DiscoverX

User Manual

PathHunter®

TrkC Bioassay Kit

For Chemiluminescent Detection of Receptor Activity

Catalog No. 93-0464Y3 Series

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Please read entire booklet before proceeding with the assay.
For additional information or Technical Support see contact information below.

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Overview

Intended Use

PathHunter® TrkC Bioassay kits provide a robust, highly sensitive, and easy-to-use, cell-based functional assay to study potency of drugs targeting Neurotrophin 3 or the TrkC receptor. The Bioassay kits contain all the reagents needed for a complete assay including cells, detection reagents, cell plating reagent, positive control agonist, dilution buffer, and assay plates. The qualified, frozen cells have been manufactured for single use and are provided in a ready-to-assay format that saves time and adds convenience.

Technology Principle: PathHunter TrkC Bioassay

These assays utilize Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, ProLink (PK) and Enzyme Acceptor (EA). Independently these fragments have no β-gal activity; however, when forced to complement through protein-protein interactions, they form an active β-gal enzyme.

In the PathHunter assay approach for tyrosine kinases, the ProLink tag is fused to the C-terminus of the receptor. The EA is fused to a phosphotyrosine SH2 domain containing protein that is able to bind the activated RTK. Ligand-induced activation of the receptor results in receptor phosphorylation. The SH2-EA fusion protein binds the phosphorylated receptor, forcing complementation of PK and EA to form an active β-gal enzyme. β-gal enzymatic activity is quantitatively measured using a chemiluminescent substrate in the PathHunter Bioassay Detection Kit (Figure 1).

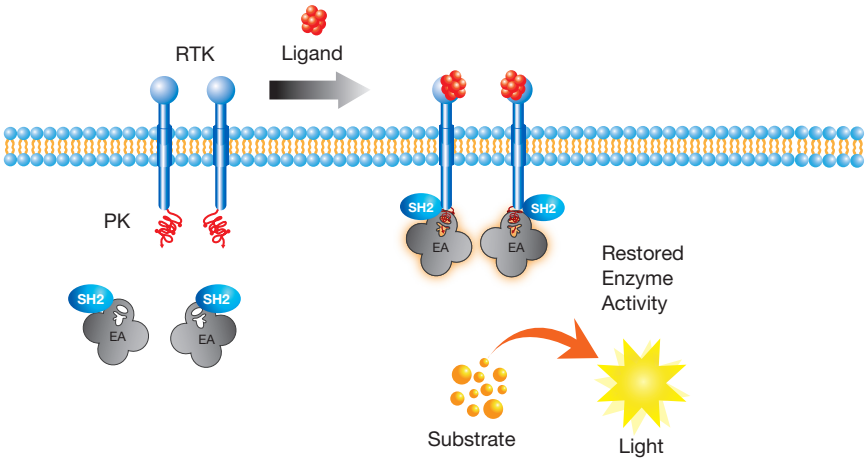


Figure 1. PathHunter TrkC Bioassay Principle

Materials Provided

List of Components	93-0464Y3-00077	93-0464Y3-00078
Description Kit Size	**Contents**	**Contents**
PathHunter U2OS TrkC Bioassay Cells	2 vials	10 vials
PathHunter Bioassay Detection Kit	200 dp	1000 dp
• Detection Reagent 1	2 mL	10 mL
• Detection Reagent 2	8 mL	40 mL
AssayComplete® Cell Plating Reagent 16	1 X 100 mL	3 X 100 mL
Protein Dilution Buffer	1 X 50 mL	2 X 50 mL
Control Agonist (NT-3)	1 vial	1 vial
96-well Clear-Bottom TC Treated, Sterile Plates w/Lid	2 plates	10 plates

Storage Conditions

PathHunter U2OS TrkC Bioassay Cells

Cells are shipped on dry ice and should arrive in a frozen state.

Immediately store the vials as follows:

- Short term (24 hours or less): Store vials at -80°C immediately upon arrival. (DO NOT store at –80°C for more than 24 hours).
- Long term (greater than 24 hours): Vials should ONLY be stored in the vapor phase of liquid nitrogen (LN_2).

Safety Warning: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. The manufacturer of the cryovial recommends storing the vials in the vapor phase above the liquid N_2. Upon thawing, if LN_2 is present in the cryovial, it rapidly converts back to its gas phase which can result in the explosion of the vial upon its removal from the liquid nitrogen tank.

PathHunter Bioassay Detection Kit

Store at -20°C. Once thawed, the detection reagents can be kept at 4°C for up to 4 days. For longer storage (up to the expiration date listed in the kit certificate of analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaws.

To make aliquots suitable for testing one assay plate each, 1 mL of Detection Reagent 1 per aliquot can be dispensed and frozen down. 4 mL of Detection Reagent 2 per aliquot can be dispensed and frozen down separately. Do not mix the two reagents during aliquoting.

AssayComplete Cell Plating Reagent 16

Once thawed, the Cell Plating Reagent can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed in the kit certificate of analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles.

To make aliquots suitable for testing one assay plate each, 30 mL of reagent per aliquot should be dispensed and frozen down.

Protein Dilution Buffer

Once thawed, the Protein Dilution Buffer can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed in the kit certificate of analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles.

To make aliquots suitable for testing one assay plate each, 10 mL of reagent per aliquot should be dispensed and frozen down. This amount may vary depending on your stock sample concentrations and should be adjusted accordingly.

Recombinant Human NT-3 Control Agonist

Store at -20°C until ready to use (up to the expiration date listed in the kit certificate of analysis). Centrifuge the vial prior to opening to maximize recovery. When ready to use, reconstitute to a concentration of 100 ug/mL by adding 100 µL of Protein Dilution Buffer to the vial containing 10 ug of lyophilized powder. Reconstituted ligand is stable for 1 week at 2-8°C. For longer storage (up to the expiration date listed on the kit certificate of analysis), it is recommended to store in working aliquots at -20 to -80°C.

96-well Tissue Culture Treated Plates

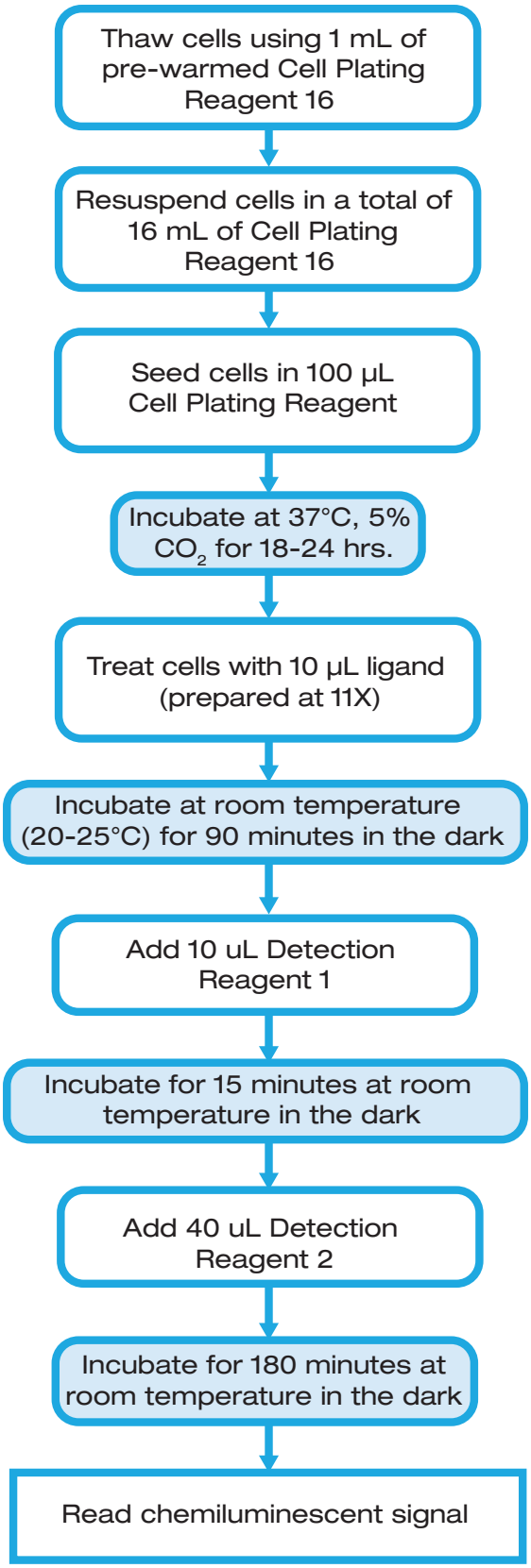
Store at room temperature.

Additional Materials Required

Equipment
The following equipment and additional materials are required to perform these assays:
Single and multichannel micro-pipettors and pipette tips
Multimode or luminescence plate reader
V-Bottom 96-well compound dilution plates (DiscoverX Cat. No. 92-0011 or similar)
Disposable Reagent Reservoir (Thermo Scientific, Cat. No. 8094 or similar)

Protocol Schematic

Quick-Start Procedure: In a white-walled 96-well tissue culture treated plate perform the following:



Detailed Protocol

Day 1: PathHunter Bioassay Cell Preparation

The following protocol is for thawing and plating frozen PathHunter U-2 OS TrkC Bioassay cells from cryovials.

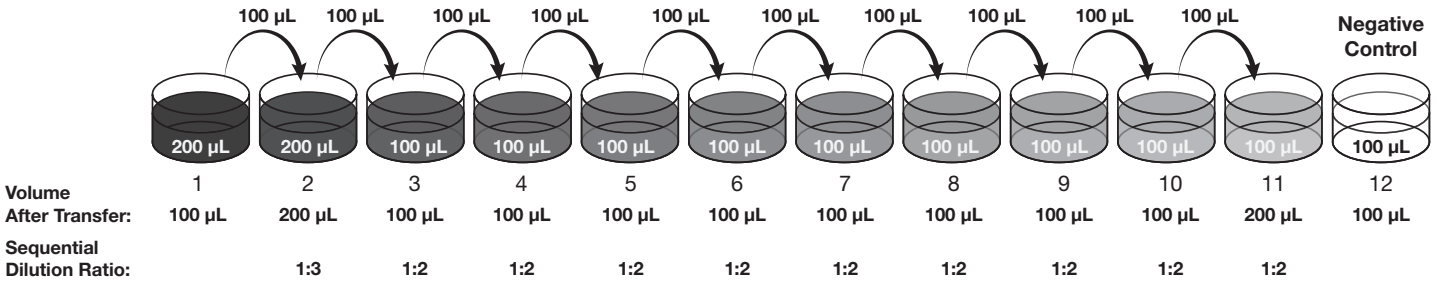
1. Before thawing the cells, ensure that all the necessary materials required are set up in the tissue culture hood. This includes:
    a. One 25 mL reagent reservoir.
    b. One 50 mL conical tube.
    c. A micropipettor (P1000) set to dispense 1 mL.
    d. A multichannel pipette and tips set to dispense 100 µL.
    e. A bottle of Cell Plating Reagent 16 (CP16, pre-warmed in a 37˚C water bath for 15min.).
    f. A white-walled, clear-bottom 96-well assay plate.
2. Dispense 16 mL of CP16 into the 50 mL conical tube
3. Remove the cryovial from liquid nitrogen and immediately place in dry ice.

DO NOT use heated water bath to thaw the vial. Wipe down the cryovial quickly with 70% EtOH, and bring it into the tissue culture hood right away. **DO NOT** touch the sides or bottom of the vial to avoid thawing of the cell pellet through body heat.

4. Thaw the pellet by immediately adding 1 mL (using P1000) of pre-warmed CP16 from the 50 mL conical tube to the cryovial, thawing the cell pellet. The medium should be added slowly along the side of the wall of cryovial tube. Mix the cells gently by pipetting up and down several times to break up any clumps. Transfer the cell suspension to the conical tube containing the remaining 15 mL of CP16. Remove any medium/suspension left in the tube to ensure complete recovery of all the cells from the vial.
5. Mix the tube by inversion to ensure the cells are properly mixed in the medium without creating any froth in the suspension and immediately pour the suspension into the 25 mL reservoir.
6. Add 100 µL of cells to each well of the 96-well assay plate using the multichannel pipette.
7. Place the assay plate to the 37°C, 5% CO_2 incubator and incubate overnight (18-24 hrs)

Day 2: Ligand Preparation

The following protocol is designed for testing purified biologics. The PathHunter assays can also be run in the presence of high levels of serum or plasma without significantly impacting assay performance. Therefore, standard curves of control can typically be prepared in neat serum or plasma and added directly to cells without further dilution. For the best results, the optimized minimum required dilution (MRD) of crude samples should be empirically determined.



1. Prepare the agonist (NT-3) dose response curve. This preparation should be sufficient to perform an 11-point dose response curve with each dose run in triplicate.

   Reconstitution of recombinant human NT-3 ligand: (DiscoverX Cat. No. 92-1025) Quick spin the vial prior to reconstituting. Add 100 µL of Protein Dilution Buffer to 0.01 mg of lyophilized powder in the vial to prepare a stock concentration of 0.1 mg/mL. Agonist is prepared at 11 X the desired final concentration as it will be diluted by adding to the 100 µL of media present in the assay plate.

   a. Add 200 µL of Protein Dilution Buffer to well A2 and 100 µL of Protein Dilution Buffer to wells A3-A12 of a master dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate, DiscoverX Cat. No. 92-0011 or similar).

   b. Add 200 µL of rhNT-3 at 11 X the final top dose of 0.5 µg/mL to well A1 of the master dilution plate.

   c. Using a clean tip, transfer 100 µL from well A1 into well A2 and mix by pipetting up and down several times. Replace the pipette tip, and transfer 100 µL from well A2 into well A3. Mix by pipetting up and down several times. Repeat this process until well A11 is reached, resulting in an eleven point dilution series. No ligand is transferred to column 12 as this will serve as a negative control.

2. Add 10 µL from each well of the agonist dose curve on the master dilution plate to the appropriate wells of the assay plate (e.g. see recommended plate map).

3. Incubate the plate at room temperature (20-25°C) for 90 minutes in the dark.

Day 2: Detection

1. Using a multichannel pipette, add 10 µL of Detection Reagent 1 to each well of the assay plate.

2. Incubate the plate at room temperature for 15 minutes in the dark.

3. Using a multichannel pipette add 40 µL of Detection Reagent 2 to each well of the assay plate.

4. Incubate the plate at room temperature for 3 hours in the dark.

5. Read sample on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube (PMT) readers of 5-10 seconds for imager.

Supplemental Information

Representative Plate Maps for Agonist Curve

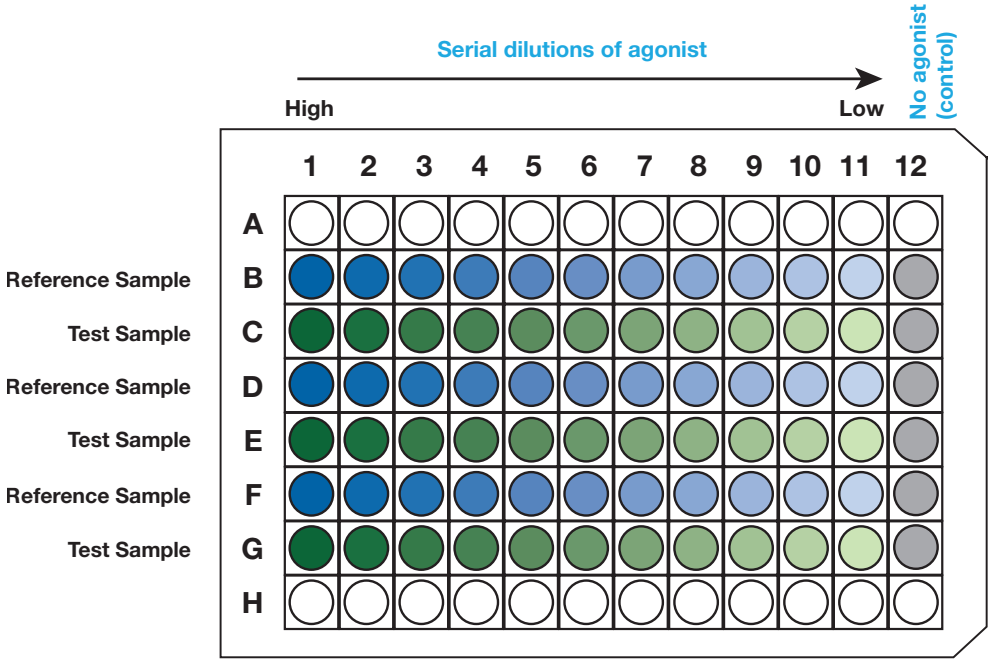


Figure 1. Serial dilution method. This plate map shows 11-point dose curves with 3 data points at each concentration for one reference and one test sample per plate.

Related Products

Description	Ordering Information
PathHunter Detection Reagents	www.discoverx.com/detectionreagents
Cell Culture Kits, Reagents, & Consumables	www.discoverx.com/cell-culture-kits-reagents-consumables
Control Ligands	www.discoverx.com/controlligands

Instrument Compatibility Chart

Assay	Instrument	Read-Out
All PathHunter® assays HitHunter® cAMP HitHunter® cGMP	**COMPATIBLE WITH ANY LUMINOMETER** **BMG:** PheraStar, Cytostar, LumiStar **Perkin Elmer:** TopCount, Victor 2 or V, Fusion, LumiCount, Envision, Micro-beta (Trilux), Viewlux, Northstar **GE:** LEAD seeker, Farcyte **Molecular Devices:** FLIPR, SpectraMax M3/M4/M5/M5e, FlexStation 3, SpectraMax L **Tecan:** Ultra, Evolution **Turner BioSystems:** Modulus Microplate **Caliper** LabChip 3000 & EZ Reader **Berthold Technologies:** Mithras LB940, CentroLIApc **Hamamatzu** FDS6000, FDSS/RayCatcher **Thermo Scientific:** Luminoskan Ascent **Biotek:** Synergy 2	Luminescence

*For other instruments not listed here, please use the information below to contact technical support.

Troubleshooting Guide

Problem	Cause	Solution
No Response	Improper thawing procedure	Refer to thawing instructions on page 5 of this user manual.
	Improper ligand used or improper ligand incubation time	Refer to the Detailed Protocols section of this manual for the recommended ligand and assay conditions.
	Improper preparation of ligand (agonist or antagonist)	Refer to vendor specific datasheet to ensure proper handling, dilution and storage of ligand.
	Improper time course for induction	Optimize time course of induction with agonist and antagonist.
Decreased Response	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using microscope.
Low or No Signal	Improper preparation of detection reagents	Detection reagents should ideally be prepared just prior to use and are sensitive to light.
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 0.1-1 sec/ well.
Experimental S:B does not match datasheet value	Incorrect incubation temperature	Confirm assay conditions as provided on the certificate of analysis.
	Improper preparation of ligand (agonist or antagonist)	Check and repeat assay at correct incubation temperature as indicated on the assay datasheet.
		Some ligands are difficult to handle. Confirm the final concentration of ligands.
EC_{50} is right-shifted	Improper ligand handling or storage	Check ligand handling requirements
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the Product Insert.
	Problems with plate type and compound stability	Hydrophobic compounds should be tested for solubility and may be diluted in Protein Dilution Buffer.
		Non-binding surface plates may be necessary for hydrophobic compounds.

For additional information or technical support, please use the contact information below.

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