1236-B

Deciphering key cancer and inflammation signaling pathways with homogeneous bioluminescent cell based kinase activity assays

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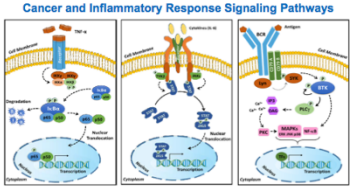
1. Introduction

Signaling pathway activation leads to a multitude of cellular responses including modulation of enzyme activity, altered gene expression, and protein translocation or degradation.

Specific phosphorylation events by specific kinases in cells constitute important **nodes** in signaling pathways. Monitoring these signaling events using cell-based methods is essential to better understand normal cell behavior and disease states.

Cancer and Inflammatory Response Signaling Pathways



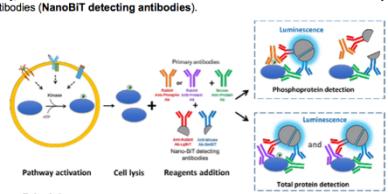
NF-κB **JAK/STAT** **BCR/BTK**

Here we describe the utility of a simple homogeneous cell-based platform to interrogate signaling pathways by detecting phosphorylation of specific proteins.

These bioluminescent cell-based pathway analysis assays can be used to study signaling pathways of interest, study the kinase cellular activity and regulation or identify specific kinase or pathway inhibitors

2. Principle of Homogeneous Cell-Based Kinase Assay

The bioluminescent cell-based kinase assays are based on NanoLuc® Binary Technology (NanoBiT) two-subunit system (SmBiT; 11 aa peptide and LgBiT; 18 kDa fragment). In this assay, the NanoBiT subunits are fused to an anti-mouse and an anti-rabbit secondary antibodies (**NanoBiT detecting antibodies**).



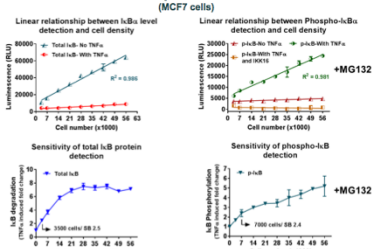
Assay Principle:

1. Activate pathway
2. Lyse Cells
3. Add antibody mix
4. Incubate 2 hours
5. Add Nano-Glo reagent
6. Read Luminescence

Pairs of 1° Abs that recognize separate epitopes on a single protein bring NanoBiT-labeled 2° Abs into proximity to form an active NanoLuc luciferase that makes light in proportion to the amount of target protein. When the 1° Ab pair includes a phosphospecific antibody, the luminescence reflects the level of target protein phosphorylation.

3. Linearity and Sensitivity of Bioluminescent Cell-Based Kinase Assay

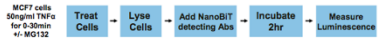
Detection of Total and Phospho IκBα in Different Cell Densities (MCF7 cells)



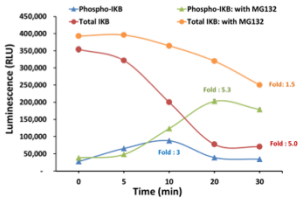
- Bioluminescent detection of IκBα protein and its phosphorylation upon NF-κB pathway activation is linear with increasing cell number.
- Assay is sensitive to detect total and phospho protein levels in low cell density.

MG132: Proteasome inhibitor. IKK16 is IKK kinase inhibitor

4. NF-κB Pathway: TNF-Induced IκBα Phosphorylation and Degradation



Detection of Total and Phospho IκBα upon TNF Treatment



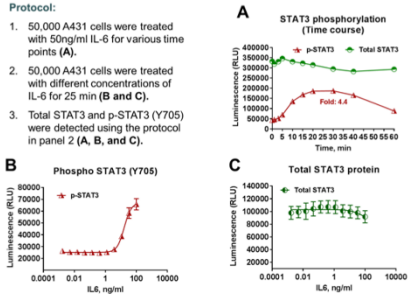
- NanoBiT IκB detecting reagents reveal the predicted biology of NF-κB signaling pathway upon TNF treatment: **IκBα phosphorylation** (pS32) immediately followed by its **fast degradation.**
- Detection of the predicted response of NF-κB pathway to proteasome inhibitor MG132 treatment: decrease in IκBα degradation and accumulation of phosphorylated IκBα.

5. JAK/STAT Pathway: Monitoring IL6-Mediated Phosphorylation of STAT3

Detection of Total and Phospho STAT3 upon IL-6 Treatment

Protocol:

1. 50,000 A431 cells were treated with 50ng/ml IL-6 for various time points **(A).**
2. 50,000 A431 cells were treated with different concentrations of IL-6 for 25 min **(B and C).**
3. Total STAT3 and p-STAT3 (Y705) were detected using the protocol in panel 2 **(A, B, and C).**



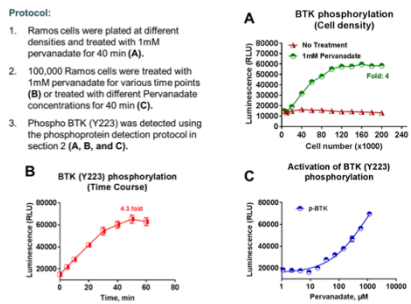
NanoBiT STAT3 detecting reagents reveal the known biology of JAK/STAT pathway after IL6 treatment: STAT3 **phosphorylation** (pY705) with **no effect** on STAT3 protein levels.

6. BTK Pathway: Activation of BTK Phosphorylation

Detection of Phospho BTK upon pervanadate Treatment

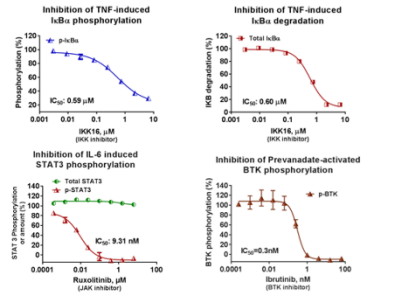
Protocol:

1. Ramos cells were plated at different densities and treated with 1mM pervanadate for 40 min **(A).**
2. 100,000 Ramos cells were treated with 1mM pervanadate for various time points **(B)** or treated with different Pervanadate concentrations for 40 min **(C).**
3. Phospho BTK (Y223) was detected using the phosphoprotein detection protocol in section 2 **(A, B, and C).**



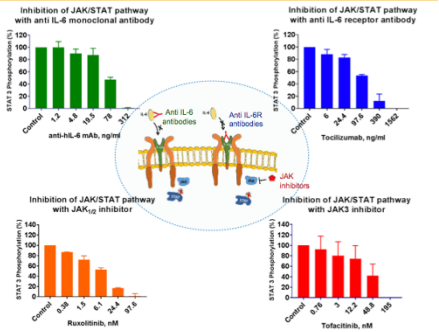
NanoBiT BTK detecting reagents show linear relationship between BTK phosphorylation (pY223) and cell number, incubation time and pervanadate (pY phosphatase inhibitor) concentration.

7. Modulation of Immune and Inflammatory Response Pathways with Small Molecules



- The bioluminescent NanoBiT pathway assays reveal the expected pharmacology of the pathway node kinase inhibitors.
- These cell-based assays can be used to screen inhibitors of cancer, immune and inflammatory response pathways.

8. Pathway Modulation with Large and Small Molecules Detected with NanoBiT Assay



Bioluminescent NanoBiT kinase assays can be used to identify small or large molecule inhibitors of signaling pathways.

9. Conclusions

Benefits of the bioluminescent cell-based NanoBiT kinase assays:

- **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 NanoBiT detecting antibodies can be adapted to any pathway of interest

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