Live Cell Multiplex Assay

For use in combination with INDIGO's
3x32-, 2x48-, or 1x96-well format
Nuclear Receptor Reporter Assay Systems

Product #

LCM-01 (1x 96 assay wells)

LCM-05 (5x 96 assay wells)

LCM-10 (10x 96 assay wells)

■

Technical Manual

(version 4.0)

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INDIGO BIOSCIENCES
The Nuclear Receptor Experts™

Live Cell Multiplex (LCM) Assay

For use in combination with INDIGO's
3x32-, 2x48-, or 1x96-well format
Nuclear Receptor Reporter Assays

I. Description

Utility and Overview of the Live Cell Multiplex (LCM) Assay

The Live Cell Multiplex (LCM) Assay provides an easy to use fluorescence-based method to quantify changes in the relative number of live mammalian cells in assay wells after treatment with test compounds. The LCM Assay is optimized to be run in multiplex with any of INDIGO's 96-well, 2x48-well, or 3x32-well luminescence-based nuclear receptor reporter assays.

The LCM assay allows a user to validate their primary Nuclear Receptor Assay data by determining if their test compound treatments exert mitogenic or cytotoxic activities on the reporter cells. Such effects will always undermine the accurate assessment of a test compound's potency and/or efficacy as a modulator of nuclear receptor function.

When screening a test compounds for *antagonist* or *inverse-agonist* bioactivity it is particularly important to also quantify changes in the relative number of live reporter cells at the assay endpoint. Test compounds that exert cytostatic, cytotoxic, or cytolytic activities invariably generate "false-positive" results in such loss-of-activity assays. When a test compound induces cytotoxicity the measured drop in luciferase activity will be incorrectly attributed to inhibition of the nuclear receptor by the test compound. In reality, the treatment has pushed the reporter cells into division arrest, apoptosis, necrosis, or lysis. APPENDIX 2 presents an example of the impact of compound-induced division arrest on ability to correctly interpret antagonist screening data.

An overview of the multiplex assay is depicted in **Figure 1**. A detailed protocol for performing the LCM and Nuclear Receptor (NR) Assays is provided in Section IV.

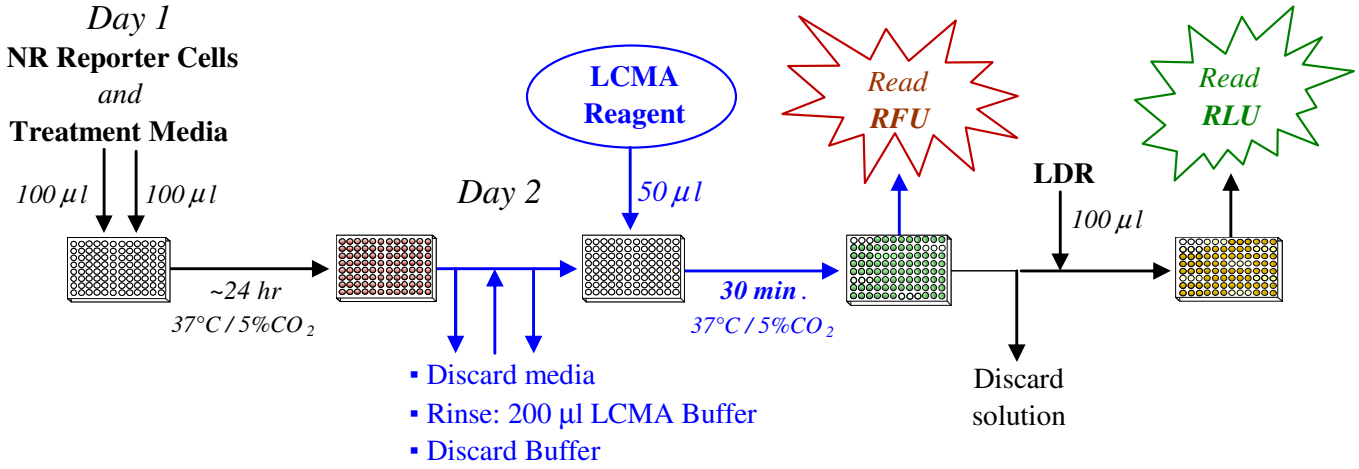


Figure 1. The fluorescence-based LCM Assay and the luminescence-based NR Assay assays are performed sequentially using the same assay wells. **Blue text** denotes the LCM Assay portion of the multiplex protocol, as described in this Technical Manual. **Black text** denotes the standard protocol used for each of INDIGO's 96-well format Nuclear Receptor Reporter Assays. (NOTE: The LCM Assay protocol is *not* compatible with INDIGO's 384-well format Nuclear Receptor Assays, which utilize a homogenous assay chemistry.)

The LCM Assay Chemistry

The LCM Assay utilizes the fluorogenic substrate Calcein-AM (AcetoxyMethyl ester of Calcein) to provide a sensitive, quantitative measure of the relative number of live Nuclear Receptor Reporter Cells remaining in assay wells following their exposure to test compounds.

Calcein-AM is a hydrophobic, non-fluorescent molecule that readily crosses cellular membranes. Once in the intracellular environment, Calcein-AM is hydrolyzed by endogenous esterases in a time- and temperature-dependent manner to generate the hydrophilic, highly fluorescent molecule Calcein (**Figure 2**). Due to the high charge density on the resulting reaction product, there is no appreciable loss of calcein from the intracellular compartment during the brief reaction period of the LCM Assay.

The wavelengths of maximal excitation and emission for calcein are **492 nm** and **513 nm**, respectively. The filter combination of [485nm$_{Ex}$ | 535nm$_{Em}$] (commonly used to quantify fluorophores such as EGFP, Fluorescein, and Rhodamine-110) may be used effectively to quantify calcein fluorescence.

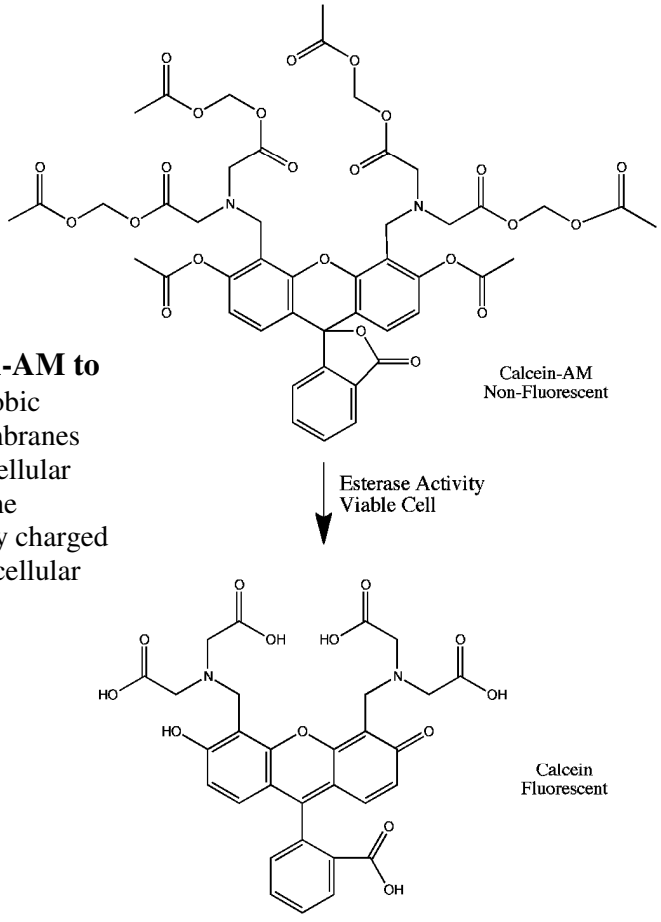


Figure 2. Conversion of Calcein-AM to Calcein. Non-fluorescent, hydrophobic Calcein-AM readily crosses cell membranes where it encounters ubiquitous intracellular esterases. Cellular esterases covert the molecule to highly fluorescent, highly charged Calcein which is retained in the intracellular space.

LCM Assay Controls

Two LCM Assay CONTROLS must be included in the plate setup:

1.) 100% Live Cells Reference. The "100% Live Cells" Reference wells for the LCM Assay will always be the same as those used as the "Untreated Control" wells in the Nuclear Receptor Assay. For example:

- When screening for NR *agonist* activities: wells containing untreated (or "vehicle" treated) NR Reporter Cells provide the Negative Control for the NR agonist assay *and* the 100% Live Cell Reference for the LCM Assay. APPENDIX 1 provides representative data.
- When screening for NR *antagonist* activities: wells containing [NR Reporter Cells + ~EC_{80} reference agonist] provide the Negative Control for the NR antagonist assay *and* the 100% Live Cell Reference for the LCM Assay. APPENDIX 2 provides representative data.

and,

2.) RFU Background Control. Values of RFU background are quantified from wells containing 200 µl of CSM media *only*. These wells are processed in identical manner to all other Control and Experimental wells. RFU Background is quantified, then subtracted from all Reference and Experimental RFU values before computing "% RFU".

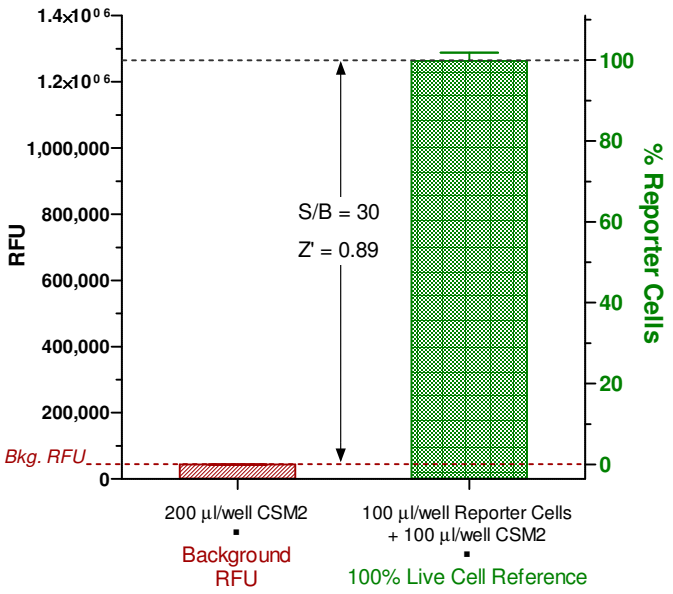


Figure 3a. Signal of the "100% Live Cell" and "Background" Controls.
The LCM Assay produces high fluorescent signal in the 100% Live Cells Reference wells, and produces minimal standard deviation between replicates, typically ≤ 5%. Despite low background fluorescence from the "0% Cells" wells, plates should always include this control. Thus, background RFU values may be determined, then subtracted from all other Reference and Experimental RFU values. A GloMax-Multi+ (Promega) plate reader fitted with the instrument's "blue" filter module [490nm_{Ex} | 510-570nm_{Em}] was used to quantify RFU.

Optional "Cytotox" Control

If desired, Staurosporine may be used as a control treatment to produce a positive cytotoxic response. For most cell types 8 µM staurosporine treatment causes near-complete cell death within the approximate 24 hr assay period.

A **4.0 mM (500x)** stock of **Staurosporine** is provided with this assay kit.

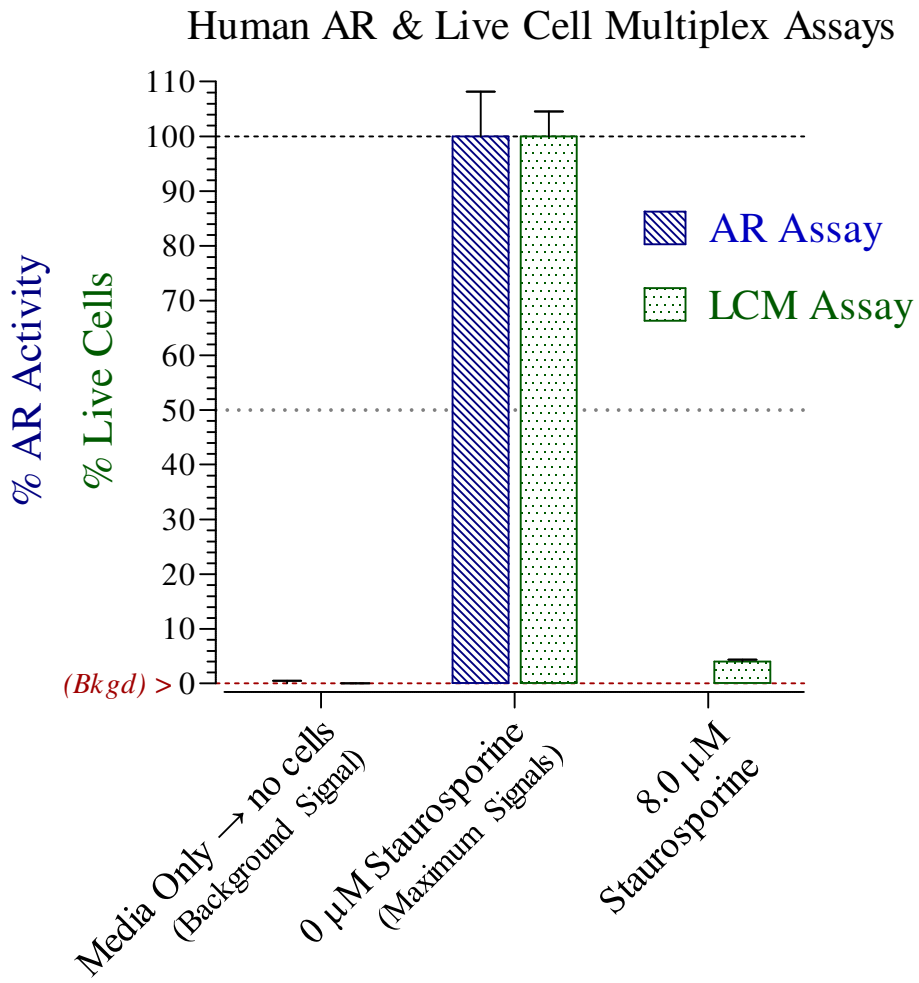


Figure 3b. Human AR and LCM Assays, including Staurosporine treatment as a 'Positive Control' for Reporter Cell death.

Human Androgen Receptor Reporter Cells were plated in Compound Screening Medium (CSM) supplemented with the agonist 6α-Fl-Testosterone (1 nM) and either 0 or 8.0 µM staurosporine. Assay wells containing media only (no Reporter Cells) provide background RFU values for the LCM Assay. 100% AR activity and 100% Live Cells derive from the "1 nM 6α-Fl-testosterone + 0 Staurosporine" treatments.

Results: 8.0 µM staurosporine is cytotoxic to Reporter Cells, resulting in ≤ 15% live Reporter Cells after the 24 hour assay period.

Data Analyses

The intensity of fluorescent signal generated in the LCM Assay is directly proportional to the number of live cells in the assay well (**Figure 4**). Therefore, the magnitude of change in fluorescence signal between the 100% Live Cells Reference wells and the wells treated with test compound(s) provides an accurate measure of the relative change, if any, in numbers of live cells per treatment set.

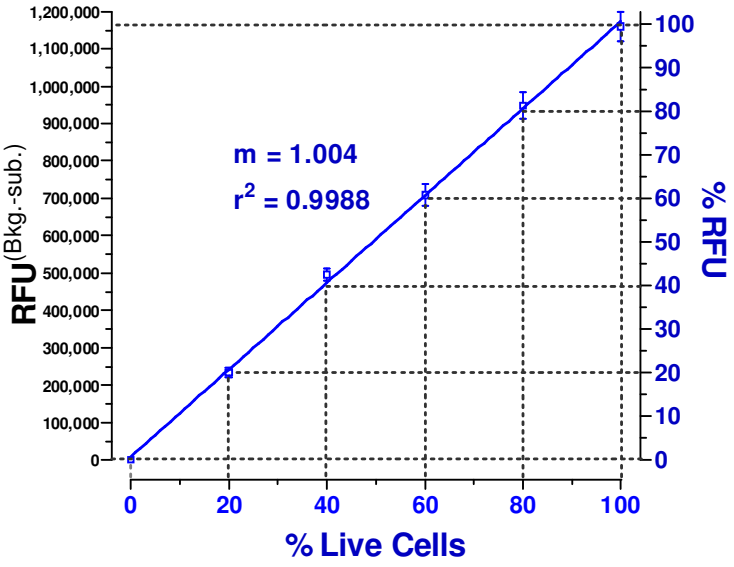


Figure 4. % RFU = % Live Cells. The LCM Assay provides a direct correlation between % RFU and % Live Cells in an assay well. To demonstrate this, a suspension of Nuclear Receptor Reporter Cells were plated at 100, 80, 60, 40, 20 and 0 µl per well. Wells containing 100 µl of cells possess the full complement of reporter cells present in INDIGO's standard NR Assay. Because these cells are untreated, they provide both the "Negative Control" for the NR Assay and the "100% Live Cells" Reference for the LCM Assay. Cells were cultured for 23 hr and the LCM Assay was performed. Average RFU values were background-subtracted, then normalized such that the wells containing the full complement of untreated cells = "100% RFU = 100% Live Cells".

The LCM Assay provides a direct correlation between % RFU and % Live Cells, and it is *not* necessary to generate a standard plot, such as depicted in Figure 4. Users may be confident in determining relative changes in Reporter Cells by simply calculating the % $RFU^{Bkg.-sub.}$ of experimental assay wells relative to the $RFU^{Bkg.-sub.}$ from the NR Assay Negative Control wells (=100% Live Cells).

Healthy NR Reporter Cells produce average RFU values with relatively low Coefficients of Variation (CV), typically ≤ 5%. Nonetheless, caution is advised against over-interpreting small differences in RFU values between the 100% Live Cell Reference and test compound treated cells. In general, a 5-12% variation between Experimental and Reference RFU values will lack statistical significance. Greater than 12% variation *may* be significant. Analyses of Variance (ANOVA) should be performed to properly assess statistical significance when only moderate differences are observed between the sets of Reference and Experimental data.

II. Product Components & Storage Conditions

LCMA Buffer is used for two distinct purposes:

i.) A portion of LCMA Buffer is combined with concentrated LCMA Substrate to generate a 1x working concentration of **LCMA Reagent**.

And,

ii.) a separate portion of LCMA Buffer is used to perform a pre-rinse of cultured cells prior to commencing the LCM Assay.

LCMA Substrate, 300x comprises a 300-fold concentration of Calcein-AM prepared in anhydrous DMSO and sealed under argon gas. LCMA Substrate may be thawed and refrozen up to three times without adverse effects. LCMA Substrate is diluted using LCMA Buffer to generate a 1x working concentration of *LCMA Reagent*.

Staurosporine, 4.0 mM (500x stock) provides users with the *option* of exposing cells to 8.0 µM staurosporine, thereby providing a "positive-control" cytotoxic response. Wear gloves and protective eyewear when handling staurosporine.

The volumes of reagents provided in a single LCM Assay kit, **#LCM-01**, are sufficient to perform 96 determinations of "% Live Cells" using any of INDIGO's standard 1x 96-, 3x 32- or 2x 48-well Nuclear Receptor assay plate configurations.

Kits **#LCM-05** and **#LCM-10** provide bulk reagent volumes sufficient to perform LCM Assays in five and ten, respectively, 96-well type assay plates.

Assay kits are shipped on dry ice. Upon receipt, kit reagents may be transferred to -20°C storage.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

Live Cell Multiplex Assay Kit Formats

# LCM-01	***Amount***	***Storage Temp.***
▪ LCMA Buffer	1 x 30 mL	-20°C
▪ LCMA Substrate (300x)	1 x 24 µL	-20°C
▪ Staurosporine, 4.0 mM (500x)	1 x 10 µL	-20°C
# LCM-05	***Amount***	***Storage Temp.***
▪ LCMA Buffer	1 x 135 mL	-20°C
▪ LCMA Substrate (300x)	5 x 24 µL	-20°C
▪ Staurosporine, 4.0 mM (500x)	1 x 10 µL	-20°C
# LCM-10	***Amount***	***Storage Temp.***
▪ LCMA Buffer	2 x 135 mL	-20°C
▪ LCMA Substrate (300x)	10 x 24 µL	-20°C
▪ Staurosporine, 4.0 mM (500x)	3 x 10 µL	-20°C

III. Materials to be Supplied by the User

The following materials must be available for use in completing the Live Cell Multiplex (LCM) Assay protocol:

- mammalian cell culture incubator calibrated to 37°C, 5% CO_2 and ~85% humidity.
- Disposable media basin, sterile.
- 8- *or* 12-channel pipette & sterile tips appropriate for the transfer of 100 µl, 200 µl, and 50 µl volumes (*Steps 1, 5 & 7,* respectively). The use of electronic pipettes capable of repeat-dispensing are recommended.
- Plate-reading Fluorometer with appropriate filters to quantify Relative Fluorescence Units (RFU) from the LCM Assay (*Step 10).* The wavelengths of maximal excitation and emission for calcein are **492 nm** and **513 nm**, respectively.

  NOTE: The commonly used filter combination of [$485nm_{Ex}$ | $535nm_{Em}$] used to quantify EGFP, Fluorescein, and Rhodamine-110 may be used effectively to quantify Calcein fluorescence.
- Plate-reading Luminometer to quantify Relative Luminescence Units (RLU) from the Nuclear Receptor Assay (*Step 13).*

IV. Assay Procedure

The LCM Assay Protocol is specifically optimized to be performed in combination with INDIGO's Nuclear Receptor Reporter Assay Systems. Please review the entire assay protocol before starting, including the Protocol NOTES, below.

Completing the multiplex LCM and NR Assays requires an overnight incubation. *Steps 1 and 2* are performed on **Day 1**, requiring 1-2 hours. *Steps 3-13* are performed on **Day 2**, requiring ≤ 1 hour to complete.

A detailed description of all steps specific to the desired Nuclear Receptor Assay is found in the Technical Manual accompanying that kit product.

Important Protocol NOTES

NOTE: Once in aqueous solution, Calcein AM undergoes a slow rate of hydrolysis that generates fluorescent calcein. Therefore, LCMA Reagent should be prepared immediately prior to its use, and only in the volume required for the intended number of assay wells. Any extra volume of the prepared LCMA Reagent can NOT be stored for later use, and should be discarded after assay setup.

NOTE: This protocol incorporates media-discard and cell-rinse steps (*Steps 4-6*) immediately prior to adding LCMA Reagent to the assay wells. This cell-rinse step is necessary because the 23 hr treatment media contain serum, a source of esterases that, if not removed, will contribute background fluorescence to the LCM Assay. Also, the treatment media may contain variable levels of esterases originating from cells killed or permeablized *via* cytotoxic effects of the user's test compounds. Extra-cellular esterase and floating dead cell debris that would otherwise generate high fluorescence background are effectively removed through a single cell-rinse prior to the LCMA assay. Use only LCMA Buffer to rinse sample wells. Do *not* use PBS or any other balanced salts or media solutions as a substitute for LCMA Buffer, as these will degrade the performance of the multiplex assays.

NOTE: The removal of [culture medium + test cmpd] from the reporter cells and brief substitution with LCMA Reagent (*Steps 7-8*) will *not* alter the performance metrics (*i.e.*, S/B, EC_{50}, Hill slope, *etc*.) of the subsequent Nuclear Receptor Assay.

NOTE: The Nuclear Receptor portion of the following multiplex protocol describes a representative ***agonist*** assay setup. When screening test compounds for ***antagonist*** activity, the specifics of the denoted (*) protocol steps will be modified, as follows:

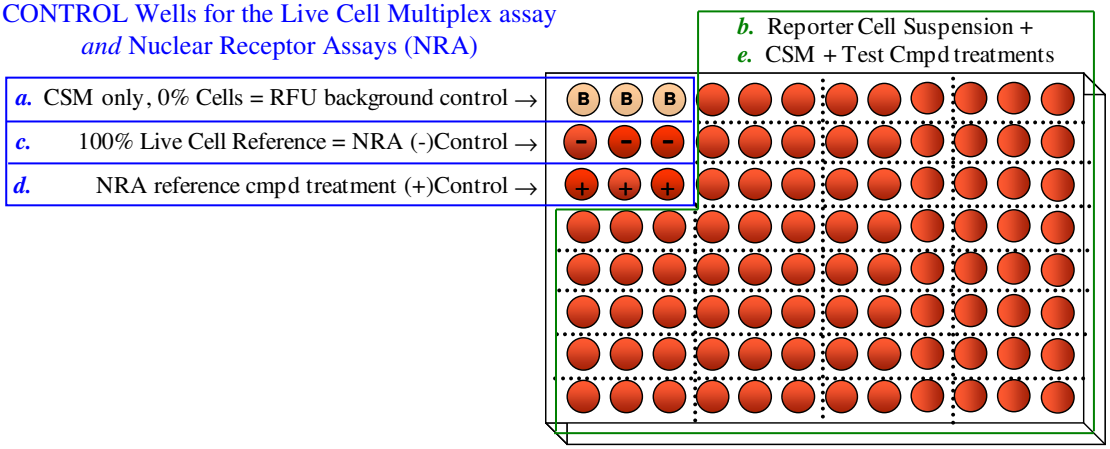
Step 1 protocol considerations when performing Antagonist Assays

c. **Negative Control for NR antagonist assay, AND "100% Live Cell" Reference for LCM Assay** (⊖): Into one set of wells containing cells, dispense previously prepared [CSM + EC80 reference agonist] (*i.e.*, *no* test cmpd).

d. **Positive Control treatment for NR antagonist Assay** (⊕): Into another set of wells containing cells, dispense previously prepared [CSM + EC_{80} Agonist + reference antagonist]; the dispensed volume will be dependent on the specific NR assay protocol.

e. **Experimental wells for LCM and NR antagonist assays:** Into all other sets of wells containing cells, dispense the previously prepared [CSM + EC_{80} Agonist + Test Cmpd]; the dispensed volume will be dependent on the specific NR assay protocol.

DAY 1 -- *Aseptic Technique Required*



Step 1. Prepare "Control" and "Experimental" assay wells for the LCM and NR Assays: * (see *NOTES, pg.9*)

☐ ***a.*** **LCMA fluorescence background Control wells:**
Into one set of replicate wells, dispense 200 µl / well of CSM *only* (Ⓑ *i.e.*, *no* cells).

☐ ***b.*** Dispense Reporter Cell suspension for nuclear receptor assay setup (●).

☐ ***c.** **Untreated Control for NR Assay (NRA) & 100% Live Cell Reference for LCM Assay** (⊖):
Into one set of wells containing cells, dispense CSM or "vehicle" supplemented CSM (*i.e.*, *no* test cmpd; volume dependent on the specific NR assay protocol).

☐ ***d.** **Positive Control treatment for NR Assay (NRA;⊕)**:
Into another set of wells containing cells, dispense previously prepared [CSM + reference cmpd]; volume is dependent on the specific NR assay protocol.

☐ ***e.** **Experimental wells for the NR and LCM Assays:**
Into all other sets of wells containing cells, dispense previously prepared [CSM + Test Cmpds]; refer to the specific NR assay protocol.

OPTIONAL: include wells with **8.0 µM Staurosporine** treatments to provoke a positive cytotoxic response.

☐ ***Step 2.*** Incubate for 22-24 hours; 37°C / 5% CO_2 / ~85% humidity.

DAY 2

DAY 2 -- *Aseptic Technique NOT Required*

☐ ***Step 3.*** Prepare the appropriate volume of **LCMA Reagent**.

# LCM Assay Wells	*300x LCMA Substrate*	+	*LCMA Buffer*	→	*LCMA Reagent*
32-wells	6.7 µl	+	2 ml	→	~ 2 ml
48-wells	10 µl	+	3 ml	→	~ 3 ml
96-wells	20 µl	+	6 ml	→	~ 6 ml

Hold LCMA Rgt. in a low-light environment for later use in *Step 7*.

☐ ***Step 4.*** After 22-24 hr incubation, discard treatment media from the assay plate.

☐ ***Step 5.*** Rinse wells with 200 µl **LCMA Buffer**.

☐ ***Step 6.*** Discard LCMA Buffer.

☐ ***Step 7.*** Dispense 50 µl / well **LCMA Reagent**. Manually rock the plate side-to-side 2-3x.

☐ ***Step 8.*** Incubate **30 minutes** at 37°C.
⇒ 37°C/humidified CO_2 incubator

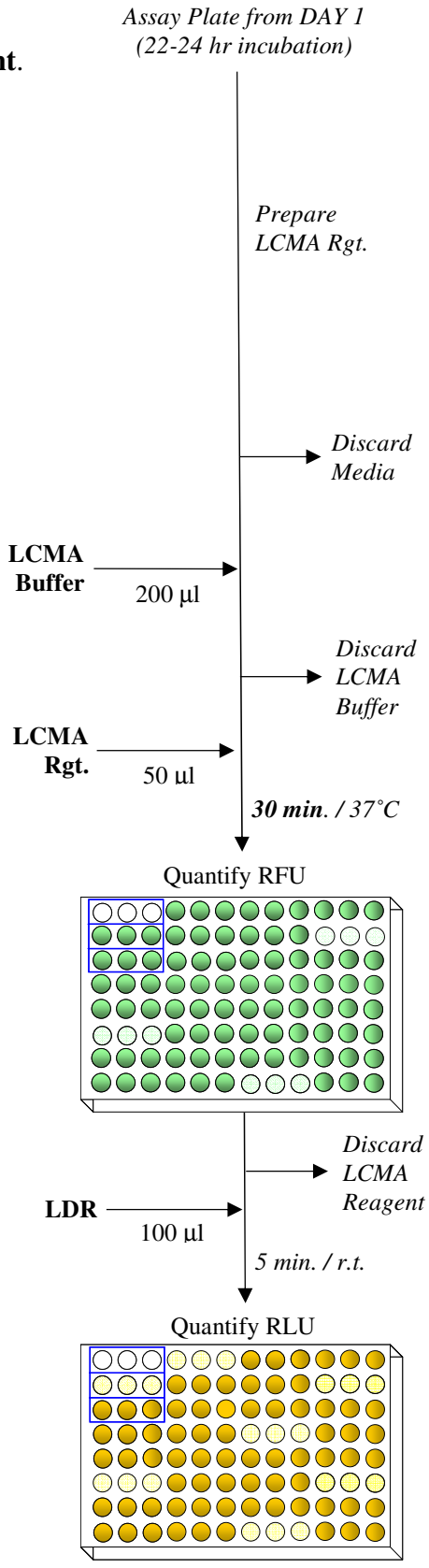
☐ ***Step 9.*** Near the end of the incubation period:
- turn on fluorometer / luminometer
- prepare **Luciferase Detection Reagent** (LDR)

☐ ***Step 10.*** At the 30 minute time point quantify fluorescence (RFU).
(Ex_{MAX} = 492 nm | Em_{MAX} = 513 nm)

☐ ***Step 11.*** Discard LCMA Reagent (*no rinse step*).

☐ ***Step 12.*** Dispense 100 µl / well **LDR** ⇒ 5 min. rest.

☐ ***Step 13.*** Quantify Luminescence.



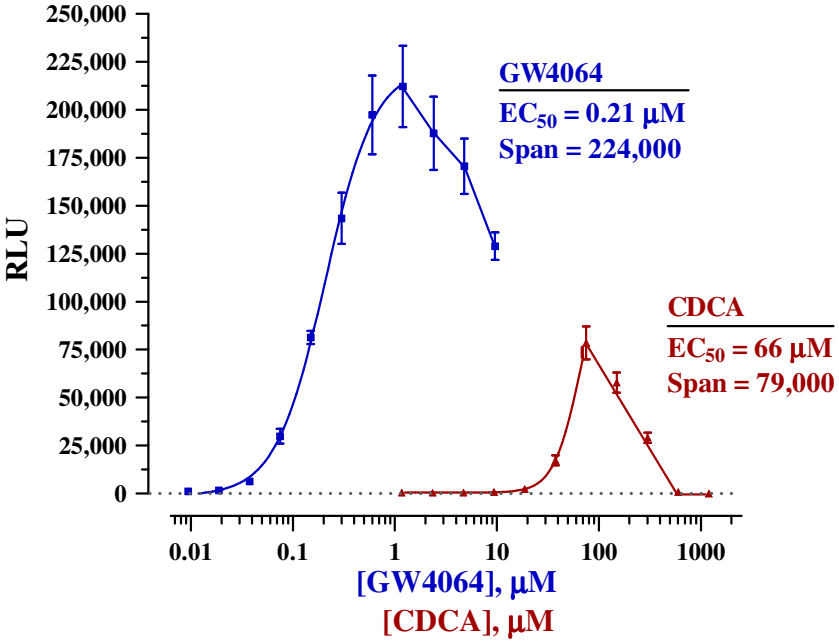
VI. Related Products

LCM Assay Products ▪ For combined use with any of INDIGO's 96-well format Nuclear Receptor Reporter Assay Systems	
Product No.	***Product Descriptions***
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in 1x96-well, *or* 2x48-well, *or* 3x32-well NR assay plates
LCM-05	Reagents in 5x bulk volumes for **480** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well NR Assay Plates
LCM-10	Reagents in 10x bulk volumes for **960** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well NR Assay Plates

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

1a. **Human FXR Assays:** GW4064 & CDCA
Dose-Responses



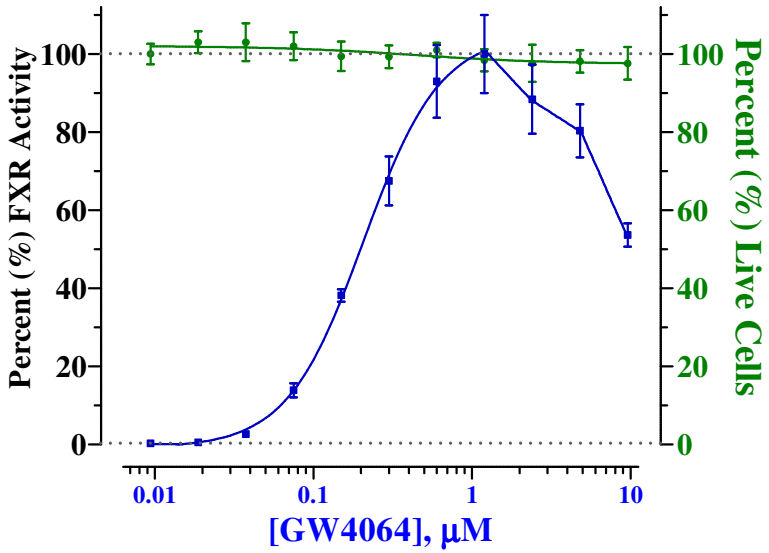
APPENDIX 1. Use of the LCM Assay to interpret FXR agonist dose-response data.

(***1a.***) FXR Reporter Cells were dosed with GW4064 or CDCA and conventional FXR Assays were performed. Both reference compounds display upper threshold concentrations, above which FXR activity plummets. Are the declines in FXR activity due to GW4064- and CDCA-induced cytotoxicity? To answer this question, the LCM Assay was performed in multiplex with the FXR Assay.

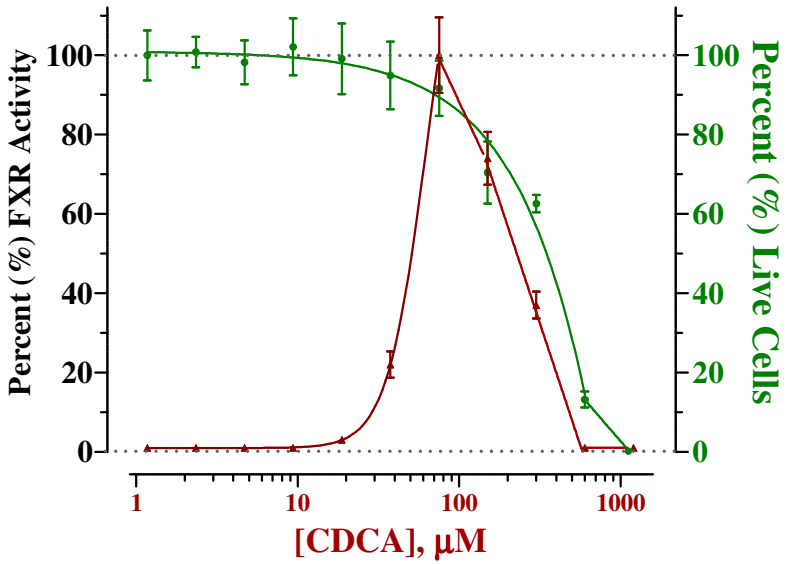
(***1b.***) The LCM Assay demonstrates that the percent Reporter Cells (—●—) are unchanged for each treatment concentration of GW4064. Hence, the observed decline in FXR activity is *not* due to cytotoxicity.

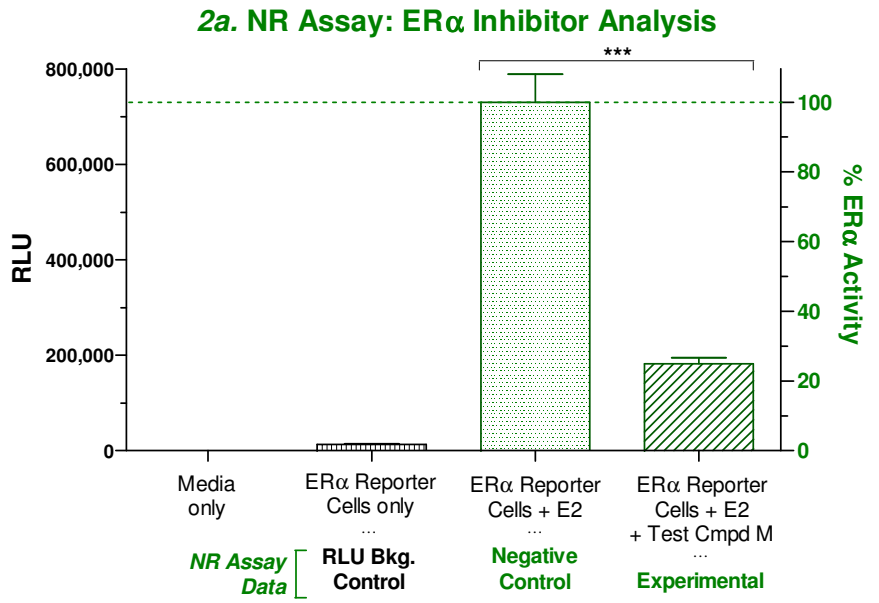
(***1c.***) Conversely, the LCM Assay reveals that CDCA exerts a profound dose-dependent cytotoxic effect on the Reporter Cells. Complete cell death is evident by 23 hr when CDCA exceeds 600 µM. Hence, the observed drop in FXR signal results from CDCA-induced cytotoxicity beginning at ~75 µM. RFU and RLU measurements were performed as described in APPENDIX 2.

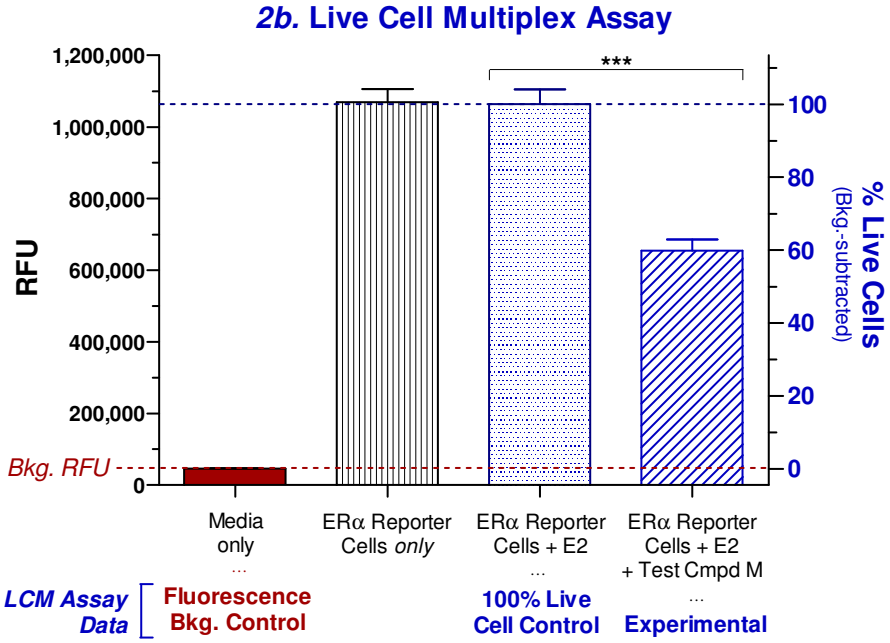
1b. FXR & LCM Assays: **GW4064** Dose-Response
(Normalized)



1c. FXR & LCM Assays: **CDCA** Dose-Response
(Normalized)







APPENDIX 2: Use of the LCM Assay to interpret NR *antagonist* screening data. Quantifying the relative numbers of live reporter cells in treated samples may reveal false-positive data.

***2a.* Nuclear Receptor antagonist assay data.** ERα Reporter Cells treated with E2[1]+"cmpd M" show significantly diminished RLU values relative to Control Cells. The question remains: Is test compound "M" an inhibitor of ERα activity, or is the observed drop in ERα activity due to non-specific events?

***2b.* LCM Assay data.** The LCM Assay reveals a significant reduction in the relative number of Reporter Cells in wells treated with "M". Thus, the apparent inhibition of ERα by "M" is, in fact, the result of induced cell toxicity.

In this example the cytostatic agent Mitomycin C was used as a mock Test Compound ("M"). Hence, the finding of ~ 60% relative numbers of cells in the "M" treated wells is attributed to non-proliferation of the reporter cells over the 23 hr treatment period; it is not the result of cell death, *per se*. However, over this period of arrested division the cells are in metabolic crisis as they commit to apoptosis. This explains why, in the "M" treated assay wells, the percent loss of ERα activity exceeds the percent reduction in the number of live cells. In essence, the "M" treated reporter cells are alive, but they are division arrested and in metabolic crisis.

Methods: 100 µl/well of ERα Reporter cells were dispensed into the 96-well plate and further supplemented with 100 µl/well of either CSM *only* (RFU Bkg. Control), CSM+1.0 nM E2 (Negative Control for ER antagonist assay & 100% Live Cell Reference for the LCM assay), or CSM+1.0 nM E2+200 µM Test Cmpd M (mock experimental). Cells were incubated for 23 hours then processed to quantify % Live Cells and ERα activity. A GloMax-Multi+ (Promega) was used in fluorescence mode using the instrument's "blue" filter module ($490nm_{Ex}$ | 510-$570nm_{Em}$) to quantify RFU of the LCM Assay. The instrument was then switched to luminescence mode to quantify RLU of the ERα Assay. ANOVA confirmed statistical significance (***, $p << 0.05$) of the data.

[1]E2: 17-β-estradiol, a potent agonist of estrogen receptors.