Implementation of Cisbio's HTRF® M1 Muscarinic Receptor Related Assays on CyBio Liquid Handling Solutions for Small and High Throughput

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Introduction

As a reference screening technology, HTRF® is commonly used by drug discovery researchers during high throughput screening operations. Homogeneous by nature, all assays can be miniaturized to high density plate formats and therefore require precise liquid handling. Besides this, HTRF "mix & read" assays are increasingly run under medium or lower throughput as an alternative to other technologies, bringing more flexibility, robustness and easiness in daily lab practice.

Acetylcholine receptors, also known as Muscarinic receptors, are classified into 5 different groups, M1, M2, M3, M4 and M5. In order to investigate the pharmacological behavior of uscarinic antagonists or agonists, Cisbio has developed a comprehensive platform including ligand binding assays for determining the affinity for the receptors, and functional assays (cAMP, IP-One and phospho-Erk) to assess compounds' efficacy.

For the purpose of the present study, Muscarinic M1 receptor, a key target in pulmonary diseases (asthma and COPD), metabolic, cardiovascular and CNS pathologies, was selected and assessed for both binding (Tag-lite®) and function studies (IP-One) with different CyBio liquid handling devices for small and high throughput. Both HTRF assays were performed with the semi-automatic pipettor yBi®-SELMA 96/25 µl which is a reliable tool to handle low sample numbers.

Selected pharmacological results were checked against data generated at our completely integrated CyBio robotic workstation for HTS to compare the reliability and reproducibility of both CyBio liquid handling solutions at different automation levels.

Material & methods

M1 receptor was chosen to exemplify the platform's HTS compatibility. The cell line overexpresses the receptor of interest bearing the SNAP-tag®. The availability of this tag allows the direct labeling of the cells for binding assay studies.The in-house cell-line was provided frozen and already labeled with the SNAP Lumi4® Tb substrate. The same cell-line was also provided frozen but non labeled for functional assay (IP-One) detecting inositol monophosphate, a downstream product of the PLC pathway.

The cells were thawed and dispensed with the robotic systems at the optimal density depending on the assay setup providing the best S/B ratio.

The fluorescent ligand used for the binding assay was a Tag-lite muscarinic red antagonist (derived Telenzepin labeled with a red HTRF fluorescent probe). Because M1 is a Gq coupled receptor, HTRF IP-One kit was used to assess the production of inositol monophosphate as a readout of the PLC pathway activation. The assay is based on a competitive immunoassay using a monoclonal cryptate labeled anti-IP1 antibody and D2 labeled IP1.

Serial dilution plate preparation

Compound serial dilution plates were prepared in parallel with the CyBi®-SELMA 96/25 µl or with the CyBio robotic workstation[1] as described below. The CyBi®-SELMA 96/25 µl was equipped with either a 96 tip tray for buffer and reagent transfers or with an 8-channel magazine for serial dilution.

- Transfer of 3 x 20 µl buffer from a 12 column robotic reservoir to the serial dilution plate, Corning 500 µl 96 well v-bottom plate (#P-96-450V-C), column 3 remained empty.
- Manual transfer of 80 µl compound in column 3 of the serial dilution plate
- 9 x 1:4 serial dilution steps with 20 µl transfers and 3 mixing cycles (from the last dilution 20 µl were discarded).
- 3 x 20 µl transfer of buffer or antagonist from an 8 line robotic reservoir to the serial dilution plate according to the plate layout.
- 5 or 7 µl transfer from the serial dilution plate to the Greiner 384sv white assay plate (#784080), in quadruplicates according to the assay description (Fig. 1).

Assay protocol

According to the assay description, the different reagents were dispensed as follows:

- The cells were aspirated from a reservoir with 96-bottom profile and homogenized by repeated resuspension prior the transfer in the assay plate.
- The red antagonist or the IP1-D2 was transferred from a 12 column robotic reservoir. In the first column the red antagonist or IP-D2 was replaced by the corresponding buffer (negative control).
- The anti-IP1 cryptate was transferred from a 96 reservoir.

	Binding assay	Functional assay
	Tag-lite	IP-One
Stimulation	5 µl compound from 96-w serial dilution plate	7 µl compound from 96-w serial dilution plate
	10 µl Tag-lite pre-labeled cells	7 µl cells
		Incubation 45 min at 37°C
Detection	5 µl red ligand at fixed concentrations (kd)	3 µl IP1-D2
		3 µl anti IP1-Cryptate
	1h00 incubation at room temperature	*1h00 incubation at room temperature*
	Readout on PHERAstar FS	Readout on PHERAstar FS

Fig. 1: Detailed assay parameters

Liquid handling

The CyBi®-SELMA is a semi-automatic pipettor for precise and reliable liquid handling in microplates (Fig. 2). It operates via a touch screen where all liquid handling parameters and heights can be adjusted and methods can be stored. The pipetting technology of the CyBi®-SELMA 96/25 µl head is exactly the same as in the CyBi®-Well vario 96/25 µl head, which is integrated in the CyBio robotic workstation (Fig. 3). Serial dilutions can be performed with the CyBi®-SELMA 96/25 µl using the corresponding 8-channel magazine. The microplate adapter 384 supports the reliable liquid transfer in 384 well plates.

Fig. 2: CyBi®-SELMA 96/25 µl (CyBi®-SELMA) with 96-tip tray in the parallel transfer mode

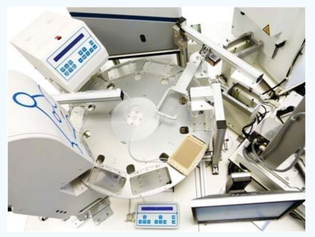

Fig. 3: CyBio robotic workstation (CyBio RWS)

Binding assay results

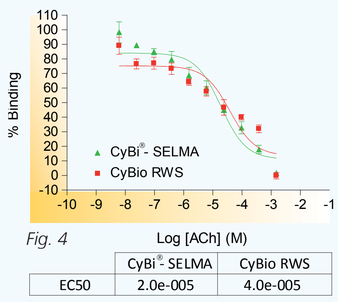

Fig. 4

	CyBi® SELMA	CyBio RWS
EC50	2.0e-005	4.0e-005

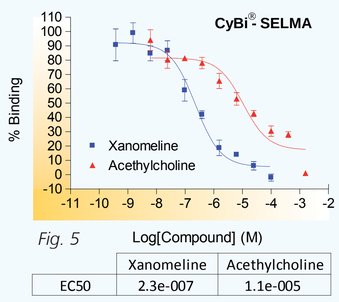

Fig. 5

	Xanomeline	Acethylcholine
EC50	2.3e-007	1.1e-005

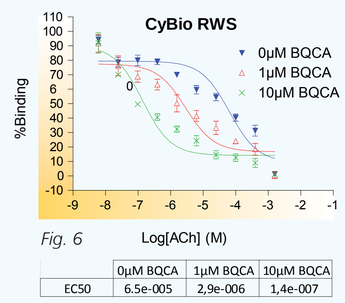

Fig. 6

	0µM BQCA	1µM BQCA	10µM BQCA
EC50	6.5e-005	2,9e-006	1,4e-007

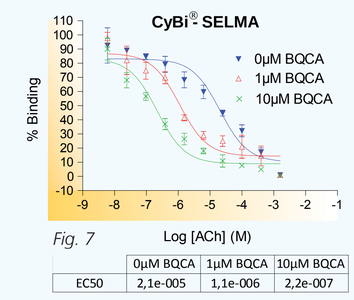

Fig. 7

	0µM BQCA	1µM BQCA	10µM BQCA
EC50	2,1e-005	1,1e-006	2,2e-007

Fig. 4: The graph compares the binding of Ach on the M1 receptor obtained using the CyBio RWS (red) or the CyBi®-SELMA 96/25 µl (green). Ach competes with Red-labeled Telenzepin (10 nM). The EC50 is around 30 µM for both systems. Very similar results were obtained on both platforms in terms of CVs and EC_{50}.

Fig. 5: Xanomeline (blue), a well known partial agonist was assayed in our Tag-lite binding assay using CyBi®-SELMA (data shown) and CyBio RWS (data not shown) in parallel with Ach (red). The EC_{50} are well correlated with literature.

Fig. 6 & Fig. 7: These two graphs show the effect of the Positive Allosteric Modulator (PAM) BQCA on acetylcholine (Ach). The assays were performed using the CyBio RWS (see Fig. 6) and the CyBi®-SELMA 96/25 µl (see Fig. 7). In both cases, Ach competes with Red-labeled Telenzepine (10 nM) in presence of 3 different concentrations of the PAM. Both robotic systems are able to provide very similar results in terms of CVs and EC_{50} and the affinity shift induced by the PAM can be clearly shown. The effect of BQCA is well correlated with literature[2].

Functional assay results

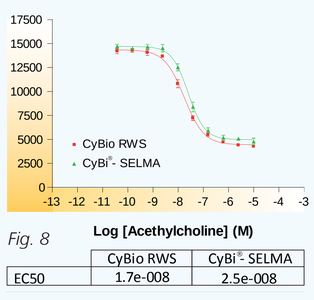

Fig. 8

	CyBio RWS	CyBi® SELMA
EC50	1.7e-008	2.5e-008

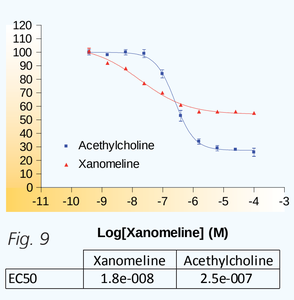

Fig. 9

	Xanomeline	Acethylcholine
EC50	1.8e-008	2.5e-007

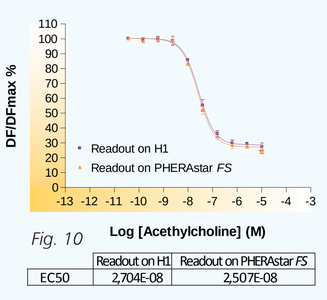

Fig. 10

	Readout on H1	Readout on PHERAstar FS
EC50	2,704E-08	2,507E-08

Fig. 8: The graph compares the dose response curves of Ach obtained on the two robotic systems (CyBi®-SELMA in green and CyBio RWS in red) using HTRF IP-One as readout. There is a perfect similarity between the two curves.

Fig. 9: The graph shows dose response curves made with CyBi®-SELMA of Xanomeline and Ach using the HTRF IP-One assay as a functional readout for receptor activation. While the full agonist Ach shows the same effect as in the binding assay, Xanomeline exhibits its well known partial agonist activity in this functional assay of the M1 receptor. This is in accordance with the literature [3].

Fig. 10: The graph shows the comparison of an IP-One assay made with CyBi®-SELMA and measured with two different readers, PHERAstar FS from BMG LABTECH and Synergy H1 from BioTek Instruments. Both readers provide comparable results.

Conclusion

The present study illustrates how different M1-related binding and functional HTRF assays were implemented and run successfully using the CyBi®-SELMA 96/25 µl and a completely integrated CyBio robotic workstation. With both liquid handling systems, comparable pharmacological results were achieved. It could be demonstrated that:

- The associations of HTRF assays and CyBio robotic systems are ideally suited for smooth transfer from assay development to screening.
- Cisbio HTRF assays and CyBio liquid handling solutions form a perfect match to offer a straightforward platform for small throughput analysis as well as for drug discovery at the HTS level.

References

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