Human Tropomysin Receptor Kinase A Reporter Assay System; TrkA

(Neurotrophic Tyrosine Kinase Receptor, type 1; NTRK1)

96-well Format Assays

Product # IB27011

▪

Technical Manual
(version 7.2i)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human TrkA Reporter Assay System
96-well Format Assays

I. Description

▪ Background ▪

Tropomysin receptor kinases A, B and **C** constitute a family of receptors denoted as TrkA, TrkB, and TrkC. Trk's are single-pass transmembrane receptors that contain an extracellular ligand-binding domain, transmembrane domain, and an intracellular tyrosine kinase domain[1].

Neurotrophins, the physiological activators of Trk receptors, comprise a group of proteins that include Nerve Growth Factor beta (NGF-β), Neurotrophin 3 (NT-3), Neurotrophin 4 (NT-4) and Brain Derived-Neurotrophic Factor (BDNF)[1, 2]. The active forms of these neurotrophins are disulfide-linked homodimer polypeptides [1, 2].

The binding of neurotrophins to Trk's triggers receptor homodimerization and autophosphorylation. The intrinsic tyrosine kinase activities of activated, dimeric Trk receptor initiate intracellular signaling cascades that include RAS-MAPK, PI3-AKT, and PLCγ pathways.

Trk's are expressed in multiple tissue types, and are primarily involved in neuronal development, neuronal proliferation, and avoidance of programmed cell death[1, 2]. Chromosomal rearrangements of *NTRK*1-3 may result in gene fusions have been clinically validated as oncogenic drivers in a wide array of human cancers[2].

Not surprising, the Trk receptors command much interest as targets for drug development and drug safety screening. Topical recombinant human nerve growth factor beta (rhNGF-β) has been FDA-approved for the treatment for patients with neurotropic keratitis[3], as well as Trk inhibitors for the treatment of patients with solid tumors harboring *NTRK* gene fusions[2]. It is also widely accepted that neurotrophins contribute to the pain suffered in osteoarthritis, and anti-NGF antibodies are efficacious in reducing these pain symptoms[4].

▪ The Assay System ▪

This assay utilizes proprietary human cells that have been engineered to provide constitutive expression of Tropomysin receptor kinases A (TrkA), which is also commonly referred to as Neurotrophic Tyrosine Kinase Receptor, type 1 (NTRK1).

Neurotrophin / TrkA activation of the PLCγ pathway leads to an increase of intracellular calcium[3] and the concomitant activation of calcineurin, a calcium-dependent phosphatase. Ca^{+2}-calcineurin acts to dephosphorylate and activate the transcription factor NFAT[5]. TrkA activation of the Ca^{+2}·calcineurin > NFAT cascade is the signal transduction pathway exploited by the reporter cells provided in this kit.

INDIGO's TrkA Reporter Cells contain the luciferase reporter gene functionally linked to tandem consensus sequences of NFAT genetic response elements upstream of a minimal promoter. Activated NFAT binds to these response elements to seed 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 surrogate measure of drug-induced changes in TrkA activity.

The principal application of this reporter assay is in the screening of test samples to quantify functional interactions, either activating or inhibitory, that they may exert against TrkA, or the coupled Ca^{+2}·calcineurin / NFAT signal transduction pathway.

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 Human TrkA. In addition to the Reporter Cells, this kit includes an optimized culture medium for use in reviving the cryopreserved cells, a culture medium for use in preparing test sample treatments, the physiological activator NGF-β, Luciferase Detection Reagents, and a cell culture-ready assay plate.

▪ 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 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 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the 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-hour 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 NGF-β 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).

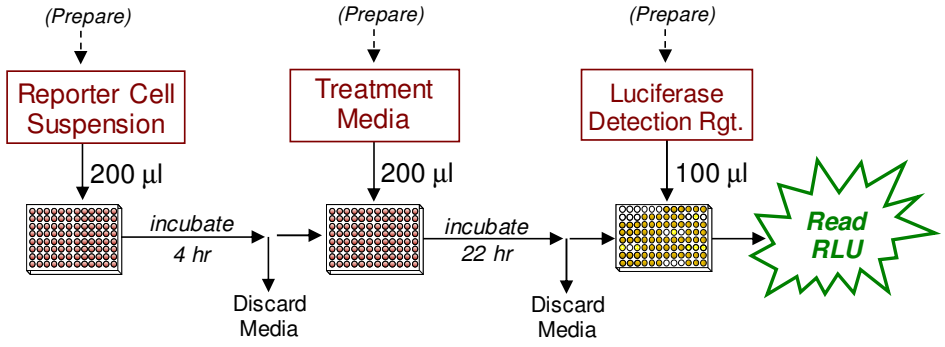
▪ Considerations for Automated Dispensing ▪

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.



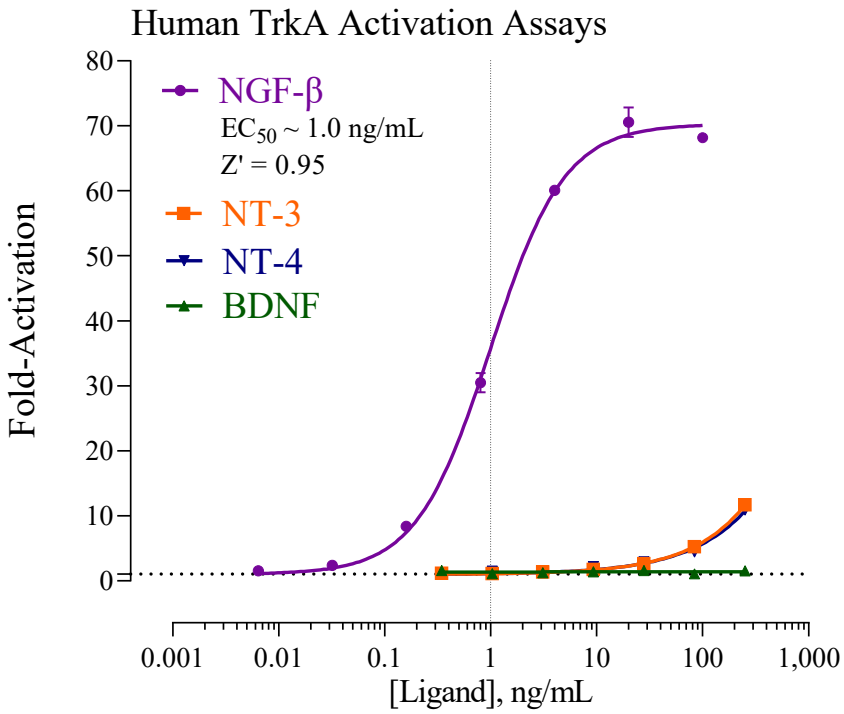


Figure 2. TrkA Activation dose response analyses. Activation dose-response assays were performed according to the protocol provided in this Technical Manual. 200 µl / well of TrkA Reporter Cell suspension was dispensed into the 96-well assay plate, which was then incubated for 4 hours. The concentrated stock of NGF-β (provided), NT-3, NT-4 and BDNF (all 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-hour 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.

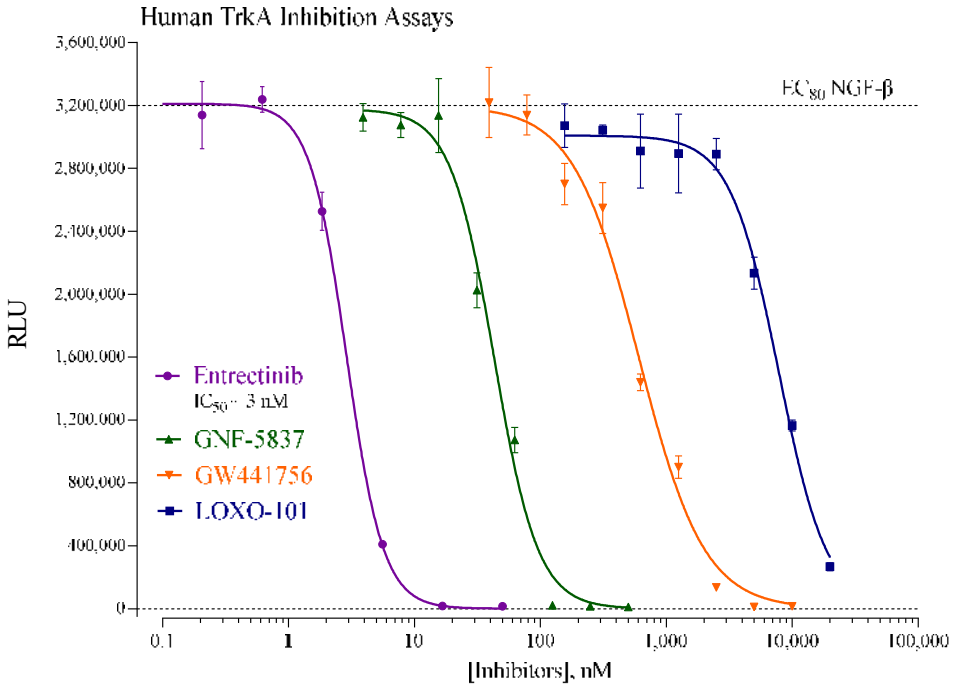


Figure 3. TrkA Inhibition dose-response analyses. TrkA reporter cells were co-treated with an EC_{80} concentration of the reference activator NFG-β and varying concentrations of the TrkA inhibitors Entrectinib, GNF-5837, GW441756 and LOXO-101 (all from Cayman Chemical, Ann Arbor MI, USA). The range 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} determination made using GraphPad Prism software.

II. Product Components & Storage Conditions

This TrkA 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.*
▪ TrkA 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
▪ NGF-β, 10.0 µg/ml (in PBS/0.1%BSA) (reference activator of TrkA)	1 x 30 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 Figure 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 ▪

When setting up receptor inhibition assays the Reporter Cells are co-treated with a fixed sub-maximal concentration (typically between EC_{50} – EC_{85}) of the reference agonist AND varying concentrations of the test compound(s). This TrkA Assay kit includes a 10 µg/mL stock solution of NGF-β, a potent physiological activator of TrkA, that may be used to set up inhibition-mode assays. ~ 3.0 ng/mL NGF-β approximates EC_{80} in this assay. Hence, it presents a suitable concentration of activator to use when screening test materials for inhibitory activities.

Add NGF-β 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 assay concentrations. This is an efficient and precise method of setting up TrkA 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 TrkA **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 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 mammalian 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 and Reference Compound treatment media: use **CSM** to prepare an appropriate dilution series of the reference and test compound stocks. 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 TrkA Assay kit includes a concentrated stock of NGF-β, 10 µg/ml prepared in PBS/0.1%BSA. The following 7-point treatment series, with concentrations generated using serial 5-fold dilutions, provides a complete dose-response: 100, 20.0, 4.00, 0.800, 0.160, 0.032, and 0.0064 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 agonist NGF-β to achieve the desired fixed assay-concentration (refer to "*A word about antagonist-mode assay setup*", pg. 9). The agonist-supplemented CSM is then used to make dilutions of test compound stock(s) to achieve the desired assay concentration series.

8.) At the end of the 4-6 hours 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. Program the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read is set to 0.5 second (500 mSec) per well, *or less*.

14.) Immediately before proceeding to *Step 15*, prepare **Luciferase Detection Reagent** (**LDR**). Combine 'Detection Buffer' and 'Detection Substrate' by pouring-over their entire volumes into a media basin; rock the basin gently to mix the reagent. The resulting volume of LDR is 12 ml.

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.

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.

18.) Analyze Data.

V. Related Products

Product No.	*Product Descriptions*
Tropomysin Receptor Kinase A (TrkA; NTRK1) Assays	
IB27011	TrkA Assay, 1x 96-well format assay
IB27012	TrkA Assay, 1x 384-well format assays
Tropomysin Receptor Kinase B (TrkB; NTRK2) Assays	
IB27021	TrkB Assay, 1x 96-well format assay
IB27022	TrkB Assay, 1x 384-well format assays
Tropomysin Receptor Kinase C (TrkC; NTRK3) Assays	
IB27031	TrkC Assay, 1x 96-well format assay
IB27032	TrkC Assay, 1x 384-well format assays
Bulk volumes of Trk Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

NFAT Assays (recommended for receptor specificity screening)	
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 96-well 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.

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VI. Citations

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[3] Bruscolini A, *et. al.* (2021) The long-term clinical efficacy of recombinant human nerve growth factor in the treatment of neurotrophic keratitis. Investigative Ophthalmology & Visual Science **62**: 728.

[4] Yang S, *et. al.* (2020) The Efficacy of Nerve Growth Factor Antibody for the Treatment of Osteoarthritis Pain and Chronic Low-Back Pain: A Meta-Analysis. Frontiers in Pharmacology, doi.org/10.3389/fphar.2020.00817.

[5] 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

[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.

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

VII. 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.

APPENDIX 1

Example scheme for the serial dilution of NGF-β and the setup of an TrkA dose-response assay.