Library Preparation and Sequencing Platform Introduce Bias in Metagenomic-Based Characterizations of Microbiomes

Casper S. Poulsen[a], Claus T. Ekstrøm[b], Frank M. Aarestrup[a] and Sünje J. Pamp[a,c]#

[a]Research Group for Genomic Epidemiology, National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark.

[b]Section of Biostatistics, Department of Public Health, University of Copenhagen, Copenhagen, Denmark.

[c]Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kongens Lyngby, Denmark

#Correspondence: sjpa@dtu.dk

Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kongens Lyngby, Denmark

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Abstract

Metagenomics is increasingly used to describe microbial communities in biological specimens. Ideally, the steps involved in the processing of the biological specimens should not change the microbiome composition in a way that it could lead to false interpretations of inferred microbial community composition. Common steps in sample preparation include sample collection, storage, DNA isolation, library preparation, and DNA sequencing. Here we assess the effect of three library preparation kits and two DNA sequencing platforms. Of the library preparation kits, one involved a polymerase chain reaction (PCR) step (Nextera), and two were PCR-free (NEXTflex and KAPA). We sequenced the libraries on Illumina HiSeq and NextSeq platforms. As example microbiomes, we assessed two pig fecal samples and two sewage samples of which aliquots were stored at different storage conditions (immediate processing and storage at -80°C). All DNA isolations were performed in duplicate, totaling 80 samples excluding controls. We found that both library preparation and sequencing platform had systematic effects on the inferred microbial community composition. The different sequencing platforms introduced more variation than library preparation and freezing the samples. The results highlight that all sample processing steps need to be considered when comparing studies. Standardization of sample processing is key to generate comparable data within a study, and comparisons of differently generated data, such as in a meta-analysis, should be performed cautiously.

Importance

Previous research has reported effects of sample storage conditions and DNA isolation procedures on metagenomics-based microbiome composition; however, the effect of library preparation and DNA sequencing in metagenomics has not been thoroughly assessed. Here, we provide evidence that library preparation and sequencing platform introduce systematic biases in the metagenomic-based characterization of microbial communities. These findings suggest that library preparation and sequencing are important parameters to keep consistent when aiming to detect small changes in microbiome community structure. Overall, we recommend that all samples in a microbiome study are processed in the same way to limit

unwanted variations that could lead to false conclusions. Furthermore, if we are to obtain a more holistic insight from microbiome data generated around the world, we will need to provide more detailed sample metadata, including information about the different sample processing procedures, together with the DNA sequencing data at the public repositories.

Introduction

Microbes are omnipresent and inhabit even the most extreme environments on Earth. Metagenomics-based analyses have provided unprecedented insight into these microbial communities. Metagenomics is applied heavily to human microbiomes, as well as animal and environmental microbiomes, and is being implemented to understand disease states (1–4), for diagnostic purposes (5), and surveillance of pathogens and antimicrobial resistance (6–9). The data from such studies are a growing resource that can be utilized in meta-analysis and data mining, revolutionizing medicine, agriculture, and food production (6, 9–12).

Findings from microbiome studies can be difficult to replicate as observed in different meta-analyses of 16S rRNA gene amplicon studies (13–16). Considering the large number of features (including functional and taxonomic) under investigation in metagenomics, it is not surprising that studies do not seem to lack significant results (17). Data dredging is a real concern in metagenomics, which brings to mind the "replication crisis" that has been highlighted in the field of psychology (18, 19). Due to the challenges of replicating results, one must not over-emphasize the results from exploratory research and keep in mind that there is a need to continually validate the robustness and ability to replicate results in microbiome studies (20, 21). With the improvement of genome reference databases and bioinformatics tools, the validation is an ongoing process (22–25).

Technical variation due to sample processing is an important factor that researchers have to minimize to make proper inferences in metagenomics studies. For example, the DNA isolation procedure has been shown to impact on microbiome composition (26–28). The effect of library preparation and sequencing platform has been investigated in metagenomics primarily on human fecal samples. Library preparation was found to affect taxonomic and functional characterization of human fecal samples and *in silico* constructed mock communities (21, 29). In a study by Costea et al. (26), the effect of library preparation was found to be lower compared with DNA isolation and intra- and inter-sample variation in general. The choice of sequencing platform also appears to have an effect on the characterization of microbiomes (30).

The aim of the present study was to assess the effect of library preparation (Nextera, KAPA PCR-free, NEXTflex PCR-free) and sequencing platform (Illumina HiSeq and NextSeq) on the metagenomics-based inference on DNA samples from two different pig feces and two different sewage microbiomes from a previous study (31). We show that library preparation and sequencing platform introduce systematic bias in the inferred microbial community composition for both sample types and that this effect is important when comparing similar samples, such as pig feces, in the present study. This highlights the need for consistent sample processing and demonstration of caution when comparing data from different studies.

Results

A subset of DNA samples was selected from a large-scale study (31), to assess the effect of library preparation and DNA sequencing on inferred microbiome composition based on metagenomics. The DNA samples originated from two pig fecal samples (Pig feces 1 and Pig feces 2) and two sewage samples (Sewage 1 and Sewage 2). For the present study, we selected DNA aliquots from fecal and sewage samples that originally were processed immediately (time point 0) and were subjected to storage at -80C, respectively, to not only assess whether one can distinguish different samples, but also samples that have the same origin but exhibit differences due to different handling conditions. The DNA aliquots underwent a total of four different strategies for library preparation and DNA sequencing (Figure 1A), namely KAPA PCR-free library preparation + sequencing on a HiSeq (KAPA HiSeq), NEXTflex PCR-free library preparation + sequencing on a HiSeq (NEXTflex HiSeq), NEXTflex PCR-free library preparation + sequencing on a NextSeq (NEXTflex NextSeq), and Nextera library preparation + sequencing on a NextSeq (Nextera NextSeq). The latter sequencing strategy was performed twice (Nextera 1 NextSeq and Nextera 2 NextSeq).

Quality control of sequencing output

The number of raw reads from the different library preparations and sequencing platforms were similar with about a factor two difference when comparing the medians. The highest number of reads was obtained from the NEXTflex HiSeq run (median: 12.1, range: 6.3 – 30.8 million reads) and the lowest from the NEXTflex NextSeq run (median: 7.6, range: 2.7 – 9.4 million reads) (Table S1). The outputs from the KAPA HiSeq run (median: 9.4, range: 7.8 – 17.4 million reads) and the Nextera NextSeq runs (median: 10.2, range: 6.5 – 16.5 million reads) were about the same. More reads were obtained from the pig fecal samples compared with the sewage, but a larger proportion of the sewage reads mapped to the reference databases. The microbial community of the sewage samples exhibited a higher Simpson diversity than the pig feces (Table S1). The number of mapped reads were higher for the sewage samples, and many of the samples had reached a plateau as observed when drawing a rarefaction curve (Figure S1). Similar results were obtained when comparing

the mean of the percent of unmapped reads of the same sample across the different library preparation and sequencing platform runs (Pig feces 1: 87.4-88.4, Pig feces 2: 89.7-90.5, Sewage 1: 70.1-74.1, Sewage 2: 54.2-59.3) (Table S1).

Sample processing impacts on inferred microbiome structure

The pairwise Aitchison distances were calculated between all the samples and visualized using PCA (Figure S2A). The sample type explained the greatest variance, and pig feces and sewage samples were clearly separated on the first axis. A clear separation of the two sewage samples was observed on the second axis, while the two pig fecal samples clustered together. Ordination of the pig feces and sewage samples separately revealed that it was possible to differentiate the two pig fecal samples (Figure S2B). However, there were also two clusters within each pig fecal sample. A clear separation of the two sewage samples was still observed (Figure S2C). Also in a boxplot visualization, library preparation, sequencing platform, and storage condition did not hamper the ability to differentiate between the two sewage samples (Figure 1B). However, we observed an overlap between pig feces 1 and 2 comparisons, relative to comparing within the two samples representing the effect of the different sample processing parameters. Nevertheless, the median suggested there is a difference between pig feces 1 and 2 (Figure 1B). In general, larger distances were calculated for the comparisons of sample processing parameters in pig fecal samples compared with sewage. The shortest distances were observed when comparing the DNA isolation replicates and the replicates of the Nextera NextSeq runs, respectively. The distances between samples that differed in library preparation and sequencing platform were greater, compared with samples that differed in storage conditions (i.e. whether they were processed directly or after freezing at -80 °C for 64 hours). The sequencing platform appeared to be a major contributor of variation when comparing the samples that were prepared with NEXTflex and sequenced on both, an Illumina HiSeq and an Illumina NextSeq (Figure 1B). Whereas, using two different preparation kits, i.e. NEXTflex and KAPA, sequenced on an Illumina HiSeq introduced a relatively lower

variation. The differences were observed to be even lower when samples were prepared with the two library preparation kits NEXTflex and Nextera and sequenced on an Illumina NextSeq (Figure 1 B).

To investigate the effect of sample processing further, principal component analyses (PCAs) were performed for the individual samples (Pig feces 1, Pig feces 2, Sewage 1 and Sewage 2). Similar patterns were observed in all samples, indicating that there was a systematic effect from sequencing platform, library preparation, and storage condition (Figure 2). The samples clustered primarily according to sequencing platform and library preparation along the x-axis that represents most of the variation. On the y-axis samples clustered according to storage condition. In general, the DNA isolation replicates were similar, as well as the two Nextera NextSeq runs (Figure 2). All the parameters had a significant effect based on permutational multivariate analysis of variance (PERMANOVA), except for storage when comparing all of the samples and in pig feces 2 (Table 1). The percent variation in pig feces attributed to sample (Pig feces 1 and Pig feces 2) (21.1%) and sequencing platform (19.1%) were at similar levels, further emphasizing the importance of sample processing when comparing communities that are more similar to each other (Table 1).

Table 1: Effect of sample origin (Pig feces 1, Pig feces 2, Sewage 1 and Sewage 2) and different parameters in sample processing (Library preparation, DNA sequencing). Statistical tests were performed by multiple permutations partitioning sum of squares (PERMANOVA). The *p-value*, as well as the percent of variation explained by the parameters, is reported, testing different sample sets (all, pig feces, sewage, Pig feces 1, Pig feces 2, Sewage 1 and Sewage 2).

Samples included	Sample *p-value* (%[a])	Storage *p-value* (%[a])	Library preparation *p-value* (%[a])	Sequencing platform *p-value* (%[a])
All	$<10^{-5}$ (81.9)	6.4×10^{-2} (0.5)	4.2×10^{-2} (1.0)	3.0×10^{-4} (1.8)
Pig feces	$<10^{-5}$ (21.1)	3.8×10^{-3} (3.3)	5.7×10^{-4} (6.2)	$<10^{-5}$ (19.1)
Sewage	$<10^{-5}$ (61.7)	2.5×10^{-2} (2.9)	3.0×10^{-2} (4.1)	4.4×10^{-3} (4.5)
Pig feces 1	NA[b]	2.8×10^{-3} (9.7)	2.8×10^{-2} (8.9)	$<10^{-5}$ (26.2)
Pig feces 2	NA	0.17 (2.7)	5.4×10^{-3} (12.3)	$<10^{-5}$ (25.3)
Sewage 1	NA	$<10^{-5}$ (15.1)	3.6×10^{-4} (14.4)	$<10^{-5}$ (12.8)
Sewage 2	NA	$<10^{-5}$ (14.0)	6.0×10^{-5} (17.8)	$<10^{-5}$ (19.6)

[a]Proportion of the variation explained in the PERMANOVA

[b]Not Applicable, no *p*-value obtained when variable subset to a single sample (Pig feces 1, Pig feces 2, Sewage 1 and Sewage 2).

Sample processing impacts on inferred microbial abundances

To investigate the effect of library preparation and sequencing platform on the abundance of specific microorganisms, an overview of the 30 most abundant genera was visualized in heatmaps (Figure 3). For the pig samples, the aliquots appeared to cluster mainly based on the sequencing platform (NextSeq vs. HiSeq) (Figure 3A). In contrast, it was possible to distinguish the two sewage samples, who clustered according to sample origin (Sewage 1 vs. Sewage 2) (Figure 3B). A clustering of samples was also observed to a certain degree for both pig feces and sewage according to storage condition and library preparation.

The pig feces contained both Gram-negative and Gram-positive bacteria, and cluster 3 exclusively consisted of Gram-negatives. There were a few Gram-negatives in the other clusters, indicating that sample processing shifts the abundance profiles for specific groups of organisms, in this case, it appeared to be associated with cell wall structure (Figure 3A). A similar pattern was observed for sewage that mainly consisted of Gram-negatives. The majority of Gram-positives were part of cluster 4, including *Clostridium*, *Faecalibacterium*, *Roseburia* and *Ruminococcus*. However, this cluster also contained Gram-negative genera (Figure 3B).

One explanation for the community differences observed by sample processing could be a possible contamination during the library preparation and sequencing steps. To elucidate this, sparse partial-least-squares discriminant analysis (sPLS-DA) was performed, assessing which genera best characterize the library preparation and sequencing platform processing methods. Components 1, 2 and 3 were included in the model containing 5, 50 and 20 different genera, respectively (Figure S3). The majority of microorganisms were abundant organisms observed across all of the sample processing methods. However, a few were clear indicators of contamination during library preparation and sequencing and were mainly present in a single processing method. This included *Methylobacterium* in the KAPA HiSeq run and *Cutibacterium* in the second Nextera NextSeq run, bacteria that previously have been associated with kit contamination (32). A heatmap of the 30 most abundant genera in the blank controls additionally revealed a high abundance of *Ralstonia* in the Nextera NextSeq runs that where performed with the same kit reagents (Figure S4). Overall, the organisms associated with contamination were limited. The separation of the

samples according to the different processing parameters therefore appeared to be real changes to the relative abundances between organisms inherently present in the microbiomes and not due to contamination.

A constrained ordination analysis, also subset according to whether samples were processed directly or after freezing, was performed to assess whether groups of organisms at a taxonomically higher level were associated with a specific library preparation and sequencing method. In the pig feces, Proteobacteria seemed associated with the HiSeq runs (Figure S5). However, this was not observed for sewage. For sewage, Archaea were associated with the HiSeq runs, but also Eukaryotes consisting of Fungi and *Cryptosporidium* seemed associated with the HiSeq runs in sewage 1 (Figure S5). Overall, it was difficult to observe a pattern when assessing this grouping of genera, highlighting that it might be difficult to generalize the effect of sample processing in different sample types and different samples of the same type.

Discussion

With the increasing amount of metagenomic data in public repositories, meta-analysis and pooling of data from different studies are exciting new opportunities to gain further insight into the microbial world (10–12, 24, 33). Data generation is usually not performed with a standard procedure across studies, and sample processing is an important factor to be aware of when trying to make inferences in these cross-study investigations (21, 26). In the present study, both library preparation and sequencing platform had a significant effect on explaining the variance in the data (Table 1). That these parameters affect the community description has also been observed previously (21, 29, 30). In the study by Costea et al. (26), DNA isolation had the largest effect compared with other technical variations. In the present study, DNA isolation was performed centrally by the same person, while library preparation and sequencing was performed in-house or at external providers, but not in any of the cases by the same person, possibly increasing variation due to DNA shipping and handling in this specific step. When performing a validation study assessing the technical variation of sample processing, the large number of methodologies and variations thereof make it impossible to test all parameters. It is likely that selecting methods that are based on different principles and for specific purposes yield results that highlight the importance of this specific step. Bowers et al. (29) investigated community changes using different amounts of input DNA, and observed that this modification had a significant effect on community description. This effect can increase bias associated with library preparation and sequencing platform in other studies were starting material is of variable quality. In the present study, investigation of sequencing platforms were limited to the NextSeq and HiSeq, which are both Illumina platforms resembling each other in technology, and which were selected due to their popularity in metagenomics with low cost relative to output (34). However, the platforms have been reported to exhibit differences in index hopping (35). In the present study, a large effect was attributed to the sequencing platform and that was also observed when using the same library preparation kit (NEXTflex PCR-Free) (Figure 1B). The library preparation included two methods that required pre-fragmented DNA that was prepared PCR-free (KAPA and NEXTflex). It was decided to include the Illumina Nextera library preparation as well, to compare with a technique that does not resemble the others in having enzymatic

fragmentation, and which involved a PCR step that is commonly applied when not enough DNA is available to prepare DNA for sequencing PCR-free. However, the two Nextera runs were relatively similar compared with the NEXTflex run when sequenced on the NextSeq (Fig. 2). The present study was not a full factorial experiment and this should be emphasized when comparing the effect sizes of specific processing parameters.

One explanation for the differences observed between the processing runs can be contamination bias. When designing a metagenomics study, it is to some extent possible to remove kit contaminations or carry-over between sequencing runs from the data *in-silico*, if for instance, blank controls are included or by rotating indexing primers between adjacent runs, respectively (36). In the present study, comparing the sPLS-DA results with the blank controls rarely identified the same genera, indicating that the genera reported to explain the specific sample processing the most were not due to contamination during DNA extraction. The general variation associated with redoing the library preparation and sequencing was low when comparing the two Nextera sequencing runs (Figures 1 and 2). The differences observed are therefore most likely due to true variation associated with the sample processing. Furthermore, it was possible to detect that these patterns were systematic in the different samples (Figure 2), and that this could be partly explained with some crude features such as distinguishing between Gram-negative and Gram-positive bacteria or at a higher taxonomic classification (Figures 3 and S5). The grouping of genera into Gram-negative and Gram-positive might be confounders of an underlying explanation that could be associated with DNA characteristics such as guanine-cytosine percent (GC%) or other specific DNA patterns. Another possibility is that DNA fragmentation during sampling, storage and DNA isolation provided DNA of different quality for specific organism groups. A shift in community structure is then reflected in the selection of different fragment sizes during the library preparation and sequencing. Practical limitations were also an issue when designing the study. To reduce the bias associated with DNA extraction, the QIAamp Fast DNA Stool Mini Kits were all ordered together ensuring that kits were from the same manufacturing batch. Another possible bias might arise from DNA samples that were frozen in between processing them for sequencing. However, only small changes were observed between the two Nextera NextSeq runs.

The Aitchison distances obtained from comparing within the two pig fecal samples separately relative to within the two sewage samples also revealed that storage, library preparation and sequencing platform has a larger effect in pig feces (Figure 1B). Since the distances between the two pig fecal samples were smaller relative to the distances between the two sewage samples, it was difficult to discern the two pig fecal samples when samples were processed differently (Figure 3). It is concerning that the variation due to sample processing might hamper the ability to differentiate between two different pig fecal samples, and this might obstruct the ability to draw meaningful conclusions when technical variations cannot be distinguished from true changes. These results should on the other hand not be overstated; the two pig fecal samples were obtained from an in-bred race raised under very similar conditions including feeding, even though they were obtained from two different healthy pigs at two different farms, the two communities are relatively similar. The finding highlights that the importance of technical variation depends on the differences that one is trying to detect (16). The technical variation did not hamper the ability to differentiate between the two sewage samples.

We show that library preparation and sequencing platform introduce systematic bias in the metagenomic-based characterization of microbial communities. These findings suggest that library preparation and sequencing are important parameters to keep consistent when aiming to detect small changes in community structure. In the present study, the bias was somewhat dependent on sample type, highlighting the importance of assessing the effect of sample processing in the specific sample type under investigation.

Materials and Methods

Sample processing

A subset of 85 DNA samples was selected from a large-scale study examining the effect samples storage conditions on inferred microbiome composition (31). The DNA samples originated from two pig fecal samples (Pig feces 1 and Pig feces 2) and two sewage samples (Sewage 1 and Sewage 2). The two pig fecal

samples were collected on different occasions from different conventional pig production farms near the laboratory. The two sewage samples were collected at a local wastewater treatment facility on different occasions. DNA isolation was performed in duplicate with a modified QIAamp Fast DNA Stool Mini Kit (Qiagen) protocol, including an initial bead beating step (MoBio garnet beads) (27) (Figure 1B). A DNA extraction (blank) control was included at each time of DNA isolation (5 controls as part of the present study). For a detailed list of all samples included in this study, see Table S1 (column H, 'Other study (Library preparation)') in Poulsen *et al.* 2021 (31). The concentration of DNA samples was measured with the Qubit dsDNA High Sensitivity (HS) assay kit on a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA) before storing the DNA at -20°C.

Library preparation and sequencing

Library preparation and sequencing were performed in the order described below and the DNA was frozen between the sequencing runs:

KAPA PCR-free on a HiSeq (KAPA HiSeq). DNA was shipped for sequencing to an external provider (Admera Health, New Jersey, USA). The DNA (500 ng) was fragmented mechanically (Covaris E220 evolution, aimed insert size=350bp) using ultrasonication. The KAPA library preparation was run PCR-free according to the manufacturer's recommendations (KAPA Hyper Prep Kit KR0961 – v6.17). Sequencing was performed on an Illumina HiSeq 4000 (2x150 cycles, paired end).

NEXTflex PCR-free on a HiSeq (NEXTflex HiSeq). DNA was shipped for sequencing to an external provider (Oklahoma Medical Research Foundation, Oklahoma, USA). The DNA (500 ng) was fragmented mechanically (Covaris E220 evolution, aimed insert size=350bp) using ultrasonication. The NEXTflex library preparation was run PCR-free according to the manufacturer's recommendations (Bioo Scientific NEXTflex PCR Free DNA Sequencing Kit – 5142-01). Sequencing was performed on an Illumina HiSeq 4000 (2x150 cycles, paired end).

NEXTflex PCR-free on a NextSeq (NEXTflex NextSeq). The DNA (500 ng) was fragmented with mechanical fragmentation (Covaris E210, aimed insert size=350bp, duty cvd=10%, Intensity=5, cycle

burst=200, treatment time=240 sec.) using ultrasonication. The NEXTflex library preparation was run PCR-free with Nextflex barcodes (NEXTflex-96 DNA barcodes) according to the manufacturer's recommendations (Bioo Scientific NEXTflex PCR-Free DNA Sequencing Kit – 5142-01). Sequencing was performed in-house on an Illumina NextSeq 500 (mid output v2, 2x150 cycles, paired end).

Nextera 1 and 2 on a NextSeq (Nextera 1 NextSeq, Nextera 2 NextSeq). The Nextera XT library preparation was performed twice. The Nextera XT protocol was carried out according to the manufacturer's recommendations (Nextera XT DNA Library Prep Kit 15031942v02). This included a tagmentation step that fragments the DNA (1 ng) and ligates adaptors, and a PCR step amplifying DNA and adding indexing primers. Library cleanup was performed with AMPure XP beads and normalized before sequencing was performed in-house on an Illumina NextSeq 500 (mid output v2, 2x150 cycles, paired end). The bioanalyzer results revealed that the aimed insert size of 350 bp was larger than expected (File S6).

Bioinformatics and statistical analysis

Pre-processing of raw reads included trimming (Phred quality score = 20) and removal of reads shorter than 50bp (BBduk2) (37). Mapping was performed with a Burrows-Wheeler aligner (BWA-mem) as implemented in MGmapper (22). Mapping was performed in the default "best mode" to 11 databases; first filtering against the human database then extracting the number of raw reads mapping to the genomes of bacteria, fungi, archaea, viruses, and Cryptosporidium. A read count correction was implemented to adjust large hit counts to specific contigs as implemented in Hendriksen et al. (9). All counts in the count table were divided by two to account for reads mapping as proper pairs, and then aggregating to genus level. The processed count table, metadata and feature data are available (File S7), and the raw reads are deposited in the European Nucleotide Archive (ENA) (Project acc.: PRJEB31650).

All statistical analyses adhered to the compositional data analysis framework and were performed in R version 3.5.2 (38–40). Alpha diversity was calculated based on the raw count table estimating richness (Chao1), evenness (Pielou's) and diversity (Simpson) using the diversity function in vegan. Initial filtering of

the count matrix was performed by removing all genera below an average count of 5. The estimation of zeroes was performed using simple multiplicative replacement (41). Centered log-ratio transformation (CLR) was used in: principal components analysis (PCA), heatmaps to perform complete-linkage clustering analysis of the samples, boxplots to calculate pairwise Euclidean distances between samples (Aitchison distance), and permutational multivariate analysis of variance (PERMANOVA, with 99999 permutations assessing the marginal effects of the terms), sparse partial-least-squares discriminant analysis (sPLS-DA, with 5 fold cross validation repeated 10 times), and constrained ordination with redundancy analysis (rda) (38, 40, 42, 43). Spearman correlation on CLR transformed data were used to cluster the genera visualized in the heatmap, but the genera abundance depicted in the cells were standardized to zero mean and unit variance after CLR transformation for comparability. Analyses performed are included (File S8) and the code is available from https://github.com/csapou/LibraryPreparationandSequencingPlatform.

Data availability

The raw reads supporting the conclusions of this article are available at the European Nucleotide Archive (ENA) (Project acc.: PRJEB31650). The processed count data, metadata and feature data are accessible in the supplemental material as File S7, and the statistical analysis performed are provided in the supplemental material in File S8. The statistical analysis is furthermore available at https://github.com/csapou/LibraryPreparationandSequencingPlatform.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

FA acquired the funding for the project. CP, FA and SP conceived and designed the experiments. CP performed the experiments, performed the literature review and wrote the paper. CP and CE analysed the data. FA, CE and SP revised the manuscript. All authors read and approved the manuscript.

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Figure legends

Fig. 1. Study design & Comparison between sample groups. A) Two pig feces samples and two sewage samples were processed directly or after storage at -80 °C for 64 hours. The DNA isolation was performed in duplicates, respectively. Library preparation and sequencing was performed in four different combinations: NEXTflex PCR-free library preparation + sequencing on a HiSeq (NEXTflex HiSeq), KAPA PCR-free library preparation + sequencing on a HiSeq (KAPA HiSeq), NEXTflex PCR-free library preparation + sequencing on a NextSeq (NEXTflex NextSeq), and Nextera library preparation + sequencing on a NextSeq (Nextera NextSeq). The latter sequencing strategy was performed twice (Nextera 1 NextSeq, and Nextera 2 NextSeq). The setup resulted in a total of 80 metagenomes plus five negative controls (i.e. DNA extraction controls). B) The boxplots displays pairwise Aitchison distances between different groupings of samples. Within the different groups, dots representing the distances were colored according to which sample the comparison was made in. Blue dots represent a distance between two different samples.

Fig. 2. Principal component analysis (PCA) subset to the different sample matrices. Euclidean distances were calculated after performing centered log-ratio transformation (CLR) of the count data (Aitchison distances). Variance explained by the two first axes are included in their labels. The same DNA samples processed differently are connected with dotted lines.

Fig. 3. Heatmaps of pig feces and sewage samples separately with the 30 most abundant genera. Complete-linkage clustering was performed to create dendograms for both genera and samples. Spearman correlation was used to cluster the genera and Aitchison distances were used to cluster the samples. Genera abundance depicted in the cells were CLR transformed counts standardized to zero mean and unit variance. Grouping of organisms were included in genera names according to cell wall structure based on Gram positive staining (G+), Gram negative staining (G-) or belonging to Archaea (Ar). (A) Heatmap of all pig feces samples, where the first branching was according to sequencing platform. The third cluster of genera

exclusively contained Gram–negatives. (B) Heatmap of all sewage samples. The fourth cluster mainly consisted of Gram–positives. A few Gram–positives were also present in the other clusters.

Supplemental Material

Table S1. Sequencing quality control and alpha-diversity overview.

Figure S1. Rarefaction curves.

Figure S2. Prinicipal component analysis (PCA) for all samples and subsetted to pig feces and sewage. Variance explained by the two first axes are included in their labels. (A) PCA were generated for all samples forming three clusters with pig feces together and sewage 1 and sewage 2 seperately, (B) PCA were generated for all pig feces samples separation were now observed between pig feces 1 and pig feces 2, and (C) PCA were generated for all sewage samples that were still easy to discriminate.

Figure S3. Sparse partial least square discriminant analysis (sPLS-DA). The sPLS-DA were run with a unique identifier for a specific DNA sample that were then processed differently in the generation of libraries and sequencing to select the most discriminative genera in explaining this aspect of sample processing.

Figure S4. Heatmap of negative controls with the thirty most abundant genera. Complete-linkage clustering was performed to create dendograms for both genera and samples. Spearman correlation was used to cluster the genera and Aitchison distances were used to cluster the samples. Genera abundance depicted in the cells were CLR transformed counts standardized to zero mean and unit variance. Grouping of organisms were included in genera names according to cell wall structure based on Gram-positive staining (G+), Gram-negative staining (G-) or belonging to Archaea (Ar).

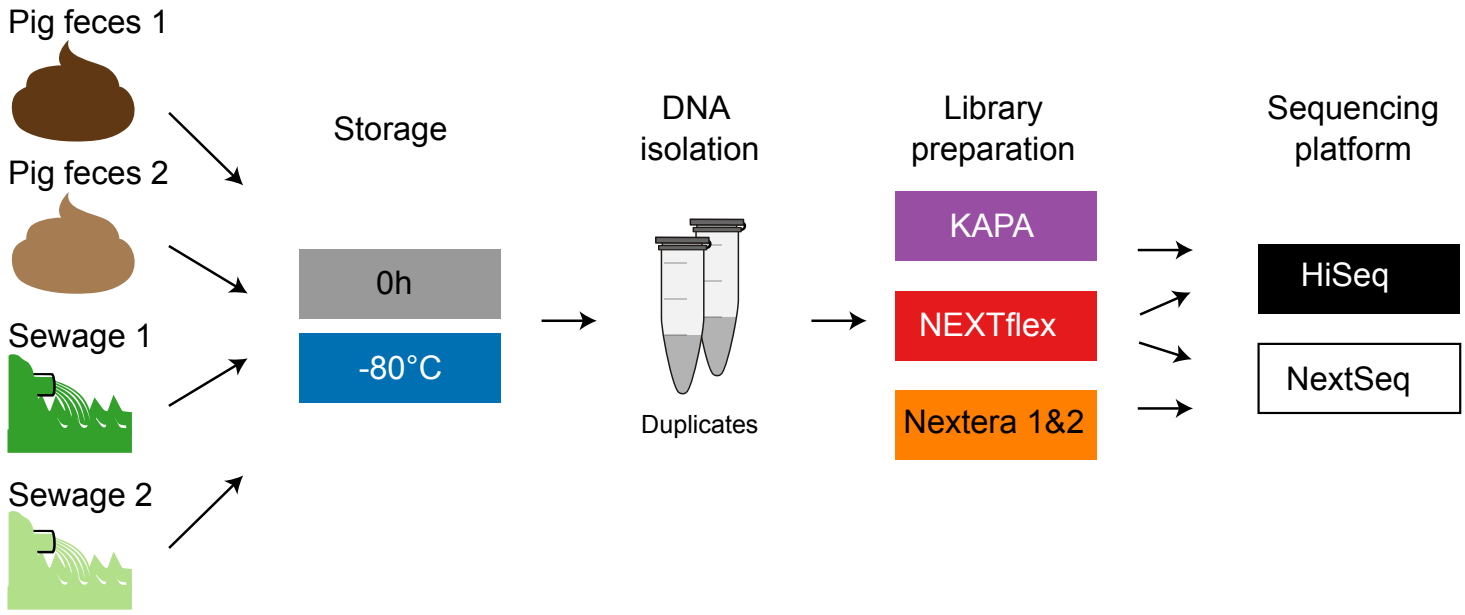
Figure S5. Redundancy analysis (rda) subsetted to sample matrix and if samples were frozen or processed directly. Taxonomic patterns were investigated by plotting genera coloured according to different taxonomic groups. DNA isolation replicates were connected with lines.

File S6. Bioanalyzer electropherograms.

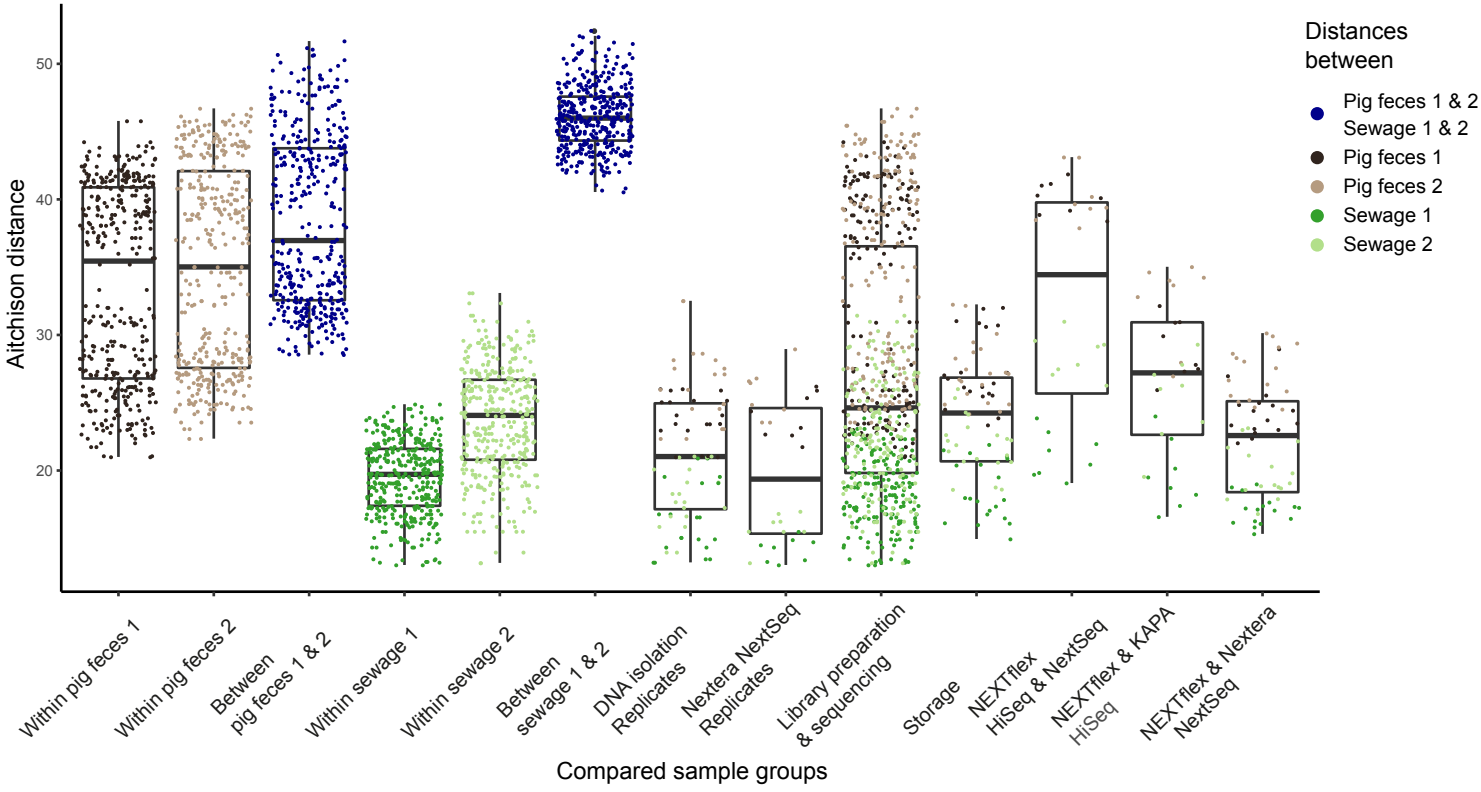
File S7. Count table, metadata and feature data used in the analysis.

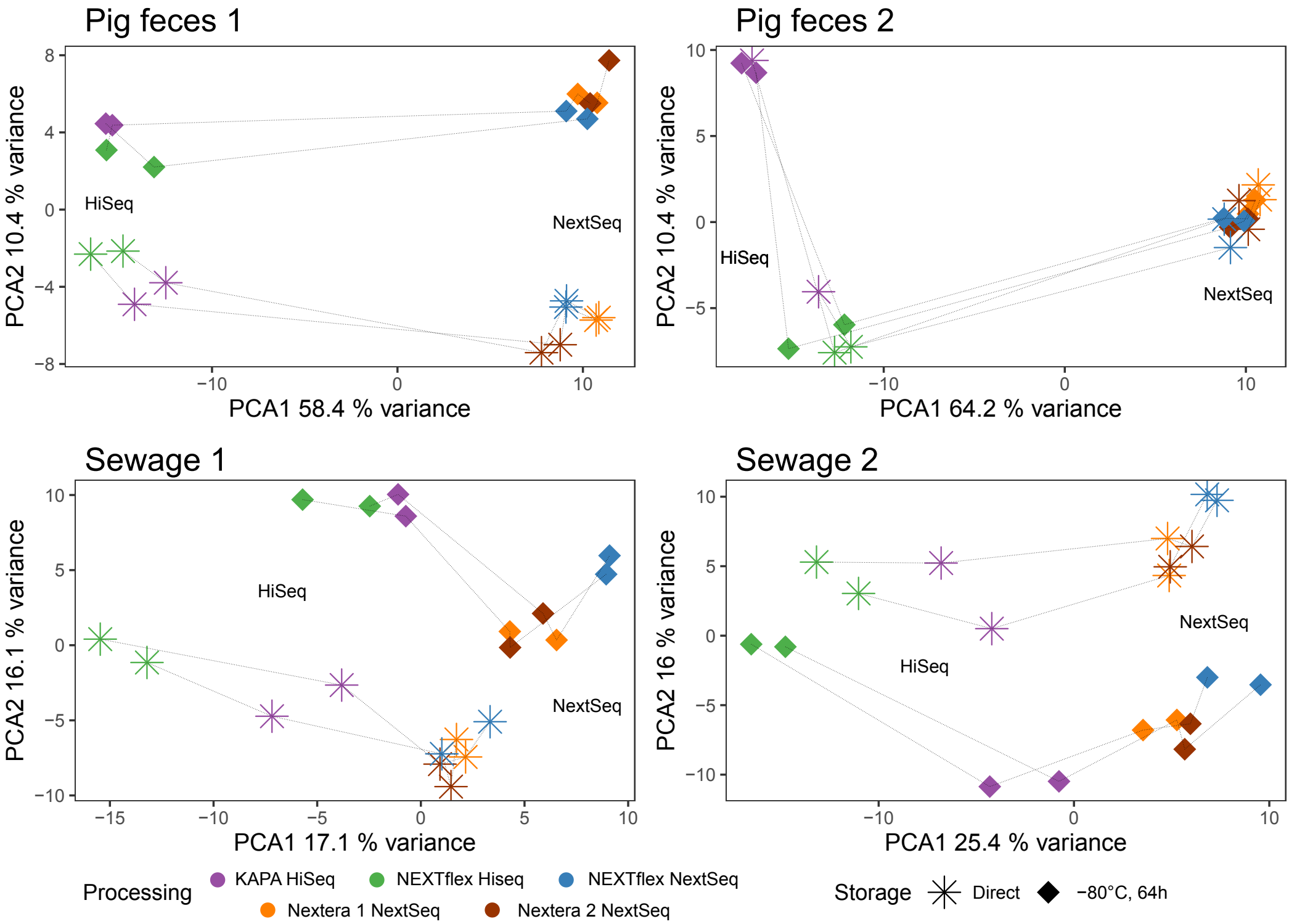
File S8. R markdown file containing the R analysis.

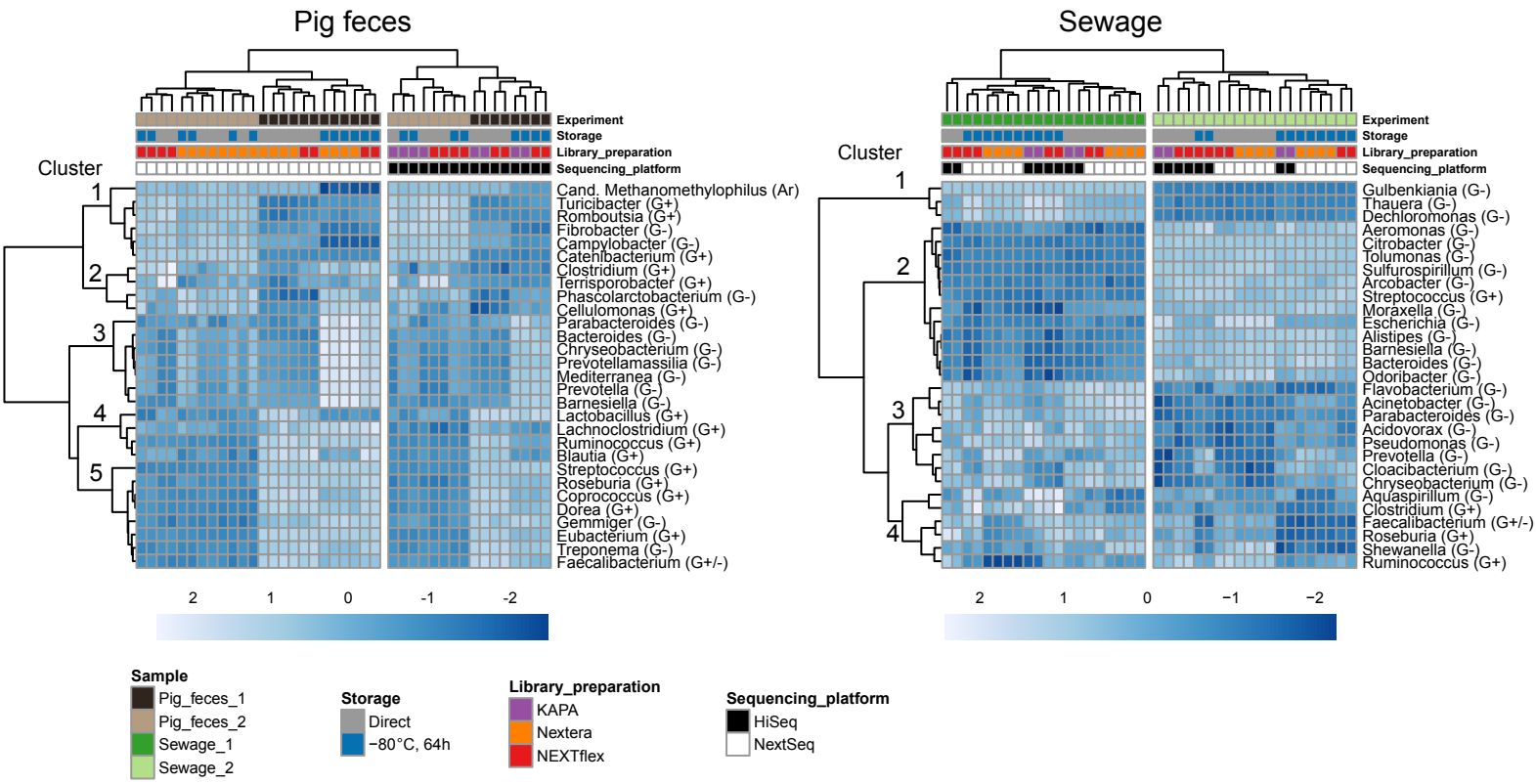
A)

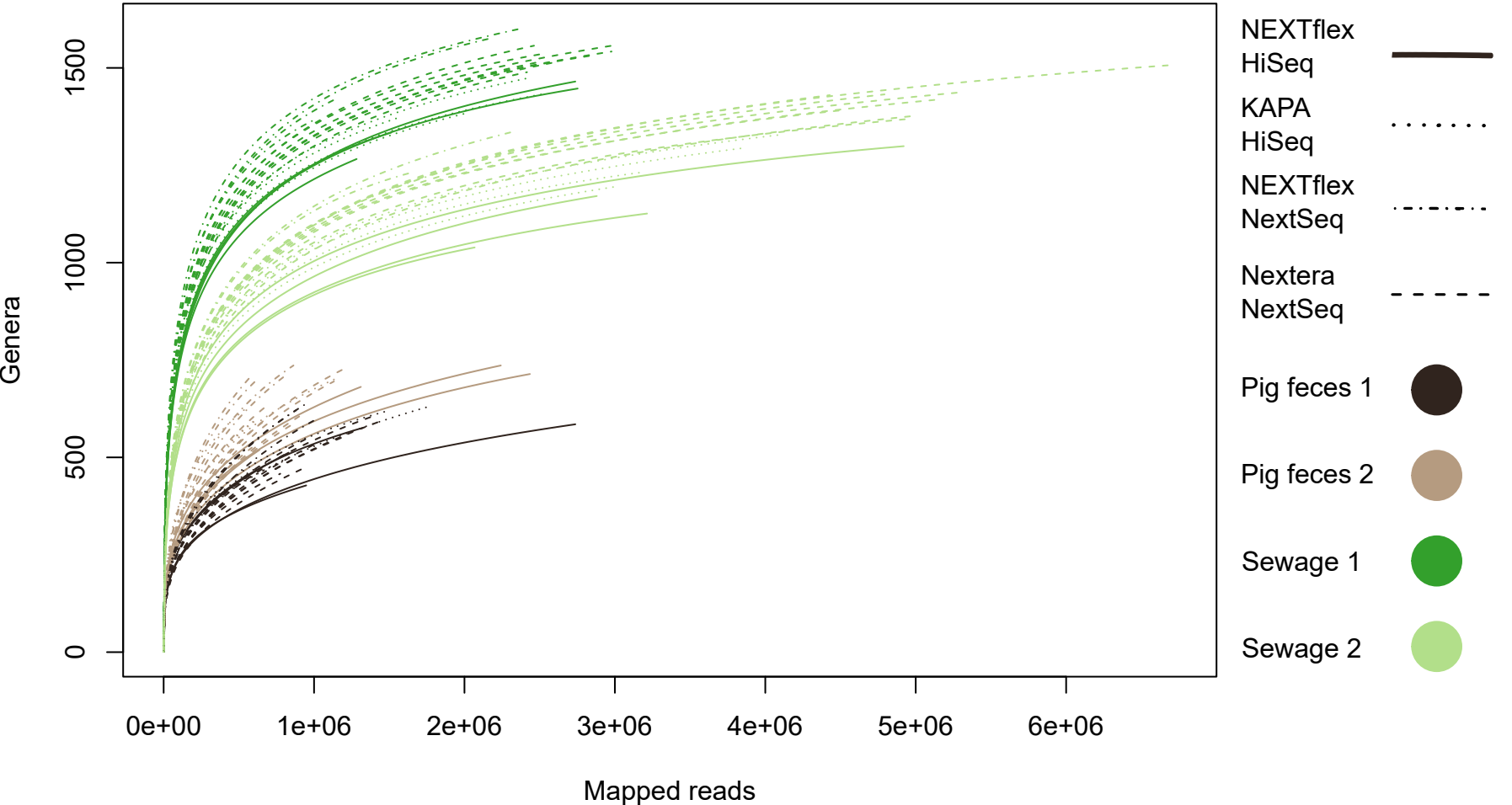

Pig feces 1
Pig feces 2
Sewage 1
Sewage 2
Storage
0h
-80°C
DNA isolation
Duplicates
Library preparation
KAPA
NEXTflex
Nextera 1&2
Sequencing platform
HiSeq
NextSeq

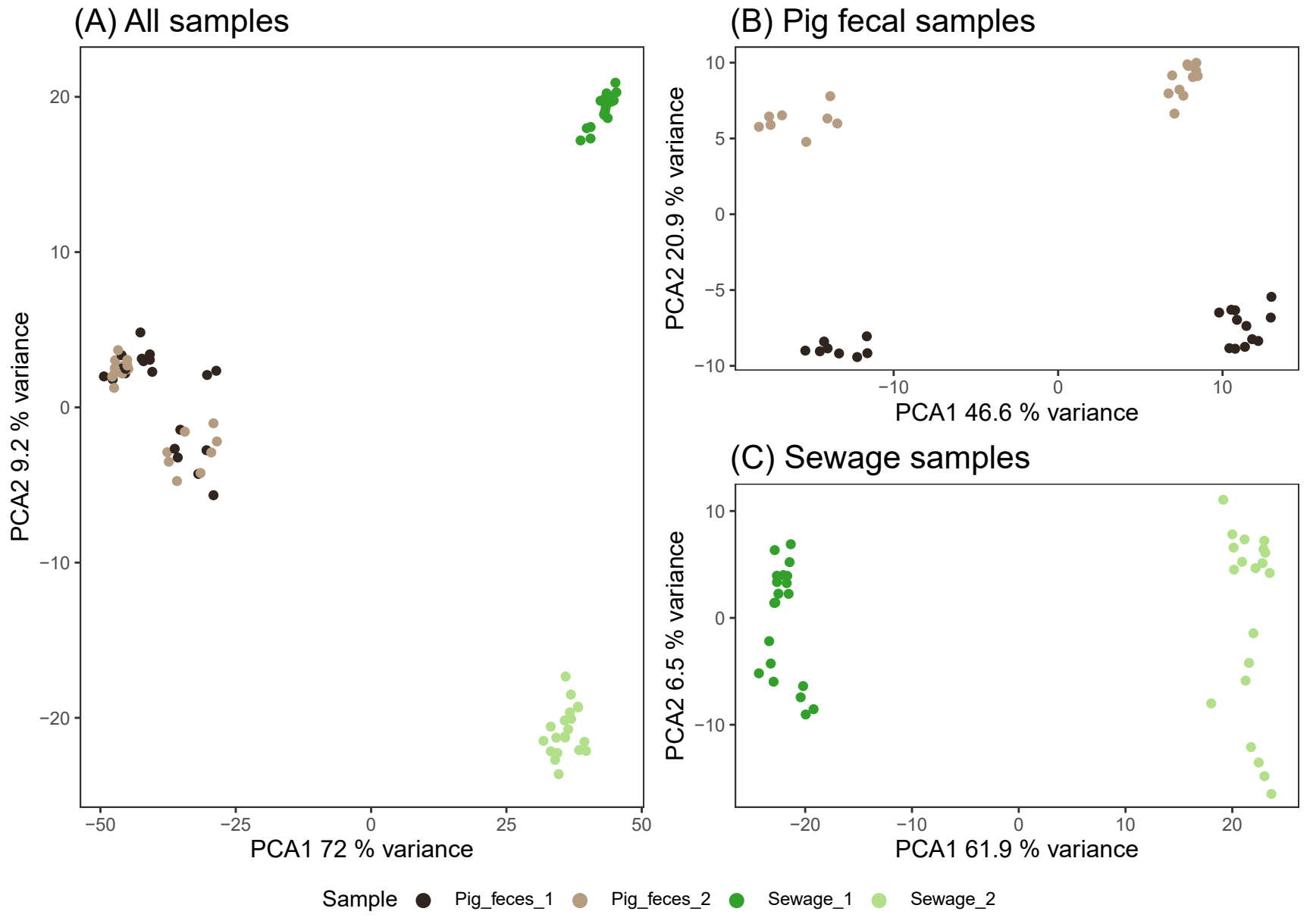
B)

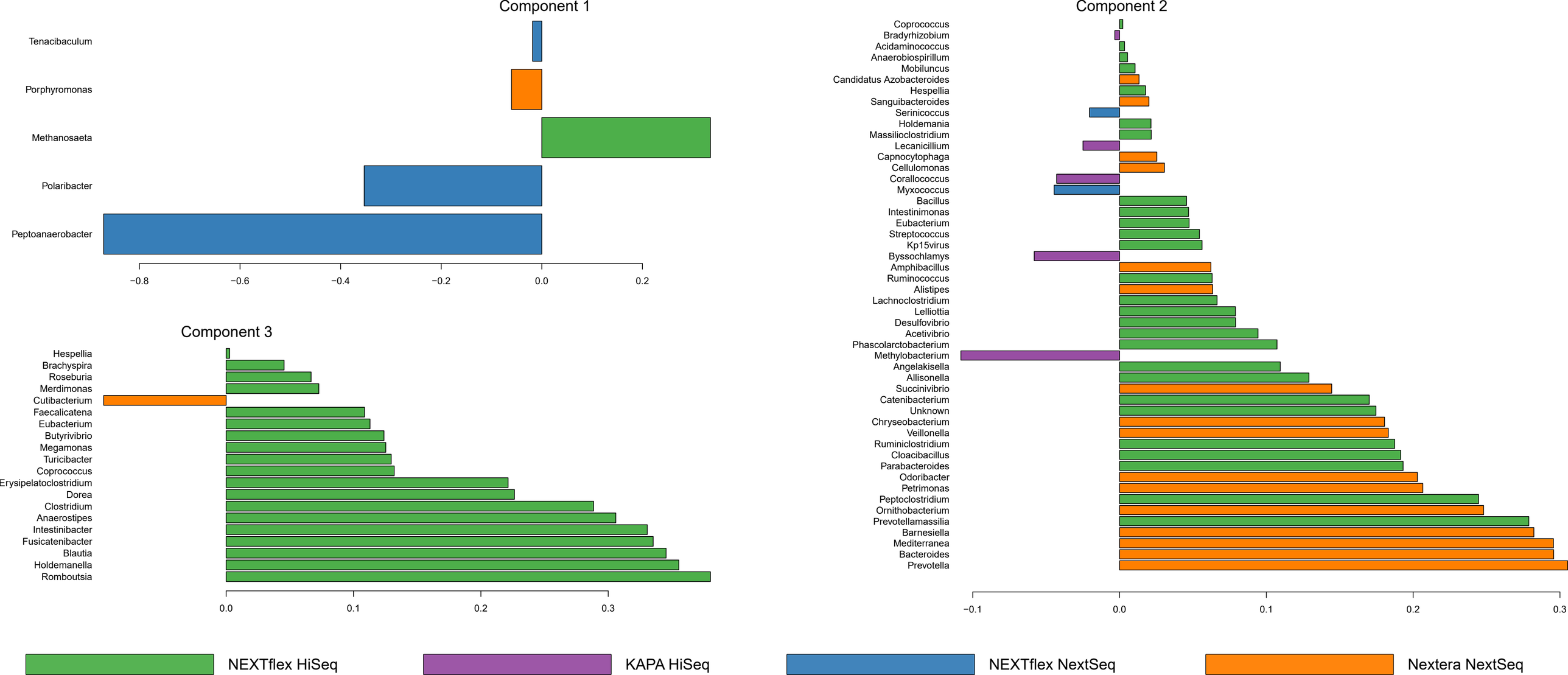

Aitchison distance
50
40
30
20
Distances between
Pig feces 1 & 2
Sewage 1 & 2
Pig feces 1
Pig feces 2
Sewage 1
Sewage 2
Within pig feces 1
Within pig feces 2
Between pig feces 1 & 2
Within sewage 1
Within sewage 2
Between sewage 1 & 2
DNA isolation Replicates
Nextera NextSeq Replicates
Library preparation & sequencing
Storage
NEXTflex HiSeq & NextSeq
NEXTflex & KAPA HiSeq
NEXTflex & Nextera NextSeq
Compared sample groups

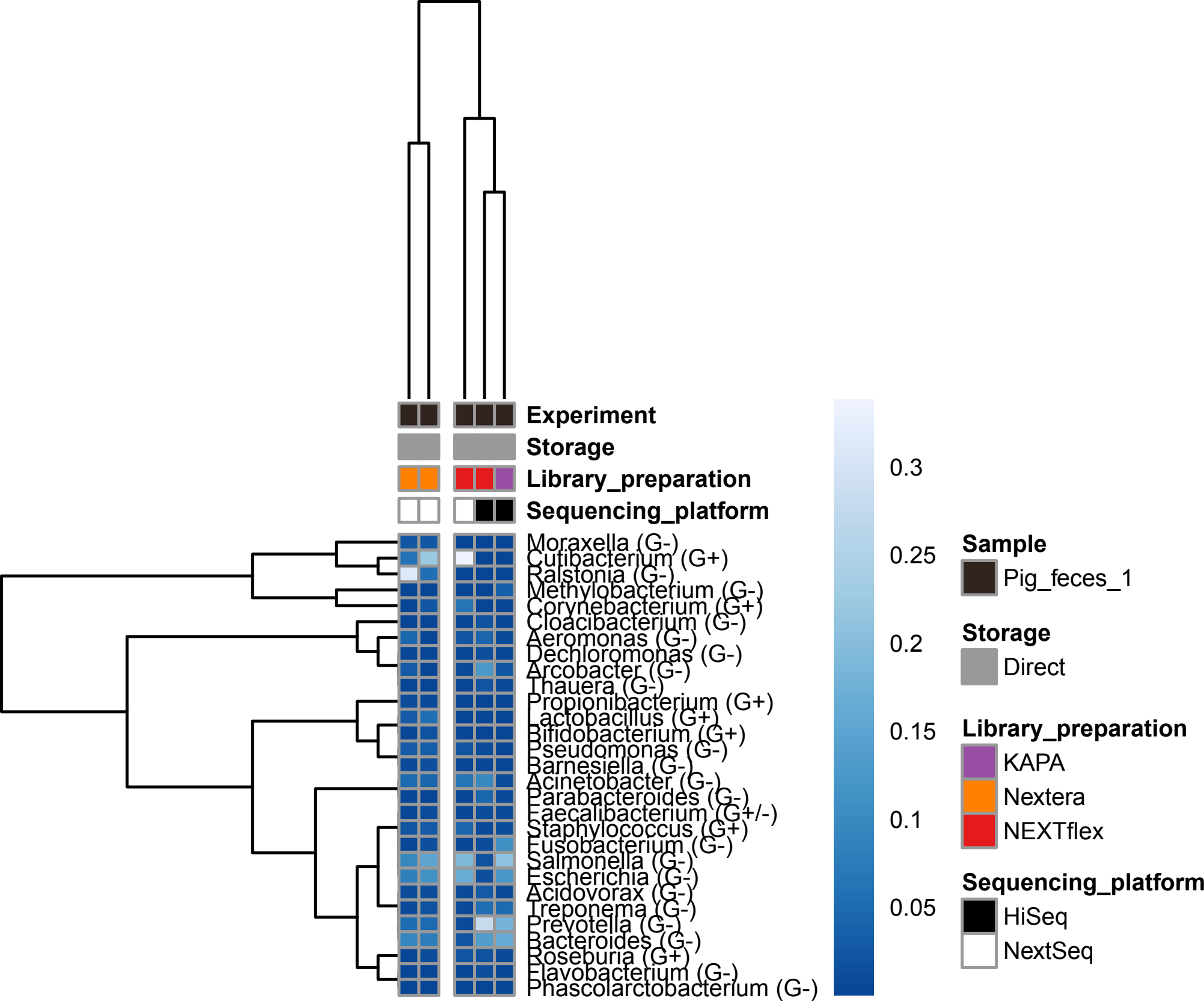

Pig feces 1
PCA2 10.4 % variance
PCA1 58.4 % variance
HiSeq
NextSeq
Pig feces 2
PCA2 10.4 % variance
PCA1 64.2 % variance
HiSeq
NextSeq
Sewage 1
PCA2 16.1 % variance
PCA1 17.1 % variance
HiSeq
NextSeq
Sewage 2
PCA2 16 % variance
PCA1 25.4 % variance
NextSeq
HiSeq
Processing
KAPA HiSeq
NEXTflex Hiseq
NEXTflex NextSeq
Nextera 1 NextSeq
Nextera 2 NextSeq
Storage
Direct
−80°C, 64h


Pig feces
Sewage
Cluster
1
2
3
4
5
Experiment
Storage
Library_preparation
Sequencing_platform
Cand. Methanomethylophilus (Ar)
Turicibacter (G+)
Romboutsia (G+)
Fibrobacter (G-)
Campylobacter (G-)
Catenibacterium (G+)
Clostridium (G+)
Terrisporobacter (G+)
Phascolarctobacterium (G-)
Cellulomonas (G+)
Parabacteroides (G-)
Bacteroides (G-)
Chryseobacterium (G-)
Prevotellamassilia (G-)
Mediterranea (G-)
Prevotella (G-)
Barnesiella (G-)
Lactobacillus (G+)
Lachnoclostridium (G+)
Ruminococcus (G+)
Blautia (G+)
Streptococcus (G+)
Roseburia (G+)
Coprococcus (G+)
Dorea (G+)
Gemmiger (G-)
Eubacterium (G+)
Treponema (G-)
Faecalibacterium (G+/-)
2
1
0
-1
-2
Gulbenkiania (G-)
Thauera (G-)
Dechloromonas (G-)
Aeromonas (G-)
Citrobacter (G-)
Tolumonas (G-)
Sulfurospirillum (G-)
Arcobacter (G-)
Streptococcus (G+)
Moraxella (G-)
Escherichia (G-)
Alistipes (G-)
Barnesiella (G-)
Bacteroides (G-)
Odoribacter (G-)
Flavobacterium (G-)
Acinetobacter (G-)
Parabacteroides (G-)
Acidovorax (G-)
Pseudomonas (G-)
Prevotella (G-)
Cloacibacterium (G-)
Chryseobacterium (G-)
Aquaspirillum (G-)
Clostridium (G+)
Faecalibacterium (G+/-)
Roseburia (G+)
Shewanella (G-)
Ruminococcus (G+)
2
1
0
−1
−2
Sample
Pig_feces_1
Pig_feces_2
Sewage_1
Sewage_2
Storage
Direct
−80°C, 64h
Library_preparation
KAPA
Nextera
NEXTflex
Sequencing_platform
HiSeq
NextSeq

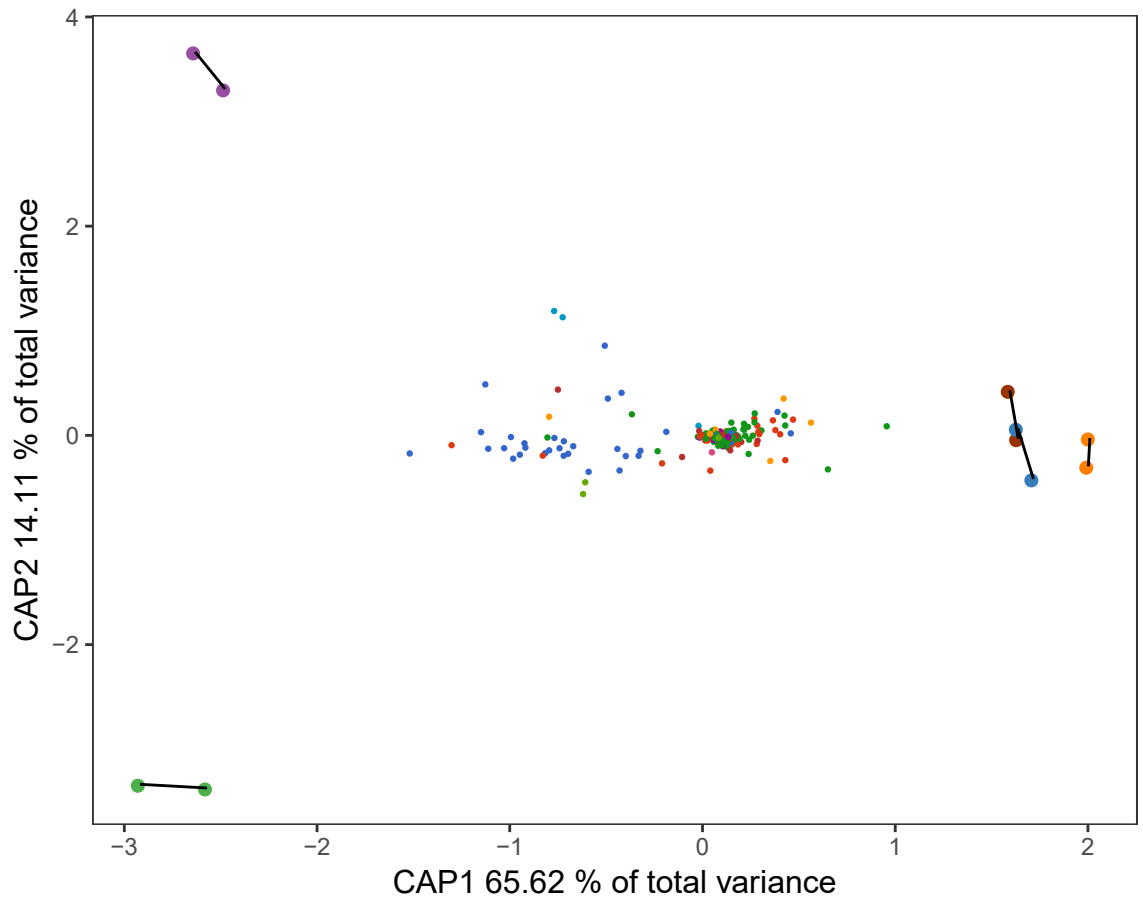

Genera
0
500
1000
1500
0e+00
1e+06
2e+06
3e+06
4e+06
5e+06
6e+06
Mapped reads
NEXTflex HiSeq
KAPA HiSeq
NEXTflex NextSeq
Nextera NextSeq
Pig feces 1
Pig feces 2
Sewage 1
Sewage 2


(A) All samples
PCA2 9.2 % variance
PCA1 72 % variance
20
10
0
−10
−20
−50
−25
0
25
50
(B) Pig fecal samples
PCA2 20.9 % variance
PCA1 46.6 % variance
10
5
0
−5
−10
−10
0
10
(C) Sewage samples
PCA2 6.5 % variance
PCA1 61.9 % variance
10
0
−10
−20
−10
0
10
20
Sample
Pig_feces_1
Pig_feces_2
Sewage_1
Sewage_2

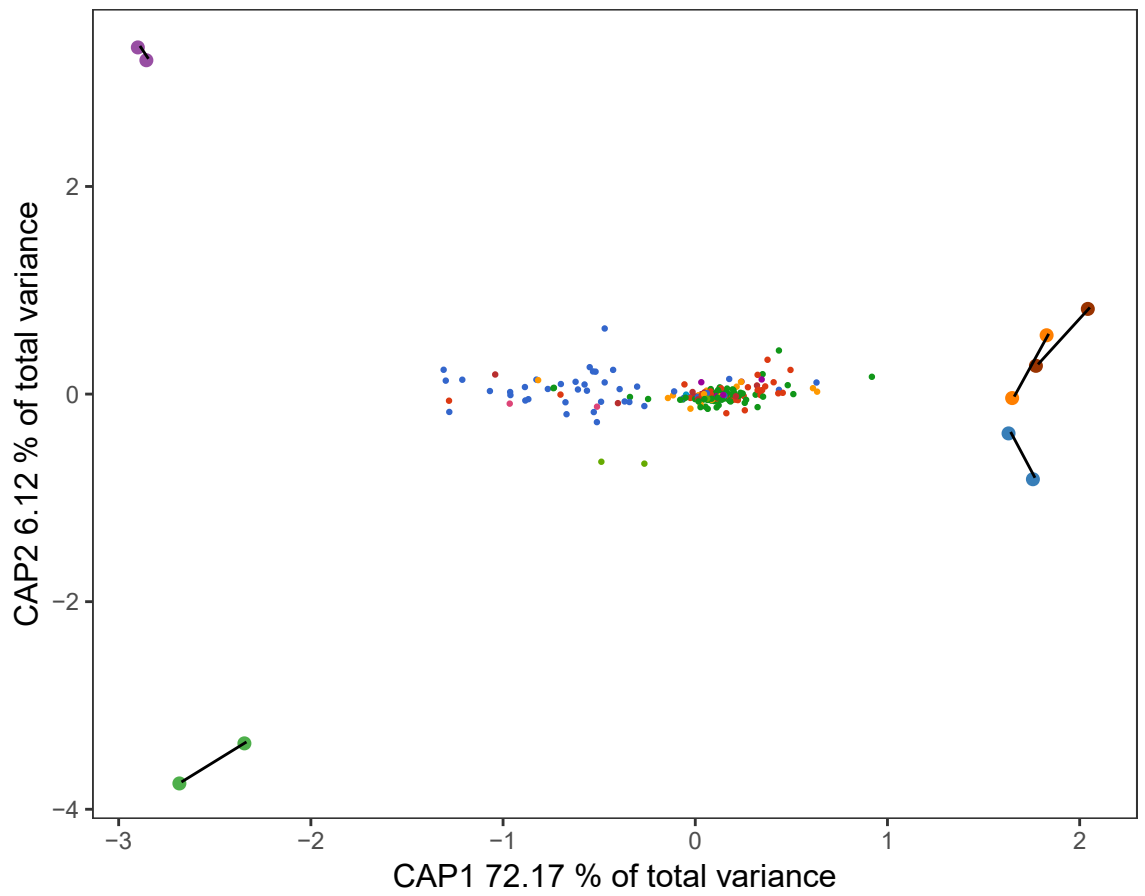

Component 1
Tenacibaculum
Porphyromonas
Methanosaeta
Polaribacter
Peptoanaerobacter
−0.8
−0.6
−0.4
−0.2
0.0
0.2
Component 2
Coprococcus
Bradyrhizobium
Acidaminococcus
Anaerobiospirillum
Mobiluncus
Candidatus Azobacteroides
Hespellia
Sanguibacteroides
Serinicoccus
Holdemania
Massilioclostridium
Lecanicillium
Capnocytophaga
Cellulomonas
Corallococcus
Myxococcus
Bacillus
Intestinimonas
Eubacterium
Streptococcus
Kp15virus
Byssochlamys
Amphibacillus
Ruminococcus
Alistipes
Lachnoclostridium
Lelliottia
Desulfovibrio
Acetivibrio
Phascolarctobacterium
Methylobacterium
Angelakisella
Allisonella
Succinivibrio
Catenibacterium
Unknown
Chryseobacterium
Veillonella
Ruminiclostridium
Cloacibacillus
Parabacteroides
Odoribacter
Petrimonas
Peptoclostridium
Ornithobacterium
Prevotellamassilia
Barnesiella
Mediterranea
Bacteroides
Prevotella
−0.1
0.0
0.1
0.2
0.3
Component 3
Hespellia
Brachyspira
Roseburia
Merdimonas
Cutibacterium
Faecalicatena
Eubacterium
Butyrivibrio
Megamonas
Turicibacter
Coprococcus
Erysipelatoclostridium
Dorea
Clostridium
Anaerostipes
Intestinibacter
Fusicatenibacter
Blautia
Holdemanella
Romboutsia
0.0
0.1
0.2
0.3
NEXTflex HiSeq
KAPA HiSeq
NEXTflex NextSeq
Nextera NextSeq


Experiment
Storage
Library_preparation
Sequencing_platform
Moraxella (G-)
Cutibacterium (G+)
Ralstonia (G-)
Methylobacterium (G-)
Corynebacterium (G+)
Cloacibacterium (G-)
Aeromonas (G-)
Dechloromonas (G-)
Arcobacter (G-)
Thauera (G-)
Propionibacterium (G+)
Lactobacillus (G+)
Bifidobacterium (G+)
Pseudomonas (G-)
Barnesiella (G-)
Acinetobacter (G-)
Parabacteroides (G-)
Faecalibacterium (G+/-)
Staphylococcus (G+)
Fusobacterium (G-)
Salmonella (G-)
Escherichia (G-)
Acidovorax (G-)
Treponema (G-)
Prevotella (G-)
Bacteroides (G-)
Roseburia (G+)
Flavobacterium (G-)
Phascolarctobacterium (G-)
0.3
0.25
0.2
0.15
0.1
0.05
Sample
Pig_feces_1
Storage
Direct
Library_preparation
KAPA
Nextera
NEXTflex
Sequencing_platform
HiSeq
NextSeq

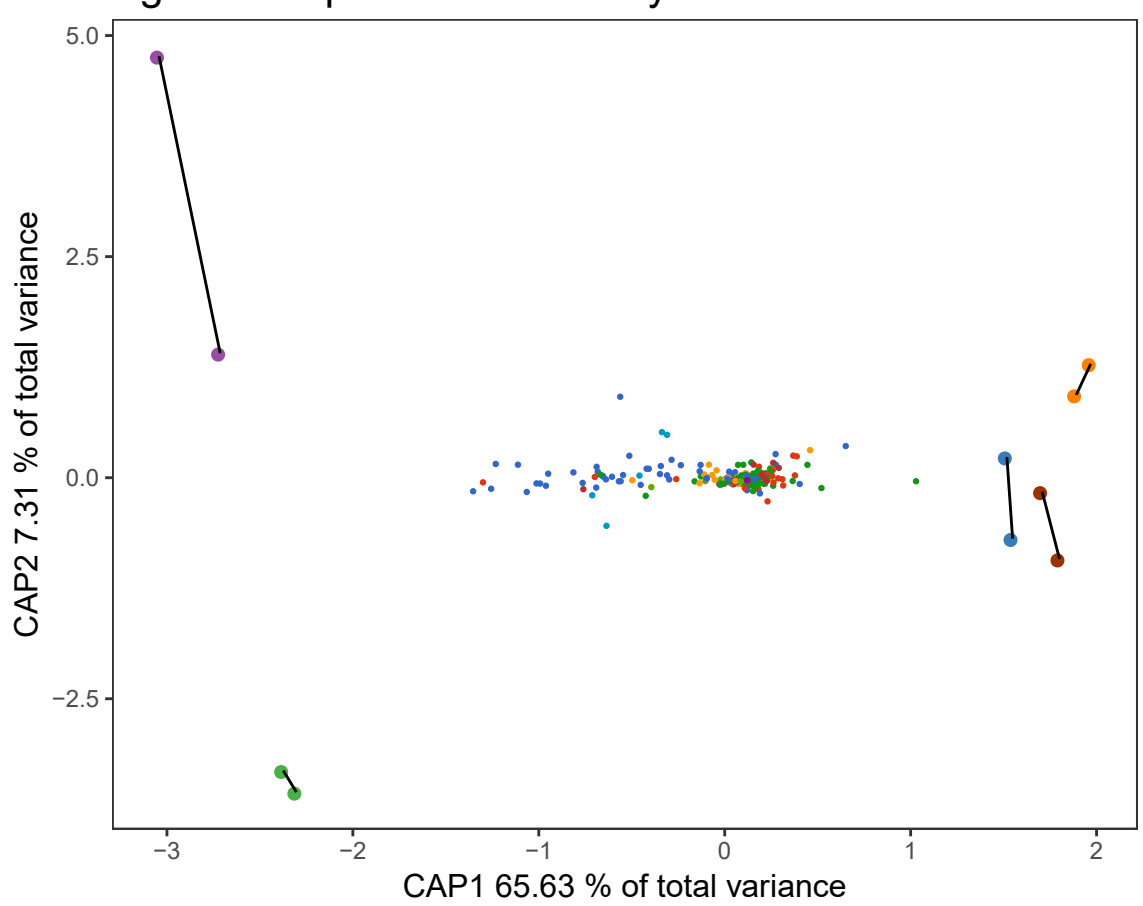
Pig feces 1 processed directly



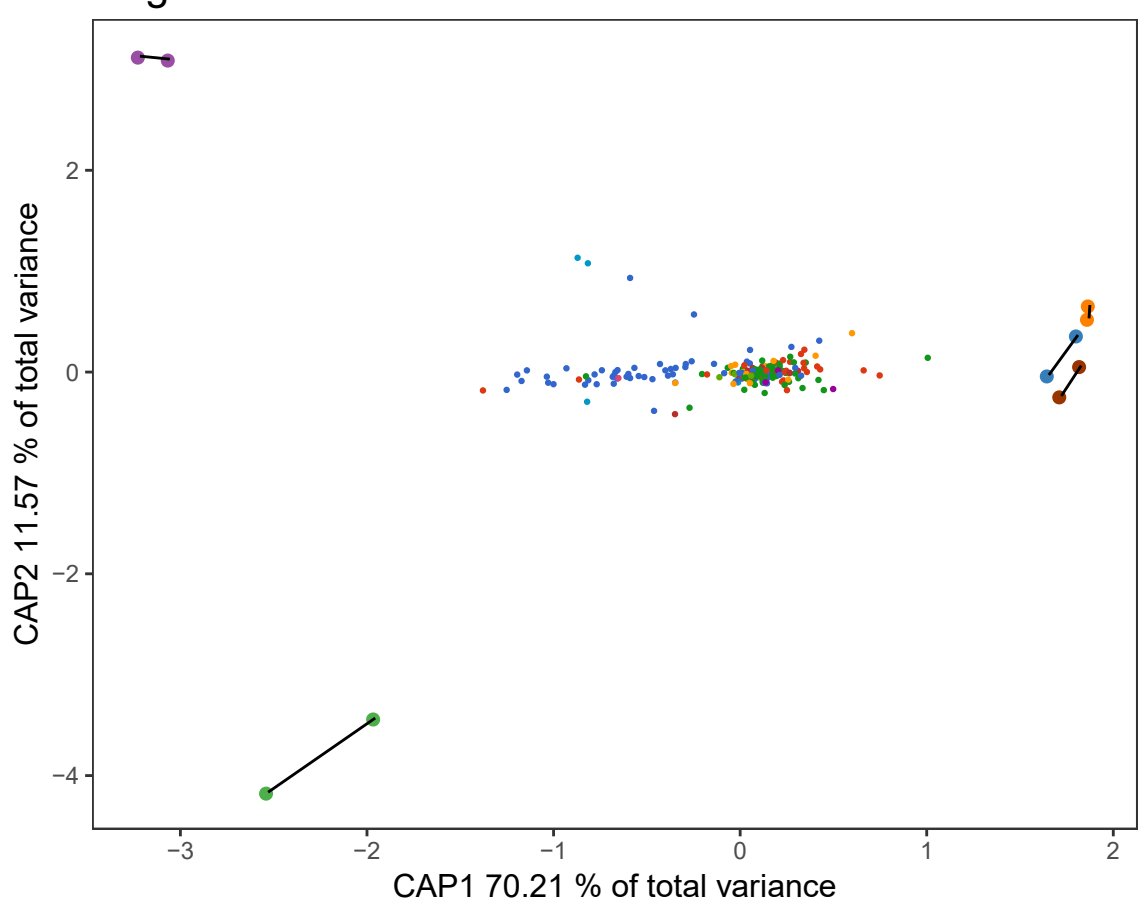
Pig feces 1 frozen



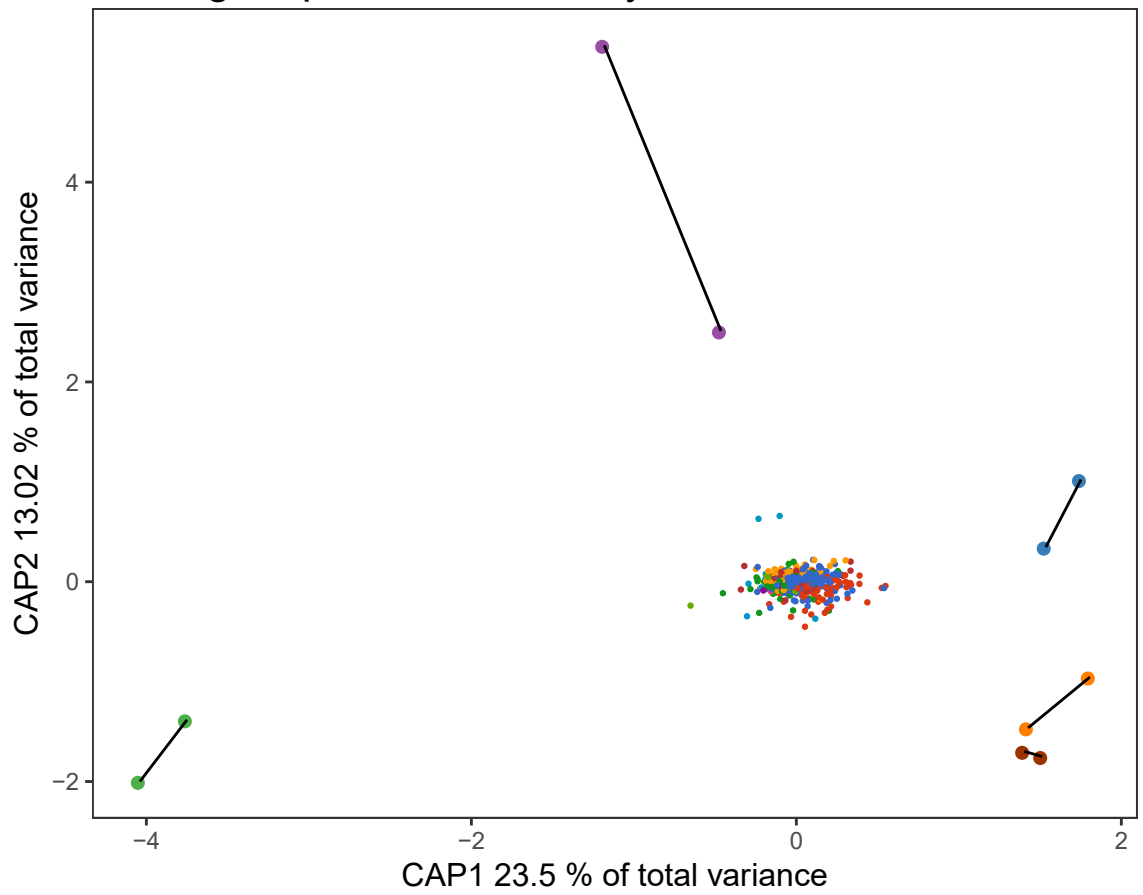
Pig feces 2 processed directly



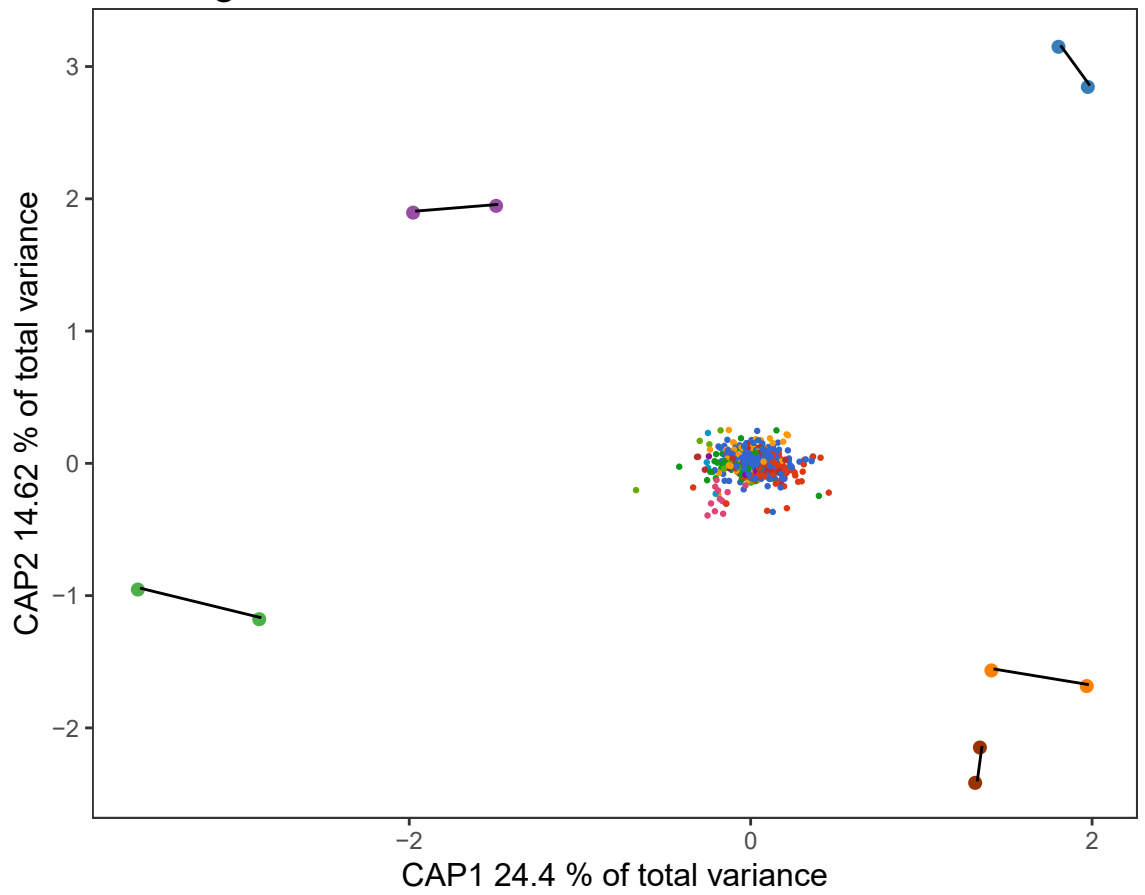
Pig feces 2 frozen



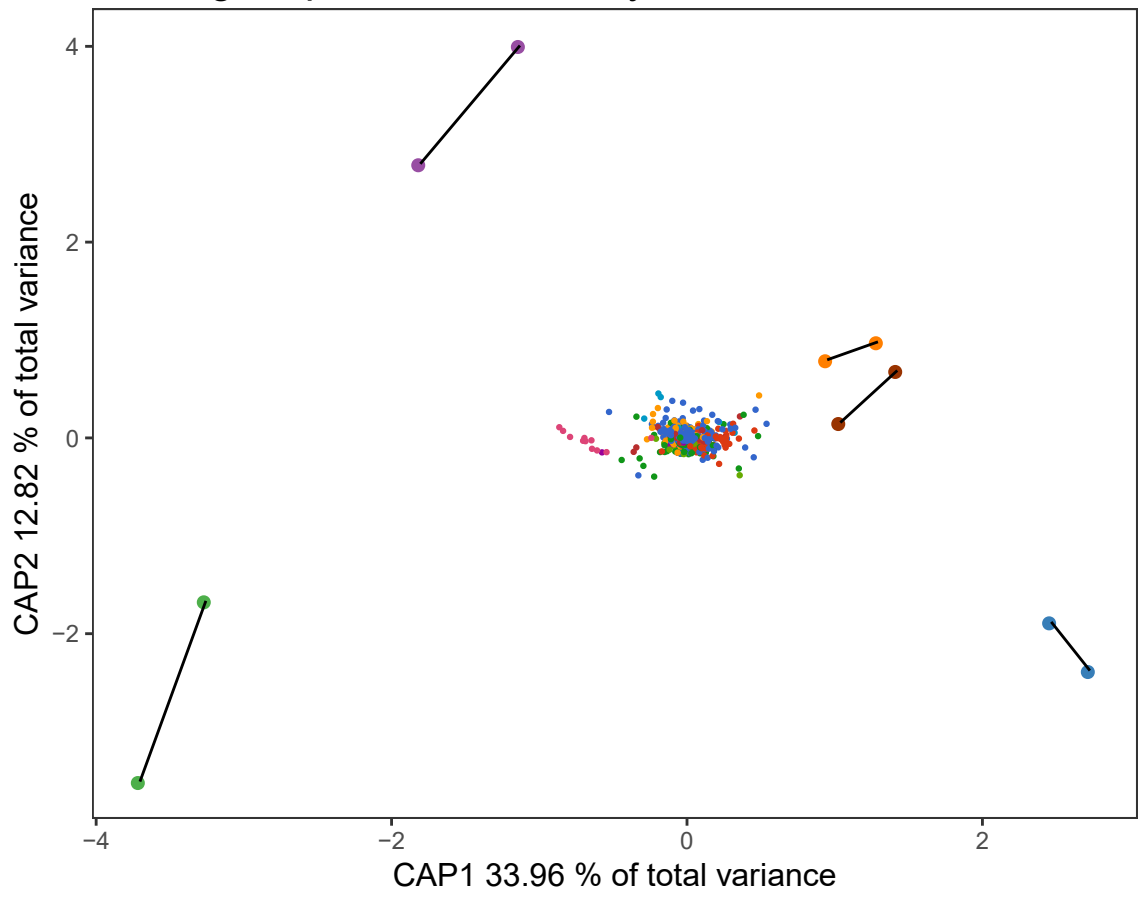
Sewage 1 processed directly



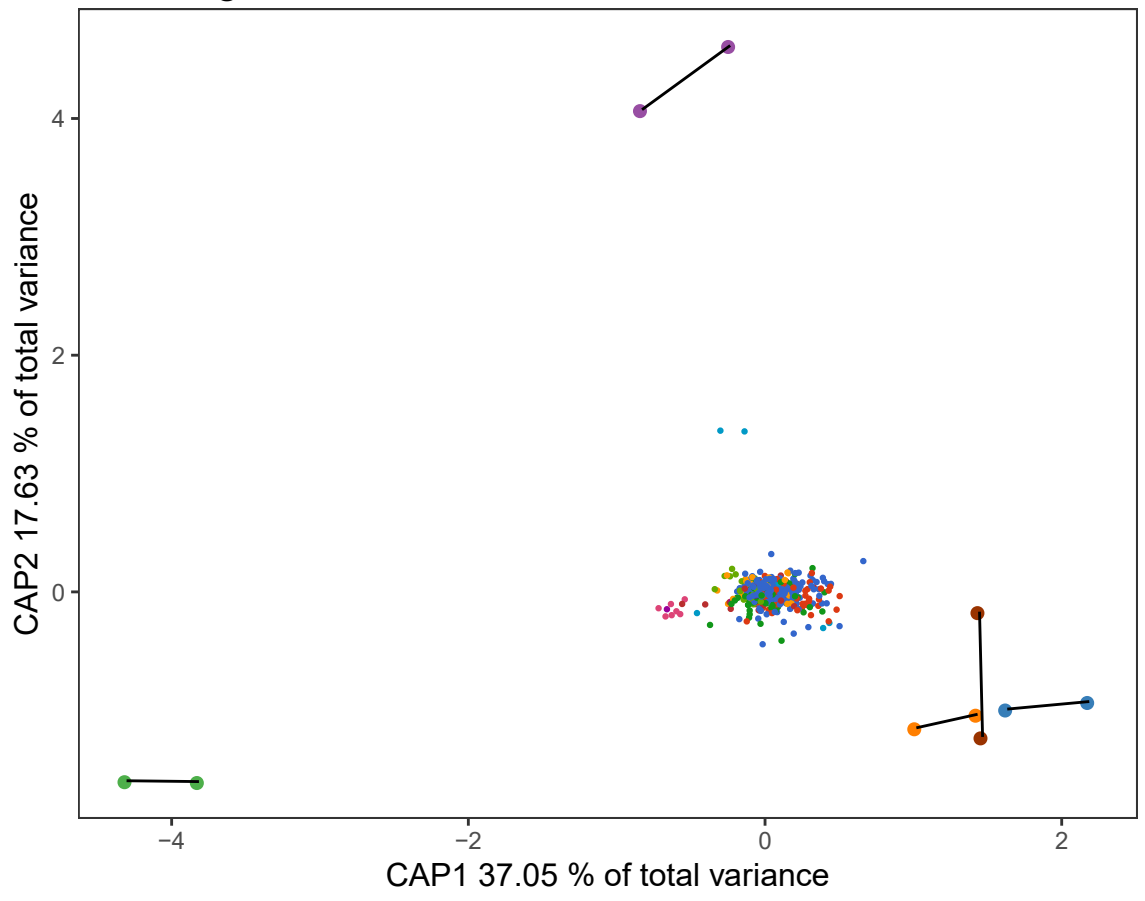
Sewage 1 frozen



Sewage 2 processed directly



Sewage 2 frozen



Processing: KAPA HiSeq, NEXTflex Hiseq, NEXTflex NextSeq, Nextera 1 NextSeq, Nextera 2 NextSeq

Organisms: Proteobacteria, Actinobacteria, Firmicutes(Negativicutes), Archaea, Other, Bacteroidetes, Firmicutes, Eukaryota, Viruses

Assay Class: High Sensitivity DNA Assay
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Modified: 22-08-2018 13:09:44

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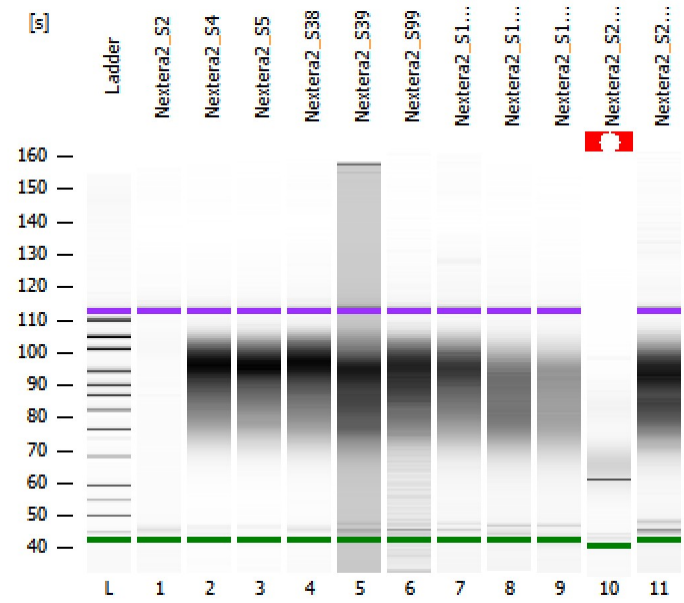

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Serial#:	DE24802594	Type:	G2938B

Assay Information:

Assay Origin Path:	C:\Program Files (x86)\Agilent\2100 bioanalyzer\2100 expert\assays\dsDNA\High Sensitivity DNA.xsy
Assay Class:	High Sensitivity DNA Assay
Version:	1.03
Assay Comments:	Copyright © 2003-2010 Agilent Technologies

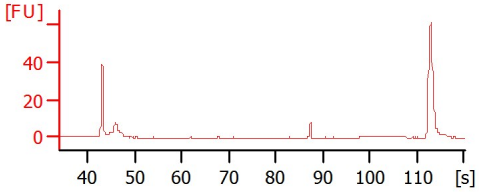
Chip Information:

Chip Lot #:

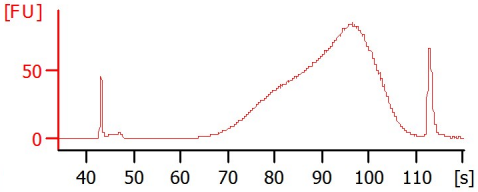
Reagent Kit Lot #:

Chip Comments:

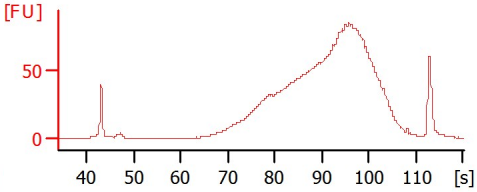
Nextera2_S2



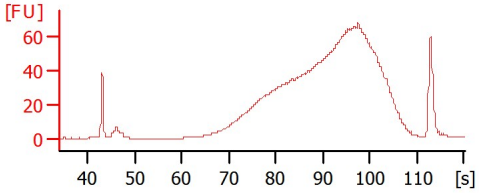
Nextera2_S4



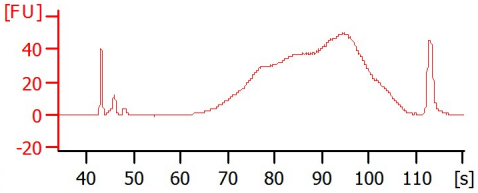
Nextera2_S5



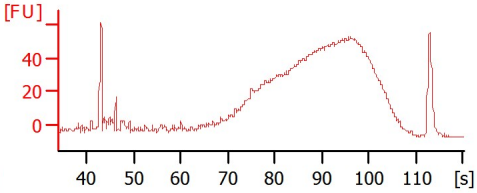
Nextera2_S38



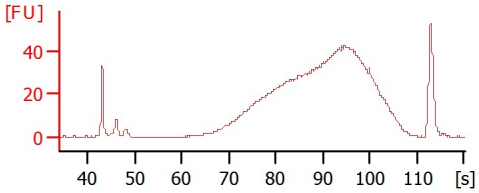
Nextera2_S39



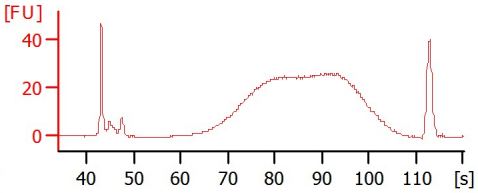
Nextera2_S99



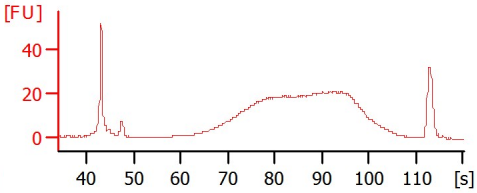
Nextera2_S101



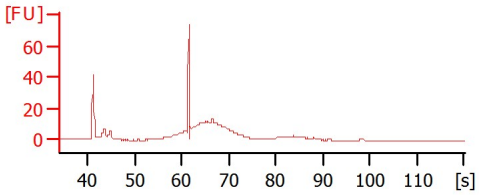
Nextera2_S160



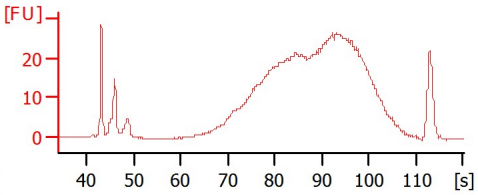
Nextera2_S161



Nextera2_S222



Nextera2_S223



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Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electrophoresis File Run Summary (Chip Summary)

Sample Name	Sample Comment	Rest. Digest	Status	Observation	Result Label	Result Color
Nextera2_S2		☐	✓			
Nextera2_S4		☐	✓			
Nextera2_S5		☐	✓			
Nextera2_S38		☐	✓			
Nextera2_S39		☐	✓			
Nextera2_S99		☐	✓			
Nextera2_S101		☐	✓			
Nextera2_S160		☐	✓			
Nextera2_S161		☐	✓			
Nextera2_S222		☐	✓			
Nextera2_S223		☐	✓			
Ladder		☐	✓			

Chip Lot # **Reagent Kit Lot #**

Chip Comments :

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Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electrophoresis Assay Details

General Analysis Settings

Number of Available Sample and Ladder Wells (Max.) : 12
Minimum Visible Range [s] : 32
Maximum Visible Range [s] : 138
Start Analysis Time Range [s] : 33
End Analysis Time Range [s] : 137,5
Ladder Concentration [pg/µl] : 1950
Uses Standard Area for Ladder Fragments
Lower Marker Concentration [pg/µl] : 125
Upper Marker Concentration [pg/µl] : 75
Used Upper Marker for Quantitation
Standard Curve Fit is Point to Point
Show Data Aligned to Lower and Upper Marker

Integrator Settings

Integration Start Time [s] : 33,05
Integration End Time [s] : 137
Slope Threshold : 0,8
Height Threshold [FU] : 5
Area Threshold : 0,1
Width Threshold [s] : 0,6
Baseline Plateau [s] : 0,5

Filter Settings

Filter Width [s] : 0,5
Polynomial Order : 4

Ladder

Ladder Peak	Size	Area
1	35	160
2	50	210
3	100	208
4	150	221
5	200	242
6	300	270
7	400	305
8	500	306
9	600	336
10	700	321
11	1000	366
12	2000	413
13	3000	411
14	7000	400
15	10380	214

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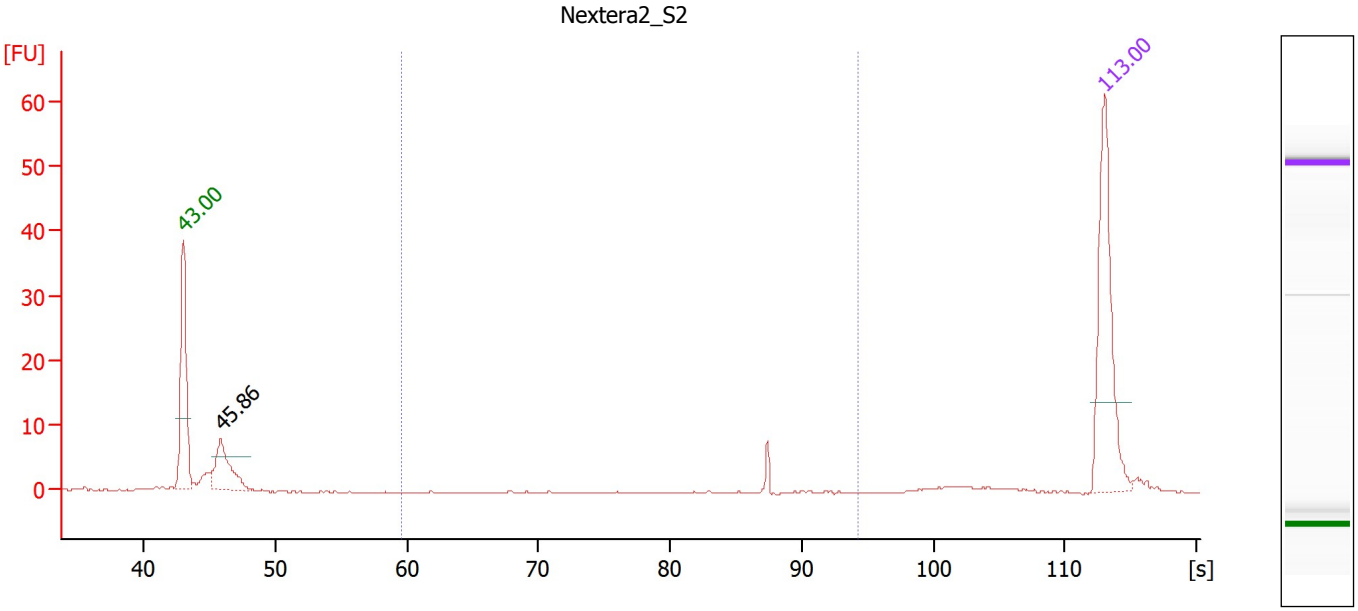
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary



Overall Results for sample 1 : Nextera2_S2

Number of peaks found:	1	Corr. Area 1:	2,5
Noise:	0,1		

Peak table for sample 1 : Nextera2_S2

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125,00	5.411,3	Lower Marker
2		56	51,43	1.379,9	
3	▶	10.380	75,00	10,9	Upper Marker

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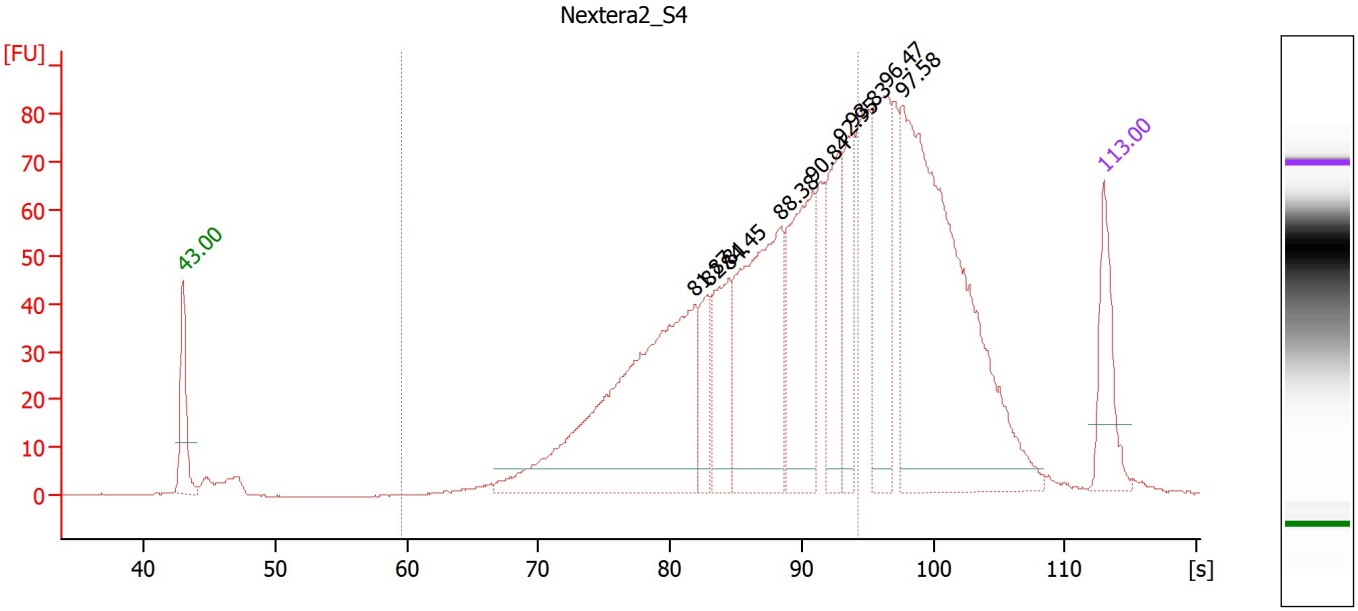
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary Continued ...



Overall Results for sample 2 : Nextera2_S4

Number of peaks found:	9	Corr. Area 1:	1.103,5
Noise:	0,1		

Peak table for sample 2 : Nextera2_S4

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125,00	5.411,3	Lower Marker
2		490	562,74	1.741,0	
3		507	67,48	201,5	
4		544	117,72	327,8	
5		646	332,01	778,7	
6		757	219,14	438,8	
7		908	131,62	219,7	
8		971	86,95	135,7	
9		1.324	159,46	182,5	
10		1.486	575,07	586,5	
11	▶	10.380	75,00	10,9	Upper Marker

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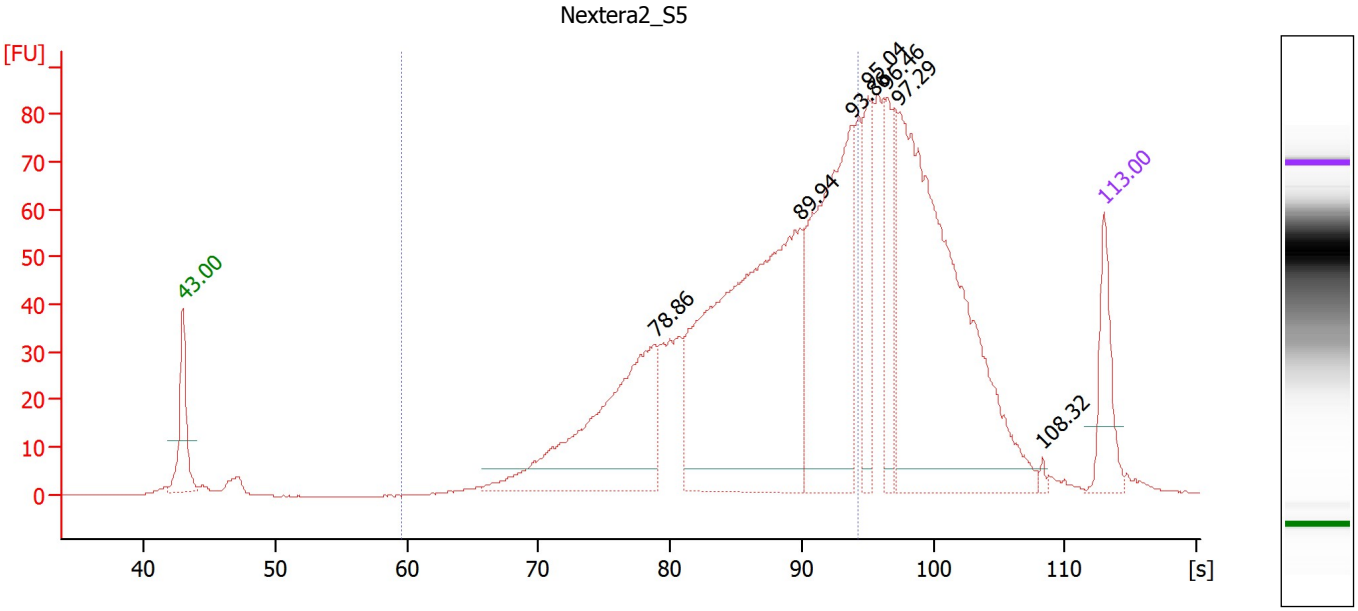
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary Continued ...



Overall Results for sample 3 : Nextera2_S5

Number of peaks found:	7	Corr. Area 1:	1.042,1
Noise:	0,1		

Peak table for sample 3 : Nextera2_S5

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125,00	5.411,3	Lower Marker
2		439	437,12	1.509,3	
3		697	841,87	1.831,2	
4		973	429,48	669,1	
5		1.117	106,56	144,6	
6		1.323	102,76	117,7	
7		1.444	677,90	711,4	
8		5.745	5,51	1,5	
9	▶	10.380	75,00	10,9	Upper Marker

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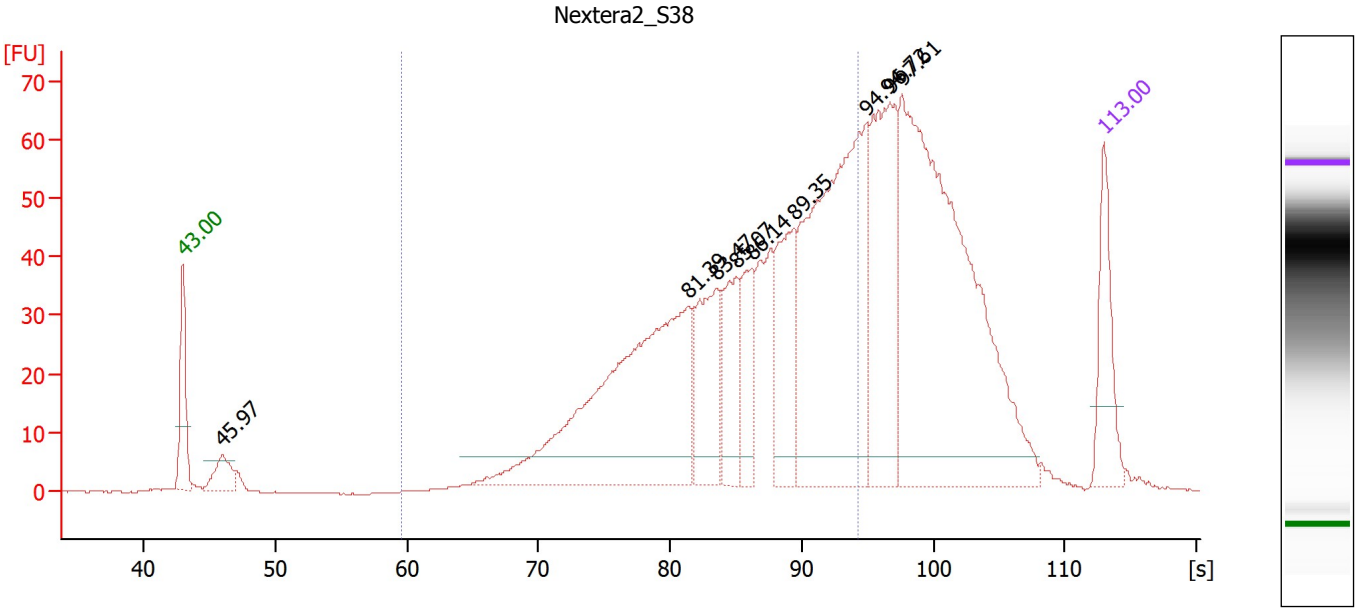
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary Continued ...



Overall Results for sample 4 : Nextera2_S38

Number of peaks found:	9	Corr. Area 1:	898,8
Noise:	0,1		

Peak table for sample 4 : Nextera2_S38

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125,00	5.411,3	Lower Marker
2		58	48,53	1.277,2	
3		482	530,43	1.668,8	
4		522	139,82	405,7	
5		558	101,30	275,0	
6		582	77,36	201,4	
7		677	137,82	308,3	
8		1.101	462,75	636,6	
9		1.360	209,87	233,8	
10		1.490	604,37	614,7	
11	▶	10.380	75,00	10,9	Upper Marker

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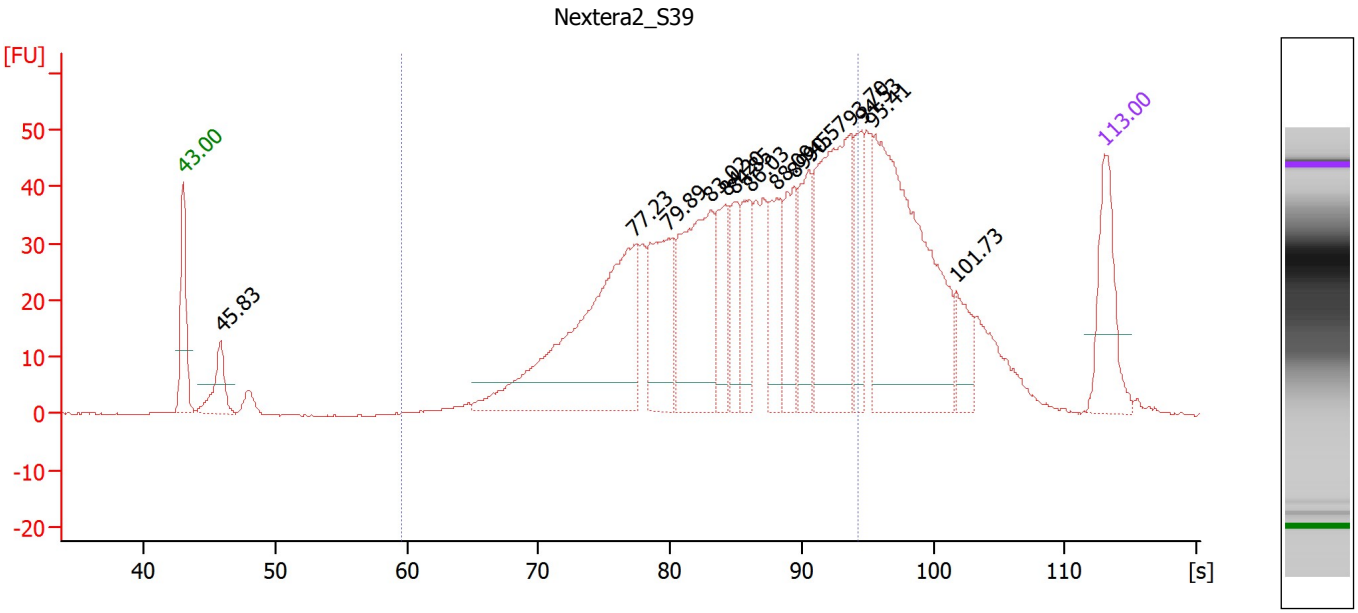
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary Continued ...



Overall Results for sample 5 : Nextera2_S39

Number of peaks found:	14	Corr. Area 1:	1.105,1
Noise:	0,1		

Peak table for sample 5 : Nextera2_S39

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125,00	5.411,3	Lower Marker
2		56	63,68	1.715,2	
3		411	375,50	1.383,3	
4		456	128,98	428,4	
5		512	209,31	619,3	
6		538	68,66	193,2	
7		553	55,87	153,1	
8		579	62,63	163,8	
9		637	73,90	175,8	
10		681	76,93	171,2	
11		737	84,68	174,0	
12		961	223,27	351,8	
13		1.042	59,15	86,0	
14		1.170	335,70	434,6	
15		2.162	33,77	23,7	
16	▶	10.380	75,00	10,9	Upper Marker

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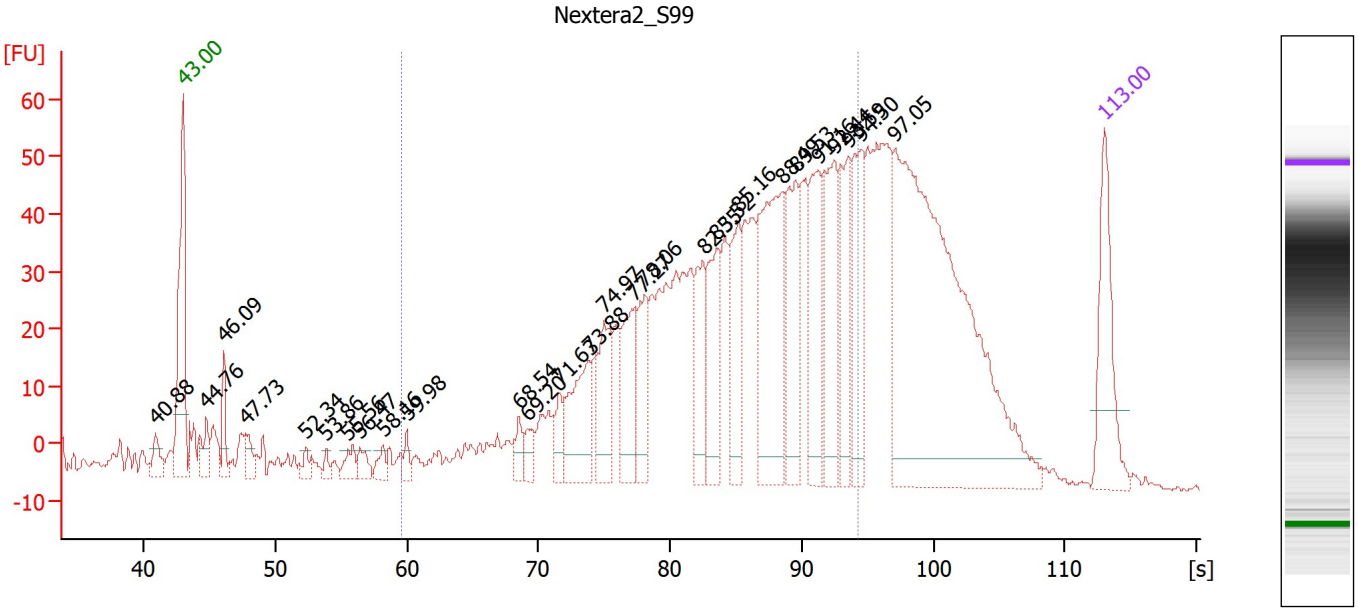
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary Continued ...



Overall Results for sample 6 : Nextera2_S99

Number of peaks found:	27	Corr. Area 1:	1.048,4
Noise:	0,2		

Peak table for sample 6 : Nextera2_S99

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	28	0,00	0,0	
2	35	125,00	5.411,3	Lower Marker
3	47	32,41	1.042,6	
4	59	36,20	933,8	
5	74	23,67	481,9	
6	121	13,58	169,8	
7	138	10,12	111,4	
8	156	20,69	200,6	
9	166	17,71	161,4	
10	185	14,62	119,7	
11	205	14,08	103,9	
12	302	19,69	98,8	
13	310	18,94	92,5	
14	340	27,45	122,4	
15	367	84,22	347,5	
16	381	67,33	268,1	
17	412	72,90	268,1	
18	425	66,32	236,3	
19	502	70,08	211,7	
20	523	80,85	234,1	
21	560	74,98	202,9	
22	650	181,24	422,6	
23	683	101,63	225,4	
24	780	95,54	185,6	
25	871	103,69	180,4	
26	954	68,78	109,3	

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Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary Continued ...

... Peak table for sample 6 : Nextera2_S99

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
27	1.038	73,82	107,8	
28	1.408	495,06	532,7	
29	10.380	75,00	10,9	Upper Marker

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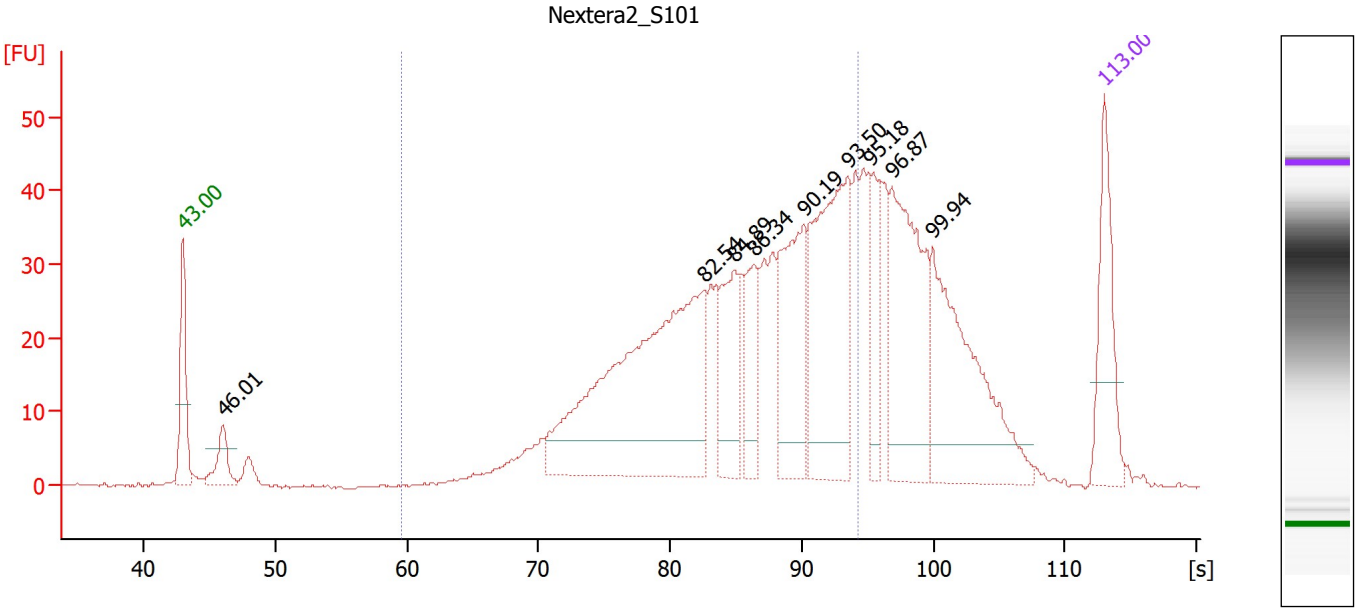
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary Continued ...



Overall Results for sample 7 : Nextera2_S101

Number of peaks found:	9	Corr. Area 1:	695,7
Noise:	0,2		

Peak table for sample 7 : Nextera2_S101

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125,00	5.411,3	Lower Marker
2		58	44,60	1.166,2	
3		502	446,23	1.348,1	
4		554	96,81	264,8	
5		586	59,66	154,2	
6		710	141,68	302,4	
7		947	219,69	351,5	
8		1.137	53,89	71,8	
9		1.382	171,92	188,5	
10		1.828	175,73	145,6	
11	▶	10.380	75,00	10,9	Upper Marker

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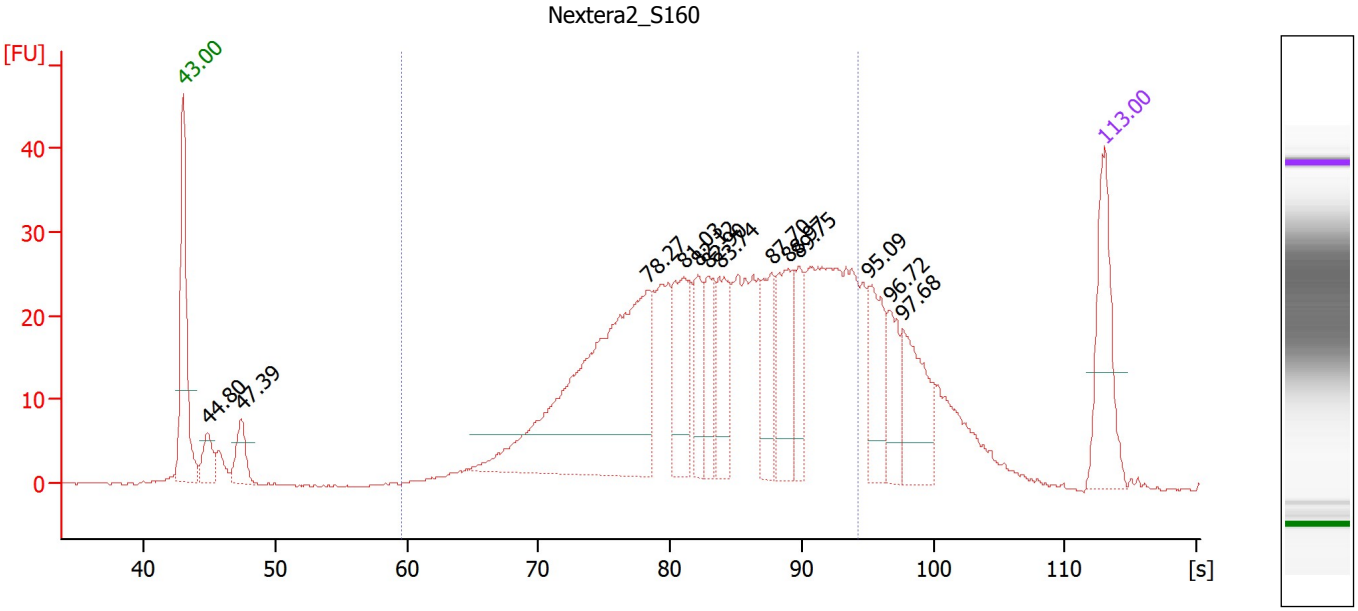
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary Continued ...



Overall Results for sample 8 : Nextera2_S160

Number of peaks found:	13	Corr. Area 1:	651,1
Noise:	0,1		

Peak table for sample 8 : Nextera2_S160

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125,00	5.411,3	Lower Marker
2		47	37,93	1.212,4	
3		71	43,98	936,8	
4		429	400,48	1.414,9	
5		476	92,17	293,6	
6		494	49,89	153,1	
7		509	49,23	146,4	
8		528	65,96	189,2	
9		624	64,22	155,9	
10		665	77,09	175,6	
11		690	46,40	101,9	
12		1.124	62,07	83,6	
13		1.360	39,18	43,6	
14		1.500	65,91	66,6	
15	▶	10.380	75,00	10,9	Upper Marker

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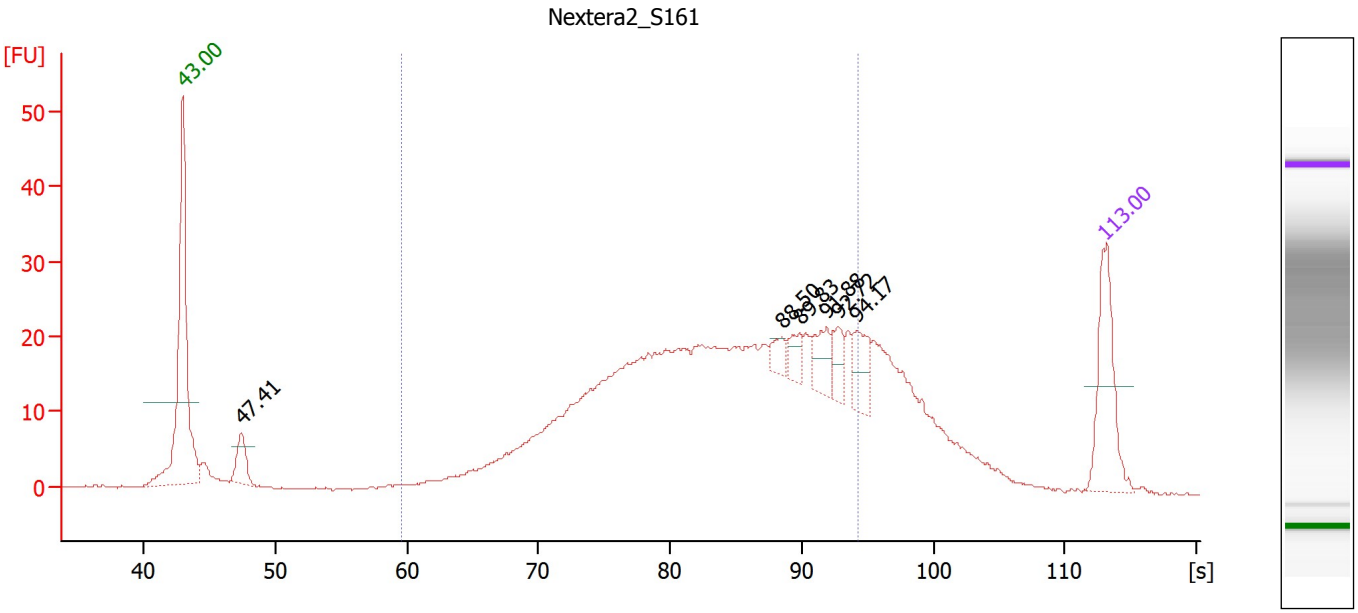
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary Continued ...



Overall Results for sample 9 : Nextera2_S161

Number of peaks found:	6	Corr. Area 1:	522,7
Noise:	0,1		

Peak table for sample 9 : Nextera2_S161

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125,00	5.411,3	Lower Marker
2		71	40,98	870,8	
3		650	15,23	35,5	
4		693	17,11	37,4	
5		831	30,81	56,2	
6		892	21,04	35,7	
7		995	33,32	50,7	
8	▶	10.380	75,00	10,9	Upper Marker

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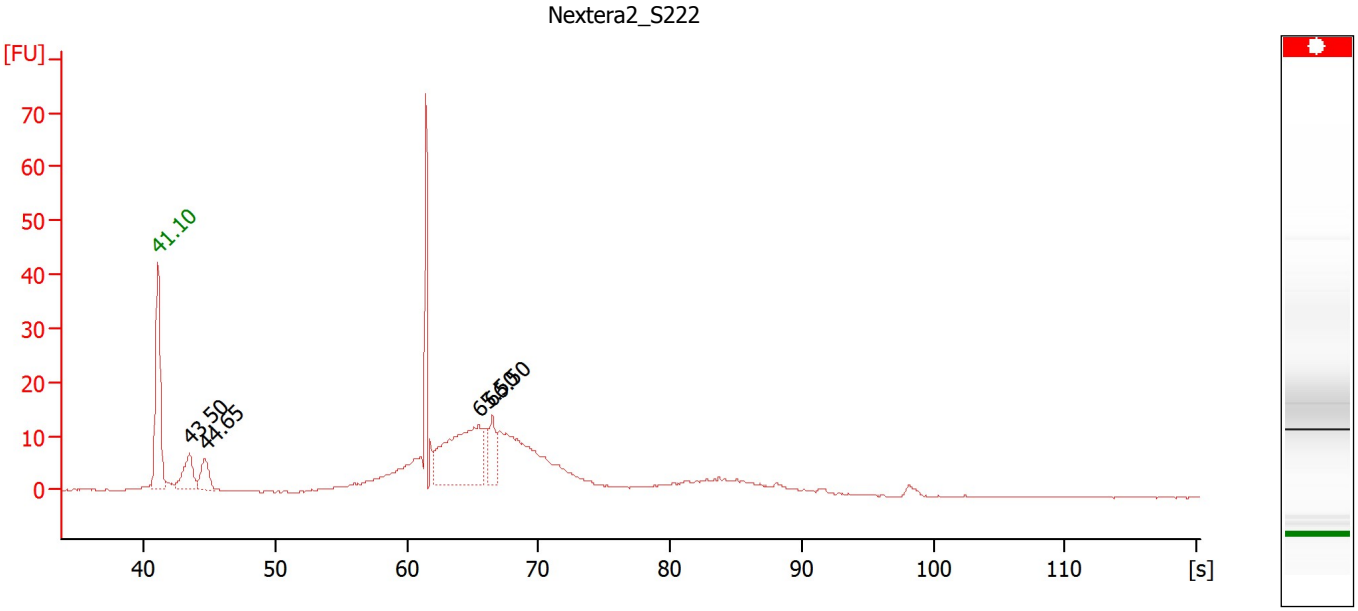
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary Continued ...



Overall Results for sample 10 : Nextera2_S222

Number of peaks found: 4 Noise: 0,1

Peak table for sample 10 : Nextera2_S222

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	0	0,00	0,0	Lower Marker
2	0	0,00	0,0	
3	0	0,00	0,0	
4	0	0,00	0,0	
5	0	0,00	0,0	

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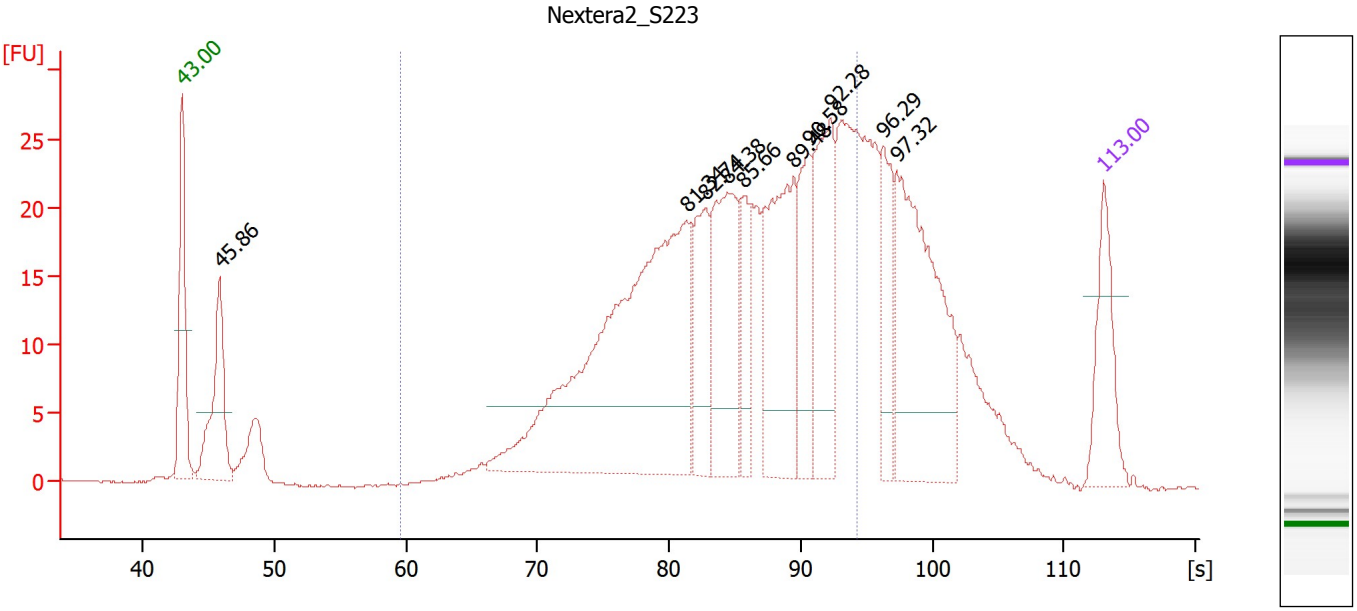
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Electropherogram Summary Continued ...



Overall Results for sample 11 : Nextera2_S223

Number of peaks found:	10	Corr. Area 1:	493,8
Noise:	0,1		

Peak table for sample 11 : Nextera2_S223

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125,00	5.411,3	Lower Marker
2		56	163,78	4.394,5	
3		481	668,15	2.105,4	
4		506	111,13	332,8	
5		543	181,12	505,8	
6		571	66,65	176,8	
7		682	222,09	493,6	
8		738	99,76	204,8	
9		860	156,68	276,1	
10		1.298	73,06	85,3	
11		1.448	249,94	261,5	
12	▶	10.380	75,00	10,9	Upper Marker

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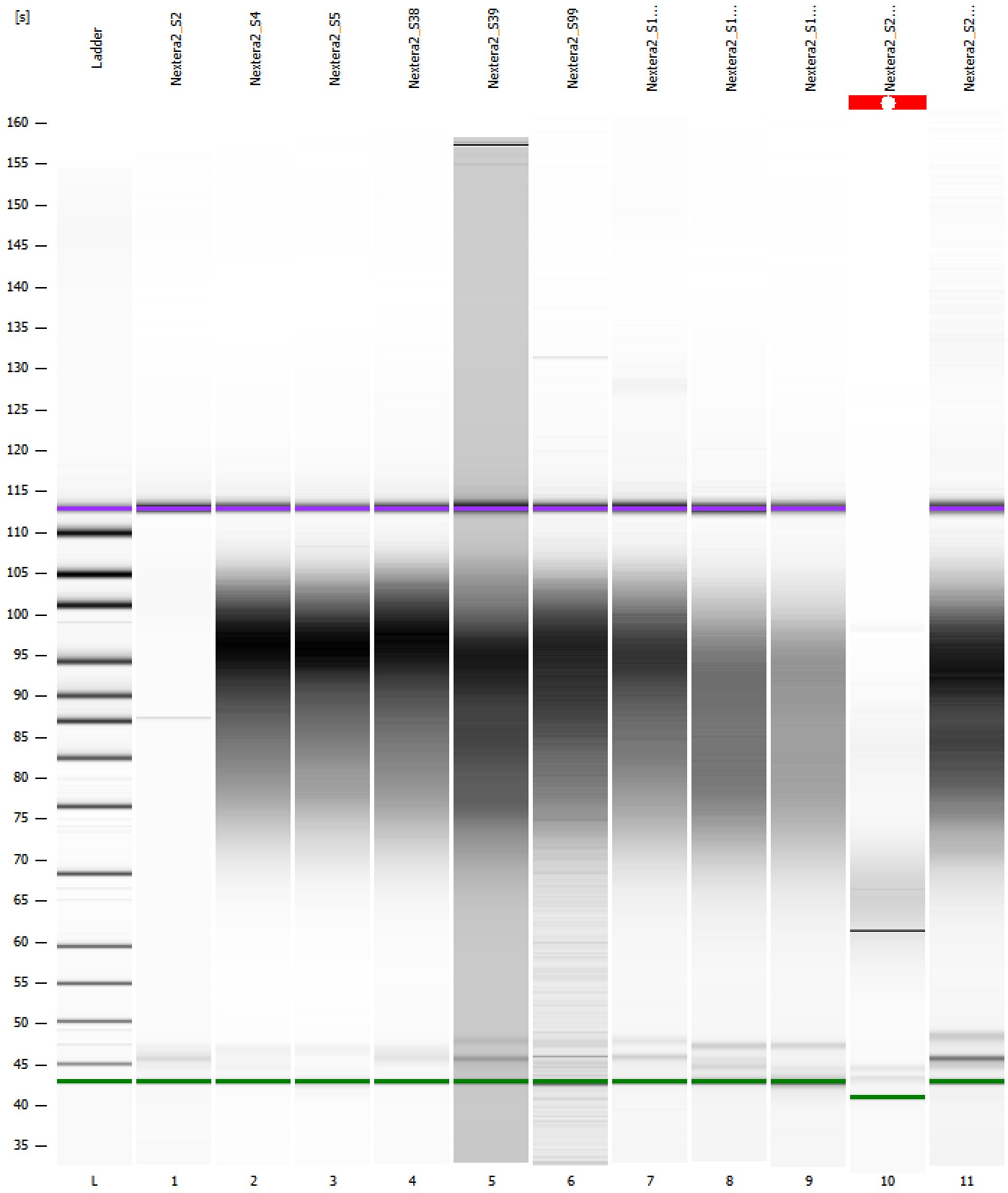
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad

Created: 22-08-2018 12:28:24
Modified: 22-08-2018 13:09:44

Gel Image



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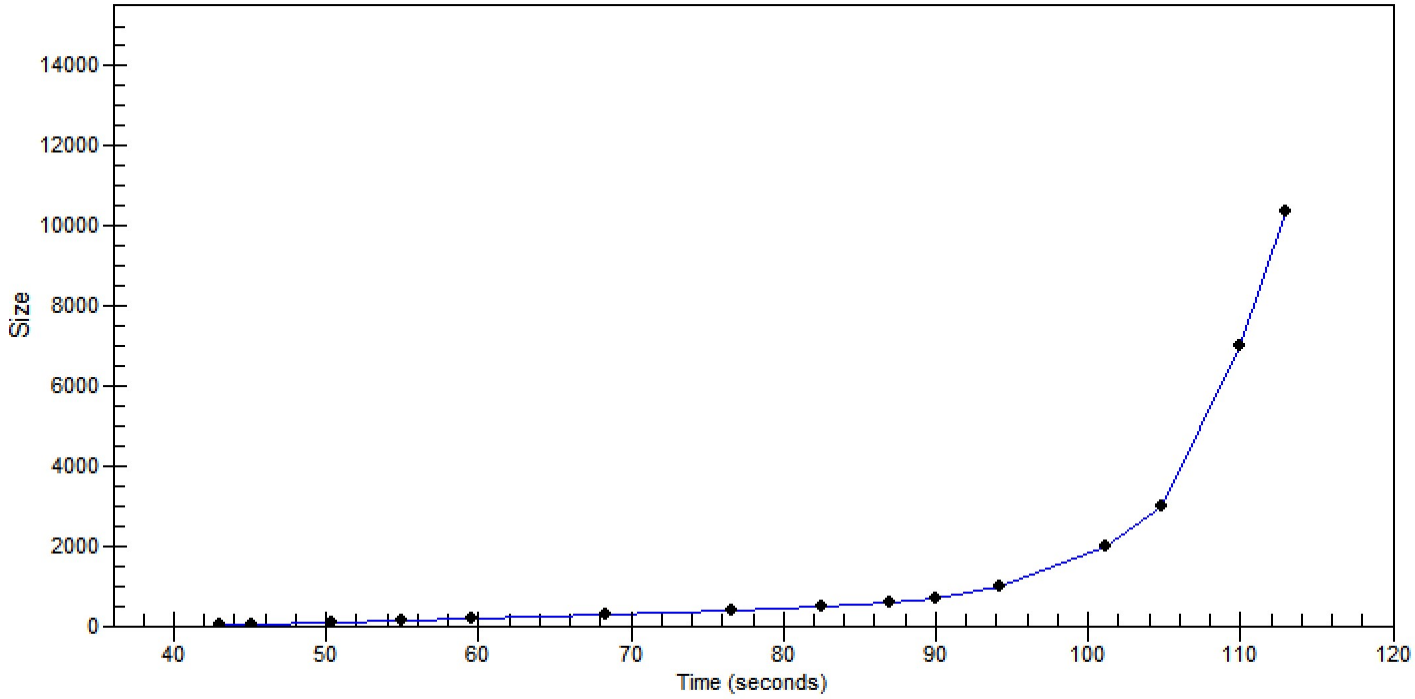
Assay Class:	High Sensitivity DNA Assay	Created:	22-08-2018 12:28:24
Data Path:	C:\...gh Sensitivity DNA Assay_DE24802594_2018-08-22_12-28-24.xad	Modified:	22-08-2018 13:09:44

Curves

Standard Curve



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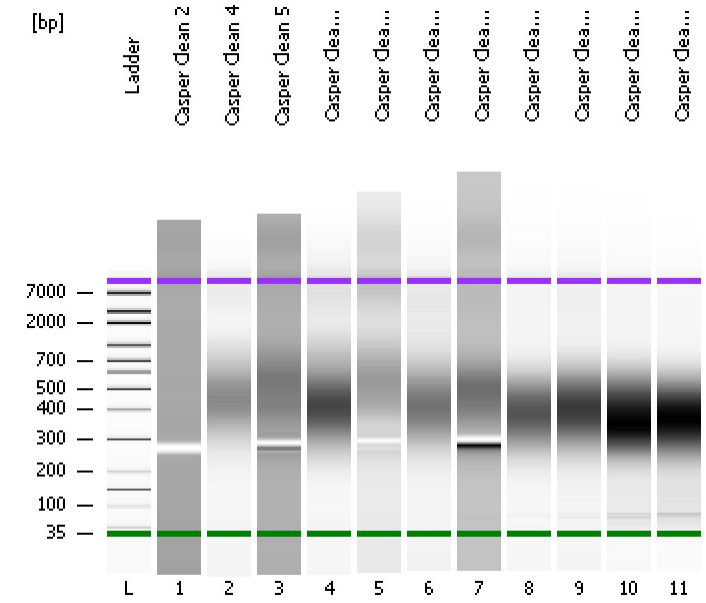
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electrophoresis File Run Summary



Instrument Information:

Instrument Name: DE72901621 Firmware: C.01.069
Serial#: DE72901621 Type: G2939A

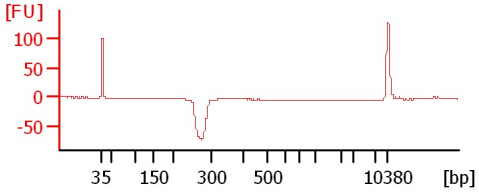
Assay Information:

Assay Origin Path: C:\Program Files\Agilent\2100 bioanalyzer\2100 expert\assays\dsDNA\High Sensitivity DNA.xsy
Assay Class: High Sensitivity DNA Assay
Version: 1.03
Assay Comments: Copyright © 2003-2010 Agilent Technologies

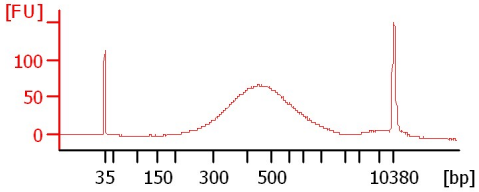
Chip Information:

Chip Lot #:
Reagent Kit Lot #:
Chip Comments:

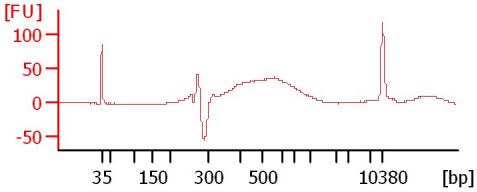
Casper Clean 2



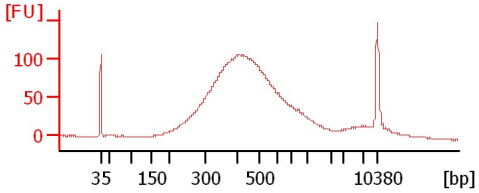
Casper Clean 4



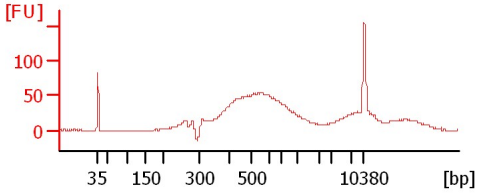
Casper Clean 5



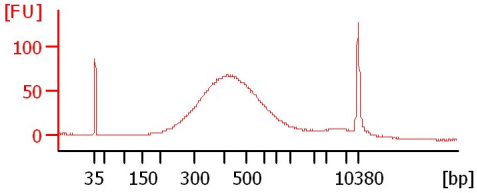
Casper Clean 38



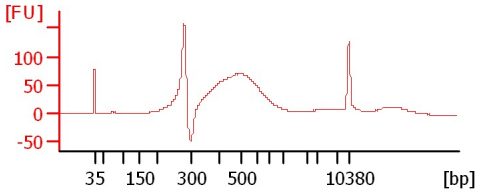
Casper Clean 39



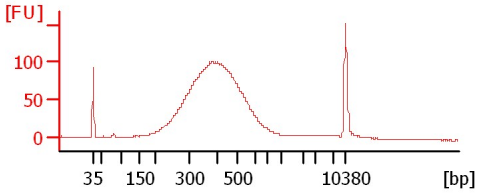
Casper Clean 99



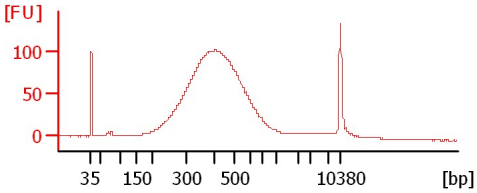
Casper Clean 101



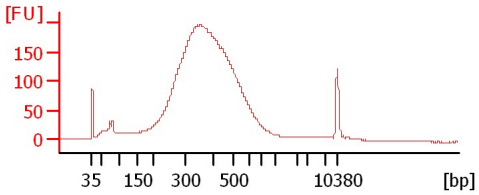
Casper Clean 160



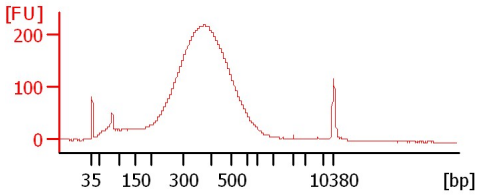
Casper Clean 161



Casper Clean 222



Casper Clean 223



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Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electrophoresis File Run Summary (Chip Summary)

Sample Name	Sample Comment	Rest. Digest	Status	Observation	Result Label	Result Color
Casper Clean 2		☐	✓			
Casper Clean 4		☐	✓			
Casper Clean 5		☐	✓			
Casper Clean 38		☐	✓			
Casper Clean 39		☐	✓			
Casper Clean 99		☐	✓			
Casper Clean 101		☐	✓			
Casper Clean 160		☐	✓			
Casper Clean 161		☐	✓			
Casper Clean 222		☐	✓			
Casper Clean 223		☐	✓			
Ladder		☐	✓			

Chip Lot #

Reagent Kit Lot #

Chip Comments :

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Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electrophoresis Assay Details

General Analysis Settings

Number of Available Sample and Ladder Wells (Max.) : 12
Minimum Visible Range [s] : 32
Maximum Visible Range [s] : 138
Start Analysis Time Range [s] : 33
End Analysis Time Range [s] : 137.5
Ladder Concentration [pg/µl] : 1950
Uses Standard Area for Ladder Fragments
Lower Marker Concentration [pg/µl] : 125
Upper Marker Concentration [pg/µl] : 75
Used Upper Marker for Quantitation
Standard Curve Fit is Point to Point
Show Data Aligned to Lower and Upper Marker

Integrator Settings

Integration Start Time [s] : 33.05
Integration End Time [s] : 137
Slope Threshold : 0.8
Height Threshold [FU] : 5
Area Threshold : 0.1
Width Threshold [s] : 0.6
Baseline Plateau [s] : 0.5

Filter Settings

Filter Width [s] : 0.5
Polynomial Order : 4

Ladder

Ladder Peak	Size	Area
1	35	160
2	50	210
3	100	208
4	150	221
5	200	242
6	300	270
7	400	305
8	500	306
9	600	336
10	700	321
11	1000	366
12	2000	413
13	3000	411
14	7000	400
15	10380	214

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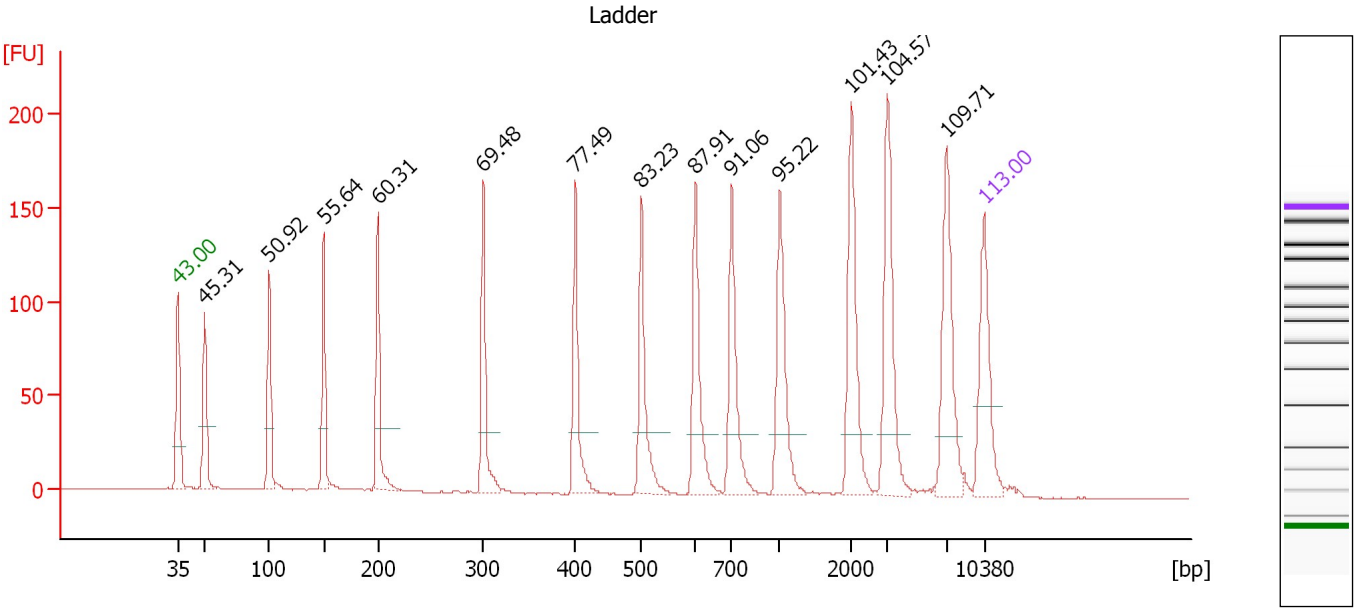
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary



Overall Results for Ladder

Noise: 0.3

Peak table for Ladder

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1		35	125.00	5,411.3	Lower Marker
2		50	150.00	4,545.5	Ladder Peak
3		100	150.00	2,272.7	Ladder Peak
4		150	150.00	1,515.2	Ladder Peak
5		200	150.00	1,136.4	Ladder Peak
6		300	150.00	757.6	Ladder Peak
7		400	150.00	568.2	Ladder Peak
8		500	150.00	454.5	Ladder Peak
9		600	150.00	378.8	Ladder Peak
10		700	150.00	324.7	Ladder Peak
11		1,000	150.00	227.3	Ladder Peak
12		2,000	150.00	113.6	Ladder Peak
13		3,000	150.00	75.8	Ladder Peak
14		7,000	150.00	32.5	Ladder Peak
15		10,380	75.00	10.9	Upper Marker

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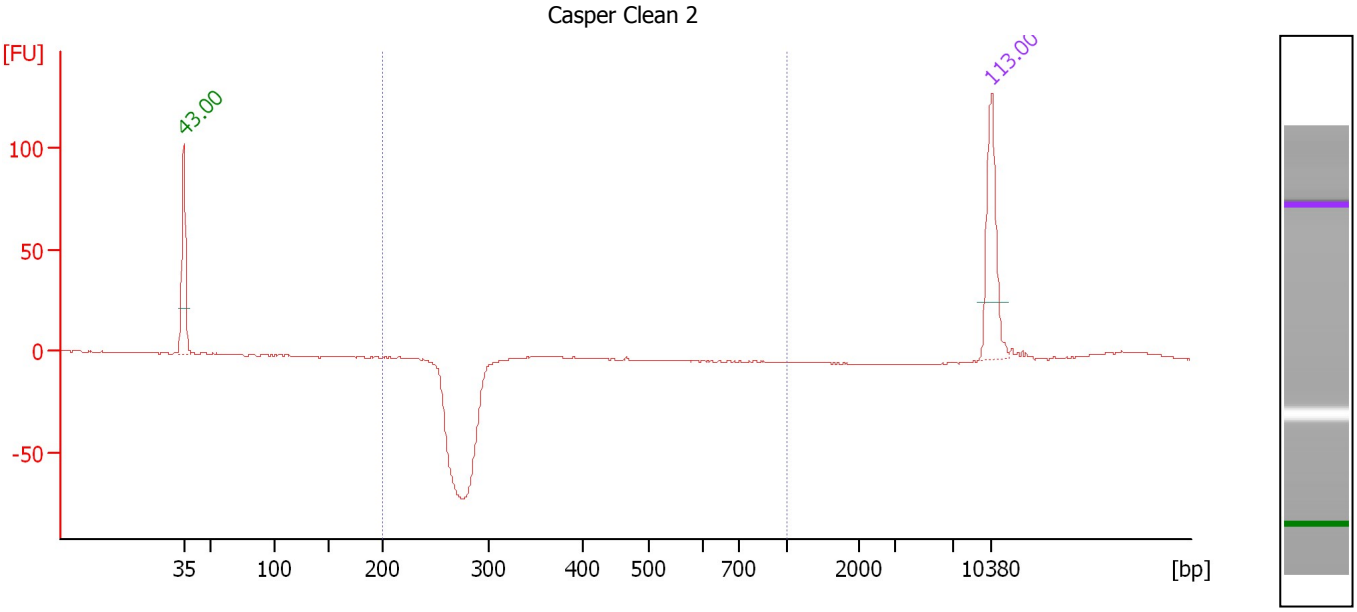
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...



Overall Results for sample 1 : Casper Clean 2

Number of peaks found:	0	Corr. Area 1:	0.0
Noise:	0.3		

Peak table for sample 1 : Casper Clean 2

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125.00	5,411.3	Lower Marker
2	▶	10,380	75.00	10.9	Upper Marker

Region table for sample 1 : Casper Clean 2

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	0.0	0	0	0.0	0.00	0.0	

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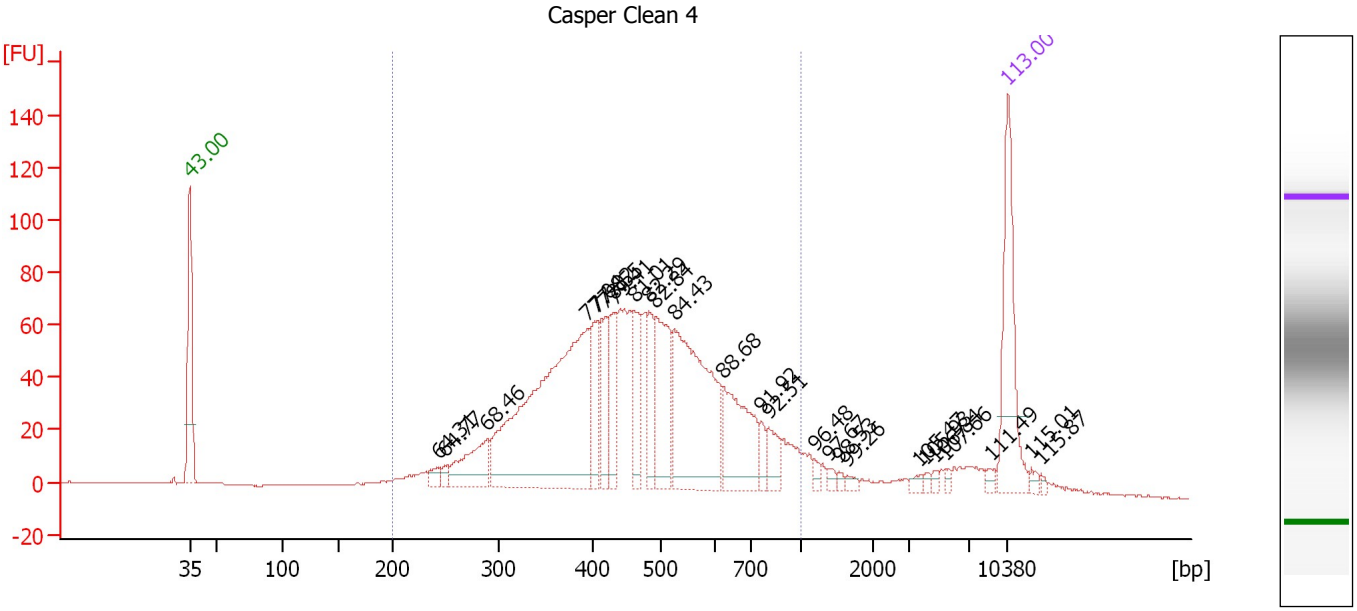
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...



Overall Results for sample 2 : Casper Clean 4

Number of peaks found:	25	Corr. Area 1:	1,657.8
Noise:	0.2		

Peak table for sample 2 : Casper Clean 4

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	244	10.41	64.7	
3	249	7.05	43.0	
4	289	55.59	291.6	
5	394	342.19	1,314.6	
6	406	42.65	159.3	
7	418	37.67	136.4	
8	434	49.85	174.2	
9	461	46.44	152.5	
10	484	39.42	123.5	
11	493	82.47	253.4	
12	526	187.51	540.4	
13	624	79.71	193.4	
14	762	11.38	22.6	
15	805	20.26	38.1	
16	1,203	4.32	5.4	
17	1,394	4.43	4.8	
18	1,534	2.80	2.8	
19	1,652	3.78	3.5	
20	3,698	3.91	1.6	
21	4,053	2.80	1.0	
22	4,763	2.55	0.8	
23	5,403	2.79	0.8	
24	8,832	3.75	0.6	
25	10,380	75.00	10.9	Upper Marker
26	12,445	0.00	0.0	

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Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...

... Peak table for sample 2 : Casper Clean 4

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
27	13,336	0.00	0.0	

Region table for sample 2 : Casper Clean 4

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	1,657.8	90	477	30.4	1,240.71	4,454.4	

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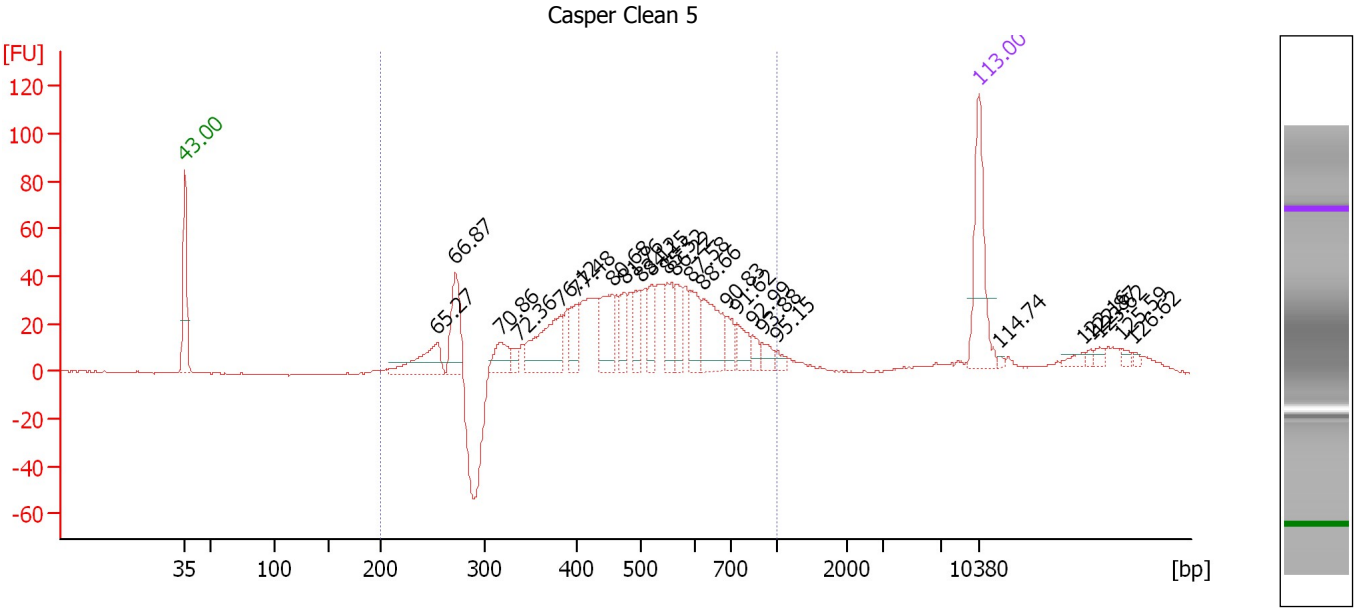
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...



Overall Results for sample 3 : Casper Clean 5

Number of peaks found:	25	Corr. Area 1:	872.4
Noise:	0.3		

Peak table for sample 3 : Casper Clean 5

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	254	60.05	358.1	
3	271	69.91	390.2	
4	317	31.82	152.0	
5	336	10.18	45.9	
6	383	87.48	346.2	
7	400	28.82	109.2	
8	456	53.20	176.9	
9	474	27.94	89.2	
10	498	28.48	86.6	
11	520	29.14	84.9	
12	549	34.67	95.7	
13	564	29.42	79.1	
14	593	35.52	90.8	
15	624	62.77	152.4	
16	693	20.49	44.8	
17	741	24.08	49.2	
18	839	11.82	21.3	
19	903	12.55	21.0	
20	995	6.65	10.1	
21	10,380	75.00	10.9	Upper Marker
22	12,167	0.00	0.0	
23	19,800	0.00	0.0	
24	20,525	0.00	0.0	
25	21,298	0.00	0.0	
26	23,327	0.00	0.0	

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Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...

... Peak table for sample 3 : Casper Clean 5

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
27	24,389	0.00	0.0	

Region table for sample 3 : Casper Clean 5

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	872.4	88	501	30.8	901.76	3,168.7	

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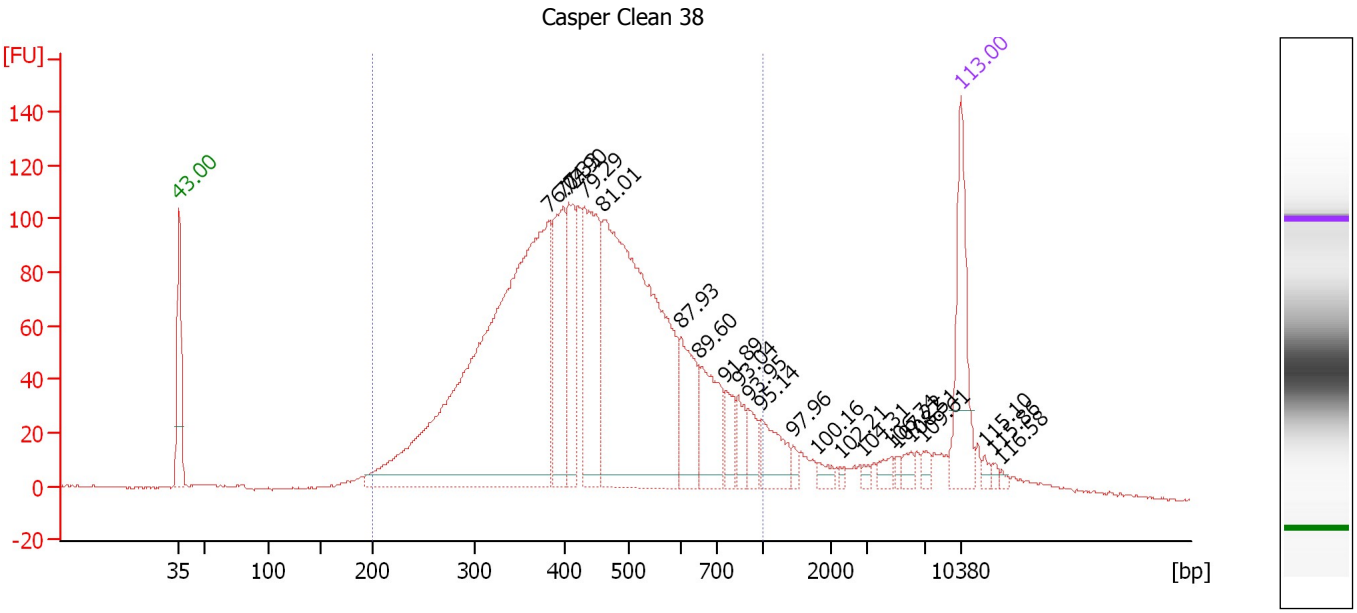
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...



Overall Results for sample 4 : Casper Clean 38

Number of peaks found:	22	Corr. Area 1:	2,752.6
Noise:	0.4		

Peak table for sample 4 : Casper Clean 38

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	382	786.04	3,118.4	
3	398	123.88	471.6	
4	407	95.63	355.8	
5	431	168.59	592.2	
6	461	531.19	1,744.8	
7	601	70.22	177.1	
8	654	73.22	169.7	
9	760	27.58	55.0	
10	843	23.67	42.6	
11	908	18.93	31.6	
12	994	38.75	59.0	
13	1,441	5.76	6.1	
14	1,795	8.04	6.8	
15	2,249	2.64	1.8	
16	2,916	3.74	1.9	
17	4,690	9.02	2.9	
18	5,061	3.97	1.2	
19	6,065	7.89	2.0	
20	6,920	6.84	1.5	
21	10,380	75.00	10.9	Upper Marker
22	12,540	0.00	0.0	
23	13,326	0.00	0.0	
24	14,062	0.00	0.0	

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Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...

... Region table for sample 4 : Casper Clean 38

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	2,752.6	89	457	32.7	2,144.15	8,145.4	

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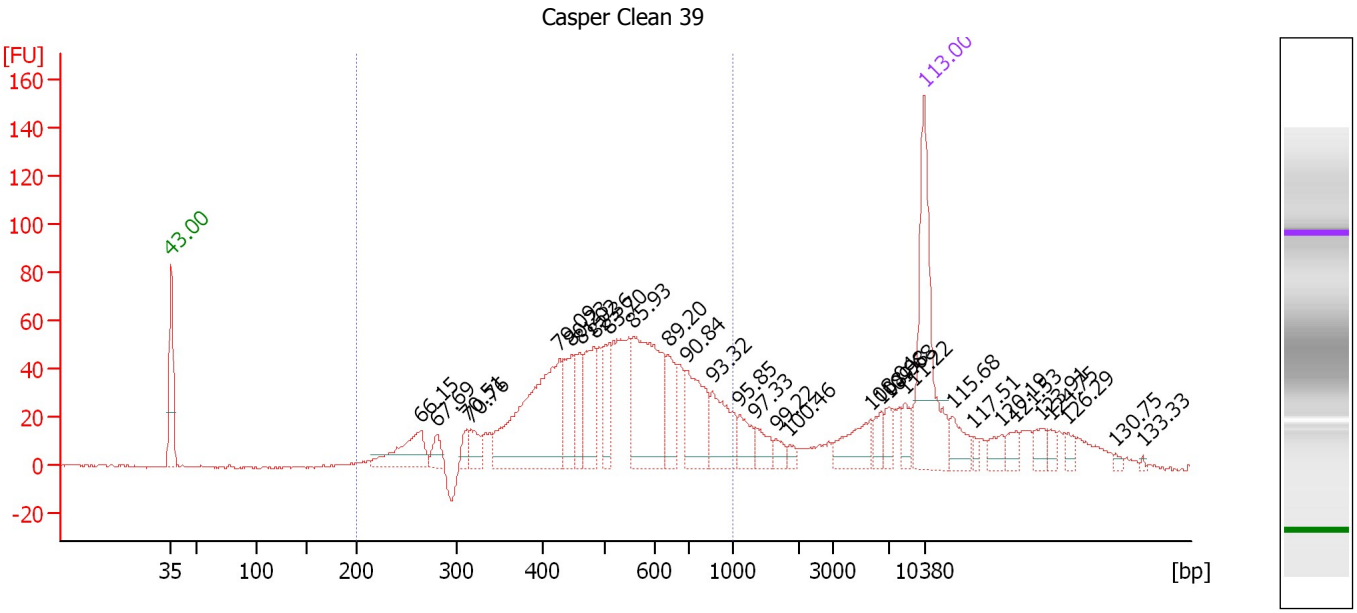
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...



Overall Results for sample 5 : Casper Clean 39

Number of peaks found:	30	Corr. Area 1:	1,206.0
Noise:	0.4		

Peak table for sample 5 : Casper Clean 39

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	264	45.05	258.9	
3	280	13.10	70.8	
4	313	8.53	41.3	
5	316	17.02	81.6	
6	428	136.40	483.0	
7	448	35.70	120.8	
8	462	24.95	81.9	
9	485	42.11	131.6	
10	510	27.48	81.6	
11	558	102.56	278.6	
12	641	29.43	69.6	
13	693	49.07	107.3	
14	863	37.22	65.3	
15	1,101	14.88	20.5	
16	1,340	11.73	13.3	
17	1,644	5.76	5.3	
18	1,844	4.01	3.3	
19	5,700	22.36	5.9	
20	6,433	8.87	2.1	
21	6,973	7.88	1.7	
22	8,545	9.37	1.7	
23	10,380	75.00	10.9	Upper Marker
24	13,133	0.00	0.0	
25	15,019	0.00	0.0	
26	17,772	0.00	0.0	

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Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...

... Peak table for sample 5 : Casper Clean 39

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
27	19,148	0.00	0.0	
28	21,595	0.00	0.0	
29	22,462	0.00	0.0	
30	24,042	0.00	0.0	
31	28,630	0.00	0.0	
32	31,281	0.00	0.0	

Region table for sample 5 : Casper Clean 39

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	1,206.0	73	529	30.6	675.56	2,235.1	

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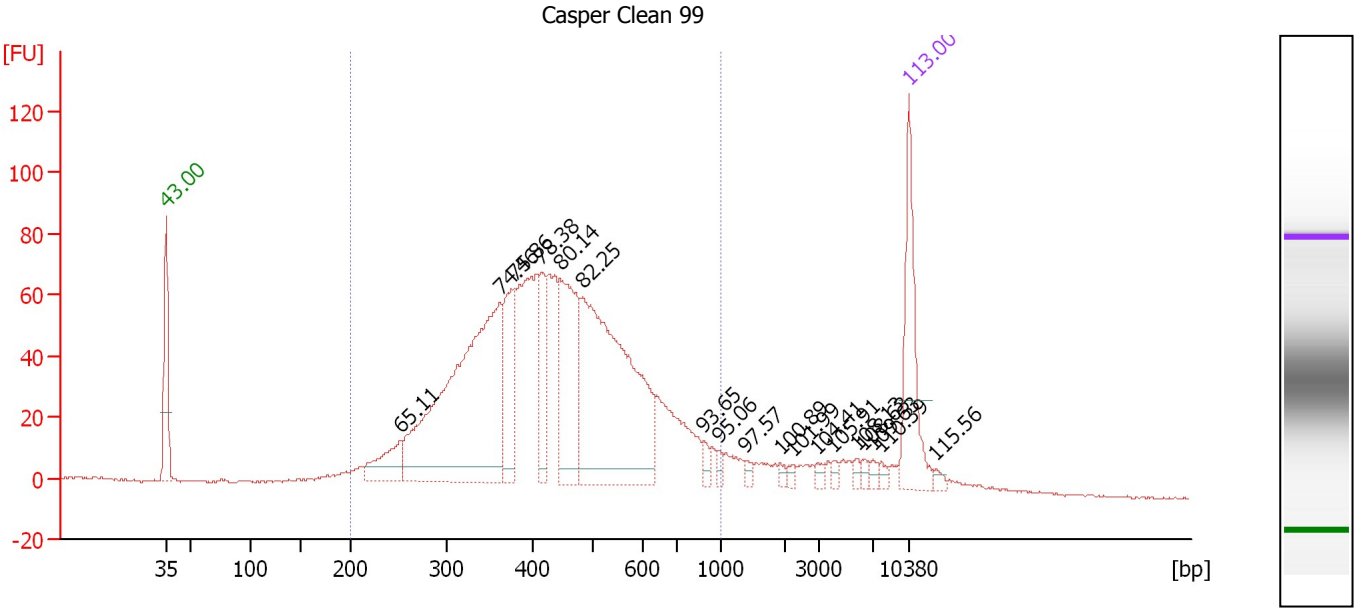
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...



Overall Results for sample 6 : Casper Clean 99

Number of peaks found:	18	Corr. Area 1:	1,707.3
Noise:	0.4		

Peak table for sample 6 : Casper Clean 99

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	252	51.65	310.2	
3	362	416.56	1,742.9	
4	380	85.82	342.4	
5	415	54.64	199.3	
6	446	139.46	473.7	
7	483	353.90	1,110.5	
8	887	8.05	13.8	
9	988	5.83	8.9	
10	1,379	5.09	5.6	
11	1,914	3.86	3.1	
12	2,181	3.25	2.3	
13	2,947	4.52	2.3	
14	4,043	4.14	1.6	
15	5,764	4.03	1.1	
16	6,156	4.03	1.0	
17	6,938	4.72	1.0	
18	7,693	4.31	0.8	
19	10,380	75.00	10.9	Upper Marker
20	13,015	0.00	0.0	

Region table for sample 6 : Casper Clean 99

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	1,707.3	90	451	31.0	1,534.71	5,839.9	

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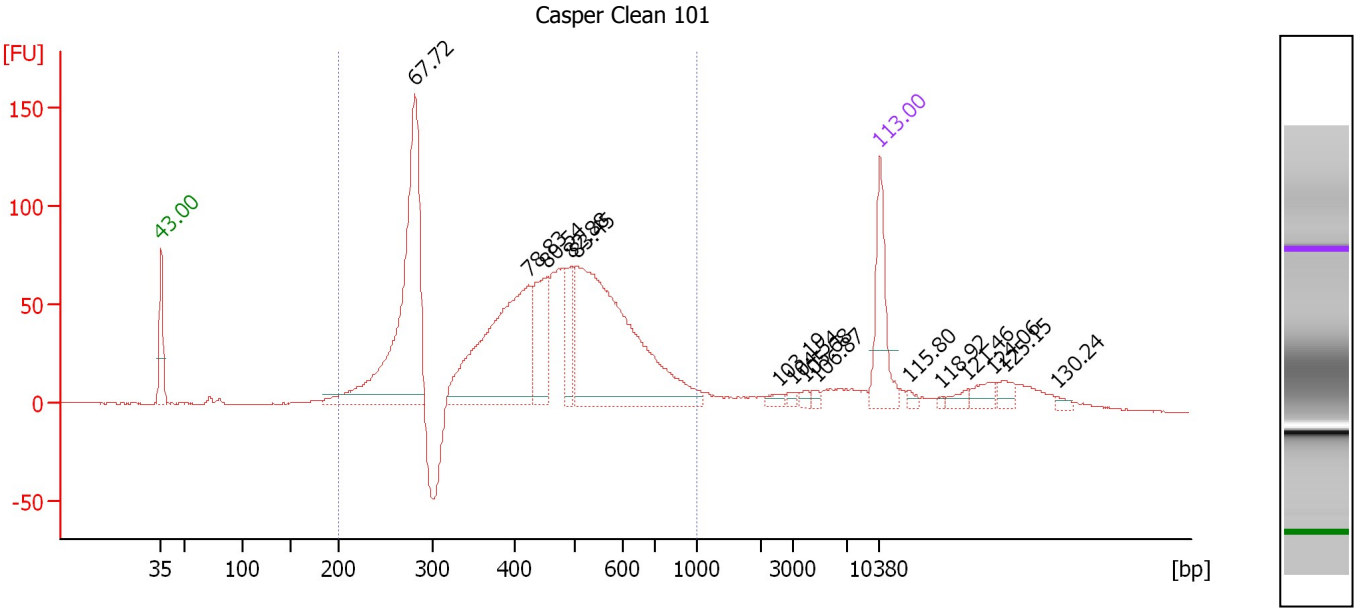
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...



Overall Results for sample 7 : Casper Clean 101

Number of peaks found:	15	Corr. Area 1:	1,787.3
Noise:	0.4		

Peak table for sample 7 : Casper Clean 101

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	281	460.71	2,486.2	
3	423	332.09	1,188.6	
4	453	102.59	343.0	
5	494	51.39	157.7	
6	505	463.83	1,392.4	
7	2,559	7.21	4.3	
8	2,988	4.80	2.4	
9	3,859	5.54	2.2	
10	4,789	4.67	1.5	
11	10,380	75.00	10.9	Upper Marker
12	13,263	0.00	0.0	
13	16,467	0.00	0.0	
14	19,084	0.00	0.0	
15	21,754	0.00	0.0	
16	22,875	0.00	0.0	
17	28,108	0.00	0.0	

Region table for sample 7 : Casper Clean 101

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	1,787.3	86	449	32.6	1,544.05	6,133.7	

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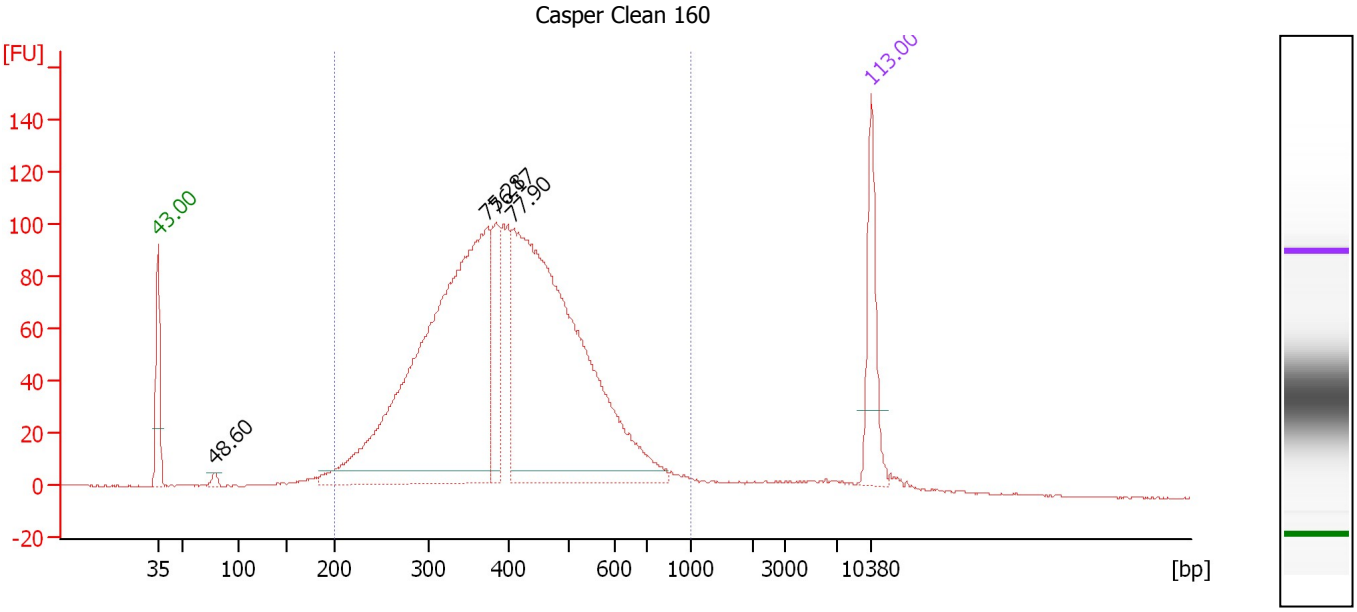
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...



Overall Results for sample 8 : Casper Clean 160

Number of peaks found:	4	Corr. Area 1:	2,364.4
Noise:	0.4		

Peak table for sample 8 : Casper Clean 160

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125.00	5,411.3	Lower Marker
2		79	9.26	176.9	
3		372	842.14	3,426.4	
4		383	102.04	403.2	
5		407	792.56	2,950.1	
6	▶	10,380	75.00	10.9	Upper Marker

Region table for sample 8 : Casper Clean 160

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	2,364.4	94	411	27.9	2,019.67	8,235.0	

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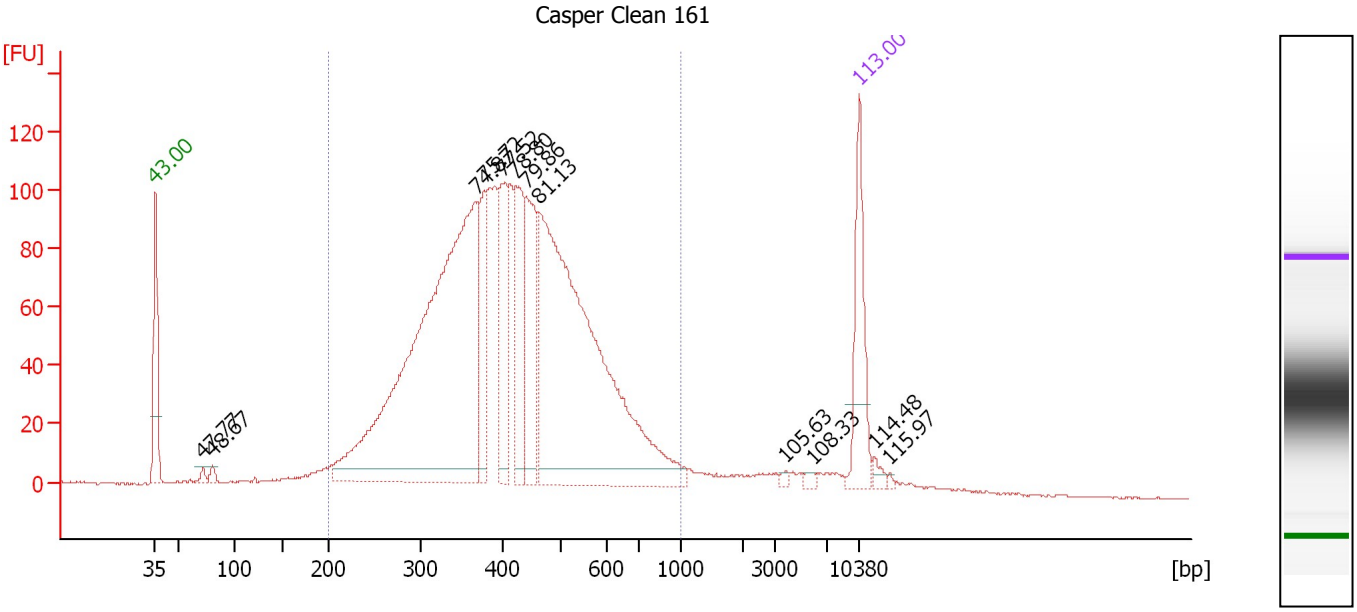
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...



Overall Results for sample 9 : Casper Clean 161

Number of peaks found:	12	Corr. Area 1:	2,459.1
Noise:	0.3		

Peak table for sample 9 : Casper Clean 161

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	72	7.64	160.9	
3	80	8.47	160.5	
4	367	799.98	3,300.1	
5	378	93.44	374.7	
6	401	123.15	465.8	
7	423	113.23	405.8	
8	441	134.47	461.8	
9	463	665.28	2,175.4	
10	3,821	3.08	1.2	
11	5,926	4.79	1.2	
12	10,380	75.00	10.9	Upper Marker
13	11,907	0.00	0.0	
14	13,434	0.00	0.0	

Region table for sample 9 : Casper Clean 161

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	2,459.1	92	429	29.1	2,250.76	8,876.4	

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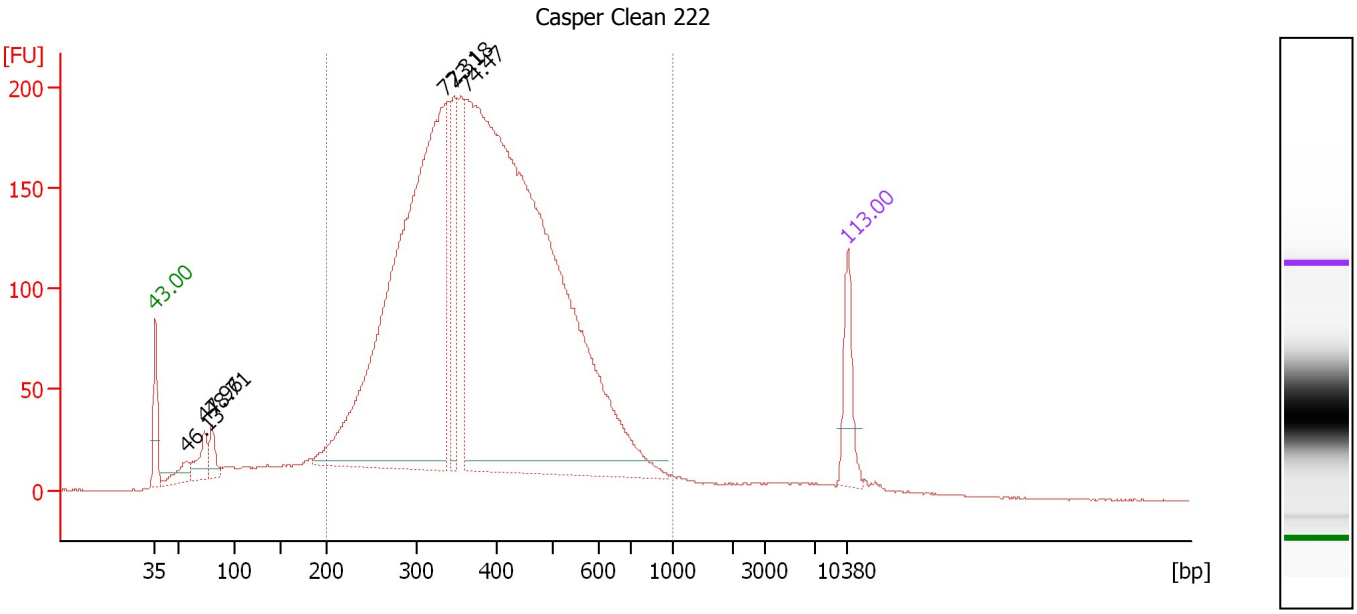
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...



Overall Results for sample 10 : Casper Clean 222

Number of peaks found:	6	Corr. Area 1:	4,834.8
Noise:	0.4		

Peak table for sample 10 : Casper Clean 222

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125.00	5,411.3	Lower Marker
2		57	53.83	1,424.9	
3		74	74.68	1,537.7	
4		80	51.58	973.0	
5		335	1,628.32	7,356.4	
6		346	186.83	817.8	
7		362	2,608.31	10,908.4	
8	▶	10,380	75.00	10.9	Upper Marker

Region table for sample 10 : Casper Clean 222

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	4,834.8	89	398	29.4	5,116.53	21,699.2	

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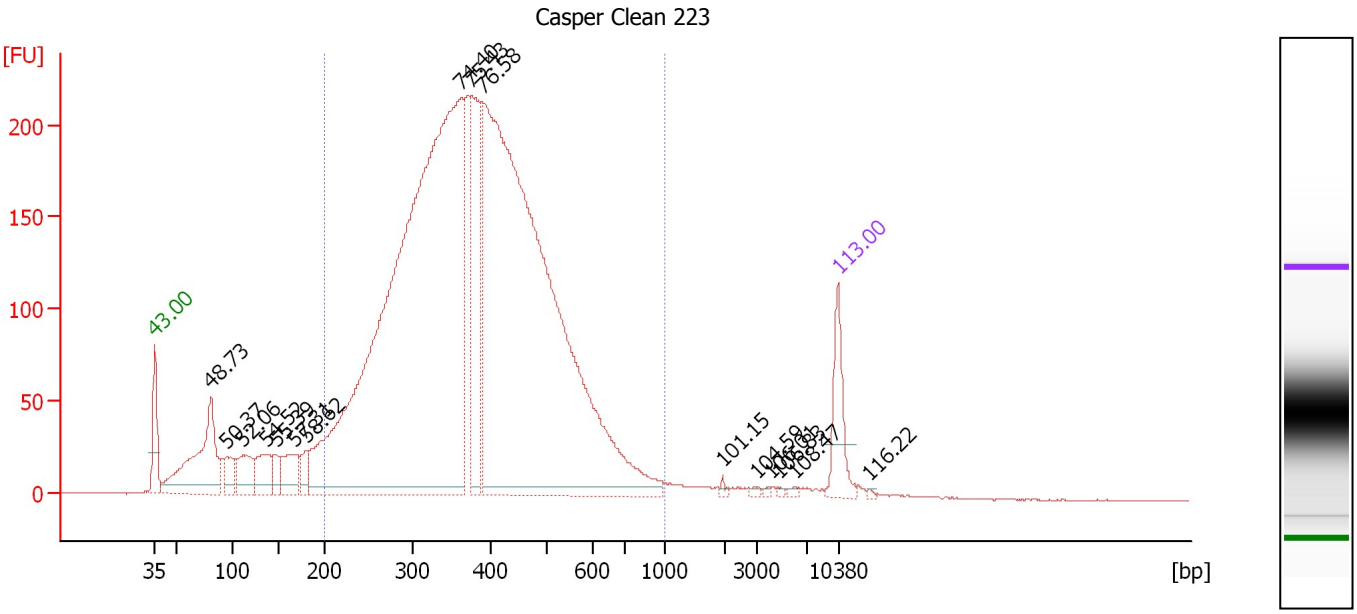
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Electropherogram Summary Continued ...



Overall Results for sample 11 : Casper Clean 223

Number of peaks found:	16	Corr. Area 1:	5,055.1
Noise:	0.3		

Peak table for sample 11 : Casper Clean 223

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125.00	5,411.3	Lower Marker
2		81	336.01	6,323.4	
3		95	56.87	905.8	
4		112	88.88	1,200.7	
5		138	88.21	967.4	
6		147	39.63	407.3	
7		168	86.80	783.6	
8		182	35.19	293.2	
9		361	2,345.87	9,835.9	
10		374	282.11	1,141.9	
11		389	2,092.27	8,157.1	
12		1,956	4.21	3.3	
13		3,013	4.39	2.2	
14		4,118	3.19	1.2	
15		4,756	2.93	0.9	
16		6,031	4.32	1.1	
17	▶	10,380	75.00	10.9	Upper Marker
18		13,693	0.00	0.0	

Region table for sample 11 : Casper Clean 223

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	5,055.1	87	392	28.0	5,031.84	21,517.5	

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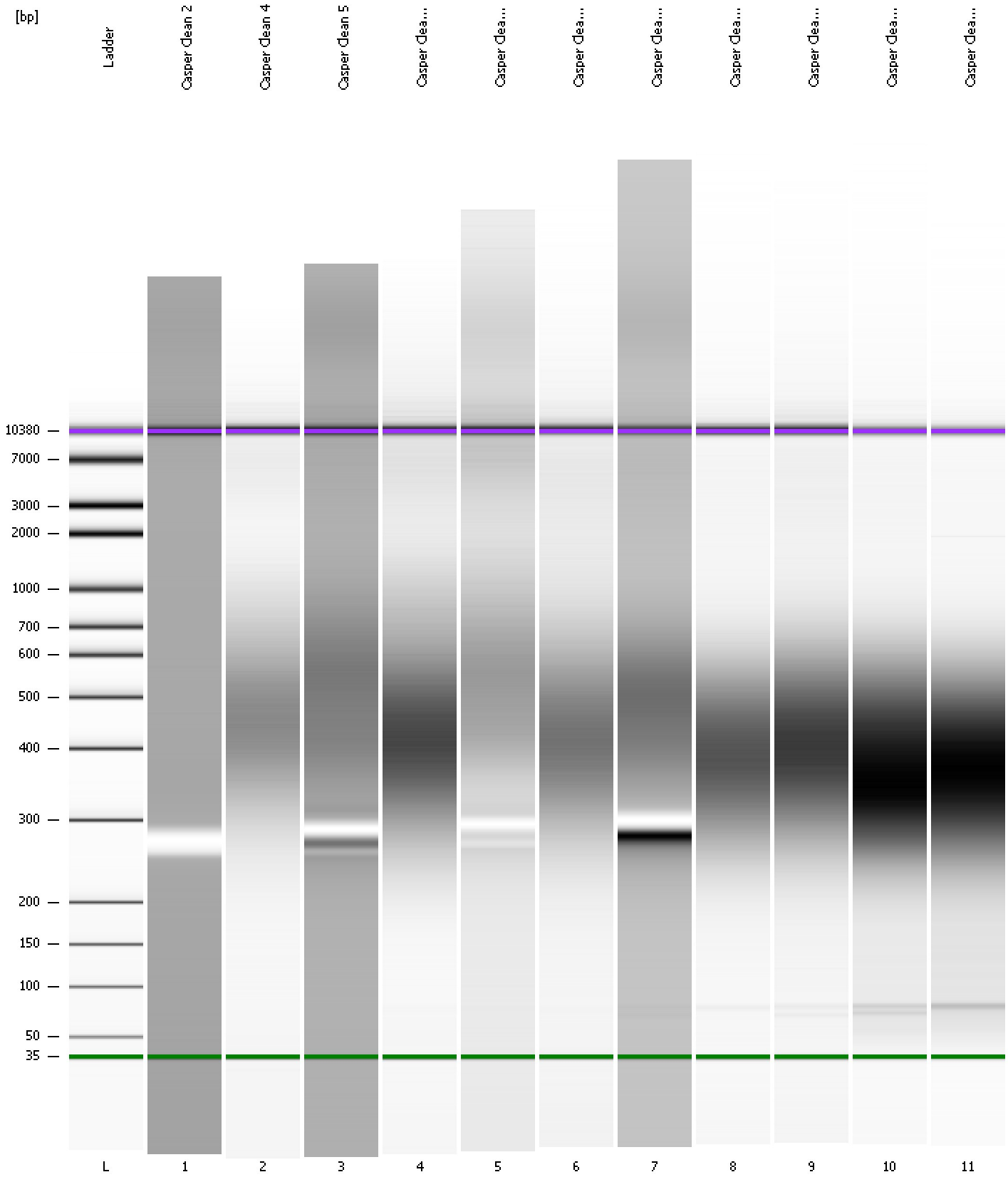
Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Gel Image



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Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad

Created: 3/16/2018 2:53:45 PM
Modified: 3/16/2018 3:34:15 PM

Run Logbook

Description	Number	Source	Category	Sub Category	Time	Time Zone	User	Host
Run ended on port 1 (Number of wells acquired: 12)		Instrument	Run		3/16/2018 3:34:12 PM	(GMT +01:00) Romance Standard Time	Lab	W-LAB02
Run started on port 1 (File: C:\Program Files\Agilent\2100 bioanalyzer\2100 expert\Data\2018-03-16\2100 expert_High Sensitivity DNA Assay_DE72901621_2018-03-16_14-53-45.xad)		Instrument	Run		3/16/2018 2:53:51 PM	(GMT +01:00) Romance Standard Time	Lab	W-LAB02
Product Number : G2939A		Instrument	Run		3/16/2018 2:53:51 PM	(GMT +01:00) Romance Standard Time	Lab	W-LAB02
Name :		Instrument	Run		3/16/2018 2:53:51 PM	(GMT +01:00) Romance Standard Time	Lab	W-LAB02
Vendor : Agilent Technologies		Instrument	Run		3/16/2018 2:53:51 PM	(GMT +01:00) Romance Standard Time	Lab	W-LAB02
Serial# : DE72901621		Instrument	Run		3/16/2018 2:53:51 PM	(GMT +01:00) Romance Standard Time	Lab	W-LAB02
Firmware : C.01.069		Instrument	Run		3/16/2018 2:53:51 PM	(GMT +01:00) Romance Standard Time	Lab	W-LAB02
Cartridge : Electrode		Instrument	Run		3/16/2018 2:53:50 PM	(GMT +01:00) Romance Standard Time	Lab	W-LAB02

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Analysis: Experiments on the effect of sequencing platform and library preparation

Casper Sahl Poulsen

08092021

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Introduction

The purpose of the following code is to assess the effect of library preparation using KAPA (PCR free), NEXTflex (PCR free), and Nextera (PCR step), and Illumina sequencing platforms (HiSeq and NextSeq) on two different microbial communities. Raw data is available online (Include the ENA reference). Samples have been quality processed and mapped with MGmapper extracting read counts mapping to the different genera.

Explanation of samples naming in the taxonomy table
DTU: Performed at the Technical university of Denmark followed by the year of sampling. LPSX: Part of the study on library preparation and sequencing platform study. HX: Part of the handling experiment study included here to represent one library preparation sequencing platform method. P1 & P2: Pig feces 1 and 2. S1 and S2: Sewage 1 and 2. KA: Kapa library preparation. NF: NEXTflex library preparation. NX: Nextera library preparation (Can both be NX1 and NX2 representing the same process to investigate variance associated with redoing library preparation and sequencing). HI: HiSeq. NS: NextSeq. 0h: Direct processing after sample collection, samples were not stored. 64h_80C: After sample collection alliquouts of the same sample as the one processed directly were stored at -80?C for 64 h. a, b, c: Indicating storage replicates, only duplicates where included in this study. MG_XXX: Internal metagenomics sample number.

Metadata
Contains information on how the samples were processed, sequencing performance, and info on mapping to different databases

Feature Contains taxonomic feature information

###Analysis notes All analysis adhere to the compositional data analysis framework by including an isometric log ratio transformation (ILR) to calculate Euclidean distances used to perform prinicipal component analysis (PCA), Heatmap sample clustering and boxplots. A centered log ratio transformation (CLR) were used when keeping genera information was important in sparse partial least square discriminant analysis (sPLS-DA) and redundancy analysis (rda). Important factors to be aware of in this analysis is that raw data is used, filtered according to an average count of 5, and zeroes are estimated with simple multiplicative replacement.

Colors In study design and heatmaps KAPA is purple (#984ea3), Nextera is orange (#ff7f00), and NEXTflex is red (#e41a1c). When performing sPLS-DA, PCA, rda the NEXTflex is run either with NextSeq is blue (#377eb8) or HiSeq is green (#4daf4a). Finally in PCA and rda Nextera is divided in 1 and 2, where 1 remains orange and 2 is brown (#993300)
...

Packages

```
#Install knitr package
#install.packages("knitr")

#Update bioconductor
#if (!requireNamespace("BiocManager"))
#    install.packages("BiocManager")
#BiocManager::install()

#install.packages("ggplot2")
library(ggplot2) #Data visualization, based on grammar of graphics.
  #help(package="ggplot2")
#install.packages("ggthemes")
library(ggthemes) #Extra themes, scales and geoms for ggplot2. help(package="ggthemes")
#install.packages("vegan")
library(vegan) #Community ecology package, ordination methods, diversity analysis
  #and other functions for community and vegetation ecologists. help(package="vegan")
library(gridExtra)
#install.packages("reshape2")
library(reshape2)
#install.packages("tidyr")
library(tidyr)
#install.packages("knitr")
library(knitr)
#install.packages("stringr")
library(stringr)
#install.packages("cowplot")
library(cowplot)
#install.packages("compositions")
library(compositions) #The package provides functions for the consistent analysis of
  #compositional data (e.g. portions of substances) and positive numbers
  #(e.g. concentrations) in the way proposed by Aitchison and Pawlowsky-Glahn.
  #Includes the clr function. help(package="compositions")
#install.packages("zCompositions")
library(zCompositions) #Include cmultRepl function to estimate zeroes
#install.packages("car")
library(car) # Companion to applied regression includes Levene's test. help(package="car")
#install.packages("robCompositions")
library(robCompositions) #help(package="robCompositions")
#install.packages("psych") #describeBy function
```

```
library(psych)

#if (!requireNamespace("BiocManager", quietly = TRUE))
#    install.packages("BiocManager")
#BiocManager::install("mixOmics", version = "3.8")
#install.packages("mixOmics") #Extends upon some of the function from robCompositions
library(mixOmics)
#install.packages("pheatmap")
library(pheatmap) # Implementation of heatmaps that offers more control over dimensions
  #and appearance help(package="pheatmap")
#install.packages("RColorBrewer")
library(RColorBrewer) #Used to make the Heatmap colors
#install.packages("FSA")
library(FSA) #A variety of simple fish stock assessment methods. Includes the Dunne
  #non-parametric follow-up test help(package="FSA")
#install.packages("Rmisc")
library(Rmisc)
#install.packages("dplyr")
library(dplyr) #A grammar of data manipulation. A fast, consistent tool for working with
  #data frame like objects. help(package="dplyr")
#install.packages("gridExtra")
library(amap) #Calculating spearman correlations
```

Read in data

```
Tax <- read.delim(file="../File_S2/TaxonomyRaw20180925.txt", check.names=FALSE,
                  stringsAsFactors=FALSE, strip.white=TRUE)
Metadata <- read.delim(file="../File_S2/Metadata20180925.txt", check.names=FALSE,
                       stringsAsFactors=FALSE, strip.white=TRUE)
Feature <- read.delim(file="../File_S2/FeatureShort20180925.txt", check.names=FALSE,
                      stringsAsFactors=FALSE, strip.white=TRUE)
```

Change naming

```
colnames(Metadata)[14] <- "Storage"
```

Analysis

Chunks can be run by themselves or consecutively

Quality control of different sequencing specific parameters

Include QC on sequencing output and alpha-diversity summarized in S1_Table and rarefaction curves S2_Fig

```
rm(list=setdiff(ls(), c("Metadata", "Feature", "Tax")))

#Subset data pig feces 1 and 2 (PF)
Subset<- "All" #All, Allspiked, Allunspiked, PF, SW, P1, P1spiked, P1unspiked, P2,
  #P2spiked, P2unspiked, S1, S1spiked, S1unspiked, S2, S2spiked, S2unspiked

#Subset experiment, Experiment_Type. (Meaningfull combinations of subset and SubExp:
  #HX=All, FTX=P1&S1, LTX=P1&S1, LPSX=All unspiked, HXFTX=P1&S1, HXLTX=P1&S1,
  #HXLPSX=All unspiked, HXLTXLPSX=P1&S1 unspiked)
```

```r
SubExp<-"LPSX" #HX, FTX, LTX, LPSX, HXFTX, HXLTX, HXLPSX, HXLTXLPSX, HXFTXLTX, All

#Subset Frozen Unfrozen
SubFre<-"Both" #Frozen, Unfrozen, Both

#Removing negative and positive controls
Metadata2<-dplyr::filter(Metadata, Sample_type_simple=="Sample")

#Subsetting Metadata2
if (Subset=="Allspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Spiked")
} else if (Subset=="Allunspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Unspiked")
} else if (Subset=="PF") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1" | Experiment == "Pig_feces_2")
} else if (Subset=="PFspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked" |
                      Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="PFunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1" |
                      Sample_type == "Pig_feces_2")
} else if (Subset=="SW") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1" | Experiment == "Sewage_2")
} else if (Subset=="SWspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked" |
                      Sample_type == "Sewage_2_spiked")
} else if (Subset=="SWunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1" | Sample_type == "Sewage_2")
} else if (Subset=="P1") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1")
} else if (Subset=="P1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked")
} else if (Subset=="P1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1")
} else if (Subset=="P2") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_2")
} else if (Subset=="P2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="P2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2")
} else if (Subset=="S1") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1")
} else if (Subset=="S1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked")
} else if (Subset=="S1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1")
} else if (Subset=="S2") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_2")
} else if (Subset=="S2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2_spiked")
} else if (Subset=="S2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2")
} else if (Subset=="All") {
```

```r
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

```
## [1] "No subsetting, all included"
```

```r
#Further subsetting Metadata2
if (SubExp=="HX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment")
} else if (SubExp=="FTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="LTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="LPSX") {
  Metadata22<-filter(Metadata2, Experiment_type == "Library_prep_seq_platform_experiment")
  VectorLPSX <- unique(Metadata22$Matching_samples)
  Metadata2<-filter(Metadata2, Matching_samples %in% VectorLPSX)
  rm(VectorLPSX, Metadata22)
} else if (SubExp=="HXFTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="HXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="HXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXLTXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXFTXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubFre=="Frozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Freezer")
} else if (SubFre=="Unfrozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Unfrozen")
} else if (SubFre=="Both") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

```
## [1] "No subsetting, all included"
```

```r
#Removing the Kappa NextSeq run
Metadata2<-Metadata2[grep("\\KANS.*", Metadata2$Sample, invert=TRUE),]

#Applying subsetting to OTU tables
Tax2<-dplyr::select(Tax, one_of(Metadata2$Sample))

#Remove orgs that are not present after subsetting.
Tax2 <- Tax2[rowSums(Tax2)>0,]

#Simple summary statistics
summary(Metadata2)
```

```
##     Sample          Sample_name        Sample_number        Number
##  Length:80          Length:80          Min.   :  4.00   Min.   :  4.0
##  Class :character   Class :character   1st Qu.: 57.75   1st Qu.:359.8
##  Mode  :character   Mode  :character   Median :113.00   Median :382.5
##                                        Mean   :112.94   Mean   :336.0
##                                        3rd Qu.:167.50   3rd Qu.:405.2
##                                        Max.   :223.00   Max.   :428.0
##
##  Seq_sample_name_SameassampleFormerginginR Experiment_type
##  Length:80                                 Length:80
##  Class :character                          Class :character
##  Mode  :character                          Mode  :character
##
##
##
##
##   Experiment           Colors          Sample_type        Sample_type_simple
##  Length:80          Length:80          Length:80          Length:80
##  Class :character   Class :character   Class :character   Class :character
##  Mode  :character   Mode  :character   Mode  :character   Mode  :character
##
##
##
##
##  Sample_type_new    Sample_type_RF          Time       Storage
##  Length:80          Length:80          Min.   : 0   Min.   :-80
##  Class :character   Class :character   1st Qu.: 0   1st Qu.:-80
##  Mode  :character   Mode  :character   Median :32   Median :-80
##                                        Mean   :32   Mean   :-80
##                                        3rd Qu.:64   3rd Qu.:-80
##                                        Max.   :64   Max.   :-80
##                                                     NA's   :40
##  FrozenUnfrozenSimple FrozenUnfrozen     StoragePlacementType
##  Length:80            Length:80          Length:80
##  Class :character     Class :character   Class :character
##  Mode  :character     Mode  :character   Mode  :character
##
##
##
##
##   Replicate          Flow_cell_lane   Concentration_DTU  ConcentrationNum_DTU
##  Length:80          Min.   : 1.000   Length:80          Length:80
```

```
##  Class :character   1st Qu.: 9.000   Class :character   Class :character
##  Mode  :character   Median :10.000   Mode  :character   Mode  :character
##                     Mean   : 9.275
##                     3rd Qu.:11.000
##                     Max.   :12.000
##
##  Date_of_collection Freeze_thaw_cycles Temperature_sampling_day Precipitation
##  Length:80          Min.   :0.0        Min.   : 7.00            Min.   : 0.0
##  Class :character   1st Qu.:0.0        1st Qu.:10.75            1st Qu.: 0.0
##  Mode  :character   Median :0.5        Median :13.50            Median : 0.0
##                     Mean   :0.5        Mean   :12.75            Mean   : 3.5
##                     3rd Qu.:1.0        3rd Qu.:15.50            3rd Qu.: 3.5
##                     Max.   :1.0        Max.   :17.00            Max.   :14.0
##
##  Precipitation_the_day_before Replicate_Groups   SpikedUnspiked
##  Length:80                    Length:80          Length:80
##  Class :character             Class :character   Class :character
##  Mode  :character             Mode  :character   Mode  :character
##
##
##
##
##  Replicate_Boxplot  Matching_samples   Library_preparation Sequencing_platform
##  Length:80          Length:80          Length:80           Length:80
##  Class :character   Class :character   Class :character    Class :character
##  Mode  :character   Mode  :character   Mode  :character    Mode  :character
##
##
##
##
##   Reads_Total        Bases_Total        Reads_AfterTrim    Bases_AfterTrim
##  Min.   : 2710623   Min.   :4.083e+08   Min.   : 2594599   Min.   :3.643e+08
##  1st Qu.: 8454797   1st Qu.:1.272e+09   1st Qu.: 7628501   1st Qu.:1.047e+09
##  Median : 9458488   Median :1.420e+09   Median : 8867728   Median :1.232e+09
##  Mean   :10702254   Mean   :1.597e+09   Mean   : 9744984   Mean   :1.334e+09
##  3rd Qu.:12032974   3rd Qu.:1.772e+09   3rd Qu.:11203132   3rd Qu.:1.516e+09
##  Max.   :30764150   Max.   :4.645e+09   Max.   :27349015   Max.   :3.526e+09
##
##     Archaea           Bacteria       Bacteria_draft    Common_animals
##  Min.   :    6.0   Min.   :  66553   Min.   : 378113   Min.   :     0
##  1st Qu.:  394.8   1st Qu.: 177899   1st Qu.: 663736   1st Qu.:     1
##  Median : 5690.0   Median : 421225   Median :1056719   Median :   354
##  Mean   : 6259.7   Mean   : 680137   Mean   :1249109   Mean   : 26519
##  3rd Qu.: 8473.5   3rd Qu.:1048261   3rd Qu.:1573516   3rd Qu.: 23313
##  Max.   :31029.0   Max.   :2529946   Max.   :3738745   Max.   :284238
##
##  Common_plants        Fungi           Human         HumanMicrobiome
##  Min.   :   1.0   Min.   :   14   Min.   :    0.0   Min.   : 21956
##  1st Qu.:  46.0   1st Qu.:  121   1st Qu.:    0.0   1st Qu.: 37859
##  Median : 168.5   Median : 2685   Median :   72.5   Median :102841
##  Mean   : 637.3   Mean   : 3711   Mean   : 6061.8   Mean   :190112
##  3rd Qu.: 984.0   3rd Qu.: 6579   3rd Qu.:11423.0   3rd Qu.:291209
##  Max.   :5672.0   Max.   :16380   Max.   :61495.0   Max.   :856979
##
```

```
##  Parasites_other    Parasites_vertebrates   ResFinder        Unmapped
##  Min.   :    1.00   Min.   : 1471         Min.   :  457   Min.   : 1496139
##  1st Qu.:    8.75   1st Qu.: 5808         1st Qu.: 1748   1st Qu.: 5832961
##  Median : 5253.50   Median : 8175         Median : 3013   Median : 6833336
##  Mean   :19597.78   Mean   : 9912         Mean   : 3585   Mean   : 7515692
##  3rd Qu.:36720.25   3rd Qu.:12636         3rd Qu.: 4619   3rd Qu.: 8058677
##  Max.   :86532.00   Max.   :35914         Max.   :13107   Max.   :24250760
##
##    Virulence VirulenceFactors      Virus             Mapped
##  Min.   :0   Min.   :   0.00   Min.   :    0.0   Min.   : 519830
##  1st Qu.:0   1st Qu.:   1.75   1st Qu.:  222.8   1st Qu.: 992124
##  Median :0   Median : 647.50   Median :  823.0   Median :1710584
##  Mean   :0   Mean   : 854.94   Mean   : 2620.8   Mean   :2194676
##  3rd Qu.:0   3rd Qu.:1468.00   3rd Qu.: 3505.2   3rd Qu.:2960078
##  Max.   :0   Max.   :3506.00   Max.   :14298.0   Max.   :7243363
##
##   MappedParsed      ReadsNotPhiX      PercentUnmapped  PercentPhiX
##  Min.   : 486505   Min.   : 2594567   Min.   :51.67   Min.   :0.0003660
##  1st Qu.: 977481   1st Qu.: 7628373   1st Qu.:66.91   1st Qu.:0.0008363
##  Median :1633466   Median : 8841916   Median :80.25   Median :0.0011655
##  Mean   :2161458   Mean   : 9710368   Mean   :76.45   Mean   :0.2824303
##  3rd Qu.:2925106   3rd Qu.:11178469   3rd Qu.:88.84   3rd Qu.:0.0018101
##  Max.   :7243155   Max.   :27087990   Max.   :91.65   Max.   :2.3724866
##
```

```
#Using the psych library to create summary for specific parameters. Used to create S1 Table.
#Make column that combine lib prep and seq platform
Metadata2$LPSP<-paste(Metadata2$Library_preparation, Metadata2$Sequencing_platform,
                      sep="_") #Be aware that Nextera 1 & 2 are analyzed as one group.
Metadata3 <- dplyr::select(Metadata2, one_of(c("LPSP", "Reads_Total", "Reads_AfterTrim",
                                               "Mapped", "PercentUnmapped")))
describeBy(Metadata3, Metadata3$LPSP)
```

```
##
##  Descriptive statistics by group
## group: Kappa_Hiseq
##                 vars  n        mean         sd     median    trimmed        mad
## LPSP*              1 16        1.00       0.00       1.00        1.0       0.00
## Reads_Total        2 16 10593744.12 2827116.71 9429034.50 10310564.4 2223801.41
## Reads_AfterTrim    3 16  9569830.19 2795390.14 8203492.00  9293024.4 1946476.63
## Mapped             4 16  2090665.31 1211173.94 1851270.50  2028484.7 1484352.43
## PercentUnmapped    5 16       77.58      13.09      80.57       78.1      12.88
##                       min         max      range  skew kurtosis        se
## LPSP*                1.00        1.00       0.00   NaN      NaN      0.00
## Reads_Total     7770085.00 17381919.00 9611834.00  1.08     0.06 706779.18
## Reads_AfterTrim 6880135.00 16134806.00 9254671.00  1.04    -0.09 698847.53
## Mapped           723943.00  4327916.00 3603973.00  0.42    -1.23 302793.49
## PercentUnmapped      56.72       91.08      34.37 -0.43    -1.54      3.27
## ------------------------------------------------------------
## group: Nextera_Nextseq
##                 vars  n        mean         sd      median     trimmed
## LPSP*              1 32        1.00       0.00        1.00        1.00
## Reads_Total        2 32 10568702.84 2171725.13 10159631.50 10540565.77
## Reads_AfterTrim    3 32  9776944.94 2023848.62  9385629.00  9744943.23
## Mapped             4 32  2592690.12 1902833.80  2078232.00  2411158.19
```

```
## PercentUnmapped    5 32       75.36      14.66       78.79       76.22
##                        mad        min         max       range  skew kurtosis
## LPSP*                 0.00       1.00        1.00        0.00   NaN      NaN
## Reads_Total     2231840.06 6497278.00 16510194.00 10012916.00  0.34     0.04
## Reads_AfterTrim 2143004.90 6022970.00 15338228.00  9315258.00  0.37     0.03
## Mapped          1681296.57  519830.00  7243363.00  6723533.00  0.73    -0.76
## PercentUnmapped      14.55      51.67       91.42       39.75 -0.40    -1.50
##                        se
## LPSP*                0.00
## Reads_Total     383910.39
## Reads_AfterTrim 357769.27
## Mapped          336376.67
## PercentUnmapped      2.59
## ------------------------------------------------------------
## group: Nextflex_Hiseq
##                 vars  n        mean         sd     median     trimmed
## LPSP*              1 16        1.00       0.00        1.0        1.00
## Reads_Total        2 16 14893656.44 8208573.04 12104649.5 14374253.43
## Reads_AfterTrim    3 16 13036491.12 7295407.53 10620802.0 12565742.64
## Mapped             4 16  2337912.56 1160028.02  2423194.0  2239130.71
## PercentUnmapped    5 16       78.07      13.05       80.9       78.59
##                        mad        min         max       range  skew kurtosis
## LPSP*                 0.00       1.00        1.00        0.00   NaN      NaN
## Reads_Total     5170282.10 6294805.00 30764150.00 24469345.00  0.85    -0.83
## Reads_AfterTrim 4614286.34 5314446.00 27349015.00 22034569.00  0.85    -0.82
## Mapped          1056514.84  820592.00  5238179.00  4417587.00  0.66     0.00
## PercentUnmapped      12.94      57.23       91.65       34.42 -0.42    -1.54
##                        se
## LPSP*                0.00
## Reads_Total     2052143.26
## Reads_AfterTrim 1823851.88
## Mapped           290007.01
## PercentUnmapped      3.26
## ------------------------------------------------------------
## group: Nextflex_Nextseq
##                 vars  n      mean         sd     median    trimmed        mad
## LPSP*              1 16       1.0       0.00       1.00       1.00       0.00
## Reads_Total        2 16 6886462.2 2308294.12 7630467.00 7002553.29 2633588.34
## Reads_AfterTrim    3 16 6564709.8 2194564.68 7266381.50 6671380.29 2450715.56
## Mapped             4 16 1359420.0  637118.33 1085288.00 1322755.93  333316.65
## PercentUnmapped    5 16      75.9      14.49      78.91      76.55      14.67
##                        min        max      range  skew kurtosis        se
## LPSP*                 1.00       1.00       0.00   NaN      NaN      0.00
## Reads_Total     2710623.00 9437027.00 6726404.00 -0.33    -1.57 577073.53
## Reads_AfterTrim 2594599.00 9041433.00 6446834.00 -0.33    -1.57 548641.17
## Mapped           679273.00 2552864.00 1873591.00  0.79    -1.05 159279.58
## PercentUnmapped      52.01      90.79      38.78 -0.42    -1.53      3.62
```

```
Metadata3 <- dplyr::select(Metadata2, one_of(c("Experiment", "Reads_Total",
                                               "Reads_AfterTrim", "Mapped",
                                               "PercentUnmapped")))
describeBy(Metadata3, Metadata3$Experiment)
```

```
##
##  Descriptive statistics by group
```

```
## group: Pig_feces_1
##                 vars  n        mean         sd     median     trimmed
## Experiment*        1 20        1.00       0.00       1.00        1.00
## Reads_Total        2 20 10778441.00 4670236.87 9430178.50 10009687.56
## Reads_AfterTrim    3 20  9802157.00 4099187.25 8571205.00  9098167.44
## Mapped             4 20  1206140.90  539057.43 1041951.50  1111624.25
## PercentUnmapped    5 20       87.73       1.23      87.76       87.72
##                       mad        min         max      range skew kurtosis
## Experiment*           0.00       1.00        1.00        0.0  NaN      NaN
## Reads_Total     2230748.87 6176773.00 26738368.00 20561595.0 1.97     4.09
## Reads_AfterTrim 2129123.31 5903434.00 23594456.00 17691022.0 1.90     3.69
## Mapped           321251.99  679273.00  3007972.00  2328699.0 1.87     3.59
## PercentUnmapped       1.48      85.84       89.74        3.9 0.05    -1.53
##                         se
## Experiment*           0.00
## Reads_Total     1044296.71
## Reads_AfterTrim  916606.13
## Mapped           120536.91
## PercentUnmapped       0.28
## ------------------------------------------------------------
## group: Pig_feces_2
##                 vars  n        mean         sd     median     trimmed
## Experiment*        1 20        1.00       0.00       1.00        1.00
## Reads_Total        2 20 12509469.65 7126520.82 9406461.00 10925461.44
## Reads_AfterTrim    3 20 11336395.85 6178439.00 8736953.50 10001248.31
## Mapped             4 20  1134489.50  624486.61  873942.00  1001051.88
## PercentUnmapped    5 20       89.96       1.15      90.23       90.01
##                       mad        min         max       range  skew kurtosis
## Experiment*           0.00       1.00        1.00        0.00   NaN      NaN
## Reads_Total     1267688.23 6497278.00 30764150.00 24266872.00  1.66     1.35
## Reads_AfterTrim 1418566.51 6022970.00 27349015.00 21326045.00  1.64     1.30
## Mapped           217198.68  519830.00  2837230.00  2317400.00  1.62     1.58
## PercentUnmapped       1.34      87.92       91.65        3.73 -0.21    -1.39
##                         se
## Experiment*           0.00
## Reads_Total     1593538.50
## Reads_AfterTrim 1381540.96
## Mapped           139639.45
## PercentUnmapped       0.26
## ------------------------------------------------------------
## group: Sewage_1
##                 vars  n       mean         sd     median    trimmed        mad
## Experiment*        1 20       1.00       0.00       1.00       1.00       0.00
## Reads_Total        2 20 9482881.20 2420426.96 9893242.00 9679118.81 2027319.84
## Reads_AfterTrim    3 20 8685531.75 2194112.26 9106574.00 8873042.19 1692583.60
## Mapped             4 20 2445230.20  653288.97 2694864.00 2496994.31  468380.03
## PercentUnmapped    5 20      71.82       1.93      71.44      71.84       2.72
##                       min         max      range  skew kurtosis        se
## Experiment*           1.00        1.00       0.00   NaN      NaN      0.00
## Reads_Total     4237170.00 13149234.00 8912064.00 -0.55    -0.39 541223.92
## Reads_AfterTrim 4026411.00 11680638.00 7654227.00 -0.63    -0.57 490618.42
## Mapped          1127982.00  3306581.00 2178599.00 -0.70    -0.85 146079.85
## PercentUnmapped      68.81       74.67       5.86 -0.07    -1.42      0.43
## ------------------------------------------------------------
```

```
## group: Sewage_2
##                 vars  n       mean         sd      median     trimmed
## Experiment*        1 20        1.0       0.00        1.00        1.00
## Reads_Total        2 20 10038222.9 3817314.50 11071639.00 10129225.06
## Reads_AfterTrim    3 20  9155852.2 3511934.45 10083087.50  9228667.19
## Mapped             4 20  3992841.9 1593867.50  4211995.00  4005999.12
## PercentUnmapped    5 20       56.3       3.01       56.48       56.29
##                       mad        min         max       range  skew kurtosis
## Experiment*          0.00       1.00        1.00        0.00   NaN      NaN
## Reads_Total     2929857.78 2710623.00 16510194.00 13799571.00 -0.32    -0.95
## Reads_AfterTrim 3082931.04 2594599.00 15338228.00 12743629.00 -0.25    -1.08
## Mapped          1652136.05 1098428.00  7243363.00  6144935.00 -0.04    -0.95
## PercentUnmapped       3.39      51.67       61.21        9.54 -0.08    -1.23
##                       se
## Experiment*         0.00
## Reads_Total     853577.47
## Reads_AfterTrim 785292.42
## Mapped          356399.61
## PercentUnmapped      0.67
```

```
#Combining both LPSP and Experiment
Metadata2$QCperf<-paste(Metadata2$LPSP, Metadata2$Experiment, sep="_")
Metadata3 <- dplyr::select(Metadata2, one_of(c("QCperf", "Reads_Total",
                                               "Reads_AfterTrim", "Mapped",
                                               "PercentUnmapped")))
describeBy(Metadata3, Metadata3$QCperf)
```

```
##
##  Descriptive statistics by group
## group: Kappa_Hiseq_Pig_feces_1
##                 vars n        mean        sd      median     trimmed        mad
## QCperf*            1 4        1.00       0.0        1.00        1.00       0.00
## Reads_Total        2 4 11651621.25 3461471.8 11254465.50 11651621.25 3550220.62
## Reads_AfterTrim    3 4 10560302.00 3568012.5 10196601.00 10560302.00 3793518.98
## Mapped             4 4  1309658.50  571317.0  1257220.50  1309658.50  634166.58
## PercentUnmapped    5 4       87.92       1.4       87.92       87.92       1.79
##                       min         max      range skew kurtosis        se
## QCperf*               1.00        1.00       0.00  NaN      NaN       0.0
## Reads_Total     8239800.00 15857754.00 7617954.00 0.16    -2.17 1730735.9
## Reads_AfterTrim 7065998.00 14782008.00 7716010.00 0.13    -2.22 1784006.3
## Mapped           774764.00  1949429.00 1174665.00 0.09    -2.31  285658.5
## PercentUnmapped      86.62       89.23       2.61 0.00    -2.42       0.7
## ------------------------------------------------------------
## group: Kappa_Hiseq_Pig_feces_2
##                 vars n       mean         sd     median    trimmed        mad
## QCperf*            1 4        1.0       0.00       1.00        1.0       0.00
## Reads_Total        2 4 10844635.8 4401150.04 9057953.00 10844635.8 1110024.10
## Reads_AfterTrim    3 4  9711395.5 4314942.75 7904844.00  9711395.5  960783.36
## Mapped             4 4   950466.2  389276.21  773461.50   950466.2   68226.29
## PercentUnmapped    5 4       90.1       0.85      90.01       90.1       0.91
##                       min         max     range skew kurtosis         se
## QCperf*               1.00        1.00       0.0  NaN      NaN       0.00
## Reads_Total     7880718.00 17381919.00 9501201.0 0.71    -1.72 2200575.02
## Reads_AfterTrim 6901088.00 16134806.00 9233718.0 0.72    -1.71 2157471.38
## Mapped           723943.00  1530999.00  807056.0 0.72    -1.71  194638.10
```

```
## PercentUnmapped      89.28       91.08       1.8 0.12    -2.25       0.42
## ------------------------------------------------------------
## group: Kappa_Hiseq_Sewage_1
##                 vars n       mean         sd     median    trimmed        mad
## QCperf*            1 4       1.00       0.00       1.00       1.00       0.00
## Reads_Total        2 4 9657517.25 1736074.40 9749314.50 9657517.25 2033616.44
## Reads_AfterTrim    3 4 8866168.25 1671035.05 9137876.00 8866168.25 1646175.26
## Mapped             4 4 2342512.25  472515.07 2435971.00 2342512.25  427269.75
## PercentUnmapped    5 4      73.63       0.67      73.48      73.63       0.53
##                        min         max      range  skew kurtosis        se
## QCperf*               1.00        1.00       0.00   NaN      NaN      0.00
## Reads_Total     7770085.00 11361355.00 3591270.00 -0.05    -2.33 868037.20
## Reads_AfterTrim 6880135.00 10308786.00 3428651.00 -0.16    -2.25 835517.52
## Mapped          1753112.00  2744995.00  991883.00 -0.23    -2.17 236257.54
## PercentUnmapped      73.06       74.52       1.46  0.35    -2.04      0.33
## ------------------------------------------------------------
## group: Kappa_Hiseq_Sewage_2
##                 vars n        mean         sd      median     trimmed
## QCperf*            1 4        1.00       0.00        1.00        1.00
## Reads_Total        2 4 10221202.25 1633177.93 10271664.50 10221202.25
## Reads_AfterTrim    3 4  9141455.00 1640000.09  9216352.00  9141455.00
## Mapped             4 4  3760024.25  535306.49  3746326.50  3760024.25
## PercentUnmapped    5 4       58.65       1.73       58.66       58.65
##                        mad        min         max      range  skew kurtosis
## QCperf*               0.00       1.00        1.00       0.00   NaN      NaN
## Reads_Total     1982074.60 8512911.00 11828569.00 3315658.00 -0.03    -2.36
## Reads_AfterTrim 1964324.91 7438475.00 10694641.00 3256166.00 -0.03    -2.37
## Mapped           649783.55 3219528.00  4327916.00 1108388.00  0.02    -2.33
## PercentUnmapped       2.07      56.72       60.57       3.85 -0.01    -2.19
##                        se
## QCperf*              0.00
## Reads_Total     816588.96
## Reads_AfterTrim 820000.04
## Mapped          267653.25
## PercentUnmapped      0.86
## ------------------------------------------------------------
## group: Nextera_Nextseq_Pig_feces_1
##                 vars n       mean         sd     median    trimmed        mad
## QCperf*            1 8       1.00       0.00       1.00       1.00       0.00
## Reads_Total        2 8 9457361.88 2298440.95 8938264.00 9457361.88 1501436.43
## Reads_AfterTrim    3 8 8740719.12 2120294.27 8190030.50 8740719.12 1318636.30
## Mapped             4 8 1102437.00  287114.11 1045559.50 1102437.00  321251.99
## PercentUnmapped    5 8      87.42       1.07      87.44      87.42       1.39
##                        min         max      range  skew kurtosis        se
## QCperf*               1.00        1.00       0.00   NaN      NaN      0.00
## Reads_Total     6640924.00 13364493.00 6723569.00  0.52    -1.35 812621.59
## Reads_AfterTrim 6192563.00 12326109.00 6133546.00  0.53    -1.37 749637.23
## Mapped           700156.00  1499122.00  798966.00  0.14    -1.59 101510.17
## PercentUnmapped      86.06       88.69       2.63 -0.03    -1.97      0.38
## ------------------------------------------------------------
## group: Nextera_Nextseq_Pig_feces_2
##                 vars n       mean         sd     median    trimmed        mad
## QCperf*            1 8       1.00       0.00       1.00       1.00       0.00
## Reads_Total        2 8 9458107.38 1713168.81 9157529.50 9458107.38 1108069.29
```

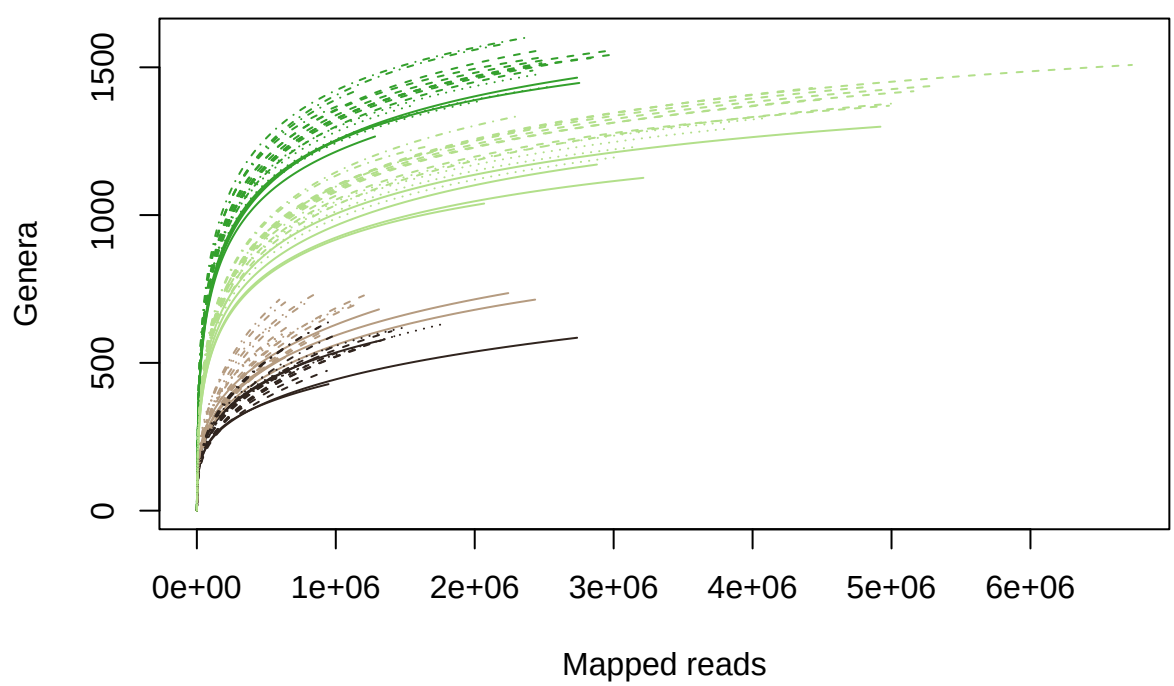
```
## Reads_AfterTrim    3 8 8760619.62 1598181.40 8491885.00 8760619.62 1040381.93
## Mapped             4 8  920752.38  281463.43  885319.00  920752.38  266939.16
## PercentUnmapped    5 8      89.69       1.46      89.72      89.69       2.01
##                        min         max      range  skew kurtosis        se
## QCperf*               1.00        1.00       0.00   NaN      NaN      0.00
## Reads_Total     6497278.00 12178541.00 5681263.00 -0.05    -0.98 605696.64
## Reads_AfterTrim 6022970.00 11295881.00 5272911.00 -0.03    -1.01 565042.45
## Mapped           519830.00  1335944.00  816114.00  0.14    -1.58  99512.35
## PercentUnmapped      87.92       91.42       3.49  0.02    -2.02      0.52
## ------------------------------------------------------------
## group: Nextera_Nextseq_Sewage_1
##                 vars n        mean         sd      median     trimmed       mad
## QCperf*            1 8        1.00       0.00        1.00        1.00      0.00
## Reads_Total        2 8 10519889.38 1009113.05 10156389.00 10519889.38 729330.23
## Reads_AfterTrim    3 8  9701089.88  930914.52  9376177.50  9701089.88 646300.92
## Mapped             4 8  2897175.50  263040.45  2768608.50  2897175.50 138119.02
## PercentUnmapped    5 8       70.11       1.14       69.96       70.11      1.48
##                        min         max      range skew kurtosis        se
## QCperf*               1.00        1.00       0.00  NaN      NaN      0.00
## Reads_Total     9403237.00 12542662.00 3139425.00 0.83    -0.70 356775.34
## Reads_AfterTrim 8720989.00 11604674.00 2883685.00 0.90    -0.57 329127.99
## Mapped          2657342.00  3306581.00  649239.00 0.55    -1.67  92998.84
## PercentUnmapped      68.81       71.51       2.69 0.08    -2.06      0.40
## ------------------------------------------------------------
## group: Nextera_Nextseq_Sewage_2
##                 vars n        mean         sd      median     trimmed
## QCperf*            1 8        1.00       0.00        1.00        1.00
## Reads_Total        2 8 12839452.75 1712890.98 12706266.00 12839452.75
## Reads_AfterTrim    3 8 11905351.12 1622143.30 11805688.00 11905351.12
## Mapped             4 8  5450395.62  795053.24  5332496.00  5450395.62
## PercentUnmapped    5 8       54.21       2.05       54.48       54.21
##                        mad         min         max      range  skew kurtosis
## QCperf*               0.00        1.00        1.00       0.00   NaN      NaN
## Reads_Total     1007403.72 10754736.00 16510194.00 5755458.00  0.95    -0.05
## Reads_AfterTrim  917257.93  9778274.00 15338228.00 5559954.00  0.86    -0.09
## Mapped           445674.75  4725786.00  7243363.00 2517577.00  1.25     0.39
## PercentUnmapped       2.51       51.67       56.24       4.57 -0.10    -2.10
##                        se
## QCperf*              0.00
## Reads_Total     605598.41
## Reads_AfterTrim 573514.26
## Mapped          281093.77
## PercentUnmapped      0.72
## ------------------------------------------------------------
## group: Nextflex_Hiseq_Pig_feces_1
##                 vars n        mean         sd      median     trimmed       mad
## QCperf*            1 4        1.00       0.00        1.00        1.00       0.0
## Reads_Total        2 4 15621734.75 7884993.68 13184081.50 15621734.75 4643123.7
## Reads_AfterTrim    3 4 13628208.00 7066040.60 11261099.50 13628208.00 3930482.3
## Mapped             4 4  1596917.25  967465.87  1239669.00  1596917.25  396462.8
## PercentUnmapped    5 4       88.44       1.34       88.45       88.44       1.7
##                        min         max      range skew kurtosis         se
## QCperf*               1.00        1.00        0.0  NaN      NaN       0.00
## Reads_Total     9380408.00 26738368.00 17357960.0 0.52    -1.88 3942496.84
```

```
## Reads_AfterTrim 8396177.00 23594456.00 15198279.0 0.52    -1.88 3533020.30
## Mapped           900359.00  3007972.00  2107613.0 0.63    -1.77  483732.94
## PercentUnmapped      87.13       89.74        2.6 0.00    -2.40       0.67
## ------------------------------------------------------------
## group: Nextflex_Hiseq_Pig_feces_2
##                 vars n        mean         sd      median     trimmed
## QCperf*            1 4        1.00       0.00        1.00        1.00
## Reads_Total        2 4 23770332.75 9017670.84 26435597.50 23770332.75
## Reads_AfterTrim    3 4 20861852.50 8043898.59 23089103.50 20861852.50
## Mapped             4 4  2005047.75  925004.42  2181184.50  2005047.75
## PercentUnmapped    5 4       90.54       0.98       90.49       90.54
##                       mad         min         max       range  skew kurtosis
## QCperf*              0.00        1.00        1.00        0.00   NaN      NaN
## Reads_Total     6028858.72 11445986.00 30764150.00 19318164.00 -0.43    -1.97
## Reads_AfterTrim 5638089.84  9920188.00 27349015.00 17428827.00 -0.42    -1.98
## Mapped           821714.00   820592.00  2837230.00  2016638.00 -0.27    -2.11
## PercentUnmapped       1.12       89.53       91.65        2.13  0.07    -2.23
##                         se
## QCperf*              0.00
## Reads_Total     4508835.42
## Reads_AfterTrim 4021949.30
## Mapped           462502.21
## PercentUnmapped       0.49
## ------------------------------------------------------------
## group: Nextflex_Hiseq_Sewage_1
##                 vars n        mean         sd      median     trimmed
## QCperf*            1 4        1.00       0.00        1.00        1.00
## Reads_Total        2 4 10102549.00 3310976.59 10178643.50 10102549.00
## Reads_AfterTrim    3 4  8855355.25 3079809.85  8987149.00  8855355.25
## Mapped             4 4  2264576.50  818886.31  2322481.50  2264576.50
## PercentUnmapped    5 4       74.05       0.54       74.08       74.05
##                       mad        min         max      range  skew kurtosis
## QCperf*              0.00       1.00        1.00       0.00   NaN      NaN
## Reads_Total     4118114.24 6903675.00 13149234.00 6245559.00 -0.01    -2.42
## Reads_AfterTrim 3727075.52 5766485.00 11680638.00 5914153.00 -0.02    -2.40
## Mapped           956465.29 1430673.00  2982670.00 1551997.00 -0.03    -2.40
## PercentUnmapped       0.46      73.36       74.67       1.31 -0.14    -1.87
##                         se
## QCperf*              0.00
## Reads_Total     1655488.30
## Reads_AfterTrim 1539904.92
## Mapped           409443.16
## PercentUnmapped       0.27
## ------------------------------------------------------------
## group: Nextflex_Hiseq_Sewage_2
##                 vars n        mean         sd    median     trimmed        mad
## QCperf*            1 4        1.00       0.00       1.0        1.00       0.00
## Reads_Total        2 4 10080009.25 3896249.17 9242607.0 10080009.25 2194594.93
## Reads_AfterTrim    3 4  8800548.75 3556361.41 8059941.5  8800548.75 2107428.43
## Mapped             4 4  3485108.75 1274511.58 3244742.0  3485108.75  899142.79
## PercentUnmapped    5 4       59.26       1.94      59.3       59.26       2.38
##                       min         max      range  skew kurtosis         se
## QCperf*              1.00        1.00       0.00   NaN      NaN       0.00
## Reads_Total     6294805.00 15540018.00 9245213.00  0.45    -1.81 1948124.58
```

```
## Reads_AfterTrim 5314446.00 13767866.00 8453420.00  0.44    -1.82 1778180.71
## Mapped          2212772.00  5238179.00 3025407.00  0.39    -1.85  637255.79
## PercentUnmapped      57.23       61.21       3.98 -0.02    -2.35       0.97
## ------------------------------------------------------------
## group: Nextflex_Nextseq_Pig_feces_1
##                 vars n       mean         sd     median    trimmed        mad
## QCperf*            1 4       1.00       0.00       1.00       1.00       0.00
## Reads_Total        2 4 7704125.25 1451339.96 7630467.00 7704125.25 1644849.07
## Reads_AfterTrim    3 4 7340836.75 1380281.27 7266381.50 7340836.75 1565567.78
## Mapped             4 4  919254.75  171186.87  962799.00  919254.75  110503.37
## PercentUnmapped    5 4      87.43       1.43      87.56      87.43       1.59
##                        min        max      range  skew kurtosis        se
## QCperf*               1.00       1.00       0.00   NaN      NaN      0.00
## Reads_Total     6176773.00 9378794.00 3202021.00  0.07    -2.21 725669.98
## Reads_AfterTrim 5903434.00 8927150.00 3023716.00  0.07    -2.22 690140.64
## Mapped           679273.00 1072148.00  392875.00 -0.48    -1.86  85593.43
## PercentUnmapped      85.84      88.77       2.93 -0.08    -2.32      0.71
## ------------------------------------------------------------
## group: Nextflex_Nextseq_Pig_feces_2
##                 vars n       mean        sd     median    trimmed      mad
## QCperf*            1 4       1.00      0.00       1.00       1.00      0.0
## Reads_Total        2 4 9016165.00 494799.79 9076559.00 9016165.00 532780.5
## Reads_AfterTrim    3 4 8587492.00 461154.61 8610059.50 8587492.00 543296.6
## Mapped             4 4  875428.75 104506.11  873942.00  875428.75 123471.7
## PercentUnmapped    5 4      89.81      1.02      89.81      89.81      1.3
##                        min        max     range  skew kurtosis        se
## QCperf*               1.00       1.00      0.00   NaN      NaN      0.00
## Reads_Total     8474515.00 9437027.00 962512.00 -0.08    -2.35 247399.90
## Reads_AfterTrim 8088416.00 9041433.00 953017.00 -0.05    -2.33 230577.30
## Mapped           760471.00  993360.00 232889.00  0.02    -2.20  52253.05
## PercentUnmapped      88.84      90.79      1.95  0.00    -2.41      0.51
## ------------------------------------------------------------
## group: Nextflex_Nextseq_Sewage_1
##                 vars n       mean         sd     median    trimmed        mad
## QCperf*            1 4       1.00       0.00       1.00       1.00       0.00
## Reads_Total        2 4 6614561.00 2522106.72 6616340.00 6614561.00 3221977.42
## Reads_AfterTrim    3 4 6303955.50 2407287.79 6310983.00 6303955.50 3066912.29
## Mapped             4 4 1824711.25  729409.98 1808999.50 1824711.25  904035.37
## PercentUnmapped    5 4      71.21       0.74      71.33      71.21       0.49
##                       min        max      range  skew kurtosis         se
## QCperf*               1.0       1.00       0.00   NaN      NaN       0.00
## Reads_Total     4237170.0 8988394.00 4751224.00  0.00    -2.42 1261053.36
## Reads_AfterTrim 4026411.0 8567445.00 4541034.00  0.00    -2.42 1203643.89
## Mapped          1127982.0 2552864.00 1424882.00  0.01    -2.39  364704.99
## PercentUnmapped      70.2      71.99       1.78 -0.35    -1.84       0.37
## ------------------------------------------------------------
## group: Nextflex_Nextseq_Sewage_2
##                 vars n       mean        sd     median    trimmed        mad
## QCperf*            1 4       1.00       0.0       1.00       1.00       0.00
## Reads_Total        2 4 4210997.75 1160693.4 4385553.00 4210997.75 1057270.97
## Reads_AfterTrim    3 4 4026554.75 1108468.9 4196444.00 4026554.75 1008951.55
## Mapped             4 4 1818285.25  563268.8 1859071.00 1818285.25  522223.61
## PercentUnmapped    5 4      55.16       2.9      55.48      55.16       3.18
##                        min        max      range  skew kurtosis        se
```

```
## QCperf*               1.00       1.00       0.00   NaN      NaN      0.00
## Reads_Total     2710623.00 5362262.00 2651639.00 -0.26    -2.04 580346.68
## Reads_AfterTrim 2594599.00 5118732.00 2524133.00 -0.26    -2.04 554234.43
## Mapped          1098428.00 2456571.00 1358143.00 -0.15    -1.92 281634.41
## PercentUnmapped      52.01      57.66       5.66 -0.07    -2.36      1.45
```

```
#Create rarefaction curves
#Adding colors to the rarecurves according to experiment, P1=#663300 (blue),
  #P2=#FF9900 (red), S1=#006600 (forestgreen), S2=#33FF33 (gold)
rare<-data.frame(t(Tax2))
rare<-add_rownames(rare, "Sample")
rare$colors<-ifelse(grepl("_P1", rare$Sample), "#30241E",
                    ifelse(grepl("_P2", rare$Sample), "#B59B80",
                           ifelse(grepl("_S1", rare$Sample), "#33a02c",
                                  ifelse(grepl("_S2", rare$Sample), "#B2DF8A",
                                         "pink"))))
rare$line<-ifelse(grepl("HX", rare$Sample), "solid",
                  ifelse(grepl("NX", rare$Sample), "dashed",
                         ifelse(grepl("KAHI", rare$Sample), "dotted",
                                ifelse(grepl("NFNS", rare$Sample), "dotdash",
                                       "longdash"))))

#Plot rarefaction curves can decrease step for final plotting
set.seed(31)
rarecurve(t(Tax2), step=10000, xlab="Mapped reads", ylab="Genera", col=rare$colors,
          lty=rare$line, label=FALSE)
```



```r
pdf(paste("S2_Fig_Rarecurve.pdf", sep=""), height=6, width=8)
rarecurve(t(Tax2), step=10000, xlab="Mapped reads", ylab="Genera", col=rare$colors,
          lty=rare$line, label=FALSE)
dev.off()
```

```
## pdf
##   2
```

```r
#Calculate alpha diversity stats
#Use vegan to calculate various diversity and richness indexes for each sample
set.seed(32)
diversityCalc <- data.frame(Shannon=diversity(t(Tax2), index="shannon"),
                            Simpson=diversity(t(Tax2), index="simpson"),
                            invSimpson=diversity(t(Tax2), index="invsimpson"),
                            fisher=fisher.alpha(t(Tax2)), richness=specnumber(t(Tax2)),
                            rarefy_min_count=rarefy(t(Tax2), sample=min(rowSums(t(Tax2)))),
                            chao1=estimateR(t(Tax2))["S.chao1",],
                            chao1SE=estimateR(t(Tax2))["se.chao1",],
                            ShannonRar=diversity(rrarefy(data.frame(t(Tax2)),
                                                         min(rowSums(t(Tax2)))),
                                                 index="shannon"),
                            SimpsonRar=diversity(rrarefy(data.frame(t(Tax2)),
                                                         min(rowSums(t(Tax2)))),
                                                 index="simpson"),
                            invSimpsonRar=diversity(rrarefy(data.frame(t(Tax2)),
                                                            min(rowSums(t(Tax2)))),
                                                    index="invsimpson"),
                            Pielou=diversity(t(Tax2))/log(specnumber(t(Tax2))))
#Merge with metadata and make S2 Table.
diversityCalc<-add_rownames(diversityCalc, "Sample")
Metadata2<-merge(Metadata2, diversityCalc, by="Sample")

alpha <- dplyr::select(Metadata2, one_of(c("Sample", "Experiment", "LPSP", "QCperf",
                                           "chao1", "Pielou", "Simpson")))
describeBy(alpha, alpha$LPSP)
```

```
##
##  Descriptive statistics by group
## group: Kappa_Hiseq
##             vars  n    mean     sd  median trimmed    mad    min     max
## Sample*        1 16    8.50   4.76    8.50    8.50   5.93   1.00   16.00
## Experiment*    2 16    2.50   1.15    2.50    2.50   1.48   1.00    4.00
## LPSP*          3 16    1.00   0.00    1.00    1.00   0.00   1.00    1.00
## QCperf*        4 16    2.50   1.15    2.50    2.50   1.48   1.00    4.00
## chao1          5 16 1142.76 383.38 1146.41 1143.62 534.46 614.76 1658.75
## Pielou         6 16    0.40   0.08    0.40    0.40   0.10   0.27    0.50
## Simpson        7 16    0.79   0.13    0.82    0.80   0.15   0.57    0.94
##               range  skew kurtosis    se
## Sample*       15.00  0.00    -1.43  1.19
## Experiment*    3.00  0.00    -1.56  0.29
## LPSP*          0.00   NaN      NaN  0.00
## QCperf*        3.00  0.00    -1.56  0.29
## chao1       1043.99  0.00    -1.88 95.84
## Pielou         0.23 -0.12    -1.25  0.02
## Simpson        0.37 -0.41    -1.43  0.03
```

```
## ------------------------------------------------------------
## group: Nextera_Nextseq
##             vars  n    mean     sd  median trimmed    mad    min     max
## Sample*        1 32   16.50   9.38   16.50   16.50  11.86   1.00   32.00
## Experiment*    2 32    2.50   1.14    2.50    2.50   1.48   1.00    4.00
## LPSP*          3 32    1.00   0.00    1.00    1.00   0.00   1.00    1.00
## QCperf*        4 32    2.50   1.14    2.50    2.50   1.48   1.00    4.00
## chao1          5 32 1237.49 416.70 1254.07 1243.28 597.91 650.45 1721.46
## Pielou         6 32    0.37   0.09    0.38    0.37   0.11   0.22    0.48
## Simpson        7 32    0.75   0.17    0.79    0.76   0.17   0.44    0.93
##               range  skew kurtosis    se
## Sample*       31.00  0.00    -1.31  1.66
## Experiment*    3.00  0.00    -1.46  0.20
## LPSP*          0.00   NaN      NaN  0.00
## QCperf*        3.00  0.00    -1.46  0.20
## chao1       1071.00 -0.03    -1.93 73.66
## Pielou         0.26 -0.33    -1.22  0.02
## Simpson        0.49 -0.51    -1.28  0.03
## ------------------------------------------------------------
## group: Nextflex_Hiseq
##             vars  n    mean     sd  median trimmed    mad    min     max
## Sample*        1 16    8.50   4.76    8.50    8.50   5.93   1.00   16.00
## Experiment*    2 16    2.50   1.15    2.50    2.50   1.48   1.00    4.00
## LPSP*          3 16    1.00   0.00    1.00    1.00   0.00   1.00    1.00
## QCperf*        4 16    2.50   1.15    2.50    2.50   1.48   1.00    4.00
## chao1          5 16 1106.89 348.63 1052.48 1105.69 429.56 581.72 1648.99
## Pielou         6 16    0.38   0.08    0.40    0.39   0.11   0.25    0.49
## Simpson        7 16    0.78   0.14    0.80    0.78   0.14   0.55    0.94
##               range  skew kurtosis    se
## Sample*       15.00  0.00    -1.43  1.19
## Experiment*    3.00  0.00    -1.56  0.29
## LPSP*          0.00   NaN      NaN  0.00
## QCperf*        3.00  0.00    -1.56  0.29
## chao1       1067.27  0.12    -1.62 87.16
## Pielou         0.23 -0.15    -1.30  0.02
## Simpson        0.39 -0.43    -1.35  0.03
## ------------------------------------------------------------
## group: Nextflex_Nextseq
##             vars  n    mean     sd  median trimmed    mad    min     max
## Sample*        1 16    8.50   4.76    8.50    8.50   5.93   1.00   16.00
## Experiment*    2 16    2.50   1.15    2.50    2.50   1.48   1.00    4.00
## LPSP*          3 16    1.00   0.00    1.00    1.00   0.00   1.00    1.00
## QCperf*        4 16    2.50   1.15    2.50    2.50   1.48   1.00    4.00
## chao1          5 16 1221.88 347.83 1171.35 1213.03 429.22 775.58 1792.15
## Pielou         6 16    0.38   0.09    0.39    0.38   0.11   0.24    0.50
## Simpson        7 16    0.77   0.15    0.80    0.77   0.16   0.53    0.94
##               range  skew kurtosis    se
## Sample*       15.00  0.00    -1.43  1.19
## Experiment*    3.00  0.00    -1.56  0.29
## LPSP*          0.00   NaN      NaN  0.00
## QCperf*        3.00  0.00    -1.56  0.29
## chao1       1016.57  0.24    -1.65 86.96
## Pielou         0.25 -0.09    -1.34  0.02
## Simpson        0.42 -0.39    -1.38  0.04
```

```r
describeBy(alpha, alpha$QCperf)
```

```
##
##  Descriptive statistics by group
## group: Kappa_Hiseq_Pig_feces_1
##             vars n   mean     sd median trimmed    mad    min    max  range
## Sample*        1 4   2.50   1.29   2.50    2.50   1.48   1.00   4.00   3.00
## Experiment*    2 4   1.00   0.00   1.00    1.00   0.00   1.00   1.00   0.00
## LPSP*          3 4   1.00   0.00   1.00    1.00   0.00   1.00   1.00   0.00
## QCperf*        4 4   1.00   0.00   1.00    1.00   0.00   1.00   1.00   0.00
## chao1          5 4 730.66 115.57 715.12  730.66 109.74 614.76 877.65 262.89
## Pielou         6 4   0.31   0.05   0.31    0.31   0.06   0.27   0.37   0.10
## Simpson        7 4   0.66   0.10   0.66    0.66   0.12   0.57   0.76   0.19
##             skew kurtosis    se
## Sample*     0.00    -2.08  0.65
## Experiment*  NaN      NaN  0.00
## LPSP*        NaN      NaN  0.00
## QCperf*      NaN      NaN  0.00
## chao1       0.23    -2.07 57.78
## Pielou      0.03    -2.39  0.03
## Simpson     0.02    -2.41  0.05
## ------------------------------------------------------------
## group: Kappa_Hiseq_Pig_feces_2
##             vars n   mean    sd median trimmed   mad    min    max  range skew
## Sample*        1 4   2.50  1.29   2.50    2.50  1.48   1.00   4.00   3.00 0.00
## Experiment*    2 4   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00  NaN
## LPSP*          3 4   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00  NaN
## QCperf*        4 4   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00  NaN
## chao1          5 4 840.68 75.04 822.19  840.68 39.32 771.09 947.26 176.17 0.51
## Pielou         6 4   0.36  0.04   0.36    0.36  0.06   0.31   0.40   0.08 0.00
## Simpson        7 4   0.69  0.09   0.69    0.69  0.11   0.61   0.78   0.17 0.00
##             kurtosis    se
## Sample*        -2.08  0.65
## Experiment*      NaN  0.00
## LPSP*            NaN  0.00
## QCperf*          NaN  0.00
## chao1          -1.80 37.52
## Pielou         -2.42  0.02
## Simpson        -2.40  0.04
## ------------------------------------------------------------
## group: Kappa_Hiseq_Sewage_1
##             vars n    mean    sd  median trimmed   mad     min     max  range
## Sample*        1 4    2.50  1.29    2.50    2.50  1.48    1.00    4.00   3.00
## Experiment*    2 4    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## LPSP*          3 4    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## QCperf*        4 4    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## chao1          5 4 1584.50 51.81 1567.07 1584.50 26.50 1545.11 1658.75 113.64
## Pielou         6 4    0.50  0.01    0.50    0.50  0.00    0.49    0.50   0.01
## Simpson        7 4    0.94  0.01    0.94    0.94  0.01    0.93    0.94   0.01
##              skew kurtosis    se
## Sample*      0.00    -2.08  0.65
## Experiment*   NaN      NaN  0.00
## LPSP*         NaN      NaN  0.00
## QCperf*       NaN      NaN  0.00
```

```
## chao1        0.57    -1.83 25.90
## Pielou      -0.62    -1.78  0.00
## Simpson     -0.05    -2.38  0.00
## ------------------------------------------------------------
## group: Kappa_Hiseq_Sewage_2
##             vars n    mean    sd  median trimmed   mad     min     max  range
## Sample*        1 4    2.50  1.29    2.50    2.50  1.48    1.00    4.00   3.00
## Experiment*    2 4    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## LPSP*          3 4    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## QCperf*        4 4    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## chao1          5 4 1415.20 77.31 1411.59 1415.20 93.26 1345.56 1492.07 146.51
## Pielou         6 4    0.42  0.01    0.42    0.42  0.01    0.41    0.42   0.01
## Simpson        7 4    0.87  0.00    0.87    0.87  0.01    0.87    0.87   0.01
##              skew kurtosis    se
## Sample*      0.00    -2.08  0.65
## Experiment*   NaN      NaN  0.00
## LPSP*         NaN      NaN  0.00
## QCperf*       NaN      NaN  0.00
## chao1        0.02    -2.41 38.66
## Pielou      -0.20    -1.94  0.00
## Simpson     -0.07    -2.31  0.00
## ------------------------------------------------------------
## group: Nextera_Nextseq_Pig_feces_1
##             vars n   mean    sd median trimmed   mad    min    max  range  skew
## Sample*        1 8   4.50  2.45   4.50    4.50  2.97   1.00   8.00   7.00  0.00
## Experiment*    2 8   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## LPSP*          3 8   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## QCperf*        4 8   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## chao1          5 8 799.48 75.68 814.84  799.48 66.04 650.45 884.51 234.05 -0.71
## Pielou         6 8   0.27  0.06   0.27    0.27  0.07   0.22   0.34   0.12  0.02
## Simpson        7 8   0.58  0.14   0.57    0.58  0.18   0.44   0.72   0.28  0.01
##             kurtosis    se
## Sample*        -1.65  0.87
## Experiment*      NaN  0.00
## LPSP*            NaN  0.00
## QCperf*          NaN  0.00
## chao1          -0.81 26.76
## Pielou         -2.20  0.02
## Simpson        -2.21  0.05
## ------------------------------------------------------------
## group: Nextera_Nextseq_Pig_feces_2
##             vars n   mean    sd median trimmed   mad    min    max  range  skew
## Sample*        1 8   4.50  2.45   4.50    4.50  2.97   1.00   8.00   7.00  0.00
## Experiment*    2 8   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## LPSP*          3 8   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## QCperf*        4 8   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## chao1          5 8 869.76 96.33 883.46  869.76 94.13 712.63 982.58 269.95 -0.33
## Pielou         6 8   0.31  0.05   0.31    0.31  0.06   0.26   0.36   0.10  0.00
## Simpson        7 8   0.61  0.09   0.62    0.61  0.11   0.53   0.70   0.17 -0.01
##             kurtosis    se
## Sample*        -1.65  0.87
## Experiment*      NaN  0.00
## LPSP*            NaN  0.00
## QCperf*          NaN  0.00
```

```
## chao1          -1.60 34.06
## Pielou         -2.20  0.02
## Simpson        -2.22  0.03
## ------------------------------------------------------------
## group: Nextera_Nextseq_Sewage_1
##             vars n    mean    sd  median trimmed   mad     min     max range
## Sample*        1 8    4.50  2.45    4.50    4.50  2.97    1.00    8.00  7.00
## Experiment*    2 8    1.00  0.00    1.00    1.00  0.00    1.00    1.00  0.00
## LPSP*          3 8    1.00  0.00    1.00    1.00  0.00    1.00    1.00  0.00
## QCperf*        4 8    1.00  0.00    1.00    1.00  0.00    1.00    1.00  0.00
## chao1          5 8 1689.45 35.69 1704.10 1689.45 25.44 1642.44 1721.46 79.02
## Pielou         6 8    0.47  0.01    0.47    0.47  0.00    0.46    0.48  0.02
## Simpson        7 8    0.92  0.00    0.92    0.92  0.00    0.92    0.93  0.01
##              skew kurtosis    se
## Sample*      0.00    -1.65  0.87
## Experiment*   NaN      NaN  0.00
## LPSP*         NaN      NaN  0.00
## QCperf*       NaN      NaN  0.00
## chao1       -0.28    -2.01 12.62
## Pielou      -0.56    -1.25  0.00
## Simpson     -0.28    -1.72  0.00
## ------------------------------------------------------------
## group: Nextera_Nextseq_Sewage_2
##             vars n    mean    sd  median trimmed   mad     min     max  range
## Sample*        1 8    4.50  2.45    4.50    4.50  2.97    1.00    8.00   7.00
## Experiment*    2 8    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## LPSP*          3 8    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## QCperf*        4 8    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## chao1          5 8 1591.28 49.18 1581.63 1591.28 50.83 1525.56 1664.41 138.85
## Pielou         6 8    0.41  0.01    0.41    0.41  0.01    0.39    0.42   0.02
## Simpson        7 8    0.87  0.01    0.87    0.87  0.01    0.86    0.88   0.02
##              skew kurtosis    se
## Sample*      0.00    -1.65  0.87
## Experiment*   NaN      NaN  0.00
## LPSP*         NaN      NaN  0.00
## QCperf*       NaN      NaN  0.00
## chao1        0.21    -1.68 17.39
## Pielou       0.01    -1.76  0.00
## Simpson     -0.02    -1.68  0.00
## ------------------------------------------------------------
## group: Nextflex_Hiseq_Pig_feces_1
##             vars n   mean     sd median trimmed   mad    min    max  range
## Sample*        1 4   2.50   1.29   2.50    2.50  1.48   1.00   4.00   3.00
## Experiment*    2 4   1.00   0.00   1.00    1.00  0.00   1.00   1.00   0.00
## LPSP*          3 4   1.00   0.00   1.00    1.00  0.00   1.00   1.00   0.00
## QCperf*        4 4   1.00   0.00   1.00    1.00  0.00   1.00   1.00   0.00
## chao1          5 4 725.50 103.91 748.52  725.50 72.80 581.72 823.25 241.53
## Pielou         6 4   0.30   0.05   0.30    0.30  0.06   0.25   0.35   0.10
## Simpson        7 4   0.64   0.11   0.64    0.64  0.14   0.55   0.75   0.20
##              skew kurtosis    se
## Sample*      0.00    -2.08  0.65
## Experiment*   NaN      NaN  0.00
## LPSP*         NaN      NaN  0.00
## QCperf*       NaN      NaN  0.00
```

```
## chao1       -0.43    -1.88 51.95
## Pielou       0.01    -2.36  0.02
## Simpson      0.01    -2.42  0.06
## ------------------------------------------------------------
## group: Nextflex_Hiseq_Pig_feces_2
##             vars n   mean    sd median trimmed   mad    min    max  range  skew
## Sample*        1 4   2.50  1.29   2.50    2.50  1.48   1.00   4.00   3.00  0.00
## Experiment*    2 4   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## LPSP*          3 4   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## QCperf*        4 4   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## chao1          5 4 868.22 84.51 892.55  868.22 58.26 753.49 934.29 180.79 -0.41
## Pielou         6 4   0.34  0.05   0.34    0.34  0.06   0.29   0.39   0.10  0.02
## Simpson        7 4   0.68  0.09   0.68    0.68  0.11   0.60   0.76   0.16 -0.01
##             kurtosis    se
## Sample*        -2.08  0.65
## Experiment*      NaN  0.00
## LPSP*            NaN  0.00
## QCperf*          NaN  0.00
## chao1          -1.99 42.26
## Pielou         -2.40  0.03
## Simpson        -2.43  0.04
## ------------------------------------------------------------
## group: Nextflex_Hiseq_Sewage_1
##             vars n    mean    sd  median trimmed   mad     min     max  range
## Sample*        1 4    2.50  1.29    2.50    2.50  1.48    1.00    4.00   3.00
## Experiment*    2 4    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## LPSP*          3 4    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## QCperf*        4 4    1.00  0.00    1.00    1.00  0.00    1.00    1.00   0.00
## chao1          5 4 1547.13 87.86 1538.30 1547.13 94.88 1462.94 1648.99 186.05
## Pielou         6 4    0.48  0.00    0.48    0.48  0.00    0.48    0.49   0.01
## Simpson        7 4    0.93  0.01    0.93    0.93  0.01    0.92    0.94   0.01
##              skew kurtosis    se
## Sample*      0.00    -2.08  0.65
## Experiment*   NaN      NaN  0.00
## LPSP*         NaN      NaN  0.00
## QCperf*       NaN      NaN  0.00
## chao1        0.11    -2.26 43.93
## Pielou      -0.06    -2.33  0.00
## Simpson      0.00    -2.43  0.00
## ------------------------------------------------------------
## group: Nextflex_Hiseq_Sewage_2
##             vars n    mean     sd  median trimmed    mad     min     max  range
## Sample*        1 4    2.50   1.29    2.50    2.50   1.48    1.00    4.00   3.00
## Experiment*    2 4    1.00   0.00    1.00    1.00   0.00    1.00    1.00   0.00
## LPSP*          3 4    1.00   0.00    1.00    1.00   0.00    1.00    1.00   0.00
## QCperf*        4 4    1.00   0.00    1.00    1.00   0.00    1.00    1.00   0.00
## chao1          5 4 1286.72 103.11 1283.13 1286.72 113.11 1170.68 1409.95 239.27
## Pielou         6 4    0.41   0.01    0.41    0.41   0.00    0.40    0.41   0.01
## Simpson        7 4    0.85   0.01    0.86    0.85   0.01    0.85    0.86   0.01
##              skew kurtosis    se
## Sample*      0.00    -2.08  0.65
## Experiment*   NaN      NaN  0.00
## LPSP*         NaN      NaN  0.00
## QCperf*       NaN      NaN  0.00
```

```
## chao1        0.06    -2.08 51.56
## Pielou      -0.54    -1.84  0.00
## Simpson     -0.13    -2.28  0.00
## ------------------------------------------------------------
## group: Nextflex_Nextseq_Pig_feces_1
##             vars n   mean    sd median trimmed   mad    min    max  range  skew
## Sample*        1 4   2.50  1.29   2.50    2.50  1.48   1.00   4.00   3.00  0.00
## Experiment*    2 4   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## LPSP*          3 4   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## QCperf*        4 4   1.00  0.00   1.00    1.00  0.00   1.00   1.00   0.00   NaN
## chao1          5 4 850.17 50.86 868.79  850.17 19.36 775.58 887.53 111.95 -0.65
## Pielou         6 4   0.29  0.05   0.28    0.29  0.06   0.24   0.34   0.09  0.03
## Simpson        7 4   0.62  0.11   0.62    0.62  0.14   0.53   0.73   0.21  0.01
##             kurtosis    se
## Sample*        -2.08  0.65
## Experiment*      NaN  0.00
## LPSP*            NaN  0.00
## QCperf*          NaN  0.00
## chao1          -1.75 25.43
## Pielou         -2.40  0.03
## Simpson        -2.43  0.06
## ------------------------------------------------------------
## group: Nextflex_Nextseq_Pig_feces_2
##             vars n   mean    sd median trimmed   mad    min     max  range
## Sample*        1 4   2.50  1.29   2.50    2.50  1.48   1.00    4.00   3.00
## Experiment*    2 4   1.00  0.00   1.00    1.00  0.00   1.00    1.00   0.00
## LPSP*          3 4   1.00  0.00   1.00    1.00  0.00   1.00    1.00   0.00
## QCperf*        4 4   1.00  0.00   1.00    1.00  0.00   1.00    1.00   0.00
## chao1          5 4 970.67 53.81 951.53  970.67 20.30 930.01 1049.61 119.60
## Pielou         6 4   0.33  0.05   0.33    0.33  0.06   0.29    0.37   0.08
## Simpson        7 4   0.67  0.09   0.67    0.67  0.11   0.58    0.74   0.16
##              skew kurtosis    se
## Sample*      0.00    -2.08  0.65
## Experiment*   NaN      NaN  0.00
## LPSP*         NaN      NaN  0.00
## QCperf*       NaN      NaN  0.00
## chao1        0.65    -1.75 26.90
## Pielou      -0.01    -2.43  0.02
## Simpson     -0.01    -2.43  0.04
## ------------------------------------------------------------
## group: Nextflex_Nextseq_Sewage_1
##             vars n    mean     sd  median trimmed    mad     min     max  range
## Sample*        1 4    2.50   1.29    2.50    2.50   1.48    1.00    4.00   3.00
## Experiment*    2 4    1.00   0.00    1.00    1.00   0.00    1.00    1.00   0.00
## LPSP*          3 4    1.00   0.00    1.00    1.00   0.00    1.00    1.00   0.00
## QCperf*        4 4    1.00   0.00    1.00    1.00   0.00    1.00    1.00   0.00
## chao1          5 4 1656.86 126.52 1664.11 1656.86 139.31 1507.06 1792.15 285.09
## Pielou         6 4    0.49   0.00    0.49    0.49   0.00    0.49    0.50   0.01
## Simpson        7 4    0.93   0.01    0.94    0.93   0.01    0.93    0.94   0.01
##              skew kurtosis    se
## Sample*      0.00    -2.08  0.65
## Experiment*   NaN      NaN  0.00
## LPSP*         NaN      NaN  0.00
## QCperf*       NaN      NaN  0.00
```

```
## chao1       -0.09    -2.15 63.26
## Pielou      -0.46    -1.88  0.00
## Simpson     -0.01    -2.42  0.00
## ------------------------------------------------------------
## group: Nextflex_Nextseq_Sewage_2
##             vars n    mean     sd  median trimmed    mad     min     max  range
## Sample*        1 4    2.50   1.29    2.50    2.50   1.48    1.00    4.00   3.00
## Experiment*    2 4    1.00   0.00    1.00    1.00   0.00    1.00    1.00   0.00
## LPSP*          3 4    1.00   0.00    1.00    1.00   0.00    1.00    1.00   0.00
## QCperf*        4 4    1.00   0.00    1.00    1.00   0.00    1.00    1.00   0.00
## chao1          5 4 1409.83 125.32 1388.94 1409.83 116.41 1293.10 1568.33 275.24
## Pielou         6 4    0.41   0.01    0.41    0.41   0.01    0.40    0.42   0.02
## Simpson        7 4    0.85   0.00    0.85    0.85   0.00    0.85    0.86   0.01
##              skew kurtosis    se
## Sample*      0.00    -2.08  0.65
## Experiment*   NaN      NaN  0.00
## LPSP*         NaN      NaN  0.00
## QCperf*       NaN      NaN  0.00
## chao1        0.24    -2.12 62.66
## Pielou      -0.22    -1.94  0.00
## Simpson      0.05    -2.08  0.00
```

```
write.table(alpha, file="S1_Table_alphaOverview.txt", quote=F, sep="\t")
```

PCA

All, pig feces both P1 and P2, and sewage both S1 and S2 creating S3_Fig

```
rm(list=setdiff(ls(), c("Metadata", "Feature", "Tax")))

#Subset data pig feces 1 and 2 (P1, P2), Sewage 1 and 2 (S1, S2), or spiked unspiked
Subset<- c("PF", "SW", "All") #All, Allspiked, Allunspiked, PF, SW, P1, P1spiked,
  #P1unspiked, P2, P2spiked, P2unspiked, S1, S1spiked, S1unspiked, S2, S2spiked,
  #S2unspiked
#i<-c("All")

#Subset experiment, Experiment_Type. (Meaningfull combinations of subset and SubExp:
  #HX=All, FTX=P1&S1, LTX=P1&S1, LPSX=All unspiked, HXFTX=P1&S1, HXLTX=P1&S1,
  #HXLPSX=All unspiked, HXLTXLPSX=P1&S1 unspiked)
SubExp<-"LPSX" #HX, FTX, LTX, LPSX, HXFTX, HXLTX, HXLPSX, HXLTXLPSX, HXFTXLTX, All

#Subset Frozen Unfrozen
SubFre<-"Both" #Frozen, Unfrozen, Both

# Create a list to hold the plot objects.
ScreeList <- list()
StressList <- list()
PCAList <- list()
vec<-vector()

for (i in Subset) {
#Removing negative and positive controls
Metadata2<-filter(Metadata, Sample_type_simple=="Sample")

#Subsetting Metadata2
```

```r
if (i=="Allspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Spiked")
} else if (i=="Allunspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Unspiked")
} else if (i=="PF") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1" | Experiment == "Pig_feces_2")
} else if (i=="SW") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1" | Experiment == "Sewage_2")
} else if (i=="P1") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1")
} else if (i=="P1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked")
} else if (i=="P1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1")
} else if (i=="P2") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_2")
} else if (i=="P2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2_spiked")
} else if (i=="P2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2")
} else if (i=="S1") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1")
} else if (i=="S1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked")
} else if (i=="S1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1")
} else if (i=="S2") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_2")
} else if (i=="S2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2_spiked")
} else if (i=="S2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2")
} else if (i=="All") {
  print("No subsetting Subset, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubExp=="HX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment")
} else if (SubExp=="FTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="LTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="LPSX") {
  Metadata22<-filter(Metadata2, Experiment_type == "Library_prep_seq_platform_experiment")
  VectorLPSX <- unique(Metadata22$Matching_samples)
  Metadata2<-filter(Metadata2, Matching_samples %in% VectorLPSX)
  rm(VectorLPSX, Metadata22)
} else if (SubExp=="HXFTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment")
```

```
} else if (SubExp=="HXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="HXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXLTXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXFTXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="All") {
  print("No subsetting SubExp, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubFre=="Frozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Freezer")
} else if (SubFre=="Unfrozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Unfrozen")
} else if (SubFre=="Both") {
  print("No subsetting SubFre, all included")
} else {
  print("Subset defined not valid")
}


#Removing the Kappa NextSeq run
Metadata2<-Metadata2[grep("\\KANS.*", Metadata2$Sample, invert=TRUE),]

#Applying subsetting to OTU tables
Tax2<-dplyr::select(Tax, one_of(Metadata2$Sample))

## Create grouping factor
#colnames(Tax2)==Metadata$Sample #Checking order
#New data adding line between same samples different lib prep and seq plat
Metadata2$Sample_LPSX <-
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*_HX_*", Metadata2$Sample),
               "NFHI",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*KAHI*", Metadata2$Sample),
               "KAHI",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*NFNS*", Metadata2$Sample),
               "NFNS",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*NX1NS*", Metadata2$Sample),
               "NX1NS",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*NX2NS*", Metadata2$Sample),
               "NX2NS",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*KANS*", Metadata2$Sample),
```

```
                "KANS",
                "Other")))))) #The seq along returns true or false where the regex
  #is fulfilled and then stores the evaluated string on the new column be aware
  #HX is actually true 1-8 don't know why


## Can use different filtering strategies. 1 Bayseian approach estimating zeroes,
  #2 offset of 1 and removing all rows containing zeroes. Only minor effects observed
# f <- codaSeq.filter(ak_op, min.reads=1000, min.prop=0.005, min.occurrence=0.2,
  #samples.by.row=FALSE) #Implemented in Gloors codaSeq.filter function
#Assessing what is the max value of rows containing a zero
print("Max in rows containing a zero")
row_sub = apply(Tax2, 1, function(row) all(row !=0 ))
Tax2[row_sub,] %>% max() %>% print()
# filtering of the Counttable depending on rowSums.
Tax2 <- Tax2[rowSums(Tax2)>0,] #Removing all rows that only contains zeroes
Tax2 <- Tax2[rowSums(Tax2)>(5*ncol(Tax2)),] #Removing all rows(Species)
  #that is below an average count of 5.
# replace 0 values with an estimate using simple multiplicative replacement
Tax2 <- t(cmultRepl(t(Tax2), method="CZM", label=0))

## Maks TSS
Tax2<-sweep(Tax2, 2, colSums(Tax2), FUN="/")

Y <- Metadata2$Sample_LPSX

PCACoDa = mixOmics::pca(t(Tax2), ncomp = 10, logratio = 'ILR')#Can change CLR and ILR
#plot(PCACoDa)

#plotIndiv(PCACoDa,
#        comp = c(1,2), # the components to plot
#        pch = 16,
#        ind.names = F,
#        group = Y,
#        col.per.group = color.mixo(1:5),
#        legend = TRUE,
#        title = 'PCA comp 1 - 2')

# Calculate the variation explained by PCoA1 and 2
# and use it to generate axis labels
eig_1 <- paste("PCA1", round(PCACoDa$explained_variance[1]*100, digits = 1), "% variance")
eig_2 <- paste("PCA2", round(PCACoDa$explained_variance[2]*100, digits = 1), "% variance")
eig_3 <- paste("PCA3", round(PCACoDa$explained_variance[3]*100, digits = 1), "% variance")
eig_4 <- paste("PCA4", round(PCACoDa$explained_variance[4]*100, digits = 1), "% variance")

##Pull out coordinates for plotting from the ca object
#Structuring to add to metadata
PCAMeta<-data.frame(Sample=PCACoDa$names$sample, PCA1=PCACoDa$variates$X[,1],
                    PCA2=PCACoDa$variates$X[,2], PCA3=PCACoDa$variates$X[,3],
                    PCA4=PCACoDa$variates$X[,4])
#Merge according to
Metadata2<-merge(Metadata2, PCAMeta, by="Sample")
#Creating column in metadata for plotting with lines between replicates
```

```r
Metadata2$Sample_name_norep<-gsub("*_a|*_b|*_c", "", Metadata2$Sample_name)
#Change Storage and time to characters for plotting
Metadata2$Storage<-as.character(Metadata2$Storage)
Metadata2$Time<-as.character(Metadata2$Time)
#Change NAs in Storage to Direct
Metadata2$Storage[is.na(Metadata2$Storage)]<-"Direct"
#Change order for coloring
Metadata2$Storage<-ordered(Metadata2$Storage, levels=c("Direct", "-80", "-20", "5", "22"))

#Create plot name
pltName <- paste( 'PCA', i, sep = '' )
#create PCA
PCAList[[ pltName ]] <- ggplot(Metadata2) +
  geom_point(aes(PCA1, PCA2, color = Experiment, group = Sample), size=3) +
  #geom_line(aes(x=PCA1, y=PCA2, group=Replicate_Boxplot)) +
  #geom_line(aes(x=PCA1, y=PCA2, group=Matching_samples), size=0.1, linetype="dotted") +
  scale_color_manual(values=c(Pig_feces_1 = "#30241E", Pig_feces_2 = "#B59B80",
                              Sewage_1 = "#33a02c", Sewage_2 = "#B2DF8A")) +
  ggtitle(paste("PCA", i, sep=" ")) +
  labs(colour="Sample", x = eig_1, y = eig_2) +
  theme_bw() +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.title=element_text(size=12), legend.position="none")
}
```

```
## [1] "No subsetting SubFre, all included"
## [1] "Max in rows containing a zero"
## [1] 1783518
## No. corrected values:  21
## [1] "No subsetting SubFre, all included"
## [1] "Max in rows containing a zero"
## [1] 1581043
## No. corrected values:  140
## [1] "No subsetting Subset, all included"
## [1] "No subsetting SubFre, all included"
## [1] "Max in rows containing a zero"
## [1] 1783518
## No. corrected values:  4863
```

```r
#Draw PCA
#No validation plots
#Have the plots stored in lists
lay <- rbind(c(1,2),
             c(1,3))
#Make pdf
pdf(paste("S3_Fig_PCAAll", ".pdf", sep=""), width=9, height=6)
grid.arrange(PCAList$PCAAll, PCAList$PCAPF, PCAList$PCASW, layout_matrix = lay)
dev.off()
```

```
## pdf
##   2
```

```r
#Make format for word
#png(paste("PCA", "GenusTall",  "NoVal", ".png", sep=""), width=900, height=600)
#grid.arrange(PCAList$PCAP1unspiked, PCAList$PCAP2unspiked, PCAList$PCAS1unspiked,
```

```
#             PCAList$PCAS2unspiked, layout_matrix = lay)
#dev.off()

#Extract legend
#legend<-ggplot(Metadata2) +
#  geom_point(aes(PCA1, PCA2, color = Sample_LPSX, group = Sample, shape = Storage),
  #size=1.5) +
#  #geom_line(aes(x=PCA1, y=PCA2, group=Replicate_Boxplot)) +
#  geom_line(aes(x=PCA1, y=PCA2, group=Matching_samples), size=0.1, linetype="dotted") +
#  scale_color_manual(values=c("#377eb8", "#4daf4a", "#984ea3", "#ff7f00", "#e41a1c")) +
#  scale_shape_manual(values=c(8,18,15,16,17,0,1,2)) +
#  ggtitle(paste("PCA", i, sep=" ")) +
#  labs(colour="Storage / ?C", shape="Processing", x = eig_1, y = eig_2) +
#  theme_bw() +
#  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
  #axis.title=element_text(size=12))
#legendplot<-get_legend(legend)
#pdf(paste("LegendPCA", ".pdf", sep=""), width=24, height=12)
#grid.arrange(legendplot)
#dev.off()
#png(paste("LegendPCA", ".png", sep=""), width=100, height=300)
#grid.arrange(legendplot)
#dev.off()

legend2<-ggplot(Metadata2) +
  geom_point(aes(PCA1, PCA2, color = Experiment, group = Sample), size=5) +
  #geom_line(aes(x=PCA1, y=PCA2, group=Replicate_Boxplot)) +
  #geom_line(aes(x=PCA1, y=PCA2, group=Matching_samples), size=0.1, linetype="dotted") +
  scale_color_manual(values=c(Pig_feces_1 = "#30241E", Pig_feces_2 = "#B59B80",
                              Sewage_1 = "#33a02c", Sewage_2 = "#B2DF8A")) +
  ggtitle(paste("PCA", i, sep=" ")) +
  labs(colour="Sample", x = eig_1, y = eig_2) +
  theme_bw() +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.title=element_text(size=12), legend.position="bottom")
legendplot<-get_legend(legend2)
pdf(paste("S3_Fig_PCAAllLegend", ".pdf", sep=""), width=10, height=1)
grid.arrange(legendplot)
dev.off()
```

```
## pdf
##   2
```

```
#png(paste("LegendPCAbottom", ".png", sep=""), width=600, height=25)
#grid.arrange(legendplot)
#dev.off()
```

PCA Fig_2

Subset to P1, P2, S1 and S2 creating Fig_2

```
rm(list=setdiff(ls(), c("Metadata", "Feature", "Tax")))

#Subset data pig feces 1 and 2 (P1, P2), Sewage 1 and 2 (S1, S2), or spiked unspiked
Subset<- c("P1unspiked", "P2unspiked", "S1unspiked", "S2unspiked") #All, Allspiked,
```

```
  #Allunspiked, PF, SW, P1, P1spiked, P1unspiked, P2, P2spiked, P2unspiked, S1, S1spiked,
  #S1unspiked, S2, S2spiked, S2unspiked

#Subset experiment, Experiment_Type. (Meaningfull combinations of subset and SubExp:
  #HX=All, FTX=P1&S1, LTX=P1&S1, LPSX=All unspiked, HXFTX=P1&S1, HXLTX=P1&S1,
  #HXLPSX=All unspiked, HXLTXLPSX=P1&S1 unspiked)
SubExp<-"LPSX" #HX, FTX, LTX, LPSX, HXFTX, HXLTX, HXLPSX, HXLTXLPSX, HXFTXLTX, All

#Subset Frozen Unfrozen
SubFre<-"Both" #Frozen, Unfrozen, Both

# Create a list to hold the plot objects.
ScreeList <- list()
StressList <- list()
PCAList <- list()
vec<-vector()

i<-c("P1unspiked")

for (i in Subset) {
#Removing negative and positive controls
Metadata2<-filter(Metadata, Sample_type_simple=="Sample")

#Subsetting Metadata2
if (i=="Allspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Spiked")
} else if (i=="Allunspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Unspiked")
} else if (i=="PF") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1" | Experiment == "Pig_feces_2")
} else if (i=="SW") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1" | Experiment == "Sewage_2")
} else if (i=="P1") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1")
} else if (i=="P1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked")
} else if (i=="P1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1")
} else if (i=="P2") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_2")
} else if (i=="P2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2_spiked")
} else if (i=="P2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2")
} else if (i=="S1") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1")
} else if (i=="S1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked")
} else if (i=="S1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1")
} else if (i=="S2") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_2")
} else if (i=="S2spiked") {
```

```r
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2_spiked")
} else if (i=="S2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2")
} else if (i=="All") {
  print("No subsetting Subset, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubExp=="HX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment")
} else if (SubExp=="FTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="LTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="LPSX") {
  Metadata22<-filter(Metadata2, Experiment_type == "Library_prep_seq_platform_experiment")
  VectorLPSX <- unique(Metadata22$Matching_samples)
  Metadata2<-filter(Metadata2, Matching_samples %in% VectorLPSX)
  rm(VectorLPSX, Metadata22)
} else if (SubExp=="HXFTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="HXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="HXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXLTXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXFTXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="All") {
  print("No subsetting SubExp, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubFre=="Frozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Freezer")
} else if (SubFre=="Unfrozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Unfrozen")
} else if (SubFre=="Both") {
  print("No subsetting SubFre, all included")
} else {
  print("Subset defined not valid")
```

```
}


#Removing the Kappa NextSeq run
Metadata2<-Metadata2[grep("\\KANS.*", Metadata2$Sample, invert=TRUE),]

#Applying subsetting to OTU tables
Tax2<-dplyr::select(Tax, one_of(Metadata2$Sample))

## Create grouping factor
#colnames(Tax2)==Metadata$Sample #Checking order
#New data adding line between same samples different lib prep and seq plat
Metadata2$Sample_LPSX <-
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*_HX_*", Metadata2$Sample),
               "NFHI",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*KAHI*", Metadata2$Sample),
               "KAHI",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*NFNS*", Metadata2$Sample),
               "NFNS",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*NX1NS*", Metadata2$Sample),
               "NX1NS",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*NX2NS*", Metadata2$Sample),
               "NX2NS",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*KANS*", Metadata2$Sample),
               "KANS",
               "Other")))))) #The seq along returns true or false where the regex is
  #fulfilled and then stores the evaluated string on the new column be aware HX is
  #actually true 1-8 don't know why


## Can use different filtering strategies. 1 Bayseian approach estimating zeroes,
  #2 offset of 1 and removing all rows containing zeroes. Only minor effects observed
# f <- codaSeq.filter(ak_op, min.reads=1000, min.prop=0.005, min.occurrence=0.2,
  #samples.by.row=FALSE) #Implemented in Gloors codaSeq.filter function
#Assessing what is the max value of rows containing a zero
print("Max in rows containing a zero")
row_sub = apply(Tax2, 1, function(row) any(row !=0 ))
Tax2[row_sub,] %>% max() %>% print()
# filtering of the Counttable depending on rowSums.
Tax2 <- Tax2[rowSums(Tax2)>0,] #Removing all rows that only contains zeroes
Tax2 <- Tax2[rowSums(Tax2)>(5*ncol(Tax2)),] #Removing all rows(Species)
  #that is below an average count of 5.
# replace 0 values with an estimate using simple multiplicative replacement
Tax2 <- t(cmultRepl(t(Tax2), method="CZM", label=0))

## Maks TSS
Tax2<-sweep(Tax2, 2, colSums(Tax2), FUN="/")

Y <- Metadata2$Sample_LPSX

PCACoDa = mixOmics::pca(t(Tax2), ncomp = 10, logratio = 'ILR')#Can change CLR and ILR
#plot(PCACoDa)
```

```r
#plotIndiv(PCACoDa,
#        comp = c(1,2), # the components to plot
#        pch = 16,
#        ind.names = F,
#        group = Y,
#        col.per.group = color.mixo(1:5),
#        legend = TRUE,
#        title = 'PCA comp 1 - 2')

# Calculate the variation explained by PCoA1 and 2
# and use it to generate axis labels
eig_1 <- paste("PCA1", round(PCACoDa$explained_variance[1]*100, digits = 1), "% variance")
eig_2 <- paste("PCA2", round(PCACoDa$explained_variance[2]*100, digits = 1), "% variance")
eig_3 <- paste("PCA3", round(PCACoDa$explained_variance[3]*100, digits = 1), "% variance")
eig_4 <- paste("PCA4", round(PCACoDa$explained_variance[4]*100, digits = 1), "% variance")

##Pull out coordinates for plotting from the ca object
#Structuring to add to metadata
PCAMeta<-data.frame(Sample=PCACoDa$names$sample, PCA1=PCACoDa$variates$X[,1],
                    PCA2=PCACoDa$variates$X[,2], PCA3=PCACoDa$variates$X[,3],
                    PCA4=PCACoDa$variates$X[,4])
#Merge according to
Metadata2<-merge(Metadata2, PCAMeta, by="Sample")
#Creating column in metadata for plotting with lines between replicates
Metadata2$Sample_name_norep<-gsub("*_a|*_b|*_c", "", Metadata2$Sample_name)
#Change Storage and time to characters for plotting
Metadata2$Storage<-as.character(Metadata2$Storage)
Metadata2$Time<-as.character(Metadata2$Time)
#Change NAs in Storage to Direct
Metadata2$Storage[is.na(Metadata2$Storage)]<-"Direct"
#Change order for coloring
Metadata2$Storage<-ordered(Metadata2$Storage, levels=c("Direct", "-80", "-20", "5", "22"))

#Create plot name
pltName <- paste( 'PCA', i, sep = '' )
#create PCA
PCAList[[ pltName ]] <- ggplot(Metadata2) +
  geom_point(aes(PCA1, PCA2, color = Sample_LPSX, group = Sample, shape = Storage),
             size=5) +
  #geom_line(aes(x=PCA1, y=PCA2, group=Replicate_Boxplot)) +
  geom_line(aes(x=PCA1, y=PCA2, group=Matching_samples), size=0.1, linetype="dotted") +
  scale_color_manual(values=c("#984ea3", "#4daf4a", "#377eb8", "#ff7f00", "#993300")) +
  scale_shape_manual(values=c(8,18,15,16,17,0,1,2)) +
  ggtitle(paste("PCA", i, sep=" ")) +
  labs(colour="Processing", shape="Storage (?C)", x = eig_1, y = eig_2) +
  theme_bw() +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.title=element_text(size=12), legend.position="none")
}
```

```
## [1] "No subsetting SubFre, all included"
## [1] "Max in rows containing a zero"
## [1] 1783518
## No. corrected values:  3
```

```
## [1] "No subsetting SubFre, all included"
## [1] "Max in rows containing a zero"
## [1] 1528286
## [1] "No subsetting SubFre, all included"
## [1] "Max in rows containing a zero"
## [1] 538286
## [1] "No subsetting SubFre, all included"
## [1] "Max in rows containing a zero"
## [1] 1581043
## No. corrected values:  24
```

```
#Draw PCA
#No validation plots
#Have the plots stored in lists
#lay <- rbind(c(1,2,3,4))
#Make pdf
#pdf(paste("PCA", "Genus", "NoVal", ".pdf", sep=""), width=24, height=12)
#grid.arrange(PCAList$PCAP1unspiked, PCAList$PCAP2unspiked, PCAList$PCAS1unspiked,
  #PCAList$PCAS2unspiked, layout_matrix = lay)
#dev.off()
#Make format for word
#png(paste("PCA", "Genus",  "NoVal", ".png", sep=""), width=1200, height=600)
#grid.arrange(PCAList$PCAP1unspiked, PCAList$PCAP2unspiked, PCAList$PCAS1unspiked,
  #PCAList$PCAS2unspiked, layout_matrix = lay)
#dev.off()


#No validation plots
#Have the plots stored in lists
lay <- rbind(c(1,2),
             c(3,4))
#Make pdf
pdf(paste("Fig2_PCASubset", ".pdf", sep=""), width=9, height=6)
grid.arrange(PCAList$PCAP1unspiked, PCAList$PCAP2unspiked, PCAList$PCAS1unspiked,
             PCAList$PCAS2unspiked, layout_matrix = lay)
dev.off()
```

```
## pdf
##   2
```

```
#Make format for word
#png(paste("PCA", "GenusTall",  "NoVal", ".png", sep=""), width=900, height=600)
#grid.arrange(PCAList$PCAP1unspiked, PCAList$PCAP2unspiked, PCAList$PCAS1unspiked,
  #PCAList$PCAS2unspiked, layout_matrix = lay)
#dev.off()

#Extract legend
#legend<-ggplot(Metadata2) +
#  geom_point(aes(PCA1, PCA2, color = Sample_LPSX, group = Sample, shape = Storage),
  #size=1.5) +
#  #geom_line(aes(x=PCA1, y=PCA2, group=Replicate_Boxplot)) +
#  geom_line(aes(x=PCA1, y=PCA2, group=Matching_samples), size=0.1, linetype="dotted") +
#  scale_color_manual(values=c("#377eb8", "#4daf4a", "#984ea3", "#ff7f00", "#e41a1c")) +
#  scale_shape_manual(values=c(8,18,15,16,17,0,1,2)) +
#  ggtitle(paste("PCA", i, sep=" ")) +
```

```
#  labs(colour="Storage / ?C", shape="Processing", x = eig_1, y = eig_2) +
#  theme_bw() +
#  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
  #axis.title=element_text(size=12))
#legendplot<-get_legend(legend)
#pdf(paste("LegendPCA", ".pdf", sep=""), width=24, height=12)
#grid.arrange(legendplot)
#dev.off()
#png(paste("LegendPCA", ".png", sep=""), width=100, height=300)
#grid.arrange(legendplot)
#dev.off()

legend2<-ggplot(Metadata2) +
  geom_point(aes(PCA1, PCA2, color = Sample_LPSX, group = Sample, shape = Storage),
             size=5) +
  #geom_line(aes(x=PCA1, y=PCA2, group=Replicate_Boxplot)) +
  geom_line(aes(x=PCA1, y=PCA2, group=Matching_samples), size=0.1, linetype="dotted") +
  scale_color_manual(values=c("#984ea3", "#4daf4a", "#377eb8", "#ff7f00", "#993300")) +
  scale_shape_manual(values=c(8,18,15,16,17,0,1,2)) +
  ggtitle(paste("PCA", i, sep=" ")) +
  labs(colour="Processing", shape="Storage / ?C", x = eig_1, y = eig_2) +
  theme_bw() +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.title=element_text(size=12), legend.position="bottom")
legendplot<-get_legend(legend2)
pdf(paste("Fig2_PCASubsetLegend", ".pdf", sep=""), width=10, height=1)
grid.arrange(legendplot)
dev.off()
```

```
## pdf
##   2
```

```
#png(paste("LegendPCAbottom", ".png", sep=""), width=600, height=25)
#grid.arrange(legendplot)
#dev.off()
```

Heatmaps

Pig feces both P1 and P2, and sewage both S1 and S2 creating Fig_3. Have added a heatmap of the negative controls S5_Fig

```
rm(list=setdiff(ls(), c("Metadata", "Feature", "Tax")))

#Subset data pig feces 1 and 2 (PF)
Subset<- "PF" #All, Allspiked, Allunspiked, PF, SW, P1, P1spiked, P1unspiked, P2,
  #P2spiked, P2unspiked, S1, S1spiked, S1unspiked, S2, S2spiked, S2unspiked

#Subset experiment, Experiment_Type. (Meaningfull combinations of subset and SubExp:
  #HX=All, FTX=P1&S1, LTX=P1&S1, LPSX=All unspiked, HXFTX=P1&S1, HXLTX=P1&S1,
  #HXLPSX=All unspiked, HXLTXLPSX=P1&S1 unspiked)
SubExp<-"LPSX" #HX, FTX, LTX, LPSX, HXFTX, HXLTX, HXLPSX, HXLTXLPSX, HXFTXLTX, All

#Subset Frozen Unfrozen
SubFre<-"Both" #Frozen, Unfrozen, Both
```

```r
#How many organisms to include in heatmap
Orgs<-30 #Write a number 30-50 seems appropiate for readability

HeatmapExplainers<-c("Sequencing_platform", "Library_preparation", "Storage","Experiment")

#Make list of annotation colors
annotation_colorsNew = list(Diagosis = c(Handling_experiment = "blue"),
                            Experiment = c(Pig_feces_1 = "#30241E",
                                           Pig_feces_2 = "#B59B80",
                                           Sewage_1 = "#33a02c",
                                           Sewage_2 = "#B2DF8A"),
                            SpikedUnspiked = c(Spiked = "Black",
                                               Unspiked = "White"),
                            Time = c("0" = "#FFFFFF", "16" = "#CCCCCC", "64" = "#666666"),
                            Storage = c(Direct = "#999999", "-80" = "#0571b0",
                                        "-20" = "#92c5de", "5" = "#f4a582",
                                        "22" = "#ca0020"),
                            Sequencing_platform = c(Hiseq = "Black", Nextseq = "White"),
                            Library_preparation = c(Kappa = "#984ea3", Nextera = "#ff7f00",
                                                    Nextflex = "#e41a1c"))

#make empty list for plots
HeatList=list()

#Removing negative and positive controls
Metadata2<-filter(Metadata, Sample_type_simple=="Sample")

#Subsetting Metadata2
if (Subset=="Allspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Spiked")
} else if (Subset=="Allunspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Unspiked")
} else if (Subset=="PF") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1" | Experiment == "Pig_feces_2")
} else if (Subset=="PFspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked" |
                      Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="PFunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1" |
                      Sample_type == "Pig_feces_2")
} else if (Subset=="SW") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1" | Experiment == "Sewage_2")
} else if (Subset=="SWspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked" |
                      Sample_type == "Sewage_2_spiked")
} else if (Subset=="SWunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1" | Sample_type == "Sewage_2")
} else if (Subset=="P1") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1")
} else if (Subset=="P1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked")
} else if (Subset=="P1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1")
```

```
} else if (Subset=="P2") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_2")
} else if (Subset=="P2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="P2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2")
} else if (Subset=="S1") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1")
} else if (Subset=="S1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked")
} else if (Subset=="S1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1")
} else if (Subset=="S2") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_2")
} else if (Subset=="S2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2_spiked")
} else if (Subset=="S2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2")
} else if (Subset=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}


#Further subsetting Metadata2
if (SubExp=="HX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment")
} else if (SubExp=="FTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="LTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="LPSX") {
  Metadata22<-filter(Metadata2, Experiment_type == "Library_prep_seq_platform_experiment")
  VectorLPSX <- unique(Metadata22$Matching_samples)
  Metadata2<-filter(Metadata2, Matching_samples %in% VectorLPSX)
  rm(VectorLPSX, Metadata22)
} else if (SubExp=="HXFTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="HXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="HXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXLTXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXFTXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment" |
```

```
                        Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubFre=="Frozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Freezer")
} else if (SubFre=="Unfrozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Unfrozen")
} else if (SubFre=="Both") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

```
## [1] "No subsetting, all included"
```

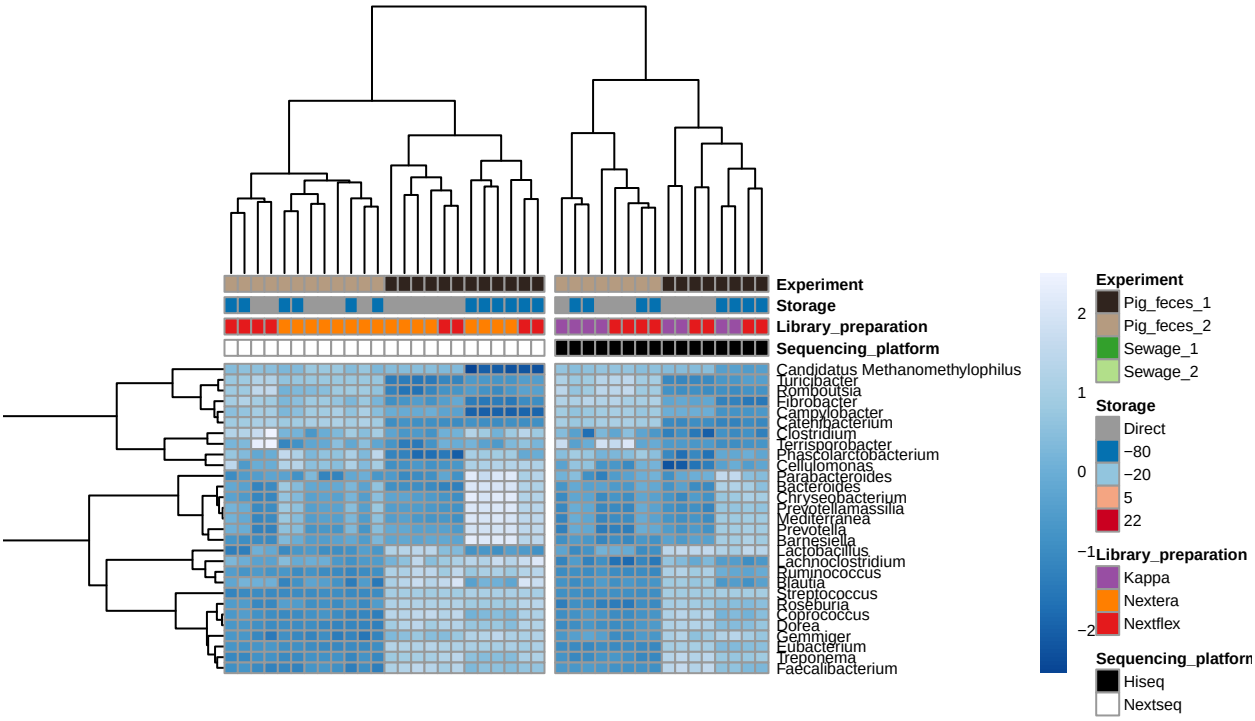
```
#Removing the Kappa NextSeq run
Metadata2<-Metadata2[grep("\\KANS.*", Metadata2$Sample, invert=TRUE),]

#Applying subsetting to OTU tables
Tax2<-dplyr::select(Tax, one_of(Metadata2$Sample))

#Remove orgs that are not present after subsetting. Need for doing standardize
Tax2 <- Tax2[rowSums(Tax2)>0,]

##Hellinger transformation
#TaxHeatmap <- data.frame(t(decostand(t(Tax2), method=Stand)))
TaxHeatmap <- Tax2

#Order genera, based on rowsums
TaxHeatmap <- Tax2[order(rowSums(TaxHeatmap), decreasing = T),]

#Impose a maximum number of plotted Species
TaxHeatmap <- TaxHeatmap[1:min(c(nrow(TaxHeatmap), Orgs)),]

##Remove Unknown from plot
TaxHeatmap <- TaxHeatmap[row.names(TaxHeatmap) != "Unknown",]

#The organisms clustering
OrgCluster<-amap::Dist(TaxHeatmap, method="spearman")

#filtering of the Counttable depending on rowSums.
TaxHeatmap <- TaxHeatmap[rowSums(TaxHeatmap)>0,] #Removing all rows that only contains
  #zeroes
TaxHeatmap <- TaxHeatmap[rowSums(TaxHeatmap)>(5*ncol(TaxHeatmap)),] #Removing all
  #rows(Species)  that is below an average count of 5.
## replace 0 values with an estimate using simple multiplicative replacement
#TaxHeatmap <- t(cmultRepl(t(TaxHeatmap), method="CZM", label=0))
#Maks TSS
TaxHeatmap<-sweep(TaxHeatmap, 2, colSums(TaxHeatmap), FUN="/")
```

```r
rn <- row.names(TaxHeatmap)

##Then I standardized the orgs into zero mean and unit variance
TaxHeatmap <- data.frame(t(decostand(clr(t(TaxHeatmap)), method="standardize")),
                         row.names = rn)
  #Can also use scale in pheatmap, but not exactly sure what scaling that is being
  #performed

##This is what I did previously. Now I changed it to do a Hellinger transformation,
  #which is total sum scaling and then square rooting each entry.
  #Doing it on total sum scaled data since then it is also normalized according to
  #genome size so in reality I'm doing total sum scaling twice.
  #Moved to the top to do it before subsetting.
#Log transform OTU table with higher base pick one.
#TaxHeatmap <- log(TaxHeatmap+1, base = exp(200))
#Log 10 transform OTU table
#TaxHeatmap <- log10(TaxHeatmap+1)


#Make dataframe with Metadata2 for heatmap annotation
colannodf <- data.frame(Metadata2[, HeatmapExplainers], row.names = Metadata2$Sample)
#Depending on if time and Storage are part of the plots make them into characters
#colannodf$Time <- as.character(colannodf$Time)
colannodf$Storage <- as.character(colannodf$Storage)
colannodf$Storage[is.na(colannodf$Storage)] <- "Direct" #Change NA to Direct

#Calculate sample-distance matrix
#Note, that this is done on the full set, not just the shown. Makes sense eventhough not
  #show in heatmap they can be in the clustering calculations, this also means samples
  #can look more similar in the heatmap but not cluster as closely. Also calculates on
  #the not log transformed data.
#filtering of the Counttable depending on rowSums.
Tax2 <- Tax2[rowSums(Tax2)>0,] #Removing all rows that only contains zeroes
Tax2 <- Tax2[rowSums(Tax2)>(5*ncol(Tax2)),] #Removing all rows(Species)
  #that is below an average count of 5.
# replace 0 values with an estimate using simple multiplicative replacement
Tax2 <- t(cmultRepl(t(Tax2), method="CZM", label=0))
```

```
## No. corrected values:  21
```

```r
#Maks TSS
Tax2<-sweep(Tax2, 2, colSums(Tax2), FUN="/")
#Calculate sample-distance matrix
#Note, that this is done on the full set, not just the shown. Makes sense eventhough not
  #show in heatmap they can be in the clustering calculations, this also means samples
  #can look more similar in the heatmap but not cluster as closely. Also calculates on
  #the not log transformed data.
distmatrix_Species <- vegdist(ilr(t(Tax2)), method="euclidean") #Previously
  #vegdist(decostand(t(Tax2), method="hellinger"), method="bray")



#Draw the heatmap
plot<-pheatmap(TaxHeatmap,
```

```
        color = colorRampPalette(rev(brewer.pal(n = 7, name = "Blues")))(100),
        margins=c(8,8),
        treeheight_row = 100,
        treeheight_col = 100,
        scale="none",
        clustering_distance_cols = distmatrix_Species,
        clustering_distance_rows = OrgCluster,
        annotation_col = colannodf,
        cutree_cols = 2,
        show_colnames = FALSE,
        cellwidth=5,
        cellheight=4,
        fontsize=6,
        annotation_colors = annotation_colorsNew[1:7],
        annotation_legend = TRUE)
```




```
HeatList[[Subset]] = plot[[4]]




#Subset data sewage 1 and 2 (SW)
Subset<- "SW" #All, Allspiked, Allunspiked, PF, SW, P1, P1spiked, P1unspiked, P2,
  #P2spiked, P2unspiked, S1, S1spiked, S1unspiked, S2, S2spiked, S2unspiked
```

```r
#Removing negative and positive controls
Metadata2<-filter(Metadata, Sample_type_simple=="Sample")

#Subsetting Metadata2
if (Subset=="Allspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Spiked")
} else if (Subset=="Allunspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Unspiked")
} else if (Subset=="PF") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1" | Experiment == "Pig_feces_2")
} else if (Subset=="PFspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked" |
                      Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="PFunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1" |
                      Sample_type == "Pig_feces_2")
} else if (Subset=="SW") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1" | Experiment == "Sewage_2")
} else if (Subset=="SWspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked" |
                      Sample_type == "Sewage_2_spiked")
} else if (Subset=="SWunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1" | Sample_type == "Sewage_2")
} else if (Subset=="P1") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1")
} else if (Subset=="P1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked")
} else if (Subset=="P1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1")
} else if (Subset=="P2") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_2")
} else if (Subset=="P2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="P2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2")
} else if (Subset=="S1") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1")
} else if (Subset=="S1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked")
} else if (Subset=="S1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1")
} else if (Subset=="S2") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_2")
} else if (Subset=="S2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2_spiked")
} else if (Subset=="S2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2")
} else if (Subset=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

```r
#Further subsetting Metadata2
if (SubExp=="HX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment")
} else if (SubExp=="FTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="LTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="LPSX") {
  Metadata22<-filter(Metadata2, Experiment_type == "Library_prep_seq_platform_experiment")
  VectorLPSX <- unique(Metadata22$Matching_samples)
  Metadata2<-filter(Metadata2, Matching_samples %in% VectorLPSX)
  rm(VectorLPSX, Metadata22)
} else if (SubExp=="HXFTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="HXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="HXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXLTXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXFTXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubFre=="Frozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Freezer")
} else if (SubFre=="Unfrozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Unfrozen")
} else if (SubFre=="Both") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

```
## [1] "No subsetting, all included"
```

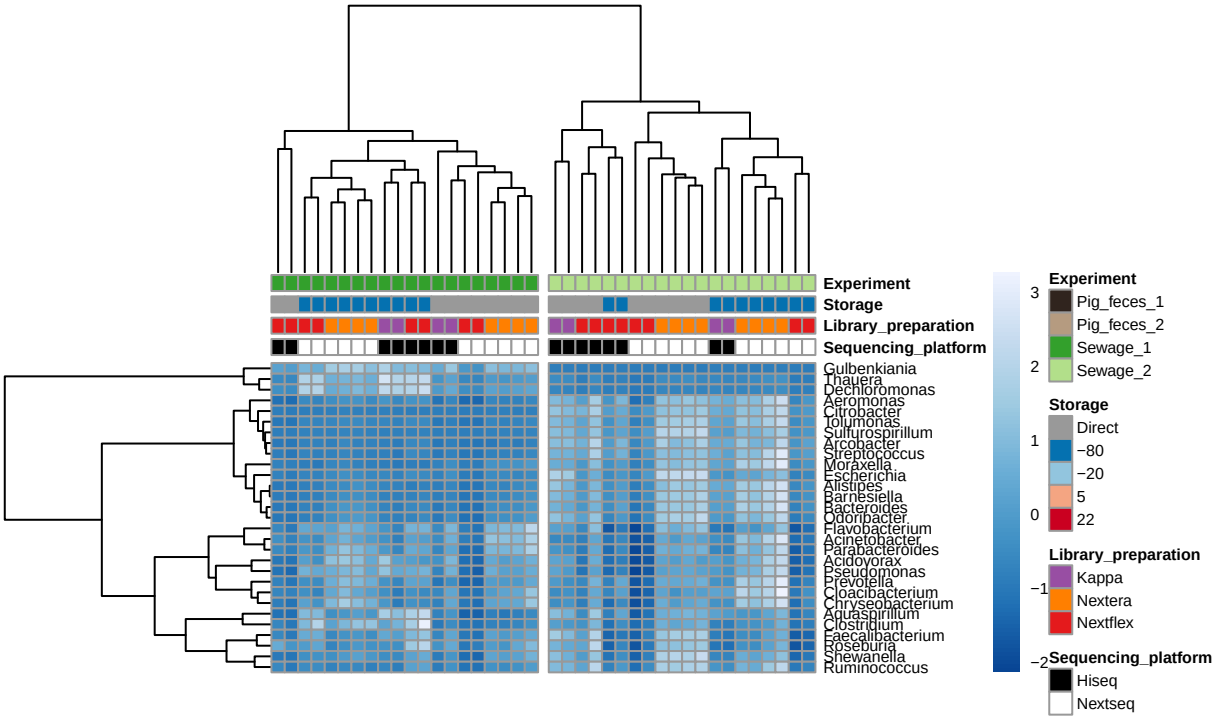
```r
#Removing the Kappa NextSeq run
Metadata2<-Metadata2[grep("\\KANS.*", Metadata2$Sample, invert=TRUE),]

#Applying subsetting to OTU tables
Tax2<-dplyr::select(Tax, one_of(Metadata2$Sample))
```

```r
#Remove orgs that are not present after subsetting. Need for doing standardize
Tax2 <- Tax2[rowSums(Tax2)>0,]

##Hellinger transformation
#TaxHeatmap <- data.frame(t(decostand(t(Tax2), method=Stand)))
TaxHeatmap <- Tax2

#Order genera, based on rowsums
TaxHeatmap <- Tax2[order(rowSums(TaxHeatmap), decreasing = T),]

#Impose a maximum number of plotted Species
TaxHeatmap <- TaxHeatmap[1:min(c(nrow(TaxHeatmap), Orgs)),]

##Remove Unknown from plot
TaxHeatmap <- TaxHeatmap[row.names(TaxHeatmap) != "Unknown",]

#The organisms clustering
OrgCluster<-amap::Dist(TaxHeatmap, method="spearman")

##Then I standardized the orgs into zero mean and unit variance
TaxHeatmap <- data.frame(t(decostand(t(TaxHeatmap), method="standardize"))) #Can also use
  #scale in pheatmap, but not exactly sure what scaling that is being performed


#Make dataframe with Metadata2 for heatmap annotation
colannodf <- data.frame(Metadata2[, HeatmapExplainers], row.names = Metadata2$Sample)
#Depending on if time and Storage are part of the plots make them into characters
#colannodf$Time <- as.character(colannodf$Time)
colannodf$Storage <- as.character(colannodf$Storage)
colannodf$Storage[is.na(colannodf$Storage)] <- "Direct" #Change NA to Direct

#Calculate sample-distance matrix
#Note, that this is done on the full set, not just the shown. Makes sense eventhough not
  #show in heatmap they can be in the clustering calculations, this also means samples
  #can look more similar in the heatmap but not cluster as closely. Also calculates on
  #the not log transformed data.
#filtering of the Counttable depending on rowSums.
Tax2 <- Tax2[rowSums(Tax2)>0,] #Removing all rows that only contains zeroes
Tax2 <- Tax2[rowSums(Tax2)>(5*ncol(Tax2)),] #Removing all rows(Species)  that is below an
  #average count of 5.
# replace 0 values with an estimate using simple multiplicative replacement
Tax2 <- t(cmultRepl(t(Tax2), method="CZM", label=0))
```

```
## No. corrected values:  140
```

```r
#Maks TSS
Tax2<-sweep(Tax2, 2, colSums(Tax2), FUN="/")
#Calculate sample-distance matrix
#Note, that this is done on the full set, not just the shown. Makes sense eventhough not
  #show in heatmap they can be in the clustering calculations, this also means samples
  #can look more similar in the heatmap but not cluster as closely. Also calculates on
  #the not log transformed data.
distmatrix_Species <- vegdist(ilr(t(Tax2)), method="euclidean") #Previously
  #vegdist(decostand(t(Tax2), method="hellinger"), method="bray")
```

```
#Draw the heatmap
plot<-pheatmap(TaxHeatmap,
         color = colorRampPalette(rev(brewer.pal(n = 7, name = "Blues")))(100),
         margins=c(8,8),
         treeheight_row = 100,
         treeheight_col = 100,
         scale="none",
         clustering_distance_cols = distmatrix_Species,
         clustering_distance_rows = OrgCluster,
         annotation_col = colannodf,
         cutree_cols = 2,
         show_colnames = FALSE,
         cellwidth=5,
         cellheight=4,
         fontsize=6,
         annotation_colors = annotation_colorsNew[1:7],
         annotation_legend = TRUE)
```




```
HeatList[[Subset]] = plot[[4]]

#grid.arrange(HeatList$PF)
#grid.arrange(HeatList$SW)

#Have the plots stored in lists
lay <- rbind(c(1,2))
```

```r
pdf(paste("Fig3_Heatmap", ".pdf", sep=""), width=15, height=5)
grid.arrange(HeatList$PF, HeatList$SW, layout_matrix = lay)
dev.off()
```

```
## pdf
##   2
```

```r
#Make heatmap of negative controls
#Subset data sewage 1 and 2 (SW)
Subset<- "All" #All, Allspiked, Allunspiked, PF, SW, P1, P1spiked, P1unspiked, P2,
  #P2spiked, P2unspiked, S1, S1spiked, S1unspiked, S2, S2spiked, S2unspiked

#Selecting negative controls
Metadata2<-filter(Metadata, Sample_type_simple=="Negative_control")

#Subsetting Metadata2
if (Subset=="Allspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Spiked")
} else if (Subset=="Allunspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Unspiked")
} else if (Subset=="PF") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1" |
                      Experiment == "Pig_feces_2")
} else if (Subset=="PFspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked" |
                      Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="PFunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1" |
                      Sample_type == "Pig_feces_2")
} else if (Subset=="SW") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1" | Experiment == "Sewage_2")
} else if (Subset=="SWspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked" |
                      Sample_type == "Sewage_2_spiked")
} else if (Subset=="SWunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1" | Sample_type == "Sewage_2")
} else if (Subset=="P1") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1")
} else if (Subset=="P1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked")
} else if (Subset=="P1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1")
} else if (Subset=="P2") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_2")
} else if (Subset=="P2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="P2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2")
} else if (Subset=="S1") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1")
} else if (Subset=="S1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked")
} else if (Subset=="S1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1")
```

```r
} else if (Subset=="S2") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_2")
} else if (Subset=="S2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2_spiked")
} else if (Subset=="S2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2")
} else if (Subset=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

```
## [1] "No subsetting, all included"
```

```r
#Further subsetting Metadata2
if (SubExp=="HX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment")
} else if (SubExp=="FTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="LTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="LPSX") {
  Metadata22<-filter(Metadata2, Experiment_type == "Library_prep_seq_platform_experiment")
  VectorLPSX <- unique(Metadata22$Matching_samples)
  Metadata2<-filter(Metadata2, Matching_samples %in% VectorLPSX)
  rm(VectorLPSX, Metadata22)
} else if (SubExp=="HXFTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="HXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="HXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXLTXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXFTXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubFre=="Frozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Freezer")
} else if (SubFre=="Unfrozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Unfrozen")
```

```r
} else if (SubFre=="Both") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

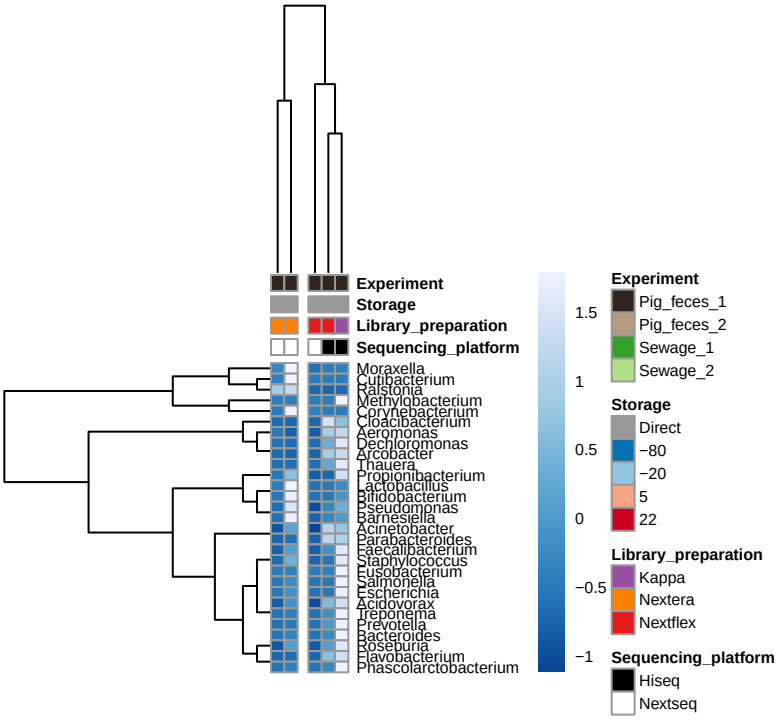
```
## [1] "No subsetting, all included"
```

```r
#Removing the Kappa NextSeq run
Metadata2<-Metadata2[grep("\\KANS.*", Metadata2$Sample, invert=TRUE),]

#Applying subsetting to OTU tables
Tax2<-dplyr::select(Tax, one_of(Metadata2$Sample))

#Remove orgs that are not present after subsetting. Need for doing standardize
Tax2 <- Tax2[rowSums(Tax2)>0,]
#Add row names to samples to manually search the organisms observed in sPLS-DA
searchMan <- add_rownames(Tax2, "Orgs")

##Hellinger transformation
#TaxHeatmap <- data.frame(t(decostand(t(Tax2), method=Stand)))
TaxHeatmap <- Tax2

#Order genera, based on rowsums
TaxHeatmap <- Tax2[order(rowSums(TaxHeatmap), decreasing = T),]

#Impose a maximum number of plotted Species
TaxHeatmap <- TaxHeatmap[1:min(c(nrow(TaxHeatmap), Orgs)),]

##Remove Unknown from plot
TaxHeatmap <- TaxHeatmap[row.names(TaxHeatmap) != "Unknown",]

#The organisms clustering
OrgCluster<-amap::Dist(TaxHeatmap, method="spearman")

##Then I standardized the orgs into zero mean and unit variance
TaxHeatmap <- data.frame(t(decostand(t(TaxHeatmap), method="standardize"))) #Can also use
  #scale in pheatmap, but not exactly sure what scaling that is being performed


#Make dataframe with Metadata2 for heatmap annotation
colannodf <- data.frame(Metadata2[, HeatmapExplainers], row.names = Metadata2$Sample)
#Depending on if time and Storage are part of the plots make them into characters
#colannodf$Time <- as.character(colannodf$Time)
colannodf$Storage <- as.character(colannodf$Storage)
colannodf$Storage[is.na(colannodf$Storage)] <- "Direct" #Change NA to Direct

#Calculate sample-distance matrix
#Note, that this is done on the full set, not just the shown. Makes sense eventhough not
  #show in heatmap they can be in the clustering calculations, this also means samples can
  #look more similar in the heatmap but not cluster as closely. Also calculates on the not
  #log transformed data.
#filtering of the Counttable depending on rowSums.
Tax2 <- Tax2[rowSums(Tax2)>0,] #Removing all rows that only contains zeroes
```

```r
Tax2 <- Tax2[rowSums(Tax2)>(5*ncol(Tax2)),] #Removing all rows(Species)  that is below an
  #average count of 5.
# replace 0 values with an estimate using simple multiplicative replacement
Tax2 <- t(cmultRepl(t(Tax2), method="CZM", label=0))
```

```
## No. corrected values:  42
```

```r
#Maks TSS
Tax2<-sweep(Tax2, 2, colSums(Tax2), FUN="/")
#Calculate sample-distance matrix
#Note, that this is done on the full set, not just the shown. Makes sense eventhough not
  #show in heatmap they can be in the clustering calculations, this also means samples can
  #look more similar in the heatmap but not cluster as closely. Also calculates on the not
  #log transformed data.
distmatrix_Species <- vegdist(ilr(t(Tax2)), method="euclidean") #Previously
  #vegdist(decostand(t(Tax2), method="hellinger"), method="bray")

#Draw the heatmap
plot<-pheatmap(TaxHeatmap,
         color = colorRampPalette(rev(brewer.pal(n = 7, name = "Blues")))(100),
         margins=c(8,8),
         treeheight_row = 100,
         treeheight_col = 100,
         scale="none",
         clustering_distance_cols = distmatrix_Species,
         clustering_distance_rows = OrgCluster,
         annotation_col = colannodf,
         cutree_cols = 2,
         show_colnames = FALSE,
         cellwidth=5,
         cellheight=4,
         fontsize=6,
         annotation_colors = annotation_colorsNew[1:7],
         annotation_legend = TRUE)
```




```
HeatList[[Subset]] = plot[[4]]

#grid.arrange(HeatList$PF)
#grid.arrange(HeatList$SW)

#Have the plots stored in lists
lay <- rbind(c(1))
pdf(paste("S5_Fig_HeatmapNegCon", ".pdf", sep=""), width=7.5, height=5)
grid.arrange(HeatList$All, layout_matrix = lay)
dev.off()
```

```
## pdf
##   2
```

Boxplots of distances between samples

Grouped according to different parameters (Sample, Storage, Library preparation, sequencing platform, Replicates) to create Fig_1

```
rm(list=setdiff(ls(), c("Metadata", "Feature", "Tax")))

Subset <- "All" #All, Allspiked, Allunspiked, PF, SW, P1, P1spiked, P1unspiked, P2,
  #P2spiked, P2unspiked, S1, S1spiked, S1unspiked, S2, S2spiked, S2unspiked

#Subset experiment, Experiment_Type. (Meaningfull combinations of subset and SubExp:
  #HX=All, FTX=P1&S1, LTX=P1&S1, LPSX=All unspiked, HXFTX=P1&S1, HXLTX=P1&S1,
  #HXLPSX=All unspiked, HXLTXLPSX=P1&S1 unspiked)
```

```r
SubExp<-"LPSX" #HX, FTX, LTX, LPSX, HXFTX, HXLTX, HXLPSX, HXLTXLPSX, HXFTXLTX, All

#Subset Frozen Unfrozen
SubFre<-"Both" #Frozen, Unfrozen, Both

#Removing negative and positive controls
Metadata2<-filter(Metadata, Sample_type_simple=="Sample")

#Subsetting Metadata2
if (Subset=="Allspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Spiked")
} else if (Subset=="Allunspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Unspiked")
} else if (Subset=="PF") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1" | Experiment == "Pig_feces_2")
} else if (Subset=="PFspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked" |
                      Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="PFunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1" |
                      Sample_type == "Pig_feces_2")
} else if (Subset=="SW") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1" | Experiment == "Sewage_2")
} else if (Subset=="SWspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked" |
                      Sample_type == "Sewage_2_spiked")
} else if (Subset=="SWunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1" | Sample_type == "Sewage_2")
} else if (Subset=="P1") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1")
} else if (Subset=="P1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked")
} else if (Subset=="P1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1")
} else if (Subset=="P2") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_2")
} else if (Subset=="P2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="P2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2")
} else if (Subset=="S1") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1")
} else if (Subset=="S1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked")
} else if (Subset=="S1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1")
} else if (Subset=="S2") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_2")
} else if (Subset=="S2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2_spiked")
} else if (Subset=="S2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2")
} else if (Subset=="All") {
```

```r
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

```
## [1] "No subsetting, all included"
```

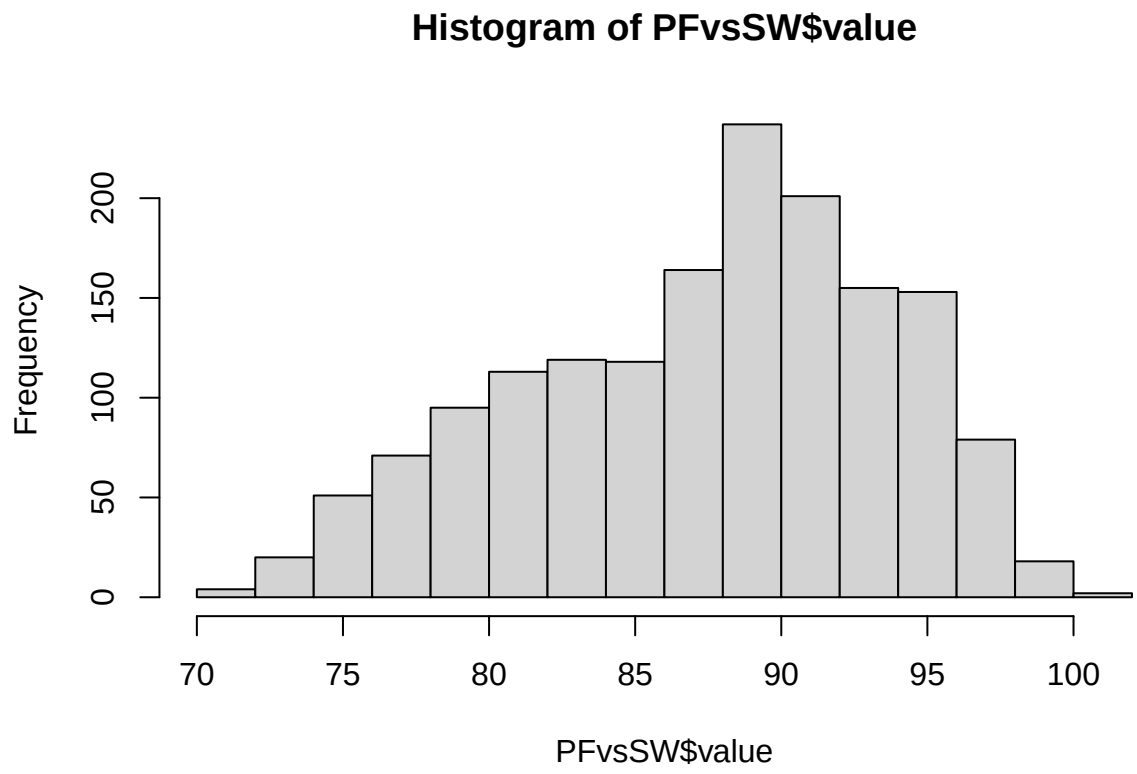
```r
#Further subsetting Metadata2
if (SubExp=="HX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment")
} else if (SubExp=="FTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="LTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="LPSX") {
  Metadata22<-filter(Metadata2, Experiment_type == "Library_prep_seq_platform_experiment")
  VectorLPSX <- unique(Metadata22$Matching_samples)
  Metadata2<-filter(Metadata2, Matching_samples %in% VectorLPSX)
  rm(VectorLPSX, Metadata22)
} else if (SubExp=="HXFTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="HXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="HXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXLTXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXFTXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubFre=="Frozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Freezer")
} else if (SubFre=="Unfrozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Unfrozen")
} else if (SubFre=="Both") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

```
## [1] "No subsetting, all included"
```

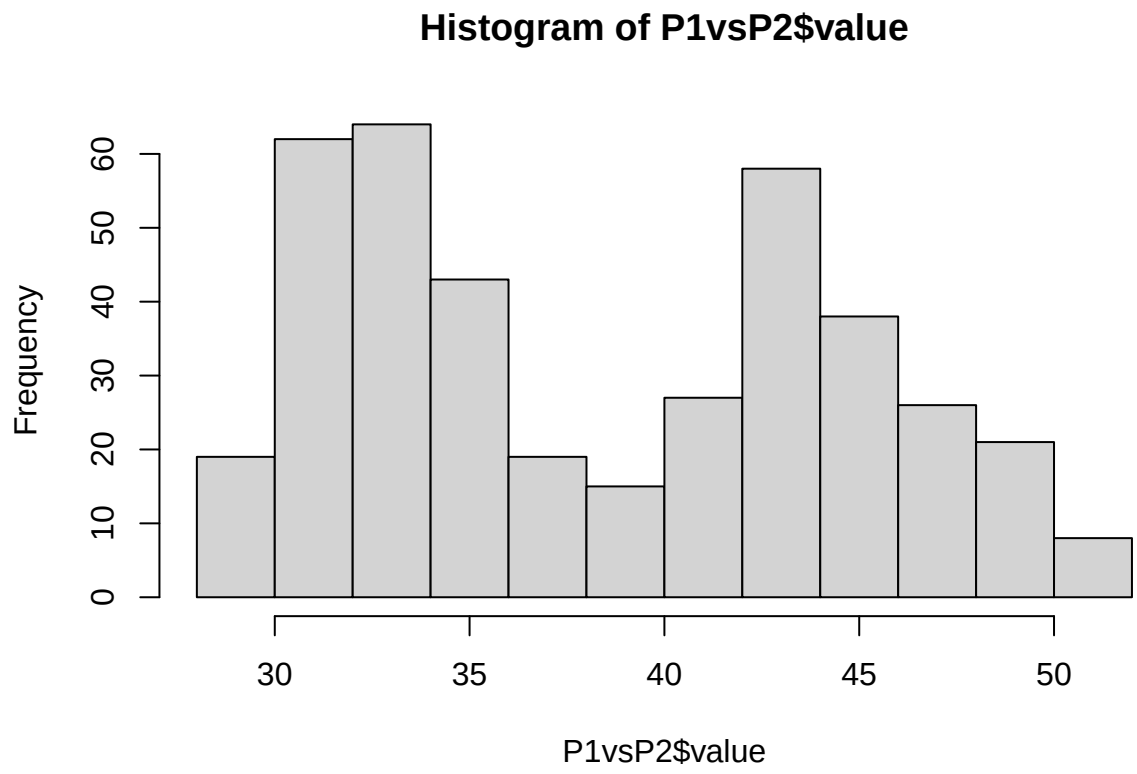
```r
#Removing the Kappa NextSeq run
Metadata2<-Metadata2[grep("\\KANS.*", Metadata2$Sample, invert=TRUE),]

#Applying subsetting to OTU tables
Tax2<-dplyr::select(Tax, one_of(Metadata2$Sample))

#Calculate sample-distance matrix
#Note, that this is done on the full set, not just the shown. Makes sense eventhough not
  #show in heatmap they can be in the clustering calculations, this also means samples can
  #look more similar in the heatmap but not cluster as closely. Also calculates on the not
  #log transformed data.
#filtering of the Counttable depending on rowSums.
Tax2 <- Tax2[rowSums(Tax2)>0,] #Removing all rows that only contains zeroes
Tax2 <- Tax2[rowSums(Tax2)>(5*ncol(Tax2)),] #Removing all rows(Species)  that is below an average count
# replace 0 values with an estimate using simple multiplicative replacement
Tax2 <- t(cmultRepl(t(Tax2), method="CZM", label=0))
```

```
## No. corrected values:  4863
```

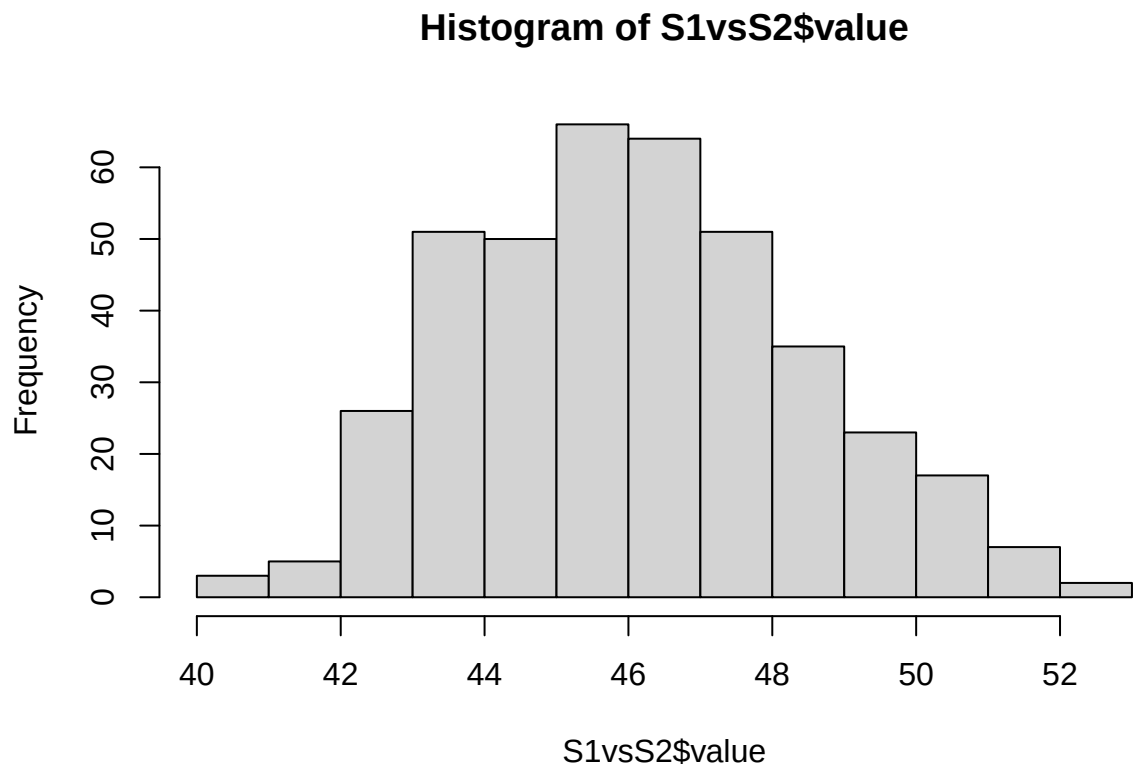
```r
#Maks TSS
Tax2<-sweep(Tax2, 2, colSums(Tax2), FUN="/")
#Calculate sample-distance matrix
#Note, that this is done on the full set, not just the shown. Makes sense eventhough not
  #show in heatmap they can be in the clustering calculations, this also means samples can
  #look more similar in the heatmap but not cluster as closely. Also calculates on the not
  #log transformed data.
distmatrix <- vegdist(ilr(t(Tax2)), method="euclidean") #Previously
  #vegdist(decostand(t(Tax2), method="hellinger"), method="bray")

#Make distances into matrix
distmatrix<-as.matrix(distmatrix)

#Contains all values except the ones equal to 0
meltdist<-subset(melt(distmatrix), value!=0)
rm(distmatrix)

##Extract info on specific comparisons
#Distance between pig feces and sewage
#Select comparisons where PF is part of the first variable
PFvsSW<-filter(meltdist, grepl('P1|P2', Var1))
#From this exclude comparisons to other pig feces samples
PFvsSW<-filter(PFvsSW, !grepl('P1|P2', Var2))
#Histogram to look at distribution
hist(PFvsSW$value)
```



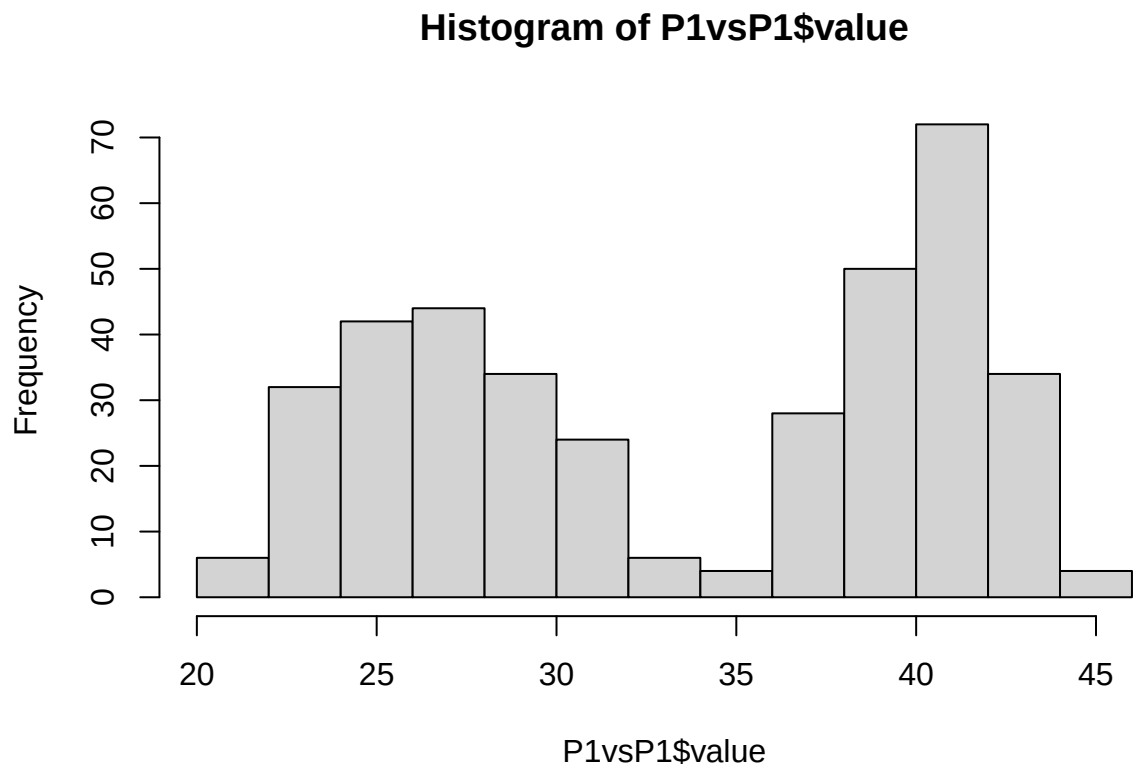

```
#Add jitter column
PFvsSW<-mutate(PFvsSW, jitter="PFvsSW")
#Add Comparison column
PFvsSW<-mutate(PFvsSW, Comparison="PFvsSW")


#Distance between the pig feces samples
#Select comparisons where P1 is part of the first variable
P1vsP2<-filter(meltdist, grepl('P1', Var1))
#From this include comparisons to P2
P1vsP2<-filter(P1vsP2, grepl('P2', Var2))
#Histogram to look at distribution
hist(P1vsP2$value)
```



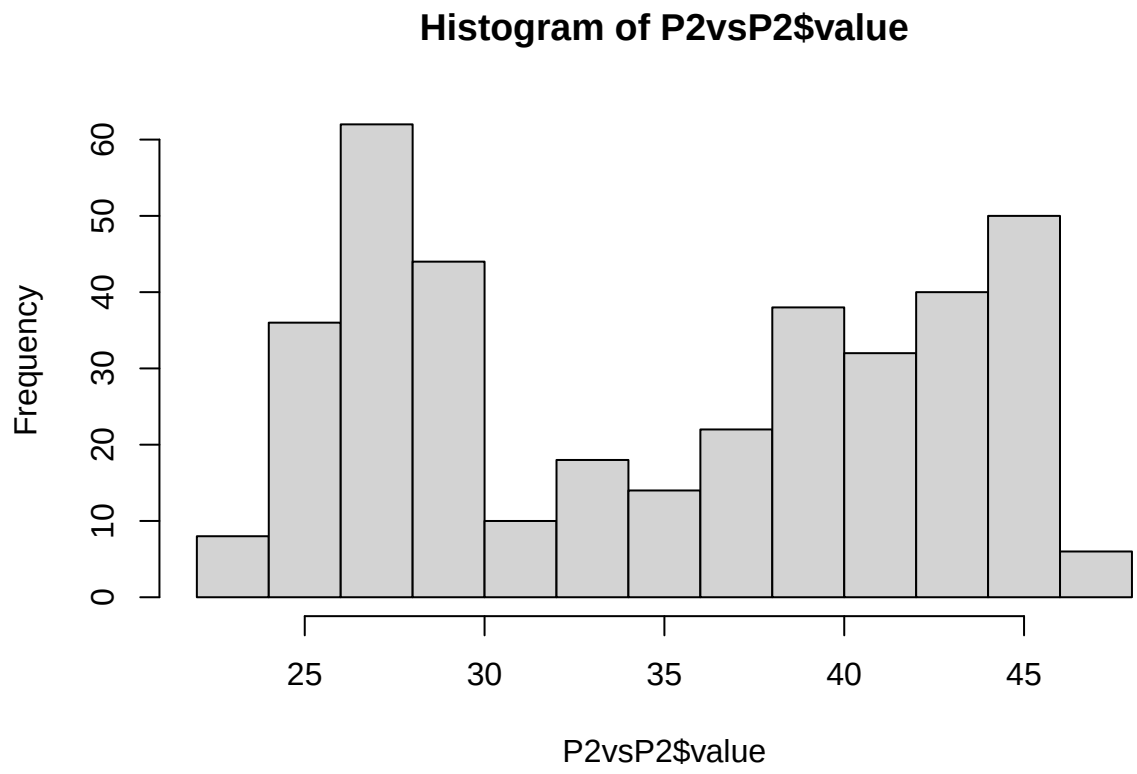

```
#Add jitter naming column
P1vsP2<-mutate(P1vsP2, jitter="P1vsP2")
#Add Comparison column
P1vsP2<-mutate(P1vsP2, Comparison="P1vsP2")


#Distance between the sewage samples
#Select comparisons where S1 is part of the first variable
S1vsS2<-filter(meltdist, grepl('S1', Var1))
#From this include comparisons to S2
S1vsS2<-filter(S1vsS2, grepl('S2', Var2))
#Histogram to look at distribution
hist(S1vsS2$value)
```



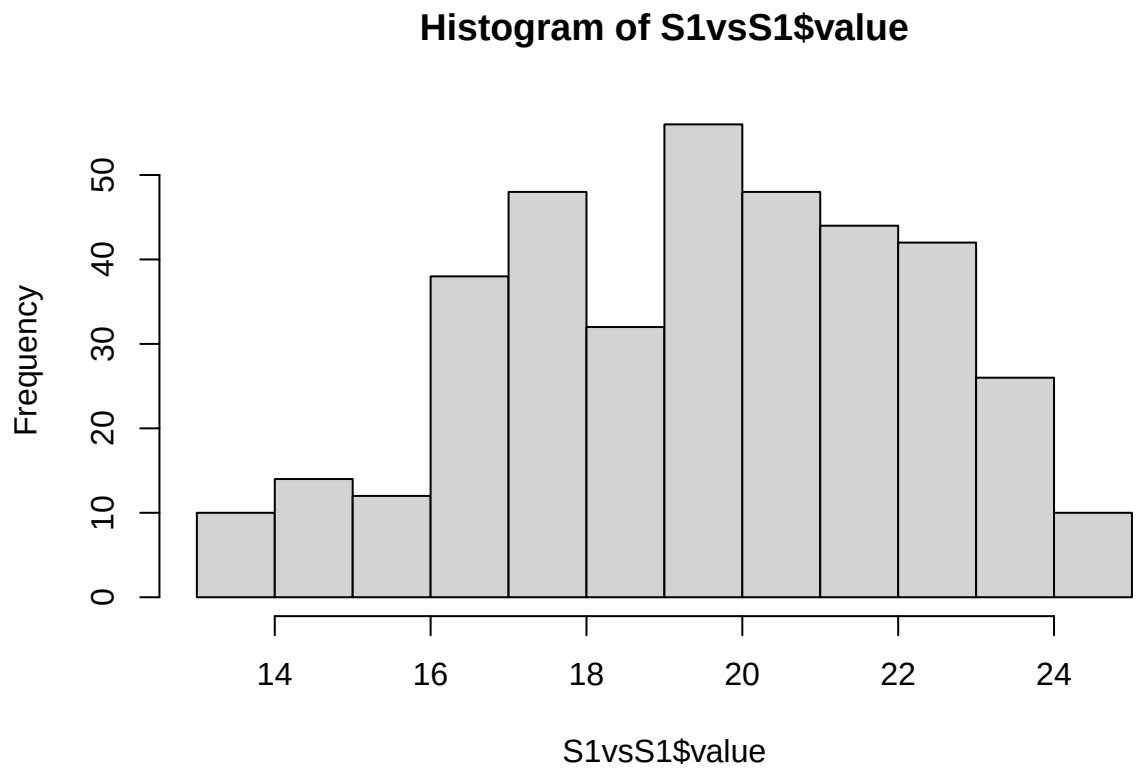

```
#Add jitter column
S1vsS2<-mutate(S1vsS2, jitter="S1vsS2")
#Add Comparison column
S1vsS2<-mutate(S1vsS2, Comparison="S1vsS2")


#Distance within P1
#Select comparisons where P1 is part of the first variable
P1vsP1<-filter(meltdist, grepl('P1', Var1))
#From this include comparisons to P1
P1vsP1<-filter(P1vsP1, grepl('P1', Var2))
#Histogram to look at distribution
hist(P1vsP1$value)
```



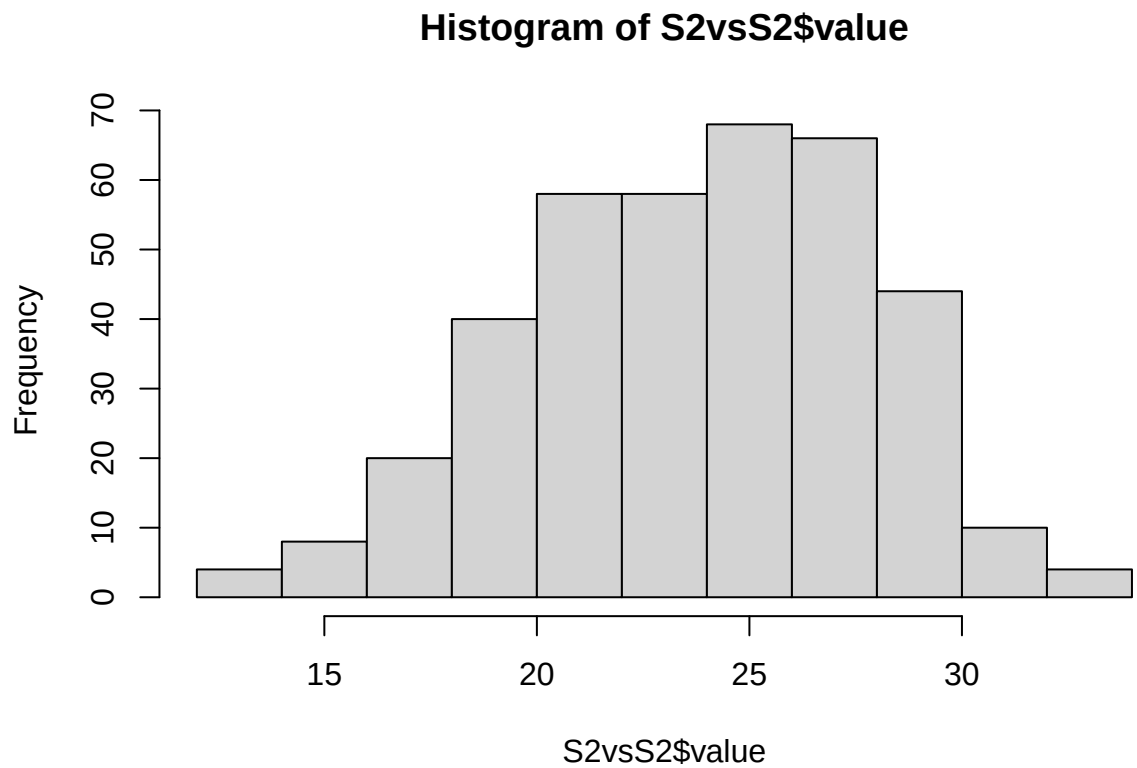

```
#Add jitter naming column
P1vsP1<-mutate(P1vsP1, jitter="P1")
#Add Comparison column
P1vsP1<-mutate(P1vsP1, Comparison="P1vsP1")


#Distance within P2
#Select comparisons where P2 is part of the first variable
P2vsP2<-filter(meltdist, grepl('P2', Var1))
#From this include comparisons to P2
P2vsP2<-filter(P2vsP2, grepl('P2', Var2))
#Histogram to look at distribution
hist(P2vsP2$value)
```



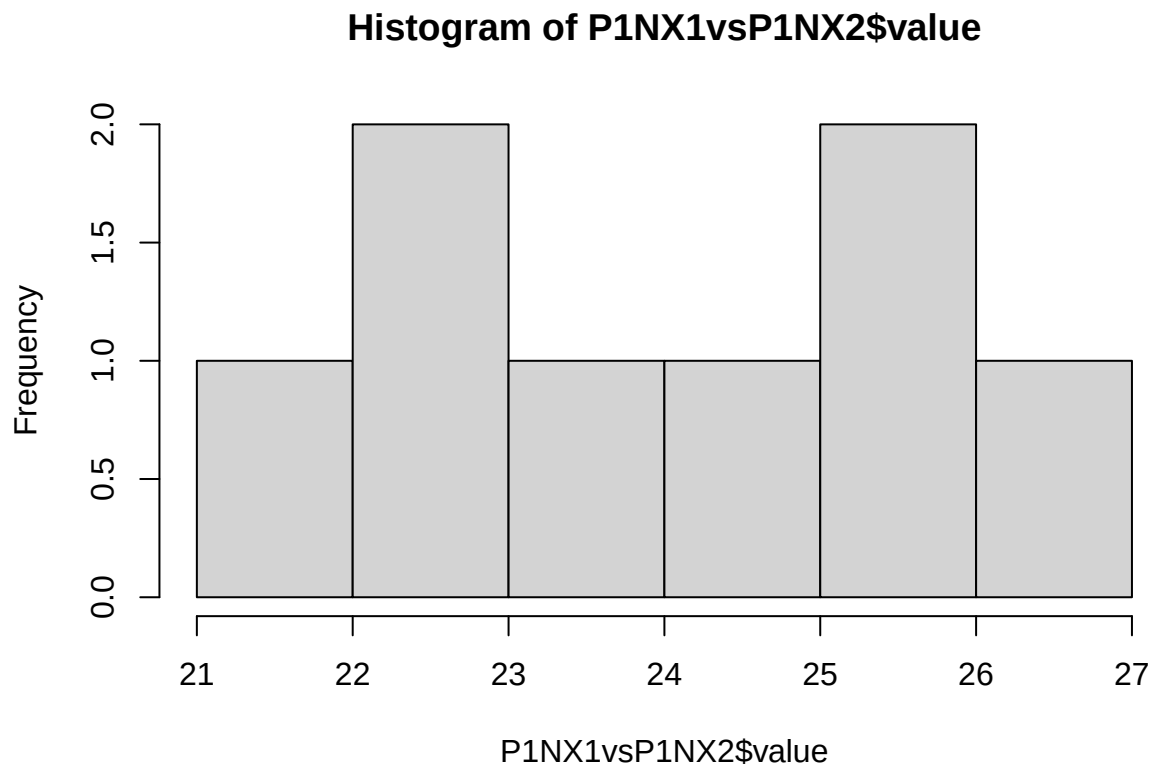

```
#Add jitter naming column
P2vsP2<-mutate(P2vsP2, jitter="P2")
#Add Comparison column
P2vsP2<-mutate(P2vsP2, Comparison="P2vsP2")


#Distance within S1
#Select comparisons where S1 is part of the first variable
S1vsS1<-filter(meltdist, grepl('S1', Var1))
#From this include comparisons to S1
S1vsS1<-filter(S1vsS1, grepl('S1', Var2))
#Histogram to look at distribution
hist(S1vsS1$value)
```



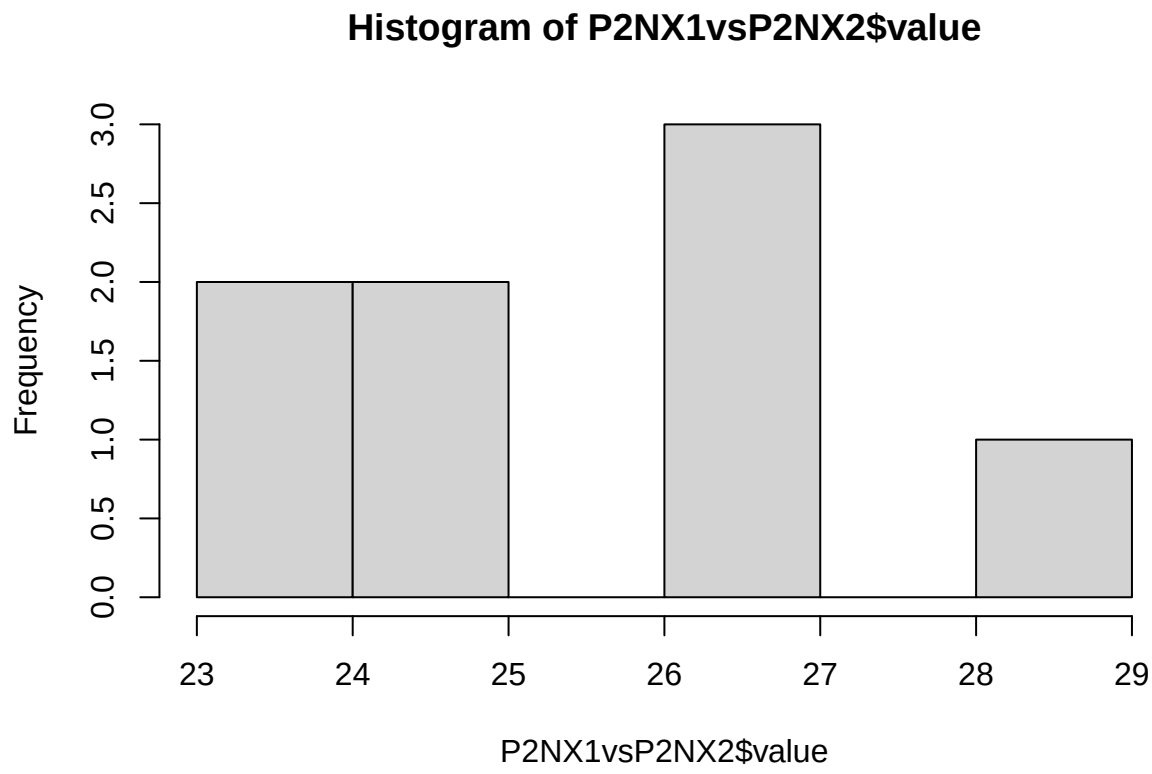

```
#Add jitter naming column
S1vsS1<-mutate(S1vsS1, jitter="S1")
#Add Comparison column
S1vsS1<-mutate(S1vsS1, Comparison="S1vsS1")


#Distance within S2
#Select comparisons where S2 is part of the first variable
S2vsS2<-filter(meltdist, grepl('S2', Var1))
#From this include comparisons to S2
S2vsS2<-filter(S2vsS2, grepl('S2', Var2))
#Histogram to look at distribution
hist(S2vsS2$value)
```



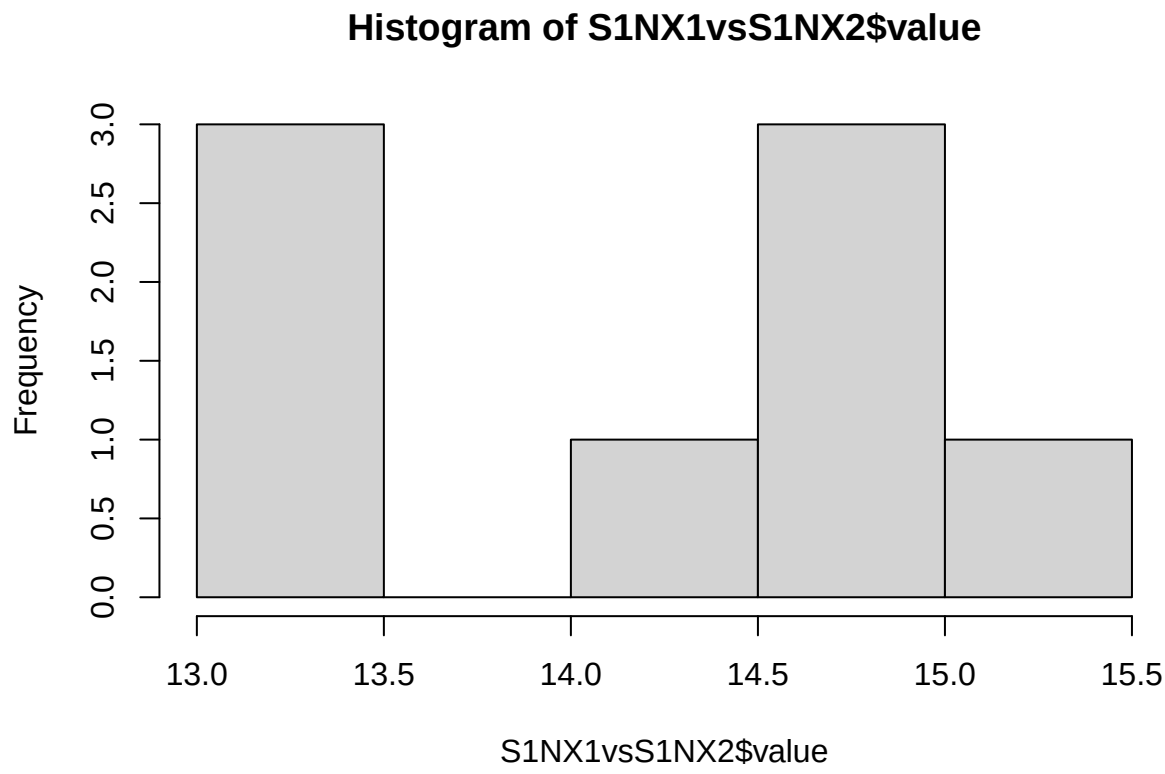

```
#Add jitter naming column
S2vsS2<-mutate(S2vsS2, jitter="S2")
#Add Comparison column
S2vsS2<-mutate(S2vsS2, Comparison="S2vsS2")


#Distance between nextera runs
#Select comparisons where P1S1 and NX1 is part of the first variable
P1S1NX1vsP1S1NX2<-filter(meltdist, grepl('NX1NS_P1_0h', Var1))
#From this include comparisons to P1S1 and NX2
P1S1NX1vsP1S1NX2<-filter(P1S1NX1vsP1S1NX2, grepl('NX2NS_P1_0h', Var2))
#Select comparisons where P1S2 and NX1 is part of the first variable
P1S2NX1vsP1S2NX2<-filter(meltdist, grepl('NX1NS_P1_64h', Var1))
#From this include comparisons to P1S2 and NX2
P1S2NX1vsP1S2NX2<-filter(P1S2NX1vsP1S2NX2, grepl('NX2NS_P1_64h', Var2))
P1NX1vsP1NX2<-bind_rows(P1S1NX1vsP1S1NX2, P1S2NX1vsP1S2NX2)
#Histogram to look at distribution
hist(P1NX1vsP1NX2$value)
```




```
#Add jitter naming column
P1NX1vsP1NX2<-mutate(P1NX1vsP1NX2, jitter="P1")
#Select comparisons where P2S1 and NX1 is part of the first variable
P2S1NX1vsP2S1NX2<-filter(meltdist, grepl('NX1NS_P2_0h', Var1))
#From this include comparisons to P2S1 and NX2
P2S1NX1vsP2S1NX2<-filter(P2S1NX1vsP2S1NX2, grepl('NX2NS_P2_0h', Var2))
#Select comparisons where P2S2 and NX1 is part of the first variable
P2S2NX1vsP2S2NX2<-filter(meltdist, grepl('NX1NS_P2_64h', Var1))
#From this include comparisons to P2S2 and NX2
P2S2NX1vsP2S2NX2<-filter(P2S2NX1vsP2S2NX2, grepl('NX2NS_P2_64h', Var2))
P2NX1vsP2NX2<-bind_rows(P2S1NX1vsP2S1NX2, P2S2NX1vsP2S2NX2)
#Histogram to look at distribution
hist(P2NX1vsP2NX2$value)
```

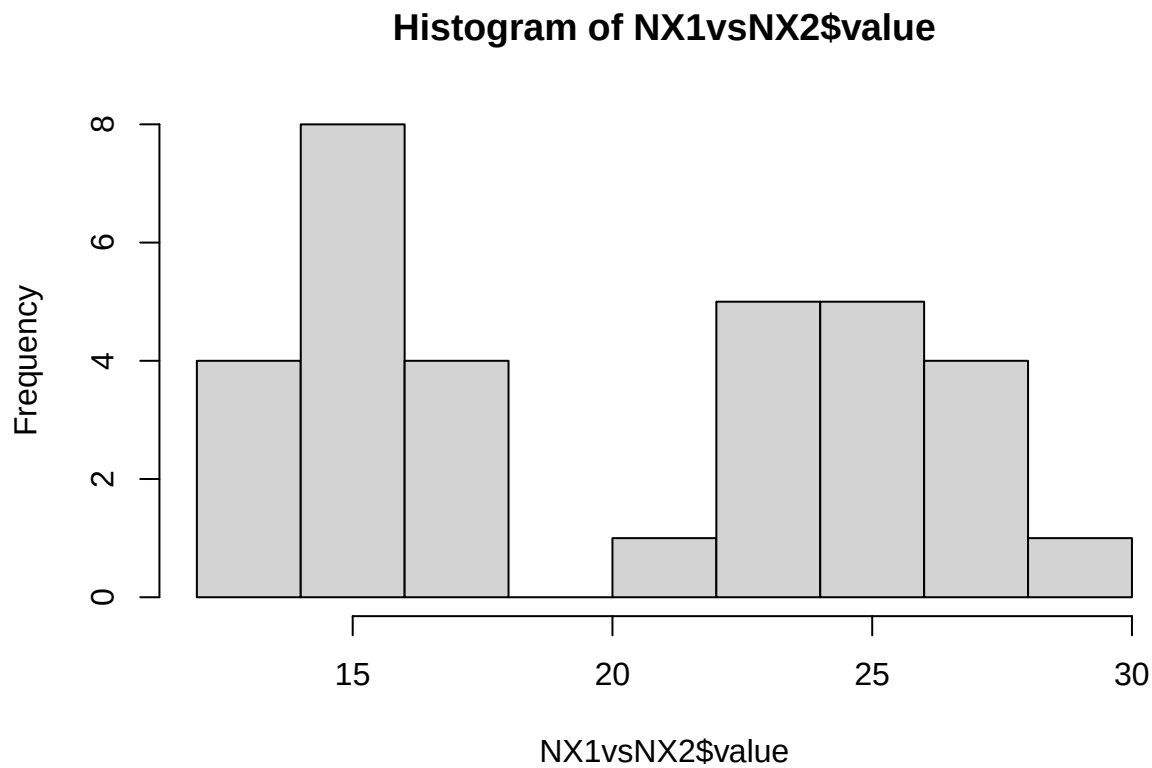



```
#Add jitter naming column
P2NX1vsP2NX2<-mutate(P2NX1vsP2NX2, jitter="P2")
#Select comparisons where S1S1 and NX1 is part of the first variable
S1S1NX1vsS1S1NX2<-filter(meltdist, grepl('NX1NS_S1_0h', Var1))
#From this include comparisons to S1S1 and NX2
S1S1NX1vsS1S1NX2<-filter(S1S1NX1vsS1S1NX2, grepl('NX2NS_S1_0h', Var2))
#Select comparisons where S1S2 and NX1 is part of the first variable
S1S2NX1vsS1S2NX2<-filter(meltdist, grepl('NX1NS_S1_64h', Var1))
#From this include comparisons to S1S2 and NX2
S1S2NX1vsS1S2NX2<-filter(S1S2NX1vsS1S2NX2, grepl('NX2NS_S1_64h', Var2))
S1NX1vsS1NX2<-bind_rows(S1S1NX1vsS1S1NX2, S1S2NX1vsS1S2NX2)
#Histogram to look at distribution
hist(S1NX1vsS1NX2$value)
```



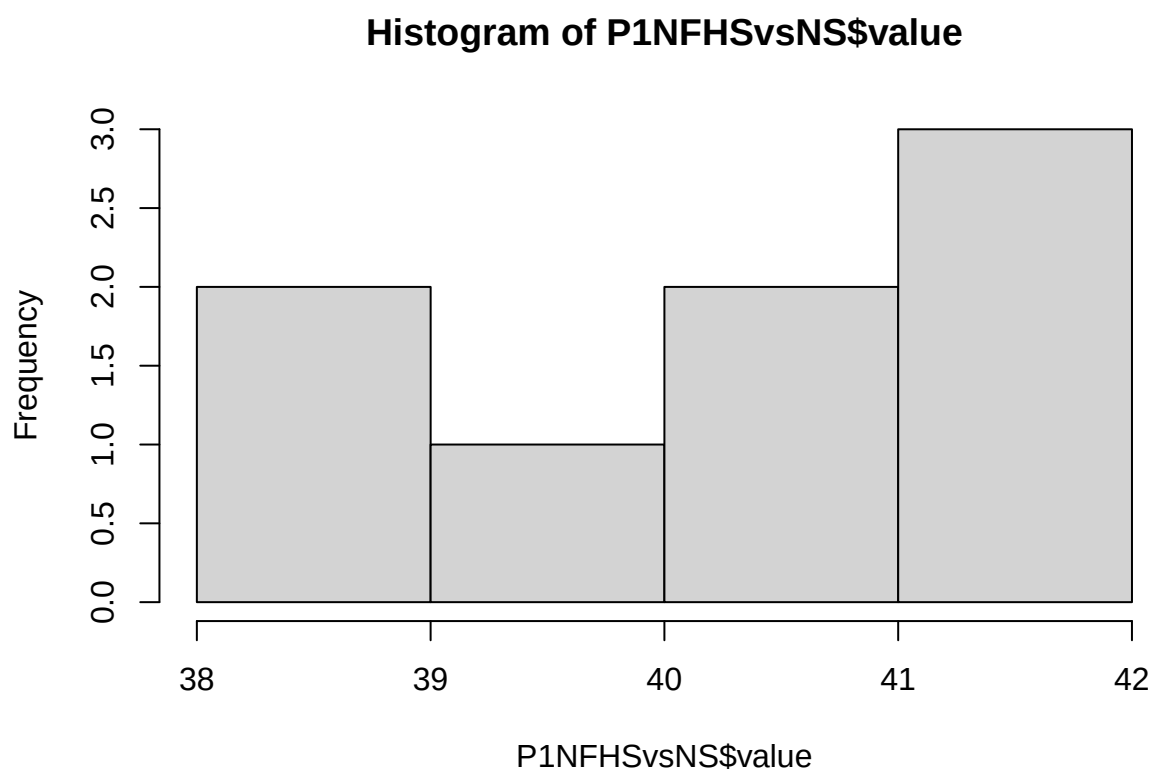

```
#Add jitter naming column
S1NX1vsS1NX2<-mutate(S1NX1vsS1NX2, jitter="S1")
#Select comparisons where S2S1 and NX1 is part of the first variable
S2S1NX1vsS2S1NX2<-filter(meltdist, grepl('NX1NS_S2_0h', Var1))
#From this include comparisons to S2S1 and NX2
S2S1NX1vsS2S1NX2<-filter(S2S1NX1vsS2S1NX2, grepl('NX2NS_S2_0h', Var2))
#Select comparisons where S2S2 and NX1 is part of the first variable
S2S2NX1vsS2S2NX2<-filter(meltdist, grepl('NX1NS_S2_64h', Var1))
#From this include comparisons to S2S2 and NX2
S2S2NX1vsS2S2NX2<-filter(S2S2NX1vsS2S2NX2, grepl('NX2NS_S2_64h', Var2))
S2NX1vsS2NX2<-bind_rows(S2S1NX1vsS2S1NX2, S2S2NX1vsS2S2NX2)
#Histogram to look at distribution
hist(S2NX1vsS2NX2$value)
```

```
#Add jitter naming column
S2NX1vsS2NX2<-mutate(S2NX1vsS2NX2, jitter="S2")
#Combine comparisons of nextera runs in all samples
NX1vsNX2<-bind_rows(P1NX1vsP1NX2, P2NX1vsP2NX2, S1NX1vsS1NX2, S2NX1vsS2NX2)
#Histogram to look at distribution
hist(NX1vsNX2$value)
```



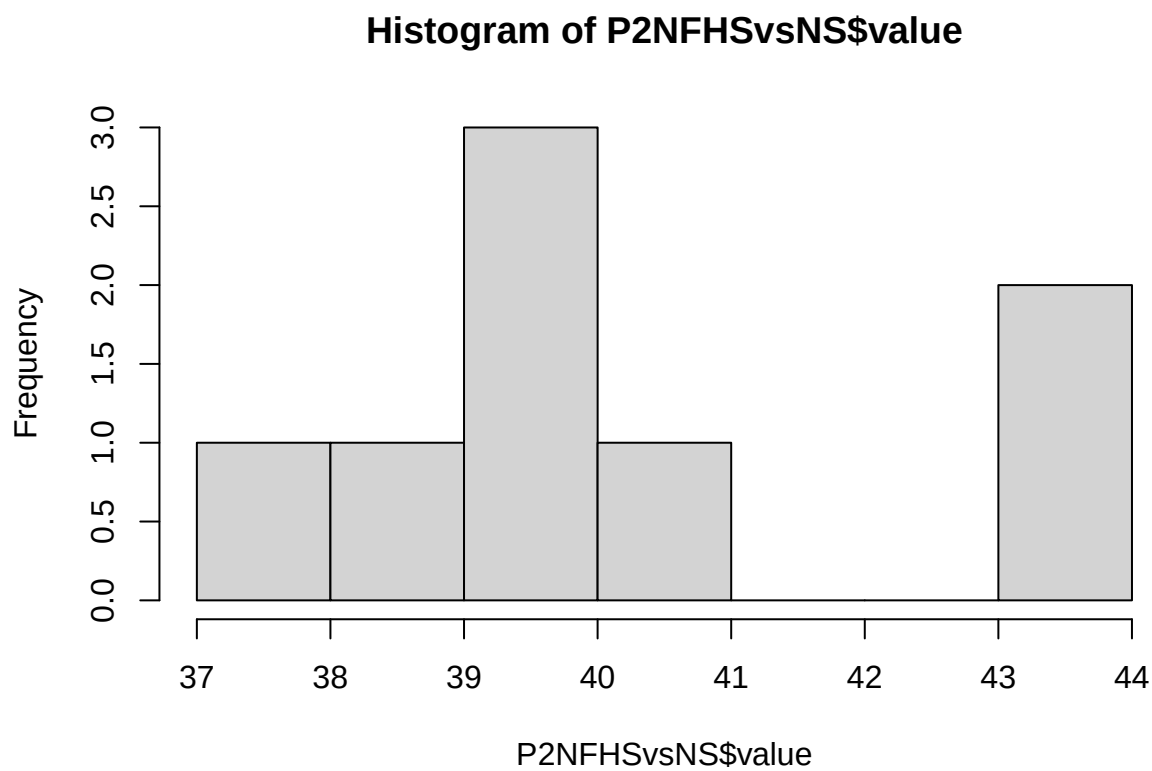

```
#Add Comparison column
NX1vsNX2<-mutate(NX1vsNX2, Comparison="NXNSRep")


#Distance between DNA extraction runs
#Makes column based on Var1 that only contain middle part of sample name
DNAExRep <- mutate(meltdist, from = str_replace(Var1,"[A-X0-9]*_[A-X0-9]*_[A-X0-9]*_","")
                   %>% str_replace("_[a-z]$",""))
#Makes column based on Var2 that only contain middle part of sample name
DNAExRep <- mutate(DNAExRep, to = str_replace(Var2,"[A-X0-9]*_[A-X0-9]*_[A-X0-9]*_","")
                   %>% str_replace("_[a-z]$",""))
#Makes a column were TRUE means from is the same as to otherwise FALSE
DNAExRep <- mutate(DNAExRep, rep = (from == to))
##Only contain samples that are replicates containing TRUE in rep
DNAExRep<-filter(DNAExRep, grepl('TRUE', rep))
#Add jitter column
DNAExRep$jitter <-
        ifelse(seq(along=(DNAExRep$Var1)) %in% grep("*_P1_*", DNAExRep$Var1), "P1",
        ifelse(seq(along=(DNAExRep$Var1)) %in% grep("*_P2_*", DNAExRep$Var1), "P2",
        ifelse(seq(along=(DNAExRep$Var1)) %in% grep("*_S1_*", DNAExRep$Var1), "S1",
        ifelse(seq(along=(DNAExRep$Var1)) %in% grep("*_S2_*", DNAExRep$Var1), "S2",
               "Other"))))
#Add Comparison column
DNAExRep<-mutate(DNAExRep, Comparison="DNAExRep")
#Remove from, to and rep column
DNAExRep<-dplyr::select(DNAExRep, one_of(c("Var1", "Var2", "value", "jitter",
```

```
                                                "Comparison")))


#Distance between Nextflex run on Hiseq and Nextseq
#Select comparisons where P1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P10hNFHSvsNS<-filter(meltdist, grepl('HX_P1_0h', Var1))
#From this include comparisons to P1, 0h storage and NFNS
P10hNFHSvsNS<-filter(P10hNFHSvsNS, grepl('NFNS_P1_0h', Var2))
#Select comparisons where P1, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P164hNFHSvsNS<-filter(meltdist, grepl('HX_P1_64h', Var1))
#From this include comparisons to P1, 64h storage and NFNS
P164hNFHSvsNS<-filter(P164hNFHSvsNS, grepl('NFNS_P1_64h', Var2))
P1NFHSvsNS<-bind_rows(P10hNFHSvsNS, P164hNFHSvsNS)
#Histogram to look at distribution
hist(P1NFHSvsNS$value)
```

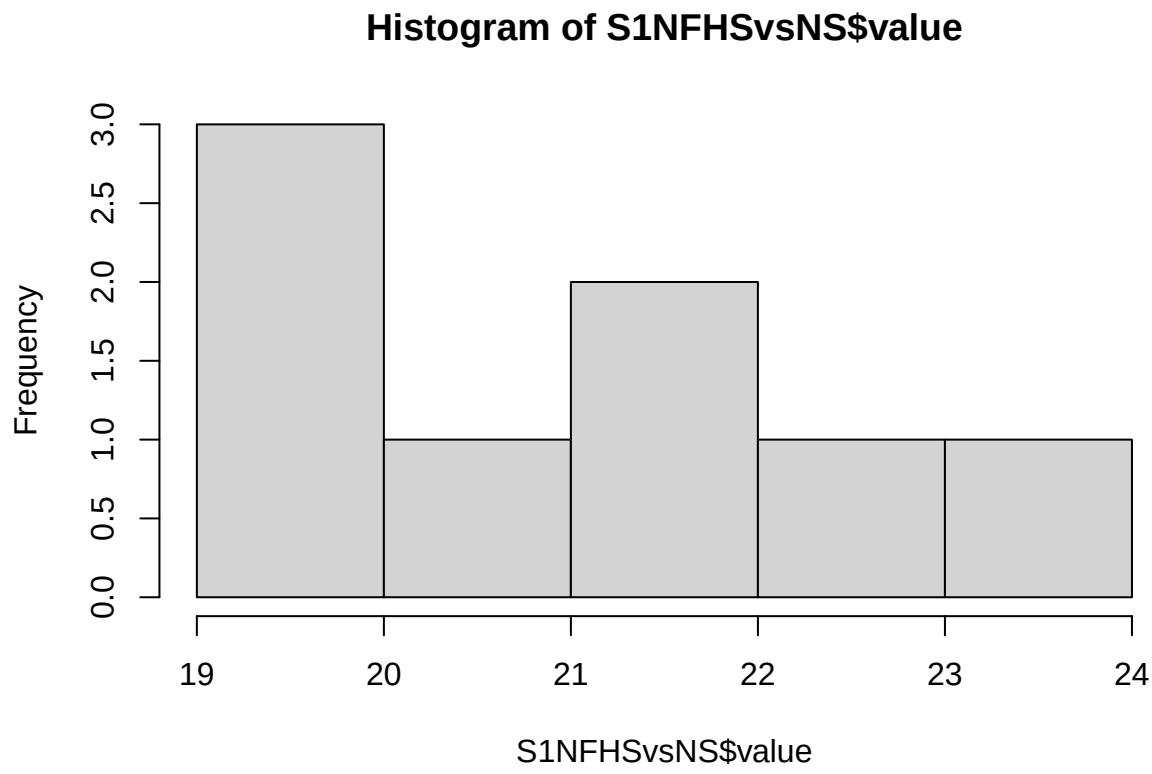



```
#Add jitter naming column
P1NFHSvsNS<-mutate(P1NFHSvsNS, jitter="P1")
#Select comparisons where P2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P20hNFHSvsNS<-filter(meltdist, grepl('HX_P2_0h', Var1))
#From this include comparisons to P2, 0h storage and NFNS
P20hNFHSvsNS<-filter(P20hNFHSvsNS, grepl('NFNS_P2_0h', Var2))
#Select comparisons where P2, 64h storage and NFHI is part of the first variable
```

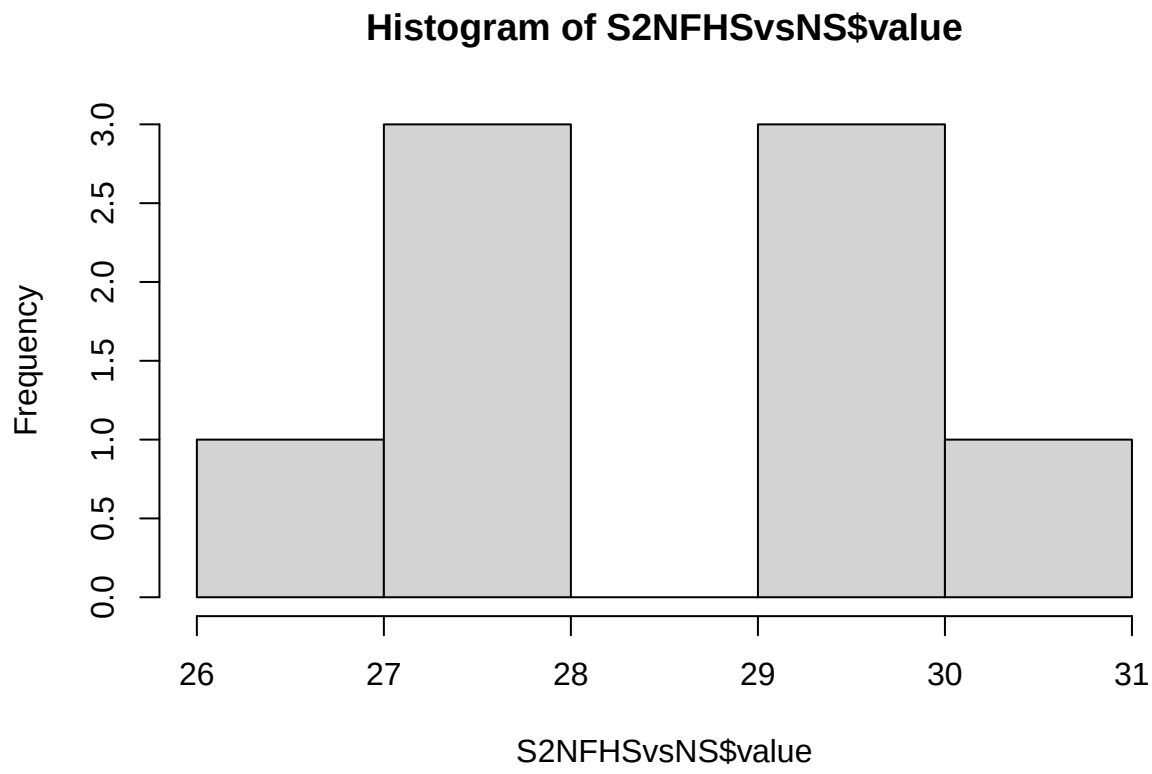
```
  #(Part of the HX experiment)
P264hNFHSvsNS<-filter(meltdist, grepl('HX_P2_64h', Var1))
#From this include comparisons to P2, 64h storage and NFNS
P264hNFHSvsNS<-filter(P264hNFHSvsNS, grepl('NFNS_P2_64h', Var2))
P2NFHSvsNS<-bind_rows(P20hNFHSvsNS, P264hNFHSvsNS)
#Histogram to look at distribution
hist(P2NFHSvsNS$value)
```



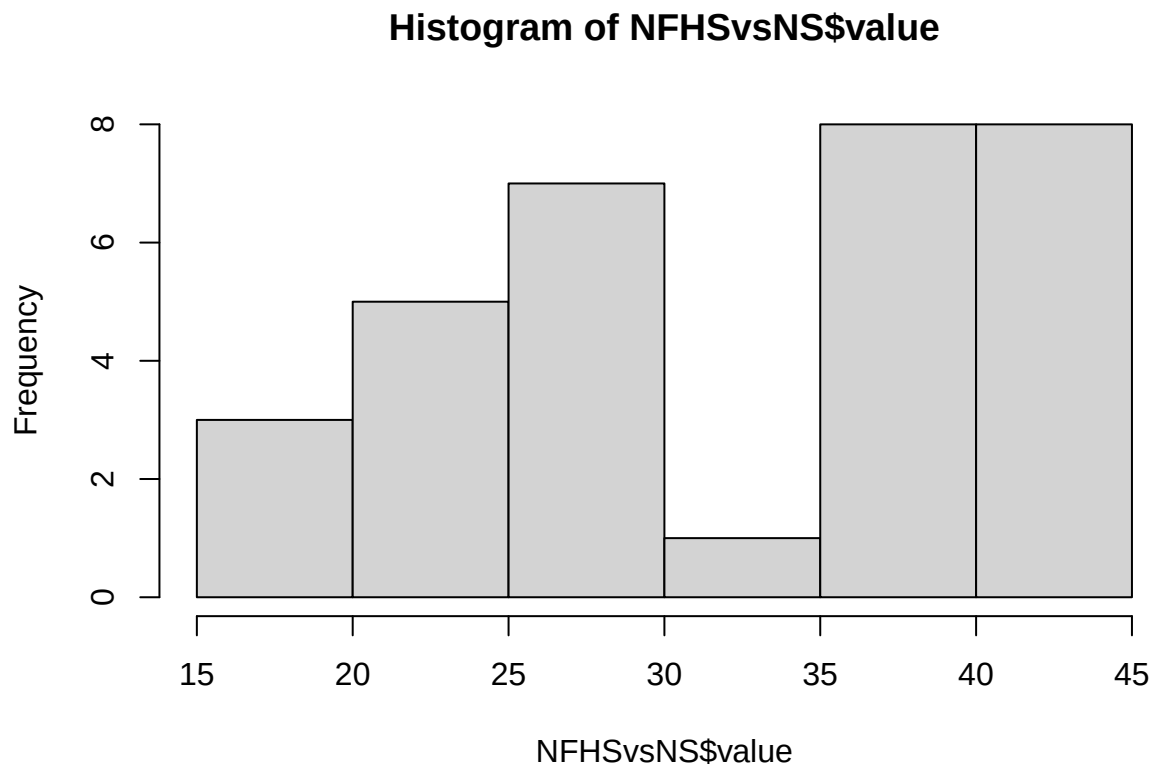

```
#Add jitter naming column
P2NFHSvsNS<-mutate(P2NFHSvsNS, jitter="P2")
#Select comparisons where S1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S10hNFHSvsNS<-filter(meltdist, grepl('HX_S1_0h', Var1))
#From this include comparisons to S1, 0h storage and NFNS
S10hNFHSvsNS<-filter(S10hNFHSvsNS, grepl('NFNS_S1_0h', Var2))
#Select comparisons where S1, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S164hNFHSvsNS<-filter(meltdist, grepl('HX_S1_64h', Var1))
#From this include comparisons to S1, 64h storage and NFNS
S164hNFHSvsNS<-filter(S164hNFHSvsNS, grepl('NFNS_S1_64h', Var2))
S1NFHSvsNS<-bind_rows(S10hNFHSvsNS, S164hNFHSvsNS)
#Histogram to look at distribution
hist(S1NFHSvsNS$value)
```



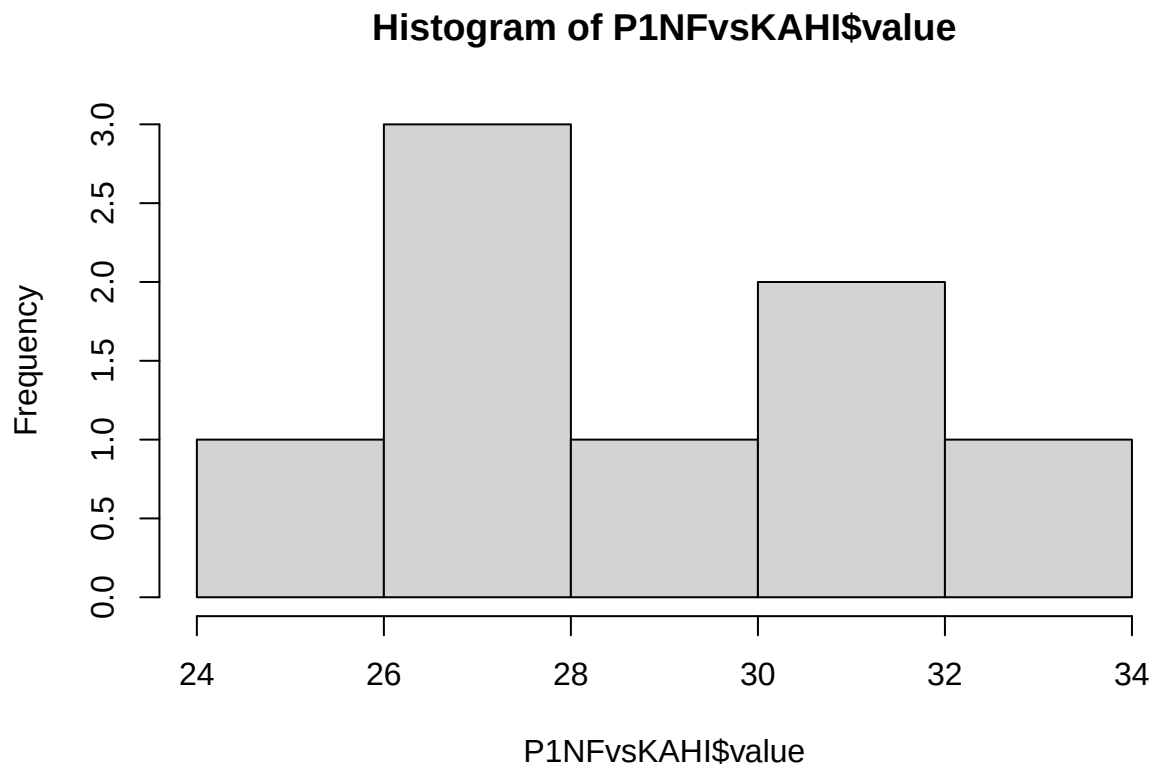

```
#Add jitter naming column
S1NFHSvsNS<-mutate(S1NFHSvsNS, jitter="S1")
#Select comparisons where S2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S20hNFHSvsNS<-filter(meltdist, grepl('HX_S2_0h', Var1))
#From this include comparisons to S2, 0h storage and NFNS
S20hNFHSvsNS<-filter(S20hNFHSvsNS, grepl('NFNS_S2_0h', Var2))
#Select comparisons where S2, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S264hNFHSvsNS<-filter(meltdist, grepl('HX_S2_64h', Var1))
#From this include comparisons to S2, 64h storage and NFNS
S264hNFHSvsNS<-filter(S264hNFHSvsNS, grepl('KAHI_S2_64h', Var2))
S2NFHSvsNS<-bind_rows(S20hNFHSvsNS, S264hNFHSvsNS)
#Histogram to look at distribution
hist(S2NFHSvsNS$value)
```



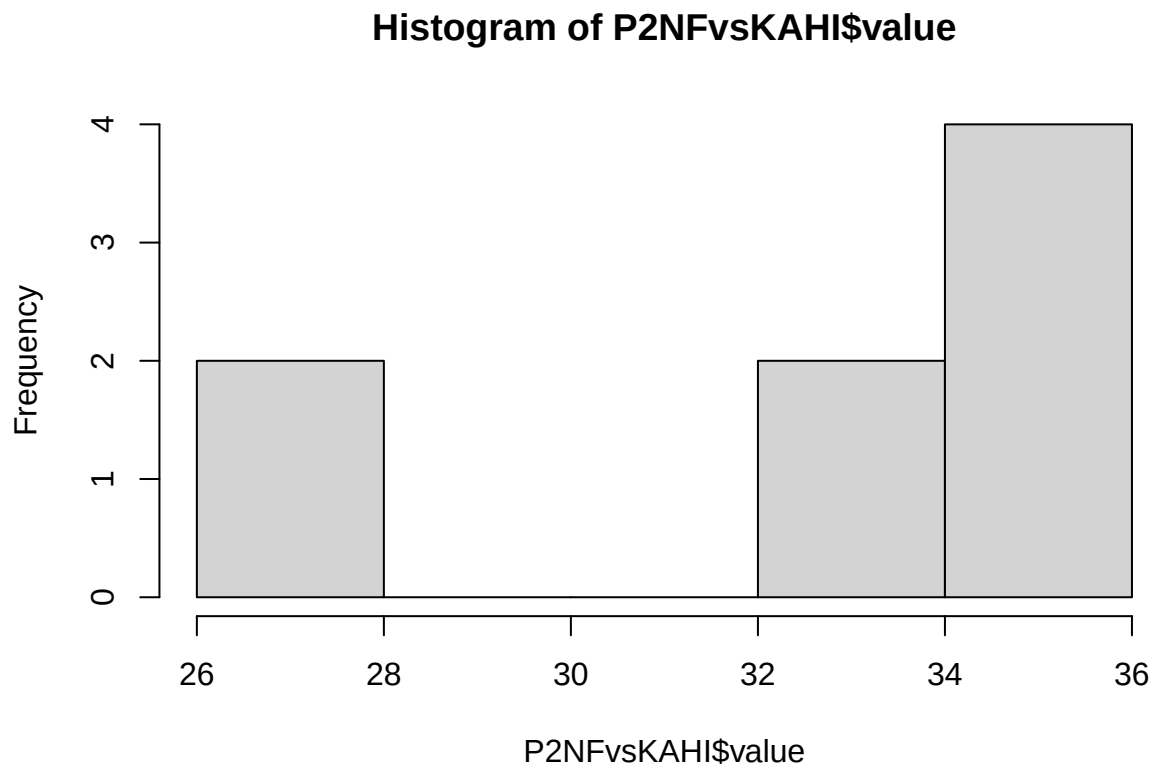

```
#Add jitter naming column
S2NFHSvsNS<-mutate(S2NFHSvsNS, jitter="S2")
#Combine comparisons of nextera runs in all samples
NFHSvsNS<-bind_rows(P1NFHSvsNS, P2NFHSvsNS, S1NFHSvsNS, S2NFHSvsNS)
#Histogram to look at distribution
hist(NFHSvsNS$value)
```



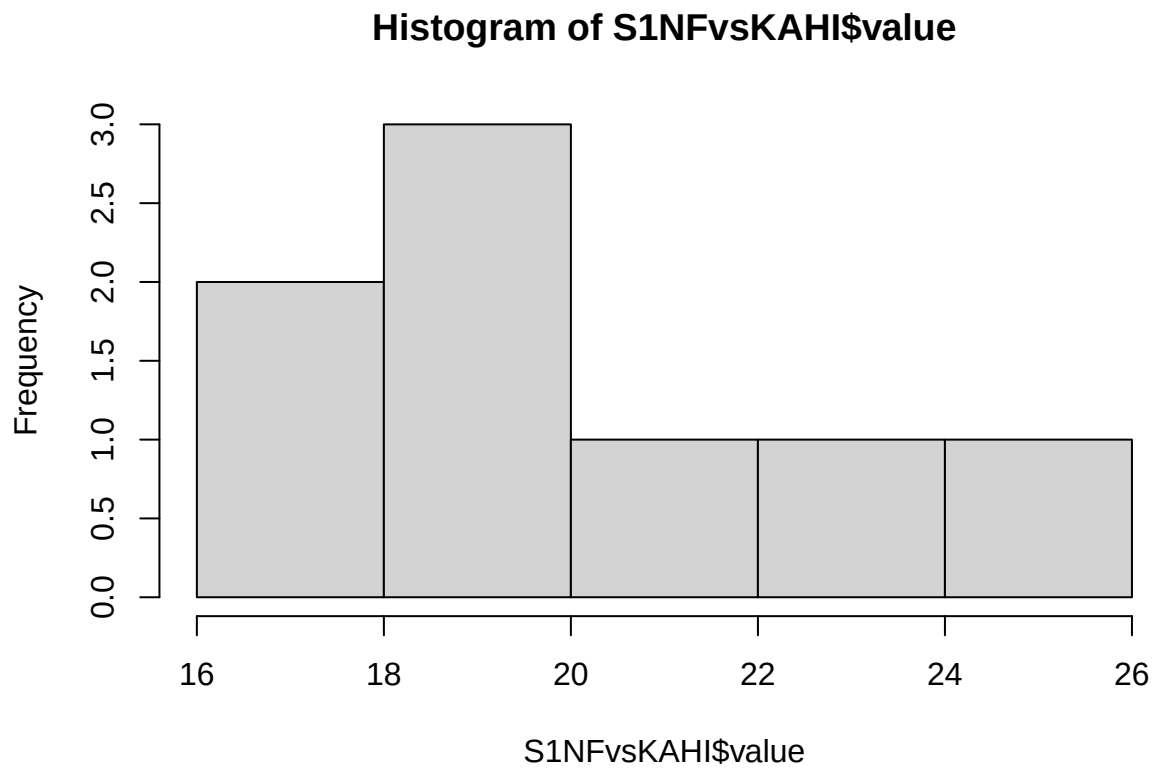

```
#Add Comparison column
NFHSvsNS<-mutate(NFHSvsNS, Comparison="NFHSvsNS")


#Distance between Nextflex and Kappa run on Hiseq
#Select comparisons where P1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P10hNFvsKAHI<-filter(meltdist, grepl('HX_P1_0h', Var1))
#From this include comparisons to P1, 0h storage and NFNS
P10hNFvsKAHI<-filter(P10hNFvsKAHI, grepl('KAHI_P1_0h', Var2))
#Select comparisons where P1, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P164hNFvsKAHI<-filter(meltdist, grepl('HX_P1_64h', Var1))
#From this include comparisons to P1, 64h storage and NFNS
P164hNFvsKAHI<-filter(P164hNFvsKAHI, grepl('KAHI_P1_64h', Var2))
P1NFvsKAHI<-bind_rows(P10hNFvsKAHI, P164hNFvsKAHI)
#Histogram to look at distribution
hist(P1NFvsKAHI$value)
```



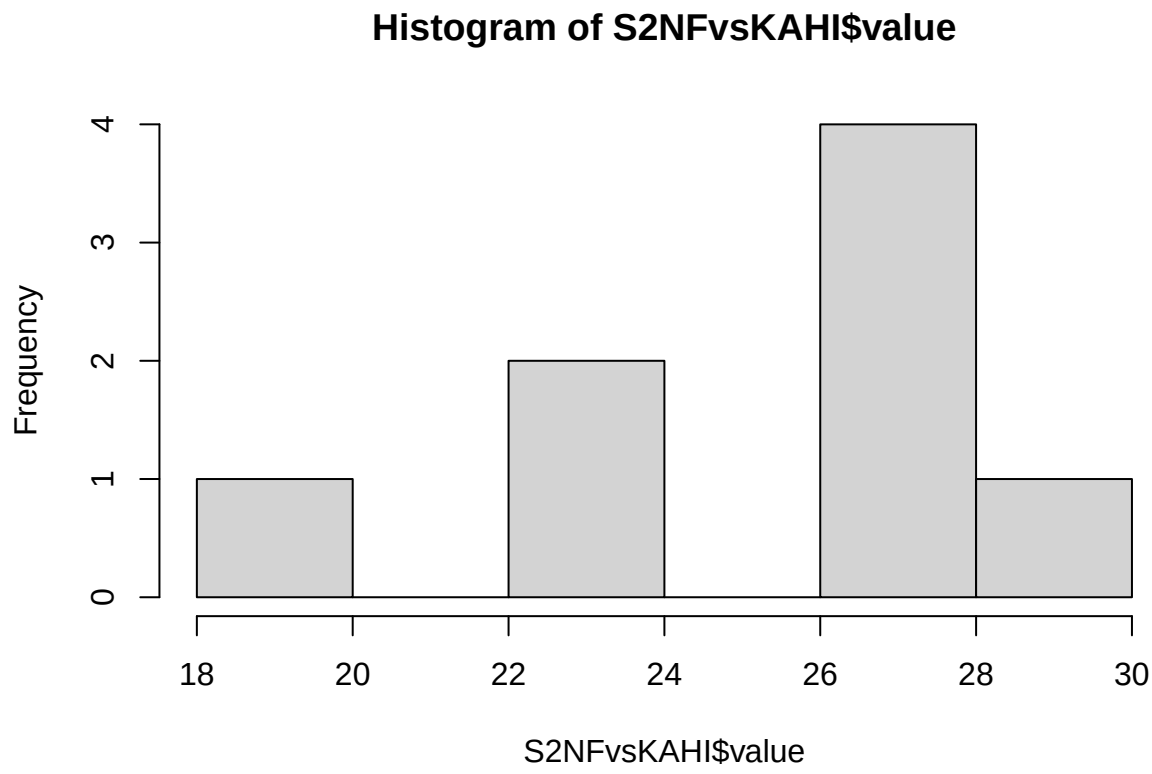

```
#Add jitter naming column
P1NFvsKAHI<-mutate(P1NFvsKAHI, jitter="P1")
#Select comparisons where P2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P20hNFvsKAHI<-filter(meltdist, grepl('HX_P2_0h', Var1))
#From this include comparisons to P2, 0h storage and NFNS
P20hNFvsKAHI<-filter(P20hNFvsKAHI, grepl('KAHI_P2_0h', Var2))
#Select comparisons where P2, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P264hNFvsKAHI<-filter(meltdist, grepl('HX_P2_64h', Var1))
#From this include comparisons to P2, 64h storage and NFNS
P264hNFvsKAHI<-filter(P264hNFvsKAHI, grepl('KAHI_P2_64h', Var2))
P2NFvsKAHI<-bind_rows(P20hNFvsKAHI, P264hNFvsKAHI)
#Histogram to look at distribution
hist(P2NFvsKAHI$value)
```



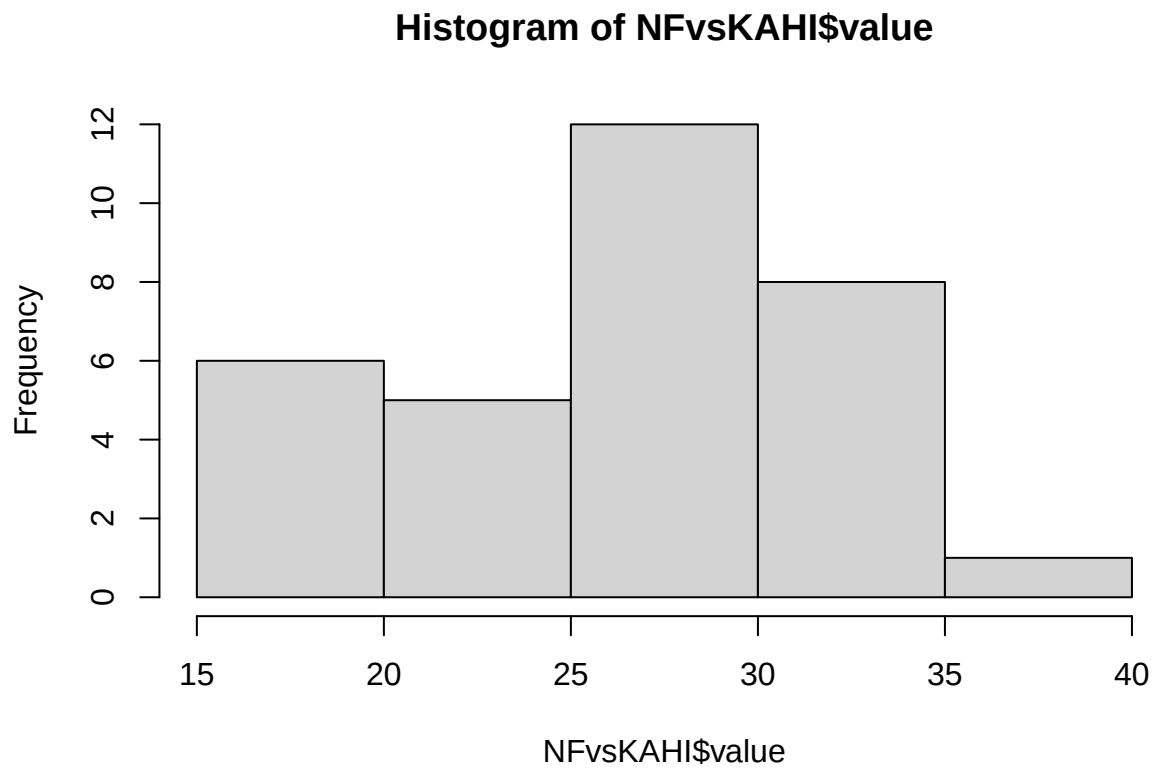

```
#Add jitter naming column
P2NFvsKAHI<-mutate(P2NFvsKAHI, jitter="P2")
#Select comparisons where S1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S10hNFvsKAHI<-filter(meltdist, grepl('HX_S1_0h', Var1))
#From this include comparisons to S1, 0h storage and NFNS
S10hNFvsKAHI<-filter(S10hNFvsKAHI, grepl('KAHI_S1_0h', Var2))
#Select comparisons where S1, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S164hNFvsKAHI<-filter(meltdist, grepl('HX_S1_64h', Var1))
#From this include comparisons to S1, 64h storage and NFNS
S164hNFvsKAHI<-filter(S164hNFvsKAHI, grepl('KAHI_S1_64h', Var2))
S1NFvsKAHI<-bind_rows(S10hNFvsKAHI, S164hNFvsKAHI)
#Histogram to look at distribution
hist(S1NFvsKAHI$value)
```



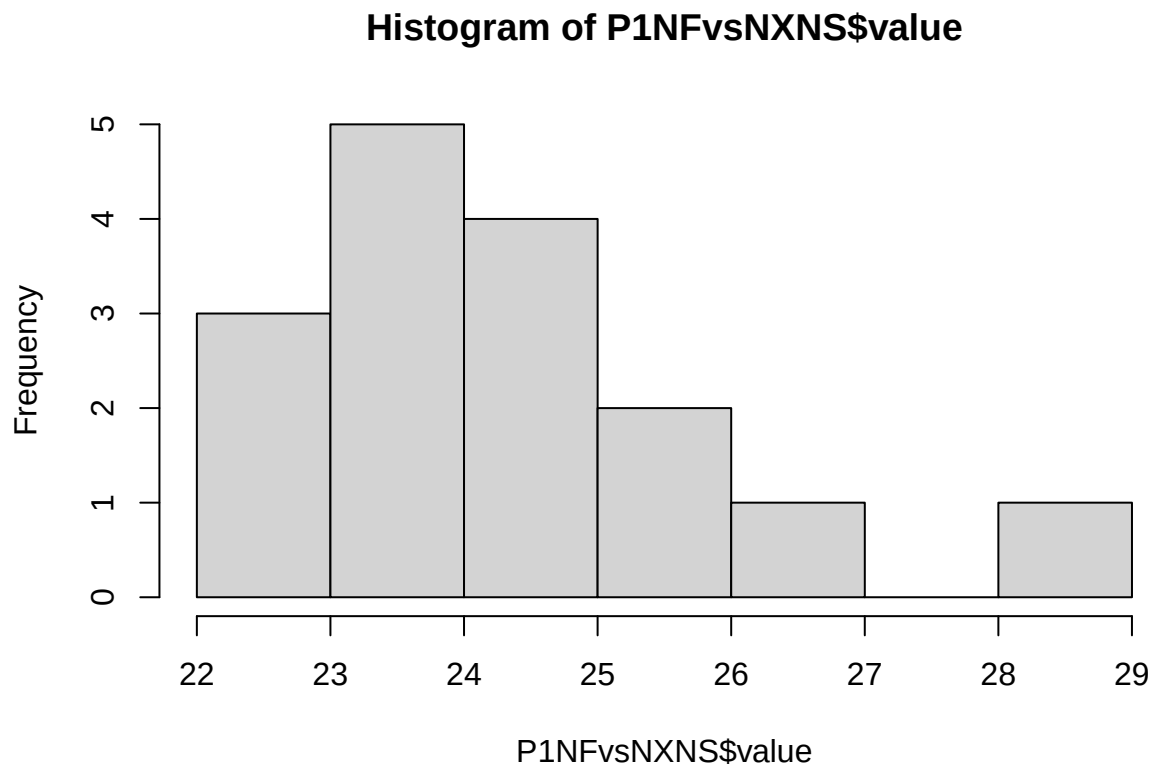

```
#Add jitter naming column
S1NFvsKAHI<-mutate(S1NFvsKAHI, jitter="S1")
#Select comparisons where S2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S20hNFvsKAHI<-filter(meltdist, grepl('HX_S2_0h', Var1))
#From this include comparisons to S2, 0h storage and NFNS
S20hNFvsKAHI<-filter(S20hNFvsKAHI, grepl('KAHI_S2_0h', Var2))
#Select comparisons where S2, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S264hNFvsKAHI<-filter(meltdist, grepl('HX_S2_64h', Var1))
#From this include comparisons to S2, 64h storage and NFNS
S264hNFvsKAHI<-filter(S264hNFvsKAHI, grepl('KAHI_S2_64h', Var2))
S2NFvsKAHI<-bind_rows(S20hNFvsKAHI, S264hNFvsKAHI)
#Histogram to look at distribution
hist(S2NFvsKAHI$value)
```



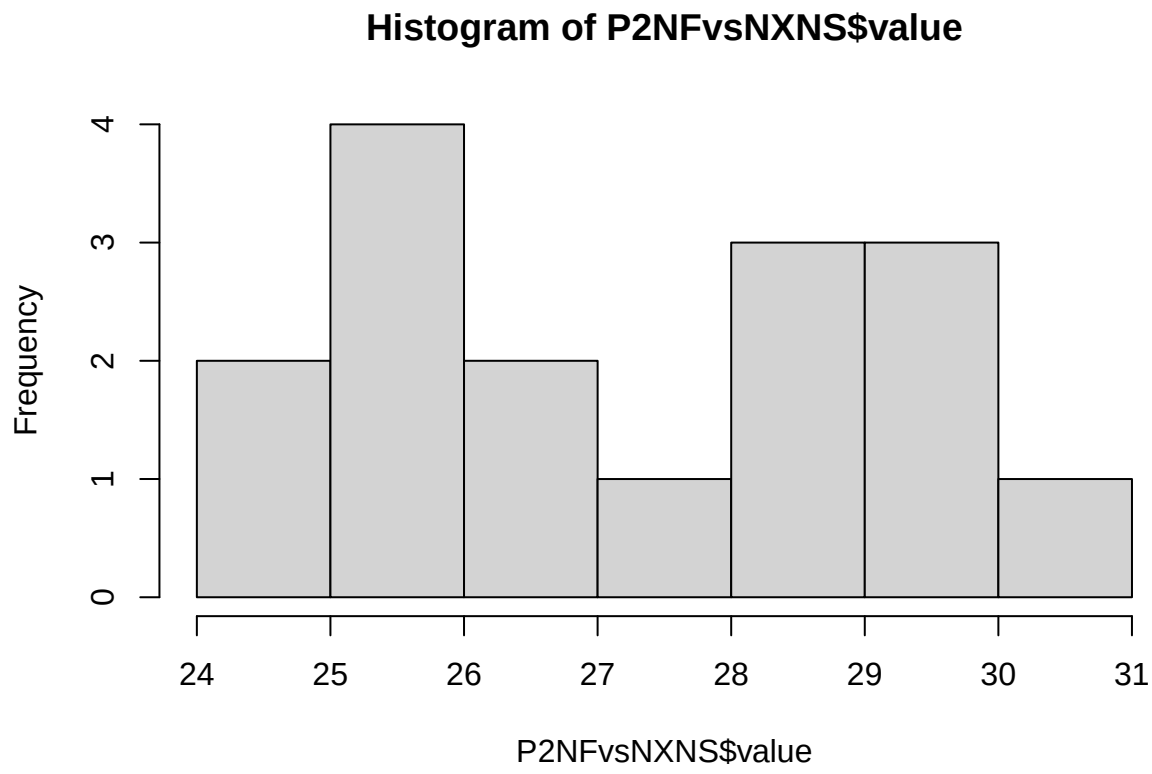

```
#Add jitter naming column
S2NFvsKAHI<-mutate(S2NFvsKAHI, jitter="S2")
#Combine comparisons of nextflex kappa runs in all samples on Hiseq
NFvsKAHI<-bind_rows(P1NFvsKAHI, P2NFvsKAHI, S1NFvsKAHI, S2NFvsKAHI)
#Histogram to look at distribution
hist(NFvsKAHI$value)
```



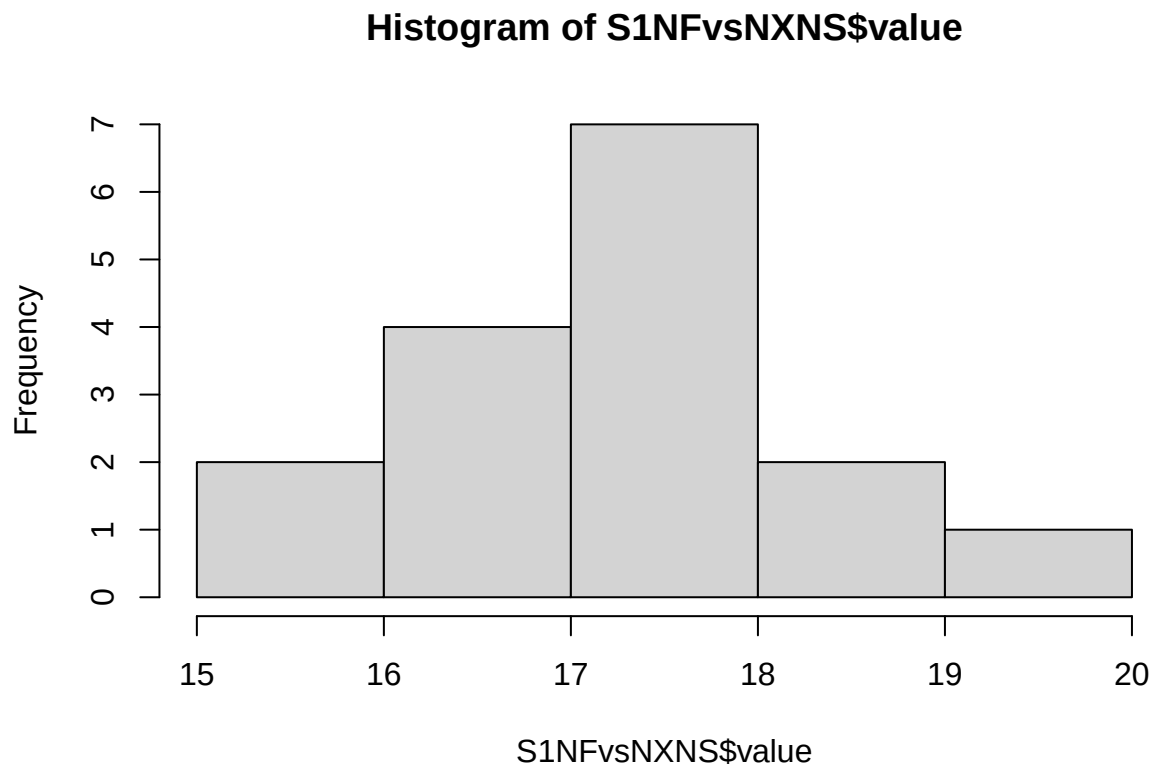

```
#Add Comparison column
NFvsKAHI<-mutate(NFvsKAHI, Comparison="NFvsKAHI")


#Distance between Nextflex and Nextera runs on Nextseq
#Select comparisons where P1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P10hNFvsNXNS<-filter(meltdist, grepl('NFNS_P1_0h', Var1))
#From this include comparisons to P1, 0h storage and NFNS
P10hNFvsNXNS<-filter(P10hNFvsNXNS, grepl('NX[0-9]NS_P1_0h', Var2))
#Select comparisons where P1, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P164hNFvsNXNS<-filter(meltdist, grepl('NFNS_P1_64h', Var1))
#From this include comparisons to P1, 64h storage and NFNS
P164hNFvsNXNS<-filter(P164hNFvsNXNS, grepl('NX[0-9]NS_P1_64h', Var2))
P1NFvsNXNS<-bind_rows(P10hNFvsNXNS, P164hNFvsNXNS)
#Histogram to look at distribution
hist(P1NFvsNXNS$value)
```



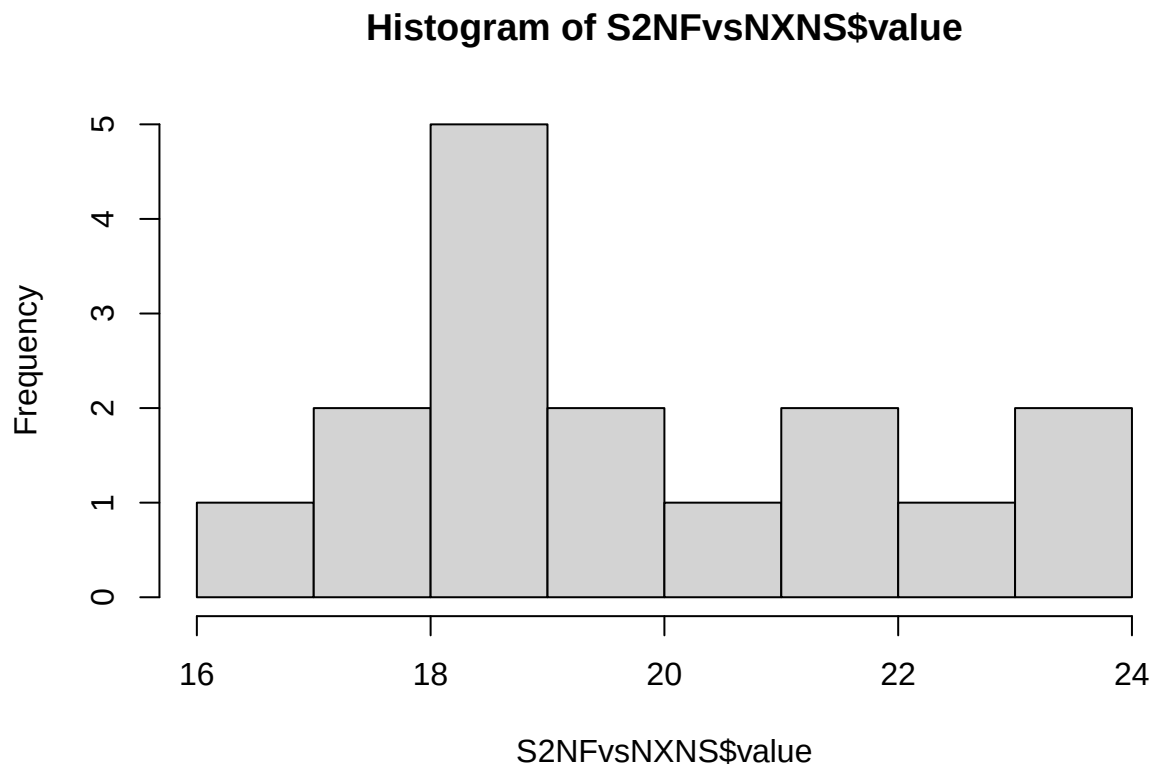

```
#Add jitter naming column
P1NFvsNXNS<-mutate(P1NFvsNXNS, jitter="P1")
#Select comparisons where P2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P20hNFvsNXNS<-filter(meltdist, grepl('NFNS_P2_0h', Var1))
#From this include comparisons to P2, 0h storage and NFNS
P20hNFvsNXNS<-filter(P20hNFvsNXNS, grepl('NX[0-9]NS_P2_0h', Var2))
#Select comparisons where P2, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P264hNFvsNXNS<-filter(meltdist, grepl('NFNS_P2_64h', Var1))
#From this include comparisons to P2, 64h storage and NFNS
P264hNFvsNXNS<-filter(P264hNFvsNXNS, grepl('NX[0-9]NS_P2_64h', Var2))
P2NFvsNXNS<-bind_rows(P20hNFvsNXNS, P264hNFvsNXNS)
#Histogram to look at distribution
hist(P2NFvsNXNS$value)
```



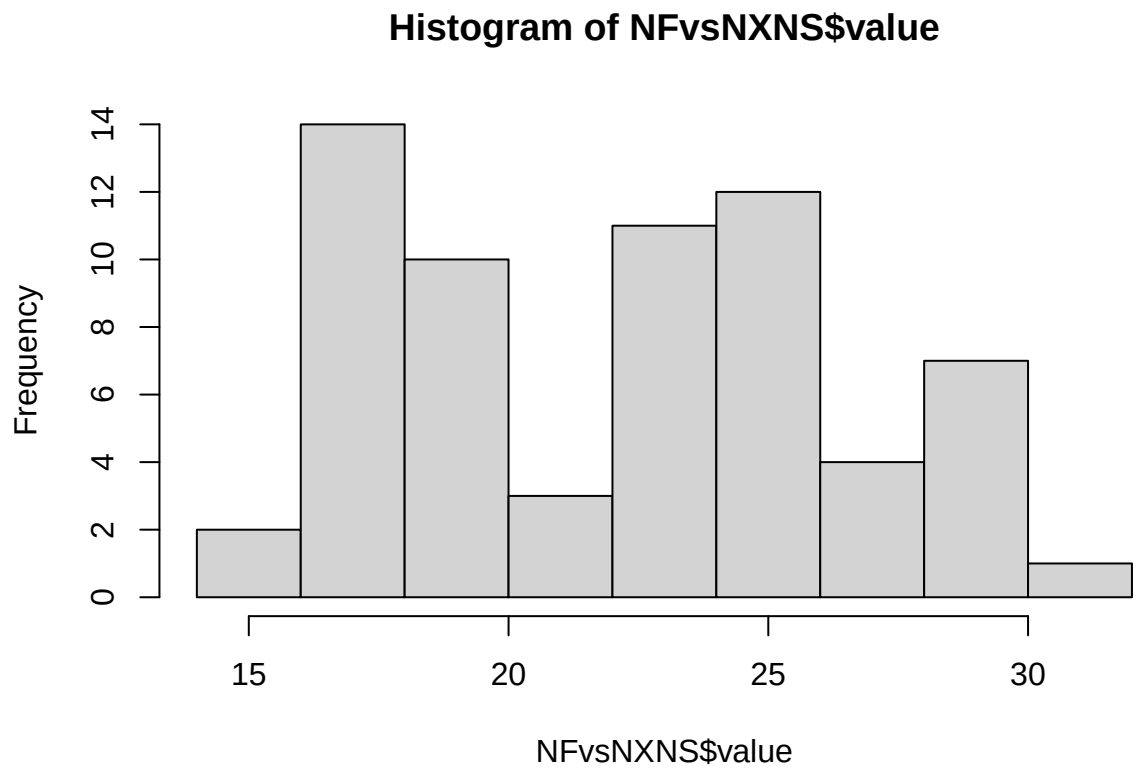

```
#Add jitter naming column
P2NFvsNXNS<-mutate(P2NFvsNXNS, jitter="P2")
#Select comparisons where S1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S10hNFvsNXNS<-filter(meltdist, grepl('NFNS_S1_0h', Var1))
#From this include comparisons to S1, 0h storage and NFNS
S10hNFvsNXNS<-filter(S10hNFvsNXNS, grepl('NX[0-9]NS_S1_0h', Var2))
#Select comparisons where S1, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S164hNFvsNXNS<-filter(meltdist, grepl('NFNS_S1_64h', Var1))
#From this include comparisons to S1, 64h storage and NFNS
S164hNFvsNXNS<-filter(S164hNFvsNXNS, grepl('NX[0-9]NS_S1_64h', Var2))
S1NFvsNXNS<-bind_rows(S10hNFvsNXNS, S164hNFvsNXNS)
#Histogram to look at distribution
hist(S1NFvsNXNS$value)
```



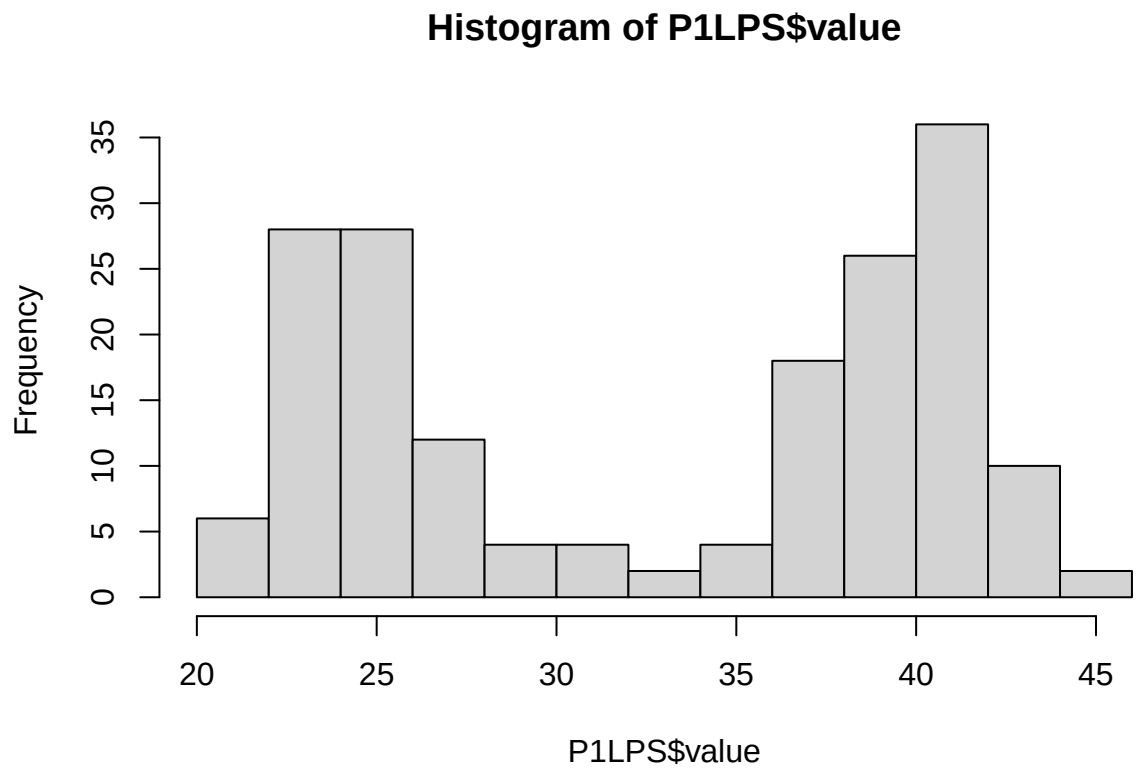

```
#Add jitter naming column
S1NFvsNXNS<-mutate(S1NFvsNXNS, jitter="S1")
#Select comparisons where S2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S20hNFvsNXNS<-filter(meltdist, grepl('NFNS_S2_0h', Var1))
#From this include comparisons to S2, 0h storage and NFNS
S20hNFvsNXNS<-filter(S20hNFvsNXNS, grepl('NX[0-9]NS_S2_0h', Var2))
#Select comparisons where S2, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S264hNFvsNXNS<-filter(meltdist, grepl('NFNS_S2_64h', Var1))
#From this include comparisons to S2, 64h storage and NFNS
S264hNFvsNXNS<-filter(S264hNFvsNXNS, grepl('NX[0-9]NS_S2_64h', Var2))
S2NFvsNXNS<-bind_rows(S20hNFvsNXNS, S264hNFvsNXNS)
#Histogram to look at distribution
hist(S2NFvsNXNS$value)
```



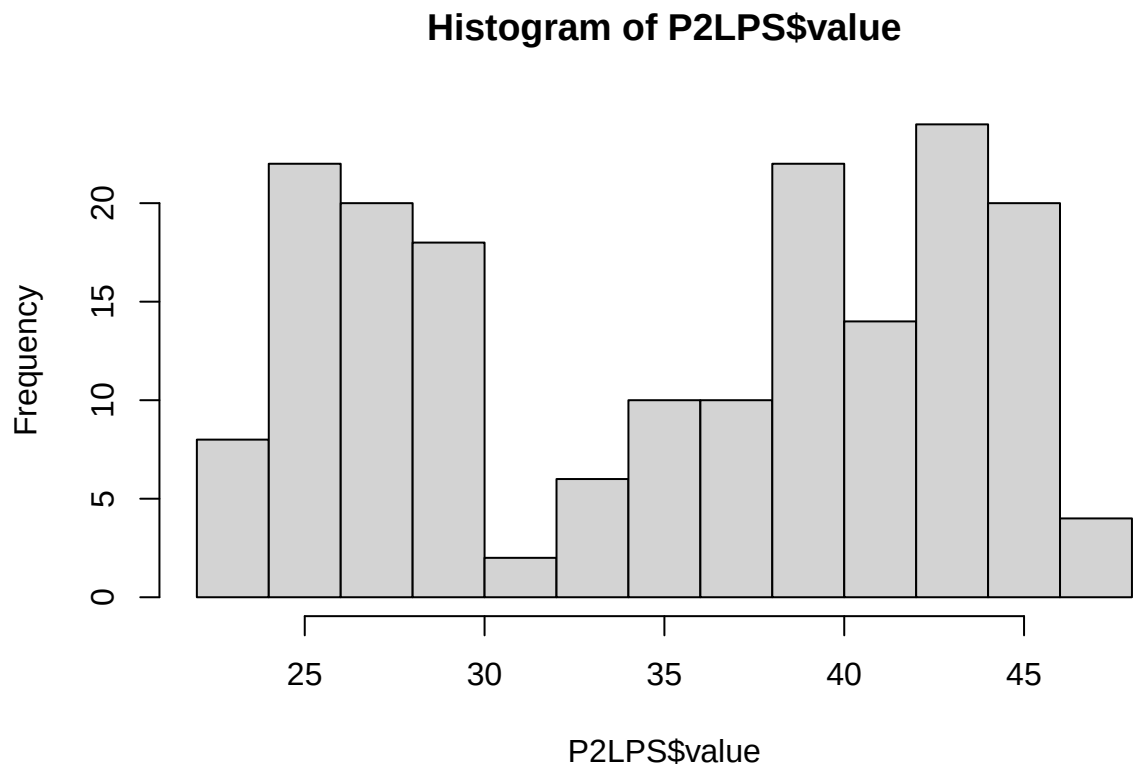

```
#Add jitter naming column
S2NFvsNXNS<-mutate(S2NFvsNXNS, jitter="S2")
#Combine comparisons of nextflex nextera runs in all samples on nextseq
NFvsNXNS<-bind_rows(P1NFvsNXNS, P2NFvsNXNS, S1NFvsNXNS, S2NFvsNXNS)
#Histogram to look at distribution
hist(NFvsNXNS$value)
```



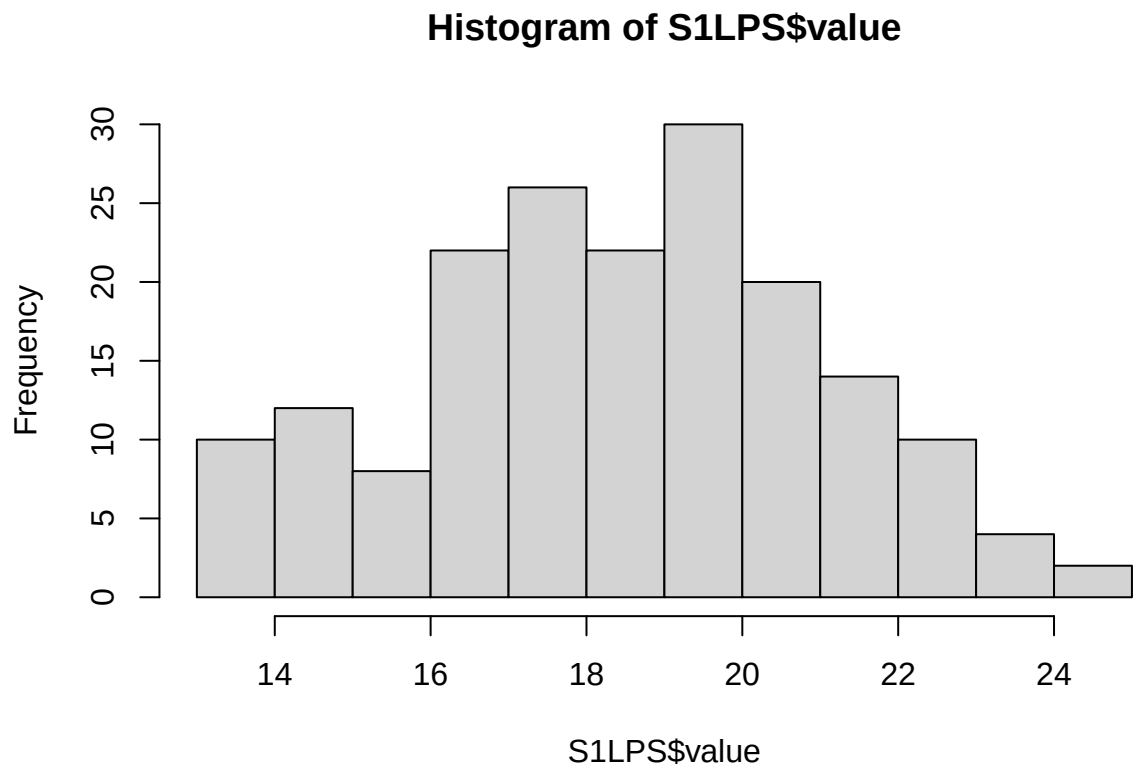

```
#Add Comparison column
NFvsNXNS<-mutate(NFvsNXNS, Comparison="NFvsNXNS")


#Distance between all LPS samples
#Select comparisons where P1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P10hLPS<-filter(meltdist, grepl('P1_0h', Var1))
#From this include comparisons to P1, 0h storage and NFNS
P10hLPS<-filter(P10hLPS, grepl('_P1_0h', Var2))
#Select comparisons where P1, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P164hLPS<-filter(meltdist, grepl('P1_64h', Var1))
#From this include comparisons to P1, 64h storage and NFNS
P164hLPS<-filter(P164hLPS, grepl('_P1_64h', Var2))
P1LPS<-bind_rows(P10hLPS, P164hLPS)
#Histogram to look at distribution
hist(P1LPS$value)
```



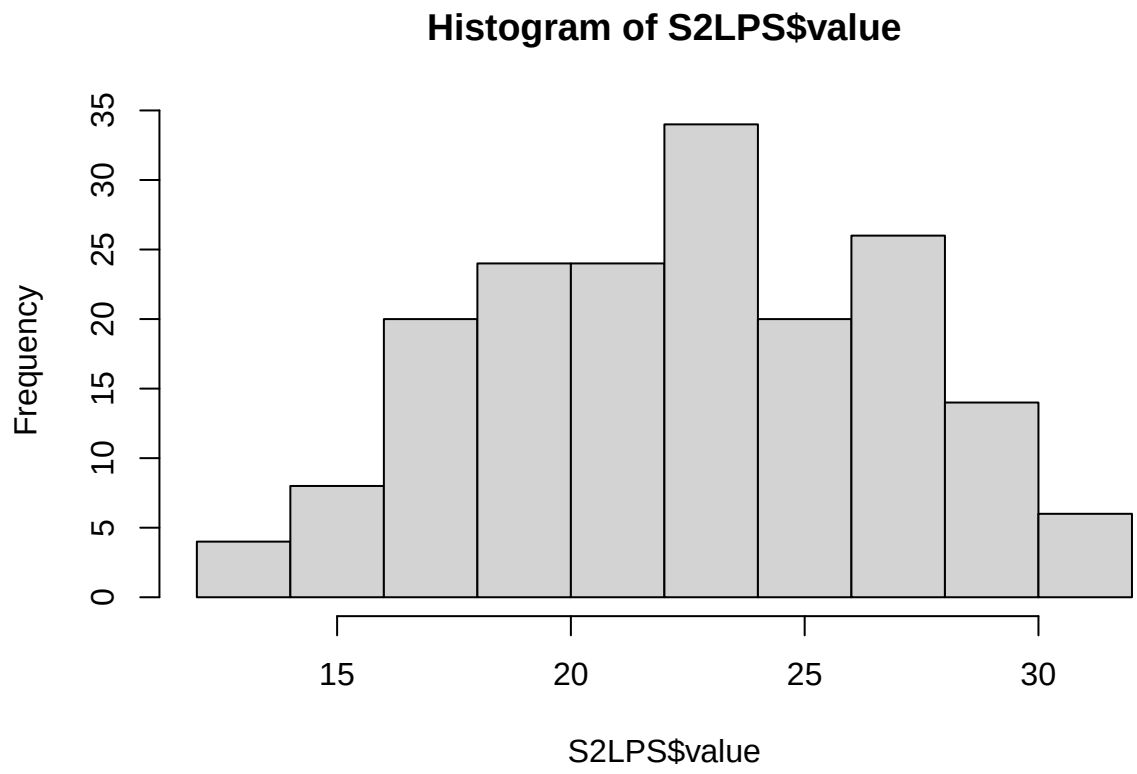

```
#Add jitter naming column
P1LPS<-mutate(P1LPS, jitter="P1")
#Select comparisons where P2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P20hLPS<-filter(meltdist, grepl('P2_0h', Var1))
#From this include comparisons to P2, 0h storage and NFNS
P20hLPS<-filter(P20hLPS, grepl('_P2_0h', Var2))
#Select comparisons where P2, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P264hLPS<-filter(meltdist, grepl('P2_64h', Var1))
#From this include comparisons to P2, 64h storage and NFNS
P264hLPS<-filter(P264hLPS, grepl('_P2_64h', Var2))
P2LPS<-bind_rows(P20hLPS, P264hLPS)
#Histogram to look at distribution
hist(P2LPS$value)
```



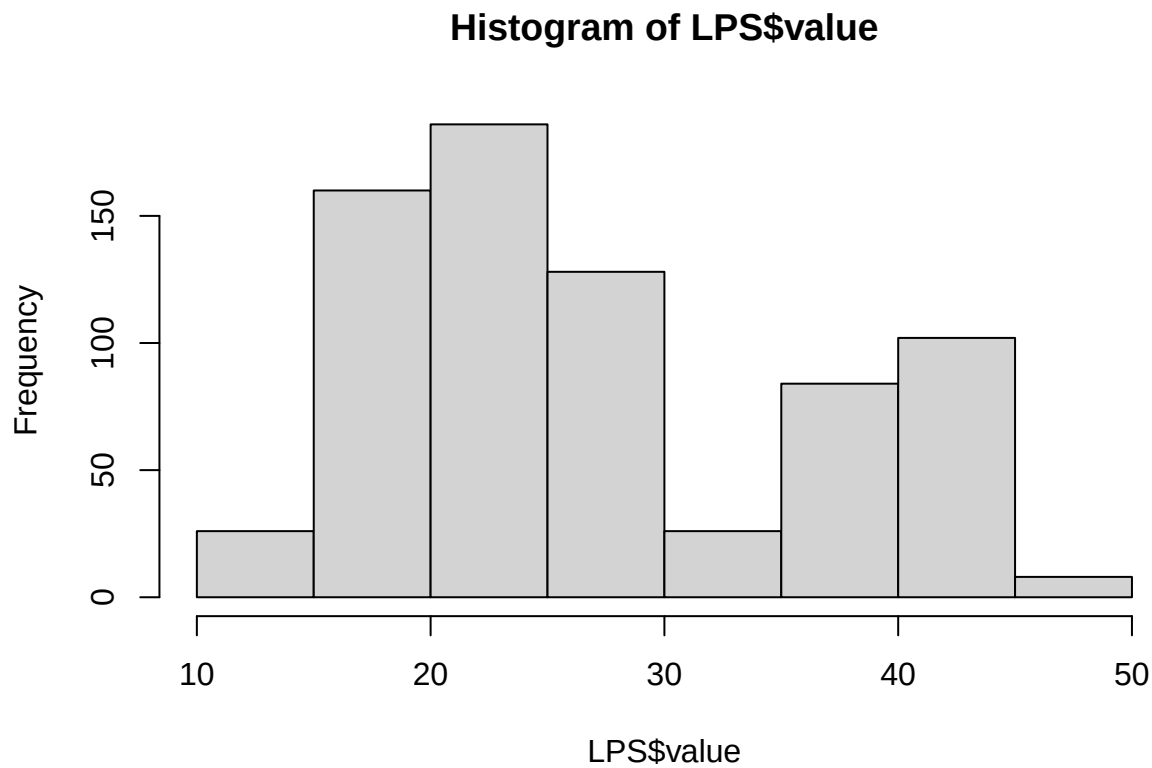

```
#Add jitter naming column
P2LPS<-mutate(P2LPS, jitter="P2")
#Select comparisons where S1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S10hLPS<-filter(meltdist, grepl('S1_0h', Var1))
#From this include comparisons to S1, 0h storage and NFNS
S10hLPS<-filter(S10hLPS, grepl('_S1_0h', Var2))
#Select comparisons where S1, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S164hLPS<-filter(meltdist, grepl('S1_64h', Var1))
#From this include comparisons to S1, 64h storage and NFNS
S164hLPS<-filter(S164hLPS, grepl('_S1_64h', Var2))
S1LPS<-bind_rows(S10hLPS, S164hLPS)
#Histogram to look at distribution
hist(S1LPS$value)
```




```
#Add jitter naming column
S1LPS<-mutate(S1LPS, jitter="S1")
#Select comparisons where S2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S20hLPS<-filter(meltdist, grepl('S2_0h', Var1))
#From this include comparisons to S2, 0h storage and NFNS
S20hLPS<-filter(S20hLPS, grepl('_S2_0h', Var2))
#Select comparisons where S2, 64h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S264hLPS<-filter(meltdist, grepl('S2_64h', Var1))
#From this include comparisons to S2, 64h storage and NFNS
S264hLPS<-filter(S264hLPS, grepl('_S2_64h', Var2))
S2LPS<-bind_rows(S20hLPS, S264hLPS)
#Histogram to look at distribution
hist(S2LPS$value)
```

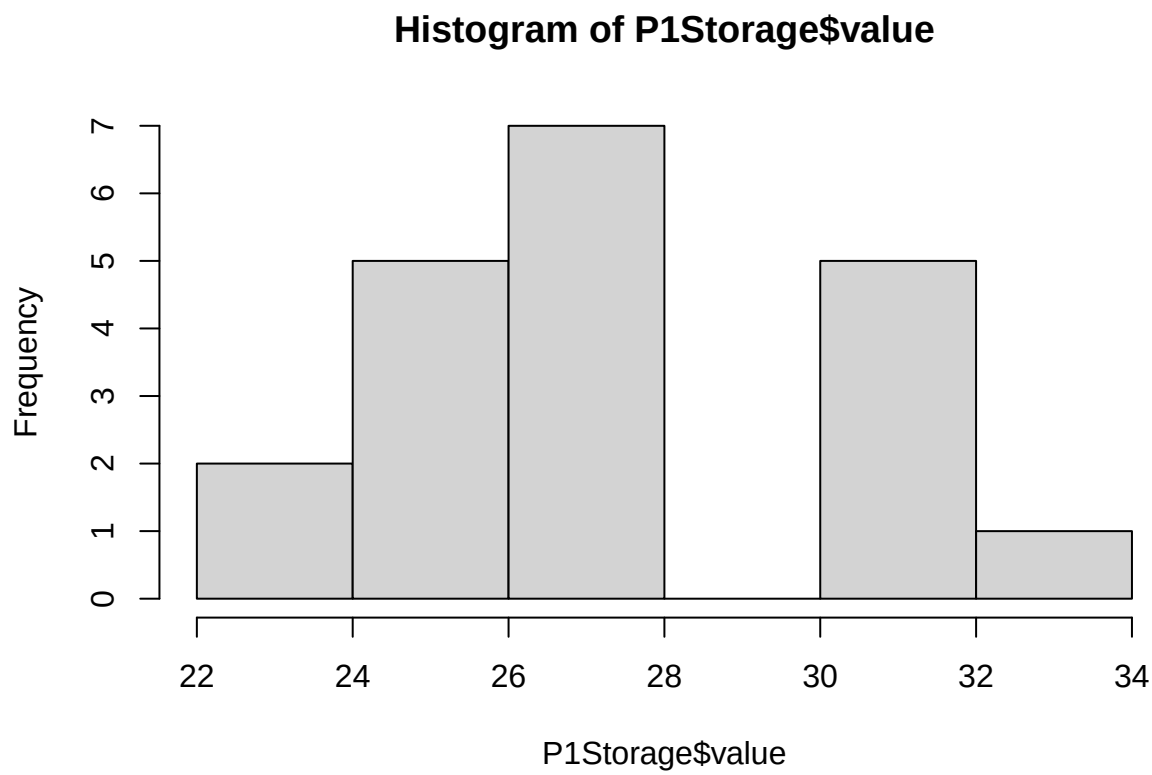



```
#Add jitter naming column
S2LPS<-mutate(S2LPS, jitter="S2")
#Combine comparisons of nextflex nextera runs in all samples on nextseq
LPS<-bind_rows(P1LPS, P2LPS, S1LPS, S2LPS)
#Histogram to look at distribution
hist(LPS$value)
```



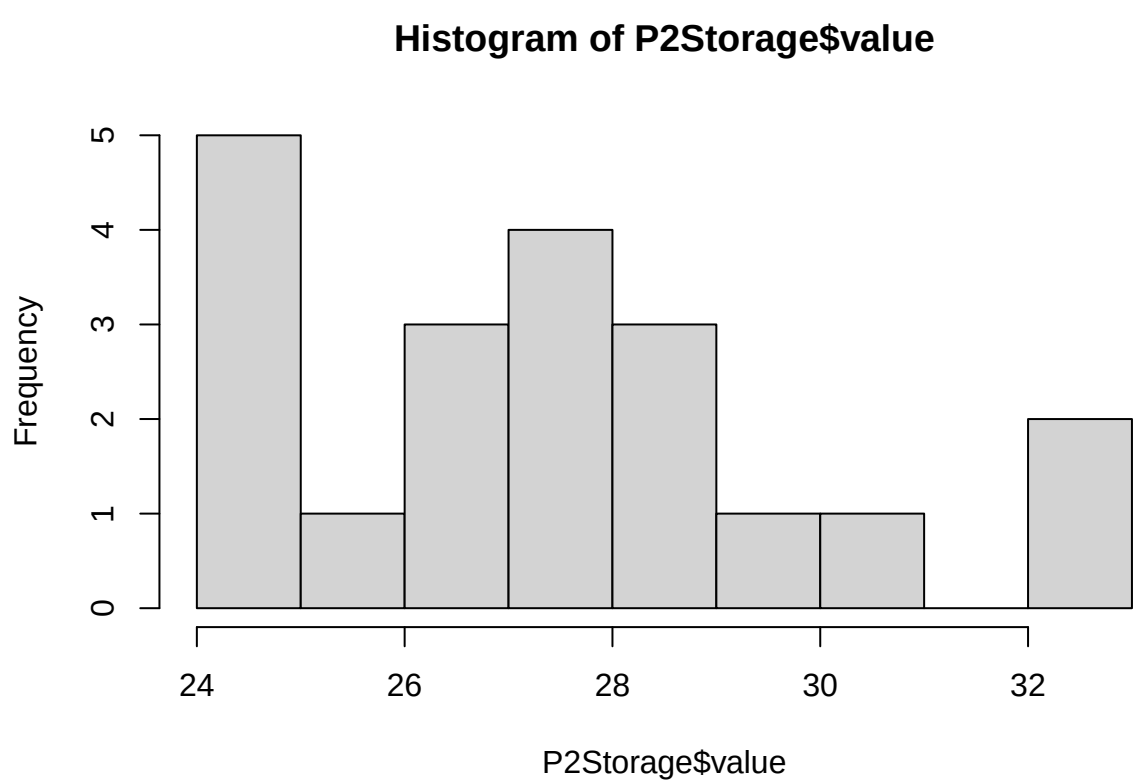

```
#Add Comparison column
LPS<-mutate(LPS, Comparison="LPS")


#Distance between storage samples
#Select comparisons where P1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P1HXStorage<-filter(meltdist, grepl('HX_P1_0h', Var1))
#From this include comparisons to P1, 0h storage and NFNS
P1HXStorage<-filter(P1HXStorage, grepl('HX_P1_64h', Var2))
#Select comparisons where P1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P1KAHIStorage<-filter(meltdist, grepl('KAHI_P1_0h', Var1))
#From this include comparisons to P1, 0h storage and NFNS
P1KAHIStorage<-filter(P1KAHIStorage, grepl('KAHI_P1_64h', Var2))
#Select comparisons where P1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P1NFNSStorage<-filter(meltdist, grepl('NFNS_P1_0h', Var1))
#From this include comparisons to P1, 0h storage and NFNS
P1NFNSStorage<-filter(P1NFNSStorage, grepl('NFNS_P1_64h', Var2))
#Select comparisons where P1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P1NX1NSStorage<-filter(meltdist, grepl('NX1NS_P1_0h', Var1))
#From this include comparisons to P1, 0h storage and NFNS
P1NX1NSStorage<-filter(P1NX1NSStorage, grepl('NX1NS_P1_64h', Var2))
#Select comparisons where P1, 0h storage and NFHI is part of the first variable
```

```
  #(Part of the HX experiment)
P1NX2NSStorage<-filter(meltdist, grepl('NX2NS_P1_0h', Var1))
#From this include comparisons to P1, 0h storage and NFNS
P1NX2NSStorage<-filter(P1NX2NSStorage, grepl('NX2NS_P1_64h', Var2))
#Select comparisons where P1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P1Storage<-bind_rows(P1HXStorage, P1KAHIStorage, P1NFNSStorage, P1NX1NSStorage,
                     P1NX2NSStorage)
#Histogram to look at distribution
hist(P1Storage$value)
```



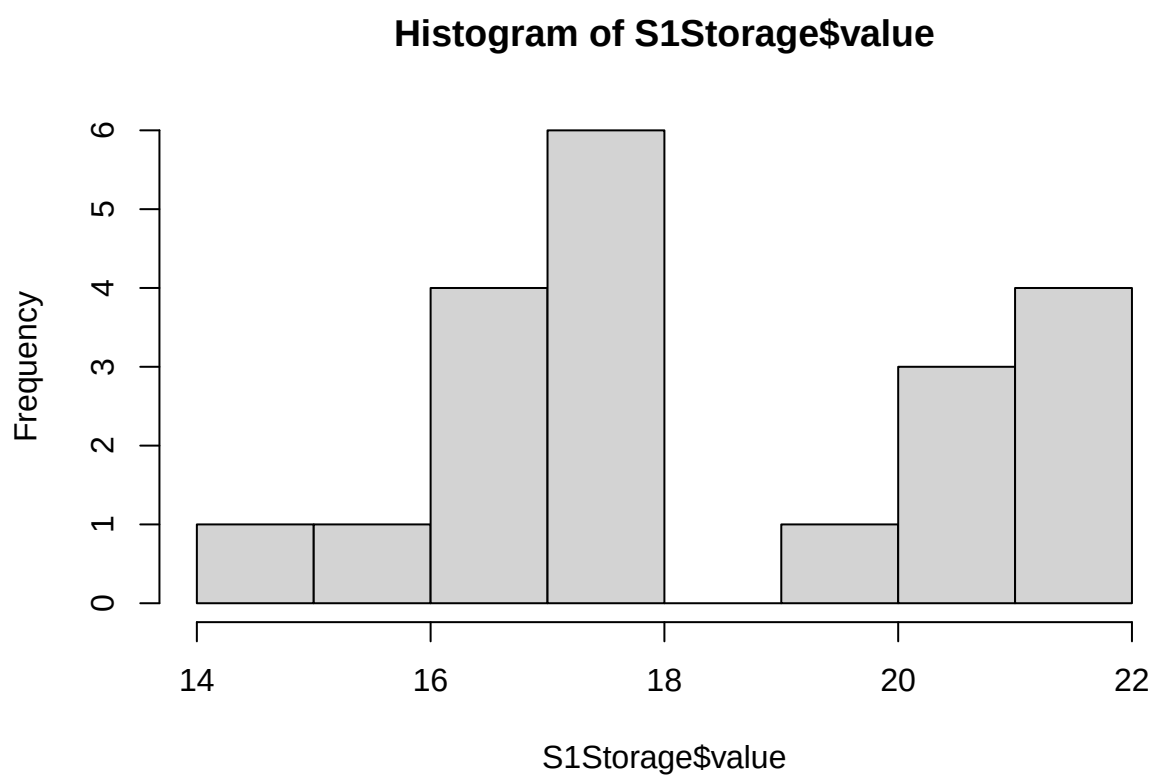

```
#Add jitter naming column
P1Storage<-mutate(P1Storage, jitter="P1")
#Select comparisons where P2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P2HXStorage<-filter(meltdist, grepl('HX_P2_0h', Var1))
#From this include comparisons to P2, 0h storage and NFNS
P2HXStorage<-filter(P2HXStorage, grepl('HX_P2_64h', Var2))
#Select comparisons where P2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P2KAHIStorage<-filter(meltdist, grepl('KAHI_P2_0h', Var1))
#From this include comparisons to P2, 0h storage and NFNS
P2KAHIStorage<-filter(P2KAHIStorage, grepl('KAHI_P2_64h', Var2))
#Select comparisons where P2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P2NFNSStorage<-filter(meltdist, grepl('NFNS_P2_0h', Var1))
```

```r
#From this include comparisons to P2, 0h storage and NFNS
P2NFNSStorage<-filter(P2NFNSStorage, grepl('NFNS_P2_64h', Var2))
#Select comparisons where P2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P2NX1NSStorage<-filter(meltdist, grepl('NX1NS_P2_0h', Var1))
#From this include comparisons to P2, 0h storage and NFNS
P2NX1NSStorage<-filter(P2NX1NSStorage, grepl('NX1NS_P2_64h', Var2))
#Select comparisons where P2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P2NX2NSStorage<-filter(meltdist, grepl('NX2NS_P2_0h', Var1))
#From this include comparisons to P2, 0h storage and NFNS
P2NX2NSStorage<-filter(P2NX2NSStorage, grepl('NX2NS_P2_64h', Var2))
#Select comparisons where P2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
P2Storage<-bind_rows(P2HXStorage, P2KAHIStorage, P2NFNSStorage, P2NX1NSStorage,
                     P2NX2NSStorage)
#Histogram to look at distribution
hist(P2Storage$value)
```

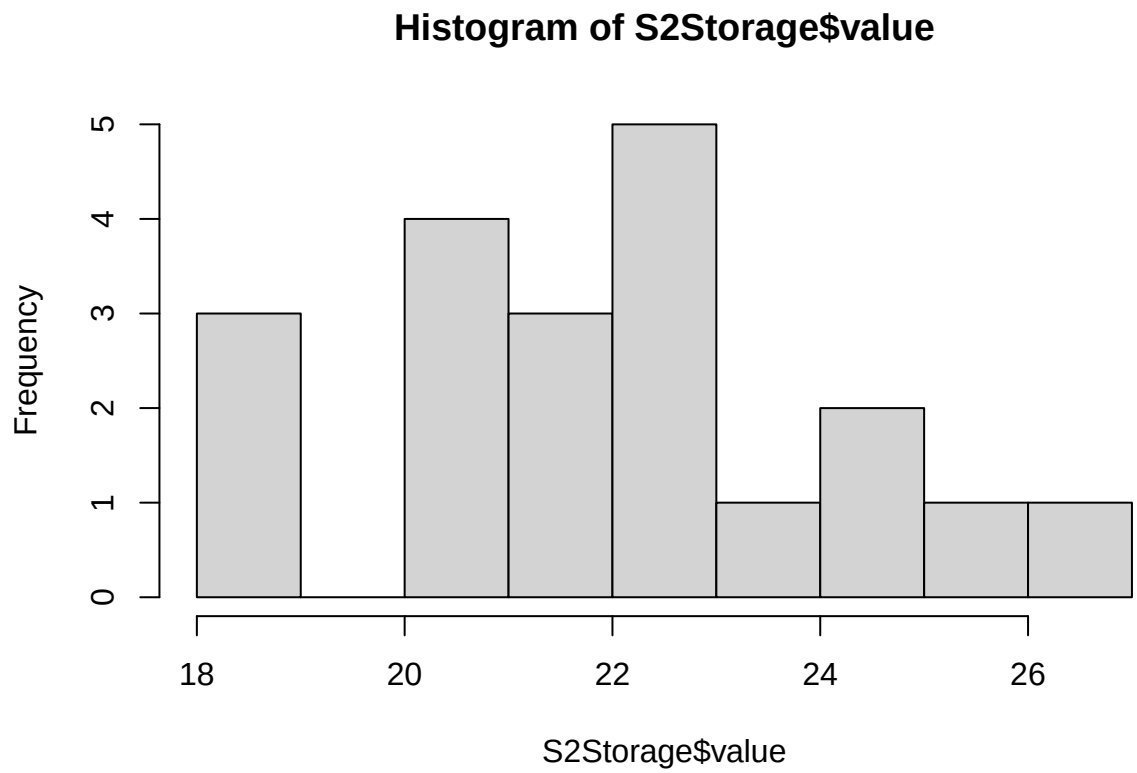



```r
#Add jitter naming column
P2Storage<-mutate(P2Storage, jitter="P2")
#Select comparisons where S1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S1HXStorage<-filter(meltdist, grepl('HX_S1_0h', Var1))
#From this include comparisons to S1, 0h storage and NFNS
S1HXStorage<-filter(S1HXStorage, grepl('HX_S1_64h', Var2))
```

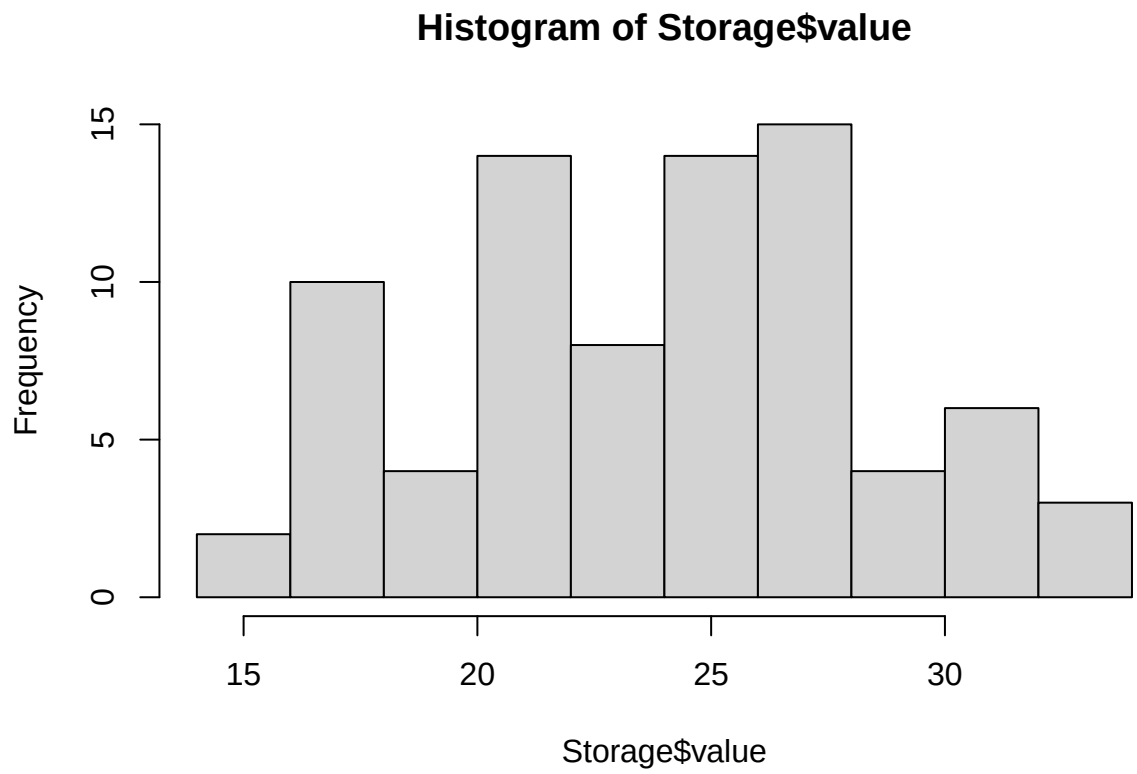
```
#Select comparisons where S1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S1KAHIStorage<-filter(meltdist, grepl('KAHI_S1_0h', Var1))
#From this include comparisons to S1, 0h storage and NFNS
S1KAHIStorage<-filter(S1KAHIStorage, grepl('KAHI_S1_64h', Var2))
#Select comparisons where S1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S1NFNSStorage<-filter(meltdist, grepl('NFNS_S1_0h', Var1))
#From this include comparisons to S1, 0h storage and NFNS
S1NFNSStorage<-filter(S1NFNSStorage, grepl('NFNS_S1_64h', Var2))
#Select comparisons where S1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S1NX1NSStorage<-filter(meltdist, grepl('NX1NS_S1_0h', Var1))
#From this include comparisons to S1, 0h storage and NFNS
S1NX1NSStorage<-filter(S1NX1NSStorage, grepl('NX1NS_S1_64h', Var2))
#Select comparisons where S1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S1NX2NSStorage<-filter(meltdist, grepl('NX2NS_S1_0h', Var1))
#From this include comparisons to S1, 0h storage and NFNS
S1NX2NSStorage<-filter(S1NX2NSStorage, grepl('NX2NS_S1_64h', Var2))
#Select comparisons where S1, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S1Storage<-bind_rows(S1HXStorage, S1KAHIStorage, S1NFNSStorage, S1NX1NSStorage,
                     S1NX2NSStorage)
#Histogram to look at distribution
hist(S1Storage$value)
```



```r
#Add jitter naming column
S1Storage<-mutate(S1Storage, jitter="S1")
#Select comparisons where S2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S2HXStorage<-filter(meltdist, grepl('HX_S2_0h', Var1))
#From this include comparisons to S2, 0h storage and NFNS
S2HXStorage<-filter(S2HXStorage, grepl('HX_S2_64h', Var2))
#Select comparisons where S2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S2KAHIStorage<-filter(meltdist, grepl('KAHI_S2_0h', Var1))
#From this include comparisons to S2, 0h storage and NFNS
S2KAHIStorage<-filter(S2KAHIStorage, grepl('KAHI_S2_64h', Var2))
#Select comparisons where S2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S2NFNSStorage<-filter(meltdist, grepl('NFNS_S2_0h', Var1))
#From this include comparisons to S2, 0h storage and NFNS
S2NFNSStorage<-filter(S2NFNSStorage, grepl('NFNS_S2_64h', Var2))
#Select comparisons where S2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S2NX1NSStorage<-filter(meltdist, grepl('NX1NS_S2_0h', Var1))
#From this include comparisons to S2, 0h storage and NFNS
S2NX1NSStorage<-filter(S2NX1NSStorage, grepl('NX1NS_S2_64h', Var2))
#Select comparisons where S2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S2NX2NSStorage<-filter(meltdist, grepl('NX2NS_S2_0h', Var1))
#From this include comparisons to S2, 0h storage and NFNS
S2NX2NSStorage<-filter(S2NX2NSStorage, grepl('NX2NS_S2_64h', Var2))
#Select comparisons where S2, 0h storage and NFHI is part of the first variable
  #(Part of the HX experiment)
S2Storage<-bind_rows(S2HXStorage, S2KAHIStorage, S2NFNSStorage, S2NX1NSStorage,
                     S2NX2NSStorage)
#Histogram to look at distribution
hist(S2Storage$value)
```

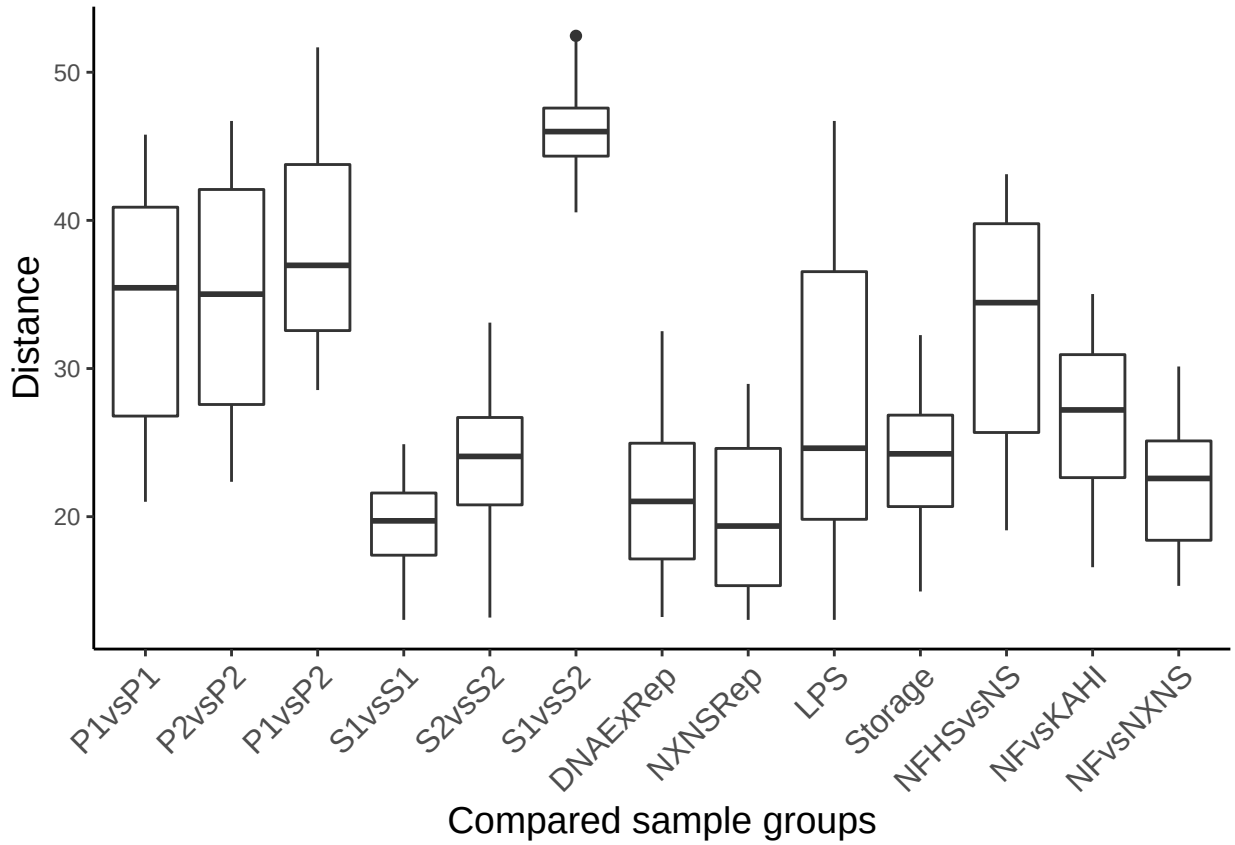



```
#Add jitter naming column
S2Storage<-mutate(S2Storage, jitter="S2")
#Combine comparisons of nextflex nextera runs in all samples on nextseq
Storage<-bind_rows(P1Storage, P2Storage, S1Storage, S2Storage)
#Histogram to look at distribution
hist(Storage$value)
```




```
#Add Comparison column
Storage<-mutate(Storage, Comparison="Storage")


#Combine selected comparisons
boxplotting<-bind_rows(P1vsP2, S1vsS2, P1vsP1, P2vsP2, S1vsS1, S2vsS2, NX1vsNX2, DNAExRep,
                       NFHSvsNS, NFvsKAHI, NFvsNXNS, LPS, Storage) #Removed PFvsSW makes
  #other differences difficult to see.

#Create boxplot
#Defining the order, can change if needed
boxplotting$Comparison<-factor(boxplotting$Comparison,
                               levels= c("P1vsP1", "P2vsP2", "P1vsP2", "S1vsS1", "S2vsS2",
                                         "S1vsS2", "DNAExRep","NXNSRep", "LPS", "Storage",
                                         "NFHSvsNS", "NFvsKAHI", "NFvsNXNS"))
boxplotting$jitter<-factor(boxplotting$jitter, levels= c("P1vsP2", "S1vsS2", "P1", "P2",
                                                         "S1", "S2"))

#pdf(paste("LPSXSFig2BoxplotPWD", ".pdf", sep=""), height=6, width=12)
ggplot(boxplotting, aes(x=Comparison, y=value)) +
  geom_boxplot() +
  labs(x="Compared sample groups", y="Distance") +
  theme_bw() +
  theme(panel.border = element_blank(), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), axis.line = element_line(colour = "black"),
        axis.text.x = element_text(size=12, angle = 45, hjust = 1),
```

```
        axis.title=element_text(size=14))
```




```
#dev.off()

#Trying to make fancy boxplots + geom_jitter(), might want to
jitter=c(PFvsSW = "Darkblue", P1vsP2 = "Darkblue", S1vsS2 = "Darkblue", P1 = "#30241E",
         P2 = "#B59B80", S1 = "#33a02c", S2 = "#B2DF8A")
pdf(paste("Fig1_BoxplotsPWD", ".pdf", sep=""), height=6, width=12)
  #PWD = Pairwise distances
ggplot(boxplotting, aes(x=Comparison, y=value)) +
  geom_boxplot() +
  labs(x="Compared sample groups", y="Distance") +
  theme_bw() +
  theme(panel.border = element_blank(), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(), axis.line = element_line(colour = "black"),
        axis.text.x = element_text(size=12, angle = 45, hjust = 1),
        axis.title=element_text(size=14)) +
  geom_jitter(aes(color=jitter), size=0.7) +
  scale_color_manual(values = jitter) +
  guides(colour = guide_legend(override.aes = list(size=5)))
dev.off()
```

```
## pdf
##   2
```

sPLS-DA

Used to find most important features for describing library preparation and sequencing platform. Overview represented as S4_Fig

```
rm(list=setdiff(ls(), c("Metadata", "Feature", "Tax")))

#Subset data pig feces 1 and 2 (P1, P2), Sewage 1 and 2 (S1, S2), or spiked unspiked
Subset <- "All" #All, Allspiked, Allunspiked, PF, SW, P1, P1spiked, P1unspiked, P2, P2spiked, P2unspike

#Subset experiment, Experiment_Type. (Meaningfull combinations of subset and SubExp: HX=All, FTX=P1&S1,
SubExp<-"LPSX" #HX, FTX, LTX, LPSX, HXFTX, HXLTX, HXLPSX, HXLTXLPSX, HXFTXLTX, All

#Subset Frozen Unfrozen
SubFre<-"Both" #Frozen, Unfrozen, Both

#make empty list for plots
sPLSList=list()

#Removing negative and positive controls
Metadata2<-filter(Metadata, Sample_type_simple=="Sample")

#Subsetting Metadata2
if (Subset=="Allspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Spiked")
} else if (Subset=="Allunspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Unspiked")
} else if (Subset=="PF") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1" | Experiment == "Pig_feces_2")
} else if (Subset=="PFspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked" |
                      Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="PFunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1" |
                      Sample_type == "Pig_feces_2")
} else if (Subset=="SW") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1" | Experiment == "Sewage_2")
} else if (Subset=="SWspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked" |
                      Sample_type == "Sewage_2_spiked")
} else if (Subset=="SWunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1" | Sample_type == "Sewage_2")
} else if (Subset=="P1") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1")
} else if (Subset=="P1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked")
} else if (Subset=="P1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1")
} else if (Subset=="P2") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_2")
} else if (Subset=="P2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2_spiked")
} else if (Subset=="P2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2")
} else if (Subset=="S1") {
```

```
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1")
} else if (Subset=="S1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked")
} else if (Subset=="S1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1")
} else if (Subset=="S2") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_2")
} else if (Subset=="S2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2_spiked")
} else if (Subset=="S2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2")
} else if (Subset=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

```
## [1] "No subsetting, all included"
```

```
#Further subsetting Metadata2
if (SubExp=="HX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment")
} else if (SubExp=="FTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="LTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="LPSX") {
  Metadata22<-filter(Metadata2, Experiment_type == "Library_prep_seq_platform_experiment")
  VectorLPSX <- unique(Metadata22$Matching_samples)
  Metadata2<-filter(Metadata2, Matching_samples %in% VectorLPSX)
  rm(VectorLPSX, Metadata22)
} else if (SubExp=="HXFTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="HXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="HXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXLTXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXFTXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

```r
#Further subsetting Metadata2
if (SubFre=="Frozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Freezer")
} else if (SubFre=="Unfrozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Unfrozen")
} else if (SubFre=="Both") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}
```

```
## [1] "No subsetting, all included"
```

```r
#Removing the Kappa NextSeq run
Metadata2<-Metadata2[grep("\\KANS.*", Metadata2$Sample, invert=TRUE),]

#Applying subsetting to OTU tables
Tax2<-dplyr::select(Tax, one_of(Metadata2$Sample))

#Note, that this is done on the full set, not just the shown. Makes sense eventhough not
  #show in heatmap they can be in the clustering calculations, this also means samples can
  #look more similar in the heatmap but not cluster as closely. Also calculates on the not
  #log transformed data.
#filtering of the Counttable depending on rowSums.
Tax2 <- Tax2[rowSums(Tax2)>0,] #Removing all rows that only contains zeroes
Tax2 <- Tax2[rowSums(Tax2)>(5*ncol(Tax2)),] #Removing all rows(Species) that is below an
  #average count of 5
# replace 0 values with an estimate using simple multiplicative replacement
Tax2 <- t(cmultRepl(t(Tax2), method="CZM", label=0))
```

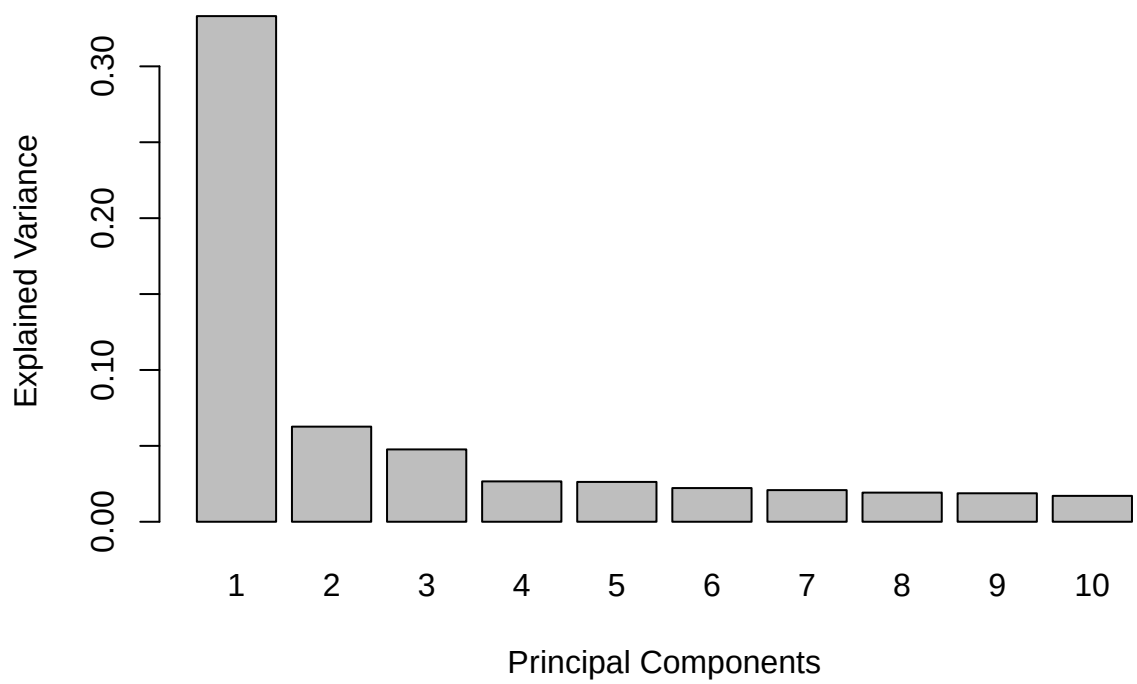
```
## No. corrected values:  4863
```

```r
#Maks TSS
Tax2<-as.matrix(t(sweep(Tax2, 2, colSums(Tax2), FUN="/"))) #Transpose to have genera as
  #columns and samples as rows

##Interest from the following sPLS-DA
#GeneraSel <- dplyr::select(data.frame(Tax2), one_of(c("Methanosaeta", "Polaribacter",
  #"Tenacibaculum", "Porphyromonas", "Gulbenkiania", "Cellulophaga")))
#row.names(GeneraSel)==Metadata2$Sample
#GeneraSel<-bind_cols(GeneraSel, Metadata2)
#Methanosaeta<-summarySE(data=GeneraSel, "Methanosaeta",
  #groupvars=c("Sample_type_RF", "Library_preparation", "Sequencing_platform"))
#Polaribacter<-summarySE(data=GeneraSel, "Polaribacter",
  #groupvars=c("Sample_type_RF", "Library_preparation", "Sequencing_platform"))
#Tenacibaculum<-summarySE(data=GeneraSel, "Tenacibaculum",
  #groupvars=c("Sample_type_RF", "Library_preparation", "Sequencing_platform"))
#Porphyromonas<-summarySE(data=GeneraSel, "Porphyromonas",
  #groupvars=c("Sample_type_RF", "Library_preparation", "Sequencing_platform"))
#Gulbenkiania<-summarySE(data=GeneraSel, "Gulbenkiania",
  #groupvars=c("Sample_type_RF", "Library_preparation", "Sequencing_platform"))
#Cellulophaga<-summarySE(data=GeneraSel, "Cellulophaga",
  #groupvars=c("Sample_type_RF", "Library_preparation", "Sequencing_platform"))

# CLR is included in the mixOmics functions
```

```r
#Is metadata the same order as Tax2
Metadata2$Sample==row.names(Tax2)
```

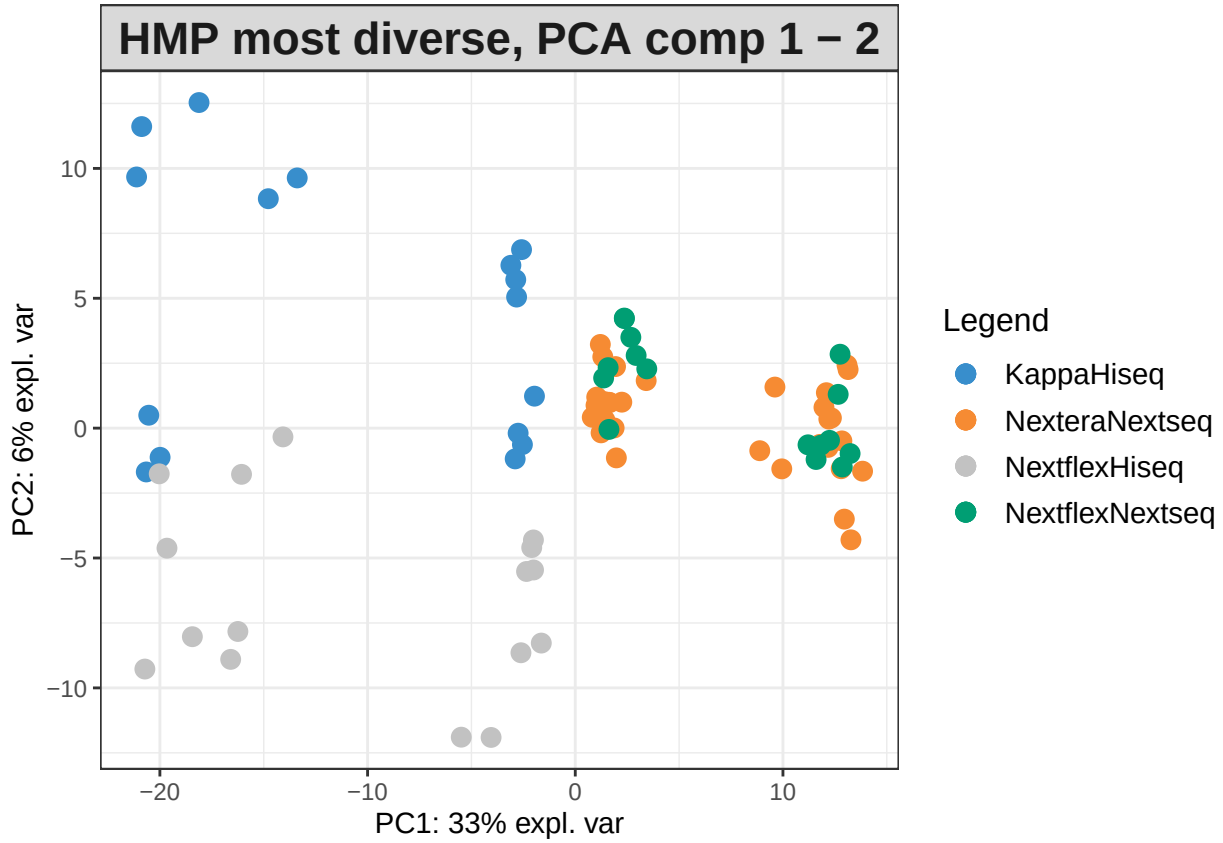
```
##  [1] TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE
## [16] TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE
## [31] TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE
## [46] TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE
## [61] TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE
## [76] TRUE TRUE TRUE TRUE TRUE
```

```r
# the outcome
Y <- paste(Metadata2$Library_preparation, Metadata2$Sequencing_platform, sep="")
# unique ID of each individual for multilevel analysis
sample <- paste(Metadata2$Sample_type_RF, Metadata2$Replicate, sep="")

#Analysis included in
  #http://mixomics.org/mixmc/case-study-hmp-bodysites-repeated-measures/
diverse.pca = mixOmics::pca(Tax2, ncomp = 10, logratio = 'CLR', multilevel = sample)
#diverse.pca
plot(diverse.pca)
```

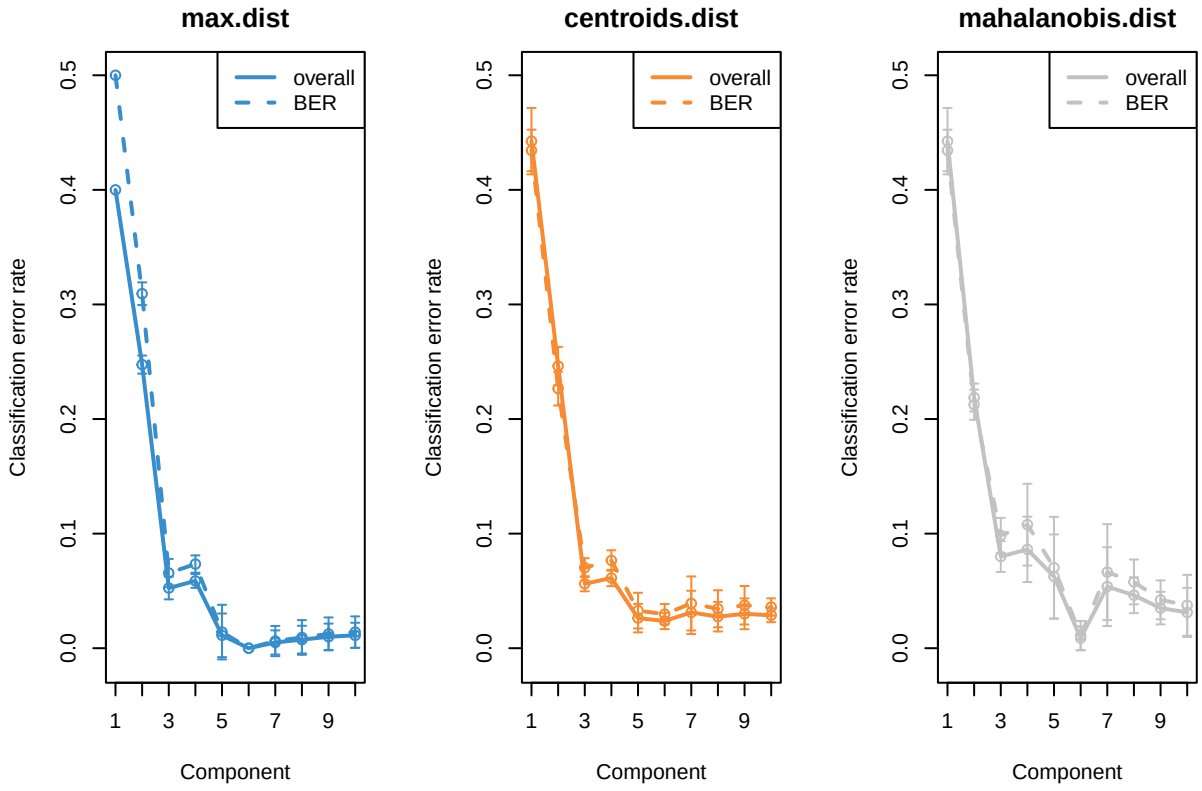



```r
plotIndiv(diverse.pca,
          comp = c(1,2), # the components to plot
          pch = 16,
          ind.names = F,
          group = Y,
```

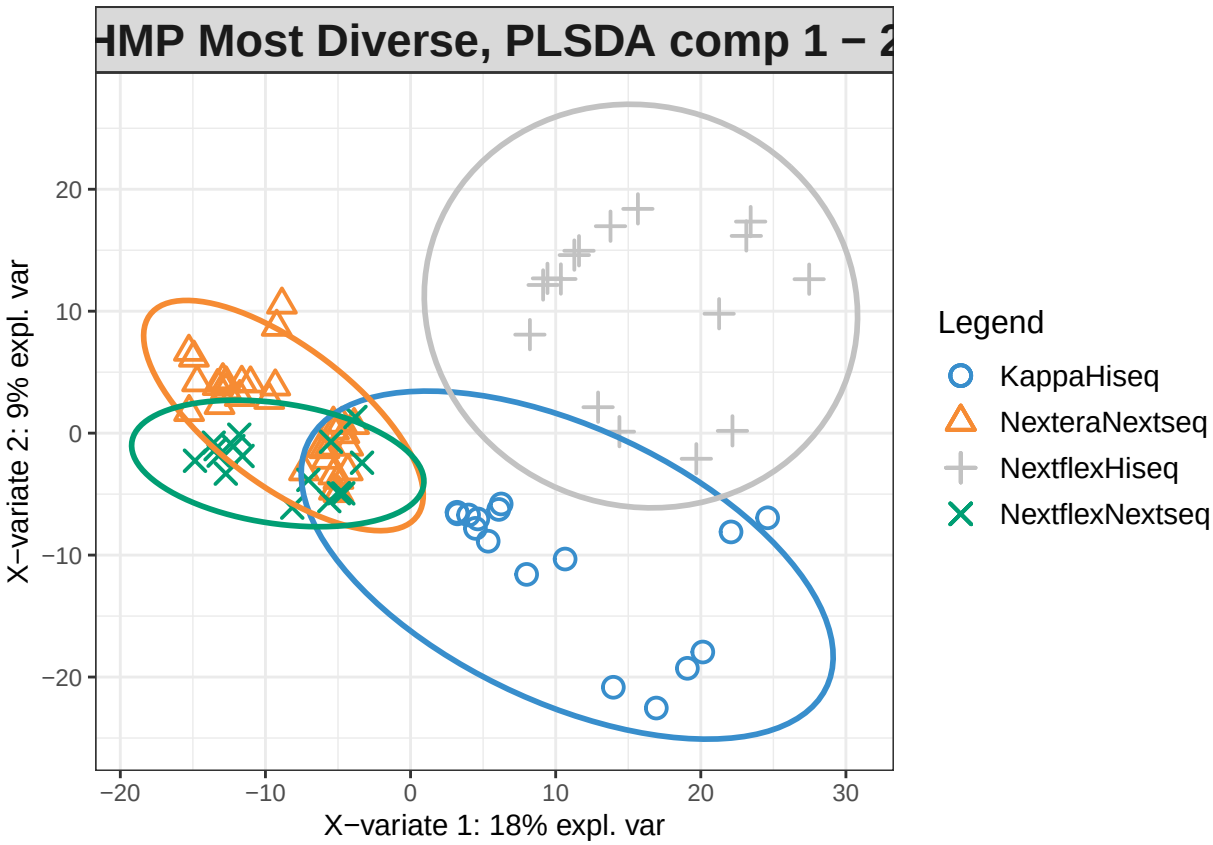
```
          col.per.group = color.mixo(1:4),
          legend = TRUE,
          title = 'HMP most diverse, PCA comp 1 - 2')
```



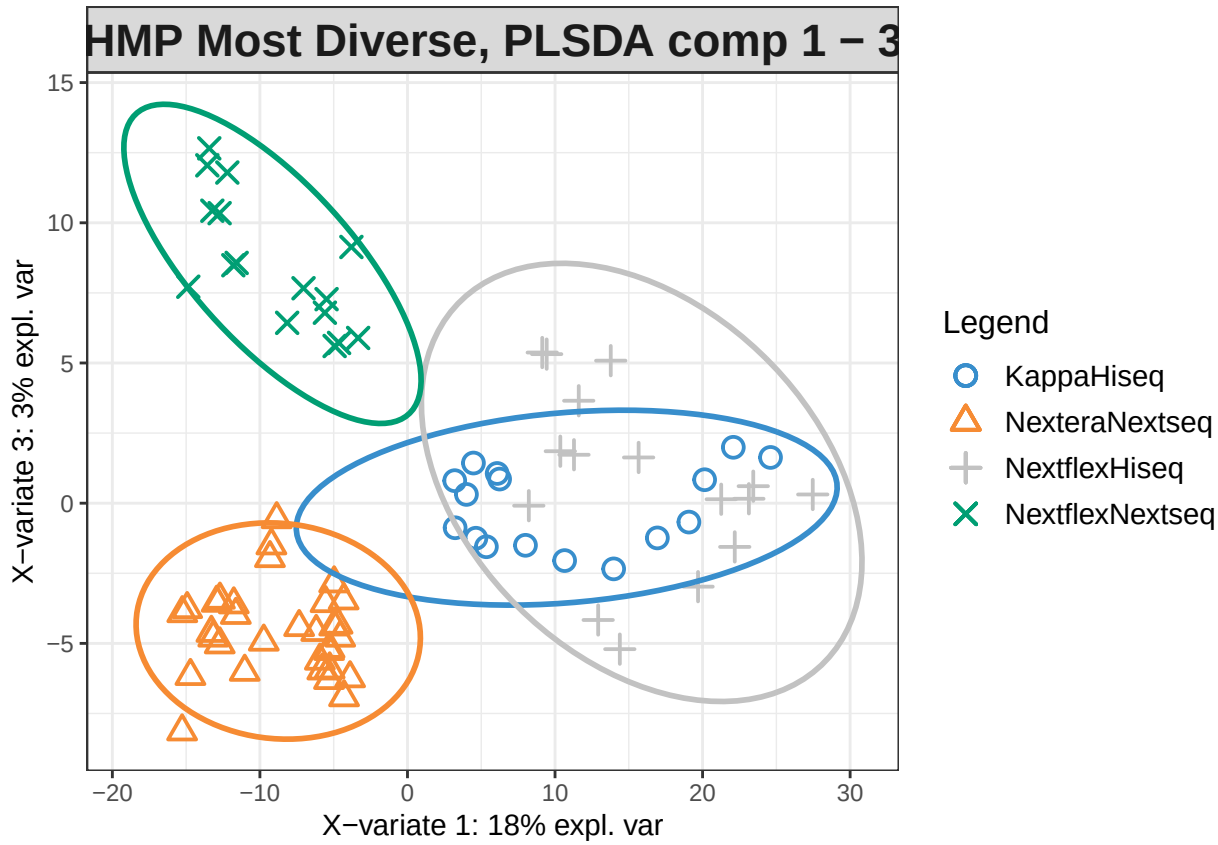

```
#Supervised analysis
diverse.plsda = plsda(X = Tax2, Y, ncomp = 10, logratio = 'CLR', multilevel = sample)
  #nlevels(Y), changed ncomp to 10

diverse.perf.plsda = perf(diverse.plsda, validation = 'Mfold', folds = 5,
                     progressBar = FALSE, nrepeat = 10)

plot(diverse.perf.plsda, overlay = 'measure', sd = TRUE)
```




```
plotIndiv(diverse.plsda , comp = c(1,2), ind.names = FALSE,
          ellipse = TRUE, legend = TRUE, title = 'HMP Most Diverse, PLSDA comp 1 - 2')
```



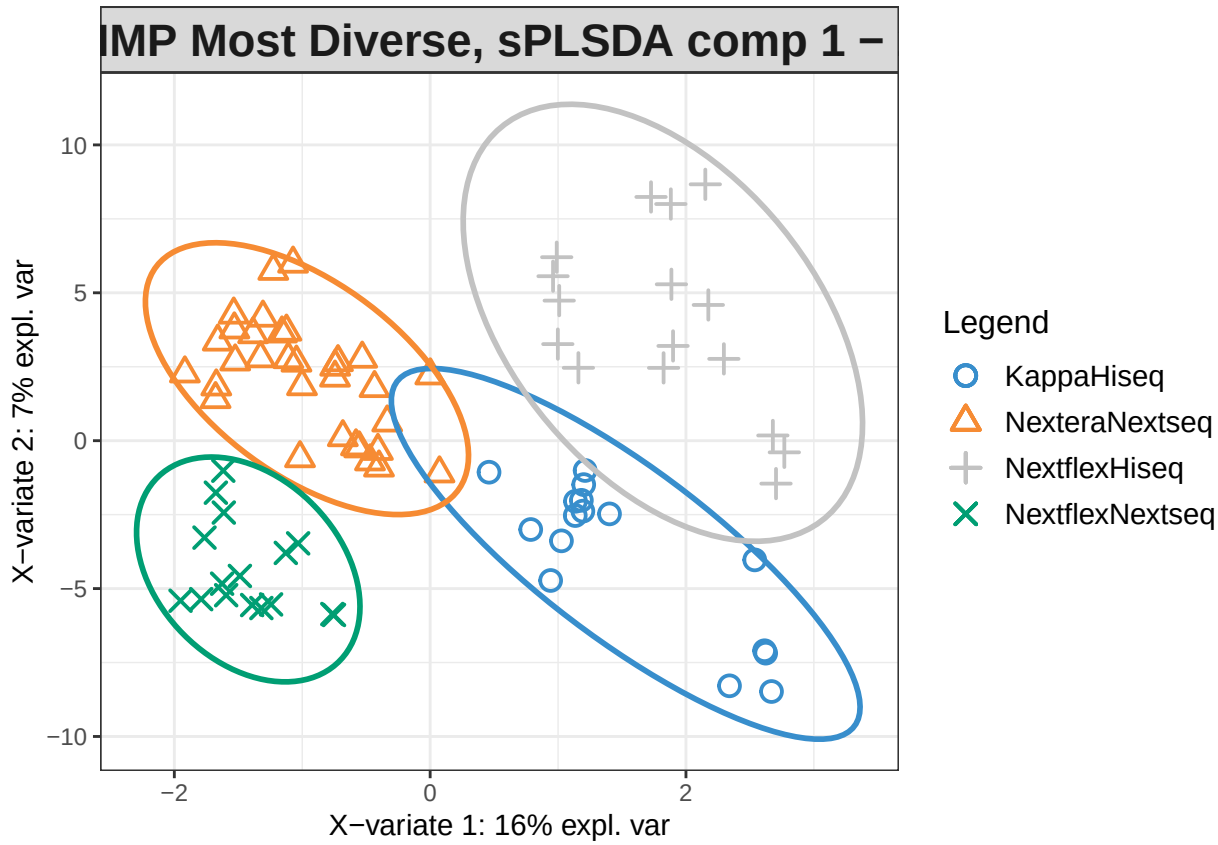

```
plotIndiv(diverse.plsda, comp = c(1,3), ind.names = FALSE,
          ellipse = TRUE, legend = TRUE, title = 'HMP Most Diverse, PLSDA comp 1 - 3')
```



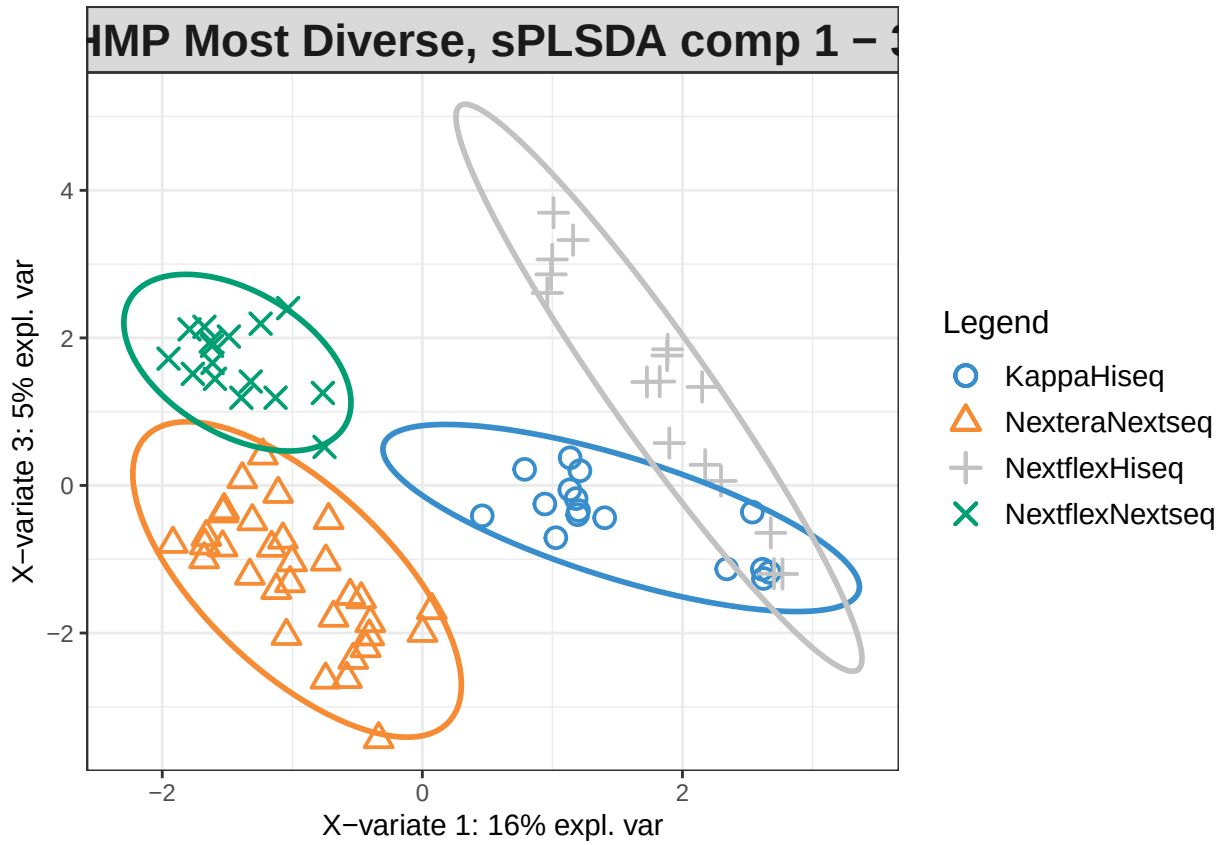

```
  #The third component seem to add info
set.seed(33)  # for reproducible results for this code
diverse.tune.splsda = tune.splsda(Tax2, Y, ncomp = 3,
                            logratio = 'CLR',
                             multilevel = sample,
                            test.keepX = c(seq(5,150, 5)), validation = 'Mfold',
                            folds = 5, dist = 'max.dist', nrepeat = 10,
                            progressBar = FALSE)
#plot(diverse.tune.splsda)

# optimal number of variables to select on 3 comps:
select.keepX = diverse.tune.splsda$choice.keepX[1:3]
select.keepX
```

```
## comp1 comp2 comp3
##     5    45     5
```

```
# select.keepX = c(50, 15, 40) # to manually choose size of selection

diverse.splsda = splsda(Tax2, Y, ncomp = 10, logratio = 'CLR', multilevel = sample,
                        keepX = select.keepX)
plotIndiv(diverse.splsda, comp = c(1,2),
          ind.names = FALSE,
          ellipse = TRUE, legend = TRUE,
          title = 'HMP Most Diverse, sPLSDA comp 1 - 2')
```




```
  #changed ncomp to 10 to see in plot(diverse.perf.splda)

plotIndiv(diverse.splsda, comp = c(1,3),
          ind.names = FALSE,
          ellipse = TRUE, legend = TRUE,
          title = 'HMP Most Diverse, sPLSDA comp 1 - 3')
```




```
set.seed(34)  # for reproducible results for this code
diverse.perf.splsda = perf(diverse.splsda, validation = 'Mfold', folds = 5,
                    progressBar = FALSE, nrepeat = 10, dist = 'max.dist')
diverse.perf.splsda$error.rate
```

```
## $overall
##        max.dist
## comp1   0.41125
## comp2   0.11625
## comp3   0.05250
## comp4   0.03500
## comp5   0.00875
## comp6   0.01250
## comp7   0.01750
## comp8   0.01750
## comp9   0.01625
## comp10  0.00750
##
## $BER
##         max.dist
## comp1  0.5140625
## comp2  0.1445312
## comp3  0.0656250
## comp4  0.0437500
## comp5  0.0109375
## comp6  0.0156250
```

```
## comp7  0.0218750
## comp8  0.0218750
## comp9  0.0203125
## comp10 0.0093750
```

```
#plot(diverse.perf.splsda)

head(selectVar(diverse.splsda, comp = 1)$value)
```

```
##                     value.var
## Peptoanaerobacter -0.87114556
## Polaribacter      -0.35319962
## Methanosaeta       0.33524745
## Porphyromonas     -0.06022969
## Tenacibaculum     -0.01835695
```

```
selected.OTU.comp1 = selectVar(diverse.splsda, comp = 1)$name
# stability of OTUs selected on comp 1
diverse.perf.splsda$features$stable[[1]][selected.OTU.comp1]
```

```
##
## Peptoanaerobacter      Polaribacter      Methanosaeta     Porphyromonas
##              1.00              0.76              0.82              0.48
##     Tenacibaculum
##              0.40
```

```
kable(data.frame(diverse.perf.splsda$features$stable[[1]][selected.OTU.comp1]), caption = '
Stability of OTUs selected on comp 1, ranked by decreasing importance in sPLS-DA.')
```

Table 1: Stability of OTUs selected on comp 1, ranked by decreasing importance in sPLS-DA.

Var1	Freq
Peptoanaerobacter	1.00
Polaribacter	0.76
Methanosaeta	0.82
Porphyromonas	0.48
Tenacibaculum	0.40

```
selected.OTU.comp2 = selectVar(diverse.splsda, comp = 2)$name
# stability of OTUs selected on comp 2
diverse.perf.splsda$features$stable[[2]][selected.OTU.comp2]
```

```
##
##                Prevotella              Bacteroides             Mediterranea
##                      1.00                     0.88                     0.88
##               Barnesiella       Prevotellamassilia         Ornithobacterium
##                      0.86                     0.98                     0.94
##          Peptoclostridium               Petrimonas              Odoribacter
##                      0.96                     0.86                     0.86
##           Parabacteroides           Cloacibacillus        Ruminiclostridium
##                      0.98                     1.00                     1.00
##               Veillonella         Chryseobacterium                  Unknown
##                      1.00                     0.86                     1.00
##           Catenibacterium            Succinivibrio              Allisonella
```

```
##                      1.00                      0.82                      1.00
##             Angelakisella     Phascolarctobacterium          Methylobacterium
##                      1.00                      1.00                      1.00
##               Acetivibrio             Desulfovibrio                Lelliottia
##                      1.00                      0.80                      0.84
##                 Alistipes             Amphibacillus         Lachnoclostridium
##                      0.74                      0.62                      0.84
##                 Kp15virus              Ruminococcus              Byssochlamys
##                      0.84                      0.74                      0.60
##             Streptococcus            Intestinimonas                Myxococcus
##                      0.78                      0.60                      0.66
##             Corallococcus                  Bacillus               Eubacterium
##                      0.52                      0.60                      0.64
##              Cellulomonas            Capnocytophaga                Holdemania
##                      0.48                      0.36                      0.54
##              Serinicoccus         Sanguibacteroides             Lecanicillium
##                      0.44                      0.38                      0.40
## Candidatus Azobacteroides       Massilioclostridium                 Hespellia
##                      0.38                      0.60                      0.58
```

```
kable(data.frame(diverse.perf.splsda$features$stable[[2]][selected.OTU.comp2]), caption = '
Stability of OTUs selected on comp 2, ranked by decreasing importance in sPLS-DA.')
```

Table 2: Stability of OTUs selected on comp 2, ranked by decreasing importance in sPLS-DA.

Var1	Freq
Prevotella	1.00
Bacteroides	0.88
Mediterranea	0.88
Barnesiella	0.86
Prevotellamassilia	0.98
Ornithobacterium	0.94
Peptoclostridium	0.96
Petrimonas	0.86
Odoribacter	0.86
Parabacteroides	0.98
Cloacibacillus	1.00
Ruminiclostridium	1.00
Veillonella	1.00
Chryseobacterium	0.86
Unknown	1.00
Catenibacterium	1.00
Succinivibrio	0.82
Allisonella	1.00
Angelakisella	1.00
Phascolarctobacterium	1.00
Methylobacterium	1.00
Acetivibrio	1.00
Desulfovibrio	0.80
Lelliottia	0.84
Alistipes	0.74
Amphibacillus	0.62
Lachnoclostridium	0.84

Var1	Freq
Kp15virus	0.84
Ruminococcus	0.74
Byssochlamys	0.60
Streptococcus	0.78
Intestinimonas	0.60
Myxococcus	0.66
Corallococcus	0.52
Bacillus	0.60
Eubacterium	0.64
Cellulomonas	0.48
Capnocytophaga	0.36
Holdemania	0.54
Serinicoccus	0.44
Sanguibacteroides	0.38
Lecanicillium	0.40
Candidatus Azobacteroides	0.38
Massilioclostridium	0.60
Hespellia	0.58

```
selected.OTU.comp3 = selectVar(diverse.splsda, comp = 3)$name
# stability of OTUs selected on comp 3
diverse.perf.splsda$features$stable[[3]][selected.OTU.comp3]
```

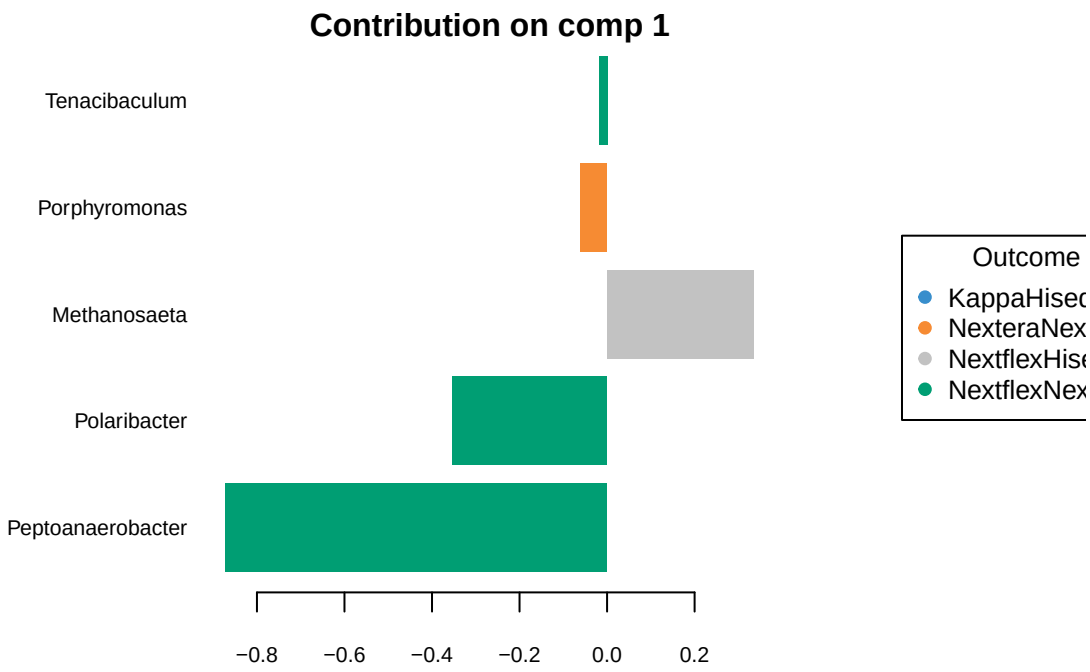
```
##
##       Romboutsia          Blautia     Holdemanella Fusicatenibacter
##             0.92             0.82             0.68             0.56
##  Intestinibacter
##             0.48
```

```
kable(data.frame(diverse.perf.splsda$features$stable[[3]][selected.OTU.comp3]), caption = '
Stability of OTUs selected on comp 3, ranked by decreasing importance in sPLS-DA.')
```

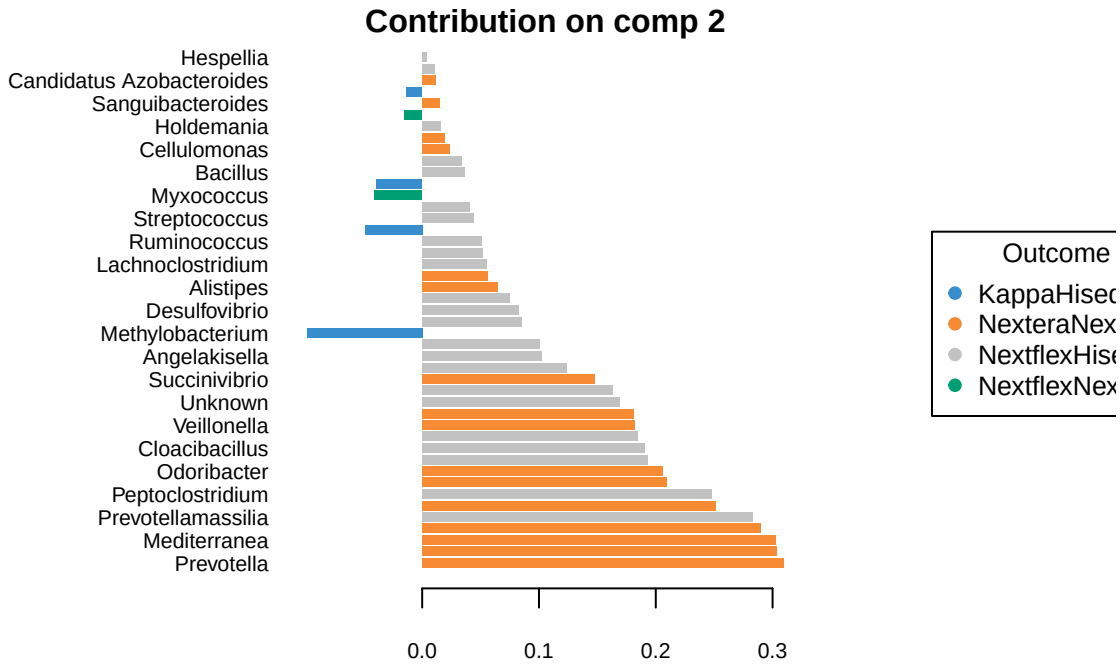
Table 3: Stability of OTUs selected on comp 3, ranked by decreasing importance in sPLS-DA.

Var1	Freq
Romboutsia	0.92
Blautia	0.82
Holdemanella	0.68
Fusicatenibacter	0.56
Intestinibacter	0.48

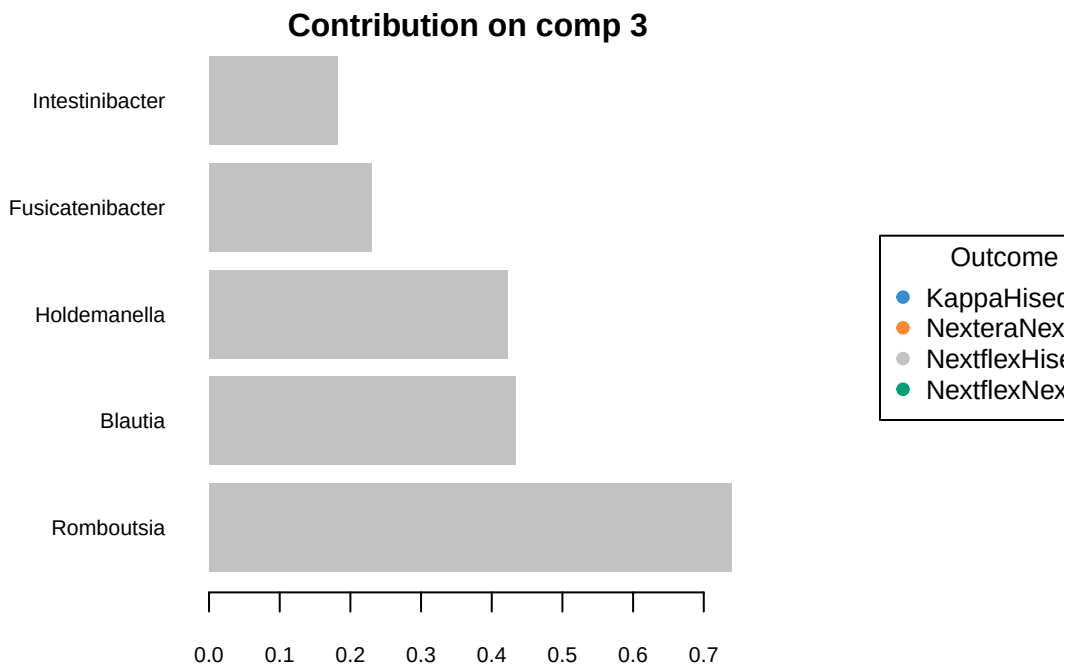
```
plotLoadings(diverse.splsda, comp = 1, method = 'mean', contrib = 'max',
             size.title = 1)
```



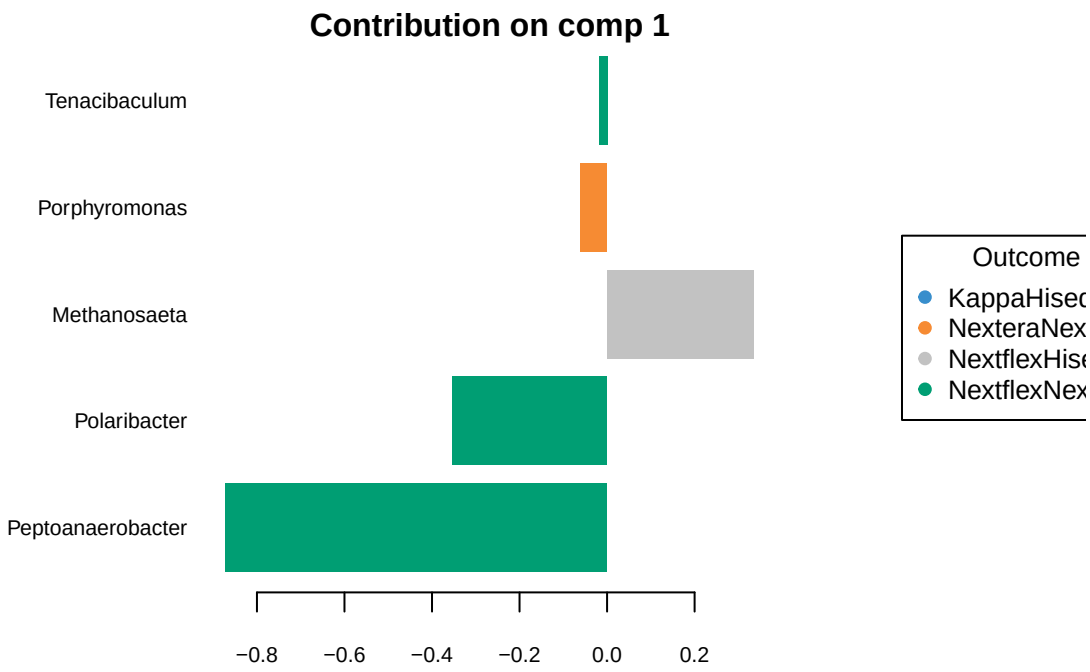

```
plotLoadings(diverse.splsda, comp = 2, method = 'mean', contrib = 'max',
             size.title = 1)
```



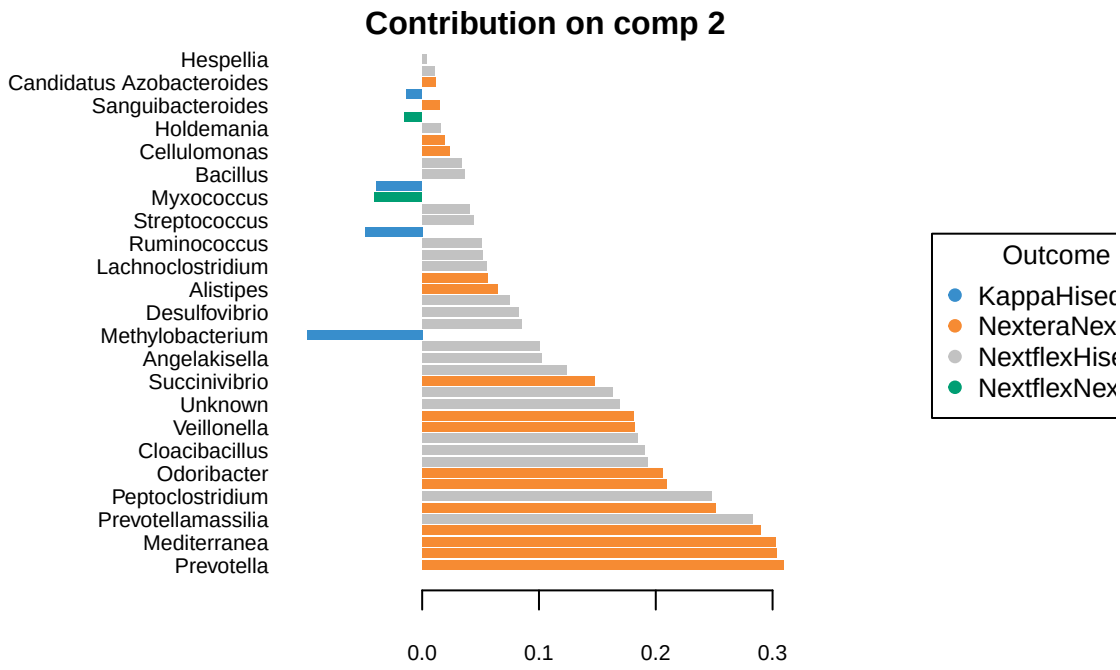

```
plotLoadings(diverse.splsda, comp = 3, method = 'mean', contrib = 'max',
             size.title = 1)
```



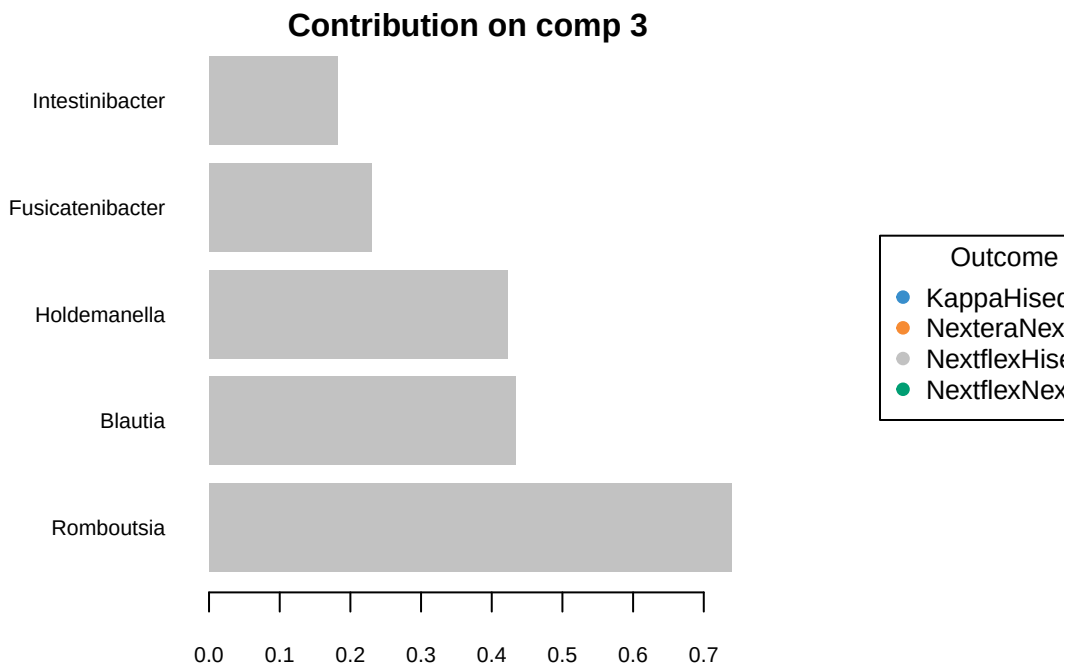

```
##Extracting barplots manually Source code
  #https://github.com/cran/mixOmics/blob/master/R/plotLoadings.splsda.R
comp1 <- plotLoadings(diverse.splsda, comp = 1, method = 'mean', contrib = 'max',
             size.title = 1)
```




```
comp2 <- plotLoadings(diverse.splsda, comp = 2, method = 'mean', contrib = 'max',
             size.title = 1)
```




```
comp3 <- plotLoadings(diverse.splsda, comp = 3, method = 'mean', contrib = 'max',
             size.title = 1)
```




```
#Create barplots with ggplot. Can not get pattern across fill unless using a hacky version
  #(Hadley Wickan writes: It's not currently possible because grid (the graphics system
  #that ggplot2 uses to do the actual drawing) doesn't support textures. Sorry!
#https://stackoverflow.com/questions/2895319/how-to-add-texture-to-fill-colors-in-ggplot2).
#Ended using base R barplot, because I could not get colors to match the entries.
comp1$genera <- rownames(comp1)
comp1$genera <- factor(comp1$genera, levels=rownames(comp1))
#Create column org grouping for colouring
comp1$color2 <- ifelse(comp1$GroupContrib=="NextflexNextseq", "#377eb8",
          ifelse(comp1$GroupContrib=="NextflexHiseq", "#4daf4a",
          ifelse(comp1$GroupContrib=="NexteraNextseq", "#ff7f00",
          ifelse(comp1$GroupContrib=="KappaHiseq", "#984ea3",
                 "Other"))))
#ggplot(data=comp1, aes(x=genera, y=importance)) +
#  geom_bar(stat="identity", fill=comp1$color2) +
#  coord_flip()

comp2$genera <- rownames(comp2)
comp2$genera <- factor(comp2$genera, levels=rownames(comp1))
#Create column org grouping for colouring
comp2$color2 <- ifelse(comp2$GroupContrib=="NextflexNextseq", "#377eb8",
          ifelse(comp2$GroupContrib=="NextflexHiseq", "#4daf4a",
          ifelse(comp2$GroupContrib=="NexteraNextseq", "#ff7f00",
          ifelse(comp2$GroupContrib=="KappaHiseq", "#984ea3",
                 "Other"))))
```

```
comp3$genera <- rownames(comp3)
comp3$genera <- factor(comp3$genera, levels=rownames(comp1))
#Create column org grouping for colouring
comp3$color2 <- ifelse(comp3$GroupContrib=="NextflexNextseq", "#377eb8",
          ifelse(comp3$GroupContrib=="NextflexHiseq", "#4daf4a",
          ifelse(comp3$GroupContrib=="NexteraNextseq", "#ff7f00",
          ifelse(comp3$GroupContrib=="KappaHiseq", "#984ea3",
                 "Other"))))

pdf("S4_Fig_sPLS-DA.pdf", height=10, width=25)
layout(matrix(c(1,2, 3,2), nrow=2, ncol=2, byrow=TRUE))
par(mar=c(4,20,4,2))
barplot(comp1$importance, horiz = T, las = 1, col = comp1$color2, axisnames = TRUE,
        beside = TRUE, names.arg=rownames(comp1))

barplot(comp2$importance, horiz = T, las = 1, col = comp2$color2, axisnames = TRUE,
        beside = TRUE, names.arg=rownames(comp2))

barplot(comp3$importance, horiz = T, las = 1, col = comp3$color2, axisnames = TRUE,
        beside = TRUE, names.arg=rownames(comp3))
dev.off()
```

```
## pdf
##   2
```

Multivariate ANOVA based on dissimilarities (Adonis)

Statistical output is presented in Table1 and S2_Table

```
rm(list=setdiff(ls(), c("Metadata", "Feature", "Tax")))

#Subset data pig feces 1 and 2 (P1, P2), Sewage 1 and 2 (S1, S2), or spiked unspiked
Subset<- c("Allunspiked", "PFunspiked", "SWunspiked", "P1unspiked", "P2unspiked",
           "S1unspiked", "S2unspiked")
#i <- c("Allunspiked") #validation purposes
#c("P1spiked", "P1unspiked", "P2spiked", "P2unspiked", "S1spiked", "S1unspiked",
  #S2spiked", "S2unspiked")
#All, Allspiked, Allunspiked, PF, PFspiked, PFunspiked, SW, SWspiked, SWunspiked,
  #P1, P1spiked, P1unspiked, P2, P2spiked, P2unspiked, S1, S1spiked, S1unspiked, S2,
  #S2spiked, S2unspiked

#Subset experiment, Experiment_Type. (Meaningfull combinations of subset and SubExp:
  #HX=All, FTX=P1&S1, LTX=P1&S1, LPSX=All unspiked, HXFTX=P1&S1, HXLTX=P1&S1,
  #HXLPSX=All unspiked, HXLTXLPSX=P1&S1 unspiked)
SubExp<-"LPSX" #HX, FTX, LTX, LPSX, HXFTX, HXLTX, HXLPSX, HXLTXLPSX, HXFTXLTX, All

#Subset Frozen Unfrozen
SubFre<-"Both" #Frozen, Unfrozen, Both

#Creating tables for all individual tests. Additional overview of p-values created.
  #To see evaluation plots remove hashtags. Using for loop to go through Subset vector

#Make empty dataframe to add p-values
dfp<-data.frame()
```

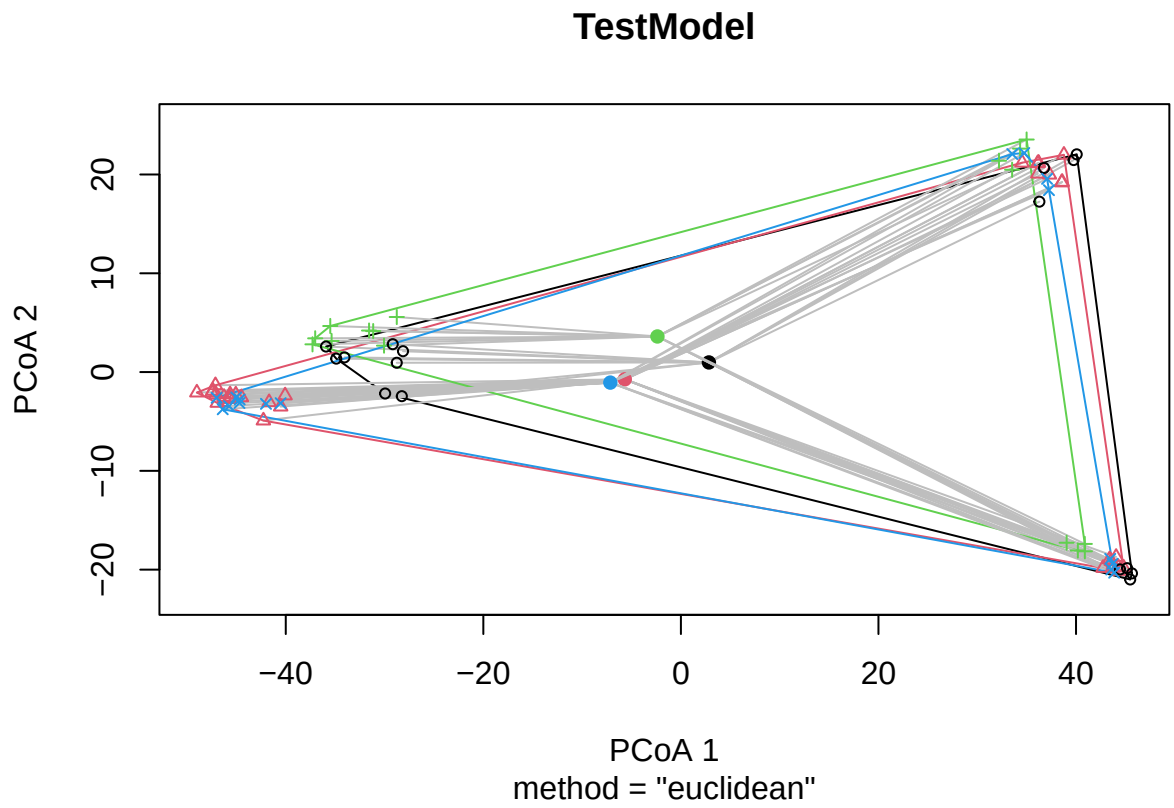
```r
dfp<-data.frame(Test=c("adonis", "betadisperAnova", "betadisperPermutest"))
dfp2<-data.frame()
dfp2<-data.frame(Test=c("adonisExperiment", "adonisStorage", "adonisLibprep",
                        "adonisSeqplat"))

for (i in Subset) {
#Removing negative and positive controls
Metadata2<-filter(Metadata, Sample_type_simple=="Sample")

#Subsetting Metadata2
if (i=="Allspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Spiked")
} else if (i=="Allunspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Unspiked")
} else if (i=="PF") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1" | Experiment == "Pig_feces_2")
} else if (i=="PFspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked" |
                      Sample_type == "Pig_feces_2_spiked")
} else if (i=="PFunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1" |
                      Sample_type == "Pig_feces_2")
} else if (i=="SW") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1" | Experiment == "Sewage_2")
} else if (i=="SWspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked" |
                      Sample_type == "Sewage_2_spiked")
} else if (i=="SWunspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1" | Sample_type == "Sewage_2")
} else if (i=="P1") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1")
} else if (i=="P1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked")
} else if (i=="P1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1")
} else if (i=="P2") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_2")
} else if (i=="P2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2_spiked")
} else if (i=="P2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2")
} else if (i=="S1") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1")
} else if (i=="S1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked")
} else if (i=="S1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1")
} else if (i=="S2") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_2")
} else if (i=="S2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2_spiked")
} else if (i=="S2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2")
```

```
} else if (i=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubExp=="HX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment")
} else if (SubExp=="FTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="LTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="LPSX") {
  Metadata22<-filter(Metadata2, Experiment_type == "Library_prep_seq_platform_experiment")
  VectorLPSX <- unique(Metadata22$Matching_samples)
  Metadata2<-filter(Metadata2, Matching_samples %in% VectorLPSX)
  rm(VectorLPSX, Metadata22)
} else if (SubExp=="HXFTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="HXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="HXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXLTXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXFTXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="All") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubFre=="Frozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Freezer")
} else if (SubFre=="Unfrozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Unfrozen")
} else if (SubFre=="Both") {
  print("No subsetting, all included")
} else {
  print("Subset defined not valid")
}

#Removing the Kappa NextSeq run
```

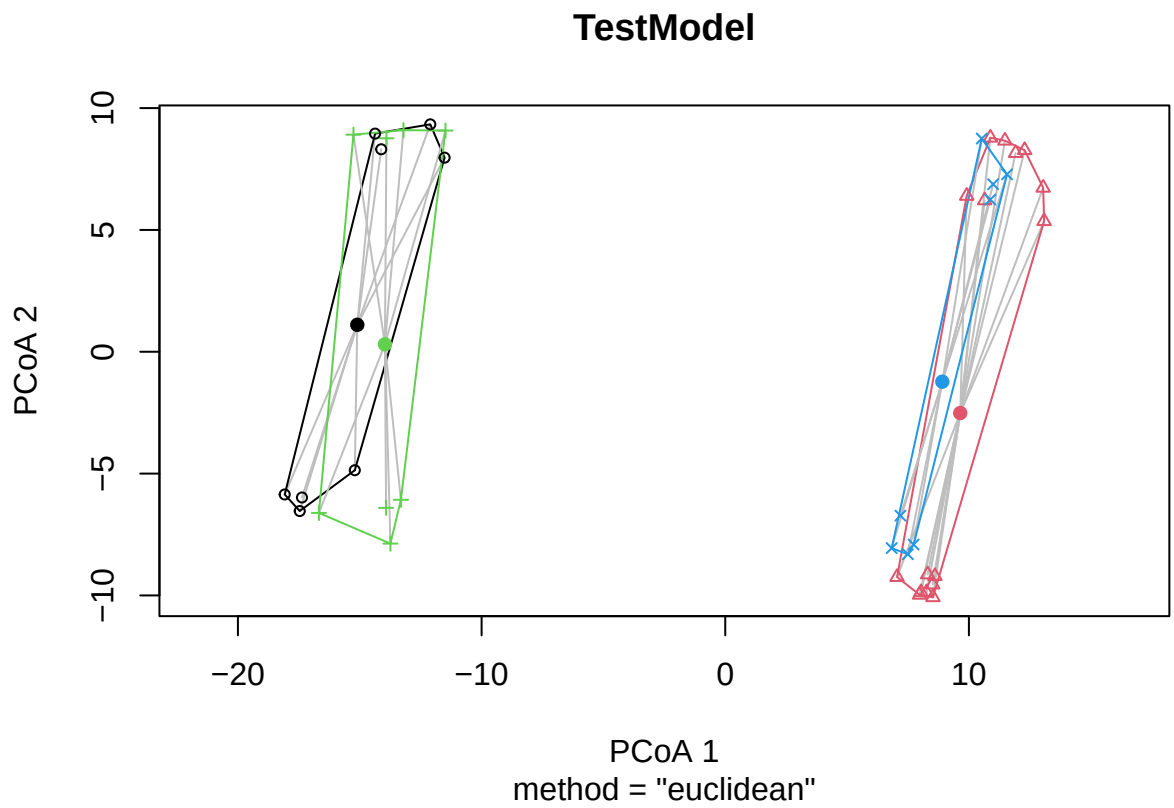
```r
Metadata2<-Metadata2[grep("\\KANS.*", Metadata2$Sample, invert=TRUE),]

#Applying subsetting to OTU tables
Tax2<-dplyr::select(Tax, one_of(Metadata2$Sample))

#filtering of the Counttable depending on rowSums.
Tax2 <- Tax2[rowSums(Tax2)>0,] #Removing all rows that only contains zeroes
Tax2 <- Tax2[rowSums(Tax2)>(5*ncol(Tax2)),] #Removing all rows(Species) that is below an
  #average count of 5
# replace 0 values with an estimate using simple multiplicative replacement
Tax2 <- t(cmultRepl(t(Tax2), method="CZM", label=0))
#Maks TSS
Tax2<-as.matrix(t(sweep(Tax2, 2, colSums(Tax2), FUN="/"))) #Transpose to have genera as
  #columns and samples as rows

#Make column that combine lib prep and seq platform
Metadata2$LPSP<-paste(Metadata2$Library_preparation,
                      Metadata2$Sequencing_platform, sep="_") #Be aware that
  #Nextera 1 & 2 are analyzed as one group,
  #you could argue that it should be done seperately

#Calculate sample-distance matrix
distmatrix <- vegdist(ilr(Tax2), method="euclidean")

#Which factor to test can pick Metadata2 columns consider including both library
  #preparation and sequencing platform individually in the model
Test_Factor <- "LPSP" #LPSP, Library_preparation, Sequencing_platform
#Test_Factor <- "Library_preparation + Sequencing_platform" #Does not work with betadisper
#Create design formula
design <- formula(paste("distmatrix"," ~ ", Test_Factor, sep=""))
if (i=="Allunspiked" | i=="PFunspiked" | i=="SWunspiked") {
  design2 <- formula(paste("distmatrix"," ~ ", "Experiment+", "Sequencing_platform+",
                           "StoragePlacementType+", "Library_preparation" , sep=""))
  print(design2)
} else {
  design2 <- formula(paste("distmatrix"," ~ ", "Sequencing_platform+",
                           "StoragePlacementType+", "Library_preparation", sep=""))
  print(design2)
}
#Have also tried to change order of model:
#"Experiment+", "StoragePlacementType+", "Library_preparation+", "Sequencing_platform"
#"Experiment+", "Library_preparation+", "StoragePlacementType+", "Sequencing_platform"
#"Experiment+", "Sequencing_platform+", "Library_preparation+", "StoragePlacementType"
#"Experiment+", "Library_preparation+", "Sequencing_platform+", "StoragePlacementType"
#"Experiment+", "Sequencing_platform+", "StoragePlacementType+", "Library_preparation"
#"Experiment+", "StoragePlacementType+", "Sequencing_platform+", "Library_preparation"
#Could also have changed the first variable Experiment ending with many combinations
#I'm getting the same results with "Experiment+", "StoragePlacementType+",
  #"Library_preparation+", "Sequencing_platform"
#and "Experiment+", "Library_preparation+", "StoragePlacementType+",
  #"Sequencing_platform", but not with
#"Experiment+", "StoragePlacementType+", "Sequencing_platform+", "Library_preparation"
#Have no idea why. I'm changing the setup to by="margin".
```

```
#design2 <- formula(paste("distmatrix"," ~ ", "Experiment*", "StoragePlacementType*",
  #"Library_preparation*", "Sequencing_platform" , sep=""))
#adonis can handle both continous and factor predictors
set.seed(999)
adonisObject<-adonis2(design, Metadata2, by="margin", perm=99999) #, perm=999
  #can increase to get exact p-values
set.seed(999)
adonisObject2<-adonis2(design2, Metadata2, by="margin", perm=99999)
#adonisObject #If significant then difference between groups
#Sys.sleep(1)
write.table(data.frame(adonisObject), file=paste(i, "Adonis", "_", Test_Factor, ".txt",
                                                 sep=""),
            sep="\t", dec=".", row.names = T, quote = F)
write.table(data.frame(adonisObject2), file=paste("All", i, "Adonis", "_", ".txt",
                                                  sep=""),
            sep="\t", dec=".", row.names = T, quote = F)

#### Evaluating the model assumptions
TestModel <- with(Metadata2, betadisper(distmatrix, LPSP)) #Also have to change here LPSP,
  #Library_preparation, Sequencing_platform. Can not run betadisper with multiple
  #independant variables
#TestModel
#plot(TestModel)
plot(TestModel, label=FALSE)
#boxplot(TestModel)
#anova(TestModel)
anovaTestModel<-anova(TestModel) #p>0.05 -> Assumption met
write.table(data.frame(anova(TestModel)), file=paste(i, "Betadisper", "_", Test_Factor,
                                                     ".txt", sep=""),
            sep="\t", dec=".", row.names = T, quote = F)
set.seed(999)
#permutest(TestModel)
permy<-permutest(TestModel)
#data.frame(permy$tab)
write.table(data.frame(permy$tab), file=paste(i, "Permutest", "_", Test_Factor, ".txt",
                                              sep=""),
            sep="\t", dec=".", row.names = T, quote = F)
#p>0.05 -> Assumption met

TukeyHSD(TestModel, perm=999) #Increasing perm does not change p adj
dfp[paste(i)]<-c(adonisObject$`Pr(>F)`[1], anovaTestModel$`Pr(>F)`[1],
                 permy$tab$`Pr(>F)`[1])
dfp2[paste(i)]<-c(adonisObject2$`Pr(>F)`[1])

if (i=="Allunspiked" | i=="PFunspiked" | i=="SWunspiked") {
  dfp2[paste(i)]<-c(adonisObject2$`Pr(>F)`[1], adonisObject2$`Pr(>F)`[2],
                    adonisObject2$`Pr(>F)`[3], adonisObject2$`Pr(>F)`[4])
} else {
  dfp2[paste(i)]<-c("Na", adonisObject2$`Pr(>F)`[1], adonisObject2$`Pr(>F)`[2],
                    adonisObject2$`Pr(>F)`[3])
}

}
```

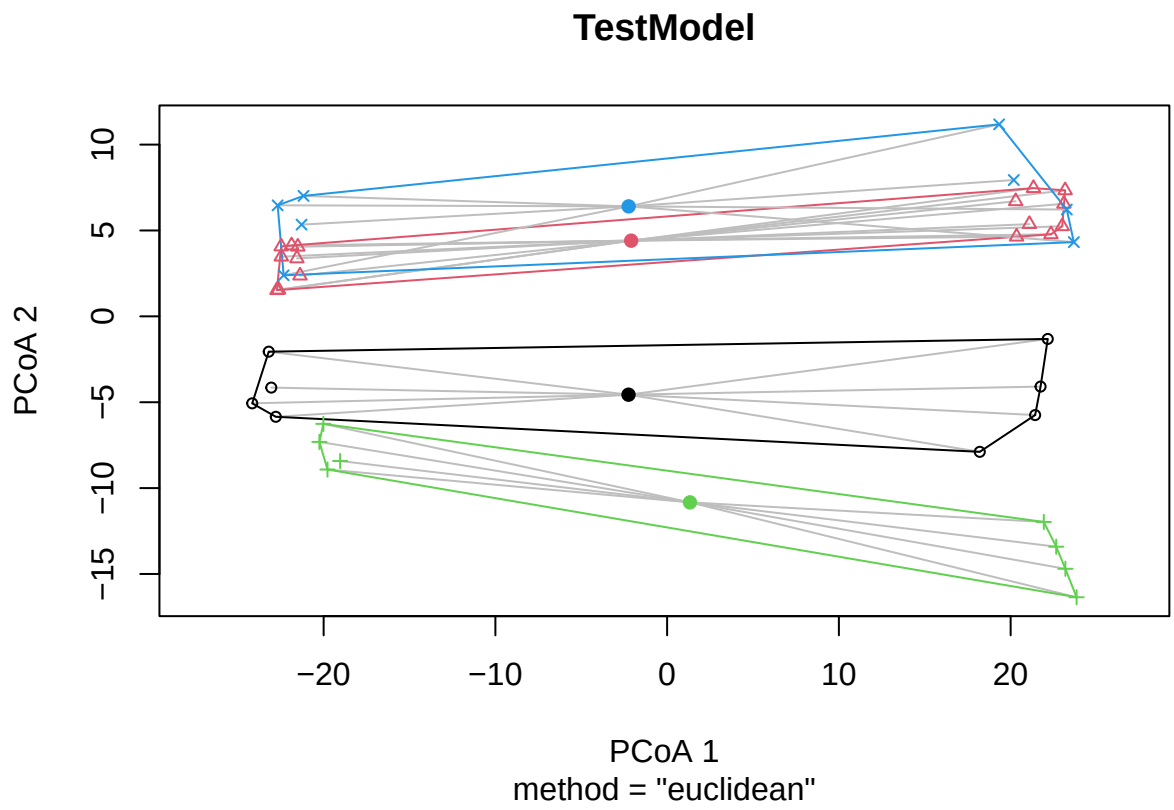
```
## [1] "No subsetting, all included"
## No. corrected values:  4863
## distmatrix ~ Experiment + Sequencing_platform + StoragePlacementType +
##     Library_preparation
```



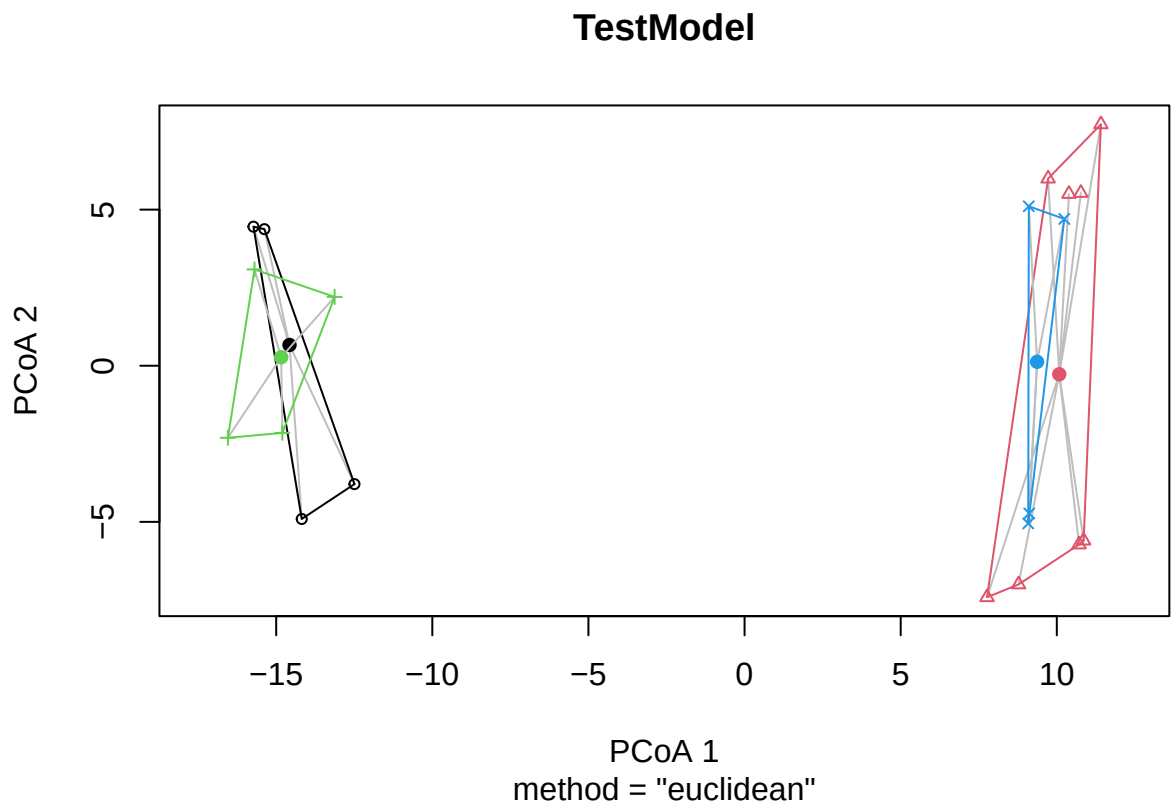

```
## [1] "No subsetting, all included"
## No. corrected values:  21
## distmatrix ~ Experiment + Sequencing_platform + StoragePlacementType +
##     Library_preparation
```



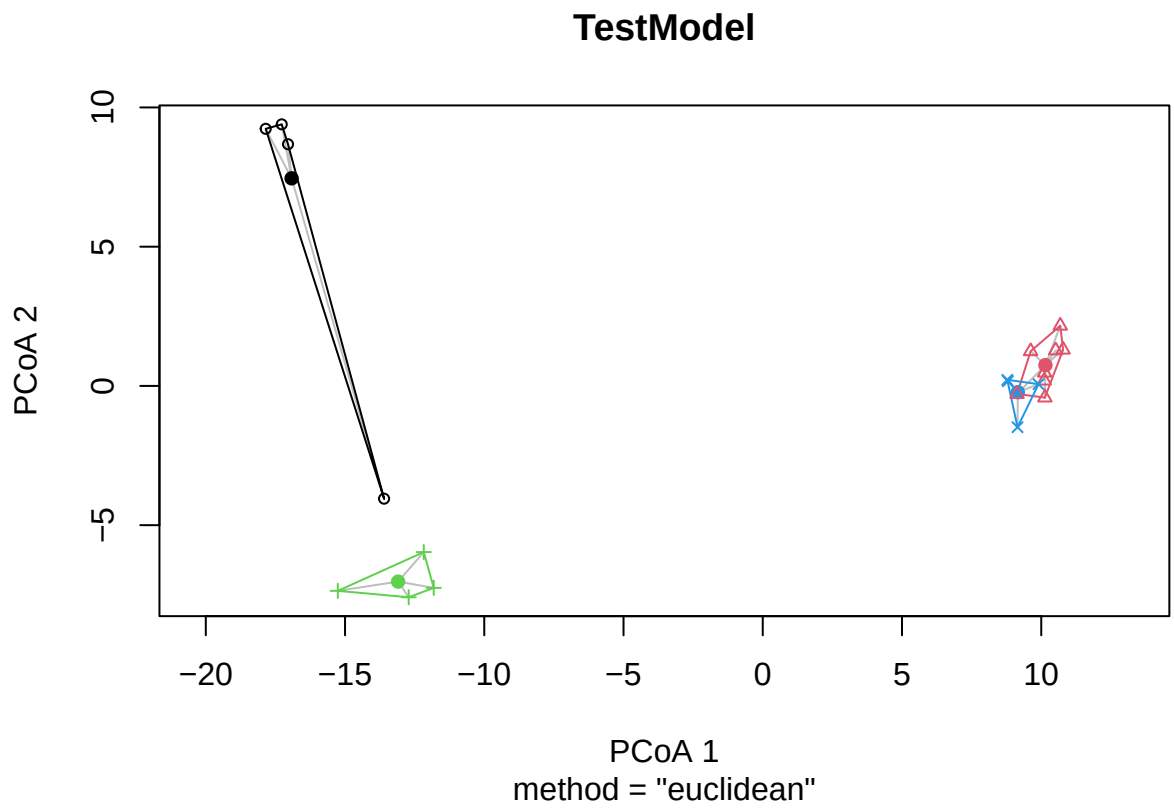

```
## [1] "No subsetting, all included"
## No. corrected values:  140
## distmatrix ~ Experiment + Sequencing_platform + StoragePlacementType +
##     Library_preparation
```



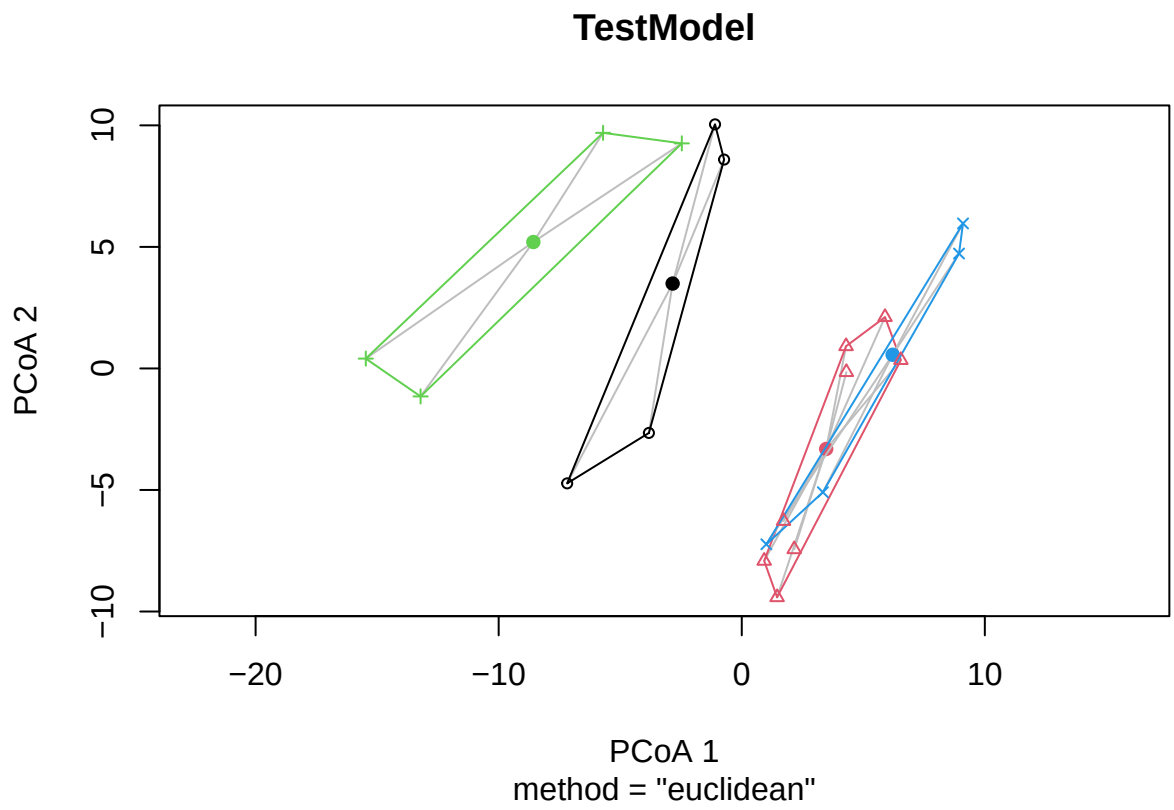

```
## [1] "No subsetting, all included"
## No. corrected values:  3
## distmatrix ~ Sequencing_platform + StoragePlacementType + Library_preparation
```



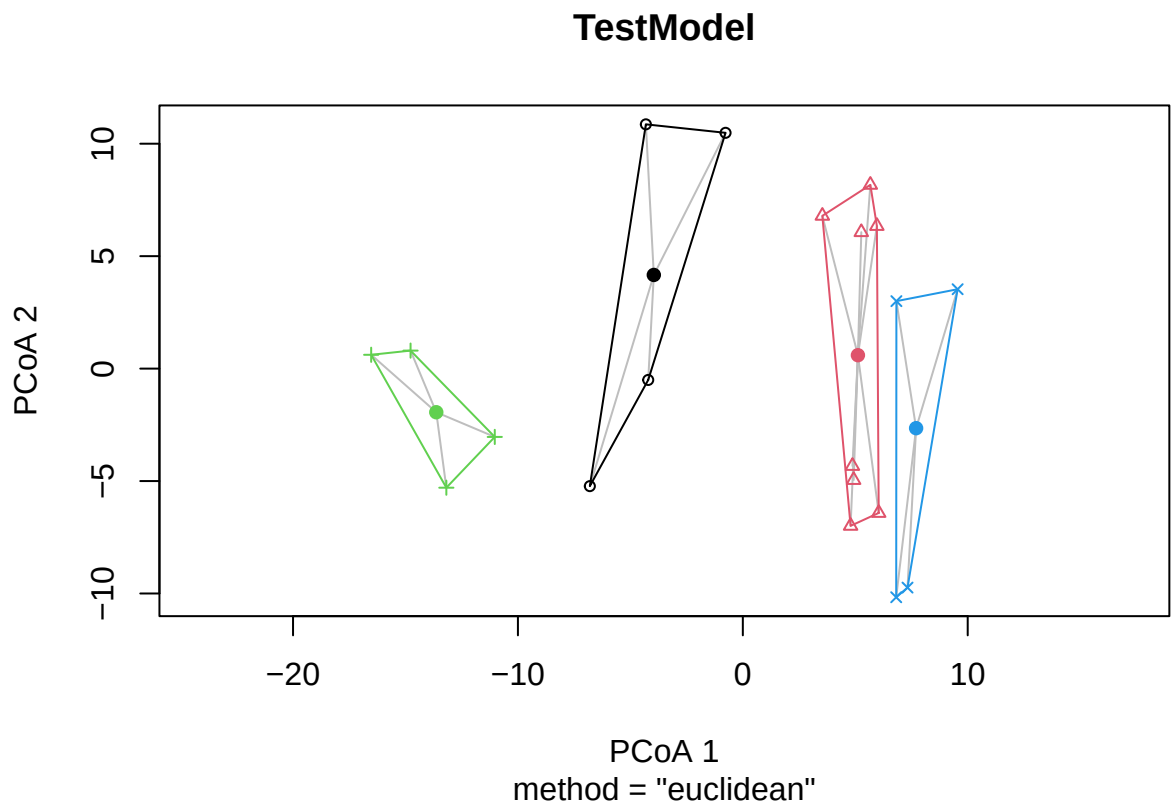

```
## [1] "No subsetting, all included"
## distmatrix ~ Sequencing_platform + StoragePlacementType + Library_preparation
```




```
## [1] "No subsetting, all included"
## distmatrix ~ Sequencing_platform + StoragePlacementType + Library_preparation
```




```
## [1] "No subsetting, all included"
## No. corrected values:  24
## distmatrix ~ Sequencing_platform + StoragePlacementType + Library_preparation
```




```
write.table(dfp, file=paste("Pvalues", Test_Factor, ".txt", sep=""), sep="\t",
            dec=".", row.names = T, quote = F)
write.table(dfp2, file=paste("AllPvalues", ".txt", sep=""), sep="\t",
            dec=".", row.names = T, quote = F)

#p-values from tests
#adonis if significant there is a difference between groups defined by Test_Factor
#betadisper - Anova and Permutest differences in group homogeneities are not violated if
  #above 0.05
kable(dfp, caption=paste(Test_Factor))
```

Table 4: LPSP

Test	Allunspiked	PFunspiked	SWunspiked	P1unspiked	P2unspiked	S1unspiked	S2unspiked
adonis	0.299100	0.000010	0.2353700	0.0000100	0.0000100	0.0000100	0.0000100
betadisperAnova	0.000817	0.081415	0.6165621	0.0061238	0.2675113	0.1009037	0.3100214
betadisperPermutest	0.002000	0.089000	0.6150000	0.0110000	0.2400000	0.0930000	0.2900000

```
kable(dfp2, caption="Testing all parameters simultaneously")
```

Table 5: Testing all parameters simultaneously

Test	Allunspiked	PFunspiked	SWunspiked	P1unspiked	P2unspiked	S1unspiked	S2unspiked
adonisExperiment	0.00001	0.00001	0.00001	Na	Na	Na	Na
adonisStorage	0.00030	0.00001	0.00444	1e-05	1e-05	1e-05	1e-05

Test	Allunspiked	PFunspiked	SWunspiked	P1unspiked	P2unspiked	S1unspiked	S2unspiked
adonisLibprep	0.06384	0.00381	0.02524	0.00284	0.16807	1e-05	1e-05
adonisSeqplat	0.04171	0.00057	0.02953	0.02804	0.0054	0.00036	6e-05

Constrained ordination with redundancy analysis (rda)

The vegan function capscale were used to create the rda

```
rm(list=setdiff(ls(), c("Metadata", "Feature", "Tax")))

#Subset data pig feces 1 and 2 (P1, P2), Sewage 1 and 2 (S1, S2), or spiked unspiked
Subset<- c("P1unspiked", "P2unspiked", "S1unspiked", "S2unspiked") #All, Allspiked,
  #Allunspiked, PF, SW, P1, P1spiked, P1unspiked, P2, P2spiked, P2unspiked, S1,
  #S1spiked, S1unspiked, S2, S2spiked, S2unspiked
#c("P1unspiked", "P2unspiked", "S1unspiked", "S2unspiked")

#Subset experiment, Experiment_Type. (Meaningfull combinations of subset and SubExp:
  #HX=All, FTX=P1&S1, LTX=P1&S1, LPSX=All unspiked, HXFTX=P1&S1, HXLTX=P1&S1,
  #HXLPSX=All unspiked, HXLTXLPSX=P1&S1 unspiked)
SubExp<-"LPSX" #HX, FTX, LTX, LPSX, HXFTX, HXLTX, HXLPSX, HXLTXLPSX, HXFTXLTX, All

#Subset Frozen Unfrozen
SubFre<- c("Frozen", "Unfrozen") #Frozen, Unfrozen, Both

# Create a list to hold the plot objects.
CAPScreeList <- list()
CAPList <- list()
CAPvec <- vector()

for (i in Subset) {
  for (j in SubFre) {
#Removing negative and positive controls
Metadata2<-filter(Metadata, Sample_type_simple=="Sample")

#Subsetting Metadata2
if (i=="Allspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Spiked")
} else if (i=="Allunspiked") {
  Metadata2<-filter(Metadata2, SpikedUnspiked == "Unspiked")
} else if (i=="PF") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1" | Experiment == "Pig_feces_2")
} else if (i=="SW") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1" | Experiment == "Sewage_2")
} else if (i=="P1") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_1")
} else if (i=="P1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1_spiked")
} else if (i=="P1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_1")
} else if (i=="P2") {
  Metadata2<-filter(Metadata2, Experiment == "Pig_feces_2")
} else if (i=="P2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2_spiked")
```

```
} else if (i=="P2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Pig_feces_2")
} else if (i=="S1") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_1")
} else if (i=="S1spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1_spiked")
} else if (i=="S1unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_1")
} else if (i=="S2") {
  Metadata2<-filter(Metadata2, Experiment == "Sewage_2")
} else if (i=="S2spiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2_spiked")
} else if (i=="S2unspiked") {
  Metadata2<-filter(Metadata2, Sample_type == "Sewage_2")
} else if (i=="All") {
  print("No subsetting Subset, all included")
} else {
  print("Subset defined not valid")
}

#Further subsetting Metadata2
if (SubExp=="HX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment")
} else if (SubExp=="FTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="LTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="LPSX") {
  Metadata22<-filter(Metadata2, Experiment_type == "Library_prep_seq_platform_experiment")
  VectorLPSX <- unique(Metadata22$Matching_samples)
  Metadata2<-filter(Metadata2, Matching_samples %in% VectorLPSX)
  rm(VectorLPSX, Metadata22)
} else if (SubExp=="HXFTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment")
} else if (SubExp=="HXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="HXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXLTXLPSX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Long_term_storage_experiment" |
                      Experiment_type == "Library_prep_seq_platform_experiment")
} else if (SubExp=="HXFTXLTX") {
  Metadata2<-filter(Metadata2, Experiment_type == "Handling_experiment" |
                      Experiment_type == "Freeze_thaw_experiment" |
                      Experiment_type == "Long_term_storage_experiment")
} else if (SubExp=="All") {
  print("No subsetting SubExp, all included")
} else {
  print("Subset defined not valid")
```

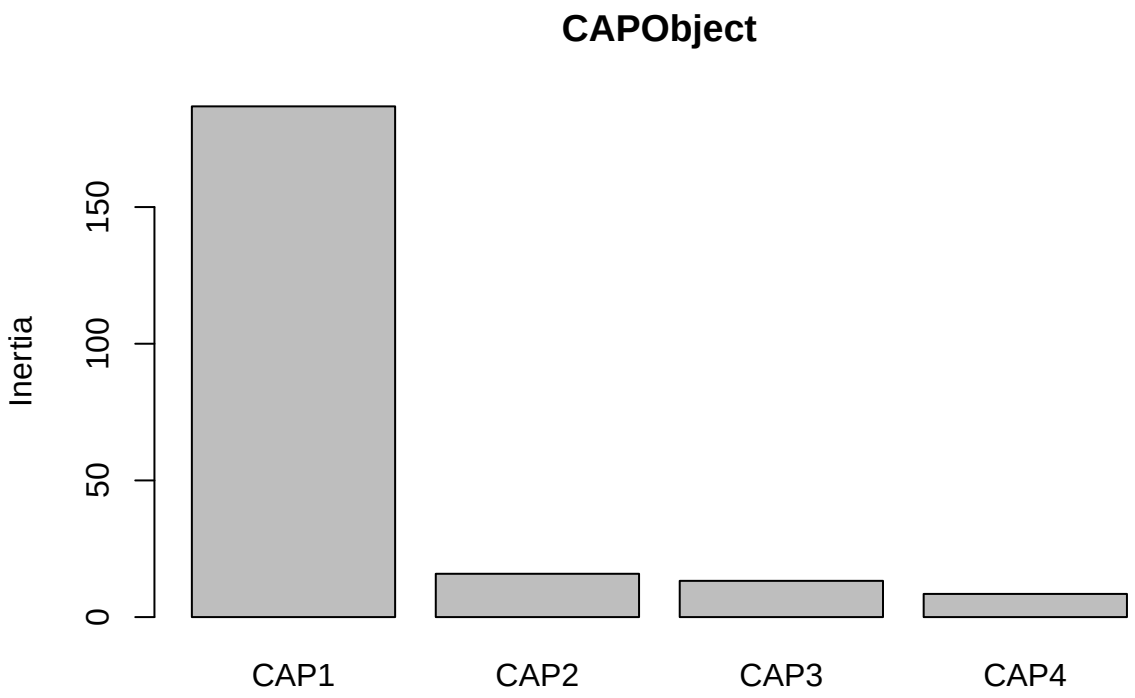
```
}

#Further subsetting Metadata2
if (j=="Frozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Freezer")
} else if (j=="Unfrozen") {
  Metadata2<-filter(Metadata2, FrozenUnfrozenSimple == "Unfrozen")
} else if (j=="Both") {
  print("No subsetting SubFre, all included")
} else {
  print("Subset defined not valid")
}


#Removing the Kappa NextSeq run
Metadata2<-Metadata2[grep("\\KANS.*", Metadata2$Sample, invert=TRUE),]

#Applying subsetting to OTU tables
Tax2<-dplyr::select(Tax, one_of(Metadata2$Sample))

## Create grouping factor
#colnames(Tax2)==Metadata$Sample #Checking order
#New data adding line between same samples different lib prep and seq plat
Metadata2$Sample_LPSX <-
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*_HX_*", Metadata2$Sample),
               "NFHI",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*KAHI*", Metadata2$Sample),
               "KAHI",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*NFNS*", Metadata2$Sample),
               "NFNS",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*NX1NS*", Metadata2$Sample),
               "NX1NS",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*NX2NS*", Metadata2$Sample),
               "NX2NS",
        ifelse(seq(along=(Metadata2$Sample)) %in% grep("*KANS*", Metadata2$Sample),
               "KANS",
               "Other")))))) #The seq along returns true or false where the regex is
  #fulfilled and then stores the evaluated string on the new column be aware HX is
  #actually true 1-8 don't know why


## Can use different filtering strategies. 1 Bayseian approach estimating zeroes, 2 offset
  #of 1 and removing all rows containing zeroes. Only minor effects observed
# f <- codaSeq.filter(ak_op, min.reads=1000, min.prop=0.005, min.occurrence=0.2,
  #samples.by.row=FALSE) #Implemented in Gloors codaSeq.filter function
#Assessing what is the max value of rows containing a zero
print("Max in rows containing a zero")
row_sub <- (apply(Tax2, 1, function(row) any(row ==0 )))
Tax2[row_sub,] %>% max() %>% print()

# filtering of the Counttable depending on rowSums.
Tax2 <- Tax2[rowSums(Tax2)>0,] #Removing all rows that only contains zeroes
Tax2 <- Tax2[rowSums(Tax2)>(5*ncol(Tax2)),] #Removing all rows(Species) that is below
```

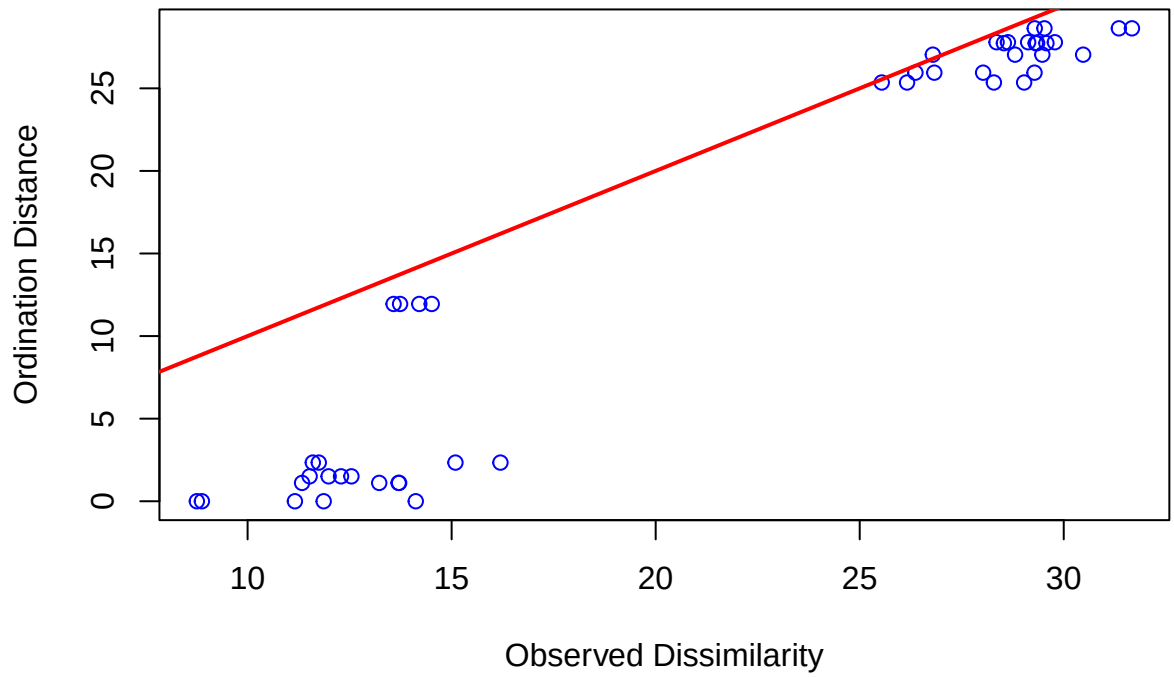
```
  #an average count of 5.
# replace 0 values with an estimate using simple multiplicative replacement
Tax2 <- t(cmultRepl(t(Tax2), method="CZM", label=0))

# Maks TSS
Tax2<-sweep(Tax2, 2, colSums(Tax2), FUN="/")

# Create distance matrix
clrmatrix <- data.frame(clr(t(Tax2))) #Previously vegdist(decostand(t(Tax2),
  #method="hellinger"), method="bray")

#Multi dimensional scaling with
#capscale https://www.rdocumentation.org/packages/vegan/versions/2.4-2/topics/capscale
#& https://www.rdocumentation.org/packages/vegan/versions/2.4-2/topics/cca.object
CAPObject<-capscale(clrmatrix~Metadata2$Sample_LPSX)

#Screeplot
screeplot(CAPObject)
screeplot<-data.frame(CAPObject$CCA$eig)
colnames(screeplot)<-c("eig")
screeplot$eig <- screeplot$eig[1:length(screeplot$eig)] / sum(screeplot$eig) * 100
screeplot<-add_rownames(screeplot, "MDS")
#screeplot$MDS <- factor(screeplot$MDS,
#                        levels=c(sprintf("MDS%d", 1:length(screeplot$eig))))
#Create plot name
pltName <- paste( 'scree', i, j, sep = '' )
#create screeplot
CAPScreeList[[ pltName ]]<-ggplot(screeplot, aes(x=MDS, y=eig)) +
  geom_bar(stat="identity") +
  labs(x ="MDS", y ="eig (%)") + ggtitle(paste("Screeplot ", i)) +
  theme_bw() +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.title=element_text(size=12), axis.text.x=element_blank(),
        axis.ticks.x=element_blank())
#ggsave(filename=paste("ScreeplotCCA", "Genus", i, OrgFlt, ".pdf", sep=""), height=6, width=12)


#Stressplot
stressplot(CAPObject)



#Redundancy analysis with capscale
#Extract scores from CAP object
scores <- vegan::scores(CAPObject, display=c("sp", "wa", "cn", "bp"), choices=c(1,2,3))

#Extract site information and add to metadata
sites <- data.frame(scores$sites)
sites$Sample <- rownames(sites)
Metadata2 <- merge(Metadata2, sites, by="Sample")
#Creating column in metadata for plotting with lines between replicates
Metadata2$Sample_name_norep<-gsub("*_a|*_b|*_c", "", Metadata2$Sample_name)
```

```
#Extract org information and add to Feature data
species <- data.frame(scores$species)
species$Genus <- rownames(species)
Feature2 <- merge(Feature, species, by="Genus")
#Create column org grouping for colouring
Feature2$col <- ifelse(Feature2$Phylum=="Firmicutes", "Firmicutes",
          ifelse(Feature2$Phylum=="Proteobacteria", "Proteobacteria",
          ifelse(Feature2$Phylum=="Actinobacteria", "Actinobacteria",
          ifelse(Feature2$Phylum=="Bacteroidetes", "Bacteroidetes",
          ifelse(Feature2$Domain=="Eukaryota", "Eukaryota",
          ifelse(Feature2$Domain=="Viruses", "Viruses",
          ifelse(Feature2$Domain=="Archaea", "Archaea",
                 "Other")))))))
Feature2$col[Feature2$Class=="Negativicutes"] <- "Firmicutes(Negativicutes)"
Feature2$col <- factor(Feature2$col, levels=c("Proteobacteria", "Bacteroidetes",
                                              "Actinobacteria", "Firmicutes",
                                              "Firmicutes(Negativicutes)",
                                              "Eukaryota", "Archaea", "Viruses",
                                              "Other"))

#Extract information on inertia
inertia <- CAPObject$CCA$tot.chi/CAPObject$tot.chi
subheader <- paste("Inertia constrained by the explanatory variables", round(inertia, digits=2))
#Extract information on eig
eig1 <- (CAPObject$CCA$eig[1]/(sum(CAPObject$CCA$eig)+sum(CAPObject$CA$eig)))*100
eig_1 <- paste("CAP1", round(eig1, digits=2), "% of total variance")
eig2 <- (CAPObject$CCA$eig[2]/(sum(CAPObject$CCA$eig)+sum(CAPObject$CA$eig)))*100
eig_2 <- paste("CAP2", round(eig2, digits=2), "% of total variance")


pltName <- paste('CAP', i, j, sep = '' )
CAPList[[ pltName ]] <- ggplot() +
  #geom_point(data=Metadata2[1:2,], aes(x=CAP1, y=CAP2, shape=StoragePlacementType), color="#377eb8", s
  geom_point(data=Metadata2[1:2,], aes(x=CAP1, y=CAP2), color="#4daf4a", size=2,
             fill="#4daf4a", stroke=1.2) +
  geom_point(data=Metadata2[3:4,], aes(x=CAP1, y=CAP2), color="#ff7f00", size=2,
             fill="#ff7f00", stroke=1.2) +
  geom_point(data=Metadata2[5:6,], aes(x=CAP1, y=CAP2), color="#993300", size=2,
             fill="#993300", stroke=1.2) +
  geom_point(data=Metadata2[7:8,], aes(x=CAP1, y=CAP2), color="#984ea3", size=2,
             fill="#984ea3", stroke=1.2) +
  geom_point(data=Metadata2[9:10,], aes(x=CAP1, y=CAP2), color="#377eb8", size=2,
             fill="#377eb8", stroke=1.2) +
  geom_line(data=Metadata2, aes(x=CAP1, y=CAP2, group=Sample_LPSX)) +
  geom_point(data=Feature2, aes(x=CAP1, y=CAP2, color=col, group=Genus), size=1) +
  scale_colour_gdocs() +
  labs(colour="Organisms", title=paste("rda ", i, j), x=eig_1, y=eig_2) +
  #scale_shape_manual(values=c(21, 22)) +
  theme_bw() +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.title=element_text(size=12), legend.position="none") #+
  #theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        #axis.title=element_text(size=12), legend.position="bottom")
```

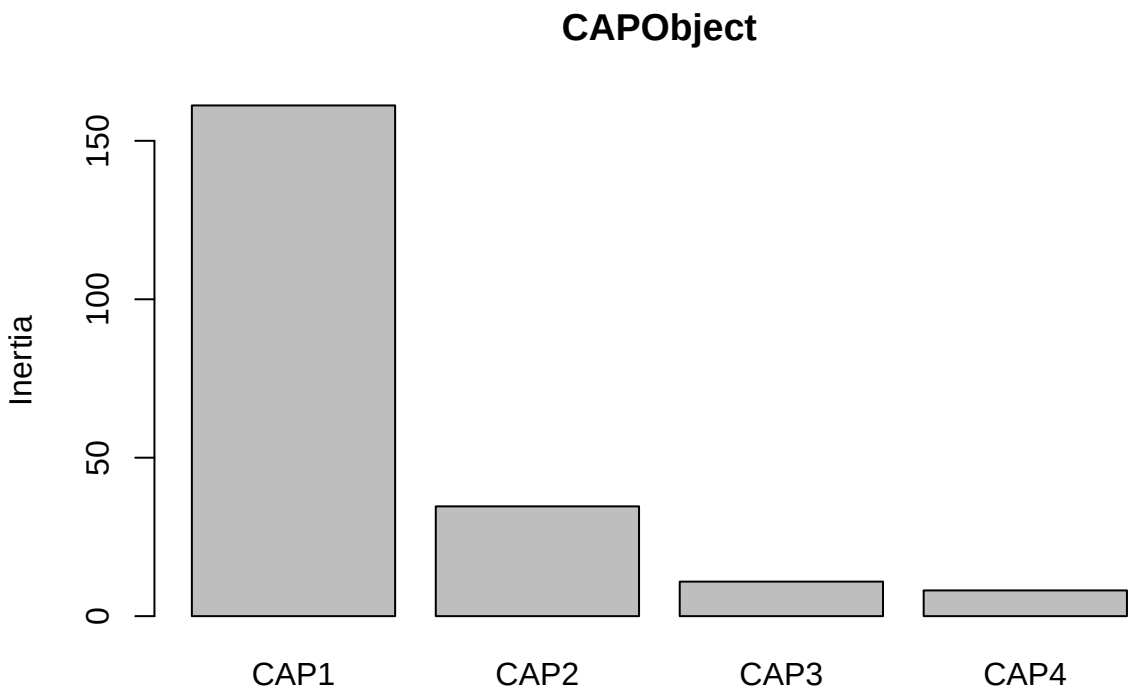
```
  }
}
```

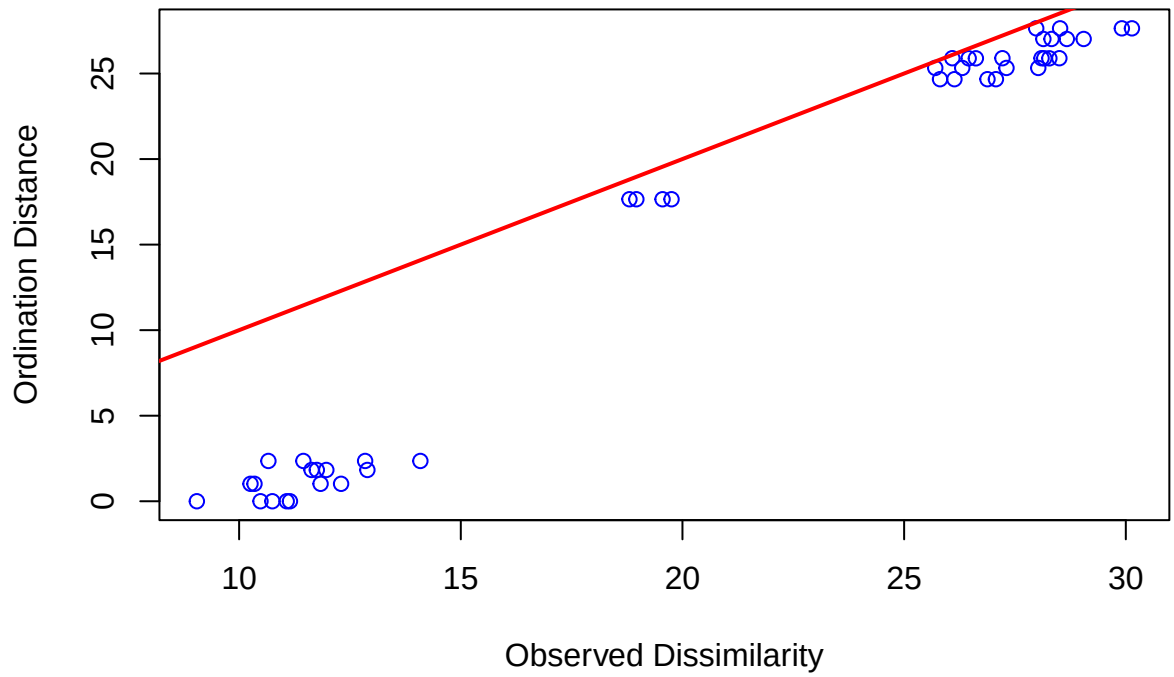
```
## [1] "Max in rows containing a zero"
## [1] 2466
```



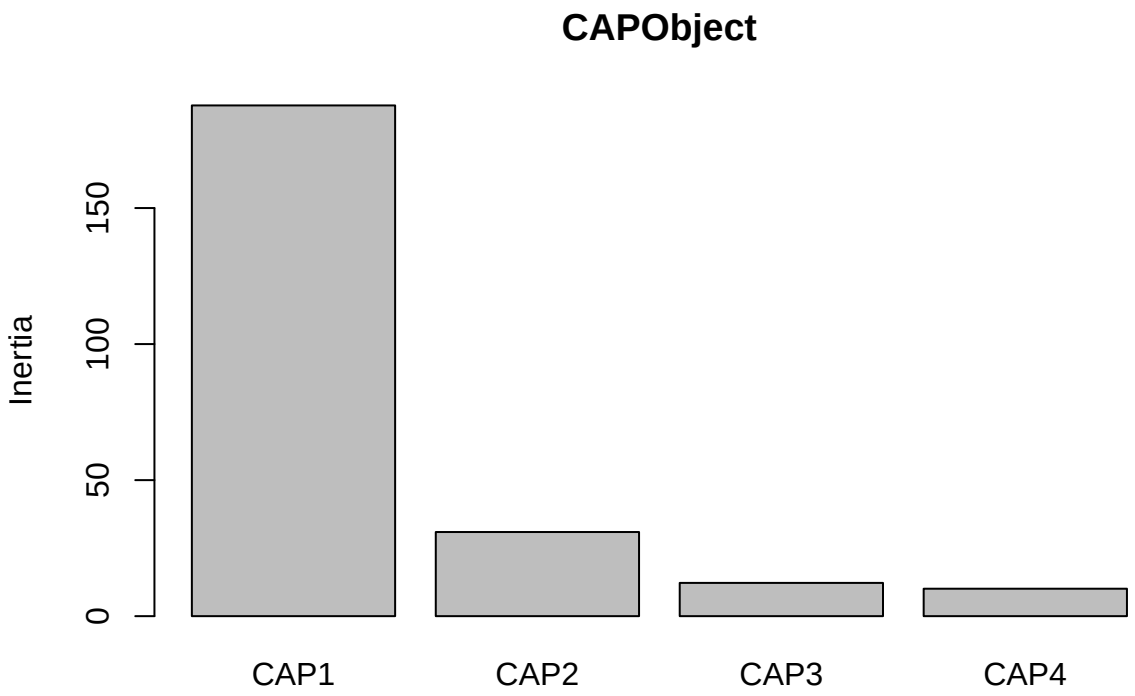


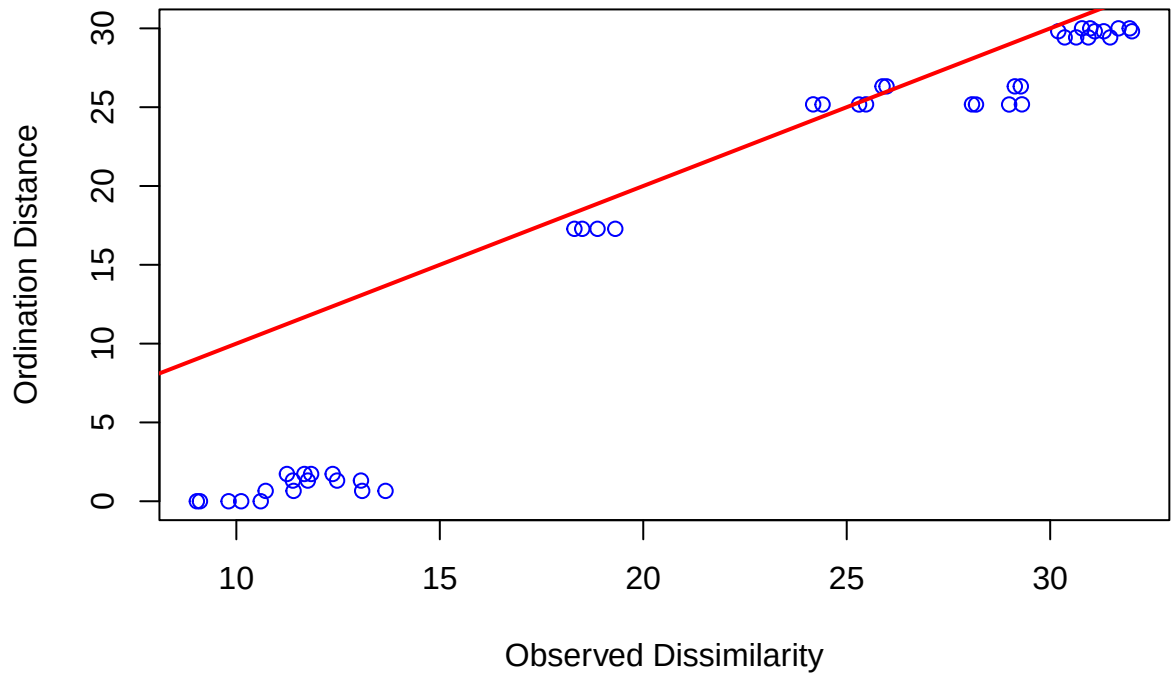

```
## [1] "Max in rows containing a zero"
## [1] 2585
```


CAPObject
Inertia
0
50
100
150
CAP1
CAP2
CAP3
CAP4

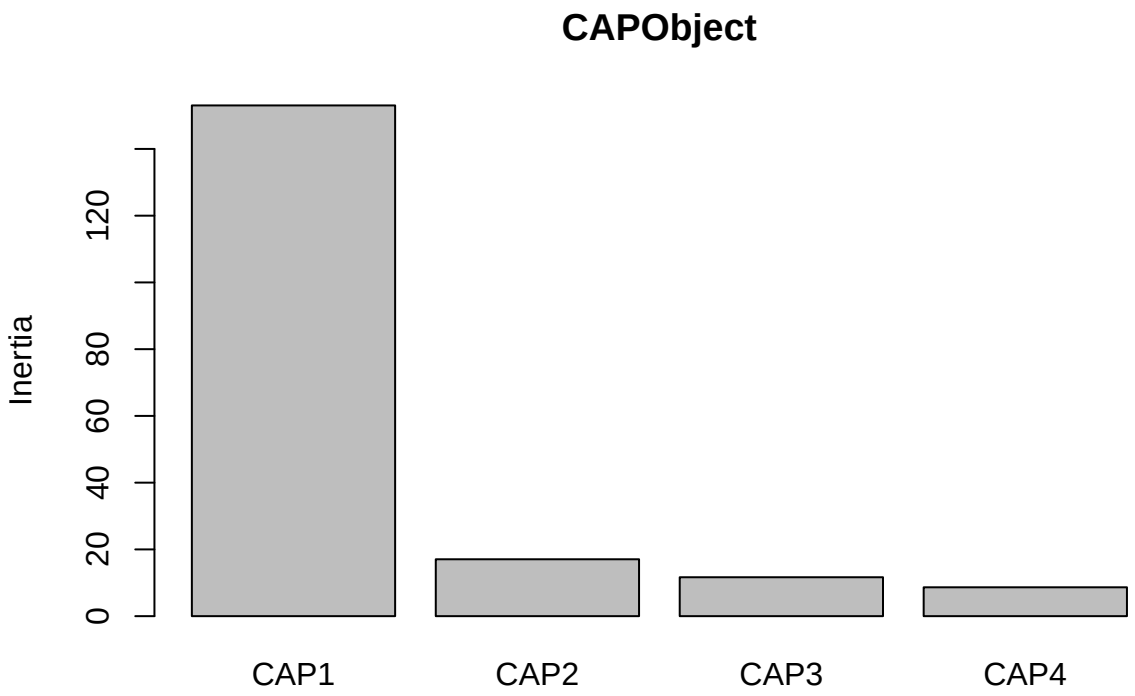


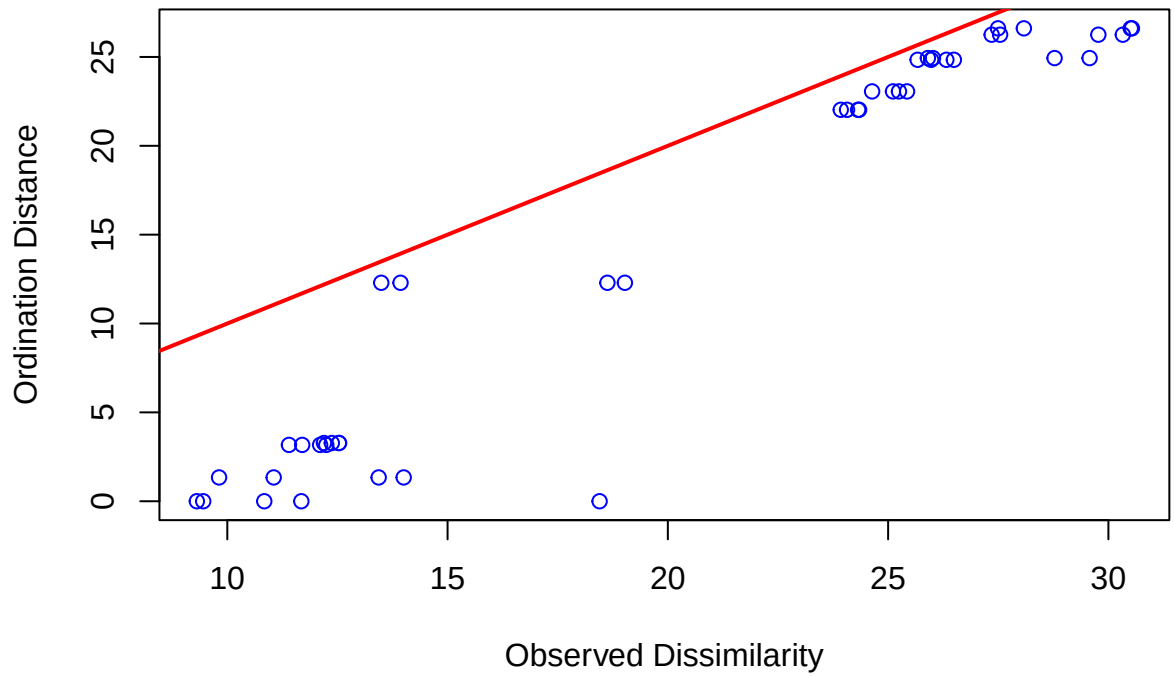

```
## [1] "Max in rows containing a zero"
## [1] 764
```


CAPObject
Inertia
0
50
100
150
CAP1
CAP2
CAP3
CAP4

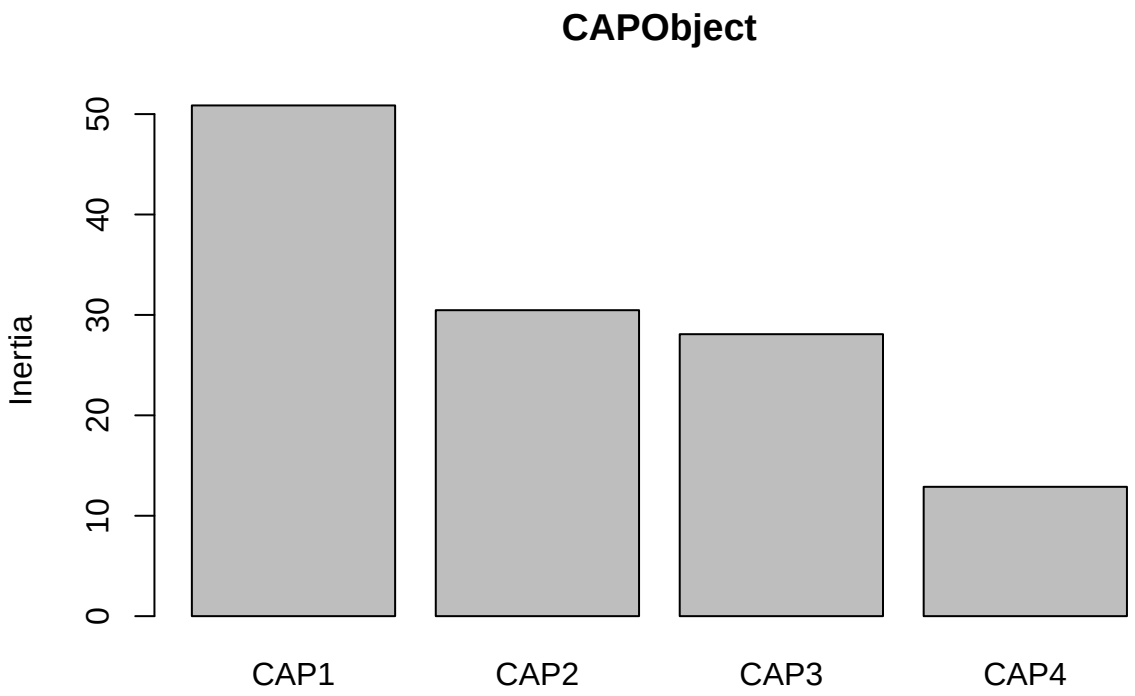


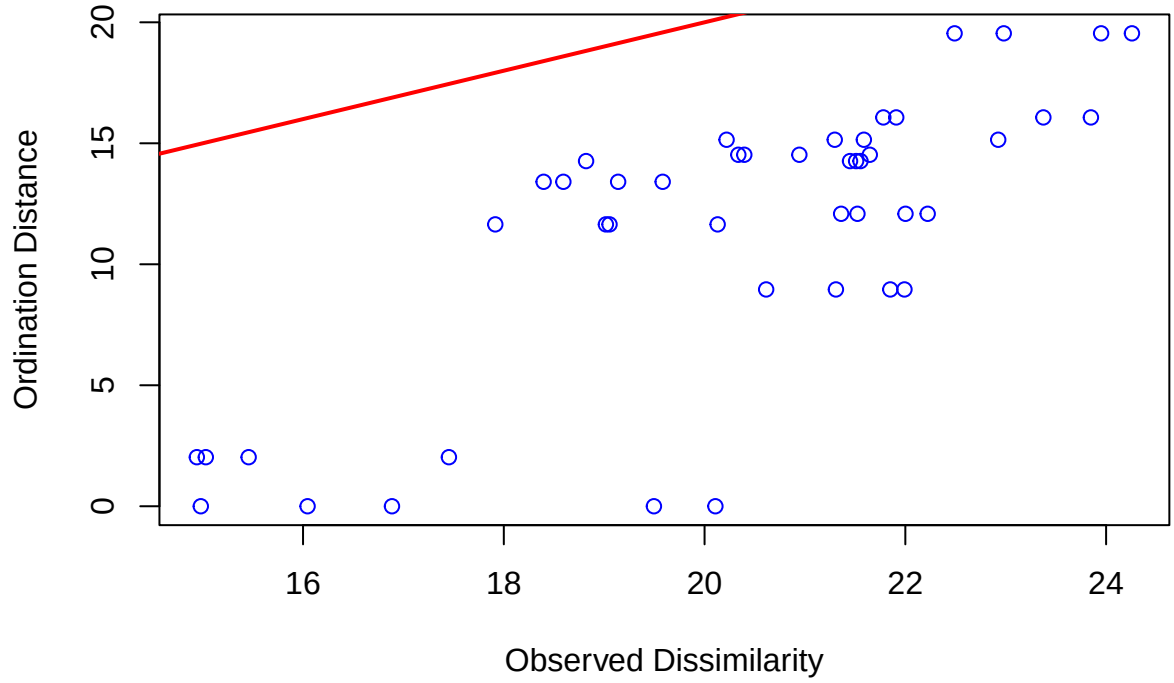

```
## [1] "Max in rows containing a zero"
## [1] 927
```


CAPObject
Inertia
0
20
40
60
80
120
CAP1
CAP2
CAP3
CAP4

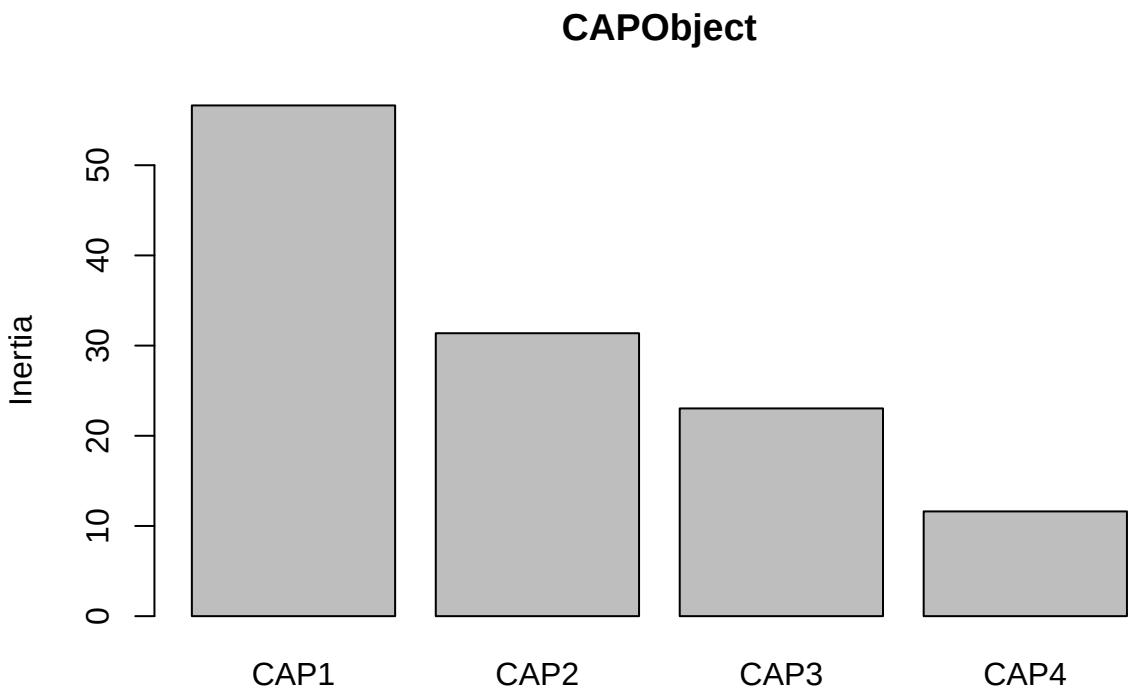


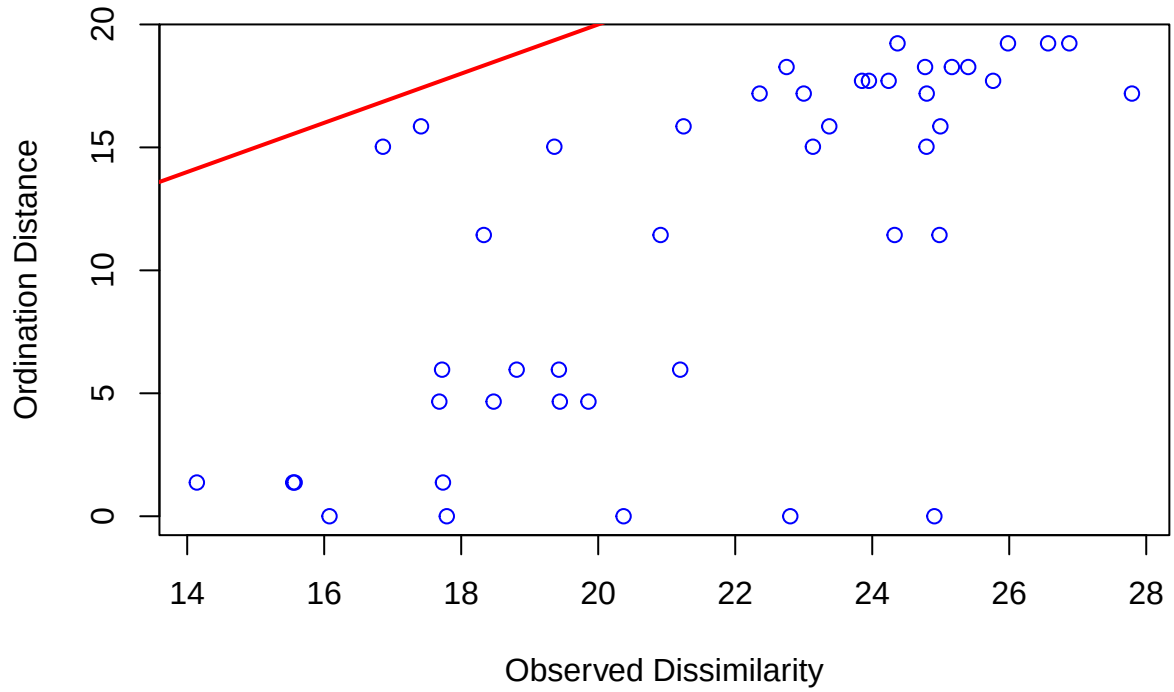

```
## [1] "Max in rows containing a zero"
## [1] 1552
```


CAPObject
Inertia
0
10
20
30
40
50
CAP1
CAP2
CAP3
CAP4

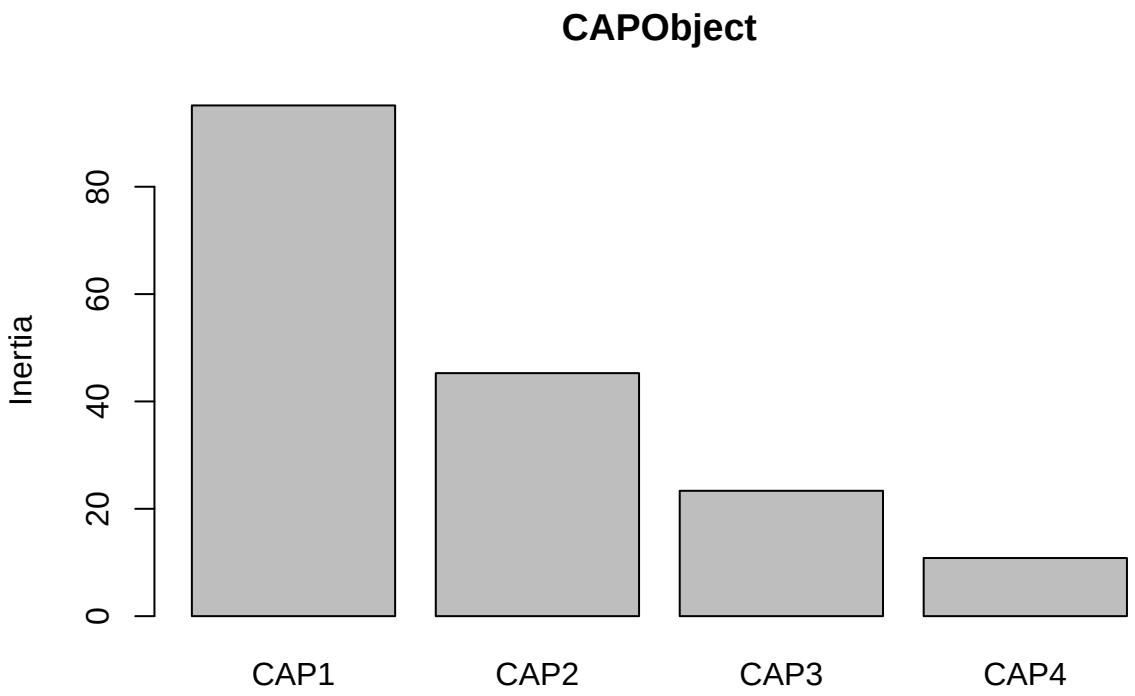


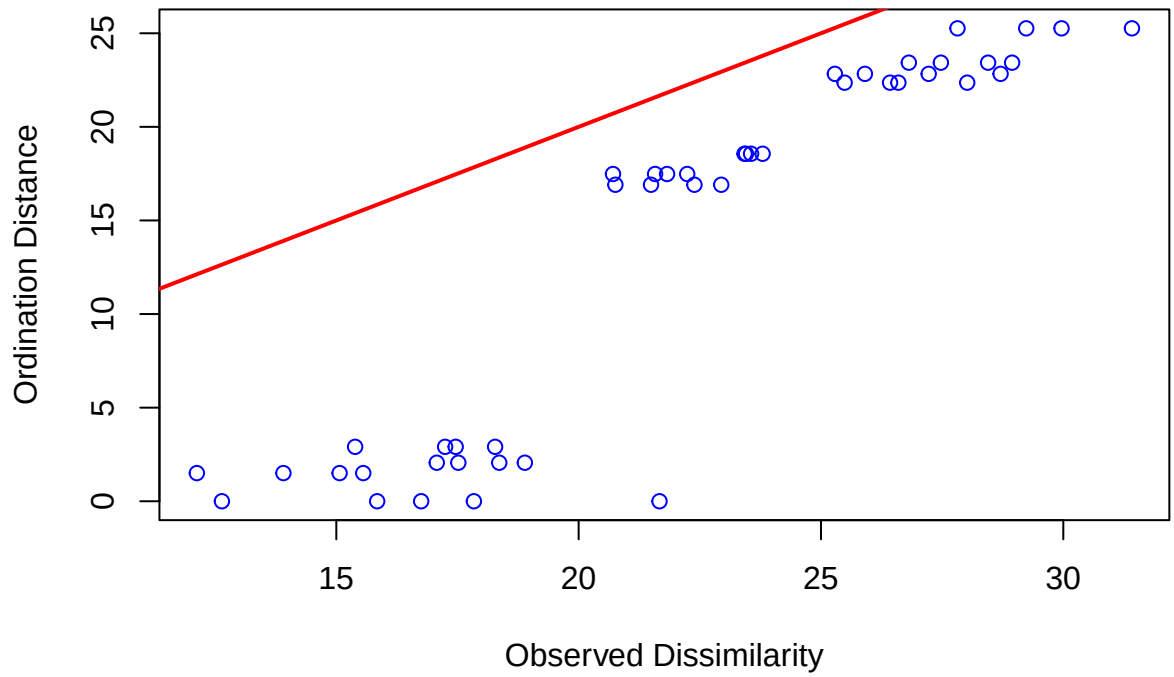

```
## [1] "Max in rows containing a zero"
## [1] 799
```


CAPObject
Inertia
0
10
20
30
40
50
CAP1
CAP2
CAP3
CAP4

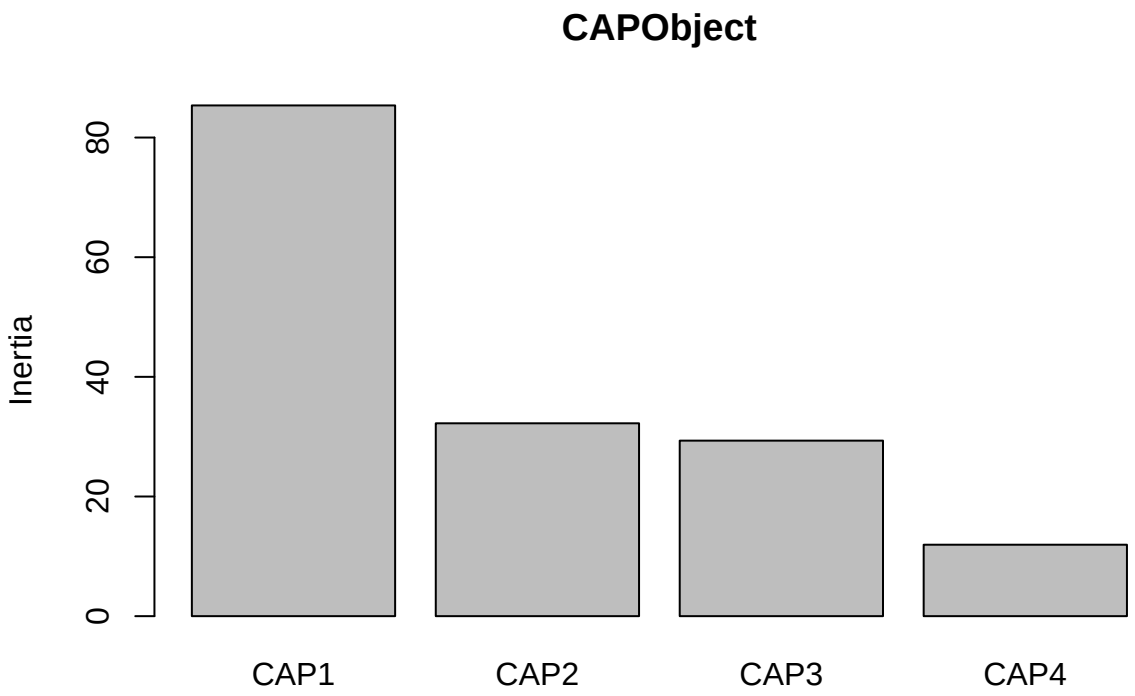


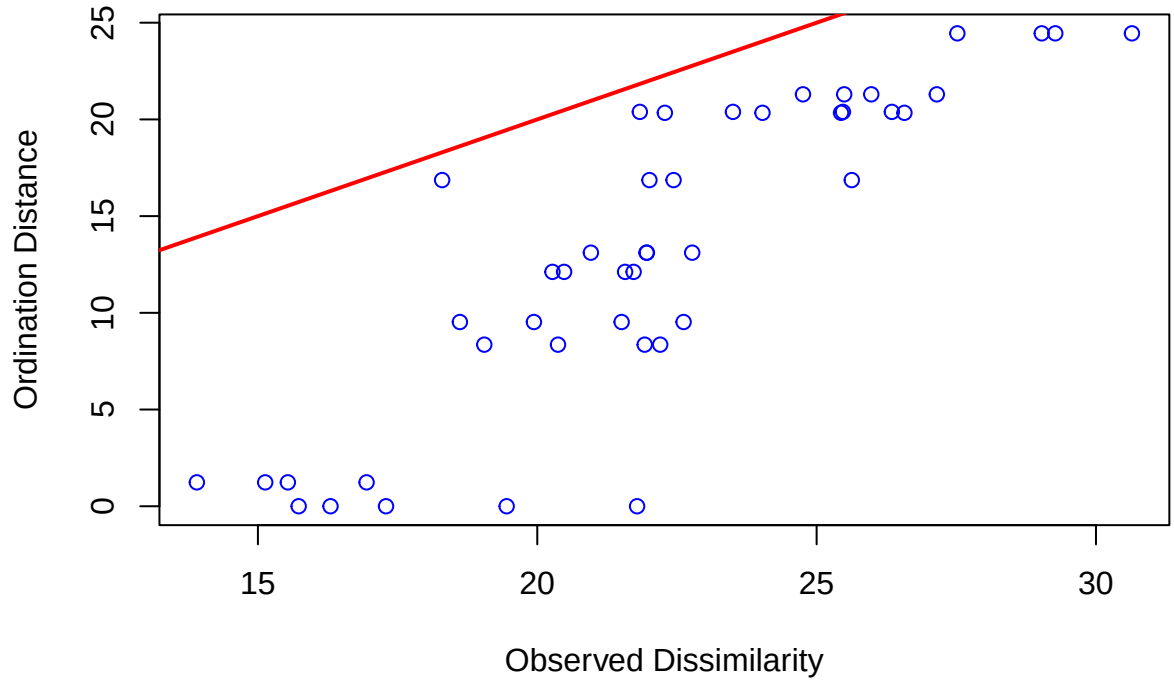

```
## [1] "Max in rows containing a zero"
## [1] 1043
## No. corrected values:  1
```


CAPObject
Inertia
0
20
40
60
80
CAP1
CAP2
CAP3
CAP4




```
## [1] "Max in rows containing a zero"
## [1] 1307
## No. corrected values:  9
```


CAPObject
Inertia
0
20
40
60
80
CAP1
CAP2
CAP3
CAP4




```
#Have the plots stored in lists
lay <- rbind(c(1,2),
             c(3,4),
             c(5,6),
             c(7,8))
pdf(paste("S6_Fig_rda", ".pdf", sep=""), width=12, height=20)
grid.arrange(CAPList$CAPP1unspikedUnfrozen, CAPList$CAPP1unspikedFrozen,
             CAPList$CAPP2unspikedUnfrozen, CAPList$CAPP2unspikedFrozen,
             CAPList$CAPS1unspikedUnfrozen, CAPList$CAPS1unspikedFrozen,
             CAPList$CAPS2unspikedUnfrozen, CAPList$CAPS2unspikedFrozen,
             layout_matrix = lay)
dev.off()
```

```
## pdf
##   2
```

```
#Create legend for SFig3
#Combine with legend in PCA
legend<-ggplot() +
  #geom_point(data=Metadata2[1:2,], aes(x=CAP1, y=CAP2, shape=StoragePlacementType),
  #color="#377eb8", size=1.5, fill="#377eb8", stroke=1.2) possible to run with a shape
    #indicating freezing or direct showing if samples are subsetted only to P1, P2, S1
    #and S2
  geom_point(data=Metadata2[1:2,], aes(x=CAP1, y=CAP2), color="#4daf4a", size=2,
             fill="#4daf4a", stroke=1.2) +
  geom_point(data=Metadata2[3:4,], aes(x=CAP1, y=CAP2), color="#ff7f00", size=2,
             fill="#ff7f00", stroke=1.2) +
```

```
  geom_point(data=Metadata2[5:6,], aes(x=CAP1, y=CAP2), color="#993300", size=2,
             fill="#993300", stroke=1.2) +
  geom_point(data=Metadata2[7:8,], aes(x=CAP1, y=CAP2), color="#984ea3", size=2,
             fill="#984ea3", stroke=1.2) +
  geom_point(data=Metadata2[9:10,], aes(x=CAP1, y=CAP2), color="#377eb8", size=2,
             fill="#377eb8", stroke=1.2) +
  geom_line(data=Metadata2, aes(x=CAP1, y=CAP2, group=Sample_LPSX)) +
  geom_point(data=Feature2, aes(x=CAP1, y=CAP2, color=col, group=Genus), size=4) +
  scale_colour_gdocs() +
  labs(colour="Organisms", title=paste("rda ", i, j), x=eig_1, y=eig_2) +
  #scale_shape_manual(values=c(21, 22)) +
  theme_bw() +
  #theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
    #axis.title=element_text(size=12), legend.position="none") #+
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
        axis.title=element_text(size=12), legend.position="bottom")
legendplot<-get_legend(legend)
pdf(paste("S6_Fig_rdaLegend", ".pdf", sep=""), width=10, height=1)
grid.arrange(legendplot)
dev.off()
```

```
## pdf
##   2
```

Additional

Session information

```
sessionInfo()
```

```
## R version 4.1.0 (2021-05-18)
## Platform: x86_64-w64-mingw32/x64 (64-bit)
## Running under: Windows 10 x64 (build 19042)
##
## Matrix products: default
##
## locale:
## [1] LC_COLLATE=English_United States.1252
## [2] LC_CTYPE=English_United States.1252
## [3] LC_MONETARY=English_United States.1252
## [4] LC_NUMERIC=C
## [5] LC_TIME=English_United States.1252
##
## attached base packages:
## [1] stats     graphics  grDevices utils     datasets  methods   base
##
## other attached packages:
##  [1] amap_0.8-18           dplyr_1.0.7           Rmisc_1.5
##  [4] plyr_1.8.6            FSA_0.9.1             RColorBrewer_1.1-2
##  [7] pheatmap_1.0.12       mixOmics_6.16.3       psych_2.1.6
## [10] robCompositions_2.3.0 data.table_1.14.0     pls_2.7-3
## [13] car_3.0-11            carData_3.0-4         zCompositions_1.3.4
## [16] truncnorm_1.0-8       NADA_1.6-1.1          survival_3.2-11
## [19] MASS_7.3-54           compositions_2.0-2    cowplot_1.1.1
```

```
## [22] stringr_1.4.0         knitr_1.33            tidyr_1.1.3
## [25] reshape2_1.4.4        gridExtra_2.3         vegan_2.5-7
## [28] lattice_0.20-44       permute_0.9-5         ggthemes_4.2.4
## [31] ggplot2_3.3.5
##
## loaded via a namespace (and not attached):
##   [1] colorspace_2.0-2    ellipsis_0.3.2      class_7.3-19
##   [4] modeltools_0.2-23   rio_0.5.27          mclust_5.4.7
##   [7] corpcor_1.6.9       proxy_0.4-26        farver_2.1.0
##  [10] cvTools_0.3.2       ggrepel_0.9.1       flexmix_2.3-17
##  [13] RSpectra_0.16-0     fansi_0.5.0         mvtnorm_1.1-2
##  [16] ranger_0.13.1       splines_4.1.0       sROC_0.1-2
##  [19] mnormt_2.0.2        robustbase_0.93-8   cluster_2.1.2
##  [22] kernlab_0.9-29      rrcov_1.5-5         compiler_4.1.0
##  [25] rainbow_3.6         assertthat_0.2.1    Matrix_1.3-3
##  [28] htmltools_0.5.1.1   tools_4.1.0         igraph_1.2.6
##  [31] gtable_0.3.0        glue_1.4.2          Rcpp_1.0.7
##  [34] cellranger_1.1.0    vctrs_0.3.8         nlme_3.1-152
##  [37] fpc_2.2-9           lmtest_0.9-38       tensorA_0.36.2
##  [40] xfun_0.23           laeken_0.5.1        openxlsx_4.2.4
##  [43] lifecycle_1.0.0     DEoptimR_1.0-9      zoo_1.8-9
##  [46] scales_1.1.1        VIM_6.1.1           hms_1.1.0
##  [49] parallel_4.1.0      yaml_2.2.1          curl_4.3.2
##  [52] reshape_0.8.8       stringi_1.7.4       highr_0.9
##  [55] pcaPP_1.9-74        e1071_1.7-8         BiocParallel_1.26.1
##  [58] boot_1.3-28         zip_2.2.0           hdrcde_3.4
##  [61] matrixStats_0.60.1  rlang_0.4.11        pkgconfig_2.0.3
##  [64] prabclus_2.3-2      bitops_1.0-7        pracma_2.3.3
##  [67] evaluate_0.14       fda_5.1.9           purrr_0.3.4
##  [70] labeling_0.4.2      ks_1.13.2           tidyselect_1.1.1
##  [73] GGally_2.1.2        magrittr_2.0.1      R6_2.5.1
##  [76] generics_0.1.0      DBI_1.1.1           pillar_1.6.2
##  [79] haven_2.4.3         foreign_0.8-81      withr_2.4.2
##  [82] mgcv_1.8-35         abind_1.4-5         RCurl_1.98-1.3
##  [85] sp_1.4-5            nnet_7.3-16         tibble_3.1.4
##  [88] bayesm_3.1-4        crayon_1.4.1        rARPACK_0.11-0
##  [91] KernSmooth_2.23-20  utf8_1.2.2          ellipse_0.4.2
##  [94] tmvnsim_1.0-2       rmarkdown_2.10      fds_1.8
##  [97] grid_4.1.0          readxl_1.3.1        forcats_0.5.1
## [100] vcd_1.4-8           digest_0.6.27       diptest_0.76-0
## [103] stats4_4.1.0        munsell_0.5.0
```

This document was processed on:

```
Sys.Date()
```

```
## [1] "2021-09-08"
```