

## Title: Lysing Methods and Reagents for Flow Cytometry Immunophenotyping

Sponsored and reviewed by ICCS Quality and Standards Committee

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### OUTLINE

Lysing erythrocytes is commonly performed as part of the processing of bone marrow, spleen and peripheral blood specimens for flow cytometry immunophenotyping. Reducing or eliminating the red blood cells (RBCs) makes it easier to isolate the white blood cells for evaluation. It also makes it easier on the cytometer by eliminating the need to acquire all of the RBC events only to have them gated out. While the threshold can be set to ignore most of the RBCs, it is important not to set the threshold too high and consequently lose lymphocytes. Collecting RBC events also increases the size of the data files significantly.

RBC lysing has numerous consequences on immunophenotyping, including possible cell loss, shifts in side scatter (SSC) and forward scatter (FSC), and changes to antibody staining for cytometric analysis. It is critical to select a method and reagent that is best for your assay and your laboratory and to validate that protocol in house.

Our goal is to compare and contrast different lysing methods and reagents, discuss the factors impacting the results, pros and cons of these methods, and provide detailed standardized protocols as a starting point for choosing and validating the optimal method for your specific application.

### INTRODUCTION

There are two basic protocols for lysing RBCs.

- 1) **Bulk lysing or Lyse / Stain / Wash (LSW):** This method involves lysing the entire sample to be used, resuspending the remaining nucleated cells in a buffer or nutrient media that keeps the cells alive, evaluating and adjusting cell concentration and staining with fluorescently conjugated antibodies.
- 2) **Tube lysing or Stain / Lyse / Wash (SLW):** This method involves staining the cells first (typically after washing) and then lysing after the staining process. Typically, the stained cells are washed again before being acquired.

There is no consensus in the flow cytometry industry on which method of lysing erythrocytes is optimal. Different protocols might be more appropriate in different situations. The Validation of cell-based fluorescence assays: Practice guidelines from the ICSH and ICCS offers this explanation; "...methodological variants of surface staining and red cell lysis are currently in use. Stain-lyse-wash methods give the best signal discrimination but should be avoided when cell-loss due to washing is an issue...Lyse-stain-wash methods are used when cell concentration has to be adjusted before staining or red cells need to be removed." [7]. Each laboratory must choose their own method, protocol and reagents based on a number of factors. Table 1 lists some goals to consider when assessing the lysing options: [1,2]

1. Low CVs on FSC and SSC
2. Large differences in mean channel values for FSC and SSC between major leukocyte populations
3. Minimal cell loss
4. Preservation of fluorochrome brightness
5. Impact on the stability of tandem fluorochromes
6. Low background staining
7. Minimal inter-instrument or inter-laboratory variation
8. Easy and fast performance
9. Microscopic assessment of effect on cells

Table 1: Goals to consider when assessing lyse protocols and reagents.

Bulk (LSW)		Tube (SLW)	
PROS	CONS	PROS	CONS
More consistency in lysing and standardization.	Some claim that it activates monocytes.	A lysing reagent with fixative can be used.	Inconsistent amount of debris and WBC from tube to tube, potentially affecting %'s reported
Ease of use to lyse one tube.	pH changes can impact performance as can exposure to O <sub>2</sub> (B. Greig).	Best signal discrimination; Better separation of populations on SSC / CD45 plot for WBC differential (Cytometry B)	Increased debris.
Left over cells suspension for add ons faster.	Some have concerns that it alters the %'s of lymphocyte subsets		Increased cell loss due to washing.
Appropriate for rare event studies (MRD, Stem Cell Enumeration), when high cell counts needed	Uses more reagent, more costly		

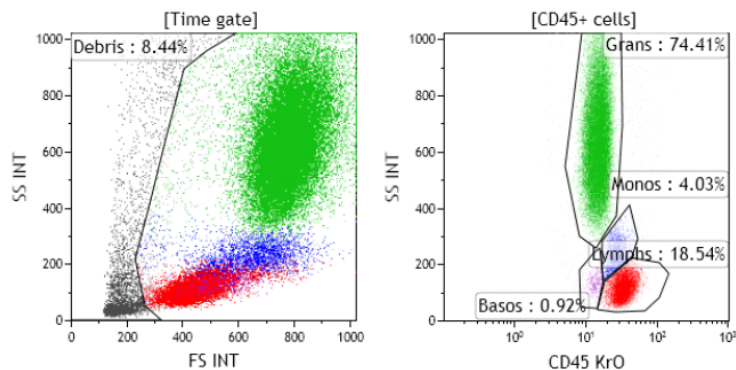
Table 2: Observed pros and cons of both Bulk (LSW) and Tube (SLW) Lyse methods.

Table 2 lists some pros and cons that have been observed and reported in an informal survey of flow cytometry experts. Each laboratory goes through a process of assessing the factors in the table above, determining the priorities of said factors, testing, selecting the right option and optimizing the protocol. Depending on the assays being performed, a laboratory might utilize multiple lyse reagents and methods to achieve optimal results for each assay.

Figure 1 (below) illustrates a comparison between the bulk lysis and tube lysis of peripheral blood (EDTA) with in-house ammonium chloride solution, demonstrating no significant difference based on the CD45 vs SS dot plot.

### Bulk lyse (LSW)

Effect of Various Lysing Methods and Reagents on Flow Cytometry Immunophenotyping



### Tube lyse (SLW)

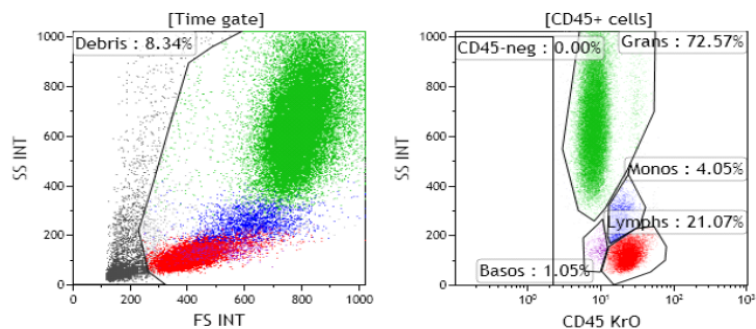
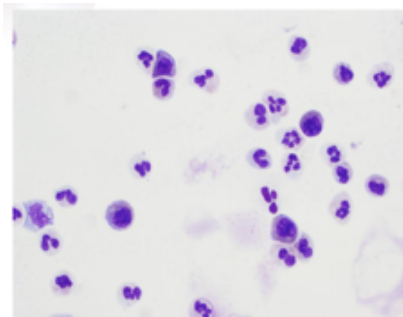


Figure 1: Comparison of the FSC vs. SSC and CD45 vs. SSC of Bulk lyse (LSW) and Tube Lyse (SLW). Provided by Andrea Illingworth, Dahl Chase Diagnostic Laboratory

Some reagents are more damaging to cells and this damage can be gauged through flow cytometric and microscopic observation. Figure 2 displays findings from the TexFlo Update for FloTex 2013; Standard Operating Procedures when comparing two commercial lyse reagents. The FACS Lyse caused significantly more cellular damage when observed microscopically as seen in Figure 2. [1]

### Pharm Lyse



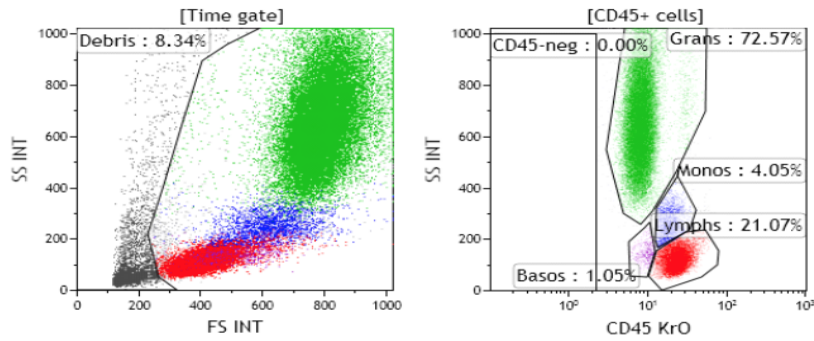
### FACS Lyse



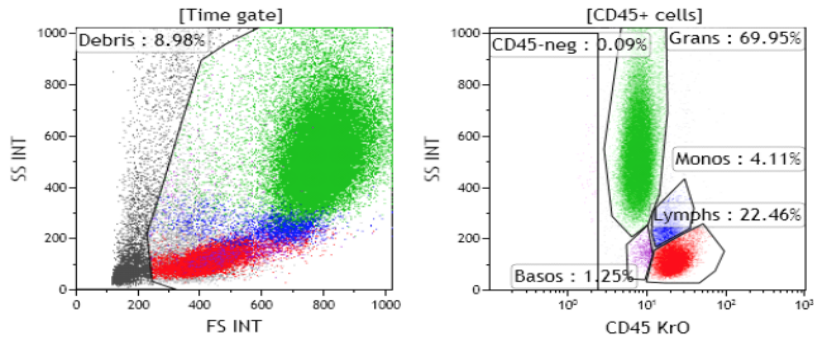
Figure 2: The FACS Lyse also exhibited increased cellular damage. [1]

The lyse reagent itself can also change the scatter and staining properties of the cells (see Figure 3).

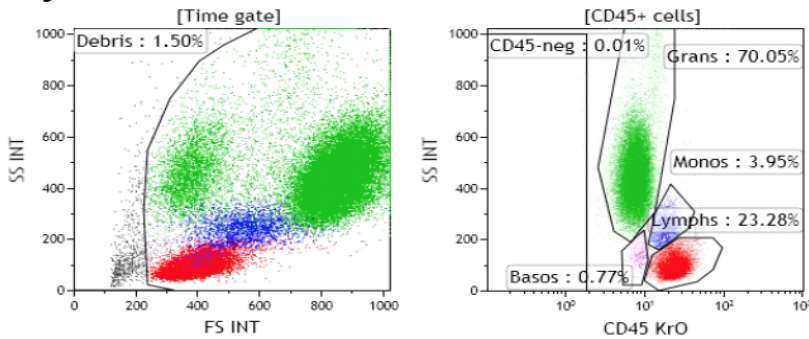
## Ammonium Chloride Lyse - Homemade



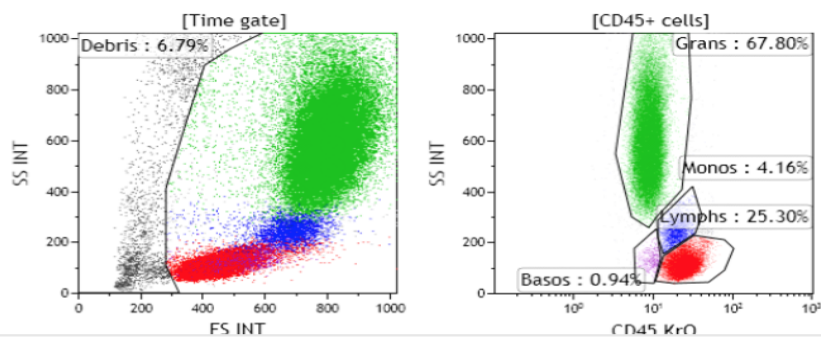
## VersaLyse



## OptiLyse

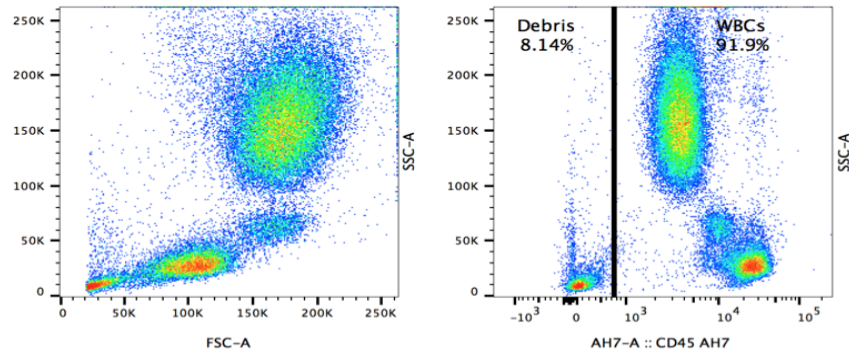


## Immunoprep Lyse



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### PharmLyse



### FacLyse

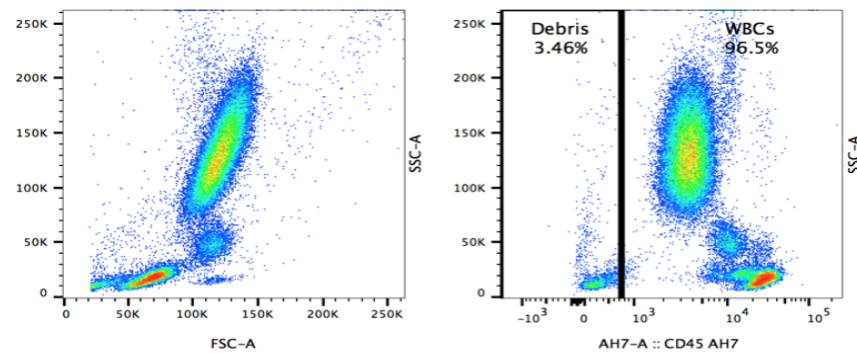


Figure 3: Comparison of homemade Ammonium Chloride lyse and OptiLyse (Beckman Coulter™), VersaLyse (Beckman Coulter™), Immunoprep Lyse (Beckman Coulter™), PharmLyse (BD Biosciences™) and FacLyse (BD Biosciences™). Provided by Andrea Illingworth, Dahl Chase Diagnostic Laboratories and Dr Paul Wallace, Roswell Park Cancer Institute).

Some laboratories have reported differences in the distribution of T and B lymphocyte subsets when using different methods and different lysing reagents, leading to the suspicion that some combinations of lyse method and reagent may selectively destroy certain subpopulations of white blood cells (see Figure 4). This emphasizes the need to validate each assay with the appropriate lysing reagent.

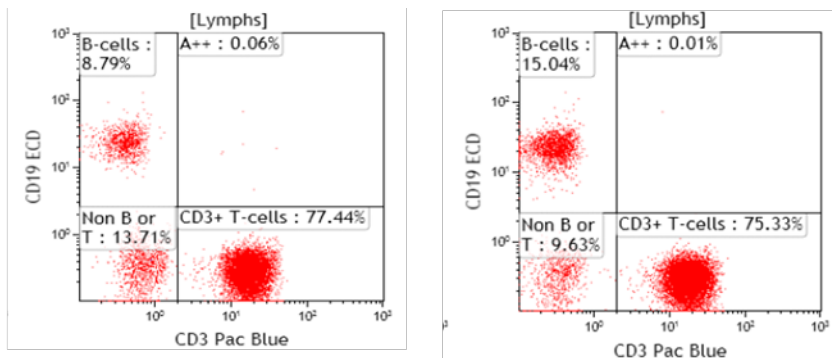


Figure 4: Comparison of the CD3 vs CD19 frequencies using Homemade Ammonium Chloride lyse and OptiLyse. In this example, the CD19 is significantly reduced with the Homemade Ammonium Chloride lyse. Provided by Andrea Illingworth, Dahl Chase Diagnostic Laboratory

## SUMMARY

It should be noted that all results of this module are based on the experiences of four different laboratories with different platforms and various antibodies from different vendors. The goal of this study was not to present one optimal lysis approach but rather raise the awareness that differences in specimen preparation present a potentially significant source of variability regarding the final results. Based on a limited number of samples our findings also suggest that bulk lysis may show a decreased proportion of CD19+ B-cells/CD3+ T-cells (see Figure 4) when compared to certain tube methods. The method used for red cell lysis can have definite impact on immunophenotyping and assays need to be validated with the assay-specific goals in mind.

## LYSE RECIPES

There are large variations throughout individual laboratories in the specific reagents used and other details in the protocol. In the sections below, we will summarize the widely accepted protocols and reagent recommendations from Current Protocols in Cytometry.

- Whether using Bulk lyse (LSW) or Tube lyse (SLW), the volume or absolute number of cells of each sample must be adjusted based on the nucleated cell count to achieve the optimal cells to antibody ratio (determined through antibody titration) before staining.
- Flow cytometry clinical tests (aside from those performed with FDA approved kits) are considered Laboratory Developed Assays (LDA's). While it is acceptable to start with recommend protocols and reagents, **it is imperative that every laboratory validate each protocol for their own site. [7]**

Ammonium Chloride lyse reagent can be prepared in the laboratory, or commercial reagents such as OptiLyse (Beckman Coulter™), VersaLyse (Beckman Coulter™), Immunoprep Lyse (Beckman Coulter™), PharmLyse (BD Biosciences™) and FacsLyse (BD Biosciences™) can be used to lyse RBCs. However, FacsLyse and some other commercial reagents contain a fixative, so staining for cell surface markers on leukocytes should be performed prior to lysis of the RBCs with these reagents. [3]

### ***Ammonium Chloride Lyse Recipe: [6]***

- **10x Solution** (Solution intended to be diluted by a factor of 10 before being used)
  - 80.2 g NH<sub>4</sub>Cl (1.5 M)
  - 8.4 g NaHCO<sub>3</sub> (100 mM)
  - 3.7 g disodium EDTA (10 mM)
  - Distilled H<sub>2</sub>O to 900 ml
  - Adjust pH to 7.4 with 1 N HCl or 1 N NaOH
  - Add distilled water to up to 1 liter total
  - Store 6 months at 4°C
- **1x Working solution**
  - Dilute 1:10 with distilled water (1 part 10x lyse, 9 parts water)
  - Make working lysing solution fresh before use and discard any unused portion.



## LYSING PROCEDURES

### **A - Tube Lyse (SLW) Protocol [3]:**

1. Wash whole specimen 3x with PBS (1 wash = add ~4mL PBS, resuspend, spin 3min at 350g, discard supernatant).
2. Add antibodies to the individual panel tubes.
3. Add specimen to the tubes with antibodies. Volume of specimen is based on cell concentration. Typically, it is considered optimal to add  $10^6$  cells to each tube for staining.
4. Incubate for 30 min at room temperature protected from light.
5. Add 2 ml Ammonium Chloride Lyse, or the correct ratio of commercial lyse as indicated on the package insert.
6. Incubate for 10 min protected from light.
7. Mix well and check that the specimens are fully lysed, see Figures 5 and 6 below).
8. Centrifuge for 3 min at 350g.
9. Discard supernatant.
10. Mix well and add 2 mL of PBS washing buffer.
11. Centrifuge for 3 min at 350g.
12. Discard supernatant.
13. Mix well and add 2 mL of PBS washing buffer.
14. Discard supernatant and resuspend cell pellet in 200uL of PBS and mix well.
15. Tubes are ready for acquisition.

Figures 5 and 6 below demonstrate partial and complete RBC lysis. The solution is opaque when incompletely lysed and becomes transparent when fully lysed. The FSC vs. SSC plots show the unlysed RBCs in the lower left corner along with debris. The partially lysed specimen shows a much larger population in this area.

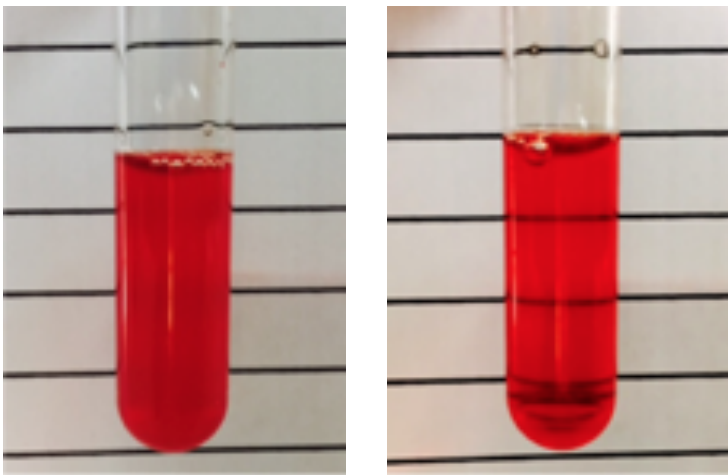


Figure 6: Partially lysed specimen on the left and fully lysed specimen on the right. Provided by Laura Johnson, Mayo Clinic Phoenix.

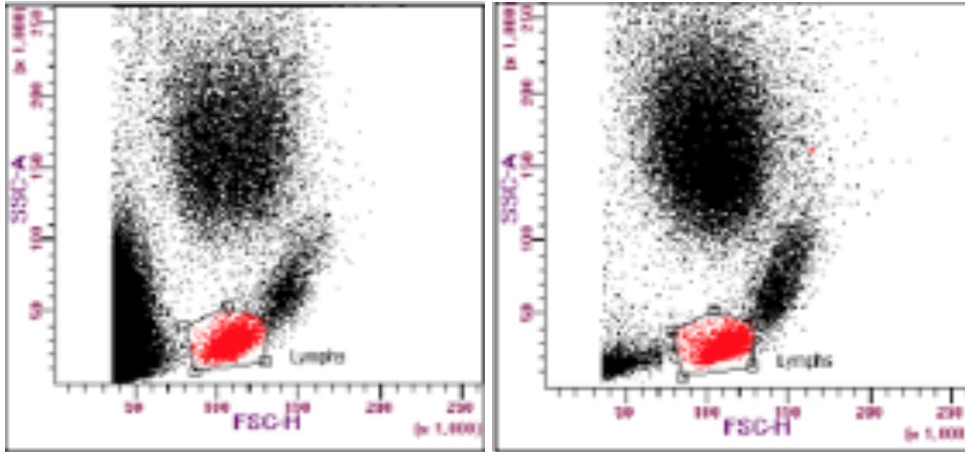


Figure 7: Partially lysed specimen on the left and fully lysed specimen on the right. Provided by Laura Johnson, Mayo Clinic Phoenix.

### **B - Bulk Lyse (LSW) Protocol [5]:**

1. Add specimen to a 15mL or 50mL conical tube.
2. Add the appropriate amount of lyse
  - a. When using ammonium chloride lyse prepared in the laboratory (see the recipe above), use a 1:5 ratio (i.e. 2mL sample, 8mL lyse) [6,7].
  - b. If you are using a commercial lyse reagent use the manufacturer's specified ratio.
3. Place conical tube on rocker for approximately 10 minutes. If a rocker is not available, invert the tube periodically during the incubation.
4. Observe for lysis (see the example above of complete lysis).
5. If the lysing appears incomplete, incubate for an additional 5 minutes.
6. Centrifuge for 5 minutes at 550g.
7. Discard the supernatant.
  - a. If a large amount of red cells are observed.
    - i. Add half as much lyse as used in step 2.
    - ii. Mix well.
    - iii. Incubate for 5 minutes.
    - iv. Repeat centrifugation and discard supernatant.
8. Resuspend pellet with PBS (Phosphate Buffered Saline), using the same volume as lyse used in step 2.
9. Mix well and centrifuge for 5 minutes at 550g.
10. Discard the supernatant.
11. Add enough Complete RPMI [6] to achieve the final cell concentration of  $10^7$  cells/mL (or  $1 \times 10^4$  cells/ $\mu$ L).
12. Pipet 100 $\mu$ L of this suspension ( $1 \times 10^6$  cells) into labeled tubes with the desired antibodies.
13. Mix well and incubate protected from light for 15 minutes.
14. Add 2mL of PBS and vortex.



15. Centrifuge for 3 minutes at 350g.
16. Discard supernatant.
17. Resuspend cell pellet in 200 $\mu$ L of PBS and mix well.
18. Tubes are ready for acquisition.

**References:**

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7. Tanqri S, et al. Validation of cell-based fluorescence assays: Practice guidelines from the ICSH and ICCS – part III – analytical issues; on behalf of ICSH/ICCS Working Group First published: 10 September 2013  
[Full publication history](#) DOI: 10.1002/cyto.b.21106

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