Human
Vascular Endothelial Growth Factor Receptor 2
Reporter Assay System
(VEGFR2, KDR, Flk-1)

3x 32 Assays in 96-well Format
Product # IB15001-32

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Technical Manual
(version 7.2)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human VEGFR2 (KDR / Flk-1) Reporter Assay System
3x 32 Assays in 96-well Format

I. Description

▪ The Assay System ▪

This assay utilizes proprietary human cells that provide constitutive expression of the **Human type II Vascular Endothelial Growth Factor Receptor,** referred to herein as **VEGFR2**, but also known as **KDR** and **FLK-1**.

VEGFR2, a member of the receptor tyrosine kinase (RTK) family, is highly expressed in vascular endothelial cells. VEGFR2 is a master regulator of the normal physiological processes of vasculogenesis, angiogenesis and lymphangiogenesis. Alternatively, dysregulated expression of VEGFR2 is strongly associated with cancer progression and tumor development.

There are several isoforms of the physiological signaling molecule vascular endothelial growth factor (VEGF); the 165aa isoform of VEGF-A is the reference activator provided with this kit. Stated simplistically, VEGF-A forms a complex with heparin (or heparan sulphate) and other co-receptors, which then bind to the extra-cellular domain of VEGFR2. Dimerization, activation of cytoplasmic tyrosine kinase domains and auto-phosphorylation ensue, followed by phosphorylation and activation of alternative associated intracellular signaling proteins.

Activated VEGFR2 may signal through several different pathways, the most prominent being Src, GRB2/Shc/SOS, PI3K, PLC-γ and JAK/STAT.[1] These various signal transduction pathways culminate in the activation of pathway-related transcription factors and the induced expression of their respective target genes.

Phosphorylation and activation of the transcription factor NFAT is one outcome of VEGFR2 activation, and it is the signaling mechanism exploited by the reporter cells included in this kit. Accordingly, INDIGO's Reporter Cells contains an engineered luciferase reporter gene functionally linked to tandem NFAT genetic response element (GRE) sequences and a minimal promoter. Activated NFAT will bind to its corresponding GRE's to initiate the formation of a complete transcription complex that drives Luc gene expression. 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- or antibody-induced changes in VEGFR2 activity.

Considering the significant involvement of VEGFR2 in sustaining tumor development it continues to command much interest as a target for the development of novel, specific small molecule inhibitory drugs and antibodies. Accordingly, the primary application of this reporter assay is to screen test materials for any functional activity, either agonistic or inhibitory, that they may exert against VEGFR2 *or* the activator protein VEGF-A.

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.

INDIGO's assay kits provide the convenience of an all-inclusive cell-based assay system. In addition to VEGFR2 Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference activator human VEGF-A (165 aa isoform, non-glycosylated), Luciferase Detection Reagents, and a cell culture-ready assay plate.

[1] Koch,S, *et. al.* (2011) Signal transduction by vascular endothelial growth factor receptors, Bi0chem. J.: **437**, 169-183.

▪ The Assay Chemistry ▪

INDIGO's nuclear 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).

Assay kits feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating a 5-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 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. Note that the final concentration of DMSO carried over into assay wells should *never* exceed 0.4%.

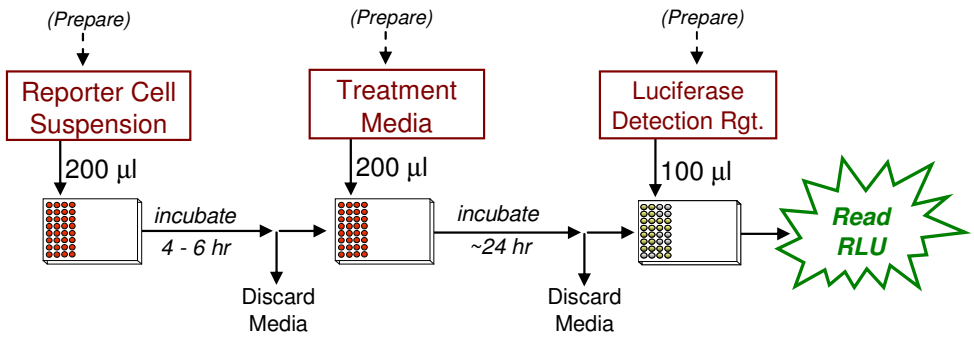
For protein or antibody test samples it is recommended to solvate the materials in aqueous buffered solutions supplemented with carrier protein (*e.g.,* PBS + 0.1% BSA) at concentrations *no less* than 10x relative to the highest desired treatment concentration. The VEGF-A_{165} stock included with this kit is prepared in PBS + 0.1% BSA at a 100x-concentration relative to the highest recommended treatment (refer to APPENDIX 1).

Immediately prior to setting up an assay the prepared stocks are serially diluted using **Compound Screening Medium (CSM+H)** to achieve the desired assay concentrations, as described in *Step 7*.

> *NOTE:* CSM+H contains heparin. In addition, it 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+H 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+H immediately prior to assay setup and are then treated as 'single-use' reagents.

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, 200 µl of Reporter Cells is dispensed into wells of the assay plate and 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 the 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.



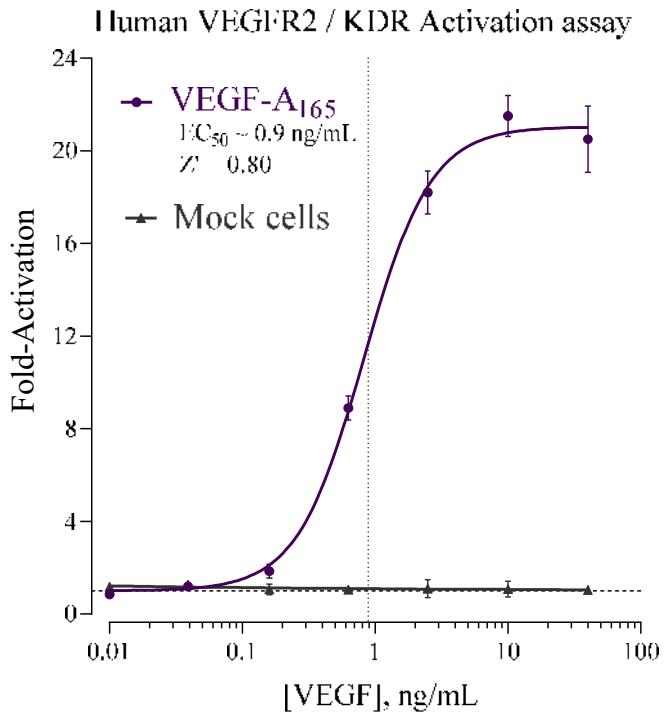


Figure 2. VEGFR2 Activation assay. The VEGFR2 activation assay was performed according to the protocol provided in this Technical Manual. 200 µl/well of VEGFR2 Reporter Cell suspension was dispensed into the 96-well assay plate, which was then incubated for 4 hours. The reference peptide, non-glycosylated VEGF-A_{165}, was further diluted using CSM+H to generate the desired assay concentrations of treatment media, as depicted in APPENDIX 1. The pre-culture media were discarded from the assay wells and 200 µl/well of treatment media were dispensed (n = 4 / conc.), including 'untreated' control wells. 'Mock' reporter cells (not included in this kit) contain the NFAT-Luc reporter gene but lack expression of VEGFR2. 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) and Z'[2] were determined. Non-linear regression analyses of Fold-Activation *vs.* VEGF-A Log_{10}[ng/mL] and EC_{50} determination were performed using GraphPad Prism software; error bars depict +/- %CV.

[2] 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.

$$Z' = 1 - [3*(SD^{Reference} + SD^{Vehicle\ Bkg}) / (RLU^{Reference} - RLU^{Vehicle\ Bkg})]$$

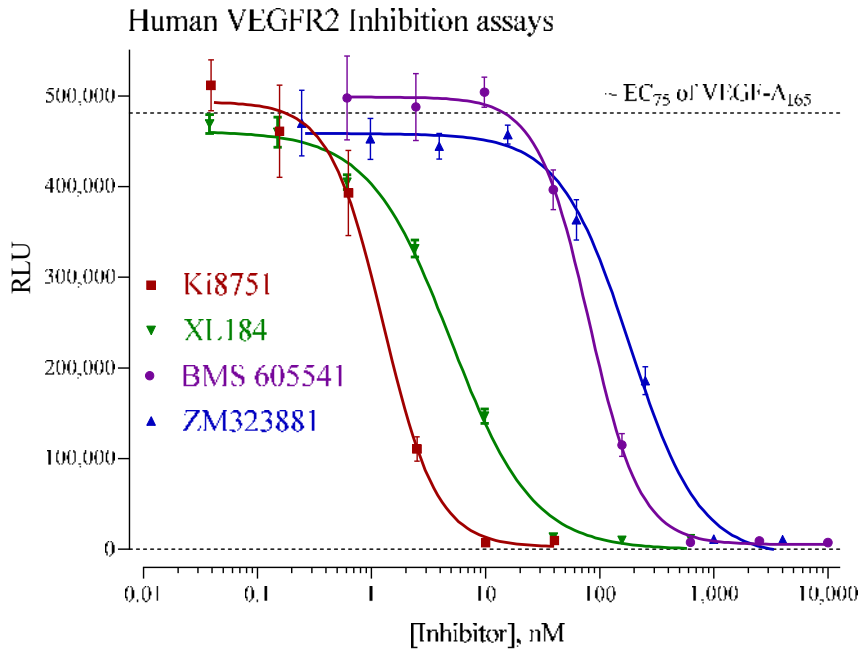


Figure 3. VEGFR2 Inhibition assays. 200 µl/well of VEGFR2 Reporter Cell suspension was dispensed into 96-well assay plates, which were then pre-incubated for 4 hours. For inhibition-mode assays, CSM+H was supplemented with 1.5 ng VEGF-A/ml. The small molecule inhibitors BMS 605541, Ki8751, ZM323881 and XL184 (Tocris) were initially prepared as concentrated stocks in DMSO, then further diluted using the VEGF-A_{165} supplemented CSM+H. Pre-culture media were discarded and 200 µl/well of the prepared treatment media were dispensed (n = 4 / conc.), including 'vehicle only' control wells. After 22 hr incubation the treatment media were discarded, Luciferase Detection Reagent was added, and RLU/well were quantified. Plots are RLU *vs.* VEGF-A Log_{10}[nM]; error bars depict +/- SD.

II. Product Components & Storage Conditions

This Human VEGFR2 Assay kit contains materials to perform three distinct groups of assays in the format of a 96-well plate. Reagents are configured so that each group will comprise 32 assays. If desired, however, reagents may be combined to perform either 64 or 96 assays.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of 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 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.*
▪ VEGFR2 Reporter Cells	3 x 0.6 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium for VEGFR assays (CSM+H)	1 x 45 mL	-20°C
▪ VEGF-A_{165}*, 4.0 µg/ml (in PBS/0.1%BSA) (physiological activator of VEGFR2)	1 x 40 µL	-20°C
▪ Detection Substrate	3 x 2.0 mL	**-80°C**
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate Frame	1	ambient
▪ Snap-in 8-well strips (white, sterile, collagen-coated wells)	12	**-20°C**

NOTE: This Assay kit contains 8-well strips that have been collagen-coated and dried; these strip wells should be stored frozen (-20°C or colder) until use.

* provided is the non-glycosylated 165aa isoform of VEGF-A, which has a molecular mass of 38.2 kDa

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:* antagonist reference compound (*e.g.,* Fig. 3)
▪ *Optional:* clear 96-well assay plate, cell culture treated, for viewing cells on *Day 2*.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

The assay protocol begins on the next page. Please review the entire Assay 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 hr 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 agonist AND varying concentrations of the test compound(s) to be evaluated for chemical inhibition or neutralizing antibody activities. This VEGFR2 Assay kit includes a 4.0 µg/mL stock solution of **VEGF-A_{165}**, a potent physiological activator of VEGFR2, that may be used to setup inhibition-mode assays. 1.5 ng VEGF-A_{165} / ml approximates EC_{75} in this assay. Hence, it presents a suitable concentration of activator to use when screening test materials for inhibitory activities.

Add VEGF-A_{165} to a bulk volume of **CSM+H**, 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 VEGFR2 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 **Reporter Cells** from -80°C storage and place them directly into dry ice to transport them to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. When ready to begin, transfer the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring **6.4 ml** of pre-warmed CRM into each tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **7.0 ml** per tube.

Third, during the 5 - 10 minutes incubation period, work in the cell culture hood to *carefully* mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

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

4.) If more than one tube of Reporter cells was thawed, combine them and gently invert several times to disperse cell aggregates and gain a homogenous cell suspension. Dispense **200 µl / well** of cell suspension into the assay plate.

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'blank' wells (meaning cell-free, but containing 'CSM') must be included in the assay plate to allow quantification of 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, collagen-coated 96-well assay plate. Continue to process the assay 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+H)** from freezer storage and thaw in a 37°C water bath.

7.) Prepare the Test Compound(s) and Reference Compound treatment media: Use **CSM+H** 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 strip wells. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM+H.

NOTE: Total DMSO, or any other organic solvent, carried over into assay reactions should never exceed 0.4%.

***a. Agonist*-mode assays**. This VEGFR2 Assay kit includes a concentrated stock of VEGF-A_{165}, 4.0 µg/ml prepared in PBS/0.1%BSA. The following 7-point treatment series, with concentrations generated using serial 4-fold dilutions, provides a complete dose-response: 40, 10, 2.5, 0.625, 0.156, 0.039, and 0.0098 ng/ml. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' (or 'vehicle') controls.

~ or ~

***b. Inhibition*-mode assays**. When setting up inhibition assays, first supplement a bulk volume of CSM+H with the challenge activator **VEGF-A_{165}** to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about inhibition-mode assay setup*", pg. 8). The 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 cell pre-culture period:*** **Discard the culture media**. Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (*e.g.,* Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus. Do *not* touch the well bottom or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the cells and greatly increased well-to-well variability.

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

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*, gently invert the tubes of Detection Substrate and Detection Buffer several times to ensure homogenous solutions, then 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, remove media contents from each well of the assay plate (as before in *Step 8*).

16.) Add 100 µl of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for at least 5 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*
Human VEGFR2 Assay Products	
IB15001-32	Human VEGFR2 Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB15001	Human VEGFR2 Reporter Assay System 1x 96-well format assay
IB15002	Human VEGFR2 Reporter Assay System 1x 384-well format assays
Bulk volumes of VEGFR2 Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

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 contained in 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays contained in 10 x 96-well assay plates

Please refer to INDIGO Biosciences website for updated product offerings.

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VI. Limited Use Disclosures

Products offered 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, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most current version available.

APPENDIX 1

Example scheme for the serial dilution of the reference agonist VEGF-A_{165}, and the setup of a VEGFR2 dose-response assay.