Platelet-Derived Growth Factor Receptors α and β Reporter Assay System

(PDGFRα/β)

96-well Format Assays

Product # IB23001

▪

Technical Manual
(version 7.2i)

www.indigobiosciences.com

3006 Research Drive, Suite A1, State College, PA 16801, USA

Customer Service:
814-234-1919; FAX 814-272-0152
customerserv@indigobiosciences.com

Technical Service:
814-234-1919
techserv@indigobiosciences.com

INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human PDGFRα/β Reporter Assay System
96-well Format Assays

I. Description

▪ The Assay System ▪

This assay utilizes proprietary human cells that have been engineered to provide constitutive expression of the **Human Platelet-Derived Growth Factor Receptors α** and **β**, referred to herein as **PDGFRα/β**. PDGFRα and PDGFRβ are both single-pass transmembrane receptors that contain respective extracellular ligand-binding domains, transmembrane domains, and intracellular tyrosine kinase domains[1].

Platelet-derived growth factor (PDGF), the physiological activator of PDGFRs, consists of four polypeptide members: A, B, C and D[1, 2]. The biologically active forms of PDGF proteins are both homo-dimers and hetero-dimers of disulfide-linked polypeptides. These function to promote cell migration, proliferation, and survival[2]. Binding of dimeric PDGF triggers conformational changes that drive the assembly of homo-dimeric (Rα:Rα, Rβ:Rβ) and/or hetero-dimeric (Rα:Rβ) receptors, and the activation of their respective cytosolic tyrosine kinase domains[1, 2]. Because these reporter cells constitutively express both PDGFRα and PDGFRβ, it is anticipated that all three forms of the activated receptor dimers are present. If desired, reporter cells expressing either homo-dimeric PDGFR can be made available (please inquire).

The tyrosine kinase activities of activated, dimeric PDGFR's initiate intracellular signaling cascades that include RAS-MAPK, PI3-AKT, PLCγ and STAT pathways[2, 3]. For example, activation of the PLCγ pathway leads to an increase of intracellular calcium[4]. One prominent outcome of the PDGF/PDGFR > PLCγ pathway is that calcineurin, a calcium-dependent phosphatase, dephosphorylates and activates the transcription factor NFAT[4]. It is PDGFR signal transduction *via* the Ca^{+2}·calcineurin / NFAT cascade that is exploited by the reporter cells provided in this kit.

INDIGO's PDGFRα/β Reporter Cells contain the luciferase reporter gene functionally linked to tandem consensus sequences of NFAT response elements upstream of a minimal promoter. Activated NFAT binds to these response elements to initiate the formation of a complete transcription complex that drives Luc gene expression.

PDGF activates PDGFRα/β in a dose-dependent manner, thereby triggering the Ca^{+2}·calcineurin/NFAT signal transduction pathway. Quantifying relative changes in luciferase activity in the treated reporter cells relative to the untreated cells provides a sensitive, dose-dependent surrogate measure of drug-induced changes in PDGFRα/β activity. Accordingly, the principal application of this reporter assay is in the screening of test materials to quantify any functional interactions, either activating or inhibitory, that they may exert against PDGFRα/β, or the coupled Ca^{+2}·calcineurin/NFAT signal transduction pathway.

The clinical use of recombinant PDGF-BB has led to the successful treatment of chronic or diabetes-related non-healing lower extremity wounds[5]. In addition, PDGF-BB has also been used in clinics for reducing Parkinsonian symptoms[5]. However, dysfunctional PDGFR signaling can lead to a range of physiological disorders. For example, enhanced signaling of PDGFRs has been implicated in the pathogenesis of atherosclerosis, pulmonary fibrosis, angiogenesis, and tumorigenesis[2]. Consequently, the PDGF receptors continue to command much interest as targets for drug development and drug safety screening.

INDIGO's Reporter Cells are transiently transfected and prepared as frozen stocks using a proprietary **CryoMite™** process. This cryo-preservation method allows for the immediate dispensing of healthy, division-competent reporter cells into assay plates. There is no need for intermediate treatment steps such as spin-and-rinse of cells, viability determinations or cell titer adjustments prior to assay setup. This assay kit provides the convenience of an all-inclusive cell-based reporter assay system for PDGFRα/β signaling. In addition to Reporter Cells, included are an optimized culture medium for use in reviving the cryopreserved cells, a culture medium for use in preparing test sample treatments, the reference physiological activator PDGF-BB, Luciferase Detection Reagents, and a cell culture-ready assay plate.

[1] Kazlauskas, K, *et al.* (2017) PDGFs and their Receptors. Gene. May 30; **614**: 1–7.

[2] Wu E, *et al*. (2008) Comprehensive Dissection of PDGF-PDGFR Signaling Pathways in PDGFR Genetically Defined Cells. PLoS One. 3(11): e3794. doi:10.1371/journal.pone.0003794

[3] Ying HZ, *et. al.* (2017) PDGF signaling pathway in hepatic fibrosis pathogenesis and therapeutics (Review). Molecular Medicine Reports **16**: 7879-7889.

[4] Park JY, *et. al.* (2020) The Role of Calcium-Calcineurin-NFAT Signaling Pathway in Health and Autoimmune Disease, Frontiers in Immunology.:doi:10.3389/fimmu.2020.00195.

[5] Niu A, *et. al.* (2020) Development of a novel reporter gene assay for platelet-derived growth factor-BB bioactivity. Biologicals **63**: 68-73.

▪ The Assay Chemistry ▪

INDIGO's receptor assay kits capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg^{+2}-dependent reaction that consumes O_2 and ATP as co-substrates to yield oxyluciferin, AMP, PP_i, CO_2, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer and is reported in terms of Relative Light Units (RLU's).

This assay kit features a luciferase detection reagent specially formulated to provide stable light emission between 10 and 90+ minutes after initiating the luciferase reaction. Incorporating a 10-minute reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.

▪ Preparation of Test Compounds ▪

Small molecule test compounds are typically solvated in DMSO at high concentrations; ideally 1,000x-concentrated stocks relative to the highest desired treatment concentration in the assay. Using high-concentration stocks minimizes DMSO carry-over into the assay plates. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

1.) Compound Screening Medium (CSM) may be used as the diluent to make serial dilutions of test compounds to achieve the desired final assay concentration series.

Alternatively, if test compound solubility is expected to be problematic,
2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the final concentration of total DMSO (or any organic solvent) carried over into assay wells should not exceed 0.4%. Significant cytotoxicity can be expected above 0.4% DMSO exposure over the 24 hr treatment period.

> *NOTE:* CSM is formulated to help stabilize hydrophobic small molecule test compounds in the aqueous environment of the treatment media. Nonetheless, high concentrations of small organic molecules diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that compound dilutions are prepared in CSM immediately prior to assay setup and are then treated as 'single-use' reagents.

Protein or antibody test samples are typically solvated in aqueous buffered solutions supplemented with carrier protein (*e.g.,* PBS + 0.1% BSA) at concentrations *no less* than 10x relative to the highest desired treatment concentration. The PDGF-BB stock included with this kit is prepared in PBS + 0.1% BSA at a 100x-concentration relative to the highest recommended treatment (refer to APPENDIX 1).

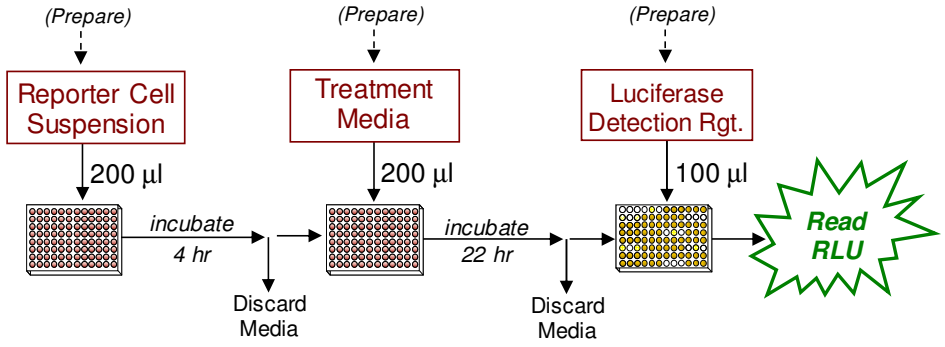
▪ Considerations for Automated Dispensing ▪

When using an automated dispensing instrument to process a small number of assay plates, first carefully consider the dead volume requirement of your instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

Stock Reagent & Volume provided	**Volume to be Dispensed** *(96-well plate)*	**Excess reagent volume available for instrument dead volume**
Reporter Cell Suspension 21 ml *(prepared from kit components)*	200 µl / well 19.2 ml / plate	~ 1.8 ml
LDR 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, 200 µl of Reporter Cells are dispensed into wells of the assay plate and incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared treatment media are added. Following 22-24 hr incubation discard treatment media and add Luciferase Detection Reagent. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



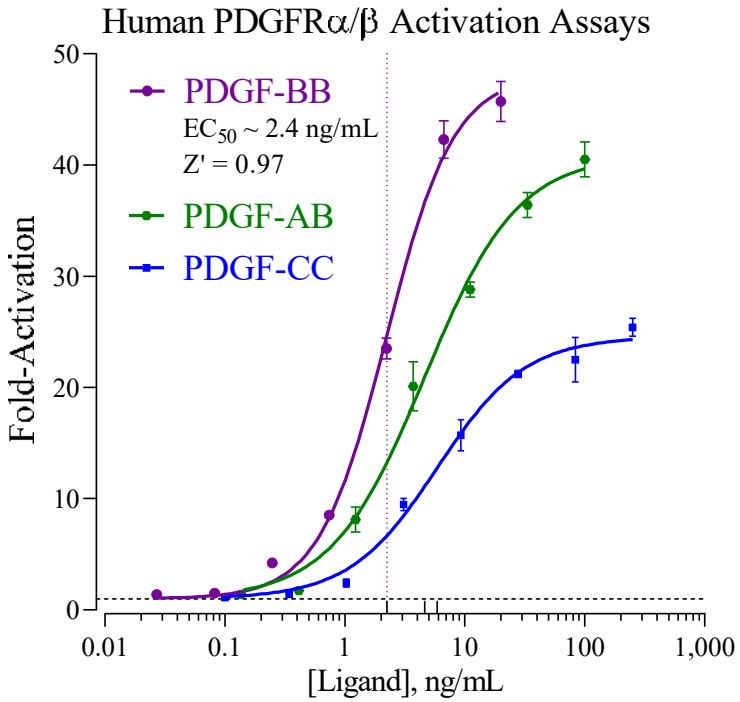


Figure 2. PDGFRα/β Activation dose response analyses. Activation dose-response assays were performed according to the protocol provided in this Technical Manual. 200 µl / well of PDGFRα/β Reporter Cell suspension was dispensed into the 96-well assay plate, which was then incubated for 4 hours. The concentrated stock of PDGF-BB (provided), PDGF-AB and PDGF-CC (both from Peprotech) were further diluted using CSM to produce treatment media at the desired assay concentrations. The pre-culture media were discarded from the assay wells and 200 µl / well of the prepared treatment media were dispensed (n = 3/conc.), including 'untreated' control wells. Following a 22 hr incubation period treatment media were discarded, Luciferase Detection Reagent was added, and luminescence intensity per well was quantified. Values of average relative light units (RLU) and corresponding values of standard deviation (SD), percent coefficient of variation (%CV), Fold-Activation and Z'[6] were determined for each treatment concentration. Non-linear regression analyses of Fold Activation *vs.* Log_{10}[ng/mL] and EC_{50} determinations were performed using GraphPad Prism software.

[6] Zhang JH, *et al.* (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen.:**4**(2), 67-73.

$Z' = 1 - [3*(SD^{Ref\ EC100} + SD^{Untreated}) / (RLU^{Ref\ EC100} - RLU^{Untreated})]$

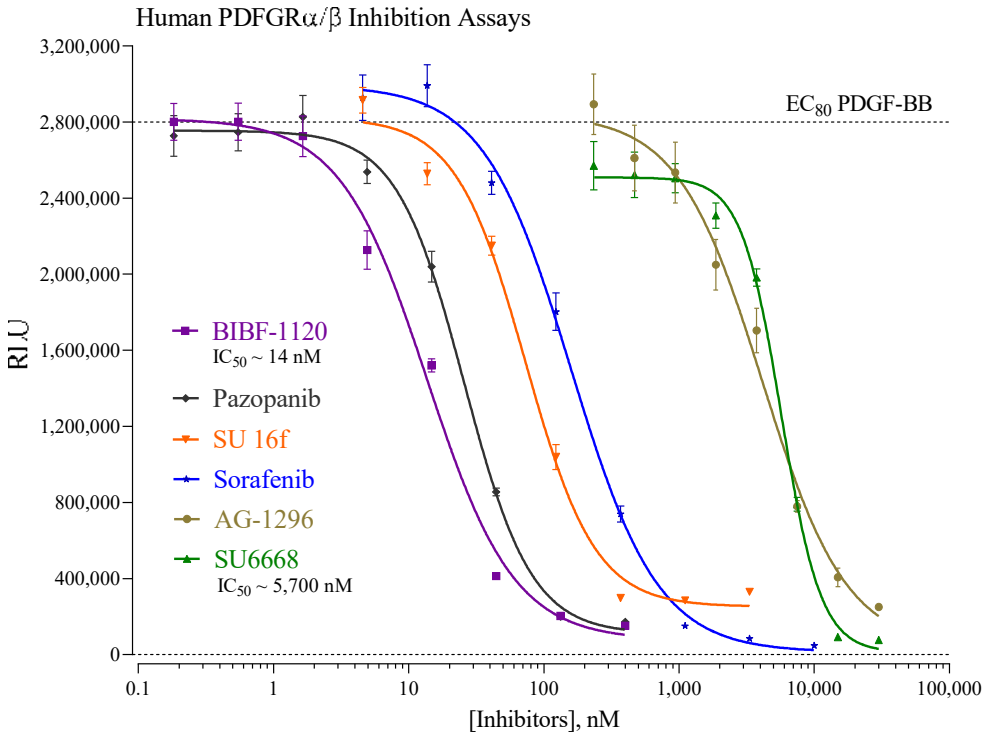


Figure 3. PDGFRα/β Inhibition dose-response analyses. PDGFRα/β reporter cells were co-treated with an EC_{80} concentration of the reference activator PDGF-BB and varying concentrations of the PDGFR inhibitors BIBF-1120, Pazopanib, SU 16f, Sorafenib, AG-1296 and SU6668 (all compounds obtained from Cayman Chemical, Ann Arbor MI, USA). The range of determined IC_{50} values is shown; (n = 3 / conc.). INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown). Non-linear regression analyses of RLU *vs.* Log_{10}[Inhibitor, nM] were plotted and IC_{50} determinations made using GraphPad Prism software.

II. Product Components & Storage Conditions

This PDGFRα/β Reporter Assay kit contains materials to perform assays in a single collagen-coated 96-well assay plate.

Cryopreserved mammalian cells are temperature sensitive! **To ensure maximal viability the tube of Reporter Cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in *Step 2* of this protocol.**

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first inspect and inventory the individual kit components, please be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen, nor can they be maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

Kit Components	*Amount*	*Storage Temp.*
▪ PDGFRα/β Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ PDGF-BB, 2.0 µg/ml (in PBS/0.1%BSA) (reference activator of PDGFR's)	1 x 40 µL	-20°C
▪ Detection Substrate	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well, *collagen-coated* assay plate (white, sterile, cell-culture ready)	1	**-20°C**

NOTE: This Assay kit contains one 96-well assay plate in which the assay wells have been collagen-coated and dried; the assay plate should be stored frozen (-20°C or -80°C) until use.

III. Materials to be Supplied by the User

The following materials must be provided by the user, and should be made ready prior to initiating the assay procedure:

DAY 1

▪ container of dry ice (see *Step 2*)

▪ cell culture-rated laminar flow hood.

▪ 37°C, humidified 5% CO_2 incubator for mammalian cell culture.

▪ 37°C water bath.

▪ 70% alcohol wipes

▪ 8-channel electronic, repeat-dispensing pipettes & sterile tips

▪ disposable media basins, sterile.

▪ sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), *or* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).

▪ *Optional:* Reference Inhibitor (refer to Fig 3.)

▪ *Optional:* clear 96-well assay plate, sterile, *collagen-coated*, for viewing cells on *Day 2*.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Please review the entire protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-11* are performed on ***Day 1***, requiring less than 2 hours of bench work and a 4 hour incubation step to complete. *Steps 12-17* are performed on ***Day 2*** and require less than 1 hour to complete.

▪ A word about Inhibition-mode assay setups ▪

Receptor inhibition assays expose the Reporter Cells to a fixed, sub-maximal concentration (typically between EC_{50} – EC_{85}) of a known activator AND varying concentrations of the test compound(s) to be evaluated for chemical inhibition. This PDGFRα/β Assay kit includes a 2 µg/mL stock solution of PDGF-BB, a potent physiological activator of PDGFR's, that may be used to set up inhibition-mode assays. ~ 4.5 ng/mL PDGF-BB approximates EC_{80} in this assay. Hence, it presents a suitable concentration of activator to use when screening test materials for inhibitory activities.

Add PDGF-BB to a bulk volume of **CSM**, as described above. This activator-supplemented medium is then used to prepare serial dilutions of test material stocks to achieve the desired respective final assay concentrations. This is an efficient and precise method of setting up PDGFRα/β inhibition assays, and it is the method presented in *Step 7b* of this protocol.

DAY 1 Assay Protocol: All steps should be performed using aseptic technique.

1.) Remove the **2 tubes** of **Cell Recovery Medium (CRM)** from freezer storage, thaw and equilibrate to 37°C using a water bath.

2.) Rapid Thaw of the Reporter Cells: *First*, retrieve the two tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve the tube of PDGFRα/β **Reporter Cells** from -80°C storage, place it directly into dry ice for transport to the laminar flow hood. When ready to begin, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 9.5 ml from ***each* of the 2 tubes** of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 minutes. The resulting volume of cell suspension will be **21 ml**.

3.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.

4.) *Gently* invert the tube of Reporter Cells several times to gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an electronic, repeat-dispensing 8-channel pipette, dispense **200 µl / well** of cell suspension into wells of the assay plate.

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'cell blank' wells (meaning cell-free but containing CSM) must be included in the assay plate to allow quantification of plate-specific fluorescence background (refer to the LCMA Technical Manual).

NOTE 4.2: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the reservoir during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

NOTE 4.3: Users sometimes wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear, preferably collagen-coated, 96-well assay plate. Continue to process this plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells. Place the assay plate into a cell culture incubator (37°C, ≥ 70% humidity, 5% CO_2) for 4 - 6 hours.

6.) Near the end of the pre-culture period, remove Compound Screening Medium **(CSM)** from freezer storage and thaw in a 37°C water bath.

7.) Prepare the Test Compound(s) and Reference Compound treatment media: Use **CSM** to prepare an appropriate dilution series of the reference and test compound stocks. Prepare all treatment media at the desired final assay concentrations. In *Step 9*, the prepared treatment media will be dispensed at 200 µl / well into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO carried over into assay reactions should not exceed 0.4%.

***a. Activation*-mode assays**. This PDGFRα/β Assay kit includes a concentrated stock of PDGF-BB, 2 µg/ml prepared in PBS/0.1%BSA. The following 7-point treatment series, with concentrations generated using serial 3-fold dilutions, provides a complete dose-response: 20.0, 6.67, 2.22, 0.741, 0.247, 0.082, and 0.027 ng/ml. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' control wells.

~ or ~

***b. Inhibition*-mode assays**. When setting up inhibition assays, first supplement a bulk volume of CSM with the challenge activator PDGF-BB to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about inhibition-mode assay setup*", pg. 9). The PDGF-BB supplemented CSM is then used to generate dilutions of test compound stocks to achieve the desired series of treatment concentrations.

8.) At the end of the 4-6 hr pre-culture period, discard the media; the preferred method is to use a 'wrist flick' to manually eject media into an appropriate waste collection container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

9.) Dispense **200 µl / well** of each prepared treatment media into the assay plate.

NOTE: If well-to-well variation due to 'edge-effects' is a concern this problem *may* be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate. Simply remove 1 tip from the 8-chanel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

10.) Transfer the assay plate into a cell culture incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 70%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

11.) For greater convenience on *Day 2*, retrieve **Detection Substrate** *and* **Detection Buffer** from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

DAY 2 Assay Protocol: Subsequent manipulations do *not* require special regard for aseptic technique and may be performed on a bench top.

12.) Approximately 30 minutes before intending to quantify receptor activity, remove **Detection Substrate** and **Detection Buffer** from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature.

NOTE: Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

13.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, *or less*.

14.) *Immediately before proceeding to Step 15*, transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a 12 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

15.) Following 22 - 24 hours incubation in treatment media, discard the media contents by manually ejecting it into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

16.) Add 100 µl of the prepared **LDR** into all wells of the assay plate. Allow the assay plate to rest at room temperature for 5-10 minutes following the addition of LDR. Do not shake the assay plate during this period.

17.) Quantify luminescence.

V. Related Products

Product No.	*Product Descriptions*
Platelet-Derived Growth Factor Receptor α and β Assay	
IB23001-32	PDGFRα/β Assay, 3x 32 assays in 8-well strips (96-well plate format)
IB23001	PDGFRα/β Assay, 1x 96-well format assay
IB23002	PDGFRα/β Assay, 1x 384-well format assays
Bulk volumes of PDGFRα/β Assay Reagents may be custom manufactured to accommodate any scale of HTS. In addition, **single receptor PDGFRα and PDGFRβ Assays can be made available upon request**. Please Inquire.	

NFAT Assays (recommended for receptor specificity screening)	
IB18001-32	NFAT Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB18001	NFAT Reporter Assay System 1x 96-well format assay

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in **5x bulk volume** to perform **480** Live Cell Assays performed in 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays performed in 10 x 96-well assay plates
INDIGlo Luciferase Detection Reagent	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents in 10 mL, 25 mL, 50 mL, and 500 mL volumes

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Limited Use Disclosures

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic, or contact use in humans or animals.

"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA)

Product prices, availability, specifications, and claims are subject to change without prior notice.

Copyright © INDIGO Biosciences, Inc. (State College, PA, USA). All Rights Reserved.

APPENDIX 1

Example scheme for the serial dilution of PDGF-BB and the setup of an PDGFRα/β dose-response assay.