Tissue-Based Multiplex Biomarker Assays and Companion Diagnostics

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COMPANION DIAGNOSTICS (CDx) and precision medicine are becoming increasingly complex and overlapping fields. With this complexity, new challenges arise, and novel approaches and techniques are needed to meet these challenges, including the discovery and development of multiplex biomarkers for companion diagnostics that indicate potential therapies for patients. Here we review the current state of multiplex biomarkers and discuss what solutions multiplexing can provide. We will also discuss regulatory and commercial considerations, along with workflow considerations, biomarker interpretation, clinical utility, market access considerations, and the importance of partnering.

What is Multiplexing and What Solutions Does it Provide?

Multiplexing can be defined as visualizing or measuring more than one biomarker in the same assay. A variety of techniques and technologies have been referred to as "multiplex" or "multiplexed" which may include, but are not limited to, DNA and RNA sequencing, mass spectrometry of proteins, microarray analysis of nucleic acids, and spatial transcriptomics of RNA. The focus of the current article is slide-based multiplexing, with a primary focus on techniques that preserve the tissue architecture, thus providing the biological and cell-specific spatial context of measured biomarkers. Examples of these tissue conserving techniques include fluorescence *in situ* hybridization (FISH) for nucleic acids and immunohistochemistry (IHC) for proteins, as well as newer techniques such as spatial transcriptomics for RNA.

Multiplexing is important because it provides solutions to several current challenges in personalized medicine. For example, as more actionable biomarkers are becoming available for patient testing across tumor types,[1] the need to perform more multiplex biomarker tests on a single specimen (such as a patient biopsy) has become increasingly prevalent. Slide-based multiplexing creates the opportunity to interpret multiplex biomarkers within the tissue and/or cell-specific

spatial context. As shown in **Figure 1**, two separate biomarkers can be visualized using two different chromogens on a single slide that contains tissue from a lung cancer (squamous cell carcinoma) specimen; the tissue section was prepared from a formalin-fixed, paraffin-embedded block using standard histological techniques.

PD-L1 as a Case in Point for Slide-Based Biomarkers

The ability to determine what cell types are expressing which biomarkers can be important both for biomarker interpretation (or scoring methods) and/or algorithms used by pathologists to interpret results from the slides. For example, in using PD-L1 IHC 22C3 pharmDx, both tumor and immune cells that stain for PD-L1 need to be considered for the scoring algorithm in certain indications.[2] A consideration when using non-tissue conserving techniques is loss of the identities of the cellular compartments or regions where the biomarker is being expressed. For some protein biomarkers nuclear or membrane localization is part of the scoring method. For example, PD-L1 in NSCLC is a biomarker that includes partial or complete cell membrane staining (when assayed using the Tumor Proportion Score with PD-L1 IHC 22C3 pharmDx).[3]

Slide-Based Multiplex and Personalized Medicine

Slide-based multiplexing can be important for personalized medicine as it creates a tissue and/or cell specific spatial context for interpreting, understanding, and extending the information captured from multiple biomarkers, which can be important in guiding treatment decisions in personalized medicine.

For example, if two biomarkers are measured on the same slide, it is possible to imagine a scenario where a patient could be positive for one biomarker and negative for another (see **Figure 2** for example scenarios). Depending on which of the two biomarkers is positive could affect the patient's eligibility for a particular therapeutic (Therapeutic A and B in **Figure 2**). Another scenario is possible with slide-based multiplexing. The patient is positive for both biomarkers and the biomarkers are expressed in close spatial proximity. This could make the patient eligible for a theoretical third therapeutic (Therapeutic C in **Figure 2**) that requires expression of both biomarkers in the same cell or in close spatially proximity within the tissue. Thus, with one slide, three potential therapeutics or therapeutic regimens could be considered based on the patient's tumor expression of biomarkers in the context of tissue architecture. This is essentially precision medicine on a slide.

Emerging Markers, Assays, and Treatments

Most new clinical trials since 2014 for anti-PD-1/PD-L1 are combination trials with other potential therapeutics.[4] Following the rise of cancer immunotherapies (for example, anti-CTLA-4 and/or anti-PD-1/PD-L1 antibodies) additional immune-oncology proteins are being targeted in clinical trials. Examples of these additional targets include LAG-3 and TIGIT.[5] IHC multiplexing facilitates visualization of multiple immune-oncology biomarkers and is a high-value tool for these therapeutics.

Multiple biomarkers that point to a single therapeutic might also be important with immuno-oncology therapy. For example, PD-L1 and deficient Mismatch Repair (dMMR) markers may prove to be a good example. dMMR markers indicate a deficiency in a series or system of mismatch repair proteins that can be visualized and/or detected by IHC. Several clinical studies are investigating the clinical utility of dMMR markers and immuno-oncology therapeutics such as PD-1/PD-L1 inhibitors.[6] Examining PD-L1 and dMMR biomarkers on the same slide could potentially aid in such trials and ultimately in diagnostics.

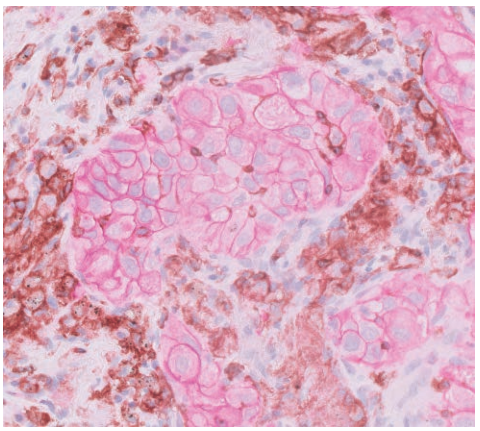

Figure 1: An example of tissue stained with two biomarkers, an anti-PD-L1 antibody (in Magenta) and an immune cell marker (in brown) in a squamous cell carcinoma lung cancer specimen. The immune cells can be visualized in the tumor area suggesting they are associated with the tumor sample. Tissue samples supplied by BioIVT (Hicksville, NY, USA).

For biomarkers to be actionable, there needs to be either a therapeutic treatment tied to the biomarker status or an actionable decision that allows the doctor and patient to plan next steps. Clinical utility is required for a CDx as approved by the FDA. Eligibility and selection of patients for treatment with a specific drug can require a CDx, which must be validated in clinical trials. To fulfill the regulatory requirements for using a CDx in clinical trials, and ultimately in personalized medicine in clinical use, a performance data package needs to be generated for the assay. Several parameters that must be analyzed for this data package include specificity, sensitivity, precision/reproducibility, robustness, and stability of the key reagents. For a multiplex assay to be used as a CDx, potential interactions of the two or more biomarkers included in the assay may need to be determined. Ultimately, clinical evidence needs to be generated that relates back to the intended use of the assay. Thus, personalized medicine is intimately coupled with companion diagnostics.

More Novel Therapies

As noted above, the advent of new therapeutic approaches that take advantage of multiple molecules may necessitate the visualization of multiple biomarkers on the same tissue sample. Combinatorial therapeutic trials with more than one therapeutic agent create scenarios where multiple biomarkers may need to be assayed in clinical trials. In addition to using multiple individual antibodies for therapies, other examples of new therapeutics include bi- and tri-specific antibodies.[7] Furthermore, technologies such as antibody drug conjugates (ADCs) and cell therapies may require measuring multiple markers in the same tissue. Tissue scarcity can also be an issue, particularly with indications that are less common.[8] Multiplexing for clinical trials, and in the post market setting following commercialization, creates the advantage of measuring multiple biomarkers from a single slide, which decreases the burden for clinical labs by reducing the number of sections needed per specimen.

How Multiplexed Companion Diagnostics Can Be Widely Adopted

For multiplexing to have worldwide commercial reach, a solution is needed that can be widely distributed. Low-plex technologies, such as IHC where 2-4 biomarkers are multiplexed, is such a solution. An example of such a technology is the Magenta chromogen offered by Agilent in combination with the brown chromogen DAB.[9] With low-plex, the reagents needed can be provided as a kit. Also, the reagents can be automated and deployed to the large, existing installed base of automated instruments.

Design control and quality systems are critical for compliance with regulations and establishing world-wide reach. Standardized instructions and controls enable the same quality of results from tests run at different sites.

Two critical components in performing an IHC assay (and several other slide-based assays) are the

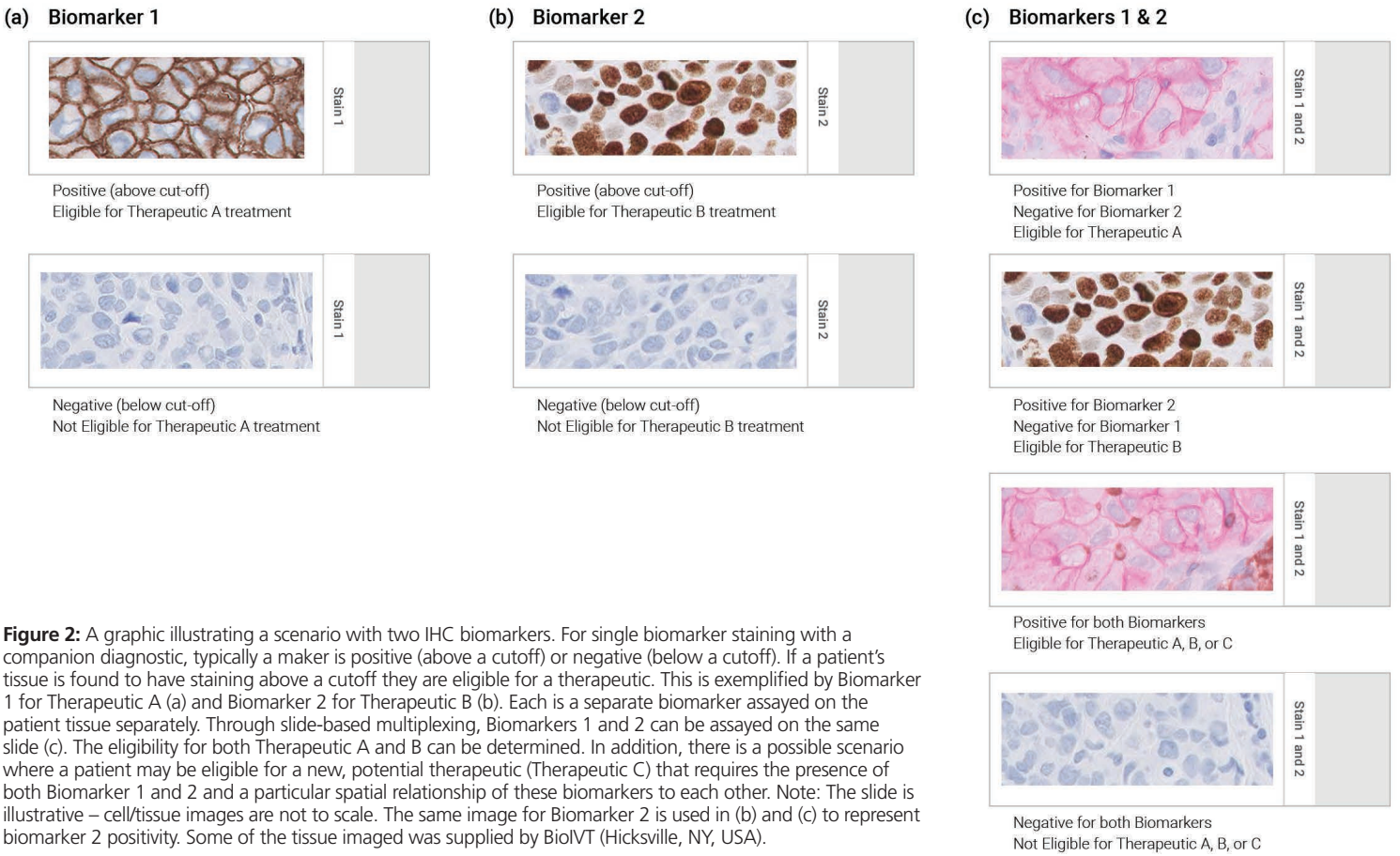

Figure 2: A graphic illustrating a scenario with two IHC biomarkers. For single biomarker staining with a companion diagnostic, typically a maker is positive (above a cutoff) or negative (below a cutoff). If a patient's tissue is found to have staining above a cutoff they are eligible for a therapeutic. This is exemplified by Biomarker 1 for Therapeutic A (a) and Biomarker 2 for Therapeutic B (b). Each is a separate biomarker assayed on the patient tissue separately. Through slide-based multiplexing, Biomarkers 1 and 2 can be assayed on the same slide (c). The eligibility for both Therapeutic A and B can be determined. In addition, there is a possible scenario where a patient may be eligible for a new, potential therapeutic (Therapeutic C) that requires the presence of both Biomarker 1 and 2 and a particular spatial relationship of these biomarkers to each other. Note: The slide is illustrative – cell/tissue images are not to scale. The same image for Biomarker 2 is used in (b) and (c) to represent biomarker 2 positivity. Some of the tissue imaged was supplied by BioIVT (Hicksville, NY, USA).

slide staining and the biomarker interpretation (scoring). Educational materials distributed with an assay should cover biomarker interpretation; such materials may include items that help make scoring consistent and reproducible, such as an interpretation manual and quick scoring guide. These materials are important since consistency and reproducibility are hallmarks for CDx utility. Manual scoring of the slides by a pathologist allows for a greater footprint of the assay as it eliminates the need for and reliance on digital pathology solutions (including costly slide scanners which many clinical labs may lack). However, it does not preclude the addition of a digital scoring solution to the manual scoring algorithm. A digital scoring solution can use the manual scoring data for training and testing sets of the digital algorithms.

A CDx assay design consideration is the ability to fit in the modern anatomic pathology laboratory workflow. To minimize disruption to the laboratory, IHC multiplexing must work with standard histology techniques. For example, the technology needs to be compatible with formalin-fixed, paraffin-embedded (FFPE) tissue and automated instrumentation. From an efficiency standpoint, slide-based mulitiplexing can be an improvement to the workflow for an assay as it decreases the overall number of slides run.

Pathologist Scoring and Interpretation Considerations – A Pathologist's Perspective

As described above, multiplexing has the advantage of doing more with less, by reducing the amount of tissue needed for a set of tests and deriving more information from a slide. For the pathology practice, maximizing information from limited tissue is a priority. Slide-based multiplexing (such as IHC) also preserves the morphology of the tissue and the spatial relationships found in the tissue, both of which are important for interpretation by the pathologist.

When considering the adoption of multiplexing within the pathology practice setting (including its effect on interpretation), another important consideration is the impact of different chromogens used and the comfort level of the pathologists with those chromogens. Historically in IHC, pathologists are very familiar with established stains such as DAB. For new combinations and/or chromogens, demonstrating the need and clinical utility of the assays will be important. Fortunately, other chromogens have already been introduced over time (such as red chromogen for heavily pigmented melanocytic lesions).[10] In addition, some of these newer chromogens have been included in "cocktail" combinations with the traditional DAB stain, such as red chromogen in both a cocktail for prostate cancer[11] and in one for lung cancer.[12] These combinations have already exposed pathologists to multiplexing in the practice setting, albeit more for primary diagnosis than for CDx up to this point. Further expansion to practicing pathologists (including a focus on the potential role to enhance CDx capabilities through multiplexing) could be accomplished through publications, journals, conferences, webinars, or combinations of all of these activities.

In addition to the use of novel chromogens, IHC algorithms and their impact on the ability to score a slide are significant elements in the adoption of multiplexing in a pathology practice setting. For example, it is more desirable to have different cellular compartments stained with each multiplexed biomarker.[13] A brown stain such as DAB could stain a biomarker in the nucleus, and a different color stain could be used for a biomarker that is located at the membrane; this compartmentalization of the staining facilitates proper scoring by making the markers visually

distinct. A more difficult scenario is where both biomarkers are localized to the membrane and the staining overlaps, potentially making single expression of the biomarkers or co-expression difficult to differentiate. In this case, careful consideration of chromogen may facilitate differentiation but needs to be determined in further testing.

Other potential uses of multiplexing for companion diagnostics (beyond primary diagnosis) include improving the readability of biomarker status determination. For example, for difficult tissues, additional markers beyond the primary biomarker could potentially be used to help differentiate tumor cells from other cell types and aid in the evaluation of the primary biomarker. Also, for additional tissues with difficult biomarkers, staining with a different biomarker could aid in interpretation by enhancing the biomarker of interest.

Thoughts on Future Directions

Currently, low-plex (2-4 biomarker) IHC assays have the advantages that they can fit into the modern pathology workflow, and they can simultaneously measure both biomarker expression and cellular and tissue morphology. However, there are also other technologies that simultaneously examine tissue morphology and the expression, function, or aberrations of genes in a highly multiplexed manner. Some of these techniques are introduced below as examples of potential future directions.

High content (or high-multiplex) IHC is often performed by having a large number of fluorescent markers stain a tissue, giving insights into multiple biomarkers as well as different cellular components. A number of different methods have been developed to perform high-multiplex IHC, with several now being offered commercially.[14] These techniques can be useful in translational research to follow multiple biomarkers in order to determine correlations of different biomarkers and/or cells with response to therapy or other endpoints. High-multiplex fluorescent IHC can also be used to investigate other aspects of tumor biology, such as the interaction of different cells within the tumor and the microenvironment.[14] However, it is unclear whether highly-multiplexed IHC (vs low-multiplexed IHC) will have a clinical need, or whether it will be mainly used to research a number of different biomarkers at once, in order to define a smaller set that will have clinical utility.

A set of techniques sometimes called spatial transcriptomics has been gaining popularity in recent years due to the ability to examine the expression of thousands of RNAs at once in a single tissue section.[15] One method, available commercially through 10X Genomics,[16] involves making cDNA from mRNA sequences inside a tissue section using spatially-barcoded primers attached to a DNA microarray, resulting in a spatially-barcoded RNA-seq library. Sequencing the library and mapping the spatial barcodes onto the tissue image gives a highly multiplexed *in situ* view of gene expression. Another method, commercially available from Nanostring,[16] involves hybridizing complementary probes to RNA in a tissue section. These complementary probes each contain a UV-cleavable barcode sequence. Regions of interest in the tissue section are defined, and UV light is used to cleave the barcodes of probes hybridized to RNA within these regions. Analysis of the barcodes reveals the identity of the genes expressed in these regions of interest. These techniques allow for a highly-multiplexed view of gene expression in a tissue section. However, while these spatial transcriptomics methodologies are currently being used for research and biomarker discovery, they may not have clinical utility in their present form due to the cost and complexity of the assays.

Market Access Considerations

For now, low-plex IHC testing is a commercially viable solution that can improve operational efficiency and provide economic benefits. Payers, government authorities, and laboratories continue to look for the highest quality testing that can improve clinical outcomes while reducing overall costs, and multiplex testing provides this opportunity for optimal care.

Utilization of low-plex IHC assays allows for efficient biomarker interpretation, tissue preservation for additional studies, and a short turnaround time (TAT). In-house testing TAT is positively impacted through the use of IHC-based assays compared to NGS testing, as a typical IHC test has a TAT of 1 to 2 days, while an NGS test takes 10 to 21 days for results to become available.[17] Additionally, the cost of IHC-based assays is low, ranging from $40-140 compared to $1,000-4,000 for NGS tests.[17]

In the United States, multiplex also allows for an overall increase in reimbursement to laboratories compared to performing multiple single-antigen tests.[18,19] Testing for biomarkers that are prevalent across multiple tumor types (for example PD-L1) further improves the overall cost-efficiency of IHC-based testing compared to molecular testing for rare genomic alterations (for example NTRK[20]) as more eligible patients will be identified for therapies of interest. Furthermore, multiplexing has the potential to optimize treatment decisions by mitigating current challenges associated with therapy selection and patient management.

Partnering Considerations

Not only can the technology for a multiplex assay be complex, but the regulatory and commercialization paths for a CDx can be as well. A close partnership between the CDx manufacturer and the therapeutic partner is an important component. A CDx partner should have experience in developing assays with reproducible results, which includes both the assay itself and the subsequent interpretation. Partners should be knowledgeable about the modern pathology laboratory, software tools, and pathologist workflows, all of which need to be considered

Table 1: Important considerations for partnerships between medical device and pharmaceutical, biotechnology, cell therapy, and/or gene therapy companies

Considerations when partnering with a CDx manufacturer
• Companion diagnostic expertise, not just technology expertise
• Experience with assay biomarker interpretation (scoring) and development
• Expertise in developing assays with consistent results
• An understanding of the precision medicine space and the companion diagnostic market
• Regulatory experience
• A proven track record with commercialization of companion diagnostic assays

Table 2: Summary of the advantages of slide-based, 2-4 biomarker multiplexing (low-plex) for CDx today

• Tissue architecture and biomarker expression can be preserved
• The spatial relationship between multiple biomarkers can be visualized (including evaluation of different cell types and/or different cellular compartments of the same cell)
• A fit in the modern anatomic pathology workflow
• The ability for pathologists to interpret the biomarkers manually, but also have the potential to incorporate a digital algorithm
• A path to commercialization

during assay development. A CDx manufacturer also needs to have regulatory experience in gaining worldwide approvals as well as commercial, global reach with products and sales channels. Important considerations for partnerships between the CDx manufacturer (medical device) and pharmaceutical, biotechnology, cell therapy, and/or gene therapy companies are summarized in **Table 1**.

Summary

Multiplexing, especially slide-based multiplexing, provides the ability to derive information on a multitude of biomarkers while preserving tissue architecture and tissue and/or cell-specific context. **Table 2** lists some of the advantages of a low-plex (2-4 biomarker) approach. In particular, low-plex slide-based multiplexing provides the advantages of preserving tissue architecture as well as biological and spatial context while paving a path to commercialization, as it can fit into the modern pathology laboratory and be scored manually by pathologists. JoPM

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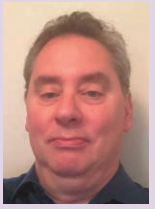
Mark Verardo has 15 years of experience in both pharmaceutical and medical device companies developing biomarker assays for research through clinical stage programs, including multiplex assays. In his current role at Agilent Technologies he part of Companion Diagnostics with a focus on strategic and technical alignment of diagnostic and therapeutic programs. He earned his PhD from UCLA and received post-doctoral training from UC Santa Barbara.

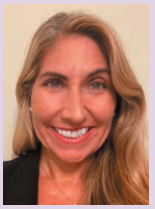
Jim Christian, MD

Jim Christian, MD is a Staff Pathologist within the Companion Diagnostics Division of Agilent Technologies, involved in multiple projects focusing on the development of biomarker assays for specific oncologic indications and their corresponding therapeutic products. Dr. Christian graduated from Wake Forest University, and obtained an MS in Anatomy and medical degree from Virginia Commonwealth University School of Medicine. He completed Pathology residency training at Wake Forest University Medical Center, and is board certified in Anatomic and Clinical Pathology. He also completed fellowships in Urologic Pathology at Bostwick Laboratories and in Surgical Pathology at the University of California, San Diego. Prior to joining Agilent Technologies, Dr. Christian practiced pathology in multiple hospital and laboratory settings.



Robert Ach, PhD

Robert Ach has over 20 years of experience in Agilent Laboratories developing novel multiplex methodologies to measure DNA and RNA in biological samples. He earned his PhD at Yale University and conducted postdoctoral studies at the University of California, Berkeley.



Dana Dilbeck

Dana Dilbeck has over 20 years of Healthcare experience primarily in the Oncology sector. Most recently in the life science industry focused on molecular diagnostic testing to advance cancer care. Currently the Senior Director Market Access, Reimbursement and Pricing for Agilent. Previously served as Director of Payer Relationships and Reimbursement for Pacific Edge Diagnostics, a New Zealand based company. In addition, Vice President for a molecular diagnostic company, Molecular Health, that provides a large next generation sequencing (NGS) panel for oncology care. Worked for McKesson Specialty Health I US Oncology for over 15 years focusing on Payer Strategy and Public Policy for the National Network of over 1000+ physicians. An expert in physician reimbursement and managed care pricing strategies to address high-cost cancer care services. Holds a bachelor's degree in Economics and Management from Rice University.

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Karina Kulangara, PhD

Karina Kulangara, PhD has over 10 years of experience in medical devices. She joined Agilent Technologies 7 years ago and held leadership positions in product development, she led the development of the first companion diagnostic device for PD-1/PD-L1 checkpoint inhibitors in partnership with Merck&Co. She was also involved in the blueprint I study comparing several PD-L1 assays in the pre-market setting. She holds a PhD degree from the Swiss Federal Institute of Technology, Lausanne, Switzerland and conducted postdoctoral studies at Duke University.

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