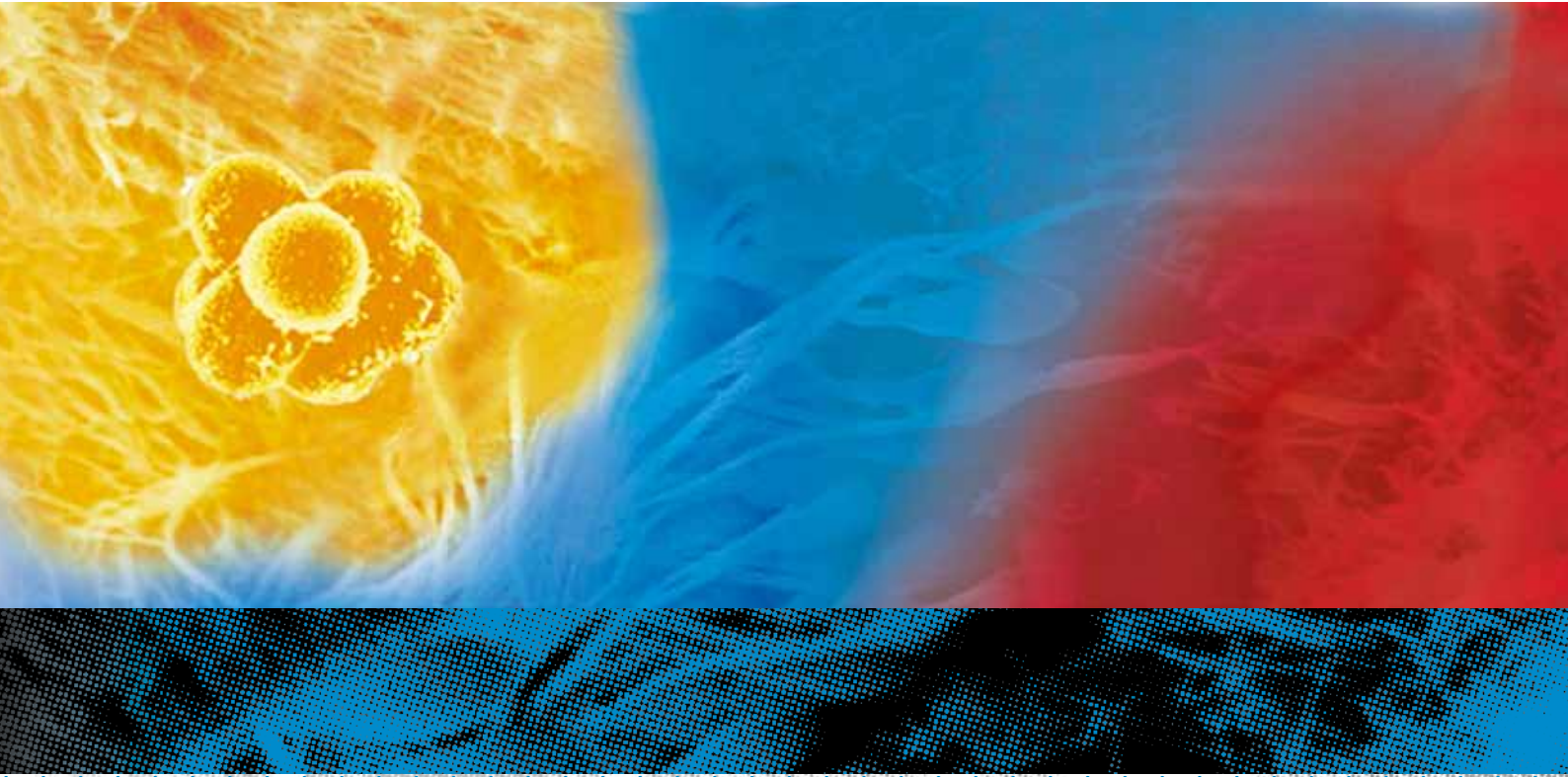


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Important steps for introducing young boars in semen production

Rudolf Großfeld and Carmen de Alba, Minitube

Introduction

The costs for new boars at an AI stud can be very high depending on their genetic value. Naturally, there is a vital interest to maximize the use of these boars in terms of semen production. After, or even during the quarantine period, the first steps for bringing a young animal into production include training the animals for semen collection and evaluating the quality of the semen. The optimal age at which a young boar to be trained for the first time varies between breeds; however, the general rule is that training should not begin before the age of 7 months.

Collection training

The most important part of boar training starts immediately on the first day when the young boar enters his new environment. His caretakers must communicate confidence. The development of a young boar's confidence is vital for his subsequent behaviour and collection performance. The handling of young boars is a task that requires experienced and dedicated staff. Stress is highly detrimental to the training process and caretakers must be able to exercise patience with the animals at all times.

The best boar trainers are patient and enjoy working with the animals. They also know and understand the basic reproductive behaviour of the animal. They also have contact with the animal before training starts in order to allow the boar to become acquainted and build trust with them. Each boar should have a dedicated trainer who spends a few minutes with the young animal each day. Scratching, rubbing and talking with the animal helps to build up confidence.

IMPORTANT: Young boars entering the stud require not only very much patience from the operator, but also the design of the training area and the training protocol are crucial for the collection performance. The environment in which the boar stays combined with the quality of the staff responsible for their care and training, are important factors that can positively or adversely affect the boars' libido.

Training should start during the calm hours in the barn and this is also the ideal time to introduce the boar to the collection pen for the first time. A suitable training area is clean, dry, well lit and free of distractions. The boar should be given adequate time to adjust to his new environment. The dummy in the collection pen should be the same kind as the one

that will be used later for semen collections. A dummy that has been used for collection by an older boar just before the training session will draw the attention of the young boar faster. The smell will stimulate the boar. If it is possible for the young boar to view the collection of another boar just before he enters the collection pen, i.e. from a preparation box, it will stimulate him significantly.

The height and width of the dummy must allow for a comfortable mounting. The height should be adjusted to an equal or slightly lower height than the boar being trained. Young animals up to the age of about 15 months need narrower dummies (25-30 cm) than older boars. The dummy should be sturdy and mounted to the ground. The floor in the collection pen must prevent slipping. A rubber mat in front of the dummy may help.

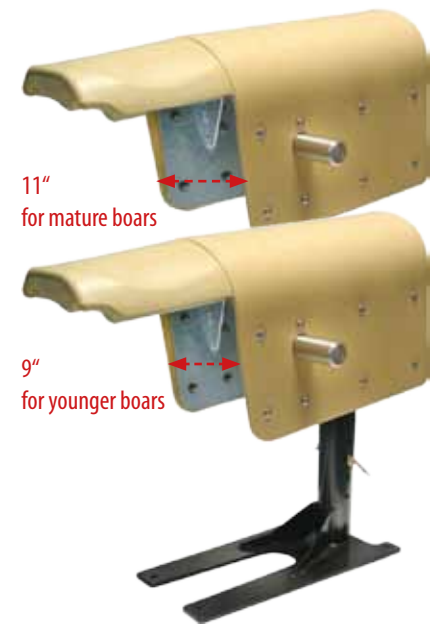


Fig. 1: Minitube boar dummy in two sizes

Each training session should be relatively short and not exceed 10-15 minutes. During this time, try to focus the attention of the boar to the dummy as he will be stimulated by viewing the shape of the dummy. This can be done by standing or crouching next to the dummy and attracting the attention of the boar by touching and talking to him (i.e. petting him or calmly slapping on the dummy). Also, semen from a previous collection, saliva of another boar or synthetic hormones can be used to impregnate the dummy before training.

Some boars will try to mount the dummy immediately; others might need several training sessions. The animal should learn from the beginning, that the collection pen means "work" only. He needs to learn, that after entering the pen, he has to be collected and then go back. Keeping the training lessons short will significantly shorten the reaction time of the boar, which is vital for the productivity of a boar stud. The reaction time is the time from entering the pen until collection starts. If the young boar has not mounted the dummy after 10 minutes, take him back into his pen and try another session on the next day. This might have to be repeated on several subsequent days. Once the boar has mounted the dummy, approach him slowly and

start to stimulate his prepuce. Slowly try to clean the prepuce. That stimulates him and is also needed to empty any preputial fluid. Grasp the penis when it starts to protrude and begin the collection.

At this moment, most of the work in training a boar is done. Once the collection is finished, show the boar that he has done well. Pet or scratch him and take him back to his pen. Give him some feed as a reward. Continue collection with the animal on the following 2-3 days in order to reinforce the positive experience of the boar. After this, allow a break of 3-4 days and then start collection with him on a weekly basis only.

If the above mentioned recommendations are followed, 90 % of the young boars should be able to be collected within 2-4 training weeks provided the training starts before the age of 10 months. If the training starts after the age of 10 months, success is normally reduced to only 70 % of the boars. After successful training, the boar can start to produce semen doses. The first useable dose will depend on the semen quality of the ejaculates. Since the first 2-3 ejaculates usually show an insufficient quality, semen quality testing should start after this.

IMPORTANT: Too young animals, lack of confidence in the operator, traumatic experience of the animal and incorrect technique may lead to total failure in the training of young boars.

Semen quality check

After the first semen collections, the young animal is usually introduced into the stud to start with semen production, provided the quarantine requirements are fulfilled. This can very well happen before the boar is 8 months old. At this time he is not yet fully sexually mature, which has an influence on his libido and especially on his semen quality. His libido depends on his genetic background but it can be boosted by applying the above mentioned techniques of boar training. The semen quality depends not only on his genetic background, but also on his age.

The minimum semen quality requirements of a young boar should fulfil the following parameters (ZDS, 2006):

- 100 ml semen volume without secretion of the bulbo-urethral gland
- 150×10^6 sperm/ml
- 15×10^9 total sperm in the ejaculate
- 70 % total motility
- Maximum 25 % abnormal sperm in total
 - < 5 % head abnormalities
 - < 10 % acrosome abnormalities
 - < 15 % plasma droplets
 - < 15 % coiled tails
 - < 15 % other abnormalities

Schulze et al. (Institute for Reproduction of Farm Animals in Schönow, Germany) have studied factors influencing semen parameters of young boars. In this study, semen from 7213 ejaculates from 5057 young boars were evaluated. The young boars were genetically verified and trained, but were not used in semen production prior to the study. Of the semen doses from these boars, 47.3 % did not fulfil the ZDS requirements. The boar age had a significant influence; most boars below the age of 8 months showed low semen quality. More than 60 % of the ejaculates of this age group did not fulfil the requirements. The reasons were primarily deficiencies in qualitative parameters, such as morphology and motility.

Besides age, the breed had an influence on the semen quality of the young animals between the age of 4.5 to 16.8 months. In this study, especially young Duroc boars showed lower semen motility and increased morphology abnormalities, whereas young Yorkshire boars showed the highest semen volume and lowest morphological abnormalities. Pietrain and Landrace boars showed the best semen motility. A seasonal influence was also evaluated and shown to have only a marginal effect.

The study concludes that young boars below the age of 8 months are more likely to fail the requirements for minimum semen quality and should only be taken into production at a higher age.

Another study (Tsakmakidis et al.), also states that the sperm chromatin instability is higher in the semen of young boars (8-10 months). In this study, AI with semen of young boars resulted in a lower farrowing rate compared to semen of older, mature boars (> 10 months of age).

Conclusion

The introduction of young boars into the stud represents a critical stage in the production life of a boar. During collection training, everything that raises the confidence and comfort of the boar is important and will lead to a shorter reaction time and more efficient semen collection, thus maximizing the productivity of the boar and ultimately that of the stud.

Semen quality of very young boars is likely to fail the minimum requirements up until the age of 8 months. This should be taken into consideration, when starting the semen production with young animals.

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Zero bioburden is consistently achievable in boar semen extender production using the Minitube HyVat Station

Rudolf Großfeld and Sandra Jobstmann, Minitube

The microbiological status of semen doses is a very important quality characteristic especially for diluted boar semen doses. The absence of contamination is important to prevent damage to the semen cells in the diluted semen and also to avoid the spread of diseases to the sow.

Sources of contamination are numerous. The primary source of bacterial contamination is the boar. Other sources that have been identified include environment, personnel, and the water used in the extender preparation (Althouse and Lu, 2005; Schulze et al., 2014). Since 95 % of the semen diluter consists of water, the quality of the water used can have significant consequences.

The main causes of contamination of the water used in extender preparation are inadequate water processing and storage and also recontamination after the extender has been prepared and is ready to use. Recontamination can occur throughout the production day when the heated extender starts to build up condensation on the cooler lid of the heated vat (Fig. 1). This condensation water drops back into the extender and can take bacteria from the environmental air, or from an inadequately cleaned lid, into the extender. Also, when the extender is removed from the vat for production, ambient air that may contain germs enters the vat.



Fig. 1: Condensation water is a potential source of contamination. When water condenses onto the lid of the heated vat, it can collect bacteria before dropping back into the extender.

To address these sources of contamination, Minitube has designed a multifunctional processing unit for boar semen extender preparation, the HyVat Station. The HyVat Station features an Ultraviolet radiation unit that disinfects the vat before and during production. In addition to this, the lid of the HyVat Station is heated to completely prevent condensation.

To prove the sterilisation effectiveness of the ultraviolet radiation, Minitube conducted several trials with the HyVat Station. Two representative trials are described below:

UV disinfection of empty vat

In a first test, the walls and lid of an empty vat were contaminated on purpose with a bacterial mixture, which resulted in a bacterial content of > 300 CFU/cm² on the walls and lid of the vat. Subsequently, the UV radiation unit of the vat was started and bacterial swabs were taken every 30 minutes to monitor the reduction in the bacterial load of the walls and lid of the HyVat Station. The UV radiation was only stopped to take the swabs and then continued. The results are presented in Table 1.

From these results, it can be concluded that the UV disinfection of the surfaces of the empty vat could completely remove any germs on the surfaces within 3.5 hours.

Time: h after UV	Bottom and walls of HyVat (cfu/cm ²)	Lid of HyVat (cfu/cm ²)
0	> 300	> 300
0,5	> 300	35
1	250	166
1,5	158	3
2	57	3
2,5	10	2
3	34	0
3,5	0	0
4	0	0
4,5	0	0

Table 1: Effect of UV radiation on bioburden of vat surfaces in the HyVat Station

UV disinfection of filled vat

In order to test if this promising result could be reproduced in a filled extender vat, the test was repeated with 100 liters of water inoculated with E.Coli, a bacteria commonly found in tap water. The test was performed as follows: The HyVat Station was filled with 100 liters of sterile water. The sterility was confirmed by taking an initial bacterial sample. Then, E.Coli bacteria were mixed into the HyVat Station until a bioburden of 195 CFU/ml of the water in the vat was reached. The lid of the HyVat Station was contaminated by thoroughly wiping it with water out of the vat. The subsequent bacterial sampling confirmed the water contamination and a bioburden of 60 CFU/cm² on the lid.

Thereafter, the UV disinfection was started and bacterial samples were taken every 60 minutes. The samples were taken from the surface of the water, from the bottom of extender vat using sterile pipettes, and swaps were taken from the lid. Table 2 summarizes the results. Already after 60 minutes zero bacteria could be detected in the vat content or on the lid.

Time: h after UV	Water from vat (cfu/ml)	Lid of HyVat (cfu/cm ²)
0	195	60
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	0	0
7	0	0
8	0	0

Table 2: Effect of UV radiation on bioburden of the water filled vat and lid in the HyVat Station

Working principle of UV light

Ultraviolet light is an electromagnetic radiation and has by definition a wave length between 100-400 nm. This wave length it is not visible to the human eye. UV-radiation is a component of sunlight and is also produced by electric arcs (i.e. during welding) and special lamps, like mercury vapour lamps.

Especially UV-C radiation is used for sterilisation purposes. Low pressure mercury vapour lamps are used to produce UV radiation with a wavelength of 254 nm. This wavelength has been proven to have the highest germicidal effect. UV-C with a wavelength of 254 nm has a photolytic effect that damages the DNA structure of bacteria, fungi and viruses. It disrupts the DNA, leaving the germ cells unable to perform vital functions and thereby killing them.

Although the UV-radiation unit of the HyVat Station can kill germs in an empty or filled vat as described, it cannot remove the residues of these killed bacteria. So, even with a UV radiation unit in use for extender preparation, it is still necessary to use water with a very low germ content. The reasons for this are twofold, as described below.

The effectiveness of the UV radiation in killing germs depends on the duration of the radiation, the energy emitted by the UV bulb and the ability of the germs to withstand UV radiation. Other factors, like turbidity of liquids, can hinder disinfection. In the HyVat Station, it is therefore important to use the disinfection unit only with the empty vat or with distilled water. UV light cannot efficiently pass through a prepared extender because the amount of energy is reduced by the higher turbidity of the extender compared to water.

Also, if germs are covered by dirt or residual particles, a successful disinfection will be prevented. Only clean surfaces can be disinfected effectively. It is therefore necessary to clean the vat of the HyVat Station

thoroughly before UV radiation starts. This is quite easy as the vat is made of stainless steel and can be tipped to allow cleaning with little effort.



Fig. 2: HyVat Station offers an automated solution to consistently achieve zero bioburden in boar semen extender preparation

Recommendations for using the HyVat Station

In order to achieve zero bioburden in boar semen extender during a full production day with different lab routines, the HyVat Station offers several programmable options. The HyVat Station can disinfect the cleaned and empty vat before filling or it can disinfect the vat after water for the extender preparation has already been added. It is also possible to start the UV radiation unit several times during the production day, so that the ambient air that flows into the vat when the extender is removed will be disinfected as well.

All these procedures are programmable which allows, with complete automation, the disinfection of the vat either before it is filled with water or afterwards as the water warms. In addition, the heated lid completely prevents condensation and therefore any risk of contamination.

Conclusion

Zero bioburden in boar semen extenders can consistently be achieved with the optimized features of the HyVat Station:

- UV radiation unit allows disinfection of the empty or water-filled vat before production and disinfected continuously during production;
- Germs on the vat surfaces, lid and in the water for extender preparation can be effectively destroyed in order to prevent contamination of the boar semen extender and of the diluted boar semen itself;
- The lid of the vat is warmed which eliminates any possibility of water condensation as a possible source of contamination;
- The procedures are programmable and allow for automation.

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Reprotoxicity in plastic materials

Carmen de Alba and Rudolf Großfeld, Minitube

Introduction

A large variety of plastic materials is used to make products for boar semen collection, processing, and insemination. The proper selection of raw materials and a suitable design of these products is necessary to provide hygienic and safe environments for boar semen. In particular, the plastics used in the manufacture of boar semen packaging units such as bottles, tubes, flat packs, or blisters, must meet the highest standards in order to protect and maintain the quality of the stored semen doses. In this context, the most fundamental prerequisite for any semen packaging unit is that it exerts no detrimental effect on the fertility of the stored semen. Exhaustive tests of the raw material to prove its neutrality with semen are therefore mandatory before manufacturing and commercializing the product.

Plastic materials used for semen packaging are polyolefins that offer great flexibility, mechanical strength, low weight, stability and high density. This group includes polyethylene (HDPE or LDPE), polypropylene (PP), polyvinylchloride (PVC), polyethylene terephthalate (PET), and other copolymers such as ethylene vinyl acetate (EVA).

Latent toxicity of multi-layer plastic films used to manufacture semen bags - a case study

Currently there are two widely varying production processes used to make boar semen packaging units. The most commonly used type of container is the boar semen tube or bottle made of pure polyethylene. The polyethylene is processed in a so-called blow-moulding machine, which melts plastic pellets and forms the semen tube or bottle from the hot and soft plastic material. No additives are needed for this straightforward production system.

Semen bags or blisters, however, are made from multi-layer plastic films which consist of 2 or more plastic layers which are glued together with an adhesive (Fig. 1). The plastic films contain plasticizers which provide the flexibility to the thin-walled bags or blisters; the most widely used plasticizers are phthalate esters. These additives have long been suspected to have a negative effect on sperm cells and living organisms in general. The EU regulations classify phthalates as potentially teratogenic substances which can impair fertility (EC Regulation 1935/ 2004, EU Commission regulation 10/2011).

The adhesives used to manufacture multi-layer plastic materials are not even regulated; however, it is obvious that their potential

bio-toxicity (Felix et al. 2012, Isella et al. 2013) warrants as much examination and control as that used for the plasticizer.

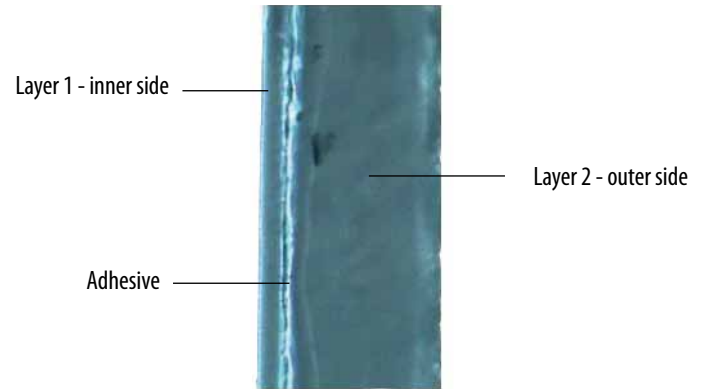


Fig. 1: Microscopic picture of a cross-section of a multi-layer semen bag wall

Although it is long known that boar semen is extremely sensitive to any chemical impurity present in extenders due to deficient water or extender component quality, boar semen bags or blisters are still being manufactured with the above mentioned risky raw materials without having adequate quality control measures in place. Nerin et al. (2014) elucidated in their study the origin and reasons behind a dramatic reproductive failure in more than 40 Spanish sow herds in the spring of 2010. It is to their merit that we understand today that it is nearly impossible to produce a safe boar semen bag when plasticizers and adhesive are involved.

Nerin et al. went through a complete analysis of all the circumstances and parameters involved in the assisted reproduction procedures used with the sow herds. They found that the only common denominator was the use of semen doses packaged in semen bags of one source. The chemical analyses of the used plastic bags revealed no less than 5 different toxic compounds:

- BADGE
- BADGE-H2O
- BADGE-2H2O
- Cyclic lactone
- Cyclic phthalates

BADGE is a derivate of Bisphenol A and long suspected to cause reproductive problems. The origin of these toxic compounds was found to be the adhesive used to manufacture the multi-layer plastic bags. It was also found that multi-layer plastic bags from the same batch could contain different amounts of adhesive and even different types of adhesive. This result explains why different concentrations of toxic compounds were found even in bags from the same batch.

The study also revealed that the toxic compounds of the plastic bags leach through intact plastic films into the extended semen and react there with the water molecules to form new compounds; namely BADGE-H₂O and BADGE-2H₂O. The total concentration of BADGE compounds and their derivatives detected by the chemical analyses conformed to the relevant European Regulation 10/2011/EU for food safety, which allows a maximum value of 9 mg/kg food. The effect on reproduction caused by toxic compounds which are in direct contact with sperm can therefore not be excluded although food toxicity levels are met. This difference can be explained by the mechanisms of toxic action.

Leaching processes depend on a variety of factors including contact time, temperature and initial concentration of the compounds in the plastic material. For this reason, the transport of toxic compounds will be higher in the semen doses stored for longer periods in plastic bags or in bags with a higher concentration of the leachable toxic compound.

Although the boar studs which used the defective semen bags performed rigorous quality control on the semen doses they produced, they could not detect any alteration in the semen parameters in their *in vitro* tests. Nerin could not even find any semen defects when her group performed *in vitro* penetration tests. Only *in vivo* fertility studies confirmed that the above mentioned compounds were indeed the main cause for the reproductive failure in the sows. The reprotoxicity did not affect conventional semen quality parameters, but it can be assumed from the presented data that it affected early embryonic development up to the blastocyst stage (d 6).

From the fertility data presented in Cristina Nerin's study, it is evident that the use of toxic semen bags resulted in an approximately 50 % loss of total born live piglets in the affected sow farms, which corresponds to a combined effect of pregnancy rates being reduced by more than 25 % and the litter size reduced by 2 piglets.

What we learned

Toxic compounds comprised in the multi-layer semen bags leach into the extended semen.

Reliable quality control of the semen bags manufactured with plasticizers and adhesives is not possible because the concentration of toxic substances varies widely between the semen bags of the same batch.

There is no *in vitro* test with semen or embryos which could reliably indicate the toxicity of a bag because most of the reprotoxic effects unfold only after fertilization during the early embryonic development.

Semen bags manufactured with plasticizers and adhesives can cause severe reprotoxicity at any time.

Boar semen tubes are safe

Boar semen tubes from Minitube are made from a pure polyethylene of pharma grade quality. Polyethylene is proven to contain neither plasticizers nor adhesives.

With comprehensive sperm safety test protocols applied to every raw material batch, Minitube assures that the fertility of boar semen packaged in its tubes will be not be impaired (Esch, 2014/2). Minitube's boar semen tubes are therefore marked with the SpermSafe label (Esch, 2014/1).



Fig. 3: Polyethylene pellets for boar semen tubes

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Sample preparation for CASA analysis

Dominika Becherer and Carmen de Alba, Minitube

When carefully validated, CASA systems provide valuable information for quality assurance of semen planned for sale, and for the understanding of the diversity of sperm responses to changes in the micro-environment in research (Amann P. and Waberski D., 2014).

Although the evaluation of semen by the CASA system has demonstrated the ability to give the best reproducibility, there still exists measurable variability among laboratories which may be due to various factors such as dilution rate and type of extender (Vizcarra JA, Ford JJ. 2006). Other factors that influence the analysis conducted by CASA include the method of sampling, sample processing, and the time span between sampling and analysis. All consumables employed for semen sampling preparation should be handled aseptically and must also be tested for sperm toxicity. Therefore, a standardised routine is the premise for reliably obtaining reproducible results (De Alba Romero C., 2011).

The preparation and homogenization of the sample is the most important step towards obtaining a correct measurement and consistent and reproducible results (Nicolae M., 2006). The essential measures leading to a standardised CASA process are:

1. Consistent mixing of the ejaculate
2. Correct drawing and handling of the sample
3. Correct measurement settings of the software (classification parameters, calibration, and chamber depth)

1. Mix the ejaculate well by inverting 5 times (by 180 degrees), without shaking.



2. The best tool for correct semen sample dilution is an electronic pipette with a dilution function.

First, draw in the extender (must be held at the same temperature as the semen sample).



Secondly, draw in the sample from the ejaculate. It is very important to draw the semen sample correctly: to be representative it must be taken from the centre of the bag or container, at approx. 5 mm depth, immediately after mixing (see step 1).



3. Wipe the outside tip of the pipette and eject the sample into an appropriate vial.



A certain number of sperm per area or field should not be exceeded. Therefore, the ejaculate sample must be pre-diluted. It is important to control the temperature not only of the semen samples but also of the extender used for pre-dilution. The following dilution rates for boar semen are recommended, depending on the semen concentration of the ejaculate:

- Standard: 200–600 million/ml (1+9 = 90 µl sperm + 810 µl extender)
- High: > 600 million/ml (1+19 = 45 µl sperm + 855 µl extender)
- Low: < 200 million/ml (1+4 = 180 µl sperm + 720 µl extender)

Before starting the analysis, ensure the following actions have been taken:

- Pre-warming all materials to 38°C / 100°F
- Preparing and pre-warming the extender to 38°C / 100°F
- Pre-warming the microscope stage to 38°C / 100°F

The procedure for the analysis is as follows:

- Mix the pre-diluted sample in its vial by inverting it at least 5 times, without shaking.
- For the removal of a sample for CASA, use a volumetric pipette. Load the counting chamber with the appropriate volume according to the chamber size and without any air bubbles.
- After filling the chamber, perform the measurement with the CASA system immediately.
- Scientific studies have shown that the measurement with a CASA-system should start 15-20 seconds after filling the chamber (Nicolae M., 2006).

When considering the possible and common mistakes of a CASA analysis, it becomes evident how important the points described above are for correct sample preparation and homogenization. Variations in sperm mobility caused by temperature differences, incompletely filled counting chambers or high and low concentrations of the same sample in two measurement fields are among the most frequent mistakes of measurement. These can be avoided by precisely observing the procedure: adequate pre-warming of all materials and solutions which will be in contact with the semen sample, the correct position of the pipette for removing the sample, a well-mixed sample before pipetting, and the correct filling of the counting chamber without any air bubbles.

Minitube conducts regular workshops for lab personnel working with CASA systems.

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