TECHNICAL MANUAL

LuxSit™ Pro Luciferase Assay System

INSTRUCTIONS FOR USE

LS0101

Rev. 07/2024/00 – Early Release

LuxSit™ Pro Luciferase Assay System

1. Introduction

The Monod Bio LuxSit™ Pro family of products are the world's smallest and highest performance de novo luciferases available on the market. They are available in multiple formats for various applications, including full length protein reporters, or the complementation system ideal for revealing protein-protein interactions. The LuxSit™ Pro family of products has been engineered to exhibit remarkable stability and has performance that exceeds that of its predecessor, LuxSit [1], by 150-fold, making LuxSit™ Pro the natural choice for research use only (RUO) and in vitro diagnostic (IVD) applications.

The Monod Bio LuxSit™ Pro products are offered as kits that enable scientists to build their desired applications. Each kit provides the following proprietary materials: (1) a plasmid encoding LuxSit™ Pro, (2) the reaction buffer, (3) substrate, and (4) substrate reconstitution buffer.

The LuxSit™ Pro products are based on the ground-breaking LuxSit luciferase [1] technology invented in the lab of Dr. David Baker at the University of Washington Institute for Protein Design (IPD). Monod Bio, Inc. has exclusive commercial rights, and the LuxSit™ Pro family of products are proprietary to Monod Bio, Inc.

The LuxSit™ Pro enzyme is a small (13.5 kDa) ATP-independent luciferase and is the world's first *de novo* Luciferase designed using state-of-the-art machine learning tools. The enzyme is monomeric, exhibits excellent solubility, thermostability, and can be expressed in both bacterial and mammalian cells. When paired with the LuxSit™ Pro Assay Buffer and LuxSit™ Pro Substrate, the LuxSit™ Pro Luciferase Assay System generates high-intensity glow-type luminescence multiple times brighter than the commonly used Firefly & Renilla luciferase systems (**Figure 1**).

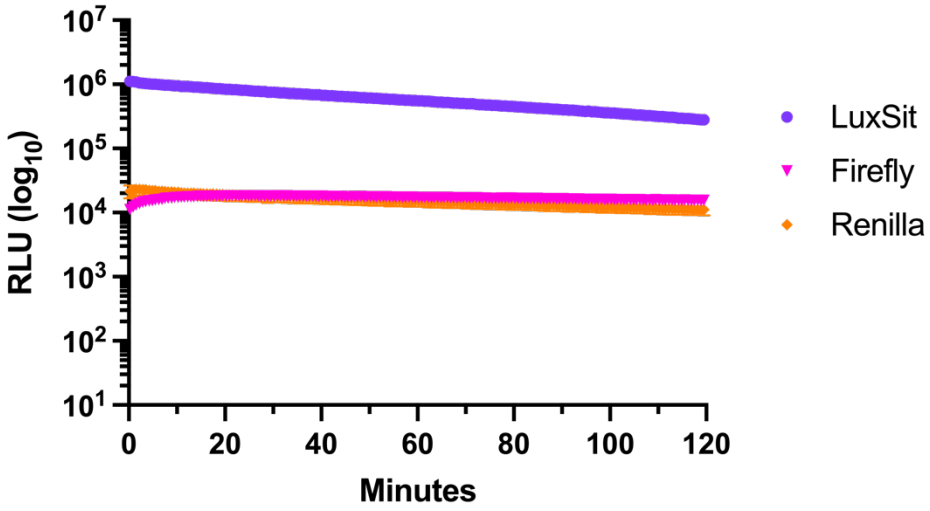

Figure 1: Comparison of LuxSit™ Pro, Firefly, and Renilla luciferases expressed in lysed mammalian cells. HEK293T cells were transfected with plasmid and incubated for 24 hours. Cells were lysed with 1x Passive lysis buffer (Promega Cat. No. E1941). The respective substrate reagents were added to the cells per manufacturer instructions and bioluminescence was measured using a standard plate reader. Bioluminescent signal (initial time point) from LuxSit™ Pro reactions was ~100x and ~50x brighter than Firefly and Renilla reactions, respectively. The means of quadruplicate transfections are graphed with error bars indicating standard deviation.

2. Product Components & Storage Conditions

Each LuxSit™ Pro Luciferase Assay System kit (REF# LS0101) provides the necessary reagents for completing up to 1,000 assays in 96-well plates. The table below lists each component, as well as the unit size and respective catalog number information:

LuxSit™ Pro Luciferase Assay System - Component Name	Catalog #	Size
L-AB010 | Assay Buffer	C-11555M	1 x 100 mL
L-RB010 | Reconstitution Buffer	C-11556M	1 x 2.5 mL
LuxSit™ Pro Substrate	S00103	5 mg

The assay kit is compatible with multiple plasmids that encode the LuxSit™ Pro Luciferase. Compatible plasmids are listed in **Table A1** of **Appendix A**, and plasmid datasheets are available from the Monod Bio website (https://www.monod.bio/resources).

2.1. Kit and Component Storage Conditions:

Upon receipt, the LuxSit™ Pro Luciferase Assay System should be stored at -20°C. The kit may be thawed at 4°C or at room temperature. When ready for use, consider the following storage recommendations for the kit components:

1. Assay Buffer may be thawed at room temperature or 4°C.
2. Once thawed, the Assay Buffer may be stored at 4°C or room temperature for up to 3 months.
   Note: *Assay Buffer stored at room temperature may experience clouding when compared to reagent stored at 4°C. Assay Buffer clouding does not affect assay performance, including similar assay brightness and signal duration when used as instructed.*
3. Once reconstituted (using Reconstitution Buffer, see **Section 3.1**), liquid LuxSit™ Pro Substrate should be stored at
   -20°C and its light exposure limited.
4. LuxSit™ Pro Luciferase Plasmid (purchased separately) should be stored at -20°C.

3. Reagent Preparation & Handling

3.1. Substrate Reconstitution:

Remove Reconstitution Buffer from -20°C storage and equilibrate to room temperature prior to use. Mix Reconstitution Buffer by inversion once equilibrated. Similarly, remove lyophilized LuxSit™ Pro Substrate from -20°C storage and bring to room temperature. Add 2.5mL of Reconstitution Buffer directly to the vial of lyophilized LuxSit™ Pro Substrate and mix by inversion until LuxSit™ Pro Substrate is fully reconstituted. The concentration of this stock LuxSit™ Pro Substrate is 5mM.

*Limit light exposure of the LuxSit™ Pro Substrate during and after reconstitution.

Store reconstituted LuxSit™ Pro Substrate and any sub-aliquots at -20°C when not in use. When making sub-aliquots consider that a full 96-well assay plate would require 120µL of 5mM stock LuxSit™ Pro Substrate to prepare enough Assay Reagent for all wells.

3.2. Assay Reagent Preparation:

Thaw or equilibrate Assay Buffer to room temperature prior to use. Assay Buffer may be thawed at 4°C or room temperature. Mix Assay Buffer by inversion once equilibrated. Allow reconstituted substrate to equilibrate to room temperature (while limiting light exposure) prior to use. Assay Reagent is prepared by diluting one volume of reconstituted Substrate with 50 volumes of Assay Buffer.

It is recommended to prepare fresh Assay Reagent for each experiment and limit light exposure prior to using. The Assay Reagent maintains up to 75% of performance (peak brightness) for up to three hours after preparation compared to that of freshly prepared Assay Reagent.

Running a full 96-well assay plate requires 10mL of Assay Reagent for example. To prepare 10mL of Assay Reagent, dilute 240µL reconstituted stock 5mM LuxSit™ Pro Substrate into 9.76mL of Assay Buffer (see example calculations below).

Example Calculations:
(5mM stock LuxSit™ Pro Substrate) * (240µL) = (120µM LuxSit™ Pro Substrate) * (10mL Assay Reagent)
10mL Assay Reagent = 9.76mL Assay Buffer + 240µL 5mM stock LuxSit™ Pro Substrate

3.3. Plasmid Preparation:

Plasmids can be purchased in addition to the LuxSit™ Pro kit (see Section 6, Related Products). Plasmids units are available in 25µg aliquot sizes. It is recommended to reconstitute plasmids to a working stock of 100ng/µL using ultrapure H2O. Based on the 25µg aliquot size, reconstitute using 250µL of ultrapure H2O to reach the recommended working stock concentration.

4. Example Protocol for Detecting LuxSit™ Pro Luciferase in Mammalian Cells (Lytic Method)

4.1. Preparing & Transfecting Cells

Prepare transfection mixes first so there is enough time for the transfection reagent and DNA complex(es) to form prior to plating the cells.

1. Calculate the amount of DNA, transfection reagent (e.g. polyethylenimine [PEI; Polysciences Cat# 24765], FuGene [Promega Cat# E5911], or Lipofectamine [ThermoFisher Cat# 18324012]), and media needed for the number of transfections being performed. Make enough for 10µL of mix per well following the manufacturer's protocol.
2. Combine all together in an appropriately sized tube(s) and mix by vortexing.
3. Allow mixture(s) to incubate at room temperature for at least 15 minutes.

Split and count the cells as you would a normal passage.

1. Based on the cell count, prepare enough volume to plate 100µL of $2x10^4$ cells per well (density of $2x10^5$ cells/mL) in each well to be tested.
2. Pipette 10µL of transfection mix into the respective wells, ensuring the transfection mix comes into contact with the media.
3. Gently mix the plate by swirling. Incubate the transfected cells for 24 hours in a normal cell culture environment (37°C/5% CO_2).

4.2. Testing Luminescence

1. Remove plate(s) from incubator and spin at 300xg for 5 minutes.
2. Remove media carefully and add 100µL per well of sterile DPBS to rinse.
3. Spin again at 300xg for 5 minutes and then carefully remove DPBS.
4. Add 20µL of 1x Passive Lysis Buffer (Prepared from 5X Passive Lysis Buffer, Promega Cat. No. E1941) per well testing and incubate for 10 minutes.
5. Prepare a 120µM stock of substrate in Assay Buffer with enough volume for each well to be tested.
6. Add 100µL of 120µM substrate per well.
7. Read luminescence on a plate reader. Refer to plate reader for appropriate settings.

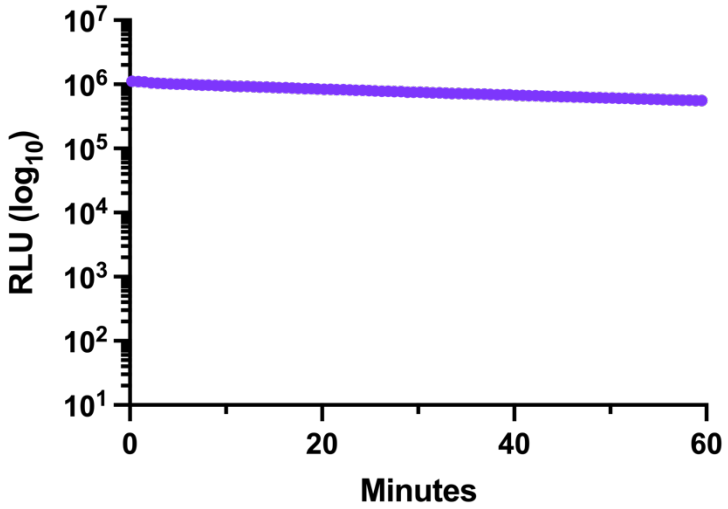

Figure 2: Detection of LuxSit™ Pro expressed in lysed mammalian cells. HEK293T cells were transfected with LuxSit™ Pro Plasmid and incubated for 24 hours. Cells were lysed with 1x Passive lysis buffer (Promega Cat. No. E1941). LuxSit™ Pro Substrate Reagent was added to the cells, and bioluminescence was measured using a standard plate reader. The means of quadruplicate transfections are graphed with error bars indicating standard deviation.

5. Considerations for Protocol Optimization Using the LuxSit™ Pro Luciferase Assay System

There are several factors to consider when trying to optimize the LuxSit™ Pro Luciferase Assay System kit for your experimental needs, including (1) plate reader/luminometer settings, (2) the kinetic performance of the LuxSit™ Pro Luciferase, and (3) experimental/protocol design.

1. **Plate Reader/Luminometer Settings.** In addition to the assay workflow optimization, it is important to consider that there may be significant differences between plate reader/luminometers, even from the same manufacturer, resulting in different relative luminescent unit (RLU) output values from equivalent experimental assay workflows. RLU output values can be modified by changing instrument integration time, gain, and read height within the settings of each plate reader/luminometer software. Users of the LuxSit™ Pro Luciferase Assay System should consider the impact of each of these settings when determining the ideal instrument configuration for your experiment.

   LuxSit™ Pro Luciferase has a peak emission wavelength of 490nm (**Figure 3**). It is not required to use a filter on the plate reader/luminometer used. However, consideration should be taken on optimizing LuxSit™ Pro RLU output if using a filter.

2. **Assay Brightness and Signal Duration.** Expression levels of luciferases can significantly impact RLU signal intensity and half-life. Higher concentrations will deplete substrate quicker leading to higher initial RLU output, but diminished signal half-life. The LuxSit™ Pro Luciferase Assay System kit demonstrates a broad range for amount of transfected DNA (ng/well) and cell density (plated cells per well) in which RLU output and signal half-life diminishes only slightly (see **Figure 4** and **Figure 5**).

3. **Experimental/Protocol Design.** Several common components of mammalian cell culture experiments were tested and evaluated for their impact on the LuxSit™ Pro Luciferase performance, showing minimal difference in luminescent output (see **Figures 6-8**). Similarly, the LuxSit™ Pro Luciferase Assay System kit shows robust signal as it pertains to total in-well assay volume allowing users to use less volume of potentially limiting sample material (see **Figure 9**).

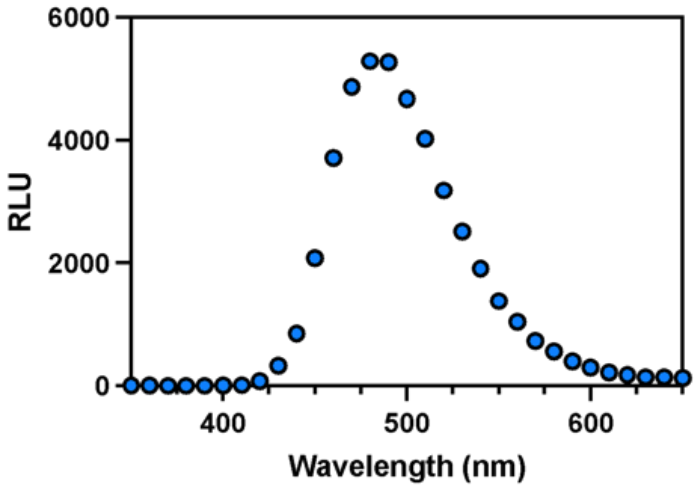

Figure 3: LuxSit™ Pro emission spectra. The LuxSit™ Pro luciferase has a peak emission wavelength of 490nm.

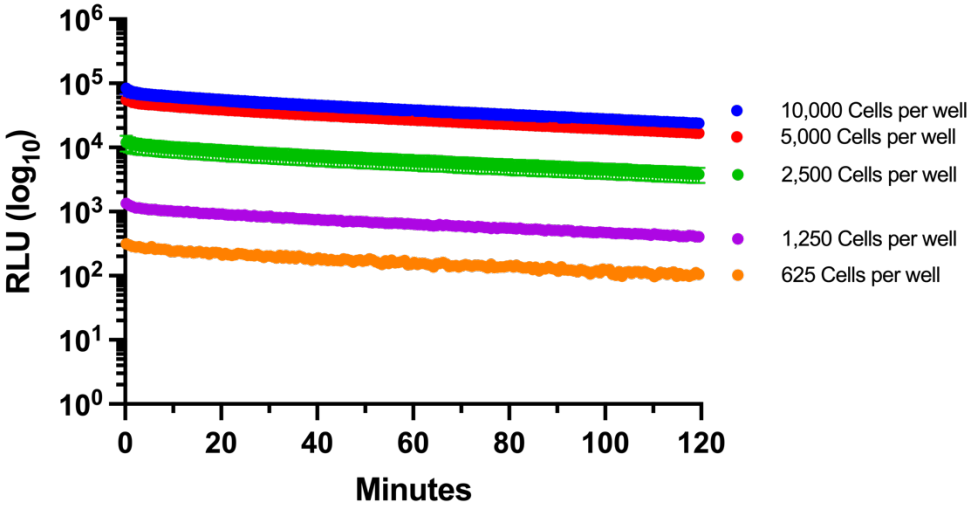

Figure 4. LuxSit™ Pro transfection with varying cell densities. Varying amounts of HEK293T cells per well were plated and transfected with 20ng/well of LuxSit™ Pro Luciferase DNA in a 96-well plate. After overnight incubation, cells were lysed for 15 minutes and then LuxSit™ Pro Substrate diluted in Assay Buffer was added to each well. Kinetics of each concentration are plotted. The average luminescent signal (n = 4) 3 minutes after Substrate addition was measured. Signal half-life decreased only by 10-15 minutes between the highest and lowest cell per well conditions.

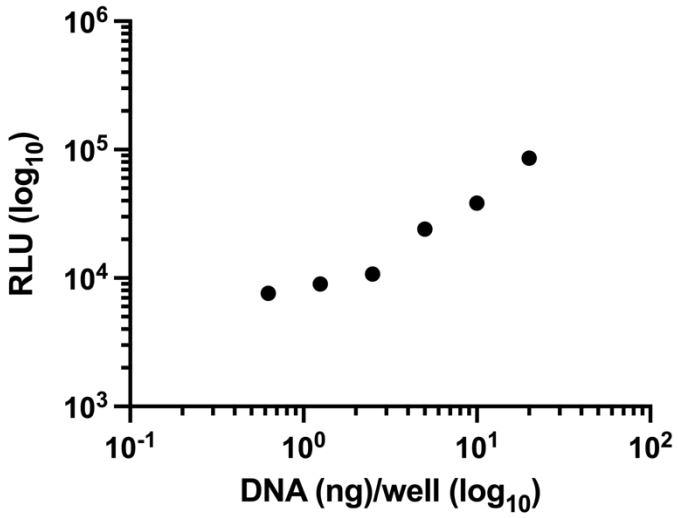

Figure 5. LuxSit™ Pro transfection with varying amounts of DNA. 10,000 cells per well of HEK293T were plated and transfected with varying amounts of LuxSit™ Pro Luciferase DNA in a 96-well plate. After overnight incubation, cells were lysed for 15 minutes and then LuxSit™ Pro Substrate diluted in Assay Buffer was added to each well. The average luminescent signal (n = 4) 3 minutes after Substrate addition was measured and plotted.

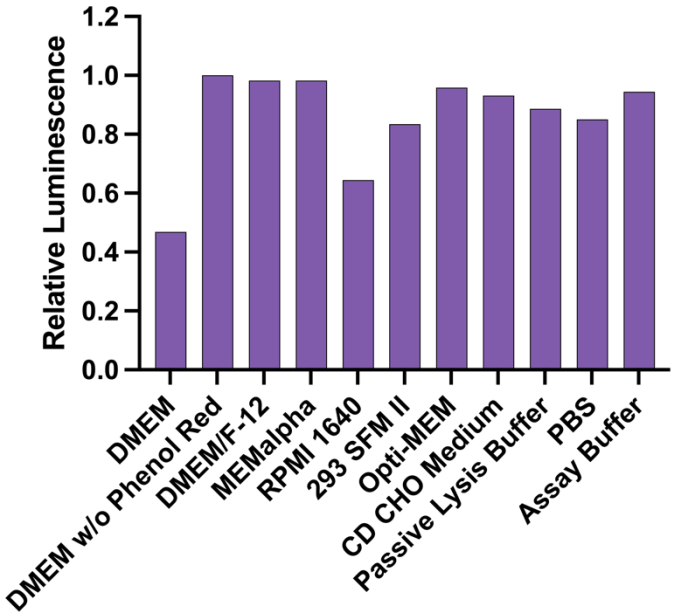

Figure 6. LuxSit™ Pro performance in cell culture media. Purified LuxSit™ Pro Luciferase was diluted to 100pM in various cell culture media and combined 1:1 with LuxSit™ Pro Substrate diluted in Assay Buffer. The average luminescent signal (n = 3) 3 minutes after Substrate addition was measured and normalized to the signal of DMEM without Phenol Red.

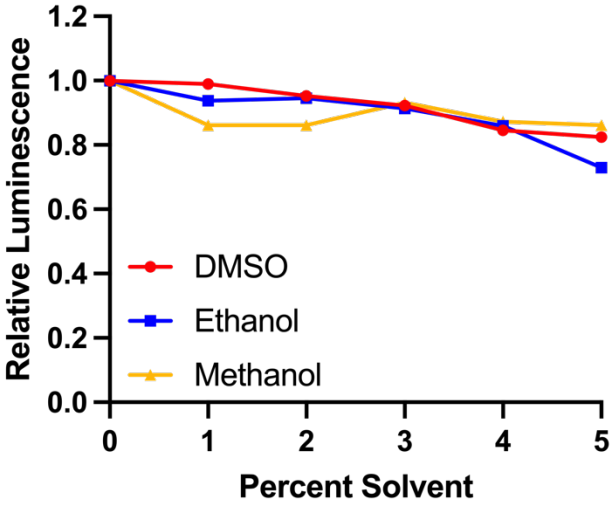

Figure 7. Impact of organic solvent on LuxSit™ Pro. Purified LuxSit™ Pro Luciferase was diluted to 100pM in Assay Buffer with various concentrations of organic solvent and combined 1:1 with LuxSit™ Pro Substrate diluted in Assay Buffer. The average luminescent signal (n = 3) 3 minutes after Substrate addition was measured and normalized to the signal of LuxSit™ in Assay Buffer alone. Values represent final in-well percentages of organic solvent.

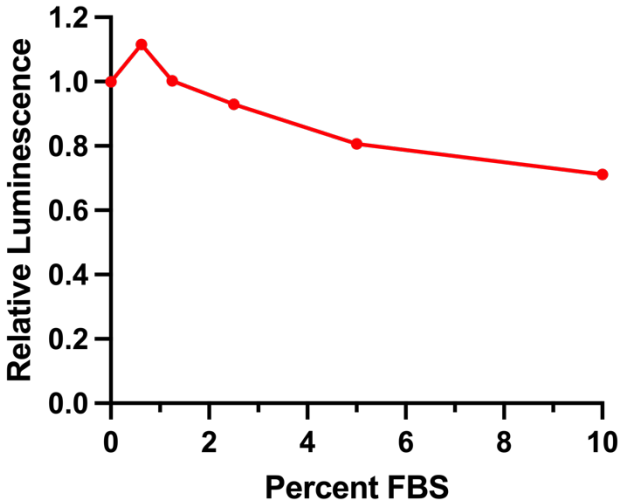

Figure 8. Impact of fetal bovine serum (FBS) on LuxSit™ Pro. Purified LuxSit™ Pro Luciferase was diluted to 100pM in Assay Buffer with titrating concentrations of Fetal Bovine Serum (FBS) and combined 1:1 with LuxSit™ Pro Substrate diluted in Assay Buffer. The average luminescent signal (n = 3) was measured 3 minutes after Substrate addition and normalized to the signal of LuxSit™ in Assay Buffer alone. Values represent final in-well percentages of FBS.

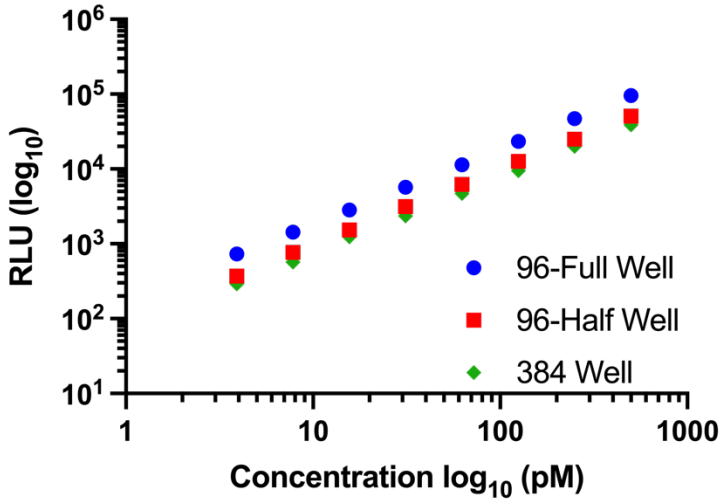

Figure 9. LuxSit™ Pro performance in various formats. Titrating concentrations of purified LuxSit™ Pro Luciferase were combined 1:1 with LuxSit™ Pro Substrate diluted in Assay Buffer in different final in-well volumes/plate formats. The average luminescent signal (n = 3) 3 minutes after Substrate addition was measured and plotted. In-well volumes of luciferase and Substrate were as follows: 96-Full Well - 50µL each, 96-Half Well – 25µL each, 384 Well - 10µL each.

6. Related Products

Compatible LuxSit™ Pro Plasmid List (available for order separately)

REF #	Description	Vector Name	Promoter	Multiple Cloning Site	N- or C-Terminal	Size	Format
P00101	LuxSit™ Pro, N-term	pcDNA3.1(+)[LuxSit™ Pro]-N	CMV	Yes	N	25 µg	Lyophilized
P00102	LuxSit™ Pro, C-term	pcDNA3.1(+)[LuxSit™ Pro]-C	CMV	Yes	C	25 µg	Lyophilized
P00103	LuxSit™ Pro Control	pcDNA3.1(+)[LuxSit™ Pro]	CMV	No	n/a	25 µg	Lyophilized

Related LuxSit™ Pro Luciferase Assay Kits (Research Use Only)

Reference	Product Name	Kit Performance Highlights
SP0101	LuxSit™ s-Pro PPI System	❖ Split LuxSit™ Pro Luciferase System ❖ Protein:Protein Interaction System ❖ Low assay background ❖ High signal-to-noise upon split enzyme reconstitution

7. Technical Support

Technical documentation, including kit instructions and plasmid datasheets, may be downloaded at **www.monod.bio/resources.**

For technical support and questions regarding protocol optimization, please email support@monod.bio

8. References

[1] Yeh, A.HW., Norn, C., Kipnis, Y. et al. De novo design of luciferases using deep learning. Nature 614, 774–780 (2023).

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Patent pending.