TECHNICAL MANUAL

LuxSit™ s-Pro Protein:Protein Interaction (PPI) System

INSTRUCTIONS FOR USE

SP0101

Rev. 05/2025/00

LuxSit™ s-Pro Protein:Protein Interaction (PPI) 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 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™ s-Pro Protein:Protein Interaction (PPI) System is composed of 2 fragments: a βit (~1.5 kDa) and a αLux (~12 kDa) and serves as a reversible complementation reporter. Designed using state-of-the-art machine learning tools, the two complementary pieces can each be expressed while linked to proteins of interest and are comprised of a Cysteine- and Lysine-free sequence to avoid unwanted post-translational modifications. When paired with the LuxSit™ Pro Assay Buffer and LuxSit™ Pro Substrate, the LuxSit™ s-Pro Protein:Protein Interaction System can generate high-intensity glow-type luminescence when complementation occurs, as illustrated using the Rapamycin system in **Figure 1**. The small size, low affinity (~90 μM, see **Figure 8**), and high stability of the 2 fragments make the LuxSit™ s-Pro PPI optimal for investigating protein-protein interactions with minimal interference and reliable results.

This technical manual provides users with a protocol to evaluate a set of PPI controls plasmids for use in mammalian cell systems, αLux-FKBP and βit-FRP, which complement in the presence of rapamycin and produce a strong luminescent signal in the presence of LuxSit™ Pro Substrate. The manual also includes

recommendations for implementing the s-Pro PPI system for novel assays and sensors. In particular, the manual details considerations for s-Pro piece orientation (N- vs. C-terminal) to the protein of interest. For technical support, questions regarding protocol optimization, and custom assay development please email support@monod.bio.

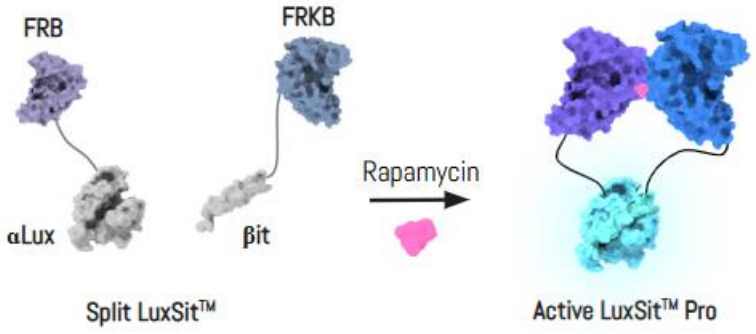

Figure 1: Illustration demonstrating complementation and luminescent signal generation of αLux-FKBP and βit-FRB in the Rapamycin system.

2. Product Components & Storage Conditions

Each LuxSit™ s-Pro PPI System kit (REF# SP0101) 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™ s-Pro PPI 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™ s-Pro Luciferase splits. Compatible plasmids are listed in **Section 6**, 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™ PPI 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 exposure to light minimized.
4. LuxSit™ s-Pro Luciferase Plasmids (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.

Important note: Limit light exposure of the LuxSit™ Pro Substrate during and after reconstitution.

Store reconstituted LuxSit™ Pro Substrate at -20°C when not in use. It is recommended to make sub-aliquots to limit the number of freeze/thaws. When making sub-aliquots consider that a full 96-well assay plate would require 240µL 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™ PPI System (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 Using the LuxSit™ PPI System 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 Assay Reagent (See **Section 3.2**) with enough volume for each well to be tested.
6. Add 100µL of Assay Reagent per well.
7. Read background luminescence for 10 minutes on a plate reader.
8. Add 10µL of Rapamycin to each well and read luminescence. Refer to plate reader for appropriate settings.

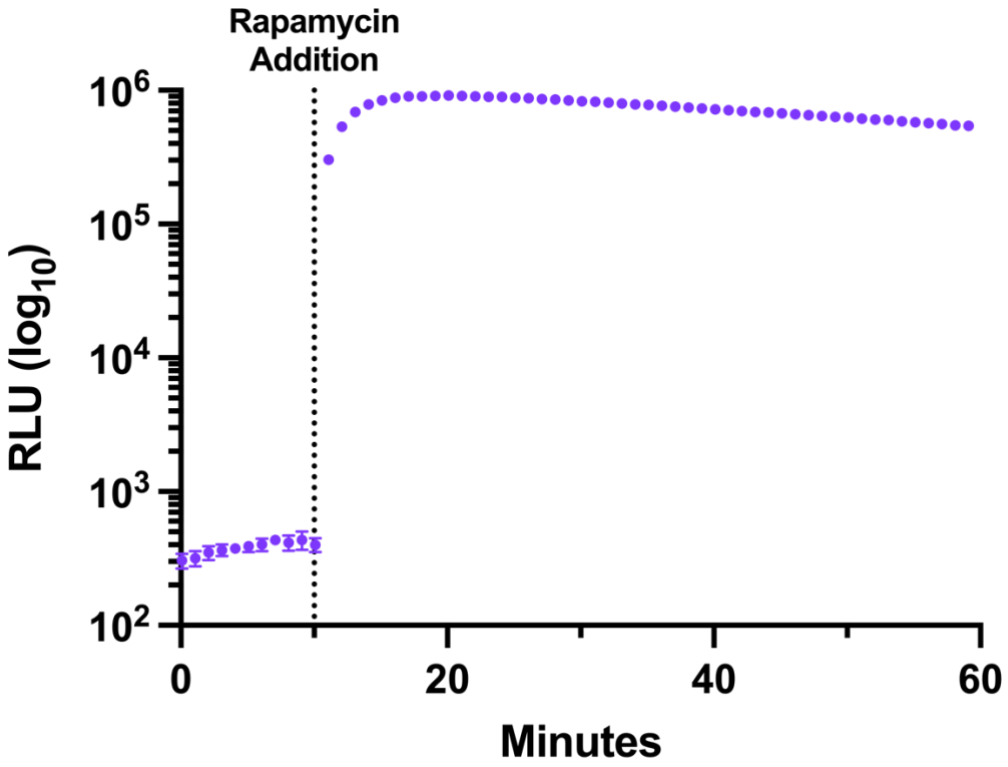

Figure 2: Detection of LuxSit™ s-Pro PPI System expressed in lysed mammalian cells. HEK293T cells were transfected with LuxSit™ s-Pro Plasmids and incubated for 24 hours. Cells were lysed with 1x Passive lysis buffer (Promega Cat. No. E1941). LuxSit™ Pro Substrate Reagent was added to the cells, and bioluminescence was measured using a standard plate reader. After a 10-minute background read, Rapamycin was added to induce signal. The means of triplicate transfections are graphed with error bars indicating standard deviation. RLU reaches its maximum response 10 minutes after Rapamycin addition.

5. Considerations for Protocol Optimization Using the LuxSit™ s-Pro PPI System

There are several factors to consider when trying to optimize the LuxSit™ s-Pro PPI System kit for your experimental needs, including (1) protein of interest/LuxSit™ piece orientation (2) plate reader/luminometer settings, and (3) the kinetic performance of the LuxSit™ s-Pro split Luciferase.

1. **Protein of Interest: LuxSit™ Piece Orientation.** Monod Bio offers expression plasmids with two orientations for each of the αLux and βit pieces of the s-Pro PPI System. Optimizing the orientation (N- vs. C-terminal) as well as which protein of interest is attached to which LuxSit™ piece is recommended to find the best luminescent response (see **Figure 3** and **Table 1**).

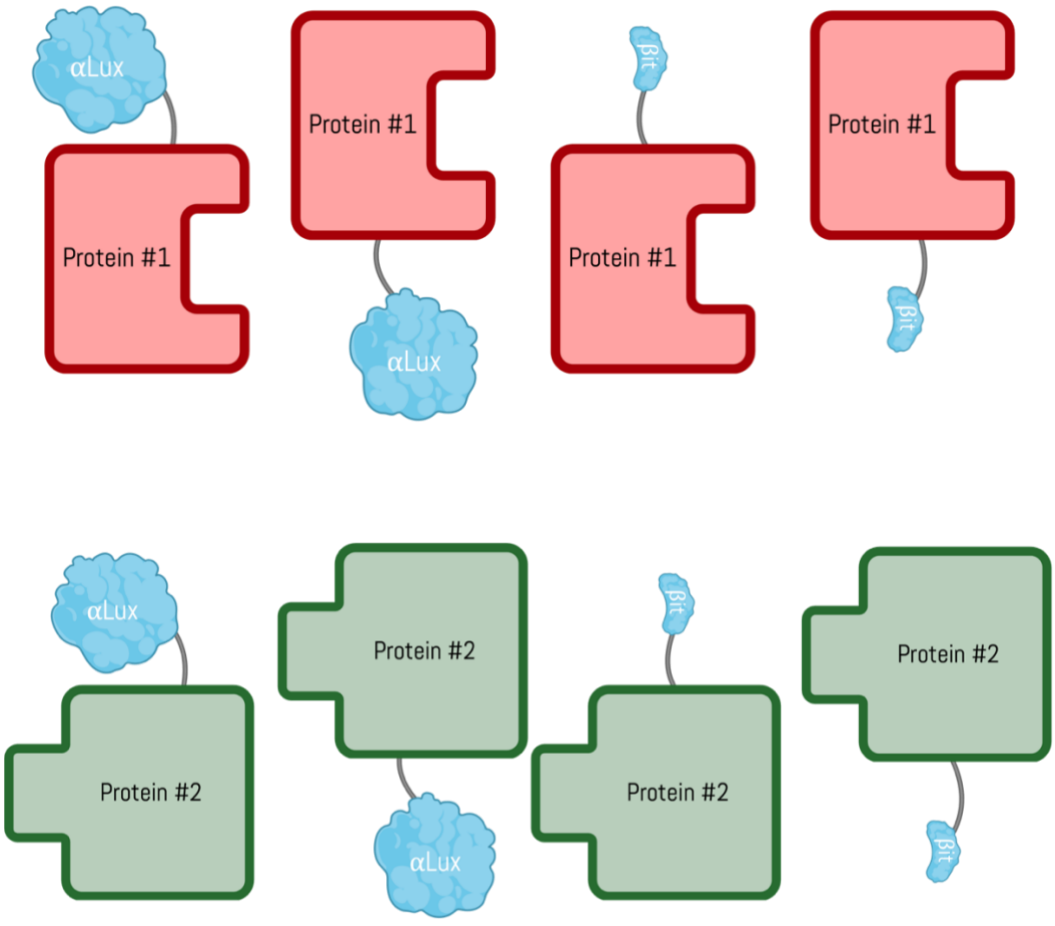

Figure 3: Illustration demonstrating potential expressed combinations and orientations of αLux, βit, Protein of Interest #1, and Protein of Interest #2.

Protein #1 N-term αLux	Protein #2 N-term βit
	Protein #2 C-term βit
Protein #1 C-term αLux	Protein #2 N-term βit
	Protein #2 C-term βit
Protein #1 N-term βit	Protein #2 N-term αLux
	Protein #2 C-term αLux
Protein #1 C-term βit	Protein #2 N-term αLux
	Protein #2 C-term αLux

Table 1. Potential pairings of theoretical Protein#1 and Protein #2 expressed with αLux or βit.

2. **Plate Reader/Luminometer Settings.** 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™ s-Pro PPI System should consider the impact of each of these settings when determining the ideal instrument configuration for your experiment.

   Upon complementation, αLux and βit have a peak emission wavelength of 490nm (**Figure 4**). It is not required to use a filter on the plate reader/luminometer used. However, consideration should be taken on optimizing LuxSit™ s-Pro RLU output if using a filter.

3. **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™ s-Pro PPI 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 5** and **Figure 6**). If using an inducer in their experimental setup, users should consider the EC_{50} concentration of inducer. For example, the EC_{50} of Rapamycin when transfecting the LuxSit™ s-Pro Control Plasmids was calculated for PPI Control Set and plotted (see **Figure 7**).

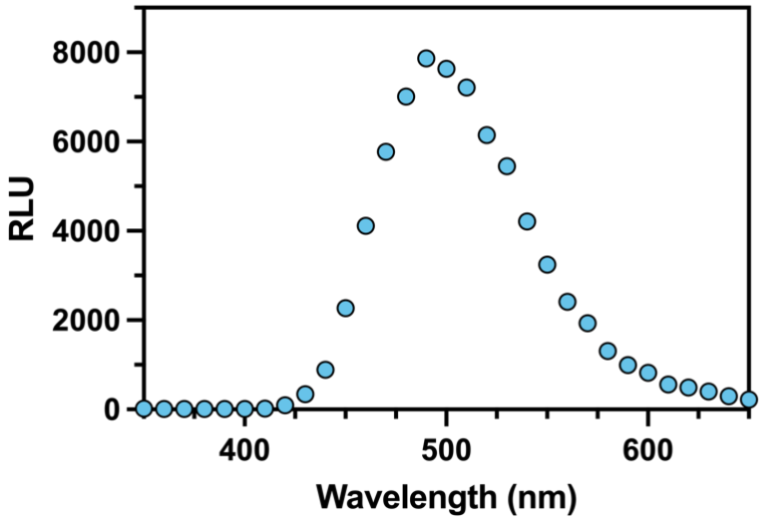

Figure 4: LuxSit™ s-Pro PPI emission spectra. The reconstituted LuxSit™ Pro luciferase has a peak emission wavelength of 490nm.

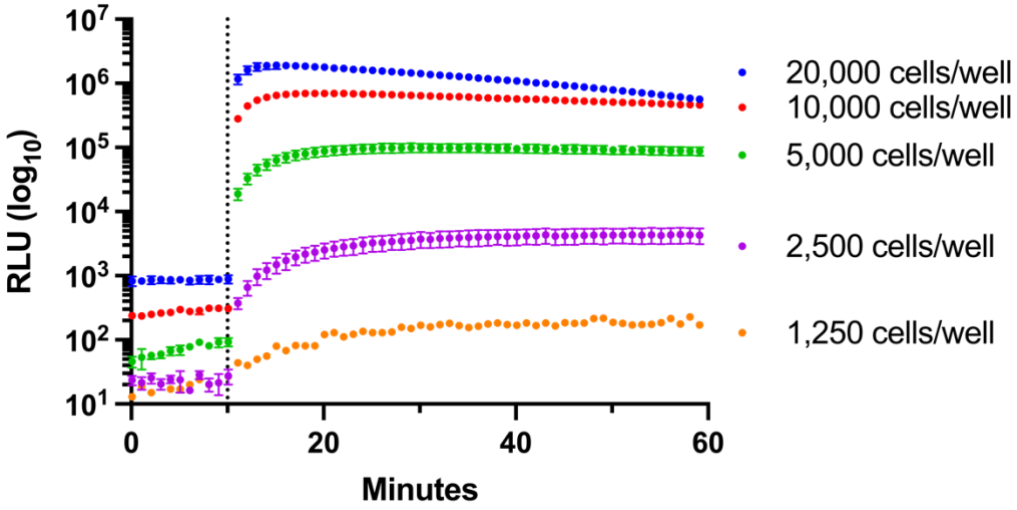

Figure 5. LuxSit™ s-Pro PPI transfection with varying cell densities. Varying amounts of HEK293T cells per well were plated and transfected with 20ng per plasmid/well of LuxSit™ s-Pro PPI Plasmids in a 96-well plate. After overnight incubation, cells were lysed for 15 minutes and then LuxSit™ Pro Substrate diluted in Assay Buffer was added to each well. Kinetics of each concentration are plotted.

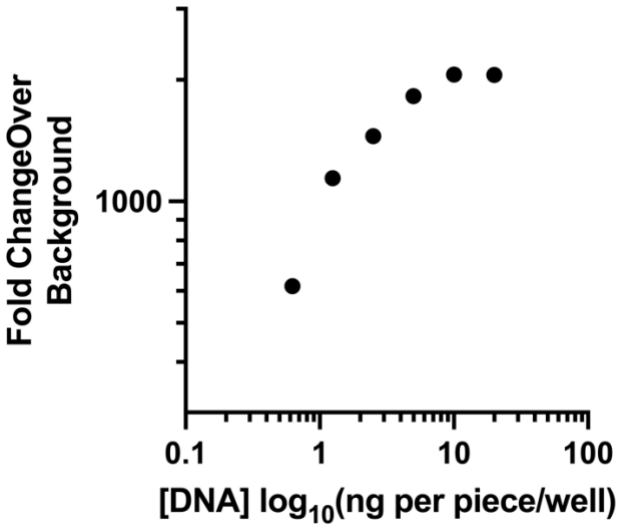

Figure 6. LuxSit™ s-Pro PPI transfection with varying amounts of DNA. 10,000 cells per well of HEK293T were plated and transfected with varying amounts of LuxSit™ s-Pro PPI Plasmids in a 96-well plate. After overnight incubation, cells were lysed for 15 minutes and then LuxSit™ Pro Substrate diluted in Assay Buffer was added to each well. The average luminescent signal (n = 4) of maximum signal after Rapamycin addition was measured and plotted as fold-change over background.

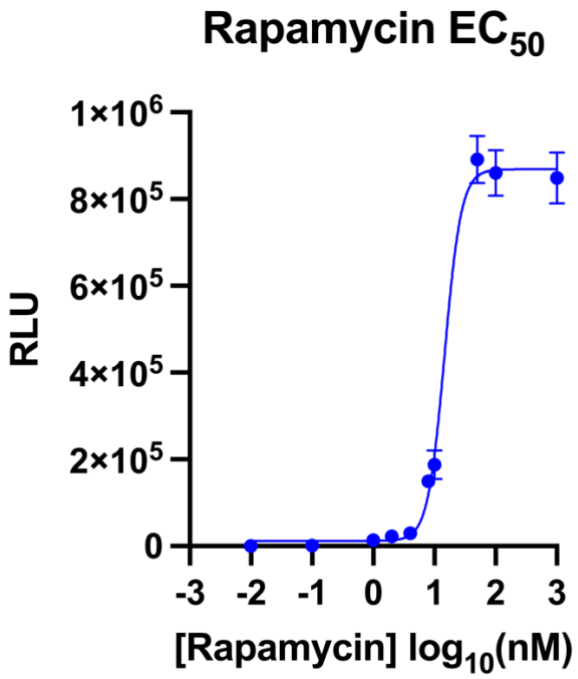

Figure 7. Determination of Rapamycin EC_{50} for LuxSit™ s-Pro PPI Control Plasmids. HEK293T were plated and transfected with LuxSit™ s-Pro PPI Control Set DNA in a 96-well plate. After overnight incubation, cells were lysed for 15 minutes and then LuxSit™ Pro Substrate diluted in Assay Buffer was added to each well. After a 10-minute background read, titrating amounts of Rapamycin were added. The maximum luminescent signal for each Rapamycin concentration over 1 hour (n = 3) is plotted. EC_{50} for this experiment was calculated to be 14nM.

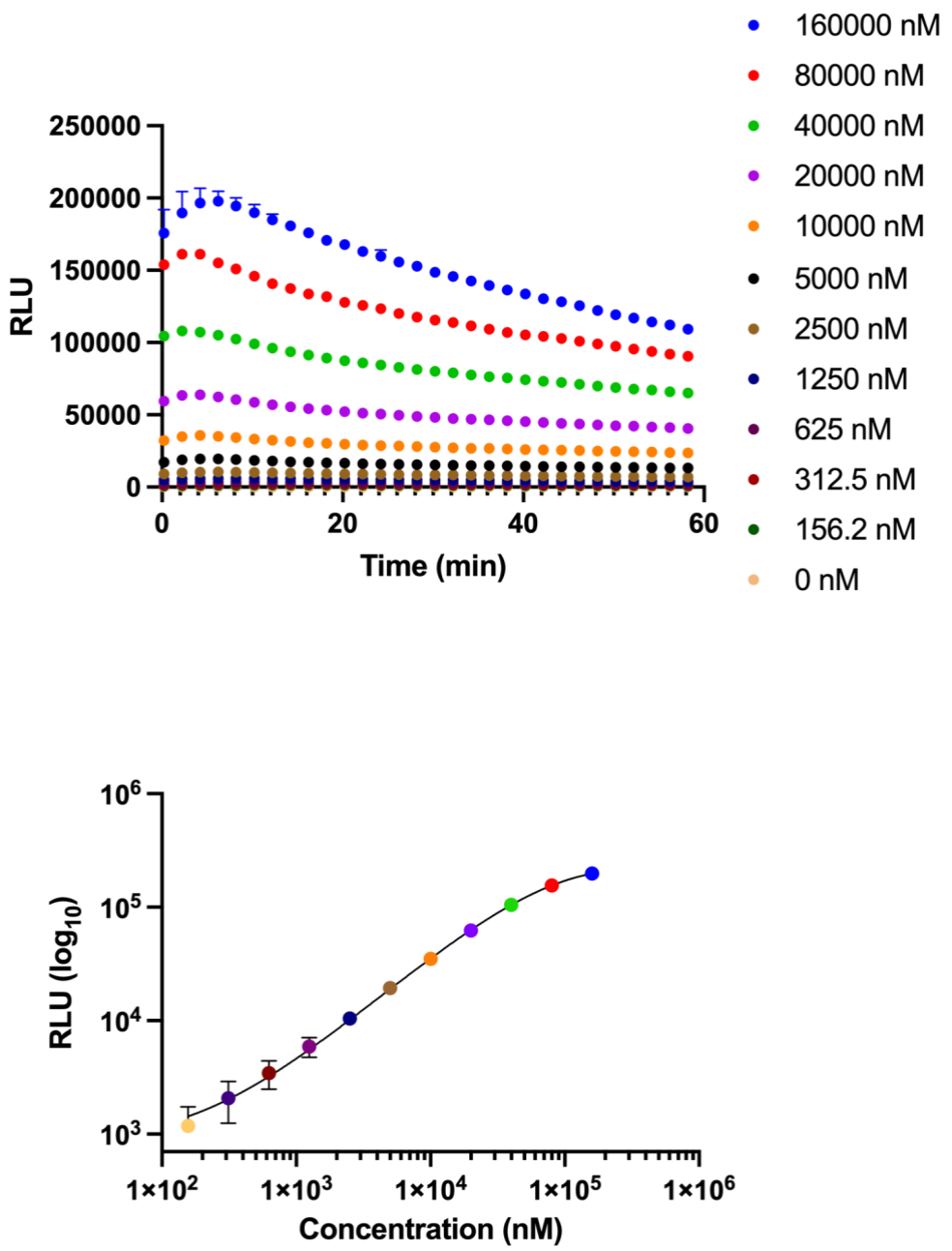

Figure 8. Affinity Determination of the Association Between αLux and βit. A) Luminescence emission kinetics for the interaction of αLux and βit. βit was titrated at concentrations ranging from 160,000 nM down to 0 nM, with a fixed αLux concentration of 4 nM. B) Correlation between the initial RLU signal from the emission kinetics and the concentration of βit. The data was fitted to a one-binding-site model (Y= Bmax*X/(Kd+X) + NS*X + Background where X is the concentration of ligand, Bmax the maximum binding signal in the same units as Y, NS the slope of the nonlinear regression in Y units divided by X units, and Background the measured signal with no added ligand) , yielding a calculated Kd value of 90 μM ± 12 μM.

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
P05101	s-Pro βit, N-term	pcDNA3.1(+)[s-Pro βit]-N	CMV	Yes	N	25 µg	Lyophilized
P05102	s-Pro βit, C-term	pcDNA3.1(+)[s-Pro βit]-C	CMV	Yes	C	25 µg	Lyophilized
P05501	s-Pro αLux, N-term	pcDNA3.1(+)[s-Pro αLux]-N	CMV	Yes	N	25 µg	Lyophilized
P05502	s-Pro αLux, C-term	pcDNA3.1(+)[s-Pro αLux]-C	CMV	Yes	C	25 µg	Lyophilized
P05901a, P05901b	PPI Control Set (FRB:FKBP)	pcDNA3.1(+)[FKBP-s-Pro αLux], pcDNA3.1(+)[FRB-s-Pro βit]	CMV	No	n/a	25 µg	Lyophilized

Related LuxSit™ s-Pro Protein-Protein Interaction Assay Kits (Research Use Only)

Reference	Product Name	Kit Performance Highlights
LS0101	LuxSit™ Pro Luciferase Assay System	❖ Small, hyper stable *de novo* luciferase ❖ Bright and linear dynamic range ❖ Highly specific proprietary synthetic substrate ❖ Peak light emission at 490nm

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.