APPLICATION NOTE

Label-free Technology

Pathway independent cell-based assays on the EnSpire Multimode Plate Reader with Epic® label-free technology

Authors

Heidi Morgan, M.S.
Miika Talvitie
Sarah Burroughs, Ph.D.
Janet Park-Bewsher
Paul Butler
Tim Cloutier, Ph.D.

PerkinElmer, Inc.
Downers Grove, IL 60515
USA

Introduction

Here we show how the PerkinElmer EnSpire® Multimode Plate Reader with Corning® Epic® label-free technology can be used in multi-well microplate formats to non-invasively identify and characterize multiple G protein-coupled receptor (GPCR) pathways in living cells using an orthogonal approach. GPCRs constitute one of the largest classes of therapeutic targets within drug discovery. By successfully monitoring the ligand-induced dynamic mass redistribution (DMR) in living cells, we demonstrate that this label-free technology is a comprehensive and versatile tool for GPCR research enabling the generation of physiologically-relevant data.

Importantly, these studies illustrate the ability to measure the pathway independent, global response from cellular network interactions which occur during signal transduction, allowing scientists to observe the entire biological picture. Most conventional technologies for studying GPCRs rely on the use of labels or dyes to quantify concentrations of specific cellular biomarkers (cAMP, Ca^{2+}, β-arrestin, etc.). These approaches focus on one of the several components within complex signaling pathways, which may result in failure to observe pharmacologically-relevant events associated with receptor activation.

To demonstrate that the EnSpire label-free platform yields comparable data to the Corning® Epic® label-free system and provides a powerful complementary assay platform relative to labeled technologies, we performed a comparative analysis to the Corning® Epic® system and labeled technologies for multiple GPCR signaling pathways. Our results show the EnSpire label-free platform compares favorably to the Corning® Epic® system in terms of sensitivity and detection, while simultaneously showing high correlation to published pharmacological data with several labeled assays. Finally, we show that the sensitivity of the EnSpire label-free platform enables the detection of cellular responses from endogenously expressed receptors obviating the need for engineered cells to over-express receptors of interest, thus eliminating the possibility of altering cellular biology and minimizing potential false negatives in screening environments. These results also strongly suggest its applicability for use with primary and stem cell applications, which have been proven previously on the Corning® Epic® system.

PerkinElmer®
For the Better

MATERIALS AND METHODS

Reagents

Ligands Isoproterenol Hydrochloride, Propranolol Hydrochloride, N6-CPA, DAMGO, Carbachol, and Atropine were purchased from Sigma Chemical Company (St. Louis, MO). DPCPX and CTOP were purchased from Tocris Bioscience (Ellisville, MO). Fluo-3 was obtained from Molecular Probes (Eugene, OR). AlphaScreen® cAMP kit (Cat # 6760625), ATPlite™ Luminescence Assay System (Cat #6016941), EnSpire label-free 384-well biosensor microplates (Fibronectin Cat# 6057428, Uncoated Cat# 6057408), white 384-well cell CulturPlates™ (Cat # 6007680), and Human adrenergic β2 Receptor; γ-irradiated frozen cells (CHO-K1 cells, Cat# ES-034-CF) and human purinergic, P2Y11 γ–irradiated frozen cells (1321N1 cells, Cat# ES-293-AF) were all obtained from PerkinElmer (Waltham, MA). Human epidermoid carcinoma cells (A431, Cat# CRL-1555), Human Embryonic Kidney 293 cells (HEK293, Cat# CRL-1573), and Chinese Hamster Ovary cells (CHO-M_1, Cat# CRL-1984) were obtained from ATCC® (Manassas, VA). All of the cell culture reagents were purchased from Invitrogen Corp. (Madison, WI).

Cell Culture

A431, HEK293 and 132N1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/L glucose, 2 mM glutamine, and penicillin-streptomycin. CHO-M_1 cells were cultured in Kaighn's F-12K Medium. All cells were grown in the presence of 5% CO_2 at 37 °C. Cells were cultured to 70% confluence, harvested using standard Cell Dissociation Buffer, and split 1:3/1:5. Cells were counted via a haemocytometer or automated cell counter.

Cell Preparation for Label-free Assays

Cultured cells were harvested at 70-80% confluency and seeded into EnSpire label-free microplates (uncoated or fibronectin-coated based on cell type) in serum containing media at an optimized density of 18,000 cells/well. For frozen cells, vials were warmed in 37 °C water bath and suspended immediately in 10 mL cell culture media. Cells were spun at 1200 RPM for 5 minutes. Cell pellet was resuspended in 10 mL media and cells were seeded into EnSpire label-free enabled microplates as described above. Post-seeding, all cell lines were incubated at room temperature for 30 minutes prior to overnight incubation in 5% CO_2 at 37 °C. The following day, cells were observed microscopically to ensure correct morphology and the absence of contamination. A431 cells were serum starved on day 2 post-seeding.

Cell Preparation for AlphaScreen cAMP Assays

Cultured cells were harvested at 70-80% confluency and seeded into CulturPlates in serum containing media at an optimized density of 10,000 cells/well. For frozen cells, vials were warmed in 37 °C water bath and suspended immediately in 10 mL cell culture media. Cells were spun at 1200 RPM for 5 minutes. Cell pellet was resuspended in 10 mL media and cells were seeded into CulturPlates as described. Post-seeding, all cell lines were incubated overnight in 5% CO_2 at 37 °C. A431 cells were serum starved on day 2 post-seeding.

Automation and Liquid Handling Parameters for Label-free and AlphaScreen cAMP Assays

Both the label-free and AlphaScreen cAMP assays were automated using PerkinElmer's JANUS® Automated Workstation. Ligand dilutions were performed using the 8-tip Varispan™ arm. The complete deck setup and detailed ligand dilution protocol are available upon request. Reagent additions, bead additions, media exchanges, buffer exchanges, ligand additions, and mixing were all performed using the 96-well or 384-well Modular Dispense Technology™ (MDT) head. Automation parameters (speed, height, mixing, etc.) were optimized to account for delicate washing, buffer exchange and ligand addition to cells adhered to assay plates. The system was also set up for light sensitive bead additions for the AlphaScreen assay. JANUS liquid handling steps were designed to account for appropriate incubation periods, which vary between the two assays. Manually performed assays provided comparable data for both precision and accuracy. JANUS Application Assistant was used to create robust and reliable easy-to-use assay protocols resulting in significant time savings off of the bench. JANUS Application Assistant screen shots and detailed protocols are available upon request.

Label-free Assay

Cells were washed 4 times using 25 µL/well assay buffer (20 mM HEPES, HEPES buffered saline solution (HBSS) and 0.1% DMSO). Washing was carried out using an aspiration wand or JANUS. The final volume was 30 µL/well. Dispensing was carried out on the side of the well to avoid disturbing the cell layer close to the sensor.

The EnSpire label-free enabled microplates were equilibrated for 2 hours at room temperature. An initial baseline measurement (5 minutes) was taken. The addition of 10 µL antagonist in assay buffer was followed by a 30 minute kinetic measurement. Another addition of 10 µL agonist in the assay buffer was followed by a 30-60 minute final kinetic measurement. Compounds were dispensed using multichannel pipettors or JANUS. Kinetic results were analyzed using MicroPlate Analyzer V2 supplied by Corning®. The difference between the last baseline measurements and the signal maximum was used to determine the EC_{50}/IC_{50} values. Graphs were generated using GraphPad PRISM® v5.01.

AlphaScreen cAMP Assay

Media was removed from cells and replaced with 25 µL of stimulation buffer (5 mM HEPES, HEPES buffered saline, 0.1% BSA, 0.5 mM IBMX) and equilibrated on the lab bench for 30 minutes at room temperature (RT). Antagonist (25 µL in stimulation buffer) was added, followed by a 30 minute incubation on the lab bench at RT. Antagonist (50 µL) was removed from plate and Agonist (5 µL in stimulation buffer) was added. Immediately following, Acceptor beads were added (5 µL in stimulation buffer) and plate was incubated for an additional 30 minutes on lab bench at RT. Donor beads (15 µL containing biotinylated-cAMP in lysis buffer-5 mM HEPES, 0.3% Tween-20, 0.1% BSA) were added for a final assay volume of 25 µL. While the beads are in use, light is limited and the plate kept covered. The plate was incubated for 1 hour at RT to allow the beads to equilibrate. The plate was then read on the EnSpire with specifications for AlphaScreen 384-well assays. Endpoint results were viewed using Excel® and graphs were generated using GraphPad PRISM® v5.01.

Fluo-3 Ca^{2+} Mobilization Assay

CHO cells at passages 3-5 were grown in Costar 96-well clear cell culture microplates (Corning Inc, Corning, NY) until ~90% confluency, washed twice with 1x HBSS (1x regular Hank's balanced salt solution, 20 mM HEPES buffer, pH 7.0) in the presence of 2.5 mM probenicid, and labeled in the same buffer containing 4 mM Fluo-3 for 1 h at RT. The cells were then washed twice with the HBSS, and maintained with 100 mL HBSS containing 2.5 mM probenicid. The assay was initiated by transferring 100 mL thrombin solution to the cell plate and calcium signal recorded over 6 minutes with a 6 second interval using the EnSpire reader with the injector option. The assays were carried out at room temperature to allow direct comparison with optical sensing data.

Viability Assay

Cell viability of cells subjected to the label-free assay was determined using the ATPlite™ Luminescence Assay System (PerkinElmer Product #6016941). The procedure was adapted to 384-well plates as follows. After the label-free experiment was completed, 15 µL assay buffer was removed from each well. Using a multichannel pipettor, 12.5 µL Lysis Buffer (provided with the kit) was added to each well and the plate was placed on a microplate shaker for 10 minutes. Next, 12.5 µL ATP substrate solution (provided with the kit) was added to each well. The EnSpire Multimode Plate Reader with label-free technology and ultra-sensitive luminescence was programmed to incorporate 10 minutes of shaking at 600 rpm (orbital, magnitude = 1, inside reader) prior to the read. This served the dual purpose of mixing and dark-adapting the plate prior to the read. A one-second integration time was used to collect the luminescence in each well. Data were exported for plotting.

RESULTS

Human epidermoid carcinoma A431, Chinese Hamster Ovary (CHO-K1), human astrocytoma 1321N1, and Human Embryonic Kidney 293 (HEK293) cells were used to test agonist and antagonist activity of either endogenously or recombinantly expressed GPCR receptors, including Adrenergic-β2 (β_2AR), Muscarinic 1 (M_1), Opioid Mu (OP_3), Purinergic $P2Y_{11}$ and Adenosine A_1, using the EnSpire with label-free technology and several leading labeled cAMP and Ca^{2+} detection technologies.

Figure 1 illustrates the workflow for the EnSpire with label-free cell-based assay. All of the liquid handling steps were performed both manually and on the JANUS Automated Workstation with comparable results. The advantage of automating the label-free steps was in providing a robust and reliable easy-to-use protocol resulting in significant time savings. The cell-based assay protocol on the EnSpire label-free platform involves a short baseline scan followed by a single compound addition step (agonist mode), and/or a second compound addition step in antagonist studies. Following compound additions, the microplate is immediately scanned for 30-60 minutes at room temperature enabling the full kinetic profile of each compound to be obtained. Conversely, the cell-based assays using the cAMP and Ca^{2+} platforms require several reagent addition steps, each followed by variable incubation steps.

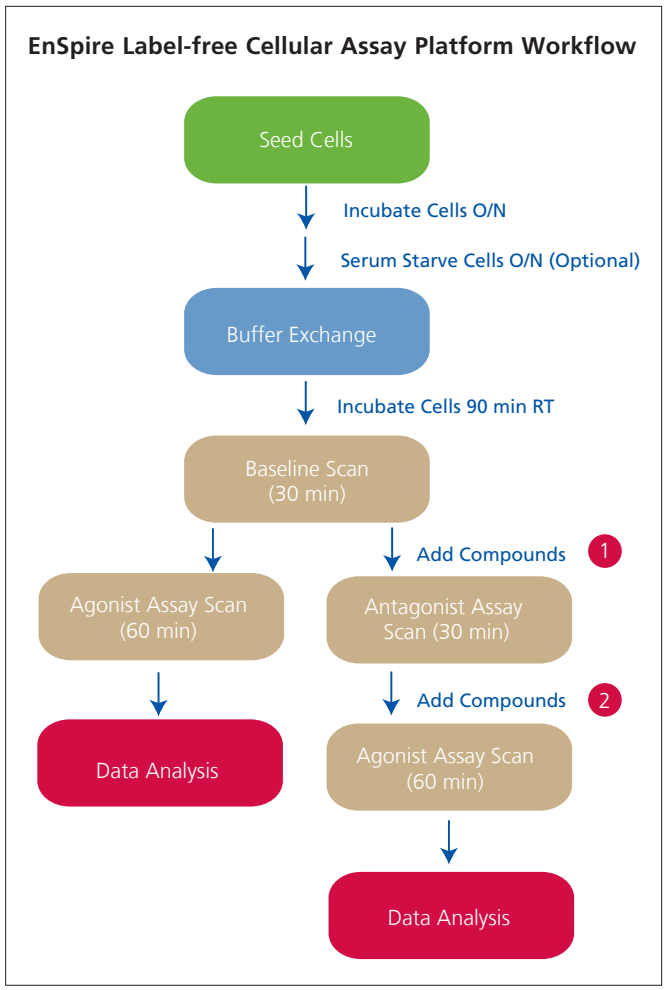


Figure 1.

Table 1. Summary of EC_{50}/IC_{50} ligand values for targets using EnSpire with label-free technology, the Corning® Epic® instrument, and EnSpire with labeled assays.

			EnSpire Label-free		Epic® Label-free		EnSpire Labeled Assay**	
Target Coupling	Host Cell	Culture Conditions	Agonist EC_{50}*	Antagonist IC_{50}*	Agonist EC_{50}	Antagonist IC_{50}	Agonist EC_{50}	Antagonist IC_{50}
β_2AR (G_s)	A431 (Endogenous)	Passaged	0.03	6.2	0.02	6.0	1**	1.8
β_2AR (G_s)	CHO-K1 (Recombinant)	Frozen	0.01	0.3	–	–	1	1.4
Opioid Mu (G_i)	CHO-K1 (Recombinant)	Frozen	0.9	43	0.5	35	2.3	0.03
A_1 (G_i)	HEK293 (Endogenous)	Passaged	47	35	100	153	–	–
M_1 (G_q)	HEK293 (Endogenous)	Passaged	5900	2.2	5000	2.0	–	–
M_1 (G_q)	CHO-K1 (Recombinant)	Passaged	177	6.3	–	–	85	14
$P2Y_{11}$ (G_q)	1321N1 (Recombinant)	Frozen	766	27000	750	31000	–	–

* Potency in [nM]

** Labeled Assays: AlphaScreen cAMP (β_2AR, Opioid Mu), Ca^{2+} Flux Assay (M_1)

The targets and ligands showcased in this application note were chosen because they have been previously characterized with the Epic® System. We demonstrate a high-level of agreement in assay sensitivity, data correlation and instrument performance between the EnSpire with label-free platform and Corning® Epic® System.

Table 1 summarizes the EC_{50}/IC_{50} ligand values for the targets studied obtained using the EnSpire, Corning® Epic® and complementary labeled assays. The EnSpire label-free platform has the ability to run complementary labeled assays in conjunction with label-free applications on the same system. The ability to interrogate ligand behavior in both a labeled and label-free capacity allows for faster and more thorough pharmacological and pharmacokinetic investigation facilitating earlier indications of target druggability, cellular accessibility and drug toxicity.

Table 1 further illustrates the ability of the EnSpire label-free platform to monitor GPCR biology in multiple cellular contexts and culture conditions (endogenous, recombinant, passaged and frozen cells), greatly increasing its value and flexibility for studying diverse biological applications.

Figure 2 shows several representative agonist and antagonist dose-response curves obtained on the EnSpire label-free platform. Several controls were added to each assay microplate in order to evaluate assay robustness (Z') and instrument performance. Assay robustness was calculated for each microplate by measuring the response of maximal agonist (positive control) vs. assay buffer alone (negative control) (Figure 3). Using the EnSpire label-free platform, the agonist Z' values were ≥ 0.7 for each target studied, with CV ≤ 5% (data not shown). These data demonstrate that the integration of Corning® Epic® technology with the EnSpire Multimode Plate Reader offers comparable results to that of the Corning® Epic® System. Thus, scientists should expect similar performance and data across other target classes and applications tested on the Corning® Epic® when using the EnSpire with label-free technology.

Similar to the results obtained in previous Corning® Epic® studies, Figures 2A, B and 2 G,H demonstrate the ability of the EnSpire with label-free technology to monitor receptor activation in both endogenously and recombinantly expressed systems for multiple receptors, β_2AR and M_1. Furthermore, Figures 2 D,E demonstrate the capacity for the EnSpire with label-free technology to robustly monitor receptor activity in G_i pathways, which are commonly difficult targets to assay using labeled systems. Together, these data highlight the added flexibility to study target and ligand behavior in a wide variety of assay and cell-type conditions, in addition to enabling more biologically-relevant cellular assay investigation.

Figures 2 C, F and I highlight the complementary capability of the EnSpire to monitor both labeled and label-free assays for all three G-protein coupling pathways. Using the Alpha detection module on the EnSpire, the PerkinElmer AlphaScreen® cAMP kit was used to study β_2AR (G_s) and OP_3 (G_i) agonist and antagonist activity in the same cell lines as run with the label-free assays. The data for all ligands tested were in strong agreement with published literature for cAMP studies while also showing excellent agreement with general compound potency and pharmacology observed in previous label-free assays (Table 1). To study G_q-mediated signaling, M_1 agonist and antagonist activation of calcium production was followed using a Fluo-3 Ca^{2+} mobilization assay. This was performed using the fluorescence module and the reagent injector option on the EnSpire

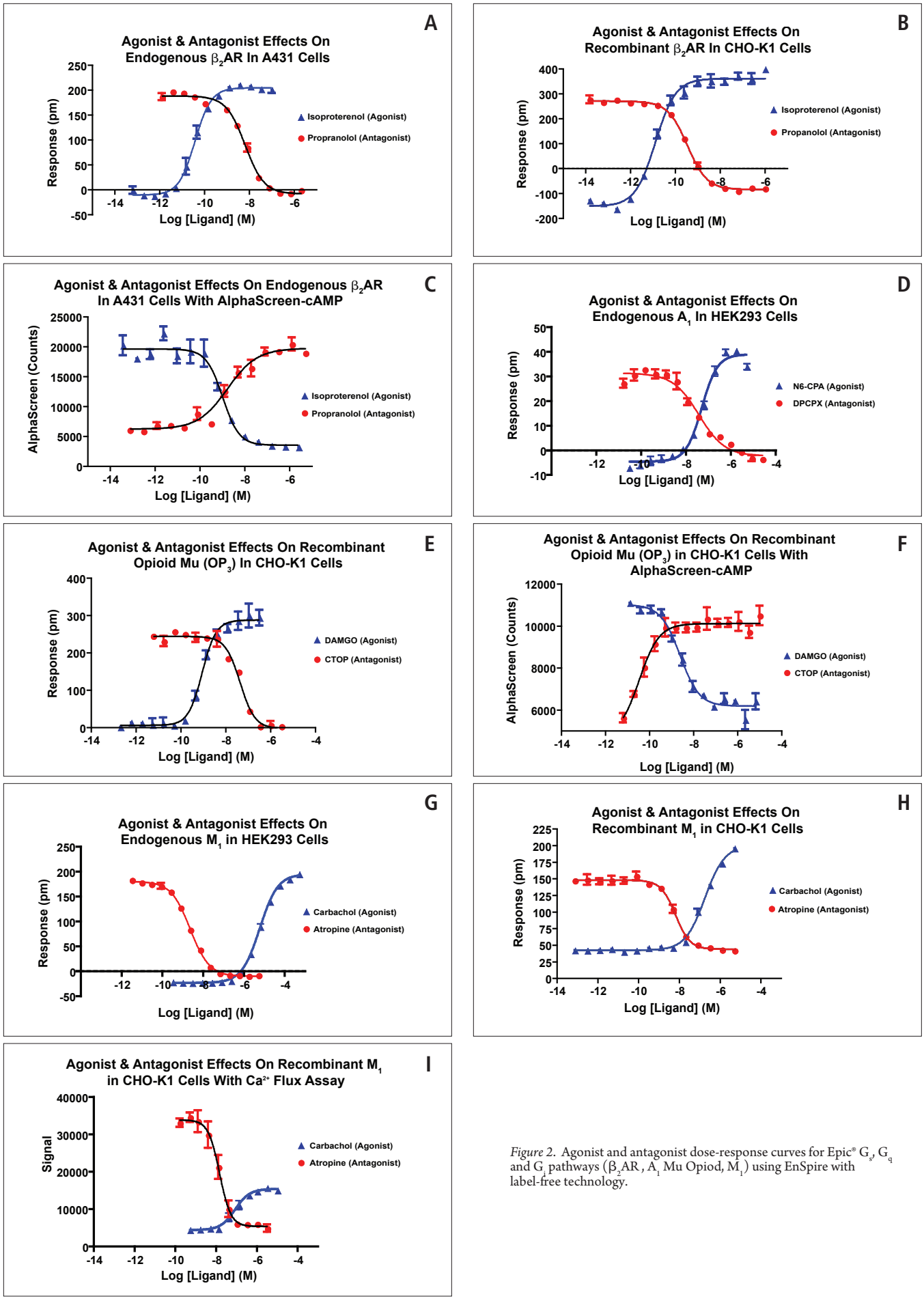
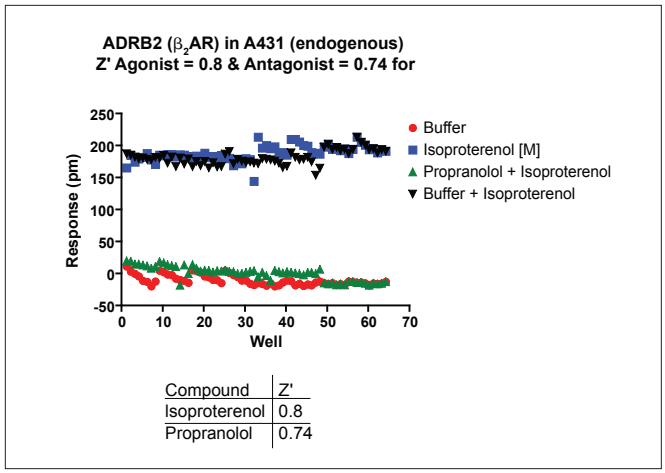
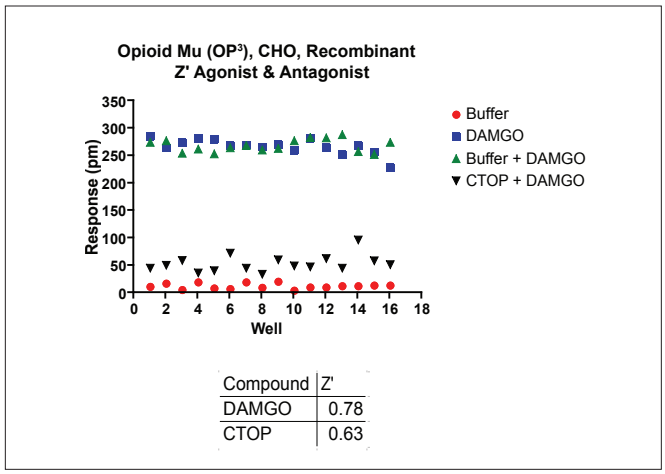


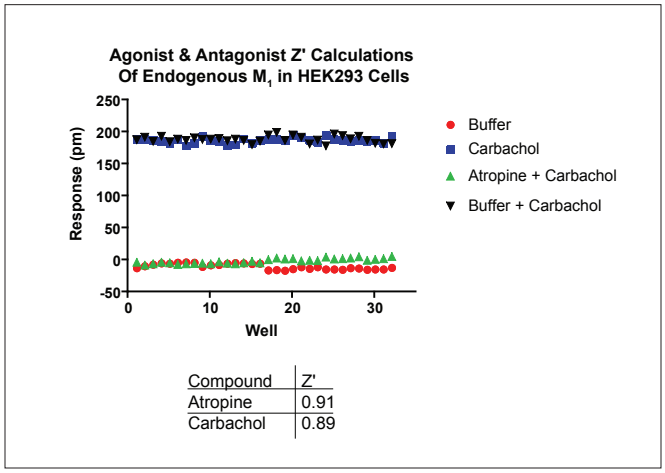
Figure 2. Agonist and antagonist dose-response curves for Epic® G_s, G_q and G_i pathways (β_2AR, A_1 Mu Opiod, M_1) using EnSpire with label-free technology.



Compound	Z'
Isoproterenol	0.8
Propranolol	0.74



Compound	Z'
DAMGO	0.78
CTOP	0.63



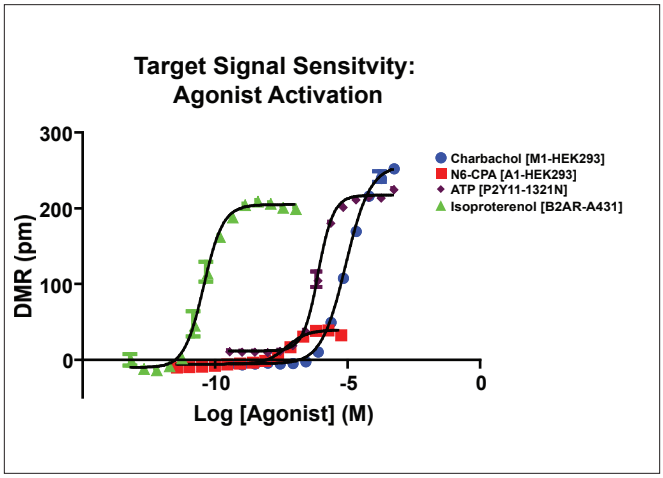
Compound	Z'
Atropine	0.91
Carbachol	0.89

Figure 3. Assay robustness on EnSpire with label-free. Agonist and antagonist Z' values are compared for 3 different host cell types expressing different targets both for endogenously expressed receptors and in recombinant cells.

instrument. Similar to the cAMP pathway studies, there was excellent correlation between published literature and accompanying label-free assays in the same cell lines.

Target Signal Sensitivity: Agonist Activation Using the EnSpire Label-free Platform

It has been demonstrated previously that Epic® technology can detect ligand activity that elicited very low DMR (<50 pm) upon receptor activation. In Figure 4 we summarize those findings by showing that the EnSpire with label-free technology can detect receptor activation for both high and low DMR signaling ligands, irrespective of endogenous or recombinant expression systems. This enhanced sensitivity demonstrates the value of the EnSpire label-free platform for accurately and robustly monitoring less potent compounds possessing low DMRs that are frequently encountered in screening environments or in characterizing small differences in potency (i.e., pathway activation) between structurally similar chemotypes, and plausibly with unique pharmacologies such as partial or inverse agonists and allosteric modulators.



Target	Coupling	EC_{50}*	DMR	A
M_1	G_q	5900	254	0.89
A_1	G_i	47	34	0.72
β_2AR	G_s	0.03	200	0.75
$P2Y_{11}$	G_q	766	226	0.80

* Potency in [nM]

Figure 4. Target Signal Sensitivity: Agonist Activation Using the EnSpire Label-free Platform.used to calculate Z'

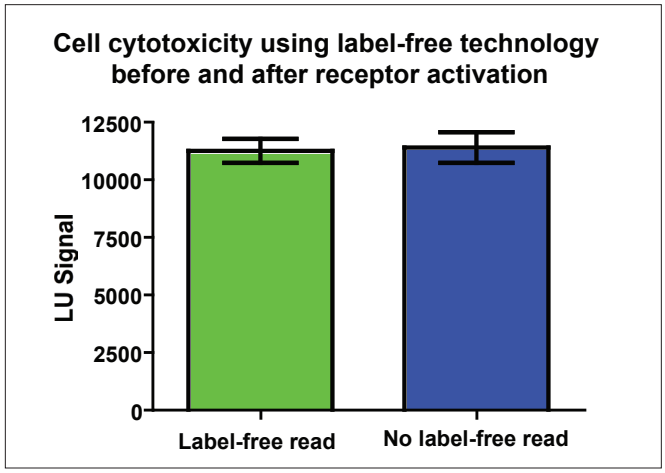


Figure 5. The EnSpire label-free platform imposed no detrimental effects to cell viability subsequent to the label-free scans as demonstrated with the ATPlite cytotoxicity assay. Experiment was conducted with A431 endogenous cells.

Label-free scans show no effect on cell viability.

Figure 5 demonstrates the non-invasive nature of label-free technology. Using the PerkinElmer ATPlite cytotoxicity assay to monitor how cells respond to the label-free detection process, we demonstrate that the EnSpire label-free platform imposed no detrimental effects to cell viability subsequent to the label-free scans. ATPlite was run on cells that had undergone a 60 minute label-free scan, both pre- and post-receptor activation, and compared to identical cells that did not undergo label-free analysis. This resulted in no measurable cytotoxicity relative to the positive control suggesting application for primary cells and cell differentiation applications such as stem cells.

SUMMARY

With labeled technologies (fluorescence, luminescence, absorbance and Alpha) and the unique capacity to work under highly variant label-free assay conditions, the EnSpire with label-free technology is well positioned relative to competing single mode label-free instruments. EnSpire offers the ability to comprehensively interrogate both pathway-dependent and pathway-independent ligand pharmacology for receptor activation, facilitating more biologically-predictive information for putative drug candidates and basic target research.

CONCLUSIONS

Experimental results presented here using the EnSpire Multimode Plate Reader with label-free technology, in combination with Epic® published literature, demonstrate the following aspects of label-free detection.

1. Label-free is a versatile tool for pathway-independent, global analysis of basic systems biological research and physiologically-relevant drug discovery.
2. Regardless of which GPCR pathway is studied, there is a clear positive response indicative of pathway activation demonstrating label-free is a universal platform for studying G_s, G_i, G_q, and $G\alpha_{12/13}$ pathways[1]. Furthermore, the label-free platform enables analysis of GPCR functional selectivity and biased agonism within a single reaction.
3. There was good correlation between the label-free analyses in endogenous vs. recombinant GPCR cell lines for multiple ligand pharmacologies.
4. Label-free is a non-invasive technology allowing cells to be run sequentially in both label-free and labeled systems. This strongly suggests that the EnSpire with label-free technology is a robust assay platform for studying embryonic stem cells without fear of triggering non-specific differentiation within cell colonies. This has been previously proven on the Corning® Epic® system.
5. There was a high-level of agreement in assay sensitivity, data correlation and instrument performance between the EnSpire label-free platform and Epic® systems.
6. There was good correlation between the pharmacological profiles obtained between label-free vs. labeled assays, demonstrating the complementary relationship of these orthogonal technologies.
7. There is good pharmacological correlation regardless of the host cell and expression level of the particular GPCR target.
8. There was excellent correlation between the label-free analyses in freshly passaged vs. frozen cell lines.

A Technical Note detailing the performance of the EnSpire with label-free technology in comparison to Epic® is also available, as well as an Application Note summarizing the use of EnSpire with label-free technology with a variety of biochemical assays previously optimized on the Corning® Epic® instrument.

1. Schröder R, Janssen N, Schmidt J, Kebig A, Merten N, Hennen S, Müller A, Blättermann S, Mohr-Andrä M, Zahn S, Wenzel J, Smith N, Gomeza, J, Drewke C, Milligan G, Mohr K, Kostenis E. Deconvolution of complex G protein–coupled receptor signaling in live cells using dynamic mass redistribution measurements. Nat. Biotechnol. Published online 15 August 2010: www.nature.com.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs

009449_01 Printed in USA Jan. 2011