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# Primer design for amplification of the *gyrA* gene in *Escherichia coli* using polymerase chain reaction (PCR)

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**Abstract**

**Background:** The *gyrA* gene is an important genetic marker associated with fluoroquinolone resistance in *Escherichia coli*. Accurate primer design is crucial for enabling the successful amplification of this gene using the polymerase chain reaction (PCR).

**Objective:** This study aims to evaluate the effectiveness of the designed primer pair in amplifying the *gyrA* gene in ciprofloxacin-resistant *E. coli* isolates.

**Method:** DNA from *E. coli* isolates was extracted using the Presto™ Mini Bacteria Kit, followed by qualitative and quantitative analysis using UV-Vis spectrophotometry. Primer design was performed using databases and tools from NCBI, OligoEvaluator, and NetPrimer. PCR amplification was optimized at an annealing temperature of 59 °C, and results were visualized by agarose gel electrophoresis using a GelDoc system.

**Results:** The qualitative test showed a clear DNA band, while the DNA purity ratio was 1.5, showing mild protein contamination. PCR amplification successfully produced a distinct DNA band at 221 bp, confirming that the primer pair was specific and effective.

**Conclusion:** The primer pair (Forward: 5′-ACTGTGAAGAAAACCGTCCT-3′; Reverse: 5′-CCTAAACGAATACCGCGAAC-3′) is effective for amplifying the *gyrA* gene in *E. coli* and is suitable for further molecular detection applications.

**Keywords:** Primer, *gyrA*, PCR, *Escherichia coli*, amplification

## 1. Introduction

*Escherichia coli* is a primary bacterial pathogen responsible for various infections in humans. Uropathogenic *E. coli* (UPEC) is the most common causative agent for urinary tract infections (UTIs), a leading cause of both community-acquired and healthcare-related infections (Mancuso *et al.*, 2023; Mohammed *et al.*, 2025). Recent studies consistently confirm that *Escherichia coli* is the predominant uropathogen isolated from urine specimens of patients with urinary tract infections. For example, Marc *et al.* (2025) reported that *E. coli* accounted for more than 66% of positive urine cultures among non-hospitalized children in Romania. Similarly, Alkhawaldeh *et al.* (2025) identified *E. coli* as the causative agent in 230 adult patients with culture-confirmed UTIs in Jordan. In addition, Bernaitis *et al.* (2024) isolated 150 *E. coli* strains from urine samples of UTI patients in India, underscoring its global role as the leading cause of urinary tract infections.

*E. coli* is a Gram-negative bacterium that normally resides in the human intestinal tract. However, this bacterium can become pathogenic when translocated from its usual habitat. Recently, antibiotic resistance has emerged as an increasingly uncontrollable global health issue, and a major contributing factor to the development of bacterial resistance is the inappropriate use of antibiotics.

Among Gram-negative bacteria, *E. coli* is the most frequent cause of UTIs, accounting for 17.01% of cases. Moreover, resistance rates to ciprofloxacin are alarming, ranging from 48.3% to 71.6% (Septiana *et al*., 2020). *E. coli* plays a significant role as a pathogenic bacterium. According to a study carried out by Al Azad *et al.* (2019), *E. coli* isolates exhibited high levels of resistance to ciprofloxacin and enrofloxacin, which is attributed to the presence of the *gyrA* gene. Recent studies have also highlighted varying levels of ciprofloxacin resistance in *E. coli* isolated from urinary tract infections. For example, Kornfält Isberg *et al*. (2024) reported a resistance rate of **9%** among male UTI patients in Swedish primary healthcare. In contrast, Bullens *et al.* (2022) found that **51.8%** of *E. coli* isolates from women with uncomplicated UTIs in Pakistan were resistant to ciprofloxacin. Similarly, Das *et al*. (2023) demonstrated an alarmingly high prevalence of ciprofloxacin resistance, with **83.6%** of human *E. coli* isolates showing resistance in a One Health study conducted in Bangladesh.

Another study by Septiana *et al.* (2020) confirmed the detection of the *gyrA* gene in *E. coli*. Polymerase chain reaction (PCR) is an important technique for detecting such genes and requires specific primers. Subsequently, primers are oligonucleotide pairs typically ranging from 18 to 30 base pairs in length. Primer design is essential for PCR and involves selecting appropriate sequences, which can be retrieved from databases such as the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov). Primer design can be performed using bioinformatics tools such as NCBI Primer-BLAST, OligoEvaluator, and NetPrimer. The present study aimed to design and evaluate a specific primer pair for the amplification of the *gyrA* gene in *Escherichia coli*, a key genetic marker associated with fluoroquinolone resistance. By optimizing primer design and validating amplification through PCR, this work sought to establish a reliable molecular tool that can be applied in subsequent studies of antibiotic resistance detection and surveillance.

## 2. Method

### *2.1. Sample preparation*

In this study, the test material used was *E. coli* isolates obtained from clinical urine specimens of patients diagnosed with urinary tract infection (UTI), who were confirmed to have resistance to ciprofloxacin. The isolates were collected from a hospital in Surakarta. The materials used included ciprofloxacin discs (Merck Oxoid CT0064B), Mueller-Hinton Agar (Merck KGaA, Germany), a DNA extraction kit (Geneaid Biotech, Taiwan), sterile NaCl, agarose gel, TBE buffer, and TE buffer. Isolates were first confirmed as *E. coli* using Gram staining and antibiotic susceptibility tests with

ciprofloxacin. Bacterial suspensions were then prepared with turbidity adjusted to the McFarland 0.5 standard before DNA extraction.

*2.2. DNA extraction procedure*

A 1.5 mL aliquot of the bacterial suspension was transferred into a microcentrifuge tube. DNA extraction was conducted using the Presto™ Mini Bacteria DNA Extraction Kit following the manufacturer's protocol, which includes cell lysis, DNA binding to a column matrix, washing, and elution steps.

*2.3. DNA quality and quantity evaluation*

*2.3.1 Qualitative analysis*

DNA quality was verified by agarose gel electrophoresis, carried out for 30 minutes under standard voltage conditions.

*2.3.2 Quantitative analysis*

a) DNA concentration and purity were determined by spectrophotometric measurement at 260 nm and 280 nm.
b) DNA was diluted in TE buffer before measurement.

*2.4. Primer design for the gyrA Gene*

The *gyrA* gene (NCBI Gene ID **946614)** nucleotide sequence of *E. coli* was retrieved from GenBank via the NCBI database (https://www.ncbi.nlm.nih.gov). Primer candidates were designed using Primer-BLAST and evaluated for secondary structures using OligoEvaluator. Final primer quality was validated using NetPrimer software.

*2.5. PCR*

*2.5.1 PCR amplification*

PCR amplification was performed in a thermal cycler with a total reaction volume of 25 µL. The reaction mixture (master mix) consisted of 21.4 ng of DNA template, 1 µL of each forward and reverse primer (final concentration 0.2 µM), 12.5 µL of 2x master mix, and nuclease-free water to reach a final volume of 25 µL.

The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 1 minute. A final extension step was carried out at 72°C for 5 minutes.

*2.5.2 Visualization by agarose gel electrophoresis*

The PCR products were visualized using agarose gel electrophoresis. A 1.5% (w/v) agarose gel was prepared by dissolving agarose powder in 1x TBE buffer. After the gel solidified, 5-10 µL of each PCR product, mixed with loading dye, was loaded into the wells. A 100 bp DNA ladder was used in the first well as a molecular weight marker. Electrophoresis was conducted at a constant voltage of 100V for approximately 45 minutes, or until the dye front had migrated a sufficient distance. The gel was then stained with ethidium bromide (EtBr) and documented using a gel documentation system (GelDoc).

## 3. Results and discussion

The DNA isolation process from ciprofloxacin-resistant *E. coli* produced a final volume of 200 µL in a microcentrifuge tube. A qualitative test was conducted to confirm the presence of extracted genomic DNA. Visualization using a Gel Documentation System (GelDoc) showed distinct DNA bands, as shown in **Figure 1**.

**Figure 1.** Visualization of qualitative test results for extracted DNA

Quantitative DNA analysis was performed using a UV-Vis spectrophotometer by measuring absorbance at wavelengths of 260 nm and 280 nm. Pure DNA typically shows strong absorption at 260 nm, while contaminants such as proteins absorb at 280 nm. Therefore, DNA purity is assessed by calculating the absorbance ratio A260/A280. According to Lesiani *et al*. (2023) and Tjoa *et al*. (2025), a ratio between 1.8 and 2.0 shows pure DNA. Ratios below 1.8 or above 2.0 suggest contamination with RNA or protein. The results of the quantitative test are shown in **Table 1**.

**Table 1.** Quantitative analysis results of extracted DNA

| A260 | A280 | DNA concentration (ng/µL) | Purity (A260/A280) |
|---|---|---|---|
| 2.859 | 1.895 | 21.442 | 1.5 |

Based on the data, the DNA concentration was 21.4 ng/µL, which was sufficient for PCR amplification. However, the purity was slightly below the ideal range, suggesting possible protein contamination. This may have resulted from suboptimal DNA isolation steps, such as incomplete protein removal or inadequate ethanol drying (Attikora *et al*., 2024; Gill *et al*., 2025). Additionally, improper use of cuvettes may affect absorbance readings (Basalla & Kendrick, 2023; Falgueras Vallbona *et al*., 2025).

Primer design was conducted using NCBI Primer-BLAST, followed by evaluation using OligoEvaluator and NetPrimer software. From 10 primer pairs generated, the best candidate based on quality and specificity was: **Forward**: 5'-ACTGTGAAGAAAACCGTCCT-3' and **Reverse**: 5'-CCTAAACGAATACCGCGAAC-3'. The designed primers fulfilled the commonly accepted parameters for PCR assays, including an optimal length range of 18–30 nucleotides, a GC content of approximately 40–60%, and melting temperatures between 50–65 °C, which are consistent with established guidelines for high-quality primer design reported in recent studies (Fulghum *et al*., 2024; Chen *et al*., 2025; Wang *et al*., 2025). The selected primer pair showed no significant secondary structures and passed all *in silico* evaluations.

PCR optimization was conducted to determine the optimal annealing temperature. The annealing step plays an important role in the success of DNA amplification. Temperatures that are too high may inhibit primer binding, while excessively low temperatures may lead to nonspecific amplification. In this study, the optimal annealing temperature was determined to be 59°C, based on the appearance of a sharp, specific DNA band without primer-dimer formation (**Figure 2**).

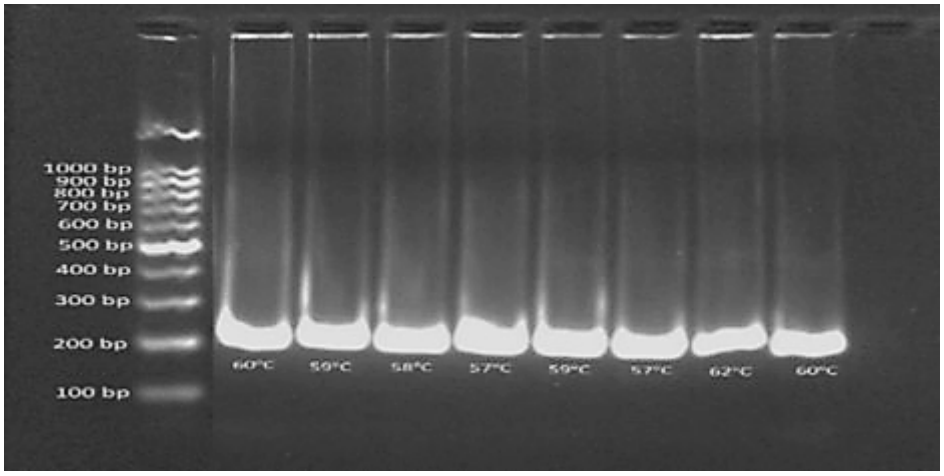


**Figure 2.** PCR optimization result showing successful amplification at 59°C, product size: 221 bp

Subsequent PCR amplification using the selected primers and optimized conditions successfully amplified the *gyrA* gene of *E. coli*, as shown by a strong DNA band of 221 bp visualized

after agarose gel electrophoresis (**Figure 3**). This result confirms the success of both primer design and PCR amplification. According to Rahmi et al. (2024), the annealing temperature should be close to the Tm of primers and adjusted to balance specificity and yield. In line with contemporary PCR optimization guidance, the annealing temperature should be set close to the primers' melting temperature (Tm) and fine-tuned to balance specificity and yield (Zhao *et al*., 2021). The clear band and absence of primer dimers in the gel image confirm that the designed primers are specific and efficient for *gyrA* amplification in *E. coli*. Consistently, recent studies indicate that a single, discrete band at the expected size and the absence of primer-dimer products on agarose gels reflect specific and efficient amplification; for example, Vijayakumar and Sakuntala (2024) reported single-band amplicons with no primer-dimer signal, and Mahdi *et al.* (2024) visualized clear *gyrA* PCR products of *E. coli* on 1.5% agarose, confirming successful target amplification.

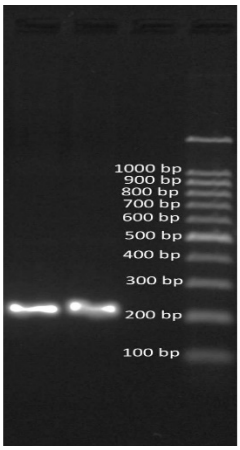


**Figure 3.** Amplification of the *gyrA* gene from *E. coli* DNA using PCR at 59°C, product size: 221 bp

## 4. Conclusion

In conclusion, this study showed that a primer pair consisting of forward primer 5'-ACTGTGAAGAAAACCGTCCT-3' and reverse primer 5'-CCTAAACGAATACCGCGAAC-3' is effective for amplifying the *gyrA* gene in *E. coli*. The amplification product of 221 bp, successfully visualized through agarose gel electrophoresis, confirms the specificity and suitability of the designed primers. These results show that designed primers can be reliably used for molecular detection and amplification of the *gyrA* gene in ciprofloxacin-resistant *E. coli* isolates.

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