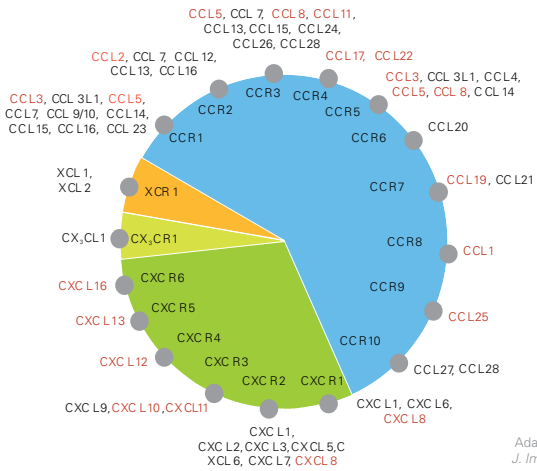
Chemokine Receptor-Ligand Binding Assays Using the 8200 Cellular Detection System

Chemokines are a large family of chemotactic cytokines that influence many aspects of the immune response via interaction with their G-protein coupled receptors. They facilitate leukocyte migration and positioning; dendritic cell function; T cell differentiation; and virus entry, including HIV-1. Due to their involvement in inflammatory, autoimmune and infectious diseases, chemokine receptors are important targets in the development of therapeutic agents. Consequently, good cell-based chemokine receptor-ligand binding assays for screening compound or antibody libraries, especially those that do not require radioactive reagents, are highly desirable. The Applied Biosystems 8200 Cellular Detection System utilizes the fluorescence-based FMAT® system technology. The accompanying software features automated IC50 graphing and z-factor analysis. The system uses non-radioactive fluorescently labeled reagents. Multiple chemokines have been labeled with the FMAT Blue® dye and tested on the 8200 system. The unique features of the Applied Biosystems 8200 Cellular Detection System enable mix-and-read cell-based receptor-ligand binding assays and generation of specific chemokine receptor ligand binding data.



Adapted from *J. Immunol. Methods*, 2002, **262:** 1-3

Figure 1. Chemokine nomenclature.There are currently 18 characterized chemokine receptors designated by chemokine subfamily name and illustrated within the circle: 10 CC-receptors (CCR1-CCR10), 6 CXC-receptor CXCR1- CXCR6, 1 C-receptor (XCR-1), and 1 CX_3C-receptor (3). All the ligands are illustrated outside the circle. CXCL4, 14, 15, and CCL6 and 18 are not shown, because their receptors are unknown. The chemokine ligands that have been labeled with FMAT Blue® dye by Applied Biosystems and tested on the 8200 Cellular Detection System are highlighted in red.

Introduction

Chemokines are a family of small (~8-14 kDa), inducible, chemotactic cytokines. Their biological activities range from the control of leukocyte trafficking in normal and inflammatory conditions to the regulation of angiogenesis/angiostasis, hematopoiesis, tissue architecture, and organ development (1). The chemokine field has developed rapidly and a systematic nomenclature for these molecules and their 7-transmembrane G-protein coupled receptors (GPCRs) became necessary as more novel chemokines have been found. Most chemokines have four characteristic cysteines in their primary structure and the new nomenclature is based on the positions (or the presence) of the first two residues. They can be divided in four groups: C, CC, CXC, and CX_3C chemokines. The same classification is adopted for their receptors (2). Figure 1 shows all currently identified chemokine receptors and the chemokines that interact with each one (3).

GPCRs are currently attractive targets for therapeutics because of their selectivity and expression in different types of leukocytes. Some receptors are shared by various cell types, but others are expressed exclusively on the membrane of certain leukocyte populations (e.g. CCR5 in monocytes, CXCR1 and CXCR2 in neutrophils) so that those cells can be recruited selectively by appropriate

chemokines. A drug targeted to a unique chemokine/receptor pair will ideally have minimal impact on the normal host immune response. Cell-based drug screening is ideal for chemokine receptors because of their surface expression and complex intra-membrane structure.

The Applied Biosystems 8200 Cellular Detection System is a fluorescence macro-confocal, biological binding event analyzer enabling high throughput cell-based screening of receptor-ligand binding. The 8200 system is based on FMAT® system technology providing users with a homogeneous assay format. The 8200 software is user-friendly and offers a wide variety of tools for data acquisition and data analysis. The 8200 system has an integrated robotic plate handler enabling walk-away automated high throughput mode operation. A red laser-dye labeled peptide/protein ligand is used as a detection probe for hit competition studies with chemical compound libraries or for IC50 analyses (4). The 8200 system uses a 633 nm HeNe laser excitation source to scan the bottom of a 96-, 384- or 1536-well plate. Cell- or bead associated fluorescence is collected by two photomultiplier tubes (PMT1, 650-685 nm; PMT2, 685-720 nm) allowing for the detection of two different color dyes. For the receptor-ligand binding assay, peptides or small proteins are directly labeled with the FMAT Blue® dye and purified. The resulting dye-protein conjugates are extensively analyzed to ensure the integrity of the protein and the presence of one dye per protein molecule. Many chemokines have been successfully labeled with FMAT Blue dye at Applied Biosystems (Fig.1).

Performing Competitive Chemokine Receptor Ligand Binding Assays

Cells expressing the receptor for the chemokine of interest are incubated in the presence of varying concentrations of unlabeled chemokine and a fixed concentration of FMAT Blue dye labeled chemokine in a 96-well plate. Following incubation the plates are read using the 8200 and the data analyzed using the 8200 Analysis software to yield IC50 curves and values.

The following protocols have been used successfully for a number of chemokines and should be used as a starting point for specific chemokine competition assays. However, parameters such as labelled and unlabeled chemokine concentration, cell density, etc. may need optimization for best performance.

Ic50 Assay Curve Protocol For Suspension Cells

1. Prepare serial dilutions of unlabeled chemokine from 300 nM to 30 pM in Hepes buffer, 0.5% BSA, 0.01% Na Azide, pH 7.4. A minimum of 5 points is required for IC50 analysis on the 8200.
2. Prepare solution of FMAT Blue dye labeled chemokine at 3 x Kd* in Hepes buffer, 0.5% BSA, 0.01% Na Azide, pH 7.4.
3. Prepare a suspension of cells expressing the appropriate receptor at 3.3 X 10^5 cells/ml in Hepes buffer, 0.5% BSA, 0.01% Na Azide.
4. Add 30 µl of each unlabeled chemokine dilution to triplicate wells of a 96 well plate.
5. Add 30 µl of the 3 x Kd FMAT Blue dye labeled chemokine to all the wells.
6. Add 30 µl of the cell suspension.
7. Incubate at 37˚C ambient atmosphere for 1-2 hours.
8. Scan the plate using the 8200 and analyze using the one-color, one-population IC50 assay set up.

*The Kd value can be found in the literature or calculated by performing a Saturation Binding Analysis experiment on the 8200 Cellular Detection System.

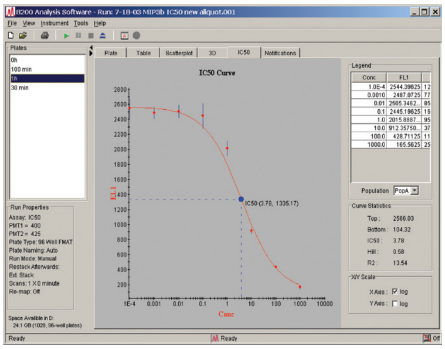


Figure 2A. FMAT Blue dye labeled MIP-3ß competition with unlabeled MIP-3ß

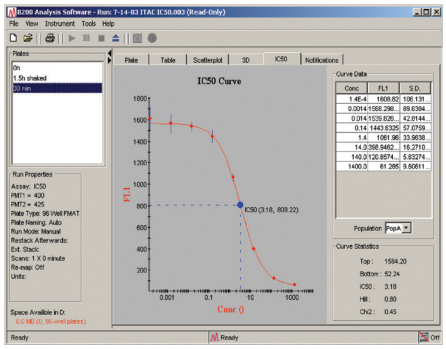


Figure 2B. FMAT Blue dye labeled I-TAC competition with unlabeled I-TAC

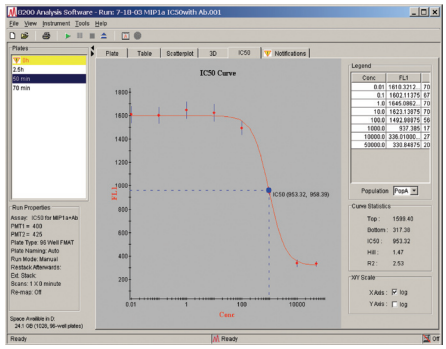


Figure 2C. MIP-1α competition with anti-CCR1

Ic50 Assay Curve Protocol For Adherent Cells

1. Remove adherent cells from cell culture flasks, dilute to 5 x10^4 cells/ml in cell medium and add 100 µl to each well. Incubate overnight at 37˚C, 5% CO_2. During incubation the cell number will approximately double to 1 x10^4.
2. Remove the cell culture medium, wash twice with 100 µl of Hepes buffer, 0.5% BSA, 0.01% Na Azide, pH 7.4 and add 30 µl of Hepes buffer, 0.5% BSA, 0.01% Na Azide, pH 7.4 to each well.
3. Prepare serial dilutions of unlabeled chemokine from 300 nM to 30 pM in Hepes buffer, 0.5% BSA, 0.01% Na Azide, pH 7.4. A minimum of 5 points is required for IC50 analysis on the 8200.
4. Prepare solution of FMAT Blue® dye labeled chemokine at 3 x Kd* in Hepes buffer, 0.5% BSA, 0.01% Na Azide, pH 7.4.
5. Add 30 µl of each unlabeled chemokine dilution to triplicate wells of a 96 well plate.
6. Add 30 µl of the 3 x Kd FMAT Blue dye labeled chemokine to all the wells.
7. Incubate at 37˚C ambient atmosphere for 1-2 hours.
8. Scan the plate using the 8200 and analyze using the one color, one population IC50 assay setup.

*The Kd value can be found in the literature or calculated by performing a Saturation Binding Analysis experiment on the 8200 Cellular Detection System.

A similar assay using either adherent or suspension cells can be performed substituting an antibody or antagonist directed against the chemokine or its receptor in place of the unlabeled chemokine.

Competitive binding assays can also be configured to screen for "hits" using the Plus/Minus Assay function of the 8200. In this assay format, binding of the FMAT Blue dye labeled chemokine is in competition with an excess of antagonist. The 8200 is configured to gate on fluorescence signal intensity. Using this parameter the user defines thresholds and any values that lie within these thresholds are designated as hits.

Results

Chemokines from Applied Biosystems are labeled with FMAT Blue dye by solution conjugation and further characterized and quantified by HPLC, Mass spectrometry and UV/VIS absorption. To test for ligand specificity, a competitive binding curve of the FMAT Blue dye labeled ligand is generated using unlabeled ligand as a competitor. The IC50 of the unlabeled ligand against the labeled should be in the Kd range expected for the receptor ligand pair. Figure 2A, 2B and 2C shows the IC50 curves and images generated on the 8200 Cellular Detection System for FMAT Blue MIP-3ß, FMAT Blue dye labeled I-TAC and FMAT Blue dye labeled MIP-1α, respectively. The IC50 of FMAT Blue MIP-3ß is 3.78 nM. This is consistent with data generated with SEAP (secreted alkaline phosphatase) labeled ligand previously reported in the literature (5). The IC50 of FMAT Blue dye labeled I-TAC is 3.18 nM which is consistent with that of radiolabeled I-TAC reported in the literature (6). The IC50 of anti-MIP-1α competing with FMAT Blue dye labeled MIP-1α is 953 ng/ml which is in agreement with the neutralization data provided by the supplier.

Figure 3A shows the results of a competitive binding Plus/Minus screening assay as displayed by the 8200 Analysis software. Receptor expressing cells and FMAT Blue dye labeled I-TAC were added to the wells of a 384 well plate. The wells in column two and five random wells were spiked with a 100 fold excess of unlabeled I-TAC. The five "hit" wells and the positive control row are automatically displayed in the run window. In this assay, the gates were set such that a low signal was designated as a hit.

Figure 3B shows the total binding cell images from a plate well containing

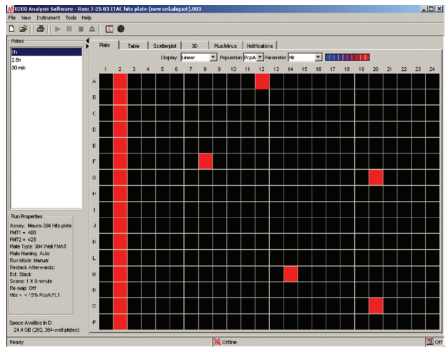


Figure 3A. I-TAC competitive binding inhibition – Plus/Minus Assay.

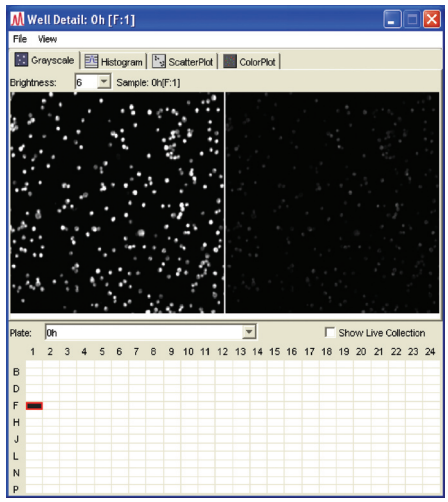


Figure 3B. Total binding Image - Cells, FMAT Blue ITAC at Kd and no unlabeled ITAC.

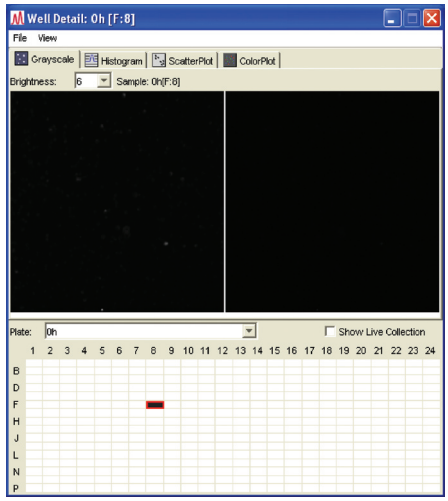


Figure 3C. Hit Image - Cells, FMAT blue ITAC at Kd and unlabeled ITAC at 100 fold excess Kd concentration.

cells, FMAT Blue® dye labeled I-TAC and no competing unlabeled I-TAC.

Figure 3C shows 8200 images of a hit well containing cells, FMAT Blue labeled I-TAC and unlabeled ITAC at 100 fold excess.

Conclusions

Chemokines have been implicated in specific disease processes and chemokine gene disruption studies have confirmed most of their biological functions. For example, the MIP-1α knockout mouse has shown reduced ability to mount an inflammatory response to influenza infection (2). RANTES and the CCR1 receptor are implicated in the pathophysiology of rheumatoid arthritis. MCP-1 and CCR2 are involved in the pathology of atherosclerosis. The 8200 system is ideal for the rapid screening of natural or synthetic compounds that are potential inhibitors of the chemokine/chemokine receptor interaction and therefore drug candidates for these diseases. The use of the FMAT Blue dye labeled chemokines with the 8200 Cellular Detection Systems eliminates the cost of disposal and safety issues associated with radio-labeled ligands. FMAT Blue dye labeled chemokines have been shown to retain their specific binding activity. The IC50s of FMAT Blue® labeled chemokines are consistent with the data found in the literature. The optimal cell line for this kind of assay should have a minimum of 50,000–100,000 active receptors per cell. Therefore, cell lines that have a Bmax of 1-20 pmol/mg are excellent candidates for use in live cell based-assays.

In addition to receptor-ligand binding, other cell-based assays that have been adapted for use with the 8200 system include apoptosis, and fixed cell immunofluorescence. Bead-based assays including immunoassays, kinase assays and protein-protein binding assays may be performed on the 8200.

References

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ORDERING INFORMATION

Description	Part Number
Instrument	
Applied Biosystems 8200 Cellular Detection System	4342920
Consumables	
FMAT® plates bar coded, cs of 100, 96-well, 384-well	4308776, 4307723
FMAT Blue®-labeled Chemokines	
FMAT Blue® MDC: 1.0 nmole, 100 pmole	4377775, 4377765
FMAT Blue® IP-10: 1.0 nmole, 100 pmole	4377776, 4377766
FMAT Blue® SDF-1α: 1.0 nmole, 100 pmole	4377777, 4377767
FMAT Blue® TCA-3: 1.0 nmole, 100 pmole	4377778, 4377768
FMAT Blue® Rantes: 1.0 nmole, 100 pmole	4377782, 4377772
FMAT Blue® MIP-1α	Available March 07
FMAT Blue® IL-8	Available March 07
FMAT Blue® BLC	Available March 07
FMAT Blue® Fractalkine	Available March 07
FMAT Blue® SRPSOX	Available March 07
Bulk quantities are available on request. Custom labeling is also available. Please inquire.	

Printed in the USA, 02/2007 113AP01-01

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