bioauxilium
BETTER TOOLS. REAL DISCOVERIES.

UPDATED: HEAD-TO-HEAD COMPARISONS OF SIX THUNDER™ PHOSPHO-PROTEIN ASSAYS WITH TWO EXISTING TR-FRET PLATFORMS

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

KEY POINTS

We compared head-to-head the performance of THUNDER™ with two existing TR-FRET assay technologies.

Six endogenous phosphorylated proteins were measured in lysates from cells treated with pathway-specific modulators.

THUNDER™ phospho-protein assays exhibit superior or comparable performance in terms of signal-to-background ratios at a lower cost per well.

INTRODUCTION

Phosphorylation is one of the most important and common post-translational modifications of proteins. Protein phosphorylation plays a critical role in cell signaling in response to extracellular stimulus and is of fundamental importance in biological regulation and human diseases [1]. Accordingly, the availability of assays capable of measuring target-specific protein phosphorylation in a physiologically relevant cellular context is important for both basic research and drug discovery.

A number of technologies are currently available for the microplate-based measurement of intracellular phosphorylated protein levels in crude cell lysates. The enzyme-linked immunosorbent assay (ELISA) was developed in the 1970s and remains the mainstay for this kind of analysis. However, ELISA has several well-known limitations. Conventional ELISAs use large volumes of sample, are time-consuming and error-prone due to a laborious protocol with multiple washing and incubation steps, have a short period for signal detection (usually within 30 minutes) and are typically performed in 96-well plates, being difficult to miniaturize to higher density plate formats.

In recent years, time-resolved Förster resonance energy transfer (TR-FRET) technology has emerged as a superior alternative to ELISA [2]. TR-FRET combines the low background and sensitivity of time-resolved fluorometry with the homogeneous assay format of FRET. TR-FRET assays are performed using a simple no-wash, "add-incubate-measure" protocol that substantially reduces assay complexity and hands-on time, while improving throughput and suitability for miniaturization and automation. In addition, TR-FRET assays have reduced sample volume requirements, higher reproducibility and long signal stability.

Bioauxilium has recently introduced THUNDER™, a new immunoassay platform based on an enhanced TR-FRET technology (see Application Note THUNDER-APP001). The THUNDER™ Cell Signaling Assay Kits are designed to measure endogenous levels of specific intracellular phosphorylated proteins with high sensitivity, specificity, robustness and cost effectiveness. The aim of this Application Note was to compare head-to-head the performance of THUNDER™ in 384-well plate format with two existing TR-FRET technologies (Companies A and B) measuring a panel of six phosphorylated proteins. The three TR-FRET assay technology platforms were evaluated for their capacity to measure relative levels of phosphorylated 4EBP1 (T37/T46), AKT pan (S473), ERK1/2 (T202/Y204) p38αβγ (T180/Y182), SLP-76 (S376) and STAT3 (Y705) in whole-cell lysates from cells treated with pathway-specific modulators. Of note, the THUNDER™ Phospho-SLP-76 and Phospho-STAT3 assay kits have been redeveloped using new antibody pairs to improve their performance.

THUNDER™ TR-FRET ASSAY PRINCIPLE

The three TR-FRET technologies use different donor and acceptor fluorophores (shown in Table 1). However, all TR-FRET assay platforms are based on the same sandwich immunoassay principle. The assay principle of THUNDER™ is shown in Figure 1. Following treatment, cells are first lysed with the specific Lysis Buffer included in the kit. The target phosphorylated protein in the whole cell lysate is then detected in a single addition step with a pair of fluorophore-labeled specific antibodies that recognize distinct epitopes on the protein. One antibody is labeled with a Europium chelate donor (Eu-Ab1), whereas the second antibody is labeled with a far-red small fluorophore acceptor (FR-Ab2). The binding of the two labeled antibodies to the target protein takes place in solution and brings the two dyes into close proximity. Excitation of the donor Europium chelate molecules at 320 or 340 nm triggers a FRET from the donor to the acceptor molecules, which in turn emit a signal at 665 nm. This signal is proportional to the concentration of phosphorylated protein in the cell lysate. In the absence of the specific target protein, the donor and acceptor fluorophores are too distant from each other for FRET to occur.

MATERIALS AND METHODS

The THUNDER™ assay kits used for this evaluation are listed in Table 2. Comparative kits were purchased from established vendors. Reagents provided with each THUNDER™ assay kit are listed in Table 3. All reagents were prepared according to each manufacturer's recommendations. Milli-Q® water was used for diluting all Lysis and Detection buffers. Other reagents and materials used in the study are shown in Table 4.

Table 2 THUNDER™ kits used in the study.

Assay Kit	Catalog number
Phospho-4EBP1 (T37/T46)	KIT-4EBP1P-100
Phospho-AKT pan* (S473)	KIT-AKTS473P-100
Phospho-ERK1/2 (T202/Y204)	KIT-ERKP-100
Phospho-SLP-76 (S376)	KIT-SLP76P-100
Phospho-STAT3 (Y705)	KIT-STAT3P-100
Phospho-p38αβγ (T180/Y182)	KIT-P38P-100

** AKT pan is AKT 1/2/3*

Table 3 Reagents supplied with each THUNDER™ Cell Signaling Assay kit.

Kit contents
Europium chelate-labeled phospho-protein antibody (Eu-Ab1)
Acceptor-labeled phospho-protein antibody (FR-Ab2)
Lysis Buffer (5X)
Detection Buffer (10X)
Positive control cell lysate
Phosphatase Inhibitor Cocktail (100X)

Table 1 Donor and acceptor fluorophores used by each TR-FRET technology.

	TR-FRET Technology		
FRET moiete	THUNDER™	COMPANY A	COMMPANY B
Donor	Eu chelate	Eu cryptate	Eu chelate
Acceptor	Far-red dye	d2 dye	ULight dye

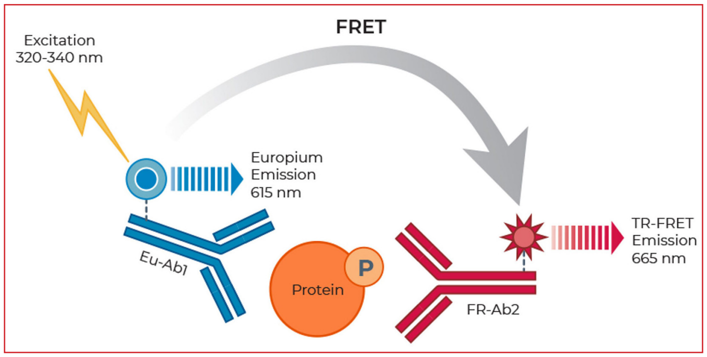


Figure 1 THUNDER™ TR-FRET cell signaling assay principle.

Table 4 Reagents and materials used in the study.

Reagents and materials	Source (catalog #)
Cell lines	
A431	ATCC (CRL-1555)
HEK293	ATCC (CRL-1573)
HeLa	ATCC (CCL-2)
Jurkat	ATCC (B-152)
MCF7	ATCC (HTB-22)
Reagents	
Anisomycin	Cayman (11308)
DMEM	Wisent (320-005-CL)
EGF	PeproTech (AF-100-15)
EMEM	Wisent (320-005-CL)
FBS	Wisent (098-050)
H_2O_2	Sigma (216763)
IFNα	ProSpec (CYT-460)
Insulin	Sigma (I9278)
PP242	Cayman (0469889-17)
RPMI	Wisent (350-007-CL)
Ultra pure water	MilliQ® water
Material	
96-well culture microplate	Costar (3595)
384-well white microplate	PerkinElmer (6007290)
Microplate seal	PerkinElmer (6050185)

The overall assay workflow is the same for all TR-FRET assays and is outlined in Figure 2. All assays were conducted manually using the standard two-plate transfer protocol for each kit, whereby the cells are seeded, treated and lysed in a 96-well culture plate, and lysates are then transferred to a low-volume 384-well assay plate for protein detection.

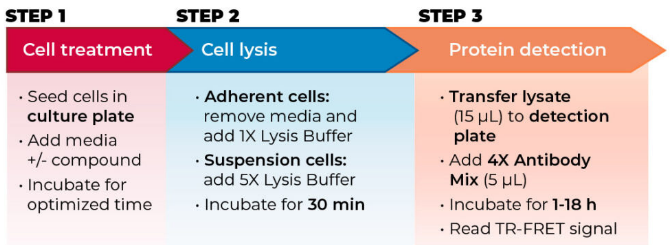


Figure 2 THUNDER™ assay workflow using the two-plate transfer protocol.

Cell lines, culture conditions and treatment protocols used for this study are listed in Table 5. For each comparison, assays were conducted side-by-side using the same batch of treated cells. Adherent cells were seeded in 96-well culture plates and treated the following day. Suspension cells were treated immediately following plating. Cells were treated with varying concentrations of pathway-specific modulators according to pre-optimized conditions. All compounds were dissolved in serum-free culture media.

All assays were conducted according to each manufacturer's protocols. The two-plate protocol used for the THUNDER™ assay platform is summarized in Figure 3. Following treatment, adherent cells were lysed with the corresponding 1X Lysis Buffer, whereas suspension cells were lysed with the corresponding concentrated Lysis Buffer. The different Lysis Buffers contained phosphatase inhibitors. Following a 30 min incubation at room temperature (RT) on an orbital shaker (400 rpm), aliquots of lysates were then transferred in triplicate to the same white 384-well plate microplate followed by the addition of the corresponding antibody detection mix (prepared in the corresponding 1X detection buffer). All assays were run in a total assay volume of 20 µL. Plates were covered with an adhesive plate sealer to reduce evaporation during incubation. The plate sealer was removed before TR-FRET reading. Plates were read at multiple incubation times on an EnVision® 2104 Multilabel Plate Reader in TR-FRET mode (lamp excitation) using an excitation filter at 320 nm and emission filters at 615 nm (620 nm for Company A) and 665 nm. TR-FRET data were expressed as emission ratios of acceptor/donor (665 nm/615 nm) signals.

Data in the figures are shown as mean ± standard deviation (three wells per assay point). Compound concentration response data were analyzed using nonlinear regression and fitted to a sigmoidal four-parameter equation with $1/Y^2$ weighting (GraphPad Prism software). Performance metrics were S/B ratio, IC50 and EC50, intra-assay variability (%CV), and stability of S/B ratio and pharmacological parameters following overnight incubation [3].

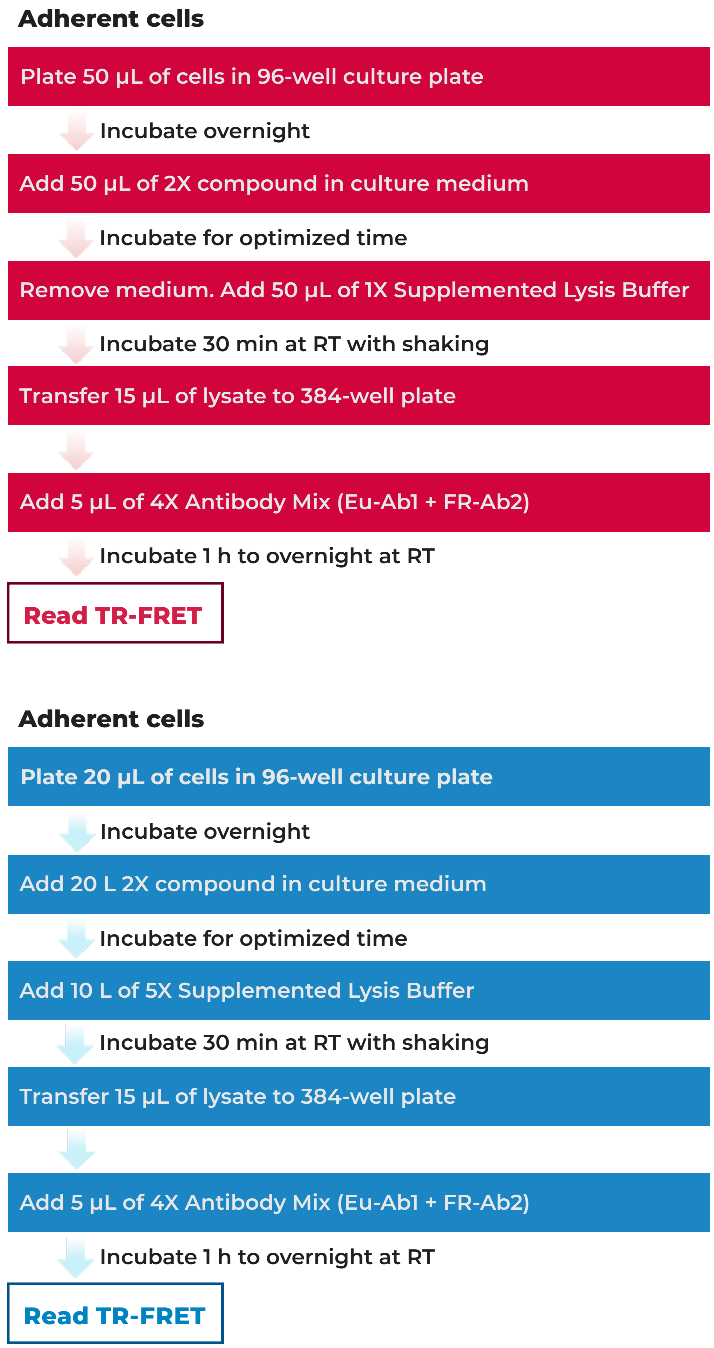


Figure 3 THUNDER™ assay protocols for adherent and suspension cells.

Table 5 Cell lines and treatment conditions used for testing the TR-FRET assay kits.

	Phospho-protein					
	4EBP1	AKT pan	ERK1/2	SLP-76	STAT3	p38αβγ
Cell line	**A431**	**MCF7**	**HEK293**	**Jurkat**	**Hela**	**Hela**
Cell seeding density	25,000 cells/well	60,000 cells/well	50,000 cells/well	400,000 cells/well	40,000 cells/well	50,000 cells/well
Cell culture conditions	DMEM +10% FBS	DMEM +10% FBS	EMEM +10% FBS	RPMI	DMEM +10% FBS	DMEM +10% FBS
Treatment conditions	PP242 3 hours at 37 °C	Insulin 10 min at RT	EGF 10 min at RT	H_2O_2 15 min at RT	IFNα2b 20 min at RT	Anisomycin 1 hour at 37 °C

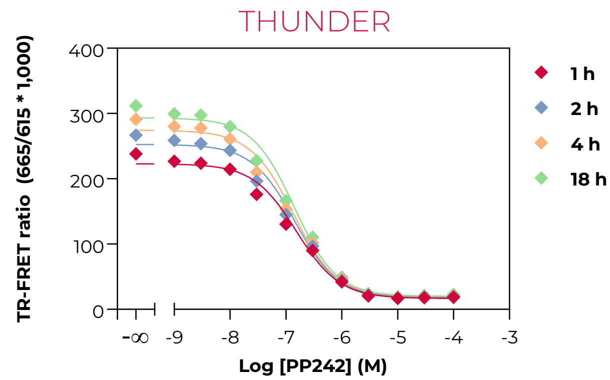
RESULTS AND DISCUSSION

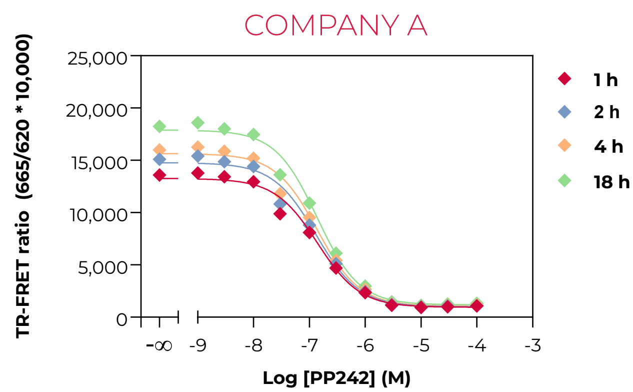
In order to adequately benchmark the performance of the new THUNDER™ TR-FRET platform against two competitive TR-FRET technologies, comparisons were conducted using real cell lysate samples, rather than recombinant proteins spiked into the corresponding lysis buffers. Indeed, the assay performance obtained with recombinant proteins is not always predictive of the performance with real cell extracts, since antibody pairs might recognize the target proteins differently in each sample matrix. In addition, it is essential to evaluate the effectiveness of each kit's lysis buffer for releasing the target protein. To better assess the sensitivity and dynamic range of each assay kit, we conducted concentration-responses curves for each target protein tested.

The results of the head-to-head comparisons are summarized in Figures 4 to 9. Overall, all three TR-FRET technologies showed an increase in the specific signal (treated cells) and S/B ratios (treated versus untreated cells) as a function of incubation time. However, there were differences in terms of the maximal S/B ratios reached by each platform. THUNDER™ assays showed the highest S/B ratios for phospho-AKT pan (S473), phospho-ERK1/2 (T202/Y204), phospho-STAT3 (Y705), and phospho-p38αβγ (T180/Y182). Comparable S/B ratios were obtained for THUNDER™ and Company A phospho-4EBP1 assay as well as THUNDER™ and Company B phospho-SLP-76 (S376) assay. Company B exhibited the lowest S/B ratios for phospho-AKT pan, phospho-ERK1/2 and phospho- STAT3.

Table 6 summarizes the S/B ratios obtained with the three TR-FRET assay platforms for each phospho-protein tested at the detection time point recommended by each manufacturer. Whereas assays from Bioauxilium and Company A exhibited acceptable S/B ratios (≥ 3) at all recommended detection times, the phospho-AKT pan (S473) assay from Company B showed sub-optimal S/B ratios (< 3).

The three TR-FRET technologies exhibited comparable inter-well variability (typical %CV <8%) and sensitivity (IC_{50} and EC_{50} values) for all six phosphorylated proteins tested (Figures 4 to 9). In addition, all technologies tolerated plate reading after overnight incubation, which allows off-line readings for increased flexibility and productivity. Of note, all THUNDER™ assays allowed plate reading after only 1 hour of incubation. This was not the case for AKT pan and STAT3 from Company B.



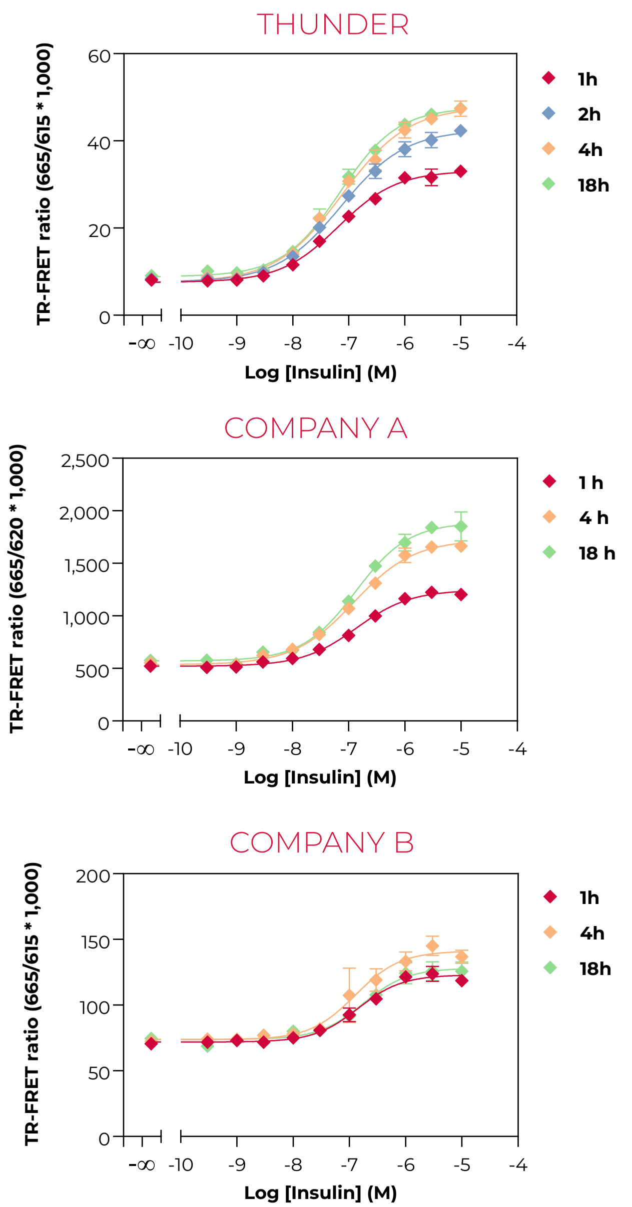


	THUNDER™		Company A	
Incubation time	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)
1 h	12.9	154	12.7	139
2 h	13.4	144	13.8	132
3 h	14	136	13.6	139
4 h	13.7	141	13.9	138

Figure 4 Head-to-head assessment of the Phospho-4EBP1 (T37/T46) assays.

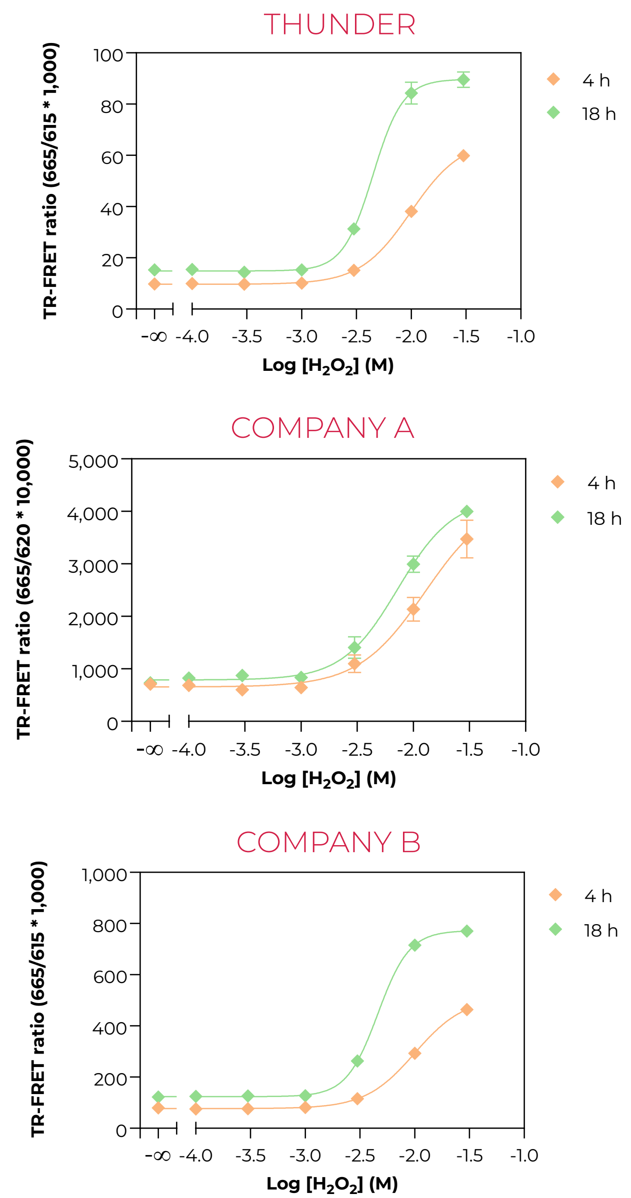
Table 6 Summary of S/B ratios obtained with each TR-FRET technology at the recommended detection time points.

Assay	S/B ratio (at manufacturer's recommended detection time)			Conclusion
	THUNDER™	Company A	Company B	
Phospho-4EBP1 (T37/T46)	13.4 (2 h)	13.8 (2 h)	Kit not available	Comparable performance between THUNDER™ and Company A kits
Phospho-AKT pan (S473)	5.7 (4 h)	3.0 (4 h)	1.9 (4 h)	Highest S/B ratio with THUNDER™ kit at all incubation timepoints
Phospho-ERK1/2 (T202/Y204)	11.2 (4 h); 14.2 (18 h)	10.8 (4 h); 10.5 (18 h)*	8.5 (18 h)	Comparable performance between THUNDER™ and Company A (Advanced Phospho-ERK1/2) kits (but highest S/B ratio with THUNDER™ kit after overnight incubation)
Phospho-SLP-76 (S376)	6.1 (4 h)	4.9 (4 h)	5.9 (4 h)	Comparable performance between THUNDER™ and Company B kits
Phospho-STAT3 (Y705)	10.3 (4 h); 15.0 (18 h)	7.4 (4 h); 14.6 (18 h)	3.6 (4 h); 6.4 (18 h)	Highest S/B ratio with THUNDER™ kit between 1 and 4 hours of incubation
Phospho-p38αβγ (T180/Y182)	18.4 (2 h)	13.5 (4 h)	Kit not available	Highest S/B ratio with THUNDER™ kit



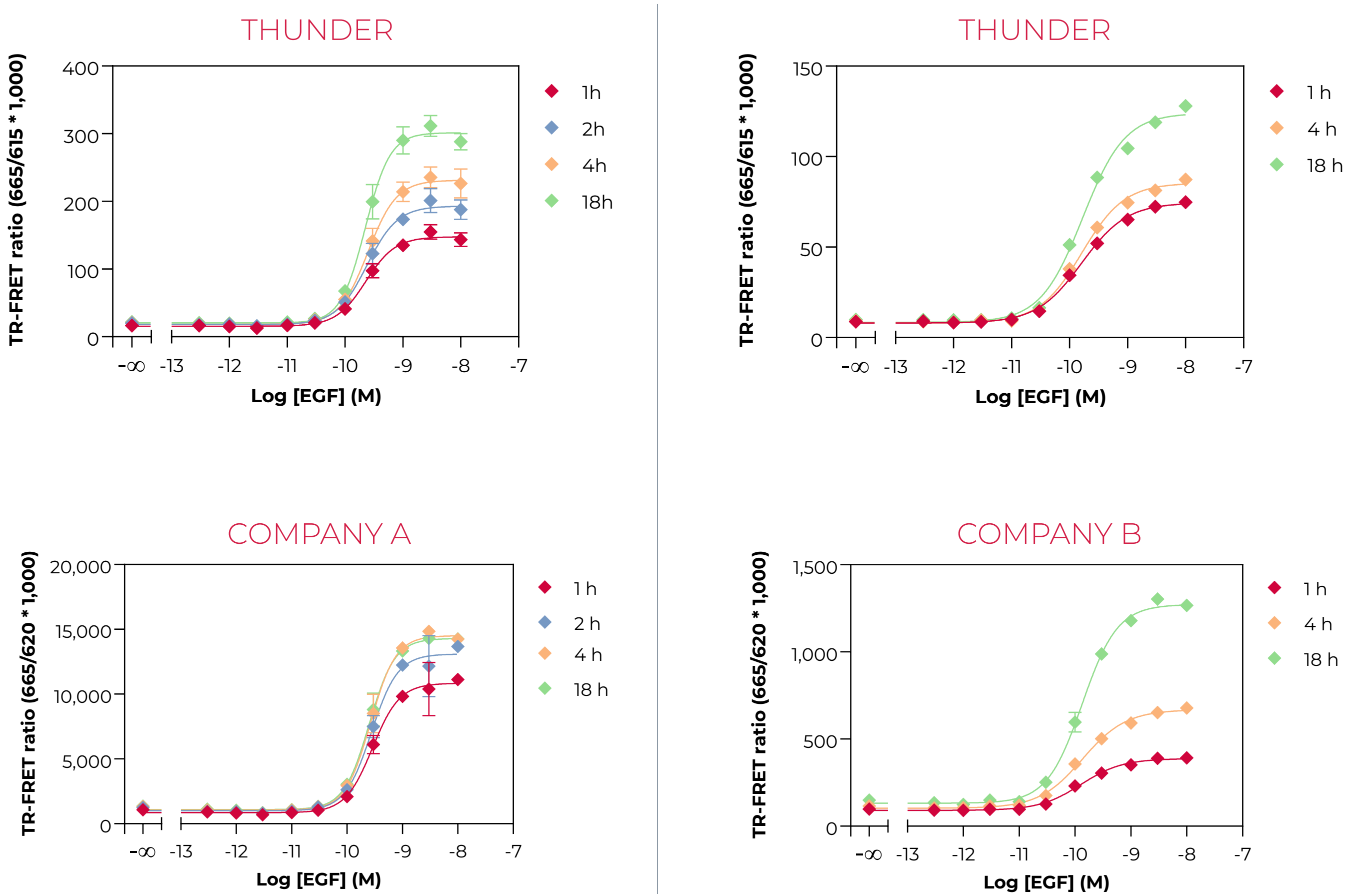
	THUNDER™		Company A		Company B	
Incubation time	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)
1 h	4.1	66	2.3	137	1.8	142
2 h	5.1	74	NM	NM	NM	NM
4 h	5.7	76	3.0	124	1.9	127
18 h	5.2	70	3.2	133	1.7	174

Figure 5 Head-to-head assessment of the Phospho-AKT pan (S473) assays.



	THUNDER™		Company A		Company B	
Incubation time	S/B	EC_{50} (mM)	S/B	EC_{50} (mM)	S/B	EC_{50} (mM)
4 h	6.1	9.9	4.9	12.6	5.9	10.0
18 h	6.0	4.5	5.5	7.2	6.3	4.6

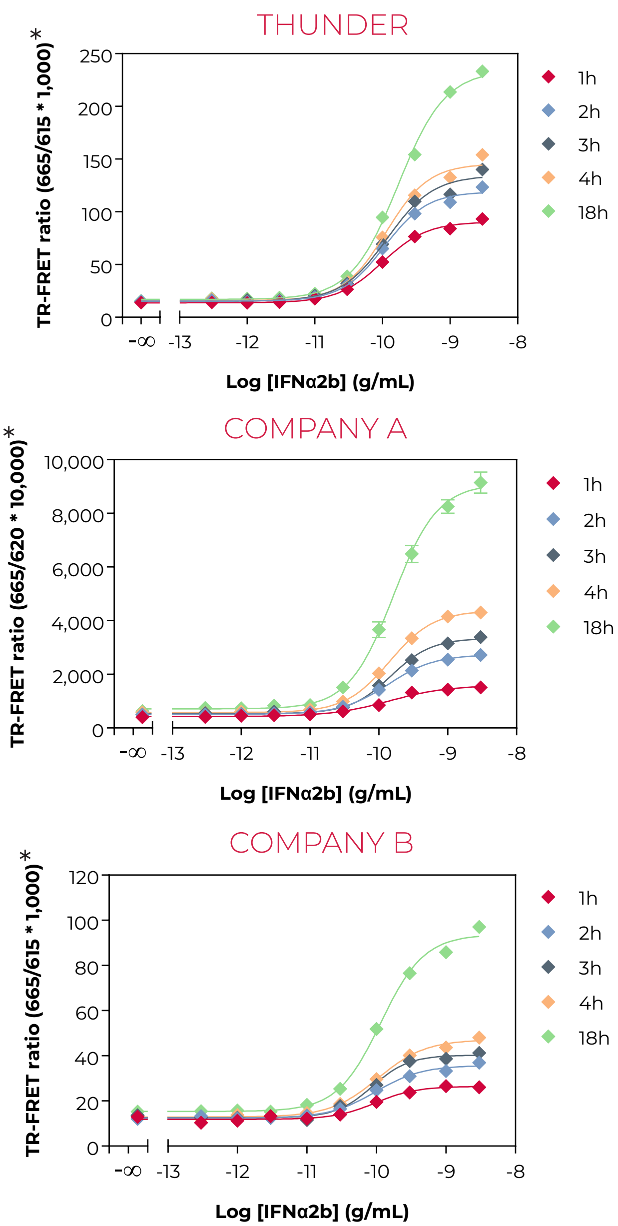
Figure 6 Head-to-head assessment of the Phospho-SLP-76 (S376) assays.



	THUNDER™		Company A	
Incubation time	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)
1 h	9.4	0.23	10.3	0.29
2 h	9.9	0.24	11.0	0.28
4 h	11.2	0.25	10.8	0.25
18 h	14.2	0.22	10.5	0.25

	THUNDER™		Company A	
Incubation time	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)
1 h	8.6	0.16	4.0	0.13
4 h	9.6	0.16	5.9	0.12
18 h	13.0	0.17	8.5	0.13

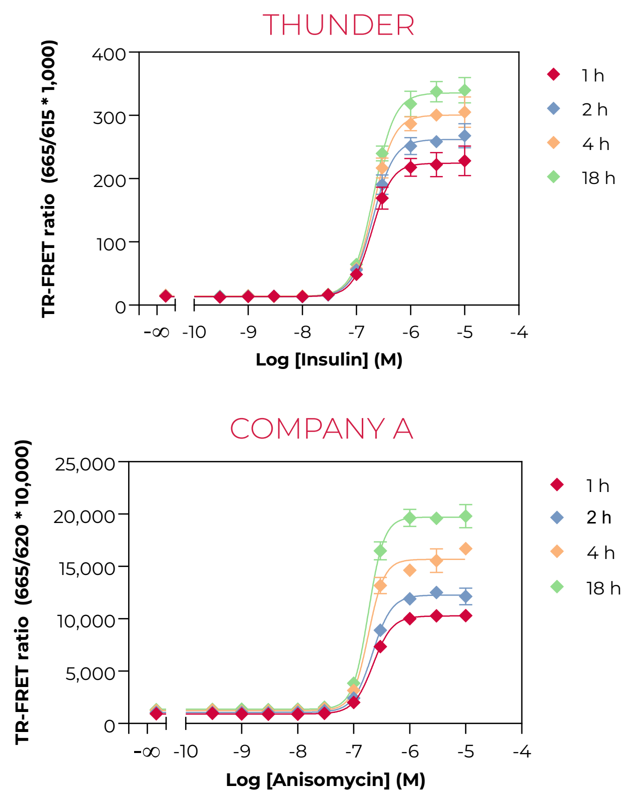
Figure 7 Head-to-head assessment of the phospho-ERK1/2 (T202/Y204) assays.



	THUNDER™		Company A		Company B	
Incubation time	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)
1 h	6.7	0.10	3.8	0.14	2.0	0.10
2 h	8.1	0.11	5.4	0.14	2.1	0.10
3h	9.1	0.11	6.5	0.15	3.0	0.09
4 h	10.3	0.11	7.4	0.14	3.6	0.11
18 h	15.0	0.17	14.6	0.16	6.4	0.11

Figure 8 Head-to-head assessment of the Phospho-STAT3 (Y705) assays.

**Results obtained with laser excitation.*



	THUNDER™		Company A	
Incubation time	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)
1 h	16.1	196	11.1	220
2 h	18.4	202	11.6	215
4 h	19.2	207	13.5	182
18 h	23.2	207	15.0	182

Figure 9 Head-to-head assessment of the Phospho-p38αβγ (T180/Y182) assays.

To serve both academic and industrial researchers, assay technologies need to deliver quality data at an acceptable cost per well. As shown in Table 7, THUNDER™ assay kits are more cost-effective than the other two commercial TR-FRET technologies.

Table 7 Assay cost analysis.

	Cost per well (USD)[1]		
Kit size	**THUNDER™**	**Company A**	**Company B**
96 tests	$3.82	$6.81	Not available
500 tests	$2.40	$4.09	$4.83
2 500 tests	$2.03	Not available	Not available
5 000 tests	$1.26	Not available	Not available
10 000 tests	$0.78	$1.19	$0.93

[1] *Assay cost per well is calculated by dividing the commercial cost of the assay (2023 price list) by the number of number of samples that can be analyzed.*
[2] *Few kits available in the 96-test format.*
[3] *When purchasing the positive control lysate (not included in the kit).*

CONCLUSIONS

This study aimed at benchmarking the performance of three homogeneous TR-FRET assay platforms on the market today for measuring the relative levels of six different endogenous phosphorylated proteins in whole cell lysates. All three platforms were studied for their capacity to deliver quality data using the two-plate assay protocol with target protein detection in 384-well microplates.

Collectively, the results of these head-to-head comparisons showed that all THUNDER™ assays tested provide superior (phospho-AKT pan (S473), phospho-ERK1/2 (T202/Y204), phospho-STAT3 (Y705), phospho-p38αβγ (T180/Y182)) or comparable (phospho-4EBP1 (T37/T46), phospho-SLP-76 (S376)) S/B ratios and comparable assay pharmacology (EC50 and IC50 values). In addition, THUNDER™ is more cost effective than the other two TR-FRET technologies.

These key advantages, combined with rigorous assay validation using cell lysates from stimulated/inhibited cells, and higher flexibility in terms of kit sizes and formats (detection of either phosphorylated, total or phosphorylated plus total proteins with the same kit), make the THUNDER™ assay platform an alternative of choice for monitoring cellular protein phosphorylation.

REFERENCES

[1] Ardito F, Giuliani M, Perrone D, et al. The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review). Int J Mol Med 40, 271-280 (2017). https://doi.org/10.3892/ijmm.2017.3036

[2] Algar WR, Hildebrandt N, Vogel SS, et al. FRET as a biomolecular research tool — understanding its potential while avoiding pitfalls. Nat Methods 16, 815–829 (2019). https://doi.org/10.1038/s41592-019-0530-8

[3] Dahlin JL, Sittampalam GS, Coussens NP, et al. Basic Guidelines for Reporting Non-Clinical Data. 2019 Oct 1. In: Markossian S, Sittampalam GS, Grossman A, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004. https://www.ncbi.nlm.nih.gov/books/NBK550206/

7171 Frederick-Banting, Suite 2115, Montréal (Québec) H4S 1Z9 CANADA **bioauxilium.com** **1-866-610-6933 x 103**