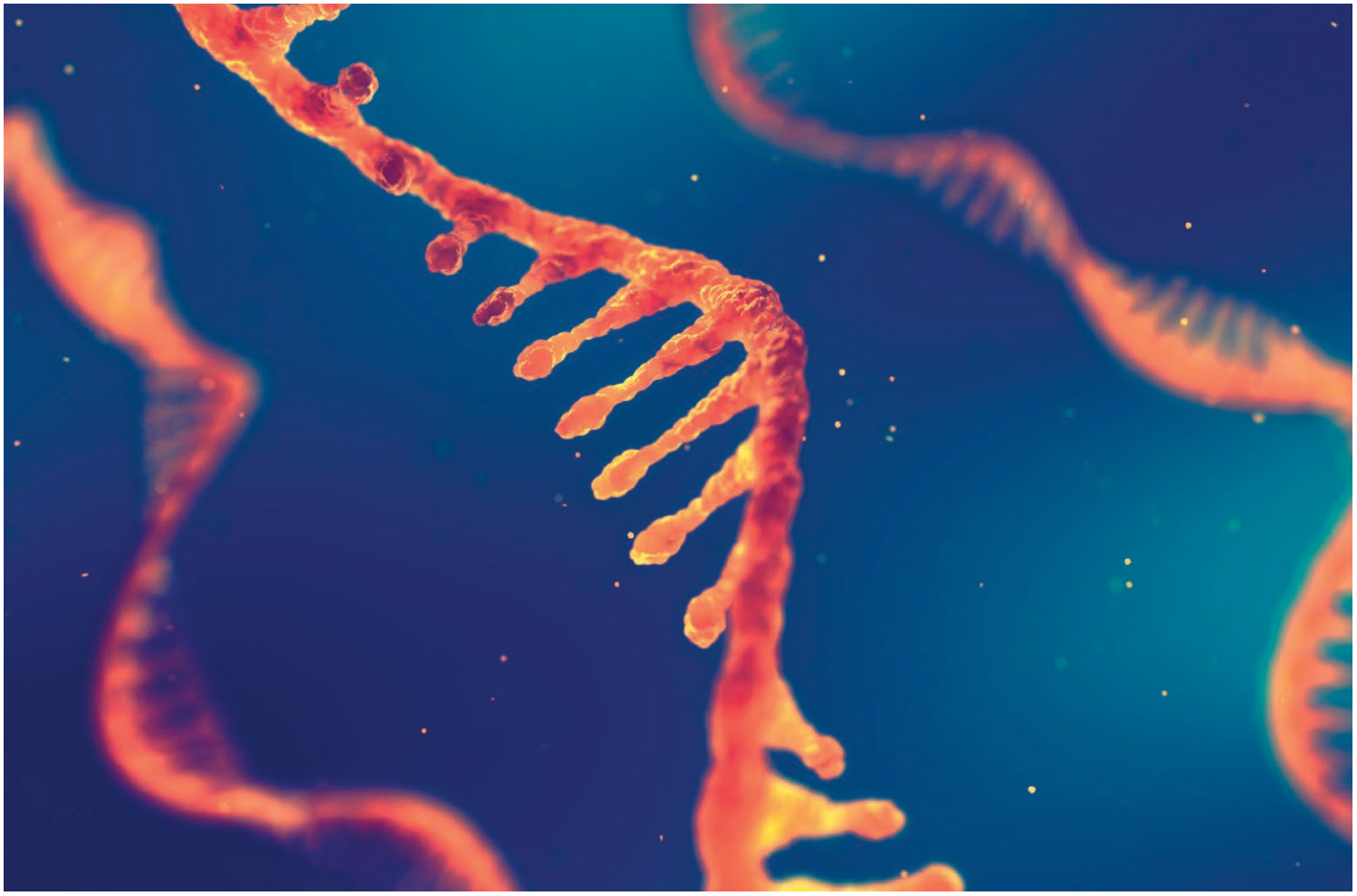

Multidimensional Predictive Diagnostics: The GPS of Clinical Practice

Moving Beyond a Static Roadmap for Diagnostics

By **Natalie A. LaFranzo, PhD**

Those in the precision medicine field recognize that measuring a single-analyte is insufficient to capture the complexity of disease and accurately chart the best path of treatment. By way of example, the European Society for Medical Oncology (ESMO, 2017) highlighted the need for a multivariate approach to predictive diagnostics, citing that:

"PD-L1 IHC positivity [alone] is an imperfect biomarker of response and currently not suitable as a definite biomarker for selection for therapy with PD-1/PD-L1 inhibitors. It is likely that a more complex, multicomponent predictive biomarker system will be required to refine appropriate patient selection for PD-1/PD-L1 blockade."[1]

In 2019, this was reinforced in an analysis of all US Food and Drug Administration (FDA) approvals of immune checkpoint inhibitors.[2] The authors found that in the 45 FDA drug approvals across 15 tumor types from 2011 until April 2019

"PD-L1 was predictive in only 28.9% of cases, and was either not predictive (53.3%) or not tested

(17.8%) in the remaining cases." The authors note that "immune-based interactions are dynamic and complex" and reinforce the need for "reliable and dynamic predictive biomarkers."[2]

The need for multivariate, predictive biomarkers has led to new approaches that are enabling diagnostics to move from discrete, static measurements to holistic, multidimensional assays. But not only are new analyses available – these approaches allow clinicians to ask more comprehensive questions that test more than one hypothesis at a time.

Powered by the dynamic nature of RNA models and the utility of machine learning to identify useful signals and patterns, Predictive Immune Modeling untangles the inherent complexity of biology first in detecting immune response and then building biomarker assays for predictive diagnostics. With Predictive Immune Modeling, we are on track to develop our own diagnostic global position system (see inset on GPS model), and help clinicians better navigate a patient's treatment path (see more details on the ImmunoPrism Assay at https://cofactorgenomics.com/immunoprism-assay-2/).

Case Study 1 – Calibrate Your Location

The first step in building any useful global positioning system is triangulating your location. How do we know we are where we think we are? In the diagnostic space, this reckoning is analogous to a technical or analytical validation.

In 2020, the first analytical validation of a Predictive Immune Modeling platform was published in the Journal of Molecular Diagnostics by the team at Cofactor Genomics.[3] Here, the authors describe how to determine immune cell composition accurately in formalin-fixed, paraffin embedded (FFPE) tissue sections by building a database of immune Health Expression Models, or iHEMs (**Figure 1**). These iHEMs are made up of hundreds of RNA signals, which uniquely define an individual immune cell or state in the heterogeneous mixture of tumor tissue and the tumor microenvironment.

To illustrate the importance of multidimensional gene expression analysis of a complex tumor environment, consider the example of T-regulatory cells or T-regs, known to be important in tumor immune escape, and "often associated with a poor clinical outcome."[4] In most studies, the cellular marker FOXP3 is used to identify this cell population using flow cytometry or immunohistochemistry (IHC). Flow cytometry, on the one hand, is highly sensitive and specific at detecting populations of cells with multiple immune markers, but requires fresh tissue for analysis. Fresh tissue is difficult to acquire and store in a clinical setting, and FFPE archiving remains the recognized clinical standard. On the other hand, IHC, while suitable for FFPE tissue analysis in the clinic, only allows for the detection of single-analytes. Multiplex imaging technologies address this, but have yet to move from the research and translational setting into clinical practice.[5]

In this context, RNA-based approaches overcome the shortcomings of both flow cytometry and IHC assays; FFPE molecular protocols are now commonly used to quantify transcriptomic signals in a highly-multiplexed fashion. Notably, RNA-based diagnostics have already been shown to have utility in the clinic, with Oncotype DX[6], Veracyte Affirma[7], and Agendia MammaPrint[8], specifically used in the oncology setting. While other software-based immune cell deconvolution methods have been reported on, they are powered primarily by public data

Figure 1: A T regulatory immune Health Expression Model (iHEM) is depicted with each point representing a unique gene, and the position of the point representing expression level.

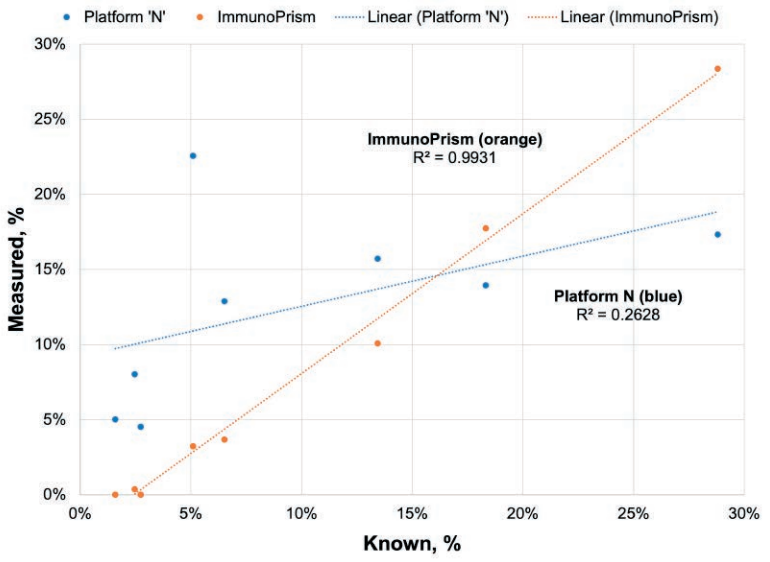

Figure 2: Results comparing the quantification of known mixtures of T-regulatory cells using single-gene approach (Platform N) and an iHEM-based approach (Cofactor ImmunoPrism®).

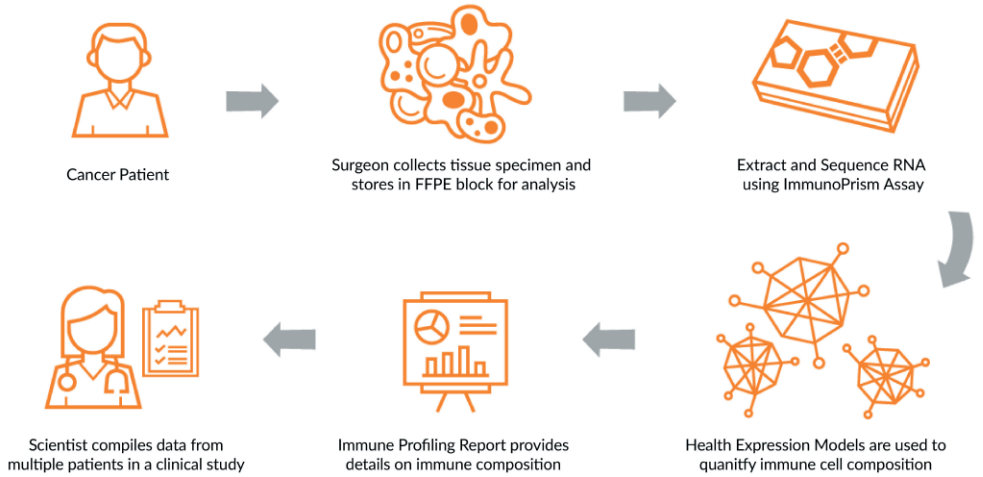

Figure 3: The workflow for a predictive immune profiling platform known as ImmunoPrism® begins by collecting FFPE tissue from a cancer patient. RNA is extracted and analyzed, and compared to a database of iHEMs to quantify immune cell composition in the tumor tissue.

sets – including microarray data – which is often very noisy. The team at Cofactor sought to build a new database and approach with a standardized molecular protocol to overcome historic challenges and build on the clinical use of RNA.[9] New ways of leveraging RNA data to measure the complex immune system are necessary to address the needs of immune oncology; this publication marked the first example of moving beyond ranked gene lists, gene profiles, and gene signatures into machine learning-derived models.

From this need, immune Health Expression Models have emerged. In short, these RNA models were built using multiple examples of isolated immune cells from deep-sequenced, transcriptomic profiles of each immune cell type. Then, using machine learning technologies, multi-gene models of RNA expression levels that uniquely define each immune cell type are generated. In the previously cited Cofactor Genomics' publication,[3] Schillebeeckx et al use increasingly complex mixtures of known cells for an analytical validation of this approach using orthogonal technologies such as flow cytometry and IHC. They found that these models enabled the quantification of immune cells in heterogeneous tissue at the same detection level as flow cytometry, yet accessible for FFPE specimens. Bringing this back to our T-regulatory cell example, data (unpublished, internal Cofactor Genomics) comparing iHEMs to another platform that uses expression levels of only one gene to measure these immune cells (FOXP3) shows that the iHEMs are more accurate in quantification of known mixtures (**Figure 2**).

In itself, this is a significant step towards better characterizing the immune response at the site of the tumor. However, knowing our "location" with higher accuracy is only one step in building a clinical map or diagnostic.[10]

"In our precision medicine "GPS" paradigm, charting a path means enabling clinicians to use this molecular information to make more informed treatment decisions."

Case Study 2 – Chart Your Route

In our precision medicine "GPS" paradigm, charting a path means enabling clinicians to use this molecular information to make more informed treatment decisions. Predictive Immune Modeling enables us to move from traditional single-analyte, 'multigene', or panel-based approaches, to true multidimensional biomarkers. In oncology, this means integrating multiple immune-related signals into a classifier. An example protocol for starting down this path of biomarker discovery to diagnostic development was published by Cofactor Genomics in the Journal of Visualized Experiments.[11] Importantly, because the assay leverages RNA data, we are also able to measure and integrate known signals such as immune escape/co-inhibitory and costimulatory gene expression. In doing so, we build a more comprehensive and more accurate predictive model.

Step 1: Collect information from individual patients

First, we can use the iHEMs to quantify the immune composition of individual patients by analyzing RNA collected from patients' solid tumor tissues. Other immune-related transcriptomic signals are also measured and reported for each patient (workflow summarized in **Figure 3**).

Step 2: Repeat for well-defined and well-annotated clinical cohort to identify and validate biomarker

Following recruitment of a well-defined and well-annotated cohort in a retrospective study, we can begin to build biomarkers or clinical decision tools with this data. For precision medicine in oncology, an example cohort would consist of responders and non-responders to immunotherapy. The signals in the individual patient profiles are considered alongside their clinical response data, and again using machine learning, a multidimensional biomarker is generated that marries together the most useful signals to uniquely define response (see process schematic **Figure 4**). The resulting biomarker may have predictive ability, reported as sensitivity, specificity, false positive, and false negative

An origin story:

A few years back, as the holidays approached, my younger sister reached out looking for gift advice for me. After some thought, I suggested she might purchase a new road atlas for my car, which resulted in much confusion. Wouldn't I be happier with a new GPS device? I politely declined, explaining the value of having maps not reliant on electricity or cellular signal. That Christmas I became the proud owner of a new Rand McNally Road Atlas. While I thought having a static roadmap for driving would give me peace of mind, the reality is that whenever I get in the car, it's my phone's GPS application that I use. I have yet to use the road atlas for its intended purpose, and in fact, it's now outdated. Today, precision medicine is in a similar situation. We've come to accept that the next generation of predictive diagnostics are necessary for making real-time clinical decisions, yet, we still struggle to let go of the classical methodologies of the past, analogous to considering one point at a time for driving instructions in analogy with viewing several possible routes on a near-real time GPS map (e.g., updates on traffic, road closures). Extending this analogy, I see many parallels between the evolution of navigation systems and building predictive diagnostics, particularly in the field of oncology. Here, with the insights gained through immune-oncology, we are much more aware of the impact of the immune system on the progression and treatment of cancer.

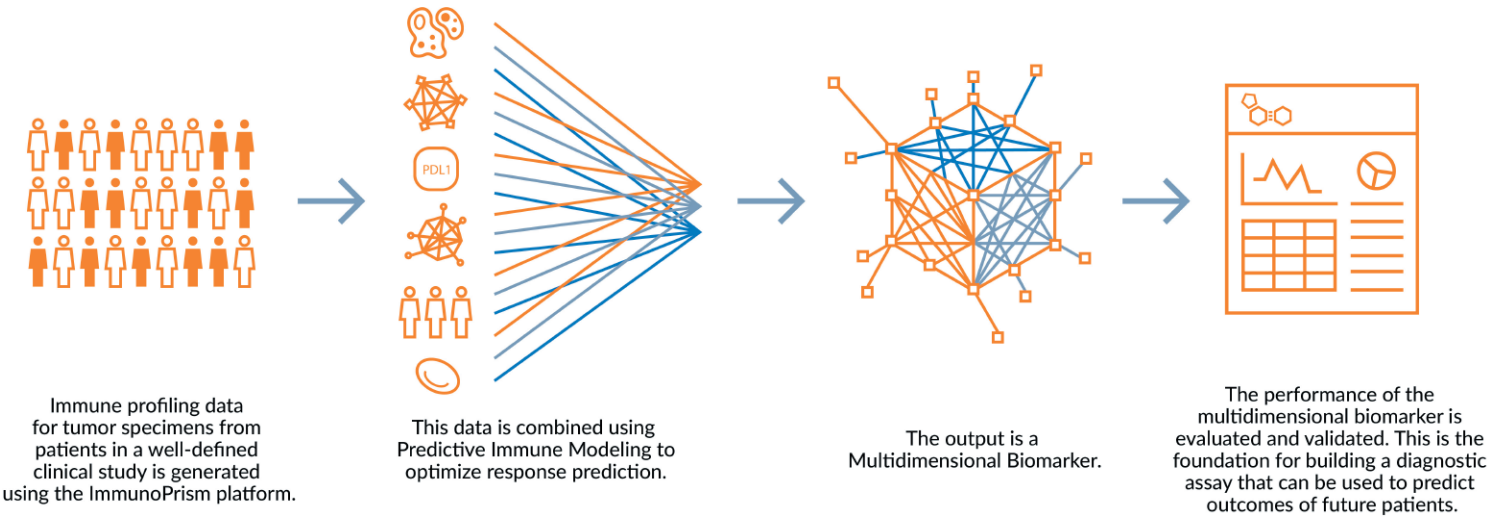

Figure 4: The process of building of building a multidimensional biomarker relies on collecting immune profiling data from a well-defined patient cohort. This data is used in machine learning software to identify the combination of analytes that will result in the highest predictive value. The performance of the biomarker is evaluated, and then validated.

values (to be further validated with an additional patient cohort). At this stage, as we formalize the process in a framework such as that supported by CAP/CLIA, we begin moving from biomarker to laboratory-developed diagnostic (LDT) with documented procedures and reports.

Step 3: Deploy Diagnostic in Clinical Setting

Once the diagnostic test is validated, we are ready to deploy in the clinic, as shown in **Figure 5**. RNA from the tumor tissue of a newly diagnosed patient may be analyzed and the results compared to our validated multidimensional biomarker to provide guidance on whether the patient is likely to respond or not respond to a specific therapy, as determined in our retrospective studies.

Together, these steps take us from defining a map, to measuring a known location, to plotting the best course moving forward, akin to the process of programming a precision medicine GPS.

Case Study 3 – Providing Directions

Thus far, we have described the utility of a multidimensional predictive diagnostic in the immune oncology setting very generally. What is a specific example of where this technology might make a significant impact? When might a clinician look to predictive diagnostics to provide information to address a clinical question?

Consider the particular example of recurrent and metastatic squamous cell carcinoma of the head and neck (or RM-HNSCC). Immune checkpoint inhibitors (ICIs) are a class of immunotherapies that have shown promise for treating RM-HNSCC, as well as across multiple indications. The specific clinical question in this setting is whether to treat a patient with an ICI. The response to Pembrolizumab alone or with chemotherapy was evaluated in the KEYNOTE-048 study.[12] Here, and for pembrolizumab therapy in general, the on-label diagnostic indication is once again PD-L1 IHC. In this study, the diagnostic is used to drive clinician confidence in prescribing pembrolizumab. If the patient's tumor tissue shows high levels (≥20 combined positive score, or CPS) then the patient is considered to be a likely responder to pembrolizumab and therefore, this is the most promising clinical path. As we now know, this assay alone does not provide adequate characterization of a patient's immune profile and has limited predictive ability. Many clinicians familiar with the study and the clinical data lack confidence that KEYNOTE-048 does not establish the measurement of a single analyte, PD-L1, as an adequate predictor of response.

As such, studies to integrate more immune markers into predictive diagnostics for this clinical decision have the potential to make an impact in patient outcomes and cost of healthcare. An unpublished economic study (contact author for detail) showed that in addition to greatly increasing confidence in predicting patient

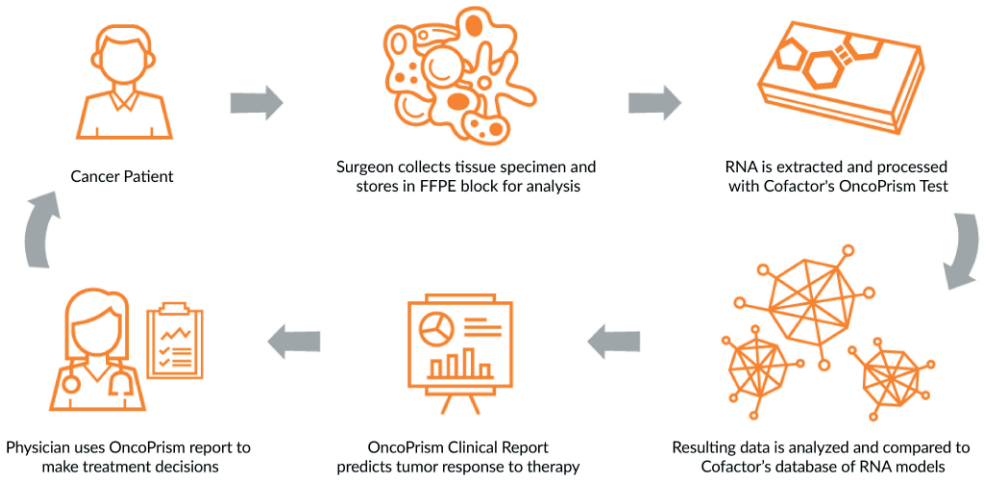

Figure 5: Once a diagnostic using a multidimensional biomarker is validated following standardized methods in a CAP/CLIA accredited laboratory, it can be deployed in the clinic as a laboratory developed test (LDT). Here, the individual patient's immune profiles are compared to the multidimensional biomarker to predict likelihood of response to therapy. These results may be used in clinical decision making.

outcomes, eliminating false positives in this specific clinical question could save the healthcare system up to $28,000 per false-positive patient avoided. By leveraging predictive multidimensional biomarkers, we can fill a need to deliver more clear and confident predictions for treatment decisions in this space. And in fact, Predictive Immune Modeling has been deployed here with promising results in a study from Washington University, not only eliminating false positives but also more accurately identifying a larger likely responder population.[13] These results were presented at the 2020 Multidisciplinary Head and Neck Cancers Symposium as a case study in supporting ICI treatment decisions.

"Predictive Immune Modeling has been deployed here with promising results in a study from Washington University"

Unique Navigational Paths

Similar to navigating any terrain, a healthcare provider considers multiple paths from which to choose, and molecular information aids the provider to select the best path depending on the circumstances. To continue with the PD-L1 case study, the path to replacing PD-L1 IHC requires "additional studies ...t o establish reliable and dynamic predictive biomarkers that may vary across tumor type and indication."[2]

By establishing Predictive Immune Modeling as a new **platform-based approach** for measuring immune signals with accuracy, and combining these signals into predictive biomarkers that may be clinically validated, the team at Cofactor Genomics are streamlining the process to more "effective and efficient clinical trials", cited as a need in the industry.[10] In fact, the Predicting Immunotherapy Efficacy From Analysis of Pre-treatment Tumor Biopsies (PREDAPT) study aims to do this, leveraging a single platform to predict tumor response across a wide range of cancers.[14] To ensure further success, this study is maximizing diverse patient recruitment by using multiple sites through the support of Curebase, a clinical research organization that enables highly virtual clinical trials with an eClinical Platform, complete with eConsent, electronic data capture, and remote monitoring capabilities. And, starting first with already-approved therapies such as immune checkpoint inhibitors, we are ensuring that the resulting predictive diagnostics have immediate utility.

The Future of Precision Medicine Relies on Predictive Diagnostics

As the future unfolds with benchtop sequencers and cloud computing growing in power and decreasing in cost, coupled with an expansion of virtual trials and study management, we anticipate that Predictive Immune Modeling studies will be launched for all immune-related diseases and therapy combinations. While the direct link to the immune system and the response to immunotherapies is obvious, there is growing evidence that shows that other standard-of-care therapies including chemotherapy and radiation therapy induce and are influenced by immune responses that impact treatment outcome.[15,16]

At Cofactor, we believe in the power of Predictive Immune Modeling. We're confident that the future of diagnostics requires the three facets of this new category including (a) predictive insights for confident treatment decisions, (b) a focus on immune response and markers, and (c) a holistic, multidimensional view enabled by machine learning. Using the approach outlined here, with RNA-based multidimensional biomarkers integrated into clinical diagnostics, the treatment decision-making paradigm can be streamlined while greatly expanding the information leveraged for each therapy decision. Predictive Immune Modeling is the key to closing the precision medicine gap that exists between powerful therapies, and the patients that will benefit most. JoPM

Natalie LaFranzo, PhD

Natalie first joined Cofactor Genomics in 2013, with a PhD in Chemistry from Washington University in St. Louis. Natalie has developed customized experimental solutions for both DNA and RNA applications at Cofactor, as well as launched and supported diagnostic reference standards as a part of Horizon Discovery's Diagnostics Division. Natalie supports Cofactor's clinical collaborations, outreach efforts, and marketing strategy as the VP of Market Development, with the goal of closing the precision medicine gap through Predictive Immune Modeling.

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Natalie LaFranzo, PhD is Vice President, Market Development of Cofactor Genomics. Using Predictive Immune Modeling and RNA, Cofactor Genomics is building multidimensional models of disease to deliver true precision medicine for improving patient outcomes.

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