



**Human Fibroblast Growth Factor Receptors 1 and 2
Assay for Paracrine FGF Signaling
(FGFR1/2)**

384-well Format Assays
Product # IB21002

▪

Technical Manual
(*version 8.0i*)

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Human FGFR1/2 for Paracrine Signaling Reporter Assay System 384-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.

Heparin and heparin sulfate proteoglycans (HSPGs) are essential cofactors for paracrine FGF interactions with FGFRs. The association between paracrine FGFs and HSPGs ensures their limited diffusion and enhanced FGFR binding specificity. In contrast, endocrine FGFs (*e.g.*, FGF-19 and -21) have minimal affinity to heparin. Rather, they require association with members of the Klotho family of proteins as cofactors for efficient binding to their cognate receptor(s)^{1,2}.

The FGFs are broad-spectrum mitogens that, through their receptor interactions, regulate a variety of cellular functions including migration, proliferation, differentiation, metabolism and survival^{1,2}. In particular, FGF/FGFR signaling plays a critical role in regulating metabolism in the kidney, liver, brain, intestine and adipose tissues^{1,2}. Perhaps not surprisingly, dysfunctional FGFR signaling can lead to a range of physiological disorders. For example, mutation, amplification, and gene fusion may result in abnormal morphogenesis and the progression of several types of cancer². Consequently, the FGF receptors continue to command much interest as targets for drug development and drug safety screening.

▪ The Assay System ▪

This assay utilizes proprietary human cells that have been engineered to provide constitutive expression of the **Human Fibroblast Growth Factor Receptors 1 and 2**, referred to herein as **FGFR1/2**. FGFR1 and FGFR2 are both single-pass transmembrane receptors that contain respective extracellular ligand-binding domains, transmembrane domains, and intracellular tyrosine kinase domains¹.

INDIGO's Compound Screening Media is supplemented with heparin, thereby enabling the formation of paracrine FGF/Heparin complexes that bind with high affinity to FGFR monomers. This binding interaction triggers conformational changes that drive the assembly of homo-dimeric (R1:R1, R2:R2) and/or hetero-dimeric (R1:R2) receptors, and the activation of their respective cytosolic tyrosine kinase domains¹.

The tyrosine kinase activities of activated FGFR's initiate intracellular signaling cascades that include RAS-MAPK, PI3-AKT, PLC γ and STAT pathways¹. For example, activation of the PLC γ pathway leads to an increase of intracellular calcium¹. One prominent outcome of the FGF/FGFR > PLC γ pathway is that calcineurin, a calcium-dependent phosphatase, dephosphorylates and activates the transcription factor NFAT³. It is FGFR signal transduction *via* the Ca⁺²-calcineurin / NFAT cascade that is exploited by the reporter cells provided in this kit.

INDIGO's FGFR1/2 for Paracrine FGF Signaling Reporter Cells contain the luciferase reporter gene functionally linked to tandem NFAT consensus response element sequences upstream of a minimal promoter. Activated NFAT binds to these response elements to initiate the formation of a complete transcription complex that drives Luc gene expression. Quantifying relative changes in luciferase activity in the treated reporter cells relative to the untreated cells provides a sensitive, dose-dependent surrogate measure of drug-induced changes in FGFR1/2 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 FGFR1/2, or the coupled Ca⁺²-calcineurin/NFAT signal transduction pathway. To re-iterate, this assay is primarily focused on the paracrine signaling activities of FGFR/FGFs.

INDIGO's Reporter Cells are transiently transfected and prepared as frozen stocks using a proprietary **CryoMite™** process. This cryo-preservation method allows for the immediate dispensing of healthy, division-competent reporter cells into assay plates. There is no need for intermediate treatment steps such as spin-and-rinse of cells, viability determinations or cell titer adjustments prior to assay setup.

This assay kit provides the convenience of an all-inclusive cell-based reporter assay system for FGFR1/2 paracrine FGF signaling. In addition to Reporter Cells, included are an optimized culture medium for use in reviving the cryopreserved cells, a culture medium supplemented with heparin for use in preparing test sample treatments, the physiological paracrine activator FGF-Acidic (*aka* FGF1), Luciferase Detection Reagent, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's nuclear receptor reporter assays capitalize on the low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg⁺²-dependent reaction that consumes O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO₂, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer and is reported in terms of Relative Light Units (RLU's).

This assay kit features a luciferase detection reagent specially formulated to provide stable light emission between 30 and 100+ minutes after initiating the luciferase reaction. Incorporating a 30-minute reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.

▪ Considerations for the Preparation and Automated Dispensing of Test compounds ▪

It is recommended that test materials that are **protein ligands** or **antibodies** 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.

Small molecule compounds are typically solvated at high concentration (ideally 1,000x-concentrated) in DMSO and stored frozen as master stocks. For **384-well format assays** these master stocks will be diluted by one of two alternative methods, the selection of which will be dictated by the type of dispensing instrument that is to 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 test compounds into assay wells (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, and as depicted in Appendix 1a.
- b.) 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 then considered to be 'single-use' reagents.

- c.) Assay setups in which an **acoustic transfer** device is used to dispense test compounds into assay wells (text highlighted in blue). Use DMSO to make a series of **1,000x-concentrated** test compound stocks that correspond to each desired final assay concentrations, as described in *Step 2b* of the Assay Protocol, and as depicted in Appendix 1b.

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

When dispensing into a small number of assay plates, first carefully consider the dead volume requirement of your tip-based dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses on a *per kit* basis. Always pool the individual reporter cell suspensions and all other respective assay kit reagents before processing multiple 384-well assay plates.

Stock Reagent & Volume provided	Volume to be Dispensed (384-well plate)	Excess reagent available for instrument dead vol.
when using tip dispensing of test cmpds Reporter Cell Suspension 7.5 ml	15 µl / well 5.8 ml / plate	~ 1.7 ml
when using acoustic dispensing of test cmpds Reporter Cell Suspension 15 ml	30 µl / well 11.5 ml / plate	~ 3.4 ml
Detection Substrate 7.8 ml	15 µl / well 5.8 ml / plate	~ 2 ml

▪ **Assay Scheme** ▪

The *Day 1* preparation, volumes, and chronology of dispensed cells and test compounds are different between assay setups using a *tip-based dispenser* (**1a**) and those using an *acoustic transfer device* (**1b**). Following 22 -24 hours incubation Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.

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

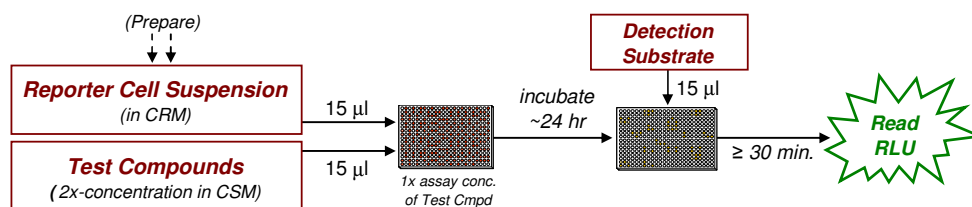
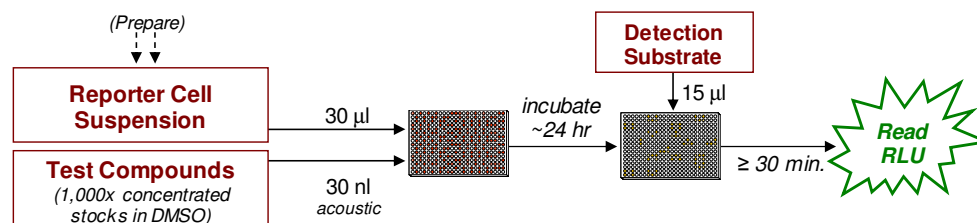


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



▪ Assay Performance ▪

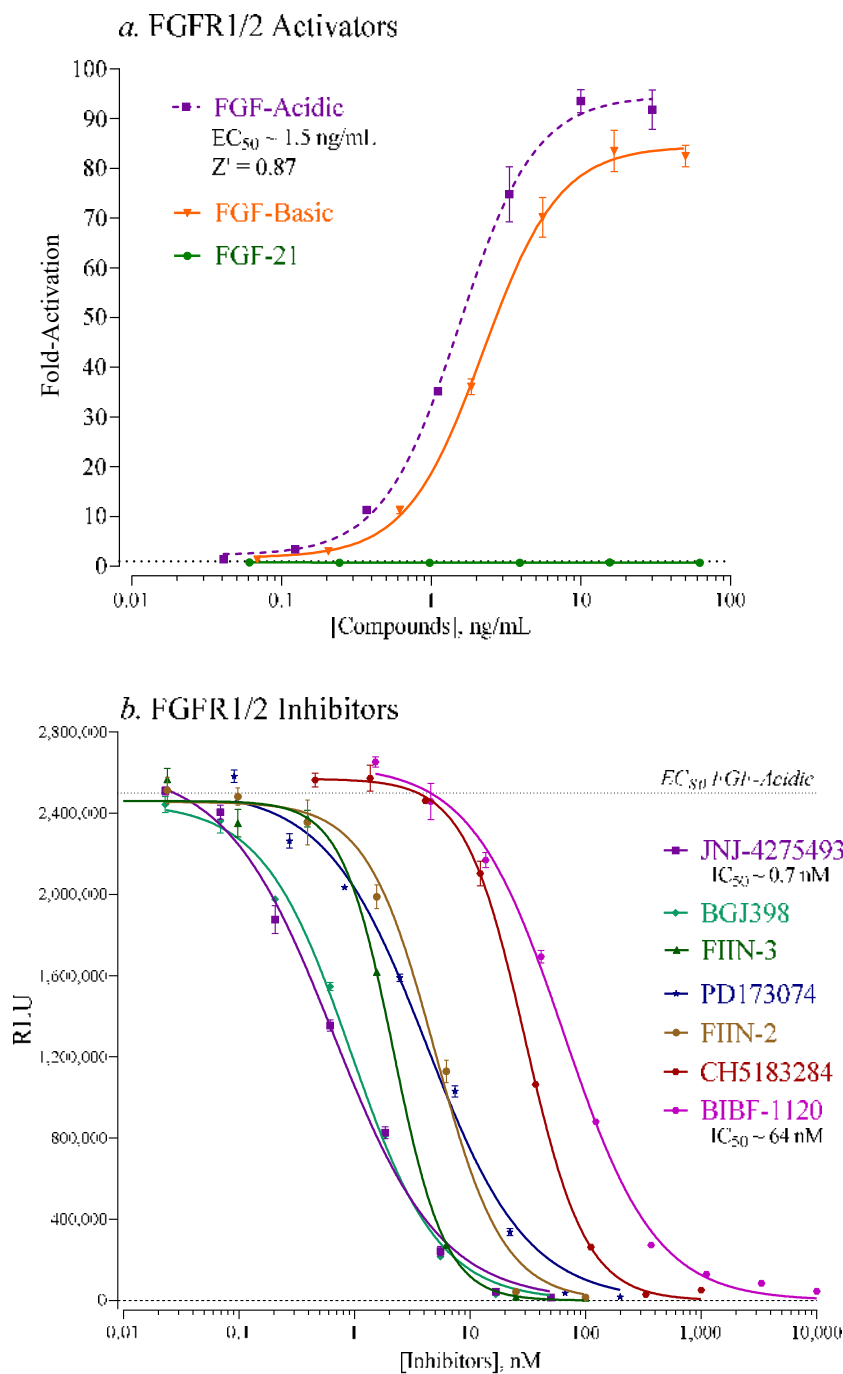


Figure 2. a.) FGFR1/2 Activation dose response analyses. Concentrated stocks of the protein activators FGF-Acidic (*aka* FGF1; provided), FGF-Basic (*aka* FGF2) and FGF-21 (all from Peprotech) were initially prepared in PBS/0.1% BSA, then further diluted using CSM+H to produce treatment media at the desired assay concentrations. As expected, the endocrine growth factor FGF-21 shows no activity.

b.) FGFR1/2 Inhibition dose-response analyses. The reporter cells were co-treated with an EC_{80} concentration of the reference activator FGF-Acidic and varying concentrations of the FGFR1/2 small molecule inhibitors JNJ-4275293, BGJ398, FIIN-3, PD173074, CH5183284 and BIBF-1120 (all compounds obtained from Cayman Chemical, Ann Arbor MI, USA). The range of determined IC_{50} values is shown. INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentrations were cytotoxic (data not shown).

Data plotting and the determination of EC_{50} / IC_{50} values were performed *via* least squares non-linear regression using GraphPad Prism software.

II. Product Components & Storage Conditions

This assay kit contains materials to perform assays in a single 384-well assay plate.

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

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

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

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

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ FGFR1/2 Reporter Cells	1 x 1.0 mL	-80°C
▪ Cell Recovery Medium (CRM)	1 x 7 mL	-20°C
▪ Compound Screening Medium (CSM+H)	1 x 35 mL	-20°C
▪ FGF-Acidic, 30 µg/mL (in PBS/0.1% BSA) (reference agonist)	1 x 80 µL	-20°C
▪ Detection Substrate	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

- dry ice container
- 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 µl volumes
- disposable media basins, sterile.
- sterile multi-channel media basins *or* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- *Optional:* antagonist reference compound / antibody (*e.g.*, Figure 3)
- *Optional:* clear 384-well assay plate, cell culture treated, for viewing cells on Day 2.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-8* are performed on **Day 1**, requiring less than 2 hours to complete. *Steps 9-13* are performed on **Day 2** and require less than 1 hour to complete.

▪ A word about Inhibition-mode assay setups ▪

Receptor inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between EC_{50} – EC_{85}) of a known agonist AND varying concentrations of the test compound(s) to be evaluated for antagonist activity. This FGFR1/2 Assay kit includes a 30 µg/mL stock solution of FGF-Acidic, the physiological paracrine activator of FGFR1/2, that may be used to set up inhibition-mode assays. Approximately 4 ng/mL FGF-Acidic approximates EC_{80} in this assay and, therefore, is a suitable *final assay concentration* of activator to be used when screening test compounds for inhibitory activity.

Adding the reference activator 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 antagonist 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 (~ 8 ng / mL) of the challenge activator FGF-Acidic.

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 (~ 4 ng/mL) of the challenge activator FGF-Acidic.

DAY 1 Assay Protocol:

All steps should be performed using proper aseptic technique.

1.) Remove **Cell Recovery Medium (CRM)** and **Compound Screening Medium (CSM+H)** from freezer storage and thaw in a 37°C water bath.

2.) **Prepare dilutions of test compounds:** Prepare Test Compound treatment media for *Agonist-* or *Antagonist-mode* screens. NOTE that test and reference compounds will be prepared differently when using tip-dispensing *vs.* **acoustic dispensing**. Regardless of the method, the total DMSO carried over into assay reactions should not exceed 0.4%.

a. *Tip dispensing method:* In *Step 6*, 15 µl / well of the prepared treatment media is added to the assay that has been pre-dispensed with 15 µl /well of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a **2x**-concentration of the test and reference material(s). Use CSM to prepare the appropriate dilution series. Plan dilution volumes carefully; this assay kit provides 35 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-molecules 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 FGFR1/2 Assay kit includes a concentrated stock of FGF-Acidic, 30 µg/mL prepared in PBS/0.1%BSA. The following 7-point treatment series, with concentrations generated using serial 3-fold dilutions, provides a complete dose-response: 30, 10.0, 3.33, 1.11, 0.370, 0.123 and 0.041 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 µl / well).

(continued ...)

APPENDIX 1b provides an example for generating such a series of 1,000x-concentrated solutions of compounds to be used when performing *acoustic dispensing* (30 nl / well). As noted in *Step 2b*, use CSM 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 cell suspension several times to gain a homogenous suspension.

a. for Agonist-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 agonist (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 **5.5 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 FGFR1/2 Reporter Cells suspension with the challenge activator **FGF-Acidic** 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.

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: 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.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
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
Bulk volumes of FGFR1/2 Assay Reagents may be custom manufactured to accommodate any scale of HTS. NOTE: Single receptor FGFR1 or FGFR2 Assays can be made available upon request. Please Inquire.	
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
NFAT Assays (recommended for receptor-specificity screening)	
IB18001-32	NFAT Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB18001	NFAT Reporter Assay System 1x 96-well format assay
LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in 5x bulk volume to perform 480 Live Cell Assays contained in 5 x 96-well assay plates
LCM-10	Reagent in 10x bulk volume to perform 960 Live Cell Assays contained in 10 x 96-well assay plates
INDIGlo Luciferase Detection Reagent	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents in 10 mL, 25 mL, 50 mL, and 500 mL volumes

Please refer to INDIGO Biosciences website for updated product offerings.

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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. Literature Citations

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

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

³ Park JY, *et al.* (2020) The Role of Calcium-Calcineurin-NFAT Signaling Pathway in Health and Autoimmune Disease, *Frontiers in Immunology* :doi:10.3389/fimmu.2020.00195.

⁴ 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.

$$Z' = 1 - [3*(SD^{EGF EC100} + SD^{Vehicle}) / (RLU^{EGF EC100} - RLU^{Vehicle})]$$

