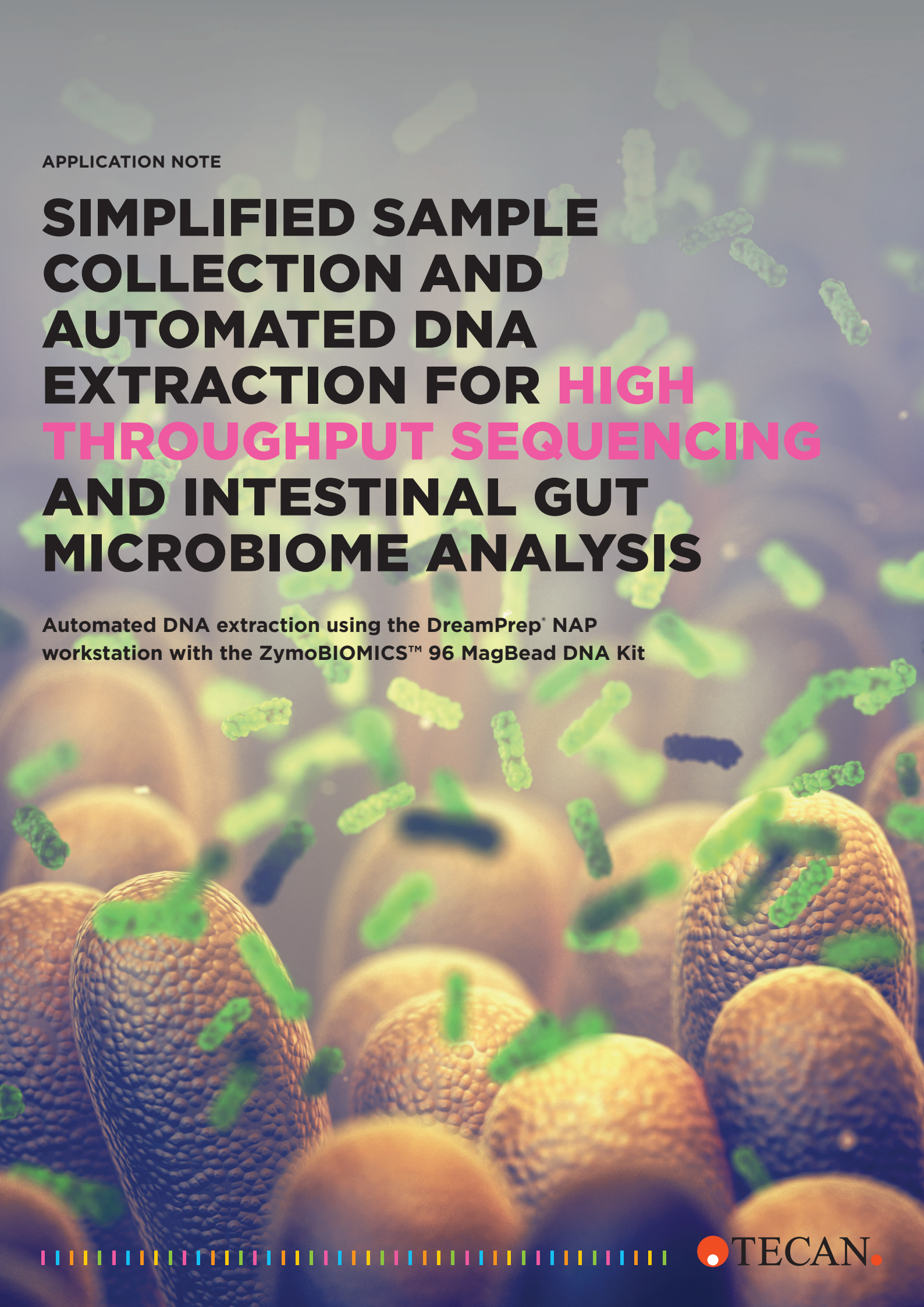
APPLICATION NOTE

SIMPLIFIED SAMPLE COLLECTION AND AUTOMATED DNA EXTRACTION FOR HIGH THROUGHPUT SEQUENCING AND INTESTINAL GUT MICROBIOME ANALYSIS

Automated DNA extraction using the DreamPrep® NAP workstation with the ZymoBIOMICS™ 96 MagBead DNA Kit



TECAN

Introduction

The intestinal microbiome is a complex ecosystem of microorganisms that reside in the gastrointestinal tract of humans and animals. It is made up of trillions of microbes – including bacteria, viruses, fungi and protozoa – which have co-evolved with their hosts over millions of years, forming a symbiotic relationship that is essential for our survival. The microbiome serves multiple functions, including aiding in the digestion of food, interacting with our immune system, synthesizing essential vitamins and nutrients – such as short chain fatty acids – and protecting us against harmful pathogens. In recent years, the study of the intestinal microbiome has gained significant attention, and there is a growing body of evidence that imbalances in this microbial community can lead to a range of diseases, including inflammatory bowel disease, obesity and even mental health disorders. As such, understanding the composition and function of the intestinal microbiome has become a major area of research, with the potential to revolutionize how we diagnose and treat a wide range of health conditions.

Next generation sequencing (NGS) technologies have transformed the study of the intestinal microbiome, by allowing comprehensive, high throughput analysis of microbial communities. This approach enables researchers to sequence the genetic material – whether DNA or RNA – of all the microorganisms present in a given sample, including those that were previously difficult or impossible to culture using traditional methods. NGS can be adjusted for either the broad analysis of whole genomes, or the specific analysis of indicative or representative parts, by using 16S, 18S or ITS rDNA clusters, respectively. The latter method has allowed accurate and complete characterization of the composition and function of microbiomes at low cost.

The goal of Ortho-Analytic – a Swiss medical laboratory for integrative medicine – is to make the medical benefits of intestinal microbiome analysis available to the general public and routine diagnostic services. The company therefore combined NGS with automated DNA extraction to create a flexible workflow offering increased throughput, reproducible analyses and reduced costs for the investigation of human stool samples.

This application note demonstrates an automated DNA extraction workflow for Ortho-Analytic's cutting-edge Calex® NGS stool sampling tubes, using the DreamPrep NAP workstation with the ZymoBIOMICS 96 MagBead DNA Kit, and compares the results with the widely used ZymoBIOMICS DNA miniprep manual extraction kit. The workflow provides the user with a significant reduction in collection-to-extraction times, highly automated hands-free operation and variable sample throughput, as well as delivering sufficient quantities of high-quality DNA for downstream applications such as NGS sequencing or qPCR analysis.

MATERIALS AND METHODS

Stool sampling was carried out using Ortho-Analytic's innovative Calex NGS sampling device (Figure 1). The Calex NGS is adapted from Bühlmann's well-known Calex Cap for calprotectin measurements. 10 mg stool specimens were preserved in a buffer solution that inactivates bacteria, viruses and yeasts, while simultaneously stabilizing RNA/DNA at room temperature for at least 30 days. Upon arrival at the laboratory, samples were ready for nucleic acid extraction without further treatment following removal of the tube caps.

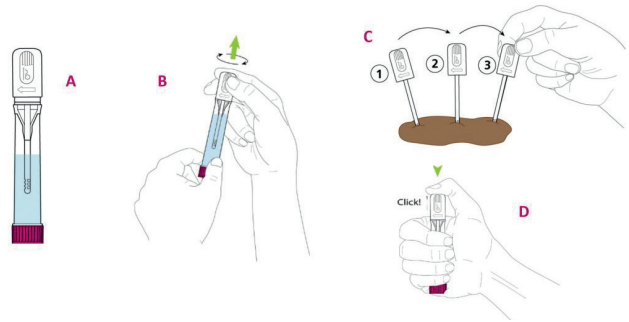


Figure 1: Sample collection with Calex NGS. For sampling, the white cap **(A)** is pulled out of the device **(B)** and pricked into the stool at 3 different places **(C)**. Afterwards, the white dosing pin is placed back into the tube, and the device sealed by firmly pressing down the cap **(D)**. A funnel inside the device makes sure that only 10 mg of stool is collected.

The DNA extraction workflow was performed using ZymoBIOMICS 96 MagBead DNA Kit on the DreamPrep NAP workstation, processing 1 to 96 samples per run. The workflow was fully automated except for the lysis, which was performed off-line. The DreamPrep NAP is equipped with an integrated barcode scanner – Fluent ID™ – which enabled automated sample identification and tracking when the Calex NGS tubes are loaded onto the Fluent platform. The system was configured to aspirate sample liquid between the dosing pin and tube walls, transferring 750 µl from each sample tube into the ZR BashingBead™ lysis tubes included in the extraction kit. The tubes were then manually transferred to a vortex mixer for physical lysis using bead beating

mechanisms, before being placed back into the DreamPrep NAP. The required sample volumes were transferred from each tube to a 96-well plate, where the fully automated, magnetic bead-based DNA extraction protocol was performed. After elution, onboard DNA quantification was performed – without any sample loss – using UV absorbance-based measurements on the integrated Frida Reader™, to allow normalization of all samples for further downstream applications as required. A standard fluorometer (Invitrogen QUBIT™ 4, Thermo Fisher Scientific) was used to compare DNA quantification results. The extracted DNA was sequenced on an Illumina MiSeq System for comparison of manual and automated extraction procedures.

RESULTS

Five unique stool samples were extracted with different lysis and elution volumes to optimize the automated DNA extraction on the Tecan DreamPrep NAP. Figure 2 displays the DNA concentration for three selected conditions. Each sample was measured 10 times with the integrated Frida Reader. The highest DNA concentration was achieved using 400 µl lysed solution, eluting the purified DNA in 35 µl elution buffer, resulting in an average of 11 ng/µl DNA. Overall, it is notable that the extracted DNA concentrations were in the lower range, due to the small sample input of only 10 mg of stool.

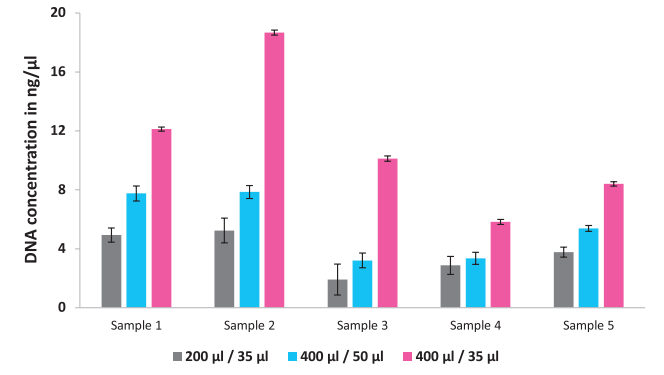


Figure 2: Measured DNA concentrations in five different samples using the integrated Frida Reader. Each sample was measured 10 times resulting in the displayed standard deviations.

The system calculated the purity of the extracted DNA by displaying 260/280 nm ratios (Figure 3A), as well as 260/230 nm ratios (Figure 3B) for each sample. The 260/280 nm ratio shows consistent results, close to the optimal ratio of 1.8, for each of the 10 repeated measurements of all 5 samples using 400 µl lysis/35 µl elution volume, indicating a low degree of protein contamination in these samples. However, the 260/230 nm ratios indicate the presence of residual salts and other contaminants for every sample, which is a common occurrence when using stool samples. It should be noted that, again, the samples extracted with 400 µl lysis/35 µl elution volumes were the purest in 4 out of 5 cases.

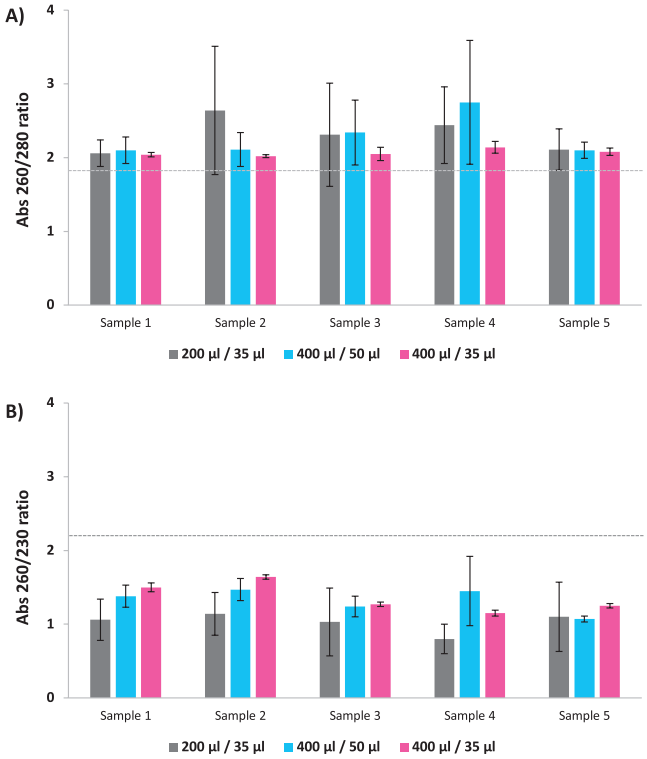


Figure 3: Measured 260/280 nm ratios (A) and 260/230 nm ratios (B) for the five previously described samples. Each ratio was measured 10 times, indicated by the respective standard deviations. The dotted lines represent the ideal ratios of 1.8 for 260/280 nm, and 2.2 for 260/230 nm.

As mentioned previously, the extracted DNA concentration was low due to the small sample input, and so the Frida Reader was operating at close to its lower detection limit of 2 ng/µl. To verify whether the measured data was accurate, a comparison was made with the highly sensitive Quant-iT™ dsDNA assay on the qubit fluorometer. The data displayed in Figure 4 shows that the Frida Reader measured systemically higher DNA concentrations compared to the Qubit fluorometer method. This can be explained by inherent characteristics of UV-based methods, where contaminants can influence the measured DNA concentrations, while Qubit measurements are highly specific for DNA, reducing the impact of contaminants on the resulting DNA concentrations.

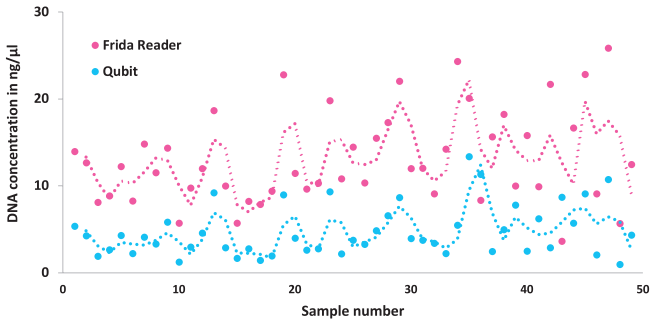


Figure 4: Comparison between Frida Reader and Qubit DNA concentration measurements in ng/µl. The dotted lines represent trend lines of the respective measurement techniques to improve readability of the plot.

Side-by-side comparison of manual and automated DNA extraction workflows

10 stool samples were extracted manually and automatically using the workflow described above. The ZymoBIOMICS Gut Microbiome Standard, an accurately quantified microbial community mimicking the human gut microbiome, was also processed as a positive control. The extracted DNA was sequenced and analyzed using the V3-V4 region of the 16S-rRNA gene. All samples resulted in high correlation values of at least 92 %, as indicated in the correlation matrix in Figure 5, with the positive control exhibiting an exceptionally high correlation of 99 %. The results show that manual and automated extraction provide comparable high-quality results. Furthermore, the data reveals that interfering factors – such as salts measured on the Frida Reader – have no influence on further downstream applications.

Sample		Manual extraction (ZymoBIOMICS DNA Miniprep Kit)										
		1	2	3	4	5	6	7	8	9	10	PC
Automated workflow	1	0.98	0.55	0.66	0.78	0.72	0.71	0.68	0.86	0.53	0.50	0.52
	2	0.54	0.97	0.58	0.60	0.54	0.60	0.63	0.65	0.49	0.39	0.34
	3	0.70	0.54	0.98	0.76	0.67	0.79	0.50	0.64	0.57	0.60	0.26
	4	0.79	0.64	0.62	0.95	0.89	0.75	0.83	0.86	0.57	0.57	0.73
	5	0.80	0.62	0.77	0.95	0.92	0.76	0.78	0.83	0.52	0.48	0.66
	6	0.73	0.56	0.82	0.75	0.65	0.92	0.55	0.64	0.60	0.64	0.33
	7	0.73	0.68	0.60	0.80	0.76	0.65	0.97	0.77	0.49	0.41	0.61
	8	0.88	0.73	0.69	0.84	0.75	0.74	0.76	0.96	0.58	0.56	0.57
	9	0.47	0.44	0.52	0.47	0.42	0.50	0.42	0.47	0.96	0.38	0.27
	10	0.68	0.52	0.77	0.71	0.59	0.78	0.50	0.59	0.63	0.94	0.24
	PC	0.60	0.45	0.33	0.68	0.68	0.45	0.68	0.70	0.32	0.23	0.99

Figure 5: Correlation matrix for manual and automated DNA extraction. All extracts were subsequently successfully sequenced and analyzed using the V3-V4 region of 16s-rRNA gene.

To further test the accuracy of this workflow, Ortho-Analytic participated in the 'Molecular Genetic Analysis of the Human Microbiome' inter-laboratory comparison for bacterial genome identification, provided by INSTAND eV. In the test in 2022, 60 out of 61 points were achieved, an overall result of 98.4 %, highlighting the excellent and robust automated protocol presented here.

Summary

The DreamPrep NAP workstation was effortlessly adapted to use Ortho-Analytics Calex NGS sampling tubes, producing a tailored workflow that can meet the demands of high throughput intestinal microbiome diagnostics laboratories. The results showed that the automated DNA extraction workflow, using ZymoBIOMICS 96 MagBead DNA kit, delivers sufficient amounts of DNA for further downstream applications, with a significant reduction in production time and higher sample throughput. Fine-tuning lysis and elution volume was key to obtaining reliable results with the best sample purity. In this setting, the integrated Frida Reader operates close to its detection limit, but still delivered concentration measurements that correlate with the highly specific and sensitive Qubit fluorometric measurements. This allowed DNA concentration measurements to be performed directly on the instrument, without wasting precious material. The subsequent normalization step saves further hands-on time, increasing daily throughput. Side-by-side comparison of manual and automated DNA extraction reveals excellent correlation between sequencing results for both methods, meaning that the manual workflow can be confidently replaced with the automated workstation. Furthermore, the accuracy of this workflow was proven by an external inter-laboratory comparison, with an overall result of 98.4 %. In summary, the workflow described here allows robust and standardized microbiome analysis, combining stabilized sampling with high quality analytics to provide the user with significant benefits, including simplifying sampling, reducing hands-on time and minimizing manual errors during DNA extraction.

Acknowledgements

This protocol was developed in close collaboration with Magne Osteras and Loïc Baerlocher from Fasteris SA, Life Science brand of Genesupport SA. Application-specific adaptations of the Tecan DreamPrep NAP were supported by Melissa Kaspar, application specialist at Tecan and Markus Wahl, former field automation specialist at Tecan. Parts of the data were created by Yvonne Graf during her master thesis.

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Axel W. Strittmatter *is one of the sales representatives for the i ntegrative services of Ortho-Analytic AG and Genesupport SA and Chief Sales & Marketing of Medisupport services, Switzerland. Axel has studied molecular microbiology, genetics and immunology and holds a PhD in these fields (awarded by the Georg-August University of Goettingen). His focus is on further development of the stool analysis portfolio for Ortho-Analytic and in parallel development of new genetic service offers for Genesupport, yet not being part of routine services or covered by the Swiss health insurance system.*

After successfully completing a master's degree in bioinformatics at the University of Geneva, Switzerland, ***Patricia Otten*** *joined the Swiss Institute of Bioinformatics. During her PhD thesis, she worked on the characterization of post-translational modifications in proteins using artificial intelligence algorithms. Following her academic training, Patricia transitioned from the realm of proteomics to the captivating field of genomics. She enrolled at Fasteris SA (now Genesupport SA) where her analytical skills have been utilized for data analysis and pipeline development.*

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