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 1.0)

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Live Cell Multiplex (LCM) Assay

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I. Description

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

The Live Cell Multiplex (LCM) Assay provides an efficient fluorescence-based method of quantifying the relative number of live cells resident in treated wells of an assay plate. While the LCM Assay may be performed as a stand-alone assay, it is has been specifically optimized to be run in multiplex with any of INDIGO's 96-well, 2x48-well, or 3x32-well Nuclear Receptor Reporter Assay System products.

The LCM assay allows user's to validate their primary Nuclear Receptor Assay data by determining if their test compound treatments exert mitogenic, cytostatic or cytotoxic activities on the reporter cells. The occurrence of such adverse non-specific 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 test compounds for *antagonist* activities it is particularly important to 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 an antagonist screen. In such cases, the observed drop in luciferase activity will be incorrectly attributed to inhibition of the nuclear receptor by the test compound. In reality, however, the treatment compound has pushed the reporter cells into division arrest, apoptosis, necrosis, or lysis. APPENDIX 2 presents an example of the deleterious effect of division arrest on interpreting 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.

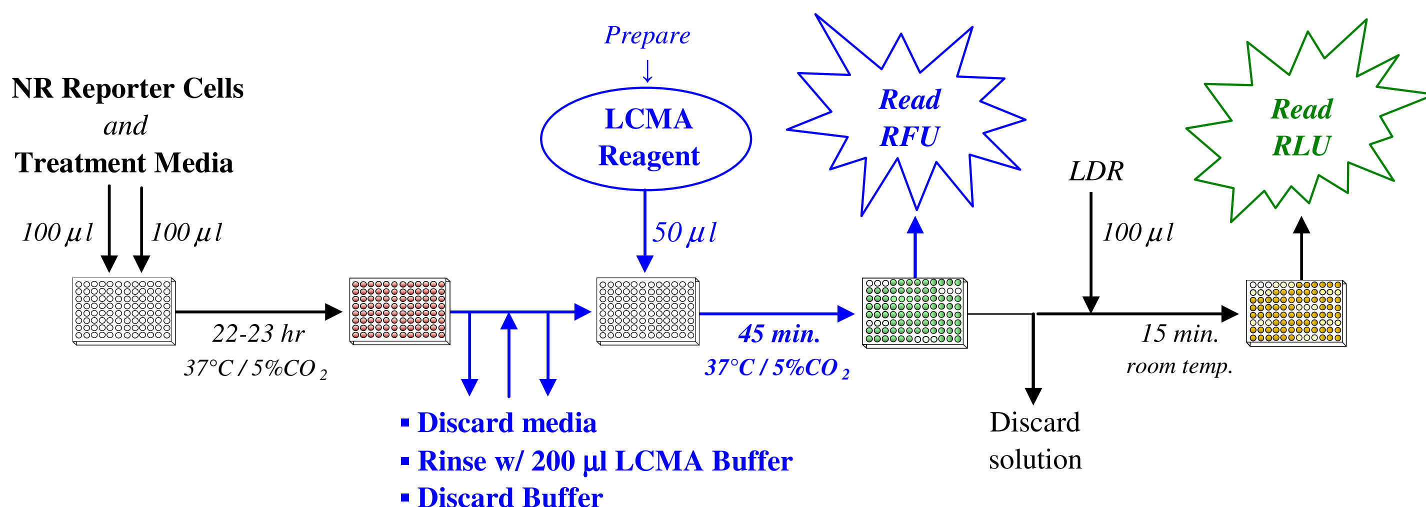

Figure 1. The fluorescence-based LCM Assay and the luminescence-based NR Assay assays are performed sequentially using the same assay wells. Blue text denotes the LCM Assay portion of the multiplex protocol, as described in this Technical Manual. Text in black denotes the standard protocol used for each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

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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 the cell membrane. 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$_{Em}$], which is commonly used to quantify fluorophores such as EGFP, Fluorescein, and Rhodamine-110, may be used to quantify Calcein fluorescence.

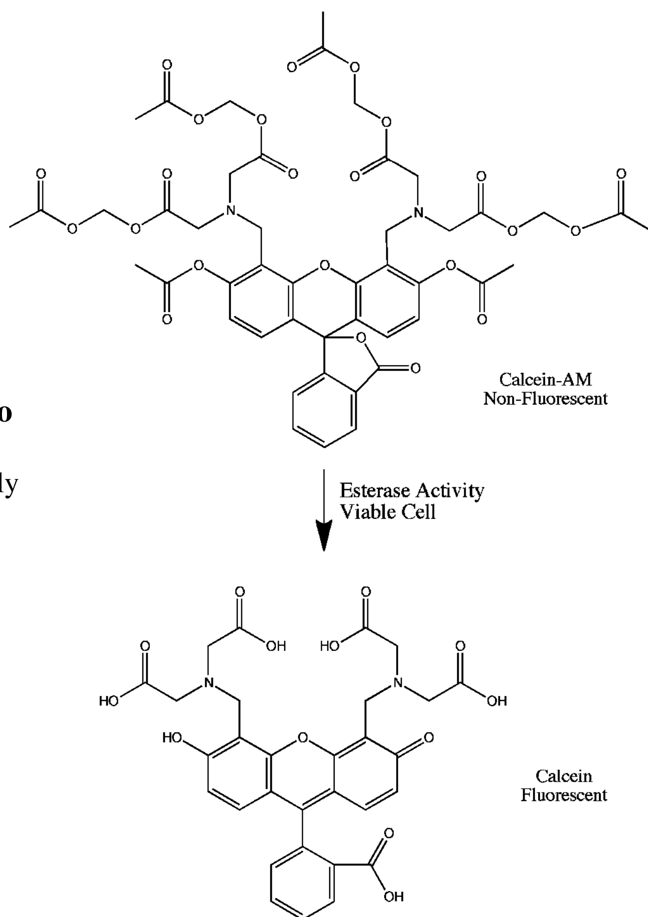

Figure 2. Conversion of Calcein-AM to Calcein. Ubiquitous intracellular esterases convert non-fluorescent Calcein-AM to highly fluorescent Calcein.

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LCM Assay Controls

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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 "Negative Control" wells in the Nuclear Receptor Assay.

▪ When screening for NR *agonist* activities: wells containing [NR Reporter Cells *only**] provide the Negative Control for the NR 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 + control agonist*] provide the Negative Control for the NR Assay *and* the 100% Live Cell Reference for the LCM Assay. APPENDIX 2 provides representative data.

* NOTE 1: (*Optional)* Users sometimes choose to add to the NR Assay Negative Control a concentration of "vehicle" (*e.g.,* DMSO) equal to the highest concentration present in the experimental wells containing test compound.

2.) RFU Background Control. Values of RFU background are quantified from wells containing 200 µl of CSM2 media *only*. These wells are processed in identical manner to all other Control and Experimental wells. RFU Background is quantified, then subtracted from Reference and Experimental RFU values before computing "% RFU".

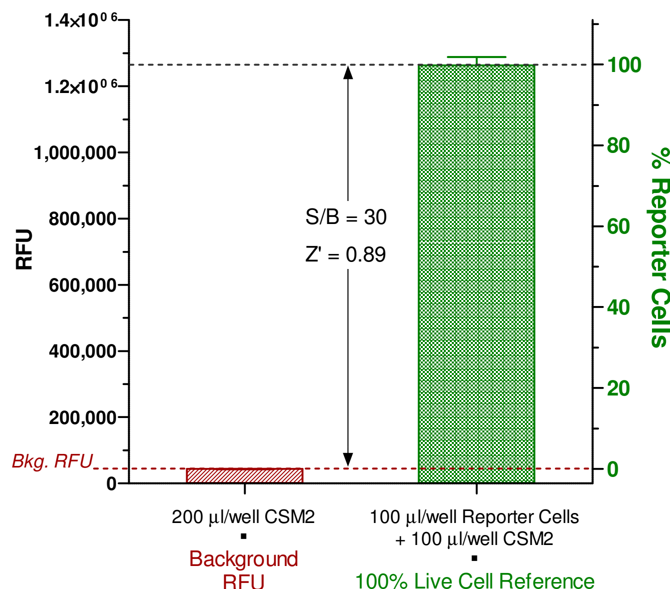

Figure 3. Signal of the "100% Live Cell" Reference and Background.
The LCM Assay produces high fluorescent signal in the 100% Live Cells Reference wells, and produces minimal standard deviation between replicates, typically ≤ 5%. Despite low background fluorescence from the "0% Cells" wells, plates should 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) fitted with the instrument's "blue" filter module [$490nm_{Ex}$ | $510\text{-}570nm_{Em}$] was used to quantify RFU.

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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.

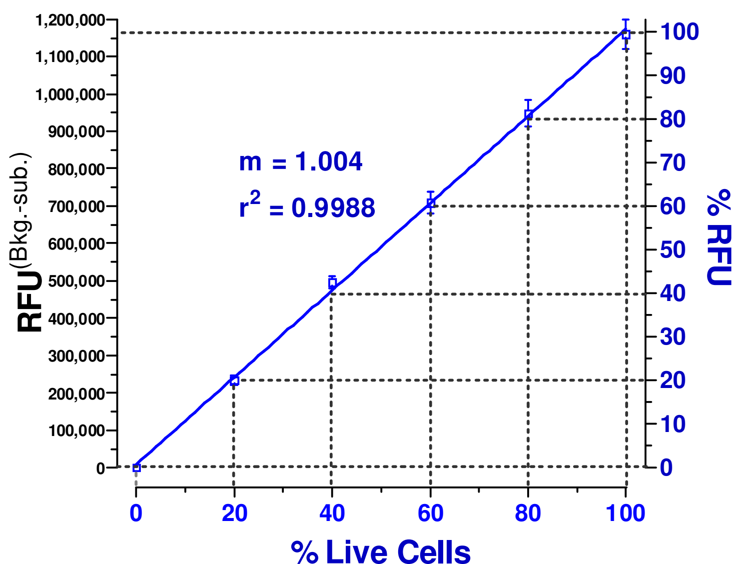

Figure 4. % RFU = % Live Cells. The LCM Assay provides a direct correlation between % RFU and % Live Cells in an assay well. To demonstrate this, a suspension of Nuclear Receptor Reporter Cells were plated at 100, 80, 60, 40, 20 and 0 µl per well. APPENDIX 3 depicts this plate set-up. Wells containing 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. Fluorescence signal was quantified as described in Figure 3. Average RFU values were background-subtracted, then normalized such that the wells containing 100 µl Reporter Cells (*i.e.,* "100% Live Cells") = "100% RFU".

The LCM Assay provides a strict correlation between % RFU and % Live Cells, and it is *not* necessary to generate a standard plot, such as depicted in Figure 4, for each experiment. Users may be confident in determining relative changes in Reporter Cells by simply calculating the % $RFU^{Bkg.\text{-}sub.}$ of experimental assay wells relative to the $RFU^{Bkg.\text{-}sub.}$ from the NR Assay Negative Control wells (=100% Live Cells). Example calculations are provided in APPENDIX 3.

NOTE 2: (*Optional*) Those users wishing to validate the performance of the LCM Assay may do so by performing a cell titration experiment, as depicted in Figure 4. APPENDIX 3 provides a guideline for generating an appropriate cell titration, handling the resulting raw RFU data, and plotting "% Live Cells *vs.* % RFU" data.

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% variation between Experimental and Reference RFU values will lack statistical significance. A 10% 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.

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II. Product Components & Storage Conditions

This LCM Assay kit contains two reagents: **LCMA Buffer** and **LCMA Substrate, 300x**. 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.

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 assay wells prior to commencing the LCM Assay.

LCMA Substrate, 300x comprises a 300-fold concentrate 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*.

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 32 mL	-20°C
▪ LCMA Substrate, 300x	1 x 24 µL	-20°C

# LCM-05	***Amount***	***Storage Temp.***
▪ LCMA Buffer	1 x 135 mL	-20°C
▪ LCMA Substrate, 300x	1 x 110 µL	-20°C

# LCM-10	***Amount***	***Storage Temp.***
▪ LCMA Buffer	2 x 135 mL	-20°C
▪ LCMA Substrate, 300x	2 x 110 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:

- 37°C, humidified 5% CO_2 incubator for mammalian cell culture.
- 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 is recommended.
- Plate-reading Fluorometer with appropriate filters to quantify RFU values from the LCM Assay (*Step 10).* The wavelengths of maximal excitation and emission for calcein are **492 nm** and **513 nm**, respectively.
- Plate-reading Luminometer to quantify RLU values from the Nuclear Receptor (NR) Assay (*Step 13).*

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IV. Assay Procedure

The Live Cell Multiplex 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 important 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.

Protocol NOTES

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***NOTE 3**:* 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 4**:* 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 rich source of esterases that will contribute high background fluorescence to the LCM Assay if not removed. 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 cells that would otherwise contribute 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 5**:* The brief substitution of [culture medium+test cmpd] with LCMA Reagent (*Steps 7-8*) will *not* alter the performance characteristics (*i.e.*, S/B, EC_{50}, Hill slope, etc.) of the subsequent Nuclear Receptor Assay.

NOTE 6**:* 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.

- ***c.* Negative Control for NR Assay, AND "100% Live Cell" Reference for LCM Assay (−)**: Into one set of wells containing cells, dispense 100 µl of previously prepared [CSM2+"2x" Agonist] (*i.e.*, *no* test cmpd).
- ***d.* Positive Control treatment for NR Assay (+)**: Into another set of wells containing cells, dispense 100 µl of previously prepared [CSM2+"2x"Agonist+"2x" Control Antagonist].
- ***e.* Experimental wells for LCM and NR Assays:** Into all other sets of wells containing cells, dispense 100 µl of previously prepared [CSM2+"2x"Agonist+"2x" Test Cmpd].

DAY 1

Aseptic Technique Required

CONTROL Wells for the Live Cell Multiplex *and* Nuclear Receptor Assays

a. CSM2 only, 0% Cells = fluor. bkg. Control →

c. 100% Cell Reference = NRA (-)Control →

d. NRA (+)Control →

b. Reporter Cell Suspension +
e. CSM2 + "2x" Test Cmpds

Step 1. Prepare "Control" and "Experimental" assay wells for the LCM and NR Assays:

* (see *NOTE 6, pg.9*)

☐ ***a.* LCMA fluorescence background Control wells:**
Into one set of replicate wells, dispense 200 µl / well of CSM2 *only* (B, *i.e.*, *no* cells).

☐ ***b.*** Dispense 100 µl NR Reporter Cell suspension into all other wells of the assay plate (●).

☐ ***c. Negative Control for NR Assay & 100% Live Cell Reference for LCM Assay (–):**
Into one set of wells containing cells, dispense 100 µl CSM2 (*i.e.*, *no* test cmpd).

☐ ***d. Positive Control treatment for NR Assay (+):**
Into another set of wells containing cells, dispense 100 µl of previously prepared [CSM2+"2x" Control Agonist].

☐ ***e. Experimental wells for the NR and LCM Assays:**
Into all other sets of wells containing cells, dispense 100 µl of previously prepared [CSM2+"2x" Test Cmpd].

☐ ***Step 2.*** Incubate for 22-23 hours; 37°C / humidified 5% CO_2.

DAY 2

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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 22-23 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 45 minutes
⇒ 37°C/humidified CO_2 incubator

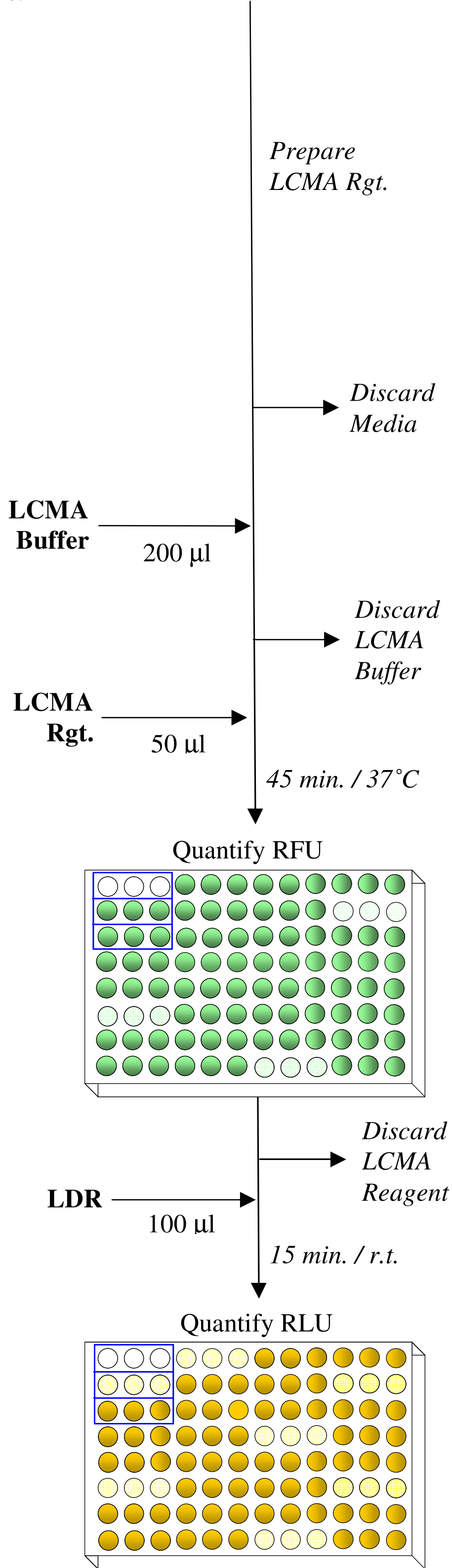
☐ ***Step 9.*** Near the end of the incubation period:
- turn on fluorometer / luminometer
- prepare **Luciferase Detection Reagent** (LDR)

☐ ***Step 10.*** At the 45 minute time point quantify fluorescence (RFU).
(Ex_{MAX} = 492 nm | Em_{MAX} = 513 nm)

☐ ***Step 11.*** Discard LCMA Reagent.

☐ ***Step 12.*** Dispense 100 µl / well **LDR** ⇒ 15 min. rest.

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



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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
Alternative volumes of LCM Assay kit reagents may be custom packaged to better accommodate Customer needs. Please Inquire.	

Please refer to INDIGO Biosciences website for updated product offerings.

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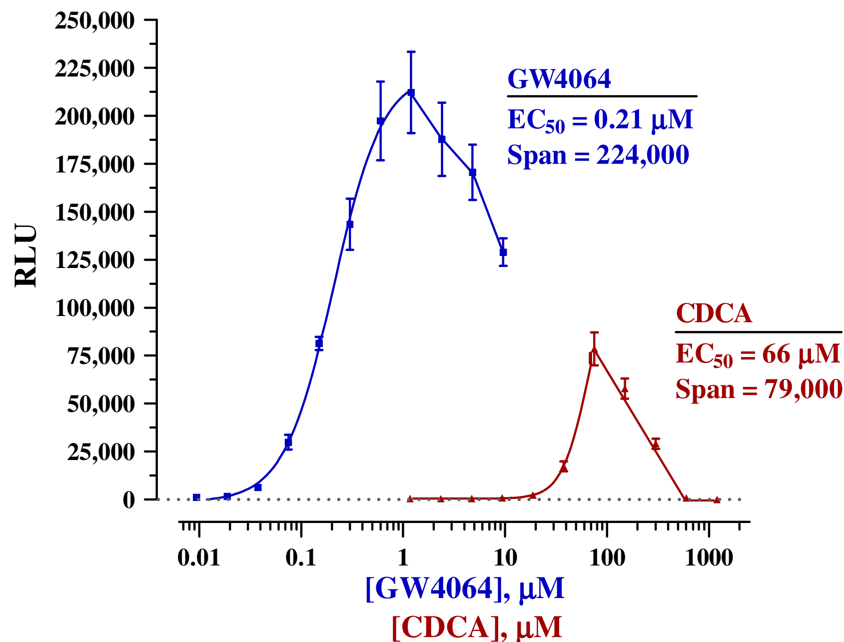
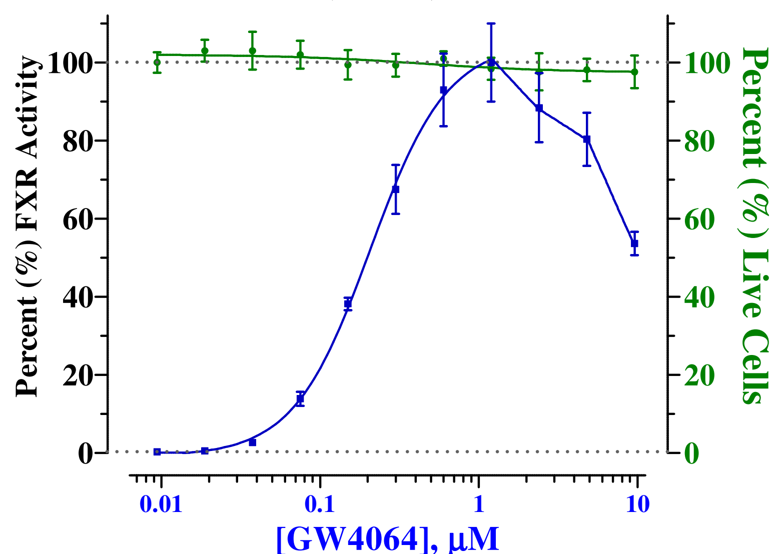
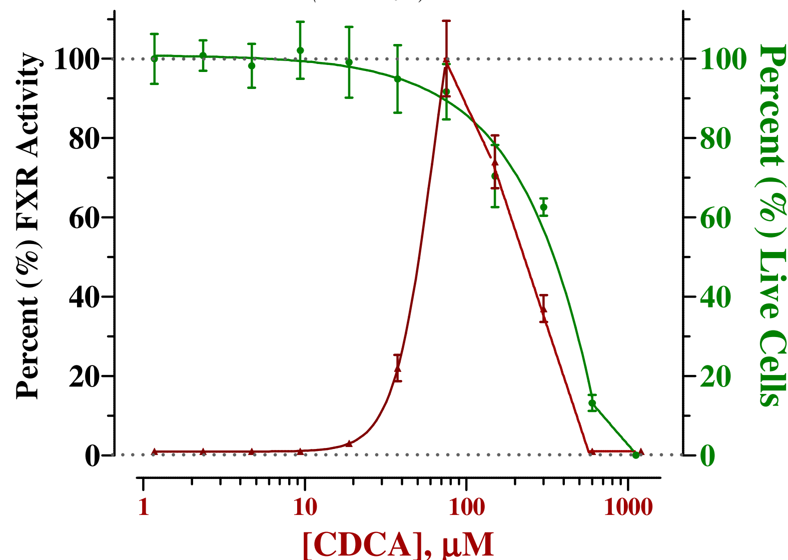
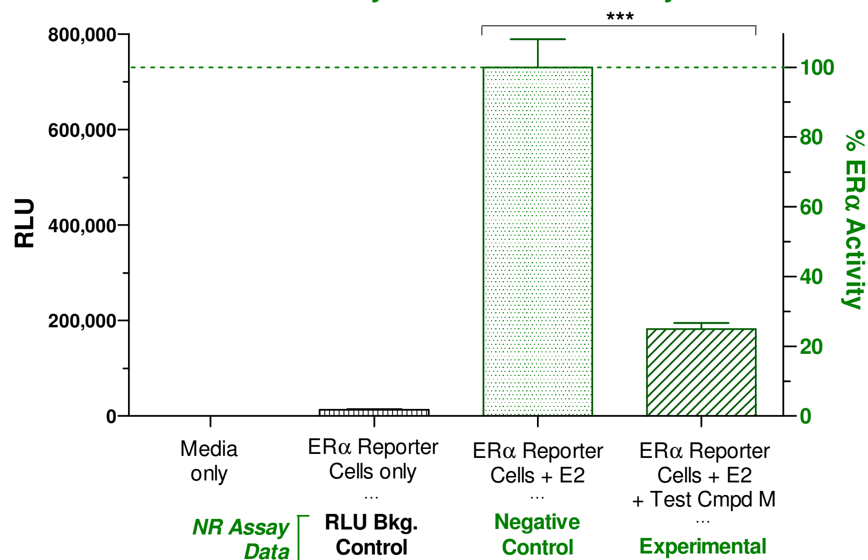
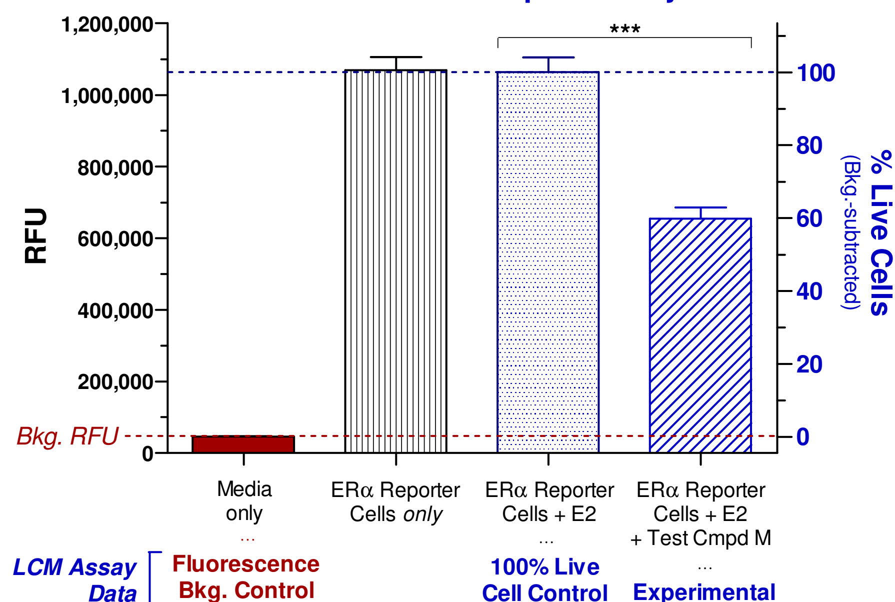


APPENDIX 1. **Use of the LCM Assay to interpret FXR agonist dose-response data.** (***1a.***) FXR Reporter Cells were dosed with GW4064 (; 0.00940-9.60 µM) or CDCA (; 1.17-1,200 µM) and FXR Assays were performed. GW4064 demonstrates significantly higher potency and efficacy relative to CDCA. Both reference 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 at 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. (The 1200 µM CDCA treatment contained ~ 0.24% DMSO which, when tested by itself, did not cause a drop in %RLU; data not shown). 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.









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]+"M" show significantly diminished RLU values relative to Control Cells. In a separate assay "M" was shown to have no significant impact on the activity of purified luciferase (data not shown), thereby ruling out the possibility that the reporter enzyme, itself, was the target of inhibition. The question remains: Is test compound "M" a specific inhibitor of ERα, or is the observed drop in ERα activity due to non-specific causes?

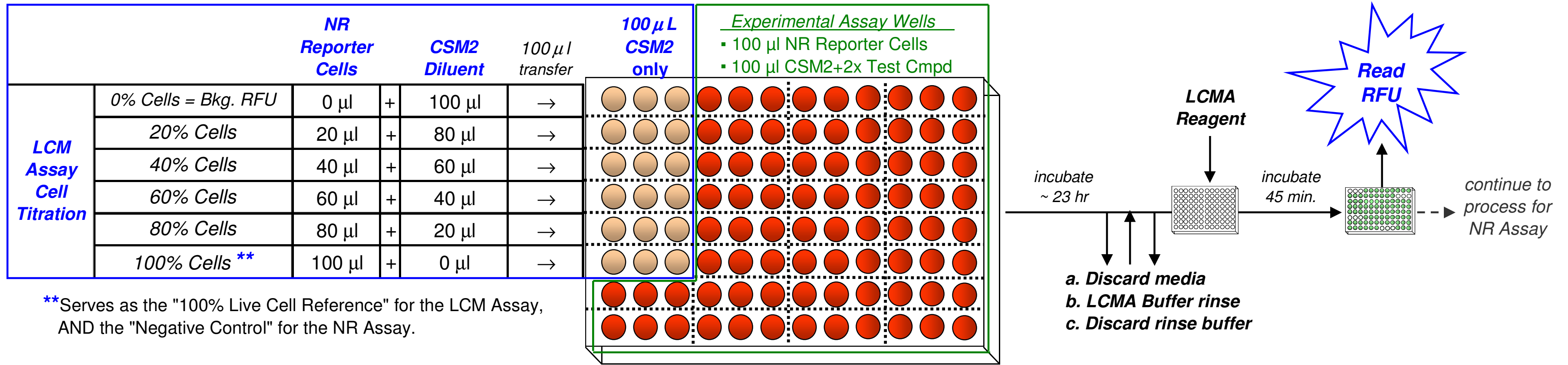
***2b.* LCM Assay data.** The LCM Assay reveals a significant reduction in the relative number of Reporter Cells in wells treated with "M". Thus, the apparent inhibition of ERα by "M" is, in fact, the result of induced cell toxicity.

In this example Mitomycin C, a potent cytostatic agent, 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 undergo metabolic shut-down 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 in metabolic crisis.

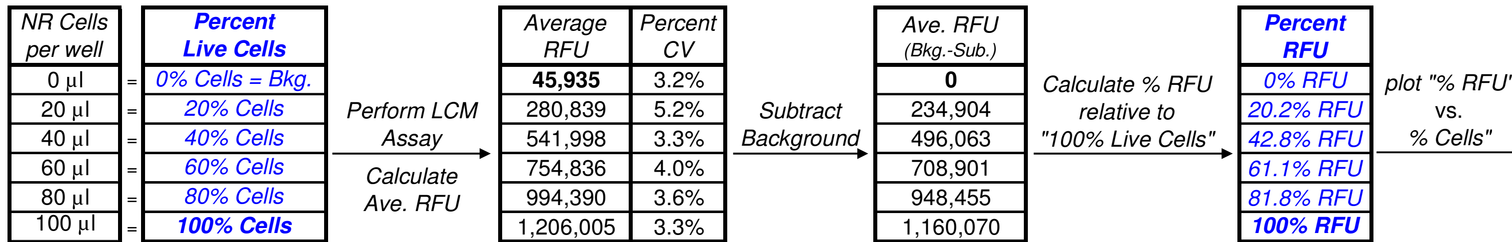
Methods: 100 µl/well of ERα Reporter cells were dispensed into the 96-well plate and further supplemented with 100 µl/well of either CSM2 *only* (RFU Bkg. Control), CSM2+1.0 nM E2 (Negative Control for ER antagonist assay & 100% Live Cell Reference for the LCM assay), or CSM2+1.0 nM E2+200 µM "M" (mock 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 ($490nm_{Ex}$ | $510\text{-}570nm_{Em}$) 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.

a. Prepare "% Cell" Titration for LCM Assay Validation



b. Background-Subtract and Normalize RFU Data



NR Cells per well		Percent Live Cells
0 µl	=	0% Cells = Bkg.
20 µl	=	20% Cells
40 µl	=	40% Cells
60 µl	=	60% Cells
80 µl	=	80% Cells
100 µl	=	100% Cells

Perform LCM Assay → Calculate Ave. RFU

Average RFU	Percent CV
45,935	3.2%
280,839	5.2%
541,998	3.3%
754,836	4.0%
994,390	3.6%
1,206,005	3.3%

Subtract Background →

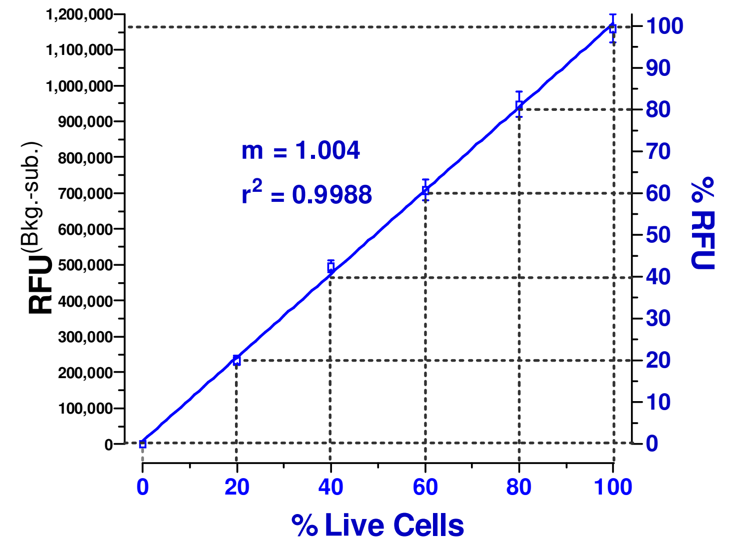
Ave. RFU (Bkg.-Sub.)
0
234,904
496,063
708,901
948,455
1,160,070

Calculate % RFU relative to "100% Live Cells" →

Percent RFU
0% RFU
20.2% RFU
42.8% RFU
61.1% RFU
81.8% RFU
100% RFU

plot "% RFU" vs. % Cells"

c. Generate the Standard Plot



APPENDIX 3: (*Optional*) Validating the LCM Assay.

The LCMA assay provides a direct correlation between % RFU and % Live Cells; it is *not* necessary to generate a standard plot for each experiment. However, if preferred, users may validate the LCM Assay by titrating NR Reporter Cells in an assay plate. (***a.***) Using CSM2 as the cell diluent, prepare cell suspensions with cell densities between 0 – 100% in 20% increments. Plate 100 µl of each cell density into wells that are pre-loaded with 100 µl of CSM2. Cell titration cultures are *not* treated further. Incubate 23 hr and perform the LCM Assay. (***b.***) Background-subtract the averaged RFU values. Percent-normalize the background-subtracted RFU values of all other sample sets to the "100% Live Cells" Reference value. (***c.***) Plot "% Live Cells" *vs.* "% RFU". A slope (m) that equals 1.0 demonstrates a perfect correlation between the calculated change in % RFU and the actual change in % Live Cells per sample wells. *Conclusion*: within standard experimental error, the calculated "% RFU" is the equivalent of "% Live Cells".