Zebrafish Thyroid Hormone Receptor Beta
(nr1a2, TRβ)
Reporter Assay System

3x 32 Assays in 96-well Format
Product # Z01101-32

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Technical Manual
(version 7.2c)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Zebrafish TRβ Reporter Assay System

3x 32 Assays in 96-well Format

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I. Description

▪ The Assay System ▪

This nuclear receptor assay system utilizes proprietary human cells engineered to provide constitutive, high-level expression of the **Zebrafish (*Danio rerio*) Thyroid Hormone Receptor beta** (nr1a2), a ligand-dependent transcription factor referred to as **zfTRβ.**

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to a TRβ-responsive promoter. Thus, quantifying changes in luciferase expression in the test sample treated reporter cells provides a sensitive surrogate measure of changes in zfTRβ activity.

The principal application of this assay is in the screening of test samples to quantify any functional bioactivity that they may exert against zebrafish TRβ. In particular, zebrafish reporter assays are frequently used in the monitoring of environmental samples for the presence of biohazardous chemical pollutants, such as endocrine disruptors.

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's Nuclear Receptor Assays are all-inclusive cell-based assay systems. In addition to zfTRβ 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 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).

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 ▪

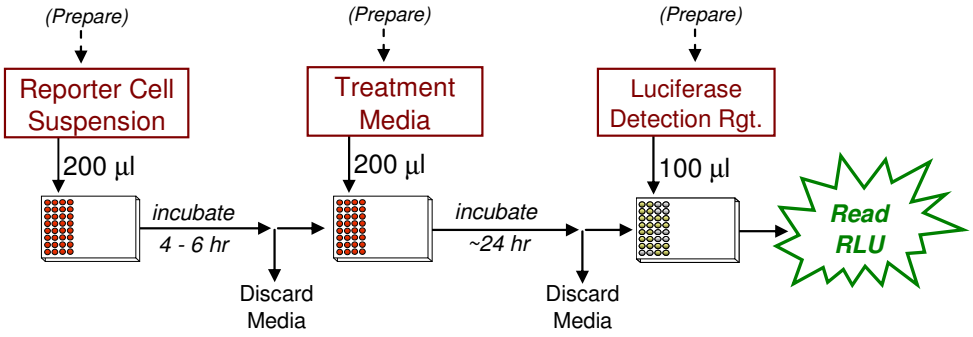
Small molecule compounds are typically solvated at high concentration (ideally 1,000x-concentrated) in DMSO and stored frozen as master stocks. Immediately prior to setting up an assay, the master stocks are serially diluted using **Compound Screening Medium (CSM**; as described in *Step 7*) to achieve the desired assay concentrations. 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. Nonetheless, high concentrations of 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 then considered to be '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.



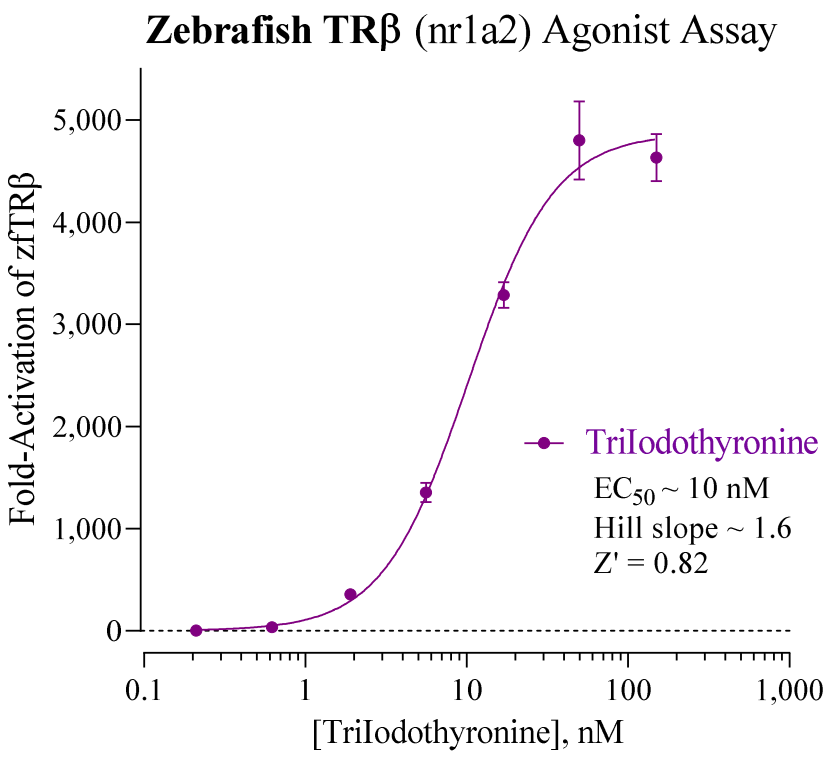


Figure 2. Agonist dose-response of Zebrafish TRβ using the reference agonist L-Triiodothyronine.
zfTRβ Reporter Cells were treated with L-triiodothyronine using a 7-point concentration range generated in 3-fold decrements: 150, 50.0, 16.7, 5.56, 1.85, 0.617, and 0.206 nM, and including 'untreated' control wells (as described in Appendix 1). Luminescence/well was quantified and the average relative light units (RLU). corresponding standard deviation (SD), percent coefficient of variation (%CV) and fold-activation values were determined for each treatment concentration (n = 4). Average fold-activation +/- %CV were plotted against their respective agonist concentrations, Log10 (nM), using GraphPad Prism software. Z' was calculated as described by Zhang, *et al*. (1999)[1].

[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.

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

II. Product Components & Storage Conditions

This Zebrafish TRβ 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*	*minimum Storage Temp.*
▪ zfTRβ 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
▪ L-Triiodothyronine, 150 µM (in DMSO) (reference agonist for TR's)	1 x 30 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 (used in *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* 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 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 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 agonist AND varying concentrations of the test compound(s) to be evaluated for antagonist activity. This assay kit includes a 150 µM stock solution of **L-Triiodothyronine**, a potent physiological agonist of TRβ that may be used to setup antagonist-mode assays. 20 nM L-Triiodothyronine typically approximates $EC_{70\text{-}80}$ in this assay. Hence, it presents a suitable concentration of agonist to be used when screening test compounds for inhibitory activity.

Add the challenge agonist to a bulk volume of **CSM** at an EC_{50} – EC_{85} concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective final assay concentrations. This is an efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 7b* of this protocol.

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 **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.

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(s) of Reporter Cells several times to gain a homogenous cell suspension. Transfer the cell suspension(s) into a reservoir. Using an electronic, repeat-dispensing 8-chanel pipette, dispense **200 µl / well** of cell suspension into the mounted strip-wells.

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'blank' wells (meaning cell-free, but containing 'CSM') must be included in the assay plate to allow quantification of 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, collagen-coated 96-well assay plate. Continue to process the assay plate in identical manner to the white assay plate.

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

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

6.) ***Near the end of the 4-6 hour pre-culture 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 strip wells. 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 zfTRβ Assay kit includes a 150 µM stock solution of **L-Triiodothyronine**, a potent reference agonist of TRβ. The following 7-point treatment series, with concentrations prepared in 3-fold decrements, provides a complete dose-response: 150, 50, 16.7, 5.56, 1.85, 0.617, and 0.206 nM. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' (or 'vehicle') control wells.

~ 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 an EC_{50} – EC_{80} 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 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** 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 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.) *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*: 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

Human TRβ Assay Products	
Product No.	***Product Descriptions***
IB01101-32	Human TRβ Reporter Assay 3x 32 assays in 8-well strips (96-well plate format)
IB01101	Human TRβ Reporter Assay 1x 96-well format assay
IB01102	Human TRβ Reporter Assay 1x 384-well format assays
Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	
Zebrafish TRβ Assay Products	
Z01101-32	Zebrafish TRβ Reporter Assay 3x 32 assays in 8-well strips (96-well plate format)
Z01101	Zebrafish TRβ Reporter Assay 1x 96-well format assay
Panel of Human TR Assays	
IB01201-48P	Human TRα and TRβ Reporter Assay PANEL 48 assays each, 8 well strips (96-well plate format)

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

"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 Technical Manual accompanying assay kit box will always be the most currently updated version.

APPENDIX 1

Example scheme for the serial dilution of L-Triiodothyronine reference agonist, and the setup of a zfTRβ dose-response assay.