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PathHunter® ProLabel® Detection Kit

Chemiluminescence Detection

For Use with PathHunter Cell Lines Expressing ProLabel or ProLink™ Fusion Proteins

User Manual: 93-0180

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:

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LEGAL SECTION

This product and/or its use is covered by one or more U.S. and/or foreign patents, patent applications, and trade secrets that are either owned by or licensed to DiscoveRx Corporation. This product is for *in vitro* use only and in no event can this product be used in whole animals. The right to use or practice the inventions in the foregoing patents (including method of use claims) by using or propagating this product is granted solely in connection with the use of appropriate Detection Reagents (protected under trade secret) purchased from DiscoveRx Corporation or its authorized distributors.

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 <u>not</u> 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:

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TECHNOLOGY PRINCIPLE

PathHunter® products are designed for cell-based or *in vitro* applications of the established Enzyme Fragment Complementation technology pioneered by DiscoveRx. In this approach, a small 4 kDa complementing fragment of β-galactosidase (β-gal), called ProLabel® or ProLink™ tag, is expressed as a fusion protein with the target gene of interest. This tag enables detection of target protein expression in cells, lysates or purified protein after complementation with the larger portion of β-gal, termed EA for enzyme acceptor, to form functional β-gal. Enzyme activity is measured using a chemiluminescent substrate. The technology can be used to measure fusion protein expression *in vitro* or in mammalian cell-based assays.

INTENDED USE

The PathHunter® ProLabel Detection Kit is for use with PathHunter Cell Lines expressing ProLabel® (PL) or ProLink™ (PK) expression or cloning vectors. The kit measures the total amount of ProLabel- or ProLink-tagged protein expressed in cells. Functional assays using PathHunter Cell Lines expressing both a ProLabel- or ProLink-tagged protein and EA require the PathHunter Detection Kit (93-0001). The assay can be used in both 96-well and 384-well microplate formats.

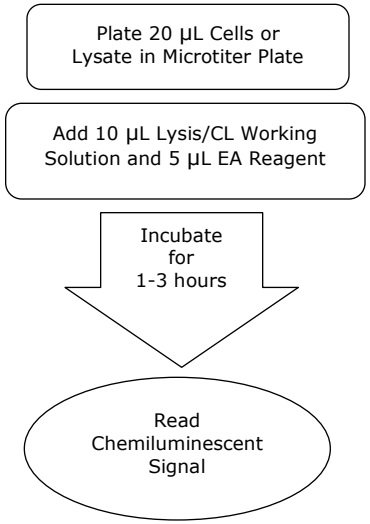
STORAGE CONDITIONS

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.

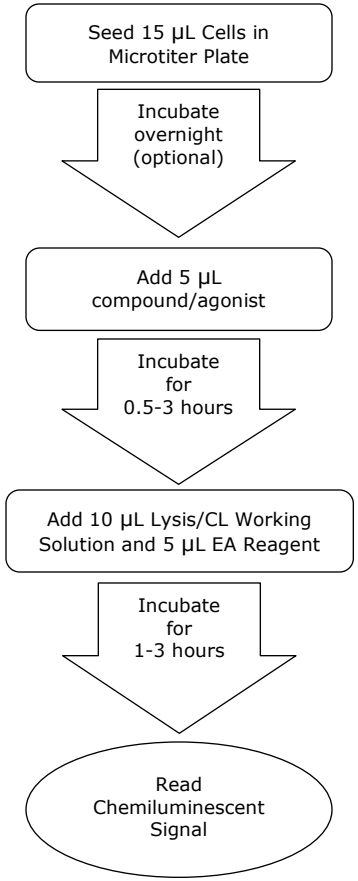
QUICK START PROCEDURE

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

ProLabel/ProLink Detection



Cell Induction Followed by ProLabel/ProLink Detection



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

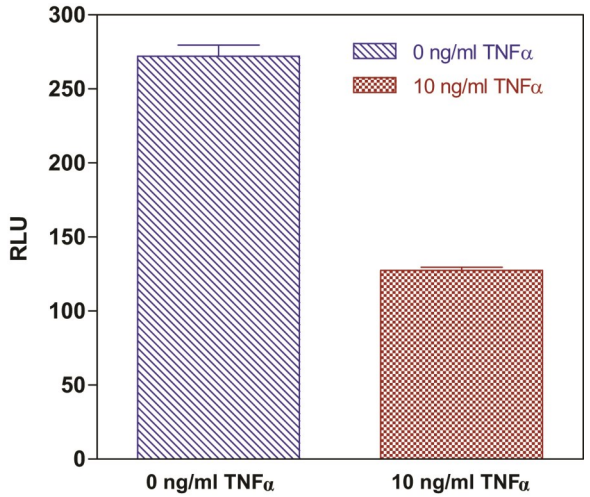


Figure 2. TNFα stimulated degradation of IκBα-PL in HeLa cells.
A HeLa cell line expressing IκBα-PL fusion protein was seeded into a 96-well plate at 10,000 cells per well and allow to adhere overnight. The cells were treated with 0 or 10 ng/mL TNFα for 30 minutes. IκBα-PL expression was measured by detecting EFC activity using the PathHunter ProLabel Detection Kit according to the assay procedure provided. Compared to control assays, a two-fold reduction in EFC activity was observed with 10 ng/mL TNFα.

MATERIALS PROVIDED

Each Detection Kit contains:

Product Code		**93-0180**	**93-0180 L**	**93-0180 XL**
Number of Tests		**800**	**10,000**	**40,000**
Item	**Description**	**Volume**	**Volume**	**Volume**
1	Cell Lysis Buffer	4 mL	50 mL	200 mL
2	EA Reagent	4 mL	50 mL	200 mL
3	CL Substrate Diluent	3.0 mL	38 mL	152 mL
4	Substrate Reagent 2	0.16 mL*	2 mL	8 mL
5	Substrate Reagent 1	0.8 mL	10 mL	40 mL
6	Positive Control Peptide	0.1 mL	0.5 mL	1 mL

NOTES:
PathHunter® ProLabel Detection Reagents are intended for use with PathHunter Cell Lines expressing ProLabel or ProLink-tagged proteins. Please read the Limited Use License for more information regarding restrictions on the number of tests that may be performed. 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 or sample containing ProLabel- or ProLink-tagged protein. • Growth medium and supplements for cells.

GUIDELINES FOR USE

The detection reagents must be used with cells or samples that express a ProLabel- or ProLink-tagged protein in order to obtain a detectable signal. A Positive Control Peptide is included and should be diluted 1 part to 49 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 by the addition of Lysis Buffer/CL Substrate Working Solution followed by addition of EA reagent to cells or protein sample. For cell-based EFC detection, the assay is compatible with standard cell culture media and reagents and can be added directly to cells without washing. For detection of EFC activity in protein samples, samples can be diluted in PBS, if necessary, prior to adding detection reagents. The compatibility of EFC detection reagents with 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, after which signal should be stable for several hours. Samples can be read on any multi-mode or microplate reader that is properly configured for detection of glow-type luminescence.

DETECTION REAGENT PREPARATION

Detection reagents must be prepared as working solutions prior to use. Once prepared, the working solutions are stable for at least 24 hours at room temperature with no impact on assay performance. The Lysis/CL Working Solution consists of the substrate and co-factors for chemiluminescence signal detection and the reagents necessary for cell lysis. Lysis/CL Working Solution is prepared by combining 1 parts Substrate Reagent 2 with 5 parts Substrate Reagent 1, 19 parts of CL diluent and 25 parts Cell Lysis Buffer. EA reagent should be used directly. Sufficient reagents are provided in each kit to perform the indicated number of assays in a 384-well, full-volume microplate, assuming an addition of 10 µL of Lysis/CL Working Solution and 5 µL EA reagent to 20 µL of cells or sample.

A Positive Control Peptide is included and should be diluted 1 part to 49 parts of culture medium or buffer. Use 20 µL of the diluted control per well of a 384-well plate. Add detection reagents as described below.

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

ASSAY PROCEDURE

The following assay procedure is designed for measuring total ProLabel-tagged fusion protein expression in mammalian cells or samples. If test compound addition is required at Step 1, the volume of cells added should be adjusted accordingly. (See the Quick Start procedure on page 8 for more information). Cell-based PathHunter functional assays require

the PathHunter Detection Kit (93-0001) and should be conducted using 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 or buffer	20 µL cells or sample in culture media or buffer
Step 2: Detection	Add 40 µL Lysis/CL Working Solution and 20 µL EA Reagent	Add 10 µL Lysis/CL Working Solution and 5 µL EA Reagent
Read chemiluminescent signal after 1-3 hours		

*Cells may also be allowed to adhere overnight before performing the assay; however, this step is not typically required. The optimal number of cells per well to use will vary depending upon cell type and must be determined experimentally.

REPRESENTATIVE DATA AND DATA ANALYSIS

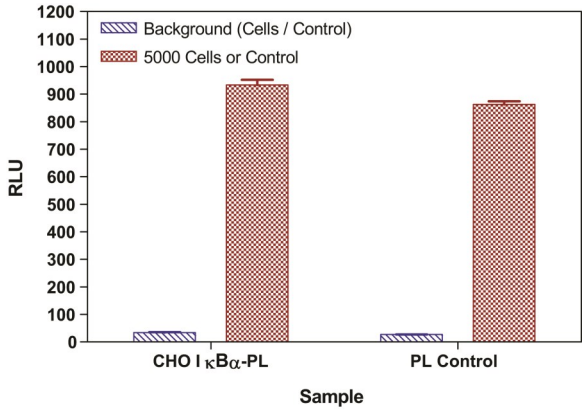


Figure 1. EFC activity of CHO-K1 Cell Line expressing IκBα-ProLabel fusion protein.
A CHO-K1 cell line expressing IκBα-PL fusion protein was seeded into a 384-well plate at 5,000 cells per well. EFC activity was measured using the PathHunter ProLabel Detection Kit according to the assay procedure. A 30-fold increase in signal over background was observed and indicated good expression of the fusion protein in the cells. The PL provided control peptide generated a comparable EFC signal at a 50-fold dilution.