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Fluorescence Calibration and Quantitative Measurement of Fluorescence Intensity; Approved Guideline

This guideline describes the basic principles, reference materials, and laboratory procedures upon which quantitative fluorescence calibration is based.

A guideline for global application developed through the NCCLS consensus process.

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Abstract

Quantitative fluorescence calibration (QFC) is an empiric system to calibrate fluorescence intensity in a way that preserves stoichiometry between the concentration of fluorochrome in solutions and the equivalent molar quantity of fluorochrome on stained measurands such as cells, gels, microspheres, and microdots. This guideline describes the basic principles, reference materials, and laboratory procedures upon which QFC is based. This guideline is intended for use with reference materials and procedures developed under the National Institute of Standards and Technology (NIST) Fluorescence Intensity Standards program. While the general principles of QFC apply to any fluorescence measurement, this guideline specifically addresses analysis of cells and microspheres by flow cytometry, including cellular immunophenotyping and suspension array technology. The current and emerging uses of these laboratory methods will have an increasing impact on public health and primary care, from large-scale screening of populations to the individual profiling of each patient's disease.

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Foreword

Over the last four decades, fluorescence has emerged as the most useful signal in biomedical science. Fluorochromes are the only labels that combine the sensitivity of radioactive tracers with the capacity for direct visualization at the submicron level. Fluorescence measurements can be made on bands, microspheres, microdots or cells in gels, suspensions, planar arrays, or three-dimensional tissue slices. The fluorescent antibody technique first developed by Coons in the 1940s[1,2] is today the basis of the most commonly used assays in the biomedical laboratory. Fluorescence microscopy remains an important tool in biology, medicine, and public health. Its heir apparent, fluorescence cytometry, is a continuing wellspring of technical advances and biomedical applications. Fluorescence imaging techniques have been harnessed to analyze high-density microarrays and "chips," each with tens of thousands of targets. As of the publication of this guideline, multiplexed fluorescence measurements are capable of resolving dozens of populations in a single suspension of microspheres, each with its own target; emerging platforms will allow simultaneous analysis of suspensions with hundreds or thousands of targets. The high-capacity, low-cost capability of these technologies for genetic and proteomic analysis presents a profound opportunity for improving human health, from large-scale screening of populations to the individual profiling of each patient's disease.

Because of its many advantages (including biosafety), fluorescence has largely supplanted radioactivity as the label of choice. However, fluorescence does not provide the same direct measure of molar quantity that radioactivity does. Units of radioactivity such as curies convert readily to molar quantities because nuclear emission is spontaneous, its decay is predictable, and it is unaffected by the chemical environment. In contrast, fluorescence requires a source of excitation, its decay is variable, and its intensity is influenced by many environmental factors including temperature, hydrophobicity, concentration, and photodegradation. While this dependence provides valuable probes for the biomolecular environment, it complicates the relationship between fluorescence signals and the molar quantity of fluorochrome. The problem of molar equivalence is even more difficult when fluorescence from fluorochrome solutions must be related to fluorescence from stained particulates like cells, microspheres, and microdots.

Despite these difficulties, an empiric system of fluorescence calibration that preserves stoichiometry between the concentration of fluorochrome solutions and their equivalent molar quantity on stained microparticles has been utilized in research applications of flow cytometry for many years.[3-7] Recent efforts show that it can be formalized and implemented in a fully standardized fashion across all fluorescence detection systems.[8] We refer to this system as quantitative fluorescence calibration (QFC).[9] The challenge and opportunity of this guideline is to establish the basic principles and the practical basis of QFC.

The earliest efforts toward QFC originated with immunologists, pathologists, and cell biologists who wanted to convert the subjective descriptions of "bright" or "dim" cell staining that reflected "high" or "low" levels of receptor expression into objective units that could be compared longitudinally across samples, experiments, and instruments.[10-19] A number of ensuing reports addressed technical issues and expanded the clinical application utility of QFC,[20-47] and an "emerging consensus" regarding the methods and uses of QFC[48] was evident from a compilation of papers published in 1998.[9,49-69] However, the lack of a formal theory and of authoritative reference materials hindered its full maturation, until the National Institute of Standards and Technology (NIST) initiated the Fluorescence Intensity Standards (FIS) program in 1999 (see Appendices B, C, and D).[8]

The NIST FIS initiative addressed both the practical and theoretical aspects of QFC. The practical need for authoritative standards led to the development of two reference materials: a fluorescein solution (see SRM 1932 in Appendix C) and a calibrated set of fluorescein-labeled microspheres (see RM 8640 in Appendix D). The parallel development of these reference materials bridges the gap between solution and particle fluorescence and serves as the model for developing future FI reference materials. While these materials have been in development, a series of papers in the NIST Journal of Research[8,70-72] (see

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Appendix B) has delineated the formal theory that supports and augments a fundamental approach to QFC first described in the cytometry literature.[33] The formalization is based upon a well-defined property of fluorochrome solutions called the fluorescence yield, which relates the molar quantity of fluorochrome and its quantum efficiency to the measured intensity of fluorescence emission. Ironically, the term fluorescence yield had been applied much earlier in fluorescence microscopy to explain the variance in staining intensity caused by environmental influences in fluorescence-stained tissues.[73]

The fluorescence yield provides the stoichiometric link between fluorochrome solutions and labeled particles. It allows fluorescence intensity measurements on such particles to be converted into equivalent molar quantity units called molecules of equivalent soluble fluorochrome (MESF).[9] The fluorescence yield of fluorochrome-labeled conjugate solutions, expressed as MESF per conjugate molecule (the "effective F/P ratio"), is analogous to the specific activity of radiolabeled tracers expressed as curies per mole. Thus, the molar quantity of conjugate on labeled particles can be determined by measuring the fluorescence yield of the conjugate solution and the MESF of the particles.

Having established the theoretical basis for QFC, this guideline addresses the operational steps required for implementing it, from characterizing primary reference materials to constructing calibration curves. Two types of fluorochromes are presented as models for QFC: organic dyes such as fluorescein, and proteins such as phycoerythrins and green fluorescent protein. These are among the most often used fluorochromes, and they pose somewhat different considerations for QFC that are representative of their different molecular properties.

Fluorescein is a small molecule which can be accurately characterized for mass purity by standard physical methods. With the availability of NIST fluorescein reference materials SRM 1932 and RM 8640, it becomes the first fluorochrome truly standardized for QFC. Its environmental sensitivities present technical difficulties for QFC, but these very properties make it a good test model for MESF calibration.

Phycoerythrin and green fluorescent protein are macromolecules that cannot be accurately characterized for mass purity by standard physical methods. This makes it difficult to establish a generic standard applicable to the wide range of possible variants. While their internalized chromophores are somewhat insulated from direct effects of the environment, these proteins are subject to changes in peptide folding that can alter fluorescence intensity. The approaches in this guideline should help bring better characterization and uniformity to the fluorescence measurements from these more complex molecules.

The subcommittee has focused on the use of QFC in ligand-binding assays in which measured fluorescence comes from a fluorochrome-conjugated ligand bound to cells or microspheres. This configuration encompasses the methods most often used in cellular immunophenotyping, suspension arrays, and microarrays. The goal of measuring fluorescence intensity (FI) from a cell-bound ligand is often to quantify the expression of its corresponding receptor, however indirect the relationship may be. Receptor binding values for unknown analytes may also be quantified by directly calibrating FI readings with cells or microspheres that have predetermined binding capacities. This approach obviates the need for MESF-calibrated fluorescence measurements. Since either approach should give the same answer, the chemistry of specific receptor-ligand interactions provides an independent reference frame for assessing the accuracy of FI measurements. The issues involved with quantifying receptor expression through QFC are detailed in Section 11.

The contents of this guideline are directed at both the suppliers and the users of fluorescence technology. The guideline should be immediately relevant to companies, researchers, and clinical specialists involved with flow and image-based cytometry and assays employing microspheres and microarrays.

Because the theoretical basis for QFC has been developed only recently, there is more background material than with most other NCCLS documents. This guideline will be most useful if it is used in concert with other NCCLS guidelines that address flow cytometry and fluorescence-based assays on other

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platforms (see the most current editions of NCCLS documents H42—*Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Lymphocytes*; H43—*Clinical Applications of Flow Cytometry: Immunophenotyping of Leukemic Cells;* H44—*Methods for Reticulocyte Counting (Automated Blood Cell Counters, Flow Cytometry, and Supravital Dyes);* and H52—*Fetal Red Cell Detection*).

Trueness in all fluorescence-based assays can be improved through QFC, and this quality assurance alone is sufficient reason for this guideline. However, the real benefit of QFC will be the direct translation of relative fluorescence measurements into standardized molar quantities, opening the way for accurate measurements of cellular expression in health and disease.[74]

A Note on Terminology

NCCLS, as a global leader in standardization, is firmly committed to achieving global harmonization in terminology wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences in terms while taking steps to achieve worldwide uniformity. NCCLS recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in NCCLS, ISO, and CEN documents; and that legally required use of terms, regional usage, and different consensus timelines are all obstacles to harmonization. In light of this, NCCLS recognizes that harmonization of terms facilitates the global application of standards and deserves immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

In keeping with NCCLS's commitment to align terminology with that of ISO, the following describes the metrological terms and their uses in I/LA24-A:

The term a*ccuracy* refers to the "closeness of the agreement between the result of a (single) measurement and a true value of a measurand" and comprises both random and systematic effects. *Trueness* is used in this document when referring to the "closeness of the agreement between the average value from a large series of measurements and to a true value of a measurand"; the measurement of trueness is usually expressed in terms of *bias. Precision* is defined as the "closeness of agreement between independent test/measurement results obtained under stipulated conditions." As such, it cannot have a numerical value, but may be determined qualitatively as high, medium, or low. For its numerical expression, the term *imprecision* is used, which is the "dispersion of results of measurements obtained under specified conditions."

Users of I/LA24-A should understand, however, that the fundamental meanings of the terms are identical in many cases, and to facilitate understanding, terms are defined in the Definitions section of this guideline (see Section 4.1).

All terms and definitions will be reviewed again for consistency with international use, and revised appropriately during the next scheduled revision of this document.

Key Words

Antibody binding capacity (ABC), calibration, effective F/P ratio, flow cytometry, fluorescence, fluorescence yield, fluorochrome, fluorochrome-ligand conjugate, fluorometer, immunophenotyping, ligand binding assays, molecules of equivalent soluble fluorochrome (MESF), reference materials, spectrophotofluorometer, standardization

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Fluorescence Calibration and Quantitative Measurement of Fluorescence Intensity; Approved Guideline

1 Scope

The scope of this document comprises the basic principles and essential procedures for quantitative fluorescence calibration (QFC): standardizing the measurement of fluorescence intensity (FI) on solutions and particles in a way that correctly maps the stoichiometry of chemical reactions. To elucidate the basic principles of QFC, the document includes background on the properties of fluorescence, the general features of instruments used to measure FI, and the assignment of molar equivalent values based on fluorescence yield. The document addresses the characterization and use of fluorochrome reference solutions, reference microparticles, and fluorochrome-labeled ligands. The document is directed toward suppliers and users of fluorescence-based technology for cellular analysis and for ligand binding assays employing microspheres, microarrays, microtiter plates, and image cytometry.

Although the general principles of QFC presented in this guideline apply to all FI measurements, the primary focus of this document is the use of suspended microparticles (microspheres and cells) to calibrate FI signals on flow cytometers. Practical methods for implementing QFC on flow cytometers are presented in the final section. QFC on imaging and scanning instruments will require additional consideration.

2 Introduction

Despite the widespread use of fluorescence in biomedical research and clinical laboratory assays, no uniform system of fluorescence calibration that maps fluorescence intensity (FI) measurements onto a scale of standardized units has yet been adopted. This overall process is referred to as quantitative fluorescence calibration (QFC).[9]

This guideline outlines the basic principles and laboratory practices involved in QFC. Because the theoretical basis of QFC has been formalized only recently, the guideline contains more detailed background material, appendices, and references than typical guidelines. It is intended for use with reference materials and procedures developed under the National Institute of Standards and Technology (NIST) Fluorescence Intensity Standards program.

At this time of rapid development in biotechnology and the need for expedient translation of research methods to the clinical laboratory, a consistent system of QFC will improve the characterization of all instruments and reagents that employ fluorochrome labels. The information provided by QFC enhances good laboratory practices (GLP)[75-79] and helps comply with U.S. CLIA regulations,[80-82] recommendations from the Centers for Disease Control and Prevention and from Health Canada for enumerating CD4 lymphocytes,[83,84] and the ISO 07025 Guidelines.[85]

Looking ahead to future applications in biomedical science and public health, QFC formalizes a property value first identified by cell biologists: the specific binding capacity for fluorochrome-labeled ligands. The accurate measurement of binding capacities to quantify cellular expression will open a whole new window to prevention and treatment through predictive medicine and disease profiling.

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