Monitoring Cbl-b autoubiquitination

Lumit™ Protein Interaction Immunoassay

The Lumit™ Protein Interaction Immunoassay using proteins tags is a homogenous bioluminescent assay to measure protein-protein or protein-small molecule interactions. It combines immunodetection and NanoLuc Binary Technology (NanoBiT®). In Lumit™ Protein Interaction Immunoassays using protein tags, NanoBiT® subunits (SmBiT and LgBiT) are conjugated to two antibodies against common protein tags. When anti-Tag-LgBiT and anti-Tag-SmBiT are added to an interacting protein pair with the corresponding tags, LgBiT and SmBiT are brought into close proximity to form an active luciferase enzyme that generates luminescence in the presence of substrate (Fig 1A). Lumit™ Protein Interaction Immunoassays have a simple, no-wash format (Fig 1B), require small sample volumes, and can be run in a high-throughput format.

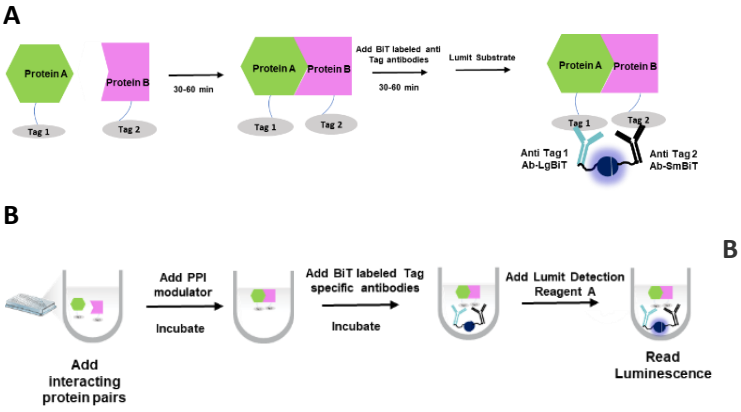

Figure 1. Schematic of Lumit™ Protein Interaction Immunoassay. A. *When LgBiT and SmBiT are brought into proximity by the interaction of Protein A and Protein B, an active luciferase generates luminescence in the presence of substrate.* ***B.*** *Overview of the simple no-wash protocol.*

Ubiquitination as a vital cellular process

Ubiquitination is the vital cellular process by which proteins are marked for degradation via the attachment of ubiquitin. The three-step process requires three enzymes: ubiquitin activating protein (E1), ubiquitin-conjugating protein (E2) and ubiquitin protein ligase (E3). To mark a protein for degradation, first, E1 establishes a thioester bond between ubiquitin and its own cysteine group in an ATP-dependent process. Next, E2 binds both E1 and the activated ubiquitin and catalyzes the transfer of ubiquitin from E1 to E2. Finally, E3 creates an isopeptide bond between the lysine of the target protein and the C-terminal glycine of ubiquitin. Additional ubiquitin molecules can be added to the monoubiquitinated protein to form polyubiquitin chains. Ultimately, the ubiquitin serves as the recognition signal for the 26S proteosome and the protein is degraded.

Humans have two E1 enzymes, approximately 40 E2 enzymes, and over 600 E3s. Thus, it is E3 that provides the substrate specificity to the ubiquitin cascade. Here, we investigate one E3 ligase, Cbl-b using Lumit™ Protein Interaction Immunoassay (Fig 2). Cbl-b function is critical to maintaining the balance between T-cell tolerance and T-cell activation. Disfunction leads to disease states ranging from autoimmune diseases to lymphoma. We monitor autoubiquitination as a proxy for monitoring ubiquitination of a target protein.

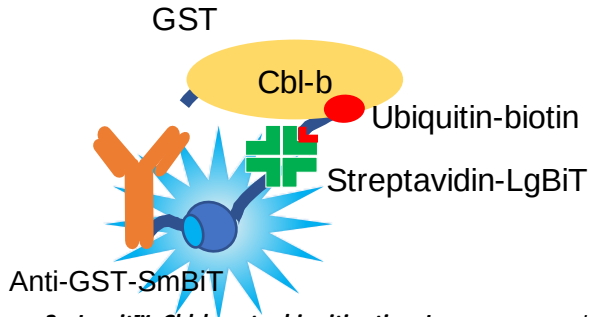

Figure 2. Lumit™ Cbl-b autoubiquitination Immunoassay. *In the presence of necessary ubiquitination components E1, E2, and ATP, Lumit™ Immunoassay can measure the interaction between biotinylated ubiquitin and GST-Cbl-b.*

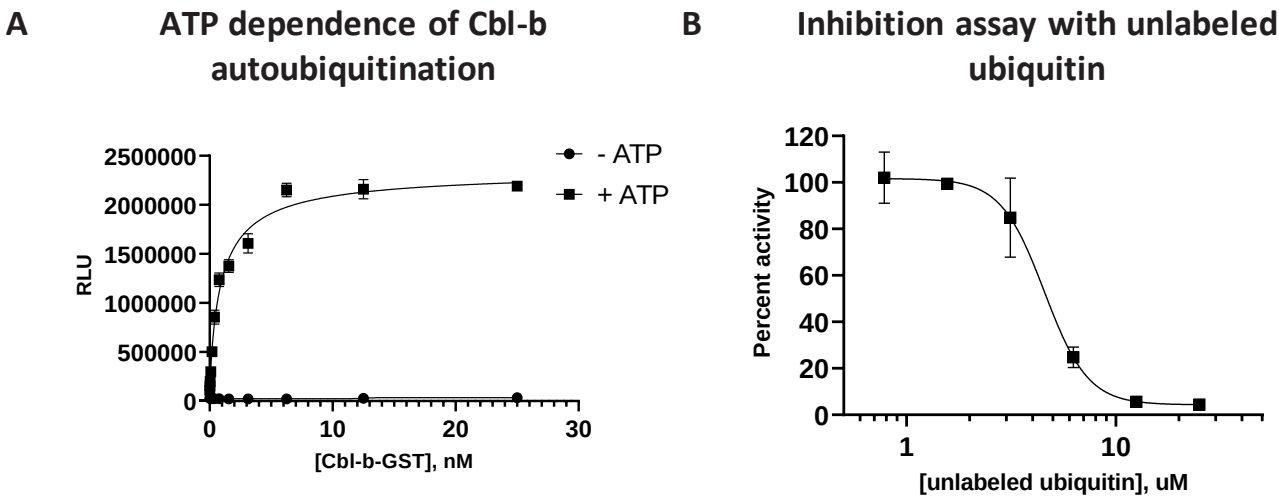

Figure 3. Measuring autoubiquitination of Cbl-b. *(A) A concentration dependent increase in Cbl-b-GST ubiquitination in the presence of ATP causes increase in luminescence signal. As the process is ATP-dependent, in the absence of ATP there is a low, background signal. (B) A dilution series of non-labeled ubiquitin was used to compete off the biotinylated ubiquitin and results in concentration dependent decrease in luminescence. Normalized RLU data are generated by assigning 100% to the maximum bioluminescent signal obtained in a reaction containing biotinylated ubiquitin only and then calculating percentage drop in signal as unlabeled ubiquitin is titrated into the reaction. Concentrations are expressed as final concentration in each well.*

Lumit™ Immunoassay for detection of Cbl-b ubiquitination

1. Prepare a reaction mixture of 42nM UBE1, 244nM UBCH5b, 20µM of ATP (or buffer alone for control), and biotinylated ubiquitin.
2. Prepare a dilution series of Cbl-b-GST.
3. Add 10µl of reaction mixture and 10µl of Cbl-b-GST to a 96-well plate.
4. Incubate at 37°C for 4hr with shaking.
5. Add 20µl of a reaction mixture containing 0.10µg/ml anti-GST-SmBiT and 0.33µg/ml Streptavidin-LgBiT diluted in Lumit™ Immunoassay Dilution Buffer A.
6. Incubate for 30 minutes with shaking.
7. Add 10µl of Lumit™ Detection Substrate A diluted 1:50 in Lumit™ Immunoassay Dilution Buffer A to each well.
8. Incubate for 2 minutes with shaking.
9. Read luminescence.

Materials

Item	Supplier	Cat. #
CBL-B TR-FRET Assay Kit	BPSBioscience	79575

Ordering Information

Item	Cat. #
Lumit™ Streptavidin-LgBiT and -SmBiT	CS332215
Lumit™ anti-GST-LgBiT and -SmBiT	CS332212
Lumit™ Immunoassay Detection Reagent A	VB2010