# IVF Handbook

*We will make you an IVF expert!*

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# About this Handbook

This handbook describes our guidelines for handling, thawing and use of both cryopreserved and fresh 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.

Female egg quality is an important factor for successful IVF, but it can sometimes be a challenge to obtain good eggs for use. Not all females are fecund at any time, and on average 1/3 of females squeezed will have good eggs (whereas males will give sperm ~90% of the time). For this reason, it may be necessary to try different set up techniques to see which will work best in your facility. Also, it is recommended to set up more than one stock of females (different age, body condition) and set up extra females to make sure that good eggs can be found.

The purpose for this handbook is to provide information to assure proper protocols are followed.
However, it is highly recommended that extraction of milt, and in vitro fertilisation (IVF) is performed in collaboration with Cryogenetics´ staff the first time. Therefore, please do not hesitate to contact us should you have any questions.

# AquaBoost® OvaCoat

**AquaBoost® OvaCoat is a product that is used to preserve egg quality after stripping and to improve results of zebrafish IVF. AquaBoost® OvaCoat is recommended for fertilisation with both fresh and cryopreserved sperm.**

**This product must be kept refrigerated, but can be stored in room temperatures for shorter periods. Open bottles should always be stored refrigerated and used within 24 hours of opening.**

*User instructions:*

Before stripping the female, prepare petri dishes for IVF by adding approximately 100 µl (2 drops) of AquaBoost® OvaCoat in each dish. Make sure the AquaBoost® OvaCoat holds the same temperature as the eggs. Eggs from one female will need approximately 100 µl AquaBoost® Ova Coat.

Use a soft paintbrush when handling the eggs!

Eggs can be kept in AquaBoost® OvaCoat for up to 30 minutes before fertilisation. When you are ready to fertilise, gently remove excess AquaBoost® OvaCoat, if needed, with a sterile pipette; be careful not to touch the eggs. It is important not to have too much AquaBoost® OvaCoat to ensure proper contact between sperm and eggs.

Add sperm and activator/water, approximately 200 µl per female. Wait 2-5 minutes before filling the dish with water and place for incubation.

**Note that AquaBoost® OvaCoat will activate the sperm!**

**It is important that both eggs and AquaBoost® OvaCoat are present before sperm is added.**

# AquaBoost® SpermCoat – for zebrafish

AquaBoost® SpermCoat for zebrafish is a sperm extender from Cryogenetics. The product is for use on fresh sperm giving the possibility to dilute the sperm and pool sperm from several males prior to IVF.

**This product is NOT a cryoprotectant solution for cryopreservation.**

AquaBoost® SpermCoat must be stored refrigerated and has an expiration of 6 months. Opened bottles should be used within 24 hours.

# Fish Set Up

Females can be identified by looking for a yellow dorsal fin and round, white belly. Fecund females sometimes have transparent skin colouration around the oviduct, which may protrude slightly.

**Set up option 1**

Females can be sorted and separated from males the day before IVF and kept overnight on running system water. Density should not exceed 5 fish per litre. Do not feed after separation!

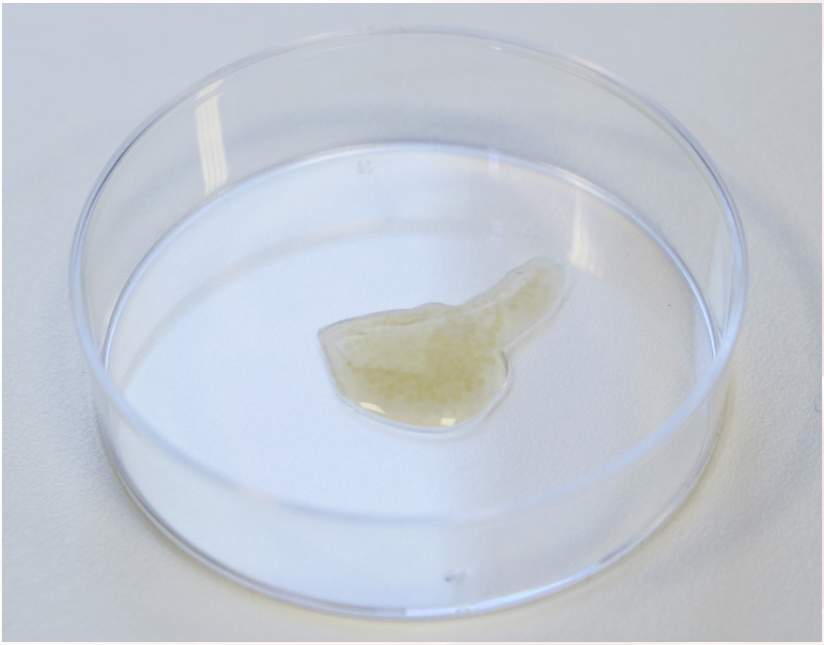
**Set up option 2**

Females can be "primed" by setting up breeding cages with a divider in place and males on the opposite side of the divider. Place 3 females and 2 males with a divider in a 1 litre breeding cage. Set up multiple cages this way. Keep in static water overnight and do not feed. Remove females the next day at the time of squeeze. It is not necessary to pull the dividers to allow males in with the females as this may cause premature egg release.

# Collection of Eggs.

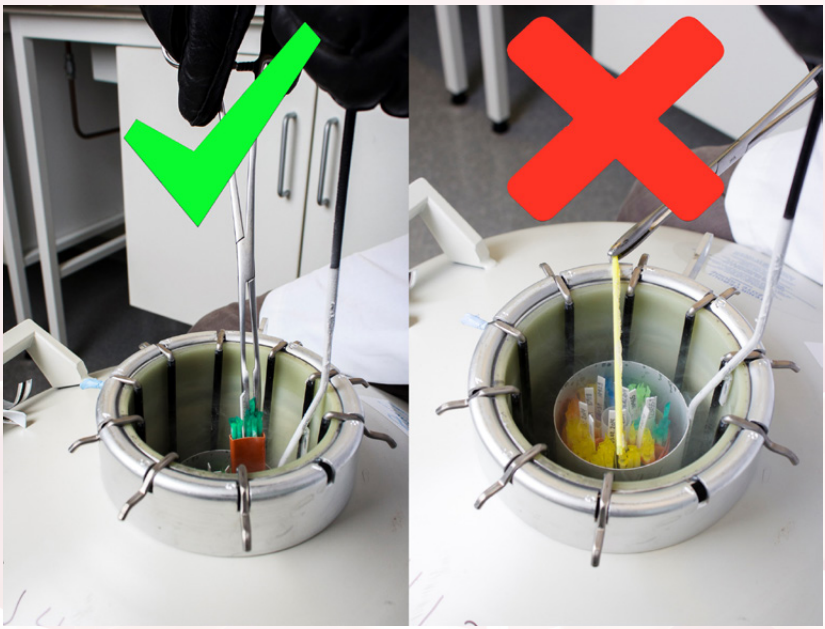
Eggs should be squeezed within 3 hours after the lights are turned on in the fish room. Good eggs are yellowish in colour and transparent with no opaque material present. The fluid around the eggs should also be transparent. Bad eggs should also be transparent or appear white and broken down. Clutches containing a mixture of good and bad eggs should not be used. The chorions of freshly stripped eggs are very fragile, so it is best to keep the handling of eggs to a minimum and use a soft brush only.

See section *AquaBoost® OvaCoat* for further description.



# *In vitro* Fertilisation with Cryopreserved Sperm

When working with cryopreserved samples: 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.



*How to correctly handle and identify cryopreserved milt straws by NOT lifting them above the neck of the dewar (left). Lifting straws above the neck of the dewar (right) leads to partial thawing and could mean poor fertilisation results.*

# Materials:

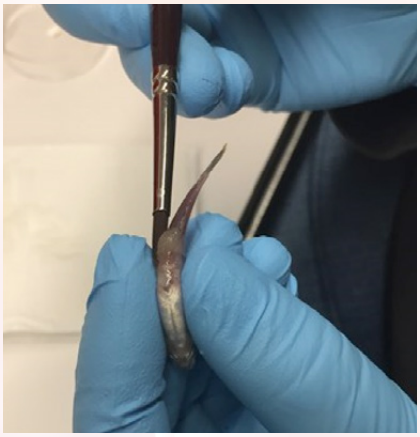
1. Scissors.
2. Tongs.
3. Cryogloves.
4. Timer set to 12 seconds.
5. Timer set to 5 minutes.
6. Temperature probe.
7. Petri dishes.
8. Spoon.
9. Paper towels.
10. Crystallising dish.
11. Paint brush.
12. Plastic plunger.
13. Syringes (1 per line).
14. Adapters (1 per line).
15. Filter forceps.
16. Liquid nitrogen.
17. Large Styrofoam cooler for liquid nitrogen bath.
18. Safety goggles.
19. AquaBoost® OvaCoat, room temperature during use.
20. 2 fish tanks partially filled with fish water (one for recovery, one for rinse).
21. Small Styrofoam cooler for water bath (or use programmable bath set to 38˚C if available).
22. Fish water or embryo medium in a squirt bottle (Embryo Medium containing Methylene Blue recommended).
23. 4.0g/L MS-222, Tricaine Stock Solution (pH 7)
    Westerfield, M. (2000). The Zebrafish Book. A guide for the laboratory use of zebrafish (Danio rerio). 4th ed., Univ. of Oregon Press, Eugene.
    *Note: To make solution of Tricaine, use 4.2 ml Tricaine Stock Solution and add to 100 ml Fish water. Mix in crystallising dish.*

# Thaw and IVF Procedure

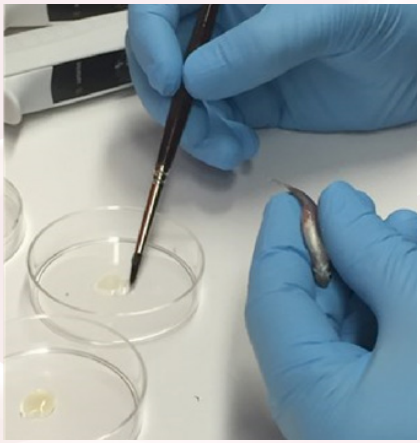
1. Fill 1 ml syringe (take adapters off) with 200 µl air, 200 µl water, 200 µl air. Fill as many syringes as necessary to match the number of straws you will be thawing (see picture 1).
2. Connect adapters to syringe and set aside for later.
3. Prepare 38˚C water bath and keep temperature probe in place to monitor temperature. A small Styrofoam cooler with a lid can be used as a water bath, adjust as needed with warm water.
4. Transfer the desired frozen straws into a bath of liquid nitrogen in a large Styrofoam cooler and bring to work area.
5. Place approximately 100 µl of AquaBoost® OvaCoat in a petri dish, use one petri dish for each straw being thawed.
6. Anesthetise females with MS-222, Tricaine working solution until gill movement slows down.
7. Remove one female with a spoon and rinse in fish water in the rinse tank.

8. Dry female by rolling gently front to back on a small stack of paper towels.
9. Fingers should be damp but not wet. Place female belly up between thumb and index finger.
10. Squeeze female gently to expel eggs and place eggs on a paintbrush with Aqua Boost® OvaCoat on (see picture 2).
11. Place female into recovery tank.
12. Dip paint brush in AquaBoost® OvaCoat in one petri dish to remove the eggs from the brush. Eggs are very delicate and should be handled with extreme care (see picture 3).
13. Repeat to combine good eggs from multiple females if larger numbers of fertilised eggs are desired.
14. Before adding the sperm gently remove excess AquaBoost® OvaCoat from eggs to ensure the sperm cells will get access to the eggs. Pipet tips or Kim wipes should be used.
15. Remove straw from liquid nitrogen bath with frozen tongs and drop immediately into 38˚C water bath, and time for 12 seconds on a timer while moving straws continuously with tongs.
16. Remove straw with tongs and dry straw thoroughly with paper towels.
17. Use scissors to cut straw on both ends and slide out metal weights. Retrieve the inner micro straw. Use plastic plunger to push micro straw out if necessary.
18. Cut one end of micro straw and place in the syringe adapter (with air, fish water, air) (see picture 4).
19. Cut the other end of micro straw while in a perpendicular angle.
20. Use syringe to expel the 200 µl sperm mixture and fish water into the clutch of eggs all at once. This will activate the sperm and eggs.
21. Time for 5 minutes after activation.
22. When timer sounds, fill the petri dish with half full of fish water from squirt bottle.
23. Keep eggs at 28˚C.
24. Repeat step 1 – 24 for additional fertilisations.
25. When fertilisations are complete, transfer 50 – 100 eggs to larger petri dishes and add additional fish water or embryo medium.
26. After 2 – 3 hours, fertilisation can be assessed, and unfertilised eggs can be removed.
27. Take good care of the embryos the first few days after fertilisation and remove any bad eggs. Replace the embryo medium daily.

***Picture 1:***
*Fill 1 ml syringe with 200 µl air, 200 µl fish water, 200 µl air.*



***Picture 2:***
*Squeeze female gently to expel eggs and place eggs on paintbrush.*



***Picture 3:***
*Dip paintbrush in AquaBoost® OvaCoat in a petri dish to remove the eggs from the brush.*

***Picture 4:***
*Place thawed microstraw in syringe adapter.*

# In vitro Fertilisation with Fresh Sperm

This chapter describes Cryogenetics´ guidelines for IVF using AquaBoost® SpermCoat.

Preparation for IVF

## Materials

1. Paper towels.
2. Spoon.
3. Sponge fish holder.
4. Filter forceps.
5. Capillaries.
6. Aspirator tube assembly or Micro-classic pipette controller.
7. Petri dishes.
8. Timer set to 5 minutes.
9. Paintbrush.
10. AquaBoost® OvaCoat, room temperature during use.
11. AquaBoost® SpermCoat (keep on ice).
12. Microcentrifuge tubes (labelled 1, 2, 3, etc. for each line and keep track).
13. Crystallising dish for Tricaine.
14. P10 Pipetteman set to 3 µl, and tips.
15. P200 Pipetteman set to 200 µl, and tips.
16. Fish water in beaker.
17. Styrofoam cooler filled with ice.
18. Fish water or embryo medium in squirt bottles (Embryo Medium containing Methylene Blue recommended).
19. Two fish tanks partially filled with fish water (one for recovery, one for rinse).
20. Dissecting microscope with direct illumination.

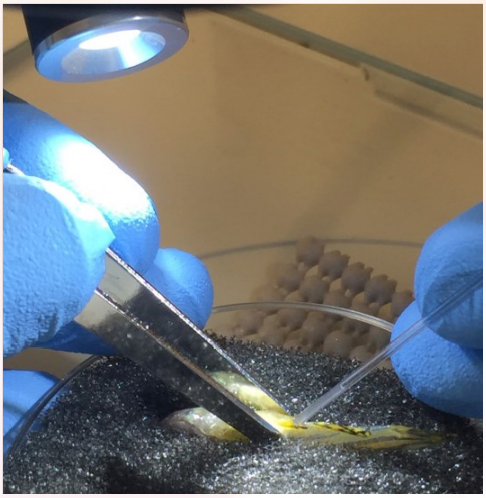
21. 4.0g/L MS-222, Tricaine Stock Solution (pH 7)
    Westerfield, M. (2000). The Zebrafish Book. A guide for the laboratory use of zebrafish (Danio rerio). 4th ed., Univ. of Oregon Press, Eugene.

    *Note: To make solution of Tricaine, use 4.2 ml Tricaine Stock Solution and add to 100 ml Fish water. Mix in crystallising dish.*

**Collection of sperm and IVF should be performed within the first 3 hours after the lights come on in your fish facility.**

## Sperm Collection

1. Anesthetise males with 4g/L MS-222, Tricaine working solution until gill movement slows down.
2. Remove one male with spoon and rinse in fish water rinse tank.
3. Dry male by rolling gently front to back on a small stack of paper towels.
4. Place the male in the sponge fish holder with the belly up, and place under dissecting microscope with direct lighting.
5. Attach capillary to aspirator tube assembly or Micro-Classic pipette controller.
6. Looking through the microscope, move pelvic fins away from the urogenital opening with the tip of the capillary.
7. Place capillary on the urogenital opening.
8. Collect sperm in capillary by applying gentle pressure with forceps on each side of fish. Use minimal pressure to aspirate up into capillary (see picture 1).
9. If white sperm is collected, dilute to 10 µl with cold AquaBoost® SpermCoat (10 µl is marked on capillaries with black line)(see picture 2).
10. Expel mixture into 0.5 ml microcentrifuge tube.
11. Add sperm from additional males from the same line, diluting each to 10 µl AquaBoost® SpermCoat.
12. When finished collecting sperm, the mixture should be cloudy white in colouration.
13. Repeat in additional labelled 0.5 ml tubes for each line and keep on ice.

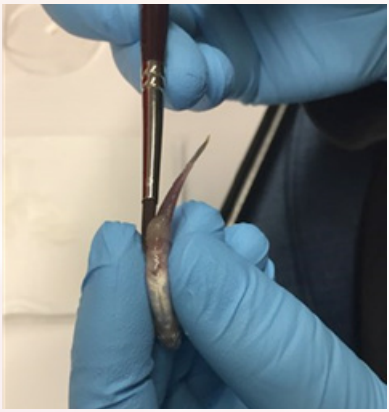


***Picture 1:***
*Collect sperm in capillary by applying gentle pressure with forceps on each side of the male.*

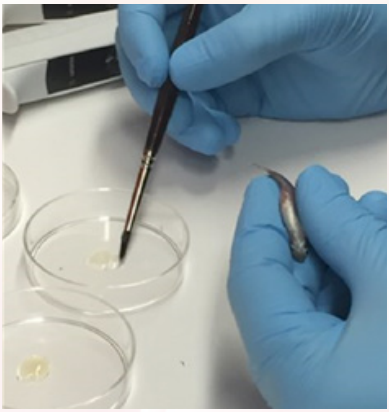

***Picture 2:***
*Dilute to 10 µl with cold AquaBoost® SpermCoat.*

# Egg Collection and IVF Procedure

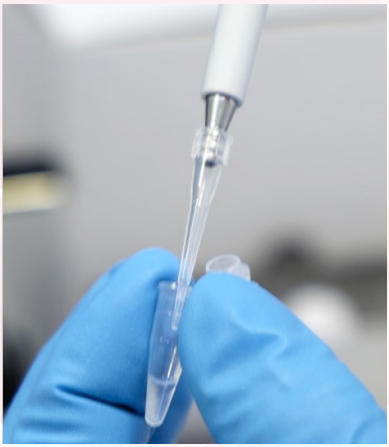
1. Place approximately 100 µl of AquaBoost® OvaCoat in a petri dish, use one petri dish for each straw being thawed.
2. Anesthetise females with MS-222, Tricaine working solution until gill movement slows down.
3. Remove one female with a spoon and rinse in fish water in the rinse tank.
4. Dry female by rolling gently front to back on a small stack of paper towels.
5. Fingers should be damp but not wet. Place female belly up between thumb and index finger.
6. Squeeze female gently to expel eggs and place eggs on a paintbrush with Aqua Boost® OvaCoat on (see picture 3).
7. Place female into recovery tank.
8. Dip paint brush in AquaBoost® OvaCoat in one petri dish to remove the eggs from the brush. Eggs are very delicate and should be handled with extreme care (see picture 4).
9. Repeat to combine good eggs from multiple females if larger numbers of fertilised eggs are desired.
10. Before adding the sperm gently remove excess AquaBoost® OvaCoat from eggs to ensure the sperm cells will get access to the eggs. Pipet tips or Kim wipes should be used.
11. Mix final sperm mixture by pipetting up and down a few times.
12. For fertilisation, pipette 3 µl of the final sperm mixture in AquaBoost® SpermCoat and expel into the clutch of eggs (see picture 5).
13. Immediately pipette 200 µl of fish water into the clutch of eggs to activate sperm (see picture 6).
14. Time for 5 minutes after fish water is added.
15. After 5 minutes, fill the dish half way up with fish water or embryo medium.
16. Keep eggs at 28˚C.
17. Repeat steps 1 – 16 for additional fertilisations.
18. When fertilisations are complete, transfer ~50 – 100 eggs to larger petri dishes and add additional fish water or embryo medium.
19. After 2 – 3 hours, fertilisation can be assessed, and unfertilised eggs can be removed.
20. Take good care of the embryos the first few days after fertilisation and remove any bad eggs. Replace the embryo medium daily.



***Picture 3:***
*Squeeze female gently to expel eggs and place eggs on paintbrush.*

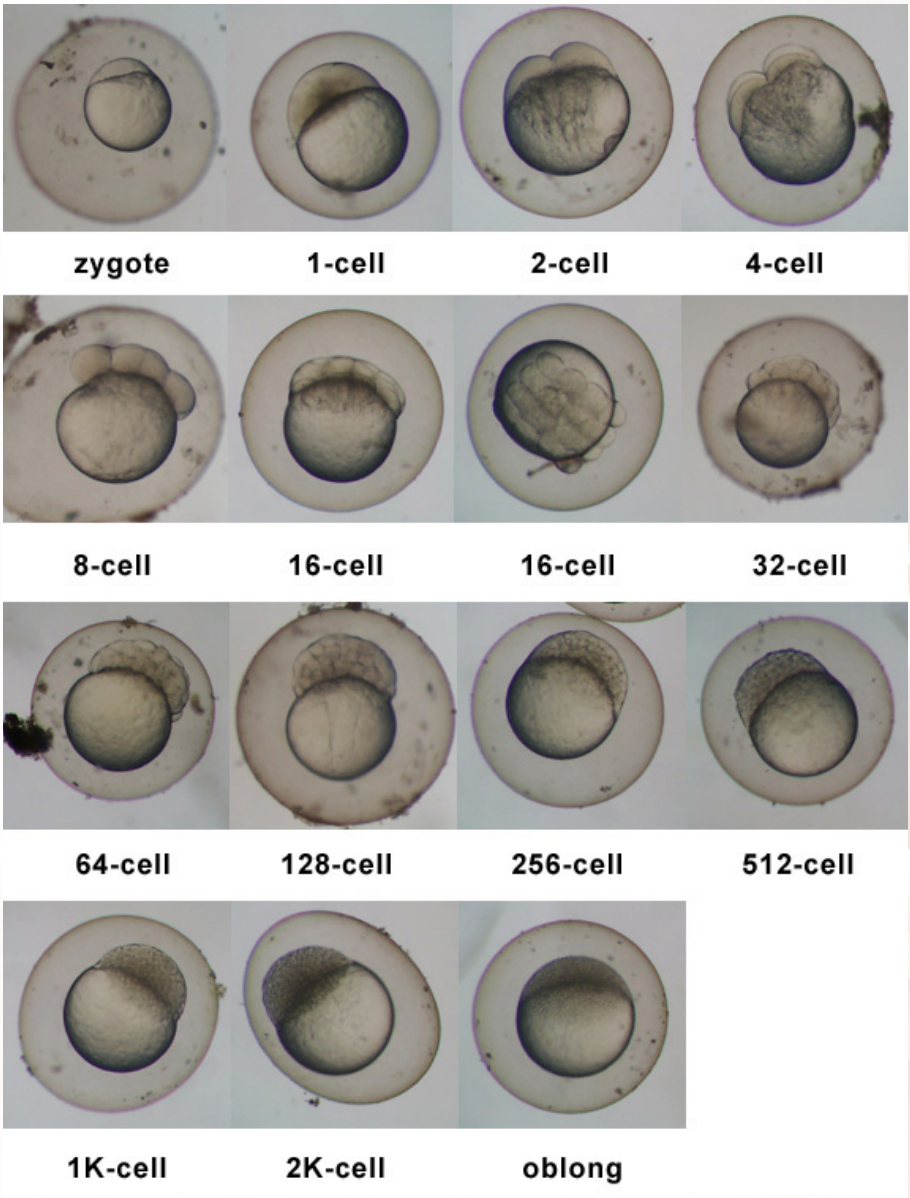


***Picture 4:***
*Dip paintbrush in AquaBoost® OvaCoat in a petri dish to remove the eggs from the brush.*



***Picture 5:***
*Pipette 3 µl of the final sperm mixture in AquaBoost® SpermCoat and expel into clutch of eggs.*

***Picture 6:***
*Immediately pipette 200 µl of fish water into clutch of eggs to activate sperm.*


zygote
1-cell
2-cell
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8-cell
16-cell
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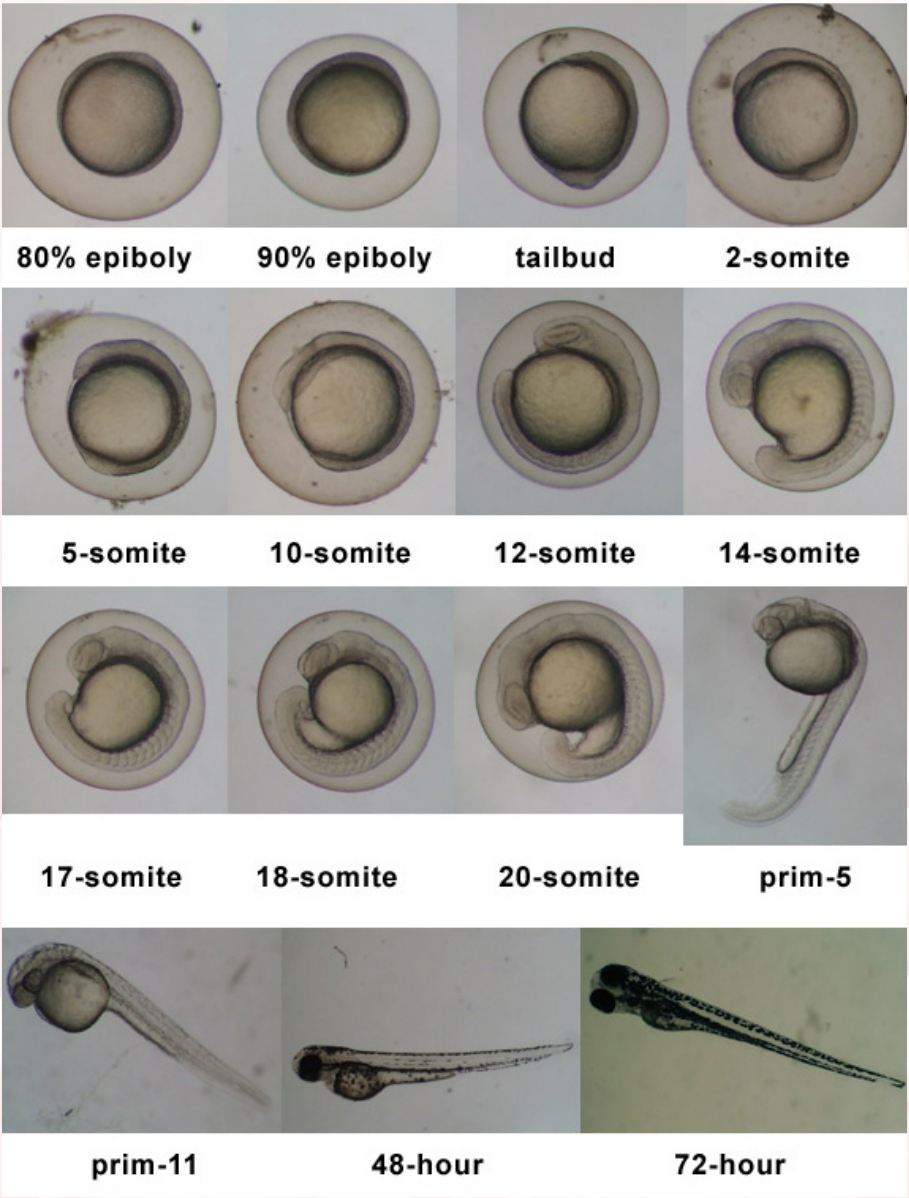
32-cell
64-cell
128-cell
256-cell
512-cell
1K-cell
2K-cell
oblong


80% epiboly
90% epiboly
tailbud
2-somite
5-somite
10-somite
12-somite
14-somite
17-somite
18-somite
20-somite
prim-5
prim-11
48-hour
72-hour

Do you have any questions regarding the IVF procedure or the collection of sperm and eggs, please do not hesitate to contact us.

We want you to achieve the best IVF possible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