Human Vitamin D Receptor
(NR1I1, VDR)
Reporter Assay System

3x 32 Assays in 96-well Format
Product # IB00701-32

Technical Manual
(version 7.2)

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

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I. Description

- The Assay System

This nuclear receptor utilizes proprietary human cells engineered to provide constitutive, high-level expression of the Human Vitamin D Receptor (NR1I1), a ligand-dependent transcription factor commonly referred to as VDR.

INDIGO's Reporter Cells express a hybrid form of human VDR. The N-terminal sequence encoding the VDR DNA binding domain (DBD) has been substituted with that of the yeast GAL4-DBD. The native VDR ligand binding domain (LBD) and other C-terminal domains remain intact and functional. Ligand interaction activates the receptor, causing it to bind to the GAL4 DNA binding sequence, which is functionally linked to a resident luciferase reporter gene. Thus, quantifying changes in luciferase activity in the treated reporter cells provides a sensitive surrogate measure of the changes in VDR activity. The principal application of this assay kit is in the screening of test samples to quantify functional activities, either agonist or antagonist, that they may exert against human VDR.

Reporter Cells are prepared using INDIGO’s proprietary CryoMite™ process. This cryopreservation 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 or viability determinations prior to assay setup.

INDIGO’s Nuclear Receptor assays are all-inclusive cell-based assay systems. In addition to VDR 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).

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 •

Test compounds are typically solvated at high-concentration 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. Do **not** use DMSO to further dilute test compound solutions. This method of dilution avoids the significant 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.

• Assay Scheme •

**Figure 1.** Assay workflow.

**NOTE:** The PXR family of assay protocols includes Day 1 steps and dispensed volumes that are different from the conventional INDIGO assay protocol that some users may be accustomed to when setting up INDIGO’s other Nuclear Receptor Assays.

*In brief,* 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.
Assay Performance

Figure 2. Agonist dose-response analyses of Human VDR.

Agonist analyses of VDR Reporter Cells using Calcitriol (provided), Calcipotriol (Cayman Chemical), Ercalcitriol, EB1089, and Doxercalciferol (each from Tocris). In addition, to assess the level of background signal contributed by non-specific factors that may cause activation of the luciferase reporter gene, “mock” reporter cells were treated with calcitriol (mock reporter cells, which contain only the luciferase vector, are not provided with assay kits). Final assay concentrations for ligand-treated cells ranged from 10,000 nM to 38 pM for Doxercalciferol, and 800 nM to 50 pM for all others. Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 6). Fold-activation and Z’ values were calculated as described by Zhang, et al. (1999). Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software. Mock reporter cells demonstrate no significant background luminescence (< 0.02% that of the reporter cells at EC_{Max} of calcitriol). Thus, luminescence results strictly through ligand-activation of VDR expressed in these reporter cells. High Z’ scores confirm the robust performance of this assay, and it's suitability for HTS.


$Z' = 1 - \frac{3 \times (SD_{\text{Control}} + SD_{\text{Background}})}{(RLU_{\text{Control}} - RLU_{\text{Background}})}$
II. Product Components & Storage Conditions

This Human VDR Assay kit contains materials to perform three distinct groups of assays in a 96-well plate format. 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.

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.

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>• VDR Reporter Cells</td>
<td>3 x 0.6 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Cell Recovery Medium (CRM)</td>
<td>2 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Compound Screening Medium (CSM)</td>
<td>1 x 45 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Calcitriol, 1.0 mM (in DMSO)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Detection Substrate</td>
<td>3 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Detection Buffer</td>
<td>3 x 2.0 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Plate frame</td>
<td>1</td>
<td>ambient</td>
</tr>
<tr>
<td>• Snap-in, 8-well strips (white, sterile, collagen-coated wells)</td>
<td>12</td>
<td>-80°C</td>
</tr>
</tbody>
</table>

 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
• cell culture-rated laminar flow hood.
• 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
• 37°C water bath.
• 70% alcohol wipes
• 8- or 12-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: antagonist reference compound.
• 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 constant, 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 VDR Assay kit includes a 1.0 mM stock solution of **Calcitriol**, a potent agonist of VDR that may be used to setup antagonist-mode assays. 10 nM calcitriol typically approximates EC$_{80}$ in this cell-based assay. Hence, it presents a reasonable assay 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. We find that 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: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. *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 of **Reporter Cell Suspension** from the water bath and sanitize the outside surface with a 70% alcohol swab.

4.) If more than one tube of Reporter cells was thawed, combine them and gently invert several times to disperse cell aggregates and gain a homogenous cell suspension. **Dispense 200 µl / well** of cell suspension into 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 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.
Near the end of the 4-6 hour pre-incubation period:

6.) 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 VDR Assay kit includes a 1.0 mM stock solution of calcitriol, a potent agonist of VDR. The following 7-point treatment series, prepared in serial 5-fold decrements, provides a suitable dose-response: 500, 100, 20.0, 4.00, 0.80, 0.16, and 0.032 nM, and including a 'no treatment' control. APPENDIX 1 provides an example 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 calcitriol 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.

   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% 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.

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.
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 VDR Assay Kit Products**

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00701-32</td>
<td>3x 32 VDR assays; strip-wells in 96-well plate frame</td>
</tr>
<tr>
<td>IB00701</td>
<td>1x 96-well format VDR assays</td>
</tr>
<tr>
<td>IB00702</td>
<td>1x 384-well format VDR assays</td>
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Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

**LIVE Cell Multiplex (LCM) Assay Products**

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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<tbody>
<tr>
<td>LCM-01</td>
<td>Reagents 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>Reagents 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.

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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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APPENDIX 1