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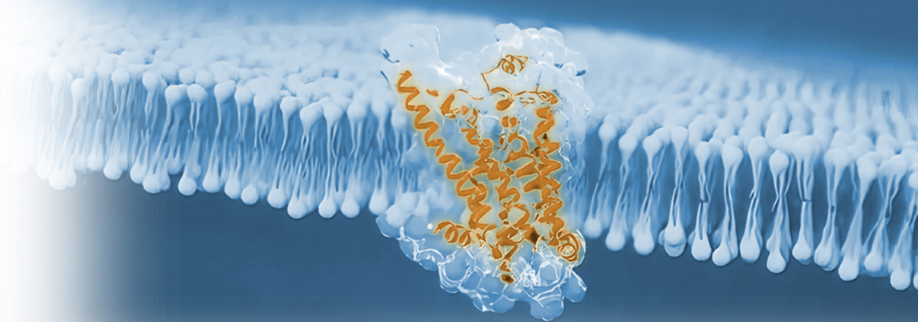

APPLICATION NOTE

Automating Cell-Based Bioassays on Tecan®'s Fluent Automation Workstation: Measuring β-Arrestin Recruitment of Cannabinoid Receptor 1

Bringing Higher Throughput, Improved Assay Consistency, and Reproducibility for Easy Implementation in Quality Testing Programs

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INTRODUCTION

Through early discovery, screening, characterization, and particularly for market release and commercialization of biologics, drug candidates require physiologically relevant, functional cell-based assays as characterization tools that attest to their therapeutic activity. This biological activity is a critical attribute for drug candidates, and can be accurately measured using cell-based potency assays. Moreover, analyzing potency is a key measurement for the final market release of a therapeutic product such as a biologic or a biosimilar.

Cell-based assays implemented in quality controlled (QC) lot-release testing programs of biologics are often indicators of any altered potency or the presence of impurities in the manufactured drug products intended for clinical use. Hence, such programs demand highly consistent and reproducible cell-based assays. There are specific requirements associated with implementing cell-based potency assays, including cell culture and handling expertise, skilled pipetting, and precise plate layout. These abilities directly contribute to an operator's ability to handle live cells and routinely run sensitive assays with a high degree of precision and accuracy. The operator is expected to execute the assay consistently and uniformly across experiments performed on different plates and across different days. QC testing typically includes multiple operators adding another variable in attaining consistent results. Thus, it can be challenging for biopharma, contract research organizations (CROs), and contract development and manufacturing organizations (CDMOs) to achieve desirable assay reproducibility, particularly with borderline assays with less reproducible results. For these reasons, bringing automation to cell-based assays can provide better assay consistency and increased throughput that can result in decreased operational costs. Introducing assay automation can also bring consistent method execution when assays are transferred between different testing sites with automation capabilities.

This Application Note presents collaborative work of Eurofins DiscoverX, the leader in cell-based assays for drug discovery and development, and Tecan, a leader in robotic lab automation. It compiles and discusses the results obtained during the automation of a Eurofins DiscoverX cell-based bioassay on Tecan's Fluent® Automation Workstation. In summary, the automation produced highly consistent and reproducible results with minor optimization. In addition, the results obtained from this automation were comparable to those obtained by an experienced QC scientist.

PathHunter β-ARRESTIN ASSAY

β-Arrestin recruitment is considered a hallmark mechanism for GPCR activation upon ligand binding. The recruitment of β-arrestin activates signaling cascades to provide a non-amplified signal ideal for antagonist mode screening, studying ligand pharmacologies, and de-orphanizing GPCRs. Using PathHunter® β-arrestin cell-based assays, researchers can run cAMP or calcium second messenger signaling assays in parallel using the same β-arrestin cell line. The benefit is this allows for easily distinguishing ligand pharmacological differences for multiple ligand mechanism-of-actions.

PathHunter β-arrestin assays utilize the well-established Enzyme Fragment Complementation (EFC) technology (Figure 1. A.) pioneered and patented by Eurofins DiscoverX.

This robust technology is based on the complementation of two inactive β-galactosidase (β-gal) enzyme fragments – a large enzyme acceptor (EA) fragment and a small enzyme donor (ED) fragment. Separately, these fragments are inactive, but they form an active enzyme that hydrolyzes a substrate to produce a chemiluminescence signal when combined. For this study, we selected the PathHunter® CHO-K1 CNR1 Bioassay that measures β-arrestin recruitment (Figure 1. B.) to demonstrate the automation of the Eurofins DiscoverX bioassays on the Tecan Fluent® instrument. CNR1, a type 1 cannabinoid receptor (CB1), encoded by the CNR1 gene, is expressed in the peripheral and central nervous systems. Endocannabinoids and phytocannabinoids such as THC, an active ingredient of cannabis, are known to activate CB1. In this bioassay, CNR1 is fused in frame with an ED fragment called ProLink™ (PK). The CNR1-PK fusion protein is then stably co-expressed in cells stably expressing a fusion protein of β-arrestin and EA. Binding of a ligand (CP 55,940, a synthetic cannabinoid) to CNR1 results in its activation stimulates recruitment of β-arrestin-EA fusion protein to CNR1-PK. This recruitment forces the complementation of the two enzyme fragments that results in the formation of an active β-galactosidase enzyme. This interaction leads to an increase in enzyme activity that can be measured using chemiluminescent PathHunter detection reagents.

For this study, the bioassay format of the assay was performed. Eurofins DiscoverX bioassays are derived from qualified stable cell lines and provide a thaw-and-use approach that alleviates any assay variability associated with cells derived from continuous culture (passage-to-passage variability). Bioassays are further optimized with a ready-to-use protocol where cells are thawed and plated directly into assay plates, thus not requiring cell culture. These bioassays are ideal for comparability studies, QC lot release testing, and are critical for accelerating the drug release into the market.

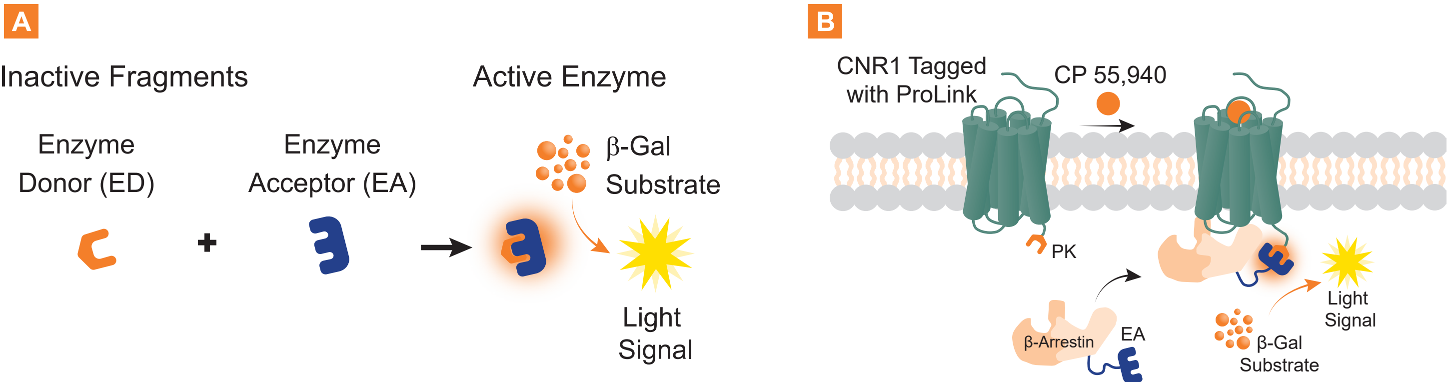


Figure 1. Eurofins DiscoverX's EFC assays. A. The EFC assay principle is based on two recombinant β-galactosidase (β-gal) enzyme fragments - an enzyme acceptor (EA) and an enzyme donor (ED). Separately, the fragments are inactive, but when combined, they form an active enzyme that hydrolyzes its substrate to produce a chemiluminescence signal. B. The PathHunter CNR1 β-arrestin recruitment assay principle: Binding of CP 55,940 results in CNR1 activation thereby stimulating recruitment of β-arrestin-EA fusion protein to CNR1-PK (noting PK corresponds to the ED of β-gal). The resulting complementation of the two enzyme fragments results in an active β-gal enzyme whose activity can be measured using chemiluminescent PathHunter detection reagents.

MATERIALS

Assay Components

PathHunter® CHO-K1 CNR1 Bioassay Kit (Cat. No. 93-0959Y2-00120), containing ready-to-use PathHunter CHO-K1 CNR1 bioassay cells, 96-well assay plate, AssayComplete™ Cell Plating 2 Reagent (CP2), protein dilution buffer, reference agonist, and detection reagent, was used from Eurofins DiscoverX.

INSTRUMENTATION

Automation Workstation

Experiments were conducted on a Tecan Fluent® 780 system that includes an eight-channel Air Flexible Channel Arm™ (Air FCA), a Multiple Channel Arm™ (MCA) with an extended volume adapter for pipetting up to 500 µl with 96 tips in parallel, and a long Robotic Gripper Arm™ (RGA) to reach below the Dynamic Deck™ (Figure 2. A.). A vertical laminar flow HEPA hood with UV light (Bigneat) was integrated with the Fluent system to ensure a clean environment. Liquid handling tasks were conducted with the Air FCA using 1000 µL and 200 µL filtered disposable tips, and with the MCA used150 µL filtered disposable tips. Sterile 15 mL Falcon™ tubes and 2 mL Eppendorf tubes were placed into tube runners for pipetting into sterile 96-well white, clear flat-bottom, TC-treated microplates with lid, and 96-well green V-bottom microplates (Eurofins DiscoverX, Cat. No. 92-0011). A Cytomat™ 2 C450-LiN Automated Incubator with two stackers (for 21 and 10 microplates, respectively) was integrated with the Fluent system; enabling the storage and incubation of CHO-K1 cells in microplates during the 48-hour incubation period with the agonist CP 55,940 (Figure 2. B.). The automated incubator parameters were set at 37°C and 5% CO_2 to provide an ideal and stable environment for the cryopreserved ready-to-use cells.

Individual scripts were developed using the FluentControl™ software for cell seeding on Day 1 and sample preparation of the agonist's serial dilutions with the subsequent addition of the detection reagents on Day 3. The chemiluminescence signal was measured with Tecan's Spark® multimode reader.

A

B

Figure 2. Tecan's Fluent Automation system. A. The Fluent Automation Workstation used to automate the PathHunter CHO-K1 CNR1 bioassay, and B. the Cytomat 2 C450-LiN Automated Incubator that was integrated with the Fluent system.

METHODS

Automation and manual experiments were performed independently. The protocol for experiments run on the Fluent® automation system is outlined in the protocol schematic (Figure 3.). For manually performed experiments, the protocol in the bioassay user manual was followed.

Cell Culture

The PathHunter® CHO-K1 CNR1 Bioassay cells were manually thawed and plated in AssayComplete™ Cell Plating 2 Reagent (CP2) at 5,000 cells-per-well in a 96-well plate and incubated overnight at 37°C and 5% CO_2.

Agonist

A 10 mM stock of CP 55,940 was prepared in DMSO as detailed in the user manual. An intermediate stock of 100 µM CP 55,940 was prepared in DMSO. Using this intermediate stock, an 11-point 1:3 dilution series of CP 55,940 was prepared with a top dose of 5 µM at 5X using CP2 as the diluent. 20 µL of the above serial dilutions were subsequently added to cells plated in 80 µL CP2 to obtain a final dose of 1 µM of CP 55,940 at 1X.

PathHunter β-Arrestin Assay

PathHunter CHO-K1 CNR1 bioassay cells were treated with increasing concentrations of CP 55,940 for 120 minutes at 37°C and 5% CO_2. Activation of a single GPCR stimulates the binding of arrestin, forcing complementation of the two enzyme fragments (ED and EA) fused with the GPCR or β-arrestin, respectively. The resultant increase in β-gal enzyme activity is measured by the addition of the chemiluminescent PathHunter Bioassay Detection Kit (Cat. No. 93-0933). Increased complementation of the two β-gal fusion subunits is directly proportional to higher chemiluminescent signal.

PROTOCOL SCHEMATIC

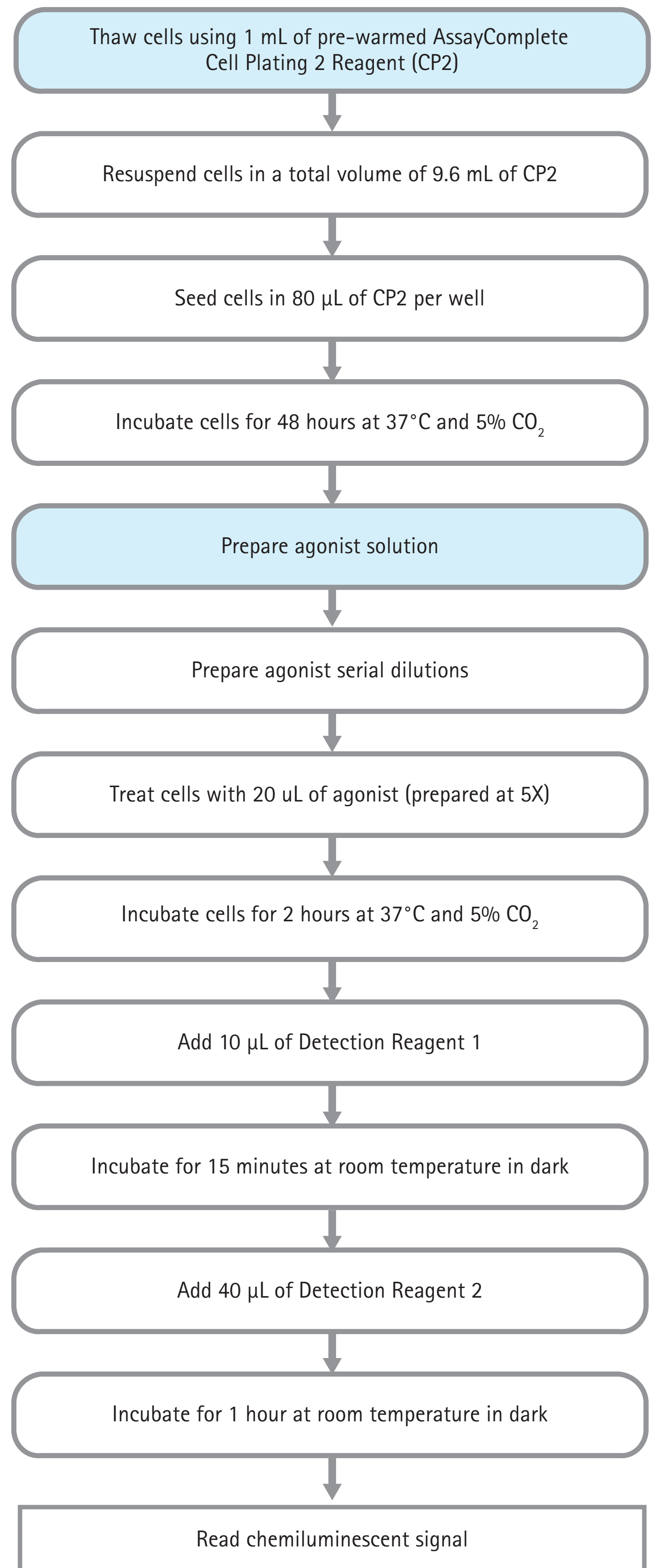


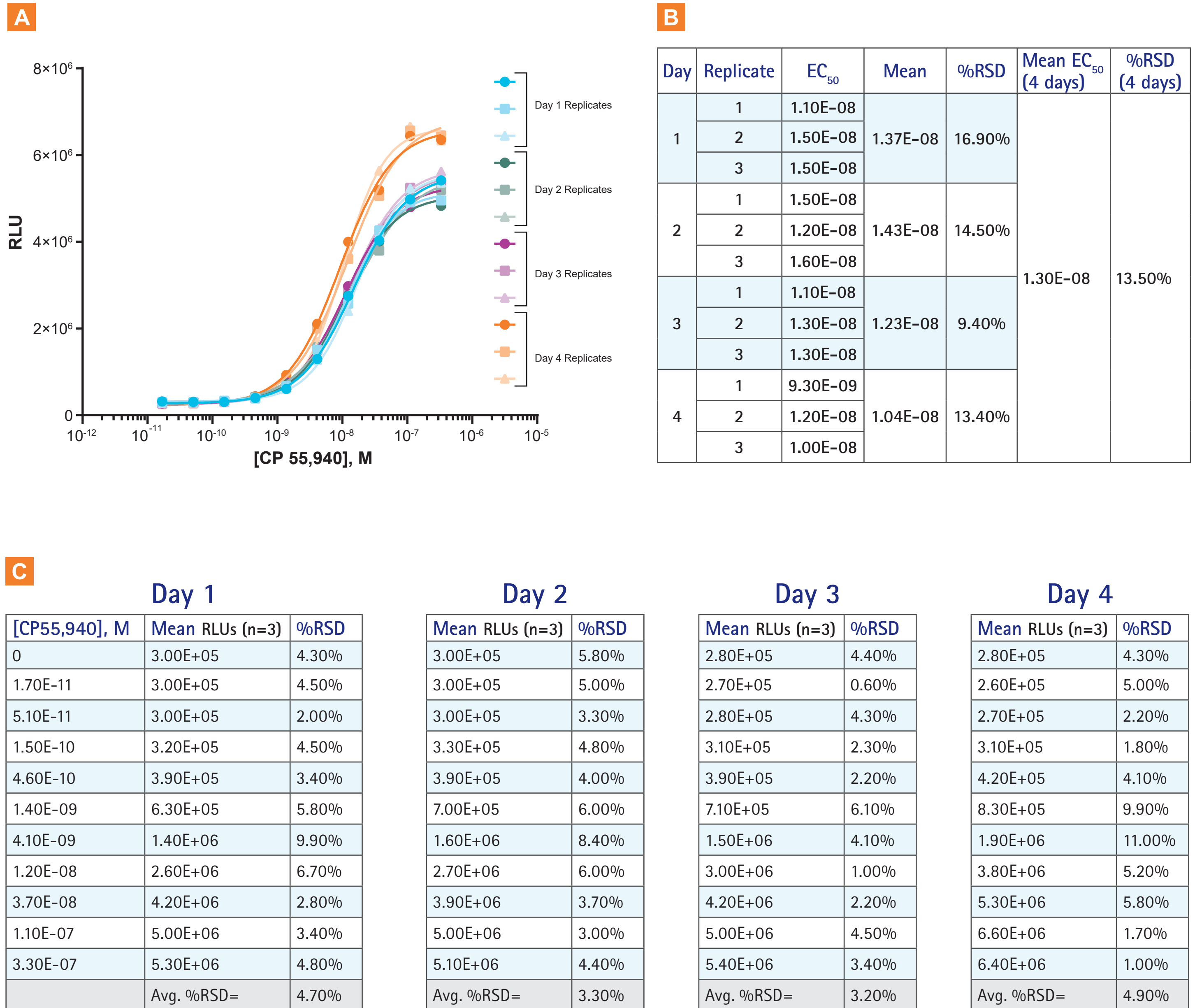
Figure 3. Protocol schematic for PathHunter CHO-K1 CNR1 Bioassay performed on Tecan's Fluent 780 System – steps highlighted in blue required minimal human intervention.

RESULTS

Automated

Inter-day results show comparable EC_{50} values across four independent experiments performed on four individual days with an average relative standard deviation (%RSD) of 13.5% (Figure 4.). Each experiment was performed in triplicate, and each replicate is plotted as a separate curve as shown below in Figure 4. A. All three curves show a high degree of overlap for every experiment, thereby demonstrating high intra-day intermediate precision.

In order to evaluate inter-day replicate consistency, the raw luminescence data from all four experiments were analyzed. As shown in Figure 4. C., the replicates for each data-point are visibly tight, with an average %RSD of less than 5%. These results demonstrate a high inter-day replicate consistency.



Day	Replicate	EC_{50}	Mean	%RSD	Mean EC_{50} (4 days)	%RSD (4 days)
1	1	1.10E-08	1.37E-08	16.90%	1.30E-08	13.50%
	2	1.50E-08				
	3	1.50E-08				
2	1	1.50E-08	1.43E-08	14.50%		
	2	1.20E-08				
	3	1.60E-08				
3	1	1.10E-08	1.23E-08	9.40%		
	2	1.30E-08				
	3	1.30E-08				
4	1	9.30E-09	1.04E-08	13.40%		
	2	1.20E-08				
	3	1.00E-08				

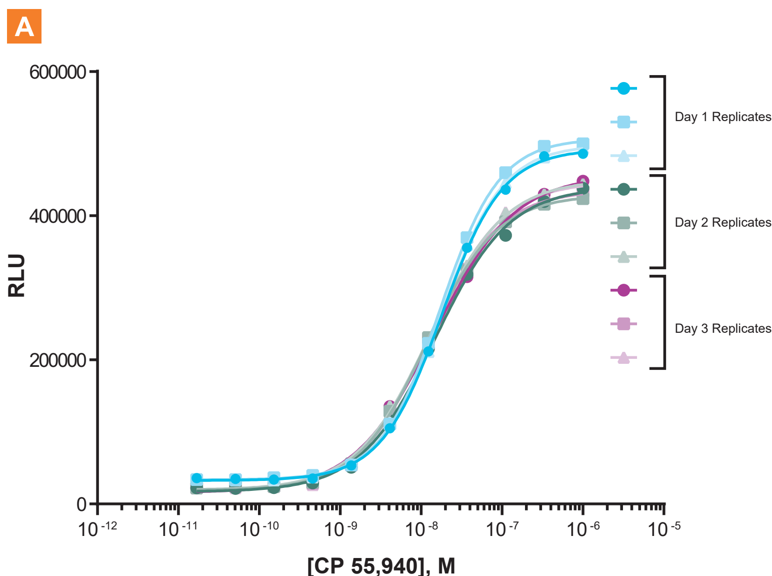
	Day 1		Day 2		Day 3		Day 4	
[CP55,940], M	Mean RLUs (n=3)	%RSD	Mean RLUs (n=3)	%RSD	Mean RLUs (n=3)	%RSD	Mean RLUs (n=3)	%RSD
0	3.00E+05	4.30%	3.00E+05	5.80%	2.80E+05	4.40%	2.80E+05	4.30%
1.70E-11	3.00E+05	4.50%	3.00E+05	5.00%	2.70E+05	0.60%	2.60E+05	5.00%
5.10E-11	3.00E+05	2.00%	3.00E+05	3.30%	2.80E+05	4.30%	2.70E+05	2.20%
1.50E-10	3.20E+05	4.50%	3.30E+05	4.80%	3.10E+05	2.30%	3.10E+05	1.80%
4.60E-10	3.90E+05	3.40%	3.90E+05	4.00%	3.90E+05	2.20%	4.20E+05	4.10%
1.40E-09	6.30E+05	5.80%	7.00E+05	6.00%	7.10E+05	6.10%	8.30E+05	9.90%
4.10E-09	1.40E+06	9.90%	1.60E+06	8.40%	1.50E+06	4.10%	1.90E+06	11.00%
1.20E-08	2.60E+06	6.70%	2.70E+06	6.00%	3.00E+06	1.00%	3.80E+06	5.20%
3.70E-08	4.20E+06	2.80%	3.90E+06	3.70%	4.20E+06	2.20%	5.30E+06	5.80%
1.10E-07	5.00E+06	3.40%	5.00E+06	3.00%	5.00E+06	4.50%	6.60E+06	1.70%
3.30E-07	5.30E+06	4.80%	5.10E+06	4.40%	5.40E+06	3.40%	6.40E+06	1.00%
	Avg. %RSD=	4.70%	Avg. %RSD=	3.30%	Avg. %RSD=	3.20%	Avg. %RSD=	4.90%

Figure 4. Results from the Tecan Fluent® 780 Automation Workstation. A. Dose response curves for CP 55,940 demonstrating β-arrestin recruitment in a dose-dependent manner for four days. B. Table summarizing EC_{50} values and %RSD for each day; and mean EC_{50} and %RSD across four days. C. Table illustrating %RSD of raw RLUs for each day. RSD = Relative Standard Deviation; RLU = Relative Luminescence Units

MANUAL

To compare assay consistency and reproducibility, the same set of experiments were performed manually by a QC scientist with more than six years of experience running similar assays. These experiments were run across three separate days in triplicate. The team plotted data of each replicate per experiment as an individual curve (Figure 5.). Data analysis of the assays performed revealed that the results obtained were very close to the automation results with the %RSD for each day being below 20%. Overall, the inter-day intermediate precision for the manually performed data was 17.5% that is slightly lower than that obtained from the Fluent® system (13.5%).

A



B

Day	Replicate	EC_{50}	Mean EC_{50}	%RSD	Mean EC_{50} (3 days)	%RSD (3 days)
1	1	1.80E-08	1.77E-08	3.30%	1.47E-08	17.50%
	2	1.70E-08				
	3	1.80E-08				
2	1	1.50E-08	1.35E-08	15.70%		
	2	1.20E-08				
	3	1.30E-08				
3	1	1.40E-08	1.30E-08	7.70%		
	2	1.20E-08				
	3	1.30E-08				

Figure 5. Results from manual experiments. A. Dose response curves for CP 55,940 demonstrating β-arrestin recruitment in a dose-dependent manner for three days. B. Table summarizing EC_{50} values and %RSD for each day; and mean EC_{50} and %RSD across three days.

The assay design of the CNR1 bioassay used in this study was automation-friendly and performed optimally out-of-the-box with minimal optimization needed for running on Tecan's Fluent system. Overall, analyzing the data obtained from automation and manually performed experiments show comparable results. Thus, assay automation can help ease congested QC testing pipelines that often result in delayed market release of critical drugs by sharing the workload without compromising the quality of results.

CONCLUSION

The work described in this Application Note demonstrates that automation of Eurofins DiscoverX's cell-based assays can consistently produce reproducible results, which is key for cell-based potency assays implemented in QC lot-release programs. The results from the assays run using automation were remarkably similar compared to the manually executed assays run by an experienced QC scientist with several years of experience. Automating cell-based potency assays in quality testing programs can play a significant role in streamlining assay workflows, maintaining assay consistency, and ultimately saving considerable time and cost by dramatically reducing the handling of repetitive tasks and increasing assay throughput. This automation is particularly helpful when operators are running several assay plates with complex plate layouts. Overall, automating assay is key in circumventing challenges and minimizing day-to-day and operator-to-operator differences producing high-quality data.

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