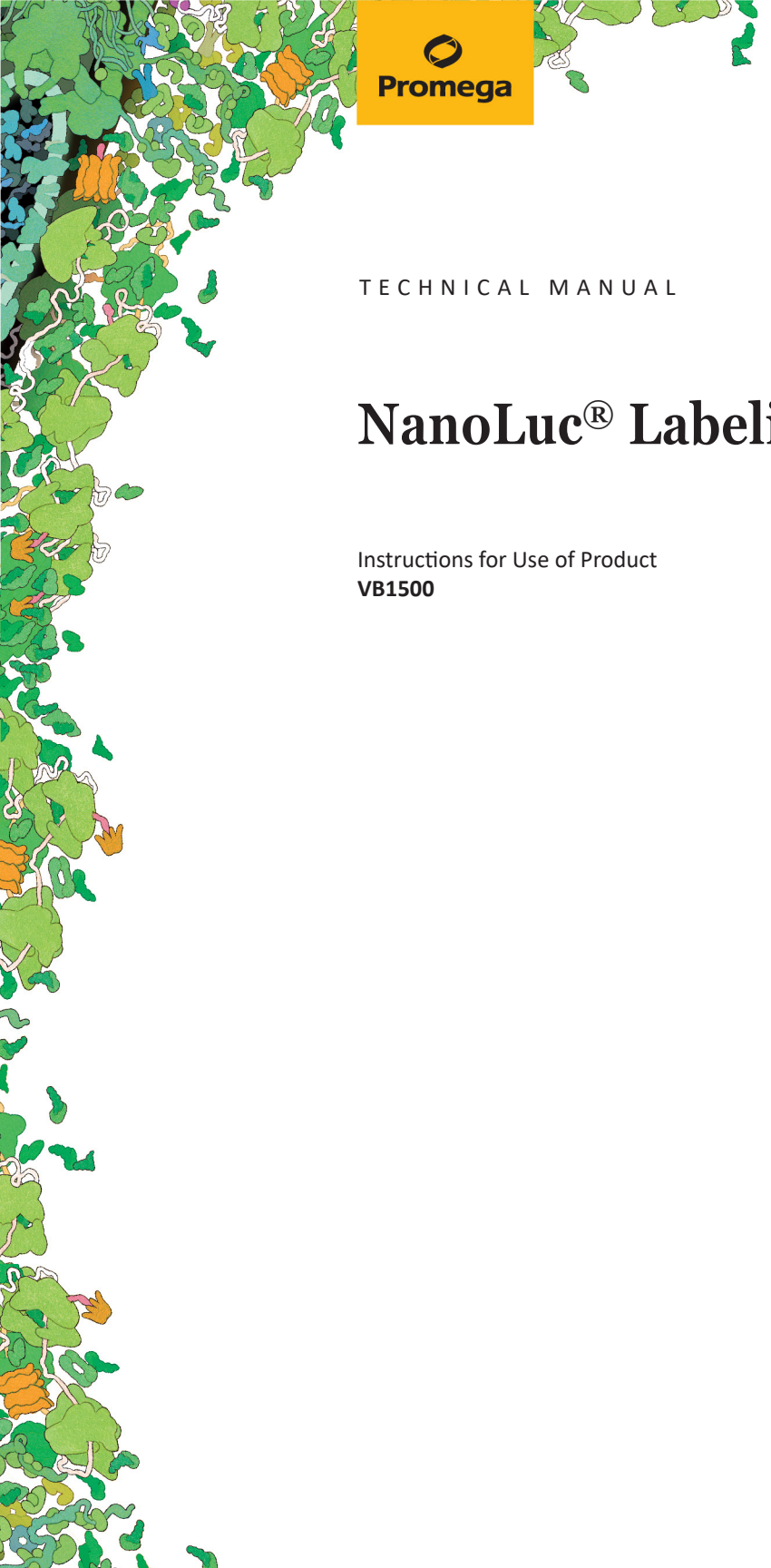

TECHNICAL MANUAL

NanoLuc® Labeling Kit

Instructions for Use of Product
VB1500

7/21
TM666

Promega

NanoLuc® Labeling Kit

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

NanoLuc® luciferase is a small (19.1kDa), stable reporter enzyme that generates a bright luminescence signal. The small size and bright signal make NanoLuc® luciferase a versatile reporter that is used in many protein analysis applications including NanoBRET™ target engagement and protein interaction applications (1). Due to its unique properties, NanoLuc® luciferase is useful as a reporter in enzyme linked immunosorbent assays (ELISA). The NanoLuc® Labeling Kit[a–d] enables labeling of antibodies and other proteins (e.g., streptavidin) with NanoLuc® luciferase for the development of bioluminescent signal-based ELISA.

In this kit, HaloTag® technology is used to label antibodies with NanoLuc® luciferase (2). HaloTag is a fusion protein that covalently binds its ligand under physiological conditions and has been used in variety of applications, including antibody labeling. Labeling is a two-step process (Figure 1) 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 with no protein preservative. Antibodies labeled with HaloTag® Ligand are then incubated with NanoLuc-HaloTag (54.2 kDa) to make a covalent conjugate of antibody-NanoLuc-HaloTag.

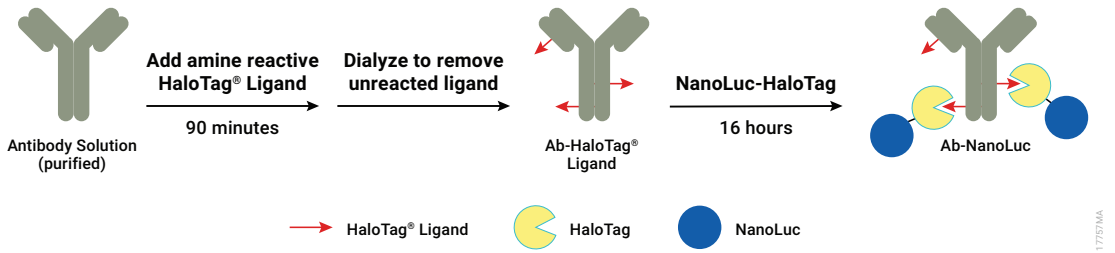

Figure 1. Schematic of antibody labeling with NanoLuc-HaloTag. A similar approach can be used to label other proteins.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
NanoLuc® Labeling Kit	**1 each**	**VB1500**

Not for Medical Diagnostic Use. Includes:

- 20µl HaloTag® Ligand (20mM)
- 100µl IGEPAL® CA-630 (100%)
- 350µl NanoLuc-HaloTag (20µM)

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

Note: Reagents provided are sufficient to label 250µg of antibody with NanoLuc-HaloTag 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.

3. Antibody Labeling with NanoLuc-HaloTag

3.A. General Considerations

This kit is designed to label antibodies and other proteins with NanoLuc-HaloTag for use in ELISA and other applications. The protocol provided in Section 3.B is for labeling antibodies, but any protein containing accessible lysines can be labeled using this kit. Section 5 provides guidelines for the setting up ELISA and should be considered before starting the labeling reaction.

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 NanoLuc-HaloTag in the subsequent step.

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 stabilizers such as BSA. Small-molecule additives and buffers like azide, Tris buffer, sucrose and others can be removed by buffer exchange using desalting columns. Follow vendor protocols carefully while using desalting columns to avoid protein loss.

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 volume of 1M sodium bicarbonate buffer at pH 8.5 to adjust the pH.

HaloTag® Ligand is a single-use reagent and should not be stored once opened.

Notes:

a. If using the Zeba™ Spin Desalting Column, follow the Zeba™ column protocol exactly as provided by the manufacturer, especially the centrifugation speed and time.

b. We recommend using two Zeba™ columns (7K or 40K MWCO) in series, to ensure complete removal of free ligand. Choose a Zeba™ column size according to reaction volume. See Materials to Be Supplied by the User, below, for column size recommendations.

Materials to Be Supplied by the User

- antibodies at 1.0mg/ml
- sodium bicarbonate buffer, 10mM (pH 8.5)
- phosphate buffered saline (PBS)
- Zeba™ Spin desalting columns: For 200–700µl samples we recommend 2.0ml Zeba™ columns (Thermo Scientific Cat.# 89889). For 30–130µl samples we recommend 0.5ml Zeba™ columns (Thermo Scientific Cat.# 89882).

3.B. Before You Begin

Prepare a 10% IGEPAL® CA-630 solution by adding 900µl of PBS to 100µl of IGEPAL® CA-630. Mix gently on a rocking platform or by rotating for 30–90 minutes at room temperature. IGEPAL® CA-630 can take several hours to dissolve, so the 10% IGEPAL® CA-630 solution should be prepared ahead of time. This 10% solution is added in Section 3.D, Step 2.

3.C. Labeling Antibodies with HaloTag® Ligand

1. Exchange the antibody buffer to 10mM sodium bicarbonate (pH 8.5) using a desalting column or by direct addition of 1/10th volume of 1M sodium bicarbonate buffer if the antibody is in PBS (pH 7.2) buffer.
2. For antibody concentrations of **1.0mg/ml or higher**, add a 20 molar excess of ligand to the antibody sample. The table below is provided as a guide.

Antibody (µg)	Ligand (µl)
100	0.7
250	1.7

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

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

Antibody (µg)	Ligand (µl)
100	1.7
250	4.25

3. Mix gently for 90 minutes at 22–25°C.
4. 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.
5. 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 NanoLuc-HaloTag to Antibodies

1. The best results have been obtained when a 4 molar excess of NanoLuc-HaloTag is added to antibody labeled with HaloTag® Ligand. For other proteins, this ratio should be optimized by the user. Use the table below as a guide for calculating the volume of NanoLuc-HaloTag to be added to antibody.

Antibody (µg)	NanoLuc-HaloTag (µl)
100	135
250	335

2. Add the 10% IGEPAL® CA-630 (Section 3.B) to the antibody-HaloTag® Ligand conjugate so that the final IGEPAL® CA-630 concentration is 0.05%.

   Note: IGEPAL® CA-630 helps minimize occasional precipitation of antibody labeled with NanoLuc-HaloTag.

3. Add the NanoLuc-HaloTag calculated in Step 1 of this section, to the antibody-HaloTag® Ligand conjugate.
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 2).

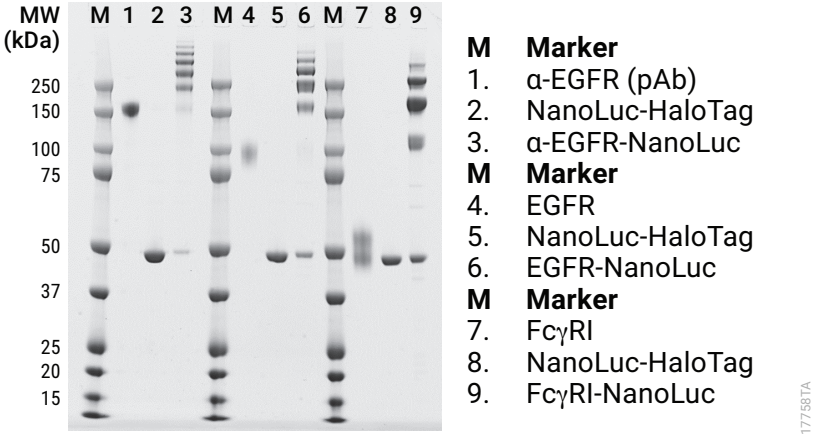

Figure 2. Confirmation of labeling by nonreducing SDS-PAGE gel. Polyclonal antibody (150kDa) against EGFR (α-EGFR (pAb)), EGFR (70.5kDa) and FcγR1 (32.5kDa) were labeled with NanoLuc-HaloTag. Multiple higher molecular weight bands indicate distribution of protein with one, two or more NanoLuc®-HaloTag® labels. Removing unreacted NanoLuc-HaloTag is not required as it will be washed away during the ELISA wash step, but if needed, NanoLuc-HaloTag can be removed as described in Section 4. Molecular weight marker (M) is Precision Plus Protein™ Dual Color Standards (Bio-Rad Cat.# 161-0374).

4. Optional: Removing Unreacted NanoLuc®-HaloTag® Protein using Magne® HaloTag® Beads

Unreacted NanoLuc®-HaloTag® protein in solution will wash off during the ELISA process. For this reason we do not recommend removing unreacted NanoLuc®-HaloTag® protein, especially if the reaction volumes are small, because of the risk of significant antibody loss during the removal process. However, the following optional protocol can be used to remove unreacted NanoLuc®-HaloTag® protein, if desired.

Materials to Be Supplied by the User

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

4.A. Protocol

1. Prepare 5ml of PBS containing 0.05% IGEPAL® CA-630 by adding 25µl of 10% IGEPAL® CA-630 (prepared in Section 3.B) to 5.0ml of PBS.
2. Resuspend the Magne® HaloTag® Beads by inverting the tube several times.
3. Use the table below as a guide to determine the volume of NanoLuc®-HaloTag® protein and Magne® HaloTag® Beads to use.

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

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

5. Case Study: ELISA for Detecting EGFR

Introduction to ELISA development methods is widely available in the published literature (3) and should be consulted before developing any new assay. Below we describe a general protocol for running a direct ELISA using NanoLuc-labeled detection antibody (Figure 3, Panel A) and an indirect ELISA using streptavidin-labeled NanoLuc (Figure 3, Panel B).

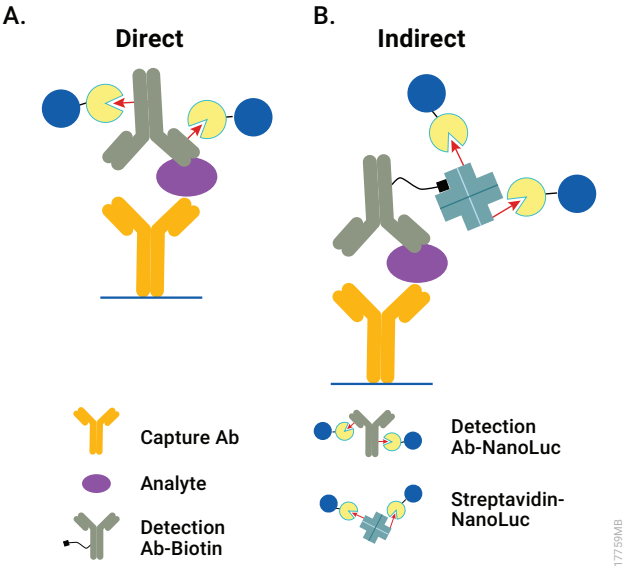

Figure 3. Schematic diagram of direct and indirect ELISA. Direct ELISA (**Panel A**) uses a primary antibody labeled with NanoLuc for detection. Indirect ELISA (**Panel B**) uses a primary antibody labeled with biotin followed by streptavidin labeled with NanoLuc. A different type of indirect ELISA uses unlabeled primary antibody followed by a secondary antibody labeled with NanoLuc.

Materials to Be Supplied by the User

- coating buffer: (100mM bicarbonate buffer (pH 9.6) or PBS)
- antibodies and proteins (Sections 5.B–D)
- wash buffer: PBS containing 0.05% Tween 20 (PBST)
- Lumit™ Immunoassay Detection Reagent A (Cat.# VB2010; contains Lumit™ Immunoassay Dilution Buffer A, 10X, and Lumit™ Detection Substrate A)
- white 96-well plate suitable for ELISA (e.g., Corning® Cat.# 3922)
- luminometer capable of reading multiwell plates (e.g., GloMax® Discover System, Cat.# GM3000)
- reagent reservoir (e.g., Thermo Scientific Cat.# 8093-11)
- plate shaker
- plate sealer

Reagents to be Supplied by the User

- purified Human EGFR Extracellular Domain (e.g., ACRO Biosystems Cat.# EGR-H5222)
- anti-EGFR recombinant Human monoclonal antibody (IgG1; e.g., R&D Systems Cat.# MAB9577)
- anti-EGFR goat polyclonal antibody (e.g., R&D Systems Cat.# AF231; to be labeled with NanoLuc)
- anti-EGFR goat polyclonal antibody-biotin (e.g., R&D Systems Cat.# BAF231)
- streptavidin (e.g., Sigma Cat.# 85878; to be labeled with NanoLuc)

5.A. Preparing Capture Plates

1. Dilute the 10X Lumit™ Immunoassay Buffer A to 1X in PBS. Approximately 50ml is needed per 96-well plate.
2. Dilute capture mAb (Anti-EGFR recombinant human monoclonal antibody; IgG1) to 2–10µg/ml in coating buffer.
3. Add 50µl of diluted capture monoclonal antibody from Step 2 to each plate well.
4. Incubate plate with shaking at room temperature (22–25°C) for 2 hours or overnight at 4°C.
5. Wash the plate three times with 200µl of wash buffer, discarding the solution after each wash.
6. To block the plate, add 200µl of 1X Lumit Immunoassay Buffer A to each well and incubate for at least 1 hour at room temperature (22–25°C) or overnight at 4°C. Other blocking buffers (e.g., 1% BSA solution in PBS) can also be used.

5.B. EGFR Binding

1. Make a dilution series of EGFR in the 1X Lumit Immunoassay Buffer A and transfer 50µl to each plate well.
2. Incubate the plate with shaking at room temperature (22–25°C) for 1 hour.
3. Wash the plate three times with 200µl of wash buffer, discarding the solution after each wash.
4. See Section 5.C for **direct** ELISA using anti-EGFR antibody labeled with NanoLuc® luciferase for detection or Section 5.D for **indirect** ELISA using streptavidin labeled with NanoLuc® luciferase for detection.

5.C. Direct ELISA with Anti-EGFR Antibody Labeled with NanoLuc

1. Dilute anti-EGFR goat polyclonal antibody labeled with NanoLuc to 0.03µg/ml in 1X Lumit™ Immunoassay Buffer A.

   Note: For other systems, the amount of NanoLuc®-labeled antibody may need to be optimized for best results. A good starting concentration is 0.1µg/ml.
2. Add 50µl of labeled antibody per well.
3. Incubate with shaking at room temperature (22–25°C) for 1 hour.
4. Wash the plate three times with 200µl of wash buffer. Discard solution after each wash.
5. Continue to Section 5.E for detection.

5.D. Indirect ELISA with NanoLuc®-Labeled Streptavidin

1. Dilute anti-EGFR goat polyclonal antibody-biotin to 0.03µg/ml in 1X Lumit™ Immunoassay Buffer A.
   Note: For other systems, the amount of biotin-labeled antibody should be optimized for best results.
2. Add 50µl of diluted antibody per well.
3. Incubate with shaking at room temperature (22–25°C) for 1 hour.
4. Wash the plate three times with 200µl of wash buffer, discarding solution after each wash.
5. Dilute NanoLuc®-labeled streptavidin to 0.03µg/ml in 1X Lumit™ Immunoassay Buffer A.
   Note: For other systems, the amount of NanoLuc®-labeled streptavidin should be optimized for best results. A good starting concentration is 0.1µg/ml.
6. Incubate with shaking at room temperature (22–25°C) for 1 hour.
7. Wash the plate three times with 200µl of wash buffer, discarding solution after each wash.
8. Continue to Section 5.E for detection.

5.E. Detecting NanoLuc® Luciferase

1. Dilute Lumit™ Detection Substrate A 1:50-fold in 1X Lumit™ Immunoassay Buffer A to create Lumit™ Detection Reagent A.
2. Add 50µl of Lumit™ Detection Reagent A to each well.
3. Wait 3 minutes and measure luminescence.

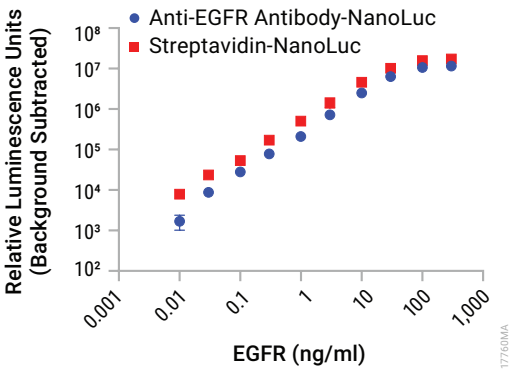

Figure 4. Dose-response curve for EGFR. Direct ELISA using anti-EGFR antibody labeled with NanoLuc, and indirect ELISA using anti-EGFR antibody labeled with biotin in combination with streptavidin-labeled NanoLuc. Each data point is the average plus or minus the standard deviation of three readings.

6. 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.
	Recoveries can be improved if higher molecular weight cutoff filters are used, e.g., 40kDa instead of 7kDa. For reference, a typical antibody molecular weight is 150kDa.
	Add IGEPAL® CA-630 during HaloTag® Ligand conjugation and during Zeba™ column buffer exchange.
Antibody-NanoLuc-HaloTag	Add IGEPAL® CA-630.
conjugate precipitate	Decrease the amount of NanoLuc-HaloTag.
No or inefficient antibody conjugation	Make sure there are no amine-containing buffers, azide, sucrose or glycerol. Use an upfront 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, free ligand will bind to NanoLuc-HaloTag and prevent it from labeling the antibody.
	Labeling efficiency is also antibody dependent; therefore, switch to another antibody if previous steps fail.
High backgrounds	Optimize the concentration of NanoLuc® detection reagent.

7. References

1. NanoLuc® Luciferase Technology web page: **www.promega.com/Resources/Technologies/nanoluc-luciferase-enzyme/** Accessed 06/15/21.
2. Nath, N. *et al.* (2017) Development of NanoLuc bridging immunoassay for detection of anti-drug antibodies. *J. Immunol. Methods* **450**, 17–26.
3. Cox, K.L. *et a*l. Immunoassay Methods. 2012 May 1 [Updated 2019 Jul 8]. In: Markossian S., Sittampalam G.S., Grossman A., *et al.*, editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences. Available from: **https://www.ncbi.nlm.nih.gov/books/NBK92434/**

8. Related Products

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

*When used with the NanoLuc® Labeling Kit, the number of assays that can be performed decreases to 200 (Cat.# VB2010), 2,000 (Cat.# VB2020) and 20,000 (Cat.# VB2030).

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[a]**NanoLuc® Labeling System Limited Use Label License**

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Researcher may use this product for research use only and may link NanoLuc®-HaloTag® fusion proteins to researcher-supplied moieties. No transfer or commercial use of this product or derivatives is allowed. Commercial use means any and all uses of this product or derivatives by a party in exchange for consideration, including, but not limited to (1) use in further product manufacture; and (2) resale of the product or its derivatives, whether or not such product or derivatives are resold for use in research. Notwithstanding the foregoing, researcher may use this product or derivatives in provision of services, information or data to third parties in exchange for consideration, provided that researcher does not transfer the product or derivatives. No other use of this product or its derivatives is authorized without the prior express written consent of Promega.

In addition, researcher must either:

(i) use Lumit™-branded luminescent assay reagents (LARs) for all determinations of luminescence activity of this product and its derivatives; or

(ii) contact Promega to obtain a license for use of the product and its derivatives with LARs not manufactured by Promega.

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[b]U.S. Pat. No. 9,540,402; European Pat. No. 2369006; Japanese Pat. No. 474868; and other patents.

[c]U.S. Pat. Nos. 9,416,353; 9,873,866; and 10,246,690; European Pat. Nos. 2341134; 2374875; 2395078; 2492342; and 2502990; Japanese Pat. Nos. 5680302 and 5840117; and other patents.

[d]U.S. Pat. No. 10,233,485 and 10,774,364; European Pat. Nos. 2990478 and 3498710; Japanese Pat. No. 6539689; and other patents and patents pending.