

Primary Human Cells and BacMam Target Delivery in a Novel, High Throughput Assay of hERG Function

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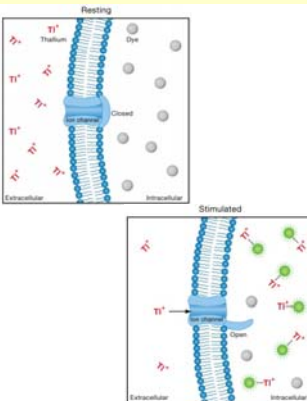
Abstract

Ion channels are an important target in drug discovery for which current assays have proven expensive and with low throughput. Potassium channels make up an important subset of these targets. A robust homogenous assay for HTS measurements of potassium ion flux would give the screening community a complementary, functional method to patch clamp analysis when screening compound libraries.

Molecular Probes has developed an equilibrium based assay for measurement of K⁺ ion flux called the FluxOR™ Thallium Assay Kit. Using thallium flux as a surrogate marker for potassium ion activity (Weaver et al), the assay is based on the TI⁺ activation of a novel fluorescent dye with a high affinity for thallium (Figure 1). Combined with BacMam Target delivery (Kost et al), many potassium channels, including hERG, are now amenable to screening in a variety of cellular backgrounds.

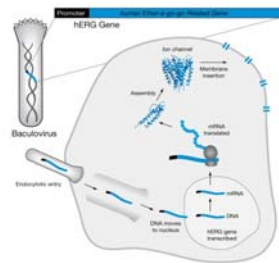
We screened the rank order of potency for several canonical hERG blockers in a variety of cellular backgrounds, including primary human aortic smooth muscle cells, to demonstrate the importance of genetic background in functional screens.

Figure 1 – FluxOR™ Assay



Cells are loaded with a thallium sensitive dye, and during the assay, a small amount of thallium is added to the outside of the cells with a stimulus. If an ion channel is opened, thallium flows into the cell and binds to the thallium sensitive dye, resulting in bright green fluorescence.

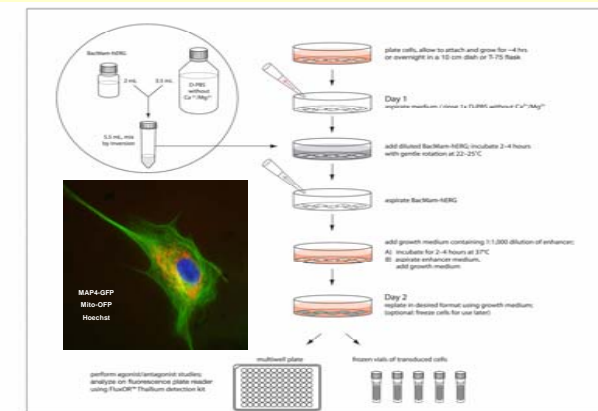
Figure 2 – BacMam Content & Delivery



The combination of BacMam target delivery of a potassium channel and the FluxOR™ assay tool provides an easy-to-use equilibrium based measurement of potassium flux.

- Biosafety Level 1, multiple day expression
- Delivery of multiple subunits

Figure 3 – BacMam Target Delivery Workflow- Fresh or Frozen?

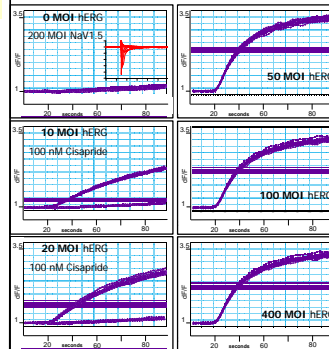


U-2 OS cell transduction and freezing method: For 1 microplate, scale as needed.

Transduction: 1 x 10⁵ U-2 OS cells (ATCC #HTB-96) are seeded into a T75 flask and allowed to adhere for 4 hours to overnight in complete medium (McCoy's + 10% FBS). Immediately before transduction, cells are rinsed with 5 mL divalent free PBS pH 7.4 (Invitrogen #10010) and replaced with 5 mL PBS containing between 0.5 and 2.0 mL high titer BacMam hERG (Invitrogen #B10019) and BacMam enhancer. Incubate 2 hours at RT out of the light, gently rotating. Aspirate and replace with 10 mL complete medium and incubate overnight at 37°C, 5% CO₂.

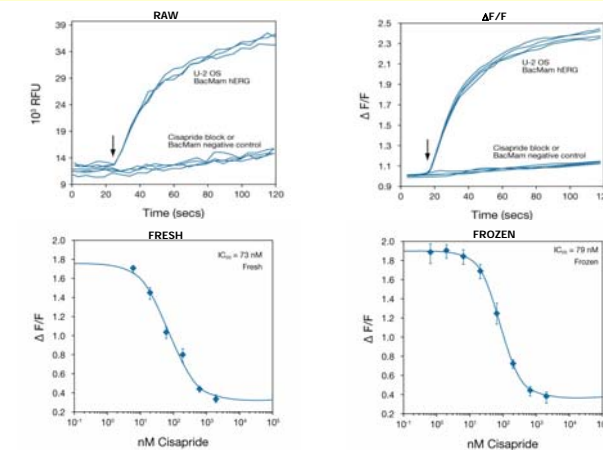
Freezing: Rinse with 5 mL divalent free PBS pH 7.4 and add 1 mL TrypLE Express (Invitrogen #12065) and incubate at RT for ten minutes. Harvest the cells in complete medium, centrifuge, and resuspend for freezing in aliquots at 1-2 x 10⁶ cells per mL in Recovery Freezing Medium (Invitrogen #12648). Freeze slowly and thaw quickly, plate minimum 2 hours before use on PDL Coated microplates. Check cell vitality if background is high.

Figure 4 – Titratable Expression



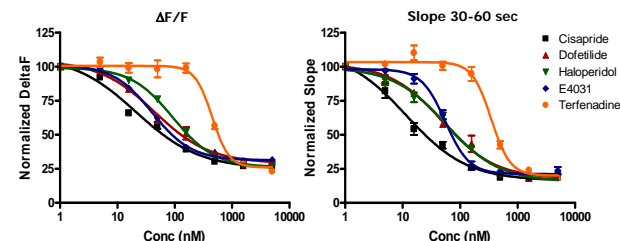
U-2 OS cells transduced with BacMam-hERG as described below. Inset shows patch clamp records from HEK 293 cells expressing BacMam NaV1.5.

Figure 5 – BacMam K⁺ Channel Delivery with FluxOR™ Assay Fresh or Frozen Cell Capabilities



U-2 OS cells were transduced with BacMam hERG. hERG activity was measured using the FluxOR™ assay kit on a Molecular Devices FlexStation™ plate reader in 384-well PCL microplates. The y-axis shows raw (RFU) and normalized (ΔF/F) fluorescence after potassium stimulation at 20 seconds shown on the x-axis. Bottom panels show the results of freshly transduced cells and cells which had been transduced and frozen in liq N₂. The frozen cells were thawed and plated in 384 well plates followed by the normal protocol for the FluxOR™ assay.

Figure 6 – Rank Order of Antagonist Potency in Human Aortic Smooth Muscle Cells Transduced with BacMam hERG



Human Aortic Smooth Muscle cells (Cascade Biologics, Invitrogen Cell Culture) or U-2 OS cells were cultured according to instructions and transduced at 200 MOI as described in Figure 3. hERG-T-REX™ 293 and CHO cells were cultured and induced to express hERG according to instructions (Invitrogen #K1236 and #K1237). Cells were plated at 1-2 x 10⁵ cells per microplate and assayed with the FluxOR™ Thallium flux kit, using 10 mM added K⁺ and 2.5 mM added TI⁺ (final concentrations, from 1:5 dilution) as a stimulus. Drugs were prepared as 1 mM stocks in DMSO and co-administered with the stimulus at 5x final concentration. Dose-inhibition curves of hERG activity were defined with either the maximum dF/F (Fig 6a) or 30 – 60 second slope (Fig 6b) of the fluorescence change elicited with stimulation, normalized against zero drug concentration. Data were acquired in 96 well plates on a FlexStation1 384 (Molecular Devices, Sunnyvale CA) and exported to GraphPad Prism for analysis and curve fitting. Each data point represents the mean of 6 - 12 determinations +/- SEM.

Figure 7 – Data Tables

Signal Window

Parameter	HASM.C	U-2 OS Fresh	U-2 OS Frozen	CHO	293
ΔF/F Max Control	2.19	2.77	2.82	2.61	2.58
ΔF/F Min Cisapride	1.32	1.47	1.38	1.26	1.27
Slope Max Control	374	441	526	475	520
Slope Min Cisapride	69	53	63	45	413

Rank Order

IC ₅₀ PM	Cisapride	Dofetilide	Haloperidol	E4031	Terfenadine
HASM.C	12	50	57	56	347
U-2 OS BacMam Fresh	34	782	85	300	623
U-2 OS BacMam Frozen	53	571	128	249	581
CHO Stable	20	149	36	98	413
293 Stable	43	69	105	45	538

Summary

- The FluxOR™ Assay is a homogenous, fluorescence based assay for HTS measurements of potassium ion flux, providing a sensitive, specific and reproducible tool to measure functional potassium channel activity.
- BacMam delivery has been demonstrated with a wide range of targets and hERG channels delivered with BacMam are compatible with the FluxOR™ assay tool. Similar results have been obtained with "resting" inward rectifier Kir2.1 (KCNJ2) channels as well as other voltage and calcium activated potassium channels.
- BacMam delivery of ion channels is possible in "classic" cell lines as well as primary cells such as Human Aortic Smooth Muscle, Vascular Smooth Muscle and Pulmonary Arterial Endothelial cells (data not shown). Human U-2 OS cells are particularly efficient at expressing BacMam targets.
- BacMam delivery also enables batch transduction and demonstrated cryopreservation of U-2 OS cells. Cells remain viable for expression of the target of interest as shown by use of the FluxOR™ assay.
- Molecular Probes will continue to develop more BacMam ion channel targets for use with FluxOR™ and Cascade Biologics™ primary cell lines for assay development and screening.

Literature Cited

- Kost, T.A. (2005) *Nat Biotech* 23:567-575
Weaver, CD et al (2004) *Journal of Biomolecular Screening* 9(8) 671-7