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Title: Lysing Methods and Reagents for Flow Cytometry Immunophenotyping - Revised version

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INTRODUCTION

Erythrocyte lysis is commonly performed as part of the processing of bone marrows, biopsies, fluids and peripheral blood specimens for flow cytometric immunophenotyping. Eliminating the red blood cells (RBCs) allows us to primarily focus on the WBCs and not to acquire events which are not needed for analysis. If an acquisition threshold is set to exclude most of the RBCs, it should not be set too high to prevent the loss of lymphocytes.

While there are other methods of removing RBCs, such as density gradient centrifugation, they introduce problems that make them sub-optimal for most flow cytometry assays. For instance, density gradient isolation causes selective loss of different leukocyte populations and lower counts of lymphocyte subsets. (1)

While largely considered the most optimal method of specimen preparation for flow cytometric analysis, RBC lysing does have numerous consequences on immunophenotyping; including possible selective white blood cell loss, shifts in side scatter (SSC) and forward scatter (FSC), and changes to antibody staining for cytometric analysis. For laboratory developed tests (LDT) 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. IVD tests must be evaluated and performed exactly as the manufacturer has specified.

Our goal is to give you the tools to evaluate and optimize your lysing protocols, compare and contrast different lysing methods and reagents, discuss the factors impacting the results, review 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.

METHOD EVALUATION

Unfortunately, there is no consensus in the flow cytometry industry on which method of lysing erythrocytes is optimal and which reagents are best. Different protocols might be more appropriate in different situations. RBC lysis is one factor in flow cytometry testing that must be assessed in conjunction with many other considerations. Sample handling, instrument

type and setup and analysis strategies can all vary. Unfortunately, these differences can all affect the results and how they are interpreted. (2)

- We recommend beginning with a literature search. Why start from scratch when there is so much information available? Countless studies and protocols exist. It is important to understand that there are conflicting studies published. This emphasizes the fact that while it is always beneficial to conduct a literature search when beginning the process of assay design, it is critical for each laboratory to perform its own comparisons during the optimization process.
- There are abundant publications and numerous standardization resources available for guidance on the evaluation, optimization and validation of flow cytometry assays. Most recently, a comprehensive CLSI document H62 (15) has been developed, which provides an excellent resource on proper standardization and validation of flow cytometric assays.
- Additional standardization efforts such as the Euroflow Consortium (3), The Human Immune Phenotyping Consortium (HIPC) (2) and the ONE study (4) have been developed and published to promote standardization of flow cytometry immunophenotyping in clinical studies and diagnostic assays, so that data could be compared across sites and studies. Other efforts have been made to standardize specific assays such as PNH, ALL MRD (COG), Multiple Myeloma MRD, AML MRD, Sezary Syndrome, MDS and more. (5,6,7).
- The Validation of cell-based fluorescence assays: Practice guidelines from the ICSH and ICCS offers this wisdom on lysing; "...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." (1) Each laboratory must choose their own method, protocol and reagents based on a number of factors. Table 1 lists some criteria to consider when assessing the lysing options: (1,2)

1. Good clustering and separation of all WBC populations (check mean channel values)
2. Minimal cell loss and cell damage, no selective loss of WBC populations
3. Preservation of fluorochrome brightness (MFI) and low background staining resulting in acceptable Signal/noise ratio
4. Does not affect the stability of tandem fluorochromes
5. Lysis should not interfere with monoclonal antibody binding
6. Does not negatively impact standardization, reproducibility and accuracy, minimal inter-instrument or inter-laboratory variation
7. Easy, fast and cost effective
8. Biosafety (fixation or unfixed)

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

- Once a thorough literature research has been performed, it is time to move on to evaluation. Evaluation should include operational factors, assay specific parameters

and quality of results. The evaluation of lyse reagents and methods should start with a look at the needs for each specific assay and operational parameters for the laboratory. Is the assay IVD? If so, you must use the reagents included in the IVD method and follow the specific protocol provided by the vendor. The most common IVD assays include TBNK and Stem Cell enumeration. There is now an IVD assay for leukemia and lymphoma immunophenotyping as well. The IVD ClearLLab 10C System from Beckman Coulter is the only FDA cleared and CE marked integrated L&L immunophenotyping solution.

How does your laboratory prioritize operational factors such as cost, complexity, overall time and hands on time? How much specimen is usually submitted for analysis? Is there a need to use fixative for your assay? Will intracellular staining be performed? Is automation available and within the capital budget?

Ultimately, quality should be the most critical factor evaluated. For this, look at the amount of debris using SS vs FS and CD45 vs SS. Evaluate the consistency of population percentages against other reagents and/or methods and between tubes. Some reagents and methods can cause loss of specific cell types, reducing the accuracy and reproducibility of the qualitative as well as quantitative results. Carefully assess the effect on the separation of populations, MFI and tandem dye stability. Keep different specimen types in mind as well. Peripheral blood, bone marrow aspirate, body fluids and various tissue samples will react differently to each combination of lyse method and reagents.

LYSE METHODS

There are three main protocols for RBC lysis:

1) **Lyse - no wash (LNW) -** This method involves staining the cells first and then lysing the samples with no washing steps. The lysing reagent remains in the tube or well with the sample during acquisition. This method is normally used for absolute cell counting, such as CD4/CD8 enumeration or hematopoietic stem cell enumeration and is easiest to automate.
2) **Bulk lysing or Lyse / Stain / Wash (LSW):** This method involves lysing the entire sample to be processed, 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.
3) **Tube lysing or Stain / Lyse / Wash (SLW):** This method involves staining the cells first (may wash prior to staining) and then lysing after the staining process. Typically, the stained cells are washed again before being acquired in order to improve the signal/noise ratio.

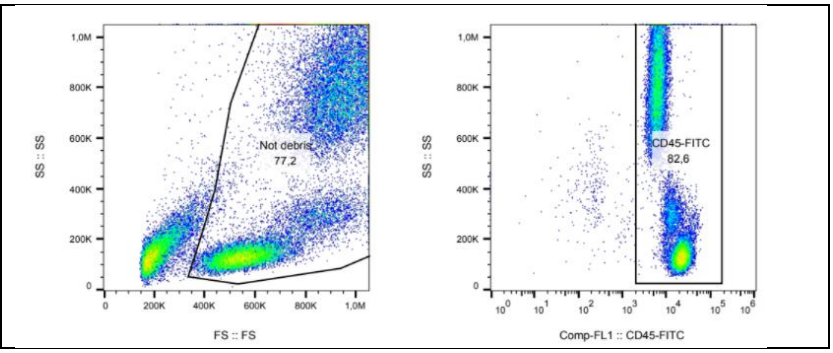
Lyse - No Wash (LNW)	
PROS	CONS
Faster - no washes / centrifugation	Increased background noise and debris
No loss of cells, ideal for absolute counts, required for single platform cell counts	Sample degradation over time, lysing agent remains in tube
Less steps, easier to standardize	Potential for decreased resolution of dim antigens and rare populations

Bulk Lyse (LSW)	
PROS	CONS
Consistent lysing, no tube to tube variation, easier to standardize	Uses more reagent, more costly
Ability to concentrate specimen useful for paucicellar specimens and rare cell assays	pH changes and exposure to oxygen can affect performance
Leftover cell suspension available for additional testing	WBC clumping; causing clogging, cell loss and WBC % changes
Cell concentration adjustable before staining	Can cause lymphocyte percent discrepancies
	Might cause monocytes activation

Tube Lyse (SLW)	
PROS	CONS
Lyse reagent with fixative can be used	Inconsistent performance, tube to tube, could affect %s reported
Good signal discrimination	Increased cell loss due to washing steps
Good separation of populations on SSC/CD45 for WBC differential	Potential for increased debris, depending on lysing reagent

Table 2: Pros and cons of each lysing method as described in literature as well as some 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 different lyse reagents and methods to achieve optimal results for each assay performed.

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.

Lyse no wash (LNW): Note the increase in FS of all populations including the debris, compared to the LSW and SLW plots run at the same voltages for FS and SS)	

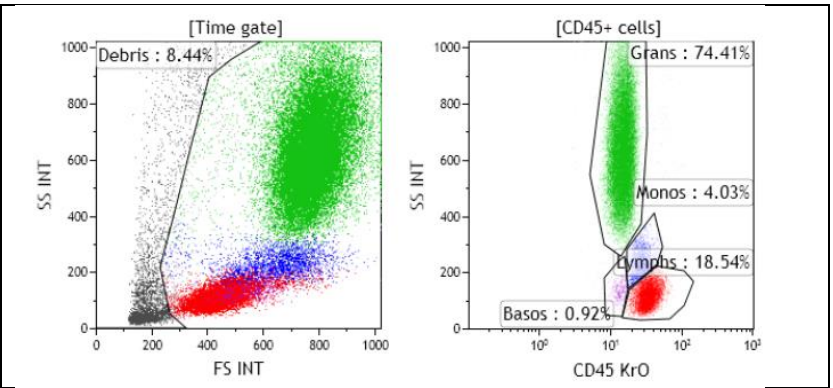
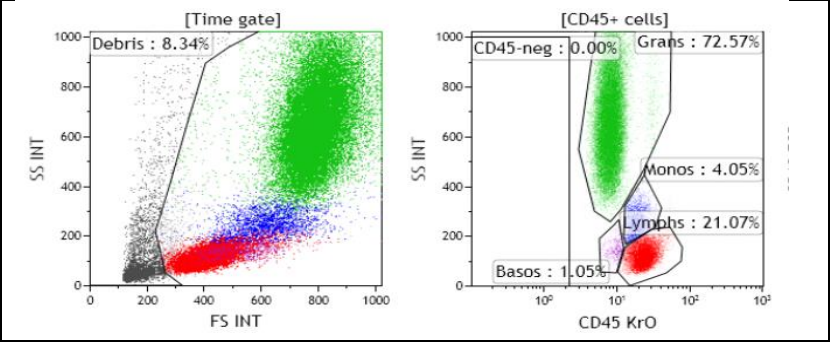
Bulk lyse (LSW)	
Tube lyse (SLW)	

Figure 1: Comparison of the FSC vs. SSC and CD45 vs. SSC plots of Lyse No Wash (LNW), Bulk lyse (LSW) and Tube Lyse (SLW) at the same voltage settings.

Additional method factors to consider:

1. A fixation step can be added to any of these methods. There are various reasons to fix specimens, which will not be covered in this module. Fixative can be added separately, but there are commercially available lyse buffers that contain fixative, allowing lysing and fixation to be done at the same time. However, outside of intracellular staining, fixative must be added after the staining occurs because the fixative can disrupt the staining process. This means that when bulk lysing (LSW), the lyse reagent used must not contain fixative because the lysing step occurs before the staining.
2. Intracellular staining, both cytoplasmic and intranuclear, affects and interacts with cell lysis in different ways depending on the combinations of reagents and particular protocols. Some laboratories perform a separate lysing step while others rely on the permeabilization step to lyse the RBCs. We won't go into this any further in this module as it is covered in depth in other modules and literature.

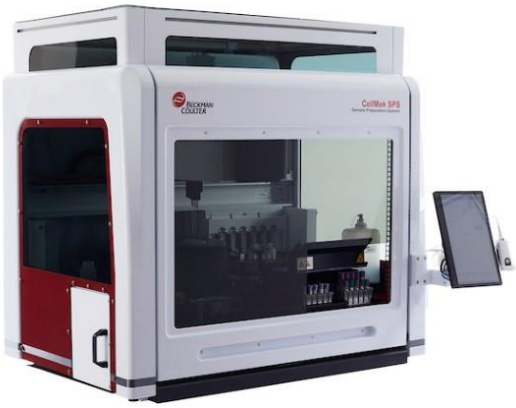
AUTOMATION

Automation is the newest significant improvement in specimen processing for flow cytometry. Automation eliminates the variability and human error that are intrinsic to manual processing. This is especially true for lysing, which contains numerous technique dependent factors. Automation also significantly reduces the risk of technologists' exposure to potentially hazardous biological materials and stress levels related to the potentially serious consequences of making errors. In an era of rapidly evolving technology, manual methods

that are risky, variable and error prone and should be phased out as much as possible in favor of automation. There are modern sample prep solutions available that can perform various workflows for a wide variety of applications with little user intervention from start to finish.

There are several major vendors with instruments that automate the lysing process:

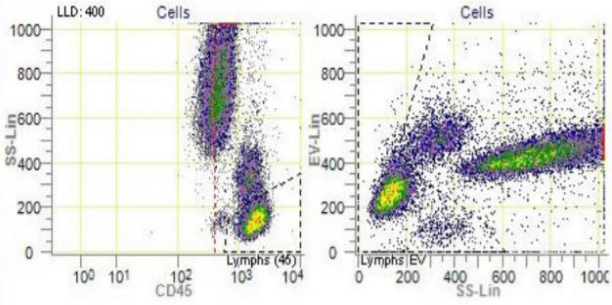
Beckman Coulter Life Sciences



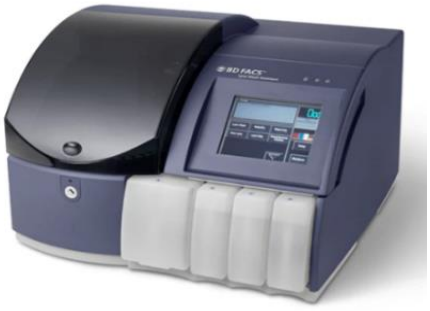
- The CellMek SPS is a sample prep system that goes from automated sample loading to fully prepared, ready to analyze samples (including on board cell washing) without user intervention. The combination of CellMek's smart processing software and dedicated Reaction Chamber Module optimizes sample preparation processes, providing efficiency through automation. This instrument allows real time access to completed samples as well. Barcoded inventory and sample management provide end-to-end traceability. On-board refrigeration and cap piercing technology reduce the daily manual handling of antibodies.

- The TQ-Prep Workstation with ImmunoPrep Reagent System provides rapid lyse-no-wash (LNW) whole-blood sample preparation. A standard 32-tube carousel allows walk-away processing in an enclosed setting. The TQ-Prep does not pipette samples, however, they can be purchased with a PrepPlus instrument that pipettes samples and antibodies. The COULTER TQ-Prep workstation is intended to prepare leukocytes from whole blood for In Vitro Diagnostic (IVD) use when paired with the COULTER ImmunoPrep Reagent System and cleared Beckman Coulter IVD applications (absolute lymphocyte subsets) and cleared Beckman Coulter flow cytometers.

- The AQUIOS CL is a flow cytometry system that combines sample preparation and analysis in one platform. Once specimens are loaded into cassettes in their original tubes, the AQUIOS uses 96 well microplates to perform every step of the specimen processing, staining and acquisition. The AQUIOS is intended for IVD use with the Beckman Coulter AQUIOS Tetra System.

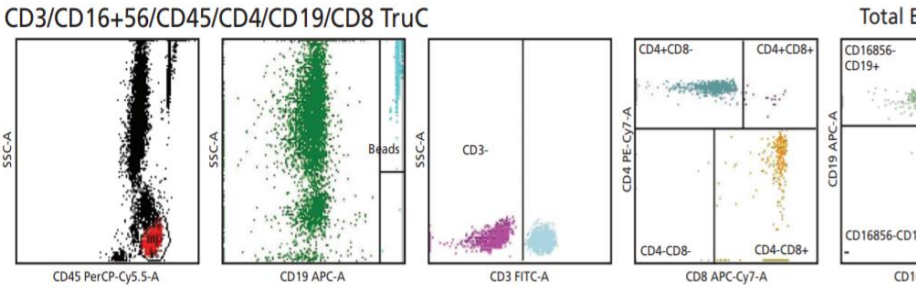


Becton Dickinson



- The BD FACS™ Lyse Wash Assistant (LWA) automates sample preparation for flow cytometry applications. It lyses, mixes, washes, and fixes samples with minimal labor. Automating incubation and washing, the LWA batch processes up to 40 patient samples per run to reduce hands-on time and operator induced variability in laboratory data. Samples can be transferred to a BD FACSCanto or BD FACSCalibur flow cytometer for analysis using the LWA carousel.

- The BD FACS™ Sample Prep Assistant III (SPA III) enables walkaway sample preparation for clinical labs. The SPA III aliquots blood and reagents into daughter tubes, adds lysing solution, and mixes the sample according to the preprogrammed or custom protocols to automate workflow and increase efficiency. This instrument is most often used with BD's IVD TBNK assays, BD Multitest™ 6-color and 4-color panels.

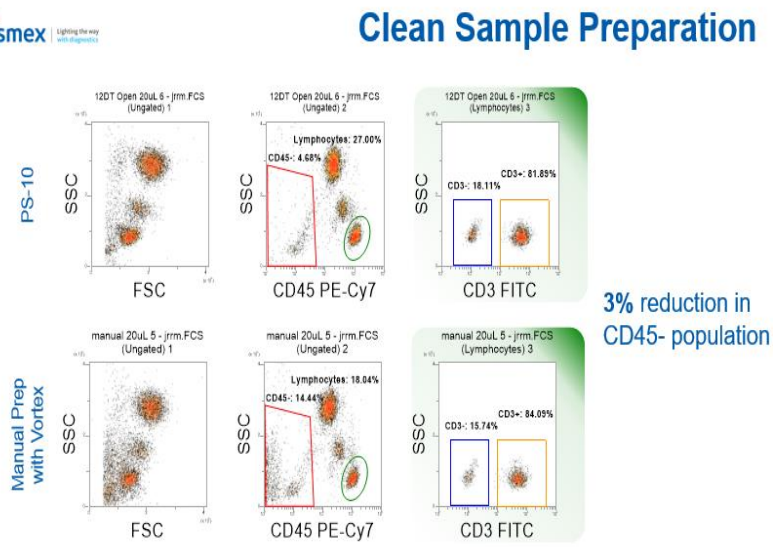


- The BD FACSDuet™ Sample Preparation System enables higher lab productivity and provides higher accuracy in results by minimizing manual intervention through its automation protocol. Physical integration between the BD FACSDuet™ Sample Preparation System and the BD FACSLyric™ Flow Cytometer allows technicians to load samples and reagents onto the BD FACSDuet™ Sample Preparation System

and obtain results once the samples are acquired and analyzed on the BD FACSLyric™ Flow Cytometer. The BD FACSLyric™ Flow Cytometer with the integrated BD FACSDuet™ Sample Preparation System is now available as an in vitro diagnostic (IVD) system in the United States as well as countries recognizing the CE-IVD certification,

Sysmex

- The PS-10 Sample Preparation System alleviates the primary bottleneck in today's busy clinical flow cytometry laboratory, while providing outstanding flexibility for the creation of complex laboratory tests. The system pipettes specimens, reagents, lyse and buffer solutions to prepare samples for flow cytometric analysis. Paired with the Helmer Ultra CW Centrifuge, the system includes the ability to customize wash steps while using the same sample carousel. The unique notable advantages of the PS-10 are that it is an open system so that any vendor or laboratory prepared, reagents can be used, and it is flexible and programmable so that user defined protocols can be utilized.

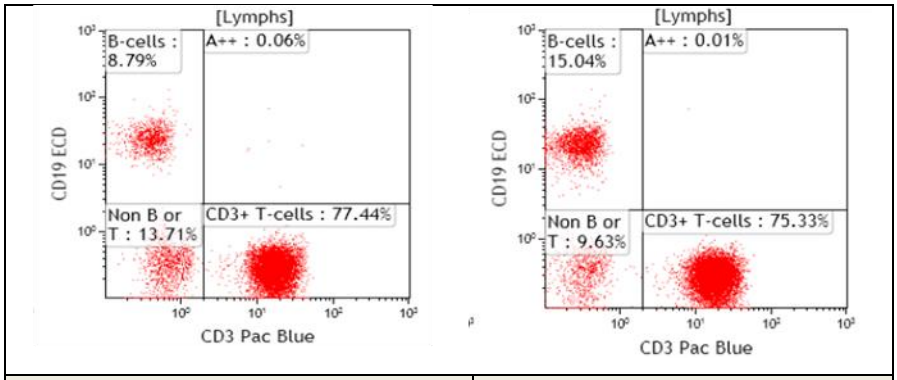


LYSE REAGENTS

It is important to select the right lyse buffer for each particular assay. There are many variables in identifying the best combination.

Evaluation by population loss:

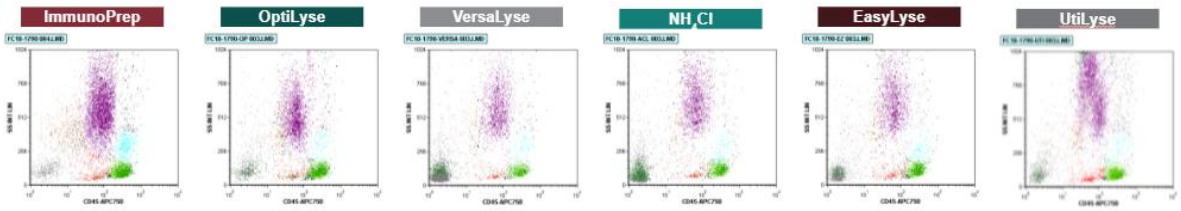
In addition to performing literature research, you must assess each reagent in your laboratory with your unique conditions. Some studies have shown that certain lyse buffers are harsher and can selectively lyse certain types of WBCs, throwing off the population percentages, while other studies find contradictory results showing that the same lyse reagent exhibits less cell loss than others. (8) Determining potential cell loss is critical because even small reductions in the number of rare cell populations can have significant deleterious effects on the diagnostic value of the results.



Ammonium Chloride (Bulk Lyse)	OptiLyse

Figure 2: Comparison of the CD3 vs CD19 percentages 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

The CD45 vs SSC plots and the table below exhibit the difference that the choice of lyse buffer can have on percentages of various cell populations.



	Lymphs	PC	Blasts	Monos	Grans	Debris
	27	6	0	3	48	7
	27	5	0	2	43	14
	11	3	0	1	22	60
	18	3	0	2	34	39
	25	5	0	2	36	27
	23	4	0	2	52	11

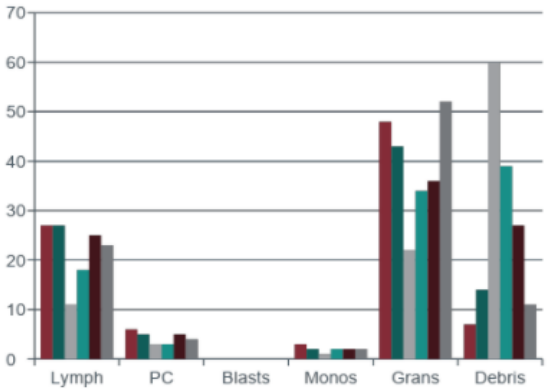


Table 3: Comparison of lysis buffer effects on the percentage of different cell populations. Plot label colors above correspond with table below.

A study published in 2013 found that the percentage of blasts after treatment with FACS Lyse was significantly smaller than with PharmLyse ($p<0.0001$), OptiLyse C ($p<0.0001$), or IOTest 3 ($p<0.0001$), respectively. The difference between PharmLyse and OptiLyse C ($p=0.93$), PharmLyse™ and IOTest 3 ($p=0.31$), and OptiLyse C and IOTest 3 ($p=0.34$) was not significant. These results emphasize the importance of harmonization of red cell lysis protocols for the application of flow cytometry in hematological neoplasms." (9) In direct conflict with the previous study, the Euroflow Consortium found that cell loss was significantly lower when FACS Lysing Solution was used (versus all other lysing reagents). (2)

Evaluation by microscopy:

In addition to comparing scatter plots for debris and cell loss, a novel method of assessing cell damage is to gauge cellular damage through microscopic observation. The figure below displays findings from the TexFlo Update for FlowTex 2013; Standard Operating Procedures when comparing two commercial lyse reagents. The FACS Lyse caused significantly more cellular damage when observed microscopically. (13)

PharmLyse FACS Lyse

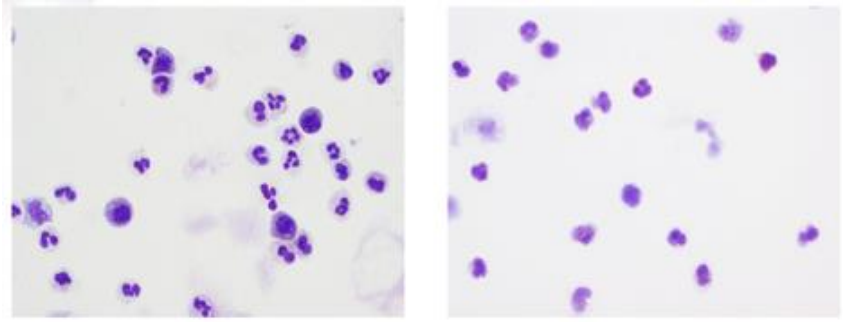


Figure 3: The FACS Lyse also exhibited increased cellular damage.

Evaluation for aggregation:

It is useful to include a gate to identify doublets utilizing the forward scatter area (FSC-A) vs forward scatter height (FSC-H) to check for lyse related aggregation of cells.

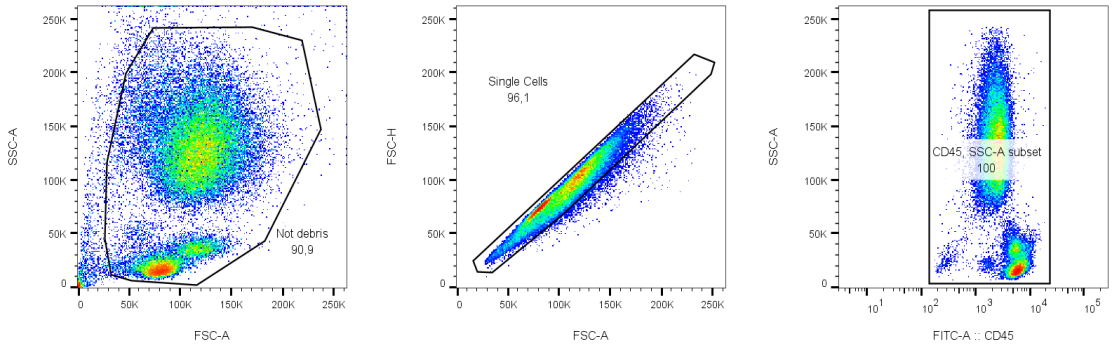
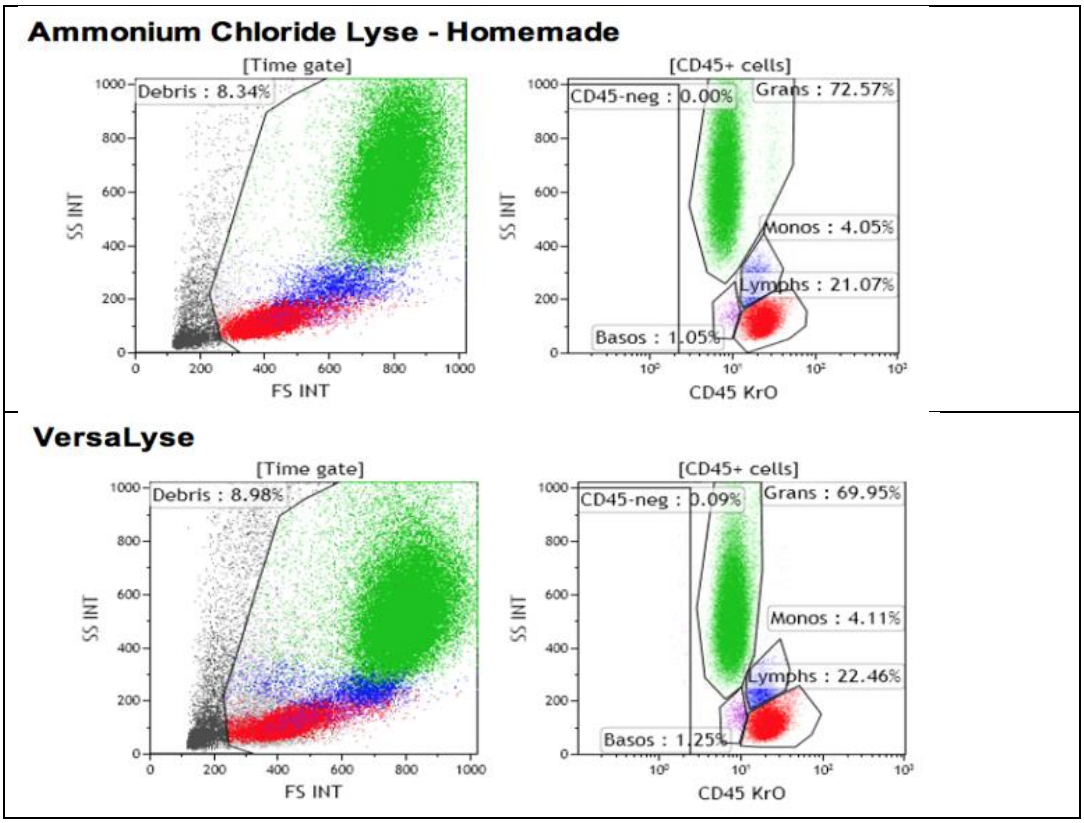


Figure 4: Typical gating method to identify doublets, or cell aggregates.

Evaluation by FSC vs SSC and CD45 vs SSC plots:

Lyse reagents can also affect the scatter and staining properties of the cells (see Figure 3). The scatter properties should be carefully evaluated and compared for quality and appropriate fit for the assay. We have included some examples below.



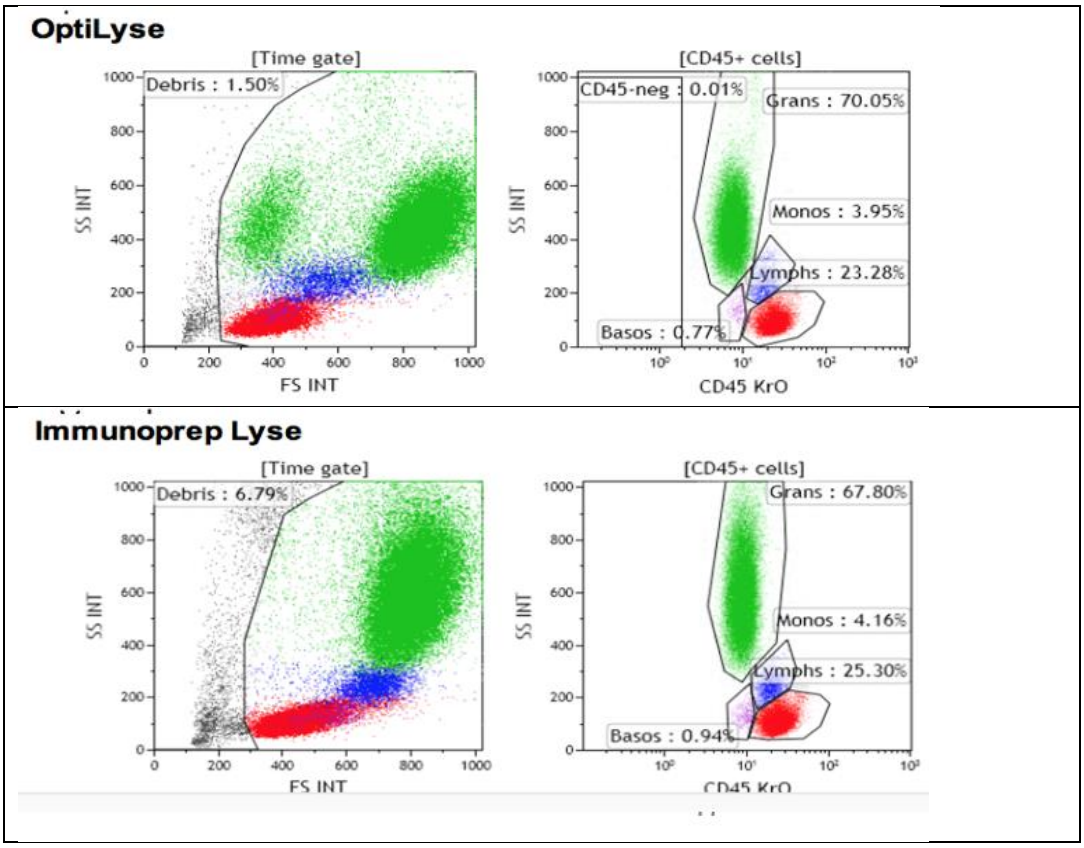


Figure 5a: Same patient, same instrument, same voltage to show difference between lysing effects on FS and SS of Beckman Coulter lysis reagents

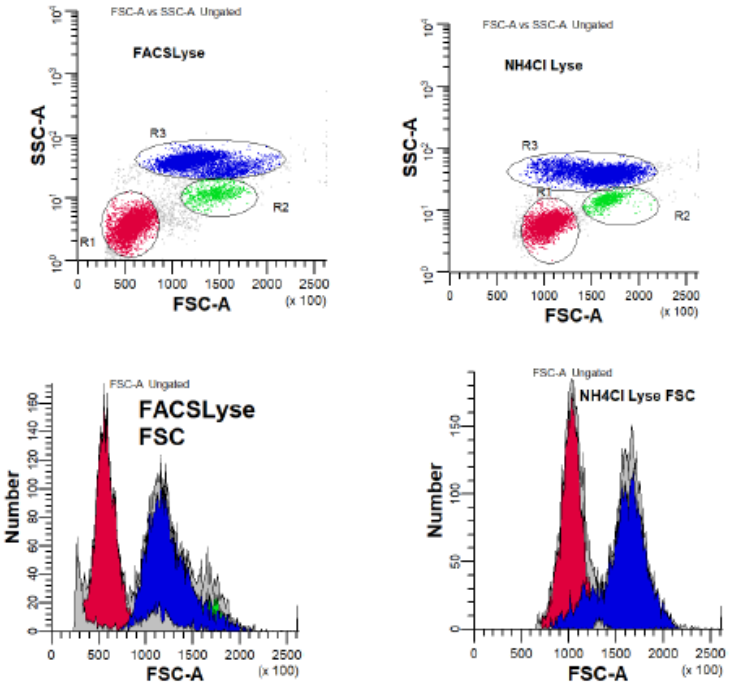


Figure 5b: Same patient, same instrument, same voltage to show difference between lysing effects on FS and SS of Becton Dickinson lysis reagents

SPECIMEN TYPES

We have looked at peripheral blood thus far in this module for consistency. However, other types of specimens are often encountered in flow cytometry laboratories. Bone marrow aspirate is a common specimen for leukemia and lymphoma immunophenotyping. Lymph nodes are tested with flow cytometry in many laboratories. A wide variety of other tissues, solid tumors and body fluids can be tested using flow cytometry. Some of them will contain enough red blood cells to require lysing, many will not.

The ratio of lyse to specimen and the length of the lysing incubation might need to be adjusted for each specimen depending on the amount of red blood cells present, the amount of nucleated red blood cells as well as the fragility of the cells of interest.

The effect of lysing will look different for every type of specimen. Bone marrow aspirate, shown below, typically contains nucleated red blood cells that are resistant to lysing as well as a more complex pattern overall representing the entire spectrum of hematopoietic development, making it more challenging to assess the effectiveness of the lyse and to identify issues.

Lymph nodes tend to contain less red blood cells and display a more homogenous scatter pattern. Typically, most of the cells in a lymph node specimen are lymphocytes. Other specimen types, such as spleen biopsies, will contain a much larger proportion of red blood cells.

Spend time in your laboratory becoming familiar with the normal and abnormal patterns of every specimen type run in your laboratory.

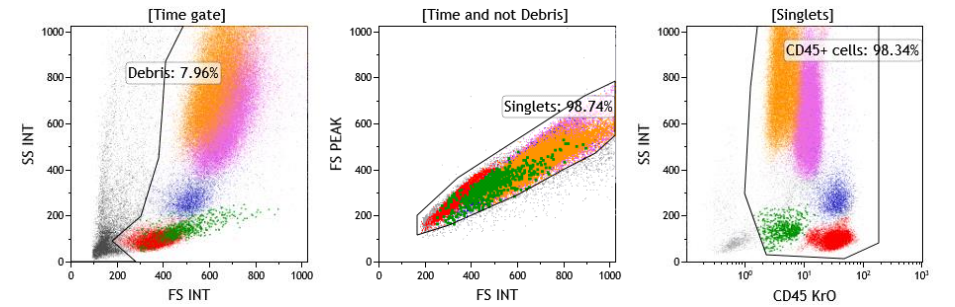
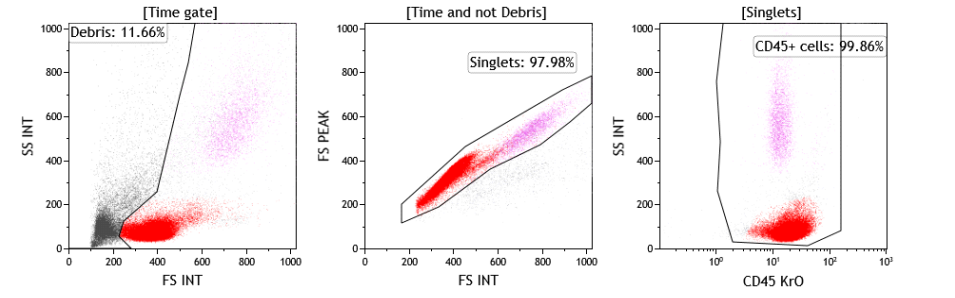
Bone Marrow Aspirate	
Lymph Node Biopsy	

Figure 6: FSC vs SSC and CD45 vs SSC plots of bone marrow aspirate and lymph node biopsy specimens.

TROUBLE SHOOTING

Common problems:

- **Insufficient lysis:** A partially lysed specimen will have opaque red color, while a fully lysed specimen will be transparent. Increased unlysed RBCs will be visible in the plots of partially lysed specimens. The FSC vs. SSC plots will display the unlysed RBCs in the lower left corner along with debris.

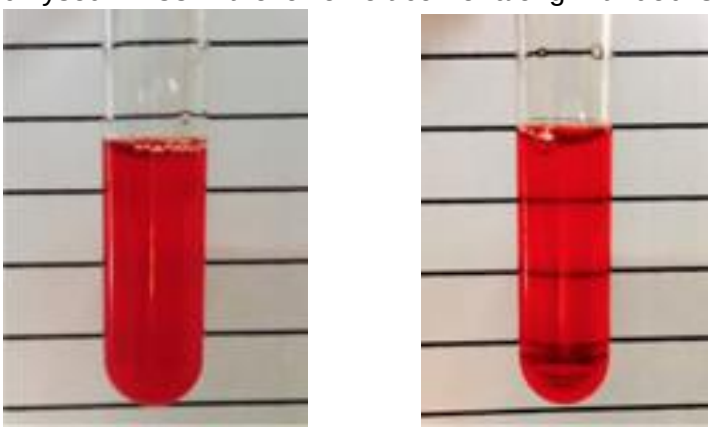


Figure 7: Partially lysed specimen on the left and fully lysed specimen on the right.

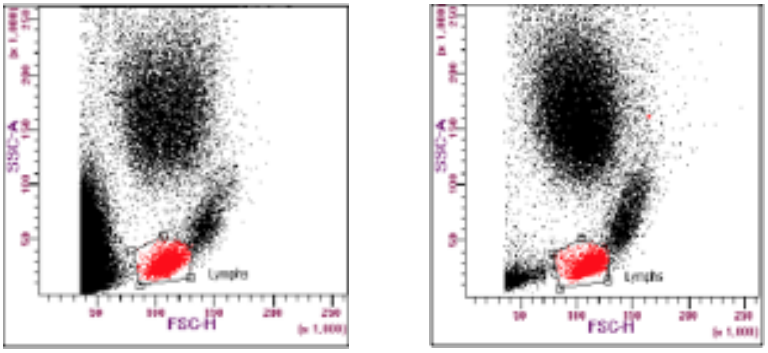


Figure 8: Insufficiently lysed specimen on the left and adequately lysed specimen on the right.

- **Excessive lysis:** Lysing with an excessive amount of reagent, an overly harsh reagent or leaving the cells in lyse for too long will destroy a portion of the white cells and cause loss of cellular integrity, resulting in poor light scatter separation of cell populations, especially monocytes and lymphocytes.

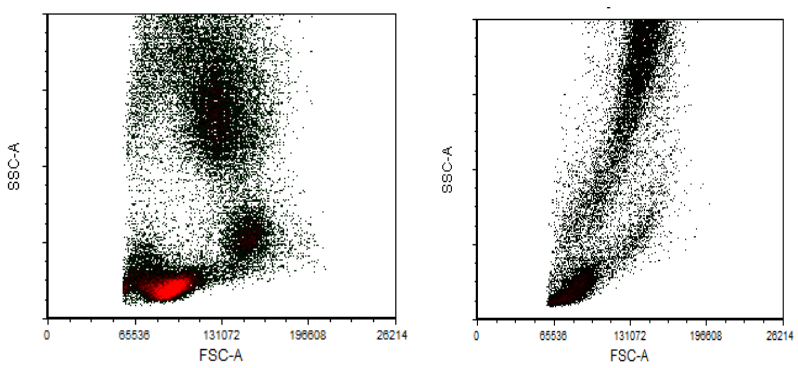


Figure 9: On the left, lyse with vendor recommended lysing time (BD PharmLyse LSW). On the right, lyse with excessive time (BD PharmLyse LSW 40 minutes)

- **<u>Skill dependent variability</u>:** As discussed above, manual processing involves person to person and day to day variability.
    - Insufficient vortexing or mixing: through either time, turbulence or position of the tube. While there is no obvious evidence shows that insufficient vortex will affect the lyse effect as long as the sample reaches the required lyse time, it is important to consider variability from technologist to technologist. Hand mixing can also be a skill dependent variable.
    - Insufficient rocking for bulk lysing (LSW): Specimens processed using bulk lyse method need to be rocked on a tube rocker for 5 to 10 minutes. Each lab must validate their own process to reach the maximum lyse effect without causing damage to the white blood cells.

- **<u>Lyse buffer pH</u>**: Most commercially available lyse reagents come as a 10x stock solution. It is recommended to prepare a 1x working reagent fresh. The pH of the 1x solution should fall within the range of pH 7.1-7.4. Adjust the pH if necessary. If the pH is too basic, the specimen might not achieve complete lyse within the lab determined incubation time. If the pH is too acidic, it will quickly destroy some of the white cell population. It is critical to follow storage recommendations for commercially available products or establish your own for laboratory made solutions.

- **<u>Temperature:</u>** i.e. room temp vs refrigerated (4^{o}C - 8 ^{o}C): Most commercial lyse reagents require use at room temperature (20^{o}C -25^{o}C). Colder temperature might prolong the lyse time needed and not separate the populations as well.

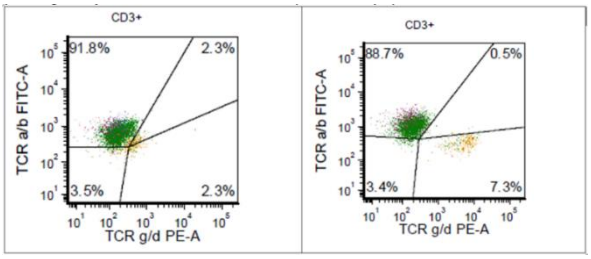


Figure 10: On the left, lyse reagent at 4^{o}C. On the right, lyse reagent at room temperature, showing larger separation of the yellow population from the green one.

- **Fixative:** Lyse reagent with fixative agent, such as formaldehyde, can also affect the lysing effect.
    - As discussed above, fixative can be harsher on cells, increasing debris and decreasing cell yield and viability.
    - Lyse reagents exhibit low efficiency on lysing nucleated red cells, and this is especially true for lyse with fixative reagent.
    - Fixative can cause reduced staining intensity for certain monoclonal antibodies.
    - Fixed cells might exhibit decreased S/N ratio, making separation of positive and negative cells challenging (see Figure 11).

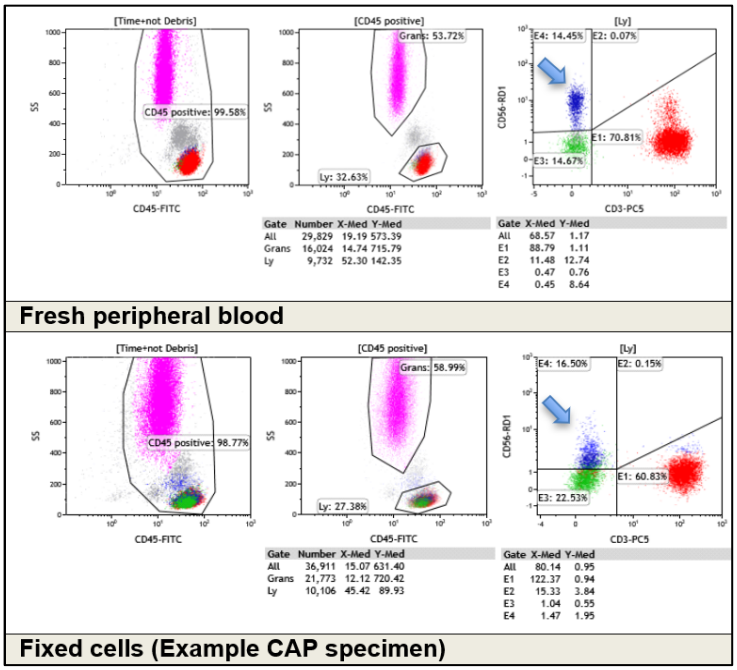
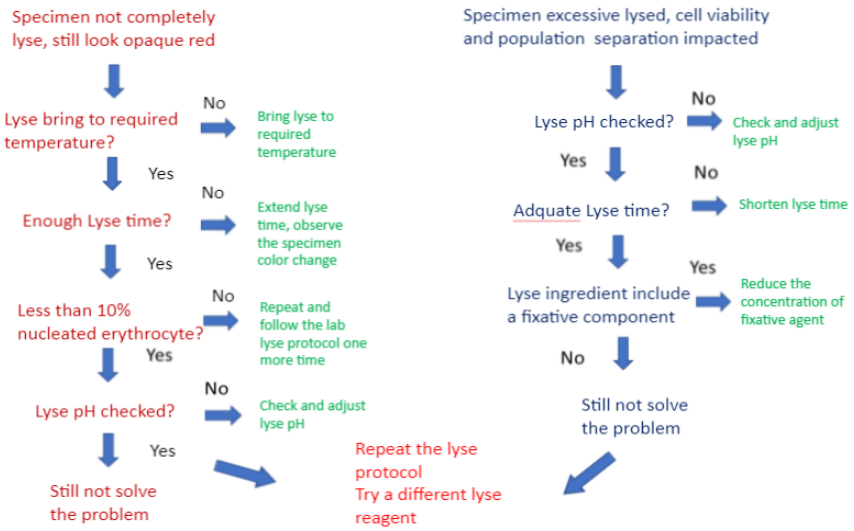


Figure 11: Fresh peripheral blood (top row) vs fixed CAP sample (bottom row) comparisons using ImmunoPrep lyse, exhibiting the poor signal/noise ratio (e.g. CD3-negative/CD56+ cells) for fixed cells.

Troubleshooting Flow Chart



LYSE BUFFER PREPARATION

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 Lyse No Wash (LNW), 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 Tests (LDTs). While it is acceptable to start with recommend protocols and reagents, **it is imperative that every laboratory validate each protocol for their own site. (1)**

Ammonium Chloride lyse reagent can be prepared in the laboratory or commercially prepared reagents, such as those discussed above, 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. (10)

Ammonium Chloride Lyse Recipe:

- *10*x Solution (Solution intended to be diluted by a factor of 10 before being used)
  - 80.2 g NH_4Cl (1.5 M)
  - 8.4 g $NaHCO_3$ (100 mM)
  - 3.7 g disodium EDTA (10 mM)
  - Distilled H2O 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 (lab needs to test and validate the stability)
- *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

Note: As discussed in the Specimen Types section, the ratio of lyse to specimen and the length of the lysing incubation might need to be adjusted for each specimen depending on the amount of red blood cells present, the amount of nucleated red blood cells as well as the fragility of the cells of interest.

A – Lyse no Wash (LNW) Protocol:

1. Add antibodies and desired reagents into a 5mL tube.
2. Add specimen to the tube.

3. Mix well and incubate protected from light for 15 minutes.
4. Add the appropriate amount of lysing agent into the tube.
5. Mix well and incubate protected from light for 15 minutes.
6. Tubes are ready for acquisition.

Note: when performing LNW using an IVD system, you must follow manufacturer's instructions.

B - Bulk Lyse (LSW) Protocol:

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 density of 10^7 cells/ml
12. Determine the correct volume of specimen to use:
    a. Create a cell suspension concentration of $1x10^7$ cells/mL (or $1x10^4$ cells/µL)
    b. Pipet 100µL into each tube." (i.e. $1x10^7$ cell/mL or $1x10^4$ cells/µL) 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 200µL of PBS and mix well.
18. Tubes are ready for acquisition.

***C - Tube Lyse (SLW) Protocol**:*

1. Wash whole specimen 3x with PBS (1 wash = add ~4mL PBS, resuspend, spin 3min at 350g, discard supernatant). Some procedures skip this step and add unwashed specimen directly to the antibodies.
2. Add antibodies to the individual panel tubes. If you are using dried down or lyophilized reagents, this step is unnecessary.
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 visually check that the specimens are fully lysed, see Troubleshooting section).
8. Centrifuge for 3 min at 350g.
9. Discard supernatant, either by decanting or aspiration. Take care to not lose the WBC pellet at the bottom of the tube. When in question, aspirate carefully.
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.

SUMMARY

The goal of this module is 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. All of the results in this module are based on the experiences of several different laboratories with different platforms and various antibodies from different vendors, exhibiting diverse combinations of methods, reagents and circumstances.

While RBC lysis is acknowledged as the most optimal method of specimen preparation for flow cytometric analysis, it is vital to consider the numerous consequences on immunophenotyping. Herein we have provided you with tools to evaluate and optimize your lysing protocols, compare and contrast different lysing methods and reagents, and evaluate the factors impacting the results, as well as standardized protocols to use as a starting point for choosing and validating the optimal method for your specific application.

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