Predictor™ Assay Setup Guide on the Tecan Safire²™ Microplate Reader

NOTE: The Tecan Safire²™ Microplate Reader was tested for compatibility with Invitrogen's 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. 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.

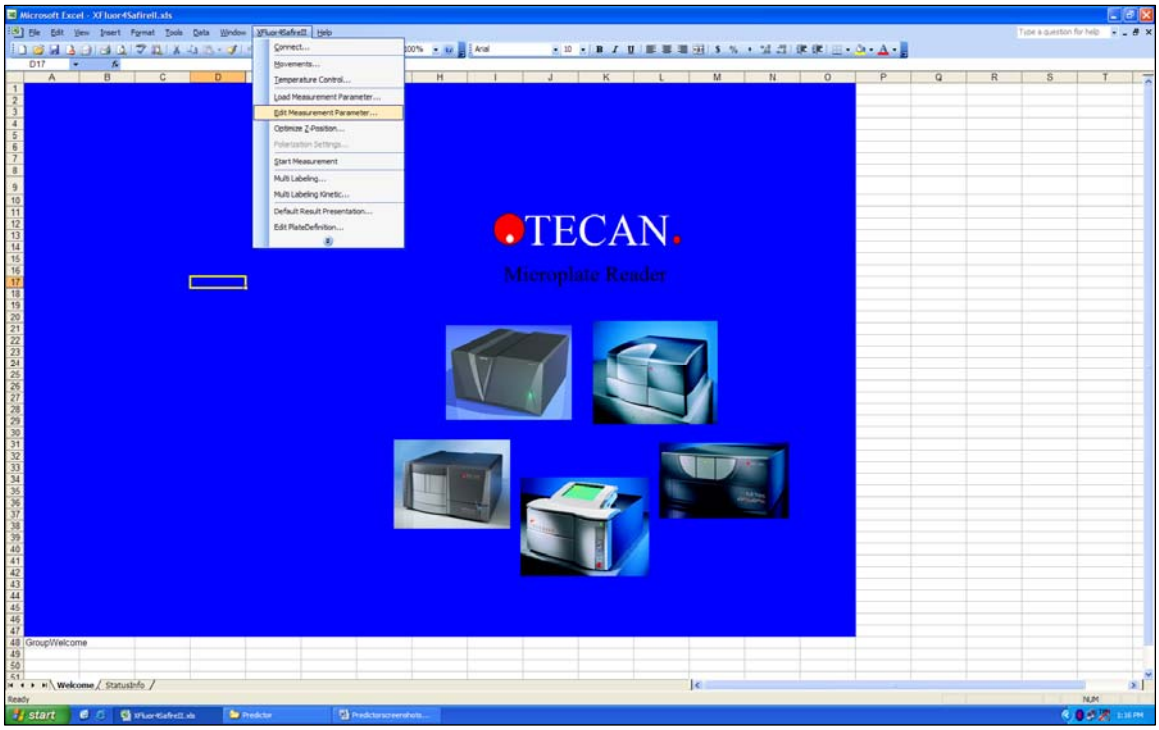
A. Recommended Optics

	wavelength (nm)	diameter (mm)
Excitation	530/20	monochromator
Emission 1	585/20	monochromator
Emission 2	585/20	monochromator

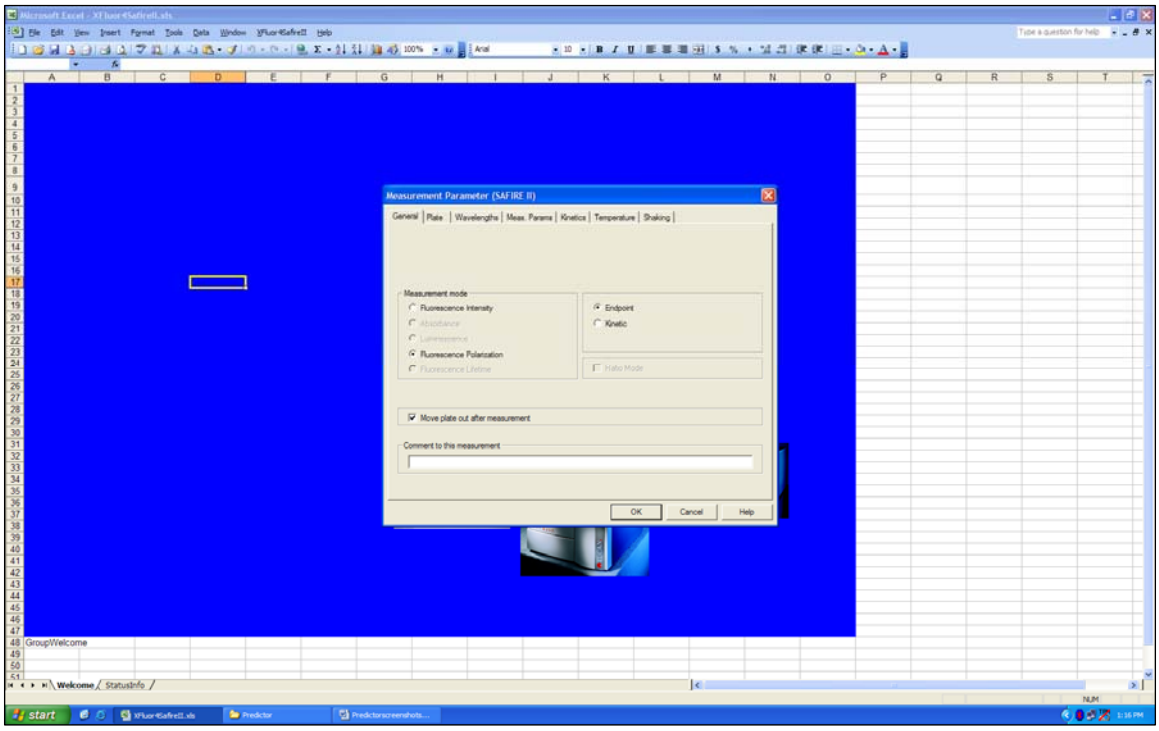
B. Instrument Setup

1. Make certain plate reader is turned on, and open up XFluor Data Manager software on computer.

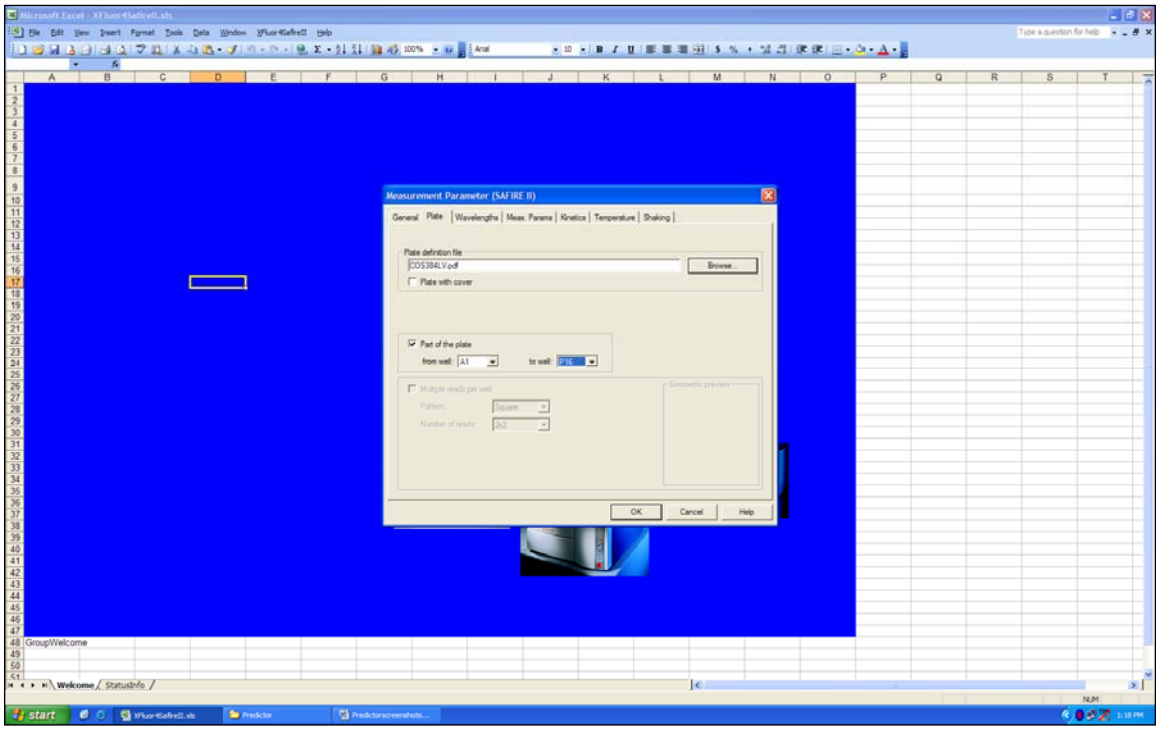
2. When XFluor opens, if you do not have a pre-existing protocol for Predictor™, select "Edit Measurement Parameter" from the drop-down menu beneath "XFluor 4SafireII" in the menu bar along the top of the screen.



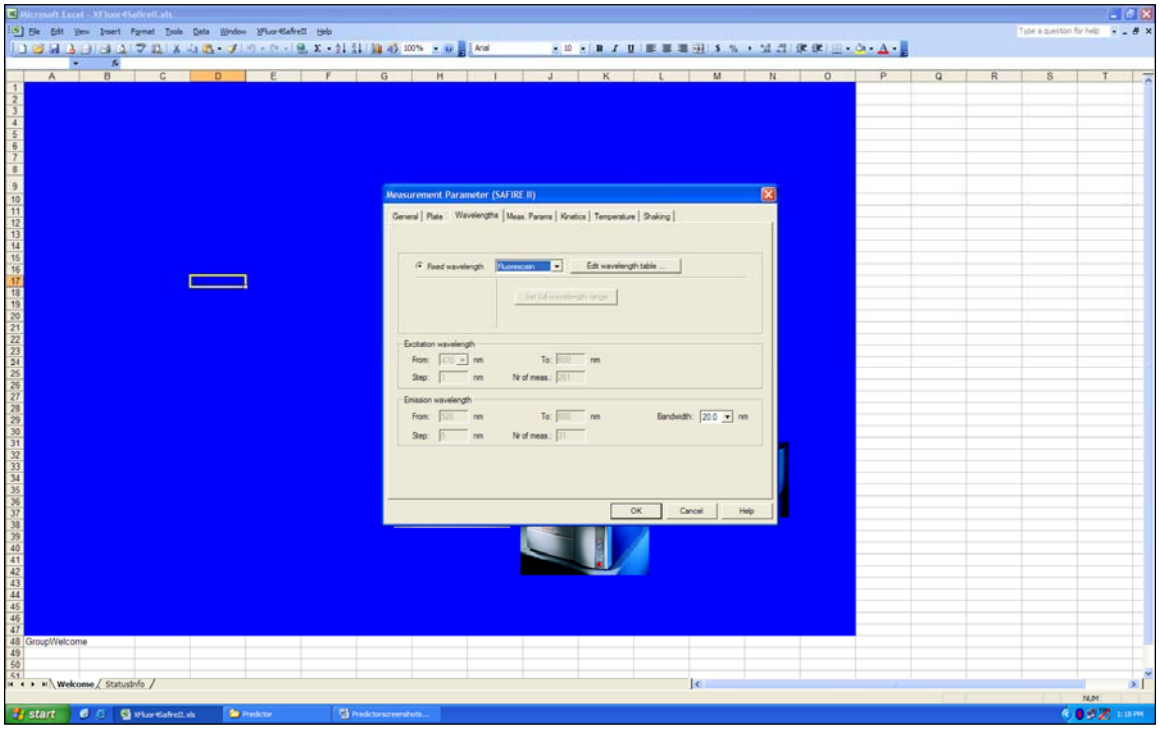
3. A popup Measurement Parameter window will appear. Under the “General” tab select the “Fluorescence Polarization” and “Endpoint” buttons.



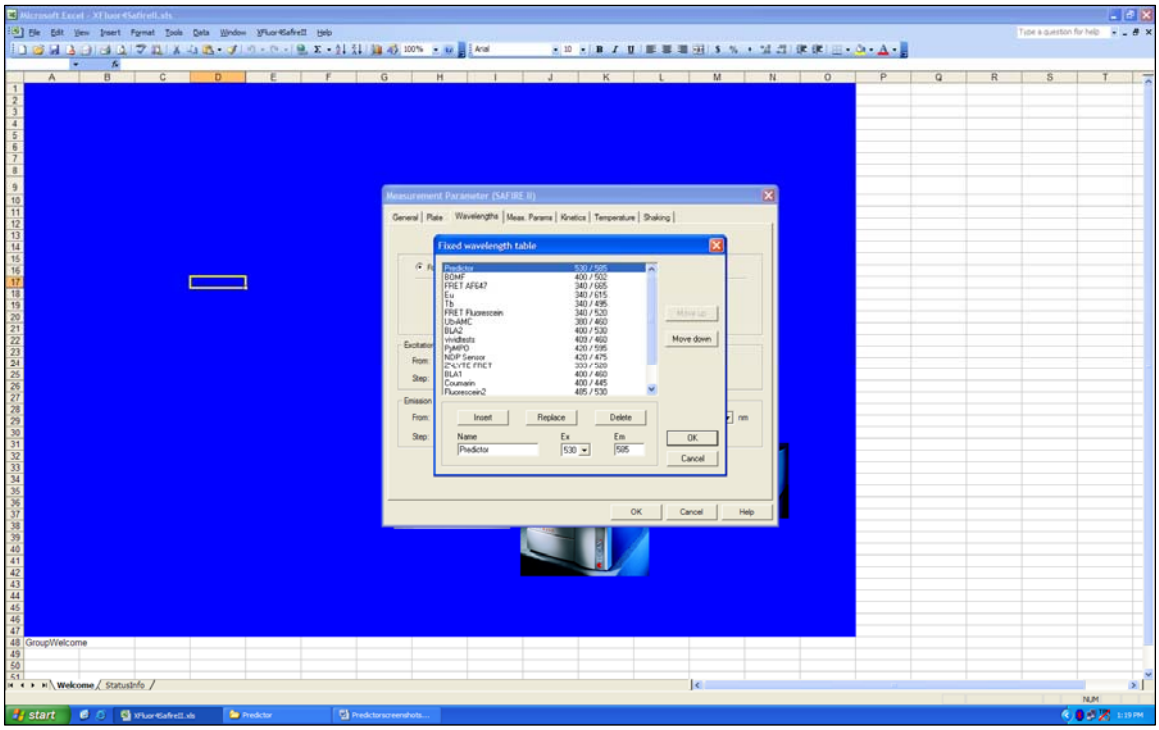
4. Now select the “Plate” tab at the top of the window. When the new tab opens, select your plate type from the drop-down list. Check the “Part of the Plate” button, and then enter the wells to be read (A1 to P16 in this example).



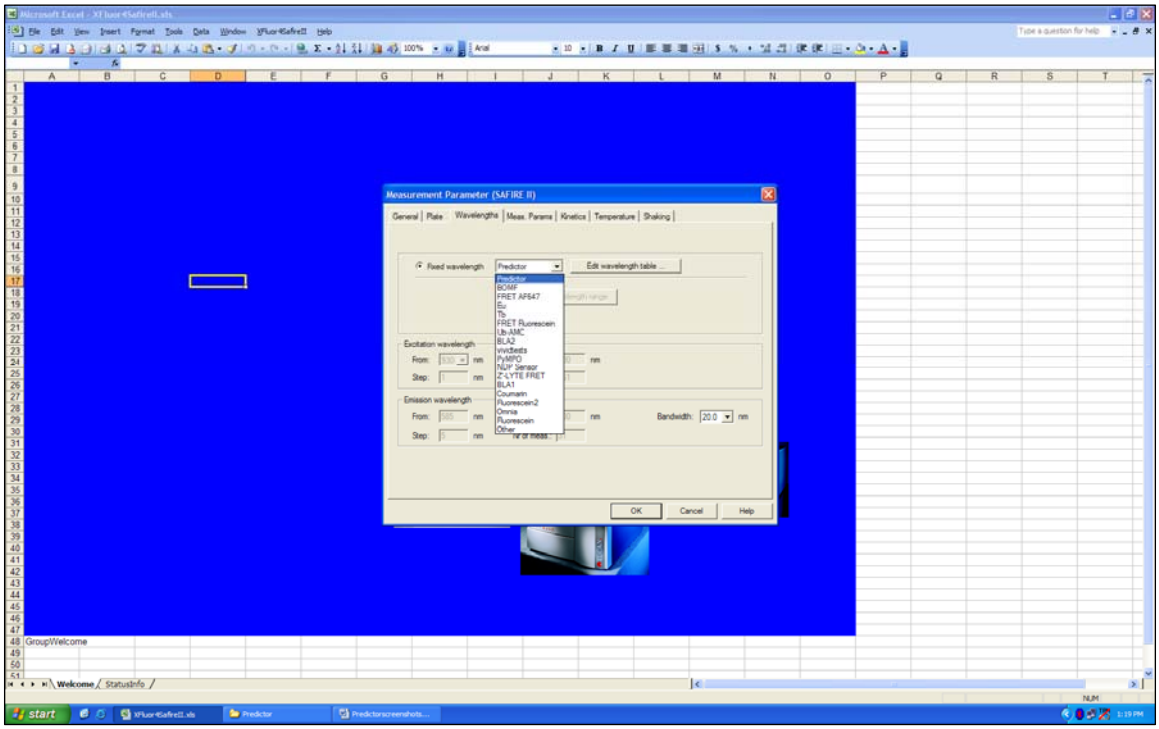
5. Select the “Wavelengths” tab. Select the “Edit Wavelengths Table” tab.



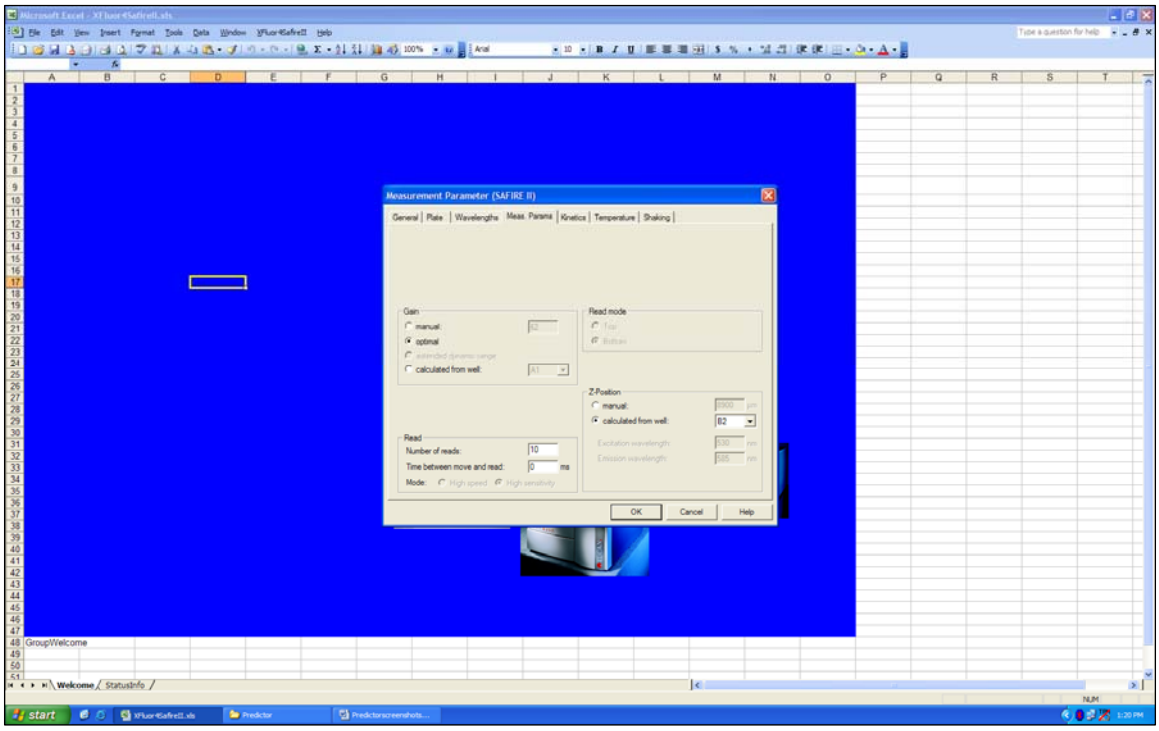
6. A new window will appear. Add a name and excitation and emission values at the bottom then press the “Insert” tab. This will save this excitation/emission set for future use. Press “OK” when finished and popup window will disappear.



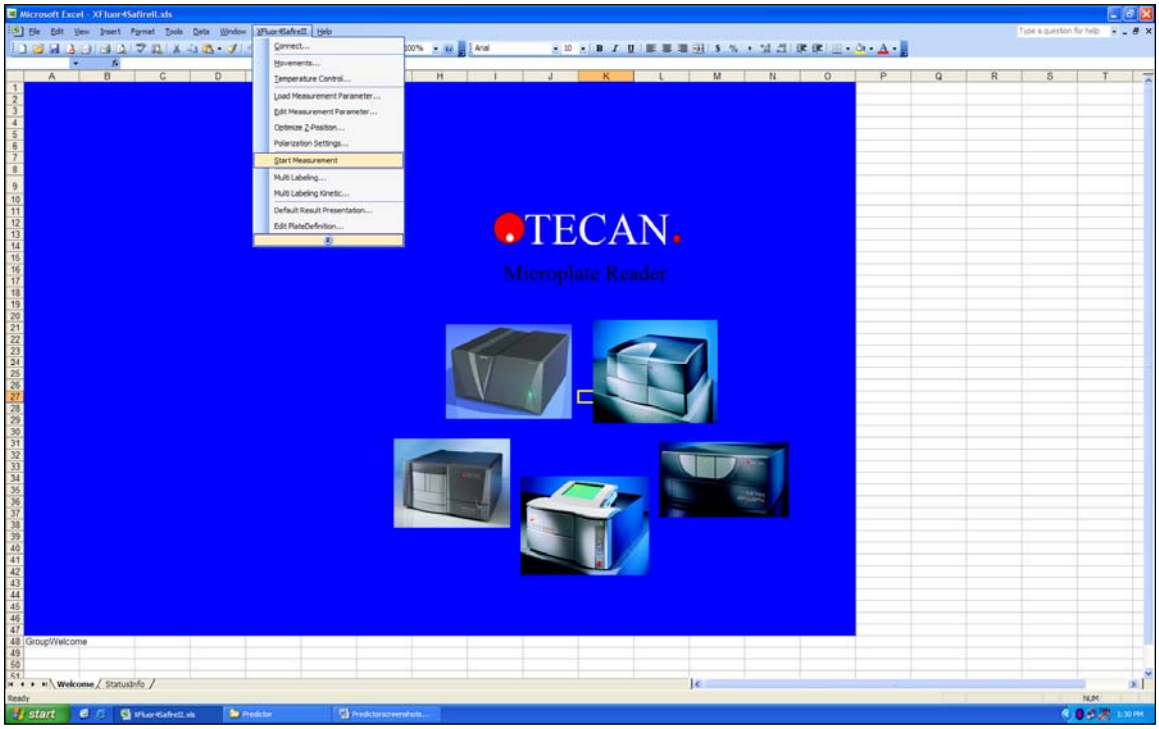
7. You will now be returned to the main Measurement Parameter window under the Wavelength tab. Select the "Fixed Wavelength" button near the top and select your wavelength settings from the drop-down menu. Make sure Bandwidth (bottom right) is set at 20nm.



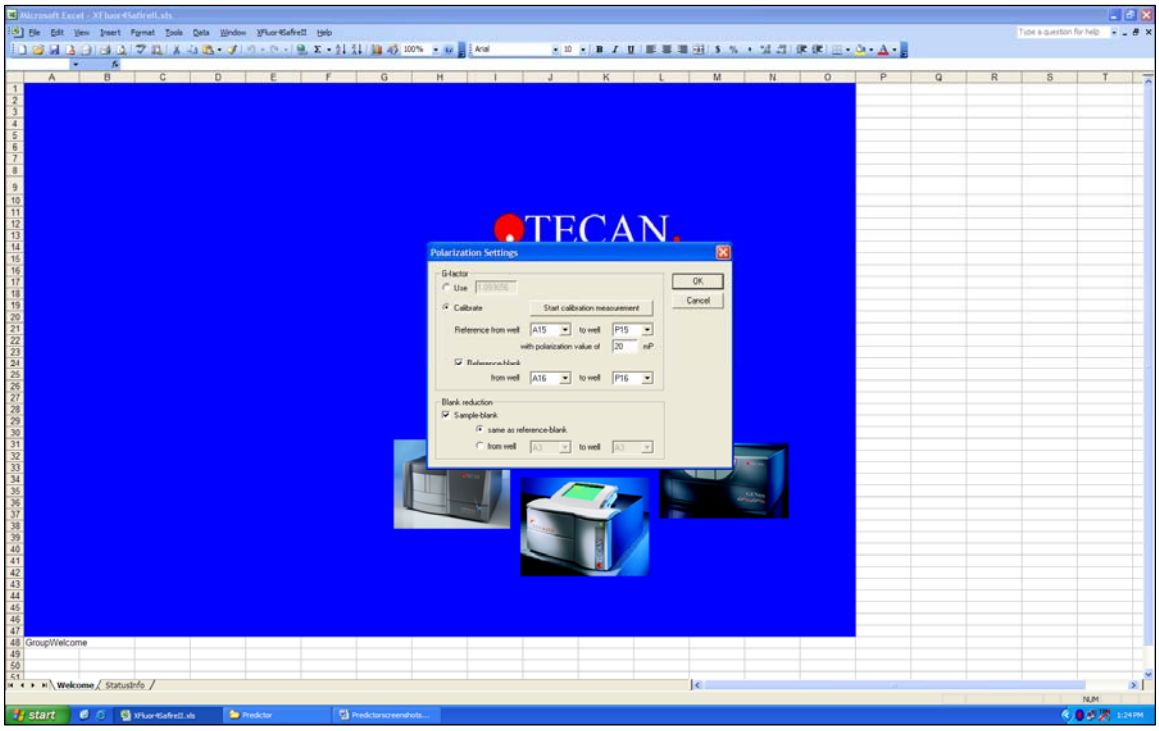
8. Select the “Measurement Parameters” tab. Set the Gain for “Optimal”, 10 reads, and set the well you want to use for Z-Position. When finished, select “OK”.



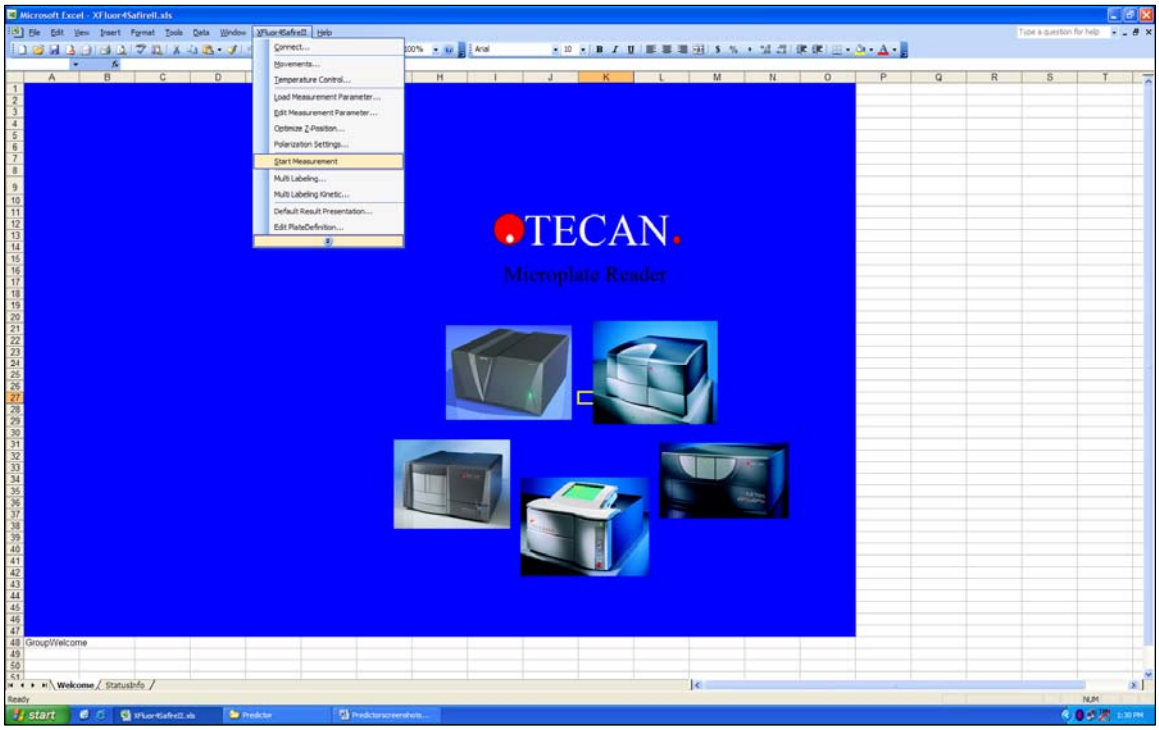
9. Go back to the drop-down XFluor menu and select “Polarization Settings”.



10. A new popup will appear. You can select a G-factor to use, but we recommend calibrating the instrument. Select the "Calibrate" button, then select your reference wells. In this example we selected Column 15 (free tracer) and set the reference to 50 mP, but alternatively the competed reference (tracer plus membrane plus saturating E-4031) column could be selected and the value set to 140 mP as directed in the protocol. When finished, select "Start Calibration Measurement" tab.



11. At this point, instrument setup is complete. From the XFluor menu bar, select "Movements" if you have not already and insert your plate, then from the same toolbar select "Start Measurement" and read assay plate.



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.

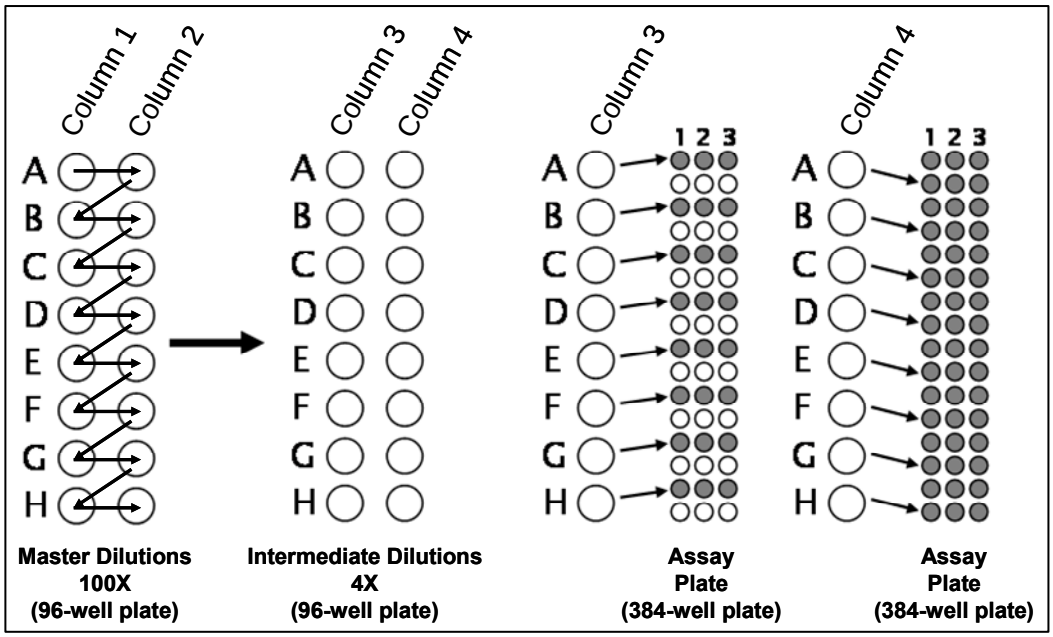


Figure 1: Schematic for Preparation of Compound Dilution. Schematic demonstrates progression of dilutions and final transfer to assay plate. Note in this case 2 compounds were titrated and each compound was finally used in replicates of 6 (3 with and 3 without saturating E-4031).

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 in Predictor™.** 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.

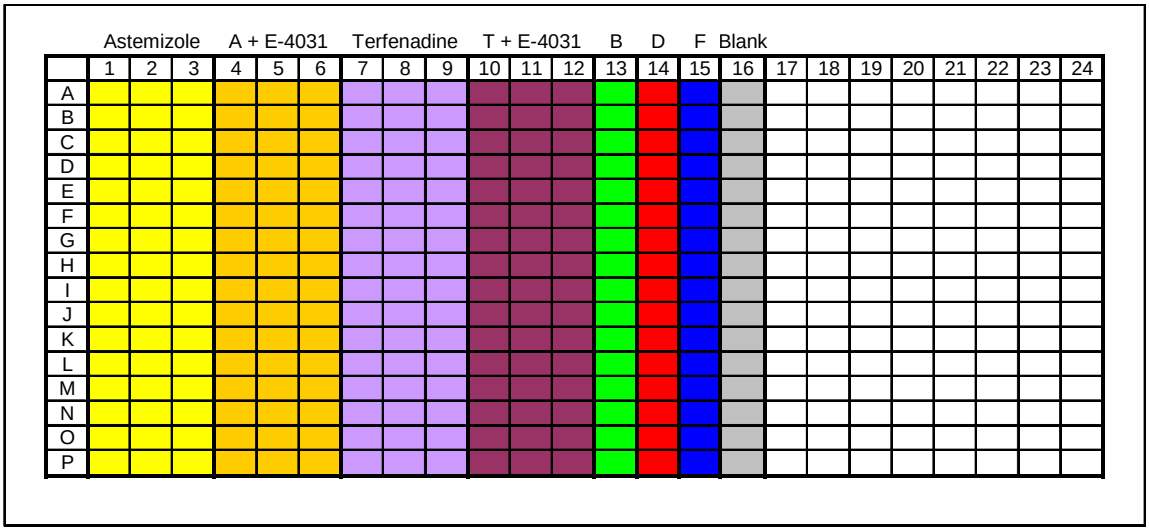


Figure 2: Schematic of Assay Plate Layout. Predictor™ hERG assay was set up with a dose-response curve of Astemizole and Terfenadine (1:3 dilution series from a starting concentration in the top row of 10 µM) prepared in replicates of 6. From this, 3 replicates were assayed for ability to disrupt Predictor™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

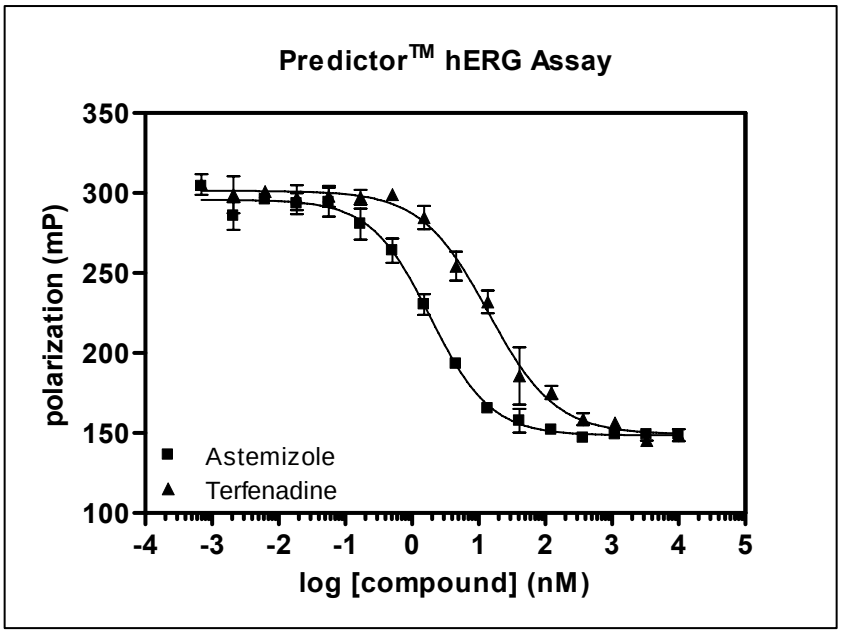


Figure 3: Predictor™ hERG Assay. Dose-Response Curves read on the Tecan Safire²™ using the Predictor™ assay and 1:3 dilution series prepared for Astemizole and Terfenadine from a starting concentration of 10 µM. Curve calculations were baseline-corrected against duplicate dilution series prepared in saturating E-4031.

	avg	std dev
free tracer	57.08	4.94
no inhibitor	299.92	6.99
30 uM E-4031	149.11	4.46
ΔmP	150.81	
Z'-factor	0.77	
Astemizole IC50	1.90 nM	
Terfenadine IC50	13.98 nM	

Table 1. Predictor™ Assay Results on the Tecan Safire²™.