Analytical Validation of Protein-Based Multiplex Assays: A Workshop Report by the NCI-FDA Interagency Oncology Task Force on Molecular Diagnostics

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Clinical proteomics has the potential to enable the early detection of cancer through the development of multiplex assays that can inform clinical decisions. However, there has been some uncertainty among translational researchers and developers as to the specific analytical measurement criteria needed to validate protein-based multiplex assays. To begin to address the causes of this uncertainty, a day-long workshop titled “Interagency Oncology Task Force Molecular Diagnostics Workshop” was held in which members of the proteomics and regulatory communities discussed many of the analytical evaluation issues that the field should address in development of protein-based multiplex assays for clinical use. This meeting report explores the issues raised at the workshop and details the recommendations that came out of the day’s discussions, such as a workshop summary discussing the analytical evaluation issues that specific proteomic technologies should address when seeking US Food and Drug Administration approval.

On October 30, 2008, members of the research and regulatory communities met for a 1-day workshop in Cambridge, Massachusetts, convened by the Clinical Proteomic Technology Assessment for Cancer (CPTAC)2 center network, a component of the National Cancer Institute (NCI) Clinical Proteomic Technologies for Cancer (CPTC). This workshop, hosted by the NCI and the US Food and Drug Administration (FDA) Interagency Oncology Task Force (IOTF), was undertaken to identify the analytical validation requirements that might apply to a proteomics technology—specifically, for mass spectrometry-based and affinity array assays—in the context of various intended uses. A unique feature of this workshop was that it focused on developing case studies that would serve as “what if” scenarios on which FDA staff and other participants could comment and provide insight for those developing new protein-based multiplex clinical assays. The workshop was designed to augment the activities of the American Association for Cancer Research-FDA-NCI Cancer Biomarkers Collaborative Assay Validation Subcommittee.

Both the NCI and the FDA recognize the promise of clinical proteomics to enable the early detection and appropriate treatment of cancer. For this reason, the NCI established the Cancer Proteomic Technologies for Cancer initiative that brings together experts in the field of proteomics to lay the foundation for the next generation of molecular (protein-based) diagnostics. As part of this initiative, NCI and FDA, through the Interagency Oncology Task Force on Molecular Diagnostics, are collaborating on several projects designed to speed cancer biomarker discovery, verification, and validation.

A crucial step in the development of new proteomic molecular diagnostics based on multiple proteins is establishing analytical performance parameters that will support regulatory filing before marketing. Protein-based assays developed on novel or complex platforms (such as multiplex mass spectrometry or multiplex immunoaffinity assays) have not been well established in the clinical context. Therefore, there has been some concern and uncertainty among transla-

mass spectrometry; LIF, laser-induced fluorescence; SID-MS, stable isotope dilution mass spectrometry.
tional researchers and developers about the type and depth of data needed to establish the performance of tests on such platforms. This workshop was intended to be a first step in developing guidance on these matters for those developing multiplex protein assays for use in clinical care.

**Overview of FDA Requirements for In Vitro Diagnostic Devices**

The FDA regulates medical devices, including those intended for use “in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals . . . ,” as stated in section 201(h) of the Federal Food, Drug, and Cosmetic Act. In vitro diagnostic devices (IVDs) are a subset of medical devices that encompass “reagents, instruments, and systems intended for use in the diagnosis of disease or other conditions, including the determination of the state of health, to cure, mitigate, treat, or prevent disease or its sequelae” (1).

Since 1976, devices must comply with FDA regulations through a risk-based classification scheme (2). Class III devices carry highest risk, and include those devices that are important in preventing impairment of human health or present a potential unreasonable risk of injury or illness to the patient or user (e.g., devices that give stand-alone diagnoses or those that specifically guide selection of therapies). Examples of class III devices include *Mycobacterium tuberculosis* nucleic acid tests and HIV, hepatitis C, and hepatitis B assays. Class II devices carry moderate risk or are devices for which performance characteristics are well understood and predictable (e.g., rubella serological tests, nucleic acid assays for aid in diagnosis of cystic fibrosis). Class I devices carry a lower risk, offering little or no potential for the unreasonable risk of injury or illness, and are generally exempt from FDA premarket review (examples include ascorbic acid tests and lactic acid tests).

Medical device submissions to the FDA can include investigational device exemptions (IDEs), premarket notifications (termed “510(k)s” after the name of the section of the Federal Food, Drug, and Cosmetic Act that establishes their use), and premarket applications (PMAs). As many IVDs have moderate risk, the most common premarket regulatory pathway for IVDs is the 510(k), which is required for some class I and most class II devices. Information on cleared and approved IVD 510(k)’s is available online at http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/LabTest/ucm126189.htm. FDA clearance of a 510(k) is based on “substantial equivalence” to a legally marketed device; this clearance requires an available predicate or device of similar type with similar intended use. More recently, the de novo 510(k) process has been developed for novel but moderate risk devices with no existing legally marketed predicate. The de novo route is used when no other device has been previously classified for a specific intended use, automatically placing the novel device in a class III category; however, use of the new device is deemed to present moderate risk to the patient, making it a candidate for down-classification. These devices are reviewed for safety and effectiveness. Once a device is down-classified via the de novo process, it can serve as a predicate for future devices of the same type with the same intended use.

Class III devices require premarket approval and a demonstration of safety and effectiveness of each device on its own merits, i.e., not compared to other legally marketed devices (predicates). Safety and effectiveness are specifically defined in FDA regulations (3), but are loosely translatable to the analytical and clinical performance of the device. Safety for IVDs is conceptualized as the benefit of a test result outweighing its risk, when the test is used as labeled. Safety can be connected to analytical performance, because the device developer must ensure that adequate instructions for use are provided, that the correct population for testing is identified, and that the expected measurement performance parameters are established. Effectiveness refers to evidence that the test result is clinically important when the test performs according to its labeling. This can be connected to the ability of the test to contribute to meaningful clinical decision-making. The type of supporting evidence required to establish a reasonable assurance of safety and effectiveness varies by device, but in general the test developer must show adequate, often statistically assessed, analytical and clinical performance of the device when used according to instructions and for its claimed intended use.

**Lessons Learned from Marketed IVDs**

The primary purpose of this meeting was to identify key areas for guidance to translational researchers and developers who may be making plans to create or market protein-based diagnostic tests. Case studies were followed by discussion. First, 2 marketed (FDA-cleared) devices were described along with the analyte- and platform-specific issues that were addressed in premarket review. This set the stage for how the FDA might go about determining which issues would need to be addressed for new proteomic technologies and assays. Next, 4 devices that are under early development were presented, to provide a framework for understanding the complexity and the extensive variety of platforms that are being envisioned for clinical use. The case studies included the following.
• MammaPrint®, the first gene expression microarray-based assay approved by the FDA (4). This marketed assay analyzes RNA extracted from breast tumor tissue to assess expression of multiple genes and create an index to aid in the prognosis of previously diagnosed breast cancer. The device was approved using a de novo 510(k) submission. The de novo route was used because no other device had been cleared for this use, and the test was judged to present moderate risk to the patient because it is intended for use in providing prognostic information to already diagnosed patients. It is not indicated to be used to select or guide therapy or to predict the outcome of using different treatment choices. The assay is qualitative (e.g., provides result as a low, low borderline, high borderline, or high risk for distant metastasis) and is performed in a single laboratory. Validation issues specific to the instrumentation, reagents, and sample type were discussed.

• A tandem mass spectrometry–based test system used widely to screen blood samples taken from all newborns for inborn errors of metabolism. The intended use of this test is to measure and evaluate amino acids, free carnitine, and acylcarnitine concentrations from newborn heel-prick blood specimens dried on filter paper. The test specifications require the use of a specific tandem mass spectrometry platform, and a variety of validation issues specific to the platform and the sample type were illustrated.

• A protein-based biomarker assay to identify individuals at high risk for developing colon cancer. The methodology being developed involves analyzing proteins in resected tumor tissue specimens, including fresh frozen and formalin-fixed, paraffin-embedded (FFPE) tissues. Analysis uses liquid chromatography–multiple reaction monitoring mass spectrometry (LC-MRM-MS) and immunohistochemistry. FFPE specimens enable a “retrospective discovery” design by selecting stage II specimens from an archive and assessing adjuvant therapy and 5-year recurrent and nonrecurrent cases.

• A MRM-MS assay of 5 candidate plasma protein biomarkers for early detection of severe preeclampsia. It is anticipated that no single marker will stratify patients into categories of increased and decreased risk. Therefore, an algorithm will be required to use this panel of 5 biomarkers and will generate a risk score.

• An assay of blood-based enzymatic activity as a cancer biomarker. The assay as envisioned would provide a conglomerate readout of proteases and could be developed toward platforms using individualized fluorophoric and/or mass spectrometry–based technologies.

• A platform based on immunological assays using interferometry or laser-induced fluorescence (LIF) to assess antigen concentration of 5–50 proteins simultaneously in plasma. The platform has 3 parts: a high-throughput detector (reader), a disposable disc with multiple immunologic arrays (up to 33 280 array elements per disc), and a data analysis system that quantifies multiple antigens. This system could enable both translational biomarker validation studies and disease-specific diagnostic assays on individual plasma samples.

**Lessons Learned from Case Study Discussions**

Among the lessons learned from discussion of cases were the following.

• The sponsor provides studies and data for analytical and clinical performance of its assay, and the FDA assesses safety and effectiveness of the device for its intended use on the basis of the submitted performance. When a clinical test is contemplated, the developer should consider carefully the necessary elements of the test, its conditions of use, and parameters of interpretation.

• An assay’s intended use is a critical factor from the FDA perspective. Level of risk as well as validation requirements are developed based on the test’s claimed intended use. It is important to lock in an intended use before initiating validation studies, so that appropriate data are generated to support that use.

• Although in most cases the FDA is not specifically concerned about methods used to generate biomarker candidates, a mechanistic understanding of the role of the analytes in health and disease may ease the validation and review process.

• Study design and a plan for statistical analysis of the data are essential, and sponsors will need to develop an appropriate study design and statistical plan to demonstrate test safety and effectiveness for the intended use planned. Assay developers are encouraged to consult early with the FDA on their proposed study plans and statistical protocols.

• The FDA requires that applicants address quality system requirements (5). A quality systems approach should include the platform (instrument) on which the assay will be performed, the reagents that will be needed for the assay, and any other items that are provided with the proposed test kit. Regulations for specific types of devices may provide additional information about the quality system requirements for particular devices.

• The FDA requires that software algorithms that are included in the assay for data and results interpreta-
tion be prespecified before analyzing study data. This means that the algorithm needs to be established and its parameters locked in up front. Alteration of the algorithm to better fit the data is generally not acceptable, and may invalidate a study.

• It is not always necessary for a sponsor to provide details of an explicit algorithm if evaluation and analysis of using that algorithm are appropriately performed, but this is determined on a case-by-case basis. Sponsors can be assured that proprietary algorithms will not be made public by the FDA.

• Independent clinical validation of a test (i.e., validation on independent data) is critical for certain types of tests. It is important for developers to understand the validation requirements for their particular tests, and to ensure that performance characteristics are determined using unbiased analysis.

• Preanalytical, specimen-related aspects of performance characteristics are becoming increasingly important, especially where analytes are labile or unstable, and the preanalytical aspects should be part of the preclinical and clinical evaluation of the device. Effects of preanalytical factors as well as any sources of assay variability should be evaluated in reproducibility studies. Preanalytical factors should be also considered in other studies such as limit of detection and limit of quantification (when the assay is quantitative) and interferences.

• The FDA requires assurance that a device is reasonably safe, which differs from the International Organization for Standardization standard that defines safety as freedom from unacceptable risk. For IVD devices, safety includes the consequences of the false positive and false negative results on the patient, as well as how diagnostic testing can harm a patient (for example, invasive collection of the specimen, level of radiation during the diagnostic procedure).

• Controls (internal, external, process, etc.) for device performance need to be established by the manufacturer before bringing the assay to the market.

• For assay standardization, use of international standards as well as well-defined and traceable quality control materials is encouraged.

• To show clinical performance of an assay, the sponsor generally needs to provide performance data on a properly sized validation set that represents a true patient population on which the test will be used. For most novel devices, this is the pivotal clinical study that will establish whether performance is adequate. In general, clinical evaluation should be carried out on the final version of the device, in multiple laboratories, and withstand a high level of analytic and statistical scrutiny. Therefore, it is critical to discuss scientific issues with the FDA so that the sponsor, researcher, and agency are on the same page.

• When a sponsor seeks clearance or approval for a test on several different instruments (e.g., mass spectrometers from multiple manufacturers), performance evaluation on each different instrument type is needed. For more complex instruments, where the performance may vary across manufacturers, the intended use of the test normally limits test use to those platforms for which performance has been established.

• Reagents that can be used in many different tests can be cleared for use with every assay and require evaluation as a part of any assay in which they are used. This is generally accomplished in the analytical and clinical validation studies, where the reagents are simply used as part of the assay to establish performance. Stability and lot-to-lot studies for reagents are often necessary for each assay, as variations in reagent performance will likely affect specific tests differently.

• Interference and cross-reactivity (reactivity with substances other than the intended analyte) in protein-based assays is an important performance issue that in many cases will require additional discussion between the proteomics experts and the regulatory community. This is because interferents may span the range of criticality depending on the test system (platform plus reagents) used. It is essential to determine the situations under which markers are likely to be used and the relevant interferences that should be tested.

• Data should be provided to show the instrument performance for the particular test. The FDA allows device master files (submissions containing necessary data to establish the performance of the instrumentation in general) for instrumentation to be submitted by the instrument manufacturer in certain cases. Once a master file is in place, multiple applicants may reference the file with the instrument manufacturer’s permission. This mechanism has primarily been used when the instrument manufacturer does not wish to share proprietary details of instrument design with the test manufacturer. In any case, whether the instrument performance is provided with the test submission or as a master file, the FDA generally reviews specific instrument performance with the first submitted test. In subsequent applications using the same instrument, unless the new assay raises new questions of instrument performance, there are generally no additional instrument-specific review issues.

• Zero-analyte samples are generally required for quantitative tests to establish the performance of the test system at low levels. For estimation of the limit of
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Next steps for the CPTAC center network include gathering additional information on the variability of proteomics platforms. CPTAC centers are testing various components of the platforms with a focus on variability, and are also developing software master files for use by the scientific community. For example, the network recently published a multisite assessment study demonstrating that multiple reaction monitoring (MRM) coupled with stable isotope dilution mass spectrometry (SID-MS) has the potential to transform how candidate protein biomarkers are evaluated as it provides a rapid way to quantify a candidate biomarker in blood (6). In addition, the FDA is following the progress of the CPTAC center network efforts and is working to understand the issues that arise, and how they may be resolved scientifically.

Workshop participants concluded that publication of a white paper, identifying issues that should be addressed for specific proteomic technologies when seeking FDA regulatory review, would benefit the research and clinical community. Early identification of these issues should allow them to be addressed as early as possible in the discovery-to-product development path. In addition to the white paper (workshop summary), the workshop also recommended that CPTAC take the lead in developing “mock 510(k)” documents (nonregulatory documents in the form of 510(k) submissions) provided to the FDA that would help orient the FDA to multiplex mass spectrometry and affinity array platforms in novel diagnostics and serve as a springboard for guidance to the proteomics community. A separate publication in this issue of Clinical Chemistry addresses outcomes of this mock submission effort (7).

Perhaps the most important lesson to come from these discussions is that the FDA encourages discussion during product development for these novel test types. The agency prefers to understand the scientific basis, the relevant issues and justifications, and the information that will be necessary to facilitate a decision. Approval failures in many cases occur because of poorly conceived studies. Before beginning the analytical and clinical validation process for complex assays, it is recommended that a sponsor consult with the FDA. In all cases, the FDA and the sponsor benefit if the agency can learn about a proposed product before it appears in a submission.

Conclusion and Action Items from the Workshop

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Because there is currently no guidance for multiplexed protein assays (such as multiplex mass spectrometry or multiplex immunoaffinity assays), the workshop provided an opportunity to involve CPTAC scientists, the FDA, and others in the proteomics community to begin to work through the issues that might be raised in regulatory review of such tests. Clearly, continued communication between the FDA and the proteomics community will be necessary to fully realize the potential of proteomics to reduce the pain and suffering due to cancer.

Appendix

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