

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

96-well Format Assays
Product # IB22001

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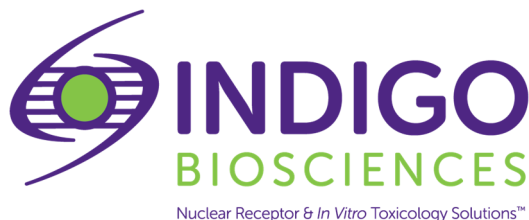
Technical Manual
(version 7.2)

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Human FGFR1c/ β -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. 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.

Recently, FGF-21 a liver secreted FGF, has been identified as a factor that has multiple beneficial effects on obesity-related disorders. For example, it is a potent activator of glucose uptake in primary human adipocytes. In addition, FGF-21 has been shown to protect diet-induced obesity and diabetic illness in animals^{2,3}. Consequently, the associated metabolic benefits of the endocrine FGFs have promoted considerable interest in therapeutic 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 1c and β -Klotho**, referred to herein as FGFR1c/ β -Klotho. FGFR1c and β -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 FGFR association with the co-receptor β -Klotho generates a scaffold that is essential for **endocrine growth factor** binding interactions, such as those with FGF-21 and FGF-19². 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 RAS-MAPK pathway leads to activation of ERK1/2 and subsequent phosphorylation and activation of the transcription factor Elk-1⁴. It is FGFR1c/ β -Klotho signal transduction *via* the RAS-MAPK-ERK1/2 cascade that is exploited by the reporter cells provided in this kit.

INDIGO's FGFR1c/ β -Klotho for Endocrine FGF Signaling 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/ 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 FGFR1c/ β -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/ 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-21, 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

- ¹ Ornitz, *et al.* (2015) The Fibroblast Growth Factor signaling pathway. *WIREs Dev Biol.* **4**:215-266.
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- ³ Xie, S, *et al.* (2020) FGF/FGFR signaling in health and disease. *Signal Transduction and Targeted Therapy* (Springer Nature) **5**:181, 1-38.
- ⁴ Ge, X, *et al.* (2011) Fibroblast Growth factor 21 Induces Glucose Transporter-1 Expression through Activation of the Serum Response Factor/Ets-Like Protein-1 in Adipocytes. *The Journal of Biological Chemistry.* **286**:40, 34533-34541.

▪ 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; preferably 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 *never* exceed 0.4%.

For protein or antibody samples it is recommended to solvate the materials in aqueous buffered solutions supplemented with carrier protein (*e.g.*, PBS + 0.1% BSA) at concentrations *no less* than 10x relative to the highest desired treatment concentration. The FGF-21 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 prepared stocks are serially diluted using **Compound Screening Medium (CSM)** to achieve the desired assay concentrations, as described in *Step 7*.

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.

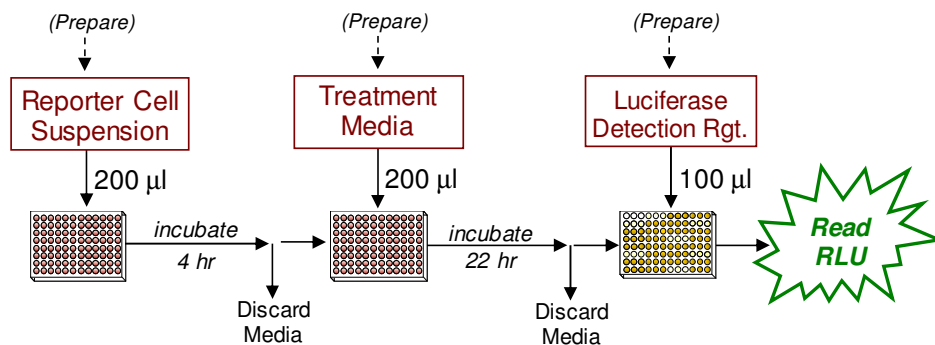
▪ Considerations for Automated Dispensing ▪

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 hr 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.



▪ Assay Performance ▪

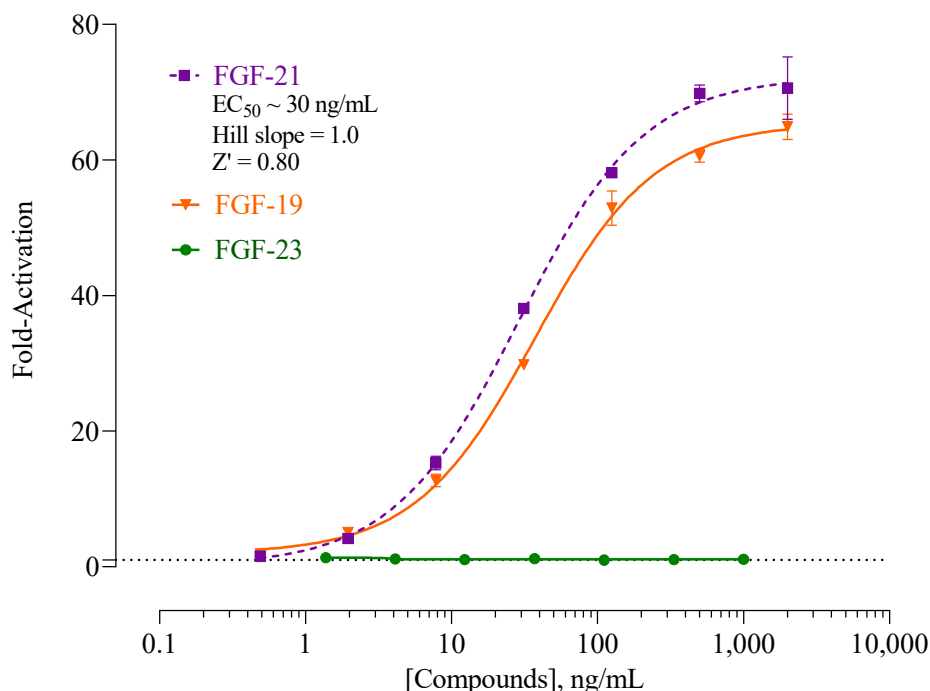


Figure 2. FGFR1c/ β -Klotho Activation dose response analyses. Activation dose-response assays were performed according to the protocol provided in this Technical Manual. 200 μ l / well of FGFR1c/ β -Klotho Reporter Cell suspension was dispensed into the 96-well assay plate, which was then incubated for 4 hours. The concentrated stock of FGF-21 (provided) and FGF-19 (Sino Biological) were further diluted using CSM to produce treatment media at the desired assay concentrations. As expected, the growth factor FGF-23, which requires α -Klotho, shows no activity. The pre-culture media were discarded from the assay wells and 200 μ l per well of the prepared treatment media were dispensed (n = 3/conc.), including ‘untreated’ control wells. Following a 22 hr incubation period treatment media were discarded, Luciferase Detection Reagent was added, and luminescence intensity per well was quantified. Values of average relative light units (RLU) and corresponding values of standard deviation (SD), percent coefficient of variation (%CV), Fold-Activation and Z'⁵ were determined for each treatment concentration. Non-linear regression analyses of Fold Activation vs. Log₁₀[ng/mL] and EC₅₀ determinations were performed using GraphPad Prism software.

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.

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

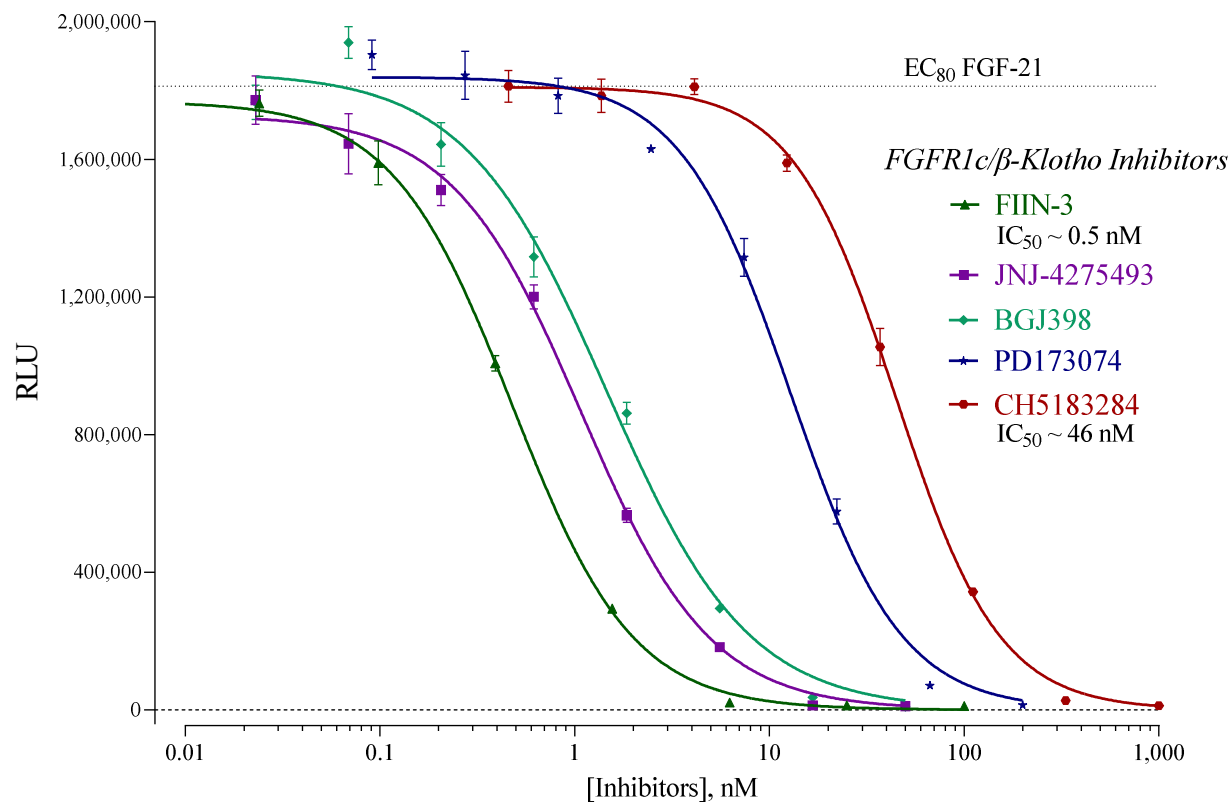


Figure 3. FGFR1c/β-Klotho Inhibition dose-response analyses. FGFR1c/β-Klotho reporter cells were co-treated with an EC₈₀ concentration of the reference activator FGF-21 (provided) and varying concentrations of the FGFR1 inhibitors FIIN-3, JNJ-4275293, BGJ398, PD173074 and CH5183284 (all compounds obtained from Cayman Chemical, Ann Arbor MI, USA). The range of determined IC₅₀ values is shown; (n = 3 / conc.). INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown). Non-linear regression analyses of RLU *vs.* Log₁₀[Inhibitor, nM] were plotted and IC₅₀ determinations made using GraphPad Prism software.

II. Product Components & Storage Conditions

This FGFR1c/ 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.

<u><i>Kit Components</i></u>	<u><i>Amount</i></u>	<u><i>Storage Temp.</i></u>
▪ FGFR1c/ 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-21, 200 μ g/ml (in PBS/0.1%BSA) (physiological activator of FGFR1c/ β -Klotho)	1 x 30 μ L	-20°C
▪ Detection Substrate	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, 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 FGFR1c/ β -Klotho for Endocrine FGF Assay kit includes a 200 μ g/mL stock solution of FGF-21, a physiological activator of FGFR1c/ β -Klotho, that may be used to set up inhibition-mode assays. ~100ng/mL FGF-21 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-21 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/ 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 FGFR1c/ 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 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 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 never exceed 0.4%.

a. Activation-mode assays. This FGFR1c/β-Klotho for Endocrine FGF Signaling Assay kit includes a concentrated stock of FGF-21, 200µg/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: 2,000, 500, 125, 31.3, 7.81, 1.95, and 0.488 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-21 to achieve an EC₅₀ – EC₈₀ concentration (refer to "*A word about inhibition-mode assay setup*", pg. 9). The FGF-21-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 hr 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.

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*, transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a 12 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

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 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.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
Fibroblast Growth Factor 1c/β-Klotho for Endocrine FGF Signaling Assay Products	
IB22001-32	FGFR1c/ β -Klotho for Endocrine FGF signaling Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB22001	FGFR1c/ β -Klotho for Endocrine FGF signaling Reporter Assay System 1x 96-well format assay
IB22002	FGFR1c/ β -Klotho for Endocrine FGF signaling Reporter Assay System 1x 384-well format assays
Bulk volumes of reporter cells and assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

<i>Product No.</i>	<i>Product Descriptions</i>
Fibroblast Growth Factor Receptor 1 and 2 for Paracrine FGF Signaling Assay Products	
IB21001-32	FGFR1/2 for Paracrine FGF Signaling Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB21001	FGFR1/2 for Paracrine FGF Signaling Reporter Assay System 1x 96-well format assay
IB21002	FGFR1/2 for Paracrine FGF Signaling Reporter Assay System 1x 384-well format assays

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

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.

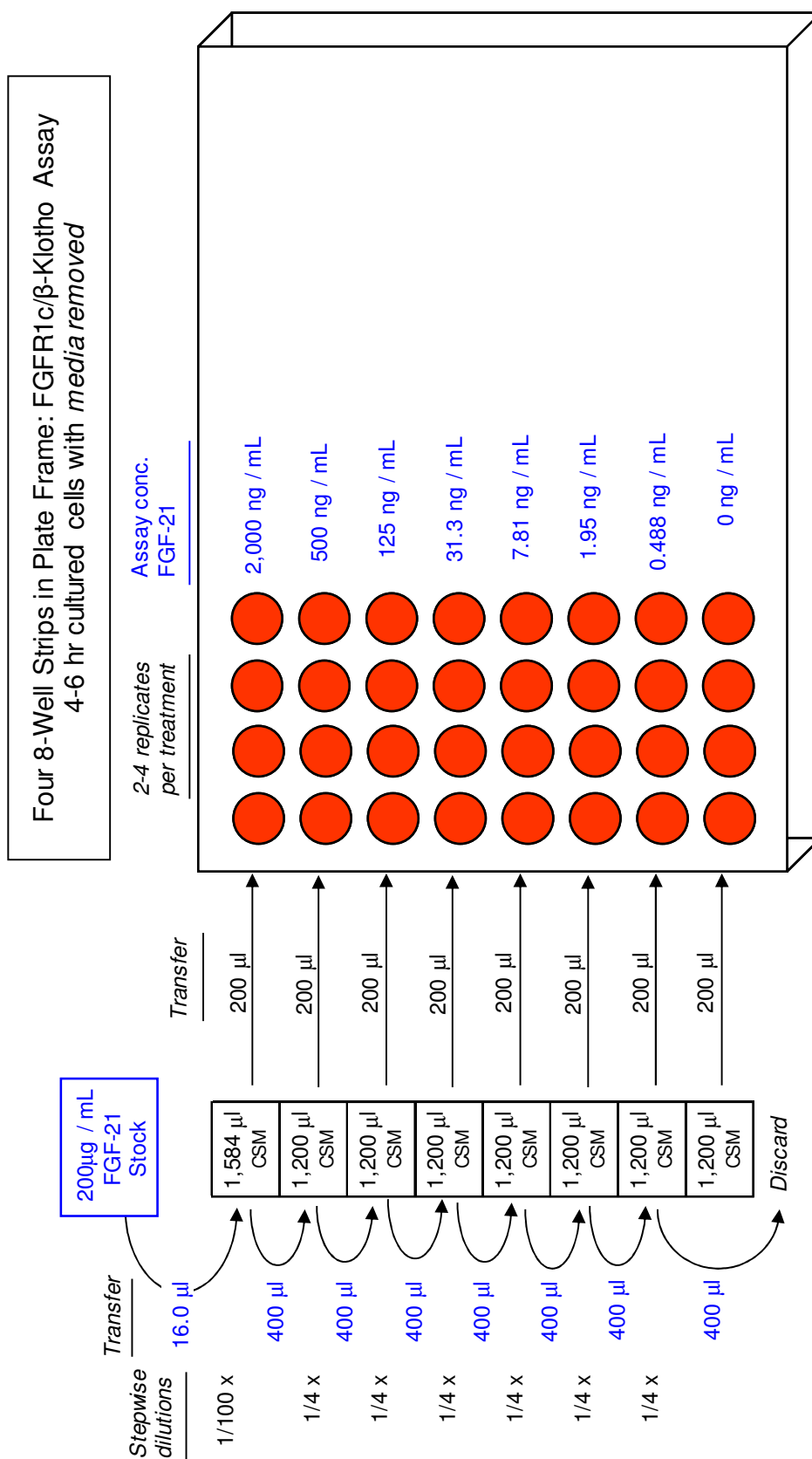
“CryoMite” is a Trademark TM of INDIGO Biosciences, Inc. (State College, PA, USA).

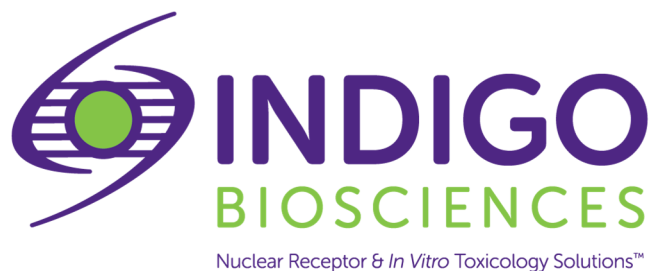
Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most current version available.

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APPENDIX 1

Example scheme for the serial dilution of FGF-21 and the setup of an FGFR1c/ β -Klotho dose-response assay.





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3x 32 Assays in 96-well Format
Product # IB22001-32

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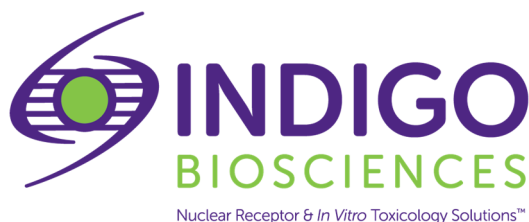
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Recently, FGF-21 a liver secreted FGF, has been identified as a factor that has multiple beneficial effects on obesity-related disorders. For example, it is a potent activator of glucose uptake in primary human adipocytes. In addition, FGF-21 has been shown to protect diet-induced obesity and diabetic illness in animals^{2,3}. Consequently, the associated metabolic benefits of the endocrine FGFs have promoted considerable interest in therapeutic development and drug safety screening.

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- ¹ Ornitz, *et al.* (2015) The Fibroblast Growth Factor signaling pathway. *WIREs Dev Biol.* **4**:215-266.
- ² Shi, *et al.* (2018) A systematic dissection of sequence elements determining β -Klotho and FGF interaction and signaling. *Scientific Reports.* **8**:11045.
- ³ Xie, S, *et al.* (2020) FGF/FGFR signaling in health and disease. *Signal Transduction and Targeted Therapy (Springer Nature)* **5**:181, 1-38.
- ⁴ Ge, X, *et al.* (2011) Fibroblast Growth factor 21 Induces Glucose Transporter-1 Expression through Activation of the Serum Response Factor/Ets-Like Protein-1 in Adipocytes. *The Journal of Biological Chemistry.* **286**:40, 34533-34541.

▪ 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; preferably 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 *never* exceed 0.4%.

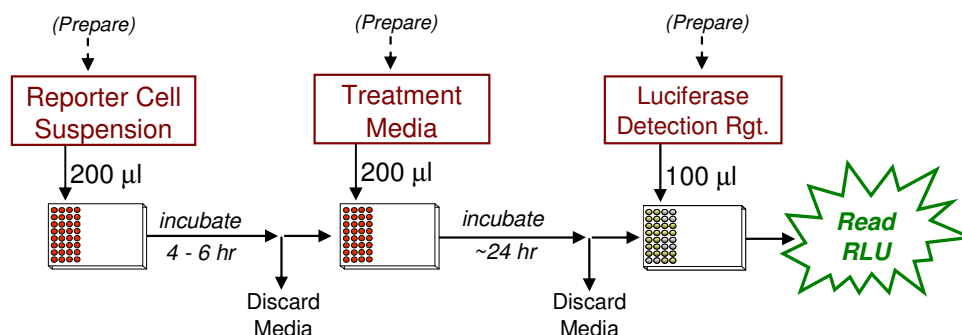
For protein or antibody samples it is recommended to solvate the materials in aqueous buffered solutions supplemented with carrier protein (*e.g.*, PBS + 0.1% BSA) at concentrations *no less* than 10x relative to the highest desired treatment concentration. The FGF-21 stock included with this kit is 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 prepared stocks are serially diluted using **Compound Screening Medium (CSM)** to achieve the desired assay concentrations, as described in *Step 7*.

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.

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, 200 μ l of Reporter Cells is dispensed into wells of the assay plate and for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 μ l/well of the prepared treatment media are added. Following 22-24 hr incubation, discard the 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.



▪ Assay Performance ▪

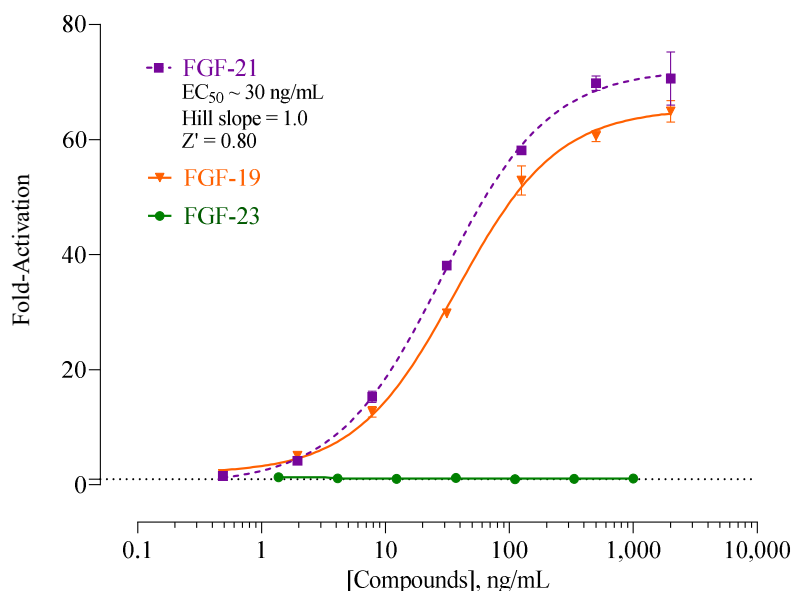


Figure 2. FGFR1c/ β -Klotho Activation dose response analyses. Activation dose-response assays were performed according to the protocol provided in this Technical Manual. 200 μ l / well of FGFR1c/ β -Klotho Reporter Cell suspension was dispensed into the 96-well assay plate, which was then incubated for 4 hours. The concentrated stock of FGF-21 (provided) and FGF-19 (Sino Biological) were further diluted using CSM to produce treatment media at the desired assay concentrations. As expected, the growth factor FGF-23, which requires α -Klotho, shows no activity. The pre-culture media were discarded from the assay wells and 200 μ l per well of the prepared treatment media were dispensed ($n = 3/\text{conc.}$), including 'untreated' control wells. Following a 22 hr incubation period treatment media were discarded, Luciferase Detection Reagent was added, and luminescence intensity per well was quantified. Values of average relative light units (RLU) and corresponding values of standard deviation (SD), percent coefficient of variation (%CV), Fold-Activation and Z' ⁵ were determined for each treatment concentration. Non-linear regression analyses of Fold Activation vs. $\text{Log}_{10}[\text{ng/mL}]$ and EC_{50} determinations were performed using GraphPad Prism software.

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.

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

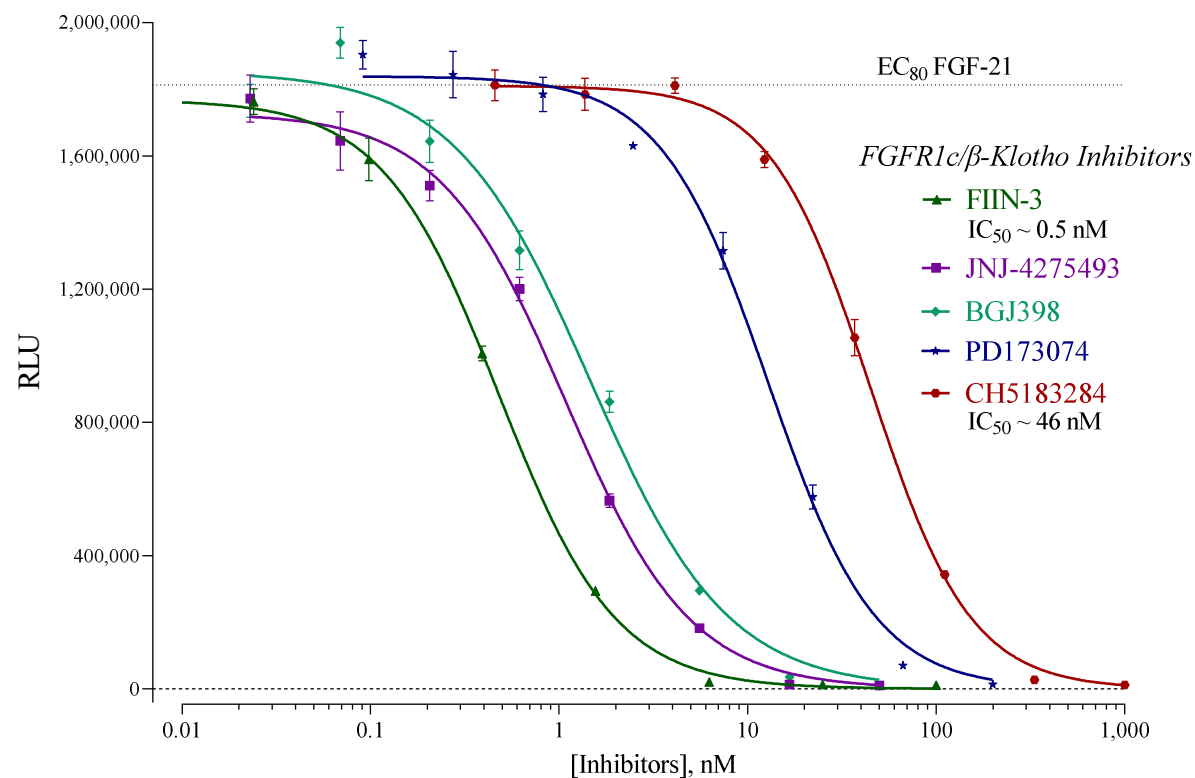


Figure 3. FGFR1c/β-Klotho Inhibition dose-response analyses. FGFR1c/β-Klotho reporter cells were co-treated with an EC₈₀ concentration of the reference activator FGF-21 (provided) and varying concentrations of the FGFR1 inhibitors FIIN-3, JNJ-4275293, BGJ398, PD173074 and CH5183284 (all compounds obtained from Cayman Chemical, Ann Arbor MI, USA). The range of determined IC₅₀ values is shown; (n = 3 / conc.). INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown). Non-linear regression analyses of RLU vs. Log₁₀[Inhibitor, nM] were plotted and IC₅₀ determinations made using GraphPad Prism software.

II. Product Components & Storage Conditions

This FGFR1c/ 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.

<u><i>Kit Components</i></u>	<u><i>Amount</i></u>	<u><i>Storage Temp.</i></u>
▪ FGFR1c/ β -Klotho Reporter Cells	3 x 0.6 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-21, 200 μ g/ml (in PBS/0.1%BSA) (activator of FGFR1c/ β -Klotho)	1 x 30 μ L	-20°C
▪ Detection Substrate	3 x 2.0 mL	-80°C
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate Frame	1	ambient
▪ Snap-in 8-well strips (white, sterile, collagen-coated wells)	12	-20°C

NOTE: This Assay kit contains 8-well strips that have been collagen-coated and dried; these strip wells 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, 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 FGFR1c/ β -Klotho for Endocrine FGF Assay kit includes a 200 μ g/mL stock solution of FGF-21, a physiological activator of FGFR1c/ β -Klotho, that may be used to set up inhibition-mode assays. ~100ng/mL FGF-21 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-21 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/ 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 **Reporter Cells** from -80°C storage and place them directly into dry ice to transport them to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. When ready to begin, transfer the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring **6.4 ml** of pre-warmed CRM into each tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **7.0 ml** per tube.

Third, during the 5 - 10 minutes incubation period, work in the cell culture hood to *carefully* mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

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

4.) If more than one tube of Reporter cells was thawed, combine them and gently invert several times to disperse cell aggregates and gain a homogenous cell suspension. Dispense **200 μ l / well** of cell suspension into the assay plate.

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

NOTE 4.2: Increased well-to-well variation 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, collagen-coated 96-well assay plate. Continue to process the assay 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% 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 strip-wells. 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 FGFR1c/β-Klotho for Endocrine FGF Signaling Assay kit includes a concentrated stock of FGF-21, 200µg/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: 2,000, 500, 125, 31.3, 7.81, 1.95, and 0.488 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-21 to achieve an EC₅₀ – EC₈₀ concentration (refer to "*A word about inhibition-mode assay setup*", pg. 8). The FGF-21 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 cell pre-culture period: Discard the culture media.

Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (*e.g.*, Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus. Do *not* touch the well bottom or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the cells and greatly increased well-to-well variability.

9.) Dispense **200 µl** of each treatment media into appropriate wells of the assay plate.

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 the appropriate number of vials of **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.

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:* To read 32 assay wells, transfer the entire volume of 1 vial of Detection Buffer into 1 vial of Detection Substrate, thereby generating a 4 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

15.) Following 22 - 24 hours incubation in treatment media, remove media contents from each well of the assay plate (as before in *Step 8*).

16.) Add 100 µl of **LDR** to each well 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.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
Fibroblast Growth Factor 1c/β-Klotho for Endocrine FGF Signaling Assay Products	
IB22001-32	FGFR1c/ β -Klotho for Endocrine FGF signaling Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB22001	FGFR1c/ β -Klotho for Endocrine FGF signaling Reporter Assay System 1x 96-well format assay
IB22002	FGFR1c/ β -Klotho for Endocrine FGF signaling Reporter Assay System 1x 384-well format assays
Bulk volumes of reporter cells and assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

<i>Product No.</i>	<i>Product Descriptions</i>
Fibroblast Growth Factor Receptor 1 and 2 for Paracrine FGF Signaling Assay Products	
IB21001-32	FGFR1/2 for Paracrine FGF Signaling Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB21001	FGFR1/2 for Paracrine FGF Signaling Reporter Assay System 1x 96-well format assay
IB21002	FGFR1/2 for Paracrine FGF Signaling Reporter Assay System 1x 384-well format assays

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

Please refer to INDIGO Biosciences website for updated product offerings.

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APPENDIX 1

Example scheme for the serial dilution of FGF-21 and the setup of an FGFR1c/ β -Klotho dose-response assay.

