The critical role of NGS reference materials in precision oncology applications

Cancers can be caused by a multitude of distinct genetic abnormalities, and each tumor is unique. Dozens of new targeted therapies have been developed in recent years, but the challenge of providing optimal care to cancer patients lies in the ability to match the genetic profile of the tumor to the most appropriate therapy. Advances in next generation sequencing (NGS) technologies have enhanced the identification of hundreds of mutations that may contribute to disease progression, and the development of new diagnostic NGS assays is continually evolving. These assays can profile genetic mutations to enable precision diagnostics, personalized treatment selection, stratification of patients for clinical trials, and improved disease monitoring.

Translating genomics research into the improvement of patient outcomes requires the development of robust assays that can perform with the accuracy, sensitivity and reproducibility required for clinical use. A crucial component of this assay development is the choice of reference material, which ensures the test is performing as expected and provides confidence in the results that will be used to monitor patient treatment and guide clinical outcomes. However, not all reference materials are created equal, and selecting the most appropriate provider is a key consideration when developing and validating a new assay.

REFERENCE MATERIALS

Reference materials are widely used in biomedical research for the development of diagnostic tests. Traditional vendors of reference materials – including Coriell Institute, ATCC, the US National Cancer Institute and many more – provide biobanked tissue samples, cell lines and nucleic acids that have gone through Institutional Review Boards (IRBs) for approval to support assay development. Prior to the advent of genomics methods, reference materials were typically single-variant cell lines and other materials of low plexity.

Figure 1: Development of assays for clinical applications

However, reference materials evolved to meet the needs of highly multiplexed methods such as NGS. In addition, it is becoming more important to use advanced, high-quality reference materials designed for genomic methods in order to translate findings from biomedical research into clinical practice.

Several third-party genomic reference material providers are working to support the development and analytical validation of NGS assays for clinical use. SeraCare (now part of LGC Clinical Diagnostics) has been manufacturing reference materials for use in clinical chemistry, molecular diagnostics, serology and immunochemistry assays for over 30 years. In 2015, SeraCare expanded this expertise to include reference materials focused on NGS assays for oncology and reproductive health. These NGS-focused genomic reference materials now cover RNA and DNA-based tumor profiling, liquid biopsy assays for disease diagnosis and monitoring, and immuno-oncology (I-O) biomarkers, such as tumor mutation burden (TMB) and microsatellite instability (MSI).

TUMOR PROFILING

Balancing sensitivity and quantity

Traditionally, tumors have been tested for a small population of biomarkers most commonly associated with specific tissues of origin. However, in many cases, iterative testing (also known as reflex testing) can delay accurate diagnosis and administration of optimal treatment. This issue is compounded by a steady recent increase in the number of targetable biomarkers for tumor profiling. In theory, this should improve patient care by offering more opportunity for diagnosis or treatment. However, when combined with the diversity of cancer cases, this presents one of the biggest challenges to oncology. Although a large proportion of tumors is driven by a small quantity of commonly mutated genes, there are a significant number of cases that are driven by rarer mutations. Many labs therefore endeavor to use comprehensive testing methods to obtain actionable information efficiently at the earliest opportunity.

The use of NGS panels to assess numerous genes in a single assay is ideally suited to tumor profiling, and provides several other key benefits to patient care decisions. Any given proto-oncogene can give rise to oncogenic mutations at multiple loci, so by providing single-base resolution of DNA or RNA, NGS can accurately identify both known and previously unidentified candidate cancer mutations. It is therefore critical for NGS assays to be able to test for a large number of distinct biomarkers with the required sensitivity and accuracy for their use in clinical care, and the performance of these tests must be rigorously validated.

Additional hurdles in tumor profiling include the limited availability of tissues for testing, and complications arising from tissue biopsies, emphasizing the importance of selecting the optimal testing method. Tissues are often preserved as formalin-fixed, paraffin-embedded (FFPE) samples, which can introduce challenges with the degradation of nucleic acids and generation of artifacts. These technical challenges necessitate the validation of assay performance on specific sample types, creating a demand for high-quality FFPE-based genomic reference materials. SeraCare therefore provides reference materials that focus on specific tumor types – as well as materials with more comprehensive applications – available in DNA, RNA or FFPE formats.

Selecting the most appropriate NGS assay

It can be a challenge to choose between a comprehensive NGS assay method – that covers most or all known cancer-related genes – or a more focused, targeted NGS panel, which tests fewer genes with known relevance to a specific tumor type. Comprehensive genomic profiling (CGP) assays enhance the probability of including the actionable gene(s), and profile large portions of the genome to routinely identify small abnormalities, such as single-nucleotide variants (SNVs) and insertions or deletions (INDELs). However, most assays do not identify all types of cancer-causing variants. For example, copy number variations (CNVs) and translocations resulting in RNA fusions can be more difficult to assess, and may require additional modifications in assay development. On the other hand, smaller targeted NGS panels provide a higher level of sensitivity for the actionable variants of interest. This enhanced sensitivity may be required when the driver gene variants exist at low frequencies. The selection of testing methods should therefore be guided by expectations based on tissue type or tissue availability.

There is no single perfect assay that can suit all applications and tissue types, especially since both the methods and our knowledge of genetic markers continue to evolve. Recent advances in scalable, high throughput NGS platforms have the potential to enable more thorough sequencing for larger NGS panels, but the associated hardware costs could also be prohibitive for labs already invested in smaller platforms. As a result, many clinical and molecular diagnostics labs would rather use NGS panels that focus on a set of known mutations in a specific tumor type, and have established their own laboratory developed tests (LDTs). These LDTs for clinical evaluation of patient disease status, which can be based on commercially available NGS assays, are validated using contrived reference samples. For example, the Hematology Lab at Sunway Medical Centre in Malaysia designed a multi-gene NGS panel targeting variants in myeloproliferative neoplasms (MPNs).[1] The team performed analytical validation on the assay, and subsequently obtained approval

from appropriate regulatory bodies to support its use in clinical decision-making and management of MPNs. This process included the use of high quality genomic reference materials – such as Seraseq Myeloid Mutation DNA Mix – as positive references for mutant alleles in mutation hotspots. However, it is important that LDTs are still fit for purpose as our understanding of the underlying biology continues to evolve, and many institutions that develop their own NGS panels in-house will find that they need to update their LDTs as research continues to discover new biomarkers, or as better resources become available.

The intended application for the assay is also an important consideration, and most commercially available NGS assays are designed to assess a variety of variant types, tumor types, or both. For example, myeloid neoplasms are commonly associated with a large number of genomic alterations that include SNVs, INDELs, and RNA fusions/translocations. The Pathology Lab at the University of Alberta Hospital selected the Oncomine Myeloid Research NGS panel, which assesses both DNA and RNA in a single assay workflow, to provide in-house disease profiling for patients with myeloid neoplasms.[2] Analytical validation of this assay was performed using both DNA- and RNA-based contrived reference materials that included commonly found or difficult to detect myeloid-associated variants, available from SeraCare.

Combining assays

Some institutions may develop NGS-based LDTs to complement other diagnostic tests. This has become common in the detection of RNA fusions, which has attracted increased interest due to the recent approval of therapeutic agents that target numerous RNA fusions. However, some fusions are difficult to detect by DNA-based NGS panels, because of technical challenges associated with sequencing intronic regions. Whole transcriptome or targeted RNA sequencing (RNA-Seq) NGS assays can therefore be used for additional diagnostic testing.

The combination of RNA and DNA assay workflows is a versatile concept that should help to improve and optimize the application of NGS testing in clinical settings. One such example was published by the Fernandes Lab at Columbia University Medical Center, which routinely uses a DNA-based hotspot NGS assay to assess solid tumors. For cases without mutations, a targeted RNA-Seq assay was developed as a reflex test. The CLIA-approved RNA fusion assay targets 17 clinically relevant fusion transcripts, and analytical validation was performed using a 16-plex contrived RNA fusion reference material from SeraCare. The lab reported that the use of a reflex RNA-Seq assay for patients with DNA mutation-negative non-small cell lung carcinoma (NSCLC) resulted in a 10% increase in diagnostic yield without increasing overall turnaround time.[3] Such findings are likely to expand and enhance the use of genomics in cancer patient management pathways to improve clinical outcomes.

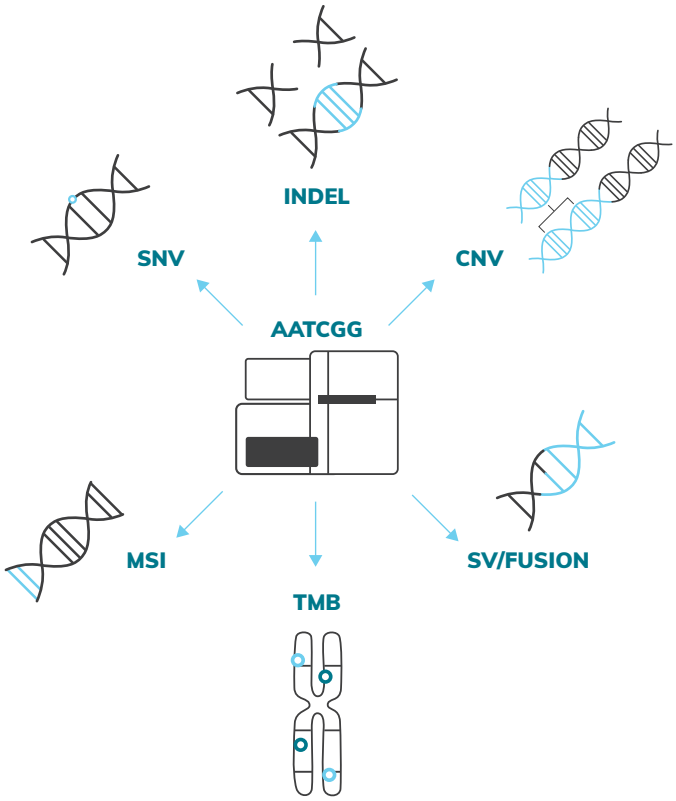

Figure 2: NGS assays can be designed to assess multiple types of cancer-driving genomic alterations.

IMMUNOTHERAPY

The use of immune checkpoint inhibitors (ICIs) is a powerful strategy to unleash the immune system to fight cancers. However, only a fraction of patients currently benefit significantly from this treatment, driving continuing efforts to develop accurate methods to determine the differences between those that respond to treatment and those that are unresponsive, including TMB (tumor mutational burden) and MSI (microsatellite instability) status. In 2020, the US FDA approved the Foundation One CDx (F1CDx) assay as an IVD test for the clinical evaluation of patients for treatment with pembrolizumab (Keytruda®), an ICI from Merck,[4] with a TMB score of more than 10 mutations per megabase of genome (TMB >10) indicating a likely positive response.

Although bespoke reference materials are critical to standardize the measurement of any biomarker, the complexity of TMB creates additional challenges. The reference material should enable laboratories to identify phases of the NGS workflow that contribute to artifact formation, be available in an FFPE matrix to represent patient-like samples, and provide TMB scores below and above clinical cutoff levels for the ICI treatment. To date, variations in TMB testing workflows between sites have resulted in varied analytical results – often contributing to inconsistent clinical outcomes – emphasizing a need for greater standardization in its clinical application.

Standardizing the analysis of biomarkers

As the clinical evaluation and validation of TMB as a biomarker are still ongoing, multiple consortia have made efforts to harmonize procedures to accurately measure these biomarkers, aiding their clinical implementation. For example, in North America, Friends of Cancer Research (FoCR) coordinated a TMB Harmonization consortium, which focused on standardizing TMB measurements across different NGS assays. This harmonization study, which involved 16 clinical laboratories, used SNP-matched normal and diseased cell lines provided by SeraCare as contrived TMB reference materials. The study provided guidance on the measurements of TMB by NGS assays, and identified some challenges in ensuring precise determination of TMB as a clinical biomarker for ICI treatment.[5]

In Europe, a collaboration between ESMO and IQNPath was initiated with the goal of harmonizing TMB testing in clinical practice. The first phase of the study evaluated the appropriateness of using contrived 100% tumor FFPE TMB reference materials. A set of ten FFPE TMB reference materials supplied by SeraCare was analyzed using four independent NGS assays that included TMB assessment (Oncomine Tumor Mutational Load, F1CDx, QIAseq Tumor Mutational Burden, and TruSight Oncology 500).[6] A follow-up EQA proficiency pilot study involving 29 clinical laboratories across Europe was then conducted, using five FFPE TMB reference materials selected from the previous study. The study compared TMB results from participating labs against scores obtained from the F1CDx assay. However, the study did not yield conclusions for the clinical application of TMB, because the labs did not provide TMB cut-offs.

"SeraCare really stepped up to the plate and helped to get us where we are now. The company served as the central laboratory that acquired the cell lines, grew them, extracted them, and provided a level of quality assurance."[5]

Mark Stewart, Vice President, Science Policy, Friends of Cancer Research

In the summer of 2018, the SeraCare TMB working group – including both clinical labs and NGS assay vendors – was established to develop fit-for-purpose TMB reference materials needed to assess TMB scores and the quantitative differences between cancer gene panels. Evaluations and analyses by the working group resulted in the commercialization of the first-to-market Seraseq TMB line of FFPE TMB reference materials, gDNA TMB reference materials, and ctDNA TMB (blood TMB) reference materials, to support further harmonization and standardization of TMB measurements.[7,8]

In addition, the first qPCR-based assay for clinical MSI status determination was developed by Promega, and has been FDA-cleared and CE-marked as an IVD medical device available in the UK and select European countries.[9] NGS assays have also been designed to evaluate MSI status due to functional loss in DNA mismatch repair genes. By analyzing a large portion of the genome, NGS allows the analysis of a greater number of microsatellite loci than PCR, presenting opportunities to identify new MSI profiles in previously uncharacterized cancer types.[10] One such assay is the F1CDx, which was recently approved by the FDA as the first companion diagnostic test for identifying MSI-high status in non-small cell lung cancer (NSCLC) patients that may benefit from pembrolizumab treatment.[11] This will drive interest in further validating this biomarker for all cancer types in other NGS panels, requiring bespoke MSI-high reference materials and samples with loss of repeats in MSI regions for additional analytical and clinical validation, respectively. SeraCare has developed contrived MSI materials (gDNA and FFPE), which were analyzed and confirmed as MSI-high using the Illumina TruSight Oncology 500 assay.[12,13]

LIQUID BIOPSY

The ability to assess cancer mutations in whole blood or plasma samples (liquid biopsy) may provide significant benefits to cancer patients in terms of early detection, disease diagnosis and treatment monitoring. Detecting DNA shed from apoptotic tumor cells into the blood, known as circulating tumor DNA (ctDNA), can serve as a surrogate for invasive tissue biopsies. The clinical utility of NGS-based liquid biopsy assays for routine cancer screening is gaining traction with both regulatory agencies and medical boards, with recent IVD and CLIA approval of ctDNA NGS assays from Foundation Medicine, Guardant Health, Thermo Fisher, Memorial Sloan Kettering Cancer Center (MSKCC), and many more. With myriad potential clinical applications, this field is likely to expand rapidly with the development of new testing platforms, each with its own set of technical challenges.

Liquid biopsy has long been desired for use in cancer diagnostics, but has only recently become a reality for many cancer applications because of the need for robust and sensitive detection methods. Although tumors can shed a significant amount of DNA, the quantity can vary widely according to the tumor type and stage. ctDNA represents only a small fraction of the total circulating cell-free DNA (ccfDNA) in the blood, so it is therefore important to implement a robust method of detection to identify somatic mutations at very low abundances. The specificity of the assay also has important clinical implications, particularly at lower limits of detection (LoD), as false positives of actionable mutations could negatively impact clinical decisions.

NGS for liquid biopsies

Recent advances in NGS chemistries and instrumentation provide options for sequencing blood-based samples at an extremely high depth of coverage, offering high sensitivity and specificity for actionable biomarkers. New methodologies, such as molecular barcoding and bioinformatics using AI tools, have also improved the accuracy of liquid biopsy assays. Unique molecular identifiers (UMIs) can be integrated into DNA library preparations to tag individual DNA molecules prior to amplification, then used in downstream data analysis to identify errors introduced during PCR. By reducing the background noise, UMIs enable the identification of true variants with high specificity. Many hospital pathology laboratories are introducing this promising approach into their practice to provide enhanced support for patient care management. However, this requires careful selection of appropriate assays, followed by rigorous analytical and clinical validation, before being deployed for routine patient testing.

Putting theory into practice

The MSKCC is an NCI-Designated Cancer Center that handles more than 10,000 outpatients annually. To address the challenge of cancer testing using whole blood or plasma, scientists at the MSKCC Molecular Pathology Lab have developed a comprehensive NGS-based liquid biopsy assay interrogating 129 clinically relevant mutations for genomic profiling. The resulting assay, MSK-ACCESS, has been CLIA approved, and is now used for the routine clinical assessment of cancer patient samples. As part of the CLIA assessment, analytical validation of assay sensitivity (LoD, VAF ~0.5%), and specificity (>98% positive predictive value) was performed using contrived ctDNA reference materials from SeraCare.[14]

Reference materials for blood-based assays

As methods for ctDNA detection improve, labs have sought to develop blood-based assays for specific tumor types. Some initiatives also strive to standardize ctDNA testing across multiple labs or platforms. Each of these endeavors requires bespoke reference materials with a broad number of actionable and rare mutations in order to verify and validate assays for endpoint use in clinical decision making.

Tissue biopsy availability from patients with non-small cell lung cancer (NSCLC) is a significant challenge, so the UK National External Quality Assessment Service (UK NEQAS) sought to address this by standardizing analysis of EGFR gene mutations in ctDNA. Using ctDNA reference materials from SeraCare, UK NEQAS conducted external quality assessments for all labs in the UK that perform EGFR mutation testing in plasma.[15] Under the umbrella of IQNPath, a broader international study was also performed to determine the standard of ctDNA testing for EGFR mutations. The international consortium included five European EQA providers – AIOM, EMQN, ESP, Gen&Tiss and UK NEQAS – which collaborated to deliver an assessment for a total of 310 laboratories from 44 countries. Many of these types of tests are conducted using contrived reference materials that, in this case, included a panel of custom manufactured plasma samples with varying EGFR mutations at a range of allelic frequencies.[16]

Testing for blood TMB (ctDNA TMB) provides a holistic view that includes both primary and metastatic tumors, however, it also presents challenges when detecting low levels of tumor burden, which may result in the underestimation of TMB scores. A prototype blood TMB reference material manufactured by SeraCare was evaluated using two liquid biopsy-based comprehensive genomic panels (CGP) at two independent clinical laboratories, Guardant and Predicine. Results of the evaluation, which showed general concordance between

these two panels, were presented by scientists from Astra Zeneca at the 2020 Society for Immunotherapy in Cancer (SITC), and are currently being collated for publication.[17,18] The reference materials have since been released for sale by SeraCare, and tested with the TruSight Oncology 500 ctDNA assay.

"It's very difficult to get hold of patient material for this type of assessment, so we have used SeraCare products to enable us to deliver this EQA to the UK-based laboratories. SeraCare was able to manufacture these exactly to our specifications."[15]

Sandi Deans, Director
UK NEQAS for Molecular Genetics

Disease monitoring

Disease monitoring is another important application for liquid biopsy assays. Not only can a patient's response to therapy be monitored over time with serialized testing, but prolonged minimal residual disease (MRD) monitoring of patients in remission can also be adequately addressed. However, although the less invasive nature of liquid biopsy is attractive for this application, innovations in NGS methods were required to lower the detection threshold of MRD assays to below the clinical decision-making threshold. It is therefore critical that LoD claims for new MRD assays are verified before they are used in clinics.

Natera leveraged its previous experience with developing ccfDNA analysis for reproductive health to expand its NGS platform for cancer diseases. The company developed the Signatera assay as the first commercially available, NGS-based MRD monitoring assay for cancers. Natera scientists performed analytical validation to determine the assay's LoD using contrived ctDNA reference materials manufactured by SeraCare. Natera titrated DNA from cancer cell lines in conjunction with Seraseq reference materials to validate its CLIA-approved MRD assay for detection of tumor DNA mutations at between 0.5% and 0.005% tumor fractions.[19]

In addition, the Translational Genomics Research Institute (TGen) developed TARDIS (Targeted Digital Sequencing), a blood-based NGS assay designed to be sensitive enough to detect mutations with only trace amounts of tumor tissue available following surgery. This assay is designed to address the challenge of MRD testing for monitoring breast cancer patients with early stage or non-metastatic disease. Analytical validation of the assay was conducted using serially diluted fractions of contrived ctDNA reference materials manufactured by SeraCare. Additional validation studies were conducted by a collaborative team from TGen, the Mayo Clinic, and the City of Hope, and reported 91% sensitivity and 53% sensitivity at 0.03% and 0.003% mutant allele fractions, respectively.[20] Exclusive licensing rights to the TARDIS assay were subsequently obtained by Exact Sciences Corporation to help strengthen the company's leadership in the field of precision oncology with this robust MRD testing solution. [21]

CONCLUSIONS

Holistic solutions for high-precision NGS assays offer robust and reproducible testing methods for cancer diagnostics, but it is clear that there is still much work ahead to address the complexity of disease profiling and monitoring. The appropriate NGS method must be selected according to each clinical setting, and even recently-validated assays may be rendered incomplete as the number of targeted therapies – and associated biomarkers – continues to increase, warranting the development and validation of new assays.

While the developments and progress in various aspects of genomics applications described here have surpassed what was considered possible even a decade ago, significant global collaborations between stakeholders in industry, academia and government will continue to increase the adoption and application of precision medicine within the healthcare industry. To fully realize the promise of this approach, it is crucial to get input from across all segments of the clinical genomics community, including drug manufacturers, NGS assay vendors, clinical laboratories, clinicians, bioinformatics solution providers, third party reference material manufacturers, regulatory agencies and insurance administrators. SeraCare (now part of LGC Clinical Diagnostics) is committed to supporting the development and validation of the emerging NGS methods through the production of both standardized and customized reference materials, helping assay developers to keep up with the rapidly evolving field of cancer diagnostics.

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ABOUT US

SeraCare offers a comprehensive portfolio of reference materials for oncology and reproductive health, designed and manufactured to meet the precision demanded by NGS assays. The portfolio includes high quality ground-truth RNA, ctDNA and genomic DNA-based reference materials that are NGS platform agnostic for tumor profiling, immuno-oncology, liquid biopsy, NIPT and germline cancer assay workflows. **For more information visit seracare.com**

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