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Performance of Rapid Clonotyping of Chronic Lymphocytic Leukemia Using Flongle Flow-Cell Nanopore Sequencing of IGH Rearrangements

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ABSTRACT

Background: Clonotyping of immunoglobulin heavy chain (IGH) gene rearrangements is critical for diagnosis, prognostication, and measurable residual disease monitoring in chronic lymphocytic leukemia (CLL). Although short-read next-generation sequencing (NGS) platforms, such as Illumina MiSeq, are widely used, they face challenges in spanning full VDJ rearrangements. Long-read sequencing via Oxford Nanopore Technologies (ONT) offers a potential alternative using the compact and cost-effective flow cells.

Methods: We evaluated IGH clonotyping in samples from 13 CLL patients using ONT MinION with Flongle flow cells and super-accuracy GPU-based base calling (Dorado), comparing results to MiSeq-based LymphoTrack assays. Amplicons were generated using the IGH FR1 assay and analyzed using Smith-Waterman alignment and IgBlast tools.

Results: All major and minor clonotypes identified by MiSeq were matched with 100% sequence identity using Nanopore sequencing. Clonal burden estimates were strongly correlated (Pearson $\rho = 0.87$, $p < 10^{-4}$), although with considerable variability. Somatic hypermutation status was reliably assessed with super-accuracy base calling (Q30: 76%). Fast or CPU-only base calling was insufficient for accurate mutation analysis.

Conclusion: Nanopore sequencing enables accurate and rapid IGH clonotyping in CLL, offering comparable performance to MiSeq with lower cost and laboratory footprint. This supports its potential utility in routine and decentralized hematopathology workflows.

1 | Introduction

At the genomic level, clonal rearrangements of immunoglobulin (IGH) or T-cell receptor (TCR) loci can often be identified in monoclonal lymphoproliferative disorders. These clonotypic rearrangements serve as stable molecular markers in differentiated B- or T-cell malignancies, providing a reliable means for disease classification, treatment response monitoring, longitudinal follow-up, and measurable residual disease (MRD) assessment. In rare cases, particularly in immature lymphoid malignancies, the clonotype resolution may be limited due to incomplete, nonproductive, or highly diverse rearrangements.

In chronic lymphocytic leukemia (CLL), the most common form of leukemia in adults, specific IGH clonotypes have been shown to hold significant prognostic value. More importantly, the mutational status of the variable (V) region genes, that is the presence or absence of somatic hypermutations, offers additional prognostic stratification [1, 2]. Historically, immunoglobulin clonotyping assays have progressed from Southern blots, fragment analysis, and Sanger sequencing, culminating in next-generation sequencing (NGS) methodologies. Although short-read NGS platforms have provided unprecedented resolution, their limitations include challenges in spanning full variable-diversity–joining (VDJ) gene rearrangements and reduced base quality at the ends. One approach to circumvent these challenges has involved paired-end sequencing with overlapping reads to improve consensus accuracy and sequence assembly.

Oxford Nanopore Technologies (ONT) sequencing has emerged as an alternative approach capable of producing long reads with real-time sequencing features. Previous studies have demonstrated the feasibility of Nanopore sequencing for clonotyping applications, including quantifying somatic hypermutation (SHM) in IGHV genes in chronic lymphocytic leukemia (CLL) in comparison to Sanger sequencing [3] and in tracing clonal trajectories across biofluids [4]. In the first study, the two methods displayed the same clonotyping success rate (88%), albeit with different samples failing. The results showed a highly concordant degree of identity to the germline V gene between the two platforms, generally differing only by 0.5%. In the latter study by Sampathi et al. [4], Nanopore sequencing of IGH rearrangements in cfDNA enabled sensitive detection of B-cell clones in acute lymphoblastic leukemia, allowing for minimally invasive monitoring of residual disease and central nervous system involvement. This approach identified leukemic clones and detected cfDNA in cerebrospinal fluid from patients classified as central nervous system (CNS)-negative. Compared to Illumina sequencing, Nanopore sequencing offered similar accuracy with lower cost and faster turnaround. Clonal dynamics tracked throughout treatment revealed evolving leukemic populations, highlighting their potential for early relapse detection. With high sensitivity and minimal DNA input requirements, this method could complement existing diagnostics and reduce reliance on invasive biopsies.

Early applications of nanopore sequencing faced challenges related to sequencing accuracy [5–7] and read-length variability. On the processing side, the base-calling algorithms have introduced multiple accuracy models (e.g., fast, high-accuracy, and super-accuracy). However, the base-calling algorithm and model choice can substantially affect read quality and downstream analysis, particularly in mutation-sensitive applications like SHM assessment. Notably, the development of super-accuracy models has significantly improved sequencing fidelity [8, 9], making it a promising tool for IGH clonotyping in clinical and research settings.

Here, we evaluate the performance of rapid and cost-effective IGH-based clonotyping using the ONT MinION Mk1C and Flongle flow cells. By leveraging a streamlined workflow with a minimal laboratory footprint, we assess the feasibility of Flongle sequencing for detecting IGH rearrangements in CLL and compare its performance to established short-read NGS methods. Our study aims to determine whether ONT-based sequencing can provide a viable alternative to Illumina MiSeq sequencing for routine clonotyping while maintaining high accuracy and cost efficiency.

2 | Materials and Methods

2.1 | Patients and Samples

We included 13 patients diagnosed with CLL, represented by a total of 18 peripheral blood samples comprising diagnostic (Dx), follow-up (FU), and selected technical replicates to assess reproducibility (Table 1). The median fraction of CD19+ cells among lymphocytes was 61% [interquartile range (IQR): 28%–92%]. Median baseline clinical parameters at diagnosis were LDH 176 U/L [IQR: 162–205], lymphocyte count 13×10^9/L [IQR: 7–38], white blood cell count 24×10^9/L [IQR: 18–59], and patient age 60 years.

Genomic DNA extracted from peripheral blood was amplified using the LymphoTrack Dx IGH FR1 Assay (Invivoscribe) according to the manufacturer's instructions. Amplicons were purified with 50 μL AMPure XP beads, incubated, and magnetically separated. The samples were washed twice with 80% ethanol, air-dried, and eluted in 30 μL of Tris–HCl 10 mM (pH 8). DNA concentrations were quantified prior to sequencing using the Qubit High Sensitivity DNA Kit.

2.2 | Library Preparation and Adapter Ligation

Amplicons underwent end repair and adapter ligation with the ONT Ligation Sequencing Kit V14 (SQK-LSK114). In brief, amplicons were incubated with the Ultra II end prep Reaction buffer and Ultra II end prep Enzyme Mix (NEBNext Ultra II End repair/dA-tailing Module; New England Biolabs) for 5 min at 20°C and then for 5 min at 65°C. They were then cleaned up using the AMPure XP bead in ethanol and eluted in nuclease-free water. The adaptors were ligated to the amplicons using the ligation buffer provided in the kit and the NEBNext Quick T4 DNA Ligase (NEBNext Quick Ligation Module; New England Biolabs) for 10 min at room temperature. The amplicons were cleaned up with AMPure XP bead in short fragment buffer and eluted in elution buffer (reagents provided in kit). Eluate concentration was quantified using

TABLE 1 | Patient characteristics and Nanopore-Miseq-paired sequencing samples.

	Patient characteristics			Paired sequencing samples			
Patient number	Sex	Age at diagnosis	Binet stage	Diagnosis (Dx)	Follow-up (FU)	Additional sampling	Major clonotype (IgBlast) IGH V/J gene usage
1	M	47	A	✓	—	—	V3-23*01/J4*02
2	M	53	A	✓	—	✓ (Dx replicate)	V3-23*01/J4*02
3	M	71	A	✓	—	—	V3-30*04/J6*02
4	M	67	B	✓	—	—	V4-38-2*02/J4*02
5	M	49	A	✓	—	—	V1-18*01/J6*02
6	M	43	A	—	✓	—	V1-69*06/J6*02
7	M	58	A	—	✓	—	V3-30*04/J4*02
8	M	60	A	—	✓	—	V4-39*01/J6*03
9	F	79	A	—	✓	✓ (2nd FU)	V3-30*18/J4*02
10	F	62	—	—	✓	—	V3-48*02/J3*02
11	M	59	B	✓	✓	✓ (Dx replicate)	V3-23*01/J4*02
12	M	70	B	✓	✓	—	V1-69*01/J6*02
13	F	62	—	—	✓	—	V3-49*04/J6*03

a Qubit 4 fluorometer with the Qubit dsDNA HS assay kit (Molecular Probes, life Technologies), and the adapter-ligated amplicons were prepared for sequencing.

2.3 | Flongle Flow Cell Loading and Sequencing

R10.4.1 Flongle Flow Cells (FLO-FLG114) were used for sequencing. Before each run, a flow cell quality check was performed to ensure the presence of at least 50 active pores. Flow cells were primed with Flow Cell Flush and Tether solutions, according to the manufacturer's instructions. A sequencing mix containing sequencing buffer, library beads (included in the Flongle Sequencing Expansion Kit, EXP-FSE002), and 10 fmol of adapter-ligated amplicon was loaded onto the flow cell. Sequencing was performed on a MinION Mk1C for approximately 24 h, generating FASTQ (Guppy) and POD5-converted FAST5 files for downstream bioinformatics analysis.

2.4 | Sequencing Data Analyses

Base recalling was performed using Dorado 0.9.1 (Oxford Nanopore Technologies) with NVIDIA Tesla V100 GPUs (SXM2 32 GB GPU, CUDA 12.4, Driver Version: 550.127.05, Intel Xeon Gold 6230, 20/40/60 vCPU, 46/92/138 GB RAM). Illumina MiSeq sequencing data were analyzed with LymphoTrack Dx Software (MiSeq 2.4.3) to detect clonal IGH rearrangements for B-cell clonality assessment. IGH V- and J-gene usage from Flongle data was determined using Smith-Waterman local alignment scoring (SWIGH-SCORE tool [10]). Clonotypes from ONT and MiSeq data were confirmed with IgBlast (NCBI, NIH) and compared to IMGT germline sequences using EMBOSS Smith-Waterman alignment (gapopen = 10, gapextend = 0.5). The 2% base mismatch threshold to germline IGHV sequences was applied for somatic hypermutation detection.

Nanopore post-sequencing data processing was performed on the UCloud high-performance computing platform, hosted by the eScience Center at the University of Southern Denmark.

3 | Results

From the technical assessment of IGH-based clonotyping using Oxford Nanopore Technologies' MinION with Flongle flow cells and super-accuracy GPU base calling (Dorado), the median number of reads generated per patient sample was 329 318 [IQR: 154 951–416 204] (Figure 1A). Although lower than the expected yield, which is close to a million in the study by Minervini et al. [3], this figure was concordant with the number of reads generated on MiSeq for LymphoTrack clonality analyses 393 805 [IQR: 144 030–541 643, Wilcoxon signed-rank test: $p = 0.67$] (Figure 1B). Also, the read length reflected the expected amplicon length yielded by the LymphoTrack IGH FR1 assay, with a median read length of 471 bases [IQR: 448–486].

In the setting of super-accuracy base calling from Nanopore Flongle sequencing, 87% of bases achieved a Phred quality score of 20 (Q20), and 75% of bases reached Q30 or more, corresponding to an estimated error rate of 0.1% [$Q30 = -10\text{Log}_{10}(0.001)$]. In contrast, 70% and 46% of reads reached Q20 and Q30, respectively, using the high-accuracy (HAC) model, only accounting for 22% and 3% and using fast base calling (Figure 1C). Unless stated otherwise, super-accuracy base calling was used for all subsequent benchmarking against Illumina MiSeq-derived clonotypes.

The major CLL clonotype was detected in all patients using Nanopore (median allelic burden of 50.7%). In addition, four of the patients displayed a distinct minor clonotype (median of 20.5%). Across all cases, the Nanopore Flongle-derived IGHVJ clonotypes matched their corresponding MiSeq counterparts with 100% sequence similarity, irrespective of whether the samples were from diagnosis, follow-up, or technical replicate. The variable and joining gene usage was confirmed using Smith-Waterman local alignment (EMBOSS Water), and clonotype assignments were validated using IgBlast (NCBI, NIH) [11]. A significant correlation was observed between the clonal burden estimated by the LymphoTrack software for MiSeq and the burden assessed from Nanopore Flongle using exhaustive parallel local alignment to the clonotype and germline genes for all sequences (Figure 2A, $\rho_{\text{Pearson}} = 0.87$, $p < 10^{-4}$). Nanopore sequencing showed a slight tendency toward underestimation of clonal burden compared with MiSeq (median difference −6%), consistent with Bland–Altman analysis indicating a modest mean bias of −3.25% but relatively wide 95% limits of agreement (−25% to +18.5%, Figure 2B).

Previously identified clonotypes indicative of somatic hypermutation (SHM > 2%) were obtained using MiSeq sequencing and LymphoTrack software, in conjunction with diagnostic clinical records. These clonotypes were successfully matched using Nanopore Flongle sequencing with super-accuracy base calling via Dorado (Figure 2C). Collectively, seven patients lacked a hypermutated clonotype, with two of these exhibiting a stereotypic IGHV1-69 usage [12]. In contrast, the neural network-based base caller Guppy (Oxford Nanopore Technologies) for fast real-time base calling on the MinION Mk1C did not achieve sufficient accuracy for reliable SHM assessment.

Characterizing the overall clonotyping precision, the Nanopore Flongle output could be described as a four-parameter logistic

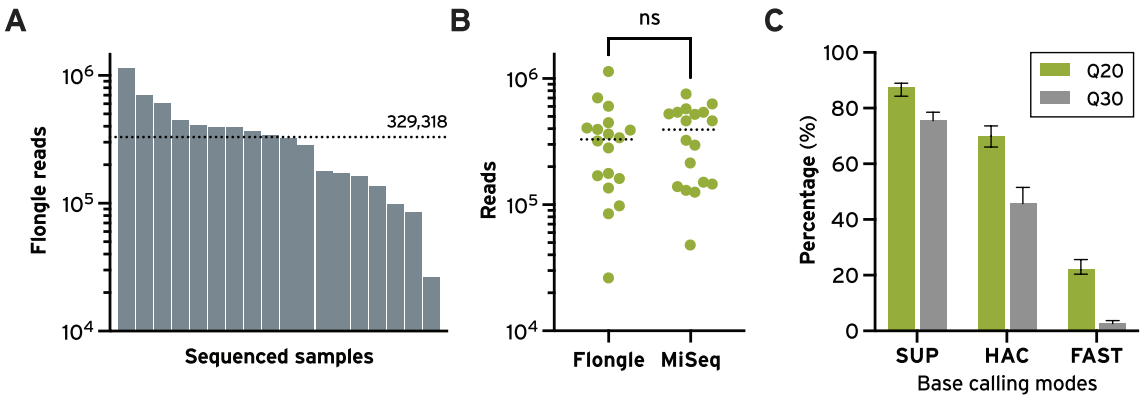

FIGURE 1 | Read yield and base-calling accuracy of IGH-based clonotyping using Oxford Nanopore Technology MinION with Flongle flow cells. (A) Distribution of Flongle read counts per sample, with a median yield of 329318 [IQR: 154951–416204]. (B) Comparison of read counts between Flongle and MiSeq platforms for LymphoTrack clonality analyses, showing no statistically significant difference (Wilcoxon signed-rank test, $p = 0.67$). (C) Base-calling performance across different modes. Super-accuracy (SUP) base calling achieved 87% of bases at Q20 and 75% at Q30. High-accuracy (HAC) base calling resulted in 70% and 46% of bases at Q20 and Q30, whereas fast base calling (FAST) yielded Q20 and Q30 at 22% and 3%, respectively.

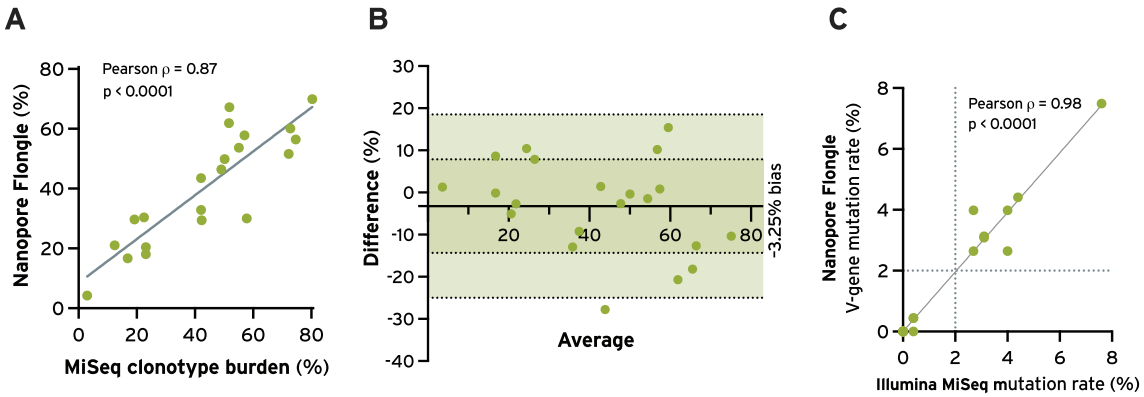

FIGURE 2 | Comparison of Nanopore Flongle and MiSeq sequencing for IGH clonotyping and somatic hypermutation assessment. (A) Correlation between clonotype burden assessed by Nanopore Flongle and MiSeq sequencing (Pearson $\rho = 0.87$, $p < 0.0001$). The paired datapoints are derived from both major and minor clones from all diagnostic, follow-up, and replicate samples combined. (B) Although clonal burden estimates from Nanopore compared to MiSeq showed no significant difference ($p = 0.22$, signed-rank test), Bland–Altman analysis demonstrates a bias of −3.25% ($SD_{bias} = 11.1$) and relatively wide limits of agreement ($LoA_{95\%}$: −25%–18.5%, $LoA_{68\%}$: −7.9%–14.3%, marked by dotted lines). (C) Concordance in V-gene mutation rates between Nanopore Flongle and MiSeq sequencing (Pearson $\rho = 0.98$, $p < 0.0001$, $n = 21$, multiple datapoints at 0).

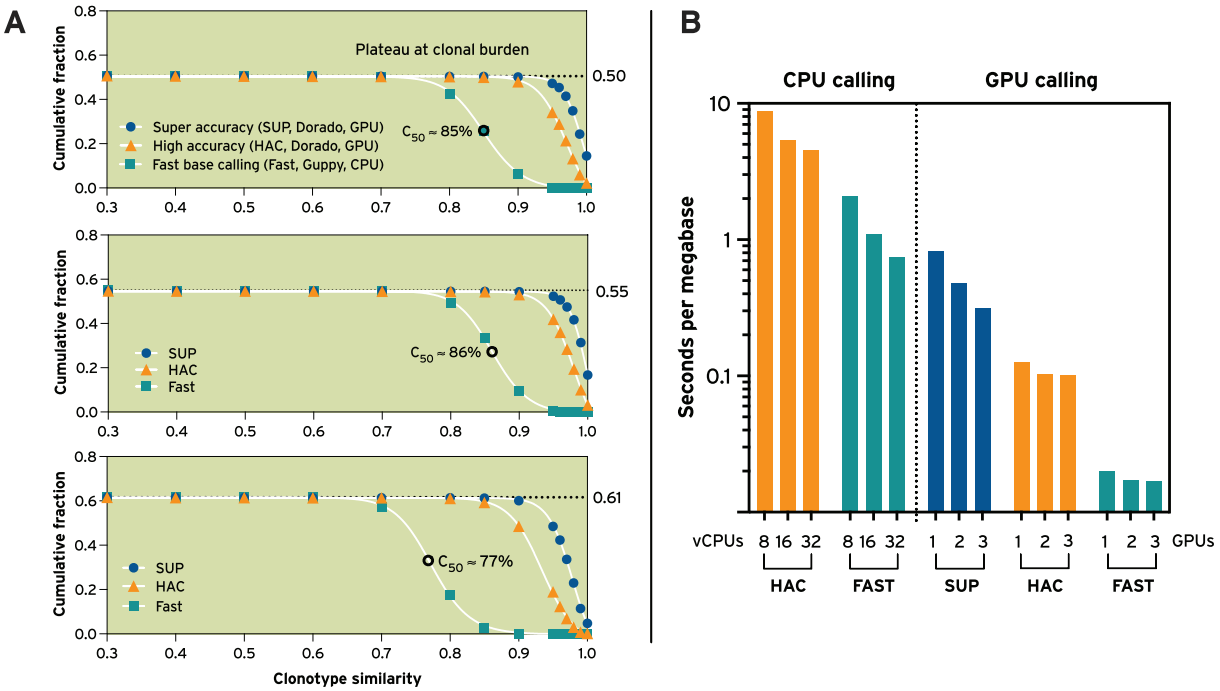

FIGURE 3 | Clonotyping precision and computational performance of base-calling models in Nanopore Flongle sequencing. (A) Cumulative fraction of clonal sequences as a function of clonotype similarity, demonstrating the performance of different base-calling models for three representative patient samples (Fast: Teal-colored points, high-accuracy [HAC]: Orange, super-accuracy [SUP]: Dark blue). Using super-accuracy base calling (Dorado), 50% of clonal sequences (C_{50}) achieved 98%–99% similarity to the reference clonotype, compared to 94%–98% using high-accuracy (Dorado) and 77%–86% with fast base calling (Guppy, CPU). The curve plateaus at 0.50–0.61 directly reflect the estimated clonal burden. (B) Computational efficiency of different base-calling models on CPU and GPU configurations. Super-accuracy (SUP) base calling was impractical on a CPU-only system, whereas a GPU-based implementation achieved a SUP processing rate of 1–3 megabases per second, with minimal gains when increasing from one to three GPUs.

regression model, regardless of sample and base calling approach. Exemplified by three representative patient samples (Figure 3A, $R^2 > 0.999$), using super-accuracy base calling (Dorado), 50% of clonal sequences (C_{50}) achieved 98%–99% identity to the referenced clonotypes (IGHV3-23*01 IGHJ4*02, IGHV3-30*04 IGHJ6*02, and IGHV4-38-2*02 IGHJ4*02). In comparison, high-accuracy (Dorado) and fast base calling (Guppy), the C_{50} reached 94%–98% and 77%–86% similarities to these clonotypes. In either case, the cumulative fraction of clonal sequences plateaued at 0.50–0.61, directly corresponding to the burden and close to the MiSeq/LymphoTrack-derived estimate. This analysis also formalizes the issue evident even with high-accuracy base calling: With a read length of nearly 500 bases, the best half of the clonal sequences may differ by many bases from the consensus clonotype (100%–C_{50}), and the dissimilarity becomes detrimental for low-accuracy *fast* base calling. Interestingly, the base-calling model, representative of the overall accuracy, had a limited effect on the ability to estimate the clonal burden, as supported by our previous error-prone base calling in CLL clonotyping [13].

Because super-accuracy base calling is now accessible on suitable hardware, we compared the wall time to high-accuracy and fast calling models. It was immediately evident that implementing the SUP model for Dorado on a pure CPU-based system, such as 32 or even 64 CPUs, was highly impractical and thus excluded. A direct log-fold increase in the required computation was observed when leveraging base-calling precision (Figure 3B), with only a minor performance gain in adding one or two parallel NVIDIA Tesla V100 GPUs to the pipeline. In this configuration, 1–3 super-accuracy megabases were resolved per second, roughly corresponding to 2000–6000 reads or PCR-amplified rearrangements (amplicons) per second.

4 | Discussion

The applicability of Oxford Nanopore Technologies' MinION Flongle flow cell sequencing for IGH-based clonotyping in CLL must be considered in the context of flexibility, cost, and accuracy. High-end sequencing platforms such as Illumina's NovaSeq 6000, MGI, or ONT's PromethION offer high throughput at a lower cost per gigabase. However, for applications requiring rapid, cost-effective, and on-demand sequencing, the Flongle or MinION flow cells provide compelling alternatives with a minimal laboratory footprint.

4.1 | Performance and Accuracy

In this study, we demonstrated that Nanopore-based sequencing using the Flongle flow cell, combined with super-accuracy GPU base calling, achieved high fidelity in clonotyping. The performance gain is attributed to the fundamentally different hardware architecture of GPUs versus CPUs: GPUs consist of thousands of parallel cores optimized for matrix and signal

processing tasks, making them particularly well-suited for real-time, high-accuracy base calling. Here, all Nanopore Flongle-derived IGH rearrangements from identified major and minor clones across the CLL patients matched their MiSeq-derived clonotypes with 100% sequence similarity. Clonal burden estimation from Flongle sequencing showed a strong correlation with MiSeq results, with a slight underestimation relative to MiSeq. These results confirm that high-accuracy Nanopore base calling can support clonotyping applications comparable to those performed on Illumina's MiSeq platform. Although the ONT platforms are rapidly shifting towards higher throughput, the general applicability is clear.

The base calling performance is a critical factor influencing clonotyping precision. Using super-accuracy base calling, which relies on a fine-tuned signal processing model, 87% of bases reached Q20, and 75% achieved Q30, corresponding to an error rate of 0.1%. This level of accuracy is sufficient to determine the mutational status of IGHV genes, a crucial prognostic marker in CLL. However, fast base calling, such as on-board Guppy processing on the Mk1C, resulted in a lower fraction of high-quality reads, precluding reliable SHM assessment.

4.2 | Considerations for Clinical Use

IGHV mutation status is a key prognostic factor in CLL, where a germline identity threshold of 98% distinguishes hypermutated cases from unmutated [1, 2]. Our findings confirm that, with high-accuracy base calling, Flongle sequencing can achieve the necessary precision for IGHV mutation analysis. Furthermore, stereotypic IGHV usage patterns are readily identifiable, such as the CLL-biased IGHV1-69 [14, 15]. Its relevance is underlined by the prognostic implications of stereotypic characterization [12] and recommendations [16], thus supporting the potential utility of Nanopore sequencing in routine hematopathology workflows.

Despite these promising results, some limitations must be acknowledged. Although a new Flongle flow cell was used for each sample, effective clonotyping was achieved with as few as 26 258 reads (see Figure 1). This suggests that multiplexing is feasible, even at the low throughput of the Flongle flow cell. However, it may be that suboptimal library DNA input in this study (~10 fmol) could have contributed to the lower-than-expected read yield, approximately half of what is reported by Jeck et al. for translocation detection in hematologic malignancies and sarcoma [17]. Optimizing DNA input could further improve sequencing efficiency.

4.3 | Advantages and Challenges

One of the key advantages of Flongle sequencing is its minimal laboratory footprint and relatively low acquisition cost compared to traditional NGS platforms. This makes it particularly attractive for laboratories with limited resources or settings requiring rapid turnaround times. Furthermore, the approach presented here accommodated both noisy and high-quality reads through exhaustive Smith-Waterman alignment scoring [10] (see Data Availability Statement), thus enabling clonotype determination robust to base-calling errors.

The sequencing depth per Flongle flow cell is limited compared to larger ONT devices such as PromethION or even MinION using standard flow cells. Additionally, high variability in sequencing yield and potential operating batch effects necessitate careful validation before clinical implementation. Signal processing and base-calling algorithms continue to improve, and further refinements may enhance the robustness of Flongle sequencing for routine diagnostic applications.

Extending the utilization of the technology, we have previously shown that Nanopore MinION sequencing is primed for leveraging cancer cytogenetics [7], which, together with Illumina's platforms, provides a leap towards comprehensive cancer cytogenomics. Other evidence demonstrates that the flexibility of the Flongle flow cell may also come in handy for rapid single-specimen detection of cancer gene fusions [17].

4.4 | Future Perspectives

Given the increasing accessibility of long-read sequencing, future improvements in base-calling models and error correction strategies will likely expand the general utility of Nanopore sequencing for IGH clonotyping. The potential for real-time data streaming and rapid processing could enable same-day diagnostic workflows, particularly in settings where rapid clonotype identification is critical, such as in acute leukemias or lymphoproliferative disorders requiring urgent clinical decisions. Generally, the acquisition of sequencing equipment may pose high relative costs, especially in developing countries, as described by Helmy et al. [18]. Thus, clonotyping using Flongle or higher throughput MinION flow cells, in perspective, holds potential for hospital laboratories with limited funds.

Our findings suggest that Nanopore sequencing could be integrated into multi-omics approaches, such as combining IGH clonotyping and whole-exome sequencing with single-cell RNA sequencing or in combination with epigenetic profiling, another direct capability of Nanopore-based sequencing [19]. Such accessible, multi-level strategies would provide a more comprehensive view of clonal evolution and disease progression in CLL and other B-cell malignancies. For a broader perspective on the potential application of Nanopore sequencing in hematology, the review by Bartalucci et al. provides a comprehensive overview [20].

4.5 | Conclusion

In summary, Nanopore sequencing, combined with GPU-based high-accuracy base calling, provides reliable IGH clonotyping with performance comparable to MiSeq sequencing while maintaining a simple and flexible workflow. The small laboratory footprint, low acquisition cost, and feasibility of multiplexing make this an attractive option for rapid clonotyping applications. Further optimization of sequencing protocols and base-calling algorithms will continue to refine the accuracy and efficiency of this approach, enhancing its clinical and research utility. Finally, we note that this study was designed as a technical proof-of-concept with a limited cohort size. Although feasibility and concordance with MiSeq sequencing were demonstrated,

larger cohorts—particularly for SHM assessment—will be required for clinical validation.

Author Contributions

M.H.H., O.C., and C.G.N. conceived and designed the study. R.G., O.C., and S.K.D. performed experiments and data acquisition. M.H.H. and R.G. carried out data analysis and interpretation. S.R.V., K.J.-J., and C.G.N. provided clinical samples and patient information. M.H.H. and O.C. established methodology and computational tools. C.G.N. and M.S. provided supervision and technical counseling. M.H.H., R.G., O.C., S.K.D., S.R.V., M.S., K.J.-J., and C.G.N. contributed to manuscript drafting and revision, and all authors approved the final version.

Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Regional Ethics Committee for the Region of Southern Denmark (#S-20160069).

Consent

Written informed consent was obtained from all participants.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

GPU-called FASTQ data have been deposited in the Harvard Dataverse and are publicly available at: https://doi.org/10.7910/DVN/V45AKI. Clonotyping implemented the source code found at the GitHub repository https://github.com/marcus-hoy-hansen/swigh-score.

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