New Cell-Based Kinase Assays for TrkA and Other Receptor Tyrosine Kinases using PathHunter™ Protein-Protein Interaction Technology

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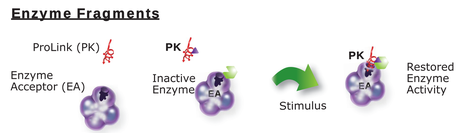
Abstract

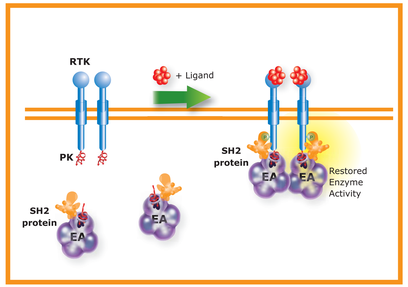
The PathHunter™ technology from DiscoveRx is an adaptation of Enzyme Fragment Complementation (EFC) that provides a novel, generic cell-based assay format for detecting protein-protein interactions. This approach has enabled development of a number of new screening and profiling assays for targets that have been difficult to address in a cell-based assay. One such class of targets, the receptor tyrosine kinases, are an example of a class of proteins for which we have now demonstrated productive, functional cell-based assays using protein-protein interaction as the detection mechanism. In this PathHunter assay approach, a small peptide epitope (ProLink) is expressed recombinantly on the target protein of interest, in this case the intracellular C-terminus of the RTK. This is co-expressed with a larger sequence, termed enzyme acceptor (EA) that is attached to a protein thought to interact with your target. A productive protein-protein interaction will generate an active beta-galactosidase enzyme, which is detected using a chemiluminescent substrate. We will present data illustrating the application of this approach to the TrkA kinase, an important target in neurological research. Our results show that this assay can be used to identify inhibitors of kinase activity in a simple, homogeneous screening assay that is cell-based and therefore more biologically relevant than traditional biochemical approaches.

Figure 1. Cellular EFC Asay for RTK Activation

EFC System to monitor Protein-Protein Interactions



EFC Assay to monitor Receptor Tyrosine Kinase Activation



Methods

- All cells lines used were from DiscoveRx Corporation and express various RTKs tagged with ProLink in cells stably expressing SH2-EA fusions.
- For all assays, 10,000 cells per well were seeded in 20 µL media and incubated overnight in 0.1% BSA and appropriate basal media.
- For agonist assays, 5 µL 5x compound was added to cells and incubated at Room Temp.
- For antagonist assays, 5 µL 5x compound was added to cells and incubated at 37°C/5% CO_2 for 10 minutes, after which 5 µL 6x agonist was added and incubated for 60 minutes.
- SH2-EA complex formation was detected with 50% (v/v) of PathHunter Detection Reagent (Dx 93-0001).
- Data was read on Packard Victor 2 or PerkinElmer ViewLux readers and analyzed using GraphPad Prism 4.

Assay Protocol

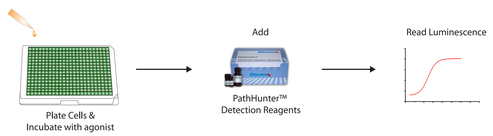
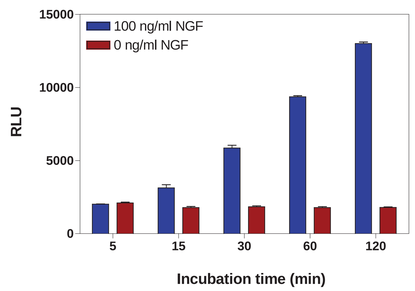
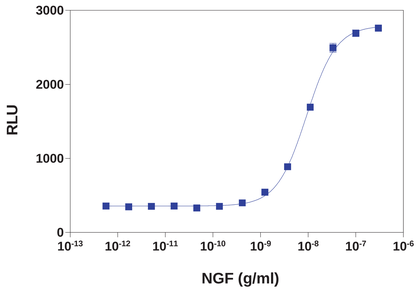


Figure 2. Activation of TrkA-PK in U2OS cells causes recruitment of SHC1-EA resulting in increased enzyme activity



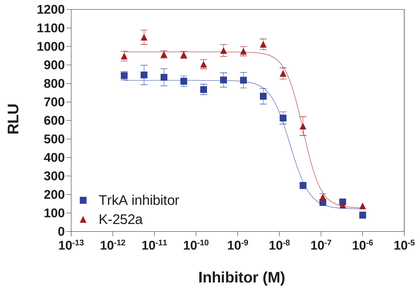
U2OS cells expressing the TrkA-PK and SHC1-EA fusion proteins were plated at 10K/well in each well of a 384-well plate in serum-free medium with 0.1% FBS. The next day, the cells were treated with either 100 ng/ml NGF or PBS+1% BSA for different time period at room temperature and assayed using PathHunter™ Detection reagent.

Figure 3. U2OS TRKA SHC1 cells show does response to NGF



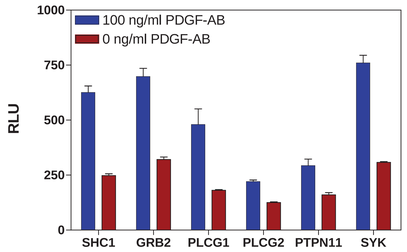
5K/well U2OS TRKA SHC1 double-stable cells were plated in each well of a 384-well plate in serum-free medium with 0.1% FBS. The next day, the cells were treated with different concentrations of NGF for 1 hr at room temperature. Then PathHunter chemiluminescent substrate was added and the signal was read 1 hr later. **EC_{50} of 9.3 ng/mL** and an **assay window of 7.8** were obtained.

Figure 4. TRK inhibitors inhibit NGF stimulated assay signal



5K/well U2OS TRKA SHC1 double-stable cells were plated in each well of a 384-well plate in serum-free medium with 0.1% FBS. The next day, the cells were treated with different concentrations of TrkA inhibitor or K-252a for 10 min at room temperature followed by 20 ng/ml NGF stimulation for 1 hr at room temperature . Then PathHunter chemiluminescent substrate was added and the signal was read 1 hr later. TrkA inhibitor gave an IC_{50} of 18 nM and an assay window of 6.7. K-252a gave an IC_{50} of 37 nM and an assay window of 7.6.

Figure 5. PDGFRB interacts with different SH2 domain containing cytoplasmic proteins



5K/well PDGFRB-PK SH2-containing protein-EA double-stable cells were plated in each well of a 384-well. The next day, the cells were treated with or without 100 ng/ml PDGF-AB for 1 hr at room temperature. Then PathHunter chemiluminescent substrate was added and the signal was read 2 hrs later.

Methods

- These data show that the PathHunter system can be used to detect intracellular phosphorylation events
- Utilizing the PathHunter EFC technology, the interaction of Receptor Tyrosine Kinases and down stream adapters such as SHC1, Grb2, and PLCG2 can be monitored in a single addition luminescent assay format
- We show here that by fusing a small tag (ProLink, 42aa) to TrkA and EA to an intracellular phosphotyrosine binding protein we can detect the phosphorylation and activation of the receptor
- The signal to noise of >7 and appropriate EC_{50} makes this assay ideal for primary and secondary screening applications
- By assaying kinase activation in cells, this novel application of the PathHunter system will enable the identification of compounds that affect all levels of receptor activation including:
  - ligand binding, receptor dimerization, receptor trafficking, and kinase activity
- Extension of this concept to alternate SH2 domains and receptors, such as the PDGFRB shown here, make this a highly flexible system that has the potential to be applied to a broad range of the 58 recep tor tyrosine kinases and many other tyrosine phosphorylation events in intact cells

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