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PathHunter® RTK Bioassay Kit

For Research Use Only

Simple Solutions for Complex Biology

CONTENTS

NOTES:

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LEGAL SECTION

This product and/or its use is covered by one or more of the following U.S. patents #6,342,345 B1, #7,135,325 B2, #8,101,373 B2, #8,067,155 and/ or foreign patents, patent applications, and trade secrets that are either owned by or licensed to DiscoveRx® Corporation.

For some products/cell lines, certain 3rd party gene specific patents may be required to use the cell line. It is the purchaser's responsibility to determine if such patents or other intellectual property rights are required.

If the purchaser has any further questions regarding the rights conferred with purchase of the Materials, please contact:

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INTENDED USE

PathHunter® RTK (Receptor Tyrosine Kinase) Bioassay Kits are ready to use kits that contain everything to perform a functional assay with live cells, without any cell culture. This kit has been designed to rapidly detect the efficacy of your biologic or presence of neutralizing antibodies against the biologic. The Bioassay Kits include single use vials of frozen cells expressing the target RTK, chemiluminescent detection reagents, control agonist, optimized cell plating reagent, protein dilution buffer, and plates. Simply thaw and plate the pre-validated cells and treat with test reagents within 24 to 48 hours. Assays are designed for 96-well plate analysis.

TECHNOLOGY PRINCIPLE

PathHunter products monitor Receptor Tyrosine Kinase (RTK) activation, phosphorylation and recruitment of SH2 domain containing proteins using β-galactosidase (β-gal) enzyme fragment complementation (EFC, Figure 1). In this system, the intracellular C-terminus of the RTK of interest is fused with the small, 4 kDa complementing fragment of β-gal called ProLink™ and co-expressed in cells stably expressing a fusion protein of the SH2 domain of an adaptor protein and the larger, N-terminal deletion mutant of β-gal (called enzyme acceptor or EA). EA is fused to many different adaptor proteins containing phosphotyrosine binding domains (such as SHC1, SHC2, Grb2, PTPN6, PLCG1 or PLCG2), depending on the RTK target. Ligand-induced activation of the receptor causes either homo- or heterodimerization of the receptor which results in cross-phosphorylation (Figure 1). The SH2-EA fusion protein then specifically binds the phosphorylated receptor resulting in complementation of the two enzyme fragments, resulting in the formation of an active β-gal enzyme. This action leads to an increase in enzyme activity that can be measured using chemiluminescent Detection Reagents. These assays provide a direct, universal platform for measuring receptor activation, thus providing an ideal tool to identify and measure the potency of various biologics and neutralizing antibodies.

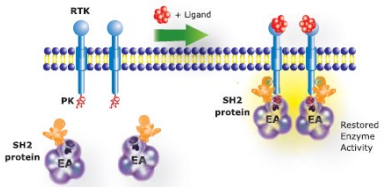


Figure 1. PathHunter® RTK (Receptor Tyrosine Kinase) Technology Principle

Activation of receptor tyrosine kinase results in dimerization and phosphorylation of RTK with subsequent interaction with SH2 protein. This interaction drives complementation of ProLink and EA epitopes generating active enzyme, which can be measured using a chemiluminescent substrate.

FREQUENTLY ASKED QUESTIONS

Q: What instruments can I use to read the plates?

A: Any bench top luminometer will work with the Bioassay kits. Below is a partial list of commercially available luminometers that have been used to validate our assays:

Turner Biosystems: Modulus Microplate
GE Healthcare Life Sciences: LEADseeker™, FarCyte™
BMG Labtech: PHERAstar Plus, LUMIstar Omega
Perkin Elmer: TopCount®, VICTOR II or V, Fusion, LumiCount, EnVision, MicroBeta® (Trilux), ViewLux, EnVision® Multilabel Reader
Molecular Devices: CLIPR™, LJL Acquest, LJL Analyst, LJL Analyst HT, LJL Analyst GT, Gemini, SpectraMax®, Flexstation™, LMax
Tecan: Ultra Evolution
Beckman Coulter – CRi
Berthold Technologies: Mithras LB 940
Hamamatsu: FDSS6000, FDSS/RayCatcher

Q: How long is the signal stable for?

A: The signal is stable for 24 hours after addition of detection reagent.

Q: Can I switch plates or should I use the plate provided?

A: You can use any clear bottom white or opaque walled plate.

Q: What if cells are not completely adherent after 24/48 hrs. incubation?

A: For certain targets, cells may not be completely adherent after 24 hours, but still greater than 80% viable. Please continue on with the protocol as described in the product insert.

Q: Why do longer incubation times with Detection Reagents lead to a higher signal?

A: The complemented ProLink and EA epitope generate active enzyme that is continually turning over the substrate. Theoretically, the signal continues to increase until the substrate is depleted. Therefore, the longer you incubate the reaction, the higher the RLU values.

Q: What if my CP reagent changes from a red/pink color to yellow after freezing/thawing?

A: If the CP reagent changes color from red/pink to yellow after thawing, please continue with the assay according to the product insert. We have observed this color change on rare occasions and have confirmed that it does not affect assay performance.

Q: Can the Bioassay kits be run in 384-well format?

A: All Bioassay kits are optimized and formatted to be run in 96-well plates. Certain assays also perform well in 384-well format but require modifications to the protocol (cell numbers, incubation times, etc.). Please contact Technical Support (techsupport@discoverx.com) for more information.

REPRESENTATIVE DATA

PathHunter RTK Bioassay Kits are able to detect neutralizing antibodies

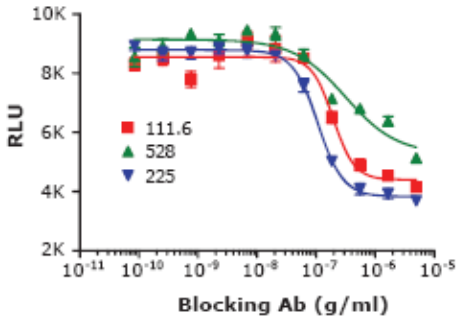


Figure 1. ErbB1 Bioassay Kit
Cells expressing human ErbB1 receptor were seeded in Cell Plating 17 Reagent and incubated with increasing concentrations of an anti-ErbB1 MAb, keeping agonist, human EGF, constant at the EC80 according to the protocol. ErbB1 Bioassay Kit is able to detect a dose dependent neutralization of receptor signaling as increasing concentrations of anti-ErbB1 mAb bind directly to the receptor and block EGF ligand signaling.

KIT CONTENTS AND STORAGE CONDITIONS:

PATHHUNTER BIOASSAY KITS COMPONENTS REQUIRE MULTIPLE STORAGE TEMPERATURES. OPEN BOXES IMMEDIATELY AND STORE CONTENTS AS INSTRUCTED.

Box 1: ASSAY-READY CELLS

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

Assay-ready cells arrive frozen on dry ice. 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 9). **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. The manufacturer of the cryovial recommends storing the vials in the vapor phase above the liquid N_2. Upon thawing, if liquid N_2 is present in the cryovial, it converts back to its gas phase which can result in the explosion of the vial upon its removal from the freezer.

Box 2A: BIOASSAY DETECTION REAGENTS: Store at -20°C

Box 2B: ASSAYCOMPLETE™ PROTEIN DILUTION BUFFER: Store at -20°C

BOX 2C: ASSAYCOMPLETE CELL PLATING REAGENT: Store at -20°C

Upon receipt, store reagents at -20°C. Once thawed, store the reagents for up to 7 days at 4°C. The reagents can tolerate up to three freeze-thaw cycles with no impact on performance. 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.

Box 3: CONTROL AGONIST: Store at -20°C

Upon receipt, store control agonist and reconstitution buffer (if provided) at -20°C. Avoid multiple freeze/thaw cycles. To avoid condensation, equilibrate the vial containing control agonist to ambient temperature before opening. Refer to the compound specific datasheet to identify a recommended working concentration and a reconstitution buffer required for the control agonist preparation.

Box 4: 96-WELL TISSUE CULTURE TREATED PLATES: Store at Room Temperature

MATERIALS PROVIDED

Description	Contents	Contents
Box 1: Assay-Ready Cells	2 vials $1.2x10^6$ cells/vial	10 vials $1.2x10^6$ cells/vial
Box 2A: Bioassay Detection Kit - Detection Reagent 1 - Detection Reagent 2 **Box 2B:** Protein Dilution Buffer **Box 2C:** Cell Plating Reagent[±]	 200 dp. 2.0 mL 8.0 mL 1 x 50mL 1 x 100mL	 1,000 dp. 10.0 mL 40.0 mL 2 x 50mL 3 x 100mL
Box 3: Control Agonist	1 vial	1 vial
Box 4: 96-well Tissue Culture Treated Plates	2 plates	10 plates

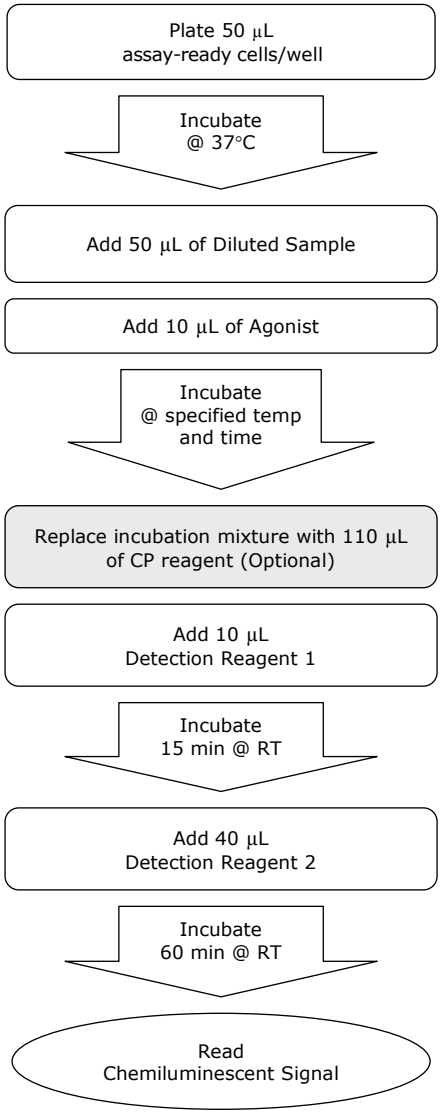
[±]Refer to cell-line specific data sheets for optimized Cell Plating Reagent included with each kit.

ADDITIONAL MATERIALS REQUIRED (NOT PROVIDED)

The following additional materials are required but not provided:

- Single- and multichannel micro-pipettors and pipette tips
- V-Bottom 384-well compound dilution plates
- Disposable Reagent Reservoir (Thermo Scientific, Cat. #8094 or similar)
- Hemacytometer
- Multimode or luminescence plate reader

QUICK-START PROCEDURE: UNPURIFIED ANTIBODY SAMPLES



*Please refer to the cell line specific datasheet for any variation in assay conditions.

g) Repeat this process 7 more times, preparing serial dilutions from right to left across the plate. **DO NOT add antibody to tubes #1 and 2.** These samples serve as the no antibody control and complete the dose curve.

h) Repeat process when testing additional antibodies.

i) Set antibody dilutions aside until they are ready to be added.

3. Remove assay ready cells (previously plated on day 1) from the incubator.
4. Transfer 50 µL of diluted antibody from tubes #1-12 to each well containing 50 µL of cells according to the plate map on page 18.
5. Incubate for 60 minutes @ 37°C.

AGONIST COMPOUND PREPARATION AND ADDITION

1. During the antibody incubation, determine the EC_{80} concentration of the agonist to be used in the assay. Prepare a **11X** EC_{80} concentration of agonist compound in CP reagent as shown below:
   Example: If the expected EC_{80} of the agonist compound is 10 nM, prepare a stock at 110 nM.
2. Add 10 µL of 11X agonist compound to each well. Add 10 µL of CP reagent to the no agonist wells (column 1).
3. Incubate for 3 hours @ room temperature (19 - 25ºC)*****.

NOTE:
*****Please refer to the cell line specific datasheet for any variation in assay conditions

ADDITION OF DETECTION REAGENTS

1. Add 10 µL of Detection Reagent 1 to the well.
2. Incubate the plate for 15 minutes at room temperature (19 - 25°C).
3. Add 40 µL of Detection Reagent 2 per well and incubate for 60 minutes at room temperature (19 - 25°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
4. Read samples on any standard luminescence plate reader.
5. Use GraphPad Prism® or other comparable program to plot your agonist dose response.

ASSAY INCUBATION AND CELL PLATING REAGENT REQUIREMENTS

Each Bioassay Kit has been validated for optimal assay performance at either 24 or 48 hours post-thaw. Although most targets perform similarly at both time points, for optimal assay performance we recommend you perform the assay according to the assay conditions provided in the cell line specific datasheet using the recommended time point, CP Reagent and control agonist.

Always use the CP Reagent and control agonist included in the kit and DO NOT substitute from an alternate kit at any time.

For optimal assay performance, we recommend you perform the assay according to the instructions provided in the cell line specific datasheet.

USE OF PLASMA OR SERUM CONTAINING SAMPLES

RTK Bioassay Kits can be run in the presence of high levels of serum or plasma without negatively impacting assay performance. Standard curves of control ligand can be prepared in high concentrations of human serum or heparinized plasma and added directly to the cells (without further dilution, i.e. 100% serum in the well). The response to high serum concentration is usually well tolerated, however, the final tolerance is receptor specific.

NOTE:
EDTA anti-coagulated plasma samples do not give a positive response in the assay. Therefore, the choice of anti-coagulant treatment is very important.

PROTOCOL 1: FOR PURIFIED ANTIBODY SAMPLES

DAY 1: THAWING AND PLATING FROZEN CELLS

The following steps outline the procedure for thawing and plating frozen assay-ready 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 phase storage and place immediately on dry ice prior to thawing. **DO NOT EXPOSE VIALS TO ROOM TEMPERATURE.**

   NOTE:
   Wait at least one minute before opening for any liquid N_2 inside the vial to evaporate.
3. Remove frozen vials from dry ice and add 0.5 mL of pre-warmed CP reagent to the cell vial under sterile conditions. Pipette up and down gently to ensure that the cells are evenly distributed.
4. Immediately transfer the thawed cells to a sterile 15 mL tube containing 11.5 mL of pre-warmed CP reagent 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, incubate for indicated amount of time at 37°C, 5% CO_2.*****

   NOTE:
   *****Please refer to the cell line specific datasheet for any variation in assay conditions.

ASSAY PROCEDURE - AGONIST DOSE RESPONSE

The steps outlined below provide the assay volumes and procedure for performing agonist assays using the assay-ready cells and Bioassay Detection Reagents.

DAY 2 OR 3: AGONIST COMPOUND PREPARATION AND ADDITION

1. Dissolve control agonist compound in the recommended vehicle at the specified concentration*.

   NOTE:
   *Please refer to the agonist specific datasheet supplied in the kit.
2. Prepare 4-fold serial dilutions of agonist compound in CP reagent or normal human serum (NHS). The concentration of each dilution should be prepared at **11X** of the final screening concentration (i.e. 10 µL compound + 100 µL of cells).

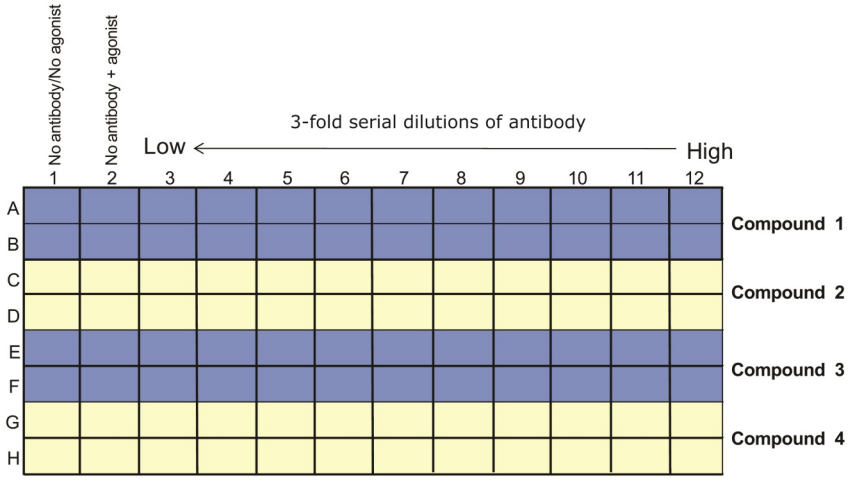
   Preparation of 12-point dose curve serial dilutions:
   We recommend starting with a concentration that is **50X** the expected EC_{50} value for the compound (**550X** EC_{50} would be the final working concentration).

   Example: If the expected EC_{50} is 10 nM, prepare the highest starting concentration at 5.5 µM.

ASSAY PROCEDURE — UNPURIFIED ANTIBODY SAMPLES

Although plate layouts and experimental designs may vary, we recommend performing a 11-point dose curve for each compound using at least *duplicate* wells for each dilution. The protocols and volumes described below are designed for a complete 96-well plate.

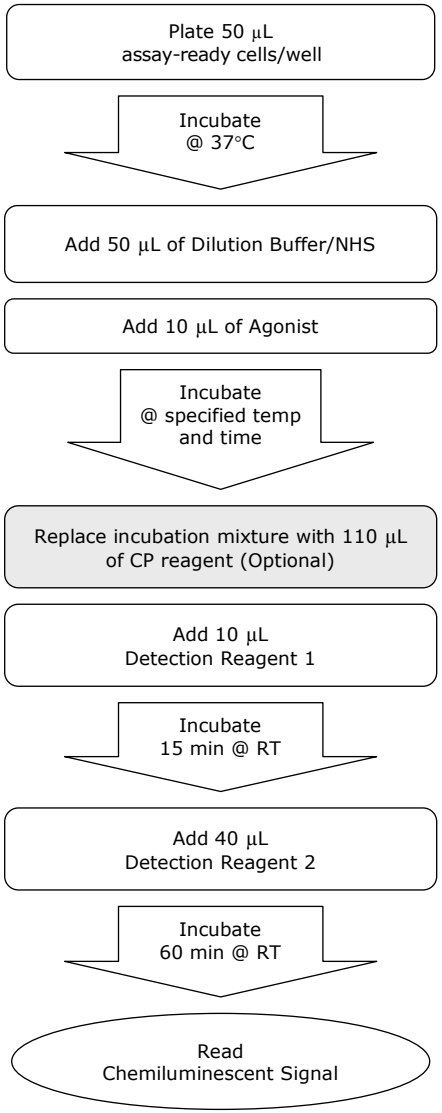
DAY 2 OR 3: ANTIBODY PREPARATION AND ADDITION



1. Dissolve antibody in the Protein Dilution Buffer
2. Prepare 3-fold serial dilutions of antibody in Protein Dilution Buffer. The concentration of each dilution should be prepared at **2.2X** of the final screening concentration (i.e. 50 µL antibody will be used in a final volume of 110 µL).

   Preparation of 11-point dose curve serial dilutions:
   a) Label tubes 1 through 12.
   b) Add 120 µL of Protein Dilution Buffer to tubes #1-11.
   c) Prepare a working stock of antibody in Protein Dilution Buffer.
   d) Add 180 µl of the working concentration of antibody to tube #12.
   e) Remove 60 µL of diluted antibody from tube #12, add it to tube #11 and mix gently by pipetting up and down. Discard the pipet tip.
   f) With a clean pipet tip, remove 60 µL of diluted antibody from tube #11, add it to the tube #10 and mix gently by pipetting up and down. Discard the pipet tip.

QUICK-START PROCEDURE: AGONIST DOSE RESPONSE



*****Please refer to the cell line specific datasheet for any variation in assay conditions.

a) Label tubes 1 through 12.
b) Add 75 µL of CP reagent or NHS to tubes #1-11.
c) Prepare a working concentration of agonist compound in CP reagent or NHS.
d) Add 100 µl of the working concentration of agonist compound to tube #12.
e) Remove 25 µL of diluted compound from tube #12, add it to tube #11 and mix gently by pipetting up and down. Discard the pipet tip.
f) With a clean pipet tip, remove 25 µL of diluted compound from tube #11, add it to tube #10 and mix gently by pipetting up and down. Discard the pipet tip.
g) Repeat this process 8 more times, preparing serial dilutions from right to left across the tubes.
h) **DO NOT add agonist compound to tube #1**. This sample serves as the no agonist control and completes the dose curve.
i) Repeat this process for each compound to be tested.
j) Set compounds aside until they are ready to be added.

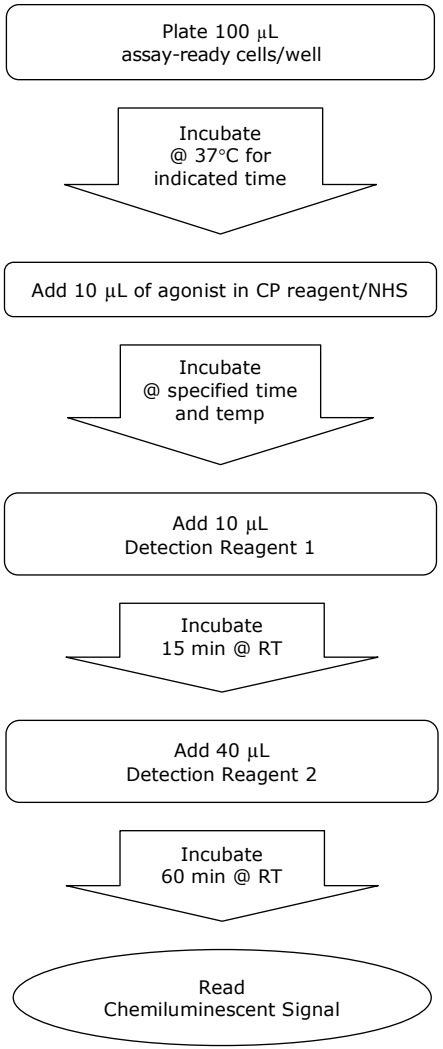
3. Remove assay-ready cells (previously plated on day 1) from the incubator.
4. Transfer 10 µL of agonist from tubes #1-12 to each well.
5. Incubate for indicated amount of time at the indicated temperature.*

NOTE:
*****Please refer to the cell line specific datasheet for specific assay conditions.

ADDITION OF DETECTION REAGENTS

1. Add 10µL of Detection Reagent 1 to the well.
2. Incubate the plate for 15 minutes at room temperature (19 - 25°C).
3. Add 40 µL of Detection Reagent 2 per well and incubate for 60 minutes at room temperature (19 - 25°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
4. Read samples on any standard luminescence plate reader.
5. Use GraphPad Prism® or other comparable program to plot your agonist dose response.

QUICK-START PROCEDURE: AGONIST DOSE RESPONSE



Please refer to the cell line specific datasheet for assay specific conditions.

a) Label tubes 1 through 12.
b) Add 75 µL of CP reagent to tubes #1-11.
c) Prepare a working concentration of agonist compound in CP reagent, containing appropriate solvent.
d) Add 100 µl of the working concentration of agonist compound to tube #12.
e) Remove 25 µL of diluted compound from tube #12, add it to tube #11 and mix gently by pipetting up and down. Discard the pipet tip.
f) With a clean pipet tip, remove 25 µL of diluted compound from tube #11, add it to tube #10 and mix gently by pipetting up and down. Discard the pipet tip.
g) Repeat this process 8 more times, preparing serial dilutions from right to left across the tubes.
h) **DO NOT add agonist compound to tube #1**. This sample serves as the no agonist control and completes the dose curve.
i) Repeat this process for each compound to be tested.
j) Set compounds aside until they are ready to be added.

3. Remove assay-ready cells (previously plated on day 1) from the incubator.
4. Add 50 µL of Protein Dilution Buffer to each well.
5. Transfer 10 µL of agonist from tubes #1-12 to each well.
6. Incubate for 3 hours @ room temperature (19 - 25°C)*.

NOTE:
***Please refer to the cell line specific datasheet for any variation in assay conditions.

ADDITION OF DETECTION REAGENTS

1. Add 10 µL of Detection Reagent 1 to the well.
2. Incubate the plate for 15 minutes at room temperature (19 - 25°C).
3. Add 40 µL of Detection Reagent 2 per well and incubate for 60 minutes at room temperature (19 - 25°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
4. Read samples on any standard luminescence plate reader.
5. Use GraphPad Prism® or other comparable program to plot your agonist dose response.

PROTOCOL 2: FOR UNPURIFIED ANTIBODY SAMPLES

DAY 1: THAWING AND PLATING FROZEN CELLS

The following steps outline the procedure for thawing and plating frozen assay-ready 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 phase storage and place immediately on dry ice prior to thawing. **DO NOT EXPOSE VIALS TO ROOM TEMPERATURE.**

   NOTE:
   When removing cryovials from liquid N2, place immediately on dry ice in a covered container. Wait at least one minute before opening for any liquid N2 inside the vial to evaporate.
3. Remove frozen vials from dry ice and add 0.5 mL of pre-warmed CP reagent to the cell vial under sterile conditions. Pipette up and down gently several times to ensure that the cells are evenly distributed.
4. Immediately transfer the thawed cells to a sterile 15 mL tube containing 5.5 mL of pre-warmed CP reagent and pour into a disposable reagent reservoir.
5. Plate 50 µL of cells into each well of the provided 96-well tissue culture plate.
6. After seeding the cells into the microplate, incubate for indicated amount of time at 37°C, 5% CO_2.*

   NOTE:
   *Please refer to the cell line specific datasheet for assay specific conditions.

ASSAY PROCEDURE - AGONIST DOSE RESPONSE

The steps outlined below provide the assay volumes and procedure for performing agonist assays using the assay-ready cells and Detection Reagents.

DAY 2 OR 3: AGONIST COMPOUND PREPARATION AND ADDITION

1. Dissolve control agonist compound in the recommended vehicle at the specified concentration*.

   NOTE:
   *Please refer to the agonist specific datasheet supplied in the kit
2. Prepare 4-fold serial dilutions of agonist compound in CP reagent or NHS. 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.

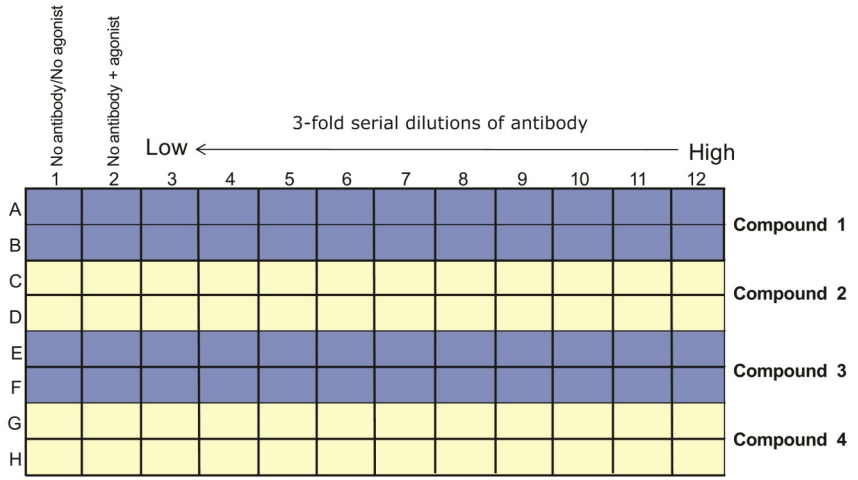
   Preparation of 12-point dose curve serial dilutions: We recommend starting with a concentration that is **50X** the expected EC_{50} value for the compound (**550X** EC_{50} would be the final working concentration).

   Example: If the expected EC_{50} is 10 nM, prepare the highest starting concentration at 5.5 µM.

ASSAY PROCEDURE — PURIFIED ANTIBODY SAMPLES

Although plate layouts and experimental designs may vary, we recommend performing a 11-point dose curve for each compound using at least *duplicate* wells for each dilution. The protocols and volumes described below are designed for a complete 96-well plate.

DAY 2 OR 3: ANTIBODY PREPARATION AND ADDITION



1. Dissolve biologic in the Protein Dilution Buffer.
2. Prepare 3-fold serial dilutions of antibody in Protein Dilution Buffer. The concentration of each dilution should be prepared at **22X** of the final screening concentration (i.e. 5 µL antibody will be used in a final volume of 110 µL). See protocol earlier for preparation of dilution curves.
3. Remove assay-ready cells (previously plated on day 1) from the incubator.
4. Transfer 5 µL of diluted biologic samples from tubes #1-12 to each well according to the plate map above.
5. Incubate for 60 minutes @ 37°C.

AGONIST COMPOUND PREPARATION AND ADDITION

1. During the antibody incubation, determine the EC_{80} concentration of the agonist to be used in the assay. Prepare a **22X** EC_{80} concentration of agonist compound in CP reagent as shown below:

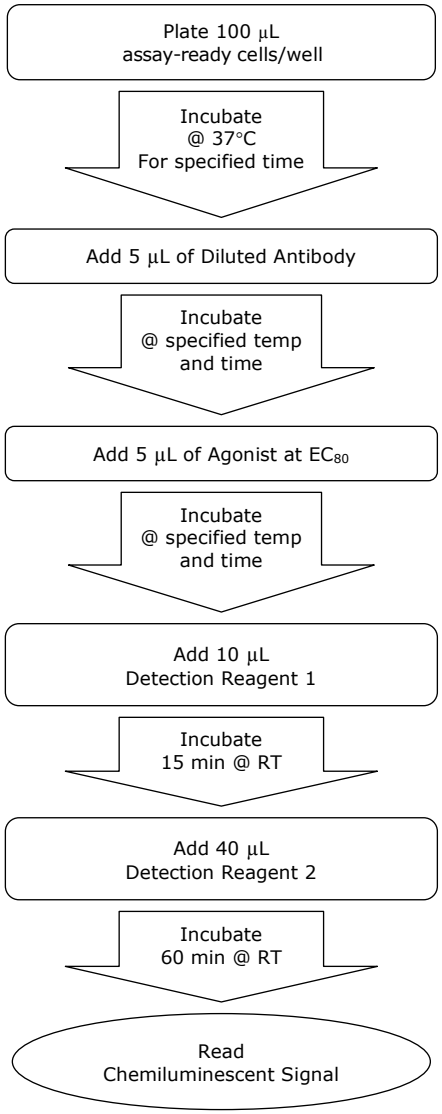
   Example: If the expected EC_{80} of the agonist compound is 10 nM, prepare a stock at 220 nM.
2. Add 5 µL of agonist compound to each well. Add 5 µL of CP reagent to the no agonist wells (column 1).
3. Incubate for 1.5 hours @ room temperature (19 - 25ºC)*****.

   NOTE:
   *****Please refer to the cell line specific datasheet for any variation in assay conditions.

ADDITION OF DETECTION REAGENTS

1. Add 10 µL of Detection Reagent 1 to the well.
2. Incubate the plate for 15 minutes at room temperature (19 - 25°C).
3. Add 40 µL of Detection Reagent 2 per well and incubate for 60 minutes at room temperature (19 - 25°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
4. Read samples on any standard luminescence plate reader.
5. Use GraphPad Prism® or other comparable program to plot your agonist dose response.

QUICK-START PROCEDURE: PURIFIED ANTIBODY SAMPLES



Please refer to the cell line specific datasheet for any variation in assay conditions.