Human Estrogen-Related Receptor, Gamma

(NR3B3, ERRγ)

Reporter Assay System

384-well Format Assays

Product # IB08022

▪

Technical Manual

(version 6.0)

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Human ERRγ Reporter Assay System
384-well Format Assays

I. Description

▪ The Assay System ▪

This nuclear receptor assay system utilizes proprietary non-human mammalian cells engineered to provide high-level expression of a hybrid form of the **Human Estrogen-Related Receptor Gamma (NR3B3)**. The N-terminal DNA binding domains (DBD) of the native ERRγ has been substituted with that of the yeast GAL4-DBD. The reporter gene is beetle luciferase functionally linked to the GAL4 upstream activation sequence (UAS).

As is true *in vivo*, these reporter cells express ERRγ that is constitutively active in the (putative) absence of a ligand binding event. Interestingly, the ligand binding domain of ERRγ may be occupied by a ligand that further elevates the constitutive activity of the receptor (an agonist response), or one that results in a dose-dependent loss of constitutive active (an inverse-agonist response)[1]. **Figure 2** demonstrates the high constitutive activity of ERRγ in the absence of added ligand, as well as agonist and inverse-agonist responses of the receptor upon ligand binding. Therefore, the principle application of this assay system is in the screening of test samples to quantify either *agonist* or *inverse-agonist* activities that they may exert against human ERRγ.

ERRγ 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 Reporter Assays are all-inclusive cell-based assay systems. In addition to ERRγ 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 reporter assay systems 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 Reporter Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 30 and 100+ minutes after initiating the luciferase reaction (see **APPENDIX 2**). Incorporating a 30 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 ▪

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using **Compound Screening Medium (CSM)**, as described in *Step 2* of the **Assay Protocol**. This method avoids the 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.

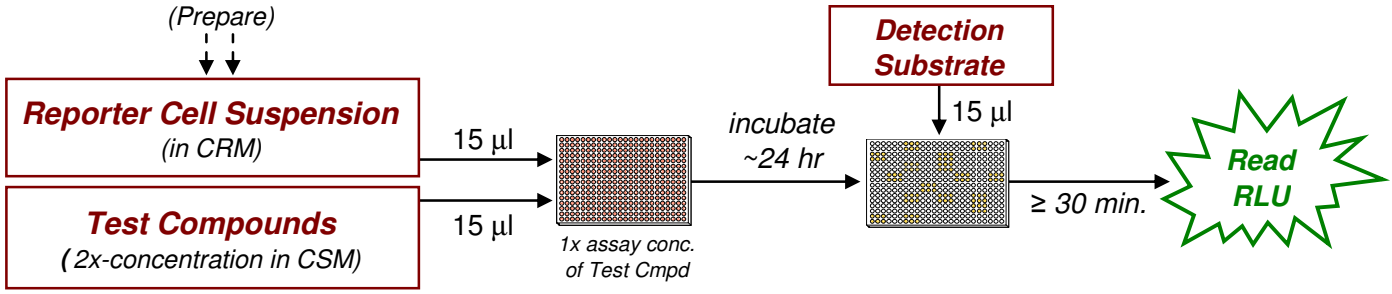
▪ Considerations for Automated Dispensing ▪

When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

Stock Reagent & Volume provided	Volume to be Dispensed *(384-well plate)*	Excess rgt. volume available for instrument dead volume
Reporter Cell Suspension 7.5 ml *(prepared from kit components)*	15 µl / well – 5.8 ml / plate	~ 1.7 ml
Detection Substrate 7.8 ml	15 µl / well – 5.8 ml / plate	~ 2 ml

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user's test compounds. Following 22 -24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each assay well is quantified using a plate-reading luminometer.



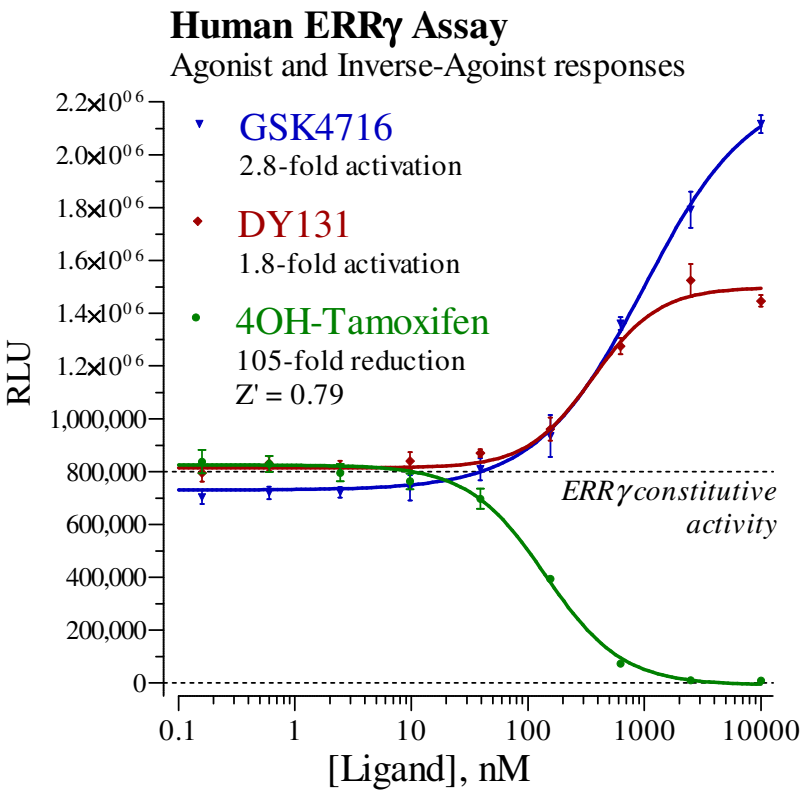


Figure 2. Agonist and Inverse-Agonist responses of ERRγ reporter cells treated with reference ligands.

ERRγ Assays were performed using manual dispensing and following the protocol described in this Technical Manual, using the agonists GSK4716[1] (Tocris) and DY131[2] (Tocris), and the inverse-agonist 4-hydroxy tamoxifen[3] (provided). Luminescence was quantified using a GloMax-Multi+ plate-reading luminometer (Promega Corp.). Values of average relative light units (RLU) and standard deviation (SD) were determined for each treatment concentration (n ≥ 6). Z' values were calculated as described by Zhang, *et al.* (1999)[4]. Non-linear regression analyses were performed and values of EC_{50} and IC_{50} were determined using GraphPad Prism software.

[1] Wang L, *et. al.* (2006) X-ray crystal structures of the estrogen-related receptor g ligand binding domain in three frunction states reveal the molecular basis of small molecule regulation. JBC:**281** (49), 37773-37781.

[2] Yu DD and Barry MF (2005) Identification of an agonist ligand for estrogen-related receptors ERRβ/γ. Bioorgan. & Med. Chem. Letters:**15**, 1311-1313.

[3] Zuercher WJ, *et al.* (2005) Identification and structure-activity relationship of phenolic acyl hydrazaones as selective agonists for the estrogen-related orphan nuclear receptors ERRβ and ERRγ. J. Med. Chem.:**48**, 3107-3109.

[4] Zhang JH, *et. al.* (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen.:**4**(2), 67-73.

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

II. Product Components & Storage Conditions

This Human ERRγ Reporter Assay System contains materials to perform assays in a single 96-well assay plate.

The aliquot of ERRγ Reporter Cells is provided as a single-use reagent. 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.

Kit Components	***Amount***	***Storage Temp.***
▪ ERRγ Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 6.0 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ 4-Hydroxy Tamoxifen, 10 mM (in DMSO) (reference agonist for ERRγ)	1 x 30 µL	-20°C
▪ Detection Substrate	1 x 7.8 mL	**-80°C**
▪ 384-well, assay plate, white, sterile	1	-20°C

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% CO_2 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).
- antagonist reference compound (optional).

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 9-15* are performed on **Day 2**, and require less than 1 hour to complete.

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

☐ **1.)** Remove **Cell Recovery Medium (CRM)** and **Compound Screening Medium (CSM)** from freezer storage and thaw.

- **CRM** should be thawed and equilibrated to 37°C using a water bath. CRM pre-warmed to 37°C is required in *Step 3*.
- **CSM** may be thawed in a 37°C water bath.

☐ **2.)** Prepare Test Compound(s) and Reference Compound stocks to be screened for *Agonist* or *Antagonist* activities.

The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

Note that, in *Step 6*, 15 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 15 µl 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. Plan dilution schemes carefully. This assay kit provides 35 ml of CSM.

This ERRγ Reporter Assay System kit includes a 10 mM stock solution of 4-hydroxy-tamoxifen, an inverse-agonist of ERRγ. The following 7-point treatment series, with concentrations presented in 5-fold decrements, provides a complete inverse-agoinist dose-response: 5000, 1000, 200, 40.0, 8.00, 1.60, and 0.320 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

☐ **3.)** *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. Perform a *rapid thaw* of the frozen cells by transferring a 5.5 ml volume of 37°C CRM into the 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 7.5 ml.

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

☐ **5.)** Invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 15 µl of cell suspension into each well of the 384-well Assay Plate.

NOTE 5.1: 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 5.2: Users sometimes prefer to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear 384-well assay plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

☐ **6.)** Dispense 15 µl of 2x-concentration treatment media into appropriate wells of the assay plate.

☐ **7.)** Transfer the assay plate into a 37°C, humidified 5% CO_2 incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 90%) 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 **Detection Substrate** from freezer storage and place it 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 ERRγ activity, remove **Detection Substrate** from the refrigerator and place them in a low-light area so that it may equilibrate to room temperature. Gently invert the tube several times to ensure an homogenous solution.

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

☐ **11.)** Following 22 - 24 hours of incubation, add 15 µl of **Detection Substrate** to each well of the assay plate.

NOTE: Perform manual reagent transfers 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 manual 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.

☐ **12.)** Allow the plate(s) to rest at room temperature for 30 minutes. Do not shake the assay plate(s) during this period.

NOTE: As discussed in APPENDIX 2, the luminescent signal is unstable during the first 30 minutes of the luciferase reaction, and will experience ~ 35% loss in intensity. However, after the initial 30 minute reaction period the luminescence signal achieves a stable emission output.

☐ **13.)** Quantify luminescence.

V. Related Products

ERRγ Assay Products	
Product No.	***Product Descriptions***
IB08021-32	Human ERRγ Reporter Assay System 3x 32 assays in 96-well format
IB08021	Human ERRγ Reporter Assay System 1x 96-well format assay
IB08022	Human ERRγ 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	
Product No.	***Product Descriptions***
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.

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VI. Limited Use Disclosures

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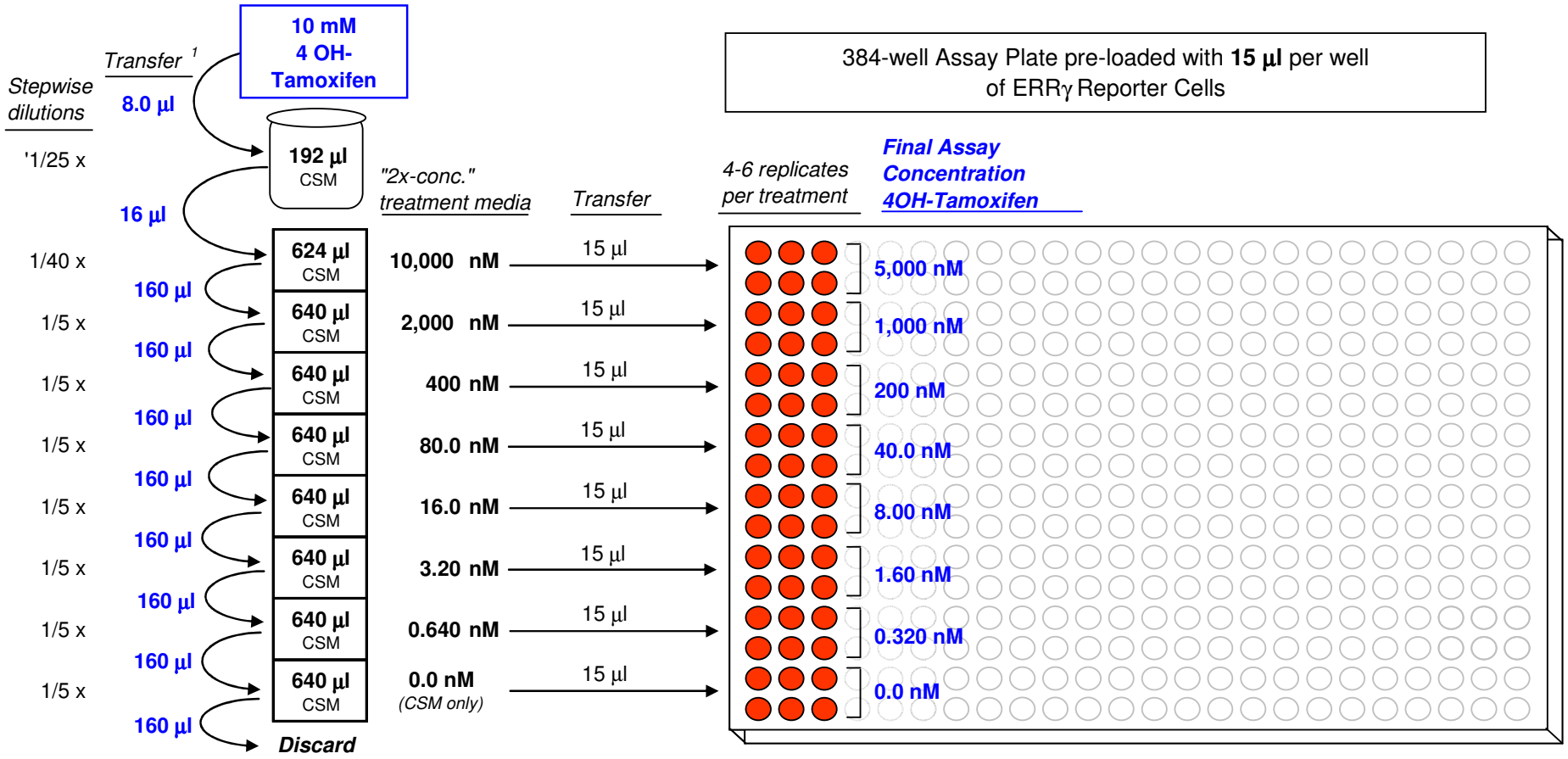
"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc.

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

APPENDIX 1

Example scheme for the serial dilution of 4OH-Tamoxifen inverse-agonist, and the setup of an ERRγ dose-response assay.



[1] For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.

APPENDIX 2
Signal Stability of the Nuclear Receptor Reporter Assay

The Human PPARα Reporter Assay System is used here to demonstrate the light emission profile of INDIGO Biosciences' homogenous assay in 384-well format.

As seen in **FIGURE 3**, within the first 30 minutes after dispensing Detection Substrate into 384-well assay plates the intensity of the luminescent reaction decays, at a variable rate, by ≥ 35%. After 30 minutes, however, the reaction stabilizes and signal intensity remains constant for, minimally, the ensuing 75 minute reaction period. From T=30 minutes to T=105 minutes, average RLU values measured from the same assay wells deviate by *less* than 3%.

Allowing a reaction-rest period of 30 minutes after the addition of Detection Substrate is important for users who elect to manually process a 384-well assay plate, *or* batch-process multiple assay plates using an automated dispenser, *or* use a luminometer that requires more than 60 seconds to read the entire assay plate. In each of these situations a significant time differential will occur between processing and/or reading the first assay well on the first plate and the last assay well on the last plate. Nonetheless, by incorporating a 30 minute reaction-rest period prior to measuring RLU values, users may be confident in directly comparing signal data from all sample wells, from the first assay plate to the last plate in the stack.

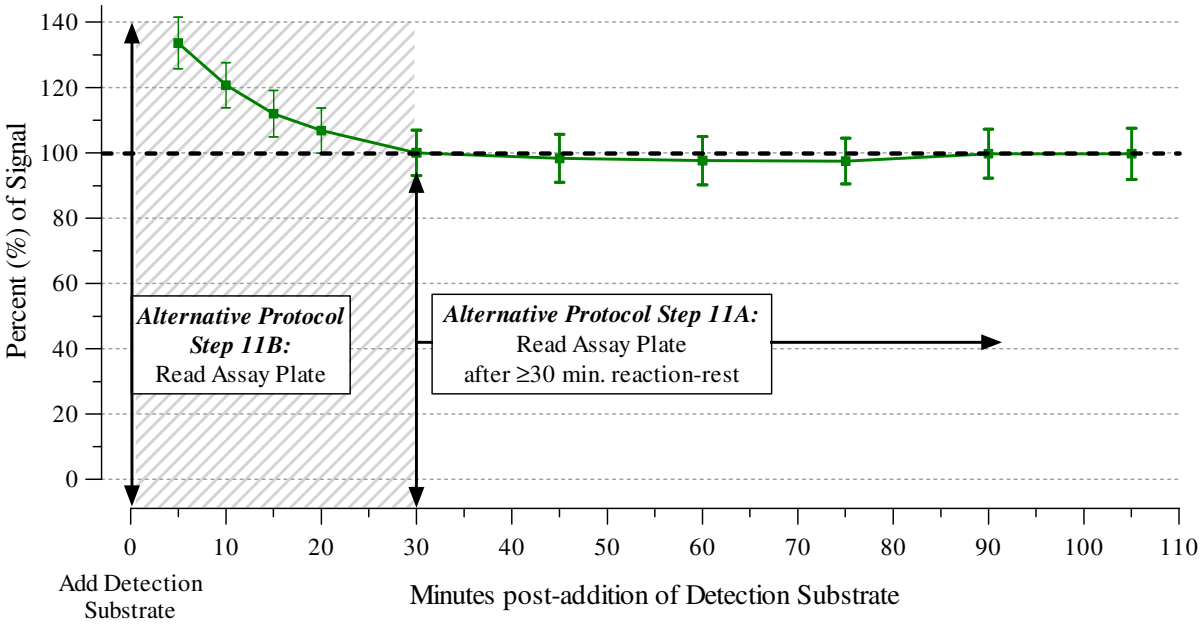


Figure 3. Stability of the luminescence signal from 384-well format, homogenous assay protocol. PPARα reporter cells were cultured in a 384-well assay plate (n=8) in the presence of 100 nM GW590735. After 24 hr incubation, Detection Substrate was added into assay wells and the assay plate was allowed to rest at room temperature. At 5, 10, 15, 20, 30, 45, 60, 75, 90 and 105 minutes post-addition of Detection Substrate, luminescence intensities were quantified by integrating photon emission over 500 mSec. Average RLU values were calculated, then normalized so that the luminescence signal at 30 minutes = 100%.