OPTIMIZED PROTOCOL FOR VIRAL METAGENOMICS STUDIES ON FAECAL SAMPLES WITH MINILYS HOMOGENIZER

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/ CONTEXT

Next Generation Sequencing (NGS) has revolutionized the study of the human gut microbiome - the community of microorganisms (including bacteria, fungi, and viruses) present within the human gut. While in recent years, our understanding of the human microbiome has greatly improved, most studies have focused on bacteria, ignoring viral genomes. For this reason, little is known regarding the human virome –the viral component of the human microbiome. One of the main challenges in studying the role of the human gut virome in health is the absence of validated methods for high throughput and reproducible virome analysis.

In this study, the quantitative effects of different steps of sample preparation for virome analysis were evaluated with qPCR and next-generation sequencing (NGS). Several procedures were tested for the homogenization, centrifugation, filtration, and random amplification steps, using a mock-virome (including 9 highly diverse viruses among which coronaviruses) and a bacterial mock-community. As a result, an optimized protocol for fecal sample preparation was created, the NetoVIR (Novel enrichment technique of VIRomes). The NetoVIR protocol makes use of the Minilys (Bertin Technologies, France) to efficiently and uniformly homogenize samples. Thanks to Minilys-powered homogenization and other optimized steps.
(Figure 1), the NetoVIR protocol can recover all viruses present in the mock-virome samples.

/ PROTOCOL

Homogenization:
Mock-virome and bacterial mock-community were homogenized using a tissue homogenizer (Minilys, Bertin technologies, France). A 200 µL stock of mock-virome was subjected to different homogenization speeds (3000 rpm or 5000 rpm) with or without the presence of ceramic beads (Ø0.1 mm (CK01–2 ml, P000919-LYSK0-A) or Ø2.8 mm (CK28–2 ml, P000911-LYSK0-A)) and compared to a non-homogenized control (Figure 2). All samples were homogenized for 1 min.

Centrifugation and filtration:
Samples were centrifuged using a bench top centrifuge (Heraeus pico 17, Thermoscientific). Two-hundred µl of mock-virome or bacterial mock-community was centrifuged at 100 g or 17000 g for 3 min or 30 min. For filtration, a 0.8-µm centrifugal (PES) filter (VK01P042, Sartorius), a 0.8-µm polycarbonate (PC) filter (ATTP14250, Millipore), as well as a 0.45-µm centrifugal filter (UFC40HV00, Millipore) and a 0.22-µm centrifugal filter (UFC40GV00, Millipore) were tested.

Nuclease treatment and DNA/RNA extraction:
Samples were treated for 2 hours at 37 °C with a cocktail of 1 µl microccocal nuclease (NEB) and 2 µl of benzonase (Millipore) and 7 µl of homemade buffer (1M Tris, 100 mM CaCl2 and 30 mM MgCl2, pH 8) and extracted with the QIAamp Viral RNA Mini Kit (Qiagen) without carrier RNA.

Random amplification:
Random amplification of nucleic acids was performed using the Whole Transcriptome Amplification Kit 2 (WTA2, Sigma Aldrich) according to manufacturer's instructions with the exception of the initial denaturation step which was performed at 95 °C instead of 70 °C in order to also denature double-stranded DNA or RNA to make it available for the amplification. In addition, the number of amplification cycles was varied between 7, 12, 17 and 22. WTA2 products were purified with the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

NGS sequencing:
NGS library preparation was performed using the Nextera XT DNA Library Preparation kit (Illumina) as described in [1].
Sequencing was performed on a HiSeq™ 2500 platform (Illumina) for 2 × 150 cycles. Sequencing reads can be seen in Figure 3.

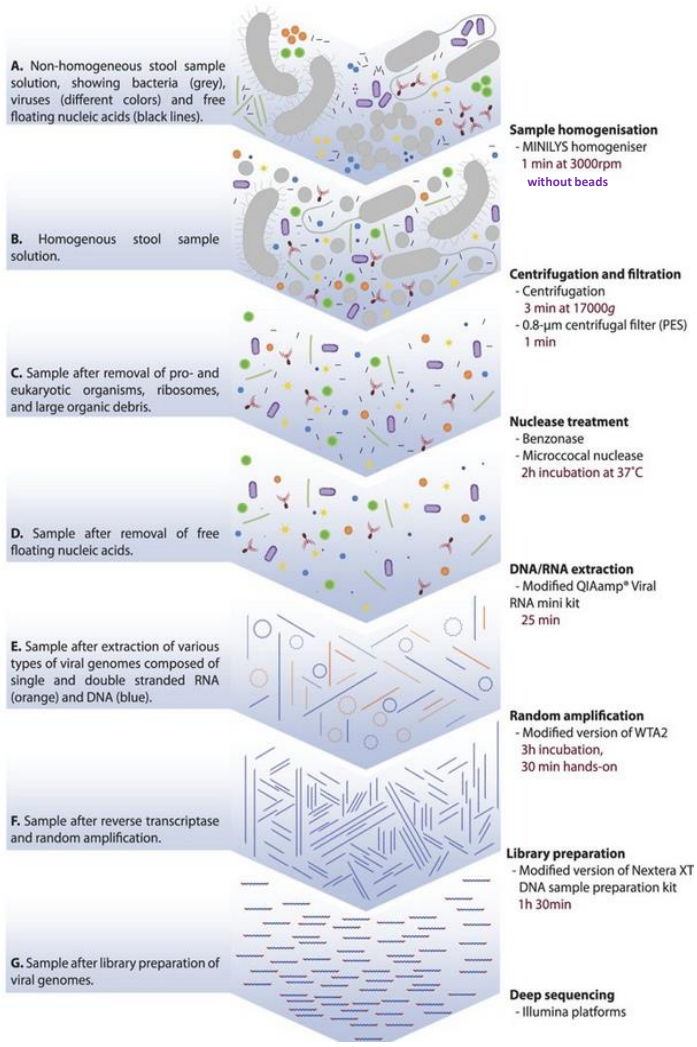

Figure 1: Description of the optimized NetoVIR protocol, On average, the protocol takes 8 h to complete. From [1]

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/ RESULTS

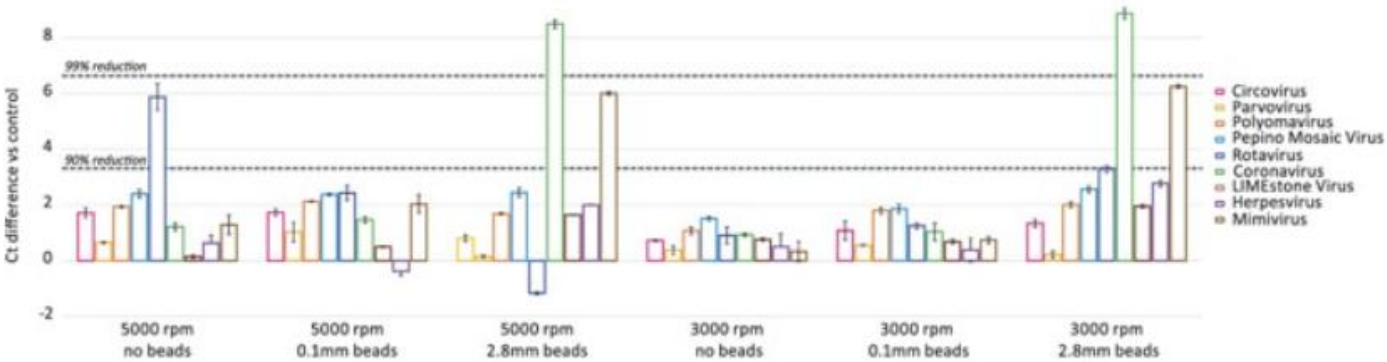

Figure 2: Ct differences vs control for different homogenization experiments performed on the mock-virome community. Standard deviations are based on three qPCR replicates. From [1].
Homogenization with Ø2.8 mm beads led to a destruction of viral particles irrespective of homogenization speed. The reduction was largest for coronavirus (99.5% and 99.6% and Ct differences of 8.5 and 8.9 for 5000 and 3000 rpm, respectively) and mimivirus (96.0% and 97.7% and Ct differences of 6.0 and 6.3 for 5000 and 3000 rpm, respectively). homogenization at 5000 rpm (without beads or with Ø0.1 mm beads) showed a larger reduction in viral particles than homogenization at 3000 rpm. Reduction of viral particles was lowest using 3000 rpm homogenization without beads.

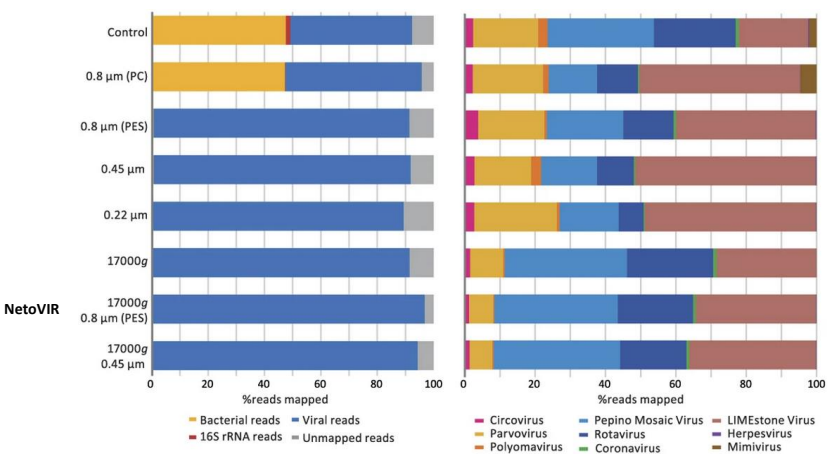

Figure 3: Left: NGS sequencing reads for sample consisting of pooled bacterial and viral communities: percentage of NGS sequencing reads for bacterial, 16S rRNA, viral and unmapped reads for the conditions tested, Right: Distribution of NGS sequencing reads for the mock-virome. From [1].
7 different workflows were tested including homogenization at 3000 rpm without beads and 17 amplification cycles, in combination with different conditions of filtration (0.8 PC/PES, 0.45 and 0.22-µm) and/or centrifugation (3 min at 17000 g).
The 0.8-µm PES filter plus centrifugation condition yielded the highest percentage of viral reads, of which most were attributed to pepino mosaic virus (33.9%), LIMEstone virus (32.9%) and rotavirus (20.6%).
The four protocols without centrifugation showed an expansion of the LIMEstone virus reads, mainly at the expense of rotavirus and pepino mosaic virus reads.
Based on these results, a favoured protocol named NetoVIR (Novel enrichment technique of VIRomes) was selected. NetoVIR consisted of homogenization at 3000 rpm for 1 min without beads, centrifugation for 3 min at 17000 g plus 0.8-µm PES filter filtration and 17 amplification cycles.

/ CUSTOMER

KU LEUVEN

[1] CONCEIÇÃO-NETO, Nádia, ZELLER, Mark, LEFRÈRE, Hanne, *et al.* Modular approach to customise sample preparation procedures for viral metagenomics: a reproducible protocol for virome analysis. *Scientific reports*, 2015, vol. 5, no 1, p. 1-14.

/ CONCLUSION

The NetoVIR protocol with Minilys homogenization allows for fast, reproducible and high throughput sample preparation for viral metagenomic studies. NGS results show that all viruses present in the mock virome sample can be recovered. It also seems like the ratio of viral versus bacterial and 16S rRNA genetic material is strongly altered in favor of viruses.

For most biological and environmental samples, obtaining an homogenous solution is essential for optimal and reproducible viral particles purification. This study shows how Minilys can homogenize fecal samples in a fast, reproductible way while introducing minimal bias.

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