GeneBLAzer® Assay Setup Guide on the Tecan Safire²™ Microplate Reader

NOTE: The Tecan Safire²™ Microplate Reader was tested for compatibility with Invitrogen's GeneBLAzer® Assay in bottom-read mode using the CellSensor® 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. 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 Tecan instruments or software, please contact Tecan at 1-888-798-0538 or info@tecan.com.

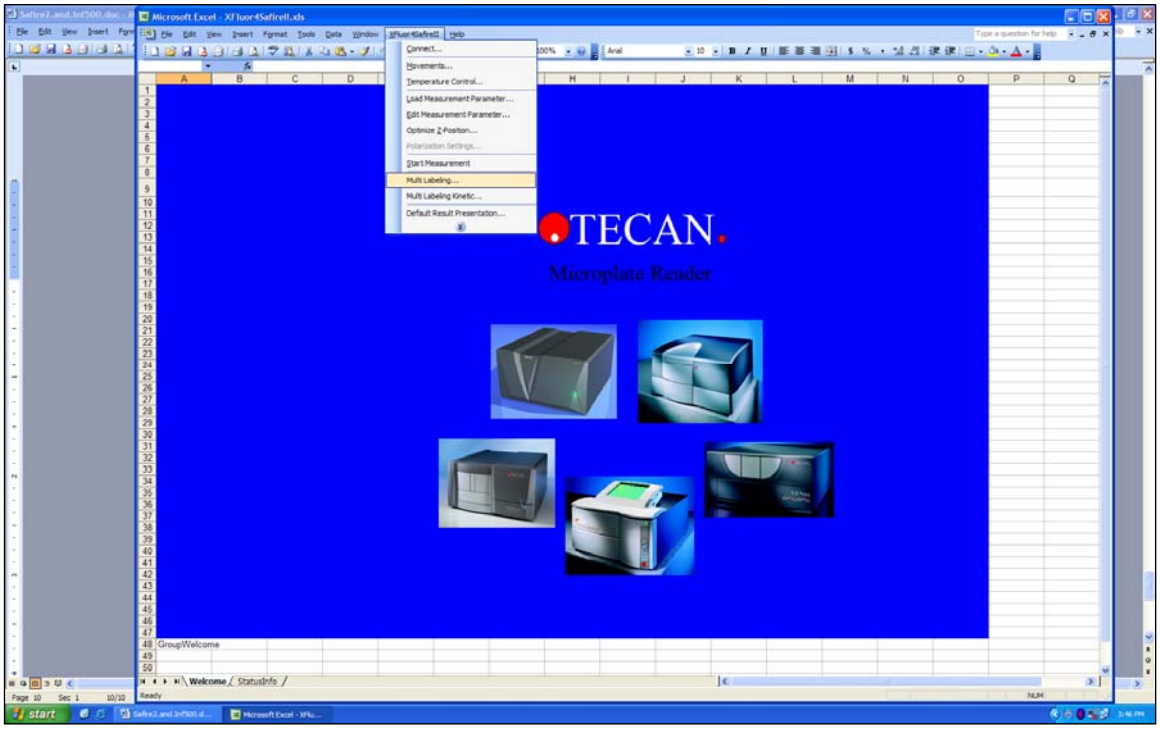
A. Recommended Optics

	wavelength (nm)	diameter (mm)
Excitation	409/12	monochromator
Emission 1	460/12	monochromator
Emission 2	530/12	monochromator

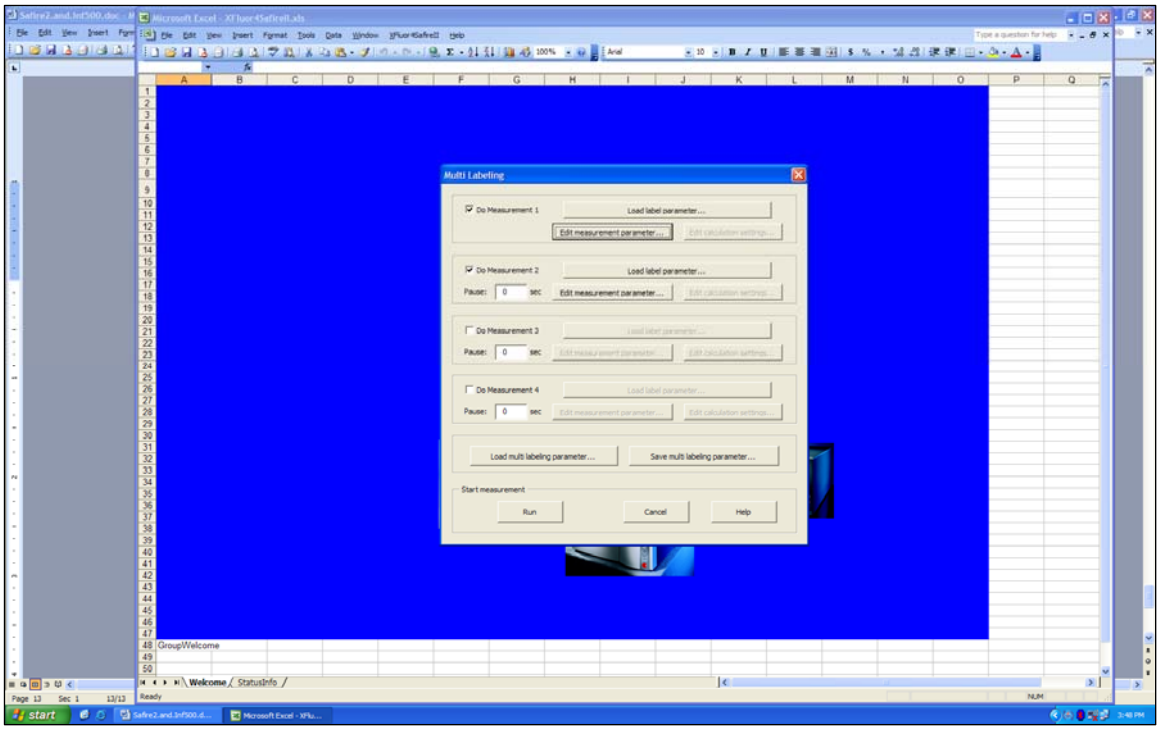
B. Instrument Setup

1. Make certain plate reader is turned on, and open up XFluor Data Manager software on computer.

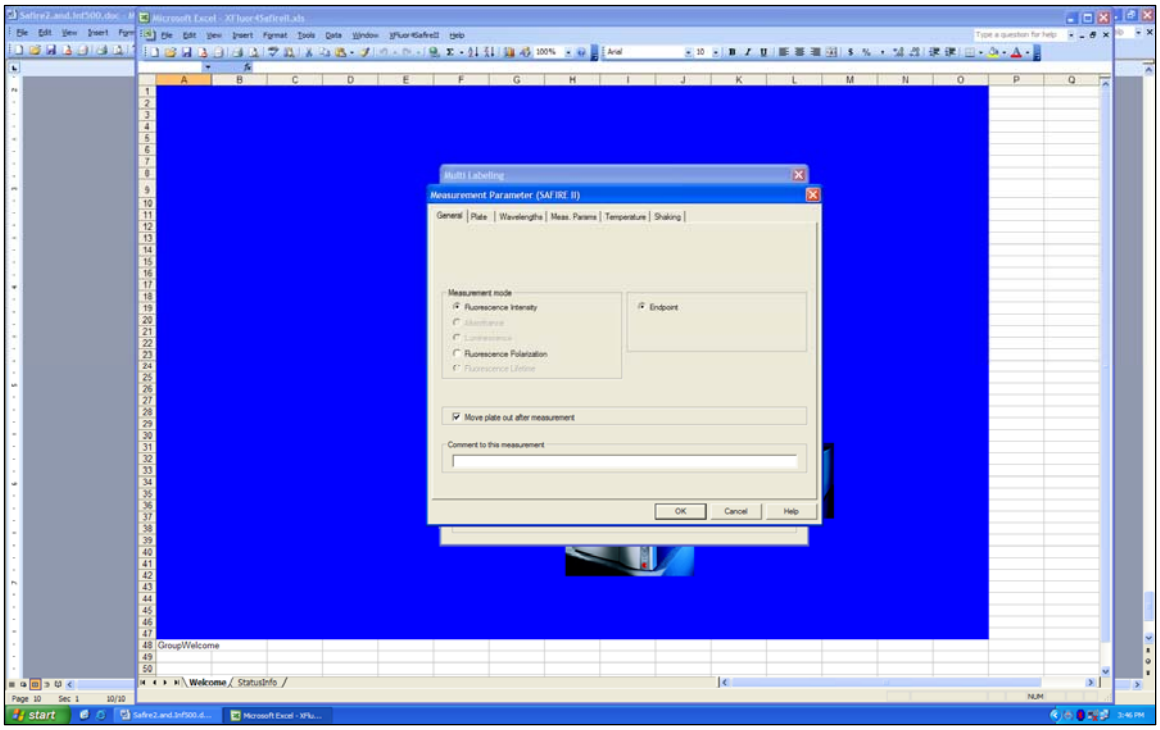
2. When XFluor opens, select "Multi-Labeling" from drop-down menu bar.



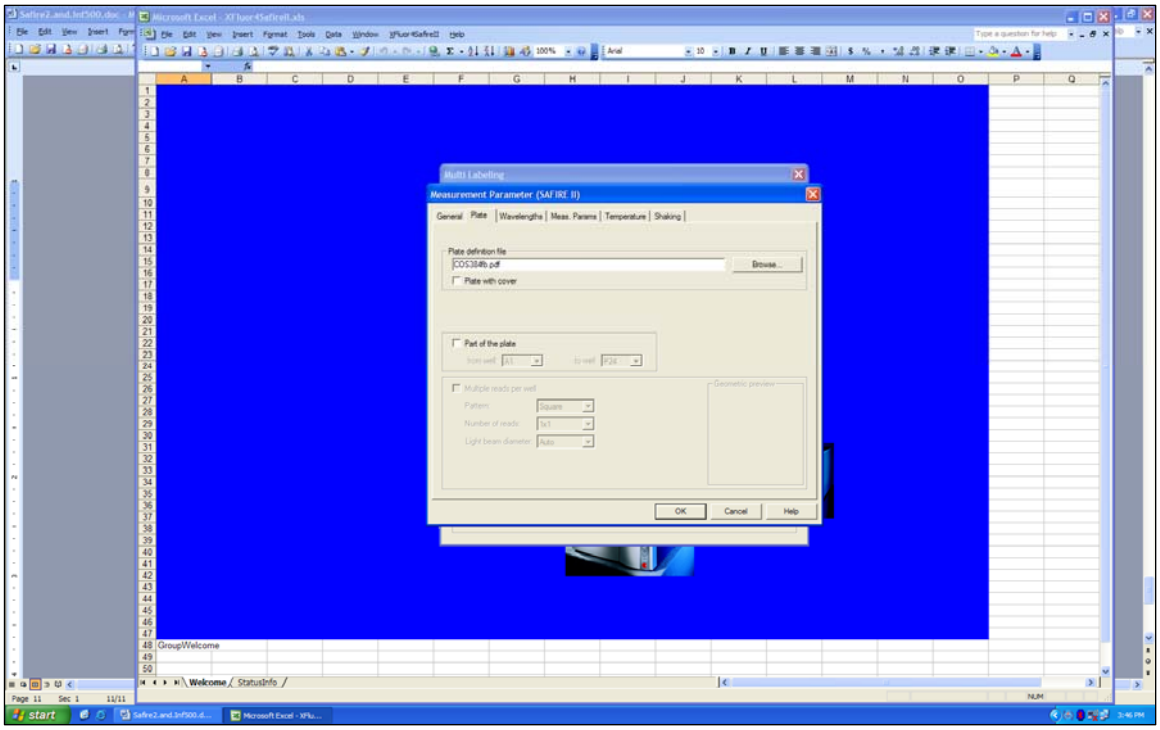
3. A Multi Labeling window will appear. Check the "Do Measurement 1" box, and then click on the "Edit Measurement Parameter" tab.



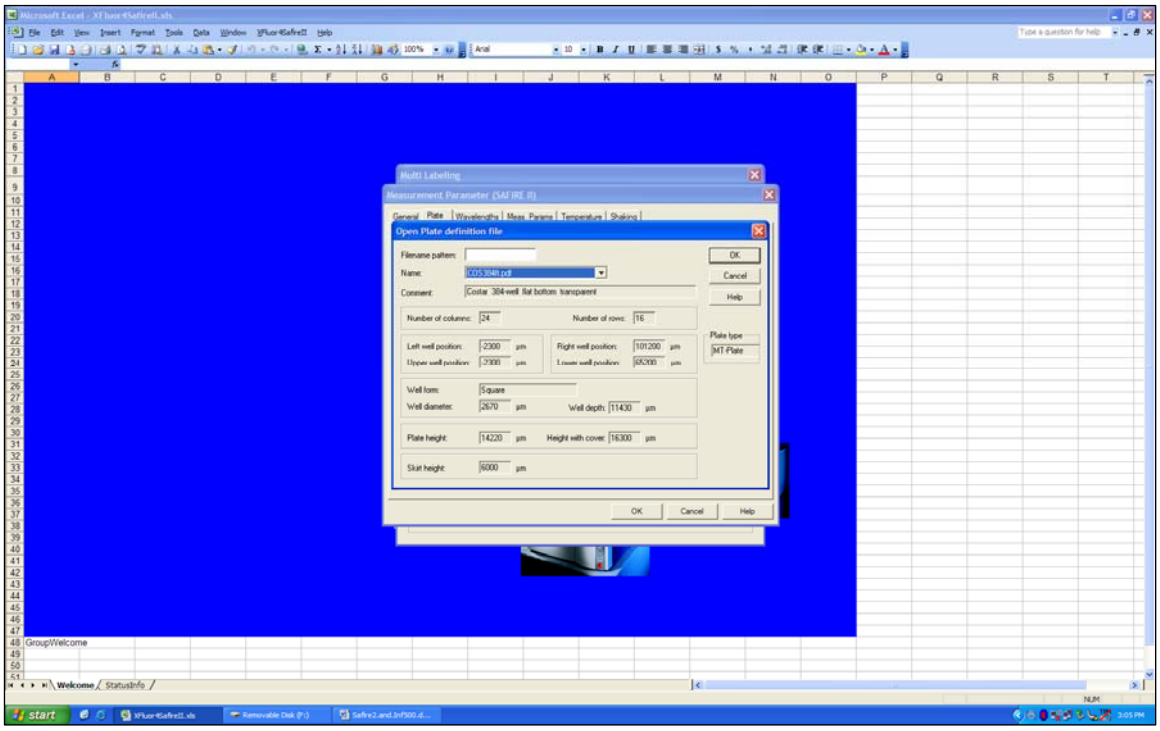
4. A popup Measurement Parameter window will appear. Under the “General” tab select the “Fluorescence Intensity” and “Endpoint” buttons.



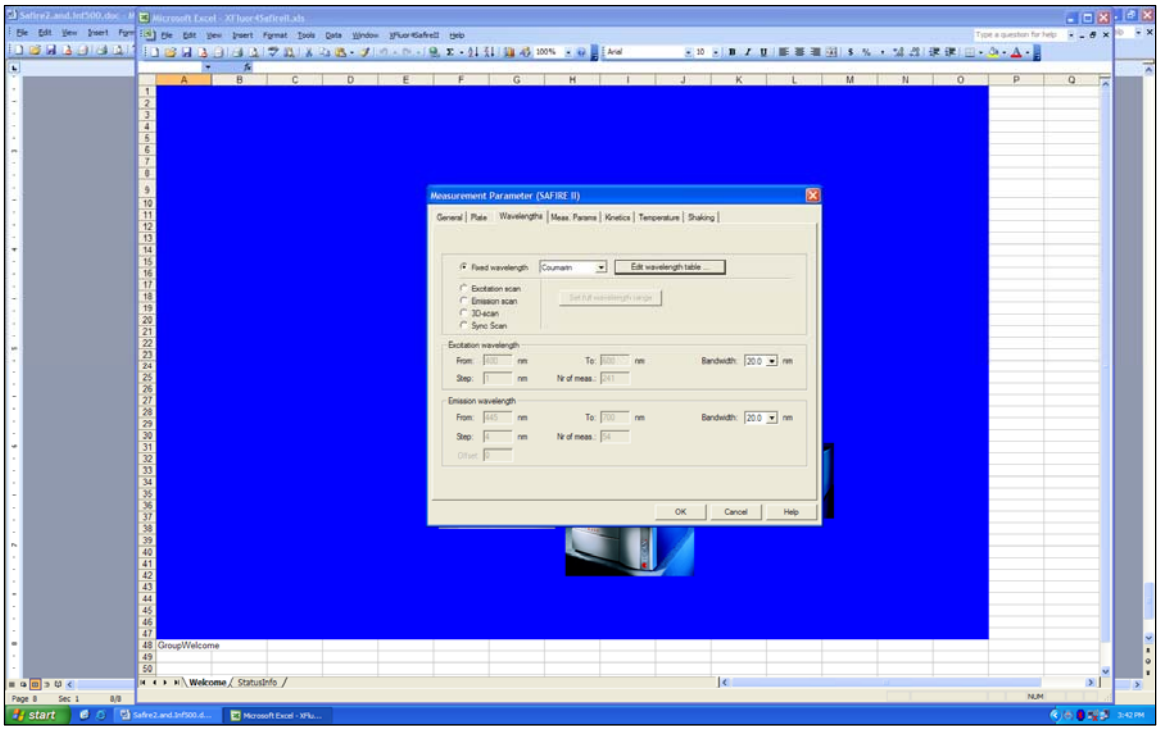
5. Now select the “Plate” tab at the top of the window. When the new tab opens, click on the "Browse" tab. Note this is also where to select which wells to read if only reading part of the plate.



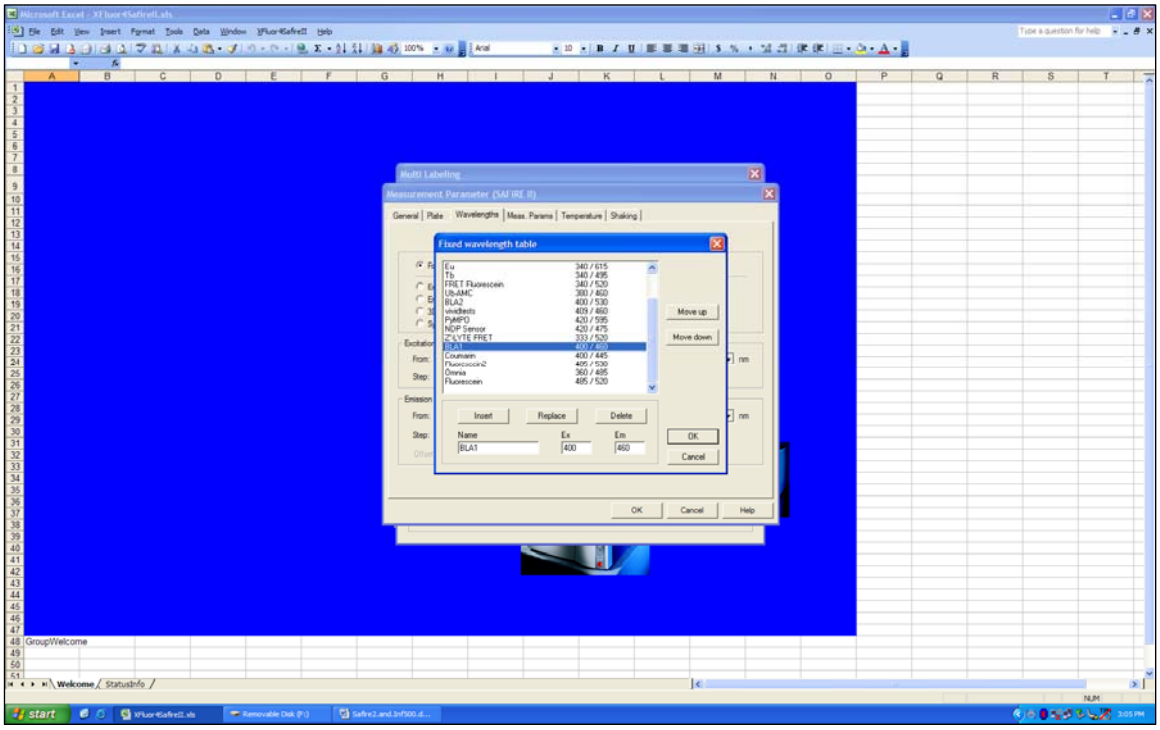
6. When the new plate window appears, select your plate as shown below. Click OK when finished to return to the main protocol setup window.



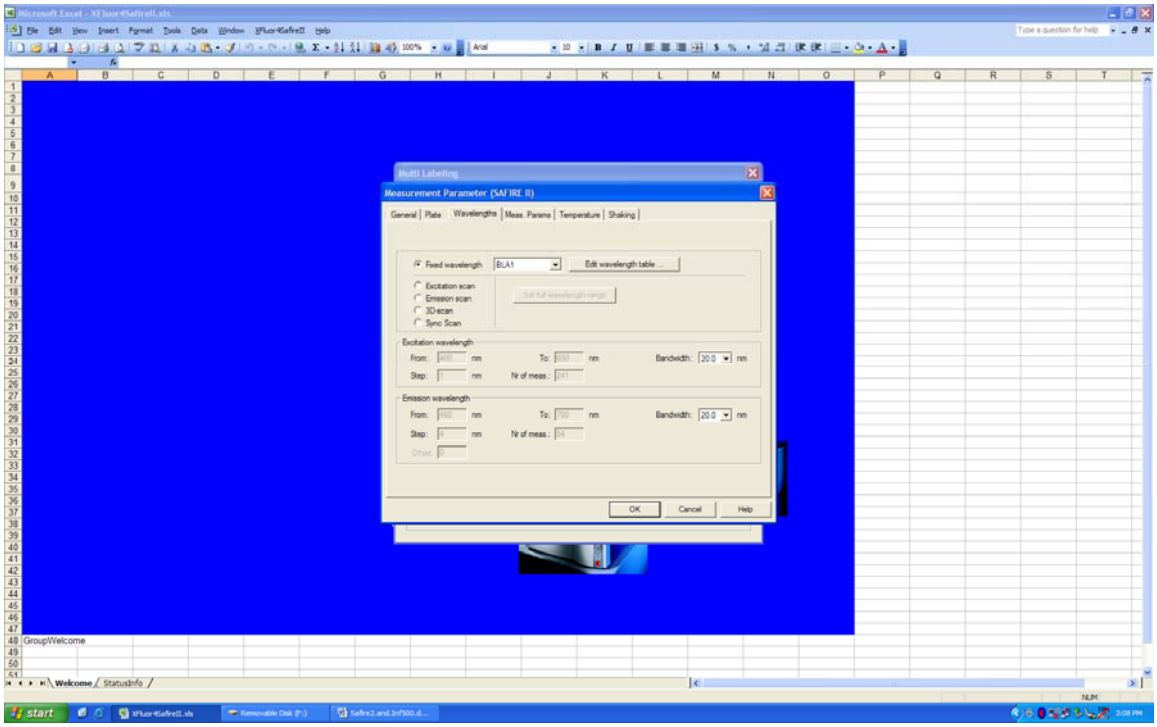
7. Select the "Wavelengths" tab from the top. Next, click the "Edit Wavelength Table" button.



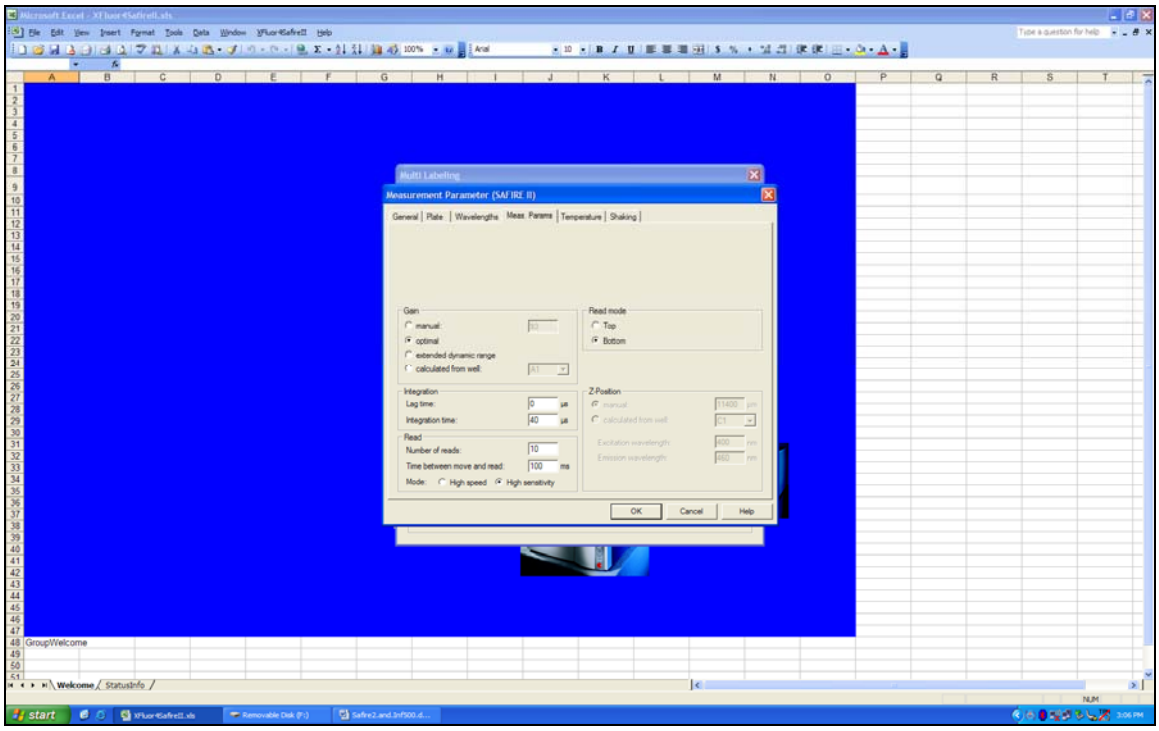
8. A new popup will appear. If you do not have suitable settings already loaded, enter your excitation and donor emission wavelengths in the blanks below "Name", "Ex", and "Em", respectively. When you have entered the desired settings, select "Insert" to enter then into the table of defined settings as shown, and then select OK when finished.



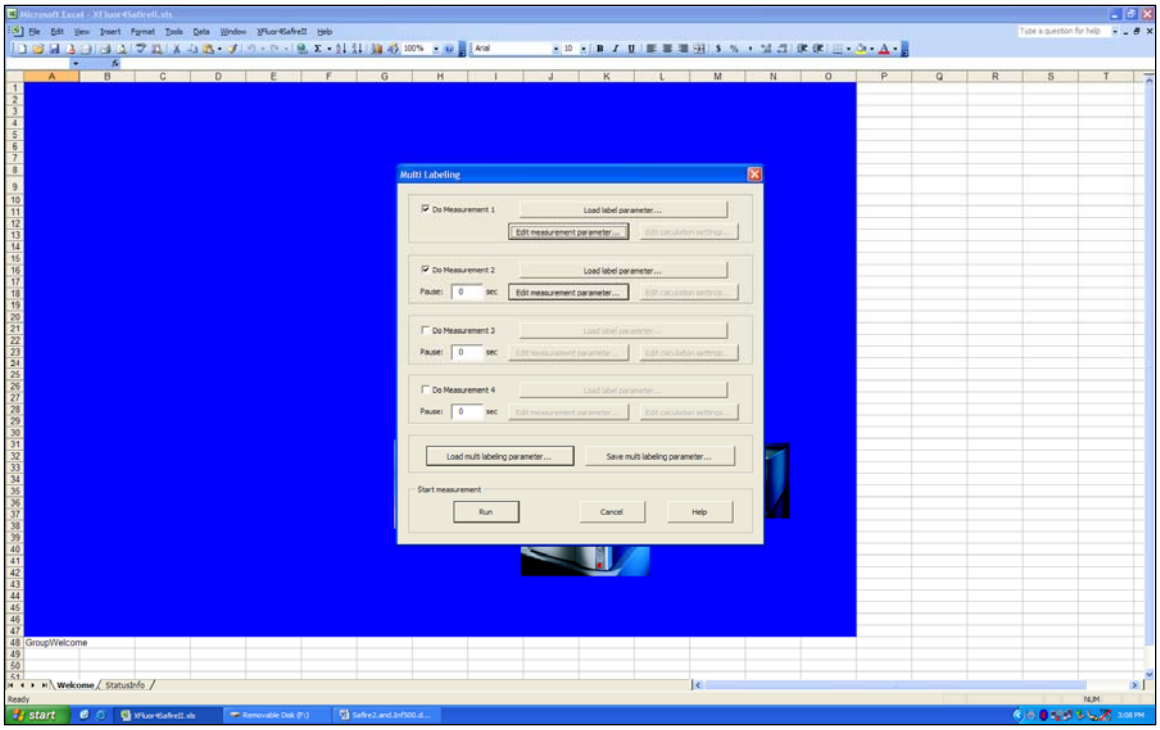
9. When returned to the main protocol window, set your excitation and emission bandwidths as shown below, then select the "Meas. Params" tab.



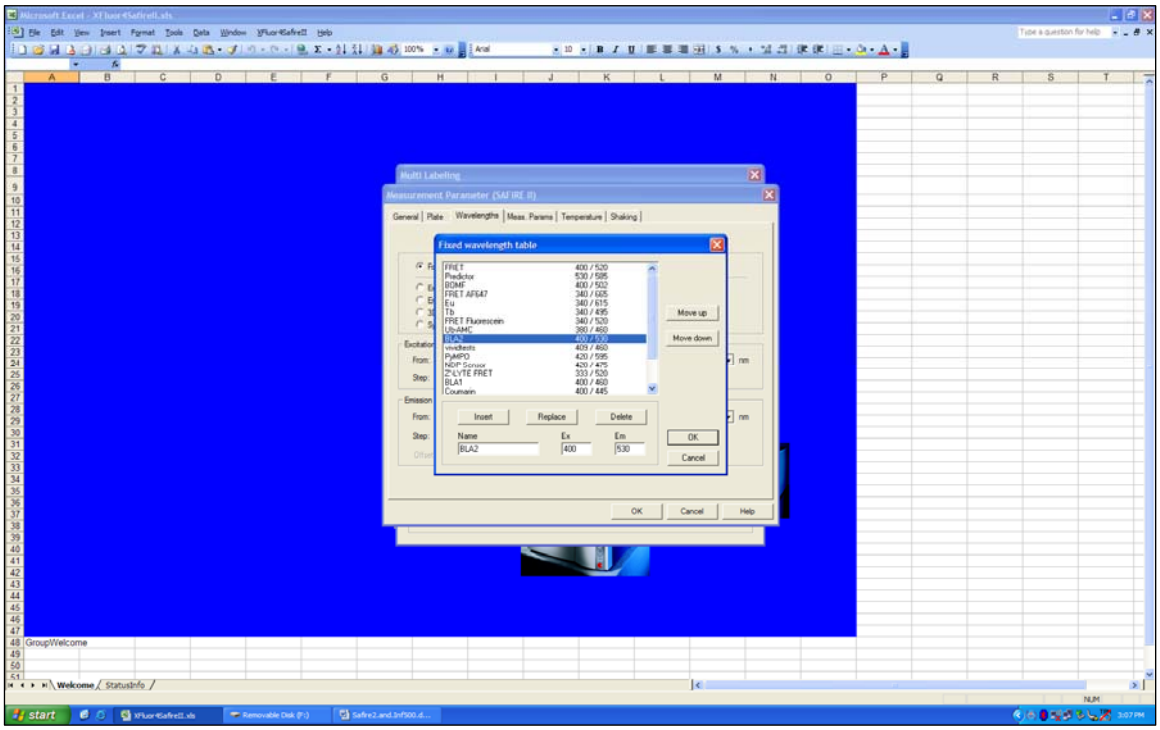
10. Set the Gain for “Optimal”, 10 reads, and set the Read Mode to "Bottom". When finished, select OK.



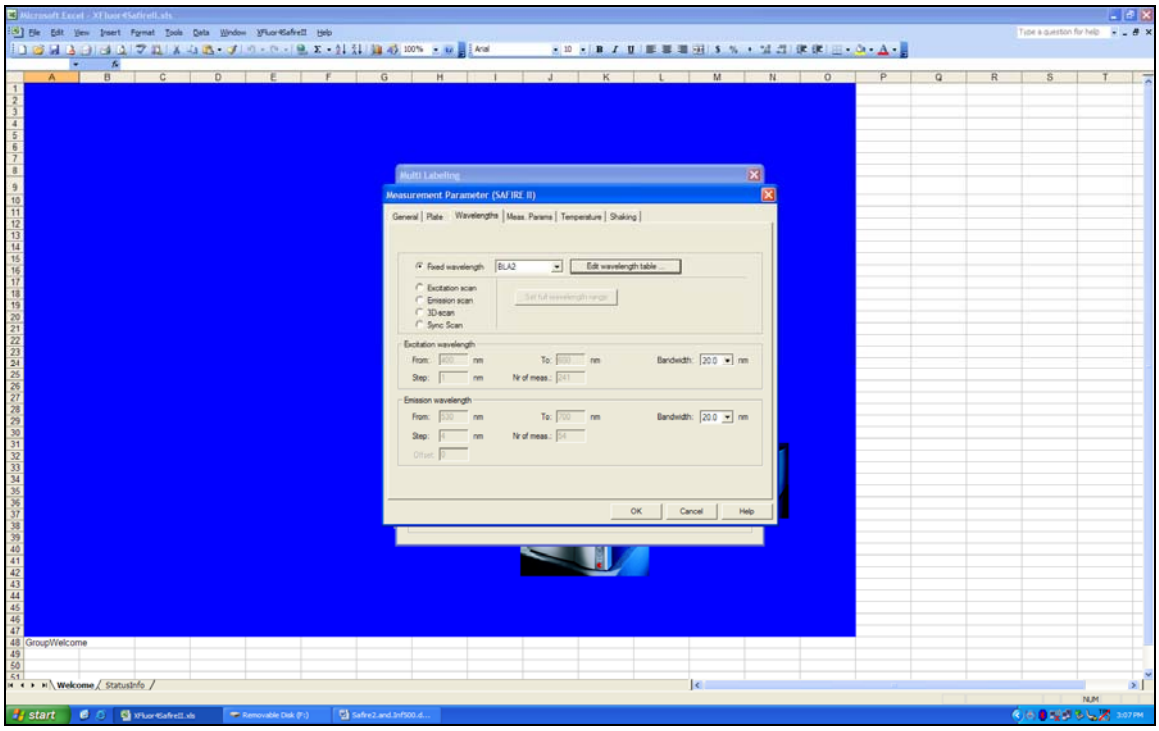
11. You will be returned to the Multi-Labeling window. Check the "Do Measurement 2" box and click the "Edit Measurement Parameter" tab to enter your excitation and acceptor settings.



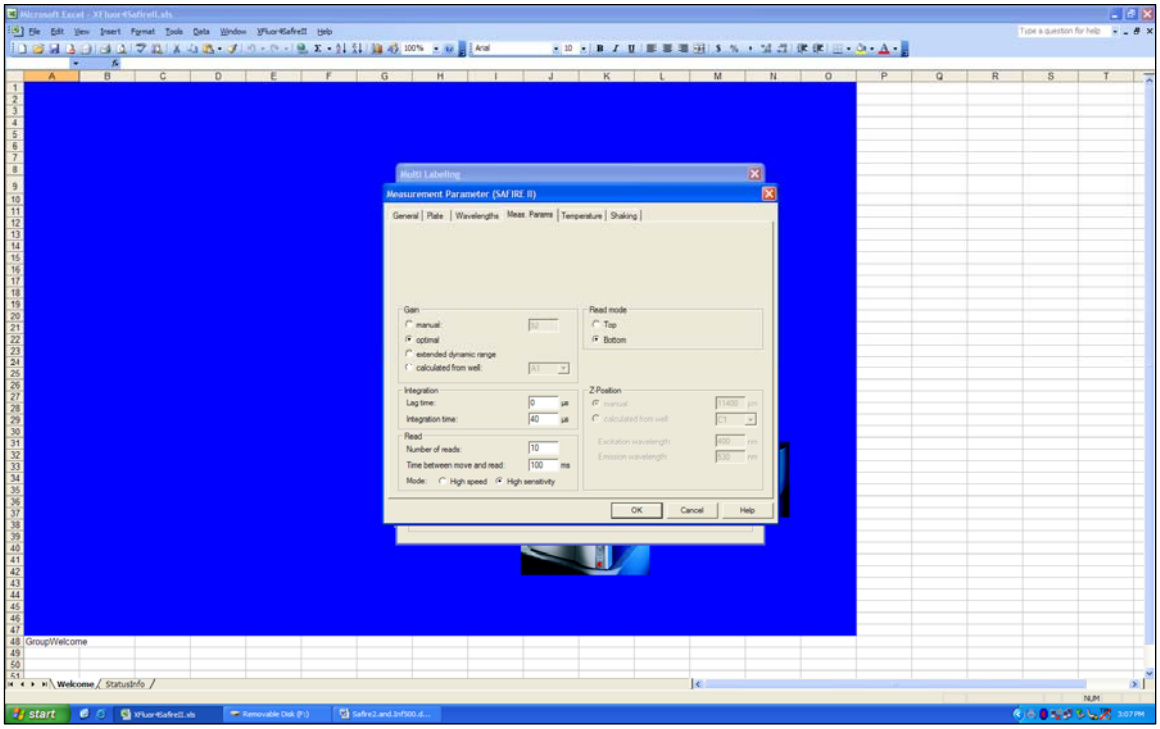
12. Both the "General" and "Plate" tabs will now be locked, as their settings were already determined by the first set of measurement parameters entered. Go straight to the "Wavelengths" tab, and select "Edit Wavelength Table" as before. In the popup, enter the values for your excitation and your ac ceptor emission as shown below, and again select "Insert" to enter them into your tables, then select OK.



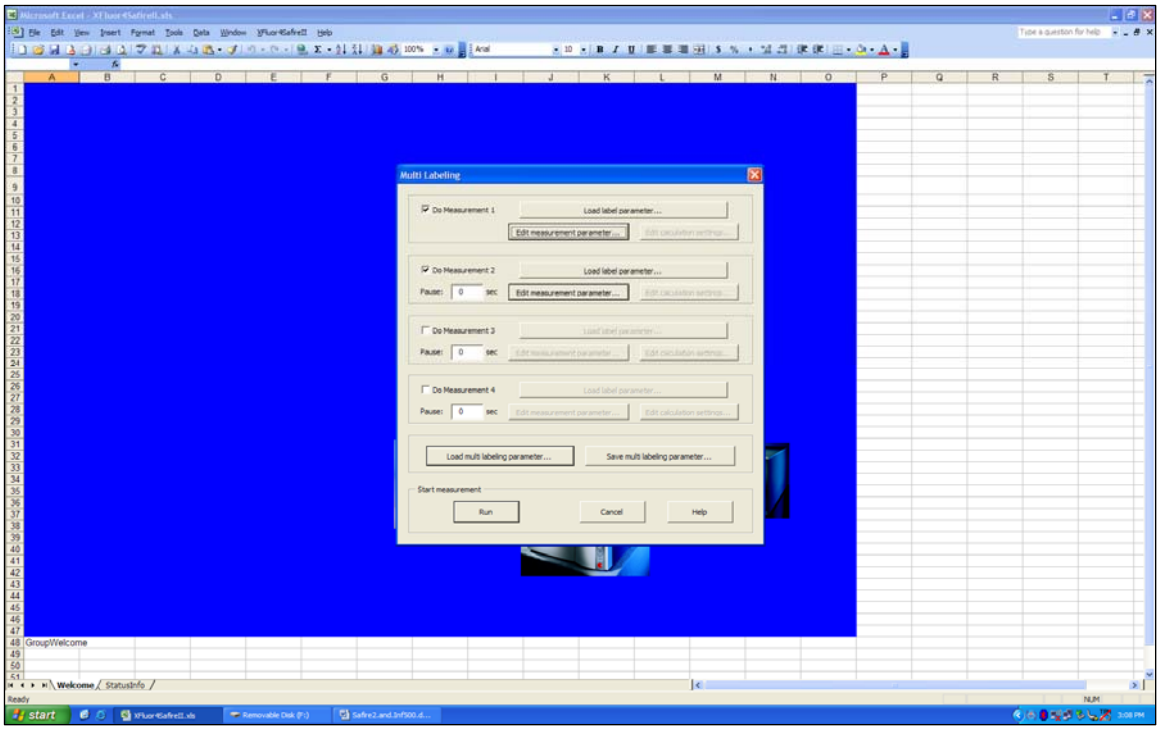
13. You will return to the main protocol page again. Enter the appropriate instrument bandwidths, then select the "Meas. Params" tab.



14. Make sure the Gain is set to "Optimal" and the Read Mode is set to "Bottom".



15. At this point you will default all the way back to the Multi-Labeling window. If you have not already, insert your plate, and select the "Run" tab to read.



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 see the appropriate line-specific protocols. Protocols for GeneBLAzer® assays can be located from the "GeneBLAzer® Portfolio" window of the following link: http://www.invitrogen.com/content.cfm?pageid=10523.

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.

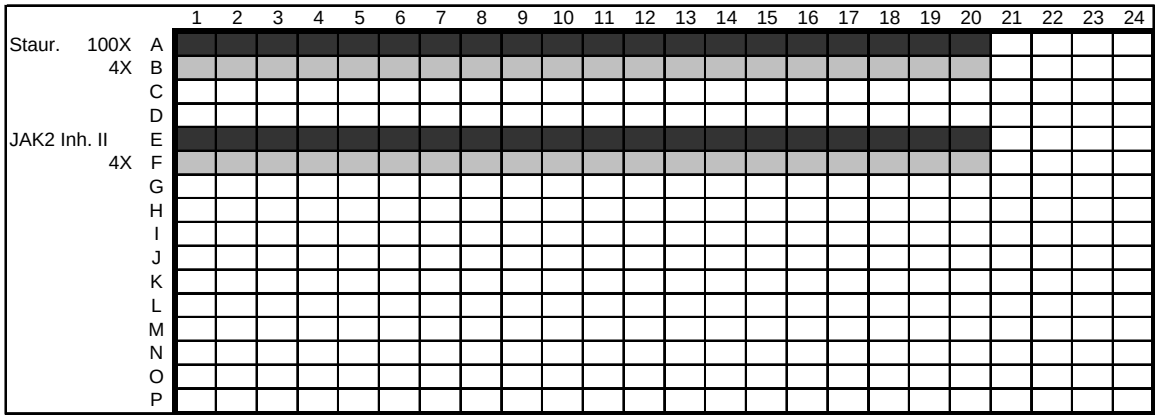


Figure 1: Schematic of initial compound dilution. Staurosporine and JAK2 Inhibitor II were titrated from a 100 µM starting concentration in the initial dilution series by preparing a 1:1 dilution curve in DMSO. A secondary dilution to 10X was then prepared in the rows below the initial dilution curve (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 1.5625×10^6 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.

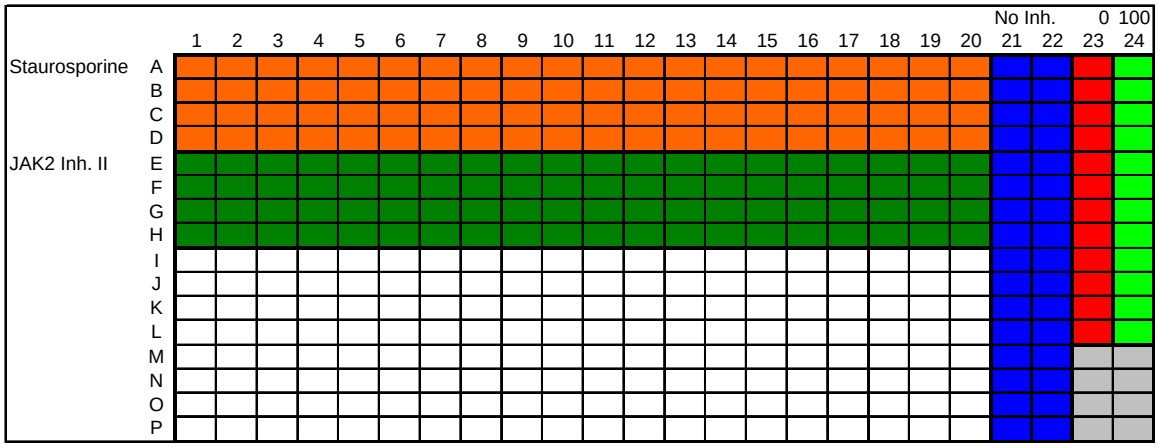


Figure 2: Assay Plate Schematic. Compound titrations shown in Columns 1-20, Columns 21 and 22 prepared without any inhibitor as 0% inhibition controls, Column 23 prepared with no CM-CSF (unstimulated 0% activity control) for the TF-1 line, but the HEL line is constitutively active so Column 23 is untreated 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

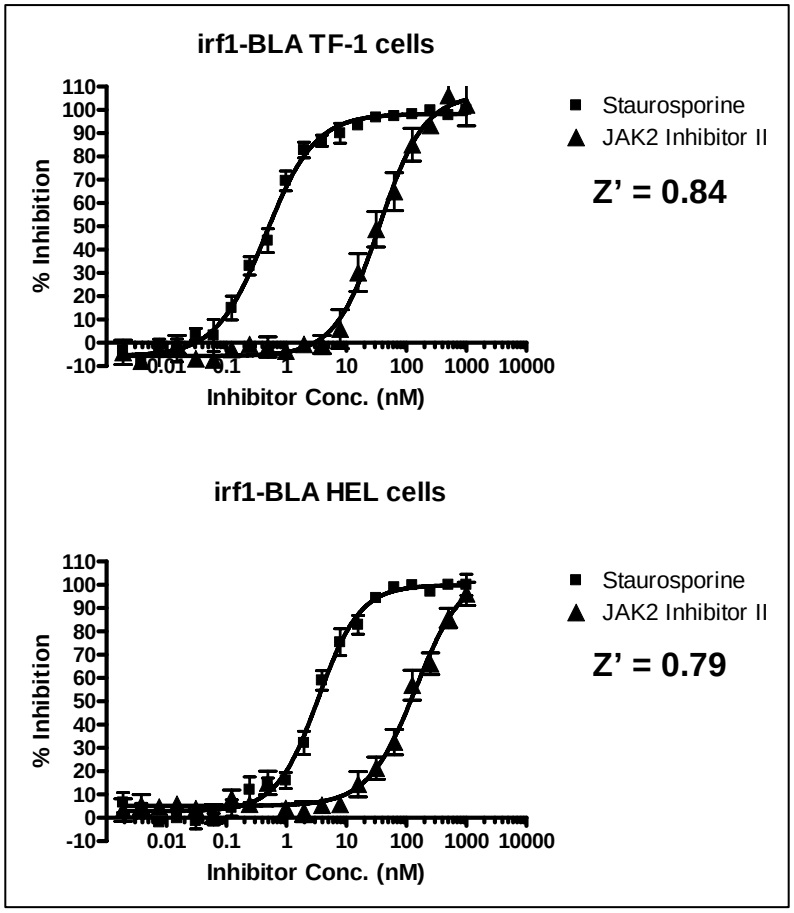


Figure 1: GeneBLAzer® Assay. GeneBLAzer® assay performed using the Tecan Safire²™.