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Sequencing of mRNA from whole blood using nanopore sequencing

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Corresponding Author:	Thomas Hoenen Friedrich-Loeffler-Institut Greifswald - Insel Riems, GERMANY
Corresponding Author's Institution:	Friedrich-Loeffler-Institut
Corresponding Author E-Mail:	Thomas.Hoenen@fli.de
Order of Authors:	Andreas Mueller Kerstin Fischer Roland Suluku Thomas Hoenen
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TITLE:

Sequencing of mRNA from Whole Blood using Nanopore Sequencing

AUTHORS AND AFFILIATIONS:

Andreas Mueller¹, Kerstin Fischer², Roland Suluku³, Thomas Hoenen¹

¹Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut, Greifswald - Insel Riems, Germany

²Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut, Greifswald - Insel Riems, Germany

³Department of Animal Science, Njala University, Sierra Leone

Corresponding Author:

Thomas Hoenen

thomas.hoenen@fli.de

Email addresses of coauthors:

Andreas Mueller (andreas.mueller@fli.de)

Kerstin Fischer (kerstin.fischer@fli.de)

Roland Suluku (nyasulukuroland2710@gmail.com)

KEYWORDS:

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SUMMARY:

Nanopore sequencing is a novel technology that allows cost-effective sequencing in remote locations and resource-poor settings. Here, we present a protocol for sequencing of mRNAs from whole blood that is compatible with such conditions.

ABSTRACT:

Sequencing in remote locations and resource-poor settings presents unique challenges. Nanopore sequencing can be successfully used under such conditions, and was deployed to West Africa during the recent Ebola virus epidemic, highlighting this possibility. In addition to its practical advantages (low cost, ease of equipment transport and use), this technology also provides fundamental advantages over second-generation sequencing approaches, particularly the very long read length, ability to directly sequence RNA, and real-time availability of data. Raw read accuracy is lower than with other sequencing platforms, which represents the main limitation of this technology; however, this can be partially mitigated by the high read depth generated. Here, we present a field-compatible protocol for sequencing of the mRNAs encoding for Niemann-Pick C1, which is the cellular receptor for ebolaviruses. This protocol encompasses extraction of RNA from animal blood samples, followed by RT-PCR for target enrichment, barcoding, library preparation, and the sequencing run itself, and can be easily adapted for use with other DNA or RNA targets.

INTRODUCTION:

Sequencing is a powerful and important tool in biological and biomedical research. It allows analysis of genomes, genetic variations, and RNA expression profiles, and thus plays an important role in the investigation of human and animal diseases alike^{1,2}. Sanger sequencing, one of the oldest methods available for DNA sequencing, is still routinely used to this day and has been a corner-stone of molecular biology. Over the past 50 years, this technology has been improved to achieve read-lengths of more than 1,000 nt and an accuracy as high as 99.999%¹. However, Sanger sequencing also has limitations. Sequencing a larger set of samples or the analysis of whole genomes with this method is time consuming and expensive^{1,3}. Second-generation (next-generation) DNA sequencing methods such as 454 pyrosequencing and Illumina technology have allowed us to significantly reduce the cost and workload required for sequencing in the last decade, and have led to a tremendous increase in the amount of biological sequence information available⁴. Nevertheless, individual sequencing runs using these second-generation technologies are expensive, and sequencing under field conditions is challenging, as the necessary equipment is bulky and fragile (similar to Sanger sequencing devices), and often has to be calibrated and serviced by specially trained personnel. Also, for many of the second-generation technologies read-lengths are rather limited, which often makes downstream bioinformatics analysis of these data challenging.

Third-generation sequencing using pocket-sized nanopore sequencing devices (see **Table of Materials**) can serve as an alternative to these established sequencing platforms. In these devices a single-stranded DNA or RNA molecule passes through a nanopore simultaneously with an ionic current that is then measured by a sensor (**Figure 1**). As the strand traverses the nanopore, the modulation of the current by the nucleotides present in the pore at any given time is detected, and computationally back-translated into the nucleotide sequence⁵. Because of this operational principle, nanopore sequencing allows both the generation of very long reads (close to 1×10^6 nucleotides⁶) and the analysis of sequencing data in real time. Barcoding is possible by attaching defined nucleotide sequences to the nucleic acids in a sample, which allows analysis of multiple samples in a single sequencing run, thus increasing sample throughput and lowering per sample costs. Due to their high portability and ease of use, nanopore sequencing devices have been used successfully in the field during the recent Ebola virus disease epidemic in West Africa, highlighting their suitability for rapid deployment into remote regions^{7,8}.

Here, we describe a detailed field-compatible protocol for the sequencing of mRNA encoding for the Niemann-Pick C1 (NPC1) protein, which is the obligate entry receptor for filoviruses such as ebolaviruses, and has been shown to limit species susceptibility to these viruses^{9,10}. The protocol encompasses extraction of whole RNA from blood samples, specific amplification of NPC1 mRNA by RT-PCR, barcoding of samples, library preparation and sequencing with a nanopore sequencing device. Data analysis cannot be discussed due to space limitations, although some basic directions are provided in the representative results; however, the interested reader is referred to a previous publication¹¹ for a more detailed description of the workflow we used, as well as to publications by others¹²⁻¹⁴ for detailed information regarding the analysis tools used in this workflow.

PROTOCOL:

Samples were collected following the Njala University Institutional Review Board (NUIRB) protocol no. IRB00008861/FWA00018924.

1. RNA extraction from blood samples

1.1. Collect 3 mL of whole blood from the species to be analyzed into a blood collection tube prefilled with 6 mL of DNA/RNA stabilizing reagent (see **Table of Materials**) and mix by inverting 5 times. Store the blood sample for up to one month at 4 °C.

1.2. Transfer the contents of the blood collection tube into a 50 mL collection tube, add 120 µL of proteinase K, and mix by vortexing for 5 s. Incubate the sample for 30 min at room temperature.

1.3. Add 9 mL of isopropanol to the mixture and vortex for 5 s.

1.4. Place a reservoir onto an RNA purification spin column (see **Table of Materials**) and place the assembly onto a vacuum manifold (see **Table of Materials**). Add the sample mixture into the reservoir. Apply a vacuum until all liquid has passed through the column.

1.5. Alternatively, if no vacuum manifold is available, the blood can be passed through the column in 700 µL portions by repeated centrifugation for 30 s at 12,000 x *g* with the flow-through discarded between centrifugation steps. However, this will require approximately 26 centrifugation steps.

1.6. Place the RNA purification spin column into a collection tube and add 400 µL DNA/RNA prep buffer (see **Table of Materials**). Centrifuge at 12,000 x *g* for 30 s and discard the flow-through.

1.7. Add 400 µL of DNA/RNA wash buffer (see **Table of Materials**) to the column, centrifuge at 12,000 x *g* for 30 s and discard the flow-through.

1.8. Mix 5 µL of DNase I (1 U/µL) (see **Table of Materials**) with 75 µL of DNA digestion buffer (see **Table of Materials**) and add the mixture onto the column. Incubate for 15 min at room temperature.

1.9. Add 400 µL of DNA/RNA prep buffer to the column and centrifuge at 12,000 x *g* for 30 s. Discard the flow-through.

1.10. Wash the column with 700 µL of DNA/RNA wash buffer and centrifuge at 12,000 x *g* for 30 s. Discard the flow-through.

1.11. Repeat step 1.9 with 400 µL of DNA/RNA wash buffer and centrifuge at 12,000 x *g* for 2 min to remove all residual wash buffer and to dry the column. When removing the column from the collection tube, make sure not to wet the underside of the column with the buffer in the

collection tube.

1.12. Place the column into a new 1.5 mL microcentrifuge tube and add 70 μ L of nuclease-free water. Incubate for 1 min at room temperature and centrifuge for 30 s at 12,000 $\times g$. Store the RNA at -80 °C until further use (or use immediately).

1.12.1. Optional: To quantify the RNA, take an aliquot and determine the concentration using a UV spectrophotometer (see **Table of Materials**).

2. Reverse transcription of NPC1 mRNA into cDNA

2.1. In a 0.2 mL reaction tube, add 8 μ L of template RNA (1 pg to 2.5 μ g of RNA) and 1 μ L each of 10x DNase buffer and DNase enzyme (see **Table of Materials**). Incubate at 37 °C for 2 min. Subsequently, centrifuge the reaction briefly and place it on ice.

2.2. Add 4 μ L of reverse transcriptase master mix (see **Table of Materials**) and 6 μ L of nuclease-free water to the reaction tube and mix gently. Incubate the reaction in a thermocycler for 10 min at 25 °C (for primer annealing), followed by 10 min at 50 °C (for reverse transcription of RNA). To inactivate the enzyme, incubate for 5 min at 85 °C.

2.3. Transfer cDNA to a new 1.5 mL microcentrifuge tube and store at -80 °C until further use (or use immediately).

3. Amplification of the NPC1 open reading frame

3.1 Initial amplification step

3.1.1. Set up a touchdown PCR^{15,16} to amplify the NPC1 cDNA with Primer Set 1 (see **Table 1**), using a hot start high fidelity DNA polymerase (see **Table of Materials**) with the appropriate reaction buffer in a 50 μ L reaction volume with 1 μ L template. If possible, set up the reaction on ice or in a 4 °C cool block.

3.1.2. Incubate the reaction in a thermocycler with an initial denaturation step of 30 s at 98 °C, followed by 10 cycles with denaturation at 98 °C for 10 s, primer annealing for 20 s at 65 °C, lowering the temperature by 0.5 °C per cycle, and elongation for 1 min at 72 °C. Subsequently, run an additional 20 cycles with 10 s at 98 °C, 20 s at 60 °C, and 1 min at 72 °C, followed by a final elongation step of 5 min at 72 °C.

3.2. PCR purification using magnetic beads

3.2.1. Transfer 50 μ L of PCR product into a 1.5 mL DNA-low binding reaction tube (see **Table of Materials**). Resuspend magnetic beads (see **Table of Materials**) thoroughly by vortexing and add 50 μ L beads to the PCR reaction. Mix well. Incubate the sample on a rotating mixer (see **Table of Materials**) for 5 min at room temperature (at 15 rpm).

3.2.2. Briefly spin down the sample and place the 1.5 mL microcentrifuge tube on a magnetic rack (see **Table of Materials**) to pellet the magnetic beads. Wait until the supernatant has been completely clarified before continuing with the next step.

3.2.3. Aspirate the supernatant without disturbing the bead pellet and discard.

3.2.4. Pipette 200 μ L of 70% ethanol into the reaction tube and incubate for 30 s. Aspirate the ethanol without disturbing the pellet and discard. Repeat for a total of two washes. Make sure that no ethanol is left. It may be necessary to first aspirate with a larger pipette (e.g., 1000 μ L), and then to remove any remaining ethanol droplets with a smaller pipette (e.g., 10 μ L).

3.2.5. Air-dry the pellet for 1 min at room temperature.

3.2.6. Remove the reaction tube from the magnetic rack, resuspend the pellet in 30 μ L of nuclease-free water and incubate for 2 min at room temperature.

3.2.7. Place the reaction tube back on the magnetic rack and wait until the beads are completely pelleted.

3.2.8. Remove the supernatant without disturbing the pellet and transfer it to a new 1.5 mL reaction tube.

3.3. Addition of barcode adapters by nested PCR

3.3.1. Set up a 50 μ L touchdown PCR with hot start High-Fidelity DNA polymerase with 5x reaction buffer and Primer Set 2 (see **Table 1**). Primers in this set consist of a target sequence-specific region to allow binding of PCR products generated in step 3.2 (inside of the primer set 1 sequences), as well as an adapter sequence that is used as target in the subsequent barcoding PCR reaction (cf. section 4). If possible, set up the reaction on ice or in a 4 °C cool block. Use 1 μ L of the purified PCR product prepared in section 3.2 as template.

3.3.2. Incubate the reaction mix in a thermocycler using an initial denaturation step of 30 s at 98 °C, followed by 10 cycles with denaturation at 98 °C for 10 s, primer annealing for 20 s at 65 °C, lowering the temperature by 0.5 °C per cycle, and elongation for 1 min at 72 °C. Subsequently, incubate the reaction for a further 30 cycles for 10 s at 98 °C, 20 s at 71 °C, and 1 min at 72 °C, followed by a final elongation step of 5 min at 72 °C.

3.3.3. Clean up the PCR product using magnetic beads as described in section 3.2.

4. Barcoding of NPC1 amplicons

4.1. For each PCR product generated in section 3, set up a barcoding PCR reaction in a 0.2 mL reaction tube using 50 μ L of Taq DNA polymerase 2x master mix (see **Table of Materials**), 2 μ L of one of the barcode primers from a PCR barcoding kit (see **Table of Materials** and **Table 2**), and 1 μ L of purified PCR product from step 3.3.2 as template. Add 47 μ L of nuclease free water to obtain

a final volume of 100 μ L.

4.2. Incubate the reaction in a thermocycler at 95 °C for 3 min as an initial denaturation. Subsequently, run 15 cycles for 15 s at 95 °C, 15 s at 62 °C, and 1.5 min at 65 °C. As a final elongation, incubate the reaction at 65 °C for 5 min.

4.2 Purify the PCR product as described under 3.2, but use 100 μ L of magnetic beads and elute in 30 μ L of nuclease-free water.

4.3 If possible, quantify the sample using a UV spectrophotometer.

5. Library preparation

5.1. Combine an equal amount of barcoded DNA from each sample for a total of 1 μ g of DNA in a volume of 45 μ L (if necessary, add nuclease-free water) in a 0.2 mL reaction tube. If no UV spectrophotometer is available, use equal amounts of each sample. For dA-tailing, add 7 μ L of End-prep reaction buffer (see **Table of Materials**), 3 μ L of End-prep enzyme mix (see **Table of Materials**), and 5 μ L of nuclease-free water. Mix gently by flicking the tube.

5.2. Incubate the reaction for 5 min at 20 °C, followed by 5 min at 65 °C in a thermocycler.

5.3. Purify the reaction product as described in section 3.2, but use 60 μ L of magnetic beads and elute in 25 μ L of nuclease-free water.

5.4. Optional: Take 1 μ L to quantify the concentration of the sample using a UV spectrophotometer. The total amount should be more than 700 ng.

5.5. Combine 22.5 μ L of purified DNA from step 5.3 with 2.5 μ L of 1D2 adapter (see **Table of Materials**) and 25 μ L of blunt/TA ligase master mix (see **Table of Materials**) in a new 1.5 mL DNA-low binding reaction tube, mix gently by flicking, and briefly spin down. Incubate for 10 min at room temperature.

5.6. Purify the reaction product as described in section 3.2, but use 20 μ L of magnetic beads, increase the incubation time for DNA binding to 10 min, perform two wash steps with 1 mL of ethanol each, and elute in 46 μ L of nuclease-free water.

5.7. Combine 45 μ L of the reaction product from step 5.6 with 5 μ L of barcode adapter mix (see **Table of Materials**) and 50 μ L of blunt/TA ligase master mix in a DNA-low binding reaction tube. Mix gently by flicking and incubate for 10 min at room temperature.

5.8. Purify the reaction product as described in section 3.2, but use 40 μ L of magnetic beads, do two wash steps with 140 μ L of ABB buffer (see **Table of Materials**) instead of ethanol, resuspend the beads by flicking and pellet on a magnetic rack. Elute in 15 μ L of elution buffer (see **Table of Materials**). Increase the incubation times for the initial binding of DNA to the beads as well as for the elution step to 10 min. Store the resulting product on ice or at 4 °C until use.

6. Quality check of flow cell

6.1. Perform a quality check on the flow cell before use. To this end, connect the sequencing device to the host computer and open the software.

6.2. Insert a flow cell (see **Table of Materials**) into the sequencing device and choose the flow cell type from the selector box and confirm by clicking **Available**.

6.3. Click **Check flow cell** in the bottom of the screen and choose the correct flow cell type.

6.4. Click **Start test** to start the quality check. A minimum of 800 active nanopores in total is required for the flow cell to be usable.

7. Loading the flow cell and starting the sequencing run

7.1. Open the priming port cover by sliding it in a clockwise direction. Set a P1000 pipette to 200 μL and insert the tip into the priming port. Adjust the pipette to 230 μL while keeping the tip in the priming port, to draw up 20-30 μL buffer and remove any air bubbles.

7.2. In a new 1.5 mL DNA-low binding reaction tube prepare the priming mix by combining 576 μL of RBF buffer (see **Table of Materials**) with 624 μL of nuclease-free water.

7.3. Carefully pipette 800 μL of the prepared priming mix into the priming port and wait 5 min. Lift the sample port cover, and pipette an additional 200 μL of the prepared priming mix into the priming port.

7.4. Pipette 35 μL of RBF buffer into a new, clean 1.5 mL DNA low-binding reaction tube. Thoroughly mix LLB beads (see **Table of Materials**) by pipetting and add 25.5 μL of the beads to the RBF buffer. Add 2.5 μL nuclease-free water and 12 μL of DNA library from step 5.8 and mix by pipetting.

7.5. Add 75 μL of the sample mixture in a slow dropwise fashion to the flow cell via the sample port.

7.6. Replace the sample port cover, close the priming port, and close the lid of the sequencing device.

7.7. Within the software, confirm that the flow cell is still available, open a new experiment, and set up the run parameters by selecting the kit used. Select live base-calling. Start the sequencing run by clicking **Begin Experiment**. Continue the sequencing run until sufficient experimental data is collected.

REPRESENTATIVE RESULTS:

In a representative experiment to test the presented protocol we extracted the RNA from 10

different blood samples of five animal species (i.e., 2 individuals per species (goat, sheep, swine, dog, cattle)) (**Table 3**). RNA yields and quality following extraction can vary widely, in particular due to differences in sample handling and storage. In our representative experiment, we observed RNA concentrations between 43 ng and 543 ng per μ L (**Table 3**). Also, after amplification by RT-PCR, gel analysis of the NPC-1 PCR-products showed various outcomes (**Figure 2**), with markedly weaker bands for samples BC01 and BC02 (both goat). These differences were most likely caused by differences in sample quality, although differences in PCR efficacy due to differences in primer binding to the NPC1 gene of different species cannot be excluded. However, these differences in yield and/or amplification efficiency did not markedly impact the overall sequencing outcome. Further, an additional non-specific PCR product occurred in sample BC10 (cattle). In contrast to Sanger sequencing, such non-specific products do not negatively influence the results of nanopore sequencing, as these reads are discarded during mapping of the obtained reads to a reference sequence as part of the data analysis.

Prior to each sequencing run, a quality check of the flow cell to be used is strongly recommended, with a minimum requirement of 800 total pores. In our representative experiment, this quality check returned 1,102 pores available for sequencing. Since the data are provided in real time and can be analyzed immediately, the length of a sequencing run can be adjusted for the individual application (i.e., until sufficient sequencing data is produced for the desired analysis). In our experiments, sequencing runs are typically performed overnight, and in the case of our representative experiment we obtained approximately 1.4 million reads during such a 14 h run.

Depending on the type of data analysis to be performed, it can be advisable to process only a subset of the obtained reads. In the case of our representative experiment, a subset of 10,000 reads was selected for further analysis. To this end, the fastq files generated during the sequencing run were further processed in an Ubuntu 18.04 LTS environment, and demultiplexed using flexbar v3.0.3 with parameters optimized for demultiplexing of nanopore sequencing data (barcode-tail-length 300, barcode-error-rate 0.2, barcode-gap-penalty -1)¹². After demultiplexing, read mapping and consensus generation can then be done using a number of different tools, but a detailed discussion of the bioinformatics aspect of nanopore sequencing goes beyond the scope of this manuscript. However, in the case of our representative results, read mapping to a reference sequence was performed using Geneious 10.2.3. Of the 10,000 reads analyzed, 5,457 showed a length between 1,750 and 2,000 nucleotides, which matches the expected sizes for the PCR fragments amplified as part of our workflow (1,769 nt, **Figure 3**). An additional peak in the length distribution of reads was observed between 250 to 500 nucleotides, which can be attributed to unspecific PCR products. Demultiplexing of reads allowed the assignment of 87.6% of the reads to one of the 10 barcodes/samples analyzed (**Figure 4**). The proportion of demultiplexed reads for each barcode ranged from 3.4% for barcode 1 to 16.9% for barcode 10; however, due to the overall large number of reads this still allowed meaningful consensus calling with a high read depth even for these lower abundance barcode datasets. Indeed, mapping of the sorted reads to a reference sequence of NPC1 resulted in between 31.7% (barcode 2) and 100% (barcode 7 and 8) of reads mapping to the reference, giving a read depth of more than 90 reads at any position for each sample. This is then more than adequate to allow confident consensus base-calling with a negligible error rate.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of DNA sequencing using nanopore technology. A single-stranded DNA molecule passes through a nanopore embedded in an electrically resistant membrane, with a helicase regulating the transition speed. An ionic current simultaneously passes through the pore and is continuously measured. Modulations of the current caused by the nucleotides present in the pore are detected and computationally back-translated into the nucleotide sequence of the DNA strand.

Figure 2: Amplification of PCR products of Niemann-Pick C1 from mRNA. mRNA was isolated from goat (BC01 and 02), sheep (BC03 and 04), swine (BC05 and 06), dog (BC07 and 08), and cattle (BC09 and 10). Nested PCR products were separated in a 0.8% agarose gel in 1x TAE buffer (prepared from 50x TAE buffer: 242.28 g of Tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 M EDTA, dH₂O to 1 L, pH adjusted to 8.0) for 45 min at 100 V and stained with Sybr Safe.

Figure 3: Read-length distribution of 10,000 reads from the representative experiment. The number of reads obtained having a given read length interval is indicated.

Figure 4: Distribution of reads after demultiplexing. The number and percentage of demultiplexed (grey) and mapped reads (black) for each barcode are shown.

Table 1: Overview of primer sets used. Initial amplification of target sequences was performed with Primer Set 1. Primer Set 2 was then used for nested amplification and adapter addition. Adapters are indicated in red.

Table 2: Overview of barcode sequences. Individual barcodes were used to identify each sequenced sample.

Table 3: RNA concentrations obtained following extraction from blood samples sequenced in the representative experiment. The RNA concentrations of two individuals from each of five species are shown, and the ratios of the optical densities at 260/280 nm and 260/230 nm are indicated.

DISCUSSION:

Over the last two decades, sequencing of biological samples has become an increasingly important aspect of studies in a wide range of subject areas. The development of second-generation sequencing systems based on the sequencing of a dense array of DNA features using iterative cycles of enzymatic manipulation and image-based data acquisition¹ has dramatically increased throughput compared to the traditional Sanger sequencing technique, and allows analysis of multiple samples as well as various nucleic acid species in a given sample in parallel⁴. However, for most of the commonly used second-generation systems, only short reads are produced, and all platforms rely on sensitive, bulky, and expensive equipment^{3,4}.

In contrast to second-generation sequencing platforms, the sequencing device used in this protocol is based on nanopore technology. Here a single-stranded nucleic acid molecule passes

through a nanopore, resulting in modulation of an ionic current that is also flowing through the same nanopore, and which can be measured and back-translated to infer the sequence of the nucleic acid molecule. This third-generation sequencing approach imparts a number of advantages over other approaches. The main advantages that are directly related to the unique working principle of this technology are the extremely long read length produced (read lengths of up to 8.8×10^5 nucleotides have been reported⁶), the ability to sequence not only DNA but also RNA directly, which was recently demonstrated for a complete influenza virus genome¹⁷, and the ability to analyze data in real-time as they are being generated, which allows rapid metagenomics detection of pathogens within minutes¹⁸. Additional practical advantages are the extremely small size of the nanopore sequencing device, allowing its use in any laboratory or on field missions to remote locations^{19,20}, and the low price in comparison to other sequencing platforms. In terms of running costs, currently a new flow cell is required for each sequencing run, which results in costs of about \$1,100 per run for the flow cell and library preparation reagents. These costs can be reduced in some cases by washing and reusing the flow cell, or by barcoding and sequencing multiple samples in a single run. Also, a novel type of flow cell is currently being beta-tested by a small number of laboratories, which will require the use of a flow cell adaptor (called a “flongle”), and should significantly reduce flow cell price and thus running costs.

The major shortcoming of nanopore sequencing remains its accuracy, with single read accuracies in the range of 83 to 86% being reported^{6,21,22}, and most of the inaccuracies being caused by insertion/deletions (indels)^{5,21}. However, high read depth can compensate for these inaccuracies, and a recent study suggested based on theoretical considerations that a read depth of >10 might increase overall accuracy to >99.8%²¹. Nevertheless, further improvements in accuracy will be needed, particularly if analysis is to be performed on a single molecule level rather than on a consensus sequence level. The use of 1D² technology as described in this protocol, which is based on the addition of the 1D² and barcode adapters (cf. section 5.5) that result in both strands of a single DNA molecule being sequenced by the same nanopore, increases read accuracy since information from both DNA strands can be used for sequence determination. Further, a workaround strategy that can be pursued in order to combine the advantages of nanopore sequencing (particularly long read length) with the higher accuracy of other sequencing technologies is to use nanopore sequencing information as a scaffold, which is then polished using sequencing data from other platforms⁶.

The most critical factor for the success of the protocol presented here is sample quality, and particularly the amount and quality of the extracted RNA. Proper storage and prompt extraction of the RNA help in achieving an adequate RNA yield. The use of appropriate blood collection tubes allows the storage of blood samples for up to one month, but blood clotting can be an issue, particularly when samples are being stored at elevated temperatures, which can be the case under field conditions. The second critical step is the amplification of target sequences, and particular under field conditions PCR reactions often perform less well than under standard laboratory conditions⁷. To this end, careful primer design and optimization is paramount to achieve robust amplification. Additionally, nested PCR approaches and touchdown PCR, as used in this protocol, can increase both specificity and sensitivity of target gene amplification^{4,7}. Indeed, in our experience in Liberia and Guinea with this technology nested protocols were required under field conditions with field samples even for primer sets which allowed

451 amplification of targets from laboratory samples and under laboratory conditions with a single
452 round of PCR (⁷ and unpublished results).

453
454 In contrast to these more critical steps, library preparation and the sequencing run itself are
455 rather robust procedures. However, under field conditions practical issues such as the availability
456 of certain pieces of equipment can be problematic. For example, a UV spectrophotometer is
457 needed to determine DNA concentrations prior to library preparation of barcoded samples.
458 However, should such a device not be available under field conditions, an equal volume of each
459 sample can simply be combined to make up the 45 µL required for library preparation, with
460 differences in sample input material then usually being mitigated by the large number of reads.
461 Similarly, the need for internet connectivity for the sequencing run can be an issue, even though
462 the base-calling no longer has to be performed online but can be done locally; however, this
463 necessity can be removed under certain circumstances by the manufacturer if required.

464
465 In summary, the presented protocol allows relatively low-cost sequencing in locations with no
466 access to traditional sequencing equipment, including in remote locations. It can easily be
467 adapted to any target RNA or DNA, thus allowing researchers to answer numerous biological
468 questions.

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475 **DISCLOSURES:**

476
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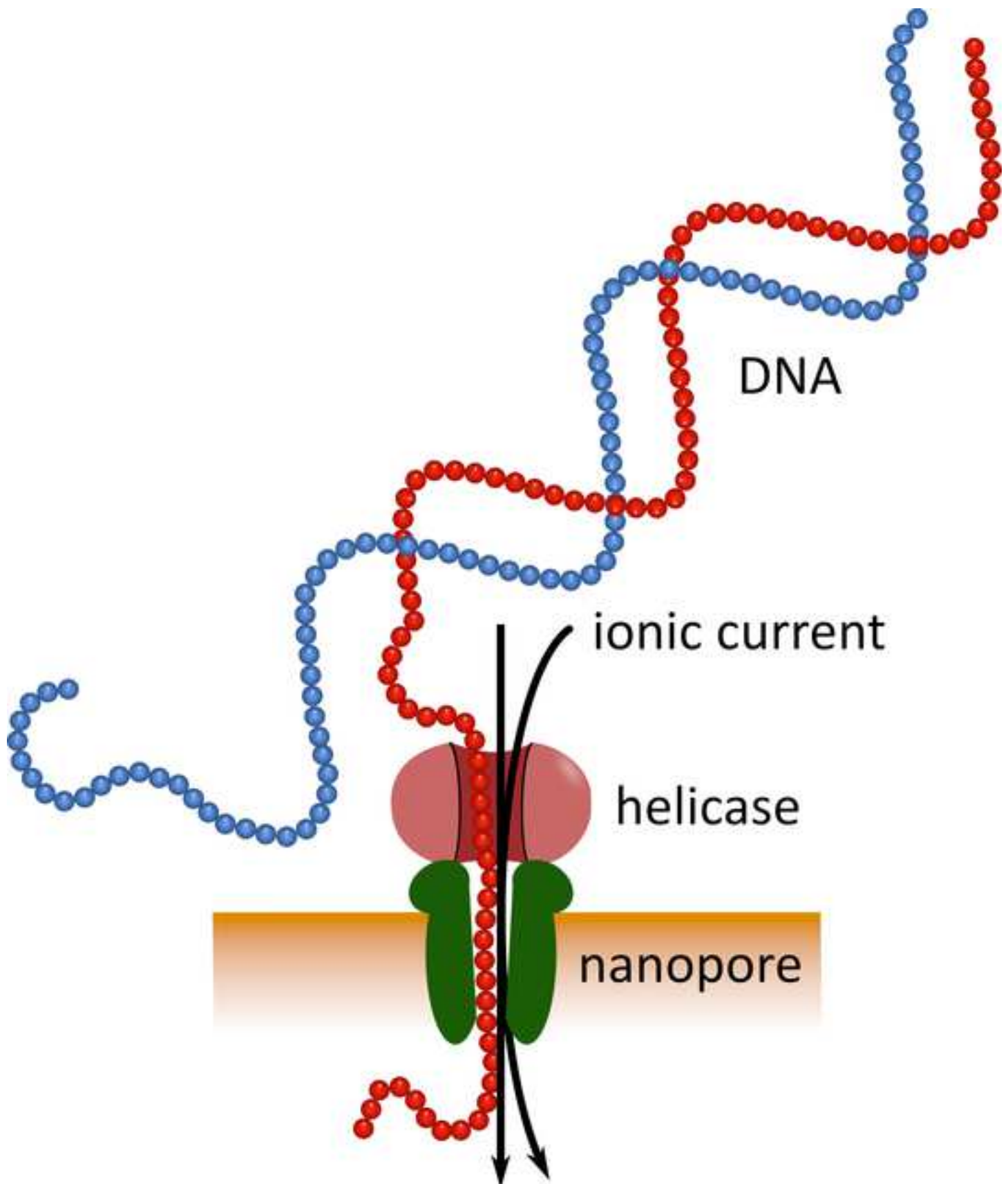


Figure 2

[Click here to access/download;Figure;figure_2.tif](#)

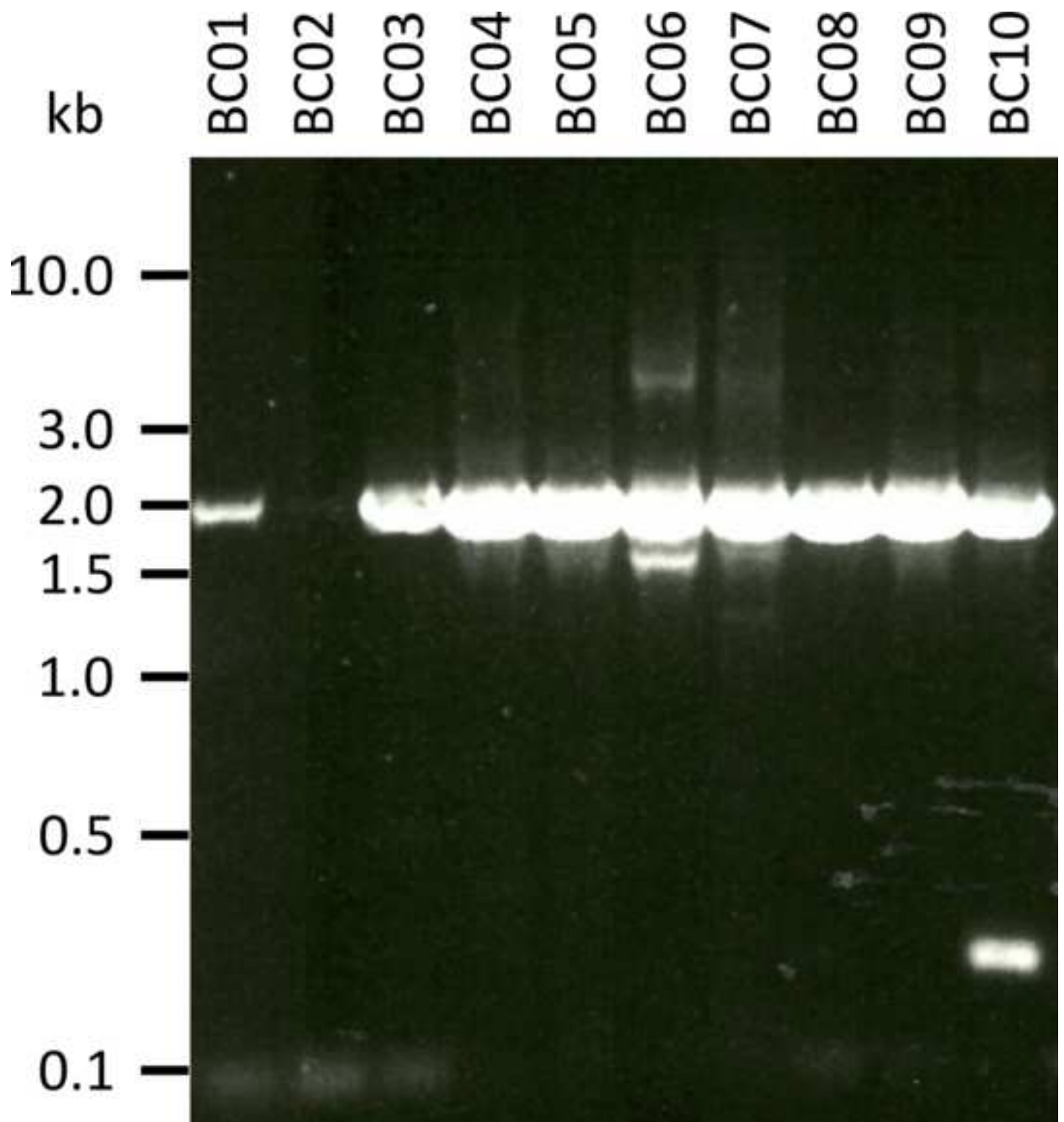


Figure 3

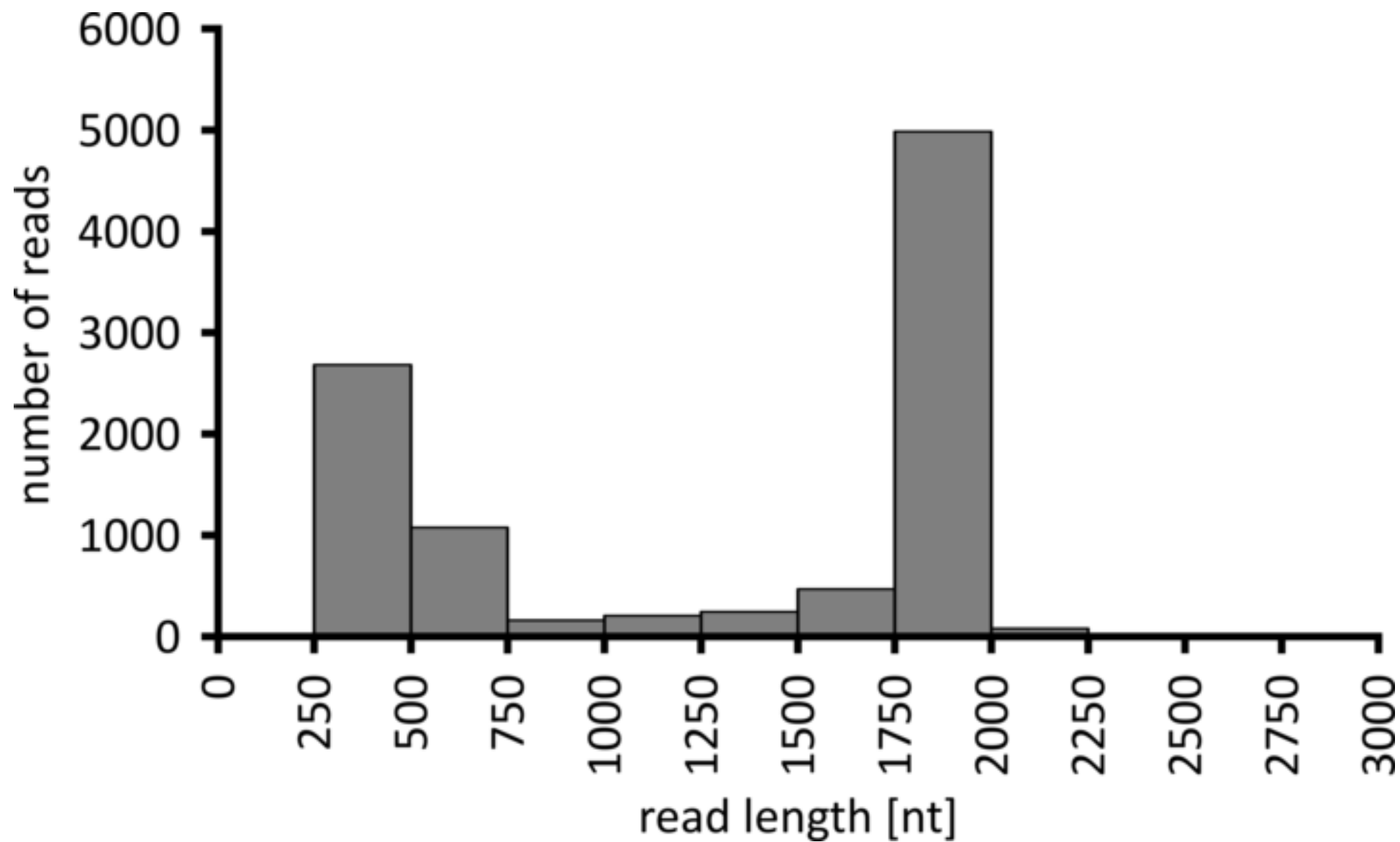
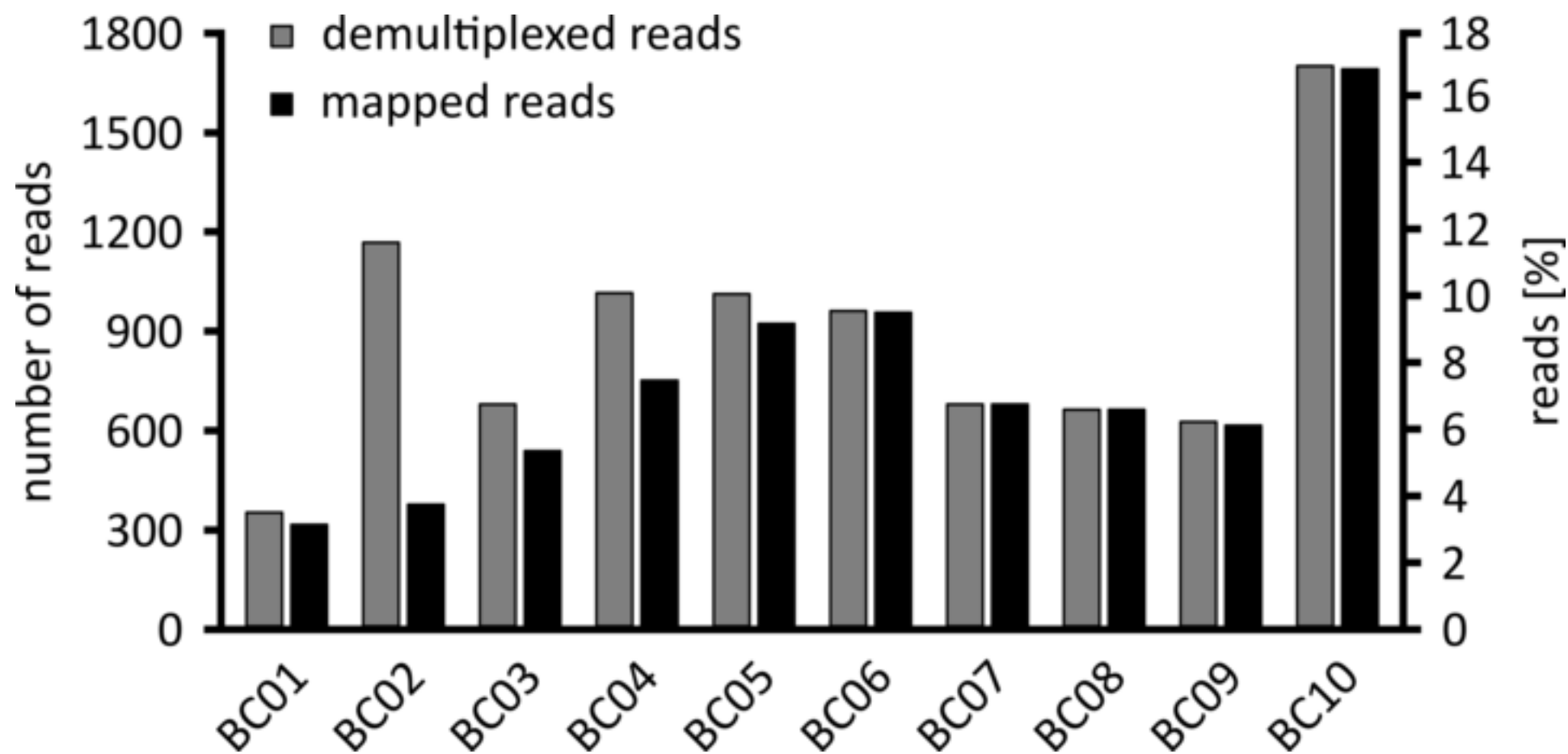


Figure 4



number	name
1	NPC1_seq_819_fwd
2	NPC1_seq_2712_rev
3	NPC1_870_adap_fwd
4	NPC1_2580_adap_rev

sequence
CCATGTATGTCATCATGTGG
GCACACCATGTTCTGC
TTTCTGTTGGTGCTGATATTGCGTGGTGCTACAGAAAACG
ACTTGCCTGTCGCTCTATCTTCGCATTGAAAGAGACTGATCC

barcode	sequence
BC01	AAGAAAGTTGTCGGTGTCTTTGTG
BC02	TCGATTCCGTTTGTAGTCGTCTGT
BC03	GAGTCTTGTGTCCCAGTTACCAGG
BC04	TTCGGATTCTATCGTGTTTCCCTA
BC05	CTTGTCCAGGGTTTGTGTAACCTT
BC06	TTCTCGCAAAGGCAGAAAGTAGTC
BC07	GTGTTACCGTGGGAATGAATCCTT
BC08	TTCAGGGAACAAACCAAGTTACGT
BC09	AACTAGGCACAGCGAGTCTTGGTT
BC10	AAGCGTTGAAACCTTTGTCCTCTC

sample	species	ng/ μ L
BC01	goat	366
BC02	goat	127
BC03	sheep	324
BC04	sheep	175
BC05	swine	543
BC06	swine	242
BC07	dog	200
BC08	dog	43
BC09	cattle	311
BC10	cattle	342

260/280	260/230
1,846	1,943
1,843	1,642
1,895	2,112
1,896	2,066
1,848	2,201
1,912	1,980
1,873	2,212
1,845	2,098
1,839	1,884
1,865	1,780

Name of Material/ Equipment	Company	Catalog Number
1D2 adapter, barcode adapter mix, ABB buffer, elution buffer, RBF buffer, LBB beads	Oxford Nanopore Technologies	SQK-LSK308
blood collection tube with DNA/RNA stabilizing reagent	Zymo Research	R1150
blunt/TA ligase master mix	New England Biolabs	M0367S
DNA-low binding reaction tube	Eppendorf	30108051
DNase buffer and DNase	ThermoFisher Scientific	11766050
flow cell	Oxford Nanopore Technologies	FLO-MIN105.24
hot start high fidelity DNA polymerase	New England Biolabs	M0493L
magnetic beads	Beckman Coulter	A63881
magnetic rack	ThermoFisher Scientific	12321D
nanopore sequencing device	Oxford Nanopore Technologies	-
PCR barcoding kit	Oxford Nanopore Technologies	EXP-PBC001
reverse transcriptase master mix	ThermoFisher Scientific	11766050
RNA purification spin column, DNA/RNA prep buffer, DNA/RNA wash buffer, DNase I, DNA digestion buffer	Zymo Research	R1151
rotating mixer	ThermoFisher Scientific	15920D
Taq DNA polymerase	New England Biolabs	M0287S

Ultra II End-prep kit	New England Biolabs	E7546S
UV spectrophotometer	Implen	-
vacuum manifold	Zymo Research	S7000

Comments/Description

1D² Sequencing Kit

DNA/RNA Shield - Blood
Collection Tube
Blunt/TA Ligase Master
Mix

DNA LoBind Tube

SuperScript™ IV VILO™
Master Mix with
ezDNase™ Enzyme

flow cell R9.4

Q5 Hot Start High-Fidelity
DNA Polymerase (500 U)
Agencourt AMPure XP
beads

DynaMag-2 Magnet

MinION Mk 1B

PCR Barcoding Kit I (R9)

SuperScript™ IV VILO™
Master Mix with
ezDNase™ Enzyme
Quick-DNA/RNA Blood
Tube Kit

HulaMixer Sample Mixer

LongAmp Taq 2X Master
Mix

NEBNext Ultra II End-
Repair/dA-tailing Modul

NanoPhotometer

EZ-Vac Vacuum Manifold



1 Aowife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

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Author(s): Andreas Mueller, Kerstin Fischer, Thomas Hoenen

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Response to Editor

> Own responses are highlighted in blue. All line number refer to the document in track-change mode.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

> We have proofread the manuscript as suggested by the editor.

2. Keywords: Please remove commercial language (MinION) and provide another keyword so that there are at least 6 keywords or phrases.

> We very much appreciate the efforts by JoVE to avoid using commercial language wherever possible. However, in this specific instance we believe that removing the keyword “MinION” would not really serve this purpose, since the name for this device is inseparable from the technology. There are no competing devices from other companies available or announced, and MinION is used both for the technology as well as for the actual device. This is a phenomenon that is quite often found in the next-generation sequencing field, where technologies are so intimately entwined with certain devices that the device name also becomes the name for the technology behind the device. Examples for this are “454 pyrosequencing” or “Illumina technology”.

> On the other hand, removing this keyword would significantly impair the visibility and impact of this protocol, which is neither in the journal’s nor our own interest, and would make it harder for interested researchers to find the protocol, since they will specifically look for the keyword “MinION” when researching this technology.

> Therefore, in an attempt to find an acceptable compromise between avoiding commercial language but also avoiding obscurity of the protocol, and to make clear that the technology platform rather than the device name is meant, we have changed the keyword to “MinION technology”.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: MinION, Oxford Nanopore Technologies, DNA/RNA Shield, Zymo-Spin IIICG, ezDNase, SuperScript, Q5, AMPure, Eppendorf, LoBind, Hula 2 mixer, LongAmp, etc.

> We have removed commercial language throughout the article wherever possible.

4. As animal blood samples are used in the protocol, please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

> As required from the editor we provide an ethical statement in our manuscript.

> line 102: "Samples were collected following the Njala University Institutional Review Board (NUIRB) protocol no. IRB00008861/FWA00018924."

5. Please provide the composition of different buffers used. If they are purchased, please cite the Table of Materials.

> In most cases buffers are commercially obtained, and we have ensured that the Table of Materials is cited. In case of TAE-buffer we now provide the composition.

> line 395: "in 1 x TAE buffer (prepared from 50x TAE buffer: 242.28 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5M EDTA, dH₂O ad 1 L, pH adjusted to 8.0)"

6. 1.1: Please specify the source of blood.

> Blood was obtained from goat, sheep, swine, dog, and cattle for our representative results, which is specified in that section; however, the protocol allows for blood from any animal or human source to be analysed. We now indicate this in the protocol.

> line 110: "Collect 3 mL of whole blood from the species to be analyzed"

7. Please revise the Protocol steps (1.4, 3.1.1, 3.3.1, etc.) so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

> We have revised our protocol and shortened individual steps where necessary.

8. Table 2: Please abbreviate liters to L (not l) to avoid confusion.

> We have changed the abbreviation of litre in table 2 to "L". Additional, we have also changed "µl" to "µL" and "ml" to "mL" throughout the text.

9. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

> As requested, we have sorted the Table of Materials in alphabetical order.

10. References: Please do not abbreviate journal titles.

> We have replaced the journal abbreviations with the full journal titles.

Response to Reviewers

> Own responses are highlighted in blue. All line number refer to the document in track-change mode.

This is a description of a protocol for amplifying a gene from RNA and performing multiplex sequencing on MinION. The motivation for the protocol is quite obvious and the method appears to be robust will care taken to optimise the amplification including the application of touch-down thermocycling conditions. The protocol focuses on the laboratory methods yet some analysis results are presented in the representative results section.

> We thank the reviewer for their positive feedback to our work.

Major comments:

There is significant detail lacking on the bioinformatics processing and results including demultiplexing results are presenting without methods. This protocol would be significantly strengthened by adding a section on analysis of amplicon sequencing using MinION as this will likely be a very popular method in future especially with the imminent introduction of the Flongle and potential to displace Sanger sequencing for this kind of work. In the current state usable results could not be reproduced by anyone not familiar with analysis of nanopore data. For example, calling the dominant base from a pileup will not be sufficient with nanopore data for best SNP calling accuracy. Citing a previous paper is not sufficient due to the fast pace of change in the analysis of nanopore sequencing, e.g. Poretools is cited but is not usable on current basecalls. There are other considerations such as adapter trimming, demultiplexing settings and primer binding site masking that must be included.

> We absolutely agree with the reviewer that the bioinformatics processing of MinION data is an important part of the workflow. At the same time, this aspect of MinION sequencing is moving at an extremely fast pace, and thus any detailed protocol of this aspect of the work will most likely be outdated by the time the paper is published. Also, for us this is not the focus of this manuscript, which rather serves to show the practical use of the MinION sequencing device, and not the downstream analysis, which certainly would warrant a detailed publication on its own. Nevertheless, we have tried to add some general comments in the representative results section towards the downstream bioinformatics analysis. Further, we have removed the citation of Poretools as suggested by the reviewer.

> line 93: "Data analysis cannot be discussed due to space limitations, although some basic directions are provided in the representative results section; however, the interested reader is referred to a previous publication ¹¹ for a more detailed description of the workflow we used, as well as to publications by others ¹²⁻¹⁴ for detailed information regarding the analysis tools used in this workflow."

> line 361: "In the case of our representative experiment, a subset of 10,000 reads was selected for further analysis. To this end, the fastq files generated during the sequencing run were further processed in an Ubuntu 18.04 LTS environment, and demultiplexed using flexbar v3.0.3 with parameters optimized for demultiplexing of nanopore sequencing data (barcode-tail-length 300, barcode-error-rate 0.2, barcode-gap-penalty -1) ¹². After demultiplexing, read mapping and consensus generation can then be done using a number of different tools, but a detailed discussion of the bioinformatics aspect of nanopore sequencing goes beyond the scope of this manuscript. However, in the case of our representative results, read mapping to a reference sequence was performed using Geneious 10.2.3. Of the 10,000 reads analyzed, [...]"

I would like to see some discussion about attempts to streamline the protocol as three-rounds of PCR is quite significant for a field method. Was a single PCR attempted using barcoded primers and if so why was this not the favourable method?

> The reviewer is correct in that three rounds of PCR represent a quite significant work load for a field method. However, in our experience it was necessary to run nested PCRs in the field, even for primer sets that worked well in a single PCR round under laboratory conditions. Similarly, we have made the observation that adding the barcode adapter to the first primer set leads to amplification problems under field conditions, at least in our hands. We now comment on this in the manuscript:

> line 475: “Indeed, in our experience in Liberia and Guinea with this technology nested protocols were required under field conditions with field samples even for primer sets which allowed amplification of targets from laboratory samples and under laboratory conditions with a single round of PCR (⁷ and unpublished results).”

Minor comments:

41 Raw read accuracy or maybe accuracy is lower than short-read sequencing platforms

> We have rephrased the sentence as suggested by the reviewer.

> line 44: “Raw read accuracy is lower than with other sequencing platforms”

78 increasing sample throughput

> “sample” has been added as requested.

> line 83: “thus increasing sample throughput”

143 Why is there two DNase treatment steps are they both required?

> The DNase treatments are part of the respective kits for RNA extraction and reverse transcription. Whether or not both steps are absolutely required for the protocol to work is something that we have not specifically investigated, but since these steps add a very limited amount of work, we believe that this is of little consequence.

180 Pipette or pipet?

> We have made the spelling of “pipette” consistent throughout the manuscript.

180 Consistency between units s and min in line 185

> We have made sure that we follow the JoVE style guide, which requires the use of “h”, “min”, and “s” as abbreviations for hours, minutes, and seconds, respectively, throughout the manuscript.

204 Annealing temperate of 71°C?

> This is correct.

324 Assume you mean 1.4 M reads?

> The reviewer is right, this was a typing error and has been changed accordingly.

> line 357: “we obtained approximately 1.4 million reads during such a 14 h run.”

332 Include the demultiplexing stringency here is that 87% requiring a single barcode to be detected?

> Unfortunately, we are not entirely sure we understand the reviewer’s comment in this instance. Demultiplexing was done using Flexbar with a barcode-error-rate of 0.2 and a barcode-gap-penalty of -1. This allowed us to assign 87.6% of the reads to a sample. We have made sure that this is specified in the manuscript.

> line 363: “and demultiplexed using flexbar v3.0.3 with parameters optimized for demultiplexing of nanopore sequencing data (barcode-tail-length 300, barcode-error-rate 0.2, barcode-gap-penalty -1)”

> line 373: “Demultiplexing of reads allowed the assignment of 87.6% of the reads to one of the 10 barcodes/samples analysed.”

347,390 ionic

> This has been changed as requested.

> line 388: “An ionic current simultaneously passes through the pore [...]”

> line 431: “[...] resulting in modulation of an ionic current [...]”

409 Cite Rang 2018

> We have added a citation to Rang 2018 as suggested by the reviewer.

411 Cite

> We have added a citation to the text at this point as requested. However, since the paper cited substantiates the statement made only with theoretical considerations, we have at the same time weakened it somewhat.

line 452: “However, high read depth can compensate for these inaccuracies, and a recent study suggested based on theoretical considerations that a read depth of >10 might increase overall accuracy to >99.8%²¹.”

Figure 1 is missing the helicase which is an essential element of the sequencing system

> We agree with the reviewer that the helicase is an essential element for the nanopore sequencing system, and have, therefore, we adjusted figure 1 accordingly.

Reviewer #2:

In this manuscript entitled "Sequencing of mRNA from whole blood using nanopore sequencing", the authors described detailed protocols of the entire procedures ranging from sample preparation to sequencing run. This is an interesting and well written protocol with some supporting data for sequencing quality. However, to increase the clarity of the protocol, the author need to explain the strategies for designing of primer set 2 used in section 3.3 and why to add 1D2 adapter and barcode adapter in section 5, or include these in the discussion section.

> We appreciate the positive assessment of our manuscript, and have addressed the constructive criticisms of the reviewer as detailed below.

> The design strategy for primer set 2 and the barcode adapter is explained in section 3.3.

> line 221: “Primers in this set consist of a target sequence-specific region to allow binding of PCR products generated in step 3.2 (inside of the primer set 1 sequences), as well as an adapter sequence that is used as target in the subsequent barcoding PCR reaction (cf. section 4).”

> With respect to the adapters, we now explain their purpose in the discussion (despite its name, the barcode adapter is not used for barcoding of different samples with different barcodes, which happens before the library preparation, but acts at a much later step of the 1D² library preparation):

> line 456: “The use of 1D² technology as described in this protocol, which is based on the addition of the 1D² and barcode adapters (cf. section 5.5) that result in both strands of a single DNA molecule being sequenced by the same nanopore, increases read accuracy since information from both DNA strands can be used for sequence determination.”