Handling Guide:
Intracellular Calcium Transient Assay
Cor.4U® Cardiomyocytes

TABLE OF CONTENTS

1. General information

This protocol covers thawing, seeding and dissociation of Axiogenesis Cor.4U® cardiomyocytes for use in **intracellular calcium transient assays**. It is recommended to use the cells earliest at day 2 post thaw, as the cells develop a syncytium and are beating synchronically after this time in culture. Please read the entire protocol and the manual for the instrument which you will record the intracellular calcium transients first before you start your experiment.

2. Safety information

Cor.4U® cardiomyocytes are produced through *in vitro* differentiation of transgenic human induced pluripotent stem cells (iPSC) and puromycin selection of the resulting cardiomyocytes. The iPSC line is generated via the Yamanaka protocol from a human skin fibroblast. The highly pure cardiomyocytes (100% purity) express cardiac-specific proteins, e.g. cardiac alpha-actinin and connexin-43, an indication of the ability for electric coupling of these cells. Patch clamp analyses, as well as multi-electrode array (MEA) recordings, demonstrate the normal electrophysiological properties of these cells. Cor.4U® cardiomyocytes are particularly useful for cell-based *in vitro* assays in pharmacology, safety, and toxicology. These cells are ideal for electrophysiological applications as well as for high content and high throughput screening applications.

- Cor.4U® cardiomyocytes are intended for *in vitro* research use only. The cells are not intended for diagnostics, therapeutic or clinical use and is not approved for human *in vivo* applications.

- Cor.4U® cardiomyocytes are genetically modified human cells and should be handled according to local directives (Biosafety level 1).

- Cor.4U® cardiomyocytes should be cultured in a sterile environment.

It is highly recommended that gloves and lab coats be worn when handling all reagents as some reagents contain chemicals that may be harmful. Please consult the CoA and material safety data sheets (MSDS) for additional safety instructions where applicable.

3. Material

3.1 Cells and media provided by Axiogenesis

Material	Container	Quant.	Content	Storage	Shelf life
Cor.4U® cardiomyocytes (Ax-B-HC02-4M)	Cryo vial	1	4 Million cells	Liquid nitrogen	1.5 years
Cor.4U® cardiomyocytes (Ax-B-HC02-1M)	Cryo vial	1	1 Million cells	Liquid nitrogen	1.5 years
seeded fresh viable Cor.4U® cardiomyocytes (Ax-C-HC02-384)	384 well plate	1	10k/ well	Incubator, 37°C	
seeded fresh viable Cor.4U® cardiomyocytes (Ax-C-HC02-96)	96 well plate	1	30k/ well	Incubator, 37°C	
Cor.4U® Culture Medium (Ax-M-HC250)	Bottle	1	250 mL	Frozen -20°C	1 year
BMCC Medium (serum-free) (Ax-M-BMCC250)	Bottle	1	250 mL	Frozen -20°C	1 year

3.2 Storage conditions

- Upon receipt of cryopreserved Cor.4U® cardiomyocytes, transfer the vial immediately to the vapor phase of liquid nitrogen. Do not expose the vials longer than one minute to room temperature as recrystallization processes might harm the cells.
- Upon receipt of fresh Cor.4U® cardiomyocytes plates, transfer to a sterile hood, carefully remove the silicon lid, aspirate the medium and add 250 µL per well (96 well plate) or 50 µL medium per well (384 well plate) of fresh pre-warmed Cor.4U® Culture Medium. Replace the lid on the flask with the new sterile filter lid shipped with the package and transfer the plates immediately to a humidified incubator (5% CO_2)
- Store Cor.4U® Culture Medium and BMCC medium at -20°C. Thaw medium overnight at 4°C and avoid excess exposure to light. Once thawed, keep medium at 4°C for up to 4 weeks.
- The media delivered with fresh Cor.4U® cardiomyocytes is non-frozen and can be stored directly at 4°C for up to 2 weeks.

NOTE: It is not recommended to store the cells at -80°C. Recrystallization can occur which may damage the cells.

3.3 Requirements

Item	Vendor	Cat. No.
Inverse microscope	various	
Imaging instrument (e.g. Hamamatsu FDSS/ µCELL)	various	
Sterile laminar flow hood (Bio-Safety level S1)	various	
Cell culture incubator (37°C, 95% humidity, 5% CO_2)	various	
Freezer (-20°C) refrigerator (+4°C)	various	
Water bath	various	
8- or 12- multi-channel pipette	various	
Sterile reservoirs	various	
Sterile 50 mL polypropylene tubes	various	
FLIPR Calcium 5 Assay Kit	Molecular Devices	R8186
* Plastic container with lid containing a damp cloth	various	
* Liquid nitrogen container	various	
* 100 µL, 1000 µL pipette	various	
* Pipettor for serological pipettes	various	
* PBS w/o Ca^{++} and Mg^{++}	various	
* PBS with Ca^{++} and Mg^{++}	various	
* 2 mM EDTA in PBS w/o Ca^{++} and Mg^{++}	various	
* 0,5% Trypsin solution	various	
* Neubauer hemocytometer	various	
* Fibronectin solution	Sigma	F1141
* Trypan blue solution	Sigma	T8154
* 384 well cell culture plate with lid	Greiner Bio-One	781091
* 96 well cell culture plate with lid	Greiner Bio-One	655087
* Centrifuge (swinging bucket rotor)	various	

Positions marked with *: Solely required for frozen cells/ cells delivered in cell culture flasks

Day 0

4. Coating of multi well plates

INFO: It is recommended to coat each well of a 384 well plate with 50 µL and each well of a 96 well plate with 100 µL of fibronectin coating solution.

1. Start > 3 hours before thawing/ plating. For a complete 384 well plate, prepare 24 mL of fibronectin coating solution by pipetting 240 µL of fibronectin stock solution (1 mg/mL) into 24 mL of PBS with Ca^{++} / Mg^{++} in a 50 mL tube and mix carefully.
2. For a complete 96 well plate, prepare 12 mL of fibronectin coating solution by pipetting 120 µL of fibronectin stock solution (1 mg/mL) into 12 mL PBS with Ca^{++} / Mg^{++}.

NOTE: Fibronectin is susceptible to shear stress, do not vortex or spin, and avoid harsh pipetting

3. Within the sterile hood: Transfer the coating solution into a reservoir and pipette 25 µL of this coating solution into each well of the 384 well plate/ 100 µL into each well of a 96 well plate, using a multichannel micropipette. Incubate the plate for at least 3h at 37°C in a standard cell culture incubator. Alternatively, the wells can be coated overnight at 4°C.

Day 1

5. Thawing and seeding of Cor.4U® cardiomyocytes

INFO: For a complete 384 well plate, one vial with 4 million plus one vial with 1 million cryopreserved Cor.4U® cells is required. For a complete 96 well plate, one vial with 4 million cryopreserved cells is required

5.1 Thawing of cryopreserved Cor.4U® cardiomyocytes (4 x 10^6 cells)

1. Transfer 3 mL Cor.4U® Culture Medium into a 50 mL tube (tube A) and warm to 37°C.
2. Before thawing the cells, warm 1 mL Cor.4U® Culture Medium in another 50 mL tube (tube B).
3. Quickly transfer the cells from liquid nitrogen on dry ice directly to a 37°C water bath and thaw the vial until the frozen cell suspension detaches from the bottom of the vial and only a small ice clump is visible (approx. 2 min).
4. Disinfect and transfer the vial to the laminar flow hood and pipette the cell suspension carefully into the tube containing 3 mL Cor.4U® Culture Medium (tube A).
5. Rinse the vial with 1 mL Cor.4U® Culture Medium from tube B and transfer to tube A. The total volume in tube A is now 5 mL.

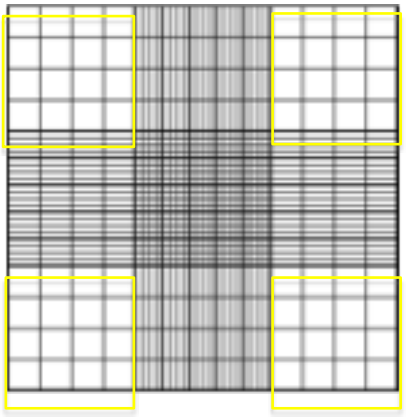
NOTE: Do not spin down the cell suspension! Centrifugation directly after thawing will damage the cells and lead to cell loss.

5.3 Counting of Cor.4U® cardiomyocytes

INFO: For a suitable cell counting procedure with a Neubauer haemocytometer of a vial containing 4 Mio. cells, withdraw a 10 µL aliquot from the cell suspension diluted in 5 mL medium.

1. Add 10 µL trypan blue solution to the 10 µL of the cell suspension in a tube. Incubate the mixture for 3 min at 37°C.
2. Apply 10 µL of the 1:1 mixture into a Neubauer haemocytometer and count viable (clear), dead (blue) and total cells.
3. Count the number of cells in each of the four outer boxes highlighted in yellow of Figure 2. Calculate the mean number of cells per yellow box.

Fig 2. Neubauer haemocytometer



4. Calculate the number of cells corrected by chamber factor (1 x 10^4), dilution factor (2), and total volume (1 mL).

   E.g.: Mean number of cells = 50

   50 x 10000 x 2 x 1 = 1000 000 (1 million cells in the cell suspension)

5.4 Seeding of Cor.4U® cardiomyocytes

1. Adjust the cell suspension to 0.4 x 10^6 viable cells per mL with Cor.4U® Culture Medium.
2. Transfer the cell suspension into a fresh sterile reservoir.
3. Immediately before seeding, discard the fibronectin coating solution from the multi well plate.

NOTE: Work quickly, do not let the wells dry; otherwise cell adhesion will be impaired.

4. Carefully mix the cell suspension.
5. Seed 10k of Cor.4U® Cardiomyocytes per well of a 384 well plate (25 µL of the cell suspension) and 30k of Cor.4U® Cardiomyocytes per well of a 96 well plate (75 µL of the cell suspension)

NOTE: Make sure that no bubbles occur during pipetting the cell suspension: use antistatic shoes/gloves to avoid air bubbles due to electrostatic charged plates during winter time. Otherwise, cardiomyocytes will not form a monolayer.

6. Close the multi well plate, transfer the plate into an enclosable plastic container containing a damp cloth (to avoid evaporation of the small volume of medium) and incubate the plate for 3 to 4 hours at 37°C and 5% CO_2 in the incubator.
7. After the incubation period, transfer 22 mL of Cor.4U® Culture Medium into a fresh 50 mL tube for a 384 well plate or 15 mL of Cor.4U® Culture Medium for a 96 well plate and warm to 37°C.
8. Transfer the warmed Cor.4U® Culture Medium into a sterile reservoir and pipette 50 µL of the medium into each well of the 384 well plate or 125 µL into each well of the 96 well plate using a multichannel pipette.
9. Transfer the plate back into the incubator at 37°C and 5% CO_2 and incubate over night.

Day 2

5.5 Medium Exchange

10. Change the complete medium every day.
11. Warm 35 mL of Cor.4U® Culture Medium for a 384 well plate or 25 mL of Cor.4U® Culture Medium for a 96 well plate to 37°C.
12. Inspect the cells at a microscope. The majority of the cells should have attached to the surface and formed a monolayer.

INFO: It is usual to have some floating (dead) cells which did not attach to the surface.

13. Transfer the multi well plate with the Cor.4U® cardiomyocytes into the laminar bench and carefully aspirate the medium from the wells.
14. Add 75 µL of fresh Cor.4U® Culture Medium to each well of a 384 well plate or 200 µL of fresh Cor.4U® Culture Medium to each well of a 96 well plate.
15. Transfer the multi well plate back into the incubator and culture the cells at 37°C, 5% CO_2, and 95% humidity.

NOTE: Aspirate the medium carefully and do not add the medium directly onto the cells to avoid damage and detachment of the cells.

INFO: Synchronous beating of the Cor.4U® cardiomyocyte monolayers will appear approximately 30h after seeding of the cells. Experiments can be started at > 72h post seeded.

Day of Experiment

6. Intracellular Calcium Transient Assay

6.1 Medium exchange to BMCC medium

1. Two hours before starting the measurements, warm 35 mL of BMCC Medium for a 384 well plate or 25 mL of BMCC Medium for a 96 well plate to 37°C.
2. Transfer the 384 well plate with the Cor.4U® cardiomyocytes into the laminar bench and carefully aspirate the medium from the wells seeded with cells. Ensure that all medium is removed from each well.
3. Add 25 µL of fresh BMCC Medium to each well of a 384 well plate or 75 µL of fresh BMCC Medium to each well of a 96 well plate.

4. Close the multi well plate, transfer the plate into a plastic container with lid containing a damp cloth (to avoid evaporation of the small volume of medium) and incubate the plate for 2 hours at 37°C and 5% CO_2 in the incubator.

NOTE: The BMCC Medium does not contain serum. Serum-free medium is a prerequisite to achieve a good signal during the intracellular calcium transient measurements.

6.2 Executing the intracellular calcium transient measurements

1. Prepare a 0.5x diluted solution of the FLIPR Calcium dye 5 in BMCC Medium according to the manufacturers instruction and warm to 37°C. Prepare 12 mL for a 384 well plate and 10 mL for a 96 well plate.

INFO: Preparing a 0.5x diluted solution of the FLIPR Calcium calcium dye 5 will result in a final concentration of 1/6 of the dye during the measurement. This most optimal concentration has been tested by Axiogenesis for lowest toxicity whilst still yielding a very good readout. It is possible to use other dyes. Then, we highly recommend to execute a dilution series/ add quencher if required, in order to find the optimal composition.

2. After the incubation period of 2 hours, add 25 µL of the calcium dye solution to each well of a 384 well plate or 75 µL of the calcium dye solution to each well of a 96 well plate.

INFO: It is normal to observe a lowered beating frequency after the application of the dye. Frequencies (about half the speed is possible). A beating frequency of 20 beats per minute can be measured adequately. If you observe lower frequencies, please contact Axiogenesis support.

3. Close the multi well plate with the lid, transfer the plate into a plastic container with lid containing a damp cloth (to avoid evaporation of the small volume of medium) and incubate the plate for 20 further minutes at 37°C and 5% CO_2 in the incubator.

INFO: If your Imaging instrument contains a temperature control unit, it is highly recommended to set the temperature to 37°C.

4. Dilute the test compounds 3x concentrated in BMCC medium. Per well of a 384 well plate 25 µL of compound solution and per well of a 95 well plate 75 µL is needed.
5. Warm the diluted compound solutions to 37°C. Ensure that the total volume is not reduced during the warming due to evaporation (use an adequate lid).
6. Start your intracellular calcium measurements: We recommend to let the plate equilibrate for further 20 minutes within the instrument and to measure the baseline.
7. Apply the compound solutions (25 µL to each well of a 384 well plate or 75 µL to each well of a 96 well plate).
8. Measure calcium transient waves repetitively every 5 minutes for 35 minutes.

NOTE: Other substance dilution protocols as proposed are possible. If another protocol is planned, ensure to calculate the correct concentration dependent on the final volume per well.

7. Appendix

7.1 Preparation of PBS/EDTA solution

1. Dilute 2 mL cell-culture tested 0.5 M EDTA solution (pH 8.0) in 500 mL PBS w/o Ca^{++}/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! Mix 100 mL of the 20 mM EDTA stock solution with 100 mL 10x PBS w/o Ca^{++}/Mg^{++} and 800 mL of distilled water. Sterile-filter the solution into a sterile 1 L bottle and store at room temperature.

7.2 Axiogenesis Label License

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