Application Note

Implementation of Cisbio's HTRF® M1 muscarinic receptor related assays on CyBio liquid handling solutions for small and high throughput

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Abstract
The present study illustrates, how different Cisbio HTRF assays were implemented and run successfully using CyBio's liquid handling solutions for different automation levels. The CyBi®-SELMA is the optimal semi-automated pipettor to handle precisely lower HTRF assay sample numbers thus ideally suited for smooth transfer from assay development to screening. The pharmacological results agreed with thus obtained with a completely integrated CyBio robotic workstation. It could be demonstrated, that Cisbio HTRF assays and CyBio liquid handling form a perfect match to offer a straightforward and flexible automated assay platform for small throughput analysis as well as for drug discovery at the HTS level.

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 such as ELISA, western blot or bead-based assays, thus bringing more flexibility, robustness and easiness in daily lab practice.
Acetylcholine (Ach) receptors, also known as Muscarinic receptors, are classified into 5 different groups, M1, M2, M3, M4 and M5. In order to investigate the pharmacological behaviour of muscarinic 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 CyBi®-SELMA 96/25 µl which is a reliable tool to handle low sample numbers. Selected pharmacological results were checked against data generated at a completely integrated CyBio robotic workstation for HTS to compare the reliability and reproducibility of both CyBio liquid handling solutions at different automation levels.
Materials and 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-IP-One antibody and D2 labeled IP-One.

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 20µl 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 detailed assay description in Fig 1.

Assay description

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<thead>
<tr>
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<th>Binding assay</th>
<th>Functional assay</th>
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<tr>
<td><strong>Tag-lite</strong></td>
<td>5µl compound from 96-w serial dilution plate</td>
<td>7µl compound from 96-w serial dilution plate</td>
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<tr>
<td><strong>Stimulation</strong></td>
<td>10µl Tag-lite pre-labelled cells</td>
<td>7µl cells</td>
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<td><em>Incubation 45min at 37°C</em></td>
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<tr>
<td><strong>Detection</strong></td>
<td>5µl red ligand at fixed concentration (kd)</td>
<td>3µl IP1-D2</td>
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<td><em>1h00 Incubation at room temperature</em></td>
<td>3µl anti IP1-Cryptate</td>
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<td>Readout</td>
<td>Readout</td>
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*Fig. 1: Detailed assay parameters*
Assay protocol

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

- The cells were aspirated from a 96 reservoir 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.

Liquid Handling

The CyBi®-SELMA is a semi-automatic pipettor for precise and reliable liquid handling in microplates (see 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 (1) (see 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.
Results

Binding assay results

Fig. 4: Tag-lite Acetylcholine binding curves obtained with both CyBio liquid handling solutions

In Fig. 4 the binding of Ach on the M1 receptor is compared between both CyBio liquid handling solutions. Ach competes with Red-labeled Telenzepin (10 nM). The EC$_{50}$ is around 30 µM for both systems. Very similar results were obtained on both platforms in terms of CVs and EC$_{50}$.

Fig. 5: Tag-lite Acetylcholine and Xanomeline binding curves obtained with CyBi®-SELMA

In Fig. 5 Xanomeline, a well known partial M1-receptor agonist, was assayed in our Tag-lite binding assay using CyBi®-SELMA (see Fig. 5) and CyBio RWS (data not shown) in parallel with Ach. The EC$_{50}$ are well correlated with literature (4).

Fig. 6: Modification of the Ach binding by the Positive Allosteric Modulator (PAM) BQCA, binding curves were obtained with the CyBio RWS

Fig. 7: Modification of the Ach binding by the Positive Allosteric Modulator (PAM) BQCA, binding curves were obtained with CyBi®-SELMA

Fig 6 & Fig 7 show the effect of the Positive Allosteric Modulator (PAM) BQCA on acetylcholine (Ach). In both cases Ach competes with Red-labeled Telenzepine (10 nM) in presence of 3 different concentrations of the PAM. Both CyBio liquid handling systems are able to provide very similar results in terms of CVs and EC$_{50}$ and show the affinity shift induced by the PAM. The effect of BQCA is well correlated with literature (2).
Functional assay results

Fig. 8: Dose response curves of Ach obtained on both liquid handling systems using functional IP-One assay

Fig 8 demonstrates the perfect similarity between the Ach dose response curves which were obtained on both CyBio liquid handling systems using the functional HTRF IP-one assay. Fig 9 shows CyBi®-SELMA dose response curves of Ach and Xanomeline using IP-One assay as 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. 9: Dose response curves of Ach and Xanomeline using the functional IP-One assay, both binding curves were obtained with CyBi®-SELMA

Fig 10: Comparison of an Ach dose response curve (functional IP-One assay) using PheraSTAR and Synergy H1 for HTRF readout, the binding curve ase obtained with CyBi®-SELMA

Fig 10 shows the comparison of an IP-One assay made with CyBi®-SELMA and measured with two different readers, PHERAstar FS (BMGLABTECH) and Synergy H1 (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 96/25µl 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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