

**Human Fibroblast Growth Factor 1c/ α -Klotho
for Endocrine FGF Signaling Reporter Assay System
(FGFR1c/ α -Klotho)**

96-well Format Assays
Product # IB40001

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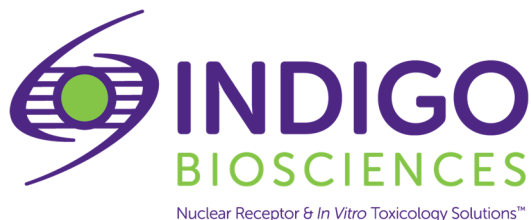
Technical Manual
(version 7.2i)

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Human FGFR1c/ α -Klotho for Endocrine FGF Signaling Reporter Assay System 96-well Format Assays

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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.

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 distant reach target distant organs².

FGFs bind and activate FGF Receptors (FGFRs) which, themselves, are members of the family of high-affinity tyrosine kinase receptors¹. The endocrine FGF-23 activates the receptor complex FGFR1c and its co-receptor α -Klotho³ (**FGFR1c/ α -Klotho**).

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 FGFR1c/ 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 provide constitutive expression of the **Human Fibroblast Growth Factor Receptor 1c and α -Klotho**, referred to herein as FGFR1c/ α -Klotho. FGFR1c and α -Klotho are both single-pass transmembrane proteins. FGFR1c has an extracellular ligand-binding domain, transmembrane domain, and intracellular tyrosine kinase domain¹. It has been established that FGFR1c association with the co-receptor α -Klotho generates a scaffold that is essential for binding of the endocrine growth factor FGF-23³. Following growth factor binding, the activated tyrosine kinase activity of FGFR initiates intracellular signaling cascades that may include RAS-MAPK, PI3-AKT, PLC γ and/or STAT pathways¹. For example, activation of the RAS-MAPK pathway leads to activation of ERK1/2 and subsequent phosphorylation and activation of the transcription factor Elk-1³. It is FGFR1c/ α -Klotho signal transduction *via* the RAS-MAPK-ERK1/2 cascade that is exploited by the reporter cells provided in this kit.

INDIGO's FGFR1c/ α -Klotho Reporter Cells contain the luciferase reporter gene functionally linked to an Elk-1 responsive promoter. Activated Elk-1 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 FGFR1c/ 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 FGFR1c/ α -Klotho or the coupled RAS-MAPK 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 FGFR1c/ 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.

▪ 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.

▪ 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. Note that the final concentration of DMSO carried over into assay wells should not exceed 0.4%.

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-23 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.

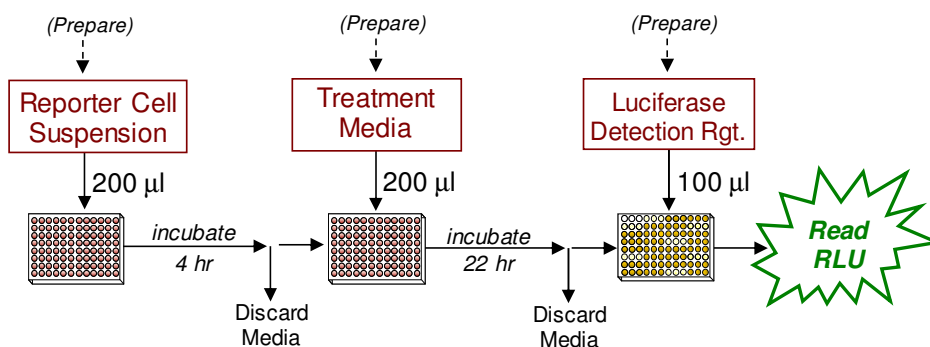
▪ Considerations for Automated Dispensing ▪

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.



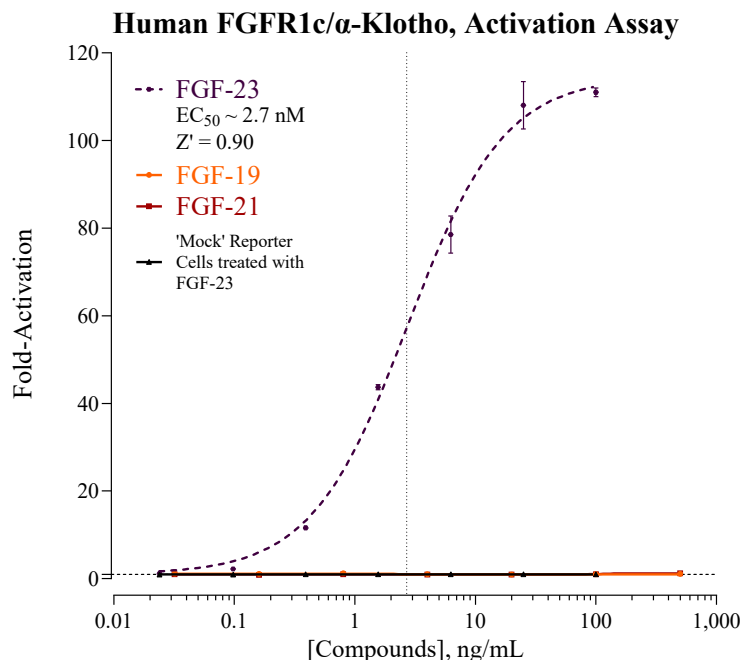


Figure 2. Activation of FGFR1c/ α -Klotho. Activation assays were performed using the reference activator FGF-23 (Prospectbio; provided). The absence of activity by the β -Klotho-dependent endocrine activators FGF-19 and FGF-21 (Peprtech) demonstrates assay specificity.

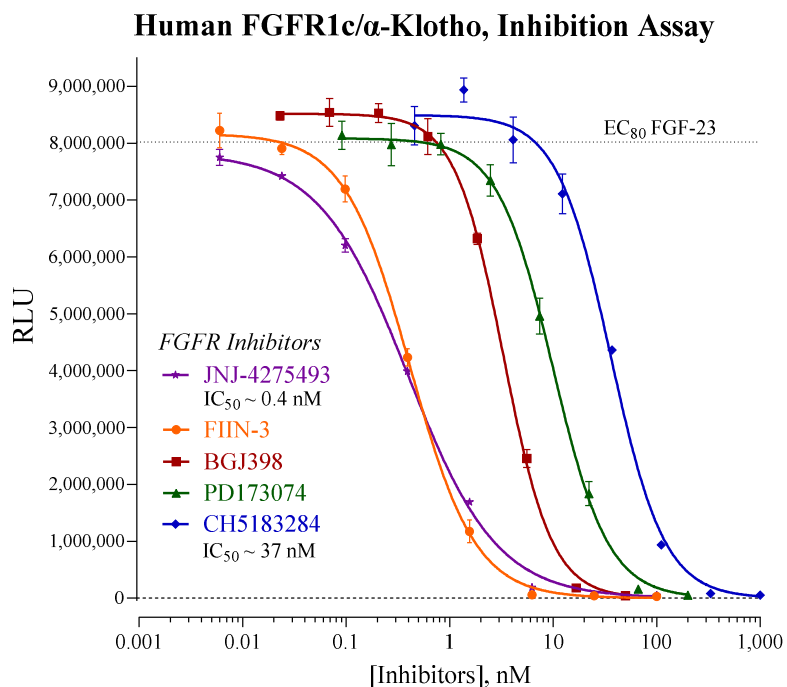


Figure 3. Inhibition of FGFR1c/ α -Klotho. FGFR1c/ α -Klotho cells were treated with an EC₈₀ concentration of the reference activator FGF-23 and varying concentrations of the FGFR inhibitors JNJ-4275293, FIIN-3, BGJ398, PD173074, and CH5183284 (all compounds obtained from Cayman Chemical). Live Cell 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. Log₁₀ [Cmpd, nM], and to determine EC₅₀ / IC₅₀ values.

II. Product Components & Storage Conditions

This FGFR1c/ α -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.

<u><i>Kit Components</i></u>	<u><i>Amount</i></u>	<u><i>minimum Storage Temp.</i></u>
▪ FGFR1c/ α -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-23, 10µg/ml (in PBS+0.1%BSA) (physiological activator of FGFR1c/ α -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, <i>collagen-coated</i> 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% CO₂ 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, collagen-coated, 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-hour incubation step to complete. *Steps 12-17* are performed on **Day 2** and require less than 1 hour to complete.

▪ A word about Inhibition-mode assay setups ▪

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 FGFR1c/ α -Klotho for Endocrine FGF Assay kit includes a 10 μ g/mL stock solution of FGF-23, the physiological activator of FGFR1c α -Klotho, that may be used to set up inhibition-mode assays. ~10ng/mL FGF-23 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-23 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 FGFR1c/ α -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 FGFR1c/ α -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 μ l / 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 an 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% CO₂) 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 not exceed 0.4%.

a. Activation-mode assays. This FGFR1c/ α -Klotho for Endocrine FGF Signaling Assay kit includes a concentrated stock of FGF-23, 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, 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-23 to achieve an EC₅₀ – EC₈₀ concentration (refer to "A word about inhibition-mode assay setup", pg. 8). The FGF-23-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. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, *or less*.

14.) Immediately before proceeding to *Step 15*, 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. Cells will remain tightly adhered to well bottoms.

16.) Add 100 μ l 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

<i>Product No.</i>	<i>Product Descriptions</i>
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 FGFR1c/ α -Klotho Assay Reagents may be custom manufactured to accommodate any scale of HTS.	

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

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

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

(continued)

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in 5x bulk volume to perform 480 Live Cell Assays contained in 5 x 96-well assay plates
LCM-10	Reagent in 10x bulk volume to perform 960 Live Cell Assays contained in 10 x 96-well assay plates

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

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. 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

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

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

³ Diener *et al.* (2015) Development of A Cell-Based Assay to Identify Small Molecule Inhibitors of FGF23 Signaling. *Assay and Drug Development Technologies*. doi: 10.1089/adt.2015.653.

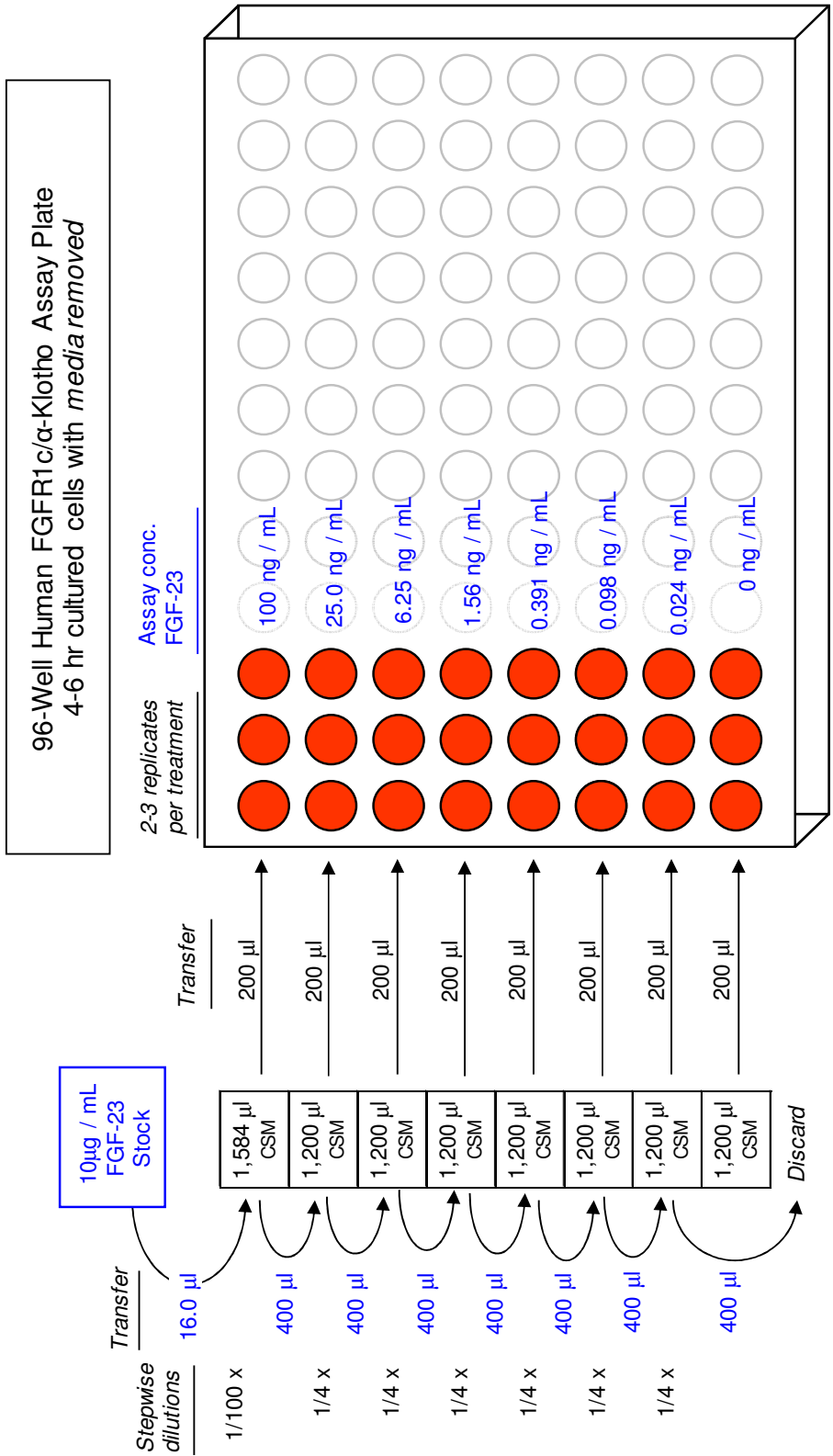
⁴ Gohil, *et al.* (2019) FGF23 and Associated Disorders. *Pediatr Endocrinol Rev.* **17**(1): 17-34.

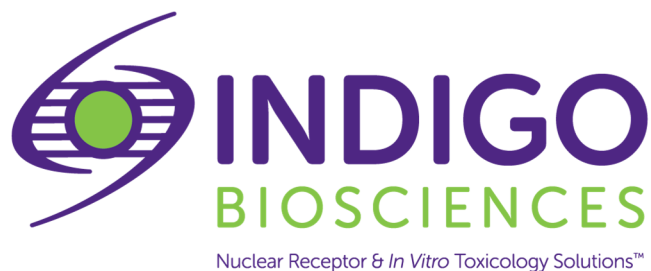
⁵ Zhang JH, *et al.* (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen.* **4**(2), 67-73.

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

APPENDIX 1

Example scheme for the serial dilution of FGF-23 and the setup of an FGFR1c/ α -Klotho dose-response assay.





**Human Fibroblast Growth Factor 1c/ α -Klotho
for Endocrine FGF Signaling Reporter Assay System
(FGFR1c/ α -Klotho)**

384-well Format Assays
Product # IB40002

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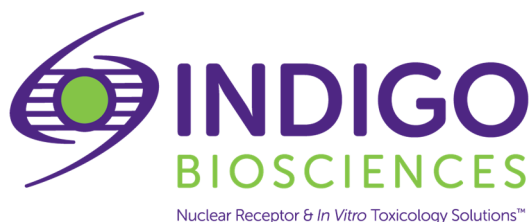
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I. Description

▪ Background ▪

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▪ The Assay System ▪

This assay utilizes proprietary human cells that provide constitutive expression of the **Human Fibroblast Growth Factor Receptor 1c and α -Klotho**, referred to herein as FGFR1c/ α -Klotho. FGFR1c and α -Klotho are both single-pass transmembrane proteins. FGFR1c has an extracellular ligand-binding domain, transmembrane domain, and intracellular tyrosine kinase domain¹. It has been established that FGFR1c association with the co-receptor α -Klotho generates a scaffold that is essential for binding of the endocrine growth factor FGF-23³. Following growth factor binding, the activated tyrosine kinase activity of FGFR initiates intracellular signaling cascades that may include RAS-MAPK, PI3-AKT, PLC γ and/or STAT pathways¹. For example, activation of the RAS-MAPK pathway leads to activation of ERK1/2 and subsequent phosphorylation and activation of the transcription factor Elk-1³. It is FGFR1c/ α -Klotho signal transduction *via* the RAS-MAPK-ERK1/2 cascade that is exploited by the reporter cells provided in this kit.

INDIGO's FGFR1c/ α -Klotho Reporter Cells contain the luciferase reporter gene functionally linked to an Elk-1 responsive promoter. Activated Elk-1 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 FGFR1c/ 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 FGFR1c/ α -Klotho or the coupled RAS-MAPK 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 FGFR1c/ 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.

▪ 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 <i>test cmpds</i> Reporter Cell Suspension 7.5 ml	15 µl / well 5.8 ml / plate	~ 1.7 ml
when using acoustic dispensing of <i>test cmpds</i> 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.

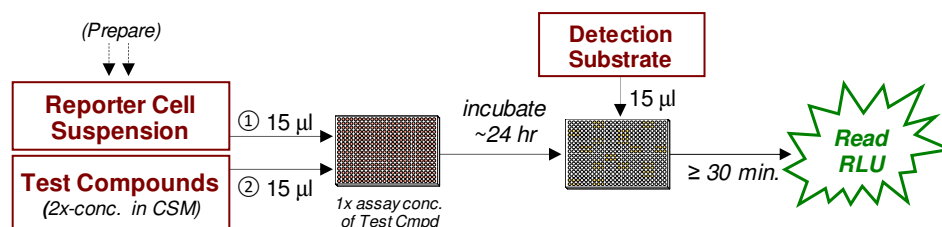
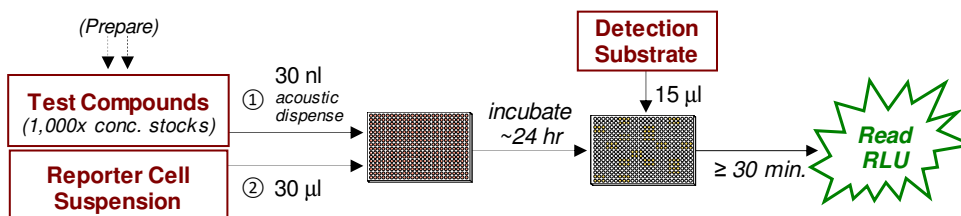


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



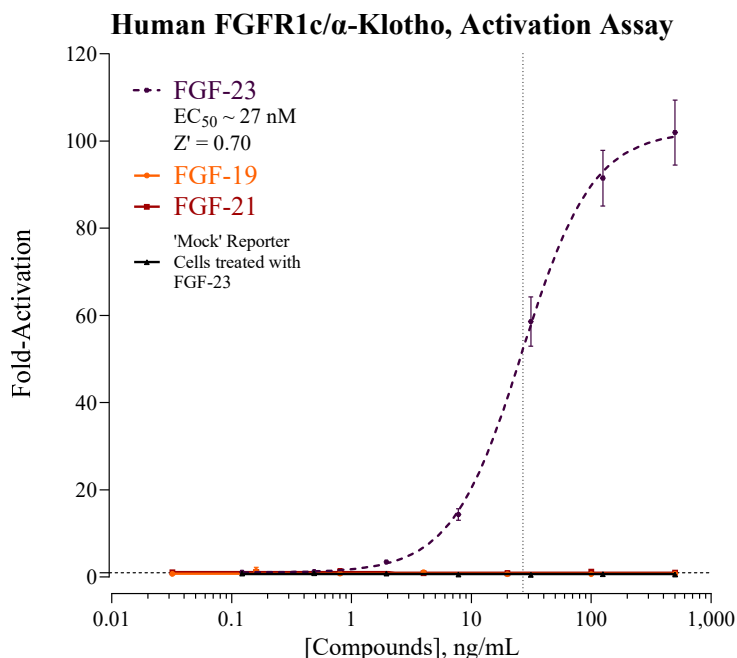


Figure 2. Activation of FGFR1c/ α -Klotho. Activation assays were performed using the reference activator FGF-23 (Prospecbio; provided). The absence of activity by the β -Klotho-dependent endocrine activators FGF-19, and FGF-21 (Peprtech) demonstrates assay specificity.

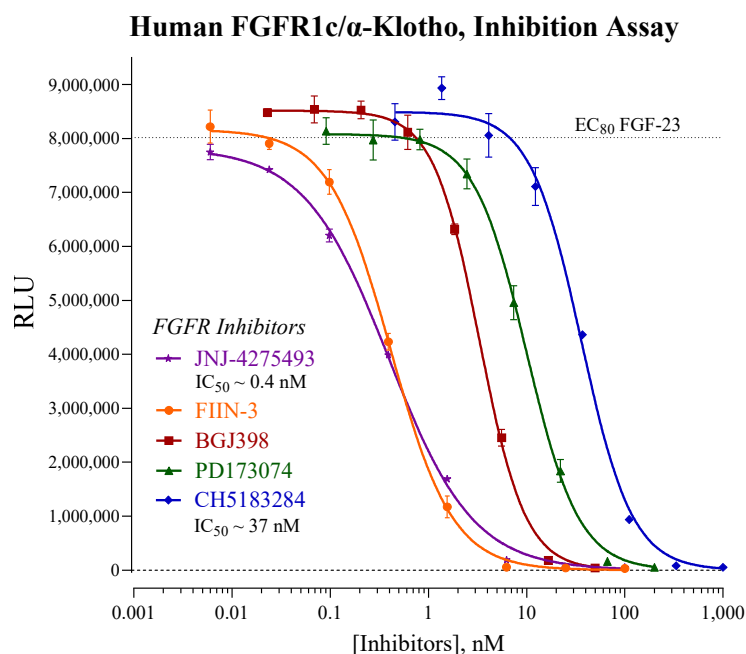


Figure 3. Inhibition of FGFR1c/ α -Klotho. FGFR1c/ α -Klotho cells were treated with an EC_{80} concentration of the reference activator FGF-23 and varying concentrations of the FGFR inhibitors JNJ-4275293, FIIN-3, BGJ398, PD173074, and CH5183284 (all compounds obtained from Cayman Chemical). Live Cell 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, nM], and to determine EC_{50} / IC_{50} values.

II. Product Components & Storage Conditions

This FGFR1c/ 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>
▪ FGFR1c/ 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, 500 μ g/ml (in PBS+0.1%BSA) (physiological activator of FGFR1c/ 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 FGFR1c/ α -Klotho for Endocrine FGF Assay kit includes a 500 μ g/mL stock solution of FGF-23, the physiological activator of FGFR1c α -Klotho, that may be used to set up inhibition-mode assays. ~70 ng/mL FGF-23 typically approximates EC₈₀ 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, 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 (~ 140 ng / mL) of the challenge activator FGF-23.

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 (~ 70 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 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 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.

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 FGF-23, 500 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: 500, 125, 31.3, 7.81, 1.95, 0.488 and 0.122 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 μ l / 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 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.

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 FGFR1c /α-Klotho Reporter Cells suspension with the challenge activator **FGF-23** to achieve an EC₅₀ – EC₈₀ 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% CO₂ incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity ($\geq 70\%$) 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

<i>Product No.</i>	<i>Product Descriptions</i>
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 FGFR1c/ α -Klotho Assay Reagents may be custom manufactured to accommodate any scale of HTS.	
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
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
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

(continued)

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in 5x bulk volume to perform 480 Live Cell Assays contained in 5 x 96-well assay plates
LCM-10	Reagent in 10x bulk volume to perform 960 Live Cell Assays contained in 10 x 96-well assay plates
INDIGlo Luciferase Detection Reagent	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents, available in 10 mL, 25 mL, 50 mL, 500 mL, and larger custom volumes

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Limited Use Disclosures

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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 current version available.

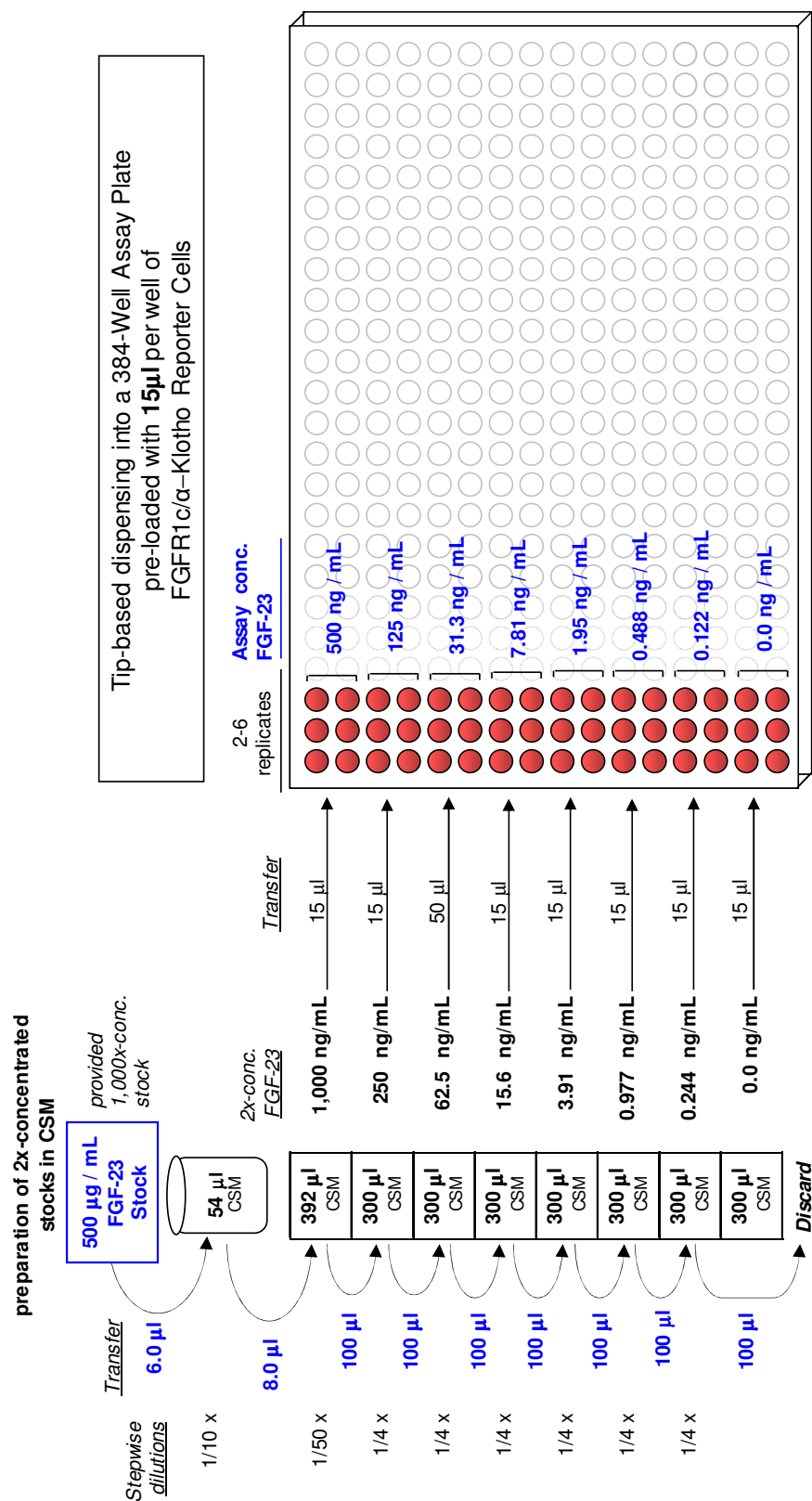
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VII. Citations

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- ⁴ Gohil, *et al.* (2019) FGF23 and Associated Disorders. *Pediatr Endocrinol Rev.* **17**(1): 17-34.
- ⁵ 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.

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

APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of the reference activator FGF-23 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 FGF-23 (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.

