Predictor™ Assay Setup Guide on the BioTek Instruments Synergy™ 2 Multi-Mode Microplate Reader

NOTE: The BioTek Instruments Synergy™ 2 Multi-Mode Microplate Reader was tested for compatibility with Predictor™ hERG FP Assay (PV5365) using controls provided within the assay kit and the known hERG channel blockers astemizole and terfenadine. 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 BioTek instruments or Gen5 software, please contact BioTek Instruments at 1-888-451-5171.

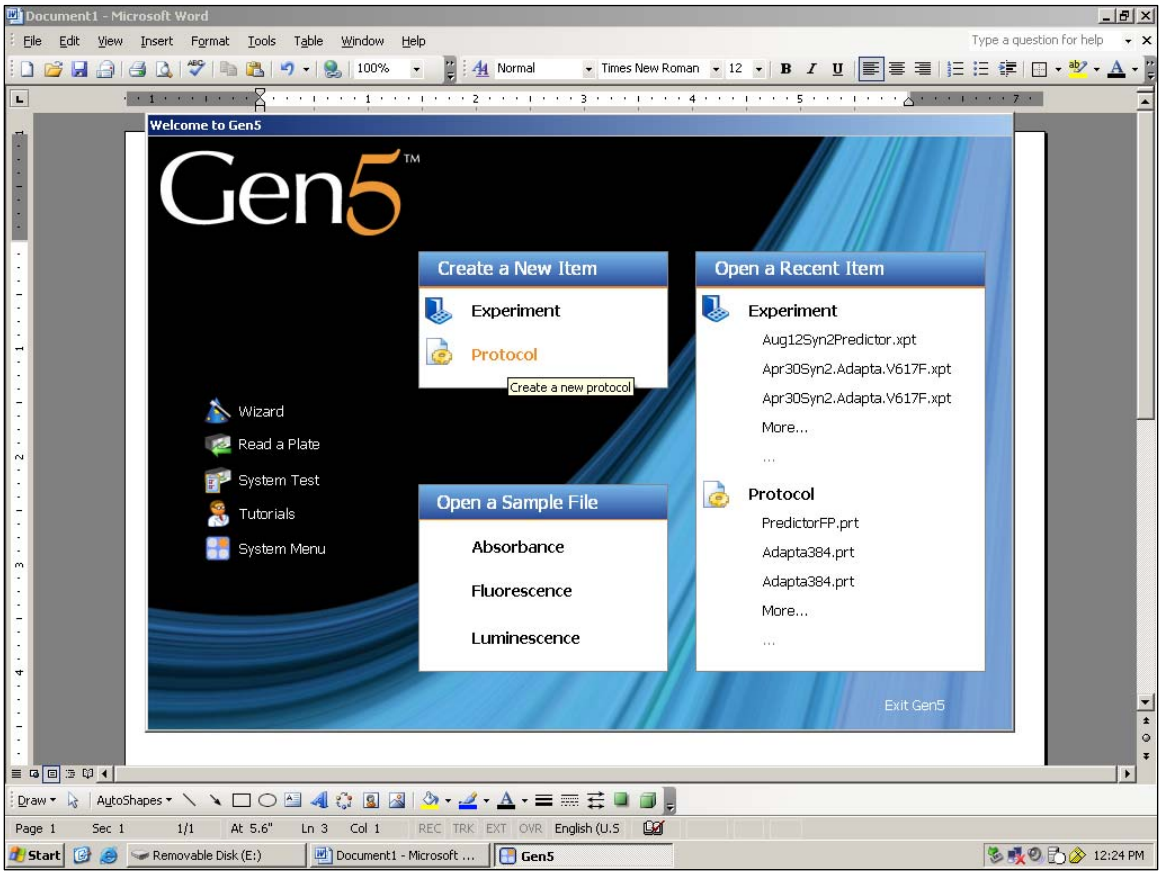
A. Recommended Optics

BioTek Instruments part number	wavelength (nm)	diameter (mm)
Excitation (7082223)	530/25	18
Emission 1 (7082224)	590/35	18
Emission 2 (7082224)	590/35	18
Dichroic Mirror (7137570)	570 (or 550)	

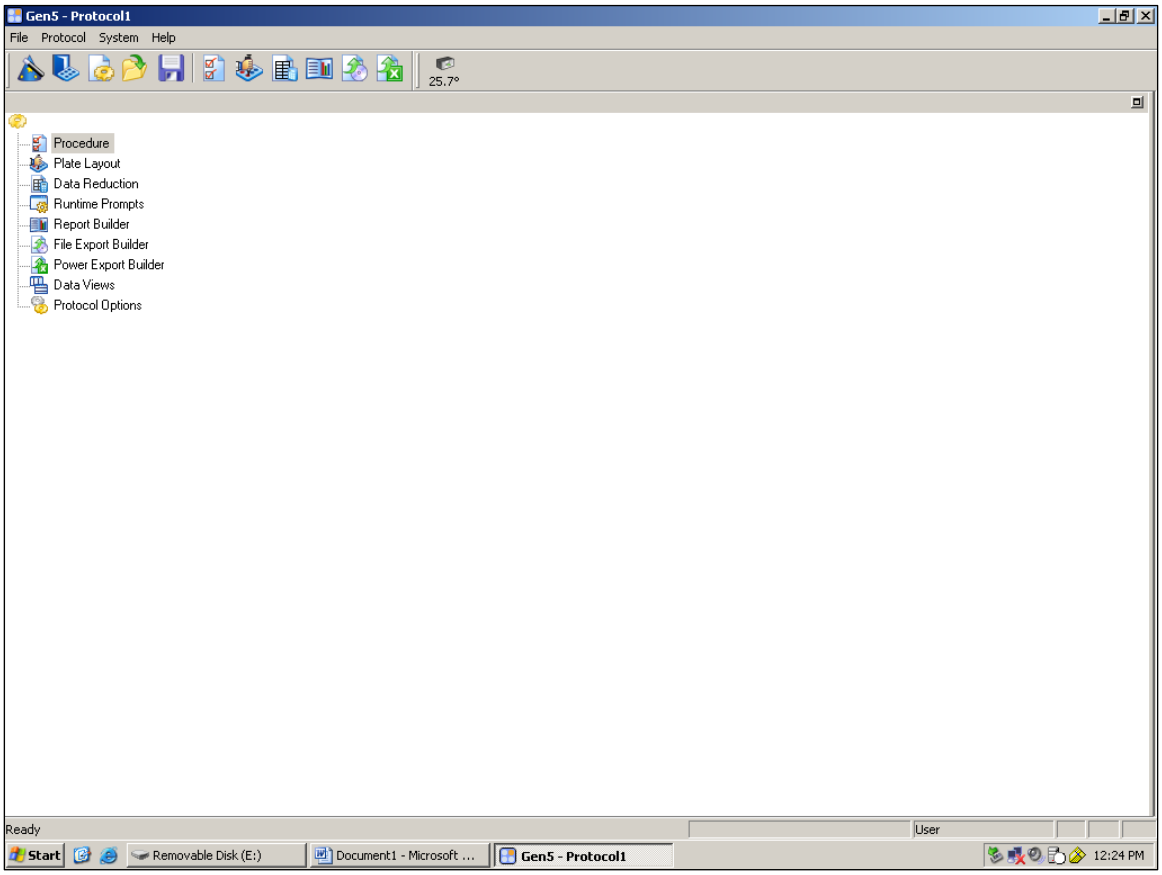
B. Instrument Setup

1. Make certain plate reader is turned on, and open up BioTek Gen5 software on computer.

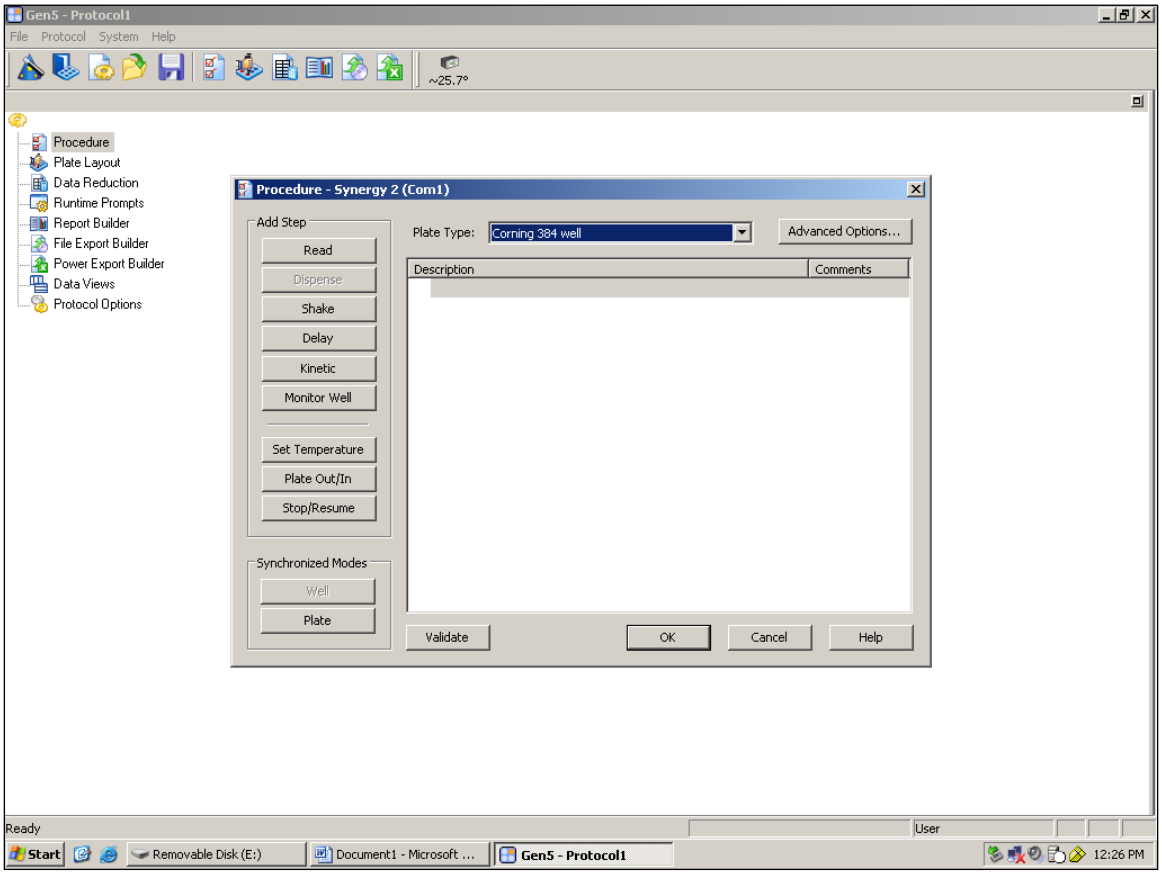
2. When Gen5 software opens, if you do not have a pre-existing protocol for Predictor™, select "Protocol" from the “Create a New Item” menu.



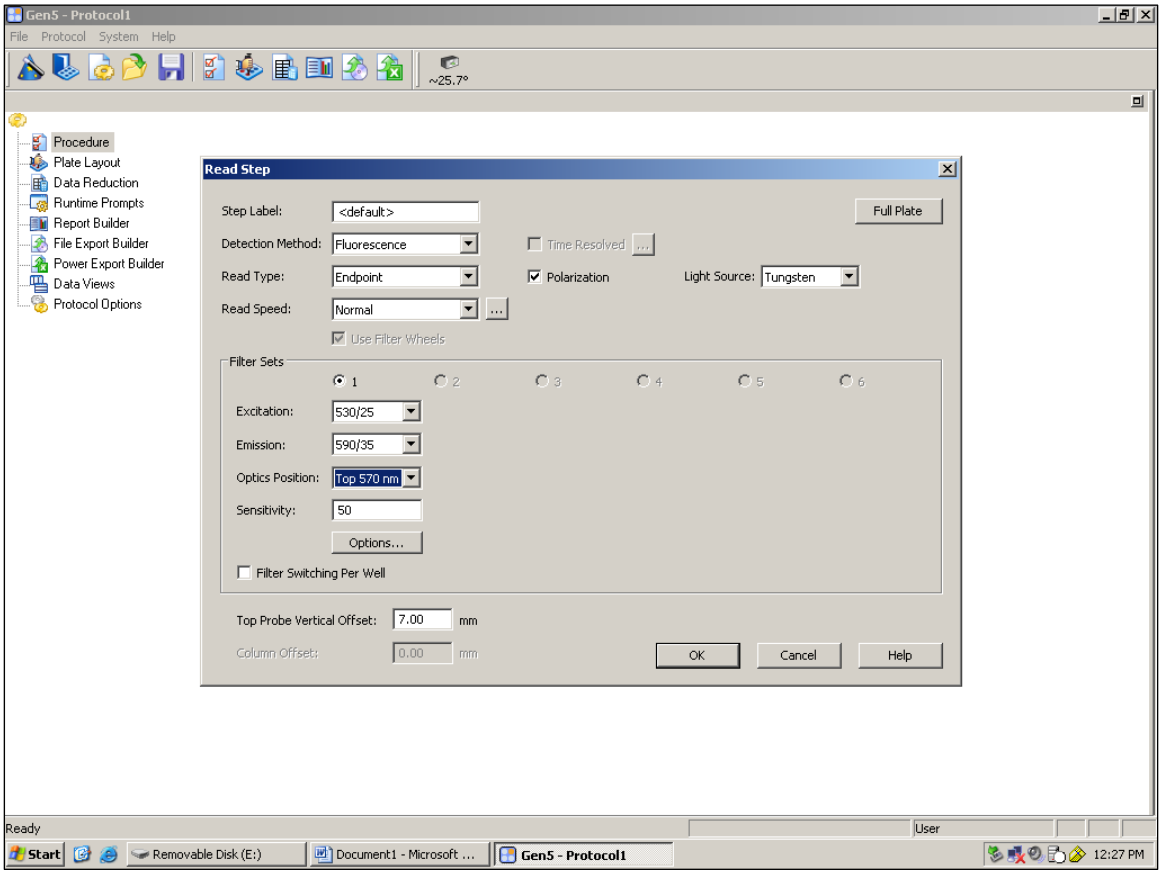
3. At this point, a new screen will open (below). Click on “Procedure” from the menu on the left side of the screen.



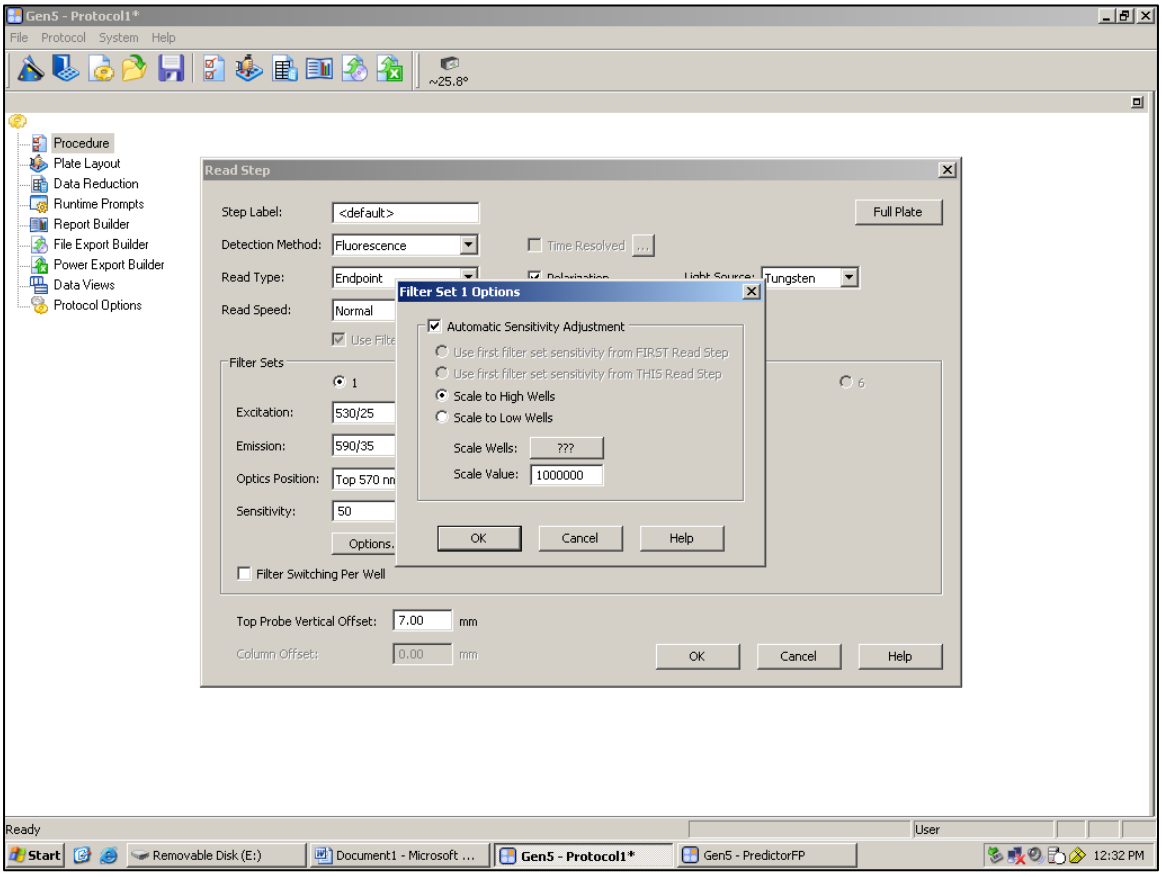
4. A new Procedure window will pop up. Select plate type from the drop-down menu. Next, click “Read” at the left side of the Procedure window.



5. A “Read Step” window will open automatically. Select “Fluorescence” for Detection Method, check the “Polarization” box, and select the appropriate filters under the “Excitation,” “Emission,” and “Dichroic” windows. When finished select “Options” just below the Sensitivity window.

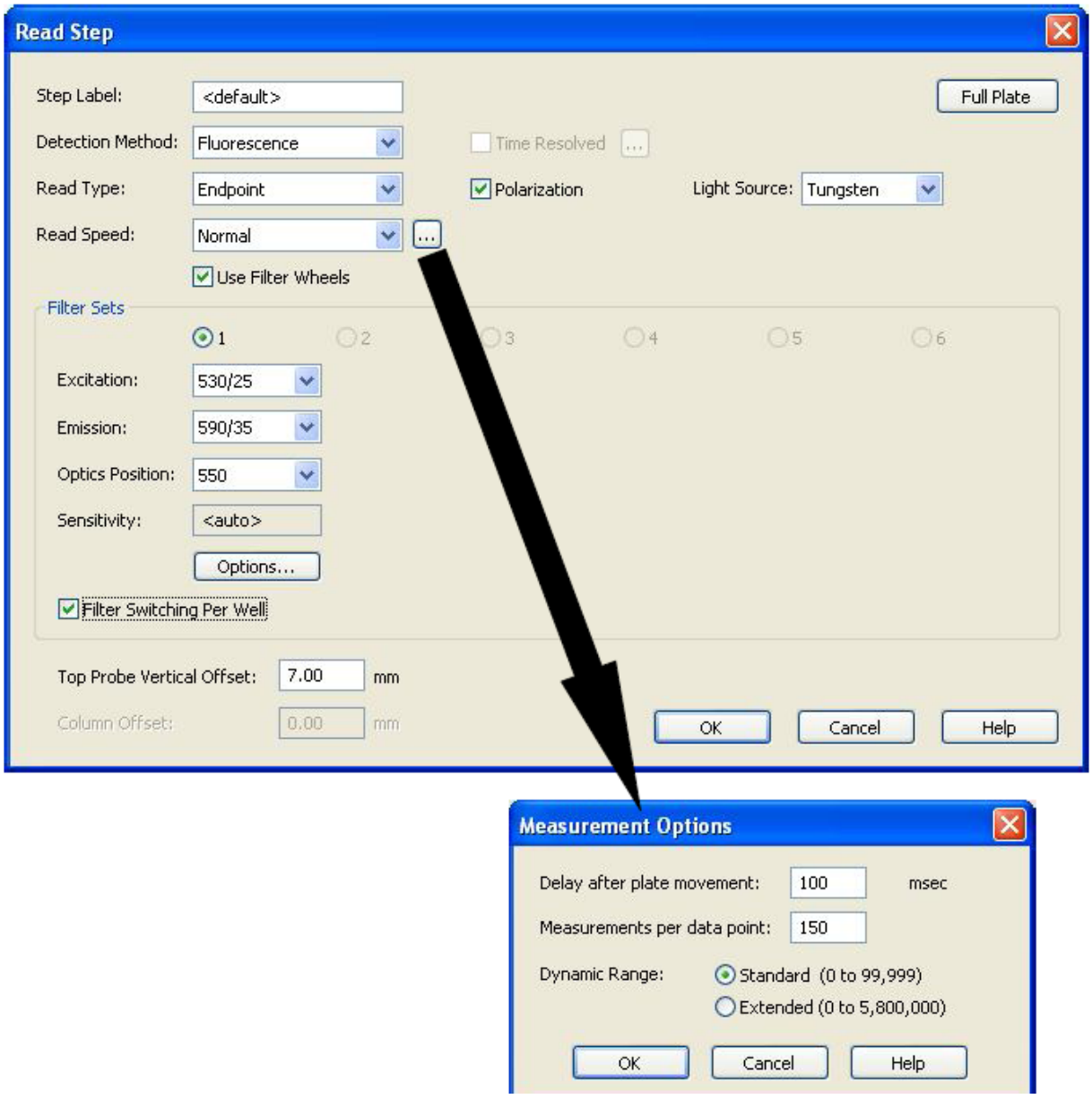


6. From the new window, check the “Automatic Sensitivity Adjustment” box and select “Scale to High Wells”. When finished, select “OK”. You may also opt to save this protocol at any point by selecting “Save Protocol As” from under File in the main toolbar at the top of the screen.

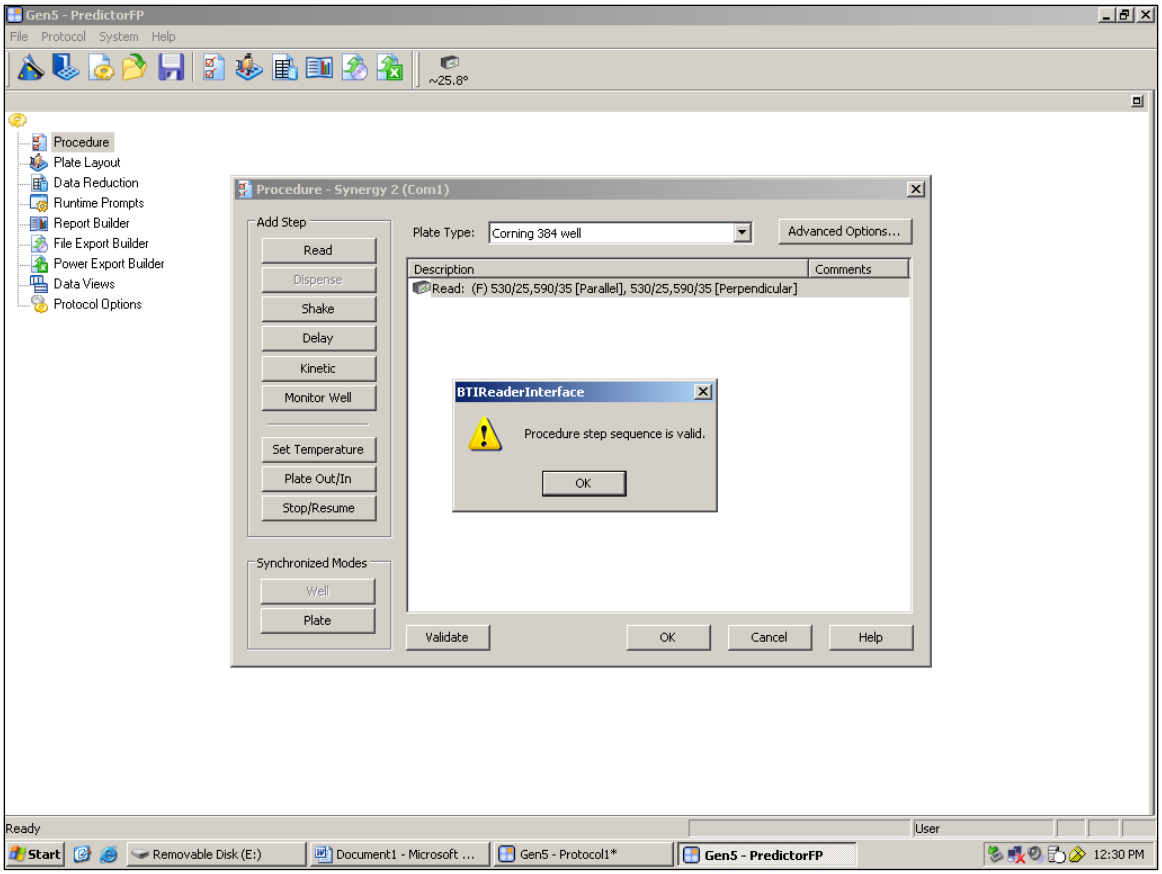


7. Adjust the read speed as shown below, and set to 150 measurements.

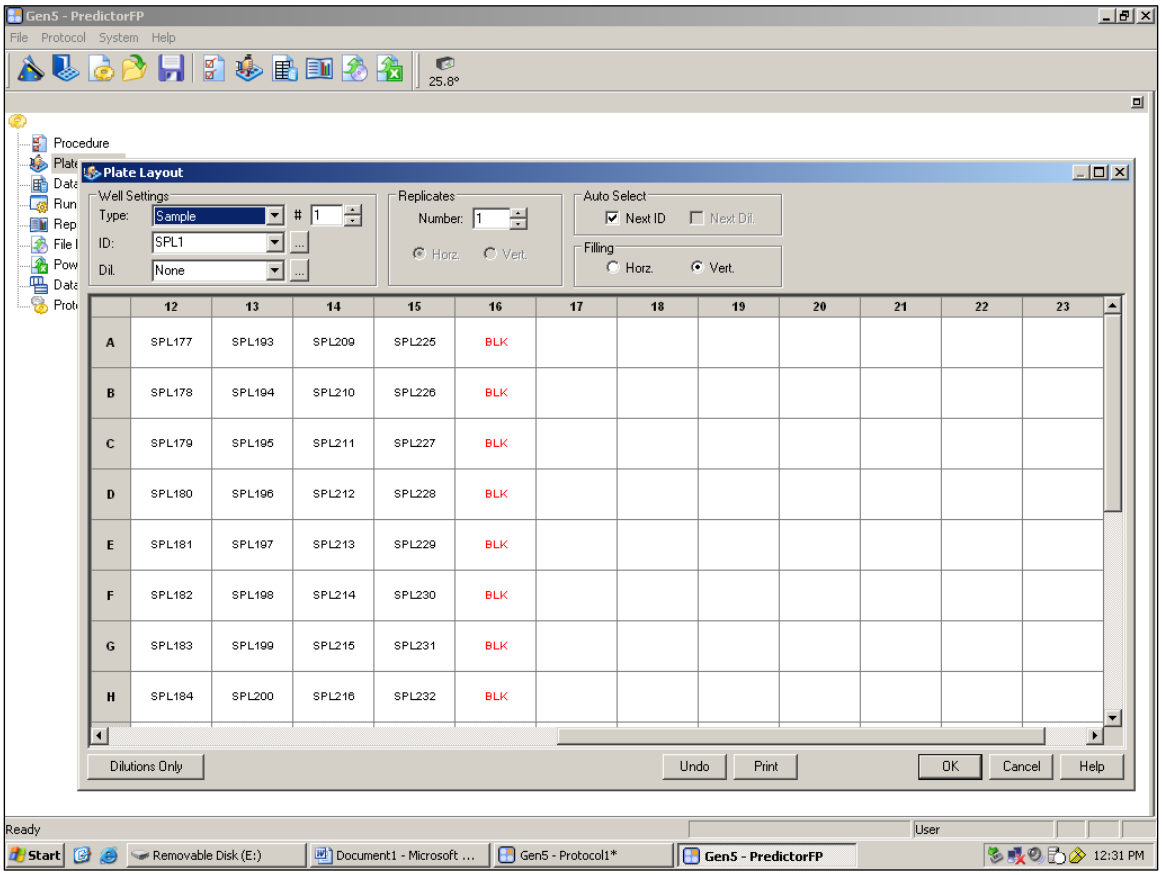
NOTE: For best assay results on this reader, using fewer measurements per data point will decrease read time but could adversely affect assay performance (e.g. %CVs, Z').



8. Returning to the “Procedure” window, click on the “Validate” tab at the bottom. The reader will run through some internal checks to verify the protocol is sound, and a message will appear. Select “OK”. Select “OK” again in the main “Procedure” window and it will close.

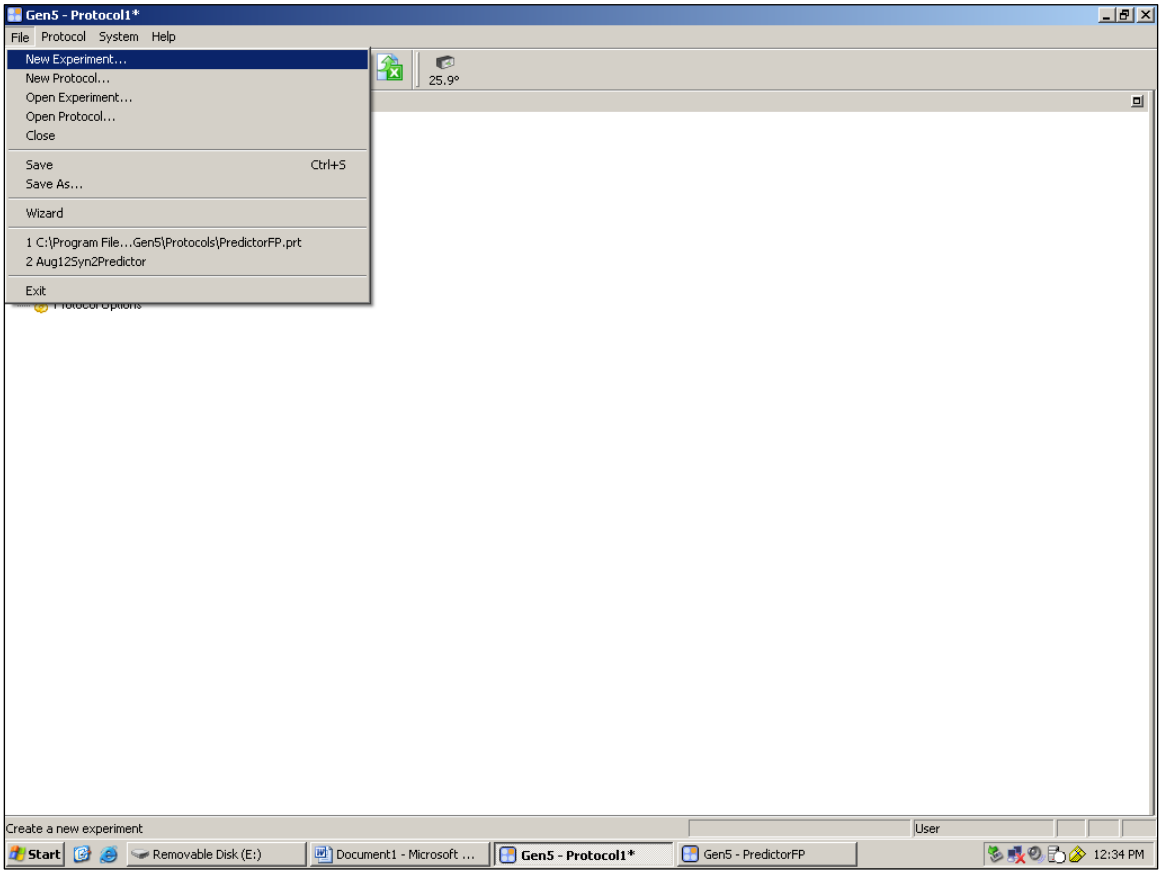


9. In the “Plate Layout” window, use the drop-down “Type” window near the top to select your sample types (here everything except Column 16 is “Sample” and Column 16 is “Blank”) and drag across the portion of the plate you wish to select. When finished, select “OK”. All sub-windows will close at this point. If you have not already, select “Save Protocol As” from under File in the main menu, and name and save your protocol.

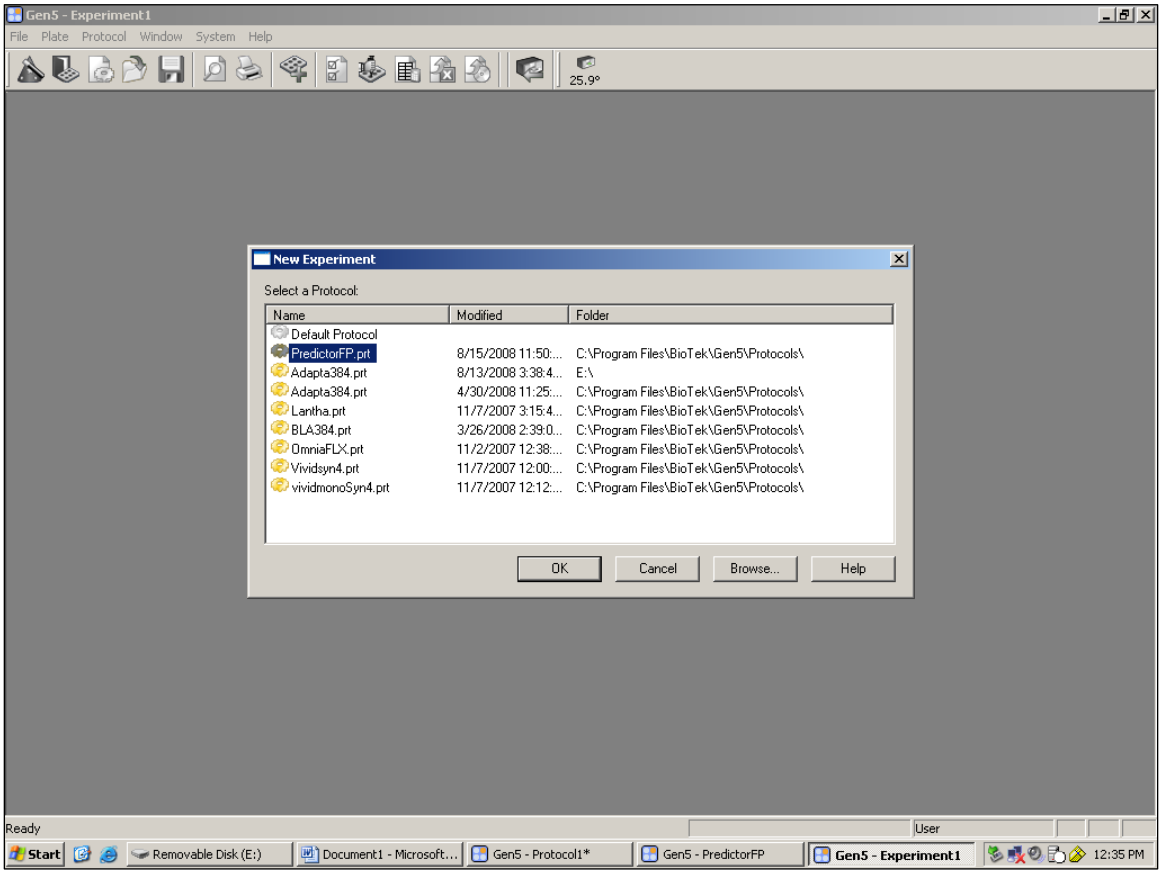


	12	13	14	15	16	17	18	19	20	21	22	23
A	SPL177	SPL193	SPL209	SPL225	BLK							
B	SPL178	SPL194	SPL210	SPL226	BLK							
C	SPL179	SPL195	SPL211	SPL227	BLK							
D	SPL180	SPL196	SPL212	SPL228	BLK							
E	SPL181	SPL197	SPL213	SPL229	BLK							
F	SPL182	SPL198	SPL214	SPL230	BLK							
G	SPL183	SPL199	SPL215	SPL231	BLK							
H	SPL184	SPL200	SPL216	SPL232	BLK							

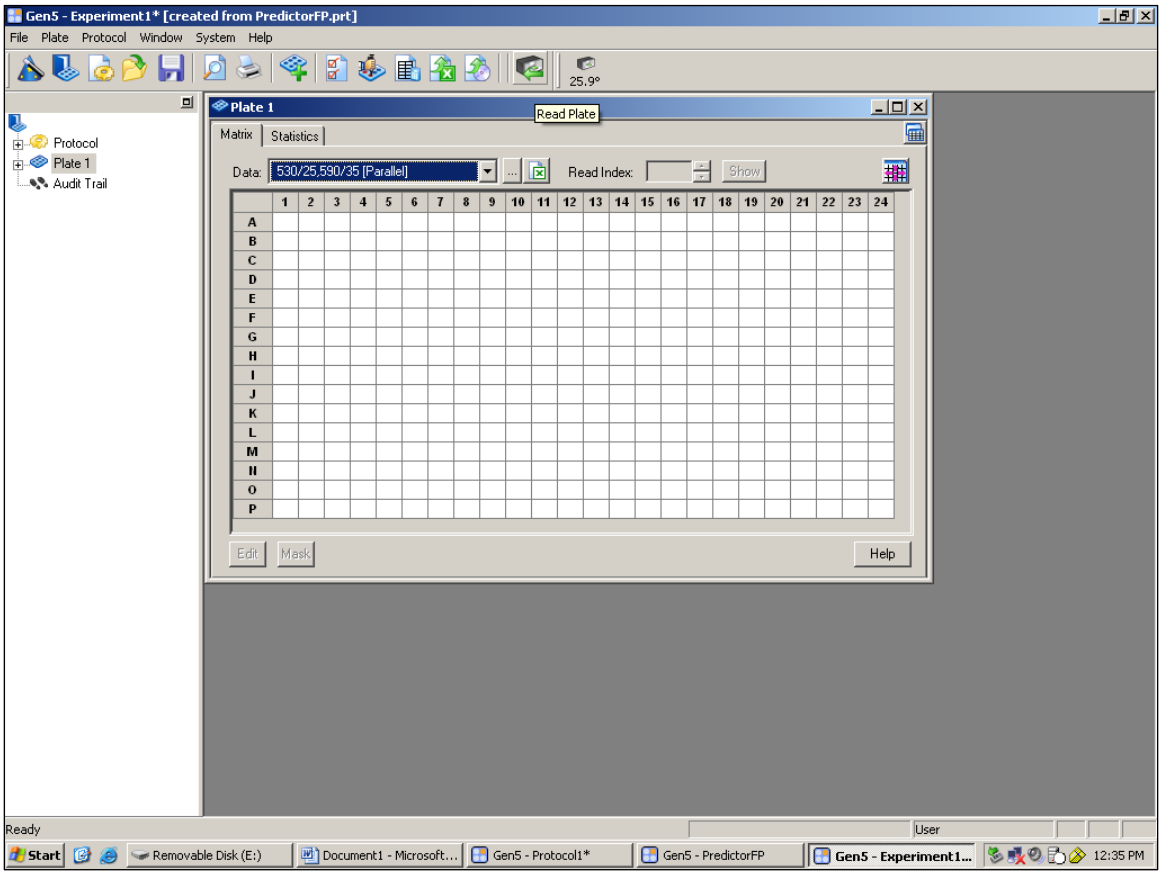
10. Select “File” and “New Experiment”.



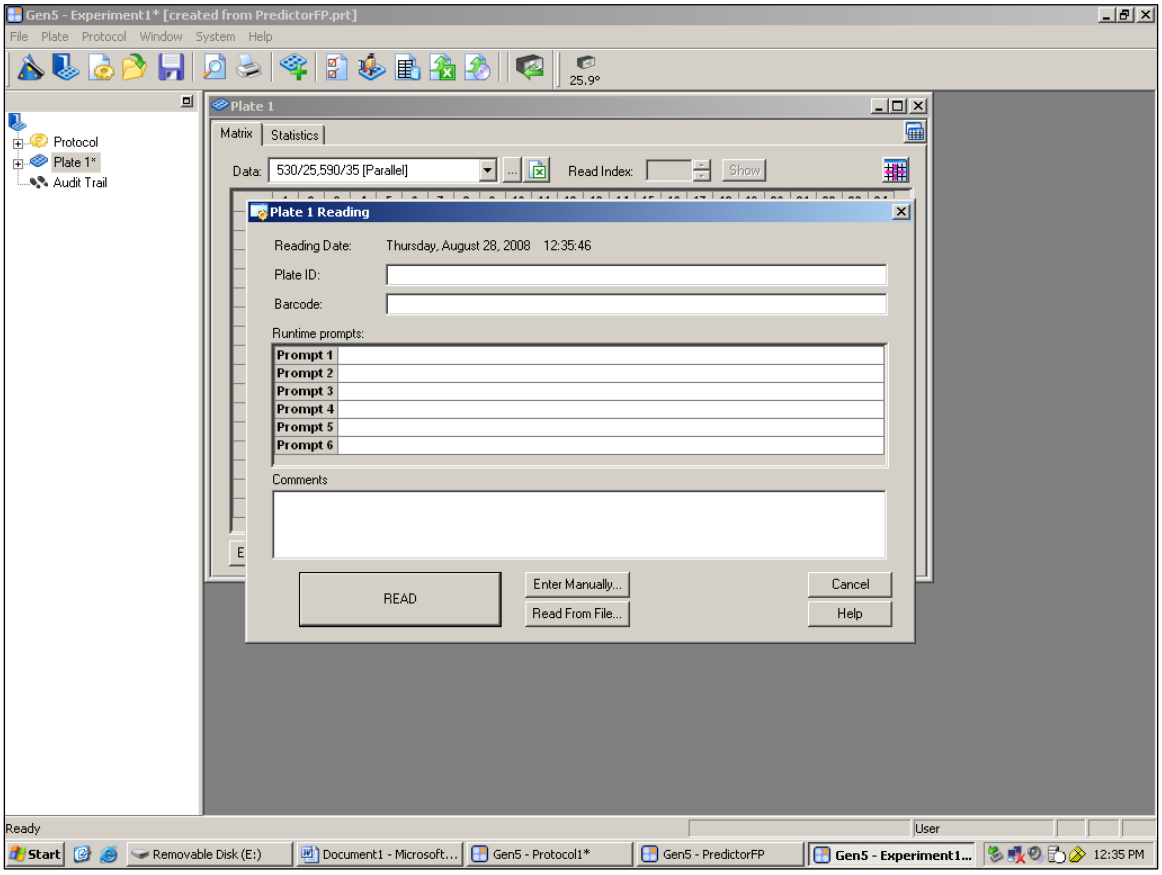
11. Select your protocol from the list.



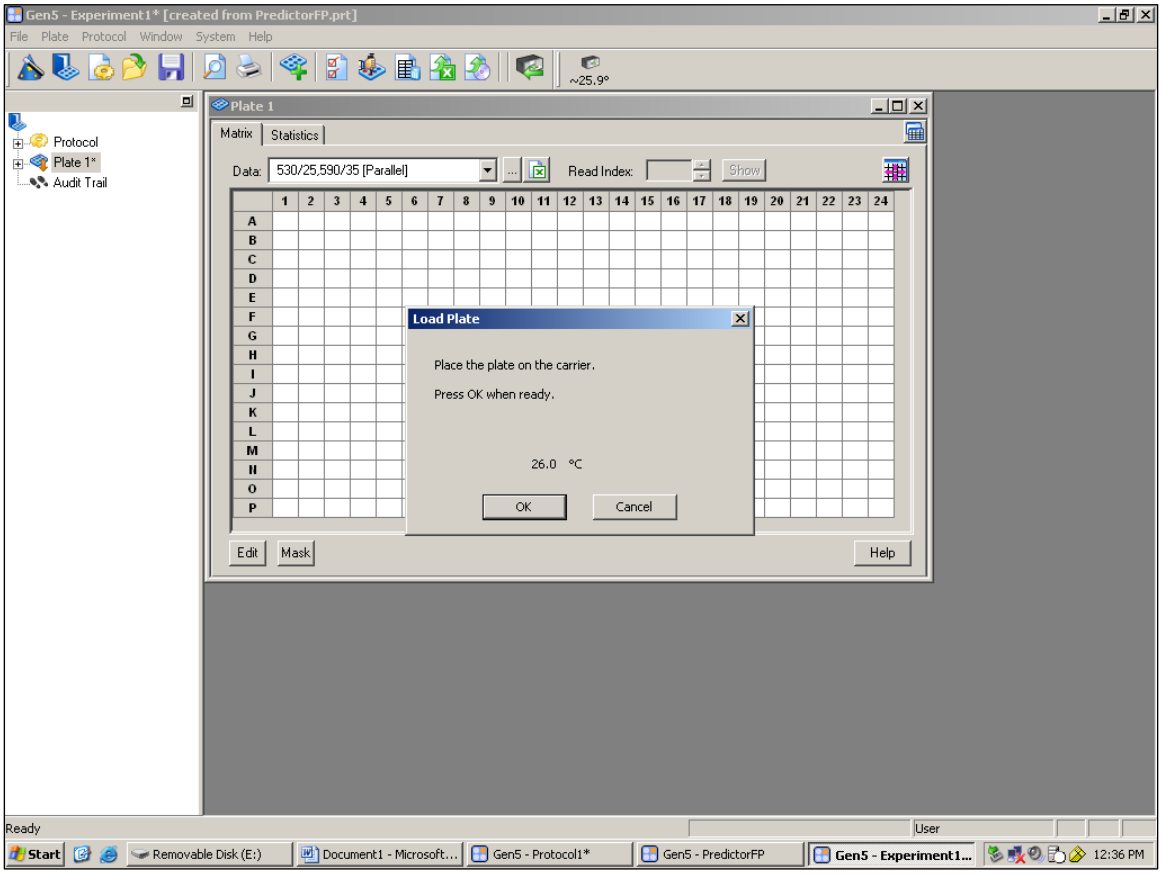
12. A new Read Step window will appear. Select the “Read Plate” icon at the top of the window.



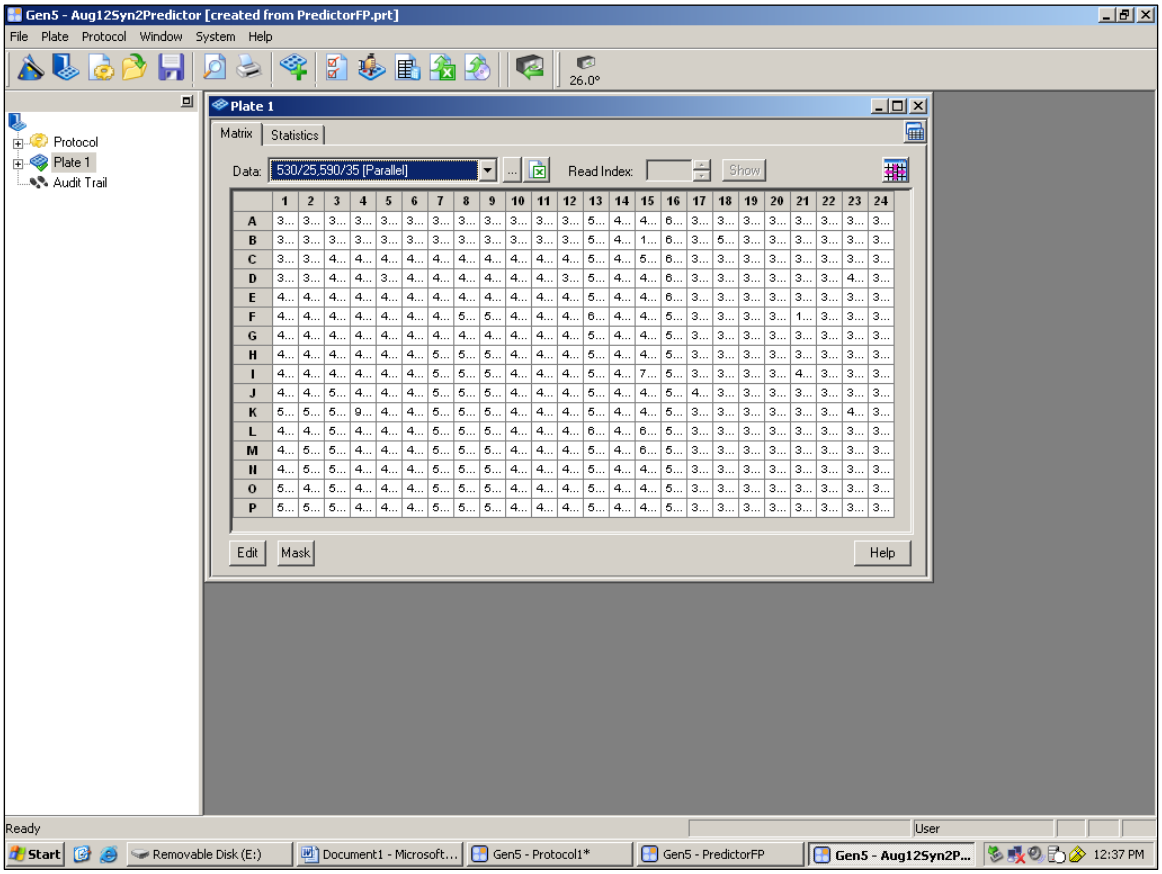
13. A second window will appear. Select “read” again.



14. A final prompt will appear. Press “OK” to initiate reading.



15. Synergy will commence reading of plate. As the instrument reads, data will begin to populate the cells as shown below. Data can be exported when finished by selecting the "Export" button or by preparing formal export settings in the export wizard.



C. Predictor™ hERG FP Assay

NOTE: The following is a sample assay performed for demonstration purposes. The instrument settings above would be sufficient for any Predictor™ assay or other Invitrogen red FP assay, the information below is provided as representative data. Assays were run in 20 µl in 384-well untreated low-volume polystyrene plates (Corning #3677). In order to demonstrate the correction of Polarization Interference at high concentrations of compound described in the Predictor protocol Section 4.5, we prepared dose-response curves of both astemizole and terfenadine with and without saturating E-4031. Also, all FP data was background-subtracted using wells containing membranes but no tracer. Note background subtraction is not required, but is strongly advised and can increase overall assay window by 10-20%.

1. Thaw all reagents as directed in protocol. Thaw compounds (Astemizole and Terfenadine, 1 mM stocks in DMSO) at room temp.

2. Prepare Predictor™ hERG membrane by sonification, dounce homogenization, or trituration to ensure a uniform suspension with no precipitate or aggregates.

3. A dilution series of both astemizole and terfenadine was prepared as follows:

   3.1. 1:10 pre-dilution: In a 96-well plate, add 40 µl of DMSO to wells B1-H1 and A2-H2 (for Astemizole pre-dilution) and B7-H7 and A8-H8 (for Terfenadine pre-dilution).

   3.2. Add 40 µl of 1 mM astemizole to well A1 and 40 µl of 1 mM terfenadine to well A7.

   3.3. Prepare Master Dilutions: Transfer 20 µl from well A1 to well A2, mix by pipetting up and down several times, then transfer 20 µl from well A2 to well B1, mix again, then transfer 20 µl from well B1 mixed into well B2, mix again, then transfer 20 µl from well B2 to well C1, repeating this pattern downwards to the bottom of plate (see Figure 1). Repeat this same procedure with terfenadine starting in well A7,

   3.4. From well A1, remove 4 µl compound in DMSO and add to well A3 in column 3. From well A2, remove 4 µl compound in DMSO and add to well A4 in column 4. Repeat to bottom of plate, transferring 4 µl aliquots from initial dilution to new column. This step can be simplified by use of a multichannel pipette.

   3.5. Repeat for terfenadine, adding 4 µl to columns 9 and 10. When finished, add 96 µl Predictor™ hERG FP Assay Buffer to each new well to create the intermediate dilution series.

4. Add 5 µl compound per well to a nontreated Corning 3677 black polystyrene 384-well plate, as follows:

   4.1. From well A3 of the 96-well plate (maximum astemizole, conc. 40 µM) transfer 6 replicates of 5 µl compound to wells A1-A6 of the 384 assay plate. In the end, column 3 of the 96-well plate will fill the first 6 wells in rows A,C, E, G, I, K, M, and O while column 4 will fill the first 6 wells of rows B, D, F, H, J, L, N, and P. Repeat same procedure was repeated for the next 6 wells in each row on the 384-well assay plate using columns 9 and 10. This step can be simplified by use of a multichannel pipette.

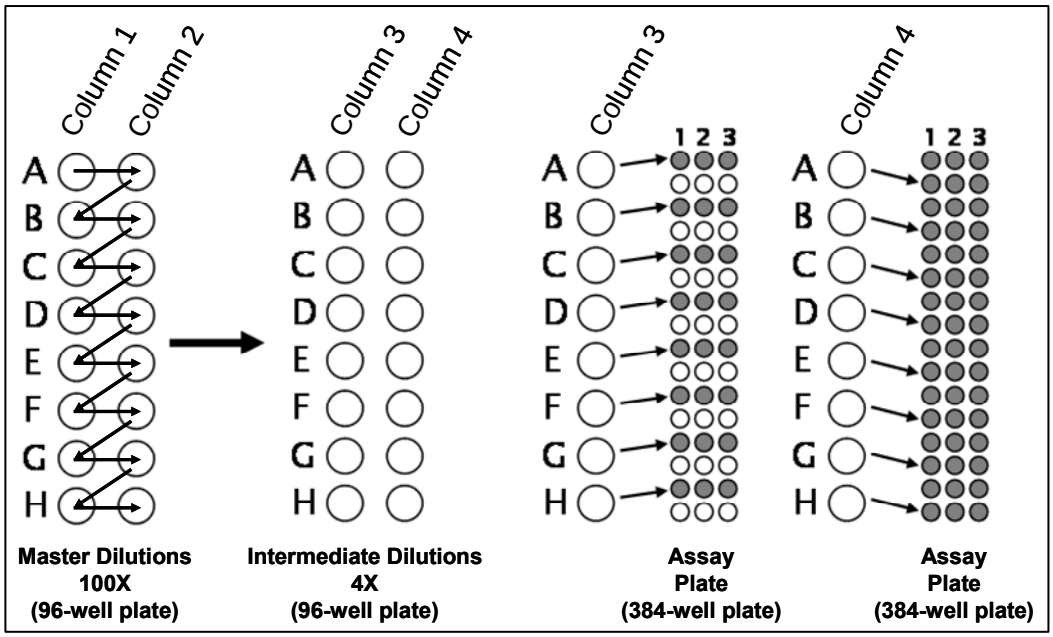


Figure 1: Schematic for Preparation of Compound Dilution. Schematic demonstrates progression of dilutions and final transfer to assay plate. Note in this case 2 compounds were titrated and each compound was finally used in replicates of 6 (3 with and 3 without saturating E-4031).

5. Once compound titration wells are prepared (columns 1-12 of assay plate), also prepare the following controls:

   - **Negative Control, Tracer fully Bound (Column 13, B).** Add 5 ul Predictor buffer containing 4% DMSO to all wells in Column 13 (to control for DMSO in titration wells). Tracer and membrane will be added later. This control will show full tracer binding activity of assay.

   - **Positive control, Tracer Displaced (Column 14, D).** Add 8 µl E-4031 to 192 µl Predictor™ hERG FP Assay Buffer also containing 4% DMSO. Add 5 µl per well of this solution to each well of Column 14 of assay plate. Tracer and membrane will be added later. This control will show maximum displacement of tracer from the hERG channels in the membrane prep.

   - **Free Tracer Control (Column 15, F).** Add 15 µl Predictor™ hERG FP Assay Buffer to each well of column 15. Tracer will be added later. This control will show fully unbound tracer in solution, as well as being used to calibrate the instrument.

   - **Assay Blank (Column 16, Blank).** Add 10 µl of Predictor™ hERG FP Assay Buffer per well to Column 16. 10 µl membrane will be added later. This control will show background fluorescence and to background subtract the rest of the assay wells prior to calculating FP values.

6. Sonicated/triturated membrane split: remove 2 ml Predictor™ membrane and add 40 µl E-4031 to these membranes.

7. Add 10 µl E-4031-containing membrane per well to columns 4-6 (astemizole with E-4031 baseline subtraction) and 10-12 (terfenadine with E-4031 baseline subtraction).

8. Add 10 µl untreated membrane per well to columns 1-3 (astemizole), 7-9 (terfenadine), 13 (Bound Tracer), 14 (Displaced Tracer), and 16 (Blank).

9. Tracer added last: Add 32 µl tracer to 1968 µl Predictor™ hERG FP Assay Buffer and add 5 µl/well of this solution to all wells in Columns 1-15.

NOTE: Predictor™ is a highly sensitive assay. Furthermore, the baseline correction wells use saturating levels of E-4031**. It is extremely important to ensure that pipetting steps are done carefully and there is no carryover/contamination of neighboring wells.** The order of wells in the plate layout may be changed to best facilitate your pipetting and minimizing carryover if desired, but all the controls above are highly recommended.

10. Shake plate on an orbital plate shaker for 30 seconds, cover with foil, and incubate 2 hours before reading.

11. Read plate on plate reader as outlined above.

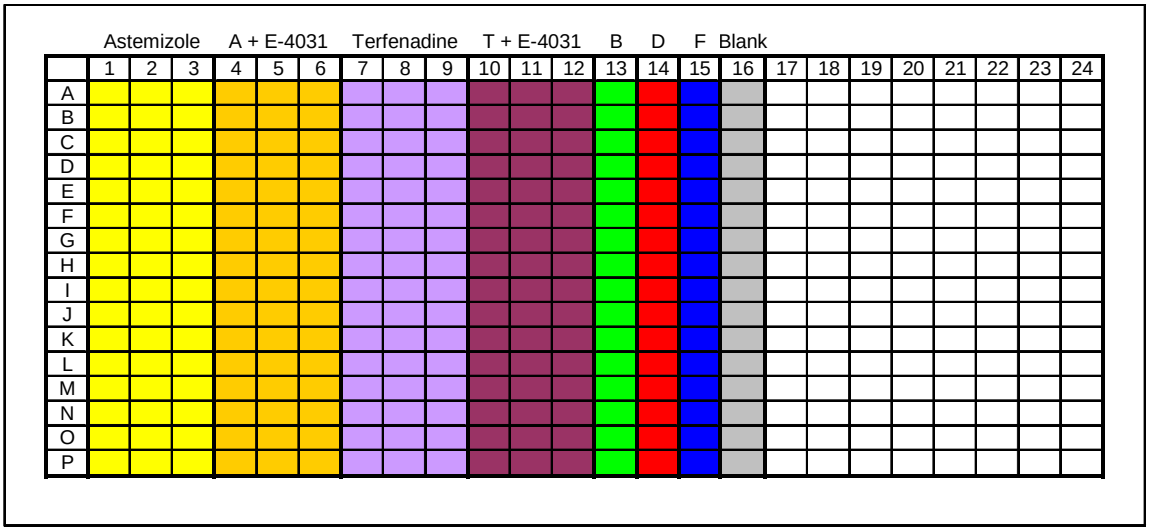


Figure 2: Schematic of Assay Plate Layout. Predictor™ hERG assay was set up with a dose-response curve of Astemizole and Terfenadine (1:3 dilution series from a starting concentration in the top row of 10 µM) prepared in replicates of 6. From this, 3 replicates were assayed for ability to disrupt Predictor™ Tracer binding and 3 were assayed in saturating levels of E-4031 in order to prepare a baseline correction for each data point.

D. Results:

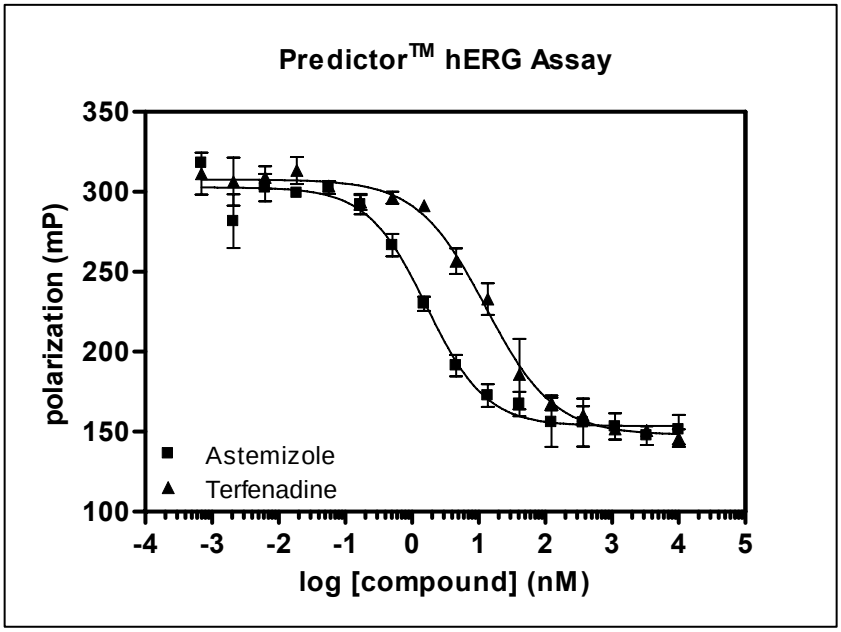


Figure 3: Predictor™ hERG Assay. Dose-Response Curves read on the BioTek Instruments Synergy™ 2 using the Predictor™ assay and 1:3 dilution series prepared for Astemizole and Terfenadine from a starting concentration of 10 µM. Curve calculations were baseline-corrected against duplicate dilution series prepared in saturating E-4031.

	avg	std dev
free tracer	55.73	6.34
no inhibitor	301.98	5.91
30 uM E-4031	149.74	5.45
ΔmP	152.74	
Z'-factor	0.78	
Astemizole IC50	1.66 nM	
Terfenadine IC50	12.74 nM	

Table 1. Predictor™ Assay Results.