FlowCellect™ Human T Cell Activation Kit

100 Tests

Cat. No. FCCH100141

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

USA & Canada Phone: 1-800-645-5476 (phone), 1-800-645-5439 (fax)
www.millipore.com

Application

Activation of T Lymphocytes is a complex and regulated sequence of events that leads to expression of cytokine receptors and secretion of cytokines and the expression of specific cell surface molecules that leads to divergent immune responses (1). Activation of immune T cells can induce proliferation, differentiation, secretion of cytokine, maturation or stimulation of other cell types and can occur in response to multiple stimuli such as antibodies, cytokines, polyclonal mitogens etc (2-3). The study of the expression of lymphocyte activation markers has thus become extremely critical in drug development, in understanding immuno-modulatory mechanisms, in understanding normal development and in research to understand the mechanisms of disease such as HIV, alzheimers, hepatitis and autoimmune diseases (4-6).

Multiple proteins are known to be upregulated during the immune activation process. The more common of these proteins include early activation marker CD69, CD71 (early), CD25 (late) and mid to late Class II, HLA-DR (7-9). While the study of each of these markers is informative and significant, CD69 is one of the most commonly studied activation marker due to its early expression in activated T, B or NK cells and its early expression in the activation process (10-11). Further CD69 is not expressed constitutively in resting T cells making the identification of activation in these cells clearer.

The FlowCellect T Cell activation kit allows for the determination of activation status of CD4 and CD8 T Cells simultaneously by allowing the detection and quantitation of CD69 marker on either whole blood or PBMC using simplified no-wash assays. The kit can be utilized on any flow cytometry system equipped with blue and red lasers. The performance of these assays on the guava easyCyte 8HT platform along with the Incyte software allows for cell count information on CD4, CD8 T cells and count and % of activated populations. Further easy identification of activated T cells can be performed using heat map features or measurement of the kinetics of activation using the dose response features. The simplified identification of activated samples can be of great utility in drug development, kinetic studies and understanding mechanism of compound action and disease.

Test Principle

Millipore's FlowCellect™ T cell Activation Assay provides a rapid and simple method to asses the level of Cd69 in activated CD4 and CD8 T cells simultaneously. The kit includes (1) Antibody Cocktail containing CD3-PECy5 CD4-FITC, CD8-APC, and CD69-PE antibodies and (2) 1X Lysing solution to lyse erythrocytes and (3) Assay buffer solutions.

The CD4-FITC/CD69-PE/CD3-PECy5 cocktail consists of three anti-human antibodies CD3-PECy5, CD4-PE, CD8-APC and CD69-PE which allow for CD4 and CD8 T cell lymphocyte detection and identification and the expression level of CD69 in activated populations. The CD3 antibody Anti-CD3, UCHT1, reacts with the ε-chain of the CD3 part of the TCR/CD3 complex. CD3 is a pan-T marker expressed by normal and neoplastic T cells and uniquely allows the identification of all T cell lymphocytes. The CD4 antibody allows the identification of human helper/inducer CD4+ T cell (HLA Class II reactive) and recognizes a 60,000 Da surface antigen. Monocytes also express CD4 but at lower density, and have no co-expression of CD3 and hence can be distinguished away from CD4 T Cells in this kit. The CD8 antibody allows the identification of CD8, a 68 kDa transmembrane glycoprotein expressed by class I major histocompatibility complex restricted, mature suppressor/cytotoxic T cells, the great majority of cortical thymocytes and approximately 30% of medullary thymocytes. In addition a proportion of γδ T cells and NK cells express CD8. Inclusion of the CD3 antibody allows for the unique identification of the CD8 cytotoxic T Cells. The Anti-human CD69 antibody (Clone FN50, IgG1, kappa) allows for the detection of CD69, an early activation antigen (12).

CD69 is a phosphorylated disulphide-linked dimer composed of two chains of 27 kDa and 33 kDa, and also known as activation inducer molecule (AIM). CD69 is the earliest inducible surface antigen expressed on lymphocytes after T or B cell activation and is absent from resting lymphocytes. Other cells, including epidermal Langerhans cells, natural killer (NK) cells, eosinophils, neutrophils and platelets may also express CD69. In vitro studies have demonstrated a transient expression of CD69 on activated T cells. After activation, surface expression can be detected within 2-4 hours, reaching a maximum after 18-24 hours followed by a gradual decrease (2). CD69 is thus detectable prior to other activation antigens like CD25 and CD71. CD69 is believed to be involved in signal transduction

The kit can thus distinguish multiple populations (1) CD3 T cells and its CD69 activation status (2) CD4 T helper cells and the level of expression of CD69 in these populations and (3) CD8 cytotoxic T Cells and the level of CD69 in these populations. The kit thus provides a complete picture of T cell activation status and its response for inducer treatment conditions or diseases. The entire assay can be performed in 45 min a simple no wash manner without loss of precious activated cells.

Sufficient reagents are provided for 100 tests. The kit includes all optimized fluorescently labeled antibodies, dyes and buffers necessary for cell preparation and analysis.

Flow chart for performing the FlowCellect™ T Cell Activation Kit

Prepare all cell samples for incubation with the antibody cocktail.

↓

Add 10 µL of cells or whole blood to 10µL of antibody cocktail

↓

Incubate at room temperature for 20 minutes

↓

Add 180µL of 1x Assay Buffer BA to PBMC samples
-or-
180uL of 1X Lysing Solution to Whole Blood Samples

↓

Incubate for 15 minutes at RT for Whole blood

↓

Acquire on a flow cytometer

Kit Components

- CD4-FITC/CD69-PE/CD3-PECY5/CD8-APC (Part No.4700-1375) One vial containing 1000 uL of cocktail.
- 1X Assay Buffer BA: (Part No. 4700-1360) One vial containing 50 mL.
- Guava 1X Lysing Solution (Part No. 4700-0082) One bottle containing 40 mL buffer.

Materials Not Supplied

1. easyCyte HT System (guava® easyCyte 8HT or easyCyte 6HT-2L) with guavaSoft™ Software or equivalent flow cytometry system with ability to detect green, red1 and red2 fluorescence
2. ViaCount™ reagent (Catalog No. 4000-0041) or ViaCount Flex reagent (Catalog No. 4700-0060)
3. PBMC or Whole Blood Samples
4. Media for cell line of interest
5. Tissue culture instruments and supplies (including 37°C incubator, growth media, plates, detachment buffer, etc.)
6. Polypropylene tubes and or bottles for sample and buffer preparation and storage.
7. 0.5-mL microcentrifuge tubes (VWR Cat. No. 16466-036 or equivalent) for sample acquisition
8. 1.5-mL microcentrifuge tubes (VWR Cat. No. 16466-030 or equivalent)

9. 96-well microplate plates, round bottom (Falcon Cat. Nos. 353910 or 353918) or flat bottom (Falcon Cat. No. 353075 or 353915), or equivalent. Refer to the appropriate Guava System user's guide for other compatible microplates.
10. Pipettors with corresponding tips capable of accurately measuring 1 – 1000 μL
11. Tabletop centrifuge capable of exceeding x300G.
12. Vortex mixer
13. Milli-Q™ Distilled Water or DI water.
14. Reagent reservoirs, optional
15. Guava® Instrument Cleaning Fluid (ICF) (Cat. No. 4200-0140), optional
16. guava easyCheck Kit (Cat. No. 4500-0025), optional
17. 20% bleach solution

Precautions

- Wear proper laboratory attire (lab coat, gloves, safety glasses) when handling or using this product.
- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.
- Some assay components included in the kit may be harmful. Please refer to the MSDS sheet for specific information on hazardous materials.
- All fluorochrome conjugated antibodies and dyes are light sensitive and must be stored in the dark at 2-8°C.
- During storage and shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For maximum recovery of product, centrifuge vial briefly prior to removing cap.
- Avoid microbial contamination of the solution, which may cause erroneous results.
- Do not use reagents beyond their expiration date.

Storage

Upon receipt, store the CD4-FITC/CD69-PE/CD3-PECY5/CD8-APC and 1X Assay Buffer BA at 2-8ºC

Upon receipt, store the Guava 1X Lysing Solution at room temperature.

Caution: *Fluorochrome conjugated antibodies should always be stored at 2-8°C. Any deviation in temperature for long periods of time may compromise the performance of the antibodies.*

Before You Begin

Specimen Collection and Preparation

The blood used for the procedure should be collected by venipuncture into a sterile K3 EDTA (lavender top) or Sodium Heparin (green top) blood collection tube.

WARNING: Blood samples that are hemolyzed, clotted, lipemic, discolored or containing interfering substances should be discarded.

NOTE: Blood should be stained within 30 hours of collection for optimal results. Unstained anticoagulated blood should be maintained at 18-25°C prior to sample processing.

NOTE: Leave the capped tubes of blood standing upright or lying on their sides if it is stored overnight. Do not rock or agitate blood in any way during extended storage.

This protocol was developed to allow direct determination of the percent of activated T cells in whole blood or PBMC's in cultures. Optimal Staining is obtained for cell concentrations between 2×10^5 and 2.5×10^4 cells/well (or 2×10^7 to 2.5×10^6 cells/mL). Millipore recommends using the ViaCount™ reagent to obtain accurate cell counts. Care should be taken to keep cell concentrations as constant as possible in all samples of an experiment. The kit may also be used for 10-30 µL of blood.

Cells should be acquired shortly after the sample preparation had been completed. While some donors have been shown to yield stable results for up to 3 hours, the stability of individual donors may vary. This time variability is a consequence of using live, unfixed cells. You should determine the stability of results for your own cells.

Time considerations: The process of staining cells with the FlowCellect™ T Cell Activation Kit takes approximately 45 minutes. Acquiring data on your guava system usually takes approximately 1 hour but can vary depending on your cell concentration. However, preparing cells for testing may require periodic maintenance and cultivation several days in advance. Once you cultivate the proper number of cells for your experiment, it may take an additional 2 to 72 hours of culture with various reagents to induce activation.

Example Cell Staining Protocol

Procedure for Staining Whole Blood Samples Using the T Cell Activation Kit

1. Prepare blood samples including positive and negative controls to cause activation of the cells.
2. Pipette 10 μL of CD4-FITC/CD69-PE/CD3-PECY5/CD8-APC Cocktail into each well or tube.
   CAUTION: Put the stock bottle of CD4-FITC/CD69-PE/CD3-PECY5/CD8-APC Cocktail back into the refrigerator or on ice immediately after use. Do not allow the bottle of Cocktail to remain at elevated temperatures for extended times.
3. Add 10 μL of blood to each well or tube.
   NOTE: Blood in the tubes should be thoroughly resuspended by gentle agitation for a few minutes before removing an aliquot for sample preparation.
4. Mix the samples thoroughly by pipetting up and down.
   CAUTION: Avoid leaving blood to dry on the side of the wells or tubes. This may cause erroneous results.
5. Incubate the samples for 20 minutes at room temperature (18 to 25ºC) in the dark.
6. Pipette 180 µL of 1X Lysing Solution directly into the wells/tubes to bring total sample volume to 200 μL.
   NOTE: If using a flow cytometer other than the easyCyte HT System, add 380uL of 1X Lysing Solution.
7. Immediately mix the sample thoroughly by pipetting up and down.
8. Incubate for 15 minutes at room temperature (18 to 25ºC) in the dark.
9. Samples are ready for acquisition and analysis on the easyCyte HT System or other flow cytometer.
   NOTE: Batch your preparations to avoid over-incubation of samples. Samples must be acquired within 3 hours after preparation.

Procedure for Staining PBMC Using the T Cell Activation Kit

1. Prepare PBMC samples including positive and negative controls to cause activation of the cells.
2. Centrifuge and resuspend cells at 5 x 10^6 cells/mL in 1x Assay Buffer BA.
3. Pipette 10 μL of CD4-FITC/CD69-PE/CD3-PECY5/CD8-APC Cocktail into each well or tube.
   CAUTION: Put the stock bottle of CD4-FITC/CD69-PE/CD3-PECY5/CD8-APC Cocktail back into the refrigerator or on ice immediately after use. Do not allow the bottle of Cocktail to remain at elevated temperatures for extended times.
4. Add 10uL of PBMC to each well or tube.
5. Mix the samples thoroughly by pipetting up and down.
6. Incubate the samples for 20 minutes at room temperature (18 to 25ºC) in the dark.
7. Pipette 180 µL of 1X Assay Buffer BA directly into the wells/tubes to bring total sample volume to 200 μL.
   NOTE: If using a flow cytometer other than the easyCyte HT System, add 380uL of 1X Assay Buffer BA.
8. Immediately mix the sample thoroughly by pipetting up and down.
9. Samples are ready for acquisition and analysis on a flow cytometer.
   NOTE: Batch your preparations to avoid over-incubation of samples. Samples must be acquired within 3 hours after preparation.

Sample Data

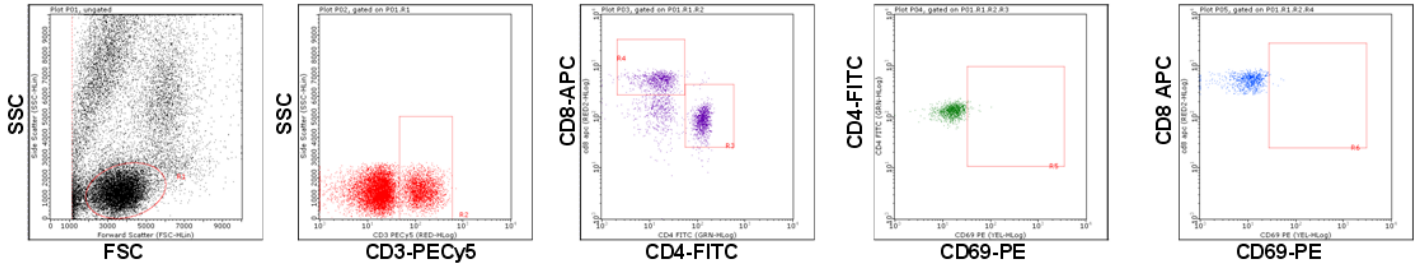

Figure 1 Display of Plots for Sample Acquisition: Set up of plots for data acquisition for samples treated with the T Cell Activation Kit. Plot 1 provides the plot of FSC (Lin) vs. SSC (Lin) which is typically used to gate and count cells. Plot 2 gates the CD3-PeCy5 (Red Channel) positive cells. 3000 CD3+ events are typically acquired. Plot 3 separated the CD3 positive cells into CD4-FITC and CD8-APC T cell subcomponents. Plot 4 provides comparison of CD4-FITC (Green Channel) vs. CD69-PE (yellow channel). Plot 5 provides the comparison of CD8-APC (Red2 channel) and CD69-PE (yellow channel). A negative control or inactivated sample should be used to set the CD69 gates for both the CD4 and CD8 populations.

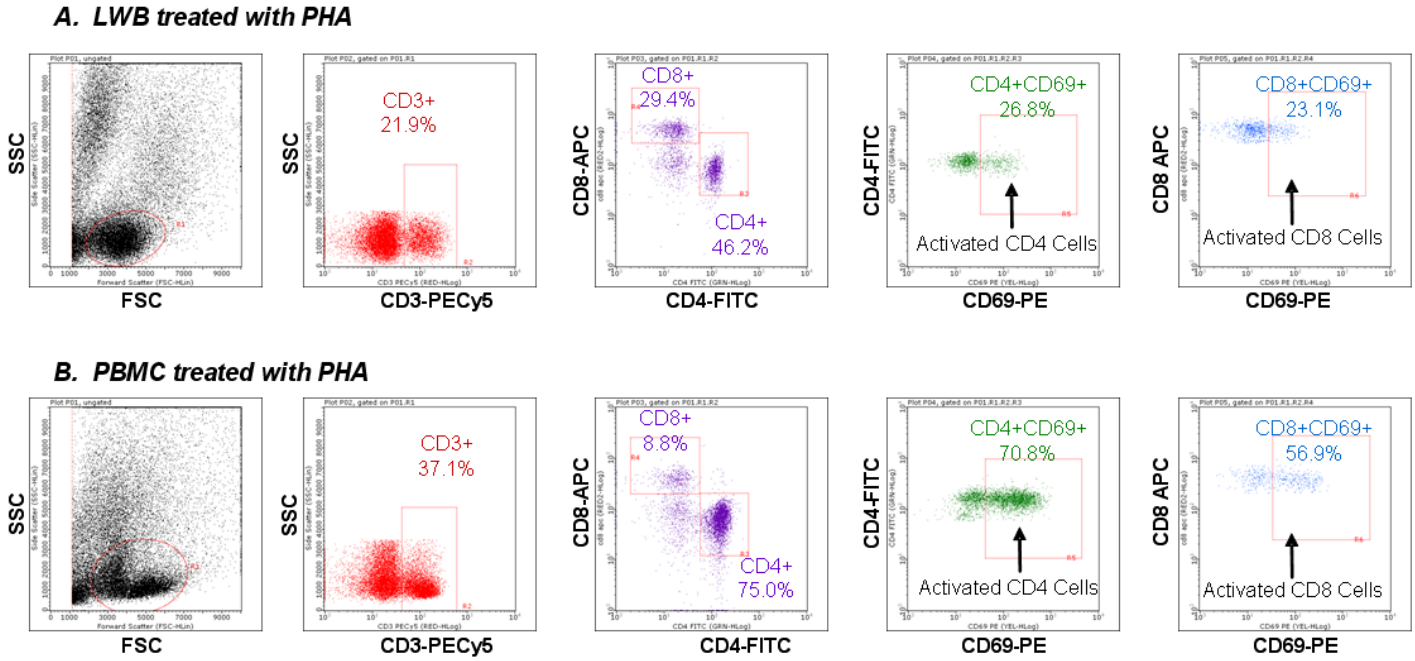

Figure 2 Analyzed Dual Parameter Data: Dot plots depicting either Lysed Whole Blood (panel A.) or PBMC (panel B.) treated overnight with *PHA (phytohemagglutinin) and* stained with the T Cell Activation Kit. Plots show the percentage of positive cells for 1) CD3 T cells, 2) CD4 T cells and CD8 T cells, 3) CD4 T cells and CD69 responsive populations, and 4) CD8 T cells and CD69 responsive populations. As seen, in the above examples, treatment with PHA causes appearance of CD69 positive populations for both CD4 and CD8 T Cells. In both cases, the gating was set up on an unstimulated control sample and applies to the stimulated samples.

Technical Hints

- All kit reagents, CD4-FITC/CD69-PE/CD3-PECY5/CD8-APC, 1X Assay Buffer BA, and Guava 1X Lysing should be brought to room temperature prior to staining and washing.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
  The easyCyte HT System and FlowCellect™ T Cell Activation Kit yield optimal results when the stained cell sample used for acquisition is between 2 x 10^7 to 3x 10^6 cells/mL cells/mL. To obtain the most accurate results, adjust the cell concentrations to within the recommended range.

Troubleshooting

Potential Problem	Experimental Suggestions
Acquisition rate decreases dramatically Instrument clogging Too many cells	• Cell concentration too high - Decrease the number of cells per microliter by diluting sample to 300 – 500 cells/uL. The Guava EasyCyte™ Plus or guava easyCyte HT systems gives the most accurate data when the flow rate is less 500 cells/uL. • Run a Clean and Rinse to clean out capillary. This procedure can be performed during or after an assay. This will wash away any material forming within the glass capillary walls.
Too few cells	• Stain an increased blood volume (up to 30µL). The assay instructions are optimized to give you a range of cells between 100-500 cells/µL in the final sample volume so accurate population count results are obtained. However, cell numbers in blood donors vary. A substantial decrease in cell numbers can lead to difficulty in adjusting settings.
Background staining and/or non-specific staining of cells	• Although the assay procedure has been optimized to function utilizing both PBMC's and Lysed Whole Blood, further antibody titrations may be necessary for some donors capture the ideal staining concentration. Non-specific staining and background may indicate that less antibody will need to be used during the staining procedure. • Although the assay procedure has been optimized so that compensation is not needed, some samples may have improved staining patterns if compensation is applied. The compensation can be performed after acquisition if needed.
Low level of staining of CD markers	• Although the assay procedure has been optimized to function utilizing both Lysed Whole Blood and PBMC's, every donor may respond differently. A lack of signal may indicate that excess antibody will need to be used during the staining procedure or that the staining time needs to be increase.
Cells do not show a shift in CD69 expression.	• Cells may not have undergone activation or the may be beyond the early activation stage. Positive control samples are recommended for each experiment. Positive controls should be appropriate for comparison with the test procedure or test cell population. Treatments to induce activation include, but are not limited to PHA and PMA/ionomycin. A time course of activation may need to be performed for different compounds to determine optimal detection conditions. • Although the assay procedure has been optimized to function utilizing both Lysed Whole Blood and PBMC's, every donor may respond differently. A lack of signal may indicate that excess antibody will need to be used during the staining procedure or that the staining time needs to be increase
Samples appear to be activated when low level of activation is expected	• Cell cultures may be compromised. Negative controls should be a sample from your cell culture, not treated to induce activation.

Variability in day to day experiments	• If the FlowCellect Cell Activation Kit results are inconsistent, check that the samples were well mixed prior to acquisition. If using an easyCyte 8HT System, be sure that the mixing option has been selected in the Worklist file used to collect data. Cells may quickly settle in your samples and your results will be inaccurate unless the cells are mixed just prior to acquisition. • Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any drop in cell numbers or viability can influence experimental results. • If there appears to be day-to-day variation of the staining pattern, ensure the easyCyte HT System is working properly. Run the easyCheck Procedure using the easyCheck Kit (Part No 4500-0025) to verify proper instrument function and accuracy.

***For further support, please contact Millipore's Technical services at 1-800-645-5476*

References

1. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. Annu Rev Immunol. 2009;27:591-619.

2. Biselli R, Matricardi PM, D'Amelio R, Fattorossi A. Multiparametric flow cytometric analysis of the kinetics of surface molecule expression after polyclonal activation of human peripheral blood T lymphocytes. Scand J Immunol. 192 Apr;35(4):439-47.

3. Kepp O, Tesniere A, Zitvogel L, Kroemer G. The immunogenicity of tumor cell death. Curr Opin Oncol. 2009 Jan;21(1):71-6.

4. Pellicanò M, Bulati M, Buffa S, Barbagallo M, Di Prima A, Misiano G, Picone P, Di Carlo M, Nuzzo D, Candore G, Vasto S, Lio D, Caruso C, Colonna-Romano G. Systemic immune responses in Alzheimer's disease: in vitro mononuclear cell activation and cytokine production. J Alzheimers Dis. 2010 Jan;21(1):181-92.

5. Barboza L, Salmen S, Peterson DL, Montes H, Colmenares M, Hernández M, Berrueta-Carrillo LE, Berrueta L. Altered T cell costimulation during chronic hepatitis B infection. Cell Immunol. 2009;257(1-2):61-8. Epub 2009 Apr 3.

6. Janke M, Peeters B, Zhao H, de Leeuw O, Moorman R, Arnold A, Ziouta Y, Fournier P, Schirrmacher V. Activation of human T cells by a tumor vaccine infected with recombinant Newcastle disease virus producing IL-2. Int J Oncol. 2008 Oct;33(4):823-32.

7. Pitsios C, Dimitrakopoulou A, Tsalimalma K, Kordossis T, Choremi-Papadopoulou H. Expression of CD69 on T-cell subsets in HIV-1 disease. Scand J Clin Lab Invest. 2008;68(3):233-41

8. Testi R, Phillips JH, Lanier LL. T cell activation via Leu-23 (CD69). J Immunol. 1989 Aug 15;143(4):1123-8. PubMed PMID: 2501389.

9. Hara T, Jung LK, Bjorndahl JM, Fu SM. Human T cell activation. III. Rapid induction of a phosphorylated28 kD/32 kD disulfide-linked early activation antigen (EA 1) by 12-o-tetradecanoyl phorbol-13-acetate, mitogens, and antigens. J Exp Med 1986;164:1988-2005.

10. Risso A, Smilovich D, Capra MC, Baldissarro I, Yan G, Bargellesi A, Cosulich ME. CD69 in resting and activated T lymphocytes. Its association with a GTP binding protein and biochemical requirements for its expression. J Immunol. 1991 Jun 15;146(12):4105-14.

11. Poggi A. CD Guide. CD69. In: Mason D, André P, Bensussan A, Buckley C, Civin C, Clark E, et al.,editors. Leucocyte typing VII. White cell differentiation antigens. Proceedings of the 7th International Workshop and Conference; 2000 Jun 19-23; Harrogate, United Kingdom. New York: Oxford University Press Inc.; 2002. p. 821-2.

12. Poggi A. CD Guide. CD69. In: Mason D, André P, Bensussan A, Buckley C, Civin C, Clark E, et al., editors. Leucocyte typing VII. White cell differentiation antigens. Proceedings of the 7th International Workshop and Conference; 2000 Jun 19-23; Harrogate, United Kingdom. New York: Oxford University Press Inc.; 2002. p. 821-2.

Related Kits

1. FlowCellect™ Human T Cell MitoDamage Kit (Catalog No. FCCH100139)
2. FlowCellect™ Human T Cell Apoptosis Kit (Catalog No. FCCH100138)
3. FlowCellect™ Human CD8 T Cell Fas Kit (Catalog No. FCCH100140)
4. FlowCellect™ Human CD4 T Cell Fas Kit (Catalog No. FCCH100154)
5. FlowCellect™ Human B Cell Fas Kit (Catalog No. FCCH100137)
6. FlowCellect™ MitoPotential Red Kit (Catalog No. FCCH100105)
7. FlowCellect™ MitoDamage Kit (Catalog No. FCCH100106)
8. FlowCellect™ MitoLive Kit (Catalog No. FCCH100107)
9. FlowCellect™ Annexin Red Kit (Catalog No. FCCH100108)
10. FlowCellect™ MitoStress Kit (Catalog No. FCCH100109)
11. FlowCellect™ Cytochrome *c* Kit (Catalog No. FCCH100110)
12. Guava® EasyCyte™ MitoPotential™ Kit (Catalog No. 4500-0250)
13. Guava Nexin® Reagent (Catalog No. 4500-0450, 4500-0455)

Warranty

Millipore Corporation (“Millipore”) warrants its products will meet their applicable published specifications when used in accordance with their applicable instructions for a period of one year from shipment of the products. **MILLIPORE MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.** The warranty provided herein and the data, specifications and descriptions of Millipore products appearing in Millipore’s published catalogues and product literature may not be altered except by express written agreement signed by an officer of Millipore. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Millipore’s sole obligation shall be to repair or replace, at its option, the applicable product or part thereof, provided the customer notifies Millipore promptly of any such breach. If after exercising reasonable efforts, Millipore is unable to repair or replace the product or part, then Millipore shall refund to the Company all monies paid for such applicable Product. **MILLIPORE SHALL NOT BE LIABLE FOR CONSEQUENTIAL, INCIDENTAL, SPECIAL OR ANY OTHER DAMAGES RESULTING FROM ECONOMIC LOSS OR PROPERTY DAMAGE SUSTAINED BY ANY COMPANY CUSTOMER FROM THE USE OF ITS PRODUCTS.**

Unless otherwise stated in our catalog or other company documentation accompanying the product(s), our products are intended for research use only and are not to be used for any other purpose, which includes but is not limited to, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses or any type of consumption or application to humans or animals.

Cat. No. FCCH100141

Nov/2010
Revision A Part No. 4600-3329MAN