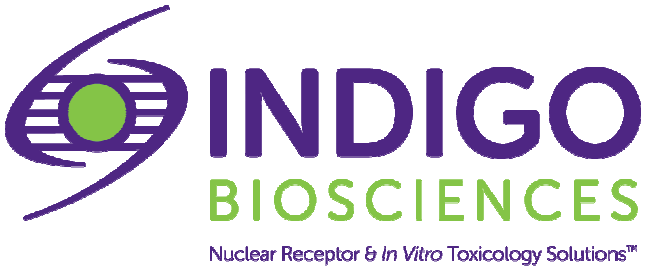

Live Cell Multiplex Assay

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 7.0)

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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 Workflow

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 assess compound-induced cytotoxicity. These are "loss-of-activity assays", and test compounds that exert cytostatic, cytotoxic, or cytolytic activities will produce "false-positive" results. Namely, the measured drop in luciferase activity will be incorrectly attributed to inhibition of the nuclear receptor by the test compound. In reality, however, the compound has induced some form of cytotoxic response by the reporter cells. APPENDIX 1 provides an example of the impact of compound-induced cytotoxicity on ability to correctly interpret antagonist screening data.

An overview of the complete multiplex assay is depicted in **Figure 1**. A detailed protocol for performing the LCM portion of this workflow is provided in Section IV. Specific protocol details for the setup of the Nuclear Receptor Assay will be found in the Technical Manual accompanying that kit product.

Figure 1. *(on the following page)*
The fluorescence-based LCM Assay and the luminescence-based Nuclear Receptor assay are performed in a multiplex format. **Blue text** denotes the LCM Assay portion of the multiplex protocol, as described in this Technical Manual.

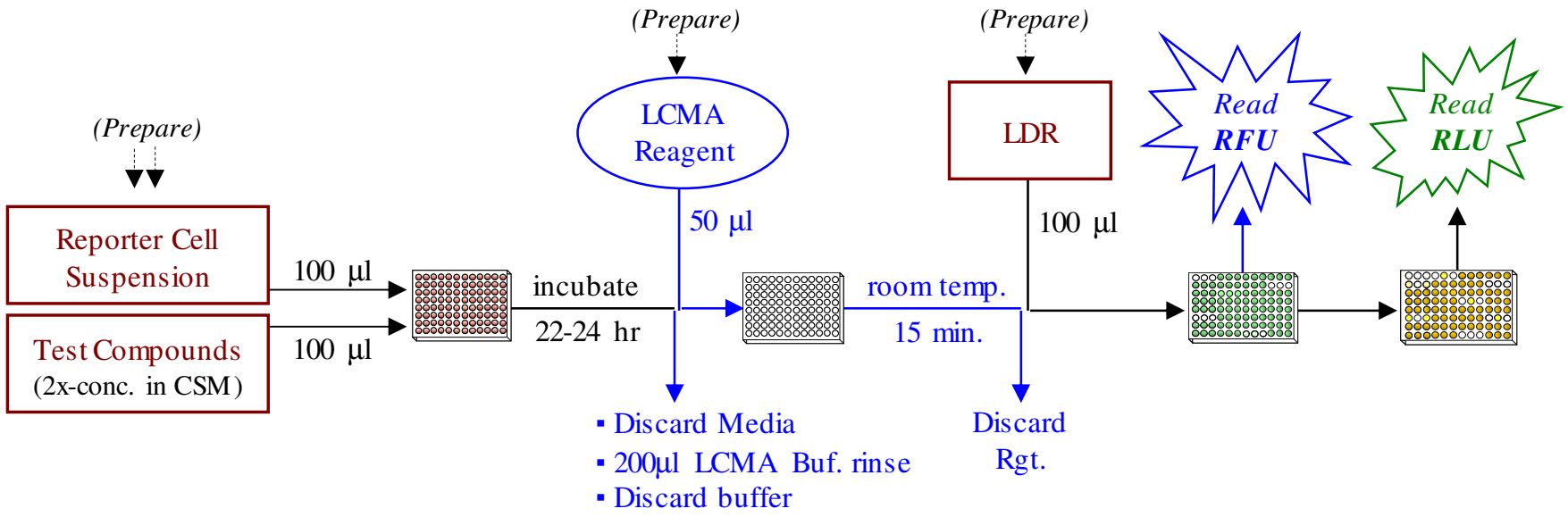
(A.) depicts in the integrated workflow of the LCM assay and one of INDIGO's 96-well *non*-preincubation format assays (*e.g.,* CAR's, ER's, FXR, GR, LRH-1, LXR's, MR, PPARδ/γ, RAR's, RXR's).

(B.) depicts in the integrated workflow of the LCM assay and one of INDIGO's 96-well *preincubation* format assays (*e.g.,* AhR, AR, EGFR, ERR's, GHR, NF-κB, Nrf2, PGR, PPARα, PXR, RORα, RORγ, TGFβR, TR's, VDR and VEGFR.)

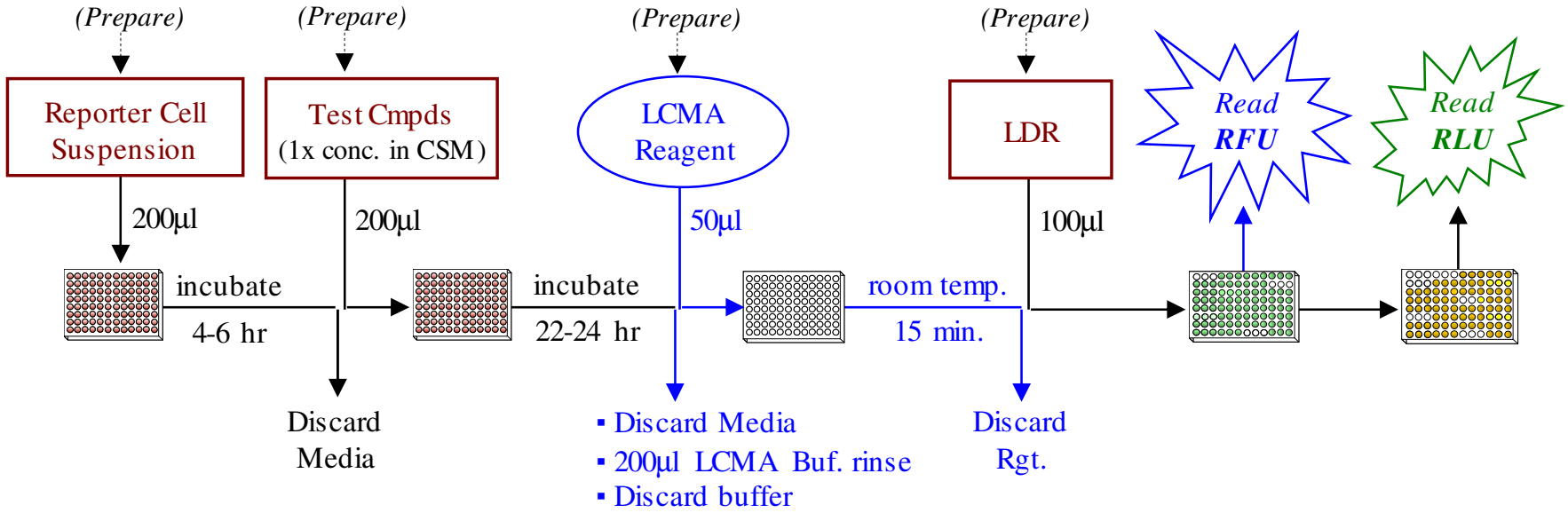
For detailed information pertaining to a receptor assay's specific setup and workflow, please refer to the respective Technical Manual.

NOTE: The LCM Assay protocol is *not* compatible with INDIGO's 384-well format Nuclear Receptor Assays, which utilize a homogenous assay chemistry.

***A.)* Workflow of the LCM assay combined with a *no-preincubation* format receptor assay**



***B.)* Workflow of the LCM assay combined with a *preincubation* format receptor assay**



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. The resulting product, calcein, is a hydrophilic fluorescent molecule (**Figure 2**). Due to the high charge density of the resulting reaction product, there will be no appreciable loss/efflux of calcein from the intracellular compartment during the short reaction period of the LCM Assay.

Instrument Parameters for the LCM Assay

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

NOTE: Calcein produces a strong fluorescence signal. Depending on the brand of instrument, some users may find that fluorescence emission from the white assay plate exceeds the optimal dynamic range of their fluorometer when reading in "auto-mode". In such cases an ERROR, or some type of WARNING, diagnostic will display. If this happens, the problem is easily addressed by manually adjusting the optical Z coordinate to a higher number (effectively dampening the efficiency of photon capture). Reread the assay plate after making the Z parameter adjustment. Alternatively, users may utilize an excitation filter with a moderately lower median wave length.

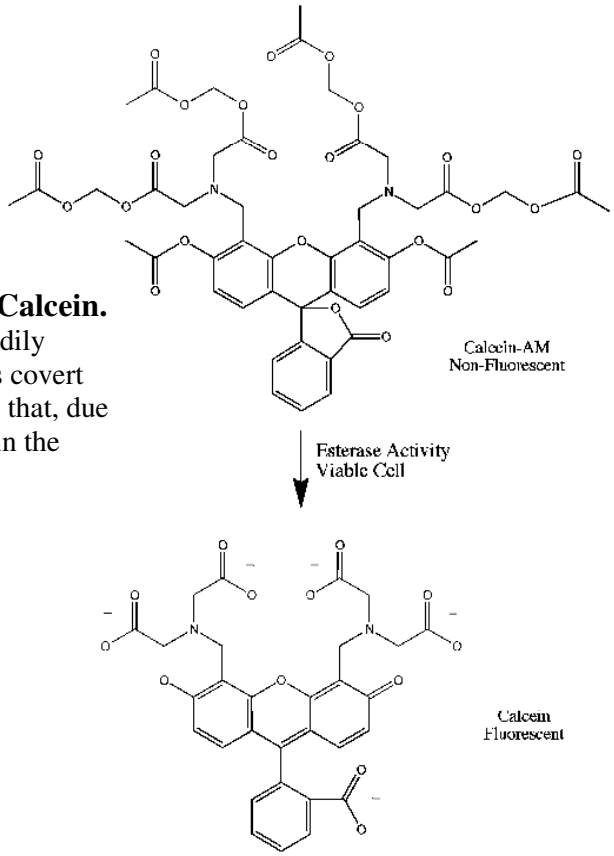


Figure 2. Conversion of Calcein-AM to Calcein. Non-fluorescent, hydrophobic Calcein-AM readily crosses cell membranes. Intracellular esterases covert the molecule to calcein, a fluorescent molecule that, due to its high negative charge density, is retained in the intracellular space.

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.
- 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 1 provides representative data.

and,

2.) RFU Background Control. Values of RFU background are quantified from wells containing 200 µl of CSM media *only* (no cells). 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".

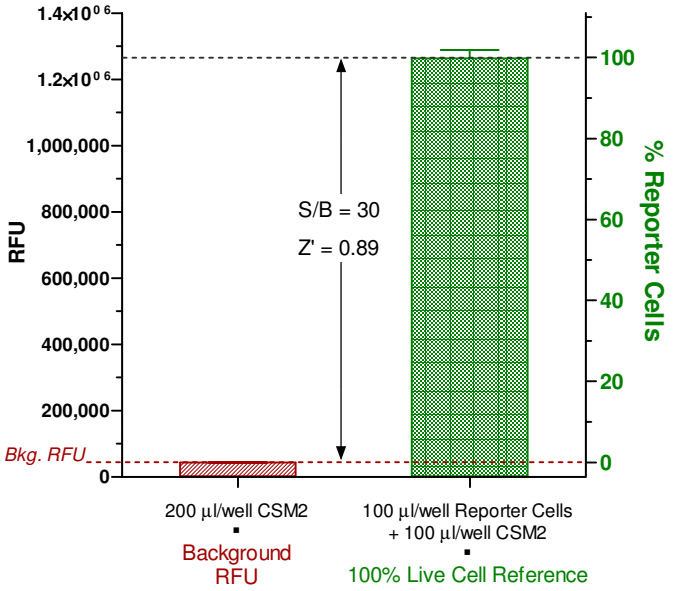


Figure 3a. Signal of the "100% Live Cell" and "Background" Controls.
The LCM Assay produces high fluorescent signal in the 100% Live Cells Reference wells, and produces minimal standard deviation between replicates, typically ≤ 5%. Despite low background fluorescence from the "0% Cells" wells, plates should always include this control. Thus, background RFU values may be determined, then subtracted from all other Reference and Experimental RFU values. RFU were quantified using a Tecan Spark plate-reader fitted with [485/20 nm^{Ex} | 535/25 nm^{Em}] filters; fixed Z =34,0000 µm; 10 flashes per read.

Optional Cytotoxicity "Positive Control"

If desired, Staurosporine may be used as a control treatment to provoke a cytotoxic response. For most cell types 8 µM staurosporine treatment causes ≥ 85% cell death within the 24 hr assay period.

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

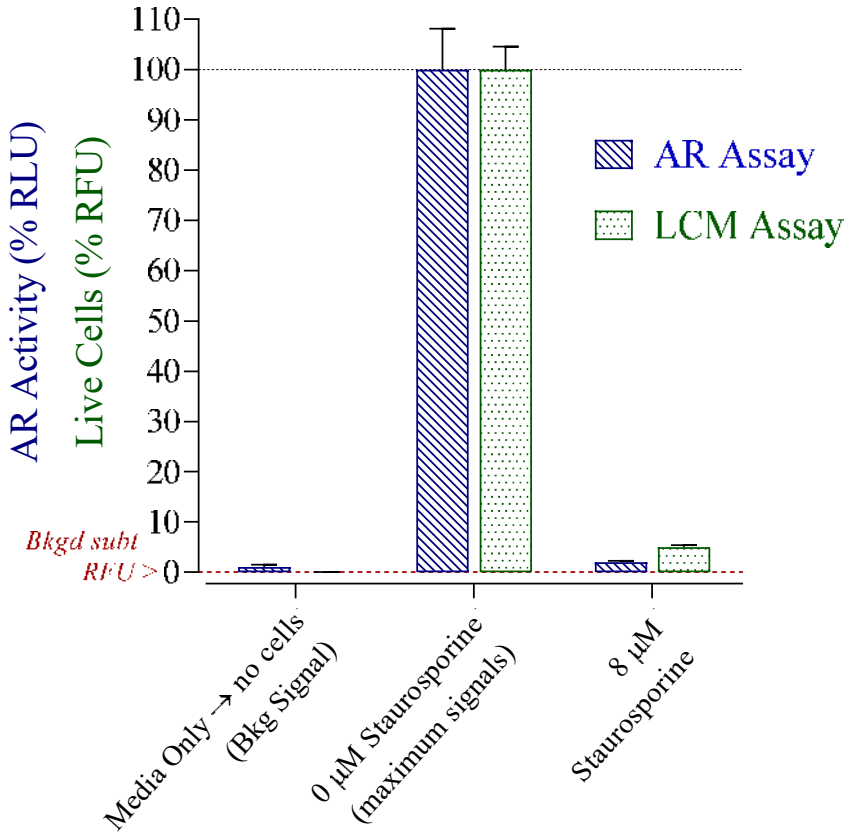


Figure 3b. Human AR and LCM Assays including treatment with Staurosporine as a 'Positive Control' for compound-induced cytotoxicity of Reporter Cells.

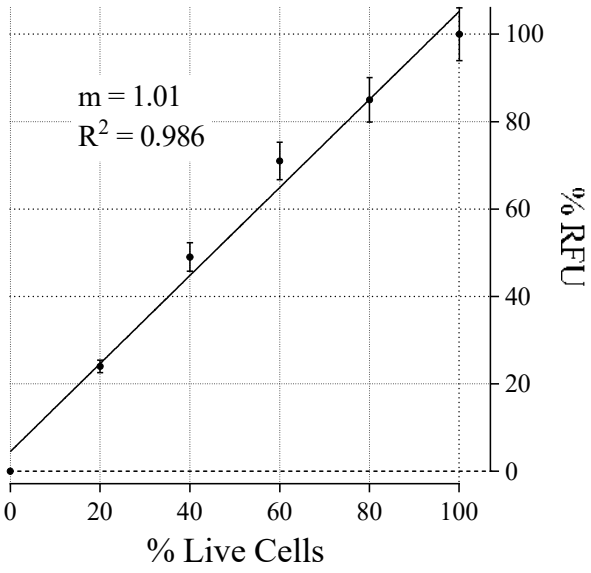
Human Androgen Receptor Reporter Cells were plated in Compound Screening Medium (CSM) supplemented with the agonist 6α-Fl-Testosterone and either 0 or 8.0 µM staurosporine. Assay wells containing media only (no Reporter Cells) provide background RFU values for the LCM Assay. 100% AR activity and 100%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

Results: 8.0 µM staurosporine is cytotoxic to Reporter Cells, typically resulting in ≤ 15% live cells after the 24 hours treatment 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 a reliable 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 Reporter Cells was plated at 100%, 80%, 60%, 40%, 20% and 0 % cells relative to the per well number specified in INDIGO's nuclear receptor assay kits. Cells were cultured for 23 hr and the LCM Assay was performed. RFU were quantified using a Tecan Spark plate-reader with the following parameters: [Ex 485nm/20nm : Em 535nm/25nm]; Z coordinate fixed at 34,000 µm. Average RFU values were background-subtracted, then normalized such that the wells containing 100% of live reporter cells = 100% RFU.



It is *not* necessary to generate a standard plot, such as depicted in Figure 4. Users may be confident in the direct correlation between % RFU and % Live Cells. Calculating % Live Cells per treatment conditions is performed using the equation below. ***Section V***: **LCMA Data Analyses** provides more detail pertaining to the specific steps in the data reduction process.

$$\%\ \text{Live Cells} = \frac{(\text{Ave. RFU}^{-\text{BKG}}\ \text{from } \textit{treated}\ \text{cells}) \times 100}{(\text{Ave. RFU}^{-\text{BKG}}\ \text{from } \textit{untreated}\ \text{cells})}$$

Healthy reporter cells will produce averaged RFU values with relatively low coefficients of variation (CV), typically ≤ 6%. However, %CV values can be expected to increase with increasing levels of compound-induced cytotoxicity.

Because of %CV, caution is advised against over-interpreting small deviations from "100% Live Cells". In general, ≤ 12% difference in % live cell values between treated and untreated assay wells will lack statistical significance. 12 - 14% difference between untreated and treated cells *may* be significant. ≥ 15% reduction in % Live Cells of treated *vs.* untreated cells is typically significant and is indicative of emerging cytotoxicity. Analyses of variance should be performed to properly assess statistical significance when only moderate differences are observed between the 'untreated' and 'test compound treated' data sets.

II. Product Components & Storage Conditions

Live Cell Multiplex (LCM) Assay Kit Formats

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.

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

All assay kits are shipped on dry ice. Upon receipt, an entire kit may be transferred to -20°C or -80°C storage.

LCM-01 Volumes sufficient to perform LCM Assays in one 96-well assay plate

▪ LCMA Buffer	1 x 30 mL
▪ LCMA Substrate (300x)	1 x 24 µL
▪ Staurosporine, 4.0 mM (500x)	1 x 10 µL

LCM-05 Volumes sufficient to perform LCM Assays in five 96-well assay plates

▪ LCMA Buffer	1 x 135 mL
▪ LCMA Substrate (300x)	5 x 24 µL
▪ Staurosporine, 4.0 mM (500x)	1 x 10 µL

LCM-10 Volumes sufficient to perform LCM Assays in ten 96-well assay plates

▪ LCMA Buffer	2 x 135 mL
▪ LCMA Substrate (300x)	10 x 24 µL
▪ Staurosporine, 4.0 mM (500x)	3 x 10 µL

Description of kit components

LCMA Buffer. This solution 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** (*Step 3*), *and*

ii.) a separate portion of LCMA Buffer is used to perform a pre-rinse of cultured cells prior to commencing the LCM Assay (*Step 5*).

LCMA Substrate, 300x. 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* (*Step 3*).

Staurosporine, 4.0 mM (500x stock). Staurosporine is provided as an optional positive control cytotoxicant. 8.0 µM staurosporine typically induces ≥ 80% cell death by the 24 hour endpoint of the receptor activation assay.

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 ~70% humidity.
- 8-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 repeat-dispensing pipettes is recommended.
- Media basins, sterile.
- 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. The filter combination of [$485nm_{Ex}$ | $535nm_{Em}$] that is commonly used to quantify EGFP, Fluorescein, and Rhodamine may be used effectively to quantify Calcein fluorescence.
- Plate-reading luminometer to quantify Relative Luminescence Units (RLU) from the receptor activity assay (*Step 13).*

IV. Assay Procedure

The LCM Assay Protocol is optimized to be integrated into the workflow of INDIGO's various receptor activity assays. Please review the entire assay protocol before starting, including the important *Protocol NOTES* on the next page.

Completing the multiplex LCM *and* receptor activity assays requires an overnight incubation. *Steps 1 and 2* are performed on **Day 1**, requiring approximately 2 hours. *Steps 3-13* are performed on **Day 2**, requiring 1-2 hours to complete.

A detailed description of all steps specific to the desired receptor assay are found in the Technical Manual accompanying that specific kit product.

General 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 ~ 24 hr treatment media contain serum, a potential source of esterases that may contribute background fluorescence to the LCM Assay. In addition, the various test compound treated wells potentially contain varying levels of esterases originating from cells degraded by induced cytotoxicity. Extra-cellular esterase and dead cell debris that would otherwise generate fluorescence background are effectively removed through the single cell-rinse step 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.

COMMENT: Immediately preceding RFU quantification (LCM assay), Luciferase Detection Reagent from the receptor assay kit is added to the assay wells (*Step 11*), thereby initiating the luciferase reaction of the receptor assay. While this may seem counter-intuitive, the concurrent photon emission from this luminescent reaction will be *miniscule* compared to the high intensity photon emission from the fluorescent LCM Assay. Also, the fluorescence emission filter used (535/20 nm) will effectively exclude luciferase light emission (562 nm median). Consequently, the concurrent luciferase reaction will *not* contribute meaningful background to the RFU measurements.

LCMA Protocol Considerations for ANTAGONIST-mode Nuclear Receptor Assays

▪

The Nuclear Receptor portion of the multiplex protocol described in this manual describes a representative ***agonist*** assay setup. When screening test compounds for ***antagonist*** activity, the steps of the protocol denoted with an asterisk (*) will have a modified description, as follows:

Step 1c. **Negative Control (no test cmpd) for Receptor 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, or if preferred 'vehicle only').

Step 1d. **Positive Control treatment for Receptor antagonist Assay** (⊕): Into another set of wells containing cells, dispense previously prepared [CSM + EC_{80} Agonist + reference antagonist]; the dispensed volume will be either 100 µl or 200 µl, depending on the specific Receptor assay protocol (and as depicted in **Fig 1**).

Step 1e. **Experimental wells for LCM and Receptor 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 either 100 µl or 200 µl, depending on the specific Receptor assay protocol (and as depicted in **Fig 1**).

DAY 1 -- *Aseptic Technique Required*

CONTROL Wells for the LIVE Cell Multiplex Assay *and* Receptor Activation (RA) Assays

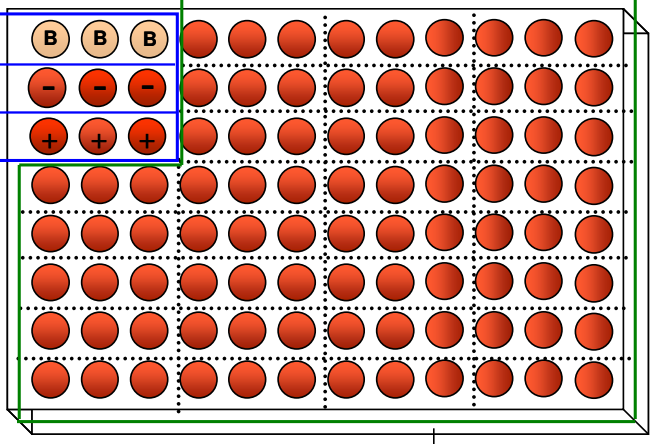
a. No cells, media only = RFU Bkg. Control →

c. Untreated = 100% Live Cell & RA (-) Control →

d. Reference Ligand = RA (+) Control →

(Optional: 8 µM Staurosporine = LCMA (+) Control)

b. Reporter Cell Suspension
e. + Test Cmpd treatment media



Step 1. Prepare "Control" and "Experimental" assay wells for the LCM and Receptor Assays (RA); *see *NOTES pg.11 for antagonist-mode assays*

☐ ***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 receptor assay setup (●); volume depends on the specific Receptor assay protocol (and as depicted in **Fig 1**). →

☐ ***c.*** **Untreated Control (⊖) for Receptor Assay *and* 100% Live Cell Reference for LCM Assay**: Into one set of wells containing cells, dispense CSM or "vehicle" supplemented CSM (*no* test cmpd); volume depends on the specific receptor assay protocol (**Fig 1**). →

☐ ***d.*** **Positive Control treatment (⊕) for Receptor Assay**: Into another set of wells containing cells, dispense previously prepared [CSM + reference cmpd]; volume depends on the specific receptor assay protocol (**Fig 1**). →

☐ ***e.*** **Test compound treatment wells for the Receptor and LCM Assays:** Into all other sets of wells containing cells, dispense the prepared Test Cmpd treatment media; refer to the specific receptor assay Technical Manual. →

OPTIONAL: include wells with **8.0 µM Staurosporine** to provoke a strong cytotoxic response (thereby providing a 'positive control' for cell death).

Step 2. Incubate for 22 - 24 hours; 37°C / 5% CO_2 / ~70% humidity.

NOTE: For greater convenience on **Day 2**, retrieve **LCMA Buffer** from freezer storage and place it in a refrigerator (4°C) to thaw overnight.

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

Transfer LCMA Rgt. to 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.*** Dispense 200 µl **LCMA *Buffer*** per well; tilt the plate side-to-side 2-3x to rinse wells

☐ ***Step 6.*** Discard LCMA Buffer.

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

☐ ***Step 8.*** Incubate **15 minutes** at **room temperature** (cover or place the plate in a drawer to avoid direct light exposure).

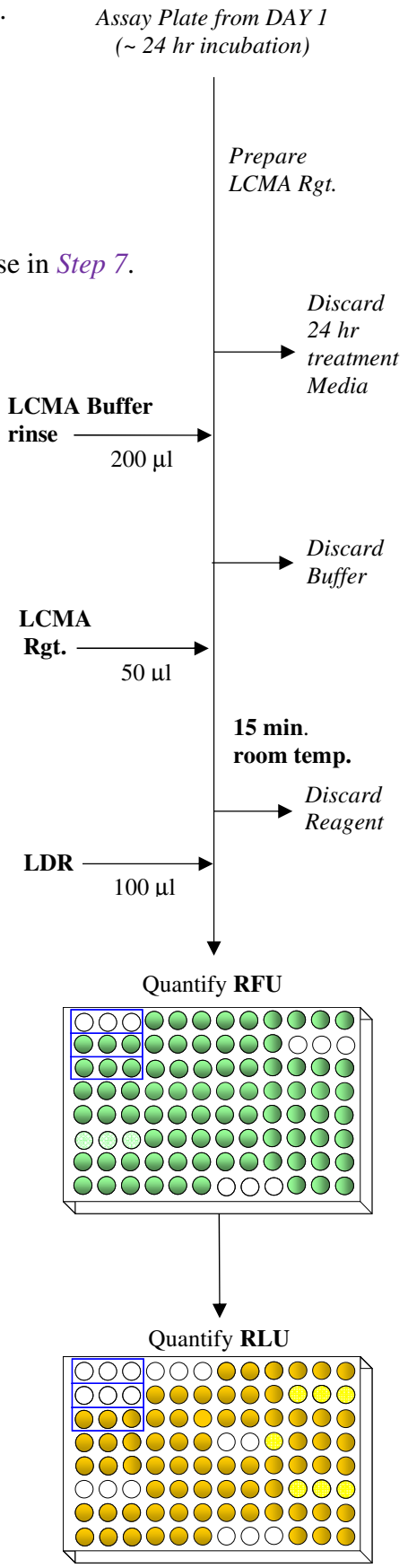
☐ ***Step 9.*** During plate incubation:
1. turn on the plate-reader and select *fluorescence* mode [485nm$_{Ex}$ | 535nm$_{Em}$]
2. prepare **Luciferase Detection Reagent** (LDR; refer to Receptor Assay Technical Manual)

☐ ***Step 10.*** At the **15-minute** time point discard LCMA Reagent (*no rinse step required*).

☐ ***Step 11.*** Dispense 100 µl / well of the prepared **LDR**

☐ ***Step 12.*** Quantify Fluorescence (RFU). If an "Error/Warning" displays, refer to the NOTE on p.4)

☐ ***Step 13.*** Convert plate-reader to Luminescence mode and quantify luminescence (RLU).



V. LCMA Data Analyses

Perform the following data reduction steps:

i. Calculate **Average RFU (aRFU)** from replicate cell treatments

ii. Calculate the **Standard Deviation (SD)** of aRFU

iii. Calculate the **Percent Coefficient of Variation (%CV)** of aRFU

iv. Calculate **Background-subtracted aRFU ($aRFU^{-BKG}$)** for each treatment condition. Recall that Background RFU derives from 'No Cells - media only' assay wells.

v. For each treatment condition, use values of $aRFU^{-BKG}$ to calculate **Percent Live Cells (%LC)** relative to the 'untreated' reporter cells, as follows:

Calculation of % Live Cells	
Untreated cells:	$\%LC^{Untreated} = (aRFU^{Untreated-BKG} / aRFU^{Untreated-BKG}) \times 100 =$ **100% Live Cells**
Test Compound treated cells:	$\%LC^{TestCmpd} = aRFU^{TestCmpd-BKG} / aRFU^{Untreated-BKG}) \times 100$
Reference Ligand treated cells:	$\%LC^{REF} = aRFU^{REF-BKG} / aRFU^{Untreated-BKG}) \times 100$
LCMA Background wells:	$\%LC^{BKG} = aRFU^{BKG-BKG} / aRFU^{Untreated-BKG}) \times 100 =$ **0% Live Cells**

Example Data Reduction & Interpretation

Treatment	*RFU 1*	*RFU 2*	*RFU 3*	aRFU	%CV	$aRFU^{-BKG}$	**% Live Cells**	Interpretation
media only, no cells	*2,950*	*3,051*	*3,023*	3,008	1.7	0	0%	RFU Background
0.1% DMSO	*48,714*	*51,237*	*53,155*	51,035	4.4	48,027	100%	healthy untreated cells
5 µM Cmpd X	*46,152*	*48,352*	*50,089*	48,198	4.1	45,190	94%	no apparent cytotox.
25 µM Cmpd X	*22,155*	*20,994*	*19,773*	20,974	5.7	17,966	37%	significant cmpd-induced cell death
(optional) 8 µM Staurosporine	*6,043*	*5,491*	*5,783*	5,772	4.8	2,764	5.8%	Postive Control cytotoxicant

VI. Related Products

Live Cell Multiplex Assay Products For combined use with any of INDIGO's 96-well format Receptor Assays (The LCM Assay is *not* compatible with INDIGO's 384-well format assays.)	
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 plates
LCM-05	Reagents in 5x bulk volumes for **480** Live Cell Assays performed in 96-well format Plates
LCM-10	Reagents in 10x bulk volumes for **960** Live Cell Assays performed in 96-well format Plates

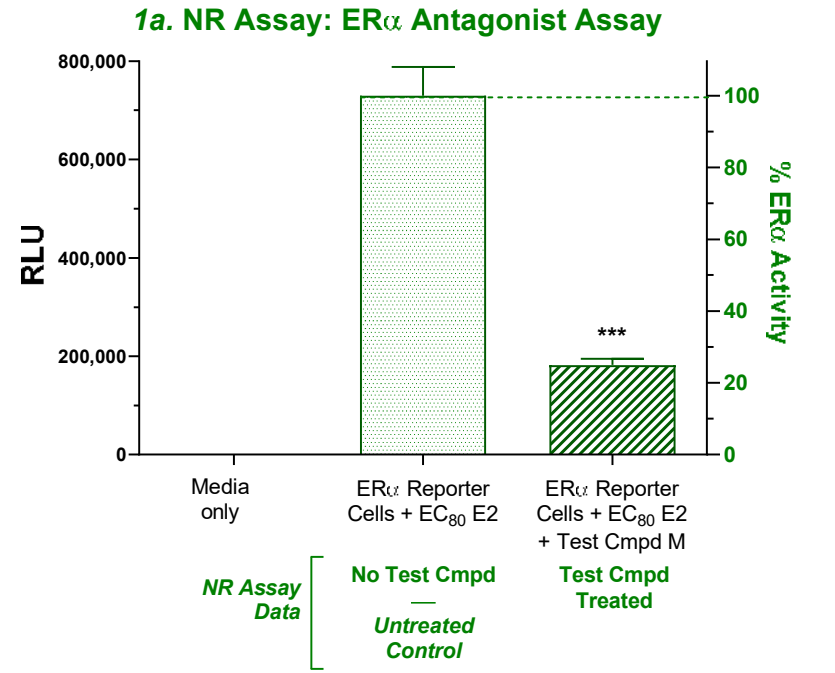
Please refer to INDIGO Biosciences' website for updated product offerings.

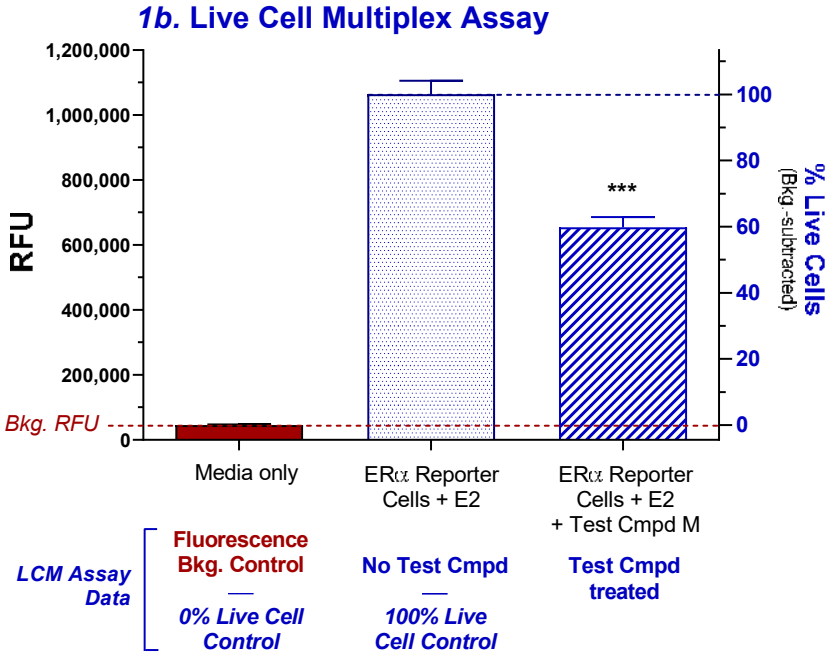
www.indigobiosciences.com

VII. Limited Use Disclosures

Products offered by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic, or contact use in humans or animals.

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





APPENDIX 1: Use of the LCM Assay to interpret receptor *antagonist* activity assay data. Quantifying the relative numbers of live reporter cells in treated samples may reveal false-positive data.

***1a.* Receptor antagonist activity data.** ERα Reporter Cells treated with E2[1] + Test Cmpd M show significantly diminished RLU values relative to untreated control cells. Is M an antagonist of ERα? Or, is the drop in ERα activity due to compound-induced cytotoxicity?

***1b.* LCM Assay data.** The LCM Assay reveals ~ 60% live reporter cells in the wells treated with M (relative to the untreated assay wells), a sure indicator of compound-induced cytotoxicity. Although the cells remaining in the M treated wells are alive, they are in metabolic crisis. This explains why the percent loss of ERα activity exceeds the percent reduction in the number of live cells. In essence, the remaining cells in the treated assay wells are alive, but they are very unhealthy, with diminished physiological processes (*e.g.,* transcription, translation, cell division) as they commit to apoptosis.

Methods: ERα Reporter cells were dispensed into the 96-well plate and further supplemented with either CSM+EC_{80} E2 (Untreated Control for ER antagonist assay & 100% Live Cell Reference for the LCM assay), or CSM+ E2+Test Cmpd M. Wells were also prepared with CSM *only* (no cells; RFU Bkg. Control in LCM assay). The assay plate was incubated for 23 hours then processed to quantify % Live Cells using the LCM assay protocol described in this Technical Manual, followed immediately by quantifying ERα activity according to the protocol described in the respective TM, and as depicted in Figure 1a of this TM.

[1] E2: 17-β-Estradiol, a potent agonist of estrogen receptors.
(***, $p << 0.05$)