Z'-LYTE® Assay Setup Guide on the Tecan Infinite® M1000 Microplate Reader

NOTE: The Tecan Infinite® M1000 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. 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.

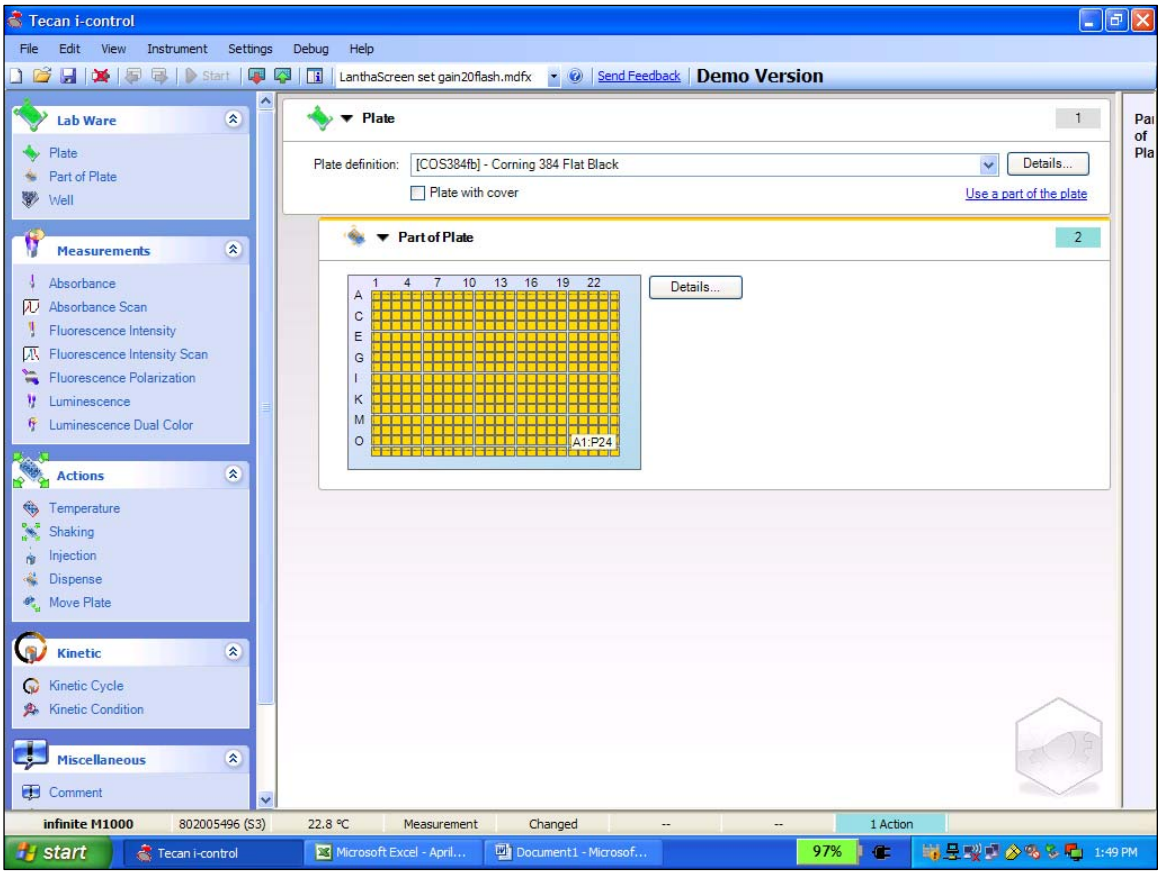
A. Recommended Optics

	wavelength (nm)	diameter (mm)
Excitation	405/12	monochromator
Emission 1	445/12	monochromator
Emission 2	520/12	monochromator

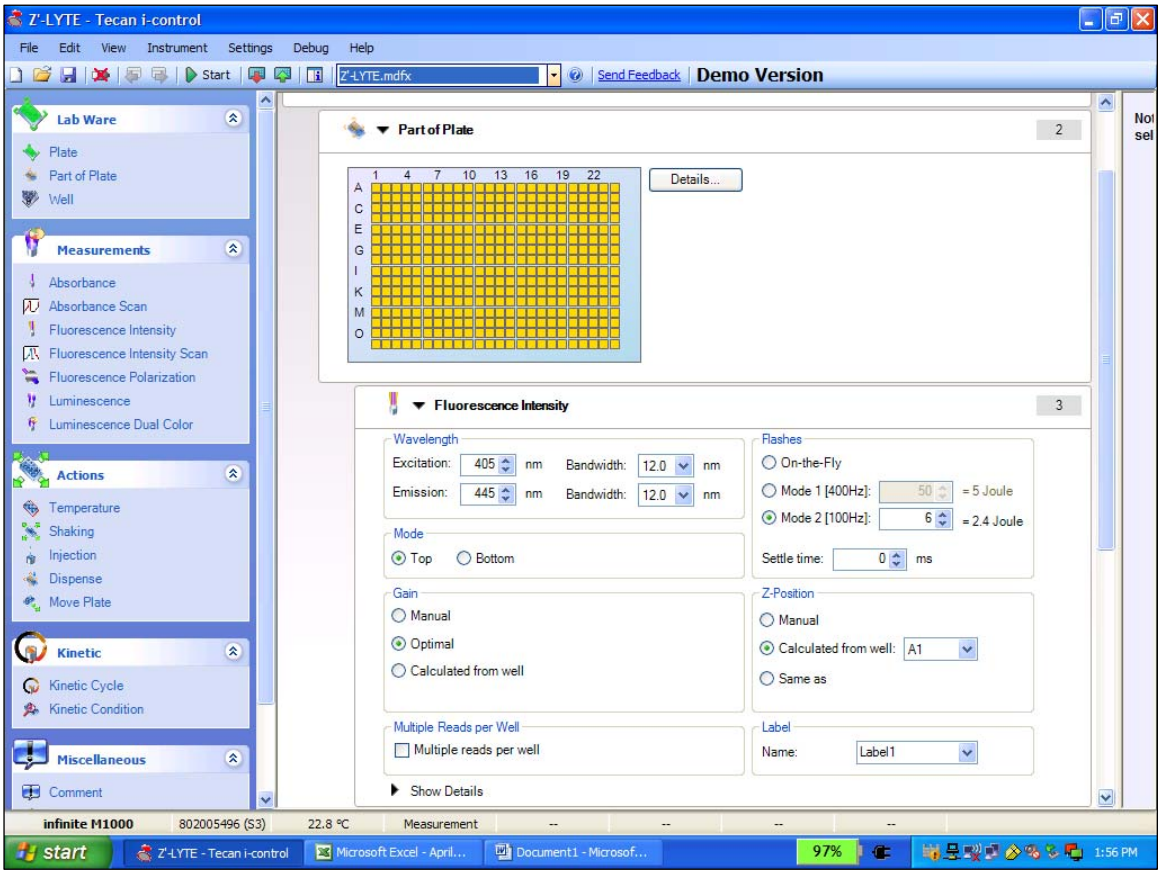
B. Instrument Setup

1. Make certain plate reader is turned on, and open up Tecan i-Control software on computer.

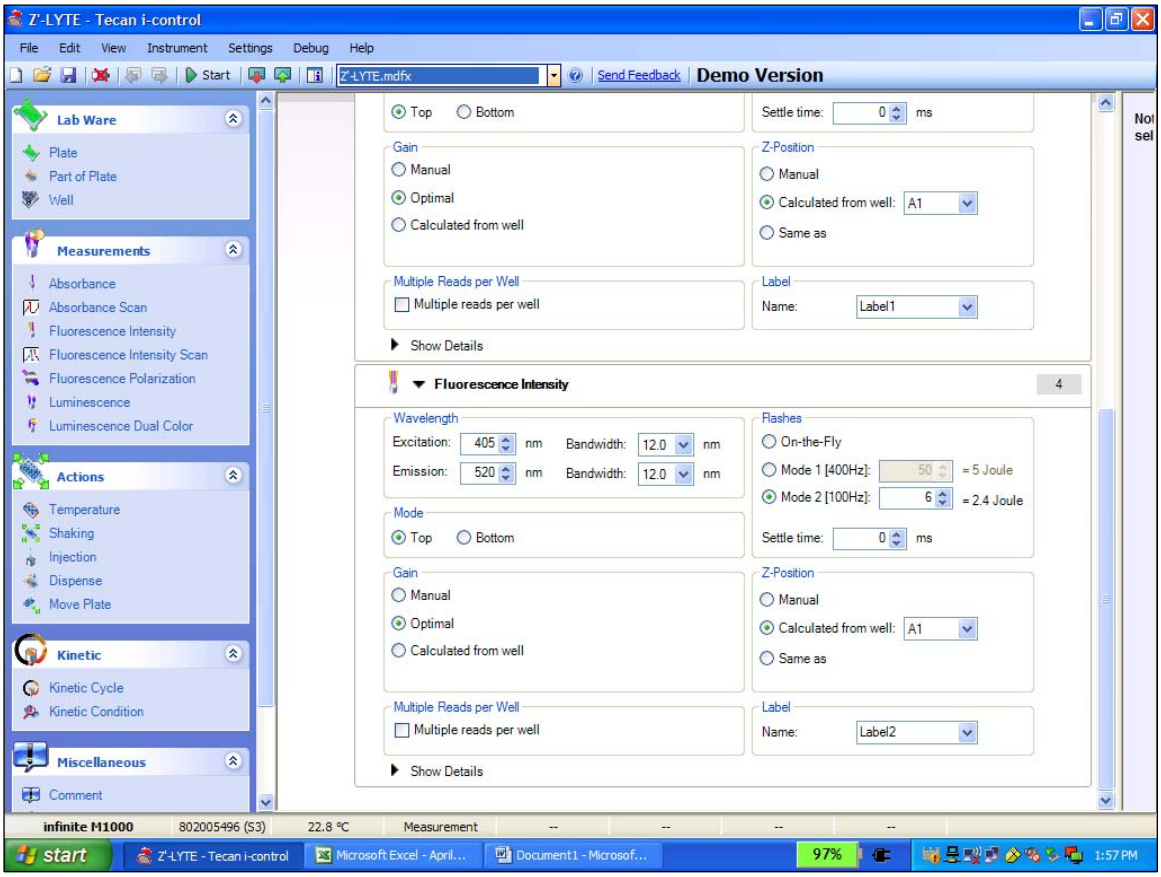
2. When i-Control opens, it will default to a generic starting page. Select "Plate Out" from the menu at the top to open the carriage, and insert your plate, then select "Plate In" to load your plate into the reader. Select your plate definition from the drop-down menu. Next, from the "Measurements" tab at the left side of the screen, select "Fluorescence Intensity".



3. At this point, a settings tab will open (below). Select the portion of the plate you wish to read by dragging across with your mouse to highlight selected wells, and in the new Fluorescence Intensity tab below, select your excitation and emission settings from the drop-down lists (selecting excitation and donor emission). Select “Optimal” for the Gain "Top" for Mode, and select an appropriate well with substrate in it for Z-positions (well A1 in this example). When finished, from the left click the "Fluorescence Intensity" tab again to open a second measurement settings tab.



4. A second Fluorescence Intensity window will open. Use the drop-down tabs to set the excitation and acceptor settings. Make sure Gain, Z-Position, Mode, and Flashes match the settings in the first tab.



5. Once all settings have been selected, and a plate is inserted and ready to read, select "Start" from the top menu bar to read.

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 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.

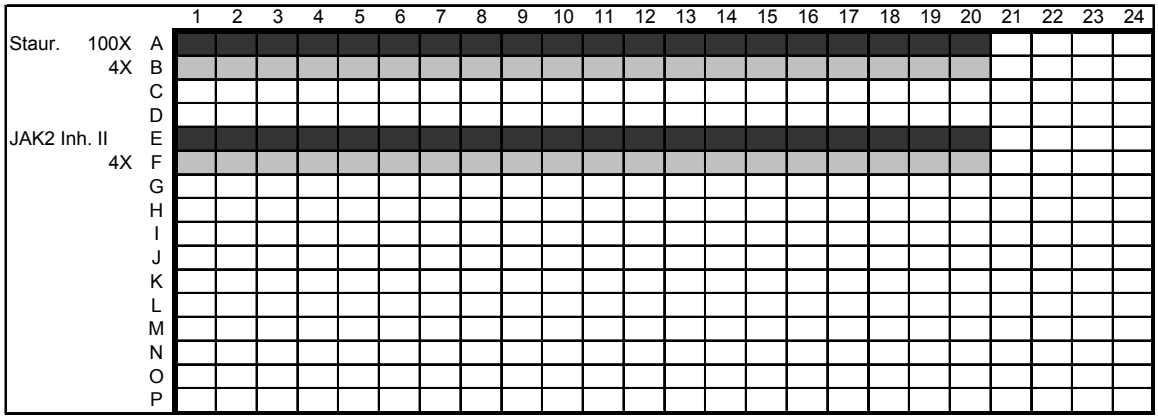


Figure 1: Schematic of initial compound dilution. Staurosporine and JAK2 Inhibitor II were titrated from a 100 µM starting concentration in the initial dilution series by preparing a 1:1 dilution curve in DMSO. A secondary dilution to 4X was then prepared in the rows below the initial dilution curve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

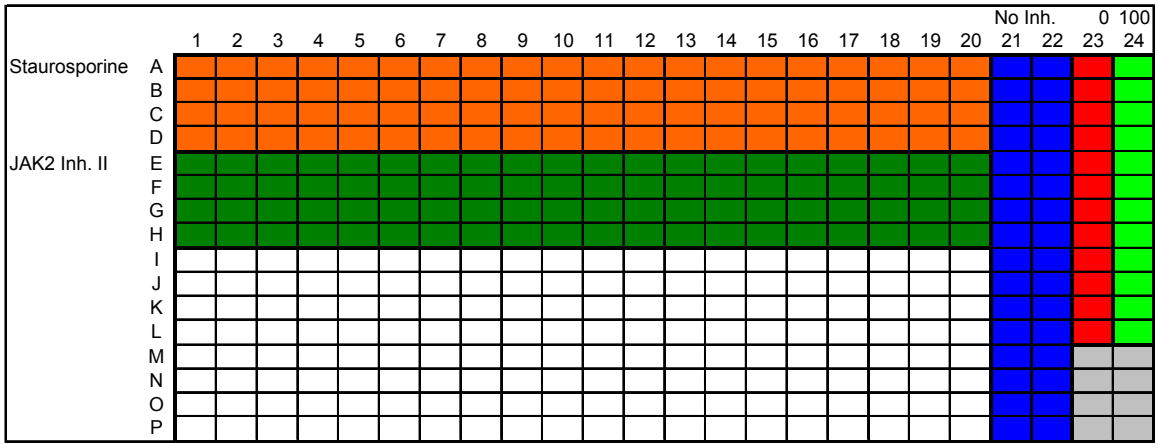


Figure 2: Assay Plate Schematic. Compound titrations shown in Columns 1-20, Columns 21 and 22 prepared without any inhibitor as kinase activity controls, Column 23 prepared with no kinase (0% phosphorylation) and Column 24 prepared using phosphopeptide control (100% phosphorylation). Note 8 wells in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

D. Results

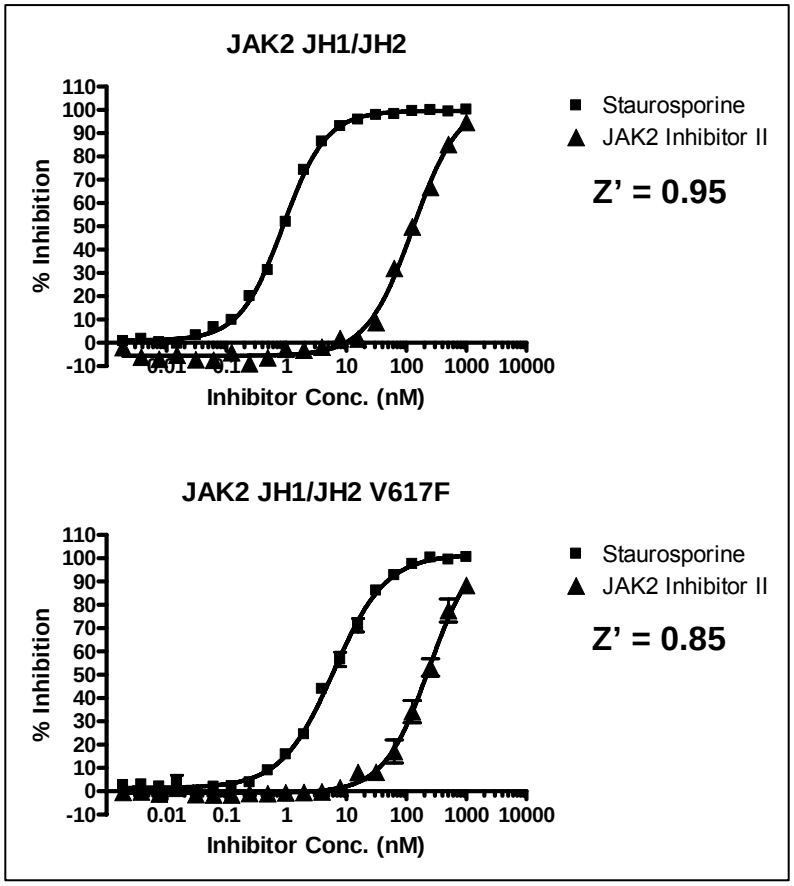


Figure 1: Z'-LYTE® Kinase Assay. Z'-LYTE® assay performed using the Tecan Infinite® M1000.