

## GeneBLAzer® Assay Setup Guide on the Thermo Scientific Varioskan® Flash Multimode Reader

NOTE: The Thermo Scientific Varioskan® Flash Multimode Reader was tested for compatibility with Invitrogen's GeneBLAzer® Assay in bottom-read mode using two CellSensor® cell lines; the *irf1-b/a* HEL and *irf1-b/a* 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 Thermo Scientific instruments or software, please contact Thermo Scientific at 1-800-522-7763 or [www.thermo.com](http://www.thermo.com).

### A. Recommended Optics

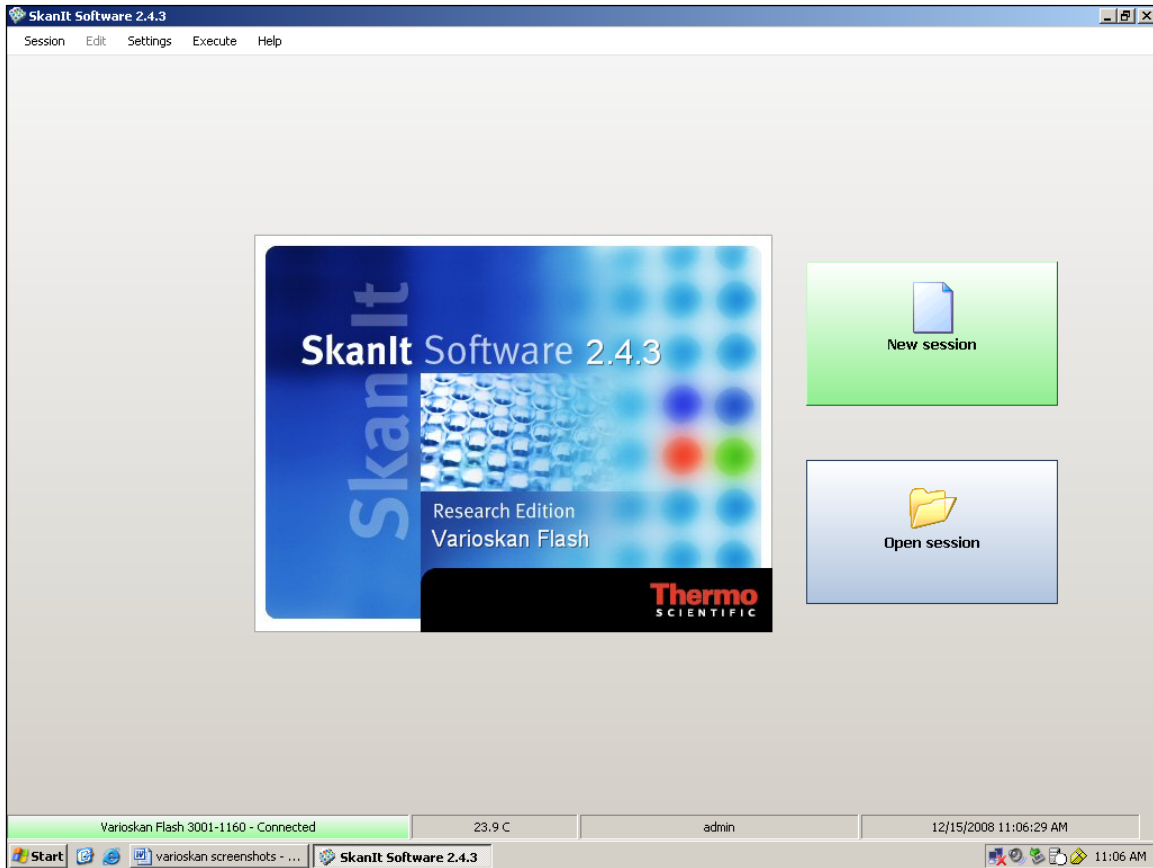
	wavelength (nm)	diameter (nm)
Excitation	409/12	monochromator
Emission 1	460/12	monochromator
Emission 2	530/12	monochromator

### B. Instrument Setup

1. Make certain plate reader is turned on, and open up Thermo Scientific SkanIt control software on computer.

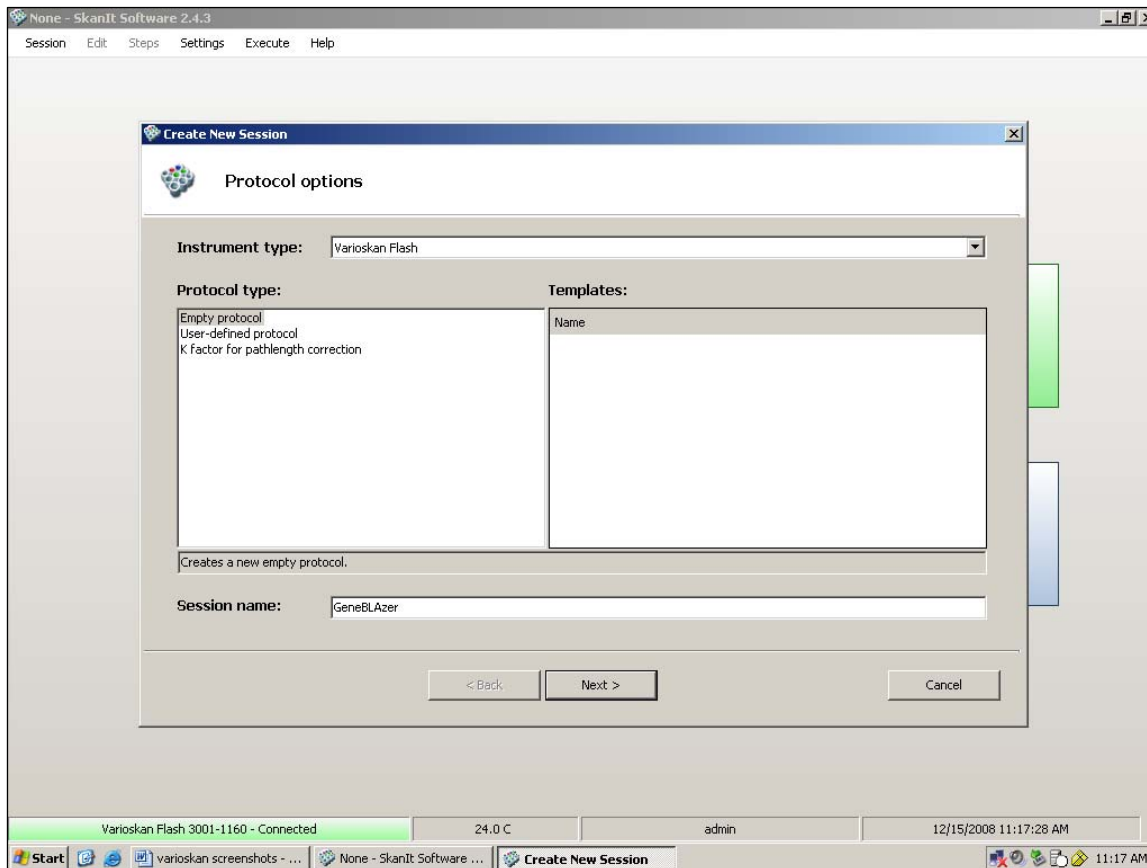
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2. When SkanIt opens, it will default to a generic starting page. Select the "New Session" icon to open a new session and create a new protocol.



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- At this point, a popup window will appear. Select "Empty Protocol" on the left, and enter a session name at the bottom. Select Next when finished.

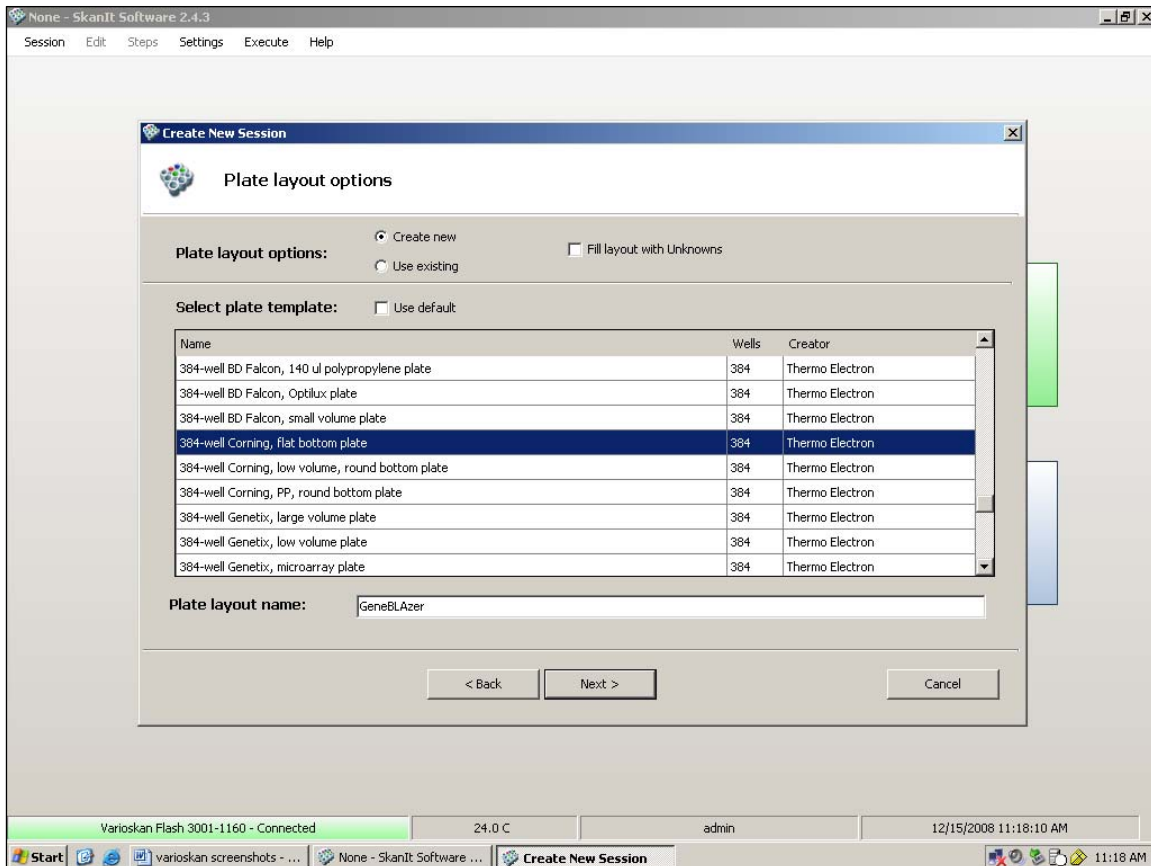


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4. Select your plate from the available list. (Note transparent Corning plates were not listed so standard flat-bottom plates were selected. Note also, because GeneBLAzer using LiveBLAzer substrates requires reading from the bottom of the plate, it is critical to have the proper carrier insert on the bottom of your Varioskan carrier tray. Inserts come in several sizes including 384- and 96-well, and using an improper insert will cause an error message. For more information on carrier inserts, call ThermoFisher.) When finished, select Next.

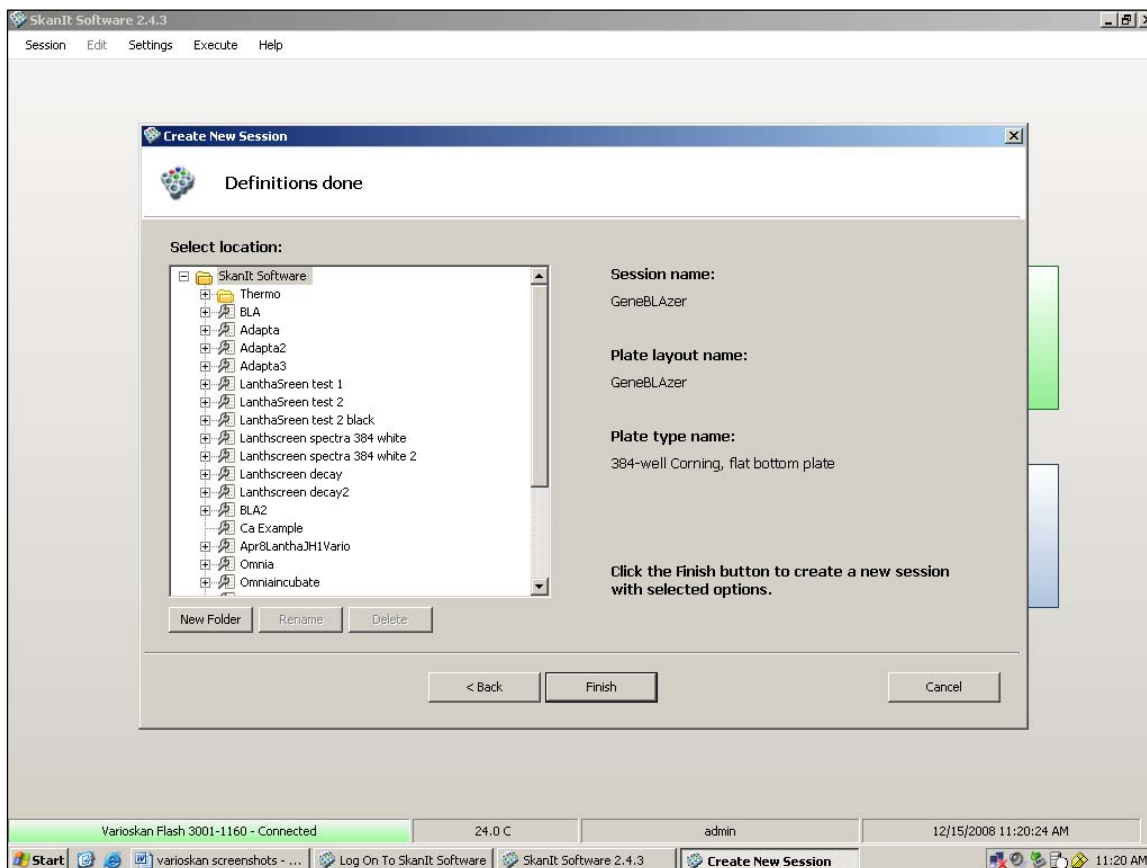


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- Once the session has been named and a plate selected, click the Finish tab to create a new protocol.

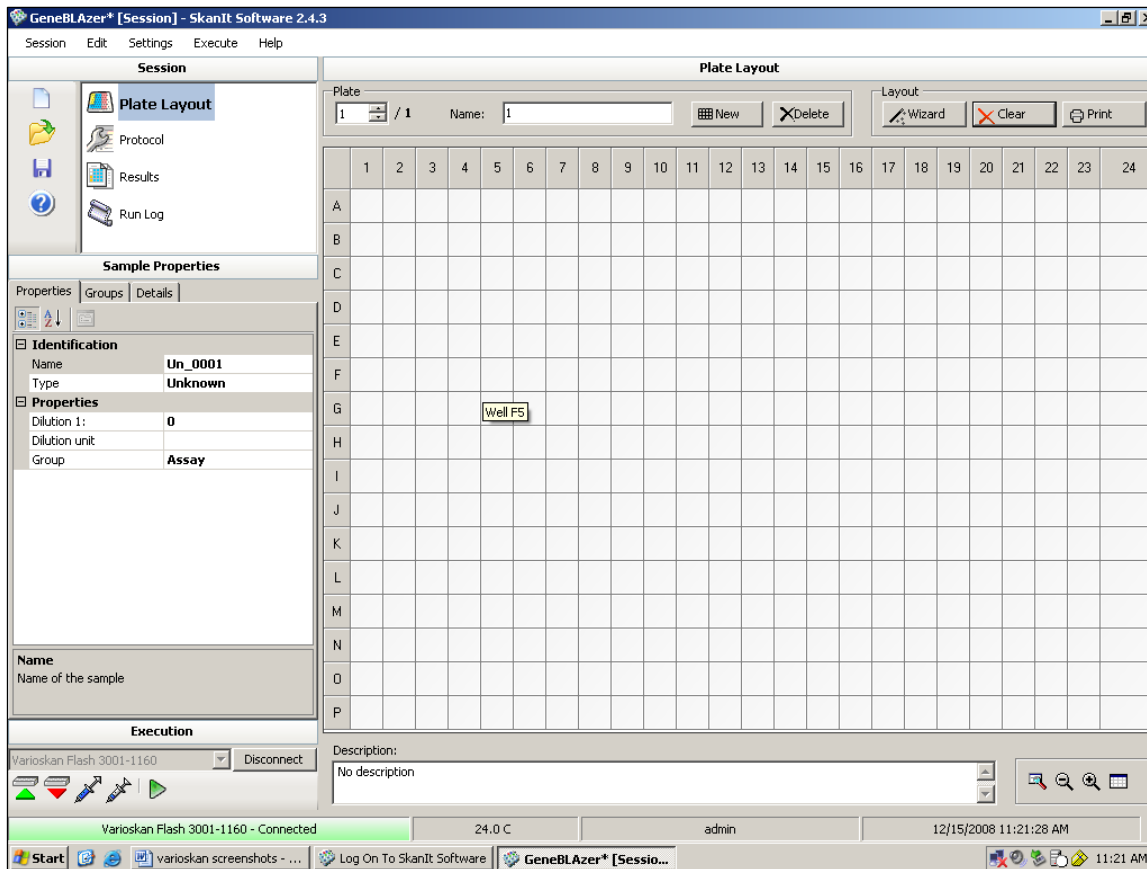


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- A new window will appear. The next step is to define the plate layout. Select the Wizard tab in the upper right.

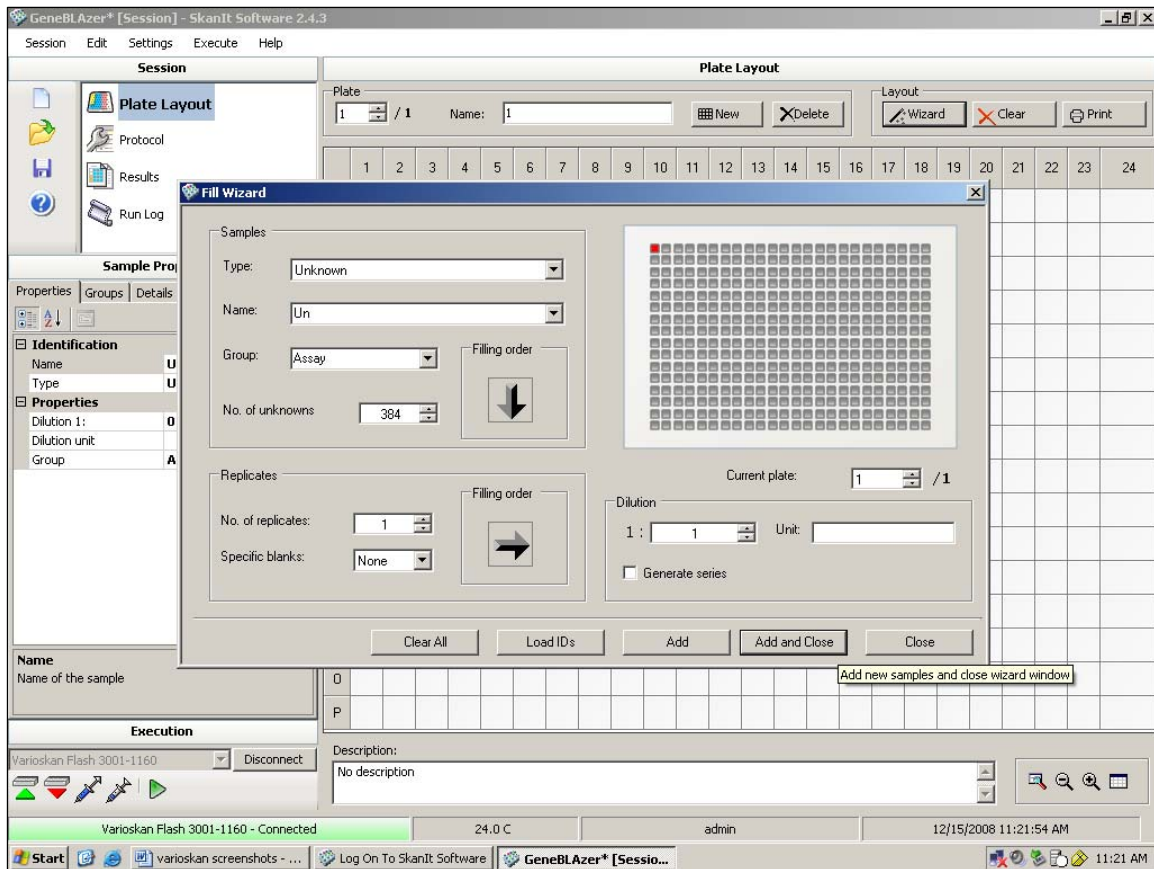


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- The Wizard popup will appear. To fill the entire plate with unknowns, enter 384 in the "No. of unknowns" blank near the center left of the popup. Select the "Add and Close" tab when finished.

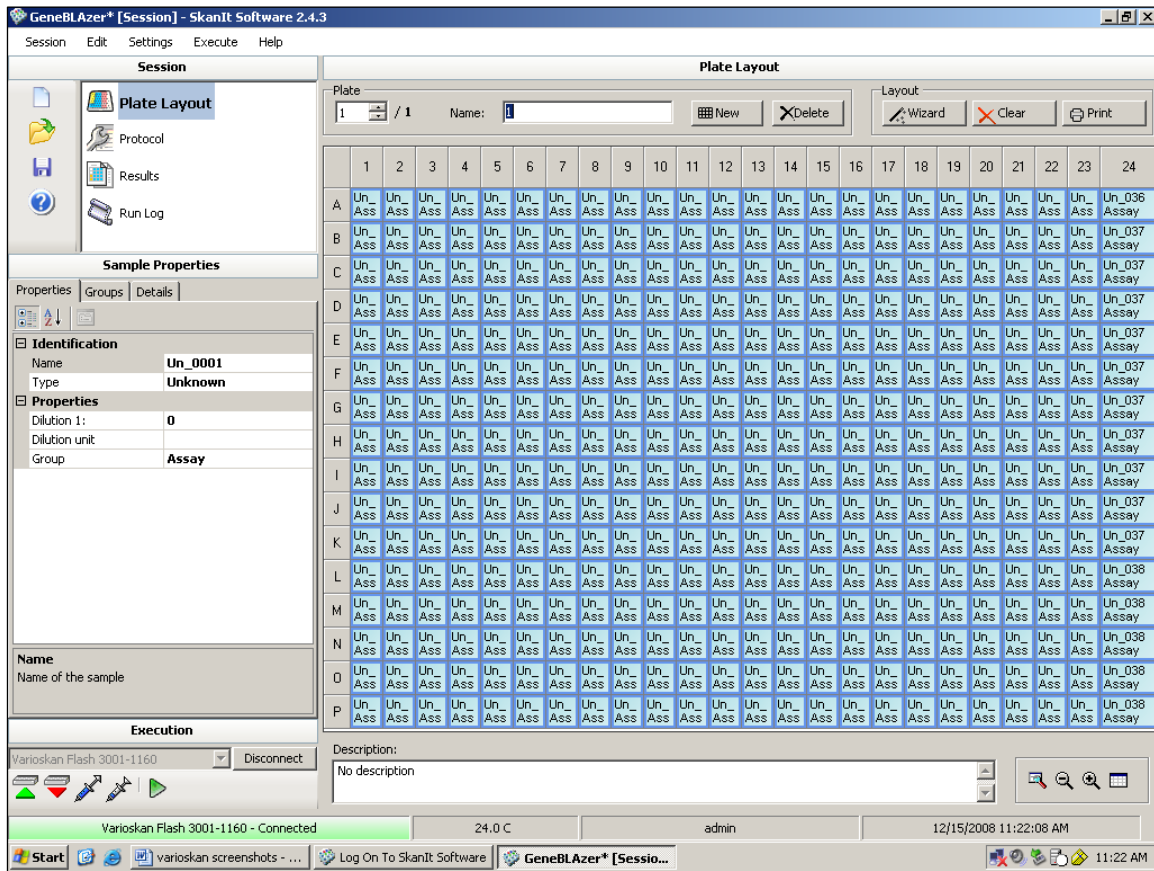


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8. The Wizard popup will close, and the plate will now be defined.



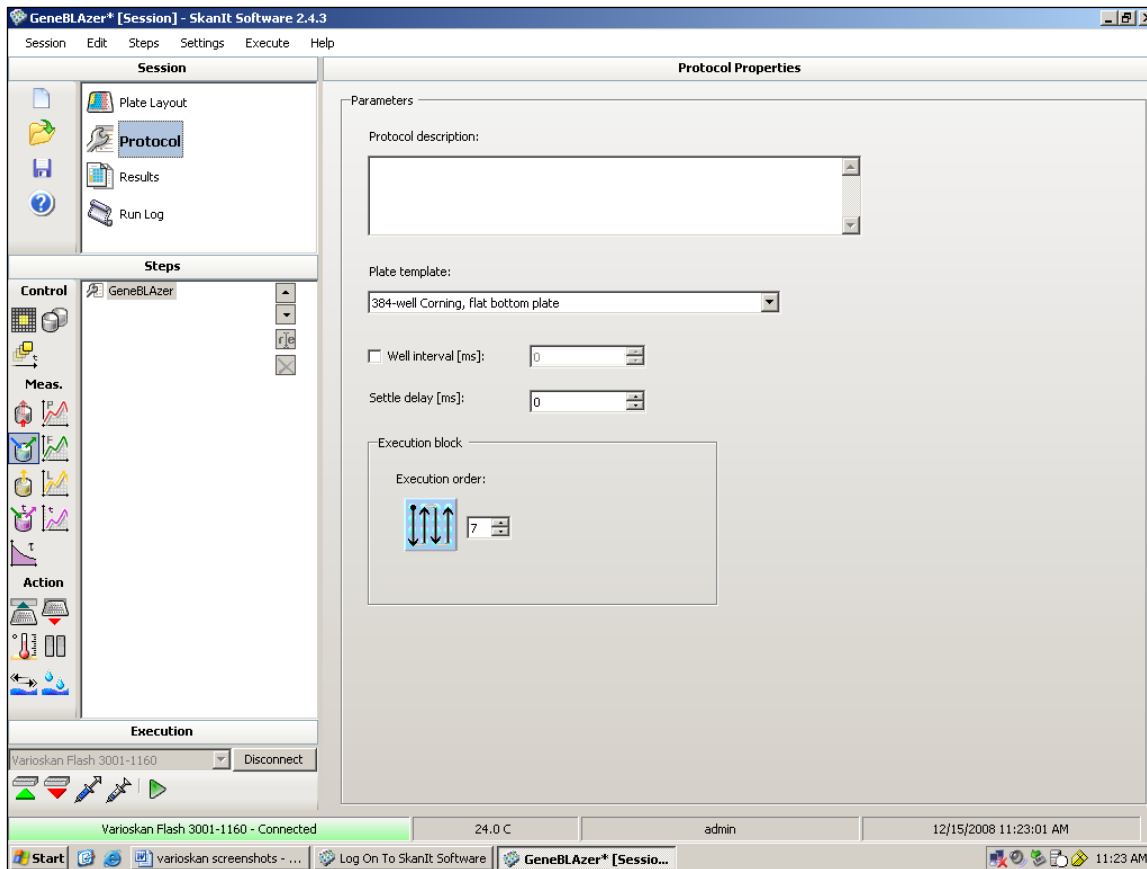
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- From the Session menu on the left, select "Protocol". The screen will change to the one below. In the "Meas." menu on the left, select the "FRET Measurement" icon to set up a FRET protocol.

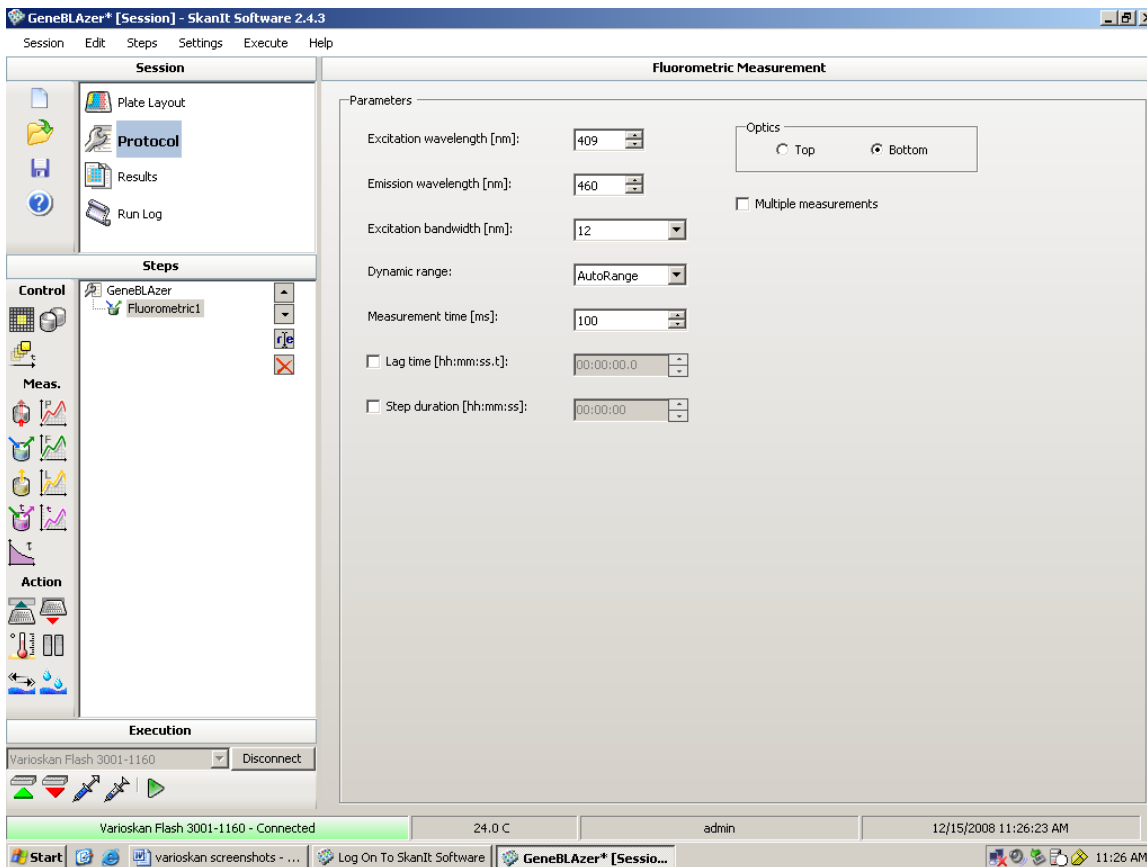


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10. Selecting the FRET icon will cause the screen to change to look like the one below. Select your excitation and donor emission wavelengths as shown below. Make sure the Optics setting is correct (GeneBLAzer® requires a bottom read).

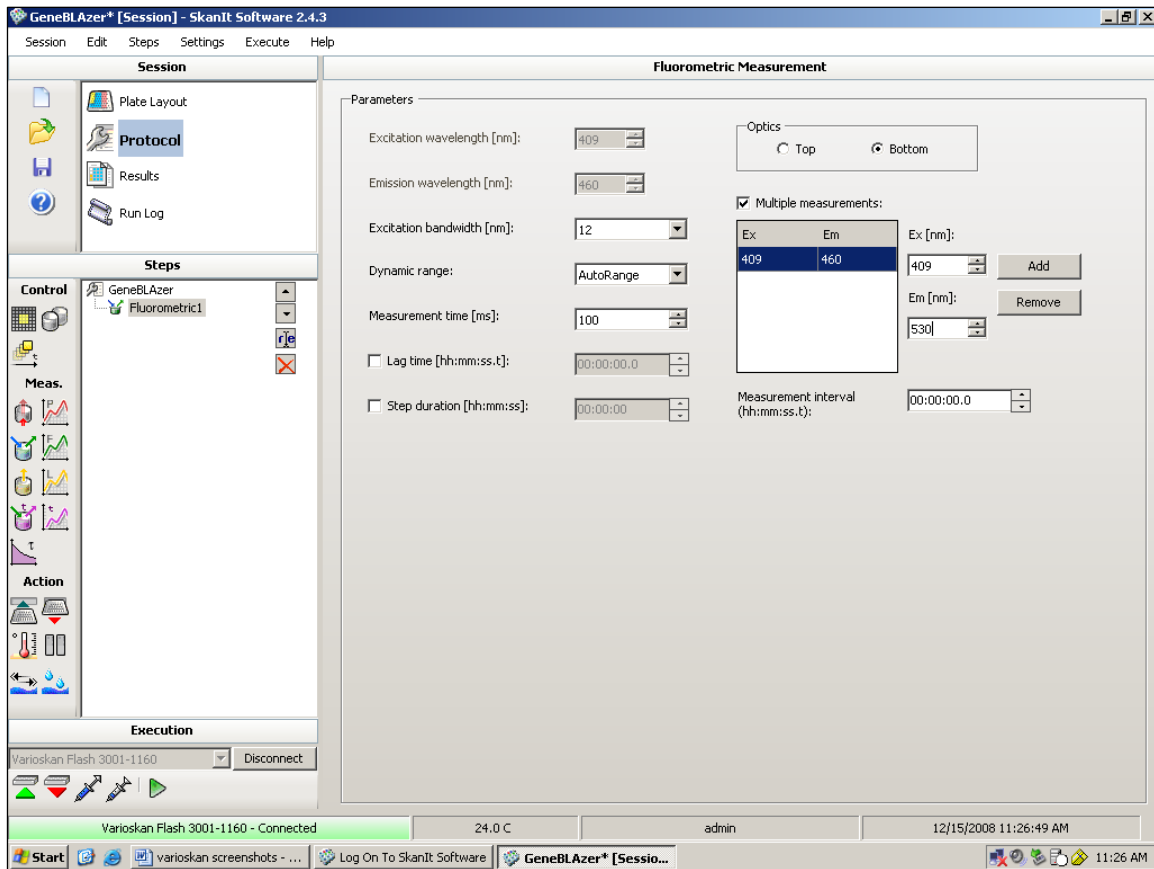


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- When finished, click on the "Multiple measurements" tab. A new set of excitation and emission windows will appear to the right; enter your excitation and acceptor wavelengths. When finished, select the "Add" tab.

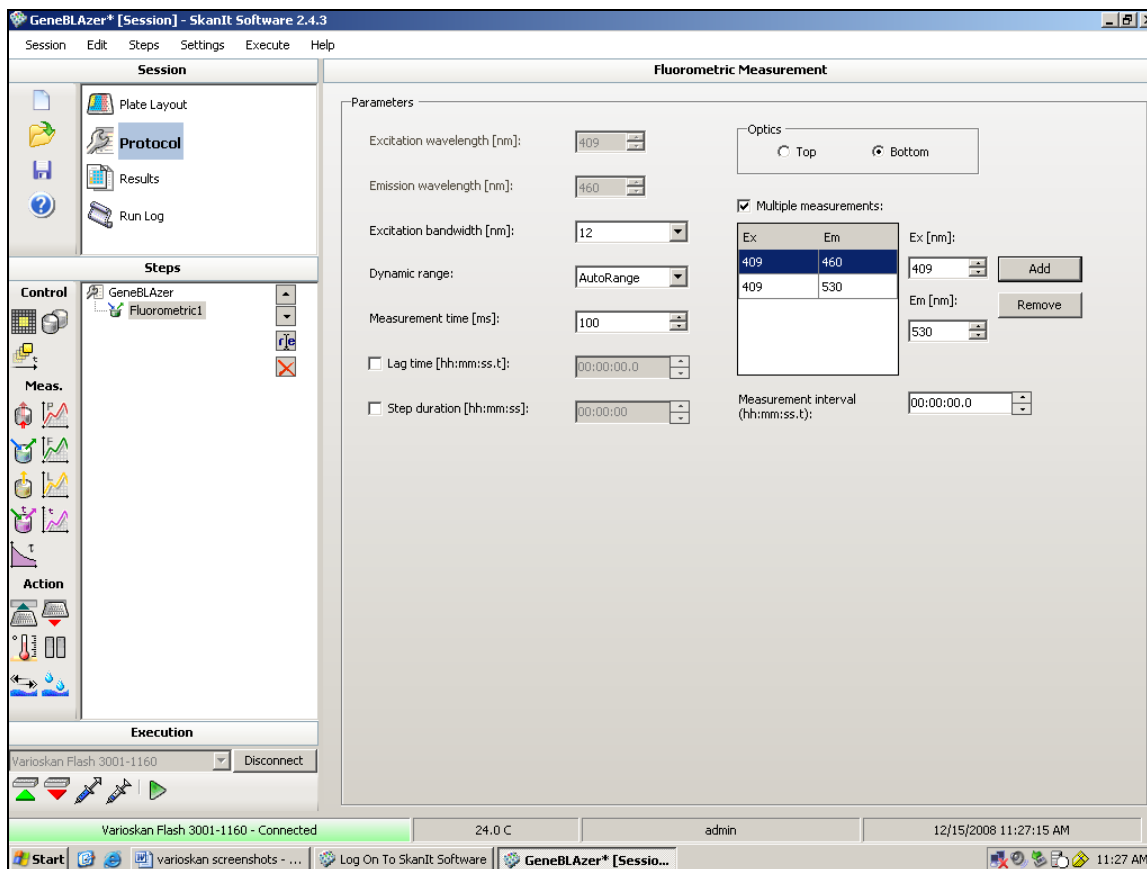


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12. Selecting the "Add" tab will now add the acceptor measurement settings into the excitation/emission chart to the left of the tab. At this point the protocol is finished, select the "Execute session" icon (small green arrow on the bottom left side) to read a plate.



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### 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  $5 \times 10^5$  cells/ml. Place cells in incubator for 16 hours.
  - In the meantime, for *Irf1-bla* HEL cells, harvest and resuspend in Assay Medium at  $9.5 \times 10^5$  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.

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		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																									
JAK2 Inh. II	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  $1.5625 \times 10^6$  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

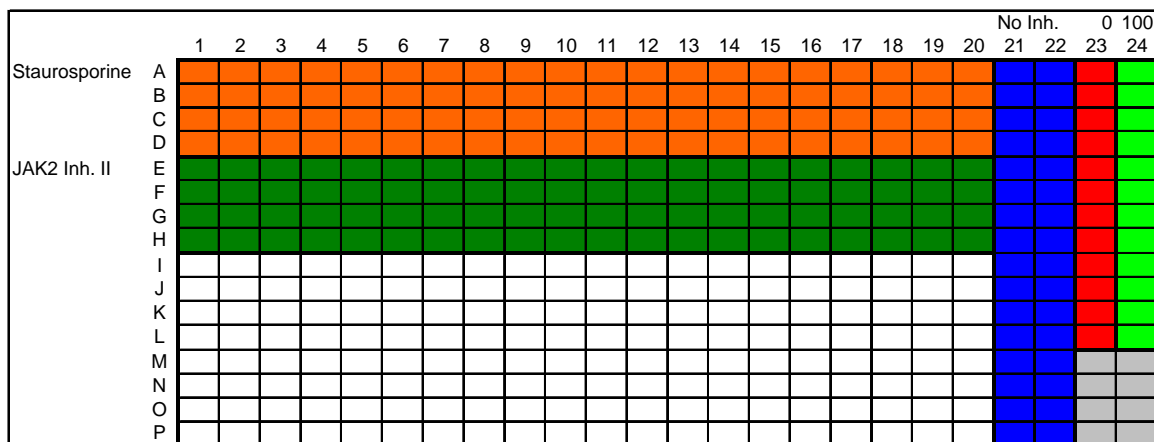
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from the next well to Column 2 Rows A through D and so on to set up an actual titration from 1 µM final starting concentration of staurosporine).

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.

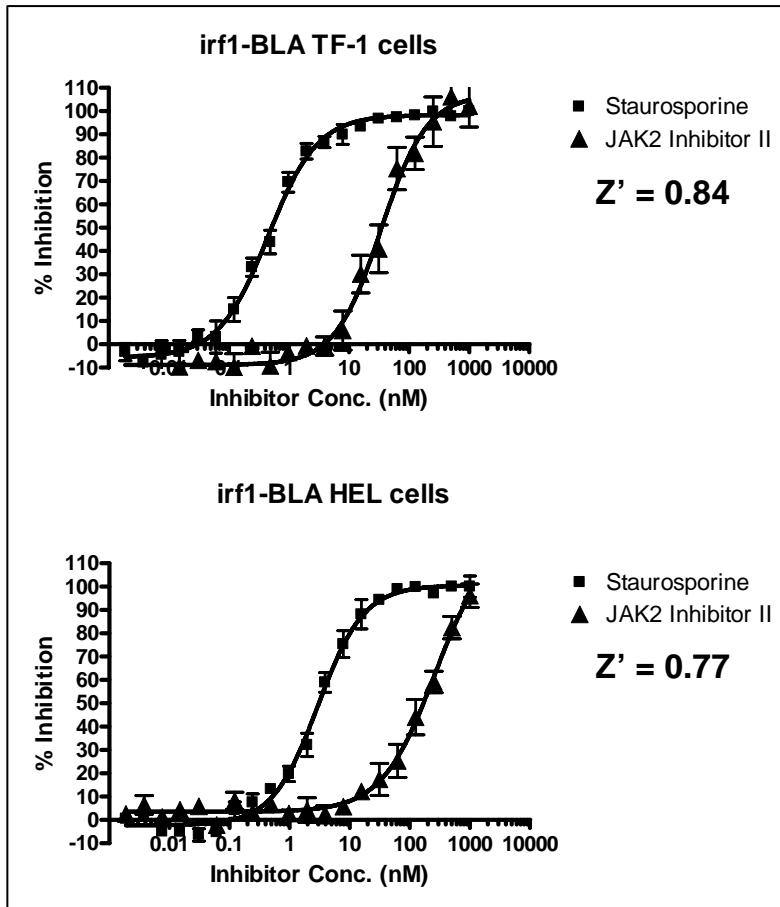
Substrate Loading

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 Thermo Scientific Varioskan® Flash.