Lumit™ Immunoassays: A Rapid and Sensitive Method for Analyte Detection

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Benefits:

- Simple add-mix-read protocol with no washing steps
- Direct analyte measurement in cell culture plates or on medium removed from cells
- No immobilization to plates, beads or other surfaces required
- Sensitive luminescence detection with wide dynamic range

Abstract

The detection of a specific analyte (small or large molecule) in a complex mixture is a common procedure in many bioanalytical workflows. Immunoassays rely on the interaction between antibodies and the analyte of interest, and heterogeneous methods—such as Western blots and enzyme-linked immunosorbent assays (ELISAs)—are widely popular. However, Western blots require multiple transfer, separation and wash steps, significantly limiting the throughput of this method. Although ELISAs are somewhat more amenable to screening applications, they also involve sample transfer and multiple washing steps. Both procedures are labor-intensive and time-consuming, requiring several hours or overnight incubations to complete.

The homogeneous Lumit™ Immunoassays provide a faster, more reliable alternative to ELISAs and Western blots, eliminating the variability associated with transfers and multiple wash steps, while offering the convenience of a simple and sensitive luminescent detection method. Because of these advantages, Lumit™ Immunoassays are easily adaptable to automation for laboratories processing large numbers of samples. This white paper presents an overview of Lumit™ Immunoassay technology and provides examples of popular applications to detect and quantify cytokines, protein phosphorylation, and antibody-Fc receptor binding.

Assay Principle and Configurations

Lumit™ Immunoassay technology is based on an adaptation of NanoLuc® bioluminescence. NanoLuc® luciferase is an engineered 19 kDa enzyme derived from the deep-sea bioluminescent shrimp, *Oplophorus gracilirostris* (1). We engineered the native enzyme to develop NanoLuc® Binary Technology (NanoBiT®), a structural complementation reporter system that is ideal for protein:protein interaction (PPI) studies.

NanoBiT® systems are based on a Large BiT (LgBiT; 18kDa) subunit and a small, complementary peptide, Small BiT (SmBiT; 11 amino acids), which has been optimized to have very low affinity for LgBiT (K_d ~190µM). The LgBiT or SmBiT subunits can be expressed as fusions to target proteins of interest. When the two target proteins interact, the NanoBiT® subunits associate to form an active luciferase enzyme and generate a bright luminescent signal in the presence of substrate (2). The small size of the NanoBiT® protein tags minimizes interference with normal host protein function, and the bright signal accommodates low, native expression levels. The negligible spontaneous association of the NanoBiT® subunits, combined with live-cell detection, enables kinetic, real-time analysis of protein interaction dynamics.

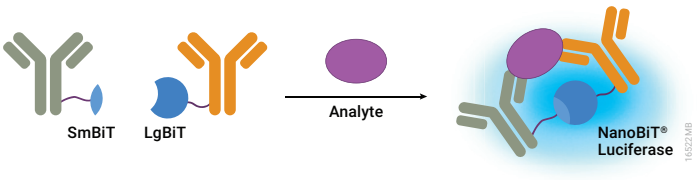

Figure 1. Principle of the Lumit™ Immunoassay. The assay uses affinity reagents and NanoBiT® complementation to deliver sensitive bioluminescence detection of analyte.

In Lumit™ Immunoassays, separate antibodies are chemically labeled with SmBiT and LgBiT, respectively. When the two antibodies come into close proximity in the presence of analyte, SmBiT and LgBiT form an active enzyme and generate a bright luminescence signal (Figure 1).

Lumit™ Immunoassays offer a completely homogeneous, faster, and less laborious workflow than ELISAs, together with sensitive luminescent detection (Figure 2). The method is also amenable to automation for high-throughput sample processing.

Lumit™ Immunoassays are available in three basic configurations: direct, indirect and competitive binding (Figure 3). In the direct configuration, SmBiT and LgBiT are conjugated directly to primary antibodies that recognize the same analyte. This approach maximizes the number of antibody pairs that can be evaluated for assay development, as there are no restrictions on the host species of either primary antibody to be combined.

In the indirect configuration, SmBiT and LgBiT are conjugated to a pair of secondary antibodies against two different species, then combined with primary antibodies that recognize the analyte. The advantage of the indirect configuration is that available, labeled secondary antibodies can be rapidly used for assay development with unlabeled primary antibodies, as long as they are from the appropriate species.

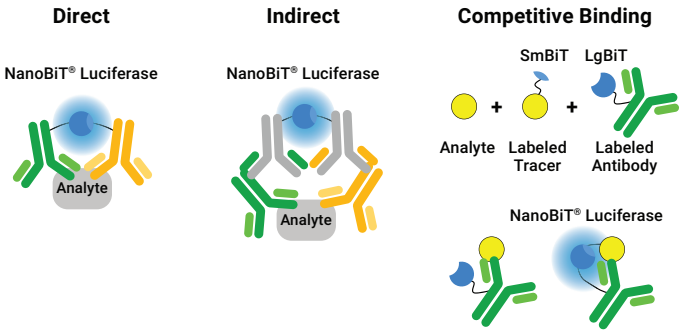

Figure 3. Lumit™ Immunoassay formats. Currently available assays use either direct, indirect or competitive binding formats.

Finally, in the competitive binding configuration, an analyte molecule is labeled with one of the NanoBiT® subunits to form a tracer. This tracer can then be used to compete with unlabeled analyte for binding to an antibody (or other affinity reagent) labeled with the complementary NanoBiT® subunit. Thus, the reaction enables sensitive determination of analyte levels and/or analyte-antibody (or other target) binding properties.

For researchers who wish to label antibodies (or other proteins) and build their own Lumit™ Immunoassays, we offer Lumit™ labeling and detection reagents. Specific kit configurations are also available with prelabeled antibodies, as illustrated in the following applications.

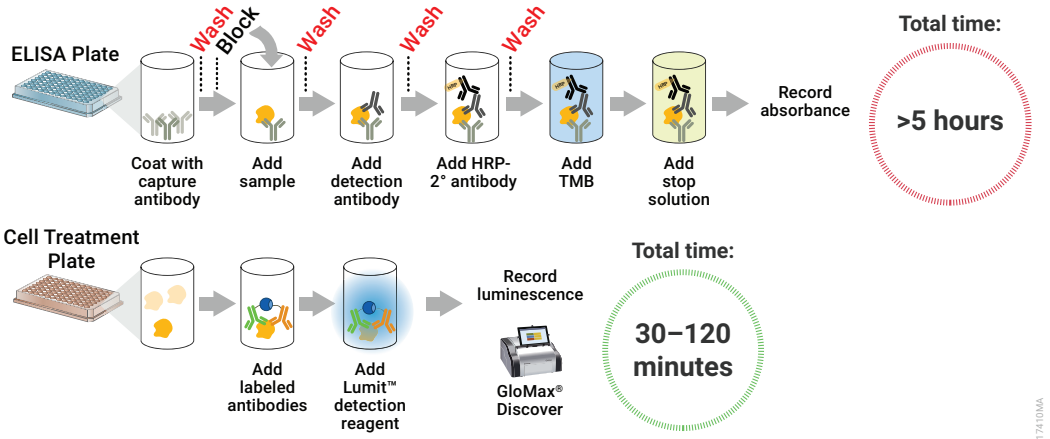

Figure 2. Lumit™ Immunoassays are a powerful alternative to conventional ELISAs. Traditional ELISAs are heterogenous, time-consuming and involve multiple steps. In contrast, Lumit™ Immunoassays involve a simple, add-mix-read protocol and can be completed in less than 2 hours.

Applications

Cytokine Detection

Cytokines are small proteins secreted by a variety of immune and nonimmune cells, and they play critical inflammatory and immunomodulatory roles in the body. They use multiple signaling pathways and define an important class of drugs and drug targets. Lumit™ Cytokine Detection Immunoassays quantitatively measure specific cytokines in cell culture samples with a simple, no-wash assay protocol. The assays can be performed directly in culture wells or on medium transferred from cell plates.

Interleukin 1 beta (IL-1β) is critical to innate immunity as a key proinflammatory cytokine that responds to microbial infection or tissue injury (3). It is processed and released subsequent to formation of the inflammasome, a multiprotein complex assembled in response to pathogens and other damage-associated molecular patterns. Mature, active IL-1β is processed from an inactive precursor, pro-IL-1β, by caspase-1, a key component of the inflammasome.

This example illustrates the use of the Lumit™ Human IL-1β Immunoassay to study modulation of the NLRP3 inflammasome, a leucine-rich repeat (LRR)-containing protein (NLR) family member (Figure 4). The NLRP3 inflammasome inhibitor, MCC950, prevents inflammasome oligomerization, caspase-1 activation and release of mature IL-1β (4). We titrated MCC950 into the culture medium of differentiated THP-1 cells (5×10^4/well), followed by addition of lipopolysaccharide (LPS; 20 EU/ml) to activate the inflammatory response via the NLRP3 inflammasome. We compared these samples to controls without LPS treatment. After treatment for 5 hours, we performed the Lumit™ Human IL-1β Immunoassay and determined an IC_{50} of 35nM for MCC950 inhibition of inflammasome activity.

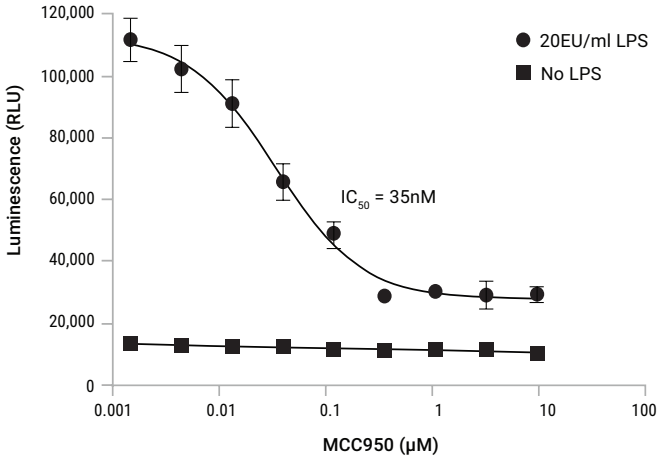

Figure 4. Measurement of the ability of an NLRP3 inflammasome inhibitor to block LPS-induced IL-1β release in THP-1 cells.

Cellular Protein Phosphorylation

Cellular signaling pathway activation can produce a variety of cellular responses, such as modulation of gene expression, protein phosphorylation or protein degradation. Reversible protein phosphorylation is a common mechanism that transduces the signal from an upstream activation event to downstream cellular responses. The Lumit™ Immunoassay Cellular System can be adapted to detect any phosphoprotein, total protein or other molecule of interest, provided that appropriate primary antibodies are available (5).

The assay system uses Lumit™ Secondary Antibodies that are conjugated to the NanoBiT® subunits, LgBiT and SmBiT. Cells in culture plates are lysed using a NanoBiT®-compatible lysis solution containing digitonin. The target protein is detected by adding an antibody mix containing two primary antibodies against the target, along with SmBiT- and LgBiT-conjugated secondary antibodies (e.g., Lumit™ Anti-Mouse Ab-LgBiT and Lumit™ Anti-Rabbit Ab-SmBiT). Binding of the primary antibody/Lumit™ secondary antibody complexes to their corresponding epitopes brings NanoBiT® subunits into proximity to form an active luciferase enzyme that generates light in proportion to the amount of target protein. When the primary antibody pair includes a phosphospecific antibody, the luminescence reflects the level of target protein phosphorylation (Figure 5).

In this example, we used the Lumit™ Cellular System to study the phosphorylation and subsequent degradation of IκBα in the NF-κB signaling pathway (Figure 6). We screened commercially available mouse/rabbit antibody pairs to determine the optimal combination and concentration of primary antibodies for detection of

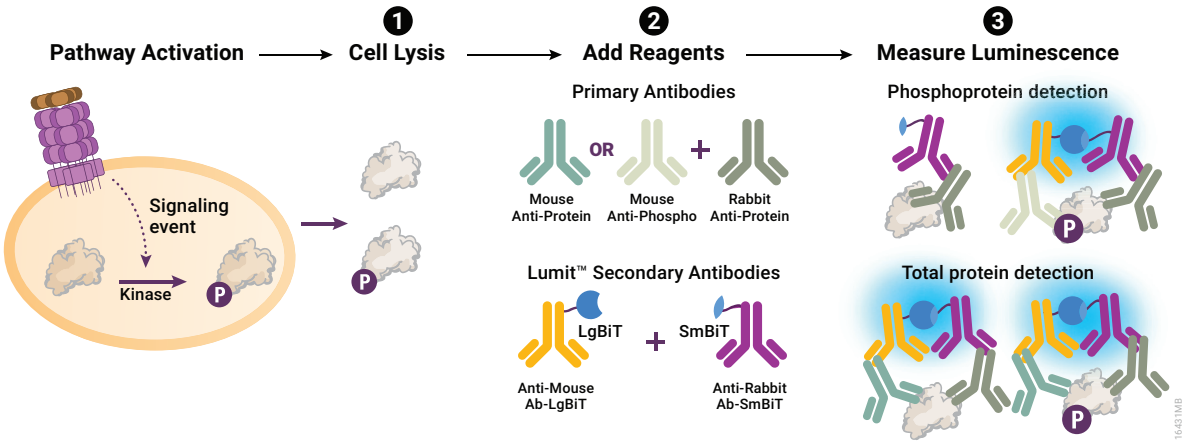

Figure 5. Principle of the Lumit™ Immunoassay Cellular System. Lumit™ tagged secondary antibodies are used to detect primary antibodies to either the normal or phosphorylated targets.

phospho-IκBα (Ser32) or total IκBα protein. We combined the selected primary antibody pairs with labeled secondary antibodies, including SmBiT-conjugated anti-rabbit IgG and LgBiT-conjugated anti-mouse IgG, to monitor modulation of the NF-κB signaling pathway in MCF-7 cells with and without TNFα treatment.

We observed that the level of phospho-IκBα was increased after TNF-α treatment, and this phosphorylation was suppressed by IKK16, an IKK complex selective inhibitor (Figure 6A). The amount of total IκBα protein also decreased due to its degradation after TNF-α treatment, and this reduction was abolished with IKK16 treatment (Figure 6B). These results serve as a proof of principle for the Lumit™ Immunoassay Cellular System, revealing the expected modulation of NF-κB signaling upon activation by TNF-α with and without a pathway inhibitor.

Fc Receptor Binding

The neonatal Fc receptor (FcRn) is a major histocompatibility complex (MHC) class I-like heterodimeric protein (6), comprised of a light chain β2-microglobulin (β2m) and a transmembrane heavy chain (α-FcRn). FcRn is expressed in the endosomal compartments of a variety of cell types, including vascular endothelium and antigen-presenting cells (APCs). FcRn binds to the Fc region of immunoglobulin G (IgG) antibodies at acidic pH within endosomes. In utero, FcRn acts to transfer maternal IgG to the developing fetus. In adults, it is involved in recycling of IgG and albumin. Recycling by FcRn is the primary reason for the long half-life (several weeks) of IgG and albumin in serum. Furthermore, a critical factor for the success of therapeutic antibodies is their extended half-life, which

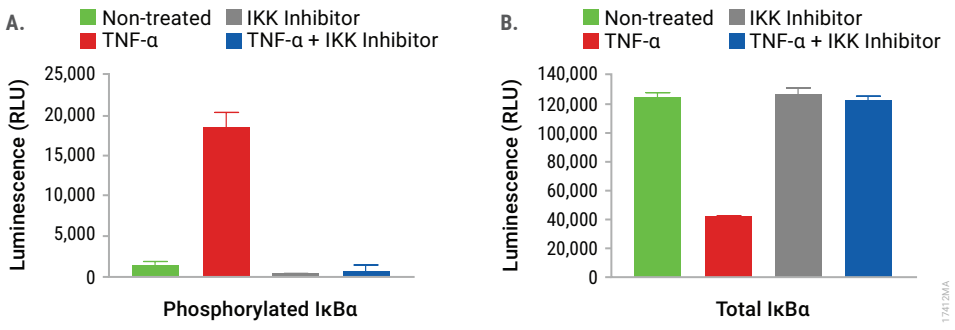

Figure 6. Detection of total and phosphorylated IκBα upon NF-κB pathway activation and deactivation. Lumit™ Immunoassays revealed the predicted biology of NF-κB signaling pathway upon TNF-α treatment: IκBα phosphorylation at S32 (Panel A) was immediately followed by its rapid degradation (Panel B).

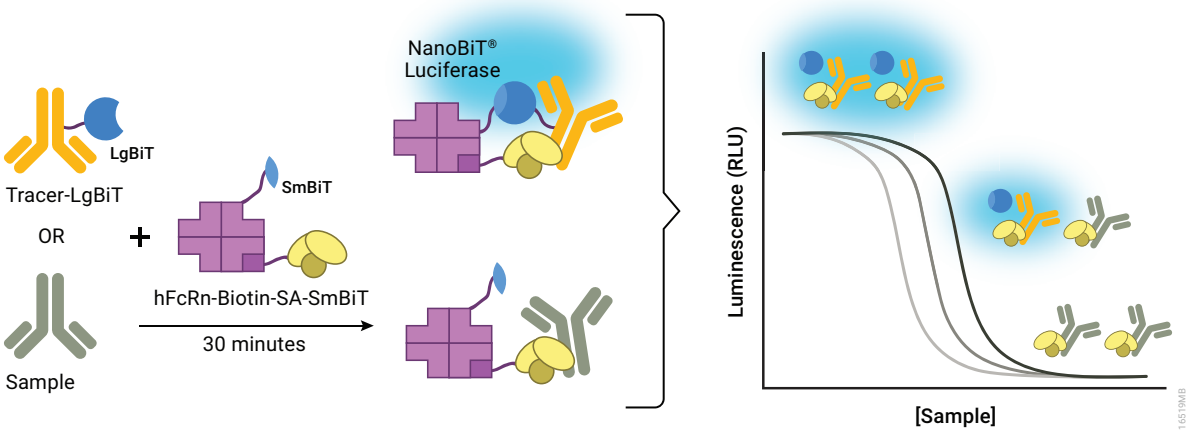

Figure 7. Principle of the Lumit™ FcRn Immunoassay. The assay uses a LgBiT-tagged tracer antibody in a competitive binding reaction with the antibody of interest and SmBiT-tagged FcRn.

contributes to efficacy and improved dosing schedule. Therefore, the FcRn-IgG interaction is a key parameter to optimize and track throughout the antibody drug development process.

In the Lumit™ FcRn Binding Immunoassay, a human IgG1 labeled with LgBiT (Tracer-LgBiT) is used as the tracer. A C-terminal biotinylated human FcRn bound to streptavidin-SmBiT (hFcRn-SmBiT) is used as the target. In the absence of an antibody analyte, Tracer-LgBiT binds to the hFcRn-SmBiT target, resulting in maximum luminescence signal (Figure 7). In samples containing analyte, nonlabeled IgG will compete with Tracer-LgBiT for binding to the FcRn target, resulting in a concentration-dependent decrease in the luminescent signal.

We used the Lumit™ FcRn Binding Immunoassay to examine the binding characteristics of three therapeutic antibodies for the FcRn target: panitumumab, trastuzumab and rituximab (Figure 8A). As expected, these antibodies exhibited similar IC_{50} values (panitumumab, 458nM; trastuzumab, 553nM; and rituximab, 471nM). Next, we examined the effect of antibody oxidation on trastuzumab binding to FcRn, using a 0.3% hydrogen peroxide solution to treat the antibody for up to 24 hours. We observed that the binding displacement curve was shifted to the right with increasing oxidation time, indicating increasing IC_{50} or decreasing affinity of the oxidized antibody for FcRn (Figure 8B). Panitumumab and rituximab displayed similar decreases in potency upon oxidation (data not shown).

The solution-based, competitive binding nature of the Lumit™ FcRn Binding Immunoassay eliminates artifacts that can occur in immobilization-based assay formats. The homogeneous assay format requires no wash steps, and the luminescence-based readout provides a wide dynamic range and large assay window. The assay can be used with low sample volumes (10–20µl) and is amenable to high-throughput formats.

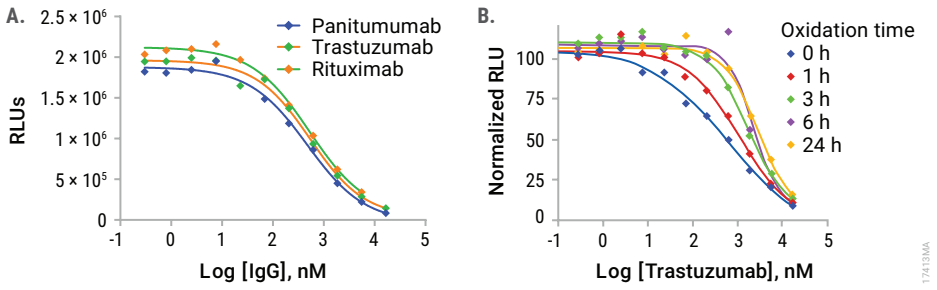

Figure 8. Application of the Lumit™ FcRn Immunoassay. Panel A: FcRn binding assessment for a panel of therapeutic antibodies. Panel B: Dose-dependent, oxidation-based loss in antibody-FcRn affinity is detectable in the assay.

Conclusions

The Lumit™ Immunoassay platform offers a rapid and convenient alternative to conventional immunodetection methods, such as ELISAs and Western blots. The no-wash, homogeneous format can be used with cells directly in culture medium and is easily adapted to high-throughput screening. The assay offers a broad dynamic range and can be used on any plate-reading luminometer. In addition to the applications described in this white paper, Lumit™ Immunoassays are in development or available as early access materials for assay of metabolic regulators, immunogenic cell death, and inhibitors of SARS-CoV2 Spike:RBD:hACE2 interaction. Antibody labeling and detection reagents for novel Lumit™ Immunoassay development are also available separately.

References

1. Hall, M.P. et al. (2012) Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem. Biol.* **7**, 1848–1857.
2. Dixon, A.S. et al. (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11**, 400–408.
3. Dinarello, C.A. (2018) Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol. Rev.* **281**(1), 8–27.
4. Coll, R.A. et al. (2015) A small molecule inhibitor of the NLRP3 inflammasome is a potential therapeutic for inflammatory diseases. *Nat. Med.* **21**(3), 248–255.
5. Hwang, B. et al. (2020) A homogeneous bioluminescent immunoassay approach to probe cellular signaling pathway regulation. *Commun. Biol.* **3**, 8.
6. Rath, T. et al. (2013) The immunologic functions of the neonatal Fc receptor for IgG. *J. Clin. Immunol.* **33**(Suppl 1), 9–17.

Ordering Information

To order Lumit™ Immunoassay products or request more information, visit: www.promega.com/lumit