EquiPure™ Product Manual

Nidacon International AB

Nidation

1. The building of a nidus, a nest, as with birds

2. Implantation of the fertilized ovum (zygote) and the building of a nest, the placenta, in the endometrium.

Conception

1. The union of male and female gametes, the sperm and egg, to form a conceptus (also known as a zygote, or a pre-implantation embryo).

2. An impression or idea.

Contents

Nidacon
International AB

Introduction

Nidacon International AB (Nidacon) manufactures and sells Medical Devices mainly for the Assisted Reproduction Technologies for both humans and animals. The company was founded in 1996 by Assoc. Prof. Paul V. Holmes MSc, PhD, DrMedSc, an embryologist and endocrinologist from the Dept. of Obstetrics and Gynaecology at Sahlgrenska University Hospital in Gothenburg, Sweden.

Nidacon considers many different factors when designing its products. We hope that the attention to detail has helped to create products which will lead to better results. We aim to work in close relation with our customers; they are the cornerstones of our research department.

One of the first products to result from the company's research and development, PureSperm®, was introduced onto the market in November 1996. It has gained rapid acceptance and is now the global market leader for isolation and preparation of sperm used in human assisted reproduction. It was the first product of its kind to achieve both 510(k)-clearance from the US FDA and CE marking with the European authorities.

EquiPure™ is based on the same scientific principles used in PureSperm® development, while optimised for horses.

Quality

Nidacon is certified according SS-EN ISO 9001 (implemented 2000-12-15) and SS-EN ISO 13485 (implemented 2003-08-15). The management system secures continued development of the organisation.

We register our products according to the valid directives and requirements in different parts of the world. This also ensures our high quality on the market and it shall continue to be our beacon. The regulations for animal products are not as rigorous as those for the human, but our productions and quality controls are equal for both.

Nidacon intends to always maintain or increase the high quality of its products and, in order to achieve this, all batches are well tested at Nidacon before they are cleared for the market. Sterility controls are performed on each batch of product manufactured, the endotoxin level is measured and biological efficacy tests are carried out. The batches are only released for sale if they meet the specified criteria.

Each batch is accompanied by a quality control certificate which records the results of the tests. Using this rigorous quality control system, we ensure that each batch meets the necessary standards.

Consequently, the customers are secure in the knowledge that our products are reliable and will provide good results when used correctly.

Shelf life

Nidacon is conscious of customer requirements and always tries to provide products which are convenient. This convenience includes ease of transportation and storage with long shelf life. Therefore, the products have a shelf life of one to two years at room temperature. All ingredients are chosen for their temperature tolerance and their stability in aqueous solution. Rigorous shelf life testing has been carried out in Nidacon's in-house laboratory to ensure that the theoretical stability of the salt formulations is matched by their actual stability when combined in the product.

Packaging

The packaging for Nidacon's products has received the same care and attention to detail as the design of the products themselves.

Bottles; for most of our products we have chosen borosilicate glass instead of sodium silicate glass to avoid the leaching of sodium from the bottles into the contents during the long shelf life. Research in our laboratory has shown that sufficient sodium ions can leach from a sodium silicate bottle to have a negative effect on the development of two-cell mouse embryos. Therefore, we avoid exposing the sperm to raised sodium-ion levels in the products by packaging in borosilicate glass.

Stoppers; Based on the embryo-toxicity testing of three types of commercially available rubber stoppers approved for pharmaceutical use today, Nidacon chose silicone rubber as the material of choice.

We found that both natural latex rubber and butyl rubber are toxic to embryos, preventing development and possibly causing embryonic death. Silicone rubber did not have any detrimental effect, allowing embryonic development and hatching to proceed normally. Therefore, stoppers made from pharmaceutical silicone rubber were chosen for our products.

Why is the composition and formulation of a product so important?

Background

Under normal physiological circumstances, sperm undergo a series of maturational changes after ejaculation, changes which enable them to negotiate the different sections of the female reproductive tract, stimulate the female tract, and eventually locate and fertilise the egg. If sperm are to be used for ART, it is essential that any product which is used for sperm preparation must match the sperm's physiological requirements as closely as possible.

If sperm are stimulated excessively, particularly ionically, they become "hyperactive", a process which results in the sperm using up their energy resources and dying before fertilisation is achieved. Therefore, the pH and osmolality of the sperm solutions must be adjusted very specifically to avoid ionic chock and subsequent hyperactivation.

Buffer

The zwitterion buffer, HEPES, is included to maintain the pH of the products while working with the sperm on the bench. Fluids designed to maintain pH in a CO2 environment, i.e. in the incubator, are unsuitable for use outside the incubator as they do not possess sufficient buffering capacity to maintain the pH.

Fluctuations in both pH and temperature are detrimental to sperm survival on the bench. In addition, HEPES has an antioxidant effect, reducing the effectiveness of reactive oxygen species (ROS) which can be damaging during sperm preparation.

Glucose

Glucose is a component of EquiPure™. Glucose is the primary energy substrate available to sperm in the female reproductive tract.

Antibiotics

Antibiotics are not included in our products for several reasons. Bacterial contamination in the ejaculate is removed by the density gradient preparation. Therefore, the absence of antibiotics in the gradient will not be detrimental to the sperm in the preparation, and avoids exposing the sperm to the potentially toxic effects of antibiotics. (refs 10-14)

Additives and Phenol Red

No preservatives or unstable ingredients are added to our products. We have also decided not to use phenol red in our media, since it has been proven to have estrogenic effects.

Gametes have receptors for estrogen and they can be affected by its presence. For instance, it has been shown that estrogen inhibits sperm motility and acrosome reaction.

EquiPure™ development

We transferred the same principals and optimised the technique for equine sperm since we have observed that the quality of the sperm varies considerably from stallion to stallion, and this presents a problem to breeders and veterinarians around the world.

We are fully aware that some specific stallions are very valuable, even though they have bad sperm quality.

EquiPure™ is a centrifugation system for the selection of fertile equine sperm and, by separating the sperm through a density gradient prior to freezing, inseminating, or semen sexing. We not only eliminate all the bad quality spermatozoa and, therefore, decrease the size of the insemination doses (ref 5), but also remove reactive oxygen species, most bacteria and many viruses (EAV). This significantly increases the sperm survival and therewith their fertilising potential.

EquiPure® is used by breeders, animal reproduction scientists and veterinarians all over the world.

EquiPure™

- Improves fertility rates, especially in sub-fertile stallions (ref 1, 2).
- Improves freezeability of equine sperm (ref 3, 4).
- Removes bacteria and other unwanted components in an unprocessed ejaculate.
- Removes Equine Arteritis Virus (EAV) particles (ref 8, 9).

Studies on the hygienic aspects of semen storage for AI demonstrate that the addition of antibiotics to commercial semen extenders is not enough to eliminate the detrimental influence of bacteria on sperm motility, viability and fertility (ref 3). This is also why we recommend EquiPure™ centrifugation prior to freezing in order to remove the bacteria.

If the sperm to be treated are already frozen, the thawed ejaculate needs to equilibrate approximately 15 minutes in the extender before centrifugation. This is necessary to allow the sperm to rehydrate and restore their correct density.

Apart from products for Equine sperm, we also carry products for bovine, porcine, ovine and many other species. If you are interested in sperm preparations for other species, please contact us!

Products

EquiPure™ Top and Bottom Layer are the two components of a density gradient system used for separating and purifying the Equine spermatozoa. This is a standard procedure used in the purification of sperm.

EquiPure™ Top Layer

EquiPure™ Top Layer is ready to use in conjunction with EquiPure™ Bottom Layer to make up the two density layers in the gradient. Together, they build two density interfaces at which the sperm will be filtered. The pellet that is retrieved after centrifugation contains only normal purified spermatozoa.

Shelf life 2 years from production date.

Components
Silane-Coated Silica
NaCl
Glucose
Na-Pyruvate
EDTA
KCl
Citrate
Lactate
HEPES
H_2O

EquiPure™ Bottom Layer

EquiPure™ Bottom layer can be used without the Top layer to form a single layer density filtration. Which method is used is the decision of the user.

Shelf life 2 years from production date.

Components
Silane-Coated Silica
NaCl
Glucose
Na-Pyruvate
EDTA
KCl
Citrate
Lactate
HEPES
H_2O

Ordering information

Cat. No	Description	Size
EPT-020	***EquiPure™ Top layer***	2 x 20 mL
EPT-100	***EquiPure™ Top layer***	100 mL

Cat. No	Description	Size
EPB-020	***EquiPure™ Bottom layer***	2 x 20 mL
EPB-100	***EquiPure™ Bottom layer***	100 mL

We have distributors in most countries. For a complete list of these distributors, take a look at our web page www.nidacon.com

General information

Background

A normal semen sample (ejaculate) is made up of seminal fluid which contains a number of different cell types, cell debris, microbiological and biological substances.

The different cell types contained in semen are normal motile sperm, juvenile and senescent sperm (no fertilisation function) and sperm with DNA breaks. In addition, epithelial cells from the male reproductive tract, male immune cells and cell debris (detritus) are present in the semen, as are also bacteria and possibly viruses.

The seminal fluid also contains biologicals such as sperm decapacitating factors and reactive oxygen species (ROS), both of which negatively affect fertilisation.

After ejaculation in vivo, normal sperm quickly migrate from the liquefied semen into the uterine cervix of the female, thereby separating themselves from the adverse affects of the seminal factors mentioned above.

An old method, still being used to prepare sperm is swim-up. Nidacon recommends using single or double layer centrifugation since this method has the following advantages, as compared to swim-Up.

Positive features of a discontinuous density gradient according to Nidacon.

Feature	Density Centrifugation	Swim-Up
Separates motile sperm from other cell types	✔	✔
Separates out immature, aged and dying sperm	✔	—
Separates out morphologically abnormal sperm	✔	—
Separates out sperm with damaged chromatin	✔	—
Removes bacteria and viruses	✔	—

General care and use

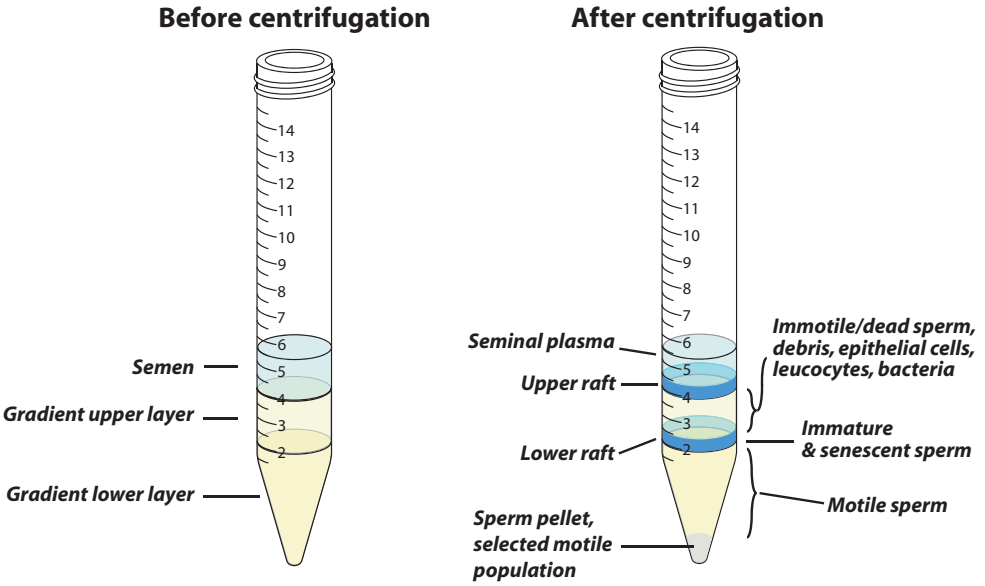
- All solutions should be brought to room temperature before use to avoid temperature fluctuations which are detrimental to sperm survival.
- Open and reseal bottles in a laminar air-flow(LAF) bench using sterile techniques to avoid contamination.
- If a LAF-bench not is available, and if not all contents is to be used, decontaminate the stopper with 70% alcohol and use a needle and syringe to aspirate the colloid.
- Store all opened bottles at 2-8°C.

Different ways of doing it

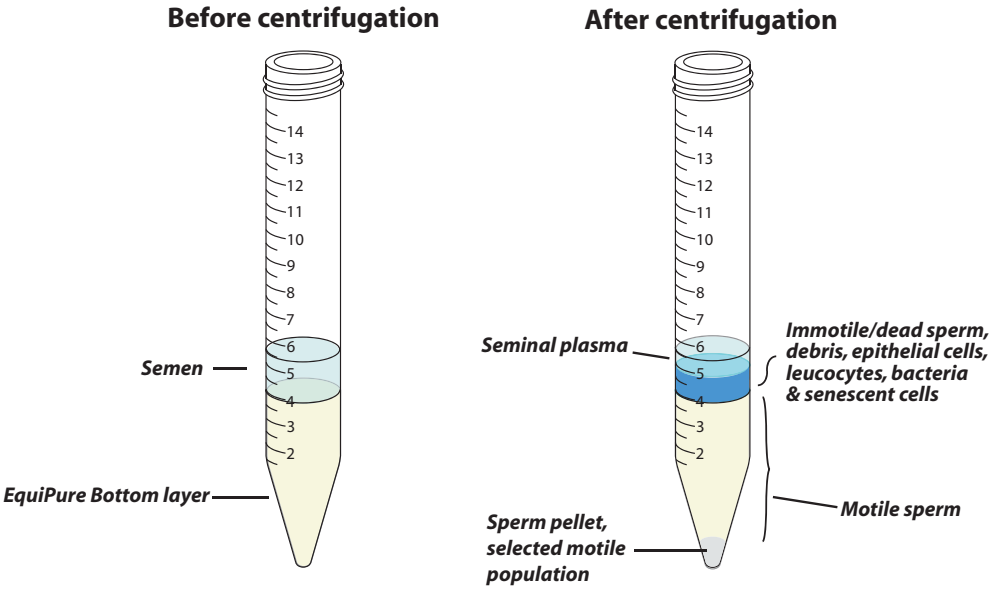
EquiPure™ has been on the market for many years and the number of protocols are now as many as there are users. It has become clear that different stallions require different methodologies. This also applies to sperm freezing protocols.

In this manual, we have described two protocols (double and single layer centrifugation with different volumes) and these should be considered suggestions; there is always a possibility to optimize the protocol to suite a specific stallion.

Double layer centrifugation



Single layer centrifugation



Calibration of the centrifuge

Calibrate the centrifuge; to achieve the correct g-force, use the equation:

Rpm = $\sqrt{[\ (g/(1.118 \times r)]} \times 10^3$

g = the centrifugal force

r = rotational radius, the distance (mm) from the centre of the rotor to the bottom of a centrifuge tube in the bucket when raised to horizontal position

For example; to achieve 300 x g when radius = 165 mm the centrifuge speed must be:

Rpm = $\sqrt{[\ (300/(1.118 \times 165)]} \times 10^3 = 1275$

1. Double layer centrifugation

Materials required

- EquiPure™ Top & Bottom Layers
- Conical centrifuge tubes
- Dispensing pipette and disposable tips
- Pasteur pipettes
- Centrifuge with swing-out rotor

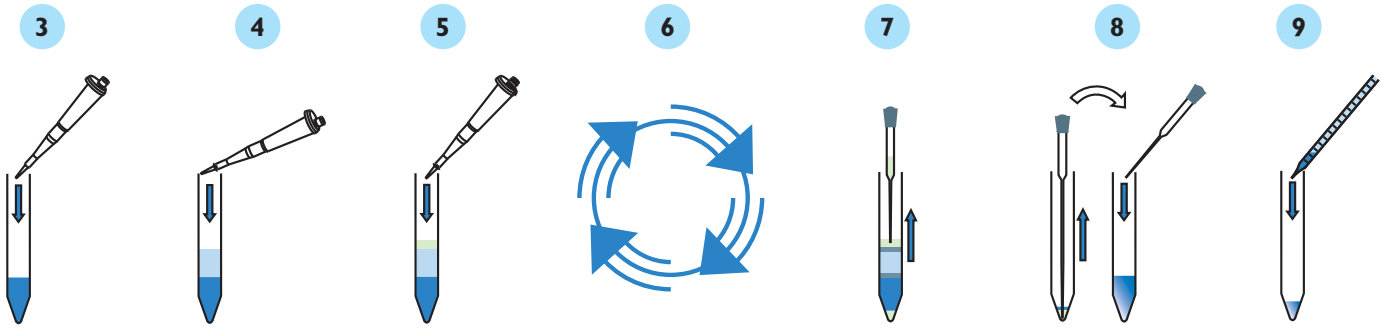
Protocol

Depending on the volume ejaculate to process, different sizes of tubes can be used. See table below.

Ejaculate size	Tube size (mL)	Bottom layer Volume (mL)	Top layer Volume (mL)	Ejaculate volume on the gradient
Small	10-15	2	2	2-3
Medium	50-60	5	5	4-6
Large	50-60	10	10	7-10

Procedure

1. Bring all materials to room temperature.
2. Extend the ejaculate 1:1 (use an extender of your choice).
3. Using a sterile pipette, transfer EquiPure™ Bottom Layer to a conical centrifuge tube (see volumes and sizes above).
4. Using a new sterile pipette, layer EquiPure™ Top Layer carefully over the EquiPure™ Bottom Layer, taking care not to disrupt the gradient layers.
5. Layer the extended semen on top of the two-layer gradient taking care not to disrupt the layers.
6. Centrifuge at 300 x g for 20 minutes at room temperature in a centrifuge with a swing-out rotor.
7. Carefully remove ejaculate, EquiPure™ Top Layer and most of the EquiPure™ Bottom Layer*.
8. Using a new sterile pipette, transfer and resuspend sperm pellet in 1 mL sperm washing medium in a new sterile tube (10-15 mL).
9. Dilute to desired sperm concentration with washing medium.



Tips

- We recommend preparing two EquiPure™ gradients for each sample, to reduce the risk of overloading a single gradient and to provide two tubes to balance the centrifuge rotor.
- If larger volumes are processed, a water vacuum system (Pasteur pipette connected to a tube connected to the water tap) can be used for step 7.

2. Single layer centrifugation

Materials required

- EquiPure™ BottomLayer
- Conical centrifuge tubes
- Dispensing pipette and disposable tips
- Pasteur pipettes
- Centrifuge with swing-out rotor

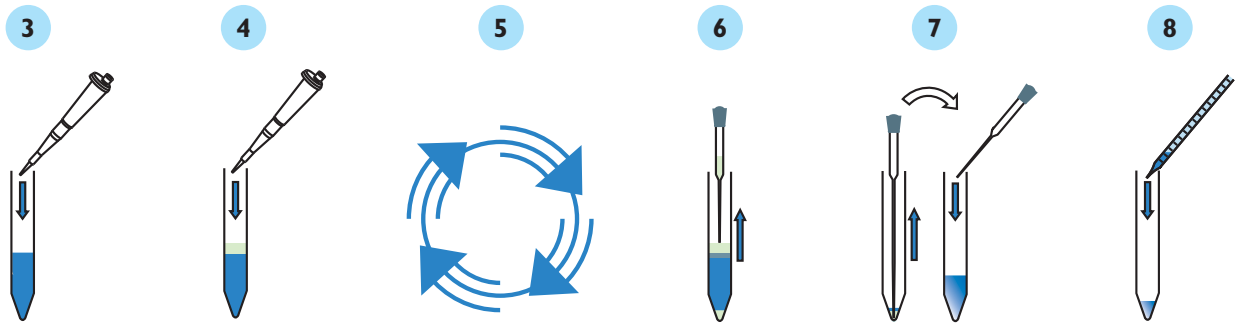
Protocol

Depending on the volume ejaculate to process, different sizes of tubes can be used. See table below.

Ejaculate size	Tube size (mL)	Bottom layer Volume (mL)	Ejaculate volume on the gradient
Small	10-15	4	2-3
Medium	50-60	10	4-6
Large	50-60	20	7-10

Procedure

1. Bring all materials to room temperature.
2. Extend the ejaculate 1:1.
3. Using a sterile pipette, transfer EquiPure™ Bottom Layer to a conical centrifuge tube (see volumes and sizes above).
4. Using a new sterile pipette, layer extended-semen on top of the EquiPure™ Bottom layer.
5. Centrifuge at 300 x g for 30 minutes at room temperature in a centrifuge with a swing-out rotor. Do not use the brake.
6. Carefully remove ejaculate and most of the EquiPure™ Bottom Layer.
7. Using a new sterile pipette, transfer and resuspend sperm pellet in1 mL sperm washing medium in a new sterile tube (10-15 mL).
8. Dilute to desired sperm concentration with washing medium.



Tips

- We recommend preparing two EquiPure™ single layer preparations for each sample. This reduces the risk of overloading a single preparation and provides two tubes to balance the centrifuge rotor.
- If larger volumes are processed, a water vacuum system (Pasteur pipette connected to a tube connected to the water tap) can be used for step 6.

Scientific references for EquiPure™

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