Cellular-HTS Assays for the Ubiquitin-Proteasome system in NFκB Signaling

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Introduction

Activation of the NFκB pathway induces a signal transduction cascade that results in the phosphorylation, ubiquitination, and proteasomal degradation of IκBα. Upon IκBα degradation, liberated NFκB translocates to the nucleus to activate target gene expression (Fig 1). We have developed a set of target-specific HTS-compatible assays capable of interrogating the ubiquitin-proteasome system. Using a clonal cell line expressing GFP-IκBα, we have developed a TR-FRET assay capable of measuring ubiquitination of endogenously expressed IκBα. We have concurrently developed a living-cell β-lactamase (*bla*) reporter assay for degradation of Bla-IκBα fusion proteins. These technologies provide a powerful means to interrogate the intermediate steps in NFκB signaling, without compromising the endogenous physiological complexity of this signaling pathway. Cellular HTS assays that interrogate these processes will provide a unique integrated approach to dissecting the ubiquitin-proteasome system in the context of NFκB signaling.

Figure 1 – NFκB Pathway

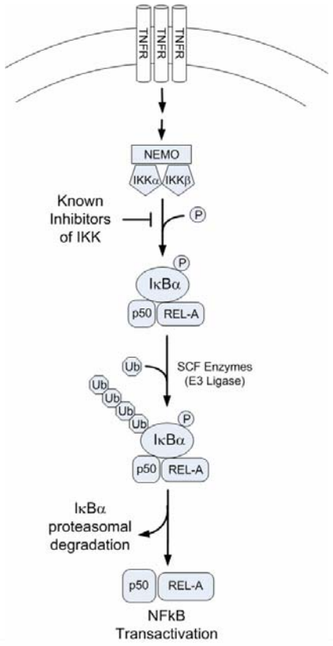


Figure 2 – Inducible degradation of GFP-IκBα in HEK-293 cells by Western analysis

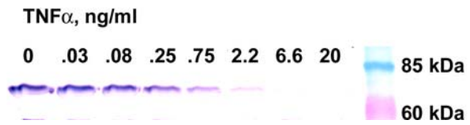


Figure 2 A clonal GFP-IκBα-expressing cell line was generated in order to probe the post-translational modifications of IκBα. We validated this construct for the inducible depletion of GFP-IκBα (approximately 65 kDa) by treating the cells with TNFα, generating lysates from each sample, and performing Western Analysis using anti-GFP antibodies.

Figure 3 – Assay schematic for ubiquitination of GFP-IκBα expressed in HEK-293 cells

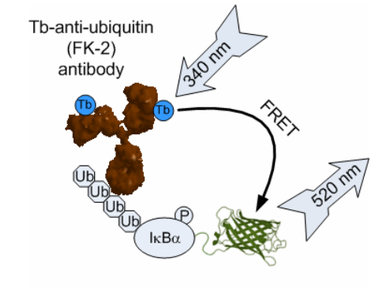


Figure 3 In cell lysates, Tb-anti-ubiquitin antibodies bind ubiquitinated GFP-IκBα, allowing the Tb and GFP fluorophores to come in close proximity for energy transfer to occur.

Figure 4 – Workflow for cellular GFP-IκBα ubiquitination assay

Treat cells with agonist or antagonist compounds

↓

Lyse cells with mild detergent

↓

Add Tb-anti-ubiquitin antibodies

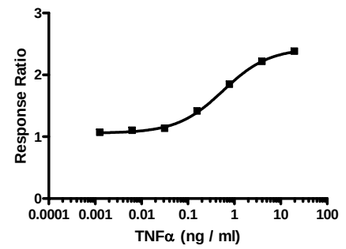
↓

Read 520nm/495nm time-gated Emission ratio

Total time = Approximately 2h

Figure 5 – Lanthascreen™ TR-FRET assays for ubiquitination of GFP-IκBα in HEK-293 cells

A TNFα-induced ubiquitination of GFP-IκBα



B Inhibition of ubiquitination of GFP-IκBα

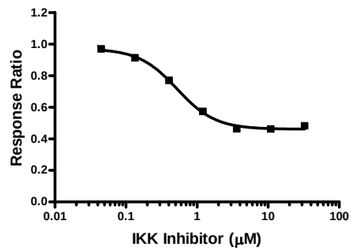
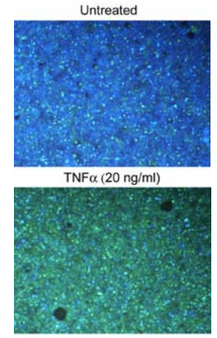


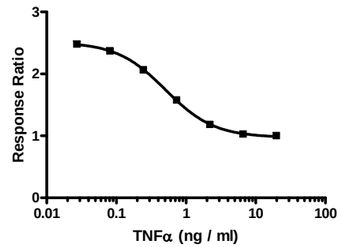
Figure 5 **a**. In cell lysates, a dose-dependent rise in TR-FRET signal is seen, concominant with an increase in ubiquitinated GFP-IκBα. Briefly, in 96 well format this cell line was treated with serial dilutions of TNFα, lysates were generated from each sample, and the lysate was probed with 10nM Tb-anti-ubiquitin antibody solution. A time-gated fluorescence emission ratio was then generated for each sample using the 520 nm (GFP) emission signal referenced against the 495 nm (Tb) emission signal. Response ratios were generated by dividing each emission ratio value by the unstimulated value (zero agonist or antagonist). Measurements were taked using a Tecan Ultra Fluorescence plate reader. **b**. Inhibition of TNFα-induced ubiquitination using IκB-kinase inhibitor IV. This cell line was pretreated with serial dilutions of inhibitor compound prior to stimulation with 1ng/mL TNFα.

Figure 6 – β-Lactamase assays for Bla-IκBα degradation in living HEK-293 cells

A Image Analysis of Bla-IκBα degradation



B TNFα-induced degradation of Bla-IκBα



C Inhibition of degradation of Bla-IκBα

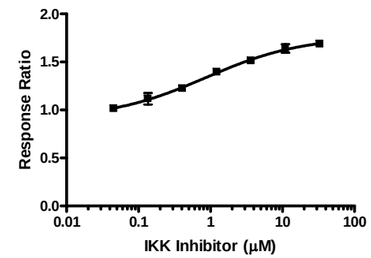


Figure 6 **a**. Color FRET images of Bla-IκBα degradation using the cell-permeable substrate for Bla, CCF4-AM. When intracellular Bla levels are above a threshold, the green-emitting CCF4-AM substrate is cleaved to generate a blue product. Thus, untreated (blue) cells become green in response to TNFα. **b**. In living HEK-293 cells, a dose-dependent depletion of Bla activity is seen, concominant with the proteolytic processing of Bla-IκBα. FRET emission ratios were generated using the 460 nm value (blue) divided by the 530 nm value (green). Response ratios were generated by dividing each blue/green ratio value by the unstimulated value (zero agonist or antagonist). **c**. Inhibition of TNFα-induced degradation using IκB-kinase inhibitor IV. Data was generated using a Tecan Safire II fluorescent plate reader.