Human Tropomyosin Receptor Kinase A Reporter Assay System; TrkA

(Neurotrophic Tyrosine Kinase Receptor, type 1; NTRK1)

384-well Format Assays

Product # IB27012

▪

Technical Manual
(version 8.0i)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human TrkA Reporter Assay System
384-well Format Assays

I. Description

▪ 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 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 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.

▪ Considerations for the Preparation and Automated Dispensing 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.

Stocks of test materials that are **Protein** or **Poly-peptide** ligands, or **Antibodies**, should be solvated in aqueous buffered solutions with carrier protein (*e.g.*, PBS + 0.1% BSA).

For **384-well format assays** the user will choose to dilute master stocks using one of two alternative methods. The selection of dispensing method to be used will be dictated by the type of instrument that will be used. This Technical Manual provides detailed protocols for each of these two alternative methods:

a.) Assay setups in which a conventional **tip-based** instrument is used to dispense **µL volumes** of for both **small-molecule** and **proteinaceous** test samples into assay wells (protocol is presented in black text). Use **Compound Screening Medium (CSM)** to generate a series of **2x-concentration** test compound treatment media, as described in *Step 2a* of the **Assay Protocol**. The final concentration of DMSO carried over into assay reactions should not exceed 0.4%; strive to use 1,000x-concentrated stocks when they are prepared in DMSO.

NOTE: CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup and are considered to be 'single-use' reagents.

and,

b.) **Acoustic transfer** or **Pin-based dispensing of nL volumes** of test compounds into assay wells (protocol is presented in blue text). Use CSM for proteinaceous test samples, or DMSO for small molecule test samples, to make a series of **1,000x-concentrated** test compound stocks that correspond to each desired final assay concentration, as described in *Step 2b* of the **Assay Protocol**.

▪ Considerations for Automated Dispensing of Other Assay Reagents ▪

When dispensing into a small number of assay plates, first carefully consider the dead volume requirement of your tip-based dispensing 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 on a *per kit* basis. Always pool the individual reporter cell suspensions and all other respective assay kit reagents before processing multiple 384-well assay plates.

Stock Reagent & Volume provided	**Volume to be Dispensed** *(384-well plate)*	**Excess reagent available for instrument dead vol.**
when using tip dispensing of test cmpds **Reporter Cell Suspension** 7.5 ml	15 µl / well 5.8 ml / plate	~ 1.7 ml
when using acoustic dispensing of test cmpds **Reporter Cell Suspension** 15 ml	30 µl / well 11.5 ml / plate	~ 3.4 ml
Detection Substrate 7.8 ml	15 µl / well 5.8 ml / plate	~ 2 ml

▪ Assay Scheme ▪

The *Day 1* preparation, volumes, and chronology of dispensed cells and test compounds are different between assay setups using a *tip-based dispenser* (**1a**) and those using an *acoustic transfer device* (**1b**). Following 22 -24 hours incubation Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.

Figure 1a. Assay workflow if using conventional **tip-based** dispensing of test compounds.

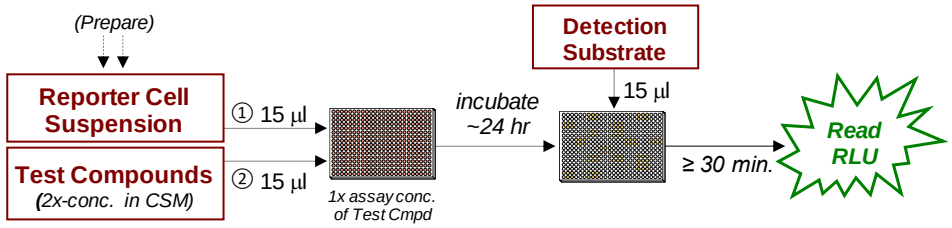
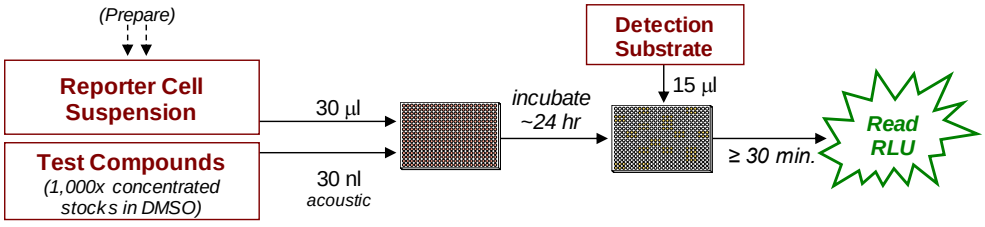


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



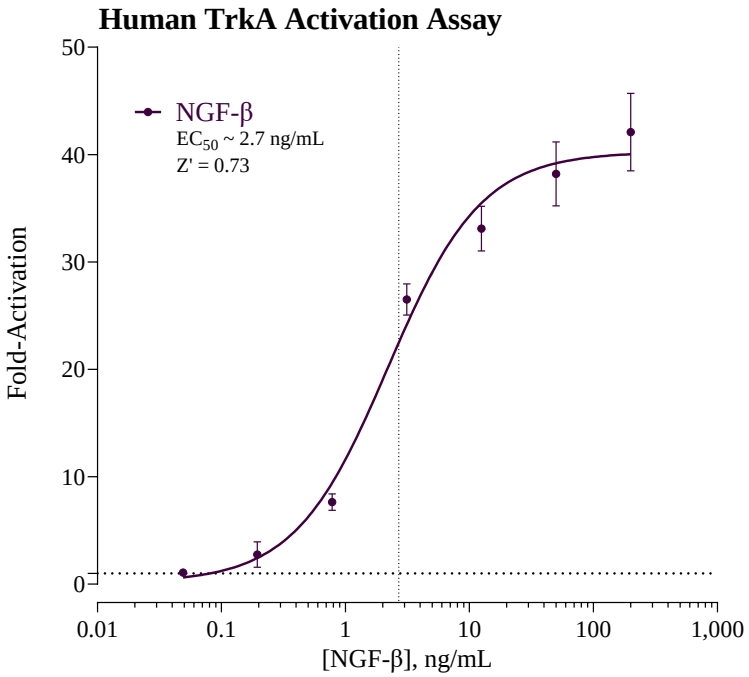


Figure 2. Activation of Human TrkA. Activation assays were performed according to the protocol provided in this Technical Manual using the reference activator NGF-β (provided).

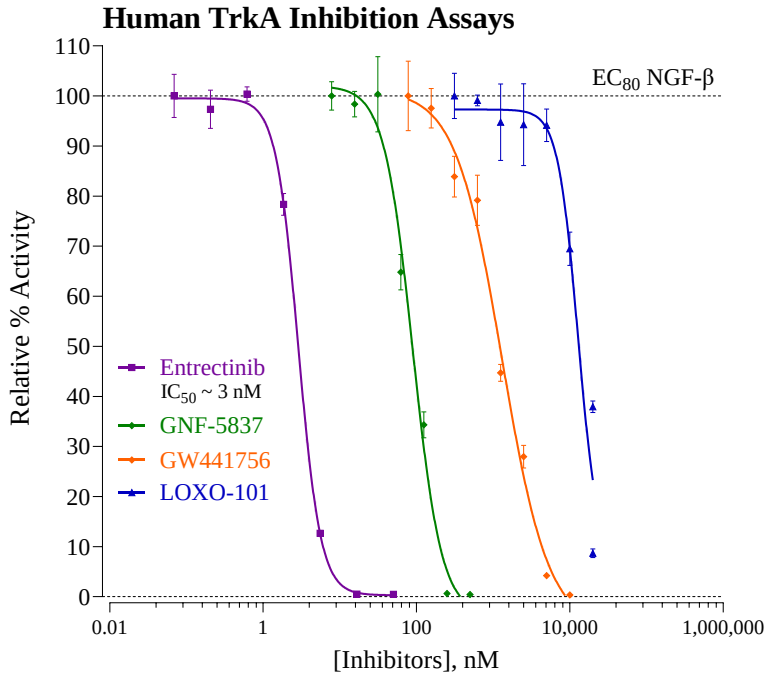


Figure 3. Inhibition of Human TrkA. TrkA reporter cells were co-treated with an EC_{80} concentration of the reference activator NFG-β and varying concentrations of the TrkA inhibitors Entrectinib, GNF-5837, GW441756 and LOXO-101 (all from Cayman Chemical, Ann Arbor MI, USA). The range of determined IC_{50} values is shown; (n = 3 / conc.).

For both the activation assay (Figure 2) and inhibition assay (Figure 3), luminescence was quantified and values of average (n = 3) relative light units (RLU), corresponding standard deviation (SD), Fold-Activation, % Relative RLU and Z'[6] values were calculated. Non-linear regression analyses of Fold-Activation or Relative % RLU *vs.* Log_{10} [Compound, nM] and EC_{50} / IC_{50} values were determined using GraphPad Prism software.

II. Product Components & Storage Conditions

This TrkA Reporter Assay kit contains materials to perform assays in a single 384-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 1.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 7 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ NGF-β, 200 µg/ml (in PBS/0.1%BSA) (reference activator of TrkA)	1 x 60 µL	-20°C
▪ Detection Substrate	1 x 7.8 mL	**-80°C**
▪ 384-well assay plate (white, sterile, cell-culture ready)	1	Ambient

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

▪ Instrumentation suitable for dispensing 15 µl volumes

▪ disposable media basins, sterile.

▪ sterile multi-channel media basins *or* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).

▪ *Optional:* inhibitor reference compound (*e.g.*, Figure 3)

▪ *Optional:* clear 384-well assay plate, cell culture treated, for viewing cells on *Day 2*.

DAY 2 plate-reading luminometer

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-8* are performed on ***Day 1***, requiring less than 2 hours to complete. *Steps 9-13* 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 TrkA Assay kit includes a 200 µg/mL stock solution of NGF-β, a potent physiological activator of TrkA, that may be used to set up inhibition-mode assays. ~ 8.0 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.

Adding the challenge activator NGF-β to the bulk suspension of Reporter Cells (*i.e.*, prior to dispensing into assay wells) is the most efficient and precise method of setting up inhibition assays, and it is the method presented in *Step 5b* of the protocol when performing tip-based dispensing, and *Step 6b* of the protocol when using an acoustic transfer device to dispense test compounds.

Note that when using a *tip-based instrument* for the dispensing of 2x-concentrated test compounds the cell suspension must also be supplemented with a **2x-**concentration (~ 16 ng / mL) of the challenge activator NGF-β.

When using an *acoustic transfer* device for the dispensing of 1,000x-concentrated test compounds the cell suspension should be supplemented with a **1x-**concentration (~ 8.0 ng/mL) of the challenge activator NGF-β.

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

1.) Remove **Cell Recovery Medium (CRM)** and **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

2.) Prepare dilutions of test compounds: Prepare Test Compound treatment media for *Activator-* or *Inhibition-mode* screens. NOTE that test and reference compounds will be prepared differently when using tip-dispensing *vs.* acoustic dispensing. Regardless of the method, the total DMSO carried over into assay reactions should not exceed 0.4%.

a. *Tip dispensing method*: In *Step 6*, 15 µl / well of the prepared treatment media is added to the assay that has been pre-dispensed with 15 µl /well of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a **2x-**concentration of the test and reference material(s). Use **CSM** to prepare the appropriate dilution series. Plan dilution volumes carefully; this assay kit provides 45 ml of CSM.

b. *Acoustic dispensing method*: In *Step 6*, 30 nl / well of **1,000x**-concentrated test compound solutions are added to the assay plate using an acoustic transfer device.

**NOTE:* Stocks of test samples that are small-molecule chemicals / drugs are typically prepared in DMSO and, for acoustic transfer dispensing, we recommend that DMSO (not CSM) is used as the diluent to generate the desired series of 1,000x-treatment concentrations. However, stocks of test samples that are solvated in aqueous solution, such as protein ligands and antibodies, should be further diluted using CSM (*not* DMSO).

Preparing the positive control: This assay kit includes a 1,000x concentrated stock of the polypeptide NGF-β, 200 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: 200, 50.0, 12.5, 3.13, 0.781, 0.195 and 0.049 ng/mL. Always include 'no treatment' (or 'vehicle') controls.

APPENDIX 1a provides an example for generating such a dilution series to be used when *tip-dispensing* compound solutions prepared in CSM (15 µl / well).

(continued …)

APPENDIX 1b provides an example for generating such a series of 1,000x-concentrated solutions of compounds to be used when performing *acoustic dispensing* (30 nl / well). As noted in *Step 2b*, use CSM to dilute sample and reference stocks that have been prepared in aqueous solutions (*e.g.*, protein ligands, antibodies, *etc.*), or use DMSO to further dilute sample stocks that were initially solvated in DMSO (*e.g.* small molecule chemicals).

When using t*ip-based* instrumentation for dispensing test compounds …

3.) *First*, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab.

Second, retrieve **Reporter Cells** from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. Perform a *rapid thaw* of the frozen cells by transferring a **6.5 ml** volume of 37°C CRM into the tube of frozen cells. Recap the tube of Reporter Cells and place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.5 ml.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab, then transfer it into the cell culture hood.

5.) Gently invert the tube of cells several times to gain a homogenous suspension.

a. **for *Activation*-mode assays**: Dispense **15 µl / well** of cell suspension into the assay plate.

~ or ~

b. **for Inhibition-mode assays**: Supplement the bulk volume of Reporter Cells suspension with a 2x-concentration of the challenge activator (refer to "*A word about Inhibition-mode assay setup*", pg. 8). Dispense **15 µl / well** of cell suspension into the assay plate.

6.) Dispense **15 µl / well** of 2x-concentrated treatment media (from *Step 2a*) into the assay plate.

When using an *acoustic transfer* device for dispensing test compounds …

3.) Dispense **30 nl / well** of the 1,000x-concentrated compounds (from *Step 2b*) into the assay plate.

4.) *First*, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab;

Second, retrieve **Reporter Cells** from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. Perform a *rapid thaw* of the frozen cells by transferring a **6.5 ml** volume of 37°C CRM into the tube of frozen cells. Recap the tube of cells and place it in a 37°C water bath for 5 - 10 minutes.

5.) Retrieve the tube of cell suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab. Add an additional **7.5 ml** of **CSM** to the tube. The resulting volume of cell suspension will be 15 ml.

6.) Gently invert the tube of cells several times to gain a homogenous cell suspension.

a. **for *Agonist*-mode assays**: Dispense **30 µl / well** of cell suspension into the assay plate that has been pre-dispensed with test compounds.

~ or ~

b. **for *Inhibition*-mode assays:** First supplement the bulk volume of TrkA Reporter Cells suspension with the challenge activator **NGF-β** to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about inhibition-mode assay setups*", pg. 8). Then dispense **30 µl / well** of the supplemented cell suspension into the assay plate that has been pre-dispensed with test compounds.

(continued …)

NOTE: Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate *will* cause well-to-well variation (= increased Standard Deviation) in the assay.

NOTE: Following the dispensing of Reporter Cells and test compounds INDIGO recommends performing a *low-speed* spin of the assay plate (with lid) for ≤ 1 minute using a room temperature centrifuge fitted with counter-balanced plate carriers.

7.) Transfer the assay plate into a 37°C, humidified, 5% CO_2 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.

8.) For greater convenience on *Day 2*, retrieve **Detection Substrate** from freezer storage and place 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**.

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

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

10.) 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. Set the read time to 0.5 second (500 mSec) per well, *or less*.

11.) Following 22 - 24 hours of incubation dispense **15 µl / well** of **Detection Substrate** into all wells of the assay plate.

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 dispensing Detection Substrate into the assay plate and throughout the following 'plate rest' period.

NOTE: Scattered micro-bubbles in the assay wells will not pose a problem. However, bubbles covering the surface of the reaction mix, or large bubbles clinging to the side walls of the well, will cause lens-effects that will degrade the accuracy and precision of the assay data. It is advised to perform a final *low-speed* spin of the assay plate (with lid) for ≤ 1 minute using a room temperature centrifuge fitted with counter-balanced plate carriers.

12.) Allow the plate(s) to rest at room temperature for 30 minutes. Do not shake the assay plate(s) during this period.

NOTE: the luminescent signal is somewhat unstable during the first 30 minutes of the luciferase reaction, however, after the initial 30-minute reaction period the luminescence signal achieves a stable emission output.

13.) Quantify luminescence.

14.) Data analyses.

V. Related Products

Product No.	*Product Descriptions*
Tropomyosin Receptor Kinase A (TrkA; NTRK1) Assays	
IB27011	TrkA Assay, 1x 96-well format assay
IB27012	TrkA Assay, 1x 384-well format assays
Tropomyosin Receptor Kinase B (TrkB; NTRK2) Assays	
IB27021	TrkB Assay, 1x 96-well format assay
IB27022	TrkB Assay, 1x 384-well format assays
Tropomyosin 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 10 mL, 25 mL, 50 mL, and 500 mL; custom volumes available

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Citations

[1] Huang EJ, *et al.* (2001) Neurotrophins: Roles in Neuronal Development and Function. Annual Review of Neuroscience **24**: 677-736.

[2] Laetsch TW, *et al*. (2021) Tropomysin Receptor Kinase Inhibitors for the Treatment of TRK Fusion Cancer. Clinical Cancer Research **27**: 4974-4982.

[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.

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

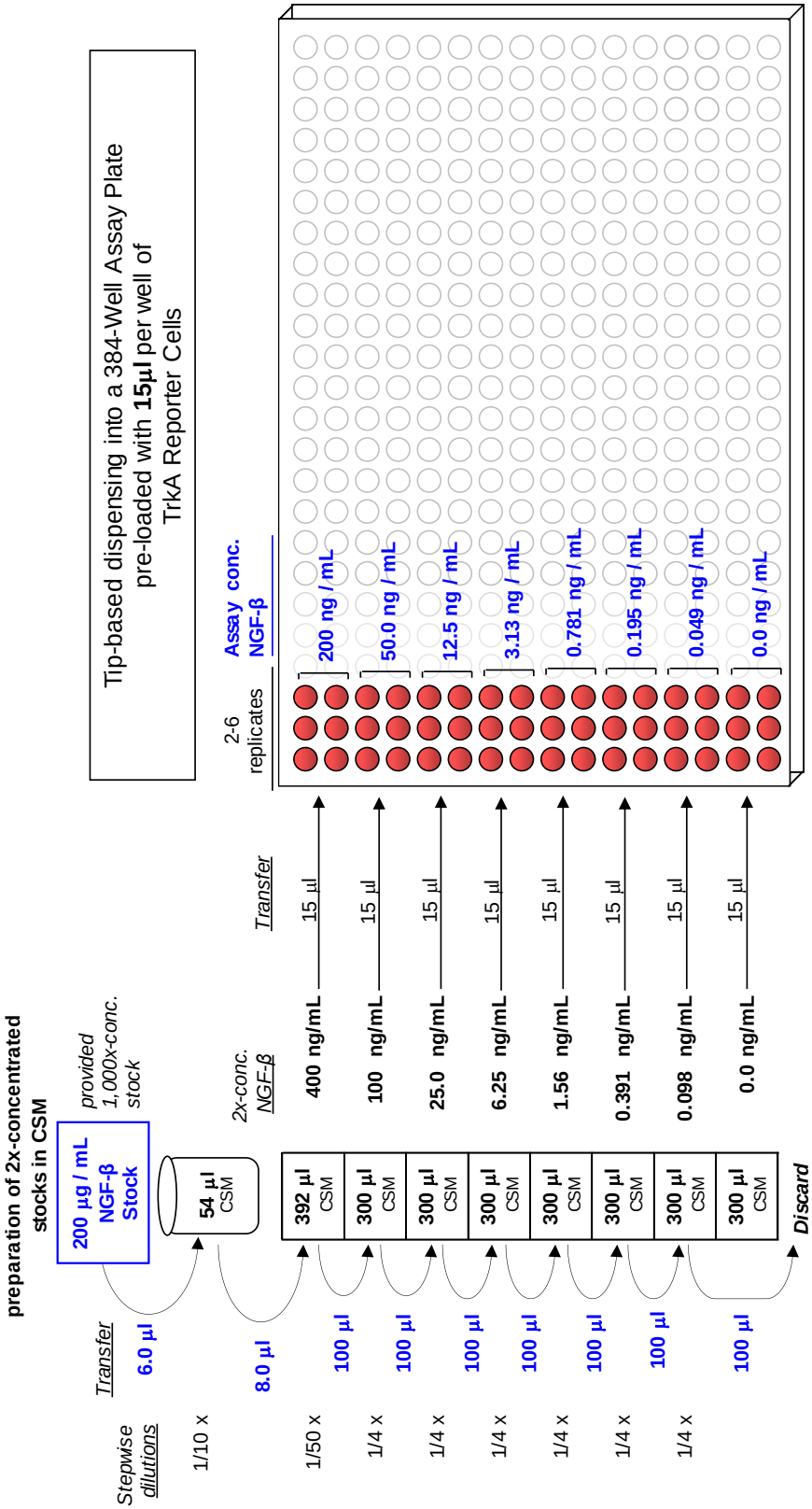
VII. Limited Use Disclosures

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

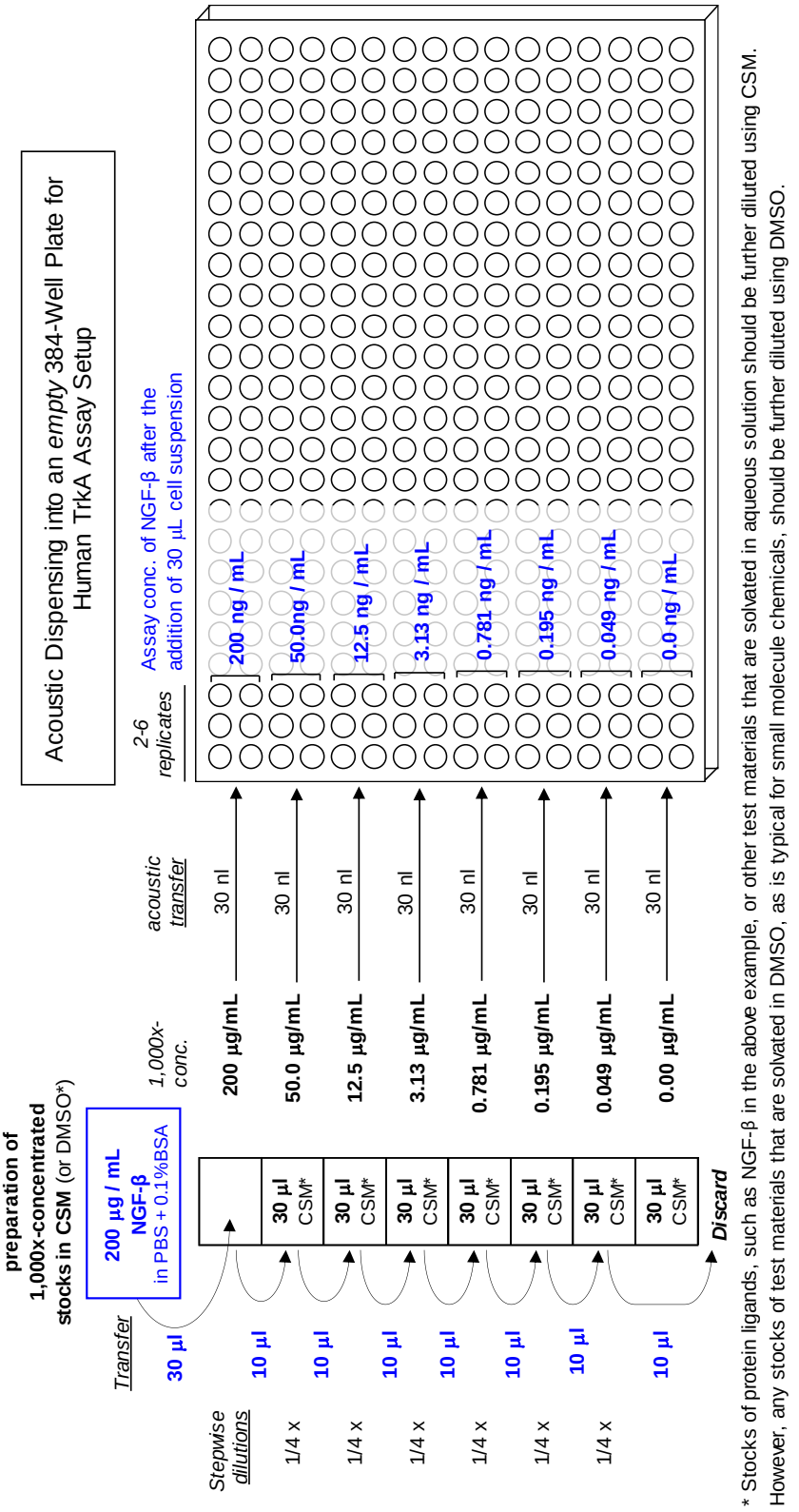
"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA)

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

APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of the reference activator NGF-β into CSM to generate **2x-concentrated** treatment media. 15 µl / well are dispensed into assay plates using a *tip-based* instrument.



APPENDIX 1b for acoustic dispensing. Example scheme for the serial dilution of the reference activator NGF-β (a protein) into CSM to generate **1,000x-concentrated** stocks. 30 nl / well are pre-dispensed into assay plates using an acoustic transfer device. **NOTE:* Stocks of small-molecule test drugs are typically prepared in DMSO, and **DMSO** (*not* CSM) should be further used to generate the desired series of 1,000x-treatment concentrations.



* Stocks of protein ligands, such as NGF-β in the above example, or other test materials that are solvated in aqueous solution should be further diluted using CSM. However, any stocks of test materials that are solvated in DMSO, as is typical for small molecule chemicals, should be further diluted using DMSO.