APPLICATION NOTE

LANCE *Ultra* TR-FRET-based detection and modulation of phosphorylated STAT3 levels in human cells.

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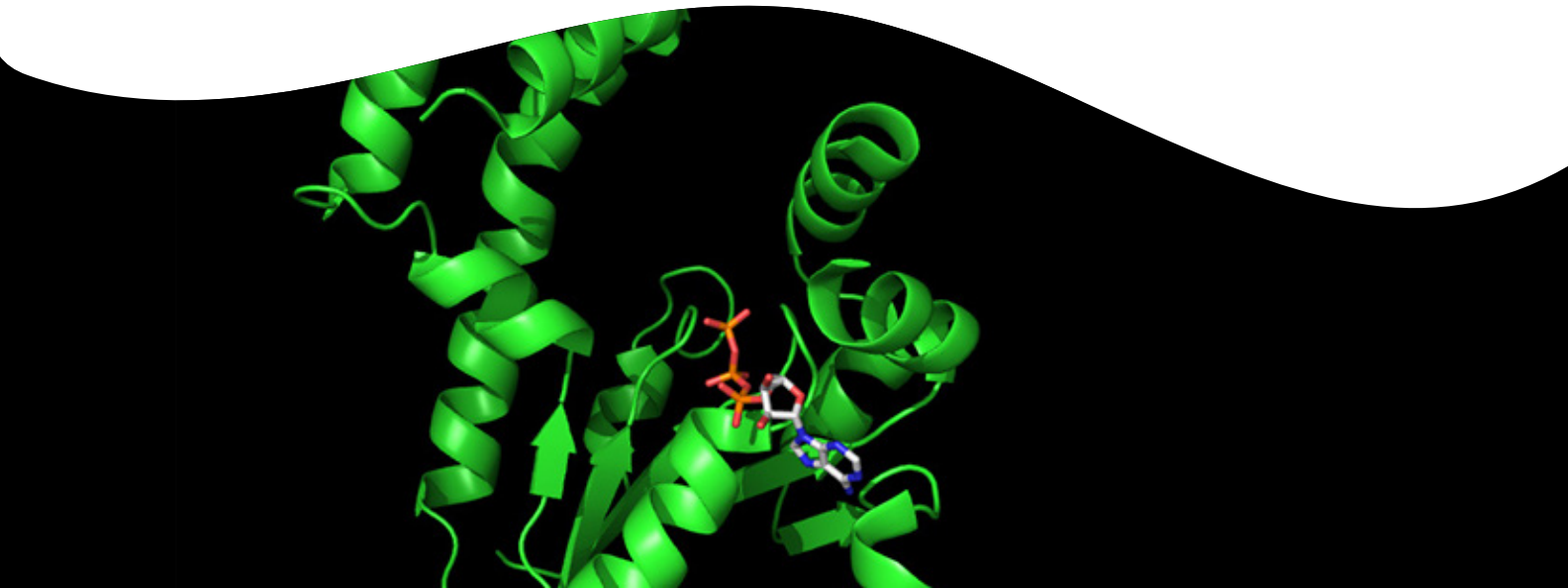
Introduction

The STAT (Signal Transducers and Activators of Transcription) family of proteins have important roles in cell survival and proliferation. They remain inactive in the cytoplasm until activated by extracellular signaling proteins such as cytokines or growth factors which bind to receptors on the cell surface.[1] The receptors dimerize and activate Janus kinases (JAK) which then phosphorylate the STAT proteins. The phosphorylated versions of STAT proteins form homo- and heterodimers, which translocate to the nucleus and bind to promoters of target genes to induce transcription. STAT3 is a particularly important therapeutic target since it is constitutively active in a range of cancer cells lines and human tumors. Therefore, fast and easy methods for screening of inhibitors of STAT3 activation are highly desirable.

LANCE® *Ultra* time-resolved fluorescence resonance energy transfer (TR-FRET) technology allows the detection of molecules of interest in a homogeneous, no-wash format.These assays utilize the unique fluorescent properties of a europium-based chelate (Eu chelate) as the donor. Eu chelates have high quantum yield, large Stokes shift, a narrow-banded emission, and a long lifetime. These properties make Eu chelates ideal energy donors in TR-FRET assays, as they are less susceptible to interference and reduce background signal. U*Light*™ is a small (<800 Da), bright, light-resistant acceptor dye that has a red-shifted emission maximum at 665 nM. LANCE *Ultra* cell-based TR-FRET products can provide fast and easy readouts of phosphorylation events within cells upon treatment with compounds.



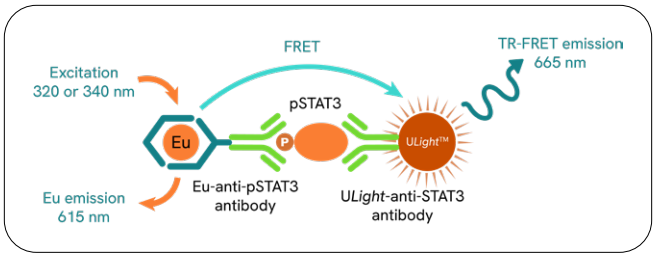


Figure 1: Phosphorylated STAT3 LANCE *Ultra* cell-based TR-FRET principle.

Materials and methods

Instrumentation

The phosphorylated STAT3 LANCE *Ultra* cell-based TR-FRET assays were measured using a Revvity EnVision® plate reader equipped with a laser, using default values for TR-FRET detection. In addition to fast, sensitive LANCE technology detection, the EnVision reader provides fluorescence intensity, luminescence, absorbance, fluorescence polarization, and AlphaLISA® and AlphaPlex™ assay technologies. The system is based on hybrid technology, combining filters and a monochromator for enhanced flexibility. It incorporates proprietary Direct Double Optics™ technology for high speed and sensitivity in simultaneous filter-based readouts, such as FRET assays, and for TRF, lamp-based excitation is standard, with the option of a high energy laser for higher speed and sensitivity.

Cell culture and treatments

Cell lines (SK-N-MC (ATCC # HTB-10), A431 (ATCC #CRL-1555), and Jurkat cells (ATCC # TIB-152)) were maintained in 75 mL cell culture flasks (Corning #430641U) in their respective media and typically passaged twice per week. SK-N-MC were grown in EMEM (ATCC #30-2003) supplemented with 10% FBS. A431 cells were grown in DMEM (ATCC# 30-2002) and supplemented with 10% FBS. Jurkat cells were grown in RPMI-1640 (ATCC #30-2001) and supplemented with 10% FBS. For assays using SK-N-MC and A431 cells, 120,000 cells/well were seeded (200 µL/well) into Revvity 96-well CulturPlates™ (Revvity # 6005680) and allowed to attach and grow overnight. The media was replaced with media lacking FBS (50 µL/well) in order to serum starve for 4 hours prior to running the assay.

Compounds used for stimulation were Epidermal Growth Factor (EGF, Sigma #E9644), Ciliary Neurotrophic Factor (CNTF, Sigma #C3710), and Interferon α-2b (IFNα-2b, Prospec #cyt-460-b). Compounds used for inhibition studies were Stattic (Tocris #2798), SD 1008 (Tocris #3035), and AG1478 (Cayman #AG-1478). All reagents were prepared and dispensed according to each manufacturer's recommendations. For the two-plate protocol, lysates were transferred to OptiPlate™-384 (Revvity #6007290) white opaque microplates prior to addition of detection reagents. For the all-in-one well assays with Jurkat cells, cells were counted and resuspended in HBSS with 10% FBS or RPMI with 10% FBS. These assays were performed directly in 384-well CulturPlates (Revvity #6007680).

LANCE *Ultra* pSTAT3 cell-based assays

All assays were performed using the LANCE *Ultra* phospho-STAT3 (Y705) kit (Revvity #TRF4004) according to the manual. However, since the signal to background was greater with an overnight incubation with the detection reagents, all data presented in this application note were measured after an overnight incubation rather than 4 hours. The two-plate protocol used is outlined in Figure 2 and the one-plate protocol is outlined in Figure 3.

Data analysis

All assays were measured on the EnVision reader using laser excitation at 320 nM. The ratio of the 665 nM signal (from *ULight)* normalized to the 615 nM (Europium) signal was calculated (and multiplied by a factor of 10,000 for easier data processing for all experiments. The data were analyzed using GraphPad Prism® version 7 software. The binding curves were generated using nonlinear regression (assuming there is one binding site and none of the signal comes from non-specific binding), using a four-parameter logistic equation (sigmoidal dose-response curve with variable slope). The inhibition curves were generated using log (inhibitor) vs. response - Variable slope (four parameters).

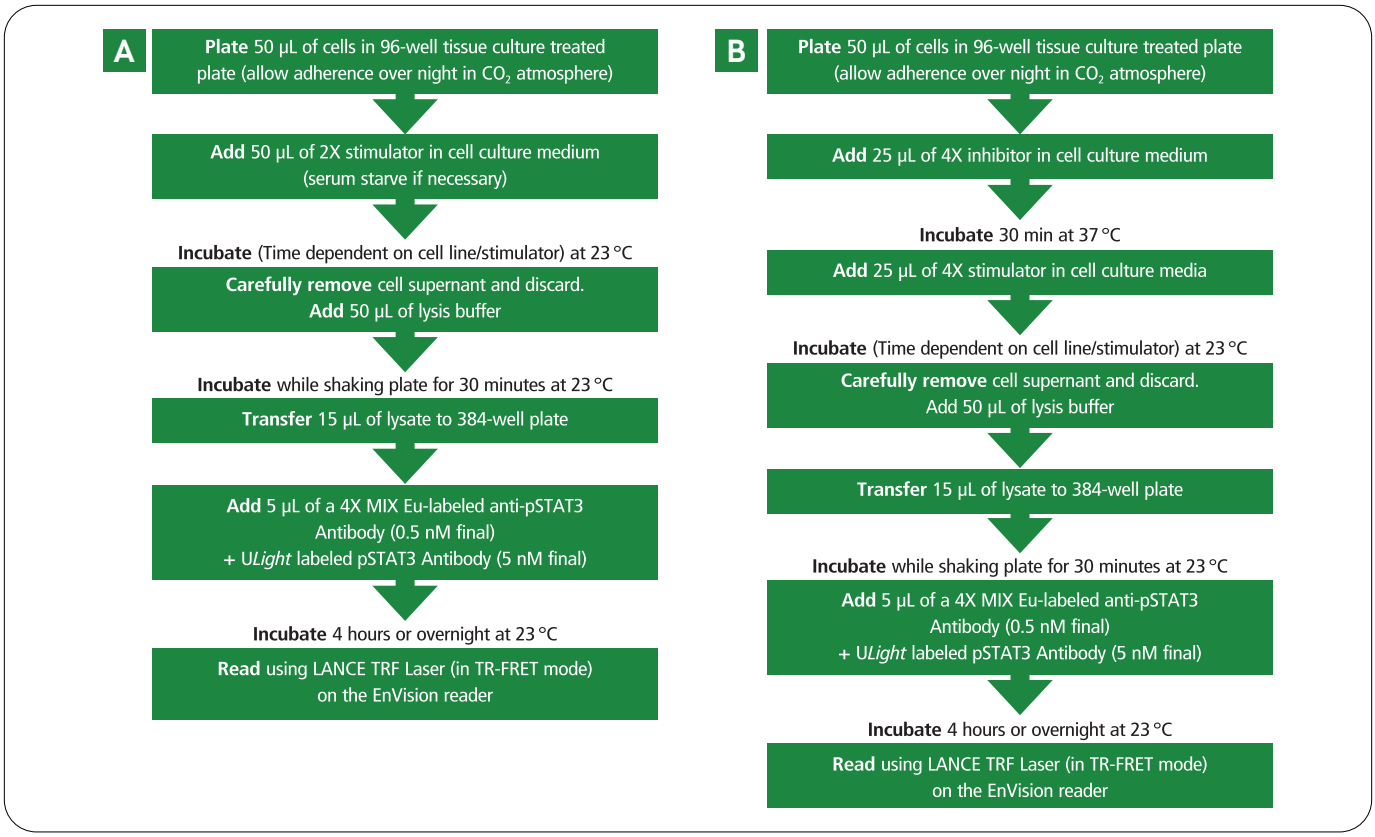


Figure 2: pSTAT3 (Y705) LANCE *Ultra* cell-based TR-FRET 2-plate protocol. A) Protocol for measuring stimulation of pSTAT3 expression. B) Protocol for measuring inhibition of pSTAT3 expression. This protocol was used for experiments with adherent cells.

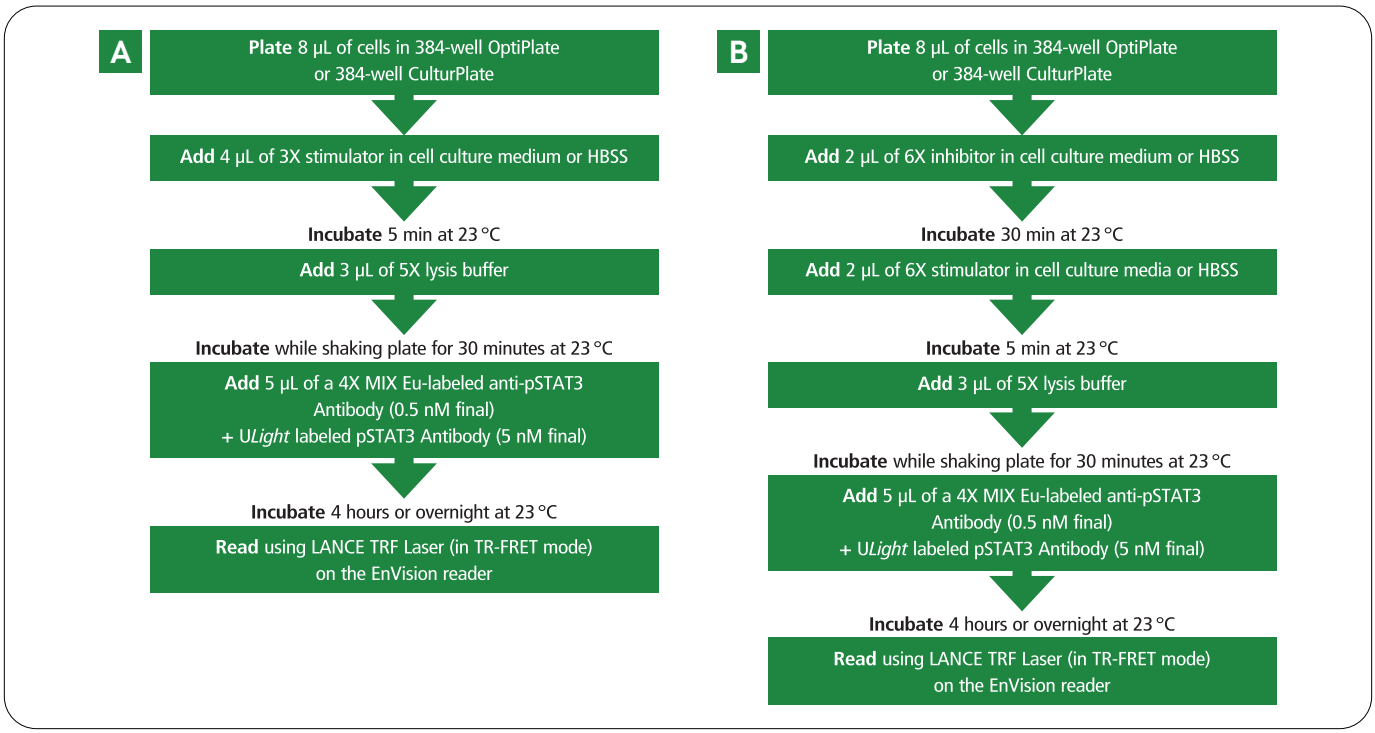


Figure 3: pSTAT3 (Y705) LANCE *Ultra* cell-based TR-FRET 1-plate protocol. A) Protocol for measuring stimulation of pSTAT3 expression. B) Protocol for measuring inhibition of pSTAT3 expression. This protocol was used for experiments with suspension cells.

Results and discussion

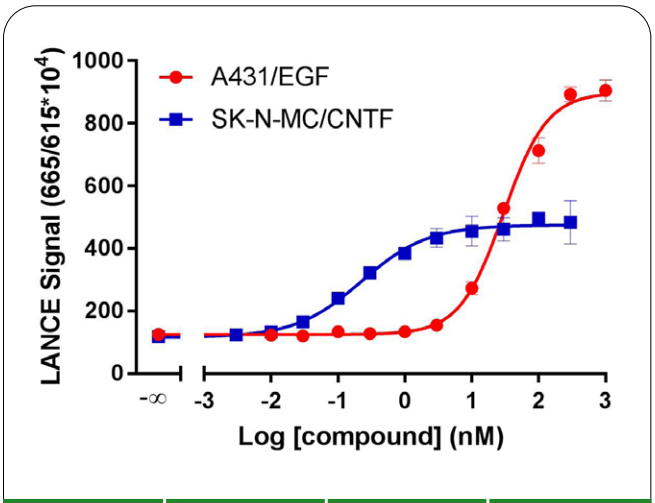
Measuring phosphorylated STAT3 expression in adherent cell lines

Changes in phosphorylated STAT3 expression levels were tested in two different adherent cells lines. For A431 cells, an epidermoid carcinoma cell line, treatment with epidermal growth factor (EGF) leads to activation of the EGF receptor (EGFR), a transmembrane protein. After binding to EGF, EGFR dimerizes which stimulates auto-phosphorylation and activates downstream signaling pathways, such as the phosphorylation of STAT proteins. In SK-N-MC cells, a neuroblastoma cell line, ciliary neurotrophic factor (CNTF), a cytokine differentiation factor, binds the CNTF receptor and activates the JAK/STAT pathway. In both cases, an increase in EGF (A431) or CNTF concentrations (SK-N-MC) should result in an increase in STAT3 activation.

Using the protocol from Figure 2A, phosphorylated STAT3 levels were measured as a function of EGF (A431 cells) or CNTF (SK-N-MC cells) concentration. As shown in Figure 4, we were able to accurately measure EC_{50} values and signal to background for both assays. The EC_{50} for CNTF is consistent with the values listed on the manufacturer's data sheet of 50 -150 ng/mL (2.2 - 6.8 nM) which was measured in a cell proliferation assay using a factor-dependent human erythroleukemic cell line, TF-1. The EC_{50} for EGF stimulation listed on the manufacturer's data sheet is 0.01 - 0.1 nM which was measured by its ability to stimulate the mouse fibroblast cell line, BALB/3T3. The EC_{50} measured here is more consistent with data shown for A431 cells, where ^{125}I-EGF bound to the EGF receptor with an apparent KI of 1.5 nM at 4 °C.[2]

Inhibition of phosphorylated STAT3 levels

Stattic is a small molecule that directly binds to the SH2 domain of STAT3 and selectively inhibits activation, dimerization, and nuclear translocation of STAT3.[3] An IC_{50} for Stattic was reported as 5.1 µM when measured in a cell-free system.[3] SD 1008 is a small molecule that specifically inhibits JAK2 activity, the enzyme that phosphorylates STAT3. The IC_{50} for inhibition of STAT3 activation was reportedly measured using a STAT3-dependent luciferase assay in the $OVCAR8_{TR}$ cell line. The IC_{50} for SD 1008 was shown to be between 10 and 30 µM.[4] In Figure 5, we show inhibition curves for Stattic and SD 1008 in both SK-N-MC and A431 cells. Slightly different IC_{50} values are seen for the two inhibitors for the two different cells lines. However, these values can be dependent on the cell line and the particular stimulator. The values obtained here are within range of the expected values.



Cell line	Compound	EC_{50} (nM)	S:B
SK-N-MC	CNTF	0.23	4.1
A431	EGF	29.9	7.2

Figure 4: Agonist titration of EGF in A431 cells or CNTF in SK-N-MC cells as measured by pSTAT3 expression levels. 120,000 cells per well were plated and allowed to adhere overnight. Cells were serum starved for 4 hours prior to running the assay. Using the protocol in Fig. 2A, A431 cells were stimulated with EGF for 5 minutes prior to lysing the cells. The SK-N-MC cells were stimulated with CNTF for 15 minutes prior to lysing the cells. Data were collected after an overnight incubation.

AG1478 is a potent and selective inhibitor of the EGF receptor.[5] Since the A431 cells were activated using EGF, it would be expected that AG1478 should reverse the EGF activation of the A431 cells as measured by phosphorylated STAT3 expression levels. As shown in Figure 6A, we see a potent inhibition curve for the titration of AG1478, with an IC_{50} of 12.5 nM and a signal to background of 5.3. This is consistent with a published value of 3 nM.[5] As a control, Figure 6B shows no inhibition of activation of pSTAT3 in SK-N-MC cells activated with CNTF.

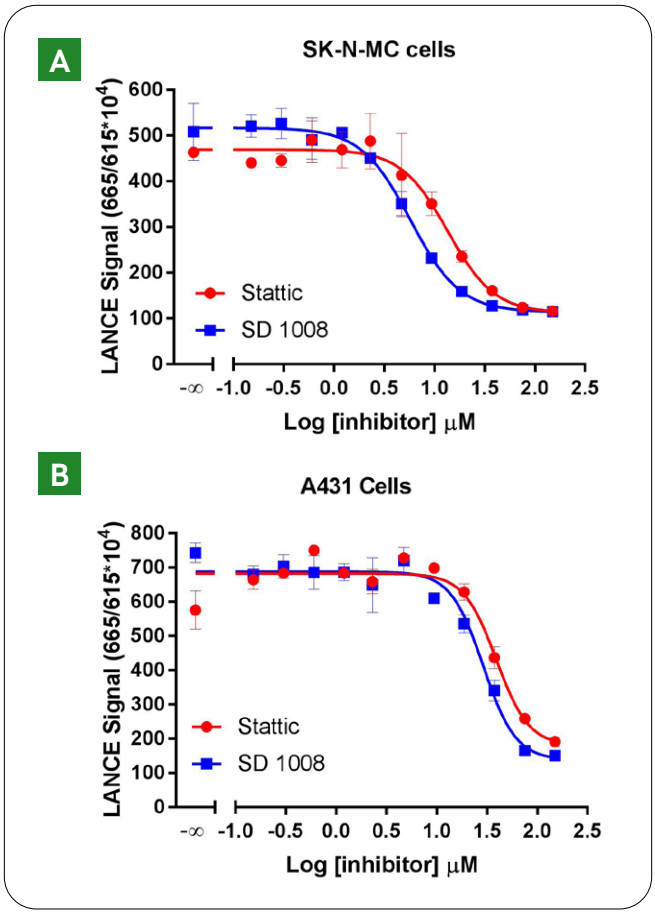


Figure 5: Inhibition of pSTAT3 expression levels in A431 cells and SK-N-MC cells.120,000 cells per well were plated and allowed to adhere overnight. Cells were serum starved for 4 hours prior to running the assay. Using the protocol in Fig. 2B, SK-N-MC cells (A) were incubated with Stattic or SD 1008 for 30 minutes and then incubated with 50 nM (final) CNTF for 15 minutes prior to lysing the cells. A431 cells (B) were incubated with Stattic or SD 1008 for 30 minutes and then incubated with 175 nM (final) EGF for 5 minutes prior to lysing the cells. Data were collected after overnight incubation.

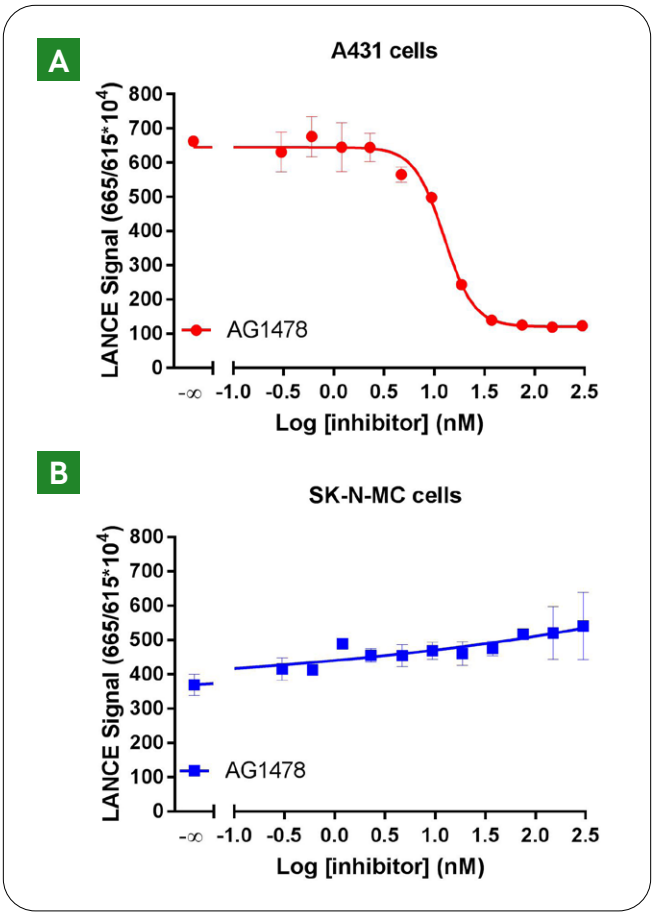


Figure 6: Inhibition of pSTAT3 expression levels using an EGFR inhibitor. 120,000 cells per well were plated and allowed to adhere overnight. Cells were serum starved for 4 hours prior to running the assay. A) Using the protocol in Fig. 2B, A431 cells were incubated with AG1478 for 30 minutes and then incubated with 175 nM (final) EGF for 5 minutes prior to lysing the cells. B) As a control, SK-N-MC cells were also incubated AG1478 for 30 minutes and then incubated with 50 nM (final) CNTF for 15 minutes prior to lysing the cells. Data were collected after overnight incubation.

Table 1 shows a summary of the inhibitor data collected with the adherent cell lines A431 and SK-N-MC using the 2-plate transfer protocol. The data show that the LANCE *Ultra* pSTAT3 cell-based kit can easily and accurately be used to assess potency of inhibitors of STAT3 phosphorylation.

Table 1: Summary of data analyzed from Figures 5 and 6.

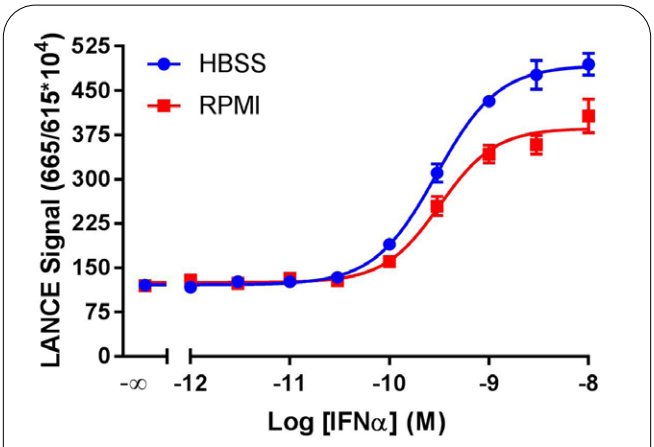
Cell line	Compound	IC_{50}	S:B
SK-N-MC	Stattic	13.6 µM	4.2
	SD 1008	5.8 µM	4.6
	AG1478	N/A	N/A
A431	Stattic	39.1 µM	3.8
	SD 1008	29.0 µM	5.0
	AG1478	12.5 nM	5.3

Modulating pSTAT3 expression levels using a one plate assay

Performing an all-in-one well assay is highly appealing for its ease and convenience. Therefore, using a suspension cell line (Jurkat cells) we looked at phosphorylated STAT3 expression levels using the one plate protocol (Figure 3). Jurkat cells are an immortalized line of human T lymphocyte cells. When treated with the cytokine, IFNα-2b, the IFNα receptor is activated and in turn activates the JAK pathway, leading to phosphorylation of STAT3. Activation of STAT3 by IFNα has been previously shown in Jurkat cells.[6]

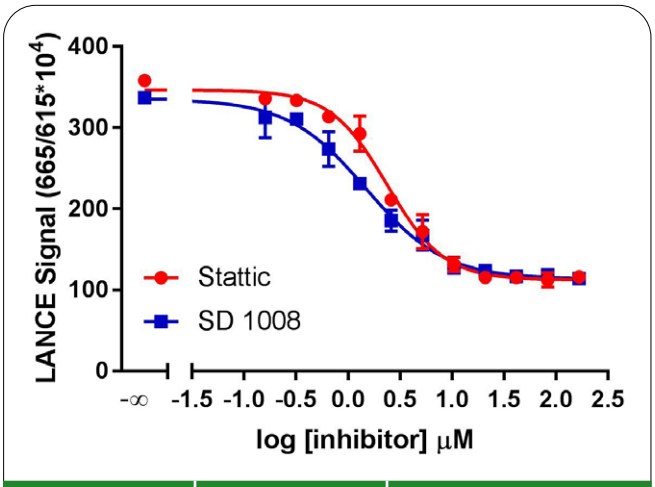
In order to assess whether cell culture medium has an effect on the assay, Jurkat cells were resuspended in either HBSS (with 10% FBS) or RPMI media (with 10% FBS) and tested with increasing concentrations of IFNα (Figure 7). Both assays gave similar EC_{50} values (0.3 nM). However, for this particular cell line, the cells resuspended in HBSS with 10% FBS gave a slightly better signal to background. It is unclear if it is the phenol red or some other component in the RPMI media that contributes to the lower signal to background. However, despite the small difference in signal to background, an excellent assay can be obtained using either cell culture medium or HBSS. In this case, the addition of 10% FBS to the HBSS slightly increased the signal to background for the Jurkat cells compared with cells incubated in HBSS without FBS (data not shown). Therefore, further experiments were done using HBSS with 10% FBS.

As with the adherent cell lines, the inhibitors Stattic and SD 1008 were titrated with IFNα-stimulated Jurkat cells using the protocol shown in Figure 3B. As shown in Figure 8, the phosphorylated STAT3 LANCE *Ultra* cellular detection kit successfully detects inhibition of pSTAT3 expression with IC_{50}s consistent with values in the literature.[3,4] The data here show that this assay can be easily adapted to a one plate protocol and used for measuring potencies of inhibitors of STAT3 phosphorylation.



Cell Line	EC_{50} (nM)	S:B
IFNα (HBSS)	0.3	4.1
IFNα (RPMI)	0.3	3.1

Figure 7: Modulating pSTAT3 expression levels with a one plate assay. Jurkat cells were re-suspended in either HBSS with 10% FBS or RPMI with 10% FBS and plated at 100,000 cells per well. Using the protocol in Fig. 3A, cells were incubated with IFNα for 5 minutes prior to the addition of lysis buffer. Data were collected after overnight incubation.

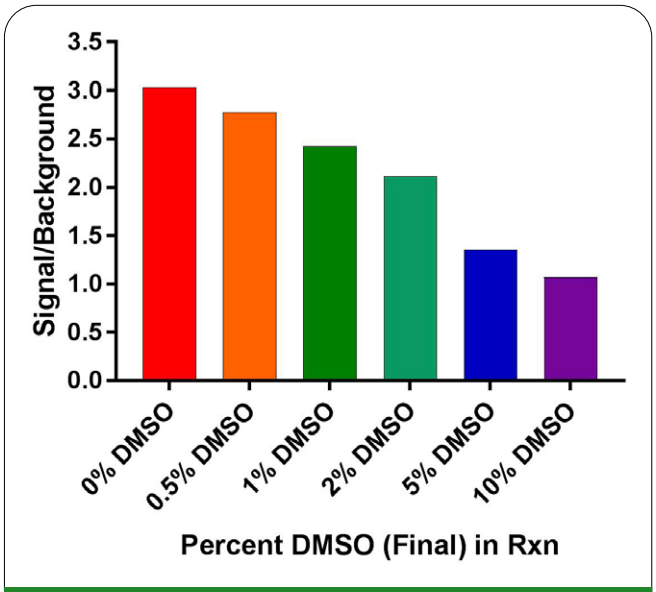


Cell line	IC_{50} (μM)	S:B
Stattic	2.4	3.1
SD 1008	1.5	3.0

Figure 8: Modulating pSTAT3 expression levels with a one plate assay. 100,000 cells per well were plated. Using the protocol in Fig. 3B, cells were incubated with Stattic or SD 1008 inhibitors for 30 minutes and then incubated with IFNα (60 nM final) for 5 minutes prior to the addition of lysis buffer. Data were collected after overnight incubation.

DMSO tolerance for one plate assay

Since many compounds used for stimulation or inhibition of kinases are soluble in DMSO, it is important to understand how the final DMSO concentration in the reaction may affect the assay. For adherent cells, any DMSO added to the cells is removed prior to lysing the cells. However, for the one plate assay, the DMSO from the stimulating or inhibiting compounds is still present in the final reaction. For the data presented in this application note, the final DMSO concentration in the reaction was 0.5%. Using the protocol in Figure 3B, we plated 8 µL of 100,000 cells/well of the Jurkat cells in HBSS with 10% FBS. We then added 2 µL of 6X the final DMSO concentration and incubated for 30 minutes. Then 2 µL of either 6X IFNα (60 nM final for stimulated cells) or HBSS with 10% FBS (unstimulated cells) was added and incubated for 5 minutes. The signal to background was calculated by dividing the LANCE *Ultra* signal for the stimulated cells by the LANCE *Ultra* signal for the unstimulated cells in the presence of various concentrations of DMSO. As shown in Figure 9, higher amounts of DMSO do progressively affect the signal to background of the assay. At 0.5% DMSO in the final reaction, the signal is still 90% of the no-DMSO control. By 10% DMSO in the final reaction, the signal cannot be distinguished from the background.



% DMSO	S:B
0	3
0.5	2.8
1	2.4
2	2.1
5	1.4
10	1.1

Figure 9: DMSO tolerance of the pSTAT3 LANCE Ultra cell-based one plate assay. 100,000 cells per well of Jurkat cells were plated. Using the protocol in Fig. 3B, cells were incubated with DMSO for 30 minutes and then were incubated with IFNα (60 nM final for stimulated) or HBSS with 10% FBS (unstimulated) for 5 minutes prior to the addition of lysis buffer. Data were collected after overnight incubation.

Z′-Factor determination

The robustness of the pSTAT3 LANCE *Ultra* cell-based assay in 384-well plate format was assessed by performing Z′-factor analysis using an adherent cell line with a two plate protocol (SK-N-MC cells) and a suspension cell line (Jurkat cells) with a one plate protocol. Cells were plated in 48 wells and half (24 wells) were stimulated while the other half (24 wells) were left unstimulated. The data are shown in Figure 10. Z′-factor, % CVs and signal to background (S/B) were determined for each assay (Table 2). After overnight incubation, high S/B ratios and Z′-factor values were obtained for the SK-N-MC cells line with the two-plate protocol (S/B= 5.5 and Z′ = 0.75) and the one plate assay with the Jurkat cells (S/B= 3.0 and Z′ = 0.53). The Z′ values and percent CV values were acceptable for cell-based assays conducted manually (> 0.4 and < 20% respectively).[7] These data, summarized in Table 2, demonstrate that pSTAT3 LANCE *Ultra* cell-based TR-FRET assays are robust and well-suited for HTS.

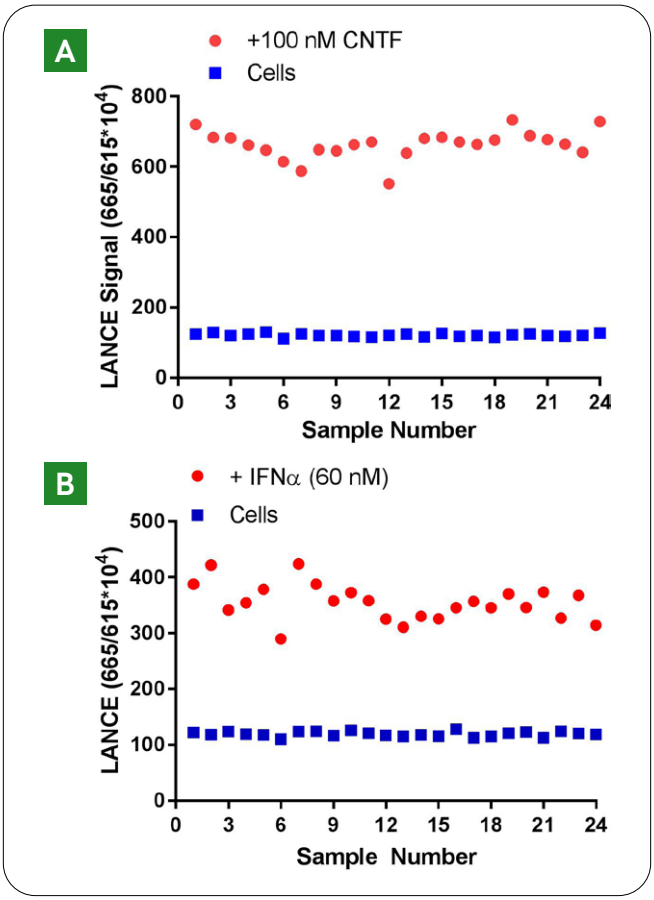


Figure 10: Z′ determination for one and two plate assays. A) Two plate assay: 120,000 SK-N-MC cells per well were plated and allowed to adhere overnight. Cells were then serum starved for 4 hours. Using the protocol in Fig. 2A, SK-N-MC cells were stimulated with either 100 nM (final) CNTF (stimulated cells) or medium (unstimulated cells) for 15 minutes prior to lysing the cells. B) One plate assay: Jurkat cells were re-suspended in HBSS with 10% FBS. Then 100,000 cells per well were plated. Using the protocol in Fig. 3A, cells were incubated with 60 nM (final) IFNα (stimulated cells) or HBSS with 10% FBS (unstimulated cells) for 5 minutes prior to the addition of lysis buffer. Data were collected after overnight incubation.

Table 2: Summary of data analyzed from Figure 10.

Cell line		Mean	StdDev	% CV	Z′	S/B
SK-N-MC	Unstimulated Cells	121	4.6	3.8	0.75	5.5
	CNTF Stimulated Cells	663	40	6.1		
Jurkat	Unstimulated Cells	120	4.6	3.8	0.53	3.0
	IFNα Stimulated Cells	355	33	9.2		

Conclusions

Modulation of pSTAT3 expression levels can be quickly and accurately assessed using the pSTAT3 LANCE *Ultra* cell-based detection kit. Here we show how LANCE *Ultra* cell-based assays provide comparable pharmacology with the expected inhibitor potency for pSTAT3. We demonstrated the suitability of the LANCE *Ultra* kits for screening compounds in a cell-based assay by showing assay precision, as calculated by Z′, which was > 0.5 in both adherent and suspension cell lines. The tolerance of the one plate assay to up to 1% final DMSO concentration allows rapid screening of DMSO-soluble compounds. LANCE *Ultra* cell-based assays provide a fast, powerful, homogeneous platform for screening potential kinase inhibitors in a variety of human cells.

References

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