GeneBLAzer® Assay Setup Guide on the Tecan Infinite® F500 Microplate Reader

NOTE: The Tecan Infinite® F500 Microplate Reader was tested for compatibility with Invitrogen's GeneBLAzer® Assay in bottom-read mode using the CellSensor® irf1-*bla* HEL and irf1-*bla* TF-1 cell lines (K1647 and K1657, respectively). 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.

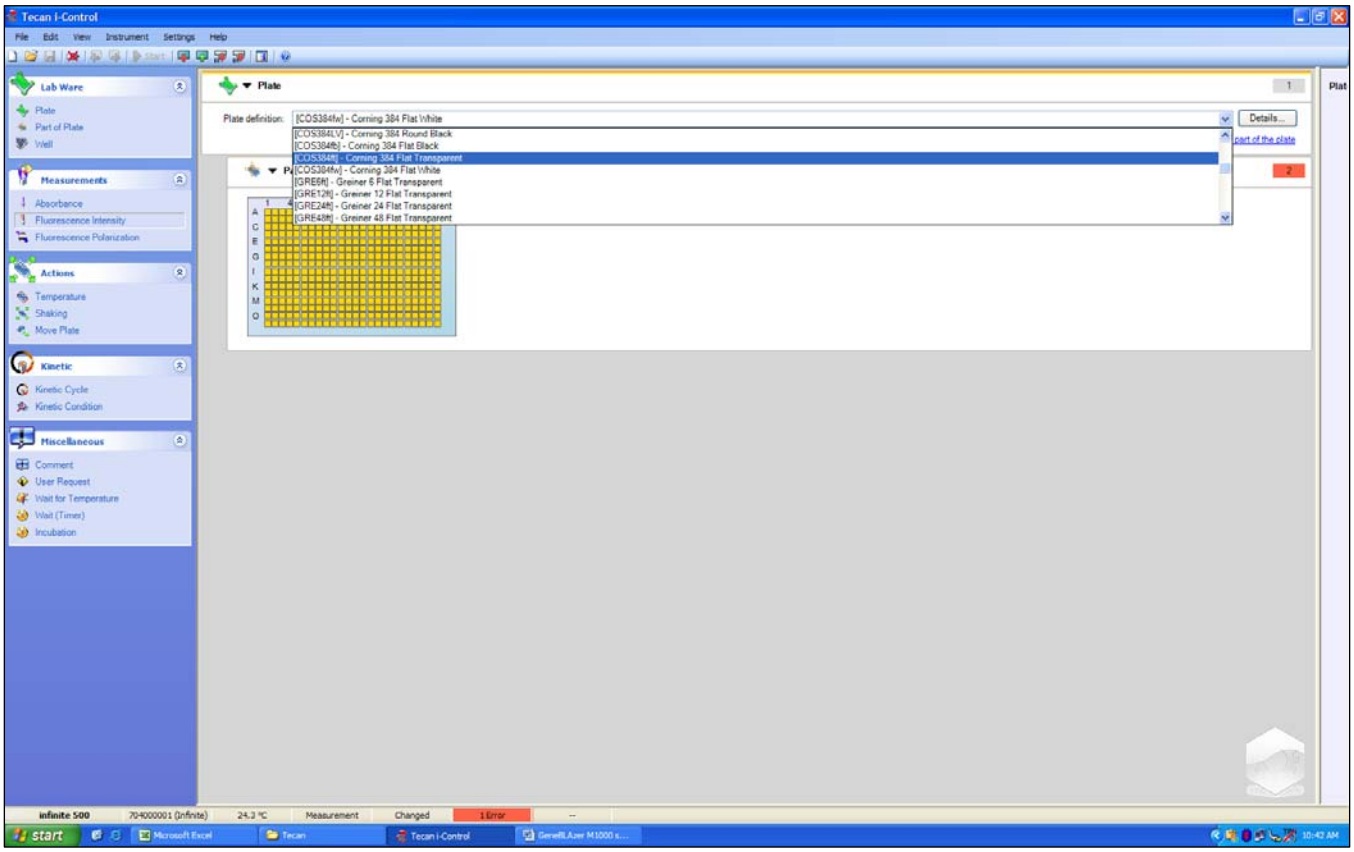
A. Recommended Optics

Tecan part number	wavelength (nm)	diameter (mm)
Excitation Filter *contact Tecan	415/20 (or similar)	12.5
Emission Filter 1 *contact Tecan	460/20 (or similar)	12.5
Emission Filter 2 *contact Tecan	535/25 (or similar)	12.5
Dichroic Mirror *contact Tecan		

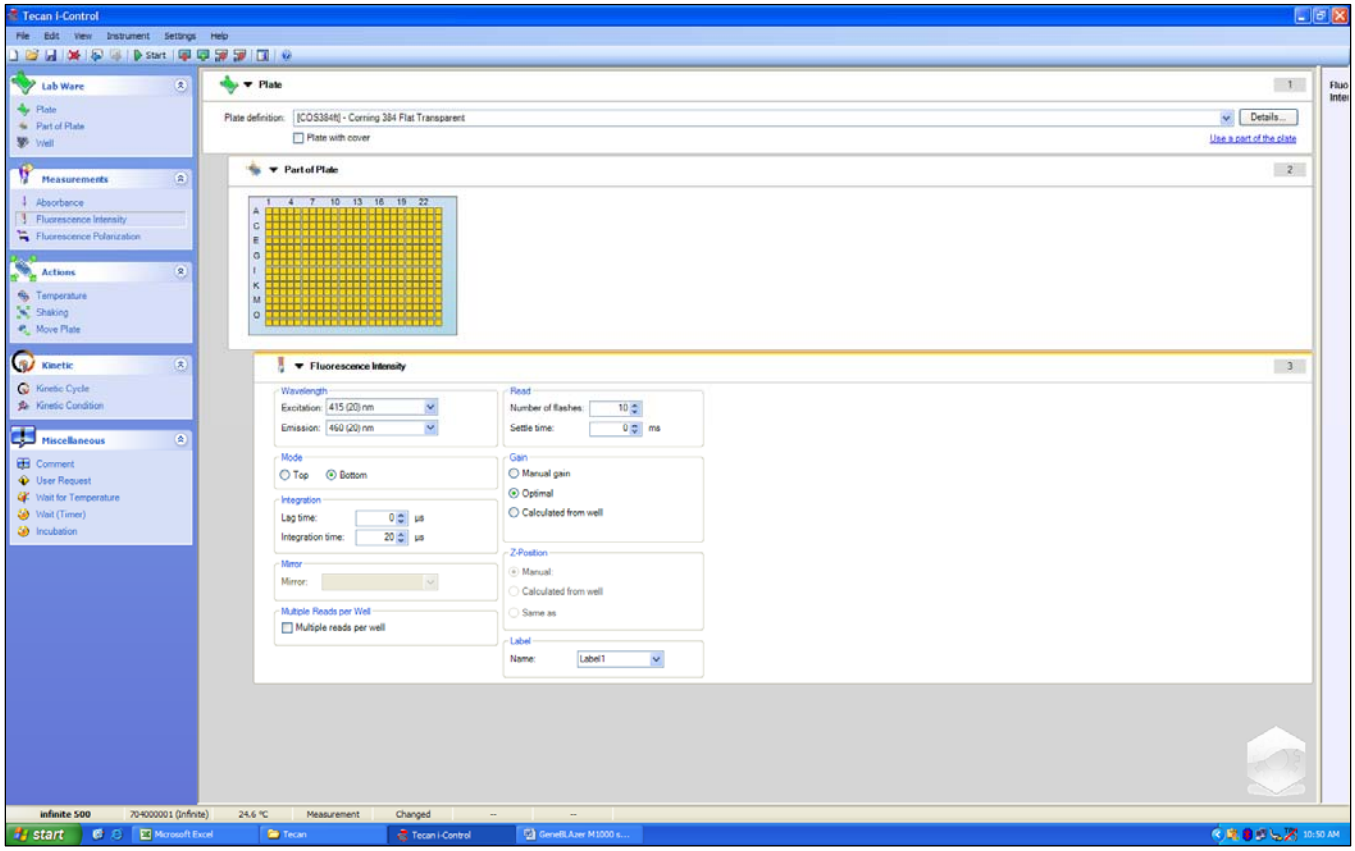
B. Instrument Setup

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

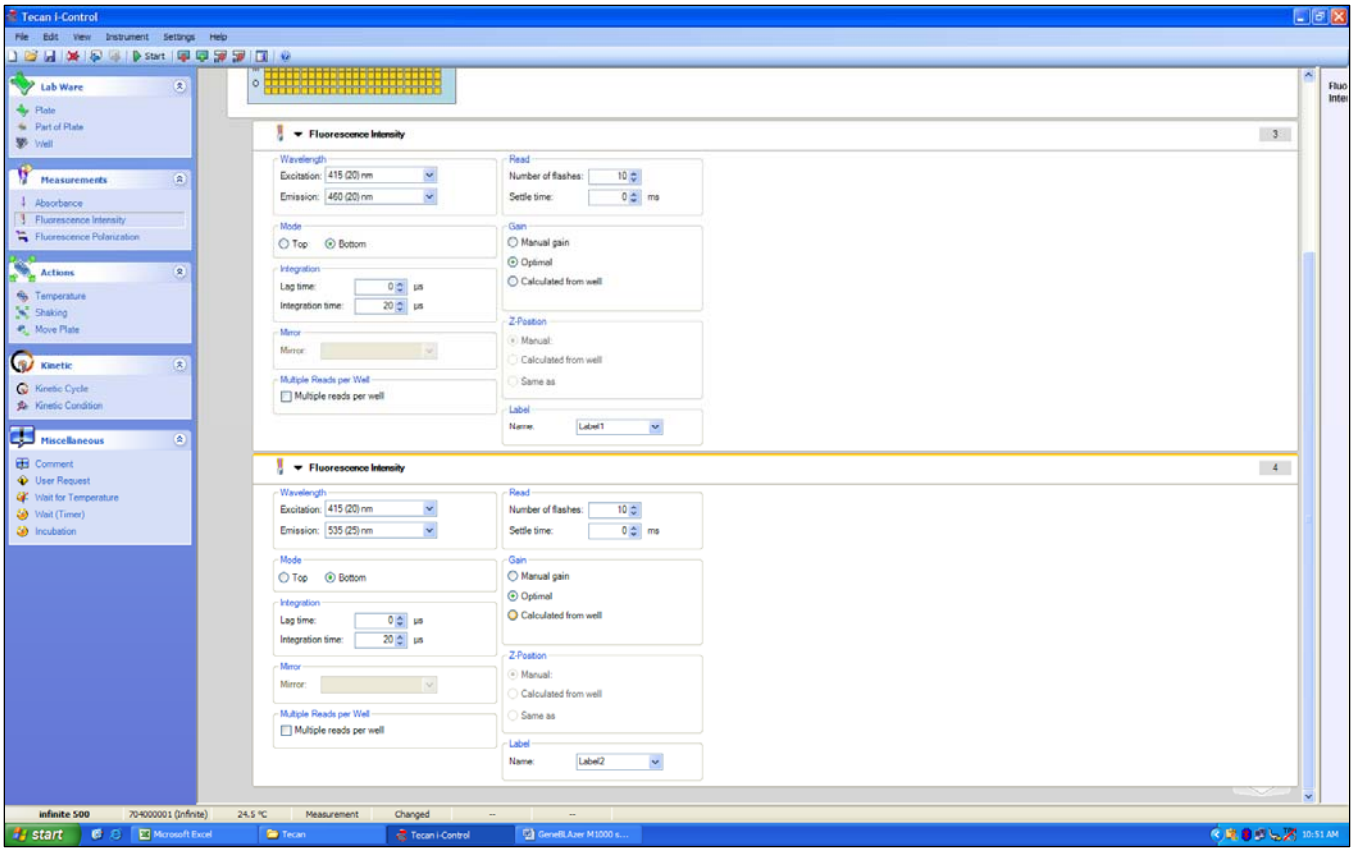
2. When i-Control opens, it will default to a generic starting page. Select “Plate Out” from the menu at the top to open the carriage, and insert your plate, then select “Plate In” to load your plate into the reader. Select your plate definition from the drop-down menu. For GeneBLAzer® assays select transparent because you will be reading from the bottom of the plate. 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). Note the filters selected below reflect the filters available at the time; the data in this document was obtained with the 415 nm excitation filter shown, but a standard 400 nm filter would also be suitable. GeneBLAzer® assays are read from the bottom of the plate, so make certain to select "Bottom" for the Mode setting. Select “Optimal” for the Gain "Top" for Mode, and select an appropriate well with substrate in it for Z-position (note in this screenshot the Z-position was set to manual). 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. GeneBLAzer® Cell-Based Assay using CellSensor® irf1-*bla* HEL and irf1-*bla* TF-1 cell lines.

NOTE: The following is a sample titration assay performed for demonstration purposes. The instrument settings above would be sufficient for any GeneBLAzer® assay, the information below is provided as representative data. Assays were run in 40 µl in 384-well black-wall, clear-bottom plates. The information below details how the validation assays were prepared and is provided as an explanation, it is not intended as a protocol. We recommend all first-time users follow the appropriate protocols and/or validation packets provided with their assays. Due to the various cellular backgrounds available, assay conditions can vary considerably. For additional details on handling and growing these and other CellSensor® cell lines, please see the appropriate line-specific protocols. Protocols for GeneBLAzer® assays can be located from the "GeneBLAzer® Portfolio" window of the following link: http://www.invitrogen.com/content.cfm?pageid=10523.

Cell-Based Assay Setup

1. On Day 1, one day prior to the actual reading step, harvest cells:
   - For Irf1-*bla* TF-1 cells count and spin down, wash once in Assay media to remove residual GM-CSF, and resuspend in Assay Media at a final density of 5 x 10^5 cells/ml. Place cells in incubator for 16 hours.
   - In the meantime, for Irf1-*bla* HEL cells, harvest and resuspend in Assay Medium at 9.5 x 10^5 cells/ml.

2. Prepare assay plate for HEL cells: Prepare a set of 1:1 serial dilutions from 100X the initial concentration (100 µM) in DMSO in a 384-well plate, starting in Rows A and E with 80 µl compound at 100 µM inhibitor in DMSO in Column 1 and 40 µl DMSO alone 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. Preparing an initial serial dilution in DMSO serves to improve accuracy and robustness, particularly with compounds which may have solubility issues at higher concentrations.

3. In Rows B and F prepare an intermediate dilution by adding 4 µl compound from the above serial dilution to each well below, then adding 36 µl Assay Buffer to produce a set of serial dilutions at 10X the desired final concentrations.

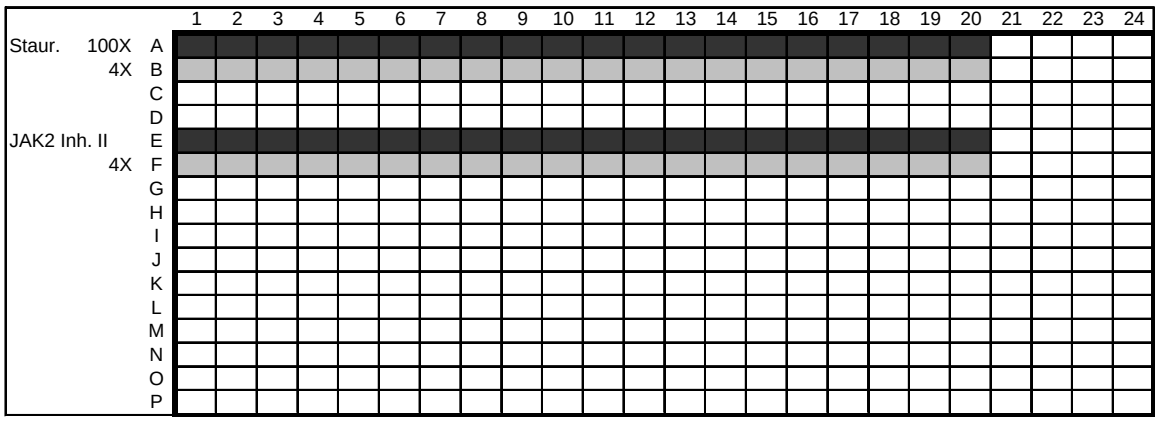


Figure 1: Schematic of initial compound dilution. Staurosporine and JAK2 Inhibitor II were titrated from a 100 µM starting concentration in the initial dilution series by preparing a 1:1 dilution curve in DMSO. A secondary dilution to 10X was then prepared in the rows below the initial dilution curve (lighter gray) using each line's specified Assay Buffer.

4. Add 36 µl per well of cell suspension to all wells except Column 23 and 24, Rows M through P. For Column 23 and Column 24, Rows M through P, add 40 µl Assay media alone. It is important to use cell-free wells in GeneBLAzer® to background subtract donor and acceptor values for optimal GeneBLAzer assay performance.

5. Now add 4 µl inhibitor from secondary dilutions above to wells in Columns 1-20 in quadruplicate (i.e. 4 µl of the 10 µM staurosporine into Column 1 Rows A through D and 4 µl of 5 µM staurosporine from the next well to Column 2 Rows A through D and so on to set up an actual titration from 1 µM final starting concentration of staurosporine). Add 4 µl Assay Buffer to Columns 21 and 22, as well as rows A-L of Columns 23 and 24, so each well has a final volume of 40 µl.

6. Incubate HEL assay plate for 15 hours.

7. The following morning, prepare an assay plate as above for the TF-1 line, except that cells are washed once more and resuspended at 1.5625×10^6 cells/ml in Assay Media. Add 32 µl cells per well to all wells of the plate except Column 23 and 24, Rows M through P. For Column 23 and Column 24, Rows M through P, add 36 µl Assay media alone, then add 4 µl Assay Buffer containing 0.8 ng/ml GM-CSF (experimentally determined, see protocol) to all wells except Column 23 (unstimulated control) to stimulate. Add 4 µl Assay Buffer to Column 23 to make up for the GM-CSF added to other wells. Now add 4 µl inhibitor from secondary dilutions above to wells in Columns 1-20 in quadruplicate (i.e. 4 µl of the 10 µM

staurosporine into Column 1 Rows A through D and 4 µl of 5 µM staurosporine from the next well to Column 2 Rows A through D and so on to set up an actual titration from 1 µM final starting concentration of staurosporine).

8. Add 4 µl Assay Buffer to Columns 21 and 22, as well as Columns 23 and 24, Rows A-L to bring all wells to a final volume of 40 ul.

9. Place TF-1 plate in incubator for 5 hours.

Substrate Loading

10. Load cells as follows(note in both cases it is critical that cells are allowed to load at room temperature.):

- For Irf1-*bla* TF-1 cells, 2.5 ml of 6X loading solution prepared by mixing 15 µl CCF4-AM with 150 µl Solution B, vortexing, then adding 2.335 ml of Solution C. Loading Solution mixed, and 8 µl per well added to plate. Plate incubated at ROOM TEMP. for 2.5 hours.
- For Irf1-*bla* HEL cells, 2.5 ml of 6X loading solution prepared by mixing 15 µl CCF4-AM with 150 µl Solution B, vortexing, and then adding 150 µl Solution D and 2.185 ml Solution C. Loading Solution mixed, and 8 µl per well added to plate. Plate incubated at ROOM TEMP. for 4 hours.

11. Read and analyze as directed in protocol.

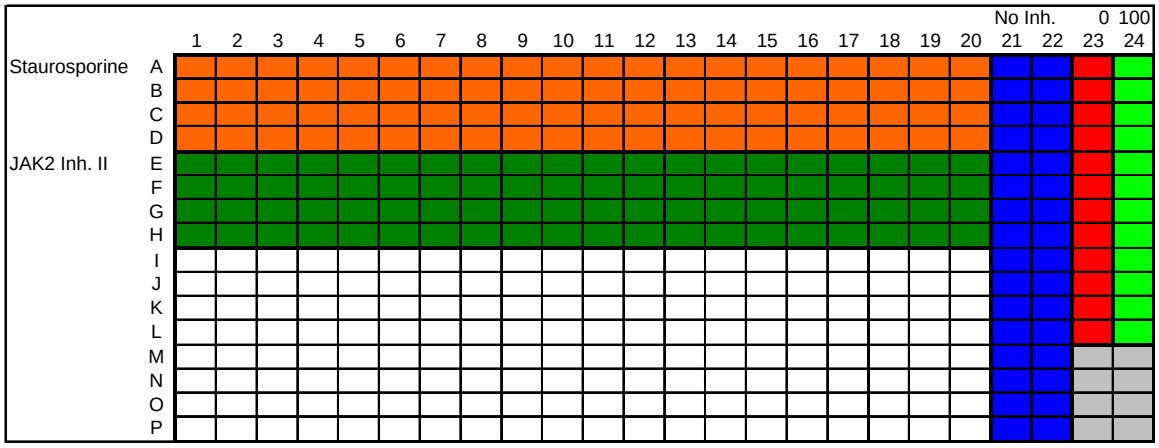


Figure 2: Assay Plate Schematic. Compound titrations shown in Columns 1-20, Columns 21 and 22 prepared without any inhibitor as 0% inhibition controls, Column 23 prepared with no CM-CSF (unstimulated 0% activity control) for the TF-1 line, but the HEL line is constitutively active so Column 23 is untreated for HEL cells, and Column 24 is also untreated in both cases as a 100% activity control. Note 8 wells in gray in bottom right, which were prepared with out any inhibitor or cells, as controls for background subtraction.

D. Results

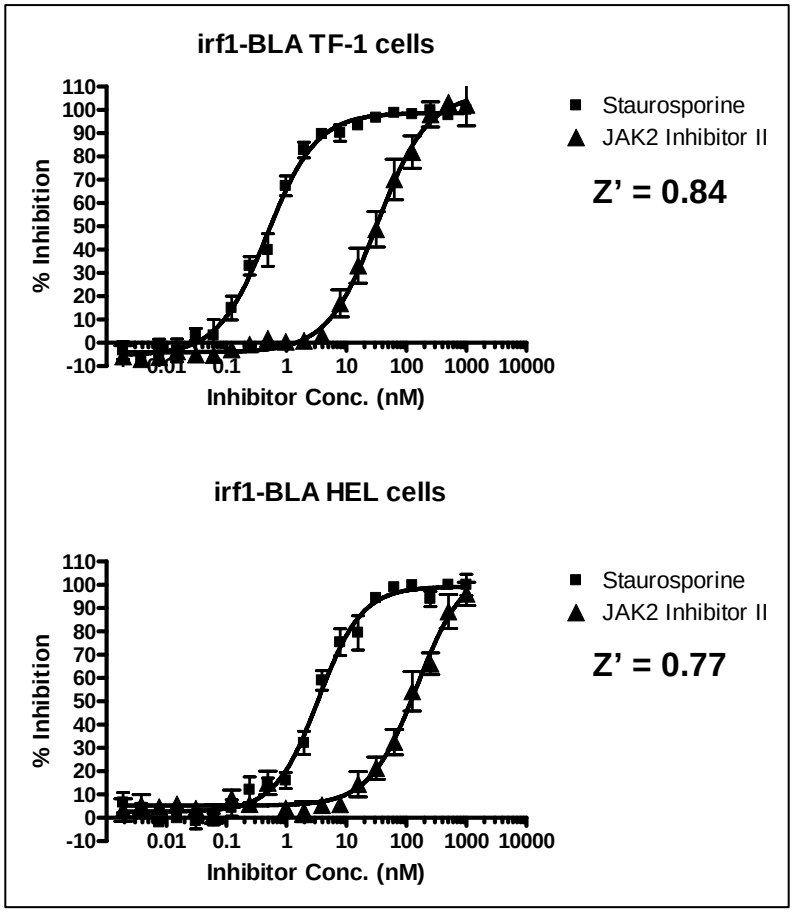


Figure 1: GeneBLAzer® Assay. GeneBLAzer® assay performed using the Tecan Infinite® F500.