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Research Article
Development of an Open System for the Detection of SARS-CoV-2 Based on Melting Curve Analysis of SYBR Green I qPCR

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Abstract

Background and Objective: Whole-genome sequencing (WGS) is currently the gold standard for genomic surveillance and monitoring epidemiological trends in the SARS-CoV-2 variants of concern (VOCs). Although efficient, operational implementation is costly and time-consuming. To overcome this problem, an efficient and economical technique is needed. The objective of the present study was to perform a cost-effective, melting-curve-based analysis of the SYBR Green I qPCR method for detecting the Δ69-Δ70 mutation in SARS-CoV-2. **Materials and Methods:** This study developed methods using synthetic genes as template targets. In addition, different long GC tails were introduced into each forward DNA primer to enhance qPCR's ability to differentiate between the wild type and the Δ69-Δ70 variant. The methods were validated against SARS-CoV-2 (hCoV-19/Indonesia/JB-BGR-BRIN-011/2020). This virus sample was identified using WGS. **Results:** Based on primer annealing optimisation, annealing at 66.7°C enabled the specific recognition of each target. The qPCR conditions were: Pre-denaturation at 95°C for 3 min, then 35 cycles for the reaction: Denaturation at 95°C for 3 sec, annealing/extension at 66.7°C for 30 sec, followed by 1 cycle of dsDNA dissociation into ssDNA, used to analyze the melting curve of each PCR product, starting at 65 to 95°C. Mt Δ69-Δ70 melting peaks with temperature melting are around 80.5-80.6°C, WT is around 77.85-77.95°C. Comparing this method's result with those of the WGS result reveals similar findings. **Conclusion:** This approach has been successfully developed and can be used as an alternative method for detecting the Δ69-Δ70 mutation in Alpha and Omicron VOC screening and SARS-CoV-2 VOC surveillance.

Key words: Whole genome sequencing; SARS-CoV-2, a variant of concern, Δ69-Δ70 mutation, SYBR Green I qPCR, melting curve

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The impact of the Coronavirus (COVID -19) pandemic on health has been highly significant. WHO, partner organizations and global researchers (including GISAID, Nextstrain and Pangolin) have monitored SARS-CoV-2 evolution since early January 2020. All viruses, including SARS-CoV-2 mutate over time. Some mutations facilitate viral adaptation. Alterations or deletions can affect the structure and function of viral proteins, influencing pathogenicity, infectivity, transmissibility, antigenicity and peptide polarity. Monitoring amino acid changes with substantial impact guides response and prevents spread. Reducing transmission is crucial to lowering mutation rates, which may have adverse public health consequences.

The above institutions develop the nomenclature for SARS-CoV-2. The WHO recommends using Greek alphabet names for variants because they are easier and more practical for widespread use. Classifications include variant under monitoring (VUM), variant of interest (VOI) and variant of concern (VOC). VOC criteria: Increased transmission and changes in COVID-19 epidemiology, increased virulence and clinical disease changes and increased virulence and clinical disease changes and decrease effectiveness of diagnostics, vaccines and disease management treatments[1].

Whole Genome Sequencing (WGS) currently remains the gold standard for genomic surveillance and variant detection in SARS-CoV-2, playing a pivotal role in tracking epidemiological trends. This method is efficient and robust, but it is also costly and time-consuming. These limitations challenge for the implementation of genome monitoring, particularly in developing countries. Therefore, a practical and affordable method is needed for broader application, especially in resource-limited settings. In addition, SARS-CoV-2 detection commonly employs TaqMan real-time PCR technology as the gold standard. However, SYBR Green I real-time PCR-based technology offers greater cost-effectiveness and widespread uses[2,3].

The Alpha variant was the first VOC to be discovered. Alpha (B.1.1.7) carries 23 point mutations. Initially identified in the UK, it is 50-100% more transmissible than earlier variants[4]. The Δ69-Δ70 deletion site is a distinct marker of the Alpha/B.1.1.7 lineage[4,5] and also occurs in Omicron[1,6]. The presence of a delta 69-70 gene deletion in the spike region resulted in spike-gene target failure (SGTF). This was in the real-time reverse transcriptase polymerase chain reaction diagnostic assay of SARS-CoV-2[7]. Additionally, other detection technologies for Alpha variants and other VOCs have also been reported, generally relying on TaqMan real-time qPCR. Zelyas *et al.*[8] developed assays for Alpha (B.1.1.7), Gamma (P.1) and Beta (B.1.351) VOCs, using TaqMan Real-time PCR technology[8]. Similar to the technology used by Zelyas *et al.*[8] VOC detection technology has been developed and applied to detect the Alpha (B.1.1.7) or Beta (B.1.351) variants, or both[9-12]. Further, Sater *et al.* developed Alpha variant VOC detection, focusing on Δ69-Δ70 and Δ106/Δ107/ Δ108 using SYBR Green I real-time PCR[5]. Despite its excellent accuracy, TaqMan technology is still prohibitively costly for numerous laboratories in developing countries to use[5]. Commercial detection leveraging melt curve analysis is available, such as the eTIB MOLBIOL VirSNiP SARS-CoV-2 Spike E484A assay (Cat. No. 53-0829-96)[12]. Therefore, this study aimed to develop and validate a cost-effective, open-system SYBR Green I real-time PCR melting curve assay for detecting the SARS-CoV-2 Δ69-Δ70 deletion associated with the Alpha variant.

MATERIALS AND METHODS

Study area and duration: The study was conducted at the Genomic and Biosafety Level 3 Laboratories of BRIN, located in the Science and Technology Park Ir. Soekarno, Cibinong, Indonesia, from January 2023 to March 2025.

Ethical approval: This study has clearance number 003/KE.06/SK/03/2024 issued by the Research Ethics Committee of BRIN.

Materials: A synthetic gene for Δ69-Δ70 and its wild-type counterpart, targeting a similar area, was used for the detection of SARS-CoV-2. The gene was inserted into the plasmid provided by Macrogen Korea. The plasmids were transformed into *Escherichia coli* DH5α competent cells for storage and plasmid amplification. The SARS-CoV-2 virus (hCoV-19/Indonesia/JB-BGR-BRIN-011/2020) served as a method validation control.

DNA primer and ssDNA design: The primers were designed to target the spike gene containing the ΔH69/ΔV70 deletion. The specific ΔH69-V70 FW-forward orientation contains 14 base GC-rich tails in the 5′ area (bold nucleotide): 5′- GCGGGCAGGGCGGCCTT GGT TCC ATG CTA TCT CTG 3′ and 6 base GC-rich tails for the forward primer of WT- ΔH69-V70 FW: 5′- GCGGGC CTT GGT TCC ATG CTA TAC ATG TCT 3′. (The underlined nucleotide in the WT-ΔH69-V70 FW indicates the location of the ΔH69/ΔV70). The reverse primer that amplified both alleles was ΔH69-V70 Rev: 5′- GGAAGCAAAATAAACACCAT3′. Melt curve pattern

prediction for both targets was performed. Furthermore, a single-stranded DNA sequence (ssDNA) probe was created for cDNA synthesis from the RNA template. The ssDNA used were: ssDNA1-rev: 5′ AAA AAT CCA GCC TCT TAT TA 3′ and ssDNA 2-rev: 5′ GAA ACT TTT TGT TAG ACT CA 3′.

SYBR Green I gradient qPCR for annealing optimisation, followed by total volume optimisation for the Δ69-Δ70 mutation and wild-type (WT): The PCR was performed in two separate PCR tubes for each target. The reaction volume was 20 µL containing: 10 µL KAPPA SYBR Fast qPCR Master Mix (#KK4600); 0.5 µL of each forward and reverse DNA primer; 8 µL MQ; and 1 µL DNA template. The real-time PCR settings included denaturation at 95°C for 3 min, predenaturation for 3 sec, annealing optimization (60, 61.6, 65.1, 66.7 and 68°C) and a dissociation step for the PCR product melt curve analysis, with the temperature increasing from 65°C to 95°C. Melting curve analysis was performed using the Bio-Rad CFX96 onboard software. The melt curves for the wild type (WT) are displayed in blue, the Δ69-Δ70 mutation in red and the no-template control (NTC) in green. Amplification was further verified by 1% agarose gel electrophoresis. Optimization was conducted using a reduced volume of 5 µL KAPA SYBR Fast qPCR master mix (#KK4600), for a total reaction volume of 10 µL. Each reaction was performed in triplicate. Additionally, the optimized detection method was validated using the ABI Fast 7500 system to ensure consistency. With the ABI Fast 7500, melting curve colours for both WT and Δ69-Δ70 mutation were generated by default.

Specificity assay for SYBR Green qPCR: The specificity test was assessed using a PCR mixture containing Fw and Rev DNA primers for WT. A plasmid containing the Δ69-Δ70 mutation as a DNA template. In another tube, a PCR mixture containing Fw and Rev DNA primers for the Δ69-Δ70 mutation was tested, with a plasmid containing WT as the DNA template. All reactions were performed in triplicate. The specificity test was optimised at annealing temperatures of 60, 61.6, 65.1, 66.7 and 68°C to determine the optimal annealing temperature for differentiating between WT and Δ69-70. The optimal temperature showed no amplification with mismatched template DNA and primer DNA, but did amplify with correctly matched primer DNA and DNA template targets.

Serial dilution of plasmid Δ69-Δ70 mutation for optimal melt curve analysis: A serial dilution of the plasmid DNA was performed starting at an initial concentration of 148 ng/µL, to the final concentration of 10^{-10}. Each dilution was then applied to a PCR tube and each dilution was repeated in triplicate. These steps were taken to achieve an optimal and ideal melt curve for analysis, confirmed by agarose gel electrophoresis, which should show a single band of the expected DNA size.

Validation of the detection of the Δ69-Δ70 mutation and the wild type (WT) using WGS methods: The SARS-CoV-2 (hCoV-19/Indonesia/JB-BGR-BRIN-011/2020), whose genome was sequence-verified using WGS methods, was used to validate the method. The virus was cultured and its RNA was extracted using a viral nucleic acid extraction kit (Geneaid #VR300), yielding a concentration of 124.9 ng/µL. cDNA was prepared using a ReverTra Ace® qPCR RT Master Mix with gDNA Remover (#Toyobo FSQ-301), combined with ssDNA as previously mentioned, which will hybridize to the target RNA. This cDNA was subsequently used to detect the target in the newly developed method. The amplification results were verified by the 1% agarose gel electrophoresis. Melting curve analysis was performed using the onboard software of the ABI Fast 7500.

RESULTS

Design and optimisation of primers for SYBR Green I qPCR -based detection of the SARS-CoV-2 Δ69-Δ70 mutation: This activity aims to provide a detection method for the Δ69-Δ70 mutation of the SARS-CoV-2 virus using a SYBR Green I qPCR-based technique. The DNA primers were designed based on a conserved region of the SARS-CoV-2 virus that differs from other viruses, particularly those prevalent in the human respiratory system (Fig. 1a). Additionally, GC-rich tails were added to both forward primers. According to the melt curve software prediction, this design may distinguish between the Δ69-Δ70 mutation and its wild type. The Δ69-Δ70 mutation is predicted to melt at 82.5°C, while the wild type is predicted to melt at 80.5°C (Fig. 1b). For virus identification, two reactions are performed in a single run. One set of tubes detects samples containing the Δ69-Δ70 mutation in SARS-CoV-2, while the other set detects wild-type samples. The detection method was developed using synthetic DNA as the target. Two synthetic genes were used in its development: one for detecting the Δ69-Δ70 mutation and the other for detecting the wild type. The real-time qPCR reagents used in each tube had the same composition, except for the forward primer DNA, which differed between tubes.

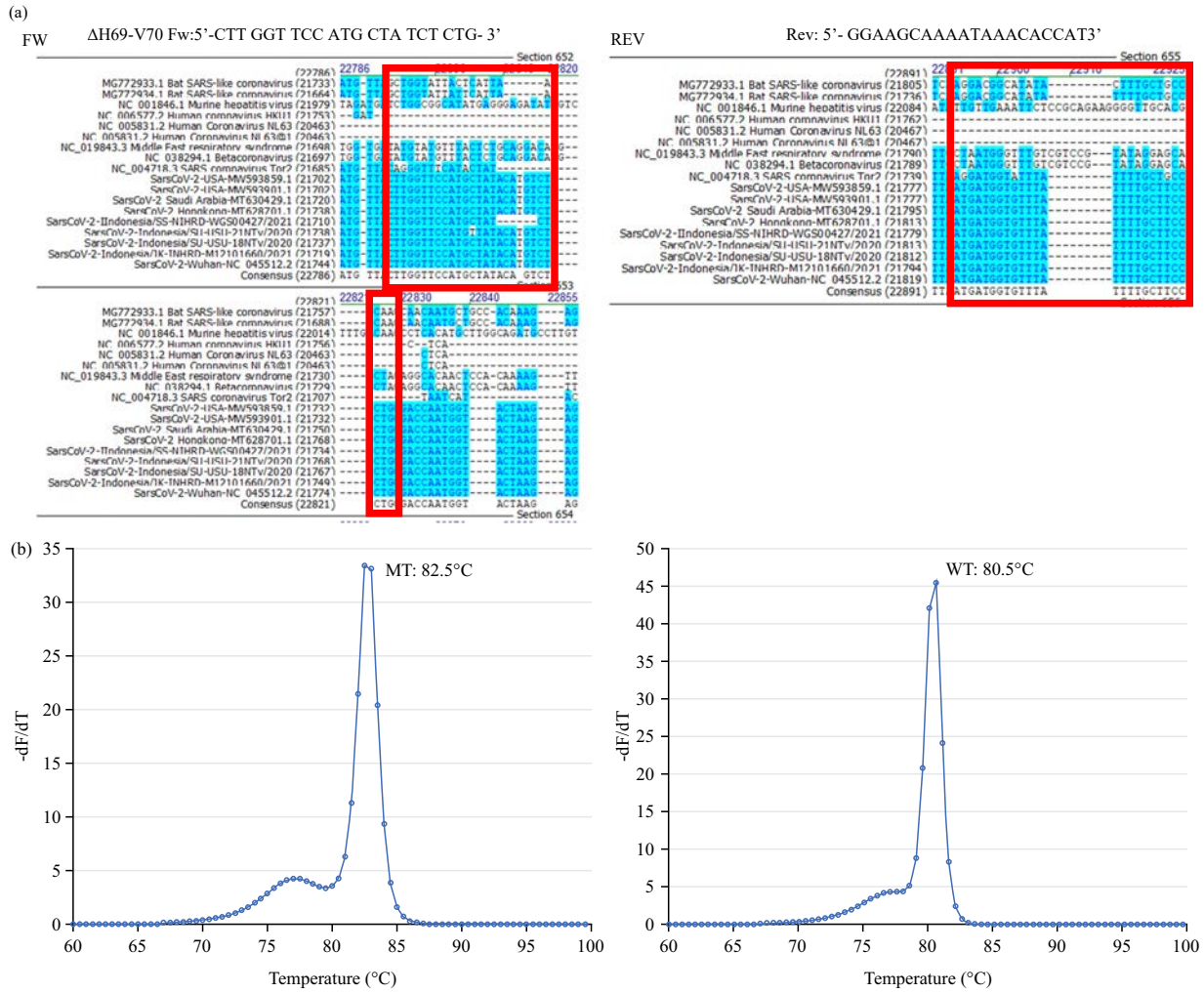


Fig. 1(a-b): Area for DNA primer design of Δ69-Δ70 and Wt detection, (a) Multiple alignment for DNA Primer specificity assay and (b) Melt curve prediction for each Δ69-Δ70 and Wt

The annealing temperature optimization using the gradient PCR technique (60, 61.6, 65.1, 66.7 and 68°C) showed that amplification was successful in each tube up to 66.7°C, enabling the detection of Δ69-Δ70 and WT (Fig. 2a-d). However, at higher temperatures, amplification did not occur (Fig. 2e). The wild-type showed a melt curve temperature of approximately 78°C (blue), while the Δ69-Δ70 variant exhibited a melt curve temperature of around 80.2°C (red). The in silico predicted melt curve temperatures for both the wild-type and Δ69-Δ70 variant displayed a similar pattern, with a difference of about 2°C between them. This key difference in melt curve temperatures allows effective differentiation between the wild-type and Δ69-Δ70 variants. These results indicate that the DNA primer design can reliably distinguish between the two variants based on their melt curve profiles. Further, comparing the real-time PCR reagent at 20 µL versus 10 µL showed similar results. To assess the consistency of the formulation, 10 µL of total volume was used again at two different temperatures, 60 and 61.6°C. Comparing of the two volumes showed similar results, indicating that a volume of 10 µL can also be used for detection, making this invention more economical (Fig. 3a-d). Therefore, further detection was performed at a 10 µL total volume.

Specificity assay of the selected SYBR Green I qPCR -based detection of the SARS-CoV-2 Δ69-Δ70 mutation: A specificity test was conducted to determine the optimal temperature at which the target could be detected. Each reaction contained a non-sample target. In the WT reaction, the Δ69-Δ70 DNA plasmid sample was added and vice versa in the Δ69-Δ70 reaction, where the wild-type (WT) plasmid sample was added. For this test, the gradient qPCR technique was performed at temperatures of 60, 61.6, 65.1, 66.7 and 68°C

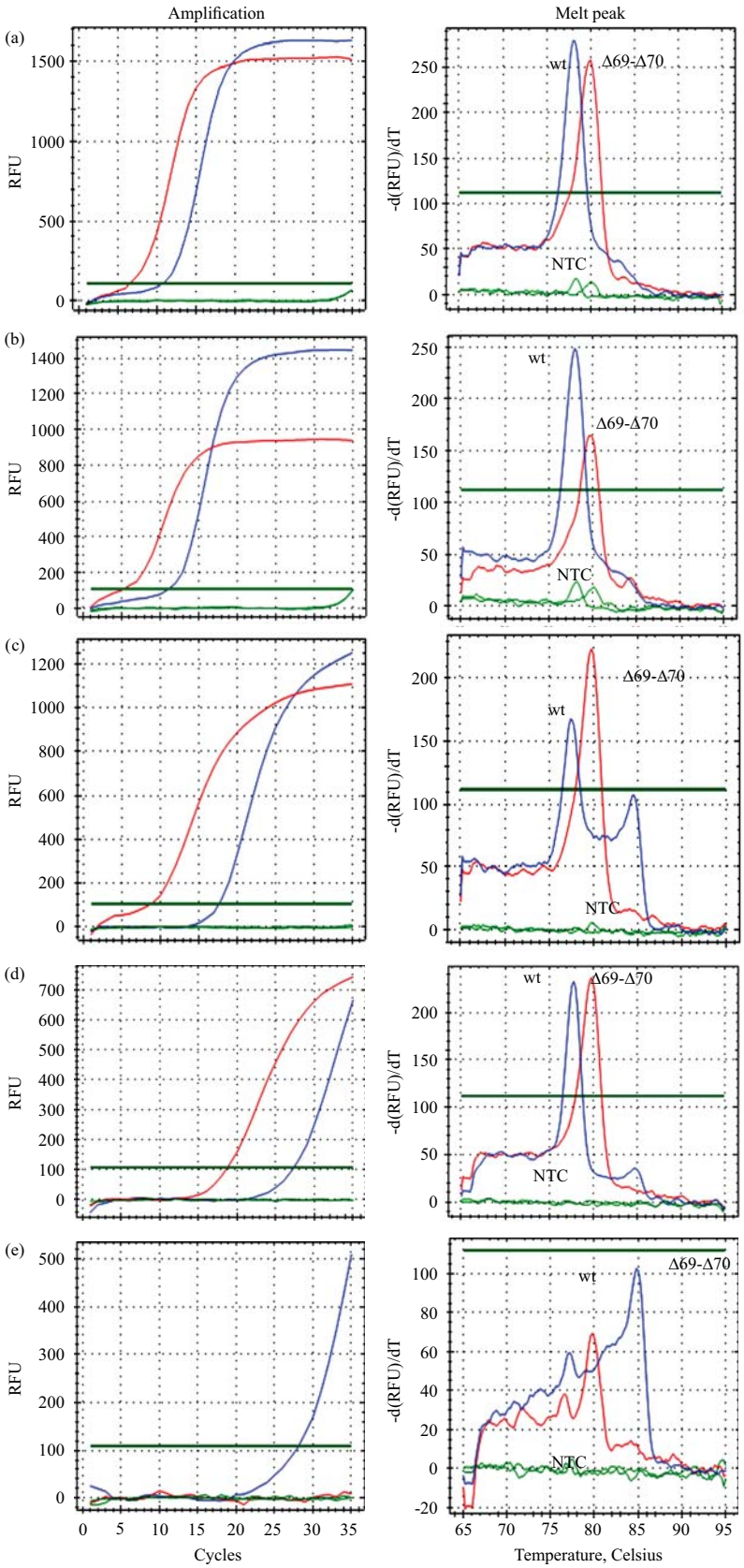


Fig. 2(a-e): Optimisation of the annealing temperature for Δ69-Δ70 and Wt detection using a Bio-Rad CFX96, (a) 60°C, (b) 61.6°C, (c) 65.1°C, (d) 66.7°C and (e) 68°C

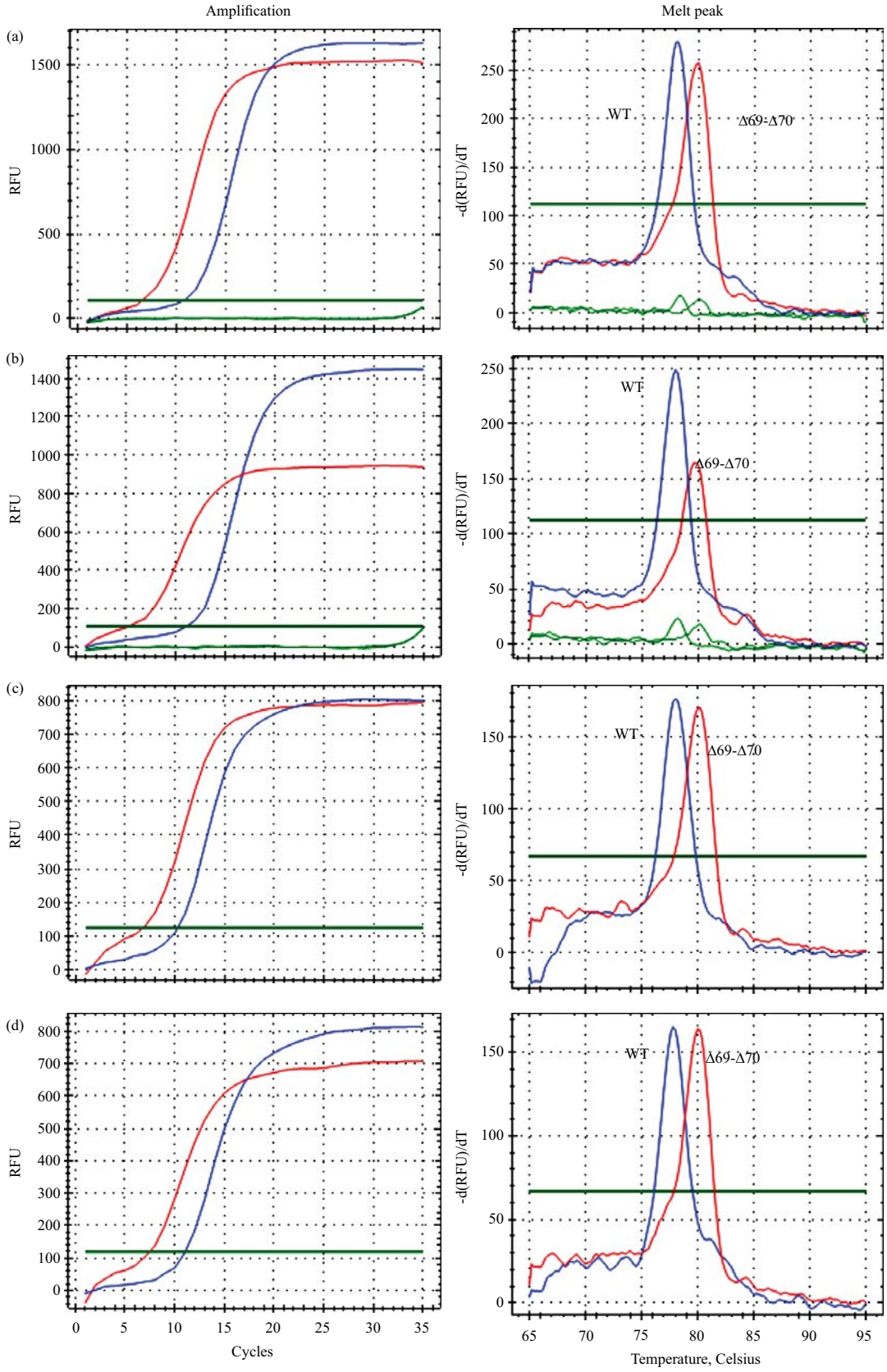


Fig. 3(a-d): Comparison of full (above) and half (below) receipts for Δ69-Δ70 and Wt detection using a Bio-Rad CFX96, (a) Full receipts at 60°C, (b) Full receipts at 61.6°C, (c) Half receipts at 60°C and (d) Half receipts at 61.6°C

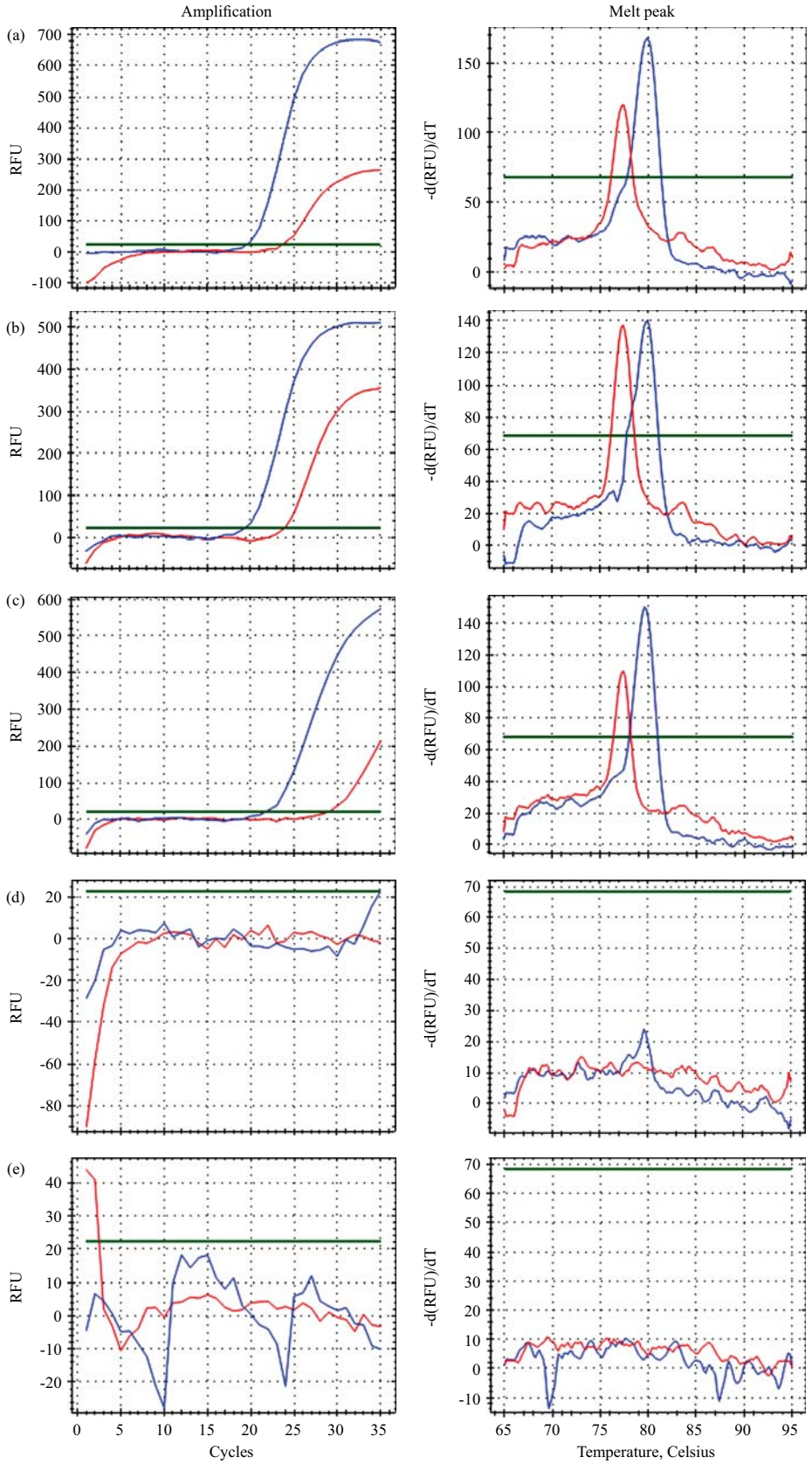


Fig. 4(a-e): Specificity assay for Δ69-Δ70 (red) and Wt (blue) detection (using Bio-Rad CFX96), (a) 60°C, (b) 61.6°C, (c) 65.1°C, (d) 66.7°C and (e) 68°C

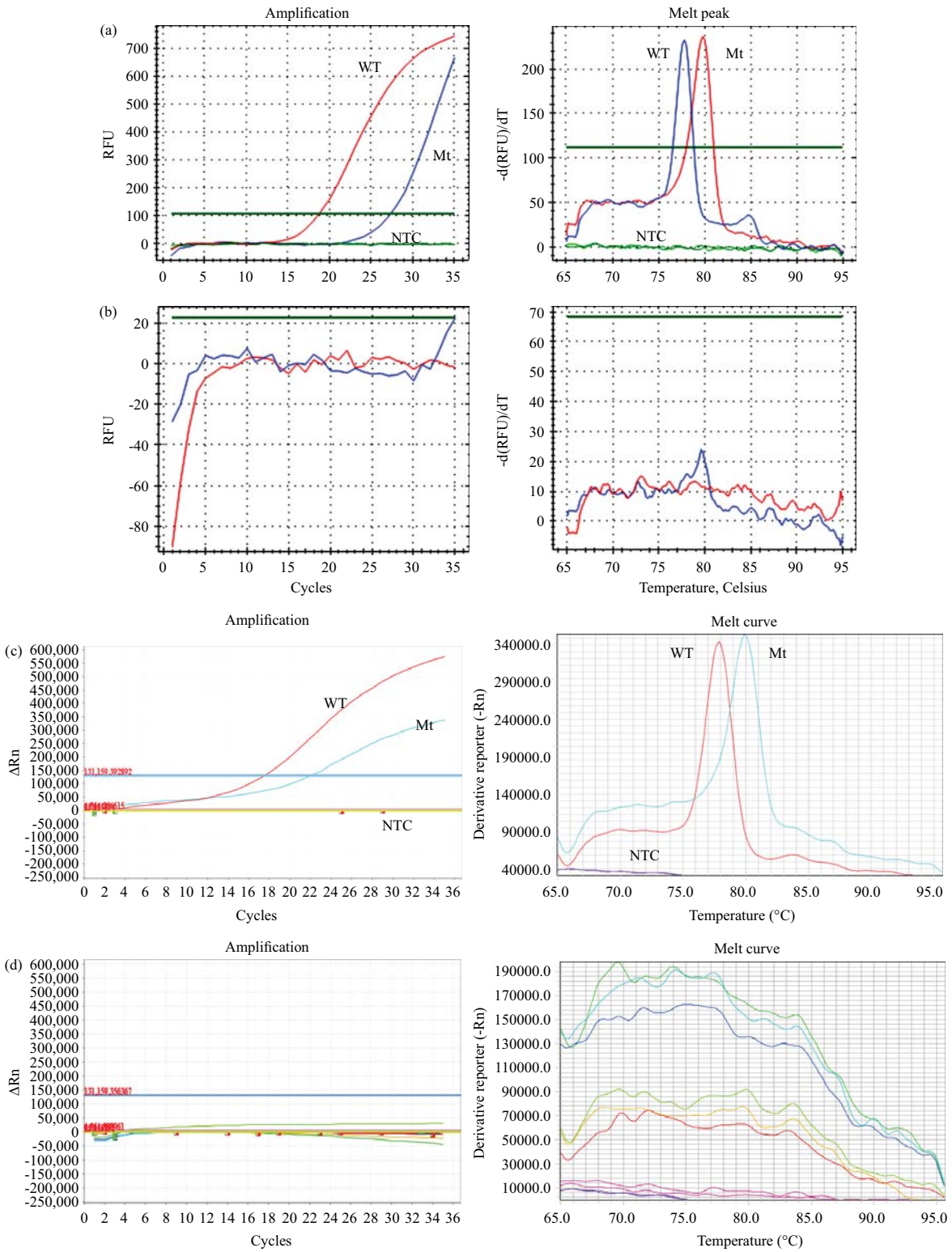


Fig. 5(a-d): Reproducibility assay for Δ69-Δ70 and wild-type (WT) detection performed at an annealing temperature of 66.7°C using two qPCR platforms, (a) Amplification of target DNA samples using the Bio-Rad CFX96 system, (b) Absence of amplification in non-target DNA samples using the Bio-Rad CFX96 system, (c) Amplification of target DNA samples using the ABI 7500 Fast system and (d) Absence of amplification in non-target DNA samples using the ABI 7500 Fast system

WT: Wild type and Mt: Mutant

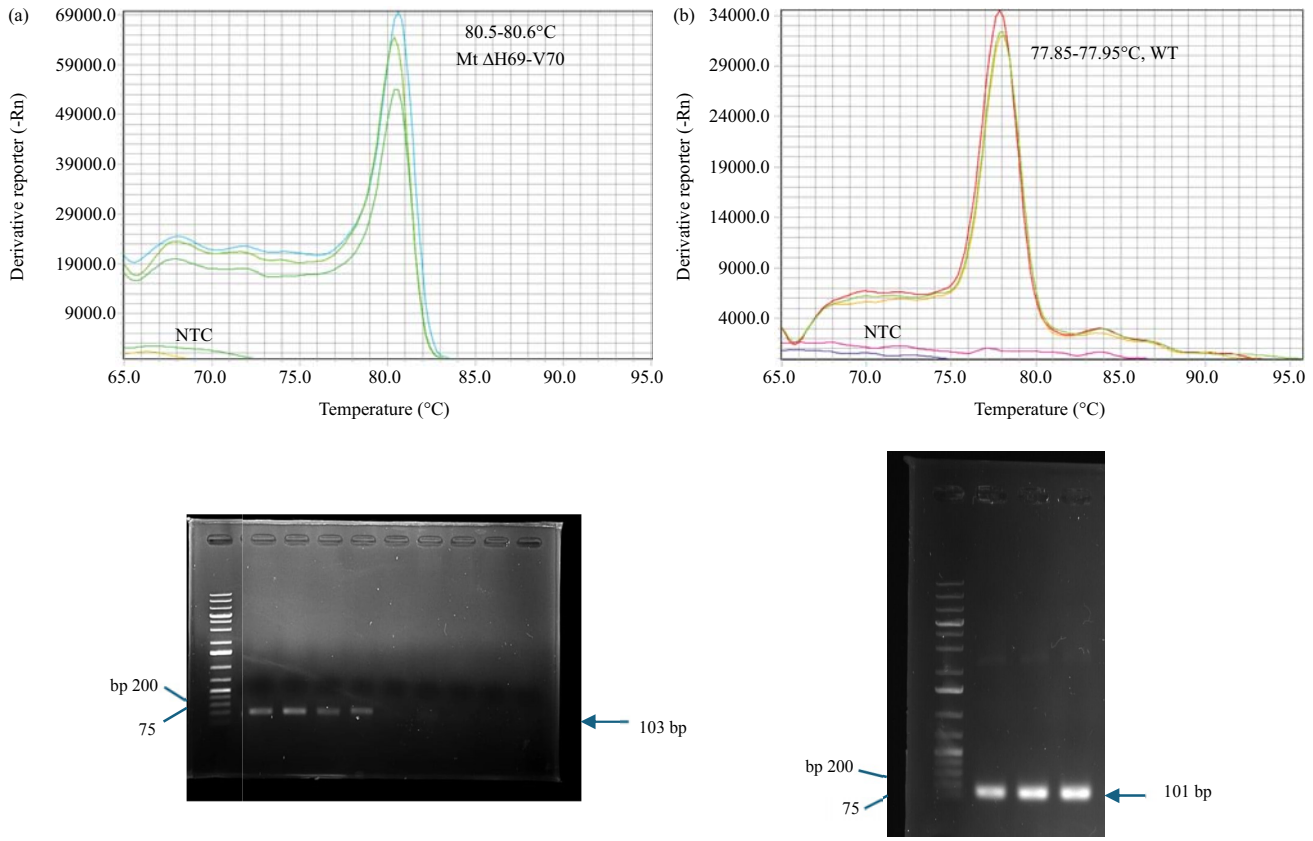


Fig. 6(a-b): Agarose gel electrophoresis (1%) verification of the optimized detection assays, (a) Detection of the Δ69-Δ70 variant showing a single specific amplification band and (b) Detection of the wild-type (WT) target showing a single specific amplification and the presence of a single band in each assay confirms the specificity of the optimized amplification conditions

(Fig. 4a-e). Based on the results, a temperature of 66.7°C yielded no amplification in each reaction (Fig. 4d). In the specificity assay, each reaction contained a non-target sample that should show no amplification. If amplification occurs, it indicates that the temperatures used to differentiate WT and Δ69-Δ70 variants were not correct. As already mentioned above, Fig. 2d shows that amplification occurred for each target at 66.7°C. Based on these two results (Fig. 2d and 4d), a temperature of 66.7°C was selected to detect and differentiate WT and Mt Δ69-Δ70. All experiments reported above were carried out with three replicates per test tube to ensure the accuracy of the results. Furthermore, similar results were obtained when switching from a Bio-Rad CFX96 to an ABI 7500 Fast, indicating high reproducibility and consistency (Fig. 5a-d). It is possible that these developed methods could be applied to other real-time PCR machines.

Since the Δ69-Δ70 sample produced a non-specific DNA band on a 1% agarose gel, the DNA concentration was optimized. This optimisation produced a single DNA target band, showing that the melting temperature of Mt Δ69-Δ70 is around 80.5-80.6°C. For WT, the DNA concentration was not optimized since it already showed a single DNA band on agarose with a melting temperature of around 77.85-77.95°C (Fig. 6a-b). The data of serial dilution were not shown.

Method validation for detecting the SARS-CoV-2 clinical sample: Further validation was performed using the SARS-CoV-2 labelled as the hCoV-19/Indonesia/JB-BGR-BRIN-011/2020 clinical sample, for which the complete genome sequence had already been confirmed using WGS methods. In addition, the position of ssDNA in the SARS-CoV-2 genome used for cDNA synthesis is shown in Fig. 7a. The validation showed that the recently developed method successfully detected and identified the absence of the Δ69-Δ70 mutation. This was performed two times in triplicate. Each one was carried out by a different operator on a different day. This was to confirm its reproducibility. This finding is consistent with the WGS results, as evidenced by the amplification and melt curve with a slightly shifted annealing temperature of the melt curve around 78.12-78.22°C. (Fig. 7b-c).

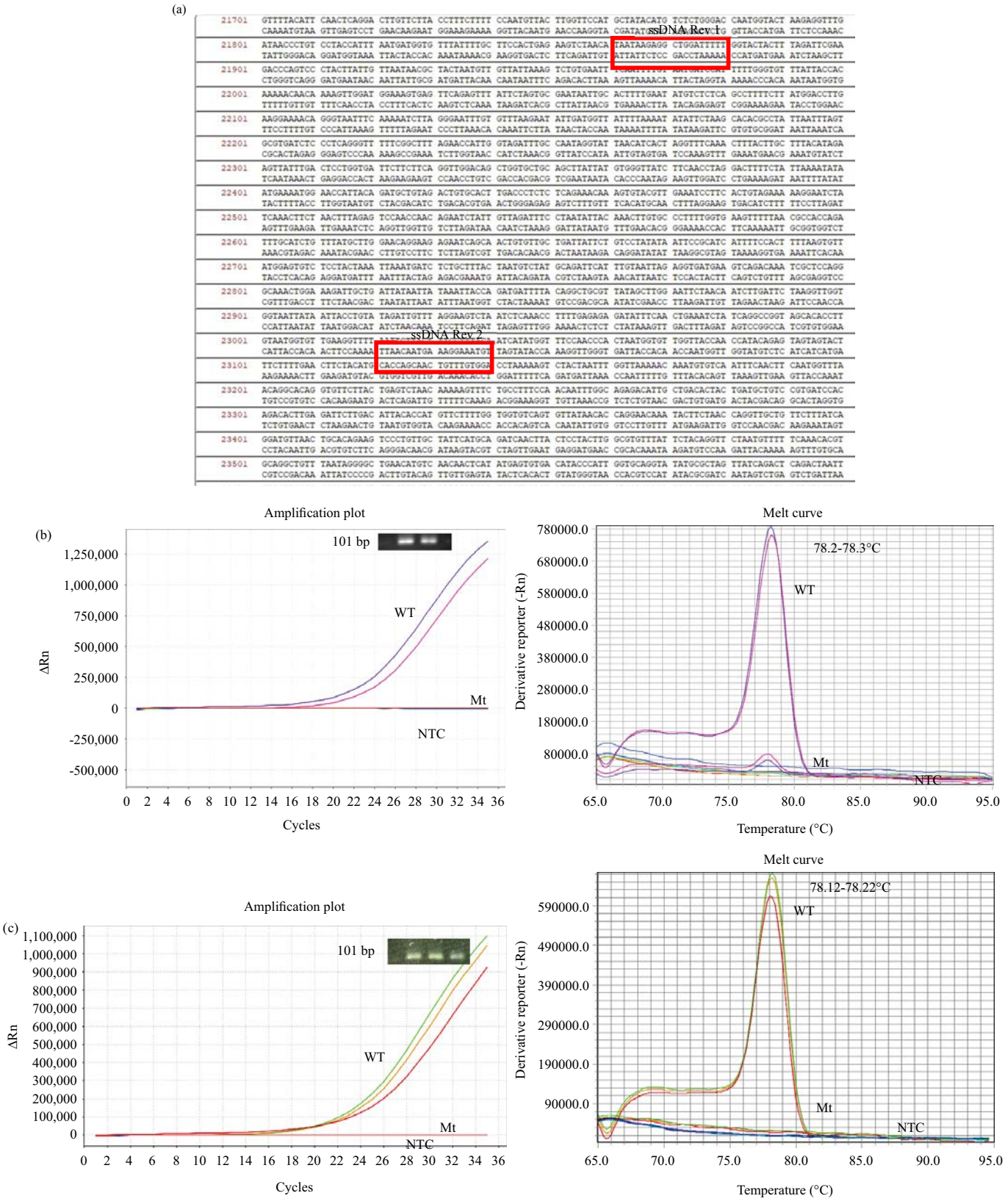


Fig. 7(a-c): Validation of Δ69-Δ70 and Wt detection with hCoV-19/Indonesia/JB-BGR-BRIN-011/2020 SARS-CoV-2 virus, (a) ssDNA attachment area and (b-c) Real-time PCR was performed using on cDNA by two different operators at different times, respectively

DISCUSSION

A robust surveillance system is necessary to detect and early identify Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) variants of concern (VOCs), which have critical public health implications. In consideration of the capacity of VOCs to potentiate transmission, increase deaths and possibly evade natural or vaccine-induced immune responses, it is crucial to identify cases of Coronavirus Disease (COVID-19) caused by VOCs. It is therefore critical to identify cases of VOC-induced disease and monitor their prevalence. Advances in technology, particularly next-generation sequencing (NGS), enable the generation of a contiguous sequence spanning the entire viral genome. This methodology facilitates the detection of mutations across the entire SARS-CoV-2 genome and can provide important insight into mutations that may affect the virus's behavior. Whole-genome sequencing (WGS) is the gold standard for diagnosing this variant and is currently the recommended method for confirming these variants. However, sequencing is generally more expensive and time-consuming than targeted detection methods.

This surveillance effort provided invaluable information on the spread and prevalence of SARS-CoV-2 VOC, eliminating the need for expensive and time-consuming genomic sequencing. A significant mutation in the S gene is a 6-nucleotide deletion at positions 207-212 (del69-70). This mutation is present in VOC Alpha (B.1.1.7) and Omicron[1,6]. The CDC suggests that S-gene target failure results can be used to identify possible B.1.1.7 cases, but sequencing should be used to confirm these results[13,14]. The accuracy of this approach has already been demonstrated and the sequencing results were entirely consistent with the RT-PCR profiles for VOC B.1.1.7.

Several approaches to detecting VOCs have been reported. These generally rely on TaqMan real-time qPCR. Despite its excellent accuracy, the cost of TaqMan technology is still prohibitive for many laboratories in developing countries. However, SYBR Green I real-time PCR technology is reported to be more cost-effective and has a wide range of applications[2,3]. In this activity, a real-time PCR assay was successfully developed to discriminate between WT and the ΔH69/ΔV70 and it was validated using the WGS method. It was found that an annealing temperature of 66.7°C was the optimum temperature for discriminating between WT and the ΔH69/ΔV70. Amplification and a melt curve occurred for both WT and ΔH69/ΔV70 when compatible DNA primers and DNA samples were used. No amplification or melt curve was produced when incompatible DNA primers and DNA samples were used at an annealing temperature of 66.7°C. The annealing temperature during PCR was one of thing that affected the specificity of the allele differentiation study[15]. Further, the primer design and formulation used in this report differ from those used by Abdel Sater *et al.*[5]. In this activity, the qPCR Tm Shift genotyping method principle was mimicked to discriminate between the wild-type (WT) and ΔH69-ΔV70 variants. The Tm Shift genotyping method uses two allele-specific primers, each containing one or two SNP allelic variants. A reverse primer is used for both DNA primers and SYBR Green I is used as a fluorescent dye[15,16]. This study uses the same reverse DNA primer to detect both the wild-type (WT) and the ΔH69-ΔV70 variants. The forward orientation of the wild-type (WT) and ΔH69-ΔV70 variants had unequal lengths of GC-rich tail added to each DNA primer. Additionally, a mismatch of more than two bases was present, particularly in the six-base nucleotide of the 3' region of the WT and ΔH69-ΔV70 DNA primers. This mismatch is located in the deletion area. Further, we use SYBR Green I as a fluorescent dye. A minimum of two mismatches in the six-base at the 3' region will prevent amplification and increase specificity. Mismatches in the middle or five-base 5' region, however, allow amplification[17,18].

Melt curve of Δ69-Δ70 mutations with a melting peak of Tm at around 80.5-80.6°C, whereas for detecting WT, around 77.85-77.95°C. These results were verified by the electrophoresis method on 1% agarose. The electrophoresis results indicate the presence of a single band of the correct size for each target DNA sample. Additional unequal GC-rich tails for each WT and H69-V70 were referred to in Wang *et al.*[16]. The unequal GC-rich tails produce different product lengths and the GC content distinguishes the Tm of each target. Adding the unequal GC tail has been proven to increase the discrimination between WT and its mutant[19-21]. Further, this method was validated using a sample that had been identified by WGS, SARS-CoV-2 (hCoV-19/Indonesia/JB-BGR-BRIN-011/2020). Comparing this method's result with those of the WGS result reveals similar findings. This indicates that the recently developed method is highly accurate. Based on the WGS results, SARS-CoV-2 (hCoV-19/Indonesia/JB-BGR-BRIN-011/2020) with the accession ID EPI_ISL_4004658 exhibits nucleotide mutations at positions C241T, C3037T, C5184T, T9127C, C14408T, C18877T, A23403G, G25563T, C26735T. The ssDNA probes designed in this research successfully hybridized with the target RNA, resulting in complementary DNA (cDNA). Both of the designed ssDNA probes were in reverse orientation. The cDNA results were confirmed using 1% agarose gel electrophoresis, which showed no DNA genome contamination (data not shown).

PCR amplification was then performed using this cDNA. In addition, ssDNA probes have been reported to hybridise effectively with RNA. McCalla *et al.*[22] developed a simple method for amplifying RNA targets (SMART), which used ssDNA probes to hybridise with the target RNA.

The Tm values obtained from the melt curve analysis slightly differed from both the predicted values and those obtained from amplification with synthetic DNA or cDNA. This difference occurred because of the different salt concentration, which contributes to the ionic strengths of the buffer used. According to Germer and Higuchi[15], the choice of polymerase used, the salt concentration and the annealing temperature during PCR affected the specificity of the allele differentiation study. Ionic strength increases the melting temperature to prevent the denaturation of DNA[23]. Additionally, changing the machine from a Bio-Rad CFX96 to an ABI 7500 Fast produced similar results. This indicates that it is an open system with high reproducibility and consistency that can operate on any instrument. The SYBR Green I approach in qPCR is reported as an open-system detection method that is compatible with any qPCR instrument. According to Rahmasari *et al.*[24], SYBR Green dye principally intercalates with any double-stranded DNA, producing a positive signal. Although the assay described here can accurately detect and differentiate between WT and ΔH69/ΔV70 deletions, with high reproducibility and repeatability, the developed methods need to be applied to more SARS-CoV-2 samples to prove its reliability.

CONCLUSION

The development of the Δ69-Δ70 and wild-type detection approach using a synthetic gene based on Melting Curve Analysis of the SYBR Green I qPCR method has been completed and validated using the SARS-CoV-2 genome (hCoV-19/Indonesia/JB-BGR-BRIN-011/2020), which has been confirmed and verified using WGS methods. The addition of different GC-rich tails in the 5' region of the forward DNA primer used to detect Δ69-Δ70 and its wild type improves the specificity of the primers, enabling differentiation between wild-type and mutant strains. The melting temperatures for Δ69-Δ70 and WT were observed to be around 80.5-80.6°C and around 77.85-77.95°C, respectively. Furthermore, the ssDNA probes designed in this study successfully hybridised with the target RNA, producing cDNA. These results demonstrate the potential of this method as an alternative for Δ69-Δ70 mutation detection, particularly for Alpha and Omicron VOC screening applications and for SARS-CoV-2 VOC surveillance.

SIGNIFICANCE STATEMENT

This study presents a cost-effective and rapid SYBR Green I qPCR melting-curve method for detecting the SARS-CoV-2 Δ69-Δ70 mutation. The assay successfully differentiated mutant and wild-type targets and showed concordance with whole-genome sequencing. This approach provides an accessible alternative for screening Alpha and Omicron variants and may support scalable SARS-CoV-2 genomic surveillance, particularly in resource-limited settings.

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REFERENCES

1. Thakur, S., S. Sasi, S.G. Pillai, A. Nag and D. Shukla *et al.*, 2022. SARS-CoV-2 mutations and their impact on diagnostics, therapeutics and vaccines. Front. Med., Vol. 9. 10.3389/fmed.2022.815389.
2. Alhamlan, F.S., A.A. Al-Qahtani, D.M. Bakheet, M.F. Bohol and S.I. Althawadi *et al.*, 2021. Development and validation of an in-house, low-cost SARS-CoV-2 detection assay J. Infect. Public Health, 14: 1139-1143.
3. Marinowic, D.R., G. Zanirati, F.V.F. Rodrigues, M.V.C. Grahl, A.M. Alcará, D.C. Machado and J.C. da Costa, 2021. A new SYBR green real-time PCR to detect SARS-CoV-2. Sci. Rep., Vol. 11. 10.1038/s41598-021-81245-0.
4. Alhamlan, F.S. and A.A. Al-Qahtani, 2025. SARS-CoV-2 variants: Genetic insights, epidemiological tracking, and implications for vaccine strategies. Int. J. Mol. Sci., Vol. 26. 10.3390/ijms26031263.
5. Abdel Sater, F., M. Younes, H. Nassar, P. Nguewa and K. Hamze, 2021. A rapid and low-cost protocol for the detection of B.1.1.7 lineage of SARS-CoV-2 by using SYBR green-based RT-qPCR. Mol. Biol. Rep., 48: 7243-7249.
6. Chrysostomou, A.C., A. Aristokleous, J.H. Rodosthenous, C. Christodoulou, G. Stathi and L.G. Kostrikis, 2023. Detection of circulating SARS-CoV-2 variants of concern (VOCs) using a multiallelic spectral genotyping assay. Life, Vol. 13. 10.3390/life13020304.
7. Pascall, D.J., E. Vink, R. Blacow, N. Bulteel and A. Campbell *et al.*, 2023. The SARS-CoV-2 Alpha variant was associated with increased clinical severity of COVID-19 in Scotland: A genomics-based retrospective cohort analysis. PLoS ONE, Vol. 18. 10.1371/journal.pone.0284187.

8. Zelyas, N., K. Pabbaraju, M.A. Croxen, T. Lynch and E. Buss *et al*., 2021. Precision response to the rise of the SARS-CoV-2 B.1.1.7 variant of concern by combining novel PCR assays and genome sequencing for rapid variant detection and surveillance. Microbiol. Spectrum, Vol. 9. 10.1128/spectrum.00315-21.
9. Yaniv, K., E. Ozer, M. Shagan, S. Lakkakula, N. Plotkin, N.S. Bhandarkar and A. Kushmaro, 2021. Direct RT-qPCR assay for SARS-CoV-2 variants of concern (Alpha, B.1.1.7 and Beta, B.1.351) detection and quantification in wastewater. Environ. Res., Vol. 201. 10.1016/j.envres.2021.111653.
10. Wollschläger, P., D. Todt, N. Gerlitz, S. Pfaender and T. Bollinger *et al*., 2021. SARS-CoV-2 *N* gene dropout and *N* gene Ct value shift as indicator for the presence of B.1.1.7 lineage in a commercial multiplex PCR assay. Clin. Microbiol. Infect., 27: 1353.e1-1353.e5.
11. Erster, O., E. Mendelson, V. Levy, A. Kabat and B. Mannasse *et al*., 2021. Rapid and high-throughput reverse transcriptase quantitative PCR (RT-qPCR) assay for identification and differentiation between SARS-CoV-2 variants B.1.1.7 and B.1.351. Microbiol. Spectrum, Vol. 9. 10.1128/spectrum.00506-21.
12. Sit, B.H.M., K.H.L. Po, Y.Y. Cheung, A.K. L. Tsang and P.K. L. Leung *et al*., 2022. Detection of SARS-CoV-2 VOC-omicron using commercial sample-to-answer real-time RT-PCR platforms and melting curve-based SNP assays. J. Clin. Virol. Plus, Vol. 2. 10.1016/j.jcvp.2022.100091.
13. Galloway, S.E., P. Paul, D.R. MacCannell, M.A. Johansson and J.T. Brooks *et al*., 2021. Emergence of SARS-CoV-2 B.1.1.7 lineage-United States, December 29, 2020-January 12, 2021. Morbidity Mortality Wkly Rep., 70: 95-99.
14. Sánchez-Calvo, J.M., J.C.A. Arboledas, L.R. Vidal, J.L. de Francisco and M.D.L. Prieto, 2021. Diagnostic pre-screening method based on N-gene dropout or delay to increase feasibility of SARS-CoV-2 VOC B.1.1.7 detection. Diagn. Microbiol. Infect. Dis., Vol. 101. 10.1016/j.diagmicrobio.2021.115491.
15. Germer, S. and R. Higuchi, 1999. Single-tube genotyping without oligonucleotide probes. Genome Res., 9: 72-78.
16. Wang, J., K. Chuang, M. Ahluwalia, S. Patel and N. Umblas *et al*., 2005. High-throughput SNP genotyping by single-tube PCR with T_m-shift primers. BioTechniques, 39: 885-893.
17. Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen and T.L. Madden, 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinform., Vol. 13. 10.1186/1471-2105-13-134.
18. Appelt, S., S.S. Aly, K. Tonooka, K. Glenn, Z. Xue, T.W. Lehenbauer and M.L. Marco, 2019. Development and comparison of loop-mediated isothermal amplification and quantitative polymerase chain reaction assays for the detection of *Mycoplasma bovis* in milk. J. Dairy Sci., 102: 1985-1996.
19. Desriani and F. Al-Ahwani, 2018. The sensitivity and efficacy method of PIK3CA exon 9 E545A as a high diagnostic accuracy in breast cancer. J. Genet. Eng. Biotechnol., 16: 71-76.
20. Desriani, Azamris, S.S. Ghaissani, S.R. Kinanti, M.A. Warisman and N. Fitria, 2021. Design and characterization of a SYBR green I-based melting curve method for investigation of HER2I655V polymorphism in breast cancer. J. Genet. Eng. Biotechnol., Vol. 19. 10.1186/s43141-020-00108-9.
21. Desriani, Azamris, P. Rustamadji, I.M. Abna and I. Ibadurrahman *et al*., 2024. Sensitive detection of PIK3CA exon 20 H1047R breast cancer based on low-cost intercalary dye SYBR Green I real-time qPCR assay. Sains Malaysiana, 53: 3683-3693.
22. McCalla, S.E., C. Ong, A. Sarma, S.M. Opal, A.W. Artenstein and A. Tripathi, 2012. A simple method for amplifying RNA targets (SMART). J. Mol. Diagn., 14: 328-335.
23. Kotin, L., 1963. On the effect of ionic strength on the melting temperature of DNA. J. Mol. Biol., 7: 309-311.
24. Rahmasari, R., M. Raekiansyah, S.N. Azallea, M. Nethania and N. Bilqisthy *et al*., 2022. Low-cost SYBR Green-based RT-qPCR assay for detecting SARS-CoV-2 in an Indonesian setting using WHO-recommended primers. Heliyon, Vol. 8. 10.1016/j.heliyon.2022.e11130.