LanthaScreen® Terbium 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 LanthaScreen® Terbium-based TR-FRET Assay using the LanthaScreen® Fluorescein-Poly GT (PV3610) and Tb-PY20 antibody (PV3552) 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.

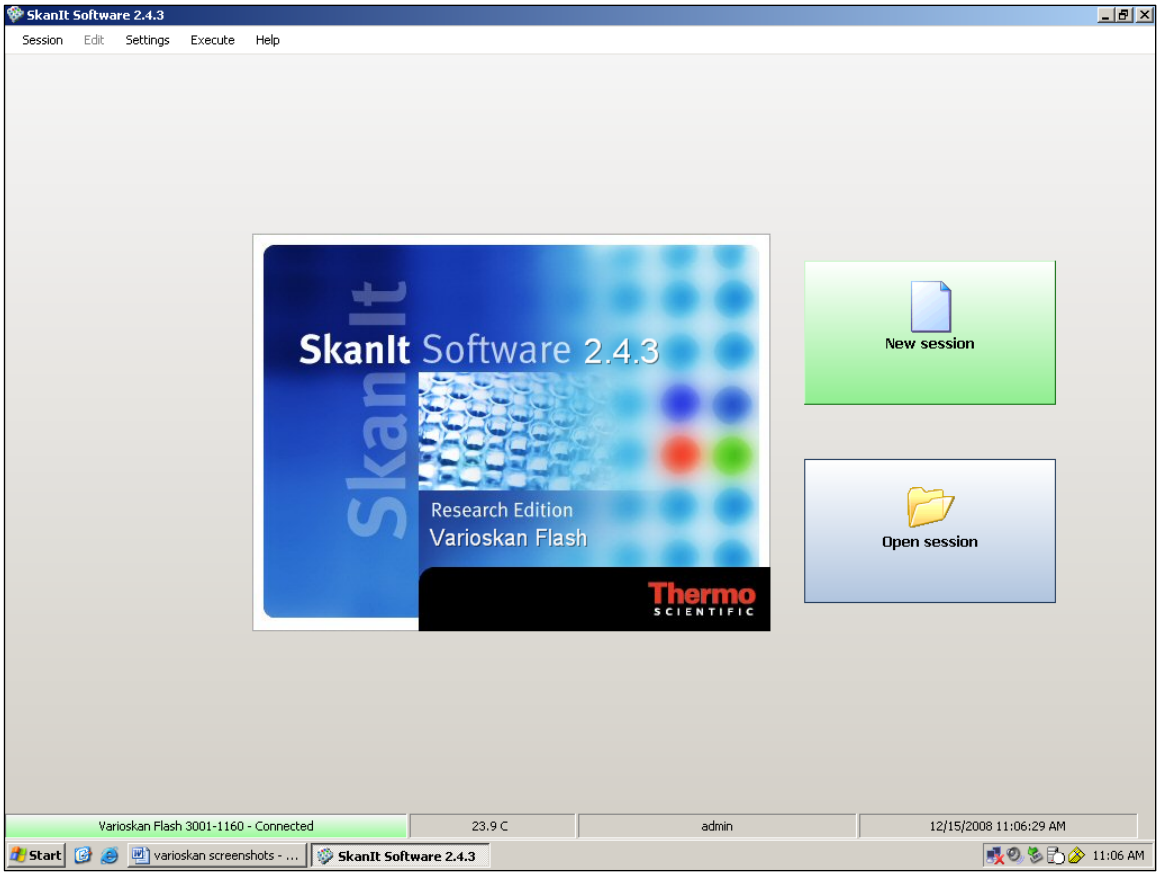
A. Recommended Optics

	wavelength (nm)	diameter (nm)
Excitation	332/12	monochromator
Emission 1	488/12	monochromator
Emission 2	518/12	monochromator

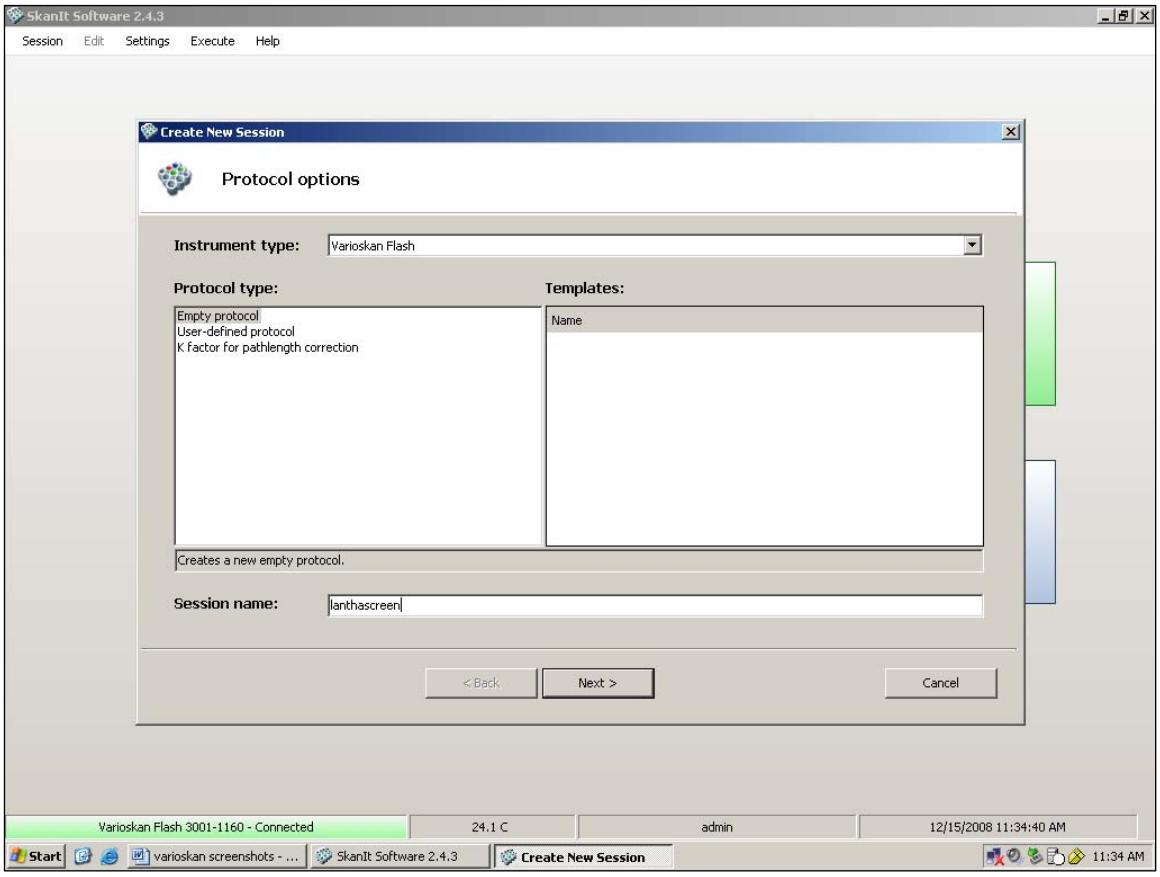
B. Instrument Setup

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

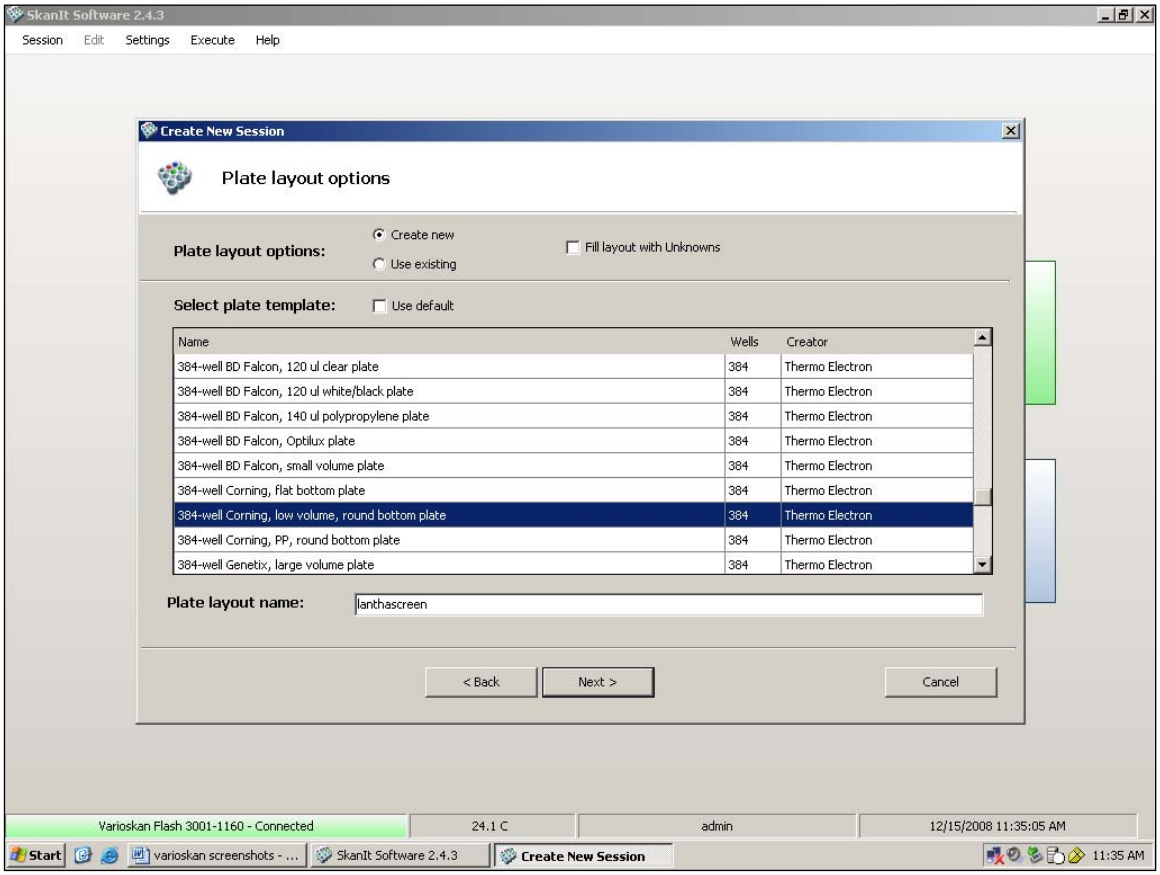
2. When SkanIt opens, it will default to a generic starting page. Select the "New Session" icon to open a new session and create a new protocol.



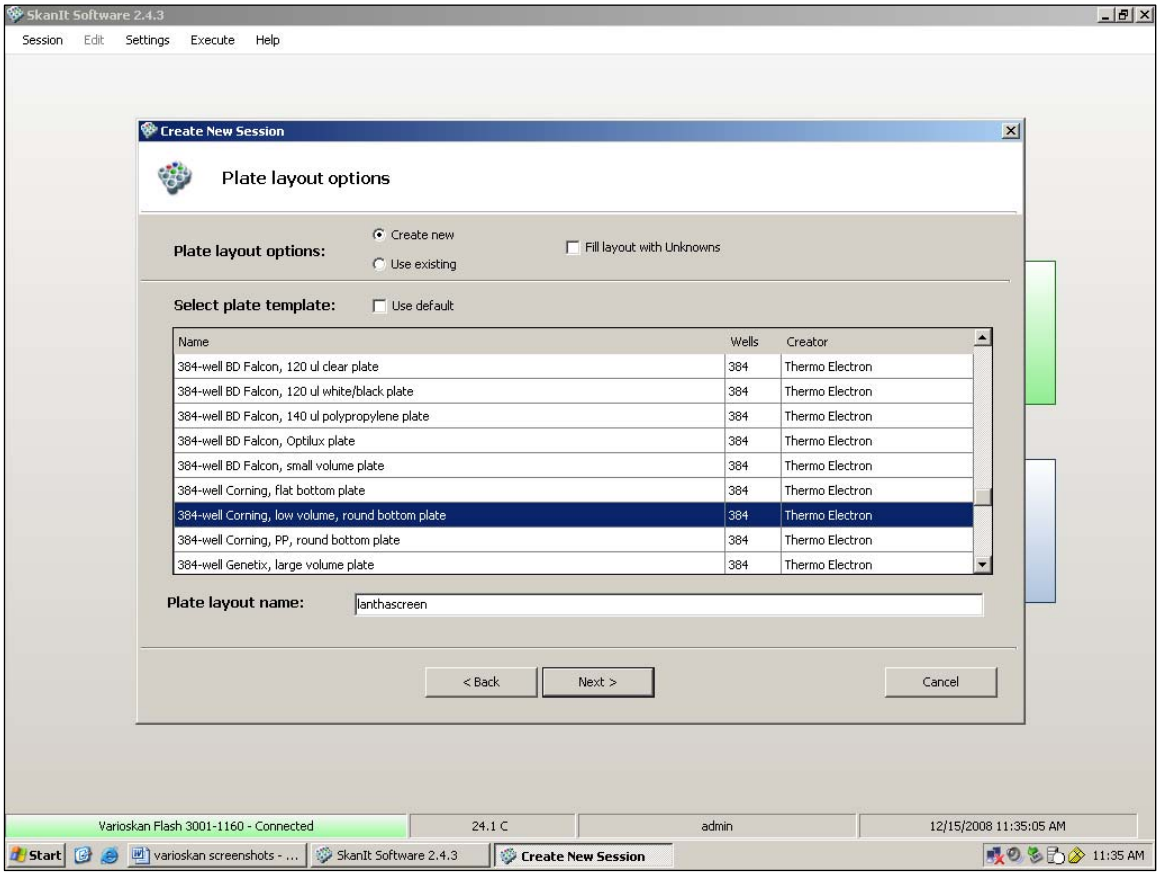
3. At this point, a popup window will appear. Select "Empty Protocol" on the left, and enter a session name at the bottom. Select Next when finished.



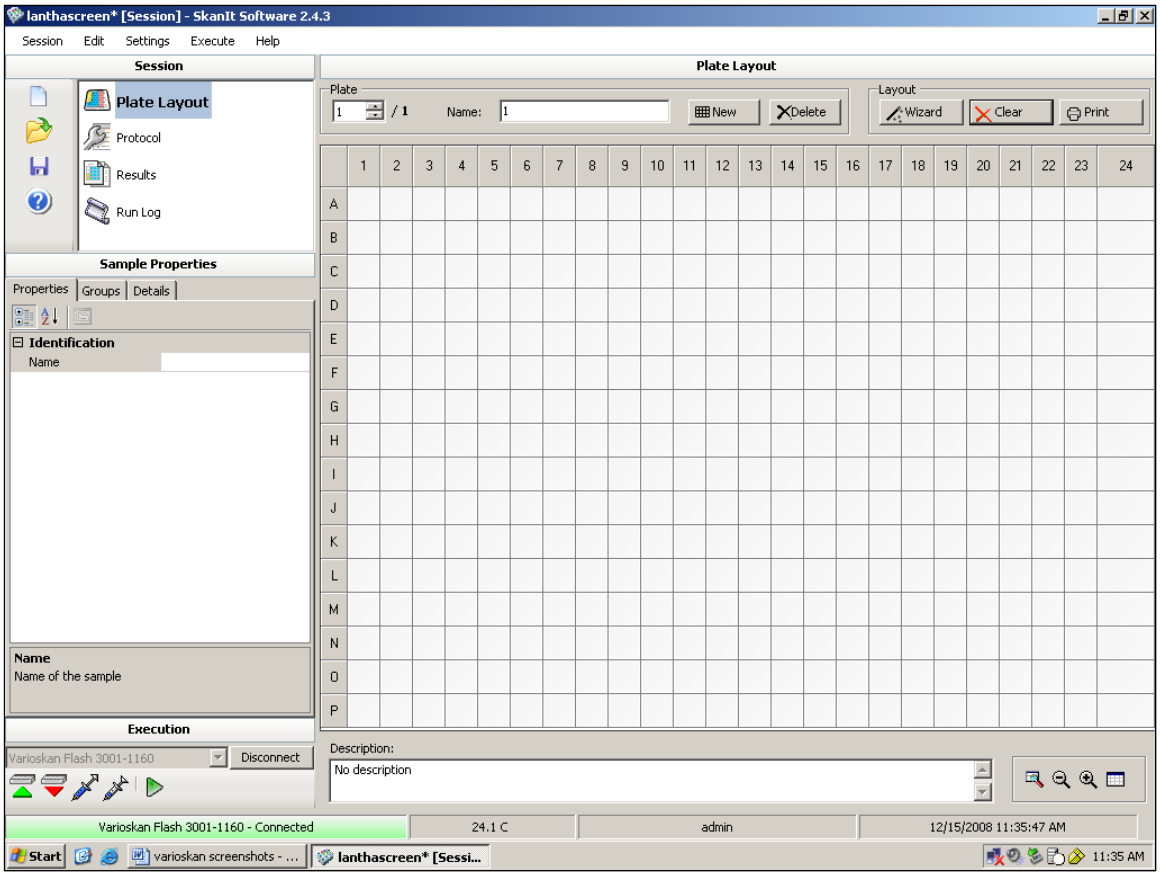
4. Select your plate from the available list. When finished, select Next.



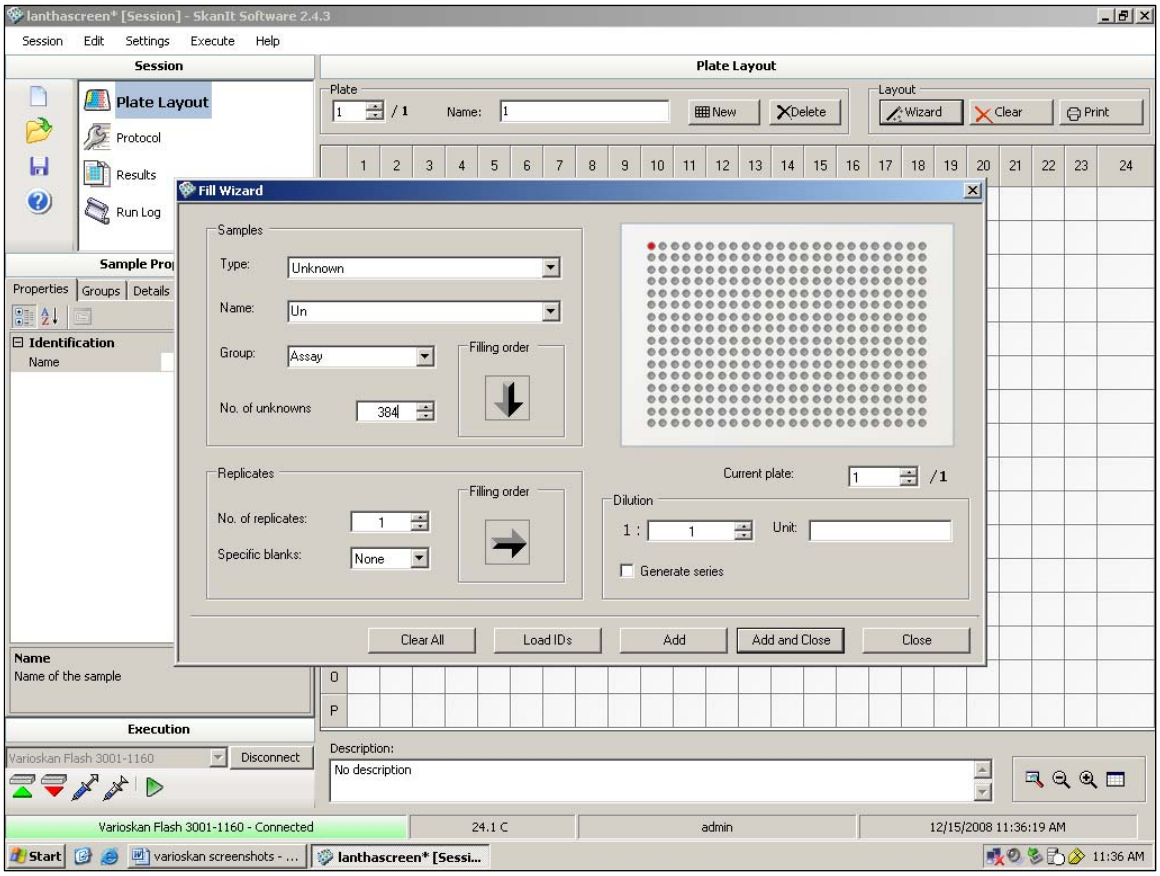
5. Once the session has been named and a plate selected, click the Finish tab to create a new protocol.



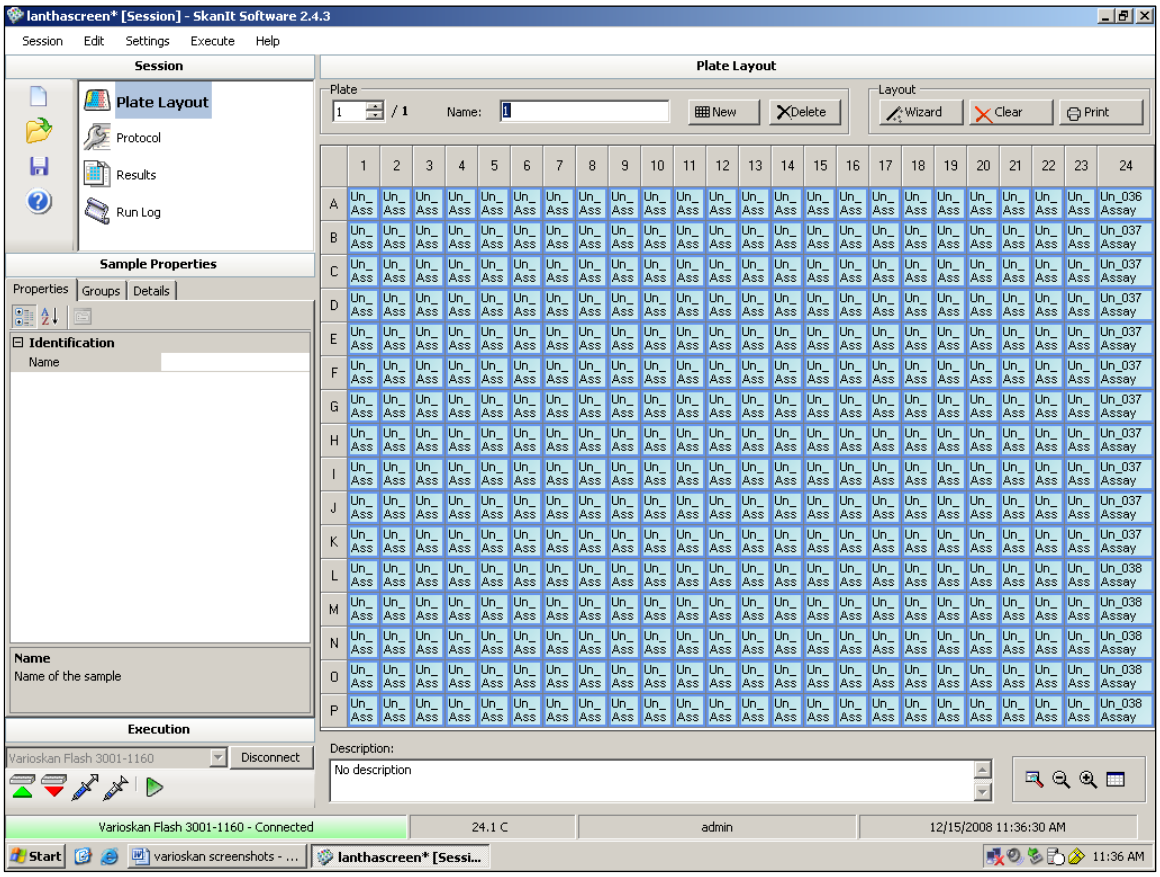
6. A new window will appear. The next step is to define the plate layout. Select the Wizard tab in the upper right.



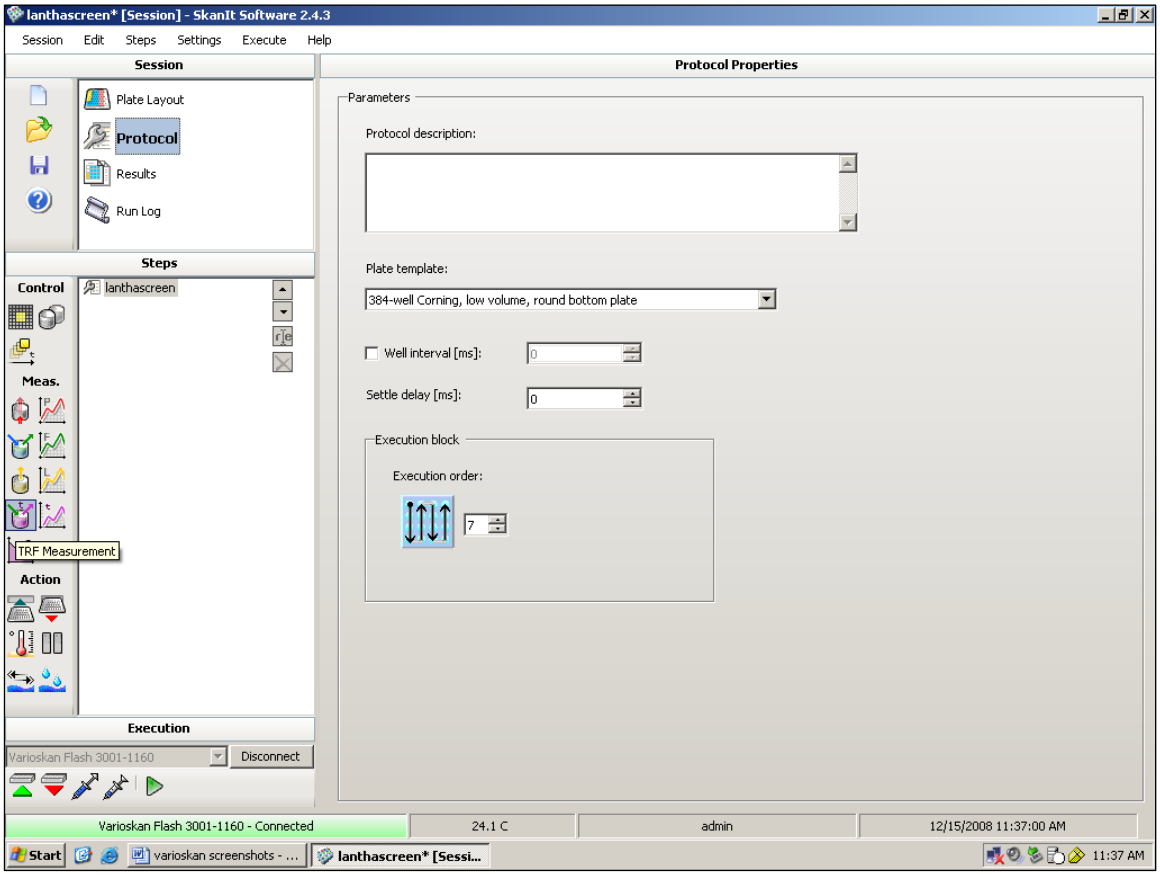
7. The Wizard popup will appear. To fill the entire plate with unknowns, enter 384 in the "No. of unknowns" blank near the center left of the popup. Select "Add and Close" tab when finished.



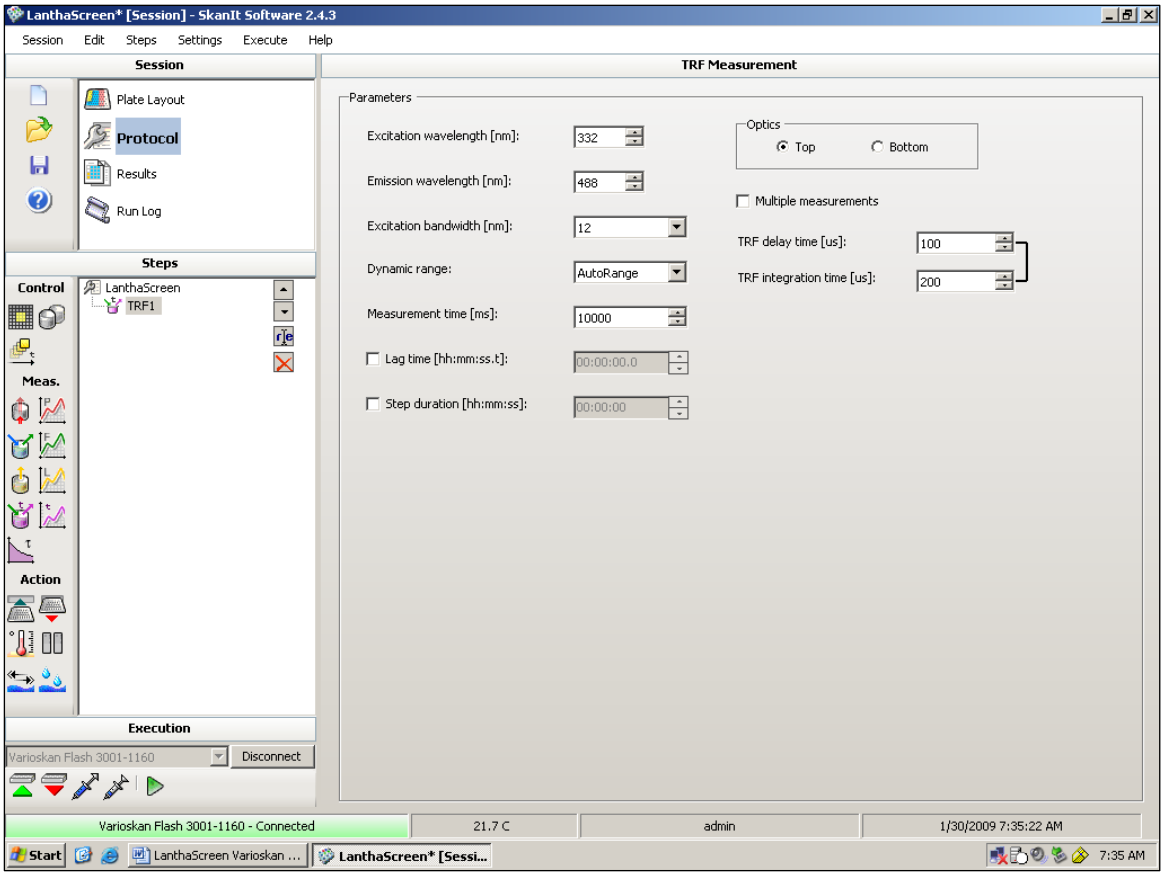
8. The Wizard popup will close, and the plate will now be defined.



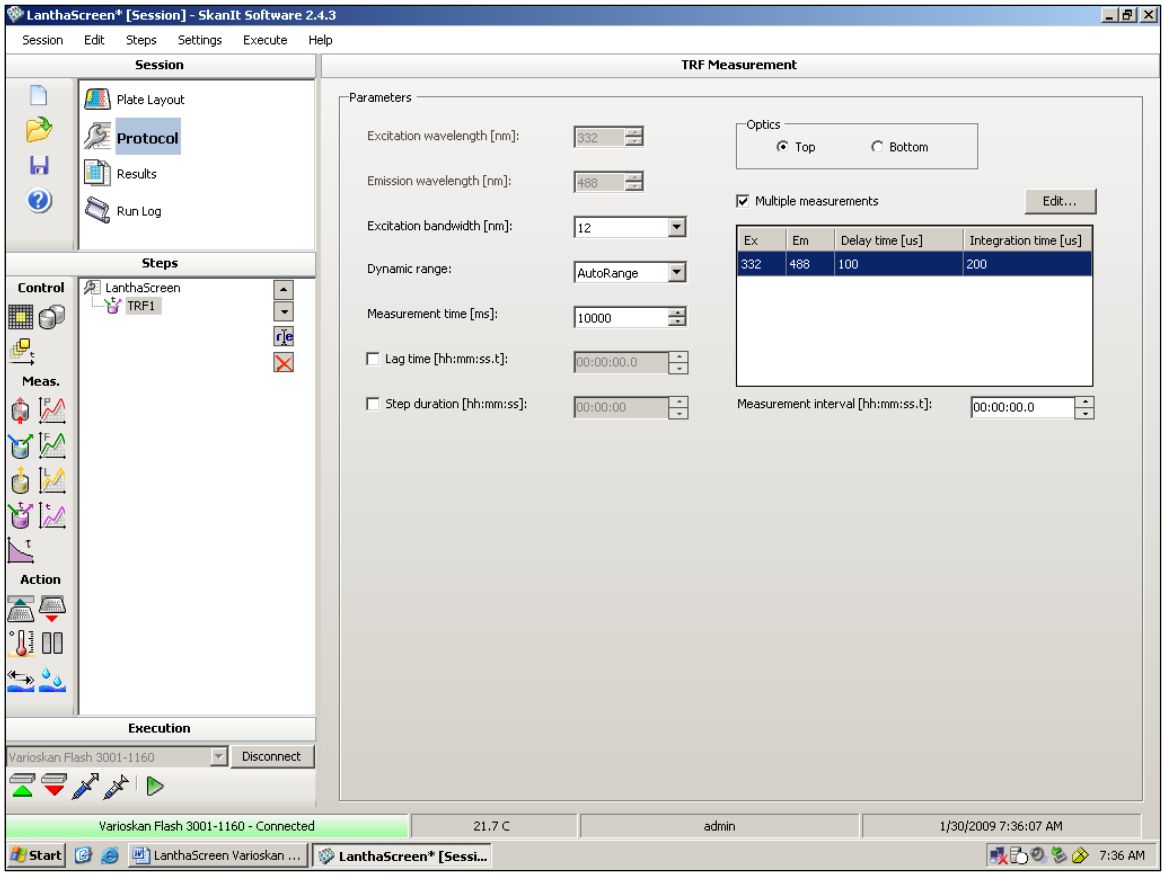
9. From the Session menu on the left, select "Protocol". The screen will change to the one below. In the "Meas." menu on the left, select the "TRF Measurement" icon to set up a Time Resolved FRET protocol.



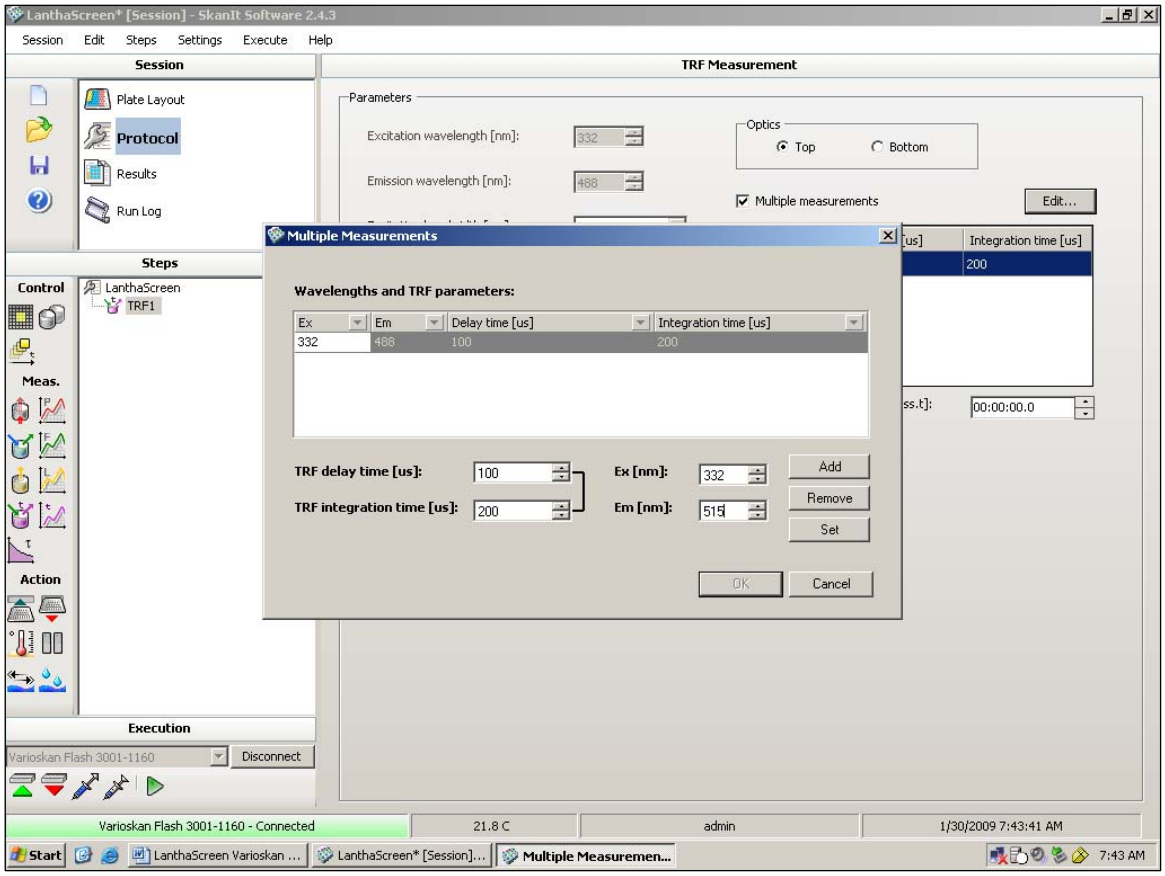
10. Selecting the TR-FRET icon will cause the screen to change to look like the one below. Select your excitation and donor emission wavelengths as shown below. Make sure the Optics setting is correct, and set the TRF delay and integration times to 100 and 200 µs, respectively. Set the Measurement time to 10000 ms. (Note increasing the measurement time serves to increase the number of flashes, and therefore increases the sensitivity by reducing variance. 10000 ms results in relatively long reads, we have also successfully used 2000 ms, which may prove more practical.)



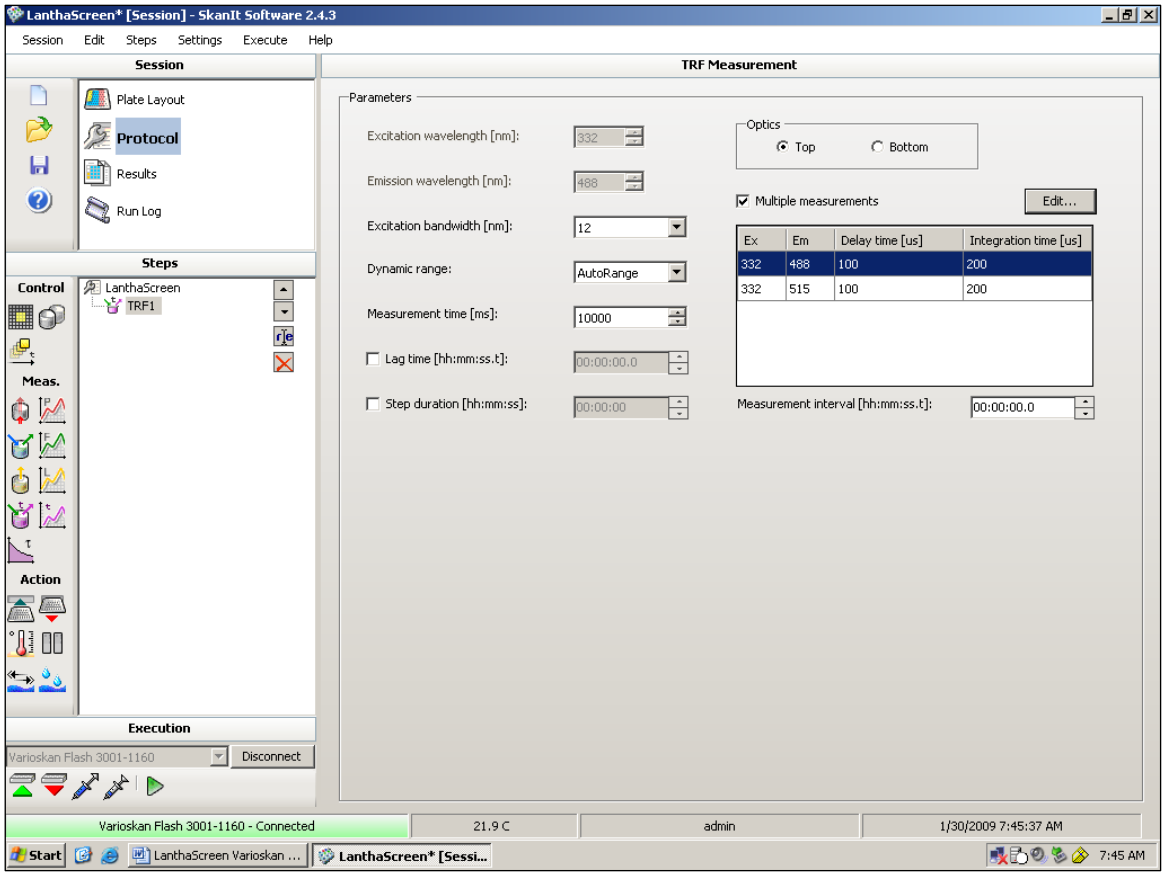
11. When finished, click on the "Multiple measurements" tab and then the "Edit" tab beside it.



12. Another popup will now appear. Add your excitation and second (acceptor) emission setting in the Ex and Em boxes shown, and when finished, select the "Add" tab.



13. The popup window will disappear, and the second set of excitation/emission parameters will now also show in the table below the "Multiple measurements" button.



14. Once all settings have been selected, and a plate is inserted and ready to read, select "Execute session" (small green arrow near bottom left) to read.

C. LanthaScreen® Kinase Assay using JAK2 JH1/JH2 and JAK2 JH1/JH2 V617F

NOTE: The following is a sample titration assay performed for demonstration purposes. The instrument settings above would be sufficient for any LanthaScreen® assay, the information below is provided as representative data only and the following section is an explanation of what was done. We recommend all first-time users begin by following the provided assay-specific protocols and/or validation packets, and include proper controls. This assay was run at ATP Km_{app} and a kinase concentration producing approximately 70-80% of maximal phosphorylation. ATP and kinase concentrations should be optimized for each kinase by the actual user and titrations/plate layout may be optimized as well. For more information on setting up assays, visit www.invtrogen.com/lanthascreenkinase.

1. Prepare initial 100X serial dilution curves in rows A, E, I, and M of a 384-well plate (Figure 1): Dilute Staurosporine, JAK2 Inhibitor 2, JAK3 Inhibitor, and AG-490 to a 100X initial concentration (100uM) in 100% DMSO. 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.

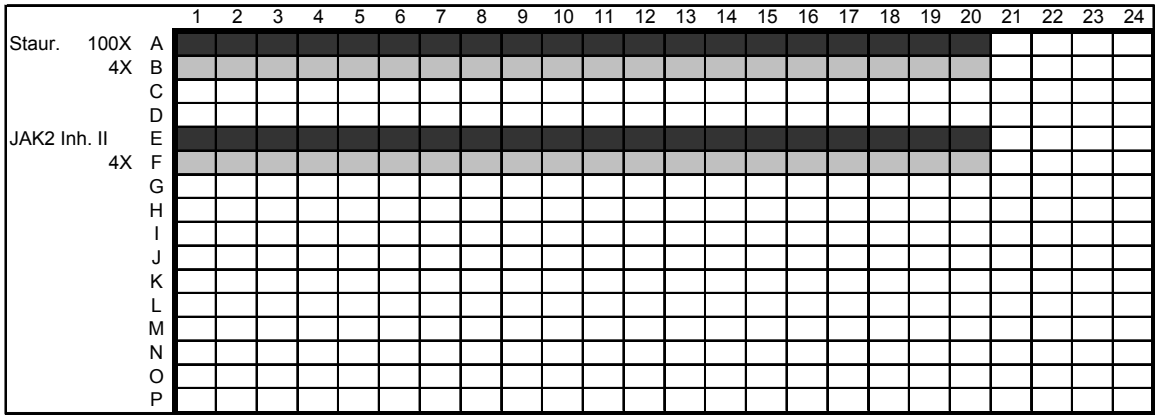


Figure 1: Schematic of initial compound dilution. Staurosporine, JAK2 Inhibitor II, JAK3 Inhibitor, and AG-490 were all titrated from a 100 µM starting concentration in the initial dilution series by preparing a 1:1 dilution curve in DMSO. A secondary dilution to 4X was then prepared in the rows below 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 (PV3189, 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 (800 nM Fluorescein-Poly GT peptide, PV3610, 222 ng/ml JAK2 JH1/JH2 or 270 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 800 nM substrate alone without kinase to Column 23, rows A-L (0% phosphorylation control) and 5 µl of 800 nM phosphopeptide control substrate to Column 24, rows A-L (100% phosphorylation control). Add 5µl 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 (20 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 10 µl per well of 20 mM EDTA, 2 nM TB-PY20 antibody (PV3552) mix diluted in TR-FRET Dilution Buffer (PV3574) per well to stop kinase reaction. Do not add to Rows M-P,Columns 23 and 24 (buffer controls only), instead replace with 10 µl TR-FRET Dilution Buffer supplemented with EDTA only.

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

11. Incubate for 30 minutes at room temperature.

12. Read and analyze as directed in the protocol.

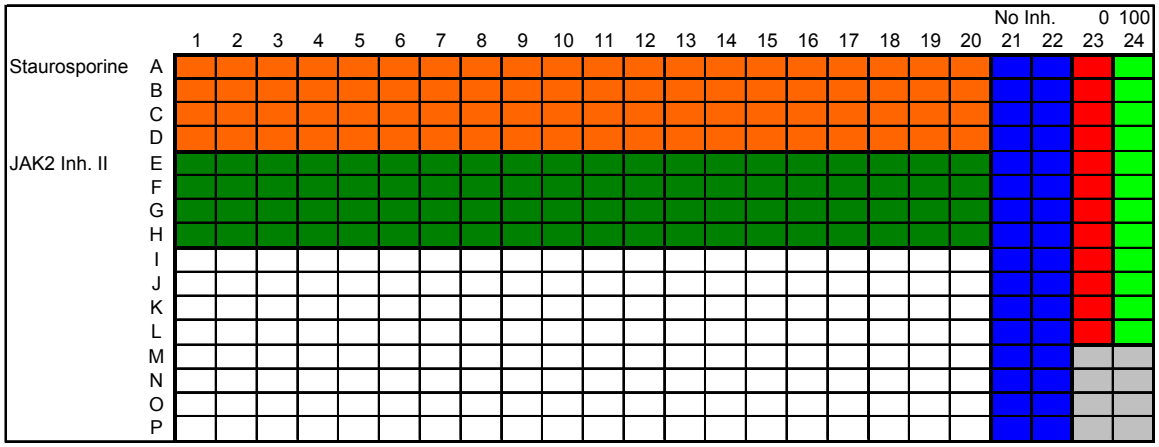


Figure 2: Assay Plate Schematic. Compound titrations shown in Columns 1-20, Columns 21 and 22 prepared without any inhibitor as kinase activity controls, Column 23 prepared with no kinase (0% phosphorylation) and Column 24 prepared using phosphopeptide control (100% phosphorylation). Note 8 wells in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

D. Results:

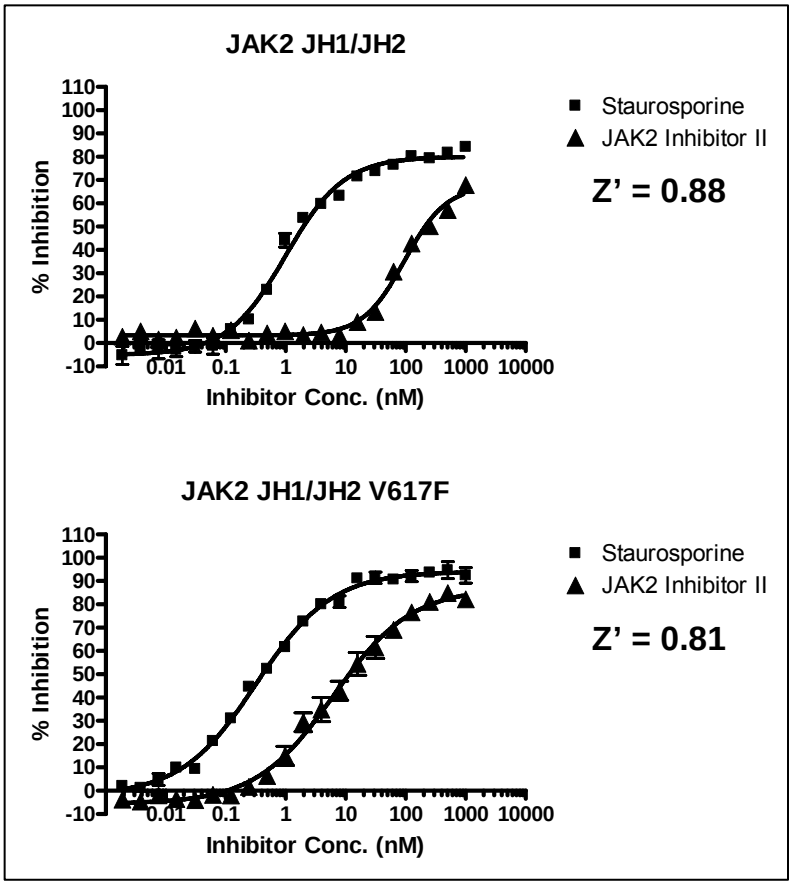


Figure 3: LanthaScreen® Assay. LanthaScreen® assay performed with the Thermo Scientific Varioskan® Flash.