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PathHunter® EA Detection Kit

Chemiluminescence Detection

For Use with PathHunter Parental Cell Lines Expressing EA

User Manual: 93-0183

Simple Solutions for Complex Biology

CONTENTS

Read the entire product insert before beginning the assay.

For additional information or Technical Support, contact DiscoveRx or visit www.discoverx.com.

NOTES:

NOTES:

LEGAL SECTION

The use of this cell line may require third party licenses. Purchaser shall have the sole responsibility for obtaining any such licenses prior to use of the cell lines.

DiscoveRx name and logo are a trademark of DiscoveRx Corporation

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:

DiscoveRx Corporation
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TECHNOLOGY PRINCIPLE

PathHunter® products feature a novel *in vivo* application of the established Enzyme Fragment Complementation technology pioneered by DiscoveRx. In this approach, the complementing fragments of the b-galactosidase (b-gal) enzyme are expressed within different compartments of stably transfected, clonally derived cell lines. The larger portion of b-gal, termed EA for enzyme acceptor, is localized specifically within the nucleus (EA-Nuc) or the cytoplasm (EA-Cyto and EA-b-Arrestin) of the cell. The small 4kDa complementing fragment of b-gal, termed ProLabel or ProLink tag, is expressed as a fusion protein to a target of interest localized within the cytoplasm. (The expressed ProLabel/ProLink peptide is also sometimes called ED for Enzyme Donor). The target protein may be, for example a transcription factor, nuclear hormone receptor, or kinase. When a signaling pathway is activated, a cascade of events ensues, resulting in the translocation of the target protein to the nucleus. Upon translocation, the two fragments of b-gal complement, forming a functional enzyme capable of hydrolyzing a substrate molecule and generating a chemiluminescent signal.

INTENDED USE

The PathHunter EA Detection Kit is used with PathHunter Cell Lines that express the Enzyme Acceptor (EA) fragment of β-galactosidase, such as EA-Nuc, EA-Cyto, or EA-β-Arrestin Parental Cell Lines. This detection reagent formulation includes ED reagent, a small ProLabel-type peptide that complements with EA during assay incubation. The assay can be performed in both 96-well and 384-well microplate formats.

NOTE:
PathHunter Cell Lines that express *both* EA and ProLabel- or ProLink-tagged proteins are designed for use with the PathHunter® Detection Kit (93-0001).

STORAGE CONDITIONS

Upon receipt, store reagents at -20°C. Thaw reagents at room temperature before use, and after thawing, store reagents for up to 7 days at 4°C. The stability of the working solution once made is 24 hours at room temperature. The reagents can tolerate up to three freeze-thaw cycles with no impact on performance.

LIMITED USE LICENSE AGREEMENT

The cells and detection reagents (collectively Materials) purchased from DiscoveRx are expressly restricted in their use. DiscoveRx has developed a NHR assay (Assay) that employs genetically modified cells and vectors (collectively, the "Cells"), and related detection reagents (the "Reagents") (collectively referred to as "Materials"). By purchasing and using the Materials, the Purchaser agrees to comply with the following terms and conditions of this label license and recognizes and agrees to such restrictions:

1. Purchaser is permitted to use and propagate the Cells only for use in the Assay and in connection with Reagents purchased from DiscoveRx Corporation or its authorized distributor.
2. The Materials are not transferable and will be used only at the site for which they were purchased. Transfer to another site owned by Purchaser will be permitted only upon written request by Purchaser followed by subsequent written approval by DiscoveRx.
3. The Reagents contain or are based upon the proprietary and valuable know-how developed by DiscoveRx, and the Reagents have been optimized by DiscoveRx to function more effectively with the Cells in performing the Assay. Purchaser will not analyze or reverse engineer the Materials nor have them analyzed on Purchaser's behalf.
4. In performing the Assay, Purchaser will use only Reagents supplied by DiscoveRx or an authorized DiscoveRx distributor for the Materials.
5. Purchaser will not use the Cells with any other reagents or substrates, other than the Reagents that are provided by or purchased from DiscoveRx or an authorized DiscoveRx distributor, in connection with the Materials.
6. The number of Assays performed will not exceed the authorized number for which Materials were purchased.

If the purchaser is not willing to accept the limitations of this limited use statement and/or has any further questions regarding the rights conferred with purchase of the Materials, please contact:

DiscoveRx Corporation
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42501 Albrae Street, Suite 100
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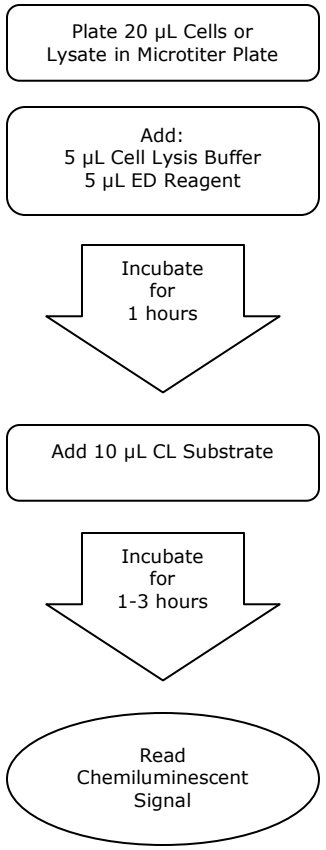
Agreements@discoverx.com

QUICK START PROCEDURE

In a White-Walled 384-well plate perform the following:



NOTE:
For best results, cell density or sample concentration and incubation times may need optimization.

MATERIALS PROVIDED

Each kit contains:

Product Code		**93-0183**
Number of Tests		**800**
Item	**Description**	**Volume**
1	Cell Lysis Buffer	4 mL
2	ED Reagent	4 mL
3	CL Substrate Diluent	6.1 mL
4	Substrate Reagent 2	0.32 mL*
5	Substrate Reagent 1	1.6 mL
6	Positive Control EA (6nM)	0.1 mL

NOTES:
PathHunter EA Detection Reagents are intended for use with PathHunter Cell Lines expressing EA. The kit is formulated for full-volume 384-well plates; however, a procedure is provided for 96-well format as well.

*Centrifuge the vial before opening to maximize recovery.

ADDITIONAL MATERIALS REQUIRED (NOT PROVIDED)

The following additional materials are required:

Equipment	**Materials**
• Pipettes and pipette tips. • 384-well or 96-well microplates (white-walled with clear bottom). • Multimode or luminescence plate reader.	• Cell line expressing EA. • Growth medium and supplements for cells.

GUIDELINES FOR USE

The reagents must be used with cells that contain EA to obtain a detectable signal. A positive EA control is included and should be diluted 1 part to 99 parts of culture medium. The assay can be run in 384-well or 96-well microplates. White plates are recommended and clear-bottomed plates can be used if desired. Assays should be run on fresh, low-passage cells that have not been allowed to reach confluency for more than 24 hours. The assay is performed in two steps: incubation of cells with lysis buffer and ED reagent followed by addition of CL substrate. The assay is compatible with standard cell culture media and reagents and so can be added directly to cells without washing. The compatibility of EFC detection reagents with other buffer components should be determined prior to testing. Assay signal will initially increase over time and will typically reach a maximum 3 hours after adding detection reagents. A stable signal should be generated for several hours thereafter. Samples can be read on any multimode or dedicated microplate reader that is properly configured for detection of glow-type luminescence.

DETECTION REAGENT PREPARATION

The CL substrate must be prepared as a working solution prior to use and is prepared by combining 1 part Substrate Reagent 2 to 5 parts Substrate Reagent 1 and 19 parts of CL diluent. Once prepared, the CL substrate is stable for at least 24 hours at room temperature with no impact on assay performance. Cell Lysis Buffer and ED Reagent should be used directly. Sufficient reagents are provided in each kit to perform the indicated number of assays in a 384-well microplate, assuming an addition of 5 µL of Lysis and ED Reagent and 10 µL CL Reagent to 20 µL of cells.

A positive EA control is included and should be diluted 1 part to 99 parts of culture medium. Use 20 µL of the diluted control per well of a 384-well plate. Add detection reagents as described below.

NOTE:
Do not substitute Lysis Buffer or CL Diluent with alternative buffers. These are formulated for optimal assay performance.

ASSAY PROCEDURE

The following assay procedure is designed for measuring total EA peptide expression in mammalian cells, such as EA-Nuc, EA-β-arrestin, or EA-Cyto Cell Lines, to verify the expression of EA in cell lines after shipping. Cell-based PathHunter functional assays, such as translocation or β-arrestin require using the PathHunter Detection Kit (DiscoveRx, Cat. # 93-0001 Series) and following the assay procedures provided in the appropriate PathHunter Cell Line product insert.

Step	96-well Plate	384-well Plate
Step 1: Add Cells*, Sample or Control	80 µL cells or sample in culture media	20 µL cells or lysate in culture media
Step 2: EFC	20 µL Lysis Buffer 20 µL ED Reagent	5 µL Lysis Buffer 5 µL ED Reagent
	Incubate 1 hour at room temperature	
Step 3: Detection	Add 40 µL CL Substrate	Add 10 µL CL Substrate
Read chemiluminescent signal after 1-4 hours		

*Cells may also be allowed to adhere overnight before performing the assay; however, this step is not typically required.

REPRESENTATIVE DATA AND DATA ANALYSIS

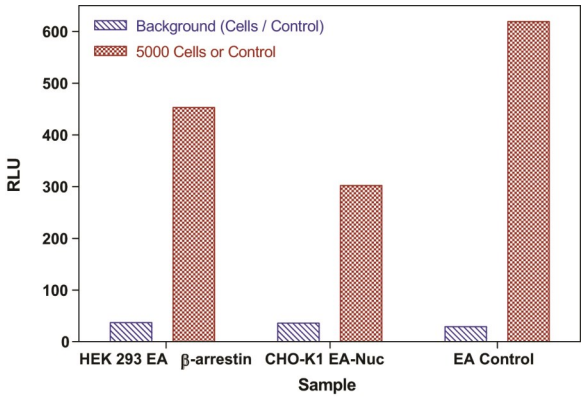


Figure 1. EFC activity of PathHunter Cell Lines.
The PathHunter HEK 293 EA-β-Arrestin Parental Cell Line and PathHunter CHO-K1 EA-Nuc Parental Cell Line were seeded at 5,000 cells per well into a 384-well plate. EFC activity was measured using the PathHunter EA Detection kit according to the assay procedure. A 10-fold increase in signal over background was observed and indicated good expression of EA in both cell lines. The EA positive control provided produced a comparable signal when used at a 100-fold dilution.