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Review Paper

Advancements in Real-Time PCR Technologies: A Comprehensive Review of Probe-Based and Non-Probe-Based Assays for Molecular Diagnostics

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Abstract
Real-Time PCR (qPCR) has become a foundational tool in molecular diagnostics, enabling accurate DNA quantification and detection. The decision between probe-based and non-probe-based qPCR assays is crucial, influenced by diagnostic goals and sample characteristics. This review provides an in-depth evaluation of these two assay types, analyzing their principles, strengths, drawbacks, and applications. A thorough review of the literature, primarily sourced from PubMed, was undertaken to explore prominent assay systems, including TaqMan, KASP, rhAmp, HRM, and SYBR Green. Probe-based qPCR assays, exemplified by TaqMan and rhAmp, are distinguished by their high specificity, aptitude for multiplex analysis, and reduced risk of false positives, making them highly suitable for SNP genotyping and pathogen detection. However, their elevated costs and intricate design requirements remain significant challenges. Conversely, non-probe-based assays, such as SYBR Green and HRM, present cost-effective alternatives with straightforward designs. HRM, in particular, is effective in identifying genetic variations like SNPs with remarkable sensitivity. Nonetheless, these methods are susceptible to non-specific amplifications, requiring careful optimization to maintain reliability. The selection of a suitable qPCR assay depends on various factors, including precision, affordability, and multiplexing capabilities, with applications spanning infectious disease detection and genetic disorder analysis. This review emphasizes the indispensable role of qPCR in molecular diagnostics while showcasing recent technological advances that aim to mitigate existing constraints and enhance diagnostic precision and accessibility.

Keywords
Real-Time PCR, TaqMan, KASP, rhAmp, HRM, Molecular Diagnostics

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1. INTRODUCTION

Molecular diagnostics has redefined the detection and management of diseases by enabling precise identification of genetic mutations and biomarkers. Despite its potential to enhance early diagnosis and personalized treatment, its implementation remains constrained in numerous regions. For instance, in Indonesia, financial limitations, insufficient infrastructure, and a lack of skilled personnel create significant obstacles to integrating molecular diagnostics into healthcare systems (Dwivedi et al., 2017; Macklin et al., 2019). As a field, molecular diagnostics leverages molecular biology techniques to examine biological markers in the genome and proteome, offering essential insights into disease mechanisms and supporting data-driven clinical decisions (Nazha et al., 2015; Schmidt et al., 2016; Sokolenko and Imyanitov, 2018).

In addition to detecting infectious agents and genetic alterations, molecular diagnostics plays a vital role in unraveling the complexities of diseases such as cancer by clarifying critical pathogenetic processes. By accurately assessing biomarkers, healthcare professionals can develop individualized therapeutic strategies and forecast disease progression, resulting in improved patient care outcomes (Fanshawe and Vazquez-Montes, 2021; Rao et al., 2018; Vetter et al., 2018). Reliable diagnostic and prognostic tools are indispensable in advancing precision medicine, enabling clinicians to implement timely and targeted interventions (Lim, 2020; Moons et al., 2015; Thomas and Fried, 2018).

Beyond healthcare, molecular diagnostics has made significant inroads into agriculture, where it enhances crop quality, boosts food security, and promotes higher yields. By analyzing plant genomes, this technology identifies desirable characteristics such as resistance to diseases and drought, which contribute to the development of robust crop varieties. Furthermore, molecular diagnostics underpins sustainable agricultural practices by reducing dependency on chemical treatments and fostering innovative solutions to global food challenges (Azizi et al., 2022; Edwards et al., 2018; Fenibo et al., 2022; Hariharan and Prasannath, 2021; Paudel et al., 2020; Travella et al., 2019).

Central to molecular diagnostics is the Polymerase Chain Reaction based (PCR), a widely used method for amplifying DNA sequences with remarkable sensitivity and specificity. This process involves three key stages—denaturation, annealing, and elongation—that enable the exponential replication of target DNA fragments (Green et al., 2015; Riet et al., 2017; Sridhar et al., 2019). PCR has proven indispensable in identifying pathogens, diagnosing genetic conditions, halal authentication and monitoring gene expression (Makrigiorgos, 2019; Müller et al., 2021; Gina et al., 2024).

Real-Time PCR (qPCR) represents a substantial advancement in this field, as it permits real-time tracking of DNA amplification using fluorescence-based detection methods (Hwang et al., 2019; Koo et al., 2022; Shi et al., 2020). By providing semi-quantitative data, qPCR offers enhanced precision, although its reliability is dependent on the incorporation of external controls during analysis (Baebler et al., 2017; Debski et al., 2017). These assays are broadly categorized into probe-based and non-probe-based technologies. Non-probe-based assays, such as SYBR Green, employ intercalating dyes that bind to double-stranded DNA during amplification. A variation of this approach, High-Resolution Melting (HRM), excels in identifying genetic variations like Single Nucleotide Polymorphisms (SNPs) without requiring sequence-specific probes (Kralik and Ricchi, 2017; Słomka et al., 2017). Meanwhile, probe-based technologies, including TaqMan and Kompetitive Allele Specific PCR (KASP), enhance specificity through the use of sequence-specific probes, making them suitable for SNP genotyping and pathogen detection.

This review critically evaluates probe-based and non-probe-based qPCR assays, examining their principles, strengths, weaknesses, and diagnostic utility. By synthesizing recent developments, it aims to provide an in-depth understanding of these methodologies and their potential to address existing challenges in molecular diagnostics.

2. METHODS

This study adopted a literature review approach, using PubMed as the primary database for sourcing information. A focused search was conducted with specific keywords, including "HRM," "TaqMan," "KASP," "rhAmp," and "molecular diagnostic," to ensure a thorough and targeted examination of relevant studies. The inclusion criteria were limited to primary research articles published within the past decade (2015–2025) that directly addressed these topics. To provide additional context and continuity, earlier publications (beyond ten years) were selectively included through a trace-back method. This involved tracing citations from recent studies to incorporate foundational research where necessary. The review emphasized primary research articles for their direct insights into advancements in molecular diagnostic technologies, particularly in qPCR assays. Secondary sources, such as review and systematic review articles, were excluded to maintain a sharp focus on original empirical data. By employing a stringent selection process, the study ensured the integration of accurate, current, and contextually relevant information regarding the principles, applications, and challenges of probe-based and non-probe-based qPCR assays. This methodological approach offers a solid and dependable basis for evaluating technological progress in Real-Time PCR diagnostics.

3. RESULTS AND DISCUSSION

3.1 Fundamental Principles of Real-Time PCR

qPCR builds upon conventional PCR by introducing the ability to quantify DNA amplification in real time. Unlike standard PCR, which relies on post-amplification detection, qPCR utilizes fluorescent dyes or probes that emit signals during DNA synthesis. The fluorescence intensity is measured at each amplification cycle, providing quantitative information about the DNA concentration in the sample (Singh and Roy-Chowdhuri, 2016; Zhang et al., 2021). This capability enhances both the accuracy and sensitivity of qPCR, making it a critical tool in molecular diagnostics (Zheng et al., 2019).

The versatility of qPCR is evident in its extensive applications, which include analyzing gene expression, detecting pathogens, and studying genetic variations. By continuously monitoring fluorescence throughout the amplification process, qPCR enables real-time tracking of DNA synthesis, generating precise quantitative data essential for these purposes (Moniri et al., 2019; Moreau et al., 2017). While qPCR shares fundamental components with conventional PCR-such as template DNA, primers, DNA polymerase, dNTPs, buffer, and magnesium ions (Mg^{2+}) it incorporates fluorescent dyes or probes alongside a detection system to measure fluorescence, providing enhanced precision and functionality (Butz and Patócs, 2019).

The qPCR workflow follows a sequential process, starting with sample collection, followed by DNA extraction to obtain purified genetic material. The reaction mixture is then assembled by combining the extracted DNA with primers, polymerase, and fluorophores, ensuring both test samples and controls are included. PCR amplification is performed in a thermal cycler, where repeated cycles of denaturation, annealing, and extension facilitate DNA synthesis. Real-time fluorescence detection tracks amplification in progress, enabling accurate identification of target sequences (Figure 1).

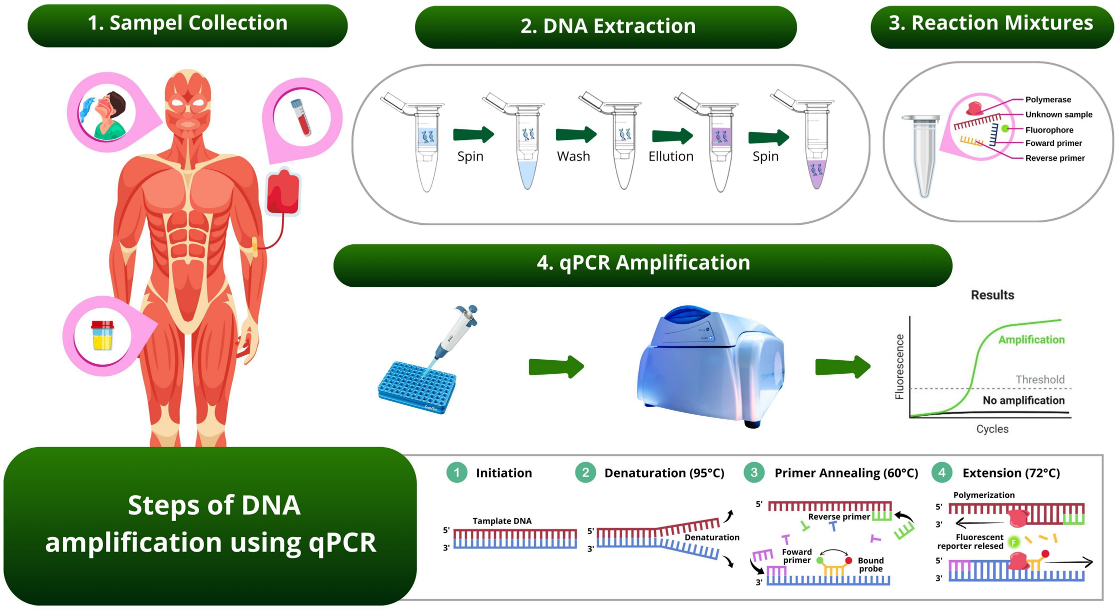

Figure 1. The Key Stages of qPCR-Based Molecular Diagnostics

The process begins with sample collection, followed by DNA extraction to isolate genetic material. The reaction mixture is then prepared by combining extracted DNA with primers, polymerase, and fluorophores, ensuring both test samples and controls are included. PCR amplification is performed in a thermal cycler, enabling DNA replication through cycles of denaturation, annealing, and extension. Real-time fluorescence detection indicates successful amplification, distinguishing positive from negative samples (Created with `Biorender.com`)

3.2 Probe-Based Real-Time PCR Assays

Probe-based assays in qPCR utilize specialized probes designed to hybridize with target DNA sequences during the annealing phase of the PCR cycle (Curk et al., 2020; Pelt-Verkuil et al., 2019). Each probe is equipped with a fluorescent reporter dye at one end and a quencher molecule at the opposite end (Hien et al., 2020). When the probe binds to its target, the quencher suppresses fluorescence by remaining in close proximity to the reporter dye (Shahmuradyan and Krull, 2016). As DNA polymerase extends the primers during amplification, its exonuclease activity degrades the probe, separating the reporter from the quencher, thereby generating a detectable fluorescence signal. This real-time increase in fluorescence is measured by the qPCR instrument, allowing for precise quantification of DNA amplification (Luthra et al., 1998; Nagy et al., 2017). Figure 2B provides a detailed mechanism probe-based qPCR assays.

One of the most commonly used probe-based assays is the TaqMan assay, which features a reporter dye at the 5' end and a quencher molecule at the 3' end of the probe (Nagy et al., 2017). During the amplification process, the Taq polymerase cleaves the probe, releasing the reporter dye from the quencher and producing a fluorescence signal. This mechanism ensures high specificity, as fluorescence occurs only when the probe binds correctly to its target sequence, thereby reducing false-positive results (Barnes et al., 2021). TaqMan assays are particularly advantageous for multiplexing, as they can utilize distinct fluorophores to target multiple DNA sequences within a single reaction, facilitating the simultaneous analysis of multiple genes or loci (Botezatu et al., 2017). Despite these advantages, TaqMan assays are relatively expensive due to the need for sequence-specific probe design, which requires rigorous optimization, particularly for multiplex applications (Alblalaihid et al., 2016; Jani et al., 2016).

Molecular beacons represent another type of probe-based assay and are characterized by their hairpin structure, which contains a fluorescent reporter dye at one end and a quencher at the other. In solution, the hairpin configuration keeps the reporter and quencher in close proximity, effectively suppressing fluorescence. When the beacon binds to its target DNA sequence, the hairpin structure unfolds, separating the quencher from the reporter and enabling fluorescence emission (Dempsey et al., 2018). This design offers high specificity, as fluorescence is only generated when the probe binds perfectly to its target sequence, minimizing non-specific signals (Del Bonis-O'Donnell et al., 2016; Roth and Seitz, 2021). Molecular beacons can also be labeled with multiple fluorophores, enabling multiplex detection within a single reaction tube, which is especially useful for complex diagnostic assays (Chandrasekaran et al., 2021). However, the cost of designing and synthesizing molecular beacons is a limitation, as the hairpin structure requires careful

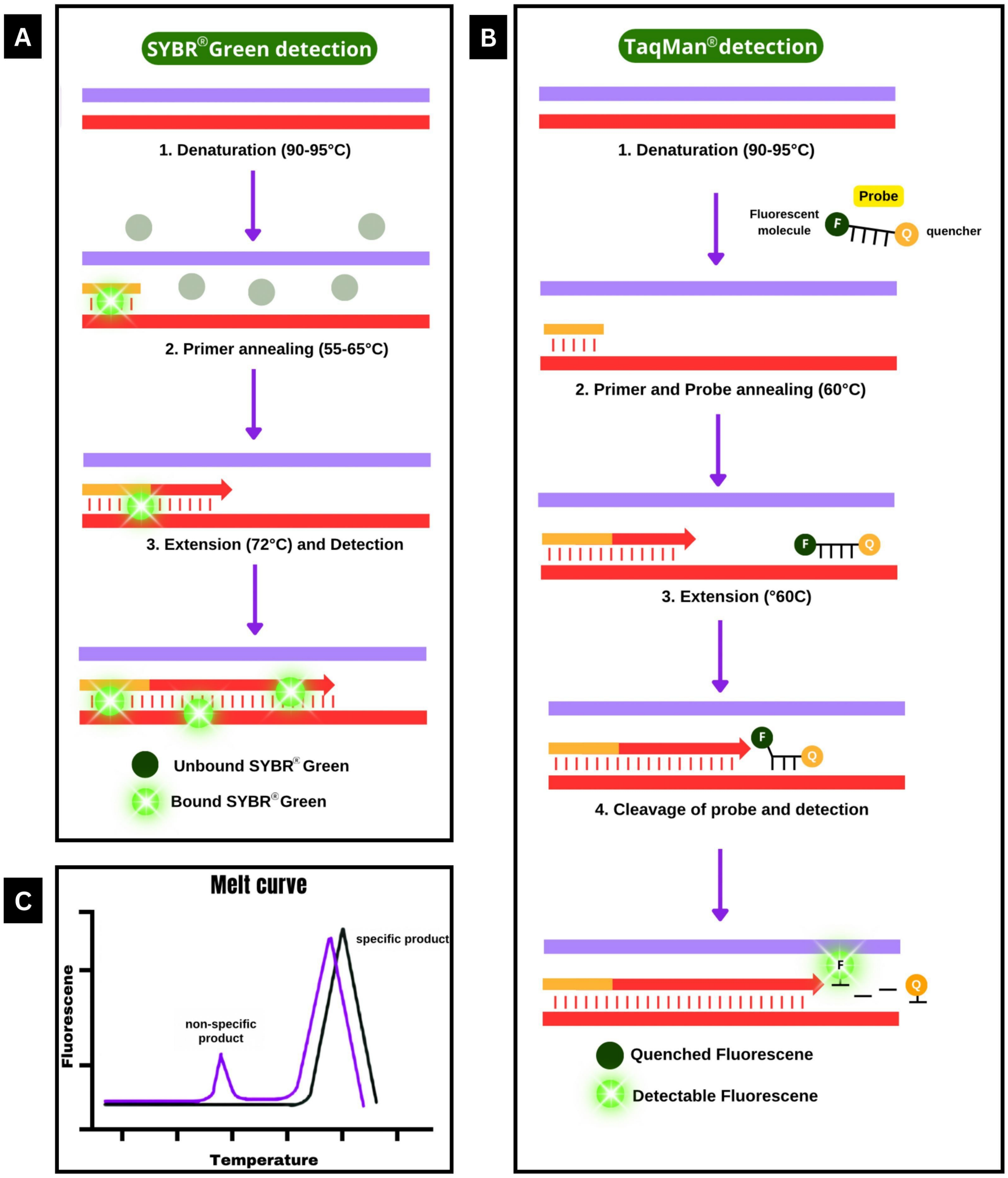

Figure 2. Illustrates the Principles of Different qPCR Detection Methods: (A) SYBR® Green (Non-Probe-Based), (B) Taqman® (Probe-Based), and (C) Melt Curve Analysis For Evaluating Primer Specificity

In SYBR® Green detection (A), fluorescence is emitted when the dye intercalates into double-stranded DNA (dsDNA) during PCR amplification. However, because SYBR Green binds indiscriminately to all dsDNA, including non-specific products like primer dimers, careful primer design and post-PCR melt curve analysis are necessary to ensure specificity. In TaqMan® detection (B), a sequence-specific probe containing a fluorophore and quencher hybridizes to the target DNA. During primer extension, DNA polymerase cleaves the probe, separating the fluorophore from the quencher and generating a fluorescence signal. This method provides higher specificity than SYBR Green, as fluorescence is only emitted upon probe degradation. Melt curve analysis (C) evaluates amplicon specificity by monitoring fluorescence changes as dsDNA dissociates with increasing temperature. A single peak (black line) represents a specific product, whereas multiple peaks (purple lines) indicate non-specific amplification or primer-dimer formation. This analysis is particularly critical in SYBR Green assays to confirm target specificity and improve data accuracy Adopted from (Adams, 2020) engineering to ensure stability and functionality (Guberman et al., 2022).

KASP is another widely used probe-based assay, developed by LGC Genomics, and is designed to detect genetic variations such as SNPs and insertions/deletions (InDels) (Dipta et al., 2024). This method employs allele-specific forward primers and a common reverse primer, with the forward primers containing unique tail sequences complementary to FRET cassettes. KASP is particularly valued for its cost-effectiveness, as it eliminates the need for labeled probes or primers, making it an economical choice in large-scale genotyping projects, such as plant breeding programs (Dipta et al., 2024). While KASP supports customization and multiplexing, it is most efficient for analyzing a limited number of SNPs (<100 SNPs) and is generally restricted to samples from diploid or haploid genomes (Dipta et al., 2024).

Similarly, rhAmp is a probe-based technology developed by IDT that integrates a thermostable RNase H2 enzyme and a mutant Taq DNA polymerase to enhance allelic discrimination (Beltz et al., 2018). The primers used in rhAmp assays contain a single RNA base and are 3'-end blocked, with allele-specific primers including tails complementary to universal probe-based reporter sequences. The RNase H2 enzyme activates the primers by removing the 3'-end block when they form a perfectly matched heteroduplex with the DNA target, enabling allele-specific PCR (Beltz et al., 2018). A significant advantage of rhAmp is its ability to work efficiently with low DNA input, making it ideal for applications with limited sample availability (Ayalew et al., 2019; Beltz et al., 2018). However, similar to KASP, rhAmp requires meticulous optimization of probe design, which may increase costs. Figure 3 provides an overview of the mechanisms underlying various probe-based qPCR assays, including TaqMan, Molecular Beacons, KASP, and rhAmp technologies. These assays employ sequence-specific probes to improve detection precision by reducing non-specific amplification. Each method utilizes a distinct probe design and fluorescence activation strategy, resulting in differences in sensitivity, specificity, and overall cost-efficiency.

3.3 Non-Probe-Based Real-Time PCR Assays

Non-probe-based qPCR assays differ fundamentally from prob e-based methods by employing intercalating dyes, such as SYBR Green (Figure 2A), which bind to double-stranded DNA and emit fluorescence upon intercalation (Bhatia et al., 2015; Debnath et al., 2025). The workflow for these assays follows the standard DNA amplification steps of denaturation, annealing, and extension, after which fluorescence is detected and monitored in real time (Saarnio et al., 2021). SYBR Green-based assays are widely recognized for their cost-effectiveness, making them particularly advantageous in resource-constrained settings (Pereira-Gomez et al., 2021; Traore et al., 2015). However, a key drawback of SYBR Green is its non-specific binding, as it intercalates with all double-stranded DNA, including unintended products such as primer-dimers. This non-specific fluorescence can lead to false-positive results due to the lack of sequence-specific probes (Peyrard, 2015; Schreier et al., 2019).

HRM analysis is another non-probe-based technique that builds on the use of intercalating dyes, such as Cyto9 and Evagreen (Figure 4), offering greater specificity than SYBR Green (Martini et al., 2015; Słomka et al., 2017). HRM involves a post-PCR analysis of DNA melting curves to differentiate DNA sequences based on their melting behavior. This method is highly sensitive and specific, enabling the detection of mutations, polymorphisms, and epigenetic changes. HRM is capable of distinguishing sequences with single nucleotide differences, providing detailed insights into genetic variations (Qiao et al., 2020; Słomka et al., 2017; Wang et al., 2016). Another notable advantage is its cost-efficiency, as HRM relies on standard qPCR reagents and intercalating dyes, eliminating the need for costly sequence-specific probes (Słomka et al., 2017).

Despite its strengths, HRM has certain limitations. It is less effective in identifying subtle genetic variations and may produce ambiguous results when analyzing samples containing closely related variants. In addition, poorly designed primers can cause non-specific amplification, complicating the interpretation of melting curves and reducing the reliability of the analysis (Zappe et al., 2021). These challenges underscore the necessity of meticulous primer design and rigorous assay optimization to maximize the accuracy and reproducibility of HRM-based methods.

3.4 Application in Molecular Diagnostics

Probe-based assays are fundamental in the diagnosis of infectious diseases and genetic disorders. TaqMan probes, for instance, are extensively utilized to detect and quantify viral and bacterial DNA or RNA and to identify specific genetic mutations and SNPs associated with various diseases. Molecular beacons, another probe-based approach, provide exceptional specificity and sensitivity, enabling precise detection of target sequences in diverse diagnostic applications (Tang et al., 2019).

Non-probe-based assays, such as those utilizing SYBR Green, also play an important role in molecular diagnostics. These assays are commonly used to amplify and quantify viral and bacterial DNA, detect gene expression levels, and identify mutations through melt curve analysis (Edwards et al., 2018; Sinha et al., 2018). Similarly, HRM analysis is widely applied to screen for mutations and SNPs linked to genetic disorders. HRM's ability to differentiate DNA sequences based on their melting profiles makes it a cost-effective and powerful tool for analyzing genetic variations (Teräsjärvi et al., 2017; Wang et al., 2016).

Both probe-based and non-probe-based assays offer distinct benefits and limitations, making them suitable for different diagnostic contexts. In infectious disease diagnostics, probe-based methods such as TaqMan and molecular beacons exhibit superior specificity, enabling accurate pathogen identification. These assays also allow for multiplexing, facilitating the simultaneous analysis of multiple targets, which is essential for monitoring disease progression and detecting co-infections

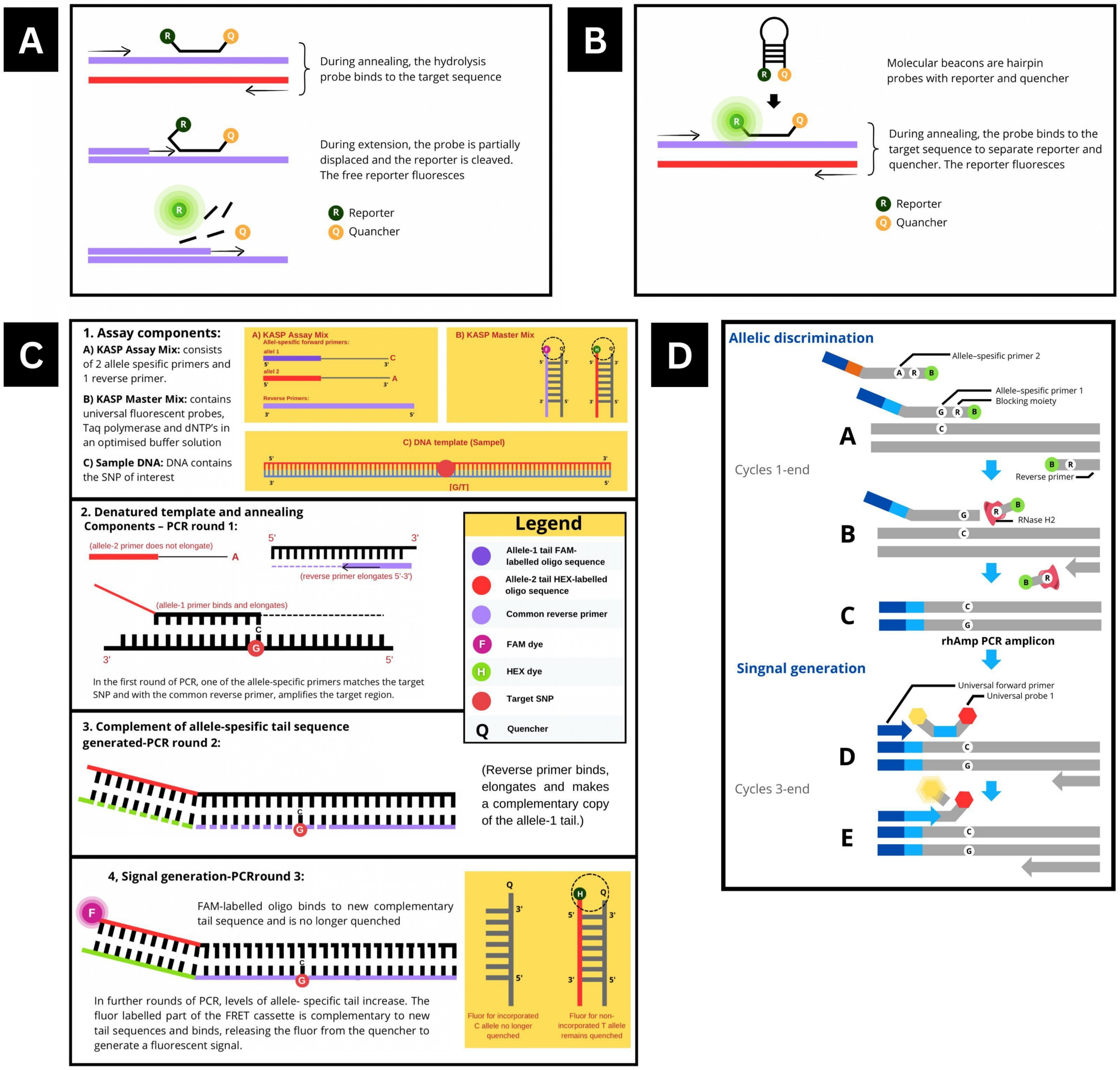

Figure 3. Ilustrates the Mechanisms of Various Probe-Based qPCR Assays, Depicts Mechanisms for Probe-Based Assays, Including Taqman (A), Molecular Beacons (B), KASP (C) and Rhamp (D)

Figures Taqman, molecular beacons, were sourced from Bio-Rad Lab's Introduction to PCR Primer & Probe Chemistries. The KASP figure was obtained from LGC Bioresearch's KASP Genotyping Explained, and the rhAmp figure is from IDT's rhAmp^{TM} SNP Genotyping System

(Grigorenko et al., 2017; Kikuchi et al., 2016; Liu et al., 2020; Onyango et al., 2017). However, probe-based assays can be expensive and require complex probe designs, making them less accessible in resource-limited settings. Non-probe-based assays, such as SYBR Green, offer simplicity and affordability, making them suitable for initial screenings and detecting a broad range of pathogens. Nevertheless, their lack of specificity and susceptibility to false positives due to non-specific binding are notable drawbacks (Marinowic et al., 2021; Sturza et al., 2021).

In the context of genetic disorders, probe-based assays are particularly effective for detecting specific mutations, such as those linked to breast cancer, offering high specificity and precise quantification (Xue et al., 2021). Conversely, non-probe-based methods like HRM are more cost-efficient and advantageous for broad mutation screening. However, HRM lacks the specificity of probe-based methods and requires skilled interpretation to ensure accurate results (Mbelele et al., 2018).

Table 1 highlights recent studies of probe-based and non-probe-based assays in molecular diagnostics. This comparison

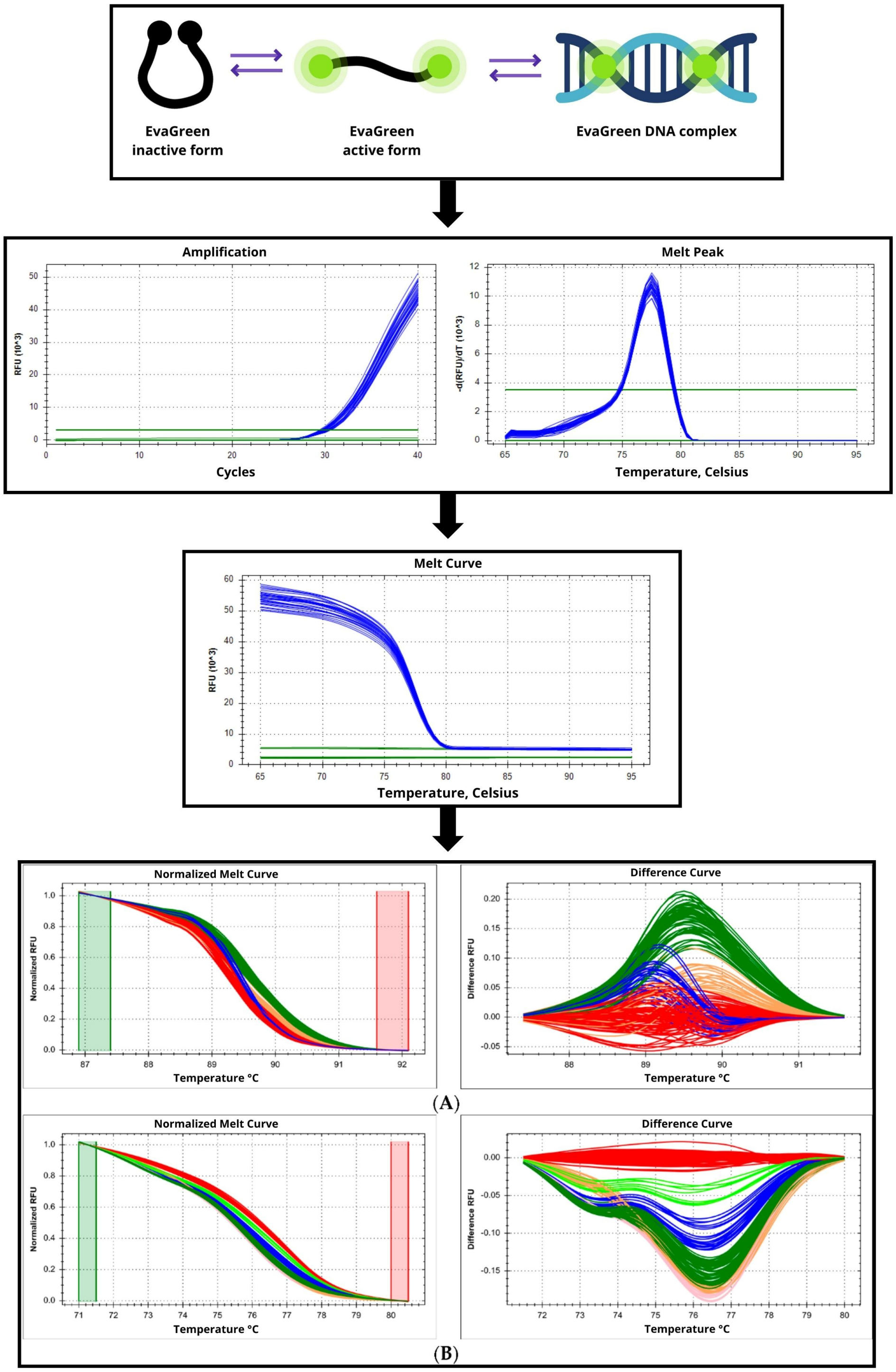

Figure 4. Illustrates the Mechanism of HRM qPCR Assays, Which Identify Genetic Variations by Analyzing Differences in DNA Melting Profiles

Following PCR amplification with a fluorescent dye such as EvaGreen, the temperature is gradually increased, leading to the denaturation of double-stranded DNA (dsDNA). As the DNA strands separate, fluorescence diminishes, producing a melt curve that represents sequence variations. Differences in melting temperature (Tm) enable the distinction of SNPs, mutations, and epigenetic modifications. Normalized melt curves and difference plots further improve resolution, allowing for highly accurate genotyping Adopted from (Rahma et al., 2025; Słomka et al., 2017) underscores the importance of selecting an assay based on diagnostic requirements, available resources, and the need to balance cost and specificity. Probe-based assays are generally preferred for high-precision applications, while non-probe-based assays are better suited for cost-sensitive or large-scale screenings, particularly in resource-limited environments.

3.5 Comparative Analysis of Probe-Based and Non-Probe-Based Assays

Probe-based and non-probe-based qPCR assays each offer distinct advantages and limitations, making them critical for various molecular diagnostic applications. HRM, a widely used non-probe-based technology, is particularly valued for its simplicity, speed, sensitivity, and cost-effectiveness. Yu et al. (2015) demonstrated that HRM reduces analysis time by nearly an hour while minimizing contamination risks and lowering overal costs, making it especially effective for applications such as identifying PPV. Similarly, Zhang et al. (2015) highlighted HRM's precision in ALDH2 genotyping, showcasing its capability to differentiate rs671 genotypes (GG, GA, and AA). Its straightforward setup and reliability in SNP genotyping establish HRM as an essential tool in molecular diagnostics.

In contrast, TaqMan assays, a commonly used probe-based technology, leverage Minor Groove Binder (MGB) modifications to improve specificity (Zhang et al., 2015). While TaqMan assays provide comparable accuracy and speed to HRM, their reliance on sequence-specific probes increases costs (Lamien-Meda et al., 2021). Although less expensive than FRET-based systems, TaqMan has limitations in SNP detection, including potential hybridization at multiple regions with minor sequence differences (Filippis et al., 2023). Furthermore, HRM's challenges in detecting certain transversions Pholwat et al. (2015), and its struggles with identifying mutations such as PIK3CA in CNS metastases is highlight the importance of selecting assays based on specific diagnostic requirements (Nicoś et al., 2016). Table 2 summarizes the critical differences between non-probe-based and probe-based qPCR assays.

When comparing HRM to KASP, HRM demonstrates notable advantages. Unlike KASP, which requires three primers (two allele-specific forward primers and one reverse primer), HRM needs only a single primer pair, simplifying the assay design and reducing costs (Kaur et al., 2023). HRM also supports multiplexing and offers more precise curve analysis, making it a superior option in many cases. However, KASP remains a practical alternative due to its simplicity in design, validation, and affordability compared to probe-based methods like TaqMan (Nunziata et al., 2020). KASP's cost-effectiveness makes it popular for large-scale genotyping projects, especially in resource-limited settings, despite HRM offering higher precision and flexibility.

Among the three probe-based assays—TaqMan, rhAmp, and KASP—rhAmp is the most cost-effective while maintaining high specificity. According to Ayalew et al. (2019), rhAmp had the lowest non-specific amplification error rate (3%) compared to KASP (6.5%) and TaqMan (7%). In terms of speed, rhAmp was also the fastest, completing analysis in 110 minutes, followed by KASP (115 minutes) and TaqMan (125 minutes). While TaqMan demonstrated higher sensitivity (detecting as low as 0.2 ng of DNA per reaction) than KASP (0.9 ng) (Broccanello et al., 2018), KASP remains significantly more affordable—approximately five times less expensive than TaqMan. However, KASP's longer protocols and higher false-positive rates can pose challenges (Landoulsi et al., 2017; Li et al., 2021). Table 3 provides a detailed comparison of key parameters across these probe-based qPCR assays.

Hybrid technologies are increasingly being developed to bridge the strengths of probe-based and non-probe-based methods. For example, HRM-RTqPCR combines qPCR with HRM analysis, reducing reagent costs compared to traditional probe-based methods. However, this hybrid technology requires more time (approximately 2.5 hours), making it less efficient than TaqMan-based methods (Ferreira et al., 2021). Another innovation, TAC-HRM, addresses HRM's limitations in detecting transversions, although it remains reliant on a real-time PCR platform (Pholwat et al., 2015).

ASQ (Allele-Specific qPCR), a FRET-based technology, provides significant cost savings—up to 10 times lower than KASP—while maintaining flexibility in SNP genotyping (Kalendar et al., 2022). Its streamlined design eliminates the hairpin loop, simplifying assay setup and further reducing costs, making it ideal for budget-conscious laboratories. Finally, Universal Probe-Based and Intermediate Primer-triggered qPCR (UPIP-qPCR) has emerged as a highly accurate and rapid SNP genotyping method. With a calling base ratio exceeding 99.1% and accuracy greater than 99.9%, UPIP-qPCR surpasses TaqMan and KASP in sensitivity, requiring as little as 0.01 ng of DNA per reaction (Li et al., 2021).

This analysis underscores the importance of carefully selecting assays based on diagnostic needs, financial constraints, and desired levels of specificity and efficiency. Both probe-based and non-probe-based technologies offer unique benefits, making them well-suited for diverse molecular diagnostic applications. To further clarify the selection process, Table 4 provides a structured comparison of widely used qPCR methods, summarizing their advantages and limitations. This comparison serves as a quick reference for selecting the most suitable method based on specificity, cost, multiplexing capability, and suitability for high-throughput applications. The information presented highlights key trade-offs that researchers and clinicians should consider when designing molecular diagnostic assays.

3.6 Cost Levels of Probe-Based and Non-Probe-Based Assays

Evaluating the cost implications of qPCR assays is essential for selecting the most appropriate technology, especially in resource-limited settings or large-scale projects. The cost of these assays is influenced by factors such as their reliance on sequence-specific probes, reagent complexity, and sensitivity

Table 1. Current Studies on Probe-Based and Non-Probe-Based Assays in Molecular Diagnostics

Assay Type	Origin of Study	Object	Summary	References
Probe-based	Bangladesh, Malaysia, Ethiopia	Human blood spots	TaqMan probes were utilized to detect *Plasmodium* spp., incurring higher costs than SYBR Green but lower than FRET. Specificity issues in identifying *Plasmodium* species were noted	(Lamien-Meda et al., 2021)
Probe-based	Tunisia	Peripheral blood from Parkinson's patients	KASP was five times cheaper than TaqMan, offering flexible primer design and higher success rates for genotyping rare Alzheimer's-related variants	(Landoulsi et al., 2017)
Probe-based	China	Human peripheral whole blood samples	The UPIP-qPCR method showed sensitivity at 0.01 ng with accuracy greater than 99.9% in SNP genotyping of the rs671 gene	(Li et al., 2021)
Probe-based	Italy	Lyophilized leaves	KASP showed high reproducibility with an error rate below 0.5% during SNP detection, providing cost-effective solutions for managing European chestnut biodiversity	(Nunziata et al., 2020)
Probe-based	USA	Wheat tissue samples	rhAmp technology was more cost-efficient and required less DNA template than TaqMan and KASP for SNP genotyping, with failure rates of 3%, 7%, and 6.5%, respectively	(Ayalew et al., 2019)
Probe-based	Italy	Sugar beet tissue samples	rhAmp showed lower costs (€0.10/sample) and faster runtime (110 minutes) than TaqMan (€0.29/sample) and KASP (115 minutes), making it suitable for SNP genotyping	(Broccanello et al., 2018)
Probe-based	Russia, Lithuania	Frozen barley leaves	ASQ technology for SNP genotyping of barley genes demonstrated high flexibility and cost reduction, requiring 2–10 times lower cost than KASP	(Kalendar et al., 2022)
Probe-based	USA	RNA samples from SARS-CoV-2	The TaqMan SARS-CoV-2 mutation panel detected and genotyped variants (e.g., Delta, Alpha) with high concordance to sequencing	(Neopane et al., 2021)
Probe-based	Hong Kong	SARS-CoV-2 respiratory specimens	An in-house TaqMan RT-qPCR assay identified the N501Y mutation with a detection limit of 1.5 copies μL, enabling high-throughput screening	(Chan et al., 2022)
Probe-based	Brazil	Cerebrospinal fluid and clinical samples	TaqMan was rapid and accurate for meningitis detection but expensive, while qPCR-HRM offered cost savings with reagent costs 20% of TaqMan's	(Filippis et al., 2023)
Non-probe-based	Japan	SARS-CoV-2 Omicron variants	HRM successfully differentiated Omicron BA.1 and BA.2 variants through melting temperature analysis of spike protein mutations	(Koshikawa and Miyoshi, 2022)
Non-probe-based	Indonesia	RNA samples from SARS-CoV-2	RT-qPCR-HRM detected the D614G mutation in SARS-CoV-2 spike protein, with results confirmed by Sanger sequencing	(Gazali et al., 2021)
Non-probe-based	Brazil	Nasopharyngeal, saliva, and serum samples	HRM-RT-qPCR reduced reagent costs compared to TaqMan and was cost-effective despite requiring an hour longer for analysis	(Ferreira et al., 2021)

Non-probe-based	China	Human blood samples	HRM proved simple, affordable, and time-efficient for ALDH2 genotyping, with minimal DNA contamination risks	(Zhang et al., 2015)
Non-probe-based	USA	Whole blood samples	HRM was fast and reproducible, taking 5 hours for completion, and was suitable for clinical and research applications in personalized medicine	(Langaee et al., 2019)
Non-probe-based	USA	Lung tumors, normal tissue, and blood plasma	MS-HRM for gene methylation analysis was cost-effective and convenient but lacked sensitivity and quantification capabilities	(Liu et al., 2017)
Non-probe-based	China	Pig tissue samples	HRM offered a rapid, sensitive, and specific alternative for detecting PPV mutations, reducing analysis time by almost an hour while minimizing contamination risks	(Yu et al., 2015)
Non-probe-based	Poland	Blood samples from rats	MS-HRM faced challenges with primer competition in CpG-rich sequences, requiring additional non-CpG sites to maintain assay accuracy for TGFB gene methylation analysis	(Bialek et al., 2021)
Non-probe-based	Poland	Formalin-fixed paraffin-embedded tissue samples	HRM-PCR was applied for PIK3CA mutation detection in CNS metastases but lacked specificity compared to TaqMan for identifying specific mutations	(Nicoś et al., 2016)
Non-probe-based	Bangladesh, Thailand, Russia, Tanzania	M. tuberculosis culture samples	TAC-HRM provided an affordable approach for detecting mutations, but its cost-effectiveness was limited by the high price of HRM-compatible PCR platforms	(Pholwat et al., 2015)
Non-probe-based	USA	Wheat tissue samples	HRM was more cost-effective and multiplex-capable than KASP for SNP genotyping in Triticum aestivum, while KASP showed limitations in detecting heterozygous alleles	(Kaur et al., 2023)

Table 2. Summarises the Key Differences Between Non-Probe-Based Real Time PCR Assays with Probe-Based Real-Time PCR Assays

Feature	Non-Probe-Based Real-Time PCR	Probe-Based Real-Time PCR
Detection Method	Uses intercalating dyes (e.g., SYBR Green or Evagreen with HRM analysis) that bind to double-stranded DNA	Utilizes sequence-specific fluorescent probes (e.g., TaqMan, Molecular Beacons, FRET, KASP, rhAmp)
Specificity	Lower specificity as dyes bind to any double-stranded DNA.	High specificity due to the sequence-specific binding of probes
Ease of Use	Simpler design: no need to design or optimize probes.	Requires careful design and optimization of sequence-specific probes
Cost	Generally lower as it only requires primers and dye.	Higher due to the cost of probes
Quantification Accuracy (Sensitivity)	Can be affected by nonspecific amplification.	Provides more accurate quantification due to high specificity
Multiplexing Capability	Limited; challenging to distinguish multiple targets.	Excellent; different fluorescent dyes can label different probes
Application	Suitable for general DNA detection and less complex assays.	Ideal for detecting specific DNA sequences and multiplex assays

Table 3. Highlights the Comparison of Key Parameters Across Various Probed-Based Real-Time PCR Assays

Parameters	TaqMan	Molecular Beacons	FRET	KASP	rhAmp
Enzyme(s)	Common Taq Polymerase	Common Taq Polymerase	Common Taq Polymerase	Common Taq Polymerase	Mutated Taq Polymerase and RNase H2
Probe	One target-specific probe	One target-specific hairpin-shaped probe	Two target-specific probes	Two universal fluorescence probes	Two universal fluorescence probes
Primers	One pair of forward and reverse primer	One pair of forward and reverse primer	One pair of forward and reverse primer	Two allele-specific forward primers labeled at the 5' ends, along with one regular reverse primer.	Two allele-specific forward primers, each with 5' ends containing allele-specific tail sequences and 3' ends blocked, along with one regular reverse primer.

Table 4. Comparative Overview of Probe-Based and Non-Probe-Based qPCR Assays

Assay Type	Advantages	Limitations	References
TaqMan Assay	High specificity, minimizes false-positive results, suitable for detecting specific targets, supports multiplexing	High cost, requires carefully designed sequence-specific probes, limited flexibility for broad screenings	(Alblalaihid et al., 2016; Barnes et al., 2021; Fang et al., 2025; Yang et al., 2025)
Molecular Beacons	Very high specificity, allows precise detection of target sequences, effective in complex samples, supports multiplexing	Expensive due to complex probe synthesis, requires extensive optimization, challenging probe design	(Chandrasekaran et al., 2021; Del Bonis-O'Donnell et al., 2016; Roth and Seitz, 2021; Yang et al., 2024)
KASP Assay	Cost-effective for SNP genotyping, does not require fluorescent probes, suitable for large-scale genotyping projects	Limited to analyzing a moderate number of SNPs, less suitable for highly complex genomic structures	(Dipta et al., 2024; Shen et al., 2025; Steele et al., 2025)
rhAmp Assay	High specificity and sensitivity for SNP detection, efficient with low DNA input, reduces non-specific amplification errors	Requires specific enzyme and probe optimization, relatively higher cost compared to KASP	(Ayalew et al., 2019; Beltz et al., 2018; Broccanello et al., 2018)
SYBR Green	Simple, cost-effective, widely available, suitable for basic gene expression and pathogen detection	Less specific due to non-target DNA binding, requires careful primer design to avoid non-specific amplification	(Cao et al., 2025; Ranaweera et al., 2025; Schreier et al., 2019; Traore et al., 2015)
HRM	Low-cost alternative for mutation screening, useful for SNP genotyping and methylation analysis, does not require labeled probes	Requires high-quality DNA and precise temperature control, less effective for complex sequence variations	(Jiang et al., 2025; Qiao et al., 2020; Taryma-Leśniak et al., 2025; Wang et al., 2016)

requirements. Table 5 provides a comprehensive ranking of qPCR assay types based on cost, ranging from high to very low, alongside their typical applications and diagnostic capabilities.

TaqMan (Probe-Based) assays represent the highest cost among qPCR technologies due to their dependence on sequence-specific probes and MGB modifications, which enhance assay specificity but significantly increase costs. These assays are extensively used in applications that demand high accuracy, such as the detection of *Plasmodium* spp, SARS-CoV-2 mutations, and diagnosing meningitis. Their precision and reliability justify their higher cost in settings where precise pathogen identification is critical (Chan et al., 2022; Filippis et al., 2023; Lamien-Meda et al., 2021).

A more cost-effective alternative is the rhAmp (Probe-Based) assay, which falls into the moderate-to-high cost category. It balances specificity and cost, making it a viable alternative to TaqMan, particularly for SNP genotyping in wheat and sugar beet tissue samples. With shorter analysis times and

Table 5. Cost Levels of Probe-Based and Non-Probe-Based Assays

Rank	Assay Type	Cost Level	Example Applications	References
1	TaqMan (Probe-based)	High	Detecting *Plasmodium* spp., SARS-CoV-2 mutations, meningitis, and HPV DNA	(Al-Ziara et al., 2025; Chan et al., 2022; Filippis et al., 2023; Lamien-Meda et al., 2021)
2	rhAmp (Probe-based)	Moderate-High	SNP genotyping in wheat, sugar beet tissue samples, and Friesian horses	(Alcaraz-González et al., 2025; Ayalew et al., 2019; Broccanello et al., 2018)
3	KASP (Probe-based)	Moderate	Alzheimer's-related variants, biodiversity in chestnut, fomesafen resistance in Amaranthus retroflexus	(Landoulsi et al., 2017; Nunziata et al., 2020; Zhang et al., 2025)
4	MS-HRM (Non-probe-based)	Moderate	Methylation analysis of TGFB, CNS metastases, and BRCA1 epimutation screening	(Bialek et al., 2021; Nicoś et al., 2016; Taryma-Leśniak et al., 2025)
5	HRM (Non-probe-based)	Low	Variant differentiation (SARS-CoV-2 Omicron, D614G mutation), ALDH2 genotyping, SNP genotyping in wheat, NX-2 chemotype detection in Fusarium graminearum	(Gazali et al., 2021; Kaur et al., 2023; Koshikawa and Miyoshi, 2022; Singh et al., 2025; Zhang et al., 2015)
6	SYBR Green (Non-probe-based)	Very Low	Affordable alternative for detecting *Plasmodium* spp. DNA, broad pathogen screening in resource-limited settings, LSDV detection in cattle, and ToBRFV detection in tomato seeds	(Sanganagouda et al., 2025; Lamien-Meda et al., 2021; Ota et al., 2025)

reduced reagent consumption, rhAmp is a practical choice for both research and diagnostic applications. Despite being more affordable than TaqMan, it remains pricier than KASP (Ayalew et al., 2019; Broccanello et al., 2018).

KASP (Probe-Based) assays further reduce costs and fall into the moderate-cost range, offering a flexible and economical solution for genotyping applications such as Alzheimer's variant research, biodiversity studies, and SNP authentication in olives. Compared to TaqMan and rhAmp, KASP is significantly cheaper; however, its longer protocols and higher false-positive rates may limit its effectiveness in high-throughput environments (Landoulsi et al., 2017; Nunziata et al., 2020).

Moving into non-probe-based methods, MS-HRM (Methylation-Sensitive High-Resolution Melting) assays offer a moderately priced option for methylation studies, particularly for genes such as TGFB and in CNS metastases. While it is an economical choice for methylation analysis, MS-HRM's complexity can limit its efficiency in high-throughput screenings, requiring careful optimization to maintain accuracy (Bialek et al., 2021; Nicoś et al., 2016).

HRM is an even more cost-effective non-probe-based method, classified as low-cost, with broad applications in SNP genotyping and mutation detection. It has demonstrated effectiveness in differentiating SARS-CoV-2 variants, such as Omicron BA.1 and BA.2, and in genotyping ALDH2 mutations. Its affordability, coupled with its ability to detect genetic variations based on melting profiles, makes it particularly suitable for resource-constrained settings (Gazali et al., 2021; Kaur et al., 2023; Koshikawa and Miyoshi, 2022; Zhang et al., 2015).

The most economical qPCR assay type is SYBR Green (Non-Probe-Based), classified as very low-cost. This method is widely used for pathogen detection and preliminary screenings in resource-limited settings due to its simplicity and lack of sequence-specific probes. However, its specificity is lower compared to HRM, making it better suited for general applications rather than high-precision diagnostics (Lamien-Meda et al., 2021). The cost hierarchy of these assays reflects a balance between specificity, sensitivity, and application demands. TaqMan remains the most expensive due to its precision and reliance on advanced probe designs, while SYBR Green provides the most affordable option for basic diagnostic needs. HRM represents an intermediate solution, combining affordability with robust accuracy, while rhAmp and KASP offer moderately priced alternatives with varying levels of flexibility and performance. This cost breakdown helps researchers and clinicians choose the most suitable qPCR assay based on their diagnostic objectives and budgetary considerations.

3.7 Recent Advances and Future Perspectives

The field of molecular diagnostics continues to evolve, with innovative technologies improving accuracy, efficiency, and accessibility in genetic testing. Emerging approaches such as ASQ and UPIP-qPCR exemplify this progress. While primarily applied to non-human samples, such as plant genotyping, these methods show significant potential for human applications, particularly in detecting disease-associated genetic variants. Their precision and cost-efficiency position them as valuable tools for expanding diagnostic capabilities in clinical settings.

Multiplex PCR platforms, including Seegene's high-through put diagnostic systems, represent another leap forward in molecular diagnostics. These systems can simultaneously detect six to seven targets in a single reaction, offering significant time and cost savings, especially for syndromic testing (Bodiybadu et al., 2023; Lim et al., 2023). The integration of such multiplex technologies in clinical workflows enhances diagnostic efficiency while conserving resources, making them highly advantageous for applications requiring the simultaneous detection of multiple pathogens or genetic markers. Future innovations in real-time PCR platforms are expected to further increase multiplexing capacities, enabling the detection of even more targets within a single reaction. These advancements are anticipated to reduce costs and expand the accessibility of molecular diagnostics, particularly in resource-constrained environments (Bodiybadu et al., 2023).

HRM technology also plays a key role in shaping the future of molecular diagnostics. Renowned for its ability to identify genetic variants, mutations, and epigenetic changes based on DNA melting profiles, HRM provides a cost-effective alternative to probe-based methods. Its affordability, coupled with its adaptability for mutation screening and SNP genotyping, makes HRM particularly valuable for resource-limited settings (Koshikawa and Miyoshi, 2022; Zhang et al., 2015). However, limitations such as reduced effectiveness in analyzing complex or heterogeneous samples need to be addressed. Research aimed at improving HRM's resolution to detect subtle genetic variations and expanding its multiplexing capabilities could significantly enhance its utility. Combining HRM with advanced multiplex PCR platforms may result in hybrid systems that deliver cost savings alongside high-throughput capabilities, thereby broadening their applicability in both clinical and research settings.

Looking forward, further advancements in molecular diagnostics should prioritize versatility and affordability, ensuring these technologies are adaptable to diverse sample types, including RNA and methylated DNA. Additionally, increasing the accessibility of these systems in resource-limited regions is critical for equitable healthcare outcomes. The ongoing refinement of technologies such as HRM, ASQ, UPIP-qPCR, and multiplex PCR platforms will continue to drive innovation, expanding the role of molecular diagnostics in fields ranging from human healthcare to agricultural genomics. These developments promise to revolutionize diagnostic strategies, ensuring improved outcomes across a wide array of applications.

4. CONCLUSIONS

qPCR technologies have profoundly transformed molecular diagnostics, delivering exceptional precision and efficiency in pathogen detection, SNP genotyping, and genetic variant analysis. This review presents a detailed evaluation of probe-based assays, including TaqMan, KASP, and rhAmp, alongside non-probe-based methods such as SYBR Green and HRM. Probe-based systems offer superior specificity and multiplexing capabilities, making them optimal for applications demanding high accuracy; however, their higher costs and complex probe design limit their applicability in resource-limited settings. In contrast, non-probe-based technologies like HRM provide cost-effective alternatives with extensive utility in SNP detection and mutation analysis, though they are prone to non-specific amplification and require expert interpretation. Emerging hybrid technologies, such as HRM-RTqPCR and UPIP-qPCR, illustrate the potential to combine the strengths of both approaches, offering enhanced accuracy, cost-efficiency, and scalability. Additionally, advancements in multiplex PCR platforms highlight the increasing capacity for high-throughput diagnostics, allowing simultaneous detection of multiple targets and streamlining diagnostic workflows. Future research must focus on creating more accessible and adaptable qPCR systems, with an emphasis on expanding multiplexing capacities and ensuring affordability. Improving the specificity and resolution of cost-effective methods like HRM will also be essential in overcoming current challenges. The integration of these innovations into clinical and agricultural workflows will significantly extend the impact of molecular diagnostics, promoting advancements in precision medicine and sustainable agriculture. By comparing existing methodologies and exploring emerging technologies, this review underscores the transformative potential of qPCR technologies in driving more inclusive, efficient, and impactful diagnostic solutions.

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