Europium-based Assay Setup Guide on the BMG LABTECH POLARstar Omega Microplate Reader

NOTE: The BMG LABTECH POLARstar Omega Microplate Reader was tested for compatibility with Invitrogen's Europium-based TR-FRET assays using the Adapta® Universal Kinase Assay Kit (PV5099) and poly E4Y substrate against JAK2 JH1/JH2 and JAK2 JH1/JH2 V617F kinases as well as the LanthaScreen™ Eu Kinase Binding Assay. 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 BMG LABTECH instruments or software, please contact BMG LABTECH at 1-877-264-5227 or www.bmglabtech.com.

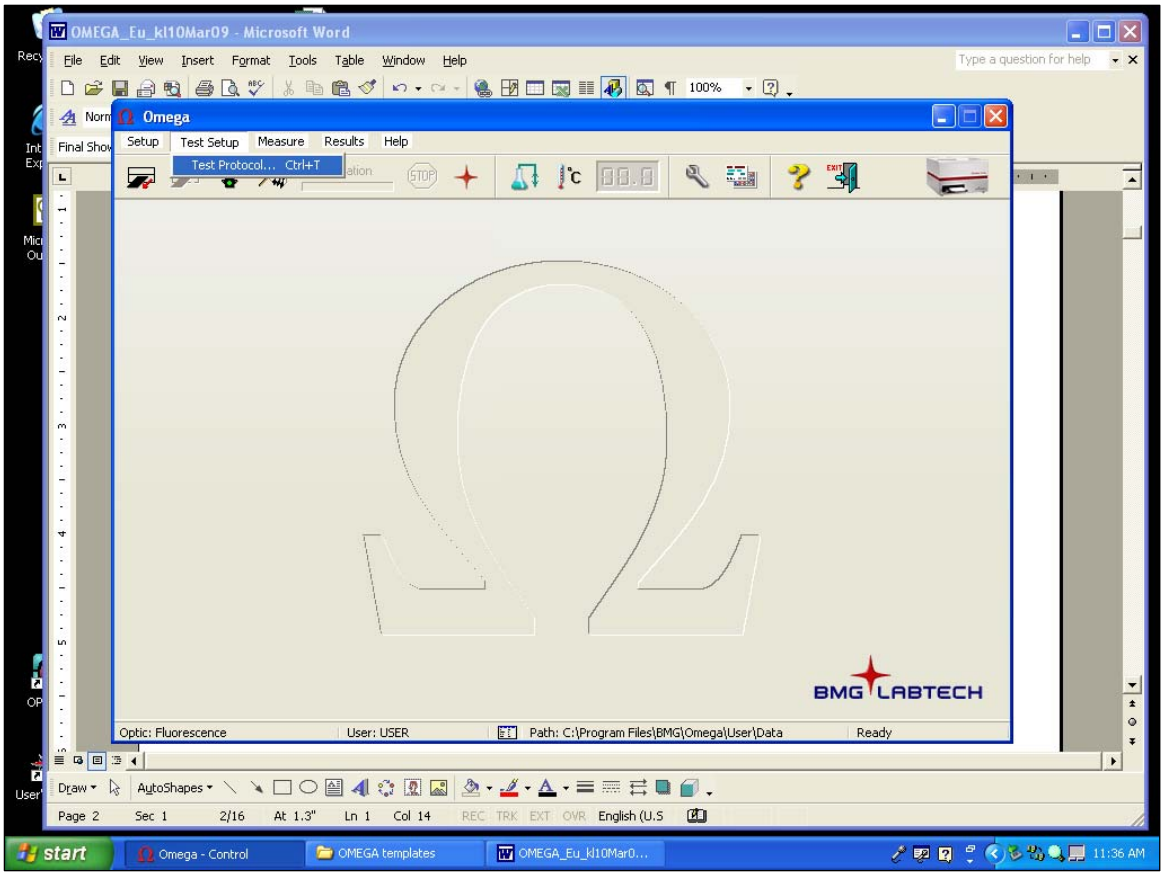
A. Recommended Optics

	wavelength (nm)	BMG LABTECH filter
Excitation	337	HTRF
Emission 1	665-10	HTRF
Emission 2	620-10	HTRF
TR-FRET Optic Module Height setting “11”	N/A	N/A

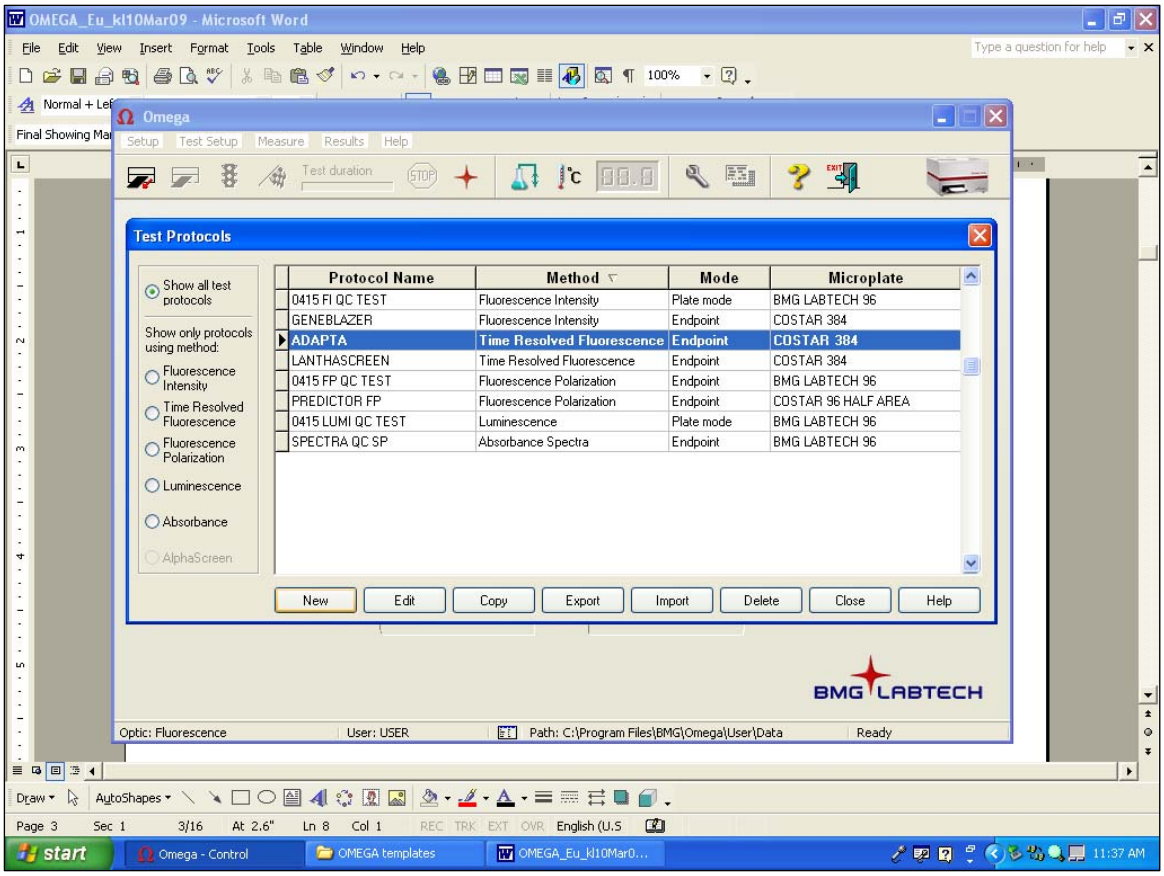
B. Instrument Setup

1. Make certain plate reader is turned on, and open up POLARstar Omega Control software on computer. Insert plate into plate reader.

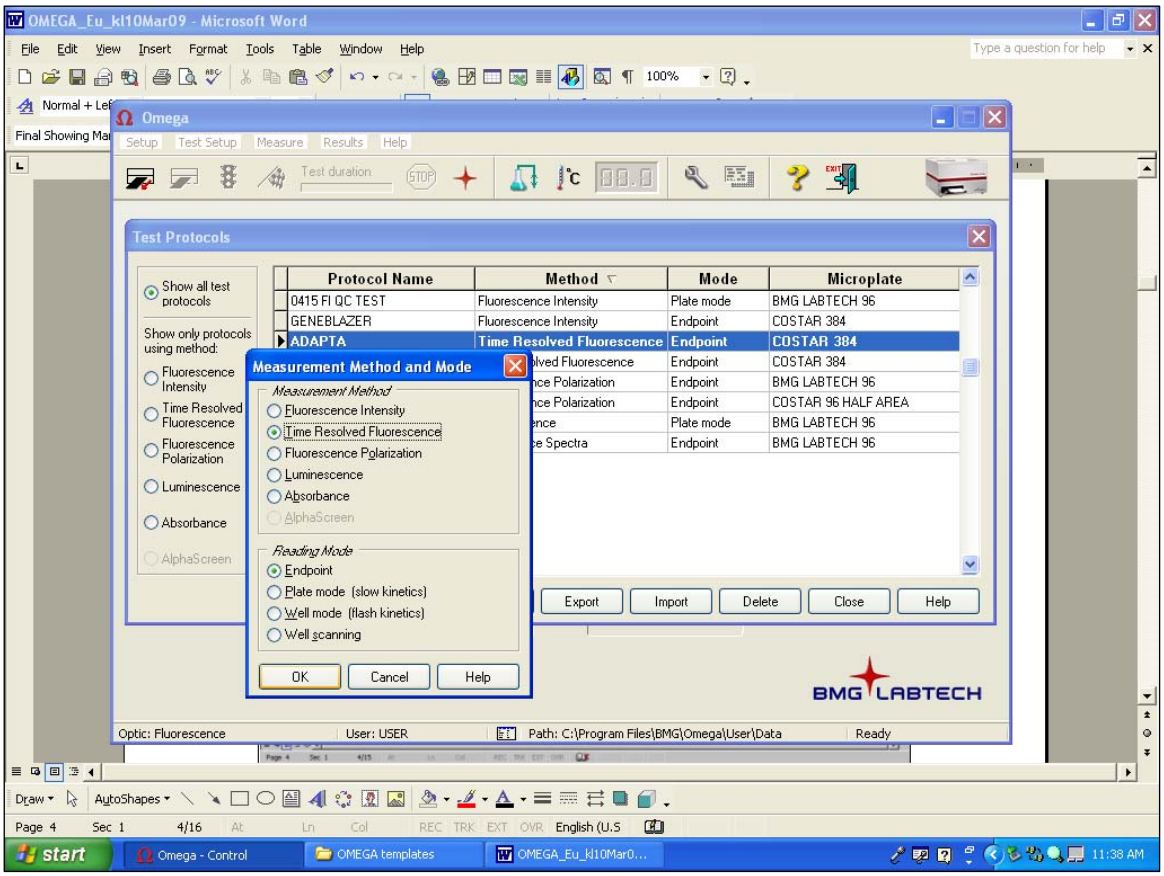
2. When Omega Control software opens, if you do not have a suitable pre-existing protocol for Europium-based TR-FRET, select "Test Protocol" from the “Test Setup” menu bar at the top of the window.



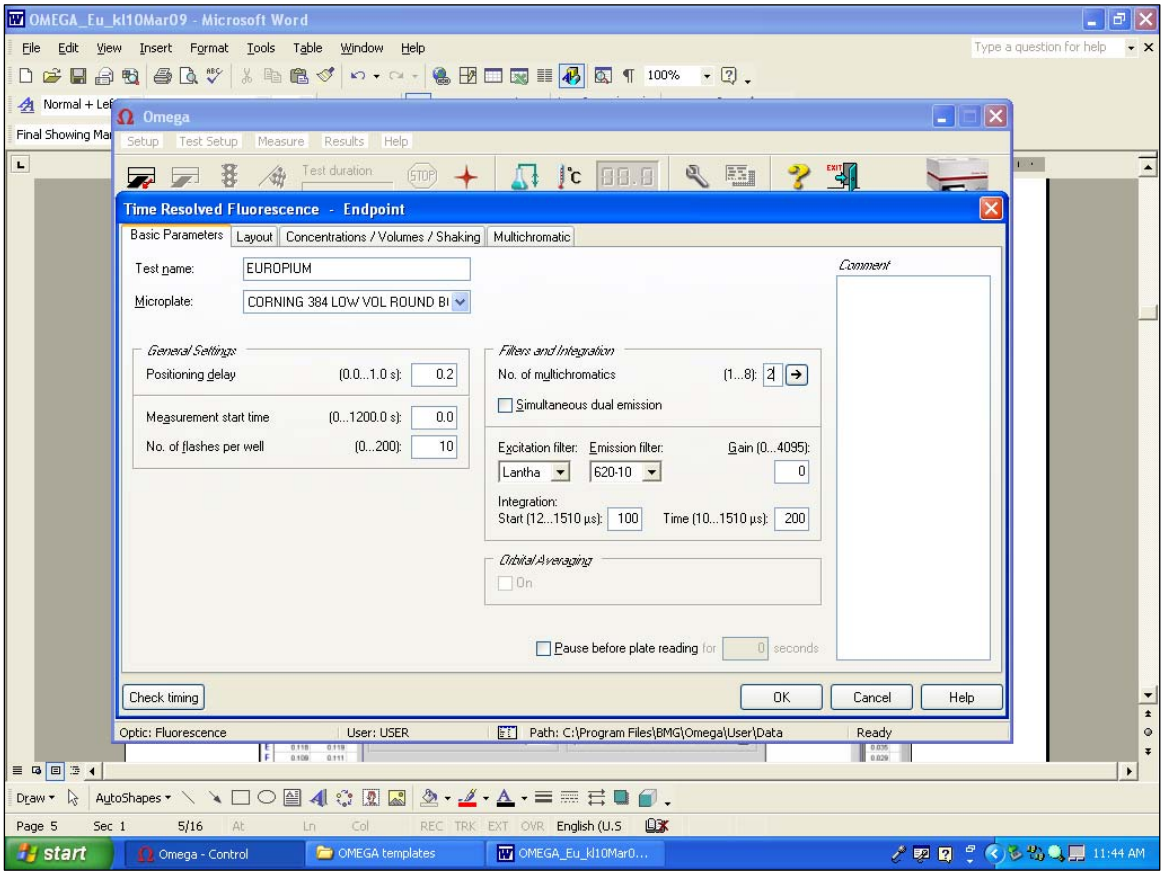
3. At this point, a new screen will open (below). Click on the “Show all test protocols” or "Time Resolved Fluorescence" button on the left side of the screen, then select “New” from the tabs at the bottom.



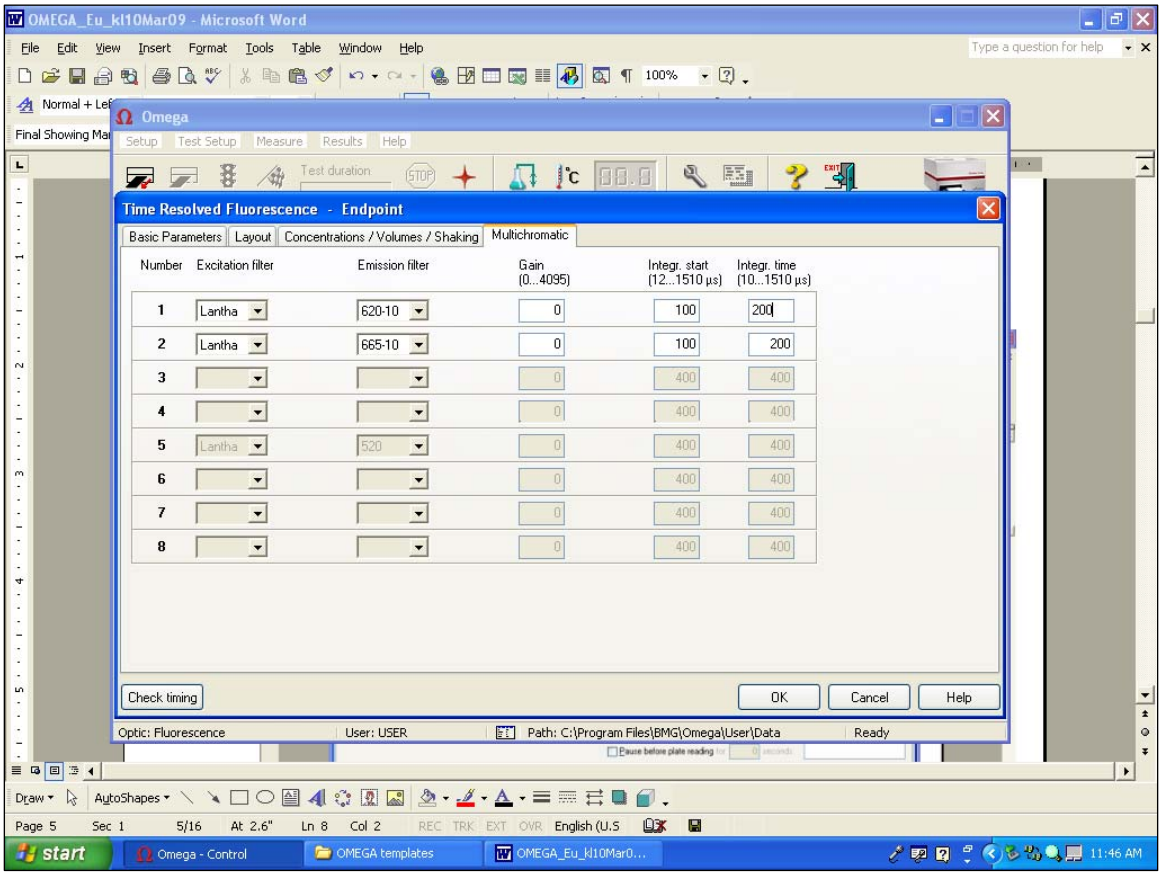
4. A new window will pop up. Select “Time Resolved Fluorescence” and “Endpoint” and then select “OK”. Note you will need to use the TRF Optic Head from BMG, and on the TRF optic the dial should be set to “11” for this application.



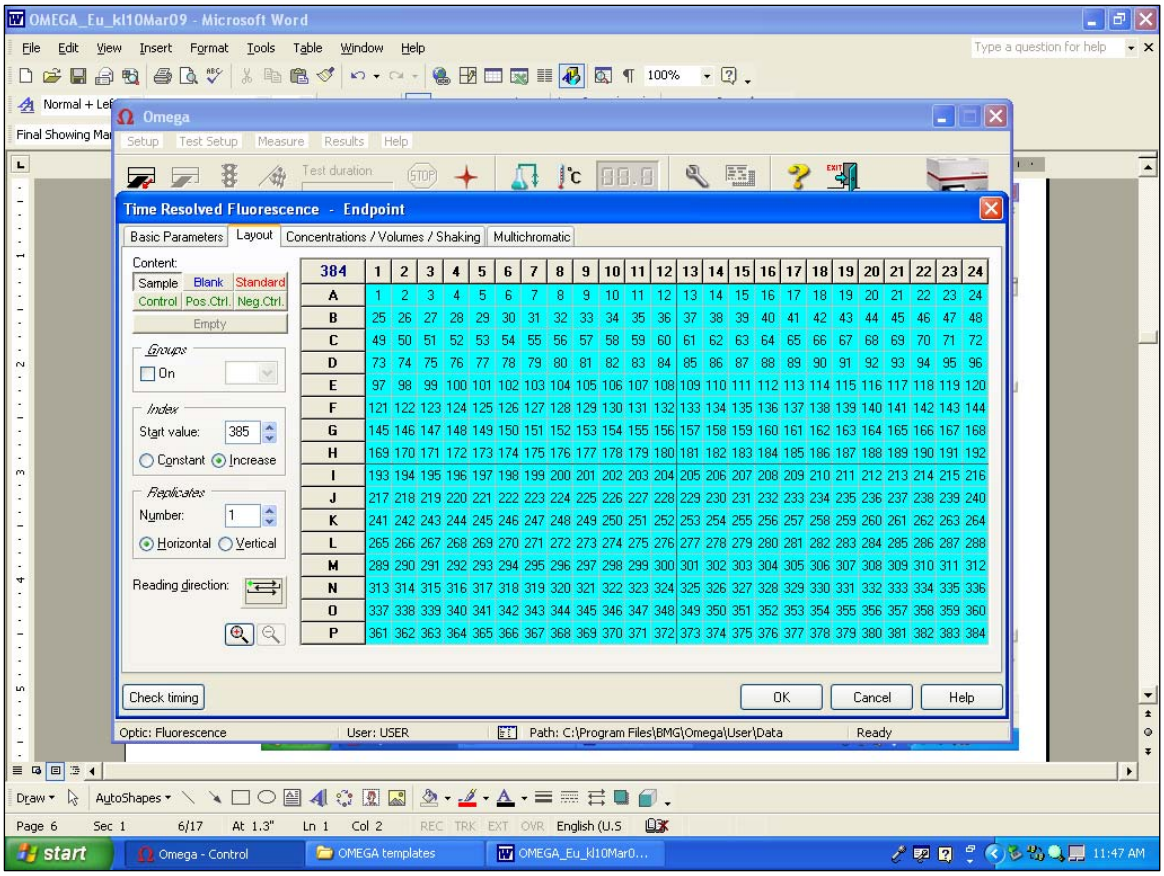
5. A new Protocol window will open automatically. Enter a test name and select plate type. Because Adapta® and the LanthaScreen™ Kinase Binding Assay are Time-Resolved FRET assays, set the "Integration: Start" and "Integration: Time" to 100 and 200 µs, respectively, as shown. When finished, enter a 2 in the "No. of multichromatics" box and then click on the arrow next to it.



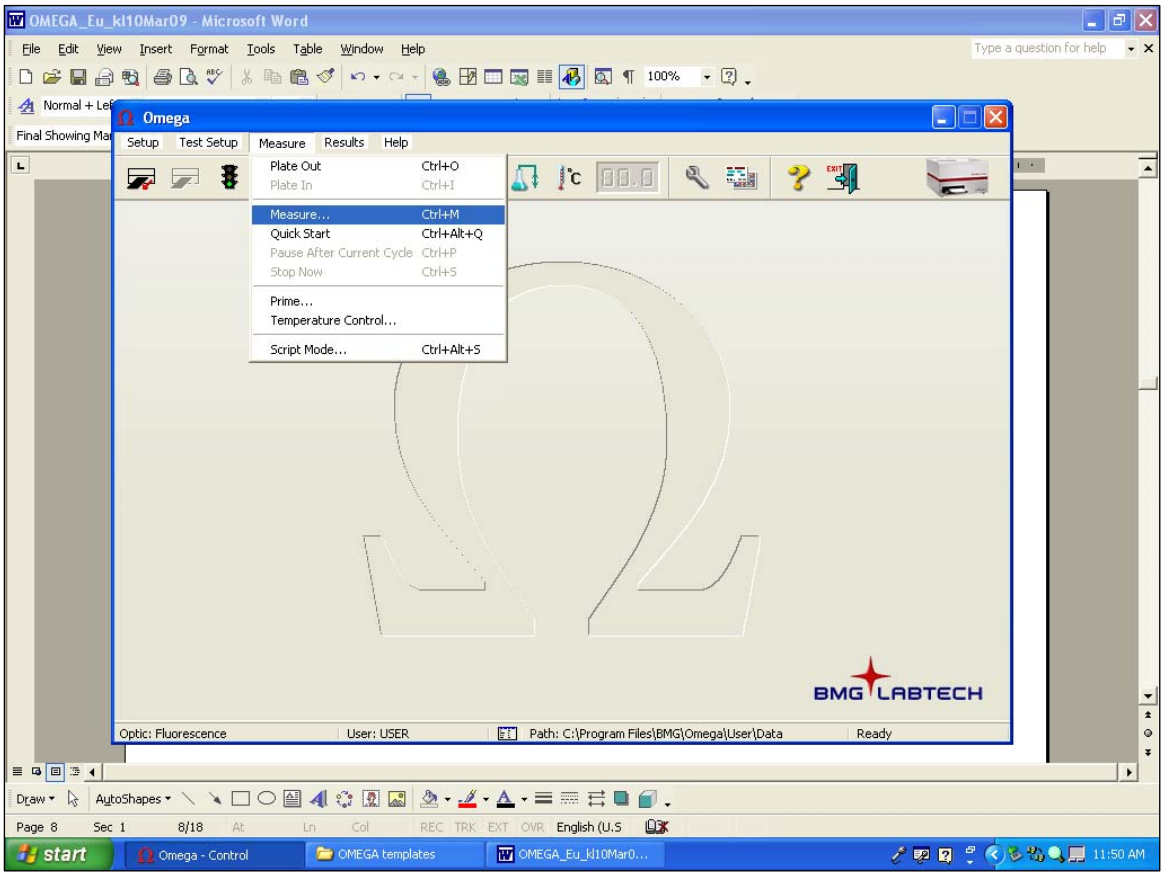
6. A new tab will open. Select your excitation and emission filters as shown, and also enter your “Integr. start” and “Integr. time” settings as shown below. When finished, select the “Layout” tab.



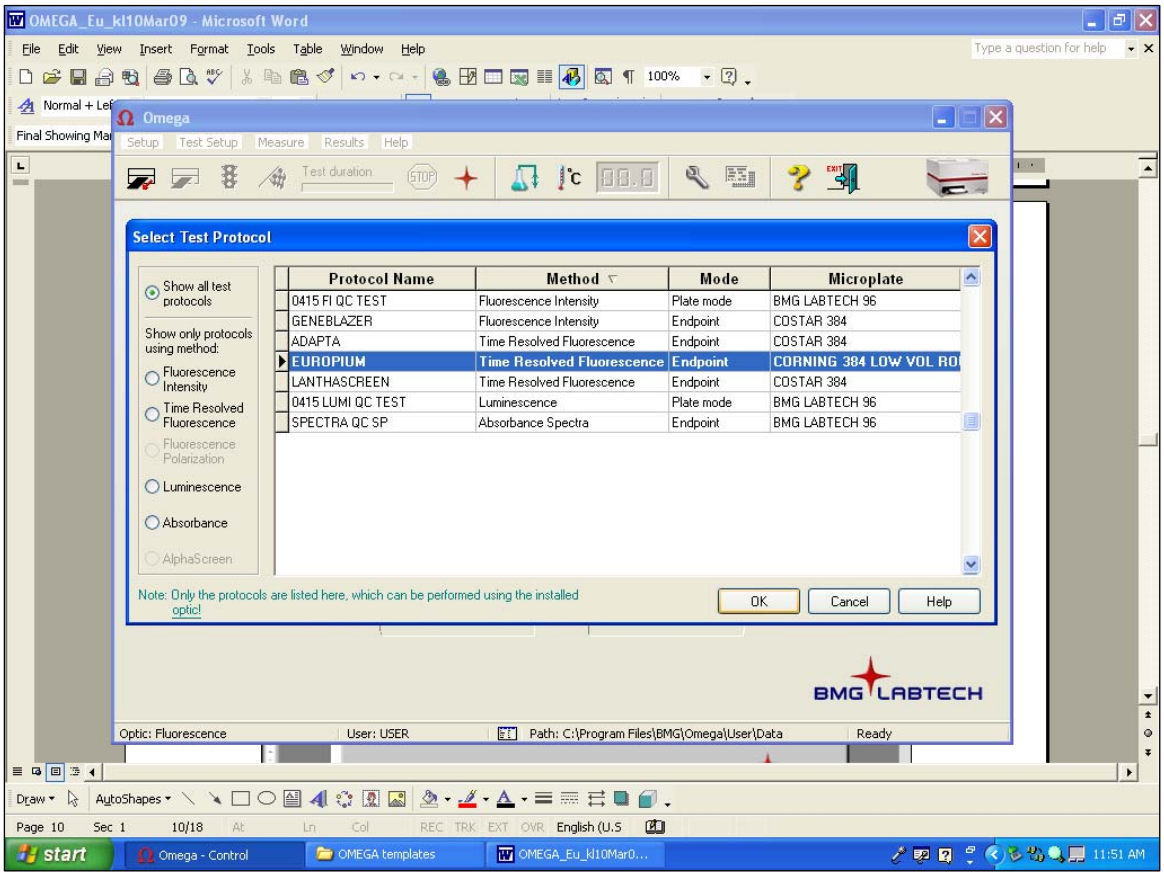
7. Select the wells you wish to read. Note in this step you can select to designate blanks, positive controls, etc. but for this case we marked all wells "Sample" and calculations were performed manually. When finished, select “OK”.



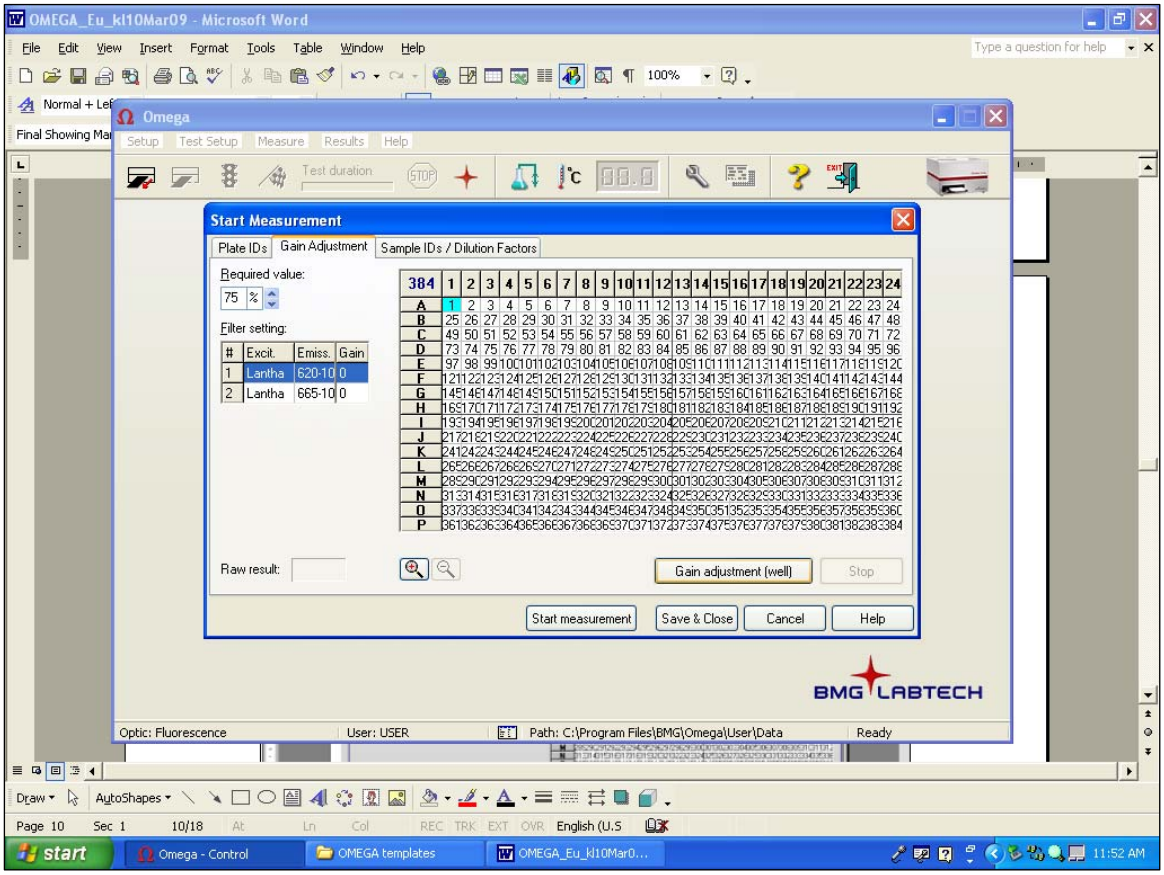
8. You will return to the initial settings window. From the drop-down menus at the top, select "Measure" and "Measure" again.



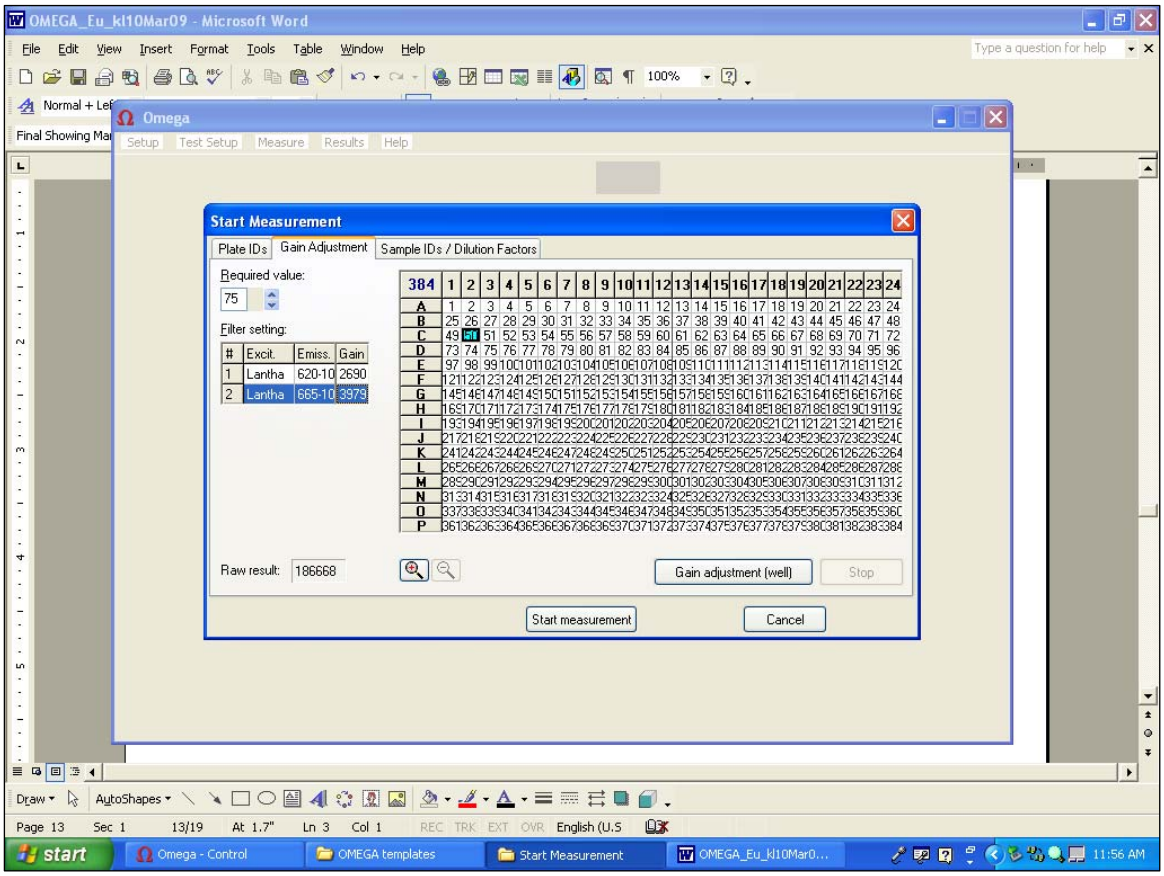
9. A new window will appear allowing you to select which of your test protocols you wish to run. Select the protocol you created, and then press “OK”.



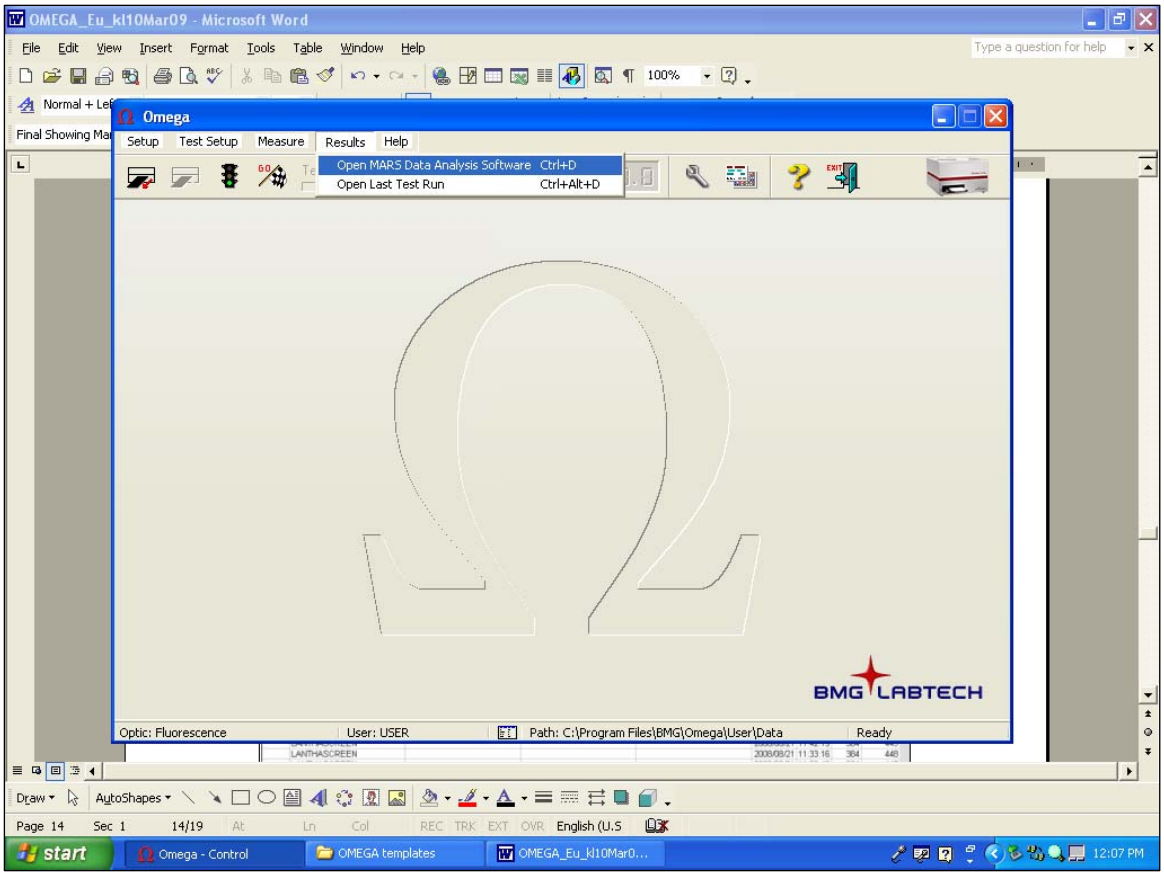
10. A new window will appear. Place your plate in the reader, and select a well to use for adjusting gain. When finished, click on the "Start Adjustment" tab. In this case we highlighted the 620 channel and selected a low-FRET well to adjust the donor gain. Next, a high-FRET well was selected to optimize the acceptor gain in the 665 emission channel. When both channels have been optimized, select the “Start Measurement” tab to measure plate.



11. In a moment, the instrument will have calculated the optimal gain for each channel. When finished, click on the "Start measurement" tab to read.



12. When Omega is done reading, you can collect your data under the “Results” tab. This will automatically redirect you to the MARS data analysis software. Select your run of interest from the list to open.



C. Adapta® 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 Adapta® assay, the information below is provided as representative data. 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 http://www.invitrogen.com/Drug-Discovery.html.

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 (100 µM) 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.

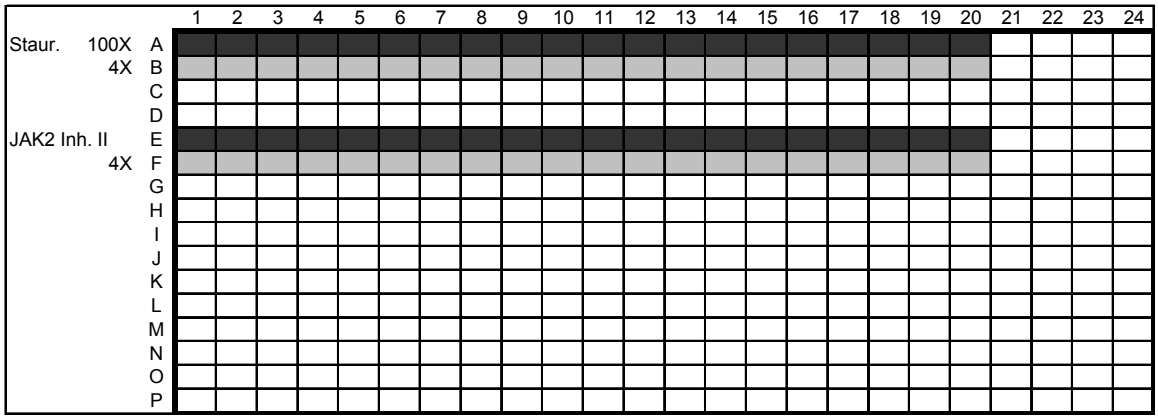


Figure 1: Schematic of initial compound dilution. Staurosporine, JAK2 Inhibitor II, JAK3 Inhibitor, and AG-490 were all titrated from a 100 µM starting concentration in the initial dilution series by preparing a 1:1 dilution curve in DMSO. A secondary dilution to 4X was then prepared in the rows below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

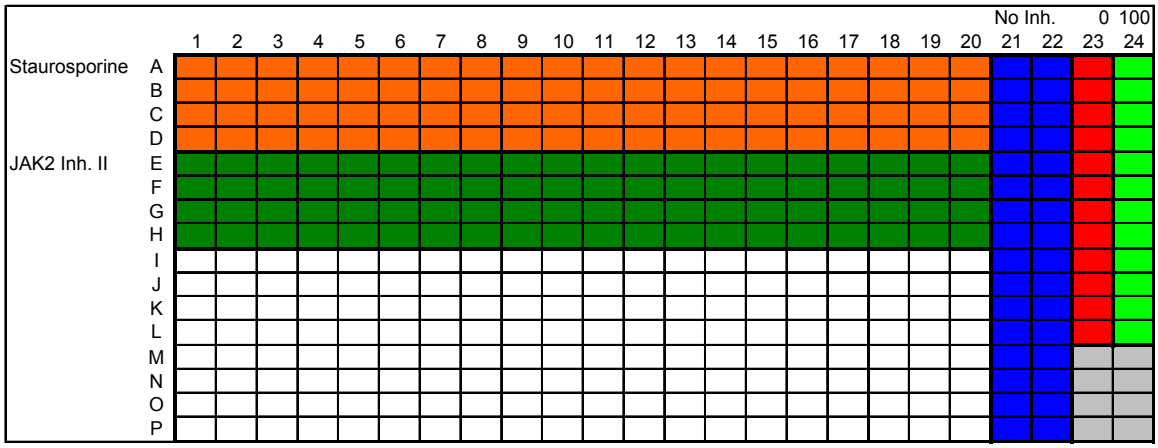


Figure 2: Assay Plate Schematic. Compound titrations shown in columns 1-20, columns 21 and 22 prepared without any inhibitor as kinase activity controls, column 23 prepared with no kinase (0% phosphorylation) and column 24 prepared using phosphopeptide control (100% phosphorylation). Note 8 wells in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

D. Results:

Note: The results shown are meant as representative data only. The actual ratio values obtained may vary from instrument to instrument. Values are dependent on multiple factors including but not limited to the specific setup of each instrument, plate type and assay target.

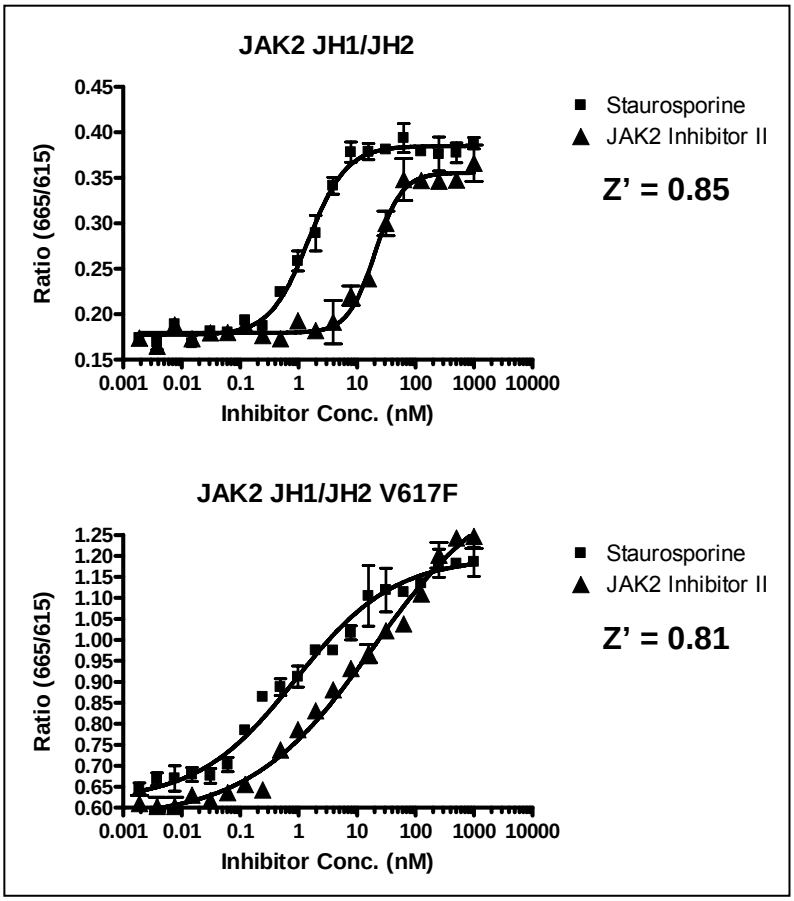


Figure 3: Adapta® Assay. Adapta® assay performed with the BMG LABTECH POLARstar Omega.

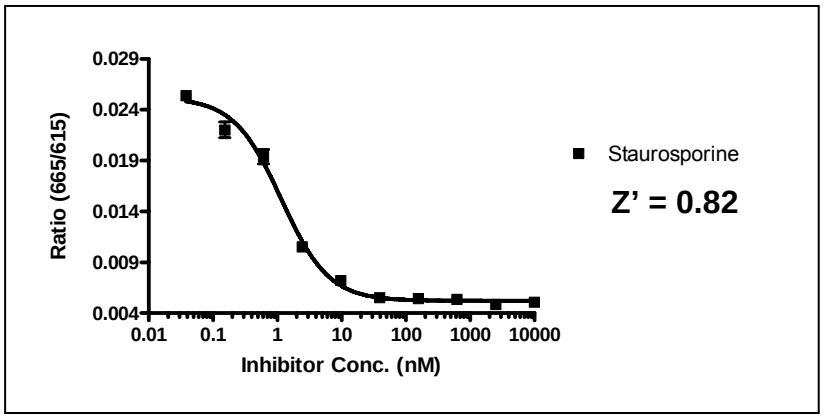


Figure 4: LanthaScreen™ Eu Kinase Binding Assay. LanthaScreen™ Eu Kinase Binding Assay performed using JAK2 JH1/JH2 and read on the BMG POLARstar OMEGA microplate reader. The assay was run as described in the JAK2 JH1/JH2 Binding Assay validation packet available online at: http://tools.invitrogen.com/content/sfs/manuals/JAK2_JH1_JH2_LanthaScreen_Binding.pdf).