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Alternative Performance Assessment in Clinical Flow Cytometry

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INTRODUCTION

Alternative Performance Assessment (APA), also termed as Alternate Proficiency Testing (ALT PT) [1,2], is used in clinical flow cytometry when formal proficiency testing (PT) is not required, available, or not appropriate for a specific assay or analyte. In the United States (US), specified regulated analytes require formal PT in accordance with the Clinical Laboratory Improvement Amendments (CLIA) regulations [3]. Some flow cytometry assays are not specifically included among these federally regulated PT analytes/tests and are therefore generally considered nonregulated for CLIA proficiency-testing purposes, although they remain subject to applicable CLIA quality requirements.

In general, APA/ALT PT is performed to support the accuracy, quality, performance, and reliability of laboratory testing. It should be performed at least semiannually, clearly defined in the standard operating procedure (SOP), and thoroughly documented. Documentation should include trend evaluation and corrective action when results fall outside established acceptance limits.

AIM

This module provides clinical flow cytometry laboratories with guidance and examples of acceptable approaches for alternative assay performance assessment.

DEFINITIONS

Alternative Performance Assessment (APA) or Alternative Proficiency Testing (ALT PT)	A documented process used to assess the reliability of laboratory testing when proficiency testing is unavailable, including the use of split-sample analysis, duplicate patient testing, comparison testing, or other laboratory-defined methods with established acceptance criteria [2,3,5].

Assay	A laboratory examination procedure (test method) designed to detect, identify, characterize, or measure one or more analytes/measurands in a specimen. It includes the complete testing procedure used to address specific clinical questions. An assay encompasses the panel(s), specimen requirements, analytical procedures, data analysis strategy, interpretation criteria, and reporting elements necessary to generate a clinical result [4].
Analyte	A component represented in the name of a measurable quantity. A related term is measurand. Thus, for a flow cytometry assay: the analyte may be *CD34+ cells;* the measurand may be *absolute CD34+ cells/µL* or*%CD34+ cells among viable leukocytes* [4].
Blind sample	A specimen that is presented to laboratory personnel as if it were a routine patient sample, while its expected result or characteristics are known to the laboratory supervisor, quality manager, or proficiency testing coordinator. The testing personnel are unaware that the specimen is being used for proficiency assessment, allowing the laboratory to evaluate the entire testing process under normal operating conditions [3].
External Quality Assessment (EQA)	EQA is a broader concept defined as an external system for evaluating the quality of laboratory performance through comparison with predefined criteria or with the performance of other laboratories. In Europe, Australia, Canada, and many international programs, the term EQA is more common [7].
Proficiency Testing (PT)	PT is an external challenge in which an organization sends unknown specimens to participating laboratories for testing, compares the reported results against assigned values or peer-group results, and provides an assessment of performance. In the United States (CAP, TJC, COLA), the term PT is used almost exclusively [2,8].
Qualification	This refers to the process of obtaining objective evidence that a system, instrument, or equipment is fit for its intended use [9].
Z-score	A standardized measure indicating how far a laboratory result deviates from the mean of a reference or peer group, expressed in units of standard deviation. It is calculated as Z = (observed value − mean) / standard deviation. The Z-score is conceptually equivalent to the standard deviation index (SDI), a term commonly used in proficiency testing and interlaboratory comparisons; both express the laboratory result's deviation from the comparison-group mean in standard deviation units.

METHODS

Several methods may be used for APA, each with distinct strengths and limitations. External APA methods, although not always required, offer peer comparison and standardized performance benchmarks. Split-sample analysis, whether external or internal, evaluates agreement between methods or laboratories and can help identify preanalytical and analytical variability. Internal split sample approaches can assess reproducibility over time but do not directly evaluate accuracy. Other options include clinical correlation by chart review, direct observation of technique-dependent procedures, and review of quality control or patient data. The selected approach should be based on assay characteristics and feasibility, with preference

given to methods that best evaluate analytical performance. Whichever method is used, it must be approved by the laboratory medical director.

External APA methods

This involves assessment of assay performance by comparing the lab's results with another laboratory or a group of peer laboratories. It provides an independent assessment of performance and may provide opportunities to identify interlaboratory variation and support harmonization of testing methods, gating strategy, data analysis, interpretation, and reporting.

A. **Interlaboratory alternative proficiency testing.** This external assessment method involves testing split samples between *two or more laboratories*, followed by qualitative and/or quantitative comparison of results (Table 1). It supports evaluation of assay performance across sites and may identify issues that may be missed if APA is performed internally. Results should be interpreted carefully because differences in reagents, instrumentation, assay design, and phenotype definitions can contribute to interlaboratory variability.

Table 1. Examples of Groups or Programs for Interlaboratory Alternative Proficiency Testing.

Interlaboratory Group or Program	Description
College of American Pathologists (CAP) Sample Exchange Registry for Alternative Assessment	A service designed for laboratories performing tests for which formal proficiency testing (PT) programs are unavailable. CAP identifies and connects laboratories performing the same or similar testing, typically involving at least three participating laboratories, and facilitates specimen exchange for interlaboratory comparison. The program enables evaluation of analytical and interpretive performance through testing of shared specimens and anonymized reporting of results. [5,7].
Diagnostic Immunology Alternate Proficiency Program led by Cincinnati Children's Hospital Medical Center (CCHMC) and Boston Children's Hospital (BCH)	This collaborative alternative proficiency testing program involves approximately eighteen clinical laboratories throughout the United States. The program was established to evaluate interlaboratory consistency and performance for specialized immunologic flow cytometry assays that are not routinely covered by commercial PT programs. Participating laboratories analyze shared specimens and compare results, interpretations, and reporting practices to identify sources of variability and promote assay harmonization.
Interlaboratory APA using PT materials for other assays (off-label use)	PT specimens may serve as an alternative source for interlaboratory comparison when dedicated PT materials are unavailable. These materials frequently consist of fresh or stabilized human hematolymphoid cells and are distributed simultaneously to multiple laboratories. Residual material remaining after completion of the intended PT challenge may be used for additional assay evaluations. Participating laboratories may coordinate testing of the same materials using pre-defined analytical methods, reporting parameters, and acceptance criteria. Advantages of this approach

	include the use of identical control material across laboratories, minimal additional shipping costs, and the ability to assess assay-specific performance characteristics that are not otherwise addressed through formal PT programs. As with all other APAs, participating laboratories should establish and document standardized comparison metrics before testing to facilitate meaningful interpretation of results. Commercially available quality control materials of the same lot can similarly be used. This requires coordinated purchase by multiple laboratories.

Interlaboratory APA is a valuable method for assessing assay performance, but it requires substantial coordination among participating laboratories especially when fresh material is used. Based on the authors' experience with the *Diagnostic Immunology Alternate Proficiency Program*, which currently includes eighteen clinical laboratories across the US, participants must follow a defined schedule of testing activities throughout the year.

Participating laboratories may volunteer to prepare challenges. Laboratories typically rotate in this coordinating role so that responsibilities are distributed more evenly across the group. The coordinating laboratory is responsible for obtaining and distributing at least two APA samples, arranging specimen shipment, collecting and analyzing participant results, and preparing a summary report. Key considerations include obtaining adequate donor specimen volume, managing shipping logistics and costs, ensuring timely submission of results, and reviewing the group results. Interpretation may be complicated by differences in assay design, reagents, instrumentation, gating strategies, analysis approaches, and reporting practices. Clear communication and predefined evaluation criteria are therefore essential for meaningful interlaboratory comparison.

B. **External split sample with another laboratory.** This external assessment method involves testing split samples between *two laboratories*. The participating laboratories may use the same or different methodology but clinically comparable approaches to evaluate assay performance. Result comparison is limited to the participating laboratories and should account for differences in assay design, gating strategy, reporting practices, and instrumentation, particularly for complex assays such as MRD testing. Participating laboratories are also responsible for defining the schedule, testing frequency, cost-sharing arrangements, result reporting format, and review process.

Internal APA methods

Internal APA methods are laboratory-developed approaches used to assess ongoing assay performance when external APA is unavailable, impractical, or not well suited to the characteristics of a particular flow cytometry assay. These approaches include *clinical validation*

by chart review, *internal split-sample assessment*, and *direct observation of technique-dependent procedures*.

A. Clinical validation by chart review. This internal method is most applicable when flow cytometry results can be correlated with other laboratory studies, such as molecular, genetic or functional testing, or with the patient's clinical presentation, diagnostic findings, and expected disease course (Table 2). Limitations include the availability and completeness of clinical records, the indirect nature of the assessment for analytical accuracy, and potential subjectivity in interpreting clinical correlations. To minimize bias, a hematopathologist, flow cytometry specialist, or other qualified reviewer should independently evaluate the relevant clinical records, laboratory results, pathology reports, and other diagnostic information without access to the original flow cytometry interpretation or report. After the blinded review is completed, the original flow cytometry findings should be compared with the final clinical diagnosis or with the clinical outcome.

Cases can then be categorized as follows:

- Concordant: Flow cytometry findings align with and support the final clinical diagnosis; for example, identifying an abnormal platelet glycoprotein expression by flow cytometry (e.g. abnormally reduced CD42b expression) that correlates with molecular findings (e.g. genetic variant found in *GP1BA* gene).
- Discordant: Flow cytometry findings are not supported by the final clinical diagnosis or outcome; for example, a slightly decreased perforin/granzyme B by flow cytometry screening result suggestive of HLH is later ruled out by subsequent clinical evaluation.

Table 2. Example format of internal APA method by clinical validation by chart review.

Case review (attach all relevant case information) Patient name: _____ Specimen ID: _____ Test performed: _____ Date performed: _____ Evaluated by: _____ Reviewed by: _____	
Clinical diagnosis (including relevant clinical history, pathology findings, cytogenetic or molecular results, and clinical outcome)	*Examples include bone marrow or tissue biopsy reports, cytogenetic or molecular findings such as FISH or NGS, consensus clinical diagnosis, and clinical outcomes.*
Flow cytometry results	*For each assessment period, review 5-10 historical cases tested by the flow cytometry assay within the preceding six months. When available, the case set should include both positive and negative or normal cases.*
Concordance rate calculation for the APA evaluation period: (Concordant cases / Total cases reviewed) × 100	*Some discrepancies may be clinically or biologically expected, such as negative peripheral blood flow cytometry in a patient with biopsy-confirmed localized, non-leukemic lymphoma. If a discrepancy is explained by known clinical*

	or biological factors, the explanation should be documented, and the case may be considered acceptable with the laboratory medical director's approval. Unexplained discrepancies, including technical errors, gating issues, or transcription errors, should be documented as laboratory deviations, with corrective action implemented and recorded when indicated.
Acceptability criteria	*Acceptable agreement of laboratory results according to predefined criteria approved by the laboratory medical director.*
Conclusion	*All discordant cases should be reviewed to determine the cause of the discrepancy and whether corrective action, additional staff education, revised interpretive criteria, or assay improvement is needed. Findings, conclusions, and any resulting actions should be documented as part of the APA or quality assessment record.*

B. Internal split sample. This method uses previously tested material/sample/control that is split internally to assess the performance across different runs, instruments, and/or operators (Table 3). Split samples are usually blinded when performed by different technologists to assess assay performance. This APA primarily evaluates repeatability and intermediate precision of the testing process, including specimen preparation, staining, acquisition, data analysis, interpretation, and reporting.

This method is less preferred because it does not directly evaluate result trueness and primarily detects changes in the performance of the assay over time. However, laboratories may select this approach when sample shipment is limited, testing is highly specialized, peer comparison is difficult because of complex reporting, or internal scheduling provides a more practical workflow for proficiency assessment. Split samples should be prepared, analyzed, and stored in the same manner as routine patient specimens.

Table 3. Example of assays selected as internal split sample method for proficiency testing.

Assay or Method	Description and rationale on selecting this APA method
CD107a mobilization assay by Flow Cytometry	This assay is a highly specialized flow cytometry test that requires fresh viable cells, time-sensitive specimen handling, and complex stimulation procedures. Because external proficiency testing materials and interlaboratory comparison programs are generally unavailable for this assay, internal split-sample testing is used as the alternative proficiency testing method.
CD19 and TCR-αβ depletion assay	This assay is performed for patients undergoing haploidentical stem cell transplantation. Flow cytometry is used to enumerate TCR-αβ+ and CD19+ cells before depletion, midway through depletion, and after completion of TCR-αβ and CD19 cell depletion. This method is selected because specimens are

	limited and time-sensitive; samples are split internally to assess performance across runs, instruments, and operators.

C. **Direct observation of technique-dependent tests.** This internal method involves observation of the complete testing process, including sample preparation, staining, gating, and other technique-dependent steps (Table 4). A qualified technologist, supervisor, or manager should observe and assess the technologist performing the procedure from start to finish. This approach is less preferred because it does not directly evaluate result trueness and primarily identifies changes in performance over time. However, it may be appropriate when other APA methods are not feasible for specific assays, such as functional flow cytometry tests that depend on precise timing, specimen handling, and consistent gating.

Table 4. Example of direct observation of technique-dependent tests for proficiency testing.

Assay or Method	Description and rationale on selecting direct observation of technique-dependent tests
Basophil activation test (BAT) by flow cytometry	Assesses basophil activation in response to allergens. Requires fresh whole blood, timely testing (approximately 30 minutes to 4 hours), and appropriate allergen reagents. As an LDT, methodologies may vary among laboratories, limiting interlaboratory comparability and external APA options. The specialized nature of testing may also require preparation of control material from healthy donor basophils.
Platelet function analysis by flow cytometry	Assessment of platelet surface glycoproteins and activation markers. Dependent on sample handling and timing (within 4 hours of collection). Testing is highly complex involving platelets that are not viable to be sent to another laboratory for external APA.

RECOMMENDED ALTERNATIVE PERFORMANCE ASSESSMENT METHODS FOR COMMON CLINICAL FLOW CYTOMETRY ASSAYS

In Table 5, commonly performed assays are listed to help laboratories select an appropriate APA method for clinical flow cytometry testing. *These are only recommendations based on the authors' experience and should be reviewed and approved by the laboratory's medical director. Although limited, published literature review should be considered when selecting appropriate methods, if available.*

Table 5. List of recommended/ preferred APA methods for common tests performed in the clinical flow cytometry laboratory when no commercial PT is available.

Assay or Method	Recommended APA Method
Hematology-Oncology (including platelet-based) assays	
1. Minimal/measurable residual disease (MRD) testing	External – interlaboratory or external split sample

2. Platelet glycoprotein expression (GpIIb, GpIIIa) or function	Internal – clinical validation by chart review or direct observation of technique-dependent test
3. Sézary enumeration testing for disease monitoring	External – interlaboratory or external split sample
4. Eosin-5- Maleimide (EMA) binding in the evaluation of hereditary spherocytosis (HS)	External – interlaboratory or external split sample
5. Chimeric antigen receptor (CAR-T) enumeration testing	External – interlaboratory or external split sample; Internal – split sample
Immune monitoring assays	
1. Neutrophil oxidative burst or Dihydrorhodamine (DHR) test	External – interlaboratory or external split sample
2. T-regulatory cells (Tregs) enumeration +/- Foxp3 expression	External – Interlaboratory or split sample
3. B-cell subset (naïve, memory, class-switched, etc.)	External – interlaboratory or external split sample
4. T-cell subset (naïve and memory T-cells, Recent Thymic Emigrants, etc.)	External – interlaboratory or external split sample
5. Autoimmune Lymphoproliferative Syndrome test (ALPS): TCRαβ- positive CD4/CD8 double-negative T-cells: DNT, B220)	External – interlaboratory or external split sample
6. Leukocyte adhesion test (CD11b, CD11c, CD18, CD15)	Internal – clinical validation by chart review; External – interlaboratory or external split sample
Cellular/ Functional-based assays	
1. Lymphocyte proliferation test by flow cytometry (antigen and/or mitogen)	Internal – clinical validation by chart review
2. Platelet function assay by flow cytometry	Internal – clinical validation by chart review or direct observation of technique-dependent test
3. Basophil activation test (BAT)	Internal – direct observation of technique-dependent test

BEST PRACTICES FOR ALTERNATIVE PERFORMANCE ASSESSMENT

The following considerations may help clinical flow cytometry laboratories identify, design, and implement an appropriate APA method. APA methods should be clearly defined in an approved standard operating procedure (SOP), supported by thorough documentation, and executed by competent designee. It is also important to note that APA samples should be prepared, tested, analyzed, interpreted, and stored in a manner representative of routine patient testing. Final review and approval of the APA method are at the discretion of the laboratory medical director.

A. **Duration:** APA is commonly required at least twice a year. In the US, this is in accordance with CLIA and enforced by regulatory/accrediting organizations [1,6]. Additional assessments may be performed at the discretion of the laboratory medical director to monitor ongoing assay performance.

B. **Number of samples:** No current requirement specifies the number of samples that must be evaluated. CAP provides recommendations based on assay type (Table 6) [6,7]. However, these recommendations may be difficult to meet for high-complexity functional or cellular assays that require substantial additional specimen volume for testing.

Table 6. Recommended number of samples for APA by CAP. This remains to be at the discretion of the laboratory medical director.

Type of Assay	Number of samples
Qualitative Assays	Recommended minimum of three (n=3) samples, including negative and positive, if possible.
Semi-quantitative Assays	Recommended minimum of three (n=3) samples across the assay's analytical measurement range (AMR).

A. **Type of samples:** For qualitative and semi-quantitative assays, laboratories should use appropriate normal/negative and abnormal/positive samples when feasible. Samples should also represent different clinically relevant result levels or span an appropriate portion of the clinically relevant measurement or reportable range. Furthermore, different specimen types should be considered if specific assays are validated with different types, such as peripheral blood, bone marrow, or body fluids.

B. **Acceptability of results:** This should be determined by the laboratory medical director using predefined criteria documented in the laboratory's quality policy or approved SOP. These criteria help ensure consistent and reliable evaluation of proficiency assessment results. Furthermore, sample size should also be considered to ensure that certain criteria can be used. For example, linear regression or z-scores may not be appropriate if only few laboratories are participating in an APA method. Recommendations outlined in Table 7 may be used, as applicable, to evaluate APA performance:

Table 7. Examples of acceptability criteria for APA methods that can be used to assess APA methods

Acceptability Criteria
For Qualitative Assays, the results should match qualitative interpretation.
For Semi-quantitative Assays, one of these criteria may be used: • Defined acceptable percent difference (e.g. ≤15% difference) compared to reference result (external interlaboratory or internal split sample). This can be used when the results are compared to another laboratory or a reference split sample. • Results are within ±2 standard deviation (SD) or SD index of peer group. • Results show a z-score of < 2 when compared to reference split sample or peer group. A z-score of > 2 usually triggers an investigation. This is aligned with ISO 13528 that describes the statistical methods for use in proficiency testing by interlaboratory comparison. • Results should demonstrate a strong linear relationship (e.g., $R^2 \geq 0.90$). Regression analysis should show minimal proportional bias and minimal constant bias. Acceptability

should be based on predefined laboratory criteria and assessment of clinical impact across the reportable range, consistent with CLSI EP09 recommendations.

- Results match qualitative interpretation. This can be used when the results are not quantitatively comparable because of assay-related differences such as gating, phenotype, or instrumentation. An example is classifying results as within reference range, above reference range, or below reference range to quality if results are comparable between two different test reporting.

Any discrepant result that does not meet the acceptability criteria defined in the procedure should be investigated to determine whether the discrepancy reflects analytical variation, specimen-related factors, gating or interpretation, transcription, or another identifiable cause, with corrective action documented when

C. **Testing personnel:** As with standard PT, APA should be rotated among qualified staff to confirm that testing can be performed reliably across personnel. It is recommended to create a schedule to ensure that this is implemented. PT or APA methods can also qualify as part of staff competency.

D. **Documentation:** APA documentation should be comparable to that required for formal PT programs. Records include evaluation report, instrument and assay controls, worksheets, analysis files or flow plots, specimen-handling documentation, assay reports, and any corrective actions, when applicable. APA records should be reviewed and signed by the laboratory medical director.

CHALLENGES

Implementing APA programs in clinical flow cytometry can be challenging because of interlaboratory variability, limited standardization, and logistical constraints. Differences in gating strategies, assay design, data analysis approaches, and interpretive criteria may contribute to significant variation in results across laboratories. Comparison of results may also be limited when laboratories have incomplete information into how data are generated, analyzed, interpreted, and reported at other institutions.

Additional challenges include identifying laboratories that perform comparable assays, coordinating parallel testing schedules, maintaining specimen stability during transport, geographical difference, and managing shipping costs. Laboratories must also determine whether patient specimens, control materials, or other challenge materials are most appropriate for a given assay. Furthermore, there may also be challenges when using leftover patient samples and if this is permitted by the laboratory's institution. It is highly important to ensure patient confidentiality with de-identification of samples and verifying institutional policies when it comes to sample sharing.

Lastly, the authors recognize the lack of standardized approaches for sample selection, testing frequency, gating templates, reporting formats, and result comparison can further limit the usefulness of interlaboratory assessment or APA methods.

SUMMARY

Alternative performance assessment may be performed using several external or internal approaches, including interlaboratory proficiency testing, external split-sample comparison, internal split-sample testing, clinical validation by chart review, and direct observation of technique-dependent procedures. External comparison methods are preferred when feasible because they provide independent assessment of laboratory performance. Internal methods may be appropriate when external programs are unavailable or impractical because of specimen stability, assay complexity, low testing volume, or limited availability of comparable peer laboratories.

Regardless of the selected APA method, laboratories should define procedures, acceptance criteria, documentation requirements, and processes for reviewing and investigating discrepant results. APA should be performed at least semiannually, similar to formal PT programs, and incorporated into the laboratory's quality management program. A risk-based, fit-for-purpose approach that considers the strengths and limitations of each method supports continued confidence in the accuracy, consistency, and clinical utility of flow cytometry assays when formal PT is unavailable. The selected APA method should be reviewed and approved by the laboratory medical director. Finally, careful review or regulatory and accreditation requirements should be determined by the laboratory based on its geographic location.

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