

# Automation of a Microplate Cell-based Assay to Measure Activity of the Histamine H1 G-protein-coupled Receptor Using A Novel 3-D Cell Culture Technique

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## Overview

- Current cell culture techniques are two-dimensional (2-D) where cells attach to the microplate surface in a single monolayer. Trypsinization is required to split cell cultures, and prepare them for downstream applications, which becomes increasingly difficult as scale-up is required for primary and secondary screens.
- 3-D cell culture techniques, where cells grow on microcarriers suspended within the culture, eliminate the need for trypsinization, and enable the growth of high-density cultures in a small volume.
- Cells frozen *in situ* on the microcarrier can be thawed and used directly in downstream applications without the need for further culturing.
- Robotic validation data, as well as agonist/antagonist data generated using the pharmacologically relevant target, Histamine H1 receptor, demonstrate the ability to use cells grown in a 3-D manner for automated drug discovery applications.

## Introduction

Cell-based assays using recombinant drug targets expressed in immortalized cell lines are today used more frequently than their biochemical counterparts in drug screening. Current cell culture methods use two-dimensional (2-D) techniques where cells attach to the microplate surface in a single monolayer. In order to maintain cell lines, complicated and time-consuming methods are used to trypsinize cells off of their growth surface, deactivate and remove the trypsin, split, and then replace cells into a new flask. This becomes increasingly difficult as scale-up is required for primary and secondary screens.

Here we show the utility of a novel three-dimensional (3-D) cell culture technique suitable for scale-up to provide primary screening quantities of cells. Cells are cultured on a magnetic alginate microcarrier coated with covalently bound coatings to promote cell adhesion, such as gelatin, laminin, or collagen. The small sub-micron magnetic particles embedded within the alginate core serve as a way to simplify culture manipulations. Since the cells grow on the microcarriers, trypsinization is not needed to split and maintain cultures. Magnetics are used to pull the microcarriers out of suspension during media changes. When cells are needed for downstream applications, an aliquot of the culture is simply aspirated from the tube used to grow the cells. As the alginate core is optically clear and non-autofluorescent, cells can remain on the microcarrier during the assay process.

In this work we show the ability of this 3-D cell culture method to deliver pharmacologically relevant data using a FRET-based assay to measure the activity of the Histamine H1 G-protein-coupled receptor. The assay was run in antagonist mode, due to the fact that a majority of the testing of this target is done to look for receptor antagonists, as evidenced by the number of antihistamine drugs on the market today, including Benadryl (diphenylhydramine) from Johnson and Johnson, and Claritin (loratadine) from Schering-Plough. The entire assay procedure was automated in 384-well format, including cell plating, compound titration and transfer, and reagent dispense, using simple, yet robust robotic instrumentation. Validation and pharmacology data demonstrate the capabilities of this novel cell culture method to be used in a high-throughput assay setting.

## BioTek Instrumentation



Figure 1 – MicroFlo™ Select Dispenser

The MicroFlo™ uses a positive displacement peristaltic pump to dispense a wide range of volumes (1 µL – 10 mL), via non-contact dispensing. Each channel is connected to an individual tip allowing for up to 8 reagents to be dispensed at a time. The instrument was used to dispense cells and LiveBLAzer™ substrate to the 384-well assay plates.

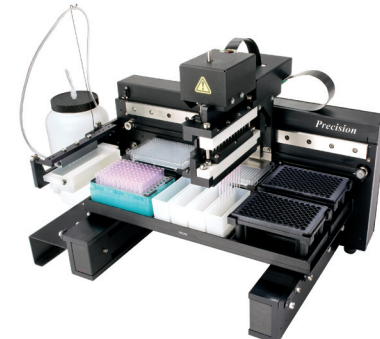


Figure 2 – Precision™ Microplate Pipetting System

The Precision™ combines a multichannel pipetting head with a multichannel bulk reagent dispenser in one instrument. The instrument was used to serially titrate agonists and antagonists across a 96-well PP plate, as well as transfer the compounds to the 384-well assay plates.



Figure 3 – Synergy™ 4 Hybrid Multi-Mode Microplate Reader

The Synergy™ 4 combines a fluorescence filter-based detection system and a monochromator-based detection system. The filter-based system can be used when high sensitivity is a requirement. The ability to use the filters for bottom reads makes it ideal for use in cell-based assays where cells are adhered to the bottom of each assay well. The filters were used in such a mode to detect the coumarin and fluorescein signals from each assay well.

## GEM™ Automated Dispensing Verification

The GEM™ (Global Eukaryotic Microcarrier) is a novel alginate three dimensional cell microcarrier. The alginate, as an unbranched polysaccharide, possesses a chemical structure similar to components of the extracellular matrix and provides a unique porous surface that mimics *in vivo*-like metabolic function. The GEM™ is non autofluorescent and optically clear, allowing for cell assay directly on the substrate, and also contains paramagnetic particles allowing for easy manipulation of cells on the microcarrier. The GEM™ is coated with a molecular layer of a protein coating allowing for attachment of unique cell types.

### Experiment 1

- GEM™ substrate in media was dispensed by the MicroFlo™ (32 µL/well) to a 384-well assay plate
- BSA in media (1 mg/mL) was dispensed manually (32 µL/well) as an assay control
- 8 µL/well of BioRad protein assay reagent was then added to each plate

**Results:** Manually dispensed BSA plate %CV=9.7%; MicroFlo™ dispensed GEM substrate plate %CV = 9. 6%

**Conclusion:** GEM™ dispensing precision statistically equivalent to soluble protein

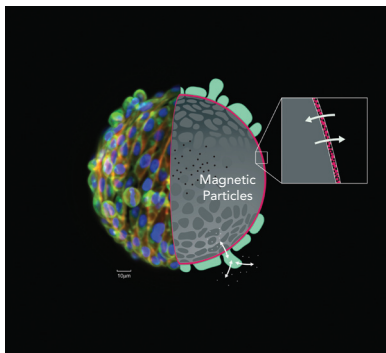


Figure 4 – GEM™ Global Eukaryotic Microcarrier

## Cell Culture Procedure

The BioLevigator™<sup>c</sup> is a benchtop hybrid of an incubator and bioreactor. The BioLevigator™ demonstrates the convenience of three-dimensional GEM™ culture with four independently controlled temperature and CO<sub>2</sub>-regulated cell culture vessels. This unique device controls positioning of the GEM™ during manual or automated handling using an internal magnet, has a touch screen interface that allows for easy data monitoring and collection, allows for increased ‘walk-away’ time by minimizing the number of manual intervening steps, and finally is easily integrated into a fully automated cell system.

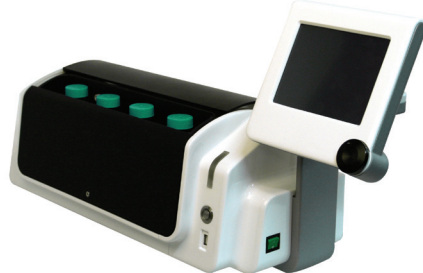


Figure 5 – BioLevigator™ Cell Culture Chamber

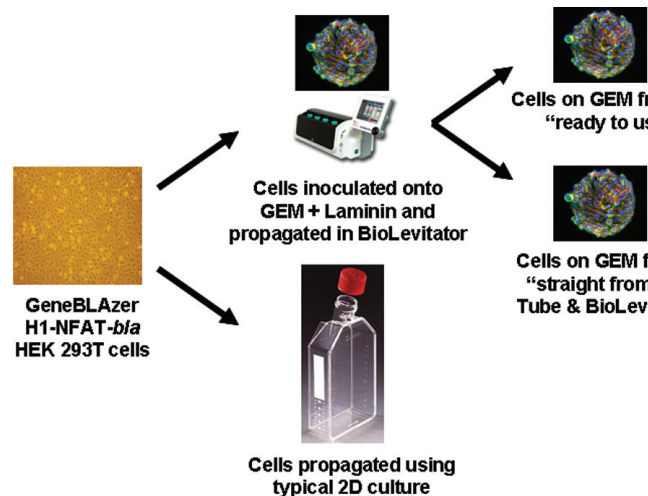


Figure 6 – Cell culture conditions

## HEK 293T Cells on GEM™ Substrate Automated Dispensing Verification

### Experiment 1

- GEM™ substrate in media plus 10 nM fluorescein was dispensed by the MicroFlo™ (32 µL/well) to a 384-well assay plate
- A standard curve was also manually dispensed containing various volumes of media plus beads, from 10-40 µL/well
- Using the standard curve, the volume dispensed per well was also calculated

**Results:** Average fluorescent value=43579.5; %CV=3.4%; Average interpolated volume dispensed/well=33.075 µL/well (3.3% accuracy)

**Conclusion:** Acceptable precision and accuracy for GEM™ dispensing using MicroFlo™

### Experiment 2

- Frozen cells on the GEM™ substrate were thawed and diluted to the proper concentration used in the GeneBLAzer® assay (5000 cells/well)
- The MicroFlo™ was used to dispense cells on the GEM™ substrate to a 384-well assay plate (32 µL/well)
- 32 µL/well of the same cells was also dispensed manually to a second plate
- 3.2 µL/well of Alamar Blue was then manually dispensed to each well of both plates

**Results:** Manually dispensed plate %CV = 8.16%; MicroFlo™ dispensed GEM™ substrate plate %CV = 8.48%

**Conclusion:** Automated protocol statistically equivalent to manual protocol

## GeneBLAzer® Histamine H1 Cell-Based Assay

- GeneBLAzer® H1-NFAT-bla HEK 293T cells contain the human Histamine Subtype 1 receptor (H1), stably integrated into the CellSensor® NFAT-bla HEK 293T cell line, which contains a beta-lactamase (bla) reporter gene under control of the NFAT response element.

- Stimulation or inhibition of the Histamine H1 receptor will be relayed through the NFAT pathway, causing increased or decreased production of beta-lactamase, respectively.

- Following addition and incubation of the beta-lactamase substrate within a well containing no bla, the substrate will remain intact. Upon excitation at 409 nm, energy is transferred from coumarin to fluorescein, and emitted at 520 nm.
- After addition and incubation of the beta-lactamase substrate within a well containing bla, the substrate is then cleaved. Upon excitation at 409 nm, FRET does not take place, and emission is seen at 447 nm.

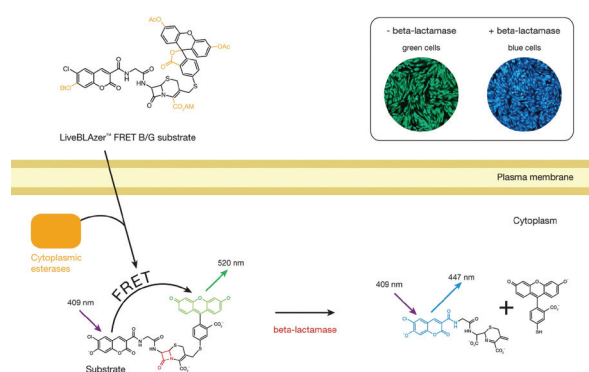


Figure 7 – Representation of the Invitrogen GeneBLAzer® Histamine H1 Cell-Based Assay

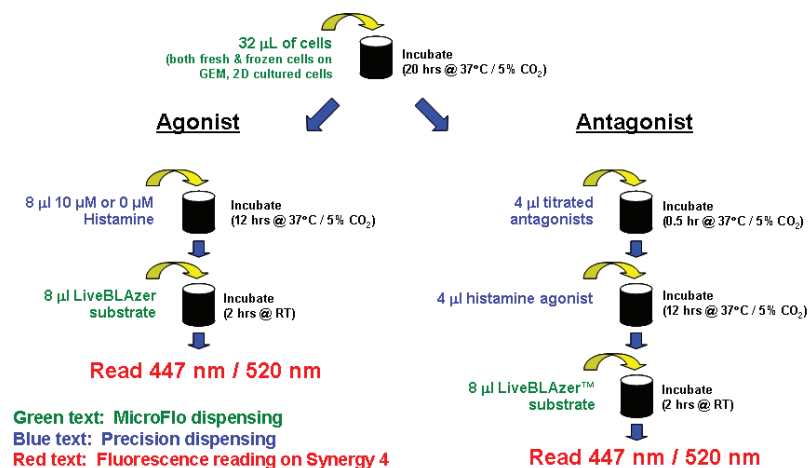


Figure 8 – Automated GeneBLAzer® Histamine H1 assay protocol

## GeneBLAzer® Histamine H1 Assay Verification

HEK 293T cells were prepared for dispensing by diluting to the proper concentration for the GeneBLAzer® assay (5K cells/well). Frozen “Cells on GEM™” were thawed and the freezing media removed by pulling the cells on the GEM substrate out of suspension using a cube magnet. Assay media, containing charcoal stripped FBS, was added to the cells. Freshly propagated “Cells on GEM™” were removed from the TPP tube and transferred to a fresh conical tube. The growth media was removed using the procedure explained above, and replaced with assay media. 2D cultured cells were removed from the T75 tissue culture flask using standard trypsinization techniques. Following centrifugation to remove the trypsin from the cells, assay media was added back to the cells. Cell counts were performed on all cells to determine cell number before dilution. Assay media was used for all dilutions.

Z'-Factor assays were set up by adding 48 replicates of either 10 µM or 0 µM Histamine to the different cell formats, and running the assay in agonist mode.

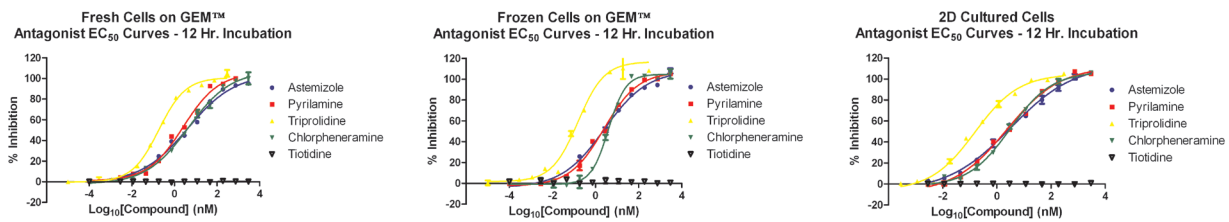
Antagonist assays were performed with four known Histamine H1 antagonists, astemizole, pyrilamine, triprolidine, and chlorpheniramine. The H2 selective antagonist, tiotidine, was also included as an assay control.

Antagonists were serially titrated 1:4 prior to addition to the 384-well assay plate. All other additions and incubations were as previously explained in the automated GeneBLAzer® Histamine H1 assay protocol.

### Z'-Factor Values

	Histamine	Fold Difference
Fresh Cells on GEM™ - 12 Hour Incubation	0.77	5.39
Frozen Cells on GEM™ - 12 Hour Incubation	0.82	2.73
2D Cultured Cells - 12 Hour Incubation	0.80	6.90
Invitrogen Z'-Factor Value	0.85	

Figure 9 – Histamine Z'-Factor values



### Antagonist IC<sub>50</sub> Values (nM)

	Astemizole	Pyrilamine	Triprolidine	Chlorpheniramine	Tiotidine
Fresh Cells on GEM™ - 12 Hour Incubation	4.197	2.603	0.1859	4.563	Not Determined
Frozen Cells on GEM™ - 12 Hour Incubation	2.613	2.48	0.1546	4.012	Not Determined
2D Cultured Cells - 12 Hour Incubation	3.278	2.309	0.1559	3.064	Not Determined
Invitrogen IC <sub>50</sub> Value	---	4.9	0.098	9.2	Not Determined
Literature IC <sub>50</sub> Value	4.0 <sup>b</sup>	---	---	---	Not Determined

Tiotidine: Negative Control (H2 Selective Antagonist)

Figure 10 – Antagonist curves and IC<sub>50</sub> values

## Conclusions

- Cells on the GEM™ substrate are able to be dispensed consistently across a 384-well plate using the MicroFlo™ Select Dispenser.
- GeneBLAzer® H1-NFAT-bla HEK 293T cells, grown and assayed on the GEM™ substrate, yield pharmacologically equivalent data for the Histamine H1 receptor, when compared to standard 2-D cultured cells.
- The combination of BioTek's instrumentation, Global Cell Solutions' novel 3-D cell culture method, and Invitrogen's GeneBLAzer® cell-based assays create an ideal solution to meet the need for easy-to-use, robust automated cell-based assays in today's drug discovery market.