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PathHunter® eXpress Cytosolic Tyrosine Kinase Functional Assay Kit

User Manual

Simple Solutions for Complex Biology

CONTENTS

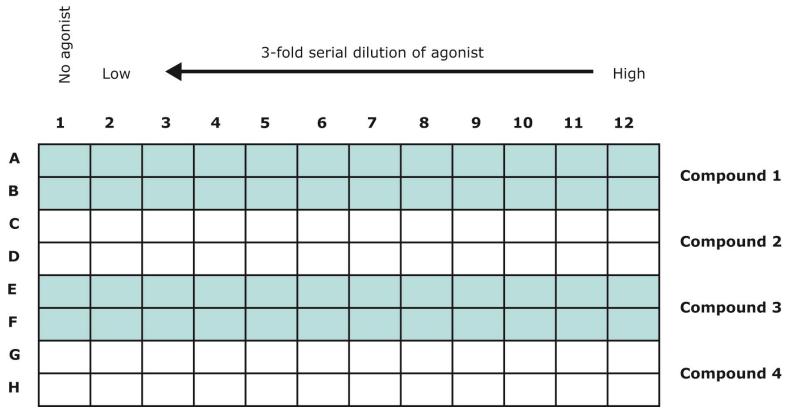
ATTENTION:

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.

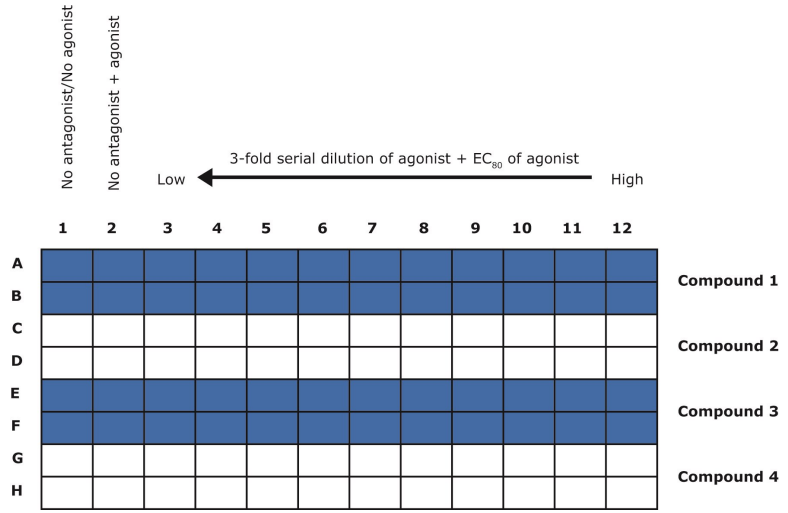
Notes

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.

B. Antibody/Inhibitor Dose Response (96-well plate layout):



This illustration shows a 10-point dose curve with 2 data points each 4 compounds per plate for a total of 8 compounds per eXpress kit.

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.

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

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INTENDED USE

PathHunter® eXpress Cytosolic Tyrosine Kinase (CTK) Functional Assay kits provide a robust, highly sensitive and easy-to-use, cell-based functional assay for monitoring cytokine receptor activation or cytosolic tyrosine kinase inhibition. The eXpress kits contain everything needed for a complete CTK experiment 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 ready-to-assay format that saves time and adds convenience. Assays have been designed for 96-well plate analyses.

*Test compounds are not included and must be provided by the researcher. Please refer to DiscoveRx ligands of choice for these assays on Appendix 1.

TECHNOLOGY PRINCIPLE

Assay-ready frozen cells are based on DiscoveRx's PathHunter technology. PathHunter cell lines feature novel *in vivo* applications of Enzyme Fragment Complementation (EFC) technology in which the β-galactosidase (β-gal) enzyme has been split into two inactive fragments. In the PathHunter assay approach for receptor tyrosine kinases, a small 4 kDa complementing fragment of β-gal (i.e., the ProLink™ tag) has been fused recombinantly to the intracellular C-terminus of the cytokine receptor of interest. The larger portion of β-gal, termed EA for 'enzyme acceptor', is fused to many different partner proteins containing phospho-tyrosine binding domains (such as SHC1, SHC2, Grb2, PTPN6, PLCG1 or PLCG2), depending on the target. This cell line also expresses the cytosolic Tyrosine Kinase (JAK), specified above in the product name. Upon ligand-induced activation, the Cytokine Receptor is phosphorylated by the Cytosolic Tyrosine Kinase, causing recruitment of the SH2-containing protein and enabling the two β-gal enzyme fragments to complement and form an active enzyme capable of hydrolyzing a substrate and generating a chemiluminescent signal.

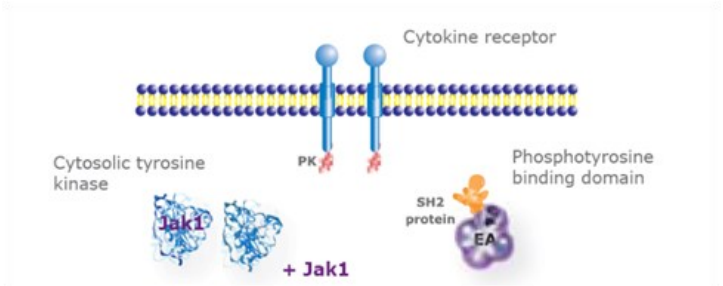


Figure 1. Path-Hunter Cytosolic Tyrosine Kinase Assay Principle.

FREQUENTLY ASKED QUESTIONS

Q: I did not see a signal with my control agonist.
A1: There may be differences in agonist purchased from different vendors.
A2: Confirm that the control agonist used is the same ligand used in the dose response shown in the provided cell-specific data sheet.
A3: Please check if appropriate compound diluent was used.

Q: I did not see a response with my compound.
A1: The concentration of DMSO or Ethanol used for dilution is too high. Maintain concentration of the agonist/antagonist diluent at ≤ 1%.
A2: Confirm that the final ligand concentration is correct. Some ligands are "sticky" and difficult to dissolve.
A3: Confirm that the cell line responds to the control agonist.
A4: Repeat the experiment using a new lot of control agonist.
A5: Please check if appropriate compound diluent was used.

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

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

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

Q: How long is the signal stable for?
A: The signal is stable for 2-3 hours after addition of detection reagent.

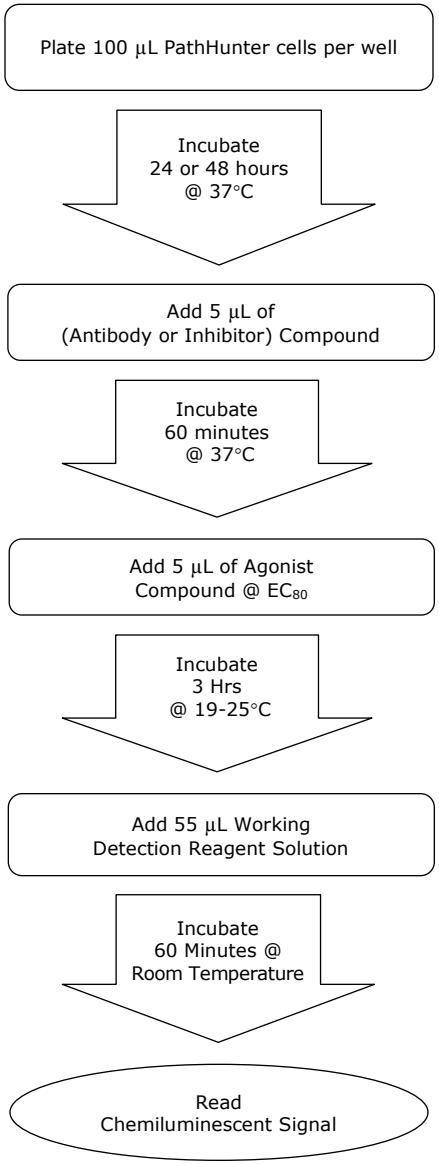
Q: My cells are floating after the 24 hours incubation.
A: The cells are not viable, contact technical support for a replacement.

Q: I ran out the cell plating reagent, can I buy it separately.
A: Yes, the reagents are available, please see page 15 for more details.

Q: How do I generate a dose curve for my agonist compound?
A: Prepare eleven serial 4-fold dilutions of the agonist compound in the desired vehicle of choice. Prepare agonist dilutions such that the concentration is **11X** of the final screening concentration (10 µl agonist + 100 µl cells). Pipette all compound dilutions following the plate map on page 14.

Q: What protocol can I use for antibody studies?
A: You can use the inhibitor protocol for a neutralizing antibody. For activating antibodies, agonist protocol can be used and for Anti-Ligand antibody, a pre- incubation of ligand with the anti-ligand antibody is recommended for 15-30 minutes before the agonist incubation of 3 hours.

QUICK-START PROCEDURE : ANTAGONIST 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 1 vial containing 1 x 10^6 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). **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. Please use these components within 6 months of receipt of this kit.**

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 (page 6).

PATHHUNTER DETECTION REAGENTS AND CELL PLATING (CP) REAGENT: Store at -20°C

NOTE:
Once thawed, store the PathHunter Detection Reagents and CP Reagent at 4°C. Avoid multiple freeze/thaw cycles.

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

MATERIALS PROVIDED
Each Shipment Contains:

Description	2-Plate Kit	10-Plate Kit
PathHunter eXpress Cytosolic Tyrosine Kinase Cells	2 vials 1 x 10^6 cells	10 vials 1 x 10^6 cells
PathHunter Detection Kit	200 dp	1000 dp
- Cell Assay Buffer - Substrate Reagent 1 - Substrate Reagent 2*	1 x 11.4 mL 1 x 3 mL 1 x 0.6 mL	1 x 57 mL 1 x 15 mL 1 x 3 mL
Cell Plating Reagent**	1 x 100 mL	2 x 100 mL
96-Well Tissue Culture Treated Plate	2 plates	10 plates

*Centrifuge vial before opening to maximize recovery.
**Cell Plating Reagent is recommended for thawing and plating the cells. It is not recommended for agonist ligand dilution.

ADDITIONAL MATERIALS REQUIRED (NOT PROVIDED)

The following additional materials are required but not provided:

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

MEDIA REQUIREMENTS

Each PathHunter eXpress Cytosolic Tyrosine Kinase Functional 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 - AGONIST DOSE RESPONSE

The following steps outline the procedure for performing an Agonist assay using the PathHunter eXpress cytosolic tyrosine kinase cells and PathHunter Detection Reagents. Although plate layouts and experimental designs will vary, we recommend performing a11-point dose curve using at least duplicate wells for each dilution. Refer to the plate map on page 14 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.

- Add 90 µl of the working concentration of antagonist compound to tube #1.
- Add 60 µL of diluent to subsequent tubes.
- Remove 30 µL of diluted compound from tube #1, add it to the second tube and mix. Label this as tube #2.
- Remove 30 µL of diluted compound from tube #2, add it to the third tube and mix. Label this as tube #3.
- Repeat this process 7 more times.
- **DO NOT** add antagonist compound to tube #11. Add only appropriate compound diluent. This sample serves as the no antagonist control and completes the dose curve.

  NOTE:
  If the compound diluent is DMSO, the final concentration of DMSO in each well should not exceed 1%.
- Repeat process when testing additional compounds.

3. Remove PathHunter eXpress cells (previously plated on day 1) from the incubator.
4. Transfer 5 µL from tubes 1-11 to each well according to the plate map on page 14. In addition, add 5 µL of compound dil to the wells in column 1.
5. Incubate for **60 minutes @ 37°C**.
6. Determine the EC_{80} concentration of the agonist from the agonist dose response curve (described on page 6).
7. Prepare the agonist compound in compound diluent (PBS + 0.1% BSA) at **22X** of the final screening concentration (i.e. 5 µL agonist compound will be used in a final volume of 110 µL).
8. Add 5 µL of agonist compound to each well. Add 5 µL of compound diluent to the no antagonist/no agonist wells (columns 1).
9. Incubate for 3 hours @ 19-25°C.

   NOTE:
   22°C was found to be optimum for this assay.

SUBSTRATE PREPARATION AND ADDITION

10. During the incubation period, prepare a working solution of the detection reagents for each plate by mixing the following reagents:
    - Cell Assay Buffer **5.7 mL** (19 parts)
    - Substrate Reagent 1 **1.5 mL** (5 parts)
    - Substrate Reagent 2 **0.3 mL** (1 part)

    NOTE:
    The working solution is stable for up to 8 hours at Room Temperature.
11. Add 55 µL of prepared detection reagent per well and incubate for 60 minutes at room temperature in the dark. **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
12. Read samples on any standard luminescence plate reader.
13. Use GraphPad Prism® or other comparable program to plot your antagonist dose response.

ASSAY PROCEDURE - ANTAGONIST DOSE RESPONSE

The following steps outline the procedure for performing an Antagonist assay using the PathHunter eXpress receptor tyrosine kinase cells and PathHunter Detection Reagents.

Although plate layouts and experimental designs will vary, we recommend performing a 10-point dose curve using at least duplicate wells for each dilution. Refer to the plate map on page 14 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 appropriate CP reagent in a 37°C water bath.
2. Remove cell vial(s) from -80°C or liquid N_2 vapor plate 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 least one minute before opening for any liquid N_2 inside the vial to evaporate.
3. Add 0.5 mL of pre-warmed CP reagent to the frozen cell pellet. Pipette up and down gently several times to ensure that cells are evenly distributed.
4. Immediately transfer the cells to 11.5 mL of CP reagent and pour into a disposable reagent reservoir.
5. Plate 100 µL of cells into each well of the provided 96-well tissue culture plate.
6. After seeding the cells into the microplate, place it in a 37°C, 5% CO_2 humidified incubator for 24 hours before testing.

DAY 2 or Day 3*: COMPOUND ADDITION
*** DEPENDING ON WHETHER THIS IS A 24 HR ASSAY OR 48 HR ASSAY**

1. Dissolve inhibitor/antagonist compound in the vehicle of choice (DMSO, Ethanol, water or other) at the desired concentration.
2. Prepare 3-fold serial dilutions of antagonist compound in appropriate diluent (DMSO, ethanol, PBS or other). The concentration of each dilution should be prepared at **22X** of the final screening concentration (i.e. 5 µL antagonist compound will be used in a final volume of 110 µL). For each dilution, the final concentration of solvent should remain constant.
   Guidelines for preparation of 10-point dose curve serial dilutions:
   - Label tubes 1 through 11.
   - Prepare a working concentration of antagonist compound in compound diluent.
     NOTE:
     We recommend starting with a concentration that is **50X** the expected IC_{50} value for the compound (**1100X** the final screening concentration).

2. Remove cell vial(s) from -80°C or liquid N_2 vapor plate 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. Place the cell vial(s) ***briefly*** (10 seconds to 1 min) in a 37°C water bath until only small ice crystals remain and the cell pellet(s) is almost completely thawed.
4. Add 0.5 mL of pre-warmed CP reagent (37°C) to the cell vial. 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 in a 37°C, 5% CO_2 humidified incubator for 24 or 48 hours prior to testing.

DAY 2 or Day 3*: COMPOUND ADDITION
*** DEPENDING ON WHETHER THIS IS A 24 HR ASSAY OR 48 HR ASSAY**

1. Dissolve agonist compound in the vehicle of choice (Usually these are peptide ligands and one can resuspend the ligand in PBS + 0.1% BSA to make up a 100 ug/ml stock solution).
   NOTE:
   Mix solution thoroughly and vortex to ensure complete resuspension of the ligand. Centrifuge the ligand solution for 30 seconds on a regular bench top centrifuge to ensure proper dissolution of the ligand. The ligand is now ready for use. If longer storage is required, aliquoting to smaller volumes are recommended. Store ligand solution as aliquots at -20°C.
2. Prepare 4-fold serial dilutions of agonist compound in CP reagent containing the appropriate solvent. The concentration of each dilution should be prepared at **11X** of the final screening concentration (i.e. 10 µL compound + 100 µL of cells). For each dilution, the final concentration of solvent should remain constant.
   Guidelines for preparation of 11-point dose curve serial dilutions:
   - Label tubes 1 through 12.
   - Prepare a working concentration of agonist compound in PBS+0.1% BSA reagent.
     NOTE:
     We recommend starting with a concentration that is **50X** the expected EC_{50} value for the compound (**550X** the final screening concentration).
   - Add 100 µl of the working concentration of agonist compound to tube #1.
   - Add 75 µL of compound diluents to subsequent tubes.
   - Remove 25 µL of diluted compound from tube #1, add it to the second tube and mix. Label this as tube #2.
   - Remove 25 µL of diluted compound from tube #2, add it to the third tube and mix. Label this as tube #3.
   - Repeat this process 8 more times.
   - **DO NOT** add agonist compound to tube #12. Add only appropriate compound diluent. This sample serves as the no agonist control and completes the dose curve.
   - Repeat process when testing additional compounds.

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 page 14.
5. Incubate for 3 hours @ 19-25°C.

NOTE:
22°C was found to be optimum for this assay.

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:
   - Cell Assay Buffer **5.7 mL** (19 parts)
   - Substrate Reagent 1 **1.5 mL** (5 parts)
   - Substrate Reagent 2 **0.3 mL** (1 part)

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

7. Add 55 µL of prepared detection reagent per well and incubate for 60 minutes at room temperature in the dark (plates can be covered with a foil) **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
8. Read samples on any standard luminescence plate reader.
9. Use GraphPad Prism® or other comparable program to plot your agonist dose response.

QUICK-START PROCEDURE: AGONIST DOSE RESPONSE