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# Development of a SYBR Green real-time PCR-based assay system for detection of *Neisseria gonorrhoeae*

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## ABSTRACT

Diagnosis of *Neisseria gonorrhoeae* infection is needed for patient therapy and for reducing this bacterial transmission in the population. The culture method is a gold standard method for *N. gonorrhoeae* detection, however, it has low sensitivity. Among molecular methods with high sensitivity and specificity, SYBR Green real-time PCR is the potential method for *N. gonorrhoeae* detection. In this study, we developed an SYBR Green real-time PCR-based system assay for *N. gonorrhoeae* detection. Several PCR conditions were optimized and analyzed including primer annealing temperature, DNA template volume, the limit of detection (LoD), cross-reaction with others (bacteria, viruses, fungus, and protozoa), and quality assurance. The results showed that the annealing temperature and DNA template volume were 60°C and 5 µL, respectively. The LoD was 29 DNA copies corresponding to 3 bacterial cells per reaction. No cross-reaction was detected for other bacteria, viruses, fungus, and protozoa. The external quality assurances enrolled in 2019 and 2021 showed 100% concordance. The preliminary testing for clinical samples was also 100% concordance. In conclusion, the SYBR Green real-time PCR-based system assay developed in this study is promising for application in clinical laboratories.

## ABSTRAK

Diagnosis infeksi *Neisseria gonorrhoeae* diperlukan untuk pengobatan pasien dan untuk menurunkan peneyebarannya dalam populasi. Metode kultur merupakan standar emas untuk deteksi *N. gonorrhoeae,* namun sensitivitasnya rendah. Di antara metode molekular dengan sensitivitas dan spesifisitas tinggi, *SYBR Green real-time PCR* adalah metode yang potensial untuk deteksi *N. gonorrhoeae*. Dalam penelitian ini sebuah metode pengujian berbasis *SYBR Green real-time PCR* telah dikembangkan. Beberapa kondisi PCR dioptimasi dan dianalisis termasuk suhu annealing primer, volume templat DNA, limit deteksi (LoD), reaksi silang dengan mikroorganism lain seperti bakteri, virus, jamur, dan protozoa serta uji jaminan kualitasnya. Hasil penelitian menunjukkan suhu *primer annealing* dan volume DNA templat berturut-turut adalah 60 °C dan 5 µL. Batas deteksi (LoD) DNA adalah 29 salinan atau sekitar 3 sel bakteri per reaksi. Tidak ada reksi silang dengan bakteri lain, virus, jamur, dan protozoa. Kesesuaian pemantapan mutu eksternal yang diikutkan pada tahaun 2019 dan 2021 mencapai 100%. Pengujian tahap awal untuk sampel klinis juga menunjukkan hasil 100%. Dapat disimpulkan, pengembangan metode pengujian berbasis *SYBR Green real-time PCR* dalam penelitian ini menjanjikan untuk diaplikasikan dalam laboratorium klinis.

***Keywords***:
*Neisseria gonorrhoeae*;
PCR;
SYBR Green;
sensitivity;
specificity

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## INTRODUCTION

Gonorrhea is a sexually transmitted disease (STD) caused by *Neisseria gonorrhoeae*.[1] In the male, the disease is usually symptomatic such as urethral discharge, dysuria, and testicular pain.[2] In females, gonorrhea is often asymptomatic and the individuals may not be aware of it.[2,3] However, some individuals are symptomatic such as vaginal discharge, dysuria, lower abdominal and/or rectal pain, dyspareunia, and abnormal uterine bleeding.[2] Untreated gonorrhea in females can cause pelvic inflammatory disease that will lead to chronic pelvic pain and cause infertility and ectopic pregnancy.[2,4]

In 2016, WHO reported 87 million new cases occurred among adolescents and adults aged 15-49 years worldwide, with a global rate of 20 per 1000 women and 26 per 1000 men.[5] The highest prevalence of gonorrhea in the African region was 1.9% (women) and 1.6% (men), followed by other regions, namely America was 0.9% (women) and 0.8% (men), Western Pacific was 0.9% (women) and 0.7% (men), and Europe was 0.3% in women and men.[6] In Europe, ECDC reported 100.673 confirmed gonorrhea cases in 2018. The highest rates (>30/100000 population) were reported in the United Kingdom, Ireland, Denmark, and Norway. Meanwhile, the lowest rates (<1 per 100 000) were reported in Bulgaria, Croatia, Cyprus, Poland, and Romania.[7] In the United States, there were 616.392 gonorrhea cases in 2019, which prevalence in men is higher than in women.[8] In South-East Asia, gonorrhea had decreased by two-thirds, from 118 million in 1990 to 39 million in 2012.[9,10] In Indonesia, the prevalence of gonorrhea is still high in asymptomatic cases in men (56.2%), women (33.0%), and trans-women (10.8%).[11]

To prevent and reduce gonorrhea transmission, screening, and testing of risk populations should be addressed.[12] Early diagnosis is important to perform for asymptomatic and symptomatic individuals.[13] There are some microbiological methods to support the diagnosis of gonorrhea, such as culture (gold standard), microscopy, biochemical test, chromogenic enzyme substrate test, immunoassay, and nucleic acid method.[12,14] The ideal diagnostic test for screening *N. gonorrhoeae* should be sensitive, specific, easy to use, rapid, and affordable.[12]

Among those alternatives, nucleic acid amplification technologies (NAATs) based on the real-time Polymerase Chain Reaction (qPCR) method are the best solution. It is a rapid, sensitive, and specific method to identify the *N. gonorrhoeae* infection.[12,14] There are two types of qPCR assays, SYBR Green and Taqman probe. The Taqman probe assay is based on double (fluorescence and quencher) labeled oligonucleotide, whereas the SYBR Green assay is based on the binding of the florescent dye to dsDNA.[15] Both methods are sensitive and specific to detect a specific gene target.[16] However, SYBR Green is simpler and more economical than the Taqman probe assay.[17] Therefore, in this study we developed an assay system based on SYBR Green qPCR for the detection of *N. gonorrhea*.

## MATERIALS AND METHODS

### Clinical specimen

Twenty cervical swab samples (Collection of Clinical Microbiology Laboratory, Faculty of Medicine Universitas Indonesia) were used for a preliminary trial of the optimized assay system developed in this study. The 10 samples have been confirmed as *N. gonorrhoeae* positive and the other 10 samples have been confirmed as *N. gonorrhoeae* negative. The confirmation

test was conducted by VITEX 2 NH (BioMerieux). This study was approved by the Ethics Committee, Faculty of Medicine, Universitas Indonesia (KET-667/UN2.F1/ ETIK/PPM.00.02/2020).

**Positive control**

Genomic DNA of *N. gonorrhoeae* ATCC 43069 was used as a positive control for optimization of SYBR Green qPCR assay developed in this study.

**DNA extraction**

DNA extractions from cervical swab samples and isolate *N. gonorrhoeae* ATCC 43069 were performed by using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instruction, with 40 μL final elution. The extracted DNA samples were stored at -35°C.

**Primers**

Forward (5'-GTT GAA ACA CCG CCC GG-3') and reverse (5'-CGG TTT GAC CGG TTA AAA AAA GAT-3') primers were used in qPCR assay as reported by Geraarts-Peters *et al.*[13]

**SYBR Green qPCR assay**

The qPCR Assay was performed by the following formulation (20 μL of reaction volume): 1x SensiFAST SYBR No-ROX Mix (Bioline, Cat. no: BIO-98005), 0.3 μM each of primer, and 4 μl of DNA template. The thermal cycles were performed by the following condition (LC96, Roche): 95°C for 3 min; 45 cycles at 95°C for 10 sec, 60°C for 30 sec, and 72°C for 30 sec; and temperature melting (Tm) analysis following standard machine setting (LC96, Roche).

**Optimization of primer annealing temperature and DNA template volume**

To obtain the optimal primer annealing temperature, a PCR gradient was performed using a range of annealing temperatures (54, 55.5, 58, 60.7, 62.9, and 64°C). A comparison of DNA template volume was performed to know the possible PCR inhibitor contained in elute solution of the DNA extraction result. The PCR inhibitor analysis was performed by comparing the DNA template volumes of 3, 4, and 5 μL.

**Limit of detection (LoD)**

LoD of the qPCR was determined by serial dilution of DNA concentration. Minimal DNA detection was defined as the lowest DNA concentration which can be detected by the qPCR. To obtain the DNA copy number as LoD, Avogadro's calculator was used (cels.uri.edu/gsc/cndna.html).

**Cross-reaction over other microorganisms**

The potential cross-reaction of the qPCR assay was evaluated against bacteria, fungi, viruses, and protozoa.

**Internal and external quality assurance**

Internal quality assurance of qPCR assay was conducted by using negative and positive controls of *N. gonorrhoeae* ATCC 43069 DNA genomes. External quality assurance was assessed by QCMD EQA Management System (Scotland, The United Kingdom) in 2019 and 2021.

## RESULTS

**SYBR Green qPCR assay**

To obtain the optimal reaction formulation and condition, several parameters were optimized, including annealing temperature, DNA template volume, LoD, and potential cross-reaction with other microbes. Based on the annealing temperature analysis

result, the melting peaks resulting from temperatures 55.5 (E), 58 (C), and 60.7°C (D) showed the overlapped peaks (FIGURE 1A). Considering the specificity of primers, we set the annealing temperature at 60°C. For DNA template volume, the melting peak from the DNA template volume of 5 μL was slightly above the melting peak from that of 4 μL (FIGURE 1B); therefore, we set the DNA template volume at 5 μL.

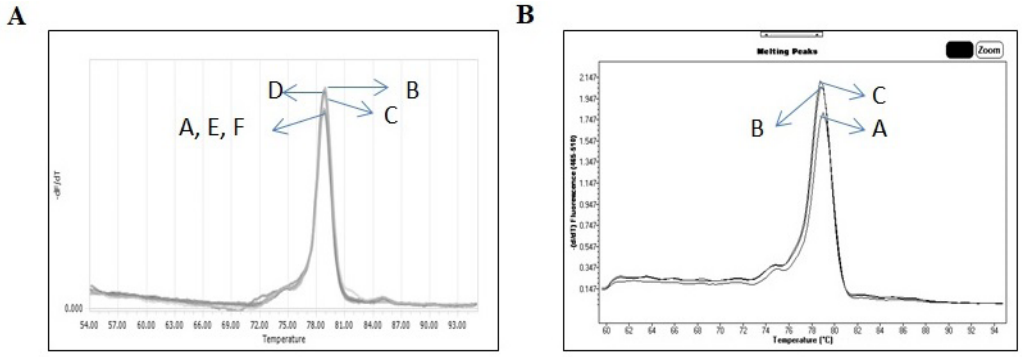


FIGURE 1. The qPCR results. **A)** the gradient annealing temperatures of 54 (A), 55.5(B), 58 (C), 60.7 (D), 62.9 (E), and 64°C (F). **B)** the DNA template volumes of 3 (A), 4 (B), and 5 μl (C).

The specificity of the primers used in the qPCR was *in silico* analyzed on Primer-BLAST GenBank. The results showed that the primers only bind with the *N. gonorrhoeae* (data not shown) target gene. Consistently, the qPCR assay was specifically detected *N. gonorrhoeae* with no cross-reaction with other organisms such as bacteria (*N. meningitidis, Mycobacterium tuberculosis, Chlamydia trachomatis, M. leprae, Bordetella pertussis, C. pneumonia, Helicobacter phylori, Legionella pneumophilae, Leptospira, Mycoplasma pneumonia, Salmonella enterica, Streptococcus pneumonia*), fungi (*Pneumocystis jirovecii*), viruses (Cytomegalovirus, Herpes Simplex Virus, Epstein-Barr Virus, Varicella Zoster Virus, *Influenza A & B, Rubella* virus), and protozoa (*Toxoplasma*) (FIGURE 2).

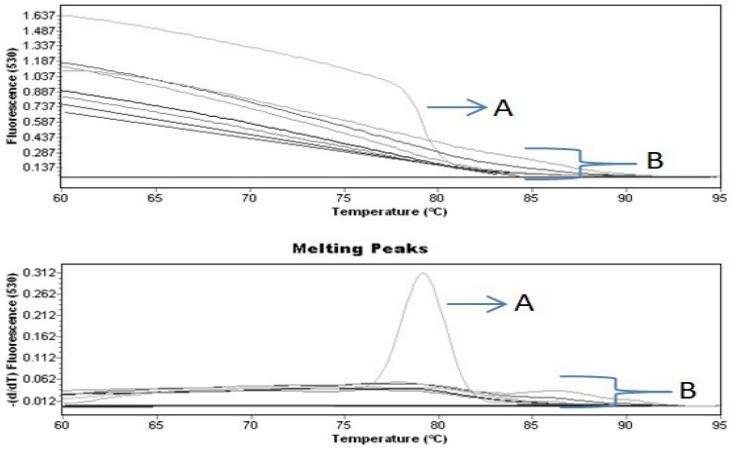


FIGURE 2. The qPCR Results (top [melting curve] and bottom [melting dynamic] images) for bacteria, viruses, fungi, and protozoa. A: The qPCR Result for the positive control (DNA genome of *N. gonorrhoeae* ATCC 49226). B: The qPCR Results for microbes (bacteria, viruses, and fungi) and protozoa.

The LoD of qPCR showed a highly sensitive assay, namely 25 x $10^{-10}$ ng DNA (FIGURE 3). Estimation using Avogadro's formulation, the 25 x $10^{-10}$ ng DNA yielded 29 DNA copies. Based on the number of *opa* gene alleles (11 copies) in *N. gonorrhoeae* (18), the LoD was thus corresponding to 3 cell/reaction.

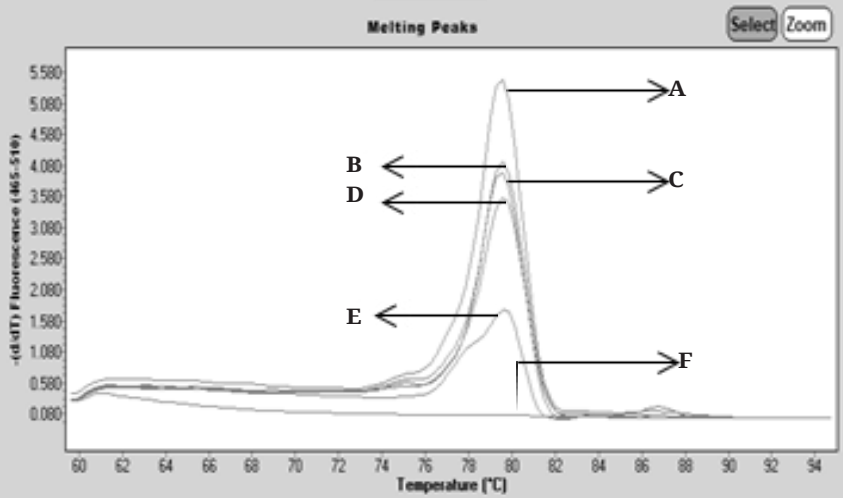


FIGURE 3. The qPCR result for determining the limit of detection (LoD). This assay was still able to detect the DNA at 25x$10^{-10}$ ng. A-F: 25x$10^{-6}$, 25x$10^{-7}$, 25x$10^{-8}$, 25x$10^{-9}$, 25x$10^{-10}$, and 25x$10^{-11}$ ng DNA respectively.

### *External quality assurance*

To validate the qPCR optimized in this study, we enrolled the external quality assurance in 2019 and 2021 by QCMD EQA Management System (Scotland, The United Kingdom). The results showed score 0, meaning the testing agreement of 100% (FIGURE 4).

### Preliminary trial for the clinical specimen

For preliminary testing, 20 cervical swab samples were used. The results showed 100% concordance with 10 positive- and 10 negative-confirmed samples (FIGURE 5).

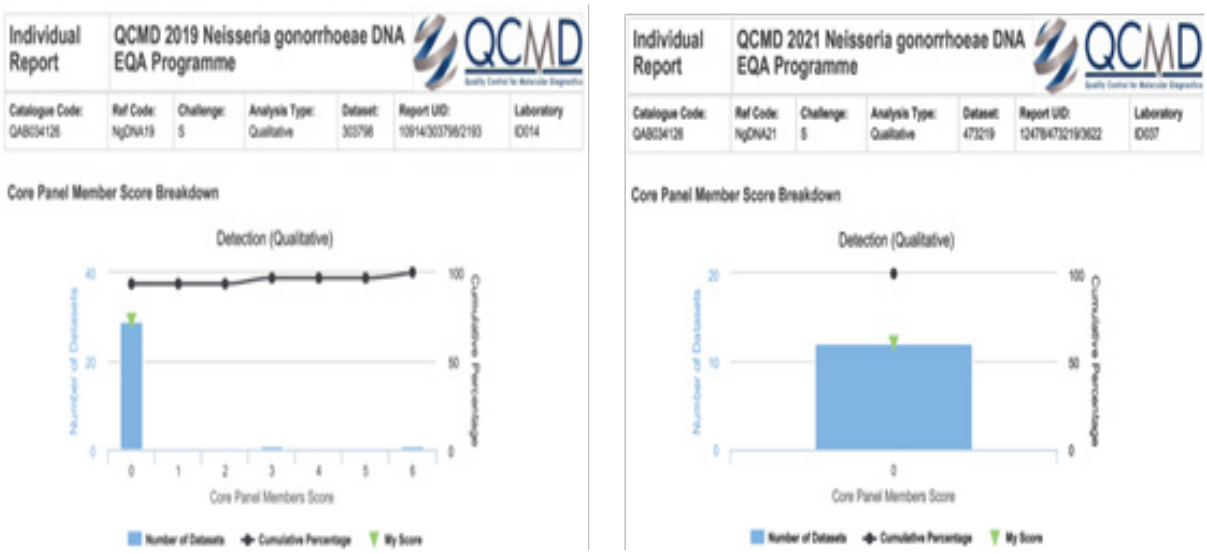


FIGURE 4. External quality assurance reports enrolled in 2019 and 2021. The score 0 is 100% concordance (The higher score, the higher discordance).

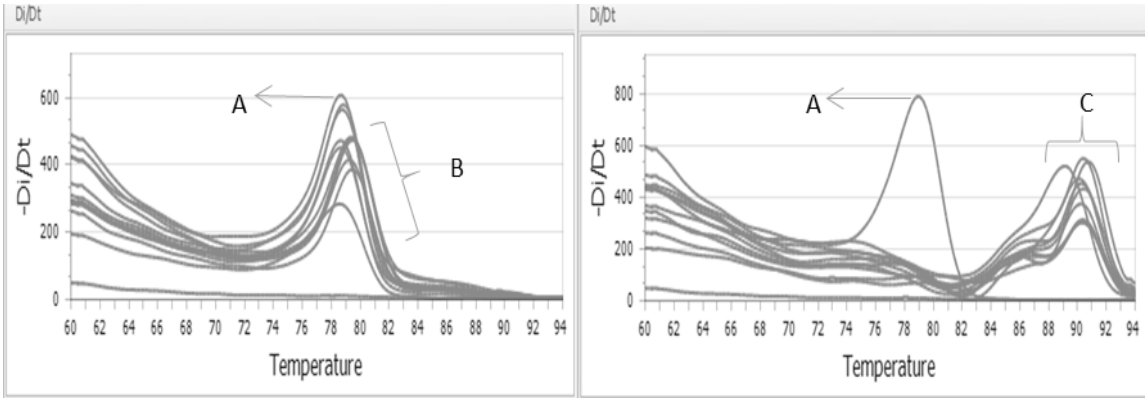


FIGURE 5. Preliminary test results for 10 positive (left)- and 10 negative (right)-confirmed samples. A: Positive control of *N. gonorrhoeae* ATCC 43069. B: Melting dynamic for 10 *N. gonorrhoeae* positive samples. C: Melting dynamic for 10 *N. gonorrhoeae* negative samples.

## DISCUSSION

For the development and establishment of PCR assay on the laboratory scale, many factors affect the sensitivity and specificity of the PCR assay, including primer design, PCR composition, annealing temperature, and PCR inhibitor.[19–21] In this study, we optimized annealing temperature and DNA template volume. The optimal annealing temperature is the temperature in which the primers attach to the DNA sequence targets completely, leading to high DNA amplification efficiency.[19] Low annealing temperature could amplify non-specific DNA fragments, whereas too high annealing temperature could reduce the PCR product purity due to poor annealing of primers.[19]

The Optimal DNA template volume is important to reduce the PCR inhibitor available in the DNA solution of DNA extraction. The inhibitor influences the efficiency of *Taq* DNA polymerase in incorporating deoxynucleoside triphosphate when the DNA strand is synthesized. The inhibitor analysis is highly important, particularly for clinical samples, such as respiratory, feces, sputum, and other body fluids (cerebrospinal fluid, blood, urine, saliva, etc.).[22] Various extraction DNA methods have a different performances to diminish the possible inhibitor in clinical samples; thus each new method has to be optimized.[23]

Another important factor that affected the PCR performance, particularly PCR specificity is primer design. In this study, we used primers specific to the *opa* gene that have been reported by Geraats-Peters *et al.*[13] Based on *in silico* and *in vitro* analysis, the primers were only reacted with the *N. gonorrhoeae* target gene, and no cross-reaction with bacteria, viruses, fungi, and protozoa that might cause the possible false-positive results. It indicates that the qPCR assay system developed in this study has high specificity.

Besides specificity, the LoD also affects the PCR performance. An important factor affecting the PCR LoD is the target gene.[24] There are several genes used for *N. gonorrhea* PCR, including *opa*, *16S rRNA*, *porA*, *cppB*, and *CMT* genes.[24] The *opa* gene is the conserved region and encodes proteins with physiological function.[13] Moreover, the *N. gonorrhoea* genome has multiple copies (about 11 alleles) of *opa* gene,[18] thus leading the PCR to be more sensitive than other genes with fewer copies. Geraats-Peters reported that the *opa* gene-based PCR is more sensitive than

16S rRNA.[13] Other study also report that *opa* gene has higher sensitivity than *porA* pseudogene.[24] The *cppB* gene and *CMT* gene have wide range sensitivity because of lacking the genes in certain *N. gonorrhea* strains.[25,26] The *CMT* gene is less specific because can cross-react with commensal *Neisseria* species.[26] Thus, the *cppB* and *CMT* genes are not suitable for *N. gonorrhoeae* detection.

The LoD of qPCR developed in this study was 29 DNA copies (25x $10^{-7}$ pg) corresponding to 3 bacterial cells per reaction. This LoD is more sensitive than what has been reported by Verma *et al*, namely 0.4 pg (*16S rRNA* and *opa* genes) and 4 pg (*porA* pseudogene) DNA of *N. gonorrhoeae.*[24] For internal quality control, this study used *N. gonorrhoeae* ATCC 43069 as positive control and nuclease-free water as the negative control. *N. gonorrhoeae* ATCC 43069 is often used as a reference strain in the diagnosis of *N. gonorrhoea* infection.[27,28] For external quality assurance (QCMD, Scotland, United Kingdom), the assay developed in this study had score 0 (100% concordance) in 2019 and 2021. Based on the preliminary trial for 20 clinical samples, the assay showed 100% concordance. Overall, the data indicate that the SYBR Green qPCR-based assay system developed in this study is highly promising for clinical laboratories application.

## CONCLUSION

We conclude that an SYBR Green qPCR-based assay system that was developed in this study has high sensitivity and specificity for *N. gonorrhoeae* detection. This assay also can be applied to cervical swab samples. However, larger cervical swab samples are required for further analysis. Also, this qPCR is needed to evaluate some types of samples such as urine, urethral swab, and other clinical samples.

## ACKNOWLEDGMENT

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