Human Fibroblast Growth Factor Receptor 4 Assay for Paracrine FGF Signaling

(FGFR4)

384-well Format Assays
Product # IB43002
▪

Technical Manual
(version 8.0)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human FGFR4 for Paracrine Signaling Reporter Assay System 384-well Format Assays

I. Description

▪ Background ▪

The family of Fibroblast Growth Factors (FGFs) comprise approximately 23 members that are related by core sequence and structure conservation, with the majority of FGFs being secreted signaling proteins. Secreted FGFs are predominantly autocrine and paracrine factors, with only three members evolved to function as endocrine factors[1].

FGFs bind and activate four FGF Receptors (FGFR1-4) which, themselves, are members of the family of high-affinity tyrosine kinase receptors[1]. Heparin and heparin sulfate proteoglycans (HSPGs) are essential cofactors for paracrine FGF (*e.g.*, FGF-1 and FGF-2) interactions with FGFRs. The association between paracrine FGFs and HSPGs ensures their limited diffusion and enhanced FGFR binding specificity.

In contrast to the paracrine ligand activators of FGFR, endocrine FGFs (*e.g.*, FGF-19, FGF-21, and FGF-23) have minimal affinity to heparin. Rather, they typically require association with members of the Klotho family of proteins as cofactors for efficient binding to their cognate receptor(s)[1, 2]. Although, FGF-23 activation of FGFR3 can occur in a Klotho independent manner, signaling through the PLCγ/calcineurin/NFAT pathway [3, 4].

The FGFs are broad-spectrum mitogens that, through their receptor interactions, regulate a variety of cellular functions including migration, proliferation, differentiation, metabolism and survival[1, 2]. In particular, FGF/FGFR signaling plays a critical role in regulating metabolism in the kidney, liver, brain, intestine and adipose tissues[1, 2]. Not surprisingly, dysfunctional FGFR signaling can lead to a range of physiological disorders. For example, mutation, amplification, and gene fusion may result in abnormal morphogenesis and the progression of several types of cancer[2]. Consequently, the FGF receptors continue to command much interest as targets for drug development and drug safety screening.

▪ The Assay System ▪

This assay utilizes proprietary human cells that have been engineered to provide constitutive expression of the **Human Fibroblast Growth Factor Receptor 4 (FGFR4)**.

INDIGO's Compound Screening Media is supplemented with heparin, thereby enabling the formation of FGF-2/Heparin complexes that bind with high-affinity to FGFR monomers. This binding interaction triggers conformational changes that drive the assembly of homo-dimeric receptors, and the activation of their respective cytosolic tyrosine kinase domains[1].

The tyrosine kinase activities of activated FGFR4 initiate intracellular signaling cascades that include RAS-MAPK, PI3-AKT, PLCγ and STAT pathways[1]. For example, activation of the PLCγ pathway leads to an increase of intracellular calcium[1]. One prominent outcome of the FGF/FGFR > PLCγ pathway is that calcineurin, a calcium-dependent phosphatase, dephosphorylates and activates the transcription factor NFAT[5]. It is FGFR4 signal transduction *via* the Ca^{+2}·calcineurin / NFAT cascade that is exploited by the reporter cells provided in this kit.

INDIGO's FGFR4 for Paracrine FGF Signaling Reporter Cells contain the luciferase reporter gene functionally linked to tandem NFAT consensus response element sequences upstream of a minimal promoter. Activated NFAT binds to these response elements to initiate the formation of a complete transcription complex that drives Luc gene expression. Quantifying relative changes in luciferase activity in the treated reporter cells relative to the untreated cells provides a sensitive, dose-dependent surrogate measure of drug-induced changes in FGFR4 activity. Accordingly, the principal application of this reporter assay is in the screening of test materials to quantify any functional interactions, either activating or inhibitory, that they may exert against FGFR4, or the coupled Ca^{+2}·calcineurin/NFAT signal transduction pathway. To reiterate, this assay is primarily focused on the paracrine signaling activities of FGFR4/FGFs.

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.

(*continued*)

This assay kit provides the convenience of an all-inclusive cell-based reporter assay system for FGFR4 paracrine FGF signaling. In addition to Reporter Cells, included are an optimized culture medium for use in reviving the cryopreserved cells, a culture medium supplemented with heparin for use in preparing test sample treatments, the physiological paracrine activator FGF-2 (*aka* FGF-Basic), Luciferase Detection Reagents, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's receptor reporter 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, and yields as products 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 30 and 100+ minutes after initiating the luciferase reaction. Incorporating a 30-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+H)** 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+H contains heparin. In addition, it 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+H (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.

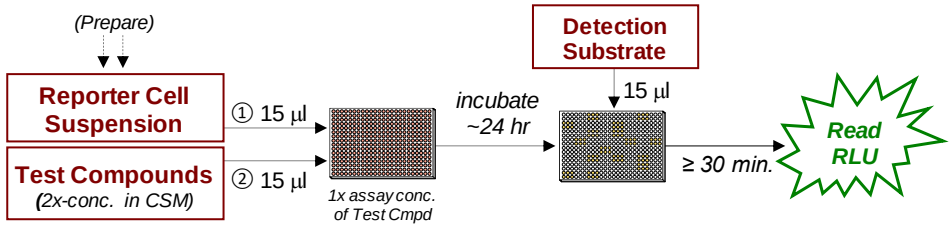
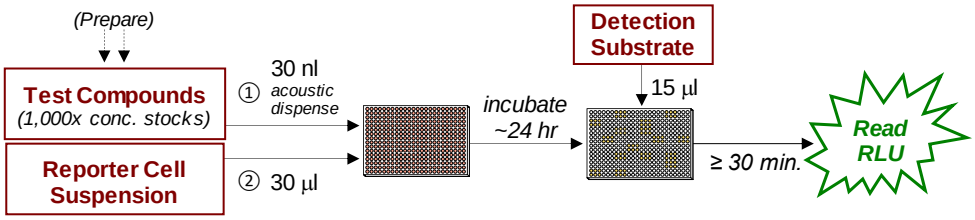


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



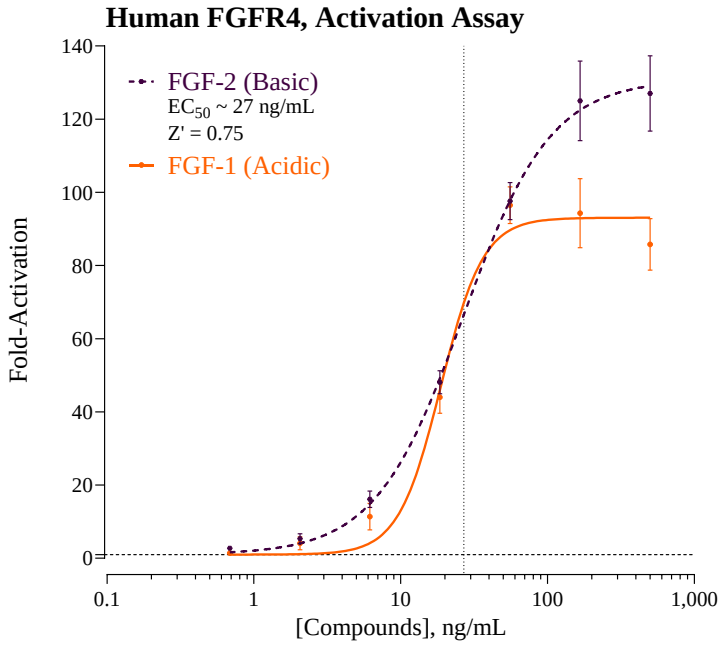


Figure 2. Activation of FGFR4. Activation assays were performed using the paracrine reference activators FGF-2 (*aka* FGF-Basic; provided) and FGF-1 (*aka* FGF-Acidic). Both polypeptide ligands were procured from Peprotech.

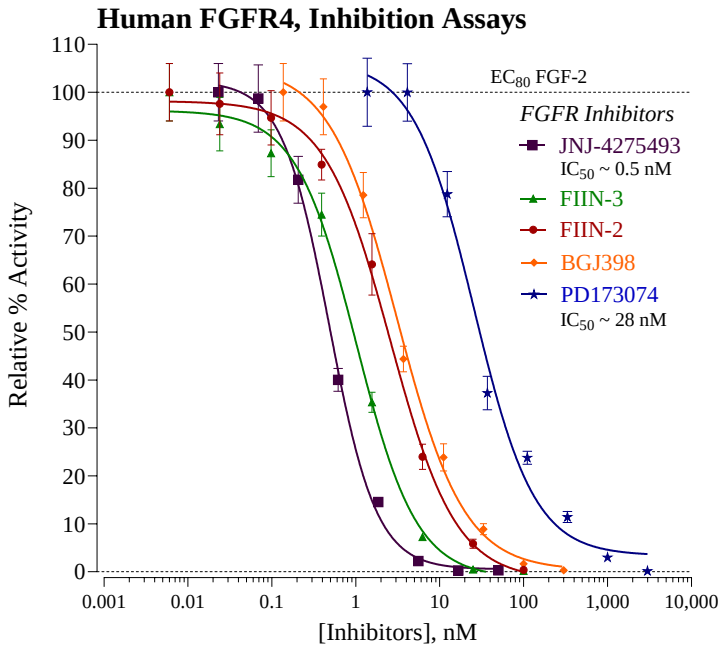


Figure 3. Inhibition of FGFR4. FGFR4 cells were treated with an EC80 concentration of the reference activator FGF-2 and varying concentrations of the FGFR inhibitors JNJ-4275493, FIIN-3, FIIN-2, BGJ398 and PD173074 (all compounds obtained from Cayman Chemical). For both Activation and Inhibition assays, luminescence was quantified and values of average (n=3) relative light units (RLU), corresponding standard deviation (SD), Fold-Activation, % Relative Activity and Z'[5] values were calculated. GraphPad Prism software was used to plot data using the least-squares method of non-linear regression for Fold-Activation or Relative % Activity *vs.* Log_{10} [Cmpd], and to determine EC_{50} / IC_{50} values.

II. Product Components & Storage Conditions

This FGFR4 for Paracrine FGF Signaling 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 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 inventory the individual kit components be sure to first transfer and submerge the tube of cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, the 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.***
▪ FGFR4 Reporter Cells	1 x 1.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 7 mL	-20°C
▪ Compound Screening Medium (CSM+H) (supplemented with Heparin)	1 x 45 mL	-20°C
▪ FGF-2, 500 µg/mL (in PBS+0.1% BSA) (physiological paracrine activator of FGFR4)	1 x 80 µL	-20°C
▪ Detection Substrate (Note: contains DTT)	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 constant, 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 inhibition activity. This assay kit includes a 500 µg/mL stock solution of FGF-2, the physiological paracrine activator of FGFR4, that may be used to set up inhibition-mode assays. ~80 ng/mL FGF-2 typically approximates EC_{80} in this assay and, therefore, is a suitable *final assay concentration* of activator to be used when screening test compounds for inhibitory activity.

Adding the challenge activator FGF-2 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 (~ 160 ng / mL) of the challenge activator FGF-2.

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 (~ 80 ng/mL) of the challenge activator FGF-2.

DAY 1 Assay Protocol:

All steps should be performed using proper aseptic technique.

1.) Remove **Cell Recovery Medium (CRM)** and **Compound Screening Medium (CSM+H)** 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+H** to prepare the appropriate dilution series. Plan dilution volumes carefully; this assay kit provides 45 ml of CSM+H.

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+H) 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+H (*not* DMSO).

Preparing the positive control: This assay kit includes a 1,000x concentrated stock of the polypeptide FGF-2, 500 µg/mL prepared in PBS+0.1%BSA. The following 7-point treatment series, with concentrations generated using serial 3-fold dilutions, provides a complete dose-response: 500, 167, 55.6, 18.5, 6.17, 2.06, and 0.686 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+H (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+H 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 *tip-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 the agonist-supplemented 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+H** 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 FGFR4 Reporter Cells suspension with the challenge activator **FGF-2** 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 agonist-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 it 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

Fibroblast Growth Factor 4 Assay for Paracrine FGF Signaling	
IB43001	FGFR4 Assay for Paracrine FGF signaling 1x 96-well format assay
IB43002	FGFR4 Assay for Paracrine FGF signaling 1x 384-well format assays

Fibroblast Growth Factor 4/α-Klotho Assay for Endocrine FGF Signaling	
IB43101	FGFR4/α-Klotho Assay for Endocrine FGF signaling 1x 96-well format assay
IB43102	FGFR4/α-Klotho Assay for Endocrine FGF signaling 1x 384-well format assays

Fibroblast Growth Factor 4/β-Klotho Assay for Endocrine FGF Signaling	
IB43201	FGFR4/β-Klotho Assay for Endocrine FGF signaling 1x 96-well format assay
IB43202	FGFR4/β-Klotho Assay for Endocrine FGF signaling 1x 384-well format assays

Fibroblast Growth Factor Receptor 1 / 2 Assay for Paracrine FGF Signaling	
IB21001-32	FGFR1/2 Assay for Paracrine FGF Signaling 3x 32 assays in 8-well strips (96-well plate format)
IB21001	FGFR1/2 Assay for Paracrine FGF Signaling 1x 96-well format assay
IB21002	FGFR1/2 Assay for Paracrine FGF Signaling 1x 384-well format assays

Fibroblast Growth Factor 1c/β-Klotho Assay for Endocrine FGF Signaling	
IB22001-32	FGFR1c/β-Klotho Assay for Endocrine FGF signaling 3x 32 assays in 8-well strips (96-well plate format)
IB22001	FGFR1c/β-Klotho Assay for Endocrine FGF signaling 1x 96-well format assay
IB22002	FGFR1c/β-Klotho Assay for Endocrine FGF signaling 1x 384-well format assays

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Fibroblast Growth Factor 1c/α-Klotho Assay for Endocrine FGF Signaling	
IB40001	FGFR1c/α-Klotho Assay for Endocrine FGF signaling 1x 96-well format assay
IB40002	FGFR1c/α-Klotho Assay for Endocrine FGF signaling 1x 384-well format assays
Bulk volumes of FGFR Assay Reagents may be custom manufactured to accommodate any scale of HTS. ***NOTE:*** **Single receptor FGFR1 or FGFR2 Assays are available upon request**. Please Inquire.	

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

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in **5x bulk volume** to perform **480** Live Cell Assays 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

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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.

VII. Citations

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[2] Xie, Y, *et al.* (2020) FGF/FGFR signaling in health and disease. Signal Transduction and Targeted Therapy (Springer Nature) **5**:181, 1-38.

[3] Bo, BB, *et. al.* (2021) FGF-23 signaling and Physiology. Journal of Molecular Endocrinology. **66**(2): R23-R32.

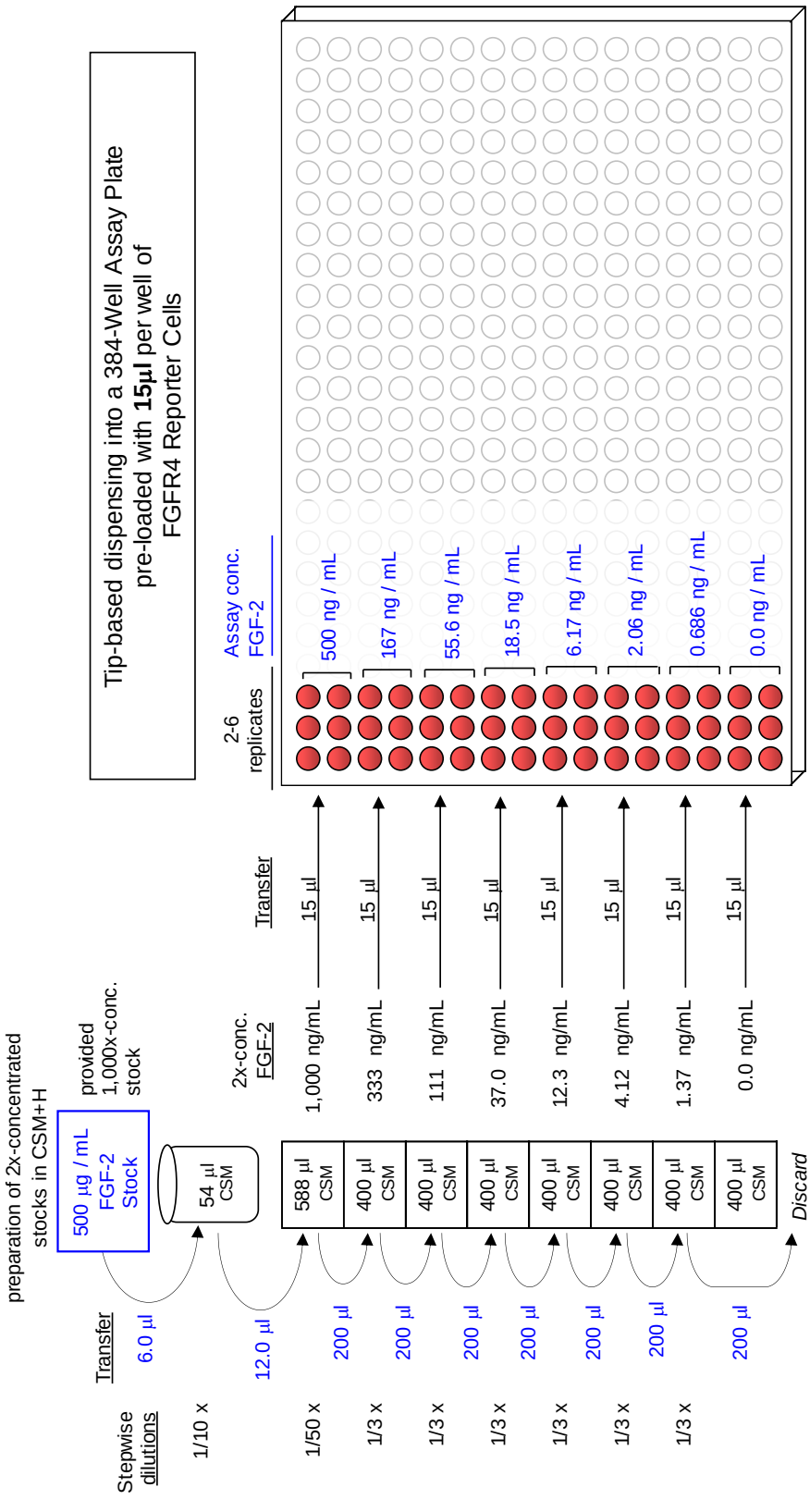
[4] Richter, B, *et. al.* (2018) FGF-23 Action on Target Tissues – With and Without Klotho. Frontiers in Endocrinology. doi: 10.3389/fendo.2018.00189.

[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.

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

APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of the reference agonist FGF-2 into CSM+H 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 agonist FGF-2 (a protein) into CSM+H 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+H) should be further used to generate the desired series of 1,000x-treatment concentrations.