



## **Live Cell Assay**

### *Product #*

LCA-01 (1x 96 assay wells)

LCA-05 (5x 96 assay wells)

LCA-10 (10x 96 assay wells)



### **Technical Manual**

*(version 1.0)*

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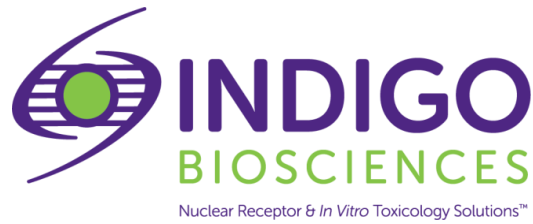
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## Live Cell (LC) Assay

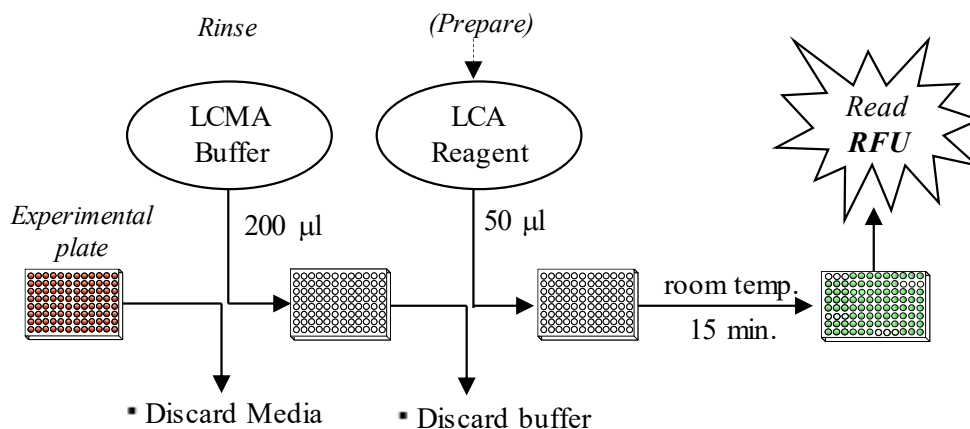
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## I. Description

### Utility and Overview of the Live Cell (LC) Assay Workflow

The Live Cell (LC) Assay is a fluorescence-based method designed to quantify relative changes in live mammalian cell number following treatment with test compounds. This assay enables the assessment of compound-induced mitogenic or cytotoxic effects on cells. The protocol is optimized for use with adherent cells cultured in black or white 96-well plates. It provides a convenient and reliable tool for evaluating cell viability as part of screening workflows, mechanistic studies, or toxicity assessments.

An overview of the complete assay is depicted in **Figure 1**. A detailed protocol for performing the LC Assay is provided in **Section IV**.

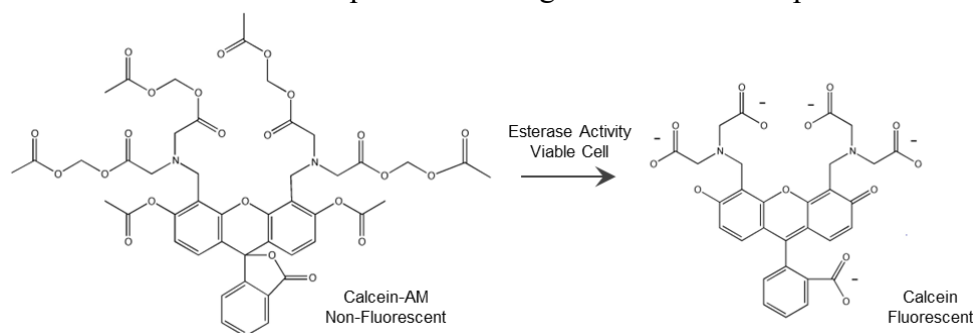


**Figure 1.** Depicts workflow of LC assay

### The LC Assay Chemistry

The LC Assay utilizes the fluorogenic substrate Calcein-AM (AcetoxyMethyl ester of Calcein) to provide a sensitive, quantitative measure of the relative number of live Cells remaining in the 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 LC Assay.



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

## Instrument Parameters for the LC Assay

The wavelengths of maximal excitation and emission for Calcein are 492 nm and 513 nm, respectively. However, the filter combination of [485nm<sub>Ex</sub> | 535nm<sub>Em</sub>] (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.

## LC Assay Controls

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

**1.) 100% Live Cells Reference.** The "100% Live Cells" Reference wells for the LC Assay will always be the same as those used as the “Untreated Control” wells.

**2.) RFU Background Control.** Values of RFU background are quantified from wells containing 200 µl of media *only* (no cells). These wells are processed in an 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”.

## 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-hour assay period.

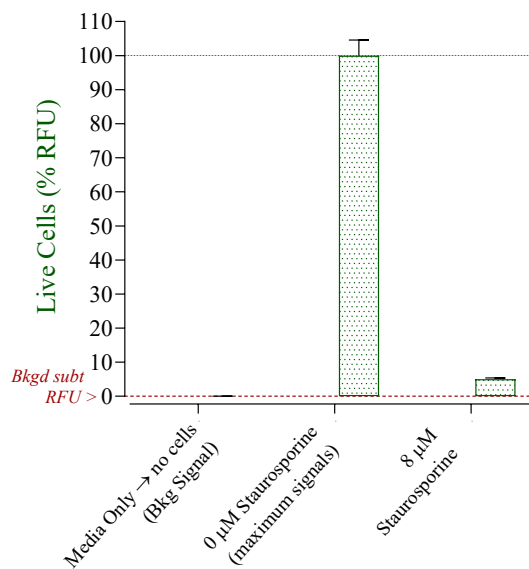
A stock of **4.0 mM Staurosporine** stock (*i.e.*, 500x concentrated) is provided with the assay kit.

### Figure 3. LC Assays, including treatment with Staurosporine as a 'Positive Control' for compound-induced cytotoxicity of Cells.

Cells were plated in Medium supplemented with either 0 or 8.0 µM Staurosporine. Assay wells containing media only (no Cells) provide background RFU values for the LC Assay. 100% Live Cells derive from the 0 µM Staurosporine treatment.

The background (Bkg) RFU values from the Media Only wells are subtracted from the Untreated (0 µM) and Experimental (8 µM) RFU values.

**Results:** 8.0 µM Staurosporine is cytotoxic to Reporter Cells, typically resulting in ≤ 15% live cells after the 24 hours treatment period.

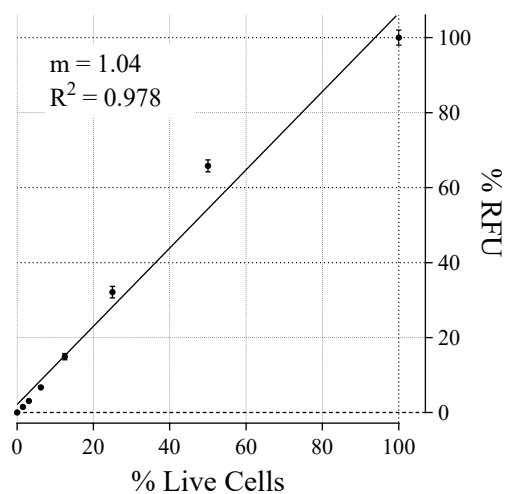


## Data Analyses

The intensity of the fluorescent signal generated in the LC 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 LC Assay provides a direct correlation between % RFU and % Live Cells in an assay well. To demonstrate this, Cells were serially diluted in a black walled plate and cultured for 24 hr. The LC Assay was performed the next day. 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  $\mu\text{m}$ . Average RFU values were background-subtracted, then normalized such that the wells containing 100% of live cells (untreated 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: LCA 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 cells will produce average RFU values with relatively low coefficients of variation (CV), typically  $\leq 6\%$ . However, %CV values can be expected to increase with increasing levels of compound-induced cytotoxicity.

## II. Product Components & Storage Conditions

### Live Cell (LC) Assay Kit Formats

The volumes of reagents provided in a single LC Assay kit, #**LCA-01**, are sufficient to perform 96 determinations of “% Live Cells”.

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, the entire kit may be transferred to -20°C or -80°C storage.

**LCA-01** Volumes sufficient to perform LC Assays in (1) 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

**LCA-05** Volumes sufficient to perform LC Assays in (5) 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

**LCA-10** Volumes sufficient to perform LC Assays in (10) 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.

- i.) A portion of LCMA Buffer is used to perform a rinse of cultured cells prior to commencing the LC Assay (*Step 3*).
- ii.) A second portion of LCMA Buffer is combined with concentrated *LCA Substrate* to generate a 1x working concentration of **LCA Reagent** (*Step 5*).
- iii.) For greater convenience on **the day before the assay**, retrieve **LCMA Buffer** from freezer storage and place it in a refrigerator (4°C) to thaw overnight. **On the day of the assay**, ensure that all reagents are brought to **room temperature** (20-25°C) before use.

**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 *LCA Reagent* (*Step 3*).

**Staurosporine, 4.0 mM (500x stock).** Staurosporine is provided as an optional positive control cytotoxicant. 8.0 µM staurosporine typically induces ≥ 85% cell death after 24-hour incubation.

### III. Materials to be Supplied by the User

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

- Adherent cells set up in a 96-well black or white flat-bottom tissue culture treated plate.
- 8-channel pipette & sterile tips appropriate for the transfer of 200  $\mu$ l and 50  $\mu$ l volumes. The use of electronic repeat-dispensing pipettes is recommended.
- Media basins, sterile.
- Phosphate Buffered Saline (PBS) solution.
- Plate-reading fluorometer equipped with filter combination of [485nm<sub>Ex</sub> | 535nm<sub>Em</sub>] that is commonly used to quantify EGFP, Fluorescein, and Rhodamine may be used effectively to quantify Calcein fluorescence.

### IV. Assay Procedure

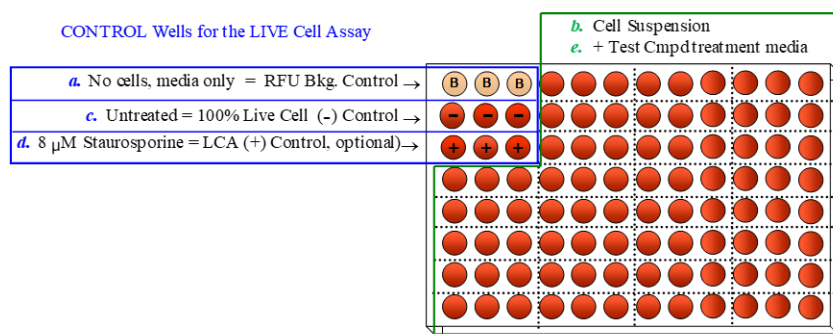
Please review the entire assay protocol before beginning including the following NOTES. Steps 1-8 should be carried out at the end of the treatment incubation period. Aseptic techniques are not required for this assay. The LC assay takes approximately 1 hour to complete.

#### General Protocol NOTES

**NOTE:** Once in aqueous solution Calcein-AM undergoes a slow rate of hydrolysis that generates fluorescent Calcein. Therefore, LCA 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 LCA Reagent can NOT be stored for later use and should be discarded after the assay setup.

**NOTE:** This protocol incorporates media-discard and cell-rinse steps (*Steps 2-4*) immediately prior to adding LCA Reagent to the assay wells. This cell-rinse step is necessary because the treatment media contain serum, a potential source of esterases that may contribute background fluorescence to the LC 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 LCA assay.

#### Example of Plate Set up and Controls



## Assay Steps

*Aseptic Technique NOT Required for the LC Assay*

**Step 1.** Prepare the appropriate volume of **LCA Reagent**.

#LC Assay Wells	300x LCMA Substrate	+	LCMA Buffer	→	LCA 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

Assay Plate

*Prepare LCA Rgt.*

Transfer LCA Rgt. to a low-light environment for later use in *Step 5*.

**Step 2.** At the end of the treatment duration, discard treatment media from the assay plate.

*Discard treatment Media*

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

**LCMA Buffer**  
rinse →  
200 µl

**Step 4.** Discard LCMA Buffer.

*Discard Buffer*

**Step 5.** Dispense 50 µl / well **LCA Reagent**, tilt the plate side-to-side 2-3x.

**LCA Rgt.** →  
50 µl

**Step 6.** Incubate **15 minutes** at **room temperature** (Cover or place the plate in a drawer to avoid direct light exposure).

**15 min.**  
**room temp.**

**Step 7.** During plate incubation, turn on the plate-reader and select *fluorescence* mode [485nm<sub>Ex</sub> | 535nm<sub>Em</sub>]

*Discard LCA Rgt.*

**Step 8.** At the **15-minute** time point, discard the LCA Rgt., Immediately dispense 100 µl of PBS and quantify fluorescence (RFU).

**PBS** →  
100 µl

Quantify **RFU**

## V. LCA Data Analysis

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<sup>-BKG</sup>)** 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<sup>-BKG</sup> to calculate **Percent Live Cells (%LC)** relative to the ‘untreated’ reporter cells, as follows:

### Example Data Reduction & Interpretation

Treatment	RFU 1	RFU 2	RFU 3	aRFU	%CV	aRFU <sup>-BKG</sup>	% 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 $\mu$ M Cmpd X	46,152	48,352	50,089	48,198	4.1	45,190	94%	no apparent cytotox.
25 $\mu$ M Cmpd X	22,155	20,994	19,773	20,974	5.7	17,966	37%	significant cmpd-induced cell death
(optional) 8 $\mu$ M Staurosporine	6,043	5,491	5,783	5,772	4.8	2,764	5.8%	Postive Control cytotoxicant

## VI. Limited Use Disclosures

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