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# Sperm Notes

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# Ovum Pick-Up and In Vitro Production of bovine embryos

Dr. Eva Held, Minitüb GmbH

## Increasing necessity for Ovum Pick-Up

Ovum pick-up followed by in vitro embryo production procedure (OPU-IVP) is strongly driven by the need of the breeding industry to continually promote genetic improvement in dairy or beef cattle especially since the advent of sexed semen. This has now become even more important with the introduction of genomic selection. Ova can be aspirated from very young heifers and biopsies are collected from the in-vitro produced embryos. Successful OPU-IVP programmes are desired in order to increase the number of embryos and offspring per donor which subsequently allows enhanced selection intensity for the next generation.

Combining genome testing and sexed semen in young heifers may also be a strategy to minimise the time required to generate replacements for elite females. The number of elite candidates is increased by using repeated flushes during the multiple ovulation embryo transfer and ovum pick-up programmes. The main advantage of using genomic selection in combination with assisted reproductive technologies is the reduction in the generation interval, which can lead to a doubling in the rate of genetic gain over conventional progeny testing systems (reviewed by Ponsart C. 2013).

Several advantages of OPU-IVF programs over conventional embryo transfer have been recently described:

- Oocytes can be collected twice a week for several weeks
- Oocyte collection from pregnant donors through the first trimester of pregnancy
- Up to 50 live calves being born per donor per year
- Division of oocytes from one donor into several groups which can be fertilised with different bulls
- More efficient use of rare, expensive or sexed semen
- Decrease of generation interval by collection of oocytes from pre-pubertal heifers

(reviewed by A.M. van Wagtenonk-de Leeuw 2006)

## Development and history of Ovum Pick-Up

In 1986, an ultrasonic-guided aspiration of bovine follicular oocytes was first proposed in Denmark by Callesen H. et al. The first successful OPU was established by the Dutch research team led by Pieterse MC. et al. by adopting human trans-vaginal ultrasound scanning procedures for bovine in 1992. The original OPU procedure includes no hormonal stimulation and is routinely performed twice a week, which allows the maximum recovery of oocytes because no dominant follicle develops when all visible follicles are aspirated during the OPU process.

In most once-a-week collection procedures, a dominant follicle will develop during successive collection. This leads to the regression and degeneration of the subordinate follicles (reviewed by Qi M. 2013).

During the last two decades, OPU research has focused on the application of hormones. The advantages of super-stimulation prior to OPU seem obvious: More follicles and more oocytes can be retrieved, resulting in more embryos produced. It was shown that the average number of follicles aspirated, oocytes collected, and embryos developed in a FSH-treated OPU procedure were all significantly higher than those produced in once-a-week non-stimulated OPU procedures on a per donor per session basis (Chaubal SA. et al. 2009).

## Equipment and procedure

To summarise the procedure, OPU is conducted using an ultrasound probe with an attached needle to penetrate the vaginal wall and aspirate the contents of each follicle on the animal's ovary. The follicular fluid containing oocytes is aspirated through a pre-warmed vacuum system. The probe holder, containing the ultrasound probe, is inserted into the vagina and placed in front of the cervix (figure 1). Using one hand in the rectum, the ovary is manipulated against the anterior section of the vagina immediately adjacent to the cervix. The follicles now become visible on the ultrasound screen. With the other hand, the probe holder is advanced into the vagina and as the needle is pushed forward it penetrates the vaginal wall and is guided into fluid filled follicles (figure 2). The oocytes are aspirated using a vacuum pump (figure 3).

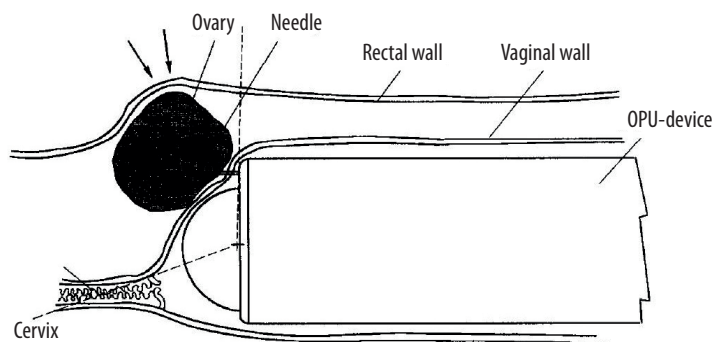


Fig. 1: Pieterse MC. 1992

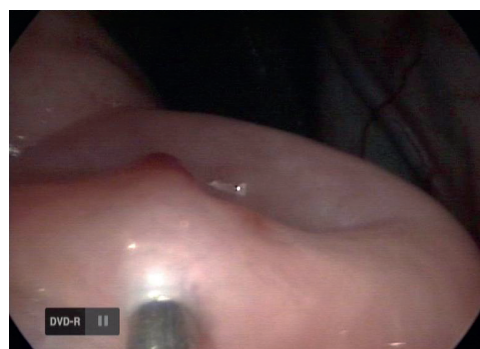


Fig. 2: Besenfelder U. and Hoelker M. not published

Since the age of the heifers selected for OPU is steadily decreasing, Minitube has developed a new probe holder with a slim and light design in order to meet the requirements of smaller animals. Furthermore, Minitube's probe holder and needle unit are especially designed to work with short needles, which impart several advantages such as inexpensive and easy replacement, easy manipulation and small dead volume. These advantages render the re-use of needles unnecessary and thus help to minimise the risk of cross contamination between treated animals (figure 4).



Fig. 3: Minitube

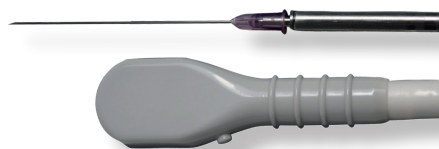


Fig. 4: Minitube

## In Vitro procedures following Ovum Pick-Up

The main steps of in vitro production of embryos are:

- Selection of oocytes
- In vitro maturation of oocytes
- In vitro capacitation of sperm cells
- In vitro fertilization
- In vitro culture of embryos

Selection of oocytes:

Oocytes used for in vitro production should have a homogenous cytoplasm and at least three layers of cumulus cells attached to the zona pellucida. Figure 5A shows an oocyte with a dense layer of cumulus cells which corresponds to a quality rating „1“. An oocyte of quality rating „2“ is shown in figure 5B. It has a nearly homogenous cytoplasm and about three layers of cumulus cells. In figure 5C, a completely denuded oocyte is shown, which typifies a quality rating „3“.

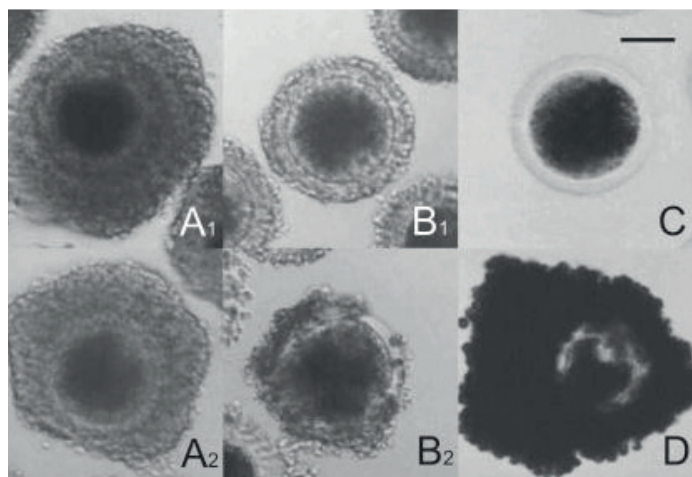


Fig. 5: Alvarez GM. 2009

Before fertilization can occur, the oocyte must reach its nuclear and cytoplasmic maturation. Nuclear maturation is the process that takes place during the resumption of meiosis. It initiates germinal vesicle breakdown (GVBD), the separation of homologue chromosomes and the extrusion of the first polar body (Grondahl C. 2008) (figure 6). Furthermore, the cumulus cells expand in response to a changing environment of gonadotrophins, growth factors, steroids, and certain other factors that become secreted by the oocyte during maturation (Buccione et al, 1990) (figure 7).

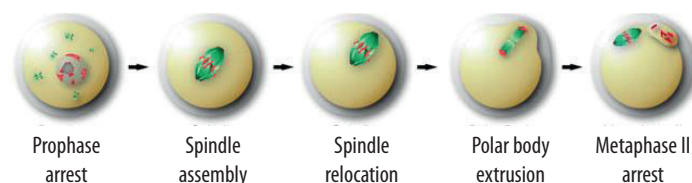


Fig. 6: M. Schuh, Nature: Meiosis in mammalian oocytes

An optimal environment is essential to maximise the number of mature oocytes after a period of 22 hours and can be defined as follows: 38.5°C, 5 % CO<sub>2</sub>, highest possible humidity, and a suitable culture media that meets the optimal nutritional and hormonal requirements.

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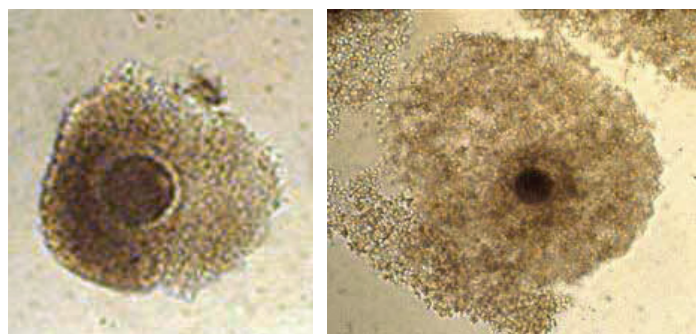


Fig. 7: University Wisconsin



After maturation, the oocytes are ready for fertilization. In order to prepare the semen for IVF, the so called swim-up procedure must be conducted. In nature, capacitation takes place during the transport of sperm cells through the oviduct and this has to be mimicked in vitro to prepare the sperm cell for the acrosome reaction occurring during fertilization. Furthermore, during swim up, vital sperm cells are separated from dead ones and unwanted residues from semen extender.

The fusion of oocyte and sperm cell is a very complex process, requiring competent oocytes and sperm cells as well as optimal culture conditions. Usually 5 to 50 oocytes are co-cultured with  $10^5$  sperm cells in droplets of 50 – 400  $\mu$ l of fertilization medium. This medium contains in addition to nutritive substances, also activators for sperm cells like epinephrine or caffeine. The actual fertilization takes between 6 to 18 hours and requires a constant temperature of 38.5°C and 5 % atmospheric CO<sub>2</sub>.

Finally, presumptive zygotes are cultured in a defined culture medium for seven days. In 1994, Gardner et al. developed a synthetic oviduct fluid (SOF) supplemented with bovine serum albumin (BSA) and essential and non-essential amino acids. This medium yielded relatively constant developmental rates of embryos. However, this medium is imprecise due to the undefined nature and variability of BSA. Therefore, one of the main challenges of today's research is to develop culture systems without animal proteins (reviewed by Thompson and Peterson, 2000). During the culture period, the embryos undergo several cleavage divisions (figure 9) until they reach the blastocyst stage on day 7 (figure 10).



Fig. 9: Rizos D. unpublished

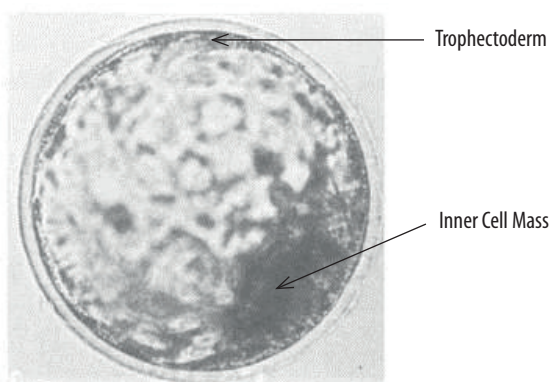


Fig. 10: George E. Seidel, Jr, FAO 1991

Since not all aspirated oocytes are developmentally competent, the blastocyst rates in the in vitro production are limited to 25 to 35 %.

## Grading of transferable embryos

Prior to embryo transfer (ET), transferable embryos have to be selected on the basis of their morphological appearance. According to the IETS Manual, a strict classification of blastocysts should be followed as described:

| Classification | Nomenclature         | Definition  |
|----------------|----------------------|---|
| I              | Excellent or good    | Symmetrical and spherical embryo mass with individual blastomeres (cells) that are uniform in size, color and density. This embryo is consistent with its expected stage of development. Irregularities should be relatively minor, and at least 85 % of the cellular material should be an intact, viable embryonic mass. This judgment should be based on the percentage of 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 might cause the embryo to adhere to a petri dish or a straw. |
| II             | 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 an intact, viable embryonic mass.  |
| III            | 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 an intact, viable embryonic mass.   |
| IV             | Dead or degenerating | Degenerating embryos, oocytes or 1-cell embryos: non-viable   |

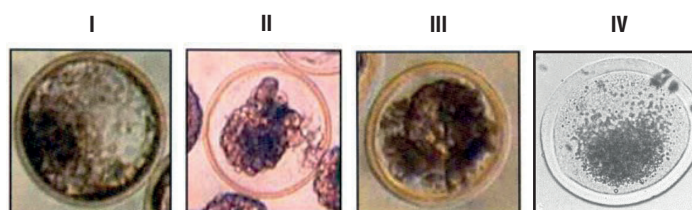


Fig. 11: IETS

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# AndroVision® - The optimal system for quantitative and qualitative analyses of bovine semen

Petra Eisenschink, Minitüb GmbH

Once semen is collected, the irreversible aging process of the sperm begins. That is why it is important to work as rapidly and effectively as possible. AndroVision® is the ideal system for quick, precise and objective determination of sperm concentration and initial motility.



Fig. 1: AndroVision®, the modular system for bovine semen analysis.

Designed as an all-encompassing semen analysis system, AndroVision® is also flexible in operation and can be easily adapted. In addition to the classic CASA, a comprehensive line of more advanced assays for sperm functionality is available, including sperm viability and acrosome integrity. Assays for mitochondria potential and DNA integrity will also be available soon.

With the unique ability to evaluate 1000 and more cells per field, AndroVision® allows for unparalleled quick and accurate semen assessments. Every single sperm cell is identified and detailed information can be queried right after analysis. The user interface, with its logical design is very easy to learn and use. The evaluation can be performed with just a few clicks which enables an uninterrupted workflow.

Furthermore, AndroVision® can identify sperm heads precisely and differentiate sperm cells effectively from other small particles in the ejaculate. This represents a major advantage when measuring diluted semen in which egg yolk or milk containing extenders have been used (figure 2).

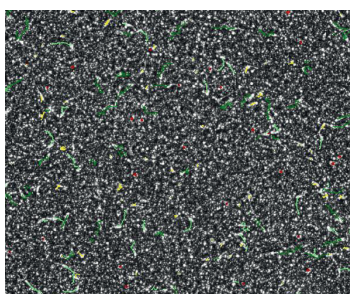


Fig. 2: Bull sperm in milk extender

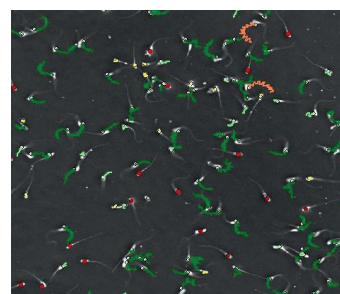


Fig. 3: Bull sperm in AndroMed® extender

The motility of a semen sample comprises three dimensions:

- Percentage of sperm showing movement
- Characteristics of movement and
- Speed of movement

Viability assessment is available as part of the Motility and Concentration Software Module. This means that after preparing two samples - and applying a stain, e.g. Hoechst 33342/PI, to one of them - it is possible to switch between the two modules during the measurement. The viability assessment result is then shown together with the relevant analysis data of the ejaculate. The results are stored in one record (figures 4, 5).

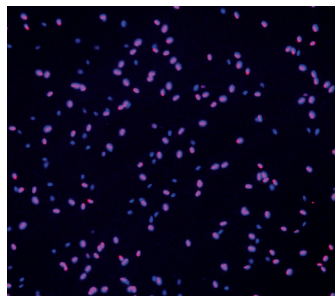


Fig. 4: Viability stain: Hoechst 33342/PI

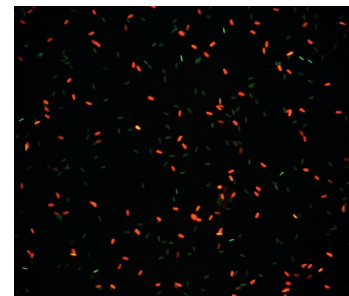


Fig. 5: Viability stain with SYBR/PI

The interactive Morphology and Morphometry Software Module assists the user with a thorough and complete analysis of morphological abnormalities of sperm. It identifies the sperm of stained and unstained samples and takes measurements of each individual cell: The Morphometry (acc. to Krüger) determines length and width of sperm head, head shape and midpiece asymmetry of each single sperm cell analysed. Sperm cells are classified into a large range of morphologic abnormalities. The classification is a self learning process or can be set to an automatic differentiation between intact and abnormal heads. Standard abnormality subclasses for manual selection are:

- Acrosome defect
- Detached head
- Abnormal midpiece
- Bent tail
- Coiled tail
- Proximal droplet
- Distal droplet
- Waste

Successfully addressing the challenges of computer assisted bull semen analysis in the lab or on the production line, AndroVision® proves to be the perfect tool for quantitative and qualitative analyses of bovine semen.



# Cryopreservation of bovine semen; optimized freezing processes to minimize cryoinjury

Dr. Eva Held, Minitüb GmbH

## Effects of cryopreservation on spermatozoa

### Physical and physiological processes

Cryopreservation of spermatozoa inevitably decreases the viability and fertility of semen due to damages occurring at the cellular level. The freezing process must therefore be understood and optimized in order to minimize these damages. Although several hypotheses exist, the intrinsic molecular mechanisms responsible for decreased fertility during in vitro storage remain unclear. Cooling and freezing procedures lead to a number of physical changes in the external environment of a cell suspension such as water and solute movement (Vishwanath V. 2000).

The three primary factors inducing cryoinjury to any cell are: osmotic stress/dehydration, oxidative stress, and intracellular ice formation. To maintain viability during freezing, the sperm cells must overcome a variety of changing osmotic and oxidative conditions. Besides the detriments of exposure to osmotic changes, the formation of intra- and extracellular ice, and the production of reactive oxygen species, the freezing process has disadvantageous effects on motility, lipid phase change, membrane integrity, mitochondrial function, DNA integrity, cell signaling and metabolism. These conditions can lead to apoptotic and necrotic cell death (reviewed by Meyers S.A. 2012 AAAA Proceedings).

One of the most significant causes of cell damage and death during freezing is the formation of intracellular and extracellular ice. Initially, reduction of temperature causes freezing of the medium between the cells. The extracellular medium freezes between  $-5^{\circ}\text{C}$  and  $-10^{\circ}\text{C}$ , depending on electrolytic concentration. In contrast, the intracellular fluids and components don't freeze but supercool in this temperature range. The extracellular ice formation leads to a higher local electrolytic concentration which affects diffusion of intracellular water. This causes dehydration of the intercellular space and leads to cell shrinkage (Mazur P. 1984). Furthermore, cryopreserved semen display signs of being in an advanced stage of capacitation after thawing, even though these sperm cells were only at the onset of capacitation prior to the freezing process (Medeiros et al. 2002).

## Steps of cooling

### Critical aspects

Generally, the freezing protocol is divided into three phases: cooling from  $+5^{\circ}\text{C}$  to  $-5^{\circ}\text{C}$ , initiation of ice nucleation, also called crystallization, and cooling from  $-5^{\circ}\text{C}$  to  $-130^{\circ}\text{C}$ .

Prior to the freezing process, the ejaculate must be cooled down to  $+5^{\circ}\text{C}$  and equilibrated at that temperature. Several studies in the past

decades have shown that the phase of cooling the extended ejaculate from  $+30^{\circ}\text{C}$  to  $+4^{\circ}\text{C}$  is crucial for post-thaw motility and viability (Robbins et al. 1976, Woelders et al. 1998). Especially between  $+20^{\circ}\text{C}$  and  $+15^{\circ}\text{C}$ , temperature-dependent structural alterations in plasma membranes have been observed (Hammerstedt et al. 1990).

Already in 1957, the highest non-return-rates were observed after an equilibration phase of 12 hours from  $+30^{\circ}\text{C}$  to  $+5^{\circ}\text{C}$  (Graham et al. 1957). In 1976, Ennen et al. compared equilibration periods ranging between 2 and 18 hours. The highest post-thaw motility was observed within the range of 4 to 10 hours.

Further experiments have shown that an equilibration period of 18 hours yields better results than a 4 hour period with respect to post-thaw motility of bovine semen when using milk based extender (Foote R.H. et al. 2002).

Cooling cells to their freezing point and beyond does not necessarily result in freezing the samples at the equilibrium freezing point when they are exposed to supercooling, intracellular ice formation, and cellular dehydration.

At a temperature between  $-5^{\circ}\text{C}$  and  $-10^{\circ}\text{C}$ , ice formation occurs within the extracellular fluid while the intracellular fluid remains supercooled. In order to maintain chemical equilibrium between intra- and extracellular fluids, the cells dehydrate. During this critical period, the cooling rate is of major importance: it must be slow enough to allow sufficient cellular dehydration, but also fast enough to freeze the remaining intracellular fluid before the cells become exposed to hyperosmotic conditions resulting from dehydration (Medeiros et al. 2002).

Consequently, cell-cryoinjury is induced at cooling rates which are either too slow or too fast. When cooling rates are too slow, cryoinjury is based mainly on solution effects such as the accumulation of higher electrolyte concentrations within the cell and severe cell dehydration. At rapid cooling rates, cryoinjury is caused mainly by insufficient dehydration and by intracellular ice formation (Mazur et al. 1972) (Picture 1).

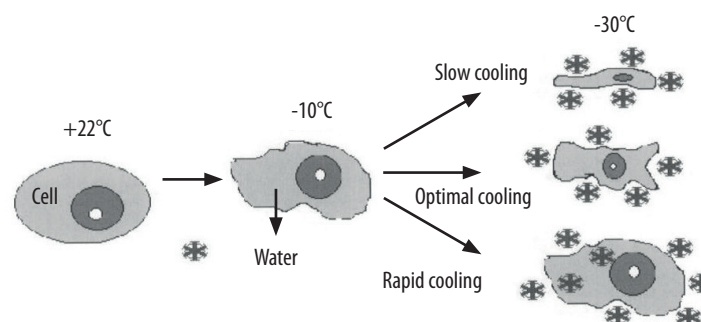


Fig. 1: Gao D. and Crister J.K. 2000

At the onset of freezing, enthalpy changes occur within the sample. As liquids have a higher internal energy than their respective solid phase, rises in temperature occur when the so-called latent heat of crystallization is released. The release of this latent heat of crystallization is observed as a sharp increase in temperature, followed by the formation of the so-called freezing plateau until the latent heat of crystallization has been removed from the sample. Several investigators have shown that a prolonged freezing plateau is detrimental to sperm survival, i.e. the reduction of this plateau improved the fertility of bull spermatozoa (Parkinson and Whitefield 1987).

## Optimisation of cooling rates

Mazur et al. 1972, suggested that the optimal cooling rate is that which is as fast as possible to avoid solution effects, but slow enough so that the cells can dehydrate sufficiently to avoid intracellular ice formation. These competing phenomena have a characteristic inverted “U” shaped cooling rate versus survival curve (Figure 2).

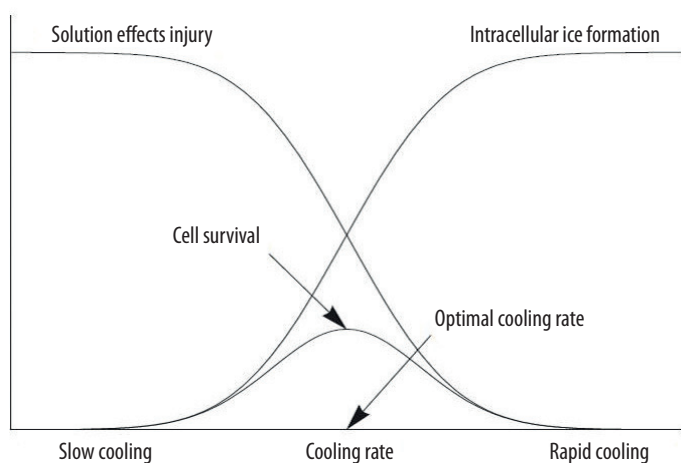


Fig. 2: Muldrew K. et al. 2004

This graph shows that at slow cooling rates, solution effects are predominant factors provoking cryoinjury. At rapid cooling rates, cryoinjury is mainly driven by intracellular ice formation. The combinations of these two effects imply that there will be an inverted “U” shape survival curve and an optimal cooling rate that minimizes both the solution effects and intracellular ice formation.

## Principle of TurboFreezer

From the above discussion, it is evident that controlling nucleation and providing temperature compensation for the release of the latent heat of crystallization results in improved post-thaw cell viability. Therefore, it is advisable to use controlled-rate freezing equipment. Temperature compensation is provided by an automated decrease of the chamber temperature that both initiates nucleation and subsequently compensates for the release of the latent heat of crystallization.

It has also proved beneficial to avoid variable degrees of supercooling in the straws of a freezing batch by deliberately inducing freezing at a

point when the samples have cooled a few degrees below their equilibrium freezing point. Despite this documented knowledge which has been well known for decades, most controlled-rate freezing processes for semen straws used in commercial set-ups today are not able to provide homogeneous cooling rates throughout all straws frozen in one cycle. The reason is mainly due to an undefined and broad temperature range within the freezing chamber and a cooling force that is not powerful enough when the latent heat of crystallization is released.

TurboFreezer is designed to meet these critical factors: four horizontally arranged fans provide a powerful nitrogen vapor stream which is forced to flow horizontally through the racks of straws. The predefined speed of vapor stream in combination with a precisely controlled temperature, leads to uniform cooling and freezing patterns within the complete batch of straws. The exceptional and unique ability of TurboFreezer to remove the latent heat of crystallization instantaneously as it is released results in an extremely short duration of the freezing plateau. No other freezer provides this feature. TurboFreezer can restore the temperature in all the straws of a complete freezing batch within less than 1 minute. This is significantly more uniform and much shorter than in conventional freezers (Figure 3).

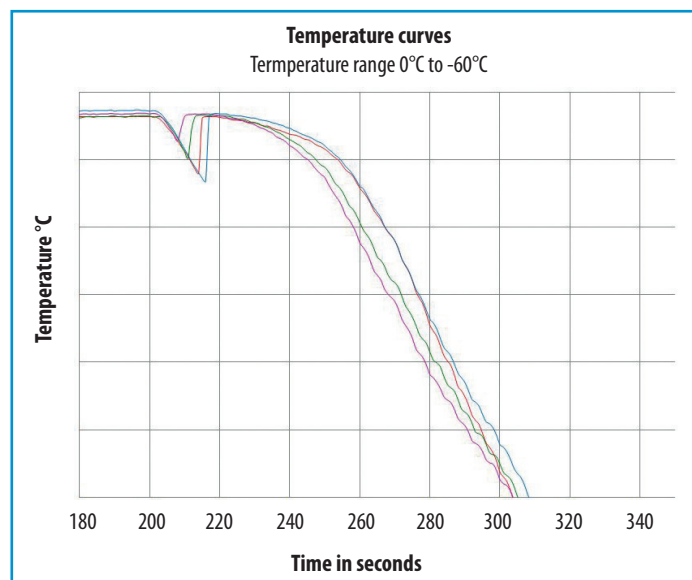


Fig. 3: Crystallization phase and plateau in the TurboFreezer

Promising results of ongoing field trials studying the correlation of freezing of bovine semen using the TurboFreezer and its impact on fertility will be published soon.

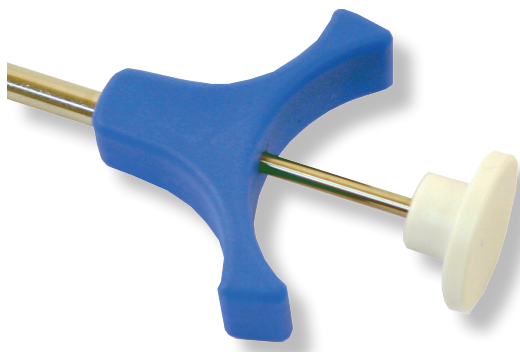
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# QuickLock Ergo - The roadster among insemination devices

A large selection of insemination devices is available on the market. Basically they differ only in the appearance of their handles.

The unique design of the Quicklock Ergo sets it apart from all other insemination devices. Not only does the ergonomically shaped handle offer the technician an optimal working procedure, QuickLock Ergo is extremely space-saving. This feature is especially convenient when several Ergos are to be loaded into the QuickLock Heater.

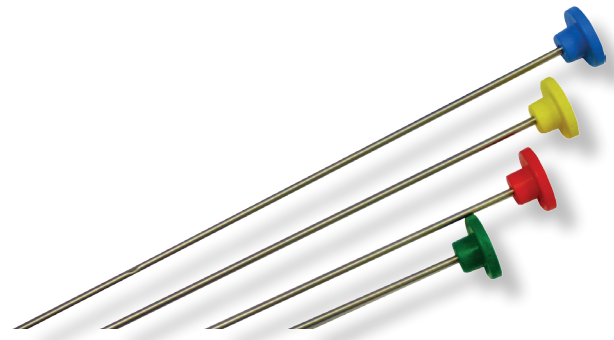


Compared to conventional designs, the QuickLock Ergo is sleek and easy to manoeuvre; like a roadster!

The sheath is easily pulled over the device. Two fixation bulges ensure a safe hold, eliminating the need for an additional clamping ring!



Mandrins are available in five different colours, enabling a clear distinction between insemination devices loaded with semen of different bulls.



## Inside Minitube: Minitube opens new logistics center

Minitube opened a new logistics center at its production location at the commercial area Asper in Tiefenbach, Germany, taking into account the rapid development of the recent years.

Since the construction of a warehouse and production building 'Asper I' in 1998, the number of employees has more than doubled and sales increased by 2.4 times. „The new 'Asper II' was therefore essential for the further development of the company and kindly funded by the Government of Lower Bavaria, as the protection and creation of jobs was one of the goals of the construction project,“ said Dr. Christian Simmet, CEO of Minitube.

With an investment volume of around 4.6 million Euros, Minitube created with the help of regional construction companies, a sophisticated, 1,000 m<sup>2</sup> comprehensive high bay warehouse with 2,260 pallet spaces. The warehouse, impressive 12 meters high, offers more than double



of the previous capacity, and features a special cooling chamber for temperature-sensitive pharmaceuticals with over 100 storage spaces. Asper II is equipped with a photovoltaic system on the south and west facades with 429 panels over an area of 721 m<sup>2</sup> and a nominal capacity of 100,345 kWp.