EarlyTox™ Cardiotoxicity Kit

The EarlyTox™ Cardiotoxicity Kit from Molecular Devices provides a fast, simple, and reliable fluorescence-based method for characterizing cardiotoxic compounds in a biorelevant assay using stem-cell derived cardiomyocytes. The kit is based on a novel calcium sensitive indicator paired with proprietary masking technology, providing mix-and-read procedures for measuring intracellular calcium flux related to cardiomyocyte contractions.

Table 1-1: Available Kits

Assay Kit	Explorer Kit	Bulk Kit
EarlyTox™Cardiotoxicity Kit	R8210	R8211

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3860 N. First Street, San Jose, California, 95134, United States of America.
Molecular Devices, LLC is ISO 9001 registered.

Chapter 1: About the EarlyTox Cardiotoxicity Kit

There is a need for cardiotoxicity and cardio-discovery assays that use more biologically relevant cell-based models to help with the development of new chemical entities and ensure drug safety. The EarlyTox Cardiotoxicity Kit has been optimized to provide a robust, high throughput method for measuring the impact of pharmacological compounds on stem cell derived cardiomyocytes, including measurement of the peak rate, amplitude, peak width, and other critical parameters.

Calcium assays from Molecular Devices employ sensitive calcium indicators and masking dyes. The EarlyTox Cardiotoxicity Kit contains an optimized dye formulation that increases the signal window while having minimal non-specific toxicity and impact on the peak rate and other peak characterization parameters. Other commercially available dyes and kits demonstrate non-specific effects on multiple parameters of cellular beating, all within the time-frame required for measurement of compound effects. The cardiotoxicity dye is mixed with the provided buffer and incubated for approximately two hours with cells, before assay. During incubation, the indicator passes through the cell membrane, and esterases in the cytoplasm cleave the AM portion of the molecule. The masking dye does not enter the cell, but significantly reduces background originating from residual extracellular fluorescence of the calcium indicator, media, or other components. This removes the requirement for washing the cells in order to obtain a robust signal window. As cardiomyocyte peak rates are sensitive to buffer changes; it is critical for the cells to remain in media to produce consistent high quality results.

After incubation with the dye, the cells are ready to be assayed. Compounds are added and measurements taken using the FLIPR® Penta High-Throughput Cellular Screening System, FLIPR® Tetra High-Throughput Cellular Screening System, or the SpectraMax® i3x Multi-Mode Detection Platform. Direct measurement of intracellular fluorescence change due to changes in intracellular calcium concentrations is enabled. This change in calcium concentration correlates to the oscillation rate and oscillation pattern of the cardiomyocyte, as further discussed in Assay Principle on page 4.

Three reference compounds are included in the kit:

- Isoproterenol, a non-selective ß-adrenergic agonist that induces positive chronotropic and inotropic effects. Isoproterenol (isoprenalin) is used for the treatment of bradycardia (slow heart rate) or heart block.
- Propranolol, a non-selective blocker of both β1-adrenergic and β2-adrenergic receptors. It inhibits the action of agonists of β-adrenergic receptors.
- Sotalol is a drug used for treatment of arrhythmias. It is a non-selective competitive β-adrenergic receptor blocker that also inhibits potassium channels. It changes the normal contraction pattern.

Assay Principle

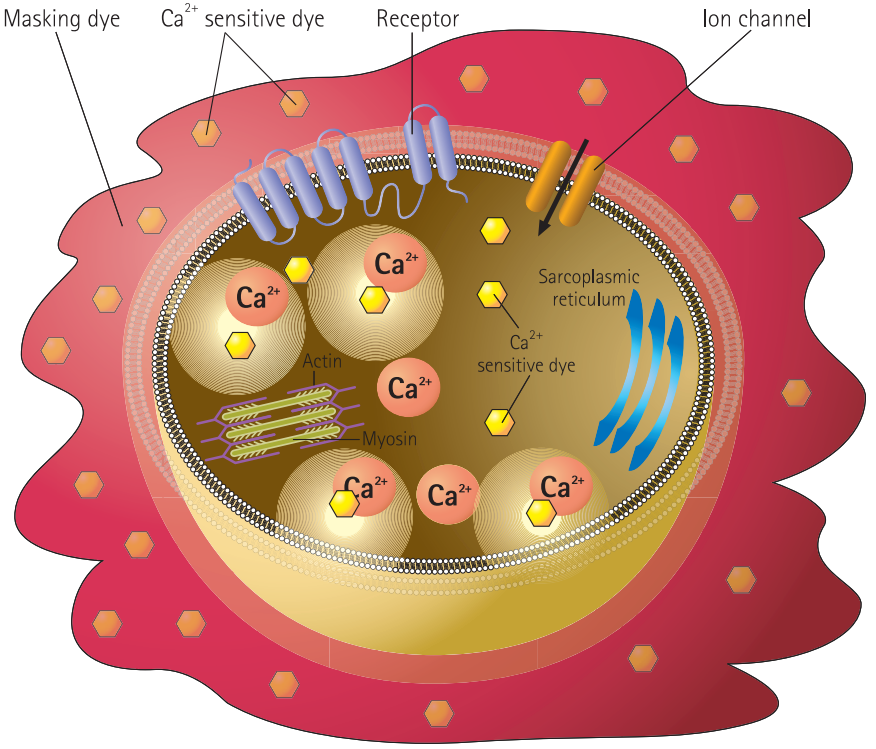


Figure 1-1: Cardiotoxicity Kit assay principle

EarlyTox Cardiotoxicity dye binds with calcium ions as they enter the cell cytoplasm enabling measurement of the change in calcium concentration. During a single calcium oscillation event, Ca^{2+} is released into the cytoplasm after the sarcoplasmic reticulum is stimulated. Calcium binds with troponin activating the sarcomere, and the cell contracts. Synchronously, there is an increase in fluorescent signal as dye binds to free calcium in the cytosol. Cellular relaxation occurs on removal of calcium from the cytosol by calcium uptake pumps of the sarcoplasmic reticulum and by calcium exchange with extracellular fluid. The fluorescent signal decreases as calcium concentration decreases. As the cycle repeats, more fluorescent peaks synchronous with calcium oscillation are observed.

Note: This is a simplified description of the cellular actions occurring. For a more detailed explanation of the methods of calcium changes and their relation to relaxation-contraction we recommend more in-depth reading of the literature related to the actions of cardiac muscle physiology. Some readings are referenced in Bibliography—Selected Readings on page 16.

Advantages

Molecular Devices developed the EarlyTox Cardiotoxicity Kit to deliver a robust, high-throughput, biologically relevant assay solution enabling earlier prediction of compound toxicity and efficacy.

- Eliminate cardiotoxic compounds and identify potential drug candidate
- Study functional profiles in a biorelevant cardiotoxicity assay
- High-throughput: measure 384 samples in minutes rather than hours
- Minimal non-specific effect of dye on calcium oscillation characteristics
- Reduced well-to-well variation, improved data quality
- Enhanced signal dynamic range
- Minimal cell perturbation (no wash required)
- Adaptable for use 96-well, 384-well, and 1536-well formats

Applications

The EarlyTox Cardiotoxicity Kit assay is designed to detect the effects of compounds on the beat characteristics of cardiomyocytes. It employs a calcium sensitive dye to monitor changes in intracellular calcium concentrations, which in turn are synchronous with cell beating. Assay results can help with the detection of efficacy or toxicity of compounds. The EarlyTox Cardiotoxicity Kit uses a newly improved calcium dye formula and patented quench technology that further enhance the signal window, minimize impact on beating rate over time, and make difficult assays more amenable for high-throughput screening.

This EarlyTox Cardiotoxicity Kit has been tried with the following types or sources of cardiomyocytes:

- iCell® Cardiomyocytes and iCell® Cardiomyocytes^2, human induced pluripotent stem (iPSC) cell-derived cardiomyocytes from Fujifilm Cellular Dynamics International.
- Cardiomyoctyes derived from H7 human embryonic stem cells (hESC).
- Cor.4U® Cariomyocytes from Ncardia, Ag.

Chapter 2: Materials and Equipment

Kit Components

Table 2-1: Components of the EarlyTox Cardiotoxicity Kits

Item	Explorer Kit (R8210)	Bulk Kit (R8211)
Component A Loading dye	2 vials	2 vials
Component B Dilution buffer: 1X DPBS (with Ca^{2+} and Mg^{2+}) plus 20 mM HEPES buffer, pH 7.4	1 bottle	1 bottle
Reference compounds: isoproterenol, sotalol, propranolol. When dissolved in 50 µL DPBS or DMSO, each aliquot will result in 20 mM concentration.	3 vials	3 vials

- The entire Explorer Kit (R8210) is sufficient for two 96-well or 384-well plates. Each vial is sufficient for one 96-well or 384-well plate.
- The entire Bulk Kit (R8211) is sufficient for ten 96-well, 384-well, or 1536-well plates. Each vial is sufficient for assaying five 96-well, 384-well, or 1536-well plates.

Storage and Handling

On receipt of the EarlyTox Cardiotoxicity Kit, store the contents at –20°C (-4°F). Under these conditions the reagents are stable for six (6) months in the original packaging.

WARNING! Reagents can contain chemicals that are harmful. Exercise care when handling reagents as described in the related safety data sheet (SDS or MSDS). The safety data sheet is available in the Knowledge Base on the Molecular Devices support web site: www.moleculardevices.com/service-support

Compatible Molecular Devices Instruments

The EarlyTox Cardiotoxicity Kit is designed to be used with the following Molecular Devices instruments:

- FLIPR Penta High-Throughput Cellular Screening System
- FLIPR® Tetra High-Throughput Cellular Screening System
- FlexStation 3 Multi-Mode Microplate Reader
- SpectraMax® i3x Multi-Mode Microplate Reader
- SpectraMax® Paradigm® Multi-Mode Microplate Reader

Chapter 3: Experimental Protocol

Quick Start Protocol

To run a cardiac calcium oscillation assay protocol:

1. Plate the thawed iPSC derived cardiomyocyte cells in 96-well or 384-well microplates as recommended by the cell provider protocol and incubate for 10 to 14 days at 37°C, 5% CO_2 or as directed by your protocol or a manufacturer's protocol. Feed the cells every second day or as recommended in the cell provider protocol.
2. Prepare the loading buffer on the same day as the assay.
3. Remove the cell plates from the incubator.
   - (Optional) Change the media in the microplates before the experiment. Add 25 µL of new media.
   - EarlyTox Cardiotoxicity Kit: Add an equal volume of loading buffer to each well, for example: 25 µL of loading buffer to 25 µL of cells and media for a 384-well plate.
4. Return the plates to the incubator and incubate two hours at 37°C, 5% CO_2.
5. For assay development, prepare the compound plates, include reference concentration response curves of reference compounds as positive controls. Molecular Devices recommends half log serial dilutions starting at: isoproterenol 10 µM, sotalol 100 µM, and propranolol 100 µM.
6. Run the experiment on a FLIPR Penta System, FLIPR Tetra High-Throughput Cellular Screening System, or SpectraMax i3x Multi-Mode Microplate Reader, or other compatible Molecular Devices instrument. See Compatible Molecular Devices Instruments on page 6.

Cell Handling

The EarlyTox Cardiotoxicity Kit assay is designed to work with stem cell derived cardiomyocytes or primary cardiomyocytes. Standard procedures vary across laboratories and Molecular Devices recognizes that a variety of cell handling conditions might be used at the discretion of the user. This section contains general guidelines for preparing cells for use with the assay kit. iPSC-derived cardiomyocytes or ES-derived cardiomyocytes are the most frequently used cells. They are generally plated 10 to 14 days before an experiment and then incubated in a 5% CO_2, 37°C incubator. See specific cell vendor protocols for recommended cell plating and handling. Molecular Devices recommends optimizing the assay and cell conditions for specific applications.

Table 3-1: General Plating Volumes and Seeding Densities

Cell Type (cells/well)	96-well microplate (100µL growth medium)	384-well microplate (25µL growth medium)
iCell Cardiomyocytes (human-iPS cell derived, from Cellular Dynamics International)	40000 to 80000	8000 to 15000
H7 cardiomyocytes (human-ESC cell derived)	Not determined at time of publication	40000 to 60000

Preparing the Loading Buffer

The following procedure is designed for the preparation of the EarlyTox Cardiotoxicity Kit loading buffer per vial of the Explorer Kit. More volumes for the Bulk Kit are included in Table 3-2.

To prepare the loading buffer:

1. Remove one vial of EarlyTox Cardiotoxicity Dye (Component A) from the freezer and equilibrate to 37°C. Also equilibrate Component B to 37°C.
2. Dissolve contents of the loading dye (Component A) vial by adding 10 mL of dilution buffer (Component B) or 1X DPBS Buffer plus 20 mM HEPES. Mix by vortexing ~1 to 2 minutes until contents of vial are dissolved.

Note: It is important that the contents are completely dissolved to ensure reproducibility between experiments.

Table 3-2: Required Volumes of DPBS plus 20 mM HEPES (or Component B)

Microplate Format	DPBS + 20mM HEPES or Component B	Explorer Kit (R8210)	Bulk Kit (R8211)
96-well or 384-well	Volume to dissolve loading dye (Component A)	10 mL	10 mL
	More required for correct volume	None	40 mL

3. Dissolve contents of the reference compounds by adding 50 µL of DMSO or 1X DPBS Buffer plus 20 mM HEPES. Mix by pipetting until contents of vial are dissolved. Final concentrations in the vial is 20 mM of each compound.

Note: As an alternative, 10 mM of compound solutions can be prepared by adding 100 µL DMSO or DPBS.

CAUTION! The components supplied are sufficient for proper cell loading. For optimal results it is important not to add other reagents or change volumes and concentrations.

Loading Cells Using Loading Buffer

To load cells using loading buffer:

1. Remove cell plates from the incubator. It is not necessary to remove the culture media. Add an equal volume of loading buffer to each well: 100 µL per well for 96-well microplates, 25 µL for 384-well microplates.
2. After adding dye, incubate cell plates for ~ 2 hours at 37 °C (loading time can be optimized for specific cell source and experiment).

CAUTION! Do not wash the cells after dye loading.

Note: Applicable compound dilutions can be prepared during this step.

3. (Optional) Read the plate after two hours of loading dye (pre-read before compound addition) as suggested below.
4. Add 5x concentrations of compounds manually or using fluidics. Incubate 10 to 30 minutes (optimal time for compound treatment can be optimized for specific cell source and experiment).
5. Read the microplate.
6. (Optional) Repeat the plate read as many times as needed with a read only protocol to monitor compound effects at different time points.

Note: Keep the plate in the incubator between reads.

Diluting Reference Compounds and Preparing the CRC Plate

To dilute reference compounds and prepare the CRC plate:

1. Dissolve reference compounds in 50 µL of DMSO or Component B buffer to make 20 mM stock concentration.
2. Dilute stock concentrations to prepare 5x solutions so that when added to the cells you have the following final concentrations:
   Molecular Devices recommends performing half log serial dilutions and to start each concentration response curve (CRC) at the following highest dose:
   - Isoproterenol at 10 µM
   - Sotalol at 100 µM
   - Propranolol at 100 µM

Running the Cardiac Beating Assay on a FLIPR Instrument

To run a cardiac beating assay:

1. Pre-warm the instrument deck to 37°C before the experiment.
2. After the incubation, transfer the microplates directly to the FLIPR instrument and start the assay as described in the instrument user guide.
3. When running a signal test before an experiment, adjust typical average baseline counts depending on the camera installed in your system.
   - For a FLIPR Tetra and FLIPR Penta instrument with an EMCCD camera, adjust typical average baseline counts to a range from 800 to 1100 RFU.
   - For a FLIPR Tetra and FLIPR Penta instrument with an ICCD camera, adjust typical average baseline counts to a range from 5000 to 7000 RFU.
   - For a FLIPR Penta instrument with an HS EMCCD camera, adjust the typical average baseline to about 5,000 RFU.
4. Run a couple of read only assays to see how the amplitude shows with the baseline you have set, and adjust as needed.
5. Molecular Devices recommends the experiment setup parameters for each FLIPR instrument as listed in Table 3-3. However, further assay development and adjustment of the volume, height, and speed of dispense are recommended to optimize the individual cell response.

Recommended Settings for the FLIPR Instrument

Before reading the microplate, set up your FLIPR instrument and using the following recommended experiment settings. Your settings depend on the camera installed in your instrument.

Table 3-3: Experiment setup parameters for the FLIPR Tetra and FLIPR Penta Instrument

Parameter	96-well microplate	384-well microplate
Camera Mode	**HS EMCCD camera:** HighSpeed	**HS EMCCD camera:** HighSpeed
Excitation LED (nm)	470–495	470–495
Emission Filter (nm)	515–575	515–575
Camera Gain	EMCCD camera: 130 HS EMCCD camera: 4 ICCD camera: Fixed at 2000	EMCCD camera: 130 HS EMCCD camera: 4 to 50 ICCD camera: Fixed at 2000

Table 3-3: Experiment setup parameters for the FLIPR Tetra and FLIPR Penta Instrument (continued)

Parameter	96-well microplate	384-well microplate
Exposure (sec)	0.05 **Note:** 0.05 second exposure or less depending upon the acquisition rate (interval) chosen. 30 Hz or 0.03 seconds with a 0.02 second exposure interval is an optimal read for peak detail of iPSC Cardiomyocytes.	0.05 or less (see note)
LED Intensity (%)	EMCCD camera: 80 HS EMCCD camera: 50 ICCD camera: 50	EMCCD camera: 80 HS EMCCD camera: 50 ICCD camera: 50
Camera Gate (%) (ICCD camera only, adjust so camera does not saturate)	22	22
Addition Volume (µL)	50	12.5
Compound Concentration (Fold)	5X	5X
Addition Height (µL)	210 to 230	35 to 45
Addition Speed (µL/sec)	Adherent Cells: 50	Adherent Cells: 30
Tip-Up Speed (mm/sec)	10	10

When using the HS EMCCD camera in HighSpeed camera mode, since biology varies, run a Protocol Signal Test with a sample plate to determine your specific protocol exposure settings. Use the recommended settings as a starting point. If your experiment could have brightness that exceeds your signal test sample plate, then reduce the exposure settings accordingly so that your brightest signal does not exceed 43,000 counts. Refer to the *FLIPR Penta High-Throughput Cellular Screening System User Guide* topic "Understanding HS EMCCD Camera Protocol Saturation."

Running the Cardiac Beating Assay on a SpectraMax Intruments

To run a cardiac beating assay:

1. Pre-warm the microplate chamber to 37°C before reading the plate.
2. After incubation, transfer the microplates directly to the SpectraMax i3/i3x readers.
3. Use the protocol named **EarlyTox Cardiotoxicity (Basic)** available in the Protocol Library. This protocol is designed for use with SpectraMax i3/i3x readers and is found in the SoftMax® Pro Software folder **Protocol Library > Molecular Devices Reagents**. If using another plate reader, use this protocol and adjust instrument settings using Table 3-4 as a guideline.
4. Molecular Devices recommends the experiment setup parameters listed in Table 3-4. However, further assay development adjustment of the parameters can be made for optimal results.

Recommended Settings for the SpectraMax Intruments

Before reading the microplate, define the settings for the using the recommended parameters listed in Table 3-4.

Table 3-4: Experiment setup parameters for either the SpectraMax i3/i3x, Paradigm, or FlexStation 3 Intruments

Parameter	Setting
Optical Configuration	Monochromator
Read Mode	FL (fluorescence intensity)
Read Type	Kinetic
Wavelengths	Excitation: 485 nm Emission: 535 nm
Bandwidth	Excitation: 9 nm Emission: 15 nm Or default bandwidth setting for other instruments
PMT and Optics	Flashes per read: 2 Read from Bottom
Timing Settings	Well Run Time: 25 to 40 sec Interval: 0.1 sec
More Settings	Read Order: Well

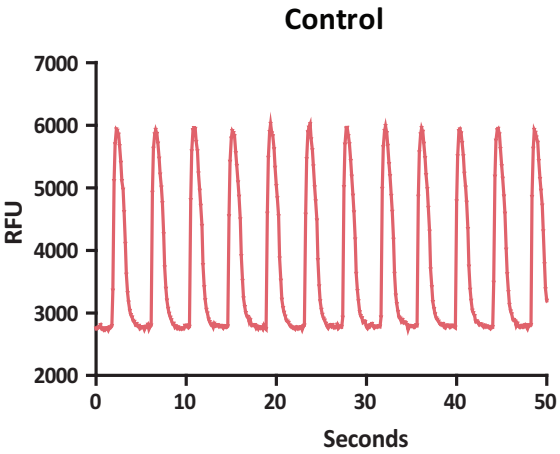
Data Analysis Examples

iPSC derived cardiomyocyte assay experimental protocol

The data below were generated using iCell® Cardiomyocytes, human induced pluripotent stem (iPS) cell-derived cardiomyocytes from Cellular Dynamics International. The cells were thawed and plated in a volume of 25 µL in a 384-well, black-wall, clear-bottom microplate based on vendor protocol. Cells were incubated and fed for 10 days before the assay. On the day of the assay, dye loading buffer was prepared and all assay preparation steps are as described in Experimental Protocol, see page 7.

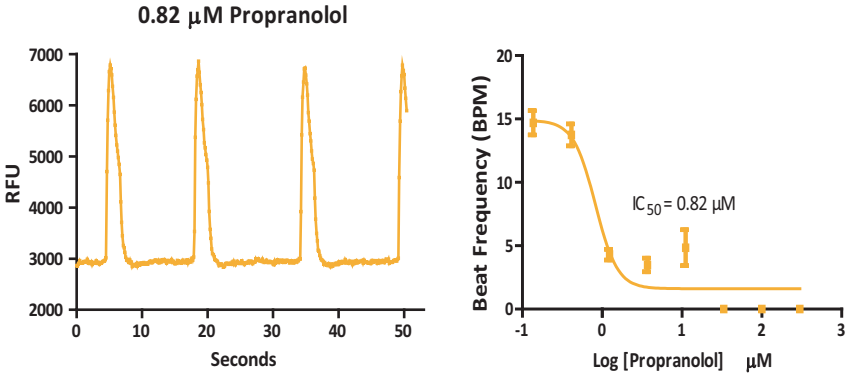
For the FLIPR Penta data and FLIPR Tetra data that follows, cell plates were transferred to the instrument, and compounds were added simultaneous with detection. Cells were returned to the incubator for 20 minutes, and then read again following a protocol for 800 reads at 0.05 second intervals without pipetting. ScreenWorks Software was used to analyze and export either beats per minute (BPM) or Time Sequence data to GraphPad Prism for graphing. EC_{50} or IC_{50} values were determined for control and each of the three reference compounds. Beat profiles were also analyzed as they can enable identification of the effects of compounds that interfere with potassium or other ion channels such as hERG blockers.

Untreated Control iPSC Cardiomyocytes 20 Minutes Post Compound Addition



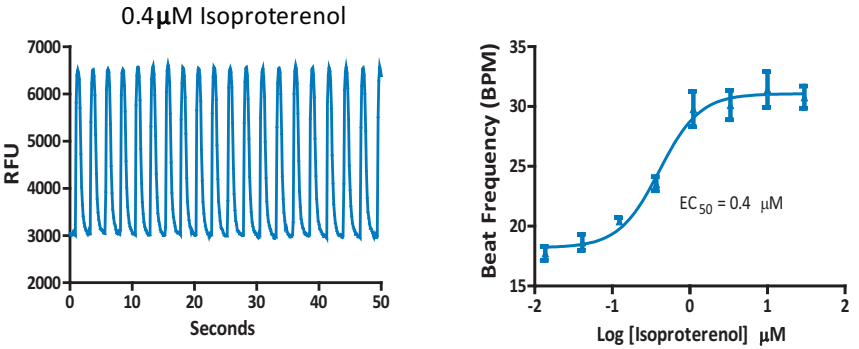
In the figure above, changes in fluorescent signal reflect the contractions or beating of the entire well of iPSC derived cardiomyocytes loaded with Cardiotoxicity Dye. As the cells contract and relax, changes in intracellular calcium concentration are reflected by the change in the fluorescent signal. EarlyTox Cardiotoxicity Kit can be used to record signal traces using the FLIPR Tetra instrument or FLIPR Penta instrument for several hours after compound addition as it has minimal non-specific toxicity compared to other available dyes. In addition, the larger signal window provided by the dye can help with determination of cell behavior when the signal is small.

iPSC Cardiomyocytes Treated with Propranolol Exhibit a Slower Peak Frequency



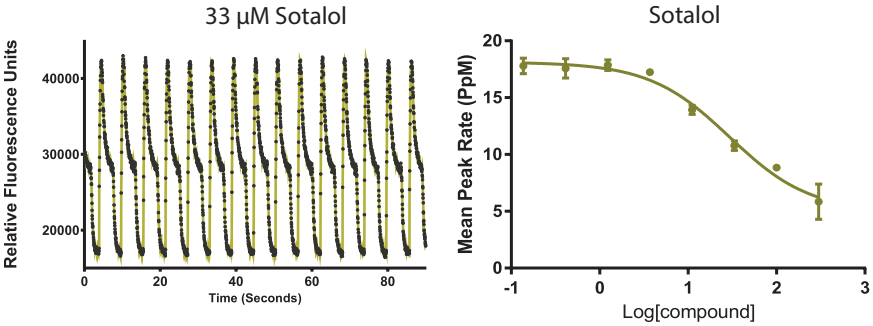
In the figure above, cells are treated with propranolol, which inhibits the action of ß-adrenergic agonists. It slows the beat frequency or contraction rate of the cells. IC_{50} values are determined from the change in contraction rate (beat frequency) vs. concentration of compound.

iPSC Derived Cardiomyocytes Treated with Isoproterenol Show Increased Peak Frequency



In the figure above, isoproterenol is shown to increase the contraction rate of the cells. Isoproterenol is a positive chronotrope that is a non-selective ß-adrenergic agonist. This data point was measured 20 minutes after compound addition, however BPM can be re-measured over several hours to determine delayed toxicity effects. EC_{50} values are determined from the peak frequency vs. concentration of compound.

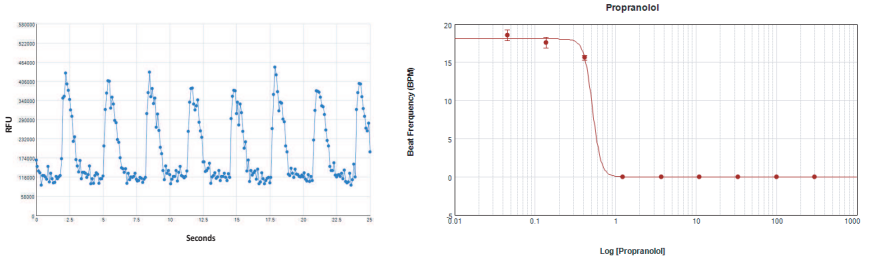
iPSC Cardiomyocytes Treated with Sotalol Show Decreased Peak Frequency and Changed Beat Pattern



In the figure above, cells were treated with sotalol, a non-selective ß blocker that also inhibits potassium channels and changes the normal beat pattern. The IC_{50} value was calculated in . EarlyTox Dye enables the identification of compounds that might have an unintended effect of affecting potassium or other ion channels early in the drug discovery process. The above experiment was run with the HS EMCCD camera at 30 Hz or an interval of .03 seconds to capture peak detail.

iPSC Cardiomyocyte Experiment on SpectraMax i3x instrument

In addition to the FLIPR Instrument, the cardiomyocyte assay has also been validated for the SpectraMax i3x instrument. The cells were prepared and dye loaded in the same way as the assay on the FLIPR Instrument. Compound was added off line and the plate was read for 25 seconds/well and an interval of 0.1 seconds on the 1 to 2 hours post compound addition. The protocol entitled **EarlyTox Cardiotoxicity (Basic)** was used to run the assay and analyze the data. Settings are summarized in Recommended Settings for the SpectraMax Intruments, see page 12.



In the figure above, cells were incubated with Cardiotoxicity dye for 2 hours. The cells were then treated with propranolol for two hours before reading on the SpectraMax i3x instrument. Each well was read for 25 seconds to determine the rate of cell beating. The EC_{50} value is determined from the average peak frequency per well. The minimal non-specific toxicity of the Cardiotoxicity Dye enables cardiomyocyte assays to be done on microplate readers, where the throughput is slower since samples are measured one well at a time.

Obtaining Support

Molecular Devices is a leading worldwide manufacturer and distributor of analytical instrumentation, software, and reagents. We are committed to the quality of our products and to fully supporting our customers with the highest level of technical service.

Our Support website, www.moleculardevices.com/service-support, has a link to the Knowledge Base, which contains technical notes, software upgrades, safety data sheets, and other resources. If you still need assistance after consulting the Knowledge Base, you can submit a request to Molecular Devices Technical Support.

Please have the instrument serial number (on the rear of the instrument), or reagent kit lot number, and any related sample data files available when you call.

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