

Endpoint Genotyping of A2063G and A2064G Mutations in *Mycoplasma pneumoniae*

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Abstract

Macrolide resistance in *Mycoplasma pneumoniae* is primarily associated with point mutations, most commonly A2063G and A2064G, in domain V of the 23S rRNA gene. Rapid detection of these mutations is important for appropriate antimicrobial therapy. In this study, we developed a fluorescence-based endpoint genotyping assay using minor groove binding (MGB) probe-based polymerase chain reaction (PCR) to detect mutations at positions 2063 and 2064 of the *M. pneumoniae* 23S rRNA gene. We designed position-specific MGB probes to selectively bind mutant sequences, enabling accurate discrimination between point mutations at adjacent nucleotide positions. In addition, we optimized the assay conditions to achieve clear genotype-specific fluorescence separation. The detection limits were 31, 27.2, and 32.7 copies/mL for wild type, A2063G, and A2064G mutations, respectively. We observed no cross-reactivity with 21 non-target respiratory pathogens. Clinical validation was performed using 44 respiratory specimens obtained from 44 patients with suspected *M. pneumoniae* infection. The developed assay demonstrated 100% overall percent agreement (44/44, 95% confidence interval, 92%–100%) with a commercially available real-time PCR assay for genotype identification, with a Cohen's κ value of 1. In summary, this multiplex MGB probe-based PCR assay could allow for a rapid and reliable detection of closely spaced macrolide resistance mutations in *M. pneumoniae*, thereby supporting timely antimicrobial decision-making in clinical settings.

Key Points

- Minor groove binder polymerase chain reaction detects macrolide resistance.
- Decreasing primer concentrations improved probe–target discrimination.
- This assay offers an alternative to Sanger sequencing for resistance genotyping.

INTRODUCTION

Mycoplasma pneumoniae is an atypical bacterium and a major cause of community-acquired pneumonia in both children and adolescents (Jiang et al. 2021; Kutty et al. 2019; Waites 2003; Waites and Talkington 2004). Owing to their efficacy and safety, macrolides are considered first-line therapeutic agents against *M. pneumoniae* infections in pediatric patients (Atkinson et al. 2008; Izumikawa 2016; Miyashita 2022). However, the extensive use of macrolides has driven the global emergence of macrolide-resistant *M. pneumoniae* (MRMP), substantially complicating its clinical management (Gdalevich et al. 2017; Sun et al. 2025; Yun 2025). The prevalence of MRMP varies geographically, where it is the highest in the Western Pacific region (53.4%), followed by Southeast Asia (9.8%), the Americas (8.4%), and Europe (5.1%) (Kim et al. 2022). MRMP infections are associated with prolonged illness, increased pneumonia severity, and a markedly elevated risk of extrapulmonary complications. Notably, macrolide resistance triples the risk of extrapulmonary manifestations and increases the likelihood of severe pneumonia by up to 5.4-fold (Zhou et al. 2014).

Macrolide resistance in *M. pneumoniae* is primarily mediated by point mutations, most commonly A2063G and A2064G, which are strongly associated with high-level resistance, in domain V of the 23S rRNA gene (Miyashita et al. 2025; Waites et al. 2017). However, additional substitutions have been described (Cao et al. 2017; Kim et al. 2017b; Phongsamart et al. 2026; Wang et al. 2024). These mutations reduce macrolide binding to the bacterial ribosome, resulting in diminished antimicrobial efficacy (Meyer Sauter et al. 2014). Importantly, macrolide-resistant and -susceptible infections could not be distinguished based on the clinical presentation or radiological findings alone, which underscores the need for the rapid molecular detection of resistance-associated mutations.

Although Sanger sequencing is widely used to identify mutations in the 23S rRNA gene, it represents a labor-intensive, costly, and time-consuming method, often requiring high target concentrations and prolonged turnaround times (Chan et al. 2013; Guo et al. 2019). These limitations restrict the utility of this approach in clinical practice and outbreak settings, where timely therapeutic decisions are critical. Real-time polymerase chain reaction (PCR)-based assays have emerged as faster alternatives, and several commercial detection kits for macrolide resistance mutations in *M. pneumoniae* have been evaluated (Leal et al. 2020; Wagner et al. 2019). However, numerous existing assays primarily target the most prevalent mutation, A2063G, whereas their ability to detect and differentiate the less common clinically relevant variants at the same hotspot (e.g., A2063T and A2063C), as well as A2064G, has not been sufficiently explored. Since the nucleotide position 2063 represents a major hotspot in domain V of the 23S rRNA gene, and multiple substitutions at this position could confer macrolide resistance, a rapid genotyping method with robust coverage of key resistance-associated mutations is required for accurate detection of macrolide resistance in routine practice. Moreover, in South Korea, where MRMP prevalence exceeds 86%, and most isolates harbor the A2063G mutation (Kim et al. 2017a; Lee et al. 2017; Park et al. 2025), clinically approved diagnostic kits capable of reliably detecting A2064G and other resistance-associated mutations remain limited or unavailable. This diagnostic gap indicates the need for a comprehensive and accessible molecular assay capable of detecting major macrolide resistance-associated variants in routine clinical settings.

To address these unmet diagnostic needs, we developed a fluorescence-based endpoint genotyping assay using a minor groove binding (MGB) probe-based PCR method for the rapid detection of mutations at positions 2063 and 2064 in the *M. pneumoniae* 23S rRNA gene (Fig. 1). Probe hybridization stability was determined based on the relationship between the probe melting and PCR annealing temperatures (T_m and T_a , respectively; Fig. 1). When the T_m exceeded the T_a , the probe hybridized stably with a perfectly matched target sequence and was cleaved during amplification, generating a fluorescent signal. In the presence of a wild-type (WT) sequence, the WT probe hybridized with the target sequence and produced a strong fluorescence signal. In contrast, when a mutation was present at the probe-binding site, the WT probe formed a mismatched duplex with reduced hybridization stability, yielding inefficient probe cleavage and minimal fluorescence. Conversely, mutation-specific MGB probes preferentially hybridized with mutant target sequences and generated fluorescence signals in the corresponding detection channels. MGB probes enhanced probe–target binding stability and single-nucleotide mismatch discrimination, allowing for reliable differentiation between WT and mutant

alleles(Kutyavin et al. 2000). As shown in a previous study(Suzuki et al. 2016), the assay was designed to detect nucleotide substitutions at positions 2063 and 2064 (2063B and 2064B variants). To facilitate future clinical implementation, we developed the assay as a laboratory-developed test compatible with automated real-time PCR platforms, with optimized conditions to achieve clear genotype-specific fluorescence separation using representative mutations (A2063G and A2064G), which ensured multiplex discrimination of mutations at these adjacent loci. The assay was subsequently subjected to analytical performance evaluation, followed by assessment of its clinical agreement with a commercially available real-time PCR assay.

MATERIALS AND METHODS

Materials and Reagents

Primers targeting the *M. pneumoniae* 23S rRNA gene and A2063B probe were adopted from a previous study on the reference strain M129 (ATCC29342, GenBank accession no. U00089)(Suzuki et al. 2016), using the following primer sequences: forward primer, 5'-TGAAATCCAGGTACGGGTGAA-3'; reverse primer, 5'-TGCTCCTACCTATTCTCTACATGATAATG-3'. The WT probe, designed for adenine (A) detection at positions 2063 and 2064 (*i.e.*, macrolide-susceptible genotype), was labeled at the 5' and 3' ends with an ATTO fluorescent reporter and an Iowa Black™ quencher, respectively, using the following sequence: 5'-(ATTO)-CGCAACGGGACGGAAAGACC-(IB™ RQ)-3'. This relatively longer WT probe was designed to achieve a T_m comparable to that of the MGB-containing mutant probes under the optimized PCR annealing conditions.

To detect MRMP harboring mutations at positions 2063 and 2064, mutant-specific probes were designed to detect nucleotide substitutions (G, C, or T) at these sites. The 2063 mutant probe (A2063B) was labeled at the 5' and 3' ends with FAM and an MGB conjugated to an Eclipse® dark quencher, respectively, using the following sequence: 5'-(FAM)-AACGGGACGGBAAGA-(MGB)-3'. The 2064 mutant probe (A2064B) was labeled at the 5' and 3' ends with HEX and an MGB Eclipse® dark quencher, respectively, with the sequence: 5'-(HEX)-AACGGGACGGABAGA-(MGB)-3'. All primers and probes were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA).

Template DNA constructs used for assay optimization and analytical performance evaluation were generated by cloning a target region of the *M. pneumoniae* 23S rRNA gene into the pUC57 plasmid vector (Bioneer Inc., Daejeon, Republic of Korea). The WT template contained A at positions 2063 and 2064. The A2063G and A2064G templates contained G substitutions at positions 2063 and 2064, respectively. Plasmid DNA was quantified, reconstituted, and serially diluted to obtain the desired concentrations prior to its use for PCR.

In Silico Primer and Probe Analysis

The specificity of all probes was verified using the Basic Local Alignment Search Tool provided by the National Center for Biotechnology Information (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Endpoint Genotyping Assay

The endpoint genotyping assay was conducted using a LightCycler® 480 PCR machine (Roche, Basel, Switzerland) and a cobas® omni optimization kit (Roche, Basel, Switzerland). Each reaction mixture contained 10.00 µL of Master Mix (MMX)-1, 14.15 µL of MMX2, 0.05 µL of 100 µM forward primer, 0.05 µL of 100 µM reverse primer, 0.05 µL of 100 µM WT probe, 0.18 µL of 100 µM A2063B probe, 0.17 µL of 100 µM A2064B probe, 27.00 µL of template DNA, with DNase-free water added to a final volume of 52 µL. Thermal cycling consisted of uracil-*N*-glycosylase incubation at 50°C for 120 s and enzyme inactivation at 94°C for 5 s, followed by a pre-PCR step (55°C for 120 s, 60°C for 360 s, and 65°C for 240 s). Amplification started with five initial cycles of 95°C for 5 s and 55°C for 30 s, followed by 45 cycles of 91°C for 5 s and 60°C for 25 s, and ended with a final cooling step at 40°C for 60 s. The endpoint fluorescence intensities were analyzed using LightCycler® 480 SW 1.5.1 (Roche, Basel, Switzerland).

Assay Optimization and Analytical Performance Evaluation

The WT, A2063G, and A2064G plasmid templates were spiked into deionized water and serially diluted to the desired concentrations for assay optimization and analytical performance evaluation. Primer and probe concentrations of 0.3, 0.2, and 0.1 µM for primers, and 0.20, 0.10, and 0.05 µM for probes, respectively, were evaluated and optimized by testing multiple combinations. Endpoint fluorescence intensity and inter-channel signal separation were assessed to determine the optimal concentrations for genotype discrimination. The optimization of primer and probe concentrations was followed by that of the WT:A2063B:A2064B probe ratio. The theoretical ratio of 1:3:3 was initially applied, and alternative ratios (1:2:2, 1:3:3, 1:3.5:3.3, and 1:4:4) were evaluated using plasmid templates at concentrations ranging from 10⁶ to 10³ copies/mL. To determine the optimal probe ratio, endpoint fluorescence intensity balance and allelic discrimination were assessed.

For fluorescence cutoff value and limit of detection (LoD) determination, WT, A2063G, and A2064G plasmid templates were tested at multiple concentrations ranging from 1 to 10⁸ copies/mL, with 20 replicates analyzed at each concentration. In addition, more than 100 no-template control reactions were included.

Analytical specificity was evaluated by testing 21 non-target organisms in triplicates. Viral and bacterial targets were tested at concentration of 10⁵ copies/mL and 10⁶ CFU/mL or 10⁶ IU/mL (as appropriate), respectively. The specificity panel included MERS-CoV; human coronaviruses OC43, NL63, HKU1, and 229E; respiratory syncytial virus type A; human parainfluenza virus types 1–4; enterovirus D68; rhinovirus; human adenovirus type 1; human metapneumovirus; human bocavirus; *Haemophilus influenzae*; *Streptococcus pneumoniae*; *Chlamydomphila pneumoniae*; *Legionella pneumophila*; *Bordetella*

pertussis; and *Bordetella parapertussis*. Organisms were obtained from Vircell (Granada, Spain), the American Type Culture Collection (ATCC; Manassas, VA, USA), and the Korea Bank for Pathogenic Viruses (KBPV; Seoul, Republic of Korea).

Statistical Analysis

Receiver operating characteristic (ROC) curve analysis was performed using endpoint fluorescence intensities corresponding to all tested concentrations to establish the channel-specific fluorescence cutoff values based on Youden's index. Detection rates were determined by applying the established fluorescence cutoff values to each genotype and concentration. LoD for each genotype was estimated using probit analysis applied to results obtained from serially diluted plasmid templates. Two-by-two contingency tables were used to evaluate the agreement between the MGB probe-based PCR and PowerChek™ Mp-DR real-time PCR assays. Positive percent agreement, negative percent agreement, Cohen's kappa values, and two-sided 95% confidence intervals (CIs) were calculated. All statistical analyses were performed using Origin Pro® 2021b version 9.0 (OriginLab Corporation, Northampton, MA, USA) and MedCalc Statistical Software version 23.0.6 (MedCalc Software Ltd., Ostend, Belgium).

Clinical Specimens

A total of 44 nasopharyngeal swab specimens tested using *M. pneumoniae* PCR (22 positive and 22 negative) at Samsung Medical Center (Seoul, Republic of Korea) between January 2024 and February 2026 were included in this study. DNA was extracted from 200 µL of each specimen using the MagMAX™ Viral/Pathogen nucleic acid isolation kit and KingFisher™ flex purification system (Thermo Fisher Scientific Inc., Waltham, MA, USA), according to the manufacturer's instructions. The extracted DNA samples were tested in parallel using both MGB probe-based PCR established in this study and a commercially available real-time PCR assay, the PowerChek™ Mp-DR real-time PCR kit (Research Use Only; KogeneBiotech Co., Ltd., Seoul, Republic of Korea). The PowerChek™ Mp-DR real-time PCR was performed following the manufacturer's instructions using CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA).

RESULTS

In Silico Primer and Probe Analysis

To evaluate the analytical specificity of the designed primers and probes, we performed BLASTn-based *in silico* analyses on publicly available nucleotide databases. We individually queried the sequences of the forward and reverse primers, as well as the WT probe against *M. pneumoniae* and *Homo sapiens* genomic sequences (Online Resource Table S1). All three oligonucleotides demonstrated 100% sequence identity and full-length query coverage with the corresponding target regions of the *M. pneumoniae* strains, including the reference strain M129, with no significant alignments detected with the *H. sapiens* genome. To further evaluate analytical exclusivity, we performed BLASTn analyses against 21 common non-target respiratory pathogens (Online Resource Table S2). The alignment analysis

demonstrated only partial sequence similarity between the designed oligonucleotides and corresponding regions of all non-target organisms. All non-target sequences contained multiple mismatches within the primer and probe-binding sites, including at least six, ten, and five mismatches in the forward and reverse primer, and WT probe regions, respectively. Since 5–6 nucleotide mismatches, particularly near the 3' end of primers, could reportedly reduce amplification efficiency by several orders of magnitude (Christopherson et al. 1997), the observed degree of sequence divergence strongly suggested a negligible risk of nonspecific amplification.

Primer and Probe Concentration Optimization

To optimize genotyping performance and establish optimal PCR conditions, we evaluated multiple primer and probe concentration combinations (0.3, 0.2, and 0.1 μM and 0.2, 0.1, and 0.05 μM , respectively; Fig. 2). At primer concentrations of 0.3 and 0.2 μM , we observed insufficient fluorescence separation between the ATTO and FAM channels for the A2063G genotype, where the A2063B probe accounted for approximately 50% of the total fluorescence signal, as well as between the ATTO and HEX channels for the A2064G genotype, with the A2064B probe contributing to approximately 50%–60% of the total fluorescence signal, resulting in suboptimal genotype discrimination. In contrast, a primer concentration of 0.1 μM yielded clear and consistent fluorescence separation across all genotypes, with WT, A2063B, and A2064B probe signals for the WT, A2063G, and A2064G genotypes, respectively, each representing approximately 70% of the total fluorescence. Among the probe concentrations, 0.1 μM yielded the most distinct fluorescence discrimination, particularly for the A2063G and A2064G genotypes, with total fluorescence accounting for 71.6% and 75.3%, respectively. At this concentration, the relative contribution of the FAM channel was most clearly separated from the ATTO channel after normalization, resulting in improved allelic discrimination. In comparison, probe concentrations of 0.2 and 0.05 μM resulted in a less balanced fluorescence distribution and reduced inter-channel separation. Based on these observations, we selected a concentration of 0.1 μM for both the primer and probe for subsequent experiments.

Probe Ratio Optimization

Following optimal primer and probe concentration selection, we further optimized the relative ratio of the WT, A2063B, and A2064B probes to achieve balanced fluorescence intensity and improved genotype discrimination. We designed the A2063B and A2064B probes to detect three possible nucleotide substitutions (*i.e.*, G, C, and T) at each position in an equimolar 1:1:1 configuration. Accordingly, the theoretical probe ratio was 1:3:3. However, since multiplex fluorescence assays might exhibit channel-dependent signal variability, we evaluated alternative probe ratios for improved inter-channel balance and cluster separation. Starting from the theoretical 1:3:3 ratio, we tested additional ratios (1:2:2 and 1:4:4), while normalizing endpoint fluorescence intensities to 100% and creating stacked plots as visual supports to compare the relative fluorescence distribution among the detection channels. To evaluate the fluorescence separation and genotype clustering under different template concentrations, we assessed the performance across a dynamic range of 10^6 to 10^3 copies/mL (Fig. 3). At a ratio of 1:2:2, the WT genotype displayed a strong predominance for the ATTO signal, compared to the other channels,

accounting for more than 75% of the total fluorescence intensity, whereas the A2063G genotype exhibited relatively limited FAM signal contribution, resulting in a relatively modest fluorescence separation. At a ratio of 1:3:3, the relative contribution of the FAM channel for the A2063G genotype increased by an average of 5.9%, compared to that observed at the 1:2:2 ratio, leading to improved allelic discrimination and reduced inter-genotype differences in fluorescence distribution. At a ratio of 1:3.5:3.3, the fluorescence contributions of ATTO, FAM, and HEX channels were the most evenly balanced across the genotypes. Under such conditions, the fluorescence separation between the A2063G and A2064G genotypes was most clearly distinguishable, and the genotype clusters were most distinctly separated. In contrast, at a ratio of 1:4:4, the relative contribution of the HEX channel increased disproportionately for the A2064G genotype, resulting in imbalanced channel representation and reduced overall signal balance.

Endpoint fluorescence intensities were normalized and visualized as 100% stacked bar plots to compare the relative contribution of each detection channel under different primer (0.3, 0.2, and 0.1 μM) and probe (0.20, 0.10, and 0.05 μM) concentration combinations. (a) Primer 0.3 μM and probe 0.2 μM , (b) primer 0.3 μM and probe 0.1 μM , (c) primer 0.30 μM and probe 0.05 μM , (d) primer 0.2 μM and probe 0.2 μM , (e) primer 0.2 μM and probe 0.1 μM , (f) primer 0.20 μM and probe 0.05 μM , (g) primer 0.1 μM and probe 0.2 μM , (h) primer 0.1 μM and probe 0.1 μM , and (i) primer 0.10 μM and probe 0.05 μM .

Endpoint fluorescence intensities were normalized to 100% and visualized as stacked bar plots to compare the relative contribution of each detection channel. WT:A2063B:A2064B probe ratios of 1:2:2, 1:3:3, 1:3.5:3.3, and 1:4:4 were evaluated to optimize the fluorescence balance and genotype discrimination. The relative fluorescence distribution among the WT (ATTO), A2063B (FAM), and A2064B (HEX) channels was assessed using different template concentrations: (a) 10^3 copies/mL, (b) 10^4 copies/mL, (c) 10^5 copies/mL, and (d) 10^6 copies/mL.

Collectively, the 1:3.5:3.3 probe ratio provided the most balanced fluorescence distribution across channels, while maintaining clear genotype discrimination. Therefore, we selected it as the optimal probe ratio under the current conditions used in this study.

Cutoff Values and the Corresponding Genotype Assignments

We performed ROC curve analysis using all tested concentrations of the synthetic template DNA and determined the channel-specific fluorescence cutoff values based on the maximum Youden's index (Ruopp et al. 2008). The cutoff values were 6.35, 6.88, and 3.58 for the ATTO (WT), FAM (A2063G), and HEX (A2064G) channels, respectively (Online Resource Fig. S1). When the fluorescence signals exceeded these cutoff values in multiple channels, we based the genotype assignment on the channel with the highest endpoint fluorescence intensity.

LoD

We estimated the LoD for each genotype using probit analysis (Table 1), yielding 31, 27.2, and 32.7 copies/mL for the WT, A2063G, and A2064G genotypes (95% CI, 20.1–42, 19.2–35.3, and 22.1–43.3), respectively.

Table 1
Limit of detection (LoD) of the endpoint genotyping assay, determined via probit analysis. Detection rates were determined for 20 replicates at each concentration. The LoD and corresponding 95% confidence intervals (CI) were estimated using probit analysis based on fluorescence cutoff criteria.

Concentration (copies/mL)	No. detected/No. replicates		
	WT	A2063G	A2064G
100	20/20	20/20	20/20
50	20/20	20/20	20/20
25	16/20	17/20	15/20
10	14/20	14/20	15/20
5	14/20	8/20	9/20
1	0/20	0/20	0/20
Probit LoD	31.0	27.2	32.7
(95% CI)	(20.1–42)	(19.2–35.3)	(22.1–43.3)

Cross-reactivity

No cross-reactivity was observed with the 21 non-target organisms included in the specificity panel. All non-target organisms tested negative, whereas positive fluorescence signals were detected only in *M. pneumoniae* samples above the established cutoff values (Online Resource Table S2).

Clinical Specimen Tests

Finally, 44 clinical specimens (22 *M. pneumoniae*-positive and 22 *M. pneumoniae*-negative specimens) were tested in parallel using the MGB probe-based PCR assay developed in this study and a commercially available real-time PCR assay (PowerChek™ Mp-DR real-time PCR kit), according to the respective assay protocols (Online Resource Table S3). Complete agreement was observed between the two assays for both *M. pneumoniae* detection and genotype assignment. The overall percent agreement was 100% (44/44; 95% CI, 92%–100%; Table 2), with a Cohen’s κ value of 1. Among the 22 *M. pneumoniae*-positive specimens, both assays identified three WT and 19 A2063G genotypes. No clinical specimens harboring the A2064G mutation were available for evaluation.

Table 2

Comparison between the genotyping results of the developed polymerase chain reaction (PCR) assay and those of the PowerChek™ Mp-DR real-time PCR kit. A total of 44 clinical specimens were tested in parallel using both assays. Genotype assignments were made according to the respective result interpretation criteria.

		PowerChek™ Mp-DR real-time PCR kit			
		WT	A2063G	A2064G	Negative
MGB probe- based PCR	WT	3	0	0	0
	A2063G	0	19	0	0
	A2064G	0	0	0	0
	Negative	0	0	0	22

DISCUSSION

Genetic mutation-driven antibiotic resistance has rendered numerous conventional antibiotics increasingly ineffective, thereby limiting available treatment options and leading to treatment failure, hospital re-admission, and increased healthcare costs and mortality rates. For certain resistant pathogens, effective therapeutic options are particularly limited, which represents a substantial global public health threat (Chinemerem Nwobodo et al. 2022; Woodford and Ellington 2007). Compared to culture-based susceptibility testing and Sanger sequencing, PCR-based mutation detection allows for a higher analytical sensitivity, a shorter turnaround time, and a precise identification of resistance-associated point mutations. These advantages enable a rapid identification of resistance-associated mutations, providing clinically actionable information to guide appropriate antimicrobial therapy (Jeong et al. 2016; Luteijn et al. 2011; Yamin et al. 2023).

Several PCR-based approaches have been developed to detect resistance-associated point mutations, including molecular beacon probes, SimpleProbe® assays, mismatch probe strategies, and high-resolution melting analysis (Table 3). However, these methods are limited by a restricted multiplexing capacity, the dependence on post-amplification melting analysis, a restricted ability to discriminate closely spaced mutations, or an increased assay design complexity (Gosse et al. 2016; van Doorn et al. 2003; Wolff et al. 2008; Wu et al. 2015). In contrast, MGB probe-based assays offer an enhanced single-nucleotide specificity, while maintaining the compatibility with multiplex real-time PCR platforms and a relatively simple assay design.

Table 3
Comparison of polymerase chain reaction (PCR)-based methods for mutation detection

Method	Principle	Single-base discrimination	Multiplexing	Assay design complexity
MGB probe	Minor groove binding	High	Moderate	Low
Molecular beacon	Hairpin structure	High	High	High
SimpleProbe®	Uncleaved probe	Moderate	Not applicable	Moderate
Mismatch probe	Intentional mismatch	Moderate	High	High
HRM	Amplicon T _m difference	Low	Not applicable	High

In this study, we developed a multiplex MGB probe-based PCR assay that could discriminate mutations at the adjacent A2063 and A2064 loci *via* endpoint fluorescence analysis. Rather than focusing solely on nucleotide substitution, our assay design prioritized the accurate discrimination of mutation positions. To achieve this, mutation-specific MGB probes were independently designed and incorporated into the assay, enabling discrimination between mutations occurring only one nucleotide apart. In addition, we designed the WT probe as a conventional TaqMan probe with a T_m adjusted slightly above the T_a (Kim et al. 2025). Under such conditions, the WT probe hybridized stably with a perfectly matched WT target sequence. However, the presence of a single mismatch lowers the T_m of the probe–target duplex below the T_a, yielding probe dissociation and a minimal fluorescence signal. In contrast, mutation-specific MGB probes exhibited relatively high T_ms despite their shorter length, allowing their selective hybridization to mutant targets, while remaining highly sensitive to single-nucleotide mismatches.

During assay optimization, we adjusted the primer and probe conditions to maximize genotype-specific fluorescence signals, while minimizing the nonspecific background. Decreasing primer concentrations improved the fluorescence separation between the genotypes, likely by increasing probe–target discrimination during the early extension steps. Moreover, the endpoint fluorescence analysis revealed relatively strong ATTO signals, compared to those of the other channels, potentially reflecting a combination of probe cross-reactivity and spectral overlap between the fluorophores. To achieve balanced fluorescence distribution among the channels, we adjusted the probe ratios, yielding an optimized ratio of 1:3.5:3.3, which improved the genotype discrimination by reducing channel dominance. We occasionally observed minor fluorescence signals in non-target channels when target templates were present, likely arising from probe cross-reactivity or spectral overlap between the fluorophores, commonly observed in multiplex fluorescence assays. Although these signals generally remained below the cutoff values, fluorescence signals in non-target channels occasionally exceeded the cutoff at high bacterial loads. In such cases, amplification curve characteristics alongside endpoint fluorescence intensities might need to be considered to further improve genotype assignment. For

example, ATTO signals exceeded the cutoff value in most clinical specimens interpreted as A2063G. This observation suggested that the WT probe, based on a conventional TaqMan design, might exhibit partial binding to A2063G target sequences. In contrast, A2063G specimens showing ATTO signals below the cutoff value (Online Resource Table S3) exhibited relatively high Ct values (≥ 28.8 cycles) using the PowerChek™ Mp-DR real-time PCR kit, consistent with lower bacterial loads.

Collectively, our optimized assay allowed for a reliable multiplex detection of closely spaced macrolide resistance mutations in *M. pneumoniae*. By combining position-specific MGB probe design with fluorescence signal optimization, this assay provided a rapid and practical alternative to sequencing-based genotyping methods for resistance detection, which could facilitate timely antimicrobial decision-making in clinical settings. Although our analytical evaluation focused on the representative mutations, A2063G and A2064G, which account for most macrolide resistance-associated variants reported in clinical isolates, further studies are warranted to validate this assay in larger clinical cohorts and expand mutation coverage to other variants, including A2063C and A2063T.

Declarations

SUPPLEMENTARY INFORMATION

Supplementary Information is available online and includes detailed descriptions of the materials, experimental protocols, and data analysis methods, comprising Supplementary Tables and Figure.

STATEMENTS AND DECLARATIONS

Competing interests

The authors declare that they have no competing interests relevant to the content of this article.

Author contributions

Conceptualization: Tae Yeul Kim, Minhee Kang, and Hyeonseek Park; Data curation: Hyeonseek Park, and Minhee Kang; Methodology: Hyeonseek Park; Validation: Hyeonseek Park; Writing – original draft: Hyeonseek Park and Minhee Kang; Writing – review and editing: Hyeonseek Park, Minhee Kang, Tae Yeul Kim, and Hee Jae Huh. All authors have read and agreed to the published version of the manuscript.

Ethics approval and informed consent

This study was approved by the Institutional Review Board (IRB) of the Samsung Medical Center. The requirement for informed consent was waived owing to the retrospective nature of the study and use of de-identified leftover specimens (IRB No.: SMC2025-08-089).

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Data availability statement

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request. Key data are also included within the article and its supplementary information (Online Resource).

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Figures

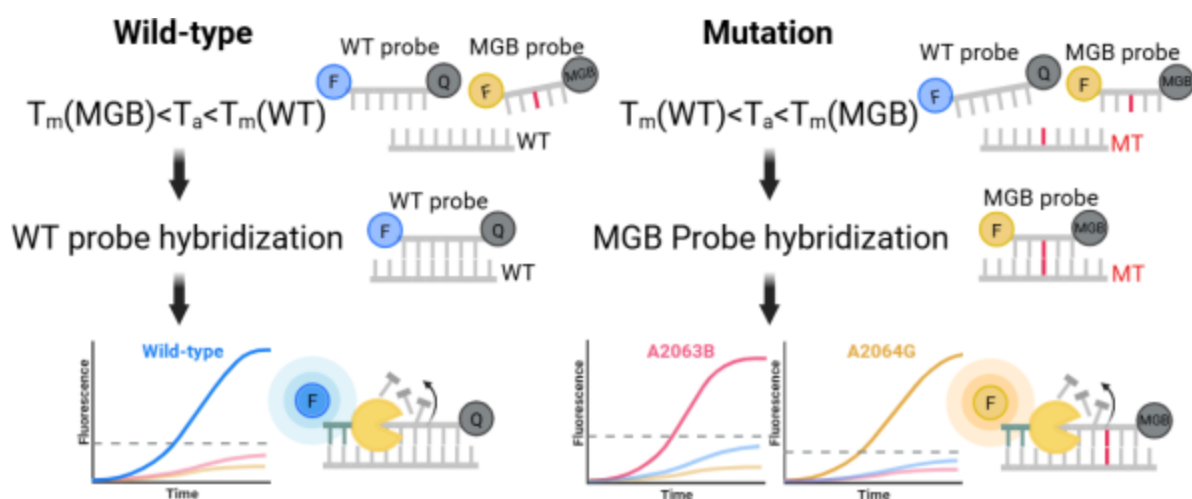


Figure 1

Principle of the minor groove binding (MGB) probe-based endpoint genotyping assay When the wild-type (WT) sequence is present, the mutation-specific MGB probe contains a mismatch and thus fails to hybridize, whereas the WT probe hybridizes with the WT target and generates a fluorescence signal. In contrast, when a mutant sequence is present, the WT probe contains a mismatch and thus hybridization

does not occur, whereas the mutation-specific MGB probe hybridizes with the mutant target, resulting in a fluorescence signal. Genotype discrimination is achieved by comparing the fluorescent signals from probes targeting the WT sequence (ATTO) and mutations at positions 2063 (FAM) and 2064 (HEX) of the *Mycoplasma pneumoniae* 23S rRNA gene.

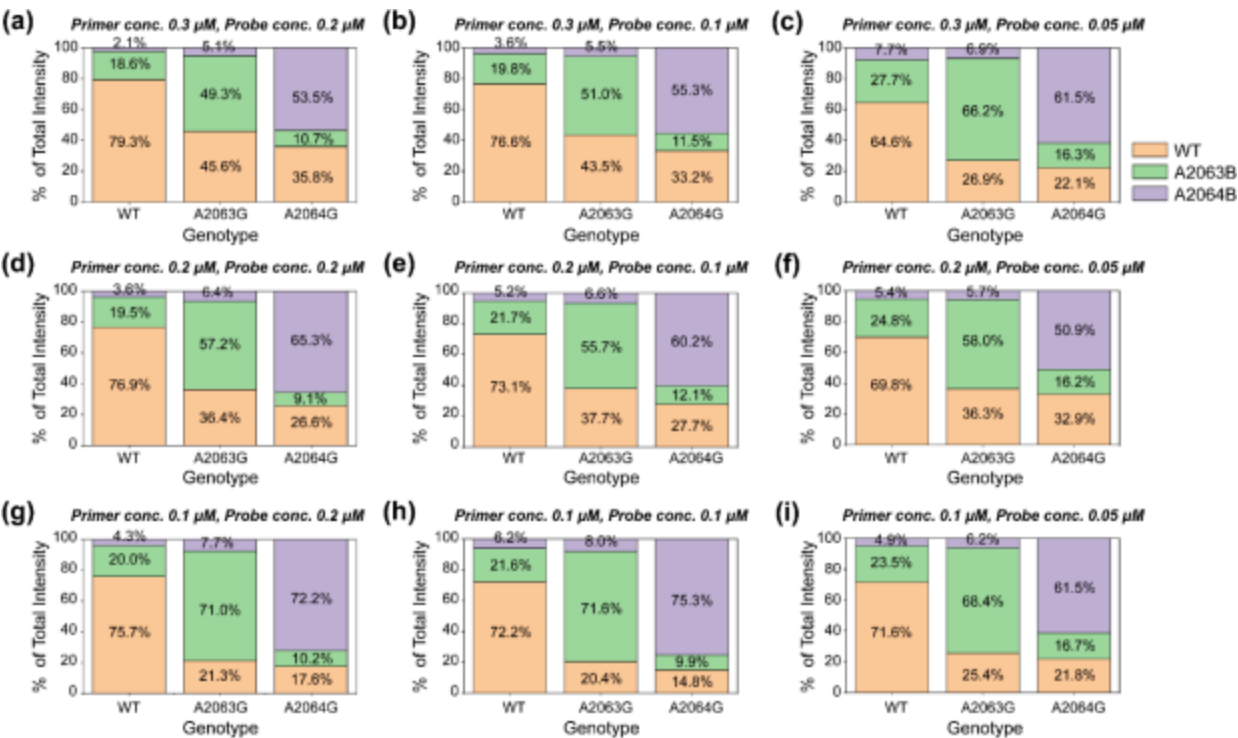


Figure 2

Primer and probe concentration optimization for genotype discrimination using normalized endpoint fluorescence signals

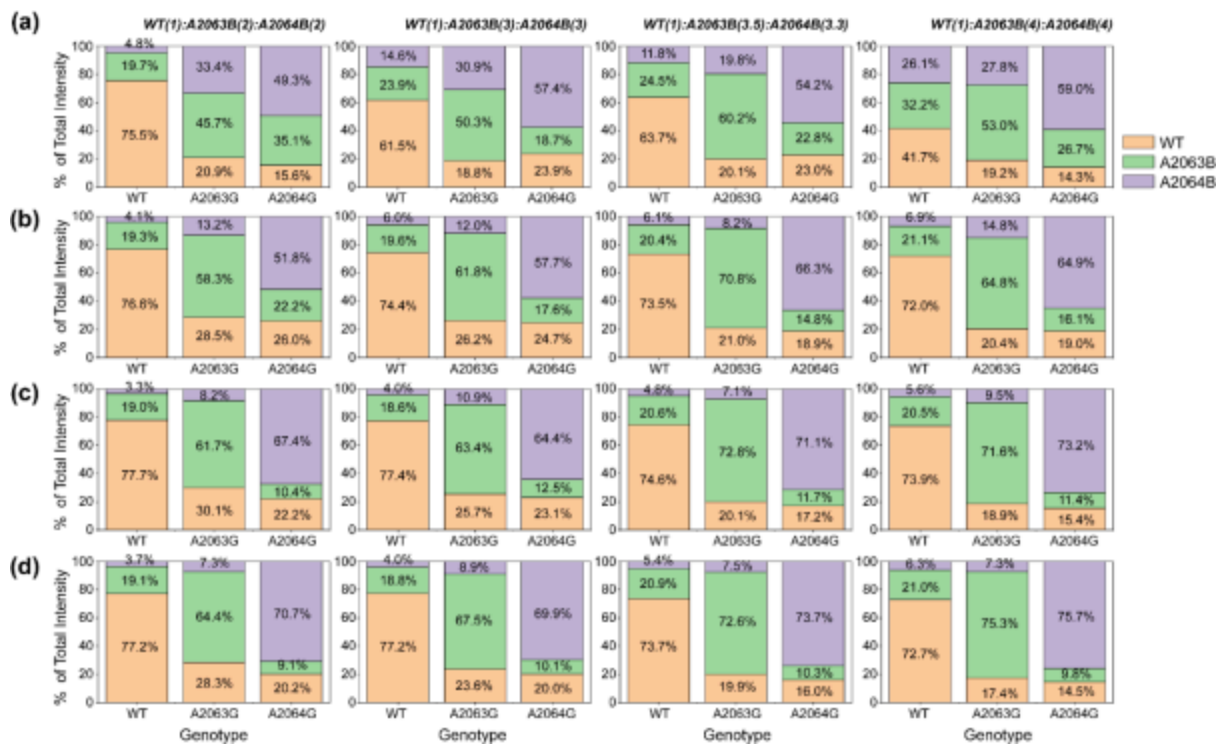


Figure 3

WT:A2063B:A2064B probe ratio optimization using normalized endpoint fluorescence intensities

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