

## HTRF Ligand Binding Assay for the Chemokine Receptor CXCR4

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### Key Words:

CXCR4  
HTRF  
GPCR  
Microplate Automation  
Cell-based Assay Automation  
Cisbio  
Tag-lite  
Receptor Ligand

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<sup>1</sup>. 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<sup>2</sup>.

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<sup>3</sup>. 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<sup>4,5</sup>.

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<sup>6</sup>.

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 K<sub>d</sub> determinations and of compound for K<sub>i</sub> determinations were done by the BioTek Precision™ Microplate Pipetting System. The new BioTek Synergy™ H1 Hybrid Multi-Mode Microplate Reader was used for all HTRF detection.

### Cisbio Tag-lite Assay Principle

Tag-lite™ is a comprehensive HTRF® cellular platform offered as ready to use kits, or as individual components that allow customized assay development specific to a target of interest including plasmids engineered to express tagged cell lines, cell lines already tagged, fluorescent donor and acceptor reagents, labeling mediums, and some compounds that can be used as controls.

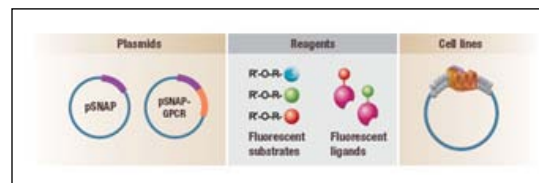
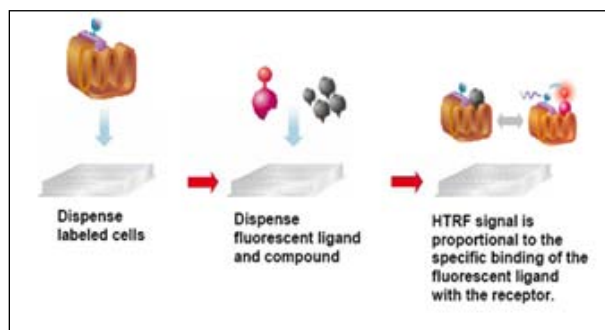


Figure 1. The Tag-lite cellular platform toolbox<sup>7</sup>.

The GPCR CXCR4 was chosen to demonstrate the automation of the Tag-lite technology, and is available as a ready to use receptor-ligand binding kit. The high natural affinity of CXCR4 for its ligand SDF1<sup>∞</sup> allows determination of compounds that might act as inhibitors to this pathway based on the competition between SDF1<sup>∞</sup> and any antagonist. In this case, the lower the HTRF signal detected, the higher the inhibition of the CXCR4 pathway by a competing compound of interest.



**Figure 2.** This assay is based on the competition between the Tag-lite fluorescent ligand (red probe) and compound.

## Materials and Methods

### Materials

#### Assay

- 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)
- SDF1 $\infty$  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 and detection settings as follows:
  - Excitation filter: 340/30
  - Emission filters: 620/10, 665/8
  - Dichroic Mirror: 400 nm
  - Read Method: Time-Resolved Fluorescence with 100 usec delay before collecting data and a data collection time of 300 uses.
  - Plate Definition: Greiner 384 Fluotrac
  - Probe Height: 7.00 mm
  - Auto sensitivity scaled to high well @ scale value of 30,000.
  - HTRF ratio value embedded as transformation formula  $DS2/DS1*10000$ , where DS1 is the 620/10 emission filter set and DS2 is the 665/8 emission filter set. This can be done after export to EXCEL also.
- MultiFlo™ Microplate Dispenser technology equipped with a 1  $\mu$ L peristaltic pump cassette and controlled by Liquid Handling Control™ PC Software programmed to:
  - Dispense 10  $\mu$ L/well of cells from a 5mL starting volume to a 384-well microplate
  - Dispense 5  $\mu$ L/well of 50 nM solution of L0012RED to a 384-well microplate

- Simultaneously dispense 5  $\mu$ L/well TLB, 5  $\mu$ L/well AMD3100, 5  $\mu$ L/well compound high concentration, and 5  $\mu$ L/well compound low concentration to 384-well microplate for Z' determination.

- Precision™ Microplate Pipetting System, 200  $\mu$ L low adhesion tips (Cat. P/N 98254 ) and standard clear 96-well microplates for serial dilutions

### Methods

#### HTS Stimulation

AZ' experiment was designed to validate automated dispensing performance of the MultiFlo technology for cells, controls, compound and tracer. Figure 3 illustrates the plate map and Figure 4 the workflow. 10  $\mu$ L/well of cells were dispensed to the plate, then a simultaneous 5  $\mu$ L/well dispense of TLB (buffer), REF1 (antagonist control), X1 (unlabeled SDF1 $\infty$  at 1  $\mu$ M), and X11 (unlabeled SDF1 $\infty$  at 1x10-10  $\mu$ M) were dispensed, followed by a 5  $\mu$ L/well dispense of the assay substrate.

The workflow shown by Figure 4 provides illustration on how 4 different compounds were dispensed to the plate simultaneously according to the plate map using the peri pump cassette technology of the MultiFlo. The dispense direction of the MultiFlo is across the plate one row at a time. Using a plate map in the LHC software of 1-24 wells of a 384-well plate this translates to each tube on the cassette dispensing to two rows of the plate (e.g. A1- B12 = 24 total wells). Therefore, each of the individual 8 tubes of the cassette were released from the tube holder and placed into a corresponding vial containing one of each of the different compounds. Tube 1 was placed into the first vial containing TLB, Tube 2 was placed into the second vial containing AMD3100, Tubes 3-5 were placed into a third vial containing the high concentration of the unlabelled SDF1 $\infty$ , and Tubes 6-8 were placed into a fourth vial containing the low concentration of the unlabelled SDF1 $\infty$ . This method was devised to optimize speed in dispensing to the plate after cell addition. Although the cells remain fairly stable at RT for a time, it is best to work quickly with the remainder of the assay following cell suspension and dispense to the plate. This assay provided a useful platform to demonstrate this technique using the peri pump cassette tubing and the MultiFlo, but the same principle in any combination can be used in a variety of applications. Resulting data of the HTS simulation is shown by Table 1.

TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB
TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB	TLB
REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1
REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1	REF1
X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1
X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1
X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1
X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1
X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1
X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1
X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11
X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11
X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11
X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11
X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11
X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11

**Figure 3.** HTS Simulation Z' Plate Map.

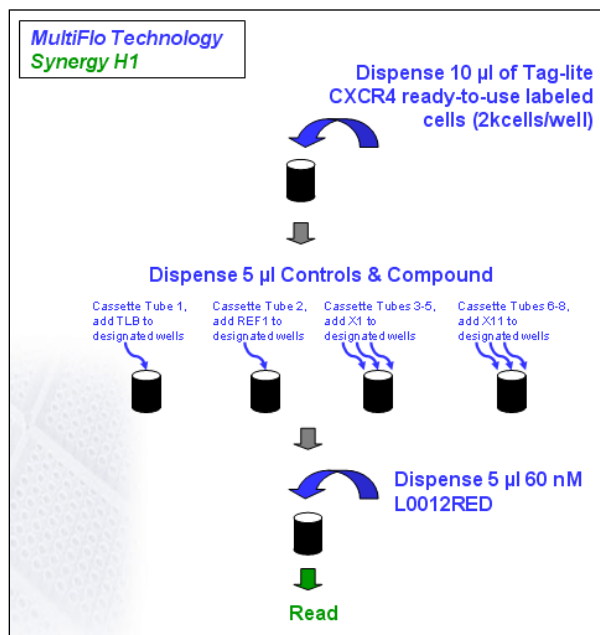


Figure 4. HTS Simulation Workflow

### 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 illustrated by Figures 5 and 6. MultiFlo™ technology was used to automate cell and substrate dispensing for both data sets. Precision™ was used to automate serial dilutions followed by addition to the assay plate of four 12-point curves at 5 µL per well in quadruplicate.

First Distribution			1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
$K_d$	Total Signal	A-D												
	Non Specific Signal	E-H												
$K_i$	Reference	I-L												
	Compound	M-P												
Second Distribution														
$K_d$	Total Signal	A-D												
	Non Specific Signal	E-H												
$K_i$	Reference	I-L												
	Compound	M-P												
Third Distribution														
$K_d$	Total Signal	A-D												
	Non Specific Signal	E-H												
$K_i$	Reference	I-L												
	Compound	M-P												

Figure 5. Assay Distribution for Pharmacology Evaluation .

An 11-point 1:2 serial dilution for the  $K_d$  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 SDF1 $_{\infty}$  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. As a reference for the automated method, manual dispensing and pipetting were done in parallel on another plate. Synergy™ H1 was used for HTRF signal detection for both the manual and automated plates. Resulting data is shown by Figures 7, 8 and Table 2.

### Results

Results of the Z' experiment with corresponding signal precision and signal to background (S/B) data are contained in Table 1. Note that the S/B and Z' values are achieved even without the total absence of inhibition from the unlabelled ligand to allow labeled ligand saturation. That the detection technology is very sensitive to the specific assay signal with little background interference can also be confirmed by low well-to-well variation on all 288 compound wells averaging only 6.2 %CV.

Cisbio uses a ratio calculation from the HTRF optical detection values to obtain 'raw' results for data reduction purposes. This ratio is obtained by dividing the 665 nm emission data by the 620 nm emission data, and then multiplying by  $10^4$  for ease of data processing. A delta ratio between the positive control (total signal) and the background (non-specific signal) is then done to obtain specific signal.

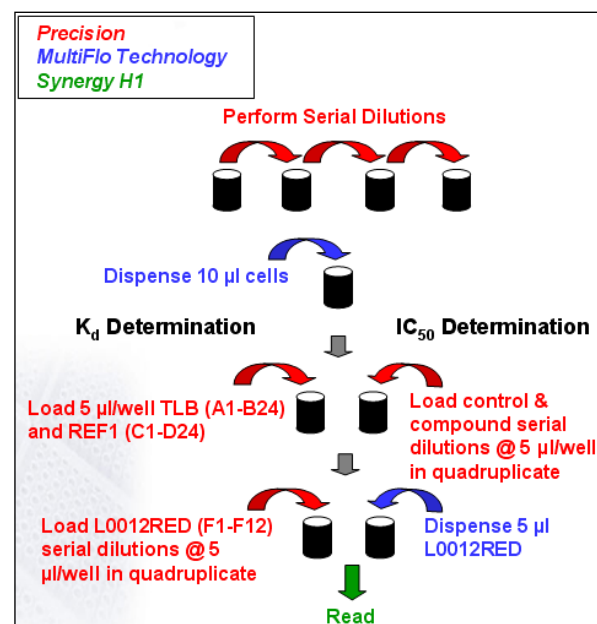


Figure 6. Pharmacology Evaluation Workflow

For  $K_d$  the total signal was a serial dilution of the labeled ligand in the presence of buffer; the non-specific signal shows a serial dilution of the labeled ligand in the presence of a single high concentration of inhibitor such that all receptor binding sites are blocked and binding of the labeled ligand in proximity to the GPCR creating an htrf signal is not associated with receptor binding. The calculated dissociation constant ( $K_d$ ) for both the manual and automated plates showed high correlation with other published data for the assay.

Compound	n	Mean	Stdev	CV%	Z'	S/B
SDF1 $\alpha$ unlabeled (1 $\mu$ M)	144	1018	48	4.7	0.73	10.88
SDF1 $\alpha$ unlabeled (1x10 <sup>-10</sup> $\mu$ M)	144	11071	854	7.7		
Negative Control (AMD3100 10 $\mu$ M)	48	1023	71	6.9	0.7	10.74
Positive Control (Tag-lite Buffer)	48	10991	940	8.6		

Table 1. Automated Assay Performance data

	Fully Automated	Manual	L0912RED Product Info*	Published Radioligand <sup>4,5</sup>
$K_d$ (nM)	20.9	19.6	18.7	
$K_i$ (nM)				
AMD3100	11.2	17.8	25	
SDF1- $\alpha$	22.5	17.9	18	
$IC_{50}$ (nM)*				
AMD 3100	18.6	29.4	34.4	106 (4)
SDF1- $\alpha$	36.0	29.0	24.2	24 (5)

\*Final [L0912RED] = 12.5 nM

Table 2. Pharmacology Data Comparison

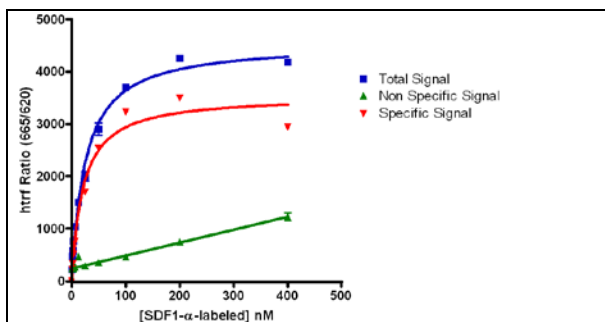


Figure 7. Automated  $K_d$  determination

Figure 8 illustrates data for the  $K_i/IC_{50}$  values shown by Table 2.  $K_{ii}$  is a property of the receptor and unlabeled compound, while  $IC_{50}$  is a property of the experiment. An  $IC_{50}$  can change based on experimental conditions like changing a ligand concentration that will not affect  $K_i$ . Lower  $K_i$  values indicate higher binding affinity, so less concentration of an unlabeled compound is required to compete for binding to the receptor.

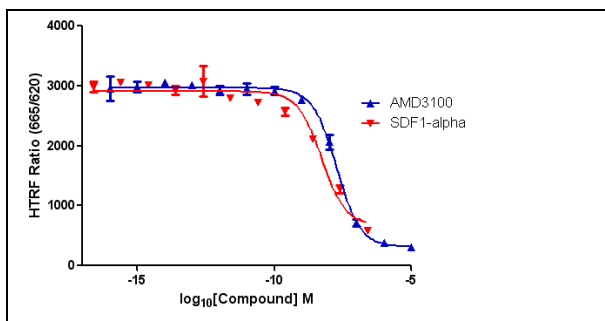


Figure 8. Automated  $IC_{50}$  Determination

The slight upward shift in the  $IC_{50}$  value for the unlabelled SDF1 $\infty$  is due to the experimental condition of starting the serial dilution at 1 nM rather than 10 nM. This was necessitated as a result of having < 10 nM of SDF1 $\infty$  remaining at the end of the experiment. To keep the 1:10 serial dilution series concurrent with the antagonist it was decided to run the compound at 1nM, that then resulted in an additional plateau point at the top of the curve. Even under these conditions the calculated  $IC_{50}$  for the compound is well within a factor of three of the comparative methods and demonstrates high binding affinity at the correlated concentration point of the dilution series.

## Conclusion

The Cisbio Tag-lite Chemokine CXCR4 receptor ligand binding assay is robust and lends itself easily to automation for both pharmacology and screening applications. Good correlation of pharmacological data was generated using BioTek liquid handling instrumentation as compared to previously published methods. The MultiFlo™ Microplate Dispenser technology is suitable for HTS applications with the Tag-lite HTRF assay method, and can be equipped with up to 2 peristaltic and 2 syringe pumps for a wide range of liquid dispensing from the same small footprint. Precision™ Microplate Pipetting System is adept in automating the serial dilutions necessary for conducting dose-response curves to evaluate ligand binding pharmacology evaluations. The Synergy™ H1 Hybrid Multi-Mode Microplate Reader provides high performance, cost-effective HTRF detection.

## References

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