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User Manual

PathHunter® Panitumumab Bioassay Kit

For Chemiluminescent Detection of Panitumumab-mediated Inhibition of EGFR/ErbB2 Dimerization

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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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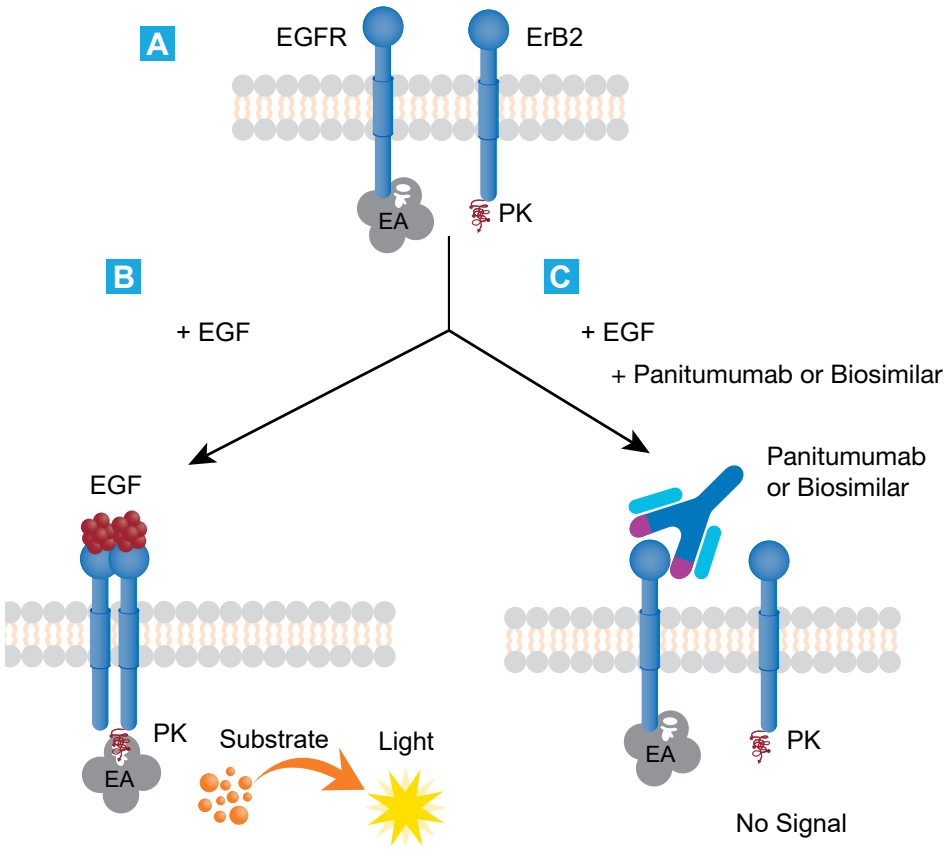
Overview

PathHunter Panitumumab Bioassay kits provide a functional, robust, highly sensitive, and easy-to-use cell-based assay to study panitumumab 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, and assay plates. The pre-validated, 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

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.

The PathHunter Panitumumab Bioassay is an application of the DiscoverX Dimerization Assay platform, which can be used to detect ligand-induced dimerization of two subunits of a receptor-dimer pair. This assay detects EGF-induced heterodimerization of the EGFR and ErbB2 receptors. The cells have been stably engineered to co-express ErbB2 fused to PK, and EGFR fused to EA. Activation of the EGFR receptor through EGF leads to receptor dimerization with ErbB2, which is an essential event in the receptor's signaling cascade. Receptor dimerization forces the two enzyme fragments to complement, resulting in the formation of a functional β-gal enzyme. The enzyme hydrolyzes a substrate to generate a chemiluminescent signal. Panitumumab binds to and inactivates the EGFR receptor preventing EGF-mediated heterodimerization of the receptors. This leads to an inhibition of the signaling event and therefore a reduction in signal.



Materials Provided and Storage Conditions

List of Components	93-1051Y3-00093	93-1051Y3-00094
PathHunter U2OS EGFR/ErbB2 Bioassay Cells*	2 vials	10 vials
PathHunter Bioassay Detection Kit	200 dp	1,000 dp
Detection Reagent 1	2 mL	10 mL
Detection Reagent 2	8 mL	40 mL
AssayComplete Cell Plating Reagent 5	1 X 100 mL	3 X 100 mL
Protein Dilution Buffer	1 X 50 mL	2 X 50 mL
Control Agonist (EGF)	1 vial	1 vial
96-Well Clear-Bottom TC Treated, Sterile Plates w/Lid	2 plates	10 plates

* Please discard the excess cells left in the reservoir, after seeding the plate.

PathHunter U2OS EGFR/ErbB2 Bioassay Cells

Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, store the vials of bioassay cells in vapor phase of liquid nitrogen as soon as possible upon receipt. Please contact technical support immediately, if the cells received were already thawed.

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's List of Components), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaws.

For the ten-plate kit, if all the plates will not be used at the same time, we recommend making five aliquots for each of the two detection reagents. Each aliquot will be adequate for two assay plates. Make five aliquots of 2.3 mL each for Detection Reagent 1, and five aliquots of 9.2 mL each for Detection Reagent 2. Sufficient reagent volumes are provided in the kit to make these aliquots.

If reagents will be used for a single plate, then the remaining Detection Reagents can be frozen. The detection reagents can be thawed and frozen for a total of three times without loss in performance.

AssayComplete Cell Plating 5 Reagent (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 on the kit's 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, 30mL of reagent per aliquot can be dispensed and frozen down.

Protein Dilution Buffer

Once thawed, the Protein Dilution Buffer can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed on 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, 10 mL of reagent per aliquot should be dispensed and frozen down. This amount may vary depending on your stock sample concentrations and should be adjusted accordingly.

Recombinant Human EGF Control Agonist

Store at -20°C until ready to use (up to the expiration date listed on the kit certificate of analysis). Centrifuge the vial prior to opening to maximize recovery. Reconstitute to a concentration of 100 µg/mL by adding 1000 µL of Protein Dilution Buffer. Reconstituted ligand is stable for 12 months at -20 to -80°C, or 1 week at 2-8°C. To make aliquots suitable for testing 1 assay plate, 10 µL of reconstituted EGF per aliquot should be dispensed and frozen down.

96-well Tissue Culture Treated Plates

Store at room temperature.

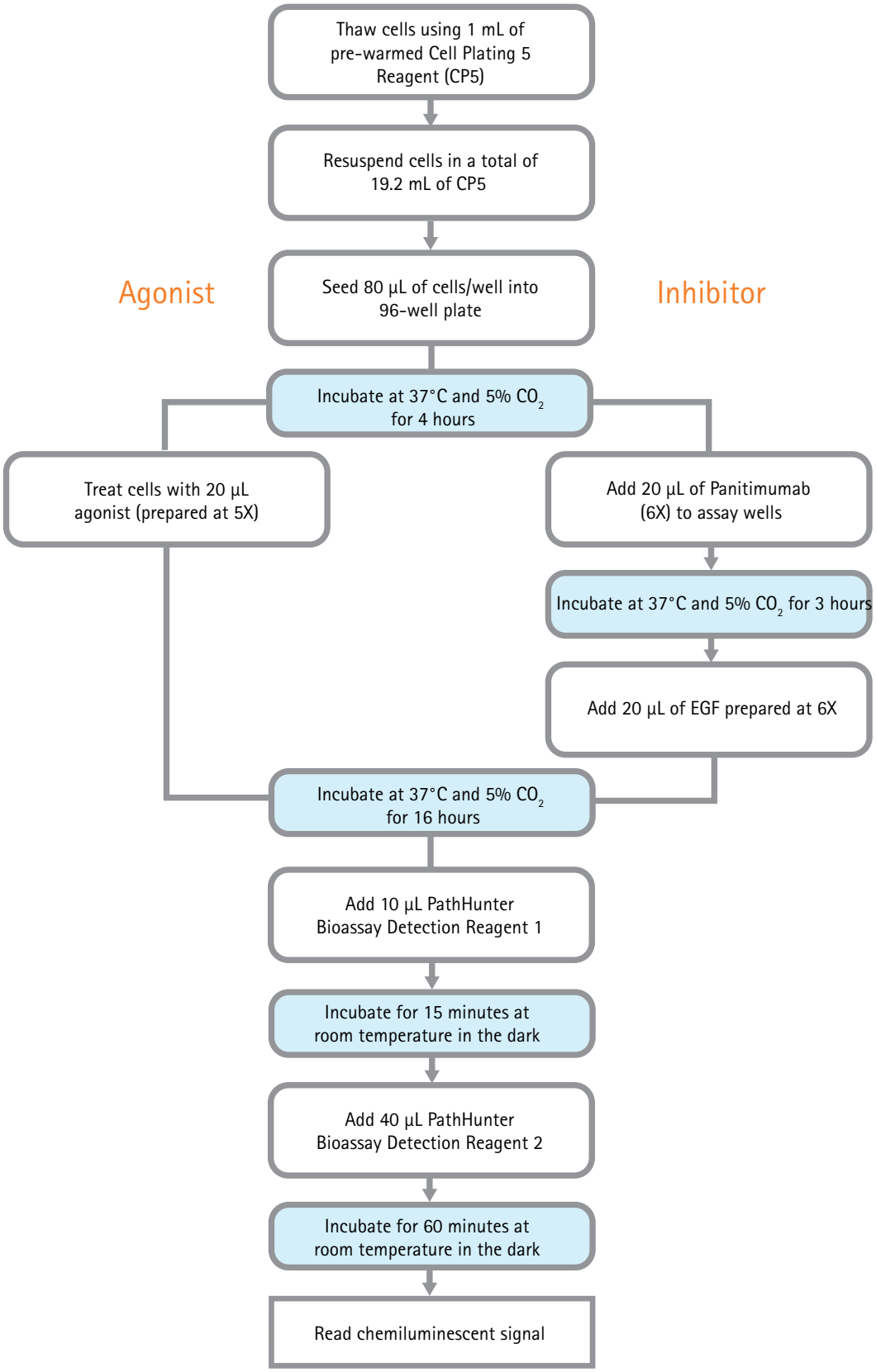
Additional Materials Required

The following equipment and additional materials are required to perform these assays:

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

Quick-Start Procedure: In a 96-well tissue culture treated plate, perform the following:



Panitumumab Bioassay Protocol

Day 1: PathHunter Bioassay Cell Preparation:

The following protocol is for thawing and plating frozen PathHunter U2OS EGFR/ErbB2 Bioassay cells from cryovials.

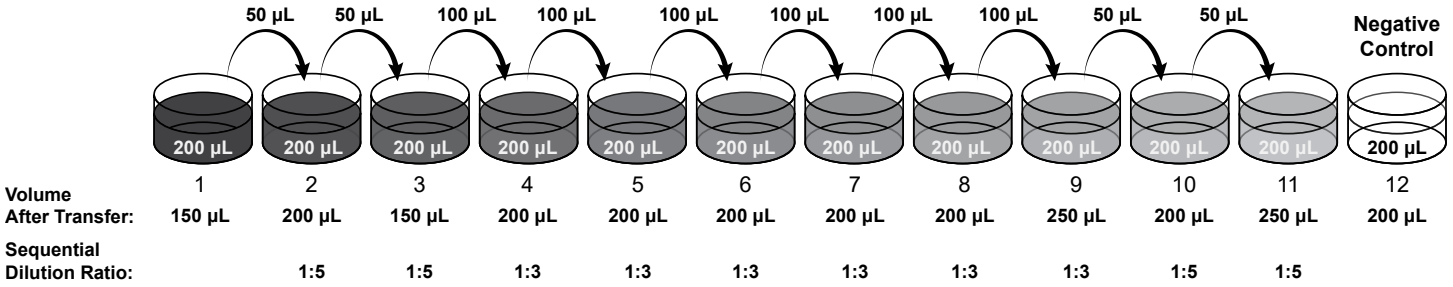
1. Before thawing the cells, ensure that all the necessary materials required are set up in the tissue culture hood. This includes:
    a. One 25 mL reagent reservoir
    b. One 50 mL conical tube
    c. A micropipettor (P1000) set to dispense 1 mL
    d. A multichannel pipette and tips set to dispense 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 19.2 mL of CP5 into the 50 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% ethanol, 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. Thaw the pellet by immediately adding 1 mL (using P1000) of pre-warmed CP5 from the 50 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.
5. Transfer the cell suspension to the conical tube containing the remaining 18.2 mL of CP5. Remove any medium/suspension left in the tube to ensure complete recovery of all the cells from the vial.
6. 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.
7. Add 80 µL of cells to each well of the 96 well assay plate using the multichannel pipette. Note: Discard the excess cells left in the reservoir after seeding the plate.
8. Let plate sit for 15 minutes at room temperature to allow cells to settle, avoiding the potential for edge effects.
9. Incubate for 4 hours at 37°C and 5% CO_2.

Day 1: Ligand 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 Panitumumab curve. Panitumumab is prepared at 6X the desired final concentration. Top dose: 50 µg/mL

    a. Add 200 µL of PDB to well B2 to B12 in a new row of the master dilution plate.

    b. Add 200 µL of Panitumumab prepared at 6X the desired final concentration (300 µg/mL) to column 1 of this row on the master dilution plate.

    c. Using a clean tip, transfer 50 µL from well B1 into well B2 for a 1:5 dilution and mix by pipetting up and down several times. Replace the pipette tip, and transfer 50 µL from well B2 into well B3 for a 1:5 dilution. Mix by pipetting up and down several times. Then transfer 100 µL from well B3 to well B4 for a 1:3 dilution. Repeat this process until well B9 is reached, then transfer 50 µL from well B9 to B10 and then from well B10 to B11 for a 1:5 dilution each. No antibody is transferred to 200 µL PDB of well B12 as this will serve as a negative control.

2. Add 20 µL from the Panitumumab curve on the master dilution plate to the appropriate wells of the assay plate.

3. Place the assay plate to the 37°C and 5% CO_2 incubator and incubate for 3 hours.

4. Prepare the reference ligand (DiscoverX EGF) dose response curve, which will serve as a positive control in this assay. EGF is prepared at 5X the desired final concentration as it will be diluted by adding to the 80 µL of media present in the assay plate.

    a. Add 200 µL of Protein Dilution Buffer (PDB) to wells A2 to A12 of a master dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate, DiscoverX 92-0011 or similar).

    b. Add 1000 µL of PDB to the EGF vial containing 100 µg of lyophilized powder to make a 100 µg/mL stock solution.

    c. Add 398 µL of PDB to an eppendorf tube. Add 2 µL of the 100 µg/mL EGF stock to this tube. Mix thoroughly by vortexing or pipetting up and down several times. This results in a 500 ng/mL solution (5X the final 100 ng/mL curve top). Transfer 200 µL of this 500 ng/mL solution from the Eppendorf tube to well A1 of the 96-well dilution plate.

    d. Using a clean tip, transfer 100 µL from well A1 into well A2 and mix by pipetting up and down several times. Replace the pipette tip, and transfer 100 µL from well A2 into well A3. Mix by pipetting up and down several times. Repeat this process until well A11 is reached, resulting in an eleven point, 1:3 dilution series. No ligand is transferred to column 12 as this will serve as a negative control.

5. Agonist challenge for biosimilar curves: The EC_{80} of the DiscoverX EGF was determined to be approximately 10 ng/mL. If EGF from a different vendor is used, the EC_{80} should be determined empirically prior to running samples. Prepare the agonist challenge at 6X the desired final concentration. For enough agonist challenge for a single biosimilar curve run in triplicate, dilute 120 µL of the 500 ng/mL solution prepared in Step 4c into an Eppendorf tube and add 880 µL of PDB. This provides 1 mL of 60 ng/mL EGF, 6X of 10 ng/mL final concentration.
6. Add 20 µL from the EGF reference curve on the master dilution plate to the appropriate wells of the assay plate.
7. Add 20 µL of EGF challenge prepared in Step 5 to all wells of the Panitumumab curve on the assay plate.
8. Place the assay plate to the 37°C and 5% CO_2 incubator and incubate overnight (16-18 hours).

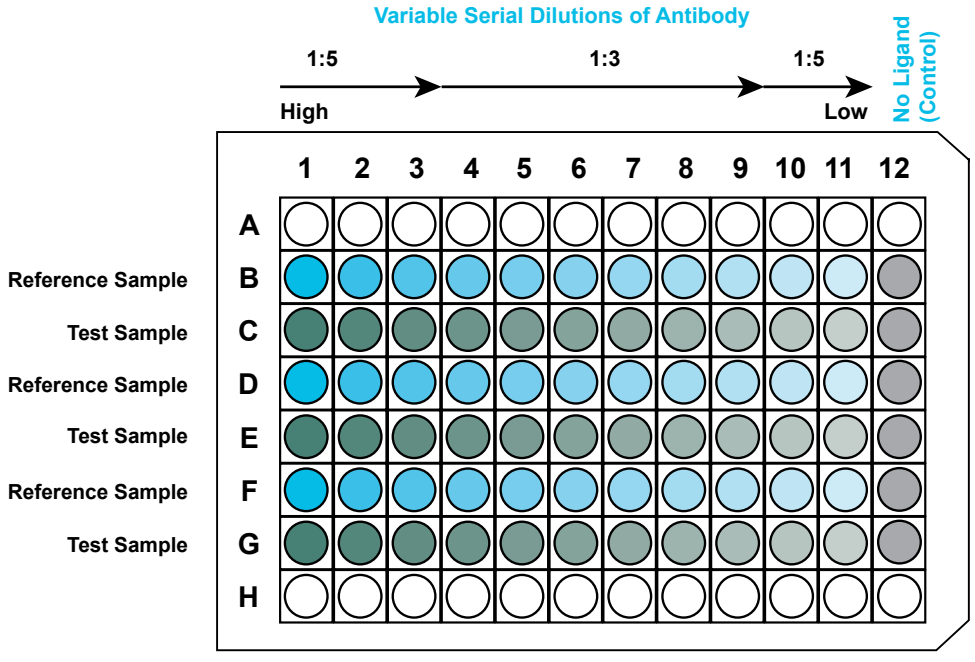
Day 2: Detection

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 1 hour in the dark.
5. Read sample on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube (PMT) readers or 5-10 seconds for imager.

PathHunter Bioassay Detection Reagents are light sensitive, thus incubation in the dark is necessary.

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.

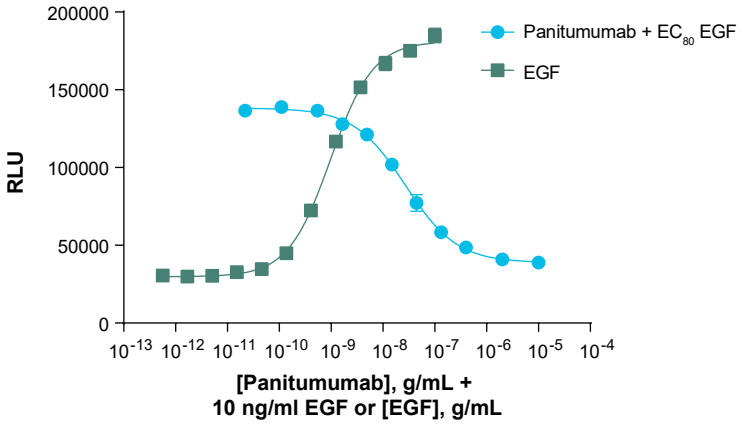
Representative Plate Map for Agonist/Inhibitor Curve



Assay 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.

Typical Results

The following graph is an example of a typical dose-response curve for the Panitumumab Bioassay, generated using the protocol outlined in this user manual. Data was acquired using the EnVision Microplate Reader (PerkinElmer).



Sample	IC_{50} / EC_{50} (ng/mL)	S/B
Panitumumab + 10 ng/mL EGF	27.2	3.7
EGF	0.98	6.0

Troubleshooting Guide

Problem	Cause	Solution
No Response	Incorrect thawing procedure	Refer to thawing instructions in this user manual as these cells are sensitive to proper thawing process
	Incorrect ligand used or improper ligand incubation time	See datasheet for recommended ligand and assay conditions
	Incorrect preparation of ligand (agonist or antagonist)	Refer to vendor-specific datasheet to ensure proper handling, dilution and storage of ligand
	Sub-optimal 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	Incorrect 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 second/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
	Incorrect 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	Check ligand handling requirements
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the Product Insert
	Problems with plate type and compound stability	Hydrophobic compounds should be tested for solubility and may be diluted in Protein Dilution Buffer
		Non-binding surface plates may be necessary for hydrophobic compounds

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

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