FLIPR® Potassium Assay Kit

Table A-1: Available Kits

Assay Kit	Evaluation Kit	Explorer Kit	Bulk Kit
FLIPR® Potassium Assay Kit	R8330	R8222	R8223

The FLIPR® Potassium Assay Kit from Molecular Devices® provides a fast, simple, and robust fluorescence-based, high-throughput assay for measuring potassium channel activity. This kit, combining a highly sensitive thallium indicator dye with a Molecular Devices proprietary masking dye technology, provides an homogeneous assay with an expanded signal window and an improved signal-to-noise ratio.

R3622 C

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MOLECULAR DEVICES

Product manufactured by Molecular Devices, LLC.
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Chapter 1: About the FLIPR Potassium Assay Kit

Assay Principle

The assay exploits the permeability of thallium ions (Tl^+) through both voltage-gated and ligand-gated potassium (K^+) channels. In this assay, a novel, highly-sensitive Tl^+ indicator dye is used. This dye produces a bright fluorescent signal upon the binding to Tl^+ conducted through potassium channels. The intensity of the Tl^+ signal is proportional to the number of potassium channels in the open state. Therefore, it provides a functional indication of the potassium channel activities. In addition, one of the Molecular Devices proprietary masking dyes is employed to further reduce background fluorescence for improved signal-to-noise ratio.

During the initial dye-loading step, the Tl^+ indicator dye as an acetoxymethyl (AM) ester enters the cells through passive diffusion. Cytoplasm esterases cleave the AM ester and relieve its active thallium-sensitive form. To activate the potassium channels, the cells are then stimulated with either a mixture of K^+ and Tl^+, or a ligand in the presence of Tl^+. The increase of fluorescence in the assay represents the influx of Tl^+ into the cell specifically through the potassium channel, providing a functional measurement of potassium channel activity.

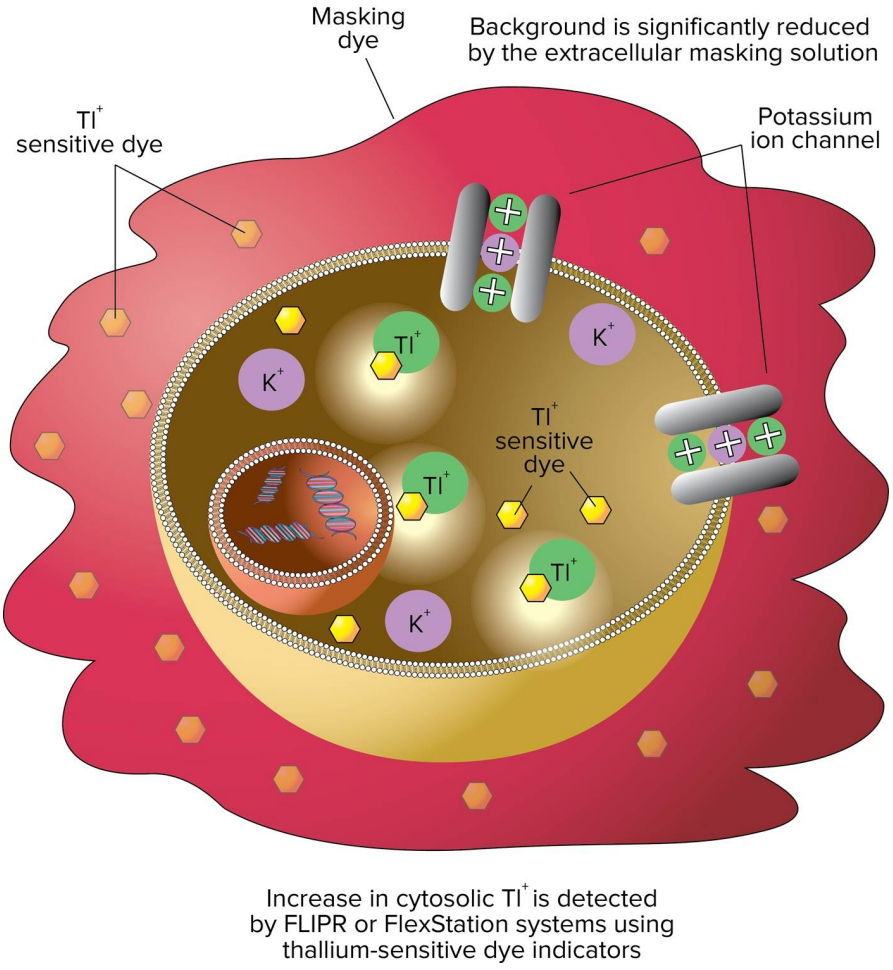

Figure 1-1: Assay principle of the FLIPR Potassium Assay Kit

Advantages

Currently, thallium-based potassium assay methodologies are slow and laborious, and can require multiple replacements of assay buffers or medium. In addition, the assay quality is often challenged by well-to-well variations and a small signal window. Therefore, a fast, easy-to-use, and sensitive cell-based assay specific to potassium channels is highly desirable for high-throughput screening. The FLIPR Potassium Assay Kit provides a true homogeneous assay using a no-wash format, with a number of key features including:

- Direct, functional measurement of potassium channel activity
- Homogeneous, no-wash protocol
- Enhanced signal window and higher signal-to-noise ratio
- Rapid procedure with less hands-on time
- Reduced well-to-well variation
- Ease of use with both adherent and non-adherent cells
- Adaptable for use in 96-well, 384-well, or 1536-well formats

Applications

The FLIPR Potassium Assay Kit provides a homogeneous assay for measuring potassium channel activities in a non-wash format. It is designed to work for the majority of ligand-gated or voltage-gated potassium channels.

Chapter 2: Materials and Equipment

Kit Components

Table 2-1: Components of the FLIPR Potassium Assay Kits

Item	Evaluation Kit (R8330)	Explorer Kit (R8222)	Bulk Kit (R8223)
Component A	2 vials	10 vials	10 vials
Component C	2 vials	10 vials	10 vials
Chloride-free Assay Buffer (5X)	1 bottle	1 bottle	1 bottle
Potassium Sulfate solution 200 mM of K_2SO_4 in water	1 bottle	1 bottle	1 bottle
Thallium Sulfate solution 50 mM of Tl_2SO_4 in water	1 bottle	1 bottle	1 bottle
Assay Buffer (Component B) 20 mM HEPES buffer + 1X Hank's Balanced Salt solution (HBSS), pH 7.4	1 bottle	1 bottle	None

- The entire Evaluation Kit is sufficient for two (2) 96-well or 384-well microplates. The entire Explorer Kit is sufficient for ten (10) 96-well or 384-well microplates. Each vial is sufficient for one (1) microplate.
- The entire Bulk Kit is sufficient for one-hundred (100) 96-well or 384-well microplates. Each vial is sufficient for ten (10) microplates.

Materials Required but Not Provided

Table 2-2: Reagents and Supplies

Item	Suggested Vendor
Dimethyl Sulfoxide (DMSO) is used for dissolving Component A. It is important that the DMSO used is of high quality and stored properly.	Sigma #D8418, or equivalent
Component B*: 20 mM HEPES buffer + 1X Hank's Balanced Salt solution (HBSS), pH 7.4 *Component B is provided for Evaluation kits and Explorer kits only.	10X Hank's Balanced Salt Solution (Gibco #14065-056, or equivalent) 1M HEPES buffer solution (Irvine Scientific #9319, or equivalent) Water for cell culture (Irvine Scientific #9312, or equivalent)
Probenecid, an inhibitor for the anion-exchange protein, might be required for some cell lines to ensure that the dye stays inside the cell and is not pumped back out. Prepare a stock solution of 500 mM in 1N NaOH, and then dilute to 250 mM in HH buffer. Prepare Loading Buffer such that the final in-well concentration of probenecid is 2.5 mM when added to cells.	Sigma #P8761, or other chemical suppliers **TIP:** Use of water-soluble probenecid is also possible following individual manufacturer instructions.

Storage and Handling

On receipt of the FLIPR Potassium Assay Kit, store Component A and Component C at –20°C, and store the other components at 4°C. Under these conditions, the reagents are stable for six (6) months in the original packaging.

After reconstitution, the Loading Buffer is stable for up to eight (8) hours at room temperature. Aliquots can be frozen and stored (without probenecid) for up to one (1) week without loss of activity.

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/support

DANGER! Thallium sulfate is harmful if swallowed. Disposal of this product, solutions, and any by-products must comply with the requirements of environmental-protection and waste-disposal legislation, and any regional local authority requirements. Exercise care when handling this product 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/support

Supported Instruments

The FLIPR Potassium Assay Kit is designed to be used with the following Molecular Devices instruments.

- FLIPR Tetra, FLIPR 3, FLIPR 384, and FLIPR 1 Imaging Microplate Readers
- FlexStation 3 Benchtop Multi-Mode Microplate Reader

Chapter 3: Experimental Protocol

Quick Start Protocol

1. Plate the cells in microplates and incubate overnight at 37°C, 5% CO_2.
2. Prepare the Loading Buffer the following day.
3. Remove the cell plates from the incubator and 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 microplate.
4. Incubate the prepared plates for one hour at room temperature in the dark.
5. Prepare the compound plates.
6. Run the experiment on a FLIPR or FlexStation 3 instrument.

Cell Handling

The Molecular Devices FLIPR Potassium Assay Kit is designed to work with many cell types, both adherent and non-adherent. Standard procedures vary across laboratories, and Molecular Devices recognizes that a variety of cell handling conditions can be used at the discretion of the user. This section provides general guidelines for preparing cells for use with the FLIPR Potassium Assay Kit.

Adherent cells are the most frequently used cells with the kits. They are generally plated the day before an experiment and then incubated in a 37°C, 5% CO_2 incubator overnight. See Table 3-1 for suggested plating volumes and seeding densities to create an 80% to 90% confluent cell monolayer for the assay in a FLIPR® or FlexStation® 3 instrument.

Table 3-1: Suggested Plating Volumes and Seeding Densities

Cell Type (cells/well)	96-well microplate (100 µL growth medium)	384-well microplate (25 µL growth medium)
Adherent cells	20000 to 80000	5000 to 20000
Non-adherent cells	40000 to 200000	10000 to 50000

For non-adherent cells, Molecular Devices recommends centrifuging cells from culture medium and re-suspending the pellet in the culture medium or applicable buffer of choice on the day of the experiment. Cells can be dye-loaded in a tube or while plated. Molecular Devices recommends that after the cells are plated, centrifuge the microplates at 100 x g for up to 4 minutes, with the brake off. As an alternative, non-adherent cells can be treated like adherent cells, plating the day before the assay using the same plating volumes and seeding densities, as long as the cells are seeded onto coated plates (for example, poly-D-lysine or collagen) to make sure that they are well attached to the microplate bottom.

Preparing the Loading Buffer

1. Remove one vial each of Component A and Component C from the freezer, and then equilibrate to room temperature.
2. Equilibrate Component B to room temperature.
   For the Bulk Kit, prepare 100 mL of 20 mM HEPES plus 1X HBSS, pH 7.4 as Component B.
3. Dissolve the contents of the Component C vial in DMSO, and then mix thoroughly by vortexing.
   - For the Evaluation Kit or Explorer Kit, use 30 µL of DMSO.
   - For the Bulk Kit, use 300 µL of DMSO.
4. Combine the vial of Component A with 10 mL of the Component B buffer.
   For the Bulk Kit, use only 10 mL of the prepared Component B buffer in this step.
5. Combine the Component C solution from step 3 to the Component A solution from step 4, and then mix by vortexing for ~1 to 2 minutes until the contents of the vial are dissolved.
   It is important that the contents are completely dissolved to ensure reproducibility between experiments.
6. For Bulk kit only, combine the solution from step 5 with the remaining 90 mL of the prepared Component B buffer, and then mix thoroughly.

Note: If the cells require probenecid (such as CHO or other cells containing an organic anion transporter), then add probenecid in the Loading Buffer to 5 mM so that the final in-well working concentration is 2.5 mM. Adjust Loading Buffer pH to 7.4 after addition of probenecid. See to the procedure for making probenecid in Materials Required but Not Provided on page 5.

Note: Do not store frozen aliquots of Loading Buffer with probenecid, and always prepare new probenecid on the day of the experiment. Use of water-soluble probenecid is also possible following individual manufacturer instructions.

Loading Cells Using Loading Buffer

1. Remove the cell plates from the incubator or centrifuge.
2. Add an equal volume of Loading Buffer to each well: 100 µL per well for 96-well plates and 25 µL per well for 384-well plates.

Note: Molecular Devices does not recommend washing cells before dye loading. However, growth medium and serum might interfere with certain assays. In this case, the supernatant can be aspirated and replaced with an equal volume of serum-free 20mM HEPES buffer + 1X HBSS, pH 7.4 before adding the Loading Buffer.

3. After adding dye, incubate the cell plates for 1 hour at room temperature. The loading time should be optimized for each cell line and target.

Note: Do NOT wash the cells after dye loading.

Preparing the Thallium Sulfate Source Plate

To introduce Tl^+ into the assay plate, prepare a 5X solution by diluting ligands or K_2SO_4 (for voltage-gated targets only) with Tl_2SO_4 in 1X Chloride-free Buffer.

Concentrations of Tl_2SO_4 and K_2SO_4 (for voltage-gated targets only) used for assays should be optimized for each target channel. Molecular Devices suggests a titration of Tl^+ or a combination of Tl^+ and K^+ at 0.5 mM to 3 mM Tl^+ and 5 mM to 30 mM K^+ (final concentration in wells). See Optimization Guidelines on page 14.

Note: Since thallium sulfate and potassium sulfate have two equivalents of cation per mole, consider them as 2X in their respective cation concentrations.

DANGER! Thallium sulfate is harmful if swallowed. Disposal of this product, solutions, and any by-products must comply with the requirements of environmental-protection and waste-disposal legislation, and any regional local authority requirements. Exercise care when handling this product 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/support

Running the FLIPR Potassium Assay on a FLIPR Instrument

After incubation, transfer the microplates to the FLIPR instrument and start the FLIPR potassium assay.

Do the assay signal test before the experiment. The LED intensity, exposure time, and the gate or gain can be adjusted to get the desired RFU range.

- For a FLIPR Tetra instrument with an EMCCD camera, adjust the typical average baseline counts to a range from 1500 RFU to 2500 RFU.
- For a FLIPR Tetra instrument with an ICCD camera, adjust the typical average baseline counts to a range from 8000 RFU to 10000 RFU.
- For a FLIPR 3, FLIPR 384, or FLIPR 1 instrument, adjust the typical average baseline counts to a range from 8000 RFU to 10000 RFU.

Update the assay signal test to transfer the adjusted settings to the protocol.

Before reading the microplate, set up the FLIPR instrument using the recommended experimental setup parameters listed in Table 3-2: Experimental setup parameters for the FLIPR Tetra instrument on page 11. and Table 3-3: Experimental setup parameters for all other FLIPR instruments on page 11.

Faster addition speeds closer to the cell monolayer are recommended to ensure better mixing of compounds and lower signal variance across the plate. Molecular Devices recommends further assay development and adjustment of the volume, height, and speed of dispense to optimize the individual cell response.

Recommended Settings for the FLIPR Tetra Instrument

Table 3-2: Experimental setup parameters for the FLIPR Tetra instrument

Parameter	96-well microplate	384-well microplate
Excitation LED (nm)	470–495	470–495
Emission Filter (nm)	515–575	515–575
Camera Gain	**EMCCD camera:** 70 to 150 **ICCD camera:** Fixed at 2000	**EMCCD camera:** 70 to 150 **ICCD camera:** Fixed at 2000
Exposure (sec)	0.1 to 0.5	0.1 to 0.5
LED Intensity (%)	50 to 100	50 to 100
Camera Gate (%) **(ICCD camera only)**	5 to 100	5 to 100
Addition Volume (µL)	50	12.5
Compound Concentration (Fold)	5X	5X
Addition Height (µL)	120 to 160	30 to 40
Addition Speed (µL/sec)	**Adherent Cells:** 60 to 100 **Non-Adherent Cells:** 20 to 40	**Adherent Cells:** 30 to 40 **Non-Adherent Cells:** 10 to 20
Tip-Up Speed (mm/sec)	20	20

Recommended Settings for the Other FLIPR Instruments

Table 3-3: Experimental setup parameters for all other FLIPR instruments

Parameter	96-well microplate (FLIPR 1 and FLIPR 384 instruments)	384-well microplate (FLIPR 384 and FLIPR 3 instruments)
Exposure (sec)	0.4	0.4
Camera Gain (FLIPR 3 instrument only)	N/A	50 to 80
Exposure (sec)	0.4	0.4
Addition Volume (µL)	50	12.5
Compound Concentration (Fold)	5X	5X
Addition Height (µL)	150 to 200	30 to 45
Addition Speed (µL/sec)	**Adherent Cells:** 50 to 100 **Non-Adherent Cells:** 10 to 30	**Adherent Cells:** 25 to 40 **Non-Adherent Cells:** 10 to 20

Running the FLIPR Potassium Assay on a FlexStation 3 Instrument

Before reading the microplate, set up the FlexStation 3 instrument using a SoftMax® Pro Software protocol. The recommended experimental setup parameters are listed in Table 3-4: Experiment Setup Parameters for the FlexStation 3 Instrument on page 12.

Molecular Devices recommends that parameters be optimized for each cell line and target to deliver the best performance for your assay.

After incubation, transfer the microplates directly to the FlexStation 3 instrument assay plate carriage and run the assay.

In an individual well or column of wells, the peak(s) should be complete within 1 to 3 minutes after addition. For an entire plate however, the protocol will not complete until all chosen columns are finished. The assays are run one column at a time.

Analyze the data using the SoftMax Pro Software.

Recommended Settings for the FlexStation 3 Instrument

Table 3-4: Experiment Setup Parameters for the FlexStation 3 Instrument

Parameter	96-well microplate	384-well microplate
Read Mode	Fluorescence	Fluorescence
Read Position	Bottom Read	Bottom Read
Excitation Wavelength (nm)	485	485
Emission Wavelength (nm)	525	525
Automatic Emission Cut-Off (nm)	515	515
PMT Sensitivity	6	6
Pipette Height (µL)	230	50
Transfer Volume (µL)	50	12.5
Compound Concentration (Fold)	5X	5X
Addition Speed (Rate)	**Adherent Cells:** 3 **Non-Adherent Cells:** 1	**Adherent Cells:** 2 to 3 **Non-Adherent Cells:** 1

Chapter 4: Data Analysis Example

Inhibition of hERG Channel

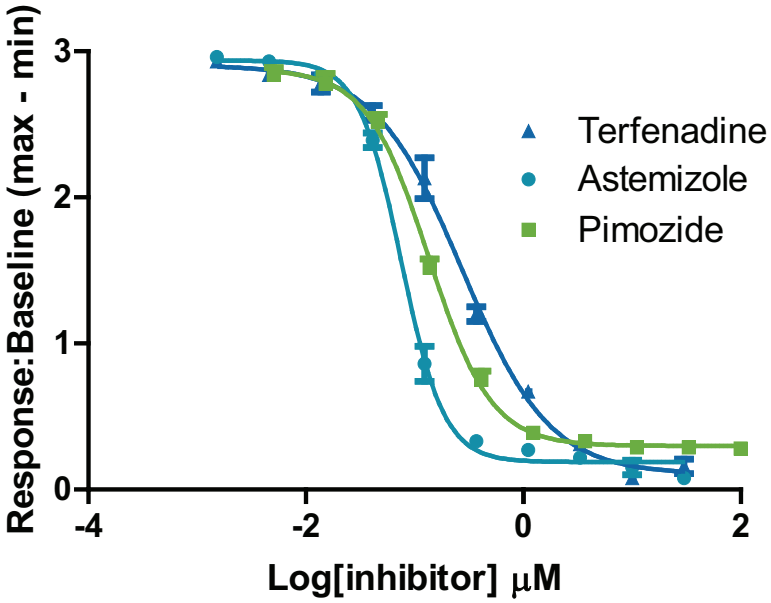

Figure 4-1: hERG Channel Blockers

Compound	IC_{50} (nM) FLIPR Instrument	IC_{50} (nM) Electrophysiology
Terfenadine	274	332
Astemizole	76	62
Pimozide	135	147

In this experiment, the concentration-dependent inhibition of hERG channels by three reference compounds was analyzed with the FLIPR Potassium Assay Kit. The hERG channel was stably expressed in the Chinese hamster ovary (CHO) cells, and all compounds were incubated with cells for 30 minutes after 1 hour of dye loading at room temperature. Stimulus buffer containing 1X chloride-free buffer and final concentrations of 10 mM K^+, and 1 mM Tl^+ was added to the wells while reading on the FLIPR Tetra Instrument. The IC_{50} values derived from this assay were compared to the electrophysiology results collected with the IonWorks Barracuda Instrument.

Chapter 5: Optimization Guidelines

Data Analysis

Correct data analysis is crucial to correctly assess the functionality of the assay. It is very important to choose the correct time point to calculate final data. In the assay, the assay background trace (optimized Thallium only or maximal inhibition condition) shows a slow upward slope over the course of the assay. Using a late time point results in a reduced assay window impacting the quality of the data and also the IC_{50} determination.

Molecular Devices recommends using a time point that results in the greatest assay window as defined as Max signal vs. Thallium only trace. Identification of the optimum time point can be done by assessing the raw data manually or by using the Negative Control Correction feature in the FLIPR® ScreenWorks® Software. This feature subtracts the assigned background curve, resulting in bell shaped traces. It can be used for identification of the optimal time point or as a general analysis tool. Using Negative Control Correction, the Max-Min analysis automatically uses the data at the optimal time point.

If other analysis methods are preferred, the optimal time point determined can be manually chosen (maximum of bell shaped curve).

Use of the Negative Control Correction feature to determine the optimal time point and signal window for the assay

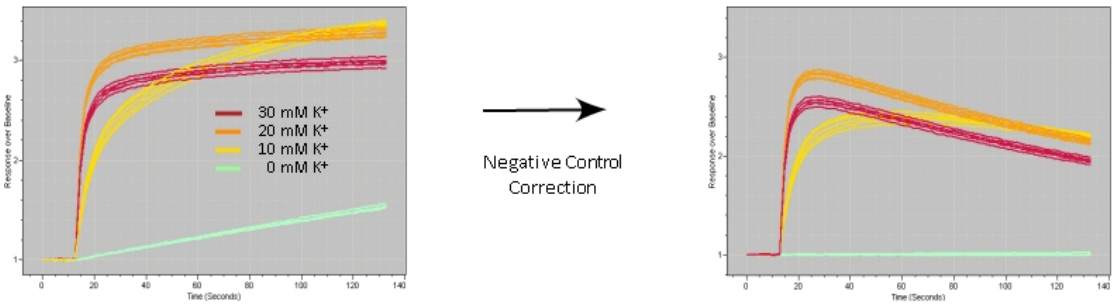

Figure 5-1: Before and after Negative Control Correction

$K_V1.3$ channel activity was measured with 2 mM Tl^+ and different K^+ concentrations. The data are displayed with and without Negative Control Correction.

Determination of Potassium and Thallium Concentrations

Potassium channels are grouped into several classes that have very different properties. It is very important to determine the target-specific optimal concentrations of Tl^+, or Tl^+ and K^+ for each assay. To determine the optimal concentrations to use for the assay, Molecular Devices recommends setting up an initial experiment with a matrix of ligand and Tl^+ or K^+ and Tl^+ at 5 mM to 30 mM K^+ and 0.5 mM to 3M mM Tl^+ (final concentration in the wells).

Titration of K^+ and Tl^+ concentrations to optimize assay performance for different targets

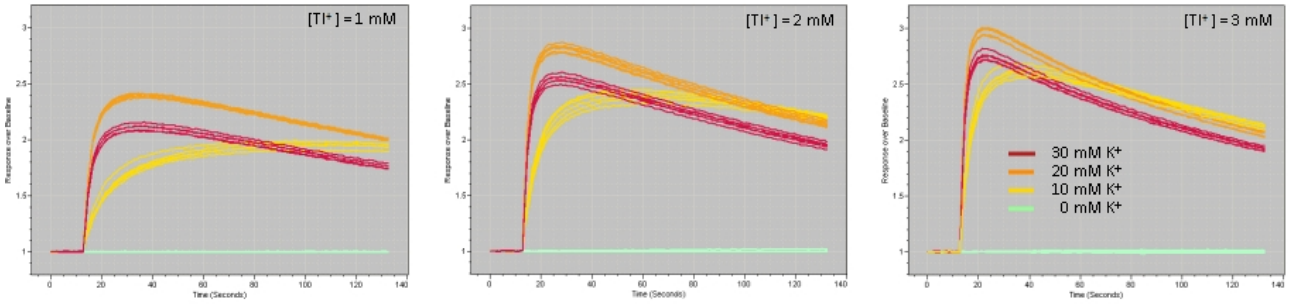

Figure 5-2: K_V1.3 Channel Activity Assay

K_V1.3 channel activity was measured with a titration of different Tl^+ and K^+ concentrations. The data are displayed with Negative Control Correction.

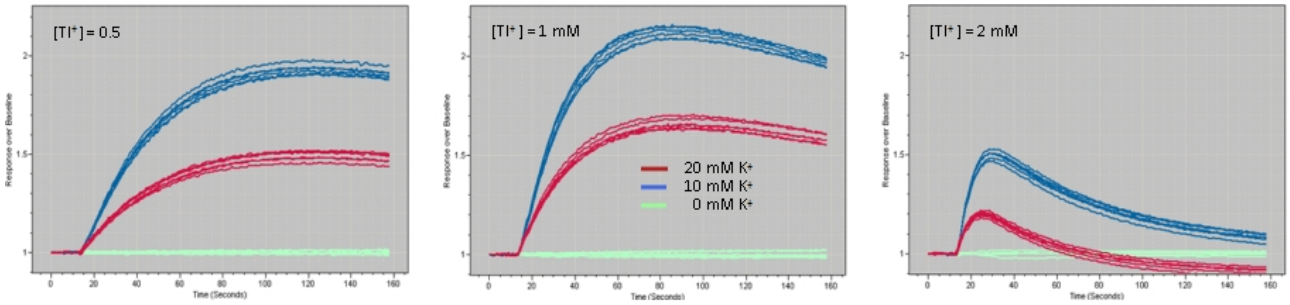

Figure 5-3: hERG Channel Activity Assay

hERG channel activity was measured with a titration of different Tl^+ and K^+ concentrations. The data are displayed with Negative Control Correction.

Chapter 6: 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 possible level of technical service.

Our support web site, www.moleculardevices.com/support , has a link to the Knowledge Base with technical notes, software upgrades, safety data sheets, and other resources. If you do not find the answers you are seeking, follow the links to the Technical Support Service Request Form to send an email message to a pool of technical support representatives.

You can contact your local representative or contact Molecular Devices Technical Support by telephone at 800-635-5577 (U.S. only) or +1 408-747-1700. In Europe call +44 (0) 118 944 8000.

Please have the product name, part number, and lot number available when you call.

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