HTRF Ligand Binding Assay for the Chemokine Receptor CXCR4

Wendy Goodrich[1], Gary Prescott[1], Peter Banks[1], François Degorce[2], Nicolas Pierre[3]

[1]BioTek Instruments, Inc., Winooski, Vermont, USA • [2]Cisbio Bioassays, Bagnols-sur-Cèze, France • [3]Cisbio US, Bedford, Massachusetts, USA

Abstract

The chemokine receptor CXCR4 has multiple critical functions in normal physiologies including embryonic development of the cardiovascular, hemopoietic and central nervous systems. It has also been implicated in disease pathologies such as HIV infection, cancer metastasis, leukemia progression and rheumatoid arthritis, and is fueling the search for small molecule CXCR4 antagonists as a means for intervention[1]. Here we demonstrate the suitability of the Tag-lite HTRF technology for CXCR4 ligand binding assays for high throughput screening applications. Automated assay performance and pharmacology will be presented.

Introduction

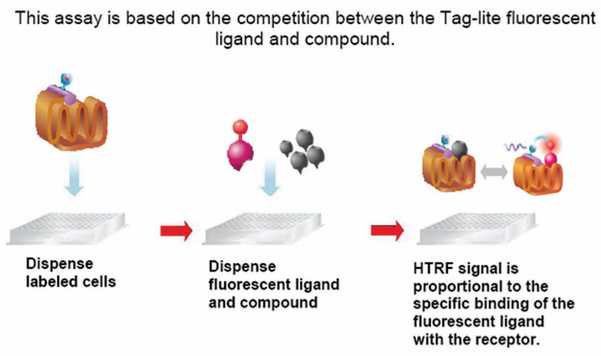
CXCR4 is a member of the G protein coupled receptor (GPCR) super family. Altered regulation of GPCR signaling can influence disease states, so the molecules involved in this process represent attractive therapeutic targets. This makes GPCR investigations among the top priorities in drug discovery research today[2].

Stromal cell-derived factor-1 (SDF1), also known as CXCL12, is a ligand for CXCR4. Multiple signal transduction pathways are activated by this receptor-ligand pair such as those that trigger the migration and recruitment of immune cells[3]. CXCR4 is highly selective to SDF1, and very few if any other agonists for CXCR4 are known. AMD3100, also known as Plerixafor (Genzyme), has been shown to compete with SDF1 acting as an identified antagonist to the CXCR4 signaling pathway. Traditionally, research into ligand binding, such as CXCR4 agonist and antagonist binding, has been done using radioactive methods[4,5].

Using the known properties of this chemokine and other GPCR subfamilies, Cisbio has developed a new 'Tag-lite' technology that provides a cell-surface based assay platform in an easy-to-use, low volume, homogenous, high-throughput capable, non-radioactive, HTRF detection format that preserves both the functionality of the receptor and the intracellular signaling pathway[6].

Here we have validated instrumentation that can provide for both high-throughput screening and pharmacological evaluation of the Tag-lite CXCR4 ligand binding kit. The high-throughput screening and pharmacological evaluation applications both used the peristaltic pump driven low-volume dispense capabilities of BioTek's new MultiFlo™ Microplate Dispenser for automated cell and reagent dispensing. Serial dilutions of both red tracer for Kd determinations and of compound for Ki determinations were done by the BioTek Precision™ XS Microplate Pipetting System. The new BioTek Synergy™ H1 Hybrid Multi-Mode Microplate Reader was used for all HTRF detection.

Cisbio Tag-lite® CXCR4 Receptor Ligand Binding Assay Technology



BioTek Instrumentation

Figure 1 – Precision Automated Pipetting System performed serial dilutions of controls and compounds and loading dilutions to the assay plate.

Figure 2 – MultiFlo Microplate Dispenser technology controlled by Liquid Handling Control™ (LHC™) PC Software equipped with a 1 µL peristalic pump cassette provided automated dispensing of cells and fluorescent labeled ligand.

BioTek Instrumentation (Continued)

Figure 3 – BioTek's newest addition to the Synergy family, the H1 Hybrid Multi-Mode Microplate Reader, was used for HTRF detection.

Materials and Methods

Assay Materials

- CXCR4 receptor binding kit materials including:
  - Tag-lite CXCR4 receptor red agonist (L0012RED)
  - Tag-lite CXCR4 labeled cells (C1TT1CXCR4)
  - Tag-lite buffer (LABMED)
- AMD 3100, antagonist (used in the competition assay, and as the non-specific binding signal referred to as REF1 in the assay protocol)
- SDF1α unlabeled, agonist (used as compound X in the competition assay and Z' validation)
- Greiner BioOne plates Cat.No. 784075 (384-well, small volume, PolyStyrene, medium binding, white)

Automation

- Synergy H1 Hybrid Multi-Mode Microplate Reader equipped with Terbium (Tb) detection based HTRF module:
  - Excitation filter: 340/30
  - Emission filters: 620/10, 665/8
  - Dichroic Mirror: 400nm 1/2
- MultiFlo Microplate Dispenser technology equipped with a 1 µL peristalic pump cassette and controlled by Liquid Handling Control PC Software programmed to:
  - Dispense 10 µL/well of cells from a 5mL starting volume to a 384-well microplate
  - Dispense 5 µL/well of 50nM solution of L0012RED to a 384-well microplate
  - Simultaneously dispense 5 µL/well TLB, 5 µL/well AMD3100, 5 µL/well compound high concentration, and 5 µL/well compound low concentration to 384-well microplate for Z' determination
- Precision XS Microplate Pipetting System equipped with single channel pipettor, 200 µL low adhesion tips (Cat. P/N 98254) and standard clear 96-well microplates for serial dilutions

Method

HTS Simulation: A Z' experiment was designed to validate automated dispensing performance of the MultiFlo technology for cells, controls, compound and tracer. Figure 4 illustrates the workflow. 10 µL/well of cells were dispensed to the plate, then a simultaneous 5 µL/well dispense of TLB, REF1 (antagonist control), X1, and X11 were dispensed, followed by a 5 µL/well dispense of the assay substrate. Results for Z' on the compound are shown by Table 1 (data is not shown for Z' on the blocked and unblocked substrate).

Pharmacology Evaluation: Using instructions provided by the assay protocol for K_d determination, competition assays (K_i, IC_{50}), and order of reagent, control, and compound addition to the plate pharmacology data was generated to validate a fully automated assay procedure outlined in Figure 5. MultiFlo technology was used to automated cell and substrate dispensing for both data sets. Precision XS was used to automate serial dilutions followed by addition to the assay plate of four 12-point curves at 5 µL/well in quadruplicate.

Briefly, an 11-point 1:2 serial dilution for the Kd determination was started from a L0012RED (F1) working solution concentration of 800 nM. AMD3100 was used as a reference ligand and prepared as an 11-point 1:10 serial dilution starting from a working solution concentration of 10 µM(REF1) to validate automated results of the K_i\$IC_{50}$ antagonist determination. REF1 was also used to produce the non-specific signal for the K_d. Unlabeled SDF1α was used as a compound and prepared as an 11-point 1:10 serial dilution starting from a working solution concentration of 1µM (X1) to validate automated results of the K_i\$IC_{50}$ agonist determination. TLB was run as a control on all dilution series as a 12th point. Data was imported to GraphPad Prism® for data analysis. Results are shown by Figures 6, 7 and Table 2.

Materials and Methods (Continued)

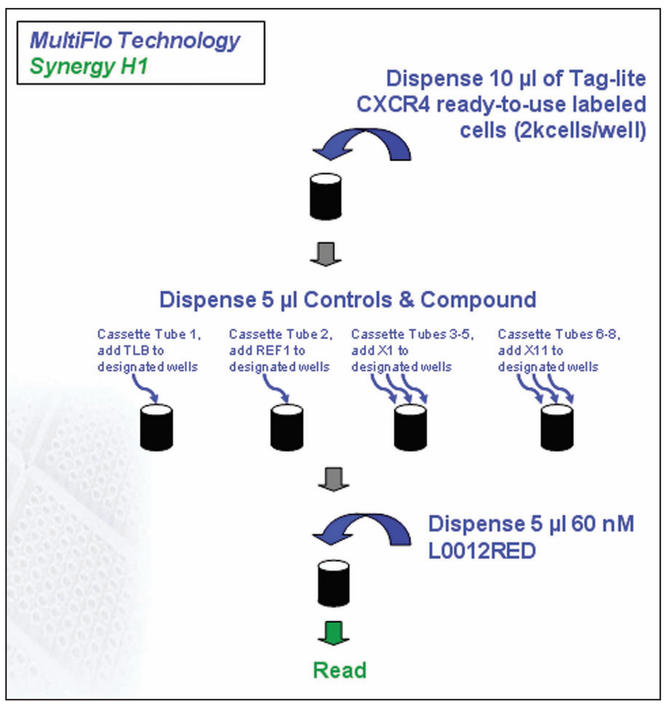


Figure 4 – HTS Simulation Workflow

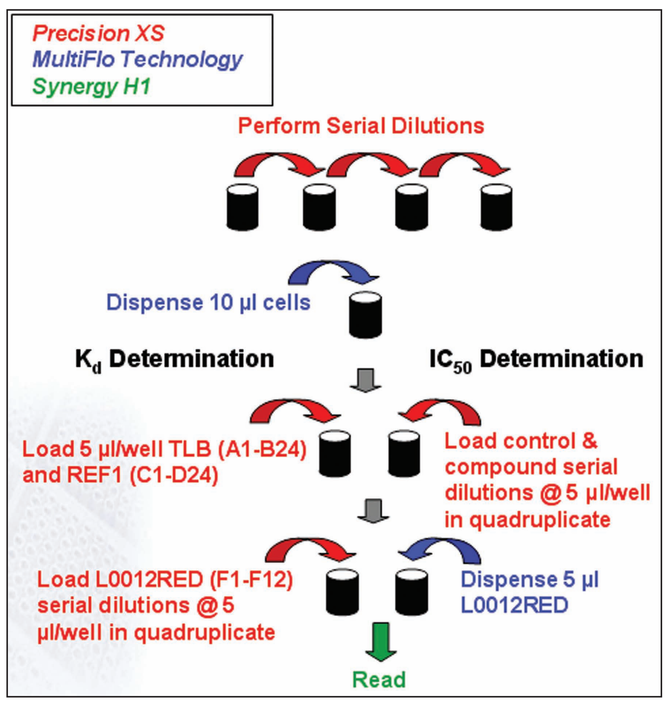


Figure 5 – Pharmacology Evaluation Workflow

Results

Compound	n	Mean	SD	CV%
SDF1-α unlabeled (X1 @ 1 µM)	144	11071	854	7.7
SDF1-α unlabeled (X11 @ 1E-10 µM)	144	1018	48	4.7

Z'	S/B
0.73	10.9

Table 1 – Automated Z' data

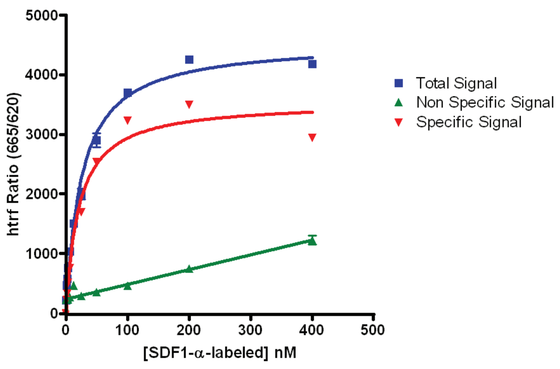


Figure 6 – Automated K_d Determination

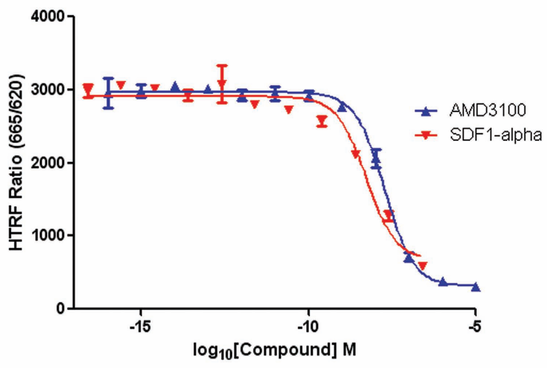


Figure 7 – Automated IC_{50} Determination

	Fully Automated	Manual	L0012RED Product Info*	Published Radioligand [4,5]
K_d (nM)	20.9	19.6	18.7	
K_i (nM)				
AMD3100	11.2	17.8	25	
SDF1-α	22.5	17.9	18	
IC_{50} (nM)*				
AMD 3100	18.6	29.4	34.4	106 (4)
SDF1-α	36.0	29.0	24.2	24 (5)

*Final [L0012RED] = 12.5 nM

Table 2 – Pharmacology Data Comparison

Conclusions

1. MultiFlo Microplate Dispenser technology is suitable for HTS applications with the Tag-lite HTRF assay method.
   - Each MultiFlo unit can be equipped with up to 2 peristaltic and 2 syringe pumps for a wide range of liquid dispensing from the same small footprint.
2. Precision XS Microplate Pipetting System is adept in automating the serial dilutions necessary for conducting dose-response curves to evaluate ligand binding pharmacolgy evaluations.
3. Synergy H1 Hybrid Multi-Mode Microplate Reader provides high performance, cost-effective HTRF detection.

[1] H. Tamamura et al. (2008), "A future perspective on the development of chemokine receptor CXCR4 antagonists," Expert Opinion on Drug Discovery, 3(10), pp. 1155-1166. • [2] http://www.htrf.com/products/gpcr/ • [3] http://www.biocarta.com/pathfiles/h_cxcr4Pathway.asp. • [4] Zhang et al. JBC 2002.
[5] Fricker et al. Biochem Pharm 2006. • [6] http://www.htrf.com/files/content/Tag-Lite-PR.pdf