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Cor.At®

Cardiomyocytes derived from Mouse Embryonic Stem Cells

Protocol

Cor.At® Cardiomyocytes Neutral Red Uptake Assay

Detection of Cardiac-specific Cytotoxicity

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1 Introduction

Cor.At® cells are cardiomyocytes derived from transgenic mouse embryonic stem cells. These cells are puromycin resistant and have the green fluorescent protein (GFP) reporter gene driven by a cardiac-specific promoter (αMHC promoter).[1]

Cor.At® cells are produced through *in vitro* differentiation of mouse embryonic stem (ES) cells and puromycin selection of cardiomyocytes. Through BrdU incorporation assays, Cor.At® cells were determined to have limited proliferative capacity similar to primary cells. These highly pure cells (>99% purity) express cardiac-specific Connexin-43, an indication of the ability for electric coupling of these cells, as shown in immunostaining. Patch clamp analyses, as well as multi-electrode array (MEA) recordings, demonstrate the normal electrophysiological properties of these cells.

1.1 Cor.At® Cardiomyocytes for Pharmacological and Toxicological Screening

Cor.At® cardiomyocytes are particularly useful for cell-based *in vitro* assays in pharmacology and toxicology. These cells are ideal for high content screening, as well as, for medium to high throughput screening.

Cor.At® cells can be used for:

- Safety pharmacology
- Toxicological analysis of compounds (cardiac-specific cytotoxicity)
- Screening for pharmacological effects
- Drug development
- Molecular and cell biology
- Research & development

1.2 Cor.At® Cardiomyocytes Compared to Cell Lines and Primary Cells

Cor.At® cardiomyocytes have many advantages over primary cells and cell lines. These cells are highly standardised and are 99.9% pure. The culture and maintenance of Cor.At® cardiomyocytes require minimal laboratory time when compared to the culture and maintenance of rat neonatal cells. Reproducible results can be expected for every assay.

These homogeneous cells are:

- Standardised from lot-to-lot
- 99.9% pure and have fully functional cardiac phenotype
- Frozen, stored and thawed with complete recovery of functionality
- An entire *in vitro*-based system

In addition to the Cor.At® Tox Kit, cells can be purchased frozen as different formulations and versions in cryo vials: Catalog numbers Ax-C-MC01 (ES derived GFP positive), Ax-C-MC02 (ES derived colorless), Ax-C-MC03 (iPS derived colorless) or pre-plated in fibronectin-coated 96-well plates: Catalog number Ax-C-MC01-96w. Cells are cultured for 48 hours to allow them to recover post thaw

and should be cultured on ***BD BioCoat™ Fibronectin***-coated dishes (Catalog # 354457 for 35 mm dishes or Catalog # 354403 for 60 mm dishes). For coating plates and dishes of alternate sizes, we recommend using ***Sigma Fibronectin*** from bovine plasma (1 mg/ml solution), Catalog # F-1141.

1.3 The principle of the Neutral Red Uptake (NRU) cytotoxicity assay

The uptake and concentration of Neutral Red (3-Amino-7-dimethylamino- 2-methylphenazin hydrochlorid) into intracytoplasmic vacuoles and granules is correlated with the viability of the cells as determined e.g. by Trypan Blue uptake. The toxic effect of a compound is correlated with the decreased uptake of Neutral Red by the cells, either due to a reduced cell number, or a reduced viability of the cells. Neutral Red stored in the viable cells is solubilized using a destain solution (1% acetic acid, 49% H20, 50% ethanol) and quantified by measuring the absoprtion at 540nm in a spectrophotometer[2].

The observed effects at different compound concentrations on Cor.At® cardiomyocytes and on the reference cell type are compared to distinguish cardiac specific cytotoxicity from general cytotoxicity.

2 Materials and Reagents

Please read the entire technical manual before beginning the culture of mouse atrial-like cardiomyocytes. Additional information on stability and storage instructions for the cells can be found in the specific Product Information Sheets (PIS).

2.1 Product Specification

Catalog #	Product	Description	Unit / Format	Storage Conditions
AX-96W	Cor.At® Plate	• 96-well plates pre-seeded with Cor.At® cardiomyocytes • ≥ 20,000 cells per well (> 99.9% pure)	Fibronectin-coated 96-well plate	-80°C* (4 weeks)*** *Plates should not be stored below –80°C!*
Ax-M-MC250E (Europe, ROW) Ax-M-MC250N (USA and Japan)	Cor.At® Media	• Culture medium for Cor.At® cardiomyocytes	250 ml bottle	-20°C* (6 months)** 4°C* (2 weeks)***
N/A	Cor.At® Thawing Media	• Thawing medium for Cor.At® cardiomyocytes	30 ml bottle	-20°C* (6 months) 4°C* (2 weeks)
N/A	Puromycin	• Puromycin stock solution (10 mg/ml)	1 ml vial	-20°C* (6 months) 4°C (1 week)
Sigma**** N-6264	Accustain buffered Neutral Red solution	• 0.5% Neutral Red (stock solution in acetate buffer, pH 5.2)	50 ml	room temperature in the dark

*Refer to PIS for additional information
**Expiry date as stated on label (i.e. 6 months from date of manufacture)
***from date of delivery of product(s)
****Refer to Sigma for more information

2.2 Additional Materials and Reagents Required for Cor.At® Tox

- Vertical laminar flow hood certified for Level I handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- 37°C water bath
- Inverted fluorescence microscope with Green Fluorescent Protein (GFP) or Fluorescein Isothiocyanate (FITC) filters
- Plate shaker for 96 well plates
- ELISA plate reader for reading absorbance at 570nm with a reference wavelength of 630nm
- Sterile 50 ml polypropylene (PP) tubes
- 8-channel or 12-channel micropipette (e.g. 8-channel Eppendorf "Research Pro" 1200)
- Sterile pipette tips
- 0.2 *μ*m Syringe filter, e.g. MliliPore
- PBS with Ca^{2+} and Mg^{2+}
- Ethanol p.a. (99.9% pure)
- Glacial acetic acid

3 Safety Instructions

- The kit is intended for *in vitro* Research Use Only
- The kit is not intended for Diagnostic, Therapeutic or Clinical Use and is not approved for human *in vivo* applications.
- Cor.At® cells are genetically modified mouse cells and should be handled according to local directives (Biosafety level 1).
- Cor.At® cells can be inactivated by autoclaving at 121°C for 20 minutes.
- Cor.At® cardiomyocytes should be cultured in a sterile environment according to good cell culture and good laboratory practices.
- It is highly recommended that gloves and labcoats be worn when handling all reagents as some reagents contain chemicals that may be harmful. Please consult the PIS and Material Safety Data Sheets (MSDS) for additional safety instructions where applicable.

4. Cor.At® Tox Test Procedure

After at least 48 hours of culture test compounds can be added to the Cor.At® cardiomyocytes. Culture period can be extended up to 14 days if a more mature phenotype is desired.

Test compounds should be added at different concentrations do obtain a dose response curve. A positive and negative control (fixed concentrations) with known effects on cardiomyocytes should be included. Doxorubicin may be used as positive control, and acetylsaliclic acid as a negative control. A positive and negative control (fixed concentrations) are included in the Cor.At® Tox kit. It may be necessary to perform a range-finding experiment in order to identify a suitable concentration range for each test compound.

Dilution of test compounds should be performed in Cor.At® medium w/o Puromycin. It is recommended to perform either 10fold dilutions to cover a broad range of concentrations, or geometrical dilutions, if an exact LC_{50} should be determined.

If compound stock solution contains solvents (e.g. DMSO, ethanol), the final concentration must not exceed 0.5%.

It is recommended to test all compounds, incl. standards, in hexaplicates. In this case, one column of 6 wells is used for each concentration or standard. Two columns of controls should be performed, as described in fig. 1.

Cor.At® cardiomyocytes can be treated with test compounds for different time periods. If the treatment period exceeds 48 h, a change of medium has to be performed.

A flow chart of the test procedure is displayed in fig. 2.

4.1 Preparation and Addition of Test Compounds

1) Prewarm 50 ml of Cor.At® medium w/o Puromycin to 37°C.
2) Take test compounds and standards out of the refrigerator and pre-warm them to room temperature. Mix well before use.
3) For each concentration to be tested, a total volume of 1.5 ml (0.2 ml per well, 6 wells) is needed.
4) Perform serial dilutions of the test compounds in Cor.At® medium w/o Puromycin. It is recommended to perform serial dilutions in a 96 well plate that mimics the Cor.At® plate layout (see fig. 1).
   Mix all compound dilutions well before use!
5) Take the 96-well plate out of the incubator and gently remove the medium using a slow-running suction pump or a multichannel pipette.
   Hold plates in a 45° angle and place the pipette tips in the inner rim of the wells to prevent the tips from touching the bottom of the wells.
6) Fill the "blank" wells and the control wells with 200 µl pre-warmed Cor.At® medium each. Transfer 200 µl of the prepared compound dilutions to the appropriate wells of the test plate.
   Hold plates in a 45° angle and place the pipette tips in the inner rim of the wells to prevent the tips from touching the bottom of the wells.

7) Incubate test plates at 37°C, 5% CO_2, 95% humidity, for 48 h, or the desired incubation period. If compounds should be incubated for more than 48 h, please change media with compound solutions on a regular basis, e.g. every 48 -h.

4.2 Quantification of cytotoxicity using Neutral Red uptake

1) Dilute Neutral Red stock solution 1:100 in 20 ml of pre-warmed Cor.At® medium w/o Puromycin medium in a 50ml tube. Pass the solution through a 0.2 μm filter.
Do not prepare more than 20 ml solution at once!

2) Take the 96-well plate out of the incubator and gently remove the medium using a slow-running suction pump or a multichannel pipette.
Hold plates in a 45° angle and place the pipette tips in the inner rim of the wells to prevent the tips from touching the bottom of the wells.

3) Carefully add 200 μl of PBS to each well of the plate using a multichannel pipette (e.g. 8-channel Eppendorf "Research Pro" 1200) at slow speed. Incubate the PBS for 2 min, then gently remove the PBS using a slow-running suction pump or a multichannel pipette.
Hold plates in a 45° angle and place the pipette tips in the inner rim of the wells to prevent the tips from touching the bottom of the wells.

4) Add 100 μl of Neutral Red solution to each well using a multichannel pipette (e.g. 8-channel Eppendorf "Research Pro" 1200) at slow speed.
Hold plates in a 45° angle and place the pipette tips in the inner rim of the wells to prevent the tips from touching the bottom of the wells.

5) Incubate the plate at 37°C, 5% CO_2, 95% humdity for 3 h. Check cells of control wells for neutral red uptake after 30 min under the microscope (fig. 3).

6) 15 min before end of incubation time, prepare desorb solution by mixing 49% MiliQ water, 50% ethanol, and 1% glacial acetic acid (e.g. for one plate a total of 15 ml: 7.35 ml H2O, 7.5 ml ethanol, and 0.15 ml glacial acetic acid).

7) After 3 h of incubation, take the 96-well plate out of the incubator and gently remove the medium using a slow-running suction pump or a multichannel pipette.
Hold plates in a 45° angle and place the pipette tips in the inner rim of the wells to prevent the tips from touching the bottom of the wells.

8) Gently wash wells with 200 μl of PBS each as described in step 4. Make sure to remove all PBS after the washing step without destroying the cell layer!
Hold plates in a 45° angle and place the pipette tips in the inner rim of the wells to prevent the tips from touching the bottom of the wells.

9) Add 150 μl of desorb solution to each well. Incubate plate on a plate shaker for 10min at maximal speed.

10) Measure absorbance at 540 nm with a reference wavelength of 690 nm.

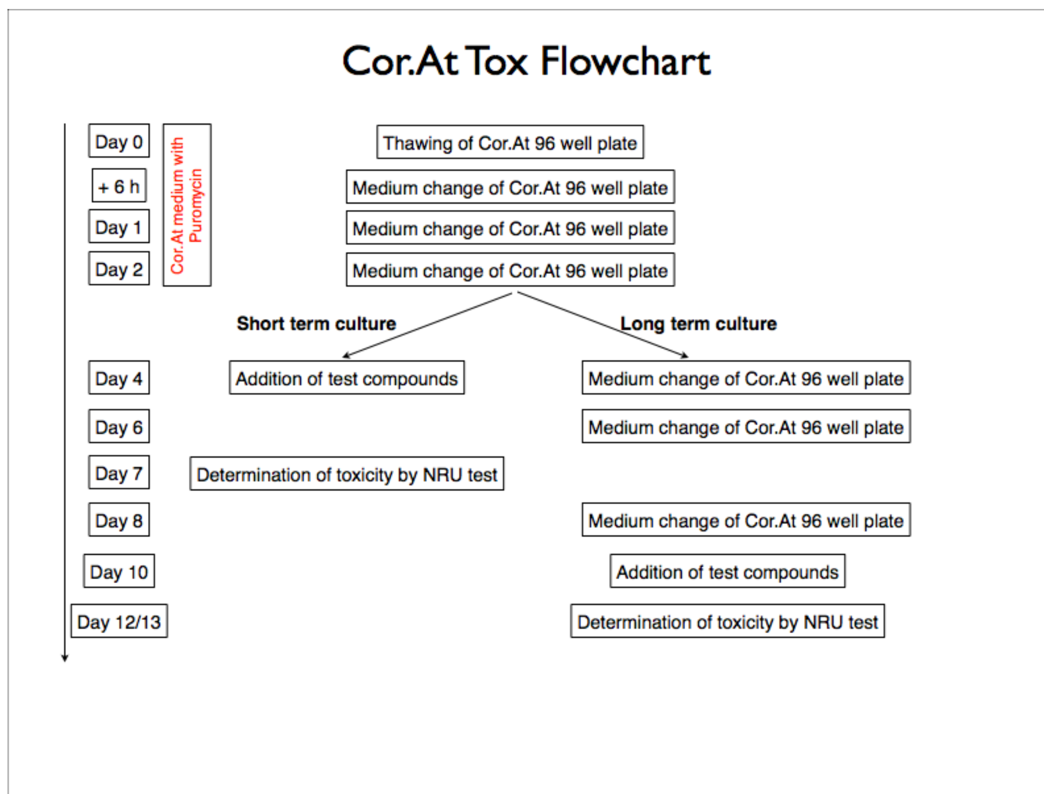
11) Subtract the mean of the blank wells (B12 -> G12) of the values from all other wells. Calculate the mean of the control wells B2-> G2 and B11 -> G11 and set it as 100%. Calculate the mean of the treated wells and display them as "% of control%". Standard A is a negative control and does not affect the cells. Standard B is a positive control exhibiting cardiac toxicity and will decrease viability by at least 60%.

5.0 Figures

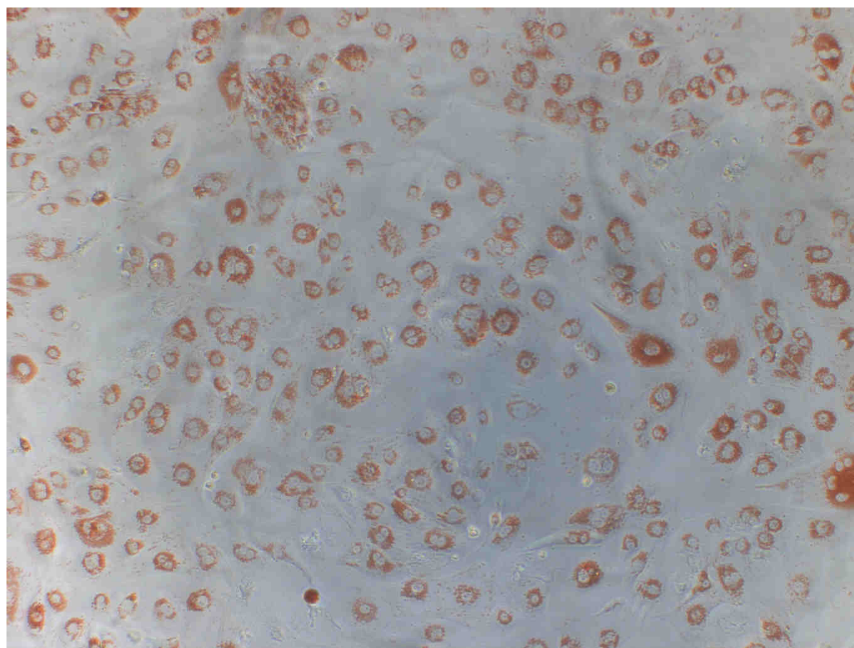
5.1 Figure 1. Layout of a Cor.At® Tox Test Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank
B	blank	control	negative control	positive control	Test compound conc. 1	Test compound conc. 2	Test compound conc. 3	Test compound conc. 4	Test compound conc. 5	Test compound conc. 6	control	blank
C	blank	control									control	blank
D	blank	control									control	blank
E	blank	control									control	blank
F	blank	control									control	blank
G	blank	control									control	blank
H	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank

5.2 Figure 2. Process Flowchart



5.3 Figure 3. Cor.At cells after absorption of Neutral Red



6 Literature

1. Kolossov E, Lu Z, Drobinskaya I, et al. Identification and characterization of embryonic stem cell-derived pacemaker and atrial cardiomyocytes. FASEB. J. 2005;19(6):577-9.

2. Borenfreund E, Puerner JA. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett. 1985; 24(2-3):119-24.

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