

# xCELLigence System

Application Note No. 13 / January 2013



## xCELLigence System RTCA HT Instrument

High-Throughput GPCR Assay  
Development

xCELLigence

For life science research only.  
Not for use in diagnostic procedures.

## Background

G-coupled protein receptor (GPCR) modulators are estimated to make up approximately 40% of currently marketed drugs. They are among the largest class of drug candidates in development.<sup>1</sup> The rich complexity of GPCR signal transduction mechanisms, involving multiple second messenger pathways, heterodimer effects, and the potential for allosteric modulation, is leading to new paradigms for GPCR drug discovery. Novel assay technologies have enabled continual elucidation of new GPCR family members as therapeutic targets.<sup>2</sup> The xCELLigence System RTCA High Throughput (HT) Instrument offers a label-free solution for high-throughput screening of GPCRs, for robust, real-time assays of both recombinant and endogenously expressed receptors.

Many cell-based, high-throughput GPCR assay systems use recombinant cell lines to couple receptor activation to an artificial readout. An effective approach uses forced calcium coupling by promiscuous G protein overexpression or binding of a beta-arrestin fusion protein to the receptor. Biologically relevant cell-based assays measure an intermediate in one of the second messenger pathways involved in signaling. On the one hand, Gq-coupled receptors may be assayed using calcium release or metabolites downstream of phospholipase C activation. On the other hand, Gs- and Gi-coupled receptors may be assayed using cyclic AMP detection reagents. Agonist-mediated GPCR activation coupled to different signaling pathways can induce a rapid morphological change that is assayable using the xCELLigence System.<sup>3</sup> The xCELLigence System allows for GPCR assays without expressing additional reporter proteins. Real-time monitoring is possible using assays with endogenously expressed receptors in appropriate cell models.

This combination of attributes in the xCELLigence System can capture all of the effects of GPCR modulation in the most biologically or disease-relevant cell types, resulting in higher-quality hits in a screening campaign.

The xCELLigence System RTCA HT Instrument consists of the RTCA HT Control Unit with RTCA HT Software, the RTCA HT Analyzer, and up to four RTCA HT Stations. The RTCA HT Station uses a 384-well, Society for Biomolecular Screening (SBS)-compatible plate (E-Plate 384) to measure cell responsiveness to GPCR stimulation using the principle of electrical impedance. Cells are seeded one day prior to stimulation for attachment to the electrodes located in the bottom of the E-Plate well. When the E-Plate 384 is placed on the station, cellular interactions with these electrodes are detected by the RTCA HT Analyzer as a change in electrical impedance due to a low-voltage current run through the microelectrode sensors in the bottom of the E-Plate. The impedance value is converted to a Cell Index (CI) by the RTCA HT Software. The CI reflects the number of cells, as well as morphological parameters, such as cell size, shape, and degree of cell attachment to the substrate.

The sampling time can be set as short as 15 seconds, allowing for real-time readout of rapid responses such as those mediated by GPCR activation. A heating unit in the RTCA HT Station ensures that all of the assays are performed at physiological temperature, making the results as biologically relevant as possible. The entire xCELLigence System platform is compact for integration into existing liquid handling and automation workflows.

## Procedure

Background impedance measurements were taken after adding 20  $\mu$ l of growth media to an E-plate 384 and spinning briefly in a centrifuge to eliminate any bubbles. CHO-K1 cells expressing the human  $\alpha$ -adrenergic 2A, histamine H1, or dopamine D1 receptors (available from Perkin Elmer) were then seeded at 12,000 cells per well, or HeLa cells at 6,000 cells per well, in a final volume of 40  $\mu$ l. The E-Plate 384 with cells was incubated at room temperature for 30 minutes for cell attachment, and then incubated in a tissue culture incubator overnight.

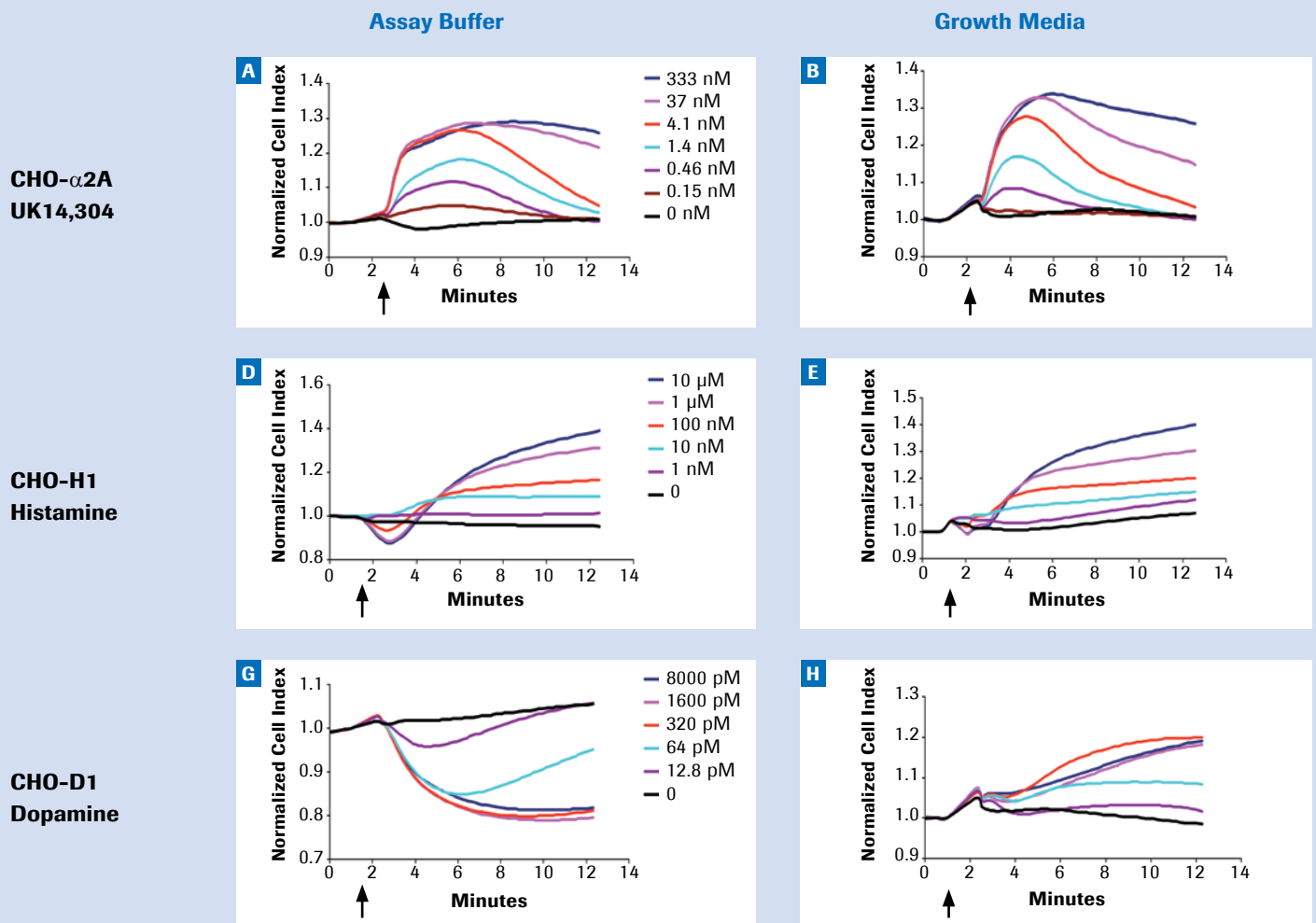
For experiments performed in assay buffer, the growth media was replaced with assay buffer (HBSS containing 0.1% BSA and 20 mM HEPES), and incubated for 15–60 minutes at +37°C in a tissue culture incubator prior to agonist treatment. E-Plates 384 with cells were then placed on the RTCA HT Station set at +37°C, and monitored using impedance recording at 30-second intervals for 2–5 minutes prior to agonist addition to determine the baseline values. Agonist was added in a volume of 5.5–6.7  $\mu$ l (as a 10x stock diluted in assay buffer) using a liquid handling instrument (Beckman FX) with continuous monitoring. Plates were measured every 15 seconds for an additional 10–20 minutes.

## Results

### Agonist Response Assay for Recombinant Receptors

To assess the ability of the RTCA HT Instrument to assay GPCR function in recombinant cell lines, representative receptors for each of the Gq-, Gi-, and Gs-coupled classes were chosen. CHO-K1 cells expressing the human  $\alpha$ -adrenergic 2A (Gi), histamine H1 (Gq), or dopamine D1 (Gs) receptors were plated on E-Plates 384, incubated overnight, then assayed using the xCELLigence System in real time under two conditions. One condition used an assay buffer similar to those used in traditional GPCR assays. This has the advantage of eliminating trace amounts of GPCR modulators present in serum-containing

media, and may, in some cases, improve assay signal-to-background and simplify buffer matching when adding agonist. The other condition avoids the media change entirely to maximize throughput. All three tested receptors showed Cell Index (CI) value changes immediately after agonist addition under both conditions (see Figure 1, A, B, D, E, G, H). The  $\alpha$ -adrenergic 2A receptor-expressing cells showed an immediate increase in CI with a maximal response observed within approximately 5 minutes. The histamine H1-expressing cells showed an immediate and transient decrease in CI followed by a gradual increase. The dopamine D1-expressing cells showed an immediate decrease in CI in assay buffer.



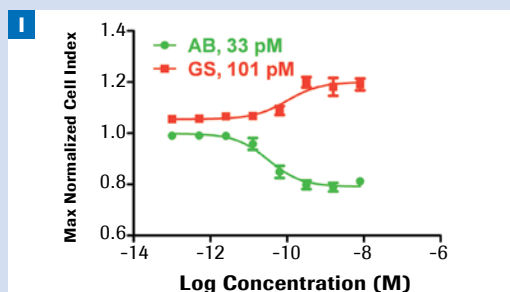
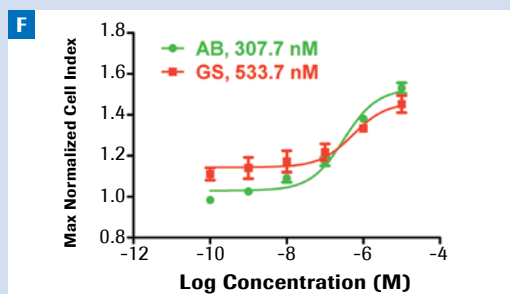
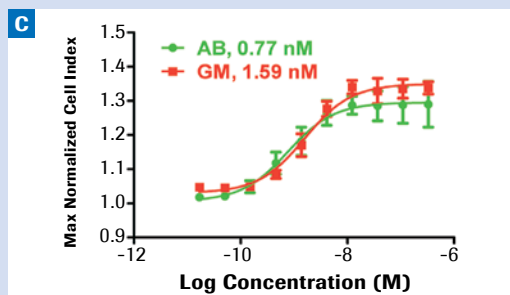
**Figure 1: GPCR assays using recombinant cells.** CHO-K1 cells expressing the  $\alpha$ -adrenergic 2A (CHO- $\alpha$ -2A), histamine H1 (CHO-H1), or dopamine D1 (CHO-D1) receptor were seeded at 12,000 cells per well on E-Plates 384, and grown overnight; growth media was changed for assay buffer (A, D, G) or left in place (B, E, H). After 15–60 minutes, cells were treated with the indicated concentrations of agonist and monitored for morphological responses using the xCELLigence RTCA HT System. Cell Index values were normalized for each well to the time point immediately preceding agonist addition, indicated by arrow and plotted as the mean value from a minimum of four replicates (A, B, D, E, G, H). Maximal, or minimal, in the case of dopamine tested in assay buffer, normalized CI values were used to plot dose responsiveness as mean values with error bars representing one standard deviation (C, F, I).

The signature responses observed will also be influenced by cell background, cell density, and assay conditions. For example, in the absence of media change, dopamine D1-expressing cells exhibited an increase in CI after agonist addition (see Figure 1H). This finding suggests that the morphological changes detected using the impedance readout can be altered by assay conditions, indicating that the direction of the CI change is not always predictive of pathway coupling.

For each recombinant cell line, assay sensitivity was assessed using the dose-response curve function of the RTCA HT Software to calculate the  $EC_{50}$  value for the agonist (see Figure 1, C, F, I). All three agonists showed  $EC_{50}$  values in the low picomolar (dopamine) to mid nanomolar (histamine) range, as expected based on cell providers' data using standard assays. In addition, assay robustness was assessed by calculating the Z factor, which takes into account both the signal-to-noise ratio and well-to-well variation.<sup>4</sup> The Z factor was calculated using the time point and agonist concentration which produced maximal CI change, and a minimum of four replicate wells (see Table 1). All three cell lines produced Z factors greater than 0.5 in at least one assay condition, indicative of a very robust assay.

For the  $\alpha$ -adrenergic 2A receptor, the maximal response was observed within the first six minutes, and for the other assays, a robust Z factor was obtained in at least one condition within the ten-minute time frame. The one exception, the dopamine D1 receptor assayed without a media change (Z factor 0.36), showed an increasing response at ten minutes, suggesting that a longer assay time may improve the Z factor. Taken together, these results indicate that the RTCA HT Instrument can be used for rapid, sensitive, and robust assays of GPCR responses in recombinant cell lines. Importantly, many receptors can be assayed without requiring additional handling steps, such as a media change prior to screening.

### Dose Response



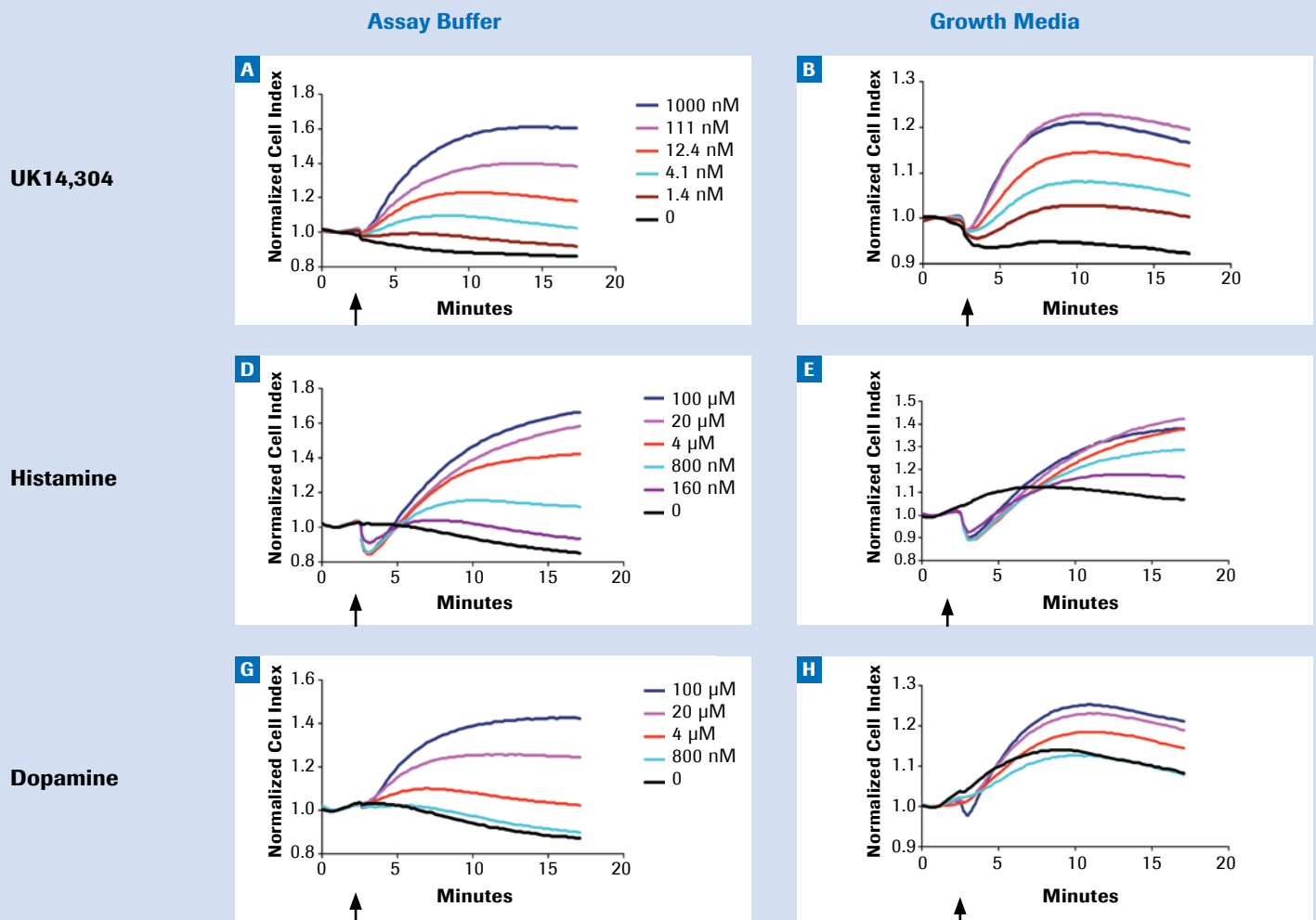
Cells	Assay Buffer			Growth Media		
	Concentration	Time (min)	Z factor	Concentration	Time (min)	Z factor
CHO- $\alpha$ 2A	12.4 nM	6.2	0.606	12.4 nM	6.2	0.600
CHO-H1	10 $\mu$ M	11.9	0.862	10 $\mu$ M	11.8	0.529
CHO-D1	1.6 pM	7.5	0.692	0.32 pM	12.3	0.341
HeLa- $\alpha$ 2A	333 nM	10.1	0.823	333 nM	10.1	0.816
HeLa-H1	20 $\mu$ M	17.1	0.802	20 $\mu$ M	17.1	0.570
HeLa-D1	20 $\mu$ M	9.6	0.619	20 $\mu$ M	9.6	0.325

Z factor calculation was performed at saturating agonist concentration and at the time point of maximal response.

### Agonist Response Assay for Endogenous Receptors

The impedance-based readout used by the xCELLigence System offers the opportunity to assay endogenously expressed receptors in their native context, which has distinct advantages over recombinant systems.<sup>2</sup> The same agonists described above were used to assay HeLa cells. HeLa cells treated with the  $\alpha$ -adrenergic 2A receptor agonist UK 14,304 exhibited a rapid increase in CI, reaching a maximum CI at approximately 5 minutes after compound addition, with or without a media change (see Figure 2). HeLa cells treated with histamine exhibited a common Gq response pattern<sup>3</sup>,

consisting of a small drop in CI followed by a large increase. Interestingly, dopamine treatment of HeLa cells generated an increase in CI under both assay conditions (see Figure 2). This differs from the decreased CI observed in the recombinant dopamine D1 receptor-expressing CHO-K1 cells assayed after a media change, and may reflect the effect of other dopamine receptors expressed in HeLa cells. For example, the dopamine D2, D2S, and D3 receptors are all Gi coupled, and all may be expressed in HeLa cells.<sup>5</sup> Alternatively, there may be differences in the way the activated signal transduction pathways couple to the morphology change in the two different cell lines, either at the level of the



**Figure 2: GPCR assays on endogenous receptors.** HeLa cells were seeded at 6,000 cells per well on E-Plates 384, and grown overnight; cell cultures were then treated with the indicated concentrations of agonist and monitored for morphological responses using the xCELLigence RTCA HT System. Cell Index values were normalized for each well to the time point immediately preceding agonist addition (indicated by arrows), and plotted as the mean value from a minimum of four replicates (A, D, G). Maximal normalized CI values were used to plot dose responsiveness as mean values with error bars representing one standard deviation (C, F, I).

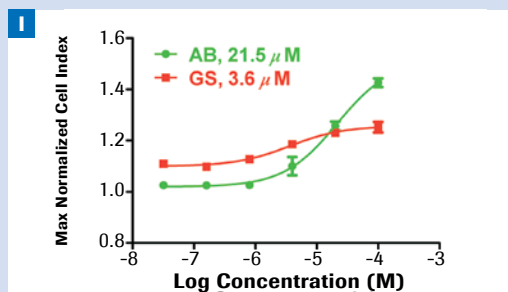
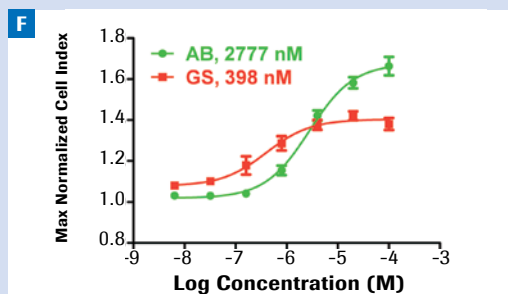
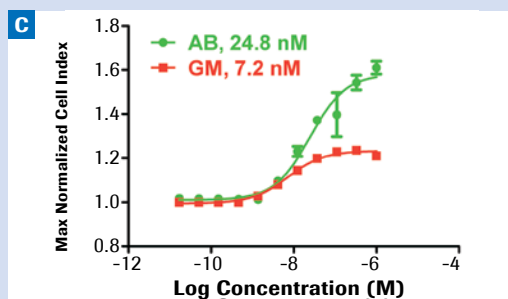
receptor/G protein-coupled step, or possibly at some point further downstream.

As with recombinant cell lines, the endogenous receptor responses assayed in HeLa cells were robust, with at least one condition exhibiting a Z factor greater than 0.5, and some as high as 0.8 (see Table 1). Again, the exception was the dopamine response assayed without a media change, suggesting an effect of trace amounts of dopamine or other modulators in the serum-containing media. Dose-response curves revealed that the xCELLigence System assay for the  $\alpha$ -adrenergic and histamine receptors endogenously expressed in HeLa cells

exhibited a similar sensitivity to that of the recombinant cell lines, with  $EC_{50}$  values in the low and high nanomolar ranges, respectively (see Figure 2, C, F, I).

In contrast, a much higher concentration of dopamine was required to activate endogenous receptors in HeLa cells than in the recombinant dopamine D1-expressing cell line under either assay condition, perhaps reflecting lower expression levels and/or a different type of receptor in the HeLa cells. Further experiments with receptor subtype-selective agonists or antagonists should be able to distinguish between these possibilities. These findings underscore the fact that, as with any assay, each receptor must be optimized individually to obtain optimal signal-to-background and Z factor values.

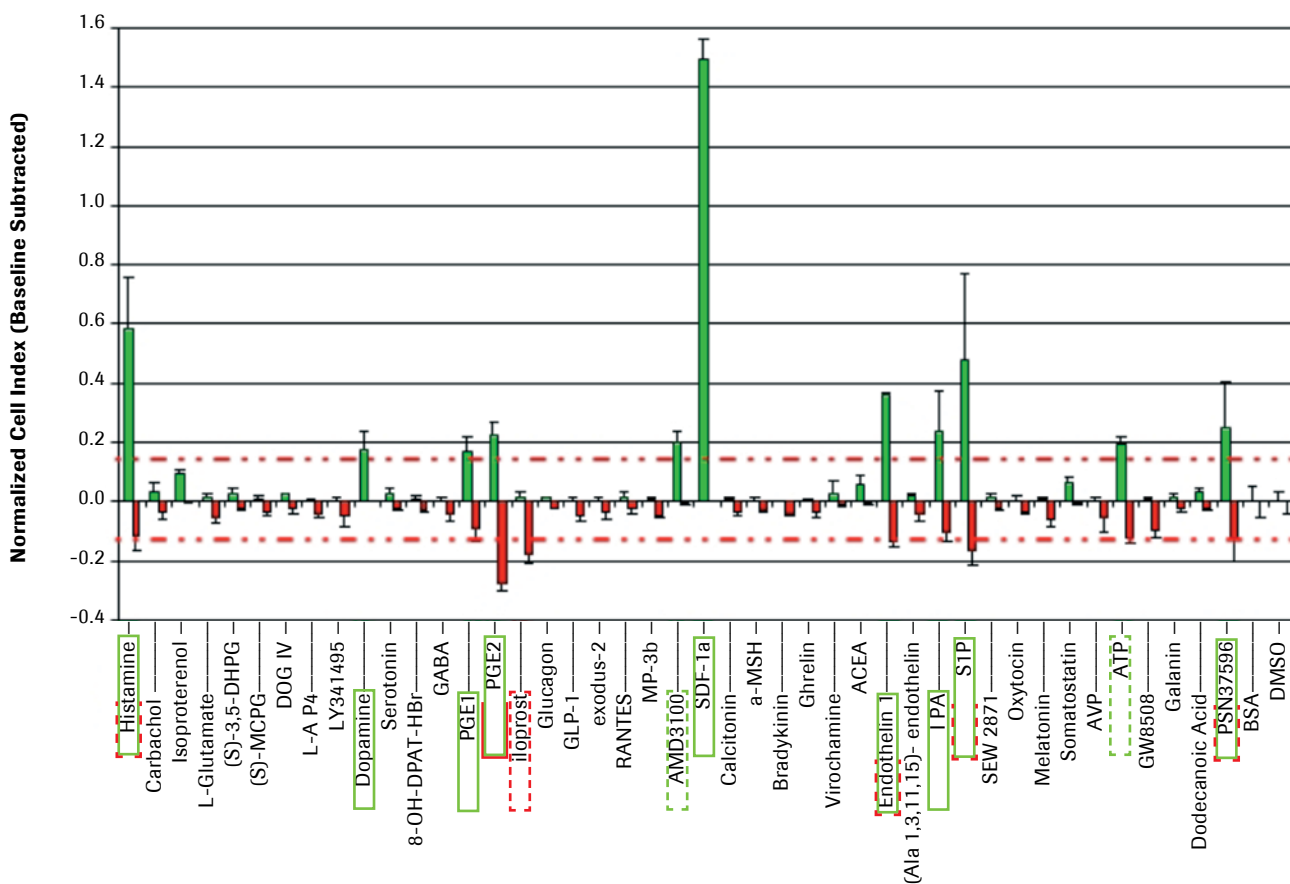
### Dose Response



### Functional Profiling of Endogenous Receptors

Directly assaying endogenous GPCRs allows for more biologically relevant assays and opens up the spectrum of cell types amenable to HT campaigns. To assess the utility of the RTCA HT Instrument for assaying a wide variety of GPCRs in the endogenous context, we assessed the function of a set of 24 GPCR receptor families in HeLa cells using a panel of 43 agonists (see Figure 3). Using the baseline subtraction feature of the RTCA HT Software, test well responses were assessed in relation to the buffer

controls for each solvent type (DMSO for small molecules, 0.1% BSA for peptides). An arbitrary cutoff of three standard deviations from the control well mean value was used. Twelve of the tested agonists produced robust responses in HeLa cells, representing eight different GPCR receptor families (histamine, dopamine, prostanoid, CXC chemokine, endothelin, lysophospholipid, purinergic, and GPR119), covering all of the major coupling classes (Gi, Gs, and Gq). This indicates that the RTCA HT Instrument can be used to assay a wide



**Figure 3: Functional profiling of endogenous receptors.** HeLa cells were seeded at 6,000 cells per well on E-Plates 384, grown overnight, and the media replaced with assay buffer. After 15 minutes, cells were treated with a panel of GPCR modulators at 7  $\mu$ M (small molecules) or 0.7  $\mu$ M (peptides), and monitored for morphological responses using the xCELLigence RTCA HT System. Cell Index values were baseline subtracted using the values of four replicate wells containing buffer controls, and then normalized to the time point immediately preceding agonist addition using the RTCA HT Software. Data were exported to Excel, and maximum and minimum CI values were determined and plotted as the mean value from four replicate wells, with error bars representing one standard deviation. The red dotted line indicates the value of three times the standard deviation of the buffer control wells used to determine active receptors; solid green boxes indicate robustly active, while dashed green boxes indicate marginally active receptors based on maximum (in green) and minimum (in red) CI values.



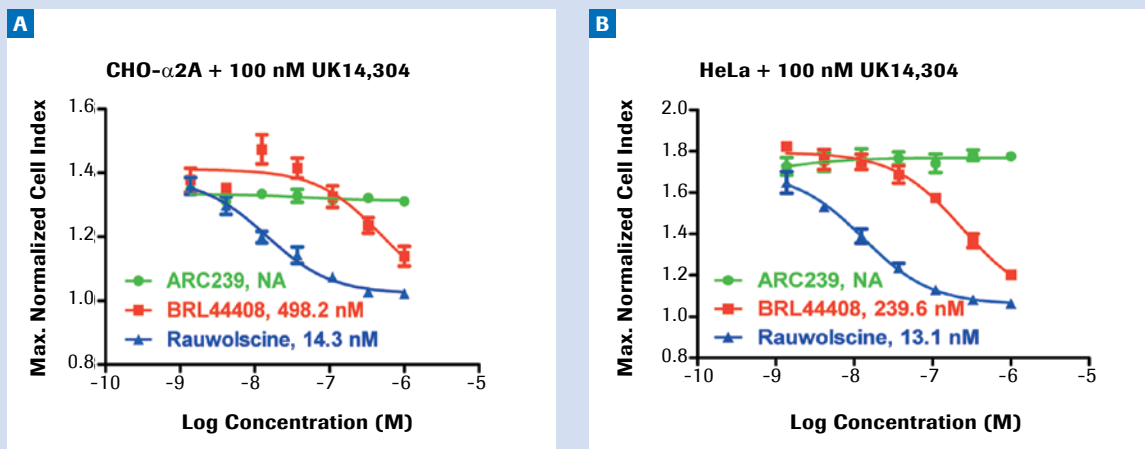
variety of endogenous GPCRs using appropriate cell backgrounds. The ability to assay GPCR function in the endogenous context is critical, because GPCR functionality can be modulated by both the repertoire and stoichiometric ratios of interacting proteins. This includes receptor heterodimers, coupled G-proteins, GPCR modulating kinases (GRKs) and beta-arrestins, all of which may exhibit altered functionality in receptor-overexpressing recombinant cell lines.

### GPCR Antagonist Assay

The potential therapeutic benefit of modulating GPCR activity is due to the antagonizing response to endogenous ligands. For example, antagonists of the  $\alpha$ -adrenergic 2A receptor are useful for treating several conditions, including hypertension. To test the utility of the RTCA HT Instrument in an antagonist screening mode, the  $\alpha$ -2A receptor-expressing CHO-K1 cell line was pretreated with different adrenergic

receptor antagonists and challenged with the  $\alpha$ 2A agonist UK 14,304. As expected, ARC 239, a selective adrenergic  $\alpha$ 2B receptor antagonist, had no effect on the morphological response to UK 14,304 stimulation, known to act through the  $\alpha$ 2A receptor (see Figure 4, A).

In contrast, both BRL 44408 (a highly selective adrenergic  $\alpha$ 2A receptor antagonist), and rauwolscine (a potent but general adrenergic  $\alpha$ 2 antagonist), exhibited a dose-dependent inhibition of the response, with  $IC_{50}$  values of 498 nM and 14 nM, respectively (see Figure 4, A). The relative potencies of these inhibitors were nearly identical when tested with respect to the endogenous adrenergic 2A receptor in HeLa cells (see Figure 4, B). These results indicate that the RTCA HT Instrument can be used to screen for selective antagonists for a given receptor, and that the degree of antagonism can distinguish among compounds with different potencies.



**Figure 4: GPCR antagonist assay.**

**A)** CHO-K1 cells expressing the  $\alpha$ -adrenergic 2A receptor were seeded at 12,000 cells per well on E-Plates 384, grown overnight, and growth media was changed for assay buffer. The indicated antagonist was added, and after 1 hour incubation, the  $\alpha$ -adrenergic 2A receptor-selective agonist UK 14,304 was added at 100 nM. Resulting dose-response relationships are plotted using mean values from four replicate wells; error bars indicate one standard deviation. ARC 239 is a selective adrenergic  $\alpha$ 2B receptor antagonist, BRL 44408 is a selective adrenergic  $\alpha$ 2A receptor antagonist, and rauwolscine is a potent, general adrenergic  $\alpha$ 2 receptor antagonist.

**B)** The relative potencies of the inhibitors BRL 44408 and rauwolscine were nearly identical when tested with respect to the endogenous adrenergic 2A receptor in HeLa cells.

## Discussion

The RTCA HT Instrument is a powerful tool for screening GPCR modulators. Sensitive and robust assays can be developed for a variety of receptor families, including the three major coupling classes (Gi, Gs, and Gq) in both recombinant cell lines and in cell lines expressing endogenous receptors. Depending on the receptor assayed, robust responses (with Z factors greater than 0.5) can be achieved in as little as 5–10 minutes. Very high throughput capacity is possible, especially when combined with parallel processing using all four RTCA HT Stations on the system at the same time. The findings show that in cases where the change of media for assay buffer produced a difference in assay quality, this effect in general improved assay robustness by increasing the signal-to-background ratio and decreasing assay variability (see Table 1 and Figures 1 and 2). Some GPCR assays showed no difference in the two different conditions performed (e.g., CHO-K1 cells expressing the  $\alpha$ -adrenergic 2A receptor). For best results, each new assay should probably be tested under both conditions, weighing the importance of assay robustness against the decrease in throughput by performing media changes.

### The present study has shown:

- The RTCA HT Instrument can be used to screen modulators of GPCR activity in recombinant cell lines as well as with endogenously expressed receptors, in up to four E-Plates 384 at one time.
- These assays can be performed on a wide variety of GPCR families, including all coupling classes.
- These assays are highly sensitive and robust.
- These assays can be performed in agonist or antagonist mode.

## References

1. Eglen RM, Reisine T. (2009).  
“New insight into GPCR function: implications for HTS.”  
*Methods Mol Biol.* **552**:1-13.
2. Kenakin TP. (2009).  
“Cellular assays as portals to seven-transmembrane receptor-based drug discovery.”  
*Nat Rev Drug Discov.* **8**:617-26.
3. Yu N, Atienza JM, Bernard J, Blanc S, Zhu J, Wang X, Xu X, Abassi YA. (2006).  
“Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors.”  
*Anal Chem.* **78**:35-43.
4. Zhang JH, Chung TD, Oldenburg KR. (1999).  
“A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays.”  
*J Biomol Screen.* **4**:67–73.
5. Elphick GE, Querbes W, Jordan JA, Gee GV, Eash S, Manley K, Dugan A, Stanifer M, Bhatnagar A, Kroeze WK, Roth BL, Atwood WJ. (2004).  
“The human polyomavirus, JCV, uses serotonin receptors to infect cells.”  
*Science.* **306**:1380-3.

## Ordering Information

Product	Cat. No.	Pack Size
<b>RTCA HT System Bundle</b>	<b>00380601070</b>	<b>1 Bundled Package</b>
<b>RTCA HT Station</b>	05919878001	1 Instrument
<b>RTCA HT Analyzer</b>	05919843001	1 Instrument
<b>RTCA HT Control Unit</b>	06200176001	1 Instrument
<b>E-Plate 384</b>	05867681001	2 × 5 Plates
	05867673001	8 × 5 Plates

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The RTCA HT Analyzer in combination with the RTCA HT Station and an RTCA HT Software Package 1.x, with the E-Plate 384, is a real-time cell based assay system covered by US patent No. 7,192,752 (exp. 11/10/2023), No. 7,470,533 (exp. 1/12/2025), No. 7,560,269 (exp. 10/24/2025).

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