DiscoverX

User Manual

PathHunter®

Insulin Glargine Bioassay Kit

For Chemiluminescent Detection of Insulin Glargine Activity

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Please read this entire user manual before proceeding with the assay.
For additional information or Technical Support, see contact information below.

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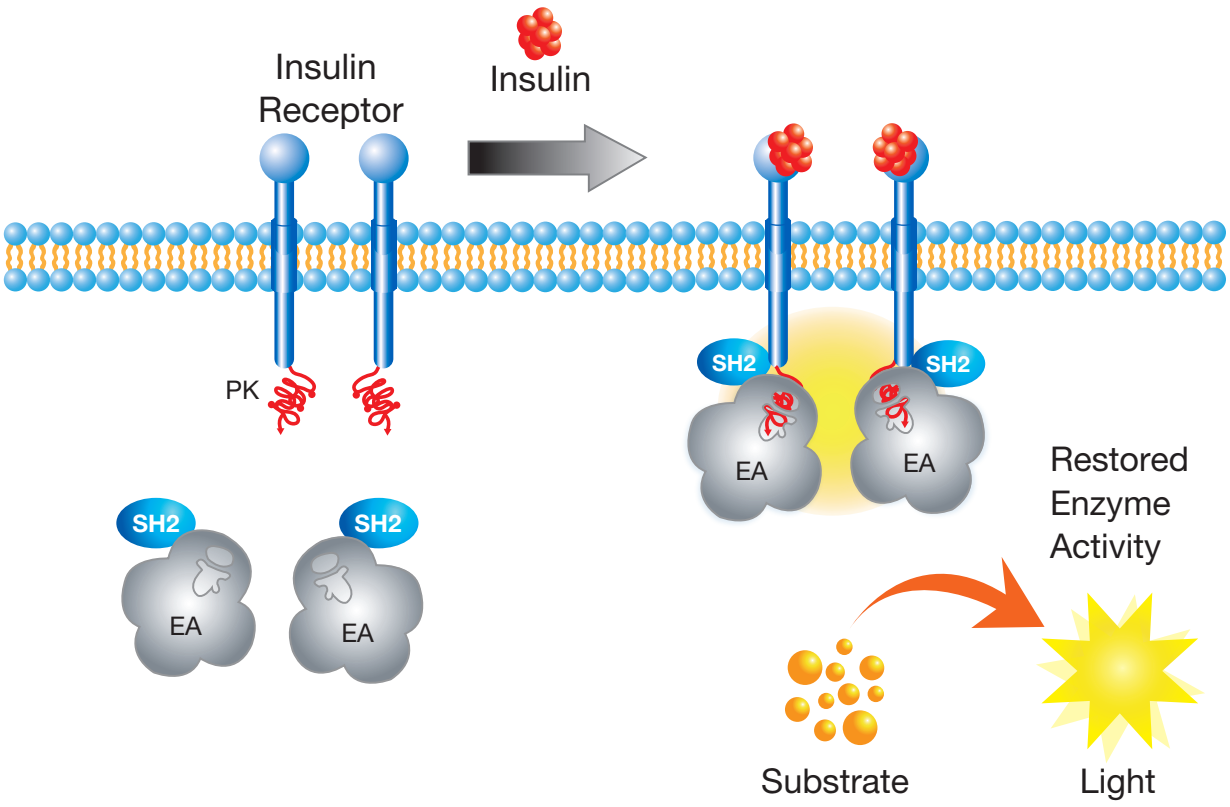
Overview

PathHunter Insulin Glargine Bioassay kits provide a robust, highly sensitive and easy-to-use, cell-based functional assay to study insulin potency and neutralizing antibodies. The Bioassay kits contain all the reagents needed for a complete assay including cells, detection reagents, cell plating reagent, positive control agonist, dilution buffer, and assay plates. The pre-qualified, frozen cells have been manufactured for single use and are provided in a ready-to-assay format that saves time and adds convenience.

Technology Principle: PathHunter Insulin Glargine Bioassay

These assays utilize Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, ProLink™ (PK) and Enzyme Acceptor (EA). Independently these fragments have no β-gal activity; however, when forced to complement through protein-protein interactions, they form an active β-gal enzyme.

In the PathHunter assay approach for tyrosine kinases, the ProLink tag is fused to the C-terminus of the receptor. The EA is fused to a phosphotyrosine SH2 domain containing protein that is able to bind the activated RTK. Ligand-induced activation of the receptor results in receptor phosphorylation. The SH2-EA fusion protein binds the phosphorylated receptor, forcing complementation of PK and EA to form an active β-gal enzyme. β-gal enzymatic activity is quantitatively measured using a chemiluminescent substrate in the PathHunter Bioassay Detection Kit.



Materials Provided

List of Components	93-0466Y3-00011	93-0466Y3-00012
PathHunter U2OS INSRb Bioassay Cells	2 vials	10 vials
PathHunter Bioassay Detection Kit		
• Detection Reagent 1 (mL)	2	10
• Detection Reagent 2 (mL)	8	40
AssayComplete™ Cell Plating Reagent 5	2 X 100 mL	4 X 100 mL
Control Agonist (Insulin)	1 vial	1 vial
96-well Clear-Bottom TC Treated, Sterile Plates w/lid	2 plates	10 plates

Storage Conditions

PathHunter U2OS INSRb Bioassay Cells

Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, thaw the vials as soon as possible upon receipt.

If continued storage of the frozen vials is necessary, store as follows:

- Short term (24 hours or less): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for more than 24 hours).
- Long term (greater than 24 hours): Vials should ONLY be stored in the vapor phase of liquid nitrogen.

Safety Warning: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. The manufacturer of the cryovial recommends storing the vials in the vapor phase above the liquid nitrogen. Upon thawing, if liquid nitrogen is present in the cryovial, it rapidly converts back to its gas phase which can result in the explosion of the vial upon its removal from the liquid nitrogen tank.

PathHunter Bioassay Detection Kit

Store at -20°C. Once thawed, the detection reagents can be kept at 4°C for up to 4 days. For longer storage (up to the expiration date listed in the kit certificate of analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles.

To make aliquots suitable for testing one assay plate each, 1 mL of Detection Reagent 1 per aliquot should be dispensed and frozen down. 4 mL of Detection Reagent 2 per aliquot should be dispensed and frozen down separately. Do not mix the two reagents during aliquoting.

AssayComplete Cell Plating Reagent 5 (CP5)

Once thawed, the Cell Plating Reagent can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed in the kit certificate of analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles. To make aliquots suitable for testing one assay plate each, 30 mL of reagent per aliquot should be dispensed and frozen down.

Recombinant Human Insulin Control Agonist

Store at -20°C until ready to use (up to the expiration date listed in the kit certificate of analysis). Centrifuge the vial prior to opening to maximize recovery. When ready to use, reconstitute to a concentration of 10 mg/mL by adding 1 mL of 0.01 N hydrochloric acid. Reconstituted ligand is stable for 1 week at 2-8°C. For longer storage (up to the expiration date listed on the kit Certificate of Analysis), it is recommended to store in working aliquots at -20 to -80°C.

96-well Tissue Culture Treated Plates

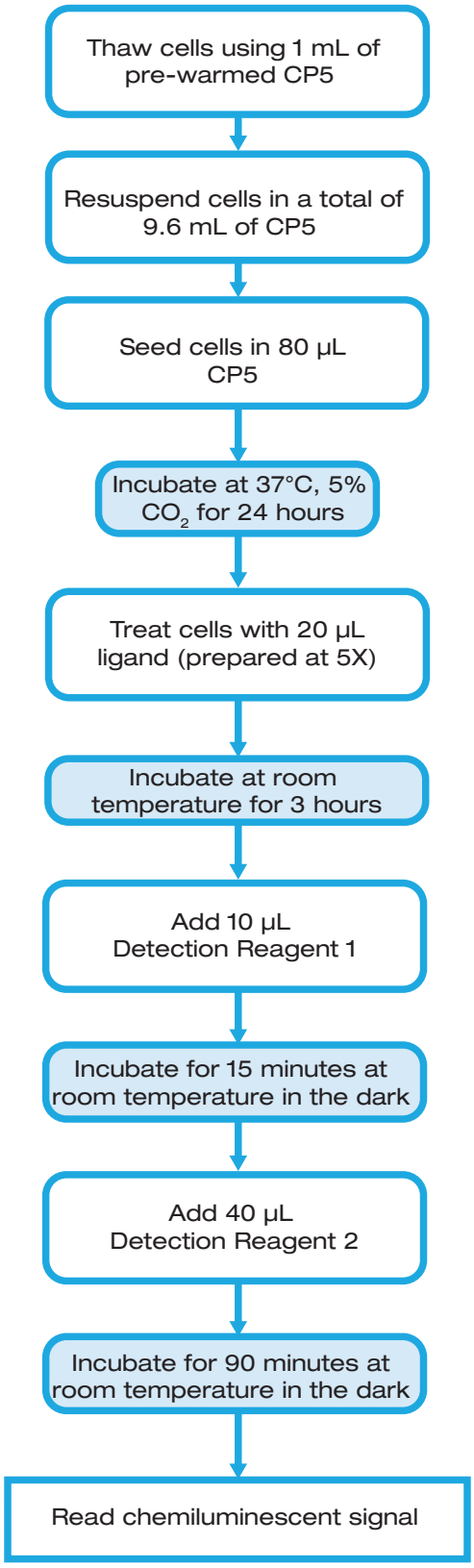
Store at room temperature.

Additional Materials Required

Material	Ordering Information
V-Bottom 96-well ligand dilution plates	92-0011
Multimode or luminescence plate reader	Refer to Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Single and multichannel micro-pipettors and pipette tips	
Disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar

Protocol Schematic

In a 96-well tissue culture treated plate provided in the kit, perform the following steps:



Detailed Protocol

Day 1: PathHunter Bioassay Cell Preparation:

The following protocol is for thawing and plating frozen PathHunter U2OS INSRb Bioassay Cells from cryovials.

1. Before thawing the cells, ensure that all the necessary materials required are set up in the tissue culture hood. This includes:
    a. One 25 mL reagent reservoir.
    b. One 15 mL conical tube.
    c. A micropipettor (P1000) set to dispense 1 mL.
    d. A multichannel pipette and tips set to dispense 80 µL.
    e. A bottle of Cell Plating Reagent 5 (CP5, pre-warmed in a 37˚C water bath for 15 minutes).
    f. A white-walled, clear-bottom 96-well assay plate.
2. Dispense 9.6 mL of cell plating reagent into the 15 mL conical tube.
3. Remove the cryovial from liquid nitrogen and immediately place in dry ice.

DO NOT use heated water bath to thaw the vial. Wipe down the cryovial quickly with 70% EtOH, and bring it into the tissue culture hood right away. DO NOT touch the sides or bottom of the vial to avoid thawing of the cell pellet through body heat.

4. Immediately transfer 1 mL (using P1000) of pre-warmed CP5 from the 15 mL conical tube to the cryovial, thawing the cell pellet. The medium should be added slowly along the side of the wall of cryovial tube. Mix the cells gently by pipetting up and down several times to break up any clumps. Transfer the cell suspension to the conical tube containing the remaining 8.6 mL of CP5. Remove any medium/suspension left in the tube to ensure complete recovery of all the cells from the vial.
5. Mix the tube by inversion to ensure the cells are properly mixed in the medium without creating any froth in the suspension and immediately pour the suspension into the 25 mL reservoir.
6. Add 80 µL of cells (20,000 cells/well) to each well of the 96-well assay plate using the multichannel pipette. Let the plate sit for 15 minutes at room temperature. This allows the cells to settle, to avoid potential edge effects. Then gently place the assay plate in a tissue culture incubator set to 37˚C and 5% CO_2. Allow the cells to recover for 18-24 hours before continuing.

Day 2: Sample Preparation

The following protocol is designed for testing purified biologics. The PathHunter assays can also be run in the presence of high levels of serum or plasma without significantly impacting assay performance. Therefore, standard curves of control can typically be prepared in neat serum or plasma and added directly to cells without further dilution. For the best results, the optimized minimum required dilution (MRD) of crude samples should be empirically determined.

1. Prepare the reference agonists (USP Insulin and DiscoverX Insulin) dose response curve. Agonists are prepared at 5X the desired final concentration as it will be diluted when 20 µL is added to 80 µL of media present in the assay plate.

    a. Prepare DiscoverX Insulin

        i. For the DiscoverX Insulin, add 1 mL of 0.01 N hydrochloric acid to the vial containing the 10 mg of lyophilized powder to make a 10 mg/mL stock solution.

        ii. Dilute the DiscoverX Insulin by adding 5 µL of the 10 mg/mL stock to 1.995 mL of 0.01 N hydrochloric acid. Mix thoroughly.

        iii. For the DiscoverX Insulin, add 235 µL of CP5 to well A1 of a master dilution plate (e.g. a V-bottom 96-well dilution plate, DiscoverX 92-0011). Add 15 µL of the diluted insulin to this well. Mix thoroughly by pipetting up and down several times. This results in an 1.5 µg/mL solution (5X the final 300 ng/mL curve top).

    b. Prepare USP Insulin

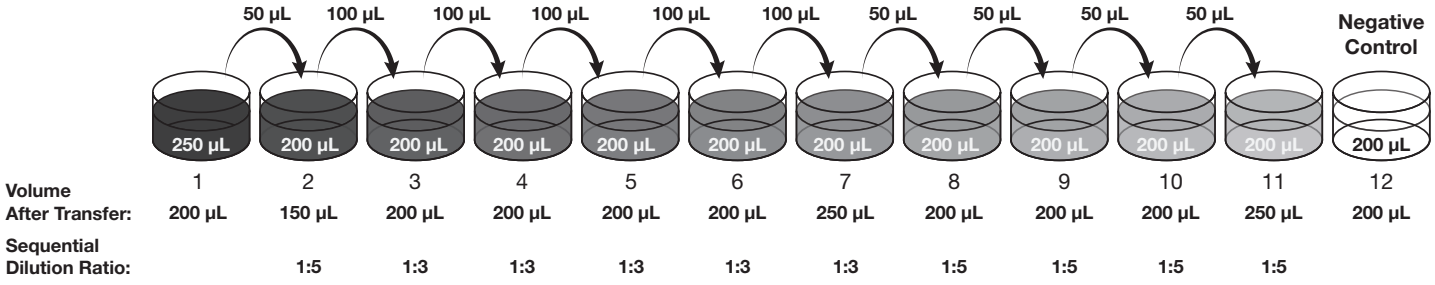
        i. For the USP Insulin, prepare 250 µL of USP Insulin in CP5 at a concentration of 300 ng/mL, 5X the top concentration of 60 ng/mL. Add 250 µL of this sample to well A1 on a different row than the DiscoverX Insulin.

    c. For both DiscoverX and USP Insulin, the following dilution process can be used. Add 200 µL of CP5 reagent to wells A2 to A12 of the master dilution plate.

    d. Using a clean tip, transfer 50 µL from well A1 into well A2 for a 1:5 dilution and mix by pipetting up and down several times.

    e. Replace the pipette tip, and transfer 100 µL from well A2 into well A3 for a 1:3 dilution. Mix by pipetting up and down several times. Repeat this process until well A7 is reached.

    f. Using a clean tip, transfer 50 µL from well A7 into well A8 for a 1:5 dilution. Mix by pipetting up and down several times and repeat this process until well A11 is reached. No insulin is transferred to well A12 as this will serve as a negative control.



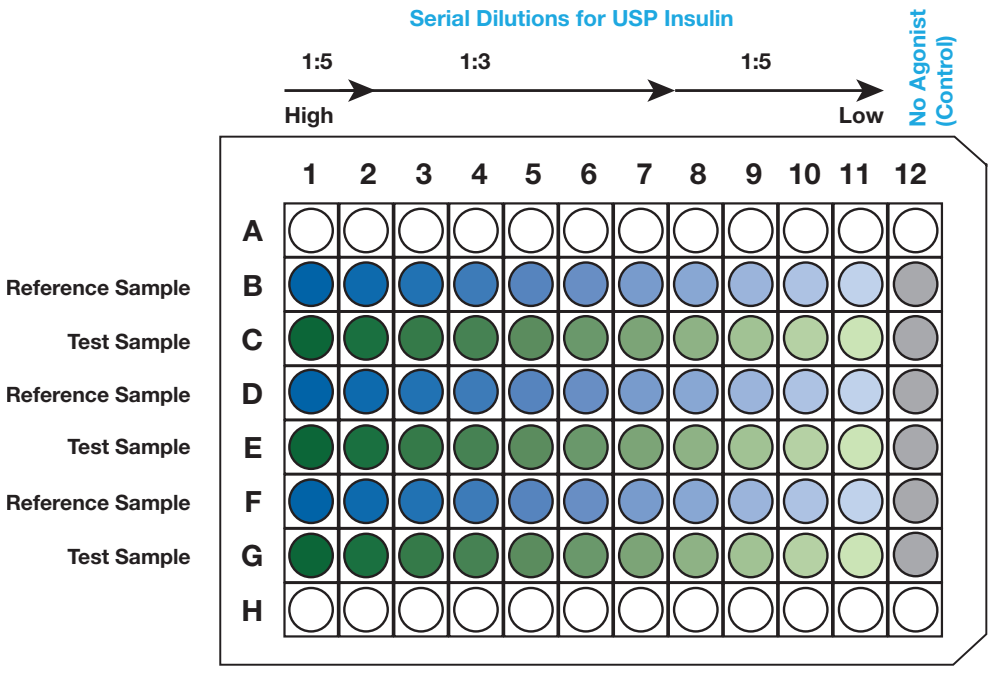
2. Remove the assay plate from the 37°C and 5% CO_2 incubator and bring into the tissue culture hood.
3. Add 20 µL from the DiscoverX Insulin and USP Insulin curves on the master dilution plate to the appropriate wells of the assay plate.
4. The assay plate is incubated for 3 hours at room temperature in the dark.

Day 2: Detection

1. Using a multichannel pipette, add 10 µL of Detection Reagent 1 to each well of the assay plate.
2. Incubate the plate at room temperature for 15 minutes in the dark.
3. Using a multichannel pipette add 40 µL of Detection Reagent 2 to each well of the assay plate.
4. Incubate the plate at room temperature for 90 minutes in the dark.
5. Read sample on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube (PMT) readers of 5-10 seconds for imager.

Note: For crude biologic samples, gently removing the liquid from all wells and replacing with 100 µL of Cell Plating Reagent before the addition of the detection reagents can result in higher signal. Additional Cell Plating Reagent is necessary for this method.

Representative Plate Maps



Serial dilution plate map. This plate map shows an 11 point dose curve with 3 data points at each concentration for one reference and one test sample per plate, with a variable serial dilution scheme.

Troubleshooting Guide

Problem	Cause	Solution
No response	Improper thawing procedure	Refer to thawing instructions in this user manual. Thawing process can have a significant effect on cell viability.
	Improper ligand used or improper ligand incubation time	See certificate of analysis for recommended ligand and assay conditions.
	Improper preparation of ligand (agonist or antagonist)	Refer to specific datasheet to ensure proper handling, dilution and storage of ligand.
	Improper time course for induction	Optimize time course of induction with agonist and antagonist.
Decreased response	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using microscope.
Low or no signal	Improper preparation of detection reagents	Detection reagents should ideally be prepared just prior to use and are sensitive to light.
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 0.1-1 seconds/well.
Experimental S:B does not match datasheet value	Incorrect incubation temperature	Confirm assay conditions. Check and repeat assay at correct incubation temperature as indicated on the assay datasheet.
	Improper preparation of ligand (agonist or antagonist)	Some ligands are difficult to handle. Confirm the final concentration of ligands.
EC_{50} is right-shifted	Improper ligand handling or storage	Make sure ligands are stored and incubated at the proper temperature.
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the certificate of analysis.
	Contamination from tips	Changing tips during serial dilutions can help to avoid carryover.
High variability between replicates	Instrument calibration	Ensure dispensing equipment is properly calibrated, and proper pipetting technique is used.

For additional information or technical support, please contact Technical Support listed below.

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