

BD Pharmingen™ Transcription Factor Buffer Set, Cat. Nos. 562725, 562574

Frequently Asked Questions

How would I use the new buffer in a bulk fix/perm procedure?

Use $\sim 2 \times 10^6$ cells per 1.0 mL of buffer or less. A helpful rule of thumb is to keep the fix/perm buffer volume < 20 mL so subsequent washing and centrifugation do not lead to additional cell loss. For adding the perm/wash buffer, after treating cells with 1X fix/perm buffer, add 1X perm/wash buffer at a volume of 20% to 100% of the original fix/perm volume.

Example: for a fix/perm treatment of 80×10^6 splenocytes, try a fixing volume of 15 mL and add a fix/perm volume of 5 mL for a total of 20 mL. Centrifuge at least 10 minutes at 350g.

Is the buffer designed for lysed whole blood?

No, the new buffer was optimized for peripheral blood mononuclear cells (PBMCs), lymphoid tissues and cell lines, and cell preparations that contain enriched cells and are generally plasma free. However, the buffer does have lytic properties. This allows for easy processing of bone marrow samples, re-lysing of poorly lysed splenocytes, and lysing of poor PBMC preparations with contaminating platelets and red blood cells. Currently modifications to the buffer and protocol are being investigated for whole blood samples.

Can you fix cells and then freeze them in standard DMSO freezing solutions for later analyses?

We have not investigated this method, but as with other fix/perm solutions, the cells will be fixed and permuted at the end of the 40–50-minute incubation time. Cells can then be processed according to your needs. For example, frozen cells could be banked and thawed for analyses at a later time, or stored overnight for staining the next day. Your storage, freezing, and thawing protocol will affect the stability of the desired epitope and would need to be optimized and investigated.

Can the buffer be used for phosphoproteins?

The buffer has not been fully validated with phosphoproteins. Tests to date indicate that, with the current protocol and design, the buffer set generally is not suitable for pStat3 and pStat5. This is because these targets require denaturing with methanol after fixation. The new buffer does not use this principle. However, we are combining phosphoantigen and nuclear transcription factor staining in one protocol in a new product, BD Pharmingen™ Transcription Factor PhosphoPlus Buffer Set, which is available upon special request.

Can the procedure be adapted to indirect staining?

Yes. Perform surface staining after primary and secondary antibodies are bound and cells have been washed. Block with appropriate serum or immunoglobulin if necessary. However, blocking may not be necessary.

Can surface proteins be stained after the perm/wash step?

Yes, most surface staining monoclonal antibodies (mAbs) tested to date stain as expected after the perm/wash step, although generally, less antibody is required. Currently a database of compatible surface staining antibodies is being compiled and is available through technical support. There are caveats with some stains, particularly APC-H7 and CCR7. For example, human CCR7 PE-Cy™7 is best stained before the fix/perm step, while human CD38 PerCP-Cy™5.5 showed better resolution when stained after the perm/wash step.

Is a permeabilization incubation step required?

No, the protocol was not verified with a perm/wash incubation time. Permeabilizing is thought to occur mostly at the fix/perm step. However, if you do not obtain resolution as expected with your antibody, try a 15-minute incubation at 4°C. We have reasonably allowed for a 15-minute perm/wash step prior to staining with antibody to ensure intracellular/intranuclear staining with good results in high-throughput assays in studies using smaller volumes of perm/wash buffer in U-bottom plates.

Why does my background staining look high for intracellular targets?

The buffer has a tendency to shift affinity curves to lower concentrations. An optimal signal to noise (S/N) can be obtained with lower concentrations of antibody. It has also been noted that the buffer allowed for the detection of baseline levels in cells that were previously difficult to resolve transcription factor signal (for example, Pax-5 in human embryonic stem cells). We have seen this especially with conjugates of Alexa Fluor® 647 and BD Horizon™ V450. The buffer was largely optimized around FoxP3 and T-bet staining, while other targets were found to be compatible in later experiments. Thus many intracellular reagents will require lower concentrations than in previous applications for optimal staining.

Try small titers with any reagent, at "test size" and at 0.25X or 0.06X test size of antibody to optimize staining. Shorter incubation times with mAb may be required to create a better workflow for some applications.

When I acquire samples, I don't see my cells by FSC-A vs SSC-A. Did the fix/perm fail?

Likely no, this is the classic problem of understanding fixation and how it interacts with optics of the cytometer. Cells will appear "smaller" by FSC vs SSC, much like if they have been treated with BD FACST™ lysing solution. Voltages on the detectors are to be increased to gate properly. When compared to primary cells and typical fixation procedures using 4% formaldehyde, the new buffer, in contrast, creates events that are "smaller," although technically this is a misnomer. The cells are more translucent and do not refract as much light as other treated cells. The instrument is important as well.

For example, a BD™ LSR II analyzer required an FSC-A voltage setting of 750 and an SSC-A voltage of 350 to visualize the PBMC population, while a BD LSRFortessa™ analyzer required 380 and 270, respectively. In general the cells have a wider coefficient of variation by FSC and SSC than primary cells. This has also provided us new information about morphology by flow cytometry yet to be investigated.

Where can I "stop" in the protocol?

Investigators should determine their own "stop" condition for their application. However, here are a few tips:

- 1. Storing fix/perm samples:** We have had success using the 1X fix/perm buffer and then storing overnight at 2–8°C with no manipulations, and then continuing with the procedure later, or the next day (tested in FoxP3 and T-bet and other stem cell related transcription factors). See the TDS for more information.
- 2. Use a perm/wash incubation:** We have found it useful to allow the cells to sit in the perm/wash buffer after staining as a "wash time." It is the developer's experience that this does not affect the protocol and might even improve S/N by allowing diffusion of unbound antibody. This step is convenient for multitasking. Rather than doing the 2X perm/wash step at the end of the protocol, an alternative is to allow stained cells to sit in 2 mL of perm/wash buffer for up to two hours, centrifuge, and then continue with acquisition. This step can be considered a long wash. A second wash sometimes is not required.
- 3. Store the sample overnight** in 150–350 µL of 1X D-PBS without calcium and magnesium for 24 hours after the fix/perm step is complete. Perm/wash and stain cells the next morning, complete the protocol, and then acquire as usual.
- 4. Storing the sample after the procedure is complete:** Remove the perm/wash buffer and resuspend the pellet in 300 µL of 1X D-PBS without Ca/Mg containing 0.1% BSA. Alternatively, if storing overnight is desired, add 300 µL of 1X D-PBS without Ca/Mg containing 0.1% formalin to fix the antigen-antibody complex. Centrifuge and remove the fixative the next day, resuspend cells in buffer, and continue to acquisition.

Should I incubate my cells on ice?

The protocol is to be performed at 2–8°C, and the cells should be cooled before applying the fix/perm buffer (for example, incubation with antibodies prior to fix/perm at 2–8°C). We have noted poor staining when fix/permed cells are stored or incubated on ice.

How long can I wait before use of the 1X fix/perm buffer after mixing the 4X fix/perm buffer with the diluent to make 1X fix/perm buffer?

The 1X fix/perm buffer is to be used within 5–20 minutes after mixing if kept on ice or in the cold. The intention is to use the solution as soon as possible after mixing with diluent. Do not store for later use.

Is the buffer compatible with intracellular cytokine analyses?

In general yes, but optimal concentrations are noted to be lower, as with the transcription factor antibodies, and the expression levels may be slightly different than BD Cytotfix/Cytoperm™ and related fix/perm buffer systems.

Is the transcription factor buffer set compatible with BD Horizon Brilliant™ Violet and BD Horizon Brilliant™ Ultraviolet conjugated antibodies?

Yes, BV and BUV conjugated antibodies can be used for both cell surface and intracellular staining. However, the BUV dyes used together often require the BD Horizon™ Brilliant Stain Buffer for the mitigation of surface immunofluorescent staining artifacts. Because intracellular environments are more prone to non-specific binding and other interfering factors at the staining step, the BD Pharmingen™ Transcription Factor Buffer Set plus Brilliant Stain Buffer may not be fully compatible with intracellular staining.

It appears that the pellet disappears after the fix/perm step. Am I losing cells?

No, unless gross technical errors have occurred. The fix/perm process will turn the cell pellet translucent; after the perm/wash step, the cell pellet turns slightly white again as expected.

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23-16564-00



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