Molecular Diagnostics in the Clinical Laboratory

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Introduction

Molecular diagnostics (MolDx) are assays that analyze genetic material (usually DNA or RNA) from a sample to indicate disease risk, diagnose a disease, predict disease course, select treatments, or monitor the effectiveness of therapies. MolDx have grown significantly in importance and scale, from a 4% market share of all *in vitro* diagnostics (IVDs) in 2000 to 24% in 2021 (Kalorama, 2021). With recent improvements in cost, effectiveness, and availability, it is likely MolDx will soon become the most common type of test run in clinical laboratories. While other diagnostic methods (immunoassays, clinical chemistry, microbiology, etc.) have advantages for certain disease states due to the protein biomarker or pathogen being detected, MolDx are typically more sensitive, specific, reproducible, and reliable. They have a similar or slightly slower assay time than immunoassays, but are much faster than microbiological methods (hours instead of days). Limitations of MolDx include their cost, instrument complexity, and the need for trained staff to perform sample preparation, operate the instruments, and analyze data.

MolDx can be used for a variety of purposes, including biomarker discovery, research, evolutionary studies, food testing, veterinary diagnosis, disease surveillance, and epidemiology; however, the scope of this paper is restricted to applications in clinical laboratories of five key MolDx technologies: real-time PCR (qPCR), digital PCR (dPCR), chromosomal microarrays (CMAs), Sanger sequencing (SS), and next-generation sequencing (NGS). **Table 1** presents a summary of these technologies, after which we explore use cases, cost-effectiveness, regulatory considerations, data interpretation, and clinical utility. We also provide three examples where a combination of different MolDx technologies are needed for the same clinical scenario. Of note, this report will offer only a limited analysis of NGS, as this technique has been widely reported in other industry publications. Moreover, in order to provide sufficient discussion regarding the aforementioned technologies, this paper will not cover certain MolDx techniques such as isothermal nucleic acid amplification techniques or fluorescence *in situ* hybridization.

Table 1. Summary of key MolDx technologies.

Technology	Purpose	Assay Time*	Capital Costs	Regulatory Concerns
Real-time PCR	Detect and/or quantify 1-100 targets quickly and inexpensively	1-3 hours	$10,000-$30,000 for 96-well instruments; >$100,000 for high- throughput automated instruments	None

Digital PCR	Detect and/or quantify 1-5 targets with high sensitivity and resolution	2-6 hours	~$50,000 - $125,000	Only one FDA approved dPCR assay; the rest are laboratory-developed tests** (LDTs)/in-house assays (as of Oct. 2023)
Microarrays	Detect hundreds to millions of targets	2 days	$500 - $1,000s for chips; $100,000 - $500,000 for instruments	Some platforms and arrays are FDA cleared, but many are operated as LDTs by clinical laboratories
Sanger sequencing by capillary electrophoresis	Sequence a single gene, including long reads, and/or amplicons	30 minutes - 2 weeks	$50,000 - $200,000	Some platforms are FDA cleared, but many are operated as LDTs by clinical laboratories
Fragment analysis by capillary electrophoresis	Analyze fragments with multiplex capability of 1-12 targets	30 minutes - 6 hours	Same as Sanger sequencing	Some platforms are FDA cleared, but many are operated as LDTs by clinical laboratories
Next generation sequencing	Detect variants (SNVs, indels, gene fusions) across many genes, for multiple samples simultaneously	1 day - 4 weeks	$100,000 - $1M+	Some platforms and assays are FDA cleared, but many are operated as LDTs by clinical laboratories

* As used herein, “Assay Time” includes ranges that cover in-house tests (i.e., tests where the sample is acquired in close proximity to the platform) and outsourced assays and analysis (i.e., samples acquired at one location, such as a doctor’s office, and shipped to an off-site clinical lab that may be near or distant).
** Per the U.S. Food and Drug Administration, a laboratory-developed test (LDT) is defined as “a type of *in vitro* diagnostic test that is designed, manufactured and used within a single laboratory.”

MolDx Technologies and Applications

Real-time PCR

Optimal use of qPCR is when an accurate diagnosis within hours to days is needed for which DNA or RNA is a clinically useful biomarker and there are known

genetic targets. qPCR is sensitive, specific, and scalable through automation, with some high-throughput instruments running thousands of samples per day. It has the ability to quantify DNA or RNA with a large dynamic range, so quantifying an unknown and highly variable amount of genetic material is often done with qPCR. If sensitivity is important (e.g., detecting low concentration samples), qPCR has a low limit of detection—100 genomic copies/mL for SARS-CoV-2 in viral transport media (Arnaout, 2020), and 50 copies/mL for epidermal growth factor receptor (*EGFR*) variants in plasma (Keppens, 2018)—and is the preferred choice.

qPCR has multiplexing capabilities, which can be performed in two possible formats: i) sample splitting, wherein the sample is divided into various compartments before amplification, with each compartment containing a different set of primers, or ii) color multiplexing, wherein 2-6 differently colored detection probes are contained within the same reaction compartment, with each color indicating a probe binding to a different target. The first method has more complex fluid handling and reduced sensitivity due to sample dilution, but simpler optical detection configurations are required (only one fluorescent dye is needed), and 10-40 targets can be detected depending on the input sample size. Color multiplexing has more optical detection complexity and is limited to 6 targets, but better sensitivity and simpler fluid handling operations as the reaction occurs in a single tube.

Applications for qPCR include the diagnosis or prognosis of cancers, liquid biopsies, detection of cancer recurrence, therapeutic monitoring, FII/FV thrombophilia diagnosis, infectious disease diagnosis from blood, stool, urine, respiratory, or cerebrospinal fluid (CSF) samples, viral load monitoring, blood bank screening, and genetic disease testing. Multiplex PCR is particularly helpful for infectious disease detection and oncology where it is necessary to identify which pathogen or genetic mutation amongst many other known possibilities is responsible for the infection or cancer. qPCR is not optimal if a quick answer is needed in an emergency situation or at the point-of-care where a clinician is willing to sacrifice accuracy for speed and cost. If a complex case arises in which there are unknown targets or >100 targets, microarrays or NGS would be the better choice.

There is evidence for the cost-effectiveness of qPCR for several applications in which a known genetic target needs to be detected. In oncology, an *EGFR* mutation assay by qPCR was found to be 9 hours quicker (3 hours vs 12 hours) and more cost-effective (Ilie, 2017) than pyrosequencing. For sepsis, qPCR was cost-saving when inappropriate antimicrobial therapy extended patient hospital stays by 4 days or more, with the incremental cost-effectiveness ratio equal to -$7,302 per death averted (Zacharioudakis, 2019). For annual screening of tuberculosis, replacing the traditional radiography exam with a sputum sample in PCR cost $543 per additional quality-adjusted life year (QALY) gained (Winetsky, 2012).

Digital PCR

dPCR is an emerging molecular diagnostic technology designed for precise and absolute quantification, which is achieved by splitting a sample into thousands to millions of parallel PCR reactions, with each reaction containing 0 or 1+ copies of target DNA or RNA. Positive and negative compartments are counted, after which a nucleic acid (NA) concentration is calculated using Poisson statistics (Diehl, 2005). Some instruments generate droplets, thermal cycle for PCR, and then read the positive/negative droplets with a flow cytometer; other technologies use microfluidic chips to split the sample into static compartments for PCR thermal cycling, after which fluorescence of the chip compartments are measured to indicate positive or negative reactions. These technologies are more complex and costly than qPCR, but offer more precise NA quantification, the ability to detect and quantify low-abundance targets, and a more robust assay against inhibitors or variations in PCR efficiency.

There are many applications of dPCR in clinical laboratories. It is an effective tool for absolute quantification of standards or reference materials for qPCR, or as a control comparator method for viral load measurements. Because a sample can be split into millions of PCR reactions, dPCR filters low-abundance NA signals from background noise well, and can detect mutant allele fractions (MAF) down to 0.1%. This ability has been useful for oncology and liquid biopsy applications by detecting rare genetic mutations and residual disease, as well as monitoring treatment effectiveness and measuring copy number variations (CNVs). Low-abundance mutations are also present in microbiology and infectious disease, where dPCR can detect rare antibiotic resistance genes, perform fold-change measurements, and accurately quantify the pathogen load. For non-invasive prenatal testing (NIPT), dPCR is adept at finding rare chromosomal abnormalities in cell-free DNA (cfDNA) as it can reliably detect low concentrations of target fetal DNA sequences in a high background of maternal DNA. It can also be used to quantify placental DNA to provide information about placental health and preeclampsia, identify fetal RhD genotype, and detect CNVs, low-level mosaic aneuploidies, specific point mutations, genetic variants, and chimerism. A recent application takes advantage of dPCR's high sensitivity by monitoring SARS-CoV-2 viral load in wastewater and environmental samples. Less common, but potentially valuable, uses of dPCR include: accurate quantification of chimerism in cellular therapies and analysis of methylation loci for cancer or pregnancy pathologies.

Given the short time period in which dPCR has been used, evidence is scarce for its cost-effectiveness. However, dPCR is expected to cost less for detecting known targets in NIPT than NGS due to the instrument and reagent cost being less expensive (Tan, 2019). Mao et al. found that dPCR could identify the ΔF508-MUT CFTR allele in cfDNA of all proband fetuses with high sensitivity and cost-effectiveness (Mao et al, 2019). Currently, there is only one FDA approval of a dPCR assay, which monitors chronic myeloid leukemia patients' molecular response to treatment. There was also an EUA granted during the COVID-19 pandemic for wastewater testing, but it has not converted into a 510(k) clearance yet. Increased usage in the clinical lab and subsequent FDA approvals will increase the body of evidence in favor of dPCR for certain applications where absolute quantification, precision, or detection of low-abundance targets in high background samples is important. When high throughput is

needed in either the number of known genetic targets or number of patient samples, other molecular technologies such as microarrays or qPCRs are more cost-effective.

Chromosomal Microarrays

Depending on the analyte of interest, different microarray technologies can be deployed in a clinical laboratory. In terms of molecular analytes, including DNA and RNA, there are multiple microarray platforms that can be used. In general, a microarray platform consists of a solid support that has thousands to millions of nucleic acid fragments (called "probes") bound thereon. These "chips" can be fabricated using a variety of methods including photolithography (creation of sequences on a base-by-base basis) and printing/spotting, in which entire probes are deposited on a predetermined location on the solid support. Moreover, microarrays can be created by third-party manufacturers for use as *in vitro* diagnostics, or they can be created in a clinical laboratory setting for use as laboratory-developed tests (LDTs).

Microarrays can be configured with different sets of probes to enable a multitude of uses by clinical laboratories. For example, one of the earlier forms of microarrays was formatted to enable quantitative analysis of gene expression. In this case, isolated patient mRNA that has been reverse transcribed and labeled is hybridized onto a microarray comprising a pre-determined set of probes. The resulting hybridized array can be imaged and the image data can be used to quantify the expression of a multitude of targets.

In terms of use cases for gene expression-based microarray analyses, there are as many combinations available as desired by a clinical laboratory. In short, laboratories have the option of purchasing either arrays with pre-selected probes (e.g., probes known to have diagnostic usage for a particular indication, such as cancer or other diseases) or custom-built arrays with a proprietary set of probes.

In addition to gene-expression analysis, microarrays can also be used in analyzing DNA sequence and chromosome structure. In some cases, these microarrays can be used in genotyping-specific applications. For example, genotyping microarrays can also be used to detect single-nucleotide polymorphisms (SNPs) as well as other mutations in particular genes, such as *CFTR* (for cystic fibrosis diagnostics), *CYP450* (for pharmacogenomic analysis), and *p53* (for cancer diagnostics and prognostics) (Shen & Wu, 2009 and Wu et al., 2005).

Next, chromosomal microarrays can be used to detect numerical chromosomal abnormalities (e.g., aneuploidy, hypodiploidy, hyperdiploidy, and polyploidy) as well as structural changes, including deletions, duplications, triplication, amplification, translocation, inversion, etc. (Shao et al., 2021). In that vein, there is also the potential to compare control or reference DNA to patient DNA using a technique known as array comparative genomic hybridization (aCGH). In this technique, reference DNA and patient DNA are labeled with different fluorescent dyes and hybridized to an array comprising a predetermined probe population. Similar to chromosomal microarrays, the

resulting relative fluorescence can reveal information regarding copy-number variation, aneuploidies, deletions, and duplications between the control and patient sample. Interestingly, these aCGH platforms can be used to study different regions of the chromosome, such as the telomeric region, subtelomeric region, pericentromeric region, and other regions of interest (Van den Veyver et al., 2019). Other potential use cases for microarrays are detailed in the charts that precede the conclusion.

Chromosomal microarrays and aCGH arrays both have significant uses in the maternal-fetal medicine field. Specifically, prenatal genetic testing can be accomplished through the use of these microarray platforms to provide for detection of significant fetal chromosomal abnormalities, such as trisomy 21 (Down syndrome), trisomy 18 (Edwards syndrome), and trisomy 13 (Patau syndrome). In fact, the American College of Obstetricians and Gynecologists (ACOG) issued a committee opinion in which it recommends the use of chromosomal microarray analysis to provide diagnostic data in the case of fetuses with one or more major structural abnormalities detected via traditional ultrasonographic examination (ACOG, 2016; affirmed in 2023). Moreover, in the same committee opinion, ACOG indicated that chromosomal microarray analysis could identify significant chromosomal abnormalities that would otherwise go undetected by traditional cytogenetic techniques, such as a karyotype analysis (ACOG, 2016; affirmed in 2023).

In addition to prenatal genetic analysis, some arrays can also be used for postnatal diagnostics. In some cases, neonates and children of various ages may display developmental delays, potential intellectual disabilities, and congenital anomalies that require genetic analysis. In these cases, chromosomal microarray analysis can be used as a first-tier evaluation tool as well (Hensel et al., 2017). The data arising from these studies demonstrate that chromosomal arrays offer significant value to patients with disorders of unknown etiology (Hensel et al., 2017).

As a final note, in the past, assay time has posed concerns for the use of microarray technologies. Specifically, early iterations of microarray technologies, including fabrication, hybridization, and scanning equipment required extensive space and computing power. As these technologies have evolved, the reduced assay times are becoming more evident. For example, in some cases with advanced arrays, turnaround times can be as low as two (2) days from sample acquisition. As such, assay time has become less of a negative consideration in electing microarray analyses.

Although outside of the scope of this article, in addition to the aforementioned molecular applications, arrays can also be deployed for non-molecular uses, such as carbohydrate arrays, kinase arrays, and antibody/antigen arrays (Lagraulet, 2010).

As with all laboratory technologies, the cost-effectiveness for a microarray depends on the application and other platform to which it is being compared. As discussed above, clinical laboratories have choices in terms of whether to purchase pre-fabricated microarrays or to select a probe set for a custom/proprietary array. Outside

of the purchase of microarrays from particular vendors, of which there are many, this section sets forth examples of cost-effectiveness of microarray platforms.

A study by Li et al. (2017) compared the cost-effectiveness of karyotyping, chromosomal microarray analysis, and next-generation sequencing for use in diagnosing idiopathic developmental delay or intellectual disability. In short, the authors found that microarray testing resulted in more genetic diagnoses at an incremental cost of $2,692 compared to karyotyping, which had an average cost per diagnosis of $11,033. Moreover, the incremental cost of adding a next-generation sequencing analysis can further add $12,295 of incremental cost.

Similarly, a Canadian group compared costs associated with whole-exome sequencing (WES) and whole-genome sequencing (WGS) compared to chromosomal microarray for the diagnosis of autism spectrum disorder (Clark et al., 2018). Clark et al. determined that the cost per sample was between 2-8 times more expensive (depending on the sequencing platform) to perform sequencing compared to a chromosomal microarray. Specifically, Clark et al. determined that "the incremental costs of CAD were $25,000 per additional positive finding if CMA [chromosomal microarray] was replaced by newer technology" (Clark et al., 2018).

As a final note on microarray economics, one consideration is reimbursement. Although public and private payers are constantly revising their reimbursement determinations, one benefit of microarrays is that the analysis is more likely to be reimbursed by public and private payers. In a 2017 report prepared for the Washington State Health Care Authority on Genomic Microarray and Whole Exome Sequencing, the author found that many large insurers reimbursed for chromosomal microarray testing for at least some specific indications (except for Medicare Fee for Service), while less than half of those same payers reimbursed for WES (Whitehead, 2017).

Sequencing

The following discussion is largely directed to the benefits and uses of "first-generation sequencing" and its associated derivatives, such as capillary electrophoresis sequencing. Comparisons are made herein to massively parallel sequencing and other forms of "next-generation sequencing" or NGS. To be clear, in a modern clinical laboratory that is strategically positioned to address near- and long-term healthcare concerns, both Sanger sequencing and NGS capabilities **should** be considered, depending on the application. The discussion herein is limited to specific use cases for which first-generation sequencing may provide a technological advantage relative to NGS. The following contents are not intended to diminish the importance of NGS in today's clinical laboratory.

Sanger Sequencing (SS)

SS is a technique pioneered by Frederick Sanger in the late 1970s and is sometimes referred to as chain-termination sequencing or dideoxy sequencing. In

short, SS relies on a polymerase-driven amplification step (cycle sequencing) in which a combination of conventional deoxynucleotides (dNTPs) are mixed with labeled dideoxynucleotides (ddNTPs), which lack a hydroxyl group needed for further nucleotide binding. As such, the addition of ddNTPs by DNA polymerase during chain extension terminates the DNA strand elongation process. Repetition during the cycling process results in amplicon fragments with labeled ddNTPs at every position, thereby identifying every nucleotide in the DNA template.

Of note, modern SS relies on fluorophore-labeled ddNTPs; however, the original methodology relied on the use of ^{32}P-containing ddNTPs, which required extensive radiation-containing protocols. ^{32}P-based SS generally involved the use of conventional acrylamide electrophoresis combined with radiographic imaging to make manual, visual calls of the amplicon's sequence. Modern SS relies on "capillary electrophoresis" in which the ddNTPs are labeled with fluorophores having different wavelengths that are automatically detected and analyzed using modern instrumentation and software. The results are then displayed as a single analog electropherogram. Moreover, conventional capillary electrophoresis-based SS often required a forward and a reverse read in order to have statistical certainty regarding the sequence; however, technological advances have enabled some single-read applications for SS.

Next-Generation Sequencing

As provided above, NGS has become a "must-have" technology for a modern clinical laboratory. Many articles summarizing NGS are available, and this educational paper will not duplicate those efforts (Sharma, 2020). Nonetheless, the breadth of NGS applications is impressive, including WGS, WES, RNA sequencing, bisulfite sequencing, and other targeted sequencing, like SNP sequencing.

Although different technologies fall under the heading of NGS, the premise is that DNA or cDNA is processed into relatively short double-stranded fragments, which are then ligated to technology-specific adapter sequences to form a library. The library is then attached to a solid surface and clonally amplified to increase signal detection. The resulting clonally amplified library is then sequenced in parallel, hence the name "massively parallel sequencing." The resulting sequence data are then assessed using a variety of algorithms and data analysis tools.

NGS vs. SS Properties

Like other technologies, pros and cons are readily discernable for each technology, depending on the application. For example, SS is capable of elucidating longer sequences, such as those greater than 500 bp, which is still a limitation for NGS. On the other hand, targeted NGS has a higher sequencing depth for increased sensitivity. In comparison, SS is the gold standard for certain sequencing applications, with over 99% accuracy. Moreover, capillary electrophoresis technology has a lower error rate, compared to other technologies, particularly for detecting rare variants or low-

frequency mutations. Of note, modern software has improved SS sensitivity such that some minor variants can be detected at a mean allele frequency of 5% or less.

In terms of economics, the scales are different between SS and NGS. For a smaller number of targets (e.g., 1-20), SS is more cost-effective with a faster turnaround time (on a per sample basis) compared to NGS. Conversely, for a greater number of targets (e.g., 20+), NGS is desirable, given the massively parallel nature of this technology. Of note, depending on the configuration of the SS platform, it may be possible to automate and expand the scale to reach efficiency with a greater number of samples, such as configurations based on 96- or 384-well plates.

Another consideration is assay time, which is challenging to compare between SS and NGS. As provided above, modern SS relies on capillary electrophoresis, which is neither complex nor time-consuming. In particular, an SS capillary electrophoresis run (i.e., after the cycle sequencing step) may last between 30 and 180 minutes. Conversely, an NGS sequencing run includes millions of parallel sequencing reactions. Depending on the NGS platform, a run can last between 180 minutes to a few dozen hours. Again, given the vastly different uses of these two technologies, the distinction in assay times is expected.

A final property for consideration is bioinformatics capabilities. As mentioned above, SS output is a relatively simple analog electropherogram, which does not require significant downstream analysis. In comparison, an NGS run can generate large volumes of sequence data, which may need to be processed by bioinformaticians. As NGS technology has advanced, ready-to-use bioinformatics software has become available, thereby reducing the need for extensive bioinformatics infrastructure to process NGS sequence data.

SS Use Cases

In spite of NGS's growing popularity, there are still multiple use cases for SS. When deciding what sequencing technology to use for clinical applications, it is important to recognize the value offered by SS, relative to NGS. As previously mentioned, significant value can be found in using SS for smaller numbers of targets, relative to NGS. For example, SS is still often used for single-gene sequencing, such as assessing *BRCA1* mutations in breast cancer diagnostics, determining certain *CFTR* variants in cystic fibrosis diagnostics, establishing mutations in a multitude of genes associated with different neuropathologies, genotyping immunologically relevant genes, such as HLA, and typing microbial species and testing for microbial resistance (Wallace, 2016; Solomon, 2018; McElhinney, 2014; and Smith, 2012). Other potential use cases for SS are detailed in the charts that follow the conclusion.

Interestingly, one recent study reported on the significant value of SS in remote, resource-challenged areas. Specifically, a group reported on the use of SS in North Kerala, India, as a mechanism for typing SARS-CoV-2 in areas without access to NGS platforms (Dhanasooraj et al., 2022). The authors indicated that SS could be readily

deployed in smaller, local laboratories that do not have significant resources to afford NGS platforms and that this use of SS ameliorated significant delays in transporting samples to larger laboratories (Dhanasooraj et al., 2022).

Fragment Analysis Use Cases

One final use case for SS does not rely on chain-termination sequencing, but rather employs capillary electrophoresis as a platform – **fragment analysis**. In short, fragment analysis employs fluorescently labeled DNA segments that are separated by capillary electrophoresis and sized by comparison to an internal standard. Fragment analysis **does not** elucidate a nucleotide sequence, rather it offers sizing, relative quantitation, and genotyping information about the target sequences. Fragment analysis can be employed for microsatellite analysis, which has applications in forensics, cell line authentication, and tracking unique DNA signatures in a mixed cell population. In addition, a modified fragment analysis procedure can be used to perform SNP genotyping, via the use of dye-labeled dNTPs, instead of dye-labeled primers.

In terms of fragment analysis use cases, this technique is highly sensitive and provides quantitative measurements, allowing detection of expansion repeats. For example, CGG repeats in the *FMR1* gene provide valuable carrier screening information and diagnostic testing of Fragile X syndrome. Also, fragment analysis can be employed for microsatellite instability analysis for applications like colon cancer screening and diagnostics. In addition, both fragment analysis and Sanger sequencing can be used for FLT3-ITD and FLT3-TKD mutation analysis for acute myeloid leukemia to determine prognostics and response to targeted therapy.

As NGS has become a first-tier technique in clinical laboratories for a variety of uses (e.g., oncology applications, companion diagnostics, microbial detection, and genotyping), this paper will not focus on use cases for NGS. Some further potential use cases for NGS are detailed in the charts that follow the conclusion.

As mentioned throughout this section, it is challenging to compare SS and NGS, as these two techniques have different potential applications. This theme is continued for cost-effectiveness, which is initially seen in capital costs. For example, equipment for capillary electrophoresis may cost between $20,000 and $100,000+, depending on if a new or a used platform is desired (Capillary Electrophoresis: Instrument Price and Cost Considerations, n.d.). On the other hand, NGS equipment may require a significantly greater capital investment, with new hardware at a cost of as much as $1.25M+ (Loftus, 2023).

Another area where there is a significant distinction in cost-effectiveness is in the preparation of samples. As provided above, SS relies on conventional nucleic acid extraction followed by PCR and cycle sequencing with the use of fluorescently labeled ddNTPs. Conversely, NGS requires extensive sample preparation. For example, in 2020, a group estimated that total costs per panel for targeted panels, WES, and WGS were $250-$300, $600-$1,930, and $2,000-$3,200, respectively (Gordon et al., 2020).

Significantly, the group found that sample/library preparation accounted for more than 75% of the costs (Gordon et al., 2020). Again, these NGS-associated costs are greater, but the clinical data revealed by NGS has significantly more impactful implications.

Overall, both SS and NGS have compelling uses in the modern clinical laboratory. SS and the associated capillary electrophoresis equipment enable high-accuracy sequence elucidation and/or valuable fragment analysis data to assess fragment size, relative quantification, and genotyping information. Conversely, NGS enables rapid sequence acquisition with the ability to simultaneously sequence millions of nucleotide segments. This can enable deep sequencing coverage, greater multiplexing capability, and higher sensitivity to rare sequence variants, relative to SS.

Examples of concomitant use of MolDx for related applications

Many clinical scenarios require a combination of MolDx technologies to accurately assess various factors throughout disease progression. There is no single "best" MolDx technology that can effectively diagnose the disease state, select treatment, and monitor therapy effectiveness. We provide three such examples here—oncology, infectious disease, and pharmacogenomics—where it would be important to have multiple types of MolDx in a clinical lab depending on type of information needed.

Table 2. Oncology MolDx combinations.

	Use case	**Other considerations**
qPCR	• SNP and drug resistance mutation detection • Companion diagnostics • Gene expression profiling • Liquid biopsies • Circulating tumor DNA (ctDNA) analysis	Fast and inexpensive, but limited to known targets
dPCR	• CNV measurements • Mutant allele fraction • Liquid biopsies: recurrence and residual disease detection, treatment monitoring	Effective for finding low-abundance mutant allele fraction for known targets
Microarrays	• Copy number analysis for chromosomal abnormalities and genetic instability • SNP detection • Companion diagnostics • Cancer profiling for liquid and solid tumors	Exceptional flexibility to detect CNVs and SNPs in a single assay, with simple analysis to reduce costs and processing times
Capillary electrophoresis	Sanger sequencing: • Companion diagnostics	Sanger sequencing: • Throughput/run is 96-384 samples and ideal for up to 20 targets/run

	• Tumor typing, e.g., *KRAS* mutation detection and variant confirmation • Verify/validate amplicons from any of the other techniques, including NGS	• Sanger is the gold-standard technique for sequencing at 99% accuracy • Used for targeted sequencing with a fast turnaround time
	Fragment analysis: • MSI analysis and MLPA • microsatellite instability analysis for oncology research and diagnostics (e.g., colon cancer)	Fragment analysis: • Throughput/run is 96-384 samples and ideal for up to 20 targets/run • Fast turnaround time through multiplexing and simplified workflows
NGS	Variant detection across multiple genetic targets for diagnosis, therapy selection, and monitoring	• Cost (consumables and capital costs) and availability of equipment in low-resource areas • Depending on use and available software, may require in-house bioinformatics expertise

Table 3. Infectious disease MolDx combinations.

	Use case	**Other considerations**
qPCR	• Pathogen detection • Drug resistance mutation detection • Viral load monitoring • Genotyping and therapy selection • Diagnosis (depending on the application)	• Effective at sensitive detection, but too expensive for screening large populations • Multiplex panels available for respiratory, blood, and CSF infections
dPCR	• Pathogen load quantification • Ultrasensitive measurements (e.g., environmental samples, wastewater) • Rare antimicrobial resistance gene detection • Diagnosis (depending on the application)	Multiplexing several low-abundance disease targets is possible with minor assay modifications
Microarrays	• Genotyping • Antibiotic resistance/susceptibility • Diagnosis (depending on the application)	• Must have known sequences to create probe set • Can create custom arrays using known sequences
	Sanger sequencing:	Sanger sequencing:

Capillary electrophoresis	• Variant analysis (HIV genotyping for drug resistance) • Microbial (viral, bacterial, and fungal) identification • Single-gene sequencing • Evolutionary studies • Antibiotic-resistance (ABR) testing • Can be used to verify/validate amplicons from any of the other techniques, including NGS • Diagnosis (depending on the application)	• Throughput/run is 96-384 samples and ideal for up to 20 targets/run • Sanger is the gold-standard technique for sequencing at 99% accuracy • Used for targeted sequencing with a fast turnaround time
	Fragment analysis: • Multiplexing capability for pathogen detection • MLPA • Diagnosis (depending on the application)	Fragment analysis: • Throughput/run is 96-384 samples and ideal for up to 20 targets/run • Fast turnaround time through multiplexing and simplified workflows
NGS	• High-throughput sequencing • WGS of infectious agents to provide data regarding genotype, ABR, and evolution (all within a single run) • Diagnosis (depending on the application)	• Cost (consumables and capital costs) and availability of equipment in low-resource areas • Depending on use and available software, may require in-house bioinformatics expertise

Table 4. Pharmacogenomics (PGx) MolDx combinations.

	Use case	**Other considerations**
qPCR	• Cytochrome genotyping • CCG repeat analysis • Detect SNPs that predict drug responses • Gene expression analysis associated with organ functions or tumor prognoses	• Most efficient for single known targets • Many regulatory approvals for qPCR-based PGx tests
dPCR	• Quantify expression levels of different alleles for genes involved in drug metabolism • Detect rare CNVs associated with pharmacogenomic traits • PGx biomarker validation	dPCR is still too expensive for most cases, which can be done with qPCR, and suffers from low usage and FDA approval

Microarrays	Cytochrome genotyping	Microarrays are a great tool for PGx screening with the ability to detect known SNPs and CNVs
Capillary electrophoresis	Sanger sequencing: Can be used to verify/validate amplicons from any of the other techniques, including NGS	Sanger sequencing: • Throughput/run is 96-384 samples and ideal for up to 20 targets/run • Sanger is the gold-standard technique for sequencing at 99% accuracy • Used for targeted sequencing with a fast turnaround time
	Fragment analysis: • Mutation/allele discovery work • Assessing difficult genes/regions, such as HLA or STRs	Fragment analysis: • Throughput/run is 96-384 samples and ideal for up to 20 targets/run • Fast turnaround time through multiplexing and simplified workflows
NGS	• Mutation/allele discovery work • Targeted panels for PGx-related markers • Can combine PGx analysis with WES or WGS clinical studies	• Potential expense • Depending on use and available software, may require in-house bioinformatics expertise

Conclusion

Molecular diagnostics provide a diverse set of technologies and applications that have revolutionized clinical laboratory practices in the past few decades. Known genetic targets can be detected within hours with high sensitivity and precision, while unknown genes can be sequenced within days—many of these technologies work together to enable personalized medicine and improve treatment outcomes. No single MolDx technology can comprehensively address all clinical needs, and laboratories will need to utilize a combination of MolDx technologies (**Tables 2-4**). There are trade-offs between cost, analytical capability, turnaround time, throughput, and practical implementation. Therefore, a strategic balance must be struck when selecting MolDx technologies to ensure optimal outcomes for patient care. The ideal approach involves using a synergistic combination of various MolDx technologies, and tailoring them to the specific requirements of the laboratory's diagnostic portfolio and patient needs. By doing so, laboratories can harness the strengths of different technologies to create a comprehensive and robust molecular diagnostic framework that aligns with the evolving demands of modern healthcare.

References

- 2021 IVD market update and covid-19 impact. (2021). Kalorama Information. https://kaloramainformation.com/product/2021-ivd-market-update-and-covid-19-impact/
- Arnaout, R., Lee, R. A., Lee, G. R., Callahan, C., Yen, C. F., Smith, K. P., Arora, R., & Kirby, J. E. (2020). *SARS-COV-2 testing: the limit of detection matters*. Cold Spring Harbor Laboratory. https://doi.org/10.1101/2020.06.02.131144
- Capillary electrophoresis: instrument price and cost considerations. https://labx.com/resources/capillary-electrophoresis-instrument-price-and-cost-considerations/1142
- Clark, M. M., Stark, Z., Farnaes, L., Tan, T. Y., White, S. M., Dimmock, D., & Kingsmore, S. F. (2018). Meta-analysis of the diagnostic and clinical utility of genome and exome sequencing and chromosomal microarray in children with suspected genetic diseases. *NPJ Genomic Medicine*, *3*(1). https://doi.org/10.1038/s41525-018-0053-8
- Dhanasooraj, D., Viswanathan, P., Saphia, S., Jose, B. P., Parambath, F. C., Sivadas, S., Akash, N. P., Vimisha, T. V., Nair, P. R., Mohan, A., Hafeez, N., Poovullathi, J. K., Vadekkandiyil, S., Govindan, S. K. K., Khobragade, R., Aravindan, K., & Radhakrishnan, C. (2022). Genomic surveillance of SARS-CoV-2 by sequencing the RBD region using Sanger sequencing from North Kerala. *Frontiers in Public Health*, *10*. https://doi.org/10.3389/fpubh.2022.974667
- Diehl, F., Li, M., Dressman, D., He, Y., Shen, D., Szabo, S., Diaz, L. A., Jr, Goodman, S. N., David, K. A., Juhl, H., Kinzler, K. W., & Vogelstein, B. (2005). Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proceedings of the National Academy of Sciences*, *102*(45), 16368-16373. https://doi.org/10.1073/pnas.0507904102
- Gordon, L. G., White, N. M., Elliott, T. M., Nones, K., Beckhouse, A. G., Rodriguez-Acevedo, A. J., Webb, P. M., Lee, X. J., Graves, N., & Schofield, D. J. (2020). Estimating the costs of genomic sequencing in cancer control. *BMC Health Services Research*, *20*(1). https://doi.org/10.1186/s12913-020-05318-y
- Hensel, C., Vanzo, R., Martin, M., Dixon, S., Lambert, C., Levy, B., Nelson, L., Peiffer, A., Ho, K. S., Rushton, P., Serrano, M., South, S., Ward, K., & Wassman, E. (2017). Analytical and clinical validity study of FirstStepDx PLUS: a chromosomal microarray optimized for patients with neurodevelopmental conditions. *PLoS Currents*. https://doi.org/10.1371/currents.eogt.7d92ce775800ef3fbc72e3840fb1bc22
- Ilie, M., Heeke, S., Butori, C., Tanga, V., Washetine, K., Lassalle, S., Long, E., Maitre, P., Bordone, O., Lespinet, V., Leroy, S., Mouroux, J., Marquette, C. H., Hofman, V., & Hofman, P. (2017). Optimization of *EGFR* mutation testing by the fully-automated qPCR-based Idylla on whole slide and biopsy tumor tissue of non-small cell lung cancer. *Journal of Clinical Oncology*, *35*(15_suppl), e20632-e20632. https://doi.org/10.1200/jco.2017.35.15_suppl.e20632
- Keppens, C., Palma, J. F., Das, P. M., Scudder, S., Wen, W., Normanno, N., van Krieken, J. H., Sacco, A., Fenizia, F., Gonzalez de Castro, D., Hönigschnabl, S., Kern, I., Lopez-Rios, F., Lozano, M. D., Marchetti, A., Halfon, P., Schuuring, E., Setinek, U., Sorensen, B., Taniere, P., Tiemann, M., Vosmikova, H., & Dequeker, E. M. (2018). Detection of *EGFR* variants in plasma. *The Journal of Molecular Diagnostics*, *20*(4), 483-494. https://doi.org/10.1016/j.jmoldx.2018.03.006
- Lagraulet, A. (2010). Current clinical and pharmaceutical applications of microarrays: from disease biomarkers discovery to automated diagnostics. *JALA: Journal of the Association for Laboratory Automation*, *15*(5), 405-413. https://doi.org/10.1016/j.jala.2010.06.011
- Li, Y., Anderson, L. A., Ginns, E. I., & Devlin, J. J. (2017). Cost effectiveness of karyotyping, chromosomal microarray analysis, and targeted next-generation sequencing of patients with unexplained global developmental delay or intellectual disability. *Molecular Diagnosis & Therapy*, *22*(1), 129-138. https://doi.org/10.1007/s40291-017-0309-5
- Loftus, P. (2023). *Illumina launches more powerful gene sequencer*. The Wall Street Journal; Dow Jones & Company. https://www.wsj.com/articles/illumina-launches-more-powerful-gene-sequencer-11664466535
- Mao, X., Liu, C., Tong, H., Chen, Y., & Liu, K. (2019). Principles of digital PCR and its applications in current obstetrical and gynecological diseases. *American Journal of Translational Research*, *11*(12), 7209-7222.
- McElhinney, L. M., Marston, D. A., Ellis, R. J., Freuling, C. M., Müller, T. F., & Fooks, A. R. (2014). Sanger sequencing of lyssaviruses. In *Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention* (pp. 159-170). Elsevier. https://doi.org/10.1016/b978-0-12-800014-4.00016-0
- *Microarrays and Next-Generation Sequencing Technology. The use of advanced genetic diagnostic tools in obstetrics and gynecology*. ACOG. https://www.acog.org/clinical/clinical-guidance/committee-opinion/articles/2016/12/microarrays-and-next-generation-sequencing-technology-the-use-of-advanced-genetic-diagnostic-tools-in-obstetrics-and-gynecology
- Shao, L., Akkari, Y., Cooley, L. D., Miller, D. T., Seifert, B. A., Wolff, D. J., & Mikhail, F. M. (2021). Chromosomal microarray analysis, including constitutional and neoplastic disease applications, 2021

revision: a technical standard of the American College of Medical Genetics and Genomics (ACMG). *Genetics in Medicine*, *23*(10), 1818-1829. https://doi.org/10.1038/s41436-021-01214-w
- Sharma, R. *NGS versus Sanger sequencing for clinical decisions*. Today's Clinical Lab. https://clinicallab.com/trends/genomics/ngs-versus-sanger-sequencing-for-clinical-decisions-23214
- Shen, Y., & Wu, B.-L. (2009). Microarray-based genomic DNA profiling technologies in clinical molecular diagnostics. *Clinical Chemistry*, *55*(4), 659-669. https://doi.org/10.1373/clinchem.2008.112821
- Smith, L. K. (2012). HLA typing by direct DNA sequencing. In *Methods in Molecular Biology* (pp. 67-86). Humana Press. https://doi.org/10.1007/978-1-61779-842-9_5
- Solomon, D. A. (2018). Integrating molecular diagnostics with surgical neuropathology. In *Practical Surgical Neuropathology: A Diagnostic Approach* (pp. 71-89). Elsevier. https://doi.org/10.1016/b978-0-323-44941-0.00005-9
- Tan, C., Chen, X., Wang, F., Wang, D., Cao, Z., Zhu, X., Lu, C., Yang, W., Gao, N., Gao, H., Guo, Y., & Zhu, L. (2019). A multiplex droplet digital PCR assay for non-invasive prenatal testing of fetal aneuploidies. *The Analyst*, *144*(7), 2239-2247. https://doi.org/10.1039/c8an02018c
- Van den Veyver, I. B., Patel, A., Shaw, C. A., Pursley, A. N., Kang, S.-H. L., Simovich, M. J., Ward, P. A., Darilek, S., Johnson, A., Neill, S. E., Bi, W., White, L. D., Eng, C. M., Lupski, J. R., Cheung, S. W., & Beaudet, A. L. (2009). Clinical use of array comparative genomic hybridization (aCGH) for prenatal diagnosis in 300 cases. *Prenatal Diagnosis*, *29*(1), 29-39. https://doi.org/10.1002/pd.2127
- Wallace, A. J. (2016). New challenges for BRCA testing: a view from the diagnostic laboratory. *European Journal of Human Genetics*, *24*(S1), S10-S18. https://doi.org/10.1038/ejhg.2016.94
- Whitehead, N. (2017). *Genomic microarray and whole exome sequencing - Washington State Health ...* Washington State Health Care Authority. https://www.hca.wa.gov/assets/program/micro-array-wes-final-rpt-20171221.pdf
- Winetsky, D. E., Negoescu, D. M., DeMarchis, E. H., Almukhamedova, O., Dooronbekova, A., Pulatov, D., Vezhnina, N., Owens, D. K., & Goldhaber-Fiebert, J. D. (2012). Screening and rapid molecular diagnosis of tuberculosis in prisons in Russia and eastern Europe: a cost-effectiveness analysis. *PLoS Medicine*, *9*(11), e1001348. https://doi.org/10.1371/journal.pmed.1001348
- Wu, L., Mickey Williams, P., & Koch, W. H. (2005). Clinical applications of microarray-based diagnostic tests. *BioTechniques*, *39*(4S), S557-S582. https://doi.org/10.2144/000112046
- Zacharioudakis, I. M., Zervou, F. N., Shehadeh, F., & Mylonakis, E. (2019). Cost-effectiveness of molecular diagnostic assays for the therapy of severe sepsis and septic shock in the emergency department. *PLOS ONE*, 14(5), e0217508. https://doi.org/10.1371/journal.pone.0217508

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