Predictor™ Assay Setup Guide on the BMG LABTECH CLARIOstar® Microplate Readers

The BMG LABTECH CLARIOstar® Microplate Readers were tested for compatibility with Life Technologies' Predictor™ hERG FP Assay (PV5365) using controls provided within the assay kit and the provided, known hERG channel blocker E-4031.

The following document is intended to demonstrate setup of this instrument and provide representative data. For more detailed information and technical support of Life Technologies' assays please call 1-800-955-6288, follow the prompts to enter 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.

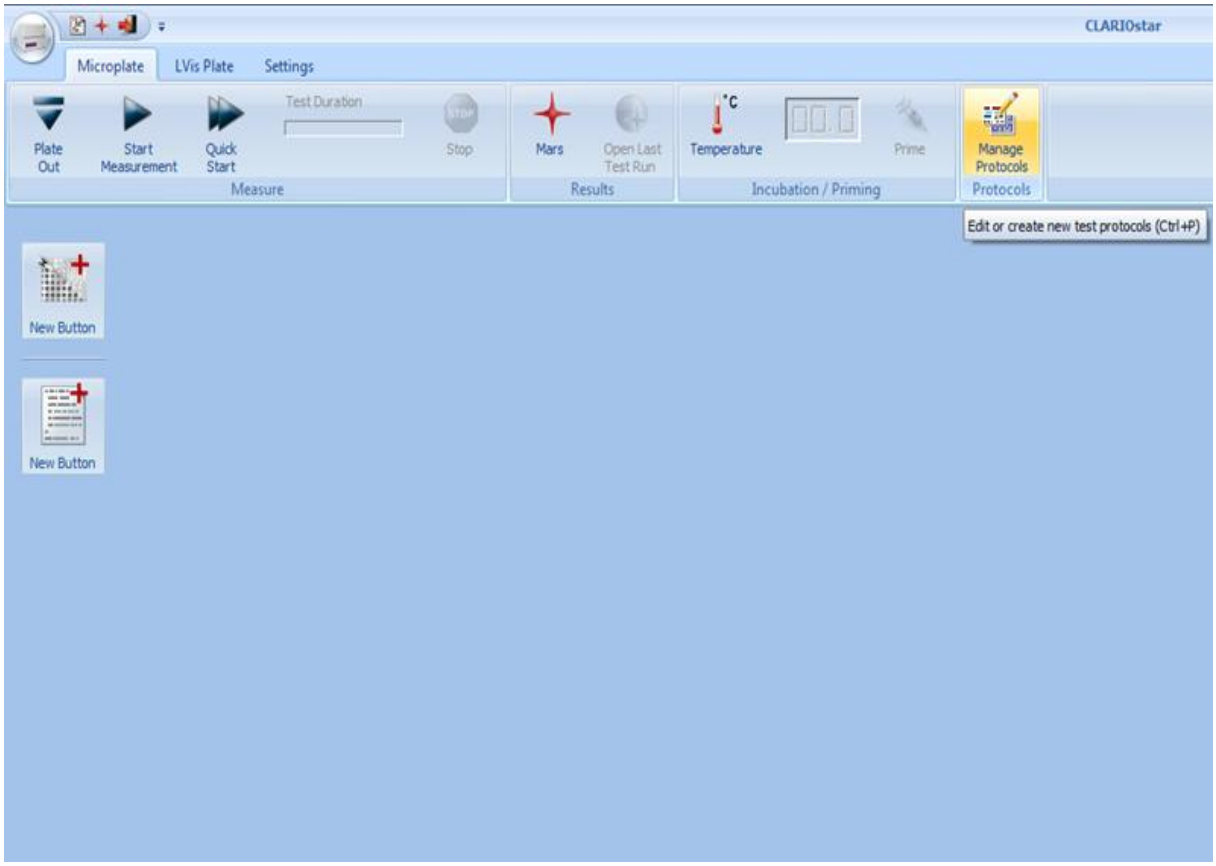
A. Recommended Optics

	Wavelength (nm)	BMG LABTECH Filters
Excitation	540	540-20
Emission 1 & 2	590	590-20
Dichroic Mirror	565	LP 565

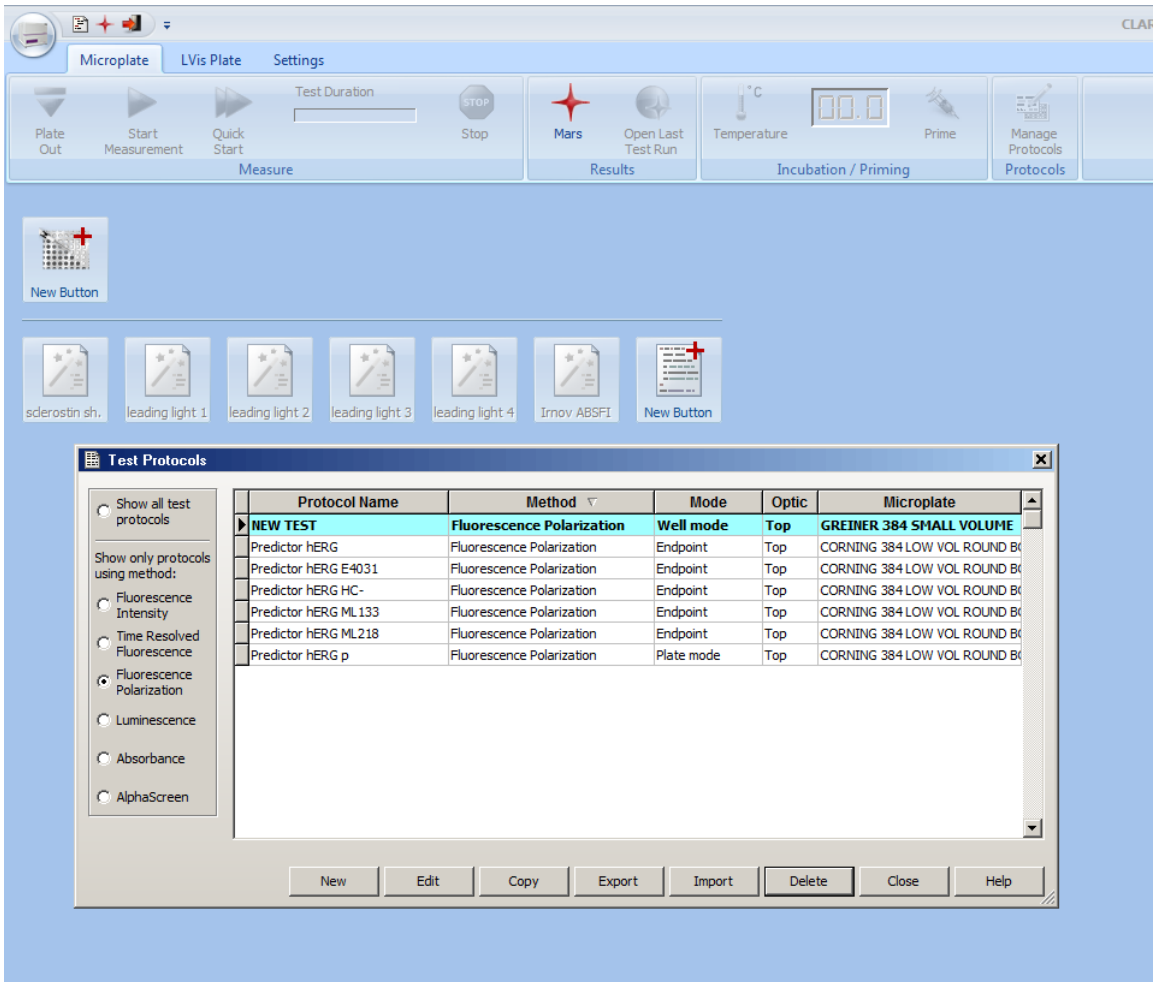
B. Instrument Setup

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

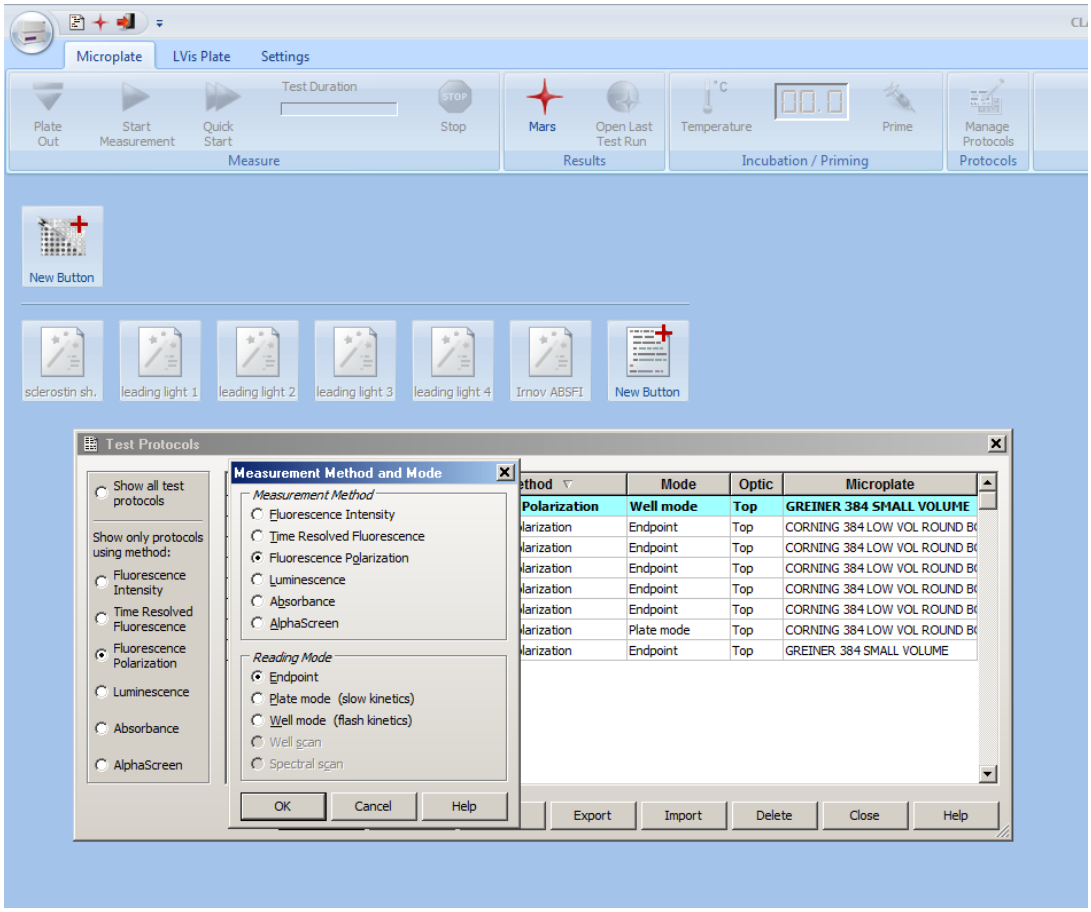
2. When CLARIOstar® Control software opens, if you do not have a pre-existing protocol for Predictor™, select "Manage Protocols" from the Protocols tab in the menu bar at the top of the window.



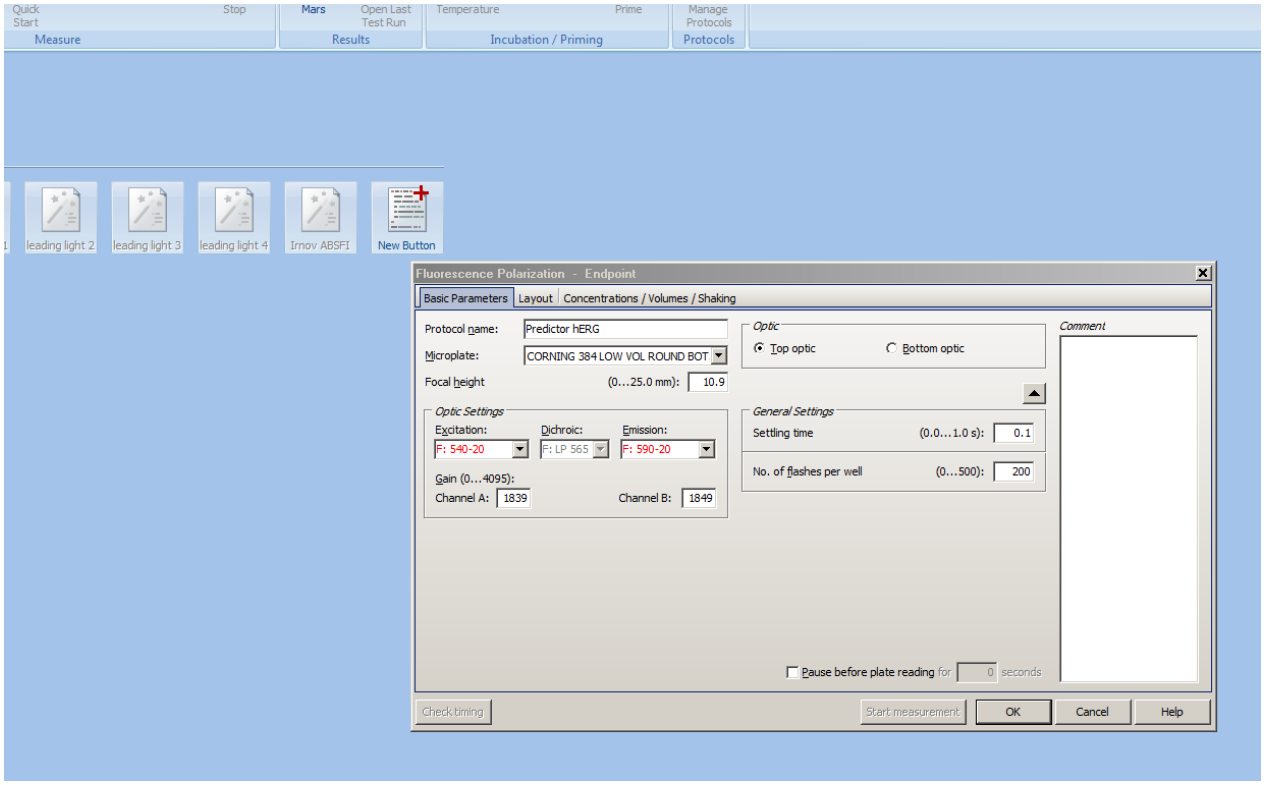
3. At this point, a new screen will open (below). Click on the “Show all test protocols” or “Fluorescence Polarization” button on the left side of the screen, then select “New” from the tabs at the bottom.



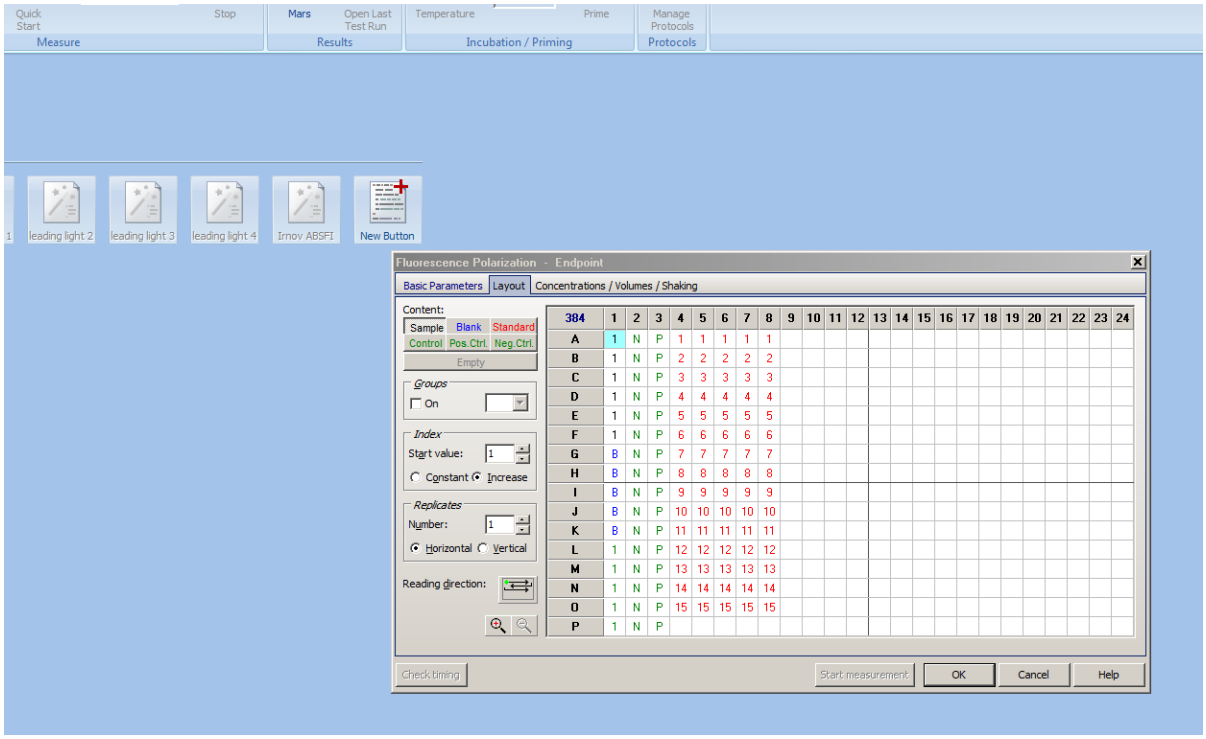
4. A new window will pop up. Select “Fluorescence Polarization” again, as well as “Endpoint:” and then select “OK.”



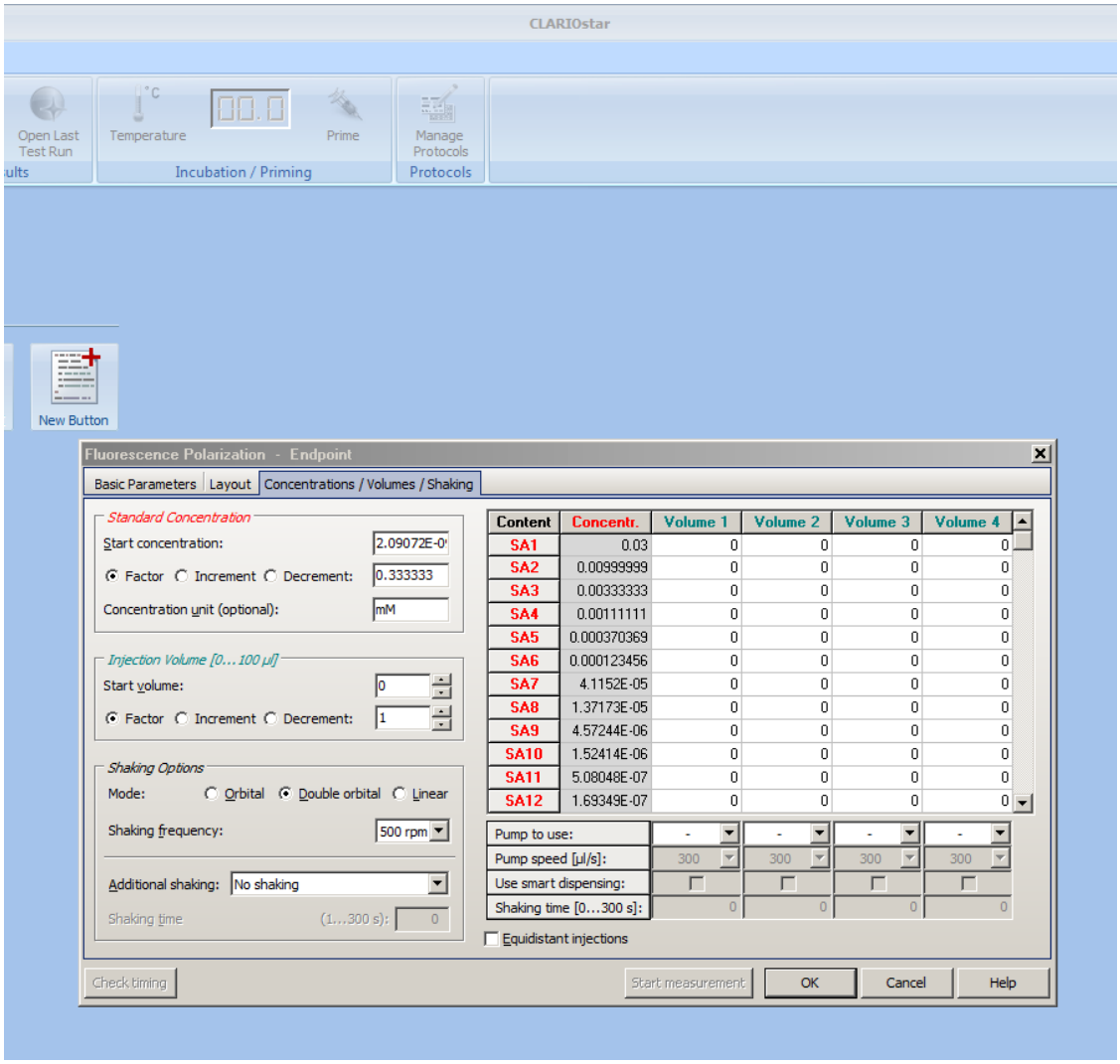
5. A new protocol will open automatically. Enter a test name, select plate type, and select the Optic Settings from the drop-down lists. Select Top optic and enter the Settling time and No. of flashes which you would like to use. When finished, click on the “Layout” tab near the top of Fluorescence Polarization window.



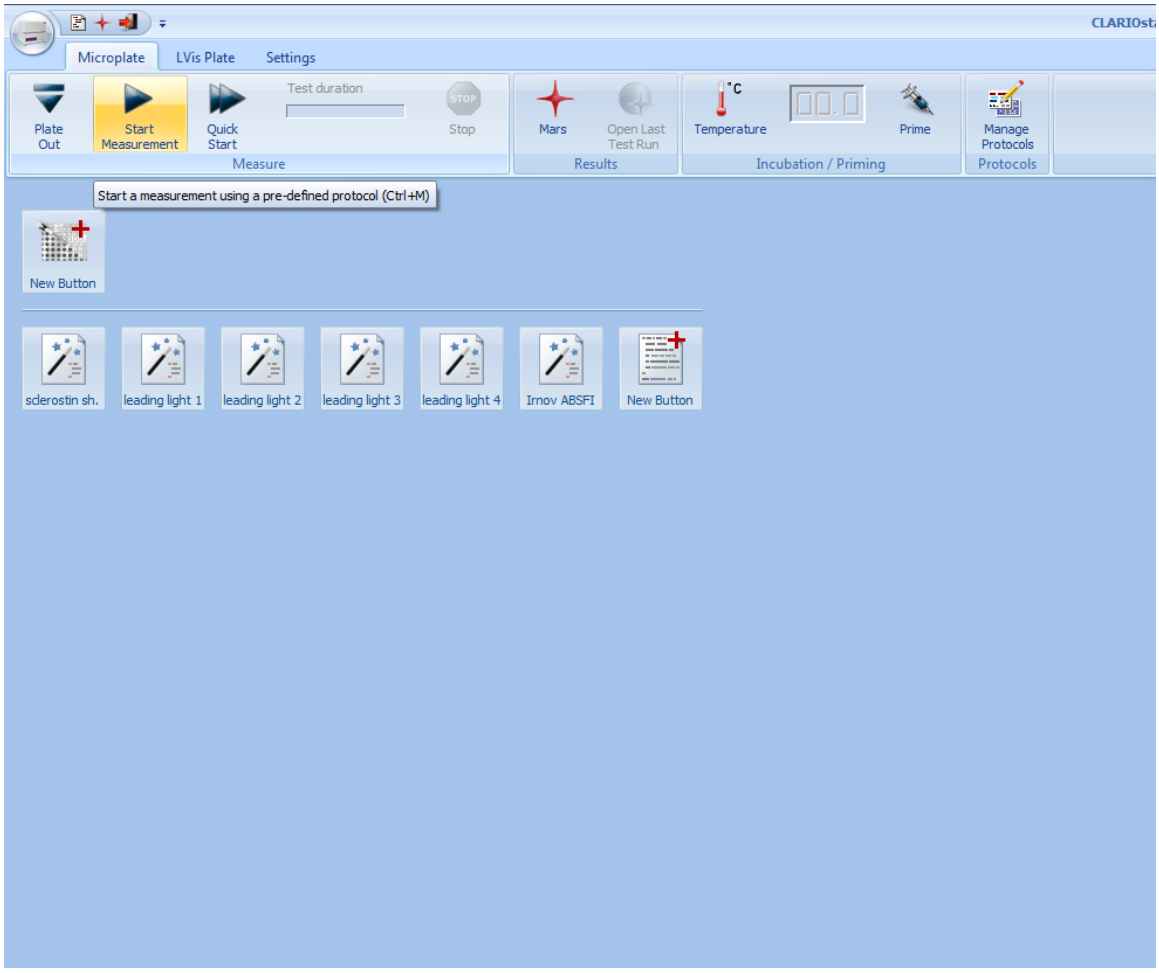
6. Using the “Content” tabs on the left-hand side of the window, select the correct layout for your plate. Note: in this case we set up the assay as one slightly modified compared to that in the Predictor™ hERG Fluorescence Polarization Assay protocol with a 15 point inhibitor titration with 5 replicates. When finished, select “OK” at bottom of window.



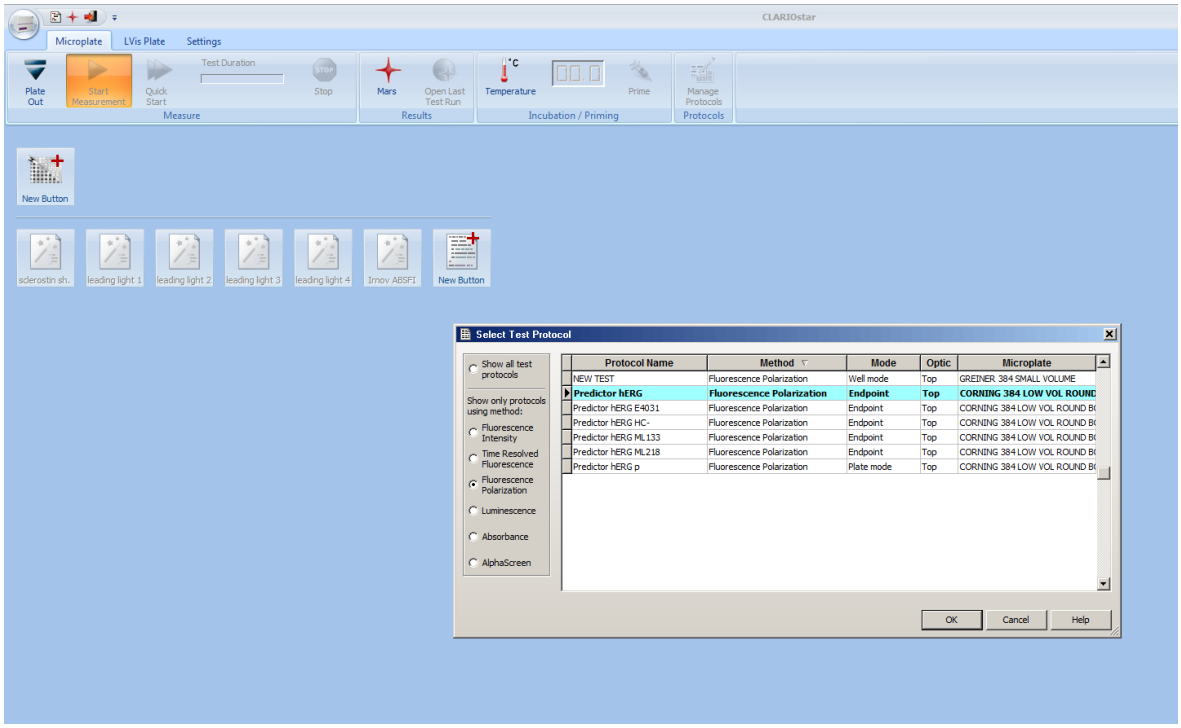
7. Because known inhibitor concentrations will be used, select the “Concentration / Volumes / Shaking.” Here one may enter the appropriate concentrations for these standards by entering the “Start concentration,” selecting “Factor” and entering the desired dilution factor, then entering the desired “Concentration unit.” Clicking on the “Concentr.” header auto-fills the table. At this point, click “Start measurement” to save settings and initiate a test run immediately. Clicking “OK” saves the protocol and closes the protocol manager.



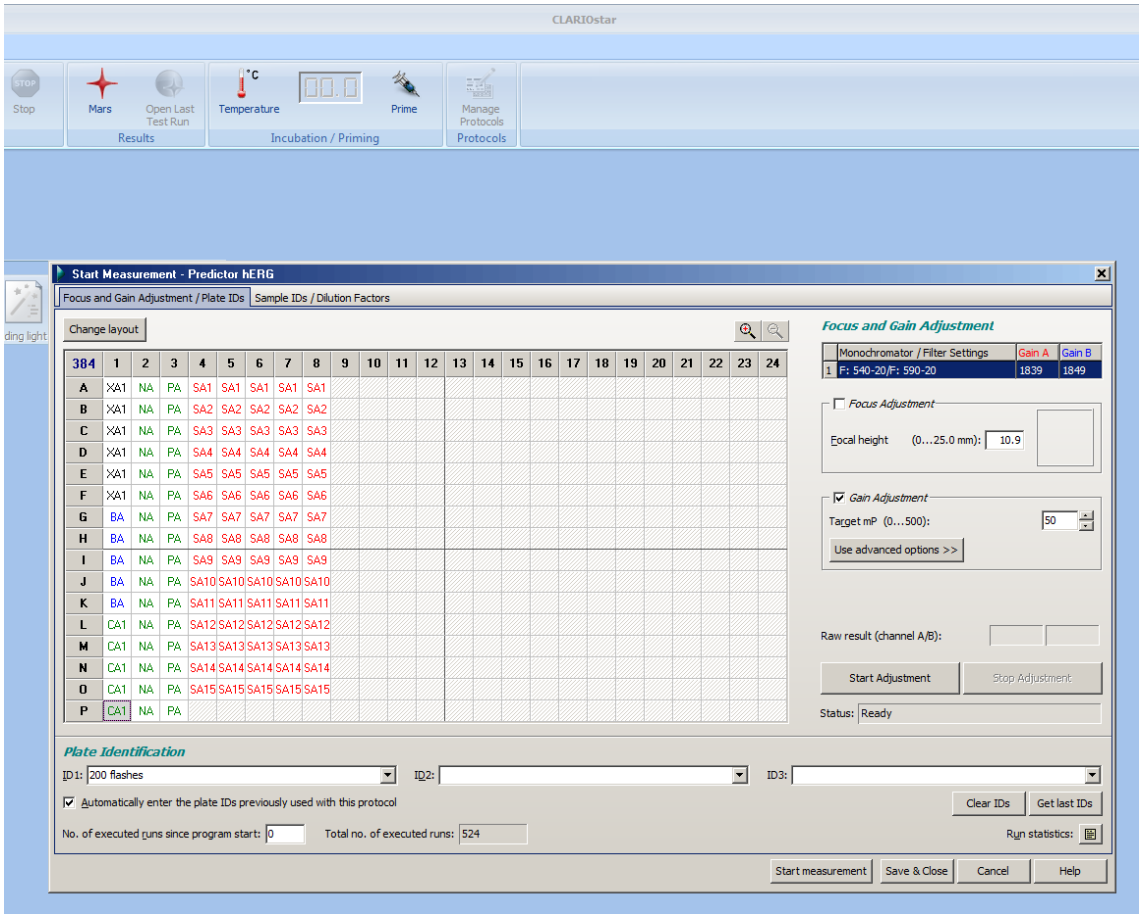
8. If you selected “OK” in the previous step you will return to the initial settings window. Select the “Start Measurement” button in the menu bar at the top of the screen.



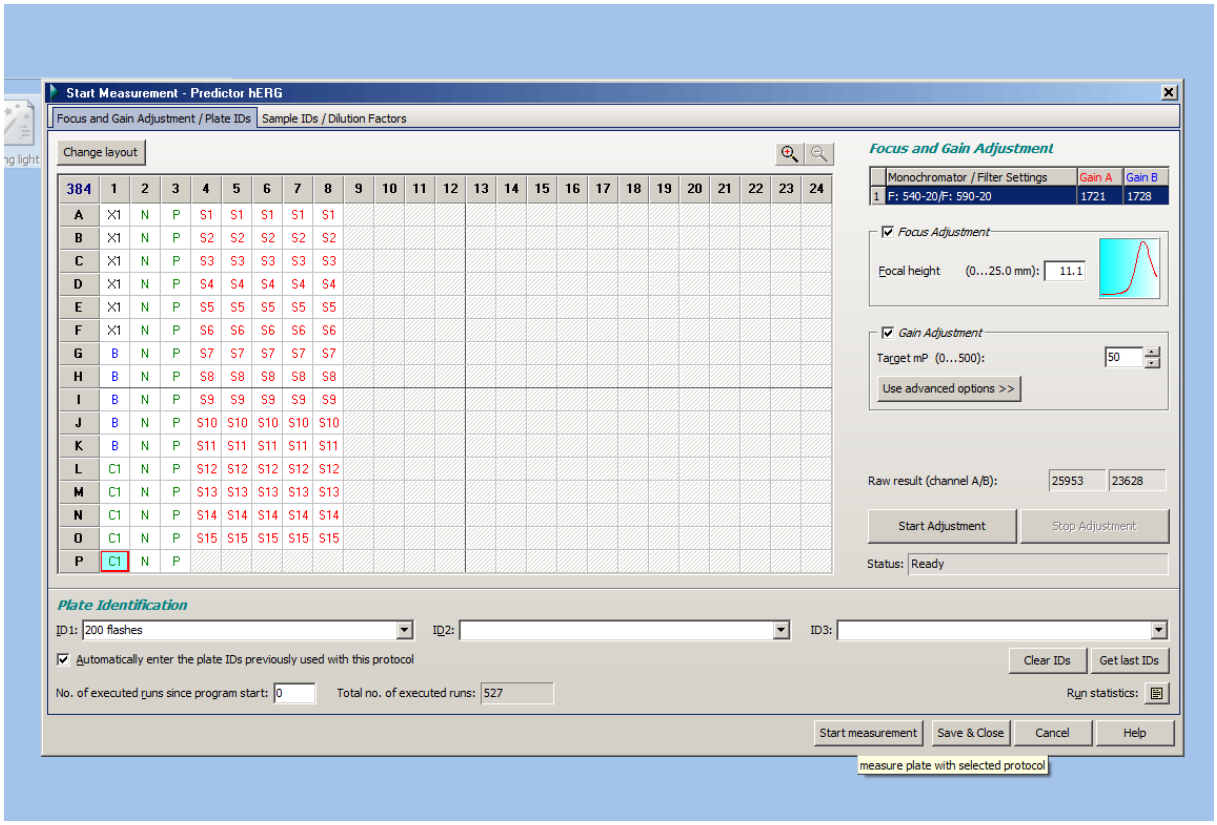
9. A new window will appear allowing selection of the test protocols to be run. Select the protocol created for Predictor hERG, and then press “OK.”



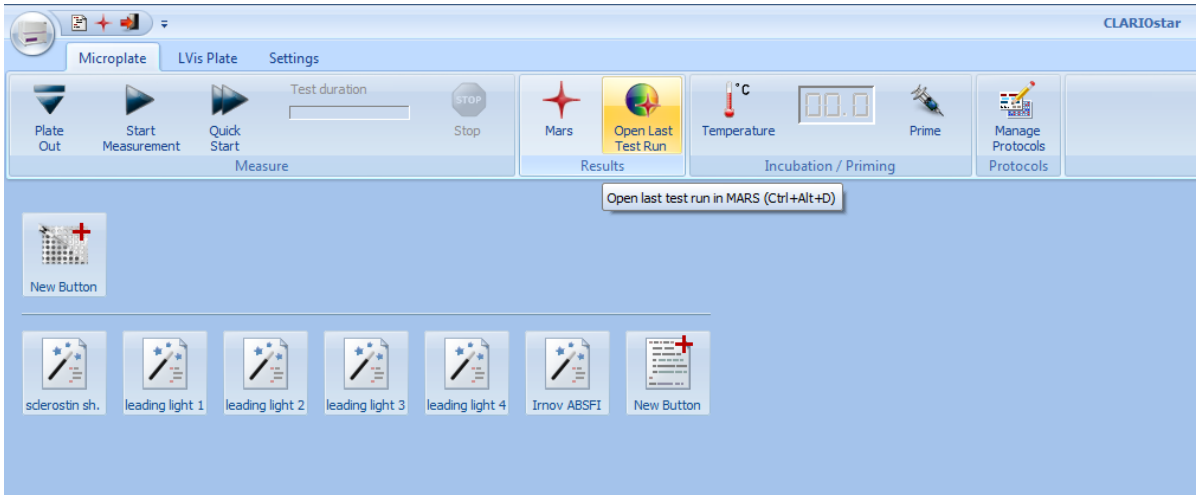
10. The following pre-measurement window will appear. Select a well for focal height and gain adjustment. Note: in this case a well from Column 1 Rows L-P was selected; these are the “Free Tracer” wells. Check “Focus Adjustment” and “Gain Adjustment” and enter “50” in the Target mP window. Select “Start Adjustment.”



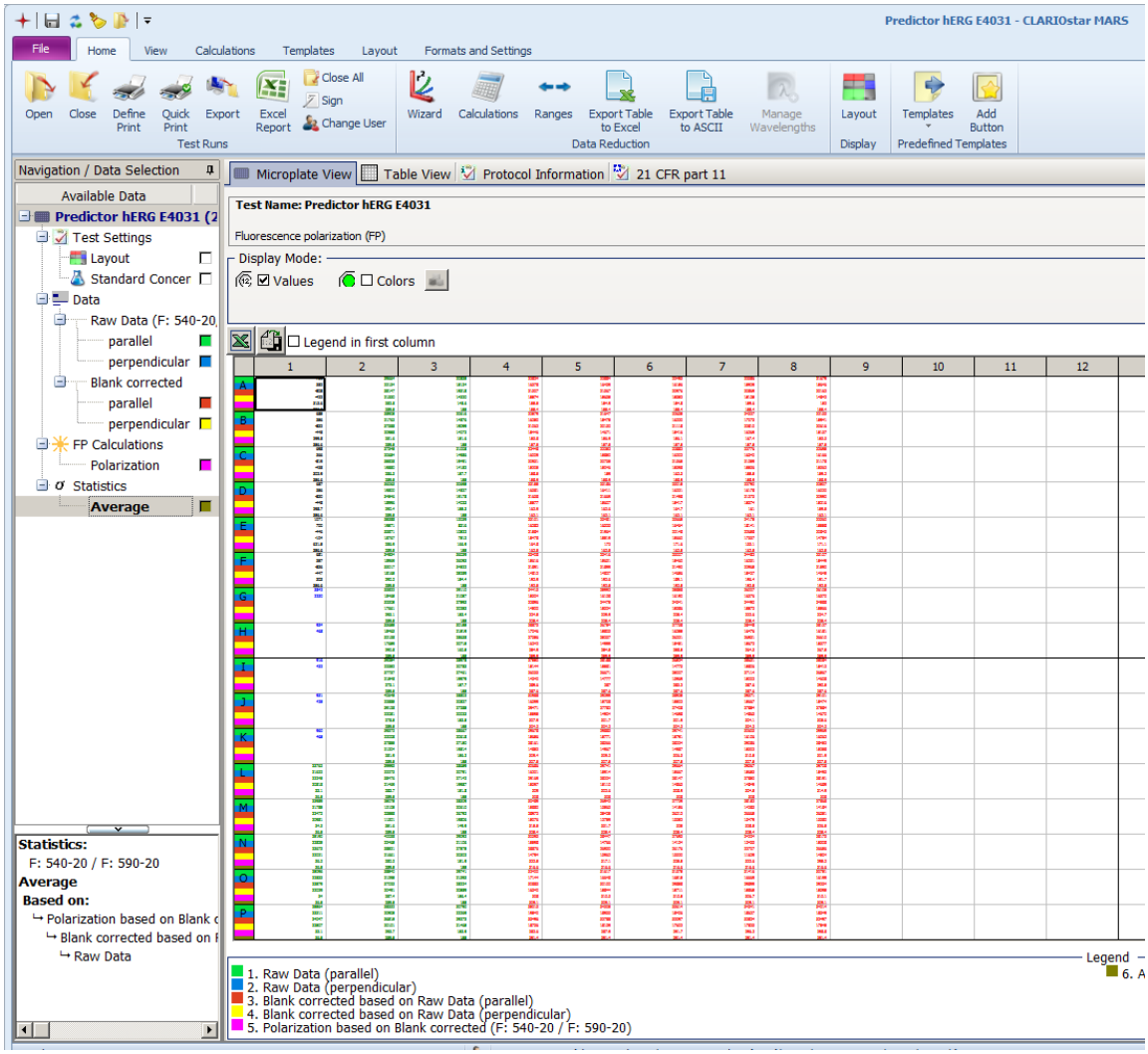
11. The CLARIOstar® will optimize its settings and the window will change to show the focal height test results (graph to right of “Focus Adjustment”) and Gain settings. At this point the instrument is ready to read, select “Start Measurement” from the bottom of the window.



12. When the CLARIOstar® has finished reading, collect the data from the just completed test run by clicking “Open Last Test Run” on the toolbar at the top of the window. This will automatically redirect to a MARS data file. Alternatively you can open MARS and select the run of interest.



13. Depending on the plate layout selections, a number of calculations may have been performed. In this case blank correction, polarization calculations, and average polarizations values have already been performed.



C. Predictor™ hERG FP Assay

Purpose

The following is a sample assay performed for demonstration purposes. The instrument settings above would be sufficient for any Predictor™ assay. Other Life Technologies FP assays might require different optical filters and dichroic mirrors, but would otherwise be the same.

The information below is provided as representative data. Assays were run in 20 µl/well 384-well black microplates (white plates are unsuitable for FP). Refer to plate outlay on page 17. We prepared dose-response curves for 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%.

At a Glance

Step 1: Setup the instrument and plate layout.

Step 2: Prepare Predictor™ hERG membrane.

Step 3: Prepare a dilution series of the channel blocker.

Step 4: Prepare controls.

Step 5: Prepare plate and read.

Materials Required

Component	Storage	Part Number	Example Reagents
Predictor hERG Tracer Red	-20°C	various	PV5365
Predictor hERG Membrane	-80°C	various	K1785
Predictor hERG FP Assay Buffer	Room Temperature	various	PV5364
E-4031	-20°C	various	PV5366

- Plate reader capable of measuring fluorescence polarization in the red spectrum.
- Pipetting devices for 1–1000 µl volumes, suitable repeater pipettors, or multi-channel pipettors.
- Qty (1) Assay Plate: Black 384-well assay plates. We recommend untreated low-volume polystyrene plates (e.g., Corning #4511 or polypropylene plates (e.g.,

Matrical #MP101-1-PP). *Important:* Plates treated with a non-binding coating should not be used. Predictor™ hERG Tracer Red binds to the coating on these plates, and this binding results in a lower assay window and increased assay variability.

- Qty (2) Dilution Plates: 384-well polypropylene plates (e.g., Corning #3657). These plates are used for preparing serial dilutions of test compounds.
- 1.5 mL polypropylene microcentrifuge tubes.
- Plate seals.

Procedure

1. Thaw all reagents as directed in the Predictor™ protocol.

2. **Prepare Predictor™ hERG membrane**. Mix thawed Predictor™ hERG Membrane by pipetting up and down ~20X with a small-bore pipette or pipette tip being careful to include all of the material in the vial.

3. **A 100X into 4X dilution series of E-4031** was prepared as follows:

Requires Qty1 Dilution Plate

3.1. In a 384-well dilution plate (I), add 20 µl of assay buffer to wells B1-P1.

3.2. Add 30 µl of 3 mM E-4031 to well A1 of the same 384-well dilution plate.

3.3. **Prepare Master Inhibitor Dilution Series:** Transfer 10 µl from well A1 to well B1, mix by pipetting up and down several times, then transfer 10 µl from well B1 to well C1, mix again. Repeat this process through well P1.

3.4. **Dilute E4031 to 4X**: Add 48 µl of Predictor™ hERG FP Assay Buffer to each well of an unused Column of the dilution plate, for example Column 24. Transfer 2 µl of master inhibitor dilution series from Column 1 of the serial dilution plate to Column 24 for the 4X dilution. Mix well by pipetting up and down.

4. **E-4031 Titration:** Transfer 5 µl of the 4X intermediate dilution from Column 24 of the 4X dilution plate to Columns 4-8 of the Assay Plate.

5. Once E-4031 titration wells are prepared (columns 4-8 of assay plate), also prepare the following controls on the assay plate.

NOTE: Predictor™ is a highly sensitive assay. **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 are highly recommended.

- **Negative Control, Tracer Fully Bound (Column 2).** Add 5 ul Predictor™ hERG FP Assay Buffer to all wells in Column 2. Tracer and membrane will be added later. This control will show full tracer binding activity of the assay. This is the high mP control and represents the maximum of the assay window.

- **Positive control, Tracer Displaced (Column 3).** Add 20 µl E-4031 to 480 µl Predictor™ hERG FP Assay Buffer in a microcentrifuge tube. Add 5 µl per well of this solution to each well of Column 3 of the 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. This is the low mP control and represents the minimum of the assay window.

- **Free Tracer Control (Column 1, L-P).** Add 15 µl Predictor™ hERG FP Assay Buffer to wells L-P of column 1. Tracer will be added later. This control will show fully unbound tracer in solution. The mP values of these wells will be below the Displaced Tracer Control as there is no viscosity from the Predictor™ Membrane. A reading near 50 mP for non-blank corrected data is expected from the target mP previously entered (step 10 on page 10).

- **Assay Blank (Column 1, G-K).** Add 10 µl of Predictor™ hERG FP Assay Buffer to wells G-K of Column 1. 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.

- **Buffer Blank (Column 1, A-F).** Add 20 µl of Predictor™ hERG FP Assay Buffer to wells A-F of Column 1. Used only when determining the G-factor with Free Tracer.

6. Membrane suspension: Add 10 µl of membrane per well to all wells except Column 1 wells A1-F1 & L1-P1.

7. Tracer added last: In a microcentrifuge tube, add 20 µl tracer to 1230 µl Predictor™ hERG FP Assay Buffer and add 5 µl/well of this solution to all wells except Column 1 wells A1-K1.

8. Cover assay plate, protect from light and incubate at room temperature (20-25°C) for 2 hours before reading.

9. Read plate on plate reader as outlined above.

	1	2	3	4	5	6	7	8
A	1	1	1	1	1	1	1	1
B	2	2	2	2	2	2	2	2
C	3	3	3	3	3	3	3	3
D	4	4	4	4	4	4	4	4
E	5	5	5	5	5	5	5	5
F	6	6	6	6	6	6	6	6
G	7	7	7	7	7	7	7	7
H	8	8	8	8	8	8	8	8
I	9	9	9	9	9	9	9	9
J	10	10	10	10	10	10	10	10
K	11	11	11	11	11	11	11	11
L	12	12	12	12	12	12	12	12
M	13	13	13	13	13	13	13	13
N	14	14	14	14	14	14	14	14
O	15	15	15	15	15	15	15	15
P	16	16	16	16	16	16	16	16

Blanks and Free Tracer Controls	Negative Control	Positive Control	E4031 titration replicate 1	E4031 titration replicate 2	E4031 titration replicate 3	E4031 titration replicate 4	E4031 titration replicate 5

D. Results:

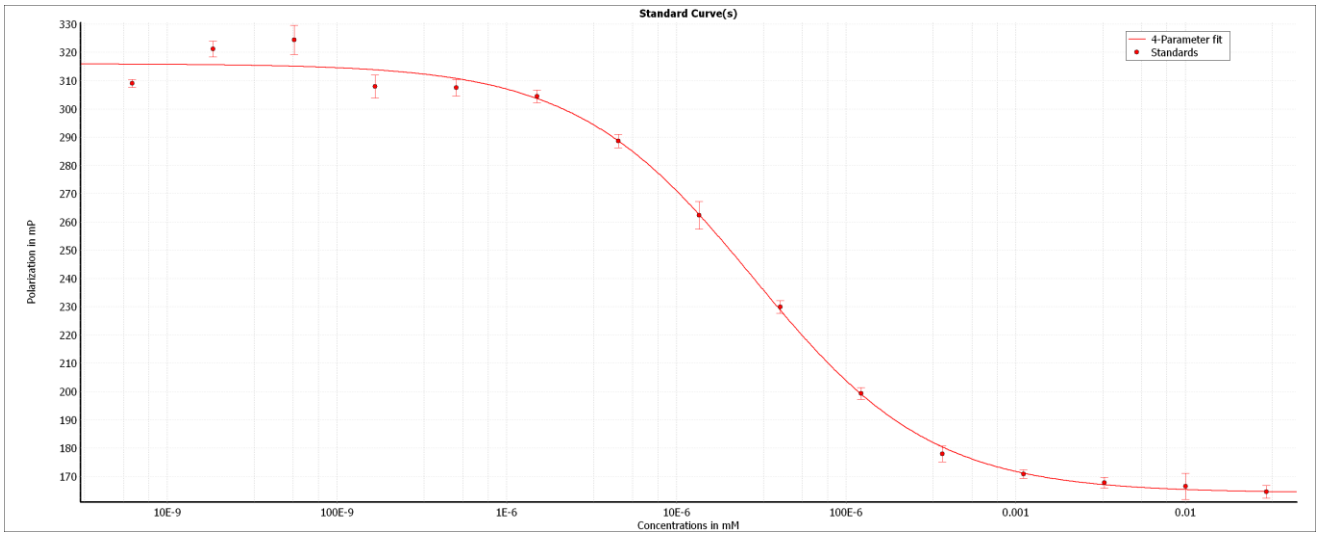


Figure 1: Predictor™ hERG FP Assay. Dose-Response Curves read on the BMG LABTECH CLARIOstar® Microplate Reader using the Predictor™ assay and 1:3 dilution series prepared for E-4031 from a starting concentration of 30 µM.

Table 1. Predictor™ Assay Results on the CLARIOstar®.

	Avg	Std Dev
Free tracer	47.1mP	1.1
No inhibitor	286.2 mP	5.6
30 µM E-4031	161.9 mP	3.1
ΔmP	124.3	
Z'-factor	0.797	
E-4031 IC_{50}	28.3 nM	