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

96-well Format Assays
Product # IB43201

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Technical Manual
(version 7.2i)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human FGFR4/β-Klotho for Endocrine FGF Signaling Reporter Assay System 96-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 FGF Receptors (FGFRs) which, themselves, are members of the family of high-affinity tyrosine kinase receptors[1].

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 organs[2]. 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].

Aberrant FGF-19 signaling mediated by its receptor, FGFR4 and co-receptor β-Klotho, is known to be a driver of hepatocellular carcinoma (HCC)[4]. Consequently, FGF-19 and FGFR4/β-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 β-Klotho**, referred to herein as FGFR4/β-Klotho. FGFR4 and β-Klotho are both single-pass transmembrane proteins. FGFR4 has an extracellular ligand-binding domain, transmembrane domain, and intracellular tyrosine kinase domain[1]. It has been established that FGFR4 association with the co-receptor β-Klotho generates a scaffold that is essential for **endocrine growth factor** binding interactions, such as those with FGF-19 and FGF-21[2, 3]. Following growth factor binding, the activated tyrosine kinase activities of the FGFR initiate intracellular signaling cascades that may include RAS-MAPK, PI3-AKT, PLCγ and/or 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/β-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/β-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/β-Klotho or the coupled NFAT 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 FGFR4/β-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-19, 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 (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.

▪ 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 **Poly-peptide** 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-Acidic 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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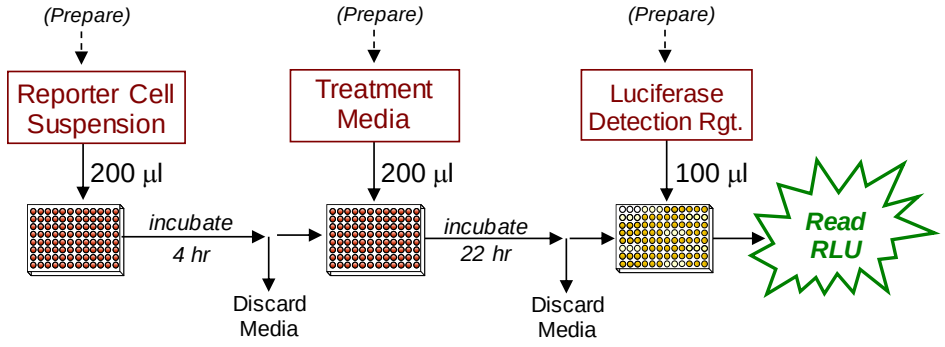
▪ Considerations for Automated Dispensing ▪

When using an automated dispensing instrument to process a small number of assay plates, first carefully consider the dead volume requirement of your instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

Stock Reagent & Volume provided	**Volume to be Dispensed** *(96-well plate)*	**Excess reagent volume available for instrument dead volume**
Reporter Cell Suspension 21 ml *(prepared from kit components)*	200 µl / well 19.2 ml / plate	~ 1.8 ml
LDR 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, 200 µl of Reporter Cells are dispensed into wells of the assay plate and incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared treatment media are added. Following 22-24 hours incubation discard treatment media and add Luciferase Detection Reagent. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



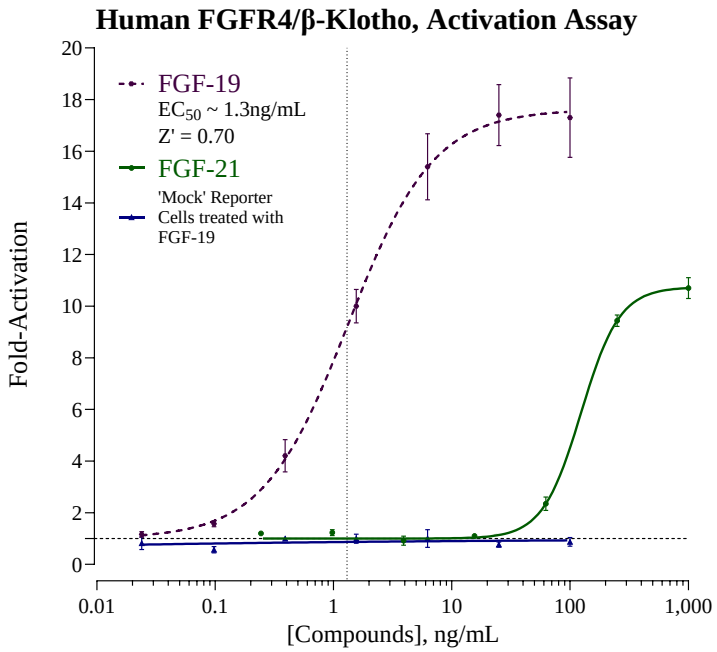


Figure 2. Activation of FGFR4/β-Klotho. Activation assays were performed using the endocrine reference activator FGF-19 (provided) and FGF-21. Polypeptide ligands were procured from Peprotech. The absence of signal in FGF-19 treated 'Mock' cells (which contains the NFAT-Luc reporter vector, but do *not* express FGFR4 or β-Klotho) confirms that the observed ligand-dependent response is specific to FGFR4/β-Klotho activation.

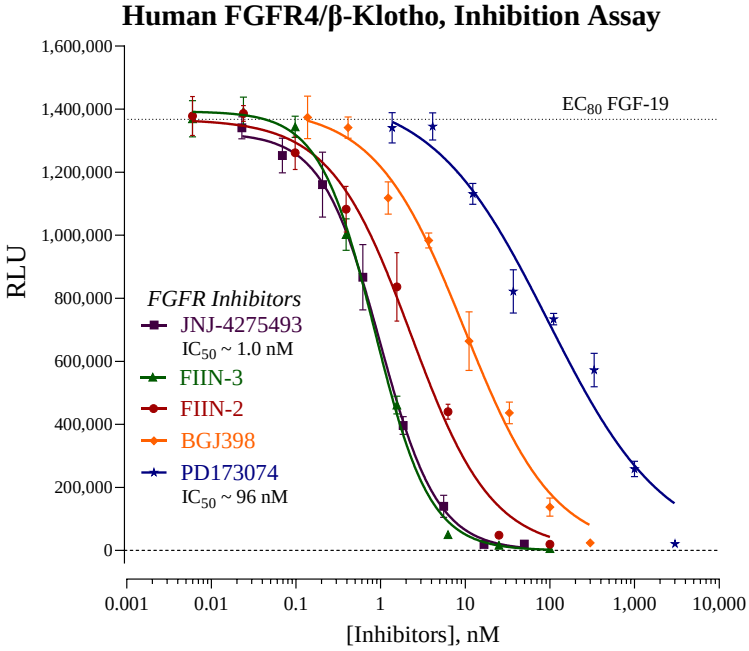


Figure 3. Inhibition of FGFR4/β-Klotho. FGFR4/β-Klotho cells were treated with an EC80 concentration of the reference activator FGF-19 and varying concentrations of the FGFR inhibitors JNJ-4275493, FIIN-3, FIIN-2, BGJ398 and PD173074 (all compounds obtained from Cayman Chemical). INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown). 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, 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 RLU *vs.* Log_{10} [Cmpd], and to determine EC_{50} / IC_{50} values.

II. Product Components & Storage Conditions

This FGFR4/β-Klotho for Endocrine FGF signaling Assay kit contains materials to perform assays in a single collagen-coated 96-well assay plate.

Cryopreserved mammalian cells are temperature sensitive! **To ensure maximal viability the tube of Reporter Cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in *Step 2* of this protocol.**

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first inspect and inventory the individual kit components, please be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen, nor can they be maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

Kit Components	***Amount***	***Storage Temp.***
▪ FGFR4/β-Klotho Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ FGF-19, 10µg/ml (in PBS+0.1%BSA) (physiological activator of FGFR4/β-Klotho)	1 x 30 µL	-20°C
▪ Detection Substrate (Note: contains DTT)	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well, *collagen-coated* assay plate (white, sterile, cell-culture ready)	1	**-20°C**

NOTE: This Assay kit contains one 96-well assay plate in which the assay wells have been collagen-coated and dried; the assay plate should be stored frozen (-20°C or 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* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).

▪ *Optional:* clear 96-well assay plate, cell culture treated, for viewing cells on *Day 2*.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Please review the entire protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-11* are performed on ***Day 1***, requiring less than 2 hours of bench work and a 4 hr 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 ▪

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 FGFR4/β-Klotho for Endocrine FGF Assay kit includes a 10µg/mL stock solution of FGF-19, a physiological activator of FGFR4/β-Klotho, that may be used to set up inhibition-mode assays. ~5ng/mL FGF-19 approximates EC_{80} in this assay. Hence, it presents a suitable concentration of activator to use when screening test materials for inhibitory activities.

Add FGF-19 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 final assay concentrations. This is an efficient and precise method of setting up FGFR4/β-Klotho inhibition assays, and it is the method presented in *Step 7b* of this protocol.

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

1.) Remove the **2 tubes** of **Cell Recovery Medium (CRM)** from freezer storage, thaw and equilibrate to 37°C using a water bath.

2.) Rapid Thaw of the Reporter Cells: *First*, retrieve the two tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve the tube of FGFR4/β-Klotho **Reporter Cells** from -80°C storage, place it directly into dry ice for transport to the laminar flow hood. When ready to begin, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 9.5 ml from ***each* of the 2 tubes** of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 minutes. The resulting volume of cell suspension will be **21 ml**.

3.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.

4.) *Gently* invert the tube of Reporter Cells several times to gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an electronic, repeat-dispensing 8-channel pipette, dispense **200 µl / well** of cell suspension into wells of the assay plate.

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'cell blank' wells (meaning cell-free but containing CSM) must be included in the assay plate to allow quantification of plate-specific fluorescence background (refer to the LCMA Technical Manual).

NOTE 4.2: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the reservoir during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

NOTE 4.3: Users sometimes wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear, preferably collagen-coated, 96-well assay plate. Continue to process this plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells. Place the assay plate into a 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 assay plate. 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 FGFR4/β-Klotho for Endocrine FGF Signaling Assay kit includes a concentrated stock of FGF-19, 10µg/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: 100, 25.0, 6.25, 1.56, 0.391, 0.098, and 0.024 ng/ml. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' control wells.

~ or ~

***b. Inhibition*-mode assays**. When setting up inhibition assays, first supplement a bulk volume of CSM with the challenge activator FGF-19 to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about inhibition-mode assay setup*", pg. 8). The FGF-19-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 4-6 hours pre-culture period, discard the media; the preferred method is to use a 'wrist flick' to eject media into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

9.) Dispense **200 µl / well** of each prepared treatment media into the assay plate.

NOTE: If well-to-well variation due to 'edge-effects' is a concern this problem *may* be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate. Simply remove 1 tip from the 8-chanel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

10.) Transfer the assay plate into a cell culture incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 70%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

11.) For greater convenience on *Day 2*, retrieve **Detection Substrate** *and* **Detection Buffer** from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

DAY 2 Assay Protocol: Subsequent manipulations do *not* require special regard for aseptic technique and may be performed on a bench top 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. Program the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time is set to 0.5 second (500 mSec) per well, *or less.*

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

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 following 'plate rest' period (*Step 16*).

15.) Following 22 - 24 hours incubation in treatment media, discard the media contents by manually ejecting it into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets.

16.) Add 100 µl of the prepared **LDR** into all wells of the assay plate. Allow the assay plate to rest at room temperature for 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*
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 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 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	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 ™ 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.

VII. Citations

[1] Ornitz, *et al*. (2015) The Fibroblast Growth Factor signaling pathway. WIREs Dev Biol. **4**:215-266.

[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] Shi, *et al.* (2018) A systematic dissection of sequence elements determining β-Klotho and FGF interaction and signaling. Scientific Reports. **8**:11045.

[4] Tao, Z, *et al.* (2022) FGFR redundancy limits the efficacy of FGFR4-selective inhibitors in hepatocellular carcinoma. PNAS **119**:40, e2208844119.

[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 1

Example scheme for the serial dilution of FGF-19 and the setup of an FGFR4/β-Klotho dose-response assay.