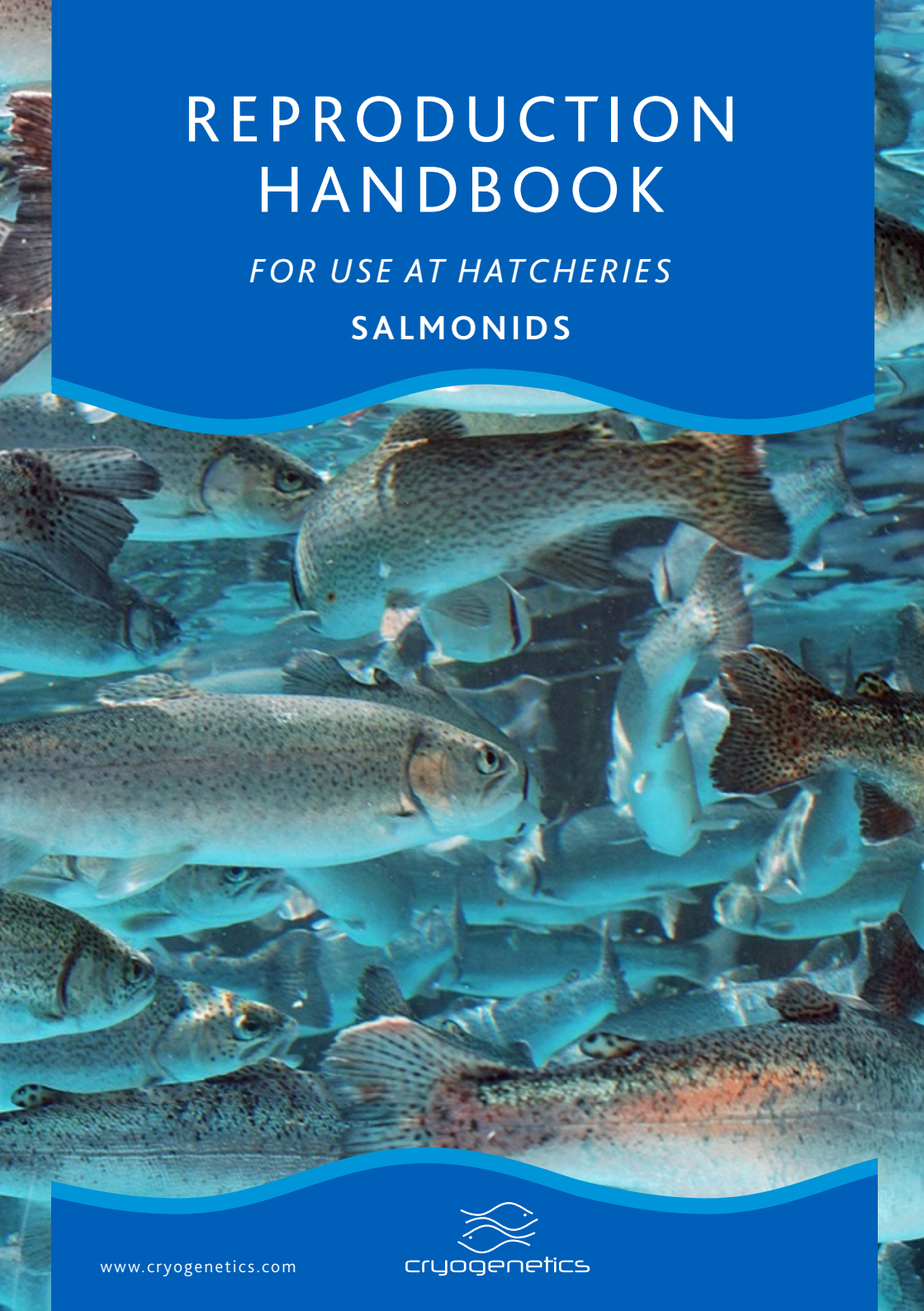
REPRODUCTION HANDBOOK

FOR USE AT HATCHERIES

SALMONIDS



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About this Handbook

This handbook describes our guidelines for handling, thawing and storing milt as well as use of cryopreserved sperm for fertilisation. However, it must be noted that these guidelines are in themselves not sufficient to guarantee good fertilisation results. It is well known that several factors may affect the fertilisation results, such as quality of the sperm (also before cryopreservation), egg quality, and conditions during egg incubation. It is important that these protocols are followed precisely, as shortcuts could give a poor fertilisation output.

Temperature plays a vital role for successful fertilisation with cryopreserved milt. Therefore, make sure that the ambient temperature and all items in direct contact with milt and eggs are at a steady temperature between 2 – 5°C at the day of fertilisation (Read more about temperature in chapter 8). These items may include (i) all chemical remedies and water, (ii) saline water, (iii) any equipment used during the process.
The purpose for this handbook is to provide information to assure proper protocols are followed.

However, it is highly recommended that extraction of gonads and fertilisation using SquarePacks® is performed in collaboration with Cryogenetics´ staff the first time. Therefore, please do not hesitate to contact us should you have any questions.

1. AquaBoost® Dilutor

AquaBoost® Dilutor is a dilution buffer used for dilution and standardisation of milt used for fertilisation. Used in combination with sperm concentration measurements from a photometer, milt can be diluted to a pre-determined sperm concentration. This allows the full fertilisation potential of the milt to be utilised by controlling the sperm-to-egg ratio. This assures a more predictable fertilisation result. By standardising the sperm concentration, you can also ensure equal genetic contribution from each male if fertilising with mixed sperm from several males.

It is important to note that AquaBoost® Dilutor is **not** a holding medium, but just a dilution buffer and must therefore be used within the same day after diluting the milt.

Instructions for use:

1. AquaBoost® Dilutor is prepared by dissolving one sachet in 1 L. (33.8 oz) distilled/deionised water.
2. Sperm concentration is determined by using a photometer (read more in chapter 5).
3. **Make sure AquaBoost® Dilutor has the same temperature as the milt before use!**
4. AquaBoost® Dilutor is added to dilute to a standard sperm concentration (contact Cryogenetics for spreadsheets designed to assist in dilution of milt)
5. Milt of unknown sperm concentration can alternatively be diluted by volume, e.g. 1-part milt + 2-parts AquaBoost® Dilutor. For Atlantic salmon, this will result in an average sperm concentration of about 2 bill. sperm/ml. However, this may vary between males, strippings and throughout the spawning season.
6. **Milt diluted with AquaBoost® Dilutor must be used within the same day.**

2. AquaBoost® Activator

AquaBoost® Activator is a sperm activation buffer that creates optimal conditions for salmonid sperm during fertilisation. AquaBoost® Activator ensures that sperm cells are fully activated and results in best possible fertilisation result.

Instructions for use:

1. AquaBoost® Activator is prepared by dissolving the contents of one sachet in 4 L./1 gallon of distilled/deionised water.
2. **Make sure the AquaBoost® Activator has the same temperature as the eggs before use.**
3. Add milt to the batch of eggs.
4. Add the appropriate amount of AquaBoost® Activator, enough to cover the eggs, while simultaneously stirring the batch of eggs. For 4000 eggs, you should use approximately 50mL. However, this may vary depending on the size of the eggs.

AquaBoost® Activator can be used when fertilising with fresh milt, diluted milt, gonad extracted milt and stored milt. **Note that for salmonid milt diluted with AquaBoost® SpermCoat, AquaBoost® Activator MUST be used during fertilisation in order for the sperm cells to be re-activated and to ensure fertilisation.**

3. AquaBoost® Quattro

Being able to assess the fertilisation outcome as early as possible is a good quality parameter in egg production. This may be particularly relevant in cases where there is doubt about the quality of the eggs or milt. The fertilisation outcome can be assessed within 150 – 200 hour-degrees after fertilisation by using AquaBoost® Quattro.
A sample of embryos is taken out of the incubator and the Quattro solution is then added. By observing the eggs under magnification, the difference between fertilised and unfertilised oocytes become apparent. The timing of the assessment is critical for the test to give a correct assessment of fertilisation outcome.

Cryogenetics has developed equipment for automatic assessment of fertility rate at 4-cell stage. This protocol will only describe how to do this assessment by hand. If you are interested in a QuattroMachine, please contact Cryogenetics directly for a quote.

Equipment

- AquaBoost® Quattro
- Circular counting dish
- Counting spoon or inoculating needle
- Microscope (10x-60x magnification) with over / sidelight

Fertilisation and incubation
Number of hour-degrees since fertilisation (hours x temperature) is critical for the accuracy of the test. You must record accurately the fertilisation timing and the water temperature in which it is incubated at. The easiest way is to use temperature data recorded with an automatic log.

Number of eggs for the assessment
We recommend that between 100-200 eggs need to be evaluated from each egg batch that have been fertilised, in order to get a good estimate of the fertilisation rate. However, this quantity depends on how accurate the estimate needs be. It you only need to see if it there has been fertilisation, then you can test with fewer eggs.

Treatment of eggs
After a certain number of hour-degrees (see table 3.1), take the required number of eggs aside and add AquaBoost® Quattro. Quattro contains components that denature proteins in cells while allowing chorion (egg membrane) to become more transparent. The treatment causes the cells in the center of the egg to become white. This process begins immediately, and the cells will become clearer after 5-10 minutes. Oocytes will be distinguished from unfertilised eggs in that cell division in a fertilised egg will be visible.

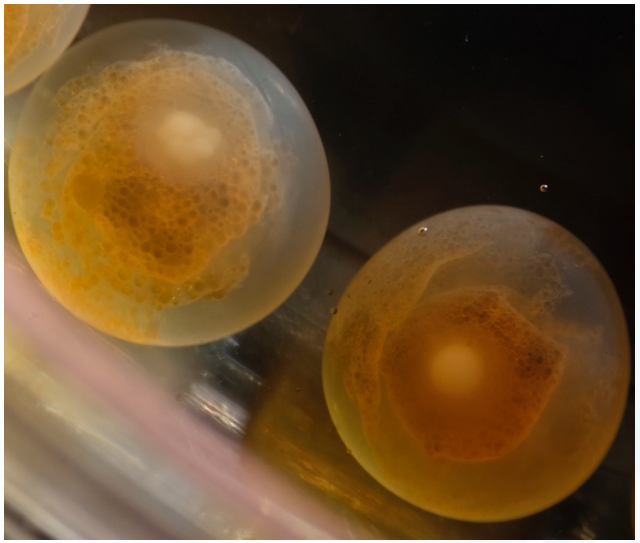


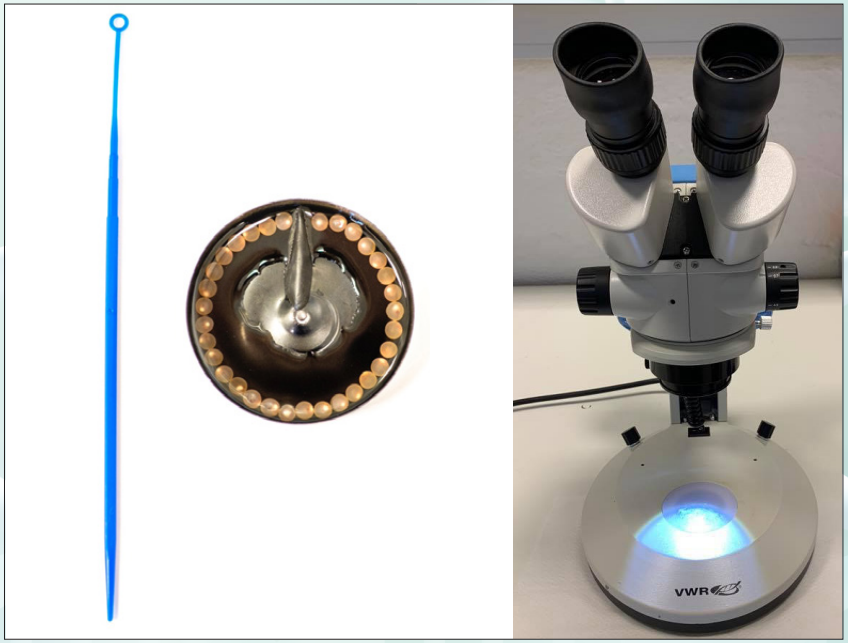
Figure 3.1: *Fertilised egg at the 8-cell stage (left) and an unfertilised egg (right). Treated with AquaBoost® Quattro after approx. 200 hours-degrees. Note that an unfertilised egg will have a triple round core, while the fertilised egg will have an irregular shape.*

Timing of the count

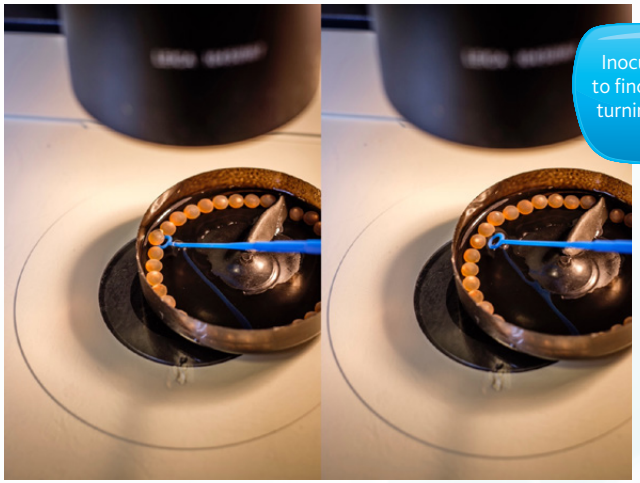
There will be a natural variation in the development pace between single eggs after fertilisation, so that some eggs will be at the 2 cells stage (1 cell division) and some at the 8-cell stage (4 cell divisions). **We recommend that assessment occurs when the majority of the cells are in the 4- and 8-cell stage.** This would allow enough time for all fertilised eggs have undergone at least one cell division. The downside is if you wait too long, the cell clump in the fertilised eggs and past 16-cells stage will have a nearly round shape and be very difficult to distinguish from oocytes.

Table 3.1.: *Hours-degrees and recommended incubation temperatures for salmonids.*

Species	Accumulated incubation temperature (hour-degrees)	Temperature °C	Hours
Atlantic Salmon *Salmo salar*	150-200	5	30-40
Rainbow Trout *Oncorhynchus mykiss*	75-100	5	15-20
Arctic Charr *Salvelinus alpinus*	150-170	5	30-34
Coho Salmon *Oncorhynchus kisutch*	120-160	8	15-20

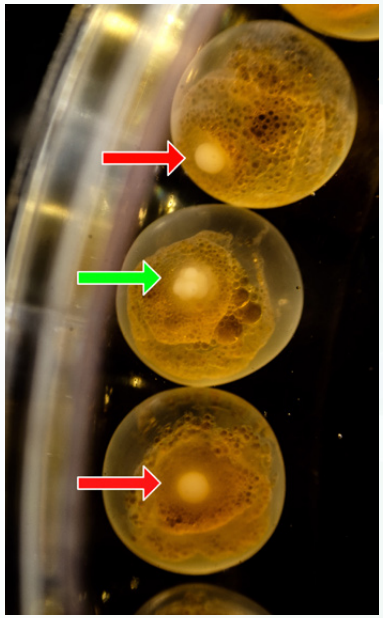


In the picture above you will see the equipment needed for counting. This includes a counting dish, an inoculating needle and a microscope with the possibility to adjust the direction and strength of the light source.



Inoculating needle to find blastodisc by turning around eggs.

Correct lighting is important in order to observe the shape of the cell nucleus. It should be downlit with a light source positioned preferrably on one side of counting bowl by a lamp.



In the picture above you will see eggs that are not fertilised (red arrow) versus fertilised (green arrow) eggs after about 200 hours-degrees.

Safety and waste management

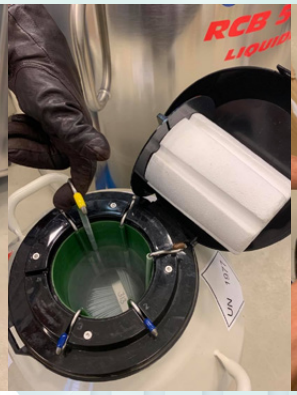
AquaBoost® Quattro can be irritating / corrosive to skin and eyes it is recommended to wear gloves and goggles during use. Eyes and skin coming into contact must be washed immediately with plenty of water. The used solution can be poured into a regular drain but requires to be rinsed with plenty of water.

4. Handling SquarePacks®

When working with cryopreserved samples: SquarePacks® or straws should **always remain immersed in liquid nitrogen** when being transferred from one container to another for use. They are extremely sensitive to temperature fluctuations and should never be held out in room temperature air before thawing.

It is important that the transfer between liquid nitrogen and water bath is done expeditiously. This is to avoid the thawing process to start whilst in the air, which could result in cellular damage.

1. Locate and identify the SquarePack® you are looking for, while the canister is still immersed in the liquid nitrogen.



2. Do not lift the cannister any higher than the bottom of the neck of the dewar, to avoid partial thawing.

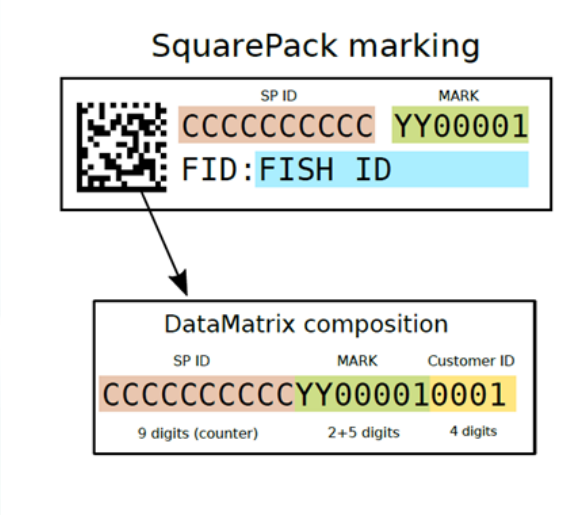
3. Grab carefully hold of the SquarePack® you need from the side of the package, and quickly move over to the thawing bath.

We highly recommend transferring the whole canister to a nitrogen bath, and work from here to choose SquarePacks® to thaws.

When cooled to -196°C, most non-metallic materials become very fragile. Thus, SquarePacks® and straws used to store cryopreserved milt is prone to being damaged during handling, such as packing and transfer of samples. Therefore, **do not** handle the SquarePacks®/straws or dewar flasks unnecessarily throughout the storage period. Any damage inflicted during storage in liquid nitrogen will usually go undetected until the samples are thawed for fertilisation and will only be apparent as milt leaks into the surrounding water, or a sudden explosion of the packaging because of heat stress during thawing (more common in straws). It is therefore important that protective eyewear and gloves are worn during the thawing process.

4.1 SquarePack® marking

Each SquarePack® will have printed IDs and a QR code, to identify each package.
FID (blue) refers to the Fish ID provided by you, whereas Mark (green) refers to an ID given to the fish by Cryogenetics. SP ID is a unique number given to each SquarePack®.



5. SDM6 Photometer

Important Information

VOLTAGE

The SDM6 photometer is delivered with an auto-switching universal type power supply that delivers 5 V DC, regardless of the input voltage (110~230 V AC).

FIRMWARE VERSION

The firmware version installed is shown on boot-up, the latest firmware per 2015 is 1.1F.

TOUCH INTERFACE

The SDM6 comes with a resistive touch screen for simple operation. Unlike capacitive touch screens that will only respond to bare skin, the SDM6 can be operated with stylus-like objects as well as gloved fingers.

CALIBRATION CURVES

Each instrument comes pre-installed with a calibration curve for Atlantic salmon (*Salmo salar L.*). Curves for other species are available on request from Cryogenetics AS for a nominal fee.

PRINTER

The SDM6 comes with a built-in printer for situations where a hard copy is useful.

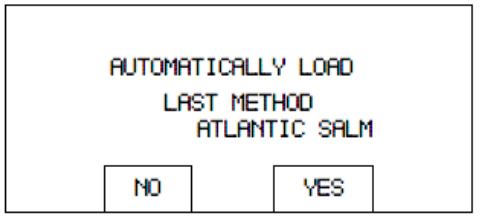
SPARE PARTS

Your SDM6 should need little to no maintenance. However, a spare light bulb is included. In case of failure, this can easily be replaced by the user. **Other spare parts are available from Cryogenetics AS.**

5.1 Startup

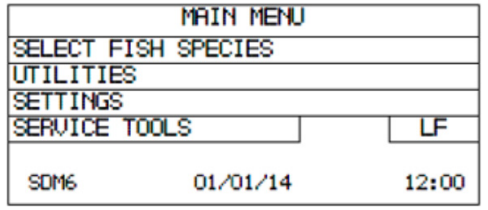
The SDM6 will load the previously used method by default. During startup, the user can choose to continue using this method or change to another species or mode of operation. Please note that the lamp needs to warm up for at least 15 minutes before you can start measuring the first zero sample

At the beginning of each spawning season. We recommend that the operator verifies that the correct mode of operation is selected by pressing NO on the first startup and then manually selecting the desired method. If the operator on the other hand knows which method was previously used, pressing YES is a quick shortcut to start measuring immediately.



The main menu

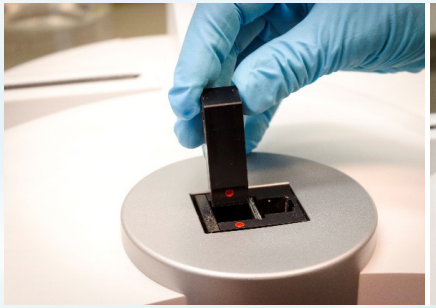
The main menu is reached by pressing NO on the start-up screen, then ESC when prompted for species. From the main menu the following options are available: User-accessible features are SELECT FISH SPECIES and UTILITIES, whereas SETTINGS and SERVICE TOOLS are password-protected. LF (line feed) is used to feed the printer paper one line at a time.



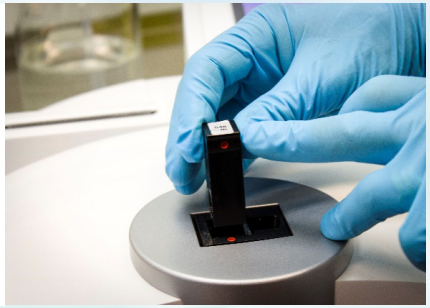
Dark level adjustment

The SDM6 needs to be calibrated before use. This is done by first doing a dark level adjustment, this should be performed every two weeks. Before adjustment, the black filter insert must be inserted with the label downwards into its chamber. From the main menu, go to UTILITIES, select DARK LEVEL ADJUSTMENT and press START. The reading will take a few seconds to complete. When completed, remember to turn the filter insert back to its normal position with the label facing up.

Dark level adjustment

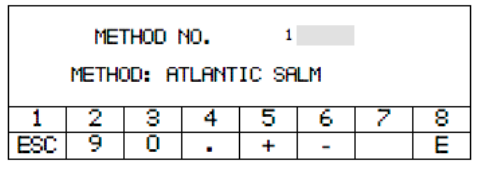


Filter position for measurement



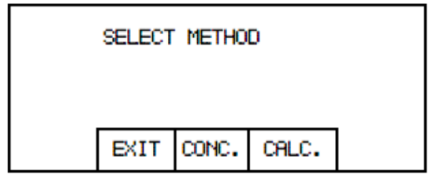
Select fish species

The SDM6 comes pre-installed with a calibration curve for Atlantic salmon (*Salmo salar* L.). This is installed as method number 1. Additional curves may be available, depending on the configuration ordered. Calibration curves for additional species can be installed later or even tailor-made on request. The method (species) is selected by typing in the species number (in this example, 1 for Atlantic salmon). The selection is confirmed by pressing E (enter).



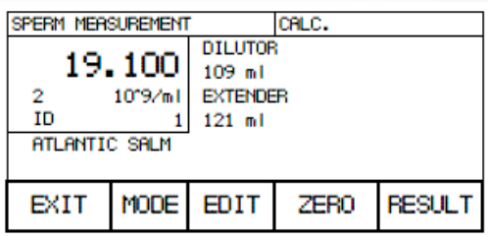
Mode of operation

Once a species is selected, the operator can choose to use the SDM6 in one of two modes, **concentration mode** or **calculation mode**. Depending on the intended use of the SDM6, a default mode of operation must be selected. This setting is automatically saved in memory and will be default mode on power-up. For routine sperm concentration measurements, concentration mode (CONC.) should be selected. For dilution of milt using AquaBoost® Dilutor, calculation mode (CALC.) should be selected. To return to the main menu, press EXIT.



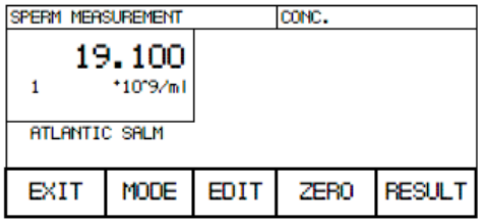
Concentration mode

In this mode, the SDM6 will estimate the sperm concentration in your sample, based on the selected calibration curve. The sperm concentration in your milt sample is given in billion (109) sperm per ml. Once a measurement has been made, the results can be printed by pressing MODE followed by PRN.



Calculation mode

When concentration measurements are done with the intention of either diluting the milt for storage or for immediate fertilisation, the Calculation mode will provide automatic calculation of volume of solution added. The user can separate values for AquaBoost® Dilutor or AquaBoost® Extender concentrations. When calculation mode is selected, the operator is prompted for ejaculate volume. Other fish information, such as FISH ID and motility can be used or left blank if not needed.



Daily use

The SDM6 needs very few adjustments in day-to-day use. The only calibrations that need to be done on a regular basis are:

1. Dark level adjustment (should be performed daily – see above)
2. Zero sample (required before each measurement series)

5.2 Maintenance

Storage and cleaning

We recommend following these guidelines when storing and cleaning the SDM6:

1. Store your SDM6 and accessories in a designated box/container in a dry environment at room temperature when not in use.
2. Clean the instrument by wiping it with a moist cloth and a non-corrosive detergent such as dish washing liquid.
3. Make sure there is an empty cuvette inserted in the measurement chamber when not in use to avoid dust, or other small particles, damaging the filter or light bulb.

Light bulb

The supplied light bulb has an expected life of at least 5000 hours, but non-ideal storage and working conditions might reduce this significantly. It is important to avoid extremes of temperatures and humidity to ensure the longest possible life span of your light bulb. Signs that the light bulb might be broken are:

1. Failure to zero the photometer
2. Negative zero or measurement values
3. Large variation in values on same sample

5.3 Preparation of 0.9% NaCl-solution

0.9 g. of NaCl is measured on the scale. Add 1 litre of distilled water and shake the bottle until it is dissolved. Fasten a dispenser to the bottle and flush the solution through several times. Make sure that the dispenser is set to the correct volume (i.e. salmonids = 4 ml). Mark the bottle with date for the preparation and change every month.

6. Gonad extraction of milt

Extraction of milt from gonads increases the efficiency and reliability of sperm collection. Instead of repeated stripping over several weeks, the male can be culled once maturation is reached, and sperm collected can be used for in fresh fertilisation or for cryopreservation purposes.

Gonad extracted milt using AquaBoost® SpermCoat can be standardised to ensure a quality-controlled process. After extraction, the sperm suspension can be treated as "normal" stripped sperm, and for instance be shipped to a facility for fertilisation or for cryopreservation off-site.

6.1 Materials and methods

We recommend the following equipment for a successful gonad extraction:

- Crushed ice or cold environment
- Sterile scalpels and/or clean scissors.
- Nitrile or vinyl gloves (NOT latex due to its spermicidic effect)
- Mechanical grinder *(provided by Cryogenetics)*
- Stainless steel sieve/strainer, and hairnet *(provided by Cryogenetics)*
- Scale
- Disposable plastic containers (approx. 1000 ml)
- Paper towels
- AquaBoost® SpermCoat. **Make sure it has the same temperature as the milt!**

For sperm concentration measurement the additional equipment is needed:

- Photometer with sperm concentration curve, e.g. the SDM6 from Cryogenetics
- Pipette, 10 µl volume

see more information in Chapter 5.

6.2 Biosecurity and hygiene

Best results are achieved when the gonad extraction is done in a hygienic manner, to avoid contamination of the sperm. Make sure to use clean equipment.

If your goal is to store gonad extracted milt over the span of several days, please contact Cryogenetics. We offer consultancy to assist you in making the most of your process.

6.3 Removing gonads

Bleed out the fish and hang the fish by the tail. Leave them head-down during the gonad removal procedure (Figure 6.1). Gonads should be excised from the abdominal cavity as soon as possible after they have bled out. Work in a cold environment (4-5°C) or keep on ice during the procedure. Avoid direct contact between gonad and ice due to the risk of water activation of the sperm cells!

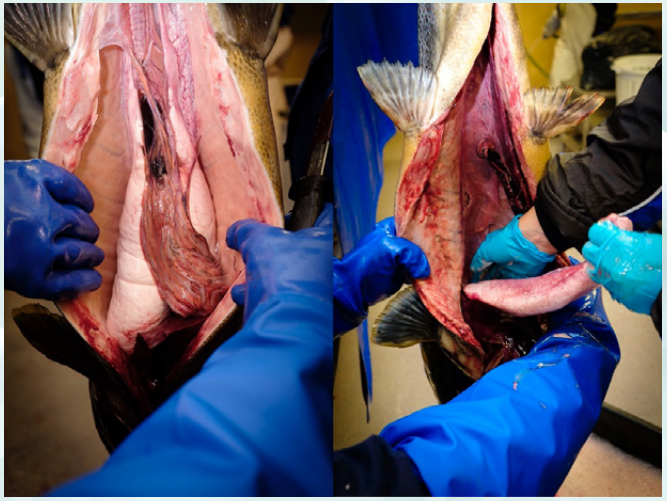


Figure 6.1 Gonads in a mature Atlantic salmon (Salmo salar L) male

6.4 Preparing the gonad

Depending on how well the fish is bled out, the internal and external blood vessels of the gonad will be more or less blood-filled. Visible blood should be removed by emptying the blood vessels thoroughly using a sterile scalpel and clean nitrile gloves. This procedure might have to be repeated since more blood may flow from vessels inside the gonad. Alternatively, if possible, you can remove the entire blood vessel using clean scissors or a scalpel (figure 6.2). The cleaned gonad should be dried lightly using paper towels.

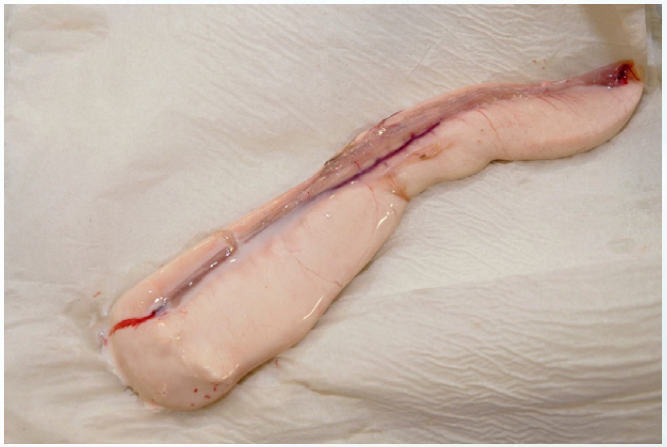


Figure 6.2 Removal of external membrane on a rainbow trout gonad. Use appropriate clean tools, disposable gloves and keep on ice (not shown). The central blood vessel should be emptied.

6.5 Homogenization

Use **simple mechanical grinders** (like the one we offer in Figure 6.3). These are disposed after use. Electric appliances such as blenders or mixers are not recommended due to the often-extensive cleaning needed, as well as risk of transferring disease, and unable to filtrate cell tissue off the homogenate. There is also a higher risk of heating up the gonad, contamination with lubricants, etc.

Figure 6.3 Disposable mechanical grinder.

Homogenise the gonad using the grinder, and cut the gonad up in smaller pieces (i.e. 3 – 6 pieces depending on the size). Collect the homogenate in a clean pre-weighed (tared) dry container and register the weight of the homogenate.

Add AquaBoost® SpermCoat at a ratio of 1 gram AquaBoost® SpermCoat per gram homogenate (ratio recommended for salmonids). **Make sure that AquaBoost® SpermCoat holds the same temperature as the milt!** Mix the solution by stirring with sterile, dry equipment, e.g., a plastic spoon. Leave for 10-15 min before filtering to make sure all sperm cells are released from the tissue.

Filter the homogenate through the metal sieves/strainer with a large mesh size first to remove gonad and membrane tissue. Then filter through a hairnet (Figure 6.4).

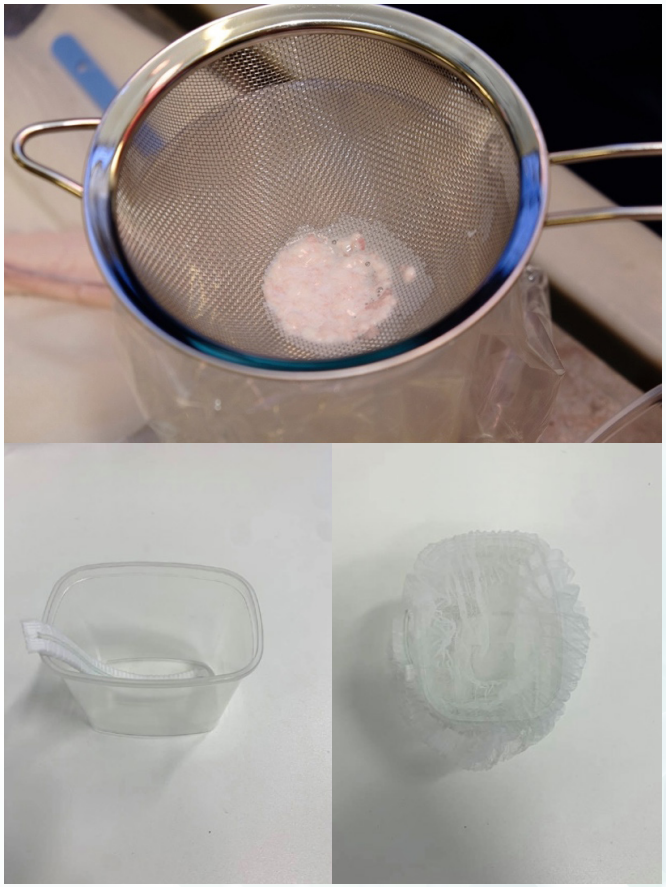


Figure 6.4 Double filtration of the homogenate through a coarse sieve (top) and subsequently fine (bottom) hairnet.

6.6 Storage conditions

The most important factor for good storage, is how hygienic you are while removing and handling the gonads, as well as temperature (see chapter 8).
We recommend storing milt in large cell culture flasks with vented caps (i.e. 650 ml). However, this depends on how much you need to store. See table below for how much milt can be stored per flask.

Size flask	Description
50 ml	Recommended for use up until 10 ml milt
250 ml	Recommended for use between 10 – 35 ml milt
650 ml	Recommended for use between 35 – 80 ml milt

The flasks containing milt should be stored in a cool environment (i.e. 1 – 3°C), but make sure temperature does not drop below 0°C causing the milt to freeze.

6.7 Using the gonad extract

Sperm concentration in the diluted homogenate may be determined using a pre-calibrated photometer, e.g. SDM6 from Cryogenetics (Figure 6.5).

Please note that microscopic evaluation of motility on gonad extracted milt can be misleading since AquaBoost® SpermCoat temporarily inhibits sperm motility. For the same reason, **AquaBoost® Activator must be used during fertilisation to reactivate the sperm and ensure optimal fertilisation conditions.**

Figure 6.5 The SDM6 photometer for measuring sperm concentration in fish milt.

The combination of gonad extraction, concentration measurement and dilution of the gonad extract with AquaBoost® Dilutor or AquaBoost® SpermCoat makes optimisation of the sperm-to-egg ratio possible. This way you can significantly increase the fertilisation potential of each male broodfish. **If you dilute the gonad extract with AquaBoost® Dilutor, make sure to use this within the same day!**

7. Fertilisation with cryopreserved milt

The following equipment is needed:

1. Forceps for handling the cryopreserved samples (25cm)
2. Thermostat-controlled water bath (15 L)
3. Digital timer
4. Sharp scissors
5. Cryo-gloves (leather or other insulated material)
6. Disposable gloves (vinyl/nitrile)
7. Protective eye wear
8. Paper towels
9. AquaBoost® Activator

AquaBoost® Activator should be prepared in advance to assure the appropriate temperature is held. This temperature should be as close as possible to the water holding temperature of the broodstock to minimise temperature shock during fertilisation.

Prepare the water bath with tap water and set the thermostat to 25 °C (± 0.5 °C). For the best results we recommend a thermostat-controlled water bath with a mechanical stirrer (provided by Cryogenetics). When large amounts of samples are thawed, the water in the bath should be changed frequently due to contamination during the process.

7.1 Thawing

Identify the milt container (straw or SquarePack®) in the dewar according to the inventory description/map provided.

Remember to COOL DOWN the pliers used for handling the SquarePack® in liquid nitrogen to avoid heat transfer before thawing.

Using the forceps, transfer the sample with a firm, but gentle grip straight from the liquid nitrogen and immediately into the water bath. It is important that the transfer between the liquid nitrogen and water bath is done expeditiously to avoid the thawing process to start whilst in the air, as this could result in cellular damage.

Once the sample is immersed into the water bath set the timer to 30 seconds and move the samples continuously within the water bath to maximise the heat transfer during thawing. Check and calibrate the water bath to keep a temperature of **25°C (± 0.5°C)**. A digital timer is also preferred, set it to **30 seconds.**

Figure 7.1 For best results we recommend a dedicated thawing bath and a digital timer.

For SquarePacks® we recommend moving your fingers in a lateral motion along the container (Figure 7.2). With a little bit of practice, several SquarePacks® can be thawed simultaneously. It is important that all SquarePacks® are separated once they are in the water, since they can stick together when thawing and cause a different thawing curve.

Figure 7.2 Thawing one SquarePack®. Move fingers in a lateral motion to ensure a correct thawing curve.

Figure 7.3 Thawing multiple SquarePacks®. Move fingers in a lateral motion to ensure a correct thawing curve, or by using a thawing rack

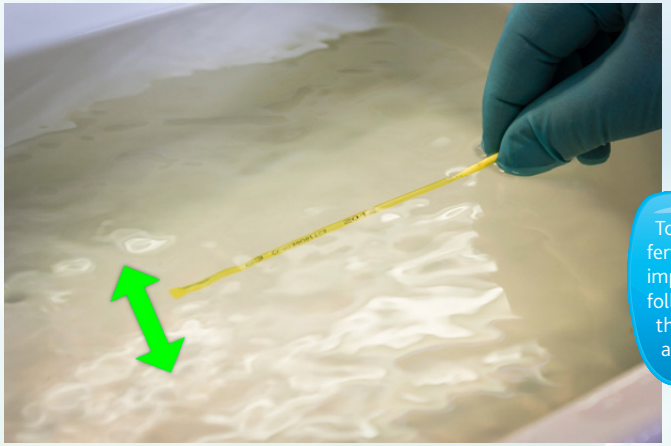


Figure 7.4 Thawing straws. Move the straw in a continuous motion by holding the end of the straw.

After exactly 30 seconds, remove the SquarePack®/straw from the thawing bath and wipe with paper towels. Quickly wipe the water off the SquarePack®/straw before opening. **DO NOT spill water into the fertilisation container before the milt is added!** Handle SquarePack®/straw by touching the edge or the ends to avoid heat transfer from your hands. Alternatively, drying the SquarePack® can be replaced by dipping the SquarePack® in saline water (4°C) for a couple of seconds. However, water should still be shaken of the exterior of the SquarePack®. Also, the milt should be used within 15 minutes after it has been thawed, if the SquarePack® is in a cold environment.

7.2 Fertilisation

7.2.1 Eggs

Between stripping and fertilisation, eggs must be kept moist in either ovarian fluid or physiological saline solution (0.9% NaCl). Before fertilisation, surplus liquid should be drained from the eggs. By minimising the liquid volume, the likelihood of a successful fertilisation will be increased.

Figure 7.6 Sufficient liquid volume for fertilisation – enough to keep eggs moist but still minimising the volume of fertilisation.

7.2.2 Fertilize

Both SquarePacks® and straws are opened by cutting the welded end of the container with scissors (Figure 7.7). The other end is sealed either with a cotton plug (straw) or sealed during manufacturing (SquarePack®).

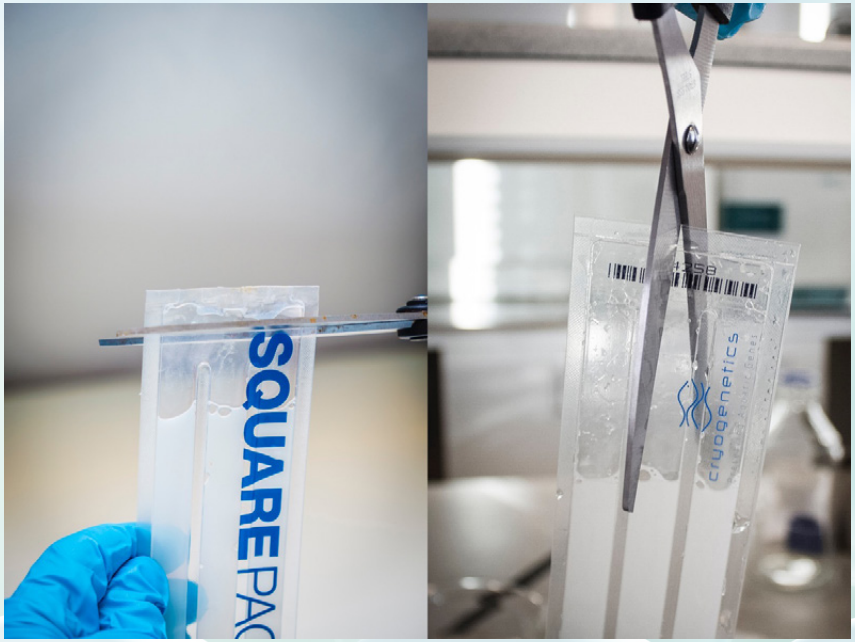


Figure 7.7 SquarePack® - Open the welded end by making a horizontal cut – pour the milt over the eggs and cut the other end to aid emptying.

Continue the process by following the description in chapter 2, AquaBoost® Activator.

8. Packing and shipment of milt

8.1 Temperature

Temperature is a key word that is mentioned in many of our protocols and standard operating procedures. It is very important that the temperature is monitored and set appropriately throughout the process, from harvest to cryopreservation to fertilisation, to ensure high quality treatment.

When preparing milt for shipment, make sure the ambient temperature does not increase above 5°C. We recommend using polyboxes (Styrofoam), with a layer of crush iced in the bottom of the box. The ice should then be covered in paper (e.g. newspaper), and the flasks on top. Then another layer of paper and ice on top. N.B. The flasks should not be in direct contact with the ice to avoid freezing.
During warmer seasons we recommend inserting cool packs with the crushed ice.

N.B. If the milt is shipped by airfreight, crushed ice must be filled in plastic bag. This to be approved as "wet cargo".

8.2 Packing

We recommend cell culture flasks to be used when shipping milt. These flasks come in three different sizes: all with a large internal surface area. This is important in order to grant sufficient access to oxygen during shipment.

Size flask	Description
50 ml	Recommended for use up until 10 ml milt
250 ml	Recommended for use between 10 – 35 ml milt
650 ml	Recommended for use between 35 – 80 ml milt

largest flask cannot be filled with more than 80 ml of milt. In these cases where one individual give more than 80 ml, we recommend using several flasks.

8.3 Labelling and documentation

Make sure to write down the fish ID on the flask using a waterproof sharpie or stick a label on to the flask with the ID. Additionally, make sure to label the Styrofoam box in which the flasks are packed, with number of flasks and fish ID.

Once your shipment is ready, make sure to email the list of males and number of flasks to Cryogenetics staff.

N.B.
For international shipment make sure to also provide Cryogenetics staff with a health certificate (i.e., Traces in EU) of the fish provided by your veterinarian, as well as the airway bill.

9. How to use RCB500 and/or RCB600

Working with larger nitrogen tanks require some practise.
These tanks are divided into 4 sectors that are colour coordinated:

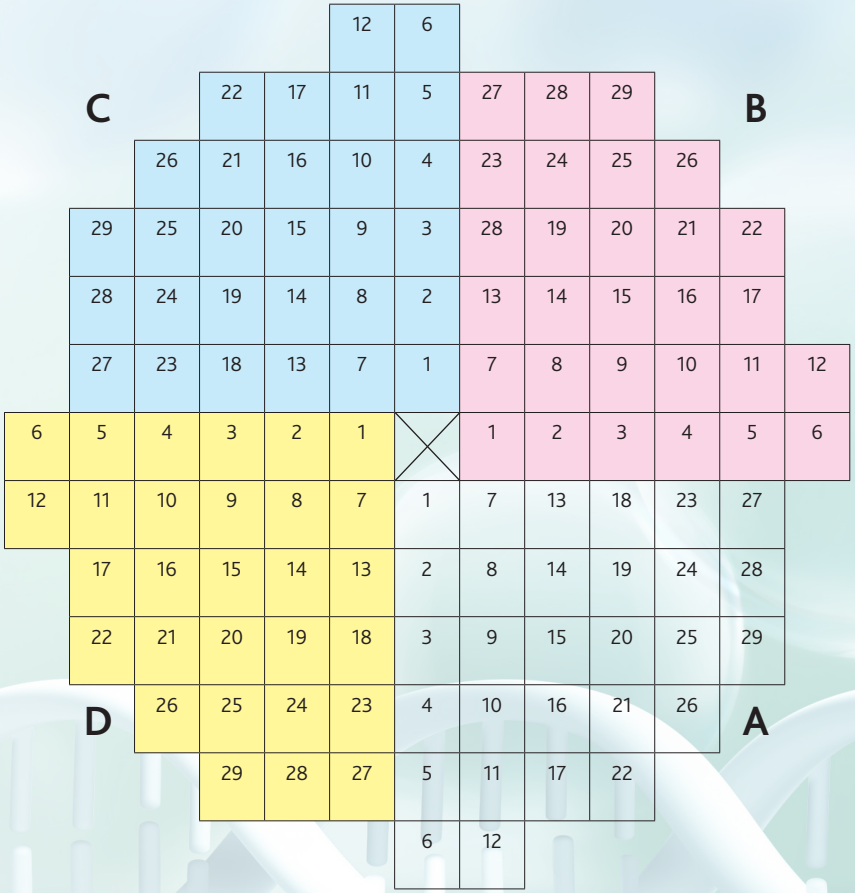
A = blank
B = Red
C = Blue
D = Yellow

Each sector has a maximum of 29 canisters, and each canister has two (i.e. RCB500) or three (i.e. RCB600) levels of SquarePack®. The bottom level is indicated as a, the next level up b, and so on. If straws are stored in the canister, the level can go all the way to h.

All locations of where the SquarePacks®/straws are placed will be provided to you, so you know where each sample is stored within the tank. This "location code" will describe to you in which sector (i.e., A, B, C, D), which cannister (i.e., 1, 2, 3…29), and what level (i.e., a, b, c…h) the biological material is stored (see 9.1. Map of the tank).

N.B.
It is important to have a nitrogen bath in close proximity while taking out the samples from the tank. Work from this bath to choose SquarePacks®/straws you want to thaw.

9.1 Map of the tank



Contact information:

Eli Sætersmoen
CEO
eli.saetersmoen@cryogenetics.com
+47 905 03 207

Inger Grevle
R&D Coordinator
inger.grevle@cryogenetics.com
+47 977 33 868

Steffen Wolla
Production Manager & Business Developer
steffen.wolla@cryogenetics.com
+47 468 48 755

Artur Zolotarev
Sales Manager
artur.zolotarev@cryogenetics.com
+47 468 49 487

Hamar Media avd. Nydal