Human Erythropoietin Receptor Reporter Assay System

(EPOR)

96-well Format Assays
Product # IB17001

▪

Technical Manual
(version 7.2)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human EPOR Reporter Assay System
96-well Format Assays

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 reporter 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 receptor assay kits capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg^{+2}-dependent reaction that consumes O_2 and ATP as co-substrates to yield oxyluciferin, AMP, PP_i, CO_2, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer and is reported in terms of Relative Light Units (RLU's).

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 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. Note that the final concentration of DMSO carried over into assay wells should not exceed 0.4%.

For protein or antibody samples it is recommended to solvate the materials in aqueous buffered solutions supplemented with carrier protein (*e.g.,* PBS + 0.1% BSA) at concentrations *no less* than 10x relative to the highest desired treatment concentration. The EPO stock included with this kit is prepared in PBS + 0.1% BSA at a 100x-concentration relative to the highest recommended treatment (refer to APPENDIX 1).

Immediately prior to setting up an assay the prepared stocks are serially diluted using **Compound Screening Medium (CSM)** to achieve the desired assay concentrations, as described in *Step 7*.

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

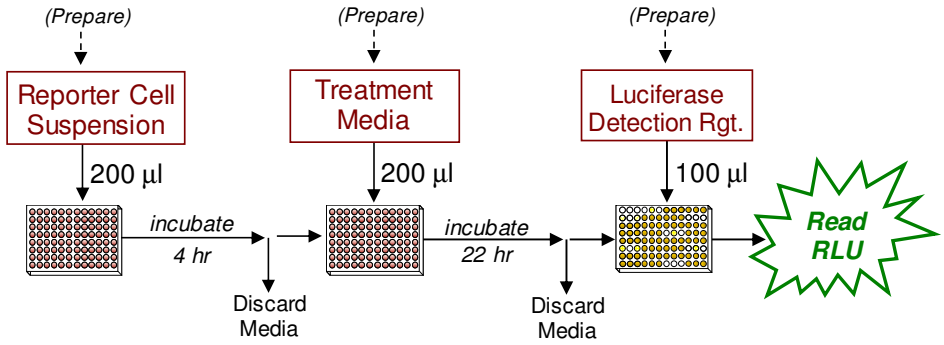
▪ Considerations for Automated Dispensing ▪

When using an automated dispensing instrument to process a small number of assay plates, first carefully consider the dead volume requirement of your instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

Stock Reagent & Volume provided	**Volume to be Dispensed** *(96-well plate)*	**Excess reagent volume available for instrument dead volume**
Reporter Cell Suspension 21 ml *(prepared from kit components)*	200 µl / well 19.2 ml / plate	~ 1.8 ml
LDR 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, 200 µl of Reporter Cells are dispensed into wells of the assay plate and incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared treatment media are added. Following 22-24 hr incubation discard treatment media and add Luciferase Detection Reagent. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



▪ Assay Performance ▪

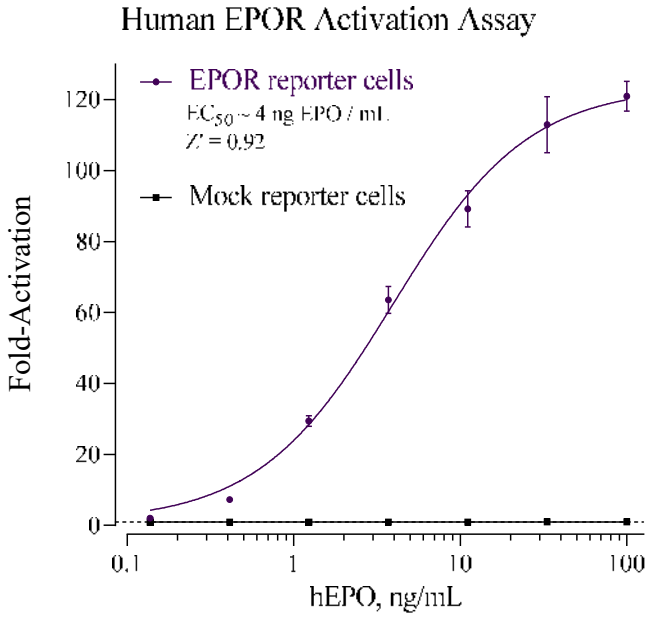


Figure 2. EPOR Activation assay. Dose-response analyses were performed according to the protocol provided in this Technical Manual. 200 µl / well of EPOR or Mock Reporter Cell suspensions were dispensed into the 96-well assay plate, then incubated for 4 hours. The concentrated stock of EPO (provided) was further diluted using CSM to produce treatment media at the desired assay concentrations. Pre-culture media were discarded from the assay wells and 200 µl per well of the prepared treatment media were dispensed (n = 4/conc.), including 'untreated' control wells. 'Mock' reporter cells, which contain the STAT5-Luc reporter gene, but lack expression of EPOR, were similarly treated with EPO. Following a 22 hr incubation period treatment media were discarded, Luciferase Detection Reagent was added, and luminescence intensity per well was quantified. Values of average relative light units (RLU) and corresponding values of standard deviation (SD), percent coefficient of variation (%CV), Fold-Activation and Z'[3] were determined for each treatment concentration. Non-linear regression analyses of Fold Activation *vs.* Log_{10}[EPO, ng/ml] and EC_{50} determination was performed 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.

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

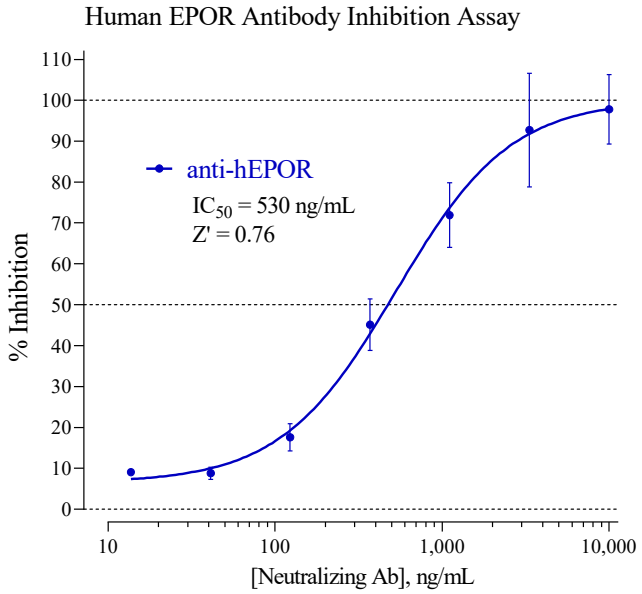


Figure 3. EOPR Inhibition assay. 200 µl / well of EPOR Reporter Cell suspension was dispensed into 96-well assay plates, which was then incubated for 4 hours. Prior to the end of the pre-culture period, CSM was supplemented with 12 ng EPO/ml (an approximate EC_{80} concentration). The EPO-supplemented CSM was then used to prepare treatment media containing varying concentrations of anti-hEPOR antibody (purified mouse monoclonal IgG, R&D Systems clone 713210). Pre-culture media were discarded and 200 µl/well of the prepared treatment media were dispensed, including 'no antibody' control wells. After 22 hr incubation treatment media were discarded, Luciferase Detection Reagent was added, and RLU/well were quantified. Data is plotted as % Inhibition of EPOR *vs.* Log_{10}[Aby; ng/ml].

II. Product Components & Storage Conditions

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

Cryopreserved mammalian cells are temperature sensitive! **To ensure maximal viability the tube of Reporter Cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in *Step 2* of this protocol.**

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

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

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

Kit Components	*Amount*	*Storage Temp.*
▪ Human EPOR 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
▪ EPO, 10 µg/ml (in PBS/0.1%BSA) (physiological activator of EPOR)	1 x 50 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* sterilized 96 deep-well blocks (*e.g.,* Axygen Scientific, #P-2ML-SQ-C-S), *or* appropriate similar vessel for generating dilution series of reference and test compound(s).

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

DAY 2 plate-reading luminometer.

IV. Assay Protocol

The assay protocol begins on the next page. Please review the entire protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-11* are performed on ***Day 1***, requiring less than 2 hours of bench work and a 4 hr incubation step to complete. *Steps 12-17* are performed on ***Day 2*** and require less than 1 hour to complete.

▪ A word about Inhibition-mode assay setups ▪

Receptor inhibition assays expose the Reporter Cells to a fixed, sub-maximal concentration (typically between EC_{50} – EC_{85}) of a known agonist 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 agonist of EPOR, that may be used to set up inhibition-mode assays. 12 ng/mL EPO approximates EC_{80} in this assay. Hence, it presents a suitable concentration of agonist to use when screening test materials for inhibitory activities.

Add EPO 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 EPOR 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 EPOR **Reporter Cells** from -80°C storage, place it directly into dry ice for transport to the laminar flow hood. When ready to begin, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 9.5 ml from ***each* of the 2 tubes** of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 minutes. The resulting volume of cell suspension will be **21 ml**.

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

4.) *Gently* invert the tube of Reporter Cells several times to gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an electronic, repeat-dispensing 8-channel pipette, dispense **200 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-incubate reporter cells. Place the assay plate into a cell culture incubator (37°C, ≥ 70% humidity, 5% CO_2) for 4 - 6 hours.

6.) Near the end of the pre-culture period: Remove Compound Screening Medium **(CSM)** from freezer storage and thaw in a 37°C water bath.

7.) Prepare the Test Compound(s) and Reference Compound treatment media: Use **CSM** to prepare an appropriate dilution series of the reference and test compound stocks. Prepare all treatment media at the desired final assay concentrations. In *Step 9*, the prepared treatment media will be dispensed at 200 µl/well into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO carried over into assay reactions should not exceed 0.4%.

***a. Agonist*-mode assays**. 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. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' control wells.

~ or ~

***b. Inhibition*-mode assays**. When setting up inhibition assays, first supplement a bulk volume of CSM with the challenge agonist EPO to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about inhibition-mode assay setup*", pg. 8). The EPO-supplemented CSM is then used to generate dilutions of test compound stocks to achieve the desired series of treatment concentrations.

8.) At the end of the 4-6 hr pre-culture period, discard the media; the preferred method is to use a 'wrist flick' to eject media into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

9.) Dispense **200 µl / well** of each prepared treatment media into the assay plate.

NOTE: If well-to-well variation due to 'edge-effects' is a concern this problem *may* be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate. Simply remove 1 tip from the 8-chanel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

10.) Transfer the assay plate into a cell culture incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 70%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

11.) For greater convenience on *Day 2*, retrieve **Detection Substrate** *and* **Detection Buffer** from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

DAY 2 Assay Protocol: Subsequent manipulations do *not* require special regard for aseptic technique and may be performed on a bench top.

12.) Approximately 30 minutes before intending to quantify receptor activity, remove **Detection Substrate** and **Detection Buffer** from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature.

NOTE: Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

13.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, *or less*.

14.) *Immediately before proceeding to Step 15*, transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a 12 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

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

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

17.) Quantify luminescence.

V. Related Products

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
Bulk volumes of EPOR 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

Please refer to INDIGO Biosciences website for updated product offerings.

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VI. Limited Use Disclosures

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

"CryoMite" is a Trademark ™ 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 1

Example scheme for the serial dilution of Human EPO and the setup of an EPOR dose-response assay.