Ultrasensitive, 3456 or 1536-Well Miniaturization-Friendly Chemiluminescent Detection of PathHunter® Cell-Based GPCR and Kinase Assays

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Abstract

DiscoveRx's PathHunter® platform is a simple, cell-based, chemiluminescent assay format that allows for screening large numbers of small molecules or hybridoma supernatants in a one-step, no-wash format making it both user-friendly and HTS-compatible. In this study, we demonstrate the 3456- and 1536-well application of DiscoveRx's PathHunter GPCR β-Arrestin assays as well as PathHunter® Receptor Tyrosine Kinase assays using the Echo® liquid handler (Labcyte Inc.) for acoustic, non-contact compound and assay reagent dispensing to AURORA high performance 3456 plates. All assays are analyzed on the new highly sensitive BMG LABTECH PHERAstar *FS* microplate reader. Precise liquid handling and ultrasensitive instrumentation further enhances the speed of processing allowing a complete 3456-well PathHunter® assay plate to be read in less than 3 minutes. Robustness of the PathHunter assay is further enhanced on a BMG LABTECH instrument and in combination with the Echo liquid handler, it reduces assay variability, improves Z' values as well as data quality. Performing a cell-based assay at <5 µL volume translates to very low compound requirement, fewer cells and low reagent usage. A highly miniaturized PathHunter assay in combination with the BMG LABTECH PHER*Astar FS* and the Labcyte Echo liquid handler provides pharma/biotech/academic consortia a faster, simpler, cost-effective and a more efficient way to screen large compound libraries thereby enabling cost effective drug discovery campaigns.

PathHunter® Protein-Protein Interaction Assay-Principle

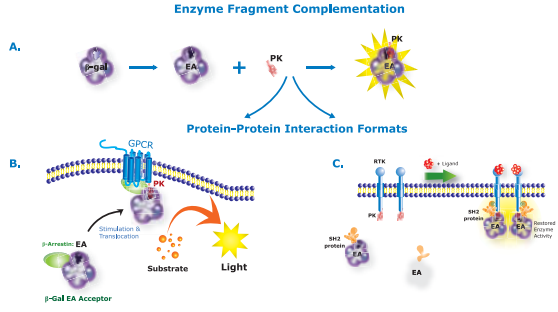

Figure 1. **(A)** DiscoveRx's proprietary Enzyme Fragment Complementation (EFC) is a homogeneous, non-radioactive detection technology based on two genetically engineered β-galactosidase fragments - a large protein fragment (Enzyme Acceptor, EA) and a small peptide fragment (Enzyme Donor, ED). Separately, the β-gal fragments are inactive, but in solution, they rapidly recombine to form active β-galactosidase enzyme that hydrolyzes substrate; producing an easily detectable chemiluminescent or fluorescent signal. The PathHunter® technology from DiscoveRx is an adaptation of EFC that provides a novel cell-based assay format for detecting protein-protein interaction. In this approach, 42 AA enzyme fragment, ProLink™ is appended to GPCR **(B)** or RTK or cytokine receptor **(C)**, while EA is recombinantly expressed as a fusion protein with β-Arrestin **(B)** or SH2 phosphotyrosine domain **(C)**. Activation of the receptor upon ligand binding allows interaction of GPCR β-Arrestin or RTK-SH2 protein resulting in a measurable Chemiluminescene signal.

1536-3456 Detection by PHERA*star FS*

The PHERAstar *FS* *(Figure 2)* is the next generation multi-detection microplate reader from BMG LABTECH. It combines rapid plate reading necessary for HTS assays with enhanced performance and sensitivity needed to measure small liquid volumes. To achieve this, the PHERAstar *FS* has separate detection systems with separate measurement electronics for each mode, allowing it to obtain extremely low background noise in all assay formats including luminescence. In addition, the advanced optical system contains a 0.1 mm z-height focus adjustment which measures the highest possible intensity in each well in all plate formats up to 3456. Other features such as three excitation sources (including two lasers), direct optic bottom reading, instantaneous full spectrum absorbance analysis, and simultaneous dual emission detection all make the PHERAstar *FS* the next generation HTS microplate reader.

Figure 2. PHERAstar *FS* (BMG LABTECH)

Liquid Handling by Echo® 500 Series

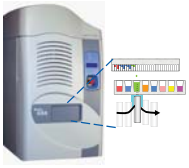

Figure 3. Echo® 520/550/555 (Labcyte Inc.)

The Labcyte Echo® 500 series revolutionizes liquid transfer by using acoustic energy to eject fluids. The Echo® 500 series allows for assay miniaturization to previously unattainable volumes. Echo® liquid handlers transfer 2.5 nL droplets repeatedly, so precision and accuracy are consistent over a larger volume range. Large volume transfer is achieved by transferring several hundred droplets per second. Transfer is non-contact and tipless, with increased cost savings from elimination of tip costs and washing fluids. Miniaturization with the Echo® liquid handler retains high assay performance, allowing quantitative results at higher assay well densities. The Echo® liquid handler can be used to transfer from any source well position to any destination well position. These can be simple fluids (media for growing cells, buffers, DMSO) or viscous solutions (lysis buffers, antibodies with glycerol, or transfection reagents).

Assay Work-flow

Cell Handling	PathHunter® Cells were diluted in Hank's Buffered Saline Solution. 40 µL were added to Echo® qualified 384-well polypropylene microplates (P-05525). PathHunter® Cells were transferred to an Aurora 1536-well or 3456-well microplate using an Echo® 555 liquid handler with a 384PP_AQ_SP calibration. Cell volumes were varied as part of the testing plan. Compounds such as pergolide or ProLactin or β-NGF was diluted in DMSO and added to an Echo® qualified 384-well polypropylene microplate and transferred at 30 nL using the 384PP_DMSO calibration.
	Incubation as per β-Arrestin and Cell-Based Kinase Assay Recommended Protocol
Addition Reagents	PathHunter® Flash Detection reagent was transferred from an Echo® qualified 384-well microplate and transferred (at a 1:1 ratio of cell volume: PathHunter® Flash Detection reagent) using Echo® 555 liquid handler with the 384PP_AQ_SP calibration.
	Incubate for 30 mins at RT
Detection	Chemiluminescence signal on both 1536 and 3456 well microplates was measured on the PHERAstar *FS* using a measurement time of 0.1 sec/well at a gain of 3600 with the Lum Plus optic module. The adjustable gain allows the PHERAstar *FS* to obtain the highest possible signal and the best signal-to-noise ratios. An automatic z-height adjustment was performed on the well with the highest signal before reading the plate.

1536-well Miniaturization of PathHunter® Cell-Based Kinase Assay

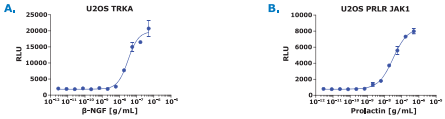

Figure 4. PathHunter® U2OS cells expressing TrkA **(A)** and PRLR-JAK1 **(B)** were seeded at 1000 cells/well in a white tissue culture coated AURORA 1536 plates. Cells were stimulated with known agonists β-NGF and prolactin. The plates were then incubated for 3 hrs at 22°C. A robust assay response was observed with an adherent 1536 protocol at a 6.03 µL total volume.

1536-well Miniaturization of PathHunter® β-Arrestin Assay (Adherent Mode)

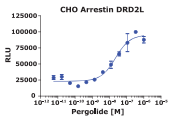

Figure 5. PathHunter® CHO-K1 cells expressing long isoform of Dopamine receptor D2 were seeded in a white tissue culture coated AURORA 1536 plates at 1000 cells/well and incubated overnight. Cells were stimulated with Pergolide for 90 minutes at 37°C and signal was detected using the PathHunter® Detection reagents (see materials and methods). These results indicate that the PathHunter® β-Arrestin assay can be successfully adapted to miniaturized to a 1536-well format.

Successful Miniaturization of PathHunter® β-Arrestin Assay (Suspension Mode)

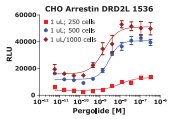

Figure 6. PathHunter® DRD2L cells 250 to 1000 cells/well cultured in suspension mode were added to 1536-well plates in a 1 µL volume and challenged with 5-10 nL of 100X pergolide (in DMSO) compound added to cells via the Echo® Liquid handler. The DRD2 1536 suspension protocol produced dose curves similar to the adherent cell experiment. The assay performance was significantly improved at 500 or 1000 cells/well. For optimal performance it is recommended that cell seeding volume using the Echo® instrument should not exceed 1 µL.

Robust Performance of PathHunter® Assay in a 3456-well Format

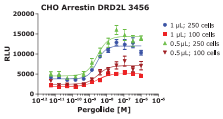

Figure 7. The cells were dispensed into 3456-well plate using Echo® 555. Varying cell densities were tested to determine the optimal cell number in the new plate format. 5 nl of 100X compound(in DMSO) was added to the cells plated in 0.5 µl (10 nl for the cells plated in 1 µl) via the Echo® instrument. Higher cell numbers performed better regardless of seeding volume.

3456-Miniaturization of PathHunter® β-Arrestin Cells

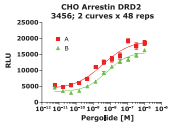

Figure 8. In this experiment, we explored the reproducibility of the assay in a 3456 plate. 500 cells/well of the PathHunter® DRD2L were added to 3456 well AURORA plates in a 0.5 µL volume, via the Echo instrument. 144 replicates for each compound dose was run. 5 nL of 100X pergolide (in DMSO) added to cells via the Echo® instrument. Equal volume (0.5 µL) of PathHunter® Flash substrate added by the Echo® Liquid handler and read on the PHERAstar *FS*, 0.1 sec/well. The assay yielded reproducible results with good correlation to 384-well and 1536 protocol.

Summary

Miniaturization with highly evolved automation technologies affords the benefit of faster screens run at much lower costs. In this presentation we have demonstrated the application of the PathHunter GPCR and cell-based kinase assays on 3456 and 1536 platforms using Labcyte's Echo® Liquid handler and BMG LABTECH's PHERAstar *FS*. Labcyte uses acoustic energy to transfer fluids in a gentle, non-contact manner, without tips, tubes, or nozzles that can create challenges in small high-throughput assays, while direct optic bottom reading, high resolution cell layer scanning, injection at the point of measurement, two excitation lasers and 3456-well microplate capability allow the PHERAstar *FS* to perform assays at lower volumes and faster speeds not possible on other readers.

- PathHunter® assays are one step, chemiluminescent, whole cell assays that allow rapid optimization for assay development and HTS needs
- PathHunter® assay can be miniaturized to < 2 µL total volume in a 3456 well suspension protocol and a 6 µL 1536 protocol
- 0.1 sec/well read time and ultra sensitive detection of low volumes demonstrates that the PHERAstar *FS* is a HTS/uHTS-friendly instrument
- Liquid handlers such as Echo® 555 can transfer 5 nL of liquids into plates greatly reducing cost and eliminating errors that arise from manual pipetting
- AURORA High performance 3456 and 1536 plates used in this assay resulted in high signal and low well-to-well crosstalk

Technology Access

DiscoveRx

DiscoveRx offers industry leading menu of GPCR and Cell-Based Kinase Assays
- PathHunter® DRD2(L) β-Arrestin Cell Line (Cat. #93-0579C2)
- PathHunter® Trk A Functional Assay (Cat. #93-0462C2)
- PathHunter® Flash Detection Reagents (Cat. #93-0247 series)
- PathHunter® PRLR-JAK1 Functional Assay (Cat. #93-0686C3)

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PHERAstar *FS* www.bmglabtech.com

LABCYTE

- Echo® 520/550/555 Liquid Handlers
- 384-well PolyPropylene Microplates (Cat. #P-05525)

www.labcyte.com

Aurora 1536-well (Cat. #300065) & 3456-well (Cat. #300062)
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