Human Hepatocyte Nuclear Factor 4 alpha
(NR2A1, HNF4α)
Reporter Assay System

96-well Format Assays
Product # IB36001

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Technical Manual
(version 7.2)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human HNF4α Reporter Assay
96-well Format Assays

I. Description

▪ The Assay System ▪

This nuclear receptor assay utilizes proprietary human cells engineered to provide constitutive, high-level expression of the **Human Hepatocyte Nuclear Factor 4 alpha** (NR2A1), a ligand-dependent transcription factor known as **HNF4α**.

In INDIGO's Reporter Cells the HNF4α receptor is expressed as a hybrid protein in which its native DNA binding domain (DBD) sequence has been exchanged with that of the DBD sequence from the yeast Gal4 gene. Additionally, the cells include the luciferase reporter gene functionally linked to the Gal4 upstream activation sequence (UAS) and a minimal promoter. HNF4α undergoes nuclear translocation, DNA binding, recruitment and assembly of the co-activators and accessory factors required to form a functional transcription complex, culminating in expression of the reporter gene. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a specific and sensitive surrogate measure of the changes in HNF4α activity. Unlike some other assay formats, the readout from INDIGO's reporter cells demands the same orchestration of all intracellular molecular interactions and events that can be expected to occur *in vivo.*

As is true *in vivo*, the expressed hybrid HNF4α receptor displays a moderate level of constitutively activity. A true ligand interaction may provoke either *i.)* an *agonist* response that delivers an additional increase in receptor activity above its already high constitutive level of activity, or *ii.)* an *inverse-agonist* response in which a loss of receptor activity is observed. **Figures 2** and **3** demonstrates these responses. The principal application of this assay is in the screening of test compounds to quantify *inverse-agonist* or *agonist* activities that they may exert against the Human HNF4α receptor.

These HNF4α Reporter Cells are prepared using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method yields high 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.

This kit is an all-inclusive assay system. As detailed on page 6, it includes cryopreserved HNF4α Reporter Cells, two optimized media for use during initial cell culture and for preparing dilutions of test samples, a control agonist, luciferase detection reagents, a cell culture-ready assay plate, and a detailed assay protocol.

▪ The Assay Chemistry ▪

INDIGO's receptor reporter assay 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 plate-reading 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 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 test 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. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

1.) As described in *Step 7* and depicted in Appendix 1 for the reference agonist, **Compound Screening Medium (CSM)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired final assay concentration series.

Alternatively, if test compound solubility is expected to be problematic,
2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the final concentration of total DMSO carried over into assay wells should not exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 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.

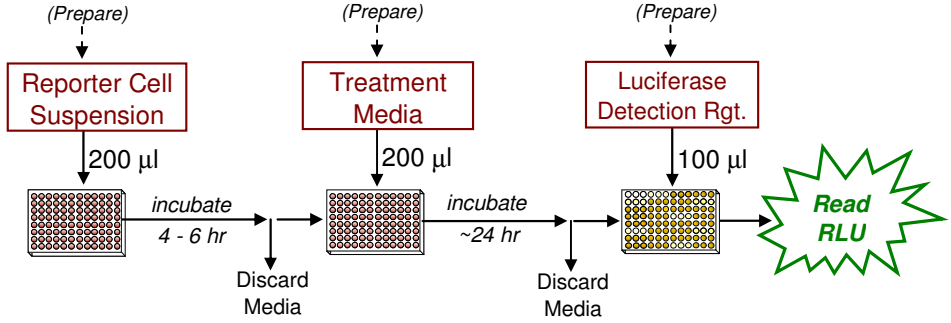
▪ Considerations for Automated Dispensing ▪

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.

Stock Reagent & Volume provided	**Volume to be Dispensed** *(96-well plate)*	**Excess rgt. 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*, the Reporter Cell suspension is dispensed into assay wells and pre-incubated for 4 - 6 hours. Following the pre-incubation period, culture media are discarded, and the prepared treatment media are added. Following 22 - 24 hours 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. *Note:* If INDIGO's Live Cell Multiplex (LCM) Assay is to be incorporated, refer to the assay workflow schematic provided in the LCM Assay Technical manual.



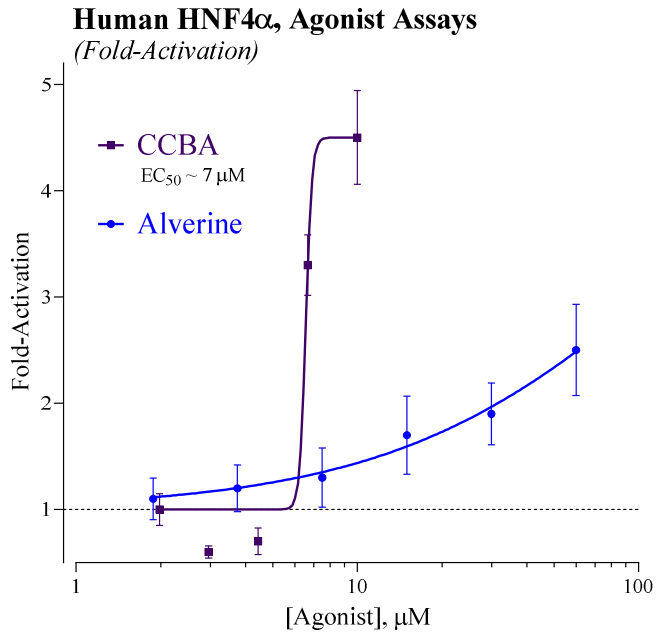


Figure 2. Agonist dose-response of Human HNF4α.

Agonist dose-response of Human HNF4α reporter cells using CCBA[1] (provided) and Alverine Citrate[2] (Millipore Sigma). Luminescence was quantified and average relative light units (RLU) and their corresponding values of standard deviation (SD) and Fold-Activation were determined for each treatment group (n = 3). GraphPad Prism software was used to curve-fit data using the least-squares method of non-linear regression, and EC_{50} values were determined.

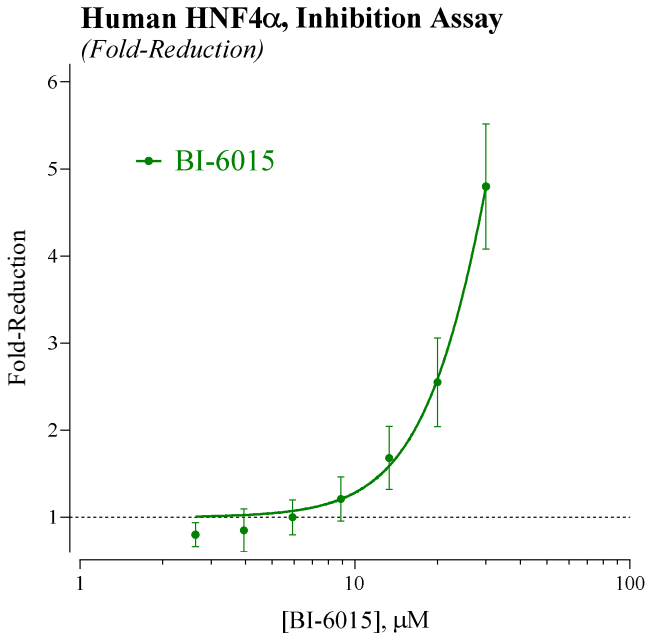


Figure 3. Inverse-Agonist dose-response of Human HNF4α.

Inverse-agonist response of HNF4α reporter cells using BI6015[3] (Cayman Chem.). Luminescence was quantified and average relative light units (RLU) and their corresponding values of standard deviation (SD) and Fold-Reduction were determined for each treatment group (n = 3). INDIGO's Live Cell Multiplex assay was performed and confirmed the absence of compound-induced cytotoxicity (data not shown).

II. Product Components & Storage Conditions

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

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 inspect and inventory the individual kit components 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***	***minimum Storage Temp.***
▪ HNF4α 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
▪ CCBA[1], 10 mM (in DMSO) (control agonist for HNF4α)	1 x 30 µL	-20°C
▪ Detection Substrate (Note: contains DTT)	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:* inverse-agonist/antagonist reference compound (*e.g.,* Figure 3)

▪ *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 to complete, but including a 4-hour incubation step. *Steps 12-17* are performed on ***Day 2*** and require less than 1 hour to complete.

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 **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 8-chanel pipette, dispense **200 µl / well** of cell suspension into 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-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 not exceed 0.4%.

Prepare the Reference Control and Test Compound treatment media at the desired final assay concentrations. This assay kit includes a 10 mM stock solution of CCBA, an agonist of human HNF4α. The following 7-point treatment series, with concentrations presented in **1.5-fold** decrements, provides a complete dose-response: 10, 6.67, 4.44, 2.96, 1.98, 1.32 and 0.878 µM. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' (or 'vehicle') control wells.

8.) At the end of the 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 or in a **fume hood.**

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. Program the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time is set to 0.5 second (500 mSec) per well, *or less*.

14.) *Immediately before proceeding to Step 15,* prepare **Luciferase Detection Reagent** (**LDR**). Combine 'Detection Buffer' and 'Detection Substrate' by pouring-over their entire volumes into a media basin; rock the basin gently to mix the reagent. The resulting volume of LDR is 12 ml.

NOTE: 'Detection Substrate' contains a high concentration of DTT, which produces a strong odor that some users may find objectionable. It is advised to work in a **fume hood** when preparing LDR and dispensing it into the assay plate, as well as during the subsequent 'plate rest' period (*Step 16*).

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 **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.

18.) Data analyses.

V. Literature Citations

[1] 5-Chloro-n-(2-chlorophenyl)-2-hydroxybenzamide (CCBA; CAS #6626-92-2)

[2] Lee, S., *et. al*., (2013) Identification of Alverine and Benfluorex as HNF4α Activators. ACS Chem. Biol.: **8**, 1730-1736.

[3] Kiselyuk, A., *et. al.* (2012) HNF4α Antagonists Discovered by a High-Throughput Screen for Modulators of the Human Insulin Promoter. Chemistry & Biology:**19**, 806-818.

[4] 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.

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

VI. Related Products

Product No.	Product Descriptions
Human Hepatocyte Nuclear Factor 4 alpha	
IB36001	Human Hepatocyte Nuclear Factor 4 alpha (HNF4α) 1x 96-well format assay
IB36002	Human Hepatocyte Nuclear Factor 4 alpha (HNF4α) 1x 384-well format assays

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 performed in 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays performed in 10 x 96-well assay plates
INDIGlo Luciferase Detection Reagent (LDR)	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents in 10 mL, 25 mL, 50 mL, and 500 mL volumes

Please refer to INDIGO Biosciences website for updated product offerings.

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VII. Limited Use Disclosures

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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 recently updated version available.

APPENDIX 1

Example scheme for the serial dilution of the reference agonist CCBA, and the setup of a HNF4α dose-response assay.