DNA AND RNA BIOMARKERS: WHY SCIENTISTS NEED TO USE THEM

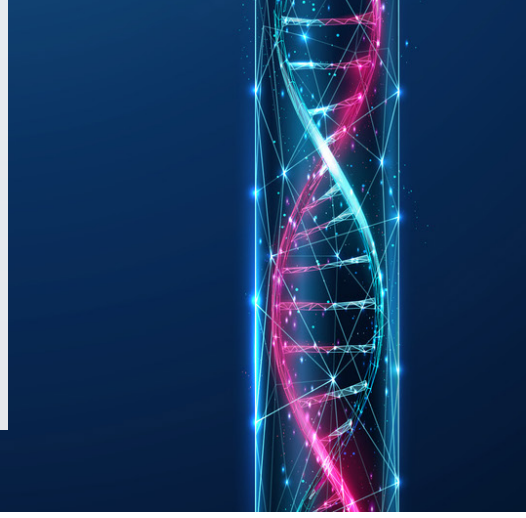
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INTRODUCTION TO NUCLEIC ACID-BASED BIOMARKERS



We are entering the era of precision medicine. In this exciting era, the goal is to optimize the effectiveness and safety of therapies by matching the treatment to the biology for which the treatment was designed. For example, a companion diagnostic ensures that the specific target that a drug is designed against is present in the individual before receiving that drug. Much work has been done to identify measurable molecular characteristics that can be used for the diagnosis, prognosis, and safety of treatments that provide minimal discomfort to the patient but maximize the chance of success. These molecular characteristics are known as biomarkers. A biomarker is a biological characteristic that is objectively measured and evaluated as an indicator of normal biological or pathological processes, or a response to a therapeutic intervention.[1] Examples include patterns of gene expression, levels of a particular protein in body fluids, or changes in electrical activity in the brain. In precision medicine, these biomarkers are often nucleic acid (DNA or RNA) based. Therefore, the importance and utilization of technologies that can analyze nucleic acid biomarkers is rapidly increasing.

Types of Biomarkers

FDA BEST biomarker definitions

There are many different ways to classify biomarkers. One approach is to classify them based on the ultimate use of the biomarker. A framework was developed by the FDA and NIH to promote consistent use of biomarker terms and concepts as they pertain to the progression from basic biomedical research to medical product development to clinical care.[1] They established the Biomarkers, EndpointS, and other Tools (BEST) Resource glossary to standardize and clarify terminology and uses of biomarkers and endpoints. For these guidelines, they defined a biomarker as a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. In this definition, molecular, histologic, radiographic, or physiologic characteristics are all types of biomarkers.[1] Importantly, a biomarker is not an assessment of how a patient feels, functions, or survives. The definitions that came out of the BEST recommendations[1] are:

- **Diagnostic biomarkers** – These are used to detect or confirm the presence of a disease or condition of interest, or to identify individuals that have a subtype of the disease.[1]
- **Monitoring biomarkers** – These are measured repeatedly for assessing status of a disease or medical condition, or for evidence of exposure to (or effect of) a medical product or an environmental agent.[1]
- **Response biomarkers** – These are used to show that a biological response, either beneficial or harmful, has occurred in an individual who has been exposed to a medical product or an environmental agent.[1]
- **Predictive biomarkers** – These are used to identify individuals who are more likely than other individuals without the biomarker to experience a favorable or unfavorable effect from exposure to a medical product or an environmental agent. Companion diagnostics fall into this category.[1]
- **Prognostic biomarkers** – These are used to identify the likelihood of a clinical event, disease recurrence, or progression in patients who have the disease or medical condition of interest. These biomarkers are often used for risk stratification.[1]
- **Safety biomarkers** – These are measured before or after an exposure to a medical product or an environmental agent to indicate the likelihood, presence, or extent of toxicity as an adverse effect.[1]

Biomarker:

A biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition. Also called molecular marker and signature molecule.

(https://www.cancer.gov/publications/dictionaries/cancer-terms/def/biomarker)

These categories are discussed in more detail in several different publications (for example, Califf).[2] Note that these categories are not mutually exclusive; a single biomarker may satisfy several of these definitions. For more information, see their website.[3]

Molecular biomarkers

Another way to classify biomarkers is on the type of molecule or characteristic that will be measured. Some of these can be based on cell- or tissue-level analyses, such as imaging, electrophysiological responses, or changes in the abundance of specific cell types. More commonly, biomarkers are subcellular molecules that can be measured with common laboratory equipment. This type of biomarker includes proteins and metabolic products that can be measured by mass spectrometry, ELISA, or other assays.

However, one of the most flexible categories of biomarkers, for reasons discussed below, is those based on nucleic acids. These analyses can analyze DNA or RNA molecules, and can be either presence/absence testing for specific sequences, or analyzing differences in relative levels of a gene or set of genes. Some of the types of nucleic acid biomarkers include:

- **Gene variant** – This is a specific sequence or sequence variation. Gene variant biomarkers are often used as companion diagnostics. Certain types of tumor treatment strategies involve knowing whether the tumor contains the PD-L1 transcript (Keytruda), is HER2 positive (Herceptin), or contains a KRAS G12C mutation [adagrasib (Krazati)]. Therefore, knowing the status of the indicating biomarker will dictate whether the treatment has a likelihood of working or not. Groups of variants found together may also be biomarkers that can help categorize individuals into high-risk or low risk groups.[4–6]
- **miRNA** – miRNAs are a type of small RNA, usually 17-23 nucleotides long, that can influence the outcome of gene expression by regulating translation of an mRNA or set of mRNAs. miRNAs are attractive candidates for biomarkers due to their small size, stability, and potential for regulating several different pathways. For example, in one study of polycystic ovary syndrome (PCOS), seven different miRNAs were found to have different levels in serum samples of women with PCOS.[7]
- **Gene expression signature** – This is a specific group of mRNAs that together are a result of an altered biological process. A recent example was published by Ma et al. They analyzed gene expression microarray data and TaqMan assays to define a set of 15 mRNAs indicating possible relapse in non-small-cell lung cancer.[8] Expression signature biomarkers may also include long non-coding RNAs (lncRNAs).[9]
- **Epigenetic mark** – These biomarkers are chemical modifications of DNA or DNA-binding histones that can affect the activation or silencing of genes. These are most frequently used for biomarkers as changes in 5-methylcytosine levels in a gene or genes.[10] Ghoreifi et al. used a set of three differentially-methylated genes and two controls to develop a urine-based epigenetic biomarker test for upper tract urothelial carcinoma (UTUC), with higher sensitivity than standard urine cytology.[11]
- **Extracellular vesicles (EVs)** – Although these themselves are not nucleic acids, many of the types of RNA and DNA molecules described above are packaged into extracellular vesicles. These EVs are present in significant amounts in circulating fluids. Because it is relatively easy to collect and purify EVs from circulating fluids, they may provide convenient avenues for biomarker development and analysis. For a recent review on how EVs are used to isolate biomarkers in for cancer research, see Mitchell et al.[12]

Why nucleic acid biomarkers over other types?

Nucleic acid biomarkers provide several advantages over other types of biomarkers. First, nucleic acids have similar biophysical properties. This means that techniques used for purification and analysis will be similar across different types of diseases and read-outs. Second, they are relatively stable and do not require specialized storage solutions or facilities. Third, if a new test or altered specificity is needed, it is easy to modify the assay by changing any querying nucleic acid sequences in the assay. Finally, the equipment needed for such analyses is readily available in academic, clinic, or hospital settings, or service provider laboratories. For these reasons, it is straightforward for researchers to develop and utilize a biomarker assay using nucleic acid targets.

Sources of molecular biomarkers

Biomarkers can be grouped based on the source of the analyte.[1] Given the diversity of pathologies, biomarkers can be developed from any type of tissue that is directly affected by the pathology. However, it is also possible to isolate biomarkers in tissues not directly affected. The affected cells might shed measurable biomarkers into systemic circulating fluids. Alternatively, a biomarker might be found that is characteristic of a systemic response to a pathology. Thus, it can be



The equipment needed to analyze nucleic acid biomarkers is readily available in academic, clinic, or hospital settings, or service provider laboratories, making it straightforward for researchers to develop and utilize biomarker assays with nucleic acid targets.

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useful to define a biomarker using a wide variety of tissue sources. Some examples that are commonly used for nucleic acid biomarkers are given below.

- **Tissue biopsies** – These are commonly used to define biomarkers at the source of the pathology. Biopsies can be fresh or frozen, but most commonly used are FFPE tissue samples. However, collecting tissue biopsies can cause significant discomfort for the patient, and in some cases it is not even possible to collect a sample of the affected tissue.[13,14]
- **Blood, serum and plasma** – These collections can be used to directly assay pathologies of circulating cells, such as leukemias. However, these blood collections are becoming commonly used as liquid biopsies. Biomarkers in liquid biopsies are likely to be based on biomolecules that are shed from the diseased tissue into the general bloodstream. These free circulating DNA or RNA molecules (cfDNA or cfRNA) provide convenient sources of analytes that can provide a snapshot of the diseased cells.[13]
- **Cerebrospinal fluid (CSF)** – CSF is the fluid that surrounds the brain and spinal cord. Collections usually rely on a lumbar puncture of the spinal cord. Like blood collections, CSF fluid can be used for liquid biopsies. However, because of its proximity to central nervous system tissues, it is a good source for biomarkers for the brain and spinal cord pathologies. Although there are usually few white blood cells in CSF, there is often cfDNA or cfRNA that can be used for biomarker development.[15,16]
- **Urine** – These collections are very easy, and therefore there is a lot of interest in developing urine as a liquid biopsy. Some systemic biomarkers may be found in urine as a result of clearance and excretion, but urine is often a source for analytes related to kidney, bladder, or reproductive organ dysfunction. Urine can be a source of cells and circulating free nucleic acids.[11]
- **Stool** – These non-invasive collections are also easy on the patient (but may be emotionally traumatic). Stool samples can contain both cells and circulating free nucleic acids that can be used to identify biomarkers. And although there could be biomarkers from distal tissues, stool samples are often used to analyze biomarkers in gastrointestinal pathologies.[17]
- **Buccal swabs** – These are scrapings from the inner cheek, and are used to collect cells for genomic DNA analysis. Buccal swabs are considered non-invasive and are therefore a good source for biomarker research of genomic DNA. When used for in this capacity, these can be used to detect specific sequence variants or epigenetic markers.[10]
- **Saliva** – This can also be used as non-invasive biofluid for detecting biomarkers. Saliva can be collected in large quantities and has many of the same analytes found in blood.[13]
- **Bronchoalveolar lavage** – This source is a way to directly obtain and query lung cells and lung-derived extracellular vesicles. Bronchoalveolar lavage is therefore useful for biomarker development for lung cancer and other lung diseases.[14]

In the last quarter century, there has been incredible progress in the discovery and application of molecular biomarkers for multiple pathologies. A comprehensive review is beyond the scope of this ebook. Nevertheless, it is useful to understand how Applied Biosystems solutions are continuing to be used for biomarker development and utilization. In the following sections, we describe some recent examples of how nucleic acid biomarkers have been discovered and used using Applied Biosystems tools for cancer and neuroscience research.

Because cancer is characterized by genomic mutations, one of the most common types of biomarkers used in cancer research is genetic variants.



It can be useful to define a biomarker using a wide variety of sources, including tissue biopsies, blood, CSF, urine, stool, buccal cells, saliva, and lung samples.

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BIOMARKERS IN CANCER

Cancer is a mutational disease of the genome. Despite more than half a century of intensive research, cancer remains an unconquered disease. Efforts to control cancer benefit from the analysis of biomarkers, by identifying those that are directly related to the tumor, or identifying those that are part of the systemic response to a tumor.

Biomarker analysis by genotyping

Because cancer is characterized by genomic mutations, one of the most common types of biomarkers used in cancer research is genetic variants. The most common genetic alteration used as cancer biomarkers are single nucleotide variants (SNVs) or small insertions or deletions (indels).

Several tumor inhibitors have been designed to work once the predominant genotype of the tumor is known. These are sometimes known as companion diagnostics, since the genotypic test must be performed before the inhibitor is given. For example, the inhibitor adagrasib was designed to covalently bind to the mutant cysteine in KRAS G12C variants, locking the mutant KRAS protein in its inactive state and preventing its growth-promoting effects.[6] This compound does not recognize wild-type KRAS, and therefore does not interfere with normal function. Many other similar compounds are currently in use.[5] Genotyping biomarkers, as companion diagnostics, are therefore critical to ensure the proper use of a molecule designed against distinct genotypes.

Another genotypic biomarker for tumor pathology that is currently under investigation is based on microsatellite instability (MSI). Microsatellite instability is often caused by mutations in or decreased expression of mismatch repair genes. Tumors with defective mismatch repair pathways produce varying lengths of microsatellite markers and larger amounts of random mutations in coding genes, also known as neoantigens. Tumors with high neoantigen burden may be sensitive to certain immune checkpoint inhibitors. Microsatellite instability is therefore a biomarker for tumors that may respond to these inhibitors.[18] Microsatellite instability is often measured by analysis of PCR fragment length by capillary electrophoresis across a collection of microsatellite loci. MSI is most commonly used as a biomarker in colorectal cancers,[19] but it can also contribute to tumor formation in other tissues. For example, using the Applied Biosystems™ TrueMark™ MSI analysis kit, Brunetti et al identified subtypes of endometrial tumors by analyzing their MSI status.[20]

Finally, genotypic biomarkers may be found in non-coding regions of genes. In an analysis of colorectal (CRC) tumors, Jeon et al. used a combination of restriction fragment length polymorphism (RFLP) analysis and Sanger sequencing to identify biomarkers in the thymidylate synthase 3'-untranslated sequence that could indicate prognosis or tumor progression.[21] They found two alleles that were significantly increased in CRC tumors.

MicroRNAs as biomarkers

miRNAs are small noncoding RNA molecules, often 17-23 nucleotides long, that help regulate the activity of genes. miRNAs act by binding to complementary sequence targets in mRNA and cause either the destruction of the mRNA or prevent protein from being made by inhibiting the translational machinery. miRNAs play vital roles in many biological pathways, and dysregulation of miRNA abundance has been linked to various pathologies, including cancer. The complementary sequence target of a miRNA may be found in many different mRNA molecules; thus, a single miRNA can regulate a suite of genes that are part of a common cellular response or pathway. And because they are short, miRNAs can be resistant to nucleases or other exogenous destabilizing factors. This facilitates recovery from difficult biological samples, such as FFPE tissue or liquid biopsies. These two properties – their multitarget regulatory capabilities and biological stability - make them highly attractive candidates for biomarker discovery in cancer research.

One of most widely studied miRNA biomarker in various types of cancer research is miR-21.[22] This molecule has been investigated as a promising diagnostic and prognostic biomarker in colorectal cancer,[23] breast cancer,[24] lung cancer,[25] and many others. Although mir-21 is a useful biomarker for understanding the biology of these cancers, other miRNAs might be more specific for other types of cell dysfunction and therefore might be better biomarkers for those cancers. For example, one study analyzed serum miR-375 levels in medullary thyroid cancers using Applied Biosystems™ TaqMan™ Advanced assays, and found that high levels of miR-375 were correlated with a poor outcome.[26]

Researchers sometimes do not have a good idea which miRNA would make a good biomarker for the pathology being investigated. In those cases, an unbiased, discovery-based strategy is usually their

first step. Researchers may begin by querying collections of common miRNAs. For one study, the TaqMan MicroRNA arrays were used to identify miRNA differences between normal and laryngeal squamous cell carcinoma (LSCC) samples. The authors identified three miRNAs that suggested they could be used as non-invasive biomarkers for LSCC.[27] Similarly, Ravegnini et al. used TaqMan microRNA cards followed by confirmation with individual TaqMan assays to find miRNAs in FFPE endometrial tumors that could differentiate mismatch-repair deficient from tumors with no specific molecular profile.[28]

For whole-miRNome analysis, investigators turn to microarrays for their discovery research. In one recent study, the Applied Biosystems™ GeneChip™ miRNA 4.0 assay was used to research candidate biomarkers in colon tumor samples and identified several candidates for further study.[29] In a study of pancreatic tumors, Mazza et al. used the GeneChip miRNA 4.0 assay to query plasma samples for circulating miRNAs.[30] They identified four candidate miRNAs and confirmed differential expression and utility of two by digital PCR. Similarly, a different study of prostate cancer started by analyzing plasma from normal and prostate-cancer FFPE samples using the GeneChip miRNA assay. They selected six for confirmation by qPCR and homed in on miR-107 as a biomarker of interest.[31]

When identifying a gene expression signature biomarker, researchers often start by performing an unbiased, transcriptome-wide survey comparing normal and pathological samples.

Gene expression signatures as biomarkers

A gene expression signature can be defined as characteristic change in expression levels of a gene or group of genes in response to a stimulus or pathology. A gene expression signature can therefore be a biomarker of what is happening to cells or tissues. Gene expression biomarkers serve many uses in cancer research. Tumor mRNA profiling can reveal mechanisms of origin, histological subtypes, cancer stages, risk stratification, and other features. Expression of immune-related genes may be important biomarkers for researching immunotherapy responses. And expression of mutant genes or fusion transcripts can be biomarkers that could point to the mechanism of the underlying pathology. In some cases, RNA expression profiling of plasma can match to the affected tissue, providing a non-invasive path to knowing tissue of origin, risk, or cancer type.[32]

When identifying a gene expression signature biomarker, researchers often start by performing an unbiased, transcriptome-wide survey comparing normal and pathological samples. In some studies, the candidate genes can be identified from public databases or projects. One commonly-used public source of gene expression information is the NCBI gene expression omnibus (GEO) collection of data sets, where the results of over 20,000 human gene expression studies using chip-based microarrays, in a wide variety of tissues and under different conditions, have been deposited.[33] The GEO database also contains results obtained by NGS RNAseq and other platforms. To mine this data for cancer biomarkers, Fauteux et al. used the GEO database to investigate a gene expression signature that could stratify different types of lung tumor cells.[34]

Once the discovery-based experiment has identified candidates, investigators will then focus on a gene or set of genes using an orthogonal technology, usually PCR, and more samples. One example of this workflow is illustrated by a study of bladder cancer. In this investigation, Olah et al. used The Cancer Genome Atlas to identify 68 mRNAs that could be a classifier for different cancer subtypes.[35] They then used custom TaqMan gene expression array cards to confirm and refine the data set, and also used the signature to show the importance of tumor-stromal interactions in the progression.

Researchers can also accumulate discovery-based transcriptomic results using their own samples. In one study, paired tumor and normal breast cancer samples were analyzed using the Applied Biosystems™ GeneChip™ Human Transcriptome microarray. They identified six genes important for the epithelial-to-mesenchymal transition that could define a gene expression biomarker for tumor metastasis.[36] In a different study to identify gene expression biomarkers in oral squamous cell carcinoma, Kengkarn et al. used expression arrays to discover candidate genes, and confirmed the top candidates using TaqMan gene expression assays.[37]

Chip-based microarrays provide an efficient mechanism for whole-transcriptome queries needed in the discovery phase of identifying gene expression signature biomarkers. For example, Ellsworth et al. investigated a gene expression signature for metastatic breast cancer. Using GeneChip HG U133A v2.0 microarrays and laser capture microdissected tissue, they identified 51 genes that showed greater than two-fold difference between primary and metastatic tumor tissue. Candidate genes were confirmed using TaqMan gene expression assays, and they found that up-regulated genes tended to function in extracellular matrix stability, while down-regulated genes might contribute to growth in new locations.[38] Another group investigated chemoresistance in malignant pleural mesothelioma (MPM). They used fluorescent activated cell sorting to disrupt intratumor heterogeneity and analyzed expression in resistant versus normal cells using the GeneChip Human Gene 2.0 for discovery, followed by TaqMan PCR for confirmation. They identified an 18-gene expression signature that had prognostic relevance for MPM research.[39] And Ibragimova et al. used the Applied Biosystems™ Clariom™ S gene expression chip for human samples to discover differences in gene expression resulting from neoadjuvant chemotherapy of breast tumors. They found that a panel of 21 genes provided a signature that could predict the occurrence of hematogenous metastasis.[40]

Other groups have used other strategies for their discovery research, but still confirm using PCR. For example, one study identified a 20-gene pituitary neuroendocrine tumor classifier signature using NGS RNAseq and validated using TaqMan PCR.[41] Finally, lncRNAs can define an expression signature biomarker.

Buishand et al. used a microarray specific for lncRNAs to query adrenocortical carcinoma tumors, confirmed with TaqMan qPCR assays, and found that they could define one lncRNA that appeared to be down-regulated when the tumor cells became malignant.[42]

Methylated DNA as biomarkers

Epigenetics is rapidly becoming recognized as a factor in cell function and dysfunction. For this reason, scientists have been using epigenomic strategies to define biomarkers in cancer research. Epigenetics is defined as mechanisms by which the utilization of the genome is altered without changing the genome sequence. Some common epigenetic mechanisms include miRNAs and lncRNAs, DNA methylation, and histone modification. With the exception of histone modification, nucleic acid analysis techniques can be used to identify epigenetic biomarkers associated with tumor formation or classification. In this section, examples of how DNA methylation can be used in cancer biomarker research are discussed.

The definition and use of epigenetic biomarkers in colon cancer research has been very successful.[17] One successful biomarker for used in colon cancer research is based on detecting methylated promoter sequences in the Septin-9 gene (SEPT9).[43] Tests that detect methylated SEPT9 DNA in liquid biopsy samples by PCR have been developed and subsequently approved for use by the FDA as a diagnostic test (Epi proColon®).[44] In lung cancer, extensive work has been done on defining and detecting methylated biomarkers in various liquid biopsy samples, including blood-based collections, sputum, bronchoalveolar lavage fluid, and urine. As a result, both prognostic and diagnostic biomarkers that can be used for lung cancer research have been identified (reviewed in Li et al.).[45] Similarly, liquid biopsy samples can be a promising source of methylated DNA biomarkers that can be used in research studies to detect ovarian cancer (reviewed in Terp et al).[46] Many of these markers can be analyzed by methylation-specific PCR or similar techniques.[46] For example, it was shown that the promoters of HOXA9 and HIC1 were hypermethylated by using PCR of tissue and serum samples from epithelial ovarian cancer tumors,[47] and the authors propose this panel would be useful as a biomarker in early detection. Finally, Huang et al. described a method for multiplexed query of methylated loci using methyl-specific PCR followed by fragment analysis by capillary electrophoresis. They were able to analyze four different loci that were differentially methylated in hepatocellular carcinoma in a single liver tissue sample.[48]

Conclusions

The research of biomarkers that can detect tumors and their response to interventions remains an important avenue for combatting cancer. No matter what is the nature of the nucleic acid biomarker, most researchers follow a similar path from unbiased, discovery-based queries, through focusing on candidate sequences, to designing specific sequence detection strategies that will ultimately give a useful readout. The Applied Biosystems portfolio provides tools that can assist these scientists every step of the way.



BIOMARKERS IN NEUROSCIENCE

The brain is an amazing and complex organ. However, with this complexity comes increased chances that things can go wrong. As with cancer, biomarkers are needed to understand the etiology, track progression of pathologies, and ultimately research treatments for brain disorders. Unfortunately, the development of biomarkers for neurological diseases has lagged slightly behind other pathologies. However, increased sensitivity and better sample collection techniques are making it easier for researchers to track brain health by analyzing biomarkers.

One of the problems associated with analyzing biomarkers in neurological research is that obtaining tissues for analysis is difficult. Research subjects are often reluctant to give brain or other neural biopsies. For this reason, researchers have been looking for biomarkers circulating in blood samples. However, this raises another problem: the blood-brain barrier makes the brain's chemistry inaccessible to convenient blood tests. The analysis of biomarkers in cerebrospinal fluid (CSF) is therefore an attractive mechanism for collecting information about brain and CNS tissues. Nevertheless, blood and CSF remain the most attractive and practical sources of biomarkers for neurological diseases (reviewed in Doroszkiewicz et al.).[16]

Nucleic acid biomarkers for neuroscience can be grouped into the same categories described above, and can be discovered and analyzed using the same techniques. Although less common than in cancer research, in some neurological diseases monitoring of genetic variant biomarkers is still useful (for example, see Prasuhn and Brüggemann).[49] Gene expression signatures[50] and methylated DNA biomarkers[51] are more common. These can be analyzed using tissue biopsies, but they are more common because they can be packaged in extracellular vesicles or in circulating free nucleic acids. Finally, miRNA analysis in circulating fluids is widely used. A recent study provided guidelines for analyzing miRNAs in CSF using TaqMan array cards.[15]

In this chapter, we illustrate how nucleic acid biomarkers are discovered and analyzed using Applied Biosystems research tools in neuroscience problems. Note this is not an exhaustive review, but focuses on major pathologies and recent studies.

Biomarkers in cancers of the nervous system

Several types of cancers, including glioblastomas, astrocytomas, and neuroblastomas, can arise in brain cells. Glioblastomas, the most common type of primary brain cell cancers, are aggressive and almost always lethal cancers that can result in death within 15 months. Characterization of the disease is often a challenge, since it mostly relies on MRI imaging and tissue biopsies. Akers et al. investigated a non-invasive miRNA signature as a biomarker for glioblastoma. They analyzed miRNAs in CSF using TaqMan Open Arrays, containing assays for 754 common miRNAs, and confirmed their results distinguished glioblastoma from other types of brain tumors using individual TaqMan assays for candidate miRNAs.[52] In another study, a set of genes was found to be selectively overexpressed in congenital glioblastoma, a tumor type affecting infants. For this, the researchers utilized frozen tissue samples and characterized changes in gene expression using the GeneChip HG-U133 Plus 2.0 microarray. A set of 31 genes were differentially expressed and could therefore be a biomarker, for congenital glioblastomas.[53]

Diffuse B-cell lymphomas can sometimes be found in the central nervous system. Ferreri et al. analyzed CSF from samples with primary central nervous system lymphoma (PCNSL). They were able to define a biomarker, consisting of a genotype at the MYD88 locus together with IL-10 expression levels, using TaqMan assays. Their biomarker displayed high sensitivity and specificity for detection of PCNSL in primary and relapsed samples.[54]

Biomarkers and Alzheimer's research

Alzheimer's disease (AD) is an incurable neurodegenerative disorder that slowly destroys cognitive function. As the global population ages, AD is increasing in frequency. There are around 50 million AD patients worldwide, but this number is projected to increase to 152 million by 2050.[55] The burdens of AD affect individuals, their families, and the economy, there is therefore tremendous interest in understanding the disease. Researchers are therefore employing multiple strategies to identify biomarkers for etiology, early detection, and monitoring of the disease.

Currently, the only confirmed genetic biomarker for sporadic AD is the APOE e4 variant. However, Babić Leko et al. showed that a specific SNV in the



The only confirmed genetic biomarker for sporadic AD is the APOE e4 variant. Researchers are employing multiple strategies to identify biomarkers for etiology, early detection, and monitoring of the disease.

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monoamine oxidase B (MOAB) gene is increased in frequency in APOE e4/e4 homozygotes using TaqMan genotyping assays, making it a candidate second biomarker for AD susceptibility.[56]

Investigation of miRNA biomarkers in AD is an important strategy for identifying at-risk or early stages of the disease. One study identified candidate miRNA biomarkers in CSF fluid using TaqMan Array Human MicroRNA A + B Set v3.0 cards.[57] They identified 36 candidate miRNAs that were further confirmed in separate studies of CSF using custom TaqMan array cards.[58] Using GeneChip arrays to discover candidate miRNAs from serum of Alzheimer patients,[59] and confirming these candidates using PCR,[60] Kumar et al. identified miR-455-3p as another peripheral biomarker, but in serum samples instead of CSF.

Another avenue of active research for AD is investigating methylated DNA (meDNA) as a biomarker. A candidate gene approach was used by one lab to identify a potential biomarker in genomic DNA from circulating peripheral blood cells. They used the Applied Biosystems™ Methyl Primer Express™ software to design primers specific to the coenzyme A synthase (COASY) promoter gene after bisulfite conversion. They then used PCR followed by high-resolution melt (HRM) to examine amount of methylation at this locus, and found that this locus had higher amounts of methylation relative to normal controls.[61] Other labs have taken discovery-based approaches to identify candidate meDNA markers. Song et al. used data in the GEO database to correlate methylation changes, analyzed on bead arrays, with changes in transcription analyzed on gene expression microarrays (GeneChip Human Gene 1.0 ST array and Human Genome U133 Plus 2.0 array) in AD brain samples. They concluded that undermethylation of the TGFBR3 gene promoter could be a risk factor for the disease.[62] Other labs have used bead chips or NGS for discovery-based research for candidate loci in blood samples that would need to be validated using an orthogonal approach, such as PCR.[63,64]

Biomarkers and Parkinson's research

Parkinson's disease is another debilitating neurodegenerative pathology. Parkinson's disease is characterized by loss of dopaminergic neurons in the substantia nigra region of the brain, controlling motor neurons. There have been a number of germline variants that may lead to predisposition to Parkinson's disease,[65] however other biomarkers are needed that can provide early detection and increase diagnostic accuracy.

One avenue of active research is to identify circulating miRNAs for Parkinson's disease. Patil et al. initiated a discovery-based study using GeneChip 4.0 miRNA arrays to query serum from Parkinson's individuals, and confirmed using PCR.[66] They found that a combination of three circulating miRNAs can discriminate between Parkinson's and normal individuals, providing a non-invasive method for querying pathology state. Another approach is to analyze gene expression signatures. One study hypothesized that circulating monocytes may have a similar gene expression signature to brain microglia in Parkinson's disease. Several genes involved in immune activation were found to be up-regulated in the early disease and may provide an early-phase biomarker.[67] Finally, the analysis of meDNA biomarkers, as either causative or indicative of the pathology, is being investigated (reviewed in Miranda-Morales et al.).[68]

Biomarkers and Huntington's research

Huntington's disease is an inherited progressive neurodegenerative brain disorder that, like Alzheimer's and Parkinson's diseases, currently has no treatment or cure. Huntington's disease pathology arises due to an expansion of CAG triplet repeats in the huntingtin gene (HTT). Because Huntington's disease arises from defects in a single gene, precise genetic diagnoses are possible. Nevertheless, because it is progressive, biomarkers are needed to assess advancement and prognosis in afflicted individuals (reviewed in Martí-Martinez and Valor).[69]

One obvious biomarker to diagnose Huntington's disease (HD) is the size of the CAG repeat cassette in the HTT gene. This is usually performed by PCR across the CAG repeat cassette, and analyzing the fragment length by capillary electrophoresis (for example, see Blanco et al.).[70] Although this biomarker can be used to determine the likelihood of the pathology, other peripheral biomarkers are

needed to track progression. For example, Romano et al. discovered candidate miRNAs using plasma from HD individuals and the GeneChip miRNA array, followed by verification using TaqMan assays. They found that the U13 small nucleolar RNA (SNORD13) was very specific for discriminating HD from normal individuals.[71]

Biomarkers and schizophrenia research

Schizophrenia is one of the most common severe mental disorders, affecting the afflicted individual's ability to think, feel, and behave. Currently, the diagnosis of schizophrenia is based on the patient's symptoms and behavioral signs. The identification of biomarkers that can be used for identifying afflicted individuals would be beneficial, and research for nucleic acid biomarkers is ongoing. Wei et al. used TaqMan Array cards to discover candidate miRNAs in serum, and confirmed using TaqMan assays. They identified two candidates that were more abundant in schizophrenia versus normal serum.[72] In a different study, genes differentially expressed in a subtype of schizophrenia were identified using a GeneChip Human Exon 1.0 ST microarray. The resulting gene expression signature was proposed as a blood-based biomarker for those who have impaired working memory phenotype.[73] And expression of genes that influence DNA methylation were found to be differentially expressed in schizophrenic blood lymphocytes and postmortem brains versus normal controls.[74]

Conclusions

The research for biomarkers that can detect brain and other neural pathologies remains important for benefitting the afflicted individuals and the people who care for them. No matter what is the nature of the nucleic acid biomarker, most neuroscience researchers follow a similar path from unbiased, discovery-based queries, through focusing on candidate sequences, to designing specific sequence detection strategies that will ultimately give a useful readout. The Applied Biosystems portfolio provides tools that can assist these scientists every step of the way.

SHAPE SHIFTING CANCER: CONNECTING TUMOR HETEROGENEITY AND ecDNA

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Above image: Courtesy of Anish Thomas, NCI Center for Cancer Research

Anish Thomas is a medical oncologist who focuses his practice and research at the National Cancer Institute on small cell lung cancer (SCLC). Thomas built a clinical and translational research program for SCLC, aiming to shift treatment paradigms for this deadly disease by uncovering the mechanisms behind tumor heterogeneity.

What is SCLC?

SCLC is the most aggressive type of lung cancer. It affects about 30,000 people annually in the US, about 250,000 individuals worldwide. Most patients diagnosed with SCLC die within a year of their diagnosis, and there are very few treatment options. So, in terms of the frequency, lethality, and lack of effective treatment options, there is a huge unmet need.

I have been fortunate to study this cancer from different angles. We have clinical trials using novel combinations and treatments, and we also apply computational approaches trying to identify what makes this cancer aggressive from a genomic standpoint. The third aspect of what we do is trying to derive hypotheses from genomic data that we test in cell lines and animal models. We try to take a comprehensive approach to understanding this deadly disease.

Why is it difficult to treat SCLC?

The big problem is tumor plasticity and heterogeneity. This is a shapeshifting tumor; when you treat it with one type of therapeutic, it shifts its characteristics. That is one of the features that make it very aggressive. Another feature is its ability to metastasize even before it causes symptoms. We think these are the reasons SCLC is so chemotherapy and immunotherapy resistant.

What causes this tumor heterogeneity?

The SCLC genome is highly altered with copy number gains and losses, amplifications, and deletions. SCLC is a smoking-associated cancer, and smoking causes a lot of genomic damage. In additional to heterogeneity driven by genomic alterations, SCLC cells are highly plastic and changes gene expression patterns based on signals from different microenvironments, and therapy pressures. We found that some patient tumors and cell lines had really high focal amplifications of specific genes. We wondered how we could explain these amplifications, especially of oncogenes such as MYC. Looking at the literature, we found that some can be explained by extrachromosomal DNA (ecDNA), which are extra-chromosomal elements that result from DNA damage and repair.

How does ecDNA contribute to tumor heterogeneity?

ecDNA segregates unevenly across daughter cells, enabling some cells to accumulate large numbers of ecDNA compared to others, producing intratumoral heterogeniety.

The high gene dosage provided by ecDNA might also allow cells to differentiate/reprogram, thus producing more intratumoral heterogeneity. For example, MYC is known to drive SCLC evolution; preclinical studies and animal models have shown that a huge transcriptional burst of MYC allows a transition between differentiation states. Extrachromosomal amplification of MYC probably underlies this switch.

How do you measure ecDNA?

We initially used AmpliconArchitect, which is a program that allows you to assemble distinct amplified and connected chromosomes. Computational approaches that identify ecDNA are still developing. Because these tumors have extremely complicated genome alterations, inferring these connections is a computational challenge.

We validated ecDNA in cell lines using fluorescent in situ hybridization (FISH) probes for MYC, MYC-L, and other genes. We also validate some junctions by PCR to confirm that they are indeed connected, and by optical mapping, which provides information on the connections between different, distant areas of chromosomes.

What research question will you explore next?

The big question is whether you can target ecDNA. ecDNA seems to fuel heterogeneity, metastasis, and resistance, which are what make cancers such difficult diseases to treat. A number of approaches are being tested to target ecDNA are still in early stages.

To target ecDNA, you also need to know which tumors contain it. For SCLC and many other tumors, it is difficult to procure tissue samples, so non-invasive approaches to detect ecDNA are important. One approach that we developed to infer ecDNA in patient plasma is called circulating free DNA ChIP-seq (cfChIP-seq). We found that ecDNA amplification profiles are very similar between tumor whole genome sequences and cfChIP-seq data. These are preliminary data, but this could be one approach to identify ecDNA amplifications non-invasively.

This interview has been condensed and edited for clarity.

BIOFLUID BIOMARKERS: MicroRNAs THAT PREDICT ALZHEIMER'S DISEASE

Julie Saugstad, PhD
Professor of Anesthesiology and Perioperative Medicine,
Oregon Health and Science University



Above image: Courtesy of Aaron Bieleck, OHSU EdCOMM

While investigating fruit fly gene transcripts during her doctoral studies, Julie Saugstad became fascinated by a small RNA that she observed at the bottom of her RNA blots. Although she never uncovered their identity, Saugstad credits her early interest in these mysterious molecules, now known to researchers as microRNAs (miRNAs), as a pivotal moment in her academic journey. Now a professor of anesthesiology and perioperative medicine at Oregon Health & Science University (OHSU), Saugstad investigates miRNAs as biomarkers for brain and neurodegenerative diseases.

How are miRNAs involved in neurodegenerative disease?

MiRNAs regulate messenger RNA translation and can communicate between specific cells by transport in extracellular vesicles, which is not random but a specific method of extracellular communication. Before working on Alzheimer's disease (AD), I studied miRNA in stroke. A small stroke does not cause much brain injury, and it seems to protect the brain from injury later on, if followed by a large stroke within a few days. That little stimulus basically tells the cells to hunker down. You hear this all your life: if a little bit of something doesn't kill you, it makes you stronger. I thought it was interesting that these mechanisms could protect your organs and cells, so we looked at miRNAs in that context. We could see changes at that protective time point, and worked to identify miRNA targets in terms of regulating mRNA translation.

Soon after I moved to OHSU, a new opportunity through the NIH arose to look at extracellular RNAs as disease markers in existing human biofluids. OHSU has an Alzheimer's Disease Research Center with a biobank that includes cerebral spinal fluid (CSF) collected by lumbar puncture. We found miRNA differences between Alzheimer's and control CSF collected from living donors, which was exciting because all previously published work was done with postmortem CSF. We found the same thing in another patient cohort at the University of California, San Diego. Then we looked at people with mild cognitive impairment (MCI), which can be a precursor to AD, and found that some of the AD miRNAs change in MCI, which was super cool.

Why is it important to develop biomarkers for early disease stages?

As we try to understand how the brain works, we find more and more players. We are trying to understand how miRNAs change in early disease stages and identify protein targets that have not been looked at before. When a patient sees a clinician for AD, there are changes in the amyloid β/tau ratio and clinicians may measure these changes as AD biomarkers, but we hope to detect other players involved earlier in the disease process.

Is there a relationship between protein biomarkers and miRNA biomarkers in AD?

If we add the amyloid β/tau ratio to our miRNA set to predict AD, this increases biomarker performance for AD, and the performance is further increased in patients with MCI. This finding tells us that the miRNAs provide more information about the MCI stage of the disease than AD, which can be an advantage for developing an early disease biomarker.

How do you measure miRNAs and are there any challenges working with CSF?

We use PCR to measure miRNAs because there is a low amount of RNA in CSF. In the beginning of the NIH study, we were asked why we were not using RNA sequencing (RNA-seq), but we only receive about half a milliliter of CSF per sample from biobanks because it is a really precious biofluid. This volume just does not contain enough RNA to do RNA-seq. However, RNA-seq allows you to discover other small RNAs, including novel miRNAs that are not represented on PCR arrays, so we presume there are more miRNAs that change in AD that we have not identified. We are now working with collaborators who have shown us that RNA-seq can work on these small volumes of CSF, which is very exciting!

What excites you about the future of AD biomarkers?

There is a large impetus to develop amyloid β/tau to use as a blood-based biomarker, as these measures have largely only been successful in CSF. But a future biomarker blood test could involve looking for changes both in miRNAs and amyloid β/tau to assess whether a patient may have AD, or to decide if they may want to see a neurologist. If you know that changes in the brain are happening earlier, one goal is to determine ways to slow them down.

This interview has been condensed and edited for clarity.

TECHNOLOGY OVERVIEW

There has been incredible progress in the discovery and application of molecular biomarkers for multiple pathologies. The Human Genome Sequencing project was instrumental for generating tools that both generated sequence information and used that sequence information to understand human health and disease. The techniques and data generated by these tools have been critical for developing new nucleic acid-based biomarkers. Applied Biosystems technologies have been at the forefront of development of these tools and has a deep portfolio of solutions that are used in biomarker research.

The Applied Biosystems solutions portfolio can be thought of as a continuum that gives researchers options based on the types of questions being asked (Figure 1). For example, some investigators might be interested in **discovery**-based experimentation, using unbiased queries spanning the entire genome to uncover new sequences or relationships between genes. These types of experiments are facilitated by Ion Torrent™ AmpliSeq™ next generation sequencing (NGS) workflows or by Applied Biosystems microarray solutions. Once a set of sequences of interest is decided, investigators might move to a focus-based experimental model, where these selected sequences are analyzed in medium-throughput analyses such as Sanger sequencing to focus on a defined region across many samples, or Applied Biosystems™ TaqMan™ panels on array cards to focus on sets containing many genes. Finally, if a very specific set of mutations or genes need to be analyzed in large number of samples with minimal effort, individual TaqMan assays may be used for **detecting** these sequences.

Importantly, these approaches and technologies are complementary to each other. For example, a researcher might start with a discovery-based approach to catalog the mutations commonly found in a tumor, then focus on those mutations by confirmatory Sanger sequencing in more samples, and finally design a TaqMan probe to the mutation(s) that can be used to stratify patients for a research program. The Applied Biosystems solutions continuum is meant to ease the research path from discovery to insight.



Figure 1. The Genetic Analysis Continuum. In oncology research, the tools and techniques used depend on the question being asked, the scale of the data that needs to be analyzed, and the logistical needs of the investigator. Applied Biosystems genetic analysis systems fall into a continuum, from very large-scale discovery based research, to medium-scale **focused** research, to very targeted **detection** of specific genes and mutations.

In this chapter, we give a brief overview of the technologies in the Applied Biosystems continuum and how they could be used for biomarker research. However, this is not a complete or comprehensive list. The flexibility of these solutions to cancer research depends only on the researcher's experimental creativity.

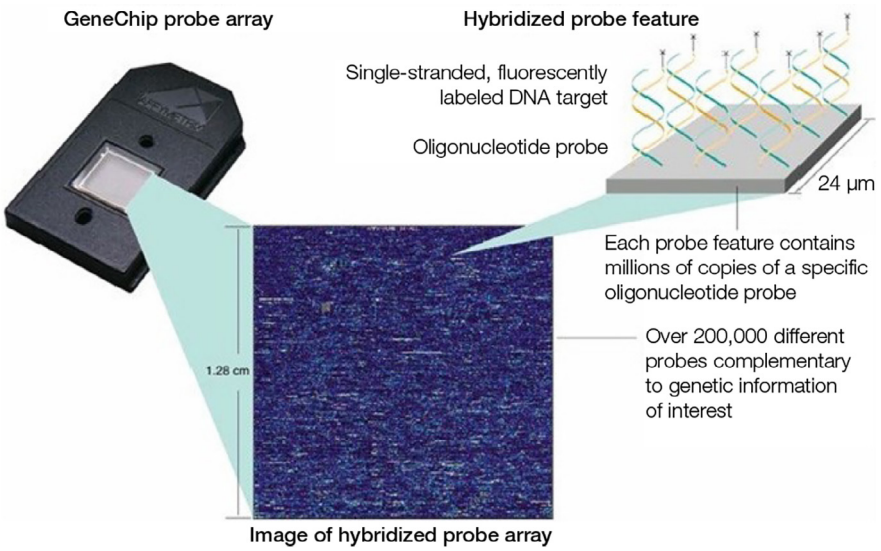
Microarray methods and tools

When a normal cell goes down a path that ultimately ends in a dysfunctional cell, it can acquire mutations that may range from single nucleotide changes, to small indels, to copy number changes, to large chromosomal rearrangements. One way to characterize these genomic changes is to use high-density DNA microarrays. For these analyses, hundreds of thousands of probes tiled across the genome are arrayed onto a single chip. Sample hybridization to these arrays can determine which sequences are present and in how many copies (Figure 2). The advantage of microarrays is that hundreds of thousands to millions of sequences can be interrogated in a single experiment. If the appropriate probes that detect specific mutations are present on the chip, they can also detect common SNP variants. Microarrays are also useful for detecting copy number changes. The resolution of the copy number sequences is determined by the number of probes available; when probes are directly designed for predefined common deletions or duplications, these can detect anomalies as small as 1,000 nucleotides. Whole-genome microarrays that cover both polymorphic (e.g., single-nucleotide polymorphisms or SNPs) and nonpolymorphic regions of the genome can be used to assess DNA copy number alterations at a much higher resolution than conventional cytogenetic analyses.

Several microarrays have been specifically designed for cancer research and can be useful for genotypic biomarker identification. The Applied Biosystems™ CytoScan™ Suite includes hybrid-SNP arrays, automated and manual target preparation options, fully kitted reagents, the Applied Biosystems™ GeneChip™ System 3000 instrument for array processing, and Chromosome Analysis Suite (ChAS) software for data interpretation and reporting. These tools support labs to generate accurate, high-quality data and maximize operational time. The Applied Biosystems™ OncoScan™ CNV Plus Assay is a whole-genome copy number microarray-based assay designed to query genomic DNA that has been degraded, such as FFPE-extracted DNA. Similarly, the Applied Biosystems™ OncoScan™ CNV Assay has the same copy number coverage as the OncoScan CNV Plus Assay, but does not include somatic SNP mutation probes. Finally, the Applied Biosystems™ Axiom™ PangenomiX Array includes over 800,000 genotyping probes. The content was selected to emphasize variants that are commonly seen in clinical research, including common cancer variants, clinically actionable variants, pharmacogenomic variants, and more. The common cancer markers were chosen from the list of published variants associated with cancer phenotypes identified via GWAS, as per NHGRI-EBI GWAS catalog as well as some recently published and unpublished cancer-associated SNPs. The clinically actionable variants include relevant variants from ClinVAR and enable assessment of actionable genetic risk across a wide range of populations.

miRNAs and other non-coding RNAs are important regulators of gene activity. It is estimated that more than 30% of protein translation of coding genes is regulated by miRNA. Because of their small size, stability, and potential for regulating several different pathways, miRNAs are ideal molecules for biomarker research. The GeneChip™ miRNA 4.0 Array keeps pace with the discovery of new and novel miRNA. This array is designed to interrogate all mature miRNA sequences in miRbase, including human, mouse and rat miRNAs. The analysis files contain host gene ID, predicted and validated miRNA target genes, and clustered miRNA information, facilitating downstream analysis.

The Clariom microarrays represent the next level of transcriptome analysis solutions. Human Clariom S Assay design provides extensive coverage of all known well-annotated genes, is compat-



Automated or manual sample prep

- **Sample types** - blood, bone marrow, FFPE, fresh and frozen tissue
- **Sample Input** - 80-250 ng DNA
- **Sample preparation** - automated on the NIMBUS Target Preparation Instrument, or manual preparation

Microarray processing

- **Flexible throughput** - High resolution whole-genome copy number analysis on the Applied Biosystems™ GeneChip™ microarrays instrument

Date analysis and reporting

- **Data analysis with automated Interpretation and reporting** - Chromosomal analysis suite (ChAS) software for genome-wide view and analysis of chromosomal aberrations, SNP variants, and copy number determinations.

Figure 2. Basics of a microarray assay.

ible with most research sample types, and is a tool well-suited for finding gene expression biomarkers with known function as quickly, easily, and cost-effectively as possible. The more advanced Clariom D Assays are based on coding and noncoding sequences culled from 16 different databases,[75] representing over 540,000 transcripts in over 6.7 million probes. Included in these sequences are probes for different splice isoforms, noncoding RNAs (pre-miRNAs, lncRNAs, Piwi-interacting RNAs (piRNAs)), and circular RNAs, as well as annotated and speculative sequences. Clariom D Assays allow translational research scientists to generate high-fidelity biomarker signatures quickly and easily.[75] Querying the multiple transcript models on the Clariom D chip in a single experiment helps ensure that important biomarkers are not missed.

Analysis of transcriptome analysis is facilitated by the Applied Biosystems™ Transcriptome Analysis Console (TAC) Software data. A report of all transcripts and the associated relative expression, using statistical and visualization tools, is provided from the data. Additionally, information regarding the role of these transcripts in biological pathways is provided. Together, the combination of microarrays and analysis software provides rapid, easy, and economical tools for identifying meaningful gene expression signature biomarkers.

Fragment analysis methods and tools

Fragment analysis is a highly flexible method that can be applied to a wide variety of research fields. The flexibility afforded with the choice of PCR primers (PCR being a necessary step in any fragment analysis experiment) means that a specifically sized fragment corresponding to a PCR target sequence is straightforward to generate (Figure 3). Coupled with the ability to label fragments with up to four different fluorophores, researchers have great flexibility in their fragment analysis experimental design. Below, we describe some examples of how fragment analysis can be used in biomarker research problems.

Mutation Detection

Biomarker researchers frequently need to investigate or verify a limited number of variations in one or more genes of interest. The Applied Biosystems™ SNaPshot™ Multiplex System is a versatile and economical method to perform SNP genotyping. Up to ten SNP markers (on different genes) can be investigated simultaneously by using PCR amplification, followed by dideoxy single–base extension (SBE) using an unlabeled primer, and then capillary electrophoresis of the resulting fragments. After electrophoresis and fluorescence detection, the alleles of a single marker appear as different colored peaks at roughly the same size in the electropherogram plot.[76]

Microsatellite Instability Assay

Microsatellite instability (MSI) is a hallmark of several cancers and is characterized by changes to the length of microsatellites due to defects in mismatch repair mechanisms (MMR). Microsatellites are genetic motifs consisting of 1-6 base pair repeats. These sequences are susceptible to replication errors that can result in deletions and insertions. Normally, these errors are corrected by the DNA MMR, however, when deficiencies in the DNA MMR system are present, microsatellite replication errors accumulate in the genome.[77] In addition to its prognostic value for certain types of cancer including colorectal[78] and endometrial, MSI can also serve as a predictive biomarker for immune checkpoint therapy response.[79]

The Applied Biosystems™ TrueMark™ MSI assay interrogates 13 mononucleotide MSI markers.[80] Eight of these markers are derived from the literature and the guidelines from the National Cancer Institute[81] and the other five markers were internally identified for monomorphism and high sensitivity in multiple cancer types. The assay also contains two sample identification markers to determine sample mix-up or contamination.[80] To facilitate analysis of MSI data, we developed the Applied Biosystems™ TrueMark™ MSI Analysis Software.

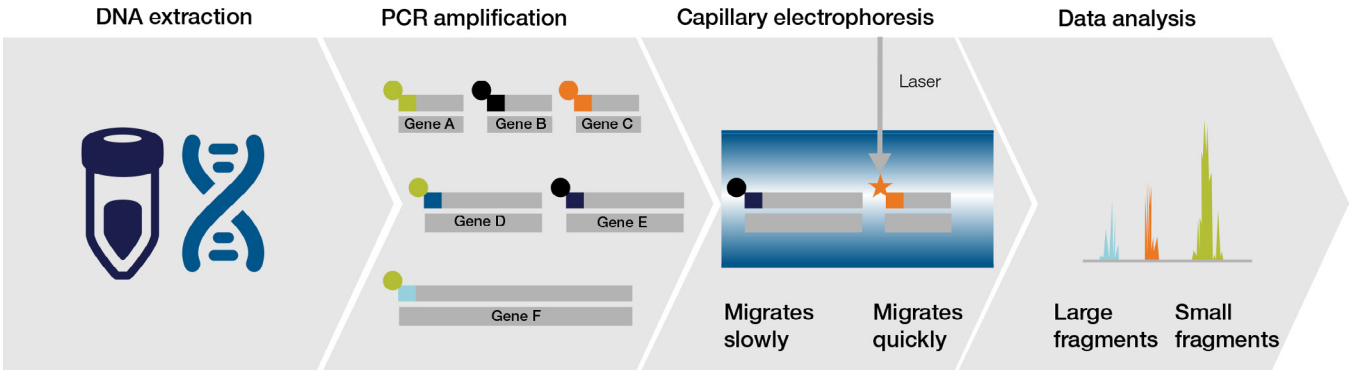


Figure 3. Basics of a fragment analysis assay. PCR primers are designed such that different target sequences are amplified with primers with different fluorophore labels, and that will generate different sized amplicons. Following PCR, the amplicons are electrophoretically separated by size in a capillary. A laser will excite the fluors as the fragments migrate through the capillary. The size and color of the resulting fragments reflects the abundance of the target sequence in the sample.

* Public database sources for Clariom D Assays: RefSeq, ccdsGene, mgcGenes, UCSC Known Genes, lincRNATranscripts, VEGA, GENCODE, NONCODE, MiTranscriptome, RNAcentral, Ensembl, circBase, Guo CircRNAs, AceView, lncRNAWiki, lncRNADB.

This is a desktop-based application that takes the fragment analysis files of the TrueMark MSI Assay and provides an easy-to-interpret analysis of the results. An advanced feature of the software is a proprietary algorithm that can analyze samples without the need for running normal sample controls concurrently, saving time and expense.

Copy Number Variability

Multiplex Ligation Dependent Probe Amplification (MLPA),[82] a technology developed and commercialized by MRC Holland B.V., is a flexible technique (www.mlpa.com) that is commonly used to detect aberrations in gene copy number such as loss of heterozygosity.[83] It is based on the ligation and PCR amplification of up to 50 multiplexed pairs of probe oligonucleotides, which hybridize to the loci of interest. Each oligonucleotide pair is designed to give an amplification product of a specific length; by using sequence-tagged ends, all ligated probes can be amplified with a single primer pair in a PCR reaction. The forward PCR primer carries a fluorescent label, allowing for the detection and quantification of size-separated probes on an automated capillary electrophoresis system.[82]

Epigenetic Modification

Changes in DNA methylation patterns are also characteristic of oncogenesis. Genome-wide hypomethylation or hypermethylation, particularly of cytosines 5′ to guanosines (CpG) within the promoter regions of tumor repressor genes, has been shown to be associated changes in gene expression that could lead to a gene expression signature biomarker.[84] The SNaPShot assay can also be used to investigate DNA methylation patterns following bisulfite treatment of genomic DNA. Sodium bisulfite converts unmethylated cytosines into uracil, which are subsequently replaced as thymine in PCR; in contrast, methylcytosines remain unchanged. This feature can be used to distinguish base differences by PCR amplification, T/C genotyping using SNaPshot SBE followed by fragment analysis.

MLPA can also be used to detect epigenetic modifications involving DNA methylation.[85] The methylation specific-MLPA (MS-MLPA) assay has probes that bind and ligate over a GCGC sequence, which is also a cleavage site for a methylation-sensitive restriction enzyme HhaI. The enzyme cleaves the probes ligated and hybridized to unmethylated DNA while the probes bound to methylated DNA remain intact and are subsequently amplified by PCR. This assay has been used to determine promoter methylation status of tumor suppressor genes p15 and p73 in acute myeloid leukemia cell lines.[85]

Cell Line Authentication and Human Sample Matching

Human cell lines are heavily relied upon for biomedical research including cancer- and neuroscience-related studies. To ensure reproducibility of scientific research, cell line authentication (CLA) is of paramount importance to confirm the cell's origin as well as check for contamination and genomic instability. CLA is performed by generating a profile of highly variable short tandem repeat (STR) markers from microsatellite loci with varying number of repeats for a particular cell line/type and then comparing it with the allelic profiles present at these loci against known standards. A study aimed at authenticating 278 human tumor cell lines used in China found that nearly 46% of the samples were either cross contaminated or misidentified.[86] Needless to say, such findings can have massive implications on the conclusion of studies that utilize such cell lines. The Applied Biosystems™ GlobalFiler™ CLA kit generates a molecular fingerprint for 24 different STR loci, while the Applied Biosystems™ Identifiler™Plus CLA kit analyzes 16 STR loci.

Sanger sequencing methods and tools

Sanger sequencing is the trusted standard for obtaining DNA sequence information. It powered the Human Genome Project and investigators continue to rely on this method to generate highly accurate reliable sequencing results. Sanger sequencing is a specialized form of fragment analysis: it relies on chain-terminating fluorescent nucleotides to generate a series of fragments that differ by one nucleotide (Figure 4). Applied Biosystems Sanger sequencing workflows provide a high degree of accuracy, long-read capabilities, and simple data analysis. Applied Biosystems™ BigDye™ Terminator v1.1 and v3.1 cycle sequencing chemistries are widely used for Sanger sequencing by CE. After cycle sequencing, various options exist for clean-up before electrophoresis, including Applied Biosystems™ ExoSAP-IT™ enzyme mix and BigDye XTerminator kits. An entire sequencing workflow can be completed in less than one workday, from sample to answer, providing the flexibility to support a diverse range of applications in many research areas.

Discovery-based genomic research, such as next generation sequencing (NGS), often uncovers novel or unexpected variants or other sequence anomalies. Investigators

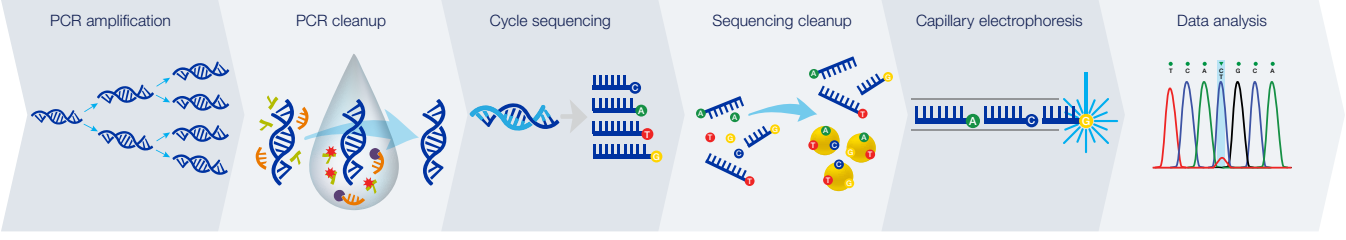


Figure 4. Basics of a Sanger sequencing workflow. The target region that will be sequenced is amplified by PCR. The primers and PCR reagents are removed before a second, linear amplification is performed. This step generates the fragments that are chain-terminated with a fluorescent dideoxynucleotide. The cycle sequencing reaction is purified, and the resulting fragments are separated by capillary electrophoresis and detected with a laser. The sequence can be read from the length of the fragments and the color of the dideoxynucleotide terminator.

look for ways to verify these new discoveries using orthogonal methods. Sanger sequencing is the method of choice for confirming NGS results studies because of its workflow simplicity and unambiguous results. For these confirmatory studies, short amplicons, usually covering only the region to be confirmed, need to be sequenced in these studies. Moreover, minor allelic variants, present in a heterogeneous sample, can be identified and confirmed by Sanger sequencing. Applied Biosystems™ Minor Variant Finder Software is simple, easy-to-use desktop software designed for the accurate detection and reporting of minor variants in Sanger sequencing traces with a detection level of minor alleles as low as 5%. On a test set of 632,452 base positions, it exhibited a 5% limit of detection with 95.3% sensitivity and 99.83% specificity.[87] Minor Variant Finder Software can also readily align sequences with the human reference genome and VCF files from NGS experiments, providing a smooth workflow for NGS confirmation with annotations in the dbSNP database.

PCR methods and tools

The TaqMan Assay family is a comprehensive set of real-time PCR (qPCR) products available for analyzing gene expression, miRNA levels, protein abundance, copy number variation, SNP genotyping, and rare allele mutation detection. The typical TaqMan assay consists of forward and reverse primers to amplify a target by PCR (Figure 5). TaqMan assays also contain a sequence-specific fluorescent probe, designed with a minor groove binder (MGB) moiety at the 3′ end that increases the melting temperature (Tm) of the probe and stabilizes probe–target hybrids. This means that TaqMan MGB probes can be significantly shorter than traditional probes, providing excellent sequence determination. In addition, TaqMan probes have a non-fluorescent quencher (NFQ) that binds to the fluor until cleaved by the polymerase and minimizes background. All TaqMan assays come with a performance guarantee such that if they do not perform as promised, they can be replaced or refunded.

One strategy that enables high-quality PCR data is that investigators follow standardized practices for performing and reporting RT-PCR results. The MIQE recommendations (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) are set of guidelines for real-time PCR (RT-PCR) experimental designs and data reporting practices, as well as standards for sharing experimental information with colleagues.[88] The standards are designed to ensure that published real-time PCR research is meaningful, accurate, and provides researchers with the information necessary to faithfully reproduce results. Applied Biosystems tools support these guidelines and provide all information necessary to ensure MIQE compliance when publishing the results of experiments with TaqMan assays. Information requested under the qPCR target, qPCR oligonucleotide, and qPCR protocol sections of the guidelines are readily available in the Applied Biosystems reagent protocol or from the Applied Biosystems website.

The TaqMan portfolio is made up of over 20 million predesigned assays. These assays can be ordered as individual assays in tubes. Alternatively, TaqMan Arrays contain TaqMan assays dried down in three array formats: 384-well TaqMan Array microfluidic cards (TACs), 96- and 384-well TaqMan Array plates, and OpenArray formats. The Taqman collection of assays includes genotyping and gene expression assays. In addition, the highly-specific TaqMan chemistry can also be used to analyze miRNAs for biomarker research.

The Applied Biosystems assay search wizard helps researchers choose the right TaqMan assays for an experiment.[89] Inputting a gene name, keyword, pathway, or disease can return a list of all the assays that meet the search criterium. The results page highlights the "best coverage" assay for each gene, publications that used the assay highlighted, and provides an opportunity to select and design a combination of assays for an array format. Furthermore, predesigned arrays are available that have been prespotted with gene expression assays targeting common pathways, diseases, and gene families, including several important in cancer and neuroscience research.

dPCR methods and tools

Digital PCR (dPCR) is a method that quantifies sequences present in a sample by counting them. The basis of dPCR is that a nucleic acid sample is segregated into thousands of parallel PCR reactions, such that each reaction well contains one target molecule on average (Figure 6). In this scenario, some reactions will not contain the target molecule at all, while others will contain one or more copies. The collection of segregated reactions is subjected to endpoint PCR, and the number of wells containing a positive signal and

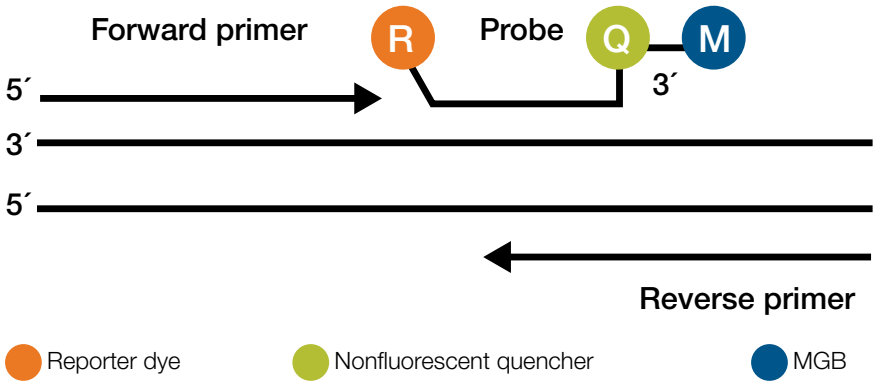


Figure 5. Anatomy of a TaqMan assay. Non-labeled forward and reverse oligonucleotide primers define the region that will be queried. A third oligonucleotide binds to the region between the primers. The probe has an end-labeled fluorophore reporter dye, a non-fluorescent quencher and a minor groove binding (MGB) moiety that stabilizes binding of the short probe sequence. When the probe is hybridized to its target sequence, the quencher greatly reduces fluorescence of the reporter. When the sequence is copied during the PCR reaction, the nuclease activity of the polymerase cleaves the reporter from the rest of the probe, releasing it so it can be detected by a laser in the instrument. The amount of fluorescence is therefore directly related to the amount of target amplified.

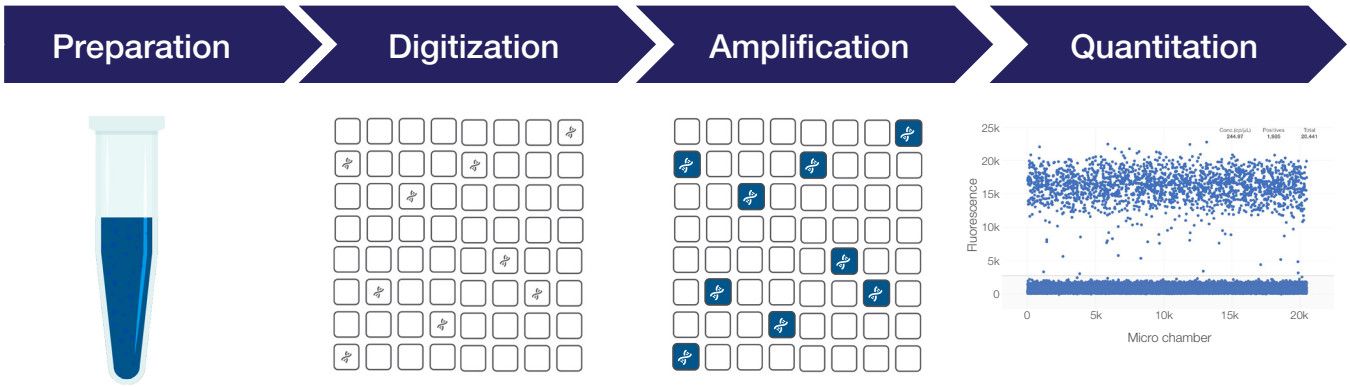


Figure 6. Basics of a digital PCR Assay. Absolute Q dPCR reactions make use of TaqMan assays to detect a target sequence. In dPCR assays, the sample is diluted and loaded into a matrix containing thousands of individual reaction chambers. If the dilution is correct, some of the chambers will have target and some will not. Subjecting the chambers to PCR will cause a positive signal in the chambers where there is a target, but no signal where there is no target. The wells that are positive and negative are counted, and the starting concentration can be calculated based on dilution and other factors. Because the sample is loaded such that there is one molecule per partition, an overabundance of one sequence will not necessarily overwhelm detection in wells that have a rare sequence.

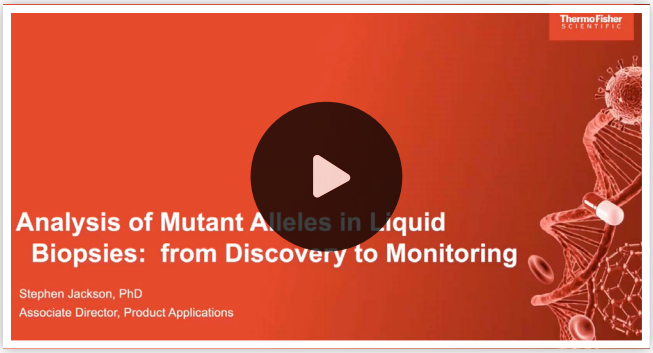
no signal are tallied. The number of target copies can be calculated from the fraction of negative reactions, based on the assumption that the segregation follows a Poisson distribution (thus accounting for the possibility that multiple target molecules occupy the same reaction). The number of individual reactions influences the sensitivity of the assay—the more reactions there are, the lower the limit of detection and the higher the accuracy.

dPCR is often used to detect rare mutant alleles in cancer samples and is used for analyzing circulating tumor DNA (ctDNA) in liquid biopsy research. Because the entire sample is segregated into individual wells, the detection of rare alleles is not masked by an overabundance of normal alleles. The sensitivity is dependent on the amount of DNA; to achieve a sensitivity of 0.1% (1 in 1,000 copies), 1,000 copies are needed. For diploid genomic DNA (gDNA), at least 6 ng of input gDNA is required for this sensitivity. Thus, the amount of recoverable DNA limits the sensitivity of the assay.

The QuantStudio Absolute Q Digital PCR System consists of just one instrument, consolidating all steps required into a single plate and transforming a multi-step multi-instrument workflow into a one-step qPCR-like workflow. And the TaqMan Absolute Q Liquid Biopsy dPCR assays detect and quantify the most common cancer-related mutations (e.g., EGFR, BRAF, KRAS, PIK3CA, JAK2), as well as therapy-resistant mutations (e.g., EGFR T790M). They have been verified to detect allele frequencies as low as 0.1% and are guaranteed to perform on the Absolute Q dPCR System. The Absolute Q Liquid Biopsy dPCR assays run on the Absolute Q Digital PCR system provide a precise, cost-effective, and rapid method for monitoring response and resistance to treatment by testing for biomarkers that may indicate tumor-driver and therapy-resistant mutations.

Learn more about Applied Biosystems solutions for cancer research at

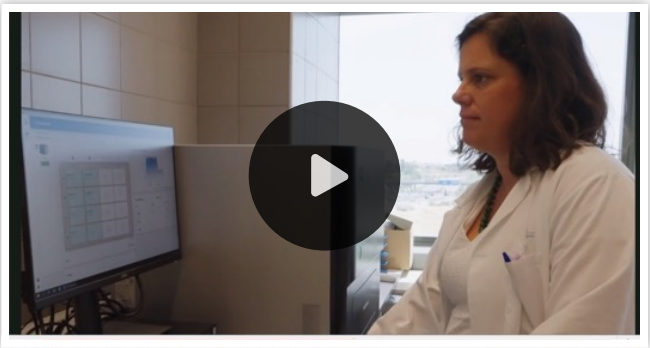
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Analysis of Mutant Alleles in Liquid Biopsies: from Discovery to Monitoring

Dr. Stephen Jackson
Associate Director, Product Applications,
Thermo Fisher Scientific

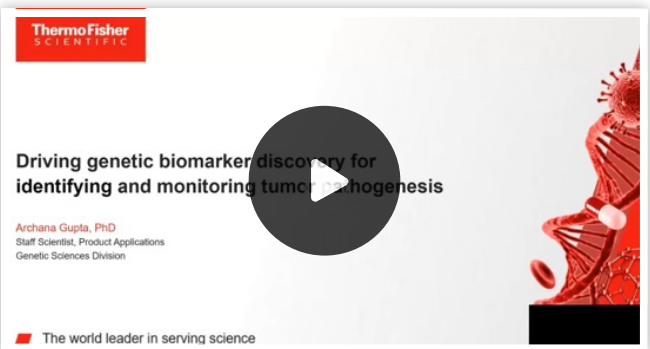
The use of quantitative PCR & Digital PCR for human disease research has increased substantially. Get insights in this on-demand webinar on how these powerful technologies have propelled some key human disease research areas and opened opportunities for new applications in the research landscape.



Analysis of Mutant Alleles in Liquid Biopsies: from Discovery to Monitoring

Dr. Atocha Romero
Head of the Liquid Biopsy Laboratory at Hospital Universitario Puerta Hierro, Majadahonda, Spain

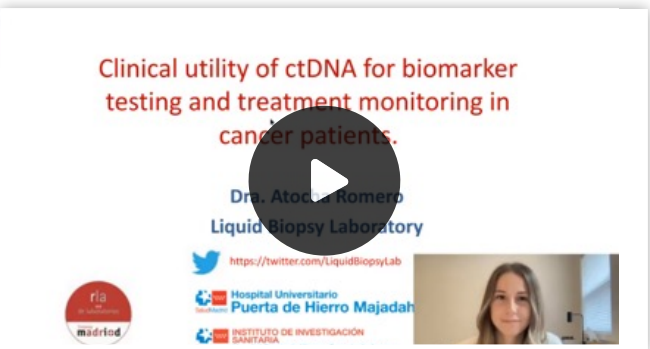
Dr. Atocha Romero from Hospital Universitario Puerta de Hierro-Majadahonda discusses her research into clinical utility of digital PCR in liquid biopsy for potential management and detection of cancer



Driving Genetic Biomarker Discovery for Identifying and Monitoring Tumor Pathogenesis

Dr. Archana Gupta
Staff Scientist, Genetic Sciences,
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Learn in this on-demand webinar on how genetic biomarkers have become increasingly important for disease prediction, early disease detection and progression, and options for intervention. Genetic markers such as microRNA, mRNAs and/or DNA either cell-free or packaged in circulating tumor cells (CTCs) and extracellular vesicles sourced from liquid biopsy provide the basis for biomarker research that contributes to precision medicine options for cancer.



Digital PCR (dPCR) in Liquid Biopsy for Management and Detection of Cancer

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Learn in this on-demand webinar on the analysis of circulating tumor DNA (ctDNA) by dPCR, which has several applications:

- ctDNA genotyping can be used for non-invasive biomarker testing, which is especially relevant in testing lung cancer samples
- ctDNA levels correlate well with tumor bulk
- ctDNA levels are of prognostic significance, as ctDNA levels have been shown to correlate well with survival outcomes

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