GeneBLAzer® Assay Setup Guide on the BMG LABTECH OPTIMA Microplate Reader

NOTE: The BMG LABTECH OPTIMA Microplate Reader was tested for compatibility with Invitrogen's GeneBLAzer® Assay in bottom-read mode using two CellSensor® cell lines; the irf1-*bla* HEL and irf1-*bla* TF-1 cell lines (K1647 and K1657, respectively). The following document is intended to demonstrate setup of this instrument and provide representative data. For more detailed information and technical support of Invitrogen assays please call 1-800-955-6288, select option "3", then extension 40266. For more detailed information and technical support of BMG LABTECH instruments or software, please contact BMG LABTECH at 1-877-264-5227 or www.bmglabtech.com.

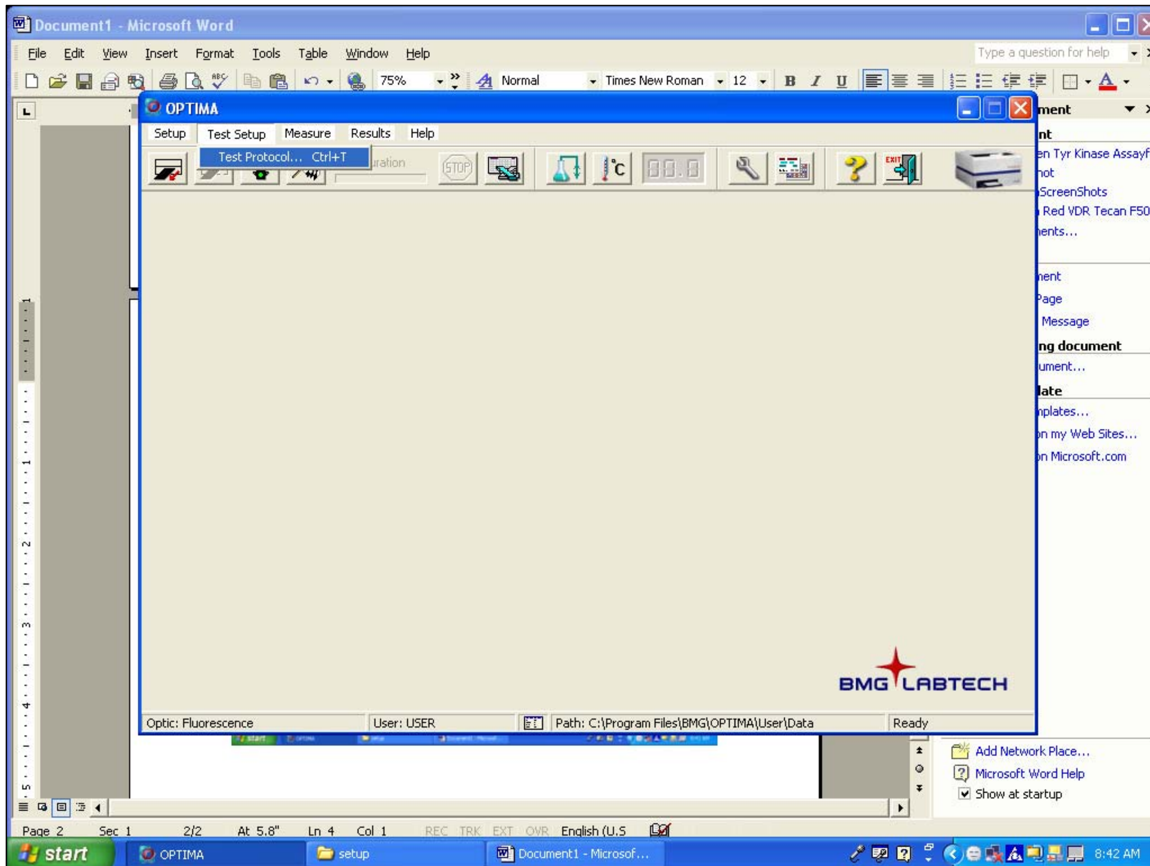
A. Recommended Optics

	wavelength (nm)	BMG LABTECH Filters
Excitation	400 (or similar)	*contact BMG LABTECH
Emission 1	440 (or similar)	*contact BMG LABTECH
Emission 2	520 (or similar)	*contact BMG LABTECH

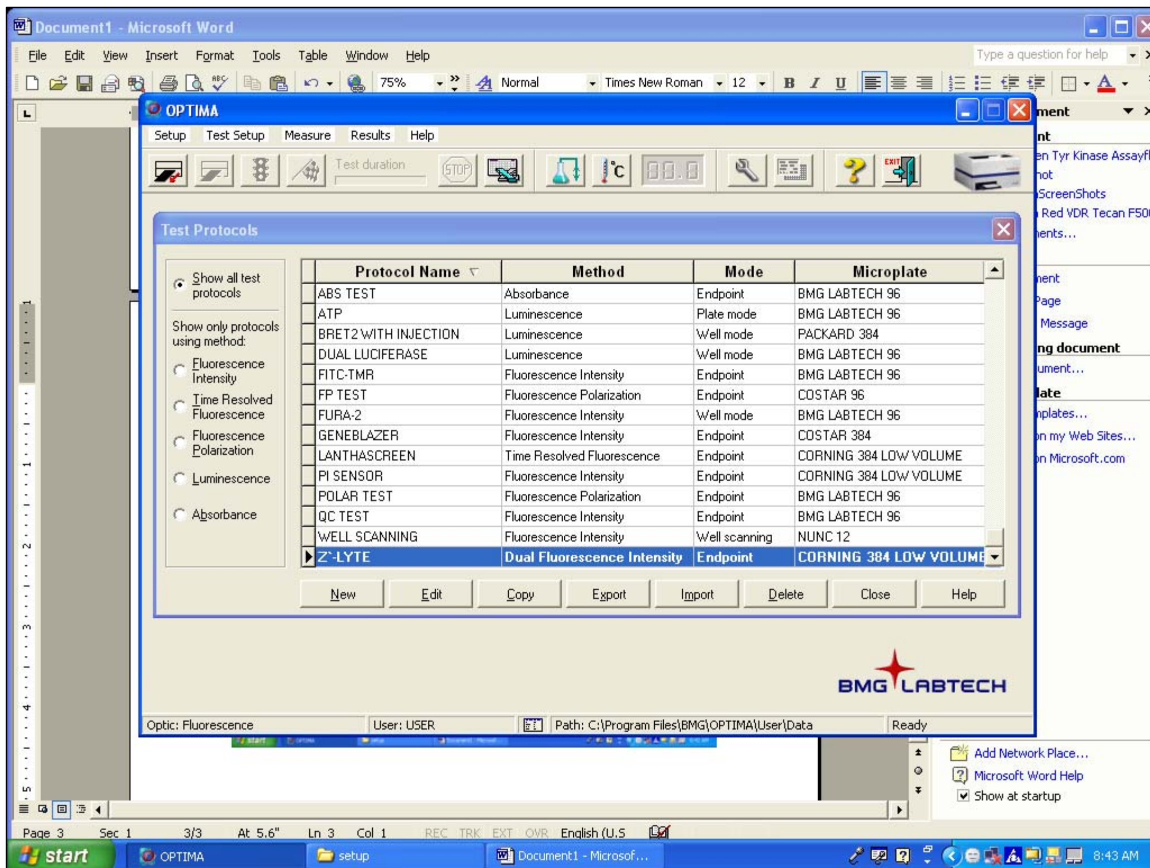
B. Instrument Setup

1. Make certain plate reader is turned on, and open up OPTIMA Control software on computer. Insert plate into plate reader.

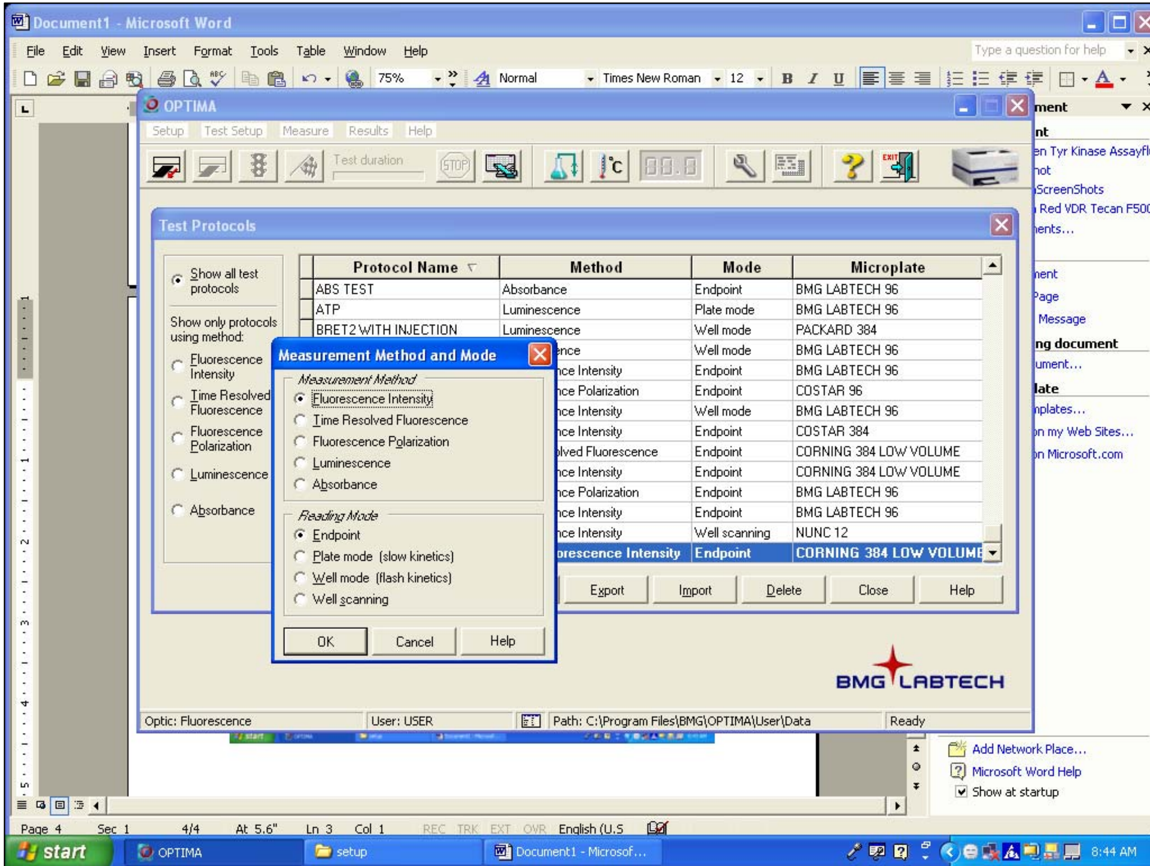
2. When OPTIMA Control software opens, if you do not have a pre-existing protocol for GeneBLAzer®, select "Test Protocol" from the Test Setup menu bar at the top of the window.



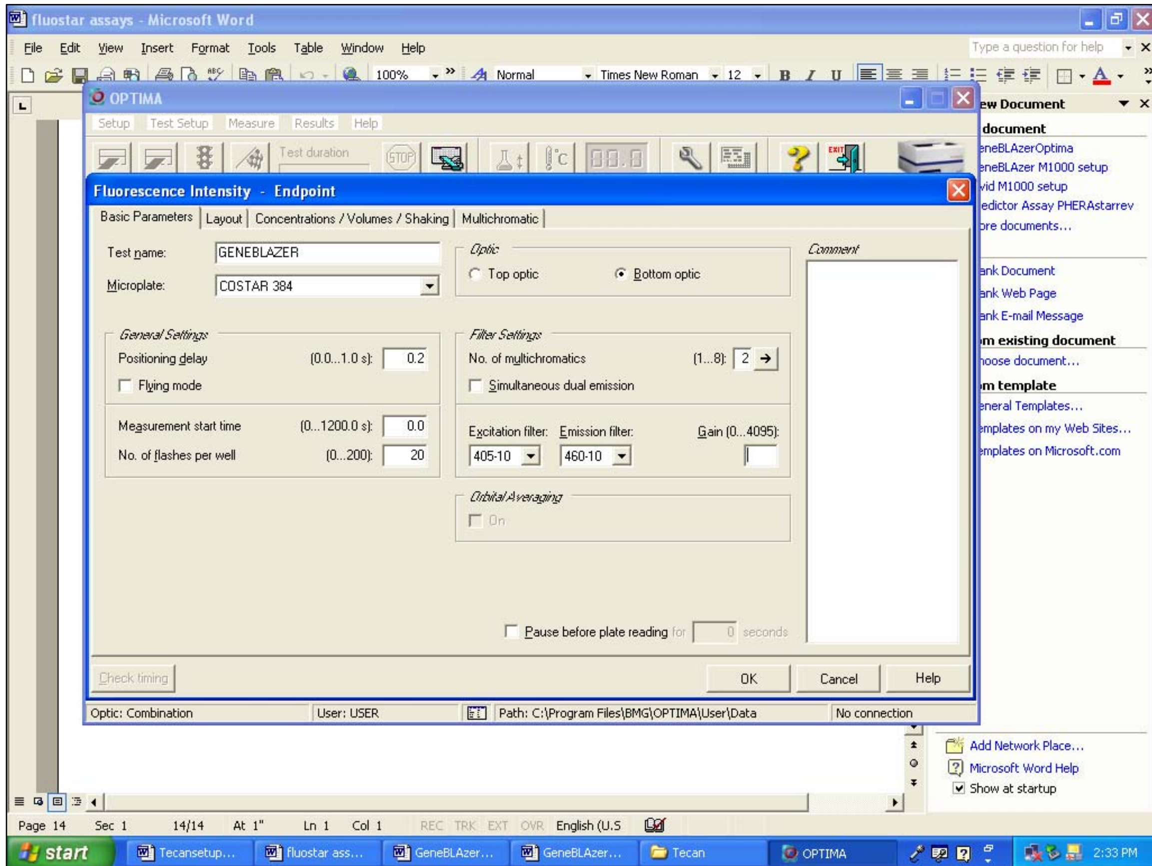
3. At this point, a new screen will open (below). Click on the "Show all test protocols" button on the left side of the screen, then select "New" from the tabs at the bottom.



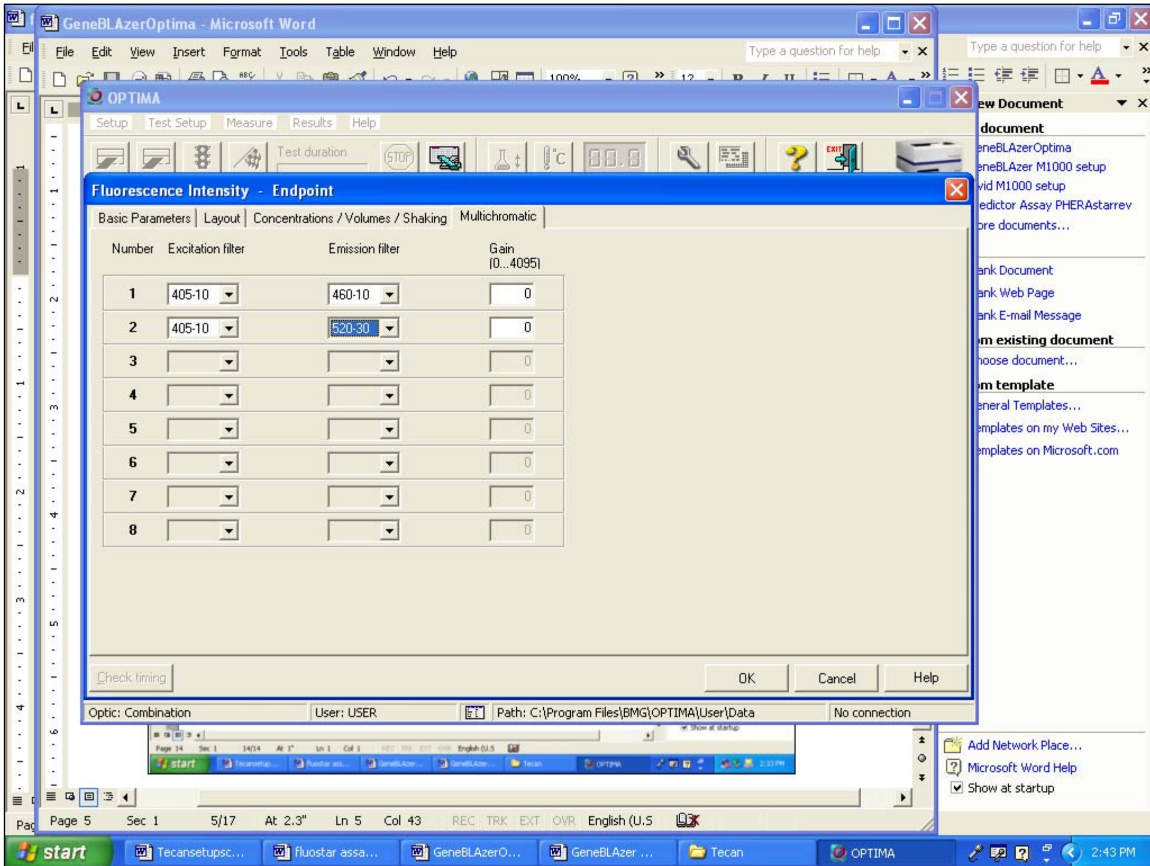
4. A new window will pop up. Select “Fluorescence Intensity” and “Endpoint” and then select “OK”.



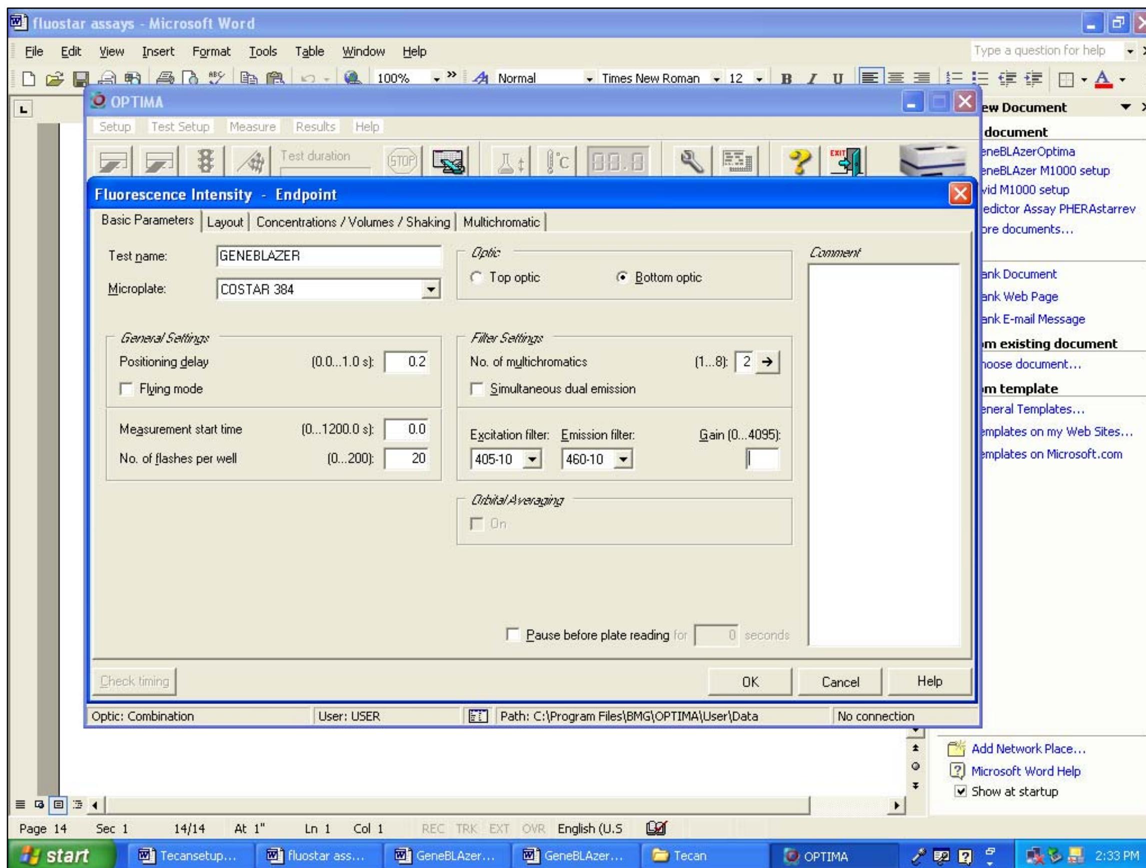
5. A new Protocol window will open automatically. Enter a test name, select plate type (there is no specification for clear-bottom plates for the equivalent 384-well plates, this does not matter), and select the **Optic (bottom)** from the drop-down list. Next, set the number of flashes per well and set up the excitation and first emission filter from the drop-down tabs. Finally, ubder "Filter Settings", enter "2" for the No. of multichromatics. This will allow you to click on the small arrow beside the box.



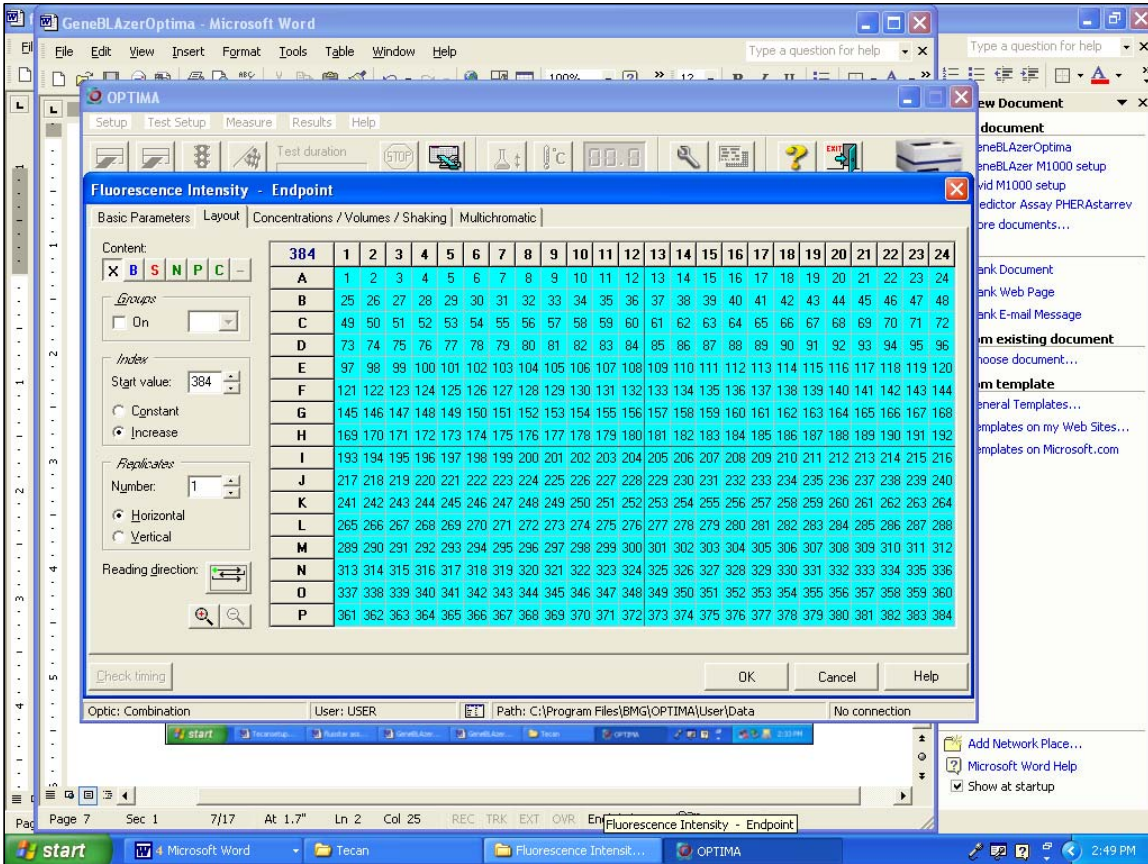
6. Clicking the arrow mentioned in 5 will open another window--select your filters here as shown below, then click "OK" when finished.



7. You will return to the initial settings window. From the tabs along the top of this window, select "Layout".



8. A new window will appear. Select the wells you wish to read by highlighting them. When finished, select "OK"



C. GeneBLAzer® Cell-Based Assay using CellSensor® irf1-*bla* HEL and irf1-*bla* TF-1 cell lines.

NOTE: The following is a sample titration assay performed for demonstration purposes. The instrument settings above would be sufficient for any GeneBLAzer® assay, the information below is provided as representative data. Assays were run in 40 µl in 384-well black-wall, clear-bottom plates. The information below details how the validation assays were prepared and is provided as an explanation, it is not intended as a protocol. We recommend all first-time users follow the appropriate protocols and/or validation packets provided with their assays. Due to the various cellular backgrounds available, assay conditions can vary considerably. For additional details on handling and growing these and other CellSensor® cell lines, please contact Invitrogen Technical Support at drugdiscoverytech@invitrogen.com.

Cell-Based Assay Setup

1. On Day 1, one day prior to the actual reading step, harvest cells:
   - For Irf1-*bla* TF-1 cells count and spin down, wash once in Assay media to remove residual GM-CSF, and resuspend in Assay Media at a final density of 5 x 10^5 cells/ml. Place cells in incubator for 16 hours.
   - In the meantime, for Irf1-*bla* HEL cells, harvest and resuspend in Assay Medium at 9.5 x 10^5 cells/ml.

2. Prepare assay plate for HEL cells: Prepare a set of 1:1 serial dilutions from 100X the initial concentration (100 µM) in DMSO in a 384-well plate, starting in Rows A and E with 80 µl compound at 100 µM inhibitor in DMSO in Column 1 and 40 µl DMSO alone in wells 2-20. Add 40 µl from well 1 to well 2, and then mix well 2, and take 40 µl from well 2 and add to well 3, mix, and so on. Preparing an initial serial dilution in DMSO serves to improve accuracy and robustness, particularly with compounds which may have solubility issues at higher concentrations.

3. In Rows B and F prepare an intermediate dilution by adding 4 µl compound from the above serial dilution to each well below, then adding 36 µl Assay Buffer to produce a set of serial dilutions at 10X the desired final concentrations.

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Staur.	100X	A																								
	4X	B																								
		C																								
		D																								
		E																								
	4X	F																								
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Figure 1: Schematic of initial compound dilution. Staurosporine and JAK2 Inhibitor II were titrated from a 100 µM starting concentration in the initial dilution series by preparing a 1:1 dilution curve in DMSO. A secondary dilution to 10X was then prepared in the rows below the initial dilution curve (lighter gray) using each line's specified Assay Buffer.

4. Add 36 µl per well of cell suspension to all wells except Column 23 and 24, Rows M through P. For Column 23 and Column 24, Rows M through P, add 40 µl Assay media alone. It is important to use cell-free wells in GeneBLAzer to background subtract donor and acceptor values for optimal GeneBLAzer assay performance.

5. Now add 4 µl inhibitor from secondary dilutions above to wells in Columns 1-20 in quadruplicate (i.e. 4 µl of the 10 µM staurosporine into Column 1 Rows A through D and 4 µl of 5 µM staurosporine from the next well to Column 2 Rows A through D and so on to set up an actual titration from 1 µM final starting concentration of staurosporine). Add 4 µl Assay Buffer to Columns 21 and 22, as well as rows A-L of Columns 23 and 24, so each well has a final volume of 40 µl.

6. Incubate HEL assay plate for 15 hours.

7. The following morning, prepare an assay plate as above for the TF-1 line, except that cells are washed once more and resuspended at 1.5625 x 10^6 cells/ml in Assay Media. Add 32 µl cells per well to all wells of the plate except Column 23 and 24, Rows M through P. For Column 23 and Column 24, Rows M through P, add 36 µl Assay media alone, then add 4 µl Assay Buffer containing 0.8 ng/ml GM-CSF (experimentally determined, see protocol) to all wells except Column 23 (unstimulated control) to stimulate. Add 4 µl Assay Buffer to Column 23 to make up for the GM-CSF added to other wells. Now add 4 µl inhibitor from secondary dilutions above to wells in Columns 1-20 in quadruplicate (i.e. 4 µl of the 10 µM staurosporine into Column 1 Rows A through D and 4 µl of 5 µM staurosporine

from the next well to Column 2 Rows A through D and so on to set up an actual titration from 1 µM final starting concentration of staurosporine).

8. Add 4 µl Assay Buffer to Columns 21 and 22, as well as Columns 23 and 24, Rows A-L to bring all wells to a final volume of 40 ul.

9. Place TF-1 plate in incubator for 5 hours.

Substrate Loading

10. Load cells as follows(note in both cases it is critical that cells are allowed to load at room temperature.):

- For Irf1-*bla* TF-1 cells, 2.5 ml of 6X loading solution prepared by mixing 15 µl CCF4-AM with 150 µl Solution B, vortexing, then adding 2.335 ml of Solution C. Loading Solution mixed, and 8 µl per well added to plate. Plate incubated at ROOM TEMP. for 2.5 hours.
- For Irf1-*bla* HEL cells, 2.5 ml of 6X loading solution prepared by mixing 15 µl CCF4-AM with 150 µl Solution B, vortexing, and then adding 150 µl Solution D and 2.185 ml Solution C. Loading Solution mixed, and 8 µl per well added to plate. Plate incubated at ROOM TEMP. for 4 hours.

11. Read and analyze as directed in protocol.

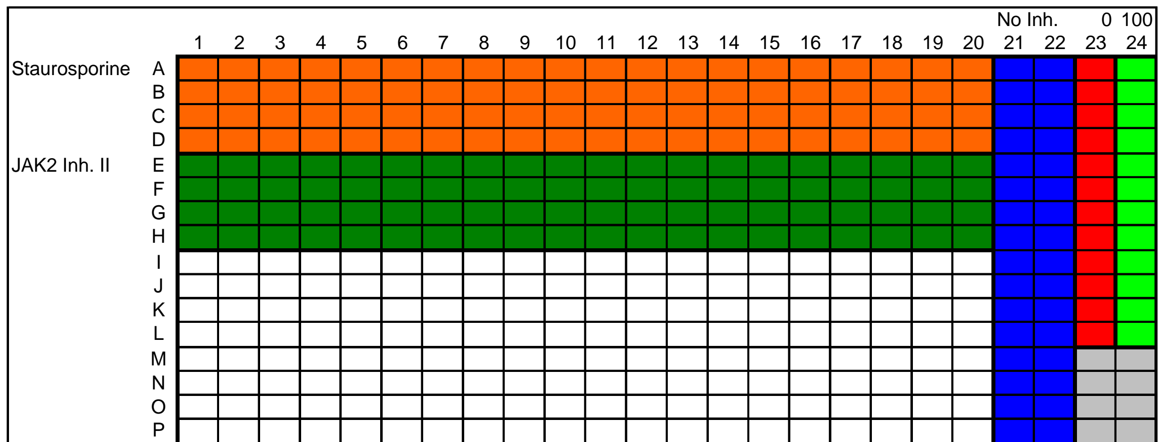

Figure 2: Assay Plate Schematic. Compound titrations shown in Columns 1-20, Columns 21 and 22 prepared without any inhibitor as 0% inhibition controls, Column 23 prepared with no CM-CSF (unstimulated 0% activity control) for the TF-1 line, but the HEL line is constitutively active so Column 23 is untreated for HEL cells, and Column 24 is also untreated in both cases as a 100% activity control. Note 8 wells in gray in bottom right, which were prepared with out any inhibitor or cells, as controls for background subtraction.

D. Results

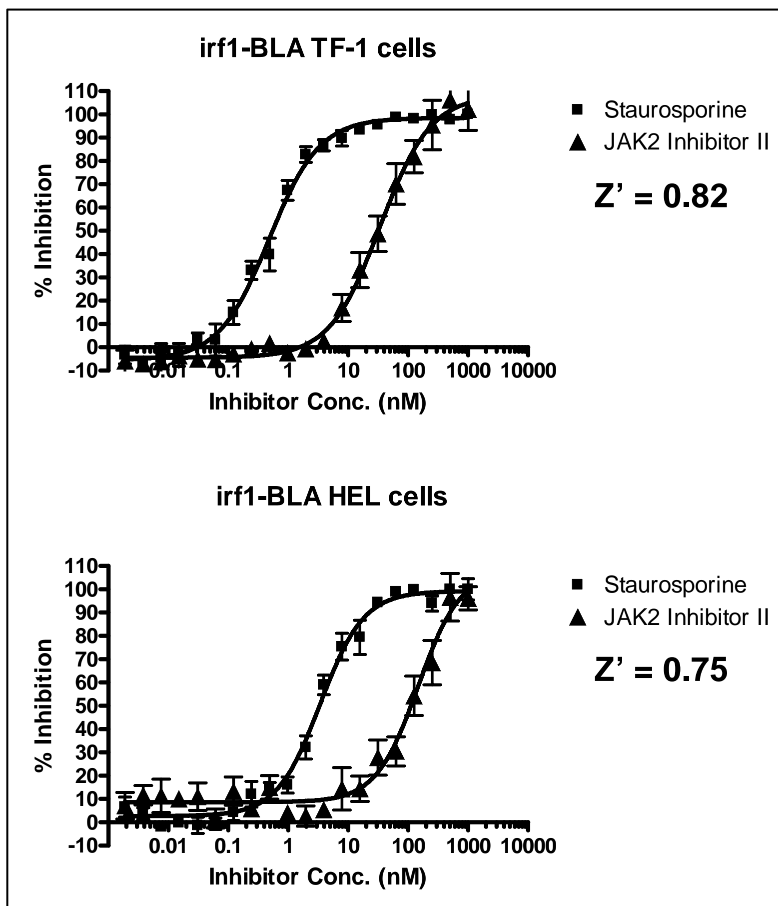

Figure 1: GeneBLAzer® Assay. GeneBLAzer® assay performed using the BMG LABTECH OPTIMA.