APPLICATION NOTE

Optimal recovery of microbial DNA for 16S sequencing from murine gastrointestinal tract tissue through bead mill homogenization and automated nucleic acid extraction.

Summary

The microbiome is a vast interconnected network of viruses, fungi, and bacteria found inside and on the surface of all entities, both living and non-living [1]. This network affects nearly all daily homeostatic functions in humans, including digestion, cognition, and immunity [1]. However, the magnitude and scale of this relationship in our everyday lives is still not fully understood, and continued research is needed to unveil the mysteries of the microbiome.

To aid in the discovery of new relationships and to delve deeper into current relationships we as humans have with our resident microorganisms, researchers must use methods that are not only repeatable and scalable but also maximize the yields of target analytes from challenging matrices. Much of the human microbiome data collection is created by 16S sequencing derived from multiple sources, including swabbing areas of interest, tissue biopsies, or stool samples [2]. Of these sample types, stool samples are often utilized for microbiome projects due to the non-invasive aspects of sample procurement, where tissue biopsy or swabbed samples may be more accurate representations or specifically targeted microbiome locations.

While easier to collect, stool samples have proven to have a host of shortcomings in microbiome research. Stool has been shown to be an inadequate sampling matrix to determine if treatments like probiotics are effectively taking residence inside a patient [3]. Additionally, stool does not allow the researcher to determine if the target organisms are present in desired concentrations at specific regions of the gastrointestinal tract (GI tract) [4].

For research use only. Not for use in diagnostic procedures.

Omni Bead Ruptor Elite™ bead mill homogenizer

To add to these conundrums, many sample preparation methods add bias to the downstream sequencing data by not offering a complete DNA extraction of all present microorganisms [5]. Some bacteria, particularly Gram-positive or acid-fast organisms, are resistant to lysis due to the thicker cell walls. These tougher to lyse organisms also resist enzymatic lysis methods and require long incubations for the same reason.

Without complete lysis and recovery of targeted intracellular analytes, tougher to lyse samples will be misrepresented as smaller percentages of populations, and other easier to lyse organisms will be overrepresented. These misrepresentations, established by the initial sample preparation, alter the accuracy and, consequently, our understanding of the data produced.

To address the need for complete lysis and provide quick and repeatable sample preparation, physical lysis of the microbes through bead beating gives optimal results. Bead beating stool samples for DNA recovery have been used for decades and are available in many typical DNA extraction kits [6,7,8]. However, not all bead beating methods provide optimal microbial lysis. When using esoteric samples like tissue that must have not only the microbial population lysed but the complete dissociation of the tissue matrix as well, particular care must be taken to use the correct methods to address the needs of each cell type.

Herein, we detail the selection of optimal bead media that address the lysis and recovery of DNA from liquid culture samples and the recovery of total microbial DNA from solid tissue samples.

Materials and methods

Equipment

- Omni Bead Ruptor Elite bead mill homogenizer (Cat # 19-042E)
- 2 mL Hard Tissue Homogenizing Mix 2.8 mm Ceramic Beads (Cat # 19-628)
- 2 mL Microbiome Homogenizing Mix 2.8 mm & 0.1 mm Ceramic Beads (Cat # 19-636D)
- Omni Bead Ruptor Elite bead mill homogenizer 2 mL tube carriage (Cat # 19-373)
- chemagic™ 360 (Cat # 2024-0020)
- chemagic DNA Stool 200 Kit H96 (Cat # CMG-1076)
- NEXTflex 16S V4 Amplicon-Seq Kit 2.0 (Cat # NOVA-4203-03)
- NEXTflex 16S V3-V4 Amplicon-Seq Kit (Cat # NOVA-4204-03)

Procedure

Optimal bead media selection

To evaluate which bead media would provide the best lysis for a wide range of microorganisms, a Gram-positive bacterium *(Staphylococcus epidermidis)*, a Gram-negative bacterium *(Escherichia coli)*, and a yeast *(Saccharomyces cerevisiae)* were chosen as target organisms to evaluate lysis. Each of these organisms was grown overnight in growth media appropriate for each target, made to the manufacturer's specifications. Either Tryptic Soy Broth (TSB) (Sigma, Cat # 21185) or Sabouraud Dextrose Broth (SDB) (Sigma, Cat. # S3306-500G) was used as a growth media. TSB was used for the propagation of both species of bacteria, and SDB was used for yeast propagation. Each organism was grown in an incubator at 37 °C for 16 hours before experimentation.

Each organism's optical density was measured at 600 nm post-incubation using an ELX808 plate reader, then standardized in 10 mL volumes by dilution in appropriate growth media to a standard OD600 of 0.1. Each organism was transferred into triplicate tubes containing either 0.5 mm ceramic, 0.5 mm glass, 0.1 mm ceramic, or 0.1 mm glass beads. The number of beads between common-sized beads was controlled by using densities provided by the suppliers. The weight between tested media (ceramic and glass) was constant between the two tested sizes. Glass bead media tubes contained 0.26 grams of bead media, and the tubes with ceramic bead media contained 0.6 grams. Three-time points (1, 2, and 3 minutes) were chosen to observe the effect of lysis on each of these organisms. Each time point would contain all four tested bead matrices. These conditions resulted in 108 tested samples. 1 mL of the tested standard was loaded into each 2 mL tube containing the target bead matrix.

All samples were processed on an Omni Bead Ruptor Elite bead mill (Cat # 19-042E) with parameters set to 4.2 m/s for 1-minute cycles. A 30-second dwell in between cycles was included on the 2 and 3-minute time points. After processing, each tube was serially diluted in 1:10 dilutions to reach 10^5 CFU/mL concentrations. For all organisms, this was roughly 10^3 CFU/mL. Next, using standard microbiological techniques, 100 µL of the 10^5 and the 10^4 CFU/mL concentrations were spread on either Tryptic Soy Agar (TSA) or Sabouraud Dextrose Agar (SDA) plates for colony counting. Plates were placed in an incubator set to 37 °C for 16 hours. After incubation, the plates were removed, and colonies were counted. These colonies were then compared to control samples plated from serial dilutions of the same standards that were not subjected to homogenization. Percent lysis was calculated as the percent reduction of visible colonies in the colony count plates. The bead media that created the highest amount of lysis across the observed time points was chosen as the most suitable media for microbial lysis. Figures 1-3 display the lysis data from each organism.

Lysis of bowel tissue

A combined bead fill was made incorporating 0.1 mm and 2.8 mm ceramic beads to evaluate the recovery of DNA from microbes in and on tissues. This new bead fill was subjected to side-by-side extractions against the original, non-optimized bead fill containing only 2.8 mm ceramic bead media. 2 fully intact, flash-frozen mouse gastrointestinal tracts were sectioned into twelve 200 mg segments (+/- 5%) starting at the colon and ending at the stomach of each mouse GI tract.

Each 200 mg GI sample was loaded into a 19-628 or 19-636D bead beating tube containing 1175 µL of lysis buffer and 25 µL of protease K from the chemagic DNA Stool 200 Kit H96 (Cat. # CMG-1076). All tubes were placed on an Omni Bead Ruptor Elite bead mill and homogenized at 6 m/s for 3, 1-minute cycles with a 30-second dwell time between each cycle. After homogenization, each tube was centrifuged at 13,000 x g for 5 minutes, and 800 µL of the supernatant was transferred to a 96-well plate compatible with the chemagic 360 instrument. DNA extraction was completed by following all steps described in the chemagic DNA Stool 200 Kit H96 protocol. The resulting extracted DNA concentrations and purity ratios were quantified with a Nanodrop 2000. DNA extraction results are displayed in Table 1.

Extraction of microbial DNA from standard community

To evaluate extraction bias from the optimal bead matrix for combined microbial and tissue lysis, a community of microorganisms was selected for extraction by purchasing a commercially available standard (Zymo, Cat. # D6300). This standard was then subjected to the same extraction performed on the bowel tissue samples. In triplicate 19-636D tubes, 75 µL of this standard was combined with 25 µL of protease K and 900 µL of lysis buffer provided in the chemagic DNA Stool 200 Kit H96 (Cat. # CMG-1076). All tubes were loaded onto the Omni Bead Ruptor Elite bead mill and homogenized at 6 m/s for 3, 1-minute cycles with a 30-second dwell time between each cycle. After homogenization, each tube was centrifuged at 13,000 x g for 5 minutes, and 800 µL of the supernatant was transferred to a 96-well plate compatible with the chemagic 360 instrument. DNA extraction was completed by following all steps described in the chemagic DNA Stool 200 Kit H96 protocol, except for reducing the elution volume to 100 µL from 250 µL. The resulting extracted DNA concentrations and purity ratios were quantified with a Nanodrop 2000.

16S sequencing

Sample DNA concentrations were determined using either a Qubit fluorometer or, for highly concentrated samples, by NanoDrop. Samples were diluted appropriately in Resuspension Buffer, and the concentration of diluted samples was again checked by Qubit fluorometry. Libraries were constructed using NEXTflex 16S V4 Amplicon-Seq Kit 2.0 (Cat # NOVA-4203-03) and NEXTflex 16S V3-V4 Amplicon-Seq Kit (Cat # NOVA-4204-03). Kits use specific primers that amplify the V4 region or V3 and V4 regions of the bacterial 16S rRNA gene. An input of 50 ng per reaction was used for DNA extracted from Zymo Community Standards samples. Library concentrations were measured using the Qubit fluorometer, and the library DNA length pattern was visualized on an electropherogram using an Agilent 2100 Bioanalyzer.

Libraries were sequenced on MiSeq (Illumina) using a 2 x 250 nt reads cartridge. Primers were trimmed by Cutadapt (https://cutadapt.readthedocs.io/en/stable/index.html). Reads were then analyzed by QIIME 2 software (https://qiime2.org/) using the newest SILVA (https://www.arb-silva.de/) datasets specific to V4 or V3-V4 regions. Population results from 16S sequencing are displayed in Figure 4.

Results

Small-diameter beads produced the highest average lysis when evaluating all tested cultured bacteria (Figures 1-3). In both *E. coli* and *S. epidermidis*, the smallest, most dense beads (0.1 mm ceramic) provided the greatest lysis at all time points. The second most effective lysing beads for these organisms was the 0.1 mm glass media. In both organisms, 0.5 mm ceramic beads produced greater average lysis at each observed time point than the 0.5 mm glass variant produced.

When evaluating the *S. cerevisiae*, some of the same trends continue. The smallest and most dense bead produced the most significant average lysis (Figure 2). However, the second-best bead media for producing lysis in yeast was the 0.5 mm ceramic bead, the largest and densest tested bead. The 0.5 mm glass beads also produced a larger average lysis than the 0.1 mm glass bead media in *S. cerevisiae*.

Homogenization of the GI tract samples with the 19-628 and the 19-636D bead kits resulted in liquified samples that could be easily pipetted into 96-well plates. However, the average DNA recovered from microbe-rich GI cultures was 12.7 times higher when incorporating the 0.1 mm ceramic beads into tubes compared to the average DNA recovered from tissue samples only using 2.8 mm ceramic beads (Table 1). Furthermore, the populations of organisms recovered from the community standard are represented in ratios equivalent to what is reported by the manufacturer for 16S genomic DNA composition (Figure 4).

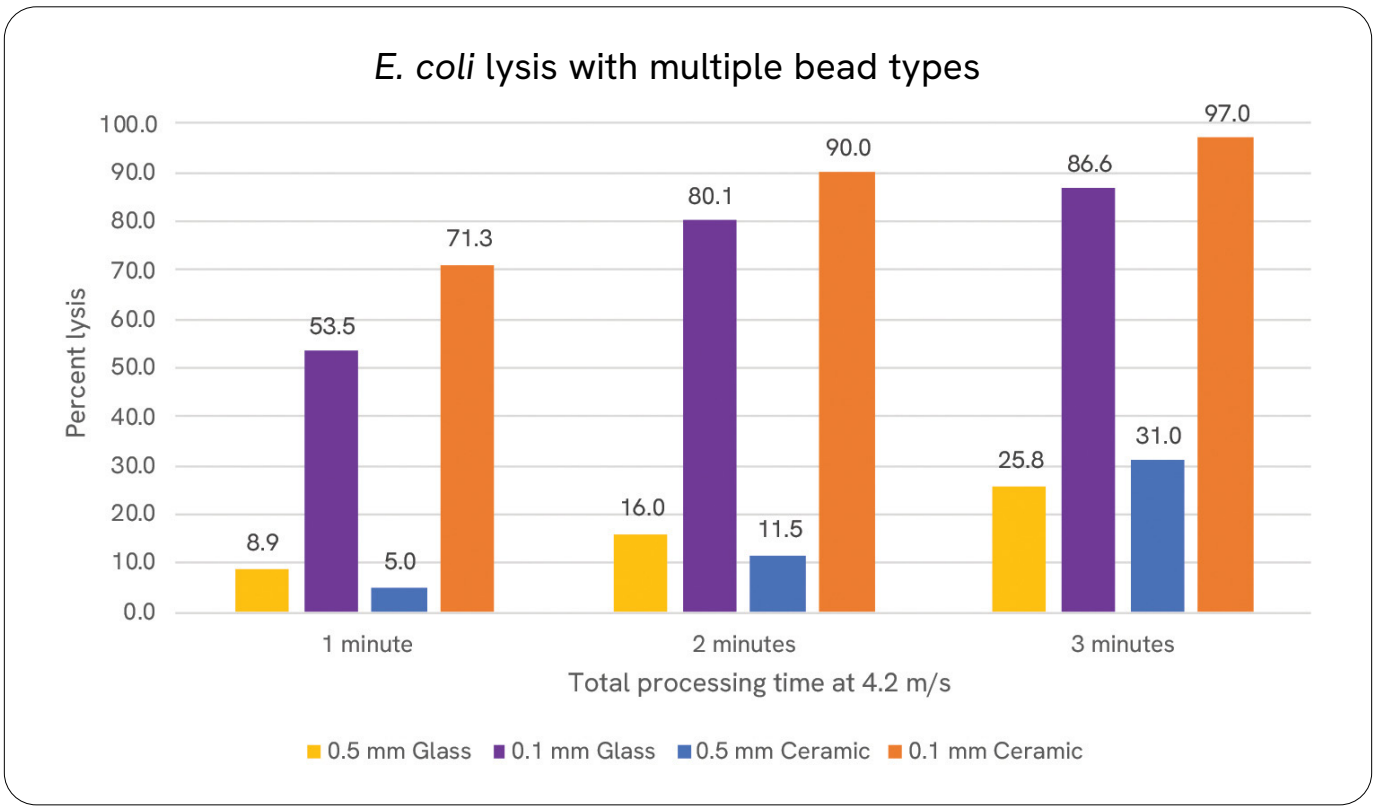

Figure 1: Lysis of *Escherichia coli* quantified by colony count.

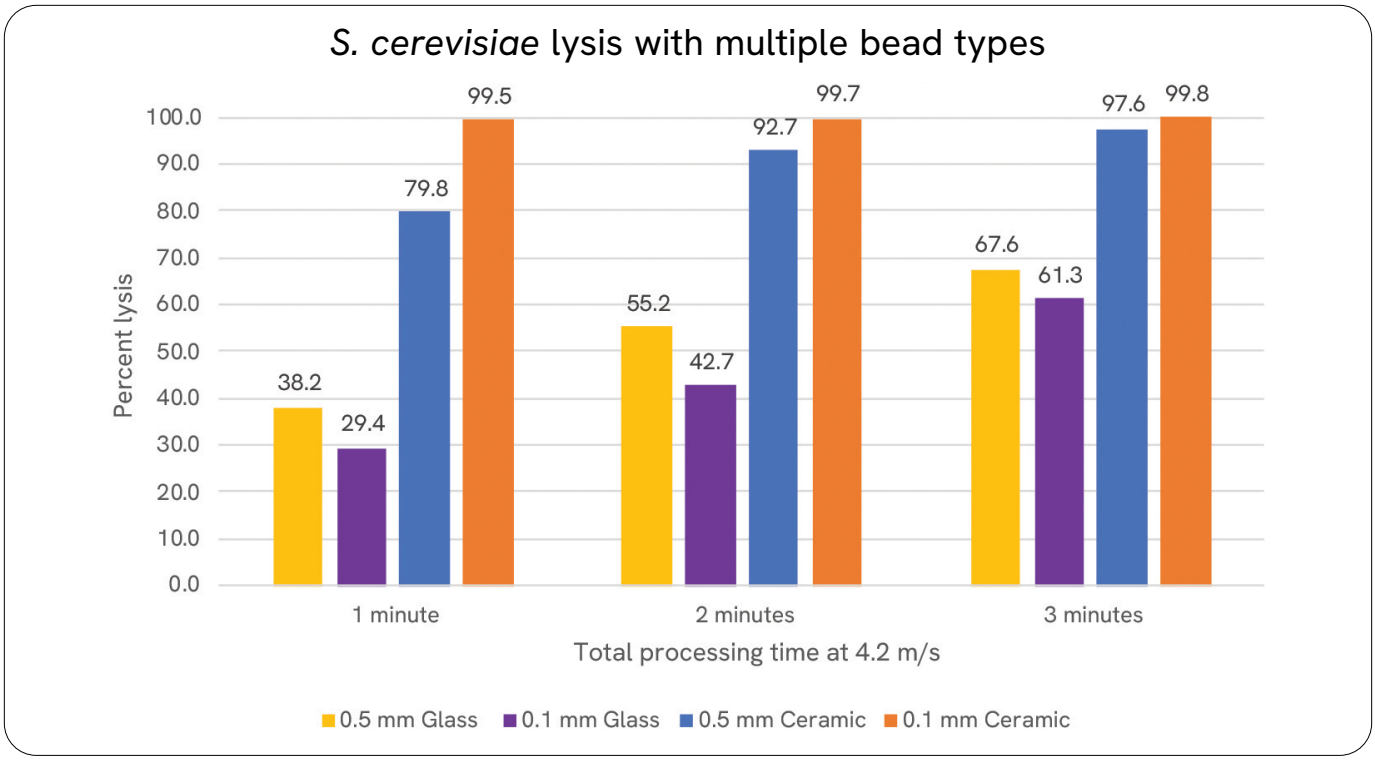

Figure 2: Lysis of *Saccharomyces cerevisiae* quantified by colony counts.

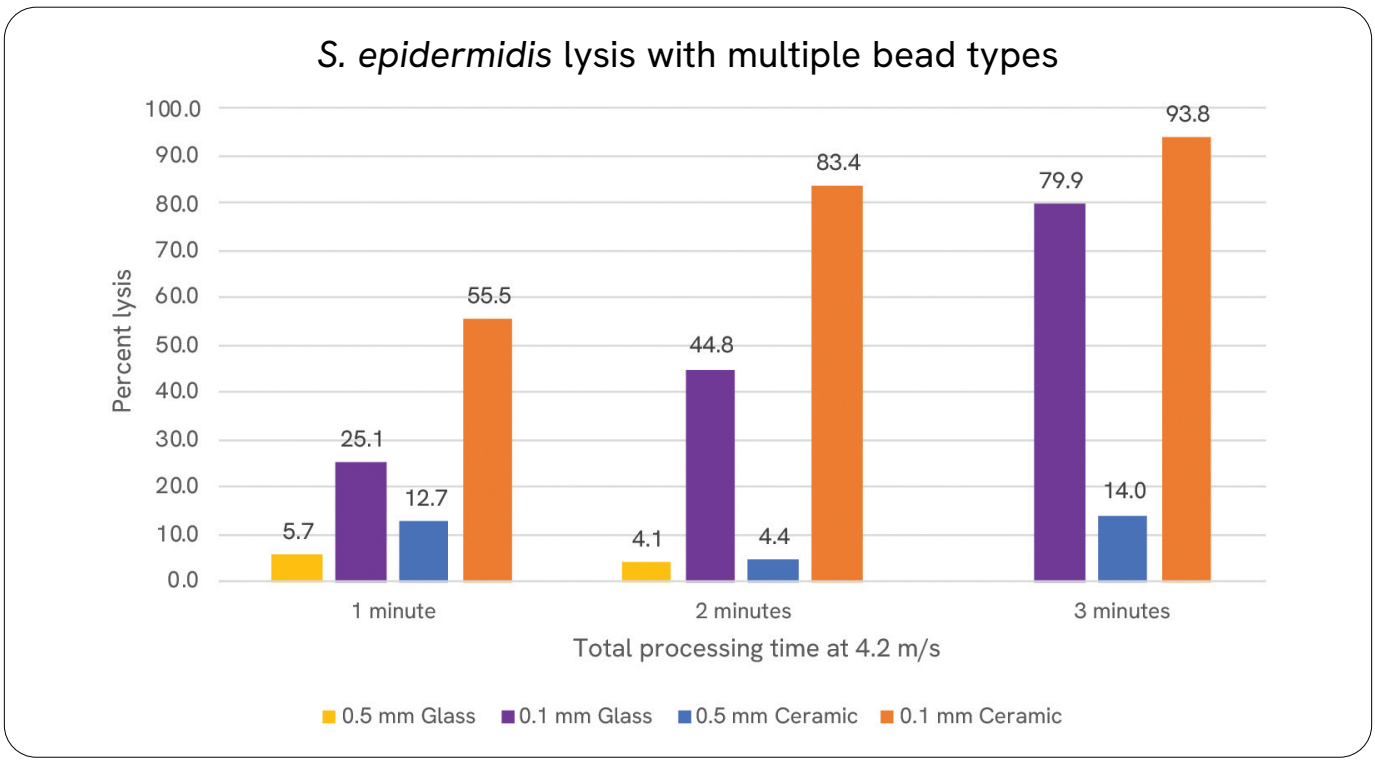

Figure 3: Lysis of *Staphylococcus epidermidis* quantified by colony counts

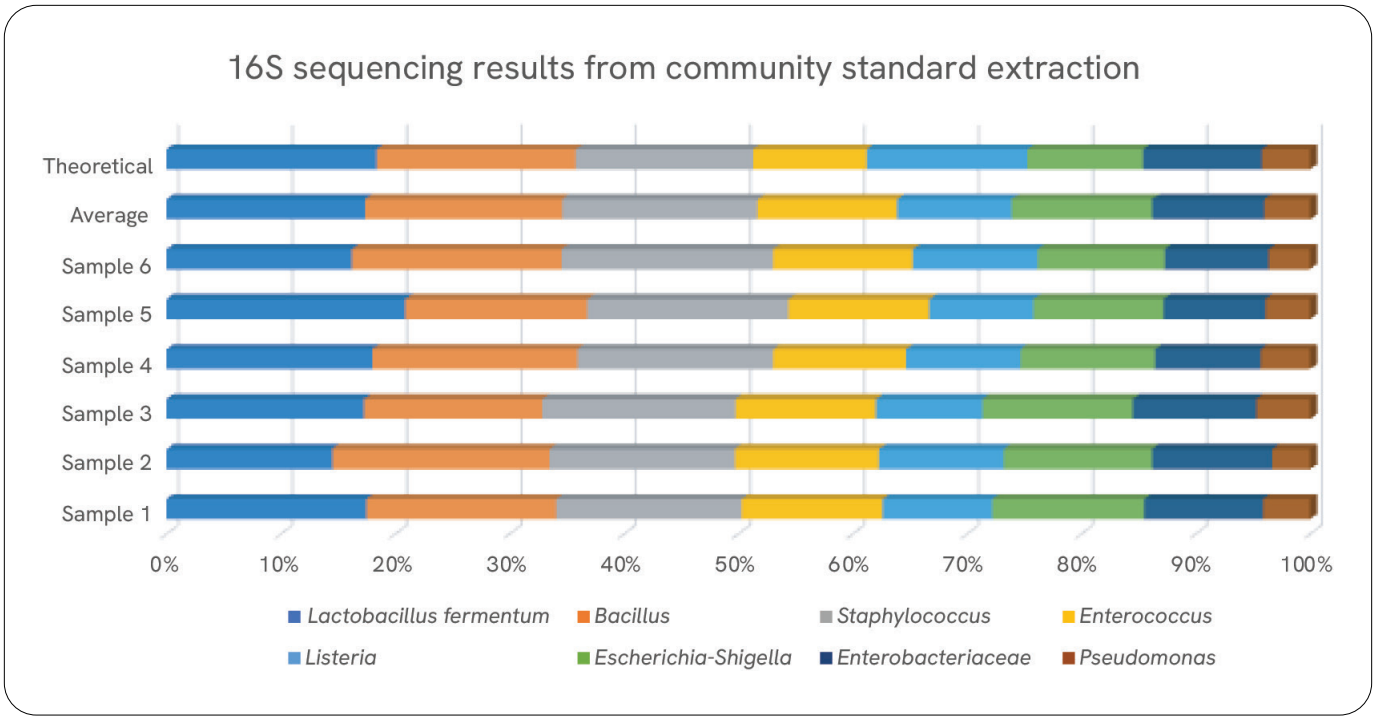

Figure 4: Population results displayed as the percentage of total 16S DNA present after sequencing the extracted Zymo Community Standard.

Table 1: Total DNA yields from mouse GI tracts after bead beating with microbial optimized and non-optimized bead fills.

Bead fill	Total yield (ng)	STD (ng)	A_{260}/A_{280}	A_{260}/A_{230}
2.8 mm ceramic beads	26,486	16,358	1.9	1.8
0.1 mm and 2.8 mm ceramic beads	338,289	104,794	2.0	2.1

Conclusion

Bead beating microbial cultures and microbe-rich tissues resulted in complete lysis and unbiased recovery of samples with high quality and high quantity of DNA. In addition, optimal lysis of all microorganisms was accomplished when using the smallest bead media evaluated, as seen in Figures 1-3.

For all tested bead types, the smallest-most dense beads provided the quickest complete lysis and the greatest total lysis compared to larger beads.

Additionally, combining the effectiveness of the optimal cultured microbial lysis beads with beads designed for the lysis of tissues and other solid samples, more total DNA is recovered from identical samples. This highlights the effectiveness of the combined bead fill in achieving high lysis efficiency and higher recovered DNA compared to lysis with only large bead media.

The sample preparation step utilizing the combined bead media is compatible with automated DNA extraction and 16S sequencing for metagenomic analysis. This analysis demonstrates the effectiveness of Omni Bead Ruptor Elite bead mill in the lysis of both tissues and microbes simultaneously. Additionally, it shows the importance of using the proper bead media in the initial lysis steps of the cellular matrix. Without the proper selection of bead media and adequate processing times tailored to the sample, complete physical disruption of the microbes cannot be accomplished.

If these diminished results are not addressed by additional steps with enzymatic or chemical lysis, the likely result would be altered data sets where easy-to-lyse organisms would be present in higher quantities than the harder-to-lyse organisms. Utilization of bead beating during the sample preparation of microbe-rich samples allows researchers to implement lysis steps that are high yielding, rapid, repeatable, and automatable.

References

1. Amon, P., & Sanderson, I. (2017). What is the microbiome? Archives of Disease in Childhood - Education & Practice Edition, 102(5), 257–260. https://doi.org/10.1136/archdischild-2016-311643

2. Qian, X.-B., Chen, T., Xu, Y.-P., Chen, L., Sun, F.-X., Lu, M.-P., & Liu, Y.-X. (2020). A guide to human microbiome research: Study design, sample collection, and bioinformatics analysis. Chinese Medical Journal, 133(15), 1844–1855. https://doi.org/10.109 cm9.0000000000000871

3. Zmora, N., Zilberman-Schapira, G., Suez, J., Mor, U., Dori-Bachash, M., Bashiardes, S., Kotler, E., Zur, M., Regev-Lehavi, D., Brik, R. B.-Z., Federici, S., Cohen, Y., Linevsky, R., Rothschild, D., Moor, A. E., Ben-Moshe, S., Harmelin, A., Itzkovitz, S., Maharshak, N., Elinav, E. (2018). Personalized gut mucosal colonization resistance to empiric probiotics is associated with unique host and microbiome features. Cell, 174(6). https://doi.org/10.1016/j.cell.2018.08.041

4. Suez, J., Zmora, N., Zilberman-Schapira, G., Mor, U., Dori-Bachash, M., Bashiardes, S., Zur, M., Regev-Lehavi, D., Ben-Zeev Brik, R., Federici, S., Horn, M., Cohen, Y., Moor, A. E., Zeevi, D., Korem, T., Kotler, E., Harmelin, A., Itzkovitz, S., Maharshak, N., ... Elinav, E. (2018) Post-antibiotic gut mucosal microbiome reconstitution is impaired by probiotics and improved by autologous fmt. Cell, 174(6). https:doi.org/10.1016/j.cell.2018.08.047

5. Li, M., Tyx, R. E., Rivera, A. J., Zhao, N., & Satten, G. A. (2022). What can we learn about the bias of microbiome studies from analyzing data from mock communities? Genes, 13(10), 1758. https://doi.org/10.3390/genes13101758

6. RamÅ¡ak, A., Peterka, M. Å., Tajima, K., Martin, J. C., Wood, J., Johnston, M. E. A., Aminov, R. I., Flint, H. J., & AvguÅ¡tin, G. (2000 Unravelling the genetic diversity of ruminal bacteria belonging to the CFB phylum. FEMS Microbiology Ecology, 33(1), 69–79. https://doi.org/10.1111/j.1574-6941.2000.tb00728.x

7. Yuan, S., Cohen, D. B., Ravel, J., Abdo, Z., & Forney, L. J. (2012). Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS ONE, 7(3). https://doi.org/10.1371/journal.pone.0033865

8. Zhang, B., Brock, M., Arana, C., Dende, C., van Oers, N. S., Hooper, L. V., & Raj, P. (2021). Impact of bead-beating intensity on the genus-and species-level characterization of the gut microbiome using amplicon and complete 16S rrna gene sequencing. Frontiers in Cellular and Infection Microbiology, 11. https://doi.org/10.3389/fcimb.2021.678522

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com