Title: **Flow Cytometric Testing for Kappa and Lambda light chains**
Sponsored and reviewed by ICCS Quality and Standards Committee

Written by: Melanie O'Donahue, Laura Johnson, Ben Hedley and Erin Vaughan

Date: Jan 15, 2018

OUTLINE

Assessment of immunoglobulin light chain (i.e., kappa or lambda) expression by flow cytometry is a key component in the diagnosis and monitoring of B cell lymphoid neoplasms. Normal and reactive B cell lymphocyte populations typically exhibit expression of both kappa and lambda light chains at an expected ratio, while neoplastic cells exhibit monotypia (over expression of either kappa or lambda).

Staining of immunoglobulin light chains for flow cytometry presents some inherent challenges; perhaps most problematic is non-specific staining. Non-specific staining is due to the cytophillic antibodies binding to the FC receptors on numerous cell types as well as antibodies being absorbed by dead and damaged cells. Elimination or mitigation of this non-specific staining is critical for proper interpretation of the results. Additionally, in some samples, equivocal light chain expression is observed and cytoplasmic (aka intracellular) staining or blocking should be performed to clarify the results. Cytoplasmic staining for light chains presents additional challenges.

The purpose of this module is to present methods to optimize both surface and cytoplasmic staining for immunoglobulin light chains. Further, as in all clinical flow cytometry assays, proper interpretation of results is dependent upon correct gating of the samples. Therefore, methods for gating are also addressed.

Introduction

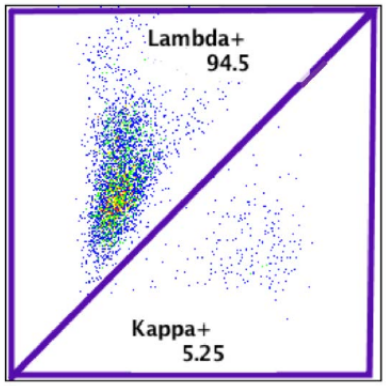
This module will discuss flow cytometric kappa and lambda light chain immunophenotyping of clinical specimens for the detection and assessment of monoclonal B cell populations which are present in B cell lymphoproliferative disorders or monoclonal lymphocytosis of undetermined significance (MLUS).

In vivo antibodies, also referred to as immunoglobulins (Ig), are secreted by B cells as part of the immune system's defense against bacterial or viral pathogens. Antibodies are secreted into the blood as soluble proteins by the B cell or and they can also be identified bound on the cell surface. The cell membrane bound form is called a surface immunoglobulin (sIg). Structurally, the antibody consists of two heavy chains and two light chains. The light chains are designated as kappa and lambda.

Each individual normal human B cell expresses either kappa or lambda light chains, not both. The overall expression of kappa to lambda in a non-diseased state exhibits a ratio of approximately 2:1[1]. A ratio that is outside of the range of 3:1 and 0.3:1 is generally considered to be monoclonal (3.0 to 0.3)[5]. It is important to note that the kappa to lambda ratio should be correlated with several other features for proper interpretation. See Diagnostic criteria for monoclonal B cell lymphocytosis. British Journal of Haematology, Marti et al[5] for more information.

Research has long supported the hypothesis that malignant cells arise from a genetic transformation and proliferation of a single cell. Thus malignant B cells consisting of a proliferating single cell line are defined as a clone and often exhibit an expansion of either kappa or lambda light chain expressing cells. This clonal

expansion is termed monoclonal light chain restriction. The detection and identification of light chain monoclonality is the cornerstone of the diagnosis of B cell lymphoproliferative disorders[6]. Therefore, while clonality by itself might not be diagnostic of a neoplastic process, monoclonal expression is one of the most important determinations in flow cytometry immunophenotyping.[2]



- Normal: K:L ratio between **3:1 and 0.3: 1**
- Monoclonal: K : L ratio **>3:1** or **<0.3:1**

Figure 1: Example of monoclonal kappa: lambda ratio; K:L = 1:18 (0.06)

The assessment of kappa or lambda monotypia is also useful in the diagnosis and monitoring of plasma cells disorders such as multiple myeloma and plasma cell leukemia. Plasma cells are terminally differentiated, non-dividing, effector cells of the B cell lineage.[4] Due to the unique intracellular proliferation of light chain immunoglobulins in plasma cell disorders, cytoplasmic kappa and lambda staining is necessary for a proper assessment of clonality. The expected and atypical ratios of kappa to lambda are the same for plasma cells as for other BCLPDs.

Optimization

Due to the particularly challenging nature of staining for kappa and lambda light chains, it is critical to carefully optimize your entire protocol; sample processing, reagents, antibody selection, permeabilization, acquisition and analysis. Potential pitfalls include autofluorescence, nonspecific/cytophillic binding, antibody excess/overly bright staining and spectral overlap (spillover/spreading).

Selecting the optimal reagents is a critical factor in optimizing your protocol. It is important to select the appropriate lysing agent, wash buffer, antibodies, blocking media, fixative, and permeabilization reagents. It is necessary to optimize the antigen: antibody ratio by titrating the antibodies[8]. To assess the performance of the system, note the median fluorescent intensities (MFIs) and stain index (SI) of the stained events. The SI is a normalized signal to background metric useful in quantitating the brightness of immunofluorescent reagents on cells. A higher SI is better for accurate differentiation between negative and positive populations.

Both polyclonal and monoclonal antibodies against kappa and lambda are commercially available; however, there is no consensus on which is preferable. Polyclonal antibodies have higher affinity and

sensitivity, while monoclonal antibodies are more specific and exhibit lower background staining[9]. It might be favorable to use polyclonal antibodies when assessing dim expression with specific BCLPDs.

	Monoclonal	Polyclonal
Binding	Single Epitope	Multiple Epitopes
Non-specificity	Less susceptible	Susceptible
Cost	$$$	$$

Table 1: Choice of Antibody: Polyclonal versus Monoclonal antibodies

It is imperative to lyse the sample to remove red blood cells for optimal analysis. There are many factors involved in this process. See more information for optimization in the Protocols section below[7].

PROTOCOLS

A - Surface staining for light chains

Although there are many different methods for surface staining of light chains, it is critical to establish an effective pre-staining protocol for proper evaluation of light chain monotypia[7] in your laboratory. Each of the following three methods removes the extracellular immunoglobulins or dead cells that can interfere with staining. The three main methods are Lyse/Wash/Stain (LWS), Wash/Stain/Lyse (WSL), and Block/Wash/Stain (BWS):

1) Lyse/Wash/Stain (LWS, aka Bulk Lysing): This method involves lysing the entire sample without fixative, washing this sample and re-suspending the nucleated cells into a buffer/media, then staining. The stained sample is then washed once more and aquired on a flow cytometer. There are many lysing agents are on the market from multiple manufacturers for laboratories to choose from. Many laboratories make ammonium chloride lyse in house. There are no guidelines stating that pre-lysing samples lowers the ability to detect monotypia, however there are unpublished observations by working groups and individual authors that state that there is a higher cell loss with pre-lysing samples when compared to lysing the red blood cells post staining.

Procedure:

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, use a 1:5 ratio (i.e. 2mL sample, 8mL lyse).
    b. If you are using a commercial lyse reagent, use the manufacturer's recommended amount.
3. Place the conical tube on a rocker for approximately 10 minutes at room temperature. If a rocker is not available, invert the tube periodically during the incubation.
4. Observe for lysis. Specimen should be a transparent cherry red color with little to no red cells observed on the white blood cell pellet.
5. If the lysing appears incomplete, incubate for an additional 5 minutes.
6. Centrifuge for 5 minutes at 550g.
7. Discard the supernatant; either by pouring off, decanting with a pipet or using a vacuum system. Different laboratories prefer different methods. If you find it difficult to pour off the supernatant

without losing white blood cells, use a decanting method. Using a vacuum system requires care and skill to avoid aspirating the white blood cell pellet.

   a. If a visible amount of red cells are observed in the pellet, add half as much lyse as used in step 2.
   b. Mix well and incubate for 5 more minutes at room temperature.
   c. Repeat centrifugation and discard supernatant.

8. Re-suspend 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 RPMI nutrient media solution or PB to achieve an optimal cell count of 10^7 cells/mL.
12. Pipet 100µL of sample into each tube.
13. Mix well and incubate protected from light for 15 minutes at room temperature.
14. Add 2mL of PBS and vortex.
15. Centrifuge for 3 minutes at 350g.
16. Discard supernatant.
17. Re-suspend cell pellet in 200mL of PBS, with or without fixative, and mix well.
18. Tubes are ready for acquisition.

2) Stain/Wash/Lyse (SWL, aka Tube Lysing): This method involves washing the aliquot of specimen adequately (at least 3 times), then staining the sample. After staining the specimen, the red cells are lysed, the sample washed and then resuspended prior to acquisition on a flow cytometer. Work published in the ICCS newsletter shows the improvement in signal on normal B cells as the number of washes increases from a single wash to multiple washes[3]. Beyond three washes the benefit in separation of negative and positive was negligible over the extra time taken to wash the sample further; although in some cases dim expression of a light chain might be resolved with an extra wash.

Procedure:

1. Wash the entire specimen 3x with PBS (1 wash = add ~4mL PBS, re-suspend, spin for 3 minutes at 350g, discard supernatant).
2. Add respective antibodies to the individual empty labeled panel tubes.
3. Add washed specimen to the tubes with respective antibodies. The 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 minutes at room temperature protected from light.
5. Add 2 ml of Ammonium Chloride Lyse, or the correct amount of commercial lyse as indicated on the package insert. Vortex
6. Incubate for 10 minutes at room temperature protected from light.
7. Mix well and check that the specimens are fully lysed.
8. Centrifuge for 3 minutes at 350g.
9. Discard supernatant.
10. Mix well and wash 2x using 2 mL of PBS and centrifuging for 3 minutes at 350g.

11. Discard supernatant and re-suspend cell pellet in 200uL of PBS (with or without fixative) and mix well.
12. Tubes are ready for acquisition.

3) Block/Wash/Stain: This method is similar to the protocols above; however, there is a blocking step in between the washing steps. Occasionally kappa and lambda expression will appear negative or too dim to discern. This lack of surface staining is thought to arise from either nonspecific binding of the antibody or a true lack of surface immunoglobulin, which comes from an abnormal modification of the immunoglobulin protein on the cell surface. In the first case, the addition of a blocking step within the washing process might allow for visualization of a dim monotypic population. However, if a suspect population does not express any surface light chains, then cytoplasmic staining can be performed as described below.

1. Add the manufacturer recommended volume of blocking reagent to the lysed cell suspension or washed, unlysed specimen. Incubate for 15 minutes at room temperature. Do not wash blocking serum off the cells.
2. Proceed with staining as outlined above

B - Cytoplasmic/Intracellular staining for light chains

Cytoplasmic staining is standard when plasma cell disorders are suspected. In addition, small populations of B cells can have equivocal light chain expression. In these dubious cases where a B cell lymphoproliferative disease is suspected, cytoplasmic staining is often used. Some disease states, particularly CLL, often exhibit very dim expression of kappa and lambda light chains. The pattern itself is indicative of CLL. But if the ratio is too difficult to assess, cytomplasmic staining can be useful. Blocking, discussed in the previous paragraph, can aid in assessment of dim or equivocal light chain expression as well.

The method for cytoplasmic staining again requires a pre-staining step prior to immunoglobulin staining which involves a process for allowing the antibody to enter the cell. This is call permeabilization. Kits are available for cytoplasmic staining which allow for surface reagents to be stained first, the sample fixed, then permeabilized and finally stained for cytoplasmic light chain immunoglobulins. Care should be taken when using these Fixation/Permeabilization kits as the antibodies might need to be re-titrated for this application. Similarly, different cytometer settings (both voltages and compensation) might be needed for correct visualization of cytoplasmic light chain staining.

Procedure:

1. Add 100uL of cell suspension to labeled tube. Use more cells if a small population of cells of interest (e.g. rare plasma cells) is present to acquire enough abnormal cells (50-100).
2. Add relevant surface marker(s), vortex and incubate for 15 minutes in the dark at 4˚C.
3. Add 100µl of fixation reagent, vortex and incubate for 15 minutes in the dark at room temperature.
4. Wash x1 for 3 minutes at 350g.
5. Add 100µl of permeabilization reagent, vortex.
6. Add antibodies for cytoplasmic staining.
7. Vortex and incubate for 15-20 minutes in the dark at room temperature.
8. Wash x1 for 3 minutes at 350g.

9. Reconstitute with 0.5ml (500uL) of PBS.

Additional Considerations:

1. Pre-washing samples: As mentioned above, is critical in decreasing the non-specific binding of light chain antibodies in flow cytometry. The figure below demonstrates the decrease in non-specific binding with 1, 2, 3 and 4 washes[3].

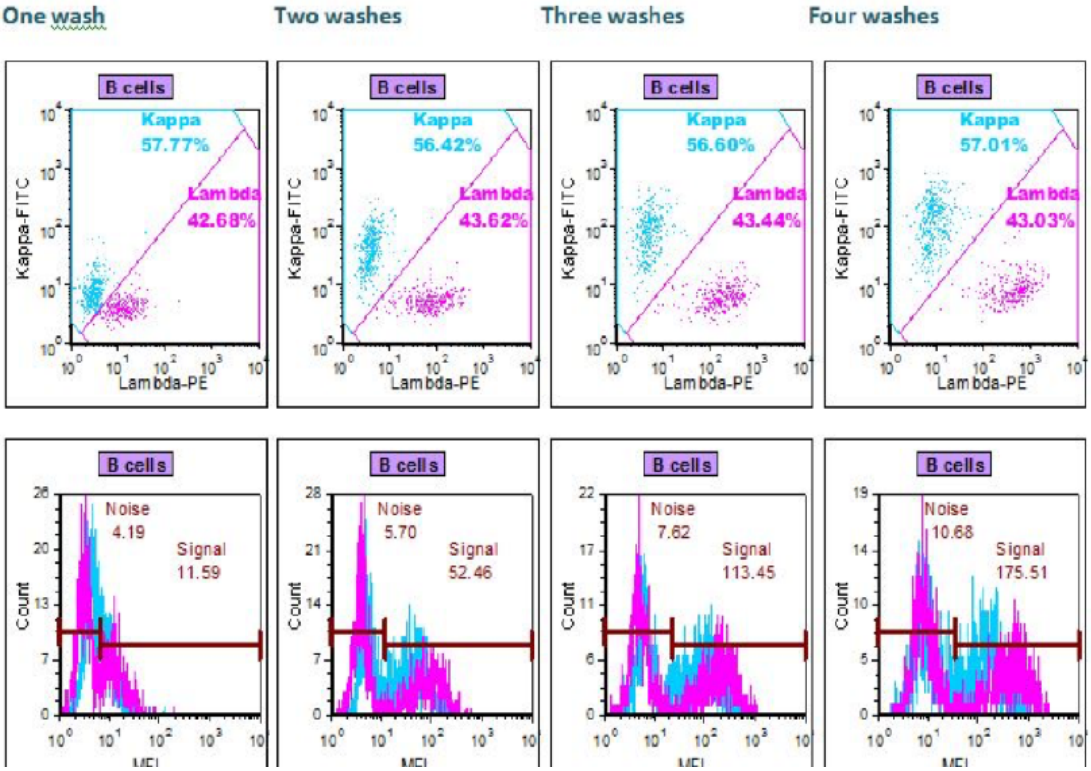


Figure 2: Surface staining with 1, 2, 3 and 4 washes

2. When to perform blocking or cytoplasmic staining for immunoglobulin light chains: The lack of surface staining is thought to arise either from non-specific binding of the antibody or a true lack of surface immunoglobulin, which comes from an abnormal modification of the immunoglobulin protein on the cell surface. In the first case, the addition of a blocking step within the washing process could allow for visualization of a dim monotypic population. However, if a suspect population does not express any surface light chains immunoglobulin then cytoplasmic staining will be more useful.

3. Staining kappa and lambda with different fluorochromes or same: When assessing cytoplasmic light chains, there are two different approaches; the first approach is to perform the assessment in a single tube, using kappa and lambda antibodies conjugated to different flurochromes. The second approach is to use kappa and lambda conjugated to the same flurochrome subsequently using two separate tubes (i.e. kappa FITC in the first tube and lambda FITC in the second). The latter method more easily allows for overlaying the signals to determine if there is truly dim cytoplasmic light chain excess.

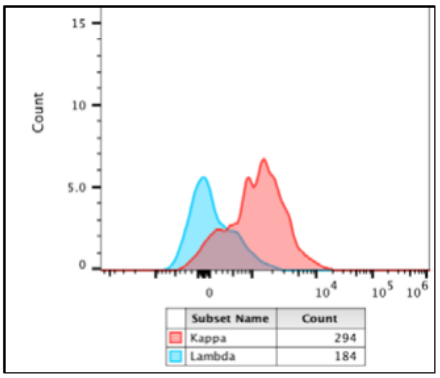


Figure 3: Kappa and Lambda overlay clearly showing fluorescence shift

Gating

The gating of B cells that are stained with antibodies to determine immunoglobulin light chain expression can be performed in several ways. As there are other cells that can bind light chains deceivingly such as NK, activated T cells, and monocytes; it is important to first isolate the B cell population. Two gating strategies are commonly used. Both methods employ sequential boolean gating to select the B cells and thereafter assess the light chain expression to determine monotypia. It should be noted that the choice of fluorochrome for CD19 is critical regardless of the strategy as accurate delineation of B cells is necessary to correctly determine the presence or absence of an abnormal population. Both strategies should employ a clean-up gate to exclude any fluctuations in the cellular flow as well as any doublets.

- The first and simplest gating strategy involves gating only B cells by using a dot plot of Side Scatter versus CD19 showing all cells.
- The second strategy outlined here uses sequential gating to firstly gate only the white blood cells on a Side scatter versus CD45 plot. This plot can be also used to gate the lymphocytes as they are low side scatter/bright CD45 on the same plot. Then this lymphocyte gate can be used as the input to a side scatter versus CD19 plot allowing for the B cells to be gated. Care should be taken if using this method as dim CD45 or higher Side Scatter populations might be excluded.

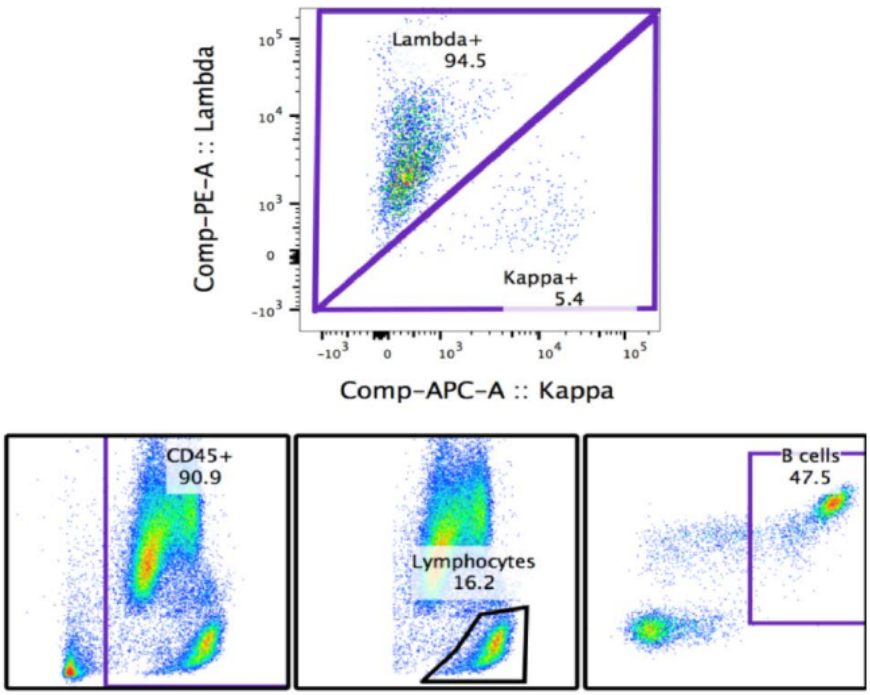


Figure 4: Example of B-cell gating, showing monoclonal surface lambda light chain restriction. Clean-up gates not shown.

*It should be noted that with modern multiparameter flow cytometry methods, it should be possible to isolate the abnormal population in most cases by a combination of markers. For example, an abnormal population of B cells positive for both CD19 and CD5 can be isolated from normal B lymphocytes which are CD5 negative. Using this gating strategy, a CD5+/CD19+ population expressing only one form of light chain can be identified, narrowing down the diagnostic picture.

There are also situations when a sample might have two monoclonal populations, one kappa and one lambda, referred to as biclonal. In these cases, the kappa to lambda ratio might be normal overall, but analysis of the discrete populations will reveal that they are indeed two separate clones.

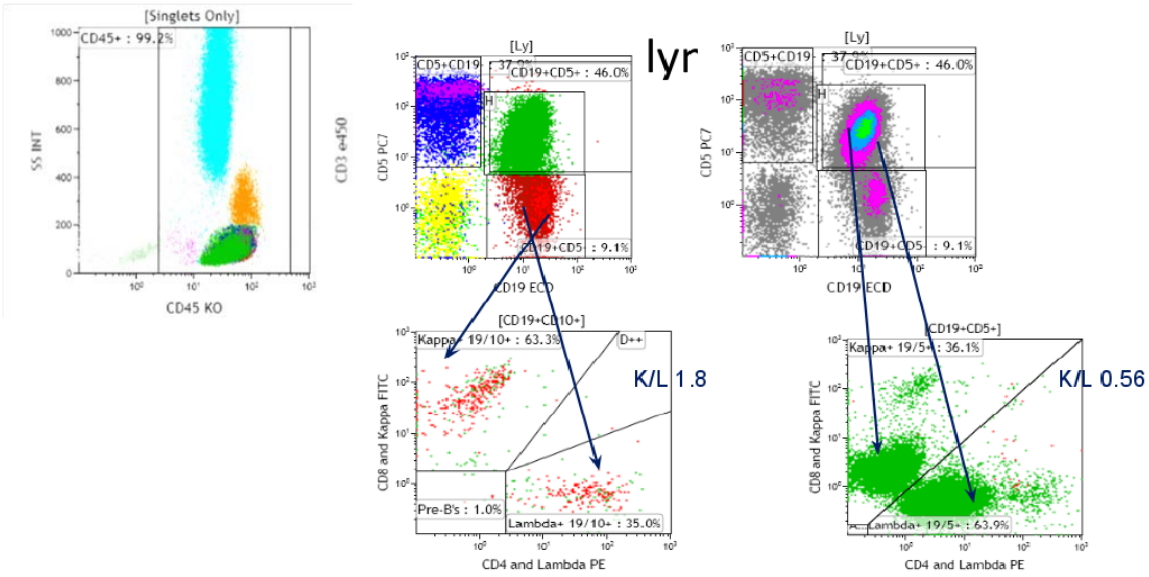


Figure 5: Example of the presence of biclonal B-cells

CONCLUSION

While kappa and lambda light chain staining is a critical factor in the diagnosis of B cell lymphoproliferative disorders, there is still a lack of consensus on the proper protocols to use, and there are numerous challenges to overcome to achieve accurate results. Optimization is essential to this process. We have provided a basis to guide laboratories through this process, but it is mandatory that each institution perform their own optimization and validations.

REFERENCES

1. The Monoclonality of Human B cell Lymphomas, Ronald Levy, Roger Warnke, Ronald F. Dorfman, and Joseph Haimovich. Journal of Experimental Medicine, Volume 145, 1977 p.1014
2. Flow Cytometric Analysis of Kappa and Lambda Light Chain Expression in Evaluation of Specimens for B cell Neoplasia, Paula I. Fukushima, Phuong Khanh Thi Nguyen, Paul O'Grady, and Maryalice Stetler-Stevenson, Cytometry 26:243-252 1996
3. Surface Staining for Kappa and Lambda, How Many Washes Are Sufficient? You Might Be Surprised. O'Donahue, M., VOL. VI, No. 3, Summer 2015
4. Immunophenotyping in multiple myeloma and related plasma cell disorders, Shaji Kumar, M.D, Teresa Kimlinger, B.A, and William Morice, M.D, Ph.D. Best Pract Res Clin Haematol. Author manuscript; available in PMC 2011 Sep 1. Published in final edited form as: Best Pract Res Clin Haematol. 2010 Sep; 23(3): 433–451. Doi: 10.1016/j.beha.2010.09.002, PMCID: PMC3005703, NIHMSID: NIHMS246587
5. Diagnostic criteria for monoclonal B cell lymphocytosis. British Journal of Haematology, Marti, G. E., Rawstron, A. C., Ghia, P., Hillmen, P., Houlston, R. S., Kay, N., Schleinitz, T. A., Caporaso, N. and The International Familial CLL Consortium (2005), 130: 325–332. Doi:10.1111/j.1365-2141.2005.05550.x
6. Flow cytometric immunophenotyping for hematologic neoplasms, Craig FE, Foon KA., Blood. 2008 Apr 15;111(8):3941-67. doi: 10.1182/blood-2007-11-120535. Epub 2008 Jan 15.
7. Lysing Methods and Reagents for Flow Cytometry, Module Immunophenotyping Sponsored and reviewed by ICCS Quality and Standards Committee, Melanie O'Donahue, MT, ASCP, CCy Date: December 1, 2016 Laura Johnson, MT, ASCP, SM, SH
8. Titration of Fluorochrome-Conjugated Antibodies for Labeling Cell Surface Markers on Live Cells, CURRENT PROTOCOLS IN CYTOMETRY, 6:6.29.1–6.29.9, Ruud Hulspas, Published Online : 1 OCT 2010, DOI: 10.1002/0471142956.cy0629s54

9. Evaluation of Polyclonal and Monoclonal Kappa and Lambda Antibody Reagents, O'Donahue, M., ICCS Annual Meeting, October 2015

Reviewed and approved by Wolfgang Kern, Jolene Cardinali and Teri Oldaker

For any questions on this module or any other suggestions, please email info@cytometry.org

The documents posted on ICCS website may contain product or vendor names which are provided for platform specific guidance. Any reference within the ICCS Quality and Standards modules to any vendor, product or educational material by trade name, trademark or manufacturer does not constitute or imply the endorsement or recommendation by ICCS.

Errata Notice: *A minor typographical error and/or editorial revision has been identified and corrected in this Quality & Standards module (11/13/26). The correction does not affect the intent, requirements, learning objectives, or overall content of the module. The module has been updated to reflect the correction.*