

**Human Estrogen Receptor Alpha
(ER α ; ESR1; NR3A1)
Reporter Assay System**

3x 32 Assays in 96-well Format
Product # IB00401-32

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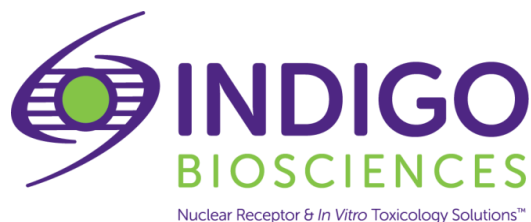
Technical Manual
(Generation 3 version 7.1i)

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

I. Description	
▪ The Assay System.....	3
▪ The Assay Chemistry.....	3
▪ Preparation of Test Compounds.....	4
▪ Assay Scheme.....	4
▪ Assay Performance.....	5
II. Product Components & Storage Conditions	7
III. Materials to be Supplied by the User	7
IV. Assay Protocol	
▪ A word about <i>Antagonist</i> -mode assay setup.....	8
▪ <i>DAY 1 Assay Protocol</i>	8
▪ <i>DAY 2 Assay Protocol</i>	10
V. Related Products	11
VI. Limited Use Disclosures	12
APPENDIX 1: Example Scheme for Serial Dilution	13

I. Description

▪ The Assay System ▪

This assay utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the full-length **Human Estrogen Receptor 1 (NR3A1)**, a ligand-dependent transcription factor commonly referred to as **ER α** .

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to an ER α -responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in ER α activity. Luciferase gene expression occurs after ligand-bound ER α 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 reporter gene. Unlike 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*.

ER α 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.

The principal application of this assay product is in the screening of test samples to quantify functional activities, either agonist or antagonist, that they may exert against the estrogen receptor. This is an all-inclusive assay system that includes, in addition to ER α Reporter Cells, two optimized media for use during cell culture and in diluting the test samples, the reference agonist 17 β -estradiol, Luciferase Detection Reagent, and a cell culture-ready assay plate comprised of twelve 8-well strips and a plate frame.

▪ The Assay Chemistry ▪

INDIGO's reporter assays 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 O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO₂, 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 reporter 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 2*, and depicted in Appendix 1 for the reference agonist, **Compound Screening Medium (CSM)** is used as the diluent to make serial dilutions of test compounds to achieve the desired assay concentration series.

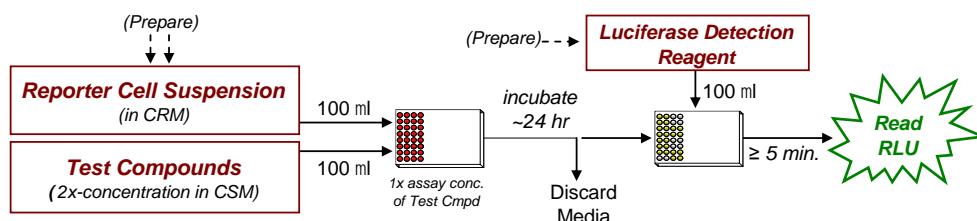
Alternatively, if test compound solubility is expected to be problematic, 2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared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

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, 100 μ l/well of Reporter Cells are dispensed into wells of the assay plate, followed immediately by dispensing 100 μ l/well of the prepared treatment media. Following 22 - 24 hours incubation, treatment media are discarded, and prepared Luciferase Detection Reagent (LDR) 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 ▪

Human ER α (NR3A1)_Agonist Assays

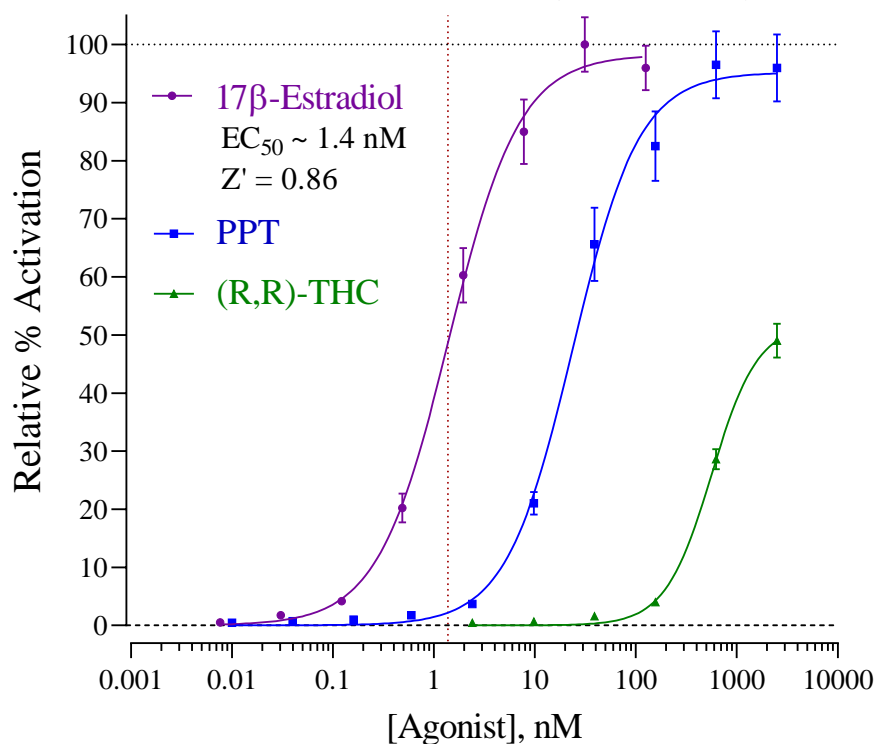


Figure 2. Agonist-mode dose-response analyses of the Human ER α assay.

Human ER α Reporter cells were treated with varying concentrations of the reference agonist 17 β -Estradiol (provided), PPT (4,4'-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol; Tocris), and (R,R)-THC ([R,R]-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol; Tocris). Concentrated stocks of agonists were prepared in DMSO; the highest concentration of DMSO in assay wells was 0.1%. Luminescence was quantified and values of average relative light units (aRLU) and corresponding standard deviation (SD) were determined for each treatment concentration ($n \geq 3$). All aRLU values were percent normalized to the aRLU value of the 17 β -estradiol EC₁₀₀ treatment concentration. GraphPad Prism was used to curve-fit data using the least-squares method of non-linear regression and to determine EC₅₀ values. Z' values were 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.

$$Z' = 1 - [3 * (SD^{E2 EC100} + SD^{Bkg}) / (RLU^{E2 EC100} - RLU^{Bkg})]$$

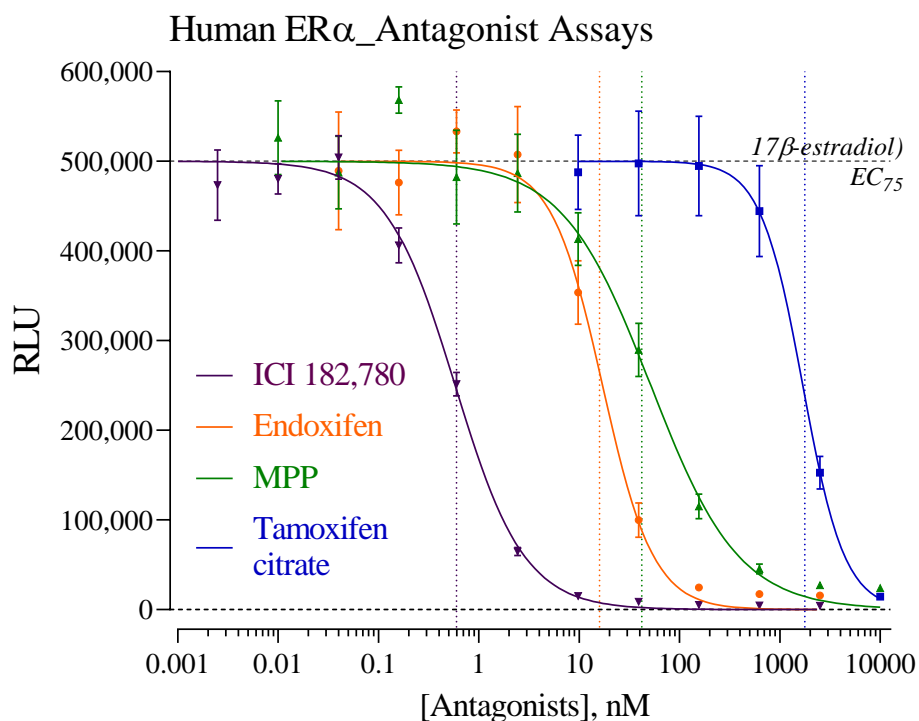


Figure 3. Validation of ER α Antagonist-mode Assays.

Human ER α antagonist assays were performed using Endoxifen, Tamoxifen citrate, ICI182780 and MPP dihydrochloride (all from Tocris). Reporter cells were co-treated with a fixed EC₇₅ concentration of the agonist 17 β -Estradiol and varying concentrations of respective challenge antagonists.

INDIGO's Live Cell Multiplex Assay (#LCM-01) was also performed to confirm that the observed drop in RLU values resulted from receptor-specific inhibition, and not from compound-induced cytotoxicity (data not shown).

II. Product Components & Storage Conditions

This Human ER α 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.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in Step 3 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 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ ER α Reporter Cells	3 x 0.60 mL	-80°C
▪ Cell Recovery Medium (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ 17 β -Estradiol, 100 μ M (in DMSO) (reference agonist for ER's)	1 x 30 μ L	-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, cell-culture ready)	12	ambient

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

- dry ice bucket (*Step 3*)
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO₂ 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*: antagonist reference compound (refer to Figure 3)
- *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 1-8* are performed on **Day 1**, requiring less than 2 hours to complete. *Steps 9-14* are performed on **Day 2** and require less than 1 hour to complete.

▪ A word about Antagonist-mode assay setup ▪

When setting up receptor inhibition assays the Reporter Cells are co-treated with a fixed sub-maximal concentration (typically between EC₅₀ – EC₈₅) of the reference agonist AND varying concentrations of the test compound(s). This ER α assay kit includes a 100 μ M stock solution of **17 β -Estradiol**, an agonist of ER α that may be used to setup antagonist-mode assays. 4 nM 17 β -Estradiol typically approximates EC₇₅ in this assay and, therefore, it is a suitable final concentration of agonist to be used when screening test compounds for inhibitory activity.

Adding a **2x**-concentration of the reference agonist (17 β -Estradiol) 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 5b* of the following protocol. Recall that, in *Step 6*, 100 μ l of treatment media is combined with 100 μ l of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a **2x**-concentration (approx. 8 nM) of 17 β -estradiol. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing a cell suspension supplemented with a desired 2x-concentration of agonist.

DAY 1 Assay Protocol: All steps must be performed using aseptic technique.

1.) Remove Cell Recovery Medium (CRM) and Compound Screening Medium (CSM) from freezer storage and thaw in a 37°C water bath.

2.) Prepare dilutions of treatment compounds (first see *Note 5.4*): Prepare Test Compound treatment media for *Agonist-* or *Antagonist-mode* screens.

Total DMSO carried over into assay reactions should not exceed 0.4%.

Note that, in *Step 6*, 100 μ l of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 μ l of Reporter Cells. Hence, to achieve the desired final assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Use **CSM** to prepare the appropriate dilution series. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

Preparing the positive control: This ER α assay kit includes a 100 μ M stock solution of **17 β -Estradiol**, a reference agonist of ER α . The following 7-point treatment series, with concentrations presented in 5-fold decrements, provides a suitable dose-response: 100, 20.0, 4.0, 800, 160, 32.0, and 6.40 nM. Always include a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

3.) Rapid Thaw of the Reporter Cells: *First*, retrieve the tube of **CRM** from the 37°C water bath and sanitize the outside 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, and 3 tubes for 96 assay wells. Place the tube(s) directly into dry ice and transport the cells to the laminar flow hood. When ready to begin, place the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring a 3.0 ml volume of 37°C CRM into each tube of frozen cells. Recap the tube(s) and immediately place it in a 37°C water bath for 5 - 10 minutes. If only one tube of reporter cells is thawed (32 assays), the resulting volume of cell suspension will be 3.6 ml.

Third, during the 5 - 10 minutes incubation period, work in the cell culture hood to *carefully* mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab.

5.) *a. Agonist-mode assays.* Gently invert the tube of Reporter Cells 2-3 times to gain a homogenous cell suspension. Without delay, dispense 100 μ l of cell suspension into each well of the assay plate.

~ or ~

b. Antagonist-mode assays. Gently invert the tube of Reporter Cells several times to gain a homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "A word about antagonist-mode assay setup", pg. 8). Dispense 100 μ l of cell suspension into each well of the assay plate.

NOTE 5.1: 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 5.2: Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate *will* cause well-to-well variation (= increased Standard Deviation) in the assay.

NOTE 5.3: 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 (100 μ l/well) into a clear 96-well cell culture treated assay plate, followed by 100 μ l/well of CSM. Incubated overnight in identical manner to those reporter cells contained in the white assay plate.

NOTE 5.4: For logistical reasons, some users find it more convenient to first plate the reporter cells and then prepare their test compound dilutions. That strategy works equally well. Once plated, cells may be placed in an incubator for up to 3 hours before proceeding to *Step 6*.

6.) Dispense 100 μ l of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a cell culture incubator (37°C, humidified 5% CO₂) for 22 - 24 hours.

NOTE: Ensure a high-humidity ($\geq 70\%$) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

8.) For greater convenience on *Day 2*, retrieve the appropriate number of sets of **Detection Substrate and Detection Buffer** (one set for each vial of reporter cells used) 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.

9.) 30 minutes before intending to quantify receptor activity, remove **Detection Substrate** from the refrigerator and place them in a low-light area so that it 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.

10.) 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 0.5 second (500 mSec) per well, *or less*.

11.) Immediately before proceeding to *Step 12* prepare **Luciferase Detection Reagent (LDR)**: 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 **LDR**. Mix gently to avoid foaming.

12.) After 22 - 24 hours of incubation, carefully remove media contents from each well.

NOTE: 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 either a single tip or 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 reporter cells, degraded assay performance, and greatly increased well-to-well variability.

13.) 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. Do not shake the assay plate during this period.

14.) Quantify luminescence.

15.) Data analyses.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
Human ERα Assays	
IB00401-32	Human ER α Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB00401	Human ER α Reporter Assay System 1x 96-well format assay
IB00402	Human ER α Reporter Assay System 1x 384-well format assays
Panel of Human ERα / ERβ Assays	
IB00421-48P	Human ER α and ER β Reporter Assay PANEL 48 assays each, in 8-well strips (96-well plate format)
Rat ERα Assays	
R00401-32	Rat ER α Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
R00401	Rat ER α Reporter Assay System 1x 96-well format assay
Zebrafish ERα Assays	
Z00401-32	Zebrafish ER α Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
Z00401	Zebrafish ER α Reporter Assay System 1x 96-well format assay
Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

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-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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VI. Limited Use Disclosures

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