



Targeting the NFkB and M1-NFAT signaling pathways using quantitative HTS (qHTS) and cell-based β -lactamase assays with a laser-scanning microplate cytometer in 1536-well plates

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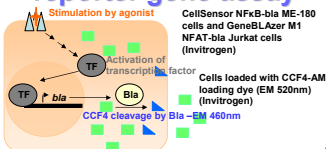
1. Introduction

The transcription factors NFkB and NFAT regulate the expression of a multitude of genes with key roles in cellular growth and differentiation, tissue development, and immune system activation and response (Lee and Park, 2006; Gilmore, 2006). Unregulated or constitutively active forms of these transcription factors leads to cellular transformation and oncogenesis (Buchholz and Ellenrieder, 2007; Gilmore and Herscovitch, 2006). Inhibiting components of these signaling pathways thus has great therapeutic potential. Using cellular-based assays with a β -lactamase reporter gene as transcriptional read-out, we perform qHTS to assess the activity of thousands of small molecules against these transcription factors. In this way, we are able to identify small molecules that down-regulate β -lactamase expression, indicating inhibition of transcription factor activation or function. The commonly used high-throughput technology for β -lactamase (*bla*) reporter gene assays relies on whole-well plate readers, in which aggregate fluorescence from thousands of cells is used to quantitate response to a small molecule. While providing robust output, this type of reader may lack sensitivity, particularly when only a fraction of the cells respond to stimulus. An alternative technology is the Acumen laser-scanning fluorescence microplate cytometer (TTP LabTech LTD), which provides information on individual cells - ideal for reporter assays in which cells may have varying responses to transcriptional stimulation or inhibition.

2. Purpose of study

- Optimize a protocol for NFkB-*bla* reporter gene assay for the Acumen eX3 in 1536-well format for an adherent cell line
- Determine if the Acumen eX3 - which scans the bottom of a well - can be reliably used in an assay involving suspension cells
- Highlight unique features of laser-scanning fluorescence microplate cytometry
- Evaluate Acumen eX3 performance in quantitative HTS

3. The β -lactamase reporter gene assay



CellSens NFkB-*bla* ME-180 cells and GeneBLAzer M1 NFAT-*bla* Jurkat cells (Invitrogen)

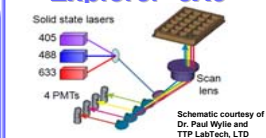
Cells loaded with CCF4-AM loading dye (EM 520nm) (Invitrogen)

We would like to acknowledge Dr. Paul Wylie and Dr. Wayne Bown of TTP LabTech Ltd. for their expert advice and assistance.

NFkB Assay Protocol

Sequence	Parameter	Description	Value
1	Reagent addition	Cell Senser NFkB- <i>bla</i> ME-180 cells (3000 cells/well)	50
2	Reagent addition	Stimulant	200
3	Reagent addition	Compound	200
4	Reagent addition	TF6	200
5	Reagent addition	CCF4-AM loading dye (EM 520nm)	50
6	Reagent addition	CCF4-AM loading dye (EM 520nm)	50
7	Excitation	Use Acumen eX3 to collect 460nm and 520nm fluorescence	

4. Acumen Explorer eX3



The Acumen Explorer eX3 is equipped with three solid-state lasers, allowing excitation of samples at 405nm, 488nm, and 633nm. In combination with four PMTs for collection of emission fluorescence, the reader has multiplexed detection capabilities.

NFAT Assay Protocol

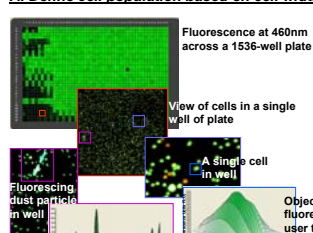
Sequence	Parameter	Description	Value
1	Reagent addition	GeneBLAzer M1 NFAT- <i>bla</i> Jurkat cells (3000 cells/well)	50
2	Reagent addition	Stimulant	200
3	Reagent addition	Compound	200
4	Reagent addition	TF6	200
5	Reagent addition	CCF4-AM loading dye (EM 520nm)	50
6	Reagent addition	CCF4-AM loading dye (EM 520nm)	50
7	Excitation	Use Acumen eX3 to collect 460nm and 520nm fluorescence	

5. High-content analysis with high-throughput capacity

- Scans entire well area of 96-, 384-, or 1536-well plate
- For 1536-well plate, approximately 2 wells/sec for 1 μ m x 8 μ m sampling resolution
- Read-time independent of well cell density

Bowen and Wylie, 2006

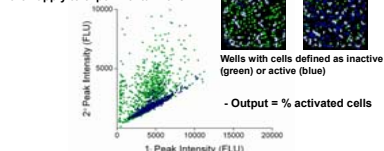
A. Define cell population based on cell width x depth



Object profile of contaminating particle - dimensions much larger than cell

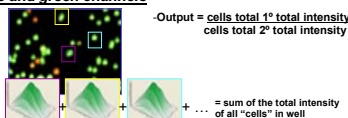
B1. Define an activated cell population (express β -lactamase) based on the ratio of blue to green peak fluorescence intensity

-Use control wells - maximally activated and no activation - to define, then apply to experimental wells



- Output = % activated cells

B2. Take the ratio of the cellular total fluorescence intensity from the blue and green channels



-Output = cells total 1 $^{\circ}$ total intensity / cells total 2 $^{\circ}$ total intensity

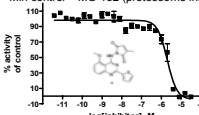
6. NFkB-*bla* assay: High performance at low cell densities

- High signal to background, low % CV, and Z' Factors > 0.5 for cell densities as low as 300 cells/well for adherent cells
- Bulk-fluorescent plate readers require significantly more cells/well (1000s) to produce data of similar quality
- Able to produce concentration curves for compound titrations to determine compound potency and efficacy

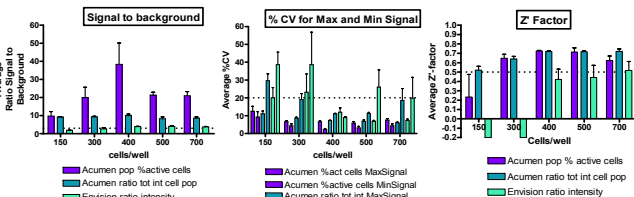


Increasing concentration inhibitor

Max control = DMSO, no inhibitor
Min control = MG-132 (proteasome inhibitor)

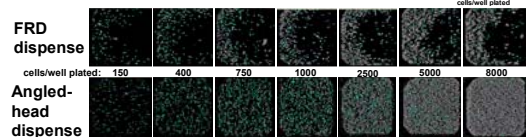
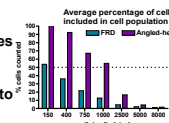


A transcriptional inhibitor of NFkB - a substituted 2-(thiophen-2-yl)quinazoline (Patanki et al., 2003) with an IC50 = 2 μ M, identical to that found in literature (Xie et al., 2007). Here, 400cells/well.



7. NFAT-*bla* assay: Optimizing a suspension cell assay - effect of reagent dispensing

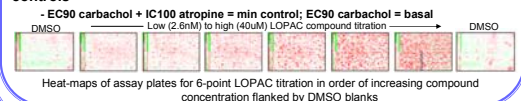
- High-velocity BioRAPTR FRD reagent dispense causes suspension cells to clump to sides of well, resulting in reduced cell count
- Kalypsys angled-head reagent dispense allows cells to remain dispersed through-out well



Picture of wells at different cell densities after delivery of reagents with different dispensers. Grey indicates cell clumps, which are not counted as part of the cell population. Green and blue indicate cells that are counted. At high cell densities, most cells exist as cell clumps, regardless of dispense. We found that 750-1000 cells/well gives the best results.

8. High performance with suspension cells in M1 NFAT-*bla* assay

- In agonist assay mode, able to achieve S:B of 10 and a Z' Factor of 0.71 for controls
- EC90 carbachol = max control; DMSO = basal
- In antagonist assay mode, have S:B of 9 and a Z' Factor of 0.67 for controls
- EC90 carbachol + IC100 atropine = min control; EC90 carbachol = basal



9. Summary

- The laser-scanning fluorescence microplate cytometer Acumen eX3 enables HTS while providing high-content analysis of cell-based assays
- Unique ability for the user to define cell populations and sub-populations, favorably influencing S:B ratio for a given assay
- This, along with the multiple excitation lasers and PMTs, allow the design of multiplexed assays with multiple fluorophores
- The high performance at low cell density saves on cell culture reagents and time
- Sensitivity to provide robust and reproducible data at very low cell densities, even in 1536-well format
- Applications are not limited to adherent cell lines - both agonist and antagonist β -lactamase assays have been developed and optimized for a suspension cell line and show high performance

Literature Cited

Bowen, WP and Wylie, PG. 2006 Assay and Drug Dev Tech 4: 209-221
 Buchholz, M., and Ellenrieder, V. 2007. Cell Cycle 6: 16-19.
 Gilmore, TD. 2006. Oncogene 25: 6680-6684.
 Gilmore, TD and Herscovitch, M. 2006. Oncogene 25: 6887-6899.
 Lee, M and Park, J. 2006. Mol. Cells 22: 1-7.
 Palanki, MS et al. 2003. Bio. & Med. Chem. Ltrs. 13: 4077-4080.
 Xie, Y. et al. 2008. Bio. & Med. Chem. Ltrs. 18: 329-335.