LanthaScreen® Terbium Assay Setup Guide on the Tecan Safire²™ Microplate Reader

NOTE: The Tecan Safire²™ Microplate 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. 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 Tecan instruments or software, please contact Tecan at 1-888-798-0538 or info@tecan.com.

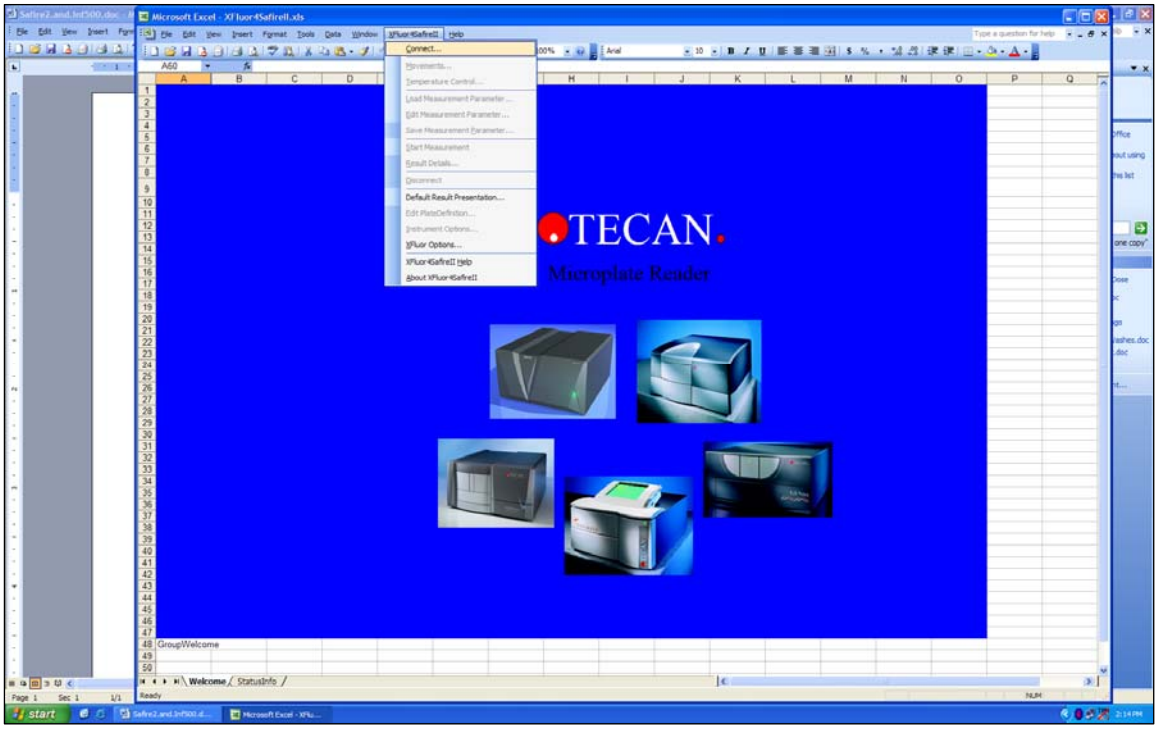
A. Recommended Optics

	wavelength (nm)	diameter (mm)
Excitation	332/20	monochromator
Emission 1	485/20	monochromator
Emission 2	515/20	monochromator

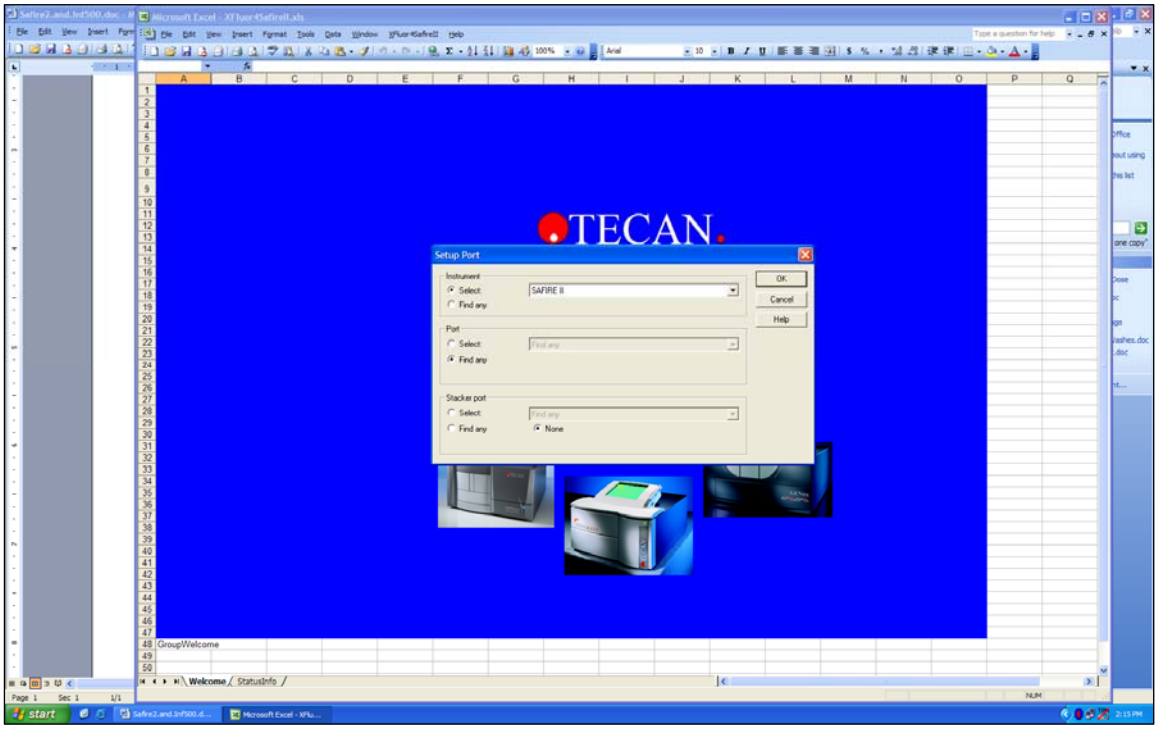
B. Instrument Setup

1. Make certain plate reader is turned on, and open up XFluor Data Manager software on computer.

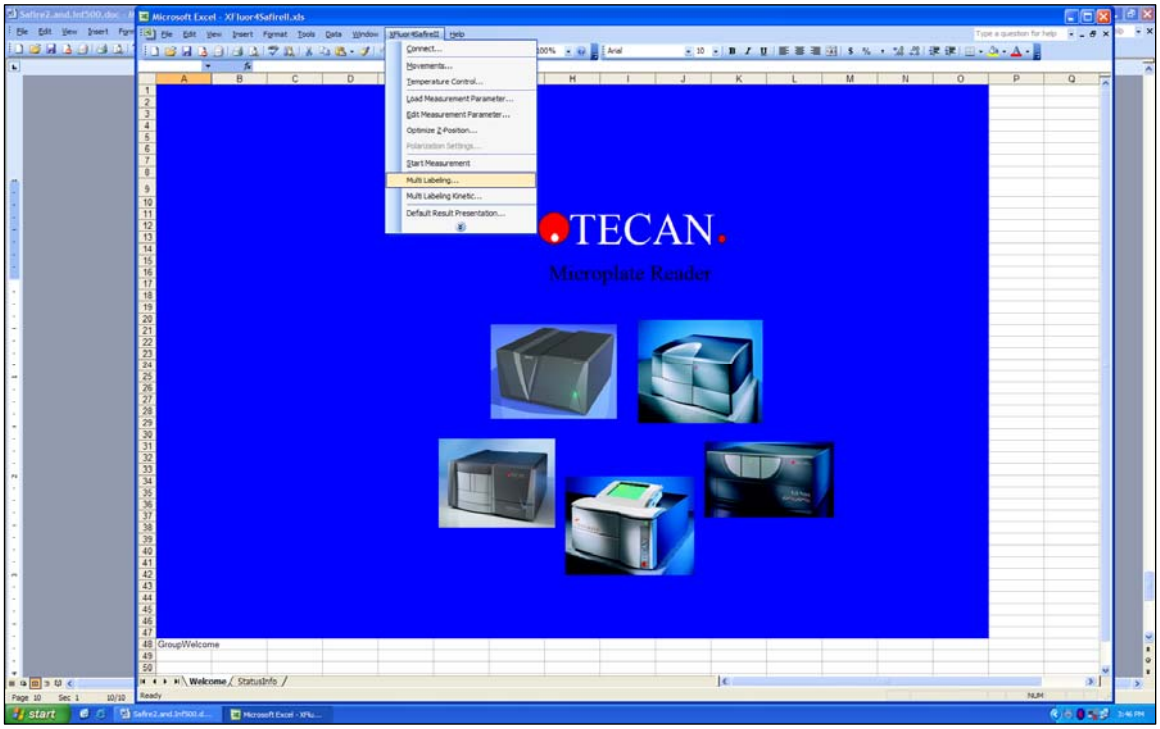
2. When XFluor opens, it will default to a generic starting page. From the menu bar on the top, go to "XFluor4SafireII" and select "Connect" from the drop-down menu.



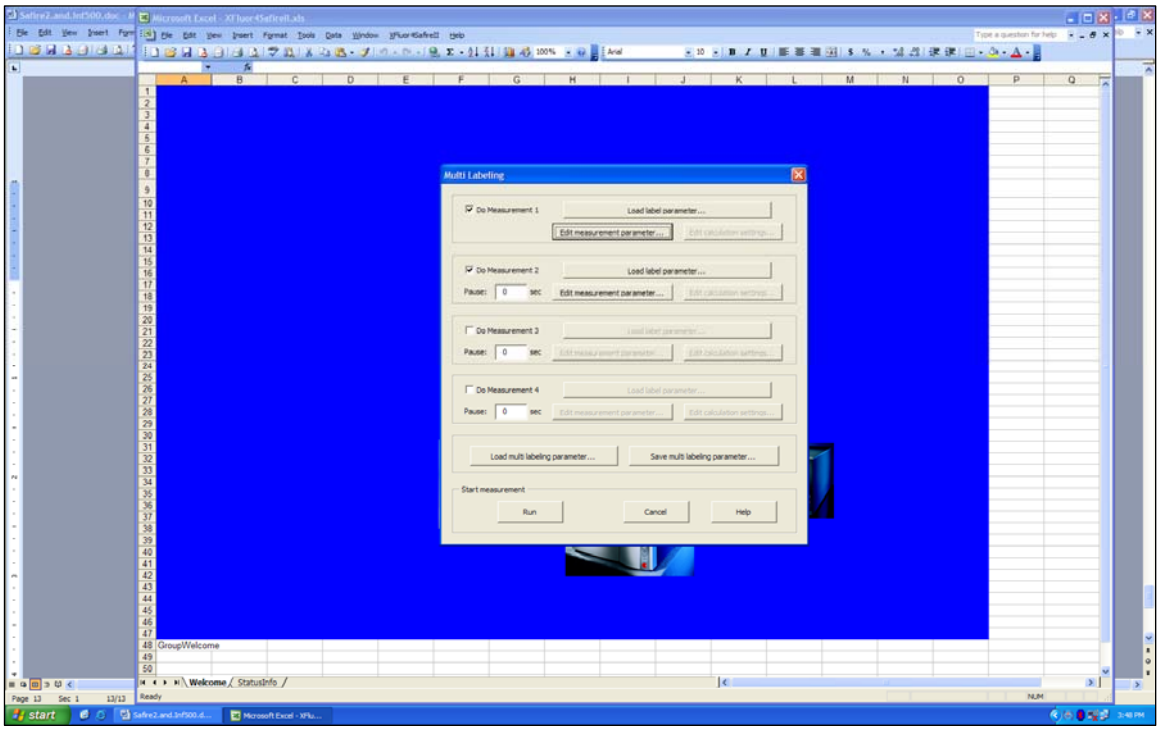
3. Select your instrument (Safire II) and click OK.



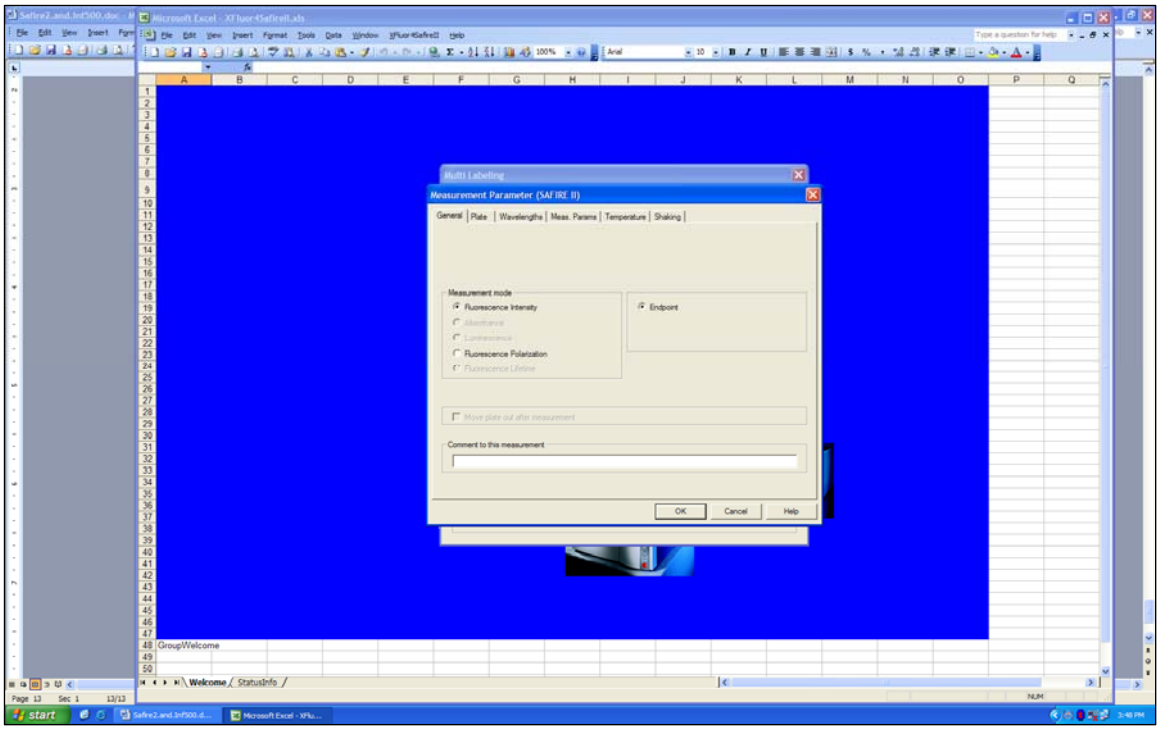
4. From the XFluor drop-down menu, select "Multi Labeling".



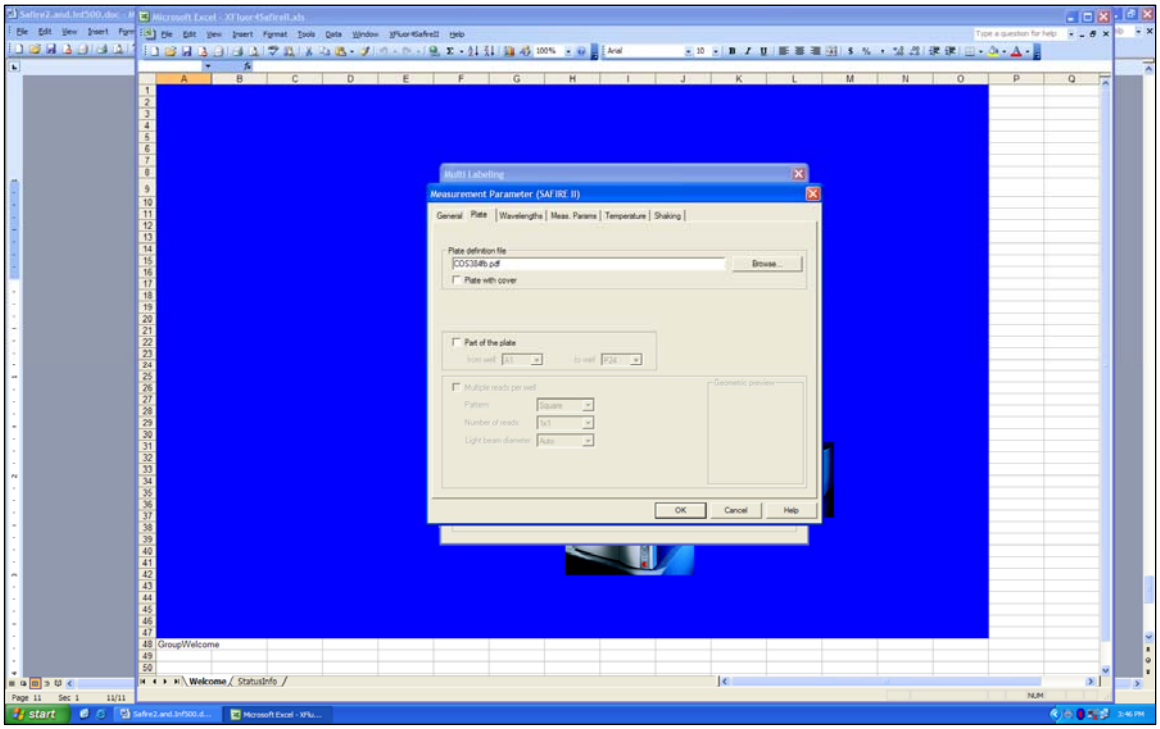
5. A new window will open. LanthaScreen® uses a donor and an acceptor, so check the boxes for "Do Measurement 1" and "Do Measurement 2". Next, click the"Edit Measurement Parameter" tab in the Measurement 1 box to set up the donor fluor measurement parameters.



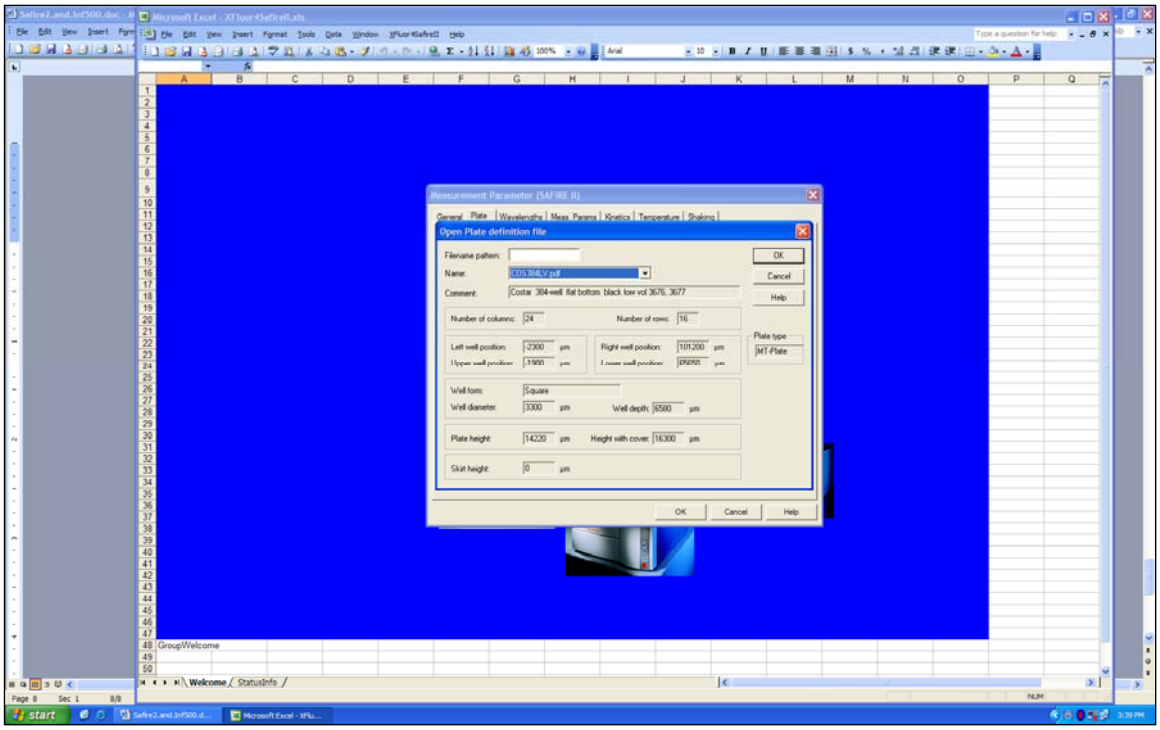
6. A new window will again open; under the General tab make sure "Fluorescence Intensity" and "Endpoint" are selected.



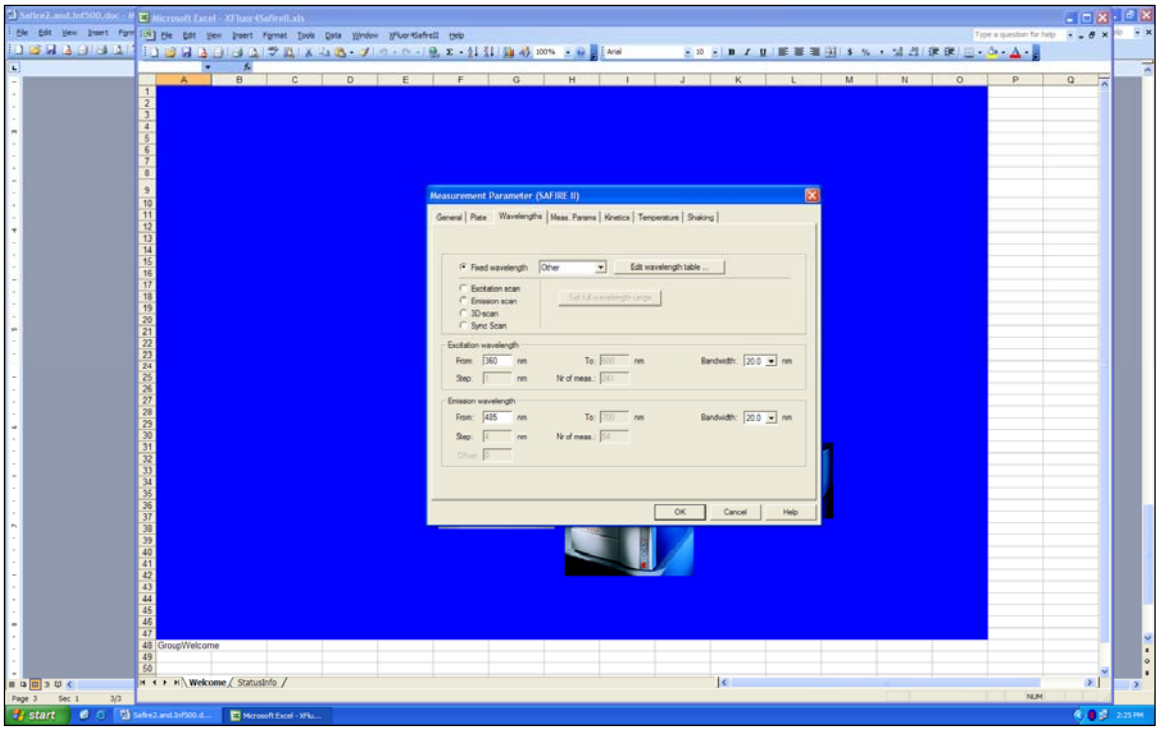
7. Select the Plate tab. Make sure the whole plate is set to be read, and click the "Browse" tab to select your plate.



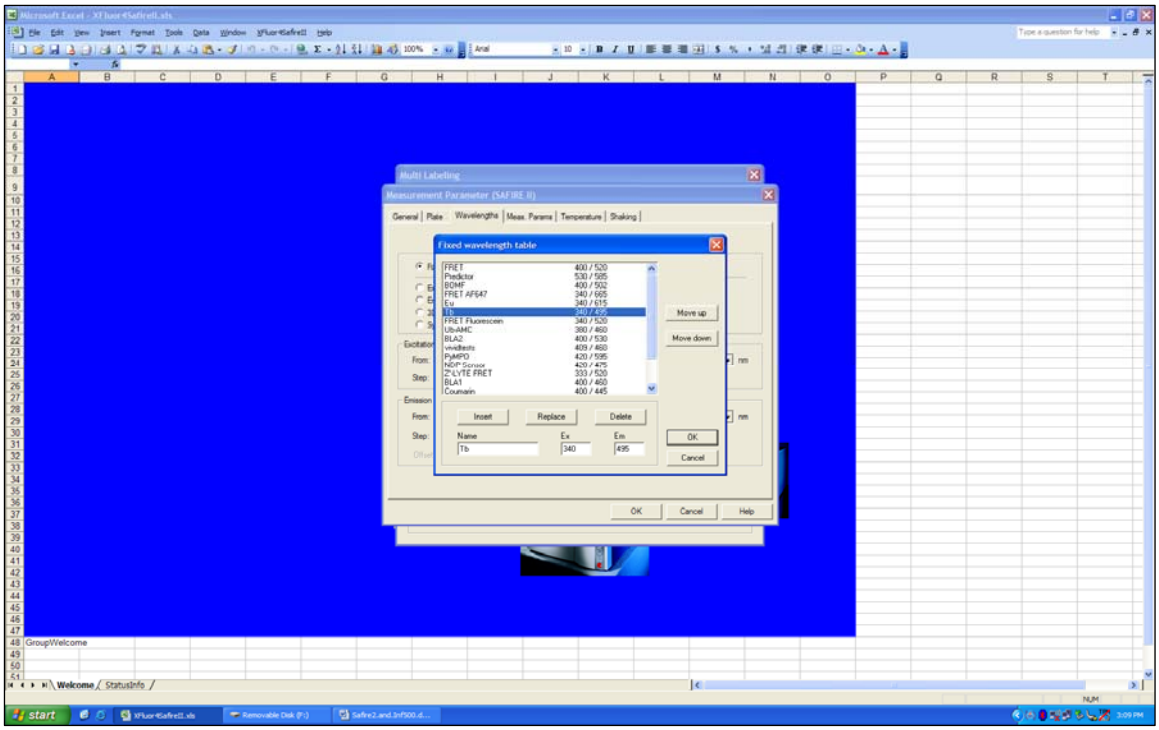
8. A new window will appear; from the drop-down menu, select your plate. Note we have run LanthaScreen® using both white and black plates following the protocol recommendations. When finished, select OK to close this window.



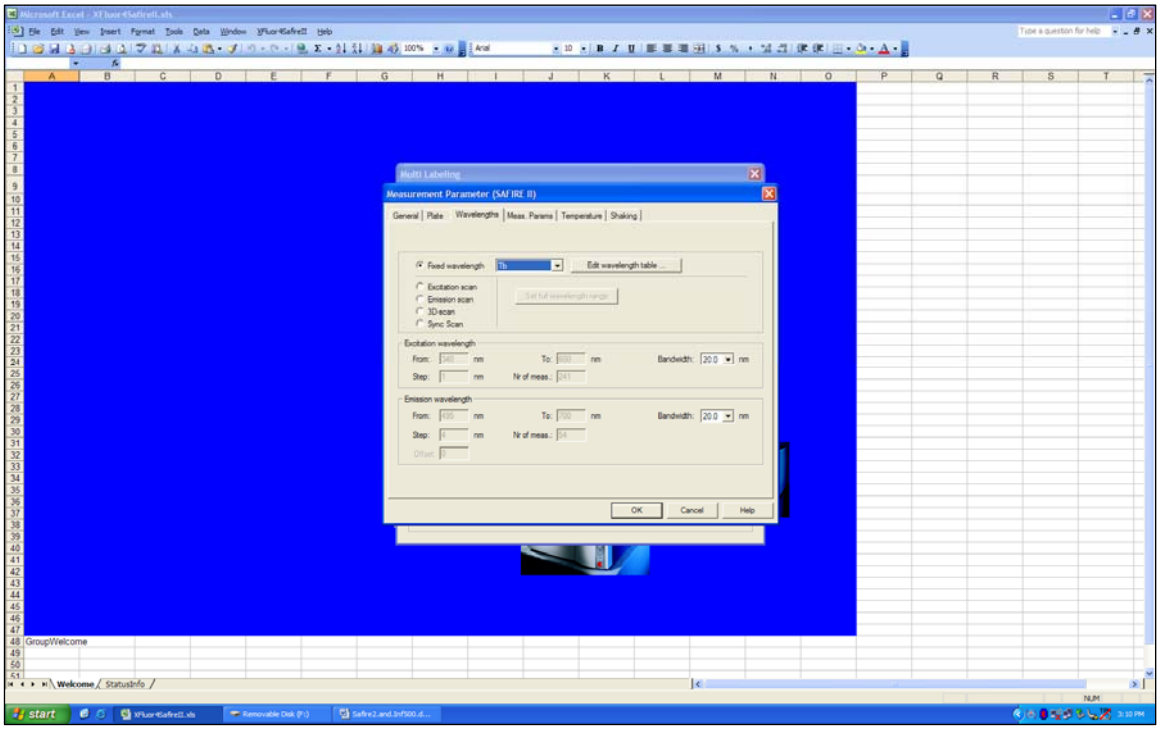
9. Select the Wavelengths tab. Check the "Fixed Wavelength" button, and then select the "Edit wavelength table" tab to enter your excitation and donor emission settings.



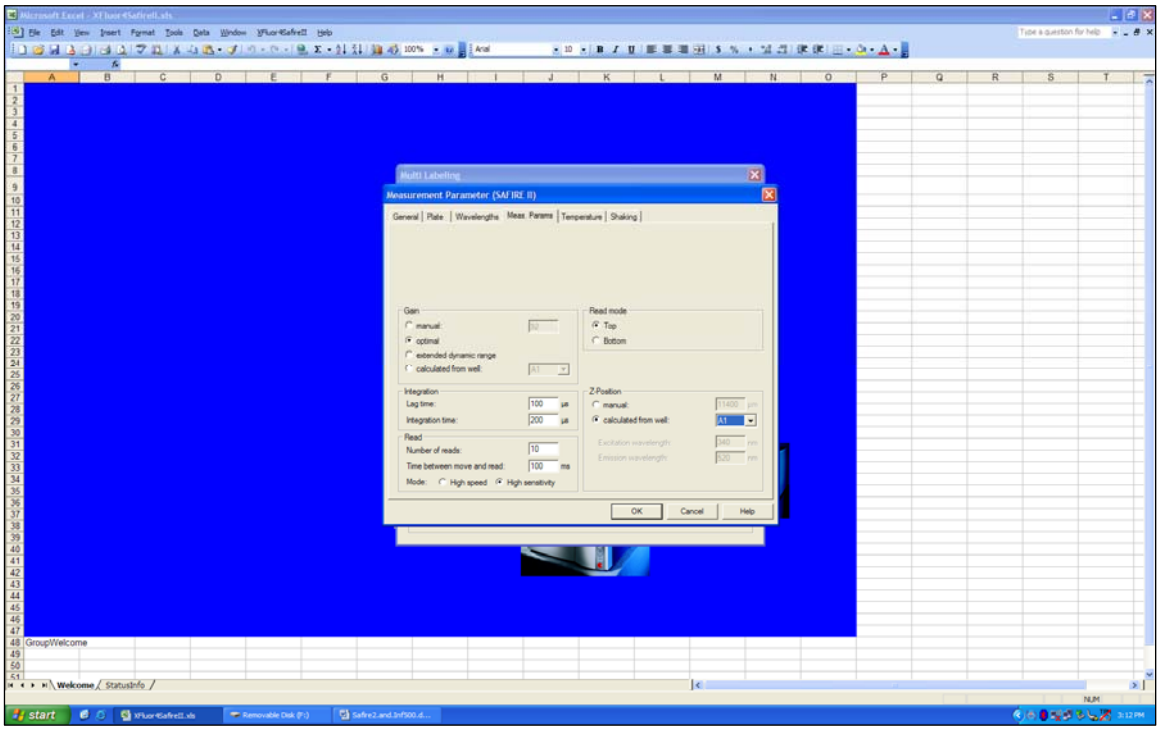
10. A list of previously defined excitations and emissions will appear. If there is not a setting for LanthaScreen® proceed to the bottom and add by inserting a name and Excitation and Emission values. When finished press the "Insert" tab to add to the list, then make sure it is highlighted, and select OK.



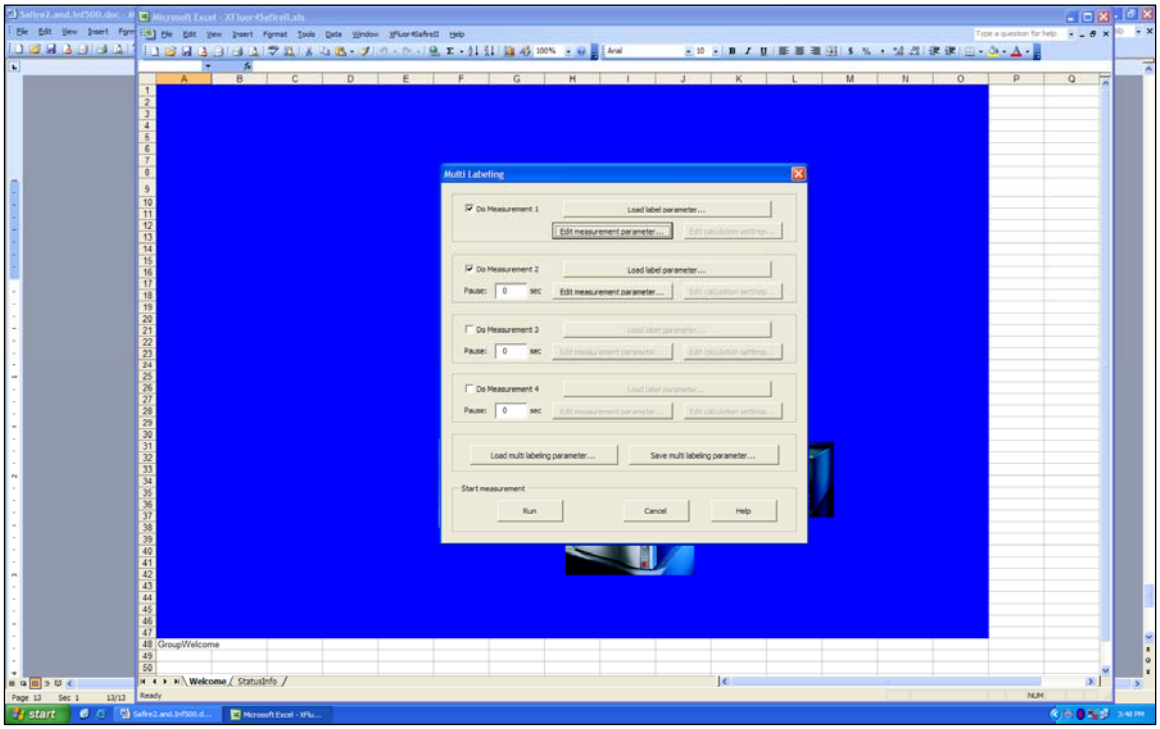
11. You will return to the Measurement Parameter window. Select the appropriate bandwidth settings and when finished select the Meas. Params. tab.



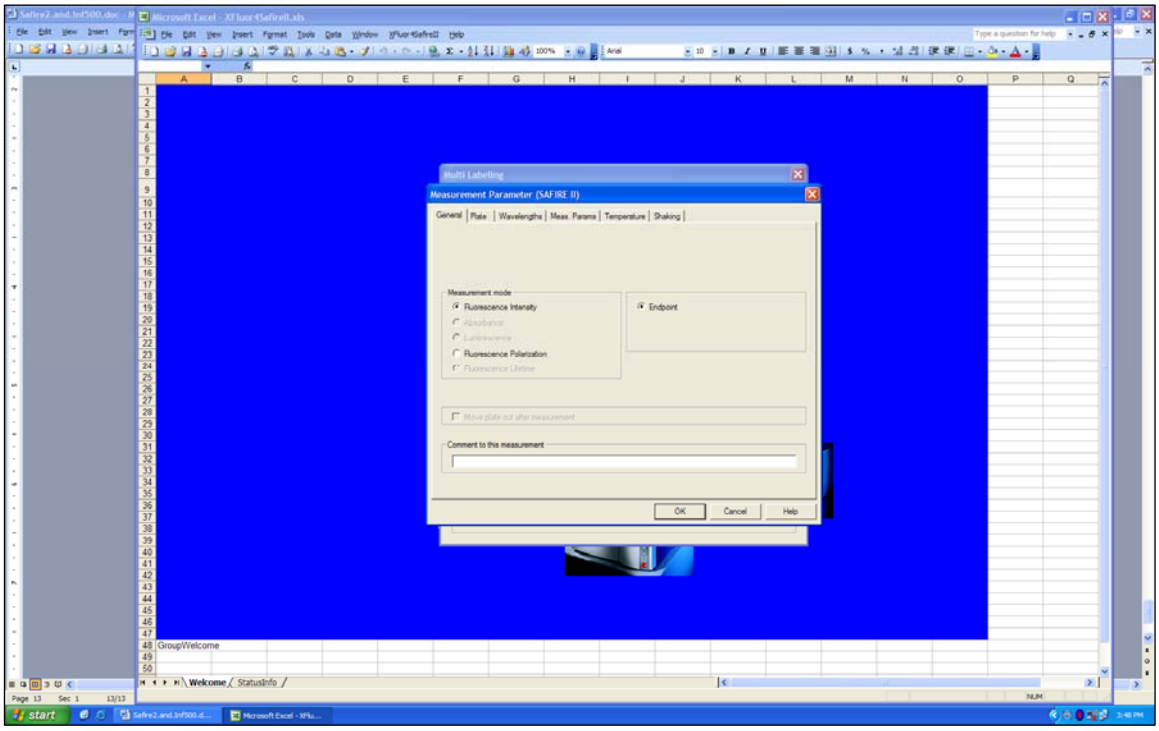
12. LanthaScreen® is a Time-Resolved FRET assay; set Lag and Integration times to 100 and 200 µs, respectively. Make sure the Read mode is set to Top, and in this case Gain was set to Optimal and Z-Position was set to well A1. When finished with all parameters, click OK at the bottom right.



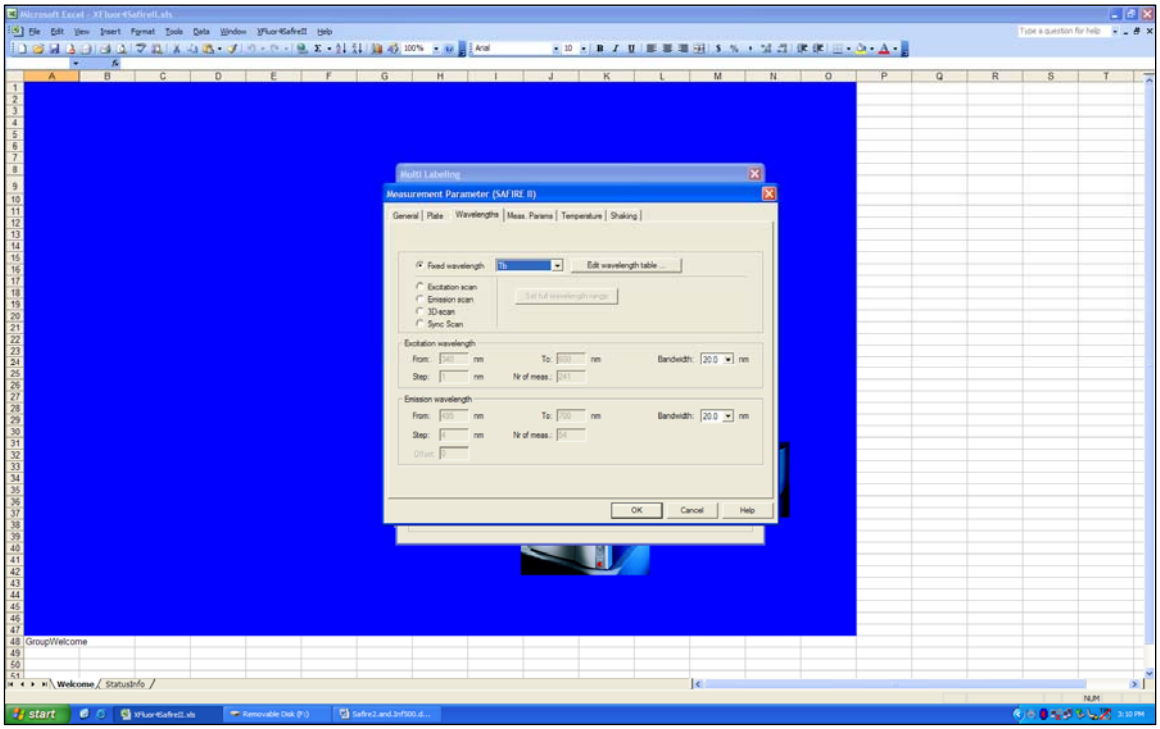
13. XFluor will default back to the Multi Labeling window. Select the "Edit Measurement Parameter" tab beneath the checked Do Measurement 2 box to edit the acceptor fluor measurement parameters.



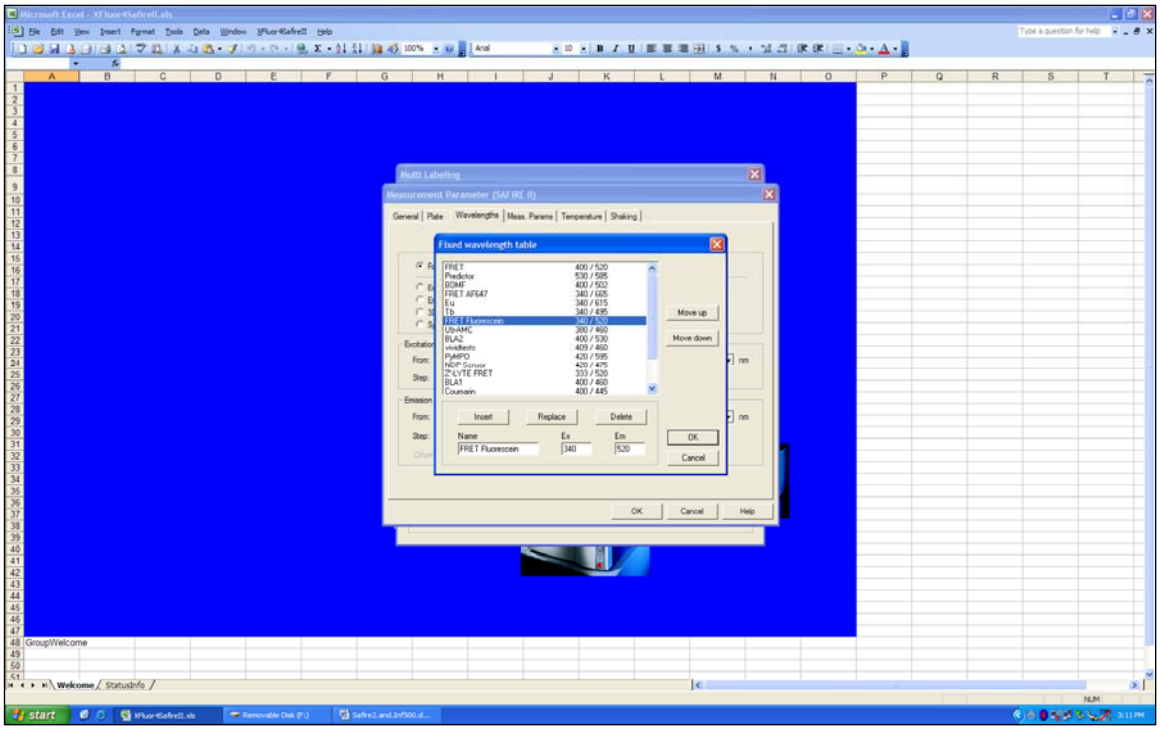
14. Under the General tab, make sure "Fluorescence Intensity" and "Wavelengths" are checked. At this point the plate window will be defaulted because of the first set of donor parameters, so skip the Plate tab and open the Wavelengths tab.



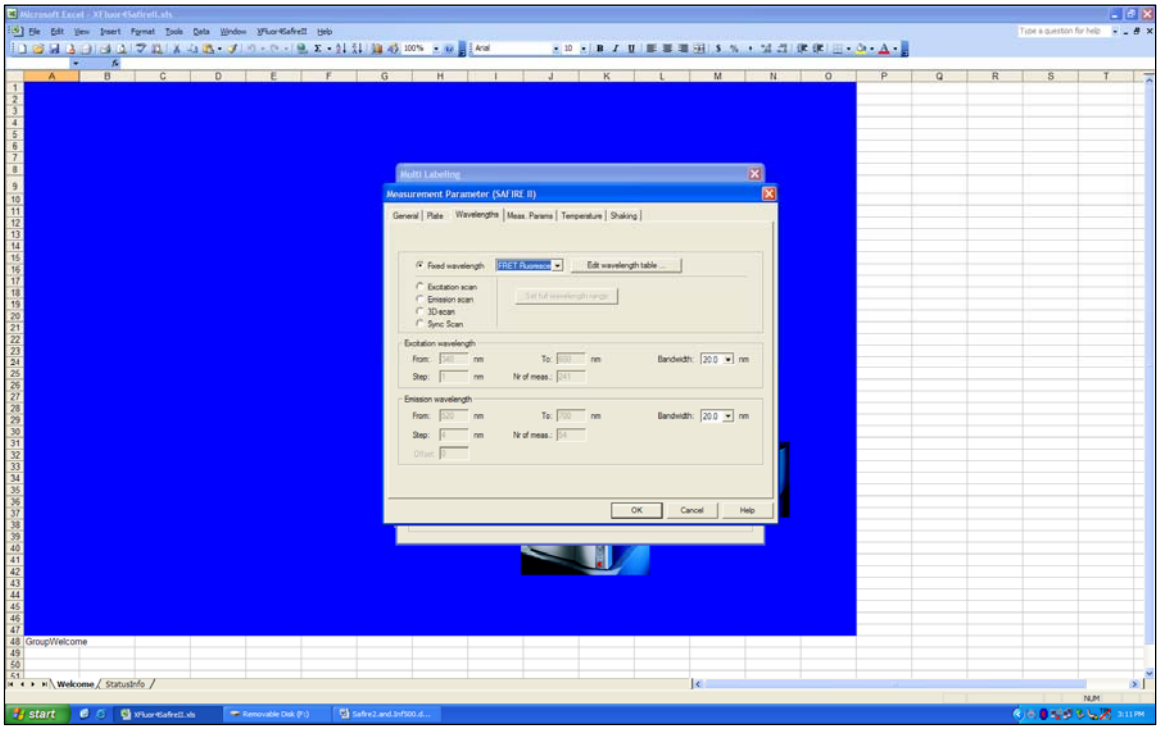
15. As before, select the "Edit wavelength table" tab.



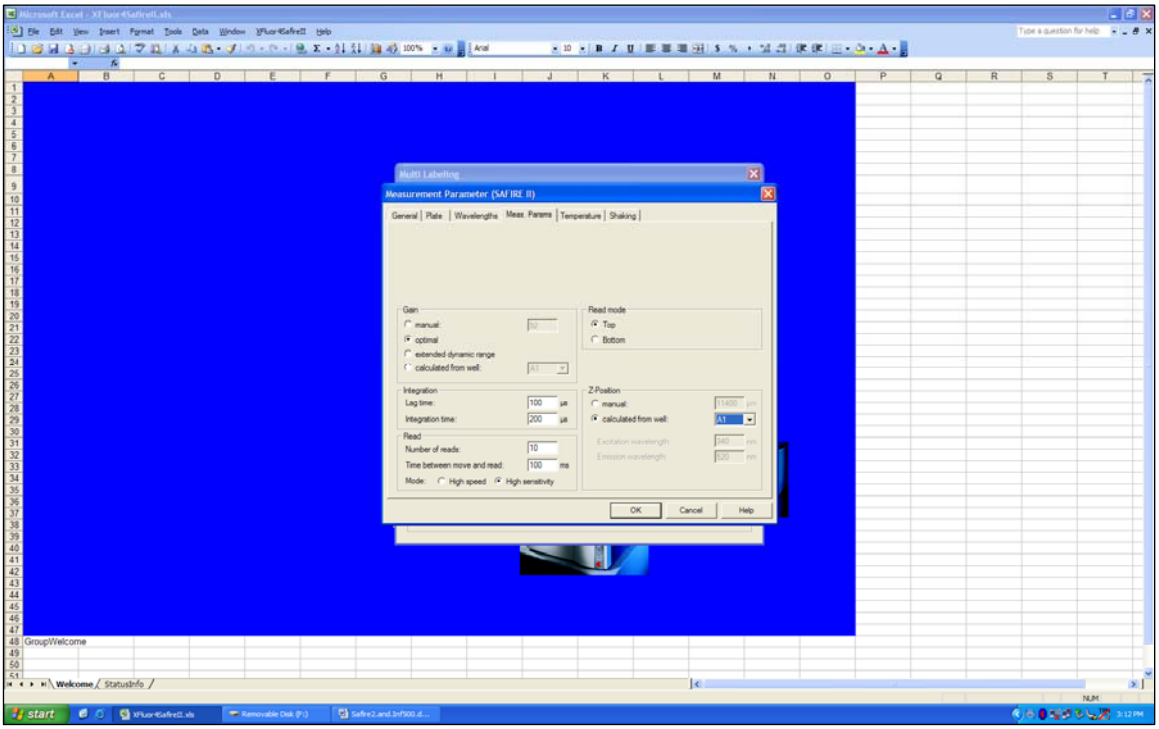
16. In the popup Fixed wavelength table enter a name and excitation and emission values for the LanthaScreen® acceptor. When finished select "Insert" to add them to the table and then select OK.



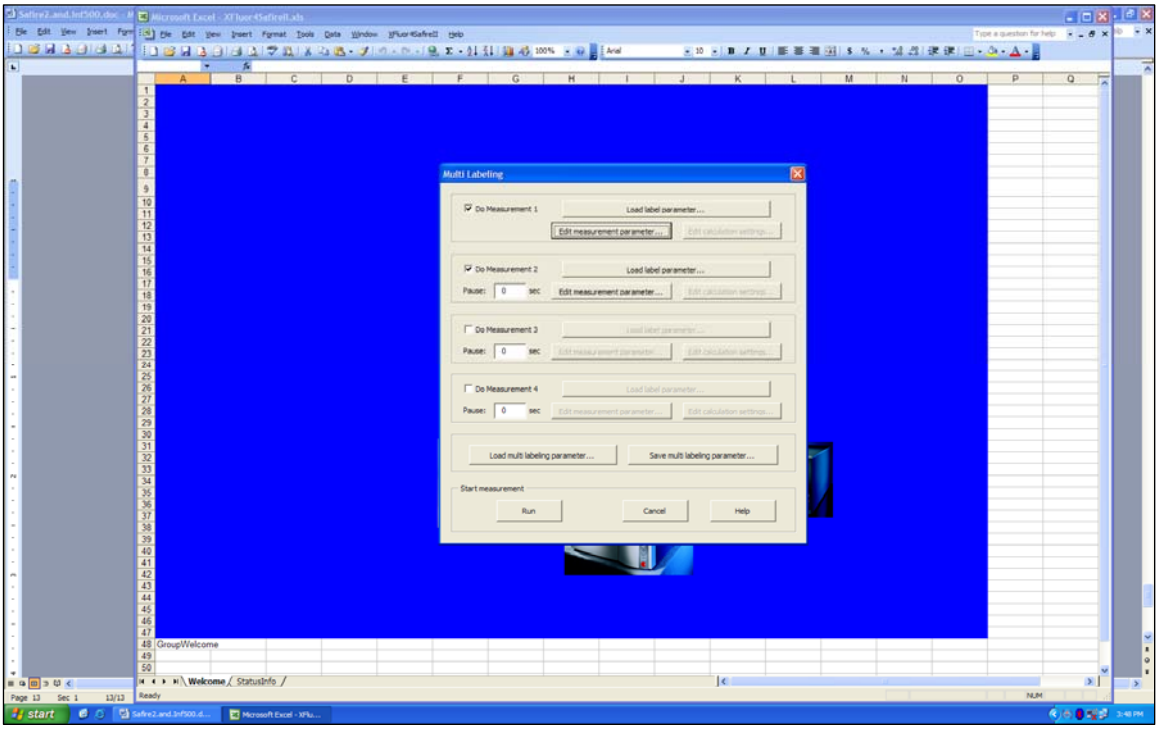
17. Back in the Measurement Parameter window, select bandwidths and then select the Meas. Params tab.



18. Set the Lag and Integration times as shown below, and set the Gain and Z-Position. Make sure Read mode is set to "Top". When finished, select OK.



19. Once again the program will default back to the initial Multi Labeling window. Make sure plate is inserted (or insert now, in "Movements" under the XFluor drop-down menu) and select "Run" to read assay plate.



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.

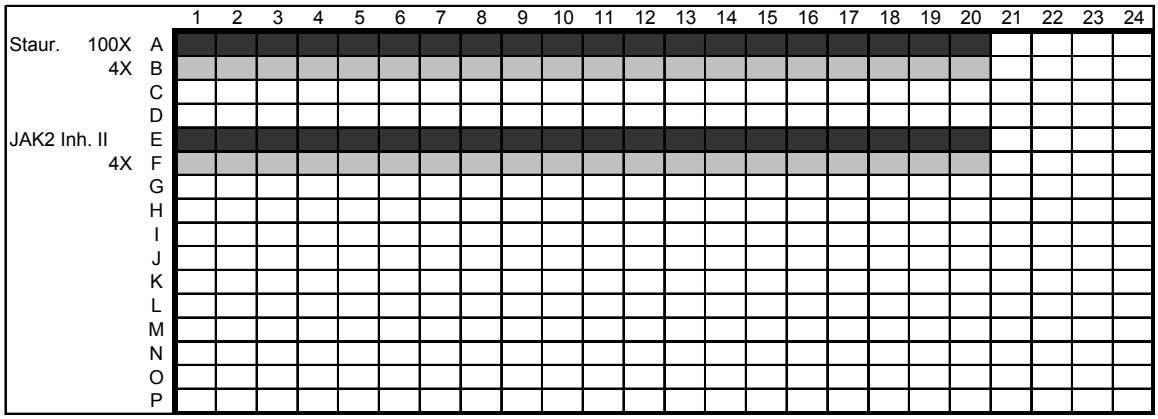


Figure 1: Schematic of initial compound dilution. Staurosporine, JAK2 Inhibitor II, JAK3 Inhibitor, and AG-490 were all titrated from a 100 µM starting concentration in the initial dilution series by preparing a 1:1 dilution curve in DMSO. A secondary dilution to 4X was then prepared in the rows below 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.

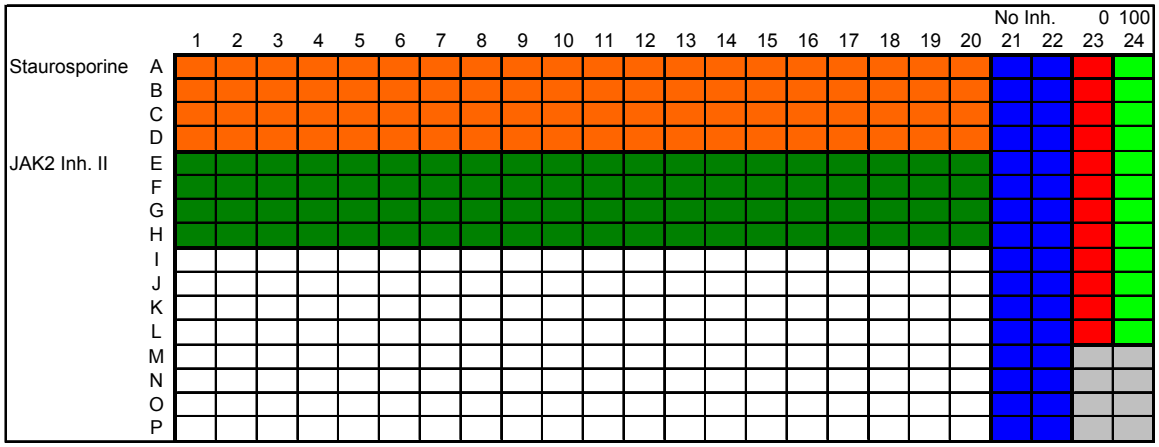


Figure 2: Assay Plate Schematic. Compound titrations shown in Columns 1-20, Columns 21 and 22 prepared without any inhibitor as kinase activity controls, Column 23 prepared with no kinase (0% phosphorylation) and Column 24 prepared using phosphopeptide control (100% phosphorylation). Note 8 wells in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

D. Results:

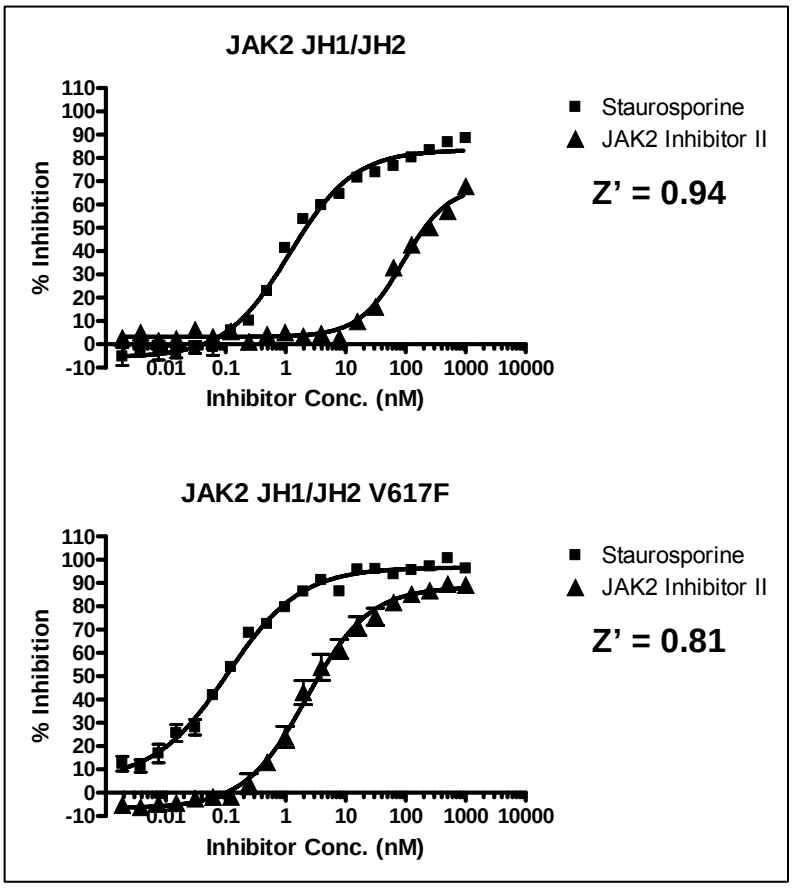


Figure 3: LanthaScreen® Assay. LanthaScreen® assay performed with the Tecan Safire²™.