TECHNICAL MANUAL

Lumit™ Immunoassay Labeling Kit

Instructions for Use of Product
VB2500

Revised 4/22
TM602

Promega

Lumit™ Immunoassay Labeling Kit

All technical literature is available at: www.promega.com/protocols/
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E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The Lumit™ Immunoassay[a–e] is based on NanoLuc® Binary Technology (NanoBiT). NanoBiT® is a structural complementation system designed for biomolecular interaction studies (1). The NanoBiT® system is composed of two subunits, Large BiT (LgBiT; 18kDa) and Small BiT (SmBiT; 11 amino acid peptide), that have been optimized for stability and minimal self-association due to weak affinity (190µM). In Lumit™ Immunoassays, antibodies are labeled with SmBiT and LgBiT subunits. Upon binding of the labeled antibodies to an analyte, the subunits come into close proximity, reassemble into a functional luciferase enzyme and generate a luminescent signal in the presence of substrate (Figure 1). Other immunoassay formats, including competition and indirect immunoassays, can also be developed using this technology (2).

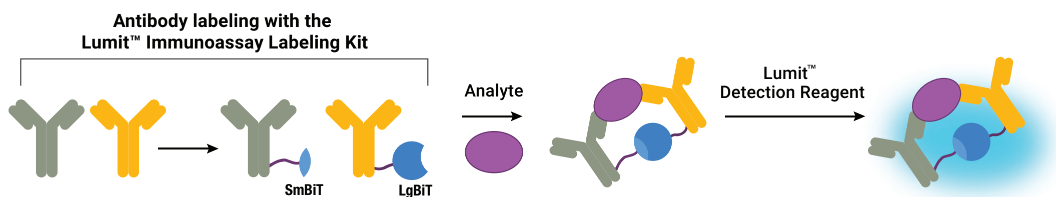

Figure 1. Schematic of Lumit™ Immunoassay.

HaloTag® technology is used to label antibodies with SmBiT and LgBiT (3). HaloTag® is a fusion protein that covalently binds its ligand (HaloTag® Ligand) under physiological conditions and has been used in a variety of applications, including antibody labeling (3). Labeling is a two-step process (Figure 2) in which amine reactive HaloTag® Succinimidyl Ester (O4) ligand (509Da) reacts with primary amines of lysine amino acids on the antibodies. For this reaction, antibodies should be in an amine-free buffer without any protein preservative. Antibodies labeled with HaloTag® Ligand are then incubated with HaloTag®-LgBiT (50kDa) or HaloTag®-SmBiT (31kDa) fusion protein to make a covalent conjugate of antibody-HaloTag®-LgBiT or antibody-HaloTag®-SmBiT.

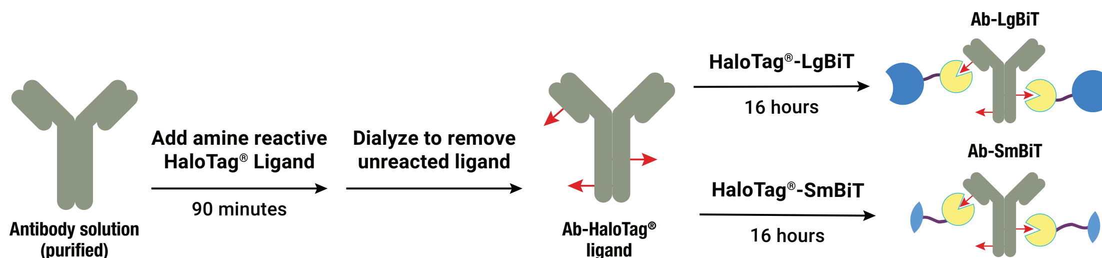

Figure 2. Schematic of antibody labeling with HaloTag®-LgBiT or HaloTag®-SmBiT. A similar approach can be used to label other proteins.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Lumit™ Immunoassay Labeling Kit	**1 each**	**VB2500**

Includes:

- 20µl HaloTag® Ligand (20mM)
- 100µl IGEPAL® CA-630 (100%)
- 350µl Lumit™ HaloTag®-LgBiT (20µM)
- 350µl Lumit™ HaloTag®-SmBiT (20µM)

Notes: The kit will allow 250µg of antibody to be labeled with HaloTag®-SmBiT and 250µg of antibody to be labeled with HaloTag®-LgBiT. The number of assays that can be performed with the reagents may change depending on the type of assay.

Storage Conditions: Store all components at –10°C to –30°C. HaloTag® Ligand is a single-use reagent and should not be stored once opened.

3. Labeling of Antibodies with HaloTag®-SmBiT and HaloTag®-LgBiT

3.A. General Considerations

- This kit is designed to label antibodies with HaloTag®-SmBiT and HaloTag®-LgBiT to enable development of a Lumit™ Immunoassay.
- Section 5 provides guidelines for the number of antibodies and amount needed during the Lumit™ Immunoassay optimization process, and should be considered before starting the labeling reaction.
- Efficiency of labeling depends on the number of accessible lysine amino acids and the protein concentration, and will require optimization. For antibodies, we recommend a concentration of 1.0–5.0mg/ml.
- Antibodies need to be free of any amine-containing buffers (e.g., Tris, Glycine), azide and stabilizer such as BSA. Small-molecule additives and buffers like azide, Tris buffer, sucrose, etc., can be removed by buffer exchange using desalting columns. Follow vendor protocols carefully while using desalting columns to avoid protein losses.
- The optimum pH for labeling the primary amine of lysine is pH 8.5. We recommend 10–100mM sodium bicarbonate buffer (pH 8.5) for labeling. Proteins in other buffer solutions may be buffer-exchanged to adjust the pH. If proteins are in PBS (pH 7.2), add 1/10th the volume of 1M Bicarbonate buffer at pH 8.5 to adjust the pH.
- HaloTag® Ligand is a single-use reagent and should not be stored once opened.
- The following protocol is for labeling antibodies, but any protein containing accessible lysines can be labeled using this kit.

3.A. General Considerations (continued)

- Reagents provided are sufficient to label 250µg of antibody with HaloTag®-LgBiT and 250µg of antibody with HaloTag®-SmBiT. At the recommended concentration of 1.0mg/ml, this amount corresponds to a volume of 250µl, which is easy to handle during buffer exchange steps.
- A critical step in labeling is the complete removal of unreacted HaloTag® Ligand after the reaction with antibody. Any leftover ligand will result in failure of the conjugation of HaloTag®-SmBiT or HaloTag®-LgBiT in the subsequent step.
    - If using the Zeba™ Spin Desalting Column, follow the Zeba™ column protocol exactly as provided by the manufacturer, especially the centrifugation speed and time.
    - We recommend using two Zeba™ columns in series (choose either 7K or 40K MWCO) to ensure complete removal of free ligand. Choose a Zeba™ column of appropriate size according to reaction volume.
        a. For samples of 200–700µl, we recommend 2.0ml Zeba™ columns (Thermo Scientific Cat.# 89889).
        b. For samples of 30–130µl, we recommend 0.5ml Zeba™ columns (Thermo Scientific Cat.# 89882). **Note:** For 250µl antibody samples, split the sample into two 125µl aliquots using 0.5ml Zeba™ columns.

3.B. Materials to Be Supplied by the User

- antibodies at 1.0mg/ml
- 10mM sodium bicarbonate buffer (pH 8.5)
- phosphate buffered saline (PBS)
- Zeba™ Spin Desalting Columns (see Section 3.A for recommendations)

3.C. Labeling Antibodies with HaloTag® Ligands

1. Exchange the antibody buffer to 10mM sodium bicarbonate, pH 8.5, using a desalting column.
2. For antibodies at concentrations of 1.0mg/ml or higher, add 20 molar excess of the ligand to the antibody sample. The table below is provided as a guide.

Antibody Amount (µg)	µl Ligand
100	0.7
250	1.7

Note: 20 molar excess ligand also works for other proteins.

3. For antibody concentrations <1.0mg/ml, add 50 molar excess of the ligand to the antibody sample. The table below is provided as a guide.

Antibody Amount (µg)	µl Ligand
100	1.7
250	4.25

4. Gently mix for 90 minutes at 22–25°C.
5. Buffer exchange the antibody into PBS using a desalting column. We recommend using two desalting columns in series to ensure complete removal of free ligand.
6. Measure the concentration of your antibody labeled with HaloTag® Ligand using absorbance at 280nm. The absorbance (280nm) of 1.0mg/ml antibody is 1.4. For recovery calculations of other proteins, use their respective extinction coefficients.

3.D. Conjugating HaloTag®-SmBiT and HaloTag®-LgBiT to Antibodies

1. The best results have been obtained when a four molar excess of HaloTag®-LgBiT or HaloTag®-SmBiT has been added to antibody labeled with HaloTag® Ligand. For other proteins, this ratio should be optimized by the user.
2. Use the table below as a guide for calculating the volume of HaloTag®-SmBiT or HaloTag®-LgBiT to be added to the antibodies.

Antibody Amount (µg)	HaloTag®-SmBiT or HaloTag®-LgBiT(µl)
100	135
250	335

3. Prepare a 10% IGEPAL® CA-630 solution by adding 900µl of PBS to 100µl of IGEPAL® CA-630. Add the 10% IGEPAL® CA-630 to the antibody-HaloTag® ligand so that the final IGEPAL® CA-630 concentration is 0.05%. IGEPAL® CA-630 may take several hours to dissolve, so the 10% IGEPAL® CA-630 solution should be prepared ahead of time. We suggest simultaneously preparing this solution during the ligand labeling step (Section 3.C).

   Note: IGEPAL® CA-630 helps minimize occasional precipitation of antibody labeled with HaloTag®-SmBiT or HaloTag®-LgBiT.
4. Incubate for 16–20 hours at 4°C with gentle mixing.
5. Save a small aliquot for gel analysis and store the rest of the conjugate at 4°C for short-term storage (1 week), or in 50% glycerol at –20 °C for long-term storage.
6. Run a nonreducing SDS-PAGE gel to confirm labeling. In a successful labeling reaction, multiple higher molecular weight bands will appear (Figure 3).

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3.D. Conjugating HaloTag®-SmBiT and HaloTag®-LgBiT (continued)

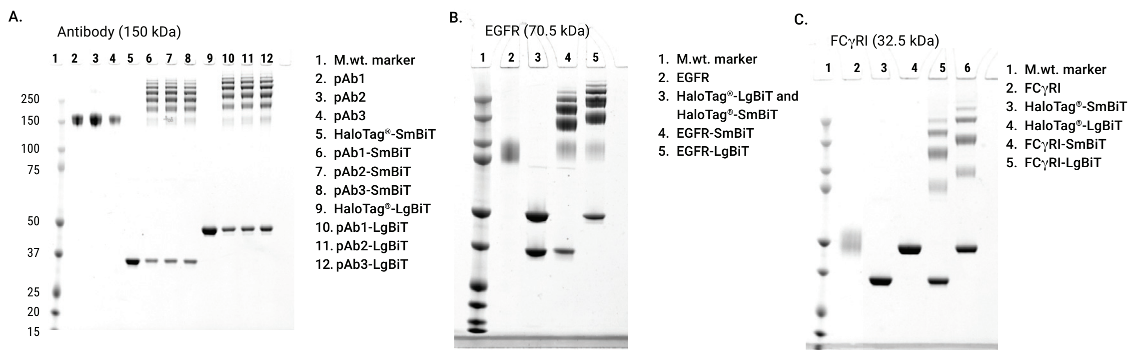


Figure 3. Confirmation of labeling by nonreducing SDS-PAGE gel. Three different polyclonal antibodies (150kDa; **Panel A**), EGFR (70.5kDa; **Panel B**) and FcγR1 (32.5kDa; **Panel C**) were labeled with HaloTag®-LgBiT and HaloTag®-SmBiT. Multiple higher molecular weight bands indicate distribution of protein with one, two, or more HaloTag®-BiT. Unreacted HaloTag®-SmBiT and HaloTag®-LgBiT do not interact due to weak affinity (190µM), but can be removed if needed.

4. Optional Step: Removing Unreacted HaloTag®-BiT using Magne® HaloTag® Beads

Unreacted HaloTag®-SmBiT and HaloTag®-LgBiT in solution do not interact with each other due to weak affinities (190µM), and therefore do not cause significant background. Therefore, we do not recommend removing unreacted HaloTag®-BiT, especially if the reaction volumes are small, because of the risk of significant antibody loss during the removal process. However, once initial experiments are performed to identify the right combination and concentration of HaloTag®-BiT labeled antibodies (see Section 5), and the labeling reaction is scaled up, then the following protocol can be used to remove unreacted HaloTag®-BiT, if desired.

4.A. Materials Needed

- Magne® HaloTag® Beads (Cat.# G7281)
- MagneSphere® Technology Magnetic Separation Stands, 1.5ml (Cat.# Z5332)

4.B. Protocol

Note: Prepare 5ml of PBS containing 0.05% IGEPAL® CA-630 by adding 25µl of 10% IGEPAL® CA-630 (prepared in Section 3.D, Step 3) to 5.0ml of PBS.

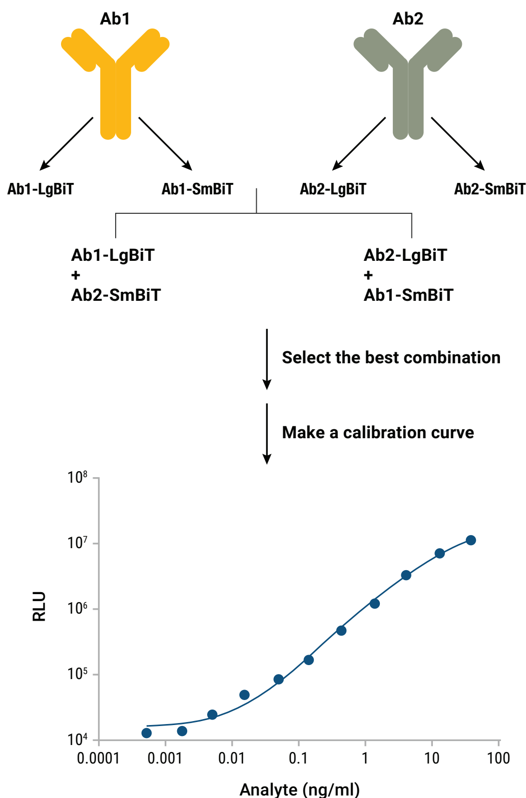
1. Resuspend Magne® HaloTag® Beads by inverting the tube several times.
2. Use the table below as a guide to determine the volume of HaloTag®-BiT and Magne® HaloTag® Beads to use.

HaloTag®-BiT (µl)	Magne® HaloTag® Beads (µl)
135	35
335	85

3. Transfer the required volume of Magne® HaloTag® Beads to a 1.5ml microcentrifuge tube.
4. Place the tube on the magnetic stand for 30 seconds to capture the beads. Carefully remove the supernatant and discard.
5. Remove the tube from the magnetic stand, and add 500µl of PBS containing 0.05% IGEPAL® CA-630.
6. Mix thoroughly for 2 minutes. Place the tube on the magnetic stand for 30 seconds. Carefully remove the supernatant and discard.
7. Repeat the wash two more times for a total of three washes.
8. Add the antibody-BiT prepared in Section 3.D to the equilibrated beads.
9. Incubate for 1 hour at room temperature (22–25°C) with constant mixing. Make sure the beads remain in suspension.
10. Place the tube on the magnetic stand for 30 seconds. Collect and save the supernatant, which contains the cleaned antibody-BiT.
11. Run a nonreducing SDS-PAGE gel to confirm the removal of unreacted HaloTag®-BiT.

5. Guidelines for Setting Up a Lumit™ Immunoassay

The Lumit™ Immunoassay has been used successfully in direct, indirect and competition assay formats. A workflow for optimizing a direct assay format in which two antibodies are labeled with HaloTag®-BiT is shown in Figure 4. This approach can be used as a guideline to optimize other assay formats.



1. Select the right pair of antibodies. Antibody pairs can be (see Section 5.A):
   a. Two mAbs that bind to different epitopes on an analyte
   b. One mAb and one pAb
   c. One pAb, split into two equal portions

2. Label antibodies (see Section 5.B).

3. Run checkerboard experiments to select the right combination and concentrations of labeled antibodies (see Section 5.C).

4. Prepare a calibration curve to determine sensitivity, dynamic range and matrix effect (see Section 5.D). RLU is Relative Light Unit.

Figure 4. A workflow for optimizing the Lumit™ Immunoassay.

5.A. Antibody Selection

The sensitivity and specificity of the Lumit™ Immunoassay depend on the antibodies; therefore, antibody selection is important to take full advantage of the Lumit™ Immunoassay format. The antibodies used in Lumit™ Immunoassays can be monoclonal, polyclonal or a combination, with each option offering distinct features as shown in the table below. A good starting point is to select antibody pairs validated in an ELISA. When using antibodies validated for ELISA or converting an existing ELISA to the Lumit™ Immunoassay format, optimization is required to decide which antibody should be labeled with which HaloTag®-BiT.

Antibody Combinations	Advantages	Comments
Two monoclonal antibodies	• Specificity • Sensitivity • Validated pairs available	• Need two mAbs that will bind two different epitopes on an analyte • Often available in small quantities and sometimes with preservatives that need to be removed before labeling
One monoclonal antibody and one polyclonal antibody	• Specificity • Sensitivity • Validated pairs available	• Polyclonal antibodies may introduce nonspecific binding • Often available in small quantities and sometimes with preservatives that need to be removed before labeling
One polyclonal antibody	• Requires only one antibody • Polyclonal antibodies are typically less expensive than monoclonal antibodies	• Polyclonal antibodies may introduce nonspecific binding • May be less sensitive than the previous two approaches

5.B. Labeling Antibodies with HaloTag®-SmBiT and HaloTag®-LgBiT

Several criteria should be considered during labeling, including number of labels per antibody, activity after labeling, stability of the labeled antibody and long-term storage. Typically, three to four labels per antibody provide the best results; however, the number of labels per antibody (or protein) can be adjusted by adding different amounts of HaloTag®-BiT during the labeling step (Section 3.C). Labeled antibodies are stable at 4°C for several weeks or months, but we recommend that, for long-term storage, antibodies should be stored in 50% glycerol at –20°C. Very rarely, some antibodies (typically monoclonal) precipitate after labeling with HaloTag® Ligand or HaloTag®-BiT. The addition of IGEPAL® CA-630 suggested in Section 3.D helps minimize precipitation. Some other steps are to: a) add IGEPAL® CA-630 throughout all steps (HaloTag® Ligand labeling, buffer exchange and conjugation with HaloTag®-BiT) of the process; and b) reduce the amount of HaloTag® Ligand and HaloTag®-BiT. In the Lumit™ Immunoassay, the typical amount of antibody required per 96-well plate is 2.0µg or less.

We recommend that each of the two antibodies be individually labeled with both HaloTag®-SmBiT and HaloTag®-LgBiT and tested in a checkerboard assay to determine which combination of antibody and HaloTag®-BiT works best. For optimization, we recommend starting with 250µg each of two antibodies and labeling them with HaloTag® Ligand. Typical recoveries after the HaloTag® Ligand labeling step are approximately 200µg for each antibody-ligand, which can be aliquoted into two 1.5ml Eppendorf tubes, with each tube containing 100µg of antibody-ligand, and then labeling one tube of antibody-ligand with HaloTag®-SmBiT and the second tube of antibody-ligand with HaloTag®-LgBiT.

Antibody #1 Labeled with HaloTag® Ligand		**Antibody #2 Labeled with HaloTag® Ligand**	
Tube #1	Tube #2	Tube #3	Tube #4
100µg for labeling with HaloTag®-SmBiT	100µg for labeling with HaloTag®-LgBiT	100µg for labeling with HaloTag®-SmBiT	100µg for labeling with HaloTag®-LgBiT

If using one polyclonal antibody, start with 250µg. After labeling with HaloTag® Ligand, split into two aliquots and label with HaloTag®-SmBiT and HaloTag®-LgBiT.

5.C. Determining Labeled Antibody Combinations and Concentrations

A checkerboard experiment can help determine the best combination and concentration to be used in an assay. Options for checkerboard experiments are provided for two scenarios: a) when a pair of antibodies is used; and b) when one polyclonal antibody is used (Figure 5).

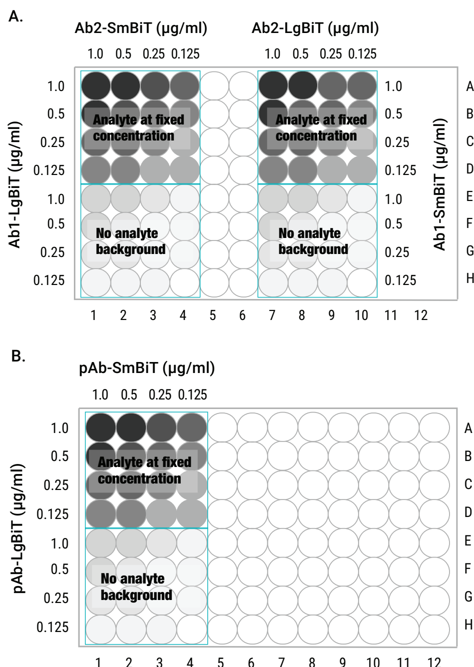


Figure 5. Example of a checkerboard titration experiment to optimize antibody-BiT combination and concentration. Panel A. Experiment to determine the right combination and concentration of two matched pair of antibodies. Matched pairs are available commercially from several vendors, or they can be two antibodies used in optimized ELISAs or other immunoassays. **Panel B.** Experiment using one polyclonal antibody to determine the right concentration. In most cases, 1.0µg/ml antibody concentration is a good starting point. The typical analyte concentration used in these experiments is 20ng/ml. Calculate the S/B ratio from the checkerboard experiment and use the combination that provides the optimum S/B and RLUs.

5.C. Determining Labeled Antibody Combinations and Concentrations (continued)

Assay Protocol for Running a Checkerboard Experiment

In the example shown below, the checkerboard experiment shown in Figure 5.A was performed to determine the optimal antibody-BiT pair and concentration. The volumes of analyte, labeled antibodies, substrate and substrate concentrations can be adjusted depending on the application, RLU and assay performance requirements. Some of the other options are described below.

Assay Type	Analyte (µl)	Ab1-SmBiT (µl)	Ab2-LgBiT (µl)	Substrate (µl)
Biochemical	20	20	20	20[1]
Cell Lysate	50	25	25	25[2]
Cell Supernatant	50	25	25	25[3]

[1]Lumit™ Immunoassay Detection Substrate A diluted 1:50-fold in Lumit™ Immunoassay Buffer A
[2]Lumit™ Immunoassay Detection Substrate A diluted 1:12.5-fold in Lumit™ Immunoassay Buffer A
[3]Lumit™ Immunoassay Detection Substrate B diluted 1:20-fold in Lumit™ Immunoassay Buffer B

If the analyte to be measured is not available in purified form for a checkerboard experiment, then other approaches may be used to determine the right antibody-BiT combination and concentrations. For example, if an intracellular protein needs to be measured, the protein can be degraded or modified using some other method and differences in expression levels can be used to optimize the antibody-BiT. In another example, if the goal is to detect post-translationally modified (PTM) proteins, then a small molecule modulator of PTM can be used for optimizing the antibody-BiT.

Materials to Be Supplied by the User

- Lumit™ Immunoassay Detection Reagent A (Cat.# VB2010)
- white 96-well plate
- multichannel pipette or automated pipetting station
- luminometer capable of reading multi-well plates (e.g., GloMax® Discover System)
- reagent reservoir (e.g., Thermo Scientific Cat.# 8093-11)
- plate shaker
- plate sealer

The following assay protocol is summarized in Figure 6.

1. Dilute the 10X Lumit™ Immunoassay Buffer A to 1X in PBS.
2. Dilute the antibody-SmBiT to 0.125, 0.25, 0.5 and 1µg/ml in 1X Lumit™ Immunoassay Buffer A.
3. Dilute the antibody-LgBiT to 0.125, 0.25, 0.5 and 1µg/ml in 1X Lumit™ Immunoassay Buffer A.
4. Prepare the analyte at 20ng/ml in 1X Lumit™ Immunoassay Buffer A.

Notes:

1. A suggested concentration is 20ng/ml. You may select a sample concentration that is in the middle of the desired dynamic range.
2. The analyte sample is prepared in 1X Lumit™ Immunoassay Buffer A; however the presence of detergent and complex sample matrix will interfere with NanoBiT® complementation (see Table 2 and Section 5.D). If desired, prepare samples in a matrix similar to the real sample and test multiple dilutions to identify any matrix effects and the minimum required dilution.

5. Choose a checkerboard plate setup from Figure 5.
6. Add 20µl of antibody-SmBiT and 20µl antibody-LgBiT to the wells.
7. Add 20µl of analyte sample to the “positive sample” wells.
8. Add 20µl of 1X Lumit™ Immunoassay Buffer A to the “no analyte” wells.
9. Mix gently and incubate for 30–60 minutes at room temperature.
10. Dilute Lumit™ Detection Substrate A 1:50 fold in 1X Lumit™ Immunoassay Buffer A.
11. Add 20µl of Lumit™ Detection Reagent A to each well.
12. Wait 3 minutes and read luminescence.
13. Use absolute luminescence readings, as well as S/B ratios, to select antibody combination and concentrations suitable for subsequent assays.

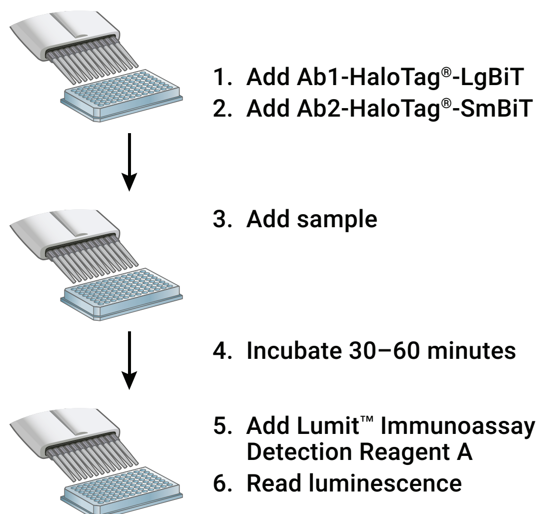


Figure 6. Lumit™ Immunoassay Protocol.

5.D. Preparing a Calibration Curve

If purified analyte is available, then the optimum antibody-BiT pair determined using a checkerboard experiment should be used to prepare a calibration curve and calculate the sensitivity and dynamic range of the assay. Calibration samples should be prepared by spiking purified analyte in the buffer and in the matrix in which analyte is to be measured to determine any matrix effect. Matrix effects may occur in biological samples like cell lysate, plasma and serum. The presence of endogenous proteins, detergents and other additives may cause nonspecific binding by antibodies and increase background. The matrix may also interfere with NanoBiT® complementation, especially if detergents are present, and reduce the signal. Matrix effects can be reduced by diluting the sample, and conditions should be optimized by the user.

5.E. Additional Considerations

Instrumentation: Assay performance will depend on the sensitivity and dynamic range of the luminometer.

Plates: Any white plate will work; however, samples in U-bottom plates are easier to handle. If nonspecific adsorption is a concern then a Corning® Non-Binding Plate (Corning® Cat.# 3600) can be used.

Throughput: The assay can be performed in 96-well plates, 96-well half-area plates or 384-well plates. We suggest keeping the ratios of the reagents and the sample the same while scaling down the assay.

Detection Reagent: Prepare just before use.

Data Analysis: To calculate the lower limit of quantitation (LLOQ), upper limit of quantitation (ULOQ) and dynamic range of an immunoassay, it is important to choose the right curve fitting model. Non-linear four-parameter logistic or five-parameter logistic regression typically provide better curve fitting and higher accuracy; however, log-log or linear plots can often provide satisfactory results.

6. Case Study: Developing a Lumit™ Immunoassay for EGFR Detection in Buffer

1. Two antibody systems were tested for this case study: 1) a combination of pAb and mAb; and 2) one pAb. Reagents used for the assay are listed in Table 1.

Table 1. Antibodies and Purified Proteins Used in the EGFR Lumit™ Immunoassay

Reagents	Supplier	Catalog Number
Purified Human EGFR Extracellular Domain	ACRO Biosystems	EGR-H5222
Goat Polyclonal Antibody (pAb)	R&D Systems	AF231
Recombinant Human mAb IgG1 (mAb) (Cetuximab Biosimilar)	R&D Systems	MAB9577

2. Both the mAb and pAb antibodies were labeled with HaloTag®-SmBiT and HaloTag®-LgBiT as described in Section 5.B.

3. A checkerboard experiment was performed for a combination of mAb-BiT and pAb-BiT and for one pAb-BiT (Figure 7). RLUs and S/B ratios were lower when a combination of mAb-BiT and pAb-BiT were used compared to using a single pAb-BiT. For mAb-BiT and pAb-BiT combinations, 0.25µg/ml of mAb-LgBiT and 0.25µg/ml of pAb-SmBiT provided the optimum combination of S/B and RLUs. For the one pAb-BiT system, 0.5µg/ml of pAb-SmBiT and pAb-LgBiT gave the optimum combination of S/B and RLUs. Other combinations may also work, depending on the intended application.

A.

	mAb-SmBiT (µg/ml)	1.00	0.50	0.25	0.125	
pAb-LgBiT (µg/ml)	1.00	361,300	268,900	186,200	118,300	20ng/ml of EGFR
	0.50	267,500	177,000	164,400	118,700	
	0.25	167,500	139,000	127,300	102,800	
	0.125	92,480	76,450	77,870	66,510	
pAb-LgBiT (µg/ml)	1.00	24,590	17,560	14,270	12,680	No EGFR
	0.50	12,720	9,505	8,834	8,454	
	0.25	11,440	7,333	5,171	4,521	
	0.125	8,794	8,554	8,123	13,660	

Signal/Background

14.7	15.3	13.0	9.3
21.0	18.6	18.6	14.0
14.6	19.0	24.6	22.7
10.5	8.9	9.6	4.9

	mAb-LgBiT (µg/ml)	1.00	0.50	0.25	0.125	
pAb-SmBiT (µg/ml)	1.00	586,300	397,500	234,700	117,300	20ng/ml of EGFR
	0.50	430,200	331,400	224,900	139,400	
	0.25	269,200	225,000	177,800	126,400	
	0.125	151,000	138,600	120,200	93,130	
pAb-SmBiT (µg/ml)	1.00	31,440	15,660	8,223	5,972	No EGFR
	0.50	17,170	9,595	5,752	3,461	
	0.25	11,600	7,633	5,081	4,811	
	0.125	11,320	8,534	8,112	12,160	

Signal/Background

18.6	25.4	28.5	19.6
25.1	34.5	39.1	40.3
23.2	29.5	35.0	26.3
13.3	16.2	14.8	7.7

B.

	pAb-LgBiT (µg/ml)	1.00	0.50	0.25	0.125	
pAb-SmBiT (µg/ml)	1.00	1,960,000	1,250,000	623,100	310,700	20ng/ml of EGFR
	0.50	1,424,000	1,011,000	574,600	298,400	
	0.25	958,900	723,200	438,600	237,500	
	0.125	514,800	431,900	284,900	156,800	
pAb-SmBiT (µg/ml)	1.00	30,070	14,560	7,593	4,481	No EGFR
	0.50	20,450	11,190	7,513	3,421	
	0.25	13,780	8,794	5,191	3,381	
	0.125	12,170	7,713	6,512	4,961	

Signal/Background

65.2	85.9	82.1	69.3
69.6	90.3	76.5	87.2
69.6	82.2	84.5	70.2
42.3	56.0	43.8	31.6

Figure 7. Checkerboard experiment to determine antibody-BiT combination and concentrations for optimum results. Panel A. The experiment was set up using one mAb-BiT and one pAb-BiT against EGFR and **Panel B.** The experiment was set up using one pAb-BiT against EGFR. EGFR at 20ng/ml was used for a positive control.

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6. Case Study: Developing a Lumit™ Immunoassay for EGFR Detection In Buffer (continued)

4. A calibration curve was generated for EGFR using the best antibody-BiT combination and concentration (Figure 8). Data were fitted to a non-linear five-parameter logistic regression equation. In this example, the pAb system was more sensitve than the combination of mAB and pAb.

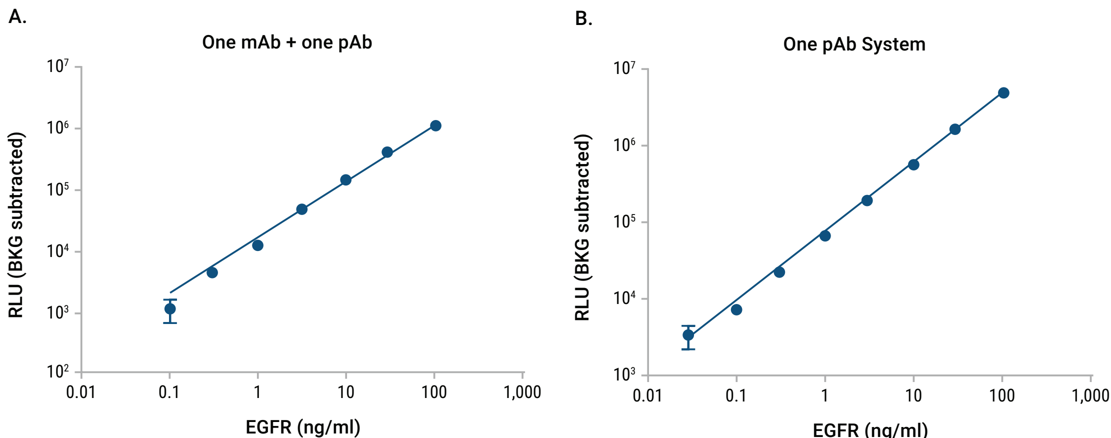

Figure 8. Dose response curve for EGFR. Panel A. Curve generated using 0.25µg/ml of pAb-SmBiT and 0.25µg/ml of mAb-LgBiT. **Panel B.** Curve generated using 0.5µg/ml of pAb-SmBiT and 0.5µg/ml of pAb-LgBiT.

7. Buffers and Additives

The presence of proteins, detergents and other additives can impact the Lumit™ Immunoassay and should be considered during assay development. Additives can prevent NanoBiT® complementation, impact activity of the luminescent enzyme, modify the binding affinities of antibodies, or alter the protein conformation.

Table 2 lists some of the additives and concentration limits for optimal performance in the Lumit™ Immunoassay. The table was generated using a direct immunoassay format and the additive concentrations are the final concentrations in the well after addition of antibody-BiT and the sample. In many cases, if the luminescent signal is sufficiently high, then higher concentrations of additives may be added without impacting assay performance. Using a higher concentration of Lumit™ Detection Reagent can also increase the light output to compensate for the decrease in RLU in the presence of additives.

Table 2. Additive Compatibility with the Lumit™ Immunoassay.

Detergent Additives	Compatibility[a]
Triton™ 100	0.002%
Tergitol™	0.002%
Tween® 80	0.01%
Tween® 20	0.02%
Digitonin	0.02%
SDS	0.001%
CHAPS	0.025%
IGEPAL® CA-630	0.005%

Protein Additives	Compatibility[a]
FBS	1.0mg/ml (0.1%)
Serum	
BSA	
Cell lysate	

Other Additives	Compatibility[a]
DMSO	1.0%
Glycerol	
Sucrose	

[a] Concentration of additives in the reaction well at which absolute signal will drop by 20%.

8. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com** E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Low recovery of antibody after Zeba™ column	Losses during buffer exchange using Zeba™ columns can happen if the vendor-provided protocol is not strictly followed. We recommend using a digital centrifuge so that centrifugation speed can be accurately controlled.
	Sometimes recoveries can be improved if higher molecular weight cutoff filters are used, e.g., 40K instead of 7K. For reference, a typical antibody molecular weight is 150K.
	Add IGEPAL® CA-630 during HaloTag® Ligand conjugation and during Zeba™ column buffer exchange.
Antibody-HaloTag®-BiT conjugate precipitate	Add IGEPAL® CA-630.
	Decrease the amount of HaloTag®-BiT.
No or inefficient antibody conjugation	Make sure there are no amine-containing buffers, azide, sucrose or glycerol. Use an up-front Zeba™ column to remove interfering components.
	Make sure the antibody does not have BSA, and is not supplied as hybridoma supernatant or ascites fluid.
	Increase the antibody concentration, as labeling efficiency is highly concentration dependent.
	If HaloTag® Ligand is not removed completely during desalting, then free ligand will bind to HaloTag®-BiT and prevent it from labeling the antibody.
	Labeling efficiency is also antibody dependent; therefore, switch to another antibody if previous steps fail.

9. References

1. Dixon, A.S. *et al.* (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11(2)**, 400–8.
2. Hwang, B. *et al.* (2020) A homogeneous bioluminescent immunoassay approach to probe cellular signaling pathway regulation. *Commun. Biol.* **3**, 8.
3. Nath, N. *et al.* (2017) Development of NanoLuc bridging immunoassay for detection of anti-drug antibodies. *J. Immunol. Methods* **450**, 17–26.

10. Related Products

Product	Size	Cat.#
Lumit™ Immunoassay Detection Reagent A	500 assays	VB2010
Lumit™ Immunoassay Detection Reagent A	5,000 assays	VB2020
Lumit™ Immunoassay Detection Reagent A	50,000 assays	VB2030
Lumit™ Immunoassay Detection Reagent B	100 assays	VB4050
Lumit™ Immunoassay Detection Reagent B	1,000 assays	VB4060
MagneSphere® Technology Magnetic Separation Stands	1.5ml	Z5332
Magne® HaloTag® Beads	1ml	G7281
Magne® HaloTag® Beads	5ml	G7282

11. Summary of Changes

The following changes were made to the 4/22 revision of this document:

1. In Section 3.A, the use of two Zeba™ columns of the same size, in series, was clarified.
2. Miscellaneous text edits were made to Sections 1 and 2.
3. The cover image was updated.

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[(b)]U.S. Pat. Nos. 9,797,889; 9,797,890; 10,107,800; and other patents and patents pending.

[(c)] U.S. Pat. Nos. 9,416,353; 9,873,866; and 10,246,690, European Pat. Nos. 2341134, 2374875, 2492342, and 2502990; Japanese Pat. Nos. 5680302 and 5840117 and other patents and patents pending.

[(d)] U.S. Pat. Nos. 9,540,402 and 10,101,332, European Pat. Nos. 1594962 and 2369006, Japanese Pat. No. 4748685 and other patents and patents pending.

[(e)]Patents Pending.

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