Protocol for iCell Cardiomyocytes² Assay on xCELLigence RTCA CardioECR System
Protocol for iCell Cardiomyocytes\textsuperscript{2} Assay on \textit{xCELLigence RTCA CardioECR System}

\textbf{Introduction}

The \textit{xCELLigence RTCA CardioECR system} enables non-invasive, label-free, real time monitoring of contractile and electrical activity of cardiomyocytes. This system delivers the ability to comprehensively evaluate viability, contractility, and electrical activity involved in excitation-contraction (EC) coupling across the cardiomyocyte monolayer. While the system works well with all cardiomyocytes, this protocol has been optimized specifically for analysis using iCell Cardiomyocytes\textsuperscript{2}. For protocols optimized for usage with cardiomyocytes from other sources, please contact ACEA support at appsupport@aceabio.com.

\textbf{Materials}

A. \textbf{Equipment}
\begin{itemize}
  \item \textit{xCELLigence RTCA CardioECR system} (ACEA Biosciences, Cat \#00380601210)
  \item \textit{CO\textsubscript{2} Incubator} (Multiple Vendors)
  \item \textit{Laminar Flow Hood} (Multiple Vendors)
  \item 8-channel Multichannel Pipettor, 20 and 200 \textmu l (Multiple Vendors)
\end{itemize}

B. \textbf{Reagents, Cell Lines & Consumables}
\begin{itemize}
  \item \textit{E-Plate CardioECR 48} (ACEA Biosciences, Cat \#00300600940)
  \item iCell Cardiomyocytes\textsuperscript{2} Kit (Cellular Dynamics International, Cat \#R1017).
  \item Fibronectin (FN) Stock (Sigma Aldrich, Cat. \#F1141)
  \item Dulbecco’s Phosphate Buffered Saline without Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (DPBS) (Multiple Vendors)
\end{itemize}
Day 0 – Experiment Setup

A: Coating the E-Plate CardioECR 48
Time: About 1.5 hours
1. Dilute the fibronectin (FN) stock 1:100 in sterile DPBS to a final concentration of 10 μg/mL.
2. Transfer 50 μL of diluted FN to each well of the E-Plate CardioECR 48.
3. Incubate the coated E-Plate CardioECR 48 at 37 °C for at least 1 hour or 4°C overnight.

B: Measure the background
Time: About 5-10 minutes
1. Replace the coating buffer in the E-Plate CardioECR 48 with 50 μL of pre-warmed iCell Cardiomyocytes Plating Medium.
2. Insert the E-Plate CardioECR 48 into the RTCA CardioECR station inside a CO₂ incubator according to the CardioECR System Operator’s Guide.
3. Start the RTCA CardioECR Data Acquisition Software and click on the Layout tab. Select the sample wells by highlighting the area in the well map corresponding to the sample wells. Enter the requested experimental information and click Apply.
4. Click on the Schedule tab, then add Step_1 for the background measurement.
   - Sweep number, interval and duration settings for Step 1 are preset for background measurements and should not be changed.
5. Click on the Start/Continue icon to obtain the background reading. The background reading step will take 1 second.
6. Remove the E-Plate CardioECR 48 from the station and place in a laminar flow hood for cell seeding.

C: Seeding iCell cardiomyocytes² to E-Plate CardioECR 48 wells
Time: About 1.5 hours
1. Refer to CDI iCell Cardiomyocyte² user’s guide on thawing, counting, and seed 50,000 cells/well in an E-plate cardioECR.
2. After cell seeding, leave the E-Plate CardioECR 48 in the laminar flow hood for 30 minutes at room temperature to ensure even cell distribution in the wells.
3. Transfer the seeded E-Plate CardioECR 48 to the incubator. Allow for 15 minutes of temperature equilibration before inserting the plate into the RTCA CardioECR station.
   - Temperature equilibration is recommended for preventing condensation on the bottom of the E-plate CardioECR 48, which will interfere with accurate assessment of the field potential signal.
4. Click on the Schedule tab and add Step_2 to monitor cell attachment and growth (optional).
   - Recommended schedule step:
     1) 100 sweeps of 20-second block duration (extra sweeps can be aborted)
     2) 6 hour interval between each sweep
     3) Sampling rate:
       Impedance: 2 or 12.8 ms
       ECR: 0.1 ms (10 KHz)
5. Click on Start/Continue icon to start Step_2.

D: Replacing Medium
Time: About 10 minutes
1. Medium should be replaced 4 hours post-seeding. Incubate the iCell cardiomyocytes Maintenance medium in a 37°C water bath.
   - It is important to use medium equilibrated to 37°C for the medium change.
2. Pause the RTCA CardioECR System monitoring by clicking on the Pause button in the RTCA CardioECR software. Remove the E-Plate CardioECR 48 from the CardioECR Station and transfer the plate to a laminar flow hood. Slightly tilt the plate, and using a multichannel pipette, gently remove the plating media completely from the wells.
3. Slowly add 100 μL of the equilibrated media by tilting the E-Plate CardioECR 48 at an angle and adding the...
medium to the side of the well.

⚠️ Do not disturb the cell monolayer during the medium change.

4. Bring the E-Plate CardioECR 48 back to the incubator. After 15 minutes of temperature equilibration, insert the E-plate CardioECR 48 into the CardioECR Station and resume measurements by clicking on the Start/Continue icon.

Tip: The iCell Cardiomyocytes beating signal should be detectable within 24-48 hours after cell seeding. However, beating frequency will be variable after switching from plating medium to culture medium. It becomes more regular 24-48 hours after switching medium.

Day 2 – 6 – Cell maintenance

E: Cell culture maintenance

Time: About 10

1. Replace the iCell cardiomyocytes Maintenance medium every 48 hours. Follow the procedure “Replacing Medium” for medium change.

2. Monitor the beating activity and field potential performance on a daily basis until the iCell Cardiomyocytes duplicate a synchronous beating profile. Coefficient of variation (CV) of beating period during each recording should be less than 10%.

Day 5-6: Cell status Quality Control (QC) Before Compound Addition

F: Measure cell beating and field potential signals

Time: 4 hours

a) If the medium needs to be refreshed on cell status QC day

1. Equilibrate the iCell culture medium in a 37°C water bath.

2. Replace culture medium following the steps described in the “Replacing Medium” section.

3. Incubate cells on the station for at least 4 hours before taking measurements for QC assessment.

4. Click Schedule tab to add Step_3 for cell status QC purposes.

Recommended schedule step for cell status QC measurement:

1) 5 sweeps of 20-second block duration

2) 1 hour interval between each sweep

3) Sampling rate:
   - Impedance: 2 or 12.8 ms
   - ECR: 0.1 ms (10 KHz)

b) If NO medium change is needed on cell status QC day

Recording can be performed right away.

Recommended schedule step for data QC measurement:

1) 5 sweeps of 20-second block duration

2) 1 hour interval between each sweep

3) Sampling rate:
   - Impedance: 2 or 12.8 ms
   - ECR: 0.1 ms (10 KHz)
Cell status Quality Control
Cardio mode test (beating activity) recommended conditions:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Recommended reading</th>
<th>Recommended CV across different recording time points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beating rate (BR)</td>
<td>BR &gt; 20 beats/min</td>
<td>CV &lt; 15%</td>
</tr>
<tr>
<td>Beating amplitude (Amp)</td>
<td>Amp &gt;= 0.07</td>
<td>CV &lt; 15%</td>
</tr>
<tr>
<td>Beating rhythm irregularity (BRI)</td>
<td>BRI &lt; 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Note:

i.  Ensure Cardio signal is robust, stable and consistent during 4 hour recording to obtain good FP signal.
ii. Stable Cardio signal is normally achieved before stable FP signal occurs.

ECR mode test (electrical activity) recommended conditions:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Recommended Reading</th>
<th>Recommended CV across different recording time points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firing Rate (FR):</td>
<td>FR &gt; 20 times/min</td>
<td>CV &lt; 15%</td>
</tr>
<tr>
<td>Sodium Spike amplitude (Amp)</td>
<td>a. 90% of electrodes detect &gt; 0.1 mV of Sodium Spike &lt;br&gt;b. 90% of electrodes have consistent amplitude of Sodium spike</td>
<td>CV &lt; 15%</td>
</tr>
</tbody>
</table>

Day 7: Compound addition

A. Cell Culture Preparation  
Time: 10 minutes

1) At least 4 hours prior to compound addition, replace the culture media with 90 µL of fresh media equilibrated to 37°C.

Note: Evaporation rates can vary across the E-Plate CardioECR 48. Changing the Maintenance Medium before compound treatment is required to ensure uniform medium volumes across the E-Plate.

B. Measure Baseline  
Time: 35 minutes

2) Measure baseline of beating activity prior to compound treatment. Click on the Schedule tab to add next Step for the baseline measurement of beating and electrical activity.

Recommended schedule step for baseline measurement:  
1) 7 sweeps of 60-second block duration  
2) 5 minutes interval  
3) Sampling rate:  
   Impedance: 2 or 12.8 ms  
   ECR: 0.1 ms (10 KHz)
C. Compound preparation and dilution

Time: About 1 hour

1. Determine the predicted effective concentration ranges for the test substances.
2. Prepare stock solutions of test compounds by dissolving the compounds in appropriate solvent. If DMSO is used as the solvent, dissolve the compound in a high stock concentration, if possible, (ideally 1000-fold of the highest test concentration) and store at -20°C.
3. Thaw the compound stock solutions and prepare 1000X concentrated serial dilutions of compounds in appropriate solvent (DMSO or H₂O).
4. Transfer solvent diluted compounds to the wells of a V-bottom microtiter plate for further dilution in culture medium (to a final concentration of 10X).

D: Compound Addition

Add the diluted compound and monitor for short-term (<1 hr) or long-term (24 hr)

1. Abort Step_3 for baseline measurement, by clicking on the Pause icon first. A pop-up window will appear asking you to confirm termination of the step. Click Yes.
2. Set up Steps for compound testing in the Schedule section, and make sure the Auto reading box is checked.

**Recommended schedule step for short-term monitoring (<1 hr)**

1) 13 sweeps of 60-second block duration
2) 5 minutes interval
3) Sampling rate:
   - Impedance: 2 or 12.8 ms
   - ECR: 0.1 ms (10 KHz)

**Recommended schedule step for long-term monitoring (<24 hr)**

1) 23 sweeps of 60-second block duration
2) 1 hr interval between each sweep
3) Sampling rate:
   - Impedance: 2 or 12.8 ms
   - ECR: 0.1 ms (10 KHz)

⚠️ Make sure to enter compound names and tested concentrations in the layout page.

3. Before removing cells from the incubator for compound treatment, disengage the E-Plate CardioECR 48 from the RTCA CardioECR Station. Transfer the plate with cells onto the stabilization core of the Temperature Tool to minimize temperature fluctuation.
4. Carefully, but quickly, add the compound to the cells. Using a multichannel pipette, transfer 10 μL of compounds diluted in culture medium from the V bottom microtiter plate to the E-Plate CardioECR 48.

⚠️ It is very important to include wells treated with the solvent only at the same final concentration as compound treated wells for the negative control.

⚠️ It is important to have a multichannel pipette, tips and tip waste box ready. This will ensure the minimum time for adding compounds and exposing cells to the ambient temperature and oxygen levels outside of the tissue culture incubator.
5. Insert the E-Plate CardioECR 48 into the CardioECR Station. Initiate the next step immediately.

⚠️ In order to capture all the information on electrical and beating activity changes after compound addition, the process of compound addition should not exceed 3-5 minutes. Ensure that all the recording schedules are pre-programmed to help minimize this time.