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PathHunter® eXpress Nuclear Translocation Assays

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 at support@discoverx.com or techsupport@discoverx.com.

NOTES:

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LEGAL SECTION

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INTENDED USE

PathHunter eXpress Nuclear Translocation assay kits provide a robust, highly sensitive and easy-to-use, cell-based functional assay for monitoring target protein translocation into the nucleus upon activation of the pathway. The eXpress kits contain everything needed for a complete 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 analysis.

TECHNOLOGY PRINCIPLE

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. The PathHunter Nuclear Translocation assay format presented here measures translocation of the target protein into the nucleus. The cells have been engineered to express two complementing fragments of β-Gal within different cellular compartments.

In this assay, the translocating protein is tagged to the ED fragment and the EA fragment is localized in the nucleus. Pathway activation results in the ED-tagged protein translocation into the nucleus. This results in the complementation of the two enzyme fragments that form active β-galactosidase enzyme. Enzyme activity is then quantitatively detected using the chemiluminescent substrate in the PathHunter Detection Kit. This assay is suitable for screening, profiling or characterizing NRF2 activators, ARE inducers or NRF2 inhibitors.

STORING & REMOVING CRYOVIALS FROM LIQUID NITROGEN

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

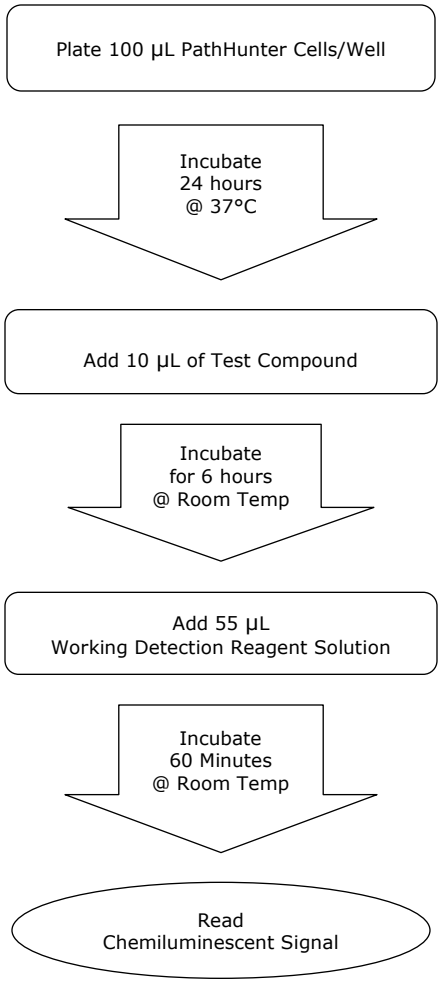
SHELF LIFE:
Use kit within 6 months from the date of receipt under proper storage conditions.

PATHHUNTER EXPRESS CELLS

STORAGE:
Short term (2 weeks or less): **Store vials at -80°C immediately upon arrival.**
Long term (greater than 2 weeks): **Place vials in the vapor phase of liquid nitrogen (N_2).**

PathHunter eXpress Nuclear Translocation cells arrive frozen on dry ice. Cells are delivered in individual vials containing 1 x 10^6 cells in 100 µL of freezing medium. Each vial contains sufficient cell numbers to generate (1) 96-well microplate prepared at the seeding density described. 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).

QUICK-START PROCEDURE: COMPOUND DOSE RESPONSE



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 (The example shown is for 1 plate, adjust volume accordingly for multiple plates):

Cell Assay Buffer	4.75 mL (19 parts)
Substrate Reagent 1	1.25 mL (5 parts)
Substrate Reagent 2	0.25 mL (1 part)

NOTE: PREPARE THE WORKING SOLUTION BEFORE USE AND STORE 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.

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.

SAFETY WARNING: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. Some cryovials can leak when submerged in liquid N_2. Upon thawing, the liquid N_2 present in the cryovial converts back to its gas phase which can result in the vessel exploding.

PATHHUNTER DETECTION REAGENTS AND CP REAGENT: Store at -20°C

Once thawed, store the Cell Plating (CP) Reagent at 4°C. Avoid multiple freeze/thaw cycles. In rare instances, the CP Reagent may be yellow in color after thawing. Although this indicates a slight change in pH, continue with the assay as this does not impact assay performance.

Thaw the PathHunter Detection Reagents at room temperature before use, and after thawing, store reagents for up to 7 days at 4°C. The reagents can tolerate up to three freeze-thaw cycles with no impact on performance. Once made, the working solution is stable for 24 hours at room temperature.

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

MATERIALS PROVIDED

Description	2-Plate Kit	10-Plate Kit
PathHunter eXpress Cells	2 vials 1 x 10^6 cells	10 vials 1 x 10^6 cells
PathHunter Detection Kit - Cell Assay Buffer - Substrate Reagent 1 - Substrate Reagent 2*	200 dp 1 x 11.4 mL 1 x 3 mL 1 x 0.6 mL	1000 dp 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)

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

RECOMMENDED MATERIALS

The following products* are recommended:

- CytoTracker™ LDH Quantification Kit (DiscoveRx, Cat. # 92-2002)
- CytoTracker™ Glutathione Quantification Kit (DiscoveRx, Cat. # 92-2003)
- CytoTracker™ DNA Damage Quantification Kit (DiscoveRx, Cat. # 92-2004M)

* Products not available in all countries. Please inquire.

MEDIA REQUIREMENTS

Each PathHunter eXpress 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 - COMPOUND DOSE RESPONSE

The following steps outline the procedure for performing compound dose response using the PathHunter eXpress Nuclear Translocation cells and PathHunter 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 subsequent pages 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 plate storage and place immediately on dry ice prior to thawing. **DO NOT EXPOSE VIALS TO ROOM TEMPERATURE.**
3. **DO NOT USE WATERBATH TO THAW THE CELLS.** 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.
4. Immediately transfer the cells to 11.5 mL of pre-warmed CP reagent, mix 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 or 48 hours* prior to testing.

* Refer to target specific datasheet for incubation time.

DAY 2: COMPOUND ADDITION

1. Dissolve agonist compound in the vehicle of choice (Usually these are small molecules, DMSO is the preferred solvent. Refer to the ligand datasheet for appropriate handling conditions).

   NOTE: MIX SOLUTION THOROUGHLY TO ENSURE COMPLETE RESUSPENSION OF THE LIGAND. IF LONGER STORAGE IS REQUIRED, ALIQUOTING TO SMALLER VOLUMES ARE RECOMMENDED. STORE LIGAND SOLUTION AS ALIQUOTS AT -20°C.

2. Prepare 3-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 compound in appropriate diluent.

   NOTE: WE RECOMMEND STARTING WITH A CONCENTRATION THAT IS **50X** THE EXPECTED EC_{50} VALUE FOR THE COMPOUND (**550X** THE FINAL SCREENING CONCENTRATION).

   - Add 75 µl of the working concentration of agonist compound to tube #1.
   - Add 50 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 plate containing PathHunter eXpress cells (previously plated on day 1) from the incubator.
4. Transfer 10 µL of the compound from tubes 1-12 to each well.
5. Incubate for the indicated time and temperature provided in the datasheet.