Rapid and sensitive single-sample viral metagenomics using Nanopore Flongle sequencing

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DOI: https://doi.org/10.1016/j.jviromet.2023.114784

Journal of Virological Methods 320 (2023) 114784

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Protocols

Rapid and sensitive single-sample viral metagenomics using Nanopore Flongle sequencing

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ARTICLE INFO

Keywords:
Viral metagenomics
Nanopore sequencing
Flongle flow cells
Clinical diagnostics

ABSTRACT

The ability of viral metagenomic Next-Generation Sequencing (mNGS) to unbiasedly detect nucleic acids in a clinical sample is a powerful tool for advanced diagnosis of viral infections. When clinical symptoms do not provide a clear differential diagnosis, extensive laboratory testing with virus-specific PCR and serology can be replaced by a single viral mNGS analysis. However, widespread diagnostic use of viral mNGS is thus far limited by long sample-to-result times, as most protocols rely on Illumina sequencing, which provides high and accurate sequencing output but is time-consuming and expensive. Here, we describe the development of an mNGS protocol based on the more cost-effective Nanopore Flongle sequencing with decreased turnaround time and lower, yet sufficient sequencing output to provide sensitive virus detection. Sample preparation (6 h) and sequencing (2 h) times are substantially reduced compared to Illumina mNGS and allow detection of DNA/RNA viruses at low input (up to 33–38 cycle threshold of specific qPCR). Although Flongles yield lower sequencing output, direct comparison with Illumina mNGS on diverse clinical samples showed similar results. Collectively, the novel Nanopore mNGS approach is specifically tailored for use in clinical diagnostics and provides a rapid and cost-effective mNGS strategy for individual testing of severe cases.

1. Introduction

The current first-line test systems used in routine diagnostics are highly sensitive, rapid, and cost-effective (Mackay et al., 2002). Targeted polymerase chain reaction (PCR) and serology are employed to specifically diagnose infectious diseases with a hypothesis-driven approach based on the clinician's differential diagnosis. While suitable for a broad range of infectious diseases, this approach potentially requires numerous diagnostic tests if the initially tested viruses are not confirmed. Indeed, a significant fraction of common human syndromes with suspected infectious causes remain of unknown or unidentifiable etiology despite extensive screening (Denno et al., 2005; Jain et al., 2015; Kapikian, 1993; Khetsuriani et al., 2002; Sivertsen and Christensen, 1996). In contrast, the shotgun-driven approach by metagenomic Next-Generation Sequencing (mNGS) is an unbiased method in which the total nucleic acid content within a given clinical sample is randomly amplified and sequenced (Delwart, 2007; Dulanto Chiang and Dekker, 2020; Mokili et al., 2012; Quince et al., 2017; Schlaberg et al., 2017). This enables the simultaneous identification of virtually any pathogen of bacterial, viral, fungal, or parasitic origin, potentially even if unknown so far, in just one analysis (Tschumi et al., 2019; Naccache et al., 2015; Cordey et al., 2016). Therefore, mNGS finds application in complex cases of infectious diseases that are difficult to diagnose and can be caused by a vast array of pathogens (Chiu and Miller, 2019; Kufner et al., 2019; Lewandowska et al., 2018; Tschumi et al., 2019; Wollants et al., 2018). mNGS has already been trialed for the diagnosis of encephalitis and meningitis (Wilson et al., 2019; Naccache et al., 2015), respiratory infections (Langelier et al., 2018; Zinter et al., 2019), joint infections (Ivy et al., 2018), and endocarditis (Million et al., 2020).

Currently applied viral mNGS protocols based on Illumina sequencing detect diverse virus families, including both DNA and RNA viruses, with high sensitivity but turnaround times and high costs remain a limiting factor for their application for single samples in severe diseases where rapid diagnosis is crucial. For example, fastest possible time-to-result of our current Illumina mNGS workflow is 24 h (Kufner et al., 2019).

Compared to other sequencing platforms, Nanopore sequencing has the advantage of longer read lengths and the possibility to analyze data

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https://doi.org/10.1016/j.jviromet.2023.114784
Received 21 April 2023; Received in revised form 11 July 2023; Accepted 25 July 2023
Available online 28 July 2023

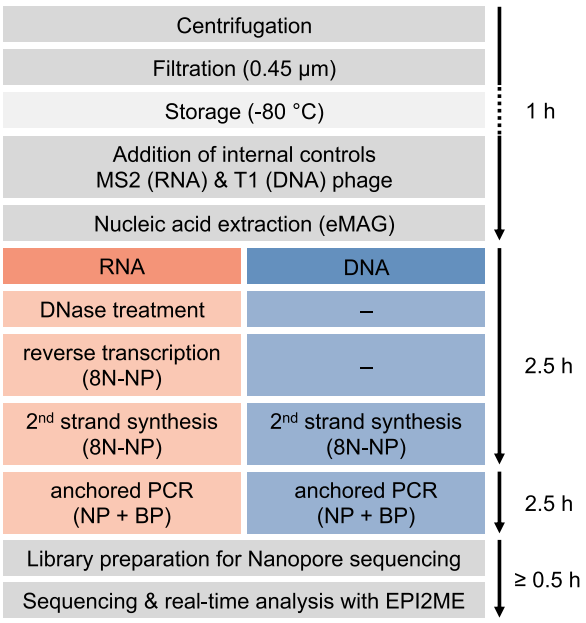


Fig. 1. Optimized workflow for unbiased and rapid virus detection using Nanopore mNGS. Sample pre-processing includes low-speed centrifugation, 0.45-µm filtration, optional storage, and addition of internal DNA and RNA controls before nucleic acid extraction. Next, the workflow is split into an RNA and DNA workflow. The former requires a DNase treatment and reverse transcription. Both workflows require second strand syntheses. The 8N-NP primers allow unbiased amplification and provide an anchor sequence for the following extension PCR with two primers, NP and BP, where the latter allows barcoding to distinguish the workflows or samples during subsequent Nanopore sequencing.

in real-time (Jia et al., 2021; Greninger et al., 2015; Li et al., 2020). The field of mNGS has also embraced this technology and diverse protocols have been developed; for bacteria (Charalampous et al., 2019), DNA viruses (Esnault et al., 2022), RNA viruses (Greninger et al., 2015; Xu et al., 2021; Claro et al., 2021; Liefting et al., 2021; Yandle et al., 2023), and DNA and RNA viruses combined (Jia et al., 2021; Fomsgaard et al., 2022). Decreasing time-to-result has been the main object in the development of all these protocols, with some of them yielding total turnaround times below six hours. Hence, mNGS on Nanopore has also been deemed suitable in the context of public health surveillance and diagnosis of infectious diseases (Schmidt et al., 2020).

In recognition of its potential as a more rapid alternative to our current Illumina mNGS approach, here, we devised a rapid and sensitive mNGS strategy to detect DNA and RNA viruses in parallel based on Nanopore sequencing. Low cost and straightforward use were achieved by flow cell dongles (Flongles), which have already been used both for specific amplicon as well as metagenomic RNA virus sequencing (Liefting et al., 2021; Gradel et al., 2022). Flongles are single-use and thus do not require a washing step or experience pore loss unlike Standard MinION and GridION Flow Cells (SFCs) when used multiple times. Our protocol can therefore be applied for single samples in situations where urgent diagnosis is needed.

2. Materials and methods

2.1. Virus stock production

Stocks of Human adenovirus 7 (HAdV-7), Human herpesvirus 5 (HHV-5), Influenza virus A/H1N1/PR8 (IAV), and Human poliovirus 1 strain LSa (EV-C) were prepared as described previously (Lewandowska et al., 2017). Aliquots were stored at − 80 °C.

2.2. Preparation of virus spiked specimen

Human plasma samples were obtained from anonymous blood donations from healthy individuals obtained by the Zurich Blood Transfusion Service (http://www.zhbsd.ch/). Negative-tested cerebrospinal fluid (CSF) samples were obtained from leftover diagnostic samples. Samples were centrifuged at 2000 rpm for 10 min and aliquots were stored at − 20 °C. Samples were spiked with HAdV-7, HHV-5, IAV, and EV-C at different concentrations by performing a 1:5-dilution series of the stock virus in phosphate-buffered saline (PBS) and extracted. Quantitative PCR (qPCR) or reverse transcription qPCR (RT-qPCR), respectively, yielded cycle thresholds (Ct) ranging from 25 to undetectable (> 45). Samples were filtered using a 0.45-µm polyethersulfone (PES) filter (TPP, Trasadingen, Switzerland).

2.3. Preparation of clinical specimen with internal controls

Clinical samples were filtered using a 0.45-µm PES filter (TPP) and spiked with two internal controls, i.e., Escherichia phage T1 (DNA control, DSM 5801) and Escherichia phage MS2 (RNA control, DSM 12767) (both DSMZ German Collection of Microorganisms and Cell Cultures GmbH, Leibniz Institute, Germany). The amount of internal control spike was determined for each stock individually. To this end, a titration series of the stock was prepared in healthy donor plasma and sequenced with our in-house Illumina mNGS protocol as described previously (Kufner et al., 2019). The amount of control stock that yielded 100 mapped reads per million quality-filtered reads per workflow (RPM) was then spiked into the samples. For CSF samples, due to their lower RNA background, ten times less MS2 was added than for blood.

2.4. Nucleic acid extraction

Samples were extracted on the NucliSENS eMAG or EasyMAG systems (both BioMérieux, Craponne, France), according to the manufacturer's instructions. 1000 µL input volume was eluted in 50 µL and 25 µL for virus spike and clinical samples, respectively. Clinical samples with volumes below 1000 µL were diluted in PBS before extraction.

2.5. Virus quantification by qPCR

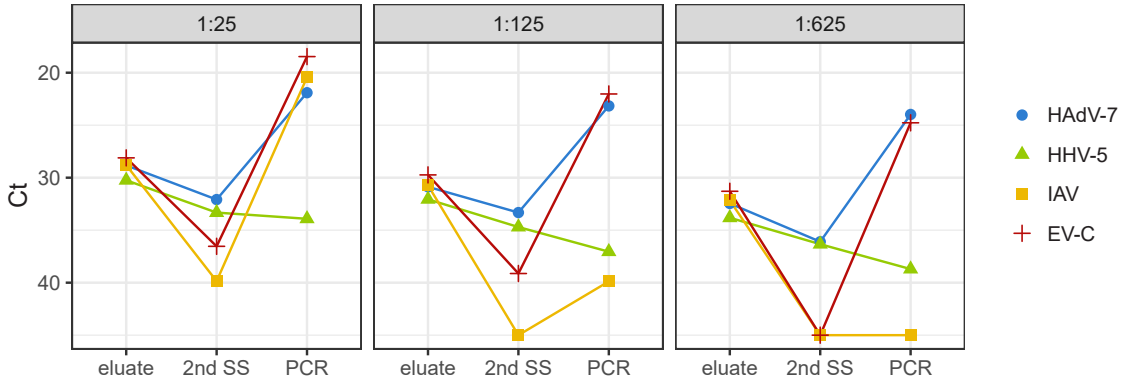
Viral loads in spiked samples were determined after nucleic acid extraction, second strand synthesis, and PCR by (RT-)qPCR as previously described for HAdV-7 (Heim et al., 2003), HHV-5 (Yun et al., 2000), IAV (CDC protocol of real-time RT-PCR for swine influenza A H1N1, 28 April 2009), and EV-C (Tapparel et al., 2009). All reactions were performed using the TaqMan RNA-to-Ct 1-Step Kit on a QuantStudio 7 Flex System (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA), with samples tested in duplicates or triplicates as previously described (Lewandowska et al., 2017). Unless stated otherwise, the reported Cts refer to the virus concentration in the eluate.

2.6. Unbiased nucleic acid amplification for Nanopore sequencing

To allow separate detection of DNA and RNA viruses, sample preparation was split into two workflows which only have varying steps at the beginning.

For the RNA workflow, 10 µL eluate was first digested using the TURBO DNA-free Kit (TURBO DNase Treatment and Removal Reagents; Invitrogen/Thermo Fisher Scientific) as described by the manufacturer. Then, 0.5 mM dNTPs and 2.5 µM primer consisting of random octamers and a "Nanopore anchor" (8N-NP, ACTTGCCTGTCGCTCTATCTTCNNNNNNNN) were added to 5 µL DNase-treated eluate in a total reaction volume of 13 µL and denatured at 65 °C for 5 min. cDNA was generated using the SuperScript IV Reverse Transcriptase (Invitrogen/Thermo Fisher Scientific) and the remaining RNA was digested using RNase H (New England Biolabs, Ipswich, MA, USA). Both kits were

a



b

	1:25		1:125		1:625	
	15 min	24 h	15 min	24 h	15 min	24 h
Total analyzed	3,982	98,780	2,971	109,284	5,947	147,734
Total classified	3,303	81,080	2,400	84,752	4,739	115,938
Human	3,234	79,663	2,388	84,370	4,733	115,745
HAdV-7	16	274	6	95	1	51
HHV-5	7	118	1	1	1	1
IAV	14	375	3	56	2	4
EV-C	28	596	2	170	4	89
other viruses	3	28	0	26	1	37

Fig. 2. Sequencing results of spiked plasma samples with decreasing viral loads. Plasma from healthy blood donors was spiked with Human adenovirus 7 (HAdV-7), Human herpesvirus 5 (HHV-5), Influenza virus A (IAV), and Human poliovirus 1 (EV-C) at different concentrations (5-fold dilution series, with starting concentration at qPCR cycle threshold (Ct) 25 for all four viruses; workflow only performed for dilutions 1:25, 1:125, and 1:625). (a) qPCR was performed after nucleic acid extraction, second strand synthesis (2nd SS) and PCR. Cts > 45 are undetectable. (b) Number of reads after 15 min and 24 h of Flongle sequencing as determined by *Fastq WIMP (Human + Viral)* by EPI2ME. All viruses from all dilutions were detected within 15 min of sequencing.

applied as described by the manufacturer. The sample was then denatured at 94 °C for 2 min.

For the DNA workflow, 5 µL eluate with added 0.5 mM dNTP and 2.5 µM 8N-NP in a total volume of 10 µL was denatured at 94 °C for 2 min.

Second strand synthesis for both workflows was performed using DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs) as described by the manufacturer. dsDNA was purified with 2X volume Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) and washed twice with 80% ethanol. DNA was eluted in 20 µL water and concentration was measured using the QuantiFluor ONE dsDNA system (Promega, Madison, WI, USA). For protocol optimization, PCRs in two different total reaction volumes of 25 µL or 50 µL were compared, containing 12.5 µL or 25 µL LongAmp Hot Start Taq 2X Master Mix (New England Biolabs), respectively, 0.1 µM "Nanopore primer" (NP, ACTTGCCTGTCGCTCTATCTTC), 0.03 µM barcoded primer (BP, i.e., BP01 to BP12 from the PCR Barcoding Kit SQK-PBK004, Oxford Nanopore Technologies, Oxford, UK) and 11.5 µL or 5 µL second strand synthesis product, respectively. The cycling conditions were 94 °C for 1 min, 10 cycles of 94 °C for 30 s, 59 °C for 30 s (annealing temperature for NP), 65 °C for 2 min, 35 cycles of 94 °C for 30 s, 62 °C for 30 s (annealing temperature for BP), 65 °C for 2 min, and 65 °C for 10 min. The final protocol uses the 50-µL reaction volume PCR (Supplementary protocol 1).

The PCR product was purified with 80% volume Agencourt AMPure XP beads (Beckman Coulter) and washed twice with 80% ethanol. The product was eluted in 15 µL water and concentration was measured as described above.

2.7. Nanopore sequencing and bioinformatic analysis

Samples from the DNA and RNA workflow were pooled and prepared to be sequenced on a Flongle or SFC (both R9.4.1 chemistry, Oxford Nanopore Technologies) as described by the manufacturer's instructions. Samples were sequenced between 15 min and 24 h, depending on the experiment, on a GridION X5 Mk1. SFCs were washed between runs using the Flow Cell Wash Kit (EXP-WSH004, Oxford Nanopore Technologies) and reads were basecalled using *guppy* (v6.1.5 onwards, https://community.nanoporetech.com) and analyzed in real-time using the *Fastq WIMP (Human + Viral)* workflow by EPI2ME (v2021.11.26, Metrichor, Oxford, UK). *Fastq WIMP (Human + Viral)* identifies viruses using complete virus genome sequences (NCBI RefSeq). One virus read was counted as a positive hit. Post-sequencing analysis was done using SAMtools (v1.10, (Danecek et al., 2021)), Burrows-Wheeler Aligner (v0.7.17, (Li, 2013)), and minimap2 (v2.24, (Li, 2018)).

2.8. Unbiased nucleic acid amplification for Illumina sequencing and bioinformatic analysis

Library preparation of clinical samples sequenced on the MiSeq platform (Illumina, San Diego, CA, USA) and bioinformatic analysis using the pipeline VirMet (https://github.com/medvir/VirMet/releases/tag/v1.1.1) was performed as previously described (Kufner et al., 2019). At least three reads of at least 75 nucleotide length distributed over at least three regions of the genome were counted as a positive hit.

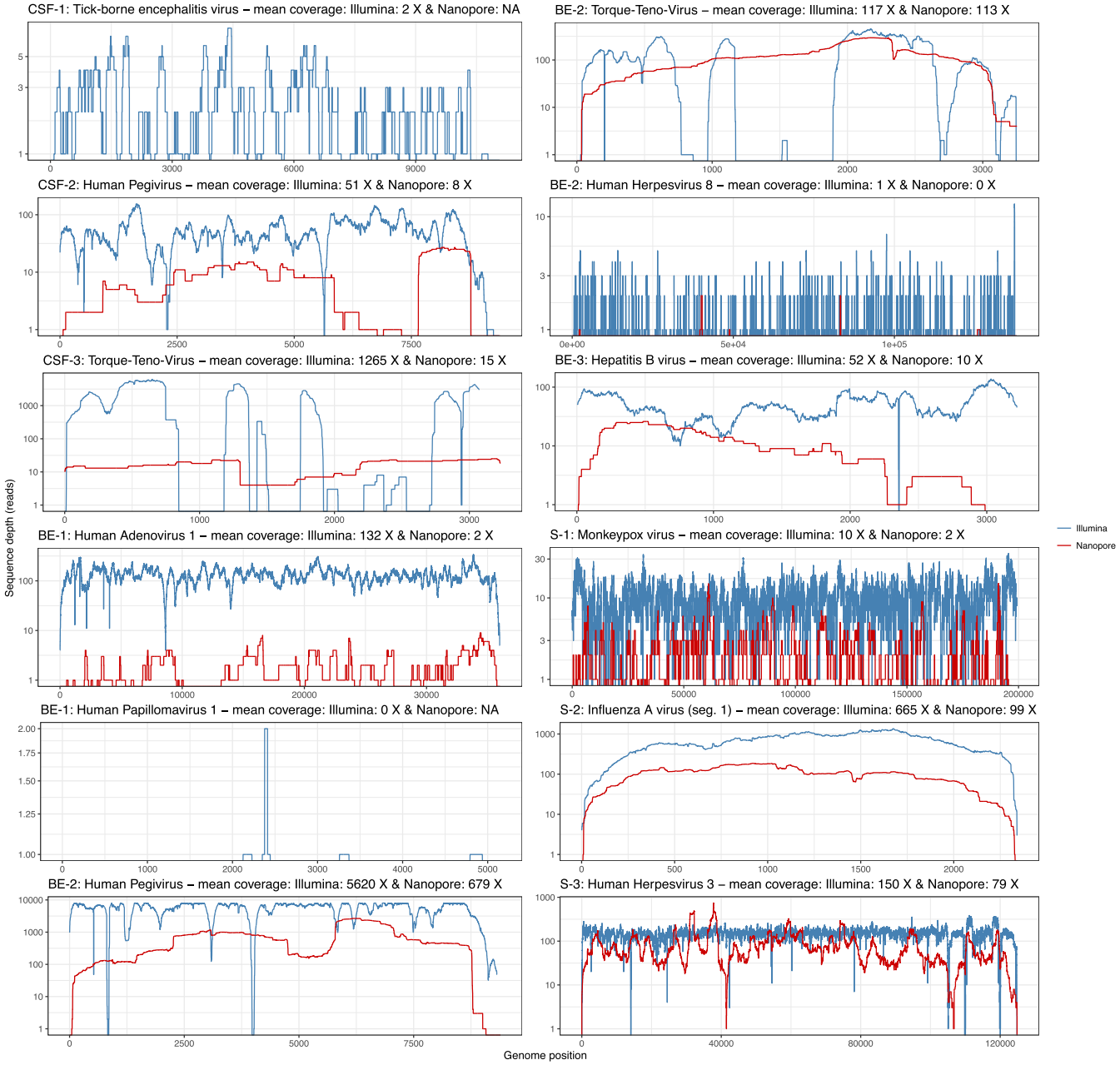


Fig. 3. Illumina and Nanopore mNGS coverage plots of viruses in clinical samples. Clinical samples of different materials, i.e., EDTA-blood (BE), cerebrospinal fluids (CSF), and swabs (S), were sequenced with the Illumina and Nanopore mNGS workflow. Reads were mapped using Burrows-Wheeler Aligner.

2.9. Analysis of results

Performance of the Nanopore mNGS approach was assessed in relation to our Illumina mNGS workflow by determining positive percent agreement (PPA) as TP/(TP + FN) and positive predictive value (PPV) as TP/(TP + FP) (Junier et al., 2019). Limits of detection (LOD) were calculated from the linear regression as "z-score * standard deviation of the residuals / slope". We used a z-score of 1.65 for a one-sided probability of 95%.

2.10. Figure generation

Figs. 2 to 4 and supplementary figures 1 to 4 were produced using the *ggplot* package (v3.4.1, (Wickham, 2016)) in RStudio (v2023.03.0.386, (Posit team, 2023)). Figures were assembled and finalized in Affinity Designer (v1.10.6).

3. Results

3.1. Development of a rapid sample preparation protocol for Nanopore mNGS

We sought to develop a sample preparation protocol for Nanopore mNGS that provides short time-to-result, is cost-effective, and through this allows single-sample application (Fig. 1, Supplementary protocol 1). For the assessment of the protocol, we used healthy donor plasma spiked with two DNA viruses (HAdV-7, naked; HHV-5, enveloped) and two RNA viruses (IAV, enveloped; EV-C, naked) at different concentrations in the eluate (Cts 25–34). We based the new method largely on our existing Illumina mNGS protocol that is in routine use in our diagnostics unit (Kufner et al., 2019). Our Nanopore mNGS workflow utilizes unique primers but otherwise follows the Illumina mNGS protocol up to second strand synthesis. The workflow is split into two to allow separate

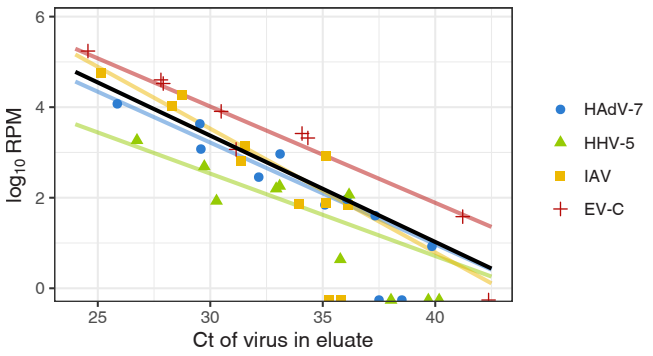


Fig. 4. Limit of detection (LOD) determination of Nanopore mNGS. Plasma from healthy blood donors was spiked with HAdV-7, HHV-5, IAV, and EV-C at different concentrations, prepared with the Nanopore mNGS workflow and sequenced on Flongles. Linear regression models were fit for all viruses individually (colored) and in combination (black). Negative Nanopore mNGS samples are not included in the regression but are still shown on the x-axis.

detection of DNA and RNA viruses. While for DNA only second strand synthesis is performed, RNA additionally undergoes DNase treatment and reverse transcription. The 8N-NP primers allow unbiased attachment to all nucleic acids in the sample (random octamer (8N)) and attach an anchor sequence for the two primers used in the following extension PCR ("Nanopore primer" (NP) and barcoded primer (BP, from the PCR Barcoding Kit SQK-PBK004)). The 8N-NP primers were tested at different concentrations with 2.5 μM yielding the best results (data not shown). The extension PCR with the two primers required optimization of concentration, cycle number and annealing temperature for both NP (0.1 μM, 10 cycles, 59 °C) and BP (0.03 μM, 35 cycles, 62 °C), as well as template volume (11.5 μL in 25-μL total volume).

Virus concentration during sample preparation was assessed by (RT-) qPCR of the four spiked viruses after second strand synthesis and PCR (Fig. 2a). All spiked viruses could be detected by sequencing on Flongles showing the validity of our protocol (Fig. 2b). Although the 1:625-dilution was qPCR-negative for IAV after the extension PCR, Nanopore mNGS still generated four reads. This difference at low viral loads can be due to the random amplification of fragments that do not include the qPCR target.

Apart from the four spiked viruses, we also found very few reads per sample of Torque teno virus (TTV), which is commonly found in plasma, and bacteriophages (Proteus phage VB_PmiS_Isfahan and Escherichia phage phiX174) and non-human viruses (granulo-, orthobunya-, and adenovirus) in all samples, which are likely reagent contaminants from library preparation (Fig. 2b, other viruses).

To further optimize the extension PCR, we increased the total reaction volume from 25 μL to 50 μL and tested different template volumes. We settled on 5 μL and compared it to the previous PCR conditions on plasma and CSF samples spiked with HAdV-7, HHV-5, IAV, and EV-C at different concentrations (Supplementary figure 1). The two PCRs perform similarly, though at lower viral loads, the 50-μL PCR detected viruses more often. In four cases viruses with Cts above 35 in the eluate (HHV-5, IAV, and twice HAdV-7) that remained undetected by the 25-μL PCR were detected by the 50-μL PCR.

3.2. Nanopore and Illumina mNGS have good agreement in diverse clinical samples

We next assessed how our Nanopore mNGS protocol (still with 25-μL PCR) performs on clinical samples. To this end we conducted a direct comparison with Illumina mNGS, making use of previously analyzed specimen from routine diagnostics. We had access to cryo-preserved eluates of different materials, i.e., cerebrospinal fluid (CSF), EDTA-blood (BE), and swab (S), and selected for each material three positive and one negative sample as determined by Illumina mNGS. We re-sequenced these samples with our Nanopore mNGS method and analyzed them with EPI2ME's *Fastq + WIMP (Human + Viral)* which uses Centrifuge to map reads in real-time (Kim et al., 2016) (Table 1). We recorded our results twice; after sequencing for thirty minutes and six hours. Among the nine positive samples, Illumina mNGS had recorded twelve viruses. Nanopore detected ten out of them after thirty minutes; a tick-borne encephalitis virus (TBEV) in sample CSF-1 and a human papillomavirus 1 (HPV-1) in sample BE-1 were not detected. In the readout after six hours, TBEV was detected but HPV-1 remained undetected. Agreement between the two sequencing methods for the clinical viruses was accordingly high (PPA = 91.67%, PPV = 100%). Considering the lower sequencing output capacity of Flongles, we were intrigued by its remarkable sensitivity. Among the three negative samples, Nanopore mNGS did not detect any virus (Table 1).

Despite its longer read lengths, the coverage achieved by Nanopore mNGS is lower compared to Illumina (Fig. 3, Supplementary figure 2). In some low-coverage cases, analysis tools provide divergent results. For instance, with sample CSF-1 Nanopore mNGS results analyzed by Centrifuge detected three TBEV reads whereas the Burrows-Wheeler Aligner found none and accordingly failed to generate a coverage plot (Fig. 3).

The internal DNA control, T1 phage, was detected in all but one sample (S-2), whereas the internal RNA control, MS2 phage, was detected in only one sample (CSF-3), highlighting the necessity to increase the input of internal controls in the final assay protocol (Table 1).

The same contaminating bacteriophages and non-human viruses as mentioned before for the spiked plasma samples were also found in the clinical samples by both Nanopore and Illumina mNGS, indicating reagent contamination during sample preparation. The non-human adenovirus was only found with Nanopore mNGS.

3.3. Nanopore and Illumina mNGS yield similar viral reads per million

As a next step, we compared the relative sensitivity between Illumina and Nanopore mNGS in more detail. Total reads passing quality filter and read lengths vary strongly between the two methods. Our in-house Illumina mNGS protocol yielded on average 4.5 M and 1.5 M reads passing the quality filter (Phred score $\geq$ 20) for the DNA and RNA workflow, respectively, whereas Nanopore mNGS yielded on average 66.5 K and 18.8 K reads passing quality filter (Phred score $\geq$ 8) for the DNA and RNA workflow, respectively (Table 1). We found that the two methods achieved very similar viral RPMs (Supplementary figure 3). This underlines that the slightly lower sensitivity of Nanopore mNGS is not an effect of sample preparation but lower sequencing output. Consequently, increasing Nanopore sequencing output for better sensitivity could potentially be attained by using multiple Flongles per sample or switching to SFCs.

3.4. Flongle to standard MinION and GridION flow cell comparison

We initially decided to use Flongles because of their lower price and the ability to use single samples, which are key factors in clinical diagnostics. However, SFCs have a higher sequencing capacity (up to 2048 active pores compared to 126 on a Flongle) and can be reused which may provide an option for higher sequencing output at lower costs, particularly if more samples are analyzed simultaneously. To probe the utility of SFCs we assessed their reusability and compared their sensitivity to Flongles. We found that sequencing for three or six hours allowed reusing the same SFC up to six times, after this the active pores were reduced substantially and SFCs only yielded read numbers comparable to Flongles (Supplementary figure 4a). Notably, Flongle and SFC sequencing resulted in very similar RPMs for each virus (Supplementary figure 4b). Comparison of read lengths and quality scores between the two flow cells also achieved similar results. On average, Flongles generated reads of 755 bases and a quality score of 10.8, whereas SFCs generated reads of 695 bases and a quality score of 11.6 (Supplementary

Table 1
Illumina and Nanopore mNGS results of clinical samples.

	Sample	**CSF-1**	**CSF-2**	**CSF-3**	**CSF-4**	**BE-1**	**BE-2**	**BE-3**	**BE-4**	**S-1**	**S-2**	**S-3**	**S-4**
	Virus(es) of interest	TBEV	HPgV	TTV	none	HAdV-1 HPV-1	HPgV TTV HHV-8	HBV_{DNA} HBV_{RNA}	none	MPXV	IAV	HHV-3	none
Illumina mNGS	**quality-filtered reads**												
	DNA workflow	4,894,244	8,079,972	10,226,947	4,494,122	4,236,550	1,922,462	3,150,792	6,362,194	2,511,547	5,918,783	2,304,245	11,794
	RNA workflow	603,330	806,318	2,150,038	236,078	1,027,602	4,458,536	733,803	1,935,788	–	4,911,155	438,089	89,988
	reads												
	Virus(es) of interest	161	3,992	50,658	–	33,451 4	336,541 2,998 445	216 1,403	–	10,306	56,091	115,850	–
	T1	399	221	35,410	4,489	62	127	10	1,548	–	20	982	4,521
	MS2	230	134	512	70	14	259	4	30	–	3	830	79
	RPM												
	Virus(es) of interest	267	4,951	4,953	–	7,896 1	75,482 1,559 231	69 1,912	–	4,103	11,421	50,277	–
	T1	82	27	3,462	999	15	66	3	243	–	3	426	383,331
	MS2	381	166	238	297	14	58	5	15	–	1	1,895	878
Nanopore mNGS	**quality-filtered reads after 6 h sequeuncing**												
	DNA workflow	95,491	104,119	109,087	139,018	36,642	52,025	33,822	33,854	95,091	26,342	39,020	33,102
	RNA workflow	7,651	9,935	12,250	24,009	22,965	24,625	5,475	9,662	15,907	85,101	4,324	4,151
	misclassified	11,179	9,148	10,620	11,651	6,010	9,575	6,181	2,471	10,398	10,734	4,245	3,118
	reads after 30 min sequencing												
	Virus(es) of interest	0	3	4	–	24 0	762 71 2	3 7	–	80	349	2,146	–
	reads after 6 h sequencing												
	Virus(es) of interest	3	42	48	–	85 0	3,918 257 6	10 30	–	436	1,996	9,206	–
	T1	7	2	181	253	1	34	1	17	–	0	132	28,657
	MS2	0	0	1	0	0	0	0	0	–	0	0	0
	RPM after 6 h sequencing												
	Virus(es) of interest	392	4,227	440	–	2,320 0	159,107 1,365 115	296 1,279	–	4,585	23,454	235,930	–
	T1	73	19	1,659	1,820	27	654	30	502	–	0	3,383	865,718
	MS2	0	0	82	0	0	0	0	0	–	0	0	0

CSF: Cerebrospinal fluid, BE: EDTA-blood, S: swab, RPM: mapped reads per million quality-filtered reads

figure 4cd). However, whereas Flongles could detect the spiked amount of HHV-5 in only 2/4 runs, the SFCs detected it in 12/15 runs, and only missed it after their sixth run.

3.5. Flongle Nanopore mNGS reliably detects viruses at Cts of as high as 33–38

Finally, we were interested in determining the LOD of our optimized Flongle Nanopore mNGS approach. To this end, we spiked plasma samples with HAdV-7, HHV-5, IAV, and EV-C at concentrations that range from a Ct of 25 to undetectable by (RT-)qPCR (> 45) and sequenced them on Flongles using the 50-µL input PCR protocol. The resulting viral RPMs (in logarithmic scale) were used to estimate the LOD (Fig. 4). The linear model predicts a theoretical LOD for 1 RPM of Ct 42 for HAdV-7, 39 for HHV-5, 40 for IAV, and 46 for EV-C. Since Flongles yield typically 10^4 to 10^5 reads passing filter per RNA or DNA workflow, RPMs of 100–10 are required, respectively. The theoretical LOD was therefore adjusted and is predicted to be as high as 33–38 Cts (HAdV-7: 38, HHV-5: 33, IAV: 33, EV-C: 37). This makes our Flongle Nanopore mNGS approach a promising tool for rapid and sensitive detection of viral infections.

4. Discussion

In this study, we developed a rapid sample preparation and sequencing protocol for viral mNGS using Nanopore Flongles. This approach allows for reliable and unbiased detection of DNA and RNA viruses with Cts of as high as 33–38 within eight hours, rendering the method of high utility for clinical diagnostics. In addition to the short turnaround time, sequencing on Flongles makes it cost-effective, allows sequencing of single samples and simplifies the workflow by omitting washing steps. Taken together, our protocol has great potential for the diagnosis of severe clinical cases, such as encephalitis or meningitis, where diagnosis can be complex and fast results are crucial (Ersoy et al., 2012). Although the lower number of total reads generated by Flongles leads to a slightly reduced sensitivity compared to Illumina mNGS in our direct comparison (PPA = 91.67%), the overall sensitivity of the method is high with even low input virus being detected.

In the development of our protocol, we worked with plasma and CSF samples spiked with HAdV-7, HHV-5, IAV, and EV-C to cover a diverse selection of viruses (DNA and RNA, naked and enveloped) and to be able to monitor their viral loads over the course of sample preparation. During this initial assessment we noted that the lower read numbers of Flongles compared to Illumina mNGS requires adjustments to the amount of internal control spiked into the sample before nucleic acid extraction.

In most Nanopore mNGS runs of clinical samples, we found bacteriophages (Proteus phage VB_PmiS_Isfahan and Escherichia phage phiX174) and non-human viruses (granulo-, orthobunya-, and adenovirus). Except for the adenovirus, reads from all these viruses were also found with Illumina mNGS, indicating reagent contamination during sample preparation. The appearance of non-human adenoviruses across multiple Nanopore mNGS runs might be explained by Nanopore-specific reagent contamination. Although not experimentally added, TTV was detected in spiked plasma samples. Prevalence of TTV has been reported to approach 100% among blood donors in some studies and thus was likely introduced in the healthy plasma diluent (Hsu et al., 2003).

Short Illumina reads (≤ 151 bases) sometimes lead to misclassification due to wrongly annotated databases. Such false positives must be eliminated by confirming uniform coverage of the reference genome. Owing to the longer read lengths in Nanopore mNGS, we never observed such misclassification. The longer read lengths also compensate for lower read quality. Although only around 92% of the bases are called correctly with Flongle using *guppy*, through the long reads confident mapping to viral reference sequences is still possible.

One downside we occasionally observed with Flongles was a sudden loss of available pores. Although the flow cell check passed, immediately upon library loading and sequencing start, no pores were available for sequencing. This extreme drop of pores was never observed with SFCs, making them more reliable than Flongles in our experience.

A challenge of using single-sample Flongles are run controls. We run positive controls as internal controls with every sample. Negative controls would ideally be included on each single flow cell as well. However, for Flongles, this would cut the sequencing output for a sample to about 75%, which directly impacts sensitivity. Additionally, by multiplexing a sample and a negative control on the same Flongle, reads can become unassignable due to barcode misclassification. Such misclassification can be disregarded in single-sample Flongle sequencing. On the other hand, as only a single sample is sequenced per flow cell, sample carry-over or index-hopping can be excluded as source of contamination. Reagent contamination or sample cross-contamination might still occur during sample preparation. For diagnostic use, we therefore sequence one negative control on a separate flow cell for each preparation of samples.

While establishing our method, two other groups also reported Nanopore-based viral mNGS protocols (Jia et al., 2021; Fomsgaard et al., 2022). While both their protocols allow sensitive DNA and RNA virus detection within eight hours of total turnaround time, they have been solely validated on SFCs. To our knowledge, our study is the first report of viral mNGS using Flongles. For a clinical diagnostics approach, SFCs are not optimal as use for a single specimen is too expensive and multiplexing several clinical samples is not always an option. Moreover, as we show, SFC reusage is limited to six times before sequencing output is limited by available pores.

In conclusion, the novel mNGS approach based on Nanopore Flongle sequencing is a more rapid, cost-effective, and simple alternative to current mNGS methods for sensitive detection of DNA and RNA viruses in severe clinical cases where a fast diagnosis is crucial.

Note added in proof

Due to the discontinuation of the PCR Barcoding Kit SQK-PBK004 during revision of the manuscript, we switched to the PCR-cDNA Barcoding Kit SQK-PCB111.24. In direct comparison, we observe equal performance which was expected due to only slight changes in the primer sequences. The new primer sequences are 8N-NP_{PCB111} 5'-TTGCCTGTCGCTCTATCTTCNNNNNNNN-3' and NP_{PCB111} 5'-TTGC CTGTCGCTCTATCTTC-3'.

Funding

Funding was provided by the Clinical Research Priority Program "Comprehensive Genomic Pathogen Detection" of the University of Zurich. The funding body did not have any role in the design of the study, in the collection, analysis, and interpretation of data, and in writing the manuscript.

CRediT authorship contribution statement

Ian Pichler: Conceptualization, Methodology, Investigation, Writing – original draft, Visualization. **Stefan Schmutz:** Methodology. **Gabriela Ziltener:** Investigation. **Maryam Zaheri:** Software. **Verena Kufner:** Writing – review & editing. **Alexandra Trkola:** Conceptualization, Writing – review & editing, Funding acquisition. **Michael Huber:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the IMV Diagnostics team and Silke Stertz for providing virus stocks.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2023.114784.

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