Human Erythropoietin Receptor Reporter Assay System

(EPOR)

96-well Format Assays
Product # IB17002

▪

Technical Manual
(version 8.0i)

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 EPOR Reporter Assay System
384-well Format Assays

I. Description

- The Assay System……3
- The Assay Chemistry……4
- Considerations for the Preparation and Automated Dispensing of Test Compounds……4
- Considerations for Automated Dispensing of Other Assay Reagents……5
- Assay Scheme……5
- Assay Performance……6

II. Product Components & Storage Conditions ……7

III. Materials to be Supplied by the User……7

IV. Assay Protocol

- A word about *Antagonist*-mode assay setup……8
  - *DAY 1 Assay Protocol*……8
  - *DAY 2 Assay Protocol*……10

V. Related Products……11

VI. Limited Use Disclosure……11

APPENDIX 1a: Example Scheme for Serial Dilution when using tip-based dispensing of test compounds……12

APPENDIX 1b: Example Scheme for Serial Dilutions when using acoustic dispensing of test compounds……13

I. Description

▪ The Assay System ▪

This assay utilizes proprietary human cells that provide constitutive expression of the **Human Erythropoietin Receptor (EPOR)**.

EPOR is a member of the class I cytokine receptor superfamily. Erythropoietin (EPO), the physiological activator of EPOR, is a glycoprotein that regulates erythropoiesis. EPO, produced by the adult kidneys, activates the cell-surface EPOR to initiate signal transduction through JAK2/STAT5, and other pathways including AKT and ERK1/2.[1] The activation of these various pathways may culminate in the activation of cytosolic targets, or in the activation of specific transcription factors and the induction of their respective target genes.

JAK2 dependent phosphorylation and activation of the transcription factor STAT5 is a prominent outcome of EPOR activation, and it is the signaling pathway exploited by the reporter cells included in this assay kit. Specifically, INDIGO's Reporter Cells contain the luciferase reporter gene functionally linked to an engineered minimal promoter sequence with upstream tandem STAT5 genetic response element (GRE) sequences. Erythropoietin activates the EPOR in a dose-dependent manner, thereby triggering the JAK2/STAT signal transduction cascade. Activated STAT5 binds to its consensus GREs to initiate the formation of a complete transcription complex that drives expression of the Luc reporter gene. Therefore, quantifying changes in luciferase activity from peptide-, drug- or antibody-treated reporter cells relative to that of 'untreated' cells provides a sensitive, dose-dependent surrogate measure of changes in the activity of EPOR.

The clinical use of recombinant human EPO has led to the successful treatment of anemia associated with conditions ranging from chronic kidney disease, chemotherapy treatment for cancer, and surgery-associated blood loss. However, side effects including hypertension, increased risk for stroke, venous thromboembolism, and death, are also risks for patients treated with hEPO and other biosimilars. This provides an opportunity for the development of additional erythropoiesis stimulating agents (ESAs) which lack these adverse effects[2]. Additionally, though erythroid progenitor cells express the highest levels of EPOR, it is also expressed on non-erythroid cells, including neural cells, myoblasts, and adipocytes, indicative of important physiological functions of EPO outside its essential role in erythropoiesis.[1] Accordingly, the primary application of this assay is to screen test materials for any functional activity, either agonistic or inhibitory, that they may exert against the human EPOR.

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 assay kits provide the convenience of an all-inclusive cell-based assay system. In addition to EPOR Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the physiological activator Erythropoietin, Luciferase Detection Reagents, and a cell culture-ready assay plate.

[1] Suresh, S, *et al.* (2020) The many facets of erythropoietin physiologic and metabolic response. Frontiers in Physiology 10:1534.

[2] Rainville, N, *et al*. (2016) Targeting EPO and EPO Receptor pathways in anemia and dysregulated erythropoiesis. Expert Opin Ther Targets 20 (3): 287-301.

▪ 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 ▪

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**. The final concentration of DMSO carried over into assay reactions should not exceed 0.4%; strive to use 1,000x-concentrated stocks when they are prepared in DMSO.

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

and,

b.) 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**.

▪ 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 rgt. volume available for instrument dead volume**
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.

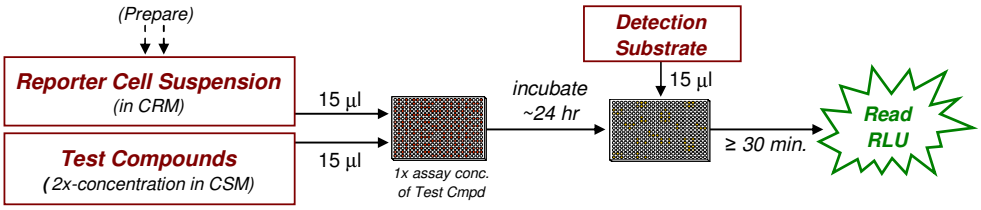
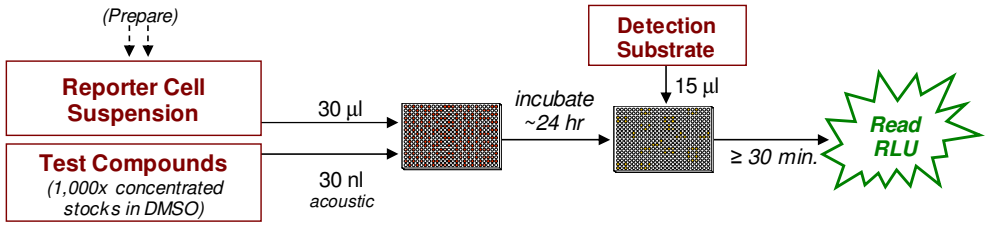


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



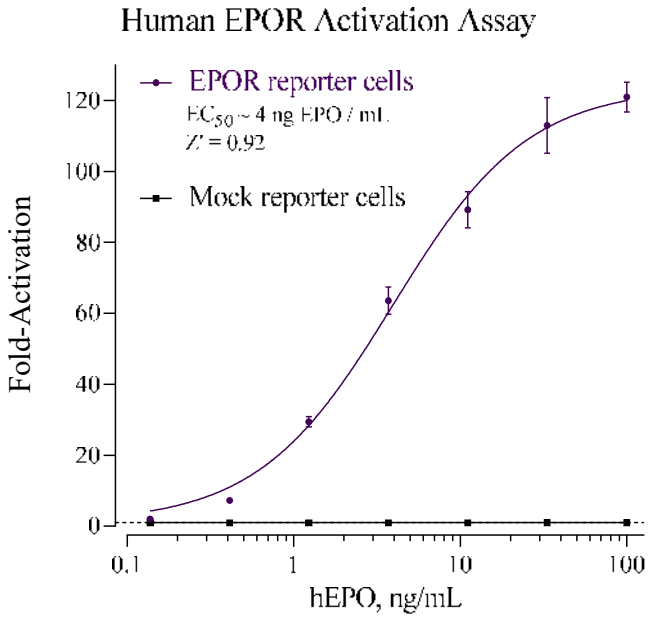


Figure 2. EPOR Activation assay. 384-well assay plates were processed using tip-based manual dispensing methods. 'Mock' reporter cells, which contain the STAT5-Luc reporter gene, but lack expression of EPOR, were similarly treated with EPO. At the 24-hour assay endpoint values of average Relative Light Units (RLU), respective Fold-Activation, and Coefficient of Variation (CV) were calculated for each treatment concentration (n =4). Z' was calculated as per Zhang, *et al*. (1999)[1]. Treatment concentrations were Log10 transformed, and values of Fold-Activation were then plotted *via* non-linear regression using GraphPad Prism software.

[3] 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.

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

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.*
▪ Human EPOR Reporter Cells	1 x 1.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 7 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ EPO, 10 µg / mL (in PBS/0.1%BSA) (physiological activator of EPOR)	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

▪ repeat-dispensing pipettes & tips or dispensing automation 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).

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 Antagonist-mode assay setup ▪

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 inhibitory activity. This EPOR Assay kit includes a 10 µg/mL stock solution of human Erythropoietin (EPO), the physiological activator of EPOR, that may be used to set up inhibition-mode assays. 12 ng/mL EPO approximates EC_{80} in this assay and, therefore, is a suitable concentration to use when screening test materials for inhibitory activities.

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 in *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 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 activator.

DAY 1 Assay Protocol:

All steps must 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 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 (prepared in DMSO) are added to the assay plate using an acoustic transfer device.

Preparing the positive control: This EPOR Assay kit includes a concentrated stock of EPO, 10 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: 100, 33.3, 11.1, 3.70, 1.23, 0.412, and 0.137 ng/mL. Always include a 'no treatment' (or 'vehicle') control.

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).

APPENDIX 1b provides an example for generating such a series of 1,000x-concentrated solutions of compounds prepared in either CSM or DMSO* to be used when performing *acoustic dispensing* (30 nL / well. *See note accompanying Appendix 1b).

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 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 *Antagonist*-mode assays**: Supplement the bulk volume of Reporter Cells suspension with a 2x-concentration of the challenge activator EPO (refer to "*A word about antagonist-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 **6.5 mL** volume of 37°C CRM into the tube of frozen cells. Recap the tube of cells and place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.5 mL.

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 *Antagonist*-mode assays:** First supplement the bulk volume of Reporter Cells suspension with the challenge activator EPO to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about antagonist-mode assay setup*", 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 1-2 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 (≥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. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Set 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** to 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: After an initial 30-minute reaction period the luminescence signal achieves a stable emission output.

13.) Quantify luminescence.

V. Related Products

Product No.	*Product Descriptions*
Human Erythropoietin Receptor Assay Products	
IB17001-32	EPOR Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB17001	EPOR Reporter Assay System 1x 96-well format assay
IB17002	EPOR Reporter Assay System 1x 384-well format assays
Human Thrombopoietin Receptor Assay Products	
IB20001-32	TPOR Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB20001	TPOR Reporter Assay System 1x 96-well format assay
IB20002	TPOR Reporter Assay System 1x 384-well format assays
Bulk volumes of all Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

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.

www.indigobiosciences.com

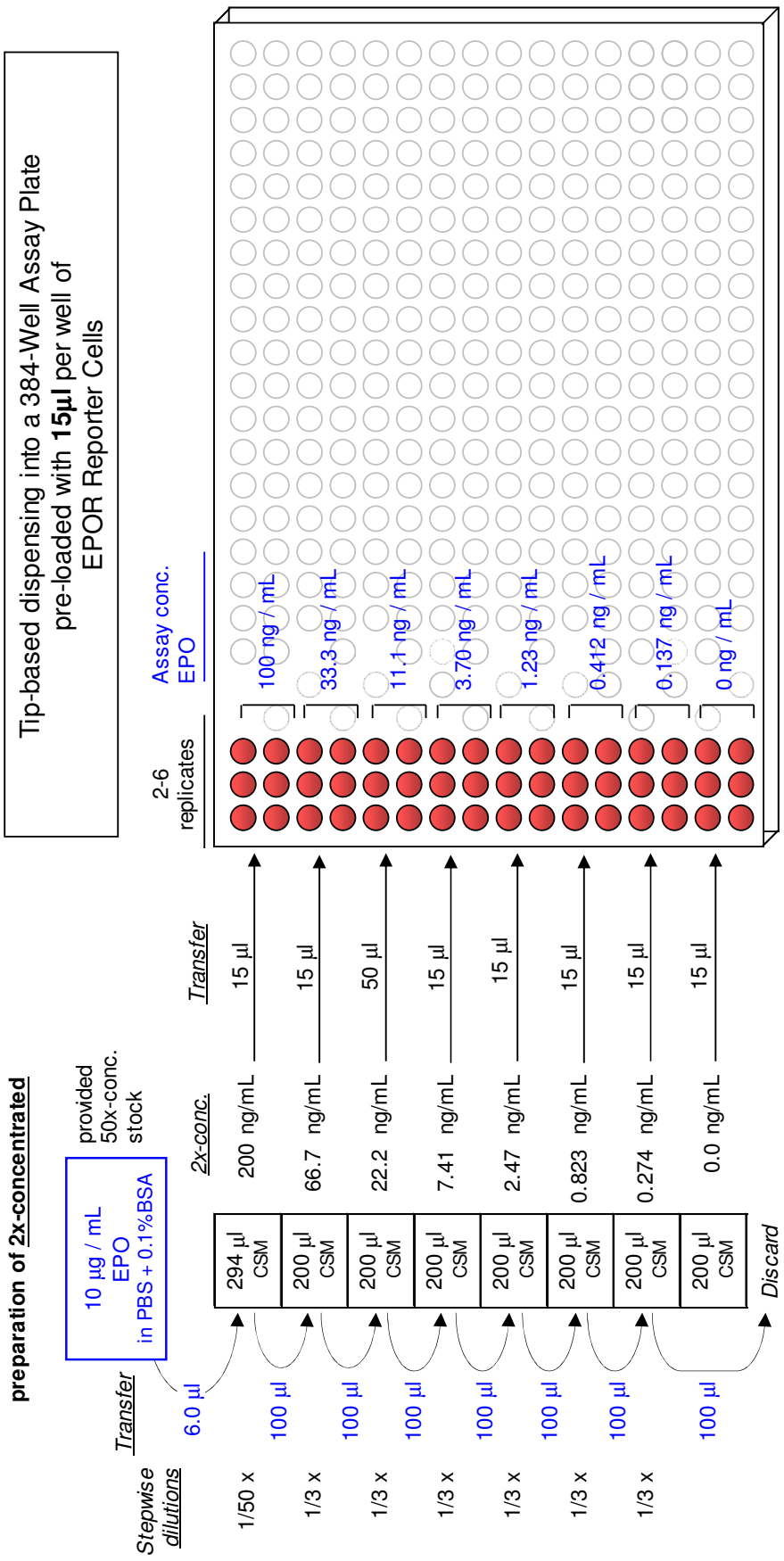
VI. Limited Use Disclosures

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

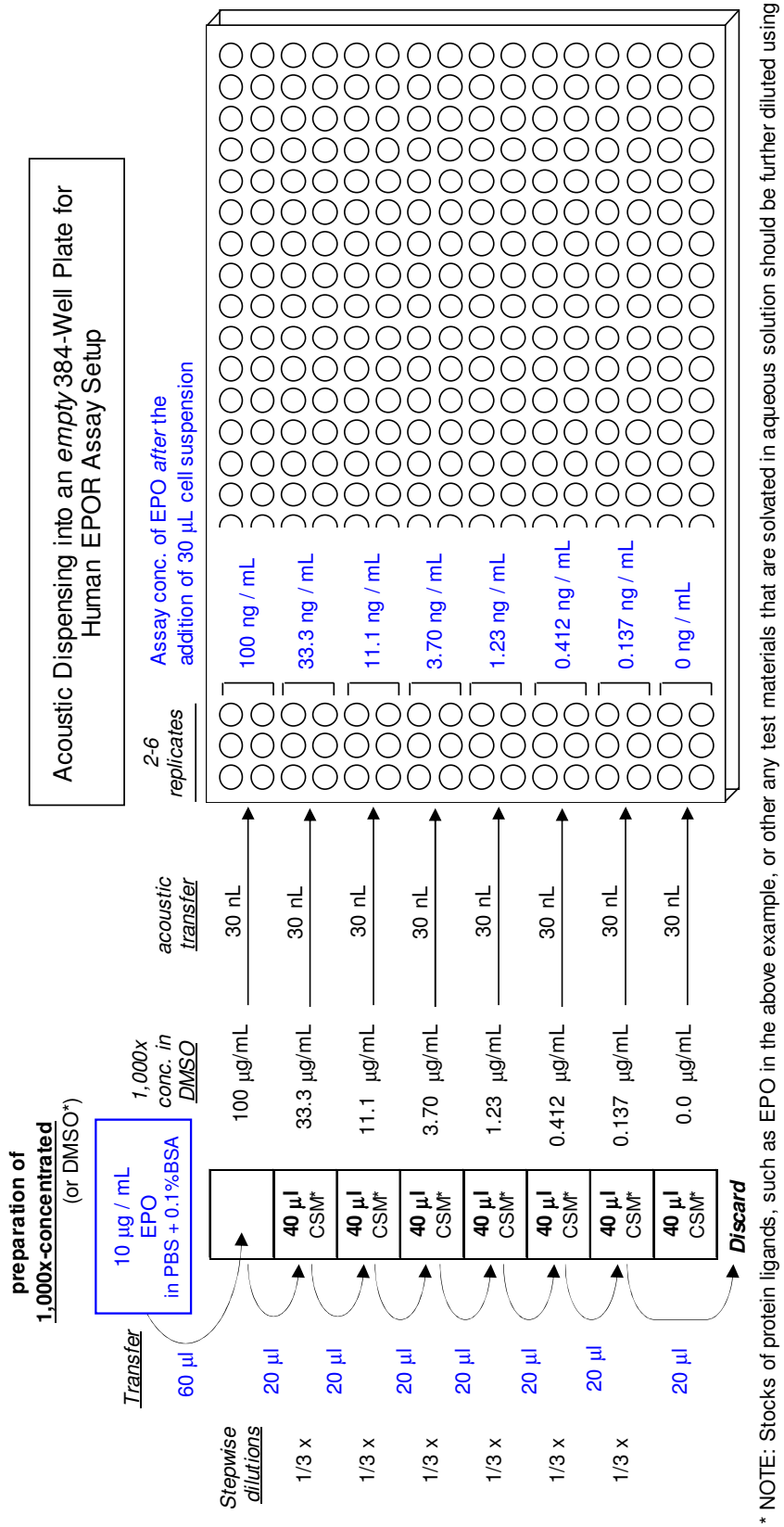
"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA).

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 currently updated version.

APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of the reference activator EPO into CSM to generate **2x-concentrated** treatment media. A *tip-based* instrument is used to dispense 15 µL/ well into an assay plate that has been *pre-dispensed* with 15 µL / well of EPOR Reporter Cell suspension.



APPENDIX 1b for acoustic dispensing. Example scheme for the serial dilution of the reference activator EPO into CSM to generate **1,000x-concentrated** intermediate stocks. 30 nL / well are then pre-dispensed into an *empty* assay plate using an acoustic transfer device.



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