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Validation of Cell-based Fluorescence Assays: Practice Guidelines from the ICSH and ICCS – Part II – Preanalytical Issues

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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 ã home brewã 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 II - Preanalytical issues. © **2013 International Clinical Cytometry Society**

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BACKGROUND

This section updates the two previous CLSI documents on fluorescence-based, clinical cellular assays (FC): (i) CLSI H43-A2, Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition (1); and (ii) CLSI H42-A2, Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition (2). CLSI H-52, Flow Cytometric Assessment of Red Cells, is currently under revision and expected to be published in late 2013; this too contributes to the background of this guideline. A recent interest in the clinical FC identification of circulating non-hematopoietic tumor cells (3,4) is also considered relevant to the recommendations presented in this document. In addition, DNA-based cytometry for S phase fraction and ploidy analysis will be briefly discussed (5,6). However, this document will not describe immunophenotypic criteria for the diagnosis of specific hematolymphoid neoplasms. Specific future guidelines are anticipated in these areas and

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well-covered in many other publications (7). Unless otherwise specified or impractical due to ethical or logistic issues, assay validations related to pre-analytical conditions should be based on ~50% normal specimens and 50% specimens containing the abnormal cell population(s) of interest, spanning the clinically relevant range of values for the analyte in question.

Sample Requirements

In all cases, there should be appropriate medical indications for the planned testing, e.g., for leukemia/lymphoma immunophenotyping (8). Appropriate samples for clinical FC include peripheral blood (PB), bone marrow aspirate (BMA), disaggregated tissue including standard soft tissue biopsies, as well as fine-needle aspirations (FNAs) and bone marrow core biopsies (BMBs), cerebrospinal fluid (CSF), other body fluids including effusions and lavage fluids and nuclei from paraffin-embedded tissue for DNA ploidy assays. PB should be collected by standard venipuncture technique, as described previously (9–11).

With the exception of formalin fixed paraffin-embedded tissue for DNA ploidy analysis, all other clinical FC specimens should be considered biohazardous and labeled as such in accordance with national or regional safety standards. These specimens must be handled with appropriate biohazard precautions as described previously (12). Several publications have also addressed specific issues relating to FC testing of HIV-positive specimens (13–15).

All samples submitted for testing must be labeled immediately at the time of specimen collection with at least two unique patient identifiers and the date and time of collection. There may be additional local regulations that apply to the labeling of patient material. When multiple specimens from the same patient are collected for analysis, the source of each specimen should be clearly indicated on each specimen container. Paraffin blocks submitted for DNA ploidy analysis should be clearly labeled with at least the referring pathology laboratory's accession number.

A test requisition form, whether printed or electronic, should accompany all specimens. This form should include unique patient identifiers, plus age, sex, diagnosis (either previously established or under consideration), name of the physician submitting the specimen, pertinent medication or recent treatment (including dates of chemotherapy or radiation), date and time of specimen collection, and source of the specimen (e.g., BMA, CSF, etc.). The requested test should appear on the specimen label or on the requisition accompanying the specimen. Relevant pathological information, including white blood cell (WBC) count and differential and/or specimen histologic, cytochemical, immunohistochemical, molecular, and/or cytogenetic findings, is desirable. If an acceptable label is not present, then the FC laboratory should follow its established policy for dealing with unlabeled or improperly labeled specimens. If adequate identification cannot be subsequently obtained after a concerted effort by the laboratory, then the specimen should be rejected under the signature of the laboratory director.

Reagents Used in the Pre-analytical Stage: Anticoagulation

Cell staining methodology, including the use of monoclonal antibodies (Mabs), will be considered elsewhere in this document, so this section is limited to reagents used in initial stages of specimen handling, including anticoagulants and tissue culture medium. At a minimum, these reagents should only be used for their intended purposes. For commercially available reagents, lot numbers and expiration dates must be recorded, and the package inserts for "active" reagents should be kept on file. For "home brew" reagents used in lab developed tests (LDTs) generated in the FC laboratory, both the creation/manufacture date and the expiration date should be kept on file, and the expiration date should be clearly affixed to all reagent containers.

The choice of anticoagulant depends upon specimen type, transportation, and storage requirements, and, in some cases, the method of sample preparation. For PB, ethylene-diaminetetraacetic acid (EDTA), sodium heparin, or acid citrate dextrose (ACD) may be used. If a complete blood count (CBC) and WBC differential are to be made from the same specimen used for FC, then EDTA is the anticoagulant of choice. Sodium heparin samples are reported to be optimally stable for 48–72 h, EDTA samples for up to 48 h, and ACD samples for 72 h. Note that any of these three anticoagulants are suitable for use with body fluids having significant PB contamination.

For BMA, sodium heparin may be the preferred anticoagulant, and is required if cytogenetic testing is to be performed on the same specimen. EDTA is acceptable for FC testing, but not for cytogenetics. ACD anticoagulation is not recommended for BMA, because if the ratio of sample to anticoagulant is not correct, the pH may be altered and the cell viability will be reduced.

The FC laboratory should, at a minimum, validate any assay likely to be applied to PB or BMA on those anticoagulants used in local practice and specify any conditions unacceptable for use with the assay. Ideally, any assay applicable to PB would also be validated on ACD-containing specimens. It is recognized that these specimens are very rare in most clinical FC laboratories. This validation may need to be performed on PB from normal volunteers drawn into ACD tubes subject to local ethical clearance.

Any FC laboratory using extended stabilization formulations that both anticoagulate and preserve specimens must first validate the use of these formulations in their FC assays. Because these formulations fix the cells to some extent, loss of antigen binding sites can occur and

cell viability by exclusion dyes cannot be measured in this setting.

Reagents Used in the Pre-Analytical Stage: Tissue Culture Medium

In general, no anticoagulant is used with tissue samples. Instead, all tissue biopsies intended for FC evaluation (e.g., lymph nodes, BMBs, and FNAs) should be immersed in an adequate volume of an appropriate transport medium (typically a tissue culture medium) in a sterile container to optimize cell viability while the specimen is being transported to the FC laboratory. Perhaps the most common medium currently used for this purpose is modified Iscove Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal calf serum, 1% glutamine, and appropriate antibiotics (RPMI+). If feasible, to maximize the exposure of the cells to RPMI and enhance cell viability, any tissue specimen (including BM) intended for FC should be finely minced with a scalpel in a plastic Petri dish in a relatively small volume of RPMI, prior to transportation to the laboratory (16). Certain disease processes may require additional special handling, such as large cell lymphomas or biopsies where infection may be a diagnostic concern. If CSF is to be submitted for FC, the specimen should be combined with an equal or greater volume of RPMI or some similar cell stabilization media as soon as it is obtained from the patient in order to maximize cell viability in these frequently paucicellular specimens. CSF samples may benefit from validated stabilization procedures (17).

Specimen Age

Because of the perishable nature of all fresh FC specimens (as opposed to FC analysis of DNA ploidy in nuclei from paraffin-embedded tissue), and the potential for antigenic alterations during the aging of specimens (18,19), tracking specimen age is a critical part of the FC procedure. Indeed, some laboratories may alter their specimen preparation somewhat depending on the reported age of the specimen at the time of receipt (e.g., "up front" utilization of a fluorescent DNA-binding dye to help exclude non-viable cells from the analysis in older specimens likely to have compromised cell viability). In general, all perishable specimens should be processed as soon as possible after collection, especially specimens from rapidly enlarging masses likely to contain tumors with high proliferative and/or apoptotic rates (e.g., Burkitt's or diffuse large B-cell lymphoma), BM samples evaluated for multiple myeloma or specimens from patients recently treated with chemotherapy and/or radiation.

The validation of any new FC assay should include documentation that the assay performs appropriately on the full range of specimen ages likely to be encountered for all specimen types accepted by the laboratory. Contemporary airborne shipping enables virtually all FC laboratories, including reference laboratories, to receive most specimens at 48 h or less ex vivo. However, due to unintended shipping delays, rare specimens may be up to 72–96 h old when received by the laboratory. A number of these specimens, particularly tissues and, for some patients, BM, will be considered irreplaceable. In such cases, if the FC laboratory has documented adequate performance of the relevant assay on such aged specimens and, if in the opinion of the laboratory director or his/her designee, the specimen is not irreparably compromised by degenerative changes then it may be appropriate to perform FC analysis in the hope of reaching a reliable conclusion about the presence or absence of malignancy. As a general rule, it is recommended that for each specimen type and each anticoagulant type, the reliability of reagent performance at specimen age greater than 4 h should be validated in at least five specimens, with time course extending to the maximum anticipated age of specimens to be received in the laboratory.

For potentially replaceable specimens such as PB, rigid specimen age cutoffs are not necessary in laboratories that have documented adequate performance of the assay at the particular specimen age. Importantly, any circumstances that may have limited/compromised the FC assay must be noted in the FC report, ideally with a statement requiring that FC results be correlated with the clinical setting and tissue morphology before a final diagnosis is made. Finally, note that some antigens show relatively greater variability than others with specimen age (e.g., CD138 and CD16 are more labile than CD45 or CD64) (19); therefore, the potential for false-negative FC results on labile antigens should be noted, as appropriate, in the FC report.

Specimen Storage, Stability, and Transportation

The integrity of PB, BM, and body fluid (including CSF and anticoagulated body fluids) samples is well maintained at room temperature (18–25°C), so these specimens should be stored and transported within this temperature range or the storage condition specified for the assay in question. Fluctuation in temperature can cause changes in membrane expression of some antigens and hence the effect of cooling and rewarming should be validated for each assay (18). Storage and transportation outside the 18–25°C range, as may occur unintentionally in particularly hot areas in summer or particularly cold areas in winter, will require validation on at least five samples of each affected specimen type with each different anticoagulant or the use of temperature monitors to enable accurate temperature exposure tracking. No convincing literature exists indicating that clinical samples age significantly different than healthy samples; hence while inclusion of target patient specimens is recommended for sample stability testing, tests involving rare diseases may be validated for specimen stability using a predominance of normal specimens. One approach to counteracting unintentional heating or cooling of specimens is to include one or two, sealed small wet ice

packs in the packaging in which the specimen is transported to the FC laboratory, albeit packaged so as not to allow direct contact between the specimen and any frozen object. Transportation of bio-hazardous specimens must be in accord with local, national (20), and international standards (21).

Assessing Specimen Viability

For all clinical FC assays, the proportion of viable cells in the sample should be estimated and recorded immediately before, or during, the assay. For assays not requiring a fixed level of cell viability, the decision to evaluate a partially degenerated specimen rests with the laboratory director.

For specimens that are not highly degenerated, fastidious forward scatter (FS) vs. side scatter (SS) gating is an effective way to estimate the percentage of viable cells, and to exclude non-viable cells from the analysis. However, for highly degenerated specimens and so-called rare event (<1%) assays, there may be uncertainty about optimal FS vs. SS gating to exclude non-viable cells, in which case utilizing a fluorescent, DNA-binding dye may be a more attractive alternative (22). Because these dyes are excluded from viable cells with intact plasma membranes, any cell in a specimen that takes up the dye and emits the characteristic fluorescence when exposed to the appropriate laser can be assumed to be dead or dying. There are many acceptable fluorescent dyes for selecting viable cells, including 7-aminoactinomycin D, propidium iodide, 4′,6-diamidino-2-phenylindole (DAPI) or equivalent reagents. Among these three dyes, DAPI offers the advantage of being well-excited by violet (405 nm) lasers, so that adequate DAPI fluorescent emission does not require use of an ultraviolet (UV) laser, and does not interfere with fluorescent emissions due to blue laser (488 nm) or red laser (633 nm) excitation. Therefore, when DAPI is used in conjunction with an appropriately designed antibody panel in a three-laser system, it is possible to exclude degenerating cells unequivocally from the analysis, while simultaneously evaluating up to nine different specific antibodies. For each dye, validation studies should be performed on an adequate number of specimens (five or more across reportable range), or site of assay performance documentation, to ensure that dyes do not alter the expression of antigens being assayed.

Cell Counting

For most fresh specimens intended for FC, cell counting is necessary prior to the addition of antibodies to cells, to ensure that an appropriate amount of antibody is used in each assay. When a cell count is required for the integrity of the assay, such as to ensure that active reagents are in saturating or optimal concentration, an automated counter is usually recommended for this purpose, but may not be suitable for samples with small volume/cell number, in which case hemocytometer counting may be performed. Adjustment of cell count should be appropriate to the application, and done with a suitable medium such as RPMI or phosphate-buffered saline (PBS) with or without bovine serum albumin (BSA). Cell counting should be performed on each day that any given specimen will be assayed, since the viable cell count will tend to decrease gradually over time, even when the specimen is being maintained in an appropriate nutrient medium (2).

Cell counting is, however, part of all single-platform analyses relying on the use of calibrating beads added to the sample, when cell numbers are within the range of antibody amount effectiveness (2).

Allowing for the Evaluation of Specimen Morphology

For PB or BMA, morphologic evaluation of freshly made Wright or May–Grünwald–Giemsa stained smears is strongly recommended for correlation with the FC data. For body fluids or disaggregated tissue, stained cytocentrifuge preparations ("cytospin") of an appropriate number of cells (to generate a monolayer on the slide) is strongly recommended. While morphologic correlation with the FC data should be a matter of course in the clinical FC laboratory, the decision whether to report the morphologic data rests with the laboratory director.

Pre-analytical Considerations for DNA Ploidy Analysis on Paraffin-Embedded Tissue

For a detailed discussion of pre-analytical considerations for DNA ploidy analysis, see Shankey et al. (5).

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