for embryo loading with plug
- Micro pipettor (20, 100 micro L)
- Micro pipette tip (20, 100 micro L)
- Mouthpiece (flexible rubber tube to fit into the micro Haematocrit tube)
- Glass test tube with screw tops / lid (for sterilization and storage of modified micro Haematocrit tube)
- Pasture pipettes
- Forceps / scissors
- Label printer (labeling on straw plug)
- 4 well tissue culture plate
- Minisart syringe filter 0.2 micron

v. For Embryo Cryopreservation

-
- LN2 storage tank (for storage of frozen embryos)
- LN2 Reserve tank for refilling

- Timer
- Embryo freezing Media 20 ml (Ethylene Glycol with sucrose)
- Funnel & Jug for LN2 transfer
- Embryo Freezer
- Tuberculin syringe
- Convertor (for fixing French mini straw to tuberculin syringe)
- French mini straw (0.25 ml) for embryo loading with plug
- Label printer (labeling on straw plug)
- Cotton & forceps for seeding
- Embryo straw holder (goblet with modified lid)
- Canister within LN2 tank

vi. For Embryo transfer in Recipient cows

-
- ET gun
- Blue sheath (over ET gun)
- Sanitary Oversheath 21 inch 80/pkt
- Thermos flask
- Towel
- Scissors
- Forceps
- AI Gloves
- Surgical gloves
- Savlon / soap
- Disposable syringe 5 ml)
- 18 G needle
- Local Anesthesia
- Thermometer

vii. For cleaning / sterilization

-
- Ultrasonic cleaner (18 Litre)
- Detergent for cleaning in the ultrasonic cleaner
- Alcohol (70%)
- Water Ultra purifier
- Hot air oven
- Autoclave 60 L

7. SELECTION OF DONORS, SIRES & RECIPIENTS

i. Selection of Donors

The first and most important step in ET is the selection of the donor cow. The donor should be selected based on the following;

- Genetic superiority: Donors should possess genetic traits that are highly desirable like milk production, milk composition, growth rates, disease resistance etc.
- Reproductive status: Donors should be free of reproductive abnormalities or genetic defects, cycling regularly with a history of high fertility (intrinsically fertile), without calving problems such as dystokia, retained placenta
- Body condition score (BCS): Moderate or better body condition having BCS 3 - 4 (based on a scale of 1 - 5). Extremely fat cows make poor donors because they do not respond well to super ovulation and reproductive tracts are more difficult to manipulate.
- Age: Donor cows should be preferably between 2nd to 5th lactation. Young cows seem to yield slightly more useable embryos than heifers (Hasler et al 1987). Heifers should be avoided as much as possible due to potential adverse reaction to ET process.
- Reproductive organs: Certain reproductive tract conditions affect usability and success as embryo donor. Some the defects such as a short cervix, excessively long vagina and deep uterus may affect fertility and are considered genetic traits that should not be propagated. Crooked cervix / kinked cervix are hindrance to flushing. Prolapsed cervical rings (caudal enlargement of cervix) & short cervix act as source of chronic infection of cervix, hindrance to flushing and compromise the cervical seal and result in chronic metritis and early embryonic loss if severe. Animals with Cervicitis (detectable by irregularity in the size and consistency of the cervix and presence of vaginal discharge), vaginitis, metritis, pyometra, should not be used as donors since this can interfere with fertility. Cows with deep uteri and long vaginas are difficult to flush embryos from and problems can result from trauma to the endometrium and cervix resulting from excess manipulation required to flush embryos from a cow with this type of uterine conformation.
- Pregnancy: If pregnancy has not advanced beyond 90 days donor cow may be aborted using PGF2α. Time for at least one natural estrus cycle prior to synchronization of estrus for flushing should be allowed (30-40 days). Pregnant cows beyond the first trimester should be skipped over as donors and used the following year.

- Conformation: the Donors should have good body conformation with good vulva position to ensure urine and dung does not enter the reproductive tract. The body conformation will decrease with the age of the animal.

- Rising plain of nutrition: The selected donor animals should be on a rising plain of nutrition with mineral supplements from one week before the protocol till the flushing. This is because with the rising plain of nutrition and comfort, the extra energy will be diverted to fertility as reproduction is not necessary for survival.
- Vaccination: The donor animals should not be vaccinated with any live vaccines and should not be treated with antibiotics from one month before the protocol till the flushing.
- Others conditions: Good donors should produce large numbers of usable embryos and donors at least two months postpartum produce more embryos than those closer to calving. Lactation in dairy cows does not decrease response to super ovulation provided that cows are cycling and not losing weight.
- Donor comes to heat in 1 – 2 weeks time after PGF2∞ administration following flush. Leave cattle for 45-60 days before undertaking synchronization for flushing of donors.

ii. Selection of Sires

- It is extremely important to use genetically superior bulls since half of the genes come from the male.
- Selecting the male is equally as important as selecting the donor females because males can be used to inseminate many donors and can be selected more accurately than females.
- It is important to select proven fertile bulls / high quality semen because sperm transport is inhibited in super-ovulated cows when inseminating.
- The sperm morphology is more important than progressive motility for fertility in both AI and ET program.

iii. Selection of Recipient animals

- Cows that are reproductively sound, that exhibit calving ease, and that have good milking and mothering ability are recipient prospects.
- They must be on a rising plane of nutrition with a BCS of 3 – 4.
- Animals cycling regularly and not exposed to bulls.
- Animals in a good state of nutrition, preferably gaining weight.
- Health status, particularly free from brucellosis, trichomoniasis and other venereal diseases.
- Cows and heifers can be used as recipients. The advantage in using cows is

there is less difficulty with calving, while heifers have higher fertility than cows, especially dairy breeds and are easier to manage (not lactating, & no calf management).

- If cows are not lactating (and not pregnant) they should not be selected as recipients since they are sub fertile.
- Generally cows (2^{nd} to 5^{th} lactation) are selected as donors (easy to manipulate the reproductive tract and pass Catheter through cervix), while heifers are good recipients (higher fertility).

8. PREPARATION OF ET RECIPIENTS

The preparation of the Recipients for ET transfer should be carried out using one of the two hormonal synchronization protocols / procedures as detailed in table below;

Day	Short protocol	Long protocol
0	Start Synchronization: Introduce CIDR-B + 2 ml Bomerol (Oestradiol Benzoate)	Start Synchronization: Introduce CIDR-B
7	1.5 ml Prostaglandins (PGF2α) + 2 ml Gonadotropin Releasing Hormone (GnRH) + Remove CIDR-B	
8	1 ml Bomerol (Oestradiol Benzoate)	
10		2 ml Prostaglandins (PGF2α)
11		Remove CIDR-B
13		Standing Heat
17	Embryo Transfer	
20		Embryo Transfer

9. SUPER OVULATION OF DONOR

Principle:

Super ovulation is the release of multiple eggs at a single estrous. The basic principle of super ovulation is to stimulate extensive follicular development (growth of multiple follicles) through the use of a hormone preparation (exogenous gonadotrophin through i/m or s/c), with follicle stimulating hormone (FSH) activity. Super ovulation results in about ten times more embryos than single ovum recovery. Without super ovulation,

a single usable embryo can be recovered about 60 % of the time from normal donors by skilled ET personnel. Under similar conditions, super ovulation usually yields an average of six or more viable / usable embryos at one estrus. Approx. 85% of all normal fertile donors will respond to the superovulation treatment with an average of 5 to 12 transferable embryos. Normally, no embryos are recovered from 20–30 % of super ovulated donors, only 1 - 3 embryos are obtained from another 20–30 %, while a small percentage of donors yield more than 20 good embryos. The two generally accepted methods of super ovulating cattle are based on two different gonadotrophins; pregnant mare serum gonadotrophin (PMSG) and follicle stimulating hormone (FSH). In recent years, FSH has surpassed PMSG as the method of choice for super ovulating cattle as FSH treatment has resulted in slightly higher numbers of usable embryos.

There is tremendous variation in the number of embryos recovered after super ovulation, which is due to variables like animal age, breed, lactation status, nutritional status, season, and stage of the estrous cycle when FSH treatment is initiated. Decreased fecundity with advancing age was reported in cattle and in other mammalian species. Fertilization rate is also reduced and quality of embryos deteriorated with the advancement of age (Agrawal et al 1992). Super ovulatory response and embryo recovery vary between different breeds of cattle. For instance, the *bos indicus* / Zebu cattle are low producers of viable embryos (39%) but had high pregnancy rates (56%) following ET (Donaldson 1984).

Opinions vary on the most effective method of administration and most effective dose rates for FSH used. There is wide variation in the dosage of FSH used for super ovulation. Since we use Jersey and Nublang (siri) cattle breeds as donors under our conditions, comparative trials need to be performed on each breed to ascertain the dosage of FSH required for optimum embryo recovery. Effective super ovulation of the donor animal is the most critical step of the ET technique as it directly affects the yield of the embryos per donor. If the dosage of FSH is low, the ovaries will not respond, while too high a dose will cause hyper response of the ovaries that can cause further complications including bleeding and adhesions of the ovary to the surrounding tissues.

Procedures of Super-ovulation

There are two protocols which can be used for super-ovulation of the donor cows.

In the short protocol; Synchronization begins with insertion of a CIDR-B (containing 1.38g Progesterone) accompanied by the injection of estrogen. Progesterone synchronizes donors to the same stage of their cycle. Estrogen helps to synchronize, by

inducing follicle atresia as well as leading to a FSH surge once it has been metabolized. FSH is injected twice daily in decreasing doses to induce multiple follicular growth. Prostaglandin is administered on day 6 to induce luteolysis, resulting in estrus occurring shortly post administration. AI is performed to take advantage of estrus and to maximize the chance of fertilization. Flushing is carried out where one would expect to see morula, Compact morula and early blastocysts.

The longer protocol seeks to exploit the natural estrus cycle of the donors, which in the normal course of action usually displays 2 'false' waves before a true ovulation occurs. This longer protocol seeks to maximize the number of follicles to coincide with ovulation. Cattle are synchronized with insertion of progesterone (CIDR-B), before removal of CIDR on day 10 and observations are made for standing heat. On the 9th day following standing heat signs the FSH treatment of the Donor is carried out. FSH is injected twice daily for 3 days (6 injections) at middle or near the end of a normal estrous cycle. Prostaglandin injection ($PGF_2\alpha$) is given on the last day of the treatment schedule which will cause CL regression and heat / estrus to occur approx. 2 – 3 days later. Donors are inseminated following final FSH and prostaglandin injection. The two different protocols are detailed in Tables below;

Short Super-ovulation protocol

DAY	PROCEDURE	VOLUME
0	Start Synchronisation: Introduce CIDR-B + 2ml Bomerol (2mg)	1.38g Progesterone & 2mg Estradiol Benzoate
4	6 AM Inject 1.8ml FOLLTROPIN V	36mg FSH
	6 PM Inject 1.8ml FOLLTROPIN V	36mg FSH
5	6 AM Inject 1.4ml FOLLTROPIN V	28mg FSH
	6 PM Inject 1.4ml FOLLTROPIN V	28mg FSH
6	6 AM Inject 1.0ml FOLLTROPIN V + 2.0ml Juramate	20mg FSH & 500µg Prostaglandin
	6 PM Inject 1.0ml FOLLTROPIN V + 1.0ml Juramate	20mg FSH & 250µg Prostaglandin

7	6 AM Inject 0.6ml FOLLTROPIN V + Remove CIDR-B	12mg FSH
	6 PM Inject 0.6ml FOLLTROPIN V + AI (if cattle displaying heat)	12mg FSH
8	7 AM AI + GnRH administration	
	1 PM AI (depending on timing of heat display)	
15	FLUSH	

Long Super-ovulation protocol

Day	Procedure	Volume
0	Insert CIDR	1.38g
9	Inject 2.0 ml Prostaglandin	500µg Prostaglandin
10	Remove CIDR	
12	Observe for standing heat	
19	Palpation for ovaries	
21	6 AM Inject 2.5ml FSH	50mg FSH
	6 PM Inject 2.5ml FSH	50mg FSH
22	6 AM Inject 1.5ml FSH	30mg FSH
	6 PM Inject 1.5ml FSH	30mg FSH
23	6 AM Inject 1.0ml FSH + 2.0ml PG	20mg FSH; 500µg Prostaglandin
	6 PM Inject 1.0ml FSH + 2.0ml PG	20mg FSH; 500µg Prostaglandin
25	AI - depending on standing heat (observe)	
26	AM - AI	
	PM - AI	
32	FLUSH	

10. INSEMINATION OF DONOR COWS

Principle

Since there is release of many ova from the multiple follicles on the ovaries, there is a greater than normal need to be certain that viable sperm cells reach the oviducts of the super ovulated females. Therefore, inseminate the super ovulated donor several times during and after estrus. Use of high quality semen with a high percentage of normal, motile cells is a very critical step. Observe the donors for heat signs to ensure AI is being performed at the right time.

Procedure

Inseminate the super ovulated donors several times (2 - 3 AIs) at 12 hours apart (12, 24, and 36 h) after the onset of standing heat as per table below (depending on whether short or long protocol is being followed).

Particulars	Short protocol	Long protocol
Commencement of AI	At time of final FSH injection, IF donor is displaying good signs of heat	48 - 60 h after $PGF_2\alpha$ inj
Specific Timing of multiple AI	1st AI: either at same time as final FSH injection, or 12 hours after final FSH injection (depending on evidence of standing heat at final FSH injection) 2nd AI: 6-12 hours after 1st injection 3rd AI: only given if donor displays good heat, given 18-24 hours following first AI	1st AI: 6 – 10 h after standing heat 2nd AI: 12 h after 1st AI 3rd AI: 12 h after 2nd AI
Flushing	Embryo flushing: 8th day after final FSH injections	Embryo flushing: 7th day after 1st AI

The correct site for semen placement is in the body of the uterus (0.5 to 1 inch in front of the cervix). If the AI gun is passed too deep and semen is deposited into one of the uterine horns, it will reduce fertility if ovulations are taking place at both ovaries. After complete flushing, the donor cow is given PGF2∞ for lysis of Corpus luteum.

11. DONOR PREPARATION & EMBRYO FLUSHING

Principle

Embryo recovery or flushing is generally accomplished through non-surgical techniques at approximately seven days after AI. In most cases, embryos are collected on day 6–8 after AI and are usually in the morula to blastocyst stage. Embryos can be recovered non-surgically as early as 4 days after oestrus from some cows, but prior to day 6 recovery rates are lower than on days 6 to 8. Embryos can also be recovered on days 9 - 14 after oestrus; however, they hatch from the zona pellucida on day 9 or 10, making them more difficult to identify and isolate and more susceptible to infection. After day 13, embryos elongate dramatically and are sometimes damaged during recovery or become entangled with each other. Procedures for cryopreservation and bisection have been optimized for day 6–8 embryos, which is another reason for choosing this time. A small percentage of embryos remain in the oviduct after day 7. Unfortunately these are not recoverable with current non-surgical procedures.

The first step in non-surgical recovery is to palpate the ovaries per rectum to estimate the number of corpora lutea. This is very difficult to do accurately if there is a large response to superovulation, although it is not critical to determine how large this response is. Even when only two or three corpora lutea are palpated by skilled personnel, occasionally four or five embryos are recovered. However, it is exceedingly rare to obtain embryos if there are no palpable corpora lutea by day 7. Under most circumstances, cows with no response are not worth flushing, although occasionally an embryo is recovered. It is rare to recover more than one embryo from cows with one palpable corpus luteum. In many situations, donors are palpated the day before recovery or the morning of recovery so that logistical plans can be made, for example, to flush those donors with poor responses first (or last) and cancel those with no response. Ultrasonography (Pierson and Ginther, 1988) provides more accurate information about responses than palpation, but currently this expensive equipment can only be justified in research contexts or in large ET programs.

There are two fundamentally different approaches to positioning the balloon of the Foley catheter for non-surgical recovery procedures. These are commonly referred to as "**body**" and "**horn**" flushes. For the body flush, the catheter is inserted into the uterine body and the balloon is inflated just past the cervix. Either air or flushing media can be used to fill the balloon. The single most common error in non-surgical recovery, especially with horn flushes, is over inflation of the balloon. This leads to rupture of the endometrium and loss of flushing fluid (and embryos) into the uterine tissue, from which recovery is impossible. Once this occurs, the only recourse is to reposition the balloon more anteriorly, precluding a body flush. The amount of air

used to inflate the balloon usually ranges from 10 to 20 cc, depending on the size of the uterus. The balloon should fit snugly, but should not rupture the endometrium. A disadvantage of the body flush is that the balloon sometimes occludes one of the horns so that only one fills.

For the horn flush, the balloon should be positioned at the palpable bifurcation of the uterine horns. The advantage of the horn flush is that a much smaller volume of the uterus is flushed, which requires less medium and theoretically results in improved embryo recovery rates. This is particularly true for older cows of large breeds with large, pendulous uteri. The major disadvantage of the horn flush is the need to reposition the catheter in the second horn after flushing the first, which requires detaching the inflow-outflow tubing, deflating the balloon and reinserting the stylet. This prolongs the flushing procedure considerably.

Procedure

- Clean the table and sanitize it with 70% alcohol. Preparation of equipments for Embryo flushing (All flushing equipments sets on surgical table)
- Use commercial flushing media (Complete flush)
- Restrain the donor in the squeeze crush
- Keep a towel/paper towel and a bucket of water for cleaning the vulva region.
- Per rectal examination of the Donor for Corpus luteum (CL) presence on the ovaries

ET Assistant's work

- Open the cover sheath of the 2 Way Foley catheter
- Check the 2 Way Foley catheter by inflating air or medium to check the balloon & by flushing some amount of flushing medium
- Cut open the Stylet (used in Foley catheter). Insert Stylet inside the 2 Way Foley catheter and lubricate with some flushing medium. Lock the Stylet to the 2 Way Foley catheter firmly (using artery forceps)
- The 2 Way Foley catheter is ready for use in the Donor cow
- Set up the Flushing media onto the IV stand
- Make sure the temperature of the flushing media is maintained at body temperature (keep it in a water bath at 35 – 37 °C. Special attention must be taken during winter months
- Cut open the Y flushing tube and connect (one end – piercing head) it to the flushing media (middle outlet)
- Flush the Y flushing tube with some flushing media using the clamp controller (In & Out) for cleansing

- Take out the Embryo filter cup from the package. Connect the long drainage tubing (connected to the Cap of Embryo Filter cup) to the bottom outlet port
- Secure Embryo filter cup with drainage tubing in vertical position on the measuring cylinder with the help of cello tape
- Also secure the measuring cylinder to the IV stand with string / cellotape
- Open the Plastic plug for proper aeration and to ensure fluid can pass freely through the cup
- Attach the Y flushing tube to the inlet port of the Embryo flushing cup

ET Personnel Work

- Use epidural anesthesia (between first and second coccygeal vertebrae) for relaxation of the rectum and local effect on the tract to aid manipulation. Embryo flushing and embryo transfer are both done after an epidural anesthesia which block contractions of the digestive tract and aid in the ease of manipulation of the cervix and the uterine horns.
- Clean the injection site with alcohol swab, clip / shave the hair from the site, scrub with iodine soap and swab with 70 % alcohol to prevent infection of the spinal column.
- Inject using 18G new needle 3 - 5 ml of lignocane (based on breed, body wt.). The correct site can be confirmed through hanging drop. A frequent error is to inject too much anaesthetic too far forward, which can cause the cow to lose control of the rear legs and fall down in the chute. The effect of anesthesia can be confirmed by monitoring flaccidity of the tail / tail drop.
- **Sedation:** Sedation with Xylazine @ 10-25 mg per donor through tail I/V should be used in very aggressive animals. The 20mg/ml concentration should be used in cattle as they are very sensitive and not 100mg/ml which is used for horses. The use of Epidural anaesthesia, needle position, preparation of the injection site and was found very useful and practical for the ET team**.**
- While the epidural anaesthesia is taking effect, the tail should be secured to one side out of the way, for example, by tying it to a cord looped loosely around the cow's neck. It should not be tied too securely to something stationary, like the chute, for fear of the tail breaking if the cow falls or if personnel forget to loosen the cord before releasing the cow.
- The rear end of the cow should be cleaned of mud, manure, loose hair, etc., and then the vulvar area scrubbed thoroughly with iodine soap and rinsed carefully with swabs of 70% alcohol. Sufficient time should be allowed for the lips of vulva to dry before inserting the recovery instrument to avoid carrying any alcohol into the uterus; disinfectants are extremely toxic to embryos. For the same reason, an assistant should open the labia gently when the cervical dilator or the recovery device is inserted.

- Insert Cervix dilator (after proper lubrication) till internal os to aid in cervix dilation

Horn Flush

- Insert 2 Way Foley catheter with Stylet through the cervix and located in place at one of the uterine horns (above the uterine bifurcation)
- Pullout the Stylet 10-15 cm out of the Catheter. Gently push further the 2 Way Foley catheter till mid uterine horn
- When the catheter is correctly located, the cuff is inflated (5 – 10 ml air for Siri breed) with Syringe (20 ml) by the Assistant & check the inflation of the uterine horn (ballooning).
- Stop inflating air once the balloon is firmly held in place in the uterine horn
- Remove the Stylet completely. Clean the Stylet with alcohol soaked in cotton, clean with flushing media and insert it into the cover sheath

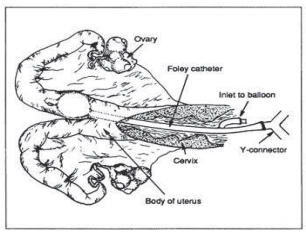
Body Flush

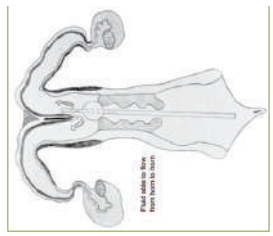
- Insert 2 Way Foley catheter with Stylet through the cervix and located in place at the uterine body (below the uterine bifurcation)
- When the catheter is correctly located, inflated the cuff with flushing media (2 – 7 ml) with Syringe (20 ml) by the Assistant & check the inflation of the cuff just above the internal os (ballooning).
- Stop inflating air once the balloon is firmly held in place in the uterine body just in front of the internal os.
- Pullout the Stylet 10-15 cm out of the Catheter. Gently pull the 2 Way Foley catheter to ensure it is lodged properly in front of the internal os and ensure the Foley catheter does not come out.

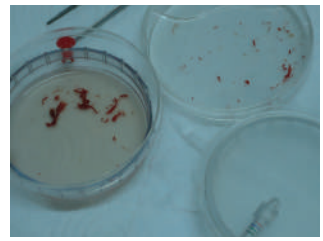
Common steps for both horn and body flushes

- Remove the Stylet completely. Clean the Stylet with alcohol soaked in cotton, let it dry for 5 min for the alcohol to take its effect, clean with flushing media and insert it into the cover sheath
- Connect the third end of the Y flushing tube into the 2 Way Foley catheter and fix it tightly. Make sure that the outlet button (to the Embryo filter cup) is properly closed and open the inlet into the 2 Way Foley catheter (into Uterine horn / body) using the control button.
- Palpate the uterine horn for the amount of flushing media and close the inlet control button once the uterine horn is sufficiently filled with the flushing media (horn flush)

- Palpate uterine body and both uterine horns for the amount of flushing media and close the inlet control button once the uterine horns are sufficiently filled with the flushing media (body flush). About 100 – 200 ml of flushing media should be used per flush, which can be repeated 5 – 10 times in body flush technique.
- Gently massage the uterine horn / horns so that the embryos will float in the flushing media
- Open the outlet control button (going into the measuring cylinder) and gently raise (by holding on the tip of the uterine horn) and shake the inflated uterine horn for proper flushing
- Repeat the procedure several times
- 1000 ml of flushing media is used per Donor, 500 ml in each horn (horn flush), while 1000 ml of flushing media is used per donor for both the horns simultaneously.
- After complete flushing of one horn, remove the air from the balloon (using syringe) and gently pull out the 2 Way Foley catheter from the uterine horn
- Raise the end of the 2 Way Foley catheter to flush all media remaining inside the tube into the embryo filter cup
- Clean the 2 Way Foley catheter by rinsing with the Flushing media
- REPEAT the same procedure on the second horn in case of horn flush
- After completing the flushing procedure, detach the Embryo filter cup from the measuring cylinder. Replace the plastic plug onto the embryo filter cup.
- Cover the Embryo filter cup with paper towel completely to protect the embryos inside from direct sunlight
- Take the Embryo Filter cup to the Laboratory asap for embryo searching
- Rub the 2 Way Foley catheter, Foleys stylet, Cervix dilator with alcohol swap
- Rinse it thoroughly with Flushing media
- Put it back in the sterilization bag & take it to the lab for further cleaning and sterilization







Horn Flushing and body flushing procedures

After complete flushing, the donor cow is given PGF2∞ for lysis of Corpus luteum.

12. EMBRYO SEARCHING

- The laboratory preparation should be completed before arrival of the embryo in the Embryo filter cup
- Cleaning & disinfection of the working bench, stereo microscopes, etc by wiping down with 70% ethanol and paper towel
- Put on the warm stage and prepare water bath to 35-37°C
- Prepare 30 mm dish with embryo holding media. Label with donors ID. Place on warm stage to reach 35-37°C
- Keep the flushing media in the water bath; syringe (20 ml and / or 50 ml, with holding media loaded) and needles (18 G) on the warm stage
- Prepare & keep ready all the pipettes, petri dishes, mouthpiece set, etc within easy reach. Parallel grid lines drawn on the bottom of the 90 mm petri dish allows it to be used as a search dish
- After the embryo filter cup reaches the Laboratory, hold firmly with one hand and open lid carefully by prying at several points between the lid and dish
- Once the lid is loosened carefully lift lid from the dish. Tilt the lid with filter to 45^0 angle with filter facing downward which will allow any drop attached to the filter to drop into the dish (make sure no media is spilled out)
- Label the 90 mm petri dishes with the donors ID for easy identification which is placed on pre-warmed heating stage.
- Gently agitate the fluid in the filter cup using a swirling motion to dislodge any material that may be stuck to bottom of membrane. In one smooth motion, empty contents into 90 mm petri dish
- Use a syringe to gently rinse the filter membrane with warmed flushing media. Hold filter cup at 45^0 over petri dish (so that cup is upside down over dish) and use force of flushing media expelled from syringe to completely wash and dislodge any material left on membrane. Care should be taken not to puncture the filter while rinsing.
- If filter fliter cup contains a large amount of sticky mucous, use 18 G needle and syringe to *gently* draw fluid from cup up and down syringe several times in an effort to break up mucous. Aggressive force could cause damage to embryos.
- Search for embryo under stereo microscopes in the 90 mm search dishes
- At least two ET technicians should search for embryos in the dishes using two separate stereo microscopes as embryos should be collected as soon as possible
- Once the embryo is found, pick up using either handheld pipette or mouth pipette and transfer it to a smaller petri dish (30 mm) containing embryo holding media kept on warm stage (warmed). Pick up all material (viable embryos, degraded embryos, unfertilized oocytes) as this needs to be recorded. Place in holding media dish corresponding to donors ID.

- Continue searching embryos in all dishes.
- After all the embryos are recovered from the search dishes, separate the identified Embryos from the mucus and other debris with the help of modified pasture pipette tips and wash for 2-3 times to remove mucus/ other debris.
- Assessed the embryo quality on appearance and stage of development, and then either prepare for fresh transfer or freeze the embryos for storage.
- Record all details as necessary using International Embryo Transfer Society (IETS) embryo retrieval / cryopreservation forms (Annexure 1).
- Evaluation of bovine embryos is normally done with a stereomicroscope at 50 to 100X magnification, with the embryo in a small holding dish. It is also necessary to "roll" the embryo on the bottom of the dish so as to view the embryo and zona pellucida from different perspectives. The overall diameter of the bovine embryo is 150 to 190 µm, including a zona pellucida thickness of 12 to 15 µm. The overall diameter of the embryo remains virtually unchanged from the one-cell stage until blastocyst stage. The best predictor of an embryo's viability is its stage of development relative to what it should be on a given day after ovulation. An ideal embryo is compact and spherical. The blastomeres should be of similar size with even color and texture. The cytoplasm should not be granular or vesiculated. The perivitelline space should be clear and contain no cellular debris. The zona pellucida should be uniform; neither cracked nor collapsed and contains no debris on its surface

13. EMBRYO GRADING

Upon collection individual embryo should be evaluated immediately under stereo microscopes for stage of development, quality and classified to determine the potential likelihood of success if transferred to a recipient cow. For many beginners, the most intimidating aspects of the embryo transfer process are morphological evaluation of embryos. Obviously, there is no profit in transferring unfertilized ova or degenerate embryos, nor in discarding perfectly normal ones. Both errors are common when people are first gaining experience. There are three elements to the successful evaluation of embryos; training, experience and proper equipment. Training includes learning the correct morphology of embryos at different stages and the meaning of deviations from normal morphology. One must also learn how to manipulate and examine embryos. Experience is gained by examining many embryos at different stages of development.

The major criteria for evaluation include:

- Regularity of shape of the embryo
- Compactness of the blastomeres (dividing cells within the boundaries of the embryo)

- Variation in cell size and diameter of the embryo
- Color and texture of the cytoplasm (the fluid within the cell wall)
- Presence of extruded cells
- Regularity of the zona pellucida (the protective layer of protein & polysaccharides around the single celled embryo)
- Presence of vesicles (small bubble-like structures in the cytoplasm)

Embryological terminology:

In mammals, the female gamete is called an egg or ovum; the correct technical term for the newly ovulated female gamete is an oocyte. Upon fertilization, the oocyte becomes a one-cell embryo, sometimes referred to as a zygote. The embryo then divides into two-cell, four-cell, etc. stages. At the 16-cell stage, the embryo becomes a morula (Latin for mulberry). When a cavity (**blastocoele**) forms between the cells of the embryo, it is termed a blastocyst. The first three divisions of the embryo are called cleavage divisions; thus, one-to eight-cell embryos are defined as cleavage stages. During this time the embryo actually decreases in weight. Only at the morula stage does the embryo begin to weigh more than at the one-cell stage. During the morula stage, cells of embryos change from spherical to polygonal in shape. This phenomenon is termed compaction. During compaction, specialized junctions form between cells, so the cells can communicate with each other. Frequently, compacted morulae are termed tight morulae. Compacted morulae are smaller than pre-compacted embryos. Compaction is an excellent sign that the embryo is developing normally; lack of compaction by six days after oestrus in cattle indicates retarded development. As the morula develops into a blastocyst, it forms a cavity, the **blastocoele**, by expending energy to pump fluid between the cells. Thus blastocyst formation also is indicative of continued normal embryonic development. Conversely, lack of blastocoele formation by 7 - 8 days after oestrus in cattle signifies retarded development.

Embryo evaluation is one of the most critical steps of the embryo transfer procedure. Embryos are usually classified based on a number code system for their stage of development (1 to 9) and for their quality (1 to 4). The IETS Manual states that embryos must be graded based on a 1 to 9 point system to determine the stage of development and a 1 to 4 system to determine embryo quality.

Embryos are evaluated for their stage of development as described below;

- Stage 1: Unfertilized oocyte / ova (UFO)
- Stage 2: 2 cell to 16 cell embryos / Undeveloped embryo / Degenerated embryo
- Stage 3: Early Morula
- Stage 4: Morula
- Stage 5: Early Blastocyst

- Stage 6: Blastocyst
- Stage 7: Expanded Blastocyst
- Stage 8: Hatching Blastocyst
- Stage 9: Hatched Blastocyst

Embryos are further classified and graded according to their quality on subjective criteria. The IETS in the early 1980s promoted and encouraged embryo transfer practitioners to grade embryos with guidelines into 4 groups. The Grade 1 would be called excellent, the Grade 2 called good, the Grade 3 would be fair and the Grade 4 are dead or degenerated as detailed below;

Grade 1 / A Grade: Excellent

- At least 85% of the cellular materials are intact / viable embryonic mass. Symmetrical and spherical / round shape embryo mass with individual blastomeres (cells) that are uniform in size, colour and density, no split cells and with normal development. This embryo is consistent with its expected stage of development and structural irregularities are relatively minor.

Grade 2 / B Grade: Good

- About 70 - 85% of the cellular materials are intact / viable embryonic mass. Symmetrical and spherical / round shape embryo mass with individual blastomeres (cells) that are uniform in size, colour & density, 10-20% separated cells / split cells & structural irregularities are relatively minor.

Grade 3 / C Grade: Fair

- About 55 - 70% of the cellular materials are intact / viable embryonic mass. Moderate irregularities in overall shape of the embryonic mass or in size, color and density of individual cells. Fair quality with 30 - 40% separated cells.

Grade 4 / D Grade: Poor / degenerated

- About 40 - 55% of the cellular materials are intact / viable embryonic mass. Major irregularities in shape of the embryonic mass or in size, colour and density of individual cells. Poor quality, dead / degenerated cells / oocytes or 1 cell embryos ≥ 60%, degenerating embryos, & these embryos are non-viable.

Each of these grades are applied to the stages of embryo development. Embryo evaluation is one of the most critical steps of the embryo transfer procedure. The IETS Manual states that embryos must be graded based on a 1 to 9 point system to determine the stage of development and a 1 to 4 system to determine embryo quality as detailed above. Grade 1 embryos survive well to the freezing/thawing procedure and are recommended for international trade; whereas Grade 2 and 3 must be transferred fresh into suitable recipients. Therefore, the decision as to whether an embryo is

worthy of transfer or freezing and whether the embryo is eligible for export will rely on the expertise and experience of the person that evaluates the embryos. The stage code signifies the relative developmental age of an embryo. Embryos that are seen at the time of flushing are typically morula (4), early blastocyst (5) or blastocyst (6). For example compact morulae (morulas) found on Day 6 are designated as a 4, early blastocysts the next stage of development are designated as a 5, blastocysts are a 6, and expanded blastocysts are designated as a 7. There of course is some overlapping in these stages, for example frequently one observes compact morulae also on Day 7. So you may see on a certificate an embryo and classification described as 4-1 which means it is a compact morula graded as a 1 with an excellent chance of becoming a pregnancy.

There is apparently no difference in pregnancy rates of fertilized cells in different stages of development assuming that they are transferred to the recipient female in the appropriate stage of the estrous cycle. Morula, early blastocyst, and blastocyst embryos endure the freezing and thawing procedures with the greatest viability. Embryo quality is also of utmost importance in the survival of the freezing and thawing stress. Grade 1 / A and Grade 2 / B are freezable embryos, while Grade 1 / A, 2 / B, 3 / C are fresh transferable embryos, yet pregnancy rates typically are reduced in grade 3 / C embryos.

The pictures of different stages & quality of embryos are annexured in Annexures 2 & 3.

14. EMBRYO LOADING (FRESH TRANSFER)

- Keep Holding media (HM) on the warm stage to keep it warm
- Transfer (using mouth piece) all the identified embryos (Compact Morula, Blastocyst,Expanded Blastocyst) from the search dish into the smaller Petri dish containing HM and wash for 2-3 times to remove mucus / other debris.
- After thorough cleaning, transfer (using mouth piece) the embryos into the four well plate (one well per Donor) containing HM.
- Draw about 3 ml of HM with syringe & needle and pour it into a small Petri dish marked as HM.
- Prepare the straw loading apparatus (French mini straws, Tuberculin syringe, adapter)
- Draw out 1 cm of HM, pull it up close to the factory sealing and then discard it for cleaning the straw. Make sure not to touch the factory seal with the HM.
- Transfer (using mouth piece) the embryo into Petri dish containing HM.
- Pull 3 cm (using tuberculin syringe) of HM into the straw
- Pull air 0.5 cm
- Pull 0.5 cm HM
- Pull air 0.5 cm
- Pull the embryo with the HM up to 1 cm

- Pull air 0.5 cm
- Pull 0.5 cm HM
- Pull air 0.5 cm
- Pull 3 cm of HM or till the media touches the factory sealing
- Check the loaded straw for the presence of embryo under the stereo microscope
- Now, the straw is ready for fresh transfer to recipient cows

15. EMBRYO LOADING (FROZEN TRANSFER)

- The Holding media (HM) are kept on the warm stage to keep it warm while the Freezing media (FM) should be kept at room temperature.
- Turn on and set up Embryo Freezer as outlined in section 14 (EMBRYO CRYOPRESERVATION).
- Transfer (using mouth piece) all the identified embryos (Compact Morula, Blastocyst, Expanded Blastocyst) from the search dish into the smaller Petri dish containing HM and wash for 2-3 times to remove mucus/ other debris.
- After thorough cleaning, transfer (using mouth piece) the embryos into the four well plate (one well per Donor) containing HM.
- Break open the seal of the FM and pour it into two small Petri dishes (Mark as FM 1, FM 2). In case we use previous stock of HM (balance of previous use) filter it with special filters / minisart Syringe filter.
- Draw about 3 ml of HM with syringe & needle and pour it into a small Petri dish marked as HM.
- Prepare the straw loading apparatus (French mini straws, Tuberculin syringe, adapter)
- Label the embryo straws with proper Identification using brady labeller
- Draw out 1 cm of HM, pull it up close to the factory sealing and then discard it for cleaning the straw. Make sure not to touch the factory seal with the HM.
- Transfer (using mouth piece) the embryo from the Petri dish containing HM to the FM 1. The process of Embryo loading and transfer into the Embryo Freezer should be completed within 10 min (timer begins when the embryos are put in FM 1).
- Make sure that the HM that are left in the mouthpiece is removed completely by gently blowing onto the paper towel.
- Transfer (using mouth piece) the embryo from the Petri dish containing FM 1 to the FM 2 for proper removal of HM from the embryos.
- NOW, the Embryos are ready for loading in the French Mini straws.
- Pull 3 cm (using tuberculin syringe) of FM into the straw
- Pull air 0.5 cm
- Pull 0.5 cm FM
- Pull air 0.5 cm

- Pull the embryo with the FM up to 1 cm
- Pull air 0.5 cm
- Pull 0.5 cm FM
- Pull air 0.5 cm
- Pull 3 cm of FM or till the media touches the factory sealing
- In case of Frozen Embryos, the embryos are suspended in the FM.
- Plug laboratory end of the straw with the plastic plug.
- Check the loaded straw for the presence of embryo under the stereo microscope
- Now, the straw is ready for freezing. Leave on paper towel at room temperature as other remaining embryos are loaded in a similar fashion.
- All embryos must be loaded within 10 minute time frame. Once all embryos are loaded within 10 minute time frame, straws can then be loaded into Embryo freezer.

Difference between loading of Fresh and Frozen embryos

Particulars	Fresh Embryos	Frozen Embryos
Medium	Only Holding medium	Holding & Freezing media
Cutting the factory seal end of the straw	Factory seal of straw pushed inside (1 cm) with a device (paper clip pin mounted on a micro-pipette tip) & cut	No need to cut the straws

*The straw is cut to adjust /fit into the length of the ET gun. In cryopreserved straws the laboratory end (sealed/plugged end) is cut after loading into the ET gun, while in fresh transfer embryos the manufacture end is adjusted and cut before loading.

16. EMBRYO CRYOPRESERVATION

Principle

Preserving embryo at sub zero temperature and change from liquid to solid state is the principle of cryopreservation. The Pre-implantation cattle embryos may be cryopreserved for short or long term storage. Pre-implantation embryos consist predominantly of water (70 – 80%), and the avoidance of intracellular ice crystal formation during the cryopreservation process is of paramount importance to maintain embryo viability. Cellular freezing constitutes a complex physiochemical process of heat and water transport between the cell and its surrounding medium (Maurer 1978, Schneider & Mazur 1985). There exists an optimum cooling rate for each cell type. It is dependent on the size of the cell, its surface to volume ratio, its permeability to water, and the temperature coefficient of that permeability (Schneider & Maurer 1983, Mazur 1977). Cells are injured during freezing and thawing primarily by solution

effects and intracellular ice formation. At high cooling rates the dehydration of the cell falls behind that of the solution and intracellular ice forms. To avoid intracellular freezing, embryos must be cooled at l°C / min or slower. However, too low a rate of cooling can also damage cells by what has been referred to as solution effect. This is especially harmful if the cells are not allowed to rehydrate during thawing. The required thawing rate depends on the freezing regimen used. There are Intracellular cryoprotectants (permeable) like glycerol, ethylene glycol, DMSO; and extracellular cryoprotectants (non-permeable) like sucrose and proteins (egg yolk, milk, blood serum). The two commonly available cryoprotectants for freezing embryos are 1.5 M Ethylene glycol with sucrose and 10% Glycerol with sucrose. Cryoprotecants are hypertonic solution (1.4 – 1.5 M) which creates an osmotic gradient that facilitates cellular dehydration. Glycerol requires stepwise exposure to the embryo before freezing and stepwise removal after thawing, while ethylene glycol allowing direct transfer after freezing and thawing and is the most widely used.

The functions of cryoprotectants are; lowering the freezing point of the media which gives cells more time to dehydrate, lower the amount of intracellular ice (at any given temperature) thus protect / reducing cell membrane from physical damage during freezing / thawing and also produce dilution effect. The more rapid techniques of freezing and thawing are preferred for field work. Embryos are normally stored in liquid nitrogen at - 196°C. The only reactions that occur at -196°C are direct ionizations from background radiation. Consequently, storage times of 200 years or so are unlikely to produce any detectable reduction in the survival of frozen embryos or cause genetic change (Schneider & Mazur 1985). There are two types of freezing machines available for slow freezing of embryos; Alcohol bath freezers and LN2 freezers, working either by vaporizing LN_2 or by a mechanical refrigerated alcohol bath.

Particulars	**Glycerol**	**Ethelene glycol**
Equilibration with cryoprotectant	10 minutes at room temperature	10 minutes at room temperature
Cooling start Temperature	20 oC	-6 °C
Cooling rate	1.0 °C /min to -7 °C, 0.1 °C / min until - 33°C,	0.5 °C / min until -35°C
Ice seeding	after 3 to 5 min	At 2 min
Holding time	10 min	10 min
Plunging into LN2	-196 °C	-196 °C

Procedure

- Set up the pre program freezing machine / programmable Freezer i.e Crysalys Cryocontroller PTC – 9500 (, Cryo Controller and Cryo Chamber Cryo Can).
- Connect the Cryo Controller (Programmer) to the Cryo Chamber.
- Lift the Cryo Chamber from the Cryo Can and Fill the Cryo Can with LN_2 (approx. 75%).
- Connect the Programmer to the power source.
- Put on the switch.
- Check all the setting on the Programmer. Set the program to Bovine.
- Once selected the Embryo Freezer will gradually decrease temperature to - 6 °C (which will be displayed on the screen).
- Once the temperature reaches -6°C the Freezer is ready for freezing the Embryo straw. The temperature is maintained at -6°C for loading the Embryo straws.
- Load embryos to be frozen as outlined in section 13 (EMBRYO LOADING – FROZEN TRANSFER)
- The straws are then placed into the Embryo Freezer, pre cooled at - 6 °C. Make sure that the straws are balanced into the Cryocan with an empty straw if necessary, similar to balancing a centrifuge (two straws per bore). Press run on Embryo Freezer. Program will hold temperature for 10 mins at -6 °C before dropping by -0.5 °C / min.
- After 2 min (alarm set for 2 min) of equilibration in the -6 °C chamber the straws are seeded. Seeding is accomplished by pulling out the straw slowly and touching / rotating straw wall with forceps (wrapped with cotton swab) dipped into LN_2 at the junction between air bubble and freezing media (without embryo). This process causes ice to form in the solution (Freezing media has lower freezing point than Holding media) and enables the required slow and uniform cooling rate to be achieved. Ice formation starts the process of cellular dehydration, thus reducing the amount of intracellular ice.
- The ice formation of the freezing media is visible (cloudy appearance) which confirm that the seeding is complete.
- After seeding, immerse the straw back into the freezer. During the whole process make sure that LN_2 is maintained at the desired level.
- The embryos in the straws will cool slowly at 0.5 °C/min (it can fluctuate briefly between 0.3° and 0.7°C) enabling further dehydration, to -35 °C. At -35 °C the Embryos changes from liquid (70 - 80%) to solid state and metabolic activities stop completely.
- Once the temperature reaches -35 °C the straws are plunged into LN_2 at -196 °C. Insert the straw in goblet with canister (with proper identification), put the cap to prevent embryo straws floating. Ensure straw is kept within LN_2 at all times.

- Remove the remaining LN_2 (can be used to top up tank in which embryos are stored), dry the Cryocan, Cryo Chamber and store it for next use.
- It is speculated that the embryos once frozen will have a storage life similar to that of frozen spermatozoa.

17. EMBRYO THAWING

According to the two different types of cryoprotectants used, the embryos are thawed as below;

For Ethylene glycol cryoptotectant:

- No special thawing procedure is required.
- Air thawing for 5 seconds
- Warm water thawing at 35°C for 10 - 30 seconds

For Glycerol cryoprotectant:

- The straw is removed from LN_2 container, placed into a water bath (35°C) and gently agitated until all ice is melted.
- The straw is then emptied into a prepared small Petri dish, containing a mixed 6% glycerol + 0.3 M sucrose in holding medium and kept for 5 min.
- Then the embryo is transferred into another small Petri dish, with 3% glycerol + 0.3 M sucrose in holding medium
- Finally the embryos are placed in normal holding medium for 10 minutes before evaluation. The thawed embryos are then transferred.

18. ET TRANSFER (FROZEN EMBRYOS)

- Ovaries of the recipient are palpated rectally to determine which ovary has ovulated (presence of prominent CL on which side of the ovaries)
- Mark on the flank region with marker on the side of the ovary which has CL with grading based on quality of CL as A, B, C
- Inject epidural anaesthesia (between first and second coccygeal vertebrae)
- Prepare the ET set for the transfer of embryo to the recipients
- Take out the Goblet with canister containing the embryos from the storage tank and immerse it completely in cool box filled with LN_2.
- Prepare the ET gun, ET sheath, sanitary sheath, forceps, thermometer, straw cutter and alcohol and thawing set
- Prepare the warm water at 35 to 37°C. Clean the straw cutter with alcohol swab. Take out the ET gun from the bag. Remove the Embryo straws from LN_2 with forceps

- Air thaw the straw for 5 - 10 seconds and thaw in warm water for 30 seconds.
- Wipe the straw with paper towel to remove the water completely.
- Gently tap the straw with your finger and invert the straw several times to mix the holding media and freezing media allowing the air bubbles to rise toward the plug end of the straw.
- Cut the straws 0.5 cm below the plug end of the straw. Load the straw into the ET gun. Make sure that the stylet is removed from the ET gun about the length of the straw.
- Put the ET sheath over the ET gun ensuring that the end of the ET sheath touches the tip of ET gun. Lock the gun with O ring firmly. Put the sanitary sheath over the loaded ET gun.
- With the aid of an assistant to hold open the vulva of the recipient cow, the ET gun is carefully passed through the cervix
- Insert the ET gun until external os and pull out the sanitary sheath once the ET gun reaches the external os
- The tip of the ET gun is then allowed to slide into the horn (pull the uterine horn over the ET gun as far as possible) on the same side of the ovary with an active CL.
- Push the plunger to gently expel the embryo in the forward tip of that uterine horn
- Take care not to cause damage to the lining of the uterus. Such inflammation and scarring would greatly reduce the probability of the pregnancy being established.
- Take out the ET gun. Clean the ET gun, stylet with alchol swab and put it back into the sanitary pack for next use.

19. ET TRANSFER (FRESH EMBRYOS)

- Per rectal examination of the recipient cows for the presence of Corpus Luteum (CL) on which side of the ovaries
- Mark on the flank region with marker on the side of the ovary which has CL with grading based on quality of CL as A, B, C
- Prepare the ET set for the transfer of embryo to the recipients
- Prepare the ET gun, ET sheath, Sanitary sheath, and alcohol.
- Take out the ET gun from the bag and keep the Embryo straws ready
- Gently tap the straw with your finger and invert the straw several times to mix the holding media allowing the air bubbles to rise toward the plug end of the straw.
- Load the straw into the ET gun. Make sure that the stylet is removed from the ET gun about the length of the straw

- Put the ET sheath over the ET gun ensuring that the end of the ET sheath touches the tip of ET gun. Lock the gun with O ring firmly. Put the sanitary sheath over the loaded ET gun
- Insert the ET gun until external os and pull out the sanitary sheath once the ET gun reaches the external os
- Insert the gun into the cervix, uterine body and pull the uterine horn (on the side where the CL was present) over the ET gun as far as possible
- Push the plunger to load the Embryo into the uterine horn. Take out the ET gun
- Clean ET gun, stylet with alchol swab and put it back into the sanitary bag for next use

20. MOUTH PIECE PREPARATION

Many devices are used to manipulate embryos. Standard Pasteur pipettes are much too large and the tips require fire polishing to prevent damage to embryos. Pipettes can be prepared from Pyrex glass tubing with a 4 mm outside diameter. Pipettes are made in batches of several hundred. They can be reused if debris does not adhere and they are washed in ultrapure water and then with 70% alcohol and kept for drying in hot air oven immediately after use.

Preparation of mouth piece

- Micro haemotocrit tube is heated in the centre with a Bunsen burner or gas burner.
- Pull both ends of heated tube until the desired diameter (outside diameter < 1 mm)
- This is then scored with a diamond pencil glass cutter / glass cutter at the desired bore size and broken to make two pipettes. The diamond cutter can be heated in flame briefly to assist with clean quick cut of pipette
- The ends of both the pipette tips are fire polished. Make sure that the cut end is smooth to prevent embryo damage during manipulation. Inspect under microscope to ensure smooth ends.
- After pipettes have been washed and rinsed thoroughly, they are placed in clean glass test tubes with screw tops. Sterilized (and dried) by dry heat.
- For use, pipettes are connected to a 0.5 or 1cc syringe or plastic mouthpiece with rubber tubing. The other end of the mouth piece is connected to the micro pipette tip. The mouth piece is ready for use.

Preparation of Embryo loading straw

- Take tuberculin syringe
- Connect the adapter
- Insert the French mini straw

21. TRYPSINIZATION

Principle

Trypisinization is the exposure of intact zona pellucida (ZP) bovine embryos to the proteolytic enzyme, trypsin to avoid transmission of pathogens by embryo transfer. Research has shown that ZP intact bovine embryos are remarkably resistant to infection (Hare 1985). This is because ZP acted as an effective barrier against infection of embryos and most pathogens are not capable of penetrating the ZP. Also gametes, somatic cells and materials of animal origin in the media are potential sources for introducing pathogens in the embryo. In order that disease agents not be transferred along with the embryo, it is essential that several criteria like examination of ZP, successive washing / processing is carried out to ensure that no pathogenic organism be introduced.

Procedure

The IETS recommended the standard procedures (Singh 1989) of washing and trypsin treatment to remove pathogens adhering to the ZP, especially for import / export of embryos to other countries. The procedure is as below;

- Pick up the embryos using mouth piece from the four well tissue culture dish and transfer through five washes of PBS without Ca^{++} and Mg^{++} but with antibiotics (penicillin) and 0.04 % bovine serum albumin in a petri dish. The embryos should be kept for 15 seconds in each droplet
- Transfer / wash the embryos through two aliquots of 0.25% trypsin at room temperature, pH 7.8 - 8.0, for a total time of less than 90 seconds in the two droplets
- After trypsin treatment, the embryos are transferred through five washes of PBS containing Ca^{++} and Mg^{++}, antibiotics (penicillin / streptomycin) and 2 % calf serum. The embryos should be kept for 15 seconds in each droplet
- Wash the embryos in Holding medium through three droplets
- After washing keep the embryos for 30 minutes in the Holding medium for healing of ZP
- After trypsinization, the embryos can be loaded / frozen as per normal procedure.

22. CLEANING & STERILIZATION

i. Ultrasonic cleaning

Materials

Foley's catheter, Y - Flushing tube, ET filter cup, plastic measuring cylinder. Plastic tubes / pipes with narrow passage where conventional cleaning is difficult

Procedures

- Clean all the used equipments thoroughly in the tap water
- Make sure to remove faeces and dirt with a brush from the equipments and rinse it with running tap water thoroughly
- Rinsed Embryo filter cup with distilled water to remove mucus and other debris and dip it in water containing 7X detergent to keep it wet so that the remaining mucus do not dry and block the filter
- 7X is a tissue culture grade and safe for cleaning the above equipments (www.mpbio.com)
- Dip all above equipments in the ultrasonic cleaner (filled with distilled water till brim). The equipments to be sterilized should be completely immersed in the solution.
- Detergent:Water ratio; 4ml of 7X detergent per litre of distilled water. Switch on the ultrasonic cleaner and keep it on for half an hour
- Drain out (using drainage tap) the water from the UC machine and wash in the ultra pure water.
- Put back the equipments in the UC machine and pour distilled water and switch on for another 15 mins for second cleaning
- Remove the equipments from UC and air dry for 25-30 min
- Now wrap them individually in sterilized plastic bags, aluminium foil for future use
- Sterilize the equipments (plastic) in Gas sterilizer and 2 Way Foley catheter (silicone) in Autoclave

i. Auto Clave

- Foley's stylet, cervical dilator, glass wares, forceps etc.
- Silicone apparatus like Foley's catheter can be cleaned with alcohol and distilled water and sterilized in Autoclave

ii. Hot Air Oven

- Plastic, Rubber, Glasswares, mouth piece

iii. Gas sterilization with Ethylene Oxide

- Embryo filter, blue sheath, 3 way's Foleys, Y flushing tube and all Plastic apparatus

iv. Water Ultra Purifier

- Resistivity of water for preparing semen extenders should be 16 MΩ·cm or more, for ET (washing materials) 15 MΩ·cm can be used. However, for preparing the media in ET/ IVF work resistivity should be 18 MΩ·cm (ideal is 18.2 MΩ·cm).

23. KEY INGREDIENTS FOR SUCCESSFUL ET PROGRAM

Sometimes ET programs are failures, usually because pregnancy rates are very low. Probably the main reason for failure is insufficient investment in training personnel. The second most common problem is insufficient animal resources. Unless large numbers of healthy, thriving cattle are available, ET will not work well, particularly when personnel are developing skills. Facilities and equipment are also important, but are frequently over-emphasized. A clean laboratory work area is needed; mobile vans can be used for this purpose. Obviously, cows must be kept separate from bulls. An unusually common error is that recipients become pregnant from natural service rather than embryo transfer, which is not discovered until calves are born one oestrous cycle late or are of the wrong breed. It is clearly necessary to be able to restrain animals for injections, AI, embryo recovery and embryo transfer. A generally successful approach is to build ET on a programme that has been successful for AI. Facilities and logistics of handling animals are similar for both techniques. Also, the skills of good oestrus detection and passing catheters through the cervix are an excellent foundation for ET. In fact, it is not recommend training people in techniques of embryo recovery and transfer until they are proficient in AI (meaning that they have inseminated well over 100 animals with good pregnancy rates).

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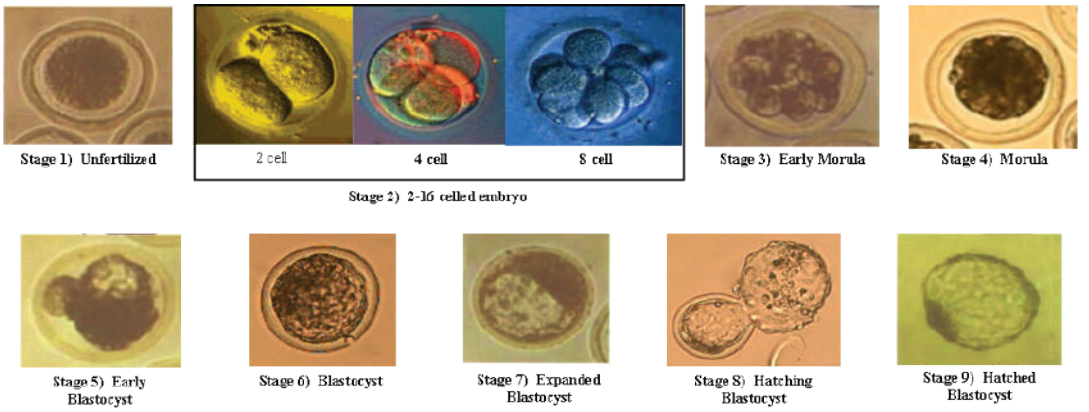
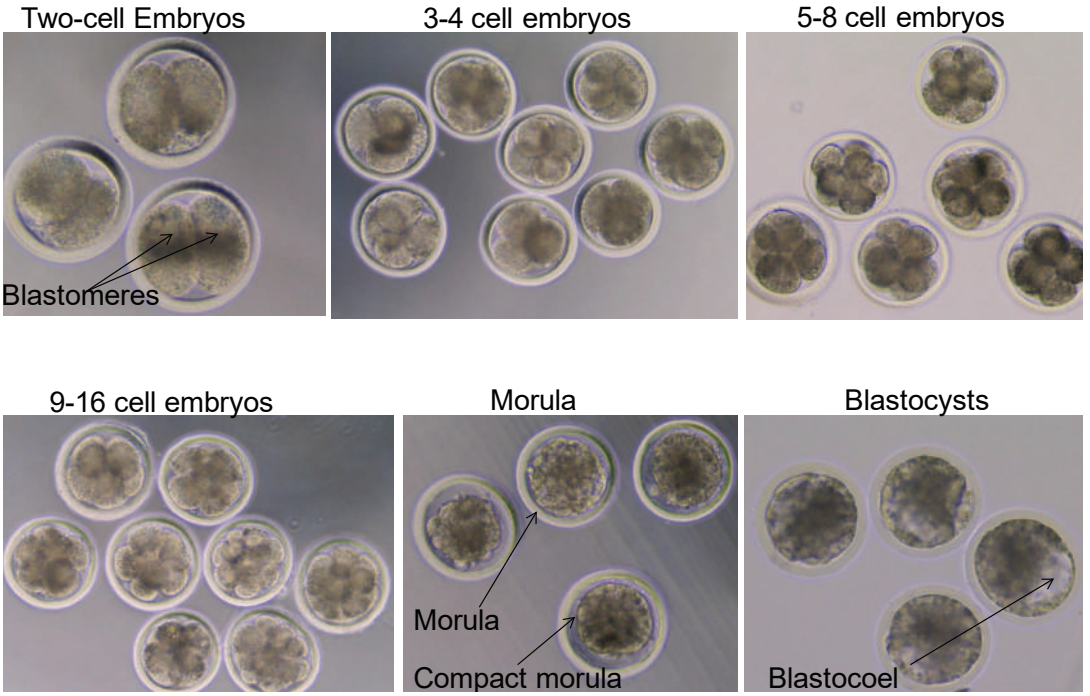
ANNEXURE 1

Annexure 1A: Stages of Bovine embryo



Annexure 1B: Stages & Quality / Grades of Bovine embryos

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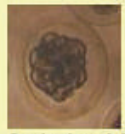
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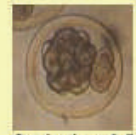
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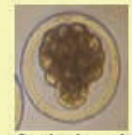
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Cycle day: 7
Stage Code: 4
Quality Code:1


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Cycle day: 7
Stage Code: 4
Quality Code:1


Cycle day: 7
Stage Code: 4
Quality Code:1


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Stage Code: 4
Quality Code:2


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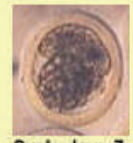
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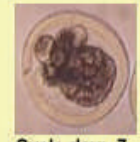
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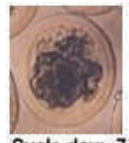
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Stage Code: 4
Quality Code:3


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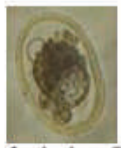
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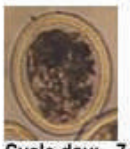
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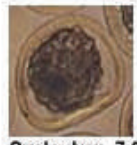
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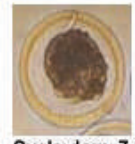
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ANNEXURE 2

Annexure 2: International Embryo Transfer Society (IETS) embryo retrieval / cryopreservation forms

A. CERTIFICATE OF EMBRYO RECOVERY

Page ___ of ___

Breed _____

Donor Name _____ No. _____ _____ Ear Tag or Tattoo
CC

Owner _____ Address _____

Onset ☐ AM
_____ Estrus Date_____ ☐ PM
Yr. Mo. Day

Service Sire _____ No. _____ _____ Breeding Date_____
CC 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 _____
CC

ID Code _____ Freeze Date or Batch No. _____ Sexed Semen X or Y ___ No. Cleaved/Degen. _____
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)

ACCOMMODATES DIRECT TRANSFER

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

IF FROZEN

Freeze Date on straw _____
Practitioner I.D. _____
Straw No.'s _____

Days since Estrus of Donor _____

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.									
9.									
10.									
11.									
12.									

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.

CONDITIONS FOR COMPLETING EMBRYO CERTIFICATES

A. Complete one or more Certificate of Embryo Recovery for each recovery. Precede registration numbers of donor dam and donor sire(s) with the International Standards Organization (ISO) three letter country codes (cc). The responsible practitioner or leader of embryo production team signing this certificate is attesting to the fact that the donor dam was identified with her certificate of registration, that the service sire information was taken from a written record of services, and that all the information is true and correct.

B. Certificate of Embryo Transfer will be completed to the extent that is necessary and/or appropriate to identify each recipient into which an embryo is transferred. If frozen embryos are transferred, the Certificate of Embryo Recovery will be completed by the responsible practitioner or by transferring from the original Certificate of Embryo Recovery or by attaching a copy. The practitioner or leader of embryo production team signing the Certificate of Embryo Transfer is attesting to the accuracy and completeness of the identification of the embryos being transferred and the identity of the recipients into which the embryos are being transferred. Days since estrus will be expressed in 1/2 day increments.

One copy of a complete Certificate of Embryo Transfer with Certificate of Embryo Recovery will be submitted to the appropriate breed office as required by the respective breed organization, which may require the record before resulting offspring can be registered or officially identified. One copy should be provided to the owner.

Should any embryo in a recipient as identified hereon change ownership, it is important that the requirements of the respective breed organization for recording the identification of the new owner be followed. This may be required before resulting offspring can be registered or officially identified. This would normally be the responsibility of the owner of record.

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

STAGE OF DEVELOPMENT

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

QUALITY OF EMBRYOS

Code 1. Excellent or Good
Code 2. Fair
Code 3. Poor
Code 4. Dead or degenerating

Refer to IETS Manual, Third Edition, Chapter 9, for description of codes.

MANIPULATION OF EMBRYOS

N - Not Manipulated
D - Divided
F - Female by Biopsy
M - Male by Biopsy
U - Sex Undetermined by Biopsy

Use comment column to describe other biopsies.

SEXED SEMEN

X - Female Y - Male

January	JA
February	FE
March	MR
April	AP
May	MY
June	JN
July	JY
August	AU
September	SE
October	OC
November	NO
December	DE

BOVINE

AN	Aberdeen Angus	BZ	Bonsmara	GS	Gascone	MR	Marchigiana
AB	Abondance	CP	Campine Red Pied	GV	Gelbvieh	ME	Maremmana
AF	Afrikaner	CN	Canadienne	GI	Gir	MI	Meuse Rhine Issel
AK	Ankole	CB	Charbray	GR	Groninger	MO	Montbeliard
AY	Ayrshire	CH	Charolais	GU	Guernsey	MG	Murray Grey
BA	Barzona	CA	Chianina	GZ	Guzera	NE	Nelore
BE	Beefalo	DB	Danish Black & White	HC	Hays Converter	NG	Nguni
BF	Beef Friesian	DJ	Danish Jersey	HH	Hereford (horned)	NM	Normande
BM	Beefmaster	RW	Danish Red & White	HP	Hereford (polled)	NR	Norwegian Red
BB	Belgian Blue	DE	Devon	SH	Highland (Scotch Highland)	PA	Parthenais
BG	Belted Galloway	DR	Dexter	HO	Holstein	PI	Piedmont
BD	Blonde D'Aquitaine	DZ	Drakensberger	HY	Hybrid (Alberta Hybrid)	PR	Pie Rouge
BX	Boran	FP	East Flemish Red Pied	IB	Ibage	PZ	Pinzgauer
BO	Braford	ER	Eringer	IN	Indu Brasil	RA	Ranger
BR	Brahman	FA	Flamand	JE	Jersey	AR	Red Angus
BH	Brahmental	FL	Fleckvieh	KB	Kobe (Wagyu)	RB	Red Brangus
BL	Braler	FR	Fribourg	LU	Luing	RD	Red Dane (Red Danish, Danish Red)
BN	Brangus	FB	Friesian (Belgian)	LM	Limousin	WW	Red Holstein
BU	Braunvieh	DF	Friesian (Dutch)	LR	Lincoln Red	RP	Red Poll
SB	Brown Swiss (beef)	GA	Galloway (beef)	MA	Maine-Anjou	RN	Romagnola
BS	Brown Swiss (dairy)	GD	Galloway (dairy)				

RO	Rotbunte						
SA	Salers						
SG	Santa Gertudis						
SE	Senapol						
MS	Shorthorn (milking)						
SS	Shorthorn (beef - Scotch)						
SP	Shorthorn (polled)						
IS	Shorthorn (Illawarra)						
SI	Simbrah						
SM	Simmental						
DS	South Devon						
SX	Sussex						
TA	Tarentaise						
TG	Tasmanian Grey						
TL	Texas Longhorn						
TU	Tuli						
WB	Welsh Black						
WF	West Flemish Red						
XX	Crossbreds						

CAPRINE

AL	Alpine	AG	Angora	CH	Cashmere	KR	Kalahari Reds
TO	Toggenburg	SA	Saanen	BG	Boer	LN	La Mancha

NU	Nubian		
SG	Savanna Goat		

EQUINE

AS	American Saddlebred	CL	Clydesdale	MN	Morgan	QH	Quarter Horse
AP	Appaloosa	HA	Hackney (Horse)	PL	Palomino	SE	Shetland
AB	Arabian	HK	Hackney (Pony)	PE	Percheron	SI	Shire
BL	Belgian	HU	Hunter	PN	Pinto	SN	Standardbred

SF	Suffolk Punch		
TW	Tennessee Walking		
TH	Thoroughbred		
WE	Welsh		

PORCINE

YO	Yorkshire	DU	Duroc	BK	Berkshire	PE	Pietrain
LA	Landrace	LC	Lacombe	SO	Spotted	TM	Tamworth
HA	Hampshire	PC	Poland Chine	CW	Chester White	WS	Wessex Saddleback

LW	Large White (British)		
LB	Large Black (British)		

OVINE

AN	Afrino	DM	Dohne Merino	IL	Ile de France	OX	Oxford
BP	Blackhead Persian	DO	Dorset	LE	Leicester	SP	Persian (Speckled/Skilder)
BC	Border Cheviot	DR	Dormer	LI	Lincoln	RA	Rambouillet
CO	Columbia	DP	Dorper	ME	Merino	RM	Romnelet
CR	Corriedale	FN	Finnish Landrace	MT	Montadale	SB	Scottish Blackface
DA	Damara	HA	Hampshire	NC	N. Country Cheviot	SR	Shropshire

MM	S. African Mutton Merino		
ST	Southdown		
SU	Suffolk		
VR	Van Rooy		
WD	White Dorper		

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A. CERTIFICATE OF EMBRYO RECOVERY

Page ___ of ___

Breed _____

Donor Name _____ No. ____ (CC) _____ Ear Tag or Tattoo

Owner _____ Address _____

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

Service Sire _____ No. ____ (CC) _____ 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. ____ (CC) _____ Total Recovered _____

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

No. Cleaved/Degen. _____

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)

ACCOMMODATES DIRECT TRANSFER

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

IF FROZEN

Freeze Date on straw _____

Practitioner I.D. _____

Straw No.'s 2 _____

Days since Estrus of Donor _____

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 donor, 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 ( ) _____

CONDITIONS FOR COMPLETING EMBRYO CERTIFICATES

A. Complete one or more Certificate of Embryo Recovery for each recovery. Precede registration numbers of donor dam and donor sire(s) with the International Standards Organization (ISO) three letter country codes (cc). The responsible practitioner or leader of embryo production team signing this certificate is attesting to the fact that the donor dam was identified with her certificate of registration, that the service sire information was taken from a written record of services, and that all the information is true and correct.

B. Certificate of Embryo Transfer will be completed to the extent that is necessary and/or appropriate to identify each recipient into which an embryo is transferred. If frozen embryos are transferred, the Certificate of Embryo Recovery will be completed by the responsible practitioner or by transferring from the original Certificate of Embryo Recovery or by attaching a copy. The practitioner or leader of embryo production team signing the Certificate of Embryo Transfer is attesting to the accuracy and completeness of the identification of the embryos being transferred and the identity of the recipients into which the embryos are being transferred. Days since estrus will be expressed in 1/2 day increments.

One copy of a complete Certificate of Embryo Transfer with Certificate of Embryo Recovery will be submitted to the appropriate breed office as required by the respective breed organization, which may require the record before resulting offspring can be registered or officially identified. One copy should be provided to the owner.

Should any embryo in a recipient as identified hereon change ownership, it is important that the requirements of the respective breed organization for recording the identification of the new owner be followed. This may be required before resulting offspring can be registered or officially identified. This would normally be the responsibility of the owner of record.

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

The complete Certificate of Embryo Transfer with Certificate of Freezing will be submitted to the appropriate breed office or as required by the respective breed organization, which may require the record before resulting offspring can be registered or officially identified. One copy will be provided the owner.

When a frozen embryo changes ownership, it is important that the requirements of the respective breed organization for recording the identification of the new owner be followed. This may be required before resulting offspring can be registered or officially identified. This would normally be the responsibility of the owner of record.

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

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

STAGE OF DEVELOPMENT

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

QUALITY OF EMBRYOS

Code 1. Excellent or Good
Code 2. Fair
Code 3. Poor
Code 4. Dead or degenerating

Refer to IETS Manual, Third Edition, Chapter 9, for description of codes.

MANIPULATION OF EMBRYOS

N - Not Manipulated
D - Divided
F - Female by Biopsy
M - Male by Biopsy
U - Sex Undetermined by Biopsy

Use comment column to describe other biopsies.

SEXED SEMEN

X - Female Y - Male

January	JA
February	FE
March	MR
April	AP
May	MY
June	JN
July	JY
August	AU
September	SE
October	OC
November	NO
December	DE

BOVINE

AN - Aberdeen Angus	BZ - Bonsmara	GS - Gascone	MR - Marchigiana	RO - Rotbunte
AB - Abondance	CP - Campine Red Pied	GV - Gelbvieh	ME - Maremmana	SA - Salers
AF - Afrikaner	CN - Canadienne	GI - Gir	MI - Meuse Rhine Issel	SG - Santa Gertudis
AK - Ankole	CB - Charbray	GR - Groninger	MO - Montbeliard	SE - Senapol
AY - Ayrshire	CH - Charolais	GU - Guernsey	MG - Murray Grey	MS - Shorthorn (milking)
BA - Barzona	CA - Chianina	GZ - Guzera	NE - Nelore	SS - Shorthorn (beef - Scotch)
BE - Beefalo	DB - Danish Black & White	HC - Hays Converter	NG - Nguni	SP - Shorthorn (polled)
BF - Beef Friesian	DJ - Danish Jersey	HH - Hereford (horned)	NM - Normande	IS - Shorthorn (Illawarra)
BM - Beefmaster	RW - Danish Red & White	HP - Hereford (polled)	NR - Norwegian Red	SI - Simbrah
BB - Belgian Blue	DE - Devon	SH - Highland (Scotch Highland)	PA - Parthenais	SM - Simmental
BG - Belted Galloway	DR - Dexter	HO - Holstein	PI - Piedmont	DS - South Devon
BD - Blonde D'Aquitaine	DZ - Drakensberger	HY - Hybrid (Alberta Hybrid)	PR - Pie Rouge	SX - Sussex
BX - Boran	FP - East Flemish Red Pied	IB - Ibage	PZ - Pinzgauer	TA - Tarentaise
BO - Braford	ER - Eringer	IN - Indu Brasil	RA - Ranger	TG - Tasmanian Grey
BR - Brahman	FA - Flamand	JE - Jersey	AR - Red Angus	TL - Texas Longhorn
BH - Brahmental	FL - Fleckvieh	KB - Kobe (Wagyu)	RB - Red Brangus	TU - Tuli
BL - Braler	FR - Fribourg	LU - Luing	RD - Red Dane (Red Danish, Danish Red)	WB - Welsh Black
BN - Brangus	FB - Friesian (Belgian)	LM - Limousin	WW - Red Holstein	WF - West Flemish Red
BU - Braunvieh	DF - Friesian (Dutch)	LR - Lincoln Red	RP - Red Poll	XX - Crossbreds
SB - Brown Swiss (beef)	GA - Galloway (beef)	MA - Maine-Anjou	RN - Romagnola	
BS - Brown Swiss (dairy)	GD - Galloway (dairy)			

CAPRINE

AL - Alpine	AG - Angora	CH - Cashmere	KR - Kalahari Reds	NU - Nubian
TO - Toggenburg	SA - Saanen	BG - Boer	LN - La Mancha	SG - Savanna Goat

EQUINE

AS - American Saddlebred	CL - Clydesdale	MN - Morgan	QH - Quarter Horse	SF - Suffolk Punch
AP - Appaloosa	HA - Hackney (Horse)	PL - Palomino	SE - Shetland	TW - Tennessee Walking
AB - Arabian	HK - Hackney (Pony)	PE - Percheron	SI - Shire	TH - Thoroughbred
BL - Belgian	HU - Hunter	PN - Pinto	SN - Standardbred	WE - Welsh

PORCINE

YO - Yorkshire	DU - Duroc	BK - Berkshire	PE - Pietrain	LW - Large White (British)
LA - Landrace	LC - Lacombe	SO - Spotted	TM - Tamworth	LB - Large Black (British)
HA - Hampshire	PC - Poland Chine	CW - Chester White	WS - Wessex Saddleback	

OVINE

AN - Afrino	DM - Dohne Merino	IL - Ile de France	OX - Oxford	MM - S. African Mutton Merino
BP - Blackhead Persian	DO - Dorset	LE - Leicester	SP - Persian (Speckled/Skilder)	ST - Southdown
BC - Border Cheviot	DR - Dormer	LI - Lincoln	RA - Rambouillet	SU - Suffolk
CO - Columbia	DP - Dorper	ME - Merino	RM - Romnelet	VR - Van Rooy
CR - Corriedale	FN - Finnish Landrace	MT - Montadale	SB - Scottish Blackface	WD - White Dorper
DA - Damara	HA - Hampshire	NC - N. Country Cheviot	SR - Shropshire	

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A. CERTIFICATE OF EMBRYO RECOVERY

Page ___ of ___

Breed _____

Donor Name _____ No. _____ (CC) _____ Ear Tag or Tattoo

Owner _____ Address _____

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

Service Sire _____ No. _____ (CC) _____ 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. _____ (CC) _____ 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 _____

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 donor, 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 ( ) _____

CONDITIONS FOR COMPLETING EMBRYO CERTIFICATES

A. Complete one or more Certificates of Embryo Recovery for each recovery. Precede registration numbers of donor dam and donor sire(s) with the International Standards Organization (ISO) three letter country codes (cc). The responsible practitioner or leader of the embryo production team signing this certificate is attesting to the fact that the donor dam was identified with her certificate of registration, that the service sire information was taken from a written record of services, and all the information is true and correct.

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

The complete Certificate of Embryo Transfer with Certificate of Freezing will be submitted to the appropriate breed office or as required by the respective breed organization, which may require the record before resulting offspring can be registered or officially identified. One copy will be provided to the owner.

When a frozen embryo changes ownership, it is important that the requirements of the respective breed organization for recording the identification of the new owner be followed. This may be required before resulting offspring can be registered or officially identified. This would normally be the responsibility of the owner of record.

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

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

STAGE OF DEVELOPMENT

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

QUALITY OF EMBRYOS

Code 1. Excellent or Good
Code 2. Fair
Code 3. Poor
Code 4. Dead or degenerating

Refer to IETS Manual, Third Edition, Chapter 9, for description of codes.

MANIPULATION OF EMBRYOS

N - Not Manipulated
D - Divided
F - Female by Biopsy
M - Male by Biopsy
U - Sex Undetermined by Biopsy

Use comment column to describe other biopsies.

SEXED SEMEN

X - Female Y - Male

January	JA
February	FE
March	MR
April	AP
May	MY
June	JN
July	JY
August	AU
September	SE
October	OC
November	NO
December	DE

BOVINE

AN	- Aberdeen Angus	BZ	- Bonsmara	GS	- Gascone	MR	- Marchigiana	RO	- Rotbunte
AB	- Abondance	CP	- Campine Red Pied	GV	- Gelbvieh	ME	- Maremmana	SA	- Salers
AF	- Afrikaner	CN	- Canadienne	GI	- Gir	MI	- Meuse Rhine Issel	SG	- Santa Gertudis
AK	- Ankole	CB	- Charbray	GR	- Groninger	MO	- Montbeliard	SE	- Senapol
AY	- Ayrshire	CH	- Charolais	GU	- Guernsey	MG	- Murray Grey	MS	- Shorthorn (milking)
BA	- Barzona	CA	- Chianina	GZ	- Guzera	NE	- Nelore	SS	- Shorthorn (beef - Scotch)
BE	- Beefalo	DB	- Danish Black & White	HC	- Hays Converter	NG	- Nguni	SP	- Shorthorn (polled)
BF	- Beef Friesian	DJ	- Danish Jersey	HH	- Hereford (horned)	NM	- Normande	IS	- Shorthorn (Illawarra)
BM	- Beefmaster	RW	- Danish Red & White	HP	- Hereford (polled)	NR	- Norwegian Red	SI	- Simbrah
BB	- Belgian Blue	DE	- Devon	SH	- Highland (Scotch Highland)	PA	- Parthenais	SM	- Simmental
BG	- Belted Galloway	DR	- Dexter			PI	- Piedmont	DS	- South Devon
BD	- Blonde D'Aquitaine	DZ	- Drakensberger	HO	- Holstein	PR	- Pie Rouge	SX	- Sussex
BX	- Boran	FP	- East Flemish Red Pied	HY	- Hybrid (Alberta Hybrid)	PZ	- Pinzgauer	TA	- Tarentaise
BO	- Braford	ER	- Eringer	IB	- Ibage	RA	- Ranger	TG	- Tasmanian Grey
BR	- Brahman	FA	- Flamand	IN	- Indu Brasil	AR	- Red Angus	TL	- Texas Longhorn
BH	- Brahmental	FL	- Fleckvieh	JE	- Jersey	RB	- Red Brangus	TU	- Tuli
BL	- Braler	FR	- Fribourg	KB	- Kobe (Wagyu)	RD	- Red Dane (Red Danish, Danish Red)	WB	- Welsh Black
BN	- Brangus	FB	- Friesian (Belgian)	LU	- Luing			WF	- West Flemish Red
BU	- Braunvieh	DF	- Friesian (Dutch)	LM	- Limousin	WW	- Red Holstein	XX	- Crossbreds
SB	- Brown Swiss (beef)	GA	- Galloway (beef)	LR	- Lincoln Red	RP	- Red Poll		
BS	- Brown Swiss (dairy)	GD	- Galloway (dairy)	MA	- Maine-Anjou	RN	- Romagnola		

CAPRINE

AL	- Alpine	AG	- Angora	CH	- Cashmere	KR	- Kalahari Reds	NU	- Nubian
TO	- Toggenburg	SA	- Saanen	BG	- Boer	LN	- La Mancha	SG	- Savanna Goat

EQUINE

AS	- American Saddlebred	CL	- Clydesdale	MN	- Morgan	QH	- Quarter Horse	SF	- Suffolk Punch
AP	- Appaloosa	HA	- Hackney (Horse)	PL	- Palomino	SE	- Shetland	TW	- Tennessee Walking
AB	- Arabian	HK	- Hackney (Pony)	PE	- Percheron	SI	- Shire	TH	- Thoroughbred
BL	- Belgian	HU	- Hunter	PN	- Pinto	SN	- Standardbred	WE	- Welsh

PORCINE

YO	- Yorkshire	DU	- Duroc	BK	- Berkshire	PE	- Pietrain	LW	- Large White (British)
LA	- Landrace	LC	- Lacombe	SO	- Spotted	TM	- Tamworth	LB	- Large Black (British)
HA	- Hampshire	PC	- Poland Chine	CW	- Chester White	WS	- Wessex Saddleback		

OVINE

AN	- Afrino	DM	- Dohne Merino	IL	- Ile de France	OX	- Oxford	MM	- S. African Mutton Merino
BP	- Blackhead Persian	DO	- Dorset	LE	- Leicester	SP	- Persian (Speckled/Skilder)	ST	- Southdown
BC	- Border Cheviot	DR	- Dormer	LI	- Lincoln	RA	- Rambouillet	SU	- Suffolk
CO	- Columbia	DP	- Dorper	ME	- Merino	RM	- Romnelet	VR	- Van Rooy
CR	- Corriedale	FN	- Finnish Landrace	MT	- Montadale	SB	- Scottish Blackface	WD	- White Dorper
DA	- Damara	HA	- Hampshire	NC	- N. Country Cheviot	SR	- Shropshire		

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D. APPLICATION FOR EMBRYO EXPORT BREED _____

Page ___ of ___

Application for International Movement. To be submitted to breed organization in exporting country.

Exporters I.D. _____

Name of Buyer _____

Address _____

Country _____

Sale Date _____ Export Date _____ Certification of DNA Genotyping/Blood Typing needed Yes _____ No _____

Donor Name _____ No. _____ _____ Ear Tag or Tattoo
CC

Owner _____ Address _____

_____ Onset ☐AM ☐PM
Estrus Date_____
Yr. Mo. Day

Service Sire _____ No. _____ _____ Breeding Date_____
CC Yr. Mo. Day
Sexed Semen X or Y

Service Sire _____ No. _____ _____ Recovery Date _____
CC Yr. Mo. Day
Sexed Semen X or Y ____

Signature of Seller _____ Total Recovered _____
Owner of Donor Dam on Date of Recovery

Address _____ No. Cleaved/Degen. _____
No. Unfertilized _____
No. Transferred _____

Signature _____ Firm _____ No. Frozen _____
Practitioner or Leader of Embryo Production Team Recovering Embryos

ET Code _____

CERTIFICATE OF FREEZING AND/OR IDENTIFICATION OF EMBRYOS (see reverse side for coding instructions)

Type of Container: **In lieu of completing this portion of this form, certificate A-C may be attached**

Straw _ _ Other _ _ Each container labeled to show firm code, breed, reg. no. of donor, 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 Embryo Production Team Freezing the Embryos

ET Code _____ Phone ( ) _____

Signature _____ Firm _____
Exporter

CONDITIONS FOR COMPLETING APPLICATION FOR EMBRYO EXPORT

Application for embryo export will accommodate all embryos from one recovery. Precede registration numbers of donor dam and donor sire(s) with the International Standards Organization (ISO) three letter country codes (cc).

Application must be signed by (1) the seller who is the owner of the donor dam on date of recovery, (2) the practitioner responsible for the recovery, (3) the practitioner responsible for freezing the embryos and (4) the person responsible for exporting the embryos.

The signature of the seller is certifying to the sales of the embryos.

The signature of the person responsible for the embryo recovery is certifying to the accuracy of the information relating to the donor dam, the sire, breeding date, date of recovery and other technical aspects.

The practitioner responsible for packaging and/or freezing the embryos is attesting to the identification of each embryo, the container labeling, as set forth on the certificate, along with the accuracy of all other information called for in this portion of the application for export.

Upon receipt of application for embryo export by the breed office, a certificate will be issued that will be forwarded to the Herd Book in the receiving country. This certificate will identify the embryos and will justify providing the DNA genotyping and/or the blood type of the donor dam and donor sire.

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

STAGE OF DEVELOPMENT

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

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D - Divided
F - Female by Biopsy
M - Male by Biopsy
U - Sex Undetermined by Biopsy

Use comment column to describe other biopsies.

SEXED SEMEN

X - Female Y - Male

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February	FE
March	MR
April	AP
May	MY
June	JN
July	JY
August	AU
September	SE
October	OC
November	NO
December	DE

BOVINE

AN	Aberdeen Angus	BZ	Bonsmara	GS	Gascone	MR	Marchigiana	RO	Rotbunte
AB	Abondance	CP	Campine Red Pied	GV	Gelbvieh	ME	Maremmana	SA	Salers
AF	Afrikaner	CN	Canadienne	GI	Gir	MI	Meuse Rhine Issel	SG	Santa Gertudis
AK	Ankole	CB	Charbray	GR	Groninger	MO	Montbeliard	SE	Senapol
AY	Ayrshire	CH	Charolais	GU	Guernsey	MG	Murray Grey	MS	Shorthorn (milking)
BA	Barzona	CA	Chianina	GZ	Guzera	NE	Nelore	SS	Shorthorn (beef - Scotch)
BE	Beefalo	DB	Danish Black & White	HC	Hays Converter	NG	Nguni	SP	Shorthorn (polled)
BF	Beef Friesian	DJ	Danish Jersey	HH	Hereford (horned)	NM	Normande	IS	Shorthorn (Illawarra)
BM	Beefmaster	RW	Danish Red & White	HP	Hereford (polled)	NR	Norwegian Red	SI	Simbrah
BB	Belgian Blue	DE	Devon	SH	Highland (Scotch Highland)	PA	Parthenais	SM	Simmental
BG	Belted Galloway	DR	Dexter			PI	Piedmont	DS	South Devon
BD	Blonde D'Aquitaine	DZ	Drakensberger	HO	Holstein	PR	Pie Rouge	SX	Sussex
BX	Boran	FP	East Flemish Red Pied	HY	Hybrid (Alberta Hybrid)	PZ	Pinzgauer	TA	Tarentaise
BO	Braford	ER	Eringer	IB	Ibage	RA	Ranger	TG	Tasmanian Grey
BR	Brahman	FA	Flamand	IN	Indu Brasil	AR	Red Angus	TL	Texas Longhorn
BH	Brahmental	FL	Fleckvieh	JE	Jersey	RB	Red Brangus	TU	Tuli
BL	Braler	FR	Fribourg	KB	Kobe (Wagyu)	RD	Red Dane (Red Danish, Danish Red)	WB	Welsh Black
BN	Brangus	FB	Friesian (Belgian)	LU	Luing			WF	West Flemish Red
BU	Braunvieh	DF	Friesian (Dutch)	LM	Limousin	WW	Red Holstein	XX	Crossbreds
SB	Brown Swiss (beef)	GA	Galloway (beef)	LR	Lincoln Red	RP	Red Poll		
BS	Brown Swiss (dairy)	GD	Galloway (dairy)	MA	Maine-Anjou	RN	Romagnola		

CAPRINE

AL	Alpine	AG	Angora	CH	Cashmere	KR	Kalahari Reds	NU	Nubian
TO	Toggenburg	SA	Saanen	BG	Boer	LN	La Mancha	SG	Savanna Goat

EQUINE

AS	American Saddlebred	CL	Clydesdale	MN	Morgan	QH	Quarter Horse	SF	Suffolk Punch
AP	Appaloosa	HA	Hackney (Horse)	PL	Palomino	SE	Shetland	TW	Tennessee Walking
AB	Arabian	HK	Hackney (Pony)	PE	Percheron	SI	Shire	TH	Thoroughbred
BL	Belgian	HU	Hunter	PN	Pinto	SN	Standardbred	WE	Welsh

PORCINE

YO	Yorkshire	DU	Duroc	BK	Berkshire	PE	Pietrain	LW	Large White (British)
LA	Landrace	LC	Lacombe	SO	Spotted	TM	Tamworth	LB	Large Black (British)
HA	Hampshire	PC	Poland Chine	CW	Chester White	WS	Wessex Saddleback		

OVINE

AN	Afrino	DM	Dohne Merino	IL	Ile de France	OX	Oxford	MM	S. African Mutton Merino
BP	Blackhead Persian	DO	Dorset	LE	Leicester	SP	Persian (Speckled/Skilder)	ST	Southdown
BC	Border Cheviot	DR	Dormer	LI	Lincoln	RA	Rambouillet	SU	Suffolk
CO	Columbia	DP	Dorper	ME	Merino	RM	Romnelet	VR	Van Rooy
CR	Corriedale	FN	Finnish Landrace	MT	Montadale	SB	Scottish Blackface	WD	White Dorper
DA	Damara	HA	Hampshire	NC	N. Country Cheviot	SR	Shropshire		

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NATIONAL DAIRY RESEARCH & DEVELOPMENT CENTRE
DEPARTMENT OF LIVESTOCK
MINISTRY OF AGRICULTURE AND FORESTS
YUSIPANG, THIMPHU, BHUTAN, POST BOX: 1058
17116985 (Direct), 17116986 (Lab), 17116976 (PABX)
www.ndrc.gov.bt