Preparing Solutions for Vitrification

1.0 Equipment

1.1 Heat-Stir CB162 Magnetic stirrer

1.2 Magnetic Followers

1.3 Plastic Funnel

1.4 1L beaker

1.5 250ml beaker

1.6 Spatulas

1.7 Analytical Balance

1.8 Electric filter pump

1.9 Gilson pipettes (10ml, 200ul)

1.10 Brady label printer

1.11 Scissors

1.12 Spectrafuge

1.13 Vortex

1.14 Safety glasses

1.15 500ml/100ml/10ml Volumetric Flasks with Lids

1.16 100ml Measuring Cylinder

1.17 E3 Eppendorf Pipettes

1.18 Conical flasks

2.0 Supplies

2.1 Water for Embryo Transfer

2.2 10ml/200µl pipette tips

2.3 E3 Eppendorf Pipette 50ml tip

2.4 1.5ml Eppendorf Tubes

2.5 Parafilm

2.6 Corning 1000ml or 250ml filter unit

2.7 14ml Falcon tubes

2.8 Weighing boats/paper

2.9 10ml Diamond Tips

2.10 200ul Pipette Tips

2.11 Dulbecco's PBS

2.12 NaCl

2.13 KCl

2.14 $MgSO_4 \cdot 6H_2O$

2.15 KH_2PO_4

2.16 Glucose

2.17 Na-Pyruvate

2.18 Penicillin G

2.19 Streptomycin

2.20 $CaCl_2 \cdot 2H_2O$

2.21 $MgCl_2 \cdot 6H_2O$

2.22 BSA (A4378)

2.23 4M DMSO (5ml/10ml)

2.24 Acetamide

2.25 Sucrose

2.26 Propylene Glycol (134368-1L)

2.27 Purified water

2.28 Cryovials

2.29 Mask

2.30 Gloves

2.31 Parafilm

2.32 KSOM

2.33 Embryo culture dishes (35:3004)

3.0 Procedure

3.1 General Information

3.1.1 DAP213 can be purchased commercially from Cosmo Bio Co. Ltd (www.cosmobio.com).

3.1.2 Sterilise the solutions by autoclaving (121°C, 15 min.) or filtration. Store in the refrigerator at 4°C.

3.1.3 Gloves should be worn when handling Acetamide and it should only be weighed out in the fume hood.

3.2 Preparation of PB1 Solution for Vitrification

3.2.1 To prepare PBS (-), add 400ml embryo transfer water to a 1L beaker and place on the magnetic stirrer on stir setting ~2.5 – no heat, with the largest magnetic follower.

3.2.2 Weigh out 4.800g Dulbecco's PBS (-) and add to beaker containing 400ml of embryo transfer water. Allow to mix until all the PBS has dissolved. This can now be referred to as the PBS (-) solution.

3.2.3 To prepare CaCl2·2H2O (100x concentration), place 5ml of embryo transfer water into a 14ml Falcon tube.

Weigh out 0.132g $CaCl_2 \cdot 2H_2O$ and add it to the embryo transfer water in the Falcon tube. Place on lid and invert until the chemical has dissolved.

3.2.4 Transfer the solution to a 10ml volumetric flask. Rinse out the Falcon tube with embryo transfer water and use this to make up the solution to 10ml. Place on lid/cover with parafilm and invert to mix. Add 5ml of this solution to the beaker from 3.2.2.

3.2.5 To prepare $MgCl_2 \cdot 6H_2O$ (100x concentration), place 5ml of embryo transfer water into a 14ml Falcon tube. Weigh out 0.100g $MgCl_2 \cdot 6H_2O$ and add it to the embryo transfer water in the Falcon tube. Place on lid and invert until the chemical has dissolved.

3.2.6 Transfer the solution to a 10ml volumetric flask. Rinse out the Falcon tube with embryo transfer water and use this to make the solution up to 10ml. Place on lid and invert to mix. Add 5ml of this solution to the beaker from 3.2.2. This solution can now be referred to as PBS (+) solution.

3.2.7 Weigh out and add (respectively) 0.018g of Na Pyruvate, 0.032g of Penicillin G and 0.500g of Glucose to the PBS (+) solution.

3.2.8 Turn off the magnetic stirrer and add 1.500g of BSA (Bovine Serum Albumin) to the PBS (+) solution, cover with parafilm and let the BSA dissolve for approximately 30 mins.

3.2.9 Once the BSA has dissolved, add the solution to a 500ml volumetric flask. Rinse out the 1L beaker with embryo transfer water and use this to make the solution up to 500ml. This is the PB1 solution with BSA.

3.2.10 Sterilise the solution by filtration using the electric filter pump and a Corning 250ml or 1000ml filter unit. Label 'PB1 with BSA' and the date. The solution can be stored in the refrigerator and has a shelf life up to 3 months at 4°C.

3.2.11 Take a 0.5ml aliquot to check osmolality; this should be 285-295mOsm/kg.

3.3 DAP213 Solution for Vitrification

3.3.1 To prepare solution A; measure out 10ml of PB1 with BSA solution into a 100ml volumetric flask using a 10ml Gilson pipette.

3.3.2 Snap off the top of a glass ampoule containing the 4M DMSO with a tissue wrapped around the top of it. Pour the 4M DMSO from the glass ampoule (by gently tapping, if necessary) into a conical flask. Do this with 1 x 5ml and 1 x 10ml ampoule or 3 x 5ml ampoules.

3.3.3 Add 14.2ml of the 4M DMSO from the conical flask to the PB1 with BSA solution in the volumetric flask. Do this by using a 10ml Gilson pipette to aspirate 2 x 7.1ml aliquots. Place on lid/cover with parafilm and invert to mix.

3.3.4 Then add 21.95ml of 6M Propylene Glycol. Do this by using a 10ml Gilson pipette to aspirate 2 x 10ml aliquots and a 1000µl Gilson pipette to aspirate 1 x 1.95ml aliquot. Replace lid/cover with parafilm and invert to mix again.

3.3.5 To prepare solution B; measure out 20ml of PB1 with BSA solution into a conical flask using a 10ml Gilson pipette. Take it to the fume hood and wearing gloves, use the balance inside the fume hood to weigh out 5.9g of Acetamide. Add the Acetamide to the PB1 with BSA solution in the conical flask. Cover the conical flask with parafilm and gently swirl the contents to mix.

3.3.6 Still inside the fume hood, add all of Solution B to the volumetric flask containing Solution A. Rinse out the conical flask with PB1 with BSA medium and use this to make the combined solutions up to 100ml in 100ml volumetric flask. Place on lid/cover with parafilm and invert to mix well. Transfer the solution to a clean conical flask before aliquoting.

3.3.7 Aliquot out 1ml into 1.5ml Eppendorfs and centrifuge at 10,000rpm for 10 mins.

NOTE: DAP213 is aliquoted into 1ml Eppendorfs prior to centrifuging rather than 25ml as larger centrifuges do not spin up to 10,000 rpm.

3.3.8 Take off 0.9ml of the supernatant using a 1000µl pipette, taking care not to disturb the pellet, and place into a conical flask. The Eppendorf containing the pellet can be discarded as clinical waste.

3.3.9 Sterilise the DAP213 solution by filtration using the electric filter pump and a Corning 250ml or 1L filter unit.

3.3.10 In a deep cleaned LAF cabinet, dispense 1ml of the solution into cryovials and label with 'DAP213' and the date. This solution has a shelf life up to 3 months at 4°C.

3.4 1M DMSO Solution for Vitrification

3.4.1 To prepare the 1M DMSO solution; snap off the top of a 10ml glass ampoule containing the 4M DMSO with a tissue wrapped around the top of it. Pour the 4M DMSO from the glass ampoule (by gently tapping, if necessary) into conical flask. Do this with a 2 x 5ml or 1 x 10ml ampoule.

3.4.2 Add 50ml PB1 with BSA media to a 100ml volumetric flask using a 10ml Gilson pipette. Using a 10ml Gilson pipette, remove 7.8ml of the 4M DMSO from the conical flask and add it to 100ml volumetric flask. Make the solution up to 100ml using the PB1 with BSA medium. Place on lid/cover with parafilm and gently invert to mix.

3.4.3 Sterilise the 1M DMSO solution by filtration using the electric filter pump and a Corning 250ml or 1L filter unit.

3.4.4 In a deep cleaned LAF cabinet, dispense 1.5ml aliquots of the solution into cryovials and label with '1M DMSO' and date. This solution has a shelf life up to 3 months at 4°C.

3.5 0.25M Sucrose Solution for Vitrification

3.5.1 Prepare the 0.25M sucrose solution by placing 50ml of the PB1 with BSA solution into a conical flask. Weigh out

and add 8.56g of Sucrose to the PB1 with BSA solution in the conical flask. Place on lid and invert until the chemical has dissolved.

3.5.2 Transfer the solution to a 100ml volumetric flask. Rinse out the conical flask with PB1 with BSA solution and use this to make the solution up to 100ml. Place on lid/cover with parafilm and invert to mix.

3.5.3 Sterilise the 0.25M solution by filtration using the electric filter pump and a Corning 250ml or 1L filter unit.

3.5.4 In a deep cleaned LAF cabinet, dispense 1ml aliquots of the solution into cryovials and label with '0.25M Sucrose' and date. This solution has a shelf life up to 3 months at 4°C.

3.6 Testing Vitrification Solutions

3.6.1 A media test requires embryos (from a passed FZ session or fresh embryos), tested dishes and tested oil to be used. The old batch of media should be used as a control.

3.6.2 Vitrify half of the embryos using the batch of vitrification media to be tested and vitrify the other half of the embryos using a proven batch of vitrification media.

3.6.3 If using frozen embryos make sure they are from a tested FZ session and thaw the embryos according to the appropriate protocol. Ensure safety glasses are worn.

3.6.4 Vitrify the embryos ensuring the tested and untested batches of vitrification media are kept separate.

3.6.5 Once the embryos have been vitrified, thaw them, making sure at all times not to mix the batches.

3.6.6 Now culture the embryos and check their development daily.