Norak Transfluor™ GPCR Assay – Q3DM EIDAQ100™ HTM Analysis

This validation report describes the performance of the NORAK Transfluor™ assay for GPCR activity on the EIDAQ™ 100 High Throughput Microscopy (HTM) system from Q3DM. The EIDAQ™ 100 HTM system performs accurate reading & measurement of GPCR activity by quantification of GPCR associated formation of GFP labeled pits or vesicles using a proprietary, multi-scale vesicle definition algorithm. The performance of this algorithm in combination with the EIDAQ™ 100 HTM system was evaluated by repeatably determining EC_{50} & IC_{50} values for agonist and antagonist well plates. The results showed very good repeatability of the GPCR analysis using the EIDAQ™ 100 HTM system and CytoShop™ software suite with the proprietary GPCR algorithm proving that the Q3DM system in combination with the Norak Transfluor™ assay presents a powerful quantification tool for GPCR activity.

Background and Significance

GPCRs mediate the activity of cell surface receptors and the transduction of a myriad of intracellular responses. They have proven to be a highly amenable class of targets for successful therapeutic intervention. In fact, of the approximately 500 drugs currently on the market today, more than 30% are mediated through the activation of GPCRs. Because GPCRs are membrane-bound proteins, they have been difficult to study in cell extracts, or to isolate and characterize. To directly ascertain GPCR activity, intact cell-based assays are quickly becoming a method of choice in high-throughput screening.

GPCRs transduce extracellular signals through the formation of protein complexes that effect both activation and subsequent desensitization of a cell surface receptor. Agonist binding to a receptor at the cell surface initiates a conformational change in the intracellular domain of the receptor that results in the phosphorylation of the receptor and subsequent binding of arrestin to the receptor. The arrestin-receptor complex is then transported to clathrin-coated pits and internalized to clathrin-coated vesicles. Finally, the entire complex is delivered to the endosomes. Some GPCRs dissociate from arrestin at or near the plasma membrane, while others remain associated and traffic into endocytic vesicles.

The combination of automated sub-micron imaging, proprietary image processing and the Norak Transfluor™ assay enables accurate measurement of GPCR activity through vesicle response and pit formation. Validated results of GPCR activity using the EIDAQ^{TM} 100 HTM system are presented with fluorescent images and dose response curves.

Experimental Methods

Norak Transfluor™ Assay Plates

In the Norak Transfluor™ assay, a cell line is developed to monitor the interaction of a given GPCR and a GFP fused to ß-arrestin. When each GPCR is activated, the ß-arrestin will bind to the membrane associated GPCR. The activated ß-arrestin-GPCR complex then enters clathrin-coated pits and migrates to intracellular vesicles via the endosomal pathway. Some GPCRs retain the arrestin molecule throughout this process, so that vesicles will fluoresce with GFP. Other GPCRs will dissociate from the arrestin such that the GFP remains with the pits, or is released to the intracellular space, and the receptors recycle back to the cell surface and bind arrestin again.

Two different plates were used, one containing a wild type cell line, the other containing an enhanced cell line. The layout of the plates was identical and is shown in Table 1.

Table 1: Norak Transfluor™ well plate layout – concentrations given in nM unless stated otherwise.

	Row	10 Point Titration																			Control
Iso	A-B	Basal																			Iso Block 50 nM
	C-H	500	500	167	167	55.6	55.6	18.5	18.5	6.17	6.17	2.06	2.06	0.69	0.69	0.23	0.23	0.08	0.08	0.03	0.03
Prop	I-N	900	900	300	300	100	100	33.3	33.3	11.1	11.1	3.70	3.70	1.24	1.24	0.41	0.41	0.14	0.14	0.05	0.05	Prop Block 1 µM
	O-P	Basal																			

EIDAQ™ 100 HTM Parameters

The two plates were scanned three times each, the first two runs were done on the same day while the third run was done a week later to show repeatability over extended time frames

The plates were imaged with the Q3DM EIDAQ™ 100 HTM system using a 20x 0.45 NA PlanFluor ELWD Nikon objective with the correction collar adjusted to the plate bottom thickness. The images were acquired using camera integration times of 16 msec for the nuclear and 1.7 sec for the GFP channel with 4 fields imaged per well.

GPCR analysis was performed using Q3DM's CytoShop™ software suite using the proprietary "Number of Vesicles" measurement. GPCR analysis using this measure is displayed in Figure 1, where the masks of the vesicles are shown for example images taken from one of the scans.

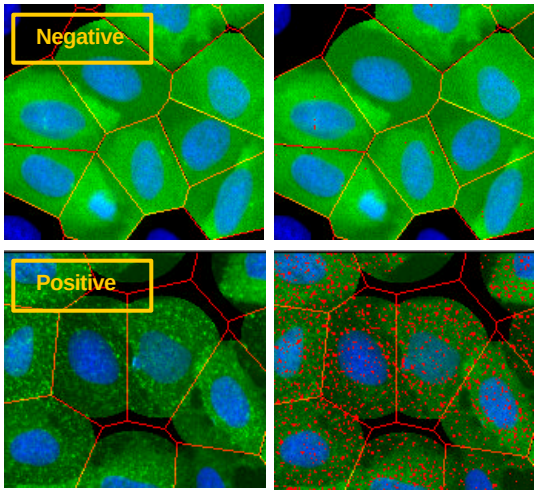


Figure 1: Cell analysis for GPCR - example images from a well plate scan; negative response (top) compared to positive response (bottom); pseudo-color overlay of nuclear dye Hoechst (blue) and GFP ß-arrestin (green) shown in left column, additionally masks for GPCR measurement (red) shown in right column.

Assay Analysis Results

The results for the validation of the GPCR analysis are shown in the next two sections. The first section displays the results as an average measurement for each well read-out using a color range display. The second section displays example EC_{50} and IC_{50} curves for each of the two plates and shows the repeatability of the EC_{50} and IC_{50} measurement over the three repeated runs per plate.

EIDAQ™ 100 Plate Read-Out

Plate read-outs of the GPCR assay analyzed with the Q3DM EIDAQ™ 100 HTM system displaying the average values for the GPCR "Number of Vesicles" measurements are shown in Figure 2 and 3. Since the color scale is relative to the minimum and maximum value of each plate, this provides a high-level overview only.

Figure 2 gives an overview of the GPCR "Number of Vesicles" measurement for the three repeated runs of the plate with the enhanced cell line.

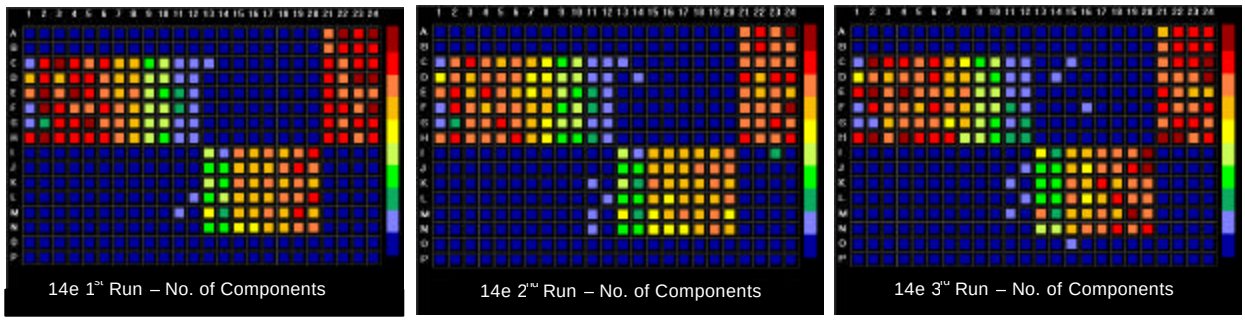


Figure 2: Plate B2AR#14e Enhanced 384 analyzed with EIDAQ™ 100 HTM and CytoShop™ Software Suite. Plate layout is described in Table 1.

Figure 3 gives an overview of the GPCR "Number of Vesicles" measurement for the three repeated runs of the plate with the wild type cell line.

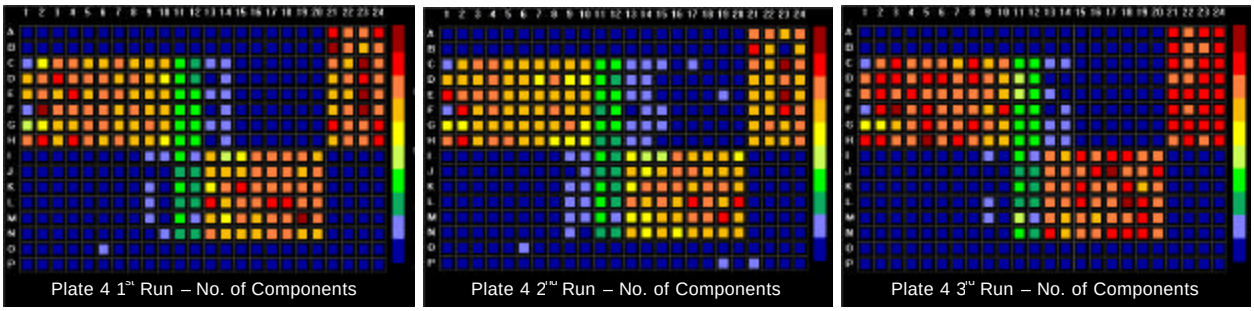


Figure 3: Plate B2AR#4 Wild Type 384 analyzed with EIDAQ™ 100 HTM and CytoShop™ Software Suite. Plate layout is described in Table 1.

EC_{50} / IC_{50} Analysis

EC_{50} and IC_{50} analysis was performed for all runs of the two plates. The results for the three runs of the two plates are summarized in Table 2 and Table 3, displaying EC_{50} / IC_{50} values for each of the 6 replicates per plate, the average EC_{50} / IC_{50} for each of the three runs of each plate and the average EC_{50} / IC_{50} for each plate. Also shown are example curves for each EC_{50} and IC_{50} for the two plates in Figures 4 and 5. The curves for all of the replicates and all runs of each plate are given in the appendix.

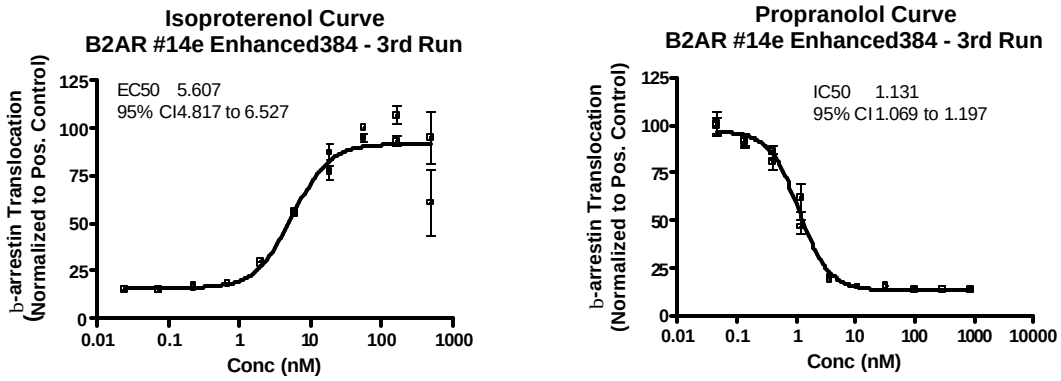


Figure 4: Examples of EC_{50} (agonist isoproterenol) and IC_{50} (antagonist propranolol) curves for wellplate B2AR#14e Enhanced 384 (3rd run). The data points in the graphs show the mean values and the error bars represent the standard deviations.

Figure 4 shows examples of EC_{50} and IC_{50} curves for the enhanced cell line. Given are the average curves with the standard deviation as error bars for run 3 of the enhanced cell plate.

In Table 2 the statistics for each of the three runs per plate are given for the enhanced cells. The mean EC_{50} and IC_{50} for each run are given in the "Plate" column, and mean EC_{50} and IC_{50} and their CVs across the three runs are given in the "Total" column.

Table 2: EC_{50} and IC_{50} statistics for multiple runs of plate B2AR#14e Enhanced 384.

		Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Rep. 6	Plate	Total (Mean±CV)
Run 1	**EC_{50}**	5.421	5.081	6.755	4.839	4.422	7.712	5.392	EC_{50} = 5.474 ± 2.12%
	IC_{50}	1.054	1.110	1.289	1.227	1.278	1.051	1.177	
Run 2	**EC_{50}**	5.451	5.578	7.197	4.716	4.279	7.089	5.424	
	IC_{50}	1.045	1.118	1.265	1.162	1.240	1.071	1.160	IC_{50} = 1.156 ± 2.01%
Run 3	**EC_{50}**	5.575	5.644	6.892	4.762	4.186	8.699	5.607	
	IC_{50}	0.9972	0.6841	1.130	1.076	1.554	1.274	1.131	

Figure 5 shows examples of EC_{50} and IC_{50} curves for the wild type cell line. Given are the average curves with the standard deviation as error bars for run 2 of the wild type cell plate.

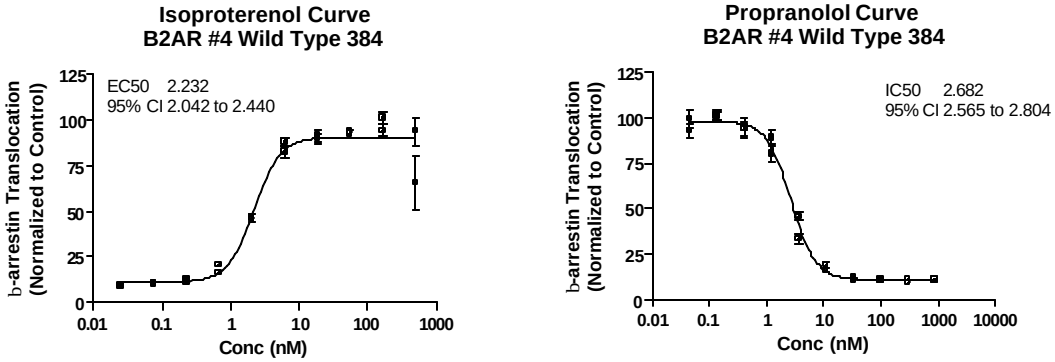


Figure 5: Examples of EC_{50} (agonist isoproterenol) and IC_{50} (antagonist propranolol) curves for wellplate B2AR#4 Wild Type 384 (2^{nd} run). The data points in the graphs show the mean values and the error bars represent the standard deviations.

In Table 3 the statistics for each of the three runs per plate are given for the wild type cells. The mean EC_{50} and IC_{50} for each run are given in the "Plate" column, and mean EC_{50} and IC_{50} and their CVs across the three runs are given in the "Total" column.

Table 3: EC_{50} and IC_{50} statistics for multiple runs of plate B2AR#4 Wild Type 384.

		Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Rep. 6	Plate	Total (Mean±CV)
Run 1	**EC_{50}**	2.238	2.474	2.254	2.111	1.918	3.081	2.244	EC_{50} = 2.200 ± 2.98%
	IC_{50}	2.521	2.822	2.890	2.597	1.991	3.047	2.628	
Run 2	**EC_{50}**	2.180	2.593	2.149	2.146	1.804	2.936	2.232	
	IC_{50}	2.503	2.841	2.864	2.662	2.031	3.179	2.682	IC_{50} = 2.709 ± 3.59%
Run 3	**EC_{50}**	1.979	2.239	2.134	2.118	1.951	2.690	2.125	
	IC_{50}	2.500	2.742	3.189	2.697	3.258	3.004	2.817	

Discussion

The two plates, plate 14 - enhanced and plate 4 - wild type, were run three times each. The first two runs were performed on the same day, while the third run was performed one week later (to show repeatability over extended time frames). The CVs for the three runs per plate for each of the EC_{50} and IC_{50} values were all better than 3.6% and the variations across plate reruns and replicates were smaller than 2.5-fold. This shows very good repeatability of the GPCR analysis using the Q3DM EIDAQ™ 100 HTM system and CytoShop software suite with the proprietary GPCR algorithm.

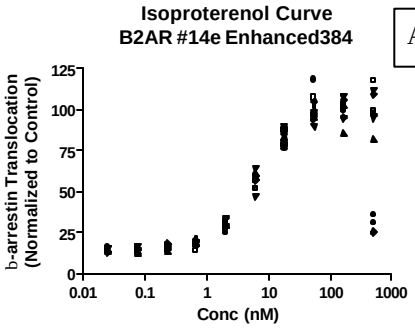
Conclusion

Q3DM's proprietary GPCR metrics provides a sensitive and accurate measurement of GPCR activation, and enables the rapid and precise quantification of fluorescent signals imaged by the EIDAQ™ 100 High Throughput Microscopy system. Use of the EIDAQ™ 100 in combination with the Norak Transfluor™ assay presents a powerful quantification tool for GPCR activity.

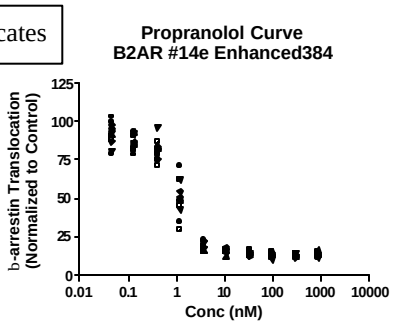
The EIDAQ™ 100 automated High Throughput Microscopy (HTM) system from Q3DM Inc. delivers an unmatched combination of speed, accuracy and detail to quantitative imaging and analysis of cell populations. The EIDAQ™ 100 is used to accelerate drug discovery, for clinical diagnostics, and in basic research.

Appendix – EC_{50} / IC_{50} Curves

GPCR Plate Analysis: 14e / 1st Run



All Replicates



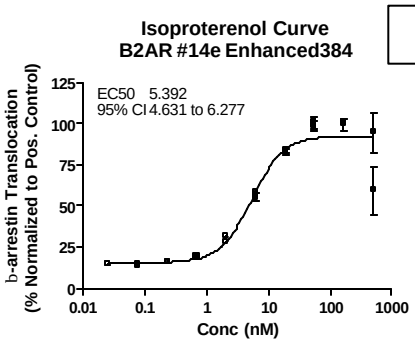
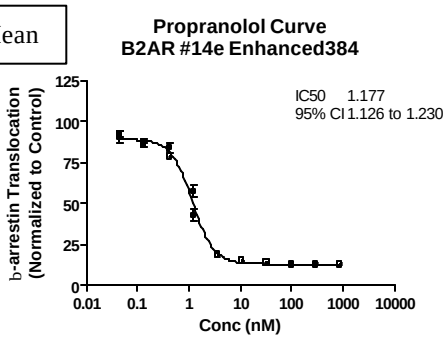
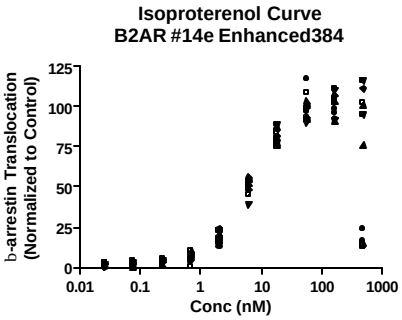


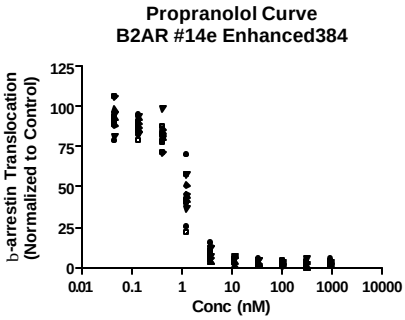
Plate Mean



GPCR Plate Analysis: 14e / 2nd Run



All Replicates



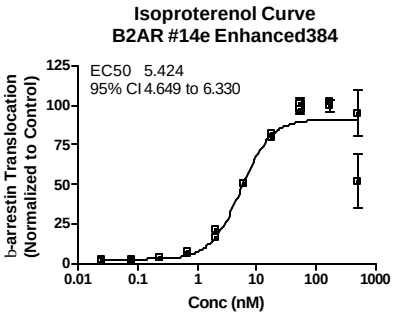
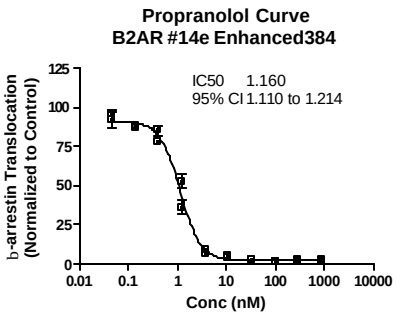
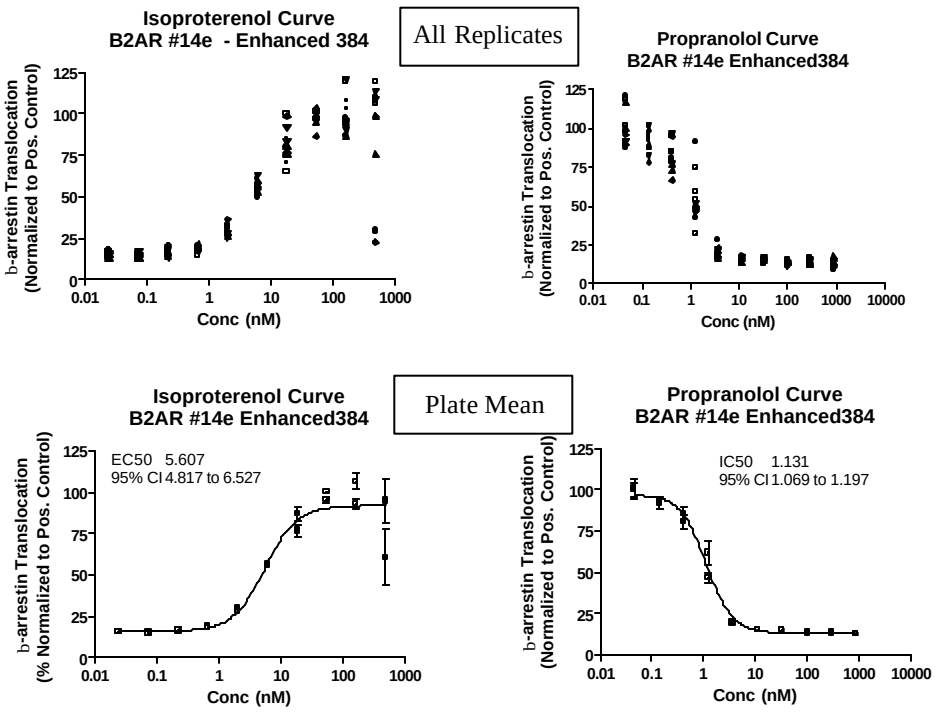


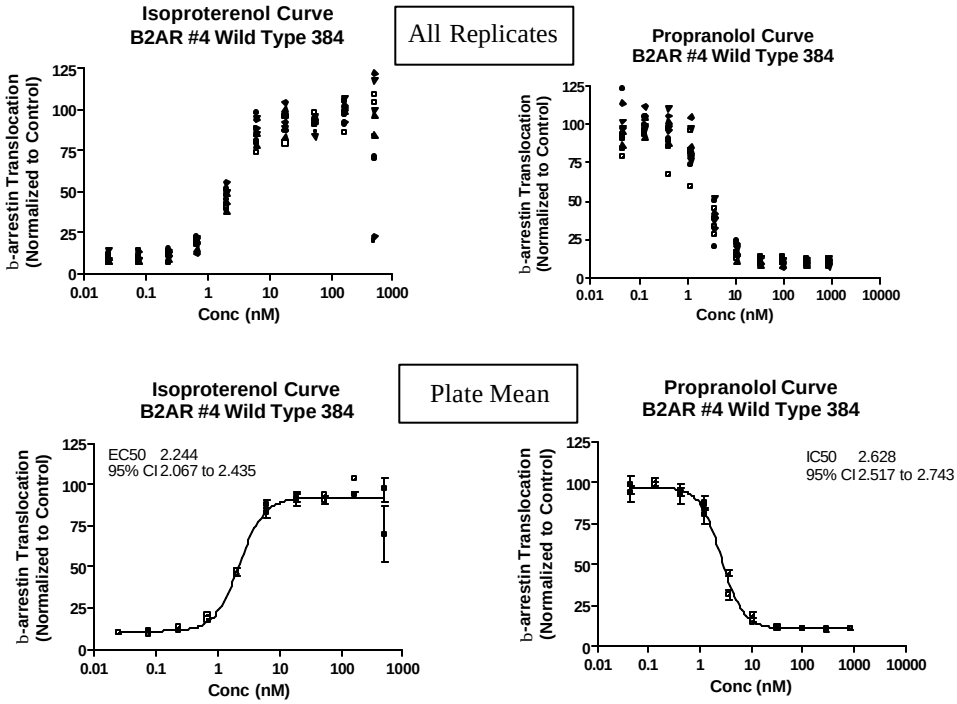
Plate Mean



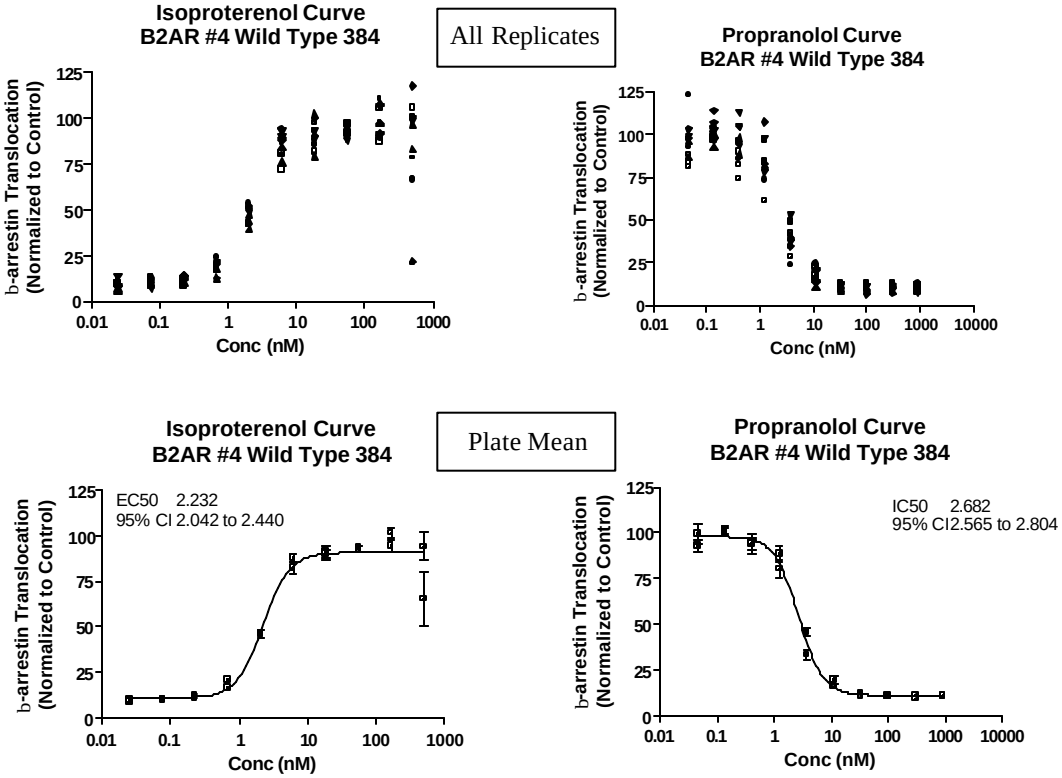
GPCR Plate Analysis: 14e / 3rd Run



GPCR Plate Analysis: 4 / 1st Run



GPCR Plate Analysis: 4 / 2nd Run



GPCR Plate Analysis: 4 / 3rd Run