Ncardia
Stem cell experts

Assessment of pro-arrhythmic effects using Pluricyte® Cardiomyocytes

on the ACEA xCELLigence® RTCA CardioECR

User Guide
Version 3.0 / March 2018

Contents

Getting Started

Please make sure to read the entire user guide carefully before you start thawing and culturing Pluricyte® Cardiomyocytes.

Pluricyte® Cardiomyocytes are for *in vitro* life science research use only.

A Material Safety Data Sheet (MSDS) for Pluricyte® Cardiomyocytes is available on our website.

Technical support and training

Our scientists are ready to help you with any questions you may have regarding this user guide or our Pluricyte® Cardiomyocytes. In addition, in-lab training is available upon request. For further information please visit our website www.ncardia.com, or contact us directly by e-mail (sci-support@ncardia).

1. Introduction

Pluricyte® Cardiomyocytes are highly suitable for ACEA xCELLigence® RTCA CardioECR MEA assays

Pluricyte® Cardiomyocytes are fully functional human induced pluripotent stem cell (hiPSC) derived ventricular cardiomyocytes that are particularly suitable for electrophysiology-based multi-electrode array (MEA) assays for predictive safety pharmacology, toxicity testing and efficacy screening in early drug discovery. The combination of Pluricyte® Cardiomyocytes and the xCELLigence® RTCA Cardio-ECR system enables detailed electrophysiological detection of potential cardiotoxic/pro-arrhythmic effects of test compounds in a 48-well plate format. The well-pronounced depolarization and repolarization peaks of Pluricyte® Cardiomyocyte monolayer field potential signals allow an easy detection of electrophysiological parameters (e.g. depolarization/repolarization peak amplitudes, beat rate, field potential duration) and facilitate efficient data analysis and interpretation of studies performed with the xCELLigence® RTCA CardioECR system.

Pluricyte® Cardiomyocytes - strengths and characteristics

Pluricyte® Cardiomyocytes exhibit a relatively high level of maturity and present the following unique characteristics:

- High purity of ventricular cardiomyocytes
- Low resting membrane potential (~-78 mV)
- Fast upstroke velocity and action potential amplitude
- Organized sarcomeric structures
- Monolayer field potential contains well-pronounced depolarization and repolarization peaks, enabling easy detection of field potential durations in MEA assays

This user guide describes our recommendations for the use of Pluricyte® Cardiomyocytes in the xCELLigence® RTCA CardioECR system (ACEA Biosciences). In addition, an application note describing the assessment of the effects of a set of pro-arrhythmic compounds in Pluricyte® Cardiomyocytes, showing the expected pharmacological responses, can be downloaded at ncardia.com.

Pluricyte® Cardiomyocytes, cultured in Pluricyte® Cardiomyocyte Medium, in combination with the xCELLigence® RTCA CardioECR system provide a highly relevant *in vitro* assay platform to study the cardiac safety profile of compounds during drug development.

2. Workflow

Day 0	- Coat E-plate® ≥ 3h before plating - Record background - Thaw and plate Pluriycte® Cardiomyocytes
Day 1	-Refresh Pluricyte® Cardiomyocyte Medium*
Day 3	-Refresh Pluricyte® Cardiomyocyte Medium*
Day 5	-Refresh Pluricyte® Cardiomyocyte Medium*
Day 7	-Refresh Pluricyte® Cardiomyocyte Medium*
Day 8-12	- Refresh Pluricyte® Cardiomyocyte Medium and incubate ≥ 1h - Record baseline and prepare compound dilutions - Add compounds and record compound effects

* Optional: in order to monitor the condition of the Pluricyte® Cardiomyocyte monolayer it is advised to perform daily measurements (≥ 1h after refreshment).

3. Important recommendations

Prior to plating the Pluricyte® Cardiomyocytes, always perform a background measurement of the E-plate® according to the xCELLigence® RTCA CardioECR Software Manual (**Section 5.2**).

- Carefully follow the thawing and seeding instructions. This step is essential for optimal cell survival and attachment (**Section 5.3**). Pluricyte® Cardiomyocytes should be directly seeded onto the E-plate®.
- We strongly recommend to use fibronectin as coating substrate for the E-plates®. Other types of coatings may reduce the signal and/or impact the condition of the cells.
- Always refresh the Pluricyte® Cardiomyocyte Medium of the cells the day after seeding the cells (**Section 5.4**). Subsequently, refresh the Pluricyte® Cardiomyocyte Medium of the cells every 2 days, or 3 days when refreshing on Friday afternoon and Monday morning to prevent weekend-work.
- First contractions of Pluricyte® Cardiomyocytes appear between 24-48 hours post-thawing. It will take 3-4 days before the cells have formed an electrically coupled monolayer. Stable beating monolayers can be observed 7-8 days post-thawing. The optimal time window to perform electrophysiology-based assays with Pluricyte® Cardiomyocytes is between 8-12 days after plating the cardiomyocytes.

4. Equipment, Materials and Reagents

Equipment, materials and reagents are described respectively in **Tables 4.1, 4.2** and **4.3.**

Equipment	Manufacturer
xCELLigence® RTCA CardioECR Instrument + software	ACEA Biosciences
Flow cabinet	Various
Incubator at 37˚C, with 5% CO_2 and humidified air	Various
P20, P200 and P1000 pipettes	Various
8-channel multichannel pipette	Various
RTCA Cardio Temperature Tool	ACEA Biosciences
Hemocytometer or automated cell counter	Various

Table 4.1: Equipment

Materials	Manufacturer	Cat#
E-plate® CardioECR 48	ACEA Biosciences	00300600940
Sterile disposable 5 ml pipettes	Various	
Sterile disposable 10 ml pipettes	Various	
Sterile disposable 25 ml pipettes	Various	
Sterile 15ml conical tubes	Various	
Sterile 50ml conical tubes	Various	
Sterile 20µl filter pipette tips	Various	
Sterile 300µl filter pipette tips	Various	
Sterile 1000µl filter pipette tips	Various	
Sterile multichannel reservoirs	Various	

Table 4.2: Materials

Reagents	Manufacturer	Cat#
Fibronectin (1 mg/ml)	Sigma	F1141
1x DPBS + Ca^{2+}+ Mg^{2+}	e.g. Life technologies	Gibco 14040
Pluricyte® Cardiomyocyte Medium	Ncardia	PM-2100-100ml
Pluricyte® Cardiomyocyte Kit	Ncardia	PCK-1.5

Table 4.3: Reagents

5. Methods

5.1 Coating of the 48-well E-plate®

The E-Plate® is coated on the day of plating the Pluricyte® Cardiomyocytes (≥3 h before plating of the cells).

Note: The volumes used below are calculated for one 48-well E-plate®. For plating more than one 48-well E-plate®, multiply the volumes used by the number of E-plates® needed.

Per E-Plate®:

1. Dilute 30 µL fibronectin solution in 3 ml sterile D-PBS (incl. Ca^{2+} and Mg^{2+}) in a 15 ml conical tube to get a 10 µg/ml fibronectin coating solution. Mix the solution carefully.

Note: Fibronectin is susceptible to shear stress, do not vortex or spin the solution, and avoid harsh pipetting.

2. Add 50 µl/well of the fibronectin coating solution to the E-Plate® to evenly coat the bottom of each well.

Note: Be careful not to touch the bottom of the plate with the pipette tips.

3. Incubate the E-plate® at 37°C for 3 hours.
   Note: Do not to let the fibronectin coating dry out.

5.2 Performing background measurement

4. Carefully aspirate the fibronectin coating solution from the wells of the E-plate® and immediately add 50 µl of Pluricyte® Cardiomyocyte Medium to each well using a multichannel pipette and sterile multichannel reservoirs. Incubate the E-plate® for 10 minutes at 37°C, 5% CO_2.

Note: Prevent the fibronectin coating from drying out and avoid touching the bottom of the wells with the pipette tips.

5. Place the E-plate® into the xCELLigence® RTCA CardioECR instrument.
6. Perform background measurement according to the xCELLigence® RTCA CardioECR Software Manual.
7. Remove the E-plate® from the instrument and leave it in the incubator until seeding of the cells.

5.3 Thawing Pluricyte® Cardiomyocytes and seeding onto the 48-well E-plate®

Note: The volumes used below are calculated for one 48-well E-plate®. For plating more than one 48-well E-plates®, multiply the number of vials used by the number of E-plates® needed. Combine the contents of the vials in the 50 ml conical tube (see step 11) and adjust the volumes of Pluricyte® Cardiomyocyte Medium to add accordingly. We recommend to thaw a maximum of 3 vials per operator at a time.

Per E-Plate®:

8. Coat the tissue culture plate with fibronectin as described in **Section 5.1**.
9. Warm 6 ml Pluricyte® Cardiomyocyte Medium to room temperature (RT).

Note: make sure to mix the medium by inverting before use.

10. Take 1 vial of Pluricyte® Cardiomyocytes from LN2 storage (optional: transport the vial on dry ice) and place the vial in a 37°C incubator for exactly 4 minutes.
11. Gently transfer the contents of the vial to a 50 ml tube using a P1000 pipette. Avoid pipetting up and down.
12. Rinse the empty vial with 1 ml Pluricyte® Cardiomyocyte Medium (at RT) and add the 1 ml Pluricyte® Cardiomyocyte Medium drop-wise to the 50 ml tube containing the cells: add 1 drop every 5 seconds using a P1000 pipette while gently swirling the cells after each drop.

Note: This step is crucial for the recovery of the cardiomyocytes. We recommend to use a timer.

13. Add 4.7 ml of Pluricyte® Cardiomyocyte Medium drop-wise to the 50 ml tube, 1 drop every 2 seconds using a 5 ml pipette.

Note: the total volume of the cell suspension is now 6 ml.

14. Take a 20 µl sample of the homogenous cell suspension and add to a micro centrifuge tube.
15. Spin down the cell suspension for 3 minutes at 250 xg.
16. Aspirate the medium and gently resuspend the cells in 1 ml Pluricyte® Cardiomyocyte Medium.
17. Determine the total cell number and cell viability as follows:
    We highly recommend to perform the cell counting manually using a hemocytometer. For in stance, by using the Fuchs Rosenthal Counting Chamber (**Figure 5.1**):
    a. Add 20 µl Trypan blue solution to the 20 µl cell sample (collected in step 14), mix carefully.
    b. Add 20 µl of the Trypan blue/cell suspension mix to the counting chamber.
    c. Calculate the total number of cells according to **equation 1**.
18. Calculate the dilution factor to reach 30,000 cells/50 µl and add Pluricyte® Cardiomyocyte Medium to the cell suspension accordingly.
19. Add the solution to a multichannel reservoir using a 5 ml pipette.
20. Transfer the coated plate(s) to the flow cabinet, do not aspirate medium from the E-plate® but add 50 µl/well of the cell suspension (=30,000 cells/well) to the side of the well using a multichannel pipette.

Note: Avoid air bubbles and gently resuspend cells in the multichannel reservoir in between pipetting steps to evenly distribute the cells.

21. Incubate the E-plate® in the flow cabinet at room temperature for 30 minutes to allow the cells to settle and ensure an even distribution.
22. Transfer the E-plate® to the incubator (37°C, 5% CO_2).

Equation 1 . Cell counting

Count 4 #2 squares according to Figure 5.1

Viable cells: __ + __ + __ + __ = ____ (#vc)

Non-viable (blue) cells: __ + __ + __ + __ = ____(#nvc)

_____ / 4 x 2 x 5000 = _____ cells/ml

[#vc]

_____ x _____ = _____ (cells in total)

[# of cells/ml] [volume after step 13]

Viability = ___: (___+ ____) x 100 = _____ %

[#vc] [#vc] [#nvc]

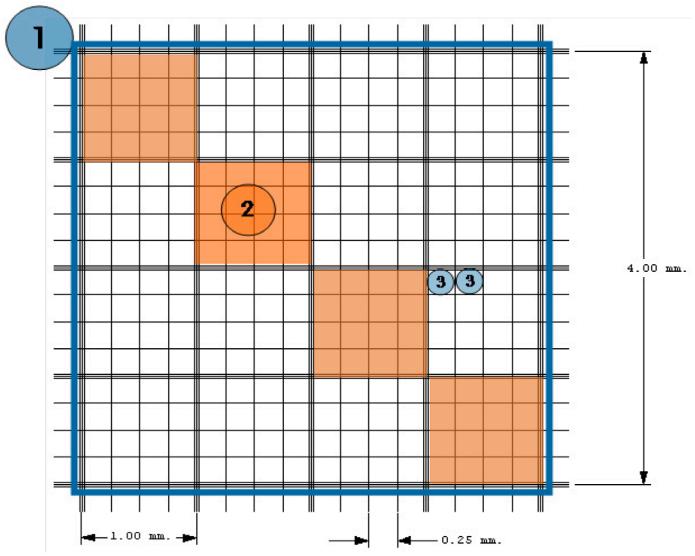

Figure 5.1. Lay-out of a Fuchs Rosenthal Counting chamber.

5.4 Maintenance of the Pluricyte® Cardiomyocytes in the 48-well E-plate®

It is crucial to always refresh the Pluricyte® Cardiomyocyte Medium of the cells one day after seeding the cells (day 1), and subsequently every 2 days (see workflow in **Section 2**).

Per E-Plate®:

23. Place the RTCA Cardio Temperature Tool in the incubator and warm the Temperature Tool to 37˚C.
24. Pipette 6 ml Pluricyte® Cardiomyocyte Medium into a sterile 15 ml conical tube and warm the medium to 37˚C for 20-30 minutes.
25. Immediately before use, transfer the warm medium into a multichannel reservoir. Transfer the E-plate® from the incubator into the pre-warmed Temperature Tool in the flow cabinet.
26. Aspirate the medium from each well using a multichannel aspirator.

Note: Avoid touching the bottom of the plate with the pipette tips to not disturb the cardiomyocyte monolayer.

27. Add 100 µl/well pre-warmed medium to the side of the well using a multichannel pipette.

Note: Avoid touching the bottom of the plate with the pipette tips to not disturb the cardiomyocyte monolayer.

28. Transfer the Temperature Tool and the E-plate® back into the incubator.

5.5 Data acquisition during maintenance

In order to monitor the condition of the Pluricyte® Cardiomyocyte monolayer, it is advised to perform daily measurements during the maintenance, starting at day 1. See the xCELLigence® RTCA CardioECR Software Manual for specific instructions on using the software for data acquisition and analysis. First contractions of Pluricyte® Cardiomyocytes appear between 24-48 hours post-thawing. It will take 3-4 days before the cells have formed an electrically coupled monolayer. The amplitudes of the Extra Cellular Recording (ECR) and Cell Index (CI) signals increase with prolonged culturing. Stable beating monolayers can be observed 7-8 days post-thawing.

For each measurement:

29. Place the E-plate® in the xCELLigence® RTCA CardioECR instrument and start a measurement (e.g. 2 sweeps of 60 seconds at 5 minutes intervals).

Note: Wait >1 hour after medium refreshments before measurement to avoid unstable signals caused by medium change.

5.6 Compound assay

The optimal time window to perform electrophysiology-based assays with Pluricyte® Cardiomyocytes is between 8-12 days after plating the cardiomyocytes. Two different protocols for drug testing, for studying acute and long term drug effects, respectively, are outlined in **paragraph 5.6.1** and **5.6.2** below.

5.6.1 Studying acute drug effects

To study acute drug effects, we recommend to dilute test compounds in Pluricyte® Cardiomyocyte Medium at ≥10x the desired final concentration and to add the compound in a volume of maximum 10% of the final volume of medium in the well (e.g. 10 µl in a final volume of 100 µl). We recommend not to use DMSO concentrations above 0.1%.

29a. Replace the Pluricyte® Cardiomyocyte Medium in the E-plate® ≥1 hour before the compound assay as described in Section 5.4, and place the plate back into the incubator.

30a. Prepare the test compounds at ≥10x the desired final concentration in a 96-well plate and place this compound-plate in an incubator at 37˚C, 5% CO_2 for at least 10 minutes.

31a. Transfer the E-plate® from the incubator into the Temperature Tool (pre-warmed at 37˚C) in the flow cabinet.

32a. Remove the chosen volume (e.g. 10 µl from a final volume of 100 µl) from each well and add the same volume from the 96-well compound plate to the E-plate®.

Note: Mix gently by pipetting 3 times.

33a. Place the E-plate® in the xCELLigence® RTCA CardioECR instrument immediately following compound addition and start measurements (e.g. 5 sweeps of 60 seconds at 5 minutes intervals).

Figure 5.2 provides an example of data acquisition for acute studies.

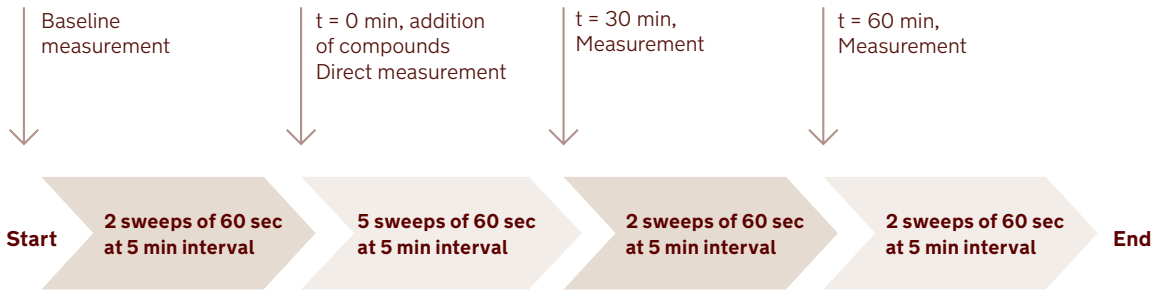

Figure 5.2. Example of a measurement protocol for studying acute drug effects. See the xCELLigence® RTCA CardioECR Instrument Operator's Guide for specific and more optional instructions. For an example with cumulative dosing of test compounds, view our Application Note.

5.6.2 Studying long term drug effects

For a long term study, we recommend to dilute the test compounds in Pluricyte® Cardiomyocyte Medium to the desired final concentration and completely replace the medium in the plate. We recommend to wait at least 30-60 minutes before performing the first measurement to avoid measuring the effects of medium change.

29b. Prepare the test compounds in the desired final concentration in Pluricyte® Cardiomyocyte Medium in a 96-well plate and place the plate in an incubator at 37˚C, 5% CO_2 for 20-30 minutes.

30b. Transfer the E-plate® from the incubator into the Temperature Tool (pre-warmed at 37˚C) in the flow cabinet and aspirate the medium from each well using a multichannel aspirator.

Note: Avoid touching the bottom of the plate with the pipette tips to not disturb the cardiomyocyte monolayer.

31b. Add 100 µl/well from the 96-well compound plate to the side of the well using a multichannel pipette.

Note: Avoid touching the bottom of the plate with the pipette tips to not disturb the cardiomyocyte monolayer.

32b. Incubate the E-plate® at 37˚C, 5% CO_2 for at least 30-60 minutes to avoid measuring the effects of the medium change.

33b. Place the E-plate® in the xCELLigence® RTCA CardioECR instrument and start measurements. **Figure 5.3** provides an example of data acquisition for long term studies.

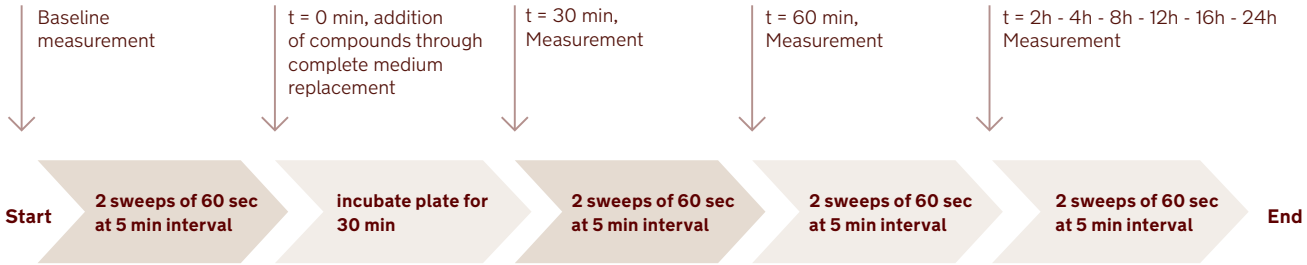

Figure 5.3. Example of an experimental timeline for studying long term drug effects. See the xCELLigence® RTCA CardioECR Instrument Operator's Guide for specific and more optional instructions.

5.7 Data analysis

34. Analyze the acquired data using the xCELLigence® RTCA CardioECR analysis software.

Note: See the xCELLigence® RTCA CardioECR Software Manual for specific instructions for data analysis.

Acknowledgement

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No. 726513.

Ncardia
Stem cell experts