INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

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

96-well Format Assays
Product # IB13002

▪

Technical Manual
(version 8.0)

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Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human EGFR1 Reporter Assay System
384-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 Reporter 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 reporter assays 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).

INDIGO's Nuclear Receptor Assays feature 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 never exceed 0.4%; strive to use 1,000x-concentrated stocks when they are prepared in DMSO.

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

and,

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 hr incubation Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.

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

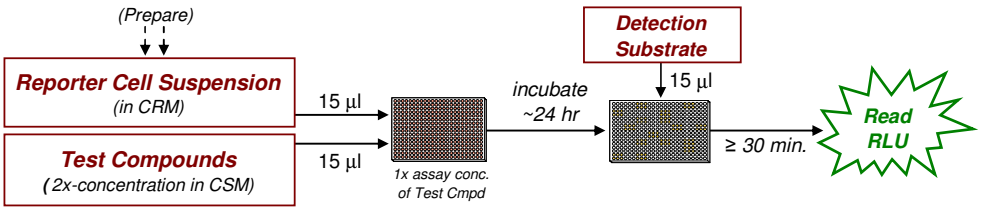
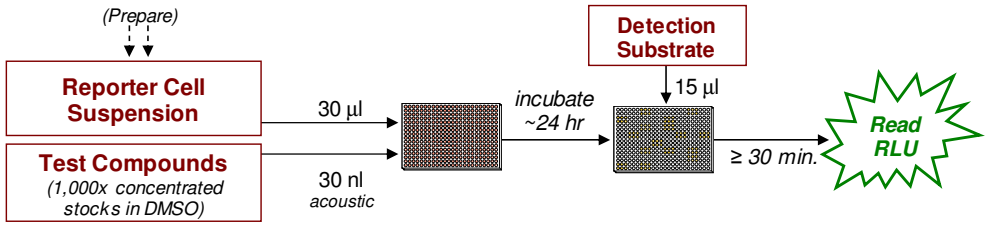


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



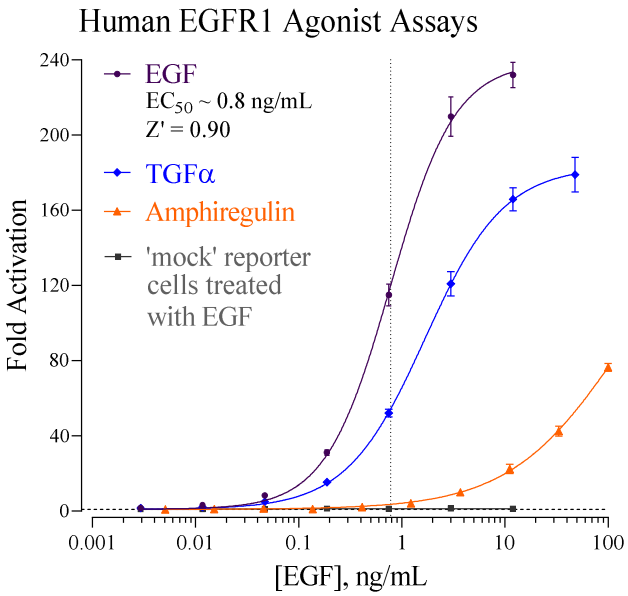


Figure 2. EGFR1 Agonist dose-response assays. EGFR1 activation assays were performed according to the protocol provided in this Technical Manual. 200 µl / well of EGFR1 Reporter Cell suspension was dispensed into the 96-well assay plate, which was then incubated for 4 hours. Concentrated stocks of the 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 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) 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.

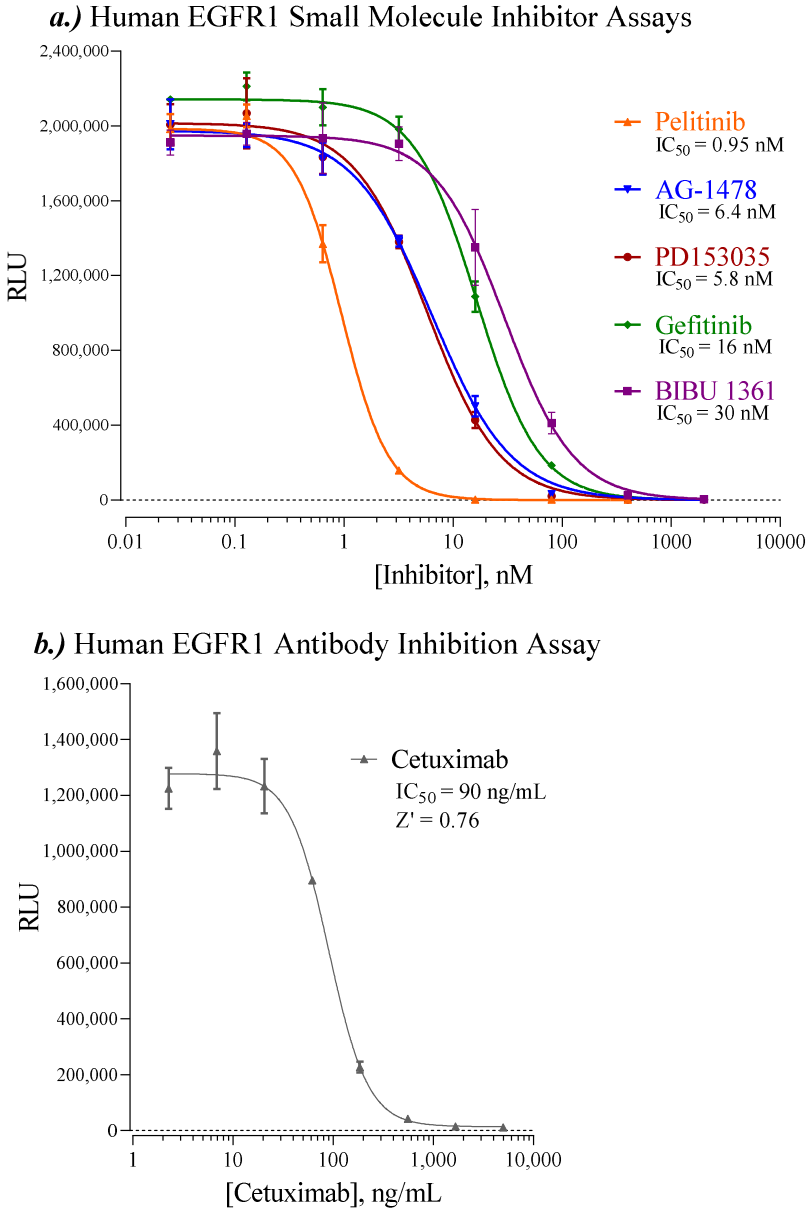


Figure 3. EGFR1 Inhibition assays. 200 µl / well of EGFR1 Reporter Cell suspension was dispensed into 96-well assay plates, which was then incubated for 4 hours. For inhibition-mode assays, prior to the end of the pre-culture period, CSM was supplemented with 1.4 ng EGF/ml (an approximate EC_{80} concentration). 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 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.

Kit Components	*Amount*	*Storage Temp.*
▪ EGFR1 Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 7 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ EGF, 12 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% CO_2 incubator for mammalian cell culture.

▪ 37°C water bath.

▪ 70% alcohol wipes

▪ 8-channel electronic, repeat-dispensing pipettes & tips suitable for dispensing 15 µl.

▪ 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.,* Fig. 3a, b.)

▪ *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 TGFβR Assay kit includes a 3.0 µg/mL stock solution of **TGF-β1**, a potent physiological agonist of TGFβR, that may be used to setup antagonist-mode assays. 0.6 ng/mL TGF-β1 typically approximates EC_{80} in this assay. Hence, it presents a reasonable *final assay concentration* of agonist to be used when screening test compounds for inhibitory activity.

Adding the reference activator TGF-β1 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 of the challenge activator.

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 of the challenge activator.

DAY 1 Assay Protocol:

All steps should be performed using proper aseptic technique.

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

2.) Prepare dilutions of treatment 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 never 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 proteins (*e.g.*, EGF and antibodies), should be further diluted using CSM (not DMSO).

Preparing the positive control: 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.

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 samples prepared in aqueous solutions (*e.g.*, EGF, antibodies) and DMSO to further dilute samples 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 5.5 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 disperse cell aggregates and 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 (in DMSO solutions, 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 disperse cell aggregates and 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 EGFR1 Reporter Cells suspension with the challenge activator **EGF** to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about inhibition-mode assay setups*", pg. 9). Then dispense **30 µl / well** of the supplemented cell suspension into the assay plate that has been pre-dispensed with test compounds.

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 1-2 minutes using a room temperature centrifuge fitted with counter-balanced plate carriers.

7.) Transfer the assay plate into a 37°C, humidified, 5% CO_2 incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 85%) 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. Gently invert the tube several times to ensure a homogenous solution.

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. 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*.

11.) Following 22 - 24 hours of incubation dispense **15 µl / well** of **Detection Substrate** into all wells of the assay plate.

NOTE: Perform this reagent transfer carefully to avoid bubble formation! Scattered micro-bubbles 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. INDIGO recommends performing a final *low-speed* spin of the assay plate (with lid) for 1-2 minutes 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 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

Human EGFR1 Assay Products	
Product No.	***Product Descriptions***
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 EGFR1 Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

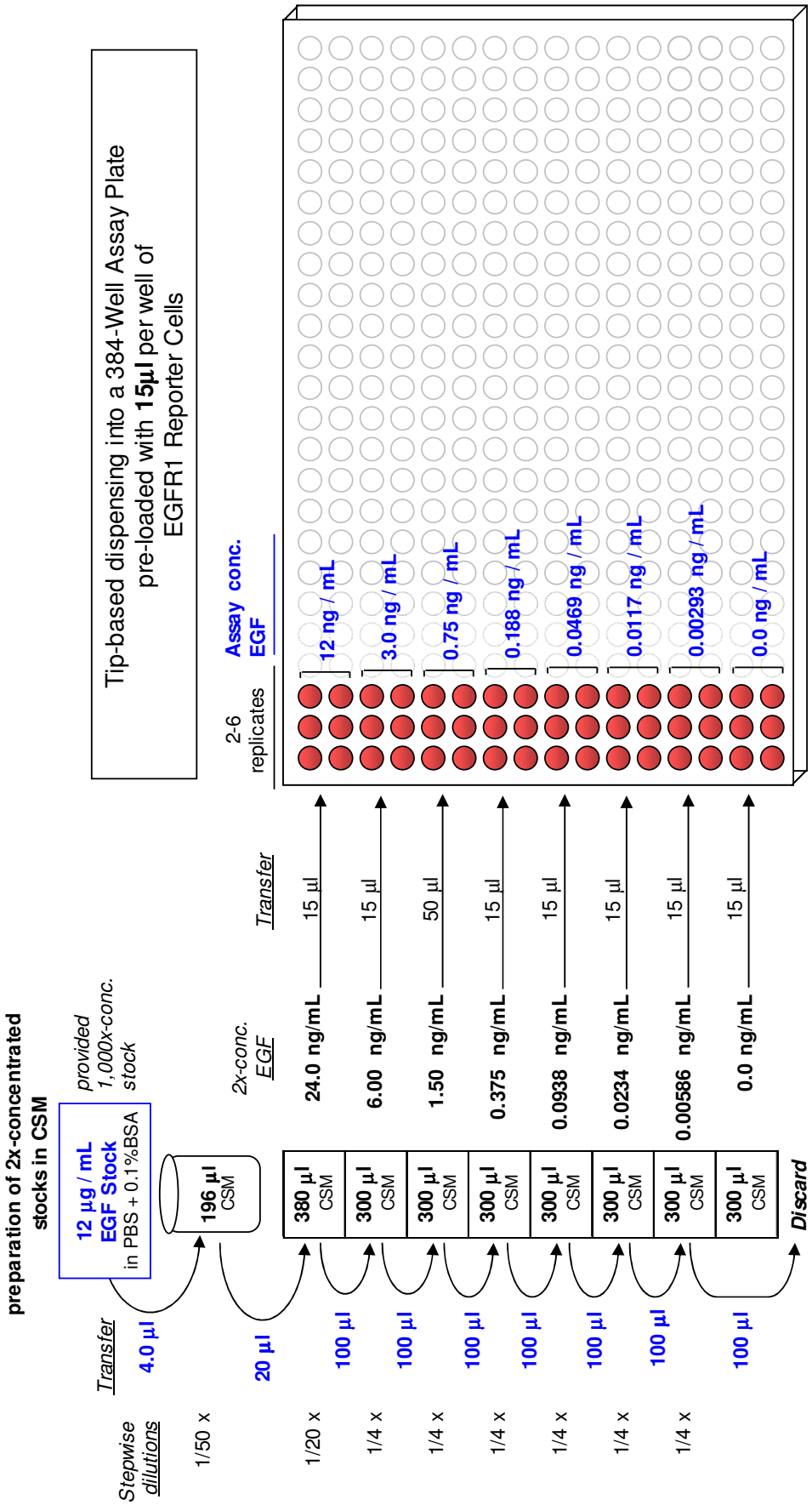
VI. Limited Use Disclosures

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

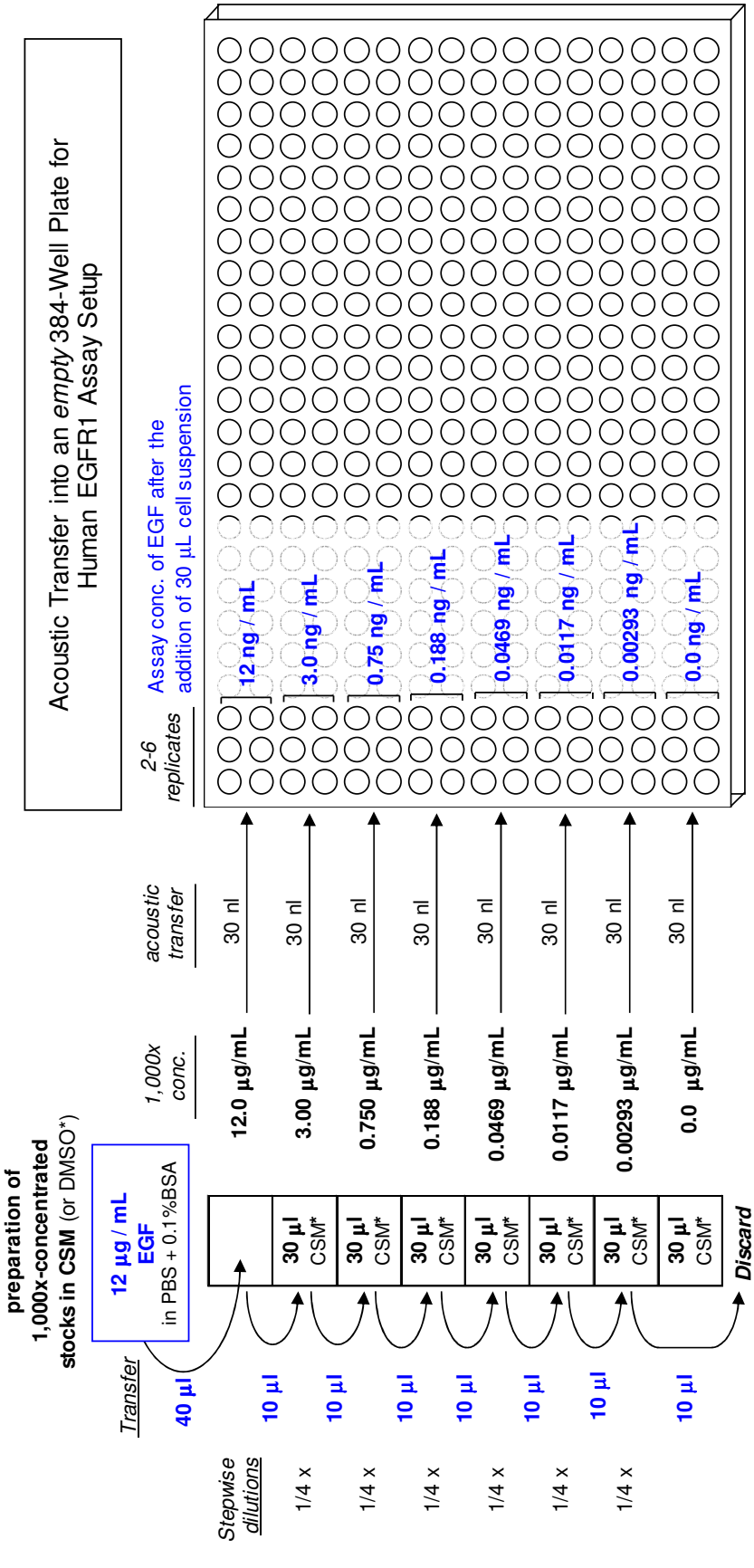
"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA).

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 current version available.

APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of the reference agonist EGF into CSM to generate **2x-concentrated** treatment media. 15 µl / well are dispensed into assay plates using a *tip-based* instrument.



APPENDIX 1b for acoustic dispensing. Example scheme for the serial dilution of the reference agonist EGF (a protein) into CSM to generate **1,000x-concentrated** stocks. 30 nl / well are pre-dispensed into assay plates using an acoustic transfer device. **NOTE:* Stocks of small-molecule test drugs are typically prepared in DMSO, and **DMSO** (*not* CSM) should be further used to generate the desired series of 1,000x-treatment concentrations.



* Stocks of protein ligands, such as EGF in the above example, or other test materials that are solvated in aqueous solution should be further diluted using CSM. However, any stocks of test materials that are solvated in DMSO, as is typical for small molecule chemicals, should be further diluted using DMSO.