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Validation of Cell-based Fluorescence Assays: Practice Guidelines from the ICSH and ICCS – Part IV – Postanalytic Considerations

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Flow cytometry and other technologies of cell-based fluorescence assays are as a matter of good laboratory practice required to validate all assays, which when in clinical practice may pass through regulatory review processes using criteria often defined with a soluble analyte in plasma or serum samples in mind. Recently the U.S. Food and Drug Administration (FDA) has entered into a public dialogue in the U.S. regarding their regulatory interest in laboratory developed tests (LDTs) or so-called home brew assays performed in clinical laboratories. The absence of well-defined guidelines for validation of cell-based assays using fluorescence detection has thus become a subject of concern for the International Council for Standardization of Haematology (ICSH) and International Clinical Cytometry Society (ICCS). Accordingly, a group of over 40 international experts in the areas of test development, test validation, and clinical practice of a variety of assay types using flow cytometry and/or morphologic image analysis were invited to develop a set of practical guidelines useful to in vitro diagnostic (IVD) innovators, clinical laboratories, regulatory scientists, and laboratory inspectors. The focus of the group was restricted to fluorescence reporter reagents, although some common principles are shared by immunohistochemistry or immunocytochemistry techniques and noted where appropriate. The work product of this two year effort is the content of this special issue of this journal, which is published as 5 separate articles, this being Validation of Cell-based Fluorescence Assays: Practice Guidelines from the ICSH and ICCS - Part IV - Postanalytic considerations.

Key words: Flow Cytometry; Quality Assessment; Quality Control; Quantitative Assays; Qualitative Assays; Cellular Analysis

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Cell-based fluorescence assays in leukemia/lymphoma evaluations typically rely on qualitative approaches for the identification and enumeration of the target cell population(s), but other equally important diagnostic assays are quantitative or semiquantitative. Qualitative assays usually give an overall picture of the composite phenotype based on the expression level of a set of antigens on particular cell lineages that render diagnostic patterns. Conversely, quantitative flow cytometry precisely measures the antigen density or absolute target cell count, and semi-quantitative assays quantify the abnormal target cell population above a certain threshold relative to its normal counterpart or total cells. The following sections attempt to provide an overview of the different types of flow cytometric evaluation of normal and pathological specimens, providing details of

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how qualitative, semiquantitative, and quantitative approaches are used. Additionally, other postanalytical aspects of clinical flow cytometry are described.

QUALITATIVE ASSAYS

Qualitative assays, such as "leukemia/lymphoma," "myelodysplasia," and "myeloma" immunophenotyping, require detailed analysis of a series of dot plots displaying a panel of markers in order to identify or exclude neoplastic populations (1–3). Because of the highly complex nature of multiparameter analysis, it is recommended that only interpreters having knowledge of malignant hematology, instrumentation, software, and data analysis perform the reporting. Close correlation with the morphologic review is particularly necessary, especially in cases with positive findings and when evaluating nonhematopoietic events. In most instances, the complete leukemia diagnostic workup is performed within one laboratory where the pathologist/hematopathologist works closely with the scientists undertaking the immunophenotyping and molecular work, in a multidisciplinary team-working approach. However, the person performing morphology review may use a reference laboratory for immunophenotyping performed independently where results without interpretation are provided. This approach optimally requires the interpreter to directly perform listmode data analysis. As optimal interpretation also includes morphologic review; reference to these findings should be included in the diagnostic comment.

Nonetheless, reagent performance still must demonstrate consistent and reproducible performance. Furthermore, sources of interference should be communicated and validated in a practical manner. For example, what staining interpretation caveats must be known in lymphoma assessment in bone marrow specimens with coexistence of multiple myeloma? In paucicellular specimens, panels designed specifically to evaluate the disease process are recommended. To mitigate the risk of a high ratio of antibody to the number of cells per tube rendering nonspecific binding and that of acquiring data with too few events, 1–2 tube antibody panel combinations should be designed tailored to the disease being evaluated. This type of "triage" selection requires consideration of clinical data as well as prior immunophenotypic data and pathology reports. For a comprehensive approach of antibody and panel combinations recent European and Bethesda guidelines provide detailed information (1–3). Also the intended use of panels might be different, if employed following or in the absence of so-called screening panels, and validation processes would be expected to differ under the different clinical practice scenarios.

Reporting of immunophenotypic analysis may include intensities of antigens that are aberrantly up-regulated or down-regulated in the abnormal population, for example bright CD10 in a B-lymphoblastic leukemia or dim CD20 expression in chronic lymphoid leukemia (CLL). Such observations are in reference to the expression pattern of the closest normal counterpart. The abnormal population should be quantified relative to the normal counterpart or within the immature population. For instance, B-lymphoid clones are described as a percentage of total lymphocytes and of the total sample. Genotypic/phenotypic correlations are increasingly being recognized, implying that comments guiding additional confirmatory testing could be included in situations where a classic immunophenotype profile is associated with an underlying mutation or translocation. For example, where aberrant CD19 expression is observed in an acute myeloid leukemia, genetic analysis for t(8;21)/AML1-ETO leukemia must be considered; lack of CD10 in B-lymphoblastic leukemia suggests a pro-B/B-I (EGIL) immunophenotype that may be associated with t(4;11); CD13 expression in B-lymphoblastic leukemia should trigger testing for t(9;22)/BCR-ABL translocation.

Aberrant and/or clonal plasma-cell clones should be described as a percentage of the total sample. If an aberrant clonal plasma cell component is identified in a polyclonal background, the presence of normal polyclonal plasma cells has prognostic relevance and should additionally be quantified relative to the clone. Aberrant and increased blast populations should be described as a percentage of the total sample.

Adoption of standardized reporting is strongly encouraged, such as that suggested by the Bethesda Consensus group (1–3). In particular, use of antigen or molecular expression using the convention of referencing to a normal cell counterpart can be applied whenever possible and using the Bethesda Consensus nomenclature where expression of the target cell population is compared in relation to normal cells such as: same Fl intensity as normal cell counterpart; dim (dimmer than normal cell counterpart), bright (brighter than normal cell counterpart (2). Describing partial or heterogeneous expression on a specific cell population may also be helpful in delineating disease states from normal. Furthermore, as reporting with color enhanced cell cytograms continues to be integrated into clinical practice, an international color coding standard of leukocyte subpopulations, such as that proposed by the French GEIL (4), with subpopulations of the CD45 versus side scatter cell cytograph immediately recognized as lymphocytes (magenta), monocytes (green), granulocytes (red), and "blast cell region" (so-called "bermudes" in cyan), is strongly supported.

QUANTITATIVE ASSAYS

Flow cytometry assays included in this category are lymphocyte subsets assessment, CD34 enumeration, enumeration of leukocytes in leukocyte-reduced apheresis products, platelet enumeration, reticulocyte counting, CD64 expression on neutrophils for infection/sepsis detection and fetal red cell enumeration for fetomaternal hemorrhage detection. For some of these assays, an absolute numerical value is assigned based on an internal bead-counting reference, as in CD34 enumeration. Alternatively, complete blood counts obtained from a hematology analyzer can be used as a reference count, such as in platelet enumeration by the platelet/RBC ratio (5). The normal range should also take into account age, sex, and regional factors that may influence the

reference range (6). It is important to stress that reference ranges may also vary depending upon the reagent, kit or analyzer used (7). Where applicable, the numerical data should include a qualifying comment relative to the reference range, i.e. normal, high or low.

For quantitation of the expression of cellular antigens, such as CD64, a bead reference can be applied to generate a calibration curve of median fluorescence intensity (MFI) to mean equivalent of soluble fluorochrome (MESF) conversion. This is used for instance for the determination of the PMN CD64 index in sepsis and other inflammatory states (8). When reporting quantitative data, it is important that normal reference ranges for the population and expected ranges in related conditions (for instance with CD64 level in sepsis, inflammatory leukemoid reaction relative to myeloproliferative neoplasms) be determined (8). As with most laboratory analytes, it is informative and relevant for assay validations to determine the reference range in healthy individuals and ensure that common physiologic factors are not compounding variables, such as patient age, sex, pregnancy, etc.

SEMIQUANTITATIVE ASSAYS

The output in semiquantitative assays is reported as a percentage of the total gated cellular events or some cellular index and reflects the disease burden. As these assays typically identify cells considered abnormal or malignant, establishing a normal reference range is not feasible, so such methods typically establish a cut-off or threshold for disease detection. Flow cytometric assays for rare event analysis, such as identification of PNH clones and minimal residual neoplasm, are included here.

Early guidelines in PNH have been published (9–11). For the identification of small PNH clones, it is recommended that 250,000 events be collected in order to identify clusters of at least 25 events for a desired sensitivity of 0.01%. When reporting the results it should clearly state on the report to what level the laboratory has determined the sensitivity level of the assay, by indicating the amount of cells counted.

PNH assays are designed to distinguish clones based on the lack of specific GPI-anchored antigens, as such appropriate validation of this assay should include positive samples that represent clones that reside in "negative" regions of dot plots. Preliminary data suggests that fluorescent aerolysin (FLAER) based flow assays are a cost-effective and sensitive method of detecting PNH granulocyte or monocyte clones (11). When small, subclinical, clones are identified in the setting of myelodysplasia or aplastic anemia, it is important to suggest follow-up testing.

Minimal residual disease (MRD) evaluation provides crucial information regarding therapeutic efficacy, the necessity of reinduction or change in therapy protocols, and transplantation considerations. In reporting MRD, the immunophenotype of the remaining neoplastic clone identified is compared with that observed in prior studies for several key reasons: (a) describing the clone in reference to normal counterparts; (b) noting possible antigen drift; and (c) lineage switch in undifferentiated and mixed phenotype leukemia; all of which have potential therapeutic implications (12). Any immunophenotypic aberrancy identifying the clone must be included in the report and a comment made on comparison to prior studies. The level of the blast population should be reported as clearly defined units, such as percentage of total nonerythroid events.

Widely accepted for therapeutic guidance in acute lymphoblastic leukemia, MRD evaluation has also been adopted in post-therapy evaluation of hematological malignancies including CLL (13). Identifying a cluster of $\geq$10 cells is desirable for accurate identification of a MRD clone, but this detail needs to be determined and verified for each specific assay. The level of the neoplastic clone is reported in CLL as a fraction of total B-lymphocytes or total leukocytes. MRD evaluation in myeloma has proven therapeutic implications (14). MRD evaluation in acute myeloblastic leukemia is also feasible and has proven similar clinical value (15).

STORAGE AND RETENTION

Data Storage and Retention

Accurate data analysis and interpretation is crucial in the quantitative determination of a reference range for flow cytometric diagnostic assays, as well as in the qualitative determination of the immunophenotypic expression profiles, which lead to the diagnosis of a range of malignant and other disease classifications. Most flow cytometry laboratories make efficient use of listmode data (LMD) files acquired by the flow cytometer followed by subsequent higher interrogative analysis at other analysis workstations in the laboratory. This improves workflow by allowing flow cytometers to be used for their primary role, data acquisition, and it encourages thorough data analysis by specialist operators using sophisticated software tools. As such, LMD files that generate these quantitative and qualitative results must be stored and secured in a traceable manner that enables retrospective reanalysis (16). This may occur in a number of settings including:

- MRD assessment, which may involve reanalysis of the original diagnostic data;
- Confirmation of original data interpretation and diagnosis if this is brought to question via subsequent sample analysis;
- Reanalysis of a quantitative result in the event of an unexpected or spurious results;
- Retrospective "look-back" of historical immunophenotypic data for medicolegal, education, research, and development purposes.

LMD files should be "backed-up" and stored in their FCS raw data format to a secured remote server or to permanent storage media (e.g., CD, DVD, and portable harddrive). The integrity of LMD back-up must be confirmed, prior to removing files from the flow cytometer that generated them. The duration of LMD file storage may vary according to national and international regulations, but generally is recommended at over two years or periods similar to anatomic pathology biopsy material.

Many software applications allow the storage of electronic gated analysis data used by laboratorians to analyze and report. Alternatively, many laboratories will print out their gated analysis for subsequent interpretation. All gated analysis, whether in paper hard-copy or electronic format (e.g., software generated PDF), that leads to quantitative and/or qualitative analysis and arriving at the reported test results generation must be stored for at least 2 years, or according to local regulations, which ever is the longer. Laboratories should ensure that all electronic record storage and transfer procedures satisfy their hospital and laboratory information technology security and privacy requirements.

Sample Storage Retention

Although testing should be completed as quickly as possible and preferably within 24 h, samples should be stored for 7 days, although less than 7 days may be sufficient to reach a definitive diagnosis. Laboratories must also adhere to local regulations. However, storage for longer than 5 days may require the use of sample preserving agents.

Once testing has been performed, sample storage at room temperature (18–22°C) is generally satisfactory, although if a delay in diagnosis or retesting is anticipated, storage at 2–8°C further prolongs cell viability. In this setting, the laboratory should validate appropriate storage conditions according to anticoagulant, sample type, and test requested, where appropriate. Extreme temperatures must be avoided. If testing is delayed to beyond 24 h, validation studies should be performed to confirm stability under the relevant storage conditions.

QC and QA Records Storage

Storage requirements for QC and QA records vary from country to country. As a minimum, they must be stored for 2 years; however, laboratories must follow the requirements of their local regulatory authority.

Equipment Maintenance Logs Storage

Storage requirements for equipment maintenance logs vary from country to country. As a minimum, they must be stored for 2 years; however, laboratories must follow the requirements of their local regulatory authority.

Standard Operating Procedures (SOP) Storage

Storage requirements for SOP vary from country to country. As a minimum, they must be stored for the period that the procedure is current plus 2 years; however, laboratories must follow the requirements of their local regulatory authority. Archival SOPs in electronic format only is acceptable, but back-up storage is recommended.

QUALITY ASSURANCE TOOLS

Introduction

Well-designed, optimized, and validated assays with appropriate QC and QA checks are building blocks of an effective quality management system. In addition to well-written and clear laboratory processes and SOPs, knowledgeable, and competent staff are necessary to maintain assay quality and process. These quality tools collectively ensure that assay accuracy and precision established at validation are consistent over time.

Training

Training is a process that provides and develops knowledge, skills and behaviors to meet the requirements, such as ISO 10015 (17). Professional training is generally the knowledge and skills required of a specific profession or job. This may include medical technology training, internships, or clinical rotations that are essentially apprenticeships in the laboratory work environment.

Employment training is provided or enabled by the employer to someone new to an organization or department where specifics on job training in policies, processes, and procedures are provided. Published guidelines are available for topics for training and education models for flow cytometry (18). However, whilst training is an important part of continued professional education, some countries, require mandatory, and current registration with professional bodies in order to practice or legally perform the job task. A laboratory training process consists of a four step process: establishing training objectives, identifying the methods used to perform the training, identification of the materials used in the training process, and criteria used to assess the effectiveness of the training. Learning objectives are defined as the expectations of the training outcome, which can be assessed or observed. These can be cognitive, affective and/or psychomotor. Each training module should have definitive learning objectives documented.

In the laboratory environment, there are numerous methods that can be used for training, which may include lectures or tutorials, self-study, instrument operator manuals, manufacturers instructions, process maps, and SOP, computer based assessments, observance of the task, practice of the task with skilled observer, testing blinded samples or listmode files and self-assessments (19,20).

Performance standards for each training module need to be determined. These standards should define expected results or behaviors. These acceptability criteria should be determined prior to the assessment. Completion of a training checklist, which documents that the trainee has been verified to show the appropriate knowledge, skills, and techniques to perform the assay or task is a regulatory requirement. Feedback from both the trainee and trainer on the training process can provide continuous improvement opportunities. These training checklists document that the trainee has demonstrated acceptable performance and can work independently.

Competency Assessment

Competency assessment is a periodic review of the staff's ability to meet the performance expectations that are stated in their job descriptions, performance and quality standards and training guidelines (18). Individual competency assessment is initially determined at the completion of the training module. Ongoing

competency for all modules that this employee performs should be assessed at least annually. This assessment must include preanalytic, analytic, and postanalytic processes as applicable. Direct observation is a valuable assessment tool and can verify that the employee is performing the task exactly as it is outlined in the SOP and work instructions. It is important to have a detailed and clear procedure that covers all aspects of the process.

Record review can consist of reviewing the equipment maintenance documents and/or quality control documentation. This assessment can determine if documentation is complete and, if nonconformances occur, that the appropriate corrective action is applied and documented. Problem solving skills can be assessed by quizzes or problem situations that are presented to the employee requiring him/her to recognize that a problem exists and determine the cause and action to be taken. This can assess the recognition of instrument, quality control, sample preparation, and analysis problems. Blind testing using specially provided materials (unknowns) can be used to assess competency. This can be done using previously tested known samples and control or blinded split samples and comparing results for equivalency. This can assess competency in sample preparation and staining techniques, but the limited stability can introduce variables not related to competency. One can also review results from external quality assurance (EQA) samples that a specific employee tested and submitted. To assess competency of gating techniques, listmode files can be used as unknowns to assess competency in the analysis (20).

Accreditation

Accreditation is a regional process that a health care institution, provider, or program undergoes to demonstrate compliance with standards developed by an official agency. The introduction of ISO-15189 will inevitably mean that international standardization regarding medical laboratory accreditation standards will improve the quality management system.

In the United States, the Clinical Laboratory Improvement Amendments (CLIA 88) dictate testing and personnel. The Centers for Medicare & Medicaid Services (CMS) regulate all laboratory testing (except research testing) performed on humans in the U.S through CLIA. CLIA covers ~200,000 laboratory entities. Other accreditations in the United States are performed by the Joint Commission (JCAHO), College of American Pathologists (CAP), AABB, and other state and federal agencies. These agencies have deemed status for CLIA and have more extensive standards.

The accrediting body in Australia is NATA, and laboratories must be NATA accredited to receive payment for testing services by the Federal Government funding body, Medicare. In France, the accrediting body is COFRAC. In 2010, a modification of the legislation established ISO 15189 accreditation as an obligation for all clinical laboratories. In the United Kingdom until recently both medical laboratories and clinical External Quality Assessment providers have been accredited through Clinical Pathology Accreditation (UK) Ltd. However, in 2011 this role was taken on by the United Kingdom Accreditation Service which will be accrediting clinical laboratories according to ISO 15189 standards and EQA programs to ISO 17043 standards.

External Assessment (Audits)

External assessments are periodic assessments of laboratory processes and procedures to ensure that requirements are met for the quality of patient testing. These assessments (audits) can be routine or based on a complaint or adverse event. An audit is defined as a planned and documented activity that is performed in accordance with written procedures and checklists to verify by examination and evaluation of objective evidence that elements of a quality assurance program have been developed, documented, and implemented.

Audits can be internal (first party) or external (second or third party). Internal audits are performed by the staff of laboratories to inspect their own system. It is better if internal audits are carried out by objective staff members trained in audit techniques. External audits can be performed by a customer, accrediting agency or regulatory body. The purpose of these audits is to verify that the laboratory or department complies with the regulatory standards of the auditing agency, including preanalytic, analytic and postanalytic activities. Review of documentation, staff credentials and verification of process should be part of an audit. Assay validation, staff training, and competency assessment records must be current. In the event of a complaint or adverse event, a focused audit can be performed reviewing a specific process to determine the root cause of the event. The auditors will discuss nonconformances and identify the magnitude of the nonconformance at the end of the audit process. A written audit report is submitted to the laboratory thereafter. The laboratory must correct and document corrective action for each nonconformance identified.

In some situations, such as audits resulting from a complaint or adverse event, documented corrective actions and possibly a cessation in testing could occur. Audits are valuable exercises and can provide insights into nonconformances and improved processes and ultimately leading to improved patient outcomes. The objective should be to audit the quality of the system rather than individual staff. The deficiencies observed should form part of a noncompliance report that should be submitted to senior management.

Quality Control and Proficiency Testing

Training, education, and quality control (QC), both internal QC (IQC) and External Quality Assessment (EQA), are necessary to ensure accurate and precise flow cytometric data. Internal quality control (IQC) should be performed on a regular basis and used to identify any potential areas for concern with variable frequency. A common QC material for flow cytometers is the use of microbeads that can be used to monitor compensations, fluidics, laser, and PMT voltages. Results from the use of beads should be plotted on a Levy–Jennings type plot

and any noticeable drift should be investigated immediately. It is also important that the instrument should be checked with beads and stabilized samples following instrument servicing to re-establish optimal settings.

It is also important to have assay specific QC checks whilst running clinical samples. This may involve using a Delta check or Levy Jennings like assessment for samples on a cumulative basis, running samples with known values at the start of each working day or each batch. In addition, acceptability limits for each test should be defined and cross checks included within a given panel. Stabilized samples can be used as a full process control as these will ensure that staining, lysing, acquisition, and analysis are consistent on a day to day or batch to batch basis. A fresh sample from different individuals should not be used as a daily instrument QC as results do not allow the operator to identify some important "drift" issues. Some assays are fortuitous in having the ability to use internal cells as the assay relevant QC.

A variety of proficiency testing programs are in existence operating at local, national, or international level. It is vital (and mandatory if ISO 15189 standard is to be achieved) that participation in a suitable EQA program is undertaken. The more common uses of flow cytometry can be subjected to EQA and many of the larger international programs such as those operated by UK NEQAS for Leucocyte immunophenotyping and the College of American Pathologists offer flow cytometric EQA programs for leukemia and lymphoma diagnosis, lymphocyte subset monitoring, feto-maternal hemorrhage assessment, paroxysmal nocturnal hemoglobinuria, and stem cell enumeration. Many of these programs now use stabilized material enabling samples to be transported long distances such that data from large international cohorts can be examined to search for any instrument or reagent bias. An EQA frequency of at least three times per annum is recommended to ensure continued performance monitoring.

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