Z'-LYTE® 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 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 Thermo Scientific instruments or software, please contact Thermo Scientific at 1-800-522-7763 or www.thermo.com.

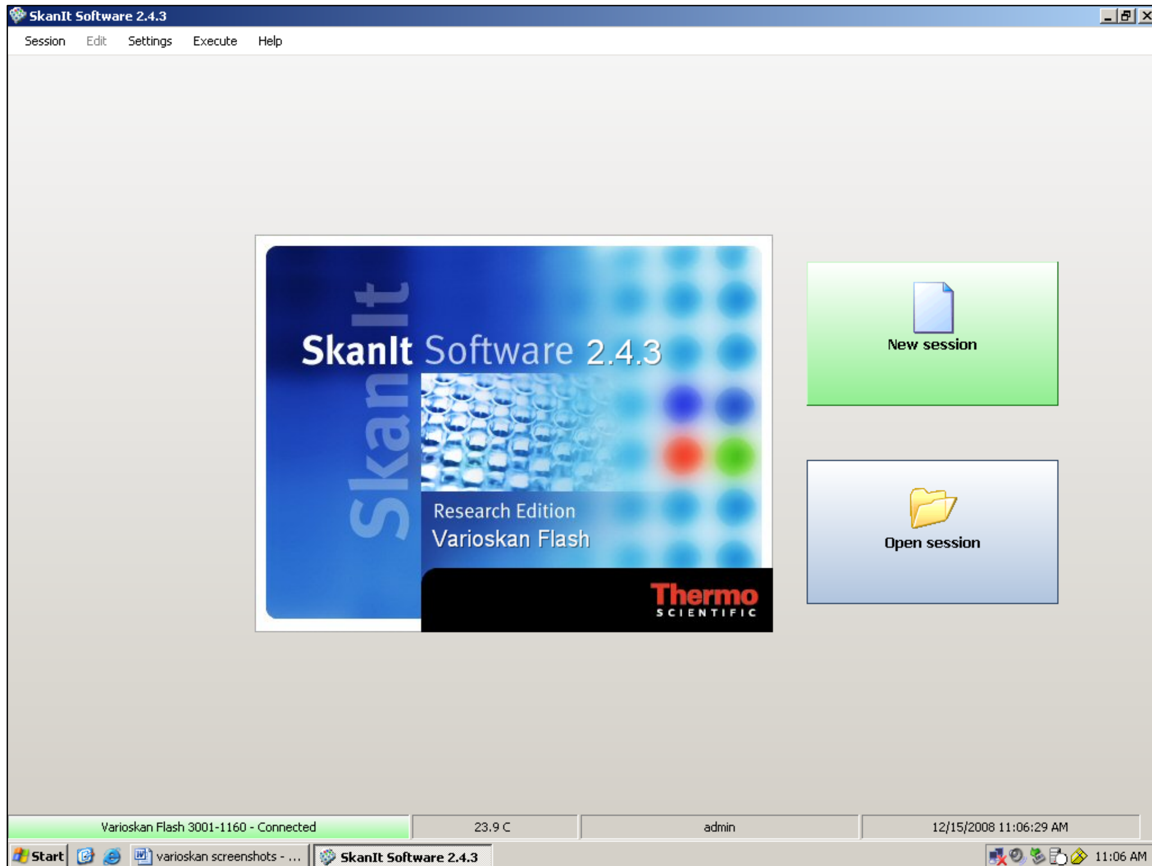
A. Recommended Optics

	wavelength (nm)	diameter (nm)
Excitation	400/12	monochromator
Emission 1	445/12	monochromator
Emission 2	520/12	monochromator

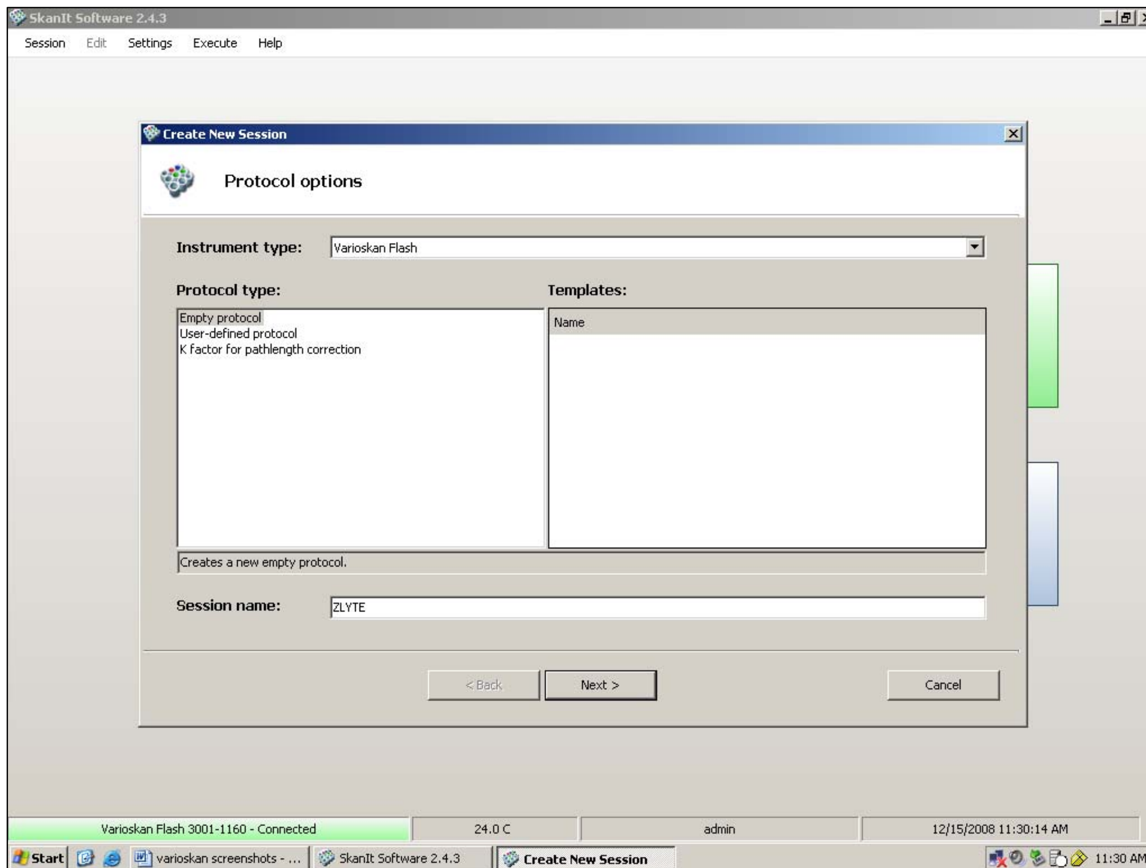
B. Instrument Setup

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

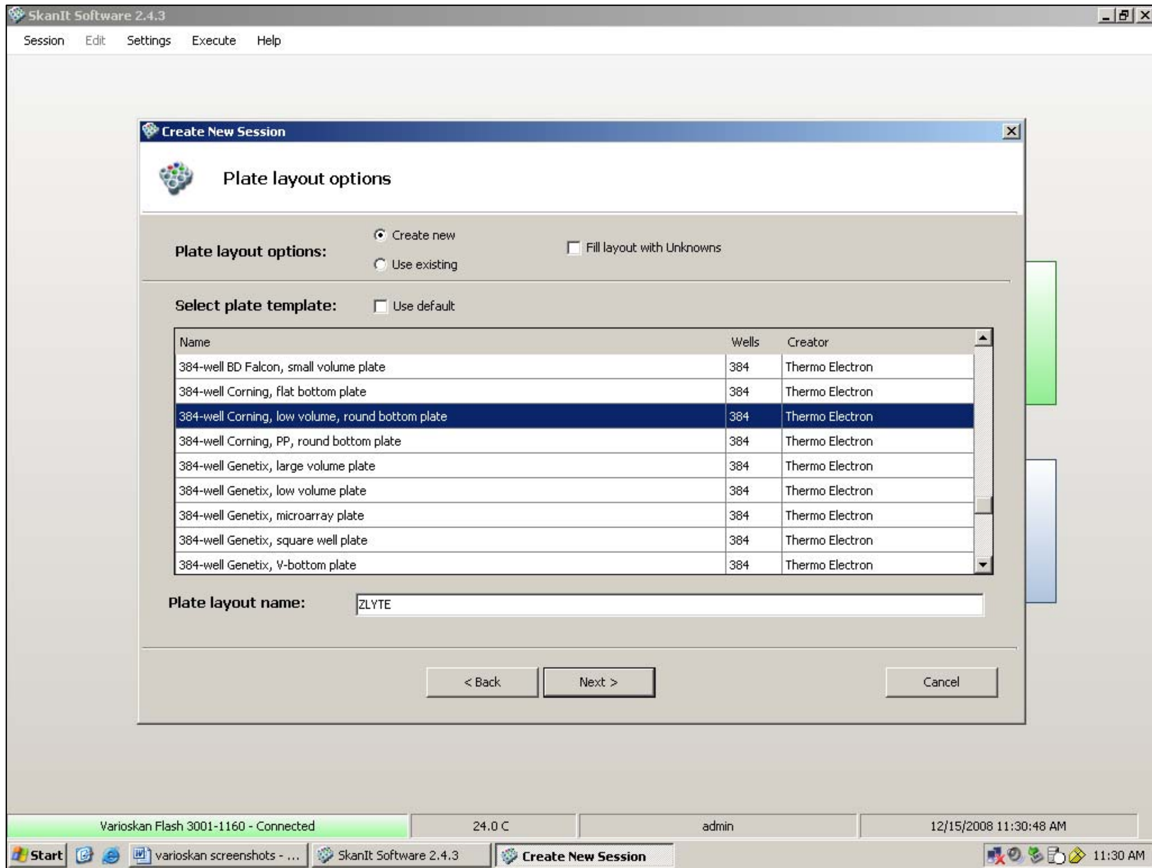
2. When SkanIt opens, it will default to a generic starting page. Select the "New Session" icon to open a new session and create a new protocol.



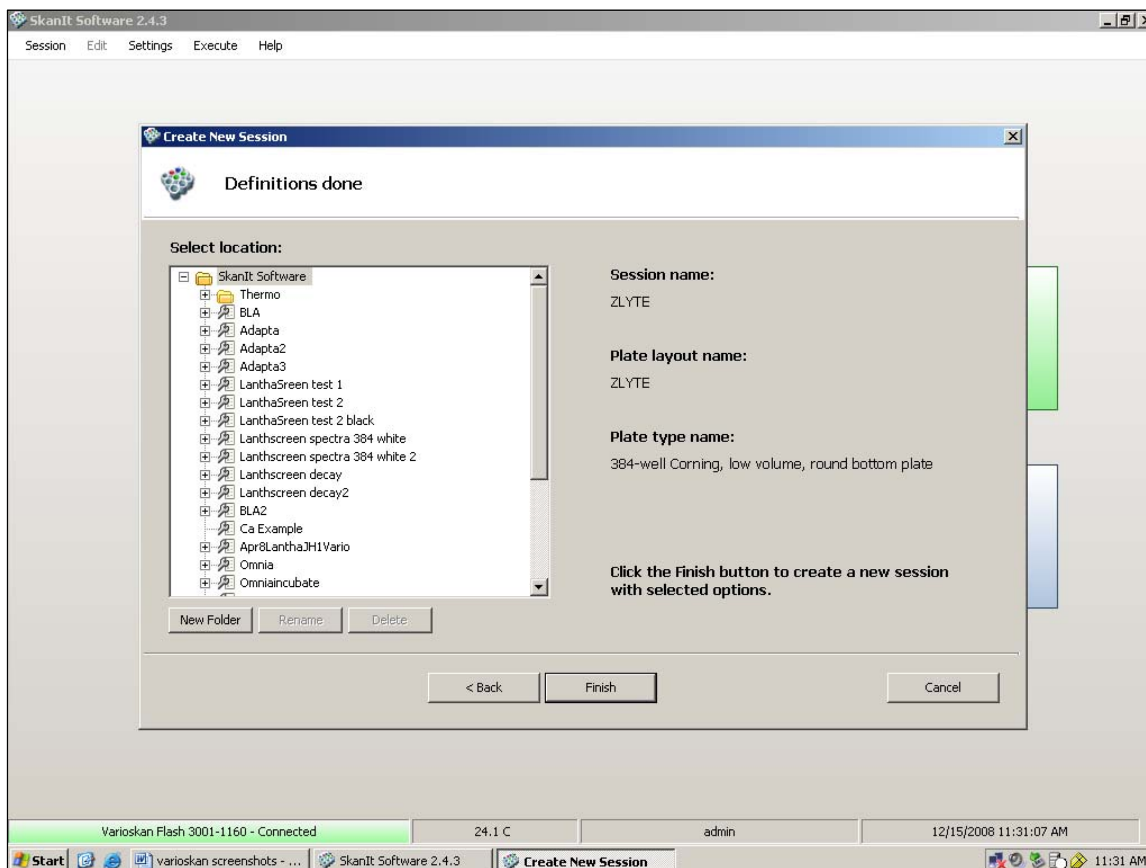
3. At this point, a popup window will appear. Select "Empty Protocol" on the left, and enter a session name at the bottom. Select Next when finished.



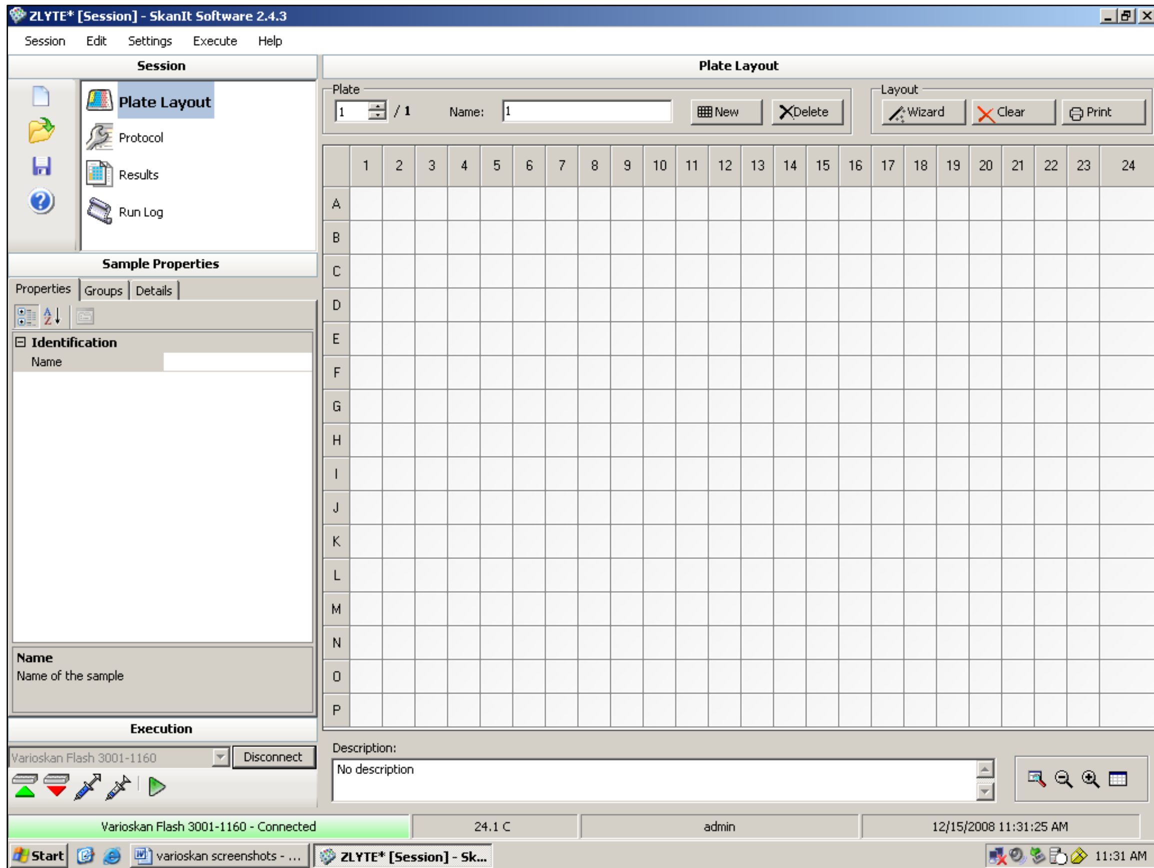
4. Select your plate from the available list. When finished, select Next.



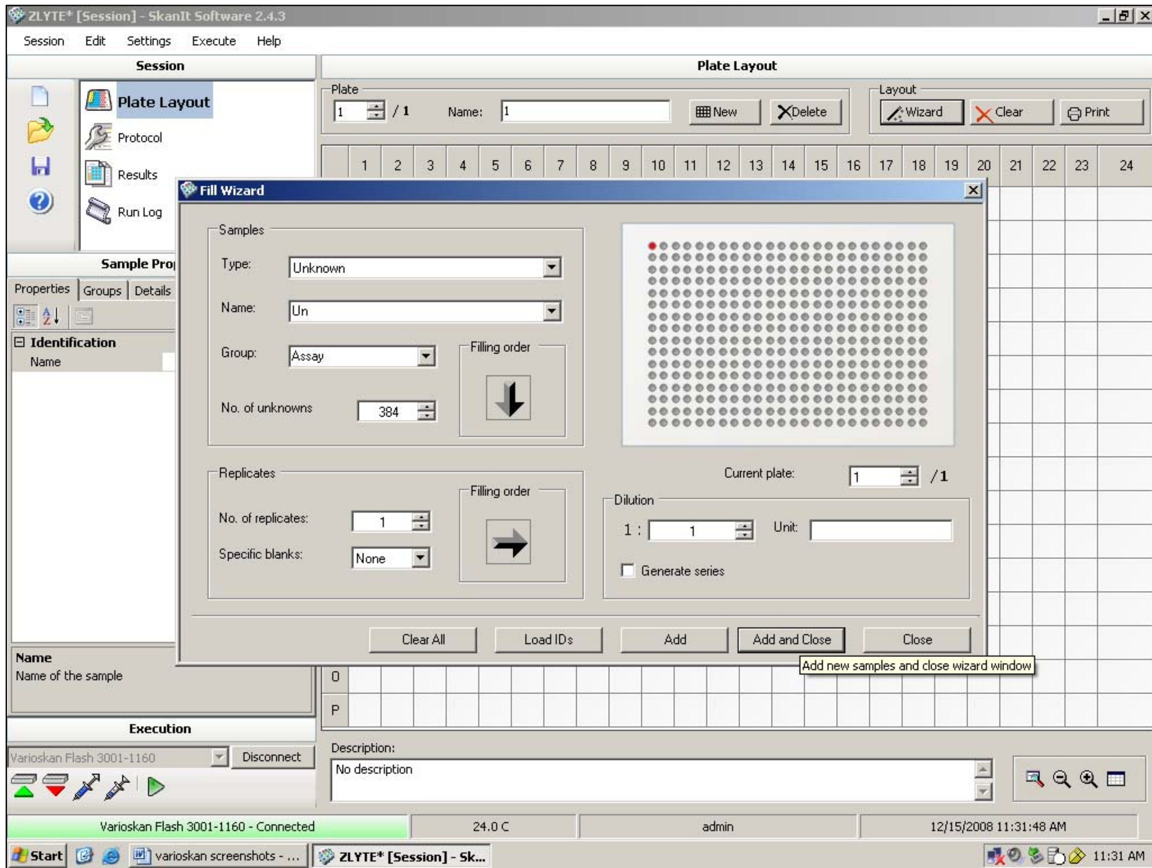
5. Once the session has been named and a plate selected, click the Finish tab to create a new protocol.



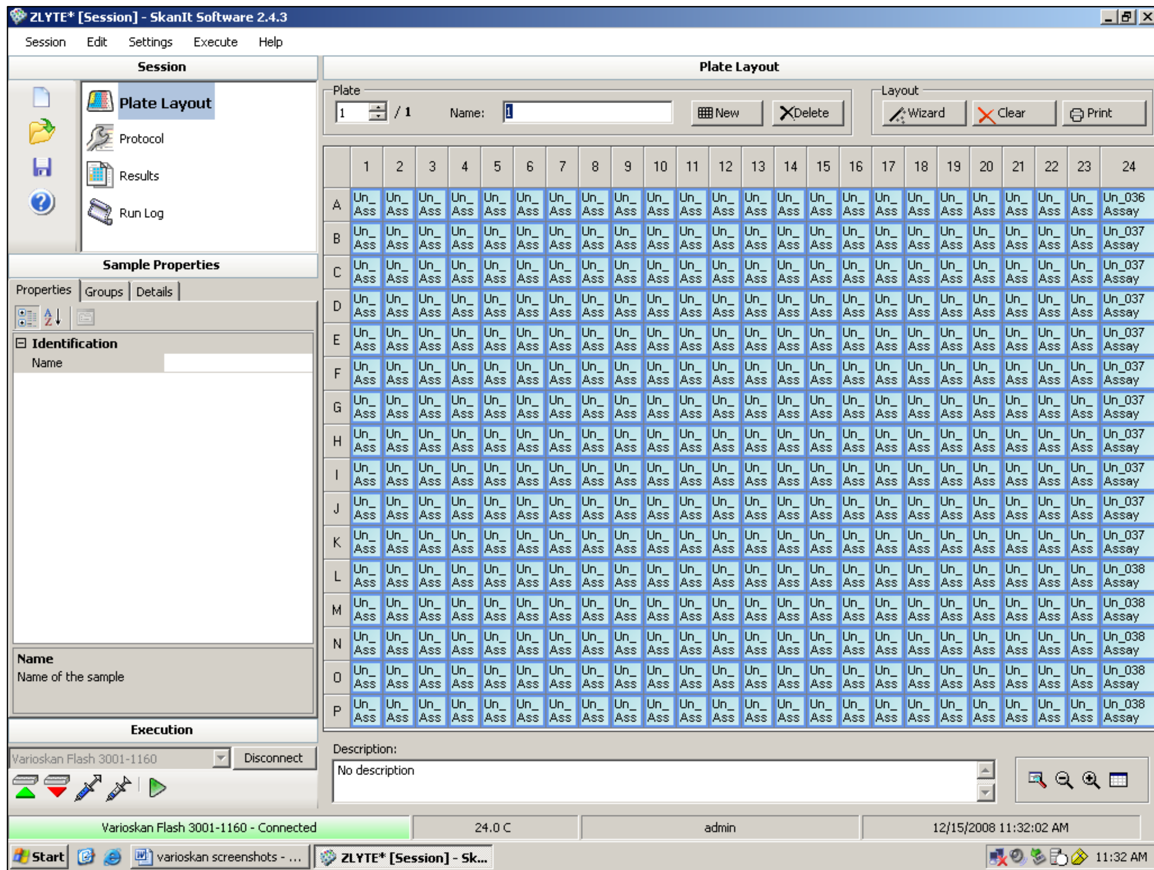
6. A new window will appear. The next step is to define the plate layout. Select the Wizard tab in the upper right.



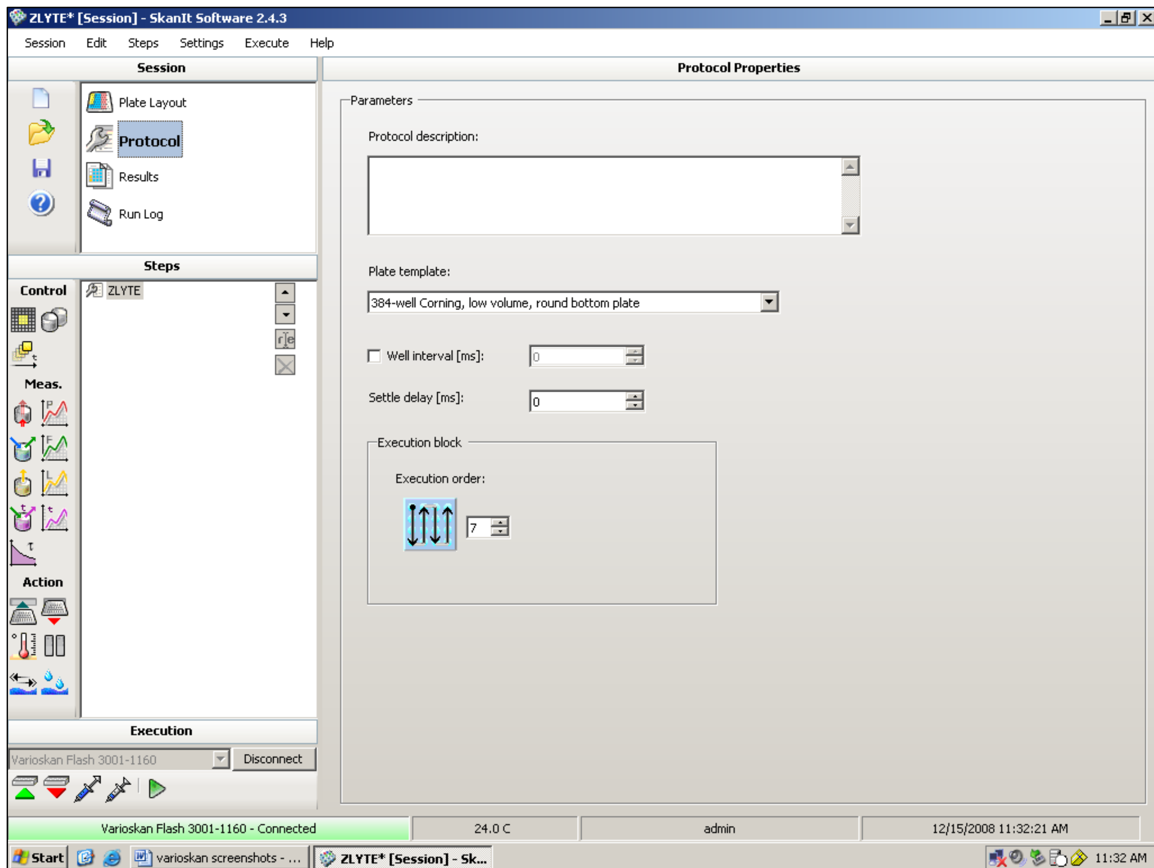
7. The Wizard popup will appear. To fill the entire plate with unknowns, enter 384 in the "No. of unknowns" blank near the center left of the popup. Select the "Add and Close" tab when finished.



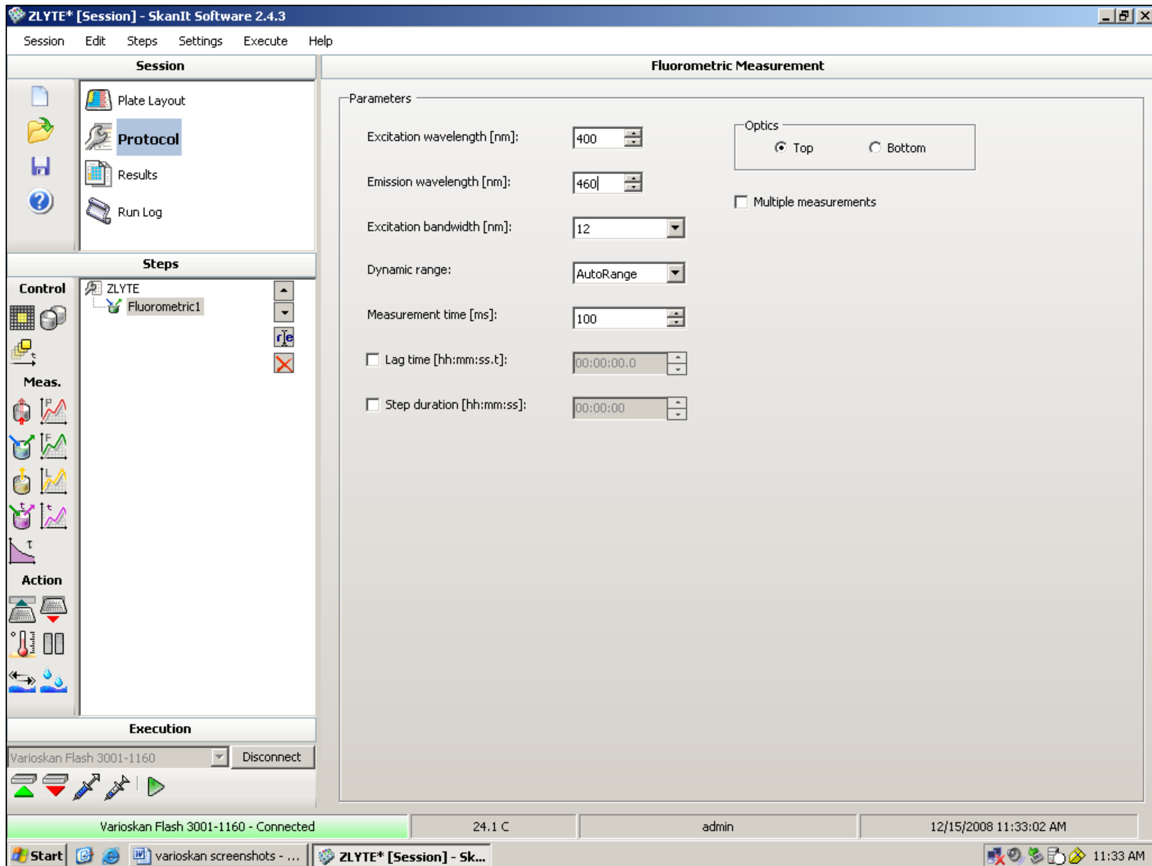
8. The Wizard popup will close, and the plate will now be defined.



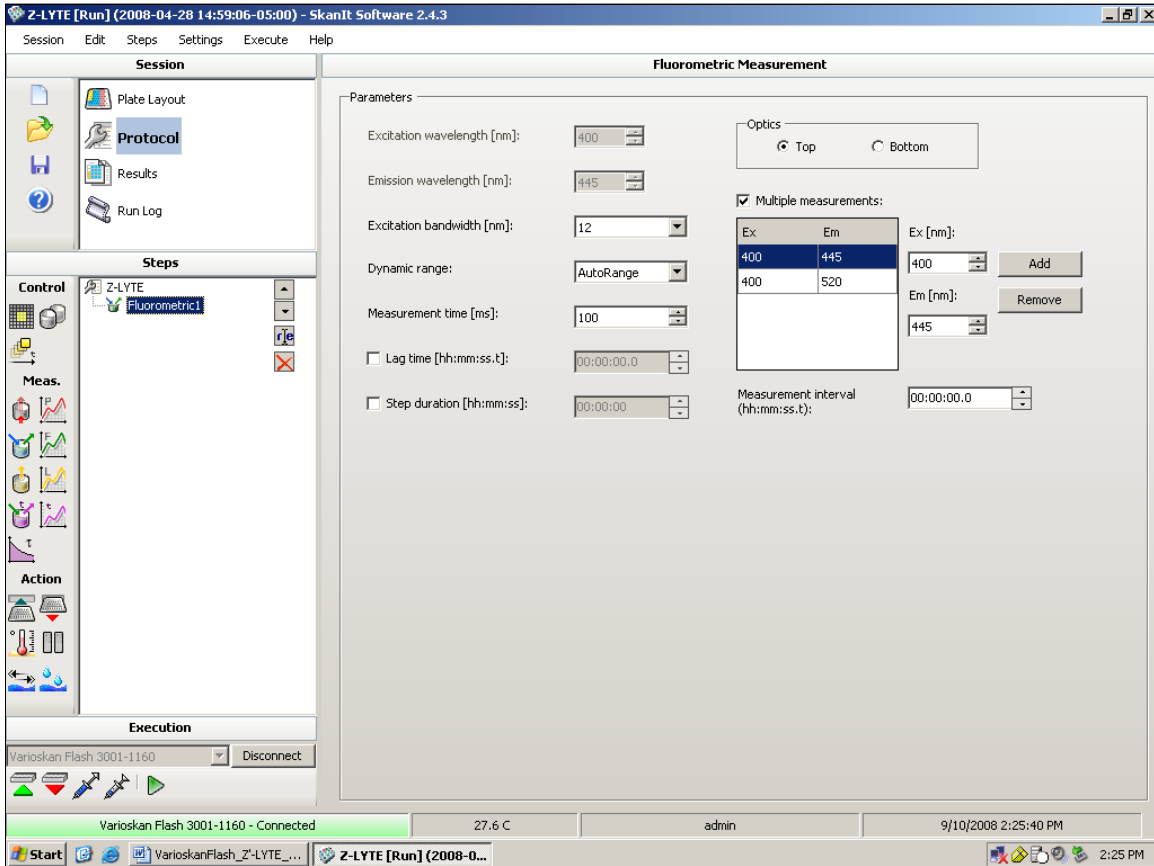
9. From the Session menu on the left, select "Protocol". The screen will change to the one below. In the "Meas." menu on the left, select the "FRET Measurement" icon to set up a FRET protocol.



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, and make sure the Optics setting is correct.



11. 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. The new excitation and emission settings will populate beneath the first set in the table to the left of the "Add" tab.



12. At this point the protocol is finished, select the "Execute session" icon (small green arrow on the bottom left side) to read a plate.

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 section below describes how the data was obtained, and is not intended for use as an assay protocol. We recommend all first-time users follow the appropriate protocols and/or validation packets provided with their specific assay kits, and include all proper controls. 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® Custom Profiling Z'-LYTE®-based kinase assay service can be seen at www.invitrogen.com/kinaseprofiling. You can find the link for Z'-LYTE Protocols and Assay Conditions under the More Information section on the right side of the web page.

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.

			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																								
		G																								
		H																								
		I																								
		J																								
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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 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 $MgCl_2$, 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.

																						No Inh.		0	100
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Staurosporine	A																								
	B																								
	C																								
	D																								
JAK2 Inh. II	E																								
	F																								
	G																								
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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

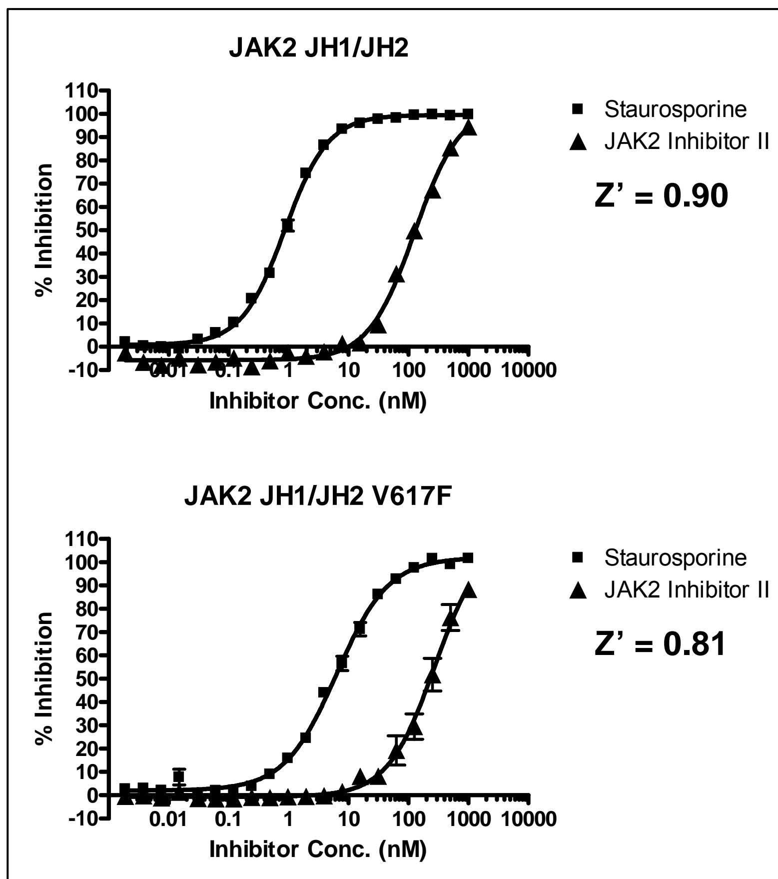

Figure 1: Z'-LYTE® Kinase Assay. Z'-LYTE® assay performed using the Thermo Scientific Varioskan® Flash.