FRET-Based Ion Channel, GPCR, and Kinase Assay Applications on the Synergy™ HT Multi-Detection Microplate Reader from Bio-Tek

Randall Hoffman and Jasmin Gibson Invitrogen Corporation • 501 Charmany Drive • Madison, Wisconsin 53719 • USA
Paul Held Bio-Tek Instruments • Highland Park • P. O. Box 998 • Winooski, Vermont 05404 • USA

abstract

Pairing fluorescent FRET-based assays with plate reader instrumentation is one of the critical first steps in drug discovery. Ideally, the initial evaluation occurs during assay development with subsequent transfer to HTS and downstream lead discovery. Keeping the same instrument throughout this process (or showing portability to other instrumentation) would be ideal. Unfortunately high-throughput screening technologies are often coupled to costly instrumentation, limiting flexibility and transfer of validated assays from assay development to screening and lead discovery groups. Our goal was to show the use of one bench top reader, the Synergy™ HT Multi-Detection Microplate Reader from Bio-Tek, that can be used for assay development and lead discovery for FRET-based technologies including Voltage Sensor Probes ion channel assays, GeneBLAzer® GPCR cell-based assays, and Z'-LYTE™ kinase assays.

introduction

Ion channels, GPCRs and kinase target classes combined account for a significant percentage of drug discovery screening:

Ion channels are important drug targets because of their critical role in nerve, cardiac, endocrine, and skeletal muscle tissues. Voltage Sensor Probes (VSP) technology can be used with any ion channel target that changes membrane potential, and is therefore well suited for sodium, potassium, calcium, chloride, and ligand-gated ion channel research. The FRET-based detection method provides ratiometric results, which significantly reduces errors arising from well-to-well variations in cell number, dye loading and signal intensities, plate inconsistencies, and temperature fluctuations. These combined features make VSP technology highly amenable for high-throughput screening (HTS) applications.

The G protein-coupled receptor (GPCR) superfamily is comprised of over 1,000 members and is the largest known class of molecular targets with proven therapeutic value. G_q, G_s, and G_i proteins mediate intracellular GPCR signaling through activation of signaling pathways leading to distinct physiological endpoints. GPCR signaling through these distinct pathways can be monitored by activation of specific transcriptional response elements placed 5' to a reporter gene. The GeneBLAzer® technology platform from Invitrogen Drug Discovery Solutions uses the beta-lactamase (bla) reporter system. The platform consists of stable cell lines used to screen for both agonists (including partial and inverse agonists) and antagonists of GPCRs, vectors for cell line development, and a membrane permeant fluorescent substrate, CCF4-AM. This FRET based substrate provides a ratiometric readout that reduces sample variability, leads to excellent Z'-factors, and allows for assay miniaturization.

Kinases play critical roles in controlling cellular processes, such as apoptosis, metabolism, transcription, cell cycle progression, differentiation, and immune responses. Since aberrations in kinase activity have been associated with more than 400 diseases, pharmaceutical companies are very interested in developing drug candidates for the various kinases associated with disease. Invitrogen's Z'-LYTE™ biochemical kinase assay employs a FRET-based, coupled-enzyme format and is based on the differential sensitivity of phosphorylated and non-phosphorylated peptides to proteolytic cleavage. Sensitive kinase assays can be quickly developed with low % CVs and high Z'-factors.

ion channel assay applications

Features and Benefits of Voltage Sensor Probes

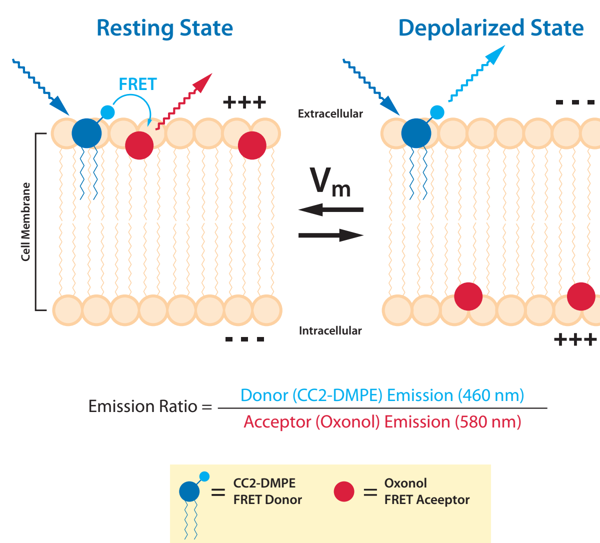
1. VSPs are universal: VSPs can be developed for any ion channel target that changes membrane potential.

2. Significant reduction of errors arising from well-to-well variations: the VSPs produce FRET-based ratiometric data.

3. There is no signal artifact due to intracellular membrane depolarization: the VSPs measure membrane potential change localized to the external surface of the plasma membrane.

Mechanism of the FRET based VSP:

The FRET donor is a membrane-bound, coumarin-phospholipid, CC2-DMPE, which binds only to the exterior of the cell membrane. The FRET acceptor is a mobile, negatively charged, hydrophobic oxonol, $DiSBAC_2(3)$, which will locate to either side of the plasma membrane in response to changes in membrane potential. At resting potential (relatively negative) the two probes associate with the exterior of the cell membrane. Exciting the CC2-DMPE donor probe (at 405 nm) generates a strong red fluorescence signal (at 570 nm) from the oxonol acceptor probe. When the membrane potential becomes more positive, as occurs with cell depolarization, the oxonol probe rapidly translocates to the other face of the membrane. This translocation separates the FRET pair, so exciting the CC2-DMPE donor probe now generates a strong blue fluorescence signal (at 465 nm).



Features and Benefits of the Synergy™ HT for VSP Assays

1. The Synergy™ HT is equipped with injectors to add depolarizing stimuli during a kinetic read. This is important for following membrane depolarization over a time course.

2. The Synergy™ HT can switch emission filters (~140 milliseconds) in well kinetic mode to give near simultaneous dual emission readout. This timing is critical for following depolarization over time.

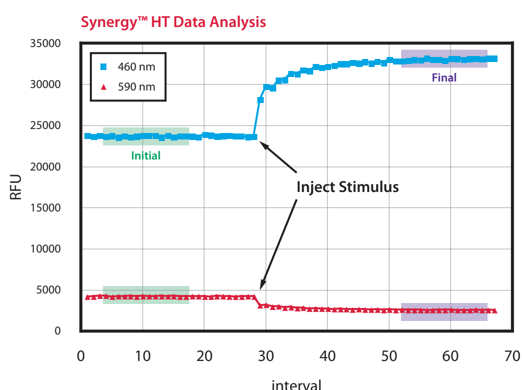
3. The Synergy™ HT can read from the bottom of clear microtiter plates. This is necessary to optimize signal intensity directly from adhered cell cultures.

4. Unlike some HTS instrumentation, the Synergy™ HT is simple to operate and compact in design, making it a suitable benchtop assay development tool.

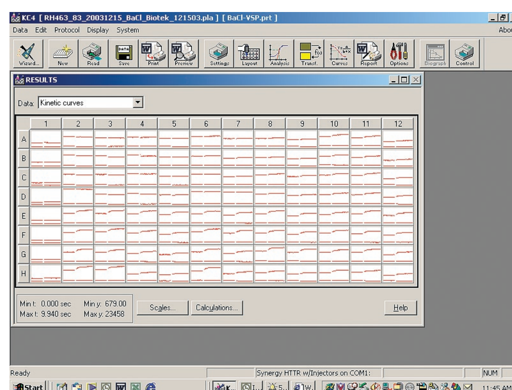
ion channel assay applications, continued

FRET-Based Ratiometric Data

One of the advantages of VSP technology is the ratiometric data analysis. A ratio can be calculated from the 460 (blue) to 590 nm (red) fluorescence signals. The data is collected in kinetic mode so a ratio can also be calculated using the blue to red ratios from both the polarized and depolarized states. This results in the Response Ratio as the final data output. The Synergy™ HT is able to rapidly switch emission filters (~140 milliseconds) enabling “dual” readout in well kinetic mode.



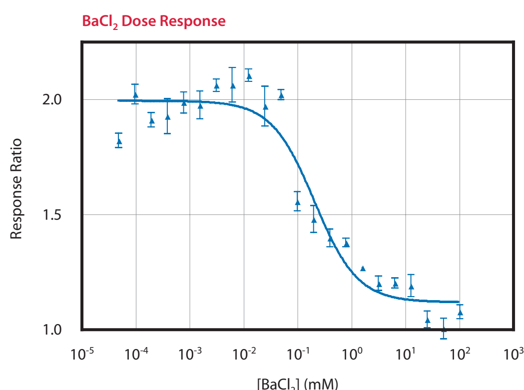
The panel above demonstrates typical raw data obtained from the Synergy™ HT during a kinetic read. This data is from an assay using rat basophilic leukemia (RBL) cells (ATCC #CRL2256) which have a high level of endogenous inward rectifying potassium (Kir) channels. The membrane potential in these cells can be changed by the addition of depolarizing stimuli (high KCl). The KCl is injected by the Synergy™ HT at the time indicated by the arrow. Note the loss of FRET upon KCl addition.



The panel above shows a screenshot highlighting a software feature of the Synergy™ HT: it allows real-time viewing of kinetic data. This is important during assay development since it is immediately evident if experimental conditions are working.

Barium Chloride Dose Response

$BaCl_2$ is known to block inward rectifying potassium (Kir) channels which are endogenously expressed at high levels in rat basophilic leukemia (RBL) cells (ATCC #CRL2256). To demonstrate functionality of VSPs in this model, RBL cells were loaded with VSPs and treated with decreasing concentrations of $BaCl_2$ followed by addition of depolarizing stimuli injected by the Synergy™ HT, n = 4 wells/concentration.



This graph shows a typical barium chloride dose response (IC_{50} = 0.21 mM) measured on the Synergy™ HT. Z′-factor (0.55) was determined in separate wells. Dose responses and Z′-factor were measured using VSP conditions of 10 µM CC2-DMPE and 4 µM $DiSBAC_2(3)$. An identical experiment was analyzed on the VIPR with similar IC_{50} values (0.22 mM). The VIPR® is an instrument developed by Aurora Discovery used for high- throughput screening of ion channel targets. Graphing and IC_{50} value calculations were done with GraphPad™ Prism®.

GPCR assay applications

Features and Benefits of GeneBLAzer® for GPCR Assays

1. GeneBLAzer® technologies are very robust and sensitive due to low CVs and high Z'-factors: the GeneBLAzer® assay system produces FRET-based ratiometric data, which significantly reduces errors arising from well-to-well variations.

2. GeneBLAzer® technology allows detection of subtle differences in gene expression not possible with other reporter technologies: another benefit of FRET-based ratiometric data.

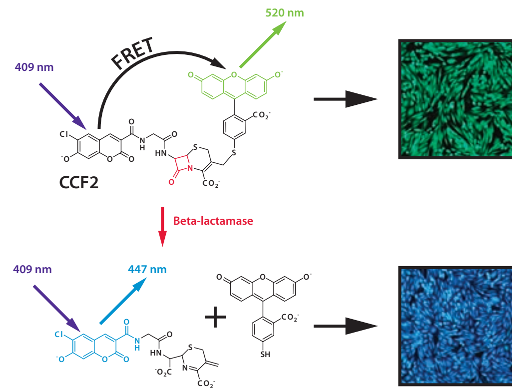
3. GeneBLAzer® uses beta lactamase (bla) combined with a cell permeable FRET-based substrate. This allows for non destructive, live cell assay loading and readout.

4. Live cell reporter assays are easily developed and miniaturized, saving time and money: Stably transfected clonal cell lines can be quickly developed from highly responsive individual cells using flow cytometry and microscopy.

5. There are no licensing fees or complicated payment requirements. GeneBLAzer® is available in an open architecture format, making it available to everyone.

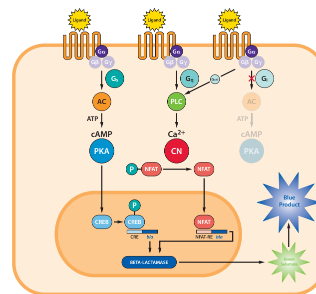
Mechanism of the GeneBLAzer® Beta-Lactamase Reporter System

CCF4-AM is a Fluorescence Resonance Energy Transfer (FRET)-based substrate for Beta-lactamase. Once CCF4-AM enters a cell, it is converted to the negatively charged CCF4 by endogenous esterases. Excitation of this substrate at 409 nm leads to efficient FRET between the coumarin and fluorescein derivatives, resulting in green fluorescence detectable at 520 nm. Cleavage of CCF4 by Beta-lactamase results in separation of the two fluorophores and loss of FRET, resulting in blue fluorescence detectable at 447 nm. The increase in beta-lactamase production by the cells is proportional to the amount of fluorescence detected at 447 nm.



GPCR Signaling monitored by Beta-Lactamase

Stable cell lines expressing the NFAT response element (monitoring Ca^{2+} flux) or the cAMP response element (CRE) linked to the beta-lactamase gene have been developed. These cell lines can be used as building blocks to develop specific GPCR assays. Upon stimulation these cell lines respond with an increase in beta-lactamase expression. This beta-lactamase response can be quantified using a FRET-based substrate, CCF4-AM, in a fluorescence plate reader.

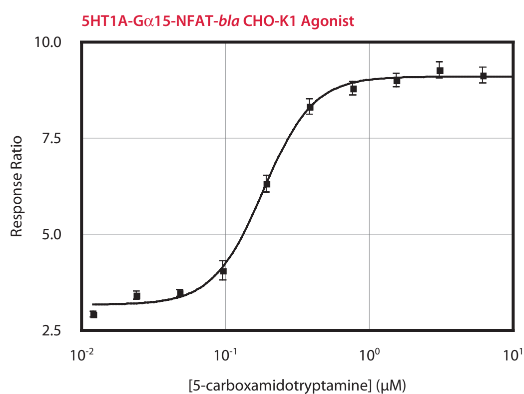


GPCR assay applications, continued

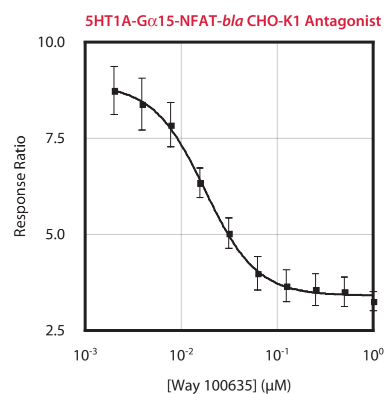
Features and Benefits of the Synergy™ HT for GeneBLAzer® GPCR Assays

1. The Synergy™ HT has excellent sensitivity in fluorescent mode. This allows detection of subtle differences in gene expression enabled by the beta lactamase reporter system.

2. Not all plate readers can read from the bottom. The Synergy™ HT has bottom read capability. This is necessary to optimize signal intensity directly from adhered or suspension cell-based assays.



5HT1A-Gα15-NFAT-bla CHO-K1 cells were stimulated with the nonspecific serotonin agonist 5-carboxamidotryptamine in the presence of 0.5% DMSO for 5 hours in DMEM based assay medium. Cells were then loaded with LiveBLAzer™-FRET B/G (CCF4-AM) for 2 hours at RT. Emission data at 460 and 530 nm were collected using an excitation wavelength of 400 nm. Data were plotted as 460/530 nm ratios versus the concentration of stimulant. EC_{50} for 5-carboxamidotryptamine was 183 nM.



5HT1A-Gα15-NFAT-bla CHO-K1 cells were stimulated with a 5HT1A selective antagonist WAY 100635 in the presence of 5-CT and 0.5% DMSO for 5 hours in DMEM based assay medium. Cells were then loaded with LiveBLAzer™-FRET B/G (CCF4-AM) for 2 hours at RT. Emission data at 460 and 530 nm were collected using an excitation wavelength of 400 nm. Data were plotted as 460/530 nm ratios versus the concentration of stimulant. Data obtained reveals a dose-dependent specific inhibition of the 5-CT response with EC_{50} = 18 nM.

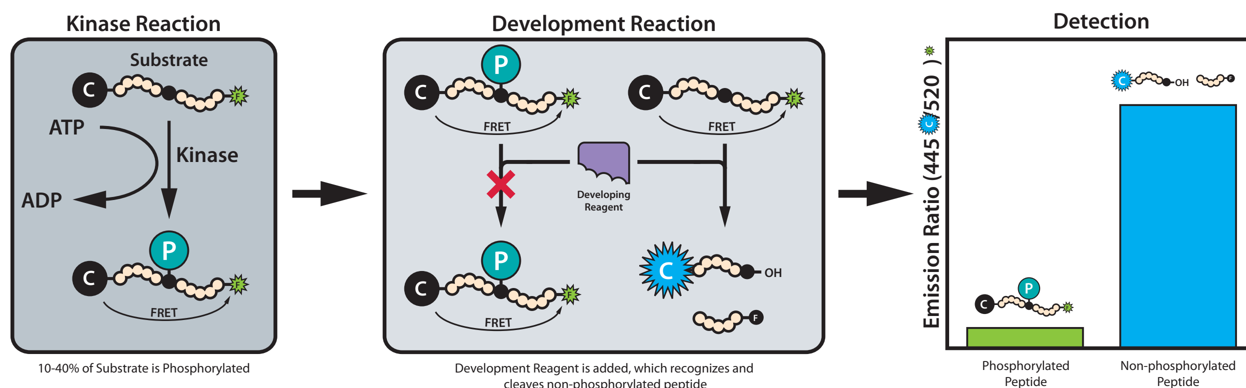
kinase assay applications

Features and Benefits of Z′-LYTE™ for Kinase Assays

1. No special instrumentation or filters required.
2. Sensitive assays can be developed using low concentrations of kinase compared to other assays: Z′-LYTE™ technology produces FRET-based ratiometric data, which significantly reduces errors arising from well-to-well variations.
3. Z′-LYTE™ technology has broad kinase coverage with >100 kinases.
4. Z′-LYTE™ assays can be developed quickly by using a substrate panel to find the best substrate for the kinase of interest. Nine Ser/Thr and four Tyr substrates are currently available.
5. Z′-LYTE™ works with kinases that require a priming phosphate such as GSK3α.

Z′-LYTE™ Technology Mechanism

The Z′-LYTE™ biochemical assay employs a FRET-based, coupled-enzyme format that is based on the differential sensitivity of phosphorylated and non-phosphorylated peptides to proteolytic cleavage. In the primary reaction (the Kinase Reaction), the kinase transfers the gamma-phosphate of ATP to a single Ser/Thr or tyrosine residue in the Z′-LYTE™ substrate. In the secondary reaction (the Development Reaction), a site-specific protease recognizes and cleaves non-phosphorylated substrate; phosphorylated substrates resist cleavage. Cleavage disrupts FRET between the donor (coumarin) and acceptor (fluorescein) on the FRET-peptide, whereas uncleaved, phosphorylated FRET-peptides maintain FRET. A ratiometric method, which calculates the ratio (Emission Ratio) of donor emission to acceptor emission after excitation of the donor at 405 nm, is used to quantitate reaction progress. The Emission Ratio will remain low if the substrate is phosphorylated (*i.e.*, no kinase inhibition) and will be high if the substrate is non-phosphorylated (*i.e.*, kinase inhibition).



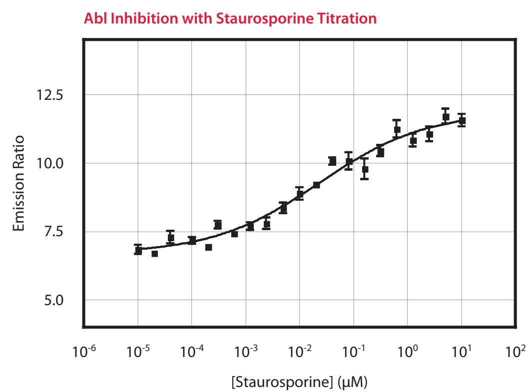
kinase assay applications, continued

Features and Benefits of the Synergy™ HT for Z'-LYTE™ Kinase Assays

The Synergy™ HT has excellent sensitivity in fluorescent mode coupled with software choices that make dual wavelength detection fast and easy.

Abl Inhibition with Staurosporine Titration

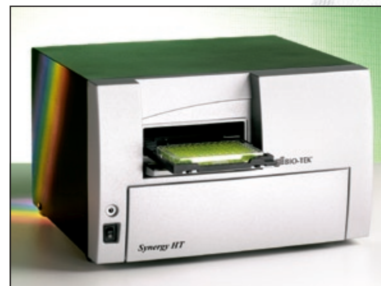
To demonstrate that the Synergy™ HT is compatible with Z' LYTE™ assays, we titrated staurosporine into Abl kinase reactions performed in triplicate in the presence of 10 µM ATP and 1.0 µM tyrosine 2 peptide substrate (Invitrogen Cat. no. PV3198). Instrument settings were excitation of 400/30 and emission of 460/40 and 530/25. The EC_{50} obtained was 23 nM.



Bio-Tek Synergy™ HT Multi Detection Microplate Reader

The Synergy™ HT Multi Detection Microplate Reader from Bio-Tek was used to help develop and validate the assays described in this poster. It offers the following highlights:

- Dual-Optical System Design for Uncompromised Multidetection Performance
- Automatic Switching of Top and Bottom Epifluorescence Read Modes
- Xenon Flash Lamp and Monochromator for Wavelength Range of 200–999 nm
- Dual Reagent Dispensers
- Automatic Probe Height Adjustment
- Compatible with 6 to 384 Well Formats



summary

1. FRET-based assay technologies provide a ratiometric readout that results in fewer experimental artifacts compared to single-wavelength dyes. This significantly reduces errors arising from well-to-well variations, enabling robust assay development for many target classes:
   a. Ion channels: Voltage Sensor Probes use FRET to measure membrane potential change localized to the outer plasma membrane.
   b. GPCRs: The GeneBLAzer™ Beta-Lactamase Reporter System uses FRET technology to allow sensitive, live cell-based GPCR assay development.
   c. Kinases: Z'-LYTE™ Technology uses FRET to enable development of highly sensitive kinase assays with broad kinase coverage.

2. Although other manufacturer's plate readers are compatible with these assays, the Synergy™ HT has demonstrated its flexibility with the following specific features:
   a. Injectors for addition of depolarizing stimuli necessary in fluorescent ion channel assays
   b. Bottom read capabilities for live cell based assays.
   c. Software that allows real time visualization of data.

3. The Synergy™ HT Multi Detection Microplate Reader from Bio-Tek is a valuable assay development tool. It has performed as well or better as other plate readers compatible with the assays highlighted here.

These products may be covered by one or more Limited Use Label Licenses (See the Invitrogen catalog or www.invitrogen.com).
By use of these products you accept the terms and conditions of all applicable Limited Use Label Licenses.

For research use only. Not intended for any animal or human therapeutic or diagnostic use.
©2005 Invitrogen Corporation. All rights reserved. Reproduction forbidden without permission. Printed in the U.S.A.

P-13913-r1 US 0305