COLLEGE of AMERICAN PATHOLOGISTS

Master

Flow Cytometry Checklist

CAP Accreditation Program

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Flow Cytometry Checklist

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ON-LINE CHECKLIST DOWNLOAD OPTIONS

Participants of the CAP accreditation programs may download the checklists by logging into cap.org and going to e-LAB Solutions Suite - Accreditation Checklists. They are available in different checklist types and formatting options, including:

- Master — contains ALL of the requirements and instructions available in PDF, Word/XML or Excel formats
- Custom — customized based on the laboratory's activity (test) menu; available in PDF, Word/XML or Excel formats
- Changes Only — contains only those requirements with significant changes since the previous checklist edition in a track changes format to show the differences; in PDF version only. Requirements that have been moved or merged appear in a table at the end of the file.

CHECKLIST ACCREDITATION RESOURCES

CAP accredited laboratories have access to additional checklist accreditation tools and resources found on the CAP website (cap.org) by logging into e-LAB Solutions Suite - Accreditation Resources. Content found in Accreditation Resources includes:

- A library of past Focus on Compliance webinars and laboratory inspection preparation videos
- Answers to the most common checklist questions
- Customizable templates and forms (eg, competency assessment, personnel, validation/verification, quality management)
- Proficiency testing (PT) frequently asked questions, forms, and troubleshooting guides
- IQCP eligibility, frequently asked questions, forms, templates, and examples
- Laboratory director education and resources
- Quality management resources
- Inspector training and inspection tip sheets
- Self and post inspection toolbox

SUMMARY OF CHECKLIST EDITION CHANGES
Flow Cytometry Checklist
12/26/2024 Edition

The information below includes a listing of checklist requirements with significant changes in the current edition and previous edition of this checklist. The list is separated into three categories:

1. New
2. Revised:
   - Modifications that may require a change in policy, procedure, or process for continued compliance; or
   - A change to the Phase
3. Deleted/Moved/Merged**:**
   - Deleted
   - Moved — Relocation of a requirement into a different checklist (requirements that have been resequenced within the same checklist are not listed)
   - Merged — The combining of similar requirements

NOTE: The requirements listed below are from the Master version of the checklist. The customized checklist version created for inspections and self-evaluations may not list all of these requirements.

Previously Cited Checklist Requirements

- The **inspector's version** of the checklist contains a listing of previously cited checklist requirements. Specific information on those citations, including the inspection date and inspector comments, is included following each related requirement within the checklist.
- Laboratories can access data on previously cited deficiencies by logging into e-LAB Solutions Suite on cap.org and going to Accreditation Reports - Inspection Summation Report.

NEW Checklist Requirements

Requirement	Effective Date
FLO.23335	12/26/2024
FLO.30595	12/26/2024

REVISED Checklist Requirements

Requirement	Effective Date
FLO.23325	12/26/2024
FLO.23737	12/26/2024
FLO.30260	12/26/2024
FLO.30275	12/26/2024
FLO.30610	12/26/2024

DELETED/MOVED/MERGED Checklist Requirements

Requirement	Effective Date
FLO.30550	08/23/2023

INTRODUCTION

This checklist is used in conjunction with the All Common and Laboratory General Checklists to inspect a flow cytometry laboratory section or department.

Flow cytometry inspectors must be pathologists, clinical scientists or medical technologists who are actively involved with or have extensive recent experience in the practice of flow cytometry, are knowledgeable about current CAP Checklist and CLIA requirements, and have completed CAP Inspector Training. Inspectors should, to the greatest extent possible, be peers of the laboratory being inspected.

***For laboratories performing only the interpretation component of flow immunophenotyping data** (the flow technical component is performed at an outside flow laboratory), the following Flow Cytometry Checklist requirements apply: FLO.18385, FLO.23706, FLO.30610, FLO.30640, FLO.30730, FLO.30790, and FLO.30820. Additionally, requirements located in the All Common Checklist addressing proficiency testing, quality management, procedure manual, specimen rejection, and results reporting are applicable.*

Policy/Procedure icon - The placement of this icon next to a checklist requirement indicates that a written policy or procedure is required to demonstrate compliance with the requirement. The icon is not intended to imply that a separate policy or procedure is required to address individual requirements. A single policy or procedure may cover multiple checklist requirements.

***Laboratories not subject to US regulations:** Checklist requirements apply to all laboratories unless a specific disclaimer of exclusion is stated in the checklist. When the phrase "FDA-cleared/approved test (or assay)" is used within the checklist, it also applies to tests approved by an internationally recognized regulatory authority (eg, CE-marking).*

PROFICIENCY TESTING

Inspector Instructions:

READ

- Sampling of peer education records

FLO.18385 Peer Education Program **Phase I**

For laboratories that perform only interpretations of flow immunophenotyping data, the laboratory participates in a peer education program in interpretive flow cytometry.

NOTE: This checklist item applies to laboratories that do not perform staining and acquisition of flow cytometry data, but which receive list mode files and/or representative dot plots from an outside laboratory for interpretation.

Programs dealing with analysis of flow data from hematolymphoid neoplasias and related benign conditions provide valuable educational opportunities for peer-performance comparisons. While not completely emulating the clinical setting involved in flow immunophenotyping, the peer

data developed by these programs can provide a useful benchmark against which laboratory performance can be evaluated.

Evidence of Compliance:

✓ Records of enrollment/participation in an educational peer-comparison program for interpretive flow cytometry **OR** records for participation in a laboratory-developed program circulating cases with other laboratories or within the laboratory's own practice with records of peer review

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*. CLSI document H43-A2. Clinical and Laboratory Standards Institute, Wayne, PA; 2007.

QUALITY MANAGEMENT

REAGENTS

Inspector Instructions:

	• Sampling of new antibody validation records • Sampling of new lot/shipment antibody and detection system reagent confirmation records
	• What procedure does your laboratory follow to ensure manufacturer's recommendations are followed regarding the use of kit reagents/controls? • How do you confirm the acceptability of new reagent lots? • How do you evaluate the performance of newly prepared antibody cocktails?

Additional requirements are in the REAGENTS section of the All Common Checklist. Reporting requirements for use of analyte-specific reagents and other reagents used in laboratory-developed tests are included in the All Common Checklist (COM.40850).

FLO.23250 Reagent Usage **Phase II**

The laboratory follows manufacturer's instructions for the proper use of reagents and controls or provides validation records if alternative procedures are used.

Evidence of Compliance:

✓ Records of method validation if alternative procedures are used

REFERENCES

1) Caldwell CW. Analyte-specific reagents in the flow cytometry laboratory. *Arch Pathol Lab Med.* 1998;122:861-864

FLO.23275 Antibody Validation **Phase II**

The laboratory has records of initial validation of new antibodies prior to use in patient diagnosis.

NOTE: Antibodies used are validated on the cell sub-population of interest in the context of the antibody combination used in an assay.

***REVISED** 12/26/2024*

FLO.23325 New Reagent Lot/Shipment Confirmation of Acceptability **Phase II**

The laboratory evaluates performance of new lots/shipments of antibodies and reagents before or concurrently with being placed into service.

NOTE: Evaluation of new lots or shipments of antibodies and reagents for positive and negative reactivity is required to ensure consistent performance. Methods for evaluation include (but are not limited to):

- *Parallel testing of old versus new lots on patient samples or control materials*
- *Testing of new lot/shipment on control material with defined criteria for acceptance*
- *Testing of the new lot/shipment on normal patient material with defined criteria for acceptance.*

Only when there is agreement between the individual old and new lots may the new lot be placed into routine use. Individual antibodies must be evaluated as a single reagent (see FLO.23335 for antibody cocktails). All reagents that interact with or alter patient samples (such as lysing agents and permeabilization reagents) must be evaluated. Inert reagents (such as sheath fluid and cleaning fluids) are exempt from this requirement.

Evidence of Compliance:

✓ Records of confirmation of new antibody and reagent lots

REFERENCES

1) Department of Health and Human Services, Centers for Medicare and Medicaid Services. Clinical laboratory improvement amendments of 1988, final rule. *Fed Register.* 2003(Jan 24): [42CFR493.1256(e)(1)].

***NEW** 12/26/2024*

FLO.23335 New Antibody Cocktail Confirmation of Acceptability **Phase II**

The laboratory evaluates performance of newly prepared antibody cocktails before or concurrently with being placed into service and assigns an expiration date for the cocktail.

NOTE: Laboratory-prepared or commercially-prepared antibody cocktails require validation to ensure proper composition and consistent performance before or concurrently with being placed into service. The laboratory must establish a stability period for laboratory-prepared cocktails during the validation of the assay for which it is used and assign an expiration date. The stability period of the prepared cocktail may be shorter than the stability of the individual components due to interaction between the components.

Because individual antibody lots/shipments require confirmation of acceptability (per FLO.23325), parallel testing of the old cocktail versus the new cocktail may be performed but is not required. Procedures must include defined criteria to accept new cocktails with both positive and negative controls for each component of the cocktail (with the exception of rare flow antigens such as CD1a, CD30, and/or CD103). Normal cells in patient samples or other standardized control material can be used for confirmation.

If antibodies or stains are not used in a cocktail, they must be evaluated individually (per FLO.23325).

Evidence of Compliance:

✓ Records of confirmation of new antibody cocktails

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Validation of Assays Performed by Flow Cytometry - Approved Guideline-First Edition.* CLSI Document H62. Clinical and Laboratory Standards Institute, Wayne, PA, 2021.
2) Tangri S, Vall H, Kaplan D, et al. Validation of cell-based fluorescence assays: practice guidelines from the ICSH and ICCS - part III - analytical issues. *Cytometry B Clin Cytom*. 2013;84(5):291-308.
3) Wood B, Jevremovic D, Bene MC, et al. Validation of cell-based fluorescence assays: practice guidelines from the ICSH and ICCS - part V - assay performance criteria. *Cytometry B Clin Cytom.* 2013;84(5):315-323.

RECORDS

Inspector Instructions:

READ	• Record retention policy (gated dot plots/histograms)

FLO.23706 Record Retention - Flow Cytometry **Phase II**

Flow cytometry data for evaluation of hematolymphoid neoplasias, PNH, and congenital immunodeficiency evaluations are retained for at least 10 years. Routine lymphocyte subset and CD34+ enumeration data are retained for at least two years.

NOTE: Stored data must include raw listmode data and final interpretation. Storage of gated data is encouraged but not required.

If the laboratory responsible for the interpretation component (interpretation only flow cytometry) does not retain the data locally, it must ensure that the data are being retained for the full retention period, such as with an agreement with the laboratory performing the flow cytometry technical component (see ANP.29670).

Evidence of Compliance:

✓ Data files with or without gated dot plots and histograms **OR**
✓ Written agreement with laboratory performing technical component for data storage

REFERENCES

1) CAP Policy PP, Retention of Laboratory Records and Materials

CONTROLS AND STANDARDS

Inspector Instructions:

READ	• Sampling of QC policies and procedures (includes acceptable control type/frequency for each flow cytometric application) • Sampling of QC records
ASK	• How do you determine when quality control is unacceptable and when corrective actions are needed? • How does your laboratory establish or verify acceptable QC ranges?
DISCOVER	• Select several occurrences in which QC is out of range and follow records to determine if the steps taken follow the laboratory procedure for corrective action

***REVISED** 12/26/2024*

FLO.23737 QC - Flow Cytometry Reagents/Stains - Qualitative Assays Phase II

The laboratory evaluates negative and positive staining patterns of the residual normal cell population for qualitative assays (eg, leukemia/lymphoma analysis) each day of patient testing.

NOTE: An evaluation of the performance of each qualitative assay must be performed on each day of patient testing. If a component/tube of an assay or panel is not run on a particular day, the evaluation does not need to be performed on that component.

The assay procedure must include the control material to be used, criteria for acceptability of the assay, and remedial action to be taken when acceptability criteria are not met. A record of the evaluation and any remedial action must be made each day of patient testing.

Most labs will use normal populations present in patient samples as an appropriate control material; however, other control materials may be used at the discretion of the laboratory director. Other examples include commercial control materials, cryopreserved cells, or normal patient/volunteer samples. The evaluation can be run concurrently with active patient samples as long as the evaluation takes place prior to release of patient results. Multiple samples may be required to evaluate all components/tubes of a panel depending on the evaluation criteria. For example, if an add-on tube is run later in the day, the patient sample on which the add-on tube is run can be used to evaluate the performance of that individual tube prior to the release of patient results.

The criteria of acceptability and form of record are at the discretion of the laboratory director, but the criteria must include a positive and negative control for each antibody/stain used in the assay. Antibodies/stains do not need to be assessed singly if they are used in a cocktail; however, each component/tube must be evaluated. If antibodies are repeated in multiple tubes, they must be evaluated in each tube. Examples of acceptability criteria:

- *CD2: normal T-cells positive, normal B-cells negative*
- *CD19: normal T-cells negative, normal B-cells positive*
- *CD45 tube 1: positive in mature lymphocytes, negative in erythroid precursors*

When positive cellular control material is not readily available (eg, rare flow antigens such as CD1a, CD30, CD103), there must be a written procedure for an alternative mechanism to evaluate for positive staining that is performed at least every six months. The laboratory procedure must define the rare flow antigens and method of evaluation.

Evidence of Compliance:

✓ Records of QC results and any corrective actions taken

REFERENCES

1) Wood BL, Arroz M, Barnett D, et al. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematolymphoid neoplasia by flow cytometry: optimal reagents and reporting for the flow cytometric diagnosis of hematopoietic neoplasia. *Cytometry B Clin Cytom.* 2007;72 Suppl 1:S14-S22.
2) Clinical and Laboratory Standards Institute (CLSI). *Validation of Assays Performed by Flow Cytometry - Approved Guideline-First Edition.* CLSI Document H62. Clinical and Laboratory Standards Institute, Wayne, PA, 2021.
3) Wood B, Jevremovic D, Bene MC, et al. Validation of cell-based fluorescence assays: practice guidelines from the ICSH and ICCS - part V - assay performance criteria. *Cytometry B Clin Cytom.* 2013;84(5):315-323.

FLO.23800 QC - Flow Cytometry Reagents/Stains - Quantitative Assays Phase II

The laboratory analyzes at least two levels of positive cellular controls for quantitative assays each day of patient testing or after an instrument restart to verify the performance of reagents, preparation methods, staining procedures, and the instrument.

NOTE: Quantitative assays have an absolute numeric reference range and include lymphocyte subset enumeration and CD34+ cell enumeration. One of the levels of these controls should be at (or near) clinical decision levels. Examples include a low CD4+ lymph count of 200 cell/uL in a HIV+ individual, or a 5 – 20 CD34+ stem cells/uL concentration in the peripheral blood of an

individual being readied for peripheral stem cell pheresis. Control testing is not necessary on days when patient testing is not performed.

There must be written guidelines defining objective criteria for acceptable performance of control material and records of the evaluation of the actual performance.

If the laboratory performs quantitative test procedures for which control materials are not commercially available, there are written procedures for an alternative mechanism to detect immediate errors and monitor test system performance over time. The performance of alternative control procedures must be recorded. "Performance" includes elements of accuracy, precision, and clinical discriminating power. Examples of alternative procedures may include split sample testing with another method or with another laboratory, the testing of previously tested patient specimens in duplicate, testing of patient specimens in duplicate, or other defined processes approved by the laboratory director.

Evidence of Compliance:

✓ Records of QC results

REFERENCES

1) Department of Health and Human Services, Centers for Medicare and Medicaid Services. Clinical laboratory improvement amendments of 1988; final rule. *Fed Register*. 1992(Feb 28):7146 [42CFR493.1256]

FLO.23925 Control Range Establishment or Verification — Phase II

The laboratory establishes or verifies an acceptable control range for each lot of control material.

NOTE: For unassayed control materials, the laboratory must establish an acceptable control range by repetitive analysis in runs that include previously tested control material. For assayed control materials, the laboratory must verify control ranges supplied by the manufacturer.

Control values supplied by the manufacturer may be used without verification for qualitative (eg, positive or negative) testing.

Evidence of Compliance:

✓ Records for control range establishment or verification of each lot

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Evaluation of Precision of Quantitative Measurement Procedures. Approved Guideline.* 3rd ed. CLSI document EP05-A3. Clinical and Laboratory Standards Institute, Wayne, PA; 2014.
2) Clinical and Laboratory Standards Institute. *Statistical Quality Control for Quantitative Measurement Procedures, Principles and Definitions.* 4th ed. CLSI guideline C24. Clinical and Laboratory Standards Institute, Wayne, PA, 2016.

FLO.24230 QC Corrective Action — Phase II

The laboratory performs and records corrective action when control results exceed defined acceptability limits.

NOTE: The actions taken must be consistent with the laboratory's quality control program (GEN.30000). Patient test results obtained in an analytically unacceptable test run or since the last acceptable test run must be re-evaluated to determine if there is a significant clinical difference in patient/client results. Re-evaluation may or may not include re-testing patient samples, depending on the circumstances.

Even if patient samples are no longer available, test results can be re-evaluated to search for evidence of an out-of-control condition that might have affected patient results. For example, evaluation could include comparison of patient means for the run in question to historical patient means, and/or review of selected patient results against previous results to see if there are consistent biases (all results higher or lower currently than previously) for the test(s) in question.

Evidence of Compliance:

✓ Records of corrective action for unacceptable control results

REFERENCES

1) Department of Health and Human Services, Centers for Medicare and Medicaid Services. Clinical laboratory improvement amendments of 1988; final rule. *Fed Register*. 2003(Oct 1):1046 [42CFR493.1282(b)(2)]

FLO.24250 QC Handling **Phase II**

The laboratory tests control specimens in the same manner and by the same personnel as patient samples.

NOTE: Personnel who routinely perform patient testing must analyze QC specimens; however, this does not imply that each operator must perform QC daily. Personnel must participate in QC on a regular basis. To the extent possible, all steps of the testing procedure must be controlled.

Evidence of Compliance:

✓ Records reflecting that QC is run by the same personnel performing patient testing

REFERENCES

1) Department of Health and Human Services, Centers for Medicare and Medicaid Services. Clinical laboratory improvement amendments of 1988; final rule. *Fed Register*. 2003(Jan 24):7166 [42CFR493.1256(d)(8)]

FLO.24300 QC Confirmation of Acceptability **Phase II**

Personnel review control results for acceptability before reporting patient/client results.

Evidence of Compliance:

✓ Records of control result approval

REFERENCES

1) Department of Health and Human Services, Centers for Medicare and Medicaid Services. Clinical laboratory improvement amendments of 1988; final rule. *Fed Register*. 2003(Jan 24):7166 [42CFR493.1256(f)]

FLO.24475 Monthly QC Review **Phase II**

The laboratory director or designee reviews and assesses quality control data at least monthly.

NOTE: The reviewer must record follow-up for outliers, trends, or omissions that were not previously addressed.

The QC data for tests performed less frequently than once per month may be reviewed when the tests are performed.

Evidence of Compliance:

✓ Records of QC review **AND**

✓ Records of corrective action taken when acceptability criteria are not met

INSTRUMENTS AND EQUIPMENT

FLOW CYTOMETERS

The checklist requirements in this section should be used in conjunction with the requirements in the All Common Checklist relating to instruments and equipment.

Inspector Instructions:

READ	• Sampling of optical alignment/laser output checks • Sampling of procedures for optical alignment, calibration, color compensation, and laser checks • Sampling of calibration records with fluorochrome standards
ASK	• How does your laboratory monitor instrument reproducibility? • How does your laboratory ensure each fluorochrome is appropriately calibrated? • How does your laboratory determine appropriate color compensation settings? • How is carryover mitigated for rare event assays and paucicellular specimens?

FLO.25150 Optical Alignment — Phase II

The laboratory monitors optical alignment (where applicable) and instrument reproducibility at least daily, or after each time the flow cytometer is restarted.

NOTE: Verifying reproducibility of instrument performance is an essential element of quality assurance within the laboratory. Instrument performance must be monitored under the same conditions used to run test samples.

Evidence of Compliance:

✓ Records for monitoring optical alignment (where applicable) and instrument reproducibility

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*. CLSI document H43-A2. Clinical and Laboratory Standards Institute, Wayne, PA; 2007.

FLO.30250 Fluorochrome Standards — Phase II

Appropriate standards for each fluorochrome (*eg,* fluorescent beads) are run each day that the instrument is used as part of the quality control to verify instrument performance, and the results are recorded and reviewed.

NOTE: These steps are necessary to optimize the flow system and the optics of the instrument.

Evidence of Compliance:

✓ Records of results, with corrective action when quality control beads fail

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition*. CLSI document H42-A2 (ISBN 1-56238-640-9). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2007

FLO.30255 Voltage Settings — Phase II

The laboratory optimizes the voltages for each specific assay.

NOTE: Voltage settings for each PMT must be optimized in order to maximize the resolution (signal-to-noise ratio) and place the antigen-negative and antigen-positive populations visibly "on -scale" for analysis. This is particularly important for dimly expressed antigens as well as visualization of antigen-negative populations. Appropriate voltage settings must be determined during validation of the specific assay; however, timing and frequency of monitoring on-going performance is at the discretion of the laboratory director.

Evidence of Compliance:

✓ Records demonstrating voltage optimization

REFERENCES

1) Illingworth A. ICCS Quality and Standards Committee. Instrument optimization - Adjusting PMT voltages and compensation on a Beckman Coulter System [module]. March 13, 2017.
2) Griffin ML, Batchelder J, Hoffman B, et al; ICCS Quality and Standards Committee. Instrument Optimization for BD FACSCanto Instruments - Creating Application Settings for White Blood Cells using Lyse/Wash, or Lyse/No Wash methods [module]. May 3, 2017.
3) Perfecto SP, Ambrozak D, Nguyen R, Chattopadhyay P, Roederer M. Quality Assurance for polychromatic flow cytometry. *Nat Protoc.* 2006;1(3):1522-30.
4) Maecker HT and Trotter J. Flow Cytometry Controls, Instrument Setup and the Determination of Positivity. *Cytometry Part A.* 2006; 69(9):1037-42.

***REVISED** 12/26/2024*

FLO.30260 Color Compensation Settings **Phase II**

The laboratory determines and monitors appropriate color compensation settings.

NOTE: For multi-color analysis there must be a procedure to ensure that cells co-labeled with more than one fluorescent reagent can be accurately distinguished from cells labeled only with one reagent. Cells stained with mutually exclusive antibodies bearing the relevant fluorochromes or singly-stained cell samples for each fluorochrome may be used to establish appropriate compensation settings. Alternatively, beads labeled with the appropriate fluorochromes may be used; these form the basis of some automated instrument setup. When beads are used, it is important that an unstained "negative" bead that approximates the autofluorescence of unstained cells is included.

Compensation settings must be determined during validation of each assay and may vary with different antibody combinations. Compensation settings must be reviewed periodically to ensure selected settings remain appropriate. Laboratories must have a documented process for checking compensation at defined intervals (such as after routine preventative maintenance or repair to the flow cytometer as these may alter the compensation). The procedure must include appropriate acceptance criteria and actions to be taken when those criteria are not met. The time interval for review of the compensation setting is at the discretion of the laboratory director.

Evidence of Compliance:

✓ Records of periodic review of color compensation settings and corrective action

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*. CLSI document H43-A2. Clinical and Laboratory Standards Institute, Wayne, PA; 2007.
2) Byrd T, Carr KD, Norman JC, Huye L, Hegde M, Ahmed N. Polystyrene microspheres enable 10-color compensation for immunophenotyping of primary human leukocytes. *Cytometry A.* 2015 Nov;87(11):1038-46. doi: 10.1002/cyto.a.22717. Epub 2015 Jul 22.

FLO.30270 Laser Performance **Phase I**

The laboratory ensures acceptable and constant laser instrument performance.

NOTE: For some instruments, current is a better gauge of laser performance than is power output, which may be relatively constant.

Evidence of Compliance:

✓ Records of laser performance checks

***REVISED** 12/26/2024*

FLO.30275 Carryover Mitigation **Phase II**

The laboratory has a process to evaluate and mitigate against carryover when applicable, including rare event assays (MRD, PNH) and paucicellular specimens.

NOTE: The laboratory must perform formal assessment of carryover during validation for assays or specimens that are particularly susceptible to it, and reassess carryover after major maintenance or repair of the instrument. Rare event assays (including MRD and PNH testing) and paucicellular specimens (such as CSF) may be affected significantly by sample carryover

introduced during sample processing or during data acquisition. The definition of a paucicellular sample may vary with sample type and composition, and the procedure must include such a definition per the discretion of the laboratory director (for example, a total cell count of 200 or less for a CSF sample).

The assay procedure must define limits for carryover during assessment and include methods for reducing possible carryover, if detected. Appropriate corrective action must be taken when defined carryover limits are exceeded. Methods for reducing this possibility include (but are not limited to):

- *Cleaning the instrument prior to running an assay*
- *Washing or flushing the sample probe between sample tubes*
- *Use of a blank tube (eg, tube containing cell-free liquid (PBS, dH20, diluent) or an unstained control tube prior to running a specimen).*

Evidence of Compliance:

✓ Records of carryover studies performed at validation and following major maintenance/repair

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Validation of Assays Performed by Flow Cytometry - Approved Guideline-First Edition.* CLSI Document H62. Clinical and Laboratory Standards Institute, Wayne, PA, 2021.
2) Oldaker TA, Wallace PK, Barnett D. Flow cytometry quality requirements for monitoring of minimal disease in plasma cell myeloma. *Cytometry B Clin Cytom*. 2016;90(1):40-46.

PROCEDURES AND TEST SYSTEMS

NOTE: Reticulocyte quantification by flow cytometry is separately covered in the Hematology and Coagulation Checklist.

IMMUNOPHENOTYPING

Inspector Instructions:

	• Select a representative assay and follow the entire process from specimen receipt to final result reporting • If problems are identified during the review of immunophenotyping procedures, further evaluate the laboratory's responses, corrective actions and resolutions.

BLOOD LYMPHOCYTE SUBSET ENUMERATION

Inspector Instructions:

	• Sampling of lymphocyte subset analysis policies and procedures (includes procedure describing method to set markers (cursors) to distinguish between negative and positive fluorescence cell populations)
	• How does your laboratory ensure specimen integrity? • How are specimens stored after initial processing? • How does your laboratory validate lymphocyte gates? • How are results of lymphocyte subset analysis corrected for gate purity?

FLO.30430 Specimen Integrity **Phase II**

The laboratory has a defined process to evaluate specimen integrity.

NOTE: The yield of lymphocytes from blood samples is affected by a number of factors. If specimens are not processed immediately after collection, the laboratory should verify that its anticoagulant, holding temperature and preparation method maintain specimen integrity. Selective loss of cell subpopulations and/or the presence of dead cells may lead to spurious results. Routine viability testing is not necessary on specimens of whole blood that are analyzed within 24 hours of drawing. Analyses on older samples are possible if the laboratory has verified the absence of statistical differences between the fresh and aged specimen phenotype fractions being evaluated.

Evidence of Compliance:

✓ Records of specimen evaluation (eg, viability results) as applicable

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition*. CLSI document H42-A2 (ISBN 1-56238-640-9). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2007

FLO.30450 Specimen Storage **Phase II**

Specimens are stored appropriately after initial processing.

NOTE: As one example, paraformaldehyde (0.5%) fixation of stained cells preserves cellular integrity and fluorescence for up to five days. Caution must be exercised in utilizing this procedure, as fluorescence may be diminished with some reagents and cytometers.

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*. CLSI document H43-A2. Clinical and Laboratory Standards Institute, Wayne, PA; 2007.

FLO.30460 Gating Techniques **Phase II**

Appropriate gating techniques are used to select the cell population for analysis.

NOTE: This may involve a combination of light scatter and/or fluorescence measurements. This is particularly important if the cell samples have a low lymphocyte count and/or a relatively high monocyte-granulocyte count. Lymphocyte gates may be verified using linear forward angle light scatter and 90-degree side scatter, and/or by using monoclonal antibodies to markers, such as CD45 and CD14.

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition*. CLSI document H42-A2 (ISBN 1-56238-640-9). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2007.
2) Clinical and Laboratory Standards Institute (CLSI). *Validation of Assays Performed by Flow Cytometry - Approved Guideline-First Edition.* CLSI Document H62. Clinical and Laboratory Standards Institute, Wayne, PA, 2021.
3) World Health Organization. WHO Protocol for Performance Laboratory Evaluation of Lymphocyte Subsets Enumeration Technologies.2017.

FLO.30470 Gate Purity **Phase II**

Results of lymphocyte subset analysis are corrected for gate purity as appropriate.

NOTE: When >5% non-lymphocyte events are included in a gate, results must be corrected for the proportion of contaminating cells. One method uses low side scatter and bright CD45 fluorescence for identification of lymphocytes, where an assumption is made that the only cells meeting these criteria are lymphocytes, and therefore the lymphocyte purity of the gate is close to 100%. Other methods may also be appropriate, and must be recorded.

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition*. CLSI document H42-A2 (ISBN 1-56238-640-9). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2007
2) Clinical and Laboratory Standards Institute (CLSI). *Validation of Assays Performed by Flow Cytometry - Approved Guideline-First Edition.* CLSI Document H62. Clinical and Laboratory Standards Institute, Wayne, PA, 2021.
3) World Health Organization. WHO Protocol for Performance Laboratory Evaluation of Lymphocyte Subsets Enumeration Technologies.2017.

FLO.30480 Markers/Cursors **Phase II**

The laboratory has defined criteria for setting markers (cursors) to distinguish fluorescence negative and fluorescence positive cell populations.

NOTE: Each laboratory must have a set of objective criteria to define the appropriate placement of markers (cursors) to delineate the population of interest. Isotypic controls may not be necessary in all cases, and cursor settings for the isotype control may not be appropriate for all markers. Cursor settings must be determined based on the fluorescence patterns from the negative and positive populations for CD3, CD4 and CD8.

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition*. CLSI document H42-A2 (ISBN 1-56238-640-9). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2007
2) Clinical and Laboratory Standards Institute (CLSI). *Validation of Assays Performed by Flow Cytometry - Approved Guideline-First Edition.* CLSI Document H62. Clinical and Laboratory Standards Institute, Wayne, PA, 2021.
3) World Health Organization. WHO Protocol for Performance Laboratory Evaluation of Lymphocyte Subsets Enumeration Technologies.2017.

CD34 STEM CELL ENUMERATION

Inspector Instructions:

READ	• Sampling of CD34 analysis policies and procedures • Sampling of CD34 records (events counted)
ASK	• How does your laboratory record CD34 cellular viability? • How does your laboratory gate to define the population of CD34+ cells? • What class of anti-CD34 monoclonal antibodies does your laboratory use, and how are they conjugated?

FLO.30564 CD34 Cellular Viability - Apheresis and Cord Blood Products **Phase II**

The laboratory measures the viability of CD34 positive cells in samples aliquoted during processing of hematopoietic progenitor cell products, apheresis products, and cord blood products.

NOTE: CD34 cell viability testing of cord blood products must be done on a sample aliquoted prior to the addition of cryoprotectant. For any hematopoietic progenitor cell product, the laboratory must define if and when additional testing for CD34 cell viability will be performed (eg, after storage). The viability dye 7-amino actinomycin-D (7-AAD) yields excellent results in this analysis. The viability assay must be performed using a flow cytometric method with the viability dye included in the same tube with the CD34 and CD45 monoclonal antibodies for the CD34+ viability determination. Estimates of total cellular viability (for example, trypan blue exclusion) may not be used as an alternative because the method can overestimate the viability of the CD34 stem cell population.

REFERENCES
1) Owens M, Loken M. Peripheral blood stem cell quantitation, In Flow Cytometry Principles for Clinical Laboratory Practice. New York, NY: Wiley-Liss, 1995:111-127
2) Keeney M., *et al.* Single platform flow cytometry absolute CD34+ cell counts based on the ISHAGE guidelines. *Cytometry.* 1998; 34:61-70
3) Hubl W, *et al.* Measurement of absolute concentration and viability of CD34+ cells in cord blood and cord blood products using fluorescent beads and cyanine nucleic acid dyes. *Cytometry.1998;* 34:121-127
4) Gratama J, *et al.* Flow cytometric enumeration of CD34+ hematopoietic stem and progenitor cells. *Cytometry.* 1998;34:128-145
5) Lee S., *et al.* Post thaw viable CD34+ cell count is a valuable predictor of haematopoietic stem cell engraftment in autologous peripheral blood stem cell transplantation. *Vox Sang* Feb: 2008: 94:46-152
6) Reich-Slotky R., *et al.* Determining post-thaw CD34+ cell dose of cryopreserved haematopoietic progenitor cells demonstrates high recovery and confirms their integrity. *Vox Sang* 2008: May 94(4):351-357
7) Ruiz-Argüelles GJ, Ruiz-Argüelles A, Pérez-Romano B, et al. Filgrastim-mobilized peripheral-blood stem cells can be stored at 4 degrees and used in autografts to rescue high-dose chemotherapy. *Am J Hematol*. 1995; 48(2):100-3.
8) Kao GS, Kim HT, Daley H, et al. Validation of short-term handling and storage conditions for marrow and peripheral blood stem cell products. *Transfusion*. 2011; 51(1):137-47.

FLO.30578 Monoclonal Antibodies Reagent Class — Phase II

Appropriately conjugated Class II or Class III anti-CD34 monoclonal antibodies are used.

NOTE: Class I reagents are not recommended. Class II reagents conjugated to FITC are not recommended.

Evidence of Compliance:
- ✓ Reagent logs

FLO.30585 CD34 Events — Phase II

A statistically valid number of CD34+ events are collected to ensure clinically relevant precision and accuracy.

NOTE: The maximum coefficient of variation for CD34+ cell counts should be 10%. To achieve this precision, a minimum of 100 CD34+ events should be counted, as recommended by the ISHAGE guidelines and European Working Group on Clinical Cell Analysis. If the CD34+ cell count in a sample is 0.13%, for example, then 75,000 events must be collected to reach a count of 100 CD34+ events. This level of precision is not required for extremely low counts, provided they are below clinical decision points. Precision is most important at clinical decision thresholds and laboratories should verify their precision at such decision points.

Evidence of Compliance:
- ✓ Records of number of events counted

REFERENCES
1) Sutherland DR, Anderson L, Keeney M, et al. The ISHAGE Guidelines for CD34+ Cell Determination by Flow Cytometry. *J Hematotherapy.* 1996;3:213-226
2) Gratama JW, Orfao A, Barnett D, et al. Flow cytometric enumeration of CD34+ hematopoietic stem and progenitor cells. European Working Group on Clinical Cell Analysis. *Cytometry*. 1998;34:128-142

FLO.30592 Sequential Gating Techniques — Phase II

Sequential (Boolean) gating techniques are used to define the CD34+ stem cells.

NOTE: Negative reagent controls (isotypic/isoclonic) are of limited, if any, utility in the enumeration of rare events, such as CD34+ cells. Some isotype controls can stain more cells nonspecifically than are stained specifically by a CD34 conjugate. Studies of a large number of normal hematopoietic samples have shown that the sequential gating approach best delineates specific from nonspecific staining, and that traditional isotype controls provide no useful information regarding the levels of nonspecific staining in the flow cytometric analysis of rare events. For this reason, the use of isotypic/isoclonic controls is not recommended. In their place, sequential Boolean gating and cluster analysis should be used to define the population of interest (CD34+ cells).

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). Enumeration of Immunologically Defined Cell Populations by Flow Cytometry: Approved Guideline-Second Edition. CLSI document H42-A2. (ISBN 1-56238-640-9). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA, 2007
2) Sutherland DR, Anderson L, Keeney M, et al. Towards a worldwide standard for CD34+ enumeration? *J. Hematotherapy.* 1997;6:85-89

*****NEW** 12/26/2024***

FLO.30595 Diluted Samples **Phase II**

Samples with high cellular concentrations are diluted to yield a result within the analytic measurement range (AMR) of the assay.

NOTE: High WBC count samples being processed for CD34 counts may require dilution prior to setting up the assay to obtain a result that falls within the AMR. A measured value that exceeds the AMR may be unreliable and should not be reported in routine practice. The measured result must be within the AMR before it is mathematically corrected by the dilution factor to obtain a reportable absolute CD34 count (CD34+cells/µl).

The composition of the diluent solution, the appropriate volumes of sample and diluent, and the maximum dilution that may be performed must be specified in the procedure manual. Specifying acceptable volumes is intended to ensure that the volumes pipetted are large enough to be accurate without introducing errors in the dilution ratio. The laboratory director is responsible for establishing the maximum allowable dilution of samples that will yield a credible laboratory result for clinical use.

Dilutions should be performed in a way that ensures that the diluted specimen reacts similarly to the original specimen in the assay system.

This checklist requirement does not apply if the CD34 cell count that exceeds the AMR is reported as "greater than" the upper limit of the AMR.

Evidence of Compliance:

✓ Patient results or worksheets

LEUKEMIA AND LYMPHOMA

Inspector Instructions:

READ	• Sampling of leukemia/lymphoma immunophenotyping policies and procedures • Sampling of patient reports and histograms (to include abnormal cell immunophenotype, interpretive comments, etc.)
ASK	• If flow leukemia/lymphoma immunophenotyping is done at an outside facility, how does your laboratory ensure that the testing is sufficiently comprehensive to facilitate accurate diagnosis, with appropriate gating and retention of records? • Under what circumstances does your laboratory measure the percentage of viable cells? • How does your laboratory distinguish neoplastic from non-neoplastic cells? • How does your laboratory distinguish between intrinsic and extrinsic immunoglobulin staining?

*****REVISED** 12/26/2024***

FLO.30610 Cellular Viability **Phase II**

The laboratory defines when the percentage of viable cells in each test specimen is measured.

NOTE: Selective loss of cell subpopulations and/or the presence of dead cells may lead to spurious results. This does not mean that all specimens with low viability must be rejected. Finding an abnormal population in a specimen with poor viability may be valuable but the failure to find an abnormality should be interpreted with caution. If specimen viability is below the established laboratory minimum, test results may not be reliable and this should be noted in the test report. Routine viability testing may not be necessary. However, viability testing of specimens with a high risk of loss of viability, such as disaggregated lymph node specimens, is required.

For those laboratories only performing the flow interpretation, the laboratory ensures that the percentage of viable cells in each test specimen is provided by the laboratory performing the flow technical component, as applicable.

REFERENCES
1) Clinical and Laboratory Standards Institute (CLSI). *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*. CLSI document H43-A2. Clinical and Laboratory Standards Institute, Wayne, PA; 2007.

FLO.30640 Appropriate Antibodies — Phase II

The panel of antibodies used is sufficiently comprehensive to address the clinical problem under consideration.

NOTE: Knowledge of the clinical situation and/or the morphologic appearance of the abnormal cells may help to guide antibody selection. Because antibodies vary in their degree of lineage specificity, and because many leukemias lack one or more antigens expected to be present on normal cells of a particular lineage, it is recommended that a certain degree of redundancy be built into a panel used for leukemia phenotyping.

Laboratories interpreting immunophenotyping data from an outside facility (ie, technical flow laboratory) must ensure that the antibody panels used for interpretation are appropriate. There must be a process by which individuals interpreting the results can provide feedback on the appropriateness of the antibody panels used. Records of such feedback and corrective action taken when problems are identified may be incorporated into the laboratory's quality management system.

Evidence of Compliance:

✓ Gated data plots, histograms, and patient reports

REFERENCES
1) Clinical and Laboratory Standards Institute (CLSI). *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*. CLSI document H43-A2. Clinical and Laboratory Standards Institute, Wayne, PA; 2007.
2) Rimsza LM, *et al*. The presence of CD34+ cell clusters predicts impending relapse in children with acute lymphoblastic leukemia receiving maintenance chemotherapy. *Am J Clin Pathol.* 1998;110:313-320
3) Siebert JD, *et al*. Flow cytometry utility in subtyping components of composite and sequential lymphomas. *Am J Clin Pathol.* 1998;110:536
4) Kampalath B, *et al*. CD19 on T cells in follicular lymphocytic leukemia/small lymphocytic lymphoma, and T-cell-rich B-cell lymphoma: an enigma. *Am J Clin Pathol.* 1998;110:536
5) Krasinskas AM, *et al*. The usefulness of CD64, other monocyte-associated antigens, and CD45 gating in the subclassification of acute myeloid leukemias with monocytic differentiation. *Am J Clin Pathol.* 1998;110:797-805
6) Wood BL, *et al*. 2006 Bethesda International Consensus Recommendations on the Immunophenotypic Analysis of Hematolymphoid Neoplasia by Flow Cytometry: Optimal Reagents and Reporting for the Flow Cytometric Diagnosis of Hematopoietic Neoplasia. Cytometry Part B (Clinical Cytometry) 2007;72B:S12-S22
7) Clinical and Laboratory Standards Institute (CLSI). *Validation of Assays Performed by Flow Cytometry - Approved Guideline-First Edition.* CLSI Document H62. Clinical and Laboratory Standards Institute, Wayne, PA, 2021.

FLO.30670 Cell Concentrations — Phase II

Cell concentrations are adjusted for optimal antibody staining.

REFERENCES
1) Clinical and Laboratory Standards Institute (CLSI). *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*. CLSI document H43-A2. Clinical and Laboratory Standards Institute, Wayne, PA; 2007.

FLO.30720 Immunoglobulin Staining — Phase II

The laboratory ensures that immunoglobulin staining is intrinsic and not extrinsic (cytophilic).

NOTE: The requirement is to ensure that the immunoglobulin light chain analysis includes only light chain synthesized by B cells (intrinsic light chain). Many cell types will bind serum immunoglobulin nonspecifically via Fc receptors (including B cells). To ensure that immunoglobulin staining detected by flow cytometry is intrinsic (on B cells) rather than cytophilic, a pan-B cell marker (eg, CD19, CD20) may be included in the same tube as one or both anti-light chain reagents. The inclusion of both lambda and kappa light chain reagents in the same tube allows a clear delineation of non-specific binding, even on B cells.

REFERENCES
1) Clinical and Laboratory Standards Institute (CLSI). *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*. CLSI document H43-A2. Clinical and Laboratory Standards Institute, Wayne, PA; 2007.

FLO.30730 Abnormal Cell Distinction — Phase II

Abnormal cells of interest are appropriately distinguished from normal cells based on their light scatter and fluorescence properties.

NOTE: Generally, both neoplastic and non-neoplastic cells are acquired in any gate used for acquisition. Attempts must be made to distinguish them at the time of analysis. Appropriate procedures include use fluorescent antibodies, fluorescent dyes, light scatter measurements, or any combination thereof to select out the relevant cell subpopulation for further analysis. Morphologic evaluation is also a valuable parameter to improve analysis.

Laboratories performing interpretation only of immunophenotyping data from an outside facility (ie, technical flow laboratory) must ensure that appropriate gating techniques are used. There must be a process by which individuals interpreting the results can provide feedback on the appropriateness of the gating techniques used. Records of such feedback and corrective action taken when problems are identified may be incorporated into the laboratory's quality management system.

REFERENCES
1) Muirhead KA, *et al*. Methodological considerations for implementation of lymphocyte subset analysis in a clinical reference laboratory. *Ann NY Acad Sci.* 1986;468:113-127
2) American Society for Microbiology. Manual of clinical immunology, 4th ed. Washington, DC: ASM, 1992
3) Sun T, *et al*. Gating strategy for immunophenotyping of leukemia and lymphoma. *Am J Clin Pathol.* 1997;108:152-157
4) Clinical and Laboratory Standards Institute (CLSI). *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*. CLSI document H43-A2. Clinical and Laboratory Standards Institute, Wayne, PA; 2007.
5) Macon WR, Salhany KE. T-cell subset analysis of peripheral T-cell lymphomas by paraffin section immunohistology and correlation of CD4/CD8 results with flow cytometry. *Am J Clin Pathol.* 1998;109:610-617
6) Dunphy CH. Combining morphology and flow cytometric immunophenotyping to evaluate bone marrow specimens for B-cell malignant neoplasms. *Am J Clin Pathol.* 1998;109:625-630
7) Clinical and Laboratory Standards Institute (CLSI). *Validation of Assays Performed by Flow Cytometry - Approved Guideline-First Edition.* CLSI Document H62. Clinical and Laboratory Standards Institute, Wayne, PA, 2021.

FLO.30760 Cell Population Distinction — Phase II

The laboratory has a defined process to distinguish fluorescence-negative and fluorescence-positive cell populations.

NOTE: This does not imply that a separate negative control sample must be run. It is possible to coordinate panels of monoclonal antibodies to compare the binding of monoclonal antibodies of the same subclass that typically have mutually exclusive patterns of reactivity of subsets of hematopoietic cells. In this way, test antibodies may also double as control reagents.

REFERENCES
1) Clinical and Laboratory Standards Institute (CLSI). *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*. CLSI document H43-A2. Clinical and Laboratory Standards Institute, Wayne, PA; 2007.
2) Clinical and Laboratory Standards Institute (CLSI). *Validation of Assays Performed by Flow Cytometry - Approved Guideline-First Edition.* CLSI Document H62. Clinical and Laboratory Standards Institute, Wayne, PA, 2021.

FLO.30790 Final Report **Phase II**

The final report includes information about the immunophenotype of the abnormal cells, if identified, and comments necessary to facilitate the interpretation.

NOTE: Clinical information and available pathologic material should be reviewed to select appropriate antibodies. In cases of suspected hematolymphoid neoplasia direct morphologic correlation of all applicable sample types should be performed when possible and clinically appropriate. In cases involving leukemia and lymphoma phenotyping, correlation should be made between the immunologic and pathologic results. The flow histograms, rather than just the percentage of positive cells, should be reviewed by the interpreting pathologist in difficult cases. The peak channel and shapes of the curves may be helpful in identifying clonal populations.

Reporting requirements for use of analyte-specific reagents and other reagents used in laboratory-developed tests are included in the All Common Checklist (COM.40850).

REFERENCES

1) Clinical and Laboratory Standards Institute (CLSI). *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*. CLSI document H43-A2. Clinical and Laboratory Standards Institute, Wayne, PA; 2007.
2) Nguyen AND, *et al*. A relational database for diagnosis of hematopoietic neoplasms using immunophenotyping by flow cytometry. *Am J Clin Pathol.* 2000;113:95-106
3) Wood BL, et al. 2006 Bethesda International Consensus Recommendations on the Immunophenotypic Analysis of Hematolymphoid Neoplasia by Flow Cytometry: Optimal Reagents and Reporting for the Flow Cytometric Diagnosis of Hematopoietic Neoplasia. Cytometry Part B (Clinical Cytometry) 2007;72B:S12-S22.
4) Sever C, Abbott CL, de Baca ME, et al. Bone marrow synoptic reporting for hematologic neoplasms: guideline from the College of American Pathologists Pathology and Laboratory Quality Center. *Arch Pathol Lab Med;* 2016;140(9):932-49.
5) Clinical and Laboratory Standards Institute (CLSI). *Validation of Assays Performed by Flow Cytometry - Approved Guideline-First Edition.* CLSI Document H62. Clinical and Laboratory Standards Institute, Wayne, PA, 2021.

RARE EVENT FLOW CYTOMETRIC ASSAYS

Inspector Instructions:

	• Sampling of rare event flow cytometric assay policies and procedures • Sampling of patient reports (to include lower limit of enumeration)
	• How does your laboratory distinguish normal populations from residual disease or abnormal PNH clones?

FLO.30800 Rare Event Flow Cytometric Assays **Phase II**

For rare event flow cytometric assays, the lower limit of enumeration has been validated.

NOTE: The detection of rare events may occur in assays, such as Paroxysmal Nocturnal Hemoglobinuria (PNH) clone testing or minimal residual disease (MRD) testing. Analytic sensitivity of the lower detection limit should be validated by performing dilutional studies using known patient or suitable reference material, such as proficiency testing material.

For high sensitivity paroxysmal nocturnal hemoglobinuria (PNH) testing (detecting and reporting clones less than or equal to 1%), validations to establish the lower limit of enumeration (quantitation) are required for red blood cells, granulocytes, and monocytes.

REFERENCES

1) Sutherland DR, Illingworth A, Marinov I, et al. ICCS/ESCCA consensus guidelines to detect GPI-deficient cells in paroxysmal nocturnal hemoglobinuria (PNH) and related disorders part 2 - reagent selection and assay optimization for high sensitivity testing. *Clin Cytom*. 2018: 94(1):23-48.

FLO.30820 Rare Event Flow Cytometric Assays — Phase I

For rare event flow cytometric assays, the lower limit of enumeration is included in the diagnostic report.

NOTE: When performing rare event flow cytometric assays (such as minimal residual disease (MRD) and/or high sensitivity PNH testing) on low cellularity samples, the number of events needed to achieve the laboratory's validated lower limit of enumeration/sensitivity may not be able to be collected. In these cases, laboratories must clearly state in the flow cytometric assay report that the sample was paucicellular and may thus have reduced analytical sensitivity.

FLO.30830 Rare Event Flow Cytometric Assays - MRD Testing — Phase I

For minimal residual disease (MRD) testing (including B lymphoblastic leukemia, acute myeloid leukemia, plasma cell myeloma, and mature lymphoid disorders), the method appropriately separates normal populations from residual disease.

NOTE: Examples include:
- *B lymphoblastic leukemia - distinguish hematogones (normal immature B-cell precursors) from B lymphoid blasts*
- *Acute myeloid leukemia - distinguish normal myeloid blasts from disease-associated/ neoplastic blasts*
- *Plasma cell myeloma - distinguish neoplastic from non-neoplastic plasma cells*
- *Non-Hodgkin lymphoma - separate normal B cells from abnormal/neoplastic B cells (or T cells if assay targets T cell non-Hodgkin lymphoma)*

Evidence of Compliance:
- ✓ Validation data showing normal and abnormal population analysis **OR**
- ✓ Examples of methods used to distinguish normal from abnormal and sample patient data from both normal and abnormal cases

REFERENCES

1) Flores-Montero J, Sanoja-Flores L, Paiva B, et al. Next Generation Flow for highly sensitive and standardized detection of minimal residual disease in multiple myeloma. *Leukemia*. 2017; 31(10):2094-2103.
2) Rawstron AC, Paiva B, Stetler-Stevenson M. Assessment of minimal residual disease in myeloma and the need for a consensus approach. *Cytometry B Clin Cytom*. 2016; 90(1):21-5.
3) Oldaker TA, Wallace PK, Barnett D. Flow cytometry quality requirements for monitoring of minimal disease in plasma cell myeloma. *Cytometry B Clin Cytom*. 2016; 90(1):40-6.
4) Gupta S, Devidas M, Loh ML, et al. Flow cytometric vs morphologic assessment of remission in childhood acute lymphoblastic leukemia: A report from the Children's Oncology Group (COG). *Leukemia*. 2017; Dec 18. doi: 10.1038/leu.2017.341.
5) Keeney M, Wood BL, Hedley BD, et al. A QA program for MRD testing demonstrates that systematic education can reduce discordance among experienced interpreters. *Cytometry B Clin Cytom*. 2017; May 5. doi: 10.1002/cyto.b.21528.
6) Schuurhuis GJ, Heuser M, Freeman S, et al. Minimal/measurable residual disease in AML: consensus document from ELN MRD Working Party. *Blood*. 2018 Jan 12. pii:blood-2017-09-801498.
7) Lacombe F, Campos L, Allou K, et al: Groupe d'Etude Immunologique des Leucemies (GEIL). Prognostic value of multicenter flow cytometry harmonized assessment of minimal residual disease in acute myeloblastic leukemia. *Hematol Oncol.* 2017; Dec 7. doi: 10.1002/hon.2488.
8) Bottcher S, Ritgen M, Kneba M. Flow cytometric MRD detection in selected mature B-cell malignancies. *Methods Mol Biol.* 2013; 971:149-74.

FLO.30840 PNH Testing — Phase I

The laboratory has a defined process to separate normal populations from abnormal PNH clones.

NOTE: For RBC analysis, the procedure must include an appropriate panel of antibodies/ reagents and methods for distinguishing normal type I RBCs from PNH type II and type III clones. This typically applies to larger RBC clones with well-defined clusters of RBC populations. Minor

clones (equal or less than 1%) may not show distinct separation between the type II and type III populations and one gate for the combined RBC PNH clone is sufficient.

Assay specific PMT settings must be established (see FLO.30255). For the red cell assay, unstained red blood cells are appropriate. For white cell assays, the method should include an appropriate panel of antibodies/reagents to distinguish normal from abnormal PNH clones in the populations of interest (granulocytes and/or monocytes). For PMT settings, it is important to ensure the unstained lymphocyte population is clearly on scale. Some WBC samples may show type II populations; current consensus is that these cells should be included in total "PNH neutrophils".

REFERENCES
1) Sutherland DR, Illingworth A, Marinov I, et al. ICCS/ESCCA consensus guidelines to detect GPI-deficient cells in paroxysmal nocturnal hemoglobinuria (PNH) and related disorders part 2 - reagent selection and assay optimization for high sensitivity testing. *Clin Cytom*. 2018: 94(1):23-48.

DNA CONTENT AND CELL CYCLE ANALYSIS

Inspector Instructions:

READ	• Sampling of DNA analysis policies and procedures (includes reference to established methodology and list of acceptable neoplasms for DNA analysis) • Sampling of specimen evaluation records • Sampling of DNA analysis linearity and QC records • Sampling of sub-optimal/specimen rejection records/log
ASK	• What is your laboratory's course of action when unacceptable or sub-optimal specimens are received? • How does your laboratory ensure debris and aggregates are excluded from consideration? • How does your laboratory ensure that the analysis contains neoplastic cells of interest? • How does your laboratory ensure detection of DNA aneuploidy?

FLO.31000 Neoplastic Cell Content **Phase II**

The laboratory ensures that specimens processed for DNA content and cell cycle analysis contain neoplastic cells of interest.

NOTE: It is critical that specimens submitted for flow cytometric analysis are representative samples of the neoplastic disorder being characterized. In specimens in which no population of abnormal DNA content is detected, it is especially important to demonstrate that neoplastic cells are present in the sample run through the flow cytometer. This generally requires microscopic evaluation of the specimen by a qualified pathologist.

Evidence of Compliance:
✓ Records of specimen evaluation

FLO.31010 Cellular Debris **Phase II**

The laboratory accounts for cellular debris and aggregates.

NOTE: Cellular debris can affect measurements of S-phase fraction, and aggregates can alter ploidy assessments; these need to be excluded from consideration. DNA analysis software programs generally provide options for debris subtraction and doublet discrimination. Each laboratory must incorporate such methods into their procedures. Confirmation with

fluorescent microscopic examination of the stained nuclear suspension may provide additional documentation of cellular aggregates.

FLO.31020 DNA Content Linearity **Phase II**

Criteria are established for determining acceptable linearity for DNA content measurement using cells or particles of known relative fluorescence.

FLO.31050 Staining Methodology **Phase II**

The staining and analytical processes described in the procedure manual are based upon established methodology (reference cited).

NOTE: Many different variables need to be controlled to ensure proper stoichiometry of dye binding to DNA. Therefore, it is essential that procedures adopted by a laboratory are based on published work.

FLO.31100 Specimen Treatment **Phase II**

Specimen treatment with nucleic acid dye includes treatment with RNAse if the dye is not specific for DNA.

NOTE: Certain dyes used to stain fixed cells, (eg, ethidium and propidium iodide) bind to RNA. Prior treatment with RNAse eliminates artifactual broadening of the DNA content distributions that would result from fluorescence of complexes of the dye with RNA.

REFERENCES
1) Shapiro HA. Practical flow cytometry. New York, NY: Alan R. Liss, 1985

FLO.31150 Neoplasm DNA Analysis Criteria **Phase I**

The laboratory uses defined criteria for the type of neoplasms acceptable for DNA analysis.

NOTE: The laboratory must show evidence that it restricts analysis to those neoplasms for which the literature supports significant independent prognostic significance for DNA ploidy and/or S-phase analysis.

REFERENCES
1) DNA cytometry consensus conference. *Cytometry.* 1993;14:471-500
2) Henson D, *et al*. College of American Pathologists Conference XXVI on clinical relevance of prognostic markers in solid tumors. Summary. *Arch Pathol Lab Med.* 1995;119:1109-1112

FLO.31200 Histogram Acceptability Criteria **Phase II**

The laboratory uses defined criteria for acceptability of histograms for interpretation.

FLO.31300 Nucleic Acid-Specific Dye Concentration **Phase II**

The concentration of nucleic acid-specific dye has been determined to be a saturating concentration.

NOTE: Standard techniques use an excess concentration of fluorochrome since concentrations below saturation will make the cells appear hypoploid.

REFERENCES
1) Shapiro HA. Practical flow cytometry. New York, NY: Alan R. Liss, 1985

FLO.31350 G0/G1 Peak **Phase II**

Control cells of known DNA content are run with each specimen or batch of specimens to establish an acceptable CV for the G0/G1 peak and to determine the DNA index.

NOTE: Repetitive analysis of the reference cells allows reference intervals to be established to determine an acceptable range of results. This can be used as a control for DNA staining and instrumental parameters used in the analysis.

Evidence of Compliance:

✓ Records of QC results

REFERENCES

1) Hiddemann W, *et al*. Convention on nomenclature for DNA cytometry. *Cytometry.* 1984;5:445-446

FLO.31400 Aneuploid Cell Population ID **Phase II**

Analytical criteria are established for identification of an aneuploid cell population in the test specimen.

NOTE: The ability to detect DNA aneuploidy by flow cytometric measurement depends upon the resolution of the DNA measurements, usually assessed by the coefficient of variation (CV) of the peaks. CVs should be reported for all clinical studies. The range of CVs is highly dependent on the tissue type and the way it is prepared. Histograms observed for clinical specimens often represent complex overlapping patterns because most tumor specimens contain a mixture of tumor cells, stromal cells, and inflammatory cells. Analysis of control cells is necessary to establish the CV for a normal diploid, G0/G1 peak. Periodic review of the CVs for control cells is necessary to ensure adequate functioning of the analytic procedure.

An international workshop recommended that cells (or nuclei) should be termed as having an "abnormal DNA stemline" or "DNA aneuploidy" when at least two separate G0/G1 peaks are demonstrated.

REFERENCES

1) Hiddemann W, *et al*. Convention on nomenclature for DNA cytometry. *Cytometry.* 1984;5:445-446
2) Coon JS, *et al*. Advances in flow cytometry for diagnostic pathology. *Lab Invest.* 1987;57:453-479