Application Note

Drug Discovery and Development

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HTRF Ligand Binding Assay for the Chemokine Receptor CXCR4

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Abstract

The chemokine receptor CXCR4 has multiple critical functions in normal physiologies including embryonic development of the cardiovascular, hemopoietic, and central nervous systems. CXCR4 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] This application note demonstrates the suitability of the Tag-lite HTRF technology for CXCR4 ligand binding assays for high-throughput screening applications. Automated assay performance and pharmacology are 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 few if any other agonists for CXCR4 are known. AMD3100, also known as Plerixafor (Genzyme, Cambridge, MA), 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 Tag-lite technology that provides a cell-surface based assay platform in an easy-to-use, low volume, homogenous, high-throughput capable, nonradioactive, HTRF detection format that preserves both the functionality of the receptor and the intracellular signaling pathway.[6]

This study 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 the Agilent BioTek MultiFlo multimode dispenser for automated cell and reagent dispensing. Serial dilutions of both red tracer for K_d determinations and of compound for K_i determinations were done by the Agilent BioTek Precision microplate pipetting system. The Agilent BioTek Synergy H1 multimode 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. Targets of interest include 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.

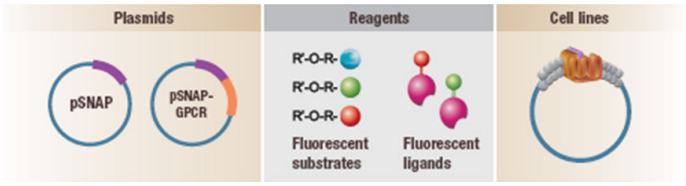

Figure 1. The Tag-lite cellular platform toolbox.[7]

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∞ allows determination of compounds that might act as inhibitors to this pathway based on the competition between SDF1∞ 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.

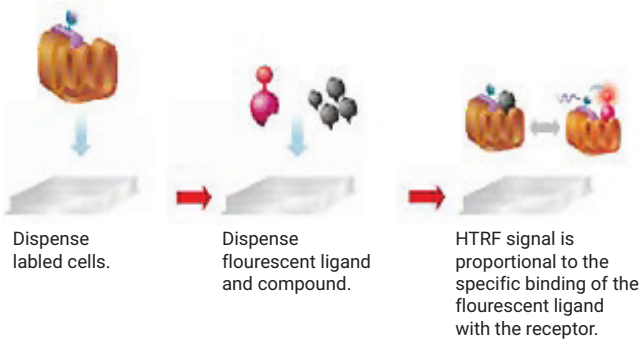

Figure 2. This assay is based on the competition between the Tag-lite fluorescent ligand (red probe) and compound.

Materials and methods

Materials

Assay

- CXCR4 receptor-binding kit materials include:
  - 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 nonspecific binding signal referred to as REF1 in the assay protocol)
- SDF1∞ unlabeled, agonist (used as compound X in the competition assay and Z' validation)
- Greiner BioOne plates (part number 784075) (384-well, small volume, polystyrene, medium binding, white)

Automation

- Agilent BioTek Synergy H1 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 µs delay before collecting data and a data collection time of 300 µs.
  - **Plate definition:** Greiner 384 Fluotrac
  - **Probe height:** 7.00 mm
  - Auto sensitivity scaled to high well at a scale value of 30,000.
  - HTRF ratio value embedded as transformation formula DS2/DS1 × 10,000, 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.
- Agilent BioTek MultiFlo multiplate dispenser microplate technology equipped with a 1 µL peristaltic pump cassette and controlled by Agilent BioTek Liquid Handling Control (LHC) PC software programmed to:
  - Dispense 10 µL/well of cells from a 5 mL starting volume to a 384-well microplate.
  - Dispense 5 µL/well of 50 nM 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.
- Agilent BioTek Precision microplate pipetting system, 200 µL low adhesion tips (part number 98254) and standard clear 96-well microplates for serial dilutions.

Methods

HTS stimulation

A Z' 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. Cells were dispensed to the plate at 10 µL/well, then a simultaneous 5 µL/well dispense of TLB (buffer), REF1 (antagonist control), x1 (unlabeled SDF1α at 1 µM), and x11 (unlabeled SDF1α at 1 × 10^{-10} µM) was done, followed by a 5 µL/well dispense of the assay substrate.

The workflow shown by Figure 4 provides illustration on how four 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 to 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 eight tubes of the cassette was 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 to 5 were placed into a third vial containing the high concentration of the unlabeled SDF1α, and Tubes 6 to 8 were placed into a fourth vial containing the low concentration of the unlabeled SDF1α. This method was devised to optimize speed in dispensing to the plate after cell addition. Although the cells remain fairly stable at room temperature (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 many applications. Resulting data of the HTS simulation are shown in Table 1.

Table 1. Automated assay performance data.

Compound	n	Mean	Stdev	CV%	Z'	S/B
SDF1 α Unlabeled (1 µM)	144	1,018	48	4.7	0.73	10.88
SDF1 α Unlabeled (1 × 10^{-10} µM)	144	11,071	854	7.7	–	
Negative Control (AMD3100 10 µM)	48	1,023	71	6.9	0.7	10.74
Positive Control (Tag-lite Buffer)	48	10,991	940	8.6	–	

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
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
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X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1	X1
X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11	X11
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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.

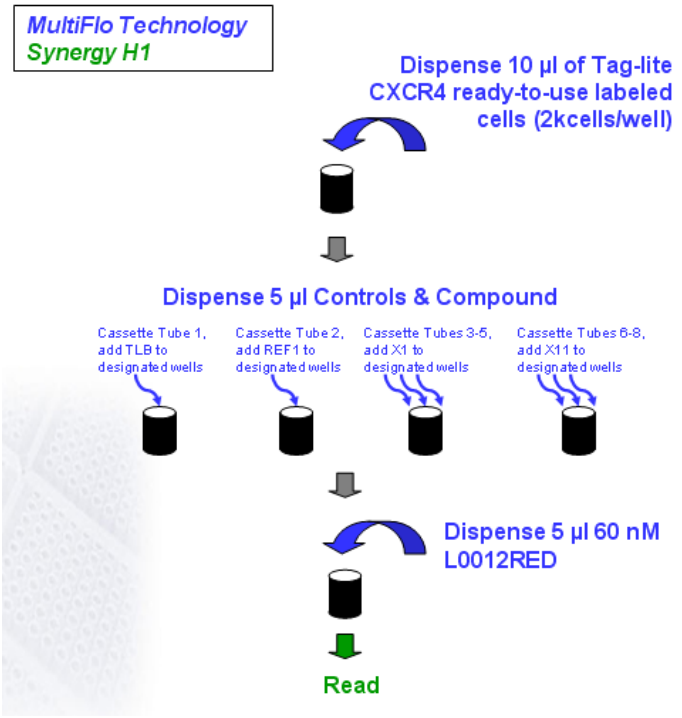
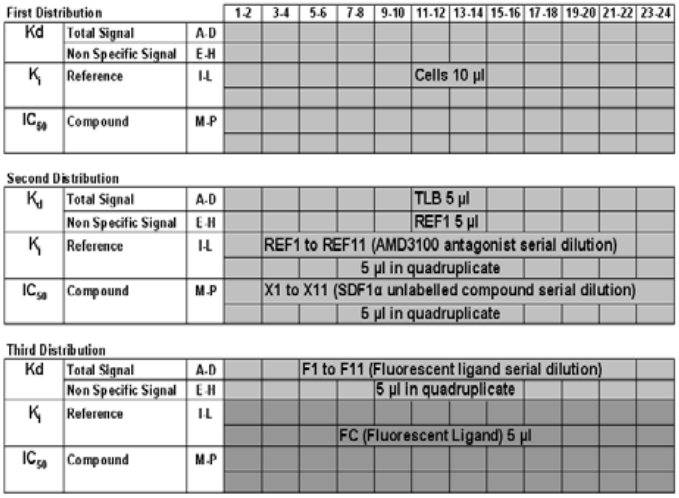


Figure 4. HTS simulation workflow.

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 were 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.

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 nonspecific signal for the K_d.



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
Kd	Total Signal	A-D												
	Non Specific Signal	E-H												
K_i	Reference	I-L						Cells 10 µl						
IC_{50}	Compound	M-P												

Second Distribution

K_d	Total Signal	A-D						TLB 5 µl						
	Non Specific Signal	E-H						REF1 5 µl						
K_i	Reference	I-L		REF1 to REF11 (AMD3100 antagonist serial dilution)										
						5 µl in quadruplicate								
IC_{50}	Compound	M-P		X1 to X11 (SDF1α unlabelled compound serial dilution)										
						5 µl in quadruplicate								

Third Distribution

Kd	Total Signal	A-D			F1 to F11 (Fluorescent ligand serial dilution)									
	Non Specific Signal	E-H				5 µl in quadruplicate								
K_i	Reference	I-L												
						FC (Fluorescent Ligand) 5 µl								
IC_{50}	Compound	M-P												

Figure 5. Assay distribution for pharmacology evaluation.

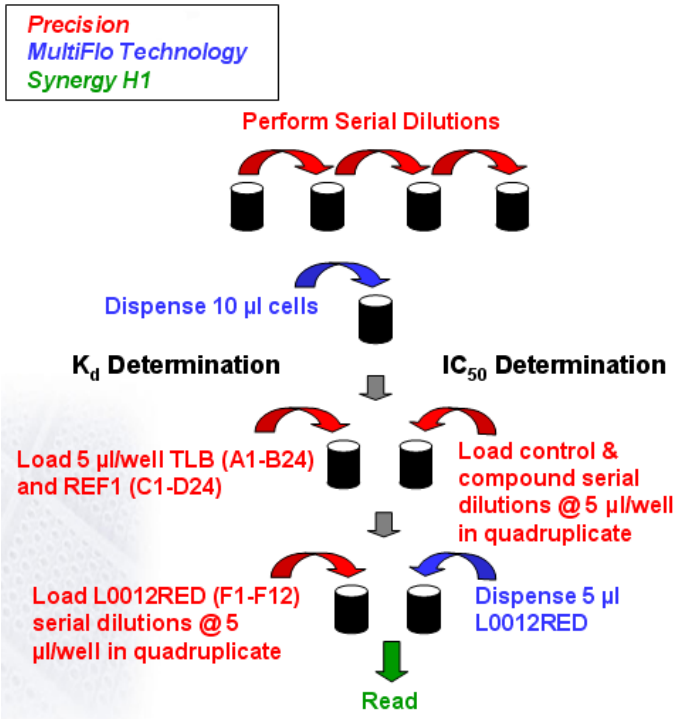


Figure 6. Pharmacology evaluation workflow.

Unlabeled SDF1∞ was used as a compound and prepared as an 11-point 1:10 serial dilution starting from a working solution concentration of 1 µM (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. The resulting data are shown in Figures 7 and 8 and Table 2.

Table 2. Pharmacology data comparison.

	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)*				
AMD3100	18.6	29.4	34.4	106 (4)
SDF1-α	36.0	29.0	24.2	24 (5)

*Final [L0012RED] = 12.5 nM

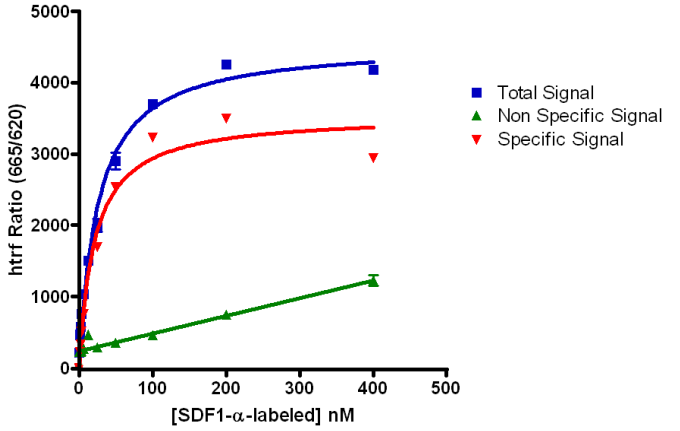


Figure 7. Automated K_d determination.

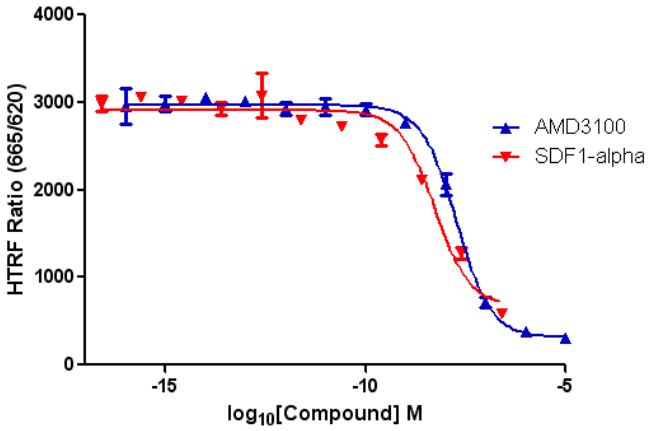


Figure 8. Automated IC_{50} determination.

Results and discussion

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 unlabeled 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, then multiplying by 10^4 for ease of data processing. A delta ratio between the positive control (total signal) and the background (nonspecific signal) is then done to obtain a specific signal.

For K_d, the total signal was a serial dilution of the labeled ligand in the presence of buffer; the nonspecific 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 and binding of the labeled ligand in proximity to the GPCR creating an HTRF signal is not associated with receptor binding. The calculated disassociation constant (K_d) for both the manual and automated plates showed high correlation with other published data for the assay.

Figure 8 illustrates data for the K_i/IC_{50} values shown in Table 2. K_i 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 such as 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.

The slight upward shift in the IC_{50} value for the unlabeled SDF1∞ is due to the experimental condition of starting the serial dilution at 1 nM rather than 10 nM. This was necessary as a result of having <10 nM of SDF1∞ 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 1 nM, which resulted in an additional plateau point at the top of the curve. Even under these conditions, the calculated IC_{50} for the compound was 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 were generated using Agilent BioTek liquid handling instrumentation compared to previously published methods. Agilent BioTek MultiFlo multiplate dispenser technology is suitable for HTS applications with the Tag-lite HTRF assay method, and can be equipped with up to two peristaltic and two syringe pumps for a wide range of liquid dispensing from the same small foot print. The Agilent BioTek Precision microplate pipetting system is adept in automating the serial dilutions necessary for conducting dose-response curves to evaluate ligand binding pharmacolgy evaluations. The Agilent BioTek Synergy H1 hybrid multimode reader provides high-performance, cost-effective HTRF detection.

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