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Reference materials for DNA-based diagnostics testing; principles, comparative analysis, contemporary applications, and future recommendation in Indonesia

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ABSTRACT

Diagnostic testing is crucial in modern healthcare, providing essential health information and influencing clinical decisions and patient outcomes. Ensuring the validity and quality of these tests is vital, with stringent quality control and assurance procedures enforced by regulatory bodies and healthcare facilities. Reference materials (RMs) are essential for the accuracy and reliability of DNA-based diagnostic tests, serving as benchmarks for error detection, test validity, and consistency. This study reviews the necessity of effective control substances for precise diagnostic testing through a narrative literature review of synthetic DNA sequences, recombinant plasmids, genomic DNA, and cell lines as RMs, sourced from PubMed, Scopus, and Google Scholar over the last decade. Each RM type has specific advantages and disadvantages impacting diagnostic performance: gBlocks are highly specific but lack genomic complexity; recombinant plasmids offer flexibility but face stability and contamination issues; genomic DNA provides comprehensive diagnostic information but is complex and costly; cell lines simulate *in vivo* conditions well but are prone to genetic drift and contamination. The review emphasizes the critical role of RMs in DNA-based diagnostics and highlights challenges faced by Indonesian laboratories, recommending national coordination and international collaboration to enhance RMs' availability, thereby improving patient outcomes and aligning with global standards.

INTRODUCTION

Diagnostic testing forms the foundation of modern healthcare practice and provides essential clinical data about an individual's health status by detecting disease conditions or infections [1]. These tests can range from simple blood analyses to comprehensive genetic screenings. They are crucial for ensuring diagnosis accuracy, guiding appropriate treatment plans, and tracking disease progression [2]. The validity and accuracy of these diagnostic tests must thus be guaranteed because they have a direct bearing on patient outcomes and clinical judgments. To preserve high standards and dependability in diagnostic testing, regulatory bodies, and healthcare facilities use stringent quality control and assurance procedures [3].

The utilization of reference materials (RMs) or external control materials (ECMs) in DNA-based diagnostic testing is pivotal for ensuring proper and reliable results. RMs act as benchmarks for testing procedures, enabling the

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detection and correction of errors and maintaining the validity of test results [4–6]. RMs and ECMs are different; RMs are a type of ECM that has been standardized and rigorously tested for specific parameters, making them vital for precise calibration and validation of diagnostic assays [7]. ECMs, which are carefully designed samples that mimic patient specimens, are used to assess the performance of diagnostic tests by providing an external standard to verify accuracy and precision [8]. Consistently incorporating both RMs and ECMs in the testing process is essential to meet quality assurance standards, particularly in healthcare and public health settings [9]. This function is critical for verifying test performance and identifying significant changes that could indicate issues with operational capabilities or equipment [10,11].

The evolution of diagnostic technology has transformed RMs from simple chemical or biological substances to more sophisticated matrices that support the immunoassay of complex substances, requiring consistent and stable RMs across various testing platforms [5,12,13]. Genetic engineering and cell culture innovations have ushered in a new era of RMs. These include synthetic DNA sequences, recombinant plasmids, genomic DNA, and cell lines with predetermined genetic compositions suited to particular diagnostic testing needs. Sanger sequencing, next-generation sequencing (NGS), Microarray and polymerase chain reaction (PCR) are examples of advanced technologies that require sensitive and specific RMs, which can improve the quality and accuracy of diagnostic tests [14–18].

Indonesia, like many other countries, faces unique challenges in its diagnostic technology sector, including limited access to high-quality RMs, variations in laboratory standards and procedures, and a need for stronger regulatory frameworks to ensure consistency and reliability in diagnostic testing [19,20]. These challenges can affect the accuracy of diagnostic results and have implications for patient care. Improving diagnostic technology in Indonesia is crucial to enhance healthcare outcomes, as accurate diagnostics are the cornerstone of effective treatment and disease management. By addressing these specific challenges and improving the quality of diagnostics, Indonesia can better manage public health, respond to disease outbreaks with greater agility, and align with international best practices. Incorporating these concerns, it is evident that there is a critical need for improvement within the diagnostic field in Indonesia to ensure precise and dependable test results, optimize patient care, and align with global healthcare standards. This review illuminates these issues and offers insights and recommendations to support Indonesian laboratories' advancement [21].

This review focuses on summarizing current knowledge about RMs and suggests ways for laboratories to improve their diagnostic protocols in accordance with international standards. The goal is to guide laboratories in the selection of appropriate RMs to enhance the quality and accuracy of their diagnostic tests. Therefore, it covers various aspects as depicted in Figure 1. It highlights the methodologies used in the study, including the narrative review approach and the inclusion criteria for selecting relevant literature. Additionally, it encompasses the discussion on applying these RMs across various diagnostic technologies such as Sanger sequencing, NGS, microarray, and PCR (both real-time and digital). The figure concludes with recommendations for enhancing the quality and availability of RMs in Indonesia through national coordination and global collaboration initiatives to assist laboratories in making informed choices.

METHODS

This narrative review approaches the necessity and effectiveness of various control substances for precise diagnostic testing. The review focused on original research articles investigating synthetic DNA sequences, artificially constructed plasmids, genomic DNA, and cell lines as external controls and RMs. Databases such as PubMed, Scopus, and Google Scholar were utilized for the literature search. Scientific papers discussing the history, evaluation, and development of RMs and their application in diagnostic technologies, including Sanger sequencing, NGS, microarray, and real-time and digital PCR (dPCR), are covered in this review. Nonscientific publications, review articles, and sources older than ten years were excluded from this review.

TYPE OF DNA-BASED RMS

The genetic testing procedure using DNA-based PCR method necessitates using RM to ensure the legitimacy and consistency of the results [22]. RMs play a crucial role in maintaining the integrity of the entire testing process by establishing essential performance standards [2]. Four commonly used RMs are gBlocks, artificial recombinant plasmids, genomic DNA, and cell lines.

gBlocks

gBlocks are synthetic, double-stranded DNA molecules designed to contain specific sequences [23]. In PCR-based protocols, gBlocks are primary controls to assess the assay's effectiveness and specificity [24]. These synthetic blocks can represent various sequences, including gene variants, SNPs, or other genetic markers pertinent to the research [12]. The high fidelity and accuracy of gBlocks make them invaluable for optimizing PCR assays, allowing scientists to evaluate primer binding efficiency and the conditions necessary for precise detection and measurement [25].

Artificial recombinant plasmids

Recombinant plasmids typically contain synthetic and bacterial-amplified plasmids [26]. Synthetic plasmids are engineered *in vitro* to contain specific gene sequences and control elements, enhancing gene expression functions or serving as standards for molecular diagnostics [27]. Bacterial-amplified plasmids involve adding foreign DNA to a natural plasmid vector, which is then replicated in bacterial host cells [28]. These plasmids are significant in PCR methods because they can be reproduced *in vitro*, facilitating the production of large DNA fragment quantities and enabling accurate gene quantification through standard curves [29].

Genomic DNA

Genomic DNA, derived from reliable sources such as cell lines or well-characterized organisms, replicates the

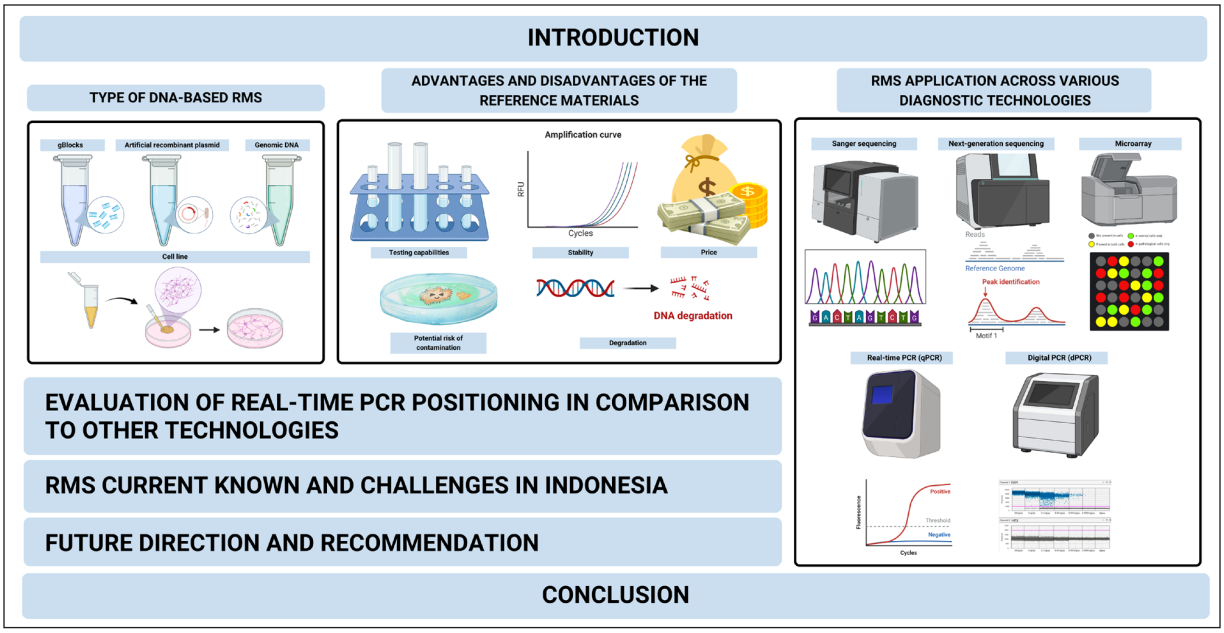

Figure 1. A comprehensive outline for the review article illustrates the various components involved in evaluating and applying reference materials (RMs) in DNA-based diagnostic testing (Created with Biorender.com).

complexity of biological samples, ensuring proper DNA extraction and amplification processes [30]. It plays a critical role in genetic testing for detecting genetic changes or assessing gene copy numbers, providing a precise comparison representing the entire human DNA complexity [31].

Cell lines

Cell lines, continuously proliferating cell cultures, offer consistent, uniform genetic material for PCR-based testing [17,32]. They can be specifically chosen or genetically altered to have relevant mutations, gene expressions, or chromosomal changes, making them ideal for validating genetic tests. Cell lines serve as relevant biological counterparts to target genes, allowing researchers to assess assay performance under conditions similar to actual patient samples, ensuring high-quality control and uniformity in routine testing [33,34].

The strategic implementation of RMs in PCR processes, including genetic material and cell lines, effectively ensures the legitimacy and reliability of test results [35]. Utilizing RMs such as gBlocks, artificial recombinant plasmids, genomic DNA, and cell lines helps laboratories identify and reduce procedural errors, optimize assay conditions, and maintain high test accuracy. The intelligent use of RMs is crucial in clinical diagnostics and genetic testing research and development, emphasizing the importance of accurate and reliable results [4,22].

ADVANTAGES AND DISADVANTAGES OF RMS

The selection of appropriate RM is crucial for ensuring the accuracy and reliability of DNA-based diagnostic testing. Different types of RMs, such as gBlocks, artificial recombinant plasmids, genomic DNA, and cell lines, each offer unique advantages and disadvantages that need to be carefully considered (Table 1). The primary goal of using RMs is to provide consistent benchmarks for testing procedures, thereby enhancing the validity and reproducibility of diagnostic results [4,22].

Four common types of RMs—gBlocks, artificial recombinant DNA, genomic DNA, and cell lines—each have distinct advantages and disadvantages. gBlocks are highly specific and adaptable, offering high stability and low contamination risk due to their synthetic nature, though they may not represent the entire genetic complexity of an organism and can degrade under certain conditions [23,24]. Recombinant plasmids provide high specificity and stability, particularly in controlled environments, but are susceptible to contamination and physical deterioration, with high costs associated with synthetic plasmids [26,36]. Genomic DNA offers a comprehensive genetic composition and high stability when properly managed, but it is prone to contamination and degradation, and its extraction and purification are costly [37,38]. Cell lines are effective for observing gene behavior and disease progression, yet they face challenges in maintaining stability and avoiding contamination, with high maintenance costs due to specialized requirements [39,40]. These considerations underscore the importance of selecting the appropriate RM based on the specific needs of the diagnostic application to ensure reliable and accurate results [4,22].

RMS APPLICATION ACROSS VARIOUS DIAGNOSTIC TECHNOLOGIES

Sanger sequencing

Sanger sequencing remains a fundamental method of DNA sequence analysis due to its high fidelity [37]. It is particularly effective for small-scale projects, such as

Table 1. Advantages and disadvantages of gBlocks, recombinant plasmid, genomic DNA, and cell line RMs.

Type of RM	Consideration	Parameter					References
		Testing capabilities	**Stability**	**Potential risk of contamination**	**Degradation**	**Price**	
gBlocks	Advantages	High specificity, adaptable to intended genetic sequences, highly dependable for precise genetic accuracy	High stability due to synthetic creation, repeated utilization without modification, detailed design and control	Lower probability of biological contamination due to synthetic nature	Exceptional stability when synthesized and stored appropriately	Often more cost-effective, especially for short sequences, lower initial costs, minimizes waste due to on-demand ordering	[1–5]
	Disadvantages	Limited in representing the genetic complexity of the organism's genome	Long-term stability issues need further research	Potential for cross-contamination with other synthetic oligonucleotides or environmental components	Vulnerable to physical deterioration from repeated freeze-thaw cycles or exposure to nucleases, and chemical stability issues under sub-par storage conditions	Can become costly for larger sequences or when multiple variants are necessary	[6–11]
Artificial recombinant plasmid	Advantages	High specificity, facilitates synthetic creation of standardized reference materials	Long-term stability, synthetic plasmids constructed without sequences causing instability, bacterial-amplified plasmids maintain stability within hosts	Lower contamination risk in synthetic plasmids due to controlled environments	Higher stability compared to linear DNA forms	Lower initial costs for bacterial-amplified plasmids, cost-effective for mass production	[12–17]
	Disadvantages	Lack of diversity and complete genomic context representation	Stability contingent on several factors, requiring careful management	Higher contamination risk in bacterial-amplified plasmids during construction, amplification, and storage	Susceptible to nuclease damage, mutations during replication, and physical deterioration from improper handling	High initial costs for synthetic plasmids, ongoing costs for maintaining bacterial cultures	[18–26]
Genomic DNA	Advantages	Provides comprehensive genetic composition, highly relevant to real patient samples, covers broader scope of genetic information	High stability when derived from biological materials	N/A	Genomic DNA does have the potential to degrade quickly if not handled and stored properly	Despite the high initial cost of extraction and purification, genomic DNA offers a long-term cost advantage due to the high quality and quantity of DNA obtained	[27–31]
	Disadvantages	Complexity and diversity across individuals, ambiguity in precision, requires accurate extraction and storage	Susceptibility to degradation during extraction, storage, and handling, dependent on proper management	High risk of contamination with other DNA species or environmental components, particularly in high-throughput environments	High risk of degradation from nuclease activity and improper storage or handling, additional fragmentation during extraction process	High cost of extraction and purification, especially from difficult or limited source materials	[2,32–35]
Cell line	Advantages	Models for dynamic behavior, effective for observing gene behavior, disease progression, and biomarker expression	Provide a consistent and controlled environment, which can lead to more reproducible results compared to primary cells.	When proper protocols are followed, cell lines can provide a reliable and consistent reference for studies, assuming that they are regularly authenticated and tested for contamination	Derived from primary cells, cell lines are modified to replicate indefinitely, ensuring consistent production over time and minimizing variability in degradation between batches.	Once established, they can be a renewable source of consistent and homogenous biological material, potentially reducing the need for sourcing new specimens which can be more costly and time-consuming.	[36–42]
	Disadvantages	Genetic and phenotypic drift over time, complexity in maintenance and quality control	Difficulty in maintaining stability, high variability due to cell culture conditions, genetic changes, or contamination	High risk of contamination with mycoplasma, viruses, or cross-contamination with other cell lines, requires extensive screening and stringent procedures	Difficulty in maintaining stability, high variability due to cell culture conditions, genetic changes, or contamination.	High cost of maintenance, requires specialized media and storage conditions, regular contamination testing increases costs	[43–51]

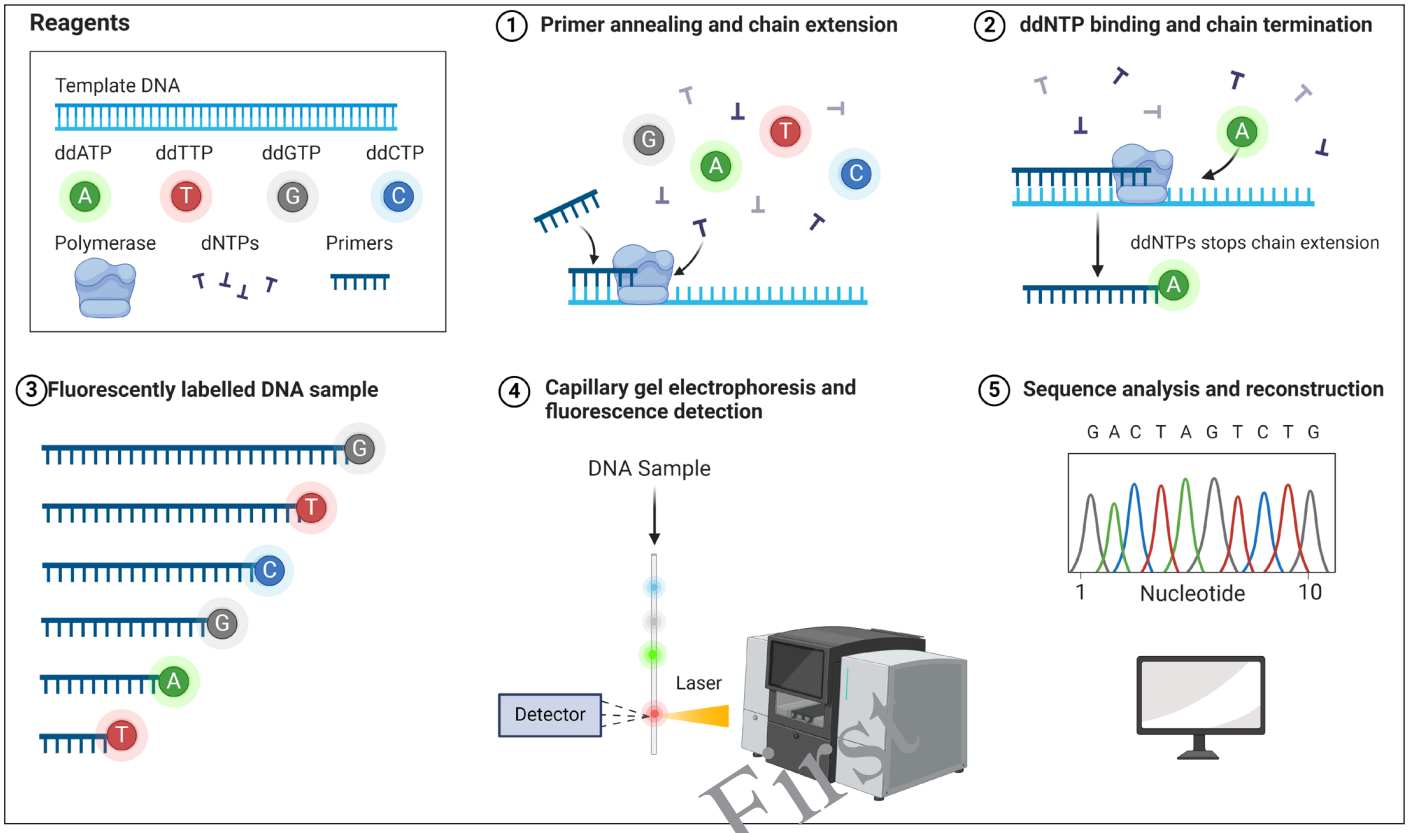

Figure 2. Sanger sequencing workflow. In Sanger sequencing, essential reagents such as ddATP, ddTTP, ddGTP, ddCTP, polymerase, dNTPs, and primers are used. Initially, primers are anneal to the DNA template, and polymerase is extended using dNTPs. Subsequently, ddNTPs are incorporated, causing chain termination. The DNA fragments produced are then labeled with specific fluorescent dyes. These fragments undergo separation by size via capillary gel electrophoresis, followed by fluorescence detection. Ultimately, the fluorescence data is analyzed to decode the DNA sequence [52].

identifying individual genes or regions of the genome involved in specific phenomena [41]. The principle of Sanger sequencing involves replicating a single DNA strand and incorporating fluorescently labeled nucleotides that terminate the growing chain (Fig. 2). These fragments are then separated by capillary electrophoresis, and the sequence is deduced from the labeled ends [42]. In practice, Sanger sequencing is used to validate results from other methods, confirm genetic variations like SNPs, and for forensic applications [43]. RMs, such as gBlocks or clones, ensure accuracy and repeatability by serving as benchmarks against which sequencing outputs are compared [44,45]. A notable case study demonstrated the use of Sanger sequencing in verifying gene mutations associated with various genetic disorders, underscoring its diagnostic precision [46].

Next-generation sequencing

NGS has revolutionized genetic analysis by enabling the simultaneous sequencing of multiple DNA strands [47]. This technology supports a variety of genomic analyses, including whole-genome sequencing, targeted re-sequencing, metagenomics, epigenomics, and transcriptome analysis [48]. NGS is characterized by its high throughput and cost-effectiveness, making it ideal for applications in personalized medicine, cancer genomics, and microbiome research [49]. The NGS workflow involves library creation, sequencing, image analysis, and data processing (Fig. 3).

RM are critical in NGS-based diagnosis because they rely on germline variant detection using high-throughput DNA sequencing for rare diseases. RMs such as standardized cell line genomes or synthetic DNA fragments ensure the quality and reliability of the data by serving as controls to validate the sequencing process and assess assay sensitivity and specificity [11]. For human whole genome sequencing, the NA12878 genome from the Coriell cell line GM12878 with a European ancestry background has been known as the sole leading human genome reference standard for various NGS applications. Despite the availability of Ashkenazi Jewish Asian and Han Chinese ancestry as genome references, as listed in the NIST-hosted Genome in a Bottle consortium, work continues to create reference genomes from various ancestries that cover all types of human genetic variation. The Genetic Testing Reference Materials Coordination Programme (GeT-RM) also provides various cell lines harboring specific mutations representing inherited diseases in ans. Additionally, for microbial reference standards, the US Food and Drug Administration (FDA) has released numerous microbial reference genomes for testing related to infectious microorganisms in the FDA-ARGOS database. Additionally, the Human Microbiome Project Consortium offers RMs for microbiome studies, assembling mock microbial communities from extracted gDNA samples.

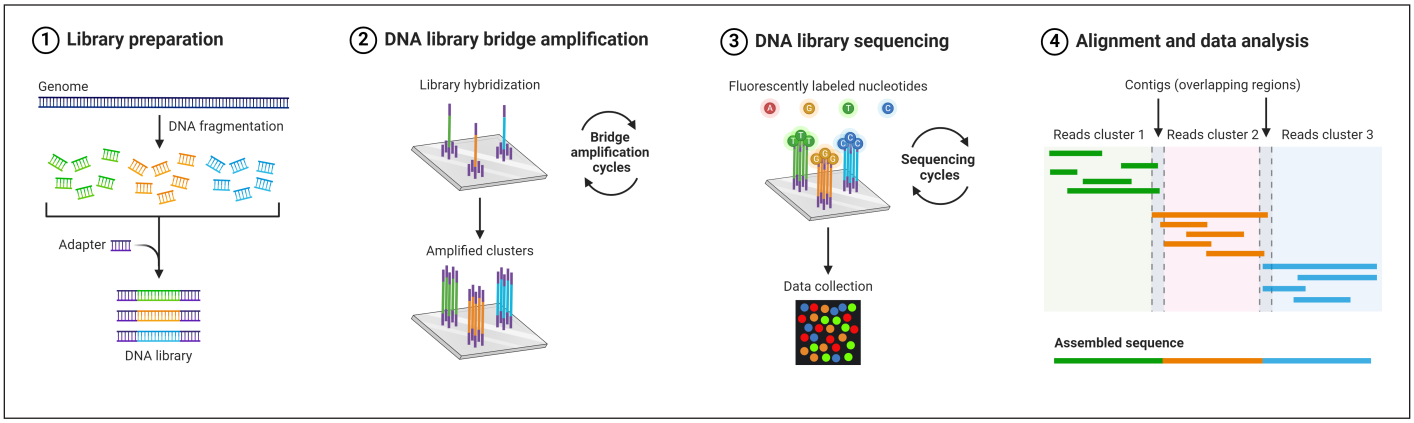

Figure 3. Next-generation sequencing workflow. The process begins with library preparation, involving DNA fragmentation, adapter attachment, and the creation of a DNA fragment library. These fragments are amplified on a flow cell during library bridge amplification, forming clusters of identical sequences. In the DNA library sequencing step, fluorescently labeled nucleotides are used to sequence these fragments, generating raw data. Finally, the data undergoes alignment and analysis to reconstruct the final sequence, including contigs and the assembled sequence [53].

Microarray

Microarray technology, also known as DNA chips, is a powerful tool for simultaneously analyzing multiple genes or DNA regions with high precision and efficiency [50]. Microarrays are solid supports, typically glass or silicon, onto densely packed DNA molecules. The principle of microarray technology, also known as DNA chips, involves the high-throughput analysis of gene expression or genetic variation. Microarrays consist of solid support, typically glass or silicon, onto which thousands of DNA probes are immobilized in a precise grid pattern. Each probe is designed to hybridize with a specific DNA or RNA sequence from the sample, allowing for the simultaneous analysis of many genes or genomic regions. During the assay, labeled nucleic acids from the sample are hybridized to the probes on the microarray. The hybridization signals are then detected and quantified using fluorescence or other detection methods, providing a comprehensive profile of gene expression or genetic variations (Fig. 4) [51]. Applications of microarrays include expression profiling, comparative genomic hybridization, and SNP detection [54].

RM like gBlocks and recombinant plasmids are used to calibrate microarrays, ensuring data accuracy and consistency across different arrays and experiments [53]. A case study on the use of microarrays for detecting chromosomal abnormalities in prenatal diagnostics illustrated the technology's utility in identifying genetic disorders early [56].

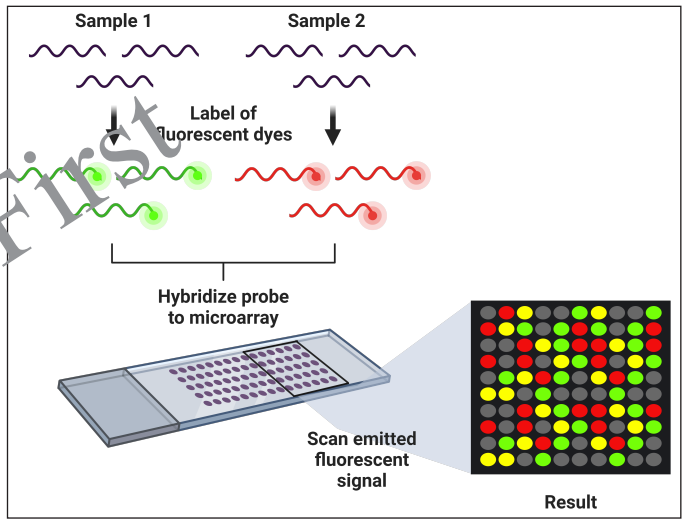

Figure 4. Microarray workflow. In microarray, samples are tagged with fluorescent dyes and matched with the microarray. Subsequently, the emitted fluorescent signals are scanned and examined to gauge gene expression levels. Different colors signify various outcomes: gray indicates absent genes, green represents genes expressed solely in normal cells, yellow denotes genes expressed in both normal and pathological cells, and red indicates genes expressed only in pathological cells. This robust technique facilitates the identification of genes implicated in disease processes and contributes to developing diagnostic and therapeutic approaches [58].

Polymerase chain reaction

Real-time PCR (qPCR) is widely used in molecular diagnostics to amplify and quantify specific DNA segments. This method employs fluorescent dyes or probes that emit fluorescence upon binding to DNA, with the fluorescence intensity being proportional to the amount of DNA amplified [57]. The principle of qPCR is based on the detection and quantification of a fluorescent signal that increases proportionally with the amount of DNA amplified during the PCR cycles. The process involves using fluorescent dyes, such as SYBR Green, which binds to double-stranded DNA, or specific fluorescently labeled probes, like TaqMan probes, which emit fluorescence upon hybridization with the target DNA sequence. As the PCR progresses, the fluorescent signal is measured in real-time at each cycle, allowing for the monitoring of DNA amplification throughout the reaction (Fig. 5).

RMs with known DNA or RNA concentrations are crucial for creating standard curves to measure unknown samples accurately [56]. qPCR is employed in various diagnostic tests, including the detection of SARS-CoV-2, malaria, and Chagas disease, and genetic variant detection crucial for personalized medicine [60–62]. A case study on

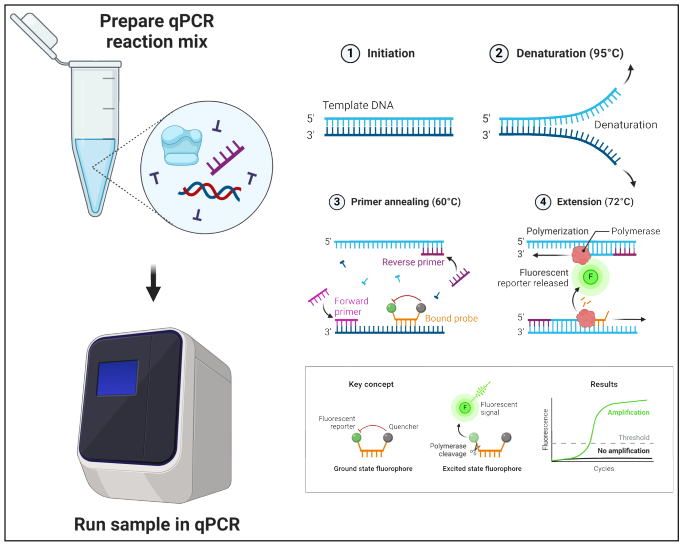

Figure 5. Real-time PCR workflow. Initially, a reaction mix is prepared with template DNA (sample), master mix (enzymes and buffers), forward and reverse primers, a fluorescent probe (reporter), and nuclease-free water (NFW). This mix is then loaded into a qPCR machine. The qPCR process involves cycles including initiation, denaturation (DNA strand separation at 90°C), annealing (primers binding to specific DNA sequences at 60°C), and extension (polymerase extending the primers and cleaving the probe at 72°C). The behavior of the fluorescent probe is crucial; in the ground state, reporter and quencher dyes emit no signal, while in the excited state, polymerase cleaves the probe, generating a fluorescent signal. The measured signal is shown as an amplification curve, where a signal below a threshold level indicates undetected target DNA (Created with Biorender.com).

qPCR's role in the rapid detection of SARS-CoV-2 during the COVID-19 pandemic highlighted its importance in managing public health emergencies [60].

On the other hand, dPCR offers exceptional precision and sensitivity in nucleic acid measurement by partitioning the DNA sample into numerous small-volume reactions, some containing the target molecule [63]. Each partition is then assessed for amplification, providing an absolute count of the target DNA without external references [64]. RMs ensure accuracy and reproducibility by validating the partitioning system and PCR efficiency [65]. dPCR is used in clinical applications such as analyzing tumor DNA, detecting drug resistance in viruses, early detection of infections, and monitoring transplant patients by measuring donor DNA levels [13,66,67]. A case study demonstrated the effectiveness of dPCR in detecting low-abundance tumor DNA, proving its value in early cancer diagnosis and monitoring [66]. Figure 6 illustrates the workflow of dPCR.

EVALUATION OF REAL-TIME PCR POSITIONING IN COMPARISON TO OTHER TECHNOLOGIES

PCR technology is a cornerstone of DNA-based genetic testing, enabling the amplification of specific gene sequences from minute DNA samples [68,69]. qPCR, an advancement of traditional PCR, is a routinely used tool in molecular diagnostic laboratories [70]. qPCR's adoption as a commonly found technology in diagnostic testing laboratories underlines its significance and thus provides a solid foundation for why this particular technology is the benchmark against which other methodologies are compared. qPCR is widely used due to its ability to quantify nucleic acids in real time, which is crucial for various applications, including disease diagnostics, genetic research, and pathogen detection [71]. This real-time quantification aspect offers a significant advantage over traditional PCR, enabling more detailed analyses of gene expression patterns, viral load determination, and genotyping [72]. Consequently, qPCR stands out as a highly sensitive, specific, and efficient diagnostic tool, making it a primary choice for comparing with other emerging genetic technologies [65].

PCR amplifies target DNA sequences through repeated cycles of temperature changes, which denature DNA, anneal primers, and extend new DNA strands [73]. This process results in an exponential increase in the target DNA region [68]. The advent of quantitative PCR (qPCR) introduced real-time DNA amplification and quantification using fluorescent dyes, enhancing genetic testing's complexity and capabilities [65]. The essential stages of DNA-based PCR diagnostic testing are illustrated in Figure 7, which details sample collection, DNA extraction, reaction mixture preparation, and PCR amplification.

The exceptional specificity, sensitivity, and quantitative capabilities of qPCR serve as the basis for molecular diagnostics. This efficacy comes from pre-designed, optimized primers and real-time fluorescence monitoring, which ensures excellent specificity and low background noise [68,74]. In addition to primer design, exact control over reaction parameters like temperature profiles and salt concentrations contributes to qPCR's specificity, which makes it possible to identify single base pair variances and clinically meaningful genetic alterations [75]. The method's superior sensitivity is achieved through efficient amplification and real-time fluorescence-based monitoring, providing accurate and reproducible quantitative data essential for applications like viral load measurement [72,76].

qPCR also offers operational simplicity, with master mixtures containing all necessary components, reducing the need for extensive optimization and minimizing potential errors. This setup simplicity, combined with user-friendly software for data interpretation, makes qPCR well-suited for rapid diagnostic environments [77,78]. This efficiency underscores qPCR's popularity for rapid and accurate diagnostics [79]. Most laboratories are equipped with qPCR technology, making it a common choice for routine molecular testing.

While qPCR is a powerful tool in diagnostic testing, it is not without competition. Table 2 compares the cost and scope of various diagnostic technologies, including Sanger sequencing, NGS, Micro Arrays, and dPCR. Each technology has unique benefits and limitations that make it suitable for specific applications. For example, Sanger sequencing excels in sequencing small DNA fragments but is costly, while NGS offers large-scale sequencing capabilities but requires substantial initial investment [48,77]. Microarrays allow simultaneous analysis of multiple genes but are expensive and complex [50]. dPCR provides high precision and sensitivity but is slightly more costly than qPCR [63,81].

Table 2. Cost comparison of diagnostic testing technologies.

Technology	Cost per reaction	Initial investment	Per-sample cost	Scope of analysis
Sanger sequencing	High	Substantial	Expensive	Relatively small DNA fragments (up to ~1000 bp)
Next-generation sequencing (NGS)	Very high	Complex	Expensive	Large-scale sequencing, including whole genomes, transcriptomes, or targeted panels
Microarrays	Very high	Intricate	Expensive	Analysis of multiple genes or regions simultaneously using specific probes
Real-time PCR (qPCR)	Low	Substantial	Minimal	Single gene targeted analysis
Digital PCR (dPCR)	Medium to high	Specialized	Moderate	Single gene targeted analysis with absolute quantification

The selection of molecular diagnostic technology depends on cost, accuracy, and scalability. Although qPCR offers significant advantages in certain areas, a comprehensive evaluation is essential to determine the most appropriate technology for specific laboratory needs [82,83].

RMS CURRENT KNOWN AND CHALLENGES IN INDONESIA

In the field of medical diagnostics, RMs play a crucial role in establishing standards and ensuring the legitimacy of tests for various diseases. RMs are essential for the validation of methods, the calibration of equipment, and the verification of diagnostic results [6,84]. Their diverse array of uses ensures consistency and reproducibility in diagnostic testing, which are fundamental to the accurate diagnosis and subsequent treatment of patients [5,10,84]. Studies employing RMs in the diagnosis of diseases have been conducted globally, covering a wide range of conditions, as summarized in Table 3.

This worldwide research promotes increased quality control in healthcare and facilitates the development of genuine and pertinent diagnostic methods. The utilization of RM's in this context is significant because of their importance in the progression of medical diagnosis and therapeutic strategies [10].

Multiple studies have concentrated on devising novel techniques to produce DNA RMs with certain attributes, which may be utilized as benchmarks for diverse purposes [85–88]. Seo *et al.* [82] reported a new bioprinting technique that can create RMs with a precise amount of target DNA, referred to as "cell number-based DNA reference material" [85]. This development offers a rigorous and replicable technique for producing DNA RMs with specified quantities, which is essential for the standardization and quality assurance of DNA analysis. In addition, the literature has emphasized the creation of RMs for certain DNA sequences or genetic markers [89–92]. In the research they conducted, [92] detailed the development of DNA molecules that functioned as model Standard RMs for the purpose of determining DNA sequences. Their findings highlighted the significance of well-characterized RMs in genetic testing. The mentioned references highlight the crucial importance of DNA RMs in upholding the quality and precision of genetic testing processes. In addition, DNA RMs have a wide range of uses, including identifying the mechanisms of antibiotic resistance in foodborne pathogens [87], creating genomic RMs for detecting meat adulteration [93], and constructing DNA hydrogels for biomedical applications [94]. Niu *et al.* [87] presented the creation of plasmid DNA RMs to identify antibiotic resistance mechanisms. They highlighted the usefulness of these materials as universal calibrators in qPCR analysis [87]. Furthermore, [93] emphasized the significance of quality control procedures in food analysis and the usefulness of DNA-based RMs in identifying adulterated meat [93]. NGS-based diagnosis heavily relies on RMs for identifying the presence of germline variants in rare diseases through the use of high-throughput DNA sequencing [95]. RMs, such as standardized cell line genomes or synthetic DNA fragments, are used to validate the sequencing procedure and evaluate the sensitivity and specificity of the assay. This ensures the data's quality and reliability [11]. The NA12878 genome from the Coriell cell line GM12878, which has a European ancestry background, is widely recognized as the primary reference standard for different NGS applications in human whole genome sequencing [95,96]. Although the NIST-hosted Genome in a Bottle consortium provides genome references for Ashkenazi Asian and Han Chinese ancestry, efforts are still ongoing to develop reference genomes from diverse ancestries that encompass all forms of human genetic variation [96]. The Genetic Testing Reference Materials Coordination Programme (GeT-RM) offers a range of cell lines that contain specific mutations associated with inherited disorders in humans [97]. In addition, the US Food and Drug Administration (FDA) has made available a large number of microbial reference genomes in the FDA-ARGOS database for the purpose of testing infectious microorganisms [95]. In addition, the Human Microbiome Project Consortium provides RMs for microbiome studies by creating artificial microbial communities using extracted gDNA samples [95]. Organizations such as the Reference Materials and Measurements of the European Commission (IRMM-EU) and the American Oil Chemists' Society (AOCS) are the main producers of commercially accessible certified RMs for matrix-based and genomic DNA. This information is supported by Li *et al.* [98]. These well-established organizations play a crucial role in standardizing and ensuring the quality of genetic testing methods by providing highly defined RMs. Collaborative ring trials have been carried out to evaluate the suitability of reference plasmid DNA calibrants in quantitative analyses of genetically modified (GM) contents. These trials

Table 3. Current RMs used in diagnostic testing.

Type of RM	Testing context (Disease)	Type of sample	Country conducting test	Reference
gBlocks	COVID-19	Nasopharyngeal or oropharyngeal swabs or sputum	South Korea	[54]
	COVID-19	Nasal swab	Canada	[55]
	COVID-19	Nasopharyngeal and nasal swabs	United States and Italy	[56]
	Monkeypox virus (MPV)	Eluat swab	United States	[57]
	Renal cell carcinoma (RCC)	Plasma cell-free DNA (cfDNA) from tumor tissue	Japan	[59]
	Non-small cell lung cancer (NSCLC)	Lung resection specimens from adenocarcinoma and sarcomatoid carcinoma	Ireland	[60]
	Non-small cell lung carcinoma (NSCLC)	Whole-blood samples	United States	[61]
	Breast cancer with PIK3CA and ESR1 mutations	Plasma	England	[6]
	Ebola virus disease	Plasma	West and Central Africa	[62]
	Tuberculosis (TB)	Sputum	Vietnam, Peru, and South Africa	[63]
	Tuberculosis (TB) and nontuberculous mycobacteria (NTM)	Sputum	Mali, West Africa	[64]
Artificial recombinant plasmid	Leptospirosis	Serum	India	[65]
	COVID-19	Nasopharyngeal swabs	Russia	[66]
	Malaria	Blood	China	[67]
	Melioidosis	Serum	Thailand	[68]
	Gastric cancer	Serum	China	[69]
	Hepatitis C virus (HCV) and influenza virus	Plasma	China	[70]
	Hepatitis D Virus (HDV)	Blood	Brazil	[71]
	Hepatitis E virus (HEV)	Serum or plasma	United States	[72]
	T cell acute lymphoblastic leukemia (T-ALL) and B-cell acute lymphoblastic leukemia (B-ALL)	Bone marrow	China	[73]
	Lassa virus (LASV)	Serum, blood, and tissue	Guinea, West Africa	[74]
Genomic DNA	Herpes simplex virus (HSV)	Cervicovaginal specimens from gynecological practices	Iran	[75]
	Malaria	Dried spots of blood obtained by finger prick	Thailand and India	[76]
	Malaria	Giemsa-stained thick blood smears and dried filter paper blood spots (DBS)	Ghana	[77]
	Nasopharyngeal carcinoma (NPC)	Plasma and nasopharyngeal epithelial cells obtained	Hongkong and Toronto	[78]
	Tuberculosis	Sputum	England	[79]
	Tuberculosis	Sputum	Peru	[80]
	Extrapulmonary tuberculosis (EPTB)	Pleural fluids and urine	India	[81]
	Lung cancer	Fresh-frozen, blood, and paraffin-embedded biopsies	Latin America (Argentina, Colombia, Brazil, Mexico, Peru)	[82]
	Hereditary colorectal cancer	Blood	Japan	[83]
	Non-small cell lung cancer (NSCLC)	Plasma	China	[84]
Cell line	COVID-19	Nasopharyngeal swabs, sputum, urine, feces, and serum samples	Australia	[85]
	COVID-19	Nasal/nasopharyngeal swab or saliva	Japan	[86]
	Non-small cell lung cancer (NSCLC)	Blood	China	[84]
	Respiratory virus infections	Nasopharyngeal swab or aspiration	Taiwan	[87]
	Human T-lymphotropic virus type 1 (HTLV-1)	Paraffin biopsy and/or blood sample	Germany	[88]
	Non-small cell lung cancer (NSCLC)	Lung biopsy, either tissue-based or cytology	Australia	[89]
	Epithelial ovarian cancer (EOC)	Serum and tissue	China	[90]
	Breast cancer	Blood	United States	[91]
	Lung cancer	Sputum	China	[92]
	Colorectal cancer	Serum	China	[93]

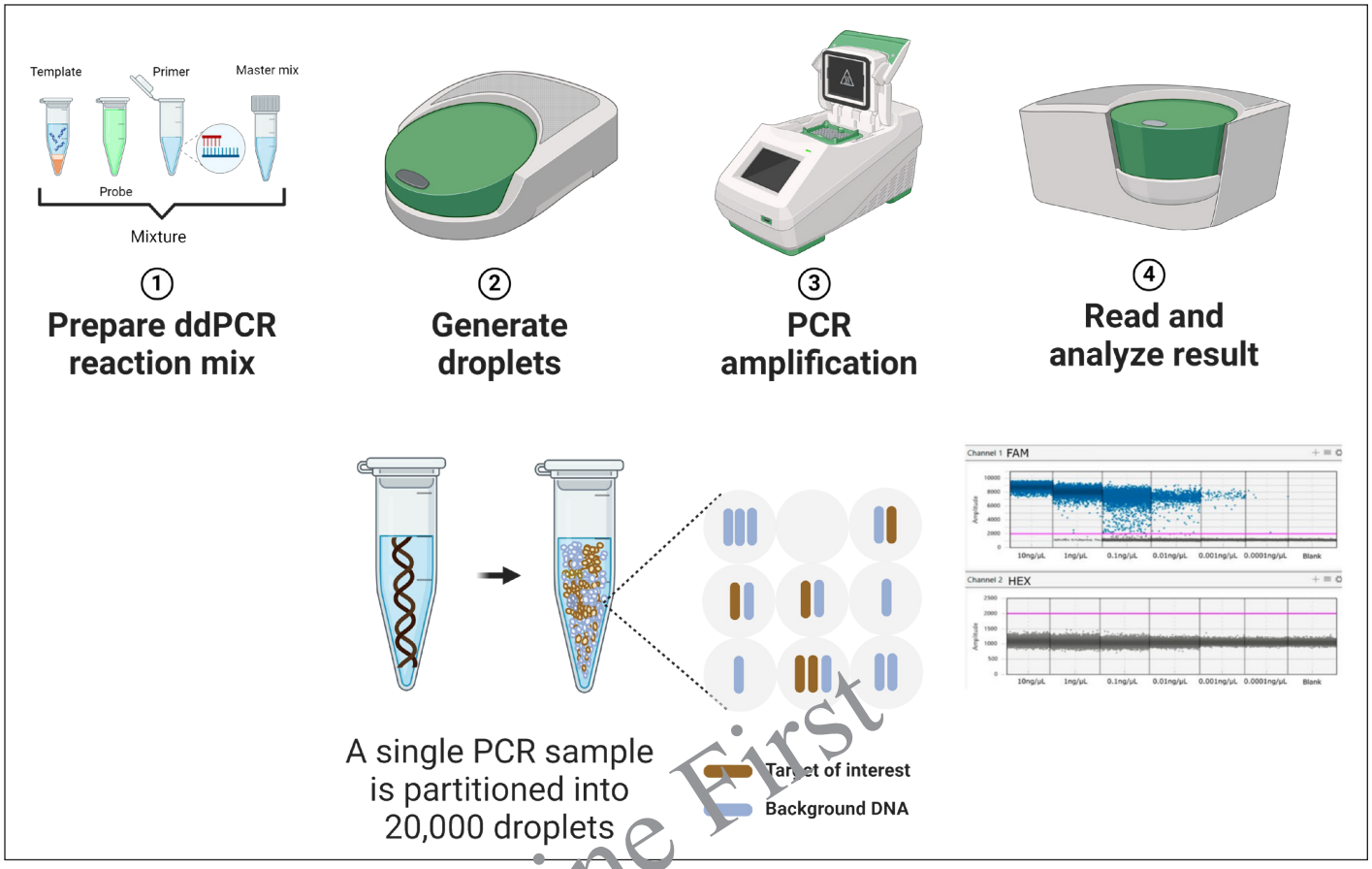

Figure 6. Digital PCR workflow. This diagram outlines the key steps in a dPCR workflow, allowing for precise quantification of target molecules. Initially, a reaction mixture is prepared with all necessary components for PCR amplification. Following this, the sample is carefully divided into numerous nanoliter-sized droplets using specialized equipment, ensuring each droplet encapsulates individual target molecules. Subsequent to droplet generation, the PCR cycling process occurs, amplifying target DNA within each droplet alongside any background DNA present. After amplification, droplets undergo analysis to differentiate positive droplets containing amplified targets from negative ones lacking. This analysis facilitates the accurate determination of the absolute abundance of target molecules in the original sample. dPCR offers researchers a robust tool with heightened sensitivity and the capability for absolute quantification, eliminating the need for standard curves (Created with Biorender.com).

emphasize the significance of certified reference materials (CRMs) in guaranteeing the precision and dependability of GM quantification methods [99]. In addition, the meticulous processes involved in certifying DNA reference RM materials to meet stringent quality standards are demonstrated through the development and characterization of quantitative RMs for specific applications, such as Legionella detection and quantification by qPCR [83].

The development of RMs has challenges, particularly in achieving interlaboratory repeatability. This issue has been emphasized in research that specifically investigates the use of new DNA RM formats in droplet digital PCR [100]. Ensuring the ability to replicate results in other laboratories is essential for the widespread acceptance of DNA RMs in diverse scientific fields. In Indonesia, the progress in developing DNA-based RMs is still in its early stages compared to international benchmarks. Although there is agreement with worldwide patterns in DNA nanotechnology and biomaterials research [101,102], there are still notable obstacles to overcome. These factors comprise restricted infrastructure, unavailability of new technological equipment, and inadequate money for comprehensive research and development projects.

To put it simply, the current situation in Indonesia regarding the establishment of DNA-based RMs encompasses the process of developing, characterizing, and certifying RMs for different purposes, such as genetic testing, and clinical diagnostics. The challenges involved in developing RMs encompass the need to guarantee consistent results across different laboratories. To address these difficulties, a significant investment in infrastructure, technology, and human resources is needed.

FUTURE DIRECTION AND RECOMMENDATION

Future research should focus on developing and standardizing RMs to improve the quality and reliability of DNA-based diagnostics. This includes the establishment of a national coordination body in Indonesia to oversee the production, validation, and distribution of RMs. Collaboration between research institutions, standardization bodies, and regulatory agencies is essential to create a robust framework for RM utilization. These efforts should prioritize the creation of standardized reference controls that align with international standards, ensuring consistent and reproducible diagnostic results [103]. By incorporating locally relevant pathogen strains

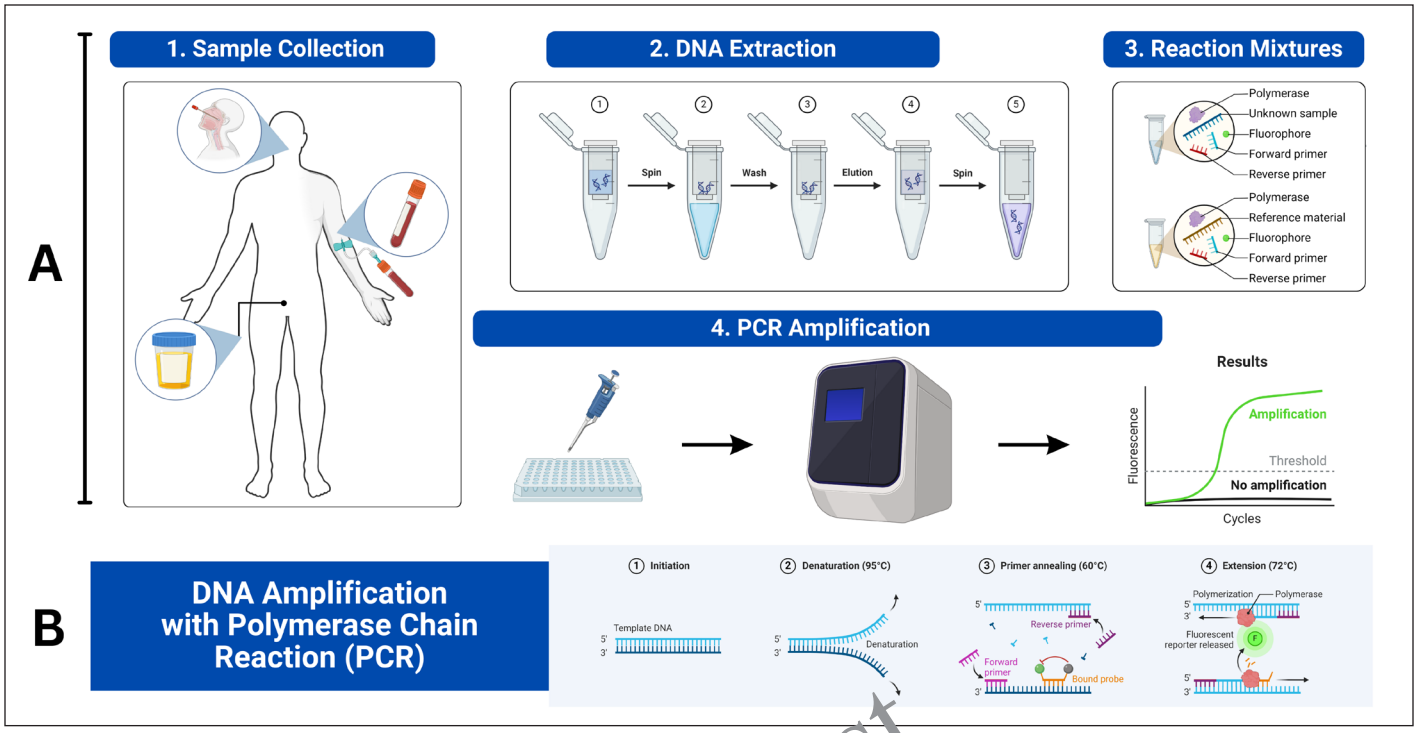

Figure 7. A) The essential stages of DNA-based PCR diagnostic testing. Firstly, sample collection involves gathering biological material like blood or urine from the patient. Next, DNA extraction isolates DNA from other cellular components. Then, reaction mixture preparation mixes the extracted DNA with primers, DNA polymerase, and a fluorophore, divided into tubes for testing (unknown samples) and controls (reference material). Lastly, PCR amplification uses a PCR machine to amplify the DNA. B) The DNA amplification process through PCR. It begins with initiation, where double-stranded DNA is denatured into single strands. Denaturation heats the mixture to separate the DNA. Annealing follows, lowering the temperature for primers to bind to the target DNA. Extension occurs as DNA polymerase adds nucleotides, amplifying the target DNA exponentially (Created with Biorender.com).

and epidemiological data, the selected RMs will enhance the accuracy and validity of diagnostic tests within the Indonesian context [104].

The selection and implementation of RMs must balance cost, capability, stability, and contamination risks. Laboratories with limited budgets can utilize cost-effective synthetic targets like gBlocks for high-volume applications, while more complex diagnostics may require genomic DNA or cell lines despite their higher costs. Continuous proficiency testing and external quality assessment are vital to validate laboratory performance against common RMs, identifying areas for improvement and ensuring high diagnostic standards [104]. Investment in infrastructure, personnel training, and policy enforcement is critical for maintaining high-quality diagnostic testing. Robust infrastructure supports effective testing, while trained personnel ensure reliable results, and policy enforcement promotes a culture of excellence [97].

Innovation and adaptability are crucial in the evolving field of diagnostic testing. Embracing new technologies and staying updated with advancements will keep Indonesia at the forefront of global diagnostic standards. This commitment to innovation will improve diagnostic accuracy, enhance patient care, and strengthen public health. By focusing on continuous improvement and aligning with international best practices, Indonesia can ensure its diagnostic testing capabilities remain advanced and effective, ultimately benefiting patient outcomes and the healthcare system as a whole.

CONCLUSION

The comprehensive review highlights the pivotal role of RMs in ensuring the accuracy and reliability of DNA-based diagnostic testing. This study identifies their unique characteristics, advantages, and limitations by examining various types of RMs—gBlocks, recombinant plasmids, genomic DNA, and cell lines. gBlocks, for example, offer high specificity and stability but may lack genomic complexity. Recombinant plasmids are flexible and stable but costly and prone to contamination. Genomic DNA provides a comprehensive genetic composition and high stability, yet it is susceptible to degradation and high contamination risk. While effective for observing gene behavior and disease progression, cell lines face challenges in maintaining stability and avoiding contamination, with high maintenance costs. The strategic selection of RMs, considering specificity, complexity, stability, and cost factors, is essential for optimizing diagnostic testing. The establishment of a national coordination body in Indonesia to standardize RM utilization and collaboration with international organizations will enhance the quality and availability of RMs, ensuring diagnostic accuracy and reliability that meets international standards.

LIST OF ABBREVIATIONS

dPCR: digital PCR; DNA: deoxyribonucleic acid; ECM: external control material; NGS: next-generation

sequencing; PCR: polymerase chain reaction; RM: reference material; qPCR: real-time PCR/quantitative PCR.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

DATA ACCESS AND RETENTION

All data analyzed during this study is included in this published article in the references section.

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