

December 2010

Detection of Cytokines, Frequently Asked Questions

Q. How important is BD Fc Block™ reagent when performing intracellular cytokine staining, and how should it be used?

A. Use of an Fc-receptor blocking antibody is a good idea, especially if you are working with monocytes or granulocytes. BD Fc Block needs to be used before the fixation and permeabilization step. We currently offer BD Fc Block for mouse (Cat. No. 553141 or 553142) and rat (Cat. No. 550270 or 550271). For intracellular staining of human cells, blocking of Fc receptors is typically not required. Under certain conditions with human samples, it might be suitable to use a general block such as irrelevant purified Ig of the same species or serum of the species of the antibody used for staining.

Q. How much recombinant cytokine is recommended for blocking intracellular cytokine staining?

A. We normally use about 0.25 µg of cytokine per 0.50 µg of antibody.

Q. What do you recommend for optimizing intracellular cytokine staining?

A. To simplify the intracellular cytokine staining protocol for flow cytometry and to reduce the possibility of non-specific interactions, we recommend using antibodies directly conjugated to a fluorescent dye. We do not recommend using biotinylated antibodies followed by a streptavidin-dye conjugate for intracellular staining for flow cytometry.

Q. Do you have a protocol for stimulation of cytokines in rat cells?

A. Stimulation of cytokines in rat cells has been difficult due to lack of viability after the suggested 2 to 5-day culture. In-house, we routinely get only about 30–60% cell viability. Here are some tips to enhance viability:

- Use whole lymph node cells instead of CD4 cells as suggested in our protocol (see section E at bdbiosciences.com/support/resources/protocols/activation_immune_cells.jsp).
- During the incubation stage with recombinant IL-2 and IL-4, look at the culture on day 2 (instead of day 3). If cells are crowded, split it 1:2 and add fresh media with recombinant IL-2 and IL-4. You can also stop the culture after 2 days instead of 3 and stimulate with PMA and ionomycin. The 2-day culturing will result in less cytokine production but greater viability of cells.

Q. I would like to perform intracellular cytokine staining of IFN-γ, IL-4, and IL-17A together with FoxP3. Could you suggest the buffers that I could use to detect them together?

A. To perform co-staining of FoxP3 together with these cytokines, we recommend using the FoxP3 buffer system. The exception is co-staining with IL-4. We have not been successful staining FoxP3 with IL-4 to date. However, both IL-17A and IFN-γ stain well. For more details and data comparing different buffers, view our webinar from April 2009 entitled: "Optimizing Intracellular Flow Cytometry: Simultaneous Detection of Cytokines and Transcription Factors" at: bdbiosciences.com/hotlines/webinars/archives/2009.jsp

Q. Does the anti-mouse IL-17 antibody (clone TC11-18H10) recognize the A or F isoform?

A. Our clone TC11-18H10 recognizes the IL-17A isoform.

- Liang SC, Tan XY, Luxenberg DP, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med.* 2006;203:2271-2279.



December 2010

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Q. What is the ATCC strain of EL4 cells used to test human IL-17A?

A. The ATCC strain for EL4 cell is TIB-39.

Q. Could you suggest reagents to detect IL-17A and Tregs using a single-step protocol?

A. We have the Th17/Treg phenotype cocktail to detect both Tregs and Th17 cells in humans (Cat. No. 560767) as well as in mice (Cat. No. 560752). For details on the protocol and reagents included, please see the technical data sheet (TDS) available at bdbiosciences.com.

Q. Can I detect Th1/Th2/Th17 secreting cells using a single-step reagent? If so, what products are recommended?

A. Yes, it is possible to detect Th1/Th2/Th17 secreting cells by flow cytometry using the Th1/Th2/Th17 phenotyping kits in both human (Cat. No. 560751) and mouse (Cat. No. 560758). For further details about these reagents and about buffer compatibility, see the TDS available at bdbiosciences.com. For a more quantitative detection of Th1/Th2/Th17 cytokines generated by cells, we recommend the BD™ Cytometric Bead Array (CBA) (Cat. No. 560484 for human and 560485 for mouse).

Q. How I can generate Th9 polarized cells in vitro using human PBMCs?

A. To generate Th9 cells in vitro:

1. Stimulate PBMCs with plate bound anti-CD3 (10 µg/mL) (Cat. No. 555329) and anti-human CD28 (1 µg/mL) (Cat. No. 555725) plus recombinant IL-2 (10 ng/mL) (Cat. No. 554603); IL-4 (50 ng/mL) (Cat. No. 554605); and TGF-β (10 ng/mL) (Cat. No. 356039) and anti-IFN-γ (10 µg/mL) (Cat. No. 554698) for 5 days.
2. Harvest the cells and restimulate with PMA (50 ng/mL) and ionomycin (1 µg/mL) in the presence of BD GolgiStop™ protein transport inhibitor.
3. Harvest the cells and stain them post fixation and permeabilization using BD Cytotfix/Cytoperm™ reagent using clone MH9A3.

Q. I would like to stain for human GATA3 staining using clone L50-823 from BD Biosciences together with IFN-γ and IL-4. What are your buffer recommendations?

A. Visualization of GATA3 by flow cytometry requires permeabilization. We have observed that GATA3 staining, together with cytokines, works well using the BD Cytotfix/Cytoperm™ fixation/permeabilization solution and BD Perm/Wash™ buffer (Cat. No. 554714).

Q. We don't see a good correlation between intracellular cytokine staining (ICS) and ELISA. Why?

A. We have found that it is difficult to directly correlate ELISA results to the percentage of positive cells detected by flow cytometry, since ELISA detects a quantity of cytokine released over a period of time (sometimes days), while detection of intracellular cytokines by flow cytometry represents a brief snapshot in time for stimulated cell populations. Therefore, it is entirely possible for a small percentage of positive cells to secrete a significant amount of cytokine over time in the culture supernatant, resulting in different ELISA results when compared to intracellular cytokine staining.



December 2010

Detection of Cytokines, Frequently Asked Questions

Q. What are the major differences between the three intracellular staining kits available from BD?

A. The main difference among the three kits is the type of cytokine transport inhibitor included. Cat. No. 554714 does not include a protein transport inhibitor, only the BD Cytotfix/Cytoperm™ fixation/permeabilization solution. Cat. No. 554715 includes monensin as the transport inhibitor together with the fixation and the permeabilization reagents. Cat. No. 555028 includes brefeldin A as the transport inhibitor together with the fixation and the permeabilization reagents.

For further information and comparison between the two protein transport inhibitors and their effects on the cytokine staining profile, please see: "Techniques For Immune Function Analysis," Application Handbook, 1st Edition, from BD Biosciences.

Q. What is the precipitate in my BD Perm/Wash buffer, and will it affect performance?

A. It is not uncommon to see a bit of precipitate in the BD Perm/Wash buffer. This precipitation is due to the saponin in the solution. Precipitation will not affect performance in any way. It can be filtered out with a 0.45-µm filter if you prefer, because permeabilization is still effective at lower percentages of saponin. However, is not necessary to do so.

Q. What is a good stopping point when following the BD Cytotfix/Cytoperm intracellular cytokine staining protocol?

A. If you are staining for intracellular cytokines for flow cytometry and need to stop at some point, we recommend doing so after fixing the cells. The cells then must be washed thoroughly in staining buffer to make sure all of the fixative is out. They can then be stored in staining buffer at 4°C for several days, and in some cases, several months. We have obtained good data from cells that were stained for CD4, fixed, and stored at 4°C for 4–6 months. However, how well they hold up in long-term storage probably depends on the antigen and antibody used.

Q. What is the difference between the BD GolgiStop™ and BD GolgiPlug™ protein transport inhibitors?

A. The major difference between these two inhibitors is where they work within a cell. BD GolgiStop (monensin) works by accumulation of protein at the endoplasmic reticulum (ER) stage, while BD GolgiPlug (brefeldin) works with accumulation of protein at the golgi complex. The use of these inhibitors leads to an enhanced ability to detect cytokine-producing cells by intracellular flow cytometry.

Q. What is the stock concentration of BD GolgiStop (monensin)?

A. BD GolgiStop is provided at 3 mM stock concentration in ethanol.

Q. What is the stock concentration of BD GolgiPlug (brefeldin)?

A. BD GolgiPlug is provided at 1 mg/mL stock in DMSO, which may become a solid at 2–8°C.



December 2010

Detection of Cytokines, Frequently Asked Questions

Q. I would like to perform intracellular staining for human Th1/Th2 selection. Which antibodies do you suggest?

A. We suggest using the following products for selection of human Th1 and Th2 cells by intracellular staining:

- **Th1:** INF- γ , clone: B27, optional IL-2, clone: MQ1-17H12;
- **Th2:** IL-4, clone: MP4-25D2, optional IL-5, clone: TRFK5

The best way to calculate the percentage of Th1-positive cells versus Th2-positive cells within a defined population is by flow cytometry. ELISA assays are quantitative and would only provide you with a positive result for a particular cytokine and would not give you the percentage of cells that are Th1 vs. Th2 positive, whereas using flow cytometry would allow you to identify the cell subsets that are Th1 vs. Th2 positive.

Q. Which anti-human IFN- γ clones work well for intracellular cytokine staining by flow cytometry? Which clone is best for neutralization of human IFN- γ ?

A. The anti-human IFN- γ clones B27 and 4S.B3 are virtually identical for intracellular staining. For neutralization, B27 is slightly better than 4S.B3.

Q. I don't see many IL-5⁺ mouse splenocytes. Why not?

A. Mouse cells produce very low levels of IL-5.

Activation procedure: Purified CD4⁺ mouse splenocytes from BALB/c or C57BL/6 mice are stimulated with immobilized anti-mouse CD3 (145-2C11, 25 μ g/mL for plate coating, Cat. No. 553057) and soluble anti-mouse CD28 (37.51, 2 μ g/mL, Cat. No. 553294) in the presence of recombinant mouse IL-2 (10 ng/mL, Cat. No. 550069) and recombinant mouse IL-4 (50 ng/mL, Cat. No. 550067) for 2 days. The cells are washed and subsequently cultured in medium containing IL-2 and IL-4 for 3 days to promote cellular proliferation and differentiation. Finally, the cells are harvested and restimulated for 4–6 h with immobilized anti-mouse CD3 (25 μ g/mL for plate coating) and anti-mouse CD28 (2 μ g/mL) in the presence of a protein transport inhibitor. Alternatively, the cells are restimulated with PMA (5 ng/mL; Sigma, Cat. No. P-8139) and ionomycin (500 ng/mL; Sigma, Cat. No. I-0634) for 4–6 hours in the presence of a protein transport inhibitor.

If PMA plus ionomycin stimulation is used, staining with the cell surface with a memory T-cell marker (such as CD44 or CD62 L) is suggested, followed by intracellular staining. Collect 5,000 to 10,000 of CD44⁺ or CD62 L⁺, and this memory T-cell gating strategy would improve detection of IL-5⁺ cells.

Q. Is lithium heparin blood collection compatible with BD FastImmune™ protocols?

A. The Application Notes entitled "Detecting Cytokines in Lymphocytes" and "Detecting Cytokines in Monocytes" generated at BD Biosciences state that only sodium heparin can be used. We have feedback from external users that lithium heparin is also compatible with whole blood BD FastImmune protocols. However, we have not performed an in-house comparison of lithium and sodium heparin to verify this information.

Q. Are your anti-TNF antibodies specific for TNF- α ?

A. Some time ago, we updated the nomenclature of our TNF products to reflect changes in naming conventions. When TNF was first described in literature, two functions were identified that seemed to be



December 2010

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coming from two related proteins which were named TNF- α and TNF- β . But as research progressed, they isolated the proteins and found they weren't related at all, and that the one that had been labeled TNF- β was the same as lymphotoxin alpha (LT- α). So, TNF- α became just TNF (α is not necessary because there is no TNF- α) and TNF- β is now called LT- α . Two related LT proteins have been found, so there is an LT- α (which was TNF- β) and an LT- β .

Q. Can you distinguish between receptor-bound, soluble, and transmembrane TNF by flow cytometry?

A. It should be possible to distinguish transmembrane TNF from membrane TNFR-bound TNF using an acid stripping protocol. In this type of experiment, cells are treated with an acid solution to desorb receptor-bound cytokines, while transmembrane cytokines remain intact. We have not performed this at BD Biosciences. However, the following publications describe this:

- Brown AR, Fishman M. Tumor necrosis factor-alpha analyzed within individual macrophages by combined immunocytochemistry and computer aided image analysis. *Cell Immunol.* 1990;130:352-363.
- Luettig B, Decker T, Lohmann-Matthes ML. Evidence for the existence of two forms of membrane tumor necrosis factor: an integral protein and a molecule attached to its receptor. *J Immunol.* 1989; 143:4034-4038.
- Renno T, Kraowski, Piccirillo C, Lin JY, Owens T. TNF-alpha expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis. Regulation by Th1 cytokines. *J Immunol.* 1995;154:944-953.

Q. How do I perform ELISA using your IL17 A ELISA pair (Cat No. 555068 and 555067)?

A. For ELISA using Mouse IL-17 A (Cat. No. 555068 and 555067), we recommend using 0.1 M Na Phosphate, pH9.0 as the coating buffer and recommend R&D Systems recombinant mouse IL-17 (Cat. No. 421-ML) as the standard.

For further details, please see our Cytokine ELISA Protocol at:

bdbiosciences.com/support/resources/elisa_elispot/index.jsp

Q: Is it possible to stain simultaneously for BrdU and intracellular cytokines?

A. Yes. We recommend that all of the antibodies be pooled together and incubated with the cells simultaneously. If you put either the BrdU or the intracellular markers first, then wash and follow with a second antibody, we have found the latter marker might be excluded and not stain the cells. You may be interested in our BrdU flow kits if you are planning on staining for BrdU and intracellular cytokines. More detailed information can be found in the kit manual at:

bdbiosciences.com/external_files/pm/doc/manuals/live/web_enabled/03-8100055-1C.pdf

Label No. 23-12929-00

