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# Time-resolved Förster Energy Transfer Assays for Measurement of Endogenous Phosphorylated STAT Proteins in Human Cells

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**TITLE:**

# Time-resolved Förster Energy Transfer Assays for Measurement of Endogenous Phosphorylated STAT Proteins in Human Cells

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**KEYWORDS:**

JAK/STAT signaling; STAT proteins; Phosphorylation; Homogeneous assays; TR-FRET; Immunoassay; Cell-based assay; Kinases; Cell signaling; High-throughput screening; Cytokine signaling

**SUMMARY:**

Time-resolved Förster resonance energy transfer cell-based assay protocols are described for the simple, specific, sensitive, and robust quantification of endogenous phosphorylated signal transducer and activator of transcription (STAT) 1/3/4/5/6 proteins in cell lysates in a 384-well format.

**ABSTRACT:**

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway plays a crucial role in mediating cellular responses to cytokines and growth factors. STAT proteins are activated by tyrosine phosphorylation mediated mainly by JAKs. The abnormal activation of STAT signaling pathways is implicated in many human diseases, especially cancer and immune-related conditions. Therefore, the ability to monitor STAT protein phosphorylation within the native cell signaling environment is important for both academic and drug discovery research. The traditional assay formats available to quantify phosphorylated STAT proteins include western blotting and the enzyme-linked immunosorbent assay (ELISA). These heterogeneous methods are labor-intensive, low-throughput, and often not reliable (specific) in the case of western blotting. Homogeneous (no-wash) methods are available but remain expensive.

Here, detailed protocols are provided for the sensitive, robust, and cost-effective measurement in a 384-well format of endogenous levels of phosphorylated STAT1 (Y701), STAT3 (Y705), STAT4 (Y693), STAT5 (Y694/Y699), and STAT6 (Y641) in cell lysates from adherent or suspension cells using the novel THUNDER time-resolved Förster resonance energy transfer (TR-FRET) platform. The workflow for the cellular assay is simple, fast, and designed for high-throughput screening

(HTS). The assay protocol is flexible, uses a low-volume sample (15 µL), requires only one reagent addition step, and can be adapted to low-throughput and high-throughput applications. Each phospho-STAT sandwich immunoassay is validated under optimized conditions with known agonists and inhibitors and generates the expected pharmacology and Z'-factor values. As TR-FRET assays are ratiometric and require no washing steps, they provide much better reproducibility than traditional approaches. Together, this suite of assays provides new cost-effective tools for a more comprehensive analysis of specific phosphorylated STAT proteins following cell treatment and the screening and characterization of specific and selective modulators of the JAK/STAT signaling pathway.

**INTRODUCTION:**

The JAK/STAT signaling pathway plays a key role in mediating cellular responses to diverse cytokines, interferons, growth factors, and related molecules[1,2]. The binding of these ligands to specific cell-surface receptors results in the activation of JAKs, which in turn activate STAT proteins by phosphorylation of specific tyrosine residues. STAT phosphorylation results in their dimerization and translocation into the nucleus, where they exert their effect on the transcription of regulated target genes. The STAT family consists of seven members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. The members play a complex and essential role in the regulation of physiologic cell processes, including proliferation, differentiation, apoptosis, angiogenesis, and immune system regulation. The abnormal activation of STAT signaling pathways is implicated in many human diseases, especially cancer and immune-related conditions[3,4]. Therefore, the ability to assess STAT protein phosphorylation within the native cell signaling environment is important for both academic and drug discovery research.

To date, the conventional methods used to measure intracellular phosphorylated protein levels, including STATs, are antibody-based and include western blotting, ELISA, and phosphoflow cytometry. These heterogeneous methods are labor-intensive, time-consuming, error-prone, low-throughput, and often unreliable (specific) in the case of western blotting[5]. In contrast, homogeneous assays require fewer experimental steps, use smaller sample volumes, and are amenable to HTS. There are five homogeneous cell-based immunoassay platforms commercially available that can be used to quantitatively monitor JAK-dependent phosphorylation of STATs in cell lysates: SureFire, HTRF, LANCE, LanthaScreen, and Lumit. Each of these platforms has its advantages and disadvantages.

SureFire is based on luminescent oxygen channeling technology, which utilizes donor and acceptor beads coated to specifically capture a pair of antibodies, one of which is biotinylated. In the presence of phosphorylated protein, the two antibodies bring the donor and acceptor beads into close proximity, enabling the generation of a chemiluminescent signal[6]. While versatile and sensitive, this technology is expensive, is affected by biotin in the culture medium, is very sensitive to ambient temperature and light, and requires a special reader for detection. HTRF and LANCE are both based on TR-FRET technology that utilizes long-lifetime luminescent lanthanide ion complexes (Europium or Terbium chelates, or Europium cryptate) as the donor molecules and far-red fluorophores as the acceptor molecules[7]. When two protein-specific antibodies labeled with either donor or acceptor molecules are brought into close proximity, FRET takes place,

causing an increase in acceptor fluorescence and a decrease in donor fluorescence. These long-lived fluorescent signals can be measured in a time-resolved and ratiometric manner to reduce assay interference and increase data quality. Other advantages of TR-FRET are that it is not light-sensitive, allows repeated readings, and exhibits long signal stability. While TR-FRET is widely implemented in HTS due to its versatility, sensitivity, and high robustness, all commercial TR-FRET-based assay platforms are expensive, thereby precluding its wide adoption in academic and small industrial laboratories. The LanthaScreen assay also uses a TR-FRET based-readout but is reliant on an engineered U2OS cell line that stably expresses green fluorescent protein (GFP)-STAT1 fusion protein combined with a terbium-labeled phospho-specific STAT1 antibody[8]. In addition to being limited in terms of choice of signaling proteins, this method requires purchasing expensive transfected cell lines, reducing its applicability and increasing the possibility of experimental artifacts. Lumit is a generic bioluminescent immunoassay platform that utilizes secondary antibodies (anti-mouse and anti-rabbit) chemically labeled with the small and large NanoBit subunits of NanoLuc Luciferase[9]. The binding of two primary antibodies to the target protein brings the secondary antibodies into proximity to form an active enzyme that generates a luminescence signal. While luminescence is generally a sensitive and robust readout, the requirement for primary antibodies raised in two different species limits the choices for assay design. In addition, the use of secondary antibodies in complex sample matrixes may be prone to assay interference.

Thus, a need still exists for a reliable, rapid, yet affordable cell-based assay platform for measuring individual phosphorylated and total STAT proteins in a manner compatible with HTS. To address this need, a new high-throughput cell-based immunoassay platform was developed based on an enhanced TR-FRET technology (THUNDER) and designed to enable simple, sensitive, robust, and cost-effective measurement of endogenously expressed intracellular proteins (phosphorylated or total) in cell lysates. The advantages of this technology stem from the combination of a donor/acceptor FRET pair exhibiting exceptional spectral compatibility and TR-FRET signal, rigorously validated antibodies, and optimized lysis buffers. These assays are formatted as sandwich immunoassays and use a straightforward, three-step workflow (**Figure 1**). Cells are first treated to modulate protein phosphorylation and then lysed with the specific lysis buffer provided in the kit. The target phosphorylated or total STAT protein in the cell lysate is detected in a single reagent addition and incubation step with a pair of fluorophore-labeled antibodies that recognize distinct epitopes on the target protein (**Figure 2**). One antibody is labeled with a Europium chelate donor (Eu-Ab1), while the second antibody is labeled with a far-red acceptor fluorophore (FR-Ab2). The two labeled antibodies bind to the protein in solution, bringing the two labels into close proximity. Excitation of the donor Europium chelate at 320 or 340 nm triggers a FRET to the acceptor, which emits a long-lived TR-FRET signal at 665 nm proportional to the concentration of target protein (phosphorylated or total) in the cell lysate.

[Place **Figure 1** here]
[Place **Figure 2** here]

Here, detailed protocols are provided for measuring, in a 384-well format, the intracellular levels of phosphorylated STAT1 (Y701), STAT3 (Y705), STAT4 (Y693), STAT5 (Y694/Y699), and STAT6

(Y641), together with total STAT1, STAT3, STAT5, and STAT6, in cell lysates from adherent or suspension cells using the THUNDER TR-FRET platform. These protocols define steps for cell treatment, lysis, and TR-FRET-based target protein detection using either a two-plate transfer protocol or a one-plate all-in-one-well protocol. These cell-based assays are applied for determining the pharmacological profile of known activators and inhibitors of the JAK/STAT pathway. The robustness and suitability of selected assays for HTS are demonstrated. Lastly, key experiments for assay optimization are discussed, along with recommendations for assay troubleshooting.

**PROTOCOL:**

**1. Cell culture**

1.1. Maintain cells in a humidified 37 °C/5% $CO_2$ incubator and culture with either DMEM supplemented with 10% fetal bovine serum (FBS) (HeLa and A431 cells) or RPMI supplemented with 15% FBS (U266B1 cells). Culture the cells until they reach 70–80% confluence, then trypsinize them and passage or use them for the assays.

NOTE: Culture media contained phenol red. No serum starvation was conducted for any cell line prior to conducting the assays.

**2. Stimulator or inhibitor titration using the two-plate assay protocol with adherent cells**

NOTE: This procedure describes how to determine stimulator or inhibitor potencies by generating a concentration-response curve from a dilution series of the test compound.

2.1. Cell seeding

2.1.1. Dispense 50 µL of cells at the pre-optimized density (40,000 HeLa cells/well for both STAT3 and STAT6; 75,000 A431 cells/well for STAT5) into a 96-well tissue culture-treated plate in the appropriate culture medium. Incubate overnight at 37 °C/5% $CO_2$.

NOTE: Optimal cell density and culture incubation time need to be determined.

2.2. Dilutions of test compounds

2.2.1. Prepare intermediate 2x dilution series of test compound(s) by serially diluting compound(s) (half-log interval dilutions) across 12 wells of a polypropylene 96-well plate into serum-free medium.

NOTE: It is recommended to conduct a 12-point, half-log interval concentration-response curve in at least duplicate for an accurate estimation of the $EC_{50}$ or $IC_{50}$.

2.2.2. Alternatively, for hydrophobic, dimethylsulfoxide (DMSO)-soluble test compounds,

perform the initial dilutions in 100% DMSO, and then dilute the compound dilution series into serum-free medium.

NOTE: The assay tolerance to DMSO must be established before conducting a test compound titration in DMSO vehicle. It is important to keep equal solvent concentrations between treated and untreated cells. In addition, when testing serial dilutions of compounds, the solvent concentrations should always remain constant across the dilution series.

2.3. Cell treatment

2.3.1. For cell stimulation, add 50 µL of serum-free medium alone (untreated cells) or containing the stimulator (2x).

2.3.2. Incubate for the pre-optimized time at either room temperature (RT) or 37 °C (interferon (IFN) α2b/20 min at RT for STAT3; epidermal growth factor (EGF)/10 min at RT for STAT5; interleukin (IL)-4/20 min at RT for STAT6). Proceed then to step 2.4.

NOTE: Optimal incubation temperature needs to be determined.

2.3.3. For cell inhibition, add 25 µL of serum-free medium alone (untreated cells) or containing the inhibitor (4x).

2.3.4. Incubate for the pre-optimized time at either RT or 37 °C (JAK Inhibitor 1/30 min at RT for STAT3 and STAT6; Erlotinib/15 min at RT for STAT5).

2.3.5. Add 25 µL of serum-free medium alone (untreated cells) or containing the stimulator (4x) at its $EC_{80}$.

2.3.6. Incubate for the pre-optimized time at either RT or 37 °C (same conditions as for step 2.3.2).

2.4. Cell lysis

2.4.1. Prepare the kit's specific 1x Supplemented Lysis Buffer as indicated by the manufacturer.

NOTE: It is mandatory to supplement the 1x Lysis Buffer with the 100x Phosphatase Inhibitor Cocktail diluted to a final concentration of 1x. The 1x Supplemented Lysis Buffer contains 1 mM sodium fluoride, 2 mM sodium orthovanadate, and 2 mM beta-glycerophosphate. Other phosphatase inhibitors are not required and should be avoided. Lysis buffers and phosphatase inhibitors other than those included in the kit are not recommended as they might contain ingredients that could interfere with the measurement.

2.4.2. Carefully remove and discard the cell culture medium by aspirating the supernatant.

2.4.3. Immediately add 50 µL of 1x Supplemented Lysis Buffer.

NOTE: Lysis Buffer volume (25–50 µL) may be optimized.

2.4.4. Incubate for 30 min at RT under shaking (orbital plate shaker set at 400 rpm; moderate agitation).

NOTE: Lysis incubation time (15–60 min) may be optimized. Lysates can be used immediately for target protein detection or frozen at -80 °C.

2.5. TR-FRET detection

2.5.1. Prepare the 4x Antibody Detection Mix in 1x Detection Buffer as indicated by the manufacturer.

2.5.2. In this transfer step, carefully pipette 15 µL of cell lysate from the 96-well culture plate to a well of a white, low-volume 384-well microplate.

2.5.3. Add 15 µL of the Positive Control Lysate and 15 µL of 1x Lysis Buffer (negative control) to separate assay wells.

2.5.4. To separate wells containing 15 µL of lysate, add either 5 µL of 4x Eu-Ab1/FR-Ab2 for the detection of the phospho-protein or 5 µL of 4x Eu-Ab3/FR-Ab4 for the detection of the total protein.

2.5.5. Cover the plate with a plate sealer and incubate for 1 h up to overnight at RT, depending on the assay kit (see the corresponding Technical Data Sheet).

NOTE: Optimal reading time needs to be optimized for each assay and cell line. The plate can be read several times without a negative effect on the assay performance.

2.5.6. Remove the adhesive plate sealer and read the plate on a TR-FRET compatible microplate reader.

NOTE: Filter-based fluorometers are recommended, though some monochromator instruments can be used. Verify that the appropriate optic module (filters and mirror) for TR-FRET is installed. Use an excitation wavelength of 320 or 340 nm to excite the Europium chelate. Read assays at both 615 nm (or 620 nm) and 665 nm to detect both the emission from the donor Europium and the acceptor fluorophore, respectively. The instrument settings will depend on the particular reader. Data presented here were obtained using lamp-based excitation, 90 µs delay, 300 µs integration time, and 100 flashes per well. The phospho-STAT4 assay, however, was read using laser excitation to generate higher signal-to-background (S/B) ratios.

**3. Stimulator or inhibitor titration using the two-plate assay protocol with suspension cells**

3.1. Dilution of test compounds

3.1.1. Prepare intermediate 2x dilution series of test compound(s) as described in steps 2.2.1 and 2.2.2.

3.2. Cell seeding and treatment

3.2.1. Dispense 20 µL of cells at the pre-optimized density (200,000 U266B1 cells/well for STAT1; 400,000 U266B1 cells/well for STAT4) into a 96-well tissue culture-treated plate in the appropriate culture medium. Directly proceed to cell treatment or incubate 2–4 h at 37 °C, 5% $CO_2$.

NOTE: This step needs to be optimized for different cell types.

3.2.2. For cell stimulation, add 20 µL of serum-free medium alone (untreated cells) or containing the stimulator (2x).

3.2.3. Incubate for the pre-optimized time at either RT or 37 °C (IFNα2b/15 min at RT for STAT1; IFNα2b/25 min at 37 °C for STAT4). Proceed then to section 3.3.

NOTE: Optimal incubation temperature needs to be determined.

3.2.4. For cell inhibition, add 10 µL of serum-free medium alone (untreated cells) or containing the inhibitor (4x).

3.2.5. Incubate for the pre-optimized time at either RT or 37 °C (JAK Inhibitor 1/30 min at RT for STAT1 and STAT4).

3.2.6. Add 10 µL of serum-free medium alone (untreated cells) or containing the stimulator (4x) at its $EC_{80}$.

3.2.7. Incubate for the pre-optimized time at either RT or 37 °C (same conditions as for step 3.2.3).

3.3. Cell lysis

3.3.1. Prepare the kit's specific 5x Supplemented Lysis Buffer as indicated by the manufacturer.

NOTE: It is mandatory to supplement the 5x Lysis Buffer with the 100x Phosphatase Inhibitor Cocktail diluted to a final concentration of 5x. The 5x Supplemented Lysis Buffer contains 5 mM sodium fluoride, 10 mM sodium orthovanadate, and 10 mM beta-glycerophosphate. Other phosphatase inhibitors are not required and should be avoided.

3.3.2. Add 10 µL of 5x Supplemented Lysis Buffer.

3.3.3. Incubate for 30 min at RT under shaking (orbital plate shaker set at 400 rpm; moderate agitation).

NOTE: Lysis incubation time (15–60 min) may be optimized. Lysates can be used immediately for target protein detection or frozen at -80 °C.

3.4. TR-FRET detection

3.4.1. Following cell lysis, conduct the TR-FRET detection step described in section 2.5 for the 2-plate assay protocol for adherent cells.

**4. Stimulator or inhibitor titration using the one-plate assay protocol with adherent or suspension cells**

4.1. Dilution of test compounds

4.1.1. Prepare intermediate dilution series of test compound(s) at either 3x (for stimulation) or at 6x (for inhibition) by serially diluting compound(s) (half-log interval dilutions) across 12 wells of a polypropylene 96-well plate into serum-free medium.

4.2. Cell seeding and treatment

4.2.1. Dispense 8 µL of cells at the pre-optimized density (160,000 U266B1 cells/well for STAT4; 80,000 HeLa cells/well for STAT6), in the appropriate serum-free culture medium, into a white, low-volume 384-well assay plate. Directly proceed to cell treatment or incubate 2–4 h at 37 °C, 5% $CO_2$.

NOTE: The requirement for a cell culture incubation period before treatment needs to be determined for different cell types.

4.2.2. For cell stimulation, add 4 µL of serum-free medium alone (untreated cells) or containing the stimulator (3x).

4.2.3. Incubate for the pre-optimized time at either room temperature or 37 °C (IFNα2b/25 min at 37 °C for STAT4; IL-4/20 min at 37 °C for STAT6). Proceed then to section 4.3.

4.3. Cell lysis

4.3.1. Prepare the kit's specific 5x Supplemented Lysis Buffer as indicated by the manufacturer.

NOTE: It is mandatory to supplement the 5x Lysis Buffer with the 100x Phosphatase Inhibitor Cocktail diluted to a final concentration of 5x.

4.3.2. Add 3 µL of 5x Supplemented Lysis Buffer.

4.3.3. Incubate for 30 min at RT under shaking (orbital plate shaker at 400 rpm).

NOTE: Lysis incubation time (15–60 min) may be optimized. Lysates can be used immediately or frozen at -80 °C.

4.4. TR-FRET detection

4.4.1. Add 15 µL of Positive Control Lysate (undiluted) and 15 µL of 1x Supplemented Lysis Buffer (negative control) to separate assay wells.

4.4.2. Add 5 µL of 4x Antibody Detection Mix (either Eu-Ab1/FR-Ab2 for the phospho-protein or Eu-Ab3-/FR-Ab4 for the total protein) prepared in 1x Detection Buffer to each of the assay wells.

4.4.3. Cover the plate with a plate sealer and incubate for 1 h up to overnight at RT, depending on the assay.

NOTE: Optimal reading time needs to be optimized for each assay and cell line. The plate can be read several times without a negative effect on the assay performance.

4.4.4. Remove the adhesive plate sealer. Read the plate on a TR-FRET-compatible microplate reader.

**5. Data analysis**

5.1. Calculate the TR-FRET ratio for each well using the following formula (**1**):

$$\frac{(Signal\ at\ 665\ nm)}{(Signal\ at\ 615\ nm)} \times 1{,}000 \qquad (\mathbf{1})$$

NOTE: Because the TR-FRET signal is read in a time-resolved mode, background subtraction is usually not necessary. If background subtraction is conducted, use the cell-free wells containing the 1x Supplemented Lysis Buffer (negative control) for background subtraction. Determine the average TR-FRET ratio from the cell-free wells and then subtract this value from the TR-FRET ratio of each well.

5.2. For concentration-response curves, analyze the data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting to generate $EC_{50}$ or $IC_{50}$ values.

5.3. For the Z' factor experiment, analyze the data according to the following formula (**2**)[10]:

$$Z' = 1 - \frac{(3\sigma p + 3\sigma n)}{|\mu p - \mu n|} \qquad (\mathbf{2})$$

Where μ and σ are the mean values and standard deviations for the positive control (*p*; stimulated cells) and negative control (*n*; untreated cells), respectively.

**REPRESENTATIVE RESULTS:**

Each THUNDER TR-FRET assay was pharmacologically validated by treating adherent (HeLa or A431) or suspension cells (U266B1) with JAK/STAT pathway-specific activators or inhibitors and then measuring the levels of specific phosphorylated and total STATs, when applicable. Assays were conducted in 384-well format using the two-plate transfer protocol and pre-optimized assay conditions. **Figure 3, Figure 4, Figure 5, Figure 6,** and **Figure 7** summarize representative concentration-response curves obtained for all STAT assays. Overall, all stimulator and inhibitor concentration–response curves for the phospho-STAT assays showed robust TR-FRET signals, wide dynamic ranges, low inter-well coefficient of variation (typically ≤5%), and acceptable S/B ratios. The $EC_{50}$ and $IC_{50}$ values reported here are within the range of expected values.

Treatment of cells with the JAK activators IFNα2b (phospho-STAT1, phospho-STAT3, and phospho-STAT4), IL-4 (phospho-STAT6), and EGF (phospho-STAT5) showed the anticipated concentration-dependent increase in STAT phosphorylation at specific tyrosine residues, while the corresponding total STAT proteins (STAT1, STAT3, STAT5, and STAT6) remained stable (**Figure 3, Figure 4, Figure 5, Figure 6,** and **Figure 7**, Panel A). The signal decrease (less than 20%) observed for total STAT5 with increasing STAT5 phosphorylation is expected and is due to steric hindrance, where phosphorylation of STAT5 hinders the binding of one of the two anti-total STAT5 antibodies to its respective antigen. The $EC_{50}$ values were in the sub-nanomolar range for IFNα2b and IL-4 (0.083 to 0.47 nM) and in the low nanomolar range for EGF (12 nM). These values are in agreement with published data[6,11].

To confirm that the signal induced by the activators was mediated by the activation of endogenous receptors, cells were pretreated with increasing concentrations of either JAK Inhibitor 1 (a pan JAK inhibitor) or Erlotinib (an EGFR tyrosine kinase inhibitor) prior to submaximal stimulation ($EC_{80}$) with the STAT activators. As anticipated, both JAK Inhibitor 1 and Erlotinib inhibited the corresponding phospho-STAT levels in a concentration-dependent manner, with $IC_{50}$ values ranging between the low nanomolar and the high nanomolar range for JAK Inhibitor 1 (29–759 nM) and in the low nanomolar range for Erlotinib (10 nM) (**Figure 3, Figure 4, Figure 5, Figure 6,** and **Figure 7**, Panel B). The $IC_{50}$ values are consistent with published data[11-13]. As was the case for stimulation experiments, the levels of corresponding total STAT were not affected by either treatment. Taken together, these results demonstrate the specificity of each assay for its endogenous target STAT protein (phosphorylated or total) and their capacity to profile activators or inhibitors exhibiting a range of potencies.

Assays in which the whole workflow (cell treatment, lysis, and protein detection) is conducted in a single well of a 384-well plate without a transfer step are more suitable for HTS. Accordingly, the phospho-STAT4 and phospho-STAT6 assays were performed using the one-plate protocol. Representative data obtained under optimized conditions are summarized in **Figure 8** and **Figure 9**. Stimulation of either phosphorylated STAT4 by IFNα2b in a suspension cell line (U266B1; **Figure 8**) or phosphorylated STAT6 by IL-4 in an adherent cell line (HeLa; **Figure 9**) was obtained with

acceptable S/B ratios and $EC_{50}$ values consistent with those obtained using the 2-plate protocol. These data show that the phospho-STAT assays can be successfully adapted from a two-plate transfer protocol to a one-plate all-in-one-well protocol.

The Z'-factor is commonly used in the HTS community for the evaluation of the suitability and robustness of an assay for HTS[10]. To further validate the phospho-STAT assays, preliminary Z'-factor studies were manually conducted using the 2-plate protocol. To assess assay stability, plates were read following both a 4-h incubation and an overnight incubation. Results obtained for the phospho-STAT1 and phospho-STAT3 assays, using a suspension cell line (U266B1) or an adherent cell line (HeLa), respectively, are summarized in **Figure 10**. Calculated Z'-factor values were 0.85 for phospho-STAT1 and 0.79 for phospho-STAT3. Following overnight incubation, the Z'-factor values remained stable (0.83 for phospho-STAT1 and 0.78 for phospho-STAT3). Similar Z'-factor values were obtained for the other phospho-STAT assays (STAT4: 0.79; STAT5: 0.78; STAT6: 0.63). A cell-based assay with a Z'-factor ≥ 0.40 is considered suitable for HTS[15]. Accordingly, these results demonstrate the robustness of these phospho-STAT assays for HTS applications.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: TR-FRET assay workflow.** The workflow consists of three steps: cell treatment, cell lysis, and protein detection using TR-FRET. In the 2-plate assay protocol, lysates are transferred to a white 384-well detection plate, whereas in the 1-plate protocol, all steps are conducted in the same white 384-well detection plate (all-in-one well protocol). Regardless of the assay protocol used, protein detection is performed in the same total volume (20 µL per well). Abbreviation: TR-FRET = time-resolved Förster resonance energy transfer.

**Figure 2: TR-FRET sandwich immunoassay principle.** One antibody is labeled with the Europium chelate donor (Eu-Ab1) and the second with the far-red small fluorophore acceptor (FR-Ab2). The two labeled antibodies bind specifically to distinct epitopes on the target protein (phosphorylated or total) in the cell lysate, bringing the two fluorophores into close proximity. Excitation of the donor Europium chelate 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 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. Abbreviations: FRET = Förster resonance energy transfer; TR-FRET = time-resolved FRET; Ab = antibody; FR = far-red; P = phosphorylation.

**Figure 3: Detection of phospho-STAT1 (Y701) modulation in U266B1 cells.** Cells (200,000 cells/well) seeded in a 96-well culture plate were treated with increasing concentrations of either (**A**) IFNα2b for 15 min at RT or (**B**) JAK Inhibitor 1 for 30 min at RT, then with 1 nM ($EC_{80}$) of IFNα2b for 15 min at RT. Following lysis, lysates were transferred to a low-volume 384-well white plate, followed by the addition of the Antibody Detection Mix. The plate was incubated for 4 h at RT and then read on a TR-FRET-compatible reader. Data are shown as the mean of triplicate wells per assay point. Error bars indicate standard deviation. Some error bars are smaller than the

symbol size. Abbreviations: STAT = signal transducer and activator of transcription; IFN = interferon; RT = room temperature; JAK = Janus kinase; TR-FRET = time-resolved Förster resonance energy transfer; S/B = signal/background ratio.

**Figure 4: Detection of phospho-STAT3 (Y705) and total STAT3 modulation in HeLa cells.** Cells (40,000 cells/well) cultured overnight in a 96-well culture plate were treated with increasing concentrations of either (**A**) IFNα2b for 20 min at RT or (**B**) JAK Inhibitor 1 for 30 min at RT then with 1.5 nM of IFNα2b for 20 min at RT. Following media removal and lysis, lysates were transferred to a low-volume 384-well white plate, followed by the addition of the Antibody Detection Mix. The plate was incubated for 4 h at RT and then read on a TR-FRET-compatible reader. Data are shown as the mean of triplicate wells per assay point. Error bars indicate standard deviation. Some error bars are smaller than the symbol size. Abbreviations: STAT = signal transducer and activator of transcription; IFN = interferon; RT = room temperature; JAK = Janus kinase; TR-FRET = time-resolved Förster resonance energy transfer; S/B = signal/background ratio.

**Figure 5: Detection of phospho-STAT4 (Y693) modulation in U266B1 cells.** Cells (400,000 cells/well) seeded in a 96-well culture plate were treated with increasing concentrations of either (**A**) IFNα2b for 25 min at 37 °C or (**B**) JAK Inhibitor 1 for 30 min at RT, then with 1 nM of IFNα2b for 25 min at 37 °C. Following media removal and lysis, lysates were transferred to a low-volume 384-well white plate, followed by the addition of the Antibody Detection Mix. The plate was incubated overnight at RT and then read on a TR-FRET-compatible reader. Data are shown as the mean of triplicate wells per assay point. Error bars indicate standard deviation. Some error bars are smaller than the symbol size. Abbreviations: STAT = signal transducer and activator of transcription; IFN = interferon; RT = room temperature; JAK = Janus kinase; TR-FRET = time-resolved Förster resonance energy transfer; S/B = signal/background ratio.

**Figure 6: Detection of phospho-STAT5 (Y694/Y699) and total STAT5 modulation in A431 cells.** Cells (75,000 cells/well) cultured overnight in a 96-well culture plate were treated with increasing concentrations of either (**A**) EGF for 10 min at RT or (**B**) Erlotinib for 15 min at RT, then with 73 nM of EGF for 10 min at RT. Following media removal and lysis, lysates were transferred to a low-volume 384-well white plate, followed by the addition of the Antibody Detection Mix. The plate was incubated overnight at RT and then read on a TR-FRET-compatible reader. Data are shown as the mean of triplicate wells per assay point. Error bars indicate standard deviation. Some error bars are smaller than the symbol size. Abbreviations: STAT = signal transducer and activator of transcription; EGF = epidermal growth factor; RT = room temperature; TR-FRET = time-resolved Förster resonance energy transfer; S/B = signal/background ratio.

**Figure 7: Detection of phospho-STAT6 (Y641) and total STAT6 modulation in HeLa cells.** Cells (40,000 cells/well) cultured overnight in a 96-well culture plate were treated with increasing concentrations of either (**A**) IL-4 for 20 min at RT or (**B**) JAK Inhibitor 1 for 30 min at RT, then with 0.5 nM of IL-4 for 20 min at RT. Following media removal and lysis, lysates were transferred to a low-volume 384-well white plate, followed by the addition of the Antibody Detection Mix. The plate was incubated for 4 h at RT and then read on a TR-FRET-compatible reader. Data are shown

as the mean of triplicate wells per assay point. Error bars indicate standard deviation. Some error bars are smaller than the symbol size. Abbreviations: STAT = signal transducer and activator of transcription; IL = interleukin; JAK = Janus kinase; RT = room temperature; TR-FRET = time-resolved Förster resonance energy transfer; S/B = signal/background ratio.

**Figure 8: Detection of phospho-STAT4 (Y693) stimulation in U266B1 cells with the one-plate protocol.** Cells (160,000 cells/well) seeded in a low-volume 384-well white plate were immediately treated with increasing concentrations of IFNα2b for 25 min at 37 °C. Following lysis, the Antibody Detection Mix was added directly to the lysate. The plate was incubated overnight at RT and then read on a TR-FRET-compatible reader using a flash lamp or laser excitation. Data are shown as the mean of triplicate wells per assay point. Error bars indicate standard deviation. Some error bars are smaller than the symbol size. Abbreviations: STAT = signal transducer and activator of transcription; IFN = interferon; RT = room temperature; TR-FRET = time-resolved Förster resonance energy transfer; S/B = signal/background ratio.

**Figure 9:** D**etection of phospho-STAT6 (Y641) stimulation in HeLa cells with the one-plate protocol.** Cells (80,000 cells/well) seeded in a low-volume 384-well white plate were immediately treated with increasing concentrations of IL-4 for 20 min at RT. Following lysis, the Antibody Detection Mix was added directly to the lysate. The plate was incubated 4 h at RT and then read on a TR-FRET-compatible reader. Data are shown as the mean of triplicate wells per assay point. Error bars indicate standard deviation. Some error bars are smaller than the symbol size. Abbreviations: STAT = signal transducer and activator of transcription; IL = interleukin; RT = room temperature; TR-FRET = time-resolved Förster resonance energy transfer; S/B = signal/background ratio.

**Figure 10: Intraplate variability study of the phospho-STAT1 (Y701) and phospho-STAT3 (Y705) assays.** (**A**) Phospho-STAT1 assay: suspension cells (200,000 U266B1 cells/well) were treated either with 10 nM of IFNα2b for 15 min or serum-free medium alone (low controls). (**B**) Phospho-STAT3 assay: adherent cells (20,000 HeLa cells/well) were treated either with 5 nM of IFNα2b for 20 min or with serum-free medium alone. For both assays, the TR-FRET signal was read after 4 h of incubation. Abbreviations: STAT = signal transducer and activator of transcription; IFN = interferon; TR-FRET = time-resolved Förster resonance energy transfer; S/B = signal/background ratio; CV = coefficient of variation.

**DISCUSSION:**

Compared to conventional methods for phosphoprotein analysis such as western blotting and ELISA-based methods, the workflow for a THUNDER TR-FRET cellular assay is simple and fast, uses a low-volume sample (15 µL), is designed for HTS in a 384-well format, and is highly amenable to automation. The assay protocol is flexible and can readily be adapted to both medium- and high-throughput applications. Assays can be run using either a two-plate transfer protocol or a one-384-well plate protocol. In the two-plate transfer protocol, cells are seeded, treated, and lysed in one cell culture plate, and lysates are subsequently transferred to a separate white detection plate (half-area 96-well or 384-well plate) for analysis. In a one-384-well plate protocol, the entire workflow is conducted in the same well, eliminating the need for a liquid transfer step. The assays

could easily be adapted to the 1536-well format by reducing the volume proportionally. Regardless of the assay protocol, there are only three reagent solutions to prepare and no washing steps, and the assays can be run following an "addition-only" protocol, requiring minimal hands-on time.

A critical step in performing any cell-based assay is the optimization of cell culture and treatment conditions[16]. Both the cell number and treatment conditions require careful optimization before running an assay, as these key parameters often vary for each cell line and each phosphoprotein. The optimization of these parameters allows maximizing the assay window, obtaining an optimum performance with a high S/B ratio and low inter-well coefficient of variation, and ensuring the robustness and reproducibility of the results. Cell number, serum-starvation (when appropriate), and stimulation or inhibition time (at either room temperature or 37 °C) should be optimized for each cell line and target protein. Too high or too low cell numbers can negatively influence the modulation of intracellular signaling pathways. Cell seeding densities of 40,000–80,000 cells/well for adherent cells or 100,000–200,000 cells/well for suspension cells are generally acceptable for most cell lines. Of note, the optimal length of time for stimulation and inhibition can vary widely among cell lines and target proteins, from a few minutes to several hours. As such, a time course study is strongly recommended to determine the optimal stimulation and inhibition incubation times, ideally at both room temperature and 37 °C, as incubation temperature affects the kinetics of target protein stimulation.

Troubleshooting a cell-based assay can be difficult and time-consuming because of the many steps involved in the workflow (cell culture, cell treatment, lysis, and protein detection). These assay kits include a positive control lysate. It is recommended to use these positive control lysates and negative control (1x supplemented Lysis Buffer alone) in every experiment. The use of proper controls facilitates troubleshooting, as problems can then be quickly attributed to either the detection step (incorrect reagent preparation and/or assay execution) or the quality of the lysates used in the experiment. The latter is generally due to the use of suboptimal cellular conditions.

An additional advantage of this platform is the availability of a suite of optimized lysis buffers with different stringencies. This allows generating more or less heterogeneous lysates from partial subcellular fractionation. This is useful because signaling proteins are located in various intracellular compartments, and some, like STAT proteins, shuttle between compartments (*e.g.*, between the cytoplasm and the nucleus). Of note, phosphorylated and total STAT1 use lysis buffer 1 because they cannot be detected well with the lysis buffer 2 used for the other STAT proteins. In contrast, other homogeneous methods rely on a single "universal" lysis buffer, which generates a more complex sample that can create non-specific interactions with the antibodies. However, the use of different lysis buffers may preclude the analysis of multiple signaling proteins from a single lysate sample. Nevertheless, one can still test lysates generated with different lysis buffers simultaneously on a single plate for parallel pathway analysis.

In conclusion, the methods presented here based on this homogeneous TR-FRET cellular immunoassay platform offer a high-throughput alternative for monitoring JAK/STAT signaling via

the detection and quantification of endogenous phosphorylated STAT1/3/4/5/6 proteins, together with total STAT1/3/5/6, in cell lysates. These assays provide new tools for a more comprehensive analysis of specific STAT proteins following cell treatment and the screening and characterization of specific and selective modulators of the JAK/STAT signaling pathways. Given its simplicity, specificity, sensitivity, reproducibility, and cost-effectiveness, this new assay platform represents an attractive alternative to traditional immunoassays, both in an academic setting and in industrial laboratories for HTS applications.

**ACKNOWLEDGMENTS:**

None.

**DISCLOSURES:**

Competing Interests: Jaime Padros, Mireille Caron, and Geneviève Chatel are employees of BioAuxilium Research, which manufactures the THUNDER TR-FRET assay kits used in this study. In addition, Jaime Padros and Mireille Caron are shareholders of Bioauxilium Research. This does not alter the authors' adherence to all JoVE policies on sharing data and materials.

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Figure 1

Click here to access/download;Figure;Figure 1-Workflow.pdf

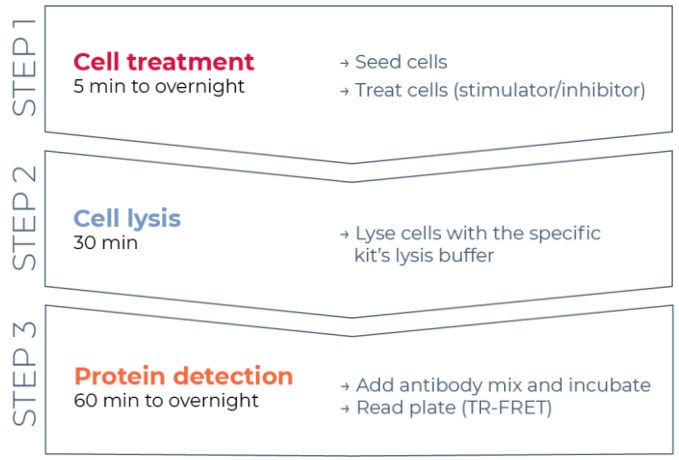

Figure 2

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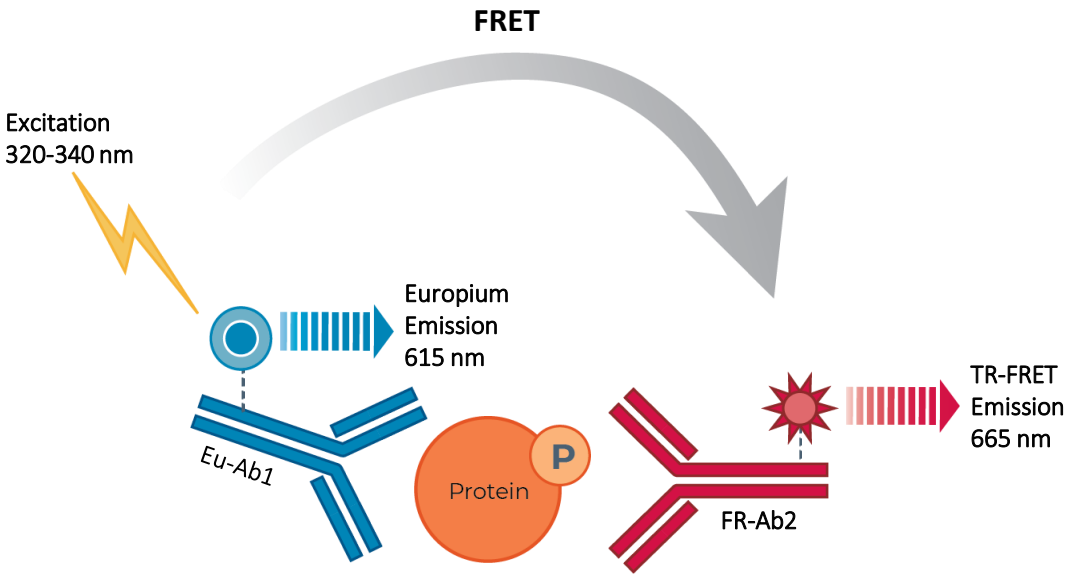

Figure 3

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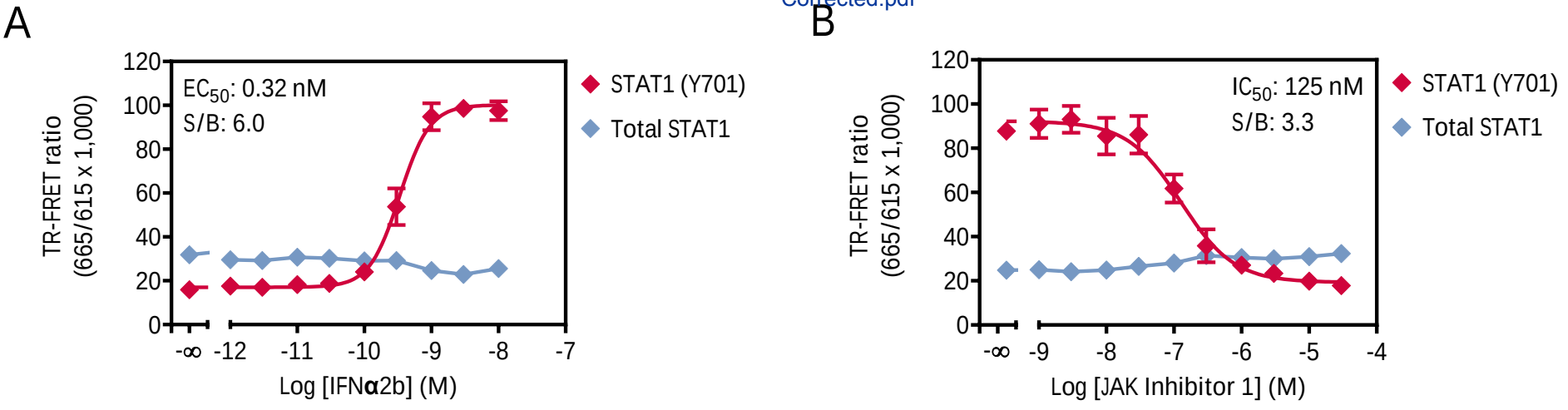

Figure 4

Click here to access/download;Figure;Figure 4-STAT3-Stim&Inhib-Corrected.pdf

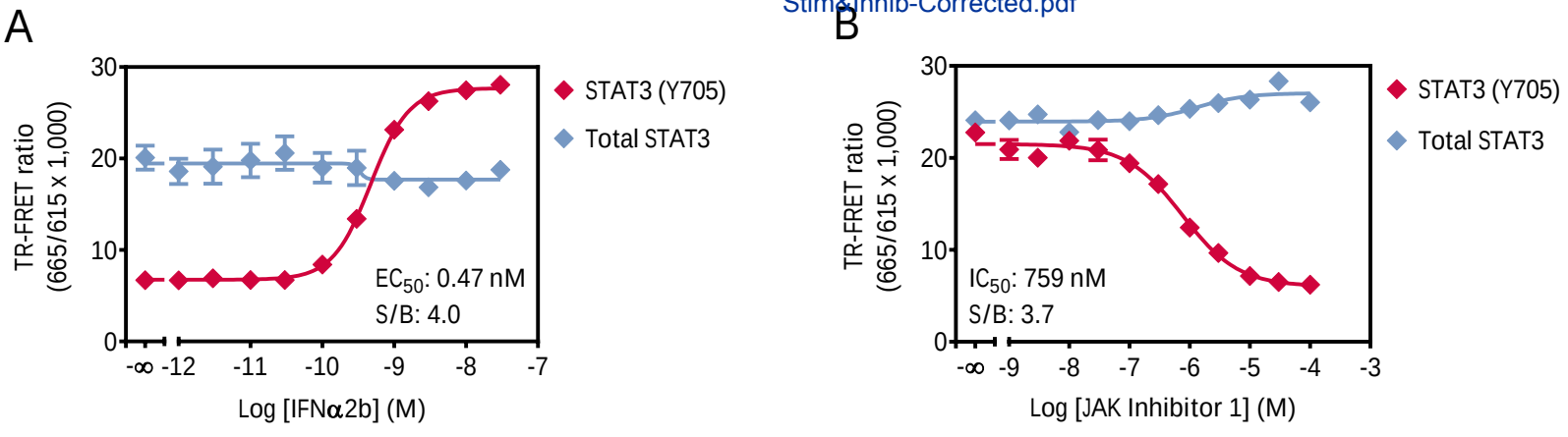

Figure 5

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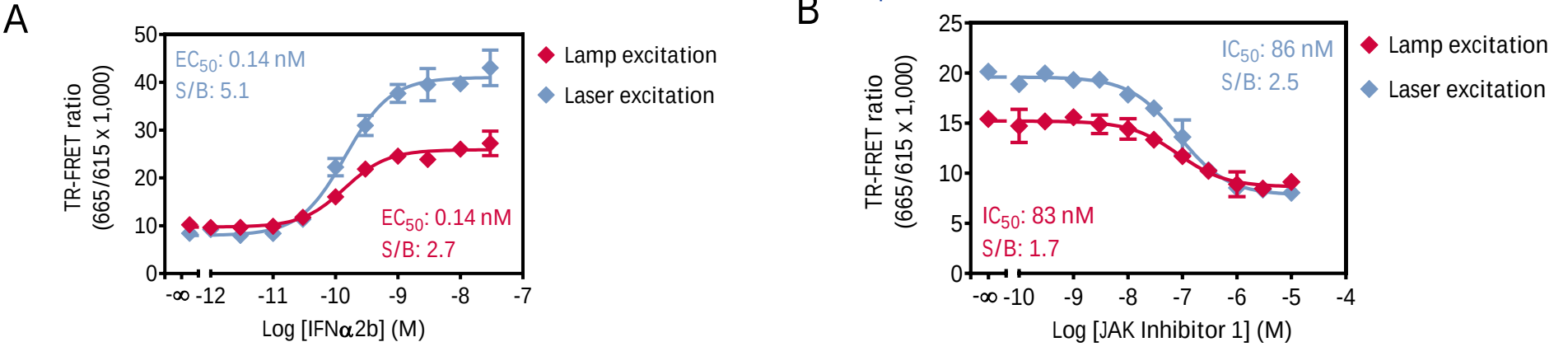

Figure 6

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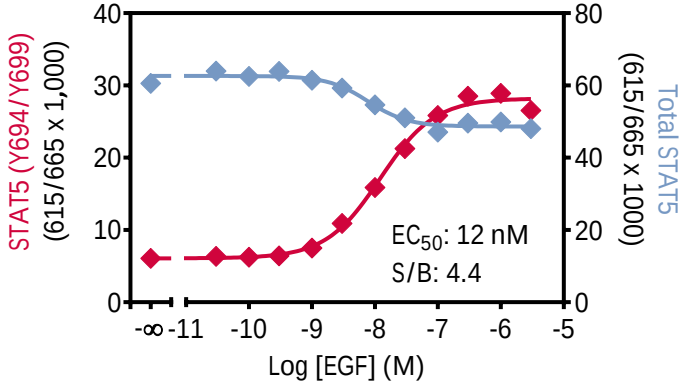
A

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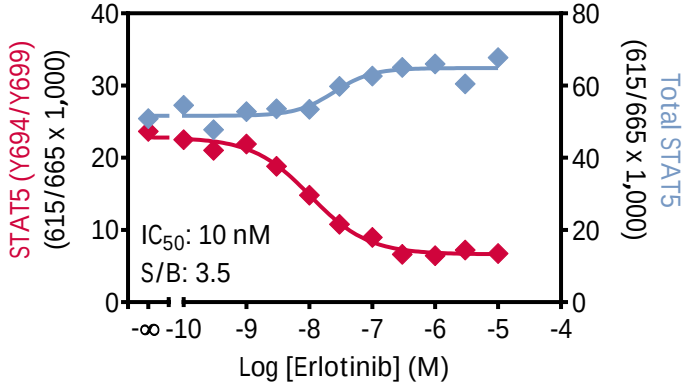

Figure 7

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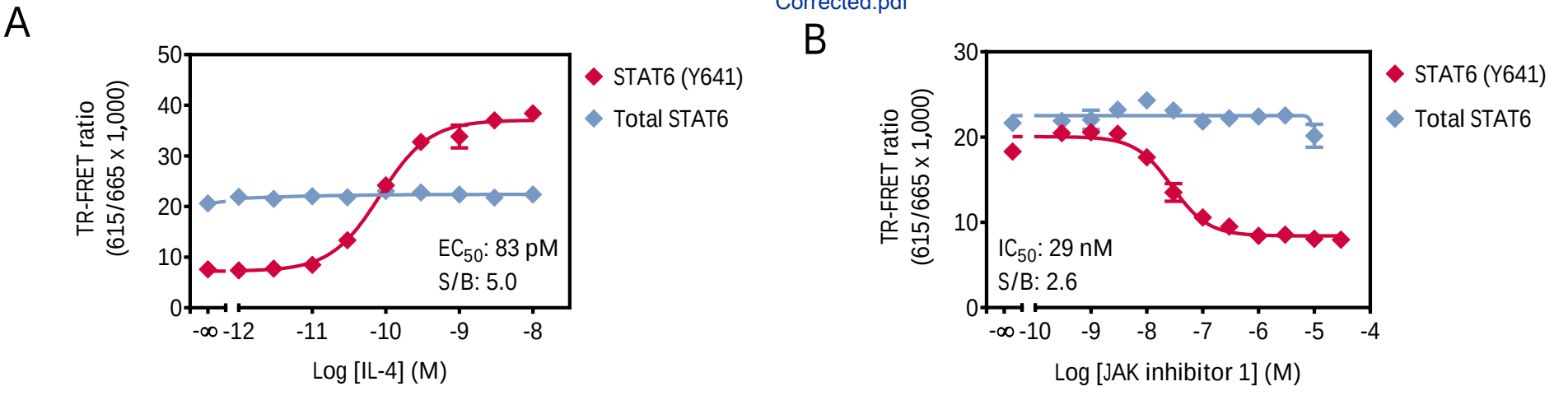

Figure 8

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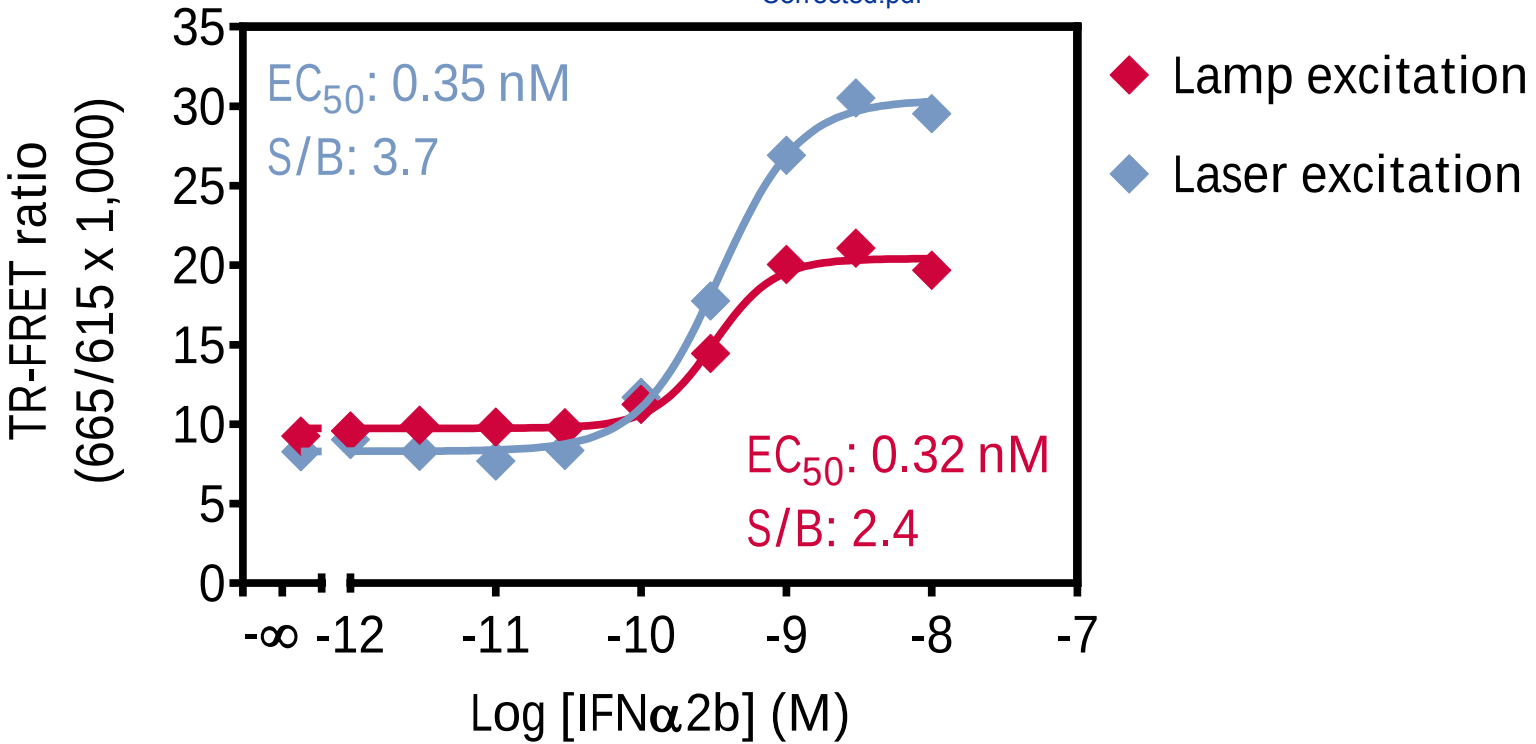

Figure 9

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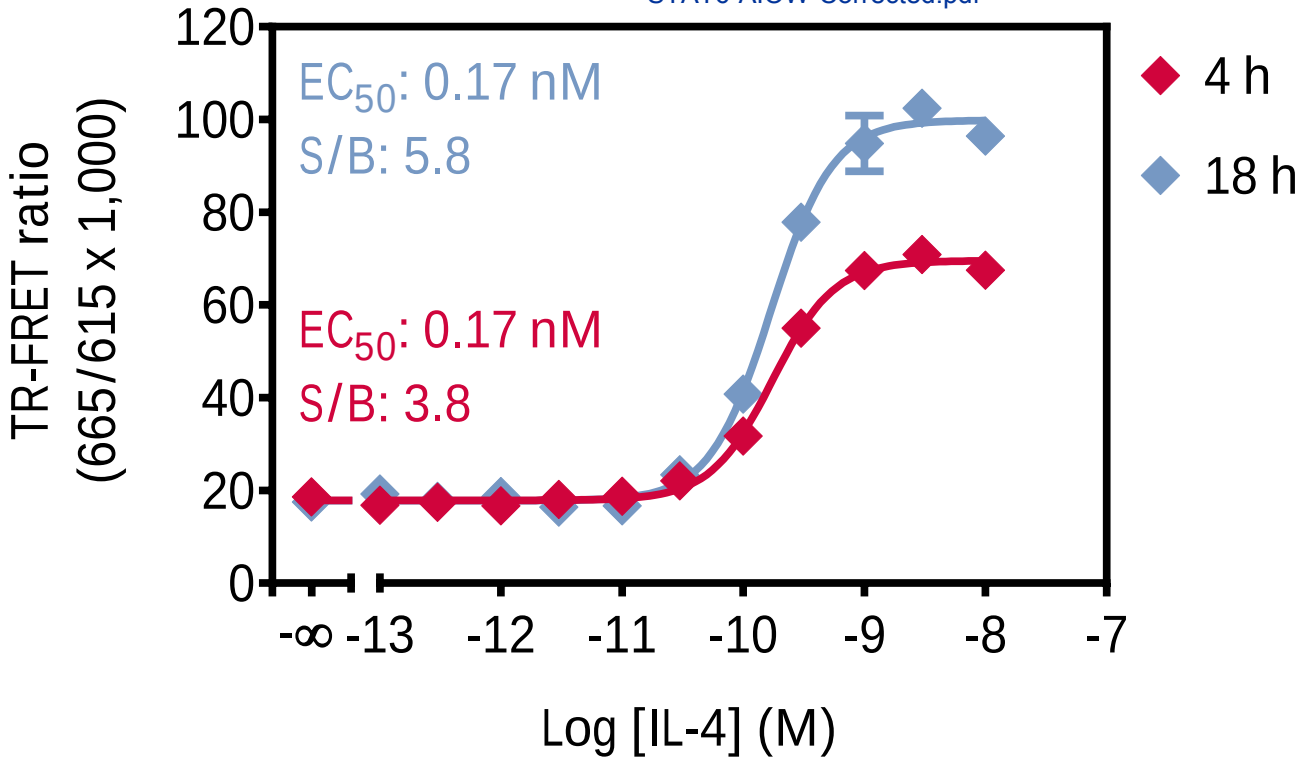

Figure 10

Click here to access/download;Figure;Figure 10-Z-factor-STAT1&STAT3-Corrected.pdf

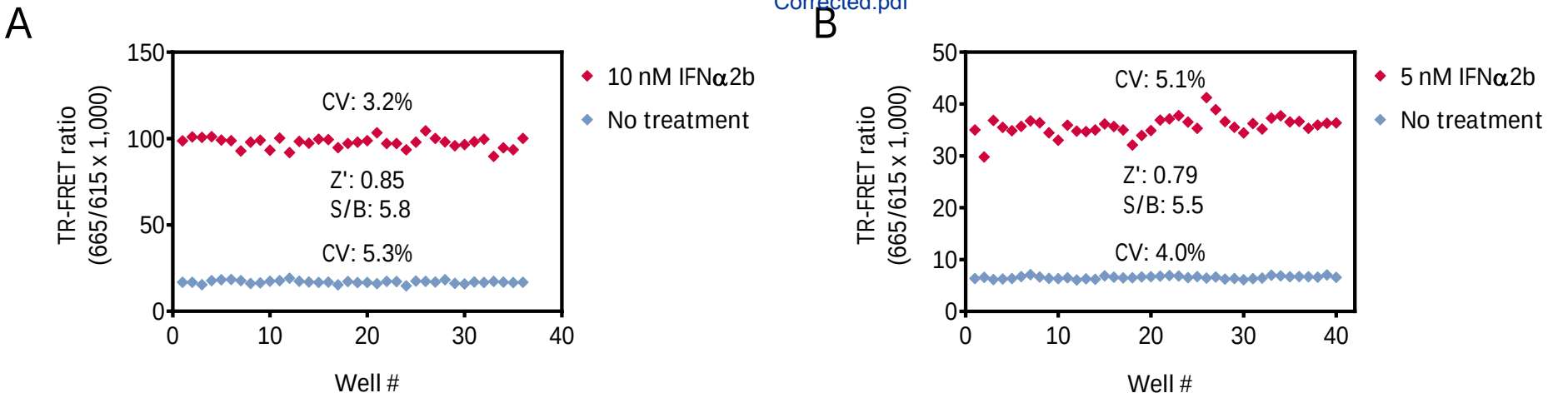

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**Table of Materials**
JoVE_Materials-BioAuxilium-R1.xls

Click here to access/download;Rebuttal Letter;Rebuttal letter-JoVE62915.docx

July 9, 2021

Amit Krishnan, Ph.D.
Review Editor
JoVE
amit.krishnan@jove.com
617.674.1888

Re: Revisions required for your JoVE submission JoVE62915 - [EMID:198569babbd115f0]

Dear Dr. Krishnan:

We have uploaded today a revised version of the manuscript entitled “THUNDER TR-FRET Assays for Measurement of Endogenous STAT Proteins in Human Cells”, along with an updated Table of Materials and corrected pdf files for the figures.

As requested, modifications to the original text have been highlighted (track changes). We hope that these modifications are acceptable.

You will find in the following pages our point-by-point responses to the reviewers’ suggestions and comments. All comments have been addressed; they are highlighted in bold.

We thank you for considering our revised manuscript and we look forward to hearing from you in the near future.

Best regards,

Jaime

Jaime Padrós, PhD, PMP
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Montréal, Québec, CANADA H4S 1Z9
Mobile: +1 514-668-7235

**Editorial comments:**
Editorial Changes
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. **Done.**

2. Please remove the commercial term "THUNDER" from the title and revise it to "TR-FRET assays for measurement of endogenous phosphorylated STAT proteins in human cells". JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. **Done**. **The new title is "TR-FRET assays for measurement of endogenous phosphorylated STAT proteins in human cells**".

3. Please revise the following lines to avoid previously published work: 46-48, 80-82,106-108, 191-193, 268-270, 451-452, 459-462, 559-560. Please refer to the iTheticate report attached. **Done for 46-48, 80-82, 191-193 (deleted), and 559-560. Note however that lines 451-452 and 459-462 correspond to iTheticate reference #2, which is a manuscript that I have co-written. In addition, lines 106-108 and 268-270 correspond to generic statements that we have written when we worked at PerkinElmer (iTheticate reference #3).**

4. Please ensure that the affiliations are complete. **Checked.**

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). **Done and corrected.**

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: THUNDER™, Jackson ImmunoResearch, etc. **Done. The manuscript and the Table of Materials have been updated accordingly.**

7. Line 166-170: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. **Done; the lines 166-170 (169-173 in the new version) have been modified as requested.**

8. Line 172-173/284-285: Please specify the cell density. Please specify the pre-optimized density used in this study as an example. **Done for the 3 protocols: 2-plate with adherent cells;**

**2-plate with suspension cells; 1-plate with suspension or adherent cells.**

9. Line 208/215/220/300/305/346/351/356: Please specify the pre optimized time used in this study. **Done for all lines.**

10. Line 231: Is the aspirated supernatant discarded? **Yes; it has been added to the text (line 251).**

11. Please include a description for Figure 1 and Figure 2. Please remove the commercial term "THUNDER" from the figure legends. **Done.**

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." **Not applicable.**

13. Figure 9: Please revise "4 hours" and "18 hours" to "4 h" and "18 h". **Done.**

14. Please replace the symbol "*" by "x" in all the figures to define multiplication. **Done for all figures.**

15. Please sort the Table of Materials in alphabetical order. **Done.**

---

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

In this manuscript, the authors describe a TR-FRET-based assay for detection of STAT protein phosphorylation in suspension and adherent cells. The assay is based on the use of directly labelled antibodies specific for total and phosphorylated STAT proteins. The principle of TR-FRET to measure STAT phosphorylation in cell lysates is not new and has been already employed by other companies, however, the authors argue that their approach is cheaper while still being reliable, rapid and compatible with HTS. While this reviewer cannot judge the cost point, the data presented are convincing and the description of the method is easy to understand. I have only a few minor points that should be addressed.

Major Concerns:

None

Minor Concerns:

Page 7: I am puzzled with the fact that in the ONE-plate protocol the authors treat adherent

cells in suspension. This seems to be odd, also because adherent cells might response differently to stimuli under these conditions. Why is this the case? **While it is true that adherent cells might respond differently to stimuli when treated immediately after seeding, the data presented in the manuscript demonstrate that at least for phosphorylated STAT6 this was not the case. Indeed, the pharmacology (EC50) obtained with both adherent HeLa cells and adherent HeLa cells "in suspension" was comparable (83 pM and 170 pM for adherent and suspended HeLa, respectively). Nevertheless, because this might not be the case for all cell types, we have now indicated the need to verify this in the protocol (Section 4.2, line 369; "Note: The requirement for a cell culture incubation period before treatment needs to be determined for different cell types.")**

2. Most media contain phenol red as a pH indicator. Since it is not explicitly mentioned I wonder if phenol red interferes with the FRET measurement. If this is the case, the authors should recommend to use phenol red free medium. **THUNDER TR-FRET is compatible with culture media containing phenol red. DMEM and RPMI used in the experiments contained phenol red. This is now indicated in both the Table of Materials and in the protocol (Section 1.1, line 171).**

3. In the discussion section, the authors mention as an advantage of their method that a set of lysis buffers with different stringencies is available, however no further details are provided and the protocol mentions only one lysis buffer. More details would be nice to have. **The formulations of the lysis buffers are proprietary. The protocol has been edited to specify that there are different, specific Lysis Buffers for the different STAT proteins (lines 242, 333, 390).** It would also be good to know if some stringent lysis buffer reagents such as SDS are excluded from use as they could interfere with the measurement. **A note has been added to the protocol (2.4.1, line 247) to indicate that lysis buffers (and phosphatase inhibitors) other than those included in the kit are not recommended, as they might contain ingredients that could interfere with the measurement.**

**Reviewer #2:**
Manuscript Summary:
This manuscript described the THUNDER™ TR-FRET cell-based assay for phosphorylated STAT1/3/4/5/6 proteins in cell lysates. This assay will help to delineate the very complicated JAK-STAT pathway. This is a very useful method to many researchers.

Major Concerns:
No

Minor Concerns:
No

**Reviewer #3:**

Manuscript Summary:
The manuscript describes a new methodology of detection of activation of different STAT family members in a simple assay. The THUNDER™ TR-FRET system presented in the manuscript appears simple, robust, and relatively easy to utilise. The assays appear suitable for JAK/STAT activation titration and inhibitor assays which can be performed in high throughput.

Major Concerns:
I have no major concerns

Minor Concerns:
One concern I had was regarding figure 6 where signal strength of total STAT5 showed some inverse correlation with phosphorylated STAT5. Does this represent some interference with increasing signal of phosphorylated STAT5 with a decrease in signal for total STAT5? **Indeed, the decrease in signal (15-20%) of the Total STAT5 protein with increasing STAT5 phosphorylation is due to steric hindrance, where phosphorylation of STAT5 hinders the binding of one of the two anti-total STAT5 antibodies to its respective antigen. This phenomenon is often observed in sandwich immunoassays measuring phosphorylated and total proteins. Because the interference was not major, and other antibody pairs tested did not generate better results, the current pair of antibodies for total STAT5 was selected. An explanation for this result has now been added to the Results section (lines 457-459).**
Of the alternative assays currently available, the assay experimental methods do not appear very different from those such as the Alpha SureFire Ultra assay. The major advantage of the THUNDER™ TR-FRET to the SureFire assay may be the reduced cost of the THUNDER™ TR-FRET, however with no indication in the manuscript of what cost difference that would likely be, the significance of this cost comparison is very difficult to judge. **Differences between THUNDER TR-FRET and Alpha SureFire Ultra assay platforms are indicated in the Introduction, and are not limited to the price (although Alpha is very expensive). In addition, TR-FRET assays exhibit higher robustness than Alpha assays due to the time-resolved and ratiometric measurement. In addition, Alpha is offered by a unique vendor (PerkinElmer), is very light- and temperature-sensitive, is affected by the presence of free biotin in the culture medium (*e.g.*, RPMI), and requires an expensive plate reader equipped with a laser-based excitation that is not widely available in small laboratories. In addition, because prices change annually, it would not be useful to indicate the prices for the different platforms available. However, the reader can always go to the respective websites to compare prices. For instance, a 500-point Alpha SureFire Ultra assay kit for a specific phospho-STAT is sold today at $1,868 USD, whereas the corresponding THUNDER TR-FRET assay kit is at $1,057 USD. The HTRF (Cisbio/PerkinElmer) price for a corresponding kit is $1,699 USD. Therefore, we indicate in the manuscript that THUNDER TR-FRET is "cost-effective".**

Line 47: suggest edit of "pathways is implicated" to "pathways are implicated". **The original sentence is correct: "The abnormal activation of STAT signaling pathways is implicated …".**

**Reviewer #4:**

I find the manuscript acceptable with the following comments:

Introduction:
Traditional (or commonly used) assay formats include also phosflow. Please refer to that as well side-by-side with western blotting and elisa. **Phosphoflow has now been added to the traditional assay formats (line 90).**
Why no pSTAT2 measurement was not set-up? **Suitable antibodies for developing a TR-FRET based phospho-STAT2 immunoassay are not commercially available.**

Representative results:
List the z'factors for pSTAT4, pSTAT5, and pSTAT6. **These values have now been added (line 495).**
Figure 4a. Error bars for STAT3(Y705) missing? **No, they are not. They are not visible because they are shorter than the size of the symbol. This has now been clarified in the figure legends.**
Could laser excitatation increase maximal TR-FRET ratio for pSTAT3 (at similar level to pSTAT4 in lamb excitation)? **Yes, reading assays with laser excitation generally generates higher S/B ratios. However, plate readers equipped with laser-based excitation are more expensive and not widely available among laboratories. This is the reason why data presented here were generated with lamp-based excitation.**
Figure3: Kindly explain the reason for lower TR-FRET ratio for total STAT1 compared pSTAT1. **The signal level depends on the antibodies used to detect the target protein, not the absolute level of protein. As such, total protein may show a lower signal compared to the phosphorylated protein, since different antibody pairs are used for detection.**
Figure5: Total STAT4 levels should be shown. **There is no assay available for total STAT4 due to the absence of suitable antibody pairs.**
Figure6: Please explain the following: the decrease in total STAT5 as EGF increases AND increase in total STAT5 as EGF-inhibitor concentration increases. **This has been explained above for Reviewer #3.** Kindly use the same y-axis for both pSTAT5 and STAT5 as is shown for other STATs/pSTATs. No error bars are visible. **The reason why no error bars are visible has already been explained. The reason why the same Y-axis was not used for both phospho and total STAT5 is that the levels of total STAT5 are 2-fold higher than those of phospho-STAT5. Therefore, if the same Y-axis is used, the curves for phospho-STAT5 are not easily visible.**