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Translating pharmacodynamic biomarkers from bench to bedside: analytical validation and fit-for-purpose studies to qualify multiplex immunofluorescent assays for use on clinical core biopsy specimens

Allison Marrero, Scott Lawrence, Deborah Wilsker, Andrea Regier Voth, Robert J. Kinders*

Pharmacodynamics Assay Development and Implementation Section, Laboratory of Human Toxicology and Pharmacology, Applied/Developmental Research Directorate, Leidos Biomedical Research, Inc, Frederick National Laboratory for Cancer Research, Frederick, MD

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ABSTRACT

Multiplex pharmacodynamic (PD) assays have the potential to increase sensitivity of biomarker-based reporting for new targeted agents, as well as revealing significantly more information about target and pathway activation than single-biomarker PD assays. Stringent methodology is required to ensure reliable and reproducible results. Common to all PD assays is the importance of reagent validation, assay and instrument calibration, and the determination of suitable response calibrators; however, multiplex assays, particularly those performed on paraffin specimens from tissue blocks, bring format-specific challenges adding a layer of complexity to assay development. We discuss existing multiplex approaches and the development of a multiplex immunofluorescence assay measuring DNA damage and DNA repair enzymes in response to anti-cancer therapeutics and describe how our novel method addresses known issues.

1. Introduction

The complexities of intracellular protein signaling, metabolic processes, and DNA replication and repair inherent in diseases such as cancer are well recognized; however, in measurements of clinical correlates from biopsies and patient specimens, analysis is still often limited to a single analyte, representing a single drug target within any one of these pathways. While this approach has the benefit of focusing preclinical development and pharmacodynamic (PD) marker selection, a critical limitation is that, in order to measure an effect, one must choose between upstream measurements of target activation and downstream measurements of pathway activation and/or intended treatment outcome at the cellular level. This, along with the additional difficulties associated with obtaining sufficient high-quality specimens for analysis, drives the current emphasis on multiplex analysis of clinical trial specimens.

There are numerous benefits to applying a multiplex format in support of a clinical trial. First, multiplex assays enable measurement of PD responses of multiple analytes on a single specimen, maximizing the amount of information obtained using a minimal amount of valuable patient tumor tissue. Second, multiplex assays can enable intracellular pathway activity reporting, measuring target engagement and the intended PD effectors and early sensors of the pathway as well as downstream markers of drug effect in the same tissue section; markers of commitment can potentially also be measured if they can be identified. A third critical aspect of a multiplex assay is that it reduces the possibility of missing a PD response due to factors such as specimen collection time, dose of the investigational agent(s), and genetic alterations in the tumor, as compared to a single marker being used as the assay readout. Finally, pathway reporting will be particularly useful in combination therapy approaches using multiple agents with different mechanisms of action.

One of the strengths of the multiplex assay is the ability to confirm a drug effect using a correlative marker in the event that there is no modulation of the primary biomarker. A lack of modulation of the primary marker measured in a single analyte assay could be interpreted as either no drug effect or a genetic defect that prevents modulation of the target. For example, when profiling a DNA repair pathway, signal from the phosphorylated form of the DNA damage sensor Nbs1 (pS^{343}-Nbs1) or histone H2AX phosphorylated at Ser139 (γH2AX) [1,2] could be absent in Ataxia telangiectasia mutated- (ATM) or DNA-dependent protein kinase (DNA-PK)–deficient models due to the genetic background. However, modulation of other markers included in a multiplex assay panel, such as Rad51 or ERCC1, could confirm a drug effect on the tumor. Importantly, the

* Corresponding author. Pharmacodynamics Assay Development and Implementation Section, Laboratory of Human Toxicology and Pharmacology, Applied/Developmental Research Directorate, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, MD 21702. Tel.: (301) 846-6410; fax: (301) 846-6536.
E-mail address: kindersr@mail.nih.gov (R.J. Kinders).

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presence of additional markers provides information that allows a negative result in one marker to be distinguished from a lack of total response, and alternate interpretations to be generated. In addition, the use of combinations of markers for a particular PD pathway can also decrease false positive calls by clarifying a spurious positive signal from only one biomarker in a measured set. Using such approaches, unexpected molecular responses in clinical samples may come to light; however, there is a separate set of challenges associated with multiplexing assays, particularly those performed on solid tissues [3,4]. Here, we will discuss some popular technologies for multiplex assays and their utilization for PD studies, enumerate the challenges inherent in multiplex immunofluorescence assays, and provide specific examples of how we dealt with these challenges during the development of a multiplex analysis of the DNA repair activation pathway in patient biopsies.

2. Multiplex assays for clinical samples

From a technical standpoint, multiplex assays can be grouped into those requiring a homogenous sample (such as tissue lysates or blood samples) and those requiring an intact tissue section for analysis. Both types of multiplex assays present specific strengths and challenges.

2.1. Assays for tissue lysates and blood samples

2.1.1. The Luminex xMAP platform

One of the most popular multiplexing technologies is the bead-based flow cytometric xMAP platform from Luminex (Austin, TX). Assays developed for this platform use the two-site or sandwich immunoassay approach, employing a monoclonal antibody (mAb) conjugated to a fluorescently labeled bead to immobilize each analyte and a second, labeled mAb against the analyte to report its concentration. An assay calibrator is required for each analyte; usually a recombinant protein version of the analyte is used. In collaboration with the National Cancer Institute (NCI), Myriad RBM (Austin, TX) has developed a number of multiplex assay panels using this technology [5,6], including Human Oncology-MAP, which surveys 130 serum proteins that have been employed as cancer markers, including established diagnostic markers such as carcinoembryonic antigen (CEA) and CA125, and a number of important growth factors [7]. This assay can be run as a service by Myriad RBM, or validated assay kits can be purchased for use on Luminex instruments. The specimen required for this assay is 500 μL of patient serum, and this technique is therefore readily applicable in most clinical situations. The Myriad RBM Cytokine-MAP A and B assays are also examples of widely used assays, in this case, for immune response modeling [8]. Their use has even been extended back into preclinical development to assist in validating biomarkers for drugs under investigation. These assays have the additional advantage of requiring only a 50 μL serum sample volume. With the current surge of interest in immunotherapy approaches to cancer treatment based on recent impressive clinical trials results, we anticipate a continuing increase in the use of these assay panels.

The NCI has recently contracted Myriad RBM to produce a Luminex platform assay for apoptosis signaling pathway proteins [9], which is now commercially available from BioRad as a BioPlex kit and can be run on their xMAP multiplex magnetic bead-separation platform. Unlike the above-mentioned assays, this assay is intended to be run on tissue extracts (or cell extracts for preclinical work) and can be performed on a good-quality 18-gauge core biopsy sample (average wet weight, 7 mg). In preclinical applications, our laboratories use a 20-mg tissue piece to provide enough material for repeat runs of all analytes. The analytes are divided into three panels; thus if a fit-for-purpose biomarker of therapy effect has been validated, the researcher may choose to use only the panel containing that biomarker, providing additional materials for repeat specimen analysis. Importantly, the kit includes a set of calibrators for each analyte in the assay.

2.1.2. MesoScale Discovery MULTI-ARRAY platform

Another popular multiplex assay platform utilizing a two-site immunoassay approach is the electro-chemiluminescent MULTI-ARRAY technology from MesoScale Discovery (Rockville, MD). Here, each analyte is bound by a capture mAb that is pre-coated on a carbon electrode plate, and a second mAb conjugated to an electro-chemiluminescent dye reports the analyte concentration when voltage is applied to the carbon electrode plate. Commercially available multiplex assay kits for this platform are designed to assess biomarkers of cardiac, liver, kidney, or muscle injury, inflammation, cytokines and chemokines, and general toxicology, among others. For example, MesoScale kits measuring human growth factors and receptors have been used to correlate growth factor receptor inhibition and treatment with the receptor tyrosine kinase inhibitors foretinib and dovitinib in clinical trials [10,11]. All kits are species-specific, include control materials for standard curve generation, and are examples of how multiplex assays consisting of a small number of established disease biomarkers can be validated and implemented. The species restriction on kit utility is a reminder that antibody cross-reactivity between model system and human homologs will affect how the preclinical development of any biomarker is approached.

2.1.3. Reverse-phase protein array (RPPA) assays

RPPA assays provide functional proteomics analysis of complex signaling pathways by probing protein tissue or blood extracts spotted onto a slide with validated monoclonal antibodies under controlled conditions [12,13]. A number of RPPA assays have been developed and validated at the NCI and at MD Anderson Cancer Center [14,15], and organizations such as Theranostics Health and the MD Anderson Proteomics Core offer services for running patient specimen analyses [16,17]. The results from this technique are best analyzed by comparison to a drug-treated control tissue analyzed on the same slide set as the clinical sample. The use of tissue controls allows for scaling of assay values across multiple experiments, because calibrators are not available for the assays and only a single antibody is used to report each analyte. This approach has demonstrated utility and is especially well-suited for discovery work in complex systems [18–21]. For example, in developing predictive molecular markers for dasatinib treatment, RPPA identified 10 potential markers that were differentially expressed in dasatinib-sensitive and -insensitive cell lines, and researchers were able to build on this information to clarify the role of CAV-1–mediated interactions between EphA2 and BRaf on dasatinib sensitivity [14].

2.1.4. Advantages of assays for tissue lysates and blood samples

A major advantage of the two-site immunoassay format is the superior analyte specificity obtained by using two separate mAbs. Strategically chosen epitopes can report, for example, only full-length or only truncated proteins. The use of analyte calibrators in these assays also allows comparisons of assay values across laboratories and over time.

2.1.5. Disadvantages of assays for tissue lysates and blood samples

Validated commercial assays tend to be very costly; unfortunately, the front-end costs of development and validation to the degree required by regulatory agencies are the primary drivers of these costs, making this limitation unavoidable. Additionally,