Cellartis Protocol
“Culturing of hES Cells”

This material was cultured and frozen using Cellartis’ protocols. WiCell recommends that stem cells should be thawed and established in the conditions in which they were initially frozen prior to transfer to alternate culture platforms. The protocols that were used to produce these cells can be found on the following pages of this document.

If you have any questions or concerns please contact WiCell’s technical support staff via www.wicell.org and we will be happy to assist you.

Thank you,

WiCell

Culturing of hES cells

General

The human embryonic stem (hES) cells are provided in straws containing approximately 10-12 pieces of hES cell colonies per straw. When culturing the hES-cells, we recommend the use of mouse embryonic fibroblast (mEF)-feeder cells seeded in centre-well organ culture dish. To reduce evaporation of the culture media in the centre-well of the culture dish, add 4 ml of medium supplemented with antibiotics to the outer well. The hES cells should be incubated at 37ºC in 5% CO_2.

Thawing of hES cells

Following instruction is designed for thawing cells in one straw; do not thaw more than one straw at a time.
NOTE! DO ALWAYS USE PROTECTIVE GLOVES AND FULL MASK WHEN THAWING.

Chemicals and material needed

Trehalose	P.No. T0167, Sigma-Aldrich
VitroPBS™	P.No. 10506, Vitrolife
hES culture medium	see Media preparation
Center-well organ culture dish	P.No. 353037 or 353653, BD Falcon
4-well dish	P.No: 176740, Nunc
Sterile filter, 0.22 µm	P.No: 166100-4433, VWR International
Stem Cell Cutting Tool	P.No: 190-210 S, Swemed by Vitrolife AB
Transfer Pipettes	P.No: H-190-210, Swemed by Vitrolife AB
Stainless steel Holder for Stem Cell Cutting Tool	P.No: H-9570, Swemed by Vitrolife AB
Plastic connector	Qosina, provided from Cellartis AB
Mitomycin C treated mEF-feeder cells	
Syringe (2 ml)	
Forceps (autoclaved)	
Pair of scissors (autoclaved)	
Cloth (autoclaved)	
Open container with liquid nitrogen (N_2)	
Container with water, 37ºC	
Stereo microscope	
Heated stage	

Method

1. Prepare 3 ml of a 0.2 M Trehalose solution in VitroPBS™ (Solution C).
2. Prepare 3 ml of a 0.1 M Trehalose solution in VitroPBS™ (Solution D).
3. Prior to sterile filtration, let 2 ml of Solution C and D respectively pass through the sterile filters and discard this volume.
4. Sterile filter Solution C and D. These solutions should be made immediately before use.
5. Pipette 0.5 ml of Solution C, 0.5 ml of Solution D and 0.5 ml hES medium into 3 separate wells in a 4-well dish.
6. Place the 4-well dish in 37˚C for 15 minutes.
7. Prepare a container with liquid N_2 and place it next to the permanent liquid tank or the transport vessel if thawed on delivery.
8. Put the Visiotube with the straw to be thawed in the container with liquid N_2.
9. Prepare a container with water, 37˚C.
10. Connect the plastic connector “Qosina” to the syringe and drawback, filling it with air. Place it on an autoclaved cloth or similar.
11. Place the 4-well dish on a heating stage under a microscope.
12. Uncap the Visiotube and use forceps to pull out the straw.

13. Hold the straw in the air in room temperature for 10 seconds.
14. Place the straw in the container with water, 37˚C for 2 seconds.
15. Wipe off the straw with an autoclaved cloth (soaked in 70% ethanol).
16. Hold the straw and use a pair of scissors to cut off the plugged seal next to the plug, in the column of air, (see Figure 2, Cut 1).
17. Connect the open end of the Plastic connector to the cut end of the straw.
18. Cut off the heat-sealed end, (see Figure 2, Cut 2). Syringe connected to straw, see Figure 3.
19. With some air in the syringe eject the cell colony pieces into Solution C. Use one well per straw.
20. Leave colonies in Solution C for 1 minute (on a heating stage).
21. Transfer the colonies to Solution D by using a transfer pipette or a Stem Cell Cutting Tool.
22. Place the 4-well dish in an incubator and leave the cell colony pieces in Solution D for 5 minutes.
23. Transfer the cell colony pieces as above to the hES medium, this is a washing step.
24. Transfer the cell colony pieces as above to plates coated with mEF cells and place in incubator.

Media Change of hES cells

Change medium every second or third day, starting on the second day after thawing or passaging. The total amount of VitroHES™ medium in the inner well should be 2.0 ml. Change 50% of the volume in the inner well organ culture dish each time. The medium in the outer well (4 ml) is changed once a week.

Chemicals and Material needed

VitroHES™ medium, (37˚C)	P.No: 10505, Vitrolife
bFGF, (4ng/ml)	P.No: 100-18B, Peprotech

Method

1. Do not change medium in more than 10 centre-well organ culture dishes at a time due to fall in temperature.
2. Remove 1 ml of medium from the inner well.
3. Add 1 ml of preheated medium to the inner well.
4. Place the centre-well organ culture dish in the incubator.
5. Discard left over preheated medium.

Culturing of hES cells

Passaging of hES cells

Passaging of hES-cell colonies should be done every 4th to 6th day. It is suitable to passage the colonies when at least 4 new pieces at the size of approximate 200 µm x 200 µm can be cut out from each undifferentiated colony. Only colonies with undifferentiated hES-cell morphology should be used. An undifferentiated hES appearance for a colony is that it has a homogenous structure. Avoid passage of the mEF feeder cells (see figure. 1).

Chemicals and Material needed

Mitomycin C treated mEF cells	
Stem Cell Cutting Tool	P.No: H-190-210 S, Swemed by Vitrolife AB
Holder for Stem Cell Knives	P.No: H-9570, Swemed by Vitrolife AB

Method

1. Place the dish with hES colonies under a stereomicroscope. Only cut one dish at a time, due to risk of temperature loss and pH change. The procedure of cutting one dish should typically take less than 10 minutes.
2. Focus the hES colonies one by one and cut a checked pattern (see Figure 1) as mentioned below.
3. Cut all colonies in one dish.
4. Use the knife to loosen all pieces one by one by carefully lifting a corner and then loosening them from the dish.
5. Use the Stem Cell Cutting Tool and holder for stem cell knives to transfer the pieces of hES cell colonies.
6. Place 10-16 pieces evenly in a new dish with mEF cells and place in incubator.

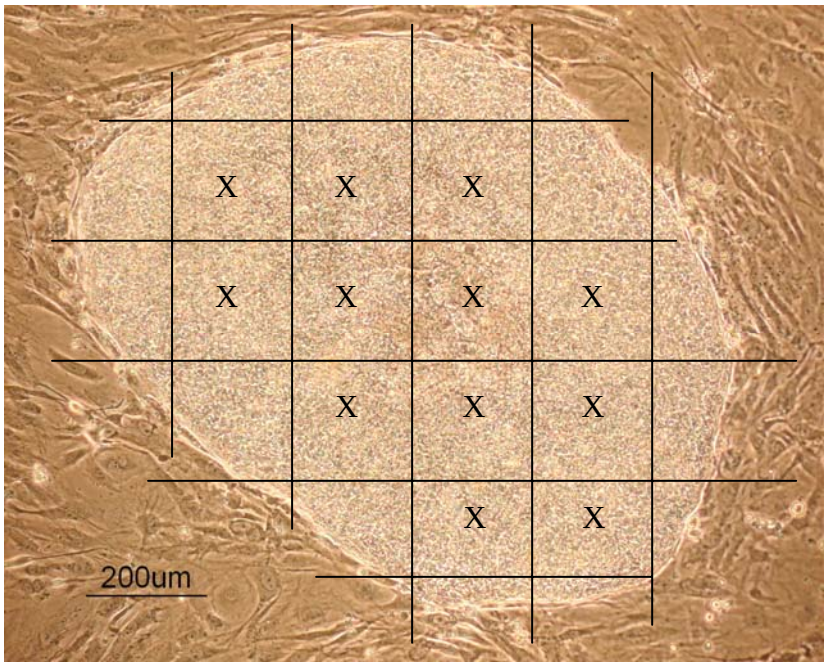


Figure 1. Preferable cutting pattern of a hES cell colony. It is recommended that only the colony pieces marked with “X” is transferred to a new culture dish, in order to avoid unwanted transfer of old mEF cells.

Culturing of hES cells

Freezing of hES cells

Following instruction is designed for freezing 3 straws.
Freeze 10-12 pieces (approximate 200µm x 200µm) of hES colonies in one straw.

Chemicals and material needed

VitroPBS™	P.No. 10506, Vitrolife
Trehalose	P.No: T0167, Sigma-Aldrich
Ethylene glycol	P.No: 102466,
DMSO	P.No: D2650, Sigma-Aldrich
Visiotube	P.No: 83000411, Air Liquide
Closed straws	P.No: 3589, Svensk Avel
Sterile filter, 0.22 µm, (DMSO safe)	P. No: 166100-4433, VWR International
4-well dish, Nunclon	P.No: 176740, Nunc
Stem Cell Cutting Tool	P.No: 190-210 S, Swemed by Vitrolife AB
Transfer Pipettes	P.No: H-190-210, Swemed by Vitrolife AB
Holder for Stem Cell Cutting Tool	P.No: H-9570, Swemed by Vitrolife AB,
Plastic connector	Qosina, provided from Cellartis AB
Syringe (2 ml)	
Forceps (autoclaved)	
Heat sealer	
Container with liquid N_2	
Cryo Pen	

Method

1. Prepare a container with liquid N_2.
2. Open the cap of the Visiotube and place in the container with liquid N_2 and submerge until the Visiotube is filled with liquid N_2.
3. Prepare 2 ml of 10% Ethylene glycol and 10% DMSO in VitroPBS™ (Solution A).
4. Prepare 2 ml of 0.3M Trehalose, 20% Ethylene glycol and 20% DMSO in VitroPBS™ (Solution B).
5. Prior to sterile filtration, let 2 ml of Solution A and B respectively pass through the sterile filters and discard this volume.
6. Sterile filter Solution A and Solution B. These solutions should be made immediately before use.
7. Pipette 0.5 ml of Solution A and 0.5 ml of Solution B in separate wells in a 4-well dish.
8. Place the 4-well dish in 37˚C for 15 minutes.
9. Cut colonies as described in Figure 1.
10. Pipette 2 drops (25 µl each) of Solution B to a sterile, hydrophobic surface for example the lid of a centre-well organ culture dish.
11. Pipette 1 ml of VitroPBS™ to the third well in the 4-well dish.
12. Connect the plastic connector to the syringe and the closed straw as described in figure 2.
13. Fill the straw with a 2-3 cm high column of VitroPBS™ by using a syringe.
14. Fill the straw with a 1-3 cm high column of air.
15. Fill the straw with a 0.5 cm high column of Solution B.
16. Transfer the cell colony pieces to Solution A.
17. Leave in Solution A for 1 minute.
18. Transfer the cell colony pieces to the first drop of Solution B and there after immediately to the second drop.
19. Immediately transfer the cell colony pieces from the second drop of Solution B into the straw, altogether Solution B with the colonies shall make a 2 cm high column.

The following steps (20-24) should be performed within 30 seconds.

20. Fill the straw with air until the VitroPBS™ makes the blue part of the plugged seal swell.
21. Use a forceps to flatten the straw 1-2 cm from the open end. Make a mechanical seal with the Heat sealer where the straw is flattened. Make two seals right next to each other (1-2 mm apart).
22. Loosen the syringe from the straw.
23. Label the straw on the "column of VitroPBS™", see Figure 2.
24. Place the straw in the Visiotube.
25. Cap the Visiotube and place it in a permanent liquid nitrogen tank for long term storage.

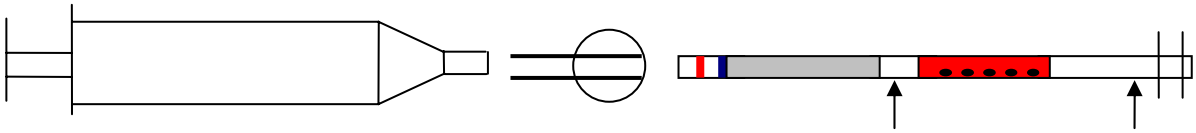
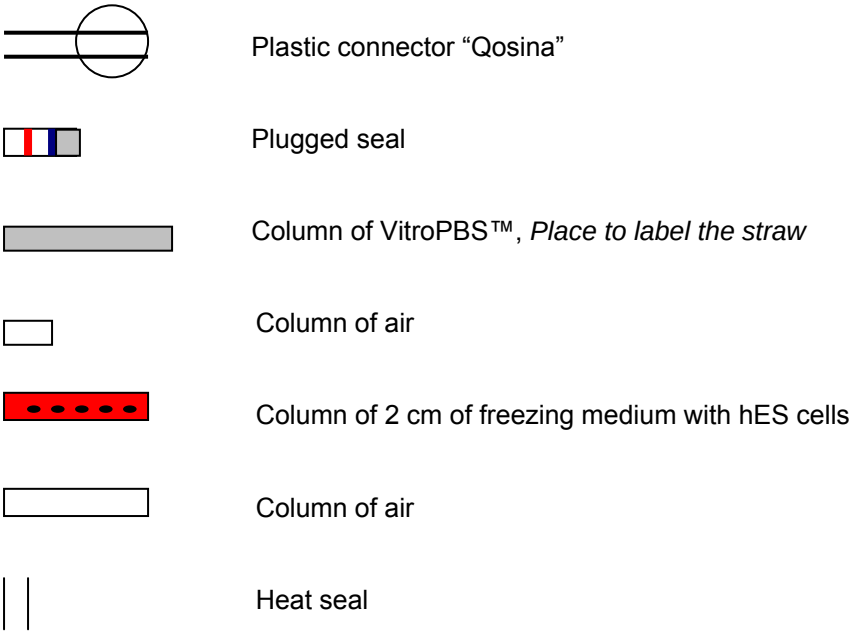


Figure 2. Syringe with closed straw.



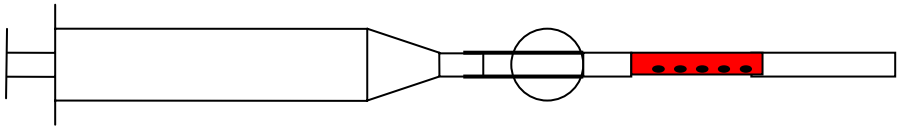


Figure 3. Syringe connected to straw.

Culturing of hES cells

mEF culture medium

Chemicals and Material needed

DMEM (Dulbecco's Modified Eagle Medium)	P.No. 61965-026, Invitrogen
FBS (Foetal Bovine Serum)	P.No. 10108-165 Gibco
PEST (Penicillin/Streptomycin)	P.No. 15140-122, Invitrogen

Method

Use mEF medium when thawing mEF cells and when preparing for seeding mEF cells in centre-well organ culture dishes. Prepare culture medium by adding 10% FBS and 1% PEST in DMEM.

hES medium

Chemicals and material needed

VitroHES™	P.No.10505, Vitrolife
bFGF	P.No. 100-18B, Peprotech

Method

Prepare the hES medium immediately before use, by adding bFGF (4ng/ml) to VitroHES™. Use the hES medium when seeding the Mitomycin C treated mEF cells, when thawing, changing medium and passaging of hES cells. Always use culture medium preheated to 37°C. Discard left over preheated medium.

Medium to the outer ring **(if using center-well organ culture dishes)**

Chemicals and material needed

Knock out DMEM	P.No. 10829-018, Gibco
PEST	P.No. 15140-122, Gibco

Method

Prepare medium to the outer ring by adding 1% PEST in Knock out DMEM.