

Analysis of ERK1/2, p38 $\alpha\beta\gamma$ and STAT3 phosphorylation with the THUNDER™ TR-FRET cellular kinase immunoassays

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- The THUNDER™ TR-FRET cellular platform was tested for compatibility on the CLARIOstar® Plus
- The phosphorylation of kinase targets ERK1/2, p38 $\alpha\beta\gamma$ and STAT3 from whole cell extracts was analyzed with TR-FRET
- Excellent assay performance was obtained in terms of signal levels, dynamic range, S/B ratio and EC₅₀

Introduction

Post-translational modification (PTM) is an essential step in cellular biology. Specific addition of residues or cleavage of existing molecular bonds are used to activate enzymes or protein-pro-forms and can even promote protein degradation. While PTM comprises a vast range of residues and cleavage sites, the phosphorylation of amino acid side chains is by far the most common modification. The understanding of protein phosphorylation is an important step, both for basic and disease-related research^{1,2}. THUNDER™ offers a TR-FRET-based high-throughput immunoassay platform designed to enable simple, rapid, sensitive, robust, and cost-effective measurement of endogenously expressed phosphorylated proteins in whole-cell lysates³.

In this application note, we describe the validation of ERK1/2, p38 $\alpha\beta\gamma$ and STAT3 THUNDER™ cellular kinase assays on the CLARIOstar^{Plus} multi-mode plate reader.

Assay Principle

THUNDER™ cellular assays are homogeneous sandwich immunoassays (Figure 1) based on an enhanced TR-FRET technology developed by Bioautilium Research. One antibody is labeled with a long lifetime Europium chelate donor [Eu-Ab1] and the second with a far-red acceptor fluorophore (FR-Ab2). Upon excitation of the Europium chelate at 320 or 340 nm, energy is transferred from the donor to the acceptor fluorophore only if the target protein is phosphorylated. The emission by the acceptor of a long-lived TR-FRET signal at 665 nm is measured after a time delay and is proportional to the concentration of phosphorylated protein in the cell lysate.

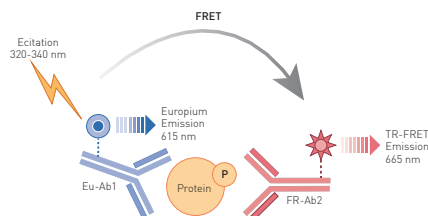


Fig. 1: THUNDER™ Cell Signaling Assay Principle.

Materials & Methods

- 96-well culture plates [Costar®] and 384-well, white low-volume plates [PerkinElmer®]
- HEK293 and HeLa cells [ATCC®]
- Epidermal growth factor [EGF; PeproTech®], Interferon-alpha [INF α ; ProSpec] and Anisomycin [Cayman Chemical]
- KIT-ERKP-100, KITP38 $\alpha\beta\gamma$ P-100 and KIT-STAT3P-100 assay kits [Bioautilium Research]
- CLARIOstar^{Plus} microplate reader

Experimental Procedure

Protocols were conducted as per Bioautilium's recommendations^{4,5,6}. Cells were cultured overnight in 96-well tissue culture-treated plates. Following treatment, the media was removed and cells were lysed with the specific 1X Lysis Buffer supplemented with the Phosphatase Inhibitor Cocktail, both included in the kit. After 15-30-min incubation at room temperature [RT] on an orbital shaker (400 rpm), lysates [15 μ L] were transferred to 384-well white assay plates followed by the addition of the Antibody Detection Mix [5 μ L] for detection of the target protein. The plates were incubated at RT for the appropriate time and read on the CLARIOstar^{Plus} using the settings recommended for THUNDER™. Data were expressed as (665/620) * 1,000 and are the mean \pm SD of three wells per point.

Instrument settings

Optic settings	Time-resolved fluorescence, plate mode endpoint	
	Filters	Ex TR Dichroic: LP TR Em 1: 665-10 Em 2: 620-10
	Gain	2300
Integration time	Delay: 60 μ sec, Time: 400 μ s	
General settings	Number of flashes	200
	Settling time	0.1 s

Results & Discussion

Figure 2 shows a concentration-response curve obtained with the Phospho-ERK1/2 (T202/Y204) assay kit. Treatment of HEK293 cells (50,000 cells/well) with EGF for 10 min at RT induced the phosphorylation of ERK1/2 at T202/Y204. The S/B ratios and EC₅₀ values were consistent with the expected values.⁴



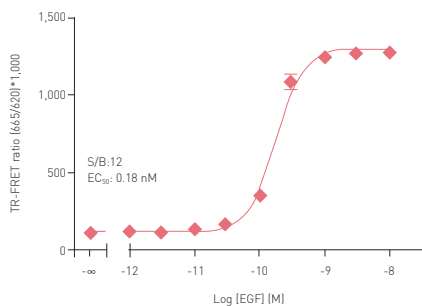


Fig. 2: THUNDER™ Phospho-ERK1/2 [T202/Y204] assay (4 hours incubation).

Figure 3 summarizes results obtained with the Phospho-p38 α (T180/Y182) assay kit. Incubation of HeLa cells (50,000 cells/well) with Anisomycin for 60 min at 37°C stimulates phosphorylation of p38 α at T180/Y182. The assay shows a very high S/B ratio and the expected EC₅₀⁵.

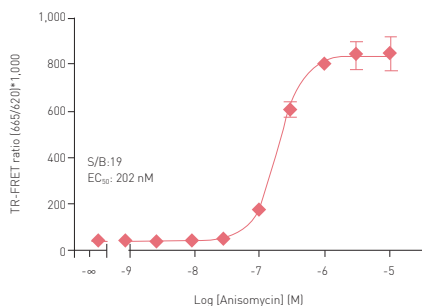


Fig. 3: THUNDER™ Phospho-p38 α [T180/Y182] assay (overnight incubation).

Results obtained with the Phospho-STAT3 [Y705] assay kit are summarized in Figure 4. HeLa cells (40,000 cells/well) treated with Interferon-alpha [IFN α] for 20 min at RT show the anticipated concentration-dependent activation of phospho-STAT3 at Y705, with a high S/B ratio and the expected EC₅₀ value⁶.

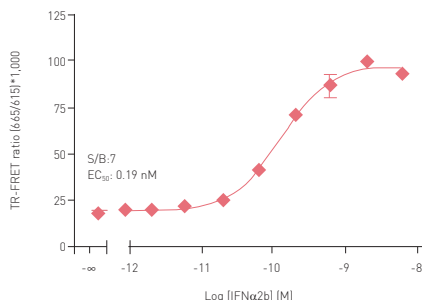


Fig. 4: THUNDER™ Phospho-STAT3 [Y705] assay (overnight incubation).

Overall, data were similar to those obtained with other TR-FRET enabled readers, demonstrating that the CLARIOstar^{Plus} is suitable for performing THUNDER™ cellular assays. In addition, results obtained using different instrument settings (gain, delay and integration time) recommended for other TR-FRET technologies had no significant impact on the overall THUNDER™ assay performance (data not shown), further highlighting the robustness of THUNDER™ cellular kinase assays.

Conclusion

The current data validate the compatibility of the CLARIOstar^{Plus} for THUNDER™ cellular kinase assays. All assays exhibited high signal levels and S/B ratios, broad dynamic ranges, and the expected potency. THUNDER™ cellular assays run on the CLARIOstar^{Plus} are well-suited for both basic research and drug discovery applications and offer a straightforward approach for the determination of protein phosphorylation levels in cell lysates.

References

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