

# Application of biomarkers in oncology clinical trials

Technological advancement and the discovery of various biomarkers have made personalized/precision medicine a reality. Thus, pharmaceutical companies have targeted many deregulated pathways for the development of specific cancer therapeutics, making the practice of precision medicine a reality for cancer patients. With the exploration of novel cancer therapies, advanced genetic- and protein-based assays are used to ensure that the correct patients are selected for each clinical trial. Technological tools are applied in order to assess the genetic makeup of each patient and changes in the expression of specific protein biomarkers. Here, we describe the utility of genetic- and protein-based technologies for the identification and monitoring of biomarkers in oncology clinical trials. Moreover, we discuss how technological advances may serve as tools for the development of future cancer therapeutics.

**Keywords:** biomarkers • diagnostic tests • nucleic acid technologies • oncology trials • protein technologies

The advancement in understanding of biomarker science and technological innovation has transformed our ability to diagnose and treat diseases at the molecular level, resulting in more informed decisions for drug development and improved clinical trial design. The proven success of targeted therapies, such as crizotinib and vemurafenib, paved the way for personalized medicine, achieving maximum benefit for the right patient while minimizing drug toxicities. From the pharmacoeconomics perspective, the high costs of new therapies and escalating health insurance premiums create strong economic incentives for new approaches to optimize therapy. Both regulatory authorities and reimbursement agencies have mandated patient selection, whenever applicable, to gain marketing approval for a therapy. The dramatic increase in the number of drug candidates in oncology clinical development pipelines and available marketed therapies have also driven pharmaceutical companies towards more personalized therapies, which fundamentally have a higher likelihood of succeeding in clinical trials, thus providing a competitive advantage.

The expansion of new molecular technologies has revolutionized biomarker discovery and clinical trial testing. Next-generation sequencing technologies that are able to comprehensively evaluate DNA and RNA changes have altered the way we characterize tumors and manage patients. Next-generation sequencing now makes it possible to assess not only single-nucleotide variation, but also DNA copy number, rearrangement, loss of heterozygosity, allele-specific amplification, methylation, transcription, aberrant splicing and RNA editing on a single high-throughput cost-effective platform. The technical utility of multiplex digital PCR (dPCR) has also been demonstrated by its ability to screen for multiple mutations simultaneously with sensitivity that is sufficient to detect mutations in circulating tumor DNA (ctDNA) obtained by noninvasive blood collection. The ability of dPCR to precisely quantitate DNA molecules, identify mutations, assess copy number variations

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and perform gene expression analysis is creating waves across the diagnostics landscape. This platform is well suited for single-cell analysis and is a promising new superior tool in the clinic due to its ability to work with a small amount of sample [1]. In addition, NanoString Technologies has developed the Prosigna<sup>TM</sup> Breast Cancer Prognostic Gene Signature Assay<sup>®</sup>, which can estimate the risk of recurrence in postmenopausal hormone receptor-positive breast cancer patients [2].

Tumor cells shed DNA into the bloodstream, which provides a noninvasive method for the detection of genetic alterations in the plasma of cancer patients. ctDNA has been evaluated as a tool for monitoring tumor progression and assessing tumor response to targeted therapies. Bettegowda et al. examined the ctDNA present in the blood of 24 colorectal cancer patients whose tumors had first responded to a therapy targeted to a specific gene, but then progressed while still being treated [3]. The ctDNA samples from these patients were screened for mutations both before and after therapy. In this study, novel somatic mutations were identified in the blood of these patients after drug treatment, which indicated a potential cause of the development of drug resistance. Furthermore, the identification of novel mutations provided new options for targeted therapy for these patients.

The functional state of the tumor may be assessed by measuring either the levels of protein biomarkers or their post-translational modifications. The most wellknown mechanism in the regulation of oncogenic pathways is protein phosphorylation and dephosphorylation via protein kinases and phosphatases, respectively. Phosphoprotein status is commonly used as a direct measure of oncogenic pathway activation state. Recent technological advances have enabled the identification and verification of the role of phosphoprotein biomarkers. These technologies are useful tools for exploring phospho-signatures unique to a specific type or subtype of cancer and as biomarkers of efficacy and toxicity [4]. While direct assessment of the phosphorylated proteins in a standardized clinical laboratory setting has not yet been fully implemented at the present time, we anticipate a paradigm shift in monitoring of labile proteins with the evolution of novel specimen preservation methods. Examples of proteins that have been established as cancer biomarkers and are used extensively in directing the right therapy to the right patient include: EGFR for lung cancer, the tyrosine-protein kinase KIT for gastrointestinal stromal tumors and HER2 (human EGFR2) for breast cancer. Phosphoproteins are currently being investigated as biomarkers for novel cancer therapeutics. For example, mTOR is a serine/threonine kinase that functions as a master switch between catabolic and anabolic metabolism and has been a target of anticancer drug development. Clinical studies have revealed that mTOR inhibition is correlated with the inactivation of ribosomal protein S6 kinase 1 and the phosphorylation status of S6 kinase 1 can be used as a biomarker for mTOR inhibitors [5].

A well-established tool for assessing protein biomarkers in hematological malignancies is flow cytometry, which plays a vital role in the diagnosis, classification and management of blood cancers, such as leukemia and lymphoma. The unique feature of this technique is its ability to perform multiparametric analysis on an individual cellular basis, which offers a distinct advantage compared with other protein-based technologies, such as western blotting, which requires upfront cell enrichment and multiple independent tests. Furthermore, flow cytometry is extremely sensitive, which allows for the detection of minimal residual disease (MRD). In the MRD setting, a small number of cancer cells remains in the patient even though other measures do not show evidence of the disease. Assessing MRD is particularly helpful in monitoring disease remission after completion of a therapeutic regimen in order to predict possible recurrence of the disease and for planning future treatments [6].

A complementary methodology that is commonly utilized to explore protein biomarkers in the solid tumor space is immunohistochemistry (IHC). While the traditional brown staining method has been adopted widely in the companion diagnostics world (e.g., ALK, HER2 and KIT), recent advancements in fluorescencebased IHCs have enabled the accurate quantitation and measurement of multiple targets in a single slide.

Here, we will review several nucleic acid- and protein-based technologies with increasing utility in the discovery and identification of biomarkers in oncology research. Figure 1 depicts how fit-for-purpose technologies can be selected to assess distinct biomarker end points in oncology clinical trials. Each of the technologies discussed provides a unique advantage that makes it the platform of choice for a specific application. Furthermore, we will discuss how each of these technologies have or will contribute to furthering the application of biomarkers in oncology clinical trials.

### New nucleic acid-based technologies

Nucleic acid testing has long been dominated by classical hybridization-based nonamplified and amplified technologies such as *in situ* hybridization (ISH) and fluorescence *in situ* hybridization (FISH) methods and quantitative PCR (qPCR), respectively.

FISH methods are widely used in the detection of gene amplifications, deletions, translocations and chromosomal instabilities [7]. Amplifications of oncogenes such as *EGFR* in lung cancer [8] and *HER2* in



Figure 1. Decision tree for selecting fit-for-purpose technologies to assess distinct biomarker end points. IHC: Immunohistochemistry; NGS: Next-generation sequencing;

breast cancer [9] are generally associated with poor prognosis for patients. Deletions of tumor-suppressor genes, namely TP53 [10] and RB1 [11], have been shown to increase the risk of metastasis. Translocations or gene fusions were first discovered in hematological diseases, but have also been detected in solid tumors [12]. ALK translocations are used as predictive markers for treatment decisions with ALK inhibitors such as crizotinib [13]. Chromosomal instabilities, including large structural rearrangements, have been implicated in disease initiation and progression [14]. Nucleic acid hybridization-based methods such as FISH have played - and will continue to play - a crucial role in the detection of genetic aberrations. A number of genetic aberrations assessed using FISH are summarized in Table 1.

The detection of cytogenetic aberrations in plasma cell neoplasms is definitive for risk stratification in a variety of hematological cancers. However, metaphase chromosome analysis is limited, due to a low proliferative rate *in vitro*. Interphase FISH dramatically improves the rate of detection. When detected by FISH, high-risk aberrations include complex karyotypes, t(4;14), t(14;16), *p53* gene deletions and hypodiploidy, standard-risk aberrations include hyperdiploidy and chromosome 13q deletions (alterations only detectable by FISH) and low-risk aberrations include normal karyotypes, t(6;14) and t(11;14). Despite the utility of interphase FISH for predicting patient outcomes, it is often difficult to detect underlying genetic changes in patients with low levels of clonal

plasma cells, such as monoclonal gammopathy of undetermined significance or in cases of MRD.

Several groups have recently reported on the use of plasma cell selection [23] or enrichment [24] based upon the cell surface antigen CD138 for the clinical study of low-level plasma cell dyscrasias, such as monoclonal gammopathy of undetermined significance, and the assessment of MRD. Plasma cell-targeted FISH can be achieved through: simultaneous cytoplasmic light-chain staining and FISH; sequential morphologic identification and FISH; immunomagnetic bead depletion of nonplasma cells; or immunomagnetic bead enrichment of plasma cells. These recent studies conclude that enrichment of specimens for CD138<sup>+</sup> plasma cells increases the likelihood of identifying cytogenetic abnormalities in the patient, thereby enabling the physician to correctly classify patients with low disease burden and allowing for accurate risk stratification and detection of MRD in patients with plasma cell neoplasms [23,24].

The detection of mRNA and miRNA biomarkers has long been a domain of quantitative real-time reverse transcription PCR due to its exquisite sensitivity and specificity [25]. Furthermore, for the miRNA biomarkers involved in critical cellular processes, qPCR might be the only viable detection method. Innovative qPCR arrays enable the analysis of hundreds of transcripts in parallel. Gene expression biomarkers are used to predict and monitor the response of a patient to a drug. The best known example of a biomarker that is measured using this method is the *BCR-ABL* 

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Table 1. Oncology biomarkers measured by FISH.					
Gene	Aberration	Tumor type	Drug	Biomarker utility	Ref.
ALK	ALK/EML4 translocation	NSCLC	Crizotinib	Predictor of drug response	[14]
EGFR	Amplification	NSCLC	Gefitinib	Predictor of drug response	[15]
MET	Amplification	NSCLC	NA	Unfavorable prognosis. Resistance to EGFR inhibitors	[16]
ROS1	Rearrangement	NSCLC	May benefit from ALK TKIs (e.g., crizotinib)	Mutually exclusive with <i>ALK</i> rearrangement. Predictive of response to crizotinib	[17,18,19]
RET	Rearrangement	NSCLC adenocarcinoma	May benefit from crizotinib treatment or RET tyrosine inhibitors	Mutually exclusive with <i>ALK</i> rearrangement, <i>EGFR</i> amplification/ mutations, <i>KRAS</i> mutations and <i>ROS</i> rearrangement	[20]
HER2	Amplification	Breast carcinoma	Trastuzumab	Increased recurrence of the disease. Unfavorable prognosis. Better response to trastuzumab	[21]
FGFR1	Amplification	Breast carcinoma and NSCLC (squamous)	FGFR1 inhibitors	Predictor of drug response	[22]
L					

NA: Not applicable; NSCLC: Non-small-cell lung carcinoma; TKI: Tyrosine kinase inhibitor.

gene fusion [26]. The presence of this biomarker is used to identify patients with Philadelphia chromosome abnormalities who can benefit from treatment with kinase inhibitors [27]. Moreover, continued assessment of the levels of expression of this fusion gene allows for monitoring of MRD in patients, thus giving physicians information on when to resume therapy.

qPCR can also be performed on DNA in order to assess the presence of certain mutations in specific biomarkers, providing tools for physicians to select the right treatment for each patient. Examples of these biomarkers are shown in Table 2.

New technological developments have opened up exciting and promising applications in molecular diagnostics for clinical use beyond the more established FISH/ISH methods and qPCR. These technologies either enable the more sensitive detection of a selected biomarker or provide the ability to assess multiple biomarkers at the same time. All of these platforms have applications in various clinical trials.

The following technologies will be discussed in more detail:

- Next-generation sequencing (NGS)
- dPCR
- NanoString technology

These three methods are based on digital technologies and enable either single-molecule detection as demonstrated by dPCR and NanoString nCounter<sup>®</sup> methods or the analysis of single-sequencing reads in NGS.

### Next-generation sequencing

Sanger sequencing has played a crucial role in decoding the genetic information from small bacteriophages to the entire human genome [48]. Despite the advantages of Sanger sequencing, such as long read lengths, low error rates and a relatively high degree of automation, large-scale sequencing projects have now been entirely replaced by NGS technologies [49]. In contrast to Sanger sequencing, NGS enables the sequencing of individual DNA molecules in a massive parallel mode, thus generating up to billions of individual sequence reads, hence the name 'deep sequencing'. This ability of NGS results in enormous improvements in scalability, enhanced throughput and a dramatic reduction in the cost of DNA sequencing. Targeted resequencing NGS applications and the resulting deep sequencing reads have improved the detection of rare alleles compared with less sensitive Sanger sequencing.

A variety of different NGS platforms are now commercially available using either sequencing-by-synthesis methodologies (e.g., Illumina, Roche, Pacific Bio-Sciences and Ion Torrent/Life Technologies) or newer nanopore-based sequencing methods (e.g., Oxford Nanopore Technologies) [50]. All of the platforms have unique characteristics regarding read lengths, sequencing accuracy, reads per run and run times.

NGS not only allows users to sequence entire genomes, but it is also an enabling technology for obtaining information about the transcriptome and DNA modifications. At a smaller scale, NGS facilitates targeted deep sequencing of a selected subset of genes, allowing not only the determination of the entire

mutation profile – including short DNA insertions and deletions – of the targeted genes, but also the detection of copy number variations and translocations. In addition, NGS provides the unique ability to pool samples using unique DNA barcodes attached to the library of DNA fragments and to sequence many individual samples in the same sequencing run, adding further efficiency to the sample testing and thus reducing the costs of DNA sequencing.

While NGS provides oncology biomarker discovery with a unique tool that no other existing technology can currently match, similarly to many other nucleic acid-based technologies, this platform has its limitations. Tumor samples provide unique challenges, such as tumor heterogeneity, which is characterized by the existence of different cellular subclones in a single sample [51]. Furthermore, cancerous cells are generally mixed with noncancerous cells, further complicating the detection of rare mutations, copy number variations and translocations. Moreover, chromosomal amplifications and losses may further modify the presence of mutations. Widely used formalin-fixed paraffin-embedded (FFPE) tumor samples and the associated degradation and crosslinking of nucleic acids provide additional challenges for any nucleic acid testing. Nevertheless, while a variety of multiple tests and technologies are usually required to detect mutations, copy number variations and translocations in a given sample, targeted NGS has unparalleled consolidative power that allows for the detection of all of these genetic aberrations in a single test.

NGS can not only be used in clinical trials for patient stratification, but also for developing a better understanding of the mode of action of a particular drug or the mechanism of development of drug resistance. In addition, poorly differentiated tumors can be better characterized by NGS because of its ability to generate a detailed molecular fingerprint [52].

As new genetic alterations continue to be discovered and new targeted therapies are developed in parallel, finding a meaningful link between the enormous capabilities of NGS and the clinical utility and actionable results for the patient presents a special challenge. The correct interpretation of a specific genetic alteration will play a very important role, not only in identifying a new biomarker for targeted therapies in human cancer and its predictive and prognostic applications, but also in the wider acceptance of NGS.

Table 2. Prognostic and predictive cancer biomarkers identified by quantitative PCR.					
Gene	Mutation	Tumor type	Drug class	Biomarker utility	Ref.
EGFR	Multiple	NSCLC	TKIs (e.g., gefitinib)	Identify patients who will benefit from TKI therapies	[28,29,30]
EGFR	T790M	NSCLC	TKIs (e.g., gefitinib)	Patients with this mutation have developed resistance to the drug	[31,32,33]
BRAF	V600E	Melanoma	Vemurafenib	Identify patients who will benefit from a particular therapeutic	[34]
BRAF	V600E	CRC, melanoma	NA (new therapies targeting this kinase are under investigation)	Unfavorable prognosis	[35]
KRAS	Mutations in codons 12 and 13	NSCLC	Examples are gefinitib and cetuximab	Poor response to therapies targeting EGFR	[36]
KRAS	Mutations in codons 12 and 13	CRC	An example is cetuximab	Poor response to therapies targeting EGFR	[37,38,39,40]
ΚΙΤ	Multiple	Melanoma	TKIs	Decreased overall survival. Better treatment response to TKIs	[41,42,43]
CALR	Multiple	ET, JAK2/MPL- negative PMF	NA	Longer survival in ET and PMF. Lower risk of thrombocytosis in ET	[44,45]
IGVH	Somatic mutation of <i>IgH</i> gene V region	CLL	NA	Lower likelihood of disease progression and better survival	[46,47]
CLL: Chronic lymphocytic leukemia; CRC: Colorectal carcinoma; ET: Essential thrombocythemia; NA: Not applicable; NSCLC: Non-small-cell lung carcinoma;					

PMF: Primary myelofibrosis; TKI: Tyrosine kinase inhibitor

Unique requirements for any nucleic acid-based analysis method in the clinical laboratory – including high throughput, fast turnaround time, automated workflow and minimal risk of contamination – have not yet been adequately addressed, but will be crucial to fulfilling the promise and potential of NGS. Consequently, the use of NGS in the clinical laboratory has been limited despite its proven capabilities and demonstrated success in other fields. The new era of precision medicine has already changed the landscape of cancer treatment, with NGS technologies being used more often for patient selection in order to predict sensitivity or resistance to targeted therapies.

A particularly interesting and emerging field in oncology is the use of NGS technologies to analyze genetic alterations in peripheral blood and other bodily fluids as a measure of the status of solid tumors [53]. This will extend the current applications of this technology (which have been primarily limited to solid tissues) to 'liquid biopsies' for analyzing ctDNA and cells. Analysis of ctDNA allows for the characterization of mutations associated with response or resistance, further informing patient care. ctDNA testing can be applied to every stage of cancer patient care. ctDNA can be detected in most types of cancer at both early and advanced stages, suggesting that it could be used as an effective screening method for most patients. A measurement of the levels of ctDNA in blood may also be used to quickly estimate a patient's stage of cancer and survival chances [3].

### **Digital PCR**

dPCR as a next-generation PCR technology has the potential to become the new 'gold standard' in nucleic acid testing [54,55]. In contrast to qPCR, in dPCR, amplified target molecules are not detected in real time as they are being generated, but by end point measurement after all amplification cycles are completed. This allows dPCR to be less dependent on different amplification efficiencies. The main difference between qPCR and dPCR is the ability of dPCR to detect and count single target molecules. This is accomplished by the partitioning of PCR reactions into millions of picoliter-containing compartments as in the case of digital droplet PCR. The ideal partitioning would generate compartments with either zero or one target molecule(s), generating either negative or positive results, respectively. Poisson distribution can correct for scenarios in which more than one target molecule is partitioned into the reaction compartments.

Fluidigm's BioMark<sup>TM</sup> instrument was the first commercial instrument using microfluidics-based partitioning. A similar strategy was used by Life Technologies with the launch of QuantStudio 3D<sup>TM</sup>. The availability of droplet-based dPCR was pioneered by Bio-Rad's QX100<sup>TM</sup> Droplet Digital PCR<sup>TM</sup> instrument and followed by the now commercially available Raindrop<sup>®</sup> instrument from Raindance. With its ability to generate millions of droplets, digital droplet PCR is uniquely positioned to detect very low target concentrations [56]. It is anticipated that the availability of different platforms and associated reagent kits will help to move this new technology from a tool primarily used in research to more sophisticated clinical applications.

The main advantages of dPCR are improved precision and accuracy in measuring nucleic acids without the need for a standard curve. In addition, dPCR is less sensitive to PCR inhibitors. This unparalleled sensitivity provides a unique opportunity for dPCR in the promising new areas of rare allele detection using ctDNA. Other dPCR applications in oncology are the more precise and accurate determination of copy number variations and the absolute quantification of expression levels of low-abundance miRNAs. One of the applications of dPCR is assessing mutations in ctDNA [1]. Due to the low ratio of ctDNA to wild-type DNA, a highly sensitive detection method such as dPCR is required for the detection and quantitation of mutations in ctDNA from the plasma of cancer patients. Furthermore, multiplex dPCR enables the testing of samples for different mutations simultaneously.

#### NanoString

NanoString's nCounter technology is based on the hybridization of hundreds of different DNA probes containing unique fluorescent 'molecular barcodes' in order to their cRNA or DNA targets [57]. After immobilization of the captured hybrids and removal of nonhybridized probes, individual hybridized probes are visualized and counted using microscopic imaging. The relatively low background of this technique provides very sensitive detection in a highly multiplexed manner [58].

The commercially available nCounter Analysis System is highly automated with the capability of processing up to 12 samples simultaneously. Major applications of the technology are in the areas of gene expression (mRNA, miRNA and lncRNA), copy number variations, translocations and chromatin immunoprecipitation.

The primary advantages are the aforementioned high sensitivity and precision of the nCounter technology. No target amplification is required to detect up to 800 targets simultaneously, which makes this technology very cost effective. A variety of different sample types can be used, including DNA, total RNA, cell lysate, FFPE samples and whole-blood lysate. The fact that no enzymes are required to detect the targets of interest makes this NanoString technology a very attractive method for working with sample matrices in which the presence of enzyme inhibitors are a concern. Ease of use, minimal hands-on time and easy data analysis are other strong advantages of this digital technology.

The relatively high cost of the nCounter Analysis System remains a challenge to its more widespread adoption. However, the technology has positioned itself in a niche between qPCR and microarray/NGS methods with respect to its multiplexing capabilities, providing an opportunity to make it cost effective when multiple samples and targets are tested at the same time.

NanoString technology enables the analysis of entire signal transduction pathways in one reaction and is uniquely positioned to detect the mRNA expression levels of multiple genes, enabling improved risk stratification of patients [59]. In 2013, NanoString received US FDA 510(k) clearance of the Prosigna Breast Cancer Prognostic Gene Signature Assay, which provided further validation of the clinical utility of this new technology.

### Protein biomarkers in cancer medicine

Due to the increasing use of biomarkers in early-phase clinical trials to make critical decisions and a growing preference from health authorities and reimbursement agencies to develop companion diagnostics, it has become imperative to develop robust biomarkers and technologies to reliably evaluate drug activity and diagnostic utility. Based on the availability of disease biopsies, various stakeholders in the oncology community have a strong desire to target relevant biomarkers directly in affected tissue and cells.

Proteins and their phosphorylated counterparts have been shown to be fundamentally responsible for the functional behavior of cancer cells and, as a result, can be used as targets of powerful anticancer agents. In recognition of this important discovery, there has been an increasing commitment of both manpower and substantial financial investments to the development of novel technologies that can reliably monitor the expression of these targets, including their presence, absence or modulation by cancer therapies, and potentially use their expression as a tool for determining the best therapeutic regimen for a patient. We summarize below the principle classes of biomarkers, specimen types and associated technologies that are used more widely in investigative oncology trials for the quantitation of protein biomarkers.

### Types of protein biomarkers explored in cancer clinical trials

Four main classes of protein biomarkers are pursued in human clinical trials with the goal of diagnosis (e.g., disease identification), prognosis (patient outcome; e.g. estrogen and progesterone receptors ), response prediction (what therapy to use; e.g., HER2 and c-KIT) or to monitor the pharmacodynamic activity of a therapeutic agent (e.g., changes in phosphorylation status). These biomarkers are typically explored in three specimen types: body fluids (e.g., surrogate biomarkers released from cancer cells such as prostatespecific antigen and carcinoembryonic antigen); blood cells (in leukemia and lymphoma settings); and tumor biopsies (solid tissue/epithelial cancers).

## Assessment of soluble protein biomarkers in oncology trials

ELISAs are among the oldest, most analytically established and widely utilized methods for the quantification of soluble protein biomarkers in body fluids and homogenized tissues and cells. Typically, three broad categories of ELISA-based technologies are utilized in human clinical trials to quantify soluble biomarkers: traditional or colorimetric assays are designed to capture single biomarkers using a pair of analyte-specific antibodies; luminescence (electrical/chemical) assays are designed to capture multiple analytes with high sensitivity via innovative printing of capture antibodies or the tagging of detection antibodies with distinct dyes; and fluorescent microspheres are conjugated to specific antibodies capable of reliably segregating a mixture of biomarkers (e.g., cytokines) in a small volume of body fluids. A more detailed description of various ELISA technologies and clinical trial testing laboratories is presented in Table 3.

It is worth mentioning one alternate technology, reverse-phase protein microarray, which has recently entered the cancer clinical trial setting due to its analytical robustness that is comparable to luminescence ELISA. The reverse-phase protein microarray workflow was designed exclusively to address a substantial unmet need of biomarkers in oncology clinical trials, namely pharmacodynamic modulation of multiple oncogenic signaling pathway proteins in freshly procured small tumor biopsies. This methodology involves proprietary protein extraction from flash-frozen or FFPE tumor tissues in a concentrated form. The extracted solution from multiple specimens is spotted onto nitrocellulosecoated slides. A unique marker is detected in each slide using a high-specificity primary antibody followed by significant signal amplification via a series of secondary (horse radish peroxidase) and tertiary (tyramidebased fluorescence system) steps, enabling quantitation of low-abundance phosphoproteins in as many as ten tumor cells [66,67]. Furthermore, platform automation enables assessment of up to 80 biomarkers and over 100 specimens in a single day on one instrument workflow. Multiple biomarker end points of a large clinical

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Table 3. Technology options for exploring soluble biomarkers in body fluids and tissues.				
Methodology	Technology principle	Suggested clinical trial applications	M and CL	
Colorimetric sandwich ELISA	Capture antibody coated on plastic, detection antibody conjugated to HRP	Well-established circulating biomarkers with diagnostic utility (e.g., PSA [60] and CEA [61])	RnD Systems (M); PPD (CL)	
SearchLight® Multiplex Sandwich ELISA	Chemiluminescent or fluorescent detection of analytes whose respective capture antibodies are spotted in arrays within each well of a 96-well microplate with higher sensitivity than colorimetric ELISA	Established and exploratory biomarker, up to 16 markers per well. Ideal for pediatric trials with small sample volumes (e.g., cytokines and angiogenesis factors [62])	Aushon Biosystems (M and CL)	
Electrochemiluminescence sandwich ELISA	Combination of electrochemiluminescence detection and patterned antibody arrays with among the highest sensitivities	Low-abundance biomarkers (e.g., phosphoproteins in cell lysates [63])	Meso Scale Discovery (M); QPS (CL)	
Cytometric bead array (flourescence multiplex ELISA)	A series of antibody-coated particles with discrete fluorescence intensities simultaneously capture multiple soluble analytes and detection is enabled via phycoerytrin-coated antibodies on a flow cytometer	Widely employed for cytokine profiling to monitor immunotherapies [64,65]. Fewer sample dilutions required to determine analyte concentration in substantially less time	BD Biosciences (M); Rules Based Medicine (CL)	
RPMA	Described in the main text	Oncogenic pathway profiling in tumor biopsy extracts (e.g., frozen and FFPE [66,67])	Theranostics Health (M and CL)	
CEA: Carcinoembryonic antigen; CL: Clinical laboratory; FFPE: Formalin-fixed paraffin-embedded; HRP: Horse Radish Peroxidase; M: Manufacturer; PPD:				

Pharmaceutical Product Development; PSA: Prostate-specific antigen; OPS: Quest Pharmaceutical Services; RPMA: Reverse-phase protein microarray

trial may typically be reported within a matter of a few weeks [66,67].

### **Exploration of cell-based biomarkers in** hematologic malignancies

Flow cytometry is among the most well-established and widely utilized technologies to support both primary (efficacy markers) and mechanistic end points (pharmacodynamic markers) in leukemia and lymphoma clinical trials due to the relative ease of accessing peripheral blood from patients and the ability to view expression in tumor cells (Table 4). Flow cytometry is uniquely suited to exploring drug activity directly in neoplastic target cells (e.g., MRD assessments) [68-72], understanding the modulation of oncogenic pathways and investigating immune cell regulation by novel therapies (e.g., anti-CTLA-4 [60,73], anti-PD-1/PD-L1 and chimeric antigen receptor T cells [74,75]) via fluorochrome-conjugated antibodies to a plethora of cell differentiation antigens expressed on the surface of cells and intricate networks of oncogenic proteins located within intracellular compartments. Furthermore, significant advancements in flow cytometry platforms (e.g., Fortessa<sup>™</sup> X-20 and FACSCanto<sup>™</sup> II from BD BioSciences ) and their adoption by many clinical trial laboratories has enabled the development of eight- to 12-color biomarker assays that provide multiple items that can be reported from a single specimen, including tumor burden, pharmacodynamic marker levels and phenotypic alterations.

Due to the recent advent of more effective cancer therapies (e.g., molecularly targeted agents used singly or in combination with standard-of-care treatment), there has been a heightened need to explore biomarkers that correlate with deeper responses in new investigational oncology trials. To this end, both tumor burden (e.g., MRD) and safety biomarkers (e.g., Th17 cells and cytokine release syndromes) have been explored by high-complexity flow cytometry assays with improved sensitivity. The results of the efficacy biomarker measurements have correlated well with clinical outcome [80]. Consequently, the MRD assays are now routinely used in oncology trials for monitoring the depth of drug response and to predict the risk of relapse. While the combination of new agents with standard-of-care treatments can result in greater benefits, in some instances, combination therapies have resulted in an increased adverse event profile. Thus, flow cytometry is being used as one of the primary tools to manage the combination immunotherapies that are becoming increasingly recognized as next-generation cancer therapeutics [64,81].

While flow cytometry helps address high-impact clinical trial end points, the need for real-time sample analysis and highly specialized training has limited its

Table 4. Common biomarker end points supported by flow cytometry. <sup>+</sup>			
End point	Assay principle	Examples	
Target engagement (receptor occupancy for dose selection and drug localization)	Fluorescently labeled antibody drug is utilized to inversely quantify target engagement on peripheral blood cells	Rituximab (CD20) [76]	
Pharmacodynamics	Blood, bone marrow and/or lymph node aspirates are stabilized in suitable fixatives for the analysis of oncogenic pathway modulation	PI3K/mTOR inhibitors (e.g., pS6 [77]); EGF family inhibitors (e.g., pMEK, pERK [78])	
Tumor burden (MRD)	Cancer cells in blood or bone marrow are sensitively detected by multiparameter analysis of differential antigen expression	B-CLL [70]; B-ALL [68,69]; MM [71]	
Phenotyping	Frequencies of T-, B- and NK-cell subsets are distinguished by the expression of distinct cell differentiation antigens	Cancer vaccines and checkpoint inhibitors (e.g., ipilimumab [CTLA-4]) [60,73]	
Pharmacokinetics (cell and gene therapies)	Proliferation kinetics and the differentiation of whole-cell therapies are sensitively monitored using fluorescently labeled antibodies to modified cells	CAR-Ts and TCRs [74-75,79]; stem cell transplants [60]	
Suggested clinical trial laboratorias, Conontin, Inc. Laboran and Quest Disconstrics			

Suggested clinical trial laboratories: Genoptix, Inc., Labcorp and Quest Diagnostics.

B-ĂLL: B-cell acute lymphocytic leukemia; B-CLL: B-cell chronic lymphocytic leukemia; CAR-T: Chimeric antigen receptor T cell; MM: Multiple myeloma; MRD: Minimal residual disease; NK: Natural killer; TCR: T-cell receptor-transduced T cell.

utility, particularly for monitoring pharmacodynamic markers (oncogenic pathways) in global clinical trials. To address this gap, our organization (Genoptix, Inc.) has developed a novel fixative, designated Novaperm-3, which enables reliable one-step fixation and freezing of blood within 20 min for flow cytometry analysis in a specialized central laboratory setting. A variety of clinical trial applications ranging from disease identification and phosphoprotein measurements to pharmacokinetic assessments have been described recently [78].

### Tissue-based biomarkers & their *in situ* quantitation

IHC is among the oldest and most widely utilized methodologies for diagnosis, treatment selection and biomarker assessment across a wide array of solid tumors and a select few blood cancers (e.g., diffuse large B-cell lymphoma). Historically, this methodology was fraught with high analytical variation due to many manual steps that were prone to individual errors. Technological innovations over the last decade have led to the introduction of fully automated and regulated platforms (e.g., Ventana's Benchmark<sup>®</sup>, an FDA cleared *In Vitro* Diagnostic system) and semiautomated open systems (e.g., Biocare's Intellipath<sup>TM</sup> and Ventana's Discovery Ultra<sup>TM</sup>), which have resulted in significant improvements in antigen retrieval and more consistent staining patterns. These innovations have also contributed to the regulatory clearance of certain cancer biomarkers as companion diagnostics, which in turn has had a significant impact on the success of many important targeted cancer therapies (Table 5).

Despite improvements in the procedural automation of IHC assays, due to the subjectivity associated with the interpretation of biomarker expression (brown stain intensity) by the human eye and the fact that only one biomarker may be examined per slide (a limitation imposed by chromogenic dyes), many laboratories have developed fluorescencebased methodologies (e.g., Automated Quantitative Analysis or AQUA®), which were shown to provide objective and reproducible quantitation of biomarkers using a combination of pattern recognition algorithms and cytokeratin and nuclear masks [86]. In some cases, the superior dynamic range and quantitative features associated with these fluorescent IHC assays produced biomarker data sets that were able to distinguish clinical responses and survival

Table 5. Immunohistochemistry-based companion diagnostics.				
Biomarker	Drug	Indication	Companion diagnostic test	
HER2	Trastuzumab	Breast cancer [82]; gastric cancer [83]	HercepTest™ (Dako)	
EGFR	Cetuximab	CRC [84]	EGFR pharmDX™ (Dako)	
ALK	Crizotinib	NSCLC [85]	Ventana, CE marked	
CE: Conformité Européene, denotes approval for use in Europe; CRC: Colorectal carcinoma; NSCLC: Non-small-cell lung cancer; .				

differences that were not decipherable by traditional IHC assays. Furthermore, some investigators and clinical laboratories described fluorescence-based multiplex assays with biomarker utility [87,88,89] and diagnostic intent [90].

### **Conclusion & future perspective**

A multitude of biomarkers have been identified in oncology and their prognostic and/or predictive roles have been characterized. Moreover, pharmaceutical companies are expanding their therapeutic portfolio to include drugs with high specificity aimed at selected genetic aberrations or altered protein expression in tumors. As a corollary, regulatory agencies have encouraged the co-development of targeted therapies and companion diagnostics to identify patients who will benefit the most from such treatments and guide patients that test negative with the companion diagnostic towards conventional therapies. These efforts have resulted in many single-biomarker diagnostic tests that have enabled the approval of novel efficacious therapies.

The FDA articulated their thought process for new cancer drug development in a recent publication called, '*The Next Phase In Oncology: FDA's Pazdur Has New Vision For Drug Development*', which suggested that oncology practice should move away from product-specific companion diagnostics to more integrated screening [91]. This drug development paradigm on pathway-based approvals as opposed to traditional histology-based approvals, requiring pharmaceutical companies, clinicians and technology developers to work together on a master protocol wherein patients would be screened for biomarkers and then assigned to a meaningful trial directed toward a molecular pathway. In such platform-based clinical trials, NGS technologies can enable patient selection. NGS tests that report out several genetic alterations at the same time are becoming more prevalent and are expected to replace single-biomarker analysis via traditional methods over the next few years. This is a leap forward not only because of the magnitude of information that can be obtained for each patient, but also with respect to the fact that this information can be obtained from the limited tissue that is often available from cancer patients. Recently, with the support of the FDA, several such platform trials involving multiple stakeholders have been launched successfully and represent the future of cancer drug development.

A major challenge in cancer management is the development of drug resistance. Cancer therapies often have short-lived benefits due to the emergence of escape mutations that cause drug resistance; biomarkers that can predict and monitor drug resistance will improve cancer treatment [92]. For example, NSCLC patients with *ALK* rearrangements can be treated with crizotinib. However, these patients frequently develop drug resistance as a result of secondary mutations in their tumors. Another mechanism for the development of resistance to treatment is the upregulation or otherwise increased function of alternative signaling mechanisms

### **Executive summary**

### Background

- Biomarkers have become a cornerstone for the development of new oncology drugs due to the rising desire from patients and payers for improved efficacy and safety. This desire has led to an explosion in biomarker discovery and technologies for their assessment in affected tissues.
- Both historical and novel technologies to monitor genetic and phenotypic changes in a wide array of cancers are described.

### Nucleic acid testing technologies

- Current nucleic acid-based technologies and their applications in cancer treatment, especially as tools for predicting the correct treatment for each patient, are presented.
- New advanced nucleic acid-based platforms and their application in oncology are described.

### **Protein biomarkers**

- Phenotypic biomarkers that define the functional state of the cancer in three major compartments body fluids, cells and tissues are explored.
- Established and novel methodologies for the detection and reliable quantification of native proteins and their post-translational modifications underlying cancer are summarized.
- Valuable insight is provided into biomarker end point assessments via specific examples.

### **Future perspective**

- Pathway-based tests rather than single-marker diagnostics will be the wave of future, enabling the delivery of right drug to the right patient at the right time.
- Technologies capable of providing information on multiple markers that are altered in cancer prior to or after therapy will have the greatest impact.
- We anticipate a renaissance in novel cancer immunotherapies over the next decade; consequently, a number of complex safety and efficacy biomarkers would need to be explored.

in tumor cells. These alternative signaling pathways replace and bypass the blockade created after the treatment, enabling uncontrolled cell growth to resume, thus making the treatment ineffective. In order to circumvent such rescue pathways, it is important to develop diagnostic tests to measure cross-talk between pathways and determine effective drug combination strategies.

The topic that dominated the 2014 Annual Society of Clinical Oncology (ASCO) conference was immuno-oncology. Enhancement of human immune responses against tumors is becoming a cornerstone in the treatment of cancer. We have witnessed the success of immunotherapy, such as CTLA-4 and PD-1/PD-L1 checkpoint blockade, as a promising approach to fighting cancer [73]. There is a high demand for standardized fit-for-purpose tests to select patients for treatment. Flow cytometry and protein-based biomarker tests are needed to fully appreciate the complexity of human cancer immune biology and to identify patients who are most likely to benefit from immunotherapies.

The application of modern technologies in patient selection strategies in clinical trials is crucial to the success of oncology drug development. All key stakeholders,

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such as pharmaceutical and diagnostic companies, must work closely together to make personalized medicine a reality. Multiple successful examples of targeted drug and diagnostic approvals have demonstrated that such approaches can dramatically shorten overall drug development and bring innovative drugs to cancer patients.

### Financial & competing interests disclosure

All authors are present or past employees of Genoptix, a Novartis company. Novartis, along with other pharmaceutical companies that have an oncology development pipeline, conduct oncology clinical trials using some of the biomarkers discussed in this article. No mention of Novartis drugs has been made in this article. Genoptix is also the owner of Novaperm-3 and the AQUA technology and a testing laboratory offering, as services, some of the tests mentioned in the article. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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