Homogeneous High Throughput Live Cell GPCR Functional and Surface Binding Assays

Oksana Sirenko[1], Estelle N'Garwate[2], Jean-Luc Tardieu[2], Marie-Laure Lebreton[2], Delphine Jaga[2], Michael Katzlinger[1], Laurence Monnet[1], Caroline Cardonnel[1], Cathy Olsen[1], Francois Degorce[2], Evan F. Cromwell[1]

[1]Molecular Devices, Inc. Sunnyvale, CA

[2]Cisbio Bioassays, Codolet, France

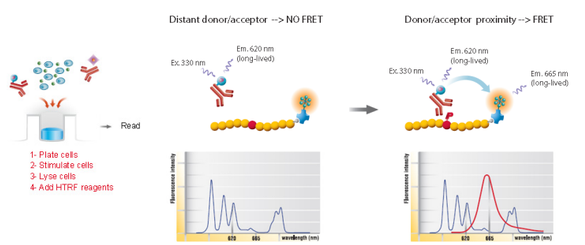
Introduction

G-protein coupled receptors (GPCR) are the largest class of cell-surface receptors and are targets for almost 40% of existing drugs. Lead discovery, testing the efficacy of prospective drugs (in the area of cardiovascular diseases and other fields), and understanding of mechanism of action of drug candidates requires assays that can measure the binding of ligands to the receptors, receptor oligomerization, and/or internalization. Accordingly, there is a real need for robust and sensitive assays of this type that are suitable for high throughput screening. The Tag-lite® cellular screening platform was designed to increase the flexibility of cell-surface receptor research. This platform is ideal for primary and secondary screening and can be applied to a variety of assay formats for pharmacological characterization and development of therapeutic antibodies. Here we show results from use of this assay platform with SpectraMax® Paradigm plate reader for characterization of GPCR agonist and antagonists in Tag-lite receptor ligand binding assays including Dopamine D3, Glucagon GLP1, and Mu Opioid assays. Excellent performance was observed as measured by Z-prime and assay window values. We also demonstrated performance for cAMP detection, pERK and pAKT kinase assays.

Method

Principles of TR-FRET measurements

HTRF® technology (Homogeneous Time-Resolved Fluorescence) is a TR-FRET based read out that uses the principles of both TRF and FRET. The HTRF donor fluorophore is either Europium cryptate (Eu3+ cryptate) or Lumi4®-Tb (Tb2+ cryptate), fruit of a recent collaboration with Lumiphore Inc. Various acceptor molecules can be used which are either Red or Green emitters. When the two fluorophores are brought together by a biomolecular interaction, a portion of the energy captured by the Cryptate during excitation is released through the acceptor. The cartoon below shows a standard assay protocol and the basic principles of TR-FRET using an Eu3+ cryptate donor and an XL665 acceptor.



SpectraMax® Paradigm and M5e Plate Reader Platforms

SpectraMax® Paradigm System

- User upgradeable high throughput multi-mode reader w/dual PMTs
- High sensitivity for all applications
- Accepts all standard microplates up to 1536 wells
- The Paradigm HTRF cartridge was used for Validation, pAKT, pERK, and Tag-lite binding assays
  - Ex 340nm, Em 616nm & 665nm
  - Read time for 384 well plate: 2.3 min

SpectraMax® M5e System

- Five modes of detection for wide range of applications
- The standard for UV/Vis absorbance
- SoftMax® Pro industry leading, all-in-one plate reader software
- Setup for Validation, pAKT, pERK,cAMP and Tag-lite binding assays:
  - Ex 340nm, Em1 616nm, Em2 665nm

Results and Discussion

Tag-lite® Live Cell GPCR Binding Assays

The Tag-lite platform allows one to efficiently label a protein of interest on a targeted site with HTRF dyes. Cisbio Bioassays offers plasmids encoding TAGs and protein of interest, or frozen cells already transfected with the constructs. The constructs lead to the expression of the tagged protein that can be labeled with Terbium Cryptate, while the receptor ligand (agonist or antagonist) is conjugated with acceptor. The Tag-lite platform is ideal for a wide range of applications, such as mechanistics and receptor dimerization, ligand binding assays, and second messenger assessment.

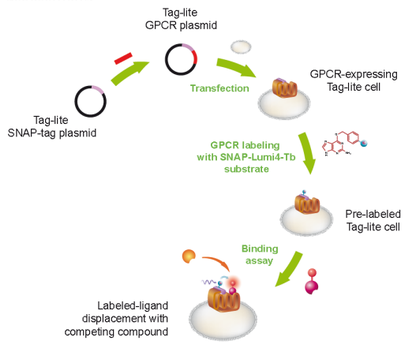


Figure 1. Depiction of the Tag-lite cell surface binding assay protocol.

cAMP Detection Assay

cAMP assay kits allow direct quantitative determination of cyclic AMP with either suspended or adherent cells using HTRF reagents. The method employs a competitive immunoassay between native cAMP produced by cells and the cAMP labeled with the dye d2. The tracer binding is visualized by a Mab anti-cAMP labeled with an Eu3+ cryptate. The capability of the assay on the SpectraMax Paradigm and M5e readers was evaluated by titration curves shown below.

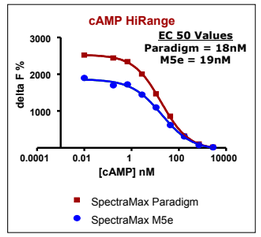
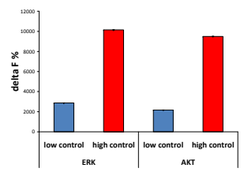


Figure 2. Cyclic AMP titration curves were evaluated using SpectraMax Paradigm and SpectraMax M5e instruments. Paradigm: W = 21.4, Z' = 0.91. M5e: W = 15.5, Z' = 0.97

HTRF Cellular Kinase Assays for phospho-AKT and phospho-ERK

Cellul'erk and HTRF phospho-Akt (Ser473) assays allow detection of activated Erk1/2 and Akt directly in whole cells. Upon receptor activation, the kinases are activated, and upon cell lysis phosphorylated kinases can be detected using the kit reagents. The assays are based on a sandwich immunoassay involving anti-kinase antibody labeled with d2, and an anti-phospho-kinase antibody labeled with Eu3+ cryptate.



SpectraMax Paradigm				SpectraMax M5e			
ERK		AKT		ERK		AKT	
low control	high control	low control	high control	low control	high control	low control	high control
2872	10140	2165	9498	1283	4225	1272	4529
2%	1%	3%	4%	6%	2%	13%	7%
3.5		4.4		3.3		3.6	

Figure 3. Stimulated and un-stimulated cell lysates provided as assay internal controls to check the quality of the results obtained. The window between high and low controls, shown in the bottom line of the table, should be greater than 2.

Dopamine D3 Binding Assay:

Dopamine receptors are a class of G protein-coupled receptors that are prominent in the vertebrate central nervous system. Dopamine receptors are implicated in many neurological processes, including pleasure, cognition, memory, and fine motor control. Abnormal dopamine receptor signaling is implicated in neuropsychiatric disorders, thus dopamine receptors are common neurologic drug targets. Antipsychotic drugs are often dopamine receptor antagonists, while psycho stimulants are typically indirect agonists of dopamine receptors. A D3 cell-based Tag-lite binding assay allows testing of receptor-selective agonists and antagonists and possibly development of novel antipsychotic drugs.

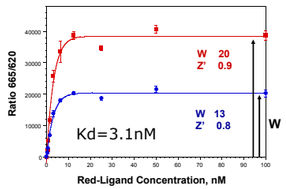


Figure 4. Binding assay: HEK293 cells expressing D3 receptor were labeled with Tb Cryptate, then plated and incubated with acceptor-conjugated ligand for 90min. Different delay and integration times for both instruments were assessed. Shorter integration time resulted in greater W and Z' values.

We have evaluated performance of the Dopamine D3 Tag-lite binding assay for the SpectraMax Paradigm and SpectraMax M5e plate readers using optimized settings. Results from a competitive inhibition assay on the two systems are shown below. The Paradigm reader shows superior results and offers unmatched flexibility. Users can purchase assay-optimized cartridges and upgrade the system at any time to enable capabilities for future applications

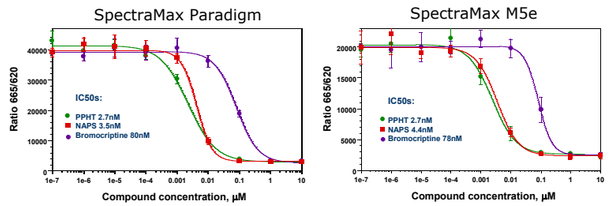


Figure 5. Competitive inhibition assay: several known dopamine receptor agonists and antagonists were tested and IC50s determined. Cells were incubated in the presence of 6nM of acceptor-conjugated ligand as well as one of the receptor agonists PPHT and bromocriptine or antagonist NAPS. Similar IC50s were determined in the assay by both plate readers.

Optimization of instrumental settings for SpectraMax Paradigm and SpectraMax M5e

Delay time and Integration times were modified during optimization. Greater assay window was achieved when shorter delay and integration times were used, for both instruments.

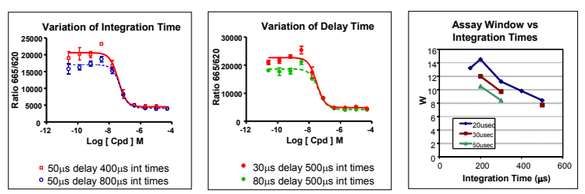


Figure 6. Characteristic titration curves (left) and plots of the assay window value W for various integration and delay times (right) for SpectraMax Paradigm. The optimum settings were found to be 20µs delay, 200µs int.

Mu Opiod Binding Assay:

The µ-opioid receptors have a high affinity to endorphins (µ = morphine). A µ receptor agonist is opium alkaloid morphine. Activation of the µ receptor by an agonist such as morphine causes analgesia, sedation, reduced blood pressure, euphoria, and decreased respiration. Long-term or high dose use of opioids may lead to mechanisms of tolerance becoming involved and opioid overdoses kill through stop of respiration and fatal hypoxia. Such overdoses can be rapidly reversed with opioid antagonists such as naloxone and naltrexone.

Here we show result from a live cell receptor binding assay using Tb / Red acceptor (Tag-lite® Dynamic TR-FRET) for the Mu Opioid receptor. Ligand binding and inhibition constants for different compounds were evaluated in the assay on the SpectraMax Paradigm plate reader.

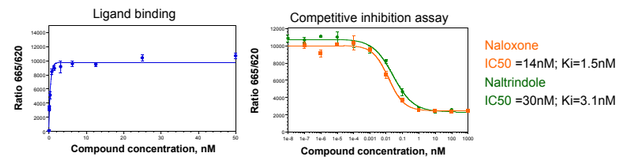


Figure 7. Results for Mu-opiod ligand binding (left) and competitive inhibition assays (right) run on the SpectraMax Paradigm system. The assay window and Z' values for the assay were 5.3x and 0.89 respectively.

Glucagon GLP-1 Binding Assay:

GLP1 receptor is known to be expressed in pancreatic beta cells. Activated GLP1R stimulates the adenylyl cyclase pathway which results in increased insulin synthesis and release of insulin. Consequently GLP1R has been suggested as a potential target for the treatment of diabetes. GLP1R is also expressed in the brain where it is involved in the control of appetite and suggested to be involved in mechanisms of memory and learning. The Glucagon GLP-1 receptor ligand binding and competitive inhibition assays were evaluated on the SpectraMax Paradigm system.

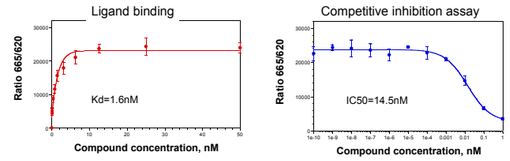


Figure 8. Results for the GLP-1 agonist peptide assay run on the SpectraMax Paradigm system. The assay window and Z' values were 19x and 0.78 respectively.

Summary

- Excellent performance of the SpectraMax Paradigm and M5e plate readers was demonstrated for several Cisbio assays: cAMP detection, pERK and pAKT kinase assays, and Tag-lite ligand binding and competition assays.
- Optimization of instrumental settings was done on both instruments for the Tag-lite assays in 384 multi-well plate format. Performance was evaluated by both assay window and Z-prime values.
- Shortening both delay and integration times resulted in better instrument performance for the SpectraMax Paradigm plate reader.
- The combination of the SpectraMax Paradigm plate reader with the Tag-lite assays is shown to be a powerful assay platform for cell surface binding and functional assays and well suited for high throughput screening.

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