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High molecular weight bacterial DNA extraction from field-collected fecal samples preserved in ethanol for long-read sequencing

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High molecular weight DNA extraction from all kingdoms
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ABSTRACT

Long-read metagenomic sequencing enables the reconstruction of better-quality assemblies compared to short-read assemblies. Long-read assemblies are typically more contiguous and this is of interest to recover full bacterial genomes. Here, we propose a protocol to extract high molecular weight bacterial DNA from mammalian fecal samples for long-read sequencing on the portable MinION instrument. DNA was extracted from feces collected in the field or from roadkill specimens and preserved in ethanol (96%) and stored at -20°C. The DNA extraction was done according to the "Genomic DNA from soil" kit (NucleoSpin Soil, Macherey-Nagel) which was optimized in three ways: i) SL1 + SX lysis buffers were used because this combination gave a high yield and degraded DNA the less, ii) two successive extractions were performed to get long fragments of DNA and iii) an additional DNA sizing step was added after the extraction to remove the remaining short DNA fragments and ensure DNA purity. The comparison of the two successive extractions showed that the obtained reads did not differ in terms of taxonomy but only the second yielded high molecular weight DNA suitable for long-read sequencing. Resulting long-read assemblies were more contiguous than Illumina short-read ones with fewer contigs (~4 000 and ~50 000 respectively) and a higher N50 (~90 000 kb and ~10 000 kb respectively) illustrating the advantage of using long reads to reconstruct bacterial genomes from ethanol-preserved fecal samples.

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KEYWORDS

high molecular weight DNA extraction, long-read sequencing, metagenomics, field-collected fecal samples

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MATERIALS TEXT

Reagents

- DNA extraction kit: Genomic DNA from soil, NucleoSpin Soil, Macherey-Nagel; reference: 740780.50
- Library preparation kit: 1D Genomic DNA by Ligation (SQK-LSK109), Oxford Nanopore Technologies
- Ethanol 96-100%
- Agencourt AMPure XP beads

Consumables

- DNA LoBind tubes 1.5 and 2 mL
- Tips P10, P20, P200 and P1000

Equipment

- Precision scale
- Vortex
- Centrifuge mini spin
- Magnetic racks
- NanoDrop spectrophotometer
- Qubit

1- DNA extraction

1 DNA extraction is done using the "**Genomic DNA from soil**" kit (**NucleoSpin Soil, Macherey-Nagel**) from feces preserved in ethanol (96%) at -20°C. **Two successive extractions** are done to retrieve high molecular weight DNA. The protocol was optimized by using **Buffer SL1 + Enhancer SX**.

Before the extraction:

- Incubate Buffer SL1 for 5 min at 50°C before use.
- Add 100 mL of ethanol (96%) to Buffer SW2 before use.

1.1 Sample preparation

1.1.1- Weigh between 500 and 700 mg of feces in a 2 mL LoBind tube.
1.1.2- Centrifuge for 5 min at full speed and remove the supernatant (ethanol). Repeat 5 times.
1.1.3- Weigh the feces without ethanol. NB: between 300 and 500 mg of fecal material remains.
1.1.4- Transfer the feces into the NucleoSpin MN Bead Tube Type A.
1.1.5- Add 700 µL of Buffer SL1.

1.2 Sample lysis

1.2.1- Add 150 µL of Enhancer SX.
1.2.2- Vortex horizontally at full speed for 5 min.
1.2.3- Centrifuge for 2 min at 12.8 rpm.
1.2.4- Transfer the supernatant (~600 µL) into a new 1.5 mL LoBind tube. **Keep the pellet in the fridge for the second extraction**.
1.2.5- Add 150 µL of Buffer SL3 and vortex for 5 sec.

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1.2.6- Incubate for 10 min at 0-4°C.
1.2.7- Centrifuge for 1 min at 12.8 rpm.

Removal of inhibitors
1.2.8- Transfer 500 µL of supernatant onto a NucleoSpin Inhibitor Removal Column (red ring) placed in a collection tube (2 mL).
1.2.9- Centrifuge for 1 min at 12.8 rpm. Keep the supernatant in a new tube and repeat steps 1.2.8 and 1.2.9 with the remaining supernatant.
1.2.10- Add 250 µL of Buffer SB and vortex for 5 sec.

DNA binding on silica membrane
1.2.11- Transfer 550 µL of supernatant onto a NucleoSpin Soil Column (green ring) placed in a collection tube (2 mL).
1.2.12- Centrifuge for 1 min at 12.8 rpm.
1.2.13- Discard the liquid and replace the collection tube below the column.
1.2.14- Repeat steps 1.2.12 and 1.2.13 with the remaining supernatant.

1.3 Whashing step

1.3.1- Add 500 µL of Buffer SB to the NucleoSpin Soil Column.
1.3.2- Centrifuge for 30 sec at 12.8 rpm.
1.3.3- Discard the liquid and replace the collection tube below the column.

1.3.4- Add 550 µL of Buffer SW1 to the NucleoSpin Soil Column.
1.3.5- Centrifuge for 30 sec at 12.8 rpm.
1.3.6- Discard the liquid and replace the collection tube below the column.

1.3.7- Add 700 µL of Buffer SW2 to the NucleoSpin Soil Column.
1.3.8- Vortex for 2 sec.
1.3.9- Centrifuge for 30 sec at 12.8 rpm.
1.3.10- Discard the liquid and replace the collection tube below the column.

1.3.11- Repeat steps 1.3.7 to 1.3.10.

Dry silica membrane
1.3.12- Centrifuge for 2 min at 12.8 rpm.

1.4 DNA elution

1.4.1- Place a NucleoSpin Soil Column in a 1.5 mL LoBind tube.
1.4.2- Add 60 µL of Buffer SE to the column.
1.4.3- Incubate for 1 min at room temperature (RT) with the lid opened.
1.4.4- Centrifuge for 30 sec at 12.8 rpm.

For the second extraction:
- Use the pellet from step 1.2.4 kept in the fridge.
- Add 700 µL of Buffer SL1 and 150 µL of Buffer SX.
- Resuspend the pellet by vortexing.
- Follow the same steps as described above from 1.2.3 to 1.3.12.

For DNA elution:
- Place a NucleoSpin Soil Column in a 1.5 mL LoBind tube.
- Add **35 µL of Buffer SE** to the column.
- Incubate for 1 min at RT with the lid opened.
- Centrifuge for 30 sec at 12.8 rpm.

2- DNA quality and purity control

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2 Quantify the DNA with Qubit fluorometer (Broad Range) and NanoDrop spectrophotometer.
Qubit/Nanodrop ratio must be between 0.6 and 1 (below 0.6 DNA is not clean enough).

Check DNA quality by migration on a 1% agarose gel.

DNA from the first extraction is more degraded than DNA from the second extraction (Figure 1).

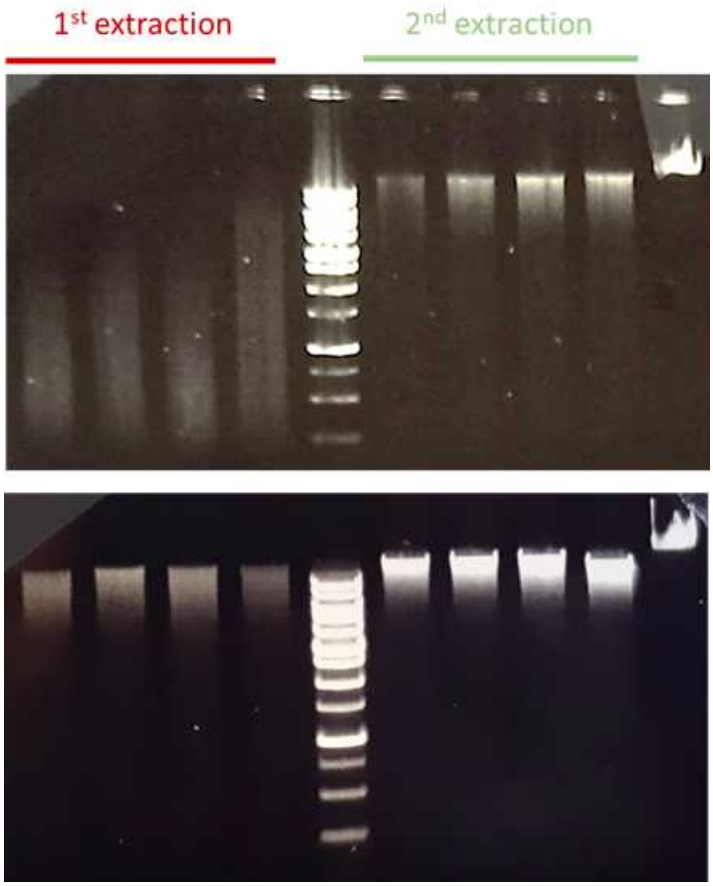

Figure 1. Agarose gel 1% in TAE 1x showing first and second DNA extractions from ethanol-preserved fecal samples of one ground pangolin (*Smutsia temminckii*, TS525, top) and one southern aardwolf (*Proteles cristatus*, TDR22, bottom). Wells 1-4: 1st extraction; well 5: 200 bp - 10 Kb ladder; wells 6-9: 2nd extraction and well 10: Lambda ladder (48.5 Kb).

3- DNA sizing with AMPure XP beads

3 This step enables to remove small DNA fragments and/or residual inhibitors.

The volume of beads (DNA volume*ratio) is adjusted according to sample degradation between 0.4 up to 1x.

3.1- Resuspend the pellet by gently shaking the tube with your finger.
3.2- Centrifuge briefly.
3.3- Incubate for 10 min at RT. In the meantime, prepare a fresh solution of ethanol 70%.
3.4- Place the tube on a magnetic rack for 4 min.
3.5- Remove the supernatant.
3.6- Add 200 µL of ethanol (70%) without disturbing the pellet.
3.7- Wait for 30 sec and remove the ethanol.
3.8- Repeat steps 3.6 and 3.7.
3.9- Centrifuge briefly, put the tube back on the magnetic rack and remove residual ethanol with P10.
3.10- Remove the tube from the magnetic rack.

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3.11- Air-dry for 1 min at RT.
3.12- Add 55 µL of H2O on the beads.
3.13- Resuspend the pellet by gently shaking the tube with your finger.
3.14- Incubate for 10 min at RT.
3.15- Place the tube on a magnetic rack.
3.16- Transfer the supernatant into a new LoBind tube.

Quantify DNA with Qubit fluorometer (Broad Range) and NanoDrop spectrophotometer. Check DNA quality by migration on a 1% agarose gel.

4- Library preparation

4 Library preparation is done according to the "**1D Genomic DNA by Ligation (SQK-LSK109)**" kit (**Oxford Nanopore Technologies**) instructions.

Quantify DNA with Qubit fluorometer (Broad Range).

5- MinION sequencing

5 Sequencing is done on MinION and Mk1C portable devices using R9 flowcells.

Comparison of first and second extractions

6 We performed short-read Illumina sequencing (150 PE) on both extractions to compare with long-read sequencing (done only on the second extraction).

Short reads (SR) were assembled for each extraction and compared to long-read assemblies (LR). Long-read assemblies were more contiguous (less contigs, higher N50, higher length of the longest contig) compared to short-read assemblies (Table 1). Short-read assemblies from first and second extractions were similar in terms of assembly statistics (Table 1).

Table 1. Comparison of short-read and long-read assemblies of 3 samples (two aardwolves, *Proteles cristatus* and one aardvark, *Orycteropus afer*). Short-read assemblies were done with *metaSPAdes* v3.15.2 (Nurk *et al*, 2017). Long-read assemblies were done with *metaFlye* v2.8.3 in strain mode (Kolmogorov *et al*, 2020). Assemblies were performed after removing adapters, low-quality reads and host and human reads. Contig statistics were computed for contigs >1 000 bp with *anvi'o* v7 (Eren *et al*, 2021).

Samples	Number of contigs			Contig N50 (bp)			Longest contig (bp)		
	SR 1	SR 2	LR	SR 1	SR 2	LR	SR 1	SR 2	LR
PRO TDR22	34 376	52 809	3 415	12 598	9 579	90 053	642 707	248 478	913 039
PRO TDR49	45 422	35 347	5 229	9 151	14 317	95 389	211 799	191 444	2 328 571
ORY TS513	82 582	62 637	4 655	8 091	9 055	82 470	953 702	959 158	3 962 646

Mapping short reads from the first extraction on assembled contigs of the second extraction from the same sample confirmed that the two extractions were similar (high percentage of mapped reads, Table 2). They were also similar to what was obtained from long-read sequencing and assembly (Table 2).

Table 2. Mapping of short reads (SR) from both extractions against contigs (C) of short-read (SR) and long-read (LR) assemblies. Short reads were mapped using *bowtie2* v2.3.4.1 (Langmead and Salzberg, 2012) with default parameters.

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Samples	% mapped reads (overall alignment rate)			
	SR against SR assemblies		SR against LR assemblies	
	SR_1 / C_{SR2}	SR_2 / C_{SR1}	SR_1 / C_{LR}	SR_2 / C_{LR}
PRO TDR22	96.36	92.99	93.56	91.65
PRO TDR49	94.45	96.04	92.31	94.7
ORY TS513	86.68	92.31	78.56	86.78

Conclusion

7 Taxonomic profiling of microbial communities associated with non-model organisms is challenging because of the incompleteness of reference databases (only few taxa could be identified). Extracting and reconstructing genomes from those unknown microbial taxa is thus of interest because they can then be placed in a phylogeny to assess their taxonomy. To do so, long reads present the advantage that they enable the reconstruction of more contiguous assemblies than short reads; this can then facilitate the recovery of contiguous bacterial genomes. With this optimized protocol, we showed that from mammalian field-collected fecal samples preserved in 96% ethanol, two successive extractions are needed to get high molecular weight DNA suitable for long-read sequencing. The two extractions only differ in the fact that the first extraction more likely yields extracellular degraded DNA whereas the second extraction allows accessing better preserved intracellular bacterial DNA. One remaining challenge with long reads is their higher error rate compared to short reads. Here, the high percentage of mapped reads on long-read assemblies suggests that short reads from each extraction could be used for polishing long-read assemblies. This will ensure that high quality contiguous assemblies are obtained and can then be used to reconstruct complete bacterial genomes.

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