Application of the PathHunter™ Protein Interaction Assay to Receptor Tyrosine Kinases (RTKs): Developing a Non-Antibody One-Step Cell-Based Kinase Activity

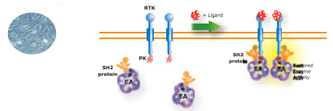
Mahesh Mathrubutham, PhD, Wei Feng, PhD, Nisha Shankaran, Martine Ercken, PhD, Tom Wehrman, PhD, and Keith R. Olson, PhD
DiscoveRx Corporation, Fremont, CA 94538, USA

Abstract

Kinase targets have been extensively studied in biochemical assays using purified protein fragments for the kinase and the substrate. However, there is an increasing need to understand how kinases function in the context of a whole cell assay. DiscoveRx has pioneered a protein interaction assay system using our established Enzyme Fragment Complementation (EFC) β-Galactosidase detection system. This is proving to be a generic assay system applicable to a wide range of protein interaction, with the key feature being the very low endogenous activity of the two fragment of our split β-Gal system, called ProLink and EA (enzyme acceptor). In our approach to a functional, whole-cell kinase assay, we express a full-length human receptor tyrosine kinase (RTK) fused at its C-terminus to the small ProLink peptide. The EA component of our assay is fused to one of a series of SH2 or PTB domain constructs that we have generated. When co-expressed in a cell, we have shown that we can detect the protein interaction that occurs in the cells when the RTK is activated and recruits an SH2/PTB domain to phospho-tyrosine residues that occur during receptor activation. We have successfully applied this assay approach to the insulin receptor, the entire Trk family, IGF1R, PDGF and FGF receptor family members, and most recently FLT3. We will present data showing both agonist and antagonist assay results for these targets, as well as our findings from a focused set of kinase inhibitors comparing TrkA analyzed in a cell-based and biochemical assay format. Furthermore, we have also shown that we can create assay systems with RTK heterodimers, with our case study example being the ErbB3 and ErbB3 activated kinase. We have recently extended these studies to show the effect of P75 co-expression with each of the Trk Family RTKs. In conclusion, the PathHunter protein interaction platform has proven to be a very useful tool in the development of a cell-based assay format for RTKs that does not require antibody or wash steps, and can be implemented in a simple one-addition HTS assay format.

PathHunter™ Receptor Tyrosine Kinase Assay Principle

Cells containing PathHunter™ Components



Typically, when tyrosine kinase receptors bind ligand, they dimerize resulting in trans-phosphorylation of tyrosine residues. The resulting phosphotyrosines provide docking sites for SH_2 containing proteins. The PathHunter Receptor Tyrosine Kinase assay monitors the interaction of tyrosine phosphorylated proteins with SH_2 containing proteins in a whole cell, homogeneous assay format using enzyme fragment complementation. In this system, a small 42 AA enzyme fragment, ProLink was appended to the C-terminus of the GPCR. The SH_2 protein is fused to the larger enzyme fragment, EA (Enzyme Acceptor). Activation of the receptor tyrosine kinase initiates binding of SH_2 proteins that forces complementation of the two enzyme fragments. This action results in an increase in enzyme activity that is measured using chemiluminescent PathHunter Detection Reagents.

PathHunter™ Detection

Methods

Agonist Protocol

- Seed 10,000 cells in 20 µL MEM + 0.5% Horse serum
- Incubate Overnight @ 37°C
- Induce cells with 5 µL Agonist
- Incubate for 3 hrs @ RT (19-25°C)
- Add 12 µL PathHunter Detection Reagent
- Incubate for 1 hr @ RT
- Read Chemiluminescent Signal

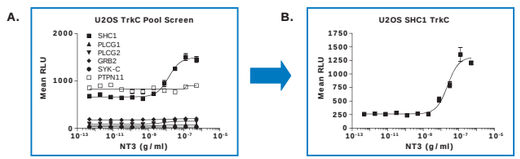
Antagonist Protocol

- Seed 10,000 cells in 20 µL MEM + 0.5% Horse serum
- incubate Overnight @ 37°C
- Treat cells with antagonist
- 1 hr @ 37°C
- Induce cells with 5 µL Agonist
- Incubate for 3 hrs @ RT (19 – 25°C)
- Add 12 µL PathHunter Detection Reagent
- Incubate for 1 hr @ RT
- Read Chemiluminescent Signal

PathHunter Cell-Based Kinase Assay Development/ Performance

SH_2 domains bind phosphorylated tyrosine residues and have preferences for the surrounding amino acids. To develop the PathHunter assays, multiple SH_2 containing proteins are initially screened for their ability to interact with the target. The SH_2 domain cell pool showing the largest assay window and accurate pharmacology is further developed into a stable clonal cell line.

Multiple SH_2 Pools **Clone**



Family-wide receptor profiling using PathHunter technology

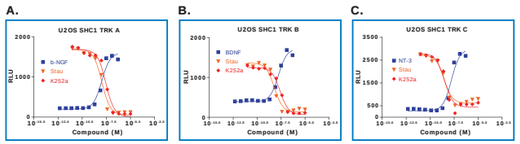


Figure 1. TrkA, B and C cell lines were initially incubated with K252a, a Trk antagonist, for 1 hr at 37°C. The cells were then challenged with EC_{90} of their respective agonists for 3 hrs at room temperature. The cells were then treated with PathHunter detection reagents and chemiluminescent signal was detected using a luminometer. Data demonstrates that robustness of the assay format simplifies agonist and antagonist determination.

PathHunter Assays Detect Multiple Mechanisms of Receptor Activation

Homodimerization **Heterodimerization** **Pre-dimerized**

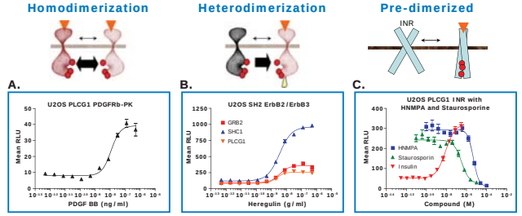


Figure 2. PDGFRB like most receptor tyrosine kinases, homodimerizes in the presence of the appropriate ligand, PDGF-BB, causing recruitment of SH_2 proteins and can be monitored using the PathHunter technology (**Panel A**). The (**Panel B**) shows that the heterodimerization and activation of ErbB3 by ErbB2 can also be detected using the PathHunter system. The insulin receptor is an example of a receptor that is constitutively dimerized but undergoes a conformational change to become active in the presence of insulin. The (**Panel C**) shows that addition of insulin to the PathHunter Insulin Receptor cells results in a 5-fold increase in enzyme activity that can be inhibited with the kinase inhibitors staurosporine and HNMPA.

Comparison of Cell-Based and Biochemical Assay Formats for Tyrosine Kinase screening

A small scale screen of TrkA with a focused kinase library was performed. TrkA was screened in a biochemical format (ADP Hunter™) using the purified kinase domain and in the PathHunter cell-based RTK assay.

% Inhibition observed at screening concentration of 1 µM			
Compound	Biochemical Assay	PathHunter™ Assay	Type of Inhibitor
Staurosporine	88.2	87.2	Pan-specific kinase inhibitor
PKC-412	88.3	88.6	PKC inhibitor
Indirubin-3'-monoxime	87.7	78.5	GSK-3-beta
Ro 31-8920	79.2	-	PKC inhibitor
5-Iodotubercidin	79.8	-	ERK2, adenosine kinase, CK1, CK2
SU 4312	70.6	-	Flk1
Quercetin dihydrate	66.9	-	PI 3-K
SP 600125	74.7	-	JNK inhibitor

PathHunter™ Assay / Biochemical Assay: 0 / 3 / 5

% Inhibition observed at screening concentration of 10 µM			
Compound	Biochemical Assay	PathHunter™ Assay	Type of Inhibitor
Staurosporine	90.1	83.7	Pan-specific kinase inhibitor
PKC-412	65.8	80.6	PKC inhibitor
PP1	59.9	70.1	Src family
AG-879	-	54.3	NGFRK
Ro 31-8220	87.0	81.6	PKC inhibitor
5-Iodotubercidin	78.4	74.6	ERK2, adenosine kinase, CK1, CK2
SU-4312	72.9	67.4	Flk1
GW 5074	64.7	73.5	cRAF
Rottlerin	-	72.3	PKC delta
BAY 11-7082	-	88.0	IKK pathway
SP 600125	82.9	75.8	JNK
Indirubin-3'-monoxime	84.3	83.0	GSK-3-beta
Terreic Acid	-	50.5	BTK
Apigenin	71.5	63.0	CK-11
AG-494	54.9	-	EGFRK, PDGFRK
LY 294002	51.3	-	PI 3-K
GF-109203X	64.8	-	PKC
LFM-A13	65.2	-	BTK
Quercetin dihyrate	72.4	-	PI 3-K

PathHunter™ Assay / Biochemical Assay: 4 / 10 / 5

Figure 3. The compound screening was performed using 1 µM or 10 µM of compound, 50 µM of ATP and 10 µM of substrate. Compound library and kinase were added together and the assay was performed at RT for 1 hour. For the PathHunter assay, the cells were initailly treated with compound for 1 hour at 37°C, and then challenged with an EC_{90} of β-NGF for 3 hours at room temperature.

Agonist and Antagonist Response for PathHunter RTK Assays

Agonist

RTK	Agonist	S/B	EC_{50} (ng/ml)
TrkA	b-NGF	8.1	10.8
TrkB	BDNF	4.4	16.8
TrkC	NT3	10.6	16.6
INSR	Insulin	6	8.3
ErbB4	NRG1	3.2	5.4
FGFR4	FGF-1	4.5	2.9
PDGFRb	PDGF-AB	14.9	1.5
IGF1R	IGF1	4.1	25
FLT3	FLT3 Ligand	3	1.2

Antagonist

RTK	Antagonist	IC_{50} (nM)
TrkA	K-252a	28
	Staurosporine	11
TrkB	K-252a	25
	Staurosporine	6
TrkC	K-252a	3.1
	Staurosporine	2.8
INSR	HNMPA	4.8E+03
	Staurosporine	320
ErbB4	PD158780	1.1E+03
	Staurosporine	NA

RTK	Antagonist	IC_{50} (nM)
FGFR4	AG1296	NA
	Staurosporine	595
PDGFRb	DMPQ	3.5E+03
	Staurosporine	14.6
IGF1R	Picropodophyllin	NA
	Staurosporine	2.3E+03
FLT3	Lestaurtinib	1.28
	Staurosporine	0.67

Table 1. The table above details the response to agonist and antagonist for each of the PathHunter RTK cell lines available from DiscoveRx. All assays demonstrate excellent assay windows and good pharmacology.

TrkA-P75 Interaction Govern Ligand Specificity

P75 is a member of the TNFR family that plays a role in Trk signaling. Here we demonstrate that co-expression of P75 with TrkA can alter ligand selectivity of the receptor.

TrkC Alone **TrkC + P75**

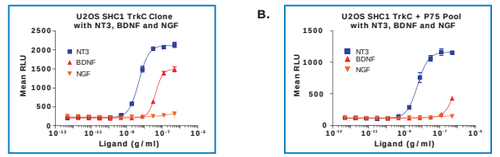


Figure 4. The reference ligand for TrkC is NT3. In the presence or absence of P75, the EC_{50} and magnitude of signal is unchanged. However the response to BDNF (which is a weaker TrkC agonist) is right-shifted at least 20-fold. As shown previously*, these results confirm that P75 co-expression can enhance the specificity of Trk receptors. Thus the PathHunter assay system can be used as a platform for assessing kinase activity as well as the effects of co-expressed proteins on receptor activation.

*Bible, Hoppe and Barde. EMBO 1999; 18(3): 616-22.

Summary

PathHunter Cell-Based Kinase Assays Provide Distinct Advantages

PathHunter Assay format uses full length receptors
- Enables the identification of ligand binding inhibitors
- Ideal for *therapeutic* ***antibody*** screening

Measured event is immediately proximal to receptor activation
- Avoids false positive that hit other downstream cellular kinases

Assays rely on natural activation process
- Unactive and active kinase forms are screened simultaneously

Widely applicable to a variety of tyrosine phosphorylation events

One Step, chemiluminescent assay format.
- Simplifies screening and pharmacology determinations

Live, Whole Cell Assays
- Provides information on cell penetrance of compounds
- Physiological ATP and substrate concentrations
- Ensure that hits identified will be functional in downstream assays

DiscoveRx
www.discoverx.com