OPTIMIZATION OF DNA EXTRACTION AND WHOLE GENOME SEQUENCING FOR *Neisseria gonorrhoeae* ISOLATES AND CLINICAL SAMPLES USING OXFORD NANOPORE TECHNOLOGY

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Abstract: Gonorrhea is a sexually transmitted infection caused by *Neisseria gonorrhoeae*. Whole-genome sequencing (WGS) is essential for genomic studies of this bacterium to understand its epidemiology and mechanisms of antibiotic resistance. The success of WGS depends on obtaining high-quality genomic DNA. This study aimed to determine optimal DNA concentration and purity from optimized extraction of two isolates from two clinical samples and to achieve optimized genomic data analysis from WGS of both sample types. DNA extraction was performed using the Qiagen *DNeasy Blood and Tissue Kit*, and WGS was conducted on the GridION platform (Oxford Nanopore Technologies) with Epi2Me software for metagenomic analysis. The results showed that isolates produced higher DNA concentrations with A260/280 ratios of 1.8–2.0, whereas some clinical samples exhibited contamination. Metagenomic analysis revealed *Neisseria gonorrhoeae* dominance in isolates but low proportions in clinical samples, where other bacteria predominated.

In conclusion, optimizing DNA extraction improved DNA quality and enabled valid WGS analysis. Metagenomic analysis revealed that *N. gonorrhoeae* dominated cultured isolates (~60–72% of reads) but was present at very low levels (<6%) in clinical samples, which were predominantly composed of *E. coli, Klebsiella spp*., and host DNA. These findings provide practical guidance for optimizing DNA extraction strategies in WGS-based studies of *Neisseria gonorrhoeae*, particularly for low-biomass clinical samples. They may support more accurate genomic surveillance and antibiotic resistance profiling to inform targeted treatment strategies.

Keywords: DNA extraction, metagenomics, *Neisseria gonorrhoeae*, optimization, whole genome sequencing

Abstrak: Gonore merupakan Infeksi Menular Seksual (IMS) yang disebabkan oleh *Neisseria gonorrhoeae*. *Whole Genome Sequencing* (WGS) merupakan metode penting dalam studi genomik bakteri untuk memahami epidemiologi dan mekanisme resistensi antibiotik. Keberhasilan WGS sangat bergantung pada kualitas DNA genomik yang diperoleh. Penelitian ini bertujuan untuk memperoleh nilai optimal konsentrasi dan kemurnian DNA hasil optimasi ekstraksi dari dua isolat pada dua sampel klinis, serta

memperoleh hasil optimasi analisis data genomik WGS dari kedua jenis sampel tersebut. Ekstraksi DNA dilakukan menggunakan Qiagen *DNeasy Blood and Tissue Kit*, sedangkan WGS dilakukan pada platform GridIon (Oxford Nanopore Technologies) dengan perangkat lunak Epi2Me untuk analisis metagenomik. Hasil menunjukkan isolat menghasilkan konsentrasi DNA yang lebih tinggi dengan rasio A260/280 mendekati 1,8–2,0, sementara beberapa sampel klinis menunjukkan adanya kontaminasi. Analisis metagenomik menunjukkan dominasi Neisseria gonorrhoeae pada isolat, namun rendah pada sampel klinis yang didominasi oleh bakteri lain. Berdasarkan hasil tersebut, dapat disimpulkan bahwa optimasi ekstraksi DNA berhasil meningkatkan kualitas DNA dan memungkinkan analisis WGS yang valid. Analisis metagenomik memperlihatkan bahwa *N. gonorrhoeae* mendominasi isolat (~60–72% bacaan), namun sangat sedikit (<6%) pada sampel klinis, yang didominasi oleh *E. coli*, *Klebsiella*, dan DNA inang. Temuan ini memberikan panduan praktis untuk mengoptimalkan strategi ekstraksi DNA dalam studi berbasis WGS pada *Neisseria gonorrhoeae*, khususnya untuk sampel klinis dengan biomassa rendah, serta dapat mendukung pengawasan genomik yang lebih akurat dan profil resistensi antibiotik untuk memberikan informasi bagi strategi pengobatan yang lebih tepat sasaran.

Kata kunci: ekstraksi DNA, metagenomik, Neisseria gonorrhoeae, optimasi, whole genome sequencing,

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Introduction

Gonorrhea is an sexually transmitted infection (STI) that poses a substantial global challenge. This is caused by the consistently high rate of gonococcal infections (estimated between 78–87 million new cases) each year globally, along with the swift emergence of *N. gonorrhoeae* resistance to antimicrobial medications, increasing the risk of untreatable gonorrhea forms. *N. gonorrhoeae* is noted for its genetic flexibility, enabling it to obtain genetic factors that confer drug resistance (Nosov *et al*., 2025).

The World Health Organization (WHO) reported approximately 82.4 million new cases of gonorrhea in 2020, with the highest incidence observed in the African and Western Pacific regions (WHO, 2024). In Indonesia, the Enhanced Gonococcal Antimicrobial Surveillance Program (EGASP) reported that 83.3% of samples tested positive for gonorrhea among 126 patients presenting with urethral discharge (Kemenkes, 2023).

N. gonorrhoeae showed extensive resistance to several antibiotics—minimum Inhibitory Concentration (MIC) values to guide treatment approaches and public health measures. The MIC values were measured for various antibiotics to analyze resistance patterns and regional differences, such as penicillin, spectinomycin, zoliflodacin, gentamicin, and fluoroquinolones. Antimicrobial resistance (AMR) in *Neisseria*

gonorrhoeae, the pathogen responsible for gonorrhea, has emerged as a significant worldwide health issue. In the past, antibiotics like penicillin and tetracycline were very successful in treating gonorrhea. Nonetheless, the rise of resistant strains has greatly diminished their effectiveness, leading to a worsening public health crisis (Ebrahimi *et al*., 2025).

The mechanisms of AMR in N. gonorrhoeae align with the idea that bacteria might be more easily spread if they lack numerous AMR mechanisms; however, current views on *N. gonorrhoeae* suggest that these AMR mutations might not inherently lead to a fitness cost, as fitness can be regained through compensatory mutations (Buckley *et al*., 2018).

The WHO has classified this bacterium as a priority pathogen due to its potential to cause infections that are increasingly difficult to treat. Whole Genome Sequencing (WGS) has emerged as a crucial tool for pathogen genome analysis, epidemiological studies, and the detection of antimicrobial resistance genes (Wiranto *et al*., 2023). Whole-genome sequencing (WGS) is revolutionizing our understanding of drug resistance, clinical management, and transmission patterns, significantly contributing to disease control efforts (Chekesa *et al*., 2024).

In this context, the present study focuses on optimizing Deoxyribonucleic Acid (DNA) extraction, WGS, and metagenomic analysis of *N. gonorrhoeae* isolates and clinical samples in Indonesia. N. gonorrhoeae expresses multiple virulence factors. These virulence factors protect the bacterium from the host immune system and complicate diagnosis by generating diverse serotypes. In recent years, whole genome sequencing (WGS) has become more affordable, enabling core genome analysis (Alam *et al*., 2025). This study aimed to determine optimal DNA concentration and purity from optimized extraction and to achieve optimized genomic data analysis from WGS for both sample types.

Materials and Methods

This study is a pilot study. The study utilized two isolates of *Neisseria gonorrhoeae* (NG) from two clinical samples (urethral swabs) obtained from Sulianti Saroso Hospital. The reagents used included the Qiagen DNeasy Blood & Tissue Kit, RBK 96 v14 (Rapid Barcoding), InTray GC media, chocolate agar, and PSB/TSB. The equipment employed included MALDI-TOF, NanoDrop, Qubit Fluorometer, and GridION (Oxford Nanopore), among others. The study was conducted at Sulianti Saroso Hospital (biomolecular and sequencing facilities) from April to July 2025.

Pretreatment Sample

Two *N. gonorrhoeae* isolates and two clinical samples were identified, cultured, and subjected to DNA extraction. Isolate samples were transported using InTray GC,

which effectively maintained *N. gonorrhoeae* viability for up to 72 hours, slightly lower than Thayer-Martin medium, but remains practical and stable (EGASP, 2023; Paris *et al.*, 2022). Subsequently, species identity was confirmed using Maldi Tof, with scores ≥ 2.0 indicating species-level identification. Sample data yielded scores ranging from 2.01 to 2.27 for *N. gonorrhoeae*, confirming strong identification (Bruker Biotyper output). DNA quantity and purity were measured using Qubit and NanoDrop, and subsequently normalized for Whole Genome Sequencing (WGS) on the GridION platform (Oxford Nanopore), followed by metagenomic analysis using the EPI2ME Lab platform.

Bacterial DNA extraction

Genomic DNA from *Neisseria gonorrhoeae* was extracted using the Qiagen DNeasy Blood & Tissue Kit, which is widely recognized for yielding high-quality DNA suitable for downstream applications, including WGS (Eagle *et al*., 2023). For isolated samples, bacterial colonies grown in TSB were transferred into 1.5 mL microtubes, centrifuged, and washed with 200 µL PBS, whereas 200 µL of urethral swab suspension was directly processed for clinical samples. Cell lysis was performed with 20 µL Proteinase K and 200 µL Buffer AL, followed by incubation at 56 °C for 10 minutes, after which 200 µL absolute ethanol was added to facilitate DNA binding to the silica membrane. The lysates were loaded onto DNeasy Mini spin columns, washed sequentially with Buffer AW1 and Buffer AW2, and eluted in 2 × 25 µL Buffer AE. DNA quality was determined by DNA yield (concentration) and purity. A quantitative spectrophotometric assay of DNA was performed to measure the DNA yield and purity suitable for sequencing. Purified DNA was stored at -80 °C for further use (Yuliarti *et al.*, 2023).

Test DNA purity and concentration

DNA concentration was quantified using a Qubit Fluorometer with the Qubit™ 1× dsDNA HS assay. The assay was prepared by mixing 199 µL working solution with 1 µL DNA sample in a 0.5 mL microtube, vortexing briefly, and incubating for 2 minutes at room temperature in the dark before measurement. DNA purity was assessed using a NanoDrop spectrophotometer, with a 1 µL blank solution used for calibration, followed by 1 µL DNA sample for measurement, yielding A260/280 and A260/230 ratios as quality indicators.

Library preparation

Library preparation for WGS was performed using the Oxford Nanopore Technologies (ONT) Rapid Barcoding Kit (RBK) with 200 ng input DNA, as recommended by the protocol. DNA concentration was quantified using the Qubit Fluorometer, and volumes were adjusted according to the formula :

$$\text{Volume DNA}(\mu L) = \frac{200}{DNA\ Concentration\ (\text{ng}/\mu L)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

Each DNA sample was aliquoted at 200 ng into 0.2 mL PCR tubes and adjusted to a final volume of 10 µL using nuclease-free water, thereby standardizing reaction conditions and reducing inter-sample variation (Chong *et al*., 2021). If the DNA concentration does not reach 200 ng, the DNA volume is increased to 2x the initial volume (20 µL) without adding NFW to ensure sufficient detection. Barcoding was performed by adding 1.5 µL of Rapid Barcode (RB01–96) to each sample. If the sample is given 2x the initial volume, then the barcoding is also given 2x the initial volume, or 3 µL rapid barcoding. followed by short heat incubations (30 °C, 2 min; 80 °C, 2 min). Barcoded libraries were pooled, purified using a 1:1 ratio of AMPure XP beads, washed with 80% ethanol, and eluted in 15 µL Elution Buffer. Finally, 1 µL diluted Rapid Adapter (RA) was added to each barcoded sample, incubated at room temperature for 5 minutes, and prepared for sequencing.

The Rapid Barcoding Kit (RBK 96 v14) was used as an alternative to conventional ligation-based library preparation. Literature supports pre-loading DNA quantification using Qubit and allocating several hundred nanograms of DNA per flow cell, with sequencing performed on the GridION platform (Zhang *et al*., 2021).

Whole Genome Sequence

High-throughput sequencing platforms for whole genome sequencing (WGS) allow the identification of various genes/pathogens in one run, potentially tackling this issue to some extent while also offering further insights by revealing phylogenetic connections among infections (Zhou *et al*., 2022).

WGS enables tracking of strain types, modeling of sexual transmission networks, and identification of genes or mutations known to contribute to reduced susceptibility to antibiotics. As WGS technologies continue to increase in speed, accessibility, and accuracy and decrease in cost, they will continue to be incorporated into workflows to enhance the public health response (Reimche *et al*., 2026).

Whole genome sequencing was performed on the Oxford Nanopore GridION platform. Flow cells were primed with a flush buffer mix (Flow Cell Flush, BSA, and Flow Cell Tether) before loading. A total of 75 µL of the prepared library was added via the SpotON sample port, followed by sequencing in Epi2Me software under low-light conditions for up to 72 hours.

Data analysis

WGS requires robust computational infrastructure to ensure fast, reliable data processing. Clinical WGS pipelines must meet a set of requirements for both the physical computational infrastructure and the software application infrastructure (Bagger *et al*., 2024). Raw FASTQ sequencing data generated by the GridION platform

were analyzed using the EPI2ME metagenomic workflow v2.12 (Oxford Nanopore Technologies). The workflow was employed for real-time taxonomic classification and metagenomic profiling, enabling the identification and relative abundance estimation of *N. gonorrhoeae* and other microbial species.

Results and Discussion

DNA quality assessment revealed marked differences between cultured isolates and clinical samples. Most cultured isolates yielded high DNA concentrations with optimal purity ratios.

Table 1. Concentration and Purity of Neisseria Gonorrhoeae Isolate Samples and Clinical Samples

Sample Name	Qubit	Nanodrop		
	Qubit HS	260/230	260/280	Concentration
84 Clinical	1.11	0.247	1.77	6.1
84 Isolate	8.44	0.543	2.07	18.1
85 Isolate	70.6	2.04	2.06	158
85 Clinical	1.05	0.387	1.8	26.7

DNA quality was assessed using both Qubit and NanoDrop spectrophotometry. From Table 1, isolate 85 represented the best example, yielding a DNA concentration of 158 ng/µL with optimal purity values (A260/280 = 2.06; A260/230 = 2.04). Conversely, clinical isolate 84A showed a poor A260/230 ratio (0.247), indicating contamination, although its A260/280 ratio (1.77) remained within the acceptable range (Svärd *et al*., 2025). The comparison between the two methods shows that NanoDrop tends to overestimate DNA concentrations due to the detection of other nucleic acids and proteins. In contrast, Qubit provides more accurate results by specifically quantifying double-stranded DNA (dsDNA). Thus, combining these two approaches is recommended to ensure accurate DNA evaluation before downstream applications, such as library preparation (Vermessen *et al*., 2024).

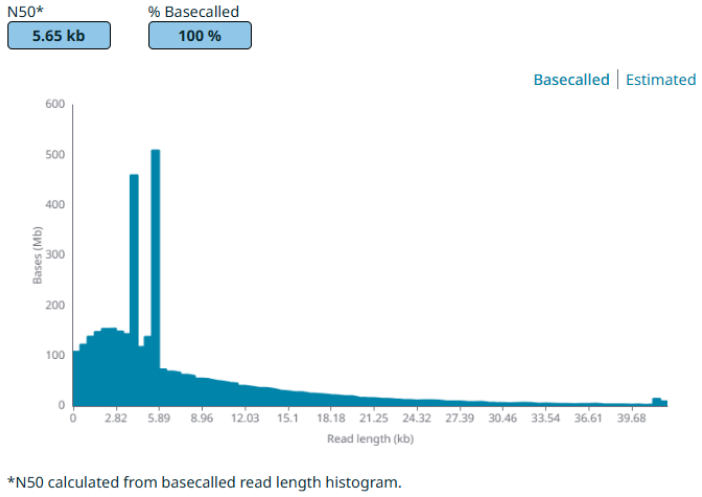


Figure 1. Library preparation

Note: READ LENGTHS OUTLIERS REMOVED. The read length graph shows the total number of bases vs the read length. The longest 1% of strands are classified as outliers, and excluded to allow focus on the main body of data.
OUTLIERS. The longest 1% of strands are classified as outliers, and aggregated into groups to show their relative amounts.

Read length (kb)	Bases (Mb)
40.96 - 303.104	45.89
303.104 - 565.248	4.06
565.248 - 827.392	2.71
827.392 - 950.272	0.95

Figure 1, the WGS results using Oxford Nanopore sequencing showed an N50 of 5.65 kb, indicating that half of the data consisted of fragments ≥5.65 kb. These long fragments facilitate genome assembly and the identification of genetic variants, while fragments >40 kb, although rare, still contribute to resolving complex genomic regions (Hong *et al*., 2024).

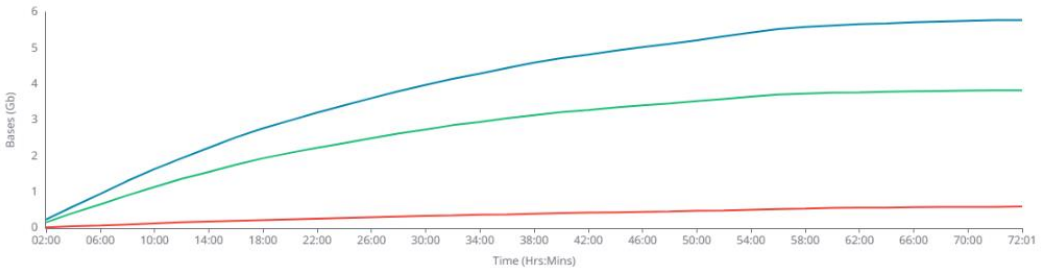


Figure 2. Cumulative Output Graph

Note: CUMULATIVE OUTPUT. The cumulative output shows the total amount of bases or reads sequenced over time by your device.

Bases Legend

- Estimated: Predicted total number of bases, prior to basecalling.
- Passed: Bases equal to or above the quality score threshold.
- Failed: Bases below the quality score threshold.

Figure 2 shows the cumulative 72-hour sequencing run generated nearly 6 Gb of data, with over 3 Gb passing quality filters for genomic analysis. High-quality reads approached one million, while failed reads were relatively few, indicating stable and reliable sequencing performance (Hong *et al*., 2024).

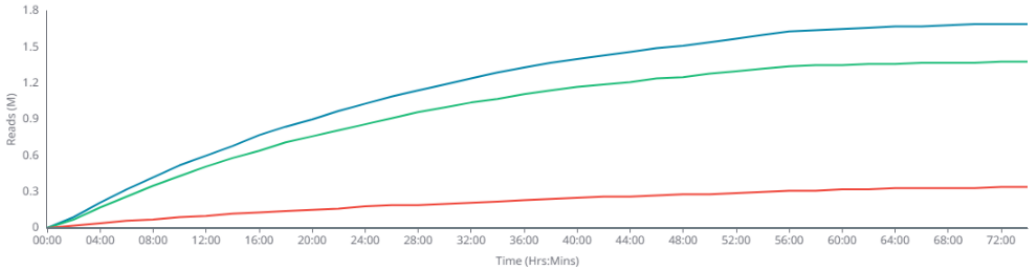


Figure 2. Reads Output

Note:

Reads Legend

- Total: Total number of reads, including passed, failed and skipped.
- Passed: Reads equal to or above the quality score threshold.
- Failed: Reads below the quality score threshold.
- Skipped: Reads that will not be basecalled. Post run basecalling is possible.

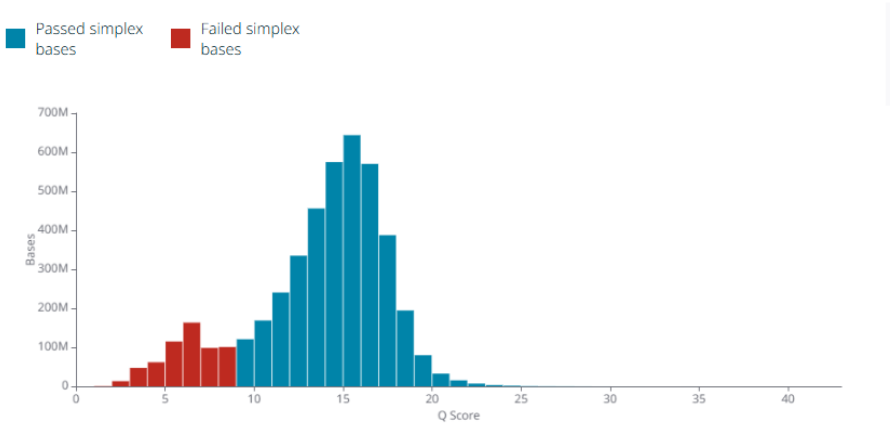


Figure 4. Quality score

Note: **QUALITY SCORE.** The quality score is calculated as basecalling is performed on your device. Reads that fall below the minimum value of 9 will be classified as failed reads. You can alter the accepted minimum quality score in MinKNOW.
Q score histogram

Filters	
Min Q score	9

Figure 3, shows that the sequencing generated approximately 1.7 million reads, of which 1.4 million passed the quality filter-sufficient for full genome analysis. The quality score is shown in Figure 4. Most reads had a Q-score of 12–16, peaking at Q15 (≈97% accuracy), indicating high and reliable quality. The decline in quality after 40 hours is typical for Oxford Nanopore sequencing (Shaskolskiy *et al*., 2022).

Metagenomic sequencing revealed a predominance of *Neisseria gonorrhoeae*, with 464,394 reads detected across all samples. Cultured isolates showed significant enrichment, yielding 119,961 reads in isolate 84 and 344,039 reads in isolate 85. In contrast, clinical samples contained only 249 reads (sample 84) and 145 reads (sample 85), representing less than 0.1% of the total *N. gonorrhoeae* sequences. This marked disparity demonstrates that isolate-based sequencing allows a substantially higher recovery of *N. gonorrhoeae* DNA compared to direct sequencing of clinical specimens, which were largely dominated by host DNA (Homo sapiens, 71,114 reads) and commensal or opportunistic bacteria such as *Escherichia coli* (54,413 reads), *Klebsiella pneumoniae* (17,483 reads), *Shigella flexneri, Salmonella enterica, and Photobacterium leiognathi*. Several clinical samples also exhibited high proportions of unclassified sequences and human DNA. WGS-based prediction of antimicrobial susceptibility works well and can be used to expedite treatment decision-making by reducing the apparent abundance of *N. gonorrhoeae* (Osman *et al*., 2024).

Patients with gonorrhea are usually treated empirically with antibiotics before the available nucleic acid amplification test is performed. This test has a time lag and shows unknown AMR patterns for patient infections, which are not ideal features for disease treatment. Thus, in cases of treatment failure and suspected ceftriaxone-resistant gonococcal infections, culture collection, antimicrobial susceptibility testing, and whole genome sequencing are recommended (Tang *et al*., 2023).

Conclusion

Optimizing DNA extraction improved DNA quality and enabled valid WGS analysis. These findings indicate that the optimized protocol is suitable for WGS of N. gonorrhoeae isolates, whereas clinical samples may require additional steps (e.g., host DNA depletion) to enhance data quality. The results of this study may serve as a reference for developing standardized laboratory protocols and metagenomic

diagnostic approaches to support pathogen genomic surveillance and gonorrhea control.

Conflict of interest

The authors declare no conflict of interest related to this study.

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