Borneo International Journal of Biotechnology (BIJB)
Vol. 3 (December 2023), 80 – 99

e-ISSN 2716-697X

Can Multiplex SYBR Green Real-Time PCR Assay Serve as a Detection and Quantification Method Comparable to the TaqMan Method for SARS-CoV-2 Diagnosis?

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https://doi.org/10.51200/bijb.v3i.
Received: 30 July 2023 | Accepted: 29 September 2023 | Published: 1 December 2023

ABSTRACT

The reopening of schools, business, and social sectors during the COVID-19 pandemic has caused a current increase in the number of COVID-19 cases and clusters all over the globe. While the COVID-19 pandemic is far from over, the reopening and resumption of all economic sectors are essential to recovering the world economy. Health experts all over the world have determined that the real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) method is the gold standard for diagnosing COVID-19 infections due to the test's high sensitivity and specificity. During the past 3 years when WHO declared the COVID-19 pandemic, the cost of laboratory diagnosis of COVID-19 using a robust RT-qPCR assay is still considerably expensive, especially for low and middle-income countries. Therefore, numerous studies have reported optimized SYBR green methods which are more economical than the qPCR probe assay. Continuous diagnostic testing is vital to mitigate the spread of COVID-19. However, there is a question as to whether SYBR Green may serve as an excellent detection and quantification method for molecular diagnosis to perform SARS-CoV-2 screening. This review summarizes the numerous studies using SYBR Green RT-PCR to detect SARS-CoV-2. The reliability of SYBR Green qPCR assays for determining gene expression based on their performance is justified and the quality is comparable to the TaqMan method.

Keywords: RT-qPCR, SARS-CoV-2, SYBR Green, multiplex, COVID-19, gold standard diagnosis

INTRODUCTION

The highly contagious coronavirus respiratory disease (COVID-19), which was first detected in December 2019 in Wuhan, China has spread fast across the globe, resulting in over 185 million positive cases and almost 4 million deaths, emerging as the most significant global health emergency since the 1918 influenza epidemic (Ritchie et al., 2021). Following prompt genomic analysis, a novel coronavirus was identified to be the disease's aetiology. COVID-19 is caused by the Coronavirus Severe Acute Respiratory Syndrome 2 (SARS-CoV-2), a major coronavirus family of RNA viruses (Hu et al., 2020). The coronavirus is responsible for both minor respiratory tract infections such as the common cold and more serious illnesses such as Middle East Respiratory Syndrome (MERS) and severe acute respiratory syndrome (SARS) (Shereen et al., 2020). On March 11, 2020, the World Health Organization (WHO) had to designate the outbreak as a pandemic due to the outbreak's rapid transmission. The virus quickly spread across the globe and has since undergone several mutations, leading to the emergence of new variants of the virus.

The diagnosis of SARS-CoV-2 is indeed still a very pertinent and crucial aspect of the ongoing global response to the COVID-19 pandemic. It is important to note that the COVID-19 infection is still ongoing in many parts of the world. With the emergence of new variants, accurate and timely diagnosis of SARS-CoV-2 infections remained essential for tracking and controlling the spread of the virus. Monitoring and diagnosing these variants are critical for understanding their impact on transmission, severity of illness, and vaccine effectiveness. Hence, research into the discovery of SARS-CoV-2 diagnosis remains pertinent as it plays a role in assessing vaccine efficacy, monitoring breakthrough infections, and determining vaccination strategies. While the RT-qPCR method is highly accurate and reliable for COVID-19 detection, it has certain disadvantages when used as a tool for mass screening such as high costs, longer turnaround time compared to rapid antigen tests, laboratory dependence and requires significant resources, including testing supplies, personnel, and laboratory space. On the other hand, while rapid antigen tests can be valuable for large-scale testing, they may not replace RT-qPCR entirely due to it being limited for qualitative diagnosis and its reduced sensitivity among asymptomatic individuals (Robinson et al., 2020). The lack of resources for COVID-19 screening and detection has been a significant challenge, especially in regions with limited healthcare infrastructure or during the early stages of the pandemic when demand for testing was high. The RT-qPCR is the gold standard for COVID-19 detection with many using TaqMan probe-based assay, which is expensive, especially for more sophisticated testing methods. The cost becomes a barrier for individuals and healthcare systems, particularly in low-resource settings. The SYBR Green methodology exhibits a straightforward configuration and is cost-effective, yet it lacks specificity. Its primary application lies in genome diagnosis and amplification. In general, the utilization of specific primers yields precise outcomes. Consequently, the adoption of efficient primers within a SYBR Green-based approach can transform it into a cost-effective alternative characterized by heightened specificity and sensitivity (Marinowic et al., 2021).

THE EMERGENCE OF SARS-CoV-2 VARIANTS

The exact origins of the SARS-CoV-2 virus are not yet fully understood, but it is believed to have originated from bats and may have been transmitted to humans through an intermediate animal host, possibly a pangolin (Gupta et al., 2022). Once the virus emerged in humans, it began to evolve and adapt to its new host. Viruses are known for their ability to rapidly mutate and evolve, especially RNA viruses like SARS-CoV-2, which have a high mutation rate (Duffy, 2018). As the virus replicated within human hosts, it accumulated genetic changes through mutations, some of which were beneficial for the virus's survival (Sanjuán & Domingo-calap, 2016). These mutations allowed the virus to become more infectious and more transmissible, as well as potentially more virulent or capable of causing more severe illness. Some mutations may have also allowed the virus to evade the immune system and cause reinfections or breakthrough infections in people who were previously infected or vaccinated (Gili & Burioni, 2023).

Variations in the viral genome's S, N, membrane (M), and envelope (E) proteins give rise to distinct viral strains. Variants of concern include Alpha, Beta, Delta, and Gamma, with Omicron being the most recent variant. The Wuhan strain was the original strain of SARS-CoV-2 that emerged in Wuhan, China and is often referred to as the "wild type" or the "original strain" (Saberiyan et al., 2022). This strain was responsible for the first wave of COVID-19 cases and was responsible for the initial global spread of the disease. The Alpha variant, also known as B.1.1.7, emerged in the UK in late 2020. This variant is believed to be more transmissible than the original strain and has mutations in the spike protein of the virus (Radvak et al., 2021). The Alpha variant quickly became the dominant strain in many countries, leading to increased concerns about the spread of the virus. The Beta variant, also known as B.1.351, was first identified in South Africa in late 2020. The Beta variant has a genetic mutation known as N501Y, which has been seen to enhance its transmissibility or ability to propagate (Lu et al., 2021). A further mutation, known as E484K, has the potential to strengthen immune evasion capabilities to the virus, hence potentially impacting the efficacy of vaccinations (Aleem et al., 2023; Wise, 2021). One of the most significant mutations in SARS-CoV-2 was the emergence of the Delta variant also known as B.1.617.2, which is thought to have originated in India in late 2020. This variant has several mutations in the spike protein of the virus, which makes it more transmissible and potentially more resistant to the immune response (Dhawan et al., 2022). The Delta variant has demonstrated a heightened transmission rate in comparison to preceding variants, including among individuals who have had full vaccination. Estimates suggest that the Delta variant is almost twice as contagious as the previous variants (Samieefar et al., 2022). It quickly became the dominant strain in many countries and is responsible for the current wave of COVID-19 cases in many parts of the world.

The Omicron variant, also known as B.1.1.529, was first identified in South Africa in November 2021. This variant has many mutations in the spike protein of the virus, which may make it more transmissible and potentially more resistant to some of the vaccines that were developed against previous strains (Ren et al., 2022). The

Omicron variant has quickly spread worldwide, leading to a surge in COVID-19 cases in many countries. However, early data suggests that the severity of illness caused by the Omicron variant may be lower than previous variants, although more research is needed to confirm this (Butt et al., 2022). Several mutations exhibit pleiotropic effects that have the potential to impact the accuracy of diagnostic procedures. Tests utilizing antibodies as recognition elements may experience false-negative results due to changes in the structure of antigens, hence rendering them ineffective for detection purposes. Significant alterations have been reported in the S protein of the coronavirus (Magazine et al., 2022). Consequently, immunoassays that target the detection of the N protein and the antibodies corresponding to it may not be influenced as compared to the S protein (Sharma et al., 2022).

Molecular diagnostic techniques, such as RT-PCR, RT-LAMP, and Microarray, have a reduced likelihood of failure due to their ability to target many molecular markers. Satisfactory results can still be obtained from other sequences that are devoid of any mutation. However, the sensitivity of diagnostic methods, including molecular diagnostics and point-of-care serological approaches, is impacted (Sharma et al., 2022). The major impact on diagnosis has been seen due to the deletion of nine nucleotide sequences in the N protein of the newly identified Omicron variation (Center for Devices and Radiological Health, 2023). COVID-19 diagnostic tests primarily target specific genetic material from the SARS-CoV-2 virus. The most common target gene is the N gene which encodes for the nucleocapsid protein of the virus and is essential for its replication. Some RT-qPCR tests also target the E gene, which encodes for the envelope protein of the virus and the RdRp gene which codes for the RNA-dependent RNA polymerase enzyme used by the virus during replication. Several RT-PCR kits specifically focus on a single gene location, which raises the concern that the currently available diagnostic approach may be targeting the deleted N-protein fragment seen in the Omicron form. Consequently, molecular testing equipment that specifically targets a single gene might be ineffective when confronted with rapidly developing variants (Sharma et al., 2022). Overall, the evolution of COVID-19 has led to the emergence of new variants of the virus that are more transmissible and potentially more resistant to some of the vaccines that were developed against earlier strains. To conclude, the diagnostic strategy for COVID-19 is playing a crucial role in ensuring effective containment and future research developments of SARS-CoV-2.

THE ISSUE OF MASS TESTING

Many individuals are tested for COVID-19 to identify infected individuals and prevent the spread of the disease. However, this procedure can be costly, and several cost considerations must be considered (Dorlass et al., 2020). The cost of tests is one of the greatest obstacles to mass screening for COVID-19. The gold standard for COVID-19 testing is the RT-qPCR method which is typically more expensive than rapid antigen tests. The term "gold standard" is used to describe a diagnostic method that is considered the most accurate and reliable among available options. In the case of COVID-19, RT-

qPCR has demonstrated high sensitivity and specificity, making it the preferred method for confirming COVID-19 infections. Cost can be a significant barrier to mass screening, particularly in low-income nations (Boro & Stoll, 2022).

In addition to the cost of tests, there are also expenses related to the apparatus and infrastructure required for mass screening. This includes the cost of acquisition, maintenance, and the employment of qualified personnel to conduct tests (Filip et al., 2022). These expenditures can be substantial, particularly in regions with limited or nonexistent testing infrastructure. Furthermore, personnel expenses are an additional cost factor for COVID-19 mass screening (Kaye et al., 2021). To collect samples, conduct tests, and interpret results, trained healthcare professionals are necessary. In certain instances, additional personnel may be required to manage crowds and preserve social distancing during the screening process. The cost of personnel can be a significant burden, particularly in regions with a shortage of healthcare professionals (Filip et al., 2022). Lastly, there are logistical costs associated with COVID-19 bulk screening. This includes the cost of transporting samples to testing facilities, managing the supply chain for testing tools, and expeditiously reporting test results.

The ability to test for SARS-CoV-2 is a big concern globally. The most common issue is the use of fluorogenic probes in the RT-qPCR methodology, the gold standard approach which can be costly, making it challenging to diagnose large populations of people, especially in low- and middle-income nations (Rahmasari et al., 2022). Thus, a more affordable alternative is necessary for the mass screening approach to be conducted more efficiently. The significance of mass testing and the advancement of multiplex SYBR Green-based quantitative polymerase chain reaction (qPCR) assays exhibit a multifaceted interconnection, particularly within the sphere of diagnostic and research applications. With proper technique, multiplex SYBR Green-based qPCR methodology can facilitate concurrent detection and quantification of multiple target nucleic acids within a single reaction vessel making large-scale mass testing more practicable (Marinowic et al., 2021). Furthermore, multiplex SYBR Green-based qPCR assays yield results for multiple targets in a single analytical cycle, thereby expediting the testing workflow. Swift turnaround times assume critical significance in the proficient management of disease outbreaks and the timely implementation of requisite interventions (Pereira-Gómez et al., 2021). Ironically, mass screening often exerts strain on resources, encompassing sample collection kits, consumables, and laboratory personnel. The implementation of multiplex qPCR serves to conserve these resources by permitting the assessment of SARS-CoV-2 with fewer materials and personnel, thereby enabling extensive testing endeavours without overburdening the infrastructure (Tao et al., 2022). Finally, vigilant surveillance of emerging variants via multiplex qPCR can be readily tailored to detect specific COVID-19 target genes, aiding surveillance initiatives aimed at tracking the dissemination of variants on public health. Thus, the development and deployment of multiplex SYBR Green-based qPCR assays evince a robust correlation with the imperative of mass screening for COVID-19. These assays proffer an efficacious, cost-efficient, and all-encompassing approach for mass screening purposes.

CURRENT COVID-19 DETECTION METHODS

The current COVID-19 detection approach primarily involves testing for the presence of specific SARS-CoV-2 antigens or antibodies to the virus (Table 1). The Polymerase Chain Reaction (PCR) is one of the most used methods for COVID-19 detection (Rong et al., 2023). Reverse transcription polymerase chain reaction (RT-PCR), a method used in PCR testing, is used to amplify and detect the genetic material of a virus using a swab taken from a person's nose or throat (Udugama et al., 2020). Antigen testing is a rapid diagnostic test that detects specific proteins on the surface of the SARS-CoV-2 virus (Pavia & Plummer, 2021). Similar to PCR testing, antigen testing involves taking a swab from the nose or throat of a person, but the results are available within a shorter turnaround time (Xie et al., 2022). On the other hand, antibody testing involves a blood test to detect antibodies produced by the body in response to a COVID-19 infection. Antibody tests are less commonly used for diagnostic purposes, as they can only detect a past infection and not an active one (Jacofsky et al., 2020). The RT-qPCR is considered the gold standard detection method for COVID-19 in many countries. It is a highly accurate and reliable diagnostic test that can detect the presence of the SARS-CoV-2 virus's genetic material in respiratory samples. It has been widely used for diagnosing COVID-19 since the beginning of the pandemic. Although many countries have granted the use of many COVID-19 self-test kits, rapid tests are not as sensitive as PCR tests, creating concerns as a false-positive vulnerable individual is placed with known positive COVID-19 patients or false-negative infected individual is allowed to carry out normal daily routine in public areas.

Table 1 COVID-19 diagnostic tests

Diasts	
Method	**Mode of detection**
Reverse Transcription Polymerase Chain Reaction (RT-PCR)	Detect the presence of viral genetic material (RNA) in a patient's sample.
Rapid Antigen Tests	Detect specific viral proteins, known as antigens, in respiratory samples.
Antibody Test	Detect antibodies (IgM and IgG) produced by the immune system in response to a SARS-CoV-2 infection.

LIMITATIONS OF RAPID TEST KIT (RTK) ANTIGEN TESTS

The RTK antigen test is a diagnostic test used to detect the presence of specific proteins (antigens) from the SARS-CoV-2 virus, which causes COVID-19. While this test has several advantages, including quick results and lower cost, it also has some disadvantages:

1. Lower sensitivity: RTK antigen tests have lower sensitivity compared to other tests like RT-PCR, which can result in false-negative results. This means that if a person has COVID-19, the test may not detect the virus, and they may be wrongly classified as negative (Brihn et al., 2021).
2. Limited specificity: The RTK antigen test can also have limited specificity, which can result in false-positive results. This means that if a person does not have COVID-19, the test may incorrectly show that they do have the virus (MDA, 2023).
3. Timing of the test: RTK antigen tests are more reliable in detecting COVID-19 when the person is symptomatic and during the early stages of infection. As the infection progresses, the viral load decreases, making it harder to detect the virus using this test (Wan et al., 2021).
4. Dependence on the operator: The RTK antigen test requires a skilled operator to perform the test correctly, and any errors in the testing process can lead to inaccurate results (Bernama, 2021).
5. Inability to identify mutated variants: RTK antigen tests are designed to detect a specific protein from the SARS-CoV-2 virus, and they may not detect new variants or mutations of the virus (Khandker et al., 2021).

RTK antigen test and RT-qPCR serve different purposes and have distinct strengths and limitations in COVID-19 detection. While the RTK-Antigen test identifies specific proteins of the SARS-CoV-2 virus, the RT-qPCR test identifies the virus's genetic material. Overall, while the RTK antigen test has several advantages, it is important to understand its limitations and potential for inaccurate results. Hence, the RTK antigen is a valuable tool for quick screening and point-of-care testing while the RT-qPCR method remains the gold standard for diagnosing COVID-19 infections for its highly sensitive and specific. Although RTKs for COVID-19 detection, including rapid antigen tests and antibody tests, have been used in clinical settings for screening and diagnosis of COVID-19, their use has certain limitations and considerations that vary by region and context. RTKs may not be the primary diagnostic tool in all clinical situations considering they are generally less sensitive than qPCR and may have limitations in detecting asymptomatic or early-stage infections. Besides that, in cases where an RTK produces a positive result, confirmatory testing using qPCR is often recommended to confirm the diagnosis, especially for individuals with symptoms or high-risk exposures. Most importantly, the performance of RTKs may be affected by the presence of new variants of the SARS-CoV-2 virus. Monitoring and validation of RTKs for variant detection are necessary.

REAL-TIME PCR, A GOLD STANDARD FOR THE ANALYSIS AND QUANTIFICATION OF COVID-19 IN CLINICAL DIAGNOSIS

Initially, the main approach to determine the presence of COVID-19 was by examining the presence of signs and symptoms, such as fever or chills (Struyf et al., 2022), cough, shortness of breath, muscle or body aches, headache, fatigue, sore throat, the new loss of taste or smell, dyspnoea (Alimohamadi et al., 2020), congestion, or runny nose (Iacobucci, 2021), nausea or vomiting, conjunctivitis (Ranzenigo et al., 2021), and gastrointestinal issues (Groff et al., 2021). Depending on an individual's age, immunological responses, and related co-morbidities, infection with SARS-CoV-2 results in reactions ranging from asymptomatic to incredibly diverse symptoms in various individuals (Hu et al., 2020). Nonetheless, in the majority of cases, young and healthy individuals may exhibit no or very moderate symptoms, acting as silent carriers and causing covert infections (Johansson et al., 2021). Therefore, multiple approaches have been developed to effectively diagnose COVID-19.

In RT-qPCR for COVID-19 detection, there are two main alternatives which are TaqMan probe-based and SYBR Green-based assays (Tao et al., 2022). Universally, TaqMan probe-based assay is preferable because it uses a fluorescent-labelled internal DNA probe that anneals within the target amplification region and a quencher molecule. The TaqMan probe assay uses a fluorescent-labelled oligonucleotide probe with 5' and 3' ends. The probe has a fluorescent reporter at the 5' ends and a fluorescent quencher at the 3' ends. The 5' reporters and 3' quenchers are near until amplification; therefore, no signal is seen (Tao et al., 2022). After separating the 5' end reporter and 3' end quencher, a fluorescent signal appears. The RT-qPCR enzymatic procedure incorporates the probe into the PCR product, separating the reporter and quencher. The TaqMan assay is more specific and sensitive because it relies on two processes: 1) primer binding to its target sequences and 2) probe binding to a complementary sequence in the downstream region of the primer (Malekshahi et al., 2022). Due to its high sensitivity and specificity, the majority rely on RT-qPCR. However, since all of these techniques entail the amplification of many genes, which necessitates the use of fluorogenic probes and one-step reagents (cDNA synthesis followed by PCR amplification in the same tube), they are limited to the use of more specialized reagents and can be quite costly (Pereira-Gómez et al., 2021). Moreover, these protocols involve the amplification of more than one gene, which implies different probes and fluorescent channels, adding to costs.

SYBR Green-based qPCR is a cheaper and more reagent-friendly alternative. When attached to the minor groove of double-stranded DNA, SYBR Green I fluoresces (Dragan et al., 2012). However, nonspecific primer binding, which results in the synthesis of primer dimers and the development of undesired PCR products, can greatly affect the sensitivity and reliability of PCR signals (Marinowic et al., 2021). By employing appropriate primer sets, the SYBR Green method demonstrated equivalent

or better performance compared to the TaqMan method. This observation holds even when accounting for the presence of recently prevalent or developing variants, such as Delta, Eta, Kappa, Lambda, Mu, and Omicron (Tao et al., 2022). When performing multiplex qPCR technique using the non-specific intercalating dye SYBR Green, a raised concern in detecting different genes found in SARS-CoV-2 is the fluorescence of gene amplicons in the same wavelength. In correlation to that, to achieve multiple target detection in SYBR Green assay, sequences are modified by manipulating the GC clamp. This alteration is necessary to obtain targets with different melting temperatures (Souza et al., 2022). By modifying the melting temperature, different peaks will be produced at the end of the qPCR run which distinguishes multiple genes detection. Understanding the importance of RT-qPCR, several studies have developed a cost-effective test using SYBR Green RT-PCR methodology by optimizing several parameters leading to a high specificity in the PCR products.

SYBR GREEN VS. TAQMAN

The TaqMan-based RT-qPCR uses hydrolysis probes that are made of sequence-specific dually fluorophore-labelled DNA oligonucleotides that allow the real-time detection of only specific amplification products. A fluorescent signal is generated when the probe is hybridized with the target DNA. The benefit of using TaqMan is that the probes can be labelled with reporter dyes that can detect more than one DNA sequence in one reaction tube. Therefore, the TaqMan-based RT-qPCR is chosen as the gold standard for diagnosing individuals infected with COVID-19 for its high specificity and sensitivity, and the ability of multiplex qPCR. The SARS-CoV-2 RT-qPCR assay frequently uses a multiplexing technique to target multiple genes in the virus genome (Corman et al., 2020). This is a crucial application as the high frequency of viral genome mutations can reduce the primer's ability to detect viruses leading to false-negative results. However, the main disadvantage of the TaqMan probe-based method is that the synthesis of dual-labelled fluorescent probes and TaqMan probe-based commercial real-time (RT) PCR kits are expensive. On the other hand, SYBR Green is a double-stranded DNA-binding dye for real-time PCR that fluoresces when bound to double-stranded DNA. The advantage of using SYBR Green is that it can be used to detect and quantify the amplification of any double-stranded DNA sequence without using any hydrolysis probes (Table 2). Hence, this can reduce the cost of PCR runs and assays, especially for massive and continuous testing of COVID-19 in the community.

Table 2 The differences between SYBR Green and TaqMan used in quantitative polymerase chain reaction (qPCR) for the detection and quantification of SARS-CoV-2 nucleic acids

qPCR Detection	SYBR Green	TaqMan
Principle	Uses a fluorescent dye that binds to double-stranded DNA.	Uses sequence-specific probes that are designed to anneal to a target DNA or RNA sequence.
Methodology	Primers specific to the target sequences are used in combination with the SYBR Green dye.	Primers in addition to the TaqMan probe. The probe is labelled with a fluorescent reporter dye at one end and a quencher at the other end.
Pros	Can detect any double-stranded DNA or cDNA (versatile) and is cost-effective.	Highly specific and provides accurate quantification of target DNA or RNA.
Cons	Prone to nonspecific amplification and amplification of unintended targets.	More expensive than SYBR Green assays due to the need for custom-designed probes. The design of specific probes can be more complex and time-consuming.

COST ISSUE OF RT-qPCR

The TaqMan-based RT-qPCR test was the most used diagnostic method for detecting the presence of COVID-19 (Sarkar et al., 2022). The cost issue associated with TaqMan-based RT-qPCR tests is mainly due to the complexity and time-consuming nature of the testing process. This involves several steps, including RNA extraction, reverse transcription, PCR amplification, and data analysis. Each step requires specific reagents, equipment, and skilled personnel, all of which can be expensive. In addition, the high demand for COVID-19 testing has put a strain on the global supply chain, leading to shortages of critical reagents and supplies, which can drive up costs even further. Furthermore, as new variants of the virus emerge, the design and validation of new probes and assays may be required, which can add to the cost and complexity of testing (Neopane et al., 2021). The cost to run a qPCR assay for COVID-19 detection can vary widely depending on several factors. These factors include the specific assay kit or reagents used, the location and country where the testing is conducted, the scale of testing, the purification level of the extracted RNA template and any associated laboratory or healthcare service fees. Generally, the cost of each sample processing using SYBR green qPCR sample ranged between ~2 and ~6 USD (Sarkar et al. 2022). Meanwhile, a rough estimation for the cost to run TaqMan qPCR can reach up to 20 USD or more, for each sample. The exact costs for both methods depend on factors like the brand, supplier, plasticware and specific assay kit used. As for the TaqMan, additional costs include the TaqMan probe master mix and probes. Overall, the cost issue associated with TaqMan-based RT-qPCR tests for COVID-19 detection is a multifactorial problem that requires a coordinated effort from the government, industry, and healthcare sectors to ensure affordable and accessible testing for all.

SYBR GREEN AS AN ALTERNATIVE

Generally, the probe-based qPCR is often preferred over SYBR Green-based qPCR for its specificity, multiplexing, quantification accuracy, better for detecting low-abundance targets and robustness. Several studies as shown in Table 1 have proven that SYBR Green qPCR assay can produce similar results to probe-based qPCR. Thus, it is important to note that SYBR Green-based qPCR also has many capabilities similar to the probe-based qPCR being cost-effective, simpler to set up, and suitable for certain applications. It has been shown to play a significant role in enabling COVID-19 testing and research in various countries for its cost-effective option especially those in resource-limited settings, contributing to the global detection response to the pandemic (Rahmasari et al., 2022). One of the main cost-saving advantages of using SYBR Green is that it eliminates the need for specific fluorescent probes. Other qPCR methods, like TaqMan probes, require expensive labelled probes for each target, whereas SYBR Green simply uses a single, more affordable fluorescent dye. The reduced need for specialized probes and simplified workflow can significantly lower the overall cost of performing RT-qPCR experiments, making it more financially feasible for resource-constrained countries and institutions. SYBR Green can be used for the detection of any PCR product, as it binds to any double-stranded DNA. This versatility allows researchers to study a wide range of genes or targets without the need for custom-designed probes. Since SYBR Green is a single dye and doesn't require target-specific probes, the experimental workflow is simplified. This reduces the overall complexity of the procedure and makes it more accessible to laboratories with limited resources and expertise.

The qPCR method is considered one of the gold standard methods for the detection of SARS-CoV-2, especially in clinical diagnostic settings. It has been widely adopted and recommended by health authorities and organizations around the world for its high sensitivity and specificity, quantitative capability, robust validation, compatibility with high throughput testing and accuracy in detecting variants. Other diagnostic methods such as rapid antigen tests have also played important roles in complementing qPCR testing. For monitoring, health authorities and experts continue to use the qPCR method to ensure results reliability. Hence, the SYBR Green method offers costs lower than TaqMan probe-based qPCR, making it a viable option in low-income countries. SYBR Green is a widely available reagent, offered by many biotechnology companies, making it easily accessible to laboratories worldwide. Here, we discuss the optimization of the SYBR Green method that increases the assay sensitivity, specificity and accuracy.

Sensitivity and Specificity

When a diagnostic test is developed to screen for a disease, we must determine how valid that test is. Does it measure accurately? Sensitivity and specificity are two strong indicators that describe how valid a test is by indicating the accuracy of the test. The accuracy of qPCR assays is often measured in terms of sensitivity and specificity.

Sensitivity refers to the ability of the assay to correctly identify positive cases (true positives), while specificity measures its ability to correctly identify negative cases (true negatives). High sensitivity and specificity are essential for reliable COVID-19 detection. The sensitivity of RT-PCR probe-based assay for COVID-19 detection can be as high as 95% or more while the specificity is also typically high, often exceeding 98%. Typical performance characteristics of qPCR tests for COVID-19 have reported sensitivities of 95% or higher, meaning they correctly identify 95% or more of the true positive cases. Many commercial tests have reported specificities of 95% or higher, indicating that they correctly identify 95% or more of the true negative cases.

In the Republic of Korea, researchers developed a probe-based RT-qPCR assay at a sensitivity of at least 98.2% and a specificity of 100% (Chung et al., 2021). Several researchers have evaluated the analytical sensitivity between the probe-based assay and the SYBR Green assay. In 2020, a study showed the effectiveness of the SYBR Green assay with successful detection in all 8 dilutions. Both tests detected the majority of the 63 samples; the conventional PCR detected 93% of the samples with detection capacity up until 10^7 dilutions, while SYBR Green was able to detect 98.42% of the positive samples (Dorlass et al., 2020). In a study by Pereira-Gómez et al. (2021), the limit of detection of SARS-CoV-2 for ORF1b-nsp14 and N targets was 10 copies/reaction (2.5 copies/µL) for the probe-based qPCR while in the SYBR Green-based assays, the limit of detection observed were 50 copies/reaction (12.5 copies/µL) for the ORF1b-nsp14 target and 250 copies/reaction (62.5 copies/µL) for N target. Although the analytical sensitivity of the SYBR Green assay with the ORF1b-nsp14 target was slightly lower than the TaqMan reference assay, the SYBR Green overall performance was considered comparable with the TaqMan probe-based assays. In another study, Rahmasari et al. (2022) adapted the WHO-recommended TaqMan-based RT-qPCR technology that targets the nucleocapsid protein (N) gene and Nsp-14 gene of SARS-CoV-2 using a SYBR Green-based methodology. The SYBR Green-based assays were able to detect up to 10 copies of *in-vitro* transcript RNA, which matched the TaqMan-based assay in clinical samples with no false-positive or false-negative signals. This supports that the SYBR Green-based assay approach is a useful alternative for SARS-CoV-2 molecular identification. Studies of the development and validation of SYBR Green-based RT-qPCR are summarized in Table 3.

Table 3 Summary of SYBR Green-based RT-qPCR assay for the detection of COVID-19

Article	Method	Gene	Sybr Green kit	Probe base kit	Slope	Sensitivity	Specificity	R^2	Detection capacity
Sarkar et al., 2022	On-step multiplex RT-PCR	E, N	Sansure SYBR Green kit	Sansure kit (Hunan, China)	−3 (E) −3.55 (N)	93%	97%	0.98 (E) 0.99 (N)	10^{0}–10^{-5} dilution
Marinowic et al., 2021	2 steps multiplex	N, E and RdRp	SYBR Green Master Mix kit (Thermo Fisher, USA)	GoTaq qPCR Master Mix kit (Promega, USA)	N/A	N/A	N/A	N/A	200 ng
Dorlass et al., 2020	One step and two-step single detection RT-PCR	E	QuantiFast SYBR® Green RT-PCR kit (Qiagen, Germany)	Pyromark OneStep RT-PCR kit (Qiagen, Germany), Invitrogen Taq Platinum Polymerase kit (Thermo Fisher, USA)	−3.497	98.4	100%	0.99	8 dilutions (10 based)
Pereira-Gómez et al., 2021	One-step single detection	ORF1b-nsp14, N	Luna Universal qPCR Master Mix (New England Biolabs, USA)	One Step RT-qPCR TaqMan (Thermo Fisher, USA)	−3.327	Comparable performance	Comparable performance	0.9608	250 copies/ reaction or 62.5 copies/µL or ~10 copies µL
Ganguly et al., 2020	One-step single detection	ORF1b, N, Rdrp	NA	Power SYBR Green RT-PCR	−3.536	NA	NA	0.97	1.7×10^{6} copies/ mL of saliva
Rahmasari et al., 2022	One-step single detection	N, Nsp-14	2019-nCoV Nucleic Acid Diagnostic Kit (Sansure Biotech, China)	iTaq Universal SYBR Green Supermix (Biorad, USA)	−3.59 (N) and -3.57 (Nsp-14)	≥95%	100%	0.995 (N), 0.998 (Nsp-14)	10 copies/µL (N), 1000 copies/µL (Nsp-14)

NA indicates not available

Accuracy of PCR (R^2)

The accuracy of RT-PCR is measured as R^2 whereby the performance of a primer set is analyzed using a serial dilution of the target either a gene-specific plasmid or a cDNA preparation in which the gene of interest is known to be present. R^2 is the coefficient of correlation obtained for the standard curve in qPCR experiments and should be >0.98 (Bustin et al., 2009). An R^2 value of 0.98 indicates that approximately 98% of the variance in the Ct values can be explained by the linear relationship with the target concentrations, suggesting a highly reliable and accurate assay. Studies have shown that both SYBR Green and TaqMan probe-based assays can provide highly accurate results when appropriately designed, validated, and performed. While TaqMan probes may offer higher specificity due to their design, reducing the risk of false-positive results compared to SYBR Green assays, several studies have shown that the SYBR Green assays can still achieve a high level of accuracy when proper controls and validation are applied (Dorlass et al., 2020; Ganguly et al., 2020; Marinowic et al., 2021; Pereira-Gómez et al., 2021; Rahmasari et al., 2022; Sarkar et al., 2022). Furthermore, SYBR Green can be used in multiplex qPCR assays because it binds to any double-stranded DNA, allowing it to detect multiple amplicons generated from different targets. In multiplex qPCR, multiple target sequences can be simultaneously amplified and detected in the same reaction using different primer sets, each specific to the target of interest. Sarkar et al. 2020 demonstrated that SYBR Green can be used in one-step multiplex RT-PCR to detect the E and N genes of COVID-19. The multiplex qPCR using SYBR Green was successful with a high accuracy of >0.98 and acceptable sensitivity and specificity of 93% and 97%, respectively. Besides that, Marinowic et al., 2021, also showed that the SYBR Green assay was suitable to be used in multiplex qPCR which successfully detected N, E and Rdrp genes of COVID-19.

LIMITATIONS OF SYBR GREEN AND STRATEGIES FOR ADDRESSING THEM

Both SYBR Green and TaqMan probe-based assays have been widely used for COVID-19 detection through RT-qPCR. However, the TaqMan probe-based assays are generally known to offer higher specificity compared to SYBR Green and thus become the gold standard for COVID-19 detection. TaqMan probes bind specifically to the target sequence, and fluorescence is detected only when the probe is cleaved during PCR, ensuring a more specific signal. On the other hand, SYBR Green has a limitation whereby it binds to any double-stranded DNA, which can lead to false-positive signals if non-specific amplification occurs. This is probably the main concern of SYBR Green as the binding to nonspecific double-stranded DNA sequences can lead to false positive signals. Interestingly, studies have found that the SYBR Green method is comparable to the TaqMan probe method by having well-designed primers that do not produce non-target sequences. Besides that, the importance of melting-curve analysis is also

emphasized in the SYBR Green approach to overcome the non-specific amplification of amplicon (Tajadini et al. 2014). The limitation of SYBR Green can be minimized using several strategies that are proven to be successful by studies stated in Table 1. One of the most important strategies is the designing of specific primer sets for each target to avoid non-specific amplification and cross-reactivity. For multiplexing qPCR using SYBR Green, primers should have similar melting temperatures (Tm) to ensure efficient amplification. Secondly, the qPCR reaction conditions for each target need to be optimized to achieve efficient and specific amplification. This may involve adjusting primer concentrations, annealing temperatures, and PCR cycling parameters. Moreover, after the qPCR reaction, melting curve analysis is crucial and needs to be done to verify the specificity of the amplified products. This helps identify any non-specific amplification or primer dimers that might interfere with accurate multiplex detection. Besides that, it is also important to include appropriate positive and negative controls to validate the multiplex assay's specificity and sensitivity. This may include known positive samples and no-template controls. Careful primer design, optimization, and appropriate controls could minimize the risk of non-specific amplification and are key to obtaining accurate and reliable results in multiplex qPCR in SYBR Green assay. The performance of the SYBR Green technique can be on par with or even better than the TaqMan approach with the right primer and PCR settings (Tao et al., 2022). As the SARS-CoV-2 virus continues to evolve, and new variants emerge, it is essential to adapt primer and probe sequences used in COVID-19 qPCR assays to account for these genetic variations. Using the SYBR Green method, scientists can modify the primer sequences to ensure they still specifically target the virus. Using multiplexing SYBR Green qPCR assays to simultaneously detect multiple regions of the SARS-CoV-2 genome, this approach can increase assay robustness by reducing the impact of mutations in a single target region. Since SYBR Green is relatively cheaper compared to TaqMan probe-based assays, adapting qPCR assays to account for the genetic variations in SARS-CoV-2 can be done proactively worldwide to ensure the continued accuracy of COVID-19 diagnostic testing.

CONCLUSION

SYBR Green has shown significant potential in COVID-19 detection because of its cost-effectiveness and versatility for detecting multiple targets in multiplex reactions. Its limitation which is lack of specificity can be minimized and even eliminated by including strategies that have shown to be successful in many studies. Overall, SYBR Green-based assays can provide highly accurate results when appropriately designed, validated, and performed. SYBR Green-based qPCR assays have been used in research and some clinical settings for COVID-19 detection and they are proven to have comparable results with probe-based assays such as TaqMan for diagnostic purposes. Therefore, it should be accepted as the gold standard of COVID-19 testing based on the test's established accuracy and reliability, supported by extensive validation and comparison with probe-based qPCR.

ACKNOWLEDGEMENTS

We would like to thank the Universiti Malaysia Sabah for its strong support of research and innovation. We would also like to thank the Research and Innovation team at the Biotechnology Research Institute for their administrative and technical support in research and grant management.

FUNDING

This research was funded by Universiti Malaysia Sabah, Grant number DKC2009.

CONTRIBUTIONS

NAY provided the concept, design, and overall supervision of this study. HRM contributed to the writing of the original manuscript. NAY contributed to the proofreading and editing of this manuscript. All authors approved and agreed to be accountable for all aspects of the work. All authors have read and agreed to the published version of the manuscript.

ETHICS DECLARATIONS

The authors declare that they have no competing interests.

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