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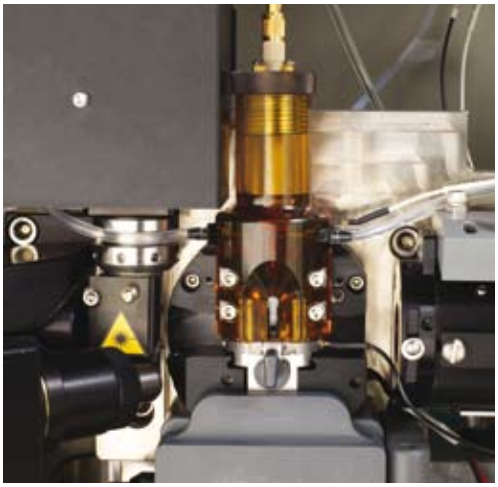
HotLines

New Generation Cell Sorters: BD FACSAria II and BD Influx Systems



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BD flow cytometers are Class I (1) laser products. For Research Use Only. Not for use in diagnostic or therapeutic procedures. Not for resale. All applications are either tested in-house or reported in the literature. See Technical Data Sheets for details.

Dear Reader,

Welcome to this new issue of our HotLines scientific forum, dedicated to you and your colleagues.

We want you to know that BD Biosciences is highly committed to providing you with relevant articles that feature new technologies and applications of interest to the Life Sciences community.

In this issue, we are pleased to announce a series of new products specifically designed to support you in your research efforts. These exciting products include:

- BD FACSAria™ II and BD Influx™ – high performance cell sorting systems
- BD™ APC-H7 reagents – tandem conjugates with a new level of stability
- BD Horizon™ V450 reagents – violet dye conjugates with exceptional performance
- BCR-ABL Protein Kit – for measuring fusion protein levels by flow cytometry
- CD107a – a flow cytometric degranulation assay
- New products for Regulatory T cell research
- BD™ Cell Cycle Kit – for determining M and S phases by bioimaging
- BD Falcon™ 50-mL Tubes with Flip-Top Cap – for easy one-handed opening and closing

Also in this issue, find an interview with Timothy Bushnell, Director of the CPBR Flow Cytometry Laboratory at the University of Rochester, New York speaking about his work and the impact flow cytometry has had on scientific discovery. Learn about a flow cytometric approach to assessing basophil activation, by Professor Dr. Didier Ebo, winner of the Clinical Cytometry award for best original paper published in 2006-2007.

Read more about fluorescent protein organelle markers and their utility in multiplexed live and fixed cell assays, and about analyzing neural differentiation of human embryonic stem cells through bioimaging and flow cytometry. And find out about a new monoclonal antibody developed at BD Biosciences for detecting acetylated p53.

For additional information on BD Biosciences instruments, research and clinical reagents, and service offerings, please visit our website at **bdbiosciences.com.**

If you have a scientific or technical question about any of our products or applications, please do not hesitate to contact our Scientific Support team – they are ready to assist you. You can reach our support team by calling the support line specific to your country, located on the back page of Hotlines, or by emailing them at **help.biosciences@europe.bd.com.**

As always, we would be pleased to hear from you with any thoughts or comments you may have. Just drop us an email at **hotlines@europe.bd.com.**

In the meantime, enjoy our new issue of Hotlines!

Sincerely,

Isabelle Gautherot
Editor - Europe

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NewProducts

BD FACSAria II Cell Sorter

The BD FACSAria™ II flow cytometry system brings simplified cell sorting and higher productivity to life science researchers. This high-performance cell sorter features hardware and software enhancements that improve overall ease of use, flexibility, and aseptic capability. And, the BD FACSAria II offers new options in lasers and nozzles to support more advanced multicolor applications.

Built on fixed-alignment technology
As in the first-generation BD FACSAria system, the flow cell of the BD FACSAria II is in true fixed alignment with the laser, to reduce startup time and improve reproducibility. Gel coupling of the flow cell to the objective lens increases the collection efficiency, sensitivity, and resolution needed for multicolor sorting applications.

New fluidics system features
A streamlined fluidics path and integrated valve manifolds make aseptic setup easier and cleaning more effective. An improved nozzle insertion design offers tight registration for a secure fit. This means a reproducible drop profile after every insertion, and reproducible instrument setup. For sorting a wide range of particle sizes, choose from four nozzles: 70, 85, 100, and 130 microns.

Better workflow and productivity
The BD FACSAria II has updated hardware and the latest software tools to optimize workflows. For example, a universal sort tube collection mount now makes it easier to insert and remove tube holders. Nozzles are more readily accessible and easy to change. Better access to controls and new software wizards simplify and automate aseptic sort setup, drop-delay determination, and clog detection. Researchers can use the new Cytometer Setup and Tracking (CS&T) feature of BD FACSDiva™ software for instrument setup and QC, further boosting productivity and helping ensure consistent, reproducible results.

375-nm Near UV laser
This new laser option expands the range of applications for the BD FACSAria II. The beam geometry of the 375-nm Near UV laser is optimized for most hematopoietic stem cell side populations. Its top hat design triples the amount of light at the sample intercept. The 375-nm Near UV laser can be operated with the red and blue lasers.

BD FACSAria to BD FACSAria II upgrades
Customers will be able to upgrade their BD FACSAria instrument to the capabilities of a BD FACSAria II. Three packages are available: a complete upgrade, a fluidics only upgrade, and a 375-nm Near UV laser upgrade. ■

Visit **bdbiosciences.com/facsaria2** for more information about the BD FACSAria II system.

BD flow cytometers are Class I (1) laser products.

BD Acquires Cytopeia

On May 13, 2008, BD announced the acquisition of Cytopeia, a privately held Washington corporation based in Seattle that develops and markets advanced flow cytometry cell sorting instruments.

"This strategic acquisition brings new technologies and capabilities that will enhance BD's ability to address growing customer needs in advanced applications, such as cell therapy research," said William A. Kozy, BD Executive Vice President. "This transaction reflects our commitment to bringing innovative tools to life scientists and will allow us to rapidly enter emerging areas of cell-based research."

Cytopeia's Influx cell sorter (now sold by BD Biosciences as the BD Influx™) offers an open, configurable cell sorting platform that can be fully optimized for researchers' unique application-specific requirements. This high level of choice and control is particularly important for life science research on the leading edge of biomedical discovery.

The BD Influx cell sorter is suited for a range of applications including cell therapy research, stem cell research, drug discovery and development, as well as marine biology. It complements the BD FACSAria™ II system—the platform of choice in research labs around the world for a broad range of immunology cell sorting and multicolor applications.

Through the acquisition, BD gains the scientific leadership and insights of Dr. Ger van den Engh, a renowned innovator in flow cytometry. Dr. van den Engh will join BD Biosciences, a segment of BD, as Vice President, Advanced Cytometry. In this role, he will manage continuing research and technology development activities in Seattle.

"In addition to access to BD Biosciences unparalleled portfolio of cellular research tools, Cytopeia's customers will benefit from our high-quality manufacturing and global customer support capabilities," Kozy said.

BD Influx Cell Sorter

Optimized for specialized applications

Over the past few years the BD Influx™ cell sorter system has been configured for a number of specialized applications. One example is its use in marine biology. In this configuration, a module to determine the polarization of scatter of fluorescent signals is used to distinguish certain organisms that cannot be identified by traditional fluorescence measurements. A full spectrum analyzer, also applicable in marine biology applications, allows researchers to measure the difference in spectral properties of natural pigments. In addition, a high dynamic range forward scatter detector can be added to the configuration to allow accurate scatter measurements of small particles.

Quick-exchange fluidics

Single-use sort nozzle assemblies and quick-exchange, gamma-irradiated tubing allow researchers to replace fluidics to achieve aseptic conditions and prevent possible cross-contamination between samples. A variety of nozzle sizes are available to accommodate a wide range of particle sizes. Researchers in cell therapy and cell cloning in industrial environments will benefit from these features.

Customized hood

A customized hood, featuring HEPA filtering and UV lamp irradiation, can be adapted to enclose BD Influx configurations, for operator and product protection.

High-speed sorting and single-cell deposition

The BD Influx system can sort at high speeds to support a variety of applications including quick enrichment of rare-event populations.

BD flow cyometers are Class I (1) laser products.

In addition, the Computerized Cell Deposition Unit (CCDU) built into the BD Influx allows for automated single cell deposition for cloning or single-cell PCR. The unit is fast and configurable and can be adapted to user-defined collection devices.

Small footprint

The BD Influx cell sorter has been kept small and can fit even in tight conditions. Built-in brackets fit ship-based research lab requirements.

For more information visit **bdbiosciences.com/influx.**

BD APC-H7 Photostable Tandem Conjugates from BD Biosciences

BD™ APC-H7 from BD Biosciences offers a new level of stability, making it easier to achieve reliable results across a broad range of multicolor flow cytometry applications.

Addresses the spillover challenge

Emission at the donor fluorochrome wavelength, caused by dye degradation and spillover, has been a challenge in the use of tandem dyes such as APC-cyanine dyes. Degradation inherent in tandem dyes leads to emission in the APC channel, which in turn leads to false positive events and reduced sensitivity. Spillover is a common issue with multicolor applications, and can result in loss of resolution sensitivity. BD APC-H7 reduces loss of fluorescence resonance energy transfer (FRET) between the donor and acceptor fluorophores to maintain integrity of signals from both channels.

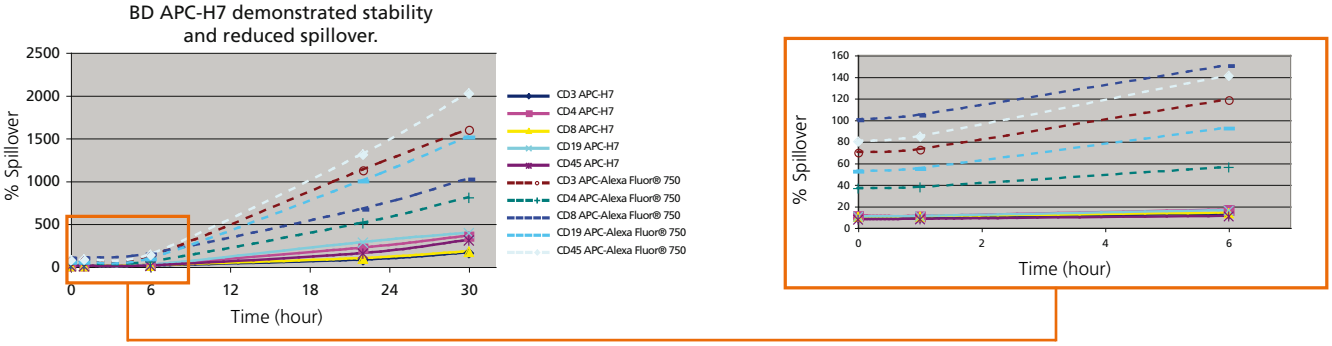

Figure 1. BD APC-H7 demonstrated stability and reduced spillover.

The experiments shown here compare the stability, as measured by % spillover, of whole blood stained with BD APC-H7 and APC-Alexa Fluor® 750 conjugated antibodies. Stained samples were stored in 1% PFA at room temperature under ambient light.

continued on next page

More reliable and reproducible

BD APC-H7 is more stable and less affected by light, temperature, and formaldehyde-based fixatives than other APC-cyanine tandem dyes such as APC-Cy™7 and APC-Alexa Fluor® 750. BD APC-H7 also is engineered to minimize spillover to the APC channel. Researchers get more reliable and reproducible staining patterns, and more reliable and reproducible results.

More flexibility and greater ease of use

BD APC-H7 improves stability in ambient light, making it easier to handle and use. Experiments can be run outside darkroom conditions with more relaxed time constraints.

Conjugates of BD APC-H7 are available for many important cell surface markers such as CD3, CD4, CD8, CD14, CD19, CD20, CD45 and HLA–DR, allowing BD APC-H7 to be used for a wide range of applications. This degree of choice, coupled with increased stability, enables greater flexibility in multicolor experiment design. ■

Visit **bdbiosciences.com/apc-h7** for more information and a complete product listing.

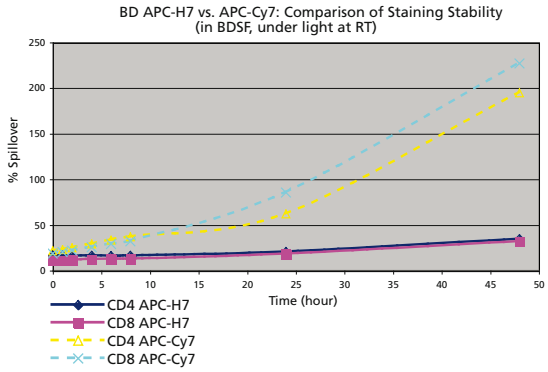

Figure 2A. APC-H7 vs. APC-Cy7: Comparison of staining stability (in BDSF, under light at RT).

Whole blood stained with CD4 APC-H7, CD8 APC-H7, CD4 APC-Cy7, or CD8 APC-Cy7. Stained samples were stored in BD Stabilizing Fixative at room temperature under ambient light for 48 hours.

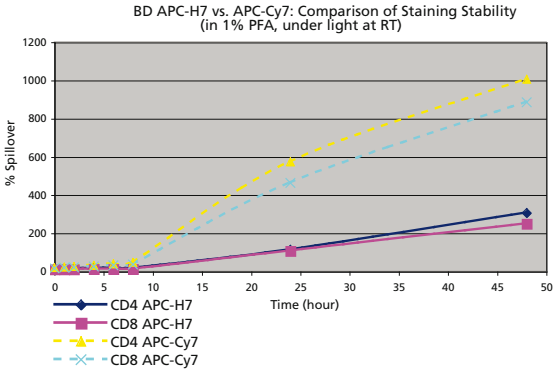

Figure 2B. APC-H7 vs. APC-Cy7: Comparison of staining stability (in 1% PFA, under light at RT).

Whole blood stained with CD4 APC-H7, CD8 APC-H7, CD4 APC-Cy7, or CD8 APC-Cy7. Stained samples were stored in 1% PFA at room temperature under ambient light for 48 hours.

BD Horizon V450 Violet Dye Reagents from BD Biosciences

The BD Horizon™ V450 reagents from BD Biosciences are high quality antibody conjugates designed to deliver exceptional performance on flow cytometers equipped with a violet laser, including the BD FACS™ brand family of flow cytometers. The first in a series of new fluorochromes from BD Biosciences, these reagents are engineered to deliver high sensitivity and consistent results.

A broad portfolio of BD Horizon V450 conjugates is available, maximizing choice and flexibility for multicolor panel design. Offering exceptional performance and compatibility, BD Horizon V450 reagents, together with online tools and resources from BD Biosciences, make the power of multicolor flow cytometry more accessible to researchers than ever before.

Optimized for the violet laser

BD Horizon V450 reagents are engineered for use with BD FACS brand instruments equipped with violet lasers, including BD™ LSR II, BD FACSAria™ II, and BD FACSCanto™ flow cytometers. With a maximum absorption of 404 nm and an emission peak of 448 nm, these reagents have improved spectral characteristics compared to Pacific Blue™ reagents when used on these instruments (**Figure 1**). Additionally, many BD Horizon V450 reagents exhibit improved performance compared to Pacific Blue™ reagents as measured by median fluorescence intensity (MFI) and normalized signal to noise (Stain Index) (**Figure 2, Table 1**).

Wide portfolio of conjugates, multiple sizes

BD Horizon V450 reagents are available in a broad array of clones. Multiple sizes address a range of requirements from small test sizes for multicolor panel design to 120-test sizes needed for high-throughput experiments. Bulk sizes and special packaging options are also available.

Tools to optimize setup, selection, and performance

Multicolor flow cytometry enables simultaneous analysis of multiple parameters in a single experiment and can quickly deliver a wealth of information about cellular composition and function.

To help advance the use of multicolor flow cytometry, BD Biosciences offers a growing library of tools and resources relevant to both experienced customers and those new to multicolor panel design (**bdbiosciences.com/colors**). In addition to this online resource, BD Biosciences offers one-on-one technical application support as part of our comprehensive customer services. Every day around the world BD flow cytometry experts help customers on the phone and on-site address a range of multicolor questions from panel design to troubleshooting.

Visit **bdbiosciences.com/colors/horizonv450** for more information and a complete product listing.

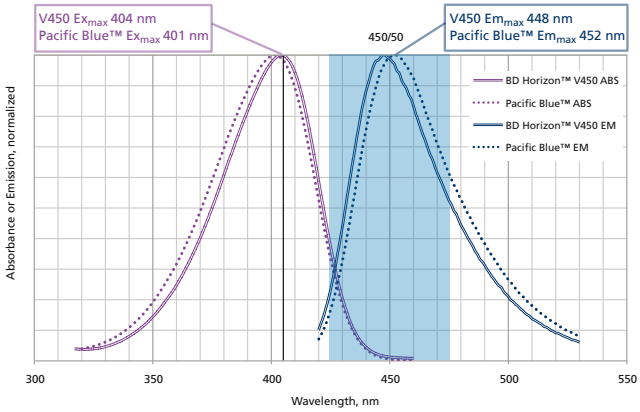

Figure 1. Spectral overlay comparison of BD Horizon V450 and Pacific Blue™

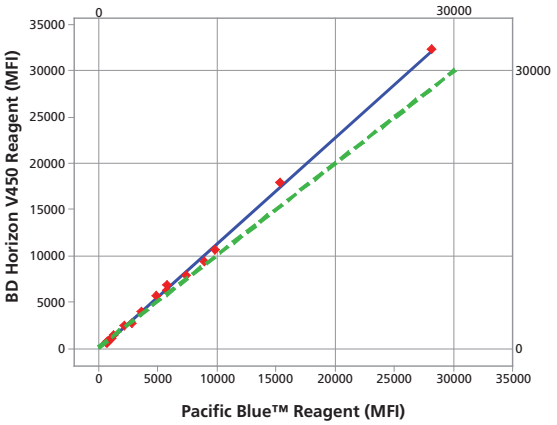

Figure 2: Comparison of fluorescence intensities of BD Horizon V450 and Pacific Blue™ reagents. Human peripheral blood cells were stained with 18 matched pairs of BD Horizon V450 and Pacific Blue™ reagents (eg, BD Horizon V450 labeled anti-human CD8 vs. Pacific Blue™ labeled anti-human CD8). The conjugates were F/P ratio matched and tested using whole blood and the lyse/wash method. The corresponding Median Fluorescence Intensity (MFI) of the positive cells for each reagent pair was measured and plotted as shown. The green dashed line represents the theoretical MFI ratio (slope = 1.0) for reagents of equivalent brightness. The blue line shows the linear regression (slope = 1.15) of the 18 reagent pairs. The data show that while there might be variation due to individual clones (see Table 1), on average the BD Horizon V450 reagents are 15% brighter than the corresponding Pacific Blue™ reagents.

	CD11c (B-Ly6)*	CD11c (B-Ly6)**	CD19 (HIB19)	CD8 (RPA-T8)	IFN-γ (B27.1)	CD3 (SP34-2)	CD3 (UCHT1)	CD45 (HI30)
BD Horizon V450	4.0	12.5	20.8	28.5	29.6	42.7	47.3	255.0
Pacific Blue™	4.2	12.3	18.8	24.6	25.7	39.0	44.8	228.0
%DIFF	-5%	2%	11%	16%	15%	9%	6%	12%

** Tested on granulocytes* *** Tested on monocytes*

Table 1: Normalized signal-to-noise (Stain Index) comparison between BD Horizon V450 and Pacific Blue™ reagents across several antibody clones. F/P matched conjugates of both BD Horizon V450 and Pacific Blue™ were tested on whole blood using the lyse/wash method with BD Pharm Lyse™ lysing buffer (Cat. No. 555899) and run on a BD LSR II system equipped with a violet laser and a 450/50 filter. Comparative stain indices between BD Horizon V450 and Pacific Blue™ were calculated to assess relative brightness of the two fluorophores. The comparison revealed that BD Horizon V450 is generally brighter than Pacific Blue™ across the tested specificities.

BCR-ABL Protein Kit from BD Biosciences

BD Biosciences has released a new BCR-ABL Protein Kit (Cat. No. 643939) for determining the presence of BCR-ABL fusion proteins. The new kit detects the presence of specific fusion proteins in cell lysates and is based on the BD™ Cytometric Bead Array (CBA) system.

About fusion proteins

Fusion proteins are a consequence of chromosomal rearrangements in a cell. Some chromosomal rearrangements lead to changes in the positions of genes, which ultimately cause the cells to generate altered proteins. Fusion proteins are absent from normal, healthy cells. Fusion proteins have antigen determinants derived from each of the original proteins that would not normally be present on the same molecular structure. By introducing two antibodies into a sample, each specific to an antigen on a different portion, it is possible to detect the presence of a fusion protein.

Principle of the assay

Flow cytometry allows for the discrimination of particles on the basis of attributes such as size and fluorescence. The BD CBA system provides a way of coupling a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes through flow cytometry.[1]
The BD™ BCR-ABL Protein Kit uses this technology to detect BCR-ABL fusion proteins in human blood research samples.[2]

About the assay kit

The BD BCR-ABL Protein Kit, a bead-based immunoassay, takes advantage of flow cytometry's excellent fluorescence detection capabilities. Key components in the kit are the capture beads, which are stained particles coated with an antibody, and the detector antibodies, which are conjugated to a fluorescent dye.

The capture beads included in this kit have been coated with an antibody specific to one part of the BCR-ABL fusion protein. The detector reagent is a phycoerythrin (PE)–conjugated antibody specific to a different part of the protein. The capture beads and detector reagent are incubated with a prepared sample. If the sample contains BCR-ABL proteins, sandwich complexes are formed, as shown in **Figure 2**.

These sandwich complexes can be studied by using flow cytometry to identify particles with attributes of both the bead and the detector. ■

Kit contents

- Pretreatment A (powder), store at –20°C.
- Pretreatment B (powder), store at 2°C to 8°C.
- Capture beads, store in the dark at 2°C to 8°C.
- Detector reagent, store in the dark at 2°C to 8°C.

Description	React	Apps	Reg	Size	Cat. No.
BD CBA BCR-ABL Protein Kit	Hu	FCM	RUO(GMP)	50 tests	**643939**

References

1. Morgan E, Varro R, Sepulveda H, et al. Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clin Immunol.* 2004;110:252-266.

2. Ben-Neriah Y, Daley GQ, Mes-Masson AM, Witte ON, Baltimore D. The chronic myelogenous leukemia- specific P210 protein is the product of the bcr/ abl hybrid gene. *Science.* 1986;233:212-214.

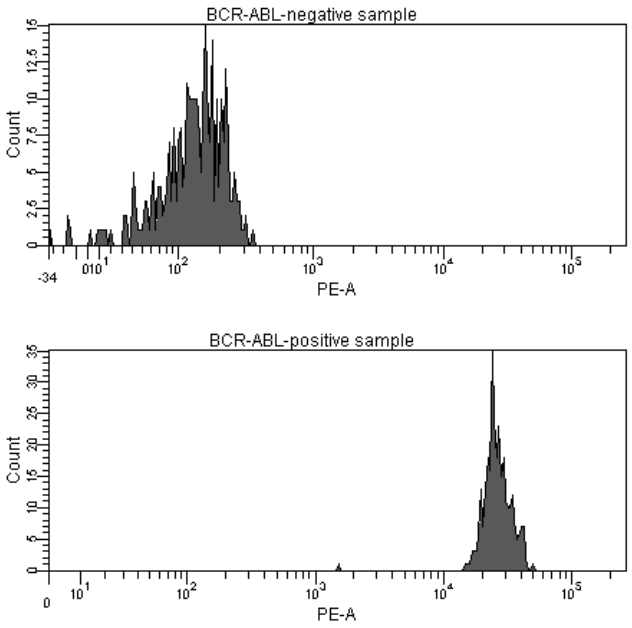

Figure 1. Once cell lysates are prepared using the BD BCR-ABL Protein Kit and samples are run in the flow cytometer, histograms show the presence or absence of BCR-ABL fusion proteins.

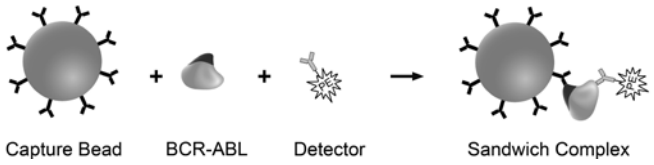

Figure 2. Formation of sandwich complexes in the BCR-ABL immunoassay.

Cytotoxic Cell Assays Taken One Step Further

Degranulating cells are identified by their surface expression of CD107a, which is a lysosomal-associated membrane protein (LAMP-1) residing in cytolytic granule membranes located within the cytoplasm. CD107a is mobilized to the cell surface following activation-induced granule exocytosis. Because of their parallel kinetics, CD107a and intracellular cytokines can be assessed at the same time in short-term-activated blood samples.

CD107a flow cytometric degranulation assay

- Designed for detection of degranulating T lymphocytes in activated whole blood
- Can be used as an alternative to ^{51}Cr release assays
- Detects cytolytic activity of $CD8^+$ T cells by measuring degranulation, a prerequisite for cytolysis ■

Description	React	Clone	Apps	Reg	Format	Size	Cat. No.
CD107a	Hu	H4A3	FCM	RUO(GMP)	APC	100 tests	**641581**

References

Betts, M, Brenchley J, Price D et al. Sensitive and viable identification of antigen-specific $CD8^+$ T cells by flow cytometric assay for degranulation. *J Immunol Met.* 2003;281:65-67.

BD Biosciences Expands Regulatory T Cell Offerings

As part of its ongoing efforts to support research in T cell regulation, BD Biosciences has expanded its portfolio with three new high quality research reagents. TGF-β1, CD39 (Clone TÜ66), and a new cocktail.

Human TGF-β1 monoclonal antibody: a powerful new tool for studying adaptive Treg populations

BD Pharmingen™-brand anti-human TGF-β1 PE conjugates are optimized for flow cytometric detection of TGF-β1, a member of the TGF-β superfamily. TGF-β1 is a key cytokine involved in a multitude of cellular processes and has recently been identified as playing an important role in the development and function of Regulatory T cells (Tregs).

Description	Clone	Isotype	Size	Cat. No.
BD Pharmingen PE Mouse anti-Human TGF-β	TB21	Mouse IgG_1, κ	100 tests	**560227**

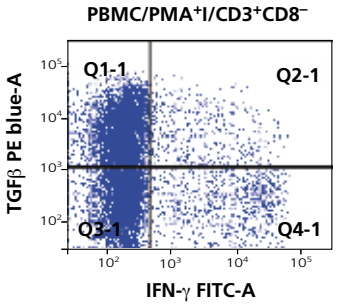

Figure 1. TGF-β expression in activated human PBMCs.

Human PBMCs were activated with PMA and ionomycin, in the presence of Monensin and Brefeldin A (BFA) for 24 hours at 37°C. Activated cells were processed by fixation and permeabilization followed by staining with FITC-conjugated IFN-γ (Cat. No. 554551) and PE-conjugated TGF-β (Cat. No. 560227) monoclonal antibodies.

Human CD39 (clone TÜ66): enhanced characterization of Tregs

CD39 is a recently identified marker for human Tregs. The BD Pharmingen-brand anti-human CD39 (clone TÜ66) monoclonal antibody is available in PE and APC formats for use in flow cytometry. TÜ66 recognizes ENTPD1, an ectoenzyme that belongs to the family of ectonucleoside triphosphate diphosphohydrolases (E-NTPDases). Recently, it has been shown that CD39 is also expressed in FoxP3 expressing $CD4^+$ Tregs in both human and mice, thereby adding to the list of cell surface markers such as CD25 and CD127 that could be used to identify $CD4^+$ Tregs.[1,2]

Description	Clone	Isotype	Size	Cat. No.
BD Pharmingen PE Mouse Anti-Human CD39	TÜ66	Mouse IgG_{2b}, κ	100 tests	**555464**
BD Pharmingen APC Mouse Anti-Human CD39	TÜ66	Mouse IgG_{2b}, κ	100 tests	**560239**

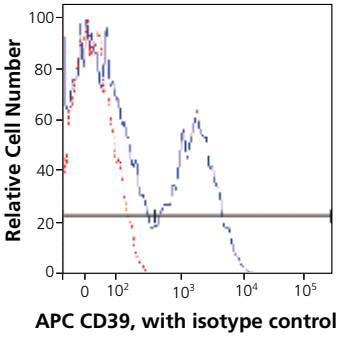

Figure 2. Analysis of human CD39 expression in whole blood.

Whole blood samples were stained at room temperature in the dark for 20 minutes with FITC CD4 clone RPA-T4 (Cat. No. 555346), PE-Cy7 CD25 clone M-A251 (Cat. No. 557741), PE CD127 clone hIL-7R-M21 (Cat. No. 557938), and APC CD39 clone TÜ66 (Cat. No. 560239). The cells were then lysed and washed twice in 1% FBS wash buffer, and samples were run on a BD FACSCanto™ system.

References

1. Borsellino G, Kleinewietfeld M, Mitri D et al. Expression of ectonucleotidase CD39 by FOXP3+ Treg cells: hydrolysis of extracellular immune suppression. *Blood.* 2007; 110:(4):1225-1232.

2. Deaglio S, Dwyer, KM, Gao W, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med.* 2007; 204:(6):1257-1265.

BD Human Regulatory T Cell Cocktail (CD4/CD25/CD127): simplify identification and isolation of Tregs

The BD Pharmingen-brand Human Regulatory T Cell Cocktail is a three-color reagent that can be used to identify $CD4^{+}CD25^{int/high}CD127^{low}$ live natural Treg populations. The reagent cocktail includes anti-human CD4 FITC (clone SK3), anti-human CD25 PE-Cy7 (clone 2A3), and anti-human CD127 Alexa Fluor® 647 (clone hIL-7R-M21). The one-step pre-mixed cocktail simplifies Treg identification and enhances enrichment of live Treg populations by 2 to 4 times compared to gating on $CD25^{high}$ alone. ■

Description	Clone	Isotype	Size	Cat. No.
BD Pharmingen™ Human Regulatory T cell cocktail:			100 tests	**560249**
CD4 FITC	SK3	Mouse IgG_1, κ		
CD25 PE-Cy7	2A3	Mouse IgG_1, κ		
CD127 Alexa Fluor® 647	hIL-7R-M21	Mouse IgG_1, κ		

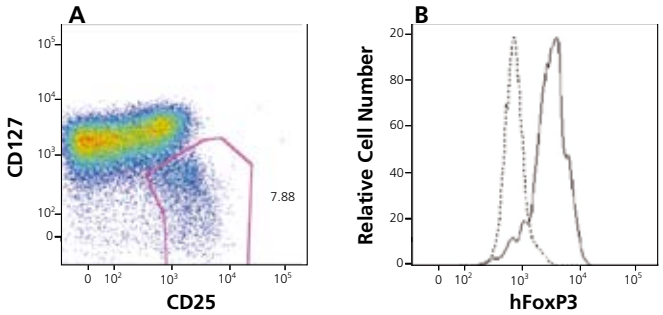

Figure 3. Three-color analysis of the expression of CD4, CD25, and CD127 on peripheral blood mononuclear cells (PBMCs).

PBMCs were stained with either an Isotype Control (Cat. No. 557872/555909, data not shown) or Human Regulatory T Cell Cocktail (Cat. No. 560249). The PBMCs were then fixed, lysed, and permeabilized using the BD Pharmingen Human FoxP3 Buffer Set (Cat. No. 560098) and stained with PE conjugated anti-human FoxP3 monoclonal antibody (Cat. No.560082). During data analysis, lymphocytes were identified by light scatter profile and CD4 positive expression. ***A)*** *Data representing the CD25 and CD127 expression profile of the CD4 positive cells.* ***B)*** *Data showing hFoxP3 expression on $CD127^{low}/CD25^{high}$ Tregs (solid line) and other T cells (dashed line). Flow cytometry was performed on a BD FACSCanto™ system.*

BD Cell Cycle Kit

BD Biosciences introduces the BD™ Cell Cycle Kit, our latest addition to the BD Bioimaging Certified Reagents product line. This cost-effective kit lets you simultaneously determine the percentage of cells in M and S phase using a one-step staining reaction. Further multiplexing can be accomplished by using additional probes for a true high-content imaging assay.

The BD Cell Cycle Kit uses antibodies that are directly conjugated with fluorochromes emitting in the green (Alexa Fluor® 488) and red (Alexa Fluor® 647) channels. This allows for an additional BD Bioimaging certified reagent in the orange channel (for example, Alexa Fluor® 555) to be multiplexed with the kit antibodies. The kit also uses Hoechst nuclear dye.

Features

- A three-color application using fluorescent antibodies in combination to determine the M and S phases of the cell cycle and specific protein expression
- Qualified for use in high-content screening (HCS) assays for generating reliable and reproducible results
- A simple protocol requiring no optimization or assay development, thus saving you time
- Validated on the BD Pathway™ 435 and 855 high-content bioimagers, and compatible with other HCS platforms and fluorescence microscopes ■

Description	Cat. No.
BD Cell Cycle Kit (96-well plate or 384-well plate)	**558662**

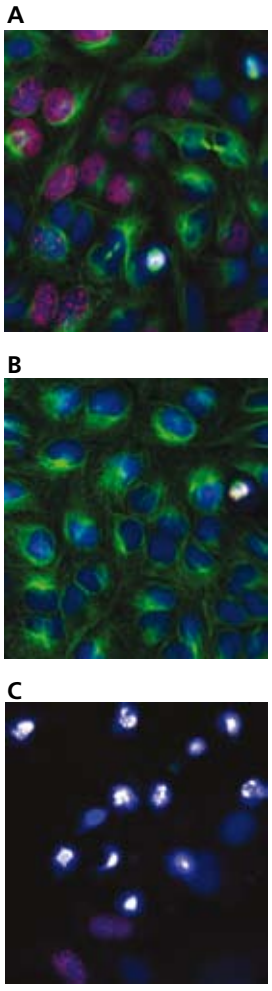

Figure 1. U-2 OS cells were either left untreated, or were treated with aphidicolin (250 ng/mL) for one hour or with colchicine (500 ng/mL) for 16 hours prior to BrdU loading.

Aphidicolin, a DNA polymerase inhibitor, blocks cells in early S phase, while colchicine, a microtubule polymerization inhibitor, blocks cells in M phase. Cells were stained according to the BD Cell Cycle Kit protocol. In addition to the two antibodies provided in the kit, an Alexa Fluor® 555 mouse anti–β-tubulin antibody (Cat. No. 558608) was used at the same time to monitor the microtubule depolymerization. Representative 20x images are shown. Hoechst staining is pseudocolored blue, BrdU staining is pseudocolored red (appears pink when co-localized with blue), Histone H3 (pS28) staining is pseudocolored yellow (appears white when co-localized with blue), and β-tubulin staining is pseudocolored green. Image analysis (data not shown) revealed that untreated U-2 OS cells (Panel A) had approximately 30% cells in S phase (pink) and approximately 3% cells in M phase (yellow/white). Aphidicolin treatment (Panel B) blocked cells from entering S phase, but there was no effect on β-tubulin or on cells entering M phase. Conversely, no β-tubulin staining (cytoplasmic intensity was measured using an 8-pixel ring dilated out from the nuclear mask identified by the Hoechst stain) was detected when cells were treated with colchicine (Panel C) and the percentage of cells in M phase increased to 50%.

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- Cell pelleting and washing
- Gradients
- Sample and buffer storage
- DNA/RNA/plasma preps
- Serial dilutions
- Sterile tissue collection
- Immunofluorescent assays
- Storage of chemicals ■

1. Flip open 2. Pipet 3. Mix 4. Ready

Description	Qty./Pack	Qty./Case	Cat. No.
BD Falcon 50-mL Conical Tubes with Flip-Top Cap	22	440	352077

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Anti-Human HLA-ABC (anti-MHC Class I) antibody (Clone DX17)

An important tool for studying inhibitory receptors on human T and NK cells is now available. Functional grade (BD NA/LE™; no azide/low endotoxin) anti-MHC class I neutralizing antibody (clone DX17) is useful for blocking HLA Class I recognition by various inhibitory receptors such as the KIR, ILT2, and CD94/NKG2A.

- Functionally disrupts both NK and $CD8^+$ T cell recognition of MHC class I
- Recognizes all human MHC class I molecules (HLA -A, -B, -C, -D, -E, -G)
- Mouse IgG_1 does not induce ADCC while blocking

In addition to the functional grade antibody, anti-human HLA-ABC clone DX17 is also available as a direct conjugate to either PE or Alexa Fluor® 488 for flow cytometric analysis.

BD Phosflow Reagents

BD Biosciences continues to expand its range of directly conjugated phosphorylation site-specific antibodies. BD™ Phosflow is an innovative flow cytometry-based technology that enables activation state analysis of multiple proteins at single cell levels. Used in combination with cell surface markers, BD Phosflow reagents make it possible to study phosphorylation events directly in small subpopulations of complex primary samples.

Top new releases include:

IRAK-4

The IRAK family consists of four identified Pelle homologs that regulate the activation of NF-κB and mitogen-activated protein (MAP) kinase pathways. IRAK4 is the closest mammalian homolog to Pelle. When overexpressed, its kinase activity allows it to activate both the NF-κB and mitogen-activated protein (MAP) kinase pathways, and block the IL-1-induced activation and modification of IRAK1. IRAK4 is also able to phosphorylate IRAK1, suggesting that IRAK4 affects the early signal transduction of Toll/IL-1 receptors before the involvement of IRAK1.

IRF-7
Interferon regulatory factor 7 (IRF-7) is a transcription factor that regulates anti-viral defenses by controlling the induction of type-I interferon (IFN) responses. IRF-7 expression is induced in lymphoid cells by virus infection, as well as by IFN, lipopolysaccharide and TNF-α. IRF-7 responses are initiated by Toll-like receptors (TLR) or the cytoplasmic protein retinoic acid inducible gene I (RIG-I).

4EBP1
The eukaryotic translation initiation factor 4E-Binding Protein 1 (4EBP1) is a phosphorylated heat- and acid-stable protein (PHAS-I or PHAS-1) that is regulated by insulin. Phosphorylation of 4EBP1 is required for protein synthesis, and it mediates the regulation of protein translation by stimuli that signal through the phosphoinositide 3 (PI3) kinase pathway.

GATA4
GATA4 (GATA-binding protein 4) is a member of the GATA family of zinc finger-containing transcription factors that bind to the GATA nucleotide sequence. Genetic studies suggest that GATA4 regulates embryonic cardiac development: in mice, disruption of the GATA4 gene leads to defects in heart tube formation, while mutations of GATA4 are associated with atrial septal defects in humans. In the adult heart, GATA4 regulates differentiated gene expression. The roles of GATA4 in endocrine and reproductive functions were recently reviewed.

SHP2 (pY542)
SHP2 is a member of the cytosolic class of protein-tyrosine phosphatases (PTPs). SHP2 reportedly contains two SH2 domains, both of which are N-terminal to the PTP catalytic domain. SH2 PTPs are believed to work in conjunction with protein-tyrosine kinases to maintain intracellular protein phosphotyrosine homeostasis and cell cycle progression.

Visit **bdphosflow.com** to learn more.

NEW Markers for Stem Cell Research

BD Biosciences has just added new antibodies to several key stem cell markers, further expanding the selection for the identification and characterization of various stem cell types. Several specificities are available as direct conjugates to facilitate multi-color analyses by imaging and/or flow cytometry.

SSEA-3
Stage-Specific Embryonic Antigen-3 (SSEA-3) is a carbohydrate epitope found on the major ganglioside, but not the neutral glycolipid, of mouse embryos and human teratocarcinoma cells. As its name implies, the expression of SSEA-3 is stage-specific and can be used to characterize embryonic cells and monitor their differentiation.

Sox2
Sox2 [SRY (sex determining region Y)-box 2] is a member of the SRY-related HMG-box (SOX) family of transcription factors. Sox2 is required for the maintenance of the undifferentiated state of pluripotent stem cells. Complexes of Sox2 with the homeobox transcription factors Oct3/4 and/or Nanog bind to the promoters of a network of genes that are involved in the maintenance of pluripotency and self renewal in stem cells.

TAZ
Taz is a 49-kDa transcriptional co-activator with a PDZ-binding motif and is regulated by binding with 14-3-3 proteins. It plays a key role in differentiation of mesenchymal stem cells into either osteoblasts or adipocytes via interactions with key transcription factors Runx2 and PPARγ. More recently, Taz was found to be a component of an E3 ubiquitin ligase involved in ubiquitin-dependent substrate proteolysis, thus showing dual functions of regulating protein degradation and transcription.

CDX-2
In embryonic development, caudal-type homeobox transcription factor 2 (CDX-2) is first expressed in the outer layer of the morula and later in the trophoblast, but not in embryonic stem cells. Along with Oct3/4, it is involved in the segregation of the trophoectoderm lineage from the inner cell mass. In the adult, CDX-2 expression is limited to the mucosa of the small and large intestines, where it regulates the synthesis of several intestine-specific proteins.

Oct3/4
Oct3/4, a member of the POU family of transcription factors, plays an important role in determining early steps of embryogenesis and differentiation. It functions in pluripotent cells of early embryonic stem cell (ES) lines and embryonal carcinomas (EC). Oct3/4 is expressed in undifferentiated cells, but is lost as cells are induced to differentiate. It is not expressed in adult tissues. The interaction of Oct3/4 with Sox2 produces an active complex that regulates expression of genes such as Nanog, UTF1 and FGF4.

Visit **bdbiosciences.com/stemcellsource** to learn more.

Bioimaging Certified Reagents

BD Biosciences bioimaging products address application needs in automated bioimaging (high content screening) and fluorescence microscopy. New specificities include:

Nanog
This transcription factor is required for the maintenance of the differentiated state of pluripotent stem cells. Nanog expression counteracts the differentiation-promoting signals induced by LIF and BMP. When Nanog expression is down-regulated, cell differentiation can proceed.

SSEA-1 (CD15) and SSEA-4
Stage-specific embryonic antigens (SSEA) SSEA-1 and SSEA-4 are related to Lewis blood group antigens and are found in a variety of embryonic and adult tissues and cancers. As their name implies, the expression of SSEA-1 and -4 is stage-specific and can be used to characterize embryonic cells and monitor their differentiation.

TRA-1-60 and TRA-1-81
The TRA-1-60 and TRA-1-81 monoclonal antibodies react with a pluripotent stem cell specific epitope on a high molecular weight transmembrane glycoprotein. The expression of TRA-1-60 and TRA-1-81 antigens is stage-specific and can be used to characterize embryonic stem cells and monitor their differentiation.

NEW Products for Regulatory T Cell Research

As part of its ongoing efforts to support research in T cell regulation, BD Biosciences has expanded its portfolio with three new high quality research reagents:

Human TGF-β1
TGF-β1, a member of the TGF-β superfamily, is a key cytokine involved in a multitude of cellular processes and has recently been identified as playing an important role in the development and function of Regulatory T cells (Tregs). BD Pharmingen™ anti-human TGF-β1 PE conjugates are optimized for flow cytometric detection of TGF-β1.

Human CD39
BD Pharmingen anti-human CD39 (clone TÜ66) monoclonal antibody recognizes ENTPD1, an ectoenzyme that belongs to the family of ectonucleoside triphosphate diphosphohydrolases (E-NTPDases). CD39 has recently been shown to be expressed in FoxP3 expressing $CD4^+$ Tregs in both human and mice. BD Pharmingen anti-human CD39 PE and APC conjugates are now available for use in flow cytometry.

Human Regulatory T Cell Cocktail (CD4/CD25/CD127)
The BD Pharmingen Human Regulatory T Cell Cocktail is a three-color reagent that can be used to identify $CD4^+CD25^{int/high}CD127^{low}$ live natural Treg populations. The one-step pre-mixed cocktail simplifies Treg identification and significantly enhances enrichment of live Treg populations compared to gating on $CD25^{high}$ alone. The cocktail includes anti-human CD4 FITC (clone SK3), anti-human CD25 PE-Cy™7 (clone 2A3) and anti-human CD127 Alexa Fluor® 647 (clone hIL-7R-M21), leaving the PE channel open for drop-in of an additional marker pre- or post-sorting.

Find more details on pages 7-8
or visit **bdbiosciences.com/treg** for more information.

NEW BD Horizon V450 reagents for the violet laser

BD Horizon™ V450 reagents are high quality antibody conjugates designed to deliver exceptional performance on BD FACS™ brand flow cytometers equipped with a violet laser. The first in a series of new fluorochromes from BD Biosciences, these reagents are engineered to deliver high sensitivity and consistent results.

- Optimized for the violet laser
- Wide portfolio of conjugates, multiple sizes
- Compatible with other BD reagents used in multicolor cocktails

In addition to the functional grade antibody, anti-human HLA-ABC clone DX17 is also available as a direct conjugate to either PE or Alexa Fluor® 488 for flow cytometric analysis.

Find more details on pages 4-5
or visit **bdbiosciences.com/colors** for more information.

NEW BD Cytometric Bead Array (CBA) Flex Set Products

BD™ CBA is a flow cytometry application that enables quantitation of multiple proteins simultaneously from a single sample. New BD CBA products include:

NEW Mouse Antibodies

RANKL/TRANCE

RANKL (Receptor Activator for NFκB Ligand), also known as TNF-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL) and ODF (osteoclast differentiation factor) has been implicated in interactions between T cells and dendritic cells. It is also important in T- and B-cell maturation. As OPGL/ODF, the same protein can also activate mature osteoclasts and mediate osteoclastogenesis.

CD172a

CD172a, also known as SIgnal-Regulatory Protein α (SIRPα), Src Homology 2 domain-containing protein tyrosine Phosphatase (SHP) Substrate 1 (SHPS-1), or Brain Immunoglobulin-like molecule with Tyrosine-based activation motifs (BIT) is an adhesion molecule of the Ig superfamily that is expressed on neurons in the central nervous system and the retina, on macrophages and on bone-marrow myeloid cells. Its ligand, CD47, is expressed by a wide variety of cells. CD172a and CD47 are proposed to mediate bi-directional signaling to modify neural synaptic activity and regulate the phagocytic activities of macrophages. ■

Description	React	Apps	Reg	Size	Cat. No.
Human IL-17A Flex Set (Bead B5)	Hu	FCM	RUO	100 tests	**560383**
Human IL-21 Flex Set (Bead B8)	Hu	FCM	RUO	100 tests	**560358**
Human soluble CD40L Flex Set (Bead C7)	Hu	FCM	RUO	100 tests	**560305**
Human soluble CD121a Flex Set (Bead B6)	Hu	FCM	RUO	100 tests	**560276**
Human soluble CD121b Flex Set (Bead B7)	Hu	FCM	RUO	100 tests	**560281**
Human Fraktalkine Flex Set (Bead C6)	Hu	FCM	RUO	100 tests	**560265**
Human IL-1α Flex Set (Bead D6)	Hu	FCM	RUO	100 tests	**560153**
Human IL-11 Flex Set (Bead D5)	Hu	FCM	RUO	100 tests	**560228**
Human IL-12/IL-23p40 Flex Set (Bead E5)	Hu	FCM	RUO	100 tests	**560154**
Human soluble TNFR I Flex Set (Bead C4)	Hu	FCM	RUO	100 tests	**560156**
Human soluble TNFR II Flex Set (Bead C5)	Hu	FCM	RUO	100 tests	**560155**
Mouse IL-1β Flex Set (Bead E5)	Ms	FCM	RUO	100 tests	**560232**
Mouse G-CSF Flex Set (Bead D4)	Ms	FCM	RUO	100 tests	**560152**
Mouse IL-1α Flex Set (Bead E4)	Ms	FCM	RUO	100 tests	**560157**
Mouse IL-12/IL-23p40 Flex Set (Bead D7)	Ms	FCM	RUO	100 tests	**560151**
Mouse IL-17A Flex Set (Bead B5)	Ms	FCM	RUO	100 tests	**560283**
Mouse IL-21 Flex Set (Bead B6)	Ms	FCM	RUO	100 tests	**560160**
Rat IL-1α Flex Set (Bead A4)	Rat	FCM	RUO	100 tests	**560159**
Phospho-Akt1 (S473) Flex Set (Bead A4)	Hu	FCM	RUO	100 tests	**560144**
Phospho-Akt1 (T308) Flex Set (Bead A4)	Hu	FCM	RUO	100 tests	**560208**
Total Akt1 Flex Set (Bead A4)	Hu	FCM	RUO	100 tests	**560206**
Phospho-Akt2 (S474) Flex Set (Bead A5)	Hu	FCM	RUO	100 tests	**560147**
Phospho-Akt2 (T309) Flex Set (Bead A5)	Hu	FCM	RUO	100 tests	**560146**
Total Akt2 Flex Set (Bead A5)	Hu	FCM	RUO	100 tests	**560207**
Phospho-BLNK (Y84) Flex Set (Bead C9)	Hu	FCM	RUO	100 tests	**560063**
Total Jnk Flex Set (Bead B5)	Hu	FCM	RUO	100 tests	**560214**
Total p38α Flex Set (Bead B6)	Hu	FCM	RUO	100 tests	**560145**

Visit **bdbiosciences.com/flexset** to learn more.

BD Biosciences Japan Hosts Thought Leaders

This past November, BD Biosciences Japan hosted a symposium that included Leonard Herzenberg, PhD, lead inventor of the Fluorescence Activitated Cell Sorter (FACS), the foundation of BD flow cytometers. Dr Herzenberg, a winner of the 2006 Kyoto prize,* and other leading researchers discussed recent findings.

Held in both Tokyo and Osaka on November 12 to 13, the meeting brought thought leaders together with researchers and students to inspire young investigators and advance research in key areas of life sciences. In addition to Dr. Herzenberg, the roster of prestigious speakers included:

Hideyuki Saya, MD, PhD, Professor in the Division of Gene Regulation of the Institute for Advanced Medical Research at Keio University School of Medicine. Dr. Saya spoke on the topic of molecular mechanisms of anti-cancer therapies.

Shin-Ichi Nishikawa, MD, Deputy Director and Group Director of the Stem Cell Lab at the Riken Center for Developmental Biology in Kobe. Dr. Nishikawa spoke on the topic of combining FACS technology, ES cell differentiation culture, and DNA microarrays.

Shigekazu Nagata, PhD, Professor in the Graduate School of Medicine at Kyoto University. Dr. Nagata spoke on the topic of clearance of apoptotic cells.

Tadatsugu Taniguchi, PhD, Professor and Chair of the Department of Immunology, Graduate School of Medicine and Faculty of Medicine, University of Tokyo. Dr. Taniguchi spoke on the topic of Interferon Regulatory Factors (IRFs) and their friends in innate immunity and oncogenesis.

Leonore Herzenberg, Dsc, Professor at Stanford University School of Medicine, who is credited as the co-inventor of FACS and is married to Leonard Herzenberg. She spoke about modern analysis and control software for FACS.

"We were very grateful to the luminaries who took time out of their busy schedules to give us their latest thinking on topics that will have a profound impact on human health in the coming years," said William Rhodes, President of BD Biosciences Cell Analysis Unit who attended the event with Vera Imper, the unit's Vice President of Business Development.

"It was a particular treat to have the Herzenbergs join us," added Frank Florio, General Manager of BD Biosciences Japan. "Their groundbreaking development of the FACS cell sorter is certainly credited as the achievement that started the whole biotech industry. I am particularly thankful for the extraordinary effort and professionalism made by the BD Biosciences Japan team to realize this event. Based on its success we plan to sponsor future gatherings to help build relationships with both students and researchers in the life sciences community."

*Herzenberg was awarded the 2006 Kyoto Prize for his outstanding contribution to life sciences with the development of a flow cytometer that uses fluorescent-labeled monoclonal antibodies. The Kyoto Prize is an international award to honor those who have contributed significantly to the scientific, cultural, and spiritual betterment of humankind. The Prize is presented annually in each of the following three categories: Advanced Technology, Basic Sciences, and Arts & Philosophy. ■

Left to right: William Rhodes (President, BD Biosciences - Cell Analysis), Leonore Herzenberg, Dsc (Professor, Stanford University School of Medicine), Leonard Herzenberg, PhD (Professor, Stanford University School of Medicine), and Frank Florio (General Manager, BD Biosciences in Japan).

From Inspiration to Application: the Unlikely Origins and Development of the Fluorescence Activated Cell Sorter (FACS)

In the early 1960s, while laboriously counting fluorescent cells one by one under a microscope, Dr. Len Herzenberg hit on the idea of developing an instrument that would minimize his labor to leave more time for discovery.

Although no instrument existed, Herzenberg learned of an instrument developed at Los Alamos National Laboratory that might be used for cell sorting and counting. Designed to measure radioactivity of fallout by sorting and analyzing particles from the lungs of animals exposed to a nuclear blast, the instrument measured particles by size. Sorting by size alone wouldn't serve Herzenberg's requirements. Instead, he suggested the instrument be adapted to measure fluorescence in addition to size.

Although Herzenberg vigorously argued for the dramatic impact on research into human disease conditions and treatment, the Los Alamos team was not enthusiastic about developing the new application themselves. However, they allowed him to take the blueprints of their work back to Stanford, where over coffee and between ping pong matches, Herzenberg and his team pieced together the machine he envisioned.

The instrument, which Herzenberg and colleagues named the Fluorescence Activated Cell Sorter (FACS), automatically sorts viable cells by their properties. By using fluorescent-labeled monoclonal antibodies as reagents with the instrument, Herzenberg made an enormous contribution toward the advancement of life sciences and clinical medicine.

Since 1970, the FACS sorter has been the cornerstone of BD instrument offerings for both clinical and research use. Today, BD FACS™ flow cytometers can process 100,000 cells per second, measure 18 fluorescent colors, and support a growing number of applications. Clinical applications include monitoring the progress of HIV by counting the number of CD4 T cells in blood, and enumerating CD34 cells. In addition, a wide range of research applications are leading to new discoveries that support the BD promise to help people live healthy lives.

Interview with Timothy Bushnell, PhD

Director, CPBR Flow Cytometry Laboratory, University of Rochester, NY, USA

Dr. Bushnell knows that discovery is no accident—it's the result of discipline, imagination, and the determination to keep on trying.

Did you always want to be a scientist?
I think back in high school I decided I was going to do some sort of science. I initially thought I'd be an MD, but that changed when I became fascinated by research.

What do you think makes a good scientist?
Perseverance. Mother Nature reveals her secrets to us very slowly, and you can't be frustrated when things don't go well. You have to believe that you're going to get there, and stick through experiments that 99% of the time don't give you anything. Then finally there's the 1% that gives you that "Eureka!" moment—that moment when you finally uncover something that no one's ever discovered before.

What's the focus of the work that you do here?
I manage a facility for flow cytometry, and we support a large group of users who are doing research on topics that range from red blood cell development to identifying the cells that we believe initiate leukemias, and how can we identify them, treat them, and eradicate them to improve outcomes. We've also been working with groups looking at prostate and bladder cancers, as well as trying to understand the new responses in the world of B cells and T cells.

Are there any particular questions you're seeking to answer?
At the moment, I support a lot of different investigators in their work. They bring me their questions, their samples, and their problems, and it's my job to help them apply flow cytometry to it all. I'm helping them answer everything from "how do I identify this very rare population and pull it out so we can study it?" to "We want to be able to measure a given reaction within a cell type under these conditions. Can we do it with flow cytometry?" I try to answer that little puzzle that they haven't worked out yet.

How do you think flow cytometry has contributed to the advancement of research in the past 20 years?
I think it has become the central tool. There are very few questions you cannot answer with flow cytometry. The technology allows you to answer questions at the single-cell level so you can look at each individual cell, probe what it is, and then understand what it's doing. You can see how the cell is working, how the cell is being affected by a drug. We have the ability to identify samples with 14, 15, 16, 17 fluorochromes at the same time.

More colors lead to more answers. More answers lead to more questions—repeat as necessary. At the end of the day we're getting to a point where we can do experiments on rare samples and understand much more completely what's happened. If you think about cytometry 20 years ago, and compare that to what we can do today, you can't really say enough about what the technology can do and where you can go with it.

What are some of those advancements?
We can do things that we were never able to do before, and that's really because of the speed of computer processing, the availability of new lasers with more power, more control over the lasers, even the size of the lasers. And now we can do 17 colors simultaneously because the processing speed of computers lets us collect the data at that speed, and the power, the lasers, and the flexibility let us identify large numbers of cells, using different fluorochromes and different areas of the spectrum that were previously inaccessible.

What technological advances do you think are needed in the future?
That is actually more of a chemical issue. We need to get more dyes that take advantage of the large spectrum. The visible color spectrum is only a small fraction of the complete spectrum. If we can move into the far infrared to get more information out, that would be great.

Improved processing is helping us do image analysis so we can correlate the morphological characteristics, which are well characterized, with the phenotype, for example. This is important in development. We're starting to open up an area that is still in its infancy, and what we accomplish there is going to be driven, I think, by the ability to use more colors and the chemical nature of fluorochromes.

Who are your heroes?
I'd have to say one of my heroes is not a scientist—it's Winston Churchill. The man persevered throughout his life, was never afraid to be wrong, but always believed in the righteousness of his ideas and his cause, and moved forward in that. If you study his history, you see that perseverance. He was not afraid to change his mind when presented with new evidence, and that's when some people get very argumentative. Yet it's important not to be dogmatic, to look at all the facts and all the data. Churchill is the type of person that I hope to emulate. That really feeds into what you were saying about what makes a good scientist. I think so, and it's interesting. You find your inspiration where you can.

Have you ever been surprised by the outcome of your research?
I think you're always surprised. You predict certain outcomes based on the data that you had, but then you look at something else and say, well, that doesn't make sense, or that doesn't fit. That's when you go back and try to understand what you missed, what assumptions were false or incorrect in some manner, and how you can revise those. It's the whole fundamental scientific principle, you know—gather data, form a hypothesis, test the hypothesis, and repeat.

Surprise is an important element of learning. We do our experiments based on our predictions, and what confirms our hypotheses, but generally that also leads to further questions. You know, we have an extra channel, we throw in an extra fluorochrome, and something comes out that you might not have predicted. If you look at it, it starts to lead to a new pathway and you find these connections that weren't necessarily obvious to you at first glance. The cell is an amazing machine, and we're still learning what it can do.

Do you work in a way that opens the way for more people to use flow?
I'm a very big proponent of showing people what you can do with it. I like to challenge people who've never done flow cytometry, never used the technology. Just let me work with them to see if we can bring something out that they haven't been able to before. I think that even the casual user needs to understand how they can make more use of flow, and I'm working hard with groups who have only done very simple experiments to try to expand what they can do.

What do you see as your most significant contribution?
I hope that I will inspire a generation of young scientists to continue moving forward. I love to see the scientists I've worked with in graduate school go on to successful careers, and know that they understand the technology and the ideas that they've worked with, and can apply them in their own work. I just hope that people see that perseverance is important, and dedication, that with that combination (and a little bit of luck), you'll move forward and do something that no one's done before. ■

Prof. Dr. Didier G. Ebo – Winner of the *Clinical Cytometry* Award for Best Original Paper published in 2006-2007

Basophil Activation in Allergy Assessment – Go with the Flow

Didier G. Ebo, PhD, MD
Department of Immunology-Allergology-Rheumatology, University of Antwerp, Belgium

Prof. Dr. Didier G. Ebo currently works as a researcher in the Department of Immunology-Allergology-Rheumatology at the University of Antwerp, Belgium. He also serves as a medical practitioner and consultant at Antwerp's University Hospital in the fields of immunology, allergology, and rheumatology, and has been certified as Specialist in Internal Medicine and Rheumatology by the Belgian Ministry of Health.

In addition to his roles as a researcher and physician, Ebo actively participates in several committees and working groups. Notably, he is member of the Working Group for Allergic Aspects of Genetically Modified Foods and Feeds at the Scientific Institute for Public Health, Biosafety Council (Brussels, Belgium), and of the Ad hoc Working Group on Medical Devices Manufactured from Natural Latex at the European Commission (Brussels, Belgium).

Ebo received his Bachelor of Science degree in Medicine and his Doctor of Medicine degree from the University of Ghent, Belgium in 1984 and 1988, respectively. He obtained his PhD in Medical Sciences from the University of Antwerp, Belgium in 2003.

Passionate about Allergology

One of the reasons why I got interested in allergology, is that I had a fascinating professor on the topic. My professor in pneumology at the University of Ghent definitely was an interesting man who could keep you busy. He introduced me and my fellow students to allergology at a time when knowledge in the field was beginning to grow. I then kept up with allergology during my studies, more specifically with clinical research in allergy, with a particular interest in methods for allergy diagnosis. One of the first topics on which I concentrated my research efforts was latex allergy; my PhD work focused on the role of IgE in the diagnosis of allergy to natural rubber latex.

Allergy assessment: when traditional tests don't tell the truth

Traditional approaches to allergy diagnosis include the recording of patient medical history, specific IgE quantitation, skin tests and challenge tests, all of which *in se* bear limitations and frequently have poor predictive value. Medical history can be inaccurate and misleading; in vivo tests, particularly challenge tests bear a risk of fatality from anaphylaxis and therefore cannot be recommended in daily practice. One of my first and major findings was that the quantitation of serum-specific IgE antibodies – one of the most widely used methods to diagnose latex anaphylaxis and IgE-mediated allergies in general – fails to demonstrate absolute diagnostic reliability,[1-3] especially when more complex reactions such as food, drug, or insect venom allergies are being considered. Specificity of IgE quantitation can indeed be relatively low, in that an individual without overt allergy may still show positive IgE test results. One of the reasons for this is that the traditional IgE test is based on monovalence. Unlike in the clinical – in vivo – situation, from the moment one IgE antibody binds to one molecule of allergen, the IgE quantitation test will be positive. In vivo, multivalent – at least bivalent – binding is needed for IgE antibodies to be able to cross-link the IgE receptors on the surface of basophils and mast cells and thereby initiate an (IgE-mediated) anaphylactic reaction. Correct diagnosis is a prerequisite for effective management of allergy. We therefore sought to develop an in vitro diagnostic test that would reflect the clinical situation and provide a better correlate of patient hypersensitivity.

The basophil activation test: a promising tool for allergy diagnosis

The discovery of basophil activation markers such as CD63 led to the development of the so-called basophil activation test (BAT), a flow cytometric approach to measure in vitro allergen-specific activation of peripheral blood basophils.[3,4] What you do is take a blood sample from your patient, mix it with your allergen of interest, and then measure activation of the basophils in your sample. Upon activation by relevant allergen through cross-linking of FceRI surface receptor-bound IgE, basophils do not only secrete bioactive mediators such as histamine and leukotriene, but also up-regulate the expression of different surface markers, notably CD63.[5] Double-staining for receptor-bound IgE and up-regulated surface CD63 therefore allows for basophil activation to be readily detected at the single-cell level using multicolor flow cytometry.[3,4] Our first application of the BAT was latex allergy. Why that? Because in a former study I had observed that latex-specific IgE was quite prevalent in individuals with pollen allergy, even though these individuals did not suffer from latex allergy.[1] We demonstrated that the BAT could constitute a highly sensitive and specific approach

Double-staining for receptor-bound IgE and up-regulated surface CD63 allows for basophil activation to be readily detected at the single-cell level using multicolor flow cytometry.

to latex allergy diagnosis and, more specifically, a most reliable tool for determining the clinical relevance of positive IgE quantitation in the absence of overt allergy.[6] What we observed in that study was that in almost 90% of the patients with false-positive latex-specific IgE, the BAT was negative; this was the starting point of the whole experience.

From allergy diagnosis to follow-up of therapy

Since its introduction in the mid to late nineties, the BAT has proven to be a safe, reliable, fast, and easily accessible approach to allergy assessment. After validating the BAT as an in vitro correlate for in vivo latex hypersensitivity, with reported specificity and sensitivity of almost 90%,[6] we have been looking to apply this novel tool to the diagnosis of more complex allergies such as food, drug, and venom allergies. We've been studying secondary food allergies, for example allergies that result from cross-reactivity to pollen allergens. In this context we demonstrated that the BAT could provide a powerful tool for the diagnosis of IgE-mediated apple allergy in patients with birch pollinosis, notably allowing for discrimination between clinically relevant and irrelevant IgE that cannot be achieved with specific IgE quantitation tests.[7] Another aspect we have focused on is hypersensitivity to drugs and related compounds. Diagnosis of drug allergies is indeed rendered difficult by the fact that different drugs – or their metabolites – can elicit both immune- and non immune-mediated pathologies, the mechanisms of which are not always clearly understood. Also, patients may be taking different drugs simultaneously, further adding to the complexity of allergy diagnosis. The BAT provides a major

The basophil activation test allows for discrimination between clinically relevant and irrelevant IgE that cannot be achieved with specific IgE quantitation tests.

advantage in this context, as it allows for simultaneous testing of different compounds. It has notably been proven useful for the diagnosis of anaphylactic (and anaphylactoid) reactions to neuromuscular blocking agents.[8] In a study on rocuronium, a muscle relaxant used in modern anesthesia to facilitate endotracheal intubation, we could demonstrate a sensitivity and specificity for the BAT of almost 92% and 100%, respectively.[9] We further showed that the test could prove helpful for identifying cross-reactivity between rocuronium and other muscle relaxants and for tailoring potentially safe alternatives. Another successful application of the BAT is hymenoptera venom allergy, with reported sensitivities and specificities of 85 to 90%.[10,11] Our recent publication in *Clinical Cytometry* – for which I was granted the award for Best Original Paper published in this journal in 2006-2007 – demonstrated that beyond wasp venom allergy diagnosis, the BAT also proves valuable for the follow-up of wasp venom immunotherapy (VIT).[12] Indeed we observed that after three years of VIT, 60% of the patients showed negative BAT results, while specific IgE had become negative in only 18% of the cases. Our investigations also were suggestive of basophil responsiveness to further decrease with time. The BAT could therefore represent a promising tool to guide the decision as to when to safely discontinue VIT.

Five reasons to "go with the flow"

A flow cytometric approach to basophil activation studies offers several advantages.

First, the technique requires only a minimal amount of sample, thus allowing for repetitive draws from the same individual.

Second, basophils do not need to be physically separated to ensure reliable and consistent data; the BAT can indeed be performed on whole blood samples. A major advantage of directly using whole blood is that one studies the cells in their natural environment. The risk of "accidentally" activating your cells or losing them is therefore much smaller.

Another major benefit of using flow cytometry in this context is the short time-to-result, as compared with other (more traditional) methods. Results are available in hours rather than days.

Also, and most important, a flow cytometric assay enables the identification of cells with different levels of responsiveness.

Last but not least, the BAT allows testing for several putative allergens simultaneously, which may be of major interest for investigating the more complex cases of allergies and cross-allergies.

***"Flow-assisted quantification of* in vitro *activated basophils in the diagnosis of wasp venom allergy and follow-up of wasp venom immunotherapy"* by Ebo et al. (*Clinical Cytometry* part B 2007;72b:196-203)**

Best Original Paper published in *Clinical Cytometry* 2006-2007

Individuals allergic to insect stings can be treated through specific venom immunotherapy (VIT). During this process, carefully calibrated injections of venom are administered over a long period of time in order to alter the patient's immune system so that it no longer reacts to the insect venom protein. VIT-treated patients may thereby attain long-term tolerance to insect venom. Currently, there is no tool to monitor the effectiveness of VIT. We basically treat patients for five years and then we stop, as we know for a fact that after three years patients already are clinically protected. It has been observed, however, that although reactivity to venom is decreasing in most treated patients, both skin tests and specific IgE tests often remain positive after five years of therapy. We showed that the BAT could in this case better reflect the clinical situation.

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Confounders: essential controls?

Study design is of major importance when validating a new method for use in clinical diagnostics. Taking the apple-mediated oral allergy syndrome (OAS) in patients with birch pollinosis as a case study, I showed that when validating a diagnostic test, it is critical that one also considers so-called potential confounders. Indeed, a number of allergies are more prevalent in atopics than in non-atopics; an adequate control group should therefore not be restricted to healthy volunteers. If one only compares completely healthy controls, having no birch pollen or apple allergy, with patients suffering from apple allergy and who by definition also suffer from a birch pollen allergy, then all tests will show a nice sensitivity and specificity. In the case of pollen-associated OAS, one would also need to test pollen allergic individuals with no clinical history of fruit allergy but the majority of which still would present apple-specific IgE antibodies.

Further potential confounders are non-responders. Our studies and those of others in the field have shown that a number of patients do not react to positive control stimulation, such as anti-IgE (activating) antibody; these patients neither show histamine release nor leukotriene production upon stimulation.[13-16] In those cases, interpretation of negative allergen stimulation becomes impossible. If the test is lost as a diagnostic tool for these particular individuals, such results nevertheless need to be considered – as false negatives – and reported accordingly. We have observed non-responders in almost all our studies; we've been publishing articles in which we reported 10 or even 15% of non-responders. In any case it needs to be stated: were non-responders observed, yes or no – otherwise the data cannot be considered as correct. Several factors may explain the occurrence of these false negative results. Recent exposure to allergen can for instance result in transient basophil refractoriness. Another explanation could be that these individuals have been taking medications, which may influence the outcome of the BAT. Finally, there is evidence that the pathway behind basophil activation might be disrupted in non-responders, with one or more signal transduction molecules being either missing or functionally inactive.

A tool for mainstream diagnostics, or for specific cases?

Most clinical studies conducted so far have been applying the BAT to cases of IgE-mediated anaphylaxis. However, a lot of allergies are not IgE-mediated. A major advantage of the BAT in this context is that the test does not require an IgE-based mechanism; it only requires basophil activation. Once the basophil gets activated, it doesn't matter what the mechanism is. The BAT could therefore, in theory, be used for a broad array of applications, from allergy diagnosis to functional analysis of allergens and allergoids. One could for instance apply this approach to search for residual allergens in food, or to functionally validate natural or recombinant allergens. Functional analysis becomes more and more general practice, and it is especially critical if one is looking to introduce a new allergen for use in allergy testing, immunotherapy, or other clinical applications. More generally, I believe the future of the BAT lies with the more complex allergies, for which no other validated – and reliable – tests are available. Drug allergies may in this context be of particular interest. Some drugs, for instance, activate the complement, which triggers basophil activation through C3a, C4a, or C5a; certain narcotics, or other compounds such as iodinated contrast, act through some other mechanism of unspecific basophil activation.

Basophil activation markers: searching for the best

Several different markers can be used for flow cytometric analysis of activated basophils, each marker bearing its advantages and drawbacks. Up to now, most groups applying the BAT have been using CD63 as an activation marker, and, to a lesser extent, CD203c. CD63 expression is not restricted to basophils, which is why an additional marker (in this case surface receptor bound IgE) needs to be used for basophil identification. In contrast, CD203c is a lineage-specific marker (ie in peripheral blood it is only expressed by basophils), thus offering the advantage of single-color basophil detection.[17] Still, quantitating basophil activation based on upregulation of CD203c appears less straightforward. CD203c is already present on resting basophils, and its upregulation upon activation is generally less prominent than that of CD63. CD63 appears to provide a better discrimination between spontaneous expression on resting cells (which is generally almost zero) and activated cells. Also, CD63 seems to be more closely related to the so-called anaphylactic degranulation, whereas CD203c appears to be linked to a different pathway. Still, for certain drug allergies for instance, which are not related to the IgE-mediated pathway, CD203c might be a better suited marker; this, however, still remains to be proven. We – as well as others in the field – are investigating various other markers for use in flow cytometric quantitation of basophil activation. Potential candidates include the newly identified basophil activation antigen CRTH2(DP2),[18,19] and the recently described activation markers CD13, CD164, and CD107a.[20] Beyond surface antigens, we are currently particularly interested in investigating potential candidate markers *inside* the basophil, ie within the signal transduction pathway behind its activation.

Could the inside picture be sharper?

The control mechanisms governing basophil activation are complex and incompletely understood. It is known nevertheless that the signal to basophil activation is rapidly propagated through consecutive phosphorylation cascades. P38 mitogen-activated protein kinase (MAPK) has been demonstrated to play a crucial role in this process.[21-26] The advent of phospho-epitope-targeted antibodies has provided a key proteomic tool for elucidating phospho-signaling networks. Signaling-related phosphorylation has traditionally been studied by Western blotting and ELISA, or other methods performed on isolated cells or lysates. Flow cytometry provides a new approach to studying signal transduction, allowing for correlation of intracellular phosphorylation events with cell phenotype. Why use flow cytometry when one can use Western blotting or ELISA? There are several good reasons for that. First of all, one doesn't need such a large sample, which makes it easier

Integrated assessment of intracellular signaling and surface phenotype by flow cytometry provides a most powerful tool for unraveling the mechanisms that govern basophil activation.

for clinical applications. Secondly, one gets single-cell data, instead of an average for all cells present in the sample, and this without having to physically separate the cells. And finally, results are obtained within hours, as opposed to days as with more traditional methods. Our most recent work consisted of developing a flow cytometric technique that combines intracellular exploration of p38 MAPK phosphorylation with

surface staining for upregulated CD63. We demonstrated that stimulation of basophils with anti-IgE or allergen induces rapid phosphorylation of p38 MAPK, peaking between one and five minutes and returning to baseline levels after one hour, whereas CD63 upregulation appears to peak a few minutes later with a more continuous expression profile.[27] Such a combined approach enables the study of basophils in their natural environment (whole blood), thus avoiding potential detrimental effects from additional manipulations. The technique furthermore allows for the identification of heterogeneously responsive cells within a sample. Finally, its short time-to-results and small sampling volume make it more accessible to clinical applications. I believe that integrated assessment of intracellular signaling and surface phenotype by flow cytometry provides a most powerful tool for unraveling the mechanisms that govern basophil activation. At the same time and most importantly, it opens new avenues to allergy diagnosis and follow-up of (allergen-specific) immunotherapy.

The next step

We have many different active projects. Our laboratory merges clinical and research activities, so we are working with patient material. Therefore we will definitely keep looking for new clinical applications of flow cytometric assessment of basophil activation. The major applications we are going to focus on will be drug, food, and venom allergies, for which conventional test results are sometimes equivocal.

The other major aspect of our future research will be to further investigate the signaling networks that are linked to basophil activation. I am particularly looking forward to working in close collaboration with BD Biosciences to investigate basophil activation signaling pathways using the BD™ Phosflow technology. We might discover better markers for basophil activation and/or gain in analytical sensitivity. We have experience with the BAT, BD has experience in flow cytometry and antibody development, and I believe a nice cooperation between our lab and the company must be possible. Such a joint venture will definitely help move the field forward.

Considering advances I would like to see to bring allergy diagnosis to the next level, I like to draw a parallel with our clinical studies: we do not only see responders and non-responders, but also believers and non-believers. Indeed, there seem to be a great number of opponents today, who are being very critical toward the BAT and more generally toward the use of flow cytometry in allergy diagnosis. I would be very happy if we could convince these non-believers that, with the BAT, we might have a new tool, which seems very promising; not a general tool for mainstream diagnosis, but certainly one for particular cases, for which we have no reliable diagnostic test to date. I am hoping that the award I received will help other projects in the field of flow cytometric allergy diagnosis gain more visibility. I certainly believe this award will help to show that, beyond hematology and oncology, flow cytometry offers new avenues to explore, notably in the fields of allergy and rheumatology. I hope the BAT will have its place in allergy diagnosis and follow-up of therapy, and possibly also in many other applications. ■

Prof. Dr. Didier Ebo, together with his co-authors, was granted the 2006-2007 *Clinical Cytometry* Award for Best Original Paper for the manuscript *"Flow-assisted quantification of* in vitro *activated basophils in the diagnosis of wasp venom allergy and follow-up of wasp venom immunotherapy."* The award was presented to Dr. Ebo at the Clinical Cytometry Society's Annual Meeting in Washington, DC, USA (October 7-9, 2007).

About the *Clinical Cytometry* award: This annual award is traditionally granted for the manuscript published under the Original Article category that is judged of the highest scientific quality and impact by the Editors and Associate Editors of *Clinical Cytometry.*

About the *Clinical Cytometry* journal: An official publication of the Clinical Cytometry Society, *Clinical Cytometry* is a journal that focuses on the development and application of analytical cytology and cytomics to the current and future practice of clinical cytometry. Published six times per year, the Journal covers techniques such as flow and image cytometry, bead-based and slide-based array analyses, as well as other cell-based spectroscopic methods. *Clinical Cytometry* also publishes clinical and translational investigations identifying and validating features and molecules that are important in diagnosis, prognosis, and therapeutic management of patients.

BD Biosciences is pleased to dedicate these few pages to Dr. Ebo in honor of his recognized work, to give him the opportunity to present his findings to our readers.

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Fluorescent Protein Organelle Markers and their Utility in Multiplexed Live and Fixed Cell Assays

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Monitoring cellular functions in live cell assays can be a challenge due to the lack of appropriate live-cell-compatible probes and reagents. Fluorescent protein (FP) tags, which are expressed as fusion proteins to a gene or domain of interest, have been used in numerous live-cell applications such as monitoring protein trafficking, detecting gene activation, tracking cellular differentiation. They also have been used as sub-cellular organelle and cytoskeletal component biomarkers. However, the utility of these FP tags is not limited to live-cell applications, since they are readily fixed using standard methods and thus allow for multiplexed antibody or dye labeling along with an FP marker in high-content applications.

BD Biosciences intoduces a collection of new BD Pharmingen™ Image Certified FP organelle marker vectors that have been validated in both live and fixed mammalian cell imaging applications. Red or green fusion FP organelle markers were transiently or stably transfected, alone and in combination, and were verified for functional location by colocalization studies using antibody or dye reagents. In addition, one FP organelle marker was used in combination with an antibody in a multiplexed end point assay to monitor the induction of apoptosis in cells.

Fluorescent protein organelle markers can be readily multiplexed with antibody or dye labeling in high-content applications.

Imaging live and stably transfected cell populations

HeLa cells (3×10^5 cells per well of a 6-well plate) were transfected with 1 µg of plasmid DNA using FuGENE® 6 Transfection Reagent (Roche Applied Science). After 48 hours, transfected cells were selected using G418 (0.5 mg/mL) for two weeks. A population of the most highly expressing cells (approximately 10% based on fluorescence intensity) was subsequently isolated by cell sorting using a BD FACSAria™ cell sorter. Sorted cells were seeded at 1×10^4 cells per well in BD Falcon™ 96-well imaging plates (Cat. No. 353219). After overnight incubation, cells were washed with Hanks' Balanced Salt Solution (HBSS) and incubated in HBSS with 2 µg/mL Hoechst 33342. Cells were imaged using a 40× objective (0.9 NA) on a BD Pathway™ 855 bioimager in confocal mode (**Figure 1**).

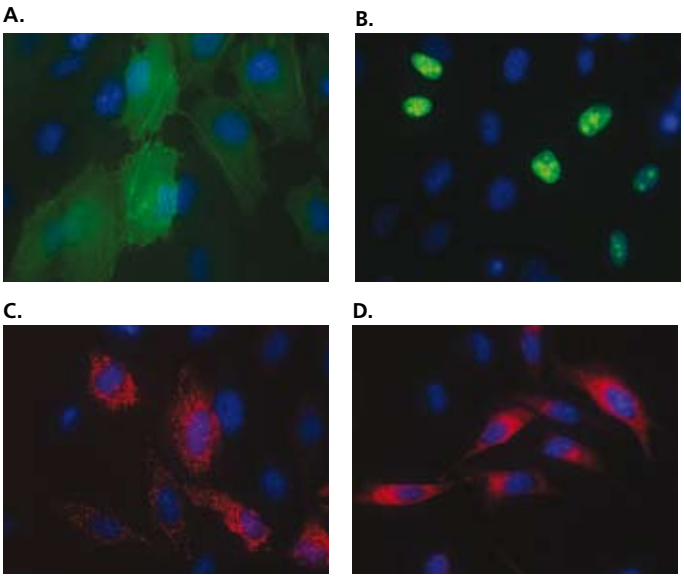

Figure 1. Live cell expression of FP-tagged organelle markers.

Live cell images were pseudo-colored and merged. The green FP signal was pseudo-colored green, the red FP signal was pseudo-colored red, and Hoechst 33342 staining was pseudo-colored blue. Panel A, BD Pharmingen Green FP - Actin; Panel B, BD Pharmingen Green FP - Nucleus; Panel C, BD Pharmingen Red FP - Peroxisome; Panel D, BD Pharmingen Red FP - Endoplasmic Reticulum.

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Imaging fixed and transiently transfected cell populations

HeLa cells (3×10^5 cells per well of a 6-well plate) were plated on glass coverslips and transiently transfected with two different FP-tagged organelle markers (1 µg plasmid DNA for each) using FuGENE® 6 Transfection Reagent (Roche Applied Science). After 48 hours, cells were fixed with 3.7% formaldehyde for 10 min, washed three times with Phosphate Buffered Saline (PBS), and mounted on slides using Vectashield™ mounting media containing DAPI (Vector Laboratories). Cells were imaged using a 40× objective (0.9 NA) on a BD Pathway 855 bioimager in confocal mode (**Figure 2**).

Verifying the intra-cellular location of the fluorescent proteins

To confirm that the fluorescent proteins were being targeted to the correct cell organelle, cells expressing the different FP organelle markers were seeded at 1×10^4 cells per well in BD Falcon 96-well imaging plates and cultured overnight. After culturing, cells were either stained with a dye that specifically stained the organelle of interest or an antibody that did the same. The BD Pharmingen Red FP - Endoplasmic Reticulum (ER) location was confirmed using an anti-ERp61 monoclonal antibody (Cat. No. 612585), which recognizes a chaperone found in the ER (**Figure 3A**). Similarly the BD Pharmingen Red FP - Mitochondrion location was confirmed using the dye MitoTracker® deep Red (Invitrogen), which stains mitochondria (**Figure 3B**). The BD Pharmingen Green FP - Actin staining was confirmed using Alexa Fluor® 647 anti-actin (Cat. No. 558624, **Figure 3C**) and the BD Pharmingen Green FP - Golgi was confirmed with Alexa Fluor® 647 anti-GM130 (Cat. No. 558712), which recognizes a 130-kDa golgi matrix protein (**Figure 3D**).

Using an FP vector in a multiplexed apoptosis assay

HeLa cells stably expressing BD Pharmingen Red FP-Mitochondrion were seeded into BD Falcon 96-well imaging

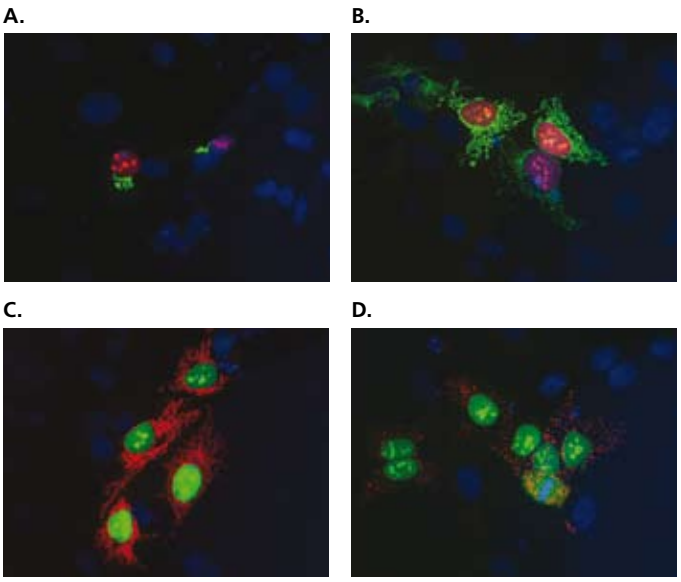

Figure 2. Transient expression of FP-tagged organelle markers in fixed cells.

Fixed cell images were pseudo-colored as described for Figure 1 and merged. The DAPI staining was pseudo-colored blue. Panel A, BD Pharmingen Green FP - Golgi and BD Pharmingen Red FP - Nucleus; Panel B, BD Pharmingen Green FP - Mitochondrion and BD Pharmingen Red FP - Nucleus; Panel C, BD Pharmingen Green FP - Nucleus and BD Pharmingen Red FP - Mitochondrion; Panel D, BD Pharmingen green FP - Nucleus and BD Pharmingen Red FP - Peroxisome.

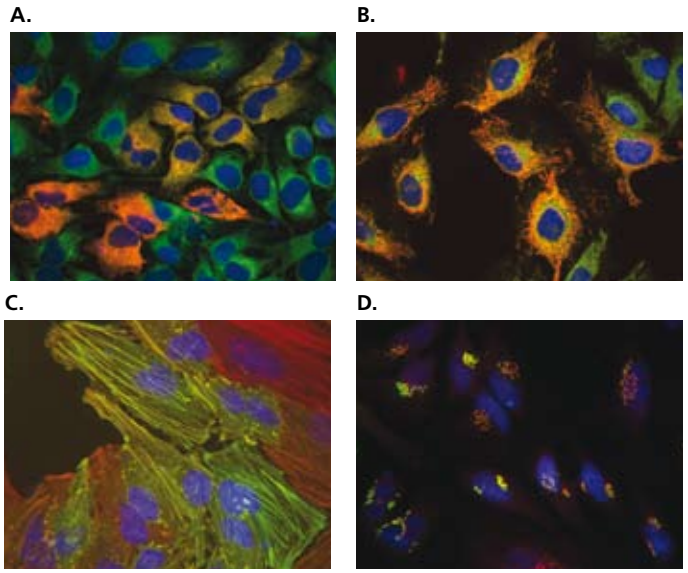

Figure 3. Intracellular location of FPs expressed from FP-tagged organelle markers.

Cell images were pseudo-colored and merged. Red FPs were pseudo-colored red and the confirmatory stain was pseudo-colored green (Panels A and B). Green FPs were pseudo-colored green and the confirmatory stain was pseudo-colored red (Panels C and D). Hoechst staining was pseudo-colored blue. Colocalization of the FP with the confirmatory stain appears yellow to orange based on the intensities of the individual signals. Panel A, BD Pharmingen Red FP - Endoplasmic Reticulum and anti-ERp61. Panel B, BD Pharmingen Red FP - Mitochondrion and MitoTracker® Deep Red. Panel C, BD Pharmingen Green FP - Actin and anti-actin. Panel D, BD Pharmingen Green FP - Golgi and anti-GM130.

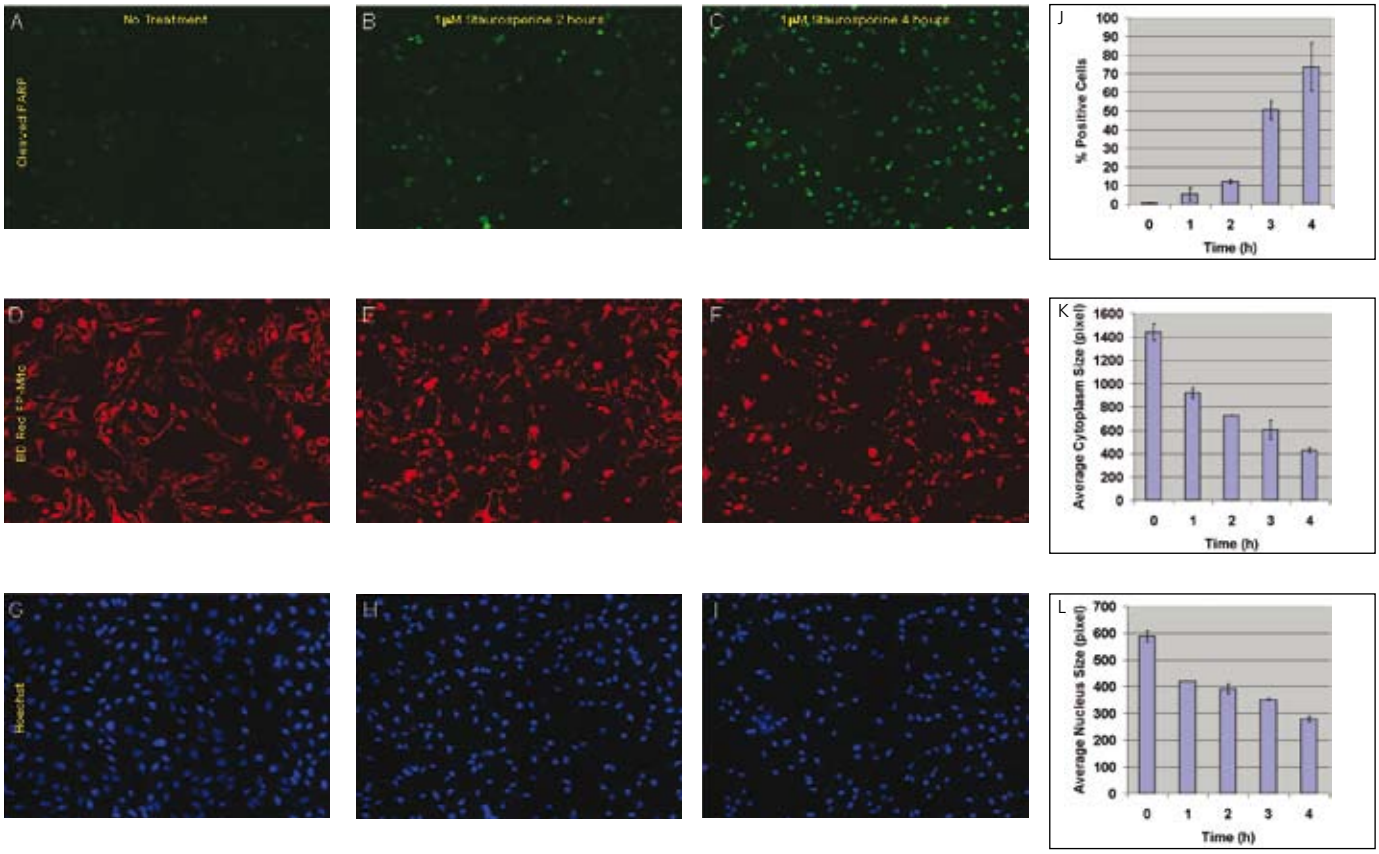

Figure 4. Multiplexed apoptosis assay using FP-tagged vectors.

Cleaved PARP staining was pseudo-colored green, the Red FP signal was pseudo-colored red, and Hoechst staining was pseudo-colored blue. For cleaved PARP, the nuclear intensity was measured using the nuclear mask identified by the Hoechst stain. The data in panel J are reported as the percentage of cells positive above a user-defined threshold, which was based on cells negative for the cleaved PARP stain. Cytoplasmic area was measured as a 50-pixel ring dilated out from the nuclear mask but within the outer boundary of the cell's cytoplasmic boundary, which was defined by the FP signal. Cells not expressing the FP were excluded from the calculation based on a user-defined minimum intensity threshold. The data in panel K are reported as the average cytoplasmic area (in pixels) per well. In panel L the data are represented as the average nuclear area (in pixels) per well, based on the Hoechst stain. Bar graphs show the mean of 2 wells ± SD.

plates (1×10^4 cells per well) and incubated with 1 µM staurosporine or vehicle control for 1 to 4 hours. After treatment, cells were fixed with 3.7% formaldehyde for 1 hour at room temperature (RT), washed twice with 1× PBS, and permeabilized with BD™ Perm/Wash buffer (Cat. No. 554723) for 30 min at RT. The cells were incubated with the Bioimaging Certified Reagent Alexa Fluor® 647 anti-cleaved PARP (Cat. No. 558710) in BD Perm/Wash buffer at 4°C overnight. The cells were washed three times with 1× PBS before being stained with 2 µg/mL Hoechst 33342 in 1x PBS.

FP tags are useful for both live cell applications and fixed cell assays.

Images were captured on a BD Pathway 855 bioimager as a 2×2 montage using a 20× (0.75 NA) objective. Image and data analysis was performed using BD Attovision™ software (**Figure 4**). Under continuous exposure to staurosporine, an increase in the percentage of cells positive for cleaved PARP was observed, as seen in the images (**panels A**, **B**, and **C**), and analyzed in **panel J**. However, as exposure time to staurosporine increased there was a decrease in cytoplasmic area as seen in the images (**panels D**, **E**, and **F**) and analyzed in **K**, and a decrease in nuclear size as seen in the images (**panels G**, **H**, and **I**) and analyzed in **L**.

Conclusions

The expression and location of BD Pharmingen Green FP and BD Pharmingen Red FP organelle markers in both transient and stably transfected cells were verified using a BD Pathway 855 bioimager. The FP organelle markers are useful for multiplexing applications. They can be used to view live cells, and can be subjected to fixation and permeabilization treatments at the end of the experiment for viewing in combination with antibodies or dyes. ■

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BD Pharmingen FP organelle marker vectors

Vector Name	Location	Fusion Tag	Ref.	Cat. No.
BD Pharmingen Green FP – Actin	Actin filaments	Whole actin fusion	1,2	558721
BD Pharmingen Green FP – Golgi	Golgi apparatus	Human β 1,4-galactosyltransferase (aa 1-81)	3,4	558719
BD Pharmingen Green FP – Mitochondrion	Mitochondria	Mitochondrial targeting sequence of cytochrome *c* oxidase subunit VIII	5	558718
BD Pharmingen Green FP – Nucleus	Nucleus	SV40 Large T antigen nuclear localization signal (in triplicate)	6,7	558720
BD Pharmingen Red FP – Endoplasmic Reticulum	Endoplasmic reticulum	Calreticulin ER targeting sequence (N-term) + KDEL ER retention signal (C-term)	8,9	558725
BD Pharmingen Red FP – Mitochondrion	Mitochondria	Mitochondrial targeting sequence of cytochrome *c* oxidase subunit VIII	5	558722
BD Pharmingen Red FP – Nucleus	Nucleus	SV40 Large T antigen nuclear localization signal (in triplicate)	6,7	558723
BD Pharmingen Red FP – Peroxisome	Peroxisomes	Peroxisomal targeting signal 1 tripeptide (SKL)	10,11	558724

BD Pharmingen Green FP and Red FP excitation and emission maxima

Fluorescent Protein	Exitation Maxima	Emission Maxima	Ref.
BD Pharmingen Green FP	475 nm	505 nm	12
BD Pharmingen Red FP	558 nm	585 nm	13

** These products are subject to license. Please contact our Scientific Support (contact numbers listed on the back page of this newsletter, or email: help.biosciences@europe.bd.com) for details.*

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Analyzing Neural Differentiation of Human Embryonic Stem Cells by Bioimaging and Flow Cytometry

Jeanne Elia and Christian T. Carson
BD Biosciences - Pharmingen

Introduction

Human embryonic stem cells (hESCs) are pluripotent stem cells which have been isolated from human embryos that were generated by in vitro fertilization and donated for research purposes. Since hESCs self-renew indefinitely and can be theoretically differentiated into any human cell type, they hold much promise for cell transplantation therapy, as well as for the generation of better model systems of human disease and human embryonic development. The recent advancements in de-differentiating human fibroblasts into pluripotent cells have fueled optimism and have brought the field one step closer to reaching these goals.[1-3]

Since the first description of hESC isolation and culture, a number of laboratories have dedicated their research focus to differentiating hESCs into a wide variety of cell types, including cardiomyocytes, blood, insulin-producing pancreatic beta islets as well as a variety of neural cells, including neural stem cells and neurons.[4] One issue that hinders these efforts is the efficacy and reproducibility of differentiation methods. Thus, there is a growing need for quick, easy-to-use assays to both quantify and qualify cell culture systems of hESCs and their differentiated progeny. In addition, there is a need to identify cell surface markers that are specific to cell types of interest so that these cells can be purified from a heterogeneous population of differentiating cells. Here we describe the use of flow cytometry and high content bioimaging for the analysis of neural differentiation of hESCs. We have created applications using fluorochrome-conjugated antibodies to facilitate and expedite multicolor analysis of these cells. These applications allow for the fast quantification of pluripotent hESCs and of hESC-derived neural stem cells. Finally, these applications are amenable to high throughput screening.

Flow cytometry and high content bioimaging allow for the fast quantification of pluripotent hESCs and of hESC-derived neural stem cells.

Characterization of hESC, H9

hESCs (H9 line) were analyzed for the presence of the pluripotent stem cell markers Nanog, Oct3/4, and SSEA-4 by bioimaging (**Figure 1**).

The majority of the cells within the hESC colony stain positive for all of these markers. These cells are cultured on a feeder layer of mouse embryonic fibroblasts that do not stain positive for these pluripotent markers. Oct3/4 and Nanog are transcription factors that control embryonic stem cell self-renewal and pluripotency, and SSEA-4 is a carbohydrate epitope that is expressed in pluripotent stem cells. All of these markers are down-regulated when hESCs differentiate.

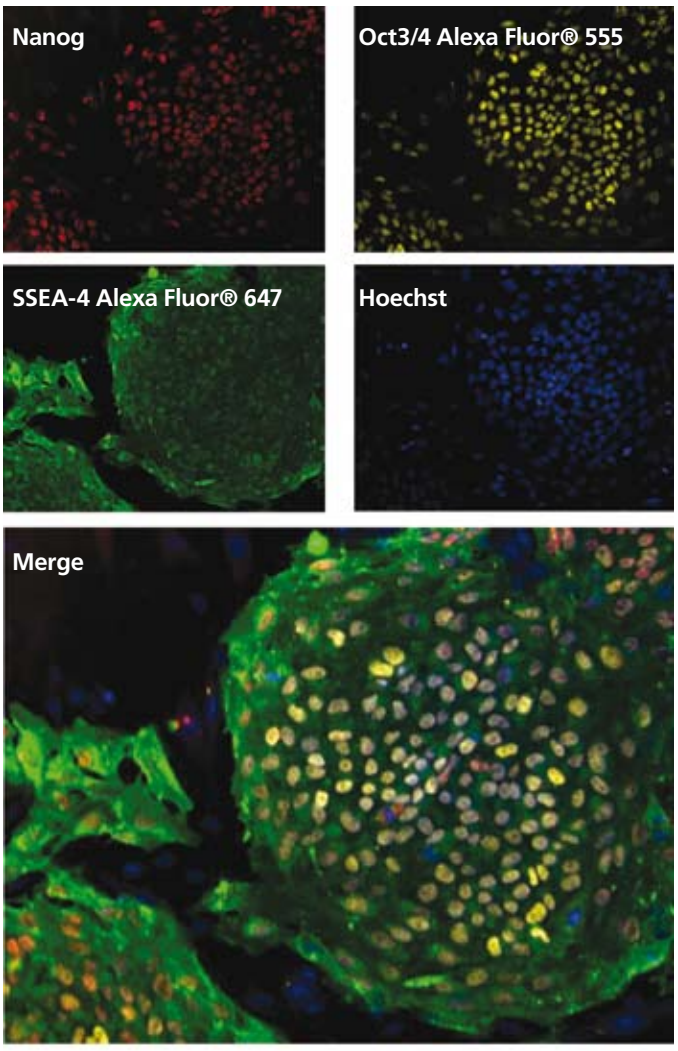

Figure 1. Image analysis of human embryonic stem cells hESC (H9), using purified and fluorochrome-conjugated antibodies to pluripotent stem cell markers.

Human embryonic stem cells hESC (H9) were plated on a mouse embryonic fibroblast (MEF) feeder layer using a BD Falcon™ 96-well imaging plate (Cat. No. 353219). Cells were washed twice with 1x PBS, fixed with 4% formaldehyde, then subsequently permeabilized with BD Perm/Wash™ buffer (Cat. No. 554723). Staining was performed using a three-step procedure since direct conjugates were not yet available for all of the antibodies. Cells were stained with a purified monoclonal antibody to human Nanog (Cat. No. 560109) followed by a secondary goat anti-mouse Alexa Fluor® 488 (Molecular Probes) (pseudo-colored red). Cells were washed twice with 1x PBS and stained with a cocktail of Oct3/4 Alexa Fluor® 555 (pseudo-colored yellow) and SSEA-4 Alexa Fluor® 647 (pseudo-colored green). Individual antibodies are shown in each of the smaller panels including Hoechst (pseudo-colored blue), which is used to identify all nucleated cells. The larger panel is a merge of all four colors. H9 colonies coexpress Nanog, Oct3/4, and SSEA-4, which are all markers for pluripotency. Feeder cells stain positive only for Hoechst

The cells were imaged on a BD Pathway™ 435 bioimager. This image is a collapsed z stack using a 10x objective.

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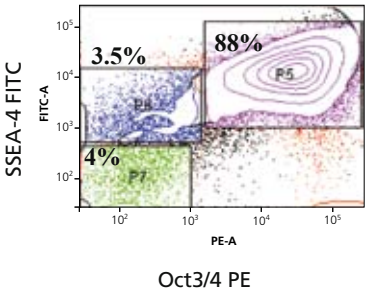

Figure 2. Flow cytometric analysis of hESC (H9).

H9 cells were grown on MEF feeder layers, then dissociated into a single cell suspension using TrypLE enzyme (Invitrogen). The cells were stained with antibodies specific for pluripotency, Oct3/4 (Cat. No. 560186) and SSEA-4 (Cat. No. 560126). Eighty-eight percent of the cells dually expressed SSEA-4 and Oct3/4. A small percentage of cells (4%) have down-regulated Oct3/4, while 3.5% of the cells were negative for both Oct3/4 and SSEA-4, which indicates that a population of the H9 cells was beginning to or had differentiated.

The cells were also analyzed by flow cytometry (**Figure 2**). hESCs were dissociated into single cells using TrypLE™ enzyme (Invitrogen) fixed in 4% PFA and permeablized with BD Perm/Wash buffer (Cat. No. 554723).

The cells were incubated with fluorescent-conjugated antibodies to Oct3/4 and SSEA-4. These data indicate that about 90% of the entire cell population are positive for Oct3/4 and SSEA-4. We speculate that the remaining 10% are cells that are positive but dim for these markers, are truly negative, or are in the process of differentiating.

Differentiation of hESC, H9 into self-renewing neural stem cells[5]

Briefly, detached hESC colonies were allowed to differentiate into embryoid bodies (EBs), which were plated on an adherent substrate. Neural rosettes were manually dissected from the EBs, dissociated into single cells, and propagated in the presence of FGF-2. These differentiated cells were analyzed by bioimaging to determine if they expressed neural stem cell markers but had lost their pluripotency. The cells stained positive for Nestin and Sox2 (neural progenitors), but stained negative for the hESC marker, Oct3/4 (**Figure 3, A** and **B**). These cells were also analyzed by flow cytometry to determine the purity of hESC-derived neural stem cells in our culture (**Figure 3, C** and **D**).

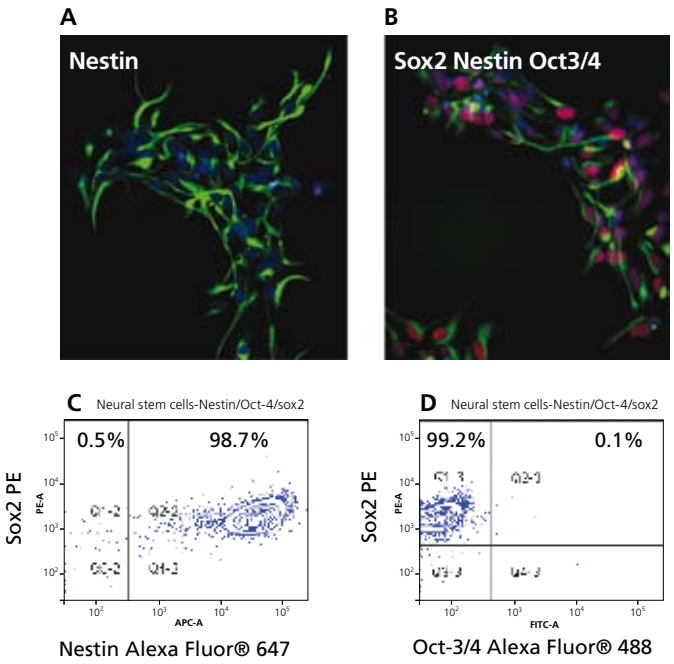

Figure 3. Analysis of hESC-derived neural stem cells by bioimaging and flow cytometry.

Panels A and B. *Neural stem cells, the multipotent stem cells that generate nerve cells, were differentiated from hESC (H9). Cells were fixed with 4% formaldehyde and subsequently permeabilized with BD Perm/Wash buffer (Cat. No. 554723).* ***Panel A*** *was stained with purified Nestin (Cat. No. 611658) followed by goat anti-mouse Alexa Fluor® 555 (pseudo-colored green) counterstained with Hoechst (pseudo-colored blue).* ***Panel B*** *was stained with purified anti-Sox2 followed by anti-mouse Alexa Fluor® 647 (pseudo-colored red), and subsequently stained with a cocktail of Nestin Alexa Fluor® 555 (pseudo-colored green) and Oct3/4 Alexa Fluor® 488 (pseudo-colored yellow). Neural stem cells were positive for Sox2 and Nestin, but were negative for Oct3/4. Nestin is an intermediate filament protein that is abundantly expressed in neuroepithelial stem cells early in embryogenesis, but is absent from nearly all mature central nervous system cells. The cells were imaged on a BD Pathway 435 bioimager. These images are a collapsed z stack using a 10x objective.*

Panels C and D*. Flow cytometric analysis of neural stem cells. Neural stem cells were dissociated using Accutase™ (Millipore). Cells were fixed using BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution (Cat. No. 554722) then washed using BD Perm/Wash Buffer (Cat. No. 554723). Cells were stained with a cocktail of Sox2 PE (Cat. No. 560291), Nestin Alexa Fluor® 647 (Cat. No. 560341), and Oct3/4 Alexa Fluor® 488 (Cat. No. 560253). Purity of neural stem cells coexpressing Nestin and Sox2 was approximately 99%. Neural stem cells were negative for Oct3/4*

Nearly all cells stained positive for both Nestin and Sox2 and were negative for Oct3/4. These results suggest that this particular differentiation process resulted in a uniform cell population and did not contain contaminating cell types. This combination of markers provides a useful tool for qualifying different batches of hESC-derived neural stem cells for purity.

Next we examined our ability to differentiate these hESC-derived neural stem cells to neurons. Cells were plated without FGF-2 and allowed to differentiate in the presence of dibutyrilcyclic AMP for 2 weeks. A high throughput image analysis was performed using a series of antibodies specific to neurons at various stages in their development. Some of these data are shown in **Figure 4**. (See figure legend for details.)

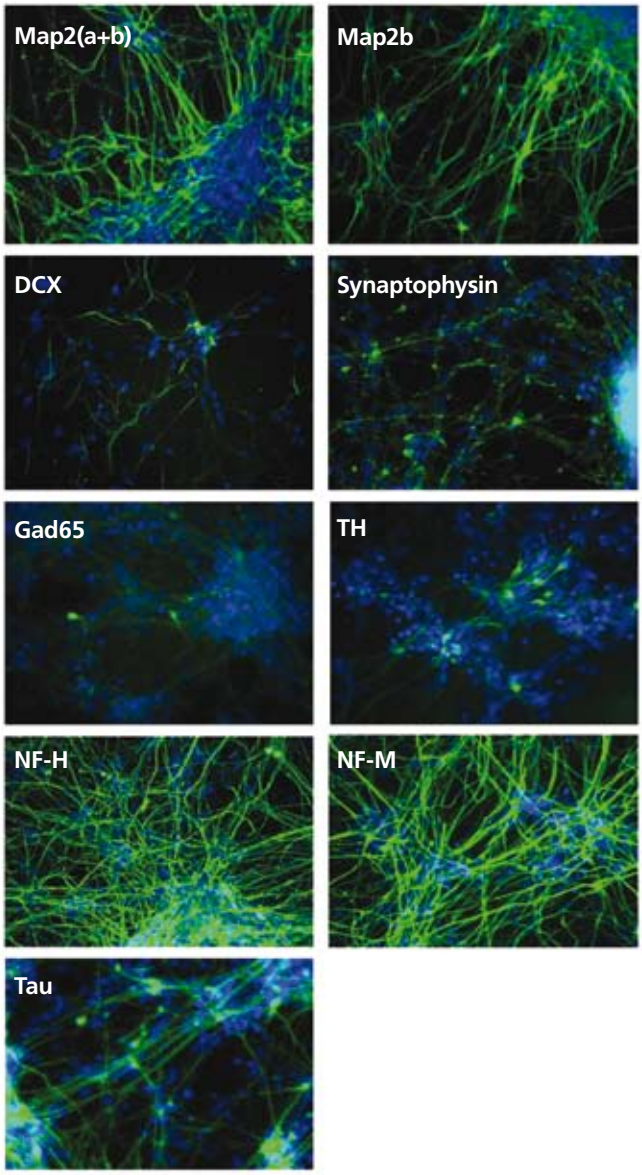

Figure 4. Differentiation of H9-derived neural stem cells into neurons.

Neural stem cells were seeded on BD BioCoat™ 96-well imaging plates coated with poly-L-Ornithine and Laminin. These cells were differentiated without FGF-2, but in the presence of dibutyrilcyclic AMP for 2 weeks. Cells were fixed using 4% formaldehyde followed by permeabilization with BD™ Phosflow Perm Buffer III (Cat No. 558050). Purified antibodies were detected with goat anti-mouse Alexa Fluor® 555 (Molecular Probes) (pseudo-colored green). Cells were washed and counterstained with Hoechst (pseudo-colored blue). The differentiated cells stained positive for a wide variety of neural markers: Doublecortin (DCX), which is expressed in immature neurons; Tau, Map2b, Synaptophysin, and Neurofilaments M and H, which are expressed in neuronal processes; Gad65 and tyrosine hydroxylase (TH), which are expressed in specific neuronal subtypes.

The cells were imaged on a BD Pathway 435 bioimager.

An image analysis was perfomed using fluorochrome-conjugated antibodies to Nestin and Map2b (**Figure 5**).

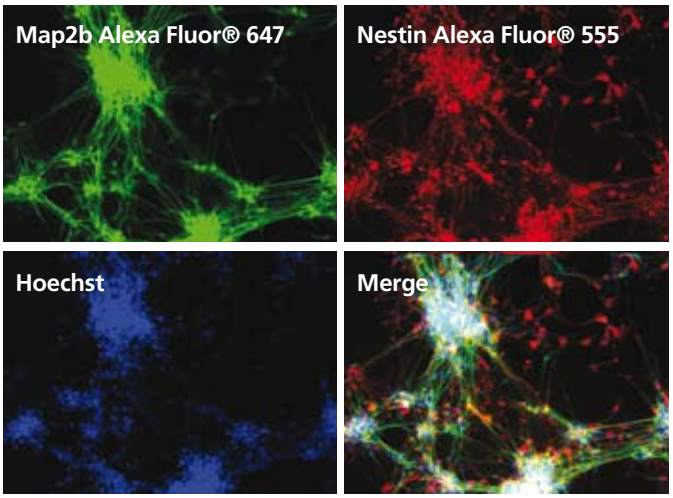

Figure 5. Map2b and Nestin expression in differentiated neural stem cells.

*Cells were plated, differentiated, and fixed as in **Figure 4**. The cells were stained with a cocktail of Nestin Alexa Fluor® 555 (pseudo-colored red) and Map2b Alexa Fluor® 647 (pseudo-colored green). Cells were washed and counterstained with Hoechst (pseudo-colored blue). The cells were imaged on a BD Pathway 435 bioimager.*

These data demonstrate that after 2 weeks of differentiation, there were mature Map2b-positive neurons as well as Nestin-positive cells in the culture. These Nestin-positive cells either represent undifferentiated neural stem cells or immature neurons. Future developments in software applications for image analysis as well as in flow cytometry will allow us to accurately quantify neural differentiation in culture. ■

Table 1: Distribution of stem cell markers at different stages of neural differentiation

Specificity	H9 (human embryonic stem cell)	Neural stem cells	Neurons
SSEA4	+	-	-
OCT3/4	+	-	-
Sox2	+	+	-
Nanog	+	-	-
Nestin	+ (low)	+ (high)	-
Map 2b	-	-	+

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Products from BD Biosciences featured in this article

Flow cytometry reagents

Description	React	Clone	Isotype	Apps	Format	Size	Cat. No.
Nestin	Rat	Rat 401	Mouse IgG_1	EM, FCM, IF, IHC(P), WB	Purified	100 µg	556309
Oct3/4	Hu, Ms	40/OCT-3	Mouse IgG_1, κ	IC/FCM	PE	50 tests	560186
Oct3/4	Hu, Ms	40/OCT-3	Mouse IgG_1, κ	FCM	Alexa Fluor® 488	50 tests	560253
Oct3/4	Hu, Ms	40/OCT-3	Mouse IgG_1, κ	FCM	Alexa Fluor® 647	50 tests	560329
Sox2	Hu, Ms	245610	Mouse IgG_{2a}	IC/FCM	PE	50 tests	560291
Sox2	Hu, Ms	245610	Mouse IgG_{2a}	IC/FCM	Alexa Fluor® 488	50 tests	560301
Sox2	Hu, Ms	245610	Mouse IgG_{2a}	IC/FCM	Alexa Fluor® 647	50 tests	560302
SSEA-4	Hu	MC813-70	Mouse IgG_3, κ	FCM	FITC	100 tests	560126
SSEA-4	Hu	MC813-70	Mouse IgG_3, κ	FCM	PE	100 tests	560128

Bioimaging Certified Reagents

Description	React	Clone	Isotype	Apps	Format	Size	Cat. No.
Nanog	Hu	L96-549	Mouse IgG_1, κ	Bioimg, WB	Purified	0.1 mg	560109
Nestin	Ms	25/NESTIN	Mouse IgG_1, κ	Bioimg, WB	Alexa Fluor® 647	100 tests	560341
Oct3/4	Hu, Ms	40/OCT-3	Mouse IgG_1, κ	Bioimg	Alexa Fluor® 488	100 tests	560217
Oct3/4	Hu, Ms	40/OCT-3	Mouse IgG_1, κ	Bioimg	Alexa Fluor® 555	100 tests	560306
Oct3/4	Hu, Ms	40/OCT-3	Mouse IgG_1, κ	Bioimg	Alexa Fluor® 647	100 tests	560307
Sox2	Hu, Ms	245610	Mouse IgG_{2a}	Bioimg	Alexa Fluor® 488	100 tests	560292
Sox2	Hu, Ms	245610	Mouse IgG_{2a}	Bioimg	Alexa Fluor® 555	100 tests	560293
Sox2	Hu, Ms	245610	Mouse IgG_{2a}	Bioimg	Alexa Fluor® 647	100 tests	560294
SSEA-4	Hu	MC813-70	Mouse IgG_3, κ	Bioimg	Purified	0.1 mg	560073
SSEA-4	Hu	MC813-70	Mouse IgG_3, κ	Bioimg	Alexa Fluor® 488	100 tests	560308
SSEA-4	Hu	MC813-70	Mouse IgG_3, κ	Bioimg	Alexa Fluor® 555	100 tests	560218
SSEA-4	Hu	MC813-70	Mouse IgG_3, κ	Bioimg	Alexa Fluor® 647	100 tests	560219

Supporting Reagents

Description	Apps	Size	Cat. No.
BD Perm/Wash™ Buffer	IC/FCM	250 tests	554723
BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution	IC/FCM	250 tests	554722
BD™ Phosflow Perm Buffer III	IC/FCM	125 ml	558050
BD Falcon™ 96-well Imaging Plate	Bioimg	8/pack, 32/case	353219

p53 Acetylation: A Call to Action

Jie Chen, Chad Sisouvanthong, Guo-Jian Gao, and David Ernst
BD Biosciences - Pharmingen

p53 (also known as tumor protein 53 or TP53) regulates the cell cycle and is involved with preserving genomic integrity.[1] This tumor suppressor gene is mutated in many human cancers, resulting in failed cellular responses to DNA damage. p53 is normally a short-lived protein that is expressed at low levels. In response to cellular or environmental stress (DNA-damaging agents, nucleotide depletion, or hypoxia) the p53 protein is transiently stabilized and accumulates in the nucleus. In addition to its differential expression, post-translational

Monoclonal antibody L82-51 specifically recognizes p53 acetylated by cell lines or normal human peripheral blood lymphocytes that are treated with DNA-damaging agents.

modifications are thought to regulate p53 actions by conversion of inert to active DNA sequence-specific binding forms of p53. Active p53 regulates the transcription of several genes including WAF1, SFN, and MDM2 that can control cell cycling (eg, induce cell cycle arrest or apoptosis). p53 coactivators, including p300 or CBP, can acetylate p53.[2-4] These acetyltransferase-containing transcriptional coactivators acetylate p53 at several sites. Lysine-382 (K382) is one of the preferentially acetylated sites (referred to as acK382). This residue resides in the C-terminal domain that is known to be critical for the regulation of p53 DNA binding. Acetylation of C-terminal p53 dramatically stimulates the sequence-specific DNA-binding activity and can promote interactions with other transcription factors, possibly as a result of acetylation-induced conformational changes.

Using a synthetic p53 peptide preparation with the lysine acetylated at the 382 position, we have generated a monoclonal antibody, L82-51 (Mouse IgG_1)(Cat. No. 560231), that is specific for the acetylated lysine 382 form of p53 (acK382). This antibody can specifically recognize p53 acetylated in cell lines (**Figure 1**) or normal human peripheral blood lymphocytes (**Figure 2**) that are treated with DNA-damaging agents such as adriamycin (also known as doxorubicin). In addition to being useful for Western blot analyses, fluorescent conjugates of this antibody have been found useful for the immunofluorescent staining and flow cytometric analysis of cell lines and normal lymphocytes as shown in **Figure 3** . Because of its versatility, this new antibody should be useful for the elucidation of roles played by p53 in cellular responses by normal and transformed cells. ■

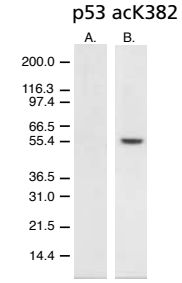

Figure 1. p53 acetylation in treated HeLa cells.

*HeLa cells (**panels A, B**) were cultured without (**A**) or were treated with (**B**) adriamycin (0.5 µM) and trichostatin A (400 nM; a deacetylase inhibitor) for 24 hours. The cells were harvested, lysed, and analyzed by Western blot using the anti-p53 acK382 antibody. p53 acK382 is identified as a major band of 53 kDa that is expressed at high levels by treated (**B**) but not by untreated (**A**) cells.*

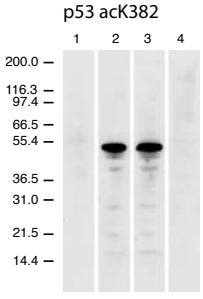

Figure 2. p53 acetylation in treated human lymphocytes.

*Human peripheral blood lymphocytes were cultured without (**lane 1**) or were treated with (**lanes 2–4**) adriamycin (0.5 µM) and trichostatin A (400 nM) for 24 hours. The cells were harvested, lysed, and analyzed by Western blot using the anti-p53 acK382 antibody alone (**lanes 1, 2**) or preincubated with non-acetylated p53 K382 peptide (**lane 3**) or with acetylated p53 acK382 peptide (**lane 4**). p53 acK382 presents as a major band of 53 kDa expressed by treated (**lanes 2, 3**) but not by untreated (**lane 1**) cells. Moreover, the blotting was blocked by the cognate p53 acetylated acK382 but not by the non-acetylated p53 K382 peptide. These results indicate that the anti-p53 acK382 antibody is highly specific for the acetylated form of p53. The specificity of the antibody was further confirmed by ELISA assay testing with cognate acetylated and non-acetylated p53 peptides that contain the lysine-382 residue (data not shown).*

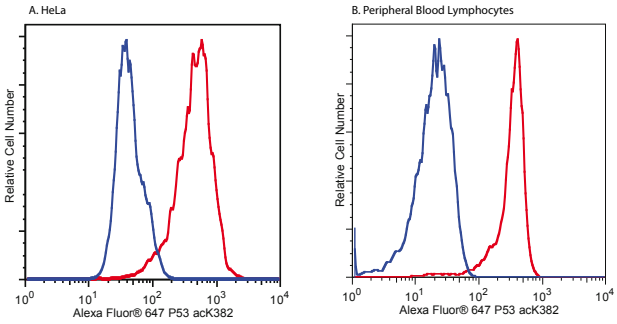

Figure 3. Flow cytometric analysis of cells expressing acetylated p53.

*The anti-p53 acK382 antibody was conjugated with Alexa Fluor® 647 and used to stain untreated (blue line histograms) or adriamycin/trichostatin A-treated HeLa (**panel A**) or human peripheral blood lymphocytes (**panel B**). The cells were cultured for 24 hours, harvested, fixed in BD Cytofix™ Fixation Buffer (Cat. No. 554655) at 37°C for 10 min, permeabilized in BD™ Phosflow Perm Buffer III (Cat. No. 558050) on ice for 30 min, stained with Alexa Fluor® 647-conjugated anti-p53 acK382 antibody. The cells were then analyzed on a BD FACSArray™ bioanalyzer.*

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p53 related products from BD Biosciences

Description	React	Clone	Isotype	Apps	Reg	Format	Size	Cat. No.
p53	Dog, Hu	80	IgG_{2b}, κ	IHC, IF, IP, WB	RUO	Purified	50 µg	**610183**
			IgG_{2b}, κ	IHC, IF, IP, WB	RUO	Purified	150 µg	**610184**
p53	Hu, Bov	DO-1	IgG_{2a}	IHC(F), IHC(Fr), IP, WB	RUO	Purified	0.1 mg	**554293**
p53	Hu, Monk, Bov	DO-7	IgG_{2b}	FCM, IHC(F), IHC(Fr), IP, WB	RUO	Purified	0.1 mg	**554294**
			IgG_{2b}	FCM	RUO	FITC Set	100 tests	**554298**
			IgG_{2b}	FCM	RUO	PE Set	100 tests	**556534**
p53	Hu	G59-12	IgG_1	IHC(Fr), IHC(F)	RUO	Purified	1 mL	**550832**
			IgG_1	IHC	RUO	FITC Set	100 test	**557026**
	Hu, Ms, Rat		IgG_1	IHC(F), IHC(Fr), IP, WB	RUO	Purified	0.1 mg	**554157**
p53 (acK382)	Hu	L82-51	IgG_1, κ	FCM	RUO	Alexa Fluor® 647	50 tests	**560231**
p53 (pS46)	Hu	I117-1091	IgG_1, κ	WB	RUO	Purified	0.1 mg	**558245**
p53 (pS37)	Hu	J159-641.15.4	IgG_1, κ	WB, IHC(F)	RUO	Purified	0.1 mg	**558369**
p53 (Wildtype) Recombinant Human Protein	Hu			WB	RUO	Purified	10 µg	**556439**
p53	Hu, Ms, Rat, Monk, Ham	PAb 122	IgG_{2b}	FCM, IF, IP, WB	RUO	Purified	0.1 mg	**554147**
p53	Hu	PAb 1801	IgG_1	IF, IHC(Fr), IHC(P), IP, WB	RUO	Purified	0.1 mg	**554169**
			IgG_1	IF, IHC(Fr), IHC(P), IP, WB	RUO	Purified	0.25 mg	**554170**
p53	Hu, Ms, Rat, Ham, Monk, Bov, Chick	PAb 240	IgG_1	IHC(Fr), IP, WB	RUO	Purified	0.1 mg	**554166**
			IgG_1	IHC(Fr), IP, WB	RUO	Purified	0.25 mg	**554167**

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Events

Date	Event	Location
October 1-4	GIC - Italian Group of Cytometry Meeting	Urbino, Italy
October 3	Regener8 Worshop - Stem Cells for Tissue Engineering	Durham, UK
October 6-8	EuroBio 2008	Paris, France
October 6-9	World Vaccine Congress	Lyon, France
October 8-10	6th European Mucosal Immunology Group (EMIG) Meeting	Milan, Italy
October 8-10	EUFEPS Conference on Drug Transport and Delivery: Impact on Drug Discovery and Development	Uppsala, Sweden
October 8-11	Italian Association for Neuroimmunology (AINI) Meeting	Naples, Italy
October 14-16	11th Annual MipTec Conference	Basel, Switzerland
October 22-24	Association Française de Cytométrie (AFC) 13th Annual Congress	Nancy, France
October 23-24	SIDEM - Italian Society of Emapheresis and Cellular Manipulation	Catania, Italy
October 28-29	BioProduction 2008	Düsseldorf, Germany
October 28-31	SIBioC 2008 40th National Congress of the Italian Society of Clinical Biochemistry and Clinical Molecular Biology	Rimini, Italy
November 11-13	7th Louis Pasteur Conference on Infectious Diseases	Paris, France
November 13-16	European Society of Gene & Cell Therapy (ESGCT) 2008	Brugge, Belgium
November 17-21	British Society for Immunology (BSI) Congress 2008	Glasgow, UK
November 26-27	Annual bioProcessUK Conference 2008	Brighton, UK
December 1-3	European Antibody Congress 2008	Geneva, Switzerland
December 1-3	Optimising Biomanufacturing Processes	Brussels, Belgium
December 2-3	Global Imaging Summit 2008	Cologne, Germany
December 4-6	Cytokines 2008	Florence, Italy
December 5-6	European Society for Animal Cell Technology (ESACT) UK 19th Annual Scientific Meeting	Cambridge, UK
December 12	GREMI Meeting on Th17-Derived Cytokines	Paris, France

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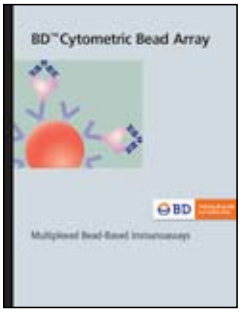

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