PathHunter™ Cell-Based Kinase Assays: A Portfolio of HTS-friendly Cytoplasmic or Receptor Tyrosine Kinase Assays for Small Molecule Inhibitors as well as Monoclonal Antibodies

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Abstract

Kinase therapeutics includes both monoclonal antibodies (mAbs) and small-molecule inhibitors. DiscoveRx's PathHunter™ protein: protein interaction system uses well-established **E**nzyme **F**ragment **C**omplementation (EFC) technology by monitoring interaction of target proteins with SH2 phosphotyrosine binding domains. We demonstrate application of PathHunter™ cell-based kinase assays in detecting anti-receptor/anti-ligand antibody and small molecule inhibitors for EGFR, c-Met, CSF3R and Trk A. Results suggest that these assays provide a robust, sensitive and one-step cell-based assay for identifying novel antibodies and small molecule inhibitors.

PathHunter™ Assay Principle

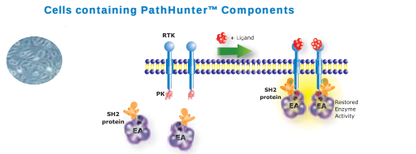


Figure 1. The PathHunter™ Tyrosine Phosphorylation assay monitors the interaction of tyrosine phosphorylated proteins with SH2 containing proteins in a whole cell, homogeneous assay format using enzyme fragment complementation. In this system, a small 42 AA enzyme fragment, ProLink is appended to the C-terminus of the receptor target. The SH2 protein is fused to the larger enzyme fragment, EA (Enzyme Acceptor). Activation of the receptor initiates tyrosine phosphorylation and binding to the SH2-EA fusion that forces complementation of the two enzyme fragments. This action results in the formation of fully complemented β-galactosidase enzyme, the activity of which is measured using chemiluminescent PathHunter detection reagents.

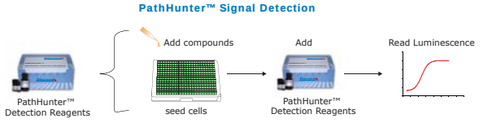
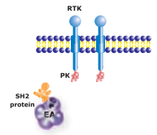


Figure 2. The PathHunter™ Detection Kit consist of a lysis buffer and substrate mixture that is added as a single solution to stimulated cells. After a short incubation, the PathHunter chemiluminescent signal can be detected using any standard luminometric plate reader.

Benefits of PathHunter™ Receptor Tyrosine Kinase Assays



Assay Attribute	Advantages
Full length Receptor	• Identify Anti-receptor antibodies • Identify Anti-ligand antibodies • Identify Activating antibodies • Identify Non-ATP Pocket Binders • Identify Ligand Binding inhibitors • Indentify Dimerization inhibitors
Whole Cell Assay	• Get cell permeability information
Single Addition Assay	• High-throughput friendly format

Assay measures ligand binding, phosphorylation and signal transduction. It measures the interaction between receptor tyrosine kinases and adaptor proteins such as SHC, GrB2, PLCG1, PLCG2 or P85.

Small Molecure Compound Library Screening of PathHunter™ RTK/CTK Assays

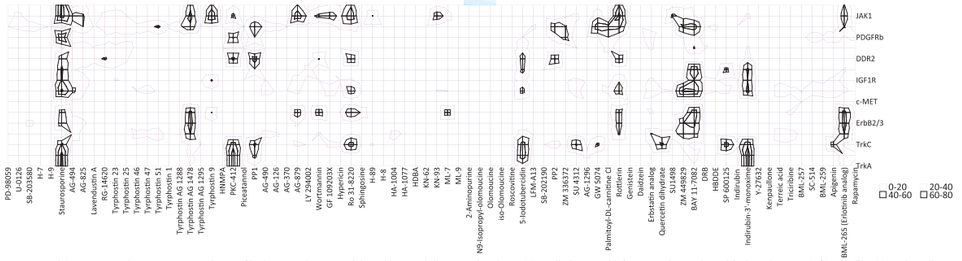


Figure 3: PathHunter™ RTK and CTK Functional assays were used to profile the BioMol Kinase inhibitor library. Cells were plated into 384 well plates and left over night in a humidified incubator at 37˚C with 5% CO_2. The cell were then treated with 10 μM of compound for 1 hour at 37˚C and then with the respective ligands for 3 hours at 23 – 25˚C. Percent inhibition of each compound against a specific RTK/CTK assay was calculated. This data was then used to generate a heat chart that illustrates the value of profiling hits identified against all receptors in a specific family as well as other related kinases in a biologically relevant cell-based assay format.

Trk A, B and C Follow-Up Inhibitor Profile

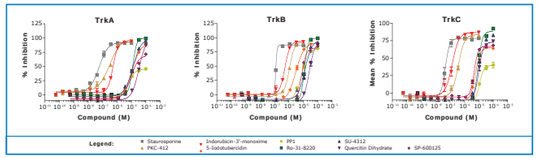


Figure 4. A follow-up study of the hits obtained from the screen was conducted on the neurotrophin receptors TrkA, TrkB and TrkC. The hits were characterized and rank order potency information was obtained. Of the kinase inhibitors in the library, 9 inhibitors affected each of the PathHunter Trk receptors. A full dose response was run on all these 9 compounds as presented above.

Trk A, B and C Follow-Up Inhibitor Profile

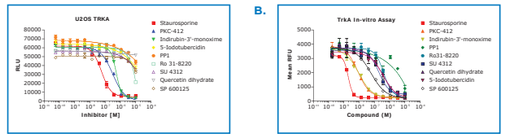


Figure 5. PathHunter™ Trk A Functional assay was compared to the in-vitro kinase assay from DiscoveRx (ADP Hunter HS). **Panel A** demonstrates the inhibitor dose response of nine compounds in the PathHunter assay, while **Panel B** demonstrates dose responses of the same compounds in an in-vitro purified kinase (Trk A) assay. Compounds were tested in a range starting from 10 uM all the way to 0.1 nM. In a biochemical assay all 9 compounds showed nM potency, while in a PathHunter assay only 3 compounds showed nM potency and the rest of the compounds showed uM potency. This variation in rank potency illustrates the difference between in vivo and in vitro assays and may afford additional information on compound permeability.

Detect Anti-Ligand and Anti-Receptor Antibodies

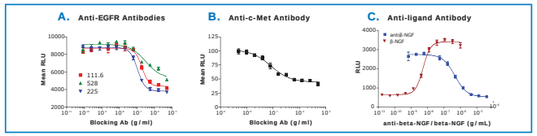
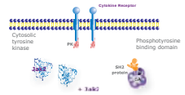


Figure 6. Panel A and Panel B: In this experiment EGFR and c-MET cells were used to screen commercially available antibodies that have the ability to block EGFR and c-MET. The experiment was performed by incubating EGFR and c-MET cells with increasing amounts of the blocking antibodies to determine which antibody had the greatest inhibitory effect. **Panel C:** The ligand for TrkA is β-NGF. In this experiment, TrkA cells were treated with an anti-β-NGF antibody prior to addition of β-NGF. This experiment demonstrates that the PathHunter assay format is capable of detecting activity of an antibody against the ligand for a receptor.

Simple, One-Step, Whole Cell Assay for Cytokine Signaling and Screening



Unlike receptor tyrosine kinases, the cytokine receptors lack kinase activity and are phosphorylated by cytosolic tyrosine kinases. To detect the phosphorylation status of cytokine receptors the ProLink is fused to the c-terminus of the target receptor, and co-expressed with a phosphotyrosine binding domain fused to the complementing fragment, EA. Upon activation and phosphorylation of the receptor by the cytosolic kinase, the SH2 domain binds to the phosphorylated residues

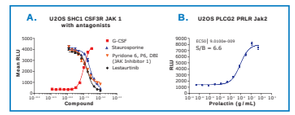


Figure 7. Panel A, PathHunter™ CSF3R cell line was incubated with and inhibitors such as staurosporine, Lestaurtinib and Pyridone 6, P6, DBI (JAK Inhibitor 1)and challenged with EC_{80} of their respective agonists (G-CSF, a known agonist). A dose dependent inhibition was observed indicating that the assay can be used to screen or profile inhibitors against the JAK1 molecule. **Panel B** describes agonist dose response of PathHunter ProLactin receptor PRLR -JAK2 responding to it's native ligand ProLactin.

Panel of RTK and CTK Assays

TARGET	PRODUCT NAME	PARTNER PROTEIN	CATALOG NUMBER
Receptor Tyrosine Kinase Assays			
C-MET	PathHunter™ C-MET Functional Assay	GrB2	93-0632C3
EphB4	PathHunter™ EphB4 Functional Assay	SHC1	93-0468C3
ErbB2/ErbB3	PathHunter™ ErbB2/ErbB3 Functional Assay	GrB2	93-0535C3
ErbB4	PathHunter™ ErbB4 Functional Assay	SHC1	93-0465C3
FGFR4	PathHunter™ FGFR4 Functional Assay	PLCG2	93-0467C3
FR3	PathHunter™ FR3 Functional Assay	P85	93-0506C3
IGFR1	PathHunter™ IGFR1 Functional Assay	SHC1	93-0505C1
INSR	PathHunter™ INSR Functional Assay	PLCG1	93-0466C3
PDGFRb	PathHunter™ PDGFRb Functional Assay	PLCG1	93-0469C3
TrkA	PathHunter™ TrkA Functional Assay	SHC1	93-0462C3
TrkA-P75	PathHunter™ TrkA-P75 Functional Assay	SHC1	93-0529C3
TrkB	PathHunter™ TrkB Functional Assay	SHC1	93-0463C3
TrkB-P75	PathHunter™ TrkB-P75 Functional Assay	SHC1	93-0530C3
TrkC	PathHunter™ TrkC Functional Assay	SHC1	93-0464C3
TrkC-P75	PathHunter™ TrkC-P75 Functional Assay	SHC1	93-0531C3
ErbB1	PathHunter™ ErbB1 Functional Assay	PLCG1	93-0681C3
Cytosolic Tyrosine Kinase Assays			
CSF3R-JAK1	PathHunter™ CSF3R-JAK1 Functional Assay	SHC1	93-0564C3
PRLR-JAK1	PathHunter™ PRLR-JAK1 Functional Assay	PLCG2	93-0686C3
PRLR-JAK2	PathHunter™ PRLR-JAK2 Functional Assay	PLCG2	93-0687C3

Summary

PathHunter cell-based kinase assays provide distinct advantages over traditional biochemical kinase screening:

- One step, chemiluminescent, whole cell assay
- Allows you to characterize your antibody for specificity
- Screen libraries for your HIT antibody
- Intensify your search for different kinds of antibodies (anti-ligand or anti-receptor)
- Screening of a small molecule compound library with all the available PathHunter cell-based kinase assays demonstrated selectivity information.
- Follow-up studies of the HIT compounds for the neurotrophin family indicated rank order potency of hits.
- The Hits correlated with the biochemical assay but differed in the rank order potency.

In this presentation we have demonstrated the application of the PathHunter cell-based kinase assays to screen and detect various small molecule inhibitors and monoclonal antibodies specific to the receptor or the ligand. This phosphotyrosine binding approach has been applied to study cytokine receptors such as CSF3R and PRLR as well as a screenable assay for JAK1 and JAK2 cytosolic tyrosine kinases.

Technology Access

PathHunter™ Cell-Based Kinase Assays
Clonal cell lines expressing ProLink-tagged Receptor Tyrosine Kinase on the membrane and EA-SH2 fusion protein in the cytoplasm. Over 20 cell lines are now available. For more information, please visit www.discoverx.com/kinases.

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 simple profiling, specificity studies or selectivity profiling against one or many receptor tyrosine kinase targets. For more information, please email profiling@discoverx.com.

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