Human Thyroid Hormone Receptor PANEL

TRα (NR1A1)
TRβ (NR1A2)

48 Assays each in 96-well Format
Product #IB01201-48P

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Technical Manual
(version 7.2b)

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INDIGO BIOSCIENCES
Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human Thyroid Hormone Receptors
TRα and TRβ Assay PANEL
48 Assays each in 96-well Format

I. Description

▪ The Assay System ▪

INDIGO's **PANEL** of **TR Reporter Assays** utilizes non-human mammalian cells engineered to express Human Thyroid Hormone Receptors Alpha (NR1A1) and Beta (NR1A2), ligand-dependent transcription factors commonly referred to as **TRα** and **TRβ**.

INDIGO's TR 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 TRα or TRβ activity. The principal application of this assay panel is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against the human TR's.

TR 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, or cell titer adjustments prior to assay setup.

INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to TR 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 reporter assay systems 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 Reporter Assay Systems 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.

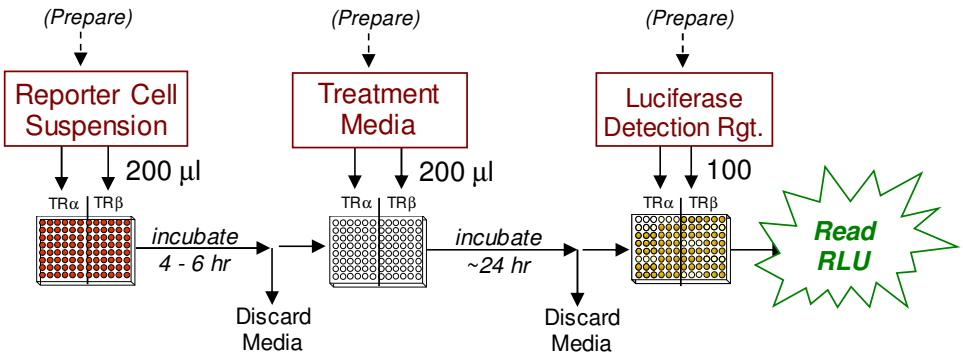
▪ Preparation of Test Compounds ▪

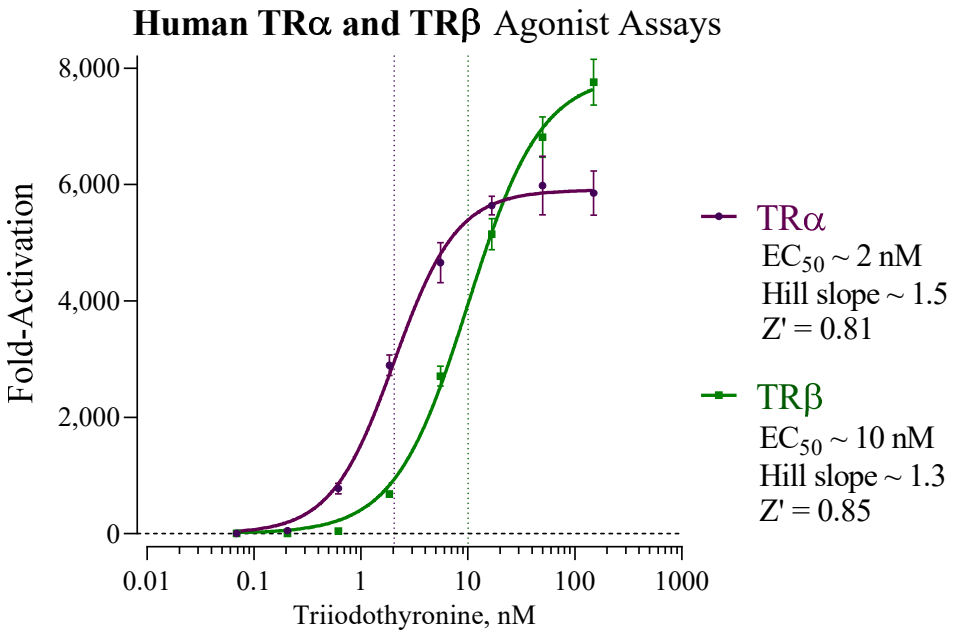
Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using **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 specially formulated to 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 regarded as 'single-use' reagents.

▪ Assay Scheme ▪

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





Figures 2. Dose-response of the TRα and TRβ Assays using the reference agonist L-Triiodothyronine.

TRα and TRβ dose-response assays were performed according to the protocol provided in this Technical Manual. **TRα** and **TRβ** Reporter Cells were both treated with 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 ($n \geq 4$). Signal-to-background (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 both the TRα and TRβ Assays and demonstrate their suitability for use in HTS applications.

[1] Zhang JH, Chung TD, Oldenburg KR. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen.:**4**(2), 67-73.

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

II. Product Components & Storage Conditions

This Human TR Reporter Assays PANEL contains materials to perform 48 TRα assays and 48 TRβ assays, all in a single 96-well plate format. All reagents are supplied with sufficient extra volume to accommodate the needs of dispensing 2 individual groups of assay reagents.

Each aliquot of TR Reporter Cells, Detection Substrate and Detection Buffer are provided as single-use reagents. Once thawed, reporter cells can NOT be refrozen 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.

Kit Components	***Amount***	***Storage Temp.***
▪ TRα Reporter Cells	1 x 1.0 mL	**-80°C**
▪ TRβ Reporter Cells	1 x 1.0 mL	**-80°C**
▪ Cell Recovery Media (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Media (CSM)	1 x 45 mL	-20°C
▪ L-Triiodothyronine, 150 µM (in DMSO) (reference agonist for TRα and TRβ)	1 x 30 µL	-20°C
▪ Detection Substrate	2 x 3.0 mL	**-80°C**
▪ Detection Buffer	2 x 3.0 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

- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO_2 incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- container of dry ice
- single channel electronic pipette suitable for the repeat-dispensing of 200 µl volumes
- 8-channel electronic pipettes suitable for the repeat-dispensing of 200 µl volumes
- 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:* clear 96-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 1-11* are performed on **Day 1**, requiring less than 2 hours of actual bench work plus a 4 hr pre-incubation step. *Steps 12-17* 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 constant, sub-maximal concentration (typically EC_{50} – EC_{85}) of a known *agonist* AND the test compound(s) to be evaluated for antagonist activity. We find that 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 7b* of the following protocol.

This TR Assay Panel kit includes a 150 µM stock solution of **L-Triiodothyronine**, the most common reference agonist for the TR's (**Figure 2**), that may be used effectively to setup receptor inhibition studies.

▪ **TRα**: 10 nM L-triiodothyronine typically approximates EC_{70} in this reporter assay.

▪ **TRβ**: 30 nM L-Triiodothyronine typically approximates EC_{80} in this assay

DAY 1 Assay Protocol: All steps must 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 tubes of TRα and TRβ **Reporter Cells** from -80°C storage, place them directly into dry ice and transport them to the laminar flow hood. When ready, transfer the two tubes 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 CRM vials (37°C) into each tube of frozen reporter cells. Place the tubes of Reporter Cells in a 37°C water bath for 5 - 10 minutes. The resulting volume of each individual cell suspension will be **10.5 ml**

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

4.) Gently invert the tubes of Reporter Cells several times to disperse cell aggregates and gain homogenous cell suspensions. Use a repeat-dispensing electronic pipette to dispense **200 µl / well** of each cell suspension into respective 48-well blocks of the assay plate.

NOTE 4.1: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling during the dispensing period. Likewise, take care to dispense uniform volumes across the assay plate.

NOTE 4.2: 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 96-well cell culture treated assay plate. Continue to process the assay plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells: Place the assay plate into a 37°C, ≥ 85% humidity, 5% CO_2 incubator for 4 - 6 hours.

6.) ***Near the end of the 4-6 hour pre-incubation period:*** Remove **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

7.) Prepare the Test Compound and Reference Compound treatment media at the desired final assay concentrations: 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 never exceed 0.4%.

***a. Agonist*-mode assays**. This assay kit includes a 150 µM stock solution of **L-Triiodothyronine**, a potent agonist of both TRα and TRβ. 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, and including 'untreated' control wells. **APPENDIX 1** provides guidance for generating such a dilution series.

~ or ~

***b. Antagonist*-mode assays**. When setting up antagonist assays, first supplement a bulk volume of CSM with the challenge agonist **L-Triiodothyronine** to achieve the desired final assay-concentration (refer to "*A word about antagonist-mode assay setup*", pg. 7). The agonist-supplemented CSM is then used to generate dilutions of test compound stocks to achieve their final assay concentrations.

8.) At the end of the cell pre-incubation period, discard the culture media by 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.

9.) Dispense **200 µl** of each treatment media into appropriate wells of the assay plate.

10.) 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.

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.) 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. Once at room temperature, gently invert each tube several times to ensure homogenous solutions.

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 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 **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

TRα Assay Products	
Product No.	***Product Descriptions***
IB01001-32	Human TRα Reporter Assay System 3x 32 assays in 96-well format
IB01001	Human TRα Reporter Assay System 1x 96-well format assay
IB01002	Human TRα Reporter Assay System 1x 384-well format assays
TRβ Assay Products	
IB01101-32	Human TRβ Reporter Assay System 3x 32 assays in 96-well format
IB01101	Human TRβ Reporter Assay System 1x 96-well format assay
IB01102	Human TRβ Reporter Assay System 1x 384-well format assays
Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay	
Product No.	***Product Descriptions***
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 in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats
LCM-10	Reagent in 10x-bulk volume to perform **960** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats

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 or diagnostic use in humans.

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

Product prices, availability, specifications and claims are subject to change without prior notice.

APPENDIX 1

Example scheme for the serial dilution of L-Triiodothyronine reference agonist, for use in both the TRα and TRβ dose-response assays.