



Society for Biomolecular Sciences 2007 Best Poster Award

High Throughput Flow Cytometric Analysis with HyperCyt®: Utilization of a 384 well microplate-based assay for the detection of ligand binding to cellular receptors

Linda S.Trinkle¹, Bruce S. Edwards², Susan M. Young², Irena Ivnitsky-Steele², Richard D. Ye², Eric R. Prossnitz², Larry A. Sklar², IntelliCyt Corporation¹, University of New Mexico Health Sciences Center and UNM Molecular Libraries Screening Center²

High-throughput flow cytometry represents a promising approach to compound library screening in addition to the ability to increase the efficiency of multiparameter flow cytometry. The HyperCyt® high-throughput (HT) flow cytometry sampling platform is an enabling technology developed under the New Mexico Molecular Libraries Screening Center and offered as a commercial product through IntelliCyt Corporation that utilizes a novel approach to specimen analysis and data collection which circumvents time delay bottlenecks of conventional flow cytometry. This approach also dramatically reduces the amount of sample aspirated for each analysis, typically requiring ~2 µl per sample while making quantitative fluorescence measurements of 40 or more samples per min with thousands to tens of thousands of cells in each microplate sample. Here, we describe a simple robust screening assay that exploits the high-content measurement capabilities of the flow cytometer to simultaneously probe the binding of test compounds to two different receptors in a common assay volume, a duplex assay format. The ability of the flow cytometer to distinguish cell-bound from free fluorophore is also exploited to eliminate wash steps during assay setup. HT flow cytometry with this assay has allowed efficient screening of tens-of-thousands of small molecules from the NIH Small Molecule Repository to identify selective ligands for two related G-protein-coupled receptors, the formylpeptide receptor and formylpeptide receptor-like 1.

This work was supported by NIH R03 MH076381-01, U54 MH074425-01, the New Mexico Molecular Libraries Screening Center, the University of New Mexico Shared Flow Cytometry Resource and Cancer Research and Treatment Center.

Introduction

Modern drug discovery involves the testing of cellular targets against millions of potentially valuable compounds that may bind cellular receptors to effect clinically therapeutic cellular responses. Because of its facility for making multiple, sensitive quantitative measurements on a per cell basis, flow cytometry is a potentially valuable platform for drug discovery. However, thus far, flow cytometry has played only an accessory role in many phases of drug discovery, because it has been severely limited in throughput rates for the serial analysis of multiple discrete cell suspensions.

One feature of conventional flow cytometry that hampers throughput is the one-sample-one-file paradigm, the practice of creating a separate data file for each cell suspension analyzed. Repetitive time delays are incurred for each sample that include one at the onset while hardware and software are initialized for data collection, and another at the end while data are saved to a file on a hard disk or other storage destination.

A second rate-limiting feature is a consequence of the traditional cell sample processing loop. The entire sample uptake/transport pathway is first rinsed with fluid to flush away the previous sample, then, the new sample suspension must be transported all the way from the source well to the point of analysis before the next data acquisition sequence can be started.

High-throughput Flow Cytometry

In contrast to the traditional sample handling paradigm, the HyperCyt[®] high-throughput flow cytometry approach is to fill the sample uptake/transport line with a stream of discrete sample particle suspensions, each typically a ~2 μ l volume (or larger, if necessary) aspirated from one of the source wells and separated one from the other by air bubbles. The entire sample stream is continuously delivered to the flow cytometer so that data from all the samples in a plate are acquired and stored in a single data file. A high resolution time parameter is also recorded during data acquisition. Temporal gaps in particle detection are created in the data stream by the passage of the air bubbles, allowing the individual particle suspensions to be easily distinguished and separately evaluated when plotted in conjunction with the time parameter.

This approach not only eliminates the time lags associated with multiple rounds of initialization, data storage and sample line filling, but also dramatically reduces the amount of sample aspirated for each analysis. Sampling rates in excess of 1 sample/sec have been successfully demonstrated for endpoint assays, but a rate of ~40 samples/min is more routinely used as the most practical for producing robust assay analysis results. It is possible to make quantitative fluorescence measurements of thousands to tens of thousands of cells in each sample under such conditions of serial sample throughput.

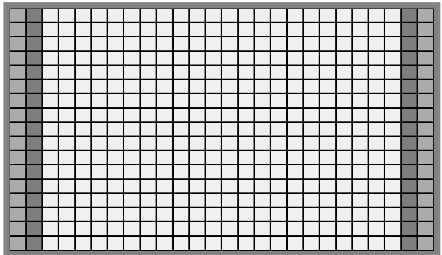
HyperCyt Ligand Binding Assay

We describe a simple robust assay that detects active compounds on the basis of their ability to block the binding of a high affinity fluorescent ligand to cellular receptors. In negative control wells or in wells containing inactive compounds, cells exhibit bright green fluorescence due to the bound fluorescent ligand. Compounds with receptor-binding activity promote displacement of fluorescent ligand from the receptor, causing a decrease in green cell fluorescence intensity. Since ligand binding is measured directly, the assay detects active compounds independently of potential complexities in the patterns of the physiological responses of cells.

The specific assay described here exploits the high-content measurement capabilities of the flow cytometer to simultaneously probe two different receptors in a common assay volume, a duplex assay format. Cells expressing different receptors are color coded to allow their distinction in a red fluorescence detection channel during the analysis. The ligand binding response is recorded in a separate green fluorescence detection channel. An important advantage of the duplex approach, and of higher levels of assay multiplexing compatible with flow cytometry, is the ability to obtain both activity and selectivity information as an integral part of a primary screen. This assay is performed in 384-well plates, facilitated by the unique capability of HT flow cytometry with the HyperCyt® platform.

This assay has been used at the New Mexico Molecular Libraries Screening Center to screen more than 25,000 compounds from the NIH Small Molecule Repository to identify selective ligands for each of the receptors (see NMMLSC assays at <http://pubchem.ncbi.nlm.nih.gov/>).

384-well Microplate Setup for HyperCyt Ligand Binding Assay



- Positive Controls- 5ul peptide blocking solution
- Negative Controls- 5ul PDP/2% DMSO
- 10 uM Peptides- 5ul

Test compounds are diluted and 5ul of each diluted compound is dispensed into the 384-well assay plate (10uM final concentration).

Into each well, dispense 5 µl of a cell mixture containing 50,000 of each cell type. The cell mixture contains U937/FPR (pre-labeled with Fura-Red) and RBL/FPRL1 cells chilled on ice prior to addition.

Seal the plate wells with ThermalSeal Film and incubate the assay plate at 4°C for 30 min, periodically inverting the plate to maintain cells in suspension.

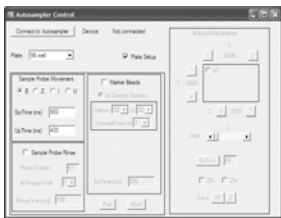
Remove the sealing film and dispense 5 µl Wpep-FITC solution into each well.

Reseal the plate wells with ThermalSeal Film and incubate the plate at 4°C for 30 min or more while rotating.

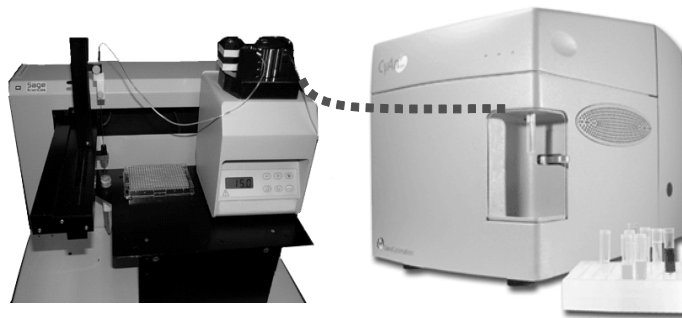
Move the plates one at a time from the rotator to the deck of the HyperCyt® autosampler. Wait until sampling of one plate is completed before moving the next to the deck.

HyperCyt[®] Workflow

1. Open worklist for 384-well plate.



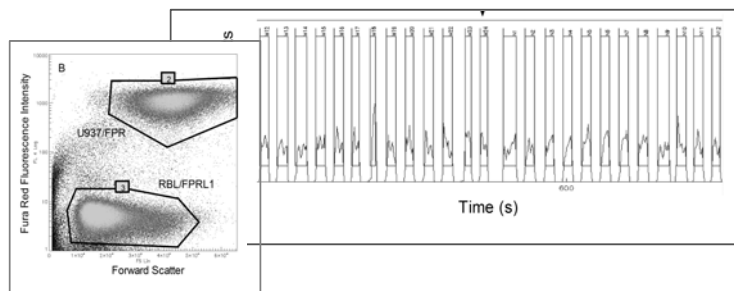
2. Connect HyperCyt[®] to flow Cytometer



3. Load FCM protocol

Sampling is continuous with each sample separated by an air bubble.

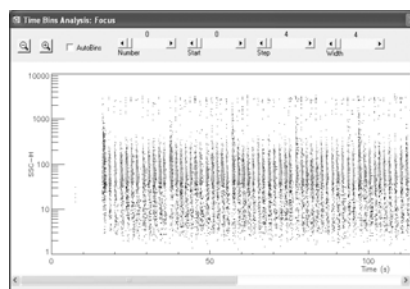
4. Acquire data



5. Analyze data with HyperCyt Data Analysis

Time and fluorescence gates identify cell and well populations.

Individual well Analysis

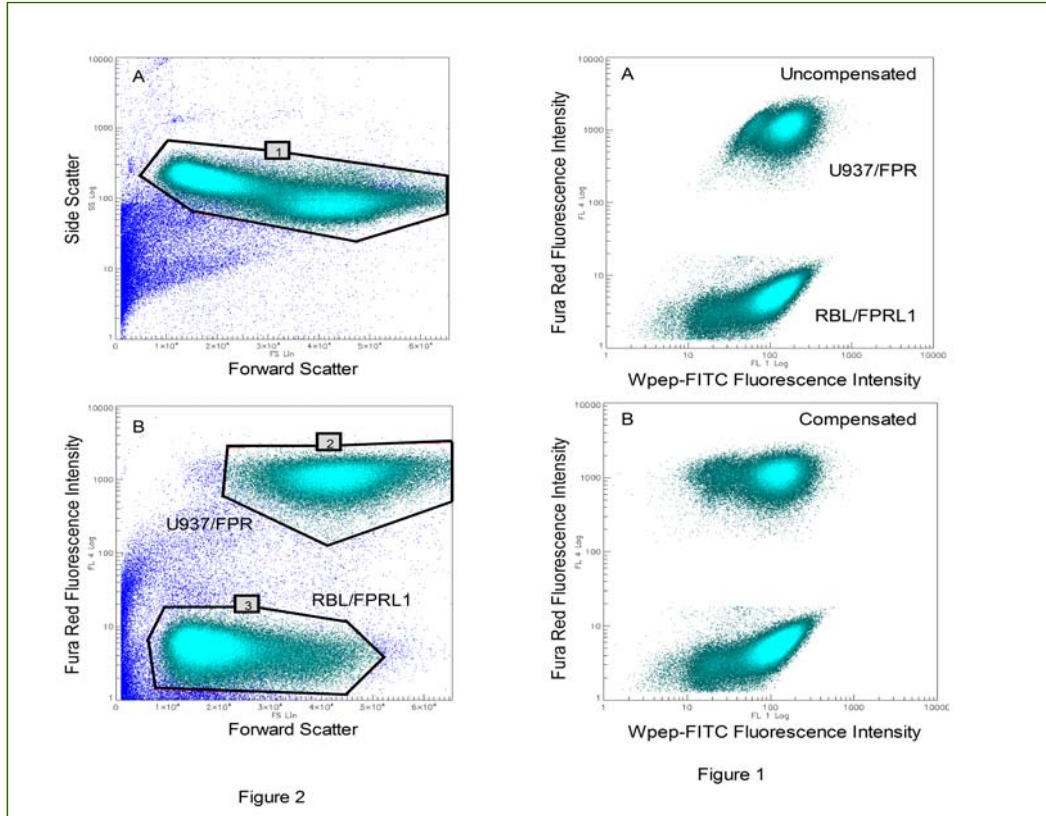


Complete plate Analysis

Save individual well data as FCS files for further analysis with HyperCyt or 3rd party software.

Analyze a plate as a whole, or export .csv files for further statistical analysis.

HTFC Duplex Assay in a 384-well Format

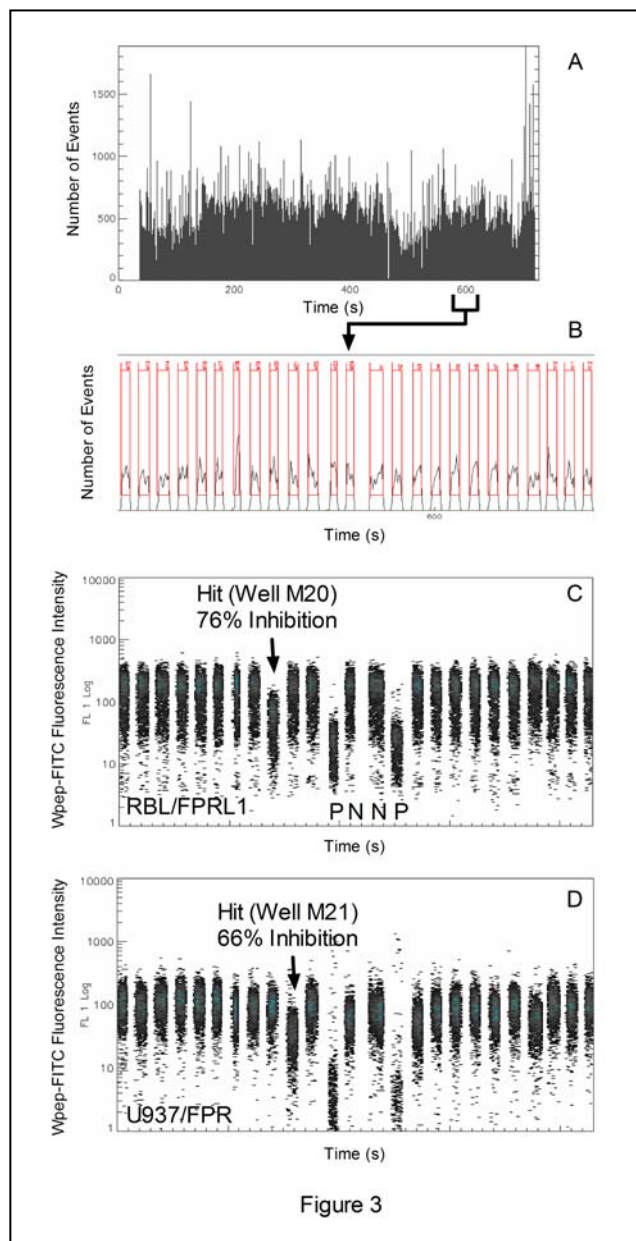


All cells from the 384 microplate are displayed with the HyperCyt[®] Analysis Software. Gates are set in order to look at specific populations from the complete data set.

Cells expressing FPR or FPRL1 receptors are color coded to allow their distinction in a red fluorescence detection channel during the analysis.

The ligand binding response is recorded in a green fluorescence detection channel.

Analysis of Ligand Binding in 384-well Microplate with HyperCyt software



(A) The entire microplate was analyzed on a CyAn flow cytometer in approximately 11 minutes and stored in a single FCS data file.

(B) HyperCyt Autobin function identifies each well and creates a well-based statistical analysis worklist that can be exported as a csv file or into an Excel spreadsheet.

(C) Display of portion of Histogram of Wpep-FITC fluorescence vs time, gated on RBL cells, indicates a Hit for the FPRL1 receptor with 76% inhibition compared to the negative control (N).

(D) Display of cells gated on U937 cells indicate a Hit with 66% inhibition for the FPR receptor.

Figure 3

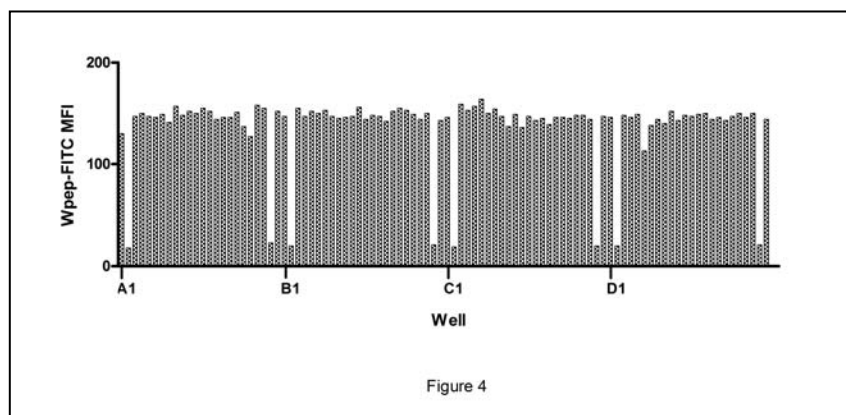


Figure 4

Green fluorescence data from the first 96 wells of a 384 well plate were exported to an Excel spreadsheet. The wells were sampled by row from well 1 to 24. Wells 2 and 23 from each row were positive controls resulting in a repetitive pattern in the data.

Statistical Analysis

The fluorescence intensity data are exported to an Excel spreadsheet template that automatically performs the calculations to quantify test compound effects on Wpep-FITC ligand binding to the two receptors.

The assay response is defined as the inhibition of fluorescent ligand binding mediated by the test compound:

$$\% \text{Inhibition} = 100 \times \{ 1 - [(\text{MfiTest} - \text{MfiPC}_{\text{AVG}}) / (\text{MfiNC}_{\text{AVG}} - \text{MfiPC}_{\text{AVG}})] \}$$

in which MfiTest , $\text{MfiPC}_{\text{AVG}}$ and $\text{MfiNC}_{\text{AVG}}$ represent the median fluorescence intensities (MFI) in individual wells containing test compounds, average MFI in positive control wells and average MFI in negative control wells, respectively.

The Z' factor is a measure of screening assay quality that reflects both assay signal dynamic range and data variation associated with the signal measurements.

$$Z' \text{ (Z-prime)} = 1 - \{ [(3 \times \text{MfiPC}_{\text{SD}}) + (3 \times \text{MfiNC}_{\text{SD}})] / (\text{MfiNC}_{\text{AVG}} - \text{MfiPC}_{\text{AVG}}) \}$$

in which MfiNC_{SD} and MfiPC_{SD} represent the standard deviation of MFI in negative and positive control wells, respectively. Z' factor values typically average ~0.65 for this assay and values of 0.4 or more are considered to indicate acceptable assay quality.

Summary

We have shown that the HyperCyt High Throughput Flow Cytometry (HTFC) platform is a robust system for automating multiparameter flow cytometric assays in a microwell format.

HyperCyt HTFC is adaptable to duplex or multiplex assays capable of detecting active compounds on the basis of their ability to block the binding of a high affinity fluorescent ligand to cellular receptors.

The HyperCyt HTFC platform has been demonstrated to routinely screen >1000 compounds in under an hour in the University of New Mexico Flow Cytometry Resource at the New Mexico Molecular Libraries Screening Center.

Optimized sample preparation coupled with HyperCyt HTFC permit sample sizes of 15ul/well and less, and sampling volumes of 2ul, thus significantly reducing the cost per result, as well as permitting replicate sampling.

Data from an entire 96-or 384-well plate can be collected and stored as single FCS data files with the HyperCyt HTFC System. Gating and compensation can be performed on plate data. The data is readily transferred to a spreadsheet for statistical analysis. Conversely, time-gated

data from individual wells can be analyzed and saved as single data files or exported for further analysis.

HyperCyt HTFC has been utilized in combination with a number of flow cytometers in a 96-well format, however, thus far, the 384-well format has been used only with the CyAn flow cytometer from Dako.

Materials

1. Tissue culture medium (TCM): RPMI-1640 medium (Mediatech MT-10-040-CVMP) supplemented with 10% fetal bovine serum (US Biotechnologies FBU 3195, heat-inactivated 30 min, 54°C), 2mM L-glutamine-10U/ml penicillin-10 µg/ml streptomycin (Mediatech MT-30-009-CI), 10 mM HEPES (Mediatech MT-25-060-CI), 4 µg/ml CIPRO (Bayer Pharmaceuticals). This may be stored at 4°C for up to 3 weeks. TCM is supplemented with 2.5 µg/ml Amphotericin B (CellGro #30-003-CF) for culture of transfected RBL-2H3 cells.
2. A solution of 0.25% Trypsin, 1mM EDTA (InVitrogen #25200-056) is used for passaging and preparation of RBL-2H3 cells.
3. *N*-formyl-methionine-leucine-phenylalanine-phenylalanine peptide (fMLFF) (Sigma, F2009) is a peptide that selectively binds FPR.
4. Tryptophan-lysine-tyrosine-methionine-valine-D-methionine (Wpep) is a hexapeptide reported to bind with high affinity to both FPRL1 and FPR, but with a $K_d < 1$ nM for FPRL1 (17, 18) as compared to 95 nM for FPR (17).
5. Fluorescein isothiocyanate-labeled Wpep (Wpep-FITC) (Bachem, special synthesis) is used to quantify free FPR and FPRL1 receptors. (*see Note 1*).
6. Peptide dilution buffer (PDB): 110 mM NaCl (SIGMA S-9625), 30 mM HEPES, 10 mM KCl (SIGMA P-3911), 1mM MgCl₂ (SIGMA M-8266), 10 mM glucose (SIGMA G-7528), and 0.1% bovine serum albumin (SIGMA A-3059). This solution is prepared fresh weekly and stored at 4°C.
7. Peptide solutions: Peptides are stored as 1 mM or 100 µM aliquots in 100% DMSO (Omnisolv Methylsulfoxide, EMD Chemicals #MX1456-6) at -20°C. On each day of assay, aliquots are freshly thawed, diluted in PDB and used immediately. The following solutions are adequate for preparation of 4 assay plates:
 - 7a. Wpep-FITC: 4.5 µl of 100 µM Wpep-FITC in 15 ml cold PDB (15 nM Wpep-FITC)
 - 7b. Blocking Control Solution: 20 µl of 100 µM Wpep + 3.6 µl of 1 mM fMLFF + 176 µl DMSO in 10ml PDB (360 nM fMLFF/200 nM Wpep/2% v/v DMSO),
8. FuraRed staining solution: Add 46 µl DMSO to each of 2 x 50 µg Fura Red™, AM (InVitrogen #F-3021, special packaging) for a 1 mM stock solution.
9. Cell Strainer: a receptacle with a 70 µM nylon mesh filter (BD Falcon # 352350) that is used to remove clumped cells from cell suspensions
10. Compound dilution plate: a polypropylene 384-well plate with conical-bottom wells (Greiner #781280) that is used for diluting compounds prior to addition to the assay sample.
11. Assay plate: a polystyrene 384-well plate with flat bottom, small volume wells (Greiner #784101) in which ligand binding assays are performed.
12. ThermalSeal Sealing Film adhesive plate well covers (Excel Scientific #100THERPLT) are used to cover assay plate wells during incubations.
13. Contrad70 (Fisher 04355), is diluted with water to 2.5% (v/v) for cleaning of microfluidics tubing.
14. Test compounds are obtained from a variety of sources and stored at -25°C in DMSO. Source 384-well plates for use in assays contain 1mM compound and are sealed with Seal and Sample aluminum foil adhesive plate well covers (Beckman Coulter #538619).

References

1. Kuckuck, F. W., Edwards, B. S., and Sklar, L. A. (2001) *Cytometry* 44, 83-90.
2. Ramirez, S., Aiken, C. T., Andrzejewski, B., Sklar, L. A., and Edwards, B. S. (2003) *Cytometry* 53A, 55-65.
3. Young, S. M., Bologna, C., Prossnitz, E., Oprea, T. I., Sklar, L. A., and Edwards, B. S. (2004) *J Biomol Screen* 10, 374-382.
4. Edwards, B. S., Bologna, C., Young, S. M., Balakin, K. V., Prossnitz, E., Savchuck, N. P., Sklar, L. A., and Oprea, T. I. (2005) *Mol Pharmacol* 68, 1301-1310.
5. Edwards, B. S., Young, S. M., Oprea, T. I., Bologna, C., Prossnitz, E., and Sklar, L. A. (2006) *Nat Protocols* 1, 59-66.
6. Migeotte, I., Communi, D., and Parmentier, M. (2006) *Cytokine Growth Factor Rev* 17, 501-519.
7. Le, Y., Murphy, P. M., and Wang, J. M. (2002) *Trends Immunol* 23, 541-8.
8. Oppenheim, J. J., Zachariae, C. O., Mukaida, N., and Matsushima, K. (1991) *Annu Rev Immunol* 9, 617-48.
9. Murphy, P. M. (1996) in *Chemoattractant ligands and their receptors* (Horuk, R., Ed.) pp 269-299, CRC Press, Boca Raton, FL.
10. Prossnitz, E. R., and Ye, R. D. (1997) *Pharmacol Ther* 74, 73-102.
11. Ye, R. D., Cavanagh, S. L., Quehenberger, O., Prossnitz, E. R., and Cochrane, C. G. (1992) *Biochem Biophys Res Commun* 184, 582-9.
12. Snipe, J. D. (1990) in *Immunophysiology: the role of cells and cytokines in immunity and inflammation* (Oppenheim, J. J., and Shevac, E. M., Eds.) pp 259-273, Oxford University Press, New York, NY.
13. Lambert, M. P., Barlow, A. K., Chromy, B. A., Edwards, C., Freed, R., Liosatos, M., Morgan, T. E., Rozovsky, I., Trommer, B., Viola, K. L., Wals, P., Zhang, C., Finch, C. E., Krafft, G. A., and Klein, W. L. (1998) *Proc Natl Acad Sci U S A* 95, 6448-53.
14. Kalaria, R. N. (1999) *Curr Opin Hematol* 6, 15-24.
15. Brown, D. R., Schmidt, B., and Kretschmar, H. A. (1996) *Nature* 380, 345-7.
16. Le, Y., Yazawa, H., Gong, W., Yu, Z., Ferrans, V. J., Murphy, P. M., and Wang, J. M. (2001) *J Immunol* 166, 1448-51.
17. Bae, Y. S., Song, J. Y., Kim, Y., He, R., Ye, R. D., Kwak, J. Y., Suh, P. G., and Ryu, S. H. (2003) *Mol Pharmacol* 64, 841-7.
18. Bae, Y. S., Yi, H. J., Lee, H. Y., Jo, E. J., Kim, J. I., Lee, T. G., Ye, R. D., Kwak, J. Y., and Ryu, S. H. (2003) *J Immunol* 171, 6807-13.
19. Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999) *J Biomol Screen* 4, 67-73.

HyperCyt® Autosampler and HyperView™ Data analysis are available from IntelliCyt™ Corporation. IntelliCyt was formerly Sage Sciences, Inc.

Contact us:

IntelliCyt Corporation
1730 Montano Road NW
Suite B1
Albuquerque, NM 87107
505-345-9075
866-782-3140
www.intellicyt.com