Human Tropomyosin Receptor Kinase A Reporter Assay System; TrkA

(Neurotrophic Tyrosine Kinase Receptor, type 1; NTRK1)

3x 32 Assays in 96-well Format
Product # IB27011-32

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Technical Manual
(version 7.2i)

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Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human TrkA Reporter Assay System
3x 32 Assays in 96-well Format

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▪ Background ▪

Tropomyosin 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 Tropomyosin 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 assays capitalize on the 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 (RLUs).

This assay kit features 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.

Stocks of test materials that are **Protein** or **Polypeptide** ligands, or **Antibodies**, should be solvated in aqueous buffered solutions with carrier protein (*e.g.*, PBS + 0.1% BSA) at concentrations *no less* than 10x-concentrated 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).

Immediately prior to setting up an assay, the above master stocks are serially diluted using one of two alternative strategies:

1.) For both **small-molecule** and **proteinaceous** test samples, **Compound Screening Medium (CSM)** may be used as the diluent to achieve the desired assay concentration series, as described in *Step 7* (pg. 9).

Alternatively, if **small-molecule** test compound solubility is expected to be problematic, *2.)* DMSO may be used to make serial dilutions to produce 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. Note: Do not use DMSO as the diluent for proteinaceous test compounds.

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%. Emerging cytotoxicity can be expected above 0.4% DMSO exposure over the 24-hour assay 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.

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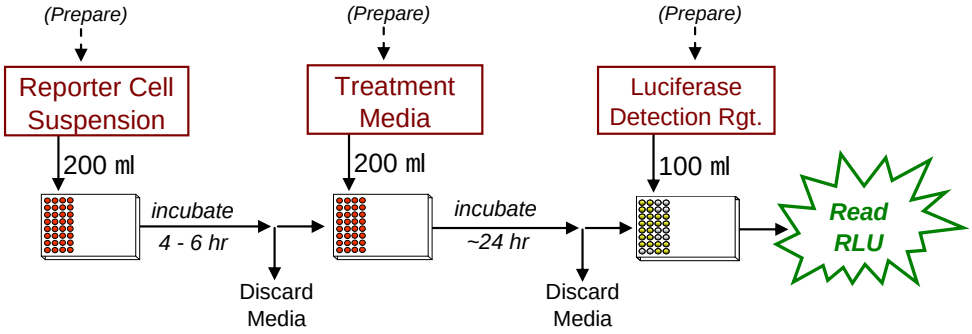
▪ Assay Scheme ▪

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.



▪ Assay Performance ▪

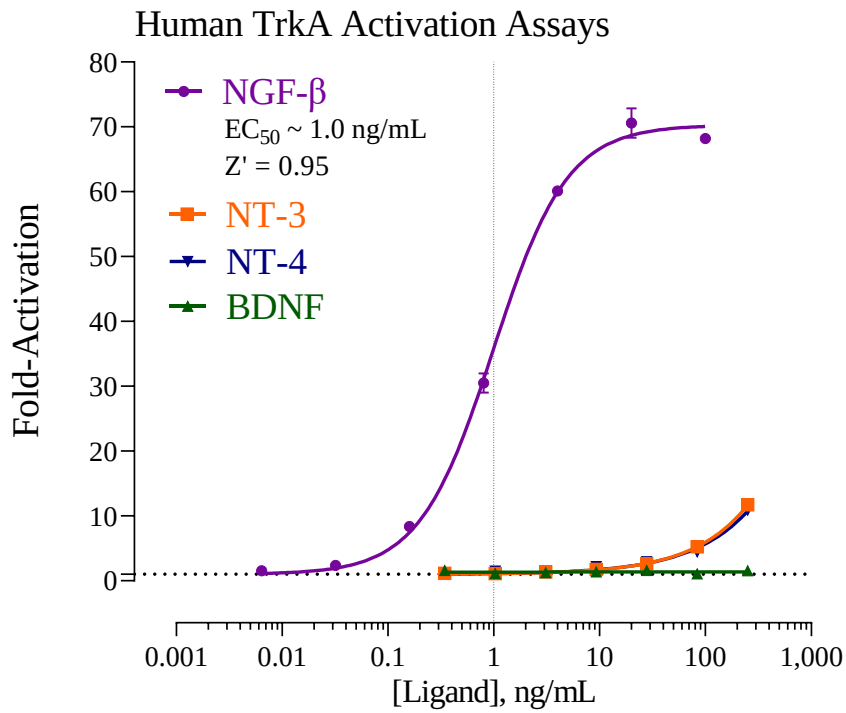


Figure 2. TrkA Activation dose response analyses. Activation dose-response assays were performed according to the protocol provided in this Technical Manual. 200 µl / well of TrkA Reporter Cell suspension was dispensed into the 96-well assay plate, which was then incubated for 4 hours. The concentrated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

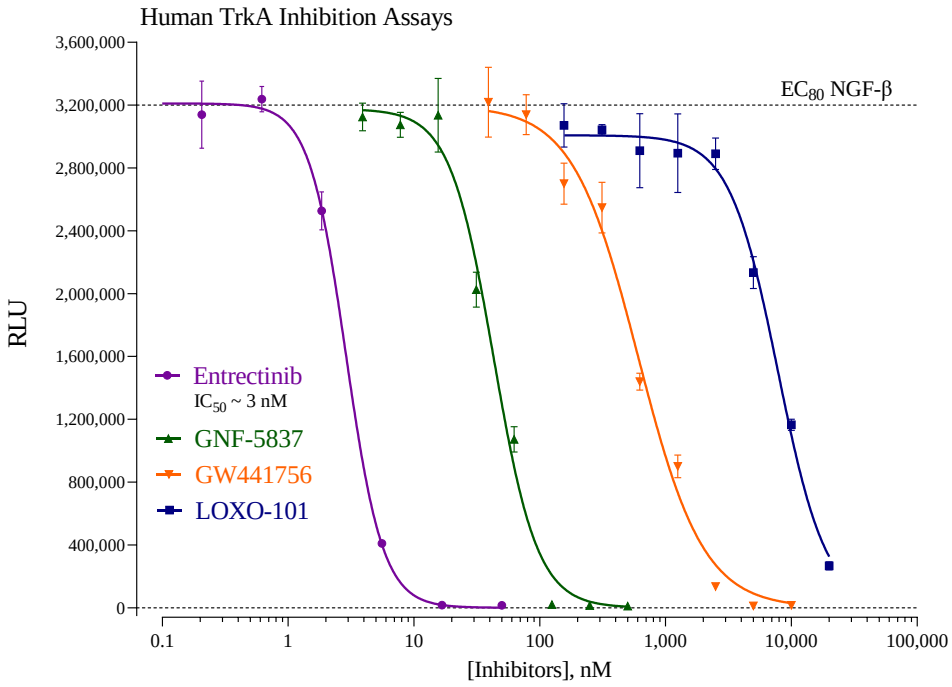


Figure 3. TrkA Inhibition dose-response analyses. TrkA reporter cells were co-treated with an EC_{80} concentration of the reference activator NFG-β and varying concentrations of the TrkA inhibitors Entrectinib, GNF-5837, GW441756 and LOXO-101 (all from Cayman Chemical, Ann Arbor MI, USA). The range 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 three distinct groups of assays in a 96-well plate format. 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.

Cryopreserved mammalian cells are temperature sensitive! **To ensure maximal viability the tubes 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 aliquots of Reporter Cells are 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	3 x 0.6 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/ (in PBS+0.1%BSA) (physiological activator of TrkA)	1 x 30 µL	-20°C
▪ Detection Substrate (Note: contains DTT)	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.

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* sterilized 96 deep-well blocks (*e.g.*, Axygen Scientific, #P-2ML-SQ-C-S), *or* appropriate similar vessel for generating dilution series of reference and test compound(s)
▪ *Optional:* inhibitor reference compound (*e.g.*, Figure 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 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 **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(s) 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 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)** 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 strip-wells. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO carried over into assay reactions should never 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.

~ or ~

***b. Inhibition*-mode assays**. When setting up inhibition assays, first supplement a bulk volume of CSM with the challenge activator NGF-β to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about inhibition-mode assay setup*", pg. 8). The NGF-β - 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** of each treatment media into appropriate wells of 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 the appropriate number of vials of **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 or in a **fume hood.**

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*. To read 32 assay wells, transfer the entire volume of 1 vial of Detection Buffer into 1 vial of Detection Substrate, thereby generating a 4 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

NOTE: 'Detection Substrate' contains a high concentration of DTT, which produces a strong odor that some users may find objectionable. It is advised to work in a **fume hood** when preparing LDR, and subsequently when dispensing it into the assay plate and throughout the 'plate rest' period (*Step 16*).

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.

18.) Data analyses.

V. Related Products

Product No.	*Product Descriptions*
Tropomyosin Receptor Kinase A (TrkA; NTRK1) Assays	
IB27011-32	TrkA Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB27011	TrkA Reporter Assay System 1x 96-well format assay
IB27012	TrkA Reporter Assay System 1x 384-well format assays
Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

Product No.	*Product Descriptions*
Tropomyosin Receptor Kinase B (TrkB; NTRK2) Assays	
IB27021-32	TrkB Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB27021	TrkB Reporter Assay System 1x 96-well format assay
IB27022	TrkB Reporter Assay System 1x 384-well format assays

Product No.	*Product Descriptions*
Tropomyosin Receptor Kinase C (TrkC; NTRK3) Assays	
IB27031-32	TrkC Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB27031	TrkC Reporter Assay System 1x 96-well format assay
IB27032	TrkC Reporter Assay System 1x 384-well format assays

NFAT Assays (recommended for receptor specificity screening)	
IB18001	NFAT Reporter Assay System 1x 96-well format assay

(Continued.)

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

INDIGlo Luciferase Detection Reagent	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents, available in 10 mL, 25 mL, 50 mL, 500 mL, and larger custom volumes

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Citations

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[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 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 recently updated version available.

APPENDIX 1

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