

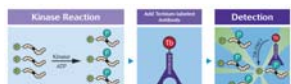
Cell Based and Biochemical Assay Tools for the Discovery of JAK Inhibitors

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Introduction

The JAK/STAT pathway is a critical signaling mechanism for a wide array of cytokines and growth factors. Inhibitors of the JAK family of protein kinases are currently being sought in the areas of oncology and immune disorders. To promote discovery of inhibitors specific to the individual family members, we have developed cell-based and biochemical assay tools suitable for HTS and hit follow-up. Using our catalog JAK kinases (JAK1, JAK2, JAK3, TYK2, and JAK2 V617F), we have produced functional biochemical assays for each enzyme using a sensitive time-resolved FRET format. We have also used the TR-FRET technology to enable determination of the phosphorylation state of STAT1, 3 and 5 in cells using the respective GFP-STAT fusions as substrates. To complement these target specific assays, we have designed cell-based assays that allow general interrogation of different JAK/STAT pathways using a beta-lactamase reporter gene downstream of relevant response elements for various STATs. Here we present the different assay technologies using JAK2/STAT5 signaling as an example.

Figure 2 – LanthaScreen™ Kinase Assays



LanthaScreen™ kinase assays are a simple, sensitive method to detect kinase activity, and due to the TR-FRET readout, are highly resistant to compound interference. A fluorescently labeled peptide substrate is incubated with the kinase and ATP. Phosphorylation of the peptide recruits a terbium-labeled phospho-specific antibody, resulting in FRET. Due to the high sensitivity, typical assays require low picomolar to low nanomolar amounts of kinase. For the JAK assays, the substrate used is a polymer of Glu-Tyr (Fluorescein-Poly GT) and the antibody is Tb-PY20.

Figure 3 – Compound Profiling of JAK2

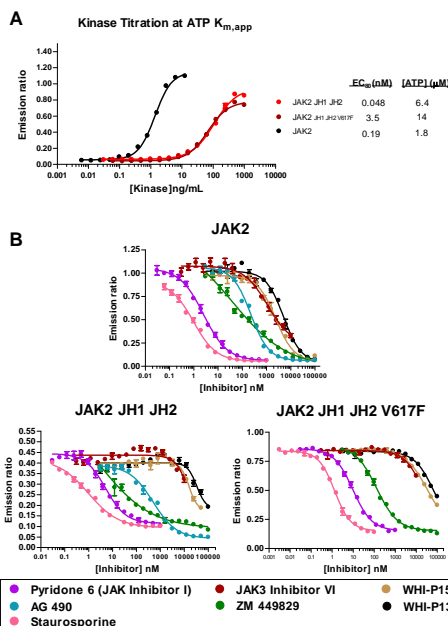


Figure 1 – Three Complementary Approaches for JAK Inhibitor Studies

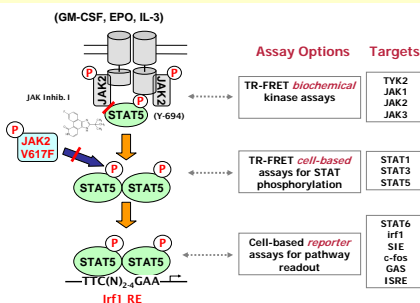
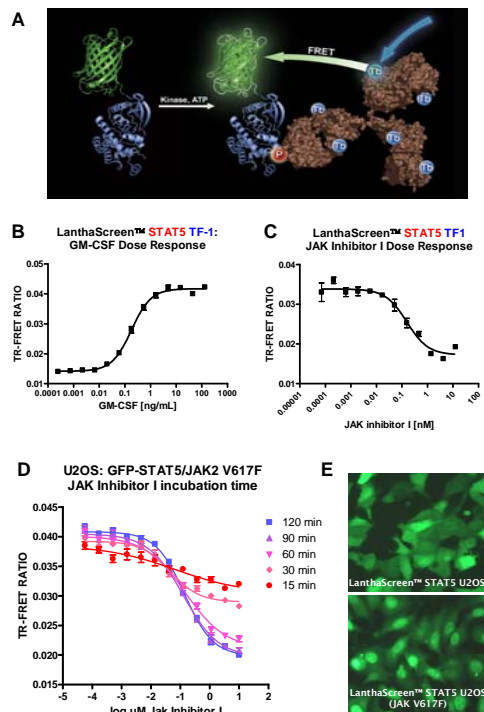


Figure 4 – TR-FRET Cell-based Analysis of STAT5 Phosphorylation



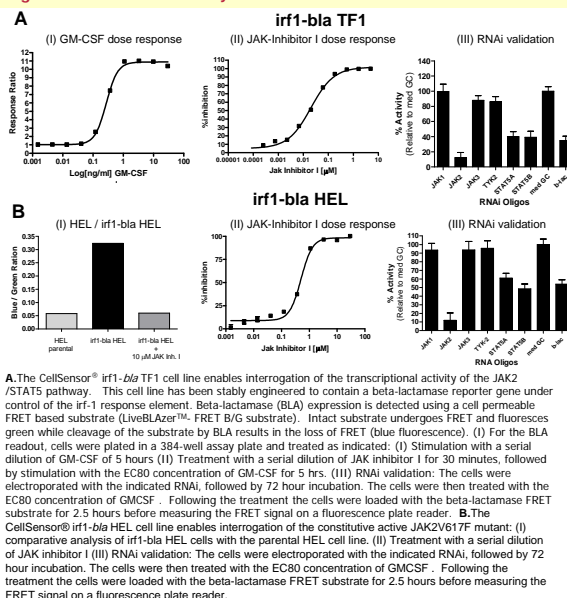
A. The LanthaScreen™ TR-FRET cell-based assay approach provides a high-throughput alternative to ELISAs for the evaluation of the phosphorylation state of the STAT5 protein. B. TF-1 cells engineered to express the GFP-STAT5 fusion protein were added to a 384-well assay plate and starved overnight. The cells were stimulated with a serial dilution of GM-CSF for 30 minutes before lysis buffer and Tb labeled anti-pY694 STAT5 antibody was added. After 60 minutes of incubation the TR-FRET signal was determined on a BMG PHERASTAR fluorescence plate reader. C. The assay was performed similar to B, except serial dilutions of the inhibitor were allowed to incubate with the cells for 90 minutes before stimulation with an EC50 concentration of GM-CSF. D. U2OS cells engineered to express GFP-STAT5 and mutant JAK2V617F were added to a 384 well plate. A serial dilution of inhibitor was added and incubated for the indicated period of time. The medium was aspirated and lysis buffer containing anti pY694 STAT5 antibody was added. After 60 minutes of incubation the TR-FRET signal was determined on a BMG PheraStar plate reader. E. GFP-STAT5 localization pattern in U2OS and U2OS-JAK2V617F cells

Table 1: Cell based Assays for JAK/STAT signaling

Primary Agonist	CellSensor™ Lines											Response Element	Cell Background
	TYK2	JAK1	JAK2	JAK2 V617F	JAK3	STAT1	STAT2	STAT3	STAT5	STAT6			
IL-4												STAT6	RA1
constitutive mouse IL-3													HEL
GM-CSF, IL-3, EPO												irf1	BlaF3
IL-2													TF-1*
IFNγ													CTL-2
IL-6													THP1
IFNγ													HEK 293T
IL-6													HEK 293T
IFNγ													ME-180*
EGF, IL-6, OSM													ME-180*
IFNγ													c-fos
IFNα, IFNβ													ME-180
IFNα, IFNβ													GAS
IFNα, IFNβ													ME-180
constitutive													HEK 293T*
													Jurkat

Primary Agonist	LanthaScreen™ GFP Cellular Assay Cell Lines			GFP-fusion	cell background
	IFNγ	IFNα	IFNβ		
IFNγ				STAT1	U2OS
IFNα				STAT3	Griptide™
IFNβ				STAT5	TF-1
constitutive				STAT5	U2OS-JAK2V617F

Figure 4 – CellSensor® Assay for JAK2 and JAK2V617F



Conclusions

- Complementary biochemical and cell-based assays for target pathways aids in data interpretation and provide a more dependable platform for kinase inhibitor discovery.
- LanthaScreen™ kinase assays enable rank order potency and selectivity determinations for all JAK kinase members with a single assay format also suitable for HTS. The inhibitor profiles for the LanthaScreen™ kinase assays show the expected patterns.
- The LanthaScreen™ GFP cellular assay provides a high throughput alternative to traditional methods for phospho-protein analysis of JAK/STAT signaling
- JAK/STAT CellSensor® cell lines provide a diverse set of relevant response elements and cell backgrounds to evaluate JAK/STAT signaling.