Adaption of Lumit p-STAT3 Cellular Immunoassay to high-throughput screening format for identification of JAK/STAT pathway antagonists

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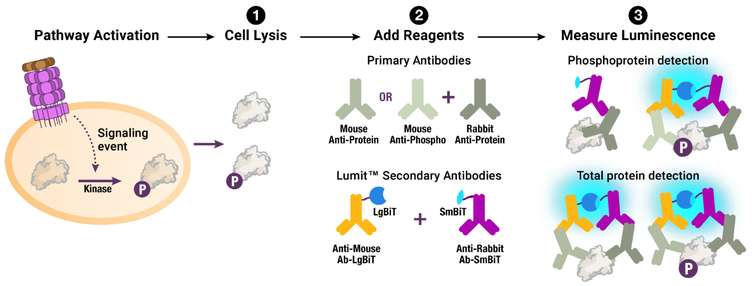
1. Introduction

The Lumit™ Immunoassay Cellular System supports an “add and read” format in cellular lysates. After desired cell treatment, cells are lysed, followed by the addition of an antibody mixture containing primary antibodies against the target of interest and secondary antibodies labeled with NanoBiT fragments. Following an incubation period, the furimazine substrate is added and plates are read on a luminometer. The assay requires no wash steps and can be completed in approximately 2 hours. Up until now, this system has not been demonstrated for use in a high throughput screen format. Here we present data showing the utility of the Lumit™ Immunoassay Cellular System for HTS.

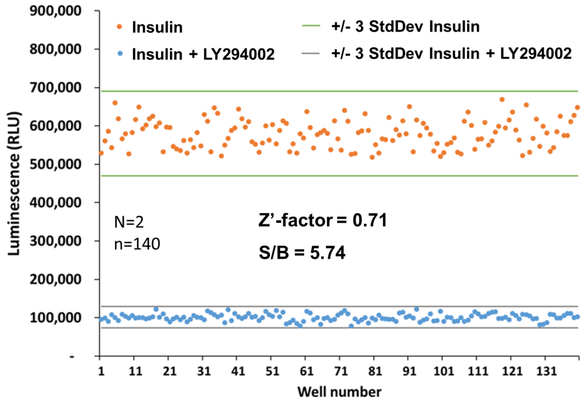


Standard 96-well Lumit™ Immunoassay Format

Treat desired number of cells in media (35 µL) → Add lysis buffer (10 µL) → 40 min → Add Antibody Mix (50 µL) → 60-90 min → Add Furimazine (25 µL) → 2 min → Measure Luminescence

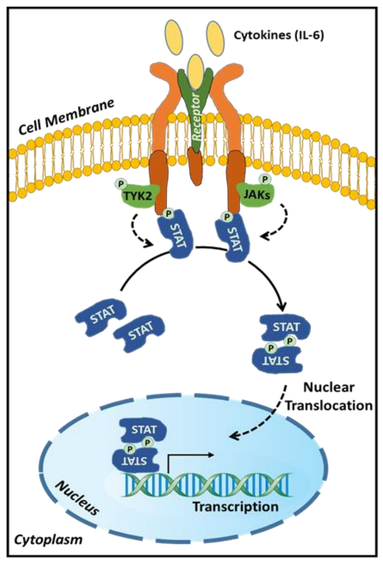
2. Feasibility of Lumit™ Immunoassay Cellular System for High Throughput Screening

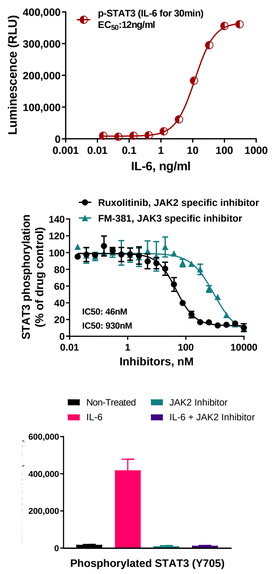
Z'-factor from test screen using the Lumit™ p-AKT assay demonstrates feasibility of using the Lumit™ Immunoassay Cellular System for High Throughput Screening.



MCF-7 cells were treated with 20 µM PI3K inhibitor for 1 hour then stimulated with 1 µM Insulin for 10 minutes prior to running Lumit™ p-AKT (S473) assay.

3. Detection of p-STAT3 (Y705) in 96-well Format



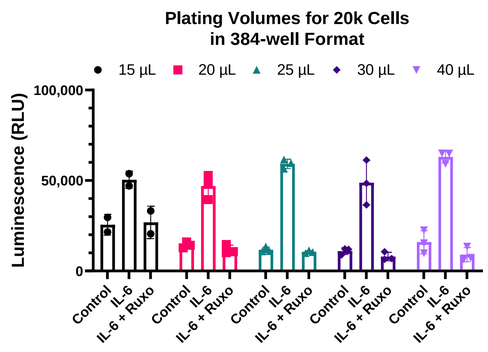


4. Flexibility of Plating Volume and Reagent Dilutions for p-STAT3 assay in 384-well plates

Initial Test Volumes for 384-well format

Treat desired number of cells in media (35 µL) → Add lysis buffer (5 µL) → 40 min → Add Antibody Mix (5 µL) → 60-90 min → Add Furimazine (5 µL) → 2 min → Measure Luminescence

20k A431 cells were plated in various volumes. Standard dilutions (1x) of Lumit™ reagents were applied, resulting in varied final concentrations of reagents.



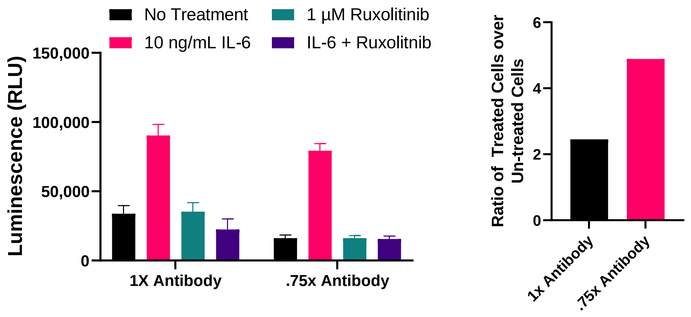
384-well Cell Plating Volumes

Reagent	15 µL	20 µL	25 µL	30 µL	40 µL
Lysis	1x	.86x	.75x	.67x	.55x
Antibody	1x	.88x	.78x	.7x	.58x
Substrate	1x	.89x	.8x	.73x	.62x

Table depicts dilution of reagents at various plating volumes. 1x concentration is standard for 96 well format, from p-STAT3 Lumit™ Early Access Kit (CS3397A11).

5. Optimizing STAT3 Antibody Conditions for 35 µL of Treated Cells in 384-well Format

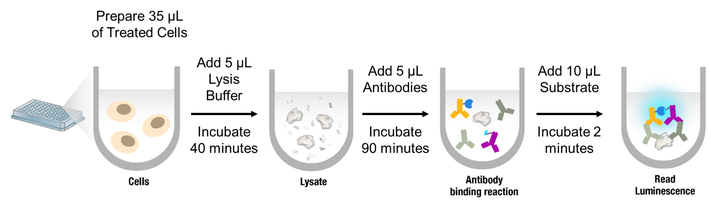
Lower concentration of antibodies for the p-STAT3 assay in 384 well format yields better signal over background without sacrificing maximum signal



20k A431 cells were plated in 25 µL of media, 1 day prior to running the assay. Cells were treated with Ruxolitinib for 1 hour following a 30-minute treatment of IL6. After IL6 treatment, the Lumit™ protocol was followed with various dilutions of antibodies.

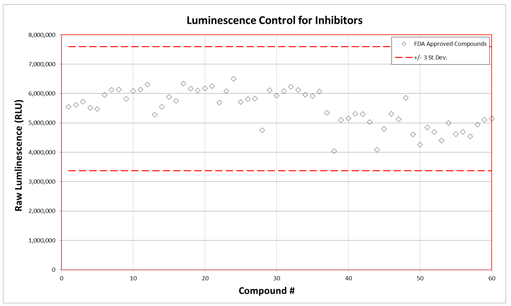
6. Lumit™ Immunoassay Cellular Systems Format for High Throughput Screening

Scheme showing Lumit™ Cellular Immunoassay protocol for HTS, and table comparing 384 well to standard 96-well volumes

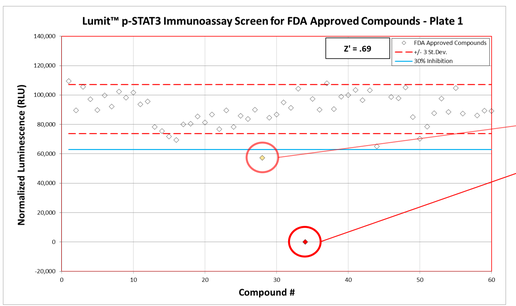


Reagent	384-well Reagent concentration	384 well addition volume (µL)	384 well final volume (µL)	96-well Reagent concentration	96-well addition volume (µL)	96 well final volume (µL)
Cells and Treatment	N/A	35	35	N/A	40	40
Lysis Buffer	.75x	5	40	1x	10	50
Antibodies	.75x	5	45	1x	50	100
Substrate	1x	10	55	1x	25	125

7. FDA Approved Compound Screen Yields Expected JAK Inhibitors



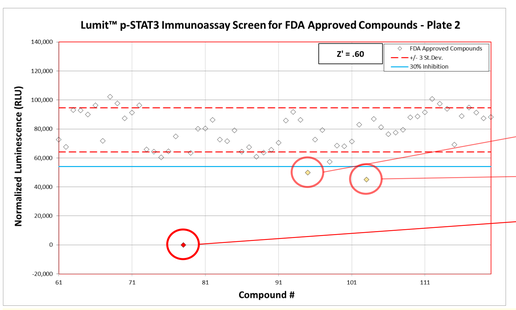
Control NanoBiT fragments that generate luminescence show no inhibition of luminescence from any of the 120 screened compounds (compounds 1-60 shown in graph).



Sunitinib malate

CP 690550 Citrate

Screen of 120 FDA approved compounds, using the HTS established format, uncovers two strong JAK inhibitors, and three other kinase inhibitors that may weakly inhibit STAT3 phosphorylation.

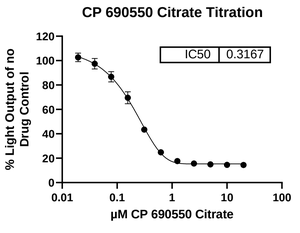


Imatinib mesylate

Bosutinib

Ruxolitinib

20k A431 cells were plated in 25 µL one day prior to screening. For screen, 5 µL of compounds were added to each well to achieve a 10 µM concentration, and cells were incubated for 1 hour. 5 µL of 70 ng/mL IL-6 was then added to each well (10 ng/mL final) and cells were incubated for 30 minutes prior to running the Lumit™ HTS protocol using the Lumit p-STAT3 (Y705) kit, and volumes shown in panel 6.



Standard format of Lumit™ in 96-well plates detects titration of CP 690550 Citrate to confirm screen results.

9. Conclusions

- Lumit™ Immunoassay Cellular System is adaptable to 384 well format for high throughput screening
- Flexibility of reagent concentrations and volumes allow screen to be tailored to needs
- Reduced concentrations of antibodies in 384 well plates allows for a reduction of background signal that results in improved assay window. This may need to be tailored for each individual assay.
- A small throughput test screen of FDA approved compounds yields expected inhibitors of JAK/STAT signaling, including Ruxolitinib and CP 690550 Citrate.

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