Human Thyroid Hormone Receptor Beta  
(NR1A2, THR, TRβ)  
Reporter Assay System

384-well Format Assays  
Product # IB01102

Technical Manual  
(version 7.1c)

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Human TRβ Reporter Assay System
384-well Format Assays

I. Description
▪ The Assay System ................................................................. 3
▪ The Assay Chemistry ............................................................ 3
▪ Preparation of Test Compounds .............................................. 4
▪ Considerations for Automated Dispensing ............................ 4
▪ Assay Scheme .................. .................................................... 4
▪ Assay Performance .............................................................. 5

II. Product Components & Storage Conditions ....................... 6

III. Materials to be Supplied by the User ................................. 6

IV. Assay Protocol
▪ A word about Antagonist-mode assay setup ........................... 7
  ▪ DAY 1 Assay Protocol ...................................................... 7
  ▪ DAY 2 Assay Protocol ...................................................... 8

V. Related Products ............................................................. 9

VI. Limited Use Disclosures .................................................. 9

APPENDIX 1: Example Scheme for Serial Dilutions .................. 10
I. Description

• The Assay System •

This nuclear receptor assay system utilizes proprietary human cells engineered to provide constitutive, high-level expression of the Human Thyroid Hormone Receptor Beta (NR1A2), a ligand-dependent transcription factor commonly referred to as THRB or TRβ.

INDIGO’s Reporter Cells include the luciferase reporter gene functionally linked to a responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in TRβ activity. The principal application of this assay is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against human TRβ.

TRβ Reporter Cells are prepared using INDIGO’s proprietary CryoMite™ process. This cryo-preservation method yields exceptional cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for cumbersome intermediate treatment steps such as spin-and-rinse of cells, viability determinations, cell titer adjustments, or the pre-incubation of reporter cells prior to assay setup.

INDIGO’s Nuclear Receptor Assays are all-inclusive cell-based assay systems. In addition to TRβ Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user’s test samples, a reference agonist, Luciferase Detection Reagent, and a cell culture-ready assay plate.

• The Assay Chemistry •

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

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (Photinus pyralis). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg²⁺-dependent reaction that consumes O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PPi, CO₂, 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 Assay kits 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.
• Preparation of Test Compounds •

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using Compound Screening Medium (CSM), as described in Step 2 of the Assay Protocol. This method avoids the adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

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

• Considerations for Automated Dispensing •

When processing a small number of assay plates, first carefully consider the dead volume requirement of your 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.

<table>
<thead>
<tr>
<th>Stock Reagent &amp; Volume provided</th>
<th>Volume to be Dispensed (384-well plate)</th>
<th>Excess rgt. volume available for instrument dead volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reporter Cell Suspension ~ 8 ml (prepared from kit components)</td>
<td>15 µl / well 5.8 ml / plate</td>
<td>~ 2.2 ml</td>
</tr>
<tr>
<td>Detection Substrate 7.8 ml</td>
<td>15 µl / well 5.8 ml / plate</td>
<td>~ 2 ml</td>
</tr>
</tbody>
</table>

• Assay Scheme •

**Figure 1.** Assay workflow. *In brief*, the prepared suspension of thawed Reporter Cells is dispensed into wells of the assay plate and then immediately dosed with the user’s test compounds. Following 22 -24 hr incubation Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.
Figure 2. Dose-response of TRβ using the reference agonist L-triiodothyronine (T3).

TRβ Reporter Cells were treated with L-triiodothyronine using an 8-point assay concentration range generated in 3-fold decrements: 150, 50.0, 16.7, 5.56, 1.85, 0.617, 0.206 and 0.0686 nM, and including ‘untreated’ control wells (as described in Appendix 1). Luminescence/well was quantified and the average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 4). Fold-Activation (i.e., S/B) and Z’ values were calculated as described by Zhang, et al. (1999)\(^1\). Non-linear regression analyses and EC\(_{50}\) calculations were performed using GraphPad Prism software. These data confirm the robust performance of this TRβ Assay and demonstrate its suitability for use in HTS applications.


\[Z' = 1 - [3*(SD_{Control} + SD_{Bkg}) / (RLU_{Control} – RLU_{Bkg})]\]
II. Product Components & Storage Conditions

This Human TRβ Assay kit contains materials to perform assays in a single 384-well assay plate.

The aliquot of TRβ Reporter Cells is provided as a single-use reagent. 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.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. Alternatively, the entire kit may be further stored at -80°C.

To ensure maximal viability, “Reporter Cells” must be maintained at -80°C until immediately prior to use.

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

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Human TRβ Reporter Cells</td>
<td>1 x 1.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Cell Recovery Medium (CRM)</td>
<td>1 x 7 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• L-Triiodothyronine, 150 µM (in DMSO) (reference agonist for human TR’s)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Detection Substrate</td>
<td>1 x 7.8 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• 384-well assay plate, white, cell-culture ready</td>
<td>1</td>
<td>ambient</td>
</tr>
</tbody>
</table>

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
• cell culture-rated laminar flow hood.
• 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
• 37°C water bath.
• 70% alcohol wipes
• electronic, repeat-dispensing pipettes & sterile tips
• disposable media basins, sterile.
• sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), or deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
• Optional: antagonist reference compound.
• Optional: clear 384-well assay plate, cell culture treated, for plating extra cells to be viewed microscopically 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 Antagonist-mode assay setup**

Receptor inhibition assays expose the Reporter Cells to a fixed, sub-maximal concentration (typically between EC50 – EC85) of a known agonist AND varying concentrations of the test compound(s) to be evaluated for antagonist activity. This TRβ Assay kit includes a 150 μM stock solution of L-Triiodothyronine, an agonist of THR’s that may be used to setup antagonist-mode assays. 20 nM L-Triiodothyronine typically approximates EC80 in this assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

For 384-well format assays, adding the reference agonist 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 following protocol. Note that, in Step 6, 15 µl of treatment media is combined with 15 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.

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**DAY 1 Assay Protocol:** All steps must be performed using 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. The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

   Note that, in Step 6, 15 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 15 µl 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. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

3.) **Preparing the positive control:** This assay kit includes a 150 μM stock solution of Triiodothyronine, the most commonly used physiological reference agonist of the thyroid hormone receptors. The following 8-point treatment series, with concentrations prepared in 3-fold decrements, provides a suitable dose-response: 150, 50, 16.7, 5.56, 1.85, 0.617, 0.206 and 0.0686 nM (final assay concentrations), and including ‘untreated’ control wells. **APPENDIX 1** provides guidance for generating such a dilution series.

4.) **Rapid Thaw of the Reporter Cells:** First, retrieve **Reporter Cells** from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. Second, when ready to proceed, retrieve the tube of CRM from the 37°C water bath and sanitize the outside surface with a 70% ethanol swab. Place the tube of cells in a rack and, without delay, perform a rapid thaw of the frozen cells by transferring the entire 7 ml volume of the pre-warmed CRM into the 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 ~ 8 ml.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface of the tube with a 70% alcohol swab.
5.) **a. Agonist-mode assays.** *Gently* invert the tube of Reporter Cells several times to disperse cell aggregates and gain a homogenous cell suspension. While taking precautions to avoid cell settling, dispense 15 µl of cell suspension into each well of the assay plate.

~ or ~

**b. Antagonist-mode assays.** *Gently* invert the tube of Reporter Cells several times to disperse cell aggregates and to gain a homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "A word about antagonist-mode assay setup", pg. 7). Dispense 15 µl of cell suspension into each well of the Assay Plate.

*NOTE 5.1:* Take special care to prevent cells from settling during the dispensing period. Cell settling will produce low precision in dispensing uniform cell numbers across the assay plate, resulting in well-to-well variation (= increased Standard Deviation) in the assay.

*NOTE 5.2:* Users sometimes prefer to examine the cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear 384-well assay plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those cells contained in the white assay plate.

6.) Dispense 15 µl of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a 37°C, humidified 5% CO₂ 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 them 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 these solutions were 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 23 - 24 hours of incubation add 15 µl of **Detection Substrate** to each well of the assay plate.

*NOTE:* Perform manual reagent transfers 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 may significantly degrade the accuracy and precision of the assay data. In the event of excessive bubble formation during manual processing, spin the assay plate (with lid) at low speed 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 post-addition of Detection Substrate, however, after this period luminescence emission achieves a stable output.

13.) Quantify luminescence.
V. Related Products

### Human TRβ Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB01101-32</td>
<td>Human TRβ Reporter Assay System 3x 32 assays in 96-well format</td>
</tr>
<tr>
<td>IB01101</td>
<td>Human TRβ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB01102</td>
<td>Human TRβ Reporter Assay System 1x 384-well format assays</td>
</tr>
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</table>

Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

### Panel of Human TR Assays

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB01201-48P</td>
<td>Human TRα and TRβ Reporter Assay PANEL 48 assays each, 1x 96-well assay plate</td>
</tr>
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</table>

### LIVE Cell Multiplex (LCM) Assay

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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<tbody>
<tr>
<td>LCM-01</td>
<td>Reagent volumes sufficient to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-05</td>
<td>Reagent in 5x-bulk volume to perform 480 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagent in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
</tbody>
</table>

Please refer to INDIGO Biosciences website for updated product offerings.

[www.indigobiosciences.com](http://www.indigobiosciences.com)

VI. Limited Use Disclosures

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

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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.

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APPENDIX 1
Example scheme for the serial dilution of Triiodothyronine reference agonist, and the setup of a TRβ dose-response assay.

[Diagram showing the serial dilution process with concentration levels and volumes indicated]