# Human Thrombopoietin Receptor Reporter Assay System

# (TPOR)

**3x 32 Assays in 96-well Format**
Product # IB20001-32

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**Technical Manual**
*(version 7.2)*

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

# Human TPOR Reporter Assay System
## 3x 32 Assays in 96-well Format

## *I. Description*

**▪ The Assay System ▪**

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

TPOR, also known as MPL or CD110, is a member of the type I cytokine receptor superfamily. Thrombopoietin (TPO), the physiological activator of TPOR, is a glycoprotein that regulates thrombopoiesis, the process of platelet production, as well as hematopoietic stem cell maintenance.[1] TPO, which is constitutively produced in the liver with some contribution by the kidneys, binds to and activates the cell-surface TPOR to initiate signal transduction through several different pathways, including JAK/STAT, MAPK and PI3K pathways.[2]

JAK2 dependent phosphorylation and activation of the transcription factor STAT5 is a prominent outcome of TPOR 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. Thrombopoietin activates TPOR 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 surrogate measure of changes in the activity of TPOR.

TPOR has been targeted successfully in the clinic to treat conditions such as chronic immune thrombocytopenia, severe aplastic anemia, thrombocytopenia in hepatitis C patients undergoing interferon-based treatments, and thrombocytopenia in adults with chronic liver disease who will be undergoing a medical procedure. Examples of FDA-approved drugs which target TPOR include Romiplostim, Eltrombopag, Avatrombopag, and Lusutrombopag.[3] 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 human TPOR.

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 TPOR Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the physiological activator Thrombopoietin, Luciferase Detection Reagents, and a cell culture-ready assay plate.

[1] Hitchcock, I, *et al.* (2021) The thrombopoietin receptor: revisiting the master regulator of platelet production. Platelets, DOI: 10.1080/09537104.2021.1925102.

[2] Varghese, L, *et al*. (2017) The Thrombopoietin Receptor: Structural Basis of Traffic and Activation by Ligand, Mutations, Agonists, and Mutated Calreticulin. Front. Endocrinol., 8:59. DOI: 10.3389/fendo.2017.00059.

[3] Gilreath, J, *et al*. (2021) Thrombopoietin Receptor Agonists (TPO-RAs): Drug Class Considerations for Pharmacists. DOI: 10.1007/s40265-021-01553-7.

## ▪ 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. 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 TPO 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).

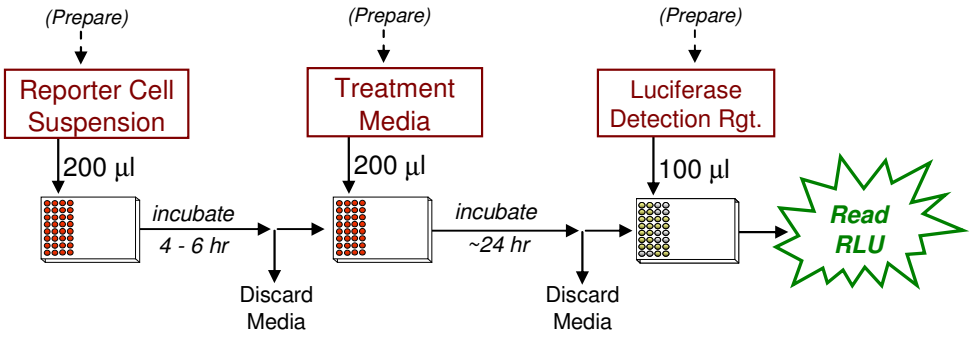
Immediately prior to setting up an assay the prepared stocks are serially diluted using **Compound Screening Medium (CSM)** to achieve the desired assay concentrations, as described in *Step 7*.

> *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.

## ▪ Assay Scheme ▪

**Figure 1.** Assay workflow.

*In brief*, 200 µl of Reporter Cells is dispensed into wells of the assay plate and 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 the 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.



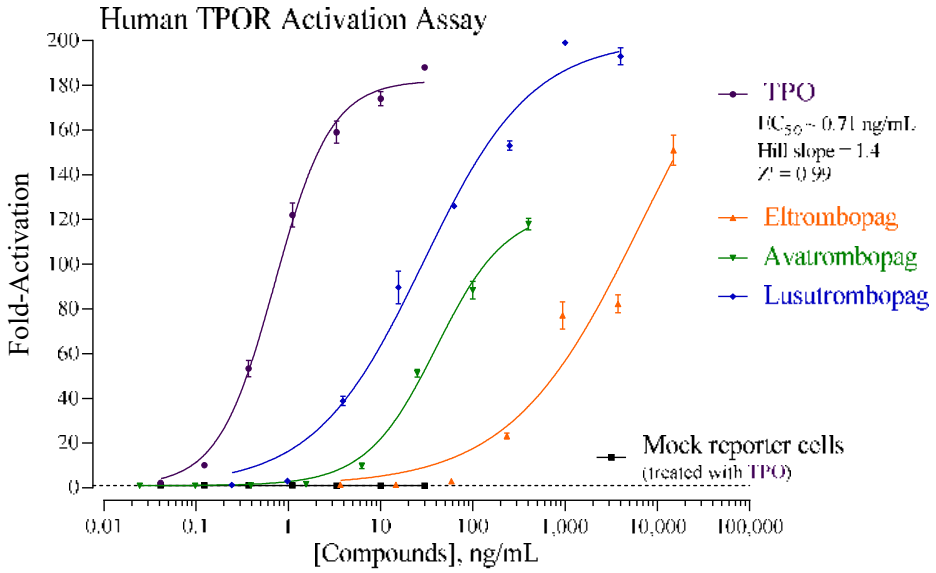



**Figure 2. TPOR Activation assay.** Agonist dose-response analyses were performed according to the protocol provided in this Technical Manual. The reference compounds TPO (provided), Eltrombopag, Avatrombopag, and Lusutrombopag (Cayman Chemical, Ann Arbor, MI, USA) were used to confirm assay performance. 200 µl / well of TPOR or 'Mock' Reporter Cell suspensions were dispensed into the 96-well assay plate, then incubated for 4 hours. The reference agonists were 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 = 3/conc.), including 'untreated' control wells. 'Mock' reporter cells, which contain the STAT5-Luc reporter gene, but lack expression of TPOR, were similarly treated with TPO. 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'[4] were determined for each treatment concentration. Non-linear regression analyses of Fold Activation *vs.* $Log_{10}$[Compounds, ng/ml] and $EC_{50}$ determination was performed using GraphPad Prism software. The absence of signal in TPO-treated 'Mock' reporter cells confirms that the response observed is specific to TPOR function.

[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.

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

## *II. Product Components & Storage Conditions*

This Human TPOR Assay kit contains materials to perform three distinct groups of assays in the format of a 96-well plate. Reagents are configured so that each group will comprise 32 assays. If desired, however, reagents may be combined to perform either 64 or 96 assays.

**Reporter 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 inventory the individual kit components be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquots of Reporter Cells are provided as single-use reagents. Once thawed, reporter cells can NOT be refrozen or 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 TPOR Reporter Cells | 3 x 0.6 mL | **-80°C** |
| ▪ Cell Recovery Medium (CRM) | 2 x 10.5 mL | -20°C |
| ▪ Compound Screening Medium (CSM) | 1 x 45 mL | -20°C |
| ▪ TPO, 3.0 µg/ml (in PBS/0.1%BSA) (physiological activator of TPOR) | 1 x 50 µL | -20°C |
| ▪ Detection Substrate | 3 x 2.0 mL | **-80°C** |
| ▪ Detection Buffer | 3 x 2.0 mL | -20°C |
| ▪ Plate Frame | 1 | ambient |
| ▪ Snap-in 8-well strips (white, sterile, collagen-coated wells) | 12 | **-20°C** |

*NOTE:* This Assay kit contains 8-well strips that have been collagen-coated and dried; these strip wells 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 activator AND varying concentrations of the test compound(s) to be evaluated for inhibitory activity. This TPOR Assay kit includes a 3.0 µg/mL stock solution of human Thrombopoietin (TPO), the physiological agonist of TPOR, that may be used to set up inhibition-mode assays. 2 ng/mL TPO approximates $EC_{70\text{-}80}$ in this assay. Hence, it presents a suitable concentration of agonist to use when screening test materials for inhibitory activities.

Add TPO 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 TPOR 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 **Reporter Cells** from -80°C storage and place them directly into dry ice to transport them to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. When ready to begin, transfer the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring **6.4 ml** of pre-warmed CRM into each tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **7.0 ml** per tube.

*Third,* during the 5 - 10 minutes incubation period, work in the cell culture hood to *carefully* mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

**3.)** Retrieve the tube(s) 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 strip-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 into the strip-wells of 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 TPOR Assay kit includes a concentrated stock of TPO, 3.0 µg/ml prepared in PBS/0.1%BSA. The following 7-point treatment series, with concentrations generated using serial 3-fold dilutions, provides a complete dose-response: 30.0, 10.0, 3.33, 1.11, 0.370, 0.123, and 0.0412 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 TPO to achieve an $EC_{50}$ – $EC_{80}$ concentration (refer to "*A word about inhibition-mode assay setup*", pg. 7). The TPO-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 cell pre-culture period discard the culture media. Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (*e.g.,* Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus. Do *not* touch the well bottom or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the cells and greatly increased well-to-well variability.

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

**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 the appropriate number of vials of **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*: To read 32 assay wells, transfer the entire volume of 1 vial of Detection Buffer into 1 vial of Detection Substrate, thereby generating a 4 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

**15.)** Following 22 - 24 hours incubation in treatment media, remove media contents from each well of the assay plate (as before in *Step 8*).

**16.)** Add 100 µl of **LDR** to each well 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 Thrombopoietin Receptor Assay Products** | |
| IB20001-32 | TPOR Reporter Assay System<br>3x 32 assays in 8-well strips (96-well plate format) |
| IB20001 | TPOR Reporter Assay System<br>1x 96-well format assay |
| IB20002 | TPOR Reporter Assay System<br>1x 384-well format assays |
| Bulk volumes of TPOR 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 TPO and the setup of a TPOR dose-response assay.