Sponsored and reviewed by ICCS Quality and Standards Committee

Title: Antibody Cocktail Validation for Flow Cytometry

Written by: Melanie O'Donahue, Fernando Ortiz, Ben Hedley, Veronica Sibing Wei, Kim Le

Date: Nov 26, 2019

_____

INTRODUCTION

The purpose of this module is to present methodology for the preparation of antibody cocktails for clinical flow cytometry assays, validation of cocktail use, stability/proper preservation and quality control. We will also discuss troubleshooting tips and methods to minimize errors when using antibody cocktails for flow cytometry.

Flow cytometry is a sophisticated technology with complex instrumentation, a wide variety of assay protocols and very specific reagents. Fluorochrome labeled antibodies against specific cellular or particulate antigens are likely the most important of these reagents. Great care must be taken to carefully select the best available antibodies, antibody conjugates and combinations thereof, and then to optimize each assay protocol to increase accuracy and decrease error and variance.

Antibody cocktails are an immensely useful tool in the clinical flow laboratory to aid in the consistent performance of an assay. An antibody cocktail is a combination of individual antibodies that are pooled into a single vessel for a specific flow cytometric assay. Using antibody cocktails allows the laboratory to reduce the time technologists spend in the setup of an experiment, minimize manual pipetting errors, standardize the results and keep track of reagent use.

Great care must be used when manually preparing antibody cocktails as any error made in the preparation of the cocktails can be very expensive and could lead to a misdiagnosis. Manual cocktail preparation creates great risk of pipetting errors and errors in individual antibody selection, missing an antibody or pipetting an antibody more than once. No matter how conscientious we are, we are human and errors do happen. Automated pipetting instruments and vendor manufactured cocktails are options that can help decrease error and save on technologist time. Automation also removes the variability inherent in human pipetting. In an era of rapidly evolving technology, manual methods are risky, largely unnecessary and should be phased out as soon as is feasible.

It is important to note that when antibody conjugates are pooled into a single reagent cocktail, interactions between the fluorochromes can lead to changes in the stability of the individual reagents

and introduce changes in expression. Initial validation and quality control of cocktail performance and stability compared to individual reagents constitutes a pre-requisite to deploying reagent cocktails in the clinical flow laboratory (1,2). There are a variety of documented examples of antibody conjugate instability within reagent cocktails, and a number of causes have been elucidated. Regardless of the causes however, results such as a loss of fluorochrome brightness, tandem breakdown or in some cases an apparent increase in brightness of a conjugate might be detected by the flow cytometer over time. (3,4)

In the United States, manufacturers are permitted to develop, optimize and manufacture antibody cocktails only for flow cytometric assays that are cleared by the FDA (in vitro diagnostic or IVD). In these situations, the manufacturer can provide diagnostic claims, instructions for use and operating procedures for the assay. Labs using FDA-cleared assays must follow the manufacturer's protocol without making any procedural alterations.

However, the majority of flow cytometric assays are not FDA cleared and fall in a category of a Laboratory Developed Tests (LDT). LDTs primarily use antibody conjugates categorized as Analyte Specific Reagents (ASR). The FDA has given some discretionary oversight to the laboratories using such ASR antibodies for use in LDTs. Laboratories may develop these individual ASRs into antibody cocktails, but they are responsible to optimize and validate these cocktails for their own use and cannot sell or give them to other labs. Some manufacturers offer a service called "contract manufacturing" of ASR antibodies into cocktails for an additional cost. It remains the laboratories' responsibility to develop and optimize the cocktail recipe and provide the contract manufacturer with that recipe. Once the contract manufacturer creates this predefined cocktail, each laboratory is responsible for the final validation.

Progress continues in the standardization of flow cytometry testing, including antibody cocktailing. Every laboratory must have its own written procedures for development, optimization and validation of antibody cocktails. It is a requirement that this process is clearly documented (1,2). In 2013 a coordinated effort resulted in a lengthy document, Validation of cell-based fluorescence assays: Practice guidelines from the ICSH and ICCS (5). More recently a document is being written that will be more detailed and comprehensive; CLSI Guideline H62--the Validation of Assays Performed by Flow Cytometry. Other efforts are currently underway to facilitate and eventually standardize this process. We are also beginning to see more manufacturers obtaining FDA (IVD) clearance for complex flow cytometric cocktails.

BACKGROUND

This module will focus on considerations for antibody cocktail validation in clinical flow cytometry. Clinical flow cytometry assays include a wide variety of tests that identify, characterize and sometimes enumerate cells or cellular components. One of the most common types of flow cytometry testing is immunophenotyping for Leukemias and Lymphomas. Clinical flow cytometry laboratories have become an essential in the detection and characterization of hematologic malignancies (leukemia, lymphoma, multiple myeloma, etc.). Clinical flow cytometry, in combination with morphology, hematology data and molecular analyses, are the standard methodologies used by Pathologists to reach accurate diagnoses.

As more complicated panels are developed (i.e. 8 antibody fluorochromes or more) and flow cytometers with increasing numbers of lasers and PMTs are utilized, the number of fluorophores that can be simultaneously detected has increased. Antibody cocktailing strategies reduce time in sample preparation for the technologists and increase the quality of data output for an increasing number of flow cytometry assays.

All the antibodies used for the cocktail need to be titrated in order to obtain the optimal separation of negative and positive expression using signal to noise ratio (S/N), stain index (SI) and median (or geometric mean) fluorescence intensity (MFI). It is not advisable to use the mean fluorescent intensity as the mean is highly susceptible to skewing due to unusually dim or bright outliers. Titration of antibodies will not only usually reduce the amount of antibody used in each cocktail, but will also optimize the signal read by the flow cytometer and the aid in the delineation and phenotypic analysis of specific target populations (6).

Sample preparation generally requires the most time from the technologists when running a flow cytometry assay. Accuracy and consistency in the staining process is critical to obtain an accurate signal on the flow cytometer. Antibody cocktailing reduces preparation time while decreasing error rates and variance.

Standardization indicates that the results obtained must be reliable between the technologists and multiple instruments used in a laboratory. This is especially true in flow cytometry laboratories with a high sample volume, where time might be limited and errors are more likely due to the increased workload.

Antibody cocktails have been utilized by flow cytometry laboratories for decades, but the lack of consensus between laboratories in the methodology used for validation continues to create variability in results. Standardization of methods between laboratories will allow accurate comparison of results,

particularly in cases where the diagnosis is complicated and interlaboratory collaboration is especially useful.

DISCUSSION

Benefits / Drawbacks

Benefits: The major benefits of using antibody conjugate cocktails are increased laboratory efficiency, the reduction or elimination of pipetting errors and standardization / reduction of variability.

- Efficiency: Dispensing a single volume of an antibody cocktail is much faster than pipetting individual reagents into a single tube.
- Error Reduction: Combining a number of tests' worth of reagents into a cocktail reduces the number of times a technologist manually pipettes reagents, thus reducing the chance of making pipetting errors; either missing an individual reagent completely, dispensing multiple aliquots of an antibody or pipetting an incorrect volume. Pipetting errors are the most common issue related to antibody cocktail preparation. While pipette calibration must be performed and monitored at least twice per year per FDA regulations, the potential for equipment error remains. Electronic pipettes do more accurately control the amount of reagent dispensed.
- Standardization: Manual pipetting creates significant opportunity for variability in staining and expression of antigenic markers. Manual antibody cocktailing reduces this variation of individual pipetting, and automation almost eliminates it.

Drawbacks:

- Possible reagent waste if the cocktails expire. Note: the entire cocktail is expired when any individual antibody used expires. All individual expiration dates must be carefully documented and monitored.
- Possibility of error and even harm to patients if an error is made in cocktail preparation, as well as significant waste of the entire prepared cocktail and necessary preparation of new cocktail. Antibody cocktailing could also increase the potential for tandem dye breakdown over time.

Options

Flow cytometry is a rapidly growing and expanding field. As the number of clinical flow cytometry laboratories grows, new assays are developed and the number of samples analyzed increases, we must keep up with innovations and constantly strive to improve quality and standardization in our processes.

Automation and vendor made cocktails are relatively new alternatives to manually prepared antibody cocktails.

Automated systems reduce the time constraints related to cocktail preparation and reduce the potential errors produced by manual pipetting and mixing. Automation is highly efficient and increases the standardization in the facility, reducing variability.

Another relatively new option is vendor made cocktails, either lyophilized or liquid. Vendor made cocktails can be costly, but they have the same benefits in that they save time, decrease errors and reduce variance. (7)

Automation:

Sysmex: The Sysmex PS-10 is the only instrument available on the market right now designed to perform antibody cocktailing. The Sysmex Sample Preparation System is a completely flexible automated specimen processing instrument that prepares cocktails as well as pipetting specimen and antibodies, lysing and washing and performing intracellular staining. The PS-10 also tracks reagent lot numbers, expiration dates and volumes (8).

Other Liquid Handlers: There are other automated pipetting systems built by a variety of vendors such as the Hamilton and Tecan, which are not flow cytometry specific but can be configured to perform automated sample and antibody pipetting.

Vendor made cocktails:

Beckman Coulter: Custom Design Services (CDS) or Contract Manufacturing Services (CMS) provide customization of single or multicolor antibody conjugates. These reagents are commercially available, but formulated, optimized and validated by the laboratory and are available in liquid or room temperature-stable dry formats. DURAClone Panels are dry pre-formulated antibody panels for rare event detection, immune function, immune system research and selected clinical applications.

BD Biosciences: create customized multi-color panels in dry or liquid form. BD Horizon™ Dri Multicolor Cocktails are premade cocktails for specific flow cytometry assays. As with the other options, these must be validated by a laboratory as part of an LDT before being used in a clinical flow cytometry assay.

VALIDATION

There are two assumptions that underscore laboratory prepared antibody cocktails in this module. One assumption is that each individual reagent that is to be used in the combination has been assessed and performs consistently with the current "in use" antibody. The other assumption is that the panel has been carefully designed and optimized to ensure proper identification and characterization of all individual antigen expression patterns and combinations of expression to accurately assess healthy, or "normal", patterns versus a variety of targeted disease states.

Once the antibodies are combined in a single vial as a cocktail, the laboratory must determine the presence of all the antigens within the vial that is consistent with individual staining (cocktail validation), establish cocktail stability, and thereafter verify the performance of this new cocktail (cocktail quality control).

One approach to validation is to stain a known sample with the new combination to be tested. The sample selected should contain relevant positive and negative populations for each of the markers within the cocktailed reagents. The selection of the sample is therefore critical in this validation and careful selection must be made if a single specimen is to be used. Occasionally two specimens might have to be used if a single specimen cannot be found that expresses all of the antigenic markers. For example, with a cocktail with CD34 in the panel, normal whole blood would be inappropriate as normal blood contains very few CD34+ cells. For rare markers that are only expressed in specific diseases, it might be necessary to wait until a specimen from a patient with this known disease comes to the laboratory. Alternatively, there are some commercial or quality assurance products, such as the CAP rare antigen survey or vendor-made quality control material that would be appropriate for validation of a cocktail.

Stability

Stability of a cocktail will vary based on individual laboratory protocols and settings as well as the antibody conjugates used within the cocktail. The aliquot size and rate of usage should be taken into consideration as well. Every time a vial is taken out of the refrigerator for use it is exposed to temperature fluctuations, ambient light and evaporation, so in general, smaller aliquots will remain stable longer than larger ones, depending on the rate of usage. Amber vials are recommended to protect the fluorochromes form photo-bleaching and breakdown.

The open vial stability of the least stable antibody conjugate will determine the maximum shelf life of the cocktail. Note that the stability of a laboratory made cocktail is usually significantly shorter than the individual vial stability. If any of the components of the cocktail expire (either shelf life or open vial

stability) before the determined stability of the cocktail, then this reagent expiration should be used as the limit of stability for this cocktail.

Great care must be taken when determining the stability of a laboratory made cocktail. The breakdown of antibodies with tandem dye conjugates into their non-energy-coupled components might show up as false positive signal. For example, a cocktail that contains CD10 APC-Alexa750 and CD34 APC, could exhibit breakdown of the CD10 fluorophore and falsely appear to express CD34 in the APC channel. This type of misleading fluorescent expression could lead to a misdiagnosis.

Documentation

Regulations and best practices dictate that detailed records be kept for tracking the reagents, preparation and quality control of antibody cocktails, whether they are made in the laboratory or are vendor made (5). For laboratory made cocktails, a record of each antibody lot number, received date, in use date and manufacturer expiration date must be documented. The laboratory should keep the initial antibody cocktail optimization, validation and stability study reports indefinitely, along with the approval of the Medical Director. Documentation specific for each prepared cocktail must include a list of each antibody conjugate used, lot number and expiration date, the preparation date, expiration date, as determined by stability studies, quality control data.

Quality control of a new combination of antibody cocktail is considered a lot-to-lot comparison to determine if a new cocktail is acceptable. Variations between lots should be minimal if the individual components have not changed. New lots of antibodies should not exhibit variation beyond the established acceptable ranges. For instance, the expression of a new lot of antibody should not exhibit an MFI that is greater than 0.5 log difference, stained with the current and new lots. Lot-to-lot variation should be monitored over time to ensure that increasing or decreasing trends in reagent performance are not noted. Levey-Jennings plots are a useful tool for visually tracking multiple lot-to-lot variations over time. Without monitoring by Levey-Jennings, it is possible that very small incremental changed over time will not be detected, possibly resulting in a large change in performance which could affect the ability to identify normal from neoplastic populations.

If large amounts of antibody cocktail are made and aliquoted into separate containers for use, only a single validation needs to be performed, as long as the entire amount will be used within the established stability range. This assumes that daily process and instrument quality controls are within established ranges. While documentation will vary depending on the regulatory agency, under which laboratory licensing is regulated, best practice would dictate the traceability for each of the components for every cocktail.

PROTOCOLS

A - Cocktail Validation Procedure

Materials

1. Amber glass bottles or vials with leak-proof caps (several vendors sell different sizes of amber glass bottles or vials. Alternatively, you can reuse empty amber antibody vials after thoroughly washing and drying them.)
2. Antibodies
3. Calibrated pipettes

Preparation

1. Determine how many tests you want to prepare for each cocktail. Amounts of antibody cocktails should not exceed the workflow volume within the defined expiration date.

   Example: B-cell cocktail for an 8-color assay

B-cell Tube	Volume	
	1 test (µL)	80 tests (µL)
Kappa FITC/ Lambda PE	5	400
CD5 PerCP Cy5.5	10	800
CD10 PE-Cy7	2.5	200
CD34 APC	2.5	200
CD19 APC-H7	2.5	200
CD20 V450	2.5	200
CD45 V500	2.5	200
Total Volume (µL)	**27.5**	**2200**

There is another option to simplify pipetting cocktails. It is acceptable to add buffer, such as PBS, to the cocktail to make it a more easily pipetted total volume. For instance, you could add 12.5 µL of buffer to bring the cocktail volume per test to 40 µL. This is especially useful if you have multiple tubes which contain different volumes per test. It would make pipetting faster and simpler to bring the volume of all cocktails to the same amount.

Depending on how you validate the stability of your antibody cocktails, you might include or exclude antibodies conjugated with certain tandem fluorochromes to prevent breakdown. In general, tandem dyes break down more easily within cocktails and can thereby shorten the shelf-life of the cocktail. Excluded antibodies can be added as a "drop in" at the time of staining. This will be determined during optimization and stability studies.

2. Label an amber glass bottle/vial with cocktail name, date of preparation, expiration date, and storage temperature (refrigerated 2-8° C).

3. Pipette the required volume of each antibody into the labeled amber glass bottle/vial.

4. Cap and mix the cocktail gently.

5. Perform lot-to-lot quality control of the new cocktail.

B - Quality Control

1. Lot-to-lot quality control must be performed before the newly made cocktail is put into use.

2. Stain the new cocktail in parallel with the previous cocktail (or fresh, individually pipetted antibodies) on the same sample.

3. Run both tubes in the same manner and compare results.

4. CAP requires lot to lot validation to have objective acceptance criteria. Examples of the criteria include but are not limited to:

   - Positive and negative expression of each antibody for a particular leukocyte population.
   - Percentage of positive population: within 10-15% difference, or less than 20% CV.
   - Median Fluorescence Intensity (MFI): within 0.5-1 log difference or the same log decade (negative, 1st, 2nd, etc.).
   - Signal to noise ratio: within 10-15% difference.

   Visual assessment of the antigenic expression patterns should not be overlooked. While visual assessment does not include numerical values for objective criteria, it provides valuable information on how a population is displayed in relation to others. It is also valuable to note a consistent pattern with the expected visual expression of normal and diseased patients for each laboratory's assays and patient population.
   Both objective measures and visual assessment should be used to define cocktail lot-to-lot acceptance criteria.

5. If the QC fails, repeat with another sample. If the QC still fails, consult with an experienced tech or supervisor for review and advice. Usually, reasons for failed QC are wrong antibodies,

contaminated antibodies or incorrect volumes. Do not use the new cocktail until the QC passes. If the issue is not able to be resolved, discard the cocktail and prepare a new one.

6. Review and approve preparation and quality control documentation.

C - Troubleshooting

- Incorrect/missing reagent. Use a table or spreadsheet to ensure that all antibodies in the panel are included.

- Cocktail stability. Prepare the appropriate amount of cocktail based on the number of tests being made so all the cocktail is used before the stability expires.

- Dim signal from one or more antigens. Check the volumes for each component prior to starting creating a cocktail.

- Validation failure. Incorrect specimen selection for validation, including lack of expression of an antibody or too few events to determine the level of staining.

- Unknown / undetermined failure or visible change in performance. Document each step in the process. It is critical in complicated assays to document traceability thoroughly.

CONCLUSION / SUMMARY

Clinical flow cytometry is a complex technology that is highly susceptible to a lack of standardization. As the technology rapidly evolves, the onus to keep up with the changes, carefully optimize/validate every aspect of each assay, perform and carefully review regular quality controls rests with each individual laboratory. Antibody cocktails provide a useful tool in the clinical flow lab to increase efficiency, decrease errors, and decrease assay variance.

New automated sample preparation systems ease the workload of technologists while potentially enhancing the quality of testing. However, these options might not be economical options for small facilities or others with financial constraints. For those without access to automated preparation devices, the deployment of the protocols detailed in this module are highly recommended.

Antibody cocktails performed with standardization and appropriate quality control will increase the accuracy of the results obtained and improve the overall performance in the flow cytometry facility.

REFERENCES

1. ICCS/ESCCA Consensus Guidelines to detect GPI-deficient cells in Paroxysmal Nocturnal Hemoglobinuria (PNH) and related Disorders Part 4 – Assay Validation and Quality Assurance, Teri Oldaker, Liam Whitby, Maryam Saber, Jeannine Holden, Paul K Wallace, and Virginia Litwin. Cytometry Part B (Clinical Cytometry) 94B:67–81 (2018).

2. Validation and quality control of immunophenotyping in clinical flow cytometry, Marilyn A. Owens , Horacio G. Vall , Anne A. Hurley , Susan B. Wormsley. Journal of Immunological Methods 243 (2000) 33–50

3. Tandem Dyes: Stability in Cocktails and Compensation Considerations, Johansson U, Macey M. Cytometry Part B 2014; 86B: 164–174.

4. Flow Cytometry and the Stability of Phycoerythrin-Tandem Dye Conjugates, Ruud Hulspas, David Dombkowski, Frederic Preffer, Derick Douglas, Brian Kildew-Shah, John Gilbert. Cytometry Part A 75A: 966-972, 2009.

5. Practice guidelines from the ICSH and ICCS – part I through V – analytical issues; on behalf of ICSH/ICCS Working Group First published: 10 September 2013 Full publication history DOI: 10.1002/cyto.b.21106, Oldaker T, Whitby L, Saber M, Holden J, Wallace PK and Litwin V. ICCS/ESCCA Consensus Guidelines to detect GPI-deficient cells in Paroxysmal Nocturnal Hemoglobinuria (PNH) and related Disorders Part 4 – Assay Validation and Quality Assurance. Cytometry Part B 2018; 94B: 67–81.

6. ICCS Quality & Standards, Module 7 Quality of Reagents – Monoclonal Antibodies Written by: Ruud Hulspas, Mike Keeney, Ben Hedley and Andrea Illingworth

7. Stabilization of Pre-Optimized Multi-color Antibody Cocktails for Flow Cytometry Applications, Chan RCF, Kotner JS, Chuang CMH and Gaur A. Cytometry Part B 2017; 92B: 508–524.

8. PS-10 Sample Preparation System; 2019 Sysmex America, Inc., Programs and specification subject to change without notice. MKT-10-1274, Rev. 2, 9.2019

Reviewed and approved by: Mike Keeney, Teri Oldaker, D. Robert Sutherland

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