

# ET/advanced repro technologies and synchronization

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## Abstract

Embryo transfer in cattle can be extremely rewarding for both the cattle producer and veterinarian. A conventional flush can be achieved through a series of shots to a donor cow, which can be stimulated to produce more offspring than she normally would in her lifetime. The process of a conventional flush will be discussed as well as grading and evaluating embryos. In addition to domestic use of embryo transfer, the International Embryo Technology Society (IETS) has a set of guidelines that allows veterinarians to follow to collect and freeze embryos for exportation either through in-vivo or in-vitro production. The process of in-vitro produced (IVP) embryos will be briefly discussed along with various synchronization protocols for both production systems.

**Key words:** embryo transfer (ET), International Embryo Technology Society (IETS), embryo grading, embryo freezing, synchronization protocols, cattle

## Introduction

Bovine reproductive advancements began in Cambridge England in 1896 by Walter Heap with his publishing of methods to recover and transfer embryos. It would not be until 1951 when Willet in the United States of America would publish a report on the first calf born from embryo transfer (ET) techniques.<sup>4</sup> On May 26, 1974, the International Embryo Technology Society was formed and developed a system to standardize record keeping, grading, and labeling of embryos.

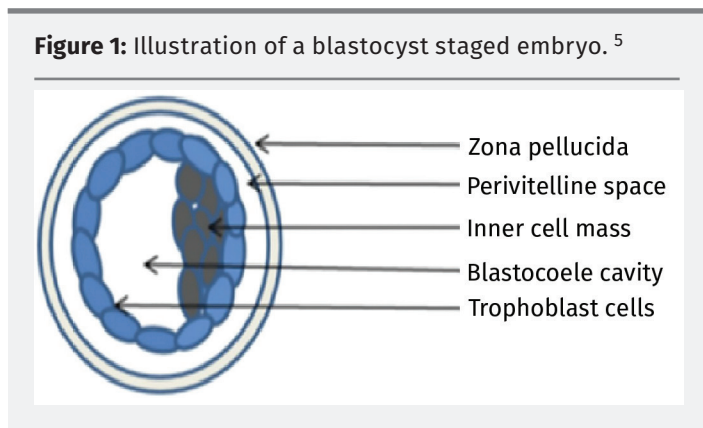
A quick overview of the bovine reproductive tract is essential for understanding the embryo production and transfer process. The bicornuate uterus of cattle has two large horns, a uterine body and a single cervix with 3 to 4 cervical rings. The importance of understanding how to pass a rod through the cervical rings and work your way through the uterine horns is important for artificial insemination, in-vivo embryo collection and embryo transfer purposes. Once the semen is deposited into the uterus, it will work its way up to the ampulla of the fallopian tube where fertilization will occur. After fertilization,

the term embryo can be applied to describes the conceptus from the fertilized 1-cell to blastocyst stages.<sup>7</sup>

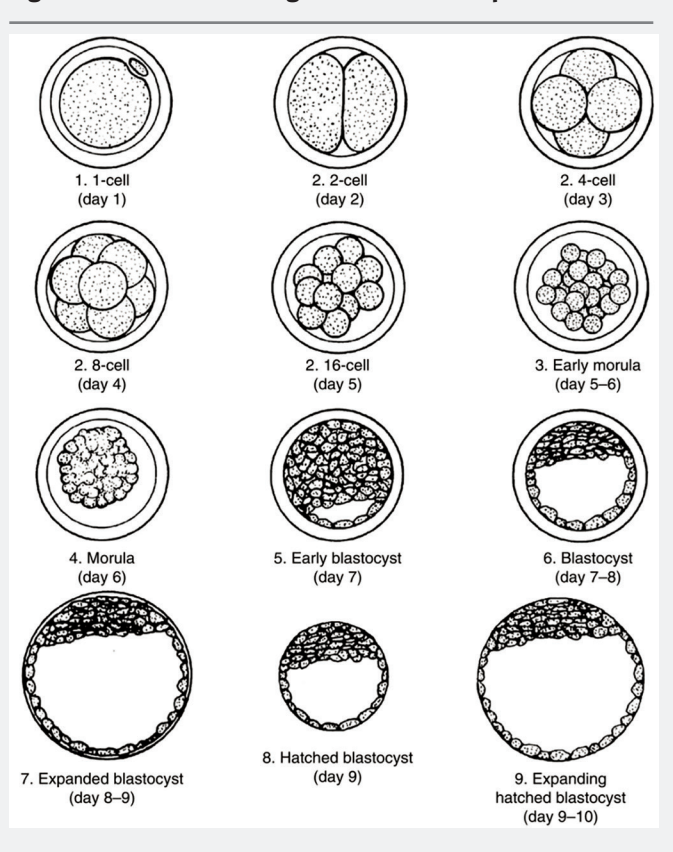
A portion of the embryo that is essential to be intact and free of any deformations is the zona pellucida (ZP) especially when being considered for exportation. It is important to understand the basic structures of an embryo, which is illustrated in Figure 1. The ZP is identified by its translucent shell and functions as a physical barrier against pathogens and contains receptors for sperm. The ZP surrounds an embryo from the 1-cell stage until the expanded blastocyst stage of development.

## Embryo classification and grading

Classification of bovine embryos begins with an evaluation of the embryo with a stereomicroscope with at least 50x zoom. When beginning an embryo evaluation, you will want to use your 10µL micropipette with a clean tip to roll the embryo to evaluate it properly. You will want to evaluate the ZP for any deformations or cracks, which will prevent the embryo from being exportable. After proper evaluation of the ZP's you will refer to Figure 2 to determine which classification code you will use for bovine embryos collected 7 days after estrus. In-vitro-produced embryos 7 days after estrus should normally be a



**Figure 2:** The numeric stage code for development.<sup>7</sup>



**Table 1:** Embryo quality code grading.<sup>7</sup>

Code 1	Excellent or good	Symmetrical and spherical embryonic mass with individual blastomere cells that are uniform in size, color and density. The embryonic stage of development is consistent with the donor's estrous cycle and day of recovery. Irregularities should be relatively minor and at least 85% of the cellular material should be intact and viable embryonic mass. That percentage should be based on the embryonic cells represented by the extruded material in the perivitelline space. The zona pellucida should be smooth and have no concave or flat surfaces that may cause it to adhere to the petri dish or straw
Code 2	Fair	Moderate irregularities in overall shape of the embryonic mass or in size, color, and density of individual cells. At least 50% of the cellular material should be intact and viable
Code 3	Poor	Major irregularities in shape of the embryonic mass or in size, color, and density of individual cells. At least 25% of the cellular material should be intact and viable
Code 4	Dead or degenerating	Degenerating embryos, oocytes, or 1-cell embryos; nonviable

stage 4 to stage 6. In-vitro-produced embryos will usually have an advanced blastocyst and range from stage 6 to stage 8.

Quality grading of bovine embryos falls into category codes ranging from 1 to 4 based on the morphological appearance of the embryo. Table 1 will describe the grading criteria for quality grading.

- **Code 1:** Excellent or Good. Symmetrical and spherical embryonic mass with individual blastomere cells that are uniform in size, color and density. The embryonic stage of development is consistent with the donor's estrous cycle and day of recovery. Irregularities should be relatively minor and at least 85% of the cellular material should be intact and viable embryonic mass. That percentage should be based on the embryonic cells represented by the extruded material in the perivitelline space. The zona pellucida should be smooth and have no concave or flat surfaces that may cause it to adhere to the petri dish or straw
- **Code 2:** Fair. Moderate irregularities in overall shape of the embryonic mass or in size, color, and density of individual cells. At least 50% of the cellular material should be intact and viable
- **Code 3:** Poor. Major irregularities in shape of the embryonic mass or in size, color, and density of individual cells. At least 25% of the cellular material should be intact and viable
- **Code 4:** Dead or Degenerating. Degenerating embryos, oocytes, or 1-cell embryos; non-viable

## Producing embryos

To produce embryos your client can either choose to do in-vivo or in-vitro derived embryos. Whichever method is chosen, it is important to educate your client to select a donor who's offspring will be able to be marketable to justify the cost of embryo production and transfer. The ideal donor is 3-10 years old with a 4 to 6 body condition score and preferable reproductively sound if going the in-vivo route. Donors used for in-vitro production can be as young as 6 to 8 months old and as far along as 120 days bred and can still be aspirated.

In-vivo embryo production is also known as a conventional flush, in which a donor is super ovulated to release as many oocytes as possible when she comes into estrus. It is best to start a donor on a reference heat even though it is possible to stimulate a donor while using controlled internal drug release (CIDR) progesterone that goes intravaginally. Synchronization drugs

used in cattle include gonadotropin-releasing hormone (GnRH), which is responsible for releasing follicle stimulating hormone (FSH) and luteinizing hormone (LH). Another synchronization drug is prosta-glandin (PG), which causes luteolysis of the corpus luteum (CL).<sup>6</sup> With the use of these synchronization drugs, you can synchronize a group of donors or recipients heat dates.

Start with a 7-day CIDR protocol to obtain reference heats on your donors. On day 0 you will insert a CIDR vaginally and administer GnRH followed by the removal of the CIDR and PG on day 7. Over the next 7 days watch for signs of standing heat on donor and record date. Once donor estrus dates are recorded, you have a 7 to 13-day window to start donors on shots. FSH will be administered in the morning and evening 12 hours apart with a decreasing dosage over 4 days. In addition, to the morning and evening shots of FSH, on the third day you will administer PG twice. Following the last shot of FSH your donor should start to show signs of heat that evening into the next morning. She will be bred 2 to 3 times over the next 24 to 30 hours from the time of the first mount depending on the semen and time she came into heat. A quick evaluation of the semen should be performed to assess the viability of the sperm being used. A shot of GnRH should be given after the first breeding. After last breeding the donor will be flushed 7 days from her breeding date.

## Recovery of embryos

A week after breeding the donor, she should be brought into a confined chute that is preferably elevated to help the reproductive tract slide back. She will need an epidural placed and will need to be palpated or ultrasounded to determine the number of corpus luteums (CL) she has present on each ovary. You will also want to evaluate the size of her cervix to determine which gauge foley catheter you will need to place. The Foley catheter should be placed in the left or right uterine horn depending which ovary contained the most CLs. After Foley placement, hook your tubing and filter up to the complete flush media that contains factory-added antibiotics. Proceed to working the horn by adding fluid into the horn and aiming to flush out all the fluid that was put in. After 3 flushes, typically on the first horn, you will pull your Foley catheter back to the cervical uterine body junction and proceed to flush 3 to 4 more times, while focusing on the opposite horn.

Once finished flushing, bring in the filter and rinse it out with rinse media and search the contents with a stereomicroscope trying to identify embryos by their translucent ZP. Recovered

em-bryos will be placed into holding media at room temperature until the flush process is completed. If the embryo count you predicted was close to the number of embryos recovered, you can pull the Foley and search the contents and then continue. Embryos will be classified based on stage and quality grade as discussed earlier.

## Cryopreservation or fresh transfer

The decision to transfer fresh or cryopreserve the embryos all depends on whether recipient cows' heat dates are available to transfer into or not. For fresh in-vivo or in-vitro embryo transfers to work, the recipients must have a heat 36 plus or minus hours of the donor heat (in-vivo) or time of fertilization (in-vitro). In-vitro embryos typically have higher conception rates when transferred on 7 to 8 day heats on fresh or frozen embryos. Embryos can be held at room temperature for 4 to 6 hours before freezing, and can still expect high survival rates after thawing and transferring, while embryos being transferred fresh can be stored at room temperature for 12 to 18 hours and have high pregnancy rates.<sup>7</sup> Whether embryos are frozen or transferred fresh, they will be washed 10 times in a hold media. If they are being frozen and are for export, they will be washed 5 times and then placed into 2 drops of trypsin for a total time of 60 to 90 seconds. Following the trypsin, they will be washed 5 more times in holding media. The essential requirements for proper washing of embryos will be showing in Table 2.

- Only embryos from a single donor washed together
- Ten or fewer embryos washed at one-time
- Only zona pellucida-intact embryos washed
- Only embryos free of adherent material washed
- Minimum of 10 washes
- Use a new sterile micropipette each time embryos are moved from one wash to the next
- Regulate volumes so that each wash is at least a 100-fold dilution of previous wash

Embryos that have a cracked ZPs, deformed ZPs or no ZPs, can still be frozen or transferred fresh into a recipient with decent success rates. After embryos have been washed, they can be loaded into 1/4cc straws. If you plan on freezing, the color of the straw you place the embryo in should determine the method used to freeze. Yellow 1/4cc straws indicate that the embryos are for direct transfer (DT), while blue 1/4cc straws indicate that the embryos are vitrified (VIT). VIT embryos require additional steps after thawing to prepare the embryo for transfer compared to the DT embryos that can be thawed and transferred immediately.

The most common method of freezing involves the use of 1.5M ethylene glycol (EG) cryoprotectant agent (CPA). There are two types of CPAs: permeating CPAs and non-permeating CPAs. Permeating CPAs involve a hyperosmotic solution where the CPA penetrates the cell and water moves out and the CPA moves into the cell (e.g. ethylene glycol or glycerol). Nonpermeating CPAs cannot move across the cell membrane, but the solution is still hyperosmotic resulting in water moving out of the cell and becoming dehydrated. Non-permeating CPAs are used in cryopreservation of advanced blastocyst embryos, such as in-vitro produced embryos.<sup>3</sup> Non-permeating CPAs still require the use of a permeating CPA after being exposed to the solution for 20 to 40 seconds. Embryos will remain in the permeating CPA solution for 10 minutes during which time they will be loaded into the appropriate straws.

Straw and cane labeling are just as important as any portion of the freezing process and Figure 3 and Figure 4 illustrate the proper labeling techniques.

Cryopreservation of the embryos will involve the use of an embryo freezer that will hold between -5°C to -7°C, while the embryos are being loaded into straws. Once embryos have equilibrated in the CPA for 10 minutes, they can be placed into the freezer that contains methanol in the embryo straw chamber. Embryo straws can be "seeded" with a metal rod to initiate crystallization. When the last embryo has been "seeded" and sat for 10 minutes in the freezer chamber, the embryo freezer can be started at that point. The freezer will drop 0.5°C per minute until it reaches between -32°C to -36°C. The last step of the freeze process will involve the "plunging" of the embryos into a liquid nitrogen bath and loaded into the appropriate canes and goblets.

## Basics to in-vitro produced embryos

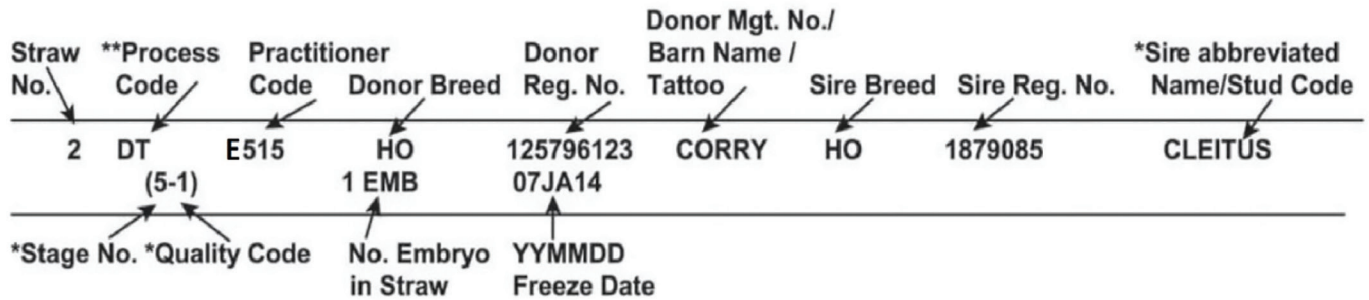
In-vitro produced (IVP) embryos can be produced as often as every 2 weeks and can be done while a donor is carrying a first trimester pregnancy. Donors do not typically need to be given FSH to stimulate them, even though it can be done. One protocol of giving shots is the one-shot FSH protocol where a CIDR is placed vaginally, and a shot of GnRH is given. Forty-eight hours later a single dose of FSH can be given, and then 48 hours later she can be aspirated. This protocol allows for a more mature oocyte and may slightly increase the overall oocyte yield.

Day 0 of the in-vitro produced embryos begins with ovum-pick up (OPU) or aspiration day. Oocytes are aspirated using an ultrasound-guided needle and sucked up into a tube using a vacuum pump. The aspirated material will then be rinsed, filtered, searched and graded with the use of a stereomicroscope. The oocytes that are healthy enough will be placed into

**Table 2:** Essential requirements for proper washing of embryos.<sup>7</sup>

Only embryos from a single donor washed together
Ten or fewer embryos washed at one-time
Only zona pellucida-intact embryos washed
Only embryos free of adherent material washed
Minimum of 10 washes
Use a new sterile micropipet each time embryos are moved from one wash to the next
Regulate volumes so that each wash is at least a 100-fold dilution of previous wash

**Figure 3.** IETS straw labeling.<sup>1</sup>



In practice, 515 HO 125796123 is one unit with only one space between segments as shown in an illustration below.

Identification information can be handwritten or printed directly on the straw or typed on slip of paper rolled so identification shows and inserted into extension of straw, or printed on a 1/4" X 3" gummed label attached to a 0.25 ml straw inserted into a 0.5 ml extension of straw.



Showing one embryo on a straw in line 2 is not necessary but when not frozen for direct transfer and the straw contains more than one embryo, the number of embryos in the straw should show on the second line at the location indicated.

**\* Optional**

**\*\*Use Appropriate Processing Code**

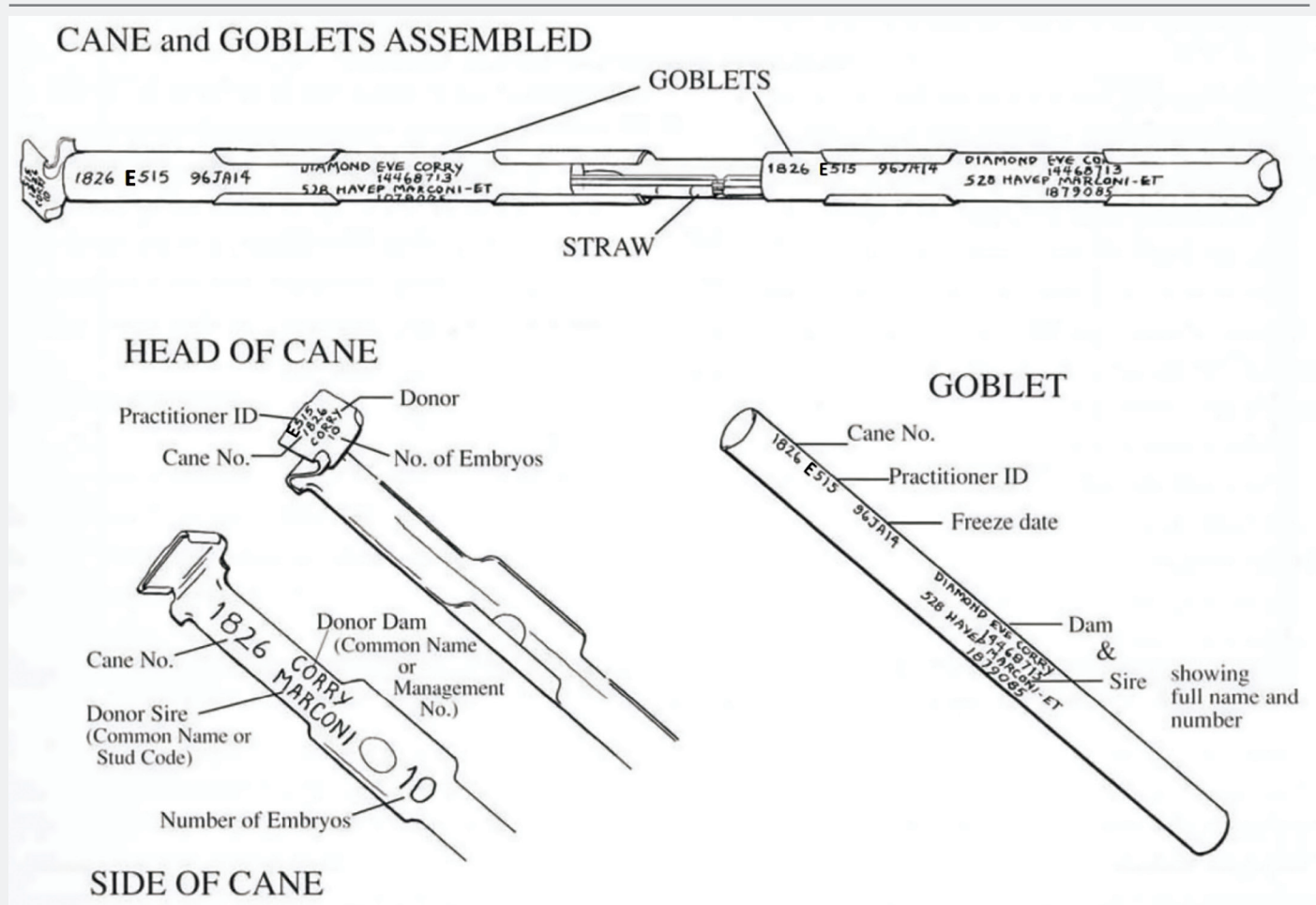
- DT Direct Transfer in vivo
- DT-IVF Direct Transfer IVF
- DTX Direct Transfer Sexed Semen - Female
- DTY Direct Transfer Sexed Semen - Male
- DTX-IVF Direct Transfer Sexed Semen - Female-IVF
- DTY-IVF Direct Transfer Sexed Semen - Male-IVF
- DTF-IVF Direct Transfer Embryo Biopsied - Female-IVF
- DTM-IVF Direct Transfer Embryo Biopsied - Male-IVF
- DTB Direct Transfer Biopsied

a maturation media in a tube and will undergo in-vitro maturation (IVM) process over the next 18 to 24 hours in an in-cubator at 38.5°C. The in-vitro fertilization (IVF) process occurs day 1 post collection (PC) and fertilization can take 8 to 24 hours. During the in-vitro culture (IVC) phase, which lasts from day 2 PC to day 8 PC, the oocytes that became fertilized will continue to mature and be monitored until day 7 PC where embryos in the blastocyst stage can be frozen, as is the case for export embryos. Export IVF embryos will be frozen from day 7 PC to day 9 PC to prevent cracked ZPs or to allow the later-maturing embryos to mature to the blastocyst stage for freezing. If the embryos are used for domestic use, day 8 PC all early blastocysts through the hatched blastocyst staged embryos can be transferred fresh or frozen.<sup>3</sup>

## Embryo transfer

The process of embryo transfer can be done with either fresh or frozen embryos. When considering the timing of transferring as stated before frozen IVP embryos should be transferred on day 7 and 8 heats for better conception rates, while in-vivo produced embryos can be transferred on day 6 to 8 heats with high conception rates. As important as it is to verify the heat dates, the size of the CL is just as important. In general, the smaller the CL the less likely a pregnancy will be maintained, while a CL around 24mm will have a higher chance in resulting in a pregnancy. When transferring embryos, especially frozen embryos, you must palpate or ultrasound the cow's ovaries to check for a viable CL. Once the CL is determined suitable, you

**Figure 4:** IETS cane and goblet labeling.<sup>1</sup>



can thaw or transfer a fresh embryo into the ipsilateral side of the horn that the CL was on. For best results, try to minimize the amount of trauma to the uterine tissue while the transfer gun is being positioned into the uterine horn.

## Useful synchronization protocols

Working in bovine reproduction, any *bos indicus* influenced breed seems to cause the most frustration when trying to obtain a standing heat, whether that be to get a reference heat to start a donor on shots, or whether it is just to artificially inseminate (AI) that female. One synchronization protocol that has worked well is the *Bos indicus* PG 5-day CIDR protocol. On day 0, insert a CIDR vaginally and give a shot of PG following a 5-day window, pull the CIDR and give another shot of PG. Timed AI can occur roughly 66 hours after CIDR removal followed by a shot of GnRH after insemination.<sup>8</sup> If using the protocol for a reference heat, the donor still will have a better chance of coming into heat over the next few days, so that you will be able to start her on shots to superovulate her.

In addition to the 7-day CIDR protocol, which was previously discussed, the 7 and 7 Synchronization protocol has shown from personal experience to increase grouping of recipient heats and has so far shown favorable embryo transfer results. On day 0 a CIDR is inserted vaginally, and a shot of PG is administered. Day 7 a shot of GnRH is given, and then on day 14 the CIDR is pulled, and another shot of PG is administered. Heats

are then observed 48 to 96 hours after CIDR pull and will be transferred roughly 7 days after the 72-hour mark post-CIDR pull.

## Conclusion

Whether embryos are being produced through in-vivo or in-vitro methods, knowing and understanding how to grade, transfer and freeze those embryos is important to your client and to your reputation as an embryologist. For those who are interested in obtaining additional training, there are various classes that are offered, but the most important thing is that you take that training and apply it right away. More times than not, you will start with your own cattle to gain confidence and then build from that point on.

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