Functional Whole-cell Assays for Tyrosine Phosphorylation Using PathHunter™ Technology

Mahesh Mathrubutham, PhD, Wei Feng, PhD, Nisha Shankaran, Bill Raab, Philip Achacoso and Tom Wehrman, PhD

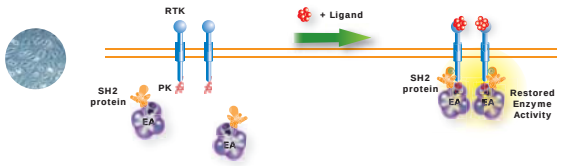
DiscoveRx Corporation 42501 Albrae Street, Fremont CA 94538, USA.

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 detection system using well-established Enzyme Fragment Complementation (EFC) technology. We show here that this system can be used to report the phosphorylation status of proteins in whole cells by monitoring the interaction of target proteins with SH2 phosphotyrosine binding domains. We have successfully applied this approach to multiple receptor tyrosine kinases including; the insulin receptor family, the Trk family, PDGF and FGF receptor family members. This approach has been extended to receptors without kinase domains that couple to cytosolic kinases. Using the G-CSFR and Prolactin receptor we demonstrate that assays specific for a receptor target or assays specific for a cytosolic tyrosine kinase, such as Jak1 or Jak2, can be rapidly developed. In conclusion, the PathHunter protein interaction platform has proven to be a useful tool in the development of a cell-based assay format for tyrosine phosphorylation that does not require antibody or wash steps, and can be implemented in a simple one-addition HTS assay.

PathHunter™ Receptor Tyrosine Kinase Assay Principle

Cells containing PathHunter™ Components



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 as 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 of the target and binding to the SH2-EA fusion 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.

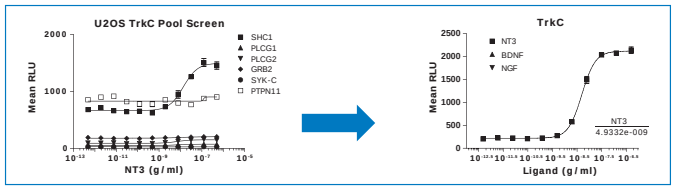
PathHunter™ Signal Detection

The PathHunter detection reagents 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.

PathHunter™ Receptor Tyrosine Kinase Assays

- > 200 SH2 containing proteins in humans
- Subset of 12 have been identified for broadest tyrosine phosphorylation coverage

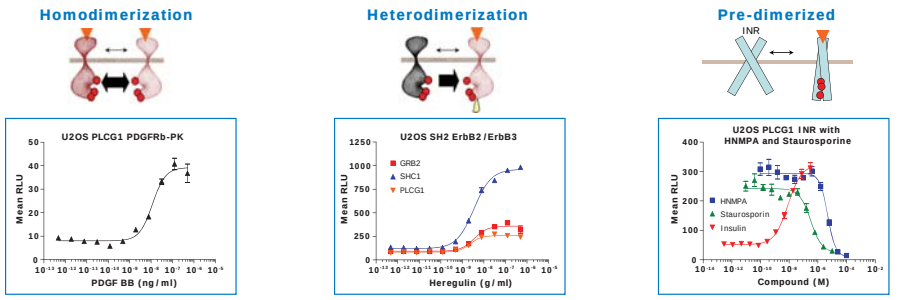
Multiple SH2 Pools **Clone**



PathHunter Receptor Tyrosine Kinase Assays. A variety of SH2 domains exist in humans and the specificity for specific phosphotyrosines is difficult to predict. In order to develop the most robust assays, each receptor tyrosine kinase, in this case TrkC, is tested with a variety of SH2 domain constructs **(left panel)**. The heterogeneous cell pools are then clonally selected for stable high-performing clones. An example for TrkC is shown in the **right panel**.

PathHunter™ Assays Detect Multiple Mechanisms of Receptor Activation

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



Left Panel, PDGFRB like most receptor tyrosine kinases homodimerizes in the presence of the appropriate ligand, PDGF-BB causes recruitment of SH2 proteins and can be monitored using the PathHunter technology. The **center panel** shows that the heterodimerization and activation of ErbB3 by ErbB2 can 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 **right panel** 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.

PathHunter™ Assays Detect Multiple Mechanisms of Receptor Activation

The following PathHunter receptor tyrosine kinase assays were tested for their ability to be induced and inhibited by the respective compounds.

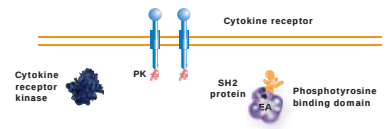
Agonist

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
EphB4	EphB2 Fc	3.1	669.7

Antagonist

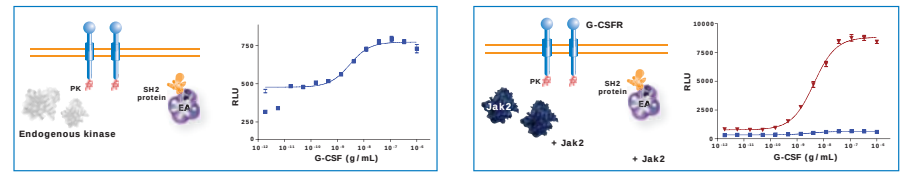
RTK	Antagonist	IC_{50} (nM)	RTK	Antagonist	IC_{50} (nM)
TrkA	K-252a	28	FGFR4	AG1296	NA
	Staurosporine	11		Staurosporine	695
TrkB	K-252a	25	PDGFRb	DMPQ	3.5E+03
	Staurosporine	6		Staurosporine	14.6
TrkC	K-252a	3.1	IGF1R	Picrpodophyllin	NA
	Staurosporine	2.8		Staurosporine	2.3E+03
INSR	HNMPA	4.8E+03	FLT3	Lestaurtinib	1.28
	Staurosporine	320		Staurosporine	0.67
ErbB4	PD158780	1.1E+03	EphB4	PP1	396
	Staurosporine	NA		PP2	277

Detection of Cytokine Receptor Phosphorylation



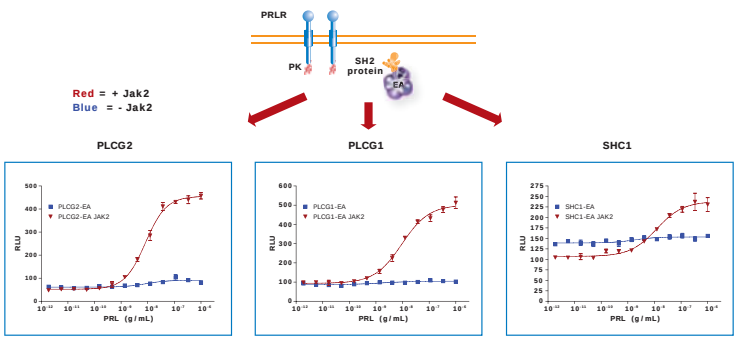
Unlike receptor tyrosine kinases, these receptors lack kinase activity and are phosphorylated by cytosolic tyrosine kinases. To detect the phosphorylation status of cytokine receptors the ProLink is fused to the c-terminus of the target receptor, and co-expressed with a phosphotyrosine binding domain 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. Specificity for a specific kinase can be obtained by co-expression of specific tyrosine kinases.

Detection of Endogenous and Engineered Kinase Targets



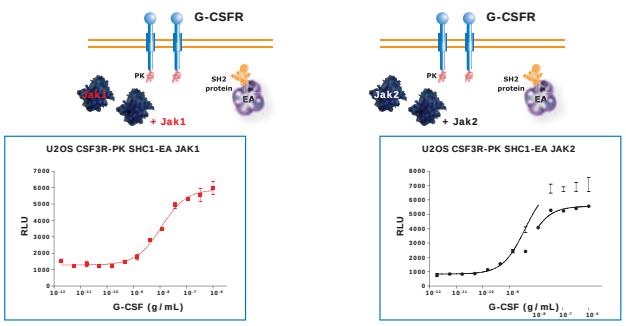
Assay for G-CSFR agonism and Jak2 activity. To detect G-CSFR activation the G-CSFR was fused to ProLink and expressed with the Shc1-EA fusion in U2OS cells. Addition of the G-CSF agonist results in phosphorylation by endogenous kinases, binding of Shc1 to the receptor and an increase in β-galactosidase activity **(left panel)**. To determine the suitability of the system for specific kinases, Jak2 which couples to the G-CSFR was co-expressed in the same cell line. Addition of G-CSF causes a 10-fold increase in enzyme activity **(red curve)**. The response of the cells without the addition of Jak2 is shown in blue. Together these results show that endogenous and specific kinase activity can be detected using the Pathhunter system.

SH2 Domain Selection Influences Assay Performance



Prolactin receptor and Jak2 activation monitored using different SH2 domains. SH2 domains show varying activities in the PathHunter system. The PRLR was fused to ProLink and expressed with the specific SH2-EA fusions. PRLR couples to Jak2 so cells were tested that overexpress Jak2 **(red curves)** or native cells **(blue curves)**. Prolactin was added to the cells in increasing doses and β-galactosidase activity was measured using PathHunter detection reagents.

Assays for Jak1 and Jak2 using PathHunter™ G-CSFR



Assays for Jak1 and Jak2 were developed using the G-CSFR-SHC1 PathHunter cell pool. These pools express the ProLink tagged receptor and the phosphotyrosine binding protein SHC1 fused to EA. The cells were transduced with plasmids expressing either Jak1 or Jak2. The G-CSFR is known to couple to both kinases. Addition of the agonist, G-CSF, induces the activation of the expressed kinase resulting in phosphorylation of the target receptor, binding of SHC1 and complementation of the enzyme that is measured using Pathhunter reagents.

Summary

- PathHunter protein-protein interaction approach has been adapted to study cell-based kinases. PathHunter Cell-Based Kinase assay monitors the interaction between wild type, full length receptor proteins with SH2 phosphotyrosine binding domains enabling the identification of therapeutic antibodies, dimerization and ligand binding inhibitors.
- The assay provides a cellular context with information on cell penetrance of compounds and can be used for primary and secondary screening applications.
- We present data on both receptor tyrosine kinases and cytokine receptors that signal through cytosolic tyrosine kinases. The receptor tyrosine kinase assays are broadly applicable across receptor sub-types and data on homodimerization, heterodimerization and pre-dimerized RTKs are shown.
- We demonstrate detection of endogenous and specific kinaseactivity with robust assay performance.

Technology Access

PathHunter™ Cell-Based Assays
Clonal cell lines expressing ProLink-tagged RTK or cytokine receptor on the membrane and EA-SH2 fusion protein in the cytoplasm. Over 15 cell lines are now available. For more information, please visit www.discoverx.com/kinases/cell_based.php

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.

 DRx_RTKP_MIPTEC1009_REV1

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