Human
Epidermal Growth Factor Receptor 1
Reporter Assay System
(EGFR1; ErbB-1; HER1)

96-well Format Assays
Product # IB13001

▪

Technical Manual
(version 7.2i)

www.indigobiosciences.com

3006 Research Drive, Suite A1, State College, PA 16801, USA

Customer Service:
814-234-1919; FAX 814-272-0152
customerserv@indigobiosciences.com

Technical Service:
814-234-1919
techserv@indigobiosciences.com

INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human EGFR1 Reporter Assay System
96-well Format Assays

I. Description

▪ The Assay System ▪

This EGFR1 assay utilizes proprietary human cells that provide constitutive expression of the **Human type I Epidermal Growth Factor Receptor (EGFR1; ErbB1; HER1)**,

EGFR1 is a single-pass transmembrane receptor, one of four members of the receptor tyrosine kinase (RTK) family. Binding interactions with extra-cellular signaling peptides such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα), or amphiregulin lead to receptor dimerization and auto-phosphorylation by the cytoplasmic tyrosine kinase domains, followed by tyrosine phosphorylation and activation of associated intracellular signaling proteins. Interestingly, EGF Receptors demonstrate two alternative signal processing modes: one *via* the membrane bound receptor and signaling at the cell surface, and the other signaling through internalized receptors, nuclear translocation, and co-association with activated transcription factors to interact directly with target gene promoter sequences[1].

Activated EGF Receptors are known to signal through several different pathways, including those mediate by Ras, PI3K, PLC-γ and JAK, culminating in the activation of specific transcription factors and the induction of respective target genes. Phosphorylation and activation of the transcription factor STAT3 is one prominent pathway utilized by EGFR1[2], and it is the signaling mechanism exploited by the reporter cells included in this kit.

INDIGO's Reporter Cells contain the luciferase reporter gene functionally linked to an upstream minimal promoter and tandem STAT3 genetic response element (GRE) sequences. Activated, dimeric $STAT3^P$ (or $STAT3^P$ associated with nuclear $EGFR1^P$) bind the STAT3 GRE's to initiate the formation of a complete transcription complex that drives Luc 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- or antibody-induced changes in EGFR1 activity.

Considering their significant role in rapid cell proliferation, and their involvement the progression of many types of cancers[3], EGFR's continues to command much interest as a target for the development of novel, specific, and predominantly inhibitory drugs and antibodies. Accordingly, the primary application of this EGFR1 Assay is to screen test materials for any functional activity, either agonistic or inhibitory, that they may exert against the EGFR1.

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.

INDIGO's EGFR1 Assay kit provides the convenience of an all-inclusive cell-based assay system. In addition to Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference agonist human EGF, Luciferase Detection Reagents, and a cell culture-ready assay plate.

[1] Carpenter G, Liao HJ. Trafficking of receptor tyrosine kinases to the nucleus. (2009) Exp Cell Res:**315**, 1556-1566.

[2] Lo H, *et al.* Nuclear interaction of EGFR and STAT3 in the activation of iNOS/NO pathway. (2005) Cancer Cell:**7**, 575-589.

[3] Lo H, Hung MC. Nuclear EGFR signaling network in cancers: linking EGFR pathways to cell cycle progression, nitric oxide pathway and patient survival. (2006) Br J Cancer: **94**, 184-188.

▪ The Assay Chemistry ▪

INDIGO's nuclear 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, and yields as products 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).

Assay kits feature 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 test samples: Chemical 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. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

1.) As described in *Step 7* and depicted in Appendix 1 for the reference activator, **Compound Screening Medium (CSM)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired final assay concentration series.

Alternatively, if test compound solubility is expected to be problematic,
2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the final concentration of total DMSO carried over into assay wells should not exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 0.4%.

Protein test samples: It is recommended that protein or antibody test samples are 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. CSM is then used as the diluent to prepare treatment media.

> *NOTE:* CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup and are considered to be 'single-use' reagents.

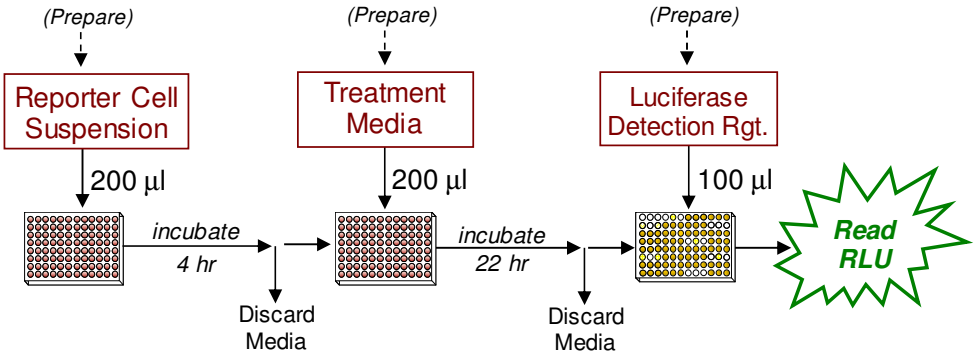
▪ Considerations for Automated Dispensing ▪

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 rgt. 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/well of Reporter Cells are dispensed into 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 hour 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.



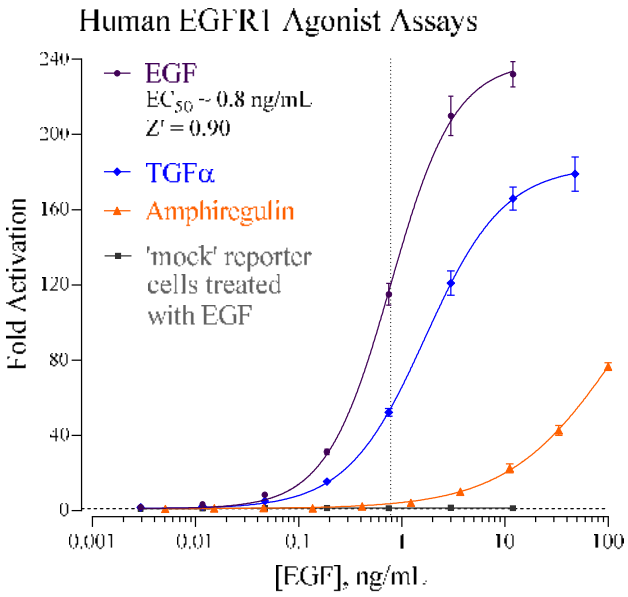


Figure 2. EGFR1 Agonist dose-response assays. EGFR1 activation assays were performed according to the protocol provided in this Technical Manual. 200 µl / well of EGFR1 Reporter Cell suspension was dispensed into the 96-well assay plate, which was then incubated for 4 hours. Concentrated stocks of the reference peptides EGF (provided), TGFα and Amphiregulin (Sigma) were prepared in PBS + 0.1% BSA, then further diluted using CSM to produce treatment media at the desired assay concentrations. The pre-culture media were discarded from the assay wells and 200 µl per well of respective treatment media were dispensed (n = 3/conc.), including 'vehicle only' control wells. 'Mock' reporter cells, which contain the STAT3-Luc reporter gene but lack expression of the EGFR1, were treated with EGF. Following a 22-hour 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) and percent coefficient of variation derived from SD (%CV) were determined for each treatment condition. Non-linear regression analyses and EC_{50} calculations were performed using GraphPad Prism software. Plots show Fold Activation *vs.* Log_{10}[ng/mL] for the various treatment materials; error bars depict +/- %CV.

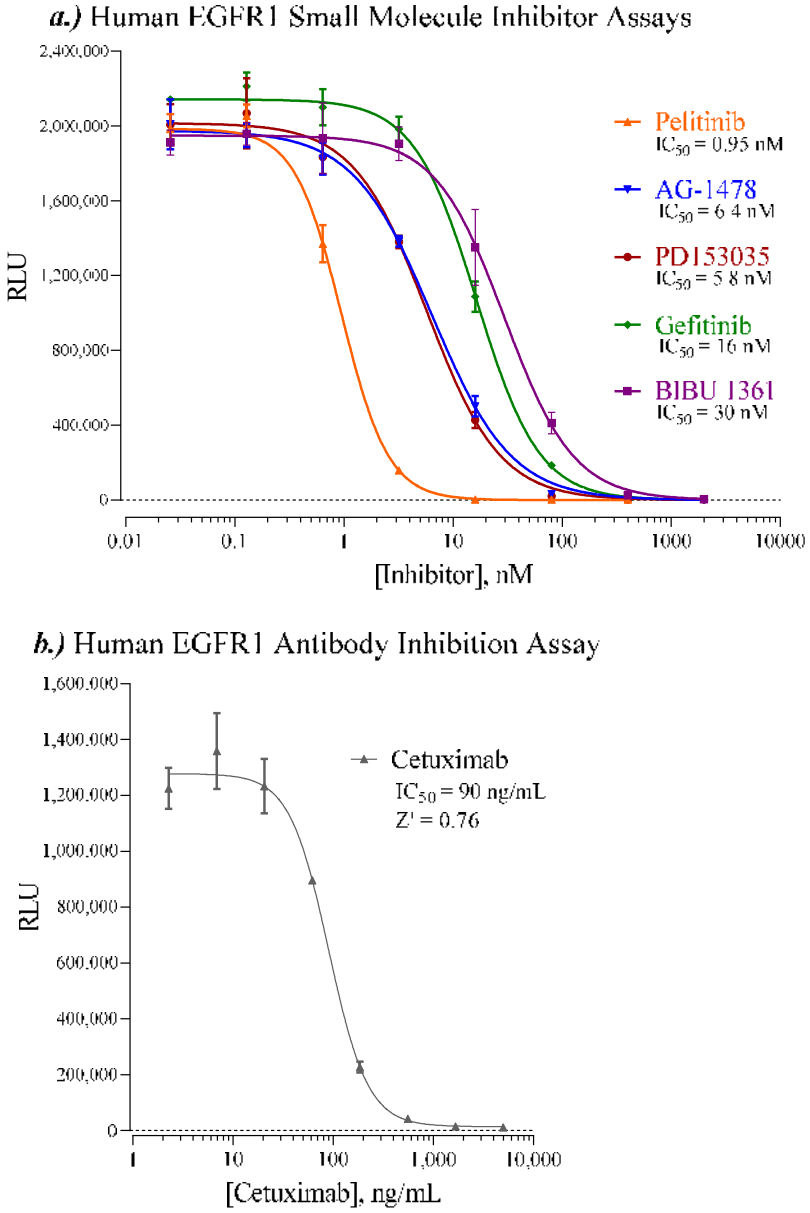


Figure 3. EGFR1 Inhibition assays. 200 µl / well of EGFR1 Reporter Cell suspension was dispensed into 96-well assay plates, which was then incubated for 4 hours. For inhibition-mode assays, prior to the end of the pre-culture period, CSM was supplemented with 1.4 ng EGF/ml (an approximate EC_{80} concentration). The EGF-supplemented CSM was then used to prepare the various treatment media. ***a.)*** The small molecule inhibitors Pelitinib and Gefitinib (Cayman Chem.), AG-1478, PD153035, and BIBU1361 (Tocris) were initially prepared in DMSO as 2 mM stock solutions that were further diluted using the EGF-supplemented CSM. Final assay concentrations began at 2 µM and proceeded with serial 5-fold decrements. Z' values[4] ranged between 0.77 and 0.84. ***b.)*** The anti-EGFR antibody Cetuximab (MedChem Express; 5 mg/mL) was diluted using EGF-supplemented CSM to produce final assay concentrations beginning at 5 µg/mL and proceeding with serial 3-fold decrements. Pre-culture media were discarded and 200 µl/well of the prepared treatment media were dispensed (n = 3/conc.), including 'vehicle only' control wells. After 22 hr incubation treatment media were discarded, Luciferase Detection Reagent was added, and RLU/well were quantified. Plots are ***a.)*** RLU *vs.* Log_{10}[nM], and ***b.)*** RLU *vs.* Log_{10}[ng/mL] for the various test materials. Error bars depict +/- SD.

[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.

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

II. Product Components & Storage Conditions

This Human EGFR1 Assay kit contains materials to perform assays in a single collagen-coated 96-well assay plate.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of 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 be sure to first transfer and submerge the tube of reporter cells in dry ice.

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

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

Kit Components	*Amount*	*Storage Temp.*
▪ EGFR1 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
▪ EGF, 12 µg/mL (in PBS/0.1%BSA) (reference activator of EGFR1)	1 x 20 µL	-20°C
▪ Detection Substrate	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well, *collagen-coated* assay plate (white, sterile, cell-culture ready)	1	**-20°C**

NOTE: This Assay kit contains one 96-well assay plate in which the assay wells have been collagen-coated and dried; the assay plate should be stored frozen (-20°C or colder) until use.

III. Materials to be Supplied by the User

The following materials must be provided by the user, and should be made ready prior to initiating the assay procedure:

DAY 1

▪ container of dry ice (see *Step 2*)

▪ cell culture-rated laminar flow hood.

▪ 37°C, humidified 5% CO_2 incubator for mammalian cell culture.

▪ 37°C water bath.

▪ 70% alcohol wipes

▪ 8-channel electronic, repeat-dispensing pipettes & sterile tips

▪ disposable media basins, sterile.

▪ sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), *or* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).

▪ *Optional:* antagonist reference compound / antibody (*e.g.,* Fig. 3a, b.)

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

DAY 2 plate-reading luminometer.

IV. Assay Protocol

The Day 1 Assay protocol begins on the next page. Please review the entire Assay 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 agonist AND varying concentrations of the test compound(s) to be evaluated for chemical inhibition or neutralizing antibody activities. This EGFR1 Assay kit includes a 12 µg/mL stock solution of **EGF**, a potent physiological agonist of EGFR1, that may be used to setup inhibition-mode assays. 1.4 ng EGF/ml typically approximates EC_{80} in this assay. Hence, it presents a suitable concentration of agonist to use when screening test materials for inhibitory activities.

Add EGF to a bulk volume of **CSM**, as described above. This agonist-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 EGFR1 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 **EGFR1 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-chanel pipette, dispense **200 µl / well** of cell suspension into wells of the assay plate.

> *NOTE 4.1:* If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'cell blank' wells (meaning cell-free, but containing 'Compound Screening Media') 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-culture reporter cells: Place the assay plate into a 37°C, ≥ 70% humidity, 5% CO_2 incubator for 4 - 6 hours.

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

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. Agonist*-mode assays**. This EGFR1 Assay kit includes a concentrated (1,000x) stock of EGF, 12 µg/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: 12, 3.0, 0.75, 0.188, 0.0469, 0.0117 and 0.00293 ng/ml. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' (or 'vehicle') controls.

~ or ~

***b. Antagonist*-mode assays**. When setting up antagonist assays, first supplement a bulk volume of CSM with the challenge agonist **EGF** to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about antagonist-mode assay setup*", pg. 8). The agonist-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 pre-culture period, discard the media by 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.

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 37°C, humidified 5% CO_2 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.) 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 room temperature **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

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

16.) Add 100 µl of the prepared **LDR** into all wells of the assay plate. Allow the assay plate to rest at room temperature for at least 5 minutes following the addition of LDR. Do not shake the assay plate during this period.

17.) Quantify luminescence.

V. Related Products

Product No.	*Product Descriptions*
Human EGFR1 Assay Products	
IB13001-32	Human EGFR1 Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB13001	Human EGFR1 Reporter Assay System 1x 96-well format assay
IB13002	Human EGFR1 Reporter Assay System 1x 384-well format assays
Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

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

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

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

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

APPENDIX 1

Example scheme for the serial dilution of the reference agonist EGF, and the setup of a EGFR1 dose-response assay.