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PathHunter® Cell-Based Assays for Kinase Receptors

Functional, Dimerization, and Translocation Cellular Assays for Receptor Tyrosine Kinases (RTKs)

OUR EXPERTISE
IN YOUR HANDS.
DISCOVER
CONFIDENTLY.

Receptor Tyrosine Kinases

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Coordinating a wide variety of cellular functions

Receptor Tyrosine Kinases (RTKs)

- ~20 Classes and ~60 known human RTKs
  - e.g. FGF, PDGF, Trk, Eph, IGF, INS
- Cell surface receptors that bind hormones, growth factors, and cytokines
- Regulators of cellular growth/proliferation, survival, development/differentiation, and disease progression
- Play a critical role the development and progression of several cancer types

Receptor Tyrosine Kinases

Understanding RTKs structure and signaling

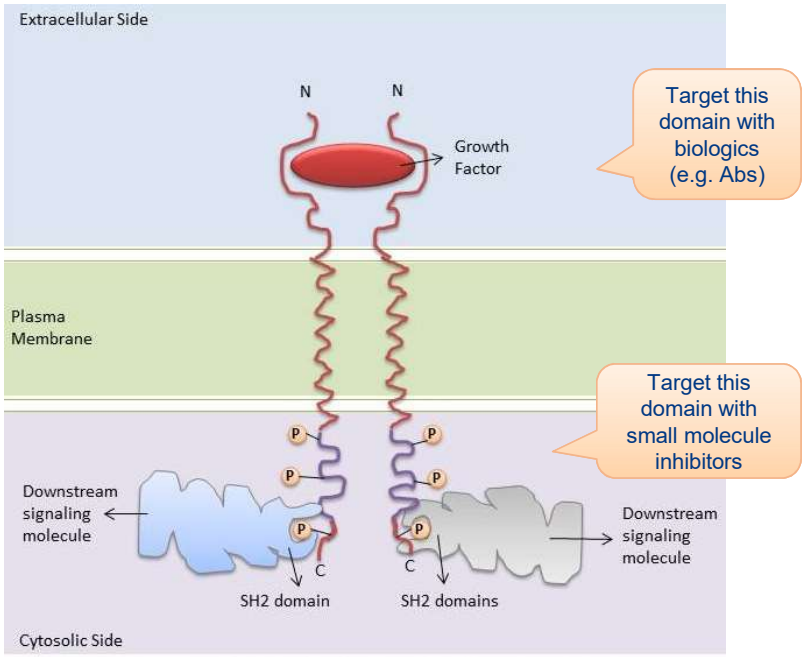
RTKs structure

- N-terminal extracellular ligand-binding domain
- C-terminal intracellular catalytic domain responsible for kinase activity
  - Autophosphorylation and tyrosine phosphorylation of RTK substrates

RTK cellular signaling

1. Ligand binding induces receptor dimerization (hetero- or homodimerization) and kinase domain activation
2. Autophosphorylation of RTKs
3. SH2-protein recruitment (at phosphotyrosine-containing motifs)
4. Promotion of further phosphorylation of downstream proteins (e.g. MAP kinases)
5. Ultimately stimulates enzymatic activity and associated with intended cellular response

Receptor Tyrosine Kinases Structure



SH2 Domains Binding to Specific Phosphorylated Tyrosine Containing Peptides

SH2 (Src Homology 2); RTK (Receptor Tyrosine Kinase), Image source.

Importance of RTKs in Drug Discovery

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Drug therapeutic relevance

RTKs are attractive targets for drug therapy due to their implications in a variety of cellular abnormalities and they are tractable targets for drug discovery medicinal chemistry

- Cancer, degenerative diseases, and cardiovascular diseases
- FDA has approved several anti-cancer drugs caused by activated RTKs

Example of FDA Approved Small Molecule Inhibitors and Antibodies Against RTKs* for Cancer Therapy

Drug	Target	Disease
Imatinib (Gleevec)	PDGFR, KIT, Abl, Arg	SML, GIST
Gefitinib (Iressa)	EGFR	Esophageal cancer, Glioma
Erlotinib (Tarceva)	EGFR	Esophageal cancer, Glioma
Sorafenib (Nexavar)	Raf, VEGFR, PDGFR, Flt3, KIT	Renal cell carcinoma
Sunitinib (Sutent)	KIT, VEGFR, PDGFR, Flt3	Renal cell carcinoma, GIST, Endocrine pancreatic cancer
Dasatinib (Sprycel)	Abl, Arg, KIT, PDGFR, Src	Gleevec-resistant CML
Nilotinib (Tasigna)	Abl, Arg, KIT, PDGFR	Gleevec-resistant CML
Lapatinib (Tykerb)	EGFR, ErbB2	Mammary carcinoma
Trastuzumab (Herceptin)	ErbB2	Mammary carcinoma
Cetuximab (Erbitux)	EGFR	Colorectal cancer, Head and neck cancer
Bevacizumab (Avastin)	VEGF	Lung cancer, Colorectal cancer
Panitumumab (Vectibix)	EGFR	Colorectal cancer

Table adapted from "Cell signalling by receptor-tyrosine kinases," by Lemmon and Schlessinger's, 2010. Cell, 141, p. 1117–1134.
* Includes non RTK examples like Abl, Raf, …

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PathHunter® Cell-Based Assays
for Kinase Receptors

Functional, Dimerization, and Translocation Assays based on EFC technology

PathHunter® Cell-Based Assays

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Investigate RTKs activity with high confidence

In general, it is difficult to create assays for cytokines and growth factors

- Available assays have downstream readouts
- Lack of assay specificity
- Long complex protocols

Highlights of PathHunter cell-based assays for receptor kinases

- Provide solutions for analyzing functional activity, dimerization, translocation, and characterization
- Allow for screening, hit ID, and lead optimization of small molecules and biologics
- Give cellular context to activation and allow for the identification of novel inhibitors and therapeutic antibodies
  - E.g. Inhibitors of receptor dimerization and/or receptor function
- Based on proprietary DiscoverX enzyme complementation fragment technology platform

Enzyme Fragment Complementation (EFC)

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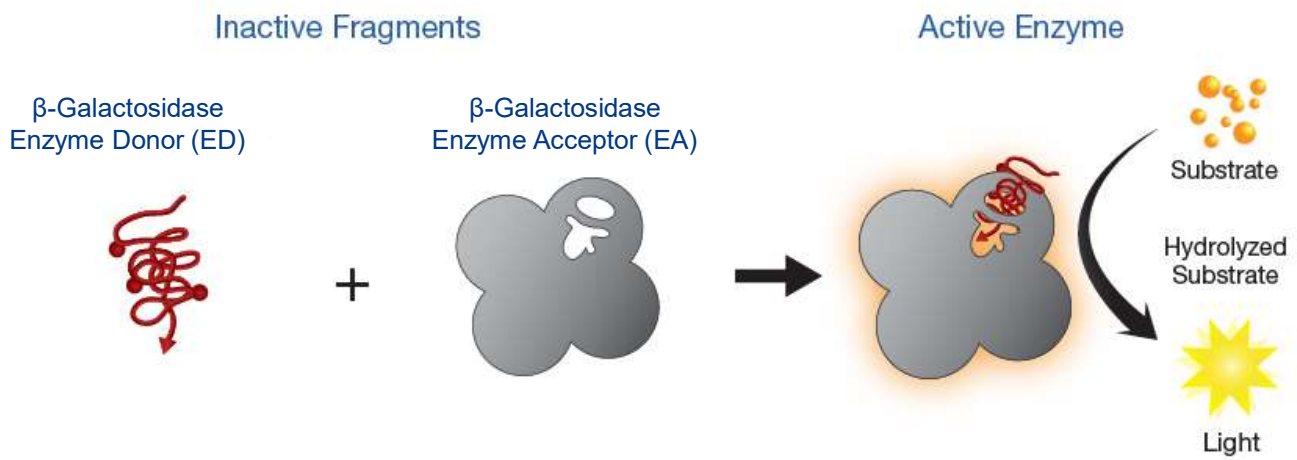
PathHunter assays are based on the proprietary DiscoverX EFC Technology



EFC is a patented detection technology based on two recombinant β-galactosidase (β-gal) fragments

- Small peptide fragment (enzyme donor, ED)
- Large protein fragment (enzyme acceptor, EA)

Separately, the fragments are inactive, but when combined, they form an active enzyme that hydrolyzes substrate to produce a chemiluminescent signal

PathHunter® Cell-Based Functional Assays

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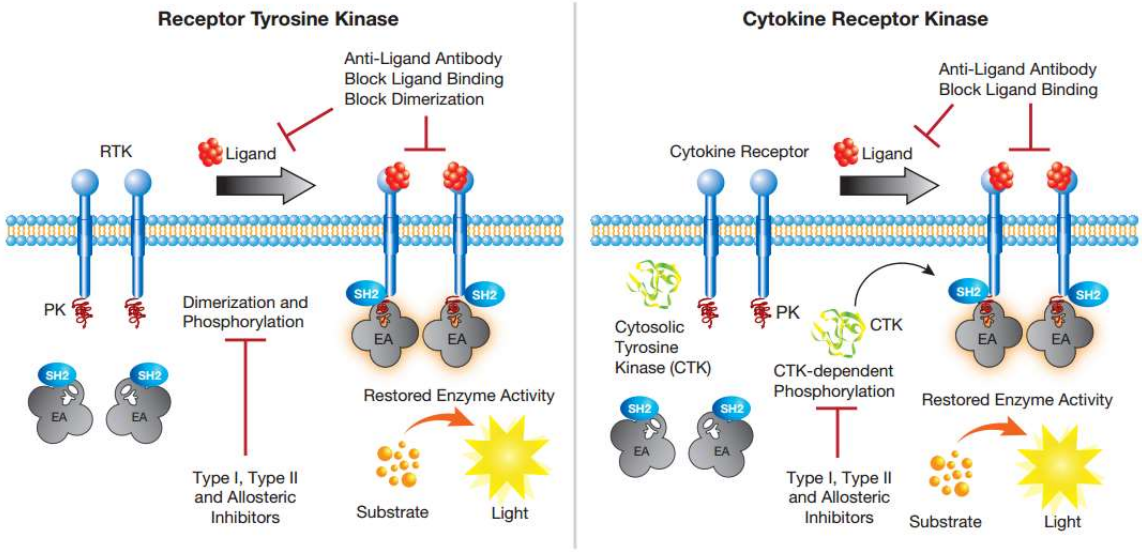
Easily analyze kinase activation for multiple tyrosine kinases receptors

PathHunter cell-based functional assays for RTKs and cytokine receptors with CTKs

- Involves the target receptor (RTK or cytokine receptor kinase) tagged with PK and one of the many different partner proteins containing SH2 domains tagged with EA
- Upon ligand-induced activation, the receptors dimerize and cross-phosphorylate
- The SH2-EA fusion protein then binds the phosphorylated receptor-PK forcing the EFC interaction which is readout as a chemiluminescent signal

EFC Cell-Based Assays for RTKs, CTKs, & Cytokine Receptor Kinases



SH2 (Src Homology 2); RTK (Receptor Tyrosine Kinase), CTK (Cytosolic Tyrosine Kinase); Kinase (RTK) page for additional acronyms and full description of images.

PathHunter® Cell-Based Dimerization Assays

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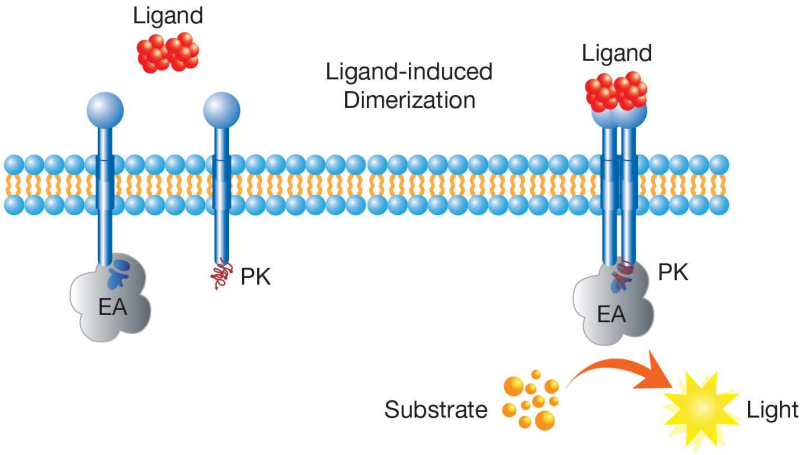
Measure heterodimerization, homodimerization, or co-receptor recruitment

PathHunter cell-based dimerization assays

- Involves two target receptors that are tagged with the EFC enzyme donor called ProLink™ (PK) or Enzyme Acceptor (EA)
- Upon ligand-induced activation (e.g. via biologics), the receptors dimerize forcing the two β-gal components to complement and create an active enzyme
- Active β-gal generates a chemiluminescent signal in the presence of substrate



Example heterodimer pairs and their therapeutic indications involvement

- EGFR-ErbB2 (Breast Cancer)
- ErbB2-ErbB3 (Cancer)

Landing page: dimerization assays
PK = EFC enzyme donor fragment; EA = EFC enzyme acceptor fragment

PathHunter® Cell-Based Translocation Assays

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Track cellular movement of proteins to multiple membrane compartments

Monitor translocation of wild type, full length RTKs in an antibody-free, non-imaging, cell-based, homogeneous (no wash), and HTS-friendly assay format

Explore receptor movements from ER to cell membrane or cell membrane to endosome

- If these localizations are altered by a ligand, protein mutations, or aberrant signaling, undesirable effects may occur, often resulting in drug tolerance, unwanted side effects, and disease

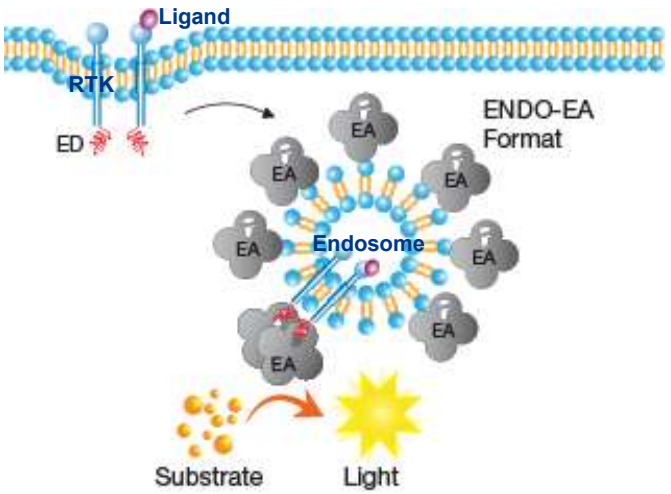
Study RTK recycling patterns

Discover compounds that have unique qualities

- e.g. agonists that behave as functional antagonists with respect to receptor internalization

Create your own quantitative cell-based assays to study translocation of any membrane protein

Tracking the movement of proteins between cellular compartments using EFC assays



Landing page: translocation assays; ENDO = endosome;
ED = EFC enzyme donor fragment; EA = EFC enzyme acceptor fragment

Products and Services for Kinases

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Technology access for analysis of RTKs, CTKs, and more

Products

- Stable cell lines
- Complete assay ready kits
  - eXpress and bioassay
- Engineered parental cell lines
  - For development of translocation and SH2-recruitment functional/dimerization assays

Services

- TK*scan*™ cell-based tyrosine kinase panel for screening and profiling
  - *tk*MAX, *tk*ELECT, *tk*E/IC50ELECT

Related products and services

- InCELL cell-based compound-target engagement assays
- ADP accumulation fluorescent assays
- Ligands and inhibitors
- KINOME*scan*®
- BioMAP® phenotypic screening

Custom assay development (CAD)

Landing pages for PathHunter RTK/CTK assays, dimerization assays, translocation assays, and kinase profiling services

Products and Services for Kinases

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Broad Range of assay targets available for analysis of RTKs, CTKs, and more

Dimerization Assays*

ACVR1/ACVR2	CSF2RB/CSF2RA
ACVR1B/BMPR2	EGFR/EGFR
ACVR1C/ACVR2	EGFR/ErbB2
ACVR1C/ACVR2B	EGFR/ErbB3
ACVRL1/ACVR2	EpoR/EpoR
ACVRL1/ACVR2B	ErbB2/ErbB3
ACVRL1/BMPR2	ErbB2/ErbB4
BMPR1A/ACVR2	ErbB4/ErbB4
BMPR1A/ACVR2B	FGFR3(G380R)/FGFR3(G380R)
BMPR1A/BMPR2	FGFR3/FGFR3
BMPR1B/ACVR2A	KDR/KDR
BMPR1B/ACVR2B	TGFBR1/ACVR2
BMPR1B/BMPR2	TGFBR1/ACVR2B
c-MET/c-MET	TGFBR1/TGFBR2
CSF1R/CSF1R	TGFBR1/TGFBR2/ENG

RTK and CTK Functional Assays

c-KIT	FGFR1	mINSRb	CTK
c-MET	FGFR1-α-Klotho	KDR	BLK
c-Ret-GFRα1	FGFR1-β-Klotho	PDGFRa	CSF3R-JAK1
c-Ret-GFRα2	FGFR2	PDGFRb	EpoR-JAK2
DDR1	FGFR4	TrkA	FGR
EphA4	FGFR4-α-Klotho	TrkA-P75	GHR-JAK1
EphA5	FGFR4-β-Klotho	rhTrkA-rhP75	GHR-JAK2
EphA7	Flt3	rTrkA-rP75	JAK3
EphB1	Flt4	Tie2	LCK
EphB2	IGF1R	TrkB	PRLR-JAK1
EphB3	INSRa	TrkB-P75	PRLR-JAK2
EphB4	INSRb	TrkC	SYK
ErbB1	cINSRa	TrkC-P75	TYK2
ErbB2-ErbB3	cINSRb	TYRO3	YES1
ErbB4	mINSRa		

Translocation and Functional Assays

DIY/Toolbox* (Parental Cell Lines)

ENDO-EA	MEM-EA
PLCG1(SH2)-EA	SHC1(SH2)-EA

Select assays shown. Please refer to the website for the current list of PathHunter RTK/CTK assays, dimerization assays, translocation assays, and kinase profiling services

* Dimerization assays: interleukin receptors and other receptors are also available but not shown here.

DIY = do-it-yourself toolbox products to create your own cell-based assays using engineered parental cell lines.

Assay Highlights

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Advantages of PathHunter® cell-based assays for kinase receptors

Accurate, Sensitive, and Reproducible - Superior quality, reproducible data with large assay windows, and robust performance

Broadly Applicable

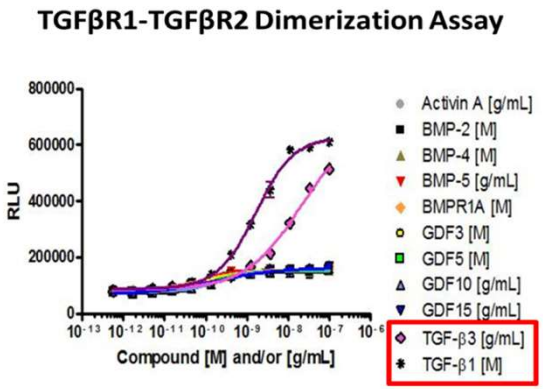
- Identify various ligands including anti-receptor, anti-ligand, activating antibodies, non-ATP pocket binders (allosteric modulators or dimerization inhibitors), or ligand binding inhibitors (ATP-competitors)
- Study hetero- and homodimers

High Specificity - Tagged, full-length tyrosine kinase eliminates non-specificity due to background from endogenous tyrosine kinases

Easy-to-Use – Simple, one-step add and read protocol that is HTS-friendly

Universal, DIY Solution – Create your own quantitative cell-based assays to study translocation or function of RTK/CTKs

Example of Assay Specificity



DIY = Do-it-yourself (toolbox products)

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PathHunter® Cell-Based Assays for Kinase Receptors

Applications and Validation data for Functional, Dimerization, and Translocation (internalization) Assays

PathHunter® Cell-Based Functional Assays

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Inhibit the kinase domain of tyrosine kinases with small molecule inhibitors

Select tyrosine kinase receptors and their small molecule inhibitors

Kinase ID	Compound ID	Historical IC_{50} (nM)
AXL	Crizotinib	195
BLK	EXEL-2880/XL-880/Foretinib	216
c-KIT	AG-013736 (Axitinib)	3.07
c-MET	EXEL-2880/XL-880/Foretinib	58.1
c-Ret-GFRa2	EXEL-2880/XL-880/Foretinib	7.88
DDR1	Dasatinib	0.679
ErbB1 (EGFR)	Gefitinib	98.8
ErbB2/ErbB3	Lapatinib	51.4
ErbB4	Lapatinib	39.4
FGFR2	AZD-4547	4.39
FGR	Dasatinib	0.997
FLT3 Activity	PKC-412	12.3
FLT3 Functional	EXEL-2880/XL-880/Foretinib	10.1
FLT4 Activity	EXEL-2880/XL-880/Foretinib	4
IGF1R	GSK 1838705A	14.2
INSR	GSK 1838705A	31.4

Kinase ID	Compound ID	Historical IC_{50} (nM)
JAK3	CP690550	1100
KDR	ABT-869	0.848
PDGFRa	AG-013736 (Axitinib)	10.2
PDGFRb	AG-013736 (Axitinib)	8.29
PRLR-JAK1	INCB018424	84.4
PRLR-JAK2	INCB018424	379
SYK	MNS	2030
TrkA	EXEL-2880/XL-880/Foretinib	9.51
TrkA-p75	EXEL-2880/XL-880/Foretinib	14.7
TrkB	EXEL-2880/XL-880/Foretinib	11.5
TrkB-p75	EXEL-2880/XL-880/Foretinib	15.4
TrkC	EXEL-2880/XL-880/Foretinib	6.56
TrkC-p75	EXEL-2880/XL-880/Foretinib	4.22
TYK2	INCB018424	1890

PathHunter® Cell-Based Functional Assays

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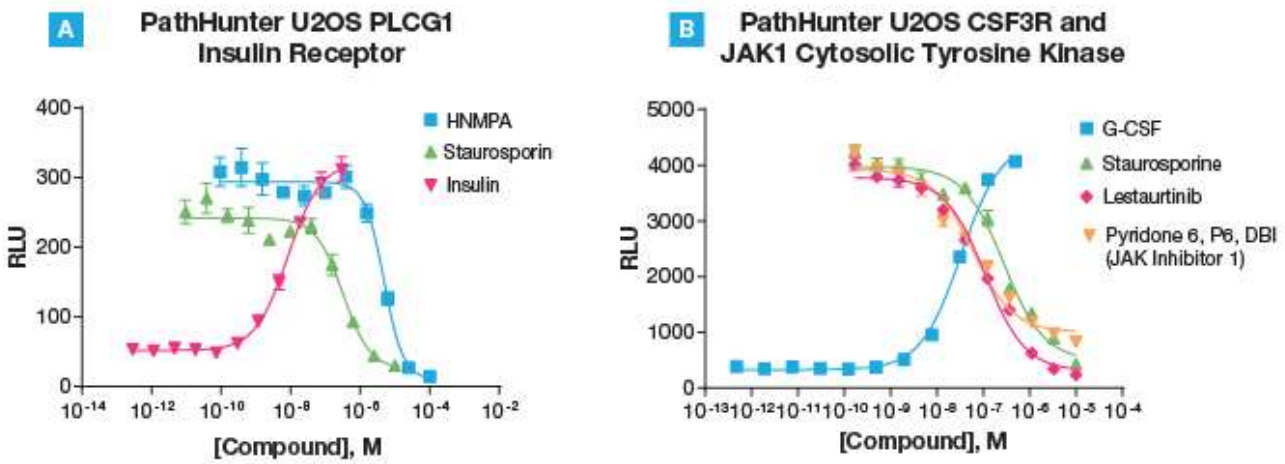
Evaluate multiple mechanisms of activation and small molecule inhibition

Insulin receptor (INSR)
(SH2-adaptor protein = PLCG1)

Colony stimulating factor 3 receptor (CSF3R)
cytokine receptor (CTK = JAK1)



A. The insulin receptor is constitutively dimerized, but undergoes a conformational change to become active in the presence of insulin (agonist) and this can be inhibited by the small molecule antagonists staurosporine and HNMPA. B. The colony stimulating factor 3 receptor (CSF3R-JAK1) cell line was preincubated with antagonists staurosporine, lestaurtinib and pyridone 6, P6, DBI (a Jak inhibitor 1) and then challenged with EC_{80} of agonist G-CSF. A dose dependent inhibition was observed with antagonist indicating that the assay can be used effectively to profile or screen inhibitors against the cytosolic tyrosine kinase JAK1.

PathHunter® Cell-Based Functional Assays

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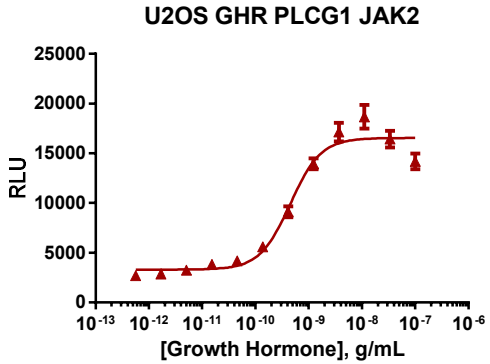
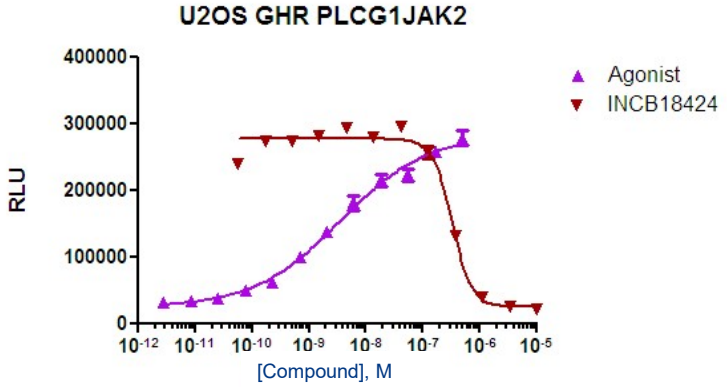
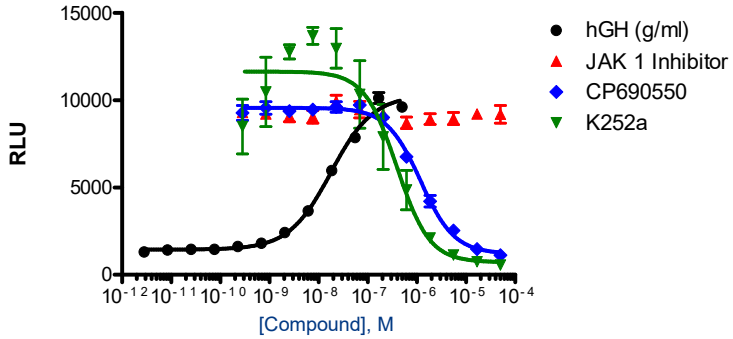
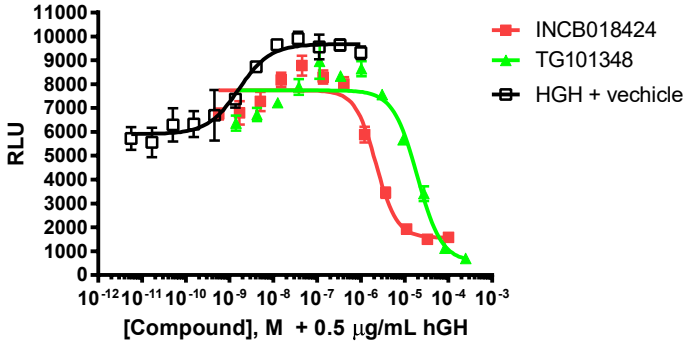
Characterize JAK-coupled cytokine receptor responses; identify JAK inhibitors

Growth Hormone Receptor examples









CTK = Jak2; SH2-adaptor protein = PLCG1; CP690550 (Tofacitinib)

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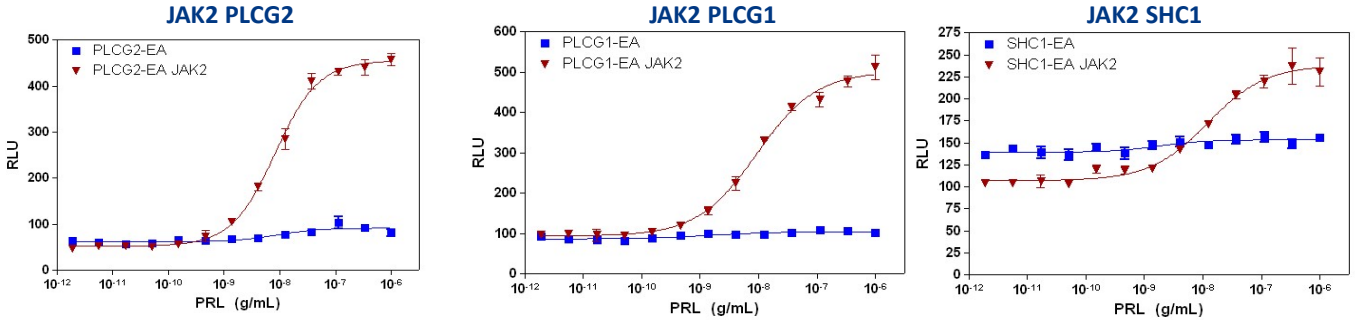
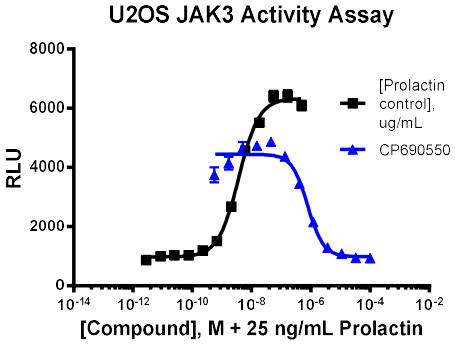
Characterize JAK-coupled cytokine receptor responses; identify JAK inhibitors

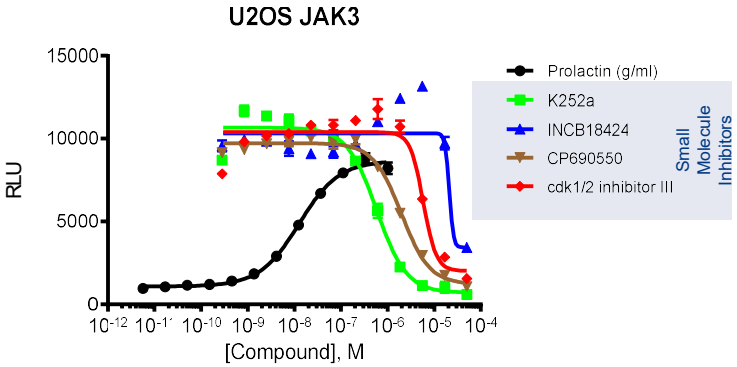
Prolactin Receptor examples

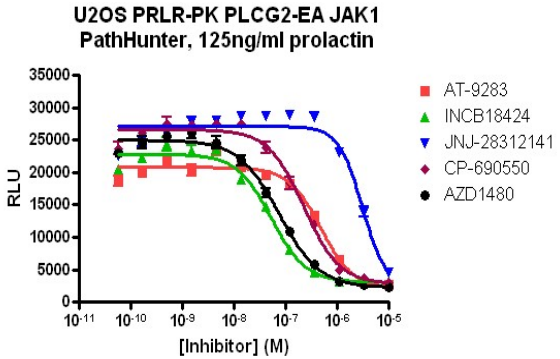


Red = + Jak2

Blue = - Jak2







Inhibitor	Potency
AT-9283	+++
INCB18424	+++++
JNJ-28312141	+
CP-690550	++
AZD1480	++++
Agonist	ProLactin

+++++ = most potent
+ = least potent

CTKs = Jak1, 2,or 3; SH2-adaptor proteins = PLCG1, PLCG2 or SHC1

Create Your Own PathHunter® Functional Assays

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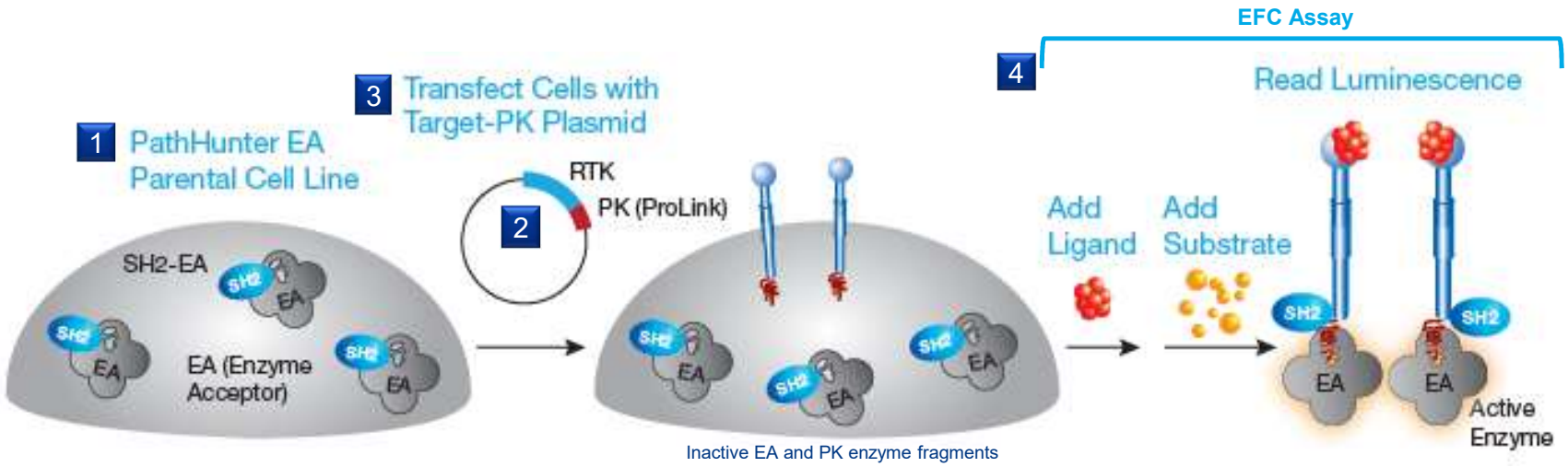
Workflow example for the development of an RTK cell-based functional assay



1. Start with an SH2-protein recruitment engineered parental cell line
2. Clone in protein of interest (e.g. RTK target) using PK (ED) cloning vector (plasmid)
   - Option to use a PK (ED) expression vector plasmid
3. Transfect plasmid into cells
4. Perform EFC assay (e.g. RTK functional assay) using ligand(s) and a PH Detection Kit (includes substrate)
   - Read chemiluminescent signal (obtain quantitative dose-response curve)

Similar workflow for translocation assays

EA = EFC enzyme acceptor fragment; ED = EFC enzyme donor fragment (e.g. ProLink™ (PK) for this example)

PathHunter® Cell-Based Dimerization Assays

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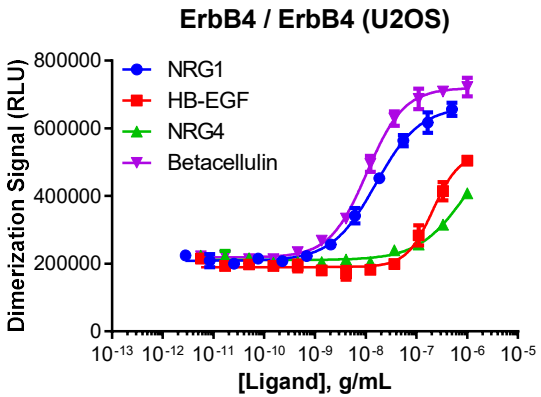
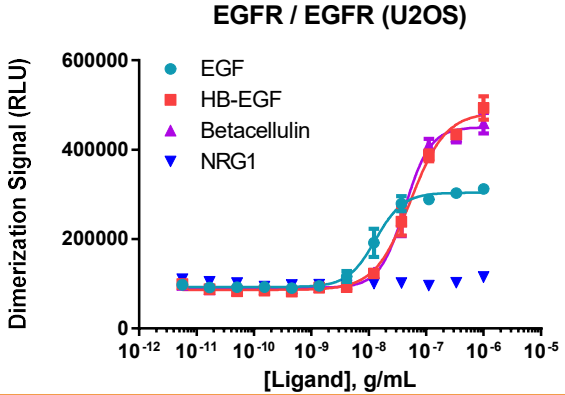
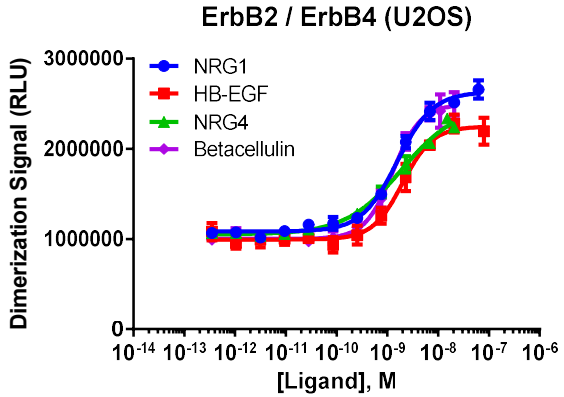
Analyze ERB and EGF RTK dimerization

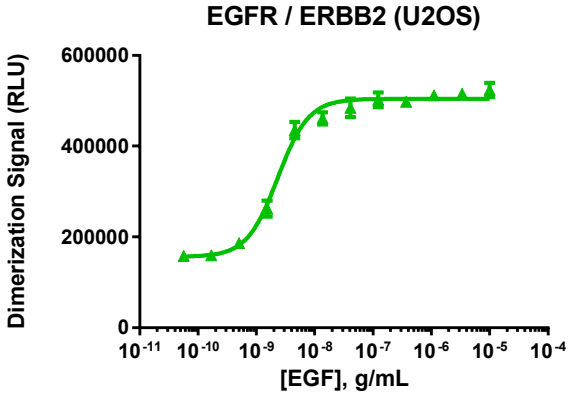
RTK Homodimers





RTK Heterodimers





PathHunter® Cell-Based Dimerization Assays

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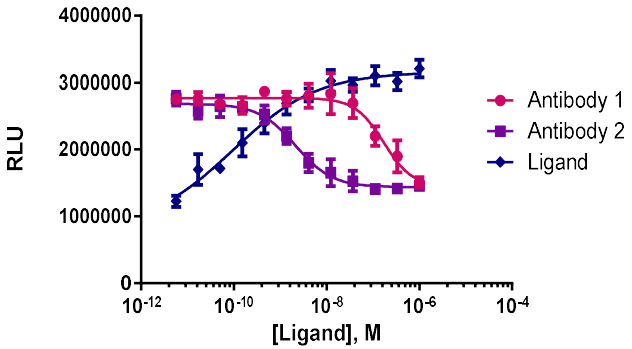
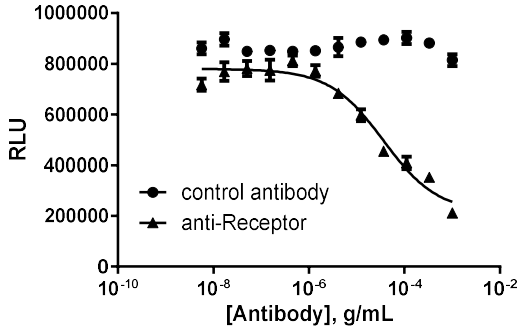
Screen and profile anti-ligand and anti-receptor antibodies (biologics)

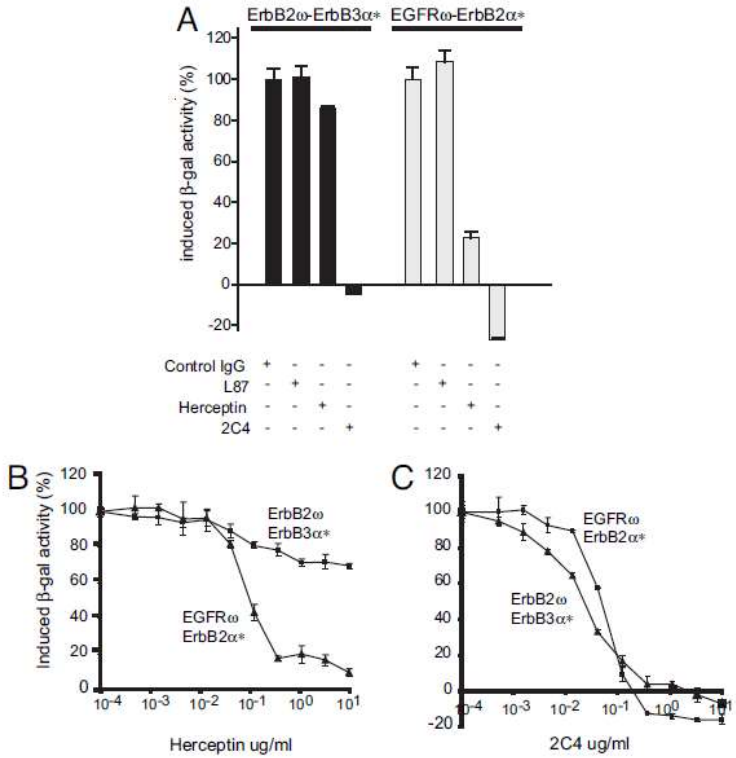
EGFR Homo-dimer Assay



TGFβR Hetero-dimer Assay



Dimerization assays demonstrate that Herceptin inhibits EGFR-ErbB2 not ErbB2-ErbB3*



*Wehrmann et al., PNAS 2006

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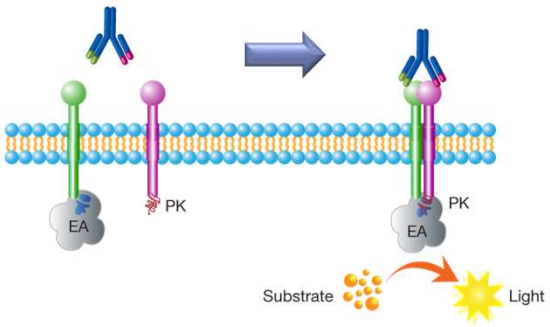
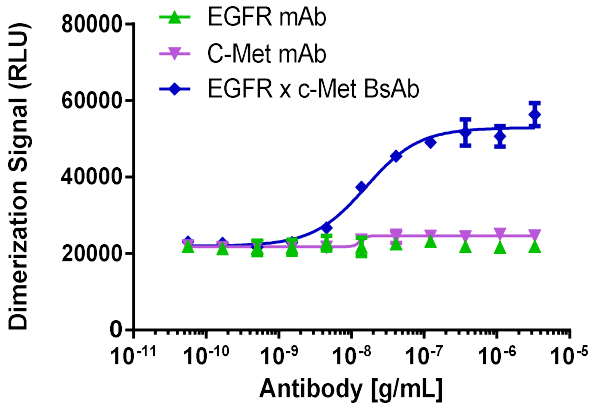
Screen and optimize bispecific antibodies (biologics). Oncology relevant example.

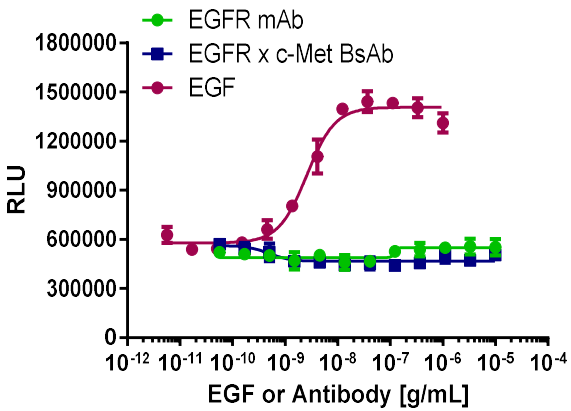
Bispecific Antibody-Induced Receptor Dimerization



EGFR/C-Met Bispecific Assay



EGFR Homodimer Assay



Assay developed to characterize bispecific antibodies

- Dimerization assay engineered to express two receptors (EGFR and c-Met) on same cell (e.g. mimic tumor model)
- Heterodimer model is specific for bispecific antibody (left panel); bispecific does not activate receptor homodimer assay (right panel)
- Published by S. W. Jarantow *et al.* in JBC (2015): Impact of Cell-surface Antigen Expression on Target Engagement and Function of an Epidermal Growth Factor Receptor × c-MET Bispecific Antibody

PathHunter® Cell-Based Dimerization Assays

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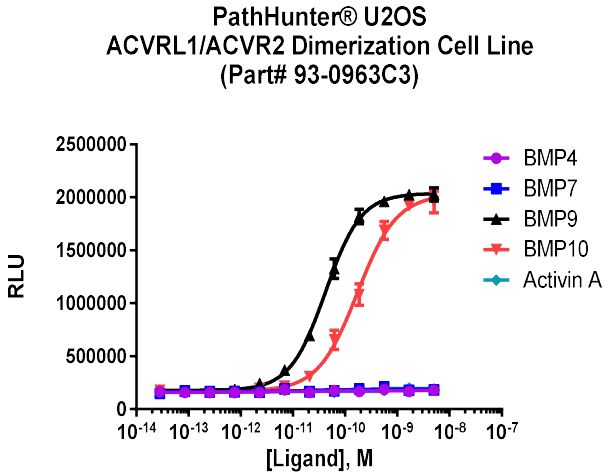
Study other kinase receptors (example of serine/threonine kinase receptors)

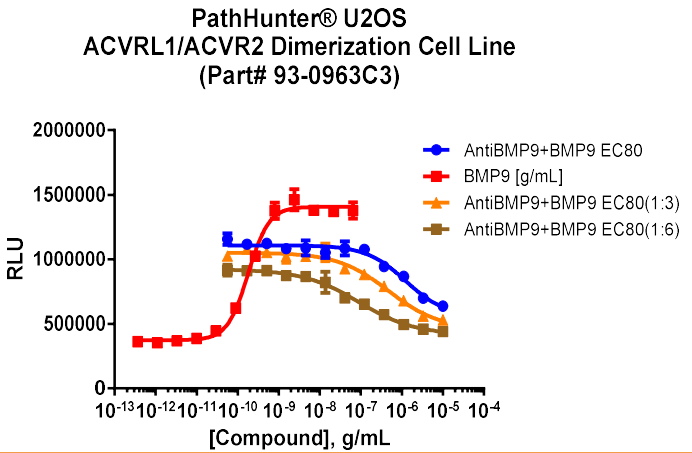
Serine/threonine-protein kinase receptors

- TGFβ superfamily receptors (e.g. BMP and ACVRL1 receptors)
- Heterodimerize with activin-binding (e.g. ACVR2) receptors to form functional receptors
- Therapeutic targets in oncology and fibrosis

Specific Ligand Profiling



Antibody Profiling
(anti-ligand, anti-receptor)



PathHunter® Cell-Based Assays for Kinase Receptors

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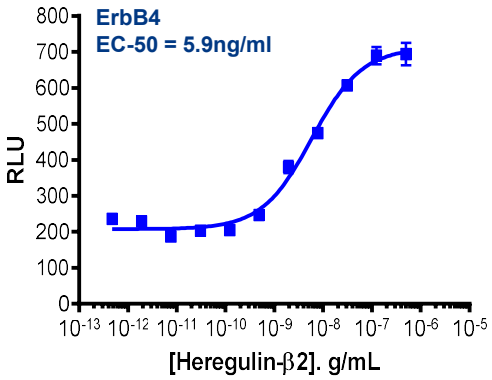
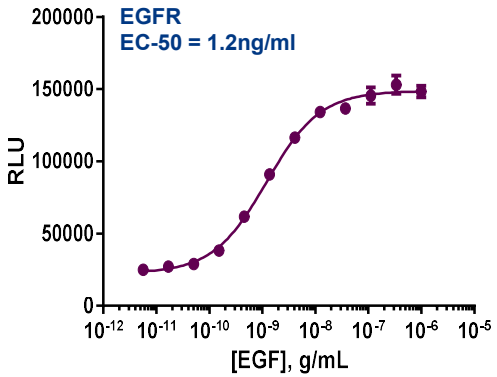
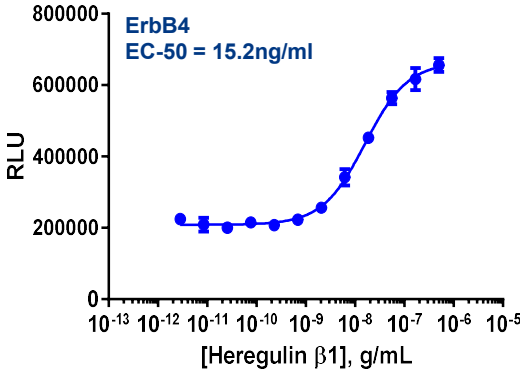
Two assay formats provide receptor-proximal pathway responses at different levels (activation via adaptor recruitment vs. dimerization)

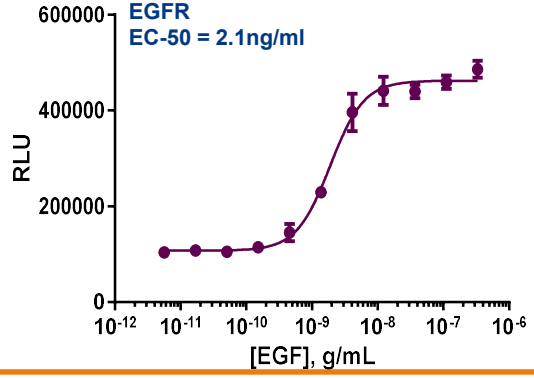
Functional Assays
(Phosphorylation-dependent SH2-Recruitment Assays)





Dimerization Assays





PathHunter® Cell-Based Translocation Assay

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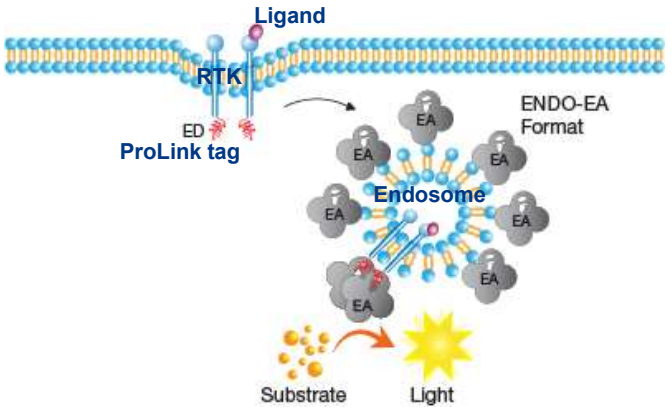
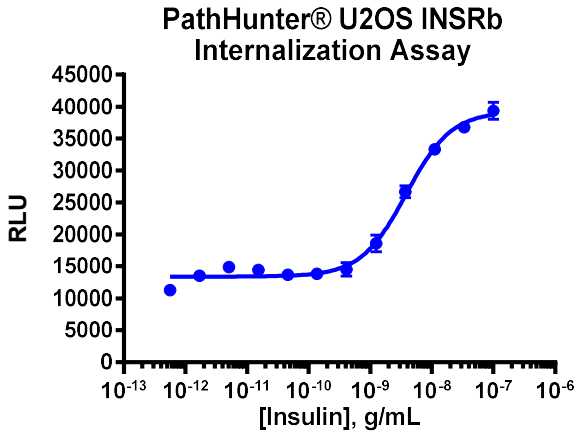
Measure internalization and trafficking of the RTKs

Provide a quantitative measurement of total endocytosed receptor protein using EFC

Example: Insulin receptor (e.g. INSRb)

- Cells are engineered to co-express the ProLink™ (PK)-tagged RTK and the Enzyme Acceptor (EA)-tagged endosome fusion proteins
- Activation of the RTK results (e.g. insulin) in internalization of the receptor and trafficking to cellular endosomes, where the two enzyme fragments complement and form a functional β-gal enzyme that is capable of hydrolyzing substrate and generating a chemiluminescent signal using the PathHunter Detection kit





Insulin receptor, isoform B (INSRb); ENDO = endosome

Create Your Own PathHunter® Translocation Assays

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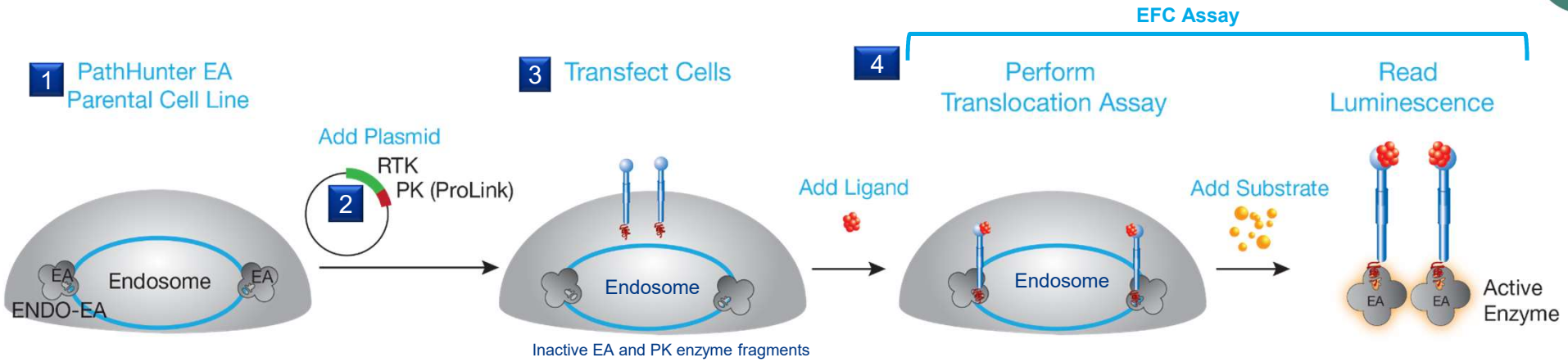
Workflow example for the development of an RTK cell-based translocation assay



1. Start with an ENDO-EA engineered parental cell line
2. Clone in protein of interest (e.g. RTK target) using PK (ED) cloning vector (plasmid)
   - Option to use a PK (ED) expression vector plasmid
3. Transfect plasmid into cells
4. Perform EFC assay (e.g. translocation) using ligand(s) and a PH Detection Kit (includes substrate)
   - Read chemiluminescent signal (obtain quantitative dose-response curve)

Similar workflow as functional assays creation

EA = EFC enzyme acceptor fragment; ED = EFC enzyme donor fragment (e.g. ProLink™ (PK) for this example)

Conclusions

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PathHunter® cell-based assays for kinase receptors

Assay Highlights

- **Accurate, Sensitive and Reproducible** - Superior quality, reproducible data with large assay windows, and robust performance
- **Broadly Applicable** - Identify different ligand classes (small molecules and biologics, monospecific vs bispecific, agonist vs antagonist) and study hetero- and homodimers
- **High Specificity** - Tagged, full-length tyrosine kinase eliminates non-specificity from endogenous tyrosine kinases
- **Easy-to-Use** – Simple, one-step add and read protocol that is HTS-friendly
- **Universal, DIY Solution** – Create your own quantitative cell-based assays to study translocation or function of RTK/CTKs

Accessing kinase assays and solutions

Kinase solutions page

Kinase targets

Kinase cell-based assays

Dimerization assays

Translocation assays

Kinase profiling services

Toolbox (DIY) solutions

DIY = Do-it-yourself (toolbox products)