



**Human Fibroblast Growth Factor 4/ α -Klotho
for Endocrine FGF Signaling Reporter Assay System
(FGFR4/ α -Klotho)**

384-well Format Assays
Product # IB43102

▪

Technical Manual
(version 8.0i)

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Human FGFR4/ α -Klotho for Endocrine FGF Signaling Reporter Assay System 384-well Format Assays

I. Description	
▪ Background	3
▪ The Assay System.....	3
▪ The Assay Chemistry.....	4
▪ Considerations for the Preparation and Dispensing of Test Compounds.....	4
▪ Considerations for Automated Dispensing of Other Assay Reagents.....	5
▪ Assay Scheme.....	5
▪ Assay Performance.....	6
II. Product Components & Storage Conditions	7
III. Materials to be Supplied by the User.....	7
IV. Assay Protocol	
▪ A word about <i>Inhibition</i> -mode assay setup.....	8
▪ <i>DAY 1 Assay Protocol</i>	8
▪ <i>DAY 2 Assay Protocol</i>	10
V. Related Products.....	11
VI. Limited Use Disclosures.....	12
VII. Citations.....	13
APPENDIX 1: Example Scheme for Serial Dilution when using tip-based dispensing of test compounds.....	14

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 factors¹. FGFs bind and activate FGF Receptors (FGFRs) which, themselves, are members of the family of high-affinity tyrosine kinase receptors¹.

Paracrine FGFs show high affinity towards the extracellular matrix (ECM) component heparin sulfate (HS) and are thus retained in the ECM and function locally. In contrast, the atypical endocrine subfamily of FGFs, that comprise FGF-19, FGF-21, and FGF-23, have reduced affinity for HS and can therefore escape from the ECM into the circulation to reach target distant organs². However, this subfamily typically requires association with members of the Klotho family of proteins as cofactors for efficient binding to their cognate receptor(s)^{1, 2, 3}.

FGF-23 is a principal regulator in the maintenance of serum phosphorus concentration by inhibiting renal tubular phosphate reabsorption⁴. Unfortunately, various FGF-23-mediated disorders exist. The most common of these is X-linked hypophosphatemia (XLH). Additional disorders of FGF-23 signaling include autosomal dominant and recessive hypophosphatemic rickets, fibrous dysplasia, and tumor-induced osteomalacia⁴. Consequently, FGF-23 and FGFR4/ α -Klotho command considerable interest as therapeutic targets in 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 and α -Klotho**, referred to herein as FGFR4/ α -Klotho. FGFR4 and α -Klotho are both single-pass transmembrane proteins. FGFR4 has an extracellular ligand-binding domain, transmembrane domain, and intracellular tyrosine kinase domain¹. It has been established that FGFR4 association with the co-receptor α -Klotho generates a scaffold that is essential for **endocrine growth factor** binding interactions, such as those with FGF-23⁴. Following growth factor binding, the activated tyrosine kinase activities of the FGFR initiate intracellular signaling cascades that may include RAS-MAPK, PI3-AKT, PLC γ and/or STAT pathways¹. For example, activation of the PLC γ pathway leads to an increase of intracellular calcium¹. One prominent outcome of the FGF/FGFR > PLC γ pathway is that calcineurin, a calcium-dependent phosphatase, dephosphorylates and activates the transcription factor NFAT⁵. It is FGFR4/ α -Klotho signal transduction *via* the Ca⁺²-calcineurin / NFAT cascade that is exploited by the reporter cells provided in this kit.

INDIGO's FGFR4/ α -Klotho for Endocrine FGF Signaling Reporter Cells contain the luciferase reporter gene functionally linked to an NFAT-AP-1 responsive promoter. Activated NFAT binds to the 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/ α -Klotho 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/ α -Klotho or the coupled NFAT pathway.

INDIGO's Reporter Cells are transiently transfected and prepared as frozen stocks using a proprietary **CryoMite™** process. This cryopreservation 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 FGFR4/ α -Klotho endocrine FGF signaling. In addition to Reporter Cells, included are an optimized culture medium for use in reviving the cryopreserved cells, a culture medium for use in preparing test sample treatments, the physiological endocrine activator FGF-23, Luciferase Detection Reagents, and a cell culture-ready assay plate. To re-iterate, this assay is primarily focused on the *endocrine* signaling activities of FGFR/FGFs.

▪ 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.

▪ 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 **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 FGF-23 stock included with this kit is prepared in PBS + 0.1% BSA at a 100x-concentration relative to the highest recommended treatment.

For FGFR4/ α -Klotho **384-well format assays** the user will dilute master stocks using one method. This Technical Manual provides a detailed protocol for a tip-dispensing method only:

Assay setups in which a conventional **tip-based** instrument is used to dispense **μ L volumes** of for both **small-molecule** and **proteinaceous** test samples into assay wells. Use **Compound Screening Medium (CSM)** to generate a series of **2x-concentration** test compound treatment media, as described in Step 2 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.

▪ **Considerations for Automated Dispensing of Other Assay Reagents** ▪

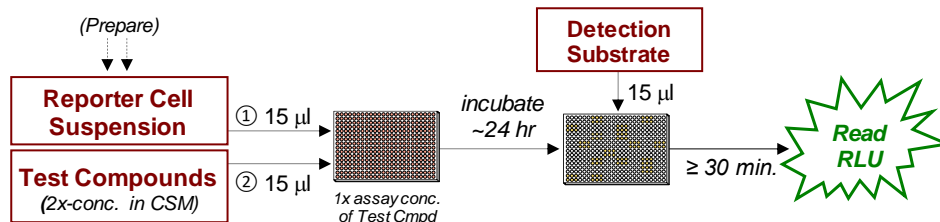
When dispensing into a small number of assay plates, first carefully consider the dead volume requirement of your tip-based dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available 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.
<i>when using tip dispensing of test cmpds</i> Reporter Cell Suspension 7.5 ml	15 µl / well 5.8 ml / plate	~ 1.7 ml
Detection Substrate 7.8 ml	15 µl / well 5.8 ml / plate	~ 2 ml

▪ **Assay Scheme** ▪

Assay workflow. *In brief*, 15 µl of Reporter Cells are dispensed into wells of the assay plate, then 15 µl/well of the prepared 2x-conc. treatment media are added. Following 22 - 24 hours incubation Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.

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



▪ Assay Performance ▪

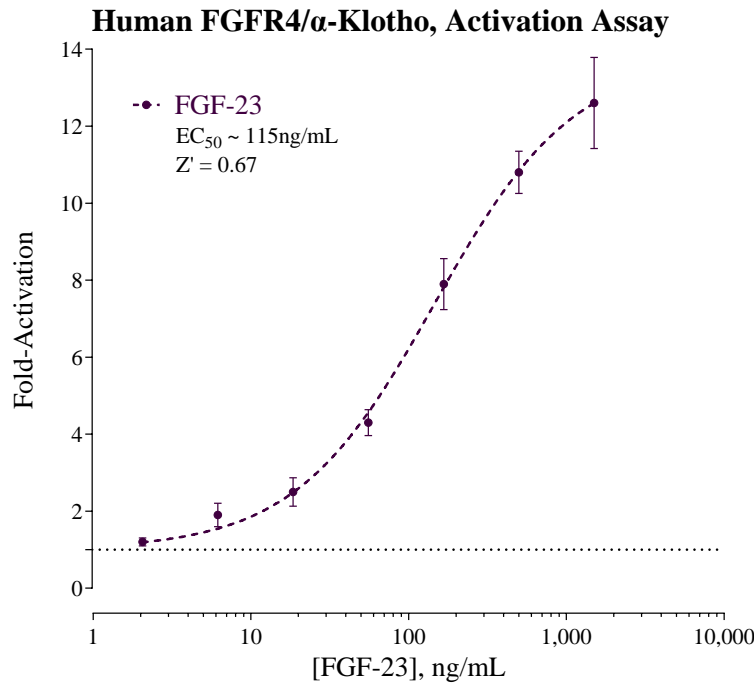


Figure 2. Activation of FGFR4/ α -Klotho. Activation assay was performed according to the protocol provided in this Technical Manual using the reference activator FGF-23 (provided; Peprotech).

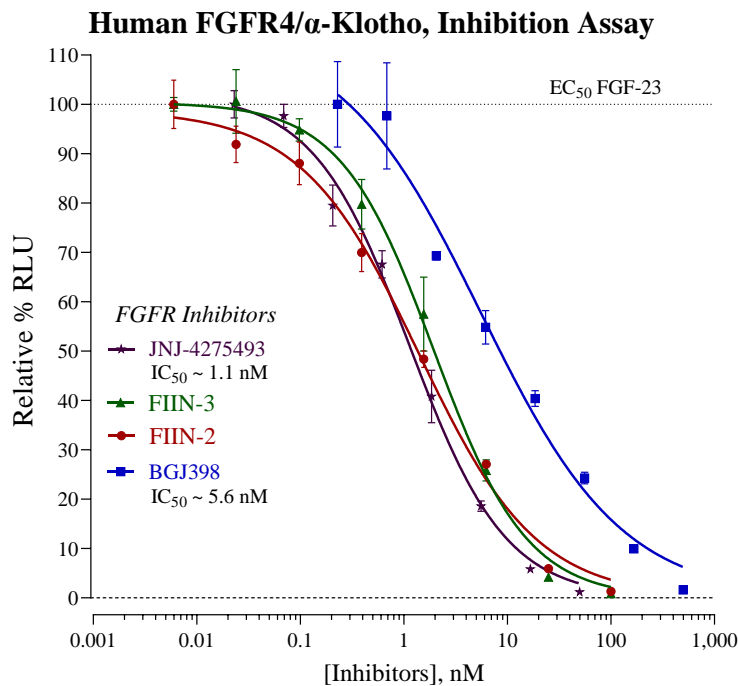


Figure 3. Inhibition of FGFR4/ α -Klotho. FGFR4/ α -Klotho cells were treated with an EC_{50} concentration of the reference activator FGF-23 and varying concentrations of the FGFR inhibitors JNJ-4275493, FIIN-3, FIIN-2, and BGJ398 (all compounds obtained from Cayman Chemical). For both Activation and Inhibition assays, luminescence was quantified and values of average ($n=3$) relative light units (RLU), corresponding standard deviation (SD), Fold-Activation, Relative % RLU 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 % RLU vs. Log_{10} [Cmpd], and to determine EC_{50} / IC_{50} values.

II. Product Components & Storage Conditions

This FGFR4/ α -Klotho for Endocrine 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 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ FGFR4/ α -Klotho 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
▪ FGF-23, 150 μ g/ml (in PBS+0.1%BSA) (physiological activator of FGFR4/ α -Klotho)	1 x 60 μ L	-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% CO₂ incubator for mammalian cell culture
- 37°C water bath
- 70% alcohol wipes
- Instrumentation suitable for dispensing 15 μ l 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 EC₅₀ – EC₈₅) of a known activator AND varying concentrations of the test compound(s) to be evaluated for chemical inhibition. This FGFR4/ α -Klotho for Endocrine FGF Assay kit includes a 150 μ g/mL stock solution of FGF-23, a physiological activator of FGFR4/ α -Klotho, that may be used to set up inhibition-mode assays. ~115 ng/mL FGF-23 typically approximates EC₅₀ 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-23 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.

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** (~ 250 ng / mL) of the challenge activator FGF-23.

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, the total DMSO carried over into assay reactions should not exceed 0.4%.

Tip dispensing method: In *Step 6*, 15 μ l / well of the prepared treatment media is added to the assay that has been pre-dispensed with 15 μ l /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.

Preparing the positive control: This assay kit includes a 100x concentrated stock of the polypeptide FGF-23, 150 μ g/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: 1500, 500, 167, 55.6, 18.5, 6.17, and 2.06 ng/mL. Always include 'no treatment' (or 'vehicle') controls.

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

(continued ...)

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 cell suspension into the assay plate.

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

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% CO₂ 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.

(continued ...)

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

<i>Product No.</i>	<i>Product Descriptions</i>
Fibroblast Growth Factor 4/α-Klotho Assay for Endocrine FGF Signaling	
IB43101-32	FGFR4/ α -Klotho Assay for Endocrine FGF signaling 3x 32 assays in 8-well strips (96-well plate format)
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 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/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

(continued)

Fibroblast Growth Factor 1c/α-Klotho Assay for Endocrine FGF Signaling	
IB40001-32	FGFR1c/ α -Klotho Assay for Endocrine FGF signaling 3x 32 assays in 8-well strips (96-well plate format)
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 counter-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

VI. Limited Use Disclosures

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

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

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

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VII. Citations

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

APPENDIX 1 for tip-based dispensing. Example scheme for the serial dilution of the reference agonist FGF-23 into CSM to generate **2x-concentrated** treatment media. 15 µl / well are dispensed into assay plates using a *tip-based* instrument.

