

**Human Progesterone Receptor
(NR3C3, PGR, PR)
Reporter Assay System**

3x 32 Assays in 96-well Format
Product # IB05001-32

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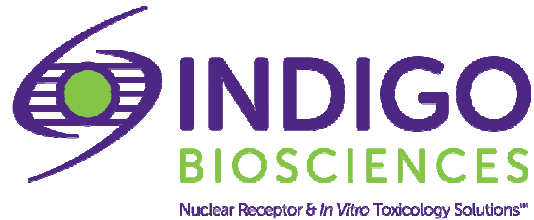
Technical Manual
(version 7.2b)

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

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I. Description

▪ The Assay System ▪

This nuclear receptor assay utilizes proprietary human cells engineered to provide constitutive, high-level expression of the full-length **Human Progesterone Receptor** (NR3C3), a ligand-dependent transcription factor commonly referred to as **PR** or **PGR**.

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to a PGR-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in PGR activity. Luciferase gene expression occurs after ligand-bound PGR 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 target gene. Unlike *in vitro* binding assays, and some other cell-based assay strategies, 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*.

PGR 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 Bioscience's Nuclear Receptor Assays are all-inclusive cell-based assay systems. In addition to PGR 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 ▪

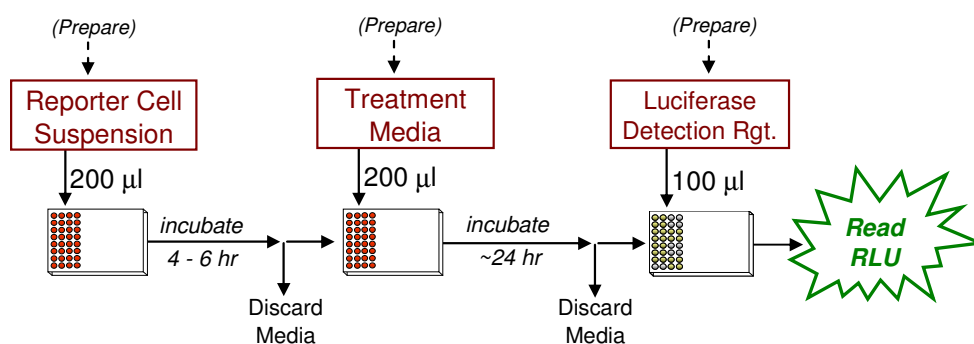
Test compounds are typically solvated at high concentration in DMSO and stored frozen as master stocks. Immediately prior to setting up an assay, the master stocks are serially diluted using **Compound Screening Medium (CSM)**; as described in *Step 7* to achieve the desired assay concentrations. Do 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.

In brief, 200 μ l 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 μ l/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 ▪

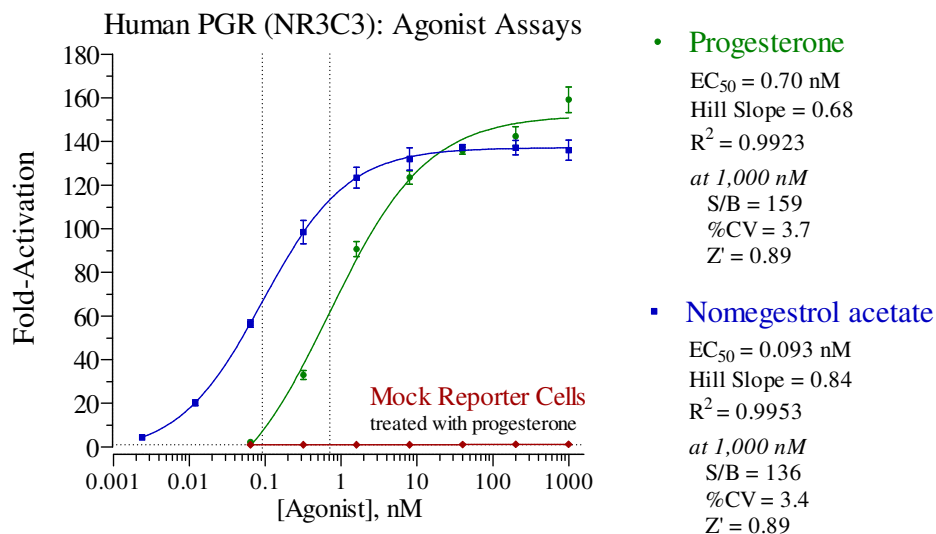


Figure 2. Agonist dose-response analyses of Human PGR.

Agonist analyses of PGR Reporter Cells using Progesterone (provided), and Nomegestrol acetate (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 Progesterone (mock reporter cells, which contain only the luciferase vector, are not provided with assay kits). Concentrated stocks prepared in DMSO were serially diluted in 5-fold decrements using CSM. Final assay concentrations for progesterone-treated cells ranged between 1,000 nM and 64 pM; assay concentrations of nomegestrol ranged between 1,000 nM and 2.4 pM. 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 ($n \geq 6$). Fold-activation (*i.e.*, signal-to-background) and Z' values were calculated as described by Zhang, *et al.* (1999)¹. Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software. Mock reporter cells demonstrate no significant background luminescence ($< 0.1\%$ that of the reporter cells at EC_{Max}). Thus, luminescence results strictly through ligand-activation of PGR expressed in these reporter cells. High Z' scores confirm the robust performance of this PGR Assay.

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.

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

Human PGR (NR3C3): Antagonist & LCM Assays

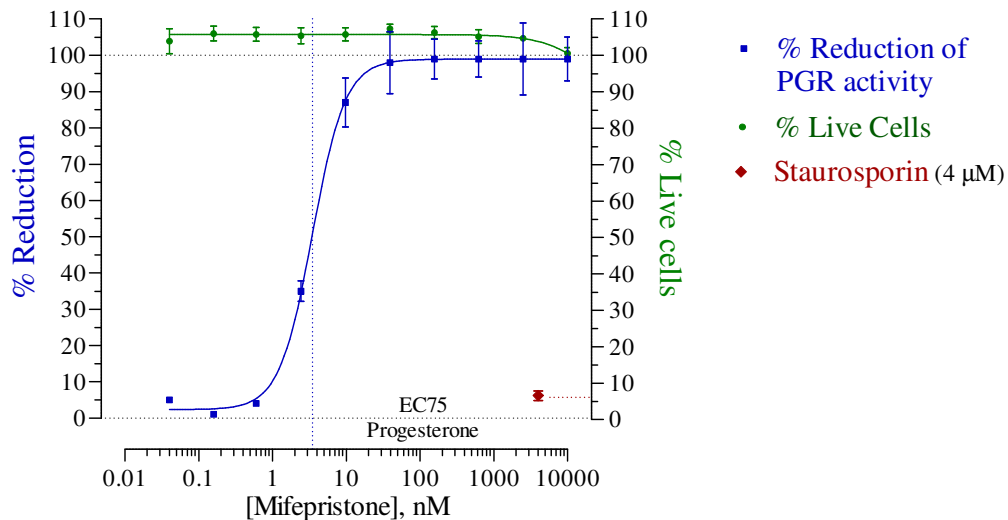


Figure 3. Validation of PGR Assay antagonist dose-response.

Antagonist analysis of PGR Reporter Cells using Mifepristone (Tocris). Assay setup and quantification of PGR activity were performed following *Protocol Variation 2* in this Technical Manual. To confirm that the observed dose-dependent increase in % inhibition resulted from PGR inhibition, not induced cell death, the relative numbers of live cells in each assay well were determined at the end of the treatment period using INDIGO's Live Cell Multiplex (LCM) Assay (#LCM-01).

In brief: CSM was first supplemented with a 2x-EC₇₅ concentration of progesterone. This medium was then used to prepare a 10-point, serial 4-fold dilution series of mifepristone to generate a range of 2x-concentration treatment media. Frozen PR Reporter Cells were then thawed in CRM, and 100 μ l of this cell suspension was dispensed into each well of the assay plate. Next, 100 μ l of the prepared series of 2x-concentration treatment media were dispensed per well, combining with the reporter cells. The final assay concentration of mifepristone ranged between 10 μ M and 40 pM, including a 'no antagonist' control ($n \geq 6$ per treatment; highest [DMSO] < 0.1% *f.c.*). Each treatment also contained an assay concentration of 8.7 nM (~ EC₇₅) progesterone as challenge agonist. Assay plates were incubated for ~23 hrs, then processed according to the LCM Assay protocol to quantify relative numbers of live cells per treatment condition. Plates were then further processed to quantify PGR activity for each treatment condition.

Results: Mifepristone produced a dose-dependent increase in % inhibition of progesterone. The LCM Assay reveals no significant variance in the numbers of live cells per assay well, even up to the maximum treatment concentration of 10 μ M. Hence, the measured increase in % inhibition of PGR activity can be attributed to dose-dependent inhibition of the progesterone receptor, and *not* to induced cell death.

II. Product Components & Storage Conditions

This Human PGR 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ PGR 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
▪ Progesterone, 1.0 mM (in DMSO) (reference agonist for PGR)	1 x 30 µL	-20°C
▪ Detection Substrate	3 x 6.0 mL	-80°C
▪ Detection Buffer	3 x 6.0 mL	-20°C
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile, collagen-coated wells)	12	-80°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

- 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, sterile, 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 hr 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 between EC₅₀ – EC₈₅) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This PGR assay kit includes a 1 mM stock solution of **Progesterone**, a potent physiological agonist of PGR that may be used to setup antagonist-mode assays. 8.0 nM progesterone typically approximates EC₇₅ 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 EC₅₀ – EC₈₅ 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 PGR 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% CO₂ 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 PGR Assay kit includes a 1.0 mM stock solution of Progesterone, a potent reference agonist of human PGR. The following 8-point treatment series, with concentrations presented in 5-fold decrements, provides a suitable dose-response: 1000, 200, 40.0, 8.00, 1.60, 0.320, 0.0640 and 0.0120 nM (final assay concentrations), and including a 'no treatment' control.

~ or ~

b. **Antagonist-mode assays.** When setting antagonist assays, first supplement a bulk volume of CSM with the challenge agonist Progesterone to achieve the desired final assay-concentration (refer to "*A word about antagonist-mode assay setup*", pg. 8). 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.

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 PGR Assay Products	
<i>Product No.</i>	<i>Product Descriptions</i>
IB05001-32	Human PGR Reporter Assay System 3x 32 assays in 96-well format
IB05001	Human PGR Reporter Assay System 1x 96-well format assay
IB05002	Human PGR 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 Products	
<i>Product No.</i>	<i>Product Descriptions</i>
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

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APPENDIX 1

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

