

Screening IL-6 pathway small and large molecule inhibitors with Lumit™ pSTAT3 (Tyr705) immunoassay

Lumit™ Immunoassay Cellular System:

The Lumit™ Immunoassay Cellular System is a homogeneous bioluminescent assay that measures levels of target proteins in cell lysates when used with the appropriate primary antibody pairs (1). It combines immunodetection and NanoLuc Binary Technology (NanoBiT®) (2). In the Lumit™ Immunoassay Cellular System, NanoBiT® subunits (SmBiT and LgBiT) are conjugated to a pair of secondary antibodies against two different species (anti-rabbit, anti-mouse, or anti-goat). Seeded cells are lysed in multi-well plates using a Lumit™ compatible lysis solution and the target protein is detected by adding an antibody mix containing two primary antibodies against the target protein along with Lumit™ secondary antibodies. Binding of the primary/Lumit™ secondary antibody complexes to their corresponding epitopes brings NanoBiT® subunits into proximity to form an active NanoLuc® luciferase that makes light in proportion to the amount of the target protein (Fig. 1).

- Hwang, B. *et al.* (2020) A homogeneous bioluminescent immunoassay approach to probe cellular signaling pathway regulation. *Commun Biol* 3, 8. doi:10.1038/s42003-019-0723-9.
- Dixon, A. S. *et al.* (2016) NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem Biol* 11, 400-408.

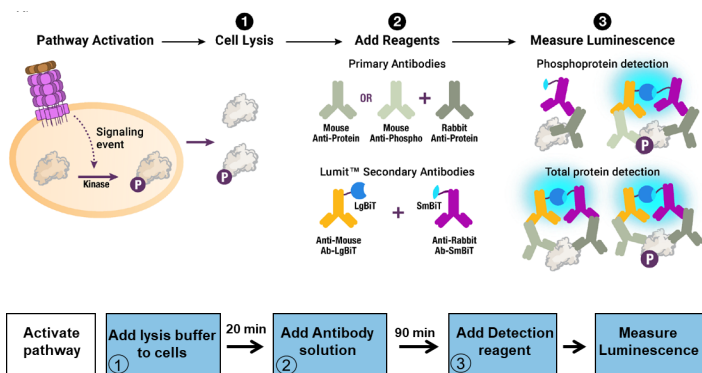


Figure 1. Illustration of Lumit™ Cellular Immunoassay. When the primary antibody pair includes a phospho-specific antibody, the luminescence reflects the level of the target protein phosphorylation (top panel). To detect total protein level, the same concept is used except both primary antibodies recognize non-phosphorylated epitopes on the protein (bottom panel). The luminescent signal generated is measured using a luminometer.

Interleukin 6 and COVID-19:

Interleukin 6 (IL-6) plays an important role in inflammatory and immune responses. It is suggested that IL-6 plays a key role in the cytokine release syndrome (CRD) that is associated with severe coronavirus disease (COVID-19) pneumonia cases. Tocilizumab (Actemra) is a humanized monoclonal antibody against the IL-6 receptor and an approved immunosuppressive drug for the treatment of rheumatoid arthritis. Recently, the FDA has approved a phase III clinical trial to evaluate its safety and efficacy for patients with COVID-19 pneumonia. In addition to Tocilizumab, several antibodies and small molecules are currently being developed for immune responses in general and COVID-19 in particular.

Here, we describe the use of Lumit Immunoassay Cellular Systems with STAT3 antibodies, including phospho-STAT3 (Tyr705), as a simple screening tool for identifying three types of IL6 signaling pathway inhibitors – JAK kinases chemical compound inhibitors, anti-IL-6 and anti-IL-6 receptor antibodies (Fig. 2).

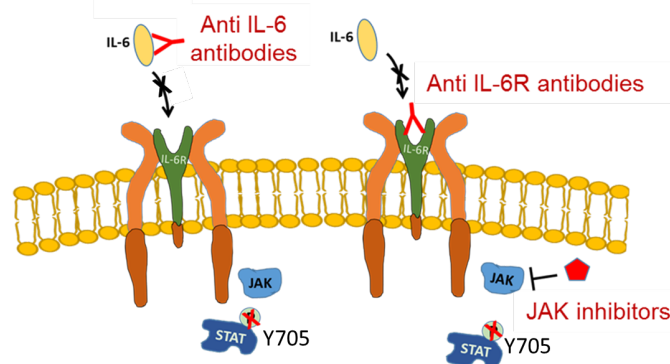


Figure 2. Phosphorylated STAT3 is a node for identification of IL-6 signaling pathway inhibitors.

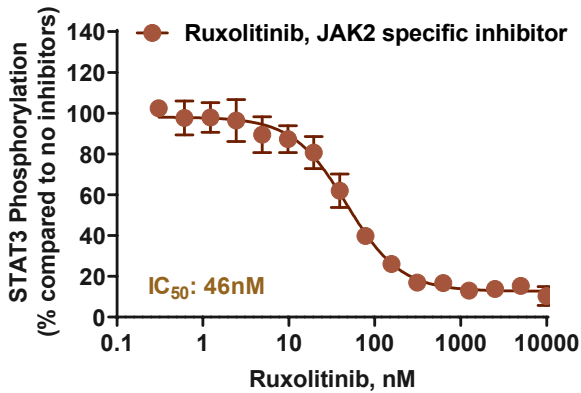
Phospho-STAT3 (Tyr 705) Immunoassay:

Upon activation of JAK/STAT pathway with Interleukin 6 (IL-6), STAT3 is phosphorylated. After lysis of the cell membrane, phospho-STAT3 (Tyr 705) can be detected using the reagents in Lumit™ Immunoassay Cellular System – Set 1 in combination with the anti p-STAT3 antibodies described in Table 1.

Lumit™ Immunoassay Cellular System Application Note

Cellular Pathway Analysis Series

A Inhibition of STAT3 phosphorylation with JAK kinase inhibitor



B Inhibition of STAT3 phosphorylation with anti-IL-6 and IL-6 receptor antibodies

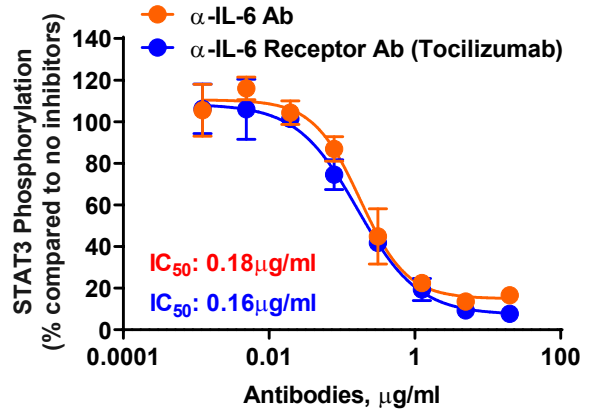


Figure 3. Inhibition of IL-6 mediated STAT3 phosphorylation with small and large molecule therapeutics. (A) 50,000 seeded A431 cells were starved overnight. After starvation, 50,000 seeded A431 cells were pretreated with various concentrations of Ruxolitinib for 1hr and then treated with IL-6 (10ng/ml, 30min) before phospho-STAT3 was measured by Lumit™ Immunoassay Cellular System – Set 1 to determine the potency of the inhibitor (IC₅₀). (B) After starvation, cells were pretreated with various concentrations of α-IL6 or α-IL6 receptor antibodies for 1hr and then treated with IL-6 (10ng/ml, 30min) before phospho-STAT3 was measured by Lumit™ Immunoassay Cellular System – Set 1 to determine the potency of the inhibitor (IC₅₀).

Lumit™ Immunoassay Cellular System Short Protocol

1. Add 10µl lysis solution to 40µl cells.
2. Incubate for 20min with shaking.
3. Add 50µl Antibody mix.
4. Incubate for 90 min.
5. Add 25µl of Lumit™ detection reagent.
6. Shake plate for 2min.
7. Read luminescence.

This is a quick reference protocol. For more details regarding cells and reagent preparation and detailed protocols see Lumit™ Immunoassay Cellular System Technical Manual TM613 at www.promega.com/protocols.

Table 1.

Antibody*	Target	Supplier	Cat. #	Working stock (µg/ml)
p-STAT3 (Rabbit)	Tyr 705	Cell Signaling Technology	9145	50
STAT3 (Mouse)	Total	Cell Signaling Technology	9139	50

*Antibodies from other suppliers may work as well. They may need optimization following Promega Technical Manual TM613.



Ordering Information:

Products	Size	Promega Cat. #
Lumit™ Immunoassay Cellular System – Set 1	100 assays	W1201
	1,000 assays	W1202
	10,000 assays	W1203