

Kit Manual

BD Cytifix/Cytoperm™
Fixation/Permeabilization Kit
(Cat. No. 554714)

BD Cytifix/Cytoperm™ Plus
Fixation/Permeabilization Kit
(with BD GolgiStop™ protein transport
inhibitor containing monensin)
(Cat. No. 554715)

BD Cytifix/Cytoperm™ Plus
Fixation/Permeabilization Kit
(with BD GolgiPlug™ protein transport
inhibitor containing brefeldin A)
(Cat. No. 555028)



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BD Biosciences offers three kits to simplify the fixation and permeabilization of cells for immunofluorescent staining of intracytoplasmic cytokines. The **BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit** provides a fixation/permeabilization solution and a permeabilization/washing solution. The **BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (with BD GolgiStop™ protein transport inhibitor)** provides these two solutions plus a protein transport inhibitor containing monensin for the treatment of freshly-explanted or cultured cells to promote intracytoplasmic cytokine accumulation. The **BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (with BD GolgiPlug™ protein transport inhibitor)** provides the two solutions, plus an alternative protein transport inhibitor containing brefeldin A. These kits provide enough of the two solutions for staining ≥ 200 cell samples.

BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (Cat. No. 554714)

This kit enables the fixation and permeabilization of cells which is necessary for staining intracellular cytokines with fluorochrome-conjugated anti-cytokine antibodies. The kit provides two reagents, fixation/permeabilization solution and BD Perm/Wash™ Buffer. After cell fixation and permeabilization, the BD Perm/Wash™ Buffer is used to wash the cells and to dilute the anti-cytokine antibodies for staining.

Note: It is important that the BD Perm/Wash™ Buffer be used for dilution of anti-cytokine antibodies, rather than a standard staining buffer, in order to maintain cells in a permeabilized state for intracellular staining.

Kit components:

- Fixation/Permeabilization solution (125 mL)
- BD Perm/Wash™ Buffer, 10 × concentrate containing Fetal Bovine Serum (FBS)* and saponin (dilute 1:10 in distilled H₂O prior to use) (100 mL)

Note 1: Although both the Fixation/Permeabilization solution and the BD Perm/Wash™ Buffer contain constituents that prevent contamination, it is recommended that the solutions be removed using sterile pipettes and the bottles closed immediately after use.

Note 2: BD Perm/Wash™ buffer is supplied as 10× stock solution that contains FBS*. The color of this product may vary from lot-to-lot, and it may contain visible precipitate. Color variation and/or precipitate do not affect product performance.

** Source of all serum proteins is from USDA inspected abattoirs located in the United States*

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BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (with BD GolgiStop™ protein transport inhibitor) (Cat. No. 554715)

In addition to the Fixation/Permeabilization solution and BD Perm/Wash™ Buffer included in the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit, the BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit provides BD GolgiStop protein transport inhibitor containing monensin. The *ex vivo* addition of BD GolgiStop™ to *in vitro*- or *in vivo*-stimulated cells blocks their intracellular transport processes. This results in the accumulation of most cytokine proteins in the Golgi complex and thereby enhances cytokine staining signals (*See figures 2-4*). Enough BD GolgiStop reagent is provided for treating up to 1 liter of cell culture at a cell density of up to 2×10^6 cells/mL.

Kit components:

- Fixation/Permeabilization solution (125 mL)
- BD Perm/Wash™ Buffer, 10 × concentrate containing FBS* and saponin (dilute 1:10 in distilled H₂O prior to use) (100 mL)
- BD GolgiStop protein transport inhibitor containing monensin (also sold as a separate component; Cat. No. 554724) (0.7 mL)

Note: BD Perm/Wash™ buffer is supplied as 10× stock solution that contains FBS*. The color of this product may vary from lot-to-lot, and it may contain visible precipitate. Color variation and/or precipitate do not affect product performance.

** Source of all serum proteins is from USDA inspected abattoirs located in the United States*

BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (BD GolgiPlug™ protein transport inhibitor) (Cat. No. 555028)

In addition to the Fixation/Permeabilization solution and BD Perm/Wash™ Buffer included in the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit, this kit provides an alternative protein transport inhibitor, BD GolgiPlug™ containing brefeldin A. *See figures 2-4*. Sufficient BD GolgiPlug reagent is provided for treating up to 1 liter of cell culture at a cell density of up to 2×10^6 cells/mL.

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Kit components:

- Fixation/Permeabilization solution (125 mL)
- BD Perm/Wash™ Buffer, 10 × concentrate containing FBS* and saponin (dilute 1:10 in distilled H₂O prior to use) (100 mL)
- BD GolgiPlug protein transport inhibitor containing brefeldin A (also sold as a separate component; Cat. No. 555029) (1 mL)

Note: BD Perm/Wash™ buffer is supplied as 10× stock solution that contains FBS*. The color of this product may vary from lot-to-lot, and it may contain visible precipitate. Color variation and/or precipitate do not affect product performance.

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Warnings and Precautions

1. *Danger*

BD Cytotfix/Cytoperm™ Buffer (Fixation and Permeabilization Solution; component 51-2090KZ) contains 4.2% formaldehyde (w/w).

Hazard statements

Harmful to aquatic life.

Causes skin irritation.

Causes serious eye damage.

May cause an allergic skin reaction.

Suspected of causing genetic defects.

May cause cancer. Route of exposure: Inhalative.

May cause respiratory irritation.

Precautionary statements

Wear protective clothing / eye protection.

Wear protective gloves.

Contaminated work clothing must not be allowed out of the workplace. Wash contaminated clothing before reuse.

Avoid release to the environment.

Do not breathe mist/vapours/spray.

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IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do.

IF ON SKIN: Wash with plenty of water.

Continue rinsing.

If skin irritation or rash occurs: Get medical advice/attention.

Storage: Store locked up.

Disposal: Dispose of contents/container to an appropriate treatment and disposal facility in accordance with applicable laws and regulations, and product characteristics at time of disposal.

2. Danger

BD GolgiStop™ Protein Transport Inhibitor (component 51-2092KZ) contains 99.61% ethanol (w/w) and 0.26% monensin, mononatriumsalz (w/w).

Hazard statements

Highly flammable liquid and vapor.

Causes serious eye irritation.

Precautionary statements

Keep away from heat/sparks/open flames/hot surfaces. No smoking.

Wear protective gloves / eye protection.

Wear protective clothing.

IF ON SKIN (or hair): Remove / Take off immediately all contaminated clothing. Rinse skin with water / shower.

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

Dispose of contents / container in accordance with local / regional / national / international regulations.

General Procedure

A. Stimulation of Cells

Various *in vitro* methods have been reported for stimulating cytokine producing cells.¹⁻⁶ Polyclonal activators have been particularly useful for inducing and characterizing cytokine-producing cells. These include the

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following: phorbol esters plus calcium ionophore or ionomycin, phytohaemagglutinin, Staphylococcus enterotoxin B, and monoclonal antibodies directed against subunits of the TCR/CD3 complex (with or without antibodies directed against costimulatory receptors such as CD28).

Note: It has been reported that cell activation with PMA alone causes a transient loss of CD4 expression from the surface of mouse T cells. Cell activation with PMA and calcium ionophore together has been reported to cause a greater and more sustained decrease in CD4 expression, and also a decrease in CD8 expression in mouse thymocytes and mouse and human peripheral T lymphocytes.⁸

1. Procedure for Using BD GolgiStop™ Protein Transport Inhibitor (contains monensin)

Add 4 µL of BD GolgiStop™ for every 6 mL of cell culture and mix thoroughly. It is recommended that BD GolgiStop not be kept in cell culture for longer than 12 hours.

Note: It is recommended that kinetic studies be performed to determine the optimal incubation time for each experimental system.

2. Procedure for Using BD GolgiPlug™ Protein Transport Inhibitor (contains Brefeldin A)

BD GolgiPlug™ contains DMSO which is a solid at 4°C. Be sure to thaw at room temperature prior to use. Add 1 µL of BD GolgiPlug for every 1 mL of cell culture and mix thoroughly. It is recommended that BD GolgiPlug not be kept in cell culture for longer than 12 hours.

Note: It is recommended that kinetic studies be performed to determine the optimal incubation time for each experimental system.

B. Protocol: Multicolor Staining for Cell Surface Antigens and Intracellular Cytokines

1. Harvest Cells

Viable cell populations can be prepared from *in vivo*-stimulated tissues or from *in vitro* stimulatory cultures treated with a protein transport inhibitor. The cells should be spun down out of the medium containing BD GolgiStop™ or BD GolgiPlug™, then resuspended in staining media, counted and transferred to plastic tubes or microwell plates for immunofluorescent staining. Cells should be protected from light throughout staining and storage.

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2. Block Fc Receptors

Reagents that block Fc receptors may be useful for reducing nonspecific immunofluorescent staining.⁷

- a. In the mouse system, purified 2.4G2 antibody, specific for Fc γ II/III receptors (BD Fc Block™; Cat. No. 553142), can be used to block nonspecific staining by fluorochrome-conjugated antibodies which is mediated by Fc receptors. To block mouse Fc receptors with Fc Block, preincubate cell suspension with 1 μ g BD Fc Block/ 10^6 cells in 100 μ L of Staining Buffer for 15 minutes at 4°C. The cells can then be washed and stained with a fluorochrome-conjugated antibody specific for a cell surface antigen of interest which should be diluted appropriately in Staining Buffer.
- b. Fc receptors on human cells can be pre-blocked by incubating cells with 10% normal human serum or an excess of irrelevant purified Ig from the same species and with the same isotype as the antibodies used for immunofluorescent staining.

3. Stain Cell Surface Antigens

- a. Stain $\sim 10^6$ cells in 50 μ L of Staining Buffer with the appropriate amount of a fluorochrome-conjugated monoclonal antibody specific for a cell surface antigen such as CD3, CD4, CD8, CD14, or CD19 (30 minutes, 4°C).

Note: Multicolor staining of different cell surface antigens can be done at this time to provide controls for setting proper compensation of the brightest fluorescent signals. Some antibodies which recognize cell surface markers may not bind to fixed/denatured antigen.¹⁴ For this reason, it is recommended that the staining of cell surface antigens be done with live, unfixed cells PRIOR to fixation/permeabilization and staining of intracellular cytokines. Altering the procedure such that cells are fixed prior to staining of cell surface antigens requires that suitable antibody clones be empirically identified.

- b. Wash cells 2 times with Staining Buffer (*e.g.*, 250 μ L/wash for microwell plates, 1 mL/wash for tubes) and pellet by centrifugation (250 \times g).

4. Fix and Permeabilize Cells

- a. Thoroughly resuspend cells and add 100 μ L per well for microwell plates (or 250 μ L for tubes) of Fixation/Permeabilization solution for 20 minutes at 4°C.

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Note: Cell aggregation can be avoided by vortexing prior to the addition of the Fixation/Permeabilization solution.

- b. Wash cells two times in 1× BD Perm/Wash™ buffer (*e.g.*, 1 mL/wash for staining in tubes and 250 µL/wash final volume for staining in microwell plates) and pellet.

Note: BD Perm/Wash™ buffer must be maintained in washing steps to keep cells permeabilized.

5. Alternative Fixation and Permeabilization Protocol

Cells can be fixed and stored to continue the intracellular staining at a later time.

a. Fixation and Storage of Cells

1. Resuspend cells in 100 µL (or 1 mL/10⁷ cells for bulk fixing) of a 4% paraformaldehyde solution at 4°C for 10-20 minutes.
2. Wash cells 2× in Staining Buffer.
3. Resuspend cells in Staining Buffer for storing cells at 4°C for up to 72 hrs or in 90% FCS/10% DMSO for storing at -80°C for longer periods of time.

b. Permeabilizing Fixed Cells

1. For frozen cells, wash 2× to remove DMSO. For cells at 4°C, pellet and remove staining buffer.
2. Resuspend cells in BD Perm/Wash™ buffer for 15 minutes.
3. Pellet by centrifugation.
4. Stain for intracellular cytokines.

6. Stain for Intracellular Cytokines

- a. Thoroughly resuspend fixed/permeabilized cells in 50 µL of BD Perm/Wash™ buffer containing a pre-determined optimal concentration of a fluorochrome-conjugated anti-cytokine antibody or appropriate negative control. Incubate at 4°C for 30 minutes in the dark.
- b. Wash cells 2 times with 1× BD Perm/Wash™ buffer (1 mL/wash for staining in tubes and 250 µL/wash final volume for staining in microwell plates) and resuspend in Staining Buffer prior to flow cytometric analysis.

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C. Flow Cytometric Analysis

Set PMT voltage and compensation using cell surface staining controls. Set quadrant markers based on isotype or blocking controls and unstained cells.

Note: Frequencies of cytokine producing cells derived from activation of human PBMCs can vary widely for a particular cytokine, depending on the donor. Cryopreserved cells from one donor can be used for longitudinal studies.^{2,6}

For proper flow cytometric analysis, cells stained by this method should be inspected by light microscopy and/or flow light scatter pattern to confirm that they are well dispersed. In order to make statistically significant population frequency measurements, sufficiently large sample sizes should be acquired during flow cytometric analysis. Bivariate dot plots or probability contour plots can be generated upon data reanalysis to display the frequencies of and patterns by which individual cells coexpress certain levels of cell surface antigen and intracellular cytokine proteins.

D. Staining Controls

1. Positive Staining Controls

The Technical Data Sheets for BD Biosciences fluorochrome-conjugated anticytokine antibodies provide specific examples of *in vitro* culture systems which can induce detectable frequencies of cytokine-producing cells at specific time points. Cells stimulated by these methods can be used as positive controls for experimental systems. Particularly important parameters for cell activation protocols include the use of protein transport inhibitors and the examination of multiple time points. Published reports of immunohistochemical staining¹¹⁻¹³ and ELISPOT analysis can also provide useful information regarding different experimental protocols for generating cytokine-producing cells.

2. Negative Staining Controls

The use of at least one of the following three controls is suggested to discriminate specific staining from artifactual staining. A combination of unstained cells and isotype control or blocking control is optimal. Investigators can choose which staining control best meets their research needs. Intracellular cytokine staining techniques and the use of blocking controls are described in detail by C. Prussin and D. Metcalf.⁶

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a. Isotype control

Stain with an isotype-matched control of irrelevant specificity.

1. Resuspend cell pellet in 50 μL of BD Perm/Wash™ buffer containing a concentration of the isotype control antibody equal to that of the anticytokine antibody.
2. Incubate 30 minutes at 4°C.
3. Wash cells using the aforementioned procedure for intracellular staining.

b. Ligand blocking control

Pre-block anti-cytokine antibody with recombinant cytokine.

1. preincubate fluorochrome-labeled antibodies with cytokine diluted to the appropriate concentration in at least 50 μL of BD Perm/Wash™ buffer at 4°C for 30 minutes.
2. Resuspend fixed/permeabilized cells in 50 μL of pre-blocked labeled anticytokine antibody (in BD Perm/Wash™ buffer) and incubate 30 minutes at 4°C.
3. Wash cells using the aforementioned procedure for intracellular staining.

c. Unlabeled antibody blocking control

preincubate cells with unlabeled antibody.

1. Resuspend fixed/permeabilized cells in 25 μL BD Perm/Wash™ buffer containing unlabeled anti-cytokine antibody diluted to the appropriate concentration, and incubate 30 minutes at 4°C.
2. After incubation, add fluorochrome-labeled anti-cytokine antibody at optimal concentration in 25 μL BD Perm/Wash™ buffer (50 μL final volume) and incubate 30 minutes at 4°C.
3. Wash cells using the aforementioned procedure for intracellular staining.

Solutions

Staining Buffer

- Dulbecco's PBS (DPBS) without Mg^{2+} or Ca^{2+}
- 1% heat-inactivated FCS
- 0.09% (w/v) sodium azide
- Adjust buffer pH to 7.4 - 7.6, filter (0.2 μm pore membrane), and store at 4°C

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Sample Data

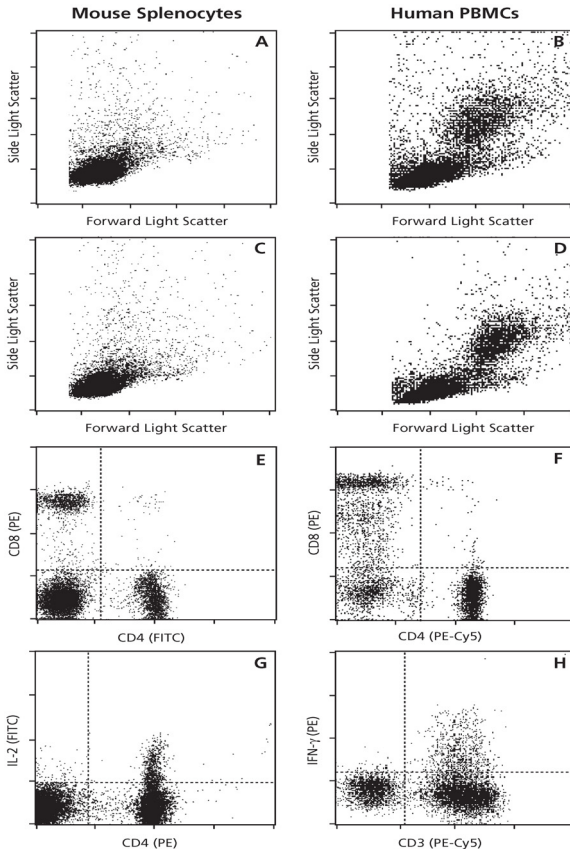


Figure 1. The effect of the BD Cytifix/Cytoperm solution on cell light scattering properties, cell surface antigen staining and intracellular cytokine staining.

Panels A and B show the forward light scatter and side light scatter profiles for fresh, untreated mouse splenocytes and Ficoll™-separated human peripheral blood mononuclear cells, respectively. Panels C and D show the forward light scatter and side light scatter profiles of the same cells (*in Panels A and B*) after they were treated with Fixation/Permeabilization solution. Panels E and F are examples of mouse and human cells, respectively, that were stained with anti-CD4 and anti-CD8 followed by incubation with the Fixation/Permeabilization solution. Panels G and H are examples of mouse and human cells, respectively, that were activated in culture in the presence of BD GolgiStop™ protein transport inhibitor, stained with PE-anti-CD4 or PE-Cy5 anti-CD3 followed by incubation with the Fixation/Permeabilization solution. The cells were then stained for intracellular IL-2 (mouse) and IFN- γ (human).

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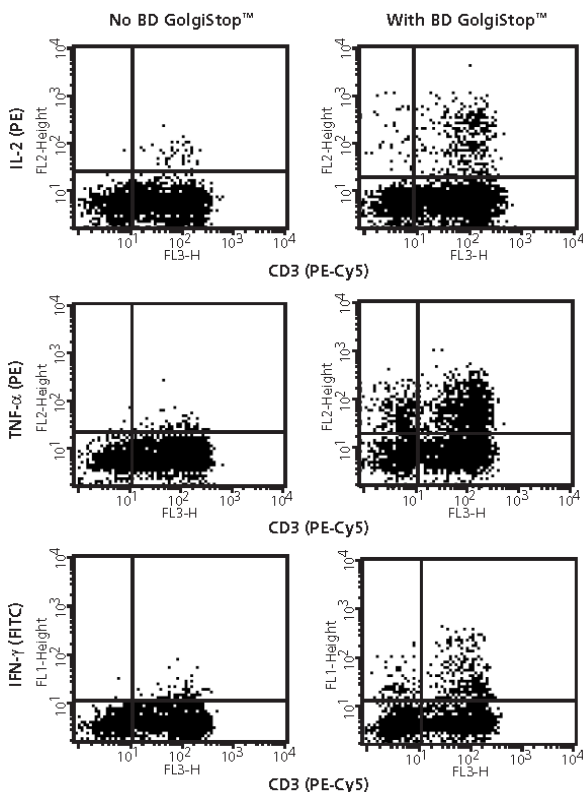


Figure 2. Effect of protein transport inhibitor on intracellular cytokine staining signal.

Human PMBCs were stimulated for 6 hours with PMA (50 ng/mL) and calcium ionophore A23187 (250 ng/mL), in the absence (*left panels*) or presence (*right panels*) of 2 μ M monensin (aka BD GolgiStop™ protein transport inhibitor). Cells were harvested, stained with PE-Cy5 anti-human CD3 (Cat. No. 555334), fixed, permeabilized, and subsequently stained with PE anti-human IL-2 (Cat. No. 554566; *top panels*), PE anti-human TNF (Cat. No. 554513; *middle panels*), or FITC anti-human IFN- γ (Cat. No. 554551; *bottom panels*).

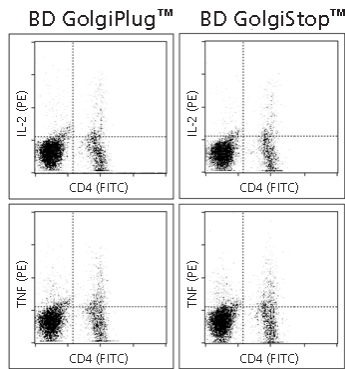


Figure 3. Comparison of the effects of BD GolgiPlug and BD GolgiStop on intracellular cytokine accumulation by activated mouse splenocytes.

IL-2 and TNF production was assessed by intracellular cytokine staining of mouse splenocytes that had been activated with immobilized anti-CD3 (25 µg/ml) + soluble anti-CD28 (2 µg/ml) for 4 hours in the presence of the protein transport inhibitor, BD GolgiPlug™ or BD GolgiStop™. In this case BD GolgiPlug and BD GolgiStop are comparably effective in inducing IL-2 and TNF accumulation.

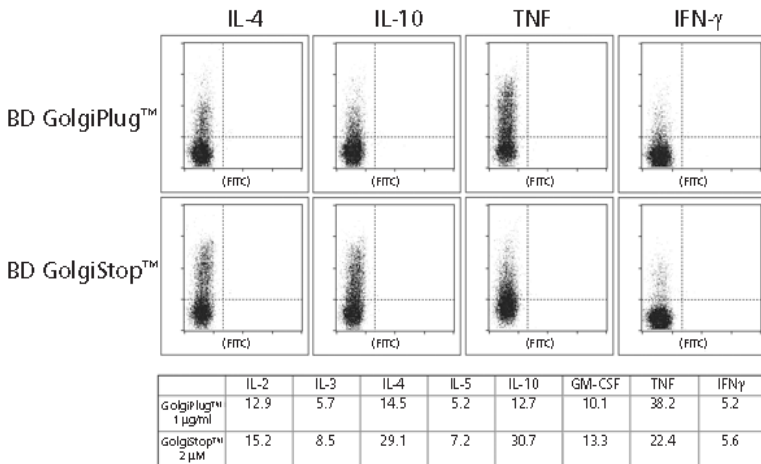


Figure 4. Comparison of the effects of BD GolgiPlug and BD GolgiStop on intracellular cytokine accumulation by re-activated purified mouse CD4⁺ cells.

Activated mouse CD4⁺ cells were restimulated with PMA (10 ng/mL) plus ionomycin (250 ng/mL) for 5 hours in the presence of BD GolgiPlug™ or BD GolgiStop™ and stained for the intracellular cytokines listed. In this case, BD GolgiPlug was more effective in inducing TNF accumulation, while BD GolgiStop was more effective in inducing IL-4 and IL-10 accumulation.

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Ficoll-Hypaque™ Purified Human PBMCs vs. Whole Blood

TH1/Th2 Cell Discrimination

PMA/A23187 - 5 hr Activation (2 μ M BD GolgiStop)

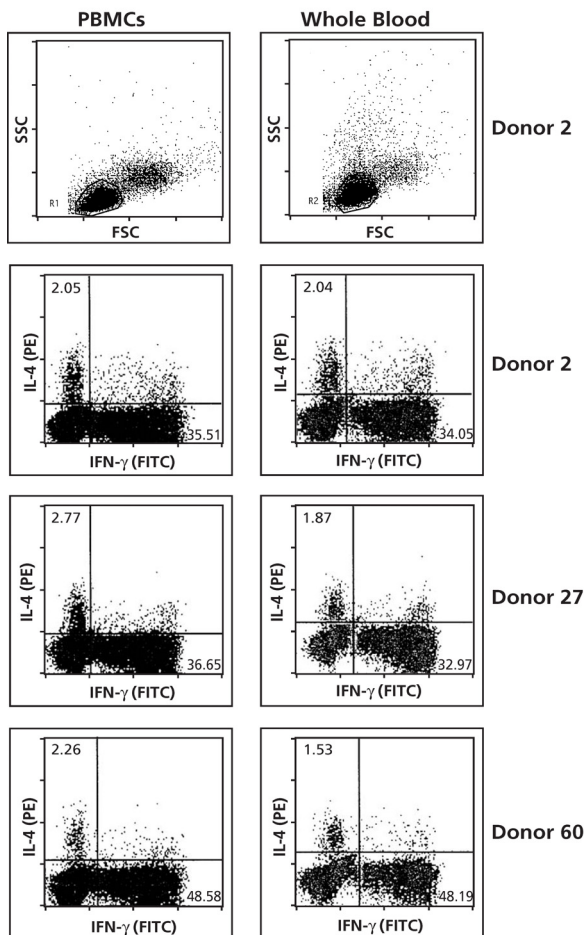


Figure 5. Frequencies of detectable cytokine-producing cells are comparable when staining activated PBMCs or activated whole blood from the same donor

Ficoll-Hypaque™ purified human PBMCs (*left panels*) and whole blood (*right panels*) from each of three donors were activated with PMA (50 ng/mL) and ionophore A23187 (1 μ g/mL) for 5 hr in the presence of 2 μ M BD GolgiStop™, fixed, permeabilized and stained with PE-anti-human IL-4 (Cat. No. 554485; 0.06 μ g) and FITC-anti-human IFN- γ (Cat. No. 554700; 0.25 μ g), according to the BD Biosciences intracellular cytokine staining protocols (Standard or Whole Blood Method).

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Alternative Protocol

Activation and Intracellular Staining of Whole Blood

1. Dilute whole blood 1:1 with IMDM. Mix well.
2. Add cell activator or mitogen to diluted blood (e.g., 50 ng/mL PMA [Sigma, Cat. No. P-8139] + 1 µg/mL calcium ionophore A23187 [Sigma, Cat. No. C-7522] or PMA + 1 µM ionomycin [Sigma, Cat. No. I-0634] - final concentration).
3. Add protein transport inhibitor:
 - BD GolgiPlug™ protein transport inhibitor, (Cat. No. 555029)
1 µL solution / 1 mL of diluted blood (aka Brefeldin A, 1.0 µg/mL final concentration) or
 - BD GolgiStop™ protein transport inhibitor, (Cat. No. 554724)
0.7 µL solution / 1 mL of diluted blood (aka Monensin, 2.0 µM final concentration).
4. Vortex briefly to mix. Aliquot into 12 × 75 mm plastic tubes, 200 µL/tube. Incubate for 4-6 hrs in 5% CO₂ at 37°C. Optimal incubation times must be investigated and incubation time should not exceed 24 hours.
5. Add 2 mL 1× BD PharmLyse™ (Cat. No. 555899), vortex, incubate 10 minutes at RT in the dark.
6. Spin 5 minutes, 500 × g.
7. Aspirate supernatant. Wash 1× in Staining Buffer. Spin 5 minutes at 500 × g. Aspirate supernatant.
8. Resuspend cell pellet in 100 µL of Staining Buffer containing an optimal concentration of fluorochrome-conjugated antibodies specific for cell surface antigens (e.g., FITC-, PE-, PE-Cy5- anti-CD3, CD4, CD8, CD14, etc.). Incubate for 15 minutes at RT in dark. Wash 1× in Staining Buffer. Spin 5 minutes, 500 × g. Aspirate supernatant.
9. Fix and permeabilize cells by adding 500 µL of Fixation/Permeabilization solution. Vortex and incubate at RT in the dark for 20 minutes. Spin 5 minutes, 500 × g. Aspirate supernatant.

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10. Wash cells by adding 2 mL BD Perm/Wash™ buffer. Incubate for 10 minutes at RT in the dark. Spin 5 minutes, 500 × g. Aspirate supernatant.
11. Resuspend cell pellet in 100 µL BD Perm/Wash™ buffer containing an optimal concentration of fluorochrome-conjugated anti-cytokine antibody for intracellular staining (e.g., ≤ 0.25 µg/test). See technical data sheet for antibody-specific recommended concentrations. Stain for 30 minutes at RT in the dark.
12. Wash cells by adding 2 mL BD Perm/Wash™ buffer. Spin 5 minutes, 500 × g. Aspirate supernatant
13. Resuspend cell pellet in 500 µL PBS/2% paraformaldehyde solution (vortex while adding PBS/PFA).
14. Analyze by flow cytometry.

Expected results

- 5 hr PMA/Ionomycin activation of normal human blood will generally yield detectable frequencies of IL-2, IL-4, IFN- γ , and TNF expressing cells (lymphocyte gate).
- 6 hr LPS activation of normal human blood will generally yield detectable frequencies of IL-1 α , IL-6, IL-8, and MIP-1 α expressing cells (monocyte gate).

Note: There is variation in typical cytokine - producer cell frequency amongst different donors.

Solutions

Staining Buffer

- Dulbecco's PBS (DPBS) without Mg²⁺ or Ca²⁺
- 1% heat-inactivated FCS
- 0.09% (w/v) sodium azide
- Adjust buffer pH to 7.4 - 7.6, filter (0.2 µm pore membrane), and store at 4°C

IMDM (BioWhittaker - Cat. No. 12-726Q) is a cell culture medium beneficial in maintaining cell viability of whole blood in culture.

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