**A versatile bioluminescent immunoassay approach to interrogate cellular signaling pathway regulation**

Hicham Zegzouti, Brian (Byounghoon) Hwang, Laurie Engel, Juliano Alves, and Said Goueli

Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711

1. Introduction

Monitoring cellular signaling events can help better understand cell behavior in health and disease. Traditional immunoassays such as ELISA or Western blot, used to study proteins involved in signaling, can be tedious, require multiple steps, and are not easily adaptable to high throughput screening (HTS). Here we describe Lumit cellular immunoassay, a novel cell-based approach where immunodetection is combined with bioluminescent enzyme subunit complementation. It is solution based, does not include washing, liquid transfer, nor immobilization steps. Therefore, cells are lysed in the same well where antibody binding and luminescence generation steps occur. Lumit immunoassays take less than two hours to complete in a homogeneous “Add and Read” format and were successfully used to monitor the activation and deactivation of multiple signaling pathways through specific nodes of phosphorylation in unmodified cells. Our results demonstrate that this technology can be broadly adapted to streamline the analysis of signaling pathways of interest or the identification of pathway specific chemical or biologic inhibitors.

3. Optimizing Lumit Immunoassay Cellular System for Total and Phosphorylated Targets

Developing Lumit immunoassay to detect IkB phosphorylation or degradation

- Selection and optimization of primary antibodies for Lumit immunoassay is fast and easy.
- Bioluminescent detection of IkB protein and its phosphorylation upon NF-κB pathway activation is linear with increasing cell number.
- Assay is sensitive to detect total and phospho protein levels in low cell density.

4. Validation of Lumit Cellular Immunoassay Detection of Phosphorylated and Total IkB

Western blot protocol (Heterogeneous)

Lumit immunoassay protocol (Homogeneous)

Comparison of Lumit immunoassay and Western blot in detecting total and phospho IkB

- Homogeneous Lumit Cellular immunoassays are easier and quicker than traditional Western to generate the same data.
- Phosphorylation or degradation of IkB can be measured using the Lumit immunoassay in small number of cells and in a more quantitative way.

5. Deciphering NF-κB Pathway Activation Through Total and Phospho IkB Detection

Detection of total and phospho IkB upon TNF Treatment

a. Lumit IkB immunoassay reveals the predicted biology of NF-κB signaling pathway upon TNF treatment: IkB phosphorylation (pS32) followed by its fast degradation. No cell engineering required: therefore same results in primary cells (d and e).

b. Lumit IkB immunoassay reveals the predicted response of NF-κB pathway to the proteasome inhibitor MG132 treatment: decrease in IkBα degradation and accumulation of phosphorylated IkBα.

c. Cytosine arabinoside inhibits de novo IkBα protein expression in response to long NF-κB pathway activation and the Lumit immunoassay can detect easily this event.

6. Detection of Diverse Signaling Target Proteins

Detection of total and phosphorylated targets upon signaling pathway activation and deactivation

- Lumit cellular immunoassays reveal the predicted biology of multiple signaling pathways upon ligand mediated activation: Quick phosphorylation of the pathway nodes such as IkBα (S32), and STAT3 (Y705) or degradation of a node target such as β-Catenin in Wnt signaling.

- Detection of the predicted response of the signaling pathways to node kinase or pathway inhibitor treatment: such as inhibitors of IKK complex, PI3K, and JAK2 abolishing IkBα/P65, AKT and STAT3 phosphorylation, respectively.

8. Conclusions

Benefits of the bioluminescent Lumit cellular immunoassays:
- Bioluminescent, less interference from chemical compounds
- Homogeneous, “Add and Read” format
- No cell engineering required, detection of endogenous substrates phosphorylation
- No special instrument or plate requirement. Only a luminometer is required
- Less complex, quicker with less steps than Western, ELISA, or fluorescent based technologies
- Amenable to HTS formatting

- "Do It Yourself" format, the Lumit detecting antibodies can be adapted to any pathway of interest


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