Handling Guide
For Cor.4U® Cardiomyocytes on MEAs

MEA Protocol v.3

General Information:

This protocol describes the seeding of pure human Cor.4U® cardiomyocytes derived from iPS cells on Microelectrode Arrays (Multi Channel Systems, Reutlingen Germany; Axion BioSystems, Atlanta USA). The protocol is set up according to the state of purchased Cor.4U® cardiomyocytes (cryopreserved or fresh) and the type of Microelectrode Arrays (1well, 6well, 9well, 12well, 48well MEAs). Please read the entire protocol before you start the experiment.

1. Material

1.1 Reagents

- 1 vial of 1 x 10^6 (1M) Cor.4U® viable cardiomyocytes (cat.-no. Ax-B-HC02-1M) (Alternatively, one T25 Flask containing 1million fresh Cor.4U Cardiomyocytes)
- 1 bottle with 10ml Cor.4U® Thawing Medium
- 1 bottle with 250ml Cor.4U® Culture Medium
- Upon request 1 bottle 250ml serum-free BMCC Medium
- 1 vial with 50µl Puromycin stock solution (10mg/ml)

1.2 Storage Conditions

- Upon receipt of cryopreserved Cor.4U® cardiomyocytes: transfer the vial immediately to the vapor phase of liquid nitrogen or store below -130°C.
- Upon receipt of fresh Cor.4U® cardiomyocytes: exchange the medium completely, place a new filter lid on the culture flask, and transfer to 37°C, 5% CO_2, and 95% humidity.
- Store Cor.4U® Medium, BMCC Medium and Puromycin at -20°C. Thaw medium and Puromycin at 4°C over night and avoid exess exposure to light. Once thawed, keep medium and Puromycin at 4°C for up to 2 weeks.
- The medium delivered with fresh Cor.4U® cardiomyocytes is non-frozen and can be stored directly at 4°C for up to 2 weeks.

1.3 Requirements

Item	Vendor	Cat. No.
10 cm sterile Petri dish	various	
Sterile laminar flow hood	various	
Freezer (-20°C) refrigerator (+4°C)	various	
LN2 storage or -130°C freezer	various	
10µl, 100µl and 1000µl pipette	various	
Pipettor for serological pipettes	various	
Neubauer Hemocytometer	various	

Microelectrode Array	MCS, Axion BioSystems	various
T25 cell culture flasks	Falcon or Nunc	various
Geltrex “ready-to-use”	Life Technologies	A15696-01
Fibronectin	Sigma	F1141
1x Trypsin/EDTA	Sigma	T3924-100ML
0.5M EDTA Solution	Life Technologies	15575-038
Trypan Blue Solution	Sigma	T8154

2. Preparations

For the handling of fresh Cor.4U cardiomyocytes, please proceed to chapter 3.3!

2.1 Thawing of Puromycin

1. Thaw Puromycin at 4°C over night the day before you thaw Cor.4U cardiomyocytes.

2.2 Coating of culture flasks with fibronectin

Attention: This step is only necessary for cryopreserved Cor.4U• cells!

1. Use a T25 flask for 1x10^6 Cor.4U® cells. Add 20µl of fibronectin stock solution (1mg/ml) to 2 mL PBS with Ca^{2+} and Mg^{2+}. The final concentration of the coating solution is now 10µg/mL fibronectin (Dilution 1:100).

2. Coat the flask with the coating solution and incubate for 3h at 37°C or over night at 4°C. Please Note: Fibronectin is very susceptible to shear stress. Avoid harsh pipetting and do not vortex or spin the solution. Avoid drying of the coating solution to prevent denaturation of the protein.

Day 0

3. Thawing and seeding of cryopreserved Cor.4U® cardiomyocytes

3.1 Thawing of cryopreserved Cor.4U® cardiomyocytes

1. Transfer 6ml Cor.4U® **Thawing** Medium into a 50ml tube (tube A), add 5µg/ml Puromycin (3µl of the 10mg/ml stock solution), and warm to 37°C.
2. Before thawing the cells, transfer 3ml of the warm Cor.4U® **Thawing** Medium from tube A into a second 50ml tube (tube B). Each tube contains 3ml.
3. Quickly transfer the cells from liquid nitrogen on dry ice directly to the laminar flow hood and add 0.5ml of tube B into the vial. Tightly screw the vial and transfer it to a 37°C water bath until the frozen cell suspension detaches from the bottom of the vial and only a small ice clump is visible (approx. 40 sec).
4. Transfer the cell suspension into tube A, rinse the vial with 1ml Cor.4U® **Thawing** Medium from tube B and transfer to tube A. The total volume in tube A is now 5ml. Please Note: Do not centrifuge the cell suspension!
5. Aspirate the coating solution of the T25 cell culture flask prepared in step 2.2.
6. Without centrifugation, pipette the entire cell suspension into the T25 flask. Incubate the cells for 3h at 37°C, 5% CO_2, and 95% humidity.
7. Prepare a 50 ml tube with 5 mL of Cor.4U® **Culture** Medium, add 5µg/ml (2,5µl of the 10mg/ml stock solution) of the puromycin stock solution, and warm to 37°C.
8. Carefully aspirate and discard 4 mL medium from the T25 flask with the Cor.4U® cells. Please Note: Do not touch the surface of the flask! Make sure to aspirate the supernatant only from the surface of the liquid at slowest speed.
9. Slowly and carefully pipette 4 ml of the warm Cor.4U® **Culture** Medium into the T25 flask. Culture the cells over night at 37°C. Please Note: Add the medium at slowest speed and avoid pipetting over the culture area of the flask!

Day 1

3.2 Medium Change

1. Warm 5 mL Cor.4U® **Culture** Medium and 5 mL of PBS with Ca^{2+} and Mg^{2+} to 37°C.
2. Inspect the cells under a microscope. The majority of the cells should have attached to the surface and form a monolayer, some floating (dead) cells are normal and will be removed with the subsequent medium change.
3. Carefully aspirate the medium from the T25 flask with the Cor.4U® cells and wash with 5 ml warm PBS. Please Note: Do not touch the surface of the flask! Make sure to aspirate the supernatant only from the surface of the liquid at slowest speed.
4. Remove the PBS and add 5ml of warm Cor.4U® culture medium. Culture the cells over night at 37°C. Please Note: Add the medium at slowest speed and avoid pipetting over the culture area of the flask!

3.3 Coating of the MEAs with Geltrex (alternatively use Fibronectin)

1. Place one sterile MEA in a 10cm Petri dish under laminar flow hood.
2. Transfer an appropriate amount of ice cold Geltrex ready-to-use solution in a sterile 50ml tube. Please refer to table 1 for volumes of Geltrex. Please note: Keep the tube with Geltrex on ice throughout the whole procedure to avoid polymerization!

Table 1: Volume of Geltrex

Microelectrode Array	Volume of Geltrex (µl)
1well (MCS)	10
6well (MCS)	5
9well (MCS)	5
12well (Axion BioSystems)	10
48well (Axion BioSystems)	10

3. Place a drop of Geltrex directly onto the electrode array area of the MEA. Please Note: Handle the coated MEAs with care to avoid drifting of the Geltrex drop.

   (Alternatively, coat the MEAs with the same volume of Fibronectin. Please refer to step 2.2 for the preparation of the Fibronectin coating solution.)
4. Incubate the coated MEA for at least 30 min at 37 °C in humidified chamber inside the incubator. (When using Fibronectin, incubate for at least 3h.)

Day 2

3.4 Dissociation of Cor.U4® Cells

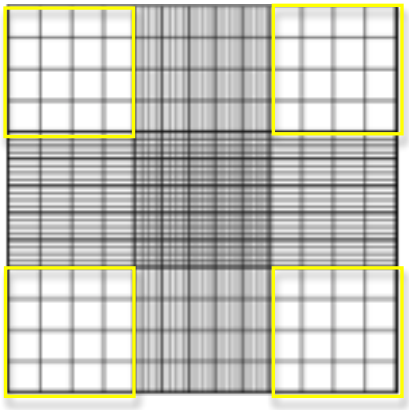
1. Prepare PBS w/o Ca^{2+}/Mg^{2+} with 2mM EDTA as described in the appendix. You need 15 mL per T-25 flask.
2. Warm 2 mL 1x Trypsin/EDTA solution for each T25 flask to 37°C.
3. Warm Cor.4U® **Culture** Medium to 37°C. You need 5 mL for the T25 flask and 1.5 mL for each MEA.
4. Aspriate the medium, and carefully wash the cells twice with 5 mL PBS/EDTA solution prepared in step 1. Incubate the cells at least for 7 min in 5 mL PBS/EDTA solution before you proceed to step 5.
5. Aspirate the PBS/EDTA solution and apply 2 mL of the warm trypsin/EDTA solution. Incubate the cells at 37°C, 5% CO_2 and 95% humidity for 2 min.
6. In the meantime, pipette 3 mL of warm Cor.4U® **Culture** Medium into a 50 mL tube.
7. Tap the T25 flask 3 -4 times within the lamina flow hood. Check with the microscope that all cells have detached. If larger amounts of cells are still attached, incubate the cells for another minute at 37°C, tap the T25 flask, and check again for detachment of the Cor.4U® cardiomyocytes.
   Please Note: Do not incubate with 1x Trypsin/EDTA for longer than 5 min! Longer treatments will cause loss of viable cells. 2 min or less is recommended.
8. Carefully detach remaining cells from the culture surface by rinsing the culture surface of the T25 flask 2 -3 times with the cell suspension.
9. Transfer the cell suspension into the 50 mL tube containing 3 mL warm Cor.4U® **Culture** Medium.
10. Rinse the flask with an additional 2 mL of Cor.4U® Culture Medium and transfer it into the 50 mL tube containing the cell suspension. This tube contains now a total volume of 7 mL.
11. Centrifuge the cell suspension for 3 min at 200 *x* g, and discard the supernatant. Resuspend the cell pellet in 0.4 mL of Cor.4U® **Culture** Medium.

3.5 Counting of Cor.4U® cardiomyocytes with a Neubauer hemocytometer

1. Pipette 10µl trypan blue solution and 10µL of the cell suspension into a tube. Count live (clear) cells, total cells, and dead (blue) cells and apply 10µl of the 1:1 mixture into a Neubauer Hemocytometer.
2. Count the number of viable cells in each of the four outer boxes highlighted in yellow of Figure1. Divide the counted number by 4 to receive the mean value.

   E.g. Number counted in for all 4 boxes: 500 → 500 / 4 = 125

Figure 1. Neubauer Hemocytometer



3. Calculate the number of viable cells correct for chamber factor (1 x 10^4), dilution factor (2), and total volume (0.4 mL),

   E.g.: Mean value of viable cells: 125

   125 x 10000 x 2 x 0.4 = 1000 000 (1 million viable cells in the cell suspension)

4. Adjust the cell suspension to 200k viable cells per 100µl (2 Mio/mL) with Cor.4U® Culture Medium.

3.6 Seeding of Cor.4U® Cells

1. Aspirate the Geltrex coating solution from the MEAs. Please Note: Do not touch the surface of the MEAs! If using multi well MEAs, aspirate Geltrex and seed cells well per well to avoid drying of the Geltrex coating!
2. Add the recommended volume of the cell suspension as a drop on the pre-coated electrode area of the MEA according to table 2. Please Note: The cell suspension should sit in a drop on the coated microelectrode area of the MEA! For an optimal monolayer avoid bubbles at anytime!

Table 2: Cor.4U seeding densities

Microelectrode Array	Cor.4U density / well	Volume of Cell Suspension (µl)
1well (MCS)	2×10^4	10
6well (MCS)	1×10^4	5
9well (MCS)	1×10^4	5
12well (Axion BioSystems)	2×10^4	10
48well (Axion BioSystems)	2×10^4	10

4. Carefully place MEAs in the 10 cm dishes back into the containers and incubate for 3h at 37°.

3.7 Add Medium

1. After 3h, transfer the dishes with the MEAs into the laminar flow hood.
2. Carefully add an appropriate volume of pre-warmed Cor.4U® **Culture** Medium to each well of the MEA. Please refer to table 3 for appropriate volumes according to the used MEA. Please Note: Do not pipette directly onto the culture area of the MEAs!

Table 3: Total Volume of Medium

Microelectrode Array	Volume of Medium (ml)
1well (MCS)	1
6well (MCS)	0.2 (round well) 0.6 (triangle well)
9well (MCS)	0.2
12well (Axion BioSystems)	0.3
48well (Axion BioSystems)	0.3

3. Place MEAs back into the containers and culture at 37°C over night.

Day 3

3.8 Change Medium

1. Warm a required amount of Cor.4U® Culture Medium to 37°C.
2. Inspect the cells under a microscope. The majority of the cells should have attached to the surface and form a monolayer. A few remaining non-adherent (dead) cells are common and will be removed with the subsequent medium exchange.
3. Carefully aspirate the medium from the MEAs and add the required amount of warm Cor.4U® Culture Medium. Please Note: Aspirate the medium from one side, and do not pipette directly onto the culture area of the MEAs!
4. Place MEAs back into the containers and incubate at 37°Cover night.

Day 4

3.9 Follow Up Information

Synchroneous beating of the Cor.4U monolayer appears around day 2. MEAs should be checked in the MEA analyzer once a day from day 2 onwards. Compounds can be added as soon as a stable baseline is obtained but it is recommended not to begin earlier than day 3 after seeding.

4. Appendix

4.1 Preparation of PBS/EDTA solution:

Dilute 2 ml cell-culture tested 0.5 M EDTA solution (pH 8.0) in 500 ml PBS or DPBS without Ca^{2+}/Mg^{2+}.

Alternatively, prepare a 20 mM EDTA stock solution in 1 L of distilled water and adjust pH to 8.2
Please Note: EDTA solves very slowly during pH adjustment; do not attempt to heat the solution!
You may use commercially available stock solutions instead.
Mix 100 mL of the 20 mM EDTA stock solution with 100 mL 10x Dulbecco's PBS w/o Ca^{2+}/Mg^{2+} (e.g. PAA H15-011) and 800 mL of distilled water. Sterile-filter the solution into a sterile 1 L bottle and store at room temperature.