INTEGRATING TECHNOLOGIES TO STUDY NEURODEGENERATIVE DISEASES

Page 2
The Many Roads that Lead to Neuronal Demise

Page 3
A Neuroscientist's Toolbox to Investigate Neurodegeneration

Page 4
Scanning for Changes to Stop ALS

Page 5
Combining Technologies to Identify a Parkinson-Specific Cell Type

Page 6
Genetic Analysis Technologies to Support Neuroscience Research

Sponsored by

ThermoFisher SCIENTIFIC



THE MANY ROADS THAT LEAD TO NEURONAL DEMISE



In the past century, major advances in science, technology, and medicine transformed how health professionals detect, treat, and prevent disease. Compared to the early 1900s, the resulting healthcare and socio-economic improvements have nearly doubled the average person's life expectancy at birth to 73.4 years, according to the World Health Organization (WHO)'s most recent report.[1] While researchers developed effective therapeutics for infectious and childhood illnesses, chronic conditions such as cancer and neurodegenerative diseases (NDs) have been more difficult to treat. As the world's population ages, the prevalence of such complex, chronic diseases will continue to rise, presenting the next big health challenge for scientists to overcome.[2]

Funding a Way out of an Impending Neurodegenerative Pandemic

Based on the Global Burden of Disease Study 2019 (GBDS-2019), researchers predict that the number of people with dementia—a group of neurological conditions characterized by a progressive decline in cognitive function—will triple by 2050, and similar increases are expected for other late-onset NDs.[3] This increased prevalence will present a major challenge for society and its already strained healthcare systems. GBDS-2019 researchers estimated global dementia-related healthcare expenses at $594 billion in 2019, and they predict these costs to surpass $1.6 trillion by 2050.[4] In 2020, the compiled economic burden of neurodegenerative diseases amounted to $655 billion in the United States (US) alone.[5]

As NDs' looming global impact becomes more apparent, public health officials and governing bodies have increased funding for research projects aimed at improving ND detection and treatment. In the US, the National Institutes of Health budgeted nearly $5 billion to support research on neurodegenerative conditions in 2022, whereas the European Commission and leading European brain research initiatives allocated €6 billion to brain-related research between 2007 and 2019.[6,7] Such continued financial support from funding bodies present scientists with the necessary resources to simultaneously attack this problem from different angles to reveal the genetic and molecular changes that cause ND in order to develop more sensitive disease biomarkers and targeted therapies.

The Genetic Revolution

NDs are a group of debilitating and largely untreatable conditions characterized by the progressive loss of various parts of the central or peripheral nervous system. These diseases exhibit substantial pathologic, genetic, and clinical heterogeneity, which complicates disease classification as well as mechanistic, therapeutic, and prognostic ND research efforts.[8] Researchers have made major strides to resolve disease heterogeneity and stratify individuals into disease subtypes based on disease manifestation and inheritance mode. To do this, geneticists and clinicians combined forces, leveraging molecular biology approaches such as polymerase chain reaction (PCR), capillary electrophoresis, and next generation sequencing to identify the genes and genomic changes that underlie neuronal loss in a growing number of hereditary ND subtypes, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's Disease (HD), and amyotrophic lateral sclerosis (ALS). However, up to 90 percent of ND patients present with a sporadic form of their disease, and scientists remain largely puzzled by these patients' environmental and genetic susceptibility factors.

Leveraging Molecular Biology to Curb Neurodegeneration

For most NDs, symptom onset occurs when a large proportion of the affected brain region has degenerated. Therefore, successful ND treatment and prevention requires developing effective therapies that prevent neuronal loss and establishing more sensitive biomarkers that detect disease prior to symptom onset. Because both approaches heavily rely on understanding the molecular changes in intra- and extracellular signaling pathways that underlie neuronal demise, scientists have leveraged RNA-based technologies such as microarrays, quantitative real time PCR, and RNA sequencing to reveal transcriptional changes in patient samples and have combined them with protein-based approaches such as western blot, multiplex immunohistochemistry, and mass spectrometry for proteomic or lipidomic analyses. Recent technological advances in single cell and spatial biology technologies now allow scientists to probe ever deeper into individual brain cells as neurons degenerate. In sum, multidisciplinary approaches that leverage these molecular biology technologies promise to open new frontiers in discovering early diagnostic markers and effective treatments for these devastating diseases.

A Neuroscientist's Toolbox to Investigate Neurodegeneration

Neurodegenerative diseases (NDs) affect the central or peripheral nervous system and cause patients to progressively lose control over their sensory, motor, or cognitive functions. This set of complex disorders displays high levels of clinical heterogeneity in terms of disease onset, severity, and progression. Overcoming NDs will require concerted research efforts on three different levels to understand the genetic and environmental causes of NDs, develop sensitive biomarkers that detect and categorize disease prior to symptom onset, and design effective therapies that prevent further neuronal loss.



Identifying Genetic Drivers of Disease

To reveal the single nucleotide variants, triplet repeats, and structural genomic changes that are responsible for various ND subtypes, researchers rely on

- Polymerase chain reaction (PCR)
- Capillary electrophoresis
- Next generation sequencing
- Chromosome capture

Developing Sensitive Biomarkers

Researchers require ultra-sensitive technologies to analyze minute changes in RNA, protein, lipids, and metabolites between patient and control groups to develop diagnostic, predictive, and prognostic biomarkers.

- Microarray
- TaqMan™ real-time PCR
- Digital PCR
- RNAseq
- Mass spectrometry
- Metabolomics
- Artificial intelligence and machine learning

Designing Effective Therapeutics

Identifying and developing therapeutic molecules that slow down or halt disease progression will require mechanistic insights into the molecular changes that cause neuronal death. Scientists combine cutting-edge technologies with conventional molecular biology technologies to obtain these insights.

- Single cell RNA sequencing
- Multiplex immunohistochemistry
- Spatial proteomic and transcriptomic technologies
- Multi-electrode electrophysiological recordings

SCANNING FOR CHANGE TO STOP ALS

Janine Kirby, PhD
Professor in Neurogenetics The University of Sheffield



Obtaining her PhD in human genetics when the field was just emerging, Janine Kirby, now a neurogenetics professor at the University of Sheffield, was eager to identify the changes in a gene's sequence or expression level that cause disease. For more than 25 years, Kirby has focused on the complex genetic puzzle that underlies motor neurone disease, particularly amyotrophic lateral sclerosis (ALS). To do this, Kirby combines microarrays with other molecular biology technologies to reveal the affected pathways in ALS patients that can be targeted to halt disease progression. She uncovered an important role for oxidative stress and nuclear factor erythroid 2-related factor 2 (NRF2), the master regulator of the antioxidant response.[1] Follow-up studies revealed that NRF2 also modulates other pathophysiological ALS pathways—RNA metabolism, inflammation, and proteostasis—and clinical trials with NRF2-activating molecules are now ongoing.

Q. How do microarrays compare to other RNA-based technologies?

Microarrays allow us to extract RNA from cells or tissues of interest, and to simultaneously quantify a sample's transcripts in a snapshot from the time of RNA extraction. They are extremely powerful for quantifying a set of genes that are expressed at a single time point and are more cost-effective compared to qPCR. While recent technologies, such as RNA sequencing (RNA-seq) or single cell RNAseq, have emerged that assess the entire transcriptome, microarrays are very robust, bioinformatic data analysis is well-developed with both open source and commercial opportunities for data analysis, and array technology has evolved such that you can now also assess splicing.

Q. How do you incorporate microarrays in your work?

We incorporate them in different ways to quickly assess the expression of a set of genes. We initially used them to understand what was going wrong in cell models that expressed a mutant or wildtype protein linked to ALS. Over time, we started using them for animal models and patient samples to identify which pathways were dysregulated, so that we could identify potential targets to slow down disease progression.

Most recently, we used them to characterize patient responses to treatment in clinical trials.[2] Neuroinflammation is one of the mechanisms that causes ALS but there are currently no approved drugs that target this pathogenic pathway. We treated patients with a low dose of interleukin (IL-2) to normalize immune function in these patients and modulate neuroinflammation.[2] We used Clariom™ D Assays to test patient responses to IL-2 because this microarray allows us to not only determine changes in gene expression, but also in splicing. In combination with other RNA-based techniques, including NanoString and quantitative PCR (qPCR), we established a dose-dependent response to IL-2 treatment and identified a biomarker signature that stratifies patients into responders and non-responders.

Q. What is the role of microarray technology for neurodegenerative disease research?

There is still an important place for microarrays in the field, but likely more during the diagnostic process to quickly screen and stratify patients for treatment options, similar to how the technology is currently used in the cancer field. The type of RNA-based technology to use during this prognostic process will depend on the number of genes that need to be tested. For example, a scientist would not perform RNAseq if they needed to test 25 genes to identify a specific disease subtype and test prognostic markers. In that case, a microarray would be much faster. If, on the other hand, one only needed to test 5 genes, then a Taqman™-based qPCR would be the go-to assay.

Q. What do you see as the most promising technology for the field?

I think single cell RNAseq is the next big thing for the ALS field as it has now become more accessible to individual researchers and allows us to profile individual motor neurons to better understand which pathways are activated or dysregulated as the neurons degenerate.

Q. How should scientists best approach their first microarray?

The most critical component of running successful microarrays is experimental design: which groups will properly test a hypothesis, how many samples and replicates are needed, how will the data be analyzed, and how will microarray findings get validated? The scientist should also make sure to collect sufficient samples, preferably collected at the same time to reduce variability, and to store them properly for later validation studies. Finally, they should run pilot experiments to familiarize themselves with the process before they start and keep an eye on quality control along the way so that they can pick up on any issues as they happen and before they have run through too many samples.

COMBINING TECHNOLOGIES TO IDENTIFY A PARKINSON-SPECIFIC CELL TYPE

Malte Spielmann, MD *Clinical Director Institute of Human Genetics*
University Hospitals Schleswig-Holstein, University of Lübeck & Kiel University



Aiming to better understand how non-coding mutations and structural genomic variants cause disease, Malte Spielmann uses CRISPR gene editing technology to generate mutant mouse models of hereditary disorders. The human geneticist characterizes these models by combining cutting-edge technologies, such as single nucleus RNA sequencing (snRNAseq) and chromosome conformation capture, with other molecular biology assays to better understand the pathways that contribute to disease pathogenesis. In a recent study, published in Brain, Spielmann discovered a unique neuronal cell type in the brains of Parkinson patients.[1]

Q. How did you identify this Parkinson-specific cell type?

My collaborator and friend, Anne Grünewald, from the Luxembourg Centre for Systems Biomedicine, had collected a large number of patient samples and wanted to maximize the information she could gain from individual sections because they are so precious and difficult to come by. We decided to perform snRNAseq on them and developed a new protocol to process frozen brain sections for this technology. Unlike most researchers in the Parkinson field who focus on the dopaminergic neurons within the substantia nigra (the affected brain region in Parkinson disease patients), we analyzed sections of the entire midbrain. The first thing we noticed, when we clustered the cells, is that we could immediately identify the major cell types of the midbrain. Because we sequenced the entire midbrain, we noticed an increased abundance of microglia and found that these cells show signs of activation. But we also noticed a small cluster of cells that were exclusive to the brains of Parkinson's patients. Anne's team next used digital PCR and immunohistochemistry to confirm that these cells, which are marked by high levels of CADPS2 expression, only appeared in patient brains.

Q. What are snRNAseq's strengths and challenges?

The biggest strength of single cell or single nucleus RNAseq is that it is completely unbiased. For low-throughput experiments, you have to formulate hypotheses up front and decide which molecules to test. But when the data disproves your hypothesis, you need to redesign your experiment and start over. In contrast, if you do snRNAseq, you sequence all cells, cluster them, and let the bioinformatic analysis tell you what the differences are. It is so powerful!

The biggest challenge is to find out how to best process and prepare your sample or your tissue so that you get high quality data out of your scRNA-seq experiment. For our study, Anne's team collected and processed samples roughly 48 hours postmortem, so that affects RNA quality. We combined several slides per patient and performed flow cytometry to collect enough viable nuclei for RNAseq. It took us more than six months to optimize the RNA extraction and sequencing workflow.

Q. What is the importance of digital PCR and qPCR technology for neurodegenerative disease research?

As for all molecular biology approaches, one technology cannot exist without another. The field has successfully adopted advanced technologies such as snRNAseq and spatial biology approaches to address more complicated research questions. But regardless of the approach, researchers always need to confirm their data with an independent technology. This is especially the case when working with large data sets, because a significant difference does not necessarily mean that it is also biologically relevant. And that is why we have to go back and validate the findings. For our studies, we leverage several technologies, including digital PCR, qPCR, in situ hybridization, and immunohistochemistry to confirm and follow up on our snRNAseq findings.

Q. What are the next steps for this project?

We plan to sequence more patients with different forms of Parkinson's to see whether CADPS2+ cells are present across familial and sporadic forms of the disease. We are also performing follow-up studies to better understand the role of CADPS2+ cells in the neurodegenerative process and to test whether we can develop a novel biomarker for Parkinson's based on this knowledge.

GENETIC ANALYSIS TECHNOLOGIES TO SUPPORT NEUROSCIENCE RESEARCH

There has been tremendous progress in understanding and treating neural pathologies. However, the link between genomic variation and dysfunction, and the etiology of these pathologies, is still an intense field of investigation. The Human Genome Project was instrumental for developing 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 contributing to the knowledge of neural pathologies. Thermo Fisher Scientific has been at the forefront of development of genetic analysis tools and has a deep portfolio of solutions that are used in biomarker research.

The Applied Biosystems™ 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 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 by medium-throughput analyses. These include Sanger sequencing to focus on a defined region across many samples and Applied Biosystems™ TaqMan™ Assay 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 a 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 Assay to **detect** the mutation(s) that can be used to stratify patients for a research program. The



Figure 1. The genetic analysis continuum. In neuroscience research, the tools and techniques used depend on the question being asked, the scale of the data that need 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.

Applied Biosystems solutions continuum is meant to ease the research path from discovery to insight.

In this chapter, we give a brief overview of the technologies in the Applied Biosystems portfolio and how they can be used for neuroscience research. However, this is not a complete or comprehensive list. The flexibility of these solutions in neuroscience 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 and small indels to copy number changes and 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 with conventional cytogenetic analyses.

Genotyping microarrays are useful for discovering variants that are linked to a pathology. For genome-wide association studies (GWAS), the Applied Biosystems™ UK Biobank Axiom™ Array

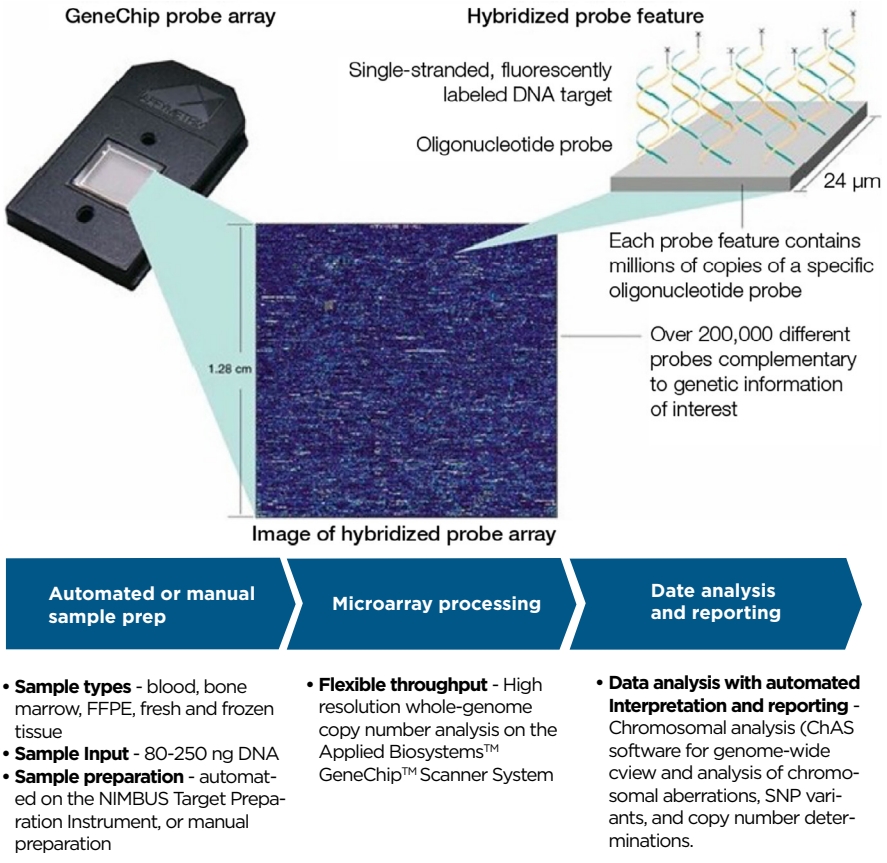


Figure 2. Basics of a microarray assay.

facilitates high-throughput, high-value genotyping of large sample cohorts with a single comprehensive low-cost solution. This assay contains one 96-array plate that allows for genome-wide analysis of large sample collections, such as those screened at biobanks, genome centers, and core labs. The Applied Biosystems™ Axiom™ Precision Medicine Diversity Research Array includes content that was selected to emphasize variants that are commonly seen in clinical research. Both arrays include variants associated with neuropsychiatric conditions, neurodegenerative diseases, clinically actionable variants, pharmacogenomic variants, and more. The markers were chosen from the list of published variants associated with phenotypes identified via GWAS, as per the 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.

Several microarrays have been designed specifically for cancer-focused, discovery-based research. The Applied Biosystems™ CytoScan™ HD Suite—comprising microarrays, reagents, and analysis software—is a comprehensive, high-resolution, whole-genome solution designed to assist in the understanding and characterization of biomarkers in hematological malignancies. 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 it does not include somatic SNP mutation probes. These arrays may be useful for brain or other neurological cancer research. Content on these arrays that query genomic CNVs may also be useful for understanding neural pathologies.

miRNAs and other noncoding RNAs are important regulators of gene activity. It is estimated that more than 30% of protein translation of coding genes is regulated by miRNAs. Importantly, miRNAs can be found in circulating fluids, such as blood or cerebral spinal fluid (CSF), and can be analyzed in liquid biopsies (Doroszkiewicz 2022). Because of their small size, stability, potential for regulating several different pathways, and location in CSF or blood samples, miRNAs are ideal biomarkers for neural pathologies. To keep pace with the discovery of novel miRNAs, Thermo Fisher offers the Applied Biosystems™ GeneChip™ miRNA 4.0 Array. 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 Applied Biosystems™ Clariom™ microarrays provide whole-transcriptome analysis solutions. The Applied Biosystems™ Human Clariom™ S Assay provides extensive coverage of all well-annotated genes, is compatible with most research sample types, and is an ideal choice for finding gene expression patterns with known function as quickly, easily, and cost-effectively as possible. The more advanced Applied Biosystems™ Clariom™ D Assays are based on coding and noncoding sequences culled from 16 different databases*, 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, long intergenic noncoding RNAs (lincRNAs), Piwi-interacting RNAs (piRNAs)), and circular RNAs, as well as annotated and speculative sequences. The Clariom D Assays allow translational research scientists to generate high-fidelity biomarker signatures quickly and easily (Thermo Fisher Scientific 2019a). Querying the multiple transcript models on the Clariom D chip in a single experiment helps ensure that important pathways and potential biomarkers are not missed.

Analysis of transcriptome data is facilitated by Applied Biosystems™ Transcriptome Analysis Console (TAC) Software. A report of all transcripts and the associated relative expression, using statistical and visualization tools, is provided for 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). Coupling this 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 (Thermo Fisher Scientific 2005).

Microsatellite Instability Assay

Microsatellite instability (MSI) is a hallmark of several cancers and is character-

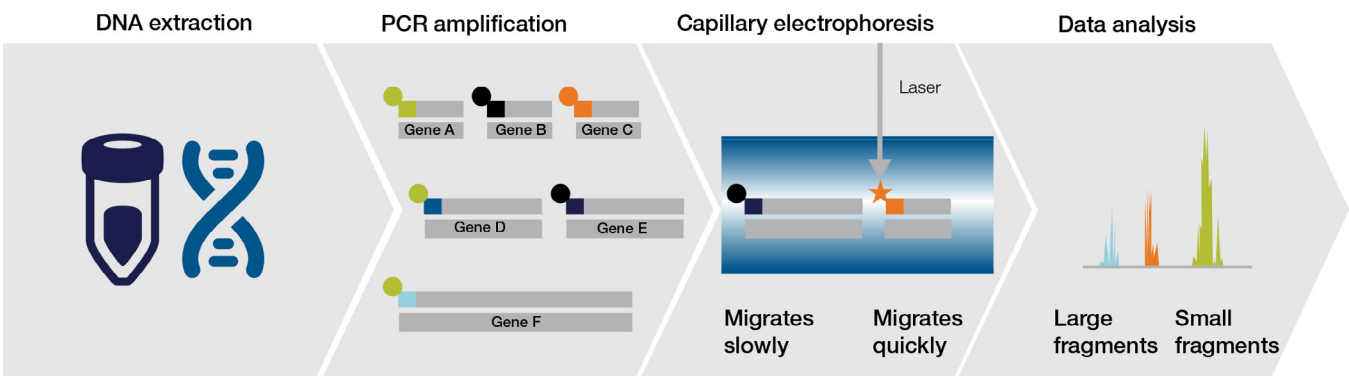


Figure 3. Basics of a fragment analysis assay. PCR primers are designed such that different target sequences are amplified with primers with different fluorophore labels that will generate different sized amplicons. Following PCR, the amplicons are electrophoretically separated by size in a capillary. A laser excites the fluorophores as the fragments migrates through the capillary. The peak intensity 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.

ized by changes to the length of microsatellites due to defects in mismatch repair (MMR) mechanisms. 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 DNA MMR; however, when deficiencies in the DNA MMR system are present, microsatellite replication errors accumulate in the genome (Reyes 2015). Although MSI is commonly seen in colorectal cancers (Kang 2018), it has been observed in pediatric glioblastoma.

The Applied Biosystems™ TrueMark™ MSI Assay interrogates 13 mononucleotide MSI markers. Eight of these markers are derived from literature and guidelines from the National Cancer Institute (Boland 1998), 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 (Thermo Fisher Scientific 2023). To facilitate analysis of MSI data, we developed Applied Biosystems™ TrueMark™ MSI Analysis Software. This is a desktop-based software that takes the fragment analysis files of the TrueMark MSI Assay and provides an easy-to-interpret analysis of the results. A groundbreaking feature of the software is a proprietary algorithm that can analyze samples without the need for running normal sample controls concurrently, which may help save time and expense.

Neuroscience researchers have long relied on fragment analysis for understanding a class of neurodegenerative diseases known as triplet repeat diseases. These diseases are linked to expansions of microsatellite repeats found within translated or untranslated regions of certain genes (Paulson 2018). The pathology often arises when the repeat sequence, which in normal alleles can range from 15 to 40 repeats, expands to exceed a disease-specific threshold, usually greater than 45 repeats. The disease severity may worsen from generation to generation because of *de novo* germline expansion of repeats. It is therefore critical to monitor the repeat length of these microsatellite sequences, especially in individuals who are at risk. Fragment analysis by CE has been recommended by the European Molecular Genetics Quality Network (EQMN) as the default analytical method for analyzing triplet repeat length in spinocerebellar ataxias (SCAs; Sequieros 2010) and Huntington's disease (Losekoot 2013). Kits that use CE to analyze repeat lengths in fragile X, Huntington's, and other neurodegenerative diseases are commercially available (asuragen.com/portfolio/genetics).

Copy Number Variability

Multiplex ligation-dependent probe amplification (MLPA™) technology, developed and commercialized by MRC Holland, is a flexible technique (mlpa.com) that is commonly used to detect aberrations in gene copy number such as loss of heterozygosity. 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, and 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 (Thermo Fisher Scientific 2019b). MLPA technology has been used to understand the role of CNV regions in Parkinson's disease (Abu 2022) and in brain development leading to differences in cognitive, neurodevelopmental, and neuropsychiatric traits (Sønderby 2021).

Epigenetic Modification

Changes in DNA methylation patterns are important in neurological diseases (Younesian 2022). 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 with changes in gene expression that could lead to a gene expression signature biomarker (Hwang 2017). The SNaPShot assay can 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 and T/C genotyping using SNaPshot SBE, followed by fragment analysis.

MLPA assays can also be used to detect epigenetic modifications involving DNA methylation (Nygren 2005). The methylation-specific MLPA (MS-MLPA) assay has probes that bind and ligate over a GCGC sequence, which is also a cleavage site for the methylation-sensitive restriction enzyme HhaI. The enzyme cleaves the ligated probes that are hybridized to unmethylated DNA, while the ligated

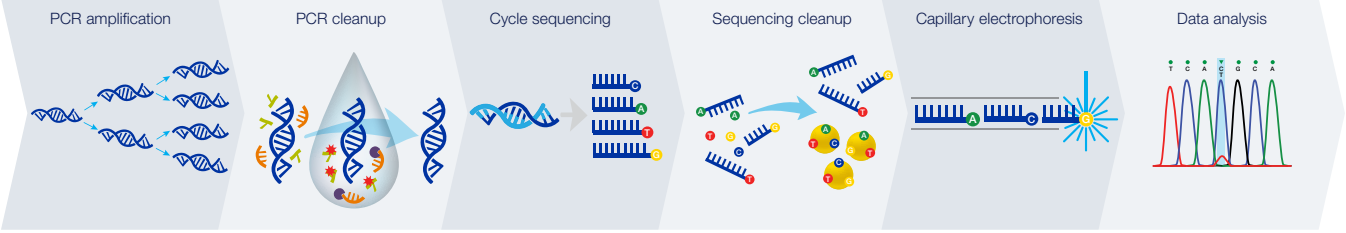


Figure 4. Basics of a Sanger sequencing workflow. The target region that will be sequenced is amplified by PCR. The primers are PCR reagents are removed before a second linear amplification is performed. This step fenerates the fragments that are chain-terminated with a fluorescent dideoxynucleotide. 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 lengths of the fragments and the colors of the dideoxynucleotide terminators.

probes bound to methylated DNA remain intact and are subsequently amplified by PCR. MS-MLPA was used to determine the status of 31 CpG islands in eight genes in multiple sclerosis, and to show that RUNX3 and CDKN2A methylation differed significantly between patients with MS and healthy controls (Sokratous 2018).

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 to 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 numbers of repeats for a particular cell line/type, and then comparing it with the allelic profiles of known standards present at these loci. 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 (Huang 2017). Needless to say, such findings can have massive implications on the conclusion of studies that utilize such cell lines. The Applied Biosystems™ CLA GlobalFiler™ kit generates a molecular fingerprint for 24 different STR loci, while the Applied Biosystems™ CLA IdentiFiler™ Plus 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 and 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). Fast and straightforward Sanger sequencing workflows using Applied Biosystems™ products 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 used for Sanger sequencing by CE. After cycle sequencing, various options exist for cleanup before electrophoresis, including Applied Biosystems™ ExoSAP-IT™ enzyme mix and Applied Biosystems™ 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.

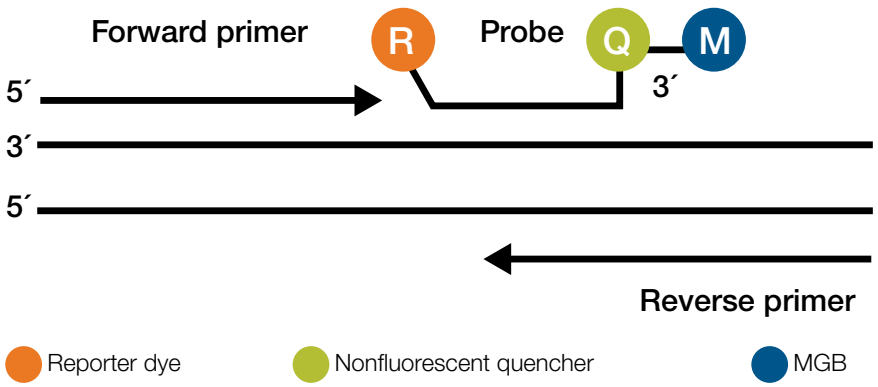


Figure 5. Basics of a TaqMan Assay. Unlabeled forward and reverse oligonucleotide primers define the region that will be queried. A third oligonucleotide binds to the region between the primers. The probe has an end-label fluorophore reporter, a nonfluorescent quencher, and a minor groove binder (MGB) moiety that stabilizes binding of the short probe sequence. When the probe is hybridized to its target sequence the quencher greatly reduces the 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 the reporter be detected. The amount of fluorescence is therefore directly related to the amount of target amplified.

The simplicity of Sanger sequencing facilitates the analysis of methylated DNA sequences (for example, see Salcedo-Tacuma 2019). Primers for sequencing can be designed using the freely available Applied Biosystems™ Methyl Primer Express™ Software (Cat. No. 4376014). After bisulfite conversion, the DNA can be sequenced using the standard Sanger sequencing workflow. Data analysis is also straightforward, since the converted sequence can be analyzed directly without needing to compare it to a specialized reference genome.

Discovery-based genomic research, such as next-generation sequencing (NGS), often uncovers novel or unexpected variants or other sequence anomalies. Investigators look for ways to verify these new discoveries using orthogonal methods. Sanger sequencing is the method of choice for confirming NGS results because of its simplicity and unambiguous results. For these confirmatory studies, short amplicons, usually covering only the region to be confirmed, need to be sequenced. 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 (Thermo Fisher Scientific 2016). 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 the most 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 of the probe and stabilizes probe–target hybrids. This means that TaqMan MGB probes can be significantly shorter than traditional probes, providing better sequence discrimination and flexibility to accommodate more targets. In addition, TaqMan probes have a nonfluorescent quencher (NFQ) that minimizes fluorescence until the reporter dye is cleaved by the polymerase.

qPCR target, qPCR oligonucleotide, and qPCR protocol sections of the guidelines are readily available in the reagent protocol or from the Thermo Fisher Scientific website.

The TaqMan Assay portfolio is made up of over 20 million predesigned assays. These assays can be ordered as individual assays in tubes. Alternatively, Applied Biosystems™ TaqMan™ Arrays contain TaqMan Assays dried down in three array formats: 384-well TaqMan array microfluidic cards, 96- and 384-well TaqMan array plates, and Applied Biosystems™ expression assays targeting common pathways, diseases, and gene families, including several that are important in 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 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.

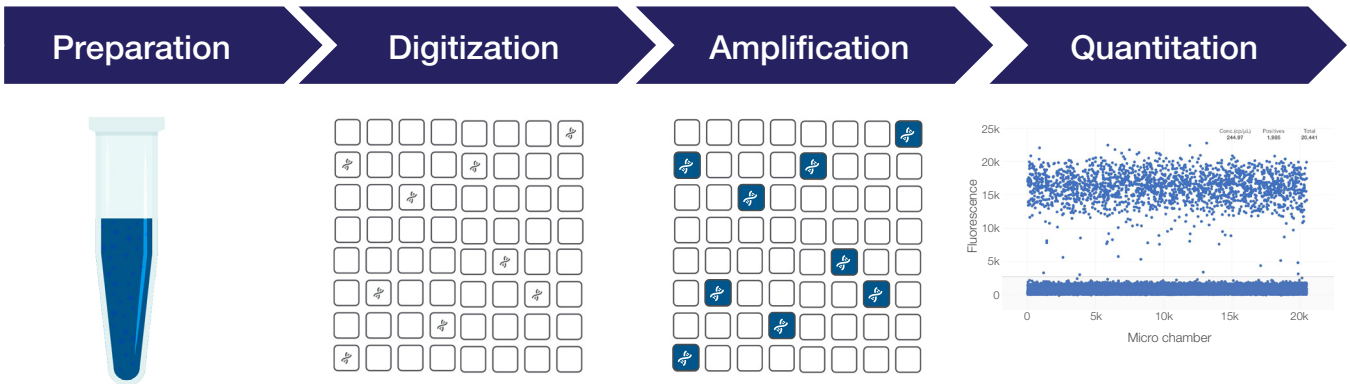


Figure 6. Basics of a digital PCR Assay. dPCR reactions on the QuantStudio Absolute Q dPCR system make use of Absolute Q dPCR assays (TaqMan chemistry) to detect a target sequence. In dPCR assays, the sample is diluted and loaded into a matrix containing thousands of individual reaction chambers. If the dilution is correct, some of the chambers will have a 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 microchamber, an overabundance of one sequence will not necessarily overwhelm detection in wells that have a rare sequence.

One strategy that helps ensure high-quality PCR data is that investigators follow standardized practices for performing real-time PCR and reporting results. The MIQE recommendations (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) are a set of guidelines for real-time PCR experimental designs and data reporting practices, as well as standards for sharing experimental information with colleagues (Bustin 2009). 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. Thermo Fisher provides necessary information to facilitate MIQE compliance when publishing the results of experiments with TaqMan Assays. Information requested under the OpenArray™ formats. TaqMan products include genotyping and gene expression assays. In addition, the highly specific TaqMan Assay chemistry can be used to analyze miRNAs for neural pathway, pathology, and biomarker research.

To help with choosing the right TaqMan Assays for an experiment, Thermo Fisher offers an assay search wizard (thermofisher.com/taqman-search-wizard). Inputting a gene name, keyword, pathway, or disease returns a list of all the assays that meet the search criteria. The results page highlights the "best coverage" assay for each gene, cites publications that used the assay, 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

dPCR is often used to detect rare mutant alleles in cancer samples and is used to analyze 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 Applied Biosystems™ 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. The Applied Biosystems™ Absolute Q™ Liquid Biopsy dPCR assays detect and quantify the most common glioma-related mutations (e.g., IDH1, TP53, EGFR, PTEN; Liu 2016) as well as therapy-resistant mutations (e.g., EGFR T790M). They have been verified to detect allele frequencies as low as 0.1%. The Absolute Q Liquid Biopsy dPCR assays run on the QuantStudio Absolute Q dPCR 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.

Conclusions and Outlook

The Applied Biosystems brand continues to offer innovative tools that facilitate research in the neurosciences. For any of the problems spanning the genetic analysis continuum, from discovery-based whole-genome research, through focused research on a distinct collection of sequences, to precise, sensitive detection of specific sequences, the Applied Biosystems portfolio has the solutions that address the research need.

Thermo Fisher is committed to providing neuroscience researchers with powerful genetic analysis solutions that enable them to pursue the understanding and intervention of neural pathologies, ultimately providing more personalized treatment strategies and better patient outcomes in the future.

Thermo Fisher can help you achieve your most exciting neuroscience research goals with a full range of genomics and transcriptomics solutions.

- Uncover cryptic genomic heterogeneity by qPCR, dPCR, NGS, or CE
- Extract precise data from limited or challenging samples such as FFPE tissue, precious solid tumor biopsy samples, or liquid biopsies, by dPCR or qPCR
- Obtain key insights from multiple perspectives provided by different technologies

For more information, contact your sales representative.

REFERENCES

Article 1: The Many Roads that Lead to Neuronal Demise

1) "Global Health Estimates: Life expectancy and healthy life expectancy," *World Health Organization*, 2019. https://www.who.int/data/gho/data/themes/mortality-and-global-health-estimates/ghe-life-expectancy-and-healthy-life-expectancy, accessed on July 6, 2022.
2) S.J. Olshansky, A.B. Ault, "The fourth stage of the epidemiologic transition: the age of delayed degenerative diseases," *Milbank Q*, 64(3):355-91, 1986.
3) GBD 2019 Dementia Forecasting Collaborators, "Estimation of the global prevalence of dementia in 2019 and forecasted prevalence in 2050: an analysis for the Global Burden of Disease Study 2019," *Lancet Public Health*, 7(2):e105-25, 2022.
4) P. Pedroza et al., "Global and regional spending on dementia care from 2000–2019 and expected future health spending scenarios from 2020–2050: An economic modelling exercise," *EClinicalMedicine*, 45:101337, 2022.
5) "Neurodegenerative disease cost exceed $655 billion a year in medical expenses and economic losses," *Partnership to Fight Chronic Disease*, 2021. https://www.fightchronicdisease.org/latest-news/neurodegenerative-disease-costs-exceed-655-billion-year-medical-expenses-and-economic, accessed on July 7, 2022.
6) "Estimates of funding for various research, condition, and disease categories," *NIH Report*, 2022. https://report.nih.gov/funding/categorical-spending#/, accessed on July 7, 2022.
7) "JPND: Towards a stronger brain health partnership," *Open Access Government*, 2022. https://www.openaccessgovernment.org/jpnd-towards-a-stronger-brain-health-partnership-europe-neurodegenerative-disease/137987/, accessed on July 7, 2022.
8) A.L. Young et al., "Uncovering the heterogeneity and temporal complexity of neurodegenerative diseases with subtype and stage inference," *Nat Comm*, 9(1):4273, 2018.

Article 2: Scanning for Changes to Stop ALS

1) J. Kirby et al., "Mutant SOD1 alters the motor neuronal transcriptome: implications for familial ALS," *Brain*, 128(Pt 7):1686-706, 2005.
2) I. Giovannelli et al., "Amyotrophic lateral sclerosis transcriptomics reveals immunological effects of low-dose interleukin-2," *Brain Commun*, 3(3):fcab141, 2021.

Article 3: Combining Technologies to Identify a Parkinson-Specific Cell Type

1) S. Smajic et al., "Single-cell sequencing of human midbrain reveals glial activation and a Parkinson-specific neuronal state," *Brain*, 145(3):964-78, 2022.

Article 4: Genetic Analysis Technologies to Support Neuroscience Research

1) M.R. Abu et al., "Genetic study of early onset Parkinson's disease in Cyprus," *Int J Mol Sci*, 23(23):15369, 2022. doi: 10.3390/ijms232315369
2) C.R. Boland et al., "A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer," *Cancer Res*, 58:5248-57, 1998.
3) R. Bonneville et al., "Landscape of microsatellite instability across 39 cancer types," *JCO Precis Oncol*, 1:1-15, 2017. doi: 10.1200/PO.17.00073
4) S.A. Bustin et al., "The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments," *Clin Chem*, 55(4):611-22, 2009. doi: 10.1373/clinchem.2008.112797
5) J. Doroszkiewicz et al., "Molecular biomarkers and their implications in the early diagnosis of selected neurological diseases," *Int J Mol Sci*, 23:4610, 2022. doi: 10.3390/ijms23094610
6) Y. Huang et al., Investigation of cross-contamination and misidentification of 278 widely used tumor cell lines," *PLoS One*, 12(1):e0170384, 2017. doi: 10.1371/journal.pone.0170384
7) J.Y. Hwang et al., "The emerging field of epigenetics in neurodegeneration and neuroprotection," *Nat Rev Neurosci*, 18(6):347-61, 2017. doi: 10.1038/nrn.2017.46
8) S. Kang et al., "The significance of microsatellite instability in colorectal cancer after controlling for clinicopathological factors," *Medicine (Baltimore)*, 97(9):e0019, 2018. doi: 10.1097/MD.0000000000010019
9) A. Liu et al., "Genetics and epigenetics of glioblastoma: applications and overall incidence of IDH1 mutation," *Front Oncol*, 6:16, 2016. doi: 10.3389/fonc.2016.00016
10) M. Losekoot et al., "EMQN/CMGS best practice guidelines for the molecular genetic testing of Huntington disease," *Eur J of Hum Genet*, 21:480-86, 2013. doi: 10.1038/ejhg.2012.200
11) A.O. Nygren et al., "Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences," *Nucleic Acids Res*, 33(14):e128, 2005. doi: 10.1093/nar/gni127
12) H. Paulson, "Repeat expansion diseases," *Handb Clin Neurol*, 147:105-23, 2018. doi: 10.1016/B978-0-444-63233-3.00009-9
13) G.X. Reyes et al., "New insights into the mechanism of DNA mismatch repair," *Chromosoma*, 124:443-62, 2015. doi: 10.1007/s00412-015-0514-0
14) D. Salcedo-Tacuma et al., "Differential methylation levels in CpGs of the BIN1 gene in individuals with Alzheimer disease," *Alzheimer Dis Assoc Disord*, 33(4):321-26, 2019. doi: 10.1097/WAD.0000000000000329
15) J. Sequieros et al., "EMQN Best Practice Guidelines for molecular genetic testing of SCAs," *Eur J Hum Genet*, 18:1173-76, 2010. doi: 10.1038/ejhg.2010.8
16) M. Sokratous et al., "CpG island methylation patterns in relapsing-remitting multiple sclerosis," *J Mol Neurosci*, 64(3):478-84. 2018. doi: 10.1007/s12031-018-1046-x
17) I.E. Sønderby et al., "ENIGMA 22q11.2 Deletion Syndrome Working Group. Effects of copy number variations on brain structure and risk for psychiatric illness: Large-scale studies from the ENIGMA working groups on CNVs," *Hum Brain Mapp*, 43(1):300-28, 2022. doi: 10.1002/hbm.25354
18) Thermo Fisher Scientific, "User bulletin: Applied Biosystems

3730/3730xl DNA Analyzers and 3130/3130xl Genetic Analyzers, 2005. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_042107.pdf
19) Thermo Fisher Scientific, "Low-level somatic variant detection in tumor FFPE samples by Sanger sequencing, 2016. https://www.thermofisher.com/de/de/home/life-science/sequencing/sanger-sequencing/applications/low-level-somatic-variant-detection-tumor-ffpe-samples-sanger-sequencing.html
20) Thermo Fisher Scientific, "Clariom D microarrays provide a deep view of our transcriptome, 2019. http://assets.thermofisher.com/TFS-Assets/GSD/Technical-Notes/transcriptome-microarray.pdf
21) Thermo Fisher Scientific, "MLPA assays on the SeqStudio Genetic Analyzer, 2019. https://assets.thermofisher.com/TFS-Assets/GSD/Application-Notes/mlpa-assays-seqstudio-genetic-analyzer-app-note.pdf
22) Thermo Fisher Scientific, "TrueMark MSI Assay for microsatellite instability analysis, 2023. https://www.thermofisher.com/us/en/home/clinical/clinical-genomics/molecular-oncology-solutions/microsatellite-instability-assay.html
23) S. Younesian et al., "The DNA methylation in neurological diseases," *Cells*, 11(21):3439, 2022. doi: 10.3390/cells11213439

Learn more at **thermofisher.com/abcomplexdisease**

For Research Use Only. Not for use in diagnostic procedures. © 2023 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. TaqMan is a trademark of Roche Molecular Systems, Inc., used under permission and license. MLPA is a trademark of MRC Holland. **COL35660 0423**