



INDIGO Biosciences, Inc.

The Nuclear Receptor Company™

**Estrogen Receptors Reporter Assay
PANEL**

ER α and ER β

48 Assays each in 96-well Format
Product #IB00421-48P

■

Technical Manual

(version 3.0)

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Human Estrogen Receptors Reporter Assays PANEL ER α and ER β 2x 48 Assays in 96-well Format

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I. Description

INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to human nuclear receptor reporter cells, kits provide an optimized cell culture medium, a medium for diluting test compounds, a positive-control agonist specific to each nuclear receptor, luciferase detection reagent, and a cell culture-ready assay plate. The primary application of these reporter assays is in the screening of test compounds to quantify functional activities, either agonist or antagonist, that they may exert against a specific human nuclear receptor.

These assay systems utilize reporter cells 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 intermediate spin-and-wash steps, viability determinations, or cell titer adjustments.

This **PANEL of Estrogen Receptors Reporter Assays** utilizes non-human mammalian cells engineered to express human **ER α** (NR3A1) and **ER β** (NR3A2), as well as the luciferase reporter gene.

INDIGO's nuclear receptor assays capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology. These nuclear receptor 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 oxyluciferin, AMP, PP₁, CO₂, and photon emission. The luminescence intensity of the reaction is quantified using a plate-reading luminometer, and is reported in terms of relative light units (RLU).

In a typical assay setup, reporter cells are dispensed into wells of the assay plate and then immediately dosed with the user's test compounds. Following an overnight incubation, the treatment media are discarded and luciferase detection reagent (LDR) is added. The intensity of light emission from the ensuing luciferase reaction provides a sensitive measure that is directly proportional to the level of ER activation in the reporter cells.

All of INDIGO's Nuclear Receptor Reporter Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 15 and 90 minutes after initiating the luciferase reaction (refer to APPENDIX 2). There is no need to sequentially process-and-read single assay plates. The unwavering signal stability of these ER Nuclear Receptor Assays enables batch processing of large numbers of assay plates, making this reporter chemistry optimally suited to meet the logistical challenges inherent to high-throughput screening (HTS).

II. Product Components & Storage Conditions

This Human ER Reporter Assays PANEL contains materials to perform 48 ER α assays and 48 ER β assays in a single 96-well plate format. All reagents are supplied with sufficient extra volume to accommodate the needs of performing 2 individual groups of assays.

The individual aliquots of ER Reporter Cells and Detection Solutions I & II are provided as single-use reagents. Once thawed, reporter cells can NOT be refrozen with any hope of retaining downstream assay performance. Extra volumes of these reagents should be discarded after each assay set-up.

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, the vials of “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.

<i><u>Kit Components</u></i>	<i><u>Amount</u></i>	<i><u>Storage Temp</u></i>
▪ ER α Reporter Cells	1 x 1.0 mL	-80°C
▪ ER β Reporter Cells	1 x 1.0 mL	-80°C
▪ Cell Recovery Media 1 (CRM-1)	1 x 10.5 mL	-20°C +4°C (\leq 5 days)
▪ Compound Screening Media 2 (CSM-2)	1 x 35 mL	-20°C +4°C (\leq 5 days)
▪ 17- β -Estradiol, 10 μ M (in DMSO) (control agonist ER α <u>and</u> ER β)	1 x 30 μ L	-20°C
▪ Detection Solution I	2 x 3.0 mL	-80°C
▪ Detection Solution II	2 x 3.0 mL	-80°C
▪ 96-well format plate frame	1	ambient
▪ snap-in, 8-well strips (white, sterile, cell culture treated)	12	ambient

III. Alternative Applications for this Reporter Assay

This PANEL of ER Reporter Assays is a sensitive and versatile research tool. As such, the user may configure these assays in several ways to achieve different research objectives.

The “Assay Procedure” begins in Section VI. It provides specific instructions for performing each ER assay, including alternative set-ups at Step 2 for the user to choose from. For example, these reporter assays may be configured to perform agonist dose-curves (Alternate 2A) or antagonist dose-curves (Alternate 2C), or to perform single-point screening of test compounds for agonist activities (Alternate 2B) or antagonist activities (Alternate 2D).

Due to the experiment-specific nature of these steps, these alternative procedures (and their attendant “NOTES”) are intended to serve as guidelines only. They are offered to assist researchers in formulating an assay design that is best suited to achieve their specific research goals.

IV. Performance Characteristics of the ER α and ER β Assays

The following figures depict the performance characteristics of the ER α and ER β reporter assays comprising this panel.

Figures 1 & 2, Part A: Representative agonist dose-response for ER α and ER β .

For each ER assay, eight agonist treatment concentrations, including “0” agonist, were tested. Treatment media were removed after 24 hr and LDR was applied directly to the cells. Luminescence was quantified using a Tecan GENios Pro plate-reading luminometer. Average relative light unit (RLU) values and respective standard deviations were determined. All numerical conversions and graphing were performed using GraphPad Prism software, as follows: Dose concentrations of 17- β -estradiol were transformed to Log₁₀ (pM). RLU values were normalized such that the lowest RLU and the highest RLU values from each data set are defined as 0% and 100%, respectively. Non-linear regression was performed using "Log (agonist) vs. normalized response - Variable slope" analyses. Error bars depict %CV. The dotted line depicts the calculated Log (EC₅₀) concentration of 17- β -estradiol in these representative assays.

Figures 1 & 2, Part B: Validation of ER Assay performance. Signal-to-background (S/B) and Z' scores were calculated for each ER assay, as described by Zhang, *et al.* (1999)¹. ER reporter cells were treated with media alone, or media supplemented with the concentration of 17- β -estradiol determined to yield RLU_{Max}. To assess the amount of background signal contributed by any other factor(s) that cause gratuitous activation of the luciferase reporter gene, “mock” reporter cells were specially prepared to contain only the luciferase vector (*i.e.*, cells withOUT the ER expression vectors. Mock reporter cells are not provided with assay kits). All cells were cultured, treated with control agonist, and processed in identical manner. Luminescence measurements were conducted as described in (A). For the purposes of these analyses, RLU values are not background-subtracted.

NOTE: RLU values will vary slightly between different production lots of reporter cells, and can vary *significantly* between different makes and models of luminometers.

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.

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

Figure 1A. Representative agonist dose-response of the ER α Reporter Assay System.

Reporter cells were treated with 17- β -estradiol at the following final concentrations: 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.81 and 0 pM. The dotted line depicts the calculated Log₁₀ (EC₅₀) concentration of 17- β -estradiol in this particular assay.

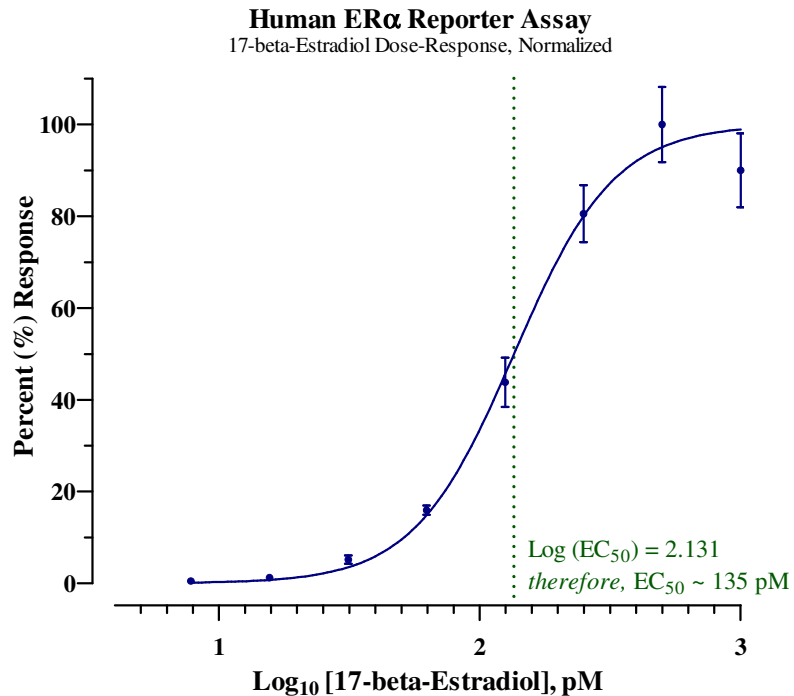


Figure 1B. Validation of the ER α reporter assay for screening applications.

In this particular experiment, ER α reporter cells treated with 500 pM 17- β -estradiol yielded S/B = 38 and a calculated Z' value of 0.74. Similarly treated mock reporter cells demonstrate only low levels of background luciferase activity ($\leq 2\%$ of EC_{Max} treatment). Thus, luminescence signal is the result of ligand-activated exogenous human ER α expressed in these reporter cells.

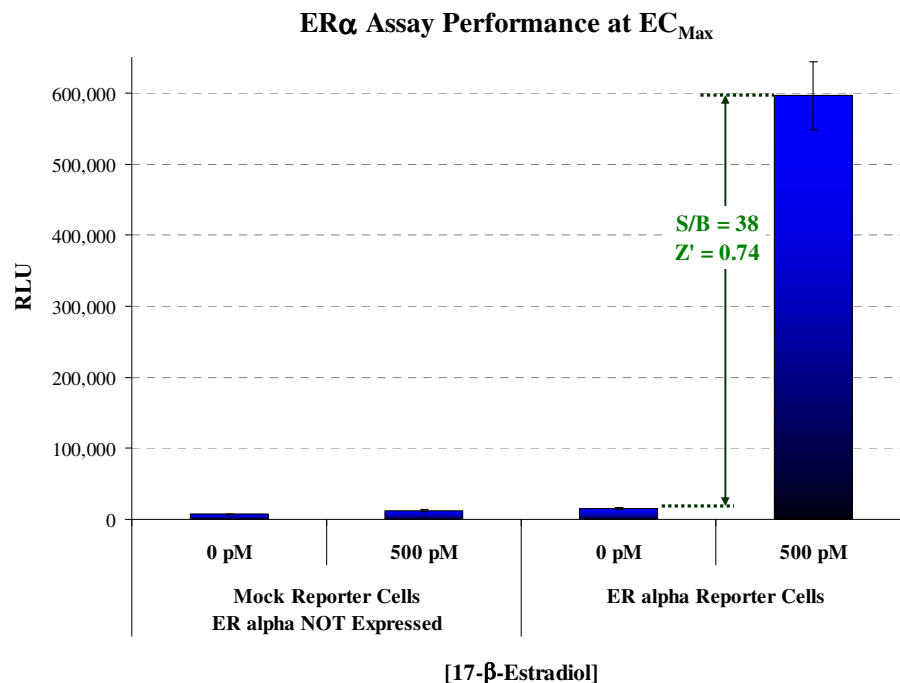


Figure 2A. Representative agonist dose-response of the ERβ Reporter Assay

System. Reporter cells were treated with 17-β-estradiol at the following final concentrations: 3000, 1000, 333, 111, 37.0, 12.3, 4.12, 1.37 and 0 pM. The dotted line depicts the calculated Log₁₀ (EC₅₀) concentration of 17-β-estradiol in this particular assay.

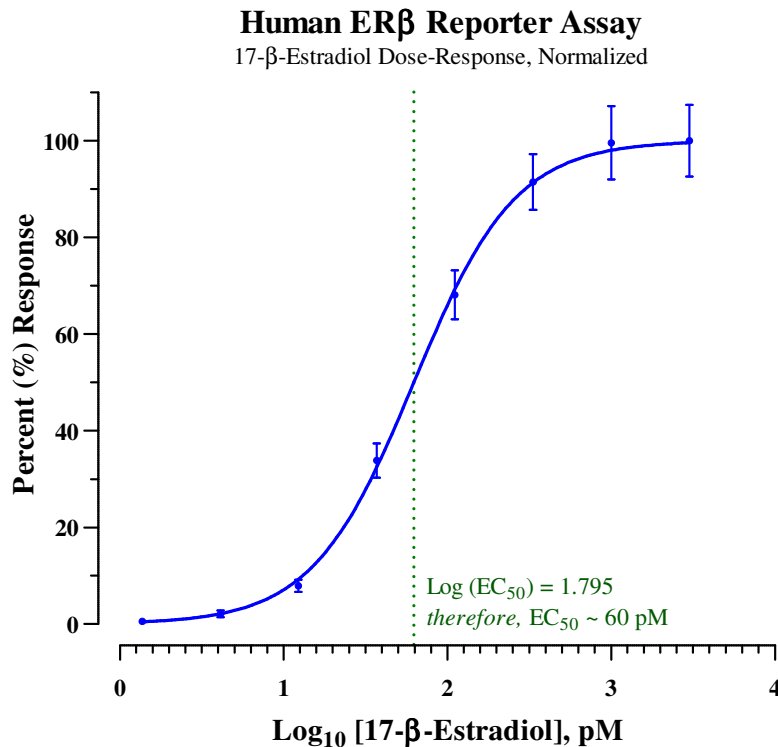
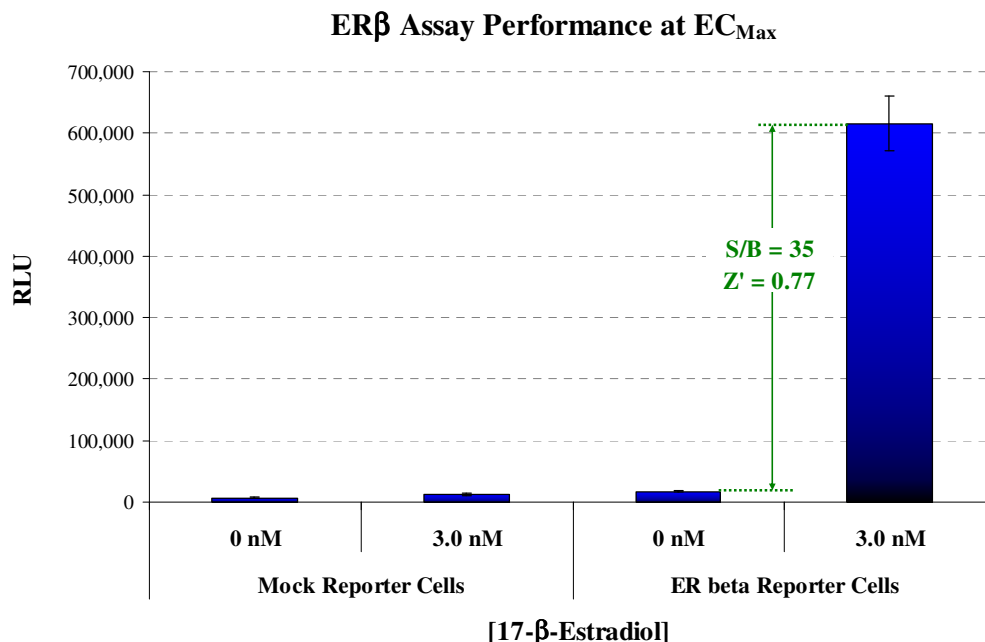


Figure 2B. Validation of the ERβ reporter assay for screening applications.

In this particular experiment, ERβ reporter cells treated with 3.0 nM 17-β-estradiol yielded S/B = 35 and a calculated Z' value of 0.77. Similarly treated mock reporter cells demonstrate only low levels of background luciferase activity (≤ 2.5% of EC_{Max} treatment). Thus, luminescence signal is the result of ligand-activated exogenous human ERβ expressed in these reporter cells.



V. Materials to be Supplied by the User

The following materials must be provided by the user, and should be made available prior to initiating the assay procedure:

DAY 1

- cell culture-rated hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath (*Step 3*).
- 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 control ligand(s) and user's test compound(s) (*Step 2*).
- *optional*: antagonist control compound (*Step 2*, Alternative Protocols C & D).
- 70% alcohol wipes (*Steps 3 & 4*).
- pipette & sterile tips appropriate for the transfer of 100 µl volumes (*Steps 5 & 6*).
The use of an electronic pipette capable of repeat-dispensing is recommended.

DAY 2

- plate-reading luminometer (*Step 10*).
- a vacuum & liquid-trap apparatus connecting to a single pipette tip or, preferably, an 8-pin manifold (*e.g.*, Wheaton Science Microtest Syringe Manifold, # 851381), for use in aspirating media (*Step 12*).
- pipette and tips, as described above (*Step 13*).

VI. Assay Procedure

Review the entire assay protocol before starting.

Completion of the assay requires an overnight incubation. *Steps 1-8* are performed on **Day 1**, requiring 1-2 hours to complete. *Steps 9-15* are performed on **Day 2**, requiring ≤ 1 hour to complete.

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



- 1) Remove **Cell Recovery Medium 1 (CRM-1)** and **Compound Screening Medium 2 (CSM-2)** from freezer storage and thaw.
 - Room temperature CSM-2 is used in the next step. A water bath may be used to facilitate rapid thawing of CSM-2.
 - Thaw CRM-1 and warm to 37°C using a water bath or incubator. Pre-warmed CRM-1 is required in *Step 3*.

ALTERNATIVE 2A: Agonist Dose-Response Assays (as in Figs. 1A & 2A.)

Use **CSM-2** to prepare a dilution series of 2x-concentrated control agonists AND an appropriate dilution series of 2x-concentrated test compound(s) to be assayed.

NOTE 2.0 In *Step 6*, 100 µl of the prepared [CSM + test cmpd] is added into an assay well already containing 100 µl of ER Reporter Cells. Hence, to achieve the desired *final* concentration of test compound in the assay one must prepare and dispense a “2x-concentration” of the test compound(s) (*i.e.*, [CSM + 2x test cmpd]).

NOTE 2.1 When generating dose-response curves, it is recommended to perform all measurements in at least triplicate. In *Step 6*, 100 µl of [CSM + 2x test cmpd] will be added per well of the assay plate. Therefore, devise an appropriate compound dilution scheme to yield a final volume of [CSM + 2x test cmpd] that is slightly greater than 300 µl, thus allowing accurate volume transfers into each of the triplicate wells of the assay plate.

(Continued on page 12)

NOTE 2.2 17- β -estradiol may be used as the positive-control agonist for both **ER α** and **ER β** . However, these two receptors display unique dose-sensitivities to 17- β -estradiol, as well as different *tolerances* for high-concentration dosing. Therefore, we recommend using discreet dose regimens, as follows:

- **ER α** . We find the ER α assay exhibits a complete dose-response to 17- β -estradiol using an *assay* concentration series of 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.81 and 0 picoMolar (pM; 10^{-12} Molar), as depicted in **Figure 1A**. However, as explained in *Note 2.0*, a 2x-concentrated dilution series of both the positive-control agonist and user's test compound(s) are required.

[*Hint: Generating the above dilution series of 17- β -estradiol for the ER α assay may be achieved by following the example presented in APPENDIX 1A. In brief, use a portion of CSM to first perform a 100-fold dilution of the provided 10 μ M 17- β -estradiol stock (e.g., mix 8.0 μ l 17- β -estradiol stock with 792 μ l of CSM). Use the resulting solution to perform a second 50-fold dilution to achieve the first desired 2x-concentration of 2000 pM. Continue by using a portion of the 2000 pM solution to perform seven sequential 2-fold dilutions to produce 1000, 500, 250, 125, 62.5, 31.2, and 15.6 pM 2x-concentrated stocks. Neat CSM (or CSM supplemented with 0.02% DMSO; i.e., the highest concentration of "vehicle") may be used to provide the "0 nM" control treatment.]*

- **ER β** . We find the ER β assay exhibits a complete dose-response to 17- β -estradiol using an *assay* concentration series of 3000, 1000, 333, 111, 37.0, 12.3, 4.12, 1.37 and 0 picoMolar (pM; 10^{-12} Molar), as depicted in **Figure 2A**. However, as explained in *Note 2.0*, a 2x-concentrated dilution series of both the positive-control agonist and the user's test compound(s) are required.

[*Hint: Generating the above dilution series of 17- β -estradiol for the ER β assay may be achieved by following the example presented in APPENDIX 1B. In brief, use a portion of CSM to first perform a 100-fold dilution of the provided 10 mM 17- β -estradiol stock (e.g., mix 10 μ l 17- β -estradiol stock with 990 μ l of CSM). Use the resulting solution to then perform one 16.66-fold dilution to achieve the first desired 2x-concentration of 6000 pM. Continue by using a portion of the 6000 pM solution to perform seven sequential 3-fold dilutions to produce 2000, 667, 222, 74.1, 24.7, 8.23, and 2.74 pM 2x-concentrated stocks. Neat CSM (or CSM supplemented with 0.06% DMSO; i.e., the highest concentration of "vehicle") may be used to provide the "0 nM" control treatment.]*

ALTERNATIVE 2B: Screening for Agonist Activities via Single-Point Assay

Use **CSM-2** to make an appropriate dilution of each test compound in the library. Also, prepare CSM-2 containing the desired positive-control agonist.

This ER Reporter Assay PANEL includes **10 μ M** stock solution of **17- β -estradiol**, an agonist of both ER α and ER β that may be used at assay-specific concentrations to provide respective positive-controls.

- **ER α** An assay concentration of 500 pM 17- β -estradiol typically provides $\geq 95\%$ activation of ER α (Figure 1A). Hence, 1000 pM 17- β -estradiol will provide a 2x-concentrated agonist that is a suitable positive-control reference. Refer to *Note 2.2* for a recommended dilution scheme to prepare the appropriate 2x-concentrated 17- β -estradiol solution.
- **ER β** An assay concentration of 3000 pM 17- β -estradiol typically provides $\geq 95\%$ activation of ER β (Figure 2A). Hence, 6000 pM 17- β -estradiol will provide a 2x-concentrated agonist that is a suitable positive-control reference. Refer to *Note 2.2* for a recommended dilution scheme to prepare the appropriate 2x-concentrated 17- β -estradiol solution.

NOTE 2.3 As a *general rule*: when an organic solvent is used to generate primary stock solutions of test compounds, we recommend that users strive to devise a dilution scheme so that the concentration of organic solvent in [CSM + 2x test cmpd] does not exceed 0.2% (*i.e.*, *minimally* a 500-fold dilution into CSM). This corresponds to a final assay concentration of 0.1% organic solvent.

NOTE 2.4 In *Step 6*, 100 μ l of the prepared [CSM + 2x test cmpd] will be added per well of the assay plate. If single-point assays are to be made, prepare a volume of each [CSM + 2x test cmpd] media that is slightly greater than 100 μ l, thus allowing accurate volume transfers into respective assay wells. This assay kit provides 35 ml of CSM to be used in preparing dilutions of control and test compounds for all 96 assays in this Panel. Plan dilution schemes *carefully*.

ALTERNATIVE 2C: Antagonist Dose-Response Assays.

A common method of performing receptor inhibition studies is to prepare a co-mix of a known agonist (at a constant concentration typically between EC₅₀ – EC₈₅) AND a dilution series of the test antagonist compound(s) to be evaluated.

See *NOTE 2.0*.

This ER Assay PANEL includes 17- β -estradiol, an agonist to both ER α and ER β that may be used to setup receptor inhibition studies. Suggested concentrations for respective assays are below. Refer to *NOTE 2.2* for dilution schemes to aid in the preparing appropriate [CSM + 2x 17- β -estradiol] treatment media.

NOTE 2.5

- **17- β -estradiol** may be used as an agonist of **ER α** (Figure 1A) to set up antagonist screens. We find that 125 pM 17- β -estradiol typically approximates EC₈₀ in this reporter assay.
- **17- β -estradiol** may also be used as an agonist of **ER β** (Figure 2A) to set up antagonist screens. We find that 111 pM 17- β -estradiol typically approximates EC₇₀ in this reporter assay.

Supplement appropriate volume portions of **CSM-2** with control agonist to generate stock volumes of [CSM + 2x 17- β -estradiol]. Use these stocks to then prepare 2x-concentrated dilution series of each test compound to be evaluated for antagonist activity. If desired, also prepare a dilution series of 2x-concentrated positive-control antagonist (supplied by the user).

NOTE 2.6 As a general rule: when an organic solvent is used to generate primary stock solutions of test compounds, we recommend that users strive to devise a dilution scheme so that the concentration of organic solvent in [CSM + 17- β -estradiol + 2x test cmpd] does not exceed 0.2% (*i.e.*, *minimally* a 500-fold dilution into CSM). This corresponds to a final assay concentration of 0.1% organic solvent.

NOTE 2.7 When generating antagonist dose-response curves, it is recommended to perform all measurements in at least triplicate. In *Step 6*, 100 μ l of [CSM + 2x 17- β -estradiol + 2x test cmpd] will be added per well of the assay plate. Therefore, devise an appropriate compound dilution scheme to yield a final volume of [CSM + 2x 17- β -estradiol + 2x test cmpd] that is slightly greater than 300 μ l, thus allowing accurate volume transfers into each of the triplicate wells of the assay plate.

ALTERNATE 2D: Screening for Antagonist Activities via Single-Point Assay.

A common method of performing receptor inhibition studies is to prepare a co-mixture of a known agonist (at a concentration typically between EC₅₀ – EC₈₅) AND a single test concentration of the candidate antagonist compound(s) to be evaluated. As previously described in ALTERNATE 2C, this ER Assay PANEL includes 17- β -estradiol, an agonist for both ER α and ER β . 17- β -estradiol may be used (at appropriate concentrations for respective ER α and ER β assays) to design such receptor inhibition screens.

See NOTE 2.5.

Supplement appropriate volume portions of **CSM-2** with an of 17- β -estradiol to generate a stock volume of [CSM + 2x 17- β -estradiol]. Refer to NOTE 2.2 for a recommended dilution scheme to prepare respective stocks of [CSM + 2x 17- β -estradiol]. Use these stocks to then prepare 2x-concentrated dilutions of each test compound to be screened for antagonist activity. If desired, also prepare an appropriate dilution of 2x-concentrated positive-control antagonist (supplied by the user).

See NOTE 2.6.

NOTE 2.8 In *Step 6*, 100 μ l of the prepared [CSM + 2x 17- β -estradiol + 2x test cmpd] will be added per well of the assay plate. If single-point assays are to be made, prepare a volume of each [CSM + 2x 17- β -estradiol + 2x test cmpd] media that is slightly greater than 100 μ l, thus allowing accurate volume transfers into respective assay wells. This assay kit provides 35 ml of CSM to be used in preparing dilutions of control and test compounds for all 96 assays in this Panel. Plan dilution schemes *carefully*.

- 3) Retrieve the **ER Reporter Cells** from -80°C storage. Retrieve **CRM-1** from the 37°C water bath and sanitize the outside of the tube with a 70% ethanol swab. Transfer the tubes into a cell-culture hood. Perform a *rapid thaw* of the frozen cells by pipette-transferring 4.5 ml of the 37°C CRM-1 into the tube of frozen cells. Recap the tubes of Reporter Cells and immediately place them in a 37°C water bath for at least 3 minutes

NOTE: During this incubation step, work in an aseptic hood to *carefully* mount the appropriate number of sterile 8-well strips into the blank plate frame. Each ER assay utilizes 6x 8-well strips, for a total of 48 reactions each. Strip-wells are fragile. Note that they have *keyed ends* (square and round), hence, they will fit into the frame in only one orientation.

- 4) Retrieve the tubes of Reporter Cell Suspensions from the water bath. Sanitize the outside surface of each tube with a 70% alcohol swab, then transfer it into the cell culture hood.

- 5) Gently invert the tubes of Reporter Cells to disperse any cell aggregates. For each ER assay, dispense 100 µl of a given cell suspension into **48 wells** of the assay plate.

NOTE: Take care to prevent cells from settling during the dispensing period. Lack of precision in transferring uniform volumes across the assay plate, and/or allowing cells to settle during the dispensing process, will cause well-to-well variation in the assay. For improved speed, precision, and ergonomic comfort, *the use of an electronic repeat-dispensing pipette is recommended.*

- 6) Add 100 µl of 2x-concentrated treatment media (as prepared in *Step 2*) to appropriate wells of the assay plate.

- 7) Replace the plate's lid and transfer it into a 37°C, humidified 5% CO₂ incubator for 22 - 24 hours.

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

- 8) For greater convenience on Day 2, retrieve **Detection Solutions I and II** from -80°C storage and place in a dark refrigerator (4°C) to thaw overnight.

NOTE: This PANEL kit contains two aliquots of Detection Solution I, and two aliquots of Detection Solution II. They are provided as individual reagents so that, if preferred, users may perform the two ER assays at different times. Thaw 1 pair of Detection Solutions I & II for each ER assay. If the user intends to perform both ER assays at the same time, place both pairs of Detection Solutions in a refrigerator to thaw overnight. In such cases, on **Day 2 (Step 11)** combine all of the separate solutions into one tube to generate a single batch volume of the final **LDR** mixture.

(Continue on DAY 2)

DAY 2: Subsequent manipulations do not require special regard for aseptic technique.

- 9) 30 – 60 minutes before quantifying ER activities, remove **Detection Solutions I and II** from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature. Gently invert each tube several times to ensure homogenous solutions.
- NOTE:* Do NOT actively warm Detection Solutions I and II 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) Turn on the luminometer. Set the instrument to perform a single 5 second “plate shake” prior to reading the assay plate. Set the read time for 0.5 second per well.
- NOTE:* Many luminometers require a “warm up” period of 15 minutes *or more* before the photo-multiplier tube (PMT) attains maximal precision in reading very low luminescence signal.
- 11) *Immediately before proceeding to Step 12*, combine the contents of Detection Solutions I and II into a single tube to generate **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.
- 12) After 22-24 hours of incubation, remove the assay plate from the incubator. Remove the plate’s lid. Remove media contents from each well.
- NOTE:* 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. Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media *via* a sweeping downward movement is NOT advised.
- 13) Add 100 µl of room temperature **LDR** to each well of the assay plate.
- NOTE:* *Pipette carefully to avoid bubble formation!* Scattered micro-bubbles will not pose a problem. However, bubbles covering the surface of the reaction mix, or large bubbles clinging to the side walls of the well, will cause lens-effects that may significantly degrade the accuracy and precision of the assay data. In the event of excessive bubble formation during processing, spin the assay plate (with lid) at *low speed* for 1-2 minutes using a room temperature centrifuge fitted with counter-balanced plate carriers.
- 14) Allow the assay plate to rest at room temperature for at least 15 minutes after the addition of LDR. Do not shake the assay plate during this period.
- NOTE:* See **APPENDIX 2** for information regarding signal stability.
- 15) Read the assay plate anytime between 15 - 90 minutes after adding LDR.

VI. Related Products

ERα Family of Assay Products	
<i>Product No.</i>	<i>Product Descriptions</i>
IB00401-32	Human ER α Reporter Assay System 3x 32 assays in 96-well format
IB00401	Human ER α Reporter Assay System 1x 96-well format assay
IB00401-B10	Human ER α Reporter Assay System (Bulk Pack) Bulk Reagent Pack for 10x 96-well plates
IB00402	Human ER α Reporter Assay System 1x 384-well format assays
IB00402-B10	Human ER α Reporter Assay System (Bulk Pack) Bulk Reagent Pack for 10x 384-well plates
ERβ Family of Assay Products	
IB00411-32	Human ER β Reporter Assay System 3x 32 assays in 96-well format
IB00411	Human ER β Reporter Assay System 1x 96-well format assay
IB00411-B10	Human ER β Reporter Assay System (Bulk Pack) Bulk Reagent Pack for 10x 96-well plates
IB00412	Human ER β Reporter Assay System 1x 384-well format assays
IB00412-B10	Human ER β Reporter Assay System (Bulk Pack) Bulk Reagent Pack for 10x 384-well plates
Alternative volumes of each ER Assay Bulk Reagents can be custom manufactured. Please Inquire.	

PANEL of ER Assays	
<i>Product No.</i>	<i>Product Description</i>
IB00421-48P	Human ER α and ER β Reporter Assay PANEL 48 assays each in 96-well plate format

Please refer to INDIGO Biosciences' website for updated product offerings.

www.indigobiosciences.com

VII. Limited Use Disclosures

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans. Other applications of this product may require licenses from others, including one or more of the institutions listed below.

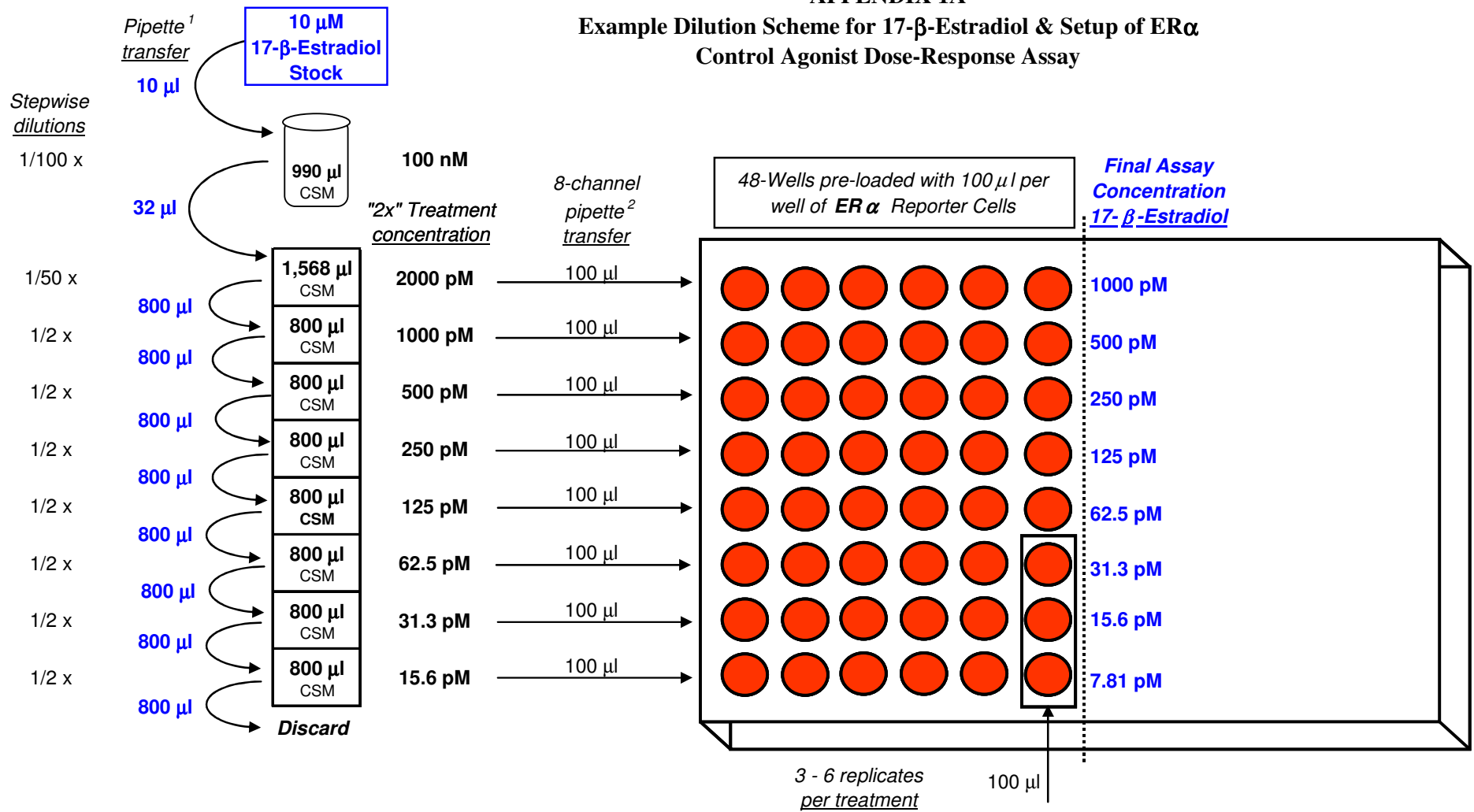
The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Patent 5,583,024. INDIGO Biosciences, Inc. has entered into a license agreement with The Regents of the University of California (Oakland, CA) for commercial application of the cDNA encoding the native luciferase of *Photinus pyralis*.

“CryoMite” is a Trademark [™] of INDIGO Biosciences, Inc.

Product prices, availability, specifications and claims are subject to change without prior notice.

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APPENDIX 1A
Example Dilution Scheme for 17-β-Estradiol & Setup of ERα
Control Agonist Dose-Response Assay

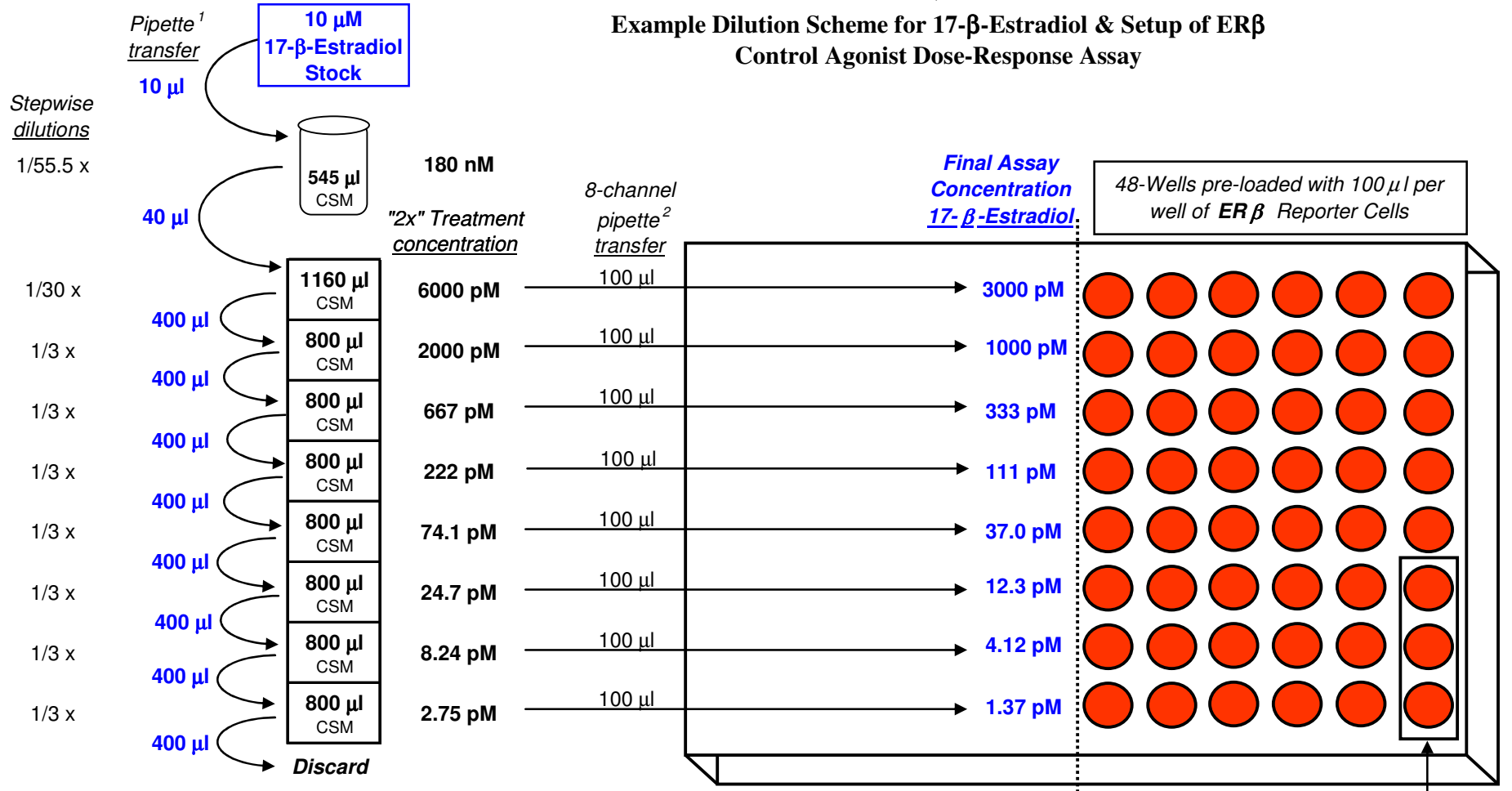


¹ To maximize accuracy, use calibrated pipettes that most closely match the desired transfer volume. For example: use a P10 to transfer 10 μl, use a P100 to transfer 32 μl, use a P1000 to transfer 800, 990, and 1568 (i.e., 2x 784) μl volumes. For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.

² To maximize speed, precision, and ergonomic comfort during manual pipetting of 100 μl volumes of reporter cells and treatment media, the use of an electronic, 8-channel, multi-dispensing P1000 pipette is recommended.

APPENDIX 1B

Example Dilution Scheme for 17- β -Estradiol & Setup of ER β Control Agonist Dose-Response Assay



¹ To maximize accuracy, use calibrated pipettes that most closely match the desired transfer volume. For example: use a P10 to transfer 10 μ l, a P200 to transfer 40 μ l, and a P1000 to transfer 400, 545, 800, and 1160 (2x 580) μ l volumes. For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.

² To maximize speed, precision, and ergonomic comfort during manual pipetting of 100 μ l volumes of reporter cells and treatment media, the use of an electronic, 8-channel, multi-dispensing P1000 pipette is recommended.

APPENDIX 2

Signal Stability of the Nuclear Receptor Reporter Assay

The human ER β Reporter Assay System is used here to demonstrate the light emission profile characteristic of INDIGO Biosciences' nuclear receptor assay system products.

As seen in **FIGURE 3**, between 5 and 15 minutes after adding LDR to assay wells the initial intensity of luminescence decays by 10-12%. However, luminescence signal stabilizes and remains essentially constant over the ensuing 75 minute reaction period. From T=15 minute to T=90 minutes, average luminescence measured from the same set of assay wells deviate by *less than 5%*

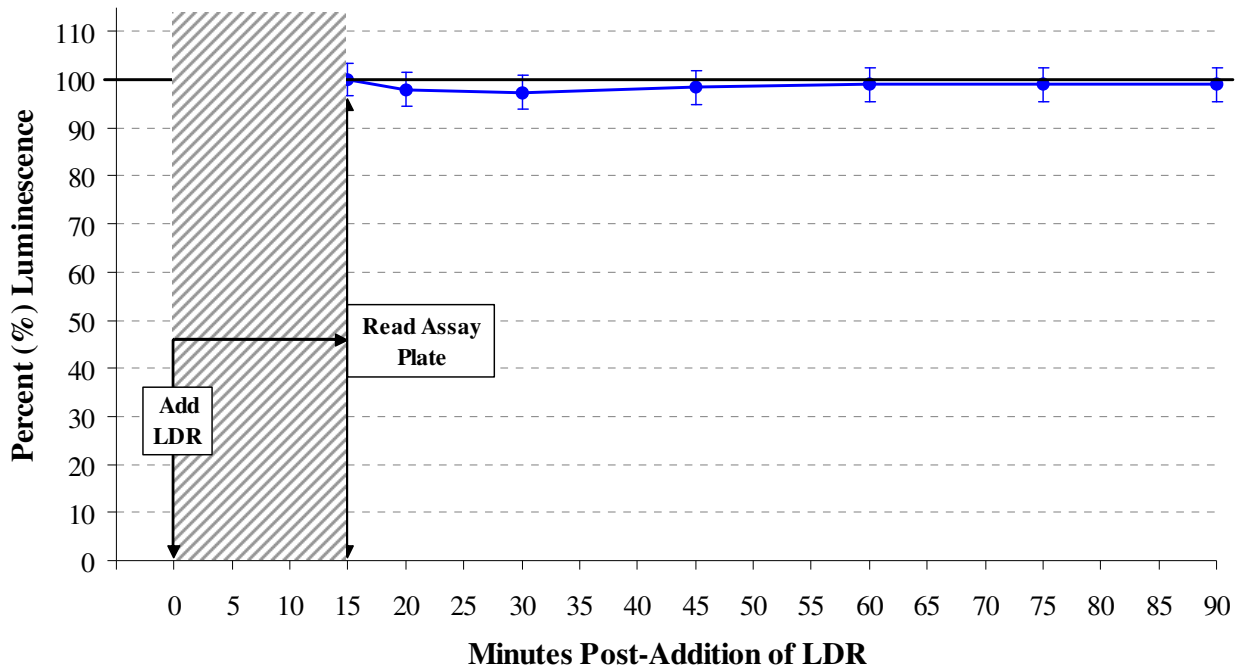


Figure 3. Stability of the luminescence signal. ER β reporter cells were cultured in 8 replicate wells of a 96-well assay plate, treated with 1 nM 17- β -estradiol for 24 hr, and media were replaced with LDR according to the protocol provided in this Technical Manual. Following an initial 5 minute rest period at room temperature, luminescence intensities were quantified by integrating light emission over 500 mSec. After the initial 5 minute time point, the assay plate was re-read at 10, 15, 20, 30, 45, 60, 75 and 90 minutes post-addition of LDR. Average RLU and respective standard deviation values were calculated, then normalized so that the luminescence signal at 15 minutes = 100%.

Allowing a *minimum* rest period of 15 minutes after the addition of LDR is particularly important for HTS users. Due to the logistics of batch-processing large numbers of assay plates, a significant time differential may occur between processing the first and last assay plates. Nonetheless, due to the stable emission profile of the luciferase reaction between 15 - 90 minutes, HTS users may be confident in comparing signal output from test samples in the first assay plate to those in the last plate in the stack.