Mix-and-Read Assays For Regulated Ubiquitination & Degradation of Proteins Using PathHunter® Technology

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ABSTRACT

Ubiquitin-mediated degradation of signaling proteins is a primary means of signal regulation in a wide variety of cellular processes including inflammatory signaling, DNA damage responses, developmental pathways, and the cell cycle. However, observation of these events with the throughput necessary for modern drug discovery has been challenging. Numerous reporter proteins exist that can be used as fusion partners, however their size and stable tertiary structure impact the half-life of the targeted protein, limiting their utility. We reasoned that a smaller tag, lacking tertiary structure could be used as an inert fusion partner that could preserve the natural turnover of the protein target. To this end we employed the DiscoveRx® enzyme complementation system. In this system the fusion partner is a peptide that is less than 5kD making the system minimally invasive. The levels of the protein fusion can be quantified with a single reagent addition making this system amenable to high-throughput screening. Here we demonstrate the utility of the system across a wide range of targets and directly compare performance with luciferase fusions.

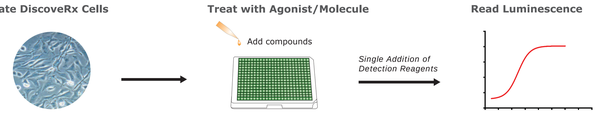
ASSAY PRINCIPLE & PROTOCOL

EFC Technology

PathHunter® assays utilize the well established Enzyme Fragment Complementation (EFC) technology pioneered by DiscoveRx®. This robust technology is based on the complementation of two inactive β-galactosidase enzyme fragments, EA which encodes the majority of the enzyme, and a small peptide termed ProLabel. The ProLabel® tag has a high affinity for EA. Here we introduce a new tag referred to as enhanced ProLabel that is smaller and has better complementation with EA than the traditional ProLabel.

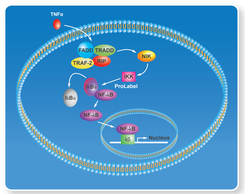
Protein Quantitation using EFC

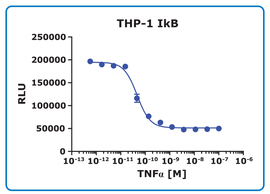
General Assay Protocol



The cells are seeded in 384-well plates and incubated overnight in assay media. The following day the cells are treated with the indicated compounds. After compound incubation which ranges from 30 minutes to 6 hours, the detection reagents are added. The detection reagents, containing lysis buffer, the EA fragment and substrate, are optimized for enzyme complementation for this assay format. After a 30 minute incubation, the plate is read on a standard luminometer.

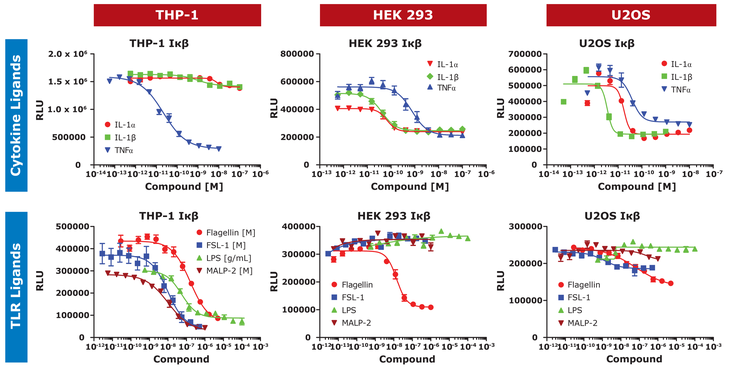
APPLICATION TO NF-κB SIGNALING





The PathHunter® HEK 293 IκB Functional Assay is used with the PathHunter ProLabel/ProLink (PL/PK) Detection Kit (93-0812) for measuring human I-Kappa-B degradation. PathHunter HEK 293 IκB Cells were plated in a 384-well plate at 10,000 cells/well and treated with the known agonist, TNFα for 30 minutes. Upon stimulation, IκB gets degraded and the prolabel-tagged IkB can be detected by the addition of PL/PK Detection Kit, which has the complimentary half of the enzyme (β-galactosidase) fragment. Plates were incubated at room temperature in the dark for 1 hour and read on a standard luminometer.

PATHHUNTER SYSTEMS CAN BE EMPLOYED ACROSS A RANGE OF CELL TYPES

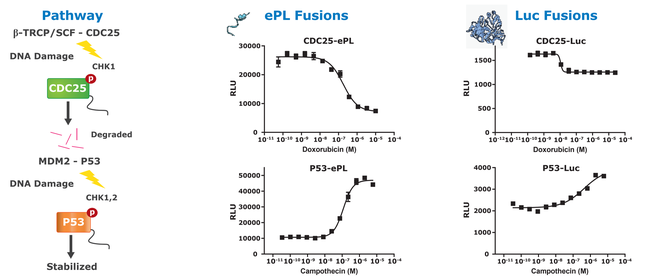


Detection of endogenous receptor activity using the ePL tag. The PathHunter Iκβ assays are stable cell lines expressing Iκβ with ProLabel (PL) fused to the C-terminus. Cells were plated in 384-well plates, 20 µL/well assay media. HEK 293 and U2OS cells were incubated overnight at 37°C/5% CO_2 to allow the cells to attach and grow. The non-adherent THP-1 cells are assayed at any time after plating. Cells were exposed to a titration of ligand for 1 hour at 37°C. PathHunter ProLabel/ProLink detection reagents were added and the assay plates were read on an Envision luminometer. The varied responses observed in the different cell backgrounds are due to the presence and absence of specific receptors indicating that the endogenous receptors can be interrogated using this approach.

SIZE COMPARISON OF COMMON FUSION REPORTERS

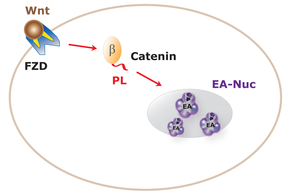
The crystal structure of the ePL tag is compared to other commonly used fusion partners, firefly luciferase and GFP. The ePL tag is less than 20% of the size of the other reporters and lacks significant tertiary structure. These properties allow it to be used as an inert fusion partner that accurately reflects target biology in comparison to the fully folded and much larger fLuc and GFP.

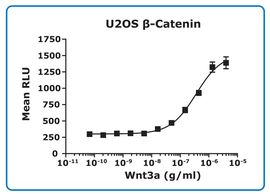
COMPARISON OF LUCIFERASE AND EPL FUSION PROTEIN PERFORMANCE

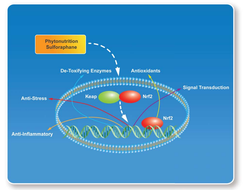


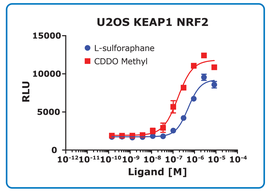
In the top panels, the wt, full length CDC25 was fused at its C-terminus to either ePL *(Left Panel)* or firefly luciferase *(Right Panel)* and expressed in U2OS cells. The cells were incubated with increasing concentrations of doxorubicin for 6 hours. In the left panel the InCELL detection reagents were used to detect the ePL signal and luciferin was used to detect the luciferase activity. The raw RLU's were then plotted. As expected, the DNA damaging agent doxorubicin caused a dose dependent decrease in the CDC25-ePL and the Luciferase fusions. However a larger change in signal is observed from the the cells expressing the ePL tagged fusion proteins. In the bottom panels, U2OS cells expressing the full length P53-ePL or the Luciferase fusions were treated with doxorubicin for 6 hours, and signal was detected in the same manner described for the CDC25 cell lines. Similar results were obtained - the stabilized P53 exhibited a much larger change of signal when fused to ePL versus luciferase.

EFC APPLICATION TO OTHER UBIQUITIN REGULATED PATHWAYS









Both β-catenin and NRF2 are regulated by continuous ubiquitination and degradation in the absence of activating stimuli by the β-TRCP/SCF and KEAP1-cul3 complexes respectively. In the presence of signal the proteins are stabilized and translocate to the nucleus. To monitor this process, cells were engineered to express the target fused to the ePL tag, with the second complementing partner (EA) expressed in the nucleus. Upon stabilization and translocation of the ePL tagged protein, complementation ensues and signal is detected using the PathHunter detection reagents. The top panel shows U2OS cells that are expressing β-catenin fused to ePL are stimulated by a titration of Wnt3A to activate endogenous Frizzled receptors, and subsequently, β-catenin. Cells were incubated with ligand for 6 hours and signal was detected using Pathhunter detection reagents. In the bottom panel, cells expressing NRF2-ePL, KEAP1, and EA (in the nucleus) were incubated for 6 hours in the presence of antioxidant compounds. Signal was also detected using the PathHunter detection reagents.

SUMMARY

Summary of the ePL tag advantages for ubiquitination and regulated proteolysis

- Minimally invasive tag = natural protein life cycle -
  - This maximizes the assay window by not imparting stability or instability from the detection tag.
- Sensitivity to low pM levels -
  - Each ePL fusion creates an enzyme, upon addition of the InCELL detection reagent, allowing low levels of the fusion proteins to be detected.
- A homogenous, whole cell, single addition assay
  - Highly miniaturizable and readily adaptable, from bench-top to HTS.
- Ready to go Biosensor cells
  - Assay ready kits, clonal cell lines, and custom assay options for your target of interest.
- Highly flexible -
  - N and C-terminal fusions perform comparably, the ePL can be used by itself for degradation assays or in conjunction with EA for translocation assays.

TECHNOLOGY ACCESS

PathHunter® Pathway Assays
Ready-to-go, biosensor cell lines with a complimentary detection system offers you convenience and saves months of assay development time.

Custom Projects
Utilize DiscoveRx's proprietary EFC technology to build your own functional cell-based kinase assays. Talk to our experts about your kinase targets, contact CAD@discoverx.com.

Custom Screening & Profiling Services
Send your compound for family-wide profiling in select targets or for selectivity studies and unique information. For more information, please email profiling@discoverx.com.

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