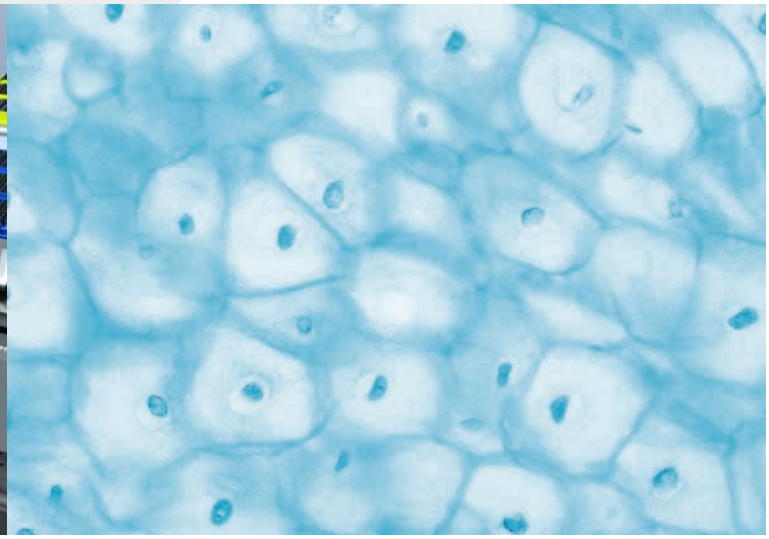
Application Note



PathHunter® β-Arrestin Human and Ortholog GPCR Assays

Easy automation for cell-based assays on the Fluent™ laboratory automation solution

Introduction

The ability to carry out cell-based G-Protein coupled receptor (GPCR) assays in high throughput is a key part of the drug discovery process. Combining a Fluent laboratory automation solution configured for cell-based assays with the DiscoveRx PathHunter β-Arrestin human and ortholog GPCR assay kits gives users the power to run 384-well plate-based assays in high throughput. This increased processing speed and capacity over current solutions, combined with extremely efficient use of laboratory space, makes this a superior system for cell-based assays.

Tecan has re-invented laboratory automation with the Fluent system, a unique instrument concept built around the application-specific needs of your laboratory. Fluent breaks new ground, delivering more capacity and increased speed; the platform provides superior throughput and walkaway time, making it easier to get more done, more easily.

DiscoveRx offers an industry-leading portfolio of over 600 naturally coupled GPCR cell lines designed to detect GPCR signaling through second messenger activation, arrestin recruitment, and receptor internalization. A broad collection of human and ortholog GPCR assays in a wide variety of functional readouts gives complete flexibility to choose the ideal screening platform to meets your specific project needs.

Regardless of the technology platform, DiscoveRx offers robust and reliable, high throughput chemiluminescent GPCR assays.
Tecan and DiscoveRx have developed protocols that demonstrate the power of their combined expertise in automated liquid handling, detection and cell-based assays, bringing a new dimension to performing fast and reliable, completely automated assays without the need for expert automation personnel.

Figure 1: The Fluent cell-based assay workstation. A Fluent 780 is shown, equipped with an 8-channel Flexible Channel Arm, a Multiple Channel Arm 384 and a Robotic Gripper Arm. A Carousel™ is integrated onto the right hand side of the instrument, along with the latest generation of CO_2 incubator. An Infinite® M1000 PRO microplate reader is located below the dynamic worktable. The dimensions of this compact system are indicated.

Materials and methods

Fluent laboratory automation solution

Fluent is the latest in Tecan's successful family of liquid handling automation platforms. The Fluent cell-based assay solution offers rapid, high precision pipetting for both the 8-channel Flexible Channel Arm (FCA) and the Multiple Channel Arm (MCA) 384.

Its patented Dynamic Deck increases worktable capacity and boosts productivity, allowing integration of a wide range of Tecan modules – including a Carousel, for the storage of various consumables; a HydroSpeed™ plate washer (not required for this assay); an Infinite M1000 PRO plate reader; and carriers for troughs, stacked disposable tips and microplates (up to six deep on the worktable, Figure 2) – as well as a third-party high capacity CO_2 incubator.

The MCA 384 uses adapters which can be automatically exchanged during a run – allowing it to act as either a 384- or 96-channel arm within the same protocol. Fully independent, task-specific arms allow parallel processing and coordinated scheduling, ensuring runs are completed faster and more efficiently. Each Fluent cell-based assay solution is equipped with:

- Flexible Channel Arm – fitted with eight pipetting channels using disposable tips that can individually access any well or tube, perfect for explicit sample and control distribution or serial dilutions.

- Multi Channel Arm – instantly swaps between 96- and 384- channel adapters during a run, offering outstanding capabilities for reagent distribution or plate replication.

- Robotic Gripper Arm (RGA) – Quickly and smoothly transfers plates and consumables between storage modules, integrated devices and the worktable without interrupting pipetting.

Figure 2: Dynamic Deck with six ANSI/SLAS positions in the depth, equipped with six boxes for disposable tips for the Flexible Channel Arm on the left. Blue colour code for 200µl and yellow colour code for 1000µl disposable tips. Top view of the Dynamic Deck on the right showing nested disposable tips, positions for troughs, active carrier for the MCA 384 and a hotel in the back.

DiscoveRx PathHunter β-Arrestin human and ortholog GPCR

Overview

β -Arrestin platform is offered as ready to go express kits or stable cell lines. PathHunter eXpress β-Arrestin human and ortholog GPCR assay kits are ready-to-use, complete kits that contain everything you need to perform a functional GPCR assay in living cells without the need for cell culture. Each kit includes single use vials of frozen cells which stably express the GPCR of interest, an optimized cell plating reagent and chemiluminescent detection reagents. Simply thaw and plate the pre-validated cells, then challenge them with a compound of interest after 24 to 48 hours. Whether you are measuring one or multiple GPCR responses to compound challenge, the ready-to-assay eXpress format eliminates the need for lengthy, expensive and time-consuming cell culture, making functional testing fast and convenient.

Assays are designed for 96-well plates, and can be run in both-single point concentration mode – allowing fast compound screening – or in a dose-response mode. Kits include enough cells and reagents for 100, 200 or 1,000 individual measurements. However, they can be easily adapted to 384-well assays, as shown here adapted volumes for 384-well formats are listed in Table 1 in the Result section.

Principle of the PathHunter technology

PathHunter β-Arrestin products monitor GPCR activity by detecting the recruitment of β-Arrestin to the activated GPCR using β-galactosidase enzyme fragment complementation (Figure 3). This is independent of G-protein coupling, providing a direct, universal platform for measuring receptor activation.

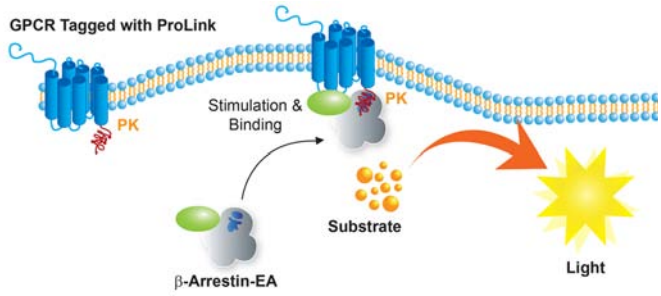


Figure 3: PathHunter β-arrestin assay principle. Activation of the ProLink-tagged GPCR results in β-arrestin recruitment and the formation of a functional β-galactosidase enzyme capable of hydrolyzing the PathHunter Detection Reagent and generating a chemiluminescent signal. (PK Proteinkinase; EA enzyme acceptor)

Automation process

Overview

The assay was designed using two different approaches, illustrating the flexibility of the system. The two versions of the assay are outlined in Figure 4 and 5, including schematic plate layouts and pipetting schemes. Version one uses 224 wells of a 384-well assay plate for a 12-point dose-response plus controls, while version two consists of a 10-point dose curve, employing all 384 wells for the assay.

Processing and analysis of a single plate took 3 hours and 15 minutes (see Figure 5). The assay was also set up to process eight 384-well plates in parallel (2,560 data points) without the need for human intervention, taking 4 hours and 36 minutes to complete the run (see Figure 5).

Detailed workflow

The frozen PathHunter eXpress cells were thawed and prepared for plating following the instructions given in the PathHunter eXpress β-Arrestin human and ortholog GPCR assays user manual. The cells were plated into eight 384-well plates (Greiner-384, white, clear bottom, CELLSTAR-TC) at a density of 2,500 cells per well (20 µl volume) in two different plate layouts. Plating was performed offline, but could also be automated. The plates containing the cells were incubated between 24 and 48 hours at 37 °C and 5 % CO_2. In preparation for the assay, the Fluent cell-based assay workstation was loaded with plates, reagents and tips. The automated process was programmed using the FluentControl™ software; once loading was complete, the system was started using the touchscreen interface.

The first step in the process was to prepare a serial dilution in a 96-well compound plate (U-bottom polypropylene, Greiner Bio-One), using the agonist exendin-4. The dilution was performed by the Flexible Channel Arm using disposable tips (1,000 µl and 200 µl, see pipetting scheme in Figure 4 and 5) to obtain either 10 or 9 different concentrations depending on the assay version.

With the compound plate ready to use, the Robotic Gripper Arm retrieved a cell plate from the incubator, brought it to the workdeck, and removed the lid. The agonist was then dispensed into the assay plate in a two-step process using the Multiple Channel Arm 384 fitted with the 96-channel adapter and 50 µl tips (see Figure 4). 5 µl of the agonist was transferred into each well per dispensing step.

In version one of the assay, the high and low controls were added directly to the assay plate containing the cells, using the Multiple Channel Arm 384 in single column pipetting mode. The lid was then replaced and the cell plate (now referred to as the assay plate) was returned to the CO_2 incubator by the Robotic Gripper Arm, and incubated for 90 minutes at 37 °C, 5 % CO_2. After incubation, the assay plate was retrieved from the CO_2 incubator by the Robotic Gripper Arm, brought to the Dynamic deck, and the lid was removed again. Using the Multiple Channel Arm 384 with the 96-channel adapter, 12 µl of the Detection Reagent working solution was dispensed into each well. With the lid replaced, another incubation took place for 80 minutes at room temperature. To measure the chemiluminescent signal, the Robotic Gripper Arm transferred the plate to the fully integrated Infinite M1000 PRO plate reader below the worktable. This version of the assay used 12 exendin-4 concentrations, but only 224 wells of a 384-well plate, resulting in duplicates for each concentration.

In version two of the assay, all 384 wells were used, but only 10 concentrations of exendin-4 where investigated. As a low control cell plating reagent 2 was used, and 150 nM exendin-4 in CP2 (resulting in a final concentration of 30 nM) was used as the high control. This resulted in quadruplicates of each exendin-4 concentration per plate.

The luminescent signal detection started after 2 minutes adaption time with automatic attenuation and an integration time of 1,000 ms.

The above processes are described for one plate only, but have been executed for eight plates in parallel, as shown in Figure 5, lower part. The time needed to complete the whole assay for all plates was around four and a half hours. The reader data can then be exported in various formats for further processing and analysis.

1st assay version

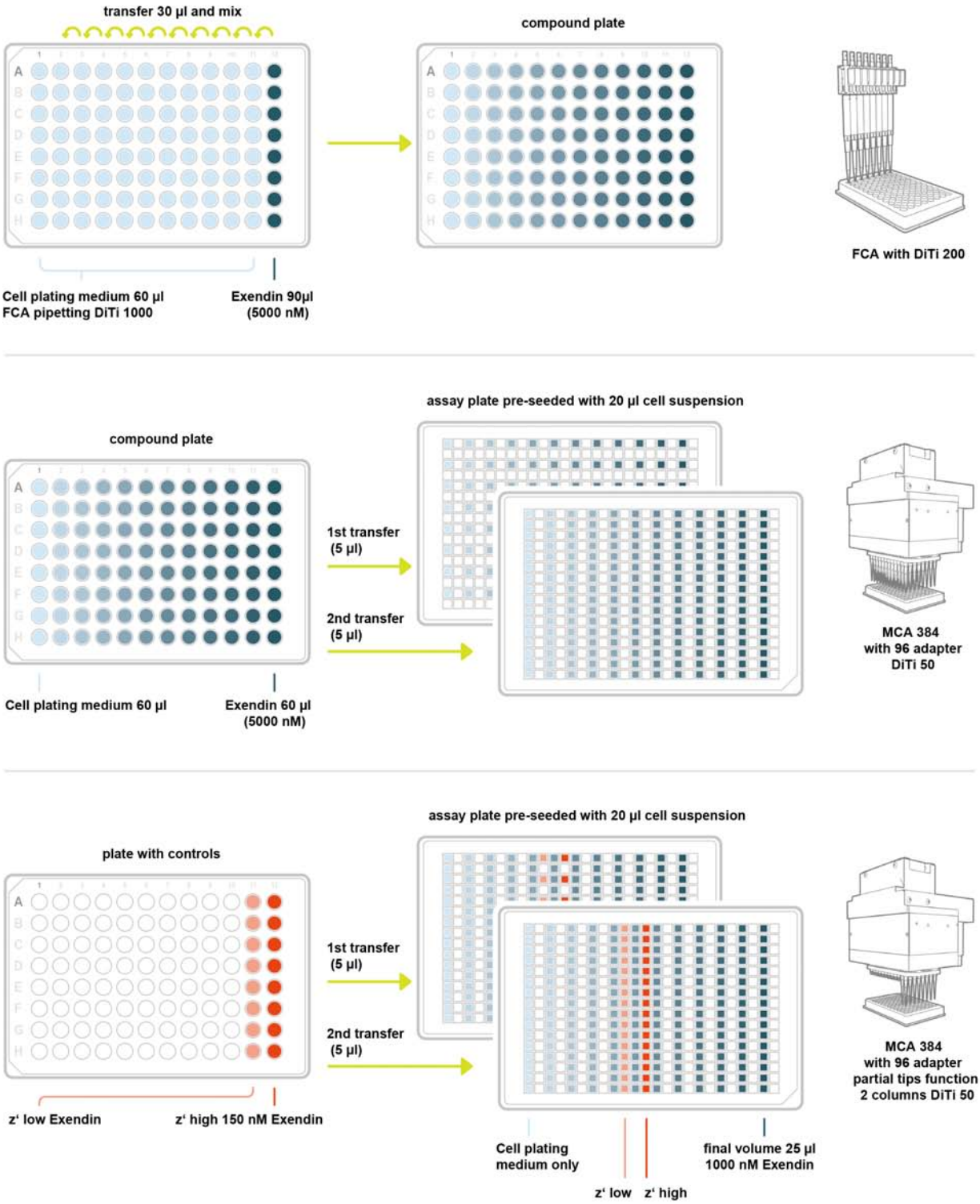


Figure 4: Plate layouts and pipetting schemes for assay version one. The experiment starts with a serial dilution of the agonist to 12 concentrations using the Flexible Channel Arm. The resulting 96-well compound plate is then transferred to the 384-well assay plate by two pipetting steps using the Multiple Channel Arm 384 fitted with the 96-channel adapter (middle). The controls are added from a half deep-well plate using the Multiple Channel Arm 384 in single column pipetting mode (lower part). Out of the 384 wells, only 224 are used, achieving a 12-point dose-response curve in duplicate with controls.

2nd assay version

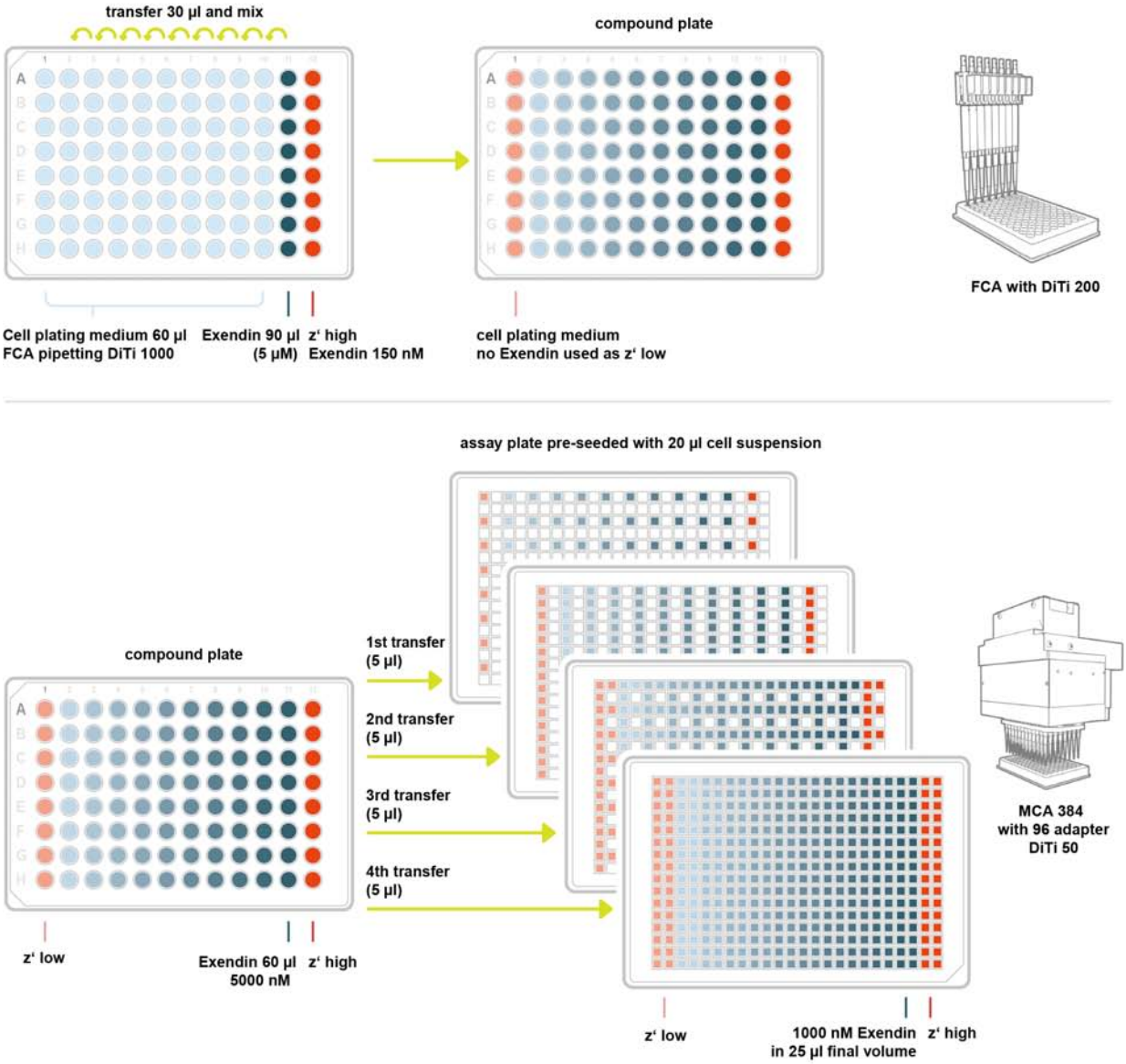


Figure 5: Plate layouts and pipetting schemes for assay version two. Upper part: The experiment starts with a serial dilution of the agonist to 10 concentrations using the Flexible Channel Arm using 200 µl tips. Middle: The resulting 96-well compound plate is then transferred to the 384-well assay plate by four pipetting steps using the Multiple Channel Arm 384 fitted with the 96-channel adapter. The controls in this version of the assay are part of the compound plate, and the assay uses all 384 wells, creating a 10-point dose-response curve in quadruplicate. Timing study shown in the lower part of the figure.

Results

Miniaturization of the assay from 96-well plates, as specified in the protocol, saves significant quantities of cells, assay reagents and compounds. However, the proportion of compound saved is less than for the other reagent savings.

Samples	96-well plate	384-well plate	Volume saved
Cell suspension	100 µl	20 µl	80%
Cell density	10‘000	2‘500	75%
Compound	10 µl	5 µl	50%
Detection reagent	55 µl	12 µl	78%

Table 1: Volumes per well for assay in 96- and 384-well format respectively.

The different assay versions tested show comparable results, proving the robustness and reliability of the automation technology. The experimental EC_{50} for Exendin-4 of 8-10 nM (Figures 6 and 7) is only slightly above the reported EC_{50} from DiscoveRx, and z’ values ranging from 0.49 to 0.69 for the various experiments verify the excellent performance of the automated assay (Table 2).

Samples	z’ Plate 2	z’ Plate 4	z’ Plate 8
Experiment 1	0.63	0.69	0.69
Experiment 2	0.55	0.49	0.69

Table 2: z’ values for the individual plates.
Low control was 0 nM exendin-4 and high control was 80 nM exendin-4.

Results of dose response experiment

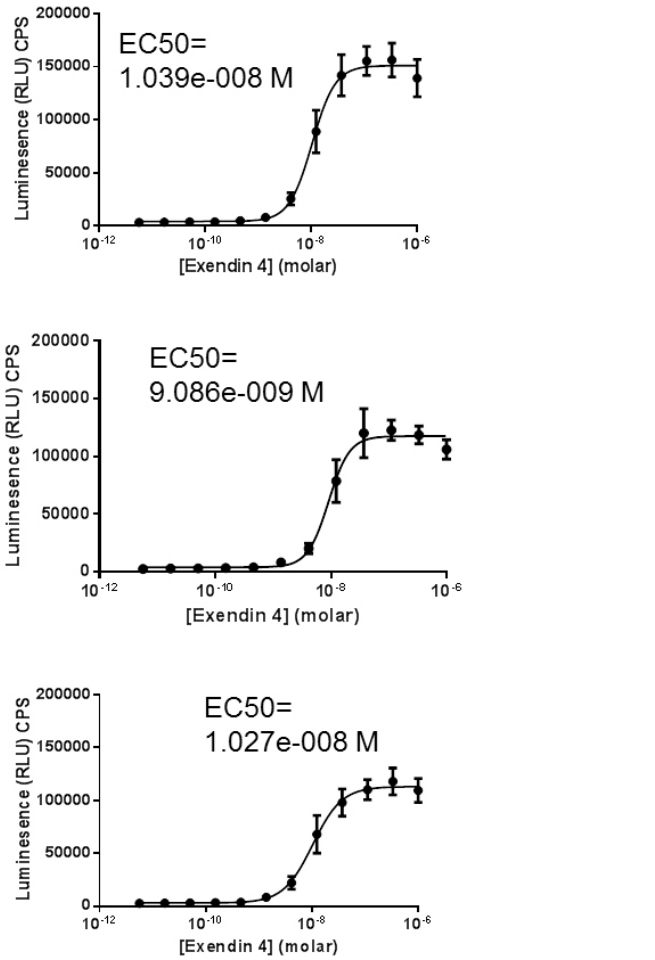


Figure 6: Dose-response curves for version one of the assay, where only 224 wells of the 384-well plate were used. The three graphs represent plates 2, 4 and 8 of a run of 8 plates processed in parallel.

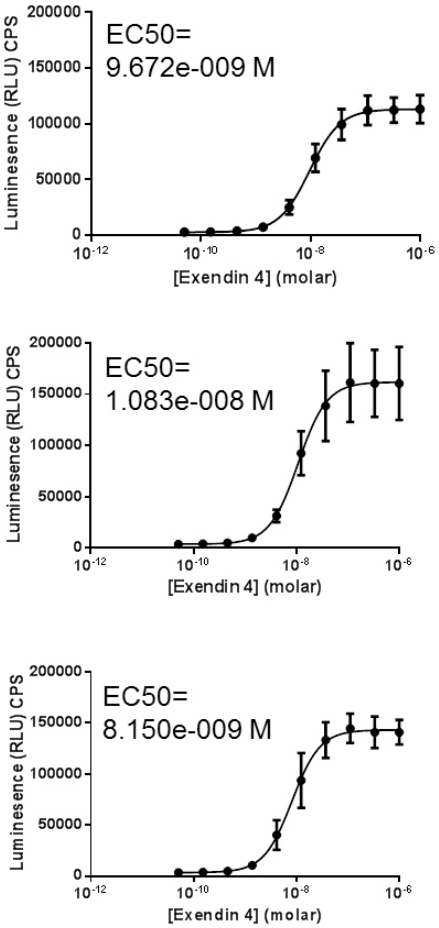


Figure 7: Dose-response curves for version two of the assay, where all 384 wells of the plate were used. The three graphs represent plates 2, 4 and 8 of a run of 8 plates processed in parallel.

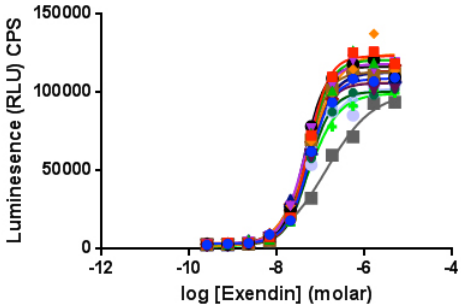


Figure 8: Exemple data from a single plate showing all 16 dose response curve replicates.

Summary

This application note describes the suitability of the DiscoveRx PathHunter β-Arrestin human and ortholog GPCR assay kits for automation in 384-well plate format with liquid handling and detection integrated in a single instrument, the Tecan Fluent laboratory automation solution.

The protocols and data show that the combination of the assay kit and the Fluent workstation offers a complete solution for, with 2,560 data points being in less than five hours. The results for exendin-4 agonist stimulation in all plate layouts and experimental settings closely follow the benchmark EC_{50} value of 6.8 nM.

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