LanthaScreen® Terbium Assay Setup Guide on the Tecan Infinite® M1000 / M1000 PRO Microplate Readers

IMPORTANT INFORMATION

Test your plate reader set-up before using LanthaScreen® Terbium and Europium assays

We have developed two technical notes which provide a method for verifying that a fluorescent plate reader is able to detect a change in time-resolved fluorescence energy transfer (TR-FRET) signal, confirming proper instrument set-up and a suitable response. The method is independent of any biological reaction or equilibrium and uses reagents that are on-hand for the LanthaScreen® assay.

For complete instructions, visit www.lifetechnologies.com/instrumentsetup and click on "Download Terbium assay application note" or "Download Europium assay application note".

The Tecan Infinite® M1000 / M1000 PRO Microplate Readers were tested for compatibility with Life Technologies 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. The M1000 PRO offers better monochromators, but would be set up in the same manner.

For more detailed information and technical support of Life Technologies assays including specific conditions for assay windows between 2-3 fold, please call 1-800-955-6288 and enter extension 40266 or email drugdiscoverytech@lifetech.com.

For more detailed information and technical support of Tecan instruments or software, please call 1-888-798-0538 or e-mail info@tecan.com .

A. Recommended Optics

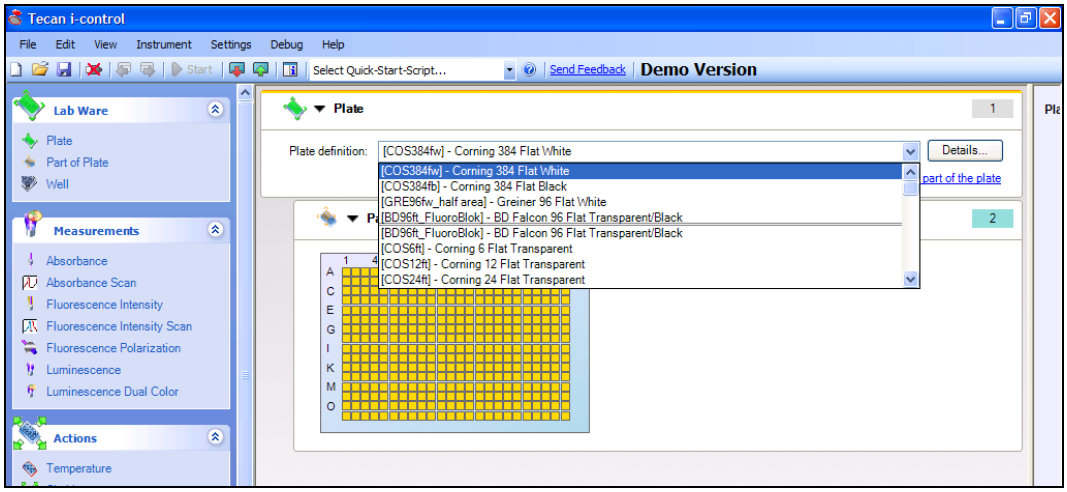
	Wavelength (nm)	Diameter (mm)
Excitation	332/20	monochromator
Emission 1	490/10	monochromator
Emission 2	520/20	monochromator

Note: The filter settings above are an update for optimal assay performance, and do not reflect the settings in the screenshots. Use the wavelengths and bandwidths above for best results.

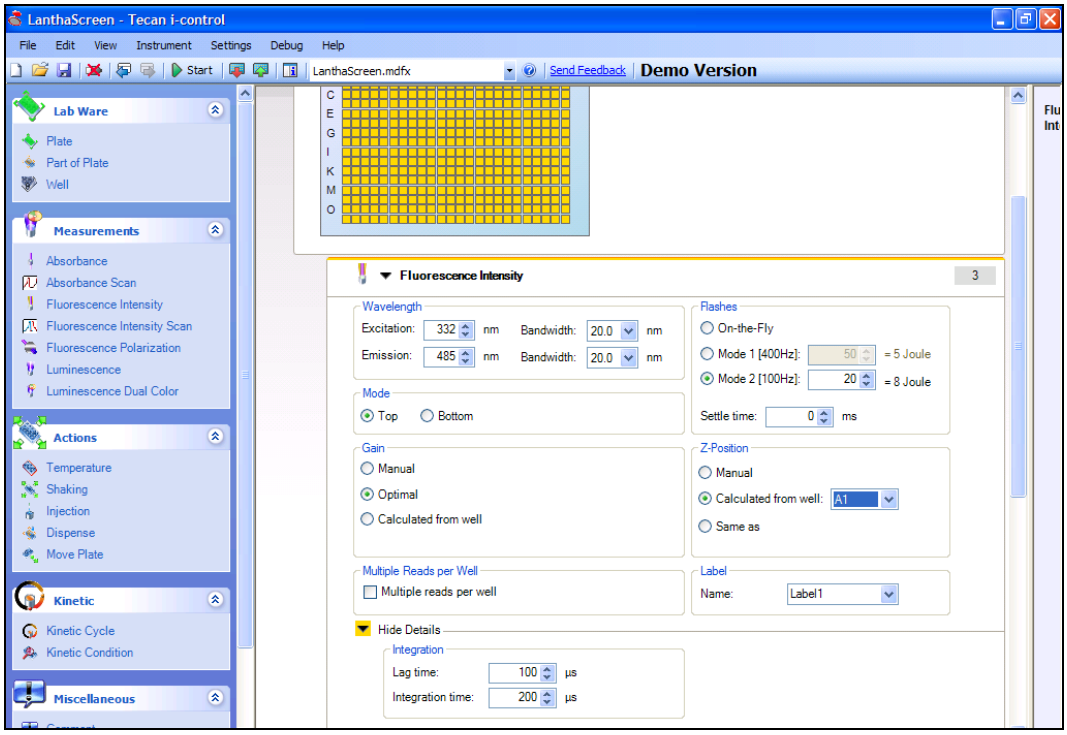
B. Instrument Setup

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

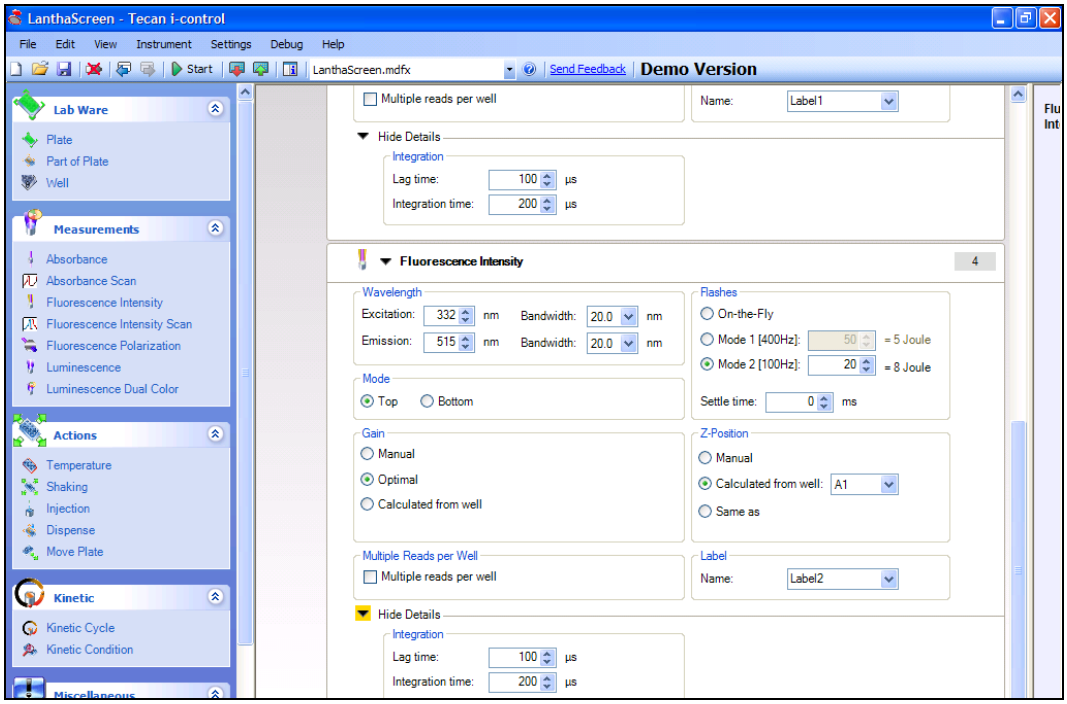
2. When i-Control opens, it will default to a generic starting page. Select “Plate Out” from the menu at the top to open the carriage, and insert your plate, then select “Plate In” to load your plate into the reader. Select your plate definition from the drop-down menu. (**Note**: in this case we have selected "Flat White" instead of black--this provided a minor assay window improvement in our hands compared with equivalent black plates in LanthaScreen® assays.) 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). Click on "Hide Details" at the bottom of the tab to open "Integration" settings for a TR-FRET assay, and set "Lag" and "Integration" times at 100 and 200 µs, respectively. 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. As before, click on "Hide Details" to open, and set Lag and Integration times as well.



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. 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.lifetechnologies.com/lanthascreen.

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.

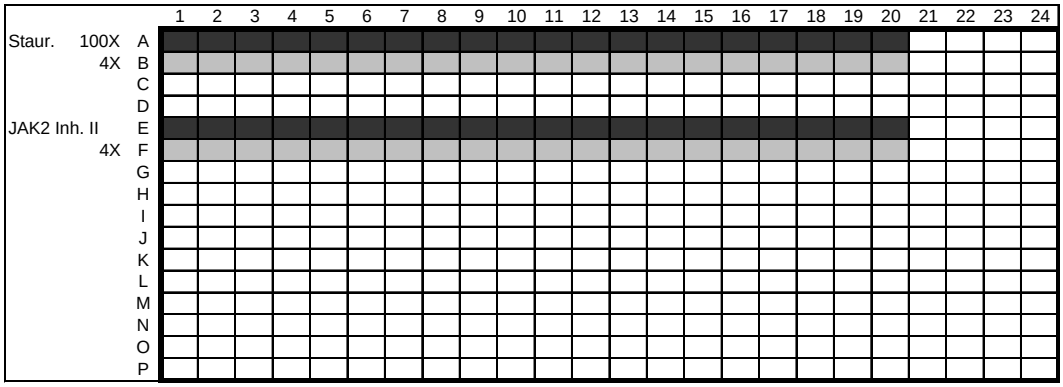


Figure 1: Schematic of initial compound dilution. Staurosporine, JAK2 Inhibitor II, JAK3 Inhibitor, and AG-490 were all titrated from a 100 µM starting concentration in the initial dilution series by preparing a 1:1 dilution curve in DMSO. A secondary dilution to 4X was then prepared in the rows below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

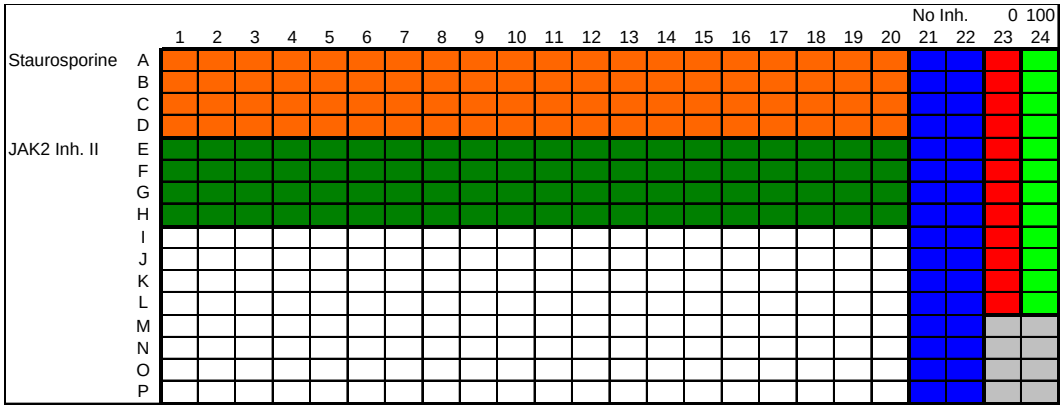


Figure 2: Assay Plate Schematic. Compound titrations shown in Columns 1-20, Columns 21 and 22 prepared without any inhibitor as kinase activity controls, Column 23 prepared with no kinase (0% phosphorylation) and Column 24 prepared using phosphopeptide control (100% phosphorylation). Note 8 wells in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

D. Results:

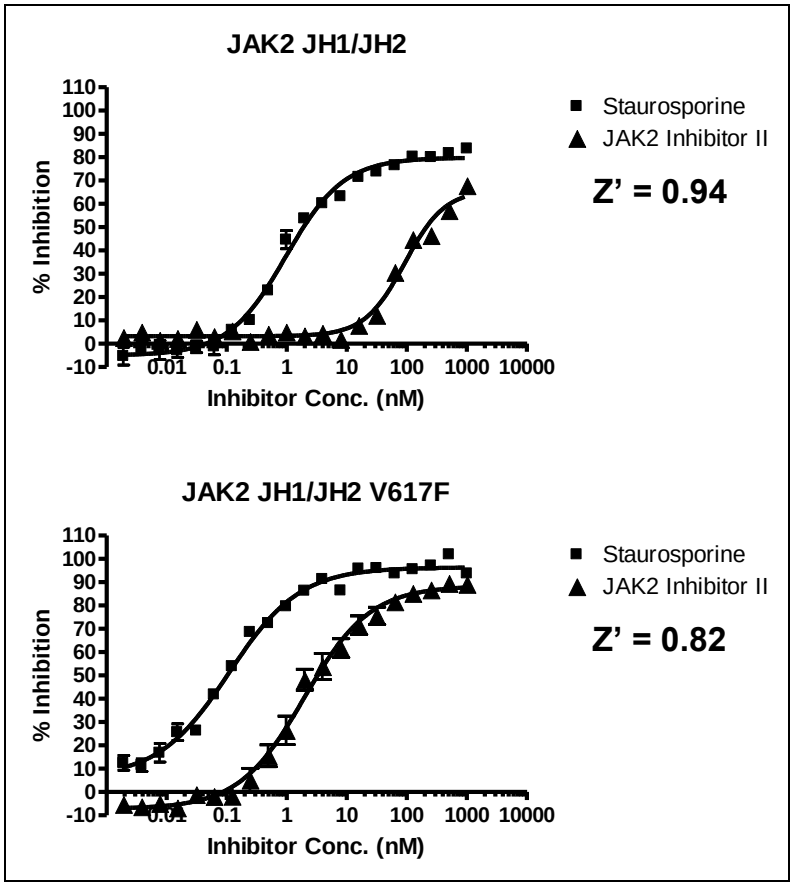


Figure 3: LanthaScreen® Assay. LanthaScreen® assay performed with the Tecan Infinite® M1000.