Contact Information

DiscoveRx Corporation
(World Wide Headquarters)
42501 Albrae Street
Fremont, CA 94538
United States

t | 510.771.3500
f | 510.979.1650
toll-free | 866.448.4864

DiscoveRx Corporation Ltd.
(Europe Headquarters)
Faraday Wharf, Holt Street
Aston Science Park
Birmingham, B7 4BB
United Kingdom

t | +44.121.260.6142
f | +44.121.260.6143

www.discoverx.com

PathHunter® eXpress HEK 293 IκB Degradation Assay

Product Booklet: 93-0538E1CP7 Series

Simple Solutions for Complex Biology

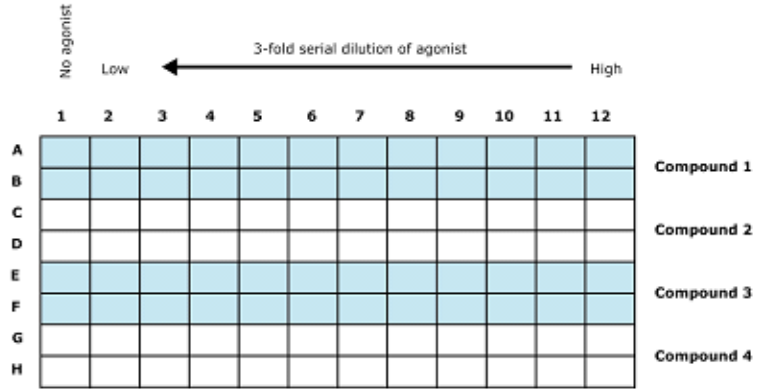
CONTENTS

ATTENTION:

Read the entire product insert prior to beginning the assay. Refer to the data sheets for additional information on cell-line specific media requirements.

For additional information or technical support, contact DiscoveRx or visit www.discoverx.com.

A. Agonist Dose Response (96-well plate layout):



This illustration shows an 11-point dose curve with 2 data points each 4 compounds per plate for a total of 8 compounds per eXpress kit.

FREQUENTLY ASK QUESTIONS & RESPONSES

Q: I did not see a signal with my control agonist.
R: There may be differences in agonist purchased from different vendors. Confirm that the control agonist used is the same ligand used in the dose response shown in the provided cell-specific data sheet.

Q: I did not see a response with my compound.
R1: The concentration of DMSO or Ethanol used for dilution is too high. Maintain concentration of the agonist/antagonist diluent at ≤ 1%.
R2: Confirm that the final ligand concentration is correct. Some ligands are "sticky" and difficult to dissolve.
R3: Confirm that the cell line responds to the control agonist.
R4: Repeat the experiment using a new lot of control agonist.

Q: My cells arrived thawed. Can I use them?
R: No. Call technical support for a replacement.

Q: How long is the prepared detection reagent good for?
R: The working detection reagent solution must be used within 8 hours of mixing.

Q: What instruments can I use to read the plates?
R: Any bench top luminometer will work with the PathHunter® eXpress assays.

Q: How long is the signal stable for?
R: The signal is stable for 5 hours after addition of detection reagent.

Q: My cells are floating after the 24 hours incubation.
R: The cells are not viable, contact technical support for a replacement.

Q: Can I switch plates or should I use the plate provided?
R: You can use any clear bottom white or opaque walled plate.

Q: How do I generate a dose curve for my Inhibitor compound?
R: First prepare ten serial 3-fold dilutions of the antagonist compound in the desired vehicle of choice. Prepare antagonist dilutions such that the concentration is **22X** of the final screening concentration (5 µl antagonist in a final volume of 110 µl). Then prepare a working stock of agonist compound at **22X** of the EC_{80} concentration. Pipette all compound dilutions per well following the plate map on page 11.

LEGAL SECTION

This product and/or its use is covered by one or more US and/or foreign patents, patents applications, and trade secrets that are either owned or licensed to DiscoveRx corporation. For some products/cell lines, certain third party gene-specific patents may be required to use the cell line. It is Purchaser's responsibility to determine if such patents or other intellectual property rights are required.

DiscoveRx® and PathHunter® are registered trademarks of DiscoveRx Corporation.

DiscoveRx name and logo are a trademark of DiscoveRx Corporation.
GraphPad Prism is a registered trademark of GraphPad Software, Inc.

All goods and services sold are subject to the terms and conditions of DiscoveRx Corporation. A copy of these terms and conditions is available on request. Contact your local DiscoveRx representative for the most current information.

For additional info or further questions regarding the rights conferred with purchase of the Materials, please contact:

Licensing Department
DiscoveRx Corporation
42501 Albrae Street
Fremont, CA 94538
t | 510.771.3527
e | info@discoverx.com

INTENDED USE

PathHunter® eXpress Pathway assay kits provide a robust, highly sensitive and easy-to-use method cell-based functional assay for IκB degradation and NFκB pathway activation. The eXpress kits contain everything needed for a complete IκB degradation assay including cells, detection reagents, media, and plates. The pre-validated, frozen eXpress cells have been manufactured for short term use and are provided in a convenient, ready-to-screen format that saves time and adds convenience. Assays have been designed for 96-well plate analyses.

TECHNOLOGY PRINCIPLE: PATHHUNTER™ PATHWAY ASSAY

DiscoveRx's proprietary Enzyme Fragment Complementation (EFC) is a homogeneous, non-radioactive detection technology based on two genetically engineered β-galactosidase fragments - a large protein fragment (Enzyme Acceptor, EA) and a small peptide fragment (Enzyme Donor, ED). Separately, the β-gal fragments are inactive, but in solution, they rapidly recombine to form active β-galactosidase enzyme that hydrolyzes substrate; producing an easily detectable chemiluminescent or fluorescent signal. The PathHunter technology from DiscoveRx is an adaptation of Enzyme Fragment Complementation (EFC) that provides a novel cell-based assay format for detecting protein-protein interactions, protein degradation and protein translocation. In this assay, small peptide fragment termed ProLabel™ is tagged to IkB protein. PathHunter IκB degradation assay measures human I-Kappa -B- (IκB, alternate name NFKBIA; NM_020529.2) degradation in response to NFkB pathway stimulation. The assay requires a specific detection kit (as the one provided in the kit) that has the EA fragment in the reagents to ensure enzyme fragment complementation.

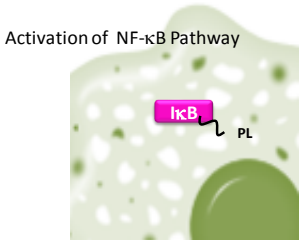
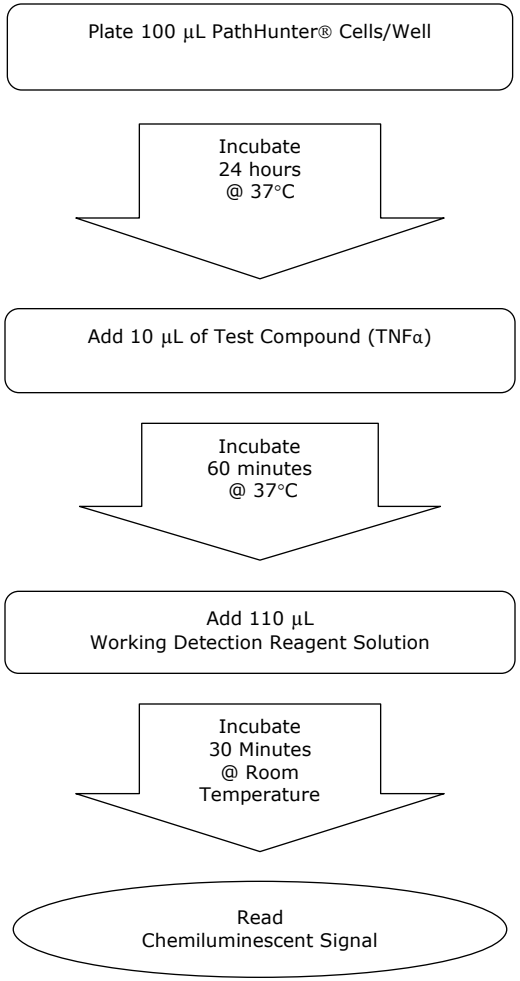


Figure 1. PathHunter TNFα Assay Principle.

QUICK-START PROCEDURE: TNFα DOSE RESPONSE



7. Add 110 µL of prepared detection reagent per well and incubate for 30 minutes at room temperature.
   This detection reagent working solution is added at a 1:1 ratio to the liquid in the well.
   DO NOT pipette up and down in the well to mix or vortex plates.

8. Read samples on any standard luminescence plate reader.
9. Use GraphPad Prism® or other comparable program to plot your inhibitor dose response.

STORAGE CONDITIONS

PATHHUNTER EXPRESS COMPONENTS REQUIRE MULTIPLE STORAGE TEMPERATURES. OPEN BOXES IMMEDIATELY AND STORE CONTENTS AS INSTRUCTED.

PATHHUNTER EXPRESS CELLS: Store at -80°C

NOTE:

- PathHunter® eXpress cells arrive frozen on dry ice. Cells are delivered in a vial containing $1x10^6$ cells in 100 µL of freezing medium. The vial contains sufficient cell numbers to generate (1) 96-well microplate prepared at the seeding density described.
- For short term storage (2 weeks or less), store vials at -80°C immediately upon arrival. For storage longer than 2 weeks, place vials in the vapor phase of liquid nitrogen (N_2). Use kit within 6 months from the date of receipt under proper storage conditions. **Do not touch the bottom of the tubes at any time to avoid inadvertent thawing of the cells. If cells are not frozen upon arrival, do not proceed. Contact technical support.**
- When removing cryovials from liquid N_2 storage, use tongs and place immediately on dry ice in a covered container. Wait at least one minute for any liquid N_2 inside the vial to evaporate and proceed with the thawing protocol (p.6).

PATHHUNTER PK/PL DETECTION REAGENT & CP REAGENT: Store at -20°C

NOTE:
Reagents can be frozen and thawed at least 2 times without affecting performance.

96-WELL TISSUE CULTURE TREATED PLATES: Store at Room Temperature

MATERIALS PROVIDED

Each Shipment Contains:

Description	2-Plate Kit	10-Plate Kit
PathHunter eXpress IkB HEK 293 Cells	2 vials $1x10^6$ cells	10 vials $1x10^6$ cells
PathHunter ProLabel®/ProLink™ Detection Kit	200 dp	1000 dp
- Lysis Buffer	2 x 2 mL	20 mL
- Substrate Reagent	2 x 8 mL	80 mL
- EA Reagent	2 x 2 mL	20 mL
- Positive Control Peptide (ED)	2 x 40 µL	200 µL
AssayComplete™ Cell Plating 7 Reagent (CP7)	1 x 100 mL	2 x 100 mL
96-well Tissue Culture Treated Plates	2 plate	10 plate

MATERIALS NOT PROVIDED

The following additional materials are required but not provided:

• Tissue culture disposables. • Disposable Reagent Reservoir (such as Thermo Scientific, Cat. #8094).	• Test molecule(s). • Multi-mode or luminescence plate reader.

MEDIA REQUIREMENTS

Each PathHunter® eXpress IκB Degradation Assay has been validated for optimal assay performance using the specific Cell Plating Reagent (CP) included in the kit. Always use the reagent included in the kit and **DO NOT substitute** from an alternate kit at any time.

ASSAY PROCEDURE - TNFα INHIBITOR DOSE RESPONSE

The following steps outline the procedure for performing an TNFα assay using the PathHunter eXpress cells and PathHunter PK/PL detection reagents. Although plate layouts and experimental designs will vary, we recommend performing an 11-point dose curve using at least duplicate wells for each dilution. Refer to the plate map on p.11 for more details.

NOTE:
Solvents can affect assay performance. PathHunter eXpress assays are routinely carried out in the presence of ≤ 1% solvent (i.e. DMSO, ethanol, PBS or other). If you use other solvents or solvent concentrations, optimize the assay conditions accordingly.

DAY 1: THAWING AND PLATING FROZEN CELLS

The following are procedures for thawing and plating frozen PathHunter eXpress cells from freezer vials:

1. Pre-warm CP reagent in a 37°C water bath.
2. Remove cell vial(s) from -80°C or liquid N_2 vapor phase storage and place immediately on dry ice prior to thawing. **DO NOT EXPOSE VIALS TO ROOM TEMPERATURE.**

   NOTE:
   When removing cryovials from liquid N_2, place immediately on dry ice in a covered container. Wait at least one minute before opening for any liquid N_2 inside the vial to evaporate.

3. Thaw cells by adding 0.5 mL of pre-warmed CP reagent directly into the cell vial.
4. Pipette up and down gently several times to ensure that the cells are evenly distributed.
5. Immediately transfer the cells to 11.5 mL of pre-warmed CP reagent and pour into a disposable reagent reservoir.
6. Plate 100 µL of cells into each well of the provided 96-well tissue culture plate.
7. After seeding the cells into the microplate, place it into a 37°C, 5% CO_2 in a humidified incubator for 24 hours prior to testing.

DAY 3: COMPOUND ADDITION

1. Make a 10-fold dilution of Recombinant Human TNFα (DiscoveRx # 92-1097; stock = 5 µg/mL) in CP7 reagent provided in the kit. The concentration is now 500 ng/mL.
2. Prepare 11 (2)-fold serial dilutions of TNFα compound in CP7. Representative data provided in the datasheet used 100 ng/mL as the highest concentration
3.
   - **Include a "no agonist" control and** Add only CP7 reagent to this tube named #12. This sample serves as the no compound control and completes the dose curve. .
3. Remove PathHunter® eXpress cells (previously plated on day 1) from the incubator.
4. Transfer 10 µL from tubes 1-12 to each well according to the plate map on p.14.
5. Incubate for 60 minutes @ 37°C.

SUBSTRATE PREPARATION AND ADDITION

6. During the incubation period, prepare a working solution of the detection reagents for each plate by mixing the following reagents:
   - **PREPARE WORKING SOLUTION BY COMBINING 1 PART OF EA REAGENT WITH 1 PART OF LYSIS BUFFER AND 4 PARTS OF SUBSTRATE REAGENT.**
   - **FOR EXAMPLE– FOR ONE 96-WELL PLATE, ADD 2 mL EA REAGENT, 2 mL LYSIS BUFFER AND 8 mL SUBSTRATE REAGENT, FOR A TOTAL OF 12 mLS.**

   NOTE:
   The working solution is stable for up to 8 hours at Room Temperature.