

GeneBLAzer® Assay Setup Guide on the BioTek Instruments Synergy™ 4 Hybrid Multi-Mode Microplate Reader

NOTE: The BioTek Instruments Synergy™ 4 Hybrid Multi-Mode 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 BioTek instruments or Gen5 software, please contact BioTek Instruments at 1-888-451-5171.

A. Recommended Optics

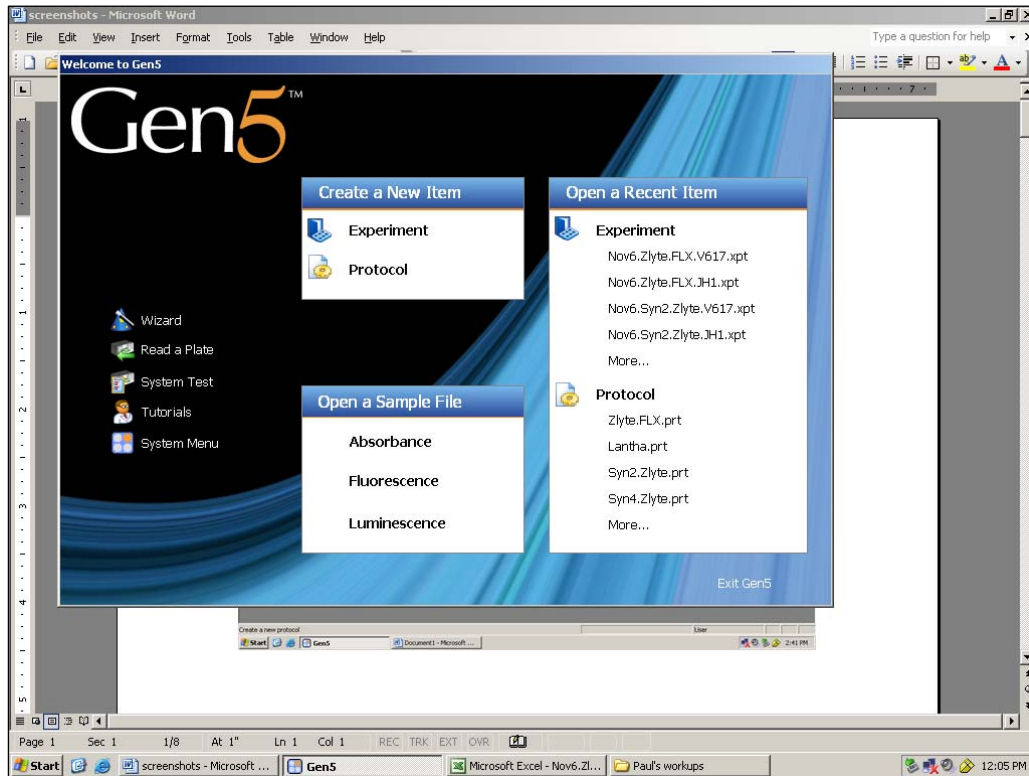
BioTek Instruments part number	wavelength (nm)	diameter (mm)
Excitation (7082205)	400/30 (or similar)	18
Emission 1 (7082222)	460/40 (or similar)	18
Emission 2 (7082247)	528/20 (or similar)	18
Dichroic Mirror	NA	

NOTE: This document shows how to set up this reader using filters, and does not read from the bottom using monochromators.

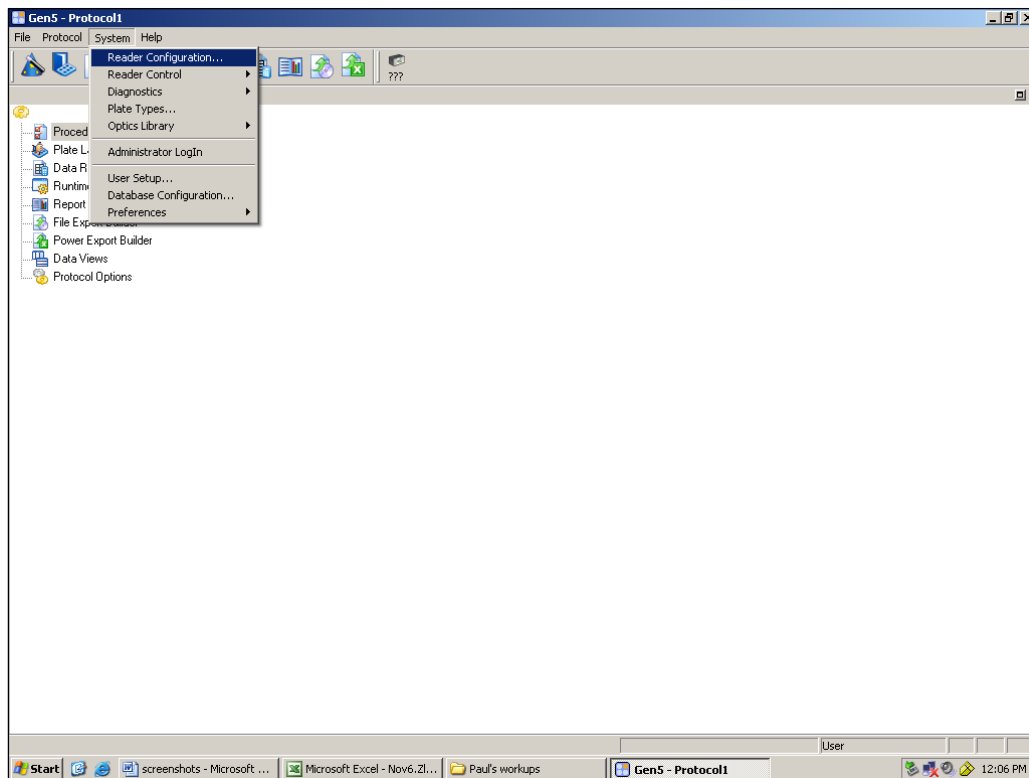
B. Instrument Setup

1. Make certain plate reader is turned on, and open up BioTek Gen5 software on computer.

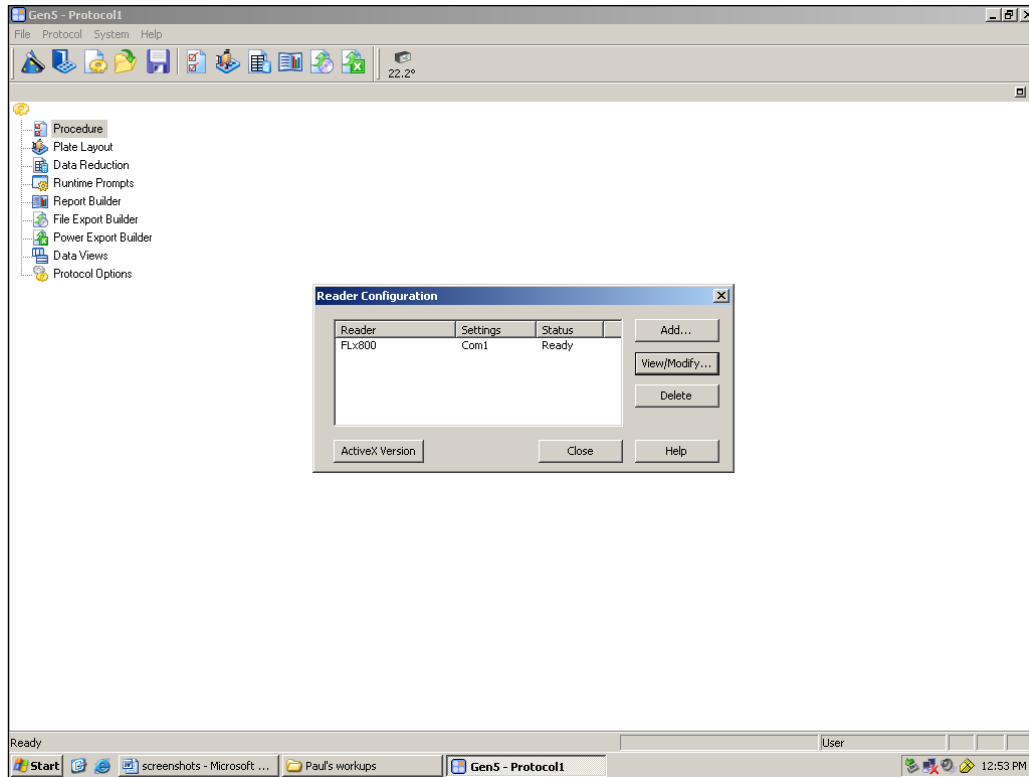
- When Gen5 opens, if you do not have a pre-existing protocol for GeneBLAzer®, select "Protocol" in the "Create a New Item" menu near the center of the screen.



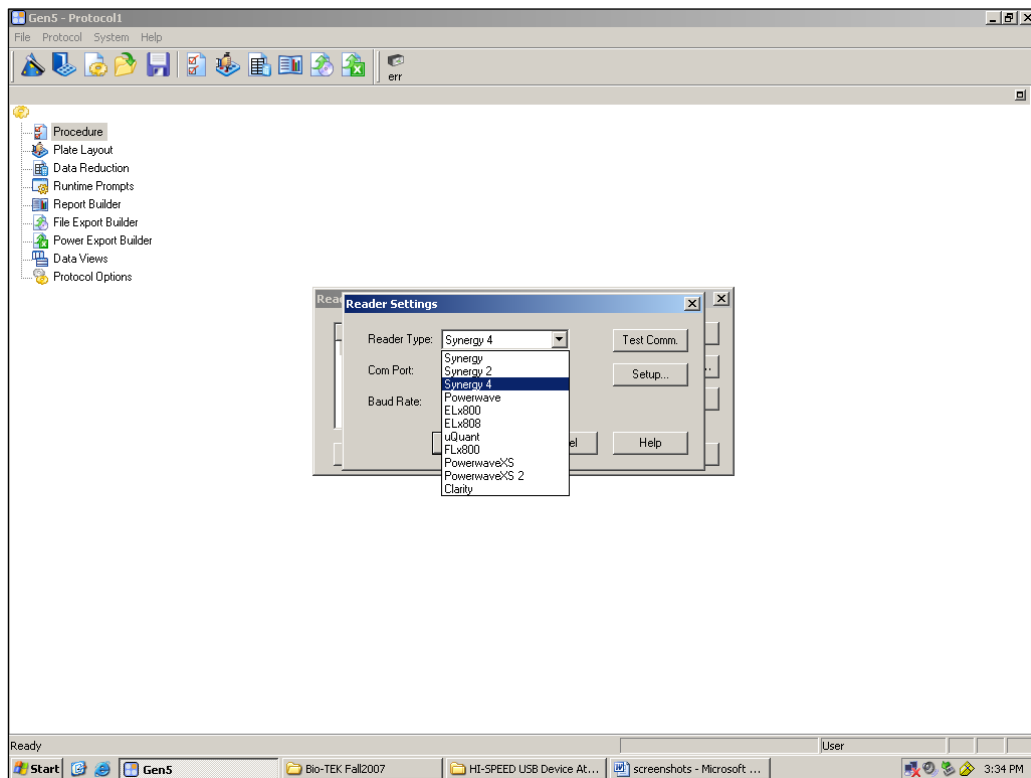
3. At this point, a blank protocol will open (below). To ensure that the correct instrument is selected, click on "Reader Configuration" under tools.



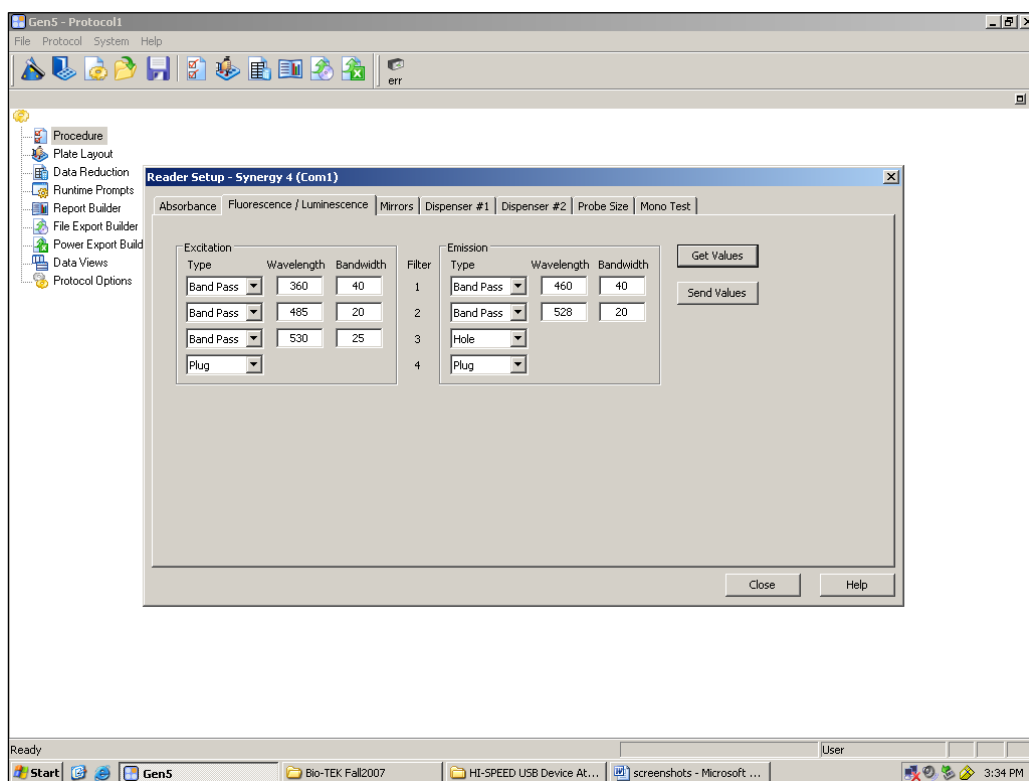
4. Check that the instrument selected is correct (below). If not, select "Delete" to remove the existing instrument, and then "Add".



5. Upon selecting "Add" a popup window will appear allowing you to select your instrument from the drop-down menu. Select your instrument here. Click on "Test Communication" to verify that the instrument is communicating properly with the software. When this is done, click on "Setup" finally to check filter and dichroic settings.

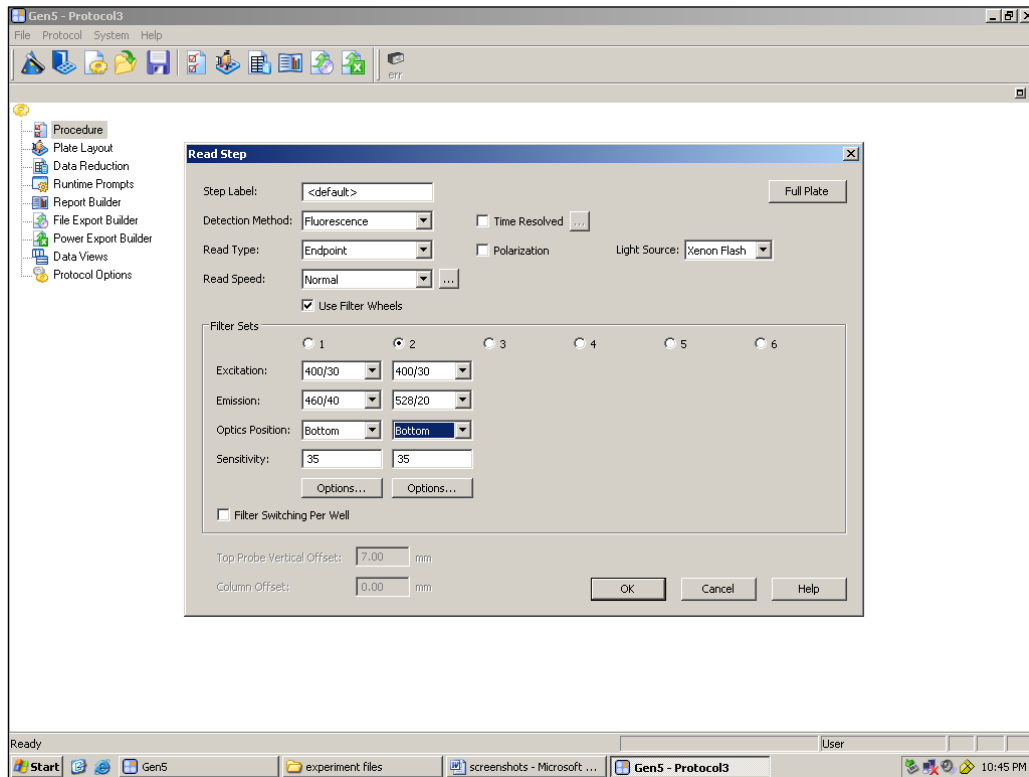


6. Select the "Fluorescence/Luminescence" tab at the top of the popup window. Enter the correct values for your filters present in your filter holder(s). Make certain to do so in the proper positions. Note if you have more than one filter holder for excitation or emission you may have to check that the correct set listed in the settings or enter the appropriate one. Click on "Send Values" to load the filter positions and settings into the instrument.

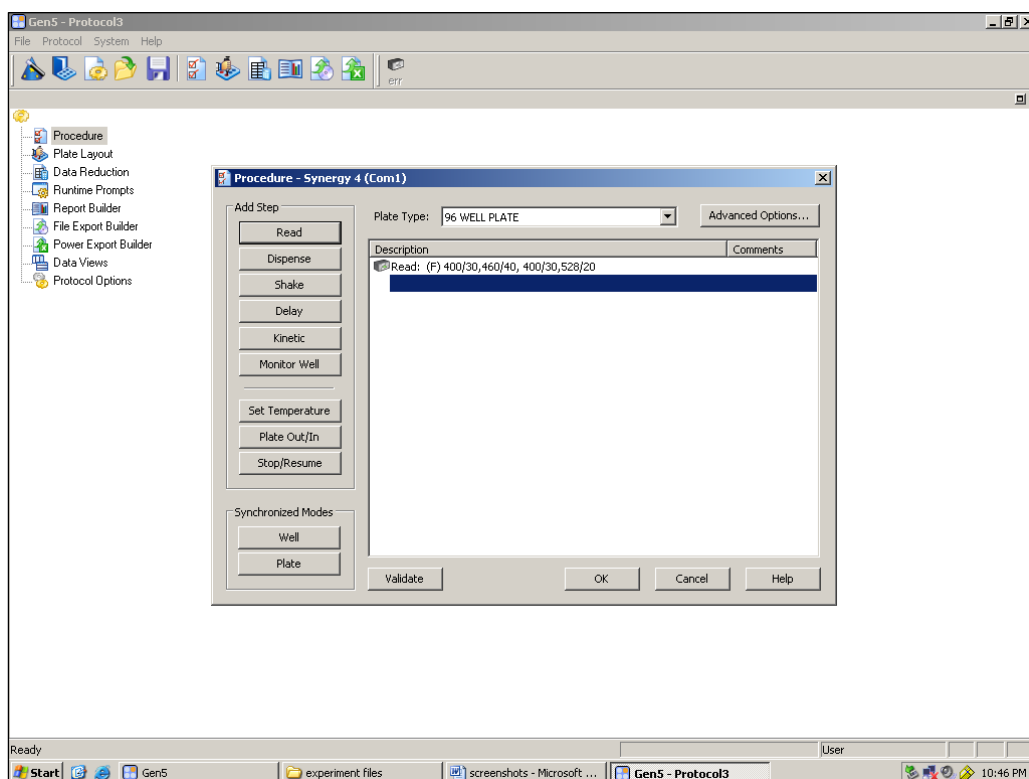


7. Select "Mirrors" and repeat for the dichroic mirror as well, if needed. Note dichroics are not used for bottom-reading, so this step may be omitted. Select "Close" when finished.

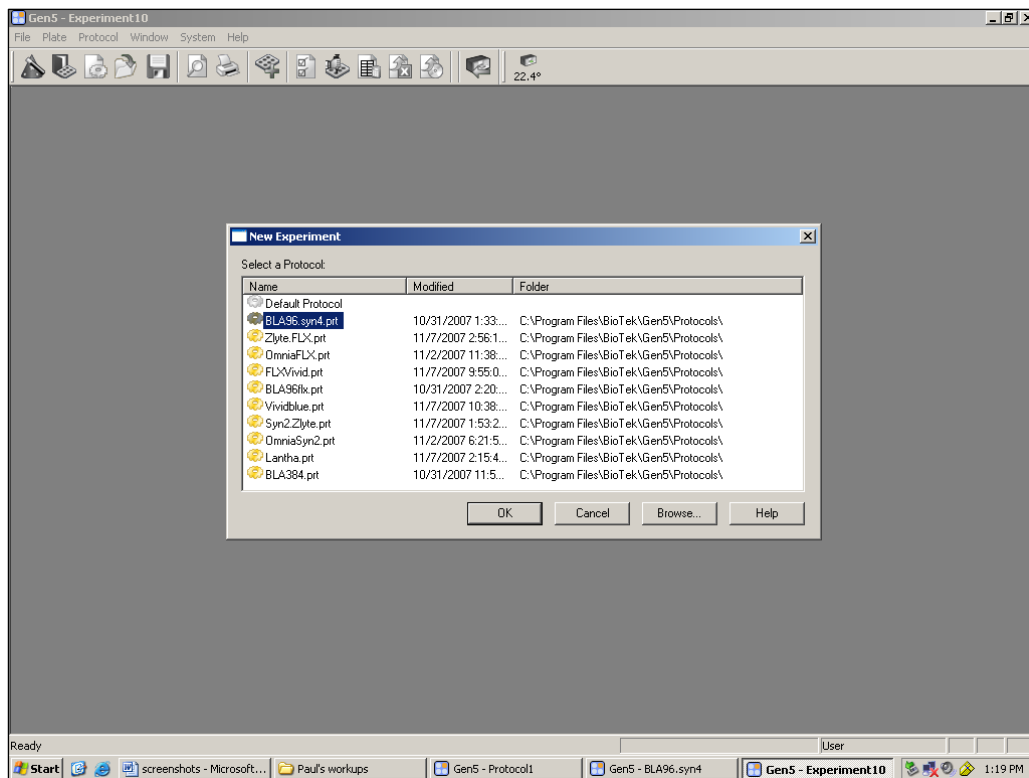
8. Since you have already selected to open a new protocol, at this point the instrument and software are ready to set up a new protocol. Select "Procedure" on the left hand side and a new window will open up. Select "Fluorescence" and "Endpoint" from the drop-down menus. Select the proper filters and "Bottom" for the optics position, as shown below. Click "OK".



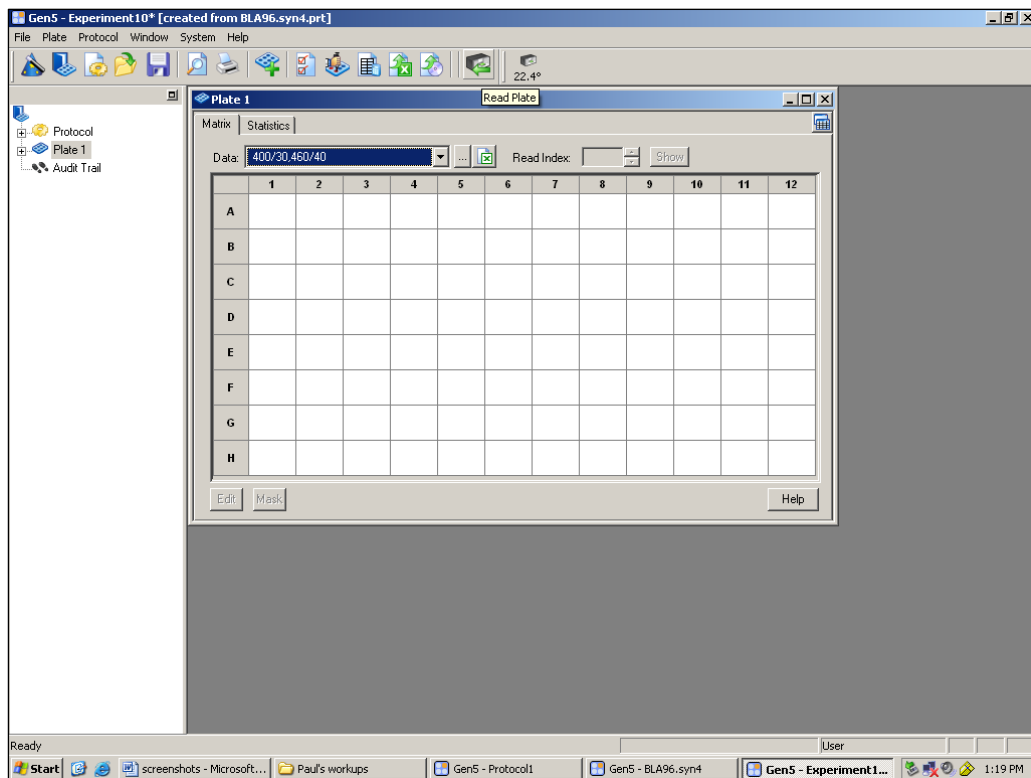
9. If you haven't already, select your plate size now. Note we have selected for a generic 96-well plate definition. Select "Validate" to ensure the protocol steps are valid with the current instrument settings. If the sequence is valid, select "OK" both in the smaller popup window and in the main screen. Select "Save As" from the "File" menu at the top of the screen, and save the protocol with an appropriate name to preserve these instrument settings.



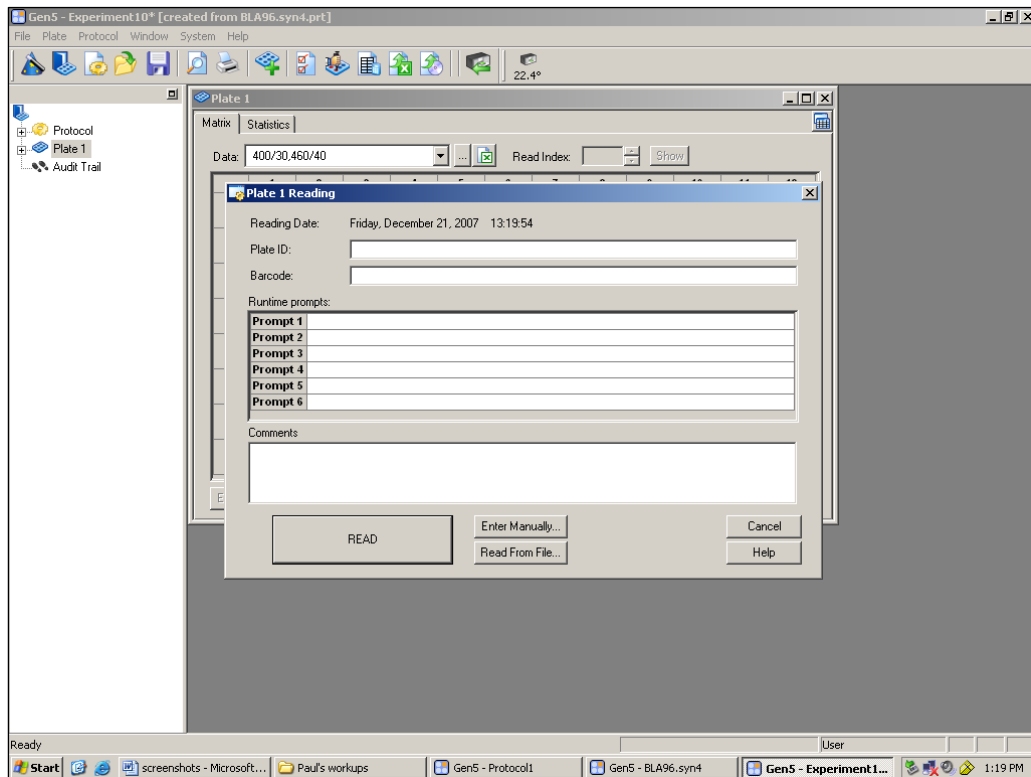
- To run an actual experiment, click on "File" again, and select "New Experiment" from the drop-down list. A smaller window will appear; select your protocol and click "OK".



11. A new window will again appear; this is the experiment window. Insert your plate to be read and click on the "Read Plate" icon with the small green arrow (second from last icon in the lower toolbar).



12. One last popup will appear. Select "Read" and instrument will commence reading your plate.



NOTE: There are several options for collecting data from the BioTek Gen5 software. Data can be exported directly to excel, or appropriate macros can be prepared within the BioTek software. For more information on data output options, please contact BioTek Technical Support.

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 100 µl in 96-well black-wall, clear-bottom plates. The information below details how the validation assays were prepared. 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>. Additionally, BioTek also offers instrument optics upgrades for bottom-read 384-well capability. We have not validated these but expect them to be suitable for GeneBLAzer® cell-based assays as well using similar settings.

Cell-Based Assay Setup

- 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 5×10^5 cells/ml. Place cells in incubator for 16 hours.
 - In the meantime, for *Irf1-bla* HEL cells, harvest and resuspend in Assay Medium at 9.5×10^5 cells/ml.
- Prepare assay plate for HEL cells: A 1:1, 12-point serial dilution was prepared for Staurosporine and JAK2 Inhibitor II beginning with 10 µM inhibitor in Assay Media. From this, 10 µl per dilution was added to an assay plate, in triplicate (See Figures 1 and 2).

		1	2	3	4	5	6	7	8	9	10	11	12
Staurosporine	A												
	B												
	C												
JAK2 Inh. II	D												
	E												
	F												
	G												
	H												

Figure 1: Schematic of Initial Compound Dilution. Staurosporine and JAK2 Inhibitor II were titrated from 10 µM by serial 1:1 dilutions, starting with 80 µl of 10 µM in well 1 and 40 µl of assay buffer in wells 2-12, and adding 40 µl from well 1 to well 2, mixing, and then adding 40 µl from well 2 to well 3, and so on.

		1	2	3	4	5	6	7	8	9	10	11	12
Staurosporine	A												
	B												
	C												
JAK2 Inh. II	D												
	E												
	F												
No Inhibitor	G												
	H									Cell-Free			

Figure 2: Schematic of Assay Plate Layout. Assay plate was prepared from the initial titration by adding 10 µl inhibitor from the dilution plate per dilution to the assay plate in triplicate, so rows A-C are Staurosporine titration replicates and D-F are JAK2 Inhibitor 2 replicates. Row G has no inhibitor added and Row H is also a no inhibitor row for then HEL line, for the TF-1 line it is GM-CSF-free (unstimulated). Last 4 wells are cell-free as loading controls for background subtraction.

3. Add 90 µl per well of cell suspension. For Row H wells 9-12, add 100 µl Assay media alone.
4. Incubate HEL assay plate for 15 hours.
5. 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 90 µl cells to Row H, wells 1-8, then add 0.8 ng/ml GM-CSF (experimentally determined, see protocol) to the remaining cells to stimulate and 80 µl cells added into Rows A-G, Columns 1-12.
6. Place TF-1 plate in incubator for 5 hours.

Substrate Loading

7. Load cells as follows:
 - 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 20 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 20 µl per well added to plate. Plate incubated at ROOM TEMP. for 4 hours.
8. Read and analyze as directed in protocol.

D. Results:

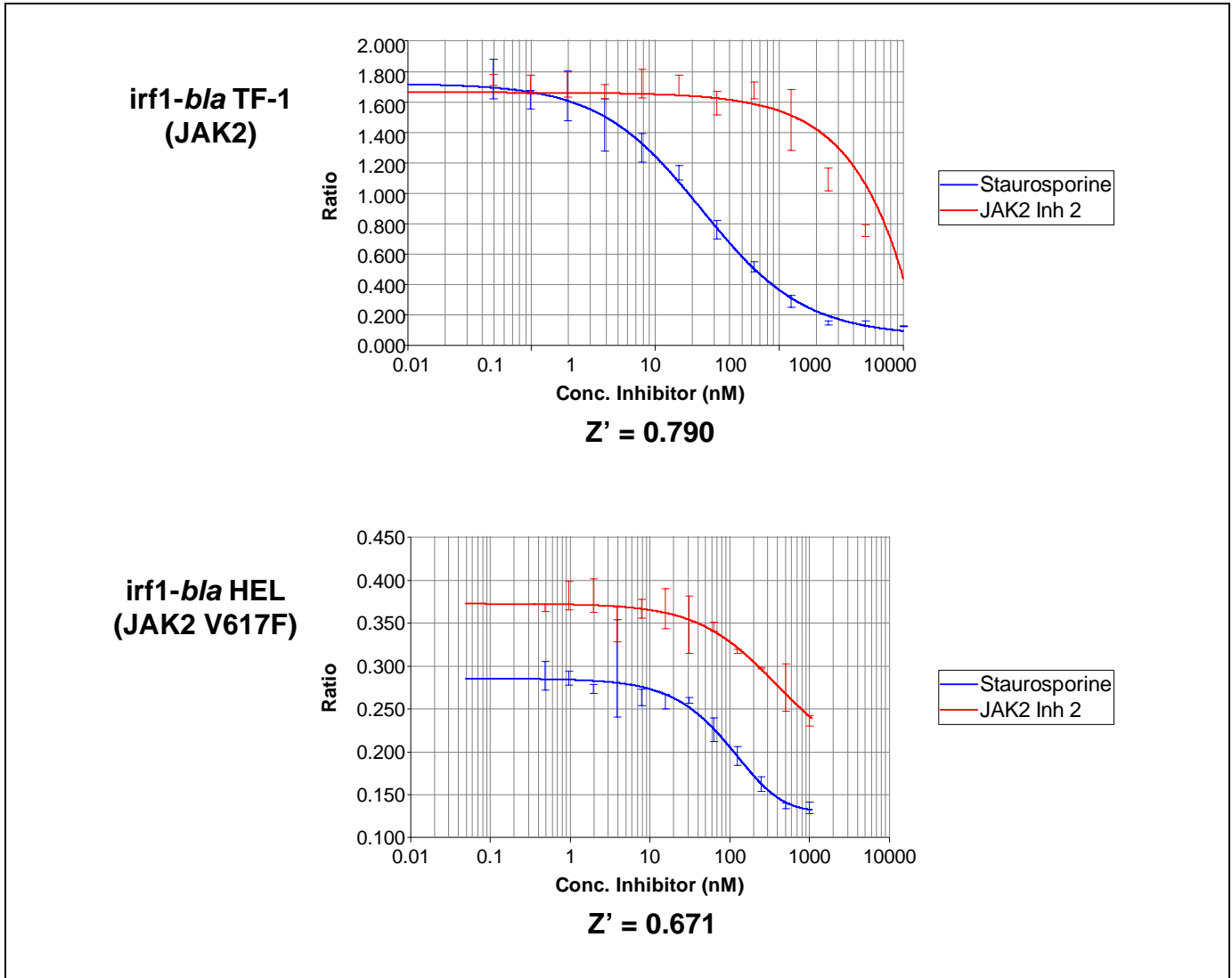


Figure 3: GeneBLAzer® Assay. Dose-Response Curves on the BioTek Instruments Synergy™ 4 using the CellSensor® Irf1-*bla* TF-1 cell line (K1647, expressing JAK2 when induced by GM-CSF stimulation) and the CellSensor® Irf1-*bla* HEL cell line (K1657, a human erythroleukemia cell line with a homozygous JAK2 V617F mutation).