IVC System

Blastocyst *In Vitro* Culture Media

Version 1.1

Components of culture media

The blastocyst *in vitro* culture protocol requires the purchase of two products: IVC-1 and IVC-2. When used in conjunction, these media can allow the *in vitro* growth and development of blastocysts beyond the implantation stage, allowing clear observation and imaging of embryo development and attachment.

Product	**Catalogue No**	**Storage**
IVC-1, 6 ml (4 x 1.5 ml)	M11-6	-20°C
IVC-1, 25 ml (5 x 5 ml)	M11-25	-20°C
IVC-2, 6 ml (4 x 1.5 ml)	M12-6	-20°C
IVC-2, 25 ml (5 x 5 ml)	M12-25	-20°C

Additional reagents

1. Phosphate-buffered saline (PBS), without calcium or magnesium
2. Mineral oil, suitable for mouse embryo culture (e.g. Sigma, cat. no. M8410)
3. Acidic Tyrode's solution (e.g. Sigma, cat. no. T1788)
4. M2 medium (e.g. Sigma, cat. no. M7167)

Use with ibiTreat µ-plates, 8-well (Ibidi, cat. no. 80826) is also recommended to provide optimum conditions for embryo attachment.

Storage conditions

To minimize freeze-thaw cycles IVC-1 and IVC-2 have been aliquoted into individual 1.5 ml or 5 ml volumes. When not in use the media should be kept at -20°C.

After thawing, these working aliquots can be kept at 4°C for up to 14 days.

The media should be kept in an incubator for a minimum of 30 minutes before use to allow pH equilibration.

Protocol

1. Blastocyst recovery from mice at E3.5

This is one method of recovering and treating embryos. Recovering the embryos at this stage and removing the zona pellucida through the use of Acide Tyrode's solution greatly increases the chance of embryo attachment.

1.1 Impregnate 5-6 week old female mice by superovulation as described in detail in Bedzhov et al, 2014[1].

1.2 Humanely cull pregnant mice at 3.5 days *post coitum* (d.p.c) by cervical dislocation.

1.3 Cut the skin and peritoneum to reveal the abdominal organs, pull back the organs and locate the ovary, oviduct and uterus underneath the fat pads.

1.4 Dissect and collect the reproductive organs in one intact piece (ovary, oviduct and two uterine horns). Transfer to 1.5 ml microcentrifuge tube filled with pre-warmed M2 medium.

1.5 Place the tissues into a 35 mm petri dish filled with pre-warmed M2 medium. Under a dissecting microscope, remove the fat pads and cut off the oviduct and ovary.

1.6 Using a 27 gauge needle attached to a 1 ml syringe filled with pre-warmed M2 medium, insert into one uterine horn and flush out the embryos with approximately 0.5 ml of M2. Flush again to release any remaining blastocysts. Repeat this step with the other horn.

1.7 Transfer the blastocysts using a mouth pipette into 10 µl drops of pre-warmed M2 medium covered with mineral oil.

1.8 Passage the embryos through several drops to wash away any residual uterine debris. Keep the plate protected from light on a heating stage at 37°C.

2. Removal of zona pellucida by Acide Tyrode's (AT) solution

2.1 Place 3 large drops (about 30 µl) of AT solution and at least 3 large drops of M2 medium in a 35 mm petri dish.

2.2 Under a dissecting microscope, transfer a group of 25-30 blastocysts into one of the M2 drops. Expunge (away from the drop) the remaining M2 medium in the mouth pipette and refill it with AT solution.

2.3 Transfer a group of 10-15 embryos into the first drop of AT solution, avoiding letting the blastocysts touch the bottom of the dish. Pipette up and down and transfer into the second drop, repeat and then transfer to the third. Observe the zona pellucida gradually disappearing.

IMPORTANT POINT: Minimize the M2 medium content while the embryos are transferred into the AT drops, since the buffers present in the M2 medium neutralize the AT solution. If a zona-free blastocyst comes into contact with the plastic dish in AT solution it may adhere tightly and be difficult to remove without damage.

2.4 Place the zona-free blastocysts into a drop of M2 to wash off and neutralize the AT solution.

2.5 Transfer the embryos in fresh drops of M2 covered with mineral oil. Keep the plates protected from light on a heating stage at 37°C.

3. Culturing embryos recovered at E3.5

3.1 Prepare a 35 mm petri dish with 3-6 drops of 20 µl IVC-1 medium under mineral oil and leave in the incubator for a minimum for 30 minutes for the pH to equilibrate. The medium will be used in a final step to wash away the M2 medium before transferring embryos to the µ-plates for subsequent culture.

3.2 Pipette 250 µl IVC-1 into each chamber of the 8-well µ-plate and place in the incubator for equilibration.

IMPORTANT POINT: Each well needs to be filled with medium even if embryos are not cultured in some of the wells. If this is not observed, micro-cracks can form in the thin bottom of the plate, due to a difference in the temperature of the optical plastic lining.

3.3 Wash the embryos in the M2 medium by transferring them into the previously prepared petri dish containing drops of IVC-1 medium, then immediate place the embryos in µ-plate wells containing IVC-1 medium.

Embryos can be cultured individually or in groups of up to 15 blastocysts per well.

IMPORTANT POINT: Embryo culture must be carried out in a humidified incubator in an atmosphere containing 5% CO_2 at 37°C.

3.2 After one day of culture, the embryos should form an expanded blastocyst. Since embryos are not yet attached to the plate, they may passively move and cluster in the center of the well. Using a mouth pipette, pick embryos individually and place them in lateral positions of the well to increase the distance between them.

IMPORTANT POINT: Once embryos attach to the plate, they cannot be moved.

3.3 Between day 2 and 3 of *in vitro* culture, the mural trophectodermal cells should differentiate into trophoblast giant cells (TGCs). As the embryos flatten and stably attach to the plate, the inner cell mass (ICM) remains as a centrally positioned clump surrounded by spreading TGCs.

3.4 When all of the embryos in the well are stably attached to the bottom of the plate, change the IVC-1 for equilibrated IVC-2 medium.

3.5 In the following days, early egg cylinders should emerge from the ICM clumps. These egg cylinders lack the parietal endoderm and Reichert's membrane that normally surround the embryo *in vivo*. Therefore, the visceral endoderm (VE) should be clearly distinguishable as an outer cell layer. As the embryos grow, a gradually expanding proamniotic cavity should become visible.

4. Treatment of blastocyst recovered at E4.5

This is the alternative method of recovering and treating embryos. Removal at the E4.5 stage maximises time of the embryos *in vivo* and can increase the efficiency of egg cylinder formation and shorten the length of *in vitro* culture. However, the efficiency of this procedure relies on the accuracy of how it is performed, depending heavily on manual dexterity.

Before sacrificing the animal, prepare plates for IVC culture as detailed in the previous section.

4.1 Superovulate mice and recover blastocysts from 4.5 d.p.c pregnant females by following steps 1.1- 1.8.

4.2 Prepare a new dish for surgical cutting of embryos. Using the lid of the 25 mm petri dish make 2 elliptical drops of M2, do not cover with mineral oil. One drop should be used to clean the pipette free of oil residue from the M2 dish, and the second to perform cutting.

4.3 Transfer one embryo into the first drop. Submerge the pipette and drag it along the drop to remove the mineral oil residue.

IMPORTANT POINT: M2 medium not covered with mineral oil is prone to evaporation. Do not use the dish for embryo surgery for longer than 10 minutes to avoid pH changes and evaporation of medium. After 10 minutes prepare a fresh dish and continue.

4.4 Transfer one embryo into the second drop. Remove the mural trophoectoderm (TE) by placing a cutting pipette at the junction between the inner cell mass and the blastocoel, then pressing down gently.

If the mural TE does not separate from the embryo, slide the cutting pipette sideways and repeat until complete separation is achieved. Alternatively use the edge of the transfer pipette as a knife and slide it along the cutting pipette on the site of the blastocoel. This will cut off the remaining mural TE.

IMPORTANT POINT: M2 medium not covered with mineral oil is prone to evaporation. Perform the procedure as swiftly as possible; ideally no more than 30 seconds per embryo.

4.5 Transfer the dissected embryo to the first drop of M2 on the bottom row of the petri dish, discard the remaining mural TE.

4.6 Repeat for each embryo, washing each in one in the remaining drops of M2 after cutting.

IMPORTANT POINT: The embryo will lose its elongated shape within 20 minutes as the cavity of the zona-free embryos becomes spherical. As the surgery can be carried out with greater precision on elongated embryos, the whole procedure should be performed in less than 20 minutes. If the blastocoel cavity collapses, the embryos in the M2 drop can be cultured in the incubator for several hours and after the cavity re-establishes it can be used for cutting.

5. Culturing embryos recovered at E4.5

5.1 Use the prepared petri dish containing IVC-1 drops. Wash out the M2 medium by transferring the embryos through all drops as described in step 3.3.

Embryos can be cultured individually or in groups of up to 15 blastocysts per well.

5.2 Transfer the embryos to a prepared µ-plate containing IVC-1 medium, as previously described, for further culture.

IMPORTANT POINT: Embryo culture must be carried out in a humidified incubator in an atmosphere containing 5% CO_2 at 37°C.

5.3 The cut embryos should attach to the bottom of the dish within 12 hours. After 12-24 hours, egg cylinders should start emerging.

5.4 On day 2 of the *in vitro* culture, replace IVC-1 medium with pre-warmed and equilibrated IVC-2 medium. The egg cylinders should continue growing and the embryonic and extraembryonic compartments together with the proamniotic cavity should become clearly distinguishable.

If too much mural TE remains, a small cavity will re-establish and the overall efficiency of the egg cylinder formation will be lower. The embryos will also attach with the same timing as described for E3.5 embryos above.

IVC Culture system trouble-shooting

If any difficulty is experienced during culture with our IVC media, please do not hesitate to contact tech@cellgs.com. Our team of experienced scientists will respond quickly and will be happy to assist you.

Blastocyst recovery and culture is a delicate and variable procedure. The following list is a sample of problems that we have anticipated may arise during this culturing protocol.

1) **Very few embryos recovered (less than 10 per mouse) after flushing the uterine horns.**
    a. Some embryos may not be in this section, also flush the connection between the uterus and the oviduct.

2) **The zona pellucida is still intact after the third drop of AT solution.**
    a. This is probably due to traces of M2 medium neutralizing the AT solution. Make fresh AT solution and repeat this washing step.

3) **During culture embryos flatten but do not attach to the ibiTreat µ-plate.**
    a. When using IVC-1 in combination with ibiTreat µ-plates, these conditions should encourage virtually all embryos to attach. Observe embryos at a higher magnification to determine if the zona pellucida remains, if so then such embryos should be discarded.

4) **Embryos show signs of apoptosis, cell shrinkage and fragmentation.**
    a. This could be due to altered pH levels. Calibrate the CO_2 content of the incubator to ensure a constant 5% CO_2 atmosphere remains.
    b. Blastocyst quality could be poor due to prolonged time for recovery. Dissect and flush the uterus and collect blastocysts as quickly as possible.

Figures

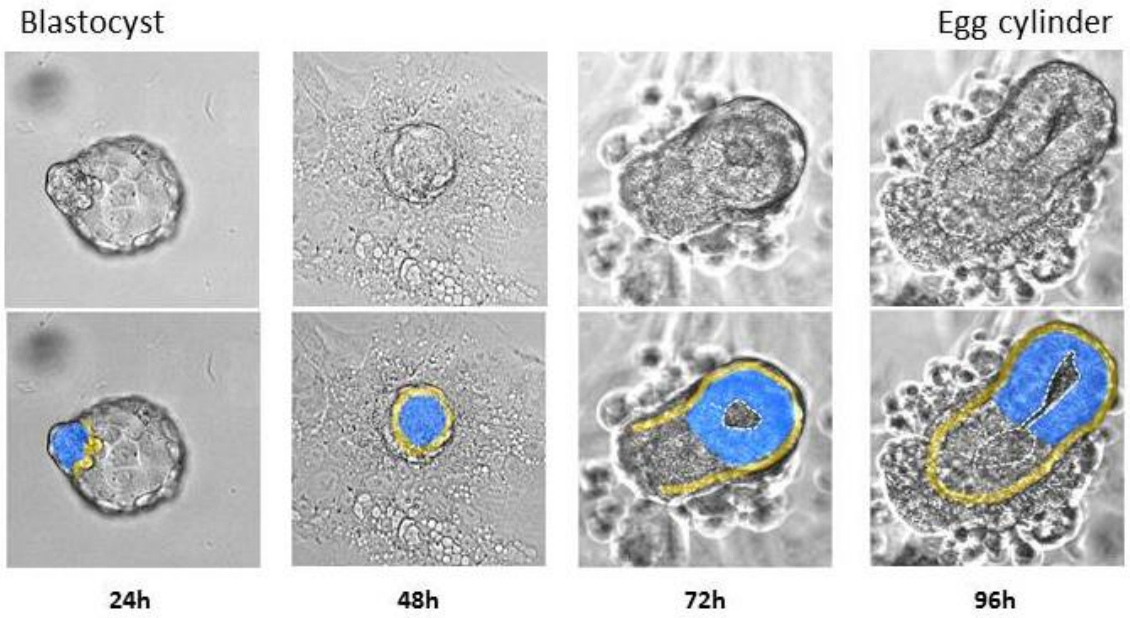

Figure 1. Blastocyst culture in IVC media. The epiblast is shown in blue whilst the primitive endoderm/visceral endoderm is shown in yellow.

References

1. Bedzhov, I., et al. 2014. *In vitro* culture of mouse blastocysts beyond the implantation stages. *Nature protocols* **9**: 2732-2739.

2. Bedzhov, I., and Zernicka-Goetz, M. 2014. Self-organizing properties of mouse pluripotent cells initiate morphogenesis upon implantation. *Cell* **156**: 1 -13.

3. Deglincerti, A. et al. 2016. Self-organization of the *in vitro* attached human embryo. *Nature* **533**(7602): 251-254.

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