Z'-LYTE® 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 Z'-LYTE® Assay using the Z'-LYTE® Tyr6 kit (PV4122) against JAK2 JH1/JH2 and JAK2 JH1/JH2 V617F kinases. 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

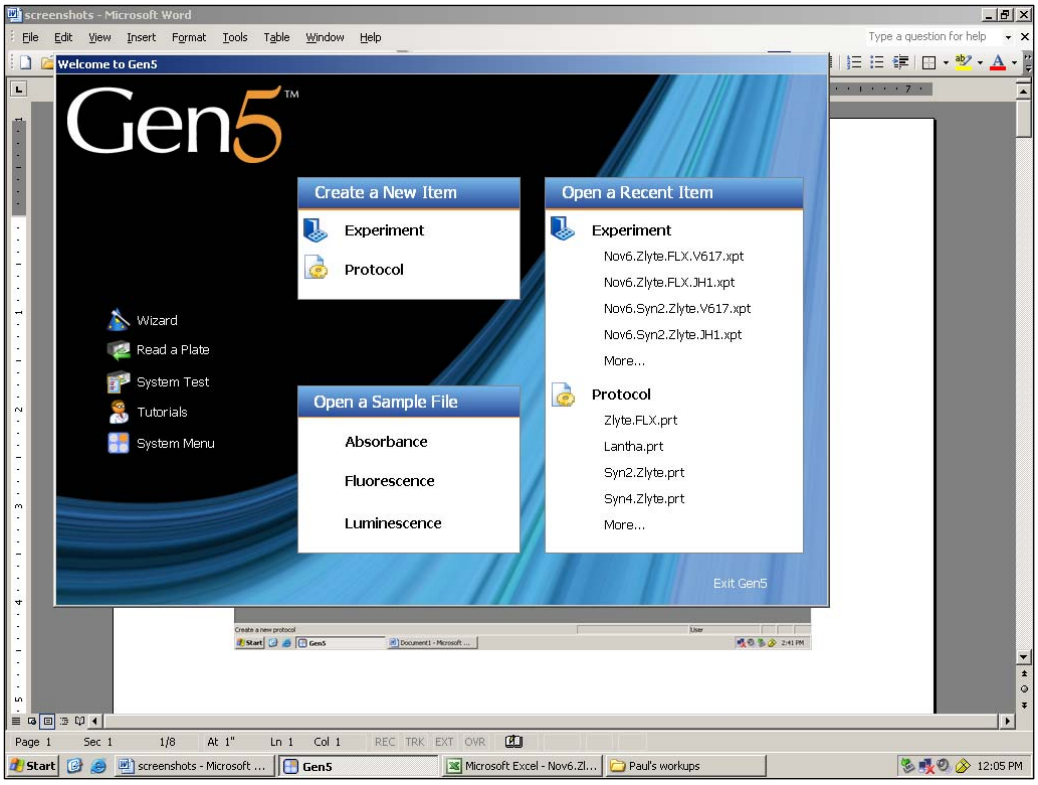
BioTek Instruments part number	wavelength (nm)	diameter (mm)
Excitation (7082205)	400/30 (or similar)	18
Emission 1 (7082222)	460/40 (or similar)	18
Emission 2 (7082247)	528/20 (or similar)	18
Dichroic Mirror (7138435)	435	

NOTE: This document shows how to set up the reader using filters, but can also perform Z'-LYTE® assays with monochromators. To use monochromators simply enter the exact wavelengths desired in Step 8 after unchecking "Filter Wheels". In monochromator experiments, we used an excitation of 400 nm and emission settings of 445 and 520 nm with a sensitivity setting of 200. Data for the same experiments read with monochromators will also be presented at the end of this document.

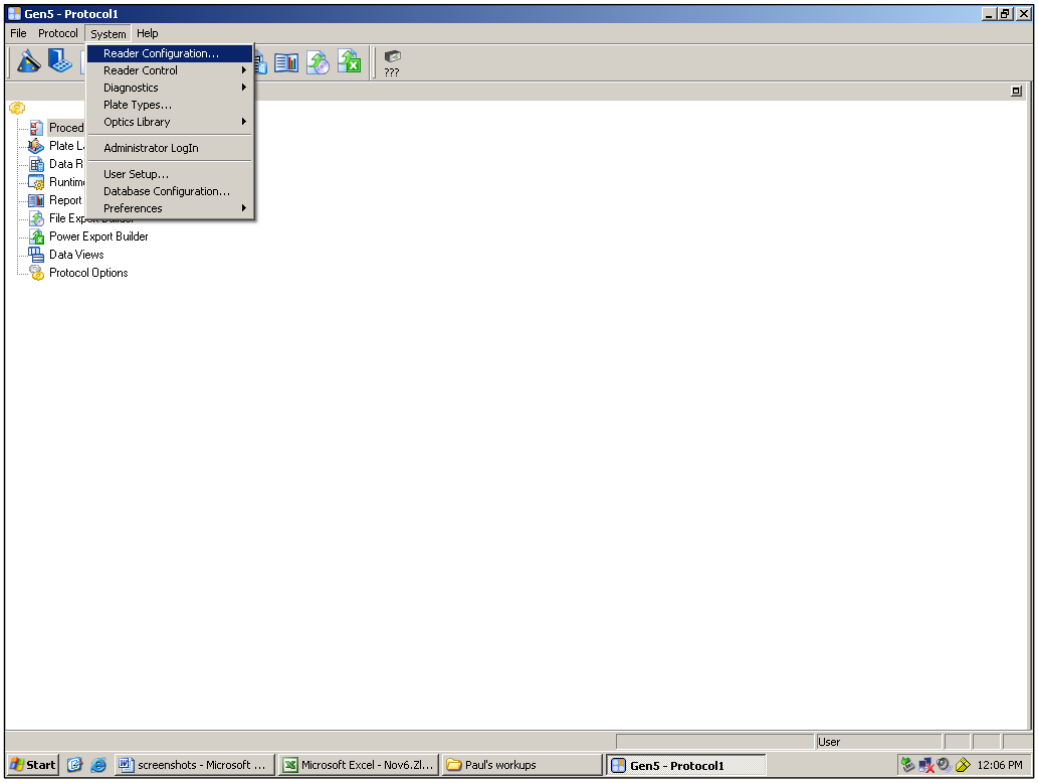
B. Instrument Setup

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

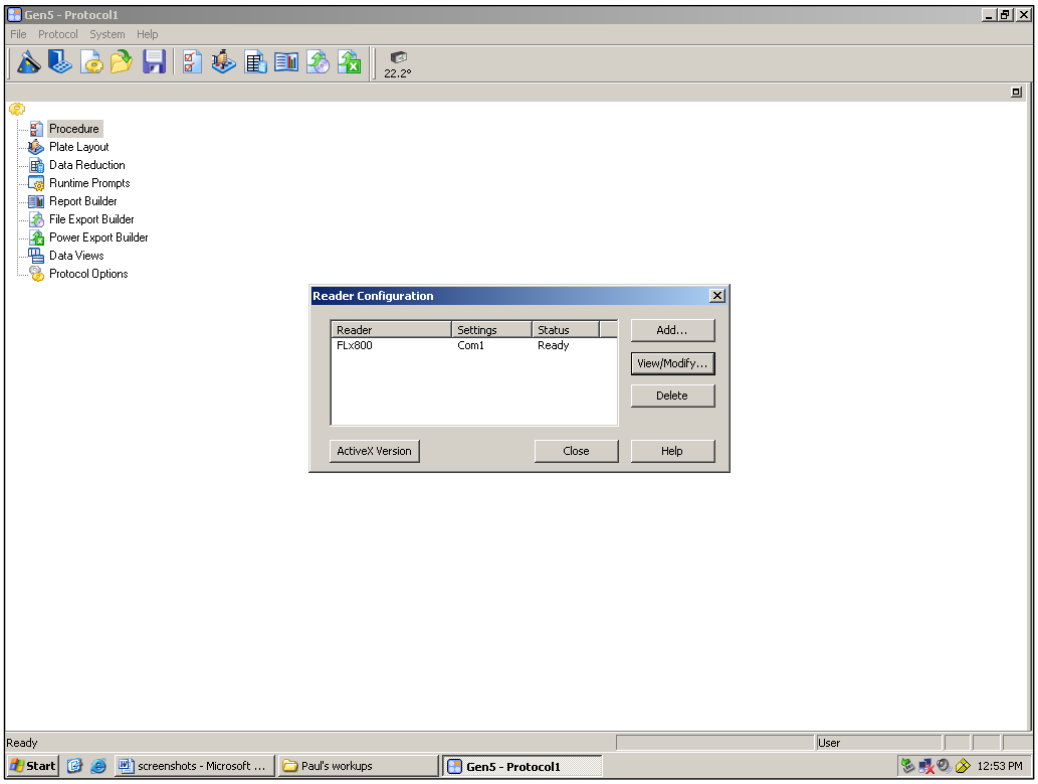
2. When Gen5 opens, if you do not have a pre-existing protocol for Z'-LYTE®, select "Protocol" in the "Create a New Item" menu near the center of the screen.



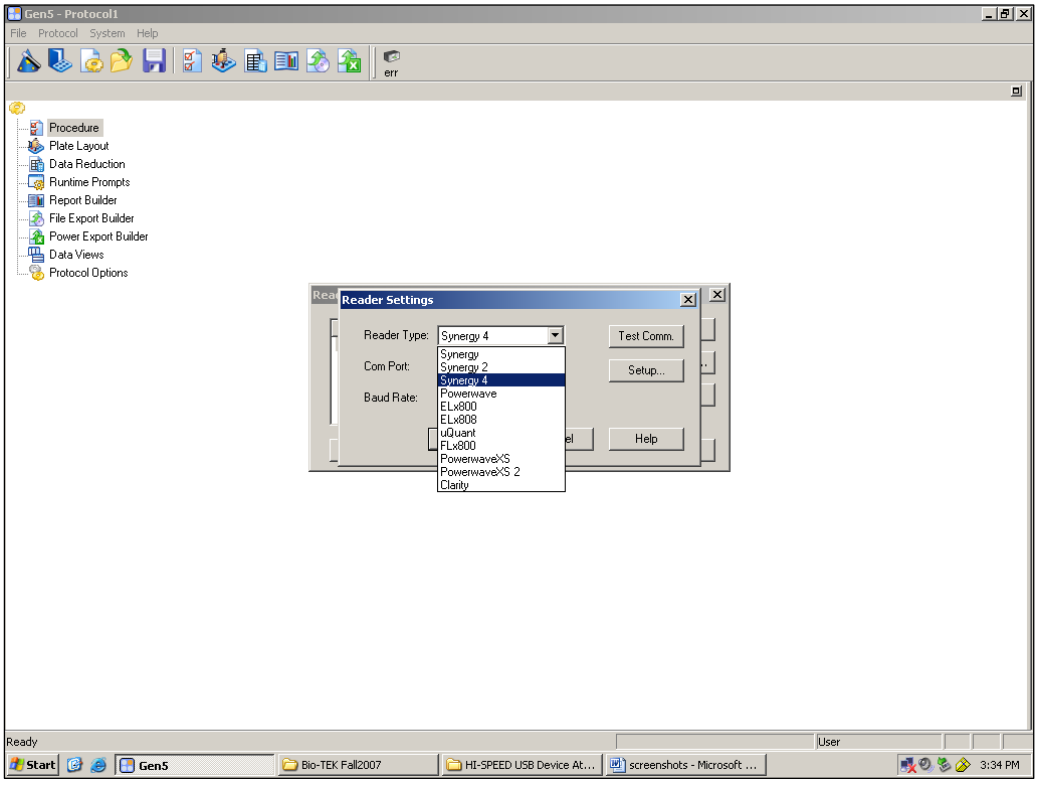
3. At this point, a blank protocol will open (below). To ensure that the correct instrument is selected, click on "Reader Configuration" under tools.



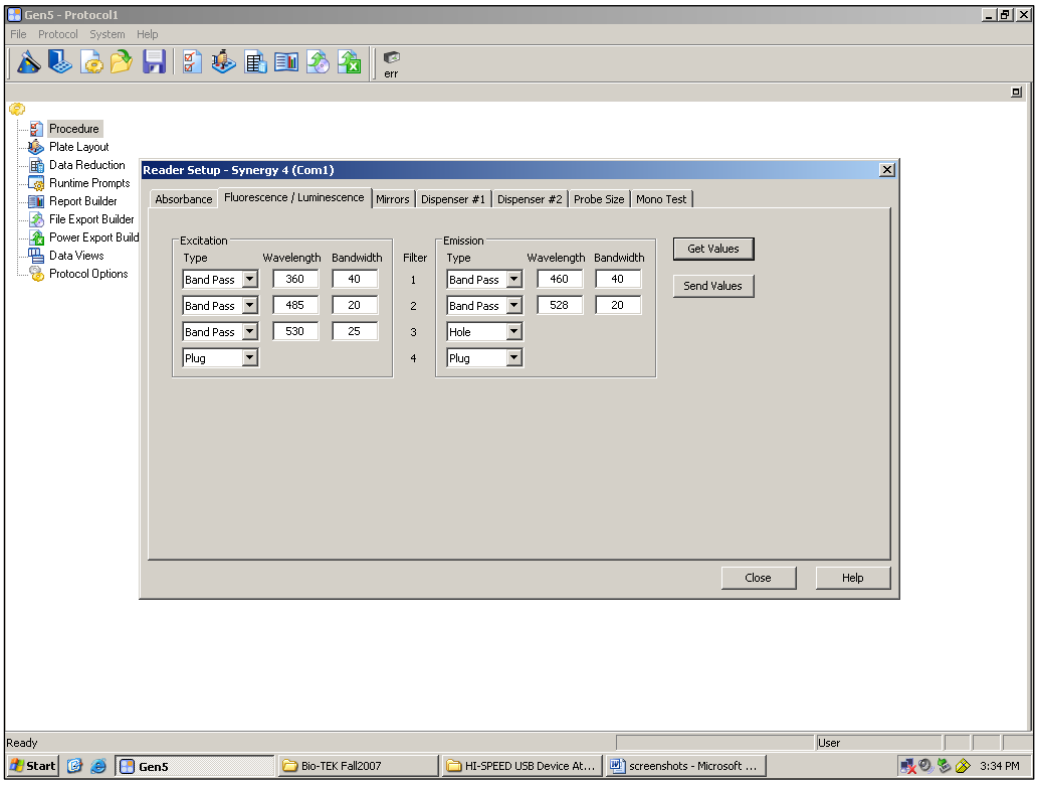
4. Check that the instrument selected is correct (below). If not, select "Delete" to remove the existing instrument, and then "Add".



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.

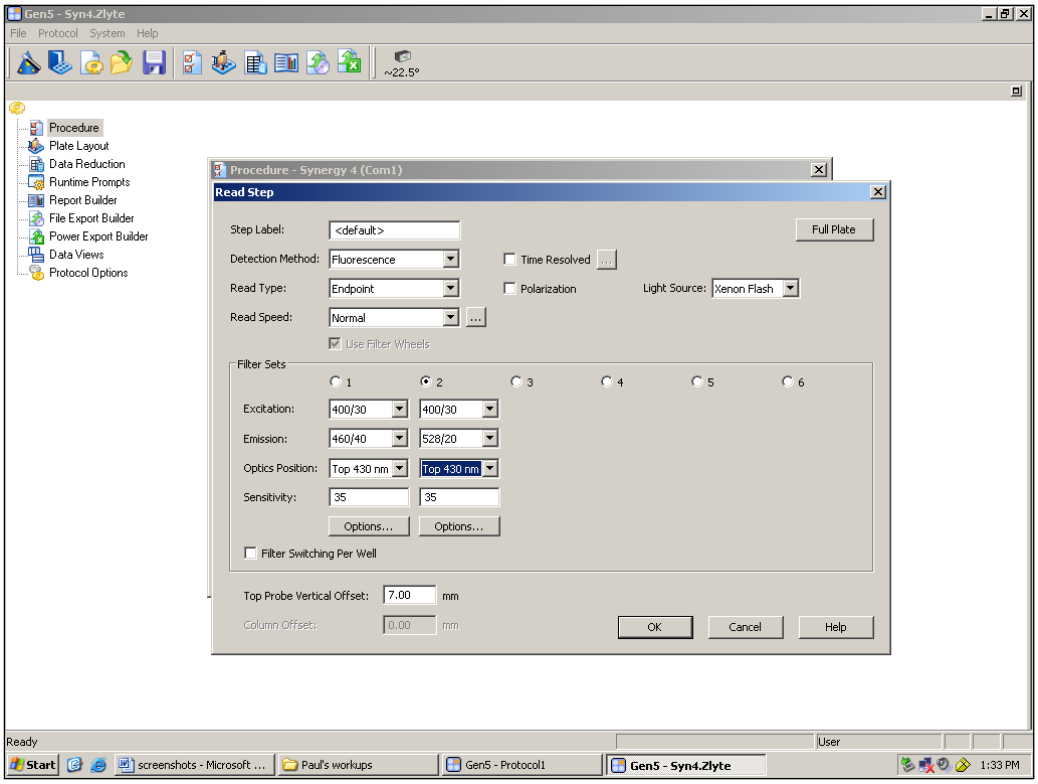


6. Select the "Fluorescence/Luminescence" tab at the top of the popup window. Enter the correct values for your filters present in your filter holder(s). Make certain to do so in the proper positions. Note if you have more than one filter holder for excitation or emission you may have to check that the 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.

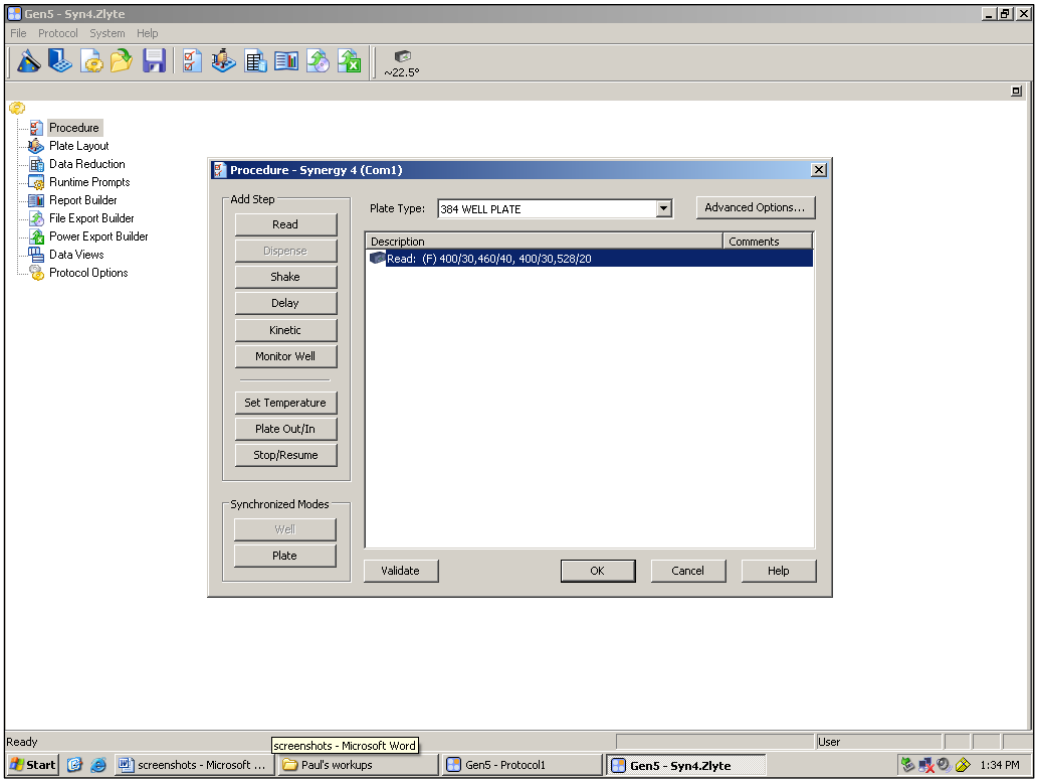


7. Select "Mirrors" and repeat for the dichroic mirror as well, if needed. Select "Close" when finished.

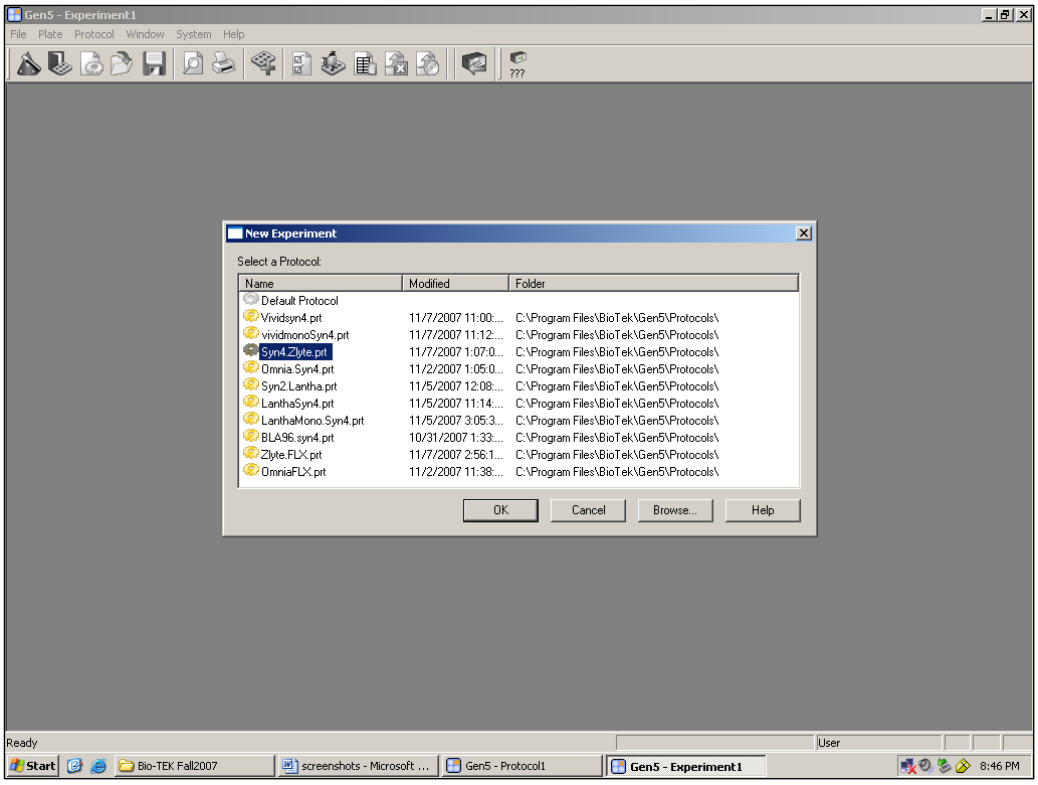
8. Since you have already selected to open a new protocol, at this point the instrument and software are ready to set up a new protocol. Select "Procedure" on the left hand side and a new window will open up. Select "Fluorescence" and "Endpoint" from the drop-down menus. Select the proper filters and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



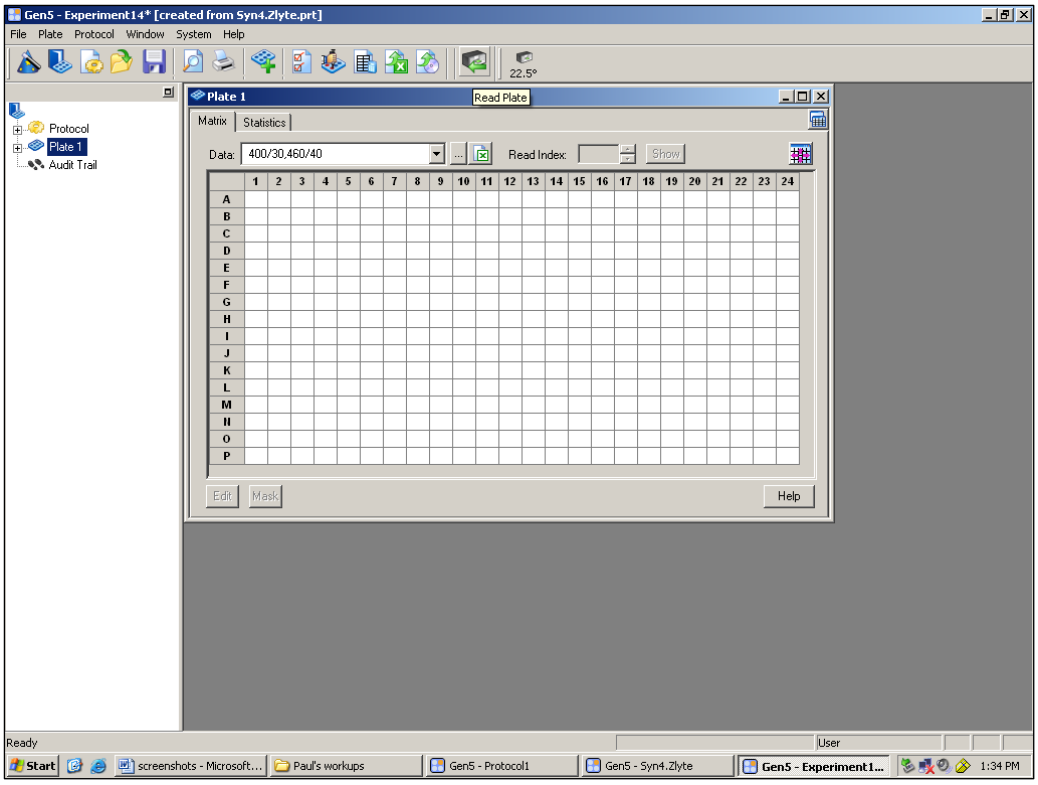
9. If you haven't already, select your plate size now. Note we have selected for a generic 384-well plate definition. Select "Validate" to ensure the protocol steps are valid with the current instrument settings. If the sequence is valid, select "OK" both in the smaller popup window and in the main screen. 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.



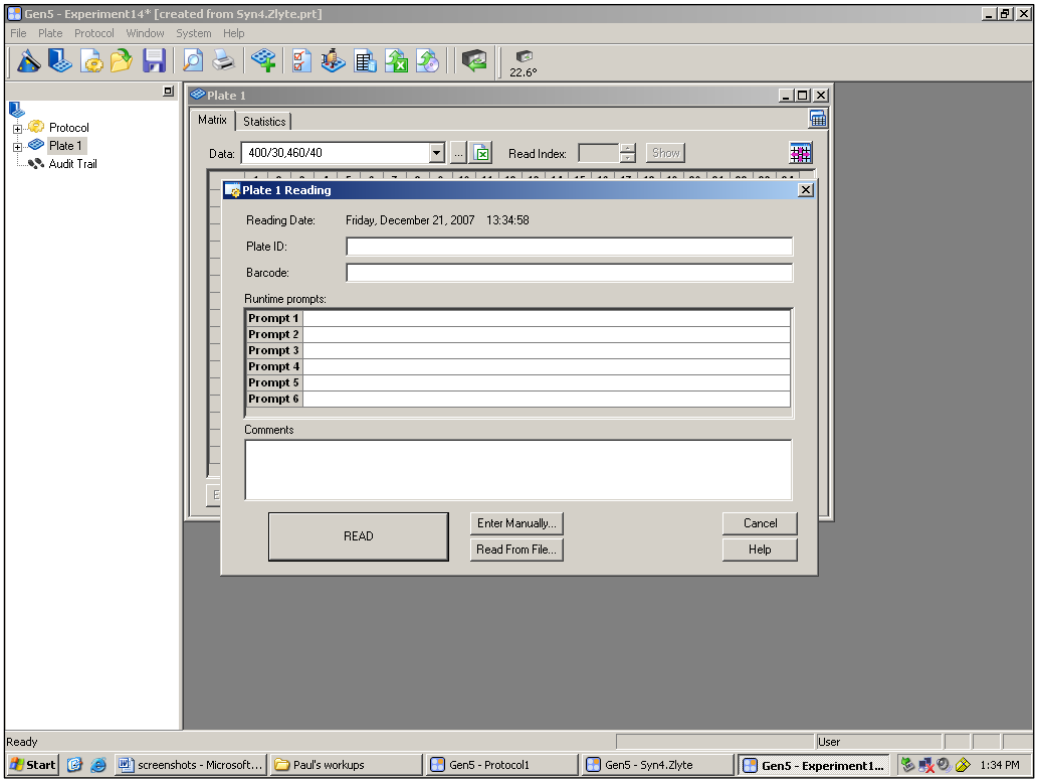
10. To run an actual experiment, click on "File" again, and select "New Experiment" from the drop-down list. A smaller window will appear; select your protocol and click "OK".



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. Z'-LYTE® Kinase Assay using JAK2 JH1/JH2 and JAK2 JH1/JH2 V617F

NOTE: The following is a sample assay performed for demonstration purposes. The instrument settings above would be sufficient for any Z'-LYTE® assay, the information below is provided as representative data. Assay was run at ATP Kmapparent and a kinase concentration producing approximately 30-40% of maximal phosphorylation, as discussed in Section 9 and 10 of the Z'-LYTE® protocols. ATP and kinase concentrations should be optimized for each kinase by the actual user. Specific Z'-LYTE® assay protocols and setup information from Invitrogen's own in-house SelectScreen® Z'-LYTE® Assay Service can be located at the following link: http://www.invitrogen.com/content.cfm?pageid=9866.

1. Prepare initial 100X serial dilution curves in rows A and E of a 384-well plate: Dilute Staurosporine and JAK2 Inhibitor II to a 100X initial concentration in 100% DMSO (100 µM). Prepare a set of 1:1 serial dilutions from the initial concentration in a 384-well plate, starting with 80 µl in column 1 and 40 µl DMSO 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.

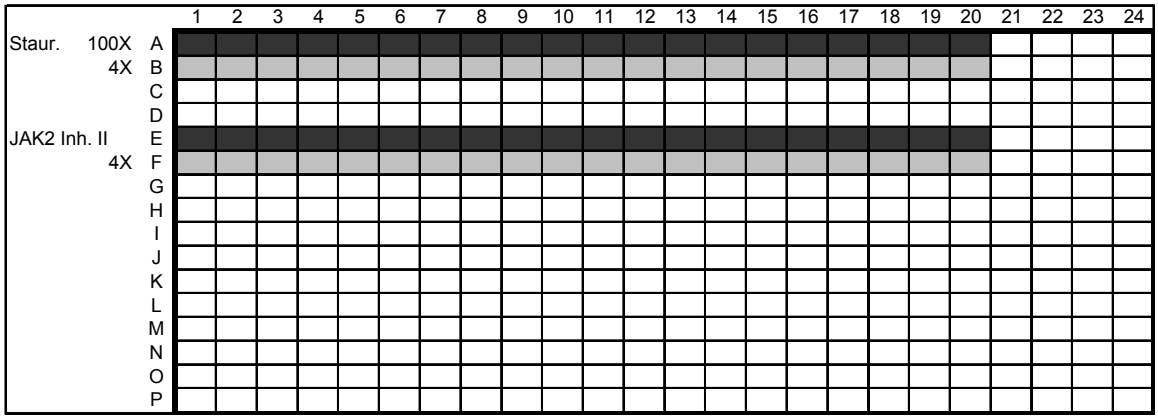


Figure 1: Schematic of initial compound dilution. Staurosporine and JAK2 Inhibitor II were titrated from a 100 µM starting concentration in the initial dilution series by preparing a 1:1 dilution curve in DMSO. A secondary dilution to 4X was then prepared in the rows below the initial dilution curve (lighter gray) using kinase buffer.

2. The 100X serial dilution set is then diluted to a 4X working concentration in Kinase Buffer (50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM MgCl2, 1 mM EGTA) in the row below by adding 2 µl of diluted inhibitor from the well above to 48 µl of kinase buffer. This will produce a final serial dilution starting at 4 µM, which will then produce a final assay concentration starting at 1 µM.

3. Begin to prepare an assay plate: Add 2.5 µL of the compound dilutions per well into a low volume NBS, 384-well plate (Corning Cat. # 3676), in quadruplicate so rows A-D are staurosporine replicates, E-H are JAK2 Inhibitor 2 replicates, etc.

4. Add 2.5 µl of kinase buffer alone to rows 21 and 22 (0% inhibition no compound control), 23 (0% phosphorylation control, no kinase added) and 24 (Phosphopeptide 100% phosphorylation positive control)

5. Add 5 µL of the 2X Peptide/Kinase Mixture (2 µM Tyr 06 peptide, 2600 ng/ml JAK2 JH1/JH2 or 1300 ng/ml JAK2 JH1/JH2 V617F, determined experimentally as outlined above) to columns 1-22. DO NOT ADD TO COLUMN 23 OR 24. Add 5 µL of 2µM substrate alone without kinase to column 23, rows A-L (0% phosphorylation control) and 5 µl of 2 µM phpsphopeptide control substrate to column 24, rows A-L (100% phosphorylation control). Add 5 µl kinase buffer alone to the remaining 8 wells (Columns 23 and 24, rows M-P) as a buffer-only reference.

6. Add 2.5 µL of 4X ATP Solution (200 µM) per well to all columns to start reaction.

7. Shake assay plate on a plate shaker for 30 seconds.

8. Incubate assay plate for 60 minutes at room temperature.

9. Add 5 µL of the Development Reagent Solution to each well. Use the lot-specific dilutions indicated on your CoA as dilution may vary based upon Z'-LYTE® peptide and Development Reagent A lot.

10. Shake plate again on a plate shaker for 30 seconds.

11. Incubate for 60 minutes at room temperature.

12. Read and analyze as directed in the protocol.

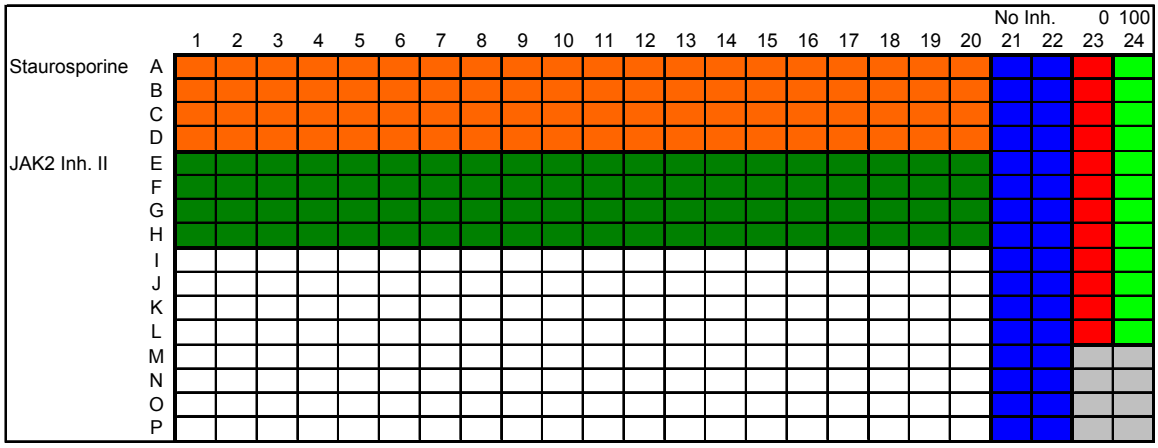


Figure 2: Assay Plate Schematic. Compound titrations shown in columns 1-20, columns 21 and 22 prepared without any inhibitor as kinase activity controls, column 23 prepared with no kinase (0% phosphorylation) and column 24 prepared using phosphopeptide control (100% phosphorylation). Note 8 wells in gray in bottom right, which were prepared with out any inhibitor or substrates, as buffer controls.

D. Results

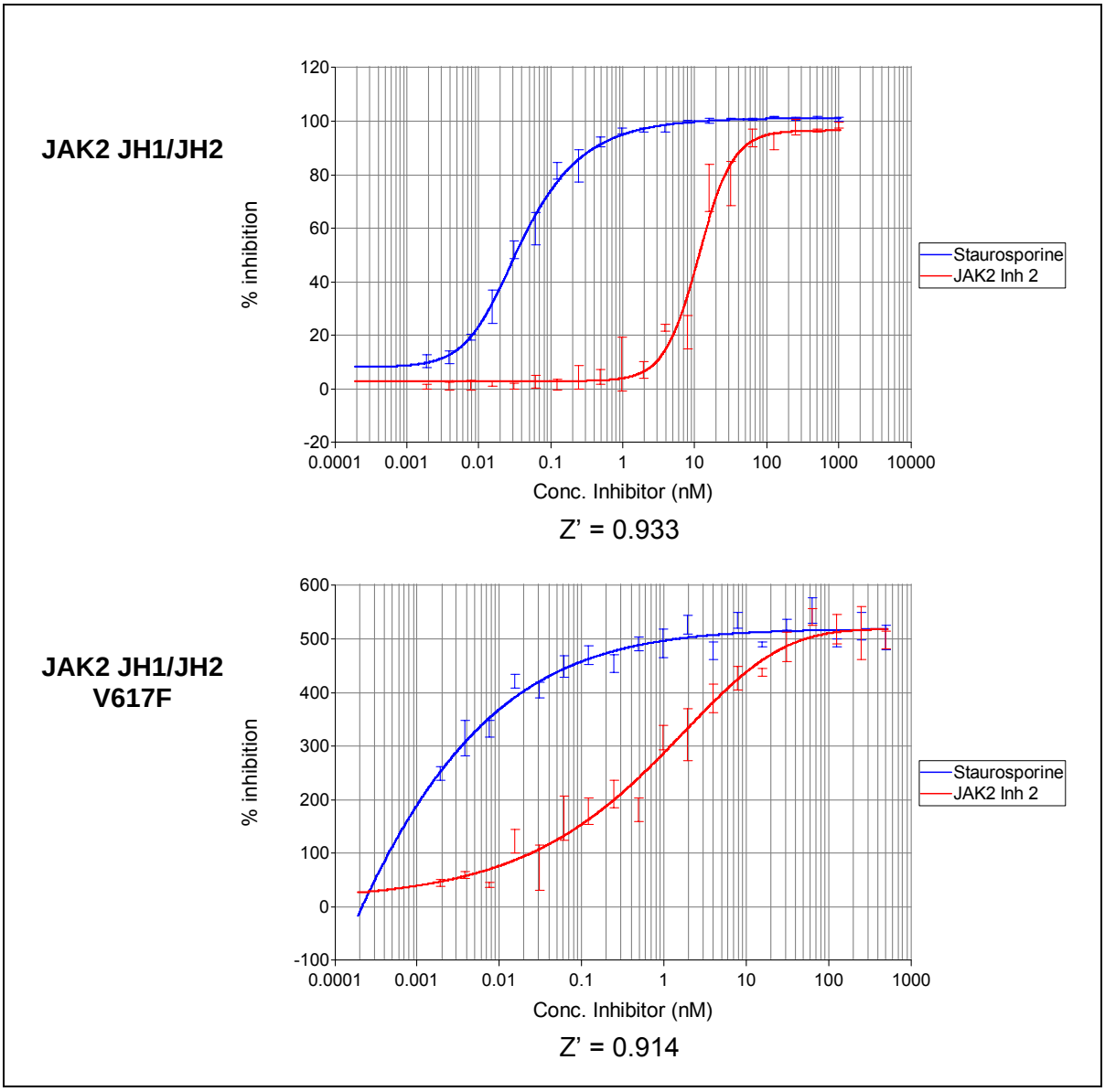


Figure 3: Z'-LYTE® Kinase Assay. Z'-LYTE® assay performed using the BioTek Instruments Synergy™ 4 with filters.

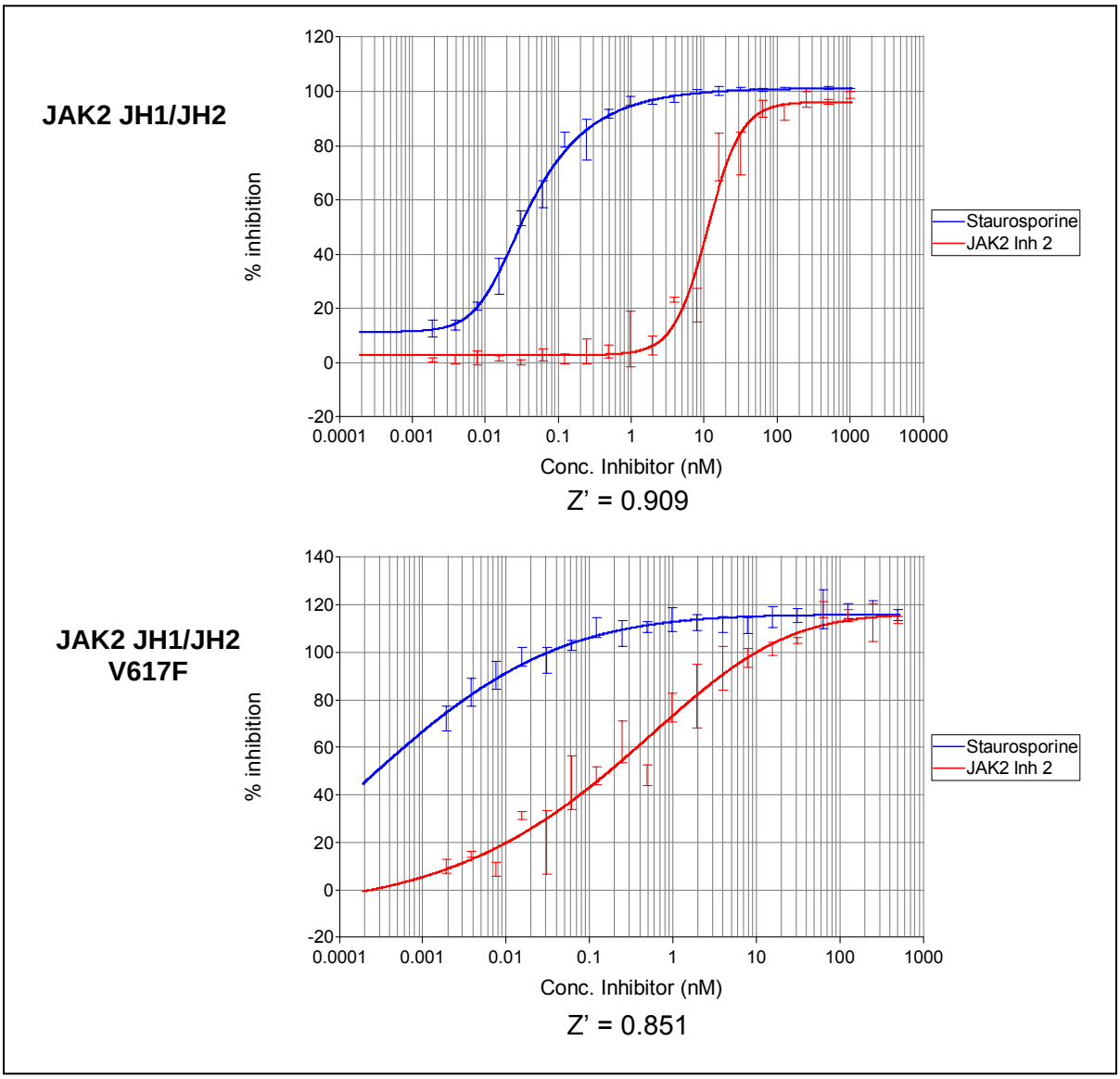


Figure 4: Z'-LYTE® Kinase Assay. Z'-LYTE® assay performed using the BioTek Synergy™ 4 with monochromators.