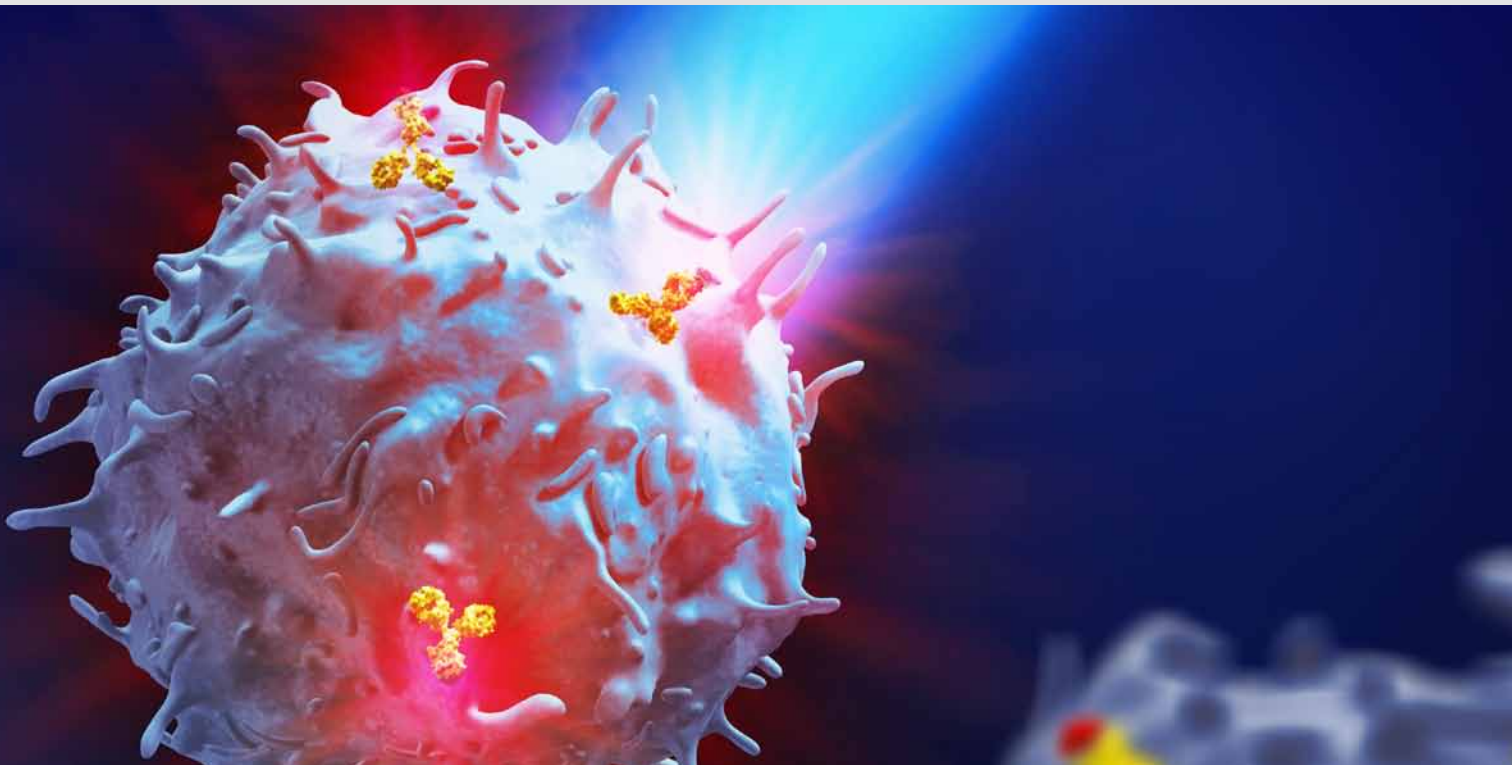# A simple no-wash method for the flow cytometric enumeration of leukocyte subsets in whole blood

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Miltenyi Biotec

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Introduction

Many diseases can affect numbers of white blood cells (leukocytes) in peripheral blood and several assays are available to determine total leukocyte numbers. However, it is far less easy to reliably and reproducibly determine changes in leukocyte subsets, where staining of cell surface markers is necessary. Traditionally, peripheral blood mononuclear cells (PBMCs) are separated from red blood cells using density-gradient centrifugation but, as this involves many wash steps to remove the separation medium, cells may be lost. Staining with antibodies for leukocyte subsets after centrifugation will only give a relative number of cells in PBMCs, rather than absolute cell numbers in the original blood sample. In addition, flow cytometers that use pressure to pass samples through flow cells can only provide relative percentages of positive cells in a sample. As the MACSQuant® Analyzer samples known volumes, it is possible to calculate total numbers of cells in the sample. With this simple whole blood lysis method, followed by analysis using the MACSQuant Analyzer, it was possible to obtain absolute cell numbers of leukocyte populations and subsets in bovine and porcine blood.

Materials and methods

Staining and lysis of bovine blood

Adult bovine blood was collected into EDTA-coated tubes. 60 µL of whole blood was transferred to a MACSQuant Analyzer–compatible sample tube. 25 µL of each pre-conjugated antibody was added to the whole blood and incubated for 20 minutes at room temperature in the dark. 500 µL of ammonium chloride lysis buffer (155 mM ammonium chloride, 0.1 mM EDTA and 10 mM sodium bicarbonate, pH 7.2) was added until the red blood cells lysed (indicated by a clearing of the sample) or 1040 µL of MACS® Red Blood Cell Lysis Solution (Miltenyi Biotec) for 10 minutes. autoMACS® Running Buffer (Miltenyi Biotec) was added to bring the total volume to 1.2 mL, thus giving a 1:20 dilution of the original sample. The samples were placed on ice and analyzed as soon as possible.

Bovine T cells were stained using CD3-Alexa Fluor® 405 (MM1a), CD4-Alexa Fluor 488 (CC30), CD8-APC (CC58), TCRγ/δ T cells using WC1-RPE (CC39), B cells using CD21-Alexa Fluor 488 (CC21), monocytes using CD14-RPE (CCG33), and granulocytes/macrophages/neutrophils using CD172a/SIRPα–APC (ILA24). All antibodies were produced by the Institute for Animal Health unless otherwise stated. CD3 mAb MM1a was

a kind gift from Bill Davies, Washington State University. Antibodies were labeled using Zenon® Labeling Kits from Invitrogen.

Fixation of stained bovine cells

Stained, lysed samples were centrifuged at 3.3 rcf for 1 minute and 1150 µL of supernatant was carefully removed (leaving 50 µL). After addition of 50 µL paraformaldehyde (4% in PBS), the pellet was resuspended and incubated for 5 minutes at room temperature. Finally, autoMACS Running Buffer was added to bring the total volume back to 1.2 mL.

Staining and lysis of porcine blood

Blood from a young pig (approximately 11 g, 4–6 weeks old) was collected into EDTA-coated tubes. 10 µL of whole blood was transferred into a MACSQuant Analyzer–compatible sample tube. 25 µL of each pre-conjugated antibody was added to the whole blood and incubated for 20 minutes at room temperature in the dark. 900 µL of distilled water was added to the blood for a few seconds (until full lysis was achieved). 100 µL of 10× Hanks balanced salt solution (HBSS) was added to bring the solution back to isotonic. autoMACS Running Buffer was added to bring the total volume to 1.2 mL, thus giving a 1:120 dilution of the original sample.

Porcine T cells were stained using CD3-Alexa Fluor 405 (1H2), B cells using CD21-Alexa Fluor 488 (JKA2), monocytes using CD14-PE (CCG33), and granulocytes/macrophages/neutrophils using CD172a/SIRPα–APC (74-22-15). All antibodies were produced by the Institute for Animal Health unless otherwise stated. CD172a mAb 74.22.15 (SWC3) was a kind gift from J.K. Lunney[1]. Antibodies were labeled using Zenon® Labeling Kits from Invitrogen.

Flow cytometry

Samples were acquired using a MACSQuant Analyzer with a low flow rate (50 µL for bovine samples and 300 µL for porcine samples). In order to visualize all cells, SSC was set on hyperlog and FSC on log2.

For analysis, a region was placed around the leukocytes to exclude any red blood cell ghosts. Total cell numbers/mL in the original sample were calculated by multiplying the number of cells per mL in the region by the dilution factor of 20 for bovine blood and 120 for porcine blood.

Results

Understanding how pathogens interact with immune cells is vital in the development of effective vaccines and prophylactics. In our work with animal pathogens we needed to develop an analysis technique with minimal wash steps, which would allow us to accurately track any changes in cell numbers during the course of disease, treatment, or vaccination. Ideally, it should also allow for neutralization of cell-associated pathogens to allow analysis outside of containment.

The combination of a 'no-wash' lysed-whole-blood method with use of the MACSQuant Analyzer was found to give accurate figures of white blood cell populations in cattle and pigs (fig. 1). Bovine blood or porcine blood was stained for specific leukocyte populations, lysed in ammonium chloride lysis buffer or water respectively, and analyzed on a MACSQuant Analyzer. A region was applied to the leukocyte population to exclude any red blood cell 'ghosts'. Gates were applied to positive cell populations. These positive cells were displayed in a density plot to obtain both the numbers of positive cells but also their positions within the leukocyte population. Total cell numbers/mL in the
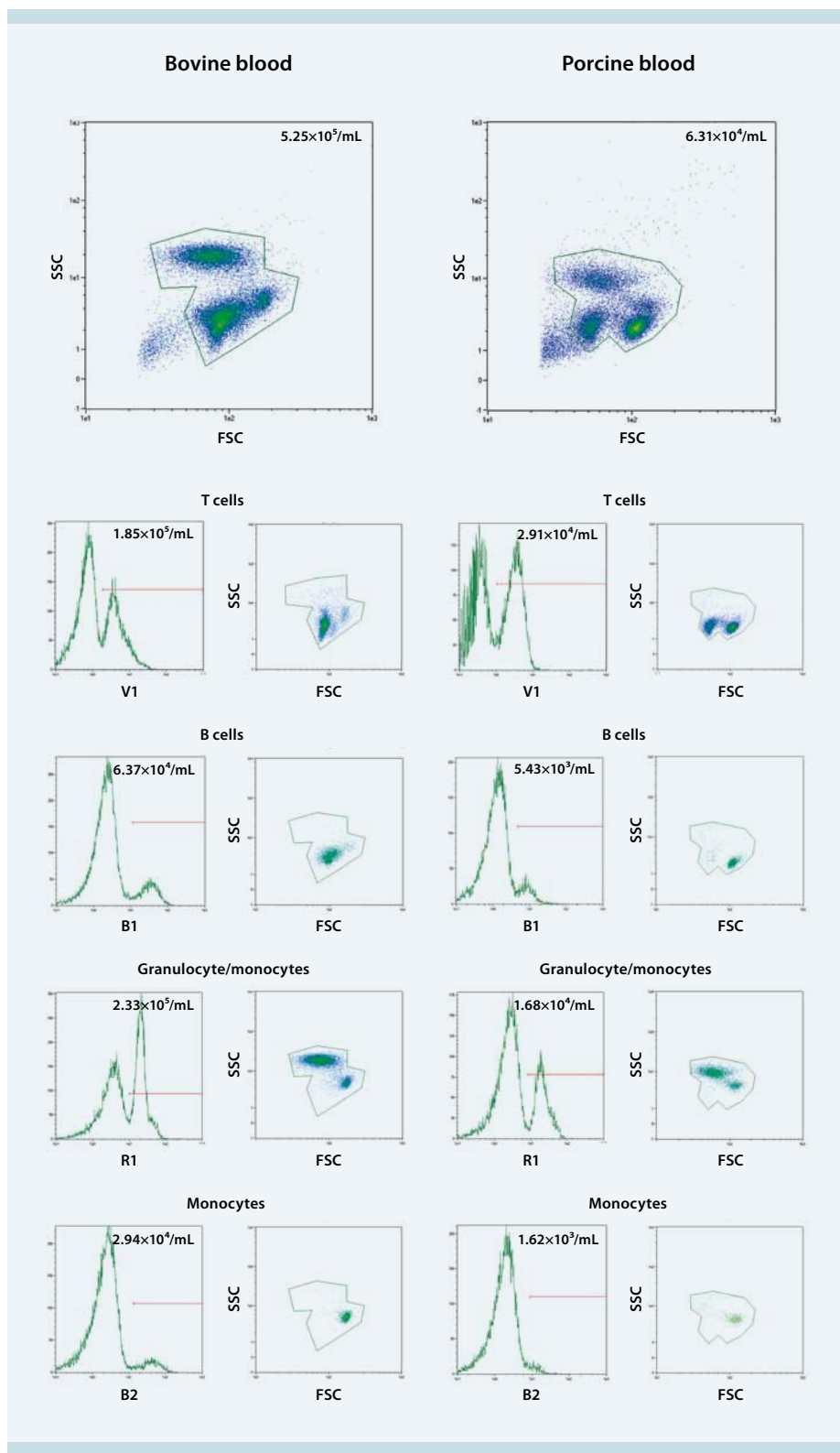

Figure 1 The combination of a 'no-wash' lysed-whole-blood method with use of the MACSQuant Analyzer can give accurate figures of leukocyte populations in cattle and pigs. 60 µL of bovine blood or 10 µL of porcine blood was stained for specific leukocyte populations, lysed in ammonium chloride lysis buffer or water respectively, and analyzed on a MACSQuant Analyzer.
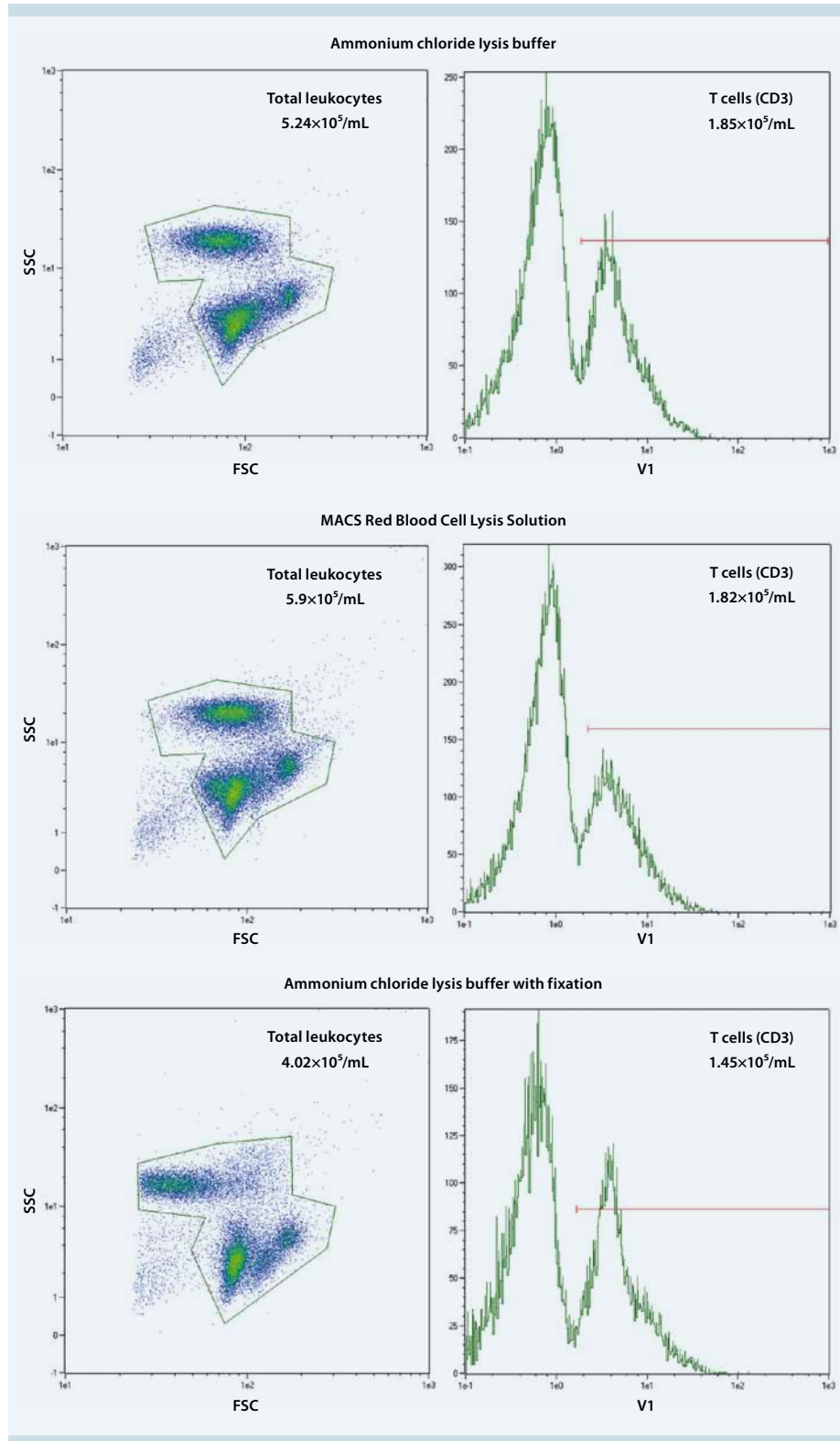
Figure 2 Comparison of lysis buffers on whole bovine blood with and without fixation. Bovine blood was stained with monoclonal CD3 antibodies to identify T cells, then lysed using either ammonium chloride lysis buffer or MACS Red Blood Cell Lysis Solution. A sample lysed with ammonium chloride was centrifuged, then fixed in a small volume of paraformaldehyde solution, and analyzed on a MACSQuant Analyzer.

Cell counts in original bovine blood sample	
Total leukocytes	1×10^7/mL
CD3$^+$	3.7×10^6/mL
CD21$^+$	1.27×10^6/mL
CD172a$^+$	4.66×10^6/mL
CD14$^+$	5.88×10^5/mL
CD4$^+$	1.62×10^6/mL
CD8$^+$	6.8×10^5/mL
WC1$^+$	9.0×10^5/mL

Table 1 Cell counts of specific leukocyte populations in bovine blood. 60 µL of bovine blood was stained for specific leukocyte populations, lysed in ammonium chloride lysis buffer, and analyzed on a MACSQuant Analyzer. Cell counts/mL were converted to cell counts in the original sample by multiplying by the dilution factor.

original sample were calculated as described above. The bovine blood leukocyte counts are in keeping with our analyses by a Sysmex® cell counter[2]. Examples of numbers for bovine blood leukocyte subpopulations are shown in table 1.

Analysis by this method can highlight marked individual differences in specific leukocyte populations. For example, the data displayed on the right hand side of figure 1 were generated using blood from a young pig (approximately 11 kg), which has large numbers of T cells (CD3), many of which are unusually small, and few granulocytes (CD172a). We have also found that young pigs have very few monocytes, which express CD14 only weakly.

We also compared lysis techniques and used ammonium chloride lysis buffer, MACS Red Blood Cell Lysis Solution, or water lysis followed by immediate addition of 10× HBSS. Water lysis is commonly used with porcine red blood cells as they are notoriously difficult to lyse using traditional buffers and require a large amount of dilution. We found that leaving porcine blood to lyse in either ammonium chloride or MACS Red Blood Cell Lysis Solution required very long lysis times (up to 1 hour) that may have an adverse effect on the whole experiment. Water lysis on the other hand, requires perfect timing for the addition of the 10× HBSS: If the buffer is added too soon, the sample will be contaminated with red blood cells; if it is

added too late, the leukocytes may suffer. MACS Red Blood Cell Lysis Solution was used successfully with bovine blood and an analysis of the two techniques is shown in figure 2. There was no significant difference in either the distribution of cells or the number of CD3-positive cells.

Many laboratories, such as our own, handle pathogens that must be analyzed inside a safety cabinet or fixed before analysis. Therefore, we also developed a technique that requires only one centrifugation step and dilutes the fixative to levels acceptable by the MACSQuant Analyzer. Bovine blood was stained, lysed with ammonium chloride, diluted with autoMACS Running Buffer, and gently centrifuged. The pellet was resuspended in a small volume of fixative and the fixative diluted back 1:24 for analysis. As can be seen in figure 2 (bottom panels), the fixation did alter the distribution of the cells (requiring a new region to be drawn) and slightly lowered the number of CD3-positive cells, which could be due to the unavoidable centrifugation step. However, if all samples are analyzed in the same manner during the course of the experiment, this should not compromise comparability of data.

Conclusion

The no-wash method in combination with the use of the MACSQuant Analyzer will allow us to more accurately analyze cell populations during the course of economically important infectious diseases of farm animals, such as foot-and-mouth disease, bluetongue disease, and African swine fever. Understanding how these viral diseases interact with immune cells is vital in the development of effective vaccines.

References

1. Alvarez, B. *et al.* (2000). Tissue Antigens 55: 342–351.
2. Windsor, M. *et al.* (2011) Vet. Res. 42: 108.

MACS Product	Order no.
MACSQuant Analyzer	130-092-197
Red Blood Cell Lysis Solution (10×)	130-094-183
autoMACS Running Buffer – MACS Separation Buffer	130-091-221