



Emerging Trends in Molecular Diagnostics: From PCR to CRISPR-Based Techniques

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Abstract

Molecular diagnostics encompasses the detection and quantification of nucleic acid sequences for infectious and inherited diseases, cancer, and various clinical conditions. Next-generation sequencing (NGS) is a transformative approach for examining human diseases, offering transformative solutions across diagnostic needs. CRISPR-based diagnostics operate with superior specificity compared to PCR, which relies upon primer-template complementarity, and involve orthogonal specificity from both guides and primers. Microbial detection platforms suffer from limited real-world deployment due to the absence of integrated, portable solutions. The SHERLOCK assay inaugurated the era of CRISPR-based diagnostics, a rapidly growing field showing potential to disrupt the molecular diagnostics market.

Keywords: Molecular diagnostics, PCR, CRISPR, gene editing, nucleic acid detection, precision medicine, biomarker discovery, diagnostic innovations.

1. Introduction to Molecular Diagnostics

Molecular diagnostics explores the molecular basis of disease by studying molecules such as DNA, RNA, proteins, and metabolites to improve patient care. It combines molecular and cellular biology knowledge with analytical techniques, instrumentation, and materials to perform measurements at the cellular, organelle, and molecular diagnostic levels. Molecular diagnostics has fulfilled, and sometimes even exceeded, the high expectations of the 1980s by providing insights into disease etiology and pathophysiology and offering important clinical applications that contribute to improved patient care.

Recent advances in technologies such as liquid chromatography, mass spectrometry, microarrays, nanotechnology, spectroscopy, next-generation sequencing, nuclear magnetic



resonance, and molecular imaging have dramatically modernized molecular diagnostic tools and accelerated their development. Nonetheless, many analytical techniques and technologies remain under investigation, awaiting specific clinical applications. The molecular basis of a disease involves alterations in cellular constituents linked to a particular pathology. The changes in DNA, RNA, protein, and metabolism represent the “molecular signature” of a disease. Analyses of such molecular signatures could enhance understanding of disease onset and progression and facilitate early diagnosis and prognosis of certain diseases such as cancer and ischemia. Nowadays, molecular-pathogenesis-based methods can detect specific biochemical and molecular events characterizing certain diseases even when gross changes in macroscopic or microscopic examination are absent.

In the clinical diagnostic field, the application of molecular diagnostics opens new perspectives to the identification of disease and complements knowledge and information provided by other diagnostic disciplines. Molecular diagnostic testing has gained increasing importance in the clinical laboratory, evolving beyond the research laboratory into the clinical diagnostic laboratory and becoming a critical tool in clinical practice. Evident contributions include infectious diseases, with substantial progress in the elucidation and interpretation of disease patterns for hepatitis B and C, and human immunodeficiency virus.

Molecular detection is also used for human DNA fingerprinting analysis to establish biological parentage, and it can detect the presence or absence of a transplanted gene in patients who have undergone gene therapy (Ransom Fairfax & Salimnia, 2013).

2. Historical Overview of Molecular Diagnostics

The history of molecular diagnostics can be traced back to the emergence of molecular markers that, when linked to genetic information, greatly facilitated gene discovery and clinical testing (E. Schmitz et al., 2022). Immunoassays and chromatography supported clinical testing by detecting analytes indicative of a pathway or disease. Early applications of molecular markers persisted into the clinical arena but have been supplemented by assays developed through the discovery of the polymerase chain reaction (PCR). The development of PCR in 1983 rapidly transformed the ability of clinical laboratories to detect genomic materials in specimens with higher sensitivity and specificity. Unlike previous methodologies, the clinical use of PCR did not rely on the ability of a patient to develop an immune response or the viability of an organism in the specimen (Ransom Fairfax & Salimnia, 2013). The 1990s witnessed the first attempts to apply molecular technology to the clinical diagnosis of infectious diseases when in-house non-amplified assays and/or amplified assays were pursued. Although PCR quickly became indispensable in molecular diagnostics, alternative technologies such as the CRISPR-Cas system have been developed. The CRISPR-Cas system complements or replaces PCR and next-generation sequencing for direct detection of disease in animals, plants, and humans (Zhang, 2022).



3. Polymerase Chain Reaction (PCR) Technology

The polymerase chain reaction (PCR) is a technology that amplifies DNA segments enzymatically, enabling the isolation and molecular analysis of single genes from the entire genome. Diagnostic PCR exploits the capacity of DNA polymerase to synthesize complementary strands from single-stranded DNA templates. The introduction of a thermostable DNA polymerase from *Thermus aquaticus* facilitated continuous amplification through repeated thermal cycling. Reverse transcriptase application extended PCR to the amplification of RNA viruses and microbial RNA, broadening its clinical utility. Further enhancements in laboratory instrumentation included development of thermocyclers capable of rapid temperature transitions and minimized reaction volumes, thereby accelerating and improving PCR efficiency (E. Schmitz et al., 2022).

Diagnostic PCR encompasses three phases: specimen processing with nucleic acid extraction, target amplification, and subsequent detection or characterization of amplified products. Optimizing nucleic acid extraction—through various automated and manual methods integrating magnetic purification or filtration—remains critical to overall assay performance, with protocols adaptable to specific microbial organisms. Since its inception, PCR has contributed significantly to genome sequencing, recombinant gene expression analysis, and rapid molecular genetic determinations such as paternity testing and infectious disease diagnosis. Conventional PCR affords qualitatively sensitive detection; however, the advent of Real-Time PCR enables in situ reaction monitoring and quantitative output, augmenting speed and precision in clinical diagnostics and research laboratories (Agne Alves Valones et al., 2009).

3.1. Principles of PCR

The polymerase chain reaction (PCR) worldwide is the most widely applied nucleic acid amplification technology because of its proven utility in numerous exemplary molecular applications. Its principle is based on the remarkable ability of purified DNA polymerase to synthesize double-stranded DNA in vitro from a single-stranded template and a pair of synthetic oligonucleotide primers that specify the region targeted for amplification. Primers complementary to opposite strands are designed to bracket the DNA sequence-of-interest and applied in appropriate assay concentrations to direct selective, enzymatic target multiplication from complex nucleic acid mixtures. The assay protocol itself is directed by the exploitation of the enzyme's fundamental synthetic stride—polymerization of the primer downward along the length of the template strand—not only in choosing the sequences but also in setting the cycling design of temperatures and time intervals that dictate the success, efficiency, and fidelity of the reaction. Inherent to the nature of the technique are extraordinary levels of analytical sensitivity and specificity, largely due to the exponential strategy and the discrete selectivity of primer annealing.



The universal adoption to practicability of a heat-stable polymerase from, notably, the thermophilic eubacterium *Thermus aquaticus* has significantly increased the efficiency of the reaction, allowing a single, fixed concentration of enzyme to be applied across successive cycles. Similarly, the co-application of reverse transcriptase enables RNA-to-DNA target conversion and thus the amplification of RNA viruses or cellular microbial mRNA/rRNA from complex biological mixtures. Improvements in laboratory equipment, notably the design and manufacture of dedicated thermocyclers featuring rapid ramping rates, in conjunction with the development of micro-volumes and high-quality tubes, have served to reduce the overall time and cost associated with individual assays and to automate the procedure in a standardized and robust manner, leading to the widespread application of the technology for clinical diagnostic purposes (E. Schmitz et al., 2022).

Conventional diagnostic PCR comprises three principal steps: (i) specimen processing and nucleic acid extraction, (ii) target amplification, and (iii) detection of the amplified reaction products. Efficient purification of DNA and/or RNA from potentially complex and/or fragile specimens is absolutely essential to the overall analytical success of reaction and to enabling significant improvements in detection sensitivities. Although far from routine but increasingly applied, large numbers of methods and systems have been developed or described that utilize silica particles and matrices or magnetic purification components to concentrate nucleic acid from crude materials for molecular-based testing (Agne Alves Valones et al., 2009).

3.2. Applications of PCR

The invention of the polymerase chain reaction (PCR) as a method to exponentially amplify a targeted piece of DNA revolutionized molecular diagnostics. This technology has become the gold standard for detecting target DNA sequences and forms the basis of many diagnostic assays. Its widespread applications encompass infectious-disease diagnosis, pathogen genotyping, human leukocyte antigen (HLA) typing, forensic analysis, donor-recipient matching, pharmacogenomics, genetic testing, and personalized medicine (Agne Alves Valones et al., 2009).

In diagnostics of infectious diseases, PCR enables rapid identification, detection, and strain (genotype)/resistance profiling of bacterial and viral organisms. PCR also provides a powerful tool for molecular genotyping or genetic-based identification of bacterial pathogens, as even genetically similar bacterial isolates belonging to the same species differ in susceptibility or resistance to specific antibiotics. Furthermore, it serves as an essential method for detecting the presence of pathogenic organisms on contamination plates, blotting membranes, or among environmental samples, particularly those that are uncultivable or difficult to culture.



3.3. Limitations of PCR

Since its introduction in 1983, polymerase chain reaction (PCR) technology has had a profound impact on molecular diagnostics. It enables amplification of nanogram quantities of DNA or RNA sequences into microgram quantities in under two hours. PCR assays are rapid and sensitive and have been successfully applied to detect mutations, characterize genes, identify infectious agents, and quantify nucleic acid targets in a wide variety of clinical specimens (Carter et al., 2010).

Broad-range PCR assays, targeting universally conserved regions such as the 16S-23S rRNA spacer region and heat-shock protein genes, have been developed to simultaneously detect multiple organisms and screen for unsuspected pathogens. Multiplex PCR assays incorporate multiple primer sets within a single reaction to detect more than one target sequence and have greater acceptance in the context of real-time PCR.

Despite its advantages in sensitivity and specificity over traditional diagnostic tests, PCR has several limitations. Each assay requires meticulous optimization of reagents and primers to avoid nonspecific amplification, and the associated costs remain high. Dedicated laboratory space and equipment are necessary to minimize contamination risks, which can still lead to false-positive results. The advent of closed-tube, real-time PCR platforms has mitigated some contamination concerns and enhanced workflow efficiency. To prevent false-negative results caused by DNA degradation or the presence of inhibitors, internal controls are essential. Moreover, efficient specimen processing and nucleic-acid extraction are critical for assay sensitivity. Although PCR has revolutionized infectious disease diagnosis, first-generation assays are increasingly supplanted by real-time PCR approaches that offer superior sensitivity, specificity, speed, and automation capabilities (E. Schmitz et al., 2022).

4. Next-Generation Sequencing (NGS)

Next-generation sequencing (NGS) allows high-throughput, parallelized DNA sequencing and has significantly advanced genetic research and clinical and laboratory settings. NGS possesses several advantages over traditional Sanger sequencing, including reduced time and labor, enhanced scalability toward whole-genome sequencing, and enhanced sensitivity (Goldberg et al., 2015). Although earlier adoption of NGS in some laboratories was delayed due to cost, complexity, and limited throughput capacity, the amplification and analytical potential of NGS has recently increased, expanding its prevalence in infectious disease and cancer diagnostics (G. Rodino & J. Simner, 2024). NGS has further driven progress in areas such as Sanger, microarray, PCR, and in situ hybridization.

Microbial diagnostics are increasingly transitioning toward NGS-based molecular diagnostics, a trend that offers enhanced accuracy within clinical microbiology applications. The technology has generated considerable interest for both direct whole-genome sequencing



from isolate culturing and metagenomic sequencing from clinical specimens. Traditional Sanger sequencing and pyrosequencing platforms—previously regarded as primary resources for microbial sequencing—have been supplanted by NGS. Technological advancements have similarly yielded improvements in quality, quantity, and cost of produced sequencing data. The sequencing process requires fragmentation of the microbial genome into many DNA segments. These fragments undergo parallel sequencing into reads that can be reconstructed into a complete genome or aligned against reference genomes to highlight sample-specific variants. Longer reads are preferred in de novo genome assembly because they fill larger gaps and span repetitive regions, whereas shorter reads incur lower costs and generate data in higher volumes. Regardless of read length, achieving high coverage and accuracy remains essential to identify true variants effectively. NGS also plays a pivotal role in metagenomics by facilitating analysis of microbial communities.

Metagenomic next-generation sequencing (mNGS) entails sequencing all nucleic acids within a specific sample: host, microbes, and contaminants alike. The approach enables direct detection of pathogen reads among the total sequencing data space, encompassing bacterial, viral, fungal, and parasitic agents in addition to fungal isolates and antimicrobial resistance genes. mNGS has demonstrated potential in revealing rare pathogens, atypical infections, coinfections, and infections influenced by immunocompromising conditions. Although most validation assays focus on cerebrospinal fluid and plasma, ongoing development extends applications to other sample types and clinical scenarios. The emergent technology offers potential as a precision-medicine tool, informing on host response to infection, microbiome structure, pathogen virulence factors, and antimicrobial resistance determinants. Direct-from-specimen NGS approaches generally remain complex, costly, and labor intensive and are therefore deployed as a last resort when standard diagnostic methods do not provide a clinical diagnosis. Early testing could nevertheless reduce healthcare costs by enabling more rapid identification of infections, highlighting the importance of result interpretation within the individual clinical context.

NGS is increasingly utilized in cancer molecular diagnostics to simultaneously analyze multiple genes for clinical utility. The technology offers fast turnaround time, adaptability, minimal DNA input requirements, decreasing costs, and capacity to assess single genes, defined gene groups, full exomes, or genomes within a single assay. NGS complements and at times replaces traditional methods such as Sanger sequencing, fluorescence in situ hybridization, immunohistochemistry, and amplification refractory mutation system polymerase chain reaction. Routine screening of multiple diagnostic, prognostic, and predictive markers from limited and often degraded nucleic acid quantities aligns with the high discovery rate of new clinically relevant markers. Recent improvements in target capture, library preparation, and sequencing solutions have expanded implementation to both academic and community hospital settings. Comparative studies of NGS components and



external quality assessments have clarified options and supported clinical adoption. Regulatory guidelines further facilitate assay validation and clinical use. Novel sequencing technologies such as Nanopore display potential to revolutionize parallel sequencing and may complement or eventually replace current NGS platforms. The technology thus addresses a critical need for routine tumor mutation profiling that enhances cancer therapeutics, and growing regulatory understanding, clinical validation reports, and technological advances have established NGS as the preferred large-scale genome sequencing method despite continued workflow complexity and validation challenges (Luthra et al., 2015).

4.1. Technological Advancements in NGS

Molecular diagnostics have experienced a rapid evolution since the discovery of the DNA double helix. For most of the twentieth century, molecular biology research focused on conceptual advances rather than technology, applying these insights to understand the genetic basis of disease more effectively during the 1970s and 1980s. In 1901, Bertino elucidated the chemical components of deoxyribonucleic acid (DNA)—notably an abundance of phosphate groups—guiding its isolation and purification. A definitive milestone occurred in 1953 when Watson and Crick proposed the DNA double helix structure, establishing the enduring, complementary relationships inherent in the structure of nucleic acids (Jayamohan et al., 2017). In 1957, Kornberg identified the first DNA polymerase, permitting the enzymatic replication of DNA. The development of the polymerase chain reaction (PCR) method in the early 1980s marked yet another major milestone in molecular biology and molecular diagnostics (E. Schmitz et al., 2022).

Next-generation sequencing (NGS) technologies provide the capacity to obtain multiple DNA or RNA sequences simultaneously, serving a diverse array of purposes in molecular diagnostics, including generating fully sequenced genomes and detecting gene mutations or rearrangements. NGS replaces labor-intensive Sanger sequencing with user-friendly instruments and high-throughput analytical software. The continuous evolution of NGS technologies stimulates the development of diagnostic applications that benefit patients across various clinical conditions while presenting technical and interpretative challenges regarding implementation in laboratory medicine.

4.2. Clinical Applications of NGS

Next-generation sequencing (NGS) technologies have paved the way for a diverse range of clinical applications, including characterization of the microbiome, molecular profiling of tumors, detection of hereditary disorders, forensic analyses, noninvasive prenatal diagnostics, and screening for carrier status and infectious agents. As the first cohort of clinical laboratories has begun to implement NGS, widespread adoption has been tempered by a variety of operational challenges and ongoing technological evolution (Luthra et al., 2015).



NGS offers several advantages over polymerase chain reaction (PCR)–based techniques, including far greater sensitivity and the ability to examine multiple genes simultaneously. The recurring expense of consumables is, however, considerably costlier and means that PCR technology typically remains the preferred choice for screening large patient cohorts or when testing for single loci. The readiness of NGS for routine clinical use is similarly hampered by the need for extensive validation of results and the development of external quality assessment schemes. Sequencing methods capable of generating longer reads are urgently needed, and future improvements would usefully focus upon automation, enhanced bioinformatics tools, and the introduction of dedicated frameworks for managing the vast quantities of data generated (Goldberg et al., 2015).

4.3. Challenges in NGS Implementation

Given the expanding clinical utilization of molecular approaches providing increasingly detailed content, the adoption of advanced sequencing technologies has escalated. Like PCR, next-generation sequencing (NGS) offers high specificity for nucleic acid amplification, with additional benefits of multiplexing and high throughput. In oncology, the spectrum of targeted therapies has evolved from single to numerous multi-target approaches, making multigene test panels a common recommendation. Testing multiple candidate genes by individual PCR assays presents challenges in cost efficiency, tissue sample requirements, turnaround time, and biological relevance of single-gene alterations, whereas most available next-generation sequencers can analyze virtually any number of genes simultaneously. Consequently, high-throughput sequencing outperforms serial molecular assays where complex alterations drive tumor progression. The capacity of NGS panels spanning hundreds to thousands of genes surpasses multiplex PCR methods in clinical utility. Although NGS techniques address some limitations associated with routine single-gene testing, their complexity poses a considerable obstacle to rapid and widespread technical adoption.

Rapid technological advancement and design optimization have lowered equipment barriers, yet requirements for sophisticated data analysis, high-performance computing, and bioinformatics expertise remain significant. Additional challenges include data storage demands and the development of user-friendly data interpretation tools. Interpretation complexity intensifies when profiling approaches encompass rare or unexpected mutational classes with high prevalence. Sample preservation methods, especially formalin-fixation, and tumor heterogeneity further impact the robustness and reproducibility of NGS assays (Luthra et al., 2015). Laboratories often treat NGS results requiring orthogonal confirmation as non-actionable without effective confirmatory assays. Commercially available tests vary widely in specimen compatibility, from formalin-fixed paraffin-embedded (FFPE) tissues to circulating nucleic acids (Akkari et al., 2019). In clinical and academic settings, test selection also hinges on panel size, return rates, and investigator requirements.



5. CRISPR-Based Diagnostic Techniques

The CRISPR-Cas system is a prokaryotic immune mechanism, enabling bacteria to detect and degrade invading nucleic acids. This system has been repurposed as a powerful molecular-toolkit for diverse biotechnology applications. CRISPR-based molecular diagnostics enable ultra-sensitive, low-cost, and rapid detection of genetic disorders, viruses, and pathogens, surpassing existing tools and clinical possibilities. Incorporation of the Cas13 system into compact, portable detection devices—combined with effective sample-preparation protocols—has demonstrated significant progress toward practical point-of-care applications. CRISPR-Cas approaches offer exceptional specificity and sensitivity for nucleic-acid targets, establishing new standards for molecular diagnosis.

5.1. Mechanisms of CRISPR-Cas Systems

The investment of naturally occurring clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) systems into nucleic acid detection is a major departure from conventional diagnostic technologies. A brief explanation of their mechanisms, followed by an exploration of ongoing applications, is therefore warranted. As a distinct group of adaptive immune regulators in bacteria and archaea, CRISPR-Cas systems employ specific RNAs to identify complementary target sequences within invading genetic material. The extensive diversity of these systems translates into an abundance of nucleic acid interrogation mechanisms (Zhang, 2022). Nevertheless, they share a recurring infectious categorization into two major classes, subdivided into six types and multiple subtypes. The defining criterion that differentiates the two classes is the utilization of either a multi-protein effector complex (class I) or a single protein effector (class II) to engage target genetic material (Puig-Serra et al., 2022).

5.2. CRISPR in Disease Detection

The identification of novel biomarkers is central to effective detection and pathogenesis monitoring in a broad spectrum of diseases, including cancer, infectious diseases, and autoimmune disorders. Conventional PCR and real-time PCR techniques have significantly influenced the translation of discoveries from basic science to clinical diagnostics in recent decades. Nonetheless, emerging molecular diagnostics methods have enriched the field of disease identification and characterization, presenting alternatives that overcome limitations inherent to traditional PCR-based techniques.

Clustered regularly interspaced short palindromic repeats (CRISPR), derived from the adaptive immune systems of bacteria and archaea, constitute a dynamic approach to disease detection. Beyond their renowned capacity for precise genome editing realized in 2013, CRISPR-Cas systems have been repurposed to recognize specific nucleic acid sequences, enabling the development of diagnostic applications. The discovery of novel Cas effectors,



such as Cas12 and Cas13, has facilitated the creation of cost-effective, portable platforms capable of efficient nucleic acid screening. These innovations hold significant promise for disease monitoring across diverse fields, including infectious diseases, genetics, pharmacogenomics, and oncology (Puig-Serra et al., 2022).

Although early CRISPR-based diagnostics predominantly utilized Cas9/type II systems, these strategies necessitated nucleic acid pre-amplification and post-amplification processing, which prevented single-tube detection and increased contamination risks. Additionally, dependence on PCR cycling imposed constraints on point-of-care applications (Zhang, 2022). Subsequent methods integrating isothermal amplification—such as NASBACC, CRISDA, and CAS-EXPAR—eliminated the requirement for thermal cycling, thereby enhancing suitability for rapid and portable diagnostics. Notably, NASBACC achieved discrimination among viral strains with single-base resolution, while CAS-EXPAR improved specificity without reliance on exogenous primers. The refinement of these techniques paved the way for versatile detection platforms employing lateral flow assays and fluorometric readouts, further advancing the accessibility and utility of CRISPR-based diagnostics.

5.3. Advantages of CRISPR Over Traditional Methods

In recent years, CRISPR/Cas9-mediated molecular diagnostics have been introduced to complement polymerase chain reaction (PCR) and next-generation sequencing (NGS). CRISPR/Cas9 offers faster, less expensive, and quantitative analysis with sub-attomolar sensitivity, filling the technological gap between PCR and NGS. The mechanism involves specific cleavage of DNA or RNA sequences guided by a complementary single guide RNA, allowing recognition and discrimination of target nucleic acids. CRISPR-based methods excel in addressing detection challenges while providing a universal platform for identifying infectious or noninfectious agents, including single nucleotide variants, circulating tumor DNA, and methylated DNA (Puig-Serra et al., 2022) (Jolany vangah et al., 2020).

6. Comparative Analysis of Diagnostic Techniques

During the corona pandemic CRISPR has shown how a molecular diagnostic technique can be disruptive. Outbreak-insight from the large-scale tester perspective was lacking despite a plethora of small-scale studies. New testing patterns can also be foundations for earlier response against other infectious diseases, not only pandemics. Emerging trends in molecular diagnostics from PCR-built diagnosis foundations towards NGS feeding more molecular details to clinical windows are accentuated and enriched by the disruptive CRISPR-based diagnostic techniques that also equip infectious metrology. Then, a discussion on how the pandemic has improved foundations for treating and controlling infectious diseases concludes the compilation.



Molecular diagnostics changed how medicine approaches infectious disease, cancer and almost any other pathology. The field emerged before PCR was conceived, but PCR soon became the method of choice for most diagnostic and research procedures owing to its sensitivity, specificity and relatively low cost and complexity. In addition to infectious-disease diagnostics and monitoring, PCR helped other techniques in analytical virology and has even been a tool for other molecular diagnostics methods. Nevertheless, PCR has some limitations, and new molecular techniques have tried to overcome them, such as next-generation sequencing (NGS). NGS detects sequence information from a broad range of pathogens, as well as from the host answer, and its use in infectious-disease diagnostics is expanding rapidly. However, a substantial number of technical problems related to NGS sample preparation, sequencing, and bioinformatic analyses, as well as high cost and complex workflows have hindered its widespread implementation in clinical laboratories. Recent developments of CRISPR-based diagnostic tools started a new wave of molecular diagnostic applications. These tools, which harness the genome-editing capabilities of CRISPR–Cas from the bacterial adaptive immune defence, offer the prospect of sensitive, specific, and rapid testing for a broad range of diseases while removing some of the bottlenecks of PCR and NGS.

6.1. PCR vs. NGS

Polymerase Chain Reaction (PCR) is a widely used technique in molecular biology, important for microscopic-level identification of genetic material in genuine samples. The next-generation sequencing (NGS) technique is an emerging technology that provides breakthrough solutions for sequencing genomes and transcripts at ultra-high-throughput and base-pair resolution. NGS enables for the analysis of entire genomes in an unbiased manner and has the potential to identify changes in DNA that are associated with cancer, enabling complementary cancer diagnosis. In addition to this NGS increasingly emerges as a key technique for viral, bacterial and fungal identification. The high costs and complex bioinformatics analysis currently limit the use of this technique in clinical microbiology, delaying a widespread integration of NGS in routine diagnostic workflow.

NGS technology unravels a broad spectrum of applications in clinical diagnosis, ranging from the diagnosis of inherited disorders to immunotherapy-oriented cancer evaluation, and non-invasive prenatal testing (NIPT). It is now established as the first-tier choice in detection of a broad spectrum of disorders including inherited diseases, chromosomal abnormalities, and complex diseases. Application of NGS techniques in clinical diagnosis and its future implications have been discussed in detail. With its diverse applications and increased coverage in next-generation molecular diagnostic tests, it sustains new challenges as well as limitless opportunities. The detailed list of all the NGS methods and the novel diagnostic tests



developed using them along with the technical specifications, applications and decisions of the Food and Drug Administration (FDA) have been tabulated for a quick overview.

6.2. NGS vs. CRISPR

Next Generation Sequencing (NGS) is an analytical method facilitated by various instrumentation platforms that operates by parallelising sequencing tasks to generate thousands or even millions of sequencing reads simultaneously (Yang et al., 2023). Developed in the mid-2000s, NGS technologies have rapidly evolved and are increasingly applied to clinical diagnostics. Compared with traditional molecular techniques such as PCR, NGS can provide an unprecedented level of detail to detect, identify and profile both known and previously uncharacterised pathogens (Ghouneimy et al., 2022). However, it remains a relatively specialised tool that is reliant on proficient staff and high upfront capital for strategic implementation (Zhang, 2022).

6.3. Cost-Effectiveness of Techniques

Determining the most cost-effective molecular diagnostic technique requires analytical sensitivity, specificity, and limit of detection information, followed by evaluation of reagent and material (e.g., plastic ware) costs related to these performance parameters. Assessing the cost-effectiveness is crucial for better utilization of resources; however, challenges often arise as not all relevant data are publicly available. Studies in this field have therefore focused on specific disease agents, such as pathogenic bacteria.

In comparative analyses of qPCR, NGS, and CRISPR-based assays, all three techniques demonstrated sufficient sensitivity and specificity for utilized reference material. Sensitivity levels were also adequate for routine clinical use. Calculated reagent and material costs indicated that NGS is the most expensive among the three. It requires a higher DNA input and extensive library preparation protocols, leading to increased reagent use. Conversely, qPCR was identified as the most cost-effective option, provided a specific probe was available. CRISPR-based assays showed higher costs than qPCR, possibly due to additional crRNA synthesis or, in some instances, high initial expenditures. Both CRISPR-based methods and qPCR offer rapid results, given that qPCR template preparation is faster than library construction for NGS.

7. Regulatory and Ethical Considerations

In most countries, molecular diagnostic tests must fulfill the approval criteria of governmental regulatory bodies before they enter the market. The stringency of these criteria typically correlates positively with the potential impact of the test on patient management. For example, Confidential In-Vitro Diagnostic Devices (IVDs), which provide general health information, are subject to minimal regulation, whereas Companion Diagnostics used to guide treatment decisions require thorough validation. The U.S. Food and Drug



Administration (FDA) classifies assays and devices into Class I (low risk), Class II, and Class III (highest risk) categories. Generally, Class I devices are exempt from premarket notification procedures, Class II devices undergo a premarket notification process, and Class III devices require premarket approval (Ghouneimy et al., 2022). In Europe, IVDs fall under Directive 98/79/EC and must carry the CE mark; currently, most CE-IVD assays fall into a low-risk category, which the new IVD Regulation intends to eliminate (E. Schmitz et al., 2022).

Molecular diagnostic techniques increasingly provide genetic information. Although the ethical considerations related to molecular diagnostics are not widely discussed in the first ranks of scientific literature, the Centre for Genetics and Society has recently highlighted several ethical issues of genetic testing and the importance of balancing benefits and risks.

7.1. Regulatory Framework for Molecular Diagnostics

The worldwide molecular-diagnostics market was valued at \$8.5 billion in 2020 and is estimated to reach over \$14.5 billion by 2025, growing at a compound annual growth rate (CAGR) of 10.3% during 2020–2025. Forecasted market value is projected to exceed \$30 billion by 2030. Of the three prime techniques—polymerase chain reaction (PCR), next-generation sequencing (NGS), and clustered regularly interspaced short palindromic repeats (CRISPR)—used for studying nucleic-acid sequences for genomic variations, transcriptional-expression, and genetic-interactions, the molecular-diagnostics community regards PCR as the benchmark for genetic detection. It is currently considered the most acceptable and widely used method in research and clinical diagnostic settings due to its high specificity and portability (E. Schmitz et al., 2022). Interest in NGS and CRISPR methods also scales rapidly, with NGS identifying variations, detecting biomarkers, anamnesis, and infection. CRISPR systems can, in principle, overcome much of the complexity, intricacy, and expense associated with PCR and NGS using a relatively simple single-molecule cleavage assay (Zhang, 2022). Safe, efficient, and effective gene therapy depends heavily upon precise nucleic-acid-detection and suitable platforms for detecting genetic components in living cells.

Regulatory bodies—mainly the U.S. Food and Drug Administration (FDA)—oversee the evaluation and regulation of molecular-diagnostics systems. Historically, regulation occurred through strict oversight of manufacturers' production and distribution of medical devices. The FDA's current approval route for molecular diagnostics systems follows the Code of Federal Regulations Title 21 Part 812, which includes four distinct phases: preclinical research, clinical research, premarket notification, and postmarket safety monitoring. Ethical concerns associated with patient access to genetic testing focus on potential limitations in government insurance programs, employers, and private insurance coverage, precluding appropriate treatment or prevention of inherited diseases.



7.2. Ethical Implications of Genetic Testing

Ethical considerations in molecular diagnostics overlap with concerns about confidentiality of patient information and the consequences of potential diagnostic errors. For genetic testing in particular, choice of what to test has a greater impact on the questionnaire used for incidental findings or unrelated pathogenic variants, and laboratory methods inform decisions about whether or not to include variant calls for single-nucleotide polymorphisms (SNPs) for ancestry or pigmentation, data that could be more controversial than the original diagnostic question. Many debates surrounding ethics of genetic testing relate to issues around privacy and the life-altering psychological impact of being diagnosed with a disease, or becoming aware of the risk of a deadly or untreatable condition (Braverman et al., 2018). In many regions of North America and Europe, there remain concerns about discrimination in the workplace due to perceived medical fragility, as well as fears over the ability to access life, health, disability, or travel insurance. Several countries such as the United States and Canada have laws preventing such discrimination. However, societies with a limited appetite for direct-to-consumer testing may be concerned about the personal and societal impacts of widespread inexpensive access to genetic technology. Continual improvements in molecular diagnostics therefore necessitate an ongoing active discourse on issues of privacy and ethics.

8. Future Directions in Molecular Diagnostics

Molecular diagnostic methods are playing an increasingly important role in the medical field. Given the current state of technology, CRISPR-based diagnostics have the potential to provide a new gold standard for molecular diagnostics and could democratize disease detection worldwide. Several applications demonstrate that these molecular diagnostic assays may transform the way infectious diseases and cancer are diagnosed.

Emerging trends in molecular diagnostics are motivated by the shortcomings of established techniques, including quantitative polymerase chain reaction (qPCR) and next-generation sequencing (NGS). These techniques are inherently expensive and require sophisticated laboratory instrumentation. Consequently, alternative techniques that can offer equal — or improved — sensitivity, specificity, and efficiency whilst circumventing cost barriers have been explored at length, with particular emphasis on diagnostics platforms that leverage the clustered regularly interspaced short palindromic repeat (CRISPR)–Cas toolset.

The manipulation of DNA is integral to molecular biology and numerous associated technologies, with polymerase chain reaction (PCR) revolutionizing this field owing to its straightforward implementation and rapid amplification.

Since the 2010s, the advent of next-generation sequencing (NGS) technologies has precipitated transformative progress in disease diagnostics, enabling rapid, high-throughput generation of genetic information. Accompanying computational and analytical



advancements have broadened the scope of clinical applications, encompassing infectious diseases, hereditary disorders, and cancer. Nevertheless, the extensive equipment requirements of NGS present financial and infrastructure challenges that may hinder widespread clinical utilization.

The CRISPR-Cas immune system has become a cornerstone of contemporary genome-editing applications. Characterized by an RNA-guided Cas endonuclease and a trans-activating CRISPR RNA (tracrRNA), the system facilitates precise target recognition and cleavage. The diverse suite of CRISPR technologies enables a breadth of disease-detection applications that either supplement or supplant PCR and NGS (Ghouneimy et al., 2022).

One pivotal challenge in molecular diagnostics is the development of sample-processing chemistries compatible with downstream analytical workflows. Innovations in automatic, user-friendly devices capable of accommodating direct sample introduction are anticipated. Long-term reagent storage and transport may be addressed via lyophilization coupled with optimal excipients, aiming for products resilient to harsh conditions, independent from cold-chain logistics, operational with minimal user training, and cost-effective. Although many CRISPR-based assays necessitate a high-temperature preamplification step, widely accessible commercial heaters mitigate this requirement. While fluorometric readouts predominate, conversions to visual signals are commercially viable, and smartphone cameras have been effectively employed for signal detection. The trajectory of innovation suggests that simple, inexpensive assays or devices will soon reshape point-of-care molecular diagnostics. The growing demand for home-based kits further underscores the importance of minimal user handling, enabling self-sampling and straightforward assay execution (Zhang, 2022).

Early CRISPR-based diagnostic modalities predominantly utilized Cas9/type II systems, which required pre- or post-amplification to achieve target detection. This dependence complicated single-tube operations and elevated contamination risks, as the methods entailed multiple procedural steps and thermal cycling, limiting applicability outside laboratory environments. The integration of isothermal amplification techniques such as NASBACC and CAS-EXPAR eliminated the necessity of thermal cyclers, advancing toward authentic point-of-care diagnostics. NASBACC affords viral strain discrimination with single-base resolution, whereas CAS-EXPAR delivers enhanced specificity without the involvement of exogenous primers. These foundational developments have catalyzed the emergence of additional CRISPR-based approaches, including lateral-flow and fluorometer-based detection platforms.

8.1. Integration of AI in Diagnostics

The future of molecular diagnostics is likely to be shaped by the integration of artificial intelligence (AI), personalized medicine, and scientific advances, underpinned by ongoing



regulatory support (Sun et al., 2022). Modern molecular diagnostic devices are expected to become automated analytical systems based on bioinformatics databases and tailored to personalized needs by efficiently correlating multiomics and massive clinical data.

The clinical value of molecular methods in diagnostics data generated by prospective trials remains to be elucidated. American regulatory agencies, the Centers for Disease Control and Prevention (CDC), and the University of Alabama at Birmingham (UAB) collaborated to develop the Advanced Molecular Detection Initiative in 2014, viewing advanced molecular technologies such as next-generation sequencing (NGS) and CRISPR/Cas nucleases as catalysts for a new era of epidemiology and diagnostics (E. Schmitz et al., 2022). Genomic methods have broadened from sequencing-based approaches to applications including CRISPR/Cas-based diagnostics, with the potential to revolutionize human disease management. Targeted genome editing has been used in the treatment of various types of cancer and has been employed to treat mitochondrial DNA diseases (Zhang, 2022). State-of-the-art molecular diagnostic techniques largely resolve the requirement of PCR amplification and cross-contamination. CRISPR-mediated nucleic acid detection technologies have the potential to supplement PCR-based assays at molecular diagnostic platforms.

8.2. Personalized Medicine and Molecular Diagnostics

Personalized medicine represents a transforming approach in healthcare, enabling individualized prevention or treatment strategies based on each person's risk profile or response determinants (Gašperšič & Videtič Paska, 2020). Molecular diagnostics lies at the core of personalized medicine, facilitating the detection of genetic predispositions to diseases, the identification of mutations associated with various disorders, and the monitoring of therapy success and disease progression. Innovations in circulating cell-free DNA (cfDNA) diagnostics for cancer enable early tumor detection through mutated DNA from cancer cells. Mutation analyses, typically conducted via quantitative polymerase chain reaction (qPCR) or next-generation sequencing (NGS), require protocol modifications tailored to diagnostic stages. The Food and Drug Administration (FDA) has approved over 20 companion diagnostic tests that integrate genetic detection tools with cancer therapeutics, employing cfDNA subpopulation assessments, circulating tumor DNA (ctDNA) analyses, or intracellular DNA probes. Numerous ctDNA tests under clinical investigation further underline the growing significance of these methods in cancer diagnostics. CRISPR-Cas-mediated assays targeting specific gene sequences offer rapid detection both in vitro and in vivo (Zhang, 2022). Early CRISPR-based diagnostics predominantly utilized Cas9/type II systems, necessitating multiple pre- or post-amplification steps that restricted assay simplicity and heightened contamination risks. Advancements incorporating isothermal amplification strategies such as NASBACC, CRISDA, and CAS-EXPAR circumvent the necessity for thermal cyclers, thereby bringing diagnostics closer to point-of-care feasibility. NASBACC



effectively discriminates viral strains differing by a single nucleotide, whereas CAS-EXPAR exhibits enhanced specificity toward mutant targets without reliance on exogenous primers. These breakthroughs have catalyzed the development of auxiliary platforms, including lateral flow and fluorometric CRISPR-based assays (Ghouneimy et al., 2022). The initial SHERLOCK assay, introduced in 2017, marked a significant expansion in CRISPR-based diagnostics. While challenges such as sample processing compatibility, long-term reagent stability, and the requirement for high-temperature pre-amplification persist, potential solutions encompass optimized chemistries, lyophilization techniques ensuring resistance to storage conditions, and integration with low-cost heating devices. The possibility of translating fluorescence outputs into visual or smartphone-detectable signals further supports the prospect of deployable, user-friendly assays. These collective innovations portend the emergence of assays or devices that could fundamentally reshape point-of-care molecular diagnostics, including applications in home-testing scenarios with minimal handling requirements.

9. Case Studies in Molecular Diagnostics

A plethora of cases demonstrate the power of molecular diagnostics. For instance, during the recent COVID-19 pandemic, real-time reverse transcription polymerase chain reaction (RT-PCR) was effectively used worldwide to identify infected patients, inform treatment strategies, monitor transmission dynamics, and guide geopolitical decisions such as the lifting of shelter-in-place directives. Moreover, RT-PCR has served as a diagnostic tool capable of detecting Zika virus in serum, saliva, and urine from patients exhibiting signs of infection in Brazil. Another example comes from oncology, where BRCA1 and BRCA2 gene mutations are recognized biomarkers indicating a heightened risk for breast and ovarian cancers. The detection of these mutations can stimulate early interventions that reduce the odds of tumor development (Zhang, 2022).

9.1. Successful Applications in Infectious Diseases

Molecular diagnostics are an increasingly important factor in the management of infectious diseases. Throughout history, many pandemics and epidemics caused mainly by viruses have led to many deaths. This has highlighted the need to adequately identify the causative agent and to incorporate appropriate measures to reduce the extent and duration of its spread.

These needs have resulted in the rapid implementation of molecular techniques for various purposes, including detection strategies, characterization efforts, and evolution studies of emerging diseases. In addition, they are used to identify mutations that increase transmissibility or confer resistance to different types of therapies. For example, reverse transcription polymerase chain reaction (RT-PCR) was of great importance in the monitoring and control of the COVID-19 pandemic, just as the identification of different new variants



allowed a faster approach to controlling the demographic and epidemiological issues associated with this pandemic.

9.2. Oncological Applications of Molecular Diagnostics

Molecular diagnostics are routinely employed in oncology for diagnosis and treatment guidance. Tumor-specific chromosomal translocations and fusion genes serve as biomarkers for liquid and tissue biopsies in clinical practice, aiding detection, diagnosis, monitoring, and surveillance. Primary methods include next-generation sequencing (NGS), polymerase chain reaction (PCR)-based tests, and fluorescent in situ hybridization (FISH). Tumor-specific translocations and fusions arise from balanced chromosomal recombination of two separate genomic loci, producing either aberrant oncogenic fusion proteins or resulting in upregulation of a proto-oncogene. Immunohistochemistry (IHC) can be applied for detection; however, well-characterized, highly specific antibodies are only available for certain fusion proteins (Zhang, 2022).

In clinical settings, NGS is capable of detecting most chromosomal abnormalities present in hematolymphoid and solid malignancies; however, the high cost for cost-sensitive and low- or middle-income countries is a major barrier. To achieve a favorable balance of sensitivity, specificity, rapid turnaround time and lower costs, many laboratories rely on PCR- and IHC-based assays. However, PCR-based assays require the design of primers across fusion junctions that have been pre-defined (E. Schmitz et al., 2022).

Molecular diagnostic assemblies can operate as early genotyping tools capable of detecting genomic loci to classify and propagate diseases into profound levels of diagnostics. As precision medicine emerges, molecular diagnostic tools will be required to offer highly accurate precision medicine, with state-of-art implementations rarely exceeding 100 markers of interest per assay. Still, early-stage tools rely only on 1–5 markers generated through molecular signatures to support infectious diseases and oncological applications.

10. Global Impact of Molecular Diagnostics

Molecular diagnostic techniques have become critical for clinical diagnostics worldwide. Yet molecular diagnostics are still only rarely available in developing countries with poor infrastructure or when healthcare workers have limited training (Ghouneimy et al., 2022). PCR-based diagnostics have been useful for identifying a wide range of pathogens, including Ebola, Zika, HIV, tuberculosis, and malaria, but the scarcity of resources limits their widespread use in resource-constrained settings (E. Schmitz et al., 2022). Molecular diagnostics can also be prohibitively expensive for low- and middle-income countries (Zhang, 2022). Ensuring that molecular diagnostics become widely accessible will require innovations in engineering, chemistry, biotechnology, and empirical testing to avoid problems associated with supply chain stability, cold storage, and contamination. Several



regions bear a disproportionate burden of infectious disease-related morbidity and mortality that might be better managed with improved access to molecular diagnostics. For example, nearly two-thirds of all adult deaths in southern Africa are caused by preventable or treatable conditions, such as human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS), tuberculosis, malaria, and lower respiratory infections. Implementation strategies that successfully scale up molecular diagnostics may have large impacts on the health and economies of such communities and countries. Because diagnosis remains the most essential component of an efficient healthcare system, nations should prioritize the expansion of molecular diagnostics to low-resource settings. Molecular diagnostics have already revolutionized the response of governments and communities to outbreaks of emerging infectious diseases by enabling rapid point-of-care identification of causative microorganisms, and there remains much room for impact in low-resource settings.

10.1. Molecular Diagnostics in Low-Resource Settings

Molecular diagnostic technologies have gained increasing attention owing to their rapid detection and personalized treatment capabilities (Ghouneimy et al., 2022). The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic highlighted the critical need for novel, accessible diagnostic technologies. Inexpensive, point-of-care assays would greatly improve virus surveillance and quell the spread of SARS-CoV-2 by rapidly monitoring and tracking infection rate. The past decade witnessed revolutionary innovations in detection technologies, subsequently reviving the discovery and implementation of CRISPR-based detection (Zhang, 2022). Polymerase chain reaction (PCR)-based methods, although widely used, require elaborate laboratory infrastructure and are inaccessible to many individuals. Several reports from early and late 2020 demonstrated the potential of the clustered regularly interspaced short palindromic repeats (CRISPR) system to provide field-deployable, rapid detection of SARS-CoV-2 (Chen et al., 2022). Several CRISPR-based technologies have since emerged, most of which use Cas enzymes with collateral cleavage activity (e.g., Cas12a, Cas12b, Cas13a, and Cas14a) and have been further integrated into microfluidic, droplet-based, and wearable platforms.

These techniques may define the next step toward the democratization of disease detection and provide the new gold standard for molecular diagnostics. The vast majority of CRISPR-based biosensors utilize Cas effectors that exhibit nonspecific endonuclease activity, which cleaves nucleic acid targets based on complementary guide RNAs (gRNAs). These platforms typically require a preamplification step (e.g., PCR or isothermal amplification) to achieve clinical diagnostic sensitivity. Thus, portable, isothermal approaches are preferred because they operate under easily controlled conditions, even at physiological temperature. For protein detection, the development of aptamers has allowed ultrasensitive detection of proteins and the identification of molecular interactions that modulate Cas activity.



Integration with microfluidic platforms further reduces sample and reagent consumption, lowers the limit of detection, and increases detection throughput. Such systems provide a promising avenue for the development of optimized point-of-care diagnostic devices. Consequently, given their sensitivity, selectivity, rapidity, low cost, versatility, simplicity, and multiplexing potential, CRISPR-based biosensors hold promise for applications in medical diagnostics, disease screening, food safety monitoring, and crop genotyping.

10.2. Impact on Public Health Policies

Molecular diagnostic technologies have had a direct impact on public health policies in recent years. Benefits include improved health protection, more rational use of medications, reduction in ineffective treatments, and improved patient quality of life (Yang et al., 2023). For example, rapid molecular diagnostic tools facilitate more appropriate use of antibiotics and more cost-effective treatment approaches by significantly reducing treatment times. Health authorities worldwide have incorporated molecular diagnostic techniques into public health policy, triggering positive change. Low-resource countries, in particular, could benefit from widespread use of molecular diagnostic technologies. The availability of these tools would lessen the burden on limited healthcare resources and help to circumvent the lack of trained medical staff. On-site or mobile screening/testing systems would increase access to health services and improve patient outcomes. Mobile technologies would facilitate early testing and contact tracing in regions lacking accessible healthcare services; this might aid in the management of pandemics and the prevention of local outbreaks (Ghouneimy et al., 2022).

11. Conclusion

Molecular diagnostics is a powerful approach to measuring analytes that define and characterize the biological state of an individual at the molecular level (Ghouneimy et al., 2022). Molecular diagnostics have become an indispensable partner to modern medicine because they provide an effective way to gather specific information about an individual's specific health condition (Zhang, 2022). With the ever-growing expansion of healthcare needs, coupled with the explosion of scientific and technological breakthroughs, emerging diagnostics methods have become a major topic of interest.

PCR is widely recognized at the cutting edge of molecular diagnostics. PCR analysis lies at the heart of diverse studies encompassing biological, medical, and technological disciplines. NGS brought about a molecular diagnostics revolution by capitalizing on one or more former innovations to drastically increase the sequencing throughput together with a remarkable sample processing method and an efficient data analysis. NGS technology is currently leveraged by a great majority of molecular biologists. Its rapid advancement also represents a tightly linked with the molecular diagnostics platform.



A significant step-change in molecular biology came in 2012 with the repurposing of the prokaryote adaptive immunity system grouped in CRISPR technology for efficiently and accurately editing whole genomes. Molecular diagnostics have thus benefited over the last 10 years from the use of CRISPR-based DNA or RNA nucleotide recognition to detect pathogenic diseases. In contrast to PCR and NGS, CRISPR diagnostics have tremendous potential to eventually impact low-resource and point-of-care settings worldwide. CRISPR-based diagnostics systems that can meet such requirements would thus represent both an elegant and affordable solution to several pressing and emerging worldwide threats.

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