Optimized Multiplex PCR for High-Performance NGS Applications with repliQa HiFi ToughMix

Keywords: Multiplex PCR, repliQa® HiFi ToughMix®, Next-Generation Sequencing (NGS), Oxford Nanopore Technologies® (ONT®), Targeted Sequencing, Long-Amplicon Sequencing, Midnight Amplicon Panel.

ABSTRACT

Multiplex PCR is a powerful technique that requires a polymerase capable of delivering balanced amplification across multiple targets. In this application note, we demonstrate the performance of repliQa HiFi ToughMix for multiplex amplification of the SARS-CoV-2 genome using the Midnight primer panel and Oxford Nanopore Technologies (ONT) sequencing. We show that repliQa HiFi ToughMix supports rapid multiplex PCR amplification with extension times as short as 30 seconds while maintaining uniform whole-genome coverage. Furthermore, increasing the extension time resulted in higher PCR product yields without affecting the SARS-CoV-2 genome coverage. Together with previously published studies, these findings establish repliQa HiFi ToughMix as a reliable polymerase for multiplex PCR applications across a wide range of organisms and sequencing-based workflows.

INTRODUCTION

The Polymerase Chain Reaction (PCR) is a fundamental molecular technique widely used to amplify specific sequences of DNA into millions of copies. It relies on a DNA polymerase and a set of primers designed to target a region of interest. While the core principles of PCR have remained the same, important innovations have expanded its capabilities, including the development of multiplex PCR for simultaneous detection of multiple targets. Multiplex PCR enables the amplification of several different DNA sequences within a single reaction simultaneously, significantly increasing the amount of information obtained per assay. This approach has revolutionized DNA testing by saving time, reducing costs, and conserving limited sample material[1]. As a result, multiplex PCR has become an essential tool across scientific research, clinical diagnostics, genetic testing, and public health surveillance, where its high-throughput capability supports the simultaneous detection of pathogens or genetic markers[2–6].

Despite its advantages, multiplex PCR presents technical challenges that must be addressed to ensure reliable and reproducible results. The design of multiple PCR primer sets that function efficiently within a single reaction is critical for achieving specific and unbiased amplification across different templates. Key primer characteristics such as internal stability, melting temperature and the potential for primer dimer formation, play a crucial role in overall assay performance[1]. In addition, factors including target sequence, amplicon length and GC content must be carefully considered to avoid competition among targets which can lead to imbalanced amplification. These considerations are particularly important for next-generation sequencing (NGS), where minimizing amplification bias and ensuring uniform representation of all targets are essential for accurate downstream sequencing data quality. Although pre-designed multiplex PCR panels are commercially available for some targets, further optimization is often required depending on the sample type. As a cost-effective alternative, many free and open-source in silico primer design tools are available to support primer design and target selection[7].

While careful primer design is essential, the choice of DNA polymerase is equally important for achieving optimal multiplex PCR performance. repliQa HiFi ToughMix is a high-fidelity PCR master mix characterized by high processivity, long-range amplification capability and high tolerance to GC-rich templates, making it ideal for multiplex PCR applications. Its performance in multiplex PCR applications has been demonstrated across multiple published studies[8–12].

Multiplex PCR Amplification and ONT sequencing of Lumpy Skin Disease Virus (LSDV)

Lumpy Skin Disease is caused by the Lumpy Skin Disease Virus (LSDV), a double-stranded DNA virus with an approximately 151 kb genome. Bajpai and colleagues (2025) developed a multiplex PCR and whole-genome sequencing workflow for rapid detection and genotyping of LSDV isolates collected from dairy cows across India[10–11]. Using the LSDV_WGSPP_3.5 primer set, a custom-designed panel of 48 primer pairs divided into two pools, generated ~3.5 kb overlapping

amplicons covering the entire viral genome. The protocol and primer set sequences are available through https://www.protocols.io/view/protocol-for-detection-and-genotyping-of-lumpy-ski-yxmvm3drbl3p/v1. Following the recommended PCR reaction setup and cycling conditions for repliQa HiFi ToughMix (Tables 1 and 2), repliQa HiFi ToughMix was the only mastermix that consistently produced strong and distinct 3.5 kb bands across all samples and primer pools, outperforming Takara Bio PrimeSTAR® GXL Premix, New England Biolabs Q5® Hot Start High-Fidelity Master Mix, and LongAmp® Taq Polymerase (Figure 1A-B). The latter two failed to amplify the multiplex reaction successfully (Figure 1B). PCR products generated with repliQa HiFi ToughMix were subsequently used for downstream sequencing on the ONT platform using the Rapid Barcoding Kit (SQK-RBK114.96). Sequencing of Indian LSDV isolates identified novel mutations compared to strains reported from other countries, reinforcing the importance of continuous genomic surveillance[10].

Component	Volume for 1 sample (µL)
repliQa HiFi ToughMix (2X)	12.5
Primer mix of Pool A or Pool B (10 µM)	3.75
Template DNA	50 ng
Nuclease-free Water	Makeup volume to 25 µL

Table 1 **Reaction setup for multiplex PCR amplification of LSDV using repliQa HiFi ToughMix and primer set LSDV_WGSPP_3.5.**

Step	Temperature	Incubation time	Cycles
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	15 sec	35
Extension	68°C	6 min	
Hold	4°C	Hold	1

Table 2 **Cycling program for multiplex PCR amplification of LSDV using repliQa HiFi ToughMix and primer set LSDV_WGSPP_3.5.**

Targeted Multiplex PCR–based Amplicon Sequencing for Genotyping Antimalarial Drug-Resistance Genes

A targeted amplicon sequencing strategy was later developed by the same research group to genotype antimalarial drug-resistance markers in *Plasmodium falciparum*. In this workflow, multiple resistance-associated genes were amplified using a multiplex PCR approach coupled with downstream ONT sequencing. To achieve this, the group developed the *Pf*MDR15 panel consisting of 37 primer pairs designed with PrimalScheme[13] and the PlasmoDB[14] reference genome for *Plasmodium falciparum*.

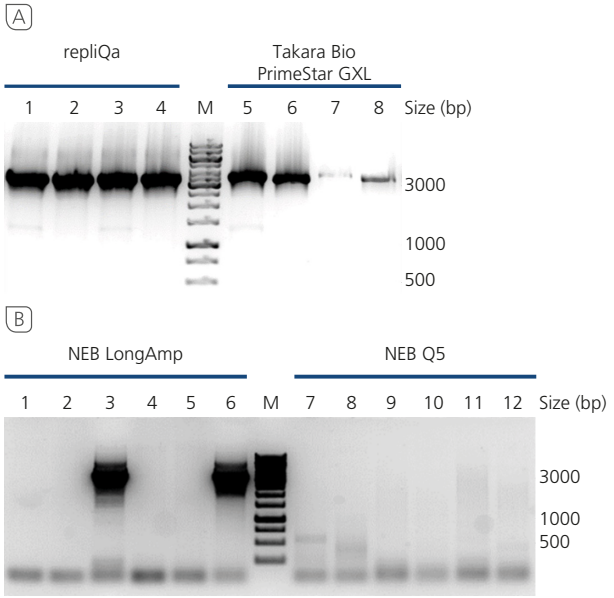

Figure 1 **repliQa HiFi ToughMix amplified ~3.5 kb amplicons from Lumpy Skin Disease Virus (LSDV) using the LSDV_WGSPP_3.5 primer panel, outperforming other polymerases.** A. The performance of repliQa HiFi ToughMix (lanes 1 - 4) and Takara Bio PrimeSTAR GXL Premix (lanes 5 - 8) is shown for LSDV genome amplification in multiplexed PCR reactions. Two field samples were analyzed; for each sample, pool A (lanes 1, 3, 5 & 7) and pool B (lanes 2, 4, 6 & 8) reactions were run. Lane M contains 1 kb DNA size ladder. B. Performance of NEB LongAmp Taq DNA Polymerase (lanes 1 - 6) and NEB Q5 Hot Start High-Fidelity 2X Master Mix (lanes 7 – 12) for LSDV genome amplification. Three field samples were analyzed for each sample, pool A (lanes 1, 3, 5, 7, 9, & 11) and pool B (lanes 2, 4, 6, 8, 10, & 12) reactions were run. Lane M contains the 1 kb DNA size ladder. The image was adapted from Bajpai *et al.*, 2025[10].

The complete primer design and workflow details are described in the "Protocol for drug resistance screening for *Plasmodium falciparum* using Oxford Nanopore Sequencing"[12]. The key component of the assay was repliQa HiFi ToughMix, which enabled reliable amplification of ~1.5 kb amplicons directly from clinical samples using two multiplexed primer pools following the recommended protocol conditions (Tables 3 and 4). The amplified products were subsequently prepared for Oxford Nanopore sequencing using the Rapid Barcoding Kit (SQK-RBK114.96). This *Pf*MDR15 multiplex PCR protocol provides a more sensitive, scalable, and sequencing-ready approach for antimalarial drug resistance surveillance directly from clinical samples.

Component	Volume for 1 sample (µL)
repliQa HiFi ToughMix (2X)	12.5
Primer mix of Pool A or Pool B (10 µM)	3.75
Template DNA	5 - 50 ng
Nuclease-free Water	Makeup volume to 25 µL

Table 3 **Reaction set up for multiplex PCR amplification of *P. falciparum* using repliQa HiFi ToughMix and primer set *Pf*MDR15.**

Step	Temperature	Incubation time	Cycles
Initial Denaturation	98°C	1 sec	1
Denaturation	98°C	15 sec	35
Extension	68°C	5 min	
Hold	4°C	Hold	1

Table 4 Cycling program for multiplex PCR amplification of *P. falciparum* using repliQa HiFi ToughMix and primer set *Pf*MDR15.

Long-Read Sequencing of Human Monkeypox (mpox) Virus

The versatility of repliQa HiFi ToughMix in long-range and multiplex PCR applications was further highlighted in a study targeting Human Monkeypox Virus. Nagy *et al*. (2025) successfully reconstructed the monkeypox virus genome from a patient skin lesion sample collected in Czechia using a long-read sequencing workflow[8]. The group designed 22 primer pairs using PrimalScheme[13] based on a MAFFT-aligned reference genome. These primers generated ~10 kb overlapping amplicons that were divided into two primer pools for multiplex PCR covering the full viral genome. repliQa HiFi ToughMix was selected to perform the amplification step due to its ability to support long-range, high-fidelity, and balanced amplification. Following multiplex PCR amplification, libraries were prepared using the Ligation Sequencing Kit (SQK-LSK110), with barcodes from the EXP-NBD196 kit developed by Oxford Nanopore Technologies. Primer sequences, amplification set up and sequencing steps are described in the protocol "Amplicon Based Sequencing of a Human Monkeypox Virus Isolate V.2"[9] (Tables 5 and 6). The assembled genome was ~198 kb in length with intact protein-coding regions and no frameshift mutations nor premature stop codons detected. A comparative analysis with the reference genome identified several deletions, insertions, and five nucleotide substitutions. Phylogenetic analysis classified the isolate within Clade IIb B.1.3, associated with the 2022 multi-country outbreak. These results demonstrate the effectiveness of repliQa HiFi ToughMix for high-quality genome reconstruction and long-read sequencing applications. Its ability to support long-range, high-fidelity and balance amplification makes it a reliable choice for sequencing applications on Oxford Nanopore Technologies platforms.

Component	Volume for 1 sample (µL)
repliQa HiFi ToughMix (2X)	12.5
Primer mix of Pool A or Pool B (10 µM)	2.5
Template DNA	2.5
Nuclease-free Water	7.5
Total Volume Reaction	25

Table 5 Reaction set up for multiplex PCR amplification of mpox virus using repliQa HiFi ToughMix.

Step	Temperature	Incubation time	Cycles
Denaturation	98°C	10 sec	35
Annealing and Extension	68°C	1 min 40 sec	
Hold	4°C	Hold	1

Table 6 Cycling program for multiplex PCR amplification of mpox virus using repliQa HiFi ToughMix.

In this application note, the Midnight Amplicon Panel was used to sequence the SARS-CoV-2 genome on an ONT platform. This workflow leveraged repliQa HiFi ToughMix for multiplex PCR starting from cDNA generated from SARS-CoV-2 RNA. repliQa HiFi ToughMix is a fast, highly processive polymerase with rapid extension times for singleplex PCR. Here we show that increasing the PCR extension time further improves its ability to successfully perform multiplex PCR. Under these optimized conditions, repliQa HiFi ToughMix generated high PCR yield while maintaining uniform genome coverage, further supporting the use of repliQa HiFi ToughMix as the best choice for multiplex PCR and sequencing-based applications.

METHODS

RNA Samples

Purified RNA from BSL-1 heat-inactivated SARS-CoV-2 strain 2019-nCoV/USA-WA1/2020 (ATCC® VR-1986HK™) was used for whole-genome sequencing.

cDNA Synthesis

First-strand cDNA synthesis was performed using the qScript® Ultra Flex Kit (Table 7).

Component	Volume for 1 sample (µL)
5x qScript Ultra Reaction Mix	4
SARS-CoV-2 RNA template (2,600 copies/µL)	5
10x Random Primers	2
Nuclease-free Water	9
Total Volume Reaction	20

Table 7 Reaction setup for cDNA synthesis using qScript Ultra Flex Kit.

Reactions were incubated at 55°C for 10 minutes, followed by enzyme inactivation at 85°C for 5 minutes, and then held at 4°C. The resulting cDNA was diluted 1:2 by adding 20 µL nuclease-free water or Low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) buffer to each 20 µL cDNA reaction prior to PCR amplification.

Midnight Primer Pools

Twenty three primer pairs (Integrated DNA Technologies) were used to generate overlapping 1200 – 1500 bp amplicons spanning the SARS-CoV-2 genome[15]. Two multiplex primer pools were prepared at a stock concentration of 100 µM. Pool 1 contained odd-numbered primer pairs (1–23), while Pool 2 contained even-numbered primer pairs (2–22). Each pool was prepared by combining 5 µL of each primer pair and subsequently diluted to a final working concentration of 10 µM in 10 mM Tris-HCl, pH 8.0. Primer pools were aliquoted and stored at –20°C for single-use applications. For the control PCR, each primer pair (1–23) was also tested individually in single-plex PCR reactions, resulting in 23 separate amplification reactions. Individual PCR products were purified using sparQ® PureMag Beads (Quantabio), quantified using Qubit® fluorometer, normalized and pooled prior to sequencing.

Multiplex PCR

For the multiplex PCR reaction, the 2 primer pools (Pool 1 and Pool 2) were used separately. Multiplex amplification of SARS-CoV-2 targets using Midnight Primer Pools and Q5 Hot Start High-Fidelity 2X Master Mix (New England BioLabs) was performed according to the published protocol: "SARS-CoV-2 genome sequencing protocol (1200bp amplicon "midnight" primer set, using Nanopore Rapid kit) V.6"[16].

Component	Volume for 1 sample (µL)
repliQa HiFi ToughMix (2X) or Q5 Hot Start High-Fidelity 2X Master Mix	12.5
Primer mix of Pool 1 or Pool 2 (10 µM)	2
cDNA Input	5
Nuclease-free Water	5.5
Total Volume Reaction	25

Table 8 Reaction set up for multiplex PCR amplification of SARS-CoV-2 using Midnight Primer Pool.

Step	Temperature	Incubation time	Cycles
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	35
Annealing	60°C	5 sec	
Extension	68°C	Variable (30 sec to 5 min)*	
Hold	4°C	Hold	1

Table 9 Cycling program for multiplex PCR amplification of SARS-CoV-2 using repliQa HiFi ToughMix and Midnight Primer Pools.
*Extension time increments used: 0.5 min, 1.0 min 1.5 min, 2 min, and 5 min

PCR Product Purification and Quality Control

PCR products generated from the two multiplex primer pools were combined and purified using sparQ PureMag Beads with Proteinase K according to Appendix A in the repliQa HiFi ToughMix product manual. DNA yield obtained at each condition was quantified using a Qubit fluorometer and the amplified products were visualized in 1% agarose gel.

Sequencing and Data Analysis

Library preparation was performed from 120 ng of purified PCR product using the Rapid Barcoding Kit (RBK004) and the ligation-based SQK-LSK109 protocol from Oxford Nanopore Technologies. The sequencing target was approximately 6,000 reads per sample.

FASTQ files were quality filtered, end-trimmed, and assembled by alignment to the SARS-CoV-2 reference genome using CLC Genomics Workbench (QIAGEN®).

RESULTS

In this application note we developed a streamlined workflow for whole-genome sequencing of the SARS-CoV-2 viral genome, which utilizes multiplex PCR using repliQa HiFi ToughMix prior to ONT sequencing (Figure 2). First, SARS-CoV-2 RNA was used as a template to generate long cDNA using the qScript Ultra Flex Kit. The cDNA was subsequently used for multiplex PCR using repliQa HiFi ToughMix and two Midnight SARS-CoV-2 multiplex primer pools to generate overlapping 1.5 kb amplicons across the viral genome. Following amplification, the PCR products from both primer pools were combined, purified using sparQ PureMag Beads, and libraries were prepared for sequencing on the ONT platform.

The recommended extension time for repliQa HiFi ToughMix when amplifying targets between 1 and 10 kb is 5 seconds per kilobase (kb). While this condition is sufficient for singleplex PCR amplification, multiplexed reactions often require longer extension times to ensure complete and balanced amplification across all targets. Therefore, extension times ranging from 30 seconds to 5 minutes were evaluated to optimize multiplex PCR performance using repliQa HiFi ToughMix and compared to Q5 Hot Start High-Fidelity 2X Master Mix, which was used in the original Midnight SARS-CoV2 sequencing protocol. Amplification performance was assessed by agarose gel electrophoresis using two multiplex primer pools designed to generate overlapping 1.2–1.5 kb amplicons spanning the SARS-CoV-2 genome (Figure 3A).

repliQa HiFi ToughMix successfully amplified both multiplex primer pools using an extension time as short as 30 seconds, demonstrating its ability to efficiently amplify long overlapping amplicons under rapid cycling conditions. When using the 5 minute extension time specified in the original publication with the Midnight SARS-CoV-2 primer panel, both polymerases produced same PCR products.

PCR yield generated at each extension time condition was quantified using Qubit fluorometer (Figure 3B). Although successful amplification was obtained at 30-second extension time, increasing the total extension time resulted in a progressive increase in multiplex PCR yield for both primer pools. PCR yield rapidly increased with longer total extension times up to 1.5 min. Further increases in the extension time did not impact the yield significantly. These results demonstrate that repliQa HiFi ToughMix supports rapid multiplex PCR amplification workflows and higher-yield applications.

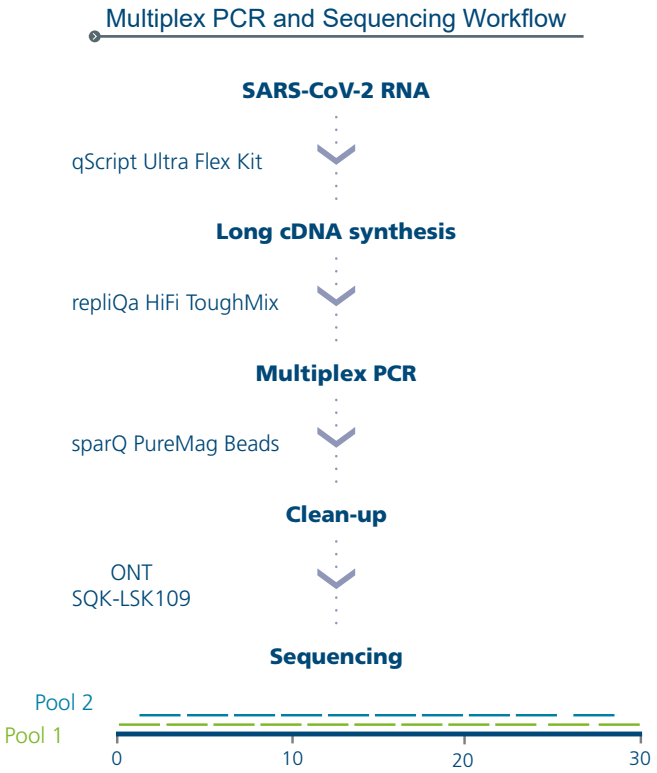

Figure 2 SARS-CoV-2 whole-genome sequencing workflow using qScript Ultra Flex Kit, repliQa HiFi ToughMix, and Midnight SARS-CoV-2 Primer Panel.

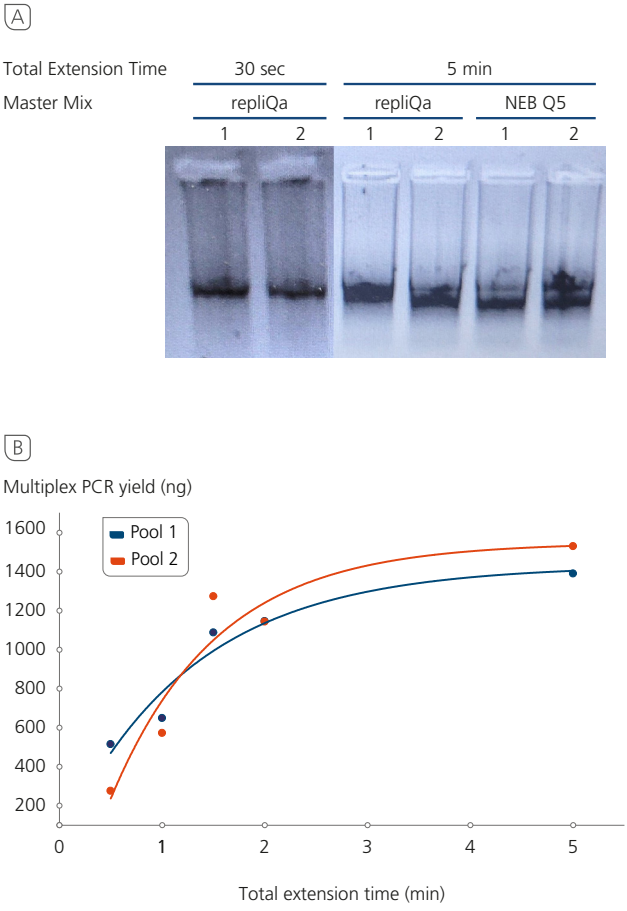

Figure 3 **Incremental increases in the extension time results in higher PCR yield with repliQa HiFi ToughMix.** A. Agarose gel analysis comparing amplification performance of repliQa HiFi ToughMix with 30 second and 5 minute extension times against Q5 Hot Start High-Fidelity 2X Master Mix using the two Midnight SARS-CoV-2 multiplex primer pools. B. Total PCR yield obtained from both multiplex primer pools across increasing total extension times (0.5, 1, 1.5, 2, and 5 minutes) was measured by Qubit.

To evaluate whole-genome coverage generated by multiplex PCR amplification using repliQa HiFi ToughMix, libraries were prepared for ONT sequencing from multiplex PCR products generated with two extension times (1.5 and 5 minutes).

As a control, singleplex PCR reactions were also performed using individual primer pairs with repliQa HiFi ToughMix with a 2-minute extension time. The resulting 23 individual PCR products were purified, normalized, pooled, and prepared for ONT sequencing.

Sample (Total Extension Time)	Total Reads	Mapped %
Pooled Singleplex PCR (2 min)	5584	96.68
Multiplex PCR (1.5 min)	5687	97.59
Multiplex PCR (5 min)	5936	97.54

Table 10 **ONT sequencing reads for multiplex and singleplex PCR amplification using repliQa HiFi ToughMix.**

Sequencing metrics, including total read number and percentage of mapped reads, were comparable across all conditions regardless of extension time or whether amplification was performed using multiplex or singleplex PCR (Table 10). Genome coverage analysis further demonstrated uniform coverage across the SARS-CoV-2 genome for both multiplex PCR conditions using repliQa HiFi ToughMix (Figure 4). Coverage profiles generated using the 1.5-minute extension time were comparable to those obtained using the longer 5-minute extension time and the singleplex control PCR, in which each amplicon was generated in an individual reaction to eliminate potential competition among primer pairs. The similarity between multiplex and singleplex coverage profiles indicates that multiplex PCR with repliQa HiFi ToughMix did not introduce amplification bias or compromise the representation of any genomic region. Together, these results confirm that repliQa HiFi ToughMix can support rapid multiplex PCR amplification while maintaining accurate whole-genome coverage and is suitable for sequencing applications.

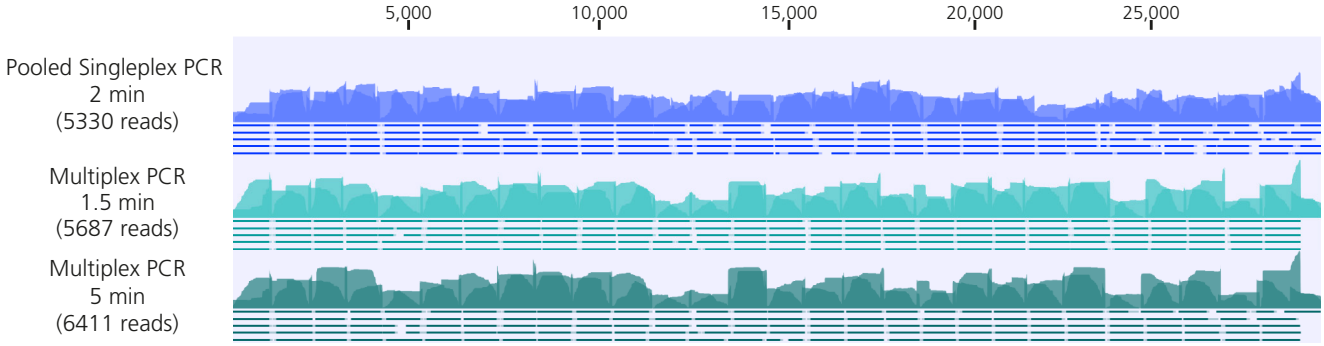

Figure 4 **SARS-CoV-2 genome coverage generated by multiplex PCR amplification using repliQa HiFi ToughMix and ONT sequencing.** Multiplex PCR with 1.5 and 5 minute total extension times was compared against pooled singleplex PCR reactions with a 2 minute total extension time. All regions in all conditions achieved a minimum read depth of 60X coverage.

CONCLUSIONS

We demonstrated that repliQa HiFi ToughMix delivers outstanding performance in multiplex PCR and supports long-range multiplex amplification workflows designed for sequencing applications, particularly ONT sequencing. repliQa HiFi ToughMix consistently allowed balanced amplification of long overlapping amplicons while maintaining high fidelity, strong amplification efficiency, and compatibility with complex multiplex PCR panels or genomes. Furthermore, we established that repliQa HiFi ToughMix supports rapid multiplex PCR amplification of the SARS-CoV-2 genome using extension times as short as 30 seconds while maintaining sequencing depth, mapping efficiency, and uniform whole-genome coverage comparable to longer extension conditions and singleplex control reactions. Together, these results highlight the ability of repliQa HiFi ToughMix to support rapid, scalable, and sequencing-ready multiplex PCR workflows for viral surveillance, pathogen genotyping, and targeted sequencing applications.

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