bioauxilium
BETTER TOOLS. REAL DISCOVERIES.

A COMPARISON OF THUNDER®, HTRF®, AND ALPHALISA™ SUREFIRE® ULTRA™ FOR MEASUREMENT OF PHOSPHORYLATED STAT3 (Y705) IN CELL LYSATES

Mireille Caron, Geneviève Chatel and Jaime Padros
Bioauxilium Research Inc., Montréal (Québec) Canada

KEY POINTS

We conducted a head-to-head assessment of THUNDER®, HTRF®, and AlphaLISA™ *SureFire® Ultra™* assays for the detection of phospho-STAT3 (Y705) in lysates from HeLa cells stimulated with interferon alpha-2b.

The High-Performance THUNDER TR-FRET assay outperformed HTRF and matched AlphaLISA in terms of assay sensitivity while being simpler, faster, and more cost-effective.

INTRODUCTION

The signal transducer and activator of transcription (STAT) family comprises STAT1 to 6. The ability to monitor the modulation of STATs by directly measuring the level of phosphorylated proteins in a cellular setting using a high-throughput screening-compatible assay is essential for drug discovery efforts and basic research. THUNDER is an advanced time-resolved Förster resonance energy transfer (TR-FRET) immunoassay platform designed to measure endogenous proteins in a cell-based format. Several THUNDER assays are available for measuring STAT1, STAT2, STAT3, STAT4, STAT5, and STAT6 proteins in cell lysates. We recently redeveloped the THUNDER assay measuring phosphorylated STAT3 [1]. This new assay exhibits a higher sensitivity and is therefore commercialized as THUNDER High-Performance Phospho-STAT3 (Y705) TR-FRET Cell Signaling Assay Kit [2].

In this Application Note, we compared head-to-head the performance of the High-Performance THUNDER Phospho-STAT3 assay to that of two other homogeneous assay platforms commonly used for drug discovery: HTRF and AlphaLISA *SureFire Ultra*.

THUNDER TR-FRET ASSAY PRINCIPLE

The three homogeneous assay platforms are based on the sandwich immunoassay principle. The assay principle of THUNDER is shown in Figure 1. One antibody is labeled with the donor fluorophore (Europium chelate; Eu-Ab1) and the second with the far-red acceptor fluorophore (FR-Ab2). The binding of the two labeled antibodies to distinct epitopes on the target protein takes place in solution and brings the two dyes into close proximity. Excitation of the donor Europium chelate molecules at 320-340 nm triggers a FRET from the donor to the acceptor molecules, which in turn emit a long-lived TR-FRET signal at 665 nm. Residual energy from the Eu chelate generates light at 615-620 nm. The signal at 665 nm is proportional to the concentration of Phospho-STAT3 (Y705) in the cell lysate.

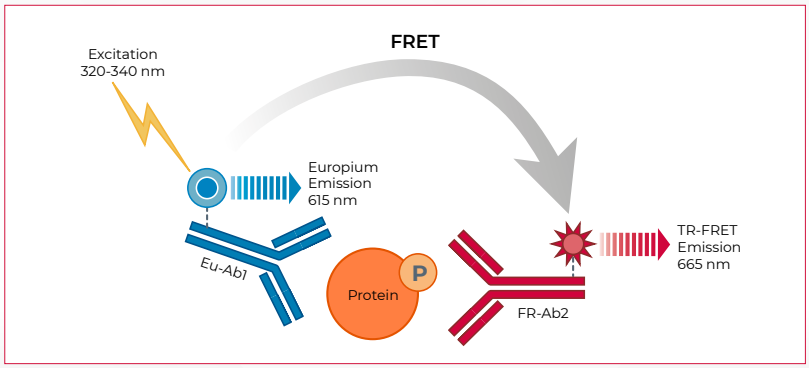


Figure 1 THUNDER TR-FRET cell signaling assay principle.

MATERIALS AND METHODS

Table 1 lists the assay kits, materials, and reagents used for this evaluation. All assays were conducted manually by the same operator using the 2-plate protocol and according to each manufacturer's instructions [3]. The three assay platforms use the same assay workflow (Figure 2), which consists of 3 steps: cell treatment, cell lysis and protein detection.

The same cell culture was used for the experiment to allow a direct comparison of the three assays.. HeLa cells were seeded at 80,000 cells/well in a 96-well culture plate and incubated overnight. Experiments were conducted previously to verify that this cell density was within the linear dynamic range of each assay. Cells were treated with varying concentrations of IFNα2b (dissolved in serum-free culture media) according to pre-optimized conditions (30 minutes at 37°C). Following removal of the culture medium, cells were lysed with the corresponding Lysis Buffer included in each kit. Lysates were then transferred to a 384-well assay plate to detect Phospho-STAT3 (Y705). Table 2 summarizes the protocol for each assay.

Following the addition of reagents, plates were covered with an adhesive plate sealer to reduce evaporation during incubation. The plate sealer was removed before TR-FRET readings. Because the AlphaLISA technology is sensitive to ambient light and temperature, protocol steps involving Donor beads were conducted in subdued light. All plates were incubated in the dark in an incubator set at 21°C.

The TR-FRET assays were read on a PHERAstar® *FSK* (laser excitation at 307 nm). The same filters and instrument settings were used for the two TR-FRET assays. TR-FRET data were expressed as emission ratios of acceptor/donor (665 nm/620 nm) signals. AlphaLISA signal was read on an EnVision® XCite 2104. To minimize photobleaching of the Donor beads, the AlphaLISA assay was only read twice.

Data in Figure 3 are shown as mean ± standard deviation (three wells per assay point). Concentration-response data were analyzed using nonlinear regression and fitted to a sigmoidal four-parameter equation with 1/Y2 weighting (GraphPad Prism software). Performance metrics were signal-to-background (S/B) ratio (Maximum signal of treated cells/Minimal signal of untreated cells), potency (EC50 value), intra-assay variability (%CV), stability of S/B ratio and EC50 value over time, ease of use, time-to-results, and cost.

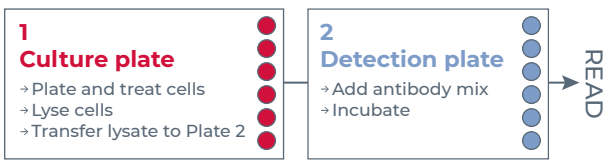


Figure 2 Assay workflow using the 2-plate (transfer) protocol.

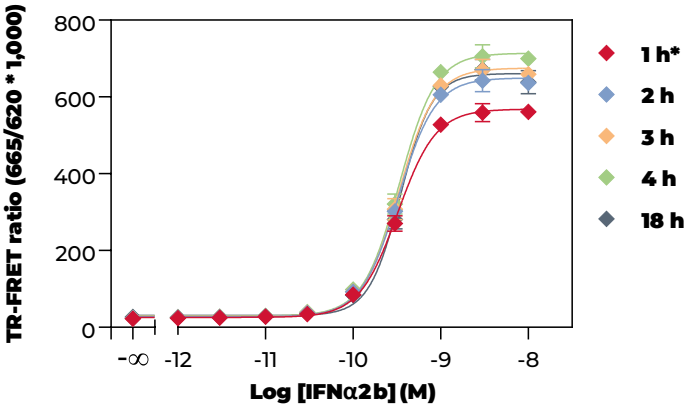
Table 1 Reagents and materials used in the study.

Reagents and materials	Source (catalog #)
THUNDER High-Performance Phospho-STAT3 (Y705) TR-FRET Cell Signaling Assay Kit, 100 points	BioAuxilium (KIT-HP-STAT3P-100)
HTRF Human Phospho-STAT3 (Tyr705) Detection Kit, 100 Assay Points	Revvity (62AT3PET)
AlphaLISA *SureFire Ultra* Human & Mouse Phospho-STAT3 (Tyr705) Detection Kit, 500 Assay Points	Revvity (ALSU-PST3-A1-HV)
HeLa	ATCC (CCL-2)
DMEM	Wisent (319-005-CL)
FBS	Wisent (098-050)
IFN-alpha 2b	ProSpec (CYT-460-a)
Ultra pure water	MilliQ water
96-well culture microplate	Costar (3595)
384-well white opaque microplate	Revvity (6007290)
Microplate seal	Revvity (6050185)

Table 2 Assay protocol for each platform.

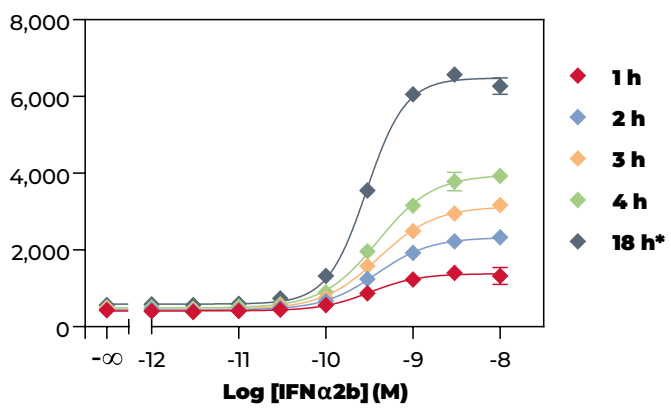
Step #	THUNDER	HTRF	AlphaLISA *SureFire Ultra*
1	Add 15 µL lysate	Add 16 µL lysate	Add 15 µL lysate
2	Add 5 µL Detection Mix	Add 4 µL Detection Mix	Add 7.5 µL Acceptor Mix
3	Seal plate and incubate 1 hour	Seal plate and incubate 18 hours	Seal plate and incubate 1 hour
4	Read	Read	Add 7.5 µL Donor Mix (in subdued light)
5			Seal plate and incubate 1 hour (in subdued light)
6			Read
Number of reagent additions	2	2	3
Number of steps	4	4	6
Total assay volume	20 µL	20 µL	30 µL
Total assay time	1 hour	18 hours	2 hours

THUNDER



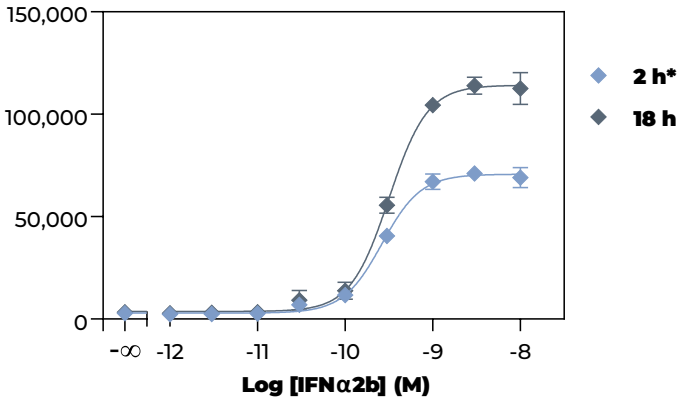
	Incubation time (h)				
	1	2	3	4	18
S/B	24.9	27.1	28.0	28.6	23.4
EC50 (nM)	0.32	0.32	0.33	0.34	0.35

HTRF



	Incubation time (h)				
	1	2	3	4	18
S/B	3.1	5.4	6.6	7.9	11.1
EC50 (nM)	0.32	0.38	0.40	0.39	0.29

AlphaLISA *SureFire Ultra*



	Incubation time (h)				
	1	2	3	4	18
S/B	-	24.6	-	-	33.9
EC50 (nM)	-	0.26	-	-	0.32

Figure 3 Concentration-response curves obtained with each assay format. Each phospho-STAT3 (Y705) assay was read at different times following the addition of all reagents. The asterisk (*) indicates the incubation time recommended by the manufacturer for each assay.

RESULTS AND DISCUSSION

Figure 3 shows the IFNα2b concentration-response curves obtained at different incubation times with each assay platform. Table 3 summarizes the key assay parameters obtained with the three assays.

Although there were clear differences in the magnitude of the assay windows, all three assays produced well-defined concentration-response curves. The EC50 values remained stable and comparable at all incubation time points and ranged from 0.26 to 0.35 nM, consistent with our reported validation values [1,2]. This result confirms that all three assays are suitable for profiling the potency of STAT3 activators. In addition, the high assay windows obtained at the manufacturer's recommended incubation times provide a suitable dynamic range for profiling both activators and inhibitors using lower cell densities.

The THUNDER assay surpassed the performance of the HTRF assay in terms of sensitivity at all incubation points (Figure 3). HTRF required overnight incubation to generate high counts and a maximal S/B ratio of 11, whereas THUNDER showed an S/B ratio of 25 after only 1 hour of incubation. Faster assay equilibrium was observed with THUNDER since a modest signal increase was observed after 2 hours of incubation. Both TR-FRET assays showed very low and comparable inter-well variation.

In a previous comparative study, the standard THUNDER Phospho-STAT3 assay generated higher S/B ratios than the HTRF assay [4]. Accordingly, the current results show that both the standard and the High-Performance Phospho-STAT3 assays outperform the HTRF assay.

The THUNDER assay required half the time of the AlphaLISA assay to generate a comparable S/B ratio (1 hour rather than 2 hours; Figure 3). Following overnight incubation, AlphaLISA generated the highest S/B ratio. However, AlphaLISA uses a longer assay protocol than THUNDER. In addition, and in contrast to TR-FRET technologies, AlphaLISA is sensitive to biotin in the culture medium, ambient light, and normal temperature variations in the laboratory and cannot be read multiple times without photobleaching. The AlphaLISA assay also showed more inter-well variation than the two TR-FRET assays , which aligns with previous reports comparing TR-FRET and AlphaLISA [5-7].

In addition to signal quality, ease of use, and speed, cost is an important factor to consider when selecting an assay platform. HTRF and AlphaLISA are 68% and 77% more expensive than THUNDER (Table 3). The high cost of these reagents precluded additional cross-platform comparisons (profiling of inhibitors; Z'-factor experiments).

CONCLUSION

All three phospho-STAT3 (Y705) assays produced robust concentration-responses curves for measuring the activation of STAT3 by IFNα2b in HeLa cell lysates. The THUNDER and AlphaLISA *SureFire Ultra* assays showed equivalent, and the highest, S/B ratios compared to the HTRF assay. However, THUNDER required fewer steps, was faster, and had the lowest assay cost per well. Accordingly, THUNDER provides a suitable alternative to both HTRF and AlphaLISA platforms.

Table 3 Comparison of assay parameters for THUNDER, HTRF, and AlphaLISA *SureFire Ultra*.

Parameter	THUNDER	HTRF	AlphaLISA *SureFire Ultra*
S/B	1 hour[1]: 25 O/N: 23	1 hour[1]: 3 O/N[1]: 11	2 hours[1]: 25 O/N: 34
EC50 (nM)	1 h: 0.32 nM 18 h: 0.35 nM	1 h: 0.32 nM 18 h: 0.29 nM	2 h: 0.26 nM 18 h: 0.32 nM
Mean inter-well CV	3.4%	3.5%	11.3%
Time-to-results	1 hour	18 hours	2 hours
Sensitivity to biotin in culture medium	No	No	Yes (biotinylated antibody/Donor bead interaction)
Sensitivity to ambient light	No	No	Yes (Donor beads)
Sensitivity to ambient temperature	No	No	Yes
Tolerance to repeated readings	Yes	Yes	Limited (Donor beads)
Cost per well[2](USD)	$2.70	$4.54	$4.78

[1] *Manufacturer's recommended incubation time (O/N: overnight incubation).*
[2] *Cost based on January 2025 prices. (kit size: 500 assay points).*

REFERENCES

[1] Technical Data Sheet: THUNDER Phospho-STAT3 (Y705) TR-FRET Cell Signaling Assay Kit.

[2] Technical Data Sheet: THUNDER High-Performance Phospho-STAT3 (Y705) TR-FRET Cell Signaling Assay Kit.

[3] THUNDER TR-FRET Cell Signaling Assay Kits – Generic User Manual – Version 2.

[4] Chatel et al. 2024. Updated: head-to-head comparisons of six THUNDER phospho-protein assays with two existing TR-FRET platforms. Application Note THUNDER-APN002.

[5] Glickman et al. 2002. A comparison of AlphaScreen, TR-FRET, and TRF assay methods for FXR nuclear receptors. J Biomol Screen 7(1):3-10. doi: 10.1177/108705710200700102.

[6] Miller et al. 2019. Quantitative high-throughput screening assays for the discovery and development of SIRPα-CD47 interaction inhibitors. PLoS ONE 14(7):e0218897. doi: 10.1371/journal.pone.0218897

[7] Wu et al. 2005. Further comparison of primary hit identification by different assay technologies and effects of assay measurement variability. J Biomol Screen 10(6):581-589. doi: 10.1177/1087057105275628.

FOR MORE INFORMATION ON DEVELOPING AND OPTIMIZING TR-FRET CELL SIGNALING ASSAYS, CONSULT THE USER MANUAL.